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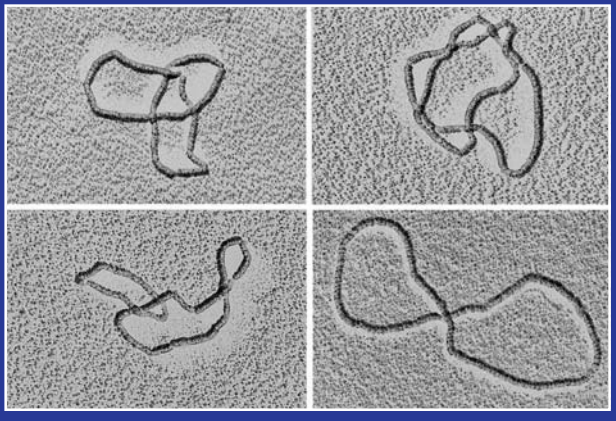
DNA TOPOISOMERASE PROTOCOLS

DNA Topology and Enzymes

Edited by Mary-Ann Bjornsti and Neil Osheroff



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Introduction to DNA Topoisomerases

Mary-Ann Bjornsti and Neil Osheroff

The helical structure of duplex DNA allows for the faithful duplication and transmission of genetic information from one generation to the next, at the same time maintaining the integrity of the polynucleotide chains. The complementary nature of the two antiparallel DNA strands enables each to serve as a template for the synthesis of the respective daughter DNA strands. The intertwining of these polynucleotide chains in duplex DNA further ensures the integrity of the DNA helix by physically linking the individual strands in a structure stabilized by hydrogen bonding and stacking interactions between the hydrophobic bases. However, these same features pose a number of topological constraints that affect most processes involving DNA, such as DNA replication, transcription, and nucleosome assembly (reviewed in ***[1–4]***).

During semiconservative DNA replication, for example, the progressive unwinding of the DNA template requires a swivel in the DNA duplex to alleviate the overwinding of the strands ahead of the moving replication fork. Of course, the replication apparatus may simply follow the helical path of the DNA template strands. However, this soon leads to a second problem of how to unlink the interwound DNA helices following the completion of DNA synthesis. This decatenation of daughter molecules is absolutely required in the case of circular genomes and plasmids, in which the template strands are physically linked circles. Similar considerations apply to the process of transcription, where the movement of a transcription complex along the DNA template may also produce a local unwinding of the DNA behind and overwinding of the DNA ahead. This may be viewed as the formation of local domains of negatively and positively supercoiled DNA, respectively ***(5)***. Indeed, the translocation of any complex that forms between the two strands of a DNA duplex (such

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as a helicase or a recombination intermediate) has the potential to generate such local changes in DNA topology.

It is relatively straightforward to imagine the consequences of these events. Without a "swivel" in the DNA, the overwinding of the DNA strands would eventually prohibit the further movement of the complex along the DNA, resulting in the inhibition of DNA replication, transcription, recombination, and so forth. Along similar lines, the inability to unlink or decatenate replicated sister chromatids would produce an extremely high rate of chromosomal breakage and/or nondisjunction during mitosis. In the case of chromatin assembly, the wrapping of DNA around the histones stabilizes negative supercoils. Because the linking number of a topologically constrained DNA molecule is conserved, this would result in the accumulation of positive supercoils in the unconstrained DNA with potentially profound effects on gene expression and DNA replication.

One solution to the topological problem lies in a family of enzymes called DNA topoisomerases ***(1,2,4,6,7)***. These enzymes catalyze changes in DNA topology by altering the linkage of DNA strands. This is accomplished via a mechanism of transient DNA strand breakage and religation. During an initial transesterification reaction, these enzymes form a covalent linkage between their active site tyrosyl residues and one end of cleaved DNA strand. This conserves the energy of the original phosphodiester backbone bond and creates a protein-linked break in the DNA. A second transesterification reaction between the free hydroxyl terminus of the noncovalently bound DNA strand and the phosphotyrosine linkage reseals the break in the DNA. Usually, this second reaction restores the original phosphodiester bond; however, under certain conditions, DNA topoisomerases may be induced to transfer one end of a DNA to a different DNA end ***(2,8)***. In the case of site specific recombinases, such as Flp in yeast, this transfer of DNA strands is precisely regulated to effect the integration and/or excision of DNA at specific sites ***(9,10)***.

DNA topoisomerases constitute an ever-increasing family of enzymes that can be distinguished on the basis of the number of DNA strands that they cleave and the covalent linkage formed in the enzyme-DNA intermediate (**Table 1**) (reviewed in ***[2,4,6,11,12]***). Type I enzymes cleave a single strand of a DNA duplex and produce changes in linking number in steps of one. The type IA enzymes, as exemplified by bacterial DNA topoisomerases I and III, and eukaryotic DNA topoisomerase III, encoded by the *topA, topB* and *TOP3* genes respectively, form a tyrosyl linkage with a 5′ phosphate. The recent discovery of DNA topoisomerase III in humans attests to the universality of this enzyme ***(13)***. In *Escherichia coli,* DNA topoisomerase I (TopA) catalyzes the relaxation of negatively supercoiled. Since the changes in DNA linking number catalyzed by bacterial DNA gyrase are opposite to that observed with TopA,

Table 1
DNA Topoisomerases*

Type	Tyrosyl linkage	Enzymes	Genes	Ref.
IA	5′ phosphate	Bacterial DNA topoisomerase I	*topA*	***(38)***
		Bacterial DNA topoisomerase III	*top*B	***(39)***
		DNA topoisomerase III	*TOP3,*	***(13,14)***
		Reverse gyrase		***(18)***
IB	3′ phosphate	DNA topoisomerase I	*TOP1*	***(20,40,41)***
		DNA topoisomerase V		***(42)***
		Vaccinia virus DNA topoisomerase I	*TOP1*	***(43)***
IIA	5′ phosphate	Bacterial DNA gyrase	*gyrA, gyrB*	***(44,45)***
		Bacterial DNA topoisomerase IV	*parC, parE*	***(46)***
		DNA topoisomerase II	*TOP2, TOP2*α,β	***(47–49)***
		T4 DNA topoisomerase II	*gn39, gn60, gn 52*	***(50)***
IIB	5′ phosphate	Archeal DNA topoisomerase VI	*top6A, top6B*	***(11)***

*Representative examples are given. This list is not meant to be exhaustive.

there appears to be homeostatic mechanism regulating the levels of expression of these enzymes to maintain the level of DNA supercoiling within a fairly narrow range. The function of DNA topoisomerase III in bacteria and in eukaryotes is less clear. These enzymes are highly related and appear to possess a potent decatenase activity. In yeast, the Top3 enzyme plays a role in suppressing recombination between repeated DNA sequences, is required during meiosis, and has been implicated in telomere maintenance ***(14,15)***. However, the enzyme does not appear to constitute a major DNA relaxation activity in the cell. Genetic studies suggest an association between Top3p and a helicase, Sgs1p, a homolog of the Bloom's and Werner's syndrome genes in human ***(16,17)***.

Reverse gyrase constitutes an additional member of the type IA family. This ATP-dependent enzyme catalyzes the positive supercoiling of DNA. Moreover, this enzyme appears to have a bipartite structure consisting of a helicase domain and a type IA topoisomerase ***(18)***.

Type IB enzymes include eukaryotic DNA topoisomerase I, the product of the *TOP1* gene. Top1p exhibits little similarity to the type IA enzymes, catalyzes the relaxation of both positively and negatively supercoiled DNA, and forms a tyrosyl linkage with a 3′ phosphate. In yeast, the *TOP1* gene is nonessential, as other cellular factors, such as DNA topoisomerase II or Trf4p, can compensate for the loss of Top1p function ***(19,20)***. Genetic studies further suggest that while DNA topoisomerase II is absolutely required to decatenate sister chromatids during mitosis, either DNA topoisomerase I or II is sufficient during other phases of the cell cycle. In *Drosophila* and mouse, DNA

topoisomerase I is absolutely required during embryogenesis and may reflect the increased requirement for a swivelase activity during periods of rapid DNA replication ***(21,22)***. Top1p is predominately associated with transcriptionally active sequences and is thought to relax the supercoils formed during DNA replication and transcription. Both DNA topoisomerase I and II have been shown to suppress the rate of rDNA recombination in yeast. Although the mechanism is unclear, it may relate to the high level of transcription of the rDNA locus ***(2)***.

Type II DNA topoisomerases cleave and religate both strands of the DNA duplex and form covalent intermediates with a 5′ phosphate. Type IIA enzymes include bacterial DNA gyrase, DNA topoisomerase IV and eukaryotic DNA topoisomerase II ***(1,2,4,23,24)***. All members of this family exhibit extensive sequence similarity and function as heterotetramers (the bacterial enzymes) or homodimers (eukaryotic Top2p). Bacterial DNA gyrase is composed of two GyrA subunits and two GyrB subunits, and is able to introduce negative supercoils into DNA or catalyze the removal of positive supercoils. DNA topoisomerase IV, encoded by the *parC* and *parE* genes, is a potent decatenase ***(25)***. Eukaryotic DNA topoisomerase II, the product of the *TOP2* gene in yeast, functions as a homodimer and catalyzes the relaxation of positively or negatively supercoiled DNA. This enzyme is essential and is required to resolved the multiply intertwined sister chromatids during mitosis. In all cases, a significant body of work suggests that these enzymes bind DNA as an ATP-dependent protein clamp ***(26–28)***. Both strands of the bound DNA are cleaved to yield staggered protein-linked nicks. A second DNA strand is then passed through this gate in the DNA, and the nicks are religated. The hydrolysis of ATP is required to drive allosteric changes in enzyme structure, rather than the cleavage or religation of the DNA. In human cells, two isoforms of the enzyme are encoded by *TOP2α* and *TOP2β*. When these two genes are coexpressed in yeast, catalytically active heterodimers are detected, suggesting that Top2α/β heterodimers may also constitute a portion of DNA topoisomerase II in mammalian cells ***(29)***.

Type IIB enzymes consist of DNA topoisomerase VI from Archea ***(11)***. These ATP dependent enzymes also catalyze the relaxation of positively and negatively supercoiled DNA, possess a potent DNA decatenase activity, and comprise heterotetramers of Top6A and Top6B. However, these enzymes exhibit little sequence similarity to the type IIA enzymes. Instead, they resemble the *SPO11* gene product, which is thought to initiate meiotic recombination in yeast by cleaving double-stranded DNA ***(30)***. The Spo11 protein becomes covalently attached to the 5-phosphate ends of the DNA. How these covalent lesions are resolved has yet to be determined.

The study of DNA topoisomerases has tremendously expanded our knowledge of all of the biological processes in which they play a role. Moreover, as described in the accompanying volume, *Protocols in DNA Topology and Topoisomerases, Part II: Enzymology and Drugs* many of these enzymes are the cellular targets for an ever-increasing number of antibacterial and anticancer agents ***(4,31,32)***. Thus, understanding the mechanism of action of these enzymes has further application in the clinical development of important therapeutic agents. Along related lines, our understanding of chromatin assembly and how alterations in nucleosome structure can profoundly affect the regulation of gene expression have been facilitated by detailing changes in DNA topology ***(33–35)***. Related studies of DNA structures, such as bending and cruciforms, have also contributed to recent models of specific protein-DNA interactions and their role in regulating promoters and enzyme function ***(36,37)***.

This volume contains numerous experimental protocols to examine various aspects of DNA structure and topology. In addition, the expression and purification of DNA topoisomerases from a wide range of experimental systems is also described. The accompanying volume details various methods for assessing DNA topoisomerase catalytic activities and sensitivities to drugs that interfere with enzyme function. Additional protocols for examining the phenotypic consequences of drug treatment and selecting drug resistant mutants are also provided. Together, these two volumes provide a comprehensive compendium of experimental protocols with which to study all aspects of DNA topology and topoisomerase function.

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2

Resolution of DNA Molecules by One-Dimensional Agarose-Gel Electrophoresis

Mary-Ann Bjornsti and Maureen D. Megonigal

1. Introduction

Agarose-gel electrophoresis is used to separate DNA molecules on the basis of size and shape ***(1–4)***. Since DNA is negatively charged, the charge-to-mass ratio is constant. Thus, migration through agarose is inversely proportional to the size of the molecule. However, the electrophoretic mobility of DNA in agarose is also affected by the shape of the DNA, the pore size of the matrix (agarose concentration), temperature, the ionic strength of the electrophoresis buffer, the applied voltage/field strength, and the presence of intercalators (reviewed in ***5,6***).

1.1. DNA Shape

Circular plasmid DNA can exist in a number of different topological conformations. Superhelical circular DNA (form I), nicked circular DNA (form II), and linear DNA (form III) of identical sequence and mol wt migrate through agarose gels at different rates ***(1)***. Owing to their compact nature, supercoiled DNA topoisomers migrate faster through agarose in comparison to linear DNA, nicked circular DNA, or relaxed DNA. For example, as shown in **Fig. 1**, negatively supercoiled plasmid DNA topoisomers (form I) migrate as a single band, whereas the same plasmid, when nicked (form II), migrates much more slowly. The frictional resistance of linear DNA is generally less than that of nicked or relaxed DNA owing to the adoption of an "end-on" orientation during migration ***(7,8)***.

The topological state of a circular DNA molecule is described by the linking number (Lk), which is the sum of two geometric properties, twist (Tw) and

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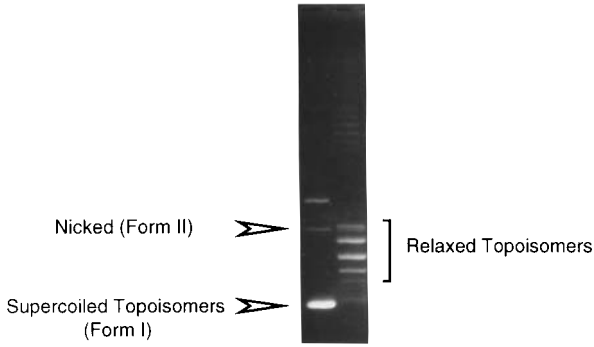


Fig. 1. Negatively supercoiled plasmid DNA and the same DNAs relaxed with DNA topoisomerase I were resolved in a 0.8% agarose gel in 100 m*M* Tris-borate buffer at 5 V/cm. The gel was subsequently stained with 0.5 mg/mL EthBr and photographed on a UV transilluminator equipped with 300-nm bulbs. The relative positions of the negatively supercoiled DNAs (form I), the nicked plasmid DNA (form II), and the relaxed plasmid DNA topoisomers are as indicated.

writhe (Wr). Tw refers to the number of times one strand passes around the other, whereas Wr describes the coiling of the helical axis. For a given closed circular DNA molecule, the linking number is invariant. Although the relative contributions of Tw and Wr may change, any change in Tw must be accompanied by an equal but opposite change in Wr. DNA molecules of different Lk can be resolved in agarose gels on the basis of differences in Wr, where adjacent bands differ by a linking number of one (**Fig. 1**) *(2)*.

When the ends of a linear DNA molecule are ligated to form a closed circle or when supercoiled plasmid DNA is treated with eukaryotic DNA topoisomerase I, a population of relaxed DNA topoisomers is formed (*see* **Fig. 1**). Under the reaction conditions used, these closed circular DNA molecules are free of torsional strain; that is, they have assumed the most energetically favored conformation. However, since the differences in energy between DNA molecules of similar linking number is quite small, a Boltzman population of the relaxed DNA topoisomers is obtained, which describes a Gaussian curve. The center of the curve defines the most relaxed form of the DNA (Lk^o). Given the constraint that the Lk for a given DNA molecule must be an integral number, the center may not correspond to a specific band in the gel. Moreover, the conditions employed for electrophoresis usually differ from those used to generate the relaxed DNA molecules. These changes in ionic strength and temperature affect the pitch of the DNA helix. This corresponds to a change in Wr and, therefore, an alteration in gel mobility. As shown for the population of relaxed DNA topoisomers in **Fig. 1**, this is manifest as a slight increase in Wr,

such that the molecules are slightly positively supercoiled in the gel. In contrast, a nicked DNA molecule is able to change conformation in response to changes in ionic strength, temperature, and so forth. Thus, under any conditions, nicked molecules will assume the most thermodynamically relaxed conformation and will migrate as a single band. The supercoiled DNA molecules in **Fig. 1** also comprise a population of topoisomers. In the absence of an intercalator, however, their compact structures preclude the resolution of discrete bands.

1.2. Applied Voltage/Field Strength

When constant field strength is applied, linear duplex molecules migrate through agarose gel matrices at a rate that is inversely proportional to the $\log_{10}$ of their mol wt ***(9)*** and proportional to the applied voltage. However, with higher voltages (5–10 V/cm), the migration of large DNA molecules (>2 kb) increases at a faster rate than that of small DNA molecules ***(5,6)***. For circular DNAs, the relative mobility of nicked and supercoiled DNA topoisomers is also affected by field strength. Indeed, in some instances, supercoiled and nicked circular DNA molecules comigrate when high voltage is employed.

1.3. Intercalator Effects

Although variations in the mobility of nicked circular and linear DNAs are dependent upon electrophoretic conditions, changes in the conformation of covalently closed circular DNA induced by intercalator binding also affect electrophoretic mobility. Binding of one molecule of the intercalator ethidium bromide (EthBr) unwinds the DNA helix by 26° ***(10,11)***. In an agarose gel, this reduction in twist would be detected as a compensatory increase in Wr, i.e., a reduction in negative supercoiling and therefore a decrease in mobility. Increasing the concentration of EthBr would result in further increments in Wr (lower mobility) until a critical concentration is reached. At this point, the original negative Wr of the negatively supercoiled molecule is effectively canceled by the EthBr-induced positive Wr. This population of DNA topoisomers would comigrate with DNA topoisomers relaxed under electrophoresis conditions. Beyond this concentration, the DNA molecules would continue to accumulate positive Wr, becoming more compact, with a corresponding increase in mobility. At ~1 µg/mL EthBr, a concentration routinely used for the resolution of DNA restriction fragments, closed circular DNA becomes saturated with EthBr ***(4)*** and acquires levels of positive Wr that are beyond the resolving capacity of the gel.

Linear and nicked circular DNA also bind EthBr. However, in this case, any reduction in twist simply results in the rotation of the free ends or the broken strand about the intact strand. Therefore, the conformation of linear and nicked

circular DNAs is not significantly altered by EthBr intercalation. In the absence of the topological constraints imposed on intact duplex DNA circles, linear and nicked circular DNA bind more EthBr than the corresponding covalently closed circular DNA. At high EthBr concentrations, the migration of these molecules may be reduced slightly owing to a neutralization of charge and an increase in rigidity that accompanies ethidium binding.

2. Materials

2.1. Plasmid DNA

Negatively supercoiled plasmid DNAs can most readily be purified from bacteria by cesium chloride/EthBr equilibrium centrifugation following alkaline lysis *(5,6)*. Alternatively, negatively supercoiled plasmid DNA can be purified by column chromatographic methods using commercially available resins, such as that supplied by Qiagen (*see* **Note 1**).

2.2. Agarose-Gel Electrophoresis

All chemicals are available from Sigma, St. Louis. All equipment is available from Fisher Scientific and Owl Scientific.

1. 10X TBE buffer: 0.89*M* Tris-borate, 20 m*M* EDTA, pH 8.0 (*see* **Note 2**).
2. 1X TBE buffer: 89 m*M* Tris-borate, 2 m*M* EDTA, pH 8.0 (*see* **Note 3**).
3. 1X TBE buffer with EthBr: 89 m*M* Tris-borate, 2 m*M* EDTA, pH 8.1, 0.5–1.0 µg/mL EthBr (*see* **Note 4**).
4. 7–10X Loading buffer: 30% Ficoll (type 400), 0.1% bromophenol blue, 0.1% xylene cyanol.
5. A horizontal gel electrophoresis apparatus consisting of a tank and a casting tray.
6. Electrophoresis-grade agarose.
7. EthBr: 10 mg/mL dissolved in dH_2O.
8. Shortwave UV transilluminator.

3. Methods

3.1. Resolution of Plasmid DNAs by One-Dimensional Gel Electrophoresis

1. Prepare a 0.8% agarose solution (0.8 g/p 100 mL 1X TBE buffer) by boiling the solution until all of the agarose is dissolved (*see* **Note 5**). This can be accomplished on a hot plate using a stir bar or in a microwave. Cool the solution to 55°C before casting the gel in a horizontal tray (*see* **Note 6**). The agarose slab used in this chapter measured 22 × 15 cm; the electrophoresis apparatus consisted of a tank measuring 29 × 16.6 cm (*see* **Note 7**).
2. Set the gel for 30 min at room temperature. Then gently remove the comb and immerse the gel tray in 1X TBE buffer.
3. Add 1/7 vol of 7X loading buffer to DNA samples. Load samples directly into submerged wells and electrophorese at ~1–5 V/cm for ~13–15 h (*see* **Note 8**).

4. Stain the gel in 1–2 L dH_2O containing 0.5 μg/mL EthBr. After 10–15 min, destain for 20–30 min in dH_2O; this decreases the background fluorescence and improves visualization of the DNA bands.
5. Visualize EthBr stained DNA by direct illumination with a UV transilluminator (*see* **Note 9**). Photograph stained gels through a Kodak Wratten #23A red filter with Polaroid Type-667 film or Type-55 positive/negative film.

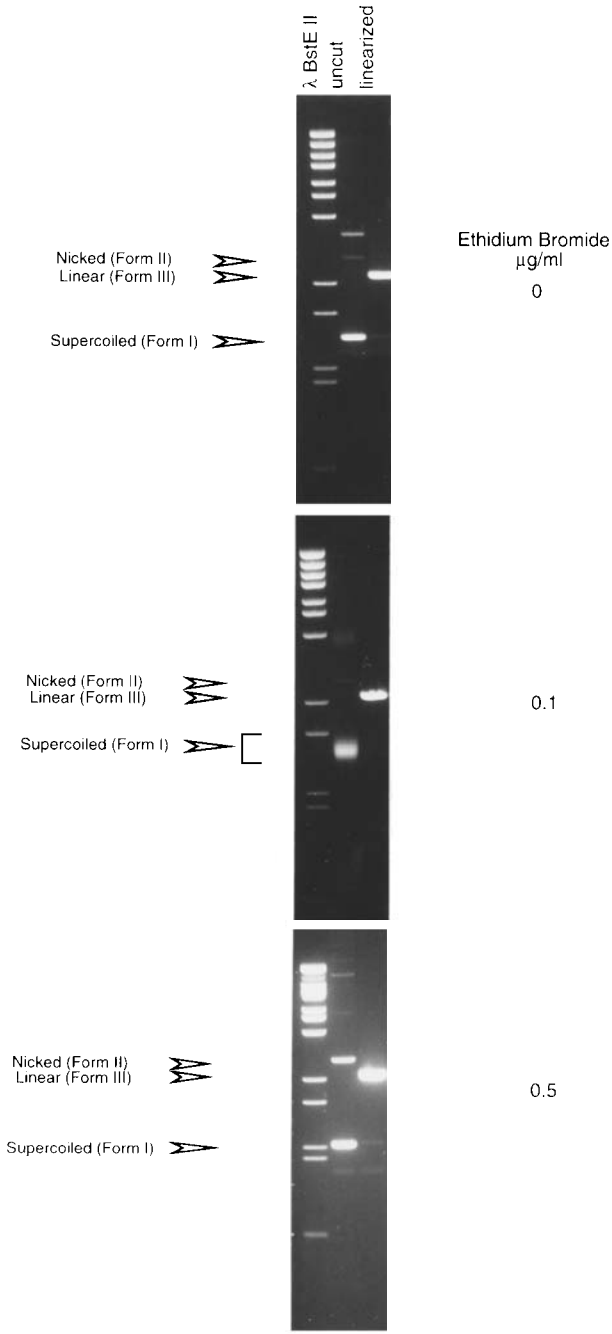
3.2. Resolution of Plasmid DNAs in the Presence of EthBr

1. When desired, 0.5–1.0 μg/mL EthBr is added to the electrophoresis buffer and agarose gel. In the case of long runs, buffer recirculation with a peristaltic pump will ensure uniform staining (*see* **Note 10**). Since EthBr is a powerful mutagen, care should always be taken to dispose properly of EthBr containing solutions.
2. DNA bands may be directly visualized during electrophoresis with a handheld UV transilluminator. Additional staining is not required to photograph the gel (*see* **Note 11**).

3.3. Analysis of Results

The effects of EthBr intercalation on electrophoretic mobility are illustrated in **Fig. 2**. In the absence of EthBr, the negatively supercoiled plasmid DNAs migrate as a discrete band between marker bands 9 and 10. When 0.1 μg/mL EthBr was added to the electrophoresis buffer and the gel, a population of topoisomers was resolved with a slightly slower mobility. This results from an increase in Wr on intercalator binding. In this case, adjacent bands differ by a linking number of one. Chloroquine, another DNA intercalator, has similar effects on DNA conformation and is also used to resolve DNA topoisomers of varying linking number. At higher EthBr concentrations (0.5 μg/mL), the negatively supercoiled DNAs have accumulated sufficient positive Wr to run as a single band, which now comigrates with marker band 10. In contrast, the nicked and linear forms of the DNA, in all cases, migrate as a single band at the same relative positions in the gel.

The mobilities of nicked and covalently closed circular DNA molecules, relative to linear DNAs, are altered by increased field strength. As shown in **Fig. 3**, in the absence of EthBr, the negatively supercoiled DNAs (form I) migrate to a position between λ DNA marker bands 9 and 10. When the field strength is increased to 5 V/cm, the mobility of the supercoiled DNA topoisomers decreases, relative to the DNA markers, and comigrates with marker band 9. In addition, the resolution of form I and II DNAs is decreased. A similar pattern of altered mobilities is seen with the DNA dimers. In both cases, of course, the linear form of the plasmid comigrates with the same marker band; however, the resolution of the higher mol wt bands is also diminished at higher voltage.


λ BstE II
uncut
linearized
Ethidium Bromide
µg/ml
0
Nicked (Form II)
Linear (Form III)
Supercoiled (Form I)
0.1
0.5

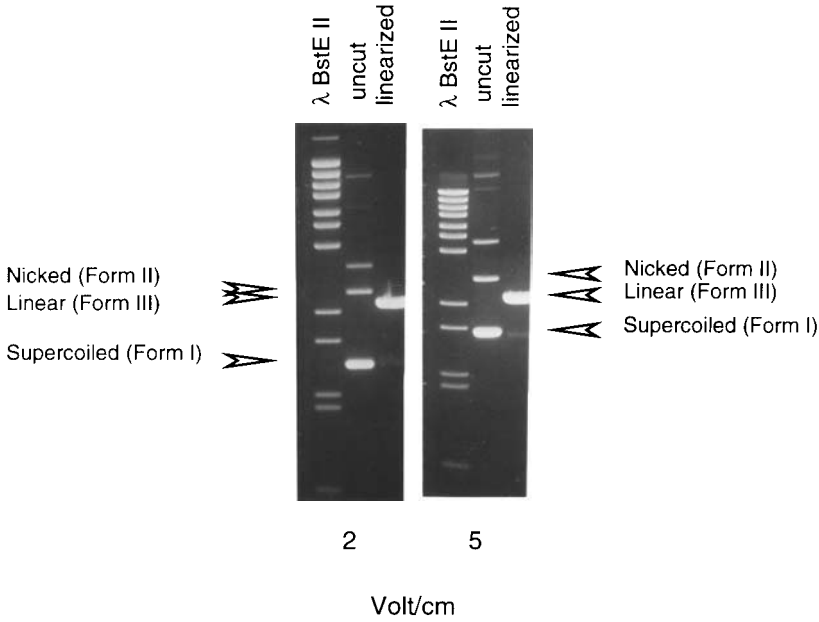


Fig. 3. The same DNAs shown in **Fig. 2** were resolved in a 0.8% agarose gel in 100 m*M* Tris-borate buffer at 2 or 5 V/cm for 15 or 2 h, respectively, in the absence of EthBr.

4. Notes

1. Although resin-purified DNAs are typically of high quality, the relative amount of nicked DNA molecules can be reduced by CsCl/EthBr equilibrium centrifugation.
2. Two commonly used buffers for the electrophoresis of native double-stranded DNA are Tris-borate EDTA (TBE) and TAE (40 m*M* Tris-acetate, 2 m*M* EDTA, pH 8.5) ***(6)***. The resolving powers of TAE and TBE are virtually identical for linear DNA, although the resolution of supercoiled topoisomers is slightly better with TAE. However, the buffering capacity of TBE is substantially greater than TAE, which tends to become exhausted during extended or high-voltage electrophoresis. Historically, TAE was preferred, since recovery of DNA from TBE-agarose gels using glass-adhesion methods was poor. Improved reagents largely circumvent this problem.

Fig. 2. *(opposite page)* Preparations of negatively supercoiled DNA, uncut and linearized with a restriction endonuclease, were resolved in a 0.8% agarose gel in 100 m*M* Tris-borate buffer. The linear, supercoiled, and nicked forms of the plasmid monomers are labeled forms III, I, and II, respectively. As indicated, a final 0, 0.1, or 0.5 mg/mL EthBr was also included in the buffer and gel. Electrophoresis was carried out at 2 V/cm for 15 h with continuous recirculation of the running buffer using a peristaltic pump. λ DNA digested with *Bst*EII served as mol wt markers.

3. Increasing the Tris-borate concentration to 100 m*M*, pH 8.3, as was done for the gels shown in **Figs. 1–3**, increases the resolution of plasmid DNA topoisomers at high-field strength.
4. The addition of EthBr alters the relative electrophoretic DNA mobilities of closed circular DNA vs nicked and linear DNA molecules. The addition of 0.5–1.0 µg/mL EthBr during electrophoresis is usually sufficient to increase the Wr of all covalently closed topoisomers of a given DNA molecule, such that they migrate as a single band.
5. The effective range of separation of DNA molecules is determined by the agarose concentration. As a general rule, agarose concentrations of 0.7–1.0% are effective for the separation of DNA in the size range of 0.5–20.0 kbp. Other matrix materials, such as polyacrylamide or chemically modified agarose, can be used to resolve effectively DNA fragments smaller than 1.0 kbp; however, supercoiled DNA molecules are excluded from polyacrylamide gels.
6. This prevents warping of the Lucite gel trays.
7. Gel electrophoretic trays and tanks of various sizes are commercially available (Owl Scientific). The use of minigels dramatically increases field strength, limiting the resolving power of the gel. For best resolution, an applied voltage of 1–5 V/cm is recommended.
8. When determining the total voltage, the distance is measured as the shortest path between the electrodes and not the length of the gel itself.
9. When only photodocumentation is desired, midrange UV wavelengths (270–340 nm) can be achieved using transilluminators outfitted with 300–nm bulbs and a UV filter. Such devices typically deliver an emission spectrum that peaks between 307 and 312 nm, the excitation peak for fluorescence of EthBr stained DNA. For preparative work, the use of longwave UV (365 nm) is recommended. This minimizes photonicking of the DNA during periods of extended viewing.
10. EthBr migrates toward the cathode. During extended runs, this will result in a progressive destaining of the gel such that smaller DNA fragments will not be visible. Buffer recirculation will prevent this.
11. Many gel devices are supplied with UV translucent trays, so the DNA may be viewed on a transilluminator directly through the gel tray.

Acknowledgments

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3

Two-Dimensional Agarose-Gel Electrophoresis of DNA Topoisomers

Ryo Hanai and Joaquim Roca

1. Introduction

Gel electrophoresis in one dimension is often insufficient to distinguish various molecular species of DNA, since different conformers, and sometimes DNA molecules of totally different structures may have the same electrophoretic mobility. These DNA species can be resolved by two-dimensional (2-D) gel electrophoresis, which involves two successive operations carried out with one gel slab under different conditions and in orthogonal directions.

In the separation of DNA topoisomers, the need for 2-D electrophoresis becomes acute as the range of the linking number becomes larger. Since the electrophoretic mobility of a duplex DNA ring is determined by its overall shape alone, DNA topoisomers with the same overall dimension but with opposite handedness cannot be separated. This problem is overcome by the addition of an intercalating agent during the second electrophoretic operation, thereby effecting a change in the mobilities of the topoisomers.

Topoisomer separation in two dimensions was first reported by Lee et al. in 1981 ***(1)***. In their study of the effects of dehydration on the helical pitch of DNA, positively and negatively supercoiled species were separated by the presence of a low concentration of ethidium bromide in the electrophoresis buffer for the second dimension. Such 2-D techniques have been routinely employed to separate and unambiguously identify DNA topoisomers. One of the clearest demonstrations of the utility of 2-D electrophoresis in the field of DNA topology was the thermodynamic characterization of the B-Z transition by Peck and Wang ***(2)***. Interconversion between the B-form and the left-handed Z-form of a plasmid segment is visualized as a break in the characteristic arch that traces

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topoisomers separated by 2-D gel electrophoresis. The 2-D technique was also particularly instrumental in the discovery of the H-form of DNA by Frank-Kamenetskii and associates ***(3)***.

The present chapter describes the utility of 2-D agarose-gel electrophoresis in the presence of a DNA intercalator and gives an example of laboratory practice. Although it is beyond the scope of this chapter, it is worth mentioning that other 2-D techniques have also been useful, e.g., in studies of DNA replication intermediates ***(4)***.

1.1. Separation of Topoisomers by 2-D Electrophoresis

The electrophoretic mobility of a DNA ring is determined by its overall dimension. As the molecule becomes more supercoiled, it compacts and migrates faster. In mathematical terms, this phenomenon is related to the observation $Wr = 0.73\Delta Lk$ ***(5)***, where Wr is the writhe of the DNA and ΔLk is the difference of the linking number from that of the relaxed state. Namely, the linking number difference results in a change of the writhe, and the writhe then translates into a difference of the electrophoretic mobility. However, there are two limitations on the electrophoretic separation of DNA topoisomers. One is that the linking number difference does not make a discernible mobility difference beyond some point. This happens because a supercoiled DNA ring tends to adapt a plectonemic fold in which the overall dimension of the molecule becomes insensitive to the change of Wr. The other is that the mobility does not reflect the sign of Wr, i.e., the handedness of the spatial curve. Both problems can be solved by 2-D electrophoresis.

In most biological systems, DNA is negatively supercoiled: the linking number of a DNA ring is smaller than that of the relaxed state. For instance, plasmids isolated from *Escherichia coli* have a typical linking number deficit of 6%; placing a histone octamer per 200 bp results in a deficit of 5%. Under standard electrophoretic conditions, DNA topoisomers in such a range of supercoiling have similar mobilities, and individual topoisomers are not resolved.

The electrophoretic mobility of a DNA ring can be altered without changing its linking number. This is possible because of the relation $Wr = Lk - Tw$ ***(5)***; a change of Tw results in a change of Wr. In the case of negatively supercoiled DNA, reduction of Tw (untwisting of the duplex) will result in a smaller $|Wr|$, thereby bringing negatively supercoiled topoisomers into a range where a difference in Lk is effectively reflected in a difference in the electrophoretic mobility. Experimentally, this is accomplished commonly by the addition of an intercalator, which inserts itself between stacked base pairs and untwists the duplex. For instance, an intercalated ethidium molecule untwists its neighbor-

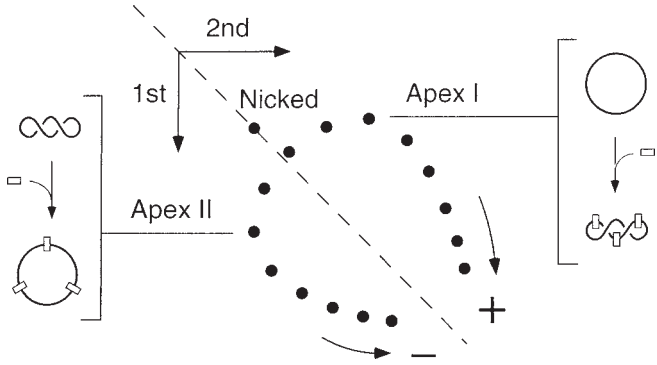

Fig. 1. Topoisomer separation by 2-D gel electrophoresis. In this schematic, topoisomers, which are represented by dots, were electrophoresed without an intercalator during the first electrophoresis and with an intercalator during the second. The apex I indicates the topoisomer that had the smallest writhe and, therefore, the smallest mobility during the first electrophoresis. Binding of intercalator, represented by open rectangles, changed the overall dimension of topoisomer such that it migrated faster in the second dimension. The apex II points to an originally negatively supercoiled topoisomer that became the most slowly migrating species in the second operation owing to intercalation.

ing base pairs by 26° ***(6)***. The corollary is that the electrophoretic mobility of a duplex DNA ring can be manipulated by the addition of an intercalator at an appropriate concentration during electrophoresis.

If DNA topoisomers are resolved by the use of an intercalator, the linking number distribution of interest may be too wide to be fit in the same sign range of *Wr*: Topoisomers of both handedness may overlap, and the order of their linking numbers cannot be determined. By performing the second electrophoresis with further changes in the mobilities of the topoisomers through an increase of the intercalator concentration, DNA topoisomers that migrated to the same distance are now separated from each other and from the other topoisomers.

This principle of topoisomer separation in two dimensions is schematically represented by **Fig. 1**. The topoisomers found at either apex had the smallest mobility during the first or the second electrophoresis. The apex I topoisomer had the smallest writhe during the first electrophoresis and assumed some writhe in the second because of intercalation. The apex II molecule initially had some negative writhe; the writhe was eliminated by intercalation in the second electrophoresis. Since intercalation has no effects on the writhe of a nicked DNA ring, which is almost zero, the nicked circle is found to the upper left of the topoisomer arch.

1.2. Structural Conversion and 2-D Electrophoresis

Some DNA sequences are known to absorb locally negative superhelical tension by adopting a conformation different from the standard B-form, such as Z-, H-, and cruciform structure (**ref. *7*** and references therein). Such structure conversions require threshold tension levels in order to occur: as the linking number of the plasmid containing such a sequence is decreased, the whole segment flips abruptly at a certain point. Since the conversion absorbs the supercoil tension, namely reduces *Tw* of the ring, *Wr* and therefore the electrophoretic mobility of the ring decrease. This transition can be clearly visualized as a break of the topoisomer arch. **Figure 2** is an illustration of 2-D electrophoresis of a plasmid containing a segment that can undergo B-Z transition. In the first electrophoresis, there is a discontinuity of the mobility between the topoisomers at the threshold. During the second electrophoresis, the presence of an intercalator removes the negative supercoil tension. Consequently, the segment assumes the normal B-form conformation, and the discontinuity in the mobility disappears. Information on the energetics of the B-Z transition can be extracted from such 2-D patterns ***(2)***.

2. Materials

2.1. Plasmid DNA

2.1.1. E. coli *Plasmid DNA*

E. coli plasmid DNA prepared by the alkali miniprep method ***(8)*** has quality high enough to be analyzed by 2-D gel electrophoresis. RNA in the preparation may be removed by treatment with DNase-free RNase A.

2.1.2. Saccharomyces cerevisiae *Plasmid DNA*

S. cerevisiae plasmid DNA can be prepared by a procedure described in **Subheading 3.1.**, which requires:

1. Toluene solution: 20 m*M* Tris-HCl, pH 8.0, 95% ethanol, 3% toluene, 10 m*M* EDTA, chilled to –20°C (*see* **step 1** of **Subheading 3.1.** and **Note 1**).
2. Spheroplasting solution: 1*M* sorbitol, 100 m*M* Tris-HCl, pH 8.8, 20 m*M* EDTA, 0.1% β-mercaptoethanol, 1 mg/mL yeast lytic enzyme (ICN) (*see* **Note 2**).
3. TE: 10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA.
4. 10% SDS.
5. 5*M* potassium acetate.

2.2. DNA Topoisomerase

Eukaryotic type I topoisomerase is commonly used to manipulate the linking number of a plasmid (*see* **Note 3**). Vaccinia topoisomerase overexpressed in *E. coli* seems to be the easiest to purify ***(9)***.

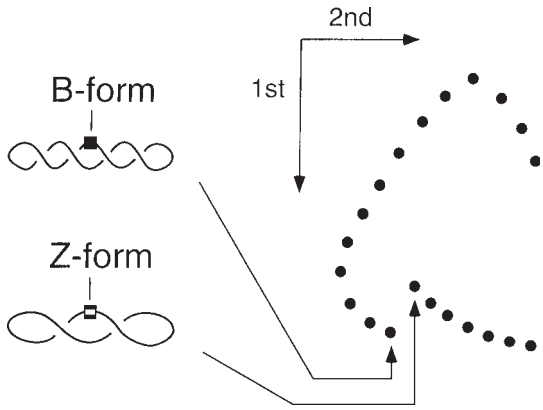

Fig. 2. A schematic representation of the 2-D electrophoretic pattern of a plasmid containing a segment that can convert to the left-handed Z-form. There is a threshold level of negative supercoiling tension for the conversion to occur. The transition to Z-form reduces the twist; therefore, the electrophoretic mobility of the DNA topoisomers, whose supercoil tension is beyond the threshold, as depicted in the left half of the figure. Consequently, in the first dimension, the topoisomers with the Z-form segment overlap other topoisomers with supercoiling tension below the threshold, thus, the segment in the normal B-form. These overlapping populations of topoisomers are separated by the second electrophoresis, in which intercalation reduces the supercoil tension and the Z-form segment assumes the B-form.

2.3. Electrophoresis

2.3.1. Apparatus

Any horizontal gel electrophoresis apparatus can be used, provided that the gel can be securely submerged in the running buffer in either orientation. A square glass plate taped at the edges can be used to cast a gel slab. For good resolution of topoisomers, samples should be loaded into holes of about 2 mm in size, which can be formed with sealed capillaries.

It is convenient to have a specialized set of apparatus, if 2-D gel electrophoresis is conducted routinely. One such set used in our laboratory consists of:

1. A 20-cm square gel-casting tray, otherwise regularly shaped: 250 mL gel solution on this tray makes a gel slab thick enough to be handled with ease.
2. A tank 35 cm long that the 20-cm tray fits in.
3. A comb made of 1.5-mm thick acrylic that has 2-mm wide teeth spaced 6.4 mm in between (*see* **Note 4**).

2.3.2. Solutions

1. 10X TBE: 1*M* Tris-borate, 20 m*M* EDTA.
2. Choloroquine diphosphate stock solution: 10 mg/mL in distilled water, stored in the dark at 4°C.
3. Ethidium bromide stock solution: 10 mg/mL in distilled water, stored in the dark at 4°C.
4. Sample loading buffer: 0.25% bromophenol blue, 0.25% xylene cyanole, 30% glycerol (*see* **Note 5**).

2.3.3. Intercalator Concentration

The following should serve as a guideline for the concentration of an intercalator added to the electrophoresis buffer.

1. For topoisomers with linking numbers close to that of the relaxed state: no intercalator for the first dimension; 2 mg/L chloroquine diphosphate for the second.
2. For topoisomers with linking number deficits around 6% (plasmids isolated from regular *E. coli* strains): 0.6 mg/L chloroquine the first dimension; 3 mg/L for the second.
3. For topoisomerase of even larger linking number deficits: 3 mg/L chloroquine for the first dimension; chloroquine at 30 mg/L or ethidium bromide at 0.5 mg/L for the second dimension.

3. Methods

3.1. Preparation of S. cerevisiae Plasmid DNA

The following describes a procedure to prepare *S. cerevisiae* plasmid DNA by spheroplasting (*see* **Note 6**). This yields sufficient material to be analyzed on several gels for detection by blot hybridization.

1. Pellet approx 10^8 yeast cells. When the topological state of the sample needs to be frozen at the time of harvesting, an equal volume of cold toluene solution is added (*see* **Subheading 2.1.2.** and **Note 1**). The fixed cells can be stored as a suspension at 4°C or at –20°C and then pelleted at the time of plasmid preparation.
2. Resuspend the cells in 1 mL of spheroplasting solution. Transfer the suspension to a microcentrifuge tube.
3. Incubate at 37°C for 15 min. Gently spin down the spheroplasted yeast cells in a microcentrifuge at 2000*g* for 5 min. Pipet out and discard the supernatant, which may be cloudy.
4. Resuspend the spheroplasts in 300 µL of TE. Add 30 µL of 10% SDS. Gently mix the suspension to lyse the cells. Let stand for 5 min at room temperature.
5. Add 200 µL of 5*M* potassium acetate to the lysate and mix well. Spin the mixture in a microcentrifuge at 16,000*g* for 5 min. Transfer the supernatant to a new microcentrifuge tube.

6. Add 1.2 mL of ethanol and mix well. Let stand at room temperature or at –20°C for 10 min, and spin at 16,000*g* for 10 min. A white pellet, mostly nucleic acids and some SDS, should be visible. Carefully discard the liquid and wash the pellet with 70% ethanol. Dry the pellet under reduced pressure.
7. Dissolve the pellet in 100 µL of TE plus DNase-free RNaseA. Let stand for 20–30 min at room temperature. Ethanol-precipitate the DNA. The pellet may be invisible this time. Dry under reduced pressure.
8. Redissolve the DNA in 25–50 µL of TE.

3.2. Generation of Topoisomers of Desired Linking Numbers

A population of topoisomers with a desired range of linking numbers can be prepared by relaxing the DNA by DNA topoisomerase in the presence of ethidium bromide (*see* **Subheading 2.2.** and **Note 3**). The right amount of ethidium has to be empirically found, although the tight binding of the compound to DNA results in an almost stoichiometric linking number deficit. A deficit of approx –1% is attained/1% (w/w) ethidium bromide added to DNA. Termination of relaxation reaction by phenol extraction also removes ethidium. Further extraction by butanol ensures the removal.

3.3. Electrophoresis

What follows is a protocol of 2-D agarose-gel electrophoresis of DNA topoisomers of various linking numbers, based on the practice in our laboratory. Only regular care, as required for 1-D agarose gel electrophoresis, is to be taken. If a more rigorous purpose, such as thermodynamic characterization of structure conversion, is served, the temperature and the buffer conditions have to be carefully controlled. In such cases and those that need a long electrophoresis time of over 24 h, the buffer needs to be circulated between the cathode and the anode buffer chamber.

1. Cast an agarose gel in TBE or 1/2X TBE (*see* **Subheading 2.3.3.** and **Note 8**). The concentration of agarose can be varied according to the size of the DNA of interest: e.g., 1% for 3-kbp rings and 0.7% for 6-kbp rings.
2. Load samples mixed with gel loading solution (*see* **Notes 4 and 5**).
3. Carry out the first electrophoresis. The field strength should not exceed 2 V/cm to attain good resolution. When using a 20 cm square gel, 1.2 V/cm for 18 h has been found to yield excellent results (*see* **Note 5**).
4. Soak the gel in the second electrophoresis buffer with gentle shaking for 1 h (*see* **Subheading 2.3.3.**).
5. Perform the second electrophoresis. The same or a field strength higher than that for the first dimension is applied. The time for the second dimension depends on the required resolution of the particular experiment.

4. Notes

1. To avoid precipitation owing to the low temperature, EDTA is added immediately prior to use.
2. The last two components are to be added immediately before use.
3. Use of eukaryotic type I DNA topoisomerase has two advantages. First, since it relaxes both positive and negative supercoils, highly negatively supercoiled DNA can be obtained by relaxing ethidium-intercalated DNA. This could not be achieved with a bacterial DNA topoisomerase I, which relaxes only negative supercoils. Second, since eukaryotic type I enzyme works without divalent cation, the risk of introducing nicks during relaxation is reduced by inhibiting possibly contaminating nuclease with EDTA.
4. A typical sample volume in a well is 5 μL. This small volume often necessitates blot hybridization for topoisomer detection.
5. Any gel loading solution containing xylene cyanole and bromophenol blue can be used to give density to DNA samples. The given formula is taken as 6X from Sambrook et al. ***(8)***. In a 1% gel, xylene cyanole has roughly the same mobility as 3-kbp DNA rings.
6. Spheroplasting is preferred to disrupting yeast cells mechanically with glass beads. The latter method breaks up chromosomal DNA, and its vast quantity gives a strong diagonal signal even with blot hybridization using a specific probe.
7. Too strong a centrifugal force would break up spheroplasts, which must be avoided at this stage.
8. Agarose can be melted in the intercalator containing buffer. Ethidium and chloroquine are apparently stable under heating by microwave.

Acknowledgments

The authors would like to express gratitude to James C. Wang, in whose laboratory this chapter was prepared, for his support.

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4

Analysis of Altered DNA Structures

Cruciform DNA

Albert J. Courey

1. Introduction

Palindromic DNA sequences have the potential to form branched structures called cruciforms, in which the interstrand base pairs within the symmetric region are replaced with intrastrand base pairs. Cruciforms can sometimes form in vivo ***(1)***, and circumstantial evidence suggests that they may serve functional roles in such processes as transcription ***(1)*** or DNA replication ***(2)***. In addition, the four-way branch at the base of the cruciform is structurally equivalent to the Holliday junction, an intermediate in homologous DNA recombination ***(3,4)***. Thus, an understanding of the thermodynamics and kinetics of cruciform formation may illuminate a number of processes in nucleic acid metabolism.

Cruciforms are intrinsically less stable than the unbranched duplex DNA from which they are derived (**5**,**6**), and measurements of the intrinsic free energy of cruciform formation have yielded values in the range of 17–19 kcal/mol at 25˚C ***(6–9)***. Therefore, cruciform formation does not occur in topologically unconstrained DNA. However, cruciform formation in negatively supercoiled DNA is associated with a favorable change in the superhelical free energy, since the process is accompanied by the unwinding of the two strands. As a result, negative supercoiling stabilizes cruciforms.

Cruciform formation can be monitored in vitro in a number of ways. First, cruciforms can be detected by changes in nuclease sensitivity that accompany the formation of these structures ***(10)***. For example, resolvases (endonucleases involved in the resolution of Holliday junctions) and single-strand specific

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endonucleases specifically recognize and cleave cruciforms. In addition, palindromic sequences that contain restriction sites at the dyad axis will become resistant to cleavage at these sites once the cruciform forms. This latter phenomenon has been used to measure accurately the rate constants associated with cruciform formation as a function of temperature and linking difference ***(6,11)***.

An alternative way to detect cruciform formation is by two-dimensional (2-D) agarose gel electrophoresis of DNA topoisomers containing palindromic sequences ***(6)***, an approach that has also been applied to other DNA structural transitions that are driven by DNA supercoiling, such as Z-DNA formation ***(12)***. Unlike methods involving the use of nucleases, this approach readily allows for the accurate estimation of cruciform stability as a function of linking difference. Thus, analyses of this kind readily yield information about the thermodynamic properties of particular cruciforms.

It is possible to monitor cruciform formation by agarose gel electrophoresis, because for moderately supercoiled DNA, the mobility of a topoisomer in an agarose gel is proportional to the magnitude of its linking difference ***(13,14)***. The linking difference of a topoisomer ($\Delta\alpha$) is the difference between the linking number of the topoisomer (α) and the linking number of the hypothetical relaxed state ($\alpha°$). $\alpha°$ is defined by the equation $\alpha° = N/h°$, where N is the number of interstrand base pairs, and h° is the helical repeat length of DNA in solution (usually about 10.5 bp/turn). Thus, linking difference is given by the equation: $\Delta\alpha = \alpha - N/h°$. When a palindromic sequence within the topoisomer assumes the cruciform conformation, N decreases by the length of the sequence in the cruciform (n). Therefore, $\Delta\alpha$ increases by the amount $n/h°$, and there is a corresponding change in the electrophoretic mobility of the topoisomer.

In 2-D agarose gel electrophoresis, a mixture of topoisomers of a plasmid containing a palindromic sequence is separated by conventional agarose gel electrophoresis. The gel is subsequently soaked in a solution containing an intercalating agent, such as chloroquine, and then rotated 90° for second-dimension electrophoresis. Chloroquine unwinds the DNA (decreases h°), altering the relative mobilities of the topoisomers. Thus, topoisomers that are poorly resolved in the first dimension (e.g., those with $\Delta\alpha \sim +2$ and $\Delta\alpha \sim -2$, under first-dimension electrophoresis conditions) are separated in the second dimension. The decrease in h° also results in a decrease in negative superhelicity and thus in the negative superhelical free energy available to drive cruciform formation. As a result, some or all of the topoisomers that contain the cruciform during first-dimension electrophoresis will lack the cruciform during second-dimension electrophoresis. If enough chloroquine is added to the gel to ensure that none of the topoisomers contain the cruciform during second-dimension electrophoresis, the mobility of the topoisomers in this

dimension will be a continuous function of linking number. On the other hand, the first-dimensional mobility of the topoisomers will exhibit a discontinuity owing to the change in $\Delta\alpha$ that accompanies cruciform formation. The position of the discontinuity gives the critical linking difference at which the cruciform becomes the stable species. In actuality, the transition from noncruciform to cruciform may be spread out over several topoisomers. Thus, the critical linking difference is more precisely defined as the linking difference at which the ratio of cruciform to noncruciform species is one. This critical linking difference can be used to calculate the intrinsic free energy of cruciform formation. Other characteristics of the transition can also be discerned from the mobility, intensity, and shape of the various topoisomer spots ***(6,11)***.

Figure 1 illustrates the technique as applied to plasmid pAC103, a 4400-bp plasmid containing a 68-bp perfect palindrome (**Fig. 1A**) ***(6)***. This plasmid also contains a unique *Eco*RI site at the center of the palindrome. In **Fig. 1B** (left), but not in **Fig. 1B** (right), the mixture of topoisomers was digested with *Eco*RI prior to electrophoresis to linearize the noncruciform species.

The spots numbered +2, +1, 0, –1, –2, and so forth, in **Fig. 1B** (right) are the topoisomers that lacked the cruciform during first-dimension electrophoresis. These numbers represent approximate values of $\Delta\alpha$ under first-dimension electrophoresis conditions. The spots numbered –13C, –14C, –15C, and so forth, are topoisomers in which the palindrome was in the cruciform conformation during first-dimension electrophoresis. That this latter array of spots do indeed represent topoisomers in the cruciform conformation is confirmed by the finding that these spots are completely resistant to *Eco*RI digestion (**Fig. 1B** [left]).

A pair of spots, such as –14 and –14C, which migrated at the same rate during second-dimension electrophoresis, represent a particular topoisomer lacking or containing the cruciform during first-dimension electrophoresis. Note that spot –14C has a first-dimensional mobility midway between that of spots –7 and –8. Thus, the shift in first-dimensional electrophoretic mobility accompanying cruciform formation in pAC103 is equal to the shift in mobility associated with a 6.5 turn change in the linking difference. This is in excellent agreement with the change in linking difference expected when a 68-bp palindrome forms a cruciform (expected change in $\Delta\alpha = n/h^\circ = 68/10.5 = 6.5$).

2. Materials

1. A closed circular plasmid 2000–6000 bp in length containing a palindrome 50–80 bp in length.
2. Ethidium bromide dissolved in water to a concentration of 1 mg/mL and millipore-filtered.
3. 4X topoisomerase I reaction buffer: 80 m*M* Tris-HCl, pH 7.5, 40 m*M* EDTA, 400 m*M* NaCl, 4 m*M* DTT, 100 µg/mL BSA.

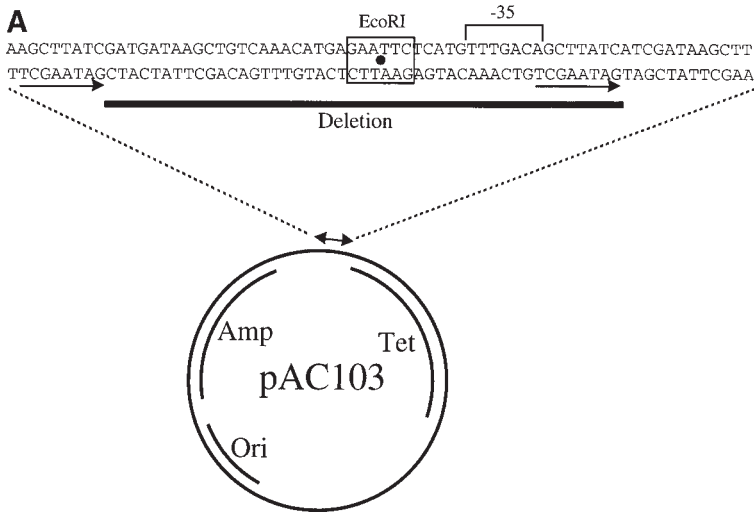

Fig. 1. Analysis of cruciform formation in plasmid pAC103 by 2-D agarose gel electrophoresis. **(A)** Structure of pAC103. This 4400-bp plasmid is a derivative of pBR322 containing a 68-bp palindrome at the 5'-end of the tetracycline resistance gene (Tet). The sequence of the palindrome is shown. The dot represents the dyad axis. The position of the –35 sequence of the Tet promoter is indicated. The thick bar beneath the sequence indicates the region lost in the spontaneous 47-bp deletion event that occurs with high frequency during growth in *E. coli*. The deleted region is flanked by 8-bp direct repeats (arrows). The deletion removes the –35 region inactivating the Tet promoter. Therefore, it is possible to select against the deletion by growing the cells harboring the plasmid in the presence of tetracycline. However, even when the plasmid is maintained in this way, 10–30% of the plasmid DNA isolated from the cells exhibits the deletion. **(B)** 2-D agarose gel electrophoresis was carried out as described in the text using two samples of a pAC103 topoisomer mixture. To induce cruciform formation, the topoisomer mixtures were incubated at 65°C for 30 min in *Eco*RI digestion buffer. Before loading, the samples were incubated for a further 30 min at 37°C in the presence (left) or absence (right) of *Eco*RI. The gel is 0.7% agarose in 0.5X TBE. After first-dimension electrophoresis (2 V/cm, 20 h), the gel was soaked in 1 L of 1.25 mg/L chloroquine. Second-dimension electrophoresis was carried out at 2 V/cm for 16 h. The numbered spots represent various closed circular topoisomers containing (–13C, –14C, and so forth) or lacking (+2, +1, 0, and so on) the cruciform. L indicates the linearized form of the plasmid produced by *Eco*RI digestion. N indicates the nicked circular form of the plasmid generated by spontaneous nicking of the plasmid during sample preparation. Minor spots paralleling and extending the curve traced out by the major noncruciform spots represent a deleted form of the plasmid lacking the palindrome that arises spontaneously during the propagation of the plasmid in *E. coli* (*see* legend to A).

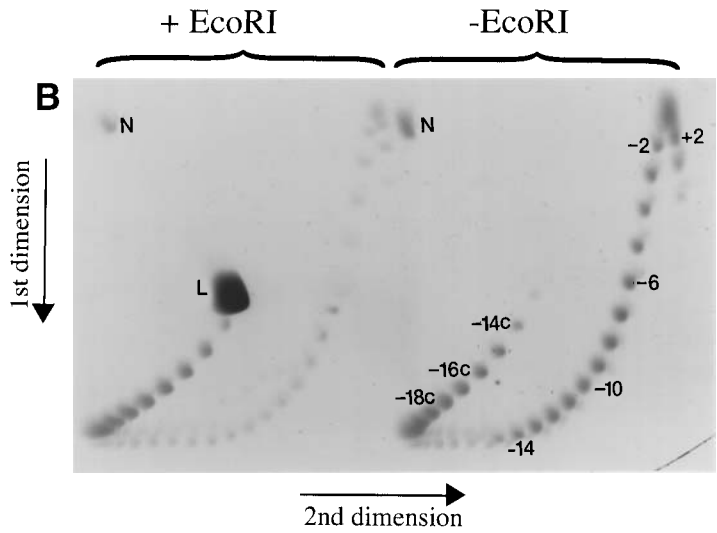

Fig. 1B.

4. Calf thymus topoisomerase I from Gibco/BRL (Grand Island, NY).
5. 0.3*M* sodium acetate.
6. Phenol saturated with 100 m*M* Tris-HCl, pH 7.5.
7. Absolute ethanol.
8. TE: 10 m*M* Tris-HCl, pH 7.9, 0.1 m*M* EDTA.
9. 5X agarose gel loading mixure: 0.25% bromophenol blue, 0.25% xylene cyanol, 15% Ficoll in water.
10. Submarine-style agarose gel electrophoresis chamber that can accommodate a gel at least 20-cm in width. The Gibco/BRL model H4 horizontal gel apparatus should be satisfactory. For this application, the casting tray is replaced with a 20 cm square glass plate. The plate is wrapped with electrical tape to hold the molten agarose during casting. The apparatus should also be equipped with a plastic slot former that will make two 1-mm square slots in the gel separated by about 6 cm. The slot former is suspended above the glass plate during the casting to make two wells along one edge of the plate. Alternatively, one can use a regular analytical gel comb that makes wells 1 mm thick × 3–5 mm wide, although this kind of comb will result in some loss of resolution in the second dimension.
11. Low-voltage electrophoresis power supply.
12. Electrophoresis-grade agarose.
13. 10X TBE: 1*M* Tris, 0.9*M* boric acid, 10 m*M* EDTA.
14. Chloroquine-diphosphate salt dissolved in water to a concentration of 10 mg/mL.

3. Methods

3.1. Construction and Maintenance of Plasmids Containing Palindromes

2-D electrophoresis is useful for studies of cruciform formation in plasmids containing palindromic sequences of at least 50 bp in length. This is because shorter palindromes will generally only adopt the cruciform conformation at superhelicities beyond the range of resolution of the agarose gel.

When designing a palindrome, it is useful to bear in mind that cruciform formation may be an extremely slow process. The rate of cruciform formation appears to be critically related to the sequence around the dyad axis. With pAC103, the relaxation time for cruciform formation near the critical linking difference is on the order of weeks at room temperature and on the order of minutes at 55°C ***(6)***. A variant of pAC103 in which the AT-rich *Eco*RI site at the center of symmetry is replaced with a GC-rich *Sma*I site has a rate of cruciform formation that is at least two orders of magnitude less than that of pAC103 ***(11)***. To ensure that the equilibrium state will be kinetically accessible, design palindromes with AT-rich sequences around the dyad axis.

Long palindromes are frequently lost from plasmids during propagation in *Escherichia coli*. These excisions are usually imprecise and occur via a recA-independent pathway ***(15)***. For example, the 68-bp palindrome in pAC103 is subject to a spontaneous 47-bp deletion. The end points of the deletion are asymmetrically disposed about the center of the palindrome (**Fig. 1A**). The deleted region is flanked by 8-bp direct repeats and the deletion leaves one copy of the direct repeat behind. It seems likely that deletion involves "slippage" during DNA replication that is aided by the formation of the hairpin. The deletion occurs at a relatively high rate. Thus, pAC103 isolated from *E. coli* typically contains about 10–30% of this deletion variant. This deletion occurs even though tetracycline selection was employed to maintain the undeleted plasmid (*see* number 3 below).

The topoisomer mixture of pAC103 used in **Fig. 1** was contaminated by about 20% of the deletion variant. This can be visualized in **Fig. 1B** (right) as a row of minor topoisomer spots just offset from the major noncruciform topoisomers spots. As expected, these minor spots are completely resistant to *Eco*RI digestion (**Fig. 1B** [left]), since the deletion event removes the *Eco*RI site.

To minimize problems associated with spontaneous deletion of palindromes, the following measures are recommended:

1. Limit palindrome length to no more than about 80 bp.
2. Avoid palindromic sequences that contain direct repeats.
3. If possible, design plasmids so that the palindrome can be maintained by positive selection. For example, the pAC103 palindrome overlaps the promoter for the

tetracycline resistance gene (**Fig. 1A**). Deletion of the palindrome results in inactivation of this gene. It might also be possible to design palindromes containing *cis*-regulatory signals essential for the translation of a critical gene or for the replication of the plasmid.
4. Avoid serial passage of cells harboring a palindrome-containing plasmid. In other words, use freshly transformed cells for each plasmid preparation.
5. Use a medium copy number vector (e.g., pBR322) rather than a high copy number vector (e.g., pUC). Spontaneous loss of palindromes is less of a problem in lower copy number plasmids, perhaps because deletion is coupled to DNA replication.
6. Use strain HB101 for propagation of the plasmid. For reasons not understood, this strain was found to yield a higher proportion of intact pAC103 than a variety of other strains tested.

If, despite these precautions, contaminating deletion variants interfere with the analysis of cruciform formation, it is possible to radiolabel the undeleted species specifically as long as the palindrome contains a unique restriction site at the center of symmetry. The plasmid preparation is cleaved at the center of symmetry, dephosphorylated with alkaline phosphatase, end labeled with polynucleotide kinase and γ^{32}P-ATP, and recircularized with DNA ligase. After gel electrophoresis, the radiolabeled topoisomer species are visualized and readily quantified by autoradiography.

3.2. Preparation of Topoisomer Distributions

Prior to analysis of a palindrome-containing plasmid by 2-D agarose gel electrophoresis, it is necessary to prepare a mixture of topoisomers ranging in specific linking difference from about 0 to about –0.05 (specific linking difference = $\Delta\alpha/\alpha°$). This is most conveniently accomplished by preparing a series of topoisomer distributions that evenly cover this range, and then mixing together equal amounts of each distribution. Topoisomer distributions with different average linking differences are prepared by relaxing plasmid DNA with topoisomerase I in the presence of various amounts of an unwinding agent, such as ethidium bromide (*see* **Note 1**).

1. Prepare a series of six mixtures containing 15 µg supercoiled plasmid DNA, 25 µL 4X topoisomerase I reaction buffer, and 0, 5, 10, 15, 20, or 25 µL of a 24 µg/mL solution of ethidium bromide (diluted from a 1 mg/mL stock). Add water to bring the volume of each mixture to 100 µL. Add 10 U of calf thymus topoisomerase I.
2. Incubate mixtures at 37°C for 2 h.
3. Stop reactions by diluting to 400 µL with 0.3*M* sodium acetate and then extracting twice with equal volumes of buffer-saturated phenol.
4. Add 1 mL of ethanol. Chill for 5 min on ice. Pellet precipitated DNA by spinning in a microcentrifuge for 10 min. Carefully decant and discard supernatant.

Resuspend pellet in 400 µL 0.3*M* sodium acetate. Reprecipitate as above with ethanol. Carefully wash pellet with 1 mL 75% ethanol. Dry pellet and resuspend in 90 µL TE (*see* **Note 2**).

5. Analyze 300 ng of each mixture by conventional agarose gel electrophoresis (**Fig. 2**) to confirm that you have generated a series of overlapping topoisomer distributions. This procedure should generate a set of topoisomer distributions with average specific linking differences of approx 0, –0.01 ... –0.05. This assumes that binding of ethidium bromide is quantitative under the relaxation conditions and that the unwinding angle of ethidium bromide is 26°. If you wish to determine more accurately the average linking difference of each distribution, this can be done by electrophoresing the distributions into a series of gels containing different amounts of an unwinding agent (e.g., chloroquine) and counting the number of topoisomer bands separating the centers of the distributions ***(14)***.

3.3. Final Sample Preparation

1. Mix together 1.8 µL of each of the six topoisomer distributions prepared as described in **Subheading 3.2.** Add 1.2 µL of 5X TBE. If you plan to digest the DNA with a restriction endonuclease prior to electrophoresis (for example, to determine the sensitivity of the various species to a restriction endonuclease that cleaves at the dyad axis), replace 1.2 µL of 5X TBE with 1.2 µL of the appropriate 10X restriction buffer.
2. Incubate the sample at a temperature that will induce cruciform formation. For most palindromes, 65°C for 30 min should be sufficient (*see* **Subheading 3.1.** for an exception) (*see* **Note 3**).
3. If desired, add 5 U of an appropriate restriction enzyme and digest for 30 min.
4. Add 3 µL of 5X agarose gel loading mixture.

3.4. Two-Dimensional Agarose Gel Electrophoresis

1. Prepare 200 mL of molten 0.7–1.1% agarose in 0.5X TBE. Use all 200 mL to pour a 20 cm × 20 cm slab gel on a glass plate with a slot former designed to create 1-mm square wells; 0.7 and 1.1% agarose have both been used successfully for ~4400-bp plasmids. Lower percentage agarose is more forgiving of overloading or of high salt concentrations in the sample, both of which can result in smearing and loss of resolution. However, higher percentage agarose can resolve topoisomers to somewhat higher levels of superhelicity, if used with care.
2. After the gel has completely cooled, remove the slot former, place the gel in a submarine-style electrophoresis chamber, and submerge in 0.5X TBE.
3. Carefully load 6 µL of a topoisomer mixture prepared as described in **Subheading 3.3.**
4. Carry out first-dimension electrophoresis at about 2 V/cm. If desired, the electrical field can be increased to 4 V/cm after the first few hours. The total time of electrophoresis depends on the agarose concentration and the size of the plasmid. With 4-kb plasmids and 0.7% agarose, it is generally necessary to electrophorese for about 20 h at 2 V/cm. The optimal time of electrophoresis can be empirically

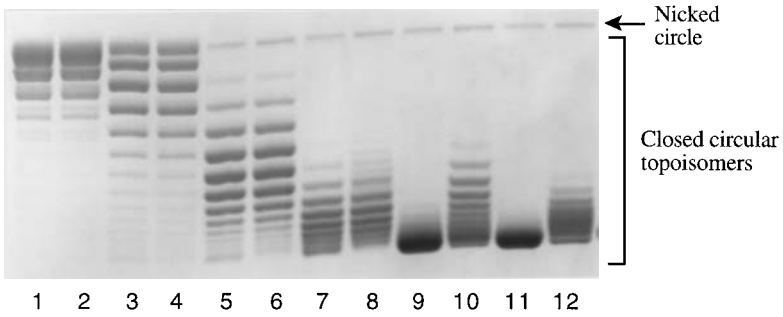

Fig. 2. Analysis of topoisomer distributions by one-dimensional electrophoresis. Topoisomer distributions of pAC103 with approximate average specific linking differences of 0 (lanes 1 and 2), –0.01 (lanes 3 and 4), –0.02 (lanes 5 and 6), –0.03 (lanes 7 and 8), –0.04 (lanes 9 and 10), and –0.05 (lanes 11 and 12) were prepared as described in the text and analyzed by electrophoresis in a 0.7% agarose gel. Electrophoresis was at 2 V/cm for 16 h. Before electrophoresis, half the samples (lanes 2, 4, 6, 8, 10, and 12) were incubated at 65°C for 30 min to induce cruciform formation in topoisomers with sufficient levels of negative superhelicity. Cruciform formation is manifested by a shift up in the topoisomer distribution of the heated samples compared to the unheated samples. This is most readily evident in lanes 10 and 12.

determined by measuring the rate at which a highly supercoiled form of the plasmid migrates through a normal one-dimensional (1-D) agarose gel. To obtain optimal separation, one should run the 2-D gel long enough to run the highly supercoiled plasmid to within a few centimeters of the bottom of the gel.

5. Carefully slide the gel off the plate into a clean Pyrex or plastic tray, and soak in 1 L of 0.5X TBE containing 1.25 mg of chloroquine for 6 h. Lower concentrations of chloroquine (down to about 0.25 mg/L) can also be used, resulting in different-shaped curves being traced out by the topoisomer spots—for an example, *see* **ref. *11*.**
6. Place the gel back onto the glass plate and then back into the electrophoresis chamber. The gel should be rotated 90° relative to its orientation during first-dimension electrophoresis. Submerge the gel in the same buffer used in **step 5**. Carry out second-dimension electrophoresis at about 2–4 V/cm. The optimal time for second-dimension electrophoresis is generally about 25% less than the optimal time for first-dimension electrophoresis.
7. Slide the gel back into the Pyrex or plastic tray, and soak for at least 1 h in water to remove most of the chloroquine. Stain for about 1 h in 0.6 µg/mL ethidium bromide. Destain for about 1 h with water. Photograph gel with UV transillumination.

4. Notes

1. As suggested in **Subheading 3.1.**, it is possible to label specifically the palindrome containing species by linearizing the plasmid at the center of the palin-

drome, end labeling, then recircularizing with DNA ligase. If this approach is being utilized, the different topoisomer distributions can be generated at the religation step by dividing the labeled DNA into multiple aliquots and carrying out the ligations in the presence of different concentrations of ethidium bromide.

2. The series of extractions and precipitations described in **steps 3** and **4** of **Subheading 3.2.** are necessary to remove quantitatively both the enzyme and the ethidium bromide. Quantitative removal of the ethidium bromide can also be achieved by two phenol extractions followed by overnight dialysis against TE containing 2*M* NaCl. This is followed by dialysis against TE.
3. As discussed in **Subheading 3.1.**, the relaxation time for cruciform formation at room temperature (and thus during electrophoresis) is frequently much greater than the time of electrophoresis. In instances where this is true, 2-D electrophoresis actually reveals the equilibrium distribution of cruciform and noncruciform species under the incubation condition used to induce cruciform formation prior to loading the gel. The average helical twist angle of the double helix (and hence h°) is a function of both temperature and salt concentration. As a result, $\Delta\alpha$ for any given topoisomer will usually be different under the electrophoresis conditions from what it was under the incubation conditions. Before using the results of a 2-D electrophoresis experiment to calculate thermodynamic parameters associated with cruciform formation, it is important to understand exactly how changes in conditions affect $\Delta\alpha$. Fortunately, the effects of temperature and salt on $\Delta\alpha$, which are largely independent of one another, can both be accurately determined.

   To correct for temperature, all one needs to do is recognize that helical twist angle is a linear function of temperature over a wide range of temperatures. Every one-degree increase in the temperature decreases the helical twist angle by 0.012° ***(13)***. Thus, the change in $\Delta\alpha$ that occurs on changing the temperature from T_1 to T_2 is given by the expression

$$\frac{0.012N}{360}\ (T_2 - T_1) = \frac{N}{3 \text{ X } 10^4}\ (T_2 - T_1).$$

   If the incubation is carried out in 0.5X TBE, no salt correction is required. However, if the incubation is carried out in restriction buffer, a salt correction is necessary. To determine the proper correction, one can relax the plasmid in question under the incubation conditions using calf thymus topoisomerase I (which is active in a variety of buffers and at a variety of salt concentrations). The average linking difference under the electrophoresis conditions of the DNA relaxed in this way is then determined by 1-D agarose gel electrophoresis ***(14)***.

   As an example, the critical linking difference for the experiment shown in **Fig. 1** can be determined. In this experiment, the incubation conditions prior to electrophoresis were 65°C in *Eco*RI digestion buffer (100 m*M* Tris-HCl, pH 7.5, 50 m*M* NaCl, 10 m*M* $MgCl_2$). In **Fig. 1B**, we can see that the topoisomer that is

present as a roughly equal mixture of cruciform and noncruciform species has a $\Delta\alpha$ of about –14 under first-dimension electrophoresis conditions. Using the temperature correction expression given above reveals that a change in temperature from the electrophoresis temperature (21°C) to 65°C results in a +6.5 turn change in $\Delta\alpha$. To correct for the change in buffer, pAC103 was relaxed in *Eco*RI digestion buffer at 21°C. When the resulting topoisomer distribution was subjected to 1-D agarose gel electrophoresis in 0.5X TBE, it was found that the average linking difference under electrophoresis conditions was +3.5. Thus, transfer from the electrophoresis buffer to *Eco*RI digestion buffer results in a –3.5 turn change in $\Delta\alpha$. Consequently, the topoisomer that is present as an equal mixture of cruciform and noncruciform species had a $\Delta\alpha$ under the incubation conditions of $-14 + 6.5 - 3.5 = -11$. This is the critical linking difference for pAC103. Using this value, one can readily show that the intrinsic free energy of cruciform formation for the 68-bp palindrome in this plasmid is 17 kcal/mol ***(6)***.

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5

Purification of Supercoiled Plasmid DNA

Kelly L. Jordan, Devon L. Evans, and David J. Hall

1. Introduction

The topology of DNA affects a number of major biological processes ***(1–4)***. For example, processive enzymes, such as the RNA and DNA polymerases, have the capability of generating both positive and negative DNA supercoils during the process of transcription and replication, respectively. These supercoils must be relaxed in order for transcription and replication to continue unaffected, as outlined in preceding chapters. The DNA topoisomerases play the central role in relaxing this supercoiling ***(1–4)***.

The study of DNA topoisomerases and DNA topology relies essentially on a two-part system: the DNA topoisomerase and the molecule of DNA. The DNA topoisomerases function as enzymes because they efficiently form covalent complexes with DNA through an active site tyrosine ***(1–4)***. The enzymes then relax supercoiled DNA. The mechanism of DNA relaxation differs depending on the type of DNA topoisomerase examined. The DNA topoisomerase then religates the cleaved strand and dissociates from the DNA. The substrate of interest in this enzymatic process is DNA, specifically supercoiled DNA. Although the DNA topoisomerases can bind to single-stranded DNA and to relaxed duplex DNA ***(2,3)***, the primary interest here is in their association with supercoiled duplex DNA, the enzymes' major substrate.

A primary source of DNA for relaxation studies is the small mol-wt multicopy double-stranded DNA plasmids (e.g., pUC-derived) ***(5,6)***. These plasmids can be amplified to high levels in *E. coli* and can be easily isolated. A very important aspect of these plasmids is that they are isolated as supercoiled molecules, and in particular, they are negatively supercoiled. It is generally considered that plasmid DNA is negatively supercoiled because of the action

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of bacterial gyrase, an *Escherichia coli* DNA topoisomerase type II enzyme that generates negative supercoils in DNA ***(2)***. For much of the work performed on DNA topoisomerases, these multicopy plasmids provide a convenient source for substrate, particularly for experiments with eukaryotic DNA topoisomerase I and II and bacterial DNA topoisomerase I, all of which can relax negatively supercoiled DNA. For particular studies in which positively supercoiled DNA is needed, it is necessary to isolate the plasmids from very specific mutant strains of bacteria usually in the presence of specific drugs that bind DNA and alter its superhelicity. This latter approach is rather involved and will not be discussed here. Instead, we will focus on the isolation methods of negatively supercoiled plasmid DNA.

2. Materials

1. Materials for growth of bacteria: yeast extract, bacto-tryptone, NaCl, antibiotics (e.g., ampicillin, chloramphenicol, kanamycin, tetracycline), 1–2 L flask, and environmental shakers (37°C; New Brunswick; Scientific, Hatsfield, UK).
2. Plasmids: multicopy, pUC-derived plasmids.
3. Chemicals for alkaline lysis: TE: 10 m*M* Tris-HCl, pH 8, 1 m*M* EDTA.
4. GTE: 50 m*M* glucose, 25 m*M* Tris-HCl, pH 7.5, 10 m*M* EDTA.
5. Sodium dodecyl sulfate (SDS) NaOH solution: 1% SDS/0.2 *M* NaOH.
6. High salt solution: 29.4 g potassium acetate, 5 mL of 100% formic acid; add water to 100 mL.
7. Chemicals for CsCl ultracentrifugation: ultrapure CsCl, ethidium bromide (10 mg/mL, Sigma), *n*-butanol (water- or TE-saturated; Fisher; Scientific, Pittsburgh, PA).
8. Centrifugation: Superspeed and ultracentrifuges (e.g., J2-21 and Optima, Beckman, Fullerton, CA). Rotors: J6, JA10, JA14, VTi65.1. Tubes: polyallomer.
9. Dialysis tubing: Spectrapore (Fisher).
10. Agarose-gel electrophoresis: Agarose (Gibco/BRL, Gaithersburg, MD), TBE: 89 m*M* Tris-borate, pH 8.0, 2 m*M* EDTA.

3. Methods

A number of methods have been previously published for plasmid purification ***(5,6)***. Here we will only focus in detail on one that seems to be the simplest, cheapest, and produces the highest yield, with comments on the effect of the isolation on the topological state of the plasmid DNA. Some methods will be mentioned as alternatives. The methods outlined below deal with large-scale preparations, with production of plasmid DNA to a high level of purity. "Miniprep" methods, although certainly enabling one to isolate plasmid DNA rapidly, usually result in a fair level of contaminating protein and RNA, which are undesirable in a detailed analysis of DNA topology.

Table 1. Bacterial Lysis and Purification Methods

Bacterial lysis	Purification
Alkaline lysis	CsCl centrifugation
Boiling method	Polyethylene glycol precipitation
Triton/detergent lysis	Qiagen[R]

Large-scale preparations of plasmid DNA usually begin with a 250–500 mL culture of *E. coli* transformed with an appropriate plasmid in a 1–2 L flask, respectively. The cells are grown at 37°C to stationary phase (usually overnight) in the presence of an appropriate antibiotic, such as ampicillin, chloramphenicol, tetracycline, or kanamycin, with vigorous shaking (300 rpm on a rotary shaking platform) to achieve the high level of aeration needed for optimal growth. At this point, the investigator must decide on the form of bacterial lysis and plasmid purification. Listed in **Table 1** are the most common forms of lysis and purification. Consult **refs.** ***(5)*** and ***(6)*** for more details on the other methods.

With regard to lysis of the bacteria, three general methods are shown in **Table 1**. The boiling method of bacterial lysis is a very fast and simple technique, but it results in a high level of contaminating protein and RNA. As with the miniprep method, this may be unsuitable for studies in DNA topology ***(5,6)***. The triton/detergent lysis method is much gentler than the other methods and should not result in denaturation of plasmid DNA ***(5,6)***. It is often used for isolation of very large plasmids (e.g., cosmids). The alkaline lysis method is most commonly used and will be discussed at length below.

In terms of purification of the DNA, three methods are also shown in **Table 1.** The CsCl centrifugation protocol will be discussed at length below in **Subheading 3.2.** A commercially available kit by Qiagen is currently used by many investigators for plasmid purification. The kit makes use of a resin that specifically binds duplex DNA. The advantages of this procedure are that the quality of the DNA is quite good, with little contamination by bacterial RNA or proteins. In addition, the entire time from beginning of lysis (alkaline lysis) to isolation of plasmid DNA is just a few hours and does not require a DNA intercalator, such as ethidium bromide, for detection of DNA or phenol for elimination of proteins. The disadvantages are that it is rather costly, and the plasmid yield can be much lower than that of alkaline lysis/CsCl centrifugation. Finally, the polyethylene glycol (PEG) method for plasmid purification is very rapid and easy to perform. However, it does require phenol extractions. One must be very careful with phenol, since it is a severe protein denaturant. Additionally, the oxidation products of phenol can cleave DNA, so phenol

extractions can have an adverse effect on DNA topology. Finally, the purity of the plasmid DNA can be variable using the PEG method, since traces of the PEG may remain after a final ethanol precipitation.

3.1. Alkaline Lysis

Alkaline lysis is the most common method of bacterial lysis. This procedure is divided into three steps. First, the bacterial cell wall is digested with lysozyme in an isotonic solution. Next, the cells are lysed in a solution of sodium dodecyl sulfate and sodium hydroxide (SDS/NaOH). Finally, proteins and chromosomal DNA are precipitated with acidic potassium acetate, and the precipitate is removed by centrifugation. The essential point of this entire procedure is that chromosmal DNA is readily denatured by the SDS/NaOH, whereas the plasmid DNA is less susceptible to denaturation, likely owing to the high level of negative supercoiling. Following addition of the acetate solution, the chromosomal DNA fails to renature, yet the plasmid DNA is duplex and supercoiled, allowing easy purification. Using the alkaline lysis/CsCl centrifugation method, hundreds of micrograms of plasmid DNA can be isolated from a 500-mL culture of bacteria.

1. Pellet bacteria from saturated culture (described above) in 0.5- or 1-L plastic bottles at 5000–6000*g* for 10 min at 4°C (J2-21 or J6 centrifuge, Beckman, JA10 or J6 rotors, respectively). Discard supernatent (treat with wescadyne or chlorox first). All remaining procedures should be performed on ice.
2. Resuspend pellet by vortexing in 10 mL of GTE. After resuspension of bacterial pellet, add 40 mg of solid lysozyme (to 4 mg/mL). Swirl tube gently to resuspend lysozyme. Let sit on ice for 5 min, and then transfer solution to a smaller 250-mL bottle.
3. Add 20 mL of SDS/NaOH solution. **Slowly and carefully** invert the bottle five to eight times. The solution should become very viscous and relatively uniform in color (clear with a brown tint) as the bacteria lyse. Be careful not to shear the chromosomal DNA. Let sit on ice for 5 min. It is important that you do not let the solution sit on ice for too long (longer than 5 min), because the plasmid DNA may begin to denature irreversibly (*see* **Note 3**).
4. Add 15 mL of high salt solution. Invert slowly until white precipitate begins to form. Invert more rapidly until brown bacterial DNA and protein are converted to white precipitate. Let sit on ice for 5 min. Pellet white precipitate by centrifugation at 10,000–15,000*g* for 10 min at 4°C (J2-21 centrifuge, JA14 rotor, Beckman). Remove the supernatent containing the plasmid DNA to a fresh 250-mL bottle to it add 2 vol of ethanol, and precipitate the DNA for at least 1 h at –20°C.
5. Pellet the nucleic acid by centrifugation at 12,000–15,000*g* at 4°C for 10 min (J2-21 centrifuge, JA14 rotor, Beckman). The resulting pellet should be very large and will consist mostly of bacterial tRNAs along with the plasmid DNA. Resus-

pend the pellet in water or TE. The volume needed for resuspension will depend of the type of purification of plasmid DNA that you will perform next but will usually be on the order of 5–11 mL.

3.2. CsCl Ultracentrifugation

Density gradient ultracentrifugation is probably the most standard way to purify plasmid DNA. For ultracentrifugation, the fixed-angle, vertical, or near-vertical rotors (Sorvall or Beckman) hold tubes of a defined volume, either 5 or 13 mL. The most rapid centrifuge runs are performed with vertical rotors, because the gradients can be generated in a very short period of time (few hours).

1. Resuspend the large pellet of nucleic acid, from **Subheading 3.1., step 5,** in 11 mL of sterile water or TE and place in a 15-mL conical tube. Measure the volume exactly (total volume should now be roughly 12 mL).
2. Add 1 g of solid CsCl for every mL of solution (e.g., if total is 12 mL then add 12 g of CsCl). Invert or vortex tubes to force the CsCl into solution. The resulting volume should now be at approx 13.5 mL.
3. Add 80 µL of ethidium bromide (10 mg/mL) to the tube. Ethidium bromide will intercalate into the nucleic acid, enabling you to detect the plasmid DNA following the centrifugation. Procedures elsewhere usually call for the addition of significantly more ethidium bromide (up to 0.5 mL) ***(5,6)***. However, we have found that a reduction in the amount of dye used still enables one to detect the DNA easily, yet is less of a health and disposal hazard.
4. The solution is added to a polyallomer "sealable" ultracentrifuge tube, capable of holding 13 mL. The centrifuge tubes have a narrow neck so that they can be heat-sealed. Place a Pasteur pipet in the opening as a funnel. Then pipet the plasmid:CsCl solution into the tube.
5. Weigh the tubes and adjust volumes so that weights are equal. Using a heat sealer, seal the tube.
6. Place it in an appropriate rotor, fixed-angle, near-vertical or vertical, and begin the centrifuge run. Before starting the centrifuge run, always make sure that the form-fitting metal caps are placed over the centrifuge tubes, then secure the tubes and caps with additional screw-on caps. For a vertical rotor (e.g., VTi65.1), choose a speed of approx 350,000*g* for 4–5 h. At this speed, the CsCl density gradient will rapidly set up, causing the plasmid DNA to band at the appropriate density. A near-vertical or fixed-angle rotor will require a longer centrifuge run, approx 12 h for near-vertical and 24 h for a fixed-angle rotor, usually at ~300,000*g*.
7. Stop the centrifuge run, and withdraw tubes from the rotor carefully, so as not to disturb the gradient. With the low concentration of ethidium bromide used, the plasmid DNA should be evident as a single band located approximately one-third of the distance from the bottom of the tube. A UV lamp should not be needed for detection. Little, if any, chromosomal DNA should be present in the preparation,

so additional bands should not be seen. If a vertical rotor is used in the centrifugation, an intense line of stained material will coat one wall of the centrifuge tube. This will be bacterial protein/RNA (tRNA), and should be avoided. If near-vertical or fixed-angle rotors are used, the intensely stained material at the bottom of the tube is bacterial protein/RNA.

8. To extract the DNA, place the tube in a rack and puncture the very top of the tube with a needle to allow air to enter the tube when the DNA is extracted. Then recover the plasmid DNA using a 3-cc syringe with a 20-gage needle. Place the needle about 1 cm below the plasmid band, and by rotating the needle (with syringe), slowly puncture the tube, being careful not to press too hard so as not to poke through the other side of the tube. It may help to practice on an empty tube. With the needle tip now inside the tube, position the tip into the band of plasmid DNA. Then, using the syringe, slowly withdraw the plasmid DNA until there is no remaining band in the tube. The volume should be equivalent to 2–3 mL. Remove the needle, and the transfer the plasmid DNA in the syringe to a 15-mL conical. Be sure to have a waste beaker handy to discard the centrifuge tube containing the remaining CsCl solution.
9. To obtain highly pure DNA that is free of RNA contamination, the sample can be easily centrifuged again (*see* **Note 1**).

3.3. Elimination of Ethidium Bromide

1. To remove the ethidium bromide from the DNA, a double extraction with *n*-butanol is performed. *n*-Butanol, equilibrated with water or TE, is not miscible in aqueous solutions and therefore forms an upper layer. Two to 3 vol of *n*-butanol (water- or TE-equilibrated) are added to the DNA-CsCl solution, which is then vortexed vigorously two times for 5–10 s.
2. The butanol and aqueous phases are allowed to separate on the bench top for a few minutes. The ethidium bromide should now have transferred to the upper butanol layer, which is then removed by pipeting and discarded appropriately.
3. The extraction is repeated one more time, then the lower DNA-CsCl solution is either dialyzed overnight against TE (against 100–200 vol of TE) to remove the CsCl, or it is diluted threefold and ethanol-precipitated directly (2 vol of ethanol are added plus NaAcetate is added to 0.1*M* final). If the plasmid is dialyzed, it usually results in a rather dilute DNA solution. It can be concentrated by ethanol precipitation.
4. Additionally, application of the ethidium bromide-stained DNA to a Dowex AG50W-X8 column will both remove the ethidium bromide and dilute the DNA enough for ethanol precipitation ***(5)***. A problem with ethanol precipitation of plasmid DNA containing CsCl is that the CsCl may precipitate out if the solutions are not diluted enough or are cooled below –20°C. Thus, it may be necessary to perform multiple precipitations or to dialyze the DNA to remove the CsCl completely.
5. Proper storage of the plasmid DNA is essential to maintain the DNA in a supercoiled state (*see* **Note 2**).

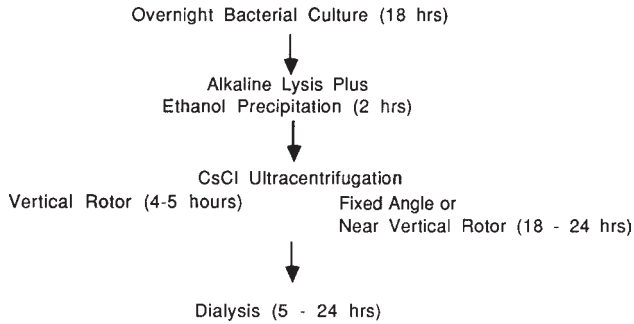

Fig. 1. Steps and time required for supercoiled plasmid isolation. Using the alkaline lysis/CsCl ultracentrifugation purification method, the above scheme shows the approximate time needed for complete isolation and purification of supercoiled plasmid DNA. Depending on some of the steps chosen, the time can vary from 29 to about 68 h.

A flowchart depicting the length of time for each step from growth of bacteria through dialysis is outlined in **Fig. 1**.

3.4. Analysis of Supercoiled Plasmid DNA Purified by Alkaline Lysis/CsCl Ultracentrifugation

1. To assay the supercoiled state of the plasmid DNA, purifed by the method above, one-dimensional (1-D) gel electrophoresis was then performed. One microgram of plasmid DNA, from the method above, was loaded onto a 1% agarose gel buffered with TBE and electrophoresed.
2. The electrophoresis run was stopped, and the gel was soaked in a dilute solution of ethidium bromide (0.25 µg/mL) to stain the DNA.
3. The gel was then exposed to short-wave UV light on a UV light box with appropriate Polaroid camera setup. Shown in **Fig. 2** is a photograph of the gel following exposure of the gel to the UV light. Indicated in the first lane on the left is the plasmid DNA immediately following purification by the above protocol. The intense fastest migrating band is negatively supercoiled DNA (SC), whereas the less intense slower migrating band is a negatively supercoiled "Dimer" of the plasmid DNA (two molecules linked). In the middle two lanes are shown the same DNA as in the first lane only following multiple freeze–thaw cycles. The additional faint band observed is nicked DNA, resulting from a break in one DNA stand, thereby allowing the DNA to relax, but remain circular. Supercoiled and nicked circular DNA have also been referred to as Form I and Form II DNA, respectively ***(5,6)***. Finally, incubation of the plasmid DNA with purified DNA topoisomerase I, capable of relaxing both negative and positive supercoiled DNA, results in a complete loss of the most supercoiled form of the DNA and generation of DNA topoisomers (lane of the right). The enzyme efficiently relaxes

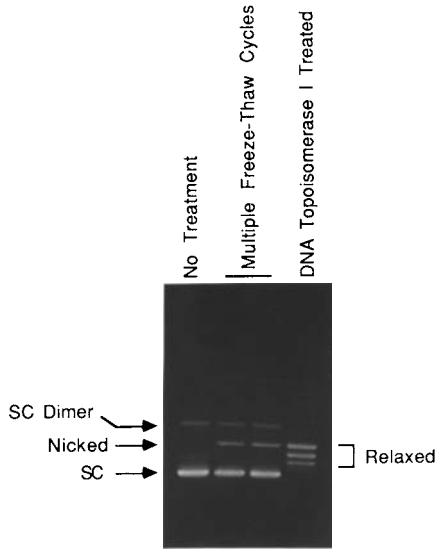

Fig. 2. Agarose-gel electrophoresis of supercoiled plasmid DNA. One microgram of plasmid DNA was electrophoresed on a 1% agarose/TBE gel. The gel was stained with ethidium bromide and photographed. The first lane on the left shows freshly isolated plasmid DNA. The two center lanes show plasmid DNA that have been subjected to multiple freeze–thaw cycles. The lane on the right shows plasmid DNA treated with purified DNA topoisomerase I prior to electrophoresis. The positions of supercoiled (SC), relaxed, and nicked DNA are shown. Also shown is the position of a supercoiled dimer of plasmid DNA (SC Dimer) (two supercoiled plasmids that are linked).

the supercoiled DNA, and as a result, the plasmid DNA migrates more slowly in the electrophoretic field, with the different topoisomers seen as distinct bands. The supercoiled dimer is also relaxed by the enzyme, and although it is difficult to see in **Fig. 2**, a pattern of very slowly migrating topoisomers of the dimer is also present in the gel. Thus, as can be seen by the analysis here, the alkaline lysis/CsCl ultracentrifugation procedure results in a purification of supercoiled plasmid DNA with no detectable contaminants.

4. Notes

1. It seems that all methods of DNA purification, including the CsCl method, can result in some low level of bacterial RNA contamination. To eliminate any detectable RNA, simply repeat the purification procedure. For example, remove the plasmid DNA from the centrifuge tube following the CsCl spin (about 2–3 mL), and simply add fresh CsCl solution (remember add 1 g of CsCl for every mL of water or TE) to bring the solution to about 13 mL. Then repeat centrifugation and process as described above.

2. DNA can be cleaved by UV light and by multiple freeze–thaw cycles (two common mechanisms). To prevent this, it is often best to freeze aliquots of the plasmid DNA in a nonfrost-free freezer, so that a single sample is protected from light and does not undergo multiple freeze–thaw cycles.
3. During the alkaline lysis procedure, it is important that you do not let the solution of sodium hydroxide/SDS stay in contact with the DNA for longer than 5 min because the plasmid DNA may begin to denature irreversibly.

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6

Purification and Use of DNA Minicircles with Different Linking Numbers

Giorgio Camilloni, Rodolfo Negri, Micaela Caserta, and Ernesto Di Mauro

1. Introduction

The structural organization of both prokaryotic and eukaryotic chromosomes has evolved following a common principle: the need for storing the genetic information in topologically independent domains consisting of one or more genes and of all the elements required in cis for their functioning. The elements that belong to the same functional unit are topologically linked: they depend on each other and cooperate.

Minimization of the reciprocal influence of adjacent sequences ***(1–6)*** requires that the structure and the role of each individual element be analyzed singly, possibly in DNA "microdomains." Different procedures can be followed to obtain small-sized circular DNA domains with various superhelical densities (topoisomers): ligation of a DNA fragment in the presence of varying concentrations of an intercalating agent, in different physicochemical conditions, or in the presence of proteins that untwist, overtwist, bend, or writhe the DNA with or without coupling this reaction with a DNA topoisomerase. These procedures can yield both positively and negatively supercoiled DNA circles, whose size goes from a minimum of about 0.1 kb to an undefined upper limit. Individual topoisomers sized between 0.1 and 2 kb can be easily isolated from gels and analyzed.

This chapter describes methods for the generation and the purification of DNA minicircles characterized by different linking numbers. Several uses can be envisaged for this particular form of genetic material, all based on in vitro assays.

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1.1. Analysis of Sequence-Dependent DNA Structural Alterations

The majority of unusual DNA structures *(7)* are stabilized by negative supercoiling. Since the superhelix density that can be reached in a small DNA ring is high, this system provides the means to study the induction of structural transitions and their topological consequences *(8)*. In addition to the well-defined *(7)* unusual DNA structures, a great variety of sequence-dependent DNA deformations exist that can be observed and studied both by chemical and enzymatic probes *(9–11)* on changing the DNA linking number.

1.2. Analysis of Sequence-Dependent DNA Curvature

When d(A) stretches or other defined sequences are repetitively and consistently present at a distance of approx 10 bp along a DNA sequence, the axis of the double helix bends. The extent of the curvature is determined by the sequence itself and can be evaluated biochemically by a circular permutation assay (*see* Chapter 14). A different method for the analysis of intrinsic DNA bends is provided by the circularization of small DNA fragments followed by DNaseI treatment *(12)*. Since this enzyme is a sensor for the DNA minor groove width, it provides a description of the rotational orientation of each part of the minicircle. If a DNA fragment is already curved in its linear form, its circularization will cause the molecule to adopt a highly preferred configuration *(12,13)*. In the absence of intrinsic bending, circularization of a small DNA fragment leads to a random orientation of the DNA duplex around its axis. In this case, binding of a DNA-bending protein fixes the rotational orientation of the minicircle *(13)*.

1.3. Analysis of Protein-Induced DNA Bending and Supercoiling

When biophysical methods cannot be applied, the ability of a protein to mediate curvature in double-stranded DNA can be tested biochemically by the circular permutation assay *(14,15)* or alternatively, by examining the effect of the protein on the DNA ligase-dependent cyclization of very short DNA fragments. This was shown for the eubacterial histone-like proteins *(16)* as well as for some HMG domain proteins *(17,18)*. In general, random-sequence DNA fragments shorter than 126 bp do not cyclize in the absence of proteins that introduce bends into the DNA duplex. Some of the proteins mentioned above introduce negative supercoiling in the DNA, indicating that they alter not only the writhing (by bending), but also the twisting component of the molecule (by unwinding). These effects can be monitored by producing DNA minicircles in the presence of increasing amounts of a certain protein: the greater the amount of the protein, the greater the extent of negative supercoiling that is introduced.

1.4. Analysis of DNA Conformation-Dependent Protein Binding

The information contained in a given segment of DNA is dictated by its sequence and by its topological status. The defined structure that characterizes each given sequence in the conditions of minimal energy (typically the B-structure observed in fragmented DNA) changes as a function of the overall topology of the closed system of which the sequence is part. Distinction between the two contributions (by the sequence and by its topology) to the actual structure of a given DNA tract is difficult, since the two properties behave as a whole.

By varying the linking number of a small DNA domain containing a defined sequence element, the rules governing the interaction of that element with proteins can be investigated. Different types of proteins exist: those that bind to specific nucleotide sequences utilizing major groove interactions ***(19)***, and those that show structural preference for supercoiled and/or bent DNA ***(20–24)*** and usually interact with the DNA minor groove ***(25)***. The very existence of multiple classes of proteins provides strong evidence for the multiplicity of the strategies for the recognition and interaction with specific DNA sites. The analysis of DNA minicircles has proven to be a valuable experimental tool for the determination of the structural and topological requirements for many proteins, among which are histone octamers and DNA topoisomerase I ***(26–28)***.

2. Materials

2.1. The DNA Fragments

The size of the fragment to be circularized is crucial in determining the ring-closure probability (the j factor), defined as the ratio of the equilibrium constants for cyclization and for bimolecular association via the cohesive ends. This factor changes as a function of the fragment length ***(29)***, varying by <10-fold between 4360 and 242 bp, but decreasing by more than 100-fold from 242-126 bp ***(29)***. Moreover, for short fragments, j depends in an oscillatory manner on DNA length with a period of about 10 bp ***(30)***. The ends of the fragment should be cohesive; blunt-ended fragments hardly yield workable amounts of circular products. Cohesive ends can be easily generated using two identical or compatible restriction sites, if necessary modifying a vector polylinker ***(26)***, or by making two 5′-protruding ends compatible by partial fill-in. This procedure offers the opportunity of using the fill-in also to label one single end at the desired specific activity. It is advisable to work with labeled fragments for several reasons (*see* **Note 1**). Labeling can be done at the 5′-end with phosphatase-kinase methods but it is advisable to work in conditions that allow high efficiency of phosphorylation of ends in order to avoid accumulation of nicked molecules during ligase. This method will label both

ends at the same time, labeling by partial fill-in being the alternative when asymmetrical labeling is needed.

The fragments used in the protocols reported below are: a 316-bp *Bss*HII-*Bss*HII fragment containg a 160-bp TG-8 repetitive sequence ***(31)*** inserted in the *Not*I-*Bam*HI sites of plasmid Bluescript KS and a 914-bp *Eco*RI-*Eco*RI fragment from plasmid pSc4816 ***(32)***, which contains the intergenic region encompassed between the GAL1 and GAL10 genes from *Saccharomyces cerevisiae*. Both fragments are labeled by T4 polynucleotide kinase after dephosphorylation of the protruding ends in standard conditions at a specific activity of .1-1 Ci/mmol.

2.2. Enzymes

T4 DNA ligase and Exonuclease III are available from Boehringer Mannheim (Darmstadt, Mannheim, Germany), and DNA topoisomerase I (calf thymus) from Gibco-BRL (Gaithersburg, MD).

2.3. Buffers and Chemicals

1. 10X ligation buffer: 0.3 *M* Tris-HCl, pH 7.8, 0.1 *M* $MgCl_2$, 0.01 *M* DTT, 5 m*M* ATP, 125 µg/mL BSA.
2. PCIA mix: phenol-chlorophorm-isoamyl alcohol (24:24:1) equilibrated with an equal volume of 0.1 *M* Tris-HCl, pH 8.0.
3. Glycogen stock solution: 2 mg/mL from Boehringer Mannheim.
4. 3 *M* Na-acetate, pH 8.0.
5. Absolute ethanol.
6. 70% Ethanol in water.
7. 5X loading buffer: 25% glycerol, 0.5% bromophenol blue, 0.5% xylen cyanol.
8. Polyacrylamide gel in TBE: 40 × 20 × 0.04 cm 5% (w/v) acrylamide/*N,N′*-methylene-bis-acrylamide, 29:1, (w/v), 90 m*M* Tris-HCl, pH 8.1, 90 m*M* boric acid, 2 m*M* EDTA.
9. Polyacrylamide gel in TBM: as above, but in 90 m*M* Tris-HCl, pH 8.1, 90 m*M* boric acid, 10 m*M* $MgCl_2$.
10. Agarose gel: 40 × 20 × 1.5 cm, vertical, 1.2% agarose in 40 m*M* Tris-HCl, 20 m*M* Na-Acetate, 1 m*M* EDTA, pH 7.9.
11. Elution buffer: 50 m*M* Tris-HCl, pH 8.0, 0.5 *M* NaCl, 10 m*M* EDTA.
12. Ethidium bromide (EtdBr) from Sigma (St. Louis, MO).

3. Methods

3.1. Preparation of Minicircles from DNA Fragments Shorter than 500 bp: Analytical Procedure

The purified fragment labeled at high specific activity (*see* **Subheading 2.**) is ligated at a DNA concentration <10 µ/mL (*see* **Note 2**) in the absence or in

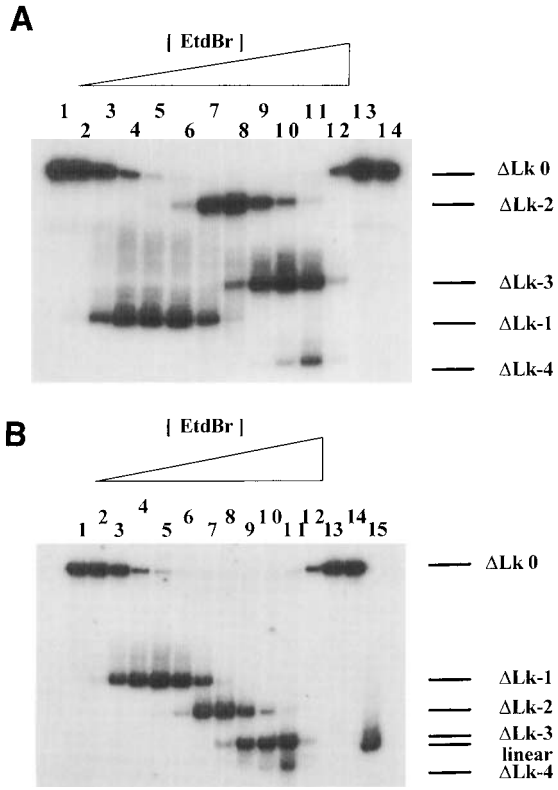

Fig. 1. Panel **(A)**: Gel electrophoresis in the absence of Mg^{2+} (TBE buffer) of samples treated as in **Subheading 3.1.** Sample 15 (linear) run out of the gel. Panel **(B)**: Gel electrophoresis in the presence of Mg^{2+} (TBM buffer) of samples treated as in **Subheading 3.1.**

the presence of increasing concentrations of EtdBr, from 0.1-2 µg/mL, at intervals as narrow as possible (*see* **Fig. 1**).

On the ligated DNA, three controls should be performed: control 1—a sample ligated in the presence of EtdBr is reacted with topoisomerase I in order to verify the relaxation of linking deficient molecules; control 2—a sample ligated in the absence of EtdBr is reacted with *Exo*III in order to evaluate the presence of linear forms and the amount of nicked DNA comigrating in the gel with relaxed circles; control 3—a sample ligated in the absence of EtdBr is treated with S1 endonuclease in mild conditions in order to reveal the possible presence and the position of single-strand DNA forms.

1. Prepare 15 aliquots containing 50 ng of the *Bss*HII-*Bss*HII 316-bp DNA fragment labeled at a specific activity of 0.2 Ci/m*M*.

2. Add to each aliquot 2 µL of 10X ligation buffer; EtdBr to 0 (sample 1), 0.1 (2), 0.2 (3), 0.3 (4), 0.4 (5), 0.5 (6), 0.75 (7), 1 (8), 1.25 (9), 1.5 (10), 1.75 (11), 1.75 (12), 0 (13), 0 (14), 0 (15) µg/mL and H_2O to 20 µL.
3. Add T4 DNA ligase (2 Weiss units) to samples 1-14.
4. Incubate all samples for 4 h at 18°C.
5. Add to all samples EDTA to 10 and 30 m*M* Tris-HCl, pH 7.8, to a final volume of 100 µL.
6. Extract all samples with PCIA mix (1 vol).
7. Add to all samples 1µL of glycogen stock solution, 11 µL of 3 *M* Na-acetate, pH 8.0, and precipitate with absolute ethanol (2 vol).
8. Wash pellet with 70% ethanol and dry.
9. Resuspend all samples in 10 µL of 10 m*M* Tris-HCl, pH 7.8.
10. Treat sample 12 with 1 unit of topoisomerase I in 20 µL of 20 m*M* Tris-HCl, pH 8, 10 m*M* $MgCl_2$, and 150 m*M* KCl for 1 h at 37°C. Stop the reaction with EDTA to 10 m*M*.
11. Treat sample 13 with 10 U of *Exo*IIIin 20 µL of 20 m*M* Tris-HCl, pH 8, 15 m*M* NaCl, and 2 m*M* $MgCl_2$ for 1 h at 37°C. Stop the reaction with EDTA to 5 m*M*.
12. Treat sample 14 with 2 U of S1 endonuclease in 20 µL of 50 m*M* Na-acetate, pH 4.5, 280 m*M* NaCl, and 4.5 m*M* $ZnSO_4$, for 10 min at 37°C. Stop the reaction with EDTA to 5 m*M*.
13. Repeat for samples 12, 13, and 14 **steps 5–9**.
14. Add to all samples 2.5 µL of 5X loading buffer.
15. Load 4 µL of every sample on an acrylamide gel in TBE. Run for 4 h at 1000 V constant voltage (xylene cyanole dye 38 cm from wells). Gel should be run in the absence of Mg^{2+}: *see* **Fig. 1,** panel A.
16. Load 4 µL of every sample on an acrylamide gel in TBM (*see* **Note 3**). Run for 4 h at 800 V constant voltage (xylene cyanole dye 38 cm from wells). Gel should be run in the presence of Mg^{2+}: *see* **Fig. 1,** panel B.

3.2. Preparation of DNA Minicircles Shorter than 500bp: Preparative Procedure

Based on the results of the analytical procedure (**Fig. 1**), set up the preparative ligation reactions, as follows:

1. Prepare four 0.5 µg aliquots of the terminally labeled DNA fragment, and ligate them in the same conditions used in the analytical procedure in **Subheading 3.1.** in the presence of 0 (sample 1), 0.3 (2), 0.8 (3), and 1.6 (4) µg/mL of EtdBr, final volume: 200 µL.
2. Add T4 DNA ligase (10 Weiss units), and incubate for 4 h at 18°C. Stop the reaction with EDTA to 10 m*M*.
3. Proceed as in **steps 6–8** of **Subheading 3.1.** (*see* **Note 4**).
4. Resuspend samples in 40 µL of 10 m*M* Tris-HCl, pH 7.8, add 10 µL of 5X loading buffer, and load on an acrylamide gel in TBM, distributing each sample in at least five wells, 1 cm wide.

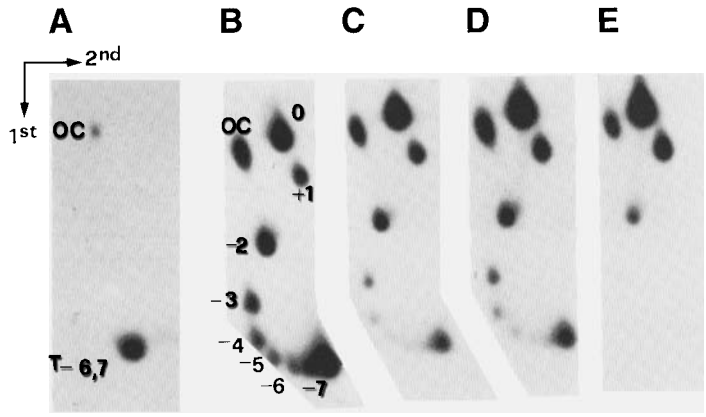

Fig. 2. Kinetics of relaxation of supercoiled topoisomers. The terminally labeled 914-bp *Eco*RI-*Eco*RI fragment is ligated under programmed conditions (**Subheading 3.2.**) mixture of topoisomers -6/-7 is purified from agarose gel after electrophoresis, recovered and treated with DNA topoisomerase I for the specified times (*see text*). The figure shows the autoradiogram of a 2-D gel run without EtdBr in the first dimension, in the presence of 0.065 µg/mL EtdBr in the second. Sample in (e) is the terminal product of the relaxation reaction. Numbers indicate the sign and the number of superhelical turns. OC is the open circular form.

5. Identify the different linkomers by autoradiography, cut the bands, and elute the desired circles in elution buffer for 12 h at 20°C in agitation.

3.3. Preparation of Minicircles from DNA Fragments Longer than 500 bp

Analytical procedures similar to those described in **Subheading 3.1.** are set up with the following specifications:

1. Analysis is performed on agarose gels (*see* **Subheading 2.**); the gel is run for 18 h at 2V/cm in a vertical apparatus. Electrophoresis temperature should be kept close to the ligation temperature in order to avoid topological rearrangements.
2. Single-dimension analysis should be complemented with a double dimension (*see* **Fig. 2**) in order to resolve completely the topoisomers distribution obtained as follows:

   Analysis of the products obtained by ligation of a 914-bp DNA fragment: two dimensional (2-D) gel electrophoresis of the topoisomers produced by topoisomerase I.

   Topoisomerase I kinetic relaxation assay: 0.05 U of calf thymus DNA topoisomerase I (from BRL) is reacted with 100 ng of internally labeled, highly supercoiled DNA (obtained as described in **Subheading 3.2.**; *see also* **Fig. 2**) in 50 µL of 150 m*M* NaCl, 10 m*M* Tris-HCl, pH 7.8 and 1 m*M* EDTA,

at 25°C for 0, 0.5, 2, 4, and 20 h. The products of topological relaxation obtained after different reaction times are extracted once with an equal volume of PCIA, ethanol-precipitated, and analyzed in 2-D gel electrophoresis. The complete ladder of topoisomers so obtained allows unambiguous (both for the sign and the number) attribution of linking value.

3.4. Preparation of Single Topoisomers

Based on the results of analytical tests (as in **Subheadings 3.1.** and **3.3.**), scale up the ligation reaction and obtain the ligated samples as in **Subheading 3.2.** After resuspension, load the sample in a 2% agarose gel, using for each sample 5-cm wide wells. Identify by autoradiography the topoisomer, and elute the corresponding band. Diffusion-driven elution should be substituted by electroelution or other methods more appropriate for large-size DNA fragments.

4. Notes

1. The use of labeled fragments is advisable because:
   a. During analytical ligation experiments, use of low amounts of DNA is often necessary, which are difficult to detect if not labeled;
   b. For most analytical purposes, the circles need to be labeled, although primer extention methods of analysis can be used as an alternative; and
   c. Even preparative ligations should be performed with labeled DNA, because other detection methods, like EtdBr staining or silver staining, damage integrity of the circles.
2. For a discussion on the appropriate DNA concentration to be used in the ligation reaction in order to favor circularization, *see* **ref. *33***.
3. In polyacrylamide gel electrophoresis in the presence of 10 m*M* Mg^{2+} cations, small circles show in general a migration proportional to their linking number. During electrophoresis in the absence of Mg^{2+} cations, a more complex correlation between linking deficiency and migration is on the contrary commonly observed (compare **Fig. 1** panel A and B and *see* **ref. *34***).
4. In certain instances, it could be important to perform a treatment with *Exo*III (as in **Subheading 3.1., step 11**) after the preparative ligation in order to eliminate the possible contaminant nicked DNA from the comigrating relaxed circles.

Acknowledgment

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7

Isolation of Kinetoplast DNA

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1. Introduction

The kinetoplast DNA (kDNA) network of the protozoan parasite *Crithidia fasciculata* is a naturally occurring gigantic catenane containing several thousand DNA minicircles. Because of its unusual structure, kDNA is an excellent substrate to use in decatenation assays of topoisomerase activity. Beyond this application, kDNA has also proven a fascinating experimental subject in its own right. Studies of the unique structure, mechanism of replication, and genetic function of kDNA have led to numerous interesting discoveries (*see* **refs. *1–5*** for reviews).

kDNA is the mitochondrial DNA of kinetoplastid parasites. Among these flagellated protozoa are human pathogens *(Trypanosoma* and *Leishmania* species) as well as *C. fasciculata*, a parasite of insects. *C. fasciculata* is easily manipulated in the laboratory; its kDNA can be isolated intact and in large quantities, and it is not a human pathogen. Therefore, its networks are the most desirable for use in topoisomerase assays. kDNA is a characteristic morphological feature in intact *C. fasciculata* cells: an electron-dense, disk-shaped structure, located within the single mitochondrion of the cell and always sited at the base of the flagellum. When isolated and examined by electron microscopy, the kDNA from each cell is in the form of a single elliptical-shaped planar network (10×15 μm). An electron micrograph of a segment of a kDNA network is shown in **Fig. 1A**. Each small loop is an individual minicircle topologically linked to several neighbors.

With the advent of topoisomerases as tools that completely disassemble the network structure ***(6)***, the components of kDNA could be easily visualized (**Fig. 1B** shows an electron micrograph of topoisomerase II-decatenated

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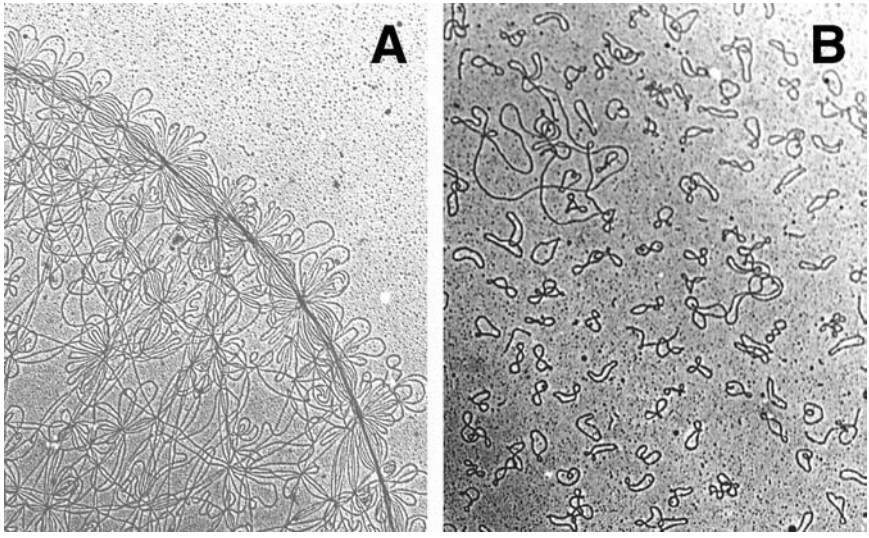

Fig. 1. Panel **(A)** Electron micrograph of part of a kDNA network from *C. fasciculata*. Panel **(B)**, Topoisomerase II decatenation yields 2.5-kb minicircles and 38-kb maxicircles (top left corner). Micrographs are at approximately the same magnification. EMs were by David Pérez-Morga **(A)** and Laura Rocco Carpenter **(B)**.

kDNA). Networks from nonreplicating cells contain about 5000 minicircles (each 2.5 kb; one major sequence class with several minor classes) and about 25 maxicircles (each ~38 kb and all identical in sequence). Like conventional mitochondrial DNAs, maxicircles encode ribosomal RNAs and several mitochondrial proteins, such as cytochrome oxidase subunits. Minicircles have the unique function of encoding small guide RNA molecules that direct the surprisingly extensive posttranscriptional editing of maxicircle messenger RNAs (editing is reviewed in **refs.** ***3,7–9***).

Recent studies have further clarified the topological organization of kDNA networks. The minicircles are arranged in a monolayer, so that the network resembles chain mail. Within the network structure, each minicircle is linked to an average of three other minicircles ***(10)*** and each linkage consists of a single interlock ***(11)***. Minicircles are unique among covalently closed circular DNA molecules in that they are not supercoiled, a characteristic that may facilitate network formation in vivo ***(11)***. The organization of maxicircles is less clear, but in networks from African trypanosomes, maxicircles form an independent catenane that is extensively interlocked with the catenane of minicircles ***(12)***.

Shortly after the report of topoisomerase II-mediated decatenation of kDNA, about 15 years ago ***(6)***, kDNA decatenation was used to monitor purification of mammalian topoisomerase II ***(13)***. Since then, kDNA decatenation has been

widely used to assay topoisomerase II. Indeed, kits for this assay are commercially available from TopoGEN Inc., Columbus, OH. Most kDNA decatenation assays are based on monitoring the release of monomer minicircles by agarose-gel electrophoresis, but quantitative methods using [^{3}H]thymidine-labeled networks have also been devised ***(14,15)***. Decatenation of kDNA is often used to distinguish type I from II topoisomerase activity. However, kDNA from replicating *C. fasciculata* contains minicircles that are nicked or gapped. These discontinuities present sites where topoisomerase I could act, in theory ***(16,17)***. To minimize any possible contribution by topoisomerase I, it is therefore advisable to use networks from stationary-phase cells.

2. Materials

2.1. Culturing C. fasciculata

1. *C. fasciculata* cell cultures may be obtained from the American Type Culture Collection, Rockville, MD (ATCC #11745) or from the authors. These organisms are not considered a biohazard, but sterile technique is required for their culturing.
2. Culture medium consists of brain heart infusion (BHI, Difco, Detroit, MI #0037-05-2) and hemin (bovine, Sigma, St. Louis, MO #H-2250). Autoclaved BHI solution (37 g/L) may be stored at room temperature; 2 mg/mL hemin in 50 m*M* NaOH should be filter-sterilized and stored at –20°C. Once hemin is added to the BHI medium (1:100, v:v), the solution should be used within 2 wk.
3. Cotton-stoppered Erlenmeyer flasks that have 5–10 times the capacity of the culture volume are convenient vessels for growing cells.
4. Inoculate to give a final concentration of ~10^6/mL. Cells grow at temperatures ranging from 20–27°C; vigorous rotary shaking appears to facilitate growth to higher cell densities. Cells should be harvested when they reach stationary phase (≥10^8/mL, roughly 24 h after inoculation). Doubling time under optimum growth conditions (27°C) is 3–4 h.
5. Microscopic examination reveals highly motile cells, each with a single flagellum. To count cells, dilute a 25-µL aliquot of the culture with 25 µL 3.7% formaldehyde in water (prepared from a 37% solution, JR Baker, Phillipsburg, NJ 2106-01) and 200 µL Gentian violet stain, and count in a Neubauer chamber. Stain is made with 0.75 mL Gentian violet (Fisher, Pittsburgh, PA SG 20-250), 0.1 g Na_2EDTA, 4 g NaCl, and water to a final volume of 500 mL. Alternatively, live or formaldehyde-killed cells can be counted without stain under a phase-contrast microscope.
6. To cryopreserve organisms, dilute a midlog-phase culture (~4 x 10^7/mL) with an equal volume of 15% glycerol in BHI (15 mL glycerol, 3.7 g BHI, water to 100 mL, filter sterilize) and dispense 1-mL aliquots into sterile 1.8-mL CryoTubes, Nunc, Naperville, IL (#363401). Freeze incrementally: on ice at 0°C for 20 min, at –20°C for 2 h, at –70°C overnight, and finally in liquid nitrogen at –195°C.

2.2. Solutions for Purification of kDNA Networks

1. NET-100: 100 m*M* NaCl, 100 m*M* EDTA, 10 m*M* Tris-HCl, pH 8.0. Add 5.8 g of NaCl, 200 mL of 500 m*M* Na_2EDTA, pH 8.0, and 10 mL of 1*M* Tris-HCl, pH 8.0, to about 700 mL water, adjust the pH to 8.0 with HCl, and bring the total volume to 1 L with water.
2. Lysis buffer: 0.1 mg/mL proteinase K (Boehringer Mannheim, Indianapolis, IN #745723), 1% sodium dodecyl sulfate (Ultrapure #811030, ICN, Costa Mesa, CA) in NET-100.
3. TE: 10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA.
4. Sucrose solution: 20% sucrose (JR Baker #4072-05) in NET-100. Store at –20°C or filter-sterilize and store at room temperature.

3. Methods

Classical methods for purifying kDNA networks from *C. fasciculata* include one or more CsCl gradients ***(18,19)***. More recently, we have replaced the CsCl gradient with a sucrose cushion ***(20)***. This method is less expensive, more convenient, and yields more networks (typically 85–250 µg from 10^{10} cells).

3.1. Purification of kDNA Networks

1. Grow *C. fasciculata* in 100 mL BHI and hemin (*see* **Subheading 2.1.**) to stationary phase (~10^8/mL, 10^{10} cells). Harvest the cells at 4000*g* (10 min, 4°C, Sorvall GSA rotor), and wash once with 50 mL NET-100. The final cell pellet may be processed immediately or stored at -70°C.
2. Lyse cells (~10^{10} total) by adding enough lysis buffer to yield a total volume of 108 mL. Shake slowly for 2 h at 50°C.
3. Prepare sucrose cushions in 38-mL ultraclear centrifuge tubes (Beckman, Fullerton, CA #344058). Allow one tube for each 18 mL of lysate (6 tubes for 108 mL). Add 20 mL sucrose solution to each tube, and then slowly overlay the 18-mL sample, being careful not to agitate the sucrose–sample interface.
4. Centrifuge samples at 47,500*g* (30 min, 20°C, Beckman SW28 rotor). Remove supernatant, leaving the pellet (which is typically invisible) in ~500 µL.
5. Resuspend and pool the pellets, rinse the tubes with ~1 mL TE, and bring the total volume to 36 mL with TE.
6. Repeat the sedimentation through sucrose, using just two centrifuge tubes. Remove supernatants, leaving each pellet in ~250 µL. Resuspend the pellets, pool, rinse the tubes, and bring the total volume to 600 µL with TE.
7. Dialyze at 4°C overnight against 200 vol of TE.
8. Store at 4°C. Networks settle during long storage; gently resuspend prior to use.

3.2. Assessing Purity and Yield of Networks

1. Dilute an aliquot of the final preparation 1:100 with TE, and measure the UV absorption at 260 and 280 nm. The A_{260}/A_{280} ratio is typically 1.9, and the overall yield can be calculated, based on an A_{260} of 0.020 for 1 µg/mL of DNA.

2. Nuclear DNA contamination is detectable by agarose-gel electrophoresis. Load 2–5 µL of the final preparation on a 1% agarose minigel, and run (70 V, 1 h) in 90 m*M* Tris-borate (pH 8.3), 2.5 m*M* EDTA, and 1 µg/mL ethidium bromide. Visualize the DNA by UV transillumination. Intact networks remain in the slot, and nuclear DNA forms a diffuse band in the compression zone of the gel (roughly at the 23-kb marker of λ *Hin*dIII DNA). Networks may wash out of the slot when the gel is processed. Therefore, this method is unreliable for quantitating kDNA yield.
3. The kDNA networks can be further evaluated by restriction enzyme digestion prior to gel electrophoresis. *Sst*II or *Xho*I cleave most *C. fasciculata* minicircles once. Complete digestion by one of these enzymes yields 2.5-kb linearized minicircles and a few percent of residual circular forms (monomers and small catenanes) that lack the restriction site.

4. Notes

1. Residual contamination by nuclear DNA can be reduced or eliminated by sedimenting the networks through a third sucrose cushion.
2. Radiolabeled kDNA networks can be prepared from *C. fasciculata* cells incubated with [^{3}H]thymidine ***(14,21–23)*** or [^{32}P]orthophosphate ***(11,20)***. ^{32}P-labeled networks are generally inconvenient to use because the half-life of the isotope is so short. However, [^{3}H]thymidine-labeled networks are readily prepared, stable, and useful for quantitative assays of topoisomerase II activity ***(14,15)***. When metabolically labeled with [^{3}H]thymidine (20 Ci/mmol, 10 µCi/mL, 60 min), the isolated networks have a specific radioactivity of roughly 10,000 cpm/mg. A 1-h labeling of log-phase cells with thymidine of higher specific activity (100 Ci/mmol, 1 mCi/mL) yields networks at 2×10^7dpm/µg.
3. After prolonged storage (months to years) networks deteriorate, releasing free minicircles or fragments into the buffer. The free circles will contribute background to a topoisomerase decatenation assay. To remove free minicircles, sediment networks from their storage buffer (12,000*g* in a microfuge, 60 min, room temperature), wash with TE, centrifuge, and resuspend in fresh TE.
4. Kinetoplast DNA networks can be isolated from kinetoplastid parasites other than *C. fasciculata*, using similar methods. The minicircle monomers released from these networks during a decatenation assay may be of a different size than the 2.5-kb minicircles from *C. fasciculata*.
5. The protocol can be modified for minipreps of kDNA ***(23)***. In this method, transfer 1 mL of *C. fasciculata* culture into a 1.5-mL microcentrifuge tube, centrifuge the cells (12,000*g* in a microfuge, 1 min, room temperature), and resuspend the pellet in 630 µL NET-100. Lyse the cells by adding 71 µL of 10% SDS and 7 µL of 20 mg/mL proteinase K, and incubate the lysate at 37°C for 1 h. Load the lysate onto a 690-µL cushion of 20% sucrose in NET-100 in a 1.5-mL microcentrifuge tube, and centrifuge (15 min, room temperature, Fisher MicroCentrifuge 5-cm rotor). Remove the supernatant, leaving the pelleted networks in 30 µL residual solution. Add NET-100 to bring the total volume of the resus-

pended pellet to 690 µL, and repeat the sedimentation through sucrose. Dissolve the second pellet in ~50 µL of supernatant, dialyze overnight against TE, and concentrate (12,000*g* in a microfuge, 60 min, room temperature).

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8

Isolation of Knotted DNA from Coliphage P4

Morten Isaksen, Bryan Julien, Richard Calendar, and Björn H. Lindqvist

1. Introduction

Type II topoisomerases readily remove knots from DNA ***(1)***. Removal of knots can be assayed by gel electrophoresis ***(1)***, and this provides a convenient assay for type II topoisomerase activity, even in crude extracts. Such assays have been useful in screening drugs that are suspected to inhibit type II topoisomerases. This chapter describes the isolation of knotted DNA from P4 phage. In the case of wild-type P4, such DNA molecules are double-stranded, hydrogen-bonded, knotted circles of 11,624 nucleotides.

P4 is a helper-dependent phage that grows lytically on *Escherichia coli* strains that carry a P2-type helper prophage. The prophage supplies genes needed for capsid and tail synthesis, and for host cell lysis ***(2,3)***. Both P2 and P4 DNA molecules have the same 19 nucleotide cohesive ends ***(4,5)***, and the noncovalent joining of these ends can produce knotted DNA. When P4 is grown on *E. coli* lysogenic for wild-type P2, a mixture of P4 phage and tailless capsids is produced, and knotted DNA can be obtained from tailless capsids, which are purified by density gradient centrifugation ***(1)***. It is not clear how knotting comes about. The original procedure for isolating P4 knotted DNA has been superceded by other procedures. Wolfson et al. ***(6)*** reported that genomic deletions increase the amount of knotted DNA that can be obtained from complete P4 phages. When P2 *lg* (large plaque type ***[7]***) is used as a prophage helper for a P4 deletion mutant, the result is a large burst of progeny phages (without free capsids) and these phages contain knotted DNA. Wolfson et al. reported that undeleted P4 phage particles contain 15% knotted DNA, whereas a deletion of 700 bp gave 50% knotted DNA, and a deletion of 1 kb gave 80% knotted DNA.

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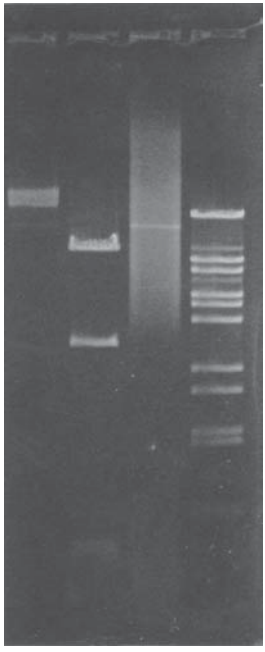

Fig. 1. Gel electrophoresis of knotted DNA. Left panel: P4 *vir1* DNA from phage particles (hydrogen-bonded circular molecules). Second panel: *Eco*RI-digested P4 *vir1* DNA from tailless capsids. Third panel: knotted P4 *vir1* DNA from tailless capsids. Fourth panel: λ phage DNA digested with *Hin*dIII. The 0.8% agarose gel containing 100 m*M* Tris, 100 m*M* borate, 2 m*M* EDTA, pH 7.6, was electrophoresed at 80 V for 90 min.

In the procedure reported here, we use a deletion of 1.7 kb, which should give more than 80% knotted DNA molecules.

We also describe here a procedure for the production of knotted P4 DNA from tailless capsids. This procedure gives 100% tailless capsids and no complete phage, since a P2 tail-gene mutant is employed to impair the assembly of complete phage particles.

Knotted DNA extracted from P4 capsids can be dried and resuspended without any loss of knots. Storage at room temperature for at least 24 h will not affect the knots. However, freezing and thawing should be avoided, since it may lead to unknotting.

Knotted DNA migrates heterogeneously on agarose gels. The presence of some unknotted DNA gives one faint band in the smear **(Fig. 1)**. Treating the DNA with topoisomerase II or linearizing with restriction enzymes results in a distinct band ***(1)***.

2. Materials

2.1. Bacteria

1. C-1895 ***(8)***, lysogenic for P2 *lg* ***(7)***, for growing P4 stocks in liquid medium (*see* **Note 1**).
2. C-2423 ***(9)***, lysogenic for P2 *lg del1*, for P4 plaque assays (*see* **Note 2**).
3. C-8001 ***(10)***, lysogenic for P2 am*H13* ***(11)***, for growing P4 capsids (*see* **Note 3**).

2.2. Bacteriophages

1. P4 *vir1* ***(12)***, a P4 phage that does not lysogenize (*see* **Note 4**).
2. P4 *vir1 del22*, containing P4's largest known deletion ***(13)***.

2.3. Media and Buffers

1. Phage buffer: 10 m*M* $MgCl_2$, 10 m*M* Tris-HCl, pH 7.5, 130 m*M* ammonium acetate (*see* **Note 5**).
2. Luria broth (LB): 1% yeast extract, 0.5% tryptone, and 0.5% NaCl.
3. LB agar: LB broth with 1% agar.
4. LB soft agar: LB broth with 0.5% agar.
5. TM buffer: 50 m*M* Tris-HCl, 10 m*M* $MgSO_4$, pH 7.5.
6. TE buffer: 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 7.6.

3. Methods

3.1. P4 Plaque Assay

1. Grow *E. coli* C-2423 into a standing culture in LB at 37°C overnight.
2. Dilute phage appropriately in phage buffer and add 100–500 PFU to 0.35 mL cells; add $CaCl_2$ to 1 m*M* and incubate for 10 min at 37°C (*see* **Note 6**).
3. Add 2.5 mL LB soft agar and spread on an LB plate.
4. Incubate at 37°C for about 8 h.

3.2. Production of Knotted DNA from P4 Deletion Mutant Stocks

1. Prepare a plate for P4 *vir1 del22* plaques.
2. On the next morning, pick one plaque into 0.25 mL phage buffer.
3. Mix this plaque with 15 mL of a culture of C-1895 grown in LB overnight at 37°C without aeration.
4. Add supplementary $CaCl_2$ to a final concentration of 1 m*M* to promote adsorption.
5. Incubate the cells and phage at 37°C for 10 min.
6. Add the infected cells to 400 mL LB broth supplemented with 0.1% glucose, 1.6 m*M* $MgCl_2$, and 0.5 m*M* $CaCl_2$ in a 2800 Fernbach flask (*see* **Note 7**).
7. Shake on a reciprocating shaker at about 65 cycles/min at 37°C for about 3-1/2 h (*see* **Note 8**).
8. At A_{600} between 0.7 and 1.5, when lysis begins, add 8 mL of 0.1 *M* EGTA (pH 8.8) to chelate the calcium ions and block phage readsorption.

9. When lysis is complete, in 45 min or 1 h, remove the cellular debris by centrifugation in a GSA rotor for 10 or more min at approx 6000*g*.
10. To precipitate the phage, add NaCl to 2.5% w/v and PEG 6000 to 8%.
11. After the PEG dissolves, keep the mixture in a refrigerator for an hour, and then centrifuge as described above.
12. Centrifuge a second time for 2 min in order to concentrate the pellet and facilitate removal of the liquid.
13. Resuspend the pellet in 5 mL phage buffer.
14. Add a small crystal of pancreatic DNase, and incubate at 37°C for 1–5 min (*see* **Note 9**).
15. Add 2 mL $CHCl_3$ and roll gently by hand 30 times to make an emulsion.
16. Centrifuge for 5 min in an SS34 rotor at 7649*g* to separate the phases. A white, solid layer will appear at the interface between the phases.
17. Remove the aqueous layer, measure its volume, and assay for P4 (*see* **Note 10**).
18. Add CsCl equal to half the weight of solution.
19. Centrifuge in the SW50.1 rotor for 18–36 h at 200,000*g*.
20. To see the phage band, use a black background and visible lighting from the side.
21. Remove the phage band with a syringe and needle.
22. Centrifuge the phage band to equilibrium in 33% w/v CsCl a second time (*see* **Note 11**).
23. Dialyze the purified phage against phage buffer (*see* **Note 12**).
24. Extract DNA twice with phenol, and then twice with phenol and $CHCl_3$.

An alternative method to cesium chloride centrifugation is DEAE-cellulose chromatography. This method was first described for the purification of λ phage ***(14)*** and can be used for the purification of P4 phage. The advantage of this procedure is that it can be completed in a few hours, and it removes all contaminating nucleic acids and proteins. The expected recovery is 90%.

1. Pour a 25-mL DEAE-cellulose column (Whatman DE 52).
2. Equilibrate it with TM buffer.
3. Apply the phage suspension from the chloroform-extracted polyethylene glycol pellet to the column.
4. Collect 2-mL fractions (*see* **Note 13**).
5. Read the A_{260}. The phage elute in the first or second peak of UV-absorbing material.
6. To check for phage particles, add 5 µL of each fraction from the first two peaks to 2 µL of 0.1 m*M* EDTA and 2 µL of 2% sodium dodecyl sulfate. Boil and subject the mixture to agarose-gel electrophoresis.
7. Pool the fractions that show phage DNA.
8. Concentrate the pooled fractions by centrifuging at 110,000*g* for 2.5 h in a Beckman-type 60 Ti rotor.
9. Decant the liquid.
10. Add 500 µL of TE buffer.

11. Extract the DNA with phenol and phenol/chloroform.
12. Precipitate the DNA by adding 1/10 vol of 3 *M* ammonium acetate, pH 5.5, and 2 vol of 95% ethanol.
13. Regenerate the column by washing with TM buffer containing 1 *M* NaCl, and then equilibrating again with TM buffer.

3.3. Production of Knotted P4 DNA from Tailless Capsids

1. Prepare a large stock of P4 *vir1* or P4 *vir1 del22* as described above.
2. Inoculate 5 mL of a fresh overnight aerated culture of C-8001 into 400 mL LB supplemented with 0.1% glucose, 1.6 m*M* $MgCl_2$ and 0.5 m*M* $CaCl_2$ in a 3-L flask.
3. Incubate at 37°C with aeration.
4. When the A_{600} reaches 0.35, add 4×10^{11} phage, to give a multiplicity of infection between 5 and 7, and continue the incubation with aeration.
5. When the cells will lyse after approx 1 h, do not add EGTA or EDTA, because this will inhibit removal of phage tails.
6. After the A_{600} has stabilized (at approx 0.4), remove the cells by centrifugation in a GSA rotor for 10 or more min at 6000*g*.
7. The remainder of the procedure is the same as that described for the production of P4 *vir1 del22* phage particles (*see* **Subheading 3.2., [first] step 10**). After banding in CsCl and dialysis, the DNA is extracted with phenol and phenol/chloroform, and precipitated with ethanol, as described above. Alternatively, the DNA can be extracted with Promega's Wizard Lambda Prep method.

4. Notes

1. For unknown reasons, *E. coli* C strain C-1895 gives the best liquid stocks for P4 and many of its mutants.
2. For unknown reasons, C-2423 gives the best plaques for P4 and many of its mutants.
3. Gene *H* encodes part of the P2 tail. Thus, an amber mutation in this gene gives tailless capsids.
4. The *vir1* mutation causes P4 early genes to be produced constitutively ***(12)***.
5. Magnesium ions prevent explosion of P4 heads.
6. Phage adsorb to bacteria during this period, because of the high concentration of cells. Calcium ions promote adsorption.
7. These flasks have large mouths that promote maximal aeration.
8. The reciprocating (back and forth) motion creates a continuous wave that is crossing the surface of the medium, maximizing aeration.
9. A decrease in viscosity is expected.
10. The yield should be about 4×10^{12} phages.
11. This step gives a more complete removal of trapped DNA.
12. The expected recovery is 50% of the initial titer.
13. It is not necessary to pretreat the phage suspension with nucleases. The cellular DNA, RNA, and proteins bind to the resin, whereas the phage particles flow through the column.

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9

Analysis of DNA Knots and Catenanes by Agarose-Gel Electrophoresis

Stephen D. Levene and Hua Tsen

1. Introduction

Supercoiling, knotting, and catenation are three common higher-order structures involving coiling of the axis of double-stranded DNA. These forms appear as a result of a number of important biological activities, including topoisomerase action, DNA replication, and genetic recombination ***(1–3)***. All of these species have mobilities in agarose gels that are distinct from those of normal open circular and linear DNA molecules of the same size. The electrophoretic properties of linking number topoisomers are dealt with elsewhere in this volume; this chapter focuses on the separation and characterization of mixtures of knotted or catenated forms.

A knot is a particular topological form of a circle in three-dimensional space; a catenane is an entity consisting of two or more topologically linked circles (mathematicians frequently refer to catenanes as links). DNA circles can be knotted or catenated by the action of topoisomerases and by site-specific recombination. A distribution of knotted or catenated products is invariably obtained rather than unique species; this distribution is a function of the topoisomerase or recombinase mechanism as well as the structure of the DNA substrate ***(4,5)***. Knots and catenanes are classified according to the minimum number and arrangement of crossings seen in a two-dimensional projection of the object. We designate the minimum number of knot crossings *Kn* and the minimum number of catenane crossings *Ca*. These are topologically invariant quantities; no deformation of the DNA short of severing and rejoining both phosphodiester backbones can change *Kn* or *Ca*. Equations that give the number of distinct knots or links as a function of the number of minimal crossings

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have remained elusive; however, all prime knots containing up to 13 minimal crossings are known. Even this restricted subset of knots contains over $1.2 \cdot 10^4$ members ***(6)***. **Figure 1** shows all of the prime knots up to $Kn = 9$, and dimeric catenanes up to $Ca = 8$. An excellent recent introduction to knot theory and its applications may be found in **ref. *6***.

This chapter will be concerned only with knotted DNA circles and dimeric DNA catenanes. Although multimeric catenanes are found in nature, as in the case of kinetoplast networks of mitochondria ***(7)***, for example, dimeric catenanes are the forms most frequently encountered. The products of DNA replication of circular genomes ***(1,8)*** and site-specific recombination systems, such as bacteriophage λ integrative (Int) recombination and Tn3 resolvase ***(5,9)***, are further restricted to the topological class known as torus catenanes. These are so called because the DNA rings can be drawn without intersection on the surface of a torus.

1.1. The Topology of "Random-Collision" Recombination

Recombination of a circular DNA molecule by λ integrative recombination results exclusively in either knots or catenanes, depending on the chemical orientation of the recombination sites. Knots are generated by recombination of inversely repeated recombination sites, whereas catenanes result from recombination of substrates bearing directly repeated sites, as shown in **Fig. 2**. The recombination sites divide the DNA contour into two domains; the value of *Kn* or *Ca* for the recombination product is proportional to the number of supercoils involving the two domains that are trapped during recombination site synapsis. Because the number of interdomainal supercoils is a stochastic quantity, the result is a distribution of product molecules with different values of *Kn* or *Ca*. Such distributions are characteristic of recombination systems that proceed according to a random collision mechanism; recombination via random collision samples all configurations of the substrate DNA molecule consistent with its tertiary structure (**Fig. 2**). Recombination in the two systems that are studied in our laboratory, the FLP system of *Saccharomyces cerevisiae* and the λ Int system, occurs by random collision of their respective sites ***(4,10)***.

This chapter describes techniques for analyzing the distribution of knots and catenanes, drawing mainly on results obtained with the λ Int system. Identical methods can be used to analyze products of FLP and other recombination systems or those generated by topoisomerases. However, mechanisms of many recombination and topoisomerase activities are processive or distributive in nature, and therefore can generate complex distributions of products that arise from multiple reaction cycles ***(5)***. This is not the case with the Int system, which carries out only one round of recombination in the absence of the excisionase protein, Xis, and therefore leads to relatively simple distributions of product topologies ***(11)***.

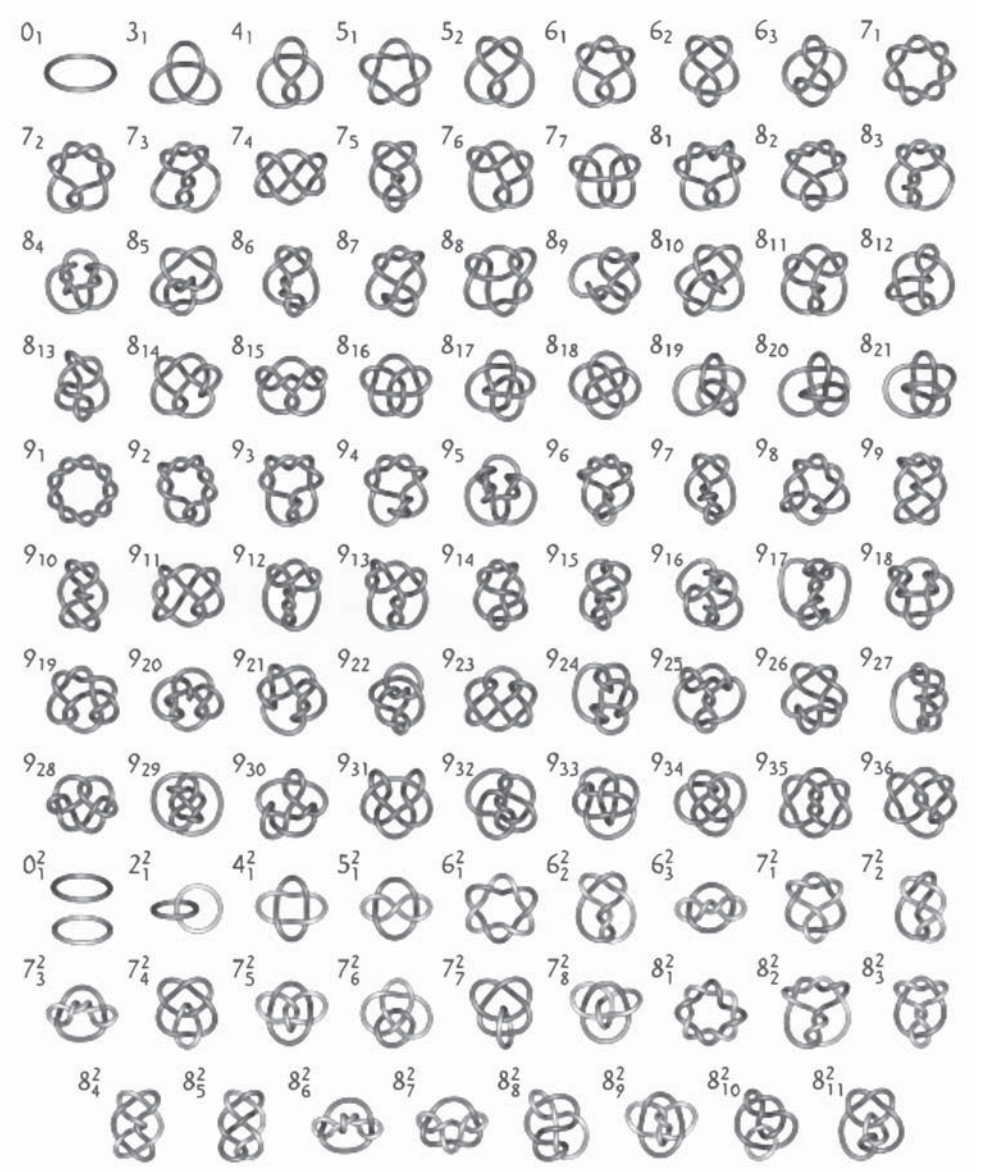

Fig. 1. The three-dimensional structures of all prime knots up to $Kn = 9$ and dimeric catenanes up to $Ca = 8$ generated using the program *KnotPlot*. Each structure is labeled above and to the left using Alexander and Briggs notation for knots and Rolfsen notation for catenanes. A similar gallery of knots and catenanes shown in color can be downloaded from the URL http://www.cs.ubc.ca/nest/imager/contributions/scharein/zoo/knotzoo.html.

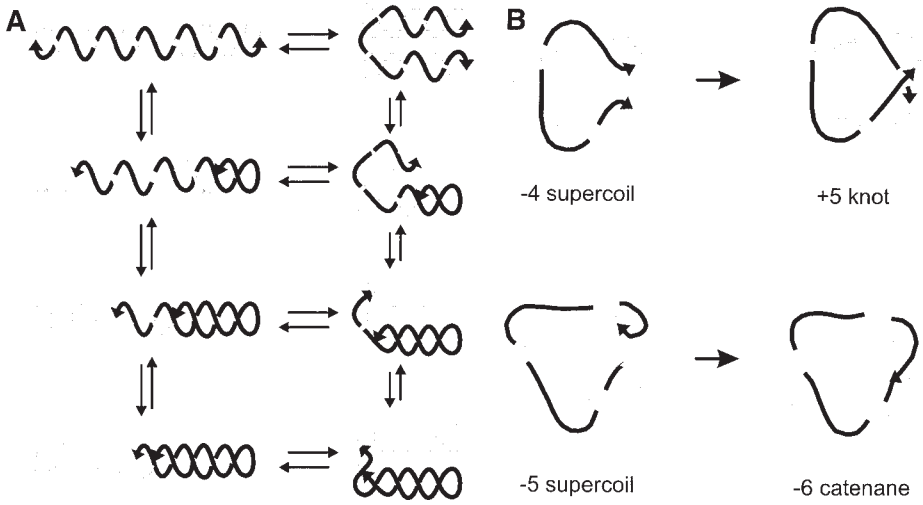

Fig. 2. Intramolecular, site-specific recombination of a supercoiled DNA substrate by a random collision mechanism. **(A)** The diagram shows a planar projection of a prototypical supercoiled DNA substrate for site-specific recombination. Recombination sites, indicated by arrows, divide the DNA contour into two domains. Relative motions of these two sites along the superhelix axis, termed slithering, generate a variable number of superhelical turns, which are trapped at site synapsis in the folded conformations shown on the right. Note that the diagram shows only approximately one-quarter period of the motion. Although slithering is shown as a unidirectional process in the figure, actual DNA motions correspond to a one-dimensional random walk. **(B)** Conversion of interdomainal superhelical turns into topologically invariant links by Int site-specific recombination. The process of strand exchange traps a variable number of superhelical turns as either knot (upper panel) or catenane (lower panel) crossings, depending on whether the sites are in inverse or direct orientation, respectively. Note that an additional crossing appears in the products as a consequence of the mechanism of strand exchange.

Bacteriophage λ integrative recombination is dependent on two proteins, the 40-kDa Integrase protein encoded by the phage genome and a 22-kDa host-encoded protein called integration host factor (IHF). Two nonidentical recombination sites are involved in the reaction: a phage recombination site, denoted *attP*, and a host site, designated *attB*. The minimal *attP* site is approx 250 bp in size and contains multiple binding sites for Int and IHF as well as other proteins involved in the excision reaction ***(11)***. In contrast, the organization of the 25-bp *attB* site is quite simple in that it contains no binding sites for the recombination proteins, but instead is recognized by the multisubunit complex of Int and IHF proteins that assembles on *attP* to comprise what is called the

"intasome" ***(12,13)***. In intermolecular recombination reactions, supercoiling of the *attP* site is required for efficient Int recombination. All of the work described here involves intramolecular recombination of supercoiled plasmid substrates bearing one copy each of *attP* and *attB*. These substrates were designed for experiments that use Int recombination as a probe of local, sequence-dependent structure on the tertiary structure of supercoiled DNA.

Nicking of the supercoiled recombination products by limited DNase I digestion is necessary in order to achieve electrophoretic resolution of knotted or catenated DNA molecules. The mixture of nicked products separates into a ladder of bands in agarose gels, as shown in **Fig. 3**. Unlike the pattern observed with linking number topoisomers in one-dimensional agarose-gel electrophoresis, the spacing between successive knot or catenane bands is nearly constant, so that the mobility of a particular product is effectively proportional to the number of minimal crossings. Although the physical basis for the gel electrophoretic separation of knotted and catenated forms is not presently understood, the resolution achievable by gel electrophoresis at low field strengths is remarkable. Knots and catenanes can be separated over a very wide range of *Kn* or *Ca* values, and there is very little compression of knot or catenane ladders even for high *Kn* or *Ca*. Moreover, agarose gels can resolve even slight mobility differences between knots that have identical *Kn* values but belong to different knot types, for example, torus and twist knots ***(14)***. In general, the structure of knots or catenanes of unknown type should be characterized by electron microscopy of RecA protein-coated DNA in conjunction with gel electrophoresis ***(15)***.

2. Materials

2.1. Plasmid DNA

DNA substrates were derived from the plasmid pGEM-7Zf(+) by inserting a 640-bp *attP*-containing *Bam*HI/*Nde*I fragment obtained from pJB3.5d ***(2)*** into the *Bam*HI/*Hin*dIII region of the multiple cloning site of pGEM-7Zf(+). An 850-bp, *Bgl*II/*Pvu*I, *attB*-containing fragment obtained from pAB7.0d ***(4)*** was modified to generate *Eco*RI ends and inserted into the *Eco*RI site of the pGEM-7 multiple cloning region. Single colonies were screened for both the presence and the orientation of the *attB* fragment. The *attB* site is positioned nearly equidistantly from the *Eco*RI sites. Therefore, the result of cloning the fragment in either orientation is a pair of 4.5-kb plasmids with the recombination sites each located approx 425 bp to either side of the *Sma*I site of the multiple cloning region. The plasmids with sites in inverse and direct orientation are designated patt4.5i and patt4.5d, respectively.

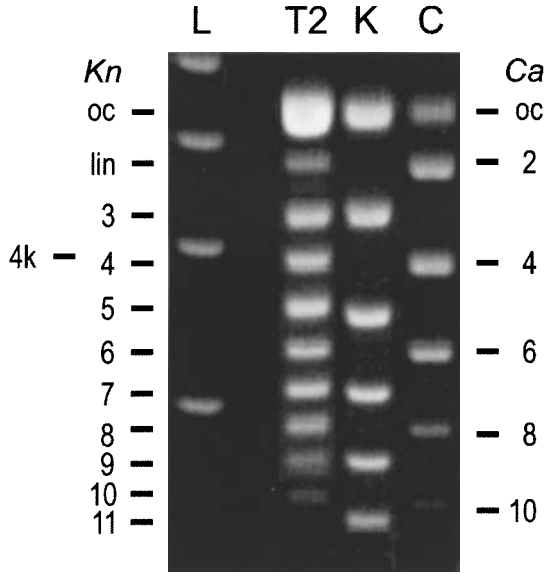

Fig. 3. Agarose-gel electrophoresis of nicked knots and catenanes. Plasmids patt4.5i and patt4.5d were incubated with Int and IHF as described, subjected to limited nicking with Dnase I in the presence of ethidium bromide, and separated on a 0.8% agarose gel. Electrophoresis was carried out for 17 h at 2.5 V/cm^{-1}. Lanes are labeled as follows: L, 1-kb ladder fragments; T2, reference knot ladder generated by incubating patt4.5d with stoichiometric quantities of T2 topoisomerase II ***(22)***; K, ladder of knotted products generated by Int recombination of patt4.5i; C, ladder of catenated products generated by Int recombination of patt4.5d. Values of *Kn* and *Ca* corresponding to the bands are indicated on either side of the figure; oc indicates the position of open-circular DNA. The Int-generated products in lanes K and C are separated by steps-of-two spacing owing to the fact that Int recombination generates knotted and catenated products belonging exclusively to the torus class; these forms have only odd and even numbers of crossings, respectively. Knots generated by T2 topoisomerase II belong to the twist class and, therefore, are separated by steps-of-one spacing. The band appearing between the *Kn* = 3 and oc bands of the T2 ladder, designated lin, consists of linear byproducts of the nicking reaction. The position of a 4-kb linear molecule is indicated on the left.

2.2. Recombination Proteins

2.2.1. λ Integrase

Int protein is purified from an inducible strain, EM424/pSX1-2 ***(16)***, according to a modification of the procedure of Nash ***(17)***. Briefly, a 3-L culture of cells is induced at an OD_{660} of 0.4–0.6 with 0.5 m*M* IPTG. The cells are pelleted, resuspended in 25 mL sonication buffer (50 m*M* Tris-HCl, 1 m*M*

2-mercaptoethanol, 10% sucrose, pH 7.6), and lysed. The crude protein fraction is recovered from the supernatant after centrifugation at 3000*g* and subjected to two differential centrifugation steps described by Nash ***(17)***. The Int-containing fraction is then applied to a 1 × 20 cm phosphocellulose column (Whatman P11) and eluted with a 10-column volume gradient of 0.6–1.2 *M* KCl in 50 m*M* Tris-Cl, 1 m*M* Na_2EDTA, 1m*M* 2-mercaptoethanol, and 10% (w/v) glycerol. Peaks in the elution profile are examined for recombination activity and pooled accordingly. This pooled fraction contains Int protein that is 65–75% homogeneous as determined by SDS-polyacrylamide gels visualized with Coomassie blue or silver stain; principal contaminants are small proteins, possibly subunits of IHF. These contaminants are removed either by hydroxyapatite chromatography as described by Robertson and Nash (personal communication) or by gel-filtration chromatography on a Sephacryl S-200HR (26/60) column. In the latter, we have found that a flow rate of 1 mL/min gives excellent resolution. The resulting Int protein is >98% homogeneous as assayed by silver-stained SDS-polyacrylamide gels.

2.2.2. Integration Host Factor (IHF)

IHF consists of two nonidentical subunits and is purified from an overproducing strain, HN880, essentially according to the published procedure of Nash et al. ***(18)***. We have found that this procedure leaves a residual nicking activity; this activity is removed by FPLC on a Pharmacia MonoS HR5/5 column, using a gradient of 0–1.2 *M* KCl in 50 m*M* Tris-HCl, 1 m*M* Na_2EDTA, 1 m*M* 2-mercaptoethanol, and 10% (w/v) glycerol, pH 7.4. IHF prepared by this procedure is at least 80–90% homogeneous.

2.3. Reagents

1. Int recombination buffers: (–Mg): 10 m*M* Tris-HCl, 50 m*M* NaCl, 5 m*M* spermidine, 1 m*M* Na_2EDTA, pH 7.5; (+Mg) 20m*M* Tris-HCl, 50 m*M* NaCl, 20 m*M* KCl, 10 m*M* $MgCl_2$, pH 7.6.
2. Dnase I nicking buffer: 20 m*M* Tris-HCl, 2 m*M* $MgCl_2$, 5% (w/v) glycerol, pH 7.6.
3. Dnase I: Pancreatic DNase I (Sigma) is suspended in 20 m*M* Tris-HCl, 5 m*M* NaCl, 0.1 mM dithiothreitol, 50% (w/v) glycerol, pH 7.6, at a concentration of 1 mg/mL. The enzyme is stored in 5-µL aliquots at –80°C, diluted immediately before use, and subsequently discarded.
4. TBE electrophoresis buffer: 50 m*M* Tris-borate, 1 m*M* Na_2EDTA, pH 8.5. To ensure reproducibility, the conductivity of each batch of electrophoresis buffer is monitored; typical values are in the range 8.8 ± mS/cm.
5. Agarose: 0.7–1.0% agarose gels are prepared from SeaKem® LE agarose (FMC Corporation) in TBE buffer. A suspension containing the appropriate amount of agarose in buffer is weighed prior to boiling. The agarose solution is cooled to

65°C in a water bath for at least 15 min, and the mass of the solution is readjusted with distilled water prior to casting the gel. Agarose gels are allowed to stand for 1–2 h at room temperature before use.

3. Methods

3.1. Plasmid Purification

Plasmid DNA can be isolated from *E. coli* strain DH5α by using the alkaline lysis method ***(19)***. After banding twice by CsCl-ethidium bromide density gradient centrifugation (*see* **Note 1**), the DNA was dialyzed extensively against TE buffer (10 m*M* Tris-HCl, 1 m*M* Na_2EDTA, pH 8.0) at 5°C and distributed into aliquots. Plasmids derived from the pGEM series of cloning vectors have a very high copy number; the overall yield of plasmid DNA is generally in the range of 4–8 mg DNA/pL of culture.

3.2. Recombination Reactions

1. Reaction mixtures containing 2 µg DNA, 200 ng Int, and 240 ng IHF in 20 µL of recombination buffer are incubated at 22°C for 30 min.
2. Inactivate the reaction by incubating at 65°C for 5 min.
3. Extract twice with phenol, once with chloroform-isoamyl alcohol (24:1), and recover the DNA by ethanol precipitation.
4. Remove residual supercoiling in the recombination products by limited nicking with DNase I in the presence of ethidium bromide ***(20)***. Nicking reactions contain 1 µg DNA, 300 µg/mL ethidium bromide, and 2 ng of DNase I in 30 µL DNase I nicking buffer. Reactions are allowed to proceed at 30°C for 15 min, and held on ice while the progress of the reaction is checked on a 0.8% agarose minigel. After significant amounts of supercoiled DNA are no longer visible, usually no more than 30 min, the reaction is inactivated at 65°C, extracted three times with phenol, once with chloroform-isoamyl alcohol, and the DNA recovered by ethanol precipitation.
5. Resuspend the pellet in 20 µL of TE buffer.

3.3. Gel Electrophoresis

1. Approximately 0.5 µg of recombination reaction products is subjected to electrophoresis in 0.7–1.0% agarose gels run in TBE buffer (50 m*M* Tris-borate, 1 m*M* Na_2EDTA, pH 8.4). Electrophoresis is carried out with an applied field of 2.0–2.5 V/cm at room temperature for 16–20 h with buffer recirculation (*see also* **Notes 2** and **3**).
2. Stain gels with 0.5 µg/mL ethidium bromide for 15 min, and destain in TBE buffer for 30–60 min.

3.4. Gel Quantitation and Analysis

Routine quantitation is carried out by capturing a digital image of the ethidium-stained gel by using a Peltier-cooled CCD camera (Biophotonics). The CCD camera has a dynamic range of over 100:1, at least fourfold greater than that for Polaroid negatives, and has spatial resolution of 640 (horiz.) × 480 (vert.) pixels. Quantitation of individual bands is obtained by analyzing the digital image using ImageQuant software (Molecular Dynamics, Inc.) (*see also* **Note 4**).

4. Notes

1. In order to maximize the efficiency of Int recombination and, therefore, the yield of knotted or catenated products, plasmid purification should be carried out with the goal of maximizing yields of supercoiled DNA. It is our experience that CsCl-ethidium bromide density gradient centrifugation remains the purification method of choice. We find that nicking of the plasmid on long-term storage can be reduced if the DNA is stored as an ethanol slurry at –20°C. The plasmid is recovered from the slurry by pelleting at 15000*g* and is subsequently reconstituted with TE or other buffer before use.
2. Incomplete nicking can occasionally leave significant traces of supercoiled DNA, which can interfere with quantitation if the supercoiled plasmid comigrates with one of the knotted or catenated products. This problem can be eliminated by inclusion of low concentrations of chloroquine phosphate (0.9 μg/mL) in the gel and electrophoresis buffer.
3. We find that the resolution obtained by using TBE-agarose gels is comparable to that obtained with Tris-acetate-SDS gels ***(1)***. Avoiding the use of SDS in electrophoresis obviates high ethidium fluorescence background levels that arise from residual detergent and interfere with quantitation.
4. Greater dynamic range and higher resolution than that available from CCD-camera detection can be achieved by Southern hybridization and quantitation using a phosphorimager ***(21)***.

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10

Sedimentation Analysis of Bacterial Nucleoid Structure

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1. Introduction

The physiology of bacterial DNA topoisomerases can be studied by examining how perturbation of intracellular enzyme activities affects the structure of extracted nucleoids. Since the few DNA nicks that occur when nucleoids are isolated ***(1,2)*** are localized by the presence of 50–100 barriers to strand rotation ***(2,3)***, it is possible to recover chromosomal DNA in which most of each molecule is topologically constrained ***(2,4)***. Consequently, intracellular changes in topoisomerase activity can be detected as differences in the average supercoiling of nucleoids isolated from cells perturbed in different ways. This general strategy has been used to show that supercoiling is relaxed by inhibition of gyrase ***(5–7)*** and that it is increased (becomes more negative) by point mutations in *topA* (the gene encoding topoisomerase I), by low concentrations of gyrase inhibitors, and by anaerobic conditions ***(6,8–10)***. Experiments of this type have contributed to the conclusions that (1) supercoiling is controlled in part by regulated expression of the gyrase and topoisomerase I genes, and (2) the overall level of supercoiling responds to growth environment (reviewed in ***[11]***).

Experimentally, changes in supercoiling are detected by titration with an intercalating dye, usually ethidium bromide ***(2)***. For this type of analysis, nucleoids are extracted and placed on a series of sucrose density gradients containing various concentrations of ethidium bromide. Nucleoid sedimentation rate decreases with increasing ethidium bromide concentration until a minimum is reached. At that point, termed the critical dye concentration, all of the supercoils are removed. As the dye concentration continues to increase, positive

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supercoils are introduced by the dye, and the sedimentation rate increases. The ethidium bromide concentration at the sedimentation minimum is related to superhelix density.

For supercoiling studies with *Escherichia coli,* nucleoids are isolated in 1 *M* NaCl at 20–25°C to minimize the contribution of protein to nucleoid sedimentation rate and to yield a nucleoid sedimentation coefficient (1500 S) close to that of a convenient sedimentation marker (bacteriophage T4; S = 1025). The major protein associated with nucleoids obtained in this way is RNA polymerase ***(1,4)***. Methods are also available for isolating membrane-associated nucleoids. They may be useful for studying topoisomerase IV, since this enzyme is thought to be membrane-associated ***(12)***. To recover membrane-associated nucleoids, cell lysis temperature is reduced to about 10°C. Under these conditions, the sedimentation coefficient of the nucleoid increases from 1500 to 3000–4000 S ***(13)***. Intact, membrane-bound nucleoids can also be isolated from cells lysed at low salt concentration if spermidine is included in the lysis mixture and if temperature is maintained at about 10°C (14). Under these conditions the sedimentation coefficient is about 5800 S ***(15)***.

One of the distinctive features of DNA gyrase and topoisomerase IV is their ability to form complexes with DNA and members of the 4-quinolone class of antibiotic ***(16–19)***. The quinolones appear to trap a reaction intermediate in which the DNA is broken, but the ends are held together by either gyrase or topoisomerase IV, preventing spontaneous relaxation of supercoils ***(20)***. Treatment of nucleoids with sodium dodecyl sulfate (SDS) after cell lysis denatures the topoisomerases and releases nucleoid DNA as large fragments. Sedimentation analysis provides an estimate of the size of the fragments and thus information about the chromosomal distribution of gyrase and topoisomerase IV ***(20,21)***. For this type of analysis, cell lysates are diluted to lower the salt concentration, then sedimented into sucrose density gradients containing SDS. Quantitative determination of DNA size can be made by comparison with marker DNA of known size ***(20)***. Qualitative comparisons can be made by mixing lysates from cells treated in different ways and labeled with different radioactive isotopes, typically [^{14}C]thymidine and [^{3}H]thymidine ***(22)***.

The following sections describe in detail methods for isolating nucleoids, for comparing the amount of negative supercoiling in different nucleoid samples, and for determining the molecular weights of large DNA fragments generated by quinolone inhibitors of gyrase and topoisomerase IV.

2. Materials

All chemicals are reagent-grade. Sodium dodecyl sulfate (SDS), egg white lysozyme and Brij-58 (polyoxyethylene 20 cetyl ether) are products of Sigma Chemical Co. (St. Louis, MO). Polyallomer centrifuge tubes ($^{1}/_{2} \times 2$ in. tubes

suitable for Beckman SW50.1 rotors [*see* **Note 1**]) are obtained from Seton Scientific (Sunnyvale, CA). A suitable scintillation fluid for determining radioactivity in sucrose-containing aqueous samples contains 4 g 2,5 diphenyloxazole (PPO) and 0.2 g 1.4-bis (4-methyl-5-phenyl-2-oxazolyl) benzene (dimethyl POPOP) dissolved in 1 L of toluene to which are added 500 mL Triton X-100 and 200 mL water.

3. Methods

3.1. Preparation of [^{14}C]-labeled Bacteriophage T4B and T4B DNA

1. Grow *E. coli* B in M9 medium ***(23)*** containing 20 µg/mL tryptophan to a density of about 6×10^8 cells/mL at 37°C.
2. Add phage at a multiplicity of infection of 1.
3. After 7 min, add another dose of phage at the same multiplicity of infection.
4. After 5 more min, add 25 µCi [^{14}C]thymidine/50 mL culture.
6. Continue growth for 3 h, and then add a few drops of chloroform.
7. Chill the lysate at 4°C for 30 min.
8. Incubate with shaking at 37°C for 10 min.
9. Chill on ice, and remove cellular debris by centrifugation at 8000*g* for 10 min.
10. Harvest phage by centrifugation at 10,000*g* for 45 min.
11. Resuspend the phage pellet in phage diluent (0.2 *M* NaCl, 0.01 *M* Tris-HCl, pH 8.0, 0.01 *M* $MgCl_2$) at 4° C with gentle shaking overnight.
12. Layer the phage suspension onto a 10–30% (w/v) sucrose density gradient containing 1 *M* NaCl, 0.02 *M* Tris-HCl, pH 8.1, and 0.01 *M* EDTA, and centrifuge at 27,000*g* (17,000 rpm with a Beckman SW50.1 rotor) for 30 min at 4°C.
13. Remove the phage band from the gradient by pipet; reduce salts and sucrose by dialysis overnight at 4°C against phage diluent (*see* **Note 2**).
14. Prepare phage DNA by extracting [^{14}C]labeled phage with a phenol/chloroform mixture containing 0.5% SDS followed by dialysis against 0.01 *M* Tris-HCl, pH 8.0, and 0.001 *M* EDTA (*see* **Note 3**).

3.2. Preparation of Sucrose Solutions and Density Gradients

3.2.1. Ethidium Bromide-Containing Sucrose Gradients

1. Prepare stock solutions of 10 and 30% (w/v) sucrose containing 1 *M* NaCl, 0.02 *M* Tris-HCl, pH 8.1, and 0.01 *M* EDTA.
2. Transfer aliquots to volumetric flasks for preparation of solutions having specified concentrations of ethidium bromide.
3. Add an appropiate amount of ethidium bromide from a stock solution at 10 mg/mL prepared in water, and add sucrose solutions to make the final volume (*see* **Note 4**).
4. Place 200 µL 60 % (w/v) sucrose in the bottom of each centrifuge tube (*see* **Note 5**).

5. Prepare 5-mL linear density gradients in subdued light with a gradient maker in which mixing occurs in the high-density reservoir that exits to the centrifuge tube.
6. Store gradients at 4°C until used later the same day.

3.2.2. SDS-Containing Sucrose Gradients

1. Prepare solutions containing 5 and 20% sucrose in 0.1 *M* NaCl, 0.05 *M* sodium phosphate buffer pH 6.8, and 0.5% SDS.
2. Place 200 µL 60 % (w/v) sucrose plus 0.5% SDS in the bottom of each centrifuge tube (*see* **Note 5**).
3. Prepare 5-mL density gradients with gradient maker in which mixing occurs in the high-density reservoir that exits to the centrifuge tube.
4. Store gradients at room temperature (gradients are generally used the same day that they are prepared).

3.3. Cell Growth and Radioactive Labeling of DNA

1. Grow cells in liquid medium (LB or M9) to midlog phase.
2. Add [^{3}H]thymidine (10 µCi/mL culture) or [^{14}C]thymidine (15 µCi/mL culture) .
3. Continue growth for about 0.5 generation to label chromosomal DNA radioactively.

3.4. Cell Lysis

The following methods have been developed for *E. coli* K-12. Adjustments will be necessary for other bacteria.

3.4.1. Membrane-Free Nucleoids

1. Rapidly chill radioactively labeled cells (4 mL), and concentrate by low-speed centrifugation (5000*g* for 5 min at 4°C).
2. Resuspend in 0.1 mL 0.01 *M* Tris-HCl, pH 8.1, 20% (w/v) sucrose, and 0.1 *M* NaCl in a glass tube on ice.
3. Immediately add 25 µL of a 4 mg/mL freshly prepared solution of egg white lysozyme in 0.1 *M* Tris-HCl, pH 8.1, 0.05 *M* EDTA.
4. Incubate on ice for about 30–60 s (*see* **Note 6**).
5. Dilute two-fold by addition of 125 µL chilled 2 *M* NaCl, 0.01 *M* EDTA, 0.4% sodium deoxycholate, and 1% Brij-58.
6. Incubate at 20–25°C until turbidity decreases substantially (*see* **Note 6**).

3.4.2. High-Salt, Membrane-Associated Nucleoids

Carry out cell lysis as described in **Subheading 3.4.1., step 6** at 10°C rather than 20–25°C ***(13)***.

3.4.3. Spermidine-Dependent, Membrane-Associated Nucleoids

1. Grow cells to midlog phase and harvest 160 mL of cells by centrifugation (5000*g* for 10 min at 4°C).

2. Resuspend on ice in 0.5 mL 0.01 *M* Tris-HCl, pH 8.2, 0.1 *M* NaCl and 20% (w/v) sucrose.
3. Add 0.1 mL of 4 mg/mL freshly prepared egg white lysozyme in 0.12 *M* Tris-HCl, pH 8.2, and 0.05 *M* EDTA.
4. Incubate on ice for 40 s.
5. Add 0.5 mL 1% Brij-58, 0.4% deoxycholate, 0.01 *M* EDTA, and 10 m*M* spermidine-HCl.
6. Incubate at 10°C for 3 min (*see* **Note 7**).

3.5. Centrifugation and Gradient Fractionation

3.5.1. Isolation of Nucleoids

1. Chill rotor, buckets, and centrifuge chamber.
2. After cell lysis is complete (**Subheading 3.4.**), place the preparation on ice.
3. Immediately layer aliquots onto chilled sucrose density-gradients (**Subheading 3.2.**; *see* **Note 8**).
4. Begin centrifugation as soon as possible, usually within 5–10 min after lysis is complete (*see* **Note 9** for conditions for different nucleoid types).
5. Fractionate gradients either from the top or the bottom into chilled tubes (*see* **Note 10).**

3.5.2. Titration of Negative Supercoils

1. Layer a small (10 µL) aliquot of [^{14}C)-labeled bacteriophage T4B onto a series of 5-mL linear 10–30% (w/v) sucrose density gradients containing 1 *M* NaCl, 0.025 *M* Tris-HCl, pH 8.0, 0.01 *M* EDTA, and ethidium bromide ranging from 0–4 µg/mL in 0.25 µg/ml increments (**Subheading 3.2.1.**; *see* **Note 11**).
2. Immediately after cell lysis, load aliquots (20 µL) of [^{3}H]-labeled, chilled lysate (**Subheading 3.4.1.**) onto each sucrose gradient.
3. Centrifuge the samples for about 30 min at 27,000*g* (17,000 rpm [Beckman SW50.1 rotor]) at 4°C (*see* **Note 12**).
4. Fractionate gradients and measure radioactivity in each fraction (**Subheading 3.5.4.**; for examples, *see [5]*).

3.5.3. Fragmented DNA (See Note 13)

1. Radioactively label, harvest, and lyse cells as described for membrane-free nucleoids (**Subheadings 3.3.** and **3.4.1.**).
2. After lysate turbidity has disappeared, chill lysates on ice and dilute 14-fold with 0.02 *M* Tris-HCl, pH 8.1, and 0.01 EDTA on ice.
3. Very gently mix the suspension.
4. Add SDS to a final concentration of 0.5%, and mix using gentle rolling to minimize DNA breakage.
5. Immediately bring solutions to room temperature to avoid precipitation of the SDS.
6. Determine the amount of sample to be applied to gradients by measuring acid-precipitable radioactivity in a small (10-µL) aliquot (**Subheading 3.5.4., steps 4–7**).

7. Load between 1000 and 20,000 cpm of acid-precipitable radioactivity onto sucrose gradients maintained at room temperature (*see* **Note 14**).
8. Perform centrifugation (*see* **Note 15**).

3.5.4. Fractionation of Sucrose Density-Gradients

1. Puncture bottom of polyallomer tubes with an 18-gauge needle passed through a rubber stopper (*see* **Notes 16** and **17**).
2. Apply positive pressure to the top of the gradient to regulate the flow.

3a. If centrifugation is used to prepare nucleoids for further analysis, collect samples in tubes on ice (*see* **Note 10**).

3b. If centrifugation is used analytically, as when supercoils are being titrated, collect fractions directly into scintillation vials for determination of radioactivity (*see* **Note 18**) or on numbered Whatman no. 2 filters (4.25-cm diameter) arranged on a filter support comparable to a "bed of nails" (*see* **Note 19**).

4. If filters are used, dry and place in a beaker.
5. Precipitate DNA on the filters by batchwise treatment with ice-cold 10% trichloroacetic acid.
6. Wash filters twice with 1 *N* HCl, once with water, and twice with 95% ethanol, all at 4°C.
7. Dry filters and place in scintillation vials for determination of radioactivity.

3.6. Analysis

3.6.1. Determination of Relative Superhelix Density

Plots of nucleoid sedimentation coefficient as a function of ethidium bromide concentration show that sedimentation coefficients decrease, reach a minimum, and then increase as ethidium concentration increases. As pointed out in **Subheading 1.**, the ethidium bromide concentration at the sedimentation minimum is related to superhelix density. To obtain values for the average nucleoid sedimentation coefficient for such "ethidium titration" plots, the distance sedimented by the nucleoids is determined relative to the distance sedimented by the bacteriophage marker (for examples, *see* ***[5]***). These distances are determined from plots of radioactivity as a function of fraction number for each density gradient. In most situations, it is adequate to assume that sedimentation rate is linear and drop size is uniform (this may not be true if lysate volumes are large, since they contain detergents that change drop size). Visual inspection of radioactivity profiles is adequate for determining sedimentation rates of nucleoids. However, the quality of the data is sensitive to the amount of care taken. For example, it is important to correct for the last fraction being only partial, as is often the case.

Although the ethidium titration curves described above can be used to detect supercoiling differences in nucleoids extracted from cells treated in different ways, analyses carried out with small circular DNAs provide the basis for

quantitative considerations ***(24,25)*** important for nucleoid studies. With small DNAs, the amount of dye bound to DNA can be readily determined, and so superhelix density can be calculated from measurement of υ_c, the amount of dye bound per nucleotide at the sedimentation minimum ***(24)***. A value for υ_c can be estimated from:

$$\upsilon_c = (\upsilon_m k C_f)/(1 + k C_f) \quad (1)$$

if C_f, the free dye concentration at the sedimentation minimum, is known (k is a constant that is taken as 0.98×10^5 L/mol for gradients containing 1 *M* NaCl; υ_m is the maximum amount of dye that can be bound, which in 1 *M* NaCl is 0.18 mol of ethidium bromide/pmol of DNA nucleotide ***[26]***). At low DNA concentration, it can be assumed that free dye concentration (C_f) equals total dye concentration (C_T). Thus, **Eq. 1** becomes:

$$\upsilon_c = (\upsilon_m k C_T)/(1 + k C_T) \quad (2)$$

In **Eq. 2**, C_T is the ethidium bromide concentration that generates the minimum sedimentation coefficient described above.

For nucleoid studies, one is generally interested in the percent change in supercoiling owing to the intracellular perturbation of topoisomerases. Since the dye bound per DNA nucleotide is not related linearly to free dye concentration or total dye concentration, the percentage difference in supercoiling cannot be calculated directly from the difference in dye concentrations at sedimentation minima for two cellular perturbation. First, **Eq. 2** must be solved for υ_c at the observed value of C_T for each cellular condition, using the values given above for k and υ_m. The resulting values of υ_c can then be used to estimate the percentage difference in nucleoid superhelix density.

It is important to stress that the data represent average values with respect to both the chromosomal population and the topologically independent domains of the chromosome ***(2,3)***. Broadening of the titration trough indicates an increase in heterogeneity, a feature that has been observed with plasmid DNA ***(27)*** and nucleoids (Drlica, unpublished observations).

3.6.2. Determination of DNA Fragment Size

The first step is to plot radioactivity carefully as a function of fraction number (counting from the top of the gradient). When DNA samples from two different treatments are sedimented in the same sucrose density gradient, even small differences will be apparent ***(22,28)***. Quantitative analysis requires determination of number average molecular weight, M_n. According to Van Holde ***(29)***:

$$M_n = \Sigma \text{ cpm} / \Sigma (\text{cpm}/M_r) \quad (3)$$

where M_n is number average molecular weight, cpm is the radioactivity (mass) of DNA at each fraction containing DNA, and M_r is the molecular weight of

DNA at each fraction in the gradient. By including a homogeneous, standard DNA of known size in each gradient, it is possible to calculate M_r for each fraction containing chromosomal DNA fragments. A suitable standard is bacteriophage T4 DNA, which has a mol wt of 132×10^6 ***(29a)***. The value of M_r at any fraction (y) is related to M_r of bacteriophage T4 DNA by the relationship:

$$y \,/\, T4 = (M_r \,/\, 132 \times 10^6)^{0.35} \tag{4}$$

where T4 is the fraction number corresponding to the midpoint of the bacteriophage T4 DNA band. The exponent 0.35 was determined experimentally ***(30)***; estimates with small DNA put the exponent at 0.38 ***(31)***.

When we compared sedimentation rates of bacteriophage T7 DNA and bacteriophage T4 DNA using 5–20% linear sucrose gradients in an SW50.1 rotor, we found a slight deviation from constant sedimentation velocity (a consideration of nonlinear sedimentation can be found in **ref. *32***). Before calculating M_n, we generally correct each fraction for nonlinear sedimentation according to the equation

$$y = 0.9438x + 0.2039 \tag{5}$$

where x is the observed fraction number (counted from the top of the gradient and corrected for the last fraction collected being only partial, if that is the case). Plots of M_n as a function of DNA concentration and rotor speed, with extrapolation to zero in each case complete the analysis. Rotor speed effects are insignificant for DNA molecules smaller than bacteriophage T4 DNA at speeds below 19,000g (14,000 rpm with Beckman SW 50.1 rotor).

4. Notes

1. Sucrose density gradient centrifugation is generally performed with swinging bucket rotors. Aged rotors sometimes have one or two buckets that do not swing freely, and that disturbs the gradients during acceleration and deceleration. Such buckets should be avoided. Rotors and centrifuge chambers should always be chilled prior to use unless solutions contain SDS.
2. Phage can be readily detected as a bluish band in the gradient when light is directed into the gradient from the top. The phage preparation procedure generally produces virus with high specific activity (500–1000 cpm/µL) such that 5–10 µL added to each sucrose gradient is sufficient for detection as a sedimentation marker. Phage particles prepared as described are stable for many months.
3. Radioactive phage DNA is much less stable than intact phage; it should be used within weeks after preparation.
4. Since it is important that the ethidium bromide concentration be accurately known, absorption at 285 nm is determined. When absorbance vs ethidium bromide concentration is plotted, all points should fall on a straight line with the absorbance of 2 µg/mL ethidium bromide being 0.275 at 285 nm. Ethidium bromide solutions stored in the dark at 4°C are stable for supercoil titrations for several months.

5. The 60% sucrose acts as a cushion that prevents rapidly sedimenting DNA from migrating to the bottom of the tube and escaping detection.
6. If turbidity fails to drop within 5 min (**step 6, Subheading 3.4.1.**), subsequent trials should include increased time of lysozyme treatment and/or increased temperature (1–2ºC) at **step 6**. If turbidity drops sharply before 3 min in high salt-detergent solution **(step 6)**, nucleoid yields are often low owing to aggregation. In such cases, it may be necessary to reduce the time in lysozyme (**step 4**). For some strains, it may be necessary to include 0.5% Sarkosyl in the detergent solution to obtain complete cell lysis. Early procedures often included a brief centrifugation step (5000*g* for 5 min) after cell lysis to remove aggregated nucleoids and debris. Since as much as 50% of the cellular DNA is discarded by this procedure, generalizations derived from such analyses are not as firm as when all of the cellular DNA is analyzed. Thus, this centrifugation step is generally omitted.
7. Often, little change in turbidity will be seen, so the success of the lysis procedure is determined only after centrifugation (**Subheading 3.5.1.**). The procedure described produces high concentrations of nucleoids suitable for enzymatic studies ***(14)***.
8. To avoid shearing the DNA, pipet tips should be cut to have an opening diameter of 1 mm or greater.
9. For isolation of membrane-free nucleoids, appropriate centrifugation conditions are 27,000*g* (17,000 rpm for a Beckman SW 50.1 rotor) for 30 min at 4°C in 5-mL linear 10–30% (w/v) sucrose density gradients containing 1 *M* NaCl, 0.025 *M* Tris-HCl, pH 8.0, and 0.01 *M* EDTA. The volumes listed for cell lysis (**Subheading 3.4.1.**) can be increased proportionately to obtain large amounts of membrane-free nucleoids ***(33)***. Up to 1 mL lysate can be loaded on a 4-mL preparative sucrose density gradient. For isolation of membrane-associated nucleoids, centrifugation time is reduced to about 17 min ***(13)***. Other aspects of the procedure are the same as for membrane-free nucleoids. For nucleoids prepared in the presence of spermidine ***(14)***, the suspension of lysed cells (about 1.2 mL) is subjected to centrifugation for 17 min at 9000*g* at 4°C through a 5-mL 12–60% (w/v) sucrose density gradient containing 0.01 *M* Tris-HCl, pH 8.2, 1 m*M* 2-mercaptoethanol, 1 m*M* EDTA, and 5 m*M* $MgCl_2$.
10. Nucleoids remain compact for several hours if maintained on ice, and under these conditions, the DNA is not broken by gentle pipeting. After centrifugation and fractionation, the position of the nucleoids in the gradient can be determined by measurement of absorption of UV light at 260 nm or by determining the radioactivity of small aliquots following acid precipitation (**Subheading 3.5.4., steps 4–7**). Spermidine-dependent membrane-bound nucleoids can be seen as a light-scattering band in the gradient by shining a light vertically through the centrifuge tube. These nucleoids can be removed by pipet.
11. The phage serves as a sedimentation marker (S_{T4} = 1025 S ***[34]***). The high concentration of NaCl is required to keep the nucleoids in a compact configuration.
12. Centrifugation time is adjusted to sediment the nucleoids slightly more than half the distance to the bottom of the tube when ethidium bromide is absent.

13. The average size of large DNA fragments can be estimated by sedimentation analysis in 5-mL 5–20 % (w/v) sucrose density gradients containing 0.1 *M* NaCl, 0.05 *M* sodium phosphate buffer (pH 6.8), and 0.5% SDS. Topoisomerase applications generally involve DNA size estimates following treatment of cells with quinolone inhibitors of gyrase and topoisomerase IV ***(20,21)***.
14. If very large DNA fragments are to be examined, DNA shearing must be minimized when samples are layered onto sucrose density gradients. In one procedure, lysates are transferred to a piece of parafilm. SDS is added, and the parafilm is placed on the top of the gradient. By gently tilting the parafilm and pulling it off the sucrose solution, the sample is left on the top of the density gradient. An alternate procedure is described in **ref. *35*.**
15. Since sedimentation coefficient varies with DNA concentration ***(32)*** and rotor speed ***(36,37)***, it is necessary to extrapolate to zero DNA concentration and zero rotor speed from a series of sucrose density gradients. The concentration range chosen depends on the accuracy required. Three or four speeds ranging from 1000–6000*g* have proved to be sufficient with Beckman SW50.1 rotors. For low rotor speeds, centrifuge tachometers are often not accurate; consequently, it may be necessary to time several thousand revolutions and calculate the revolutions per minute.
16. Gradients can be fractionated from either top or bottom. In ethidium bromide titration experiments, many gradients must be processed; in our experience, manual collection from the bottom of the tubes is the quickest (about 24 fractions are collected from each gradient, which requires about 5 min).
17. Care should be taken to avoid disturbing the gradients; a bubble passing through a gradient will generally render the data unusable.
18. For sucrose-containing aqueous samples, a Triton X-100-containing scintillation cocktail (**Subheading 2.**) is used. Sucrose gradient fractions of 200 µL plus 4 mL of scintillation cocktail are clear after a brief, mandatory shaking. If precipitation occurs, change temperature. After storage, concentrated stock solutions of Triton X-100 tend to be more concentrated at the bottoms of containers. Always shake thoroughly before use in preparation of scintillation cocktails. For filters (**Subheading 3.5.4., step 7**), a cocktail lacking Triton X-100 and water is suitable. In this case, the vials need not be completely filled with scintillation fluid.
19. Under some conditions nucleoid sedimentation rates will be so slow that unincorporated [^{3}H]thymidine, located at the top of a gradient, will mask the nucleoids. In that case, it will be necessary to measure acid-precipitable radioactivity.

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11

Coating DNA with RecA Protein to Distinguish DNA Path by Electron Microscopy

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1. Introduction

To understand, at the molecular level, the mechanism of enzymes that act on DNA, it is highly informative to know the topology of their substrates and products. To describe fully the topology of a DNA knot or catenane, it is necessary to know the overpassing and underpassing segments when two DNA helices cross. Conventional microscopy rarely allows such resolution. Coating DNA with RecA protein ***(1,2)*** allows the unambiguous determination of a DNA crossover ***(3–5)***.

The method described in this chapter has been enormously useful in determining the stereostructure of DNA products of site-specific recombinases and topoisomerases, and thus in elucidating the mechanisms of these enzymes ***(3,5–14)***. Recently, the method was used to determine the structure of catenated DNA replication intermediates in vivo ***(15,16)***.

Knotted or catenated DNA molecules are common intermediates or products of several DNA metabolic processes in vivo. Indeed, analyses of these intermediates have provided a sensitive measurement of DNA structure, DNA effective concentration, and enzyme function in vivo ***(11,15,17–25)***.

The purpose of this chapter is to provide the reader with a detailed protocol for coating DNA with RecA protein for visualization in the electron microscope. We will not discuss classifications of DNA knots or catenanes ***(26,27)***, or the use of one-dimensional gel electrophoresis to separate various knots and catenanes (*see* Chapter 9). We highly recommend the review by Dröge and Cozzarelli for general tips for these methods ***(26)***. Before attempting this protocol, the reader should become well acquainted with the use of the evaporation chamber and the electron microscope ***(28)***.

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2. Materials

2.1. RecA Coating

1. Variable-temperature water bath.
2. Ring stand with small clamp.
3. Razor blade.
4. Wooden applicator stick: Bevel the tip with a razor blade.
5. Kimwipes.
6. Microcentrifuge tubes (0.4-mL capacity, 4 mm inner diameter, 5 cm height from USA/Scientific Plastics, Ocala, FL).
7. Nylon mesh (70 µm from Spectrum, Los Angeles, CA): Cut into squares of 1 cm^2.
8. Pipetman tips (1 mL, cut ~3 mm off tip with razor blade).
9. Eppendorf tubes (0.5-mL capacity).

2.2. Electron Microscopy

1. Copper grids with tabs (300 or 400 mesh) from Ted Pella (Redding, CA).
2. 0.25–0.5% Formvar solution in ethylene dichloride available from Ernest Fullam (Latham, NY).
3. Glass microscope slides (75 × 25 mm).
4. Glass dish (150 × 75 mm) (Kimax #23000).
5. Dissecting needle.
6. Parafilm or plastic wrap.
7. Petri dishes (60 × 15 mm).
8. Forceps (fine-tipped #3C, three or four pair).
9. Tungsten wire, 0.020-in. diameter.
10. Evaporator for glow discharge, carbon coating, and tungsten shadowing.
11. Pasteur pipet drawn out in a flame to <0.5 mm diameter.
12. Vacuum desiccator.

2.3. Reagents

All reagents should be made with the highest purity water available and solutions should be filter-sterilized.

1. 100% Ethanol.
2. 0.3 *M* potassium phosphate, pH 7.6, 30 m*M* EDTA, pH 8.0.
3. Glyoxal (40% aqueous solution from Sigma, St. Louis, MO) deionized using analytical-grade mixed-bed resin AG501- X8 (D) (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Store as small aliquots (10µL) at –20°C.
4. TE buffer: 10 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA, pH 8.0.
5. 40 m*M* triethanolamine (TEA), pH 7.6.

6. Sepharose CL-4B resin from Pharmacia (Piscataway, NJ): Keep at 4°C.
7. RecA protein (Pharmacia): Dilute to 1 mg/mL in 20 m*M* Tris-HCl, pH 7.5, 10% glycerol (w/v), 1 m*M* dithiothreitol, 0.1 m*M* EDTA, pH 8.0. Store in 10-µL aliquots at –70°C. Freeze and thaw only two to three times.
8. 5 m*M* Mg acetate.
9. 100 m*M* TEA, pH 7.6.
10. 8% Glutaraldehyde ampules (Poly Sciences, Warrington, PA). Just before use, dilute with 100 m*M* TEA, pH 7.6, to give 2% glutaraldehyde in 75 m*M* TEA, pH 7.6.
11. 100 m*M* NH_4 acetate.
12. 10 m*M* NH_4 acetate.
13. 5% Uranyl acetate.

3. Methods

3.1. Preparation of the Formvar-Coated Grids

Keep the formvar solution refrigerated. Allow the solution to come to room temperature before opening the bottle to avoid condensation. The formvar solution will absorb water over time and will have to be replaced every several months to avoid pits or holes in the film on the grids.

1. To prepare formvar films, put enough formvar solution to be approx 1 in. deep in a small beaker or wide mouth reagent bottle into which a glass microscope slide can be inserted. Wipe off the glass slide with a Kimwipe. Do not use water or ethanol to clean the slide or the formvar will stick to the glass. Dip the slide into the formvar, remove it, and let the slide air-dry. Score with a dissecting needle all four edges of each film on each side of the slide. Breathe, as if fogging glass, onto the formvar films, to aid removal from the glass, and slowly insert the slide perpendicular to the water surface into a large dish of water. The formvar films should float off the slide onto the surface of the water.
2. Carefully place the grids onto the floating film with the shiny side of the grids facing the film (down). With practice one is able to fit around 10–15 grids on a 1×1 in. film. To remove the grids and formvar film from the water, use a piece of Parafilm larger than the film. Touch it quickly to the grids and underlying formvar, and lift out of the water with the grid side up. The grids and formvar will be stuck to the Parafilm. Alternatively, depending on the humidity in the room, cover a 50-mL beaker with plastic wrap, dip the wrap against the grids and formvar, and lift up. Let the formvar-covered grids dry on the Parafilm or plastic wrap. Using forceps, transfer the grids, with the formvar-coated side up, to a clean glass slide. Do not put the Parafilm or plastic wrap in the evaporator. Stabilize the formvar film with a thin coating of carbon in the evaporator. Grids can be stored indefinitely at room temperature without desiccation. Protect the grids from dust by keeping the slides in a box or plastic Petri dish.

3.2. Denaturation of DNA

1. Denaturation reaction:
   6.0 µL of 100% ethanol
   4 µL of 0.3 *M* phosphate buffer$_4$, pH 7.6, 30 m*M* EDTA, pH 8.0
   2.1 µL of deionized glyoxal
   DNA
   Water to 30 µL
2. For plentiful DNA, use a DNA concentration of 10–20 µg/mL in the reaction. Use TE as the running buffer for the Sepharose CL-4B column.
3. For scarce DNA, use a DNA concentration of about 2.5 µg/mL in the reaction. Use 40 m*M* TEA as the running buffer for the column, so that the maximum volume of denatured DNA can be added to the RecA coating reaction.
4. Incubate the denaturation reaction at 62–63°C for 90 min. Use an expanded scale thermometer, if possible, to set the temperature accurately.
5. Run the reaction mix over a Sepharose CL-4B column in a small clamp on a ring stand.
   a. Set up the column in a 0.4-mL microcentrifuge tube. From the bottom of the tube, measure 3.5 cm and make a line with a marker. Then cut off a few millimeters of the bottom of the tube with a razor blade until the diameter of the hole is about 1 mm.
   b. Plug the bottom of the column with a piece of nylon mesh of about 1 cm^2. Use a wooden applicator stick (beveled at the tip) to wedge the nylon tightly into the bottom of the tube. Cut off any excess nylon that gets pushed out the hole. Wear gloves or minimize handling.
   c. Bring the Sepharose CL-4B to room temperature to avoid bubbles in the resin bed. It is convenient to keep a short-term supply of the resin at room temperature in a tube containing 0.02% Na azide to prevent bacterial growth.
   d. Fill the column roughly halfway with buffer. Add enough Sepharose CL-4B to fill the mini-Eppendorf tube column to the line. Wash the resin with at least 3–4 column volumes of the running buffer, TE, or 40 m*M* TEA. It is convenient to use a blue pipetman tip as a buffer reservoir for these columns. Cut off the bottom of the tip, so it fits tightly in the tube. Do not let the column run dry. Since it is difficult to cap the bottom of the column to prevent buffer flow, it is best to pour the column shortly before use and to keep it washing. Add or remove resin, if necessary, so that the top of the column bed is at the 3.5 cm mark on the tube.
   e. When you are ready to run the column, remove the buffer reservoir. When the buffer reaches the top of the resin bed, carefully add the 30 µL denaturation reaction. After the sample enters the resin, add 15 µL of running buffer and let it run in. Add a second 15-µL wash and let it run in. Then add a 75-µL wash. When this final wash reaches the top of the resin, quickly wipe off the drop of buffer hanging from the bottom of the column with a Kimwipe. Then add 40 µL of buffer, and collect the eluate in a 0.5-mL Eppendorf tube This

final 40-µL eluate should contain the DNA, which can be stored at –20°C. It is useful to calibrate a trial column with radiolabeled plasmid DNA.

3.3. Coating DNA with RecA

1. RecA-coating reaction (add the reagents in the order listed):
   6 µL of 100 m*M* TEA, pH 7.6, if the DNA is in TE. If the DNA is in 40 m*M* TEA, pH 7.6, do not add 100 m*M* TEA.
   Water, if needed.
   Denatured DNA. For scarce DNA, add up to 17 µL of the DNA in 40 m*M* TEA, pH 7.6, as eluted from the denaturation column.
   2 µL of RecA protein at 1 mg/mL (=83 µg/ml or 2.21 µ*M* in the reaction).
   5 µL 5 m*M* Mg acetate.
   24 µL final volume.
2. Adjust the amount of DNA in the reaction to give a molar ratio of RecA:DNA of about 70:1 (the molecular weight of RecA is 37.6 kDa). For larger DNA substrates, you may need to decrease the amount of DNA to get complete coating of the DNA.
3. Incubate for 15 min at 37°C.
4. Add 2.7 µL of 2% glutaraldehyde in 75 m*M* TEA, pH 7.6.
5. Incubate for 10 min at 37°C. (do not put on ice after this step).
6. Run the reaction over a Sepharose CL-4B column as described for the denaturation reaction, with the following modifications:
   a. Use 5 m*M* Mg acetate as the running buffer.
   b. Final elution is with 25 µL of buffer rather than 40 µL.

3.4. Loading the DNA onto EM Grids

1. Each 25 µL of RecA-coated DNA is enough to prepare four grids. Immediately before applying the samples to the grids, glow discharge the grids for 30–60 s at 80 mtorr. With the grid tabs (shiny, formvar-coated side up) held in the forceps, bend the grid to about 45° relative to the tab to make washing the grid easier. Some researchers prefer crossaction tweezers or forceps (Roboz, Rockville, MD) to hold the grids or use the small band to hold the regular forceps closed onto the grid tabs. Apply a 6–10 µL drop of DNA to each grid surface. Leave the sample on the grid at least 1 min. The time is not critical.
2. Fill a small Petri dish to the top with 100 m*M* NH_4 acetate. Draw the shiny side of the grid across the surface of the solution seven times. Be careful not to submerge the grid. Only the grid surface should contact the solution.
3. Touch the grid sequentially to two 25-µL drops of 5% uranyl acetate. Let sit for 15–30 s. Then draw the grid seven times across a solution of 10 m*M* NH_4 acetate in another small Petri dish.
4. Aspirate the liquid off the grid surface with a vacuum using a drawn-out Pasteur pipet. Be careful not to touch the surface of the grid. Allow the grids to air-dry. Bend the tabs back to be coplanar with the grid.

3.5. Tungsten Shadowing of the Grids

1. Shadow the grids at an angle of about 7°.
2. Adequate shadowing is essential to score crossovers of the DNA strands. Aim for 1.5 min of rotary shadowing and 1.5 min of directional shadowing. Do not take shortcuts here, especially with the directional shadowing. The intensity of shadowing will depend on the time and the current (as measured by the amp meter). If the current is not high enough, the shadowing will be too light. If the current is too high, the wire will burn out too quickly. Ideally, each wire will burn for about 1 min. The relationship between the Variac setting and the amp meter is variable. The Variac setting should be between 30 and 32, with 31 usually being best.
3. Store the grids in a vacuum desiccator. View and photograph as soon as possible after preparing them, since the quality may deteriorate over time.
4. For best resolution, take photographs at 50,000 magnification.

4. Notes

1. Coating of single-stranded DNA with RecA protein shrinks the DNA to approx 60% of its length. This is useful with long substrates but is a disadvantage with short substrates or complex knots and catenanes. Two modifications to the method can be used to make the DNA longer. One is to coat the single-stranded DNA with RecA protein in the presence of ATP-γS. This elongates the DNA to approx 160% of its length. Follow the protocol as described, except add ATP-γS to a final concentration of 0.5 m*M* in the RecA-coating reaction as the final reagent.
2. Another modification is to coat double-stranded DNA in the presence of ATP. Omit **Subheading 3.2.** The RecA coating reaction should contain: 25 ng DNA, 1 µg of RecA protein, 25 m*M* TEA, pH 7.6, 2 m*M* ATP, and water to a final volume of 20 µL.
   a. Incubate at 37°C for 5 min.
   b. Add ATP-γS to 0.5 m*M* to stabilize the complexes.
   c. Incubate for 30 min at 37°C.
   d. Add glutaraldehyde to 0.2% final concentration.
   e. Incubate for 15 min at 37°C.
   f. Run on a Sepharose CL-4B column with 5 m*M* Mg acetate as the running buffer.
3. We have found that the RecA coating of single-stranded DNA in the absence of ATP-γS gives the most reproducible results. Examples of DNA coated using this method are shown in **Fig. 1**.

Acknowledgments

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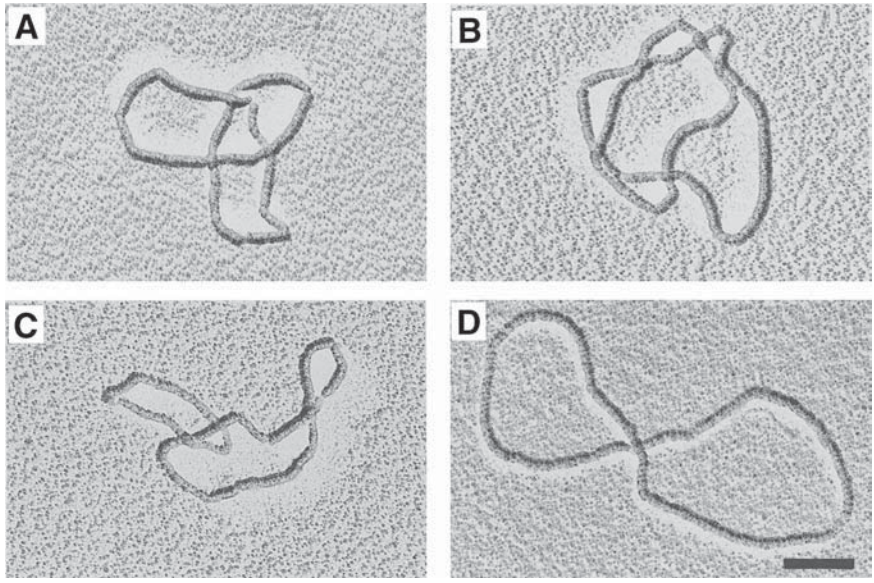

Fig. 1. (**A–C**) Single-stranded DNA coating with RecA. **(A)** A three-noded (+) knot with a 7-kb plasmid. **(B)** A five-noded (+) torus knot with an 8.5-kb substrate. **(C)** A singly-linked (2-noded) catenane. The lengths of the two rings are 4.6 and 2.4 kb. (**D**) A 3.5-kb plasmid coated by the double-strand coating technique. Note the dramatic increase in length compared to the single-strand coating technique. All negatives were shot at 50,000 magnification. The bar represents 100 nm. (Photos by N. J. C.)

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12

Methods for Analyzing DNA Bending

Jason D. Kahn

1. Introduction

DNA bending is observed in all DNA transactions, including replication, transcription, recombination, repair, and packaging. DNA bending can be sequence-directed, as in kinetoplast minicircle DNA and many synthetic sequences, or protein-induced, as in the nucleosome and in protein–DNA complexes formed with the catabolite activator protein (CAP), the TATA binding protein (TBP), and the integration host factor (IHF). In addition, the "bendability" of DNA is sequence-dependent; that is, some sequences demonstrate an increased propensity to adopt a bent conformation under stress, even when the intrinsic shape is essentially straight. The biological functions of bending and flexibility include apposition of sites that would otherwise be far apart on the stiff DNA duplex, creation of a recognition site for other proteins, organization of supercoiling geometry, decreasing DNA duplex stability, and compaction of the DNA in chromatin.

Methods for detection and quantitation of DNA (and RNA) bending include X-ray crystallography and NMR, electron microscopy and atomic force microscopy, electric birefringence, fluorescence energy transfer, "bend swap" experiments, DNA ring closure, and various gel electrophoretic methods. The latter methods require only small amounts of material and no unusual equipment, and data analysis is reasonably straightforward; consequently, these are the experiments most commonly performed. However, it has become clear that the gel methods can give unreliable results in some cases, so the results must be interpreted with some caution. The ring-closure method appears to be resistant to some of these artifacts and is not much more difficult to perform; its use is also discussed here.

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The physical basis of gel-retardation methods for analysis of bending is that bent DNA has been observed to migrate more slowly than straight DNA of the same length in polyacrylamide gels. This can be interpreted in terms of the reptation model for migration of DNA, which holds that the mobility is proportional to h_x^2/L^2, where h_x, the average end-to-end distance of a DNA with contour length L, is decreased in bent DNA relative to straight DNA. However, we do not have a quantitative understanding of the effect of bending on gel mobility ***(1)***.

Four experiments are described below. The ligation ladder experiment exploits gel retardation by multimerizing oligonucleotides, which may be only mildly bent, into polymers with substantial overall curvature. The circular permutation and phasing experiments are based on changing the position of DNA bends relative to the ends of the DNA fragment or relative to a test bend, so as to change the overall end-to-end distance of the DNA without changing its contour length substantially. The assumption is made that the relative mobilities in a family of constructs will change depending on the existence and direction of a DNA bend, even though the absolute mobility cannot be understood theoretically. Finally, ring-closure experiments are based on solution properties, the idea that bringing the DNA ends together will increase the probability of ligation of those ends. *See* **Note 7** for more discussion on the choice of experiment.

2. Materials

2.1. Equipment

The equipment and supplies used for these experiments are generally readily available in molecular biology laboratories, with the exception of temperature-controlled electrophoresis apparatuses. The materials and techniques needed for routine cloning of small DNA restriction fragments will not be described here, except insofar as bent DNA requires unusual adaptations.

1. Temperature-controlled gel apparatus: The mobility of bent DNA and the stability of protein–DNA complexes depend on temperature, so it is best to control the gel temperature actively during a run. Either an apparatus in which the gel is immersed in the running buffer (e.g., the Hoefer/Pharmacia SE600, Pharmacia, Piscataway, NJ) or one with a temperature-controlled water jacket (e.g., the Owl Polar Bear™, Owl, Woburn, MA) can be used. A refrigerated circulating water bath is required. Alternatively, gels can be run at low power in a cold room or chromatography refrigerator.
2. Gel dryer, Whatman 3MM paper for backing, and autoradiography supplies: If ethidium bromide staining is to be used to visualize gels bands, a transilluminator and Polaroid camera setup will be used instead.

3. DNA: synthetic oligonucleotides should be gel- or HPLC-purified before use. For studying protein-induced bending, a strong binding site for the protein in question must be available for cloning into bending vectors.
4. Thermal cycler (especially for **Subheading 3.5.**).
5. A Phosphorimager (Molecular Dynamics, Sunnyvale, CA) or equivalent β-particle scanner is extremely useful for binding constant or ligation kinetics measurements, as in **Subheading 3.5.** It is not necessary for qualitative gel-mobility experiments.

2.2. Supplies

1. Electrophoresis chemicals: acrylamide, *N,N′*-methylene bis-acrylamide, ammonium persulfate, *N,N,N′,N′*-tetramethylethylenediamine (TEMED), 5× TBE buffer: 450 m*M* Tris base, 450 m*M* boric acid, 10 m*M* EDTA.
2. Reagents for manipulating DNA: Restriction enzymes, polynucleotide kinase, T4 DNA ligase, 10 m*M* ATP, [γ^{32}P]-rATP, [α–^{32}P]-dATP, PCR reagents, DNA sequencing reagents, phenol equilibrated with 10m*M* Tris-HCl, pH 8.0, 24:1 chloroform: isoamyl alcohol, absolute ethanol.
3. PCR supplies, especially for **Subheading 3.5.** It may be necessary to optimize Mg^{2+} concentration for each new primer if insertions or deletions are being introduced. PCR yields from A-tract-bearing templates tend to be low, though when we have checked the products, they have had the correct sequence.
4. Cloning vectors, hosts, and supplies: We have typically used pBluescript II KS+ (Stratagene, La Jolla, CA) for cloning and XL-1 Blue *Escherichia coli* cells. Plasmids bearing A tracts can be difficult to clone, and anecdotal evidence suggests that they mutate more readily than random-sequence DNA. In our hands, dideoxy sequencing through A tracts is also difficult, and cleaner results are obtained with thermostable DNA polymerases (e.g., Vent exo$^-$, New England Biolabs, Beverly, MA) than with Sequenase 2.0 (Amersham/USB, Arlington Heights, IL).

2.3. Sample and Electrophoresis Buffers

1. For preparative ligations and restrictions, buffers supplied with the enzymes are typically adequate. For gel-shift analysis, a buffer appropriate for the protein–DNA interaction at hand should be used. If the protein does not appear to bind well in initial experiments, higher protein concentration can be used, or additives, such as ≤0.1% NP-40 detergent, ≤100 µg/mL gelatin, ≤100 µg/mL BSA, ≤10% glycerol, or 1–10 m*M* $MgCl_2$ may improve the results. For example, for *E. coli* RNA polymerase, a typical buffer includes 40 m*M* HEPES, pH 8.0, 75 m*M* potassium glutamate, 5 m*M* $MgCl_2$, 1 m*M* DTT, 0.01% NP-40, and 50 µg/mL BSA. If substantial nonspecific binding is observed, it can be alleviated using competitor DNA, such as 1–100 µg/mL sonicated calf thymus DNA or poly d(I-C). In some cases, it may be necessary to include stabilizing components, such as $MgCl_2$ and DTT, in the gel and electrophoresis buffers as well as the sample buffer.

2. Detailed buffer conditions: Native sample loading buffer (6X): 30% glycerol, 10 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA, and 0.025% each bromophenol blue and xylene cyanole. Kinase buffer: 70 m*M* Tris-HCl, pH 8.0, 10 m*M* $MgCl_2$, 5 m*M* DTT. PCR components: 10 m*M* Tris-HCl, pH 8.4, 50 m*M* KCl, 2 m*M* $MgCl_2$, 200 µCi/mL [α-^{32}P]dATP (NEN), 200 µ*M* each dNTP, 100 µg/mL gelatin, 6 U Ampli*Taq* polymerase (Perkin Elmer/Roche, Alameda, CA), 10% glycerol, 0.5 µ*M* each primer. Ligation kinetics buffer: 50 m*M* Tris-HCl, pH 7.5, 5 m*M* KCl, 3.5 m*M* $MgCl_2$ (the minimum needed to support ligase activity), 40 µg/mL BSA, 0.01% NP-40 (essential for accurate dilution of the ligase), 1 m*M* ATP, 1 m*M* DTT. Ligation quench solution (3X): 75 m*M* EDTA, 2 mg/mL proteinase K, 15% glycerol, and 0.025% tracking dyes.
3. Mobility shift gels (*see* **Note 1**) are routinely run at low ionic strength ($^1/_2$X TBE: 45 m*M* Tris, 45 m*M* borate, 1 m*M* EDTA, pH 8.3) to increase the binding affinity of the protein–DNA complexes under study and to allow the use of higher voltage at a given power. If nonspecific binding is a problem or the protein–DNA interaction is less electrostatic, a high-salt buffer can be used (25 m*M* Tris, 190 m*M* glycine, 1 m*M* EDTA, pH 8.9). The electrophoretic separation of free DNA and protein-DNA complex is a nonequilibrium process, meaning that dissociation of complexes is irreversible, so it is advantageous to perform the experiment as rapidly as possible, though without sample heating or band smearing.

3. Methods

3.1. General Considerations for Electrophoretic Methods

1. Polyacrylamide gels are prepared as follows: acrylamide and bis-acrylamide are mixed with concentrated electrophoresis buffer and adjusted to the appropriate volume with ddH_2O, $^1/_{20}$ vol of 10% ammonium persulfate is added, $^1/_{200}$ vol of TEMED is added, and the gel is poured between clean glass plates and allowed to polymerize. Typically gels are 20 cm wide × 20 cm or 40 cm long × 0.8 mm thick. Typical acrylamide: bis ratios are 29:1 or 40:1 for analysis of sequence-dependent bending and 75:1 for protein–DNA complexes, with a gel percentage around 8% for ligation ladders and from 5–12% for other experiments, depending on the mobility range of interest. We take pains to make the wells only a few millimeters deep, since this aids in careful gel loading (**Subheading 3.1., item 4**).
2. Gels are prerun thoroughly, until constant current is reached, with the temperature maintained at the temperature of the final run using a thermostated apparatus like those discussed in **Subheading 2.1.** above. We typically run at constant voltage at about 10 V/cm, being sure that the temperature in the electrophoresis chamber does not increase.
3. Gels are typically run at room temperature for analysis of sequence-directed bends and at 10°C to room temperature or occasionally 30–37°C for protein–DNA complexes. If additives such as $MgCl_2$ are present, they are added to gel stock before polymerization and to the electrophoresis buffer, and the buffer is recirculated during the run. Divalent cations have been shown to affect the curva-

ture of some DNA sequences ***(2)*** and the stability of some protein–DNA complexes (*see* **Note 1**).

4. Samples are prepared with a glycerol or Ficoll loading buffer (**Subheading 2.3., item 2**) and loaded in as small a volume as possible at the bottom of the sample well, using a gel-loading pipet tip, if necessary, with care being taken to avoid excessive dilution with electrophoresis buffer. Gel-shift gels are usually loaded while running at decreased voltage if careful mobility comparisons will be done; the intent here is to minimize the time the sample spends in solution before entering the gel and to obtain tight bands whose mobility is precisely measurable.

3.2. Preparation and Analysis of Ligation Ladders

The ligation ladder experiment is primarily designed to study DNA bending as a consequence of sequence changes, base changes (e.g., methylation), or adduction with drugs ***(3,4)***. The principle of the experiment is that as the length of concatemers increases, they will be progressively more retarded in gel mobility relative to "normal" DNA (*see* **Note 2**). It is important to use oligonucleotides whose sequence repeat is approximately equal to their helical repeat to avoid generating molecules with substantial writhe, which can migrate more rapidly than expected or have unexpected properties ***(5)***. Often a range of sequence repeats is explored in order to measure both the helical repeat and the maximum mobility effect. These issues have been reviewed in detail ***(4,6)***.

1. Synthetic oligonucleotides are phosphorylated and annealed as follows ***(5)***: 8 µg of each single strand are labeled in a 20-µL reaction with 20 µCi of γ-^{32}P-rATP, in kinase buffer (**Subheading 2.3., item 2**), with 15 U T4 polynucleotide kinase, for 30 min at 37°C. After this time, cold ATP is added to 0.5 m*M*, a further 10–20 U of T4 kinase are added, and incubation is continued for 30 min. Complementary oligonucleotides are then mixed, heated to 90°C for 1 min, and allowed to cool to room temperature over 1–2 h. The mixture can be used directly in ligation.
2. At least 1–2 µg of phosphorylated, annealed oligonucleotide are ligated with 800 U T4 DNA ligase, in kinase buffer with 1 m*M* ATP added, overnight at 4–16°C. The amount of ligase may need to be optimized to obtain the desired length distribution, or a time-course can be performed. The ligation is quenched with EDTA to 25 m*M* and the material is analyzed by on a 20 cm × 40 cm × 0.8 mm 8% (29:1) gel run at 10 V/cm for several hours. A 10-bp *Bam*HI linker can be phosphorylated and ligated in parallel as a control unbent DNA ladder.
3. The mobility of each bend in the multimer set is measured. The relative mobility R_L is then calculated according to $R_L \cong$ apparent length/actual length, where the apparent length is determined from a calibration curve using the unbent DNA ladder. The gel-mobility anomaly is then characterized by $(R_L - 1)$. In general, $(R_L - 1)$ increases quadratically with increasing curvature and becomes significantly larger than 0 at lengths grater than about 100 bp; R_L values for curved DNA range from 1.2–2.5, depending on DNA length and gel percentage ***(7)***. It is

not clear how to interpret R_L in terms of an absolute bend angle; usually the values are compared to those derived from well-characterized A-tract DNA ladders *(8)*.

3.3. Circular Permutation Assay

The circular permutation assay *(9)* was designed to identify the presence of a bend and to determine its position; the assay has since been used extensively to estimate both absolute *(10)* and relative *(11)* bend magnitudes. Experimental designs, typical data obtained, and the interpretation of the data are schematized in **Fig. 1** (*see* **Note 3**). The basis of the assay is that simple geometric arguments show that a bend in the center of the fragment will have a larger effect on end-to-end distance and, therefore, on mobility than a bend near the end.

1. The DNA source of circularly permuted fragments is prepared by cloning the site of interest into either the pBend2 vector designed for bending studies *(12)* or into a restriction-site-rich sequence (e.g., a multiple cloning site), which is then recloned as a tandem copy or cyclized. The set of probes is then generated by restriction with a set of enzymes (A-H in **Fig. 1**). If necessary, the probes are then labeled using calf intestinal alkaline phosphatase and T4 polynucleotide kinase or by the Klenow fill-in reaction. It is often unnecessary to purify these fragments before performing the gel shift, unless plasmid DNA interferes with DNA binding.

   The intermediate cyclization method in **Fig. 1** has been applied to nonclonable DNA, such as a mismatched region *(13)* and a cisplatin crosslink *(14)*. The monomeric DNA fragment is ligated overnight at a DNA concentration of ≤1n*M* to avoid excessive bimolecular ligation. The reaction is analyzed by gel electrophoresis; if a reasonable yield of monomeric circle is obtained, the reaction mixture can be restricted to give circular permutants without intermediate purification of the circle.
2. Electrophoresis is performed as in **Subheading 3.1.**
3. The migration distance of each band from the well is measured from an autoradiogram or photograph of the gel. If the free DNA shows significant mobility variation, the mobility of the bound DNA should be normalized to the mobility of the corresponding free DNA. The data are graphed and fit to the equation below by nonlinear regression (we use KaleidaGraph, Synergy Software):

$$\mu = \mu_m + \frac{1}{2}(\mu_e - \mu_m)\left[\cos\left(\frac{\textit{cut site} - \textit{bend center}}{\textit{fragment length}} \times 360^\circ\right) + 1\right] \qquad (1)$$

   where μ_m is the mobility of the molecule with the bend in the center (minimum mobility), μ_e is the mobility with the bend at the end (maximum), *cut site* is the position of the restriction cut relative to site A, *bend center* is the position of the

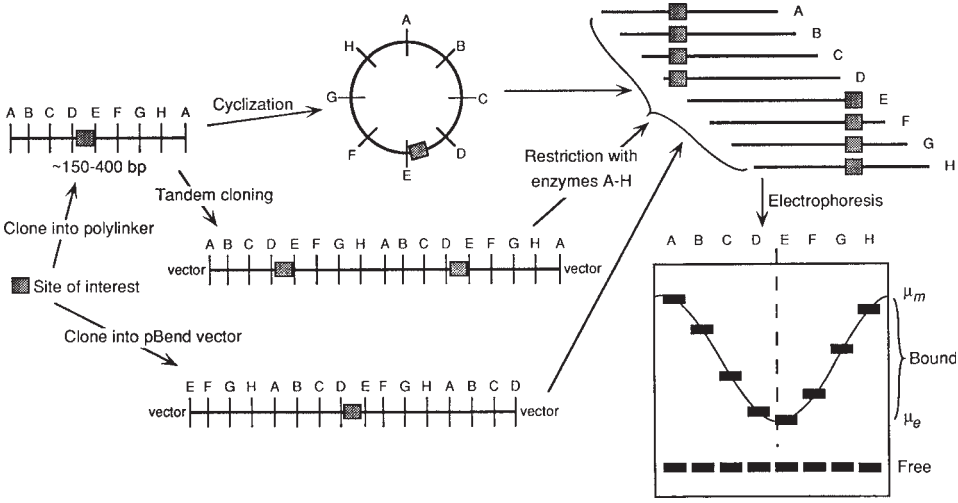

Fig. 1. Circular permutation assay, illustrated for a protein–DNA complex. The letters A–H indicate restriction sites. Three methods of constructing precursors to the circularly permuted set of fragments are illustrated. A schematic autoradiogram is illustrated in the box, with a sine curve fit to the mobilities of protein–DNA complexes. The dashed line indicates the position of the bend center, determined from the point of maximum mobility on the curve.

bend center relative to site A, and *fragment length* is in base pairs. Values for μ_m, μ_e and *bend center* are determined from the curve fit, and the estimated bend angle α is then calculated according to the empirical equation ***(15)*** below:

$$\mu_m/\mu_e = \cos(\alpha/2) \quad (2)$$

3.4. Helical Phasing Assay

The helical phasing assay ***(16,17)*** is used to determine bend direction, which is not available from circular permutation. The basis of the assay, as diagrammed in **Fig. 2**, is that when a molecule contains two bends, they can either cooperate or neutralize each other, forming cis and trans isomers, respectively (*see* **Note 4**). The former run much more slowly on a gel. This situation is realized experimentally by inserting a variable-length phasing linker between the two bends. Usually one bend is sequence-directed, a series of phased A tracts, and the other is induced by the protein of interest.

1. Bend phasing variants are prepared by cloning the site of interest into each of a set of several bend phasing vectors ***(10,18)***. It is necessary to vary the spacer

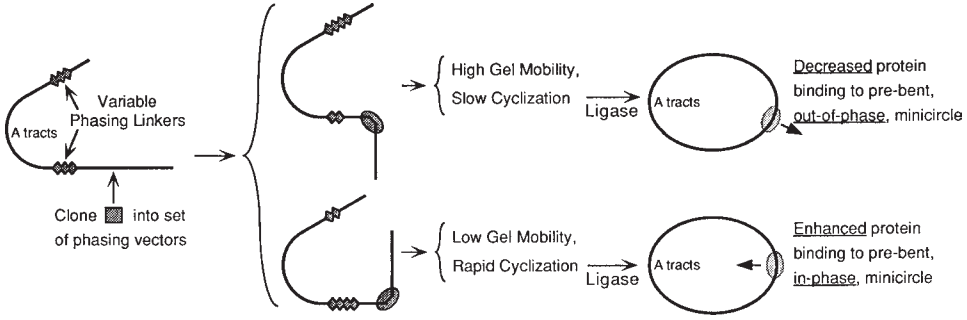

Fig. 2. Phasing, cyclization kinetics, and minicircle binding assays. The same DNA constructs can be used for all of these experiments. A protein binding to the site of interest is denoted by the oval. The use of a distal phasing linker is necessary only for cyclization experiments. For simplicity, in the example given here, the length of the distal linker is changed to maintain a constant total length of the molecule, so twist changes do not affect cyclization or minicircle binding. The trans configuration or S shape migrates more rapidly through a gel and cyclizes more slowly than the cis configuration or C shape. In the right-most figures, the arrows indicate the preferred bending direction for the protein. When the prebending in the circle enforces this direction on the free DNA, binding is enhanced ***(18,23)***.

length over one helical turn to observe the full extent of mobility variation with phasing length. Gel electrophoresis is performed as above.

2. The mobilities of the phasing constructs are measured as above. It is usually necessary to normalize the mobility of the bound species to that of the free DNA. The data from phasing experiments often do not fit to a simple model, but in general, the cis and trans isomers can be identified. Bend direction is determined as follows, based on the knowledge that A tracts are bent into the minor groove essentially ***(4)*** at the center of the A tract (at the caret AAA^AAA): If a molecule in which the bend center (identified by circular permutation) is an integral number of helical turns ($n \times 10.5$ bp) from the center of the first A tract is in phase with the A tract bend (i.e., gel mobility is at a minimum, the cis isomer), then the induced bend is into the minor groove. Similarly, if the induced bend-first A tract distance for the cis isomer is a half-integral number of helical turns ($[n + 0.5] \times 10.5$ bp), the induced bend is into the major groove. Quantitative equations suitable for measuring apparent bend angles in favorable cases have been derived ***(10)***.

3.5. Ring Closure Methods

The application of ring-closure methods to protein–DNA interaction was developed to address some of the difficulties in the electrophoretic experiments. The DNA ring closure experiments of concern here monitor the appearance of DNA minicircles (<100–300 bp) and compare results with and without binding

proteins, or with and without intrinsic DNA curvature. Since ring closure requires bringing the ends of the DNA together, it is extremely sensitive to DNA bending and flexibility changes. This can be exploited qualitatively, in that DNA fragments that are too small to cyclize on their own can do so when bound by bending proteins ***(19,20)***, or quantitatively (*see* **Note 5**), by measuring the cyclization probability or *J* factor ***(18)***. Ring closure has three main advantages over the gel methods ***(21)***:

1. The reaction is done entirely in solution, and data analysis does not require assumptions about how electrophoresis works.
2. The method is applicable to larger protein–DNA complexes than can easily be studied by gel methods ***(22,23)***.
3. Results can be quantitatively simulated by Monte Carlo simulation.

Our system for applying ring closure to bending uses constructs similar to standard phasing constructs ***(18)***, with the addition that a second phasing linker is needed to allow varying the overall length of the molecule (*see* **Fig. 2**). This is necessary because the cyclization probability is strongly dependent on torsional phasing of the DNA ends. In practice, the length variation at the second linker position can be introduced by PCR, using primers bearing insertions or deletions. We use two fundamental types of experiments: (1) Ligation kinetics (*see* **Note 5**) gives measurements of the *J* factor ***(24)***, which is the ratio of rate constants for the cyclization and bimolecular ligation reactions. (2) Binding of protein to minicircle templates is studied using gel-shift competition assays (*see* **Note 6**). The two methods give complementary results, in that a molecule that is bent so as to accelerate cyclization gives a product that is "pre-bent" so as to enhance protein binding. The theoretical expectation is that for any DNA sequence, the ratio of the binding constants to the circular DNA and the linear DNA will be equal to the ratio of *J* factors for cyclization with and without protein ***(18)***. The method can also be used in a more qualitative way, as described in **Notes 5–7**.

We note that ring closure is often observed in ligation ladder experiments, and this has been used to derive bend angles for protein-induced and sequence-directed bends. The appearance of circles made up of a smaller number of oligonucleotide segments than in control experiments is diagnostic for bending. This mixed ligation method is very sensitive and does not require cloning, but quantitative interpretation can be difficult; it has recently been reviewed ***(25)*** and will not be discussed further here.

1. Preparation of cyclization substrates for measurements on a single molecular species ***(18)***. PCR from plasmid templates bearing phasing constructs is used to generate body-labeled substrates and length variants. A 150-µL reaction as in **Subheading 2.3., item 2** is subjected to 30 cycles of 94°C 1 min/55°C 1 min/

72°C 2 min. PCR products are phenol-extracted, ethanol-precipitated, and then restricted to regenerate sticky ends; we use *Cla*I ends, using 2–5 U of *Cla*I/100 µL of PCR reaction mix, overnight at 37°C. Restricted products are gel-purified on 8% acrylamide 40 cm long native gels. It is important to purify the DNA as carefully as possible, since the integrity of the 5′ ends is essential for ligation (*see* **Note 5**). Specific activity is measured after electroelution from the gel by measuring the amount of radioactivity by scintillation counting and the DNA concentration by UV absorbance, using a 100-µL cell (Hellma). An accurate measurement of concentration is needed for quantitation of the *J* factor.

2. Sample buffer, protein concentration, competitor DNA, and electrophoresis conditions are established, which provide and demonstrate specific and stoichiometric protein binding, typically by gel-shift titration experiments (*see* **Notes 1** and **5**).
3. Ligation kinetics (**Note 5**) are measured by adding 7.5 µL of diluted T4 DNA ligase (New England Biolabs) to a 67.5 µL reaction mix containing the protein–DNA complex, quenching 8-µL aliquots into 4 µL of quench solution at 1, 2, 3, 4, 6, 9, 12, 15, and 120 min, and analyzing by gel electrophoresis on 6% 20 cm long native gels to separate monomeric, circular, and dimeric products. Samples are incubated at 55°C for 10 min before loading. The reaction is done at 21°C; varying this substantially will alter the A tract geometry. Typical final concentrations are as follows: 0.5–10 n*M* protein–DNA complex (larger concentrations make bimolecular ligation easier to measure), 1–3000 U/mL ligase (the larger amounts for molecules that cyclize slowly), in the buffer described (**Subheading 2.3., item 2**). In our studies of CAP, 100 µ*M* cAMP was also present. If competitor DNA is included, it may be necessary to increase ligase concentration.
4. The amount of DNA at each time present as starting material, circular product (verified by BAL31 digestion), and total bimolecular products (there may be several, as linear dimers with A tracts in different positions separate, and trimers and circular dimers are also included) are quantitated with a β-particle scanner. The rate constants for cyclization and bimolecular ligation are calculated by fitting the observed amount of radioactivity to the equations below. In the absence of bimolecular ligation:

$$[C]_t = [M]_0 (1 - e^{-k_c t}) \tag{3}$$

If bimolecular ligation is observed:

$$[\mathrm{C}]_t = \frac{k_c}{4k_b} \ln\left[1 + \frac{4[\mathrm{M}]_0 k_b}{k_c}(1 - e^{-k_c t})\right] \tag{4}$$

$$[\mathrm{B}]_t = \frac{1}{2}\left[[\mathrm{M}]_0 - [\mathrm{C}]_t - \frac{[\mathrm{M}]_0 k_c e^{-k_c t}}{1 + 4[\mathrm{M}]_0 (1 - e^{-k_c t}) k_b / k_c}\right] \tag{5}$$

where $[\mathrm{C}]_t$ is the concentration of circle at time t, $[\mathrm{B}]_t$ is the total concentration of bimolecular products, k_C is the rate constant for cyclization, k_b is the rate constant for bimolecular ligation, and $[\mathrm{M}]_0$ is the initial concentration of ligatable DNA, from the total DNA concentration and the fraction converted at the 2-h time-point

(*see* **Note 5**). The curves are fit (in KaleidaGraph) by initially fitting the cyclization data to **Eq. 3** above to estimate k_c, using this value in **Eq. 5** for $[B]_t$ to estimate k_b, substituting k_b into **Eq. 4** for $[C]_t$ to refine the estimate for k_c, and iterating to self-consistent values of the rate constants using **Eqs. 4** and **5**. The *J* factor is then given by k_c/k_b. The larger the *J* factor, the more efficient the cyclization. It can be interpreted semi-quantitatively ***(22)*** or by using Monte Carlo simulation ***(21)***.

5. Minicircle binding experiment (*see* **Note 6**). This is simply a gel shift, but in order to assess the extremely tight binding expected for in-phase prebent minicircles, it is usually necessary to perform competitive binding experiments, with linear DNA as competitor. The fold excess of unlabeled linear competitor needed to remove half the protein from the labeled circular DNA gives a reasonable estimate of relative binding constants; quantitative details are given in **ref. *18*.** These experiments can be technically demanding, since the gel mobility difference from binding a protein to a circle can be very small; therefore, the gels may need to be run for a very long time ***(18)***. This can cause problems with complex stability; at a minimum, it may be necessary to recirculate running buffer or to experiment with stabilizers like glycerol and DTT in the gel. Decreased binding to the out-of-phase minicircle is difficult to quantitate for the opposite reason: binding may be so weak as to be undetectable. In this case, the binding to out-of-phase circle can be estimated by using it as a competitor to remove protein from labeled linear DNA.

4. Notes

1. Gel-mobility shift conditions can vary markedly with different DNA binding proteins. If no protein–DNA complex is observed, steps should be taken initially to minimize protein absorption to tubes or aggregation. These include the use of siliconized microcentrifuge tubes and the addition of NP-40, other detergents, BSA, or gelatin as in **Subheading 2.** above. The addition of Mg^{2+}, DTT, or glycerol may be necessary. Varying the temperature of the gel, the gel percentage, and the acrylamide:bis ratio is best done later to improve resolution or stability. For many complexes, a 10% gel with a 75:1 acrylamide:bis ratio has been observed to give optimum results.
2. Ligation ladders are often observed to give smears, especially if the gel temperature or buffer conditions vary during the run, or the oligonucleotide starting material is not pure enough. Purification by HPLC instead of PAGE has been recommended in this regard ***(6)***. Extra bands between the expected bands may be caused by excess of one single strand. This can be corrected either by annealing the two strands at a range of relative concentrations or by purifying the annealed double strand before ligation. Circular molecules can be identified in a ligation ladder by resistance to exonucleases (e.g., BAL31) or simply by their appearance at high intensity in a region of the gel where the amount of linear concatemers is small. Very small circles (<100 bp) appear to run anomalously rapidly, nearly as fast as the corresponding linear DNA, whereas larger circles run much more slowly than linear molecules of the same length ***(20)***.

3. A negative result in the circular permutation assay is good evidence for a lack of bending, but a false-positive result or exaggerated bend angle can arise as a consequence of DNA "flexure" or of an unusual binding protein shape ***(10,26)***. For this reason, the absolute electrophoretic mobility of a protein–DNA complex does not appear to correlate reliably with conformation. Bend angles determined by the circular permutation method described above are often overestimated when compared to X-ray crystal structures. The assay can be used more effectively as a relative measure of the extent of bending induced by the same protein on different DNA sequences ***(11,27)***.
4. The phasing experiment, owing to its enhanced sensitivity (a consequence of the quadratic relationship between bend angle and mobility retardation), often detects DNA bending in "free DNA." If such bending is substantial, it complicates interpretation, since it is then not clear whether changes on protein addition are owing to a protein-induced change in an existing bend or a new DNA bend. The phasing experiment may also be subject to artifact if the binding protein has an unusual shape (e.g., elongated, as for bZIP and bHLH proteins), either because the shape of the complex as a whole depends on phasing or because there is direct interaction between the protein and the A tract bend ***(28)***, as in **Fig. 3**. In the latter case, a phasing assay using a larger separation between the induced bend and the intrinsic bend will give a more accurate result. Ring closure has recently been used to show that earlier electrophoretic results on a variety of bZIP transcription factors are probably in error because of this problem, though this remains controversial ***(28,29)***. Phasing can also give a false-negative result if a protein induces a very large DNA bend, because there is then very little difference between the overall shape of cis and trans molecules; this situation is, however, quite unusual. Finally, phasing is quite insensitive to DNA flexure ***(13)***; although it is usually observed that apparent bend angles derived from phasing are less than those from circular permutation, it is therefore not clear whether this is owing to flexibility ***(28,30)***.
5. The two most common problems with the ligation kinetics experiment are as follows: (a) the requirement for efficient, but specific protein binding, and (b) the observation of large amounts of nonligatable DNA, owing presumably to phosphatase or exonuclease contamination, restriction enzyme star activity, or PCR primers with chemical lesions. If a high level of binding (e.g., 80%) cannot be obtained, there will be a background level of reaction from free DNA, which will make quantitation difficult, and if nonspecific binding is present, the results can be strongly affected by rapid cyclization of a small fraction of doubly bound material. Ideally, *J* should be measured and should be constant over a range of protein concentrations. It is probably better to err on the side of low binding, since the consequences are more predictable than those of nonspecific binding. If more than about 60% of the starting material is not ligated at long times (typically 2 h), the rate of the bimolecular reaction can be substantially overestimated owing to the bimolecular ligation of "single-ended" molecules, which cannot cyclize, leading to an underestimate for *J*. Quantitative simulation ***(31)*** suggests

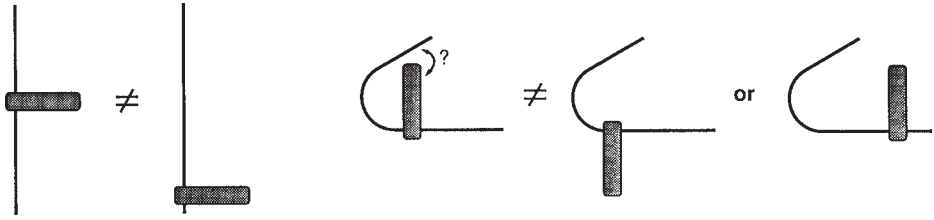

Fig. 3. Possible sources of artifacts in electrophoretic experiments. In the circular permutation experiment with an elongated protein, the T shape may migrate differently from the L shape even if the protein does not bend the DNA ***(26)***. Similarly, in the phasing assay, interaction between the protein and neighboring A tract DNA may affect mobility, and the overall hydrodynamic shape of the protein may differ for in-phase and out-of-phase even if the protein does not bend. The latter problem is probably not as severe as it is with the circular permutation assay, because mobility is determined mainly by the disposition of the DNA arms.

that as long as at least 30% of the DNA is cyclizable (requiring ~45% ligatable ends for random damage at the two ends), the apparent *J* will be no more than fourfold lower than the true value. At 50% cyclizability, the error is no more than twofold, comparable to experimental variability.

6. A major advantage of the minicircle binding experiment is that it does not require stoichiometric binding to the linear DNA and therefore may be technically more feasible than ligation kinetics. Here, it is important to (a) ensure that the minicircle topoisomer under study is the same as that formed by cyclization with singly bound protein, which may not be the same as the topoisomer formed from free DNA, and (b) allow the protein–DNA complexes to reach equilibrium. This can require a long time (days) if the protein has a very slow off-rate from an in-phase circle. In this case, it may be technically necessary to estimate binding constants from kinetic data ***(23)***.
7. A recommended course of action for solution characterization of a suspected DNA bending protein is as follows:
   a. Perform circular permutation experiment. If it is negative, stop.
   b. Clone phasing constructs, perform phasing assay.
   c. Perform qualitative ring-closure experiment as follows: Use PCR to generate phasing constructs with three different bend phasings, but the same overall length (two phasings could in principle be equally, but oppositely, out of phase and give the same result). Characterize the end point of the cyclization reaction vis à vis the product distribution between bimolecular and circular products and the appearance of any new topoisomers.
   d. If there is reason to proceed further, use the ligation kinetics experiment and/or the minicircle binding experiment to measure the length dependence of cyclization with and without protein (to address any twist changes), and proceed to measurement of *J* factors or binding constants.

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13

Formation of Extrachromosomal DNA Rings in *Saccharomyces cerevisiae* Using Site-Specific Recombination

Marc R. Gartenberg

1. Introduction

A hindrance to the study of structure and function of DNA elements is that sites of interest always lie within the context of other DNA sequences. This is particularly limiting when attempting to examine elements embedded within chromosomes inside intact cells. Analysis of the *Saccharomyces cerevisiae* genome has shown that genes are densely packed and dispersed among multiple replication origins, as well as other functional loci. The importance of context is exemplified by the phenomenon of transcriptional silencing, where regions of inactive chromatin repress the expression of proximal genes. A solution to the problem of context has been to relocate elements of interest to naturally occurring or synthetic plasmids. Though small in size and simple in organization, biologically sustainable plasmids are still complex. Shuttle vectors used in both yeast and bacteria must contain sequences necessary for replication and selection in both hosts. Many shuttle vectors also contain an additional DNA element that determines whether the plasmid will be maintained at high or low copy.

A practical solution to the problem of context in vitro is simply to use restriction endonucleases to isolate the DNA sequence of interest. Use of nucleolytic enzymes in vivo, however, is not a practical option: severed DNA ends become substrates for degradative or repair pathways ***(1)***. This chapter describes a protocol for altering DNA context in vivo using inducible site-specific recombination. We have used the methodology to make nonreplicating extrachromosomal DNA rings. The simple DNA circles have proven to be

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valuable tools to study the influence of transcription and DNA immobilization on DNA topology ***(2)***. Minimal requirements for the reaction are two chromosomal recombination targets sites and the recombinase enzyme. In *S. cerevisiae*, excision is nearly quantitative and sufficiently rapid to permit the study of events within a given cell cycle. Inducible site-specific recombination is becoming an increasingly popular tool to rearrange genomic elements in a broad spectrum of organisms, including bacterial, plant, and mammalian cells. Entire chromosomal regions can be inverted, deleted, or transferred to other chromosomes ***(3–5)***. From a technological standpoint, the reaction can be used to remove unwanted vector sequences and to regenerate selectable markers ***(6)***. More importantly, genes can either be turned on or off by removal of inhibitory or necessary sequences, respectively ***(7–9)***. In appropriately modified strains or cell lines, site-specific recombination can be used to integrate new sequences into chromosomes ***(10)***. *See* **refs.** ***(11,12)*** for comprehensive reviews of applications.

The following procedure outlines the use of the R site-specific recombinase to form DNA rings in *Saccharomyces*. However, the method is equally suited for DNA inversions and translocations with appropriately designed recombination substrates.

1.1. The Integrase Family of Recombinases

The three principal site-specific recombinases currently used for genome rearrangements, R, Cre, and Flp (pronounced "flip"), belong to the λ integrase family of recombinases ***(13,14)***. Though the class is defined by absolute conservation of just four amino acids, these enzymes perform similar types of DNA rearrangements. Cre, encoded by bacteriophage P1, assists in the stability of the circular P1 genome by resolving DNA dimers into plasmid monomers. Flp and R recombinases are encoded by nonessential plasmids of the yeasts *S. cerevisiae* and *Zygosaccharomyces rouxii*, respectively. The yeast enzymes play a novel role in amplifying plasmid copy number by catalyzing a DNA inversion event. The R recombinase is the least well characterized of the three, but similarity to Flp in amino acid sequence, substrate organization, and function suggest that the two enzymes are closely related ***(14,15)***. All three enzymes function without cofactors or accessory proteins on linear and circular substrates, which makes them ideally suited for function in heterologuous organisms. Both the Cre and R recombinases are active in *Saccharomyces* and do not crossreact with the Flp system ***(4,16)***. Use of a heterologous recombinase is advantageous, because the endogenous Flp-encoding 2-μ plasmid need not be evicted; the 2-μ provides beneficial *trans*-acting factors, which assist in the stabilization of other 2-μ-based vectors ***(17)***.

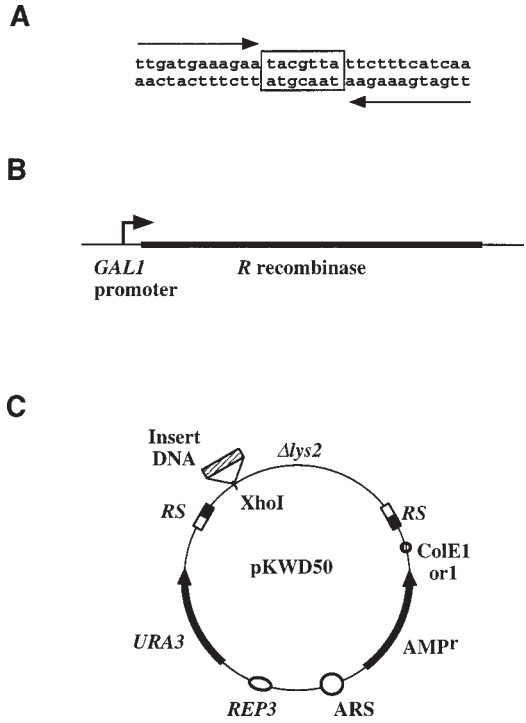

Fig. 1. Inducible site-specific recombination components. (**A**) Mimimal *RS* target site for the R recombinase of the yeast *Zygosaccharomyces rouxii*. The core sequence is boxed. (**B**) *GAL1* promoter-*R* recombinase gene fusion. (**C**) Recombination substrate pKWD50.

1.2. DNA Requirements for Site-Specific Recombination

Recombination target sites for R, Cre, and Flp recombinases are small in size (31–34 bp) and similar in structure. The R recombinase target, *RS*, contains a 7-bp core that is flanked by inverted 12-bp binding sites for recombinase protomers **(Fig. 1A)** ***(15)***. The Cre and Flp target sites, termed *loxP* and *FRT*, respectively, contain an asymmetric 8-bp core, which is flanked by two inverted 13-bp repeats. The core sequences must be homologous between pairs of reacting target sites, and the relative orientation of the core sequences between pairs of sites determines the outcome of intramolecular recombination events: when cores are inverted, the recombinase catalyzes an inversion of the intervening DNA; when cores are directly repeated, the intervening DNA is excised and religated into a circle (**Fig. 2**). Recombination between sites on separate DNA molecules leads to reciprocal translocation if both DNAs are linear or integra-

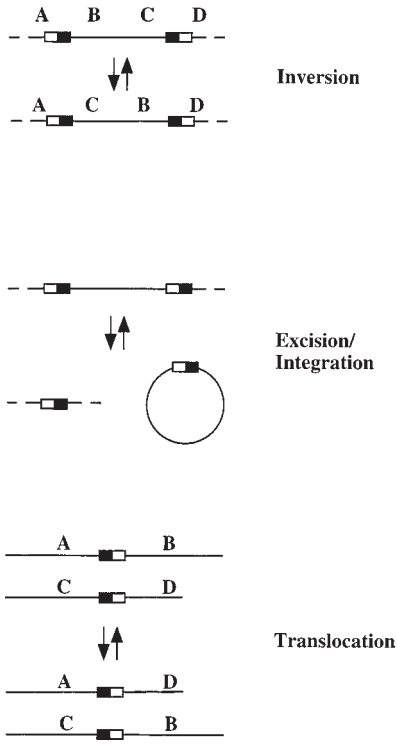

Fig. 2. Reactions catalyzed by the R site-specific recombinase. *RS* site asymmetry is indicated by half-filled rectangles.

tion if at least one of the molecules is circular. Point mutations in the core sequence of the Flp target do not block recombination if equivalent changes are made to both sites. The same is probably true for R and Cre systems. Thus, simultaneous yet independent recombination events can be performed with the same recombinase, if pairs of recombination sites do not share the same core sequence. Moreover, controlled expression of more than one recombinase could permit sequential yet independent recombination events. The reaction is fully reversible, thus, the extent of excision depends on the effective concentrations of the reactants and products. This property has been used to estimate the effective concentration of intracellular DNA in *E. coli* ***(18)***.

1.3. Induction of Site-Specific Recombination

Controlled recombinase expression is critical for most applications of site-specific recombination. The standard approach has been to fuse the protein coding sequence to a heterologous promoter that can be induced quickly and efficiently, such as the *GAL1* promoter in yeast. In this case, rapid induction is

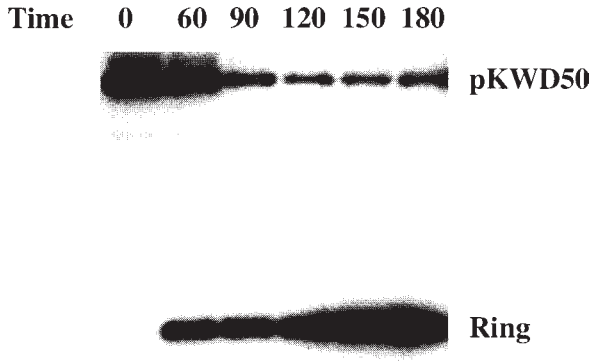

Fig. 3. Formation of DNA rings by site-specific excision of pKWD50. Time elapsed after galactose addition indicated above each lane. Both substrate and recombination product were linearized by digestion with *XhoI* prior to electrophoresis. pKWD50 and the resulting ring were visualized selectively with a probe to the excision cassette. The *GAL1-R* recombinase fusion was integrated at the chromosomal *top1* locus.

achieved by the addition of galactose to cells grown on a nonrepressing carbon source, such as raffinose. More recently, hormone receptor-recombinase fusion proteins have been developed, which are continuously expressed, but dormant until the addition of ligand ***(19,20)***. In large eukaryotes, recombination has been achieved by direct microinjection of FLP mRNA ***(21)*** or Cre protein ***(22)***. Constitutively expressed recombinases have also been introduced by transfection with viral-encoded recombinases or by mating. These techniques may not be sufficiently rapid or controlled to yield significant levels of an unstable recombination product, such as a nonreplicating extrachromosomal ring.

2. Materials

2.1. R Recombinase Expression Vector

To obtain regulated expression of the R recombinase, we have utilized a construct that contains the *GAL1* promoter linked directly to the *R* recombinase gene ***(4)*** (**Fig. 1B**). Although the promoter supports low basal level transcription, we have not detected recombination products by Southern hybridization prior to induction under standard conditions (*see* **Fig. 3**). Nevertheless, rare noninduced recombination events have been observed. In the procedure described here, the recombinase gene fusion is carried by the multicopy 2-μ-based vector, pHM153 (LEU2) ***(4)***. We have also generated strains with an integrated copy of the gene fusion ***(2)***. Both chromosomal and plasmid-based expression systems yield comparable levels of excision. However, kinetics of excision may vary with gene dosage.

2.2. Recombination Substrates

We have taken two approaches to designing recombination substrates for excision. When the region to be circularized is large (2500 bp or more), we flank the sequence directly with *RS* sites. The *RS* site we use is contained within a 58-bp subclone in plasmid pHM401 ***(15)***. Smaller biologically active sequences can probably be generated with synthetic oligonucleotides.

When the region to be circularized is small (1500 bp or less), we embed the sequence within a larger "excision cassette." The cassette we use is composed of a 2.5-kb fragment of yeast *LYS2* internal coding sequence flanked by two directly repeated *RS* targets. No known *cis*-acting elements reside in the fragment. In the procedure described here, the cassette is carried by the multicopy vector, pKWD50 (URA3) (**Fig. 1C**) ***(2)***. We have also generated strains with the recombination substrate integrated at selected chromosomal locations by targeted gene replacement (*see* **Note 1**).

2.3. Strain and Media Requirements

1. A yeast strain with a fully competent galactose induction pathway (*see* **Note 2**). The strain should also have mutations in the nutritional markers *LEU2* and *URA3*.
2. Synthetic media for selective growth of yeast cultures containing plasmids pHM153 (LEU2) and pKWD50 (URA3): (in 900 mL) 6.7 g yeast nitrogen base with ammonium sulfate, but lacking amino acids (Difco); 1.3 g ura-/leu- dropout powder ***(23)***. After sterilization, supplement media with appropriate carbon source to a final concentration of 2%. Use either 20% dextrose (w/v), 20% raffinose (w/v), or 20% galactose (w/v) (*see* **Note 3**).

2.4. Reagents to Terminate Cell Growth Rapidly

1. 500 m*M* EDTA, pH 8.0.
2. Toluene-EtOH cocktail: 95% EtOH (v/v), 3% toluene (v/v), 20 m*M* Tris-HCl, pH 8.0 (v/v).

2.5. Reagents for Isolation of Closed-Circular DNA

1. Spheroplasting solution (freshly made): 0.96*M* sorbitol (use 20% sorbitol [w/v] stock, which is roughly 1*M*), 25 m*M* EDTA, 50 m*M* β-mercaptoethanol (Bio-Rad), 1 mg/mL yeast lytic enzyme (ICN #152270).
2. IR buffer (Intermediate resuspension buffer): 50 m*M* Tris-HCl, pH 8.0, 20 m*M* EDTA.
3. 10% SDS.
4. 5*M* KOAc (pH adjustment unnecessary).
5. 100% EtOH.
6. 10 mg/mL DNase-free RNase.
7. PCI: Equilibrated phenol/chloroform/isoamyl alcohol (24:24:1) ***(23)***.
8. 7.5*M* NH_4OAc.

9. TE: 10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA.
10. Agarose-gel electrophoresis equipment and reagents ***(23)***.
11. Southern blotting reagents ***(23)***.

3. Methods

3.1. Galactose Induction of Site-Specific Recombination

1. Transform strain of interest with the recombinase expression vector pHM153 and recombination substrate pKWD50 using standard published procedures ***(23)***.
2. Inoculate 5 mL of synthetic ura-/leu- media containing 2% dextrose with a freshly restreaked transformant.
3. When the culture reaches an absorbance between 0.5 and 1.0 at 600 nm, dilute a cell aliquot (1/75) in synthetic ura-/leu- media containing 2% raffinose (*see* **Note 4**). We typically inoculate 30 mL of raffinose-supplemented media.
4. When the culture reaches an absorbance between 0.5 and 1.0 at 600 nm, initiate recombinase expression by adding galactose to a final concentration of 2%. Allow the induction to proceed between 60 and 120 min (*see* **Note 1**).
5. Terminate the induction with the following step-wise additions:
   a. EDTA to a final concentration of 20 m*M*;
   b. An equal volume of ice-cold Toluene-EtOH cocktail. Invert to mix after each addition.
6. Pellet cells in a tabletop centrifuge (1000*g*) at room temperature for 5 min. Discard the supernatant (*see* **Note 5**).
7. Resuspend cell pellet in 1 mL of IR buffer, and transfer to an Eppendorf tube. Pellet cells with a brief microcentrifuge spin (16,000*g*), and discard supernatant.
8. Cell pellet can be stored for extended periods at −20°C until DNA isolation.

3.2. Isolation of Closed-Circular DNA

1. Resuspend pellet in 0.5 mL of spheroplasting buffer. Incubate at 37°C for 20 min.
2. Pellet spheroplasts with a brief microfuge spin (16,000*g*), and remove supernatant.
3. Resuspend spheroplast pellet in 0.36 mL of IR buffer. Add 40 µL of 10% SDS, and mix by inversion. Incubate at room temperature for 5 min.
4. Add 100 µL of 5*M* KOAc, and mix by inversion. Incubate on ice for 20 min with occasional mixing.
5. Pellet cell debris with a 5-min microfuge spin (16,000*g*). Transfer supernatant to a new Eppendorf tube. Add 1 mL of 100% EtOH to precipitate DNA.
6. Resuspend pellet in 0.2 mL TE. Add 3 µL of 10 mg/mL DNase-free RNase. Incubate at 37°C for 10 min.
7. Extract aqueous phase repeatedly with PCI until interface is clear. This may require two to three extractions.
8. Add 0.5 vol of 7.5*M* NH_4OAc. Mix. Add 2.5 vol of 100% EtOH to precipitate DNA.
9. Resuspend in 40 µL TE. DNA is ready for electrophoresis. We typically use 5–10 µL for each lane on an agarose gel. DNA rings are detected by southern

hybridization. A typical excision analysis is shown in **Fig. 3**. After addition of galactose, a band corresponding to an extrachromosomal DNA ring appears in a time-dependent fashion.

4. Notes

1. When using a single-copy recombinase expression vector, 70–90% excision from pKWD50-based substrates was achieved in 150 min in the strain used (*see* **Fig. 3**). When using a multicopy expression vector, 70–90% excision from a single-copy chromosomal excision cassette was achieved in 60 min. Excision rates may be affected by the sequence content and context of the excision cassette, and strain-dependent variation (*see* **Note 2**).
2. Strains that are auxotrophic for tryptophan owing to the *trp1-Δ1* mutation are also missing sequences required for activation of the adjacent *GAL3* gene (galactokinase). In *trp1-Δ1* strains, kinetics of galactose induction are greatly reduced. Furthermore, older strains originating from the progenitor strain S288C have a mutation in *GAL2* (a galactose transporter) and do not grow well on low concentrations of the sugar, especially when respiration is compromised. Some of the recent derivatives of S288C are corrected for this defect.
3. Sterilize raffinose solutions by filtration. Do not autoclave.
4. The cell density at this step is not critical. However, if cultures are at midlog phase prior to transfer, a growth lag associated with dilution in the new media will be minimized.
5. A harmless precipitate forms at low temperatures in media treated with the EtOH-Toluene cocktail. The precipitate partitions to the supernatant if centrifugation is performed with chilled samples.

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Overexpression and Purification of Bacterial DNA Gyrase

Anthony Maxwell and Alison J. Howells

1. Introduction

1.1. DNA Gyrase

DNA gyrase is the bacterial type II topoisomerase that can introduce negative supercoils into DNA using the free energy of ATP hydrolysis ***(1,2)***. The enzyme from *Escherichia coli* consists of two proteins, A and B (termed GyrA and GyrB), of molecular masses 97 and 90 kDa, respectively; the active enzyme is an A_2B_2 complex. All DNA topoisomerases are able to relax negatively supercoiled DNA, but only gyrase can also catalyze the introduction of negative supercoils, in a reaction coupled to ATP hydrolysis. Mechanistic studies have identified the steps involved in the supercoiling reaction. Briefly, this involves the wrapping of DNA around the A_2B_2 complex, cleavage of this DNA in both strands (involving the formation of DNA–protein covalent bonds), and passage of another segment of DNA through this double-stranded break. Resealing of the break results in the introduction of two negative supercoils. Catalytic supercoiling requires the hydrolysis of ATP.

Both GyrA and GyrB have been shown to contain distinct functional domains. The A protein consists of an N-terminal domain (59–64 kDa) involved in DNA breakage and reunion, and a C-terminal domain (33 kDa) involved in DNA–protein interactions ***(3–5)***. The B protein consists of an N-terminal domain (43 kDa) containing the ATPase activity, and a C-terminal domain (47 kDa) involved in interactions with the A protein and DNA ***(6–9)***. The structure of the 43 kDa N-terminal domain complexed with an ATP analog has been solved to 2.5-Å resolution by X-ray crystallography ***(10)***.

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The gyrase supercoiling reaction can be inhibited by a number of compounds, including the quinolone and coumarin groups of antibacterial agents ***(1,11–14)***. The quinolones (e.g., nalidixic acid and ciprofloxacin) interrupt the DNA breakage and resealing reaction of gyrase, whereas the coumarins (e.g., novobiocin and coumermycin A_1) inhibit the ATPase reaction. The structure of a complex between a 24-kDa N-terminal fragment of GyrB and novobiocin has recently been solved ***(15)***.

Given its importance as a drug target, one of the main interests in expressing the gyrase subunits is for screening potential antibacterial compounds. Currently, it is possible to obtain gyrase from commercial sources (*see* **Note 1**), but the availability of a number of overexpressing clones and relatively straightforward purification procedures means that inhouse production is not too difficult.

1.2. DNA Gyrase Clones

DNA gyrase was discovered in 1976 by Gellert and coworkers ***(16)*** and was shown to introduce supercoils into closed-circular DNA. It was later established that the enzyme is composed of two proteins, GyrA and GyrB, which could be purified independently ***(17)***. Although it is possible to purify gyrase from wild-type strains of *Escherichia coli*, it is now more convenient to use strains that have been engineered to overexpress the GyrA and GyrB proteins. Mizuuchi et al. ***(18)*** cloned the *gyrA* and *gyrB* genes under the control of the λP_L promoter in plasmids pMK90 and pMK47. Protein production is switched on by a temperature shift from 32–42°C, and yields of 12.5 mg of GyrA and 1.5 mg of GyrB/L are reported ***(18)***. Hallett et al. ***(19)*** have described plasmids in which the gyrase genes are cloned under *tac* promoter control. Strains harboring these plasmids synthesize the GyrA and GyrB proteins to about 40% of soluble cell protein (typical yields are 50–150 mg/L). The GyrA and GyrB plasmids (pPH3 and pAG111) are based on vector pTTQ18 ***(20)*** and are shown diagrammatically in **Fig. 1**. Protein expression is induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) during the midlog phase of bacterial growth. The plasmids are normally carried in *E. coli* strain JM109, and the resultant strains are termed JMtacA and JMtacB.

Although JMtacA and JMtacB produce large amounts of the gyrase proteins, some practical problems with these strains have been encountered. The vector pTTQ18 contains an identical 54-bp sequence both in the *lacI*^{q} gene and between *ori* and *taq* (**Fig. 1**). This can lead to homologous recombination and loss of the cloned gene. We have sometimes found this to be a problem during the construction of derivatives of GyrA or GyrB. This problem has been solved by creating the vector pTTQ18* in which the 54-bp sequence between *ori* and *taq* has been deleted ***(5)***.

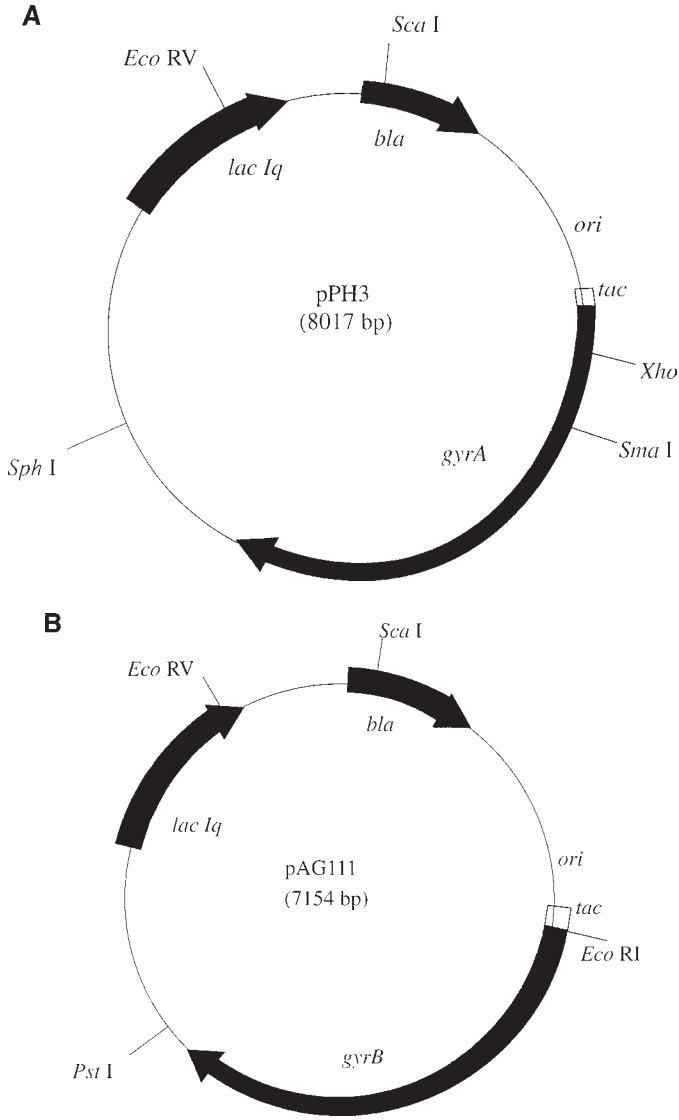

Fig. 1. Plasmids pPH3 **(A)** and pAG111 **(B)** for the overexpression of GyrA and GyrB ***(19)***. Some unique restriction enzyme sites are shown, and the approximate positions of genes are indicated.

It is well known that the supercoiling specific activity of GyrA generally exceeds that of GyrB ***(18)***. This problem seems to be exacerbated when GyrB is overexpressed to a high level, as in JMtacB, and protein from this strain can also show a high degree of uncoupling as manifested by high DNA-independent ATPase activity ***(21)***. These problems appear to be owing to misfolding of GyrB as a consequence of overloading of the chaperone system in JMtacB when it is induced; solutions to this problem are currently under investigation. Nevertheless, the GyrB produced from strain JMtacB is suitable for most purposes.

In addition to clones expressing the full-length GyrA and GyrB proteins, clones expressing various fragments of GyrA and GyrB have been generated. These include N-terminal fragments of GyrA (58–66 kDa ***[4]***), the 33-kDa C-terminal domain of GyrA ***(5)***, a 24-kDa N-terminal subdomain of GyrB ***(22)***, and the 43-kDa N-terminal domain of GyrB ***(9)***. Clones expressing the 47-kDa C-terminal domain of GyrB have not been described.

2. Materials

2.1. Media

1. Luria-Bertani broth (LB): 5 g yeast extract (Oxoid), 10 g tryptone (Oxoid), 10 g NaCl/L.
2. LB + Amp: LB containing 50 µg/mL ampicillin.

2.2. Buffers

1. TGED: 50 m*M* Tris-HCl (pH 7.5), 10% (w/v) glycerol, 1 m*M* EDTA, 2 m*M* DTT.
2. TED: 50 m*M* Tris-HCl (pH 7.5), 1 m*M* EDTA, 2 m*M* DTT.
3. Enzyme buffer (EB): 50 m*M* Tris-HCl (pH 7.5), 100 mM KCl, 10% (w/v) glycerol, 1 m*M* EDTA, 2 m*M* DTT.
4. Coupling buffer: 0.1 *M* $NaHCO_3$ (pH 8.3), 0.5 *M* NaCl.

3. Methods

3.1. FPLC-Based Purification

Conventional purification procedures using low-pressure chromatography for preparing DNA gyrase and the GyrA and GyrB subunits have been described by Mizuuchi et al. ***(17,18)***. These procedures have now been adapted to take advantage of developments in high-pressure chromatography; the procedures described below use the Pharmacia FPLC system. The preparations described are for 12 L of bacterial culture grown in a fermenter but can easily be scaled for other culture volumes and cultures grown in shaker flasks (*see* **Note 2**). It is also feasible to extract GyrA or GyrB from small culture volumes (5–10 mL) for diagnostic purposes (*see* **Note 3**). A gel showing stages in the purification of GyrA and GyrB is shown in **Fig. 2**.

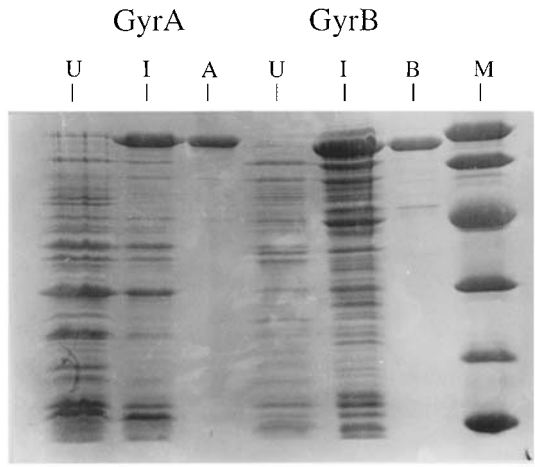

Fig. 2. SDS-polyacrylamide gel showing protein extracts from JMtacA (GyrA) and JMtacB (GyrB) in the absence (U) and presence (I) of IPTG. Tracks (A) and (B) contain purified GyrA and GyrB, respectively, and (M) contains mol-wt markers (94, 67, 43, 30, 20, and 14 kDa).

3.1.1. Preparation of Gyr A

1. Streak out strain JMtacA onto an LB + Amp agar plate. Grow at 37°C overnight (O/N).
2. Pick a single colony and grow O/N in 5 mL LB + Amp. (At this stage, it is advisable to carry out small-scale inductions on 5-mL cultures (*see* **Note 3**) to check that the strain is okay).
3. Use the 5-mL culture to inoculate 500 mL prewarmed LB + Amp and grow O/N at 37°C.
4. Inoculate the fermenter (containing 12 l LB + Amp) with the 500-mL culture, and grow until the A_{595} is 0.5. Add IPTG to a final concentration of 200 μ*M*, and grow for a further 4 h or until the growth curve plateaus.
5. Harvest the cells by centrifugation, and resuspend in a small volume (~30 mL) of 50 m*M* Tris (pH 7.5) and 10% sucrose. Quick freeze in liquid nitrogen and store at −70°C.
6. Thaw the cells and add DTT, EDTA, and KCl to the following final concentrations: 2, 20, and 100 m*M*. Disrupt the cells using a French press, and then spin for 1 h at ~100,000*g* in a precooled rotor (e.g., 34,000 rpm/TFT 50.38 rotor/Sorvall OTD65B centrifuge). Decant the supernatant. (Can be quick-frozen and stored at −70°C at this stage, if necessary.)
7. Thaw the supernatant and add solid ammonium sulfate (finely ground, enzyme grade–low in heavy metals) to a final concentration of 0.31 g/g solution, ensuring that it is added slowly over a period of 15 min at 0°C. (This can be achieved by placing a beaker containing the solution in an ice bath over a stirrer in the cold

room.) Stir for 15–30 min at 0°C. Spin at ~7500*g* (e.g., 10,000 rpm/SS34 rotor/Sorvall RC5B centrifuge) for 15–20 min (4°C). Retain the supernatant. (Check both supernatant and pellet by SDS-polyacrylamide gel electrophoresis [SDS-PAGE]; most of the GyrA protein should be in the pellet.)

8. Resuspend the pellet in a small volume of TGED and dialyze O/N into TGED at 4°C, making sure that the volume of dialysis buffer is at least 200-fold greater than the sample. Change the buffer at least twice.
9. Load the protein solution onto a Hi-Load Q-Sepharose column (Pharmacia 16/10) at 2.5 mL/min, and develop using a shallow (~250 mL) 0–450 m*M* NaCl gradient in TED. Check the conductivity of the protein solution before loading onto the column. (TED is ~12 µS, so the sample should be 10–18 µS.) GyrA should elute at 300–400 m*M* salt. Peak fractions can be identified by SDS-PAGE, and should be dialyzed into EB before quick freezing and storing at −70°C.
10. If extra purity is required, the protein may be applied to a Mono-Q column (Pharmacia 10/10) as described in **step 9** and developed with a shallow gradient (180 mL).

A typical yield from 12-L culture is 1 g purified protein.

3.1.2. Preparation of Gyr B

Steps 1–6 are exactly as described for GyrA, except strain JMtacB is used.

7. Pour a large Heparin-Sepharose (350 mL, Pharmacia) column and prerun with TGED + 200 m*M* NaCl (~3 column volumes) at ~80–100 mL/h. Load sample diluted 50/50 in TGED + 200 m*M* NaCl (the conductivity should be <30 µS) and recirculate for 1 h. Wash column with TGED + 200 m*M* NaCl until no more protein is detected. Elute by stepping off with 400 m*M* NaCl in TGED (at least 1.5 column volumes), and then wash with 1.5 column volumes of 1 *M* NaCl in TGED. Collect 10-mL fractions. Peak fractions can be identified by SDS-PAGE; pool all fractions containing GyrB, and dialyze into TGED O/N at 4°C.
8. To separate Gyr B from its remaining contaminants, the material is further purified using a Mono-Q (10/10, Pharmacia) or Hi-Load Q-Sepharose column (16/10, Pharmacia), and eluting with shallow gradients. For the Mono-Q column, this should be a 0–400 m*M* NaCl gradient in TED in at least 10 column volumes (100–150 ml). For the Hi-Load, it should be at least 200 mL. Gyr B should elute at 230–330 m*M* salt. A good way to remove the majority of the contaminant quickly is to have a fairly steep initial gradient from 0–150 m*M* NaCl over a small volume and then a much shallower gradient from 150–400 m*M*. Collect small fractions (~5 mL). Peak fractions can be identified by SDS-PAGE and should be dialyzed into EB before quick freezing and storing at −70°C. Since Gyr B tends to be unstable when pure, it is a good idea to keep the final concentration between 0.1 and 1 mg/mL.

A typical yield from 12-L culture is 0.8 g purified protein.

3.2. Affinity Column-Based Purification

Although the FPLC-based methods for preparing gyrase described above are entirely satisfactory, it is possible to make GyrA and GyrB using affinity chromatography methods. These tend to be more rapid, but do not necessarily achieve such high levels of purity.

3.2.1. Novobiocin-Affinity Columns

This method was first introduced by Staudenbauer and Orr ***(23)***, and is based on the high affinity of GyrB for the antibiotic novobiocin. It has been used to extract gyrase from various sources, including *E. coli* ***(23)***, *Bacillus subtilis* ***(24)***, and *Streptomyces sphaeroides* ***(25)***. Affinity columns can also be made using other coumarin drugs, such as coumermycin A_1 and chlorobiocin ***(22)***. The method outlined below is based on that of Staudenbauer and Orr ***(23)***.

1. Swell 5 g of epoxy-activated Sepharose 6B (Pharmacia) in 500 mL water for 1 h at room temperature.
2. Wash the Sepharose on a sintered glass filter with distilled water (approx 200 mL water/g sepharose powder).
3. Mix the gel with 2 g novobiocin (Sigma) dissolved in 100 mL of water, and shake gently for 16 h at 37°C. Do not use a magnetic stirrer.
4. Decant uncoupled novobiocin, and rinse the gel with water to remove excess novobiocin.
5. Block excess epoxy groups by transferring the gel to 1 *M* ethanolamine (pH 8.0) and let it stand overnight at 37°C.
6. Pour the novobiocin-sepharose onto a sintered funnel, and wash alternately with 0.1 *M* sodium acetate (pH 4.0), 0.5 *M* NaCl, and then 0.1 *M* Tris-HCl (pH 8.0) and 0.5 *M* NaCl. Repeat the washes at least three times.
7. Resuspend the gel in EB (or similar), degas, and pack into a column.
8. Apply the protein extract in EB (at least 1 mg of GyrB will stick to a 20-mL column), and wash the column with several column volumes of EB (most proteins will not stick to the column). GyrA may be eluted with salt (e.g., 0.8 *M* KCl), and GyrB may be eluted with urea (>5 *M*). It is worth washing with lower concentrations of urea to remove contaminant proteins.
9. Renature the protein by dialyzing into EB with three changes (*see* **Note 4**).

3.2.2. GyrA- and GyrB-Affinity Columns

As an alternative to novobiocin, affinity columns can also be prepared with either the GyrA or GyrB proteins covalently coupled to the matrix. The method is essentially the same for both proteins.

1. For a 1.75-mL column, weigh out 0.5 g dry CNBr-activated sepharose 4B (Pharmacia), and resuspend in 10 mL 1 m*M* HCl. Then, wash immediately with

1 m*M* HCl (100 mL/0.5 g gel) in several aliquots on a sintered glass filter for 15 min. Then wash with 2.5 mL coupling buffer, and immediately transfer to the protein solution (*see* **step 2**).

2. Dialyze the protein (5–10 mg of protein/mL gel) into coupling buffer, and add the washed gel. (Dilute with coupling buffer if necessary to acheive a gel:protein volume ratio of 1:2.) Mix gently on a rotating table (do not vortex) O/N at 4°C, or for 1 h at room temperature.
3. Wash excess ligand away with ~10 mL coupling buffer.
4. Pour the gel into a column (e.g., Bio-Rad 10-mL Econo-Pac column), and wash with 0.1 *M* Tris-HCl (pH 8.0) to block any remaining active groups. Allow the column to stand in the Tris buffer for 2 h at room temperature.
5. Wash the column alternately with 0.1 *M* sodium acetate (pH 4.0), 0.5 *M* NaCl, and then 0.1*M* Tris-HCl (pH 8.0) and 0.5 *M* NaCl (at least 5 column volumes each). Repeat this three times. Finally, wash the column with EB, and store at 4°C in 0.05% thimerosol in EB.
6. To use the column, pre-equilibrate with at least five column volumes of EB or TGED.
7. Load the protein extract (a 1.75-mL column should bind ~0.6 mg protein) diluted in the same buffer, keeping the conductivity fairly low (12–15 μS).
8. Wash the column with the same buffer until no more protein is eluted.
9. Develop the column with three to five column volumes of 500 m*M* NaCl in the running buffer (KCl can be used but has the disadvantage of making the fractions more difficult to load onto an SDS gel, since it forms a complex with the SDS). Collect 1.5-mL fractions.
10. Wash the column with 2 *M* NaCl (about three column volumes) to remove any remaining protein, and then wash extensively with EB.
11. To regenerate the column, wash with alternating high- and low-pH buffers (0.1 *M* Tris-HCl [pH 8.5], 0.5 *M* NaCl, and 0.1 *M* sodium acetate [pH 4.5] and 0.5 *M* NaCl). This cycle should be repeated three times followed by re-equilibration in the running buffer. If the protein preparation was fairly clean before loading, regeneration is simply a matter of washing in high salt followed by re-equilibration in the running buffer.

4. Notes

1. At the time of writing, DNA gyrase is available from two commercial sources: *Micrococcus luteus* gyrase is available from Gibco/BRL/Life Technologies (USA/Europe), and *E. coli* gyrase is available from Lucent Ltd. (UK).
2. We use an LH 2000 Series I fermenter with a 20-L vessel containing 12 L of broth, stirred at ~650 rpm with an air sparge rate of 12–15 L/min. We find that yields are generally higher in shaker flasks, but it is not as easy to grow large volumes in these.
3. A 10-mL culture of LB + Amp is inoculated with 0.2 mL from an O/N culture of JMtacA or JMtacB. At ~A_{595} = 0.5, split the culture into two aliquots and add

IPTG to one of these. Allow to grow for ~4 h, and pellet the bacteria. Resuspend the pellet in ~0.2 mL 50 m*M* Tris (pH 7.5), 10% sucrose, and analyze ~10 µL by SDS-PAGE. This will quickly show if the strain is overproducing protein.

4. It should be noted that urea-treated protein can have a low specific activity and high DNA-independent ATPase activity ***(26)***. However, such GyrB is suitable for most purposes, such as routine DNA supercoiling and DNA cleavage assays (*see* ref. *3* for details of assays).

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Overexpression and Purification of Bacterial DNA Topoisomerase I

Chang-Xi Zhu and Yuk-Ching Tse-Dinh

1. Introduction

In order to carry out studies on the structure and mechanism of enzymes, substantial quantities of purified proteins are often needed for many of the commonly used biophysical methods. This is especially true for three-dimensional structure determination using X-ray crystallography or NMR. Structure–function analysis by site-directed mutagenesis requires that a large number of mutant enzymes be expressed and purified readily, so that their properties can be compared to those of the purified wild-type enzyme. Biophysical characterizations of a mutant enzymes are desirable to assess if the mutation has altered the folded conformation of the enzyme. Therefore, it is necessary to overexpress the protein of interest to maximize the yield and facilitate the purification process. For these reasons, *Escherichia coli* DNA topoisomerase I and several of its partial fragments have been purified previously after overexpression ***(1–5)***. The methods involved should in general be applicable for overexpression and purification of bacterial topoisomerase I.

The genes coding for a number of other topoisomerase I of bacterial origins have also been cloned and sequenced ***(6–12)***. There are regions in the coded amino acid sequences that are highly conserved. The information from these sequences should facilitate the design of degenerate PCR primers for isolation of other homologous bacterial topoisomerase I genes in future. Overexpression by recombinant methods and purification of these enzymes will facilitate the investigation of their properties and potential interactions with inhibitors that may be of therapeutic use.

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Detailed procedures are given here for the overexpression of the *E. coli* DNA topoisomerase I under the control of the *lac* promoter. In an *E. coli* host strain with a *lacI*^{q} genotype suppressing the expression of the enzyme until induction by IPTG, the presence of the expression plasmid does not appear to confer a significant disadvantage for growth. In *E. coli* host strains lacking the *lacI*^{q} genotype, the *lacI*^{q} function can be conferred by contransformation with the plasmid pMK16-*lacI*^{q} ***(1)*** encoding the *lac* repressor. Alternatively, a version of the expression plasmid that includes both the *topA* gene and the *lacI*^{q} gene ***(13)*** can be used. This reduces the uninduced expression of the topoisomerase I effectively, avoiding potential selection of mutant plasmids that correspond to lost or reduced topoisomerase I activities.

2. Materials

2.1. Overexpression Plasmids and Hosts

The *E. coli topA* gene was first isolated in the laboratory of J. C. Wang (Harvard University) ***(6)***. There, it was cloned into two different expression plasmids. The plasmid pJW312 ***(1)*** has the *amp* gene for ampicillin resistance in addition to the *topA* coding sequence under the control of the *lac* promoter. A second plasmid ***(13)*** has the *amp*^{r} gene, the *topA* coding sequence under the control of the *lac*UV5 promoter, as well as the *lacI*^{q} repressor gene. Therefore, it has more tightly regulated *topA* expression than pJW312 and can be grown in virtually any *E. coli* strain. A *lacI*^{q} host is required for the maintenance of pJW312. We have used both MV1190 (available from Bio-Rad, Hercules, CA) and JM103 (available from Stratagene, La Jolla, CA) for the overexpression of *E. coli* topoisomerase I. MV1190 transformed with pJW312 has an excellent growth rate and yields about 10 mg of >95% pure enzyme from each liter of culture. *E. coli* JM103 transformed with pJW312 overexpresses slightly more topoisomerase I, but the growth rate is slower. *E. coli* topoisomerase III is known to copurify with topoisomerase I ***(14)***. It may therefore be desirable to use a host strain with a mutation in *topB*. We have constructed a derivative of JM103 selected for resistance to kanamycin after P1 transduction with phage prepared from *E. coli* K38 (*topB*::kan^r, obtained from K. J. Marians, Sloan-Kettering Institute). Although it is possible to overexpress topoisomerase I in this strain (**Fig. 1**), some of the transformants screened did not show satisfactory overexpression. There may be selection against overexpression of topoisomerase I in the absence of topoisomerase III activity.

A *topA* expression plasmid utilizing the bacteriophage T7 promoter in plasmid pET-3c has also been previously constructed along with an *E. coli* BL21 *topB*::kan^r host ***(14)***. The 67-kDa N-terminal fragment of *E. coli* topoisomerase I was overexpressed in *E. coli* as a fusion protein with glutathione-S-

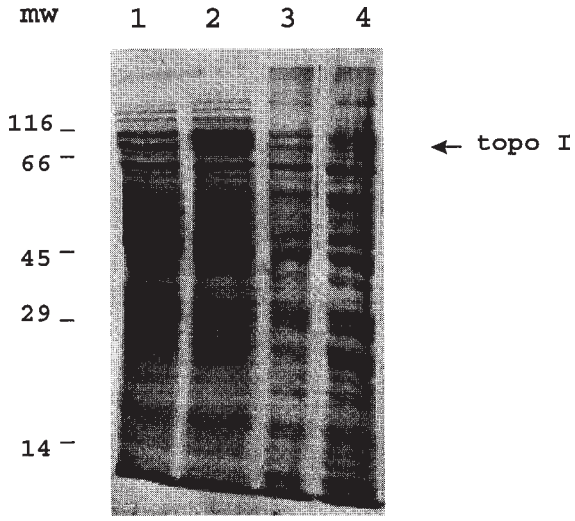

Fig. 1. Overexpression of *E. coli* topoisomerase I. Soluble lysates of *E. coli* cells were analyzed by a 10% SDS-polyacrylamide gel. The gel was stained with Coomassie brilliant blue. The lysates shown were prepared from (1) AS17 cells, (2) AS17 cells transformed with pJW312 coding for the C662H mutant enzyme, (3) JM103 *topB*::kan^r cells) and (4) JM103 *topB*::kan^r cells transformed with pJW312 coding for the wild-type topoisomerase I.

transferase that can be cleaved with thrombin ***(4)***. The 14-kDa C-terminal fragment of *E. coli* topoisomerase I was also overexpressed under the control of the bacteriophage T7 promoter using plasmids pET-3a and pET31f1+ ***(5)***. These examples illustrate the variations of vectors that can be used to express bacterial topoisomerase I. The *topA* coding region in pJW312 can be excised by digestion with *Bgl*II and *Hin*dIII ***(1)*** and replaced by a compatible restriction fragment coding for another topoisomerase I. Cloning into the pET vectors for expression under the bacteriophage T7 RNA polymerase usually requires that the starting ATG be part of a *Nde*I or *Nco*I site ***(15)***, and a *Bam*H1 site is usually available on the vector for ligation of the 3'-end of the restriction fragment to be cloned. If the appropriate restriction sites are not present on the bacterial topoisomerse I genes to be cloned, they can be generated by oligonucleotide-directed mutagenesis ***(14)***. Alternatively, primers with the appropiate restriction sites present can be designed for PCR amplification of the coding sequence to be cloned ***(5)***. However, caution should be made for potential introduction of errors into the coding sequence of the clone owing to PCR. A high-fidelity DNA polymerase, such as the Pfu DNA polymerase (from Stratagene), should be used for the PCR synthesis.

Owing to the efficient overexpression, the level of topoisomerase I expressed from the plasmid pJW312 is >10^4-fold higher than that from the chromosome. Therefore, if topoisomerase I from another bacterial species is to be expressed in *E. coli*, or if a mutant topoisomerase I has to be purified, copurification of the wild-type *E. coli* topoisomerase I should not normally be a significant problem. Nevertheless, there are two possible approaches to limit the potential copurification. The exogeneous or mutant topoisomerase I can be expressed as a fusion protein, as in the case of the 67-kDa N-terminal fragment ***(4)***. A number of amino acids would remain added to the topoisomerase I sequence after cleavage of the fusion. The other approach involves using the *E. coli* strain AS17 ($topA_{am}$ pLL1[Tc^R $supD^{ts}$]), from R. E. Depew, Northeastern Ohio University) that has a reduced level of expression from chromosomal *topA* at temperatures above 37°C owing to the presence of a temperature-sensitive suppressor ***(1)***. This was used to overexpress and purify several mutants of *E. coli* topoisomerase I (**Fig. 1**) ***(16)***.

2.2. Expression of Topoisomerase I

1. Luria broth (LB) base (from GIBCO/BRL, Gaithersburg, MD).
2. Ampicillin.
3. Isopropyl-β-D-thiogalactoside (IPTG): 100 m*M* solution.

2.3. Purification of Topoisomerase I

1. Lysozyme, from egg white, ultrapure-grade.
2. 200- and 20-mL chromatography columns.
3. DEAE-51 (from Whatman, Clifton, NJ).
4. Phosphocellulose P11 (from Whatman).
5. Single-stranded DNA agarose (from GIBCO/BRL).
6. Buffer I: 20 m*M* potassium phosphate, pH 7.4, 0.2*M* KCl, 1 m*M* dithiothreitol (dTT), 1 m*M* EDTA, 10% glycerol.
7. Buffer A: 20 m*M* potassium phosphate, pH 7.4, 1 m*M* dTT, 1 m*M* EDTA, 10% glyercol.
8. Storage buffer: 0.1*M* potassium phosphate, pH 7.4, 0.2 m*M* dTT, 0.2 m*M* EDTA, 50% glycerol.

3. Methods

3.1. Expression of Cloned Topoisomerase I

1. Prepare a fresh overnight culture of the *E. coli* cells transformed with the topoisomerase expression plasmid in LB medium with 100 μg/mL of ampicillin at 37°C (*see* **Note 1**). The overnight culture should be diluted at least 100-fold into LB with ampicillin for the large-scale culture. A volume of 500 mL in a 2-L flask would provide adequate aeration in a shaker incubator.
2. For pJW312 in MV1190 or JM103, topoisomerase I expression is induced by the

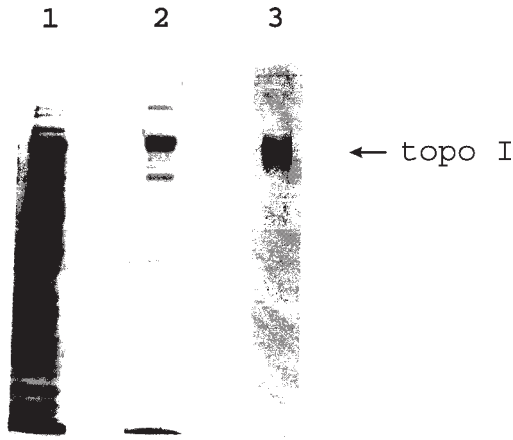

Fig. 2. Purification of *E. coli* topoisomerase I expressed in JM103 transformed with pJW312. The Coomassie-stained 10% SDS gel showed (1) fraction I before phosphocellulose P11 column chromatography (2) fraction II after phosphoceullose P11 purification and (3) pure enzyme after ssDNA agarose column chromatography.

addition of IPTG to 400 µ*M* when the absorbance of the culture reaches an OD of 0.4–0.6 at 595 nm. The timing of induction should be optimized for each recombinant clone. Growth is continued for another 3 h. Pellet the cells by centrifugation in a GSA-3 rotor at 4°C at 5080*g* for 10 min. A small amount of the cell pellets from each flask should be checked for overexpression after small-scale lysis before the cell pellets are combined. The cell pellets can be stored at −70°C.

3.2. Purification of Topoisomerase I from MV1190/pJW312 or JM103/pJW312 Cells

1. Resuspend the cell pellet from each 500 mL of culture in 10 mL of 10 m*M* Tris-HCl, pH 8.0. Add 1/10 vol of 4*M* KCl and 1/10 vol of 10 mg/mL lysozyme (dissolved in 10 m*M* Tris-HCl, pH 8.0). Keep on ice for 1 h.
2. Freeze cells rapidly in dry ice or −70°C freezer. Thaw tubes at room temperature until no longer frozen. Do not leave at room temperature beyond that point. Immediately freeze cell lysates again. After freezing and thawing for a total of three times, spin lysate at 5080*g* in a Ti45 rotor at 4°C for 3 h. Discard pellets.
3. Dialyze the crude extract against buffer I overnight at 4°C. Mix 100 mL of crude extract with 100 mL of DEAE-51 to remove nucleic acids. Stir gently at 4°C for 1 h. Spin at 5080*g* in a GSA rotor at 4°C for 10 min. Save supernatant. Mix pellet with another 30 mL of buffer I, and stir gently for 10 min at 4°C. Repeat centrifugation. Combine supernatants (Fraction I, *see* **Fig. 2**).
4. Load Fraction I onto 175 mL phosphocellulose (P11) column equilibrated with buffer I. Wash with buffer I until the column fractions has no absorbance at 280 nm. Elute with 1800 mL gradient of buffer A with 0.2–1*M* KCl. Assay

fractions for relaxation activity (*see* **Note 2**), and check protein elution profile with SDS-gel electrophoresis followed by staining with Coomassie brilliant blue. The enzyme should elute at around 0.4–0.5*M* KCl. Pool the active fractions (Fraction II, *see* **Note 3**).

5. Check the conductivity of Fraction II to estimate the KCl concentration. Dilute fraction II with buffer A to achieve a KCl concentration of 0.1*M*, or dialyze against buffer A + 0.1*M* KCl. Load onto a 10-mL ssDNA agarose column (*see* **Note 4**) equilibrated with buffer A + 0.1*M* KCl. Wash with 50 mL buffer A + 0.1 *M* KCl. Elute with 100 mL gradient of buffer A with 0.1–1*M* KCl. Check purity by SDS gel. The high-purity enzyme fractions are eluted toward the end of the gradient. Active but impure fractions can be combined for repeated chromatography on the ssDNA agarose column. Dialyze the pooled enzyme fractions into enzyme storage buffer, and keep at −20°C.

4. Notes

1. The MV1190/pJW312 and JM103/pJW312 transformants expresses soluble *E. coli* topoisomerase I well when grown at 37°C. However, for some other recombinant topoisomerases, it may be necessary to alter the growth temperature for optimal expression level. Many recombinant proteins can be recovered more easily as soluble proteins if they are expressed at 30°C.
2. Bacterial topoisomerase I requires Mg(II) for relaxation activity. Therefore, when assays for activity are carried out for the crude lysate, and during the early stages of purification, it will be necessary to include 5 µg of tRNA in each reaction to inhibit the degradation activities of nucleases, so that conversion of supercoiled DNA to relaxed topoisomers will not be obscured by nicking of DNA by nucleases.
3. *E. coli* topoisomerase I purified by the above procedures appeared homogeneous when stained with Coomassie brilliant blue after SDS-gel electrophoresis. To ensure that even very low-level contaminants not visible by Coomassie staining are removed, an extra chromatography step with hydroxylapatite column can be carried out between the P11 and ssDNA agarose column steps. The column is equilibrated with buffer A and eluted with a linear gradient of buffer A with potassium phosphate concentration increasing from 20–450 m*M*.
4. The ssDNA agarose can be regenerated by extensive washing with 10 vol of 2*M* NaCl, followed by re-equilibration with 10 vol of buffer A + 0.1*M* KCl. The affinity matrix can be used two or three times without significant loss of performance.

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Overexpression and Purification of *Escherichia coli* DNA Topoisomerase III

Russell J. DiGate

1. Introduction

The development of recombinant DNA techniques and protein expression systems has been critical to the understanding of the structure and catalytic mechanism of topoisomerases. The ability to overexpress and purify large quantities of these molecules has led to the elucidation of the crystal structures of the amino-terminal fragment of the GyrB subunit of *Escherichia coli* DNA gyrase ***(1)***, a large fragment of yeast topoisomerase II (topo II) ***(2)***, and of the first 596 amino acids of *E. coli* DNA topoisomerase I (topo I) ***(3)***. In addition to the obvious advantages that high-yield protein expression systems have in terms of the absolute quantity of enzyme obtained, these systems also provide a sufficient amount of starting material to allow the purification of an enzyme from any possible contaminating activity(ies). This has been clearly demonstrated for *E. coli* DNA topo I and topoisomerase III (topo III) ***(4)***. Early studies, using topo I preparations purified from cells containing the gene encoding topo I (*topA*) on a multicopy plasmid showed that topo I was capable of resolving plasmid DNA replication intermediates in vitro ***(5)***. It was not until the purification and characterization of topo III that it was realized that certain catalytic properties previously ascribed to topo I may be owing to contamination of topo I preparations with topo III. Subsequently, *topA* was cloned into an overexpression vector, induced, and purified from cells in which the gene encoding topo III (*topB*) had been disrupted ***(4)***. Topo I purified in this manner was incapable of fully resolving plasmid DNA replication intermediates in vitro ***(4)***.

The bacteriophage T7 transient expression system, described by Studier and colleagues ***(6)***, has been particularly useful in the overexpression of proteins of

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both eukaryotic and prokaryotic origin. The genes encoding both topo I and III have been cloned into this system ***(4,7)***. The overexpression vectors designed for use with the system, pET vectors place a gene of interest directly downstream of a consensus ribosome binding site and a strong bacteriophage T7 promoter. Expression of the cloned gene product is minimal until T7 RNA polymerase is supplied to the cell. This is accomplished by induction of the T7 RNA polymerase, using Isopropyl B-D- Thiogalactoside (IPTG), in a host in which the gene encoding T7 RNA polymerase has been placed in the *E. coli* chromosome under the control of the *lac*UV5 promoter (strains designated DE3), or by infecting cells (that do not have an endogenous T7 RNA polymerase gene) with bacteriophage λ CE6, which contains the T7 RNA polymerase gene under the control of the phage P_L promoter ***(6)***. A pET vector that contains a gene encoding harmful polypeptide cannot be maintained in DE3 hosts, and the polypeptides must be induced by bacteriophage λ CE6 infection. This is presumably owing to the "leakiness" of the *lac*UV5 promoter in the absence of IPTG. Topo III is an example of a polypeptide that requires phage induction ***(4,7)***. Interestingly, a pET vector containing *topA* can be maintained in DE3 strains.

The protocol in this chapter describes the purification of *E. coli* topo III. Topo III, a type 1 enzyme, is the smallest of the *E. coli* topoisomerases (73.2 kDa) ***(7)***. This topoisomerase was originally purified as a DNA relaxation activity from cells lacking topo I activity (Δ*topA*) ***(8,9)***. Topo III is a potent decatenase in vitro ***(10)*** and a site-specific binding protein that binds specifically to its cleavage site ***(11)***. Topo III is unique among topoisomerases in its ability to bind and cleave RNA as well as DNA ***(12)***.

This purification protocol makes use of a soybean trypsin inhibitor column. This column has been found useful to limit proteolysis of both topo I and topo III ***(4)***. These enzymes are particularly sensitive to proteolysis by a trypsin-like protease, since the carboxyl-terminal domains of both enzymes contain a large number of lysine and arginine residues ***(7,13,14)***. The procedure has been designed to allow the purification of relatively large quantities of topo III in a 3–4 d time period. An assay for topoisomerase activity is provided; however, topo III constitutes the majority of the protein after the first purification step and the enzyme can be purified by protein assays.

2. Materials

2.1. Plasmid Vector and Bacterial Strains

1. Topo III expression plasmid pDE1 ***(7)***, which consists of the *topB* gene cloned into the bacteriophage T7 transient expression vector pET3c ***(6)***, is available from this laboratory for noncommercial, academic use.

2. *E. coli* strain BL21, ED8739, and bacteriophage λ CE6 may be purchased from Novagen, Madison WI.

2.2. Expression of DNA Topo III

1. Luria broth (LB): 10 g tryptone, 5 g yeast extract, 10 g NaCl/L. Adjust the broth to pH = 7.5 by the addition of 10 *N* NaOH (~200 µL/L of broth). Plates: add 15 g agar/L LB media.
2. Ampicillin: 100 mg/mL (sodium salt) made in sterile dH_2O. Available from Sigma (St. Louis, MO).
3. Maltose: 20% solution made in dH_2O and filter-sterilized.
4. Glucose: 40% solution made in dH_2O and filter-sterilized.
5. Magnesium sulfate: 1*M* solution made in dH_2O and filter-sterilized.

2.3. Buffer Preparation

1. Prepare 200 mL of buffer A: 50 m*M* Tris-HCl, pH 8.0 (at 22°C), 10% sucrose.
2. Prepare 4 L of buffer B: 50 m*M* Tris-HCl, pH 8.0 (at 22°C), 1 m*M* dithiothreitol, 1 m*M* EDTA, 10% sucrose.
3. Prepare 1 L of buffer C: 50 m*M* Tris-HCl, pH 8.0 (at 22°C), 1 m*M* dithiothreitol, 1 m*M* EDTA, 50 m*M* NaCl, 40% glycerol.

2.4. Purification of DNA Topo III

1. Brij-58: 10% solution made in dH_2O.
2. DE-52 cellulose: available from Whatman (Maidstone, UK).
3. Soybean inhibitor agarose: available from Sigma.
4. Single-stranded DNA cellulose: available from Sigma.
5. Sephacryl S-200-HR: available from Sigma.
6. Centriprep 30 concentrator: available from Amicon (Beverly, MA).
7. SDS-PAGE equipment ***(15)***.

3. Methods

3.1. Preparation of Bacteriophage λ CE6

1. Streak an LB agar plate with *E. coli* ED8739, and grow overnight at 37°C.
2. Pick a single colony, and grow overnight at 37°C in 5 mL of LB supplemented with 5 m*M* magnesium sulfate.
3. Add 1 mL of the overnight culture and 0.1 mL of bacteriophage λ CE6 lysate ($1–5 \times 10^{10}$ PFU/mL) to 200 mL of prewarmed LB containing 5 m*M* magnesium sulfate. Swirl to mix, and let stand for 15 min at 37°C.
4. Shake at 200 rpm at 37°C, until lysis occurs (usually within 7 h).
5. Add 2 mL of chloroform to the flask after lysis has occurred, and shake for an additional 5–10 min.
6. Centrifuge the supernatant fluid in a GSA(or JA14) rotor at 8000 rpm (10,000*g*) for 20 min.

7. Remove the supernatant fluid (only ~150 mL to avoid chloroform contamination), and transfer to a sterile glass bottle. Cover the bottle with aluminum foil, and store in the refrigerator until needed. This commonly results in a bacteriophage lysate of $1–5 \times 10^{10}$ PFU/mL (which can be confirmed by titering the lysate).

3.2. Chromatographic Resin Preparation

1. Hydrate 20 g of DE-52 cellulose in 200 mL of dH_2O. Define the cellulose five times by allowing the resin to settle, pouring off the supernatant fluid, and resuspending the resin in the same volume of dH_2O. Resuspend the resin in 5 vol of 0.5*M* Tris-HCl (pH 8.0 at 22°C). Degas the resin using a vacuum pump until few gas bubbles are observed. After degassing, allow the resin to settle and pour off the supernatant fluid. Add another 5 vol of the 0.5*M* Tris-HCl (pH 8.0 at 22°C) and mix. Let the suspension stand for 15 min, and then transfer the preparation to a centrifuge tube (GSA or JA14 tube) and centrifuge the resin at 2500*g* for 5 min. Resuspend the resin in 5 vol of buffer B, and allow to stand 15 min. Repeat the centrifugation step and re-equilibrate the resin again in 5 vol of buffer B. Repeat this equilibration procedure a total of five times to ensure that the DE-52 resin is completely equilibrated in buffer B. After the final equilibration step, resuspend the resin in a 50% v/v slurry in buffer B. The equilibration of DE-52 cellulose may be performed days in advance of the purification, and the final equilibrated resin stored in the refrigerator until needed.
2. Combine ~15 mL soybean trypsin inhibitor agarose slurry and 100 mL of buffer B in a side arm flask, and completely degas resin using a vacuum pump. Store in the refrigerator until needed.
3. Hydrate ~2 g of powdered single-stranded DNA cellulose in buffer B supplemented with 2*M* NaCl. Store in the refrigerator until needed.
4. Pour enough of a Sephacryl S-200-HR slurry to yield 150 mL of resin. Pour the resin and 250 mL of buffer B, supplemented with 0.5*M* NaCl, into a side arm flask, and completely degas the mixture using a vacuum pump. Store in the refrigerator until needed.

3.3. Expression of DNA Topo III

1. Transform *E. coli* expression strain BL21 (either electroporate or use chemical methods ***[16]*** to obtain competent cells) with topo III expression plasmid pDE 1, and plate transformation on LB plates containing 200 µg/mL ampicillin. Incubate overnight at 37°C (*see* **Note 1**).
2. Inoculate 50 mL of LB broth (in a 250-mL flask) containing 1 m*M* magnesium sulfate, 0.2% maltose, and 0.5 mg/mL ampicillin with a single colony of BL21 that contains plasmid pDE1. Incubate overnight in a gyratory water shaker (or air shaker) at 37°C. Read the optical density of the grown culture at 590 nm (the OD_{590} is usually between 2 and 3).
3. Dilute the overnight culture to a final $OD_{590} = 0.1$ in 500 mL LB media (in a 2-L flask) supplemented with 1 m*M* magnesium sulfate, 0.2% maltose, 500 µg/mL ampicillin, and grow at 37°C, 250 rpm, until culture reaches an $OD_{590} = 0.3$.

Add magnesium sulfate to a final concentration of 10 m*M*, and glucose (40%) to a final concentration of 0.4%. Continue growth at 37°C until culture attains OD_{590} = 0.6–1.0.

4. Add 100 mL of a bacteriophage λ CE6 lysate (~2–5 × 10^{10} PFU/mL) to the culture and shake slowly (~50 rpm) for 15 min to allow the bacteriophage to adsorb to the cells. After 15 min, shake cells for 3 h, 37°C, 250 rpm (*see* **Note 2**).
5. Transfer the induced culture to an ice bath, and shake the culture until the media has cooled to < 5°C. Collect the induced cells by centrifugation in a Sorval GS3 rotor (Beckman JA10 or equivalent) at 8700*g*, for 10 min at 2°C.
6. Resuspend the cell pellet to a final OD_{590} = 200 with buffer A (this should be ~2.5 mL final volume). Transfer the cells to a 15-mL screw-cap polypropylene tube, and freeze the cells in a dry ice–ethanol bath for ~20 min. Store the cells in a −70°C freezer until needed.

3.4. Purification of DNA Topo III

The following purification is designed for the purification of topo III from 500 mL of induced cells; however, the capacities of the chromatographic resins used in the purification are provided so that the scale of preparation can be increased or decreased.

3.4.1. Crude Extract Preparation

1. Place frozen cells on ice until completely thawed. The cells should be well lysed after thawing. Add 1/100 vol of 10% Brij-58 to the cells, and invert several times to mix. Place lysed cells in a 15-mL polypropylene centrifuge tube (for an SS-34 or JA20 rotor) and centrifuge at 39,000*g* for 60 min in an SS-34(JA20) rotor at 2°C.
2. Remove supernatant fluid, and measure volume and protein concentration (using Bio-Rad protein assay kit or equivalent). Calculate total amount of protein present in the crude extract. Approximately 30–50 mg of protein can be expected at this stage of the purification.

3.4.2. DE-52 Cellulose Batch Chromatography

1. Dilute the crude extract to a final concentration of 10 mg/mL using buffer B.
2. The DE-52 batch step is performed at a ratio of 10 mg protein/mL of DE-52 resin. Remove the appropriate volume of the pre-equilibrated 50% DE-52 slurry to a 40-mL screw-cap SS-34 (JA20) tube, and centrifuge the slurry for 5 min at 3000*g* in an SS-34 (or JA20 rotor) at 2°C. Remove the equilibration buffer from the resin, add the crude extract to the resin pellet, and cap the tube. Mix the extract and the resin thoroughly using a nutator rocker shaker in a 2–4°C cold room. Shake the mixture for 60 min.
3. Centrifuge the slurry in an SS-34 (JA20) rotor at 3000*g* for 10 min at 2°C. Remove the supernatant fluid, and store in capped bottle on ice. Resuspend and wash the remaining resin with 1 vol of buffer B. Repeat the centrifugation step,

and pool the wash with the unbound protein pool. Attach a sintered glass funnel to a side arm flask, and pass the pooled flowthrough and wash fraction through the funnel using a vacuum source to remove any remaining DE-52 resin.

4. Measure the volume, and calculate the protein concentration of this fraction. Approximately 10–15 mg of protein can be expected at this stage of the purification. Keep the fraction on ice until the next step in the protocol (*see* **Note 3**).

3.4.3. Trypsin Inhibitor Agarose Chromatography

1. Pour a 10 mL (1.77 $cm^2 \times 5.6$ cm) soybean trypsin inhibitor agarose column (i.e., ~2 mg protein/mL of resin).
2. Equilibrate the column by washing the column with 10 column volumes of buffer B.
3. Load the combined DE-52 flowthrough, wash onto the trypsin inhibitor column, and collect 2-mL fractions. Begin collecting fractions immediately, since topo III does not bind to this column. Elute the column at a flow rate of 10 mL/h.
4. Wash the column with two column volumes of buffer B once the protein fraction has been completely loaded. Continue to collect 2-mL fractions.
5. Determine the protein concentration for each fraction, and pool all fractions that contain protein. Measure the volume and protein concentration of the pooled fractions. Approximately 9–14 mg of protein can be expected at this stage in the purification (*see* **Note 4**).

3.4.4. Single-Stranded DNA Cellulose Chromatography

1. Pour a 2-mL (0.4 $cm^2 \times 5$ cm) single-stranded DNA cellulose column (i.e., ~5 mg protein/mL of resin).
2. Equilibrate the column with 10 column volumes of buffer B supplemented with 50 m*M* NaCl.
3. Adjust the trypsin inhibitor flowthrough fraction to 50 m*M* NaCl by the addition of the appropriate amount of solid NaCl to the fraction. Mix slowly in the cold until the NaCl is completely dissolved.
4. Load the trypsin inhibitor agarose flowthrough fraction through the single-stranded DNA column using a flow rate of 2 mL/h.
5. Once the flowthrough has been completely loaded through the column, wash the column with two column volumes of buffer B (supplemented with 50 m*M* NaCl) at a flow rate of 2 mL/h.
6. Elute the column with a 20 mL 50 m*M* → 600 m*M* NaCl gradient (prepared in buffer B) at a flow rate of 2 mL/h. Collect 100 × 0.2 mL fractions.
7. Measure the protein concentration of every fifth fraction (use ~5 µL for each fraction for the determination). Once the approximate location of the protein peak has been found, determine the protein concentration of every other fraction around the peak. Pool all fractions that contain at least one-half of the protein contained in the peak fraction.
8. Measure the volume and protein concentration of the pooled single-stranded DNA cellulose fractions. Approximately 4–6 mg of protein can be expected at this stage of the purification.

3.4.5. Sephacryl S-200-HR Chromatography

1. Pour a 100-mL (0.8 $cm^2 \times 125$ cm) Sephacryl S-200-HR column.
2. Equilibrate the column with 10 column volumes of buffer B supplemented with 0.5*M* NaCl.
3. Concentrate the single-stranded DNA cellulose pool to a final volume of 0.7 mL using a Centriprep 30 apparatus.
4. Remove buffer from the top of the S-200-HR column, and load the concentrated single-stranded DNA cellulose pool atop the resin. Allow the sample to enter the resin slowly. After the sample has entered the resin, apply a small amount of buffer atop the resin and begin to elute the column at a flow rate of 5 mL/h. Collect 100×1 mL fractions.
5. Measure the protein concentration of every fifth fraction (use ~5 µL for each fraction for the determination). Once the approximate location of the protein peak has been found, determine the protein concentration of every other fraction around the peak. Pool all fractions that contain at least one-half of the protein contained in the peak fraction.
6. Measure the volume and protein concentration of the pooled S-200-HR fractions. Approximately 1–2 mg of protein can be expected at this stage of the purification
7. Dialyze the pooled S-200-HR fraction overnight against 1 L of buffer C. Store sample at –20°C.
8. Examine the protein present from each of the pools using SDS-PAGE ***(15)***. An example the polypeptides present in each fraction is illustrated in **Fig. 1** (*see* **Notes 5–7**).

4. Notes

1. We have found that maximal overexpression of topo III in *E. coli* BL21 requires the use of a freshly transformed colony of BL21. We recommend performing a transformation of BL21, with plasmid DNA pDE1, just prior to beginning the purification.
2. In order to prevent the waste of time, we recommend that a small aliquot of the induced cells be lysed and examined by SDS-PAGE prior to the beginning of the purification. We commonly pellet 1 mL of the induced cell culture in an Eppendorf tube and resuspend the pellet in 40 µL of buffer A. Fifty microliters of SDS denaturation buffer ***(15)*** are added, and the sample is denatured for 5 min at 95°C. Electrophorese 15 µL of this sample through a 10% SDS gel, and stain with Coomassie brilliant blue. If an intense 73-kDa protein band is not observed, the induction did not work properly and one should discard the cells.
3. The recovery of protein after the DE-52 cellulose batch step is also indicative of the efficiency of topo III overexpression. If there is good induction, the recovery of protein from the DE-52 cellulose step should be 25–40%. A recovery of <15% is indicative of a poor induction.
4. The trypsin inhibitor agarose step is included in this purification because it appears to bind a protease that cleaves the carboxyl-terminus of topo III.

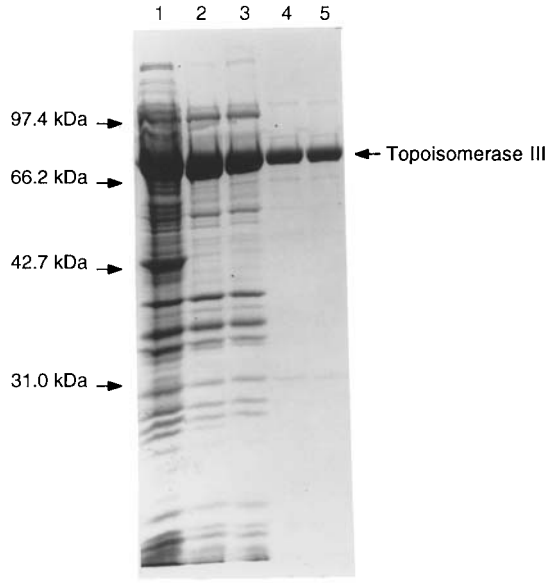

Fig. 1. SDS-polyacrylamide gel analysis of polypeptides present at different stages of the topo III purification. Lane 1, crude extract; lane 2, DE-52 cellulose pool; lane 3, trypsin inhibitor agarose pool; lane 4, single-stranded DNA cellulose pool; lane 5, Sephacryl S-200-HR pool. The position of DNA topo III is indicated as well as the positions of mol-wt markers run in an adjacent lane.

However, this resin is particularly expensive; therefore, if full-length topo III is not absolutely required, one may omit the trypsin inhibitor agarose chromatography step. This results in the purification of partially proteolyzed, but active topo III.

5. This purification protocol may also be used for the purification of *E. coli* topo I ***(6)***. The only change to the protocol would be the use of a 50 m*M* → 2*M* NaCl gradient for the elution of the enzyme from single-stranded DNA cellulose. A topo I expression plasmid (pTI1), cloned into plasmid pET3c, is also available for noncommercial, academic use from this laboratory.
6. The purification of topo III does not require the use of a topoisomerase assay; however, the final fraction should be assayed for topoisomerase activity. A topo III DNA relaxation is as follows: reaction mixtures (25 µL) contain 40 m*M* HEPES-KOH buffer (pH 8.0 at 22°C), 1 m*M* magnesium acetate (pH 7.0), 0.1 mg/mL bovine serum albumin, 40% (v/v) glycerol, 200 ng ϕX174 form I DNA. Reactions are incubated at 52°C for 10 min, and the reaction products are separated through a vertical 1% agarose gel (using TAE buffer) and visualized by staining with 1 µg/mL ethidium bromide ***(11)***.
7. The approximate amount of protein to expect at each stage of the purification is provided; however, this may vary. The capacity at which we use each chromato-

graphic resin is also provided to facilitate scale-up (or down) of the preparation. In the case of the scale-up (or down) of the Sephacryl S-200-HR chromatography step, a general rule of thumb is to load no more protein (mg) than 10% of the total volume of the column. For example, do not load more than 10 mg of protein onto a 100-mL S-200-HR column. In addition, for maximum separation, never elute a gel-filtration column at >1/10 column volume/h.

8. Column volumes are also provided as dimensions for each chromatographic step in the form of πr^2 (cm^2) × h (cm), where r is the radius of the column and h is the height of the resin.

Acknowledgment

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Overexpression and Purification of Bacterial Topoisomerase IV

Hong Peng and Kenneth J. Marians

1. Introduction

Escherichia coli topoisomerase IV (topo IV) was discovered by Kato et al. ***(1)***, who showed that the predicted open reading frames from the *parC* and *parE* genes encoded proteins with a high degree of amino acid similarity to *gyrA* and *gyrB*, respectively. A new superhelical DNA relaxation activity could be demonstrated when extracts prepared from strains overproducing the *parC* and *parE* gene products were mixed together. ParC and ParE were subsequently purified ***(2,3)***, and it was demonstrated that they formed a heterotetramer ***(3)*** with ATP-dependent, type II topoisomerase activity ***(2,3)***.

Characterization of topo IV activity in vivo and in vitro has shown convincingly that it, and not DNA gyrase, is the enzyme responsible for decatenating replicating daughter DNA molecules ***(4,5)***. Whereas topo IV is gyrase-like based on amino acid sequence comparisons ***(1)***, the enzymes have distinct substrate preferences. Gyrase acts preferentially on supercoiled DNA, whereas topo IV acts preferentially on catenated DNA ***(6)***. On the other hand, both enzymes are inhibited by the quinolone and coumarin antibiotics in vitro ***(2,3)***, and it has been shown recently that topo IV can be a target in vivo for the quinolones ***(7)***.

2. Materials

2.1. Bacterial Growth

1. Luria broth (LB): 10 g tryptone, 10 g NaCl, 5 g yeast extract, 3 mL 1 *N* NaOH/L.
2. Ampicillin stock solution: 50 mg/mL.
3. Thiamine stock solution: 2 mg/mL.

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4. Chloramphenicol stock solution: 25 mg/mL in C_2H_5OH.
5. Glucose stock solution: 40%.
6. Isopropyl-β-D-thiogalactoxide (IPTG) stock solution: 40 m*M*.
7. The host strain *E. coli* BL2l(λDE3)pLysS was from Novogen (Madison, WI).
8. The expression plasmid vector pET3c was from Novogen.
9. Tris-sucrose buffer: 50 m*M* Tris-HCl (pH 8.0 at 4°C), 10% sucrose.

2.2. Protein Purification

1. Tris buffer stock solution: 1*M* Tris-HCl (pH 7.5 at 50 m*M* and 4°C).
2 EDTA stock solution: 0.5*M*.
3. DTT stock solution: 1*M*.
4. Polymin P stock solution: 10% in 50 m*M* Tris-HCl (pH 7.5) (*see* **Note 1**).
5. Lysozyme stock solution: 10 mg/mL.
6. Brij-58 stock solution: 10% (*see* **Note 2**).
7. Buffer A: 50 m*M* Tris-HCl (pH 7.5 at 4°C), 5 m*M* DTT, 1 m*M* EDTA, 20% (v/v) glycerol.
8. Topo IV storage buffer: 50 m*M* Tris-HCl (pH 7.5 at 4°C), 10 m*M* 2-mercaptoethanol, 1 m*M* EDTA, 150 m*M* NaCl, 40% glycerol.

2.3. Superhelical DNA Relaxation

1. Tris buffer stock solution: 1*M* (pH 7.8 at 50 m*M* and 37°C).
2. $MgCl_2$ stock solution: 1*M*.
3. DTT stock solution: 1*M*.
4. ATP stock solution: 20 m*M*.
5. KCl stock solution: 1*M*.
6. Spermidine-HCl stock solution: 100 m*M*.
7. BSA stock solution: 5 mg/mL (*see* **Note 3**).
8. Superhelical plasmid DNA stock solution: 400 µg/mL (*see* **Note 4**).

3. Methods

3.1. Overexpression of ParC and ParE

1. Overnight cultures of BL21(λDE3)pLysS-pET3c-*parE* or *parC* are grown at 37°C in LB supplemented with 0.5% glucose, 20 µg/mL thiamine, 0.5 mg/mL ampicillin, and 25 µg/mL chloramphenicol.
2. The overnight is diluted into fresh medium to give an $OD_{600} = 0.1$. Four liters of culture should be grown. This provides enough material for proper lysis and sufficient purification. Grow the cultures in 2-L flasks (baffled flasks are preferred) with only 0.5 L of media/flask. Grow at 37°C with vigorous aeration (grow on a rotary shaker at 10,000*g*) to $OD_{600} = 0.4$, add IPTG to 0.4 m*M* and continue the incubation for an additional 2–3 h (*see* **Note 5**).
3. Chill the cells in an ice-water bath, and harvest using a Sorval GS-3 rotor at 8000 rpm for 10 min.

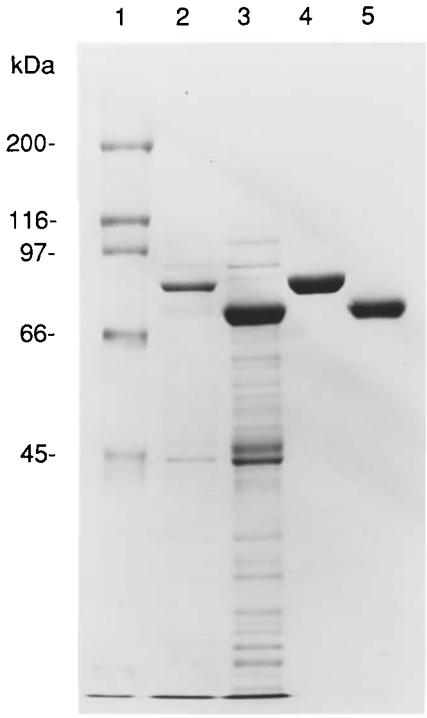

Fig. 1. SDS-PAGE analysis of fraction 1 ParC (lane 2) and ParE (lane 3) and purified ParC (lane 4) and ParE (lane 5).

4. Resuspend the cells in 8 mL of Tris-sucrose/L of culture. At this stage, the cell suspension can be frozen in liquid N_2 and stored at −80°C for later use or lysed directly (*see* **Note 6**).

3.2. Cell Lysis and Extract Preparation

1. Cell suspension (24 mL/tube) is distributed into two tubes capable of being centrifuged at 100,000*g* (Sorvall A-841 tubes or Oakridge type 30 tubes) and is adjusted to 50 m*M* Tris-HCl (pH 8.4 at 4°C), 20 m*M* EDTA, 150 m*M* NaCl, 0.1% Brij, and 0.02% lysozyme. The suspension is incubated at 0°C for 20 min and then centrifuged at 100,000*g* for 1 h. The supernatent is fraction 1a (**Fig. 1**).
2. Fraction 1a is made 0.07% in Polymin P by the slow addition (over 10 min) with rapid stirring of a 1% Polymin P stock solution. The suspension is stirred an additional 10 min, and precipitated nucleic acid is removed by centrifugation in the Sorvall SS-34 rotor at 47,000*g* for 10 min. The supernatant is fraction 1b.
3. Protein is precipitated from fraction 1b by the addition (over 10 min) with rapid stirring of 0.29 g $(NH_4)_2SO_4$/mL. The suspension is stirred an additional 30 min,

and the precipitate collected by centrifugation as in **step 2** above. The protein pellet is dissolved in a minimal volume of buffer A. The resuspended protein (fraction II) can be frozen in liquid N_2, and stored at −80°C or used directly for protein purification (*see* **Note 7**).

3.3. Purification of ParE

1. Fraction 2 is dialyzed against 100 vol of buffer A overnight. Conductivity of the dialyzate should be equal to that of buffer A. Any precipitated protein is cleared by centrifugation.
2. Fraction 2 is loaded onto a DE-52 column at a ratio of 10 mg protein/mL of packed column. The column is equilibrated beforehand with buffer A (*see* **Note 8**).
3. The column is washed with five column volumes of buffer A and then eluted with a 10-column volume linear gradient of 0–200 m*M* NaCl in buffer A. Fractions (one-tenth column volume) are collected and protein concentration determined. ParE elutes at 90–100 m*M* NaCl, and its elution pattern is identical to that of the total protein. Alternatively, ParE can be localized by SDS-PAGE or activity assay. Fractions equivalent to one-half peak height or greater are pooled to give fraction 3.
4. Fraction 3 is diluted with an equal volume of buffer A and applied to a heparin agarose column at a ratio of 5 mg protein/mL of packed column. The column is equilibrated beforehand with buffer A + 50 m*M* NaCl. The column is washed with five column volumes of the equilibration buffer and eluted with a 10-column volume linear gradient of 50–400 m*M* NaCl in buffer A. Fractions are collected and ParE (which elutes at 200 m*M* NaCl) localized as in **step 2** above. Pooled fractions are fraction 4.
5. Fraction 4 is adjusted to 1 *M* NaCl by the addition of solid and loaded onto a hydroxlapatite column (*see* **Note 9**) at 3 mg protein/mL of packed column. The column is equilibrated with buffer A + 1 *M* NaCl beforehand. The column is washed with five column volumes of equilibration buffer, and eluted with a 10-column volume gradient of 0–400 m*M* $(NH_4)_2SO_4$ in buffer A + 1 *M* NaCl. Fractions are collected, and ParE is localized as in **step 2** above. ParE elutes at 200 m*M* $(NH_4)_2SO_4$. This fraction (fraction 5) is dialyzed against 100 vol of topo IV storage buffer overnight. ParE (**Fig. 1**) is stable for at least 2 yr at either −80 or −20°C in this buffer at protein concentrations higher than 2 mg/mL. However, for long-term storage, we recommend dividing the pool into small aliquots, freezing in liquid N_2, and storing at −80°C. If the material is going to be used to form isolated topo IV heterotetramer, then dialyze against buffer A + 150 m*M* NaCl, and use directly.

3.4. Purification of ParC

1. Fraction 2 is dialyzed against 100 vol of buffer A + 100 m*M* NaCl overnight. The conductivity of the dialyzate is determined and adjusted, if necessary, by the addition of buffer A to that equivalent to buffer A + 100 m*M* NaCl. ParC is insoluble at <50 m*M* NaCl, so it is important to track the conductivity.

2. Fraction 2 is applied to a BioRex 70 column that had been previously equilibrated with buffer A + 100 m*M* NaCl at 10 mg protein/mL of packed column. The column is washed with five column volumes of equilibration buffer and eluted with a 10-column volume linear gradient of 100–500 m*M* NaCl in buffer A. Fractions are collected, and ParC is localized as for ParE. Peak ParC fractions (eluting at 250 m*M* NaCl) are pooled (fraction 3).
3. Fraction 3 ParC is diluted with buffer A to give a conductivity equivalent to that of buffer A + 150 m*M* NaCl and loaded onto a heparin-agarose column that had been equilibrated previously with buffer A + 150 m*M* NaCl at 5 mg protein/mL of packed column. The column is washed with five column volumes of equilibration buffer and eluted with a 10-column volume linear gradient of 150–600 m*M* NaCl in buffer A. Fractions are collected and ParC localized as above. Peak ParC fractions (400 m*M* NaCl) are pooled to give fraction 4.
4. Fraction 4 is diluted with buffer A to give a conductivity equivalent to that of buffer A + 100 m*M* NaCl and applied to a phosphocellulose column that had been equilibrated previously with buffer A + 100 m*M* NaCl at 3 mg protein/mL of packed column. The column is washed with five column volumes of equilibration buffer and eluted with a 10-column volume gradient of 100–600 m*M* NaCl in buffer A. Fractions are collected, and ParC localized as above. Peak ParC fractions (**Fig. 1**) (350 m*M* NaCl) are pooled to give fraction 5. ParC is stored as for ParE.

3.5. Isolation of Reconstituted Topo IV Heterotetramer

1. ParC and ParE associate readily to form active topo IV. For most circumstances, mixing the appropriate amounts together in the assay reaction mixture is adequate. If isolated heterotetramer is required, the following procedure works well. For best results, it is recommended that ParC and ParE be at least 15 mg/mL in buffer A. In this example, ParC was 23 mg/mL and ParE 18 mg/mL.
2. ParC (120 µL) is combined with ParE (150 µL) and incubated on ice for 1 h. The mixture is then injected onto a 25-mL Pharmacia-LKB Superose 6 FPLC gel-filtration column equilibrated in buffer A + 100 m*M* NaCl. The column is developed with the same buffer at a flow rate of 0.2 mL/min. The first 10 mL of eluate are discarded, and fractions (0.2 mL) are then collected. The topo IV heterotetramer elutes before the excess ParE (**Fig. 2**). Peak fractions (one-half peak height) are pooled. The pooled material is dialyzed against storage buffer, and the enzyme frozen in liquid N_2 in small aliquots and stored at -80°C.

3.6. Assay for Topo IV Superhelical DNA Relaxation Activity

Reaction mixtures (20 µL) contain 50 m*M* Tris-HCl (pH 7.8 at 37°C), 6 m*M* $MgCl_2$, 10 m*M* DTT, 1 m*M* ATP, 20 m*M* KCl, 1 m*M* spermidine-HCl, 100 µg/mL nuclease-free bovine serum albumin, and superhelical plasmid DNA (0.4 µg). Incubation is at 30°C for 30 min. If assaying ParC or ParE individually, the reaction mixtures should contain 50 ng of, e.g., ParC, with ParE titrated between 1 and 20 ng. Complete DNA relaxation should occur by about 10 ng

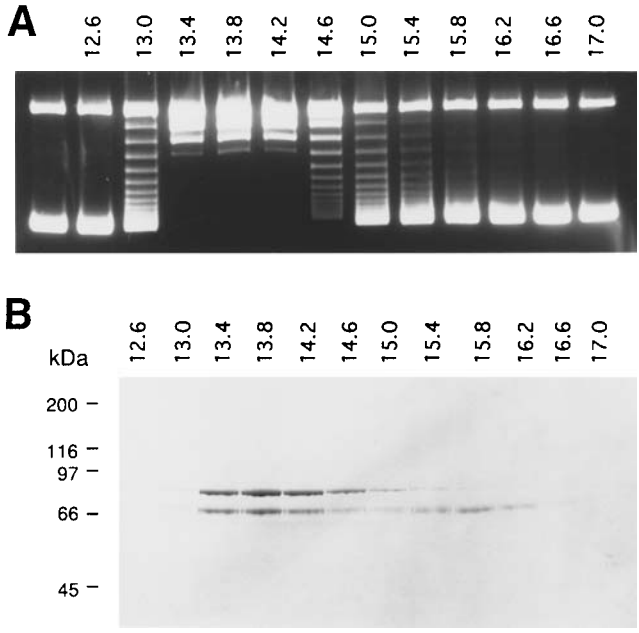

Fig. 2. Isolation of the topo IV heterotetramer by gel filtration. ParC (280 µg) and ParE (270 µg) were incubated in buffer A + 100 m*M* NaCl (300 µL) for 1 h at 4°C. This mixture was loaded onto a 25-mL Pharmacia-LKB Superose 6 FPLC column that was developed with the same buffer. Fractions (0.2 mL) were collected. **(A)** 0.33 µL of the indicated fractions was assayed for superhelical DNA relaxation activity. **(B)** SDS-PAGE analysis (10% gel) of the polypeptides present in the indicated fractions. Fractions are denoted by the eluate volume.

of the titrated subunit. Isolated, reconstituted topo IV heterotetramer is about two-fold more active, and complete relaxation occurs by about 10 ng of heterotetramer. The assay is analyzed by electrophoresis through an agarose gel (*see* **Note 10**), followed by visualization by ethidium bromide straining.

4. Notes

1. The 50% stock solution of Polymin P from suppliers like BDH (Poole, United Kingdom) is very acidic. In preparing the 10% stock, even though the Tris buffer is present, it is necessary to neutralize the solution by adding NaOH.
2. The Brij precipitates rapidly from solution. Thus, the working solution should be prepared immediately before use. Long-term storage is only effective at 37°C.
3. BSA should be nuclease-free. Any endonuclease present will nick the superhelical plasmid DNA. It is a good idea to test most commercial sources of "nuclease-free" DNA for nuclease by investigating whether they will nick the plasmid DNA under the conditions of the relaxation assay.

4. The assay works best with small (2–5 kb) plasmids. For best results, the DNA preparation should be >90% superhelical DNA.
5. Inducing at a higher OD is nonproductive. Overexpression generally decreases at ODs > 0.4.
6. pLysS cells tend to lyse spontaneously after thawing, making a mess. The best tack is to lyse the cells immediately after resuspension without freezing them.
7. This is the place to stop, if so desired.
8. Maximum flow rates are obtained when the ratio of column diameter to height is between 0.2 and 0.33.
9. Because of the colloidal nature of a suspension of hydroxylapatite, columns can have very slow flow rates. To alleviate this problem partially, use a mixture of hydroxylapatite (Biogel HTP) and cellulose powder (Whatman CF11) of 60:17 (w/w).
10. Either vertical or horizontal gels can be used. Resolution of the vertical gels is superior to that of the horizontal gels.

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Purification of the Bacteriophage T4 Type II DNA Topoisomerase

Kenneth N. Kreuzer and Sue H. Neece

1. Introduction

Bacteriophage T4 encodes a type II topoisomerase with properties more similar to those of the eukaryotic class of enzymes than to those of the bacterial DNA gyrase ***(1,2)***. Indeed, the discovery of the T4 topoisomerase provided the first example of an ATP-dependent relaxing enzyme ***(3,4)***, and an understanding of the properties of the T4 enzyme rapidly led to the discovery of similar enzymes from eukaryotic cells ***(5–7)***.

Over the past 15 years, phage T4 and its type II topoisomerase have provided an excellent model system for analyzing the mechanism of action of topoisomerase inhibitors ***(8–10)***. The T4 enzyme is sensitive to many of the same anticancer agents that inhibit the eukaryotic enzyme and is also moderately sensitive to the antibacterial quinolones ***(11,12)***. Thus, the mechanism of action of both groups of compounds can be analyzed using T4, and studies with T4 provided some of the strongest evidence that the anticancer and antibacterial agents inhibit topoisomerases by a common mechanism ***(12,13)***.

The T4 topoisomerase consists of two copies of each of three subunits, the products of phage genes *39*, *52*, and *60*. Because these three genes are expressed from early and middle-mode promoters, a modest overproduction of the enzyme during phage infection is achieved by blocking the transition to late gene expression. Thus, the enzyme is generally prepared from nonsuppressing cells that are infected with a phage containing amber mutations in each of two genes (*33* and *55*) required for late transcription. The standard purification procedure described in this chapter yields from 3–10 mg of highly purified topoisomerase/200 g (wet) of infected cell paste (*see* **Note 1**). The procedure can be scaled up

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or down for different amounts of starting cell paste, but we have not been successful trying to purify the enzyme from very small amounts of infected cells (e.g., 1–5 g paste).

2. Materials

1. Strains: *Escherichia coli* CR63 (*supD*) is used for growing the large T4 stock necessary as starting material, and *E. coli* B^E (nonsuppressing) is used for the infection in which topoisomerase is overproduced and subsequently purified. The T4 double-amber mutant phage *amN134 amBL292* (amber mutations in genes *33* and *55*) is used for the generation of wild-type T4 topoisomerase. Genetic crosses can be used to introduce topoisomerase mutations into the double-amber mutant background for the purification of mutant topoisomerases ***(14,15)***.
2. Growth medium: Phage stocks are grown using Luria broth (LB) (10 g bacto-tryptone, 5 g yeast extract, and 10 g NaCl/L). Medium for the fermenter is made by adding to the deionized water, prior to sterilization, the following components (all quantities are per L): 0.395 g KH_2PO_4, 1.62 g $K_2HPO_4 \cdot 3H_2O$, 10 g NaCl, 8.8 g nutrient broth, 5.5 g bacto-peptone, 10 g glucose, and 0.025 mL antifoam B (Sigma Chemical Co., St. Louis, MO). After the medium in the fermenter has been sterilized and cooled down to 37°C, presterilized solutions of $MgSO_4$ (1/100 vol of 10% [w/v]) and tryptophan (1/100 vol of 2 mg/mL) are added. During growth of the culture, 10-fold dilute antifoam B is added as needed, and the pH is maintained at 7.1–7.2 with NaOH.
3. Buffer A: 200 m*M* NaCl, 40 m*M* Tris-HCl, pH 8.1, 10 m*M* $MgCl_2$, 2 m*M* $CaCl_2$, 1 m*M* Na_3EDTA, 1 m*M* 2-mercaptoethanol, 1 m*M* phenylmethylsulfonyl fluoride (PMSF), and 10 m*M* benzamidine-HCl (the last two compounds are added immediately before use).
4. Buffer B: 100 m*M* NaCl, 20 m*M* Tris-HCl, pH 8.1, 5 m*M* Na_3EDTA, 1 m*M* 2-mercaptoethanol, and 1 m*M* PMSF (added immediately before use).
5. C Buffers: 20 m*M* Tris-HCl, pH 8.1, 1 m*M* Na_3EDTA, 1 m*M* 2-mercaptoethanol, and 10% (v/v) glycerol. Buffer C1 also contains 0.15*M* NaCl, C2 contains 0.25 *M* NaCl, C3 contains 0.6*M* NaCl, and C4 contains 2*M* NaCl.
6. D Buffers: Equimolar solutions of KH_2PO_4 and K_2HPO_4 (mixed together to give a pH of ~6.8), 10 m*M* 2-mercaptoethanol, and 10% (v/v) glycerol. The final potassium phosphate concentrations are: buffer D1, 0.1*M*; D2, 0.3*M*; D3, 0.7*M*.
7. E Buffers: 40 m*M* Tris-HCl, pH 7.8, 20 m*M* NaCl, 0.5 m*M* Na_3EDTA, 1 m*M* 2-mercaptoethanol, 10% (v/v) glycerol, and either 0% (buffer E1) or 25% (w/v; buffer E2) $(NH_4)_2SO_4$.
8. Buffer F: 30 m*M* potassium phosphate, pH 7.2, 10 m*M* 2-mercaptoethanol, 0.5 m*M* Na_3EDTA, and either 10 or 50% (v/v) glycerol, as specified.
9. Column matrices: The ssDNA cellulose (Sigma) is prepared in buffer C1, hydroxyapatite (HTP; Bio-Rad Laboratories, Hercules, CA) in buffer D1 (this matrix can be mixed with Whatman [Maidstone, England] CF-11 cellulose [20% w/w] to improve flow), and norleucine-Sepharose ***(16)*** in buffer E2. For

the standard purification described here (200 g [wet] of infected cell paste), use an ssDNA-cellulose column with a bed volume of approx 120 mL, a hydroxyapatite column with a volume of about 100 mL, and a norleucine-Sepharose column of about 2 mL. For all columns, the height of the bed should be about 10 times the diameter.

10. Relaxation assay buffer: 40 m*M* Tris-HCl, pH 7.8, 60 m*M* KCl, 10 m*M* $MgCl_2$, 0.5 m*M* dithiothreitol, 0.5 m*M* ATP, and 30 µg nuclease-free albumin/mL.
11. Relaxation assay stop solution: 5% (w/v) SDS, 20% (w/v) Ficoll-400, and 0.1% (w/v) each bromophenol blue and xylene cyanole.
12. TBE running buffer: 89 m*M* Tris base, 89 m*M* boric acid, and 2.5 m*M* Na_3EDTA (ethidium bromide should not be used during electrophoresis).

3. Methods

3.1. Growth of T4-Infected Cells

1. A large stock of the T4 double-amber mutant phage (approx 10^{15} PFU for a run in a 220-L fermenter) is prepared by multiple growth cycles in *E. coli* CR63 (*supD*). Procedures for growing and titering T4 phage are described in the recent monograph on the phage ***(17)***.
2. *E. coli* B^E (nonsuppressing) is grown at 37°C in a fermenter until the A_{560} reaches 0.75 (corresponds to a cell density of approx 6×10^8/mL). The T4 double-amber mutant phage (or derivative thereof) is then added at a multiplicity of 5–10 PFU/cell (*see* **Note 1**). This infection does not produce viable phage particles (owing to the absence of a suppressor in the bacterial host), preventing contamination of the fermenter facility (for future runs) with phage.
3. After 2.5 h, the cells are collected by centrifugation at 10°C. The cell paste is transferred into plastic bags on ice. The bags are then sealed, frozen, and stored at −75°C. A typical run in a 220-L fermenter (170 L of infected cells) yields about 500–600 g (wet) cell paste. The procedure described below uses 200 g cell paste but can be modified accordingly for smaller or larger amounts.

3.2. Preparation of Cleared Lysate

1. The frozen cell paste (200 g) is first broken into fragments with a wooden mallet, and the fragments are added to 600 mL buffer A on ice. The mixture is stirred at low speed, and pipeted up and down until the solution is homogenous and contains no ice crystals.
2. The mixture is then divided into two or three aliquots, and each aliquot is sonicated at maximum power, keeping the temperature at or below 10°C. Sonication is complete when the turbidity (A_{560}; measured with diluted samples) drops about sevenfold from the original suspension; complete sonication generally takes 5–10 min/aliquot.
3. The aliquots are combined, 16 mg of pancreatic DNase I (Worthington Biochemical Corp., Freehold, NJ) are added, and the mixture is incubated for 15–30 min at 15°C (which should greatly reduce the viscosity).

4. The lysate is then clarified by a 45-min centrifugation at 18,000*g* at r_{max}; 4°C in a Sorvall GSA rotor, and the supernatant is poured off the pellet.
5. The supernatant from the first spin is further clarified by a 2.5-h centrifugation at 186,000*g* at r_{max}; 4°C in a Beckman 45 Ti rotor, and the resulting supernatant is carefully removed from the loose pellet.
6. The second supernatant is then dialyzed in multiple small dialysis bags against 15 L of buffer B overnight at 4°C, followed by an additional 15 L for at least 4 h in the morning. After collecting the dialysate, prechilled ultrapure glycerol is added to a final concentration of 10% (v/v) (*see* **Note 2**).

3.3. Single-Stranded DNA Cellulose Chromatography

1. The dialyzed lysate is applied at a flow rate of no more than 100 mL/h to the ssDNA cellulose column.
2. The column is washed with buffer C1 until the eluate is free of protein, and the topoisomerase along with some contaminating proteins are then eluted with buffer C2 (*see* **Note 3**). The column can be regenerated (and other T4 proteins can also be recovered if desired) by subsequent washes with buffers C3 and C4 (*see* **Note 4**).
3. The fractions of the C2 wash that contain topoisomerase are most easily determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). These fractions are pooled and applied directly (without dialysis) to the next column (*see* **Note 2**).

3.4. Hydroxyapatite Chromatography

1. The ssDNA-cellulose pool is loaded onto the hydroxyapatite column at a flow rate of no more than 100 mL/h, and the column is washed with 200 mL of buffer D1.
2. The column is then developed with a 1-L linear gradient of 0.3–0.7*M* potassium phosphate (using buffers D2 and D3). Topoisomerase elutes at about 0.4*M* in the gradient (*see* **Note 3**).
3. Fractions containing the enzyme are identified by SDS-PAGE and pooled. If the profiles of any contaminating proteins trail into the topoisomerase peak, two pools ("clean" and "dirty") can be made and processed separately (note that the next column provides very little purification from contaminating proteins) (*see* **Notes 2, 4,** and **5**).

3.5. Norleucine-Sepharose Chromatography

1. The norleucine-Sepharose column is used to concentrate the protein into a small volume. Solid $(NH_4)_2SO_4$ is added to the hydroxyapatite pool to a final concentration of 25% (w/v).
2. The enzyme is then loaded onto the norleucine-Sepharose column at a flow rate of about two column volumes per hour.
3. After washing the column with several volumes of buffer E2, the protein is eluted by applying buffer E1 (*see* **Note 4**).
4. The topoisomerase-containing fractions are identified either by SDS-PAGE, A_{280} measurement, or Bradford protein assay ***(18)*** (*see* **Note 2**).

3.6. Final Dialysis and Measurement of Specific Activity

1. The norleucine-Sepharose pool is dialyzed twice against buffer F containing 10% glycerol (4 h each) and then once against buffer F containing 50% glycerol (6 h).
2. The enzyme is stored at –20°C, where it is stable for at least several years.
3. The concentration of topoisomerase in the final pool is measured using the Bradford ***(18)*** protein assay (reagents from Bio-Rad Laboratories) with bovine serum albumin as the standard. Albumin binds about 1.2-fold more dye than an equivalent amount of the T4 topoisomerase, so the measured topoisomerase concentration from the albumin curve should be multiplied by 0.83 ***(15)***.
4. The activity of the purified topoisomerase is verified by measuring the relaxation of 0.3 µg supercoiled pBR322 DNA in 20-µL reactions using the relaxation assay buffer. Fresh serial dilutions of the enzyme are prepared using ice-cold buffer F supplemented with 50% (v/v) glycerol and 50 µg bovine serum albumin/mL. After incubating for 30 min at 30°C, the reaction is terminated by adding 5 µL relaxation assay stop solution. The reaction products are then separated by electrophoresis through a 1% (w/v) agarose gel in TBE running buffer. One unit is defined as the amount of enzyme that catalyzes half relaxation of the substrate under these conditions. The final purified topoisomerase pool should have a specific activity of about 4×10^6 units/mg.

4. Notes

1. Singer and Gold ***(19)*** described a phage construct that overproduced the product of gene *52* owing to the insertion of an upstream T7 promoter (when T7 RNA polymerase was induced in the host cells prior to infection). After this chapter was submitted, we succeeded in overproducing all three subunits using a similar strategy, improving the yield of the topoisomerase purification.
2. Samples of the original lysate and all relevant pools should be saved for measurement of total protein and for a final comparative SDS-PAGE.
3. For each column, check the flowthrough and other fractions for topoisomerase in case the column did not work properly. SDS-PAGE analysis is sufficient, except for the ssDNA-cellulose flowthrough (which has too many proteins for easy visualization of the topoisomerase). A very significant loss of topoisomerase can occur at the ssDNA-cellulose step if the column does not have a high capacity for protein binding. In practice, if the total yield from salt elution of the ssDNA-cellulose column appears low, try to recover additional enzyme from the flowthrough by loading it onto another ssDNA-cellulose column. As with the original column, wash extensively with buffer C1. Then, if buffer C2 elutes a significant amount of additional topoisomerase, the two C2 buffer eluates can be combined for subsequent steps or can be purified separately.
4. All of the columns can be regenerated and stored at 4°C in the appropriate buffer containing 1 m*M* Na_3EDTA and 0.02% (w/v) sodium azide.
5. If any contaminating proteins remain after the final column, a gel-filtration column (Sephacryl S-300) can be used for additional purification ***(1)***. In this case,

the enzyme is reconcentrated after gel filtration with another norleucine-sepharose column

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Overexpression and Purification of DNA Topoisomerase I from Yeast

Mary-Ann Bjornsti and Jolanta Fertala

1. Introduction

The mechanism of action of DNA topoisomerase I in catalyzing the relaxation of supercoiled DNA and how this reaction is perturbed by the antitumor drug camptothecin have been the subject of intense investigation (reviewed in ***(1–3)***. Much of this effort has focused on structure/function studies of wild-type and mutant forms of the enzyme, derived from a variety of sources ***(1,4–20)***. The budding yeast *Saccharomyces cerevisiae* has proven particularly amenable to the genetic manipulations required to overexpress and purify wild-type and mutant forms of eukaryotic DNA topoisomerase I ***(3,4,12,13)***.

In yeast, the gene encoding DNA topoisomerase I (*TOP1*) is nonessential ***(21,22)***. Yeast strains deleted for *TOP1* (*top1*Δ) are viable because other gene products, such as DNA topoisomerase II, can compensate for the loss of DNA topoisomerase I ***(21,23)***. Thus, in yeast, unlike other eukaryotic systems, it is possible to purify a plasmid encoded DNA topoisomerase I to homogeneity free of any contaminating endogenous enzymes. In addition, the use of tight, strong, inducible promoters, such as the galactose inducible *GAL1-10* promoters ***(24)***, allows for the regulated overexpression of the enzyme at levels that might otherwise prove lethal or toxic to cells. This coupled with the availability of single copy and multicopy vectors and the ease with which yeast can be genetically manipulated, stably transformed with plasmids, and grown in liquid culture, provides distinct advantages over more complicated baculovirus and vaccinia virus expression systems.

One major drawback to the expression of DNA topoisomerase I in yeast is the susceptibility of the enzyme to proteolytic degradation. However, this can

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be largely circumvented with protease-deficient strains ***(25)***, and the inclusion of protease inhibitors during cell lysis and protein purification ***(12,13)***. Indeed the specific activity of intact enzyme prepared from yeast, on the order of 5×10^6 U/mg, compares favorably with enzyme purified from higher eukaryotic sources ***(12,13)***. Two protocols are presented below for purifying DNA topoisomerase I from yeast cells to homogeneity. Although this chapter focuses on the overexpression and purification of yeast DNA topoisomerase I, similar approaches may be used to purify any eukaryotic DNA topoisomerase I.

2. Materials

2.1. Yeast Media

1. YPD_A media: 10 g yeast extract, 20 g bacto-peptone, 0.7 g adenine in 900 mL dH_2O. Autoclave to sterilize, and then add 100 mL of 20% dextrose.
2. 20% Raffinose: 20 g raffinose/100 mL dH_2O, filter-sterilized through a 0.45-µm filter (*see* **Note 1**).
3. 20% Galactose: 20 g galactose/100 mL dH_2O, filter-sterilized through a 0.45-µm filter.
4. 20% Dextrose: 20 g dextrose (glucose)/100 mL dH_2O, filter-sterilized through a 0.45-µm filter.
5. Synthetic complete media lacking uracil (S.C. ura-media): 1.7 g yeast nitrogen base without amino acids and ammonium sulfate (Difco Laboratories, Detroit, MI), 5 g ammonium sulfate, 0.72 g ura-dropout mix, 1 mL 2*N* NaOH, 900 mL dH_2O. Autoclave or filter-sterilize (*see* **Note 2**), and then add 100 mL of the requisite sugar solution to give a final 2% (2 g/100 mL).
6. Ura-dropout mix ***(26)***: 0.5 g adenine sulfate, 2.0 g L-tryptophan, 2.0 g L-arginine, 2.0 g L-asparagine, 2.0 g L-aspartic acid, 2.0 g L-cysteine, 2.0 g L-glutamic acid, 2.0 g L-glycine, 2.0 g L-histidine, 2.0 g inositol, 2.0 g L-isoleucine, 10.0 g L-leucine, 2 g L-lysine, 2.0 g L-methionine, 0.2 g *para*-aminobenzoic acid, 2.0 g L-phenylalanine, 2.0 g L-proline, 2.0 g L-serine, 2.0 g L-threonine, 2.0 g L-tryptophan, 2.0 g L-tyrosine, 2.0 g L-valine.

2.2. Yeast Transformation

1. 10X LiOAc solution: 1*M* LiOAc.
2. 10X TE buffer: 100 m*M* Tris-HCl, pH 7.5, 10 m*M* EDTA.
3. 50% PEG solution: 50 g PEG 3350/100 mL dH_2O. Filter-sterilize through a 0.45-µm filter.
4. 1X TE- LiOAc: 10 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA, 0.1*M* LiOAc. Make fresh from 10X stocks just prior to use (*see* **Note 3**).
5. 1X TE- LiOAc-PEG: 10 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA, 0.1 *M* LiOAc, 40% PEG. Make fresh from stock solutions just prior to use (*see* **Note 3**).
6. Salmon sperm DNA: 10 mg/mL salmon sperm DNA, sonicated and boiled.

7. S.C. ura-plates: 1.7 g yeast nitrogen base without amino acids and ammonium sulfate (Difco), 5 g ammonium sulfate, 0.72 g ura-dropout mix, 1 ml 2*N* NaOH, 20 g agar, 900 mL dH_2O. Autoclave, cool to 55°C, and then add 100 mL 20% dextrose.

2.3. Cell Lysis

1. Acid-washed 425–600 µm diameter glass beads.
2. 5X TEEG–1*M* KCl buffer: 250 m*M* Tris-HCl, pH 7.4, 5 m*M* EDTA, 5 m*M* EGTA, 1*M* KCl, 10% (v/v) glycerol.
3. TEEG buffer: 50 m*M* Tris-HCl, pH 7.4, 1 m*M* EDTA, 1 m*M* EGTA, 10% (v/v) glycerol. KCl or ammonium sulfate is added to the final concentration indicated.
4. 3*M* KCl.
5. Ultrapure ammonium sulfate.
6. 100X protease inhibitor stock: 100 µg/mL chymostatin, 200 µg/mL aprotinin, 100 µg/mL phosphoramidon, 700 µg/mL E-64, 20 µg/mL benzamidine, 100 µg/mL pepstatin, 50 µg/mL leupeptin, 250 µg/mL antipain, 50 µg/mL bestatin.
7. 80 mg/mL Sodium fluoride.
8. 10 mg/mL Sodium bisulfite.
9. 20 mg/mL PMSF in 2-propanol.
10. Phosphate buffers: 0.05*M* phosphate, pH 7.5, and 0.6*M* phosphate, pH 7.5.

2.4. Column Chromatography

1. P-11 resin (Whatman, Clifton, NJ) activated as per manufacturer's instructions.
2. DEAE-cellulose.
3. Heparin agarose.
4. Hydroxylapatite.
5. Phenyl-Sepharose.
6. Gradient maker.
7. Fraction collector (Gilson, Middletown, WI).
8. Various-sized columns with bed supports, such as Econo-columns (Bio-Rad Laboratories, Hercules, CA), fitted with two-way stopcock valves.
9. Conductivity meter.

3. Methods

3.1. Yeast Transformation

In order to purify yeast DNA topoisomerase I, or any other eukayotic DNA topoisomerase I, it is first necessary to transform yeast cells lacking the *TOP1* gene (*top1*Δ strains) with the appropriate expression vector. The most common expression vectors, such as YEpGAL1-TOP1 ***(12,13)***, contain the *TOP1* coding region cloned under the galactose-inducible *pGAL1* promoter as well as a selectable marker, such as *URA3*. The plasmid is introduced into the appropri-

ate cells using a modified LiOAc procedure *(27)*. Subsequent selection on S.C.-ura plates ensures plasmid maintenance.

1. Grow protease-deficient, *top1*Δ yeast strains in 40 mL YPD_A media to an OD_{595} = 1.0 at 30°C (*see* **Note 4**).
2. Pellet cells by centrifugation at 4000*g* for 10 min, wash with 1/4 the original culture volume of freshly prepared 1X TE-LiOAc, and resuspend the cells in 600µL 1X TE-LiOAc. This will give a final 2×10^9 cells/mL.
3. Add 200 µL of the cell suspension to microcentrifuge tubes containing 150 µg salmon sperm DNA plus 200–500 ng expression vector DNA (*see* **Note 5**). Mix thoroughly.
4. Add 700 µL 1X TE-LiOAc-PEG, mix thoroughly with a pipet, and incubate at 30°C for 30 min with gentle shaking.
5. Heat-shock for 15 min at 42°C. Spin the cells at 15,000*g* for 30 s. Aspirate off all but 100 µL of the supernatant, resuspend the cells in the remaining 1X TE-LiOAc-PEG, and plate the cell suspension on S.C.-ura plates. Individual transformants should be visible as distinct colonies following 2–3 d of incubation at 30°C.

3.2. Induction of TOP1 Expression and Cell Lysis

1. For large-scale protein purification, several transformants are first grown in 100 mL S.C. ura-media containing dextrose at 30°C, with aeration. At an OD_{595} = 1.0–2.0, the culture is diluted 1:100 into 6 × 1.5 L of S.C. uar-media containing raffinose in 4-L flasks to alleviate glucose repression (*see* **Note 6**).
2. Once the cells have reached an OD_{595} = 2.0, each culture is induced with 150 mL 20% galactose for 6–8 h (*see* **Note 7**). The cells are then collected by centrifugation at 4000*g* for 10 min at 4°C, washed with 1/5 vol chilled dH_2O, and resuspended in a final 2 mL/g wet cells of TEEG + 0.3*M* KCl buffer. The cells are then flash frozen in a dry ice/ethanol slurry and stored at −80°C (*see* **Note 8**).
3. The cell suspension is thawed at 4°C and supplemented with a final 100 µg/mL PMSF, 800 µg/mL sodium bisulfite, 100 µg/mL sodium fluoride, and a 1:100 dilution of the 100X protease inhibitor stock solution (*see* **Note 9**). From this point forward, all steps should be carried out at 4°C, with prechilled tubes, centrifuges, and buffers.
4. The cell suspension is distributed in 15-mL aliquots into 50-mL Oak Ridge centrifuge tubes, mixed with 0.6 vol of acid-washed glass beads, and vortexed for 20 × 1 min intervals (*see* **Note 10**).
5. Clarified cell extracts are prepared by centrifugation at 15,000*g* for 30 min and pooled (*see* **Note 11**). The proteins in the supernatant are then subjected to successive ammonium sulfate fractionations. Solid ammonium sulfate is added to a final 35% saturation (19.4 g/100 mL), and dissolved by gently rocking the extracts at 4°C for 30 min. The precipitates are removed by centrifugation at 15,000*g* for 30 min, and the supernatant is then adjusted to 75% saturation with solid ammonium sulfate (25.4 g/100 mL) and gentle rocking at 4°C (*see* **Note 12**).

6. The precipitates are then collected by centrifugation at 15,000*g* for 30 min and resuspended in TEEG buffer supplemented with a 1:1000 dilution of the 100X protease inhibitor stock solution. As needed, additional buffer is then added to adjust the conductivity of the sample to match that of TEEG + 0.2*M* KCl.

3.3. Top1 Protein Purification

1. The proteins are then fractionated over a 50-mL phosphocellulose column (P-11) equilibrated with TEEG + 0.2*M* KCl buffer. To prepare the column, 80 mL of resin are suspended in 400 mL 5X TEEG–1*M* KCl buffer. A volume of slurry sufficient to give 50-mL packed column volume is poured into an 80-mL column and washed with 3–5 column volumes of TEEG + 0.2*M* KCl buffer. Once the protein sample has been applied to the column, wash with 3–5 column volumes of TEEG + 0.2*M* KCl.
2. The proteins are then eluted with a 500-mL linear gradient of 0.2–0.8*M* KCl in TEEG buffer and collected in 6-mL fractions. Fractions containing DNA topoisomerase I (as determined by plasmid relaxation assays and/or Western blot analysis) are pooled, dialyzed against TEEG + 0.05*M* KCl, and applied to a DEAE-cellulose column (10 mL) equilibrated with the same buffer.
3. The column is washed with 10 mL TEEG + 0.05*M* KCl. The flowthrough and wash fractions, which contains DNA topoisomerase I, are applied directly to a 10-mL heparin-agarose column equilibrated with TEEG + 0.2*M* KCl buffer (*see* **Note 13**). DNA topoisomerase I is eluted with a 100-mL linear gradient of 0.2–0.8*M* KCl in TEEG buffer.
4. The 2-mL fractions containing DNA topoisomerase I are pooled, dialyzed against 0.05*M* phosphate buffer, and fractionated over a 10 mL hydroxylapatite column equilibrated with the same buffer. The column is developed with a 100-mL linear 0.05–0.6*M* phosphate gradient. The 2-mL fractions containing DNA topoisomerase I are adjusted to a final 50% glycerol, aliquoted, and stored at −80°C.

3.4. Alternative Purification Protocol

To avoid the complications sometimes attendant with protein purification via hydroxylapatite chromatography, the chromatographic steps involving DEAE-cellulose, heparin-agarose, and hydroxylapatite can be replaced with a single phenyl-sepharose chromatographic step.

1. Fractions containing DNA topoisomerase I eluted from the phosphocellulose column are pooled, adjusted to a final 0.9*M* ammonium sulfate (*see* **Note 14**), and applied to a 50-mL phenyl-sepharose column equilibrated with TEEG + 0.9*M* ammonium sulfate (*see* **Note 15**).
2. DNA topoisomerase I is eluted with a 500-mL gradient of 0.9–0*M* ammonium sulfate in TEEG buffer in 6-mL fractions. DNA topoisomerase I is then concentrated by adjusting the conductivity of the relevant fractions to match that of TEEG + 0.2*M* KCl and applying the proteins to a 1-mL phosphocellulose column

equilibrated with TEEG + 0.2*M* KCl. The protein is eluted with 2 mL of TEEG + 0.6*M* KCl, and individual fractions are adjusted to 50% glycerol, aliquoted, and stored at −80°C.

4. Notes

1. All sugar solutions are filter-sterilized into sterile bottles to avoid carmelization during autoclaving.
2. Several amino acids are unstable with prolonged autoclaving. Filter-sterilization will avoid this problem. Alternatively, the indicated final concentration of sterile solutions of the following components can be added to media lacking the dropout mix following autoclaving ***(26)***: 20 mg/L adenine, 20 mg/L tryptophan, 20 mg/L histidine, 20 mg/L arginine, 20 mg/L methionine, 30 mg/L tyrosine, 100 mg/L leucine, 30 mg/L isoleucine, 30 mg/L lysine, 50 mg/L phenylalanine, 100 mg/L glutamic acid, 100 mg/L aspartic acid, 150 mg/L valine, 200 mg/L threonine, 400 mg/L serine.
3. The use of older solutions typically decreases transformation efficiency.
4. The protease-deficient yeast strain, JEL1-Δ*top1*, also expresses a chromosomal copy of the *GAL4* gene from the promoter *pGAL10* ***(12,25)***. The use of this strain increases the yield of intact Top1 protein.
5. The inclusion of single-stranded salmon sperm DNA increases transformation efficiency.
6. Transformants should not be inoculated directly into S.C.-ura, raffinose media. Since raffinose is not a terrific carbon source, pregrowth in dextrose-containing media will ensure more rapid cell growth prior to galactose induction.
7. Though longer induction times result in the production of more Top1 protein, there is a significant decrease in protein stability after 8 h. The time of induction should be empirically determined for each strain and *TOP1* expression vector used.
8. The inclusion of a freeze–thaw cycle improves cell lysis. Moreover, the frozen cells may be stored for 1–2 wk without any loss in Top1 protein integrity or specific activity.
9. PMSF is very unstable at 4°C, so it should be added just prior to cell lysis. In all subsequent steps, the 100X protease inhibitor stock is added to the buffers at a final 1:1000 dilution.
10. As an alternative to vortexing by hand, the cells may also be lysed in a bead beater by Biospec. In this case, the glass bead–cell suspension is mixed for 3 × 1 min with 30-s intervals.
11. At this point, the concentration of total protein in the extracts should be 4 mg/mL or higher. If not, additional TEEG + 0.3*M* KCl buffer can be added to the cell/glass bead pellets, which are then vortexed for additional 10 × 1 min intervals.
12. It may take several hours or overnight for the ammonium sulfate to dissolve. DNA topoisomerase I is extremely stable in ammonium sulfate, so prolonged incubation at 4°C is not a problem.

13. Although DNA topoisomerase I does not bind DEAE-cellulose, a number of major contaminants do. Thus, this chromatographic step effects a major purification of the enzyme.
14. Care must be taken to add solid ammonium sulfate slowly, since the proteins will precipitate at concentrations exceeding 0.9*M*. This is particularly true of human DNA topoisomerase I expressed in yeast.
15. In our hands, DNA topoisomerase I binds with different affinities to phenyl-sepharose and phenyl-agarose. The latter resin is not recommended.

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Overexpression and Purification of *Saccharomyces cerevisiae* DNA Topoisomerase II from Yeast

Janet E. Lindsley

1. Introduction

Mechanistic and structural studies require large quantities of highly purified enzyme. Unfortunately, traditional expression strategies using *Escherichia coli* are often not successful for eukaryotic proteins, especially large ones. Despite numerous attempts, overexpression and purification of *Saccharomyces cerevisiae* DNA topoisomerase II from *E. coli* proved unsuccessful (Worland and Wang, personal communication). This failure may be owing to the common occurrence of the rare *E. coli* codons CTA (leucine) and AGG (arginine) in the yeast gene, particularly in the carboxy-terminal half. In response, Worland and Wang developed an expression and purification system for topoisomerase II in yeast ***(1)***. This procedure and its variations have been immensely useful; 5 mg of wild-type or mutant topoisomerase II can generally be purified from 1 L of cells grown in selective media. This chapter describes methods for growing and inducing the yeast cells, and purifying the highly expressed type II DNA topoisomerase. Researchers who are unfamiliar with yeast may find several chapters in ref. ***(2)*** useful.

2. Materials

2.1. Overexpression Plasmid

All of the overexpression plasmids that we have used are based on the plasmid YEpTOP2PGAL1 described by Giaever et al. ***(3)***. This plasmid is a shuttle vector that includes required sequences for replication and selection in both

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E. coli and yeast. The ampicillin resistance gene and ColE1 origin were taken from the *E. coli* plasmid pBR322. The autonomously replicating sequence from the endogenous 2-μ*M* plasmid and the auxotrophic URA3 gene were taken from YEp24. The promoter for topoisomerase II has been replaced by the galactokinase (GAL1/10) promoter. This promoter is induced in the presence of galactose and repressed in the presence of glucose (dextrose) ***(4)*** (*see* **Note 1**). Use of an inducible promoter was found to be essential because high levels of continuous topoisomerase II overexpression are lethal to yeast. We have altered YEpTOP2PGAL1 to express many different mutant or tagged topoisomerases ***(5–7)***. Interestingly, as long as the sequences immediately surrounding the initiating ATG are left unchanged, we obtain very high levels of protein expression.

2.2. Yeast Strain

An appropriate strain must be *ura3*$^{-}$ for use with this plasmid, and it should have mutations in proteinase A *(pep4*$^{-}$*)* and proteinase B *(prb1*$^{-}$*)* to help prevent proteolysis. Additionally, strains that have a second integrated copy of the GAL4 gene fused to the GAL1/10 promoter can give higher levels of topoisomerase expression owing to additional GAL4 protein ***(8)***. We generally use either BCY123 (*a pep4::HIS3 prb1::LEU2 bar1::HISG lys2::GAL1/10-GAL4 can1 ade2 trp1 ura3 his3 leu2-3*, *112 cir GAL*$^{+}$*RAF*$^{+}$*SUC*$^{+}$*)* or JEL1 (α leu2 trp1 ura3-52 prb1-1122 pep4-3 his3::GAL1/10-GAL4*)* strains for expression. Yeast strains can be stored indefinitely in 15% glycerol (v/v) at −70°C.

2.3. Nonselective Media for Growth of Untransformed Yeast

Typically, untransformed yeast cells can be grown and stored for several months on nonselective media plates. YPD is a complex media for routine growth. To make 500 mL of YPD (sufficient for ~20 standard plates), mix:

5 g Yeast extract;
10 g bacto-peptone;
10 g dextrose (D–(+)–glucose);
10 g bacto-agar; and
distilled water to 500 mL.

Autoclave for 15 min at 121°C and 15 lb/sq. in. of pressure. The agar is omitted for liquid media.

2.4. Media for Selection and Growth of Transformed Yeast

Synthetic complete (SC) media is made with the appropriate "dropout" powder to provide selection for a desired plasmid. For example, after the

topoisomerase II expression vector YEpTOP2PGAL1 has been transformed into yeast, cells that contain the plasmid are selected by growth on media lacking uracil.

2.4.1. Ura⁻ Dropout Powder

In a blender, mix together all of the following:

Adenine	1.0 g
Tryptophan	1.0 g
Histidine	1.0 g
Arginine	1.0 g
Methionine	1.0 g
Phenylalanine	2.5 g
Tyrosine	3.0 g
Lysine	3.0 g
Leucine	4.0 g
Isoleucine	4.0 g
Glutamic acid	5.0 g
Aspartic acid	5.0 g
Valine	7.5 g
Threonine	10 g
Serine	20 g
Total	69 g

2.4.2. Ura⁻ SC Plates

1. Autoclave together the following ingredients for 15 min:
   0.85 g Yeast nitrogen base w/o amino acids and ammonium sulfate (Difco).
   2.5 g Ammonium sulfate.
   0.7 g Ura⁻ dropout powder.
   10 g bacto-agar.
   Distilled water to 450 mL.
2. Add 50 mL of sterile 20% dextrose and pour ~20 plates.

2.4.3. Ura⁻ SC Liquid Media

Mix the following together with distilled water to a final volume of 900 mL and autoclave:

1.7 g Yeast nitrogen base w/o amino acids and ammonium sulfate.
5.0 g Ammonium sulfate.
1.4 g Ura⁻ dropout powder.
10.0 g Succinic acid.
6.0 g NaOH.

Check that the final pH is 5.5–6.0.

2.4.4. 10X Carbon Sources to Mix with Ura⁻ SC Media

1. 20% Dextrose, filter-sterilized.
2. 30% Glycerol/20% lactic acid, pH 6.0, filter-sterilized.
3. 20% Galactose, filter-sterilized.

2.5. Buffers for Cell Storage and Protein Purification

1. Buffer I: 50 m*M* Tris-HCl, pH 7.7 (at 25°C), 1 m*M* EDTA, 1 m*M* EGTA, 10% glycerol (v/v), 1 m*M* phenylmethylsulfonyl fluoride (PMSF), and 5 m*M* β-mercaptoethanol. The last two ingredients should be added immediately prior to use (*see* **Note 2**).
2. Wash buffer: buffer I plus 25 m*M* sodium fluoride and 1 m*M* sodium bisulfite.
3. Protease inhibitor stocks:
   100 m*M* PMSF (100X) in 100% ethanol. Make fresh daily.
   100 m*M* benzamide (100X) in 100% ethanol. Store frozen in small aliquots.
   0.7 mg/mL pepstatin and 0.5 mg/mL leupeptin (1000X) in DMSO. Store frozen in small aliquots.

2.6. Cell Lysis and Materials for Both Purification Methods

1. Glass beads: 425–600 μm diameter, washed with nitric acid and enough water to return the pH to neutrality, and baked in a drying oven.
2. Protein concentration determining assay: Colorimetric assays, particularly those purchased as stock solutions, are easiest.
3. Phosphocellulose (Whatman, Hillsboro, OR): Prepare and store as directed by the manufacturer. Equilibrate the phosphocellulose to buffer I + 150 m*M* KCl prior to using.

2.7. The Worland Method of Purification

1. Polyethyleneimine (also called polymin P): Make a 10% (v/v) stock solution, adjusted to pH 7.0–8.0 by adding HCl dropwise, fresh the day of the purification. Generally 1–2 mL of the 10% stock solution are sufficient.
2. Diatomaceous earth (Celite 545, Fluka, Ronkonkoma, NY): Prepare by rinsing with water, removing any fines, and baking to dryness.
3. Ammonium sulfate: Use only ultrapure, enzyme, or molecular biology grade ammonium sulfate. Make a solution of buffer I that is 100% saturated with ammonium sulfate (add ammonium sulfate and stir until no more goes into solution); store cold.

2.8. The Berger Method of Purification

For each 5 mg of protein in Fraction II_B, 1 mL of packed Q-Sepharose fast-flow resin (Sigma, St. Louis, MO) is required. Starting with 20 g of cell pellets, one generally has ~30 mg of Fraction II_B and a 6-mL (0.78 $cm^2 \times 8$ cm) column works well.

2.9. Further Purification

Either a high-trap heparin column (1 mL, Pharmacia, Piscataway, NJ) or a Poros HE1 column (1.6 mL, Perseptive Biosystems, Framingham, MA) equilibrated with buffer I + 150 m*M* KCl can be used.

3. Methods

3.1. Yeast Transformation

Transform yeast with the topoisomerase II expression vector (*see* **Subheadings 2.1.** and **2.2.**) by either electroporation ***(9)*** or LiAc ***(10)***. Select for transformants by plating on Ura$^-$ SC plates (*see* **Subheading 2.4.2.**) and incubating at 30°C for 2–4 d.

3.2. Growth and Induction of Transformed Yeast

1. Add several transformed colonies to 10 mL of Ura$^-$ SC media (*see* **Subheadings 2.4.3.** and **2.4.4.**) supplemented with 2% (w/v) dextrose, and grow them at 30°C on a shaker platform or roller.
2. When these cultures reach late log phase (usually after 24 h), dilute them 100-fold into 1 L of Ura$^-$ SC media supplemented with 3% (v/v) glycerol and 2% (v/v) lactic acid. These cells should be shaken as fast as possible (>2000 rpm) in either 4-L flasks, 2.8-L Fernbach flasks, or 2-L baffled flasks to obtain maximum aeration. There is generally a lag of 8–10 h before the cells resume growing after dilution.
3. When the culture reaches an optical density (at 600 nm) of 0.8–1.2, add 100 mL of 20% galactose to induce topoisomerase II production.
4. Six to 8 h after induction, harvest the cells by centrifugation at 14,000*g*. Resuspend the cells from each liter of culture in 100 mL of chilled wash buffer (*see* **Subheading 2.5., step 2**), and repellet them in a preweighed bottle.
5. Weigh the cells and resuspend them in an equal volume of chilled wash buffer.
6. Instantly freeze the suspension as small pellets by dripping directly into liquid nitrogen (*see* **Note 3**). These pellets are stored for no longer than 6 mo at −70°C.

3.4 Purification

There are two basic methods that we routinely use to purify topoisomerase II from yeast cells. One was developed by Worland and Wang ***(1)*** and the other by Berger and Wang (unpublished); henceforth, they will be referred to as either the Worland or the Berger methods, respectively. Subscripts "W" and "B" are used to distinguish fractions from each preparation procedure. The same Fraction I, clarified lysed cell supernatant, is used for both preparations. The descriptions below are slight variations of the original procedures. Additionally, some general tips on protein purification are provided for the novice (*see* **Notes 4–8**).

3.4.1. Cracking the Cells

1. Thaw 20 g of cell pellets dispensed into two 40-mL centrifuge tubes rapidly in a warm water bath with agitation just until all of the pellets have thawed; transfer the tubes immediately to an NaCl-H_2O/ice bath (~–5°C).
2. Add protease inhibitors to the thawed cells (*see* **Subheading 2.5., step 3**) and 10 m*M* fresh β-mercaptoethanol.
3. Add an equal volume of glass beads and agitate the tubes vigorously on a vortex mixer for 20 pulses of 20 s each. Between the pulses, return the tubes to the NaCl-H_2O/ice bath to chill for 40 s. The efficiency of cracking can be checked by visualizing cells under a light microscope; cracked cells appear as empty "ghosts," whereas whole cells appear as bright spheres. One can expect 50–80% lysis by this method (*see* **Note 9**).
4. Decant the lysed cells into a clean set of chilled centrifuge tubes. Wash the glass beads with several changes of buffer I + 150 m*M* KCl. Combine the washes with the lysed cells, and centrifuge at 40,000*g* for 30 min to remove the cell debris.
5. Determine the total protein concentration of the supernatant (Fraction I), and if necessary, dilute to 2.5 mg/mL using buffer I +150 m*M* KCl. A small aliquot of Fraction I, and all subsequent fractions, is frozen for future analysis.

3.4.2. Worland Method (1)

This method uses polyethyleneimine (also known as polymin P) to precipitate nucleic acids along with any bound proteins. The precipitated solution is mixed with a nonspecific support (Celite) and poured into a column. This column format allows unbound and weakly bound proteins to be washed off in low salt. A high-salt wash elutes a fraction containing highly enriched topoisomerase II. This fraction is further purified over a phosphocellulose column. The final fraction is 90–95% pure and essentially free of any type I topoisomerase or contaminating ATPases.

1. While Fraction I is stirring on ice, add 10% polyethyleneimine (*see* **Subheading 2.7., step 1**) dropwise to a final concentration of 0.2%. Continue stirring for an additional 30 min.
2. Meanwhile, suspend 3 g of Celite (*see* **Subheading 2.7., step 2**) in 20 mL of buffer I + 150 m*M* KCl. Pour this suspension into a 5-cm diameter column, and allow it to settle slowly by gravity at a flow rate of ~1 mL/min.
3. Mix dry Celite with the precipitated Fraction I (8 g/100 mL), and gently pour this mixture onto the settled Celite plug. While this mixture is settling, allow the column to drip only very slowly (~1 mL/min); this slow initial settling appears to prevent the column from becoming blocked during the washing phase.
4. Once the column has fully settled and the original liquid has drained through, wash it at 2–4 mL/min with 1 vol of buffer I + 150 m*M* KCl, and then with buffer I + 400 mM KCl until the eluant has no detectable protein (~3–4 column volumes).

5. Elute the topoisomerase II in one column volume of buffer I + 1 *M* KCl, and combine only the peak fractions.
6. Precipitate these peak fractions by adding ammonium sulfate to 65% saturation. Add an equal volume of buffer I 100% saturated with ammonium sulfate (*see* **Subheading 2.7., step 3**). Then for each 100 mL of this 50% saturated solution, add 9.9 g of solid ammonium sulfate slowly while the solution is stirring on ice. Once all of the solid has dissolved, the solution is stirred an additional 30 min.
7. Collect the precipitate by centrifugation for 40 min at 25,000g in a 4°C centrifuge and rotor.
8. Dissolve the ammonium sulfate pellet in sufficient buffer I to make the conductivity equal to that of buffer I + 250 m*M* KCl. This is Fraction II_W.
9. Load Fraction II_W onto a phosphocellulose column pre-equilibrated with buffer I + 250 m*M* KCl (*see* **Subheading 2.6., step 3**). The optimal size of the column will vary with the amount of protein in Fraction II_W; for each 3 mg of protein, use 1 mL of packed phosphocellulose (*see* **Note 10**).
10. Wash the column with buffer I + 250 m*M* KCl until the eluant has no detectable protein.
11. Elute the bound proteins with a linear gradient 10 times the column volume from buffer I + 250 m*M* KCl to buffer I + 1 *M* KCl. The main peak should be at ~0.5 *M* KCl and contain the topoisomerase II.
12. Check these peak fractions by SDS-PAGE prior to combining them. These combined fractions are Fraction III_W. Fraction III_W is 90–95% pure full-length topoisomerase II; many of the contaminating proteins are proteolytic fragments since they are reactive with topoisomerase II polyclonal antibodies on immunoblots ***(1)***. Further purification and concentration steps are discussed in **Subheadings 3.4.4.** and **3.4.5.**

3.4.3. The Berger Method of Purification

The Berger method uses phosphocellulose as a first step and Q-sepharose fast flow (Sigma) as a second step. This method is effective because few other proteins will bind to both a cation- and an anion-exchange column at the same pH.

1. Adjust fraction I (*see* **Subheading 3.4.1.**, **step 5**) to a conductivity equal to that of buffer I + 150 m*M* KCl and a total protein concentration ≤2.5 mg/mL
2. Add 10 mL of settled phosphocellulose (*see* **Subheading 2.6., step 3**) per 100 mg of protein. Stir this slurry gently on ice for 45 min.
3. Pour the slurry into a 5-cm diameter column, and allow it to pack at a flow rate of 2 mL/min.
4. Wash the column with 1 vol of buffer I + 150 m*M* KCl, followed by buffer I + 300 m*M* until the eluant has no detectable protein (3–5 column volumes).
5. Elute tightly bound proteins (including topoisomerase II) with two column volumes of buffer I + 1 *M* KCl.
6. Combine the peak fractions and dilute them with buffer I until the conductivity equals that of buffer I + 150 m*M* KCl (Fraction II_B).

7. Load Fraction II_B onto a Q-Sepharose fast-flow column pre-equilibrated with buffer I + 150 m*M* KCl (*see* **Subheading 2.8.**) at 1 mL/min.
8. Increase the flow rate to 2 mL/min, and wash the column with 2 vol of buffer I + 150 m*M* KCl.
9. Run a 10-column volume linear gradient from buffer I + 150 m*M* KCl to buffer I + 1 *M* KCl. Topoisomerase II will elute in the main peak during the gradient at ~400 m*M* KCl. Careful running of the gradient is essential for separating topoisomerase I from II. Again the topoisomerase II is 90–95% pure at this step (Fraction III_B).

3.4.4. Further Purification of Topoisomerase II

The topoisomerase II purified by either of the above techniques is contaminated with casein kinase II ***(11)***. One method for removing the casein kinase II uses a glycerol gradient as described by Cardenas et al. ***(11)***. We have instead used the high affinity of casein kinase II for heparin to separate the proteins ***(12)***.

1. Dialyze or dilute up to 2 mg of Fraction III topoisomerase II (from either the Worland or Berger procedures), so that the conductivity equals that of buffer I + 150 m*M* KCl.
2. Load the protein on either a high-trap heparin column or a Poros HE1 column (*see* **Subheading 2.9.**) equilibrated with buffer I + 150 m*M* KCl at a flow rate of 1 mL/min.
3. Wash with 10-column volumes of buffer I + 150 m*M* KCl at 2 mL/min.
4. Run a linear gradient from 150 m*M* to 1 *M* KCl in buffer I. The topoisomerase II elutes at ~400 m*M* KCl and casein kinase elutes just after at ~500 m*M* KCl; to ensure kinase-free topoisomerase II, combine only the fractions from the first half of the topoisomerase peak.

3.4.5. Concentration of Topoisomerase II

Many standard protein concentration techniques have been unsuccessful when applied to topoisomerase II because the protein has a tendency to stick irreversibly to many surfaces, including most membranes. We have lost >50% of the protein using Centricon spin concentrators (Amicon, Beverly, MA) and collodion bag vacuum concentrators (Satorius, Gottingen, Germany). Berger et al. used a Schleicher and Schuell (Keene, NH) vacuum dialysis concentrator to reach 12 mg/mL of topoisomerase II ***(13)***. A threefold concentration can be achieved by dialyzing the Fraction III topoisomerase II in 10% glycerol vs a buffer containing 50% glycerol; dialysis only takes a few hours if 50,000 M_r cutoff tubing is used. Another simple method uses a very small phosphocellulose column (1 mL/5 mg of protein) equilibrated with buffer I + 200 m*M* KCl.

1. Dilute Fraction III topoisomerase II so that the conductivity equals that of buffer I + 200 m*M* KCl.
2. Load the protein onto the miniphosphocellulose column at a flow rate of 1 mL/min.
3. Bump the topoisomerase II off in a very small volume by running buffer I + 1 *M* KCl over the column; combine only the drops containing high concentrations of protein. This method typically yields topoisomerase II at concentrations of 3–5 mg/mL.

3.4.6. Determination of Topoisomerase II Concentration

Using the method of Lohman et al. ***(14)*** and the knowledge that a monomer of *S. cerevisiae* topoisomerase II has 15 tryptophans and 60 tyrosines, an extinction coefficient at 280 nm of 162,150 M^{-1}/cm has been determined. This means that a 1 mg/mL solution of topoisomerase II has an absorbance at 280 nm of 1.0. Use of a colorimetric reagent (Bio-Rad) to determine the concentration by comparison to a bovine serum albumin standard curve gives values within 10% of those determined by absorbance at 280 nm.

3.4.7. Storage of the Purified Protein

Like many proteins, topoisomerase II is most stable when stored concentrated. To maintain full activity for several months, we only store topoisomerase II at ≥1 mg/mL. However, at high concentrations, *S. cerevisiae* topoisomerase II will precipitate at low ionic strength; it should always be stored in buffer with ≥150 m*M* KCl or NaCl. If the protein is in 10% glycerol, it is divided into small, single-use aliquots, frozen in liquid nitrogen, and stored at −70°C. Since topoisomerase II looses activity on repeated freezing–thawing, any unused protein from a thawed aliquot is discarded. Topoisomerase II that has been dialyzed into 50% glycerol can stored at −20°C and is treated like a restriction enzyme.

4. Notes

1. The GAL1/10 promoter includes four binding sites for the transcriptional activator GAL4. A second protein, GAL80, binds to GAL4. In the absence of galactose, GAL80 masks the GAL4 activation domain. When the yeast is induced with galactose, the GAL4/GAL80 complex undergoes a conformational change that reveals the transcriptional activation domain; if no glucose is present, transcription increases >1000-fold over uninduced levels ***(15)***.
2. Generally, buffer I plus a given concentration of KCl is used. Therefore, we mix a 10X stock of the Tris, EDTA, and EGTA with 100% glycerol and 2 *M* KCl to generate the final buffer.
3. Storing the cells as frozen pellets allows one to easily check expression levels and purify the topoisomerase II from any desired quantity of cells.

4. During a protein preparation, keep everything at 0–4°C largely because contaminating proteases are less active at lower temperatures. Protein fractions left even for a short period of time at higher temperatures can result in increased proteolysis. Keep everything on ice. Run columns in a cold room or cold box. Prechill any centrifuge bottles, flasks, or cylinders into which you will pour your protein.
5. Work quickly, especially at the start of the preparation. Even in the presence of protease inhibitors and at 0–4°C, there are still active proteases until they are separated away from your protein. For the topoisomerase II preparation, the first column should be completed within 4 h of cracking the yeast cells to avoid excessive proteolysis.
6. In general, proteins stick more to glass than to plastic; avoid use of glass especially for later, purer fractions. When the protein is expected to be dilute, collect and store it in silanized microfuge tubes. Silanized tubes can be prepared by filling and emptying the tubes with Sigmacote (Sigma), rinsing them with ethanol, and allowing them to dry. The Sigmacote can be used repeatedly to silanize many tubes.
7. Do not vigorously stir, vortex, or shake proteins; bubbles denature protein.
8. Save all fractions of the preparataion at 0–4°C until you are certain where your desired protein is.
9. Other laboratories have successfully used the Bead Beater (Biospec, Bartlesville, OK) with glass beads to lyse the yeast cells rapidly and efficiently. The Bead Beater provides better agitation than a vortex mixer, but also produces considerably more heat. We have found it difficult to cool the Bead Beater consistently to prevent large-scale proteolysis during cell cracking and, therefore, prefer to lyse the cells as described above. Other methods for lysing yeast are described by Jazwinski ***(16)***.
10. In a preparation starting with 20 g of cell pellets, one would expect ~30 mg of protein in Fraction II_W. In this case, a 10-mL, 1.8 $cm^2 \times 6$ cm column will be sufficient.

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Purification of DNA Topoisomerase II from *Drosophila melanogaster*

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1. Introduction

To characterize properly protein function and enzymatic activity, it is highly desirable to perform experiments with purified protein preparations. This is especially true in the case of topoisomerase II, because many of the enzymatic assays critical to the topoisomerase field (such as DNA cleavage) require enzyme levels in excess of the DNA substrate ***(1–5)***. As a result of the high topoisomerase II concentrations routinely used in these assays, even minor contamination by topoisomerase I or other enzymes that affect DNA structure (such as nucleases) may pose significant technical problems or may lead to erroneous conclusions.

In this era of enzyme overexpression, purification of topoisomerase II from native sources has become less common. However, in spite of the potential for high enzyme yields, such problems as gene rearrangements and protein truncation have been reported for systems that overexpressed topoisomerase II or expressed the enzyme in non-native systems ***(6–8)***. Furthermore, since alternative patterns of post-translational modification have been reported for topoisomerase II in different species ***(9)***, it is possible that cross-species expression may not yield native modification patterns. Finally, even when yeast topoisomerase II is overexpressed in yeast, it appears to be under-phosphorylated ***(3,5,10)***. Consequently, isolation of topoisomerase II from native species may be necessary to define accurately many of the properties of the enzyme as it exists in vivo.

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1.1. Overview of Purification

Topoisomerase II was first purified from HeLa cells in 1981 ***(11)***. The first "large-scale" purification scheme was developed two years later by Shelton et al. ***(12)***, who used *Drosophila melanogaster* as the source material. Although a number of purification protocols for topoisomerase II from native sources have been reported since the *Drosophila* procedure was published (reviewed in **ref.** ***13***), none has proven to be more reproducible or to produce consistently enzyme of higher quality. Not only is the *Drosophila* enzyme free of topoisomerase I, but it is also devoid of the protein kinase activity that contaminates some other topoisomerase II preparations ***(14)***.

Purification of *Drosophila* topoisomerase II has been achieved from both embryos and Kc embryonic tissue-culture cells. The protocol described below is based on the original procedure of Shelton et al. ***(12)*** and routinely produces 2–3 mg of topoisomerase II from 500 g of starting material. The resulting enzyme is >95% homogeneous and has a high specific activity. Although the conditions described below have been optimized for the purification of *Drosophila* topoisomerase II, many aspects of the purification scheme have been successfully applied to the isolation of the enzyme from other species ranging from yeast to mammals ***(15,16)***.

A general flowchart of the purification scheme is depicted in **Fig. 1**. Overall, this procedure purifies topoisomerase II >1000-fold with a final yield of 5–10% (*see* **Note 1**). The purification protocol as outlined takes ~6 d, but it should be noted that nearly 3 d of the preparation time are consumed by centrifugation of the glycerol gradients. Unless otherwise stated, the protocol is written for tissue-culture cells.

The underlying rationale for each step of the purification follows. The first day affords a crude purification of topoisomerase II as a prelude to column chromatography. Initially, cells are lysed to separate nuclei from cytosolic fractions. Once nuclei have been washed, they are disrupted using a salt concentration suffient to release topoisomerase II from chromosomes. This extract is cleared of nuclear membrane and membrane-bound chromosomal fragments. Residual DNA and RNA, as well as many DNA binding proteins and ribonucleoproteins (that potentially interfere with later chromatographic steps) are removed by precipitation with polyethyleneimine. Following fractionation of topoisomerase II by differential ammonium sulfate precipitation, the enzyme is further purified by column chromatography on hydroxylapatite and phosphocellulose. These media are ion-exchange resins. Furthermore, since they both contain phosphate groups, it is believed that DNA binding proteins display an increased affinity for these resins. Topoisomerase II is then concentrated on a phosphocellulose collection column to provide an appropriate vol-

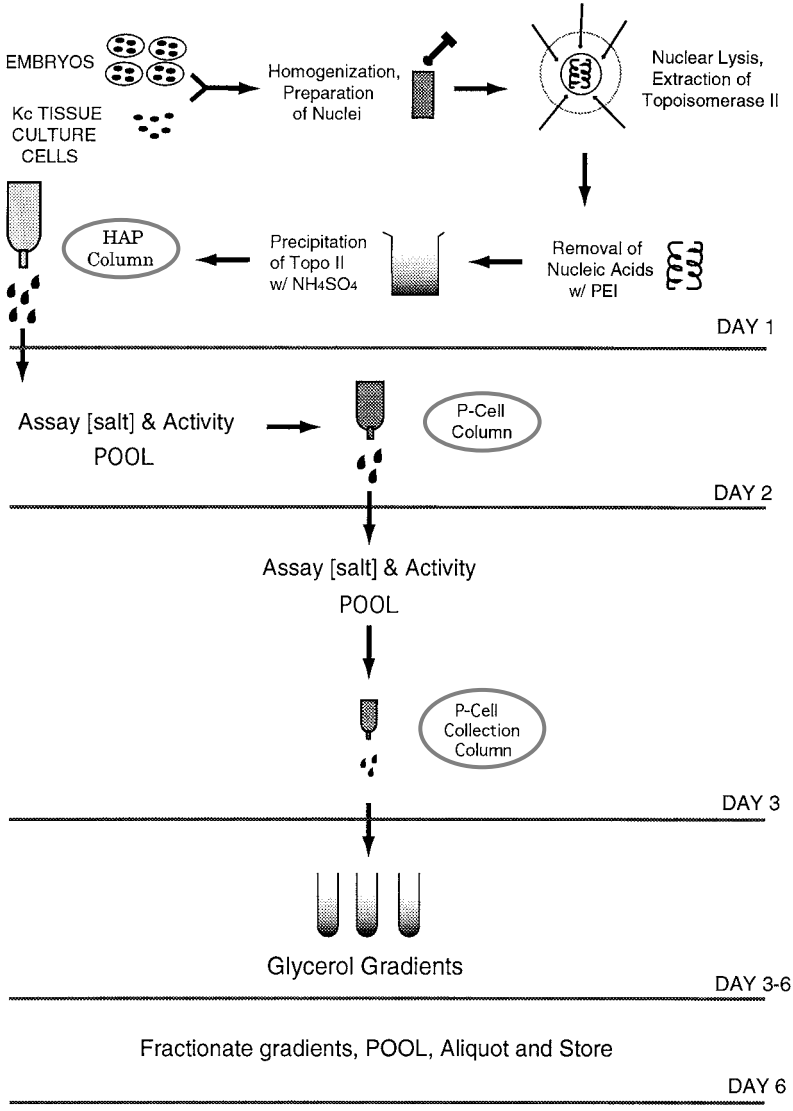

Fig. 1. Flowchart for the preparation of topoisomerase II from *D. melanogaster* embryos or Kc tissue-culture cells.

ume for application to glycerol gradients. The final purification step of sedimentation velocity through glycerol gradients fractionates topoisomerase II on the basis of its hydrodynamic properties and removes the final traces of topoisomerase I and protein kinase.

2. Materials

2.1. Starting Material

Use 500 g of Kc tissue-culture cells (*see* **Notes 2** and **3**) or 12- to 18-h-old embryos (*see* **Note 4**).

2.2. Stock Solutions

All solutions should be made with H_2O that is either filtered (such as from a MilliQ system) or glass-distilled. It is advisable to make all solutions fresh for each preparation. These stocks are used to make the other buffers listed below. Solutions are stored at room temperature, 4°C, or -20°C as noted.

1. 500 mL of 1.0*M* Tris-HCl, pH 7.9 (4°C).
2. 100 mL of 1.0*M* KCl (room temperature).
3. 300 mL of 5.0*M* NaCl (room temperature).
4. 100 mL of 1.0*M* $MgCl_2$ (room temperature).
5. 100 mL of 0.25*M* NaEDTA, pH 8.0 (4°C).
6. 50 mL of 10% Triton X-100 (4°C).
7. 15 mL of 0.5*M* dithiothreitol (DTT) (–20°C).
8. 500 mL of 0.5*M* Na phosphate, pH 7.1 (room temperature).
9. 500 mL of 0.5*M* K phosphate pH 7.1 (room temperature).
10. 750 mL of 0.5*M* dibasic Na phosphate (room temperature).
11. 2 L of Kc cell storage buffer (Kc cell prep only) (4°C), prepared as follows: Dissolve 7.94 g of L-glutamic acid and 4.04 g of glycine in H_2O, adjust pH to 7.0 with 10*N* KOH, and bring to a volume of 50 mL; dissolve 13.82 g of L-glutamic acid and 7.04 g of glycine in H_2O, adjust pH to 7.0 with 10*N* NaOH and bring to a volume of 50 mL; dissolve 2.0 g of $MgCl_2 \cdot 6H_2O$ and 2.0 g of $MgSO_4$, and bring to a volume of 300 mL; dissolve 0.83 g of $NaHPO_4 \cdot H_2O$ in H_2O, and bring to a volume of 10 mL; combine solutions 1–4, add 400 mL of 50% glycerol, adjust pH to 6.7, and bring to a final volume of 2 L.
12. 2.5 L of 1*M* sucrose (4°C).
13. 2.5 L of 50% glycerol (v/v) (4°C).
14. 8 L of Triton-salt solution (embryos only) (room temperature): 0.005% Triton, 6 m*M* NaCl.
15. 50 mL of 5% polyethyleneimine (v/v) (may be purchased from Sigma (for example) as a 50% solution of polyethyleneimine (mol wt 50,000) in H_2O) (4°C) prepare as follows: Add 5 mL (5.35 g) to ~30 mL of H_2O and stir slowly, adjust pH to 7.8 with HCl, and bring to a final volume of 50 mL. Filter through a scintered glass filter.
16. 1 L of 50% Clorox/H_2O (v/v) (embryo prep only) (room temperature).
17. 150 mL of 1*M* Na_2SO (4°C).
18. 150 mL of 60 m*M* phenylmethylsulfonylfluoride in isopropanol (PMSF) (–20°C).

2.3. Cell Fractionation Buffers

All buffers should be stored at 4°C. DTT, Na_2SO_5, and PMSF should be added to buffers on the day of use.

1. 1 L of 10X homogenization buffer (10X HB): 150 m*M* Tris-HCl, pH 7.9, 50 m*M* KCl, 5 m*M* $MgCl_2$, 0.5 m*M* EDTA, pH 8.0.
2. 1.5 L of extraction buffer: 30 m*M* Tris-HCl, pH 7.9, 0.5 m*M* EDTA pH 8.0, 350 m*M* NaCl, 10% glycerol, 0.5 m*M* DTT, 0.6 m*M* PMSF, 10 m*M* Na_2SO_5.
3. 4 L (embryo prep) or 3 L (Kc cell prep) of HB-0.35*M* sucrose buffer: 1X HB containing 0.35*M* sucrose, 0.5 m*M* DTT, 10 m*M* Na_2SO_5, 0.6 m*M* PMSF, 0.05% Triton X-100 (Triton X-100 can be omitted in embryo prep).
4. 1.2 L of HB-0.8*M* sucrose buffer: 1X HB containing 0.8*M* sucrose, 0.5 m*M* DTT, 10 m*M* Na_2SO_5, 0.6 m*M* PMSF.

2.4. Hydroxylapatite Column Buffers

All buffers should be prepared the day of use using freshly boiled H_2O. (Boiling removes dissolved CO_2 from the H_2O. If the CO_2 is not removed, it can adversely affect column flow rates by forming a carbonate precipitate within the chromatographic resin.) All buffers should be stored at 4°C.

1. 500 mL of H_0 + Triton X-100 buffer: 15 m*M* Na phosphate, pH 7.1, 10% glycerol, 0.01% Triton, 0.6 m*M* PMSF, 0.5 m*M* DTT, 10 m*M* Na_2SO_5.
2. 1.6 L of H_{100} + Triton X-100 buffer: 15 m*M* Na phosphate, pH 7.1, 10% glycerol, 0.01% Triton X-100, 100 m*M* NaCl, 0.6 m*M* PMSF, 0.5 m*M* DTT, 10 m*M* Na_2SO_5.
3. 600 mL of H-KP200 buffer: 15 m*M* Na phosphate, pH 7.1, 10% glycerol, 200 m*M* K phosphate, pH 7.1, 0.6 m*M* PMSF, 0.5 m*M* DTT, 10 m*M* Na_2SO_5.
4. 400 mL of H-KP_{600} buffer: 15 m*M* Na phosphate, pH 7.1, 10% glycerol, 600 m*M* K phosphate, pH 7.1, 0.6 m*M* PMSF, 0.5 m*M* DTT, 10 m*M* Na_2SO_5.

2.5. Phosphocellulose Column Buffers

All buffers should be stored at 4°C. DTT should be added to buffers on the day of use.

1. 500 mL of P_0 buffer: 15 m*M* Na phosphate, pH 7.1, 0.1 m*M* NaEDTA, pH 8.0, 10% glycerol, 0.5 m*M* DTT.
2. 1.2 L of P_{200} buffer: 15 m*M* Na phosphate, pH 7.1, 0.1 m*M* NaEDTA, pH 8.0, 10% glycerol, 0.5 m*M* DTT, 200 m*M* NaCl.
3. 100 mL of P_{700} buffer: 15 m*M* Na phosphate, pH 7.1, 0.1 m*M* NaEDTA, pH 8.0, 10% glycerol, 0.5 m*M* DTT, 700 m*M* NaCl.
4. 50 mL of P_{700} + 5% glycerol (collection column): 15 m*M* Na phosphate, pH 7.1, 0.1 m*M* NaEDTA, pH 8.0, 5% glycerol, 0.5 m*M* DTT, 700 m*M* NaCl.

2.6. Glycerol Gradient Buffers

Buffers should be made shortly before use and stored at room temperature.

1. 100 mL of 15% GG buffer: 15 m*M* Na phosphate, pH 7.1, 0.1 m*M* NaEDTA, pH 8.0, 15% glycerol, 0.5 m*M* DTT, 700 m*M* NaCl.
2. 100 mL of 40% GG buffer: 15 m*M* Na phosphate, pH 7.1, 0.1 m*M* NaEDTA, pH 8.0, 40% glycerol, 0.5 m*M* DTT, 700 m*M* NaCl.
3. Combine ratios of the 15% GG and 40% GG buffers to generate 24 mL each of buffers with a final concentration of glycerol of 21.25, 27.5, or 33.75%.

2.7. Reagents/Special Equipment

1. 75-μm Nitex screen (embryo prep only).
2. Two metal dounces.
3. Glass dounce (Wheaton Duragrind, 40 mL, Millville, NJ).
4. Hydroxylapatite (Bio-Rad Bio-Gel HTP, Bio-Rad, Hercules, CA).
5. Phosphocellulose (Whatman [Maidstone, UK] P-11 resin).
6. 2.5 × 10, 1.5 × 10, and 0.7 × 2.5 cm Econocolumns (Bio-Rad).
7. 1-L and 250-mL gradient makers.
8. Peristaltic pump.
9. Gradient sipper (Haake Buchler, Saddle Brook, NJ).
10. Fraction collector.
11. Conductivity meter.
12. Bradford protein assay reagents (Bio-Rad).
13. Phast gel-electrophoresis system (Pharmacia, Piscataway, NJ) (optional).

3. Methods

3.1. Preparation of Kc Tissue-Culture Cells for Topoisomerase II Purification

1. Thaw *Drosophila* Kc tissue-culture cells (500 g) in room temperature cell storage buffer. The total volume should not exceed 3 L (to accommodate rotor capacity). The temperature of the thawing cells should remain cold through out this process.
2. Divide the cell suspension among 500-mL bottles, and centrifuge in a JA-10 (or equivalent) rotor for 10 min at 5000 rpm (4400*g*) at 4°C.
3. Aspirate the supernatant, and resuspend the cell pellets as described in **Subheading 3.3., step 1**. All subsequent steps of the preparation should be performed at 4°C or on ice.

3.2. Preparation of Embryos for Topoisomerase II Purification

1. Thaw *Drosophila* 12- to 18-h-old embryos (500 g) to room temperature in Triton-salt solution, and collect them on a Nitex screen.
2. Dechorionate the embryos at room temperature with sufficient 50% Clorox to keep them covered. Depending on the size of the Nitex screen, this may have to

be performed in more than a single batch. After 2 min, rinse well (three to four times) with Triton-salt solution. All subsequent steps of the preparation should be performed at 4°C or on ice.

3. Separate intact from damaged embryos by resuspending the dechorionated embryo preparation to a volume of ~2 L with cold Triton-salt solution. (Intact embryos settle, but damaged embryos float.) Allow embryos to settle for 15 min with occasional stirring of the upper layer. Approximately 95% of the embryos should be intact.
4. Aspirate the damaged embryos and the Triton-salt solution above the settled embryos, resuspend the intact embryos in Triton-salt solution, and repeat **step 3**. Collect the intact embryos by filtration through a Nitex screen.

3.3. Preparation of Nuclear Extract

1. Resuspend prepared cells or embryos in 1.6 L of HB-0.35*M* sucrose and disrupt them by six to seven strokes using a metal dounce. If initial passes with the metal dounce are too difficult, initial cell disruption may be carried out in a glass dounce (*see* **Notes 5–7**).

2a. For Kc cells, crude nuclei are pelleted by centrifugation in a JA-10 rotor for 15 min at 7600 rpm (10,000*g*) at 4°C. Remove the cytosolic supernatant by aspiration, and wipe any lipids from the walls of the tubes (*see* **Note 8**).

2b. For embryos, remove debris by filtration through a Nitex screen. Resuspend any remaining solids in 400 mL of HB-0.35*M* sucrose, and dounce again as described in **step 1**. Filter through Nitex screen, combine flowthrough fractions, and pellet nuclei as in **step 2a**.

3. Resuspend the loose nuclear pellet in a small volume (50–100 mL) of HB-0.35*M* sucrose using three to five strokes in a glass dounce. Dilute to a final volume of 1 L with HB-0.35*M* sucrose and mix.
4. Wash the crude nuclei by pouring 125 mL of the preparation into each of eight 250-mL centrifuge bottles, underlay with 115 mL of HB-0.8*M* sucrose, and centrifuge (in two batches) in a JS7.5 (or equivalent) swinging bucket rotor for 15 min at 4000 rpm (3000*g*) at 4°C. Aspirate the supernatant above the washed nuclear pellet.
5. Lyse the washed nuclei by resuspending the pellet in 500 mL of extraction buffer (*see* **Note 9**), place the suspension in centrifuge bottles, and mix occasionally by gentle inversion for 45 min. Centrifuge in a JA-10 rotor for 15 min at 9800 rpm (17,000*g*) at 4°C. Pour off the nuclear extract supernatant and retain.
6. Repeat the extraction of the gel-like pellet by resuspending with 250 mL of extraction buffer, and mix occasionally by gentle inversion for 30 min. Centrifuge as described in **step 5**, and combine the supernatants from the first and second nuclear extractions.
7. If necessary, the hazy nuclear extract supernatant may be further clarified by one additional centrifugation as described in **step 5** (*see* **Note 10**). Retain the supernatant and determine its volume.

3.4. Removal of Nucleic Acid and Precipitation of Topoisomerase II

1. Remove nucleic acids from the nuclear extract by slowly adding 10 μL of 5% polyethyleneimine for each milliliter of nuclear extract while stirring. Following this addition, the extract should become turbid and white. Stir for 30 min. Pellet nucleic acids by centrifugation in a JA-10 rotor for 10 min at 9800 rpm (17,000*g*) at 4°C. Retain the polyethyleneimine supernatant, and determine its volume.
2. Further fractionate topoisomerase II by adding ground ammonium sulfate (0.197 g ammonium sulfate/mL of supernatant) slowly and with stirring to the polyethyleneimine supernatant to obtain a final saturation of 35%. Once the ammonium sulfate is in solution, stir for 30 min. Pellet insoluble protein by centrifugation as described in **step 1**. Retain the supernatant and determine its volume.
3. Precipitate topoisomerase II by adding ground ammonium sulfate (0.295 g ammonium sulfate/mL of supernatant) slowly and with stirring to the 35% ammonium sulfate supernatant to obtain a final saturation of 80%. Once the ammonium sulfate is in solution, stir for 30 min. Pellet topoisomerase II by centrifugation as described in **step 1**. Immediately pour off the supernatant and wipe the centrifuge bottle walls dry to remove excess liquid. Redissolve the 80% ammonium sulfate pellet (which contains topoisomerase II) for column chromatography as described below (*see* **Note 11**).

3.5. Hydroxylapatite Column Chromatography

1. During cellular fractionation, boil 4 L of water (to remove dissolved CO_2), and use it to prepare hydroxylapatite column buffers listed in **Subheading 3.**
2. Prepare ~20 g (dry wt) of hydroxylapatite in H_{100} + TX buffer following the Bio-Rad protocol (*see* **Notes 12** and **13**). After the resin settles, decant the supernatant, and resuspend the hydroxylapatite in fresh buffer. Allow the resin to settle a second time, decant the supernatant, and add enough buffer to make an ~2:1 slurry of buffer:resin.
3. Pour a 2.5 × 8 cm (35–40 mL) hydroxylapatite column according to the manufacturer's specifications. Ensure that the column does **not** run dry (it will form a carbonate crust that may dramatically decrease column flow rates).
4. Wash the column with at least 2 column volumes of H_{100} + TX buffer, and adjust the flow rate to ~2–3 column volumes/h (~100 mL/h).
5. Resuspend the 80% ammonium sulfate pellet in Ho + TX buffer (~>100 mL) and check its conductivity. Adjust the final volume such that the conductivity of the solution is lower than that of the H-KP_{200} buffer (this may require >400mL)
6. Load the sample onto the column at a flow rate of ~100 mL/h.
7. Wash the column with 2–3 column volumes of H_{100} + TX buffer followed by 3–4 column volumes of H-KP_{200} buffer (at ~100 mL/h).
8. Elute topoisomerase II with a 16 column volume linear salt gradient of H-KP_{200} to H-KP_{600} buffer. Collect ~4 mL fractions (~160 fractions) at a flow rate of 50–100 mL/h.

9. Assay the salt concentration across the gradient by sampling the conductivity of every tenth fraction (use 10-µL samples diluted to 1 mL in H_2O).
10. Monitor the elution of topoisomerase II by assaying fractions (typically diluted 1:25) for enzymatic activity using a DNA catenation (12), decatenation (11), or unknotting (11) assay. If a Phast gel-electrophoresis system is available, topoisomerase II may be visualized by either Coomassie or silver staining on denaturing polyacrylamide gels. *Drosophila* topoisomerase II generally elutes at a salt concentration between 280 and 360 m*M*.
11. Pool the peak topoisomerase II fractions (hydroxylapatite column pool), and prepare them for the phosphocellulose column as described below.

3.6. Phosphocellulose Column Chromatography

1. Regenerate ~10 g (dry wt) of phosphocellulose resin following the Whatman protocol (*see* **Note 14**).
2. To adjust the pH of the regenerated phosphocellulose, scoop the resin into a 600-mL beaker (with as little water as possible, ~25 mL). While slowly mixing with a stir bar, add sufficient 0.5*M* dibasic Na phosphate to reach pH 7.1 (this will require ~200–300 mL of Na phosphate). Remove the stir bar, and allow the resin to settle at room temperature (to avoid crystallization of the dibasic Na phosphate). Decant and discard the supernatant.
3. Equilibrate the phosphocellulose by resuspending it in P_{200} buffer, and store at 4°C. Allow the resin to settle and exchange into fresh P_{200} at least three additional times. Resuspend the phosphocellulose with sufficient P_{200} to make an ~2:1 slurry of buffer:resin.
4. Pour a 1.5 × 8.5 cm (~15 mL) phosphocellulose column according to the manufacturer's specifications. Pack the column at 100 mL/h, and equilibrate using at least 2 column volumes of P_{200}.
5. Prepare the hydroxylapatite column pool for chromatography on the phosphocellulose column by diluting it with P_0 (slowly and with stirring) such that the average salt concentration is ~200 m*M*. Some flocculence may appear at this step, but it does not interfere with the chromatography.
6. Load the sample onto the column at a flow rate of 50–100 mL/h.
7. Wash the column with ~3 column volumes of P_{200} buffer at a flow rate 50–100 mL/h.
8. Elute topoisomerase II using a 10 column volume linear gradient of P_{200} to P_{700} buffer. Collect 1.8-mL fractions (~80 fractions) at a flow rate of 50 mL/h.
9. Monitor the elution of topoisomerase II by assaying fractions as described in **Subheading 3.5., step 10**. In addition, an ATP-independent relaxation assay may be employed to detect the presence of topoisomerase I in the preparation. If a Phast gel-electrophoresis system is available, topoisomerase II may be visualized by silver staining on denaturing polyacrylamide gels. *Drosophila* topoisomerase II generally elutes at a salt concentration between 350–400 m*M*.
10. Pool the peak topoisomerase II fractions (phosphocellulose column pool), and prepare them for the phosphocellulose collection column as described below.

3.7. Phosphocellulose Collection Column

1. To concentrate the phosphocellulose eluent for centrifugation through glycerol gradients, pour a 0.7 × 2 cm (~2 mL) phosphocellulose column at ~100 mL/h and equilibrate with 2 column volumes of P_{200} buffer.
2. To prepare the sample for the collection column, dilute the phosphocellulose column pool with P_0 (slowly and with stirring) such that the average salt concentration is ~200 m*M*. Some flocculence may appear at this step, but it does not interfere with the chromatography.
3. Load the column at a flow rate of 50–100 mL/h.
4. Wash the column with ~3 column volumes of P_{200} buffer at a flow rate of 50–100 mL/h.
5. To elute topoisomerase II, allow the buffer meniscus to just enter the top of the resin, wash with a few drops of P_{700} + 5% glycerol, and then fill the column with the same high-salt buffer. (This procedure will prevent topoisomerase II from diffusing back into the buffer reservoir.) Collect 3 drop fractions (~12 fractions) by hand. (The peristaltic pump and fraction collector are removed to minimize dead volume.) Fractions may be collected either by gravity or by applying positive air pressure to the buffer reservoir at the top of the column with the peristaltic pump.
6. Assay 1-µL aliquots from each fraction for protein content by Bradford assay, and pool the samples with peak protein for loading onto glycerol gradients as outlined below.

3.8. Glycerol Gradients

1. Pour glycerol step gradients in five layers (15, 21.25, 27.5, 33.75, and 40% glycerol) in SW41 (or equivalent) tubes at room temperature (*see* **Note 15**). Form gradients using 2.25 mL of each solution. Start with the 15% solution and underlay each successive solution.
2. Allow the gradients to sit covered at 4°C for 8–15 h before use.
3. Layer samples (0.2–0.4 mL/tube or 2–4 mg of protein/tube) on glycerol gradients. Centrifuge samples in an SW41 (or equivalent) rotor for 65–70 h at 39,000 rpm (185,000*g*) at 4°C.
4. Using a gradient sipper that collects from the top of the tube, collect ~200 mL fractions (~55 fractions) at a rate of ~1.5 mL/min (*see* **Note 16**). Fractions may be assayed for topoisomerase II activity. However, it is generally sufficient to monitor protein content by assaying 5-mL aliquots of fractions using a Bradford assay (*see* **Subheading 3.7.**). Topoisomerase I and other contaminating proteins typically sediment in a minor band toward the top of the gradient, whereas topoisomerase II should compromise the major band toward the bottom (**Fig. 2**).

3.9. Storage of Purified Topoisomerase II

1. Pool topoisomerase II-containing fractions, and assay the final protein concentration using a Bradford assay. Typically, this preparation yields 1.8–3.0 mg of *Drosphila* topoisomerase II with a final concentration of 0.5–1.0 mg/mL (*see*

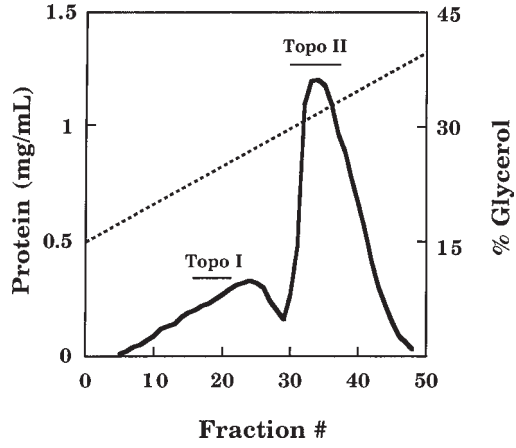

Fig. 2. Glycerol gradient (final step) of the *Drosophila* topoisomerase II preparation. The concentration of glycerol is denoted by the dashed line.

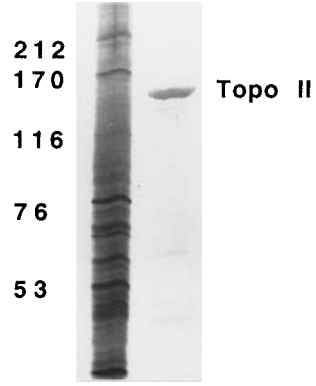

Fig. 3. Silver-stained denaturing polyacrylamide gel of Kc tissue-culture cell initial homogenate (lane 1) and purified *Drosophila* topoisomerase II (lane 2). Molecular mass standards (kDa) are indicated.

Notes 17 and **18**). A silver-stained denaturing polyacrylamide gel of a typical enzyme preparation is shown in **Fig. 3**.

2. Aliquot and store the preparation in cryotubes in liquid nitrogen until use. Generally, topoisomerase II stored in this fashion is active for a minimum of 1 yr. If liquid nitrogen is not available, topoisomerase II is also stable when stored at −80°C.
3. To use stored topoisomerase II, thaw rapidly and store as a liquid at -20°C. The thawed enzyme is stable for >6 wk under these conditions (*see* **Note 19**). The enzyme may be refrozen in liquid nitrogen once without apparent loss of activity. However, multiple freeze–thawing is not recommended

4. Notes

1. This protocol is designed for large-scale preparations, and is not as successful with small-scale preparations. Purification utilizing FPLC protocols ***(17,18)*** may be more effective for small-scale purification.
2. Kc tissue-culture cells are embryonic in origin and are undifferentiated ***(19)***. They can be grown in large-scale cultures at high density and do not require serum for growth ***(19)***. Consequently, the production of these cells is relatively economical (<$1.00/g).
3. Kc cells must go through one freeze–thaw cycle prior to use in this purification protocol. Owing to their elastic nature, fresh cells are not readily lysed by mechanical douncing.
4. Topoisomerase II has been successfully prepared from 6- to 12- or 12- to 18-h-old embryos. However, embryos up to 24-h-old may be used. Early embryos (0–3 hr) are not advised because of their low nuclei count.
5. Rotary dounces should be avoided for cell homogenization because they shear nuclei.
6. Embryos are considerably more difficult to dounce than Kc cells. Therefore, the initial passes should be carried out using a glass dounce.
7. Cell disruption following douncing can be monitored by trypan blue exclusion.
8. The cytosol, which can be stored at −80°C, is a rich source of cellular material and can be used for the preparation of other proteins.
9. An alternative nuclear fractionation protocol has been used in other successful preparations of topoisomerase II ***(20)***. In these preparations, nuclei are lysed under low-salt conditions in which topoisomerase II is not released from chromatin. The enzyme is coprecipitated with nucleic acids and subsequently is extracted from the precipitate with salt.
10. **Step 7** in **Subheading 3.3.** usually is only necessary when Kc cells are used as the starting material.
11. The first day of the purification protocol generally requires 10–12 h from the time the starting material is thawed until the hydroxylapatite column loading begins.
12. It is suggested that the lot numbers of column resins be recorded. Each lot will have slightly different chromatographic properties.
13. Care should be taken when handling the hydroxylapatite resin owing to its fragile nature. Proteins are eluted most effectively with phosphate buffers. If nonphosphate buffers are used, the salt concentrations necessary to elute topoisomerase II may differ significantly.
14. The phosphocellulose resin requires special attention during preparation. The stability and capacity of the resin are dependent on the buffering anion used. The resin is significantly more stable in phosphate buffers and should not be exchanged into Tris buffers until a few days prior to use.
15. The gradients should be generated in buffer that contains at least 500 m*M* salt to avoid aggregation and anomalous sedimentation of topoisomerase II.

16. Glycerol gradients may be collected from the bottom of the tube, but collecting from the top of the gradient minimizes diffusion of the sample and allows the gradients to be fractionated more quickly.
17. The enzyme is most stable when stored in a concentrated state. It is further stabilized by the high-salt and glycerol concentrations of the gradients.
18. When the preparation is complete, it is suggested that the purity of topoisomerase II be checked by electrophoresis on a denaturing polyacrylamide gel (*see* **Fig. 3**). The enzymatic activity of the topoisomerase II preparation should be evaluated by any one of a number of assays, including DNA relaxation, catenation, decatenation, or unknotting ***(1,3,5)***. Regardless of which assay is employed, ATP-independent relaxation should be determined to test for potential topoisomerase I contamination.
19. In some −20°C freezers, freeze–thaw cycling of topoisomerase II preparations may be problematic. If this is the case, the enzyme should be moved to a freezer that is either slightly warmer or is not frost-free. Alternately, the glycerol concentration in the storage buffer may be increased to ~50%.

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22

Purification of DNA Topoisomerase I from Human Placenta

Joseph A. Holden

1. Introduction

DNA topoisomerase I (topo I) has been firmly established as the molecular target of the camptothecin group of anticancer drugs. These drugs include camptothecin, topotecan, 9-amino-camptothecin, and irinotecan ***(1)***. Many of them are now in clinical trials and are showing activity against a wide variety of solid human malignancies ***(2)***.

Much experimental data indicate that the toxicity of drugs targeted against topo I is directly related to the ability of the drug to stimulate topo I-dependent DNA cleavage. Drugs promoting extensive DNA cleavage in the presence of topo I possess much more antitumor activity than drugs that cause little DNA cleavage in the presence of topo I ***(3)***. Thus, the potential antitumor activity of a topo I-targeted drug might be easily screened by testing the drug's ability to cause DNA cleavage in the presence of topo I. Assays to measure drug-stimulated topo I-dependent DNA cleavage require nanogram amounts of purified enzyme ***(4)***.

Because of the extensive interest in topo I as an anticancer drug target, the human enzyme has been purified from several sources, including HeLa cells ***(5)***, human KB-3 cells ***(6)***, human Burkitt lymphoma cells ***(7)***, human breast cancer cells ***(8)***, and human daudi cells ***(9)***. In addition, expression vectors for human topo I have been developed that allow for the overexpression and purification of the human enzyme in yeast ***(10)*** and baculovirus infected insect cells ***(11)***.

Human placenta has served as a rich source of human enzymes over the years. It contains abundant topo I and can also serve as a source of this enzyme

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(12). There are several advantages to using placenta as a source of topo I. First, for investigators on a limited budget, there is no expense in growing and maintaining cultured cells. Fresh normal placentas are readily available in labor and delivery rooms. Second, topo I is known to undergo posttranslational modifications, which can affect the enzyme's activity *(13,14)*. Such modifications may not be present in the recombinant enzymes isolated from yeast and baculovirus expression systems to the same extent as they might in the enzyme isolated from a human tissue. Third, as described below, active human topo I can be purified in a simplified two-step purification from placenta. The amount of enzyme obtained is enough to perform hundreds of topo I-mediated DNA cleavage assays.

2. Materials

2.1. Preparation of a Nuclear Extract

1. Buffer A (4000 mL): 30 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl, 0.3*M* sucrose, 0.2 m*M* ethylenediaminetetra-acetic acid (EDTA), 15 m*M* mercaptoethanol, 1 m*M* phenylmethylsulfonyl fluoride (PMSF).
2. Buffer B (500 mL): 30 m*M* Tris-HCl, pH 7.5, 0.3*M* sucrose, 4 m*M* $CaCl_2$, 1 m*M* PMSF, 2 m*M* dithiothreitol (DTT).
3. Buffer C (500 mL): 10 m*M* Tris-HCl, pH 7.5, 10 m*M* NaCl, 1 m*M* PMSF, 2 m*M* DTT.
4. Buffer D (150 mL): 50 mM Tris-HCl, pH 7.5, 2*M* NaCl, 1 m*M* PMSF, 2 m*M* DTT.
5. Buffer E (150 mL): 50 m*M* Tris-HCl, pH 7.5, 1 *M* NaCl, 18% (w/v) polyethylene glycol (PEG), 1 mM PMSF, 2 m*M* DTT.
6. DTT: 1 *M* stock in H_2O stored at –20°C.
7. Phenylmethylsulfonyl fluoride: 100 m*M* in isopropanol stored at –20°C.
8. PEG (mol wt 8000 from Sigma, St. Louis, MO).
9. Tissue grinder: This consists of a stirrer (model 102, Talboys Engineering Corporation) and pestle and tube (size code 0025). The stirrer, pestles, and tubes are available from Kontes Scientific, Vineland, NJ. The pestle should be attached to the stirrer with a piece of heavy vacuum tubing and a screw clamp.
10 Light microscope.
11. Safety goggles.
12. Cheesecloth.
13. Medium-speed centrifuge.
14. 10% Neutral buffered formalin (Richard-Allan Medical, Richland, MI).
15. Examination gloves.
16. Hand-driven meat grinder (available at local antique stores).

2.2. Hydroxylapatite Chromatography

1. Hydroxylapatite (Bio-Gel HTP gel, Bio-Rad, Hercules, CA).
2. Syringe (30 mL).

3. Glass wool.
4. 18-gage needles.
5. Polypropylene tubing.
6. Rubber stoppers.
7. 0.2*M* potassium phosphate, pH 7.0 (200 mL).
8. 1.0*M* potassium phosphate, pH 7.0 (100 mL).
9. Equilibration buffer (150 mL): 50 m*M* Tris-HCl, pH 7.5, 1*M* NaCl, 6% (w/v) PEG, 1 m*M* PMSF, 2 m*M* DTT.
10. Fraction collector.
11. Buffer F (250 mL): 0.2*M* potassium phosphate, pH 7.0, 10% glycerol, 1 m*M* PMSF, 2 m*M* DTT.
12. Buffer G (50 ml): 0.7*M* potassium phosphate, pH 7.0, 10% glycerol, 1 m*M* PMSF, 2 m*M* DTT.
13. Linear gradient maker: This can be conveniently made by using two 50-mL beakers connected by glass tubing.
14. Magnetic stirrer.

2.3. Mono S Chromatography

1. Fast protein liquid chromatography (FPLC) system with a mono S column (Pharmacia, Alameda, CA).
2. Mono S diluent: 10% glycerol, 0.1 m*M* EDTA, 1 m*M* PMSF, 2 m*M* DTT.
3. Buffer H (200 mL): 50 m*M* HEPES, pH 7.5, 0.2*M* NaCl, 10% glycerol, 0.1 m*M* EDTA, 1 m*M* PMSF, 2 m*M* DTT.
4. Buffer I (200 mL): 50 m*M* HEPES, pH 7.5, 1.5*M* NaCl, 10% glycerol, 0.1 m*M* EDTA, 1 m*M* PMSF, 2 m*M* DTT.
5. 0.2-µm filters (Pierce, Rockford, IL).
6. Vacuum funnel filter (Pierce).

2.4. Superdex 200 Gel Chromatography

1. FPLC system (Pharmacia).
2. Superdex-200 resin (Pharmacia).
3. XK-16/100 column (Pharmacia).
4. Buffer J (300 mL): 50 m*M* Tris-HCl, pH 7.5, 250 m*M* NaCl, 10% glycerol, 0.1 m*M* EDTA, 1 m*M* PMSF, 2 m*M* DTT.
5. 0.2-µm filters (Pierce).
6. Vacuum funnel filter (Pierce).

3. Methods

3.1. Preparation of a Nuclear Extract

Note: All procedures described in **Subheading 3.** are performed with ice-cold buffers and done in a cold room unless otherwise noted.

1. A fresh placenta is obtained immediately (*see* **Note 1**) after delivery, and the umbilical cord and membranes are removed and discarded in neutral buffered formalin (*see* **Note 2**).

2. The remaining placental tissue is washed with buffer A in 500-mL aliquots. The washed placenta is then passed through a hand-driven meat grinder and resuspended in 400 mL of buffer B.
3. To prepare a preparation of nuclei, the placental suspension in buffer B is homogenized in a Kontes ground-glass tissue homogenizer in 50-mL aliquots. Placenta contains a fair amount of connective tissue, and it requires 6–10 strokes with the homogenizer to produce a uniform suspension of placental nuclei. A good homogenate has the appearance of tomato juice. An industrial or autoclave glove should be worn while holding the pestle, and safety goggles should be worn during the homogenization. The adequacy of the homogenization can be checked by visualizing the nuclei by light microscopy.
4. The suspension of nuclei is filtered through cheesecloth to remove connective tissue, and the nuclei pelleted by centrifugation at 2000*g* for 20 min.
5. The pelleted nuclei are resuspended in 350 mL of buffer C and centrifuged again at 2000*g* for 20 min.
6. The nuclear pellet from the second centrifugation is resuspended in 150 mL of buffer C, made 4 m*M* in EDTA, and then stirred for 15 min. Buffer D (150 mL) is then added to lyse the nuclei, and after stirring for 15 min, buffer E (150 mL) is added to precipitate the DNA. After stirring for an additional 15 min, the suspension is centrifuged at 20,000*g* for 30 min. Topo I is recovered in the supernatant, which is referred to as the nuclear extract (*see* **Note 3**).

3.2. Hydroxylapatite Chromatography

1. A slurry of hydroxylapatite is made by suspending the dry resin in H_20. The slurry is decanted several times to remove fine particles, and then poured into a 30-mL syringe containing a plug of glass wool at the bottom and equipped with an 18-gage outlet needle connected to polypropylene tubing. The resin is allowed to settle to a bed volume between 10 and 15 mL. The column is topped with a rubber stopper containing an inlet 18-gage needle connected to polypropylene tubing, which in turn is hooked up to a buffer reservoir.
2. The column is washed with three successive washes (100 mL each) of 0.2*M* potassium phosphate, pH 7.0, 1*M* potassium phosphate, pH 7.0, and finally 0.2*M* potassium phosphate, pH 7.0. The column is then washed with 150 mL of equilibration buffer prior to use (*see* **Note 4**).
3. The nuclear extract prepared in **Subheading 3.1.** (about 450 mL) is applied to the hydroxylapatite column at a flow rate between 25 and 35 mL/h (*see* **Note 5**).
4. The column is then washed with 150 mL of buffer F, and topo I is eluted with a 100-mL linear gradient of buffer F to buffer G at a flow rate of about 25 mL/h. Fraction size is 2.5 mL. Topo I elutes near the end of the gradient at about 0.6*M* potassium phosphate (*see* **Note 6**).

3.3. Mono S Chromatography

1. The mono S column is washed first with buffer H, then with buffer I, and finally re-equilibrated with buffer H (*see* **Note 7**).

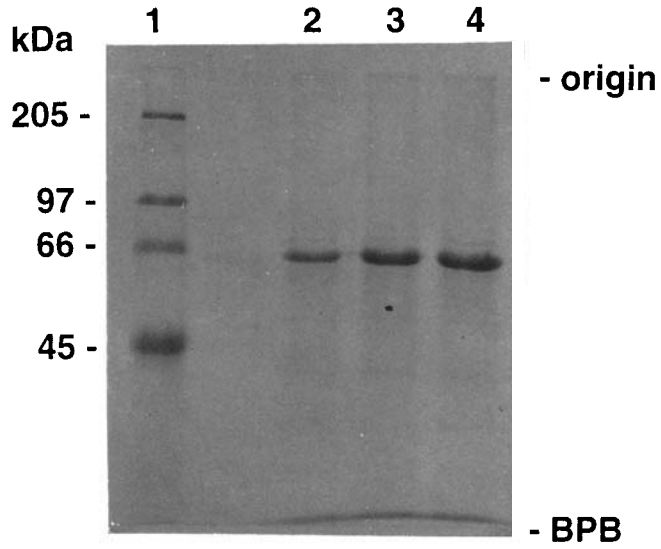

Fig. 1. SDS gel of DNA topo I isolated from human placenta. DNA topo I was isolated from human placenta by hydroxylapatite and mono S column chromatography. The final mono S preparation was subjected to SDS gel electrophoresis on a 10% polyacrylamide gel. The gel was stained with Coomassie blue. Lane 1 shows the migration of the standard proteins; myosin heavy chain (205 kDa); phosphorylase b (97 kDa); BSA (66 kDa); ovalbumin (45 kDa). Lane 2 contains 3 µg of placental topo I. Lane 3 contains 5.5 µg of placental topo I. Lane 4 contains 11 µg of placental topo I. The position of the tracking dye, bromophenol blue (BPB), is indicated.

2. Active fractions from the hydroxylapatite column are pooled, centrifuged at 5000*g* for 10 min, and then diluted fivefold with mono S diluent. This yields between 40 and 90 mL of diluted topo I.
3. The diluted topo I is applied manually to the mono S column through pump A at a flow rate of 0.5 mL/min.
4. After the enzyme is applied, the mono S column is washed with 20 mL of buffer H. Topo I is eluted with a linear 10-mL gradient of buffer H to buffer I. Fraction size is 0.25 mL. The bulk of topo I is in the 67-kDa form and elutes from the mono S column at about 800 m*M* NaCl. This preparation yields a single Coomassie staining protein species on an SDS gel as shown in **Fig. 1**. The purification is summarized in **Table 1**. The enzyme is catalytically active and sensitive to camptothecin. It is stable for several weeks when stored in the mono S buffer at −70°C. It contains no nuclease or type II topoisomerase activity. The purification can be easily completed in three working days (*see* **Notes 8** and **9**).

3.4. Superdex 200 Gel Chromatography

1. Although the bulk of topo I from placenta is in the 67-kDa form and can be isolated as a pure protein by mono S chromatography, some of the enzyme is

Table 1
Purification of DNA Topo I from Human Placenta

Fraction	Volume mL	Protein mg	Units x 10^{-6}[a]	Specific activity U x 10^{-6}/mg	Yield %	Purification, fold
Nuclear extract	450	585	36	0.06	100	1
Hydroxylapatite	8	0.88	12.8	14.5	36	241
Mono S	0.5	0.15	6	40	17	666

[a]One unit of topo I activity is the amount of enzyme that relaxes one-half of the input supercoiled plasmid DNA (500 ng) in a standard topo I relaxation assay.

present as the intact 100-kDa molecule. This form of the enzyme elutes several fractions ahead of the 67-kDa form during mono S chromatography. Unfortunately, it is contaminated with nontopo I proteins. The 100-kDa form can be purified, however, by subsequent gel-filtration chromatography on Superdex 200.

2. A fully packed superdex 200 gel-filtration column suitable for FPLC can be purchased (Pharmacia), but it is less expensive to buy the resin separately and pour it into a column. An XK-16/100 column (Pharmacia) is recommended, since it can accommodate both a large bed volume as well as the pressure of the FPLC. A suitable column has a bed height of 80–90 cm.
3. The superdex column is equilibrated with buffer J, and the 100-kDa form of the enzyme obtained from the mono S column (0.25–0.5 mL) is applied to the superdex column and eluted at a flow rate of 0.5 mL/min with buffer J. Fraction size can range from 0.5–1.0 mL. The 100-kDa form of topo I elutes in front of yeast alcohol dehydrogenase (mol wt 150,000), suggesting the enzyme has a somewhat asymmetric conformation in agreement with recent data ***(11)***.
4. The superdex 200 column is useful for separating the 100-kDa form of topo I from the 67-kDa form and from the intermediate proteolytic fragments between them as shown in **Fig. 2**.

4. Notes

1. Although it would be optimal to obtain the placenta just as it is being delivered, in reality, this is not always possible. Topo I is fairly stable in placenta, and adequate amounts of enzyme can still be isolated, even if the tissue has been sitting at room temperature for 30 min or so.
2. Because placenta is a human tissue and at delivery contains a fair amount of fresh blood, it would be prudent to double glove during the initial preparation of the nuclear extract. All tissue byproducts are discarded in neutral buffered formalin.
3. It takes roughly 5–6 h to prepare a nuclear extract from a fresh placenta. If a placenta is received late in the day, it can be washed with buffer A, and then the tissue immediately frozen in a bath of liquid nitrogen and stored at –70°C. Topo I is stable in frozen placenta for at least 3 wk. To isolate the topo I from the frozen tissue, the placental tissue should be allowed to thaw at room temperature, passed

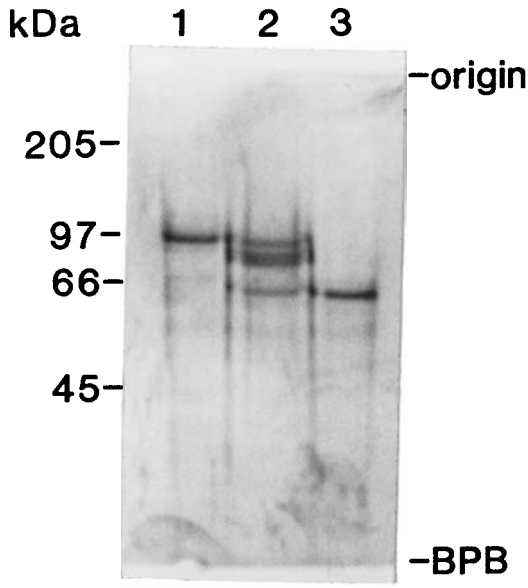

Fig. 2. SDS gel electrophoresis of DNA topo I fragments separated by gel filtration. A partially purified fraction from human placenta containing a mixture of topo I fragments was applied to an FPLC superdex column (1.6 × 83 cm) and eluted in buffer J at a flow rate of 0.5 mL/min. The fractions (1.0 mL) were assayed by measuring the topo I-catalyzed relaxation of supercoiled plasmid DNA. Three activity peaks were observed and the active fractions subjected to SDS gel electrophoresis. The first peak of activity eluted in front of yeast alcohol dehydrogenase (mol wt 150,000) and represents the intact 100-kDa form of topo I (Lane 1). The second peak of activity eluted between yeast alcohol dehydrogenase and BSA, and consists of topo I proteolytic fragments (Lane 2). The last peak of activity eluted from the column one fraction ahead of BSA and represents the 67-kDa form of topo I (Lane 3). The proteins were detected by silver staining.

through a meat grinder, and then resuspend in buffer B. Homogenization can then proceed as described in **Subheading 3.1.**

4. A fresh hydroxylapatite column is recommended for each purification. Although the columns can be reused after re-equilibration, the yields tend to decrease somewhat, and the flow rate falls.
5. It is convenient to allow the nuclear extract (450 mL) to pass though the hydroxylapatite column overnight. To prevent the column from running dry, the outlet tubing should be positioned above the column bed height.
6. Fractions from the hydroxylapatite column should be frozen by immersion in liquid nitrogen and then stored at −70°C. The hydroxylapatite fractions are stable for several weeks when frozen in this manner. They can be thawed at a later date, and the purification continued.

7. All buffers for FPLC should be filtered through 0.2-μm filters and degassed for at least several hours prior to use.
8. In addition to purification, the mono S column also serves to concentrate topo I. Because of this, it is simpler to identify fractions containing topo I by running a small aliquot of each fraction on an SDS gel rather than by assaying the fractions for topo I activity.
9. The two-step procedure ending with the mono S column yields a pure protein. It is, however, the 67-kDa form. Although not the intact molecule, this form is catalytically competent and is sensitive to topo I-targeted drugs. It has recently been demonstrated that the enzymatic properties of the proteolytic fragments of topo I are indistinguishable from the intact molecule ***(11)***. Therefore, the 67-kDa form obtained from placenta is a valuable reagent to study drugs that target the enzyme and has an added advantage in that it can be obtained easily with minimal expense and time. Some intact 100-kDa topo I is present in placental nuclear extracts and can be isolated by gel-filtration chromatography, which can be performed subsequent to the mono S column. The proportion of the 100-kDa form of topo I to the 67-kDa form varies from placenta to placenta. It is not clear whether degradation of the 100-kDa form to the 67-kDa form occurs during delivery, during the purification, or reflects the amount of time elapsing between delivery and the procurement of the tissue.

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Purification of Baculovirus-Expressed Human DNA Topoisomerase I

Lance Stewart and James J. Champoux

1. Introduction

DNA topoisomerase I (topo I) can be isolated from cultured human cells in quantities that are more than sufficient for investigations into the ability of topo I to relax supercoiled DNA (250 µg/10^9 cells) ***(1,2)***. However, the production of human topo I (htopo I) in this manner becomes both costly and labor-intensive if milligram quantities are needed for structural studies. Although active htopo I has been overexpressed in mammalian cells ***(3)***, yeast ***(4)***, and *Escherichia coli* ***(5,6)***, these systems have not proven capable of providing large quantities of the protein. In *E. coli*, the htopo I gene was found to be highly toxic to most strains ***(3)*** and appears to contain cryptic prokaryotic promoter elements that lead to constitutive expression of truncated forms of the protein (Madden and Champoux, unpublished observations). In addition, the *E. coli*-expressed htopo I is very unstable, with proteolytic breakdown products nearly as abundant as those of the full-length protein ***(6)***. This is true for htopo I constructs fused to either the T7 gene 10 translation initiation signal ***(6)*** or to glutathione-S-transferase (GST). Furthermore, expression in protease-deficient strains of *E. coli* does not appreciably reduce the proteolytic breakdown of htopo I (unpublished observations).

When numerous attempts to overproduce the htopo I in *E. coli* failed, we turned to the baculovirus-insect cell system ***(7)***, which is one of the most efficient and versatile systems for overproducing recombinant proteins in a eukaryotic setting ***(2)***. Based in part on the domain structure of htopo I ***(8)*** (**Fig. 1A**), we have generated eight different recombinant baculoviruses that express various forms of htopo I ***(2,9)***. These include wild-type and active-site

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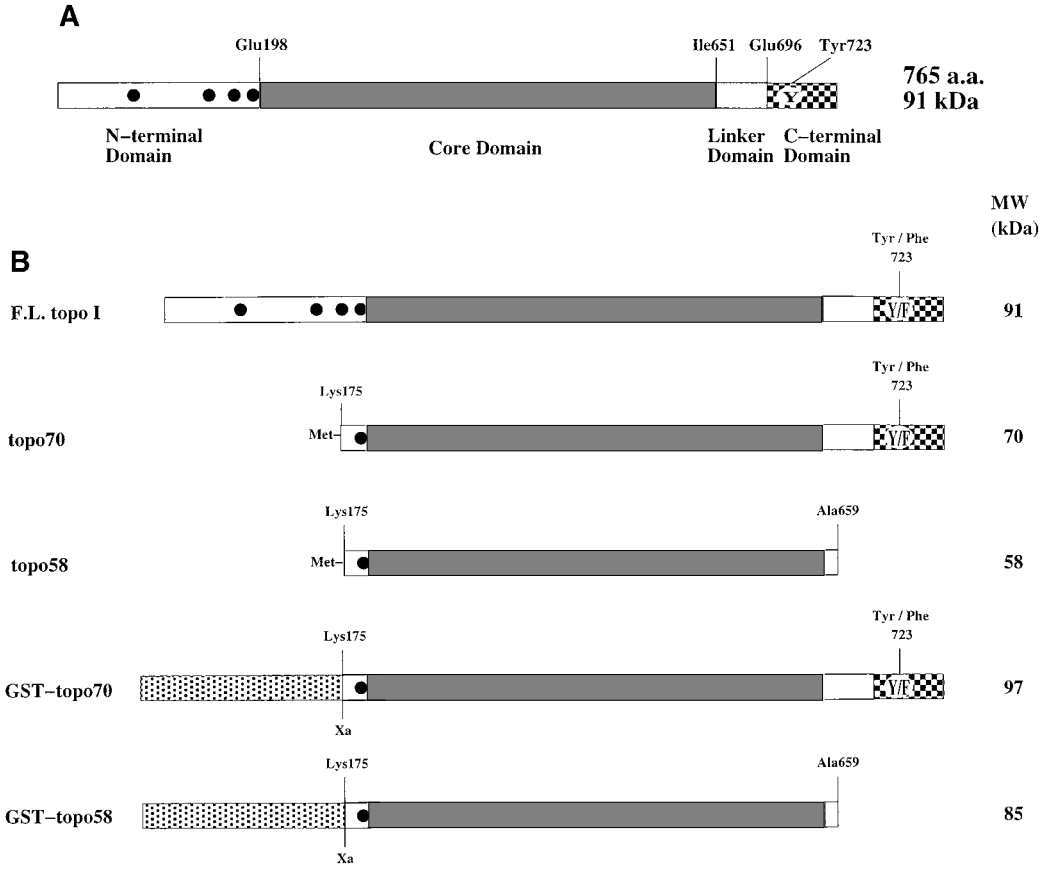

Fig. 1. Domain Structure of htopo I and recombinant proteins. **A.** Based on amino acid sequence comparisons of cellular eukaryotic topo I proteins ***(13)***, the human enzyme can be divided into four domains. Listed below each domain is the calculated molecular mass for that domain. Filled areas represent regions that are highly conserved, whereas open areas represent the unconserved regions. Residues M1-K197 comprise the unconserved amino-terminal domain. Residues E198-I651 make up the conserved "core" domain. Residues D652-E696 form an unconserved "linker" domain. The conserved C-terminal domain, residues Q697-F765, contains the active-site tyrosine at position 723 and is represented by the letter Y. The locations of four potential nuclear localization signals (residues K59-E65, K150-D156, K174-D180, and K192-E198) are represented by filled circles ***(14)***. **B.** Baculoviruses were engineered to express the following proteins: (1) wild-type and active-site mutant (Y723F) full-length htopo I (F.L. topo I), (2) wild-type and Y723F mutant versions of a 70-kDa N-terminally truncated htopo I (topo70), which initiates translation with an engineered methionine immediately upstream of K175, (3) an N- and C-terminally truncated

mutant (Y723F) versions of the full-length, truncated, and GST-fused proteins. As outlined below, the baculovirus-insect cell system has enabled the production and purification of tens of milligram quantities of each of the various htopo I proteins shown in **Fig. 1B**. Zhelkovsky and Moore ***(10)*** have also described a recombinant baculovirus that expresses htopo I.

2. Materials

2.1. Insect Cell-Culture Medium and Recombinant Baculoviruses

1. *Spodoptera fuigiperda* Sf9 cells (ATCC, Rockville, MD, CRL-1711).
2. Complete TC100 medium: TC100 (Gibco/BRL, Gaithersburg, MD) prepared with 0.35 g/L $NaHCO_3$ (pH 6.2, pH adjusted with NaOH), and supplemented with 10% fetal calf serum, an additional 2.22 g/L NaCl, yeastolate (3.33 g/L), lactalbumin hydrolysate (3.33 g/L), penicillin (100 U/mL), streptomycin (100 µg/mL), and nystatin (100 U/mL).
3. Recombinant baculoviruses: generated by cotransfecting Sf9 cells with linearized wild-type *Autographica californica* multiple nucleocapsid nuclear polyhedrosis virus (AcMNPV) DNA (Invitrogen, Carlsbad, CA) together with transfer vector DNAs (pBlueBac-based), and plaque purified according to standard procedures described by Invitrogen.

2.2. Buffers and Chemicals

1. 10X Phosphate-buffered saline (10X PBS): 40 g NaCl, 1 g KCl, 3 g Na_2HPO_4, and 1 g KH_2PO_4 dissolved in 500 mL of water. The final 1X PBS is a 10-fold dilution of the concentrated stock.
2. Phenylmethylsulfonyl fluoride (PMSF): prepared fresh as a 10 mg/mL stock in isopropanol.
3. Aprotinin: prepared fresh as a 10 mg/mL stock.
4. Dithiothreitol (DTT): prepared fresh as a 1 *M* stock.
5. Reduced glutathione: prepared fresh as a 1 *M* stock.
6. Lysis buffer: 50 m*M* KCl, 10 m*M* Tris-HCl, pH 7.5, 2 m*M* $MgCl_2$, 1% Triton X-100, 15 m*M* DTT, 0.15 mg/mL PMSF, 0.05 mg/mL aprotinin.
7. Resuspension buffer: 50 m*M* KCl, 10 m*M* Tris-HCl, pH 7.5, 2 m*M* $MgCl_2$.

Fig.1. *(see opposite page)*58-kDa form of htopo I (topo58), which has the same initiating methionine as topo70, but is terminated after residue A659, (4) wild-type and Y723F mutant topo70 fused to GST (GST-topo70), and (5) topo58 fused to GST (GST-topo58). The GST domain is represented by the stippled area(s). The fusion region of both GST-topo70 and GST-topo58 is comprised of a factor Xa cleavage site followed by seven extraneous amino acids (Gly-Ile-Asp-Pro-Ile-Asn-Met). The predicted molecular mass (kDa) for each protein is indicated at the right.

8. Ethylenediaminetetraacetic acid (EDTA): 0.5 *M* EDTA stock, pH 8.0.
9. 2X Nuclear extraction buffer: 2 *M* NaCl, 80 m*M* Tris-HCl, pH 7.5, 20% glycerol, 2 m*M* EDTA.
10. PEG buffer: 18% PEG 8000, 1 *M* NaCl, 10% glycerol.
11. Potassium phosphate buffer (PPB): 250 m*M* potassium phosphate, pH 7.4, 1 m*M* DTT, 1m*M* EDTA, 0.1 µg/mL PMSF.
12. PC elution buffer: 700 m*M* potassium phosphate, pH 7.4, 1 m*M* DTT, 1m*M* EDTA, 0.1 µg/mL PMSF.
13. K100 buffer: 100 m*M* potassium phosphate, pH 7.4, 1 m*M* DTT, 1 m*M* EDTA, 0.1 µg/mL PMSF.
14. S buffer A: 25 m*M* potassium phosphate, pH 7.4, 1 m*M* DTT, 1 m*M* EDTA, 0.1 mg/mL PMSF.
15. S buffer B: 1 *M* potassium phosphate, pH 7.4, 1 m*M* DTT, 1 m*M* EDTA, 0.1 µg/mL PMSF.
16. SP20 buffer A: 10 m*M* Tris-HCl, pH 7.5, 1 m*M* DTT, 1 m*M* EDTA, 0.1 µg/mL PMSF.
17. SP20 buffer B: 1 *M* KCl, 10 m*M* Tris-HCl, pH 7.5, 1 m*M* DTT, 1 m*M* EDTA, 0.1 µmg/mL PMSF.
18. Bovine serum albumin (BSA): purified 10 mg/mL stock (New England Biolabs, Beverly, MA).
19. Storage buffer: 50% glycerol, 10 m*M* Tris-HCl, pH 7.5, 1 m*M* DTT, 1 m*M* EDTA.
20. Cation-exchange buffer A: 7 m*M* monohydrate [2-(*N*-morpholino)-ethanesulfonic acid] (MES), 7 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 7 m*M* Na acetate, pH 7.5.
21. Cation-exchange buffer B: 1 *M* NaCl, 7 m*M* MES, 7 m*M* HEPES, 7 m*M* Na acetate, pH 7.5.

2.3. Chromatography Matrices

1. Phosphocellulose (P11, Whatman, Clifton, NJ): prepared according to manufacturer's specifications.
2. Phenyl Sepharose CL-4B (Pharmacia, Piscataway, NJ).
3. Glutathione Sepharose 4B (Pharmacia).
4. Mono-Q HR 5/5 (Pharmacia).
5. Mono-S HR 5/5 (Pharmacia).
6. POROS SP20 (4.6 mm R/100 mm L) (PerSeptive Biosystems, Framingham, MA).

3. Methods

3.1. Culture of Insect Cells

1. Sf9 cells are cultured in 100 mL or 1-L spinner flasks (Bellco, Vineland, NJ), with maximum volumes of 80 or 500 mL of complete TC100 medium, respectively. The 1-L flasks are assembled with microcarrier impellers (Bellco, Cat. #1965-01000), adjusted to break the air–liquid interface (Graber, personal communication). Cells are seeded at 0.5–0.8 $\times$ 10^6 cells/mL and cultured by

stirring at rate of 60 rpm in an atmosphere of 50% O_2/50% air at 27°C. When a density of 3–3.5 × 10^6 cells/mL (~3 d) is reached, the cells are diluted with complete TC100 back to the seeding density of 0.5–0.8 × 10^6 cells/mL.
2. If an oxygentated environment is not available, then Sf9 cells should be split 1:4 every 2 d such that maximum densities of no >2 × 10^6 cells/mL are reached.
3. Insect cells are easily broken if stirred too vigorously. Therefore, the stir rate should be set just fast enough to ensure that the cells are fully in suspension (~60 rpm).
4. When Sf9 cells are growing well, the doubling time should be 24–30 h.
5. Sf9 cells do not grow well at temperatures above 30°C.

3.2. Preparation of High-Titer Virus Stock

1. The original plaque-purified virus stock (P1) is used to inoculate two 100-mm diameter plates each containing 1 × 10^6 Sf9 cells in 10 mL of complete TC100 (100 µL of P1/plate). After 5 d of incubation at 27°C, the culture supernatant (P2 virus stock) is harvested. The cells should be visibly lysed from the infection. Cell debris is removed from the P2 stock by centrifugation at 1000*g* at room temperature for 5 min.
2. The P2 virus stock (10 mL) is used to infect a 1-L spinner flask containing 500 mL of Sf9 at a density of 1 × 10^6 cells/mL. At 1 d postinfection, the cells are split 1:2 into two 1-L flasks each containing 500 mL. At 6 d postinfection, the medium is harvested, and cell debris is removed by centrifugation at 1000*g* at room temperature for 5 min. This P3 virus stock is maintained at 4°C and used for large-scale infections. The virus stock can be titered using a plaque assay developed by Invitrogen (not described here). Typically virus titers are on the order of 10^{10} PFU/mL.

3.3. Large-Scale Sf9 Infection

1. Large-scale Sf9 infections are initiated with Sf9 cells that have been doubling every 24–30 h for at least 2 d.
2. Pellet the cells at room temperature by centrifugation at 600*g* for 5 min. Discard the spent medium and resuspend the cells in complete TC100 at a density of 1 × 10^7 cells/mL.
3. Add a volume of P3 or P4 virus stock, which is 1/5th the volume of concentrated cell suspension. This ensures a multiplicity of infection (moi) of at least 10 PFU/cell, with the actual moi being on the order of 100–1000 PFU/cell. No adverse effects on htopo I expression have been observed with such a high moi.
4. Stir for 1 h at room temperature.
5. Dilute the infected cells to 3 × 10^6 cells/mL with complete TC100 medium. In the absence of oxygenation, the cells should be resuspended at 1 × 10^6 cells/mL.
6. Harvest the infected cells at 48 h postinfection by centrifugation for 5 min at 1000*g*. The virus supernatant (P4) can be saved and used as high-titer virus for subsequent infections. However, repeated use of culture supernatants from large-scale infections to carry out subsequent large-scale infections is not recom-

mended, since this leads to reduced yield of recombinant protein. The best high-titer virus stocks are those obtained by infecting cells at a low moi and allowing the infection to proceed for 6 d (**Subheading 3.2.**).

3.4. Purification of htopo I from Baculovirus-Infected Insect Cells

The following purification protocol applies to the wild-type and active-site mutant (Y723F) forms of the full-length and N-terminally deleted topo70 proteins (*see* **Fig. 1**). All purification steps are carried out at 4°C, except those involving room temperature high-pressure liquid chromatography (Mono-Q, Mono-S, and POROS columns).

1. The starting material for the purification of baculovirus-expressed htopo I is a cell paste of approx 3×10^9 Sf9 cells harvested 48 h postinfection.
2. Wash the cells three times with a total of 1 L of ice-cold 1 × PBS. This involves resuspension by shaking and pelleting by centrifugation for 5 min at 400*g*.
3. Resuspend the washed cells in 180 mL of lysis buffer by vigorous shaking for 1 min on ice.
4. Pellet the nuclei by centrifugation at 600*g* for 10 min. Discard the cytoplasmic supernatant. For each of the htopo I proteins shown in **Fig. 1**, approx 10% of the total baculovirus-infected cell protein is the recombinant htopo I, and >95% of this material is located in the nucleus.
5. Resuspend the nuclei in 120 mL of resuspension buffer containing 15 m*M* DTT, 0.15 mg/mL PMSF, and 0.05 mg/mL aprotinin by vigorous shaking (*see* **Note 1**).
6. Pellet the nuclei by centrifugation at 600*g* for 10 min.
7. Repeat **steps 5** and **6**.
8. Resuspend the washed nuclei in 50 mL of resuspension buffer containing 25 m*M* DTT, 0.4 mg/mL PMSF, and 0.12 mg/mL aprotinin.
9. Adjust the nuclei to 10 m*M* EDTA by adding 800 µL of 0.5 *M* EDTA.
10. With stirring, add 50 mL of 2X nuclear extraction buffer to lyse the nuclei.
11. Use a stir bar to stir the lysed nuclei at a high enough speed to get the entire viscous solution moving well, but not foaming.
12. While stirring, slowly (dropwise) add 50 mL of PEG buffer.
13. Stir for 30 min. The mix will appear milky gray owing to PEG-mediated precipitation of nucleic acid.
14. Pellet the precipitated nucleic acid by centrifugation at 10,000*g* for 10 min. Discard the pellet. The htopo I remains in the supernatant where it comprises about 40% of the soluble protein.
15. Dialyze the supernatant (~150 mL) overnight against 4 L of PPB.
16. Clarify the dialysate (~170 mL) by centrifugation at 10,000*g* for 10 min. Discard the pellet, which contains proteins that precipitate during dialysis. All of the htopo I remains soluble following dialysis and, after clarification, represents about 50% of the total soluble protein.
17. Pass the clarified, dialyzed PEG supernatant through a 7-mL bed volume of phenyl sepharose (PS) equilibrated with PPB (1 mL/min). The htopo I flows through

the PS column and is collected together with a 10-mL wash in a total volume of about 200 mL. This simple step removes a large quantity of contaminating baculoviral and cellular proteins. At this point, htopo I represents ~90% of the total protein. Discard the PS matrix (*see* **Note 2**).

18. Load the PS flowthrough at 0.5 mL/min onto a 15-mL bed volume of phosphocellulose (PC) that has been equilibrated with PPB. After washing with 50 mL of PPB, step elute the htopo I with 30 mL of PC elution buffer. Discard the PC matrix (*see* **Note 2**).
19. Dialyze the PC eluate against 2 L of K100.
20. Filter the dialyzed PC eluate through a 0.22-µm syringe filter (Millex-GV, Millipore). This serves to remove contaminating dust as well as some proteins that precipitate during dialysis.
21. Pass the filtrate over a Mono-Q (5H/R, Pharmacia) column that has been equilibrated with K100 (1 mL/min). The vast majority (>99%) of htopo I flows through the Mono-Q column (*see* **Note 3**), whereas most of the remaining contaminant proteins bind tightly.
22. Load the Mono-Q flowthrough onto a Mono-S column (5H/R) that has been equilibrated with a 9:1 mix of S buffers A:B (*see* **Note 4**). After washing with 10 mL of the equilibration buffer, elute the column with a 25-mL salt gradient from 100–200 m*M* potassium phosphate (from 9:1 to 8:2 S buffers A:B). The htopo I is the first protein to elute from mono-S (~150 m*M* potassium phosphate). Subsequent peaks are owing to the elution of small quantities of contaminant proteins.
23. Pool the peak htopo I fractions from the Mono-S chromatography.
24. Load the mono-S pool onto (3 mL/min) a self-packed POROS SP20 (4.6 mm R/100 mm L) column (PerSeptive Biosystems) that has been equilibrated with 300 m*M* KCl (7:3 mix of SP20 buffers A:B) (*see* **Note 5**). After washing with 10 mL of the equilibration buffer, elute the column with a linear 25 mL KCl gradient (1 mL/min) from 300–800 m*M* KCl (from 7:3 to 2:8 SP20 buffers A:B). The htopo I elutes at ~450 m*M* KCl.
25. Pool the peak htopo I fractions from the SP20 chromatography.
26. Concentrate the htopo I to 5 mg/mL with an Amicon Ultrafiltration Cell Model (Amicon, Beverly, MA) 52 using compressed nitrogen at 20 psi and a stir rate of 60 rpm (*see* **Note 6**).
27. Dialyze the concentrated htopo I into storage buffer.
28. Assay the final protein concentration by the method of Bradford ***(11)*** using BSA as a protein concentration standard (Bio-Rad Protein Assay kit cat. no. 500-0001, Bio-Rad, Hercules, CA).
29. Store the final htopo I in sealed microcentrifuge tubes at –20°C. Do not freeze the concentrated htopo I at –80°C, since this will lead to irreversible precipitation.

3.5. Yield and Activity

1. The final yield of htopo I is 20–30 mg from 3×10^9 cells. The differences in yield depend on how well the infection proceeds. It is critical that the cells are dou-

bling every 24–30 h prior to infection, and that the virus stock used is of sufficient titer to ensure an moi of at least 10 PFU/cell.
2. Approximately 0.5 ng of purified recombinant htopo I will fully relax 1 µg of a CsCl-purified supercoiled 3.0-kbp plasmid DNA in 10 min at 37°C in 150 m*M* KCl, 10 m*M* Tris-HCl, pH 7.5, 1 m*M* DTT, 1 m*M* EDTA, and 0.1 mg/mL BSA (New England Biolabs) (*see* Vol. 94, Part II, Chapter 2). If $MgCl_2$ is included in the reaction at 10 m*M*, the htopo I is approximately 16-fold more active. This activity is equal to or better than that reported for htopo I purified from either HeLa cells or placenta ***(1,12)***.

3.6. Modified Protocol for Purification of Recombinant topo58

The topo58 protein, an N- and C-terminally deleted version of htopo I (**Fig. 1**), can be purified from baculovirus-infected cells using the following modified version of the protocol of **Subheading 3.4.**

1. The initial steps in the purification of topo58 are identical to **steps 1–21** of **Subheading 3.4.**
2. The resulting Mono-Q flowthrough material is loaded onto a Mono-S column (5H/R) that has been equilibrated with a 9:1 mix of S buffers A:B. After washing with 10 mL of the equilibration buffer, elute the column with a 25-mL salt gradient from 100–300 m*M* potassium phosphate (9:1 to 7:3 S buffer A:B mix).
3. Analyze the fractions by SDS-PAGE and Coomassie blue staining (**Fig. 2**). Approximately 70% of the topo58 elutes from the Mono-S at 200 m*M* potassium phosphate, whereas the remainder elutes at 250 m*M* potassium phosphate.
4. Pool the peak topo58 fractions that elute from Mono-S at 200 m*M* potassium phosphate.
5. Carry out SP20 chromatography on the Mono-S pool as described in **step 24** of **Subheading 3.4.** The topo58 elutes at ~400 m*M* KCl.
6. Pool the peak SP20 fractions.
7. Concentrate, dialyze, quantify, and store the topo58 according to **steps 26–29** of **Subheading 3.4.**

3.7. Modified Protocol for Purification of HeLa topo I

Native htopo I can be purified from suspension cultured HeLa S3 (ATCC # CCL 2.2) cells using the following modified version of the protocol of **Subheading 3.4.**

1. The starting material for purification of native topo I is 3×10^9 HeLa S3 cells that were doubling every 20–24 h in S-MEM (Gibco/BRL) supplemented with 10% fetal calf serum, 100 U/mL of penicillin, 100 µg/mL of streptomycin, and 50 U/mL of nystatin
2. Carry out **steps 1–16** of **Subheading 3.4.** The initial steps in purification of HeLa topo I, up to the point of isolating the clarified dialyzed PEG supernatant, are identical to that described above for the recombinant enzyme.

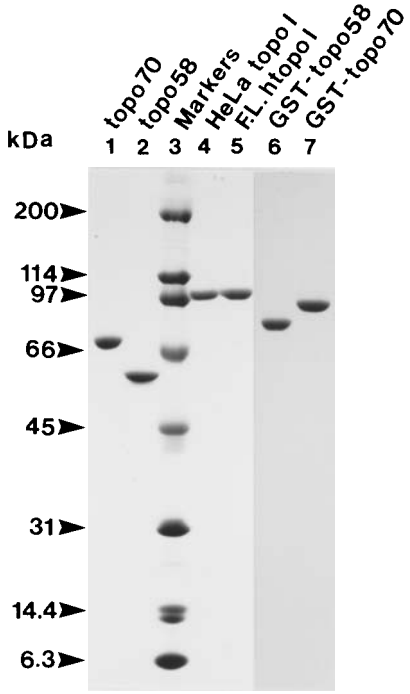

Fig. 2. Purified proteins. Purified proteins (5 μg each) were fractionated by 9–17% SDS-PAGE and visualized by Coomassie blue staining. Lane 1, Y723F topo70. Lane 2, topo58. Lane 3 contained molecular mass markers (Bio-Rad) myosin (200 kDa), β-galactosidase (114 kDa), phosphoylase b (97 kDa), BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), lysozyme (14.4 kDa), and aprotinin (6.3 kDa). Lane 4, HeLa topo I. Lane 5, Y723F full-length topo I. Lane 6, GST-topo58. Lane 7, GST-topo70.

3. Dilute the dialyzed PEG supernatant with an equal volume of water, and filter through a 0.45-μm filter.
4. Load (3 mL/min) the filtrate onto a POROS SP20 (4.6 mm R/100 mm L) column that has been equilibrated with cation-exchange buffer with 100 m*M* NaCl (9:1 mix of cation-exchange buffers A:B). After washing with 10 mL of the equilibration buffer, elute the SP20 column with a 30-mL linear salt gradient (1 mL/min) from 100–800 m*M* NaCl (from 9:1 to 2:8 cation-exchange buffer A:B mix).
5. Perform plasmid relaxation assays to identify the peak htopo I fractions. The htopo I elutes from the SP20 column at ~700 m*M* NaCl.
6. Pool the peak fractions, and dialyze against 2 L of PPB.
7. Pass the dialyzed SP20 pool over PS according to **step 17** of **Subheading 3.4.**
8. Dilute the PS flowthrough with an equal volume of water.
9. Carry out Mono-S chromatography according to **step 22** of **Subheading 3.4.**

10. Pool the peak Mono-S fractions, and dialyze into storage buffer.
11. Concentrate, dialyze, quantify, and store the HeLa topo I according to **steps 26–29** of **Subheading 3.4.**

3.8. Modified Protocol for Purification GST–htopo I Fusion Proteins

We have generated recombinant baculoviruses that express N-terminal GST fusions of wild-type topo70 (GST-topo70), active-site mutant topo70 (GST-topo70 Y723F), and topo58 (GST-topo58) ***(9)***. Each of the GST fusions are purified according to the following modified version of the protocol of **Subheading 3.4.**

1. Carry out **steps 1–16** of **Subheading 3.4.** Therefore, the initial steps in purification of the GST fusions, up to the point of isolating the clarified dialyzed PEG supernatant, are identical to those described above for the full-length enzyme.
2. Load the clarified dialyzed PEG supernatant onto a 5-mL bed volume column of glutathione Sepharose 4B that has been equilibrated with PPB. After washing with 15 mL of PPB, elute the column with 10 mL of PPB containing 5 m*M* reduced glutathione.
3. Dialyze the eluate against 2 L of K100.
4. Filter the dialyzed eluate through a 0.22-µm syringe filter.
5. Perform Mono-Q and Mono-S chromatography according to **steps 21** and **22** of **Subheading 3.4.** The GST-topo70 elutes at ~150 m*M* potassium phosphate, whereas the GST-topo58 elutes as two distinct peaks at ~150 and 170 m*M* potassium phosphate.
6. Pool the peak fractions.
7. Concentrate, dialyze, quantify, and store the GST fusions according to **steps 26–29** of **Subheading 3.4.**

4. Notes

1. The first ~200 residues of htopo I are extremely sensitive to proteolysis. Consequently, both PMSF and aprotinin are included during the initial stages of purification to prevent proteolysis of the full-length protein (**steps 3–8** of **Subheading 3.4.**). However, to reduce cost, the aprotinin can be excluded from these purification steps when the N-terminally truncated topo70, topo58 (**Subheading 3.6.**), or GST-fused versions of htopo I are being prepared (**Subheading 3.7.**).
2. Rather than being washed and re-equilibrated between uses, the PC and PS matrices are discarded after a single use, since they are relatively inexpensive. Furthermore, some proteins bind irreversibly to PS, making its repeated use undesirable.
3. If a salt gradient is applied to the Mono-Q column, the contaminating proteins elute in tight uniform peaks, whereas the small amount of remaining htopo I (<1%) elutes in a very broad peak from 100–400 m*M* potassium phosphate. This htopo I does not differ in its activity from that which flows through Mono-Q.

Furthermore, if the Mono-Q flowthrough is reapplied to the Mono-Q a second, third, or even a fourth time, a small amount of htopo I will invariably bind to the matrix. Since the physical basis for the low-level interaction of htopo I with Mono-Q is not understood, and since that which binds to mono-Q is only a small fraction of the total, it is not included in further purification steps.

4. Often the Mono-S column will become overloaded during the first pass of the Mono-Q flowthrough containing recombinant htopo I. If this occurs, excess htopo I will flow through the column. In addition, some of the bound htopo I will be displaced from the Mono-S by incoming contaminant proteins that bind to Mono-S with a higher affinity. In fact, the htopo I that is displaced in this manner is very pure. Any htopo I that flows through on the first loading is rechromatographed on a fresh column until all of it has been eluted from the Mono-S with a salt gradient
5. The POROS SP20 chromatography (**step 24** of **Subheading 3.4.**) of recombinant htopo I serves to remove only very small traces of remaining contaminants, and for most purposes, this step could be eliminated.
6. Centrifugal concentration devices should not be used to concentrate htopo I, since they can generate concentration gradients (from high at the bottom to low at top) that can lead to precipitation of the protein. This is especially true for the less-soluble N-terminally deleted forms of htopo I ***(2).***

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24

Rapid Purification of DNA Topoisomerase II Containing a Hexahistidine Tag by Metal Ion Affinity Chromatography

Harald Biersack, Sanne Jensen, and Ole Westergaard

1. Introduction

Since DNA topoisomerases have become a major focus for scientists, the purification of these proteins from all sources represents one of the basic hurdles on the way to investigating this important class of enzymes. A variety of methods consisting of numerous steps have been applied to obtain a reasonable amount of pure enzyme ***(1–4)***.

The recent advantage of expressing recombinant topoisomerases to higher levels in *Saccharomyces cerevisiae* ***(2,5)*** has further accentuated the requirement for a rapid and easy purification procedure. This led to the idea of using metal chelating chromatography as a single-step purification to obtain recombinant protein of more than 80% purity.

In 1975, immobilized metal chelate affinity chromatography was introduced for the first time to purify proteins ***(6)***. During the 1980s, this purification method taking advantage of the high affinity of histidine residues for metal ions was established and became a widely used technique. The first matrices, where iminodiacetic acid (IDA) ***(7,8)*** was used as the chelating ligand, were usually charged with nickel, copper, or zinc ions. In the late 1980s, a nitrilo-triacetic acid resin (Quiagen Ni-NTA) was introduced ***(9)***. In this resin, the metal ion is held by four chelating sites, resulting in a stronger binding to the matrix compared to the former matrices containing only three sites (*see* **Fig. 1**). Thus, the binding of the histidine-tagged protein to the Ni-NTA matrix is more efficient, and there is less release of heavy metal ions from the column.

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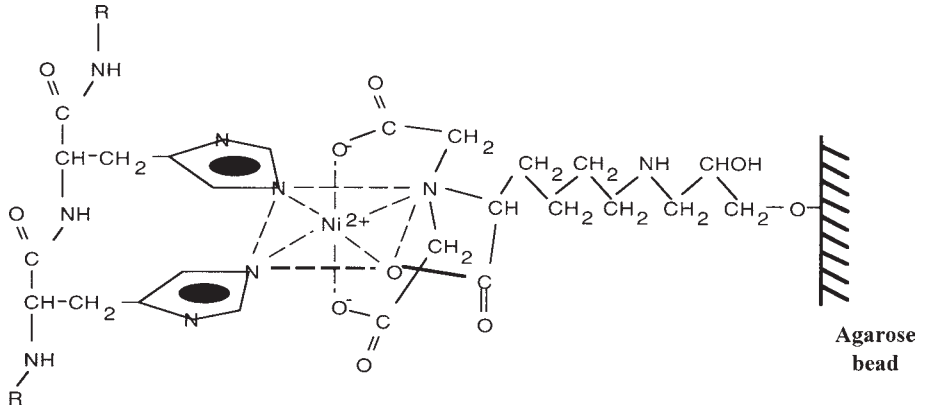

Fig. 1. Interaction between hexahistidine tag and Ni-NTA resin. Four chelating sites interact with the metal ion. Two of the six ligand binding sites are available for the interaction with the hexahistidine-tagged protein. The figure is reproduced with the kind permission of Diagen GmbH.

The binding of nontagged proteins to the column material can be reduced considerably by using buffers containing high salt (up to 1 *M* NaCl) and/or glycerol (up to 30%). Copurification of other proteins, which might have formed disulfide bonds to the tagged proteins, can be avoided by the addition of β-mercaptoethanol (up to 10 m*M*) to all buffers. The strong binding of the tagged proteins allows purification under native as well as denaturing conditions ***(10)***. Agents, such as guanidine hydrochloride (up to 6 *M*) and urea (up to 8 *M*), do not influence the binding properties of the tagged protein to the matrix. The binding capacity of most of the commercially available nickel matrices ranges from 5–10 mg histidine-tagged protein/mL of resin.

Proteins bound to the resin can be eluted in several ways:

1. For purification of topoisomerase II (topo II), we have employed an imidazole gradient. The structure of imidazole at its binding site is so similar to histidine that the tagged proteins can be competitively removed by imidazole. The presence of imidazole in the purified enzyme sample does not affect the activity of the enzyme;
2. In cases where elution is achieved by a decreasing pH gradient, the low pH of the eluted sample containing topoisomerase protein can influence its catalytic activity; and
3. It is also possible to strip the column of the Ni^{2+} ions by the chelating agent EDTA. However, under these conditions, the eluted protein is highly contaminated with heavy metal ions.

In principle, the hexahistidine tag can be cloned to either the N-terminus or the C-terminus of the protein of interest. For many purified enzymes, it has been described that addition of the hexahistidine tail at either end of the protein does not influence the enzymatic activity. In our case, the affinity tag was fused to the C-terminal end of topo II from various origins, resulting in enzymes that possess normal catalytic activities.

A number of expression vectors, containing a hexahistidine sequence in the polylinker region, are available nowadays for expression in yeast, *Escherichia coli*, or baculovirus. Our topo II constructs were originally cloned by introducing the *TOP2* cDNAs into a modified version of the *LEU2*/ARS-CEN plasmid pRS315 ***(11)***. In these constructs, topo II is expressed behind the constitutive yeast *t*riose *p*hosphate *i*somerase *(TPI)* promoter. A bicomposite tag consisting of a c-*myc* epitope and a hexahistidine tail was fused to the 3'-end of the *TOP2* cDNA using PCR. In addition, constructs were made containing topo II under the control of a galactose-inducible promoter on a multicopy plasmid with the selection markers *LEU2* or *URA3* (R&D Systems).

The purification of histidine-tagged topo II is a fast and well-reproducible method resulting in active enzyme. However, the technique does not lead to an ultrapure sample in the way it is described here, and it is therefore often necessary to combine it with either an ammonium sulfate precipitation or another chromatographic step. For further purification and/or concentration of the material from the nickel column, it can successfully be submitted to an ion-exchange (e.g., Source S) or a heparin sepharose column after appropriate dilution.

A further advantage of the affinity chromatography technique is its applicability as an assay for protein–protein interaction, where one of the proteins of interest is histidine tagged ***(12,13)***. The described assay has already been applied in our laboratory to investigate the dimerization of human topo II subunits ***(14)***.

2. Materials

1. Yeast cells overexpressing recombinant topo II carrying a histidine tag (*see* **Notes 1** and **2**).
2. Extraction buffer: 50 m*M* Tris-HCl, pH 7.8, 1 *M* NaCl.
3. PMSF, 100 m*M* stock (should be made fresh each time to reduce protein degradation) (*see* **Note 13**).
4. Glass beads (425–600 μm, acid-washed).
5. Bead beater (optional) or vortex unit where several 50-mL tubes can be mounted on.
6. Buffer A: 1 *M* NaCl, 10 m*M* phosphate buffer, pH 8, 10 m*M* β-mercaptoethanol, 10% glycerol.
7. Buffer B: Buffer A + 250 m*M* imidazole-HCl, pH 8.

8. Buffer C (5X): 1 *M* NaCl, 50 m*M* phosphate buffer, pH 8, 50% glycerol, 100 m*M* imidazole-HCl, pH 8.
9. Nickel matrix (Ni-NTA, Quiagen [Chatsworth, CA] or equivalent) (*see* **Notes 9** and **12**).
10. Empty columns ranging from 2–10 mL (optional HR 5/10 or HR10/10) (Pharmacia, Uppsala, Sweden).
11. FPLC system (optional) (Pharmacia).
12. Nitrocellulose filter pore size 0.65 µm.

3. Methods

1. Collect cells from yeast cultures grown in selective media by centrifugation and extract the yeast cells according to the following procedure.
   a. For large-scale extraction, it is recommended to use the bead beater (Biospec Products Inc.). To 1 vol of cells add 1 vol glass beads and 2–4 vol extraction buffer containing a final concentration of 0.1 m*M* fresh PMSF. Do five times a 1-min burst interrupted by a 1-min pause on ice. Add fresh PMSF every 5 min to avoid protein degradation (*see* **Note 8**).
   b. For small-scale extraction, alternatively 50-mL conical tubes can be used. Add cells, glass beads and buffer in the same ratio as in (a). Vortex for 30 min at 4°C while adding fresh PMSF every 5 min. To achieve an optimal extraction, it is preferable that the tubes do not contain more than 30 mL.
2. For both procedures of extraction, remove the glass beads by centrifugation for 10 min at 4000*g*, transfer the supernatant to 30-mL corex tubes, and spin for an additional 30 min at 15,000*g*.
3. Filter the supernatant through a 0.65-µm filter. Save a sample of extract as a control for expression.
4. In parallel, prepare a nickel column (optional: an HR 5/10 column containing 2 mL or an HR 10/10 column containing 8–10 mL matrix) (*see* **Note 11**). Equilibrate the column with 5–10 vol of buffers A and B using 8% buffer B corresponding to 20 m*M* imidazole (*see* **Notes 4** and **10**).
5. During loading, it is recommended to avoid binding of other undesirable proteins exhibiting a lower affinity to the matrix, instead of binding these to the column and removing them in the following wash. Therefore, mix the filtered extract in a 4:1 ratio with buffer C to have a final concentration of 20 m*M* imidazole in the sample. This will result in more binding sites for the target protein during loading and less background caused by other proteins (*see* **Notes 3–14**).
6. Load the filtered diluted extract to the equilibrated column with a flow rate between 0.1 and 1 mL/min depending on the column size. The interaction between the Ni^{2+}-resin and histidine-tagged proteins is not taking place as fast as the binding to e.g., an ion-exchange matrix. Thus, a low flow rate is recommended. If using an FPLC system, the loading can be done overnight.

7. After loading, wash the column with approx 5–10 vol of equilibration buffer (A + 8% B) (*see* **Note 10**). If using an FPLC system, wash until a steady baseline is reached.
8. Elute the column with a gradient from 8–100% buffer B (20–250 m*M* imidazole) in 15–70 mL depending on the column size. The vast majority of topoisomerase II will be eluted in the first half of the gradient at approx 50–100 m*M* imidazole.
9. Save samples from run-through, wash, and gradient for SDS gel analysis followed by Coomassie staining or immunostaining. **Figure 2** shows the results of a purification of histidine-tagged human topoisomerase IIβ.
10. For storage of the fractions of interest, the concentration of glycerol should be adjusted to 50%, after which the enzymes can be stored at −20°.

4. Notes

As a guideline for troubleshooting, we have listed a number of problems, that might occur when the nickel column is used for purification of recombinant topo II containing a hexahistidine sequence. Further complications can arise in general when using this purification method for other types of recombinant protein. In these cases, it is recommended to refer to manuals delivered with the resin.

4.1. If Histidine-Tagged Topoisomerase II Does Not Bind to the Metal Chelating Resin or Appears in the Wash

1. Check by sequencing that the histidine tag is intact.
2. The histidine tag might be hidden owing to folding under native conditions. In the case of topo II, the affinity tag is well presented when fused to the C-terminal region. However, the histidine tail might not be sufficiently exposed when cloned to the more compact N-terminal part of the enzyme. Try under denaturing conditions, or move the tag to the other end of the protein.
3. Check composition and pH of all buffers. The pH determines the binding efficiency of the tagged protein to the resin. With respect to topo II, a pH between 7.0 and 8.0 is recommended.
4. The concentration of imidazole during loading and wash should not exceed 30 m*M*; otherwise the stringency will be too high.
5. No chelating agents (EDTA, EGTA) should be present during purification. Even the lowest concentration of these compounds is able to strip the Ni^{2+} ions from the column.
6. Eliminate reducing agents, such as DTT and DTE, during the procedure, since they reduce the Ni^{2+} ions dissociating them from the resin.
7. Higher concentrations of β-mercaptoethanol (>10 m*M*) should be avoided during purification.

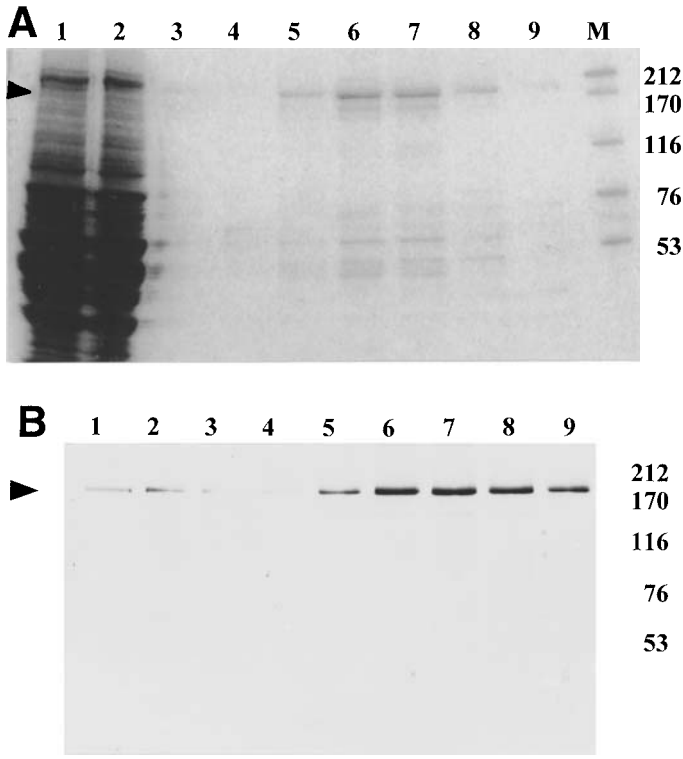

Fig. 2. Purification of full-length human DNA topoisomerase IIβ expressed in yeast. The recombinant enzyme is expressed from a multicopy plasmid carrying the selectable marker *LEU2* and the *TOP2* β cDNA under the control of a galactose-inducible promoter. After growth for 2 d and induction for 16 h the cells from 3-L culture were harvested and extracted according to **Subheading 3.** Extract was loaded on a 10-mL Ni-NTA column, and following wash, the column was developed in a 70-mL gradient. Five-milliliter fractions were collected during the elution step. **(A)** The samples were analyzed on a 4–20% SDS-PAGE gradient gel followed by Coomassie blue staining. Lanes: 1, extract in a 1:5 dilution; 2, run-through in a 1:5 dilution; 3, wash; 4–9, first part of the gradient containing topoisomerase IIβ. Lane M contains protein markers with the molecular masses indicated in kilodaltons on the right margin (Pharmacia HMW). The position of topoisomerase IIβ is indicated by an arrowhead. Lanes 6 and 7, each containing 8 µg protein, represent the peak fractions of the gradient. **(B)** Immunostaining of fractions from A run on a similar gel and transferred to nitrocellulose membrane. The antibody used is the commercially available MYC1-9E10.2 recognizing the human c-*myc* epitope, which has been fused to the C-terminal end of the protein together with the hexahistidine sequence. Similar results have been obtained using anti-topoisomerase IIβ antibodies. From the immunostaining, it is clear that a complete binding cannot be achieved, since the run-through fraction always contains a certain amount of topoisomerase IIβ. However, the vast majority of the enzyme is present in the gradient.

8. Use of the less toxic protease inhibitor Pefabloc instead of PMSF prevents any binding to the column matrix.
9. The column material should not be reused more than three to four times.

4.2. If Contaminating Proteins Appear Together with Topoisomerase II in the Elution

10. The stringency during loading and wash must not be too low. Binding of undesirable proteins with lower affinity to the matrix occurs if <15 m*M* imidazole is present.
11. Make sure that the ratio between column size and volume of extract correlates to avoid additional binding sites for unspecific binding proteins.
12. In our hands, less contamination appears when the column material has already been used once. However, the material should not be reused more than three to four times.
13. Check by immunostaining whether the contamination is a degradation product of Topo II containing the histidine tag. Degradation can result if PMSF is not added as described above or is not prepared fresh.
14. Increase the concentrations of salt, glycerol, and β-mercaptoethanol to the maximum level (1 *M*, 30%, and 10 m*M*, respectively) to reduce unspecific binding.

Acknowledgments

We are grateful to Yong Wang for his skillful help. This work was supported by the Danish Cancer Society grant 93-004 and 78-5000, the Danish Center for Human Genome Research, the Danish Natural Science Research Council, grant no. 11-0972, and the Danish Center for Molecular Gerontology. H. B. was supported by the Deutsche Forschungsgemeinschaft (Bi 541/1-1).

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Metabolic Labeling, Immunoprecipitation, and Two-Dimensional Tryptic Phosphopeptide Mapping of Human Topoisomerase II

Nicholas J. Wells and Ian D. Hickson

1. Introduction

Protein phosphorylation is almost certainly the most important posttranslational mechanism of enzyme regulation in eukaryotic cells (reviewed in ***1***). The equilibrium between phosphorylation by protein kinases and dephosphorylation by protein phosphatases modulates the activity, subcellular localization, or DNA/RNA/protein binding properties of numerous proteins. Indeed, it appears that the majority of intracellular proteins in human cells are phosphorylated to some degree under certain conditions of cell growth. However, interest in protein phosphorylation is more generally directed toward a study of the alterations in phosphorylation status that either accompany a change in cell physiology or are invoked by exposure to an extracellular stimulus (reviewed in ***2***).

Studies on the phosphorylation state of cellular proteins generally involve the combined use of metabolic labeling of the protein of interest with radioactive phosphate (almost always ^{32}P-orthophosphate), purification of the protein by immunoprecipitation, and an analysis of the sites of phosphorylation by phosphopeptide mapping (reviewed in ***3***,***4***). In general, the procedures for metabolic labeling of proteins with ^{32}P-orthophosphate (or with ^{35}S-methionine to detect total protein levels) are relatively straightforward, but require rigorous attention to the safety of personnel, since the use of 5–10 mCi of ^{32}P-orthophosphate is somewhat common ***(4)***. We use 1-cm thick perspex boxes to hold all ^{32}P-labeled material and work behind a 1-cm thick perspex screen. However, certain pitfalls inherent in the procedure do present them-

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selves, in particular, the tendency for the phosphate-depleted medium used for maximizing uptake of radiolabeled orthophosphate to inhibit cell proliferation. This is obviously a serious limitation for studies in which the continued progress of cells through a particular phase of the cell division cycle is a requirement. It may be necessary to titrate down the amount of "cold" phosphate in the culture medium to a level that facilitates uptake of sufficient ^{32}P-orthophosphate into cells, without preventing a near-normal rate of cell-cycle progression. Some cell lines are also particularly susceptible to the damage caused by ^{32}P-induced radiation. In such cases, it may be necessary to use very short labeling periods with high concentrations of orthophosphate in order to minimize losses owing to cell death.

In order to purify the protein of interest away from all other phosphoproteins following metabolic labeling, it is almost always necessary to immunoprecipitate the antigen. Although the extensive use of epitope tagging (fusion to a recombinant protein of a short peptide epitope that is recognized by an available antibody) has reduced the need to raise antibodies in certain cases, many procedures still require an antibody that is specific for the protein of interest (in our case, DNA topoisomerase II). In order to carry out some of the procedures outlined in this chapter, there is a requirement for an antibody that can immunoprecipitate an antigen efficiently, extracting a substantial fraction of the total topoisomerase II present in the cell nuclei. Although the recovery of 0.1% (or less) of an antigen may be sufficient in those cases where the immunoprecipitate is subjected to subsequent Western blotting, yields of 10–50% of the total antigen may be required for certain phosphorylation analyses or where the antigen is of a very low abundance in cells.

Determining whether a particular protein is phosphorylated in a given cell line is usually only the first step in the analysis of a phosphoprotein. Many proteins are phosphorylated on more than one residue (serine, threonine, or tyrosine) and at multiple sites throughout the protein. As a result, it is usually necessary to digest the protein into small fragments using trypsin or a similar protease in order to analyze phosphorylation at a particular site (*see* **ref. *3*** for a discussion of reagents available for cleavage of proteins).

The phosphopeptide mapping procedure utilizes immunoprecipitated antigen for the identification of phosphorylated residues in a protein and relies on an ability to detect very small quantities of a phosphopeptide by autoradiography. The procedure requires, therefore, that the isotope used (usually either ^{32}P-orthophosphate for metabolic labeling of cultured cells, or [γ-^{32}P]-ATP for in vitro phosphorylation reactions) is of a very high specific activity. In general, depending on the number of phosphorylation sites in a given protein, the final product used for two-dimensional (2-D) peptide separation should have an activity in excess of 250 cpm/sample loaded. Little progress has been

made in utilizing other isotopes for metabolic labeling of phosphoproteins, such as ^{33}P, which would be attractive alternatives on personal safety grounds alone.

Identification of specific sites of phosphorylation can be achieved in several ways, including direct sequencing of phosphopeptides. This, however, requires the availability of a significant quantity of purified peptide. The advent of recombinant DNA technology has simplified the procedure for phosphorylation site mapping by allowing in vitro phosphorylated recombinant proteins (and site-specific mutant derivatives of these proteins) to be compared directly with in vivo labeled proteins.

Metabolic labeling, in conjunction with immunoprecipitation of the antigen, can also be used to investigate whether a given protein can form a stable complex with any other phosphoproteins. Coimmunoprecipitation has proven a highly valuable method for identifying functional protein:protein interactions in vivo, including those between the SV40 large T-antigen and p53 ***(5)***, and between cyclin-dependent kinases and cell-cycle regulatory molecules, such as $p21^{CIP1/WAF1}$, which can inhibit the activity of these kinases ***(6)***.

2. Materials

2.1. Metabolic Labeling of Adherent HeLa Cells

1. 10 mCi/mL carrier-free [^{32}P]-orthophosphate (e.g., from Life Sciences, Amersham, UK).
2. Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 3 m*M* L-glutamine and 5–10% fetal bovine serum (FBS).
3. Phosphate-free DBEM, supplemented as above.
4. Dulbecco's phosphate-buffered saline (PBS).

2.2. Cell Synchronization Studies

1. This requires thymidine stock solution of 200 m*M* in distilled water (dH_2O), filter-sterilized.

2.3. Flow Cytometry

1. PBS.
2. Ice-cold 70% ethanol/30% PBS.
3. RNase A stock solution at 10 mg/mL in dH_2O.
4. Propidium iodide stock solution of 4 mg/mL in dH_20.

2.4. Immunoprecipitation of Topoisomerase IIα

2.4.1. Preparation of Nuclear Extracts

1. Nuclear isolation buffer (NIB): 30 m*M* Tris-HCl, pH 7.5, 1.5 m*M* $MgCl_2$, 10 m*M* KCl, 20% (v/v) glycerol.
2. Tris-buffered saline (TBS): 20 m*M* Tris-HCl, pH 7.5, 0.9% (w/v) NaCl.
3. Triton X-100.

4. 5*M* NaCl stock.
5. Cell lifter/scraper (e.g., Costar, Cambridge, MA, cat. no. 3008).
6. Stock solutions of 100 m*M* phenylmethylsulfonyl fluoride (PMSF), 2mg/mL leupeptin, 1 mg/mL aprotinin, 1 mg/mL pepstatin A, 1 mg/mL soybean trypsin inhibitor, 1 m*M* benzamidine, 1 mg/mL antipain; 50 mg/mL L-1-chloro-3-(4-tosylamido)-7-amino-2-heptanone hydrochloride (TLCK), 0.1 m*M* β-glycerophosphate; 100 m*M* *p*-nitrophenyl phosphate, 500 m*M* glucose-1-phosphate, 10 m*M* microcystin, 200 m*M* sodium orthovanadate, 1*M* sodium fluoride (e.g., from Sigma, St. Louis, MO or Boehringer Mannheim, Germany).

2.4.2. Immunoprecipitation Reactions

1. Immunoprecipitation buffer (IPB): 100 m*M* Tris-HCl, pH 8.0, 500 m*M* NaCl 0.75% (v/v) Triton X-100, 10 m*M* EDTA, 0.02% (w/v) NaN_3.
2. An isoform-specific antiserum (e.g., from Cambridge Research Biochemicals, Cambridge, UK).
3. Cyanogen bromide-activated protein A sepharose (e.g., from Sigma, St. Louis, MO).
4. Stock solution of 20% SDS in dH_2O.

2.5. Tryptic Phosphopeptide Mapping

1. Ammonium bicarbonate freshly prepared at 50 m*M* (e.g., from Sigma).
2. 30% Methanol.
3. L-1-Chloro-3-(4-tosylamido)-4-phenyl-2-butanone- (TPCK) treated trypsin at 1 mg/mL in 0.1 m*M* HCl (e.g., from Worthington Biochemical Corporation, Freehold, NJ USA).

3. Methods

3.1. Metabolic Labeling of Adherent HeLa Cells

3.1.1. Asynchronous Cultures

1. Exponentially growing HeLa cells (*see* **Notes 1** and **2**) are washed in PBS, transferred to phosphate-free DMEM supplemented with 3 m*M* L-glutamine and 5% (v/v) normal FBS, and are then exposed to [^{32}P]orthophosphate (to a final concentration of approx 100 μCi/mL).
2. Return cultures to a humidifed 37°C incubator for 3–14 h.

3.1.2. Synchronous Cultures

1. Add 2 m*M* thymidine to growth media of exponentially growing HeLa cell cultures (*see* **Notes 1** and **2**).
2. Incubate for 14 h.
3. Aspirate media, and wash cell monolayer with PBS (*see* **Note 3**). Repeat this step to ensure complete removal of thymidine.
4. Release cells into fresh media, and incubate for 11 h.

5. Reapply thymidine at 2 m*M* to the cell cultures, and incubate for a further 15 h.
6. Release cells into fresh growth media following removal of thymidine, as described in **step 3**, to enable cells to continue cell-cycle progression (*see* **Note 2**).
7. Following a 7–8 h incubation, change media and add radiolabel as described in **Subheading 3.1.1.**, **step 1**.
8. Examine an identically treated cell culture into which no label has been added in order to observe synchronous entry into M phase. In the case of HeLa cells, this occurs approx 1–3 h after addition of the phosphate-free medium. The cells in these control dishes may be harvested for flow cytometry if required (as discussed in **Subheading 3.2.**).

3.2. Flow Cytometry

1. Remove cells from culture dishes using PBS containing 0.5 m*M* EDTA and trypsin (or an equivalent method), and harvest by centrifugation.
2. Fix the cells for 30 min in ice-cold 70% ethanol/30% PBS; harvest by centrifugation.
3. Resuspend cells in PBS containing 100 µg/mL RNase A and 40 µg/mL propidium iodide. Incubate suspension at 37°C for 30 min.
4. Cell-cycle distribution may then be determined by flow cytometry, e.g., using a FACScan (Becton-Dickinson, Oxford, UK). A typical example is shown in **Fig. 1**.

3.3. Immunoprecipitation of Topoisomerase IIα

3.3.1. Preparation of Nuclear Extracts (Based on Glisson et al. [7])

1. All procedures from this point should be carried out at 4°C with the inclusion of all protease and phosphatase inhibitors (*see* **Note 4**). Remove media and add 2 mL TBS (per 9-cm dish) containing 1 m*M* EDTA. Harvest the cell monolayer by scraping with a cell lifter.
2. Centrifuge cell suspension at 1000*g* for 2 min before washing in harvesting buffer.
3. Resuspend cell pellet in 0.36 mL NIB to which 40 µL of 10% (v/v) Triton X-100 are added. Mix the suspension, and incubate on ice for 5 min (*see* **Note 5**).
4. Pellet nuclei by centrifugation at 1000*g* for 90 s, and then resuspend the pellet in 0.36 mL NIB containing 0.35*M* NaCl. Incubate on ice for 30 min (*see* **Note 6**).
5. Centrifuge samples at 10,000*g* for 5 min to remove cellular debris. Retain supernatant for immunoprecipitation of topoisomerase IIα.

3.3.2. Immunoprecipitation Reactions

1. Preclear nuclear extracts by incubation with 0.1 vol of 50% preswelled protein A-sepharose beads on a rotating wheel at 4°C for 60 min.
2. Add precleared nuclear extracts to an equal volume of IPB containing the antihuman topoisomerase IIα specific antibody, e.g., CRB, at a dilution of 1:40 ***(8)***. Incubate on ice for 1–2 h.
3. Add 0.1 vol of 50% preswelled protein A-Sepharose beads, and place the mixture on a rotating wheel at 4°C for 1–2 h.

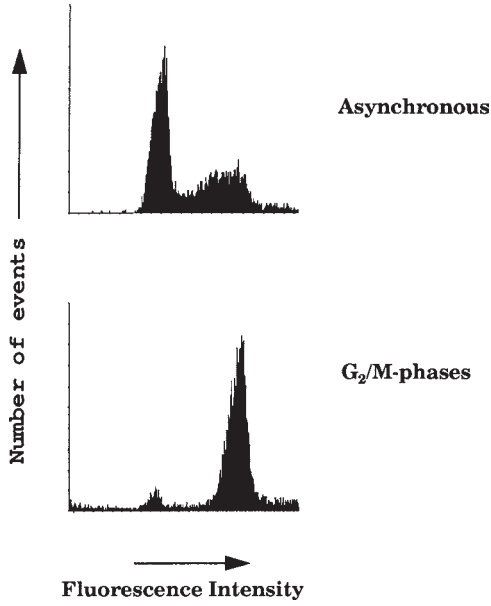

Fig. 1. Flow cytometric analysis of asynchronous and G2/M phase-enriched HeLa cell populations was determined on a Becton-Dickinson FACScan using propidium-iodide-stained cells. The G2/M phase-enriched sample contained 91% cells with a 4n DNA content.

4. Harvest the beads by centrifugation at 10,000*g* for 10 s and wash three times in IPB, containing 0.1% (w/v) SDS, before a final wash in 10 m*M* Tris-HCl, pH 7.5.
5. Resuspend the immunoprecipitates in 30 µL 2X SDS sample buffer, and heat at 98°C for 3 min. Resolve by electrophoresis on a standard 7.5% SDS-polyacrylamide gel.
6. Dry gel onto Whatman 3MM filter paper using a heated vacuum gel dryer.
7. Detect immunoprecipitated human topoisomerase IIα by autoradiography (if cells were initially metabolically labeled). A typical example is shown in **Fig. 2**.

3.4. Preparation of Samples for Two-Dimensional Tryptic Phosphopeptide Mapping

Two protocols may be followed for the generation of tryptic phosphopeptide maps. van der Geer et al. ***(3)*** summarize the approach pioneered by Hunter (*see* **Note 7**). However, an alternative method, outlined by Morgan et al. ***(9)***, may also be utilized.

1. Utilize autoradiography (and Stratagene Glogos™ Autorad Markers) to locate the radiolabeled protein bands, and excise relevant area of dried gel.

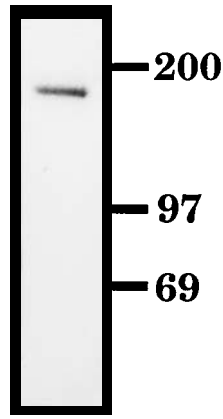

Fig. 2. Immunoprecipitation of human topoisomerase IIα protein from HeLa cell nuclei. The topoisomerase IIa protein was immunoprecipitated from cells metabolically labeled with ^{32}P-orthophosphate, and the immunoprecipitate was run on a 7.5% SDS-polyacrylamide gel. Radiolabeled proteins were detected by autoradiography. The sizes of mol-wt standards (in kDa) are shown on the right. The single 170 kDa phosphoprotein is topoisomerase IIα.

2. Rehydrate gel for 5 min in 30% methanol, and wash the gel twice for 15 min each in fresh 50 m*M* ammonium bicarbonate.
3. The Whatman 3MM filter paper used in the drying procedure can be easiliy removed with forceps at this stage.
4. Add 1 mL of 50 m*M* ammonium bicarbonate to the rehydrated gel, before addition of 20 µL of 1 mg/mL TPCK-treated trypsin. Incubate at 37°C on a rotating wheel for 8 h.
5. Add an additional aliquot of trypsin as above. Continue incubation for another 8 h (or overnight).
6. Transfer ammonium bicarbonate to a fresh microcentrifuge tube.
7. Add 0.5 mL of 50 m*M* ammonium bicarbonate to the tube containing the rehydrated gel slice, and incubate on the rotating wheel for a further 2 h.
8. Pool the eluates and centrifuge at 10,000*g* for 10 min.
9. Transfer 90% to a fresh microcentrifuge tube avoiding any particulate matter
10. Lyophilize in a centrifugal evaporator (e.g., Savant Speedvac).
11. Resuspend in 1 mL distilled water, centrifuge as before, and transfer to a fresh microcentrifuge tube being careful to avoid particulate matter. Lyophilize.
12. Resuspend sample in 500 µL distilled water and lyophilize.
13. Resuspend sample in 100 µL electrophoretic buffer of choice, and lyophilize once again.
14. *See* van der Geer et al. ***(3)*** and Woodgett ***(4)*** for a detailed description of the two-dimensional separation of phosphopeptides by electrophoresis and chromatogra-

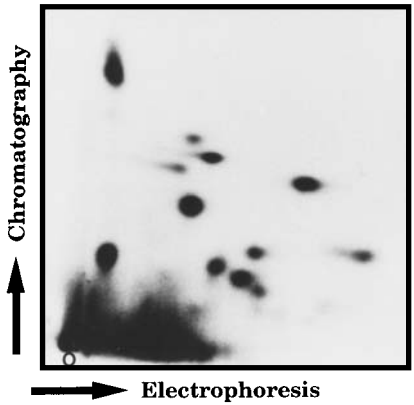

Fig. 3. Analysis of phosphopeptides on thin-layer cellulose plates. Phosphopeptides were separated in the horizontal dimension by electrophoresis at pH 1.9 and in the vertical dimension by chromatography. The position of the origin (O) is indicated. The radiolabeled peptides were detected by autoradiography.

phy on cellulose thin-layer chromatography plates. A typical example is shown in **Fig. 3** (*see* **Notes 8** and **9**).

4. Notes

1. Adherent cell cultures must be labeled at subconfluence in order to prevent downregulation of topoisomerase IIα expression through contact inhibition of cell proliferation.
2. The use of phosphate-free growth media in combination with dialyzed, low-phosphate, FBS for metabolic labeling cells is not advised, since it can inhibit cell proliferation. If phosphate-free medium is employed, the addition of 5% regular FBS prevents this problem from arising.
3. Cells may be washed in either TBS or PBS, although phosphate buffers should be avoided in metabolic labeling studies.
4. All procedures should be carried out at 4°C, with the inclusion of all the following protease and phosphatase inhibitors: 1 m*M* PMSF, 2 µg/mL leupeptin, 1 µg/mL aprotinin, 1 µg/mL pepstatin A, 1 µg/mL soybean trypsin inhibitor, 1 m*M* benzamidine, 1 µg/mL antipain, 50 µg/mL TLCK, 0.1 m*M* β-glycerophosphate, 0.1 m*M* *p*-nitrophenyl phosphate, 0.5 m*M* glucose 1-phosphate, 10 n*M* microcystin, 1 m*M* sodium orthovanadate, and 1 m*M* sodium fluoride. The addition of microcystin has been demonstrated to protect the MPM-2 phospho-epitope, which is particulary sensitive to dephosphorylation ***(10)***.
5. The efficiency of cell lysis following Triton X-100 treatment should be assessed by microscopy prior to performing the immunoprecipitation.
6. For an analysis of nuclear antigens, such as topoisomerase II, it is generally preferable to work with nuclear rather than whole-cell extracts, since this partial

purification step removes a lot of "unwanted" protein that can adversely affect the quality of the subsequent two-dimensional phosphopeptide separation.

7. Comparison of the methods for 2-D tryptic phosphopeptide mapping described by Morgan et al. ***(9)*** and van der Geer et al. ***(3)*** indicates that the former method leads to the persistence of a proportion of radiolabeled material that fails to migrate on the thin-layer plate during chromatographic resolution. Therefore, this material smears horizontally on the plate and may obscure phosphopeptides that lie close to the origin.

   A second major difference between the two methods is that complete oxidation of methionine and cysteine residues occurs during the course of processing samples using the method described by van der Geer et al. ***(3)***. This ensures that only a single radiolabeled spot is obtained in cases where a cysteine or methionine residue is present in the peptide. In the absence of full oxidation, multiple spots representing the same peptide displaying different oxidation states can be obtained. Therefore, this protocol is recommended if candidate phosphopeptides are likely to contain either methionine or cysteine residues
8. Interpretation of 2-D tryptic phosphopeptide maps is complicated by the observation that trypsin is a poor exopeptidase. Therefore, two or more phosphopeptides may arise via alternative cleavage around a single phosphorylated residue. This occurs particularly at positions where two or more basic residues are adjacent in the primary sequence ***(11)***. For example, tryptic cleavage around the mitotic phospho-acceptor residues Ser^{29} and Ser^{1212} (residue numbers taken from Tsai-Pflugfelder et al. ***[12]***), of human topoisomerase IIα, yields two phosphopeptides in each case ***(13,14)***.

   A second difficulty that can arise is that phosphorylated residues can inhibit recognition of adjacent potential cleavage sites by trypsin ***(15)***. The influence of phosphorylation on tryptic digestion has been observed in human topoisomerase IIα. Cleavage at sites of basic residues situated between the phospho-acceptor residues Ser^{1353} and Ser^{1360} is inhibited when these residues are phosphorylated ***(14)***.
9. The choice of electrophoresis buffer is dependent on the proportion of acidic and basic phosphopeptides present in the sample. Work in our laboratory indicates that the electrophoretic separation in pH 1.9 Buffer (50 mL formic acid [88% w/v], 156 mL glacial acetic acid, 1794 mL deionized water) produces acceptable resolution of tryptic phosphopeptides derived from both of the isoforms of human topoisomerase II.

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Immunoblot Analysis and Band Depletion Assays

Scott H. Kaufmann and Phyllis A. Svingen

1. Introduction

Western blotting has been widely utilized to detect various polypeptides or polypeptide epitopes (e.g., posttranslational modifications) within cells (reviewed in ***1–4***). If the signals in samples being analyzed are compared to a suitable standard curve and appropriate internal standards are utilized to confirm equivalent loading of various samples, Western blotting appears to be a suitable method of quantitating polypeptides as well.

A related approach can also be utilized to assess the formation of covalent topoisomerase (topo) DNA complexes in intact cells. As described in Chapter 1 of this volume, topoisomerases form transient covalent adducts (termed "cleavage complexes") with DNA. In simplistic terms, this interaction can be represented by the following equilibrium:

$$\text{Free topo} + \text{DNA} \rightleftharpoons \text{topo–DNA covalent complexes} \quad (1)$$

In this equation, the pool of "free topo" actually represents a complicated mixture that includes (1) polypeptide molecules that are not bound to DNA and (2) molecules bound noncovalently to DNA. What these molecules have in common is the fact that they will migrate at the subunit molecular weight of the topo molecule on SDS-polyacrylamide gels. In contrast, the covalent topo–DNA complexes are larger in size and will exhibit a lower mobility after denaturation.

In intact cells, there are few covalent topo–DNA complexes, and these complexes are probably short-lived. In other words, the equilibrium lies far to the left under ordinary conditions. Treatment of cells with certain antineoplastic agents, however, increases the number of covalent topo–DNA complexes (reviewed in ***5–7***). Specifically, the epipodophyllotoxins, aminoacridines, and

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antineoplastic quinolones increase the number of covalent adducts between mammalian topo II and DNA; and the camptothecin analogs increase the number of covalent adducts between mammalian topo I and DNA. In other words, these agents shift the equilibrium depicted in **Eq. 1** toward the right. As a consequence, if samples are rapidly denatured, fewer topo molecules are free to migrate at the subunit molecular weight on SDS-polyacrylamide gels after drug treatment. These considerations form the basis for the band depletion method described in this chapter.

Over the past 15 years, this band depletion assay has been employed for a number of purposes. Tricoli and Kowalski ***(8)*** initially utilized a band depletion approach to examine the DNA binding specificity of chicken erythrocyte topo I. In these experiments, the authors added increasing amounts of test DNA to purified topo I in an attempt to force the equilibrium to the right. They then terminated the reaction in 15% trichloroacetic acid and utilized Coomassie blue staining to assess the amount of topo I that remained free to migrate at M_r ~100,000 on SDS-polyacrylamide gels. In a later adaptation of this approach to intact cells, Hsiang et al. ***(9)*** treated cells with camptothecin and demonstrated that the signal for topo I at M_r ~100,000 was diminished, whereas the signal for topo IIα at M_r ~170,000 was unaltered. Zwelling and coworkers ***(10)*** subsequently utilized this approach to demonstrate that the stabilization of covalent topo IIα-DNA complexes required higher amsacrine concentrations in HL-60/AMSA cells, which contain a mutant topo IIα, than in parental HL-60 cells. Likewise, Hendricks et al. ***(11)*** utilized this approach to demonstrate that the stabilization of covalent topo I–DNA complexes in P-glycoprotein-expressing cells required higher extracellular topotecan concentrations than were required for the same complex stabilization in parental cells. These references illustrate the diversity of applications of this method as well as some of the factors that can potentially affect the band depletion assay in intact cells.

2. Materials

2.1. Standards

1. For quantitation of topoisomerases in untreated clinical samples, purified enzymes are available:
   - Purified topo I can be purchased from TopoGen (Columbus, OH) or Gibco/BRL (Gaithersburg, MD).
   - Purified topo IIα can be purchased from TopoGen.
2. It is also advisable to include a standard in the band depletion assay, e.g., a tissue-culture cell line that is treated in parallel with each sample. K562 human leukemia cells (available from American Type Culture Collection, Rockville, MD) are a suitable control line, because they are resistant to drug-induced apoptosis ***(12,13)*** but do not have any identified defect in drug accumulation.

2.2. Drugs for Stabilizing Topo–DNA Adducts (Required for Band Depletion Assay Only)

These agents can be prepared as concentrated stocks in dimethylsulfoxide and stored at −20°C. We find it convenient to prepare a stock that is 200-fold more concentrated than the highest desired concentration as well as several serial (two- or threefold) dilutions from these stocks.

1. Topo II-directed drugs: Etoposide can be purchased from Sigma Chemical Company (St. Louis, MO). Amsacrine is available from the Drug Synthesis Branch of the National Cancer Institute (Bethesda, MD).
2. Topo I-directed agents: Camptothecin can be purchased from Sigma Chemical Company. Topotecan and SN-38 (7-ethyl-10-hydroxycamptothecin) are available from SmithKline Beecham Pharmaceuticals (King of Prussia, PA) and the Upjohn Pharmacia Company (Kalamazoo, MI), respectively.

2.3. Buffers for Isolating Cells

1. Ficoll-Hypaque solution is available from various suppliers (e.g., Histopaque-1077 and Histopaque-1119 from Sigma).
2. RPMI-HEPES: RPMI 1640 medium, 10 m*M* HEPES, pH 7.4.

2.4. Buffers for Rapidly Denaturing Cells

1. Based on previously published results ***(14)***, we prefer 6 *M* guanidine hydrochloride containing 250 m*M* Tris-HCl (pH 8.5 at 21°C) and 10 m*M* EDTA. Immediately prior to use, each aliquot of this buffer is supplemented with 1% (v/v) β-mercaptoethanol (electrophoresis-grade, available from Bio-Rad, Richmond, CA) and 1 m*M* phenylmethylsulfonyl fluoride (available from Sigma). To avoid rapid hydrolysis ***(15)***, the latter compound is prepared as a 100 m*M* stock using isopropanol that has been dried over molecular sieves, which are available from Aldrich (Madison, WI).
2. Alternatively, cells can be rapidly denatured using an appropriate SDS-containing sample buffer. The one that we have utilized consists of 4 *M* urea (deionized over Bio-Rad AG1X-8 mixed-bed resin to remove charged breakdown products), 2% (w/v) electrophoresis grade SDS, 62.5 m*M* Tris-HCl (pH 6.8 at 21°C), and 1 m*M* EDTA.

2.5. Supplies for SDS-PAGE and Blotting

1. Paper support for transferring polypeptides:
   a. Nitrocellulose.
   b. Nylon (e.g., Genescreen from New England Nuclear, Boston, MA, or Nytran from Schleicher and Schuell, Keene, NH).
   c. Polyvinylidene fluoride (PVDF) (e.g., Immobilon-P, Millipore, Bedford, MA).
2. Fast green FCF (e.g., Aldrich, Madison, WI) for staining polypeptides after transfer to solid support.

3. Reagents for electrophoresis (acrylamide, bis-acrylamide, 2-mercaptoethanol, SDS) should be electrophoresis grade (e.g., Bio-Rad).
4. All other reagents (Tris, glycine, urea, methanol) are reagent grade.
5. Bicinchoninic acid for protein determination is available from Pierce (Rockford, IL).
6. Antibodies to topo I are available from TopoGen and Alpha Antigens (San Leandro, CA) (Scl-70 positive control).
7. Antibodies to topo IIα and topo IIβ are available from TopoGen and Cambridge Research Biologicals (Wilmington, DE).
8. Radiolabeled secondary antibodies and radiolabeled protein A are available from Amersham (Arlington Heights, IL) or DuPont/NEN (Boston, MA).
9. Enzyme-coupled secondary antibodies are available from multiple suppliers (e.g., peroxidase-coupled or alkaline phosphatase-coupled affinity-purified secondary antibodies from Kirkegaard and Perry, Gaithersburg, MD).
10. Chemicals for enhanced chemiluminescence using enzyme-coupled secondary antibodies and luminescent substrates are available from a variety of suppliers (e.g., ECL from Amersham; Western-Light from Tropix, Bedford, MA). These chemiluminescent substrates appear to yield a signal that is easier to quantify than the chromogenic substrates that are precipitated on blots as a consequence of enzyme action.
11. Blocking solution, e.g., 10% (w/v) powdered nonfat milk, 150 m*M* NaCl, 10 m*M* Tris-HCl (pH 7.4 at 21°C), 100 U/mL penicillin G, 100 µg/mL streptomycin, and 1 m*M* sodium azide.
12. Phosphate-buffered saline: 137 m*M* NaCl, 2.7 m*M* KCl, 1.5 m*M* KH_2PO_4, 8 m*M* Na_2HPO_4, pH 7.4, at 21°C. This can be prepared as a 10-fold concentrated solution and stored indefinitely at 4°C or room temperature.
13. Wash buffer consisting of PBS and 0.05% (w/v) Tween 20. Prepare 900 mL/blot. Alternatively, prepare 300 mL of PBS containing 2 *M* urea (deionized as described above) and 0.05% Tween 20 for washing after the primary antibody and 600 mL of PBS-0.05% Tween 20 for washing after the secondary antibody.

3. Methods

3.1. Preparation and Solubilization of Samples for Quantitation (Skip to Subheading 3.2. *for Band Depletion Assays)*

1. Wash specimen in serum-free buffer.
   a. Solid tumor specimens should be washed in ice-cold PBS to remove serum proteins.
   b. Leukemia cells can be harvested from the interface of ficoll-Hypaque step gradients ***(16)***, diluted with serum-free RPMI 1640-10 m*M* HEPES (pH 7.4 at 21°C), sedimented at 200*g*, resuspended, counted, and sedimented at 200*g* for 10 min.
2. Solubilize sample by sonication in 6 *M* guanidine hydrochloride denaturing buffer (**Subheading 2.4., item 1**).

3.2. Formation of Topo–DNA Complexes In Vitro for Band Depletion Assay

1. Prepare a single-cell suspension of the cells to be assayed (*see* **Notes 1** and **2**). For clinical leukemia samples, sediment blood or bone marrow on ficoll-Hypaque gradients ***(16)*** and harvest the interface(s) from the step gradient.
2. Dilute cells with RPMI-HEPES and pellet at 200*g* for 10 min. Remove supernatant containing ficoll, Hypaque, and traces of serum (*see* **Note 2**).
3. Resuspend cells in a suitable volume of RPMI-HEPES. We find it convenient to resuspend cells at a concentration of 1–10 × 10^6/mL in 6.5 mL. This allows for six aliquots plus a small amount of sample for cell counting.
4. Add drug or diluent (e.g., 5 µL dimethylsulfoxide) to 1-mL aliquots of cells (*see* **Notes 3** and **4**). Mix samples gently, but thoroughly.
5. Incubate for 45 min at 37°C (see **Note 5**).
6. Sediment the cells (e.g., 3200g for 1 min or 200g for 10 min).
7. Remove as much of the supernatant as possible. Immediately add denaturing agent, and rapidly disrupt the cells by vigorous agitation or sonication. In our laboratory, we proceed one sample at a time, adding 1000 µL of guanidine hydrochloride-based denaturing solution (**Subheading 2.4., item 1**) and immediately vortexing until all turbidity has disappeared (*see* **Notes 6** and **7**). Samples are then sonicated to diminish viscosity (e.g., 40 bursts of $^1/_3$ s each at $^2/_3$ the maximal output of a microtip).

3.3. SDS-PAGE and Immobilization of Polypeptides

1. Prepare samples for SDS-PAGE. If samples have been lysed in 6 *M* guanidine hydrochloride, sample preparation consists of the following:
   a. React samples for 1 h at 21°C with 154 m*M* iodoacetamide to block free sulfhydryl groups, which could otherwise reoxidize to form large disulfide crosslinked polypeptide oligomers that will fail to enter an SDS-polyacrylamide gel.
   b. Transfer samples to dialysis bags and dialyze at 4°C against four to five changes of 4 *M* deionized urea. Each buffer change should be 10–100 times the total volume of the samples in the dialysis bags; and sufficient time (≥90 min) should be permitted for equilibration to occur before each buffer change. Tris-HCl (50 m*M* final concentration, pH 7.4, at 4°C) should be added to the first aliquot of 4 *M* urea to prevent the pH from rising above 9.0 when the temperature of the samples is decreased to 4°C.
   c. Dialyze the samples against three changes of 0.1% (w/v) SDS.
   d. After completion of dialysis, a small aliquot can be removed for protein determination by the bicinchoninic acid method ***(17)***, which is unaffected by 0.1% SDS.
   e. Transfer samples to test tubes, and dry using a lyophilizer or Speedvac.
   f. Resuspend samples in a convenient volume of SDS sample buffer, e.g., a volume that yields 2–3 × 10^5 cells/10 µL or 20–50 µg of protein/10 µL.

2. Pour SDS-polyacrylamide gels using standard techniques ***(18)***. Apply samples to adjacent wells. For quantitation of topoisomerase levels, the following order is appropriate:
   a. Full loading of control sample (cell line or purified topoisomerase) followed by $^1/_2$ loading, $^1/_4$ loading, $^1/_{10}$ loading, and $^1/_{20}$ loading to provide a standard curve (*see* **Note 8**).
   b. Full loading of unknown samples in which topo levels are being quantitated.

   For band depletion assays, the following loading is appropriate:
   a. Full loading of control sample (i.e., sample treated with diluent) followed by $^1/_2$ loading, $^1/_4$ loading, and $^1/_8$ or $^1/_{10}$ loading to provide a standard curve (*see* **Note 8**).
   b. Full loading of samples treated with various concentrations of topo-directed drug in ascending or descending order (*see* **Fig. 1**).
3. Separate polypeptides by electrophoresis and transfer them to a solid support, such as nitrocellulose, nylon, or PVDF, using standard techniques (*see* **Notes 9** and **10**). These techniques are described in detail in ref. ***(4).***
4. After transfer, stain the immobilized polypeptides with a nonspecific protein stain to confirm appropriate loading and efficient transfer of samples. We prefer to stain nitrocellulose or PVDF membranes with 0.1% Fast green FCF in 50% (v/v) methanol–5% (v/v) acetic acid and destain the blots in 50% (v/v) methanol–5% (v/v) acetic acid (*see* **Note 11**). The treatment with acidified methanol also appears to fix polypeptides on the nitrocellulose, preventing their unintended elution during subsequent treatments ***(19,20)***.
5. Block nonspecific binding sites by incubating with a protein solution. For most antibodies, we treat blots for 6 h at 21°C with 10% (w/v) nonfat powdered milk in 10 m*M* Tris-HCl (pH 7.4 at 21°C) containing 150 m*M* NaCl (*see* **Note 12**).

3.4. Immunodetection of Topoisomerase Molecules with Monomer Molecular Weight

1. From this point onward, it is convenient to have the blot in a Ziplock bag.
2. Add an appropriate dilution of antibody in blocking solution, and incubate overnight (10–15 h) at room temperature with gentle agitation (*see* **Notes 13** and **14**).
3. Remove antibody solution, and save for reuse (*see* **Note 15**).
4. Wash nitrocellulose with the following solutions (100 mL/wash for each blot): PBS containing 2 *M* urea and 0.05% (w/v) Tween 20 (three washes, 15 min each); PBS (two washes, 5 min each) (*see* **Note 16**).
5. Add 25 mL 3% (w/v) powdered milk in PBS. Add a suitable amount of enzyme-coupled secondary antibody (*see* **Note 17**). For peroxidase-coupled affinity-purified secondary antibodies, we routinely use a final concentration of 0.1 µg/mL. Incubate for 60 min at room temperature with gentle agitation.
6. Remove and discard secondary antibody. Wash blots with PBS containing 0.05% Tween 20 (100 mL/wash) as follows: two washes of 5 min each, two washes of 15 min each, two washes of 5 min each.

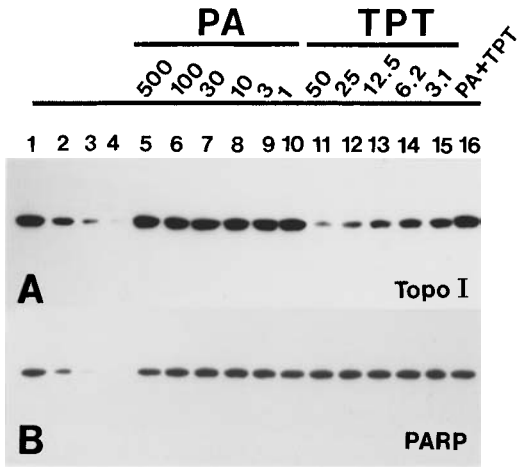

Fig. 1. Western blot showing topo I band depletion. K562 human leukemia cells were treated with decreasing concentrations of pyrazoloacridine (500–1 μ*M*, lanes 5–10) or topotecan (50–3.1 μ*M*, lanes 11–15) or with 500 μ*M* pyrazoloacridine and 50 μ*M* topotecan (lane 16). Aliquots containing 2×10^5 cells were loaded in lanes 5–16. To provide an indication of the relationship between topo I signal and topo I content, lanes 1–4 contained protein from 2×10^5, 1×10^5, 0.5×10^5, and 0.2×10^5 untreated cells, respectively. After polypeptides were transferred to nitrocellulose, blots were probed with MAb to topo I **(A)** or poly(ADP-ribose) polymerase **(B)**, a nuclear polypeptide that has a molecular weight and subnuclear distribution similar to that of topo I ***(33,34)***. Qualitatively it appears that the signal for topo I is unaffected by treatment with pyrazoloacridine (lanes 5–10), but decreases in a dose-dependent manner after treatment with topotecan (lanes 11–15).

7. Prepare enzyme substrate and apply to the blot as instructed by the supplier. For Amersham ECL reagent, combine equal volumes of solution 1 and solution 2 (3–4 mL of each should be sufficient for the usual blot). Discard the last wash, and incubate the blot with the substrate for 1 min (*see* **Note 18**).
8. Drain as much substrate as possible from the blot. Seal the Ziplock bag, and expose the blot to Kodak Xomat AR-5 or RP-5 film (*see* **Notes 19** and **20**).

3.5. Quantitation of Topoisomerase Levels in Untreated Samples (Skip to Subheading 3.6. *for Band Depletion Assays)*

1. Using a suitable scanner and computer program, quantitate the signal in each lane of the X-ray film (*see* **Note 21**).
2. Using values obtained with serial dilutions of the control sample (**Subheading 3.3., step 2a**), construct a standard curve of signal vs relative amount of topo loaded in the gel lanes (*see* **Note 22**). An example is shown in **Fig. 2**.

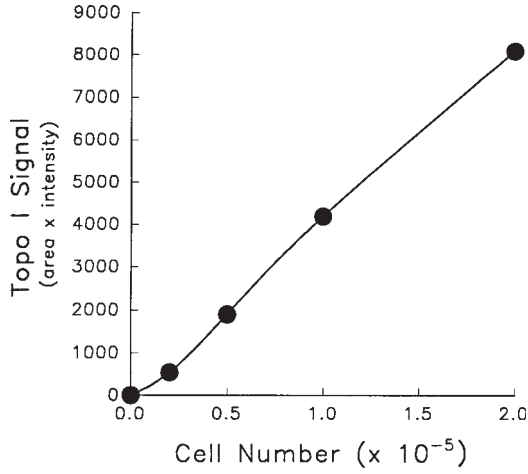

Fig. 2. Standard curve showing ECL signal vs amount of sample present. Lanes 1–4 of the autoradiograph shown in **Fig. 1A** were digitized and quantitated as described in **Subheading 3.5.**

3. By interpolation on this standard curve, determine the relative amount of unit mol-wt topo present in each of the unknown samples.
4. Reprobe the blot with an antibody against a polypeptide that would be expected to be constant from cell to cell among the unknowns. The most suitable polypeptide would appear to be a histone, which would be expected to be present in equal amounts in all diploid cells.

3.6. Quantitation of Complex Formation in Band Depletion Assays

1. Using a suitable scanner and computer program, quantitate the signal in each lane of the X-ray film (*see* **Note 21**).
2. Using values obtained with the serial dilutions of the control sample (**Subheading 3.3., step 2a**), construct a standard curve of signal vs relative amount of topo loaded in the gel lanes (*see* **Note 22**). An example is shown in **Fig. 2**.
3. By interpolation on this standard curve, determine the relative amount of unit mol-wt topo present in each of the drug-treated samples.
4. Reprobe the blot with an antibody against a polypeptide that would not be expected to be directly affected by drug treatment (**Fig. 1B**). Suitable examples include actin, histones, or lamins. This control is utilized to confirm that all undiluted samples have been equally loaded or to correct for slight differences in loading from sample to sample (*see* **Notes 23** and **24**).
5. Use the data from **step 4** to construct a curve showing the relative topo I signal remaining at each drug concentration (**Fig. 3A**) or the % of initial topo molecules

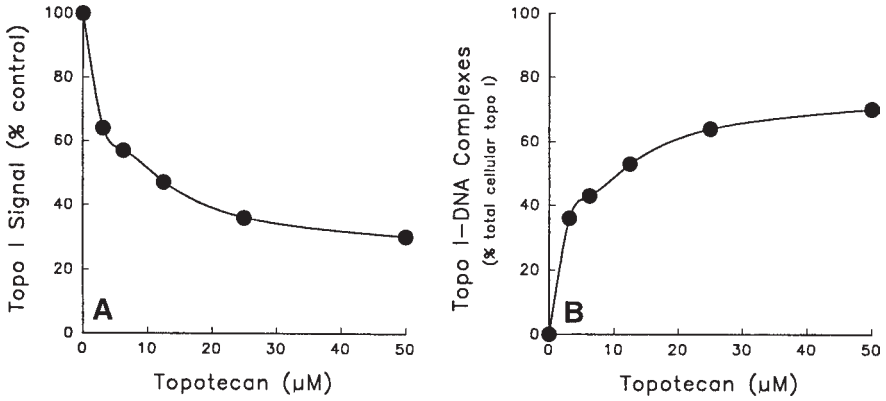

Fig. 3. Plot of topo I–DNA complexes vs drug concentration. The signals in **Fig. 1** (lanes 11–15) were compared to the standard curve in **Fig. 2**. At each drug concentration, the signal at 100 kDa was compared to the signal expected in 2×10^5 cells (lane 1). This can then be plotted as the relative topo I signal remaining on the blot vs drug concentration **(A)** or the % of topo I depleted from the blot as a function of drug concentration **(B)**.

that are covalently bound to DNA at each drug concentration (**Fig. 3B**; *see* **Notes 25** and **26**).

4. Notes

4.1. Formation of Topoisomerase–DNA Complexes In Vitro for Band Depletion Assay

1. The band depletion technique is not suitable for solid tumor specimens. The underlying assumption is that all cells are equally exposed to the chemotherapeutic agent. This assumption cannot be verified in solid tumor specimens.
2. Because samples will be lysed under denaturing conditions without being washed (*see* **Subheading 3.2., step 7**), it is important that cells be freed of serum proteins and resuspended in serum-free medium prior to the start of the assay.
3. The concentrations utilized in the band depletion assay will vary with the drug under consideration and, to a smaller extent, with the cell line being studied. For topotecan or camptothecin, concentrations of 1–50 μ*M* result in depletion of the topo I signal at M_r ~100,000 in a variety of human leukemia cells ***(21)***. Likewise, 7–700 μ*M* etoposide results in substantial (although incomplete) depletion of the topo II signals at M_r ~170,000 and M_r ~180,000 ***(21)***.
4. Concentrations used in the band depletion assay are 100- to 1000-fold higher than concentrations required to produce cytotoxicity (lack of colony formation or induction of apoptosis) with prolonged (≥24 h) exposure. Higher concentrations are employed in the band depletion assay because this assay requires the forma-

tion of large numbers of topo–DNA adducts to produce a signal (loss of signal on Western blots), whereas cytotoxicity can result from the stabilization of small numbers of adducts if these adducts are converted into cytotoxic lesions ***(5)***.

5. The recommended incubation time represents a compromise between the time required for stabilization of topo–DNA complexes and the possibility of inducing apoptosis in susceptible cell types. Flow cytometry experiments ***(11)*** indicate that camptothecin derivatives rapidly enter and exit from mammalian cells ($t_{1/2}$ ~2 min in K562 human leukemia cells). In contrast, maximal accumulation of topo II–DNA adducts in etoposide-treated human leukemia cells appears to require a ≥30-min incubation (S. H. K., unpublished observations). On the other hand, treatment with high concentrations of etoposide or camptothecin also induces apoptosis with its attendant protease activation in as little as 2 h in some human leukemia cell lines ***(22)***. A suggested incubation of 30–60 min appears to be a reasonable compromise between these competing considerations.
6. If cells are washed in drug-free medium, topo-mediated religation of the DNA and concomitant loss of the topo–DNA adducts can occur. These events have even been detected at 4°C ***(23)***. For this reason, we prefer to perform the drug incubation in serum-free medium and lyse the cells without any washing step. Because the mammalian topoisomerases have turnover life-times of >24 h ***(24)***, it is unlikely that significant alterations in topoisomerase protein levels occur during this 45-min incubation under serum-free conditions.
7. Cells must be lysed rapidly so that lysosomal proteases do not degrade the topoisomerases and yield a false-positive assay. Myeloid cells (white blood cells of the granulocyte lineage) have particularly high protease contents; proteolysis of abundant cellular polypeptides has been demonstrated when these cells are lysed in SDS under conditions where proteases are inadequately inhibited ***(25)***. Previous studies from this laboratory ***(14)*** suggest that use of the denaturing agent guanidine ***(26)*** yields samples that have a higher signal for topo II isoforms.

4.2. SDS-PAGE and Immobilization of Polypeptides

8. Serial dilutions are required to provide an appropriate standard curve for quantitation of the relative amount of topo that migrates at the subunit molecular weight (*see* **Subheading 3.5., step 2**).
9. Nitrocellulose has the advantage of ease of use. It is compatible with a wide variety of staining procedures. With multiple cycles of blotting and erasing, however, nitrocellulose tends to become brittle. Derivatized nylon has the advantage of greater protein binding capacity and greater durability, but avidly binds many nonspecific protein stains (reviewed in ***4***). The higher binding capacity of nylon is also said to contribute to higher background binding despite the use of blocking solutions containing large amounts of protein. PVDF membranes are durable, compatible with a variety of nonspecific protein stains, and capable of being stripped of antibody and reutilized.
10. Importance of SDS in transfer buffer if topo II will be assessed: Although the size cutoff varies with the porosity of the gel ***(1)***, polypeptides above 100–120 kDa

transfer poorly in standard transfer buffer consisting of 192 m*M* glycine, 25 m*M* Tris, and 20% (v/v) methanol. Addition of low concentrations of SDS (0.01–0.1% w/v) to this buffer facilitates transfer of larger polypeptides, but also results in considerable heating of the transfer apparatus owing to increased current flow. We routinely perform electrophoretic transfers at 90 V for 4–6 h in a Hoefer TE52 or TE 62 transfer apparatus containing sample buffer consisting of 0.02% (w/v) SDS, 192 m*M* glycine, 25 m*M* Tris, and 20% (v/v) methanol. To prevent excessive heating, the transfer is performed at 4°C with the transfer apparatus packed in ice.

11. Alternative staining procedures utilize Coomassie blue, Ponceau S, Amido black, India drawing ink, colloidal gold, or silver (reviewed in ***1–3***).
12. Alternative proteins utilized to block unoccupied binding sites on nitrocellulose include 3% (w/v) bovine serum albumin, 1% hemoglobin, and 0.1% gelatin (reviewed in ***2,3***).

4.3. Western Blotting

13. Unfortunately, the appropriate dilution of antiserum or antibody must be determined empirically. Some antisera are useful for blotting at a dilution of greater than >1:20,000, whereas others are useful at a dilution of 1:10 or 1:100.
14. A variety of incubation times with primary antibodies have been recommended (reviewed in ***3***). Preliminary studies have revealed that the signal intensity obtained with some antibodies is much greater when blots are incubated with primary antibody overnight rather than 1–2 h (G. Humphrey and S. H. K., unpublished observations).
15. Diluted antibody solutions can be reused multiple times. They should be stored at 4°C after additional aliquots of penicillin/streptomycin and sodium azide have been added.
16. Some antisera give high backgrounds on Western blots. This background can be diminished by adding 2 *M* urea to the initial three washes after the primary antibody. Preliminary studies (S. J. McLaughlin and S. H. K.) indicate that most antigen–antibody complexes are stable in 4 *M* urea once they have formed. Alternatively, other investigators include a mixture of SDS and nonionic detergent (e.g., 0.1% [w/v] SDS and 1% [w/v] Triton X-100) in the wash buffers. On the other hand, for antibodies with low avidity (especially certain monoclonal antibodies [MAb]), the use of 2 *M* urea or SDS should be avoided because these agents diminish the signal intensity.
17. Alkaline phosphatase-coupled antibodies, which are also utilized in conjunction with chemiluminescent detection (although with different substrates), can be employed using the procedures described here. [^{125}I]-labeled secondary antibodies can also be utilized in conjunction with autoradiographic detection. Protocols for the use of radiolabeled secondary antibodies have been previously described in detail ***(4,11,27)***.
18. If multiple blots are being probed simultaneously, this solution can be poured from one bag to the next. We routinely use the same aliquot for three or four blots.

19. The optimum exposure time will vary with the abundance of the antigen and the dilution of the primary antiserum. Using the recipes described here, useful signals have been developed in as little as 5 s or as long as 2 h. We usually expose the first piece of X-ray film for 2–10 min and then adjust the subsequent exposure times based on results of this first exposure.
20. Trouble-shooting the ECL reaction—signal too low or background too high.
    a. The Amersham ECL reagent appears to continue giving luminescence for up to 4 h, albeit with decreasing intensity after the first 30–60 min. Therefore, long exposures (even overnight) are sometimes useful.
    b. Because the luminescence decreases over time, the most intense signals are usually obtained in the first hour. If it is necessary to recreate this high intensity several hours later, it is possible to reincubate the blot with reagents 1 and 2 (**Subheading 3.4., step 7**) and expose a new piece of X-ray film.
    c. Occasionally blots will have an extremely high background. It was recently reported that washing the blot twice with PBS and incubating for 1 min with a 1:10 dilution of solutions 1 + 2 can diminish this background ***(28)***.

4.4. Quantitation of Topo Levels in Untreated Samples and Quantitation of Complex Formation in Band Depletion Assays

21. A variety of scanners or digitizing devices are suitable. These range from widely available inexpensive scanners (e.g., Apple U730) coupled with commonly used quantitation programs (Collage, Image Dynamics Corp.) to more expensive dedicated computer systems (e.g., Visage from Bio-Rad; BioImage from Millipore). A recent comparison suggests that comparable results are obtained with both types of systems ***(29)***.
22. Potential pitfalls in quantitation of autoradiographic signals:
    a. Although some standard curves are linear (e.g., **Fig. 2**), other standard curves are not. As a result, the commonly utilized practice of running gels without a serial dilution of purified topoisomerase molecules or untreated cells and assuming that the integrated signal strength on Western blots is a linear function of the amount of antigen present is strongly discouraged.
    b. Likewise, because the area of the signal as well as its intensity can vary from lane to lane (**Fig. 1**), the practice of scanning the center of each lane with a gel scanner (e.g., Hoefer model GS300 densitometer) and determining optical density as an index of signal strength is discouraged. The recommended practice of determining signal area and signal intensity appears to present a more accurate measurement of signal strength.
23. If it is necessary to strip antibody off the blot prior to probing with the second antibody, methods for stripping and reusing blots have been recently reviewed in detail ***(4)***.
24. The underlying assumption is that loss of signal at the unit molecular weight of the topoisomerase is a reflection of the formation of covalent topo–DNA complexes (*see* **Eq. 1**) and not a reflection of protein degradation within the cells. Two points need to be considered in this regard:

a. The assumption that the total topo content within cells is constant during this type of assay has been confirmed by heating samples of teniposide-treated HeLa cells to 65°C just prior to lysing the cells under denaturing conditions, and showing that the topo II signal returns to baseline values as a consequence of this treatment ***(30)***. Similar results were recently observed with topo I ***(31)***. This heat treatment inhibits the nicking activity of topoisomerase, but not the religation activity, shifting the equilibrium in **Eq. 1** to the left.
b. Reprobing the blots with a second antibody is a useful means of correcting for differences in loading. Different polypeptides have differing susceptibilities to proteolysis, particularly in cells undergoing drug-induced apoptosis ***(22)***. Accordingly, probing a blot with a second antibody does not address the question of proteolysis unless the antibody recognizes one of the polypeptides that is cleaved early in the apoptotic process.

4.5. General Notes

25. Construction of the curve shown in **Fig. 3** should not be considered an end unto itself. When two cell lines are shown to require different drug concentrations to induce the formation of the same number of topo–DNA adducts, there are multiple factors that might account for this difference including:
    a. Differences in steady-state drug accumulation;
    b. Differences in drug metabolism; and
    c. Differences in ability of the drug to shift the equilibrium shown in **Eq. 1** as a consequence of genetic or posttranslational alterations in the topo molecule. Examples of each of these causes of drug resistance are reviewed in recent references ***(5,6,32)***.
26. With minor modification, this method can also be utilized to assess the formation of cleavage complexes in adherent tissue-culture cell lines. Cells are washed several times in serum-free medium to remove serum proteins (*see* **Note 2**) and then incubated with drug in serum-free medium. At the conclusion of the incubation, the drug-containing medium is aspirated and cells are lysed. It is particularly convenient to lyse the cells in guanidine hydrochloride-based denaturing agent (**Subheading 3.2., step 7**). The large volume of buffer needed to cover a plate (2 mL for 60-mm tissue-culture plate, 3 mL for 100-mm tissue-culture plate) is not a problem, because the sample preparation method involves lyophilization (**Subheading 3.3., step 1**), permitting an opportunity to resuspend the samples in a smaller volume.

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27

Visualization of DNA Topoisomerases by Electron Microscopy

Piero Benedetti and Alessandra Silvestri

1. Introduction

Eukaryotic DNA Topoisomerase II (Topo II) is an essential enzyme that catalyzes the relaxation of supercoiled DNA and the segregation of newly replicated chromosomes ***(1–3)***. The enzyme is highly conserved through evolution, and appears to result from the fusion of the A- and B-subunits of bacterial DNA gyrase ***(4)***. It has an A_2 dimeric structure, whereas the bacterial enzyme has an A_2B_2 tetrameric arrangement.

Although the large size of most topoisomerases was a problem for crystallographic studies, recently fragments have been crystallized and their structure solved ***(5,6)***. The large size, however, makes possible their direct observation using electron microscopy ***(7–10)***.

This chapter will discuss some methods to visualize Topo II by electron microscopy. The use of this technique is most powerful when used in conjunction with biochemical and structural methods. In the case of this enzyme, its overall shape is so particular that it renders possible some mechanistic studies that are supported by a well-known biochemical analysis.

We will describe in detail the rotary shadowing technique of specimens dried in the presence of glycerol. This method of preparation of single molecules has been widely used to determine the shape of several protein, such as spectrin, myosin, actin, fibrinogen, and DNA gyrase ***(11–13)***. The strong advantage of this technique is that molecules are dried in a mild way, with consistently little shearing effect. In the case of eukaryotic topo II, the enzyme appears consistently as a heart-shaped molecule composed of a larger central structure connected to two symmetric spherical masses. Negative staining technique will

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also be described, and the difference in molecule shape obtained with this method will be discussed ***(13,14)***.

2. Materials

2.1. Electron Microscopy Supplies

1. Vacuum evaporator (procedures in this chapter were carried out with an Edwards 306 Apparatus).
2. Spray apparatus (we use a modified artist airbrush from Paasche, model H-1, Chicago, IL; *see* **Fig. 1**).
3. Straight tungsten electrodes (Ted Pella 3-strand CA96099).
4. Platinum wire 0.2 mm diameter.
5. Carbon rod for evaporation.
6. N_2 pressure tank with valve.
7. 300- and 400-mesh grids (Ted Pella).
8. Mica (Polysciences Cat# 0861).
9. Whatman circles filter paper (No. 1).
10. Double-stick tape.
11. Rimless pipetman tips.
12. 1-mL pipet-man tips cut with a razor blade at ~6 mm of tip for glycerol pipeting.
13. Stopwatch/timer.
14. Protective dark goggles.
15. Crystallization dish 70 × 50 mm covered with black tape.
16. Lens cleaning tissues.

2.2. Reagents

1. Purified DNA Topo II at 0.3–1 mg/mL stock solution in 10% glycerol, 50 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA, 1 m*M* EGTA, 100–400 m*M* KCl.
2. Glycerol (pure-grade).
3. 1 *M* NH_4 acetate stock.
4. Methanol.
5. Ethanol 100%.
6. Uranyl acetate 1% in double-distilled water.

3. Methods

3.1. Preparing Vacuum Evaporator for Shadow

1. Wear gloves.
2. Cut 2.5-cm platinum wire, and twist it tightly on the central part of a tungsten filament that has been cleaned with methanol. Put the clean platinum/tungsten filament on a lens tissue before mounting it on the evaporator electrodes.
3. Prepare the carbon gun (according to the apparatus manual).
4. Set the rotary stage.
5. Clean well the rotary stage with methanol.
6. Place double-stick tape to hold the mica sheets.

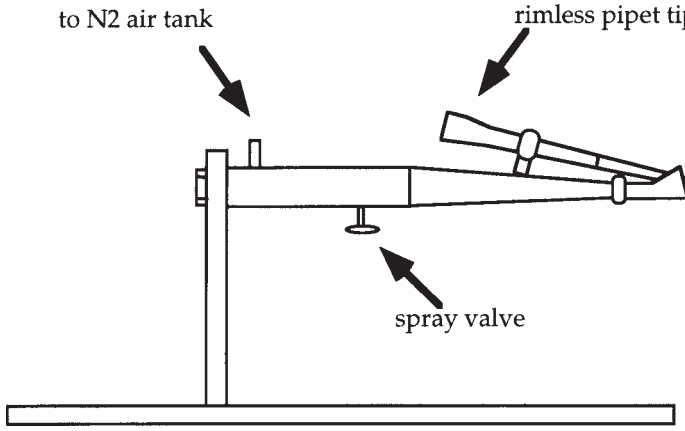

Fig. 1. Spraying apparatus consisting of an aluminum support with a modified Paasche H1 airbrush. The pipetman tip is positioned to be just at the edge of the air gun. Mica is held 30–40 cm far from the air gun.

7. Place a bent piece of a filter paper facing the Pt/W filament at the same distance of the rotating table center (color will indicate the Pt thickness) (*see* **Note 1**).
8. Place a piece of filter paper on the main table of the evaporator with some vacuum grease to monitor carbon film thickness (grease will not be stained by the carbon, and a brown color will indicate carbon thickness.
9. Place the platinum/tungsten filament at a 7° angle with the center of the rotating table (*see* **Note 2**).
10. Place carbon evaporation gun at a 90° angle with the rotating table.
11. Cut mica into small 6 × 6 mm pentagons with one pointed edge (this helps the carbon replica to float off the mica), and number them with a water-resistant marker.

3.2. Preparing Protein Samples for Spray

Some salts, detergents, and buffers tend to affect the grain size and retreat of the solution toward the center of the droplet. High concentrations of ATP, Tris, and KCl tend to layer over a large part of the droplet leaving free protein molecules in a very small area. For this reason, we use an NH_4 acetate that is sufficiently volatile as the spray buffer. We have not noticed any major difference in enzyme shape with NH_4 acetate concentrations ranging from 50–600 m*M*.

We perform protein assays in standard topo II buffer, and dilute the reaction mixture in spray buffer just before mounting the samples for electron microscopy (EM).

Optimal final protein concentration for spray is between 2 and 30 μg/mL, which corresponds to $3.4–50 \times 10^{12}$ mol/mL.

We prepare our glycerol NH_4 acetate solution fresh before use. In a standard spray experiment, we dilute enzyme in 100 m*M* acetate solution and 60% glycerol. We use 40 µL for spray.

1. Prepare spray apparatus.
2. Open nitrogen tank valve, and adjust pressure to ~32 psi (~2.2 kg/cm^2).
3. Open valve to airbrush, and check with few bursts proper operation.
4. Take 40 µL with a rimless pipetman tip, and place in the airbrush (*see* **Fig. 1**).
5. Cleave a mica square with fine-tip tweezers in two halves, and place the fresh cleaved surface ~30 cm away from the tip of the airbrush (*see* **Note 3**).
6. Spray the protein glycerol solution, with quick short bursts, onto the mica.
7. Place sprayed mica on the rotary table, close the bell jar, and start pumping.
8. Add liquid N_2 to cold trap.

3.3. Platinum Shadow

When the vacuum has reached ~3–5 × 10^{-6} torr, start shadowing.

1. Add again liquid N_2 to cold trap.
2. Turn on the rotary table at ~100 rpm.
3. Have protective goggles ready.
4. Slowly dial up current until the filament turns red. Then stop to allow vacuum recovery. Try to maintain vacuum values close to 10^{-5} during evaporation.
5. Increase current slowly, waiting for stable vacuum until the filament melts (greater vacuum loss at this stage).
6. When filament melts, start the stopwatch and count down 1.5 min, increasing current slowly 2% of the meter scale, every 5 s (Never look at the filament without goggles.) After the elapsed time, shut off current.
7. Reset vacuum to 10^{-5} and start evaporating carbon by increasing the current at a rate of 1%/s until the carbon begins evaporating. After 1 s, rapidly turn off current. You must be able to see a pale brown color on the filter paper on the main table. We aim for a carbon film thickness of about 10 nm.
8. Vent evaporator, and collect mica in a clean Petri dish.

3.4. Mounting Replica on EM Grids

1. Have very clean 300- or 400-mesh grids (*see* **Note 4**).
2. Fill to overflow with deionized distilled water a crystallization dish that has been covered outside with black tape (this helps to see replicas on water surface).
3. Clean the surface of the water by swiping with lens cleaning tissue to remove debris.
4. Pick up the mica with tweezers, and float off the carbon replica by slowly submerging the mica at an angle of 30°. Depending on humidity condition, sometimes replicas do not float off in the first immersion. In very dry days, keep replicas in a humid atmosphere for 30 min before floating them off.

5. Gently pick up portions of the film replica from below the water surface with 400-mesh grids, and slowly blot the water excess from one edge of the grid with pieces of filter paper.
6. Let grids dry for few minutes before observing them in the electron microscope.

3.5. Negative Staining

For negative staining, a protein solution at 50 μg/mL in NH_4 acetate was absorbed on glow discharged carbon-coated grids for 1 min, stained with 1% aqueous uranyl acetate, and dried with filter paper (**Note 5**).

3.6. Observation in the Electron Microscope

Locate the protein droplets in the replica by looking at the salt residues in the center of the droplet using a magnification of 5000x. The best protein images are generally located in the area immediately outside the droplet residue in which salts and sugar are concentrated by the retreat of the glycerol front.

We observe samples between 20.000 and 50.000x, and we take pictures at 50.000x magnifications. For magnification calibration, we use tropomiosin paracrystals, which have repeating units of ~400 Å, or a DNA fragment of known length.

Figure 2 shows a typical field with *Saccharomyces cerevisiae* DNA topo II. The majority of the proteins present in the field are composed of a large central core connected to two symmetrical spherical masses, but four dotted structures with a central "hole" are also visible. In negative stained samples, shown in **Fig. 3**, the four dotted structure is the most abundant.

In the rotary shadowed images, we find molecules in which the two spherical masses form different angles in relation to the central core, varying from 160–0°. Few molecules, however, show a four globular structure. On binding of a nonhydrolyzable ATP analog to the enzyme, this angle is significantly reduced as the two spherical masses swing into contact. We think that the variability in the shape of the protein is owing to different conformational states of the enzyme and not to a shearing effect related to the technique.

If a DNA fragment is mounted with the same method as a control, molecules do not show any tendency to align, suggesting that the shearing forces are minimal. **Figure 4** shows the open and closed conformation that can assume the fragment spanning from aa 660 to aa 1204 of yeast DNA topo II.

If a negative staining method is used, we observe two protein images, either four globular regions with an apparent hole in the middle, the largest number, or U-shaped molecules that resemble the three globular structure obtained with rotary shadowing. Recently, Shultz et al. ***(9)*** have used a combination of nega-

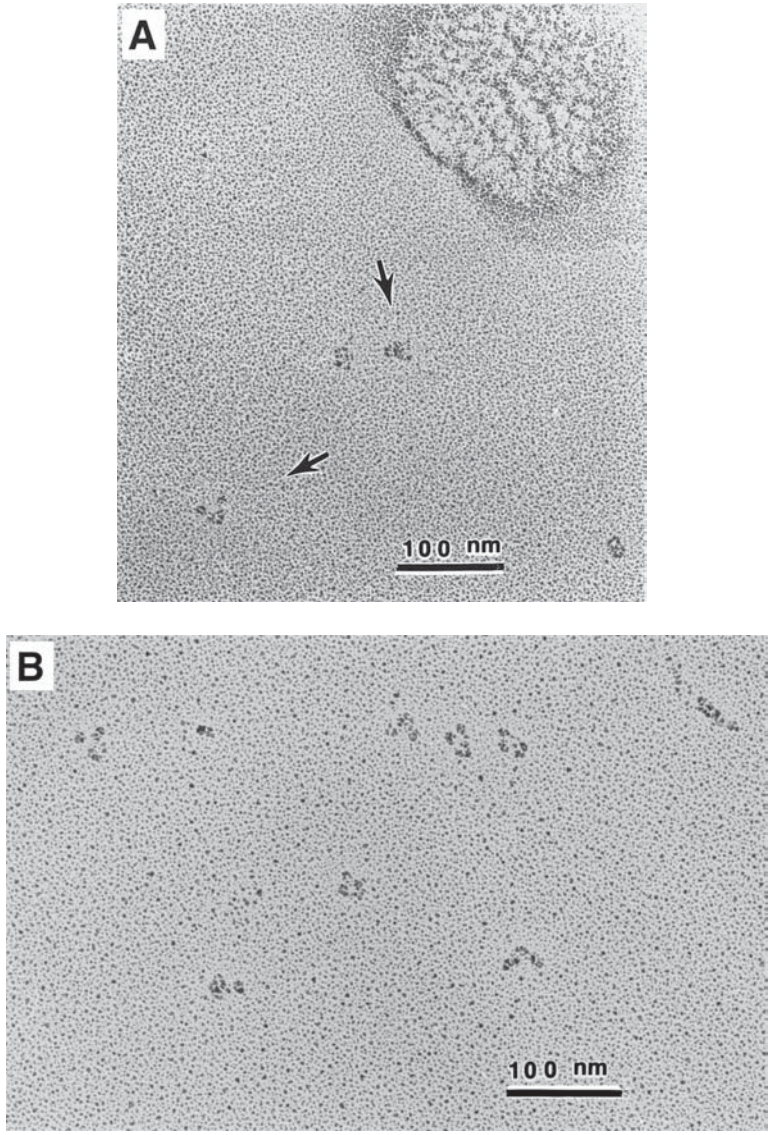

Fig. 2. **(A)** Region of rotary shadowed replica showing *S. cerevisiae* DNA topo II. Molecules, indicated by arrows, are visible in a clear zone outside the central droplet residue, where salts are concentrated by the retreating of the glycerol front. **(B)** Molecules of topo II with different shape arrangement.

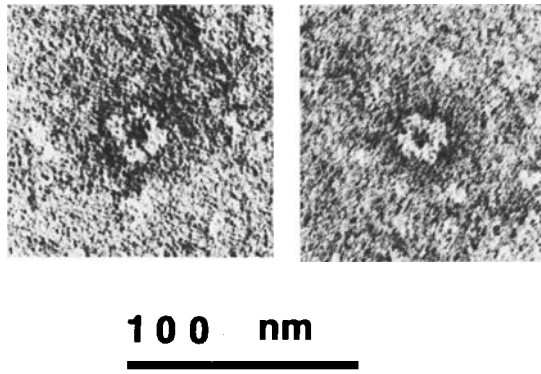

Fig. 3. Electron micrograph showing a negative-stained molecule.

tive stain and Cryo electron microscopy to analyze the shape of human DNA topo II, and the molecule's structure is comparable to the rotary shadowed images.

4. Notes

1. We cut a piece of filter paper in such a way that could be taped on the holder of the rotating table, in a fixed position facing the platinum filament gun. The gray color on this paper after the evaporation of the metal will indicate the platinum thickness.
2. We use a plastic ruler to measure the distance of the center of the rotating plate with the platinum filament gun. A 7°angle is obtained by placing the filament at 10 cm from the center of the table and at 1.4 cm high.
3. Do not leave the mica uncovered, and use shortly after the separation of the fresh layers.
4. Wash grids using a dish liquid detergent, and rinse them thoroughly in tap water. Rinse in deionized and in double-distilled water. Leave grids in acetone for 1 h and then dry them by inverting on a filter paper.
5. Several procedures are used for negative stain. For an extensive review on this matter, *see* **ref.** ***(13)***.

Acknowledgments

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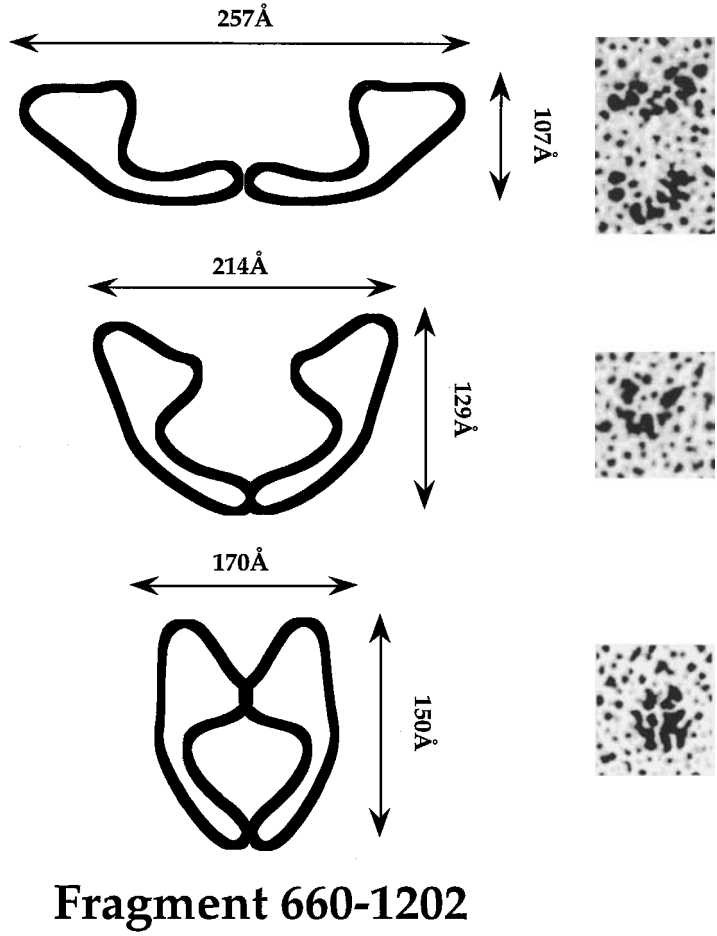

Fig. 4. Fragment 660–1202. Open and closed conformation of the fragment of yeast DNA topo II spanning from aa 660 to aa 1204. The schematic images on the left are redrawn from crystal structure of the fragment solved by Berger et al. ***(6)***.

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Appendix: Compendium of DNA Topoisomerase Sequences

Paul R. Caron

DNA topoisomerases can be grouped in to three families based on biochemical properties and amino acid sequence. Following are multiple protein sequence alignments of the members of each of these families: the eukaryotic DNA topoisomerase I type, the DNA topoisomerase II type, and the DNA topoisomerase III/eubacterial DNA topoisomerase I type. These sequences were obtained from data in the public data bases and represent the most reliable data available as of GenBank release 95.0, June 15, 1996 ***(1)***. These alignments are updates of alignments published previously ***(2)***.

Residues that are either identical or replaced by conservative changes in over 70% of the available sequences are bold. Each alignment is accompanied by a table that provides an NCBI unique identifier number for a representative data base entry for each sequence. One method for sequence retrieval would be to use the Entrez program, which can be accessed on the web at <http:www3.ncbi.nlm.nih.gov/Entrez>. Go to the protein data base and search using the NCBI ID number. This will provide links to the corresponding nucleotide sequences and related publications.

In some cases, such as the N-terminal regions of the eukaryotic DNA topoisomerase I sequences and the C-terminal regions of the DNA topoisomerase II sequences, there is no significant sequence homology encompassing all of the sequences. The sequences are presented in the alignment for the sake of completeness, and the alignments presented should not be considered statistically significant.

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Topoisomerase I Reference Table

Code	Organism	NCBI ID
At	*Arabidopsis thaliana*	16557
Ce	*Caenorhabditis elegans*	1236752
Cg	*Cricetulus griseus*	297078
Dm	*Drosophila melanogaster*	158642
Fv	Shope fibroma virus	333617
Hs	*Homo sapiens*	339805
Mm	*Mus musculus*	220617
Or	Orf virus	521137
Pf	*Plasmodium falciparum*	790481
Sc	*Saccharomyces cerevisiae*	173003
Sp	*Schizosaccharomyces pombe*	5118
Um	*Ustilago maydis*	474908
Vc	Vaccinia virus	295419
Vr	Variola virus	623595
Xl	*Xenopus laevis*	214833

Topoisomerase II Reference Table

Code	Organism	NCBI ID
HsTop2a	*Homo sapiens*	292830
CgTop2a	*Cricetulus griseus*	191218
MmTop2a	*Mus musculus*	220616
RnTop2a	*Rattus norvegicus*	57964
HsTop2b	*Homo sapiens*	37231
ClTop2b	*Cricetulus longicaudatus*	790988
MmTop2b	*Mus musculus*	1066004
RnTop2b	*Rattus norvegicus*	506869
DmTop2	*Drosophila melanogaster*	8711
CeTop2b	*Caenorhabditis elegans*	1228961
CeTop2c	*Caenorhabditis elegans*	1330372
CeTop2	*Caenorhabditis elegans*	156413
AtTop2	*Arabidopsis thaliana*	474890
ScTop2	*Saccharomyces cerevisiae*	887623
SpTop2	*Schizosaccharomyces pombe*	5121
CpTop2	*Cryptosporidium parvum*	913611
TbTop2	*Trypanosoma brucei*	162305
TcTop2	*Trypanosoma cruzi*	162307

(continued)

Topoisomerase II Reference Table (*continued*)

Code	Organism	NCBI ID
CfTop2	*Crithidia fasciculata*	11001
ASFVTop	African swine fever virus	262135
EcGyrB	*Escherichia coli*	41646
HiGyrB	*Haemophilus influenzae*	1222504
PmGyrB	*Proteus mirabilis*	150881
BaGyrB	*Buchnera aphidicola*	551761
PpGyrB	*Pseudomonas putida*	45694
NgGyrB	*Neisseria gonorrhoeae*	150257
CcGyrB	*Caulobacter cresentus*	392774
BsGyrB	*Bacillus subtilis*	467326
SpGyrB	*Streptococcus pneumoniae*	1052804
SaGyrB	*Staphylococcus aureus*	296395
HfGyrB	*Haloferax alicantei*	149024
ScGyrB	*Spiroplasma citri*	49348
MgGyrB2	*Mycoplasma genitalium*	1045888
BsGyrB2	*Bacillus subtilis*	1405461
BbGyrB	*Borrelia burgdorferi*	454038
BbGyrB2	*Borrelia burgdorferi*	520781
McGyrB	*Mycoplasma capricolum*	533332
MpGyrB	*Mycoplasma pneumoniae*	44484
MaGyrB	*Mycoplasma gallisepticum*	603237
MgGyrB	*Mycoplasma genitalium*	1045671
MhGyrB	*Mycoplasma hominis*	453419
MlGyrB	*Mycobacterium leprae*	1262356
MtGyrB	*Mycobacterium tuberculosis*	1107468
MsGyrB	*Mycobacterium smegmatis*	1213062
SoGyrB	*Streptomyces coelicolor*	436027
SsGyrBs	*Streptomyces spheroides*	581743
SsGyrBr	*Streptomyces spheroides*	581742
EcParE	*Escherichia coli*	882560
StParE	*Salmonella typhimurium*	154238
HiParE	*Haemophilus influenzae*	1205762
T4Gn39	*Bacteriophage T4*	728617
T2Gn39	*Bacteriophage T2*	
T4Gn60	*Bacteriophage T4*	215846
EcGyrA	*Escherichia coli*	41634
HiGyrA	*Hemophilus influenzae*	1205505
ErGyrA	*Erwinia carotovora*	525202
VsGyrA	*Vibrio salmonicida*	832878

(continued)

Topoisomerase II Reference Table (*continued*)

Code	Organism	NCBI ID
KpGyrA	*Klebsiella pneumoniae*	43808
StGyrA	*Salmonella typhi*	1419297
AsGyrA	*Aeromonas salmonicida*	1019146
AbGyrA	*Acinetobacter baumannii*	558547
NgGyrA	*Neisseria gonorrhoeae*	529408
PaGyrA	*Pseudomonas aeruginosa*	459929
BsGyrA	*Bacillus subtilis*	40019
BsGyrA2	*Bacillus subtilis*	1405462
SaGyrA2	*Staphylococcus aureus*	561880
McGyrA	*Mycoplasma capricolum*	530421
McGyrA2	*Mycoplasma capricolum*	530409
SaGyrA	*Staphylococcus aureus*	296396
AtGyrA	*Agrobacterium tumefaciens*	1296444
CfGyrA	*Campylobacter fetus*	818859
CjGyrA	*Campylobacter jejuni*	144206
HpGyrA	*Helicobacter pylori*	508471
ScGyrA	*Spiroplasma citri*	49349
HfGyrA	*Haloferax sp.*	43485
MlGyrA	*Mycobacterium leprae*	1122296
RpGyrA	*Rickettsia prowazekii*	409961
MpGyrA	*Mycoplasma pneumoniae*	44485
MgGyrA	*Mycoplasma genitalium*	1045672
MgGyrA2	*Mycoplasma genitalium*	1045889
MaGyrA	*Mycoplasma gallisepticum*	551907
MtGyrA	*Mycobacterium tuberculosis*	466275
MsGyrA	*Mycobacterium smegmatis*	1122892
SoGyrA	*Streptomyces coelicolor*	436028
SsGyrA	*Synechocystis sp.*	1001649
SeGyrA	*Staphylococcus epidermidis*	240997
FsGyrA	*Fibrobacter succinogenes*	402872
EcParC	*Escherichia coli*	882549
StParC	*Salmonella typhimurium*	154235
HiParC	*Hemophilus influenzae*	1205764
AbParC	*Acinetobacter baumannii*	1212749
T4Gn52	Bacteriophage T4	728620
BbGyrA	*Borrelia burgdorferi*	49296

Topoisomerase III Reference Table

Code	Organism	NCBI ID
EcTop1	*Escherichia coli*	415338
KaTop1	*Klebsiella aerogenes*	
HiTop1	*Haemophilus influenzae*	1205601
SsTopA	*Synechococcus sp.*	288126
MtTopI	*Mycobacterium tuberculosis*	1395205
BaTop1	*Bacillus anthracis*	478996
BsTop1	*Bacillus subtilis*	520753
TmTop1	*Thermotoga maritima*	881494
MgTop1	*Mycoplasma genitalium*	1045802
RP4TraE	Plasmid RP4	437697
BfTop1	*Bacillus firmus*	39484
SaTrsI	*Staphylococcus aureus*	310616
pAMb1	*Enterococcus faecalis*	S45077
BT223g	*Streptococcus pyogenes*	456366
EcTopB	*Escherichia coli*	148026
HiTop3	*Hemophilus influenzae*	1204694
ScTop3	*Saccharomyces cerevisiae*	173002
HsTop3	*Homo sapiens*	1292913
SaRevG	*Sulfolobus acidocaldarius*	152943
MkRevGB	*Methanopyrus kandleri*	1173903
MkRevGA	*Methanopyrus kandleri*	1173901

References

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2. Caron, P. R. and Wang, J. C. (1994) Alignment of primary sequences of DNA topoisomerases. *Adv. Pharmacol.* **29B,** 271–297.

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Hs  MSGD.......HLHNDSQIEADFRL.................NDSHKHKDKHKDREHRHKEHKK.......EKDREKSKHSNSEHKDSEKKHKEKEKTKH   69
Cg  MSGD.......HLHNDSQIEADFRL.................NDSHKHKDKHKDREHRHKEHKKDK.....EKDREKSKHSNSEHKDSEKKHKEKEKTKH   71
Mm  MSGD.......HLHNDSQIEADFRL.................NDSHKHKDKHKDREHRHKEHKKDK.....DKDREKSKHSNSEHKDSEKKHKEKEKTKH   71
Xl  MSED.......HVQNDSQIEAVFRV.................NDSHKHKKDKEHRHKEHKKDKDRE.....KSKHNNSEHRDPSEKKHKDKHKNNDKHRE   71
Ce                                                                            MMKNQFLKRERKNERIREKRKSSAKNGKK   29
Dm  MSGDVAAENSIHIQNGGSCEVVQSNGVTTNGHGHHHHHHSSSSSSKHKSSSKDKHRDRREREHKSSNSSSSSKEHKSSRDKDRHKSSSSSSKHRDKDKE  100

Hs  KDGSSEKHK.....................................................DKHKDRDKEKRKEEKVRASGDAKIKKEKE  107
Cg  KDGSSEKHK.....................................................DKHKDRDKEKRKEEKIRASGDAKIKKEKE  109
Mm  KDGSSEKHK.....................................................DKHKDRDKEKRRKEEKIRAAGDAKIKKEKE  109
Xl  KDGEKHRERDGEKHRDKNGEKHRDGEKHKEKDIEKHKEVEKHRVKDGEKHKEKDVEKHKEKDVEKHRDGEKHKHRDKDREKKKEEKMKSSSGGVKVKK.E  170
Ce  KDVGSDSED.....................................................DYKPEKKKSKKNNKKKAQESSEDDDEESEG   68
Dm  RDGSSSNSHRSG......SSSSHKDKDGSSSSKHKSSSGH.....................HKRSSKDKERRDKDKDRGSSSSSRHKSSS  162
Pf                                                                  MQSMEINDNNSIKNESTSDDDILINKIKQNLGNNKSCNSR   40
At          MGTETVSKPVMDNGSGDSDDDKPLAFKRNNTVASNSNQSKSNSQRSKAVPTTKVSPMRSPVTSPNGTTPSNKTSIVKSSMPSSSSKASPAKSPL   94

Hs  NG.........................FSSPPQ...IKDEPEDDGYFVPPKEDIKPLKRPRDEDDVD.....................................  146
Cg  NG.........................FSSPPR...IKDEPDDDGYFAPPKEDIKPLKRPRDEDDAD.....................................  148
Mm  NG.........................FSSPPR...IKDDPEDDGYFAPPKEDIKPLKRLRDEDDAD.....................................  148
Xl  NG.........................FSSPVR...VKDEPEDQGFYVSPKE.NKAMKRPR.EDDED.....................................  207
Ce  DVSEEDVKP...................QIHSDD...ELEEEDEAPTTDDEEEQKRKEKERRKKEKREKKKRKEKKRLEKENRKI.................  131
Dm  SSRDKERSSSSHKSSSSSSSSKSKHSSSRHSSSSS...SKDHPSYDGVFVKPEPVSQQLMHSGSVDAFQMQQLGSYEAAAAGTNFNGNGNVAGANYKNGY  259
Pf  SS.....................KKESIKKQKSNSELGIKKNTKKSLGIKKEEEK.KKQISKRKSNELK.....................................   87
At  RNDMPSTVKDRSQLQKDQSECKIEHEDSEDDRPLSSILSGNKGPTSSRQVSSPQPEKKNNGDRPLDRASRIIKDESDDETPISSMFRKKIDSGMSGGNQL  194
Sp                                                                                  MSSSDSVSLSIRRRQRRGSSKRI   23

Hs  ..........................................................................................................  146
Cg  ..........................................................................................................  148
Mm  ..........................................................................................................  148
Xl  ..........................................................................................................  207
Ce  ..........................................................................................................  131
Dm  EESIVDIKKEEESFNNLSQASSCDYSMSQFRADEPPFVVKHEQSYAEEDSTMNYNDHDDDADEMNDDEEDVPLAMRKRKQEATDRPDGGMDNDDDDDIPL  359
Pf  ..........................................................................................................   87
At  SNDEKKPLVQKLHQNGSTVKNEVPNGKVLGKRPLEKNSSADQSSLKKAKISASPTSVKMKQDSVKKEIDDKGRVLVSPKMKAKQLSTREDGTDDDDDDDV  294
Sc            MTIADASKVNHELSSDDDDDVPLSQTLKKRKVASMNSASLQDEAEPYDSDEAISKISKKKTKKIKTEPVQSSSLPSPPAKKSATSKPKKIK   91
Sp  SMKESDEESDSSENHPLSESLNKKSKSESDEDDIPIRKRRASSKKNMSNSSSKKRAKVMGNGGLKNGKKTAVVKEEEDFNEIAKPSPKHKRVSKANGSKN  123
Um        MNSIQVKNEPMLASFASTSTNGKAKRSAKPSLFSGSEVSSDDDEEKPLAKRPKVEDSDSDAPLTSTVSSQNGVQKRSGSSNNDDNDDDSDSDSDA   95

Hs  .....YKPKKIKTEDTKKEKKRKLEEEEDGKLKKPKNKDKDKKVPEPDNKK.....KKPKKEEEQKWKWWEEERYPEGIKWKFLEHKGPVFAPPYEPLPE  236
Cg  .....YKPKKIKTEDIKKEKKRKLEEEEDGKLKKTKNKDKDKKGAESDNKK.....KKPKKEEEQKWKWWEEERYPEGIKWKFLEHKGPVFAPPYEPLPE  238
Mm  .....YKPKKIKTEDIKKEKKRKSEEEEDGKLKKPKNKDKDKKVAEPDNKK.....KKPKKEEEQKWKWWEEERYPEGIKWKFLEHKGPVFAPPYEPLPE  238
Xl  .....YKPKKIKSEDDKKGKKRKQEEEDIKPKKKSKAKGNEEGVKKK..........KVKKEEEEKWKWWEEERHRDGIKWKFLEHKGPVFAPPYEPVPD  292
Ce  ...KEEDDEDSDDEDDEKAKKKKRKSKGAEKSKPSTSKKDAGGKKEPPKKK.....VKKEEDIEDIWEWWKEEKKPAGVKWNSLQHCGPLFAPPYIPLPS  223
Dm  LARKKVVKEKIKKESKEKSKKRVKEEPSDDYGNVKPKKKKMKKEPEPAVSPGKRQKAKAKVEEEEVWRWWEEEKRADGVKWSTLEHKGPVFAPRYERVPR  459
Pf  .....EKNNLKEGKKKYVEKKSRTVKDETKLTNVIKKETQNNKKPKKLL.......KKSEENFEPINRWWEKIDDQTDIQWNYLEHRGLIFSPPYV..QH  173
At  PISKRFKSDSSNSNTSSAKPKAVKLNSTSSAAKPKARNVVSPRSRAMTKNT....KKVTKDSKYSTSSKSSPSSGDGQKKWTTLVHNGVIFPPPYK..PH  388
Sc  KEDGDVKVKTTKKEEQENEKKKREEEEEEDKKAK.....................EEEEEYKWWEKENEDDTIKWVTLKHNGVIFPPPYQPLPS  164
Sp  GAKSAVKKEESDTDDSVPLRAVSTVSLTPYKSELPSGASTTQNR..........SPNDEEDEDEDYKWWTSENIDDTQKWTTLEHNGVIFAPPYEPLPK  212
Um  PLTALVKKSNGSDDDEDDDDDDDEGDDDDEEDDDDDDDDDDK............PLSKSSKSNRKPPKTMSITGSGEKKWDVLIHKGPRFPDPYQPLAK  182
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Fig. 1. Part 1, page 1.

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Hs  NVKFYYDGKVMKLSPKAEEVATFFAKMLDHEYTTKEIFRKNFFKDWRKEMTNEEKNI......ITNLSKCDFTQMSQYFKAQTE.....ARKQMSKEEKL  325
Cg  GVKFYYDGKVMKLSPKAEEVATFFAKMLDHEYTTKEIFRKNFFKDWRKEMTNDEKNV......ITNLSKCDFTQMSQYFKDQSE.....ARKQMSKEEKL  327
Mm  SVKFYYDGKVMKLSPKAEEVATFFAKMLDHEYTTKEIFRKNFFKDWRKEMTNDGKNT......ITNLSKCDFTQMSQYFKAQSE.....ARKQMSKEEKL  327
Xl  NVKFYYDGNLVKLSPKAEEVATFFAKMLDHEYTTKDIFRKNFFKDWKKEMTTDERNL......ITNLSKCDFNAMSLYFKEQSE.....ARKNMTKEEKL  381
Ce  HVHFKYGGEKMKLTLETEEIAQFYAGVLDHEYSTKEAFNKNFMKDWRKVMTVEERER......IHDLKKCDFRAIDAYQKEQRE.....IRKAMTKEEKL  312
Dm  NVRFYYDGKPLELSEETEEAATFYAKMLNHDYCTKEVFNNNFFKDFRKSMTPRERERI......IKDFRKCNFQEMFNYFQAESE.....KRKAASKEEKL  548
Pf  HVPIFYKSIKIELNAKSEELATYWCSAIGSDYCTKEKFILNFFKTFINSLENDNIIKQENETKLKKGDISNFKFIDFMPIKDHLLKLREEKLNKTKEEKE  273
At  GIKILYKGKPVDLTIEQEEVATMFAVMRETDYYTKPQFRENFWNDWRRLLGKKHV........IQKLDDCDFTPIYEWHLEEKE.....KKKQMSTEEKK  475
Sc  HIKLYYDGKPVDLPPQAEEVAGFFAALLESDHAKNPVFQKNFFNDFLQVLKESGGPLN..GIEIKEFSKCDFTKMFDYFQLQKE.....QKKQLTSQEKK  257
Sp  NVKLIYDGNPVNLPPEAEEVAGFYAAMLETDHAKNPVFQDNFFRDFLKVCDECNFNH.....NIKEFSKCDFTQMFHHFEQKRE.....EKKSMPKEQKK  302
Um  DVKLKYDGRPVDLPCQTEEEIAMFYAVKLETQHAQNAIFNRNFFDDFKTDLKKYPPRD...GTQIKSFDKLDFRDMYNYWRSLKD..AELERKKALAPSAR  277

Hs  KIKEENEKLLK.EYGFCIMDNHKERIANFKIEPPGLFRGRGNHPKMGMLKRRIMPEDIIINCSKDAKVPSPP..PG.HKWKEVRHDNKVTWLVSWTENIQ  421
Cg  KIKEENEKLLK.EYGFCVMDNHRERIANFKIEPPGLFRGRGNHPKMGMLKRRIMPEDIIINCSKDAKVPSPP..PG.HKWKEVRHDNKVTWLVSWTENIQ  423
Mm  KIKEENEKLLK.EYGFCVMDNHRERIANFKIEPPGLFRGRGNHPKMGMLKRRIMPEDIIINCSKDAKVPSPP..PG.HKWKEVRHDNKVTWLVSWTENIQ  423
Xl  KIKAENERLLQ.EYGYCIMDNHKERIANFRIEPPGLFRGRGDHPKMGKLKKRIMPEDIIINCSKDSKIPVAP..AG.HKWKEVRHDGKVTWLVSWTENIQ  477
Ce  KIKEEKEAEVK.IYGIAIIDGHRQKVANFRIEPPGVFRGRGGHPKMGLIKKRIMPEDVIINCGKDTEIPKPP..PG.HKWKEVRHDNTVTWLCSWTESVL  408
Dm  IKKNENEALMK.EFGFCMIDGHKEKIGNFRLEPPGLFRGRGEHPKMGMIKRRIQASDVSINCGKDSKVPSPP..PG.SRWKEVRHDNTVTWLASWIENVQ  644
Pf  EEKKMRMEKEL.PYTYALVDWIREKISSNKAEPPGLFRGRGEHPKQGLLKKRIFPEDVVINISKDAPVPRLYDNMCGHNWGDIYHDNKVTWLAYYKDSIN  372
At  ALKEEKMKQEE.KYMWAVVDGVKEKIGNFRVEPPGLFRGRGEHPKMGKLKKRIHPCEITLNIGKGAPIPECP.IAG.ERWKEVKHDNTVTWLAFWADPIN  572
Sc  QIRLEREKFEE.DYKFCELDGRREQVGNFKVEPPDLFRGRGAHPKTGKLKRRVNPEDIVLNLSKDAPVPPAP..EG.HKWGEIRHDNTVQWLAMWRENIF  353
Sp  AIKEKKDEEEE.KYKWCILDGRKEKVGNFRIEPPGLFRGRGSHPKTGSLKRRVYPEQITINIGEGVPVPEPL..PG.HQWAEVKHDNTVTWLATWHENIN  398
Um  KREIEERKAEETKWKICLVDGVEQRVGNVNVEPPGLFLGRGAHPKAGKVKRRISPGDITINHSGDHPAPQPP..AGMGDWAEVVEKKDVTWLAYWKENIN  375
Fv                                                MRAFTYKDGKLYEDKELTIPVHCSNPTYEILKHVKIPSH...LTDVIVYEQTYEQSLSRLIFVGLDSK   65
Vr                                                MRALFYKDGKLFTDNNFLNPVSDNNPAYEVLQHVKIPTH...LTDVVVYEQTWEEALTRLIFVGSDSK   65
Vc                                                MRALFYKDGKLFTDNNFLNPVSDDNPAYEVLQHVKIPTH...LTDVVVYEQTWEEALTRLIFVGSDSK   65
Or                                                MRALHLSDGKLFFDKELTQPVPDDNPAYAVLAKIRIPPH...LSDVVVYEQDLESAQQGLIFVGRDAK   65

Hs  G.SIKYIMLNPSSRIKGEKDWQKYETARRLKKCVDKIRNQYREDWKSKEMKVRQRAVALYFIDKLALRAGNEKEEGETADTVGCCSLRVEHINL......  514
Cg  G.SIKYIMLNPSSRIKGEKDWQKYETARRLKKCVDKIRNQYREDWKSKEMKVRQRAVALYFIDKLALRAGNEKEEGETADTVGCCSLRVEHINL......  516
Mm  G.SIKYIMLNPSSRIKGEKDWQKYETARRLKKCVDKIRNQYREDWKSKEMKVRQRAVALYFIDKLALRAGNEKEEGETADTVGCCSLRVEHINL......  516
Xl  G.SIKYIMLNPSSRIKGEKDWQKYETARRLKMCVEKIRNTYKEDWKSKEMKVRQRAVALYFIDKLALRAGNEKEEGETADTVGCCSLRVEHINL......  570
Ce  G.QNKYIMLNPSSKIKGEKDFEKYETARRLKKKIGGIRERYTDDFKSKEMRVRQRATALYFIDKLALRAGNEKDVDEAADTVGCCSLRVEHIKL......  501
Dm  G.QVKYIMLNPSSKLKGEKDHIKYETARRLDKVIDKIRATYRDEWKSKEMRVRQRAVALYFIDKLALRAGNEKDEDQ.ADTVGCCSLRVEHVQL......  736
Pf  D.QIKYTFLSAQSKFKGYKDLMKYENARRLKSCVHKIREDYKNKMKNKNIIDKQLGTAVYLIDFLALRVGGEKDIDEEADTVGCCSLRVEHISFAHDIPF  471
At  PKEFKYVFLGAGSSLKGLSDKEKYEKARNLTDHIDNIRTTYTKNFTAKDVKMRQIAVATYLIDKLALRAGNEKDDDE.ADTVGCCTLKVGNVEC......  665
Sc  N.SFKYVRLAANSSLKGQSDYKKFEKARQLKSYIDAIRRDYTRNLKSKVMLERQKAVAIYLIDVFALRAGGEKSEDE.ADTVGCCSLRYEHVTL......  445
Sp  N.NVKYVFLAAGSSLKGQSDLKKYEKSRKLKDYIDDIRKGYRKDLKNELTVERQRGTAMYLIDVFALRAGNEKGEDE.ADTVGCCSLRYEHVTL......  490
Um  G.QYKYVFLDATSNFKTNSDREKFEKARKLDTVVKQIRRDVNKNLKSKVRHERQIATIVCLIDNFSLRAGNEKGEDE.TETYGVCSLRCEHAQI......  467
Fv  G.RRQYFYGKMHVQRRNSARDTIFIKVHRVIDKIHKFIDDTIE...HKNDVLFQLGVFMLMETSFFIRMGKVKYLKE.NDTVGLLTLKNKNIVR......  154
Vr  G.RRQYFYGKMHVQNRNAKRDRIFVRVYNVMKRINCFINKNIK..KSSTDSNYQLAVFMLMETMFFIRFGKMKYLKE.NETVGLLTLKNKHIEI......  155
Vc  G.RRQYFYGKMHVQNRNAKRDRIFVRVYNVMKRINCFINKNIK..KSSTDSNYQLAVFMLMETMFFIRFGKMKYLKE.NETVGLLTLKNKHIEI......  155
Or  G.RKQYFYGRGHVERRTAVRNAVFVRVHRVMNKINAFIDDHLA...SGSEAEAQMAAFLLMETSFFIRVGKTRYERE.SGTVGMLTLRNKHLAE......  154

Hs  ......................HPELDGQEYVVEFDFLGKDSIRYYNKV.PVEKRVFKNLQLFMEN.............KQPEDDLFDRLN.........  569
Cg  ......................HPELDGQEYVVEFDFPGKDSIRYYNKV.PVEKRVFKNLQLFMEN.............KQPEDDLFDRLN.........  571
Mm  ......................HPELDGQEYVVEFDFPGKDSIRYYNKV.PVEKRVFKNLQLFMEN.............KQPEDDLFDRLN.........  571
Xl  ......................FQELDGQEFVVEFDFPGKDSIRYYNKV.PVEKRVFKNLQLFMEN.............KQPDDDLFDRLN.........  625
Ce  ..................FDSAKLNEDDKKEKEFVVEFDFLGKDSIRYFNRV.SVEKRVYKNLKIFMEG.............KAPSDDLFDRLD.........  563
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Fig. 1. Part 2, page 1.

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Dm  .....................HKELNGKENVVVFDFPGKDSIRYYNEV.EVEKRVFKNLELFMEH.............KKEGDDLFDRLN.....  791
Pf  KSVDSKEQKTNDEKVNKIPLPTNLESISSEDCYITLDFLGKDSIRYFNTV.KIDKQAYINIIIFCKN.............KNRDEGVFDQIT.....  549
At  ..........................IPPNKIKFDFLGKDSIQYVNTV.EVEPLVYKAIGQFQAG.............KSKTDDLFDELD.....  715
Sc  ........................KPPNTVIFDFLGKDSIRFYQEV.EVDKQVFKNLTIFKRP.............PKQPGHQLFDRLD.....  496
Sp  ........................KPPRTVVFDFLGKDSIRYYNEV.EVDPQVFKNLKIFKRP.............PKKEGDLIFDRLS.....  541
Um  ........................KMPDTIHLEFLGKDSMKFEEDLKITNPDVFKNIAMFLKSNGMKDKSGNYVRKKPSDPIFCAPESGSGKMQPL  539
Fv  ..........................ENRKILIHFVGKDKIIHNFTV.HSSNRLYKPLLRLIGR.............KEPDSFLFHKLS.....  203
Vr  ..........................SPDKIVIKFVGKDKVSHEFVV.HKSNRLYKPLLKLTDD.............SSPEEFLFNKLS.....  204
Vc  ..........................SPDEIVIKFVGKDKVSHEFVV.HKSNRLYKPLLKLTDD.............SSPEEFLFNKLS.....  204
Or  ........................AEGGEEIRVAFVGKDRVAHEFAV.REGQRLFAALRRLWDP.............GAPDRLLFDRLS.....  205

Hs  .TGILNKHLQDLMEGLTAKVFRTYNASITLQQQL.....................................................KELTAPDENIPA.KIL...  617
Cg  .TGILNKHLQDLMEGLTAKVFRTYNASITLQQQL.....................................................KELTAPDENVPA.KIL...  619
Mm  .TGILNKHLQDLMEGLTAKVFRTYNASITLQQQL.....................................................KELTAPDENVPA.KIL...  619
Xl  .TSILNKHLQDLMEGLTAKVFRTYNASITLQQQL.....................................................DELTNSDDNVPA.KIL...  673
Ce  .TATLNDHLRSLMDGLTVKVFRTYNASITLQEQL.....................................................IKLTNPKDNVAA.KIL...  611
Dm  .TQVLNEHLKELMEGLTAKVFRTYNASKTLQSQL.....................................................DLLTDPSATVPE.KLL...  839
Pf  .CSKLNEYLKEIMPTLSAKVFRTYNASITLDQQLKRIKEVYGKTTYSLYSGETELHKSKKRKSSHLTSDTNILSDASDSTINDVNNEYDENGINKKLSYA  648
At  .TSKLNAHLKELVPGLTAKVFRTYNASITLDEML.....................................................SQETK.DGDVTQ.KIV...  762
Sc  .PSILNKYLQNYMPGLTAKVFRTYNASKTMQDQL.....................................................DLIPN.KGSVAE.KIL...  543
Sp  .TNSLNKYLTSLMDGLSAKVFRTYNASYTMAEEL.....................................................KKMPK.NLTLAD.KIL...  588
Um  QPNSVNQFLSKYMKGLSAKVFRTYNASVTFQGLL.....................................................EQTEEWLKSRPNAAE.REI...  591
Fv  .EKKVYKAVQQF..GIRLKDLRTYGVNYTFLYNF.....................................................WTNVKSLNPIPPIKKM...  250
Vr  .ERKVYECIKQF..GIRIKDLRTYGVNYTFLYNF.....................................................WTNVKSISPLPSPKKL...  251
Vc  .ERKVYECIKQF..GIRIKDLRTYGVNYTFLYNF.....................................................WTNVKSISPLPSPKKL...  251
Or  .ERRVYTFMRRF..GIRVKDLRTYGVNYTFLYNF.....................................................WSNVRSLEPRPSVKSL...  252

Hs  ..........................SYNRANRAVAILCNHQRAP.PKTFEKSMMNLQTKIDAKKEQLADARRDLKSAKADAKVMKDAKTKKVVESKK  688
Cg  ..........................SYNRANRAVAILCNHQRAP.PKTFEKSMMNLQSKIDAKKDQLADARRDLKSAKADAKVMKDAKTKKVVESKK  690
Mm  ..........................SYNRANRAVAILCNHQRAP.PKTFEKSMMNLQSKIDAKKDQLADARRDLKSAKADAKVMKDAKTKKVVESKK  690
Xl  ..........................SYNRANRAVAILCNHQRAP.PKTFEKSMMNLQGKIDAKKDQLADARREFKSAKADAKVRRDEKTKKLVESKK  744
Ce  ..........................SYNRANRQVAILCNHQRAV.SKGFDESMQKLEQKIKDKKKEVKEAEAALKSARGAEKEKAQKK.........  673
Dm  ..........................AYNRANRAVAILCNHQRSV.PKSHEKSMENLKEKIKAKREAIEKCESEYHSRDEKKGKQ.............  897
Pf  TTVGKENDVDDKNSPIEVDVSNINELINFYNNANREVAILCNHQRSI.PKQHDTTMSKIKKQIELYNEDIKEYKKYLQHLKKNSDKKFIFVSKVSTLDGT  747
At  ..........................VYQKANKEVAIICNHQRTV.SKTHGAQIEKLTARIEELKEVLKELKTNLDRAKKGKPPLEGSDGKKIRSLEP  833
Sc  ..........................KYNAANRTVAILCNHQRTV.TKGHAQTVEKANNRIQELEWQKIRCKRAILQLDKDLLKKEPKYFEEIDDLTK  614
Sp  ..........................FYNRANRTVAILCNHQRSV.TKNHDVQMERFAERIKALQYQRMRLRKMMLNLEPKLAKSKPELLAKEEGITD  659
Um  ....................NQTNLRLAYNEANRQVAILCNHQKTVNPMLLNRNLERTQDKIFQIRYEIMKEQQKILTFHKVSELKKEFKVKEHPFMKQ  670
Fv  ..........................ISTSIKQTADIVGHTP.....................................................  266
Vr  ..........................IALTIKQTAEVVGHTP.....................................................  267
Vc  ..........................IALTIKQTAEVVGHTP.....................................................  267
Or  ..........................ICTSVRQTAETVGHTP.....................................................  268
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Fig. 1. Part 1, page 2.

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Hs  KA..........................................................................................  690
Cg  KA..........................................................................................  692
Mm  KA..........................................................................................  692
Xl  KA..........................................................................................  746
Ce  ............................................................................................  673
Dm  ............................................................................................  897
Pf  LRPNKVKENMKEESC.............................................................................  762
At  NAW.........................................................................................  836
Sc  EDEATIHKRIIDREIEKYQRKFVRENDKRKFEKEE.LLPESQLKEWLEKVDEKKQEFEKELKTGEVELKSSWNS.....................  687
Sp  SWIVKHHETLYELEKEKIKKKFDRENEKLAAEDPKSMLPESELEVRLKAADELKKALDAELKSKKVDPGRSS.....................  731
Um  FDKIMQKQDLDAEKVKQYEEQMISDKKSKLESTFKRQQSELQYQLEQKGLTGDDGTPKKGKKAKNVEEEVRSSIKGFKDKKQVDEELKALNETAKRLEKE  770
Fv  ............................................................................................  266
Vr  ............................................................................................  267
Vc  ............................................................................................  267
Or  ............................................................................................  268

Hs  ............VQRLEEQLMKLE.......VQATDREENKQIALGTSKLNYLDPRITVAWCKKWGVPIEKIYNKTQREKFAWAIDMADEDYEF          765
Cg  ............VQRLEEQLMKLE.......VQATDREENKQIALGTSKLNYLDPRITVAWCKKWGVPIEKIYNKTQREKFAWAIDMTDEDYEF          767
Mm  ............VQRLEEQLMKLE.......VQATDREENKQIALGTSKLNYLDPRITVAWCKKWGVPIEKIYNKTQREKFAWAIDMTDEDYEF          767
Xl  ............VQRIEEQLMKLE.......VQATDREENKQIALGTSKLNYLDPRISVAWCKKYGVPIEKIYNKTQREKFAWALDMADDDFKF          821
Ce  ............YDRLKEQLKKLK.......ISRTDKDENKQIALGTSKLNYIDPRITVAWCKKFEVPLEKVFTKTHREKFRWAIDMTNSSDEEYVF  751
Dm  ............LERLRDQLKKLE.......LQETDRDENKTIALGTSKLNYLDPRISVAWCKKHDVPIEKIFNKTQRTKFLWAVHMADENYRF          972
Pf  ............KKKLITLIKKVE....LLNNQMKVRDDNKTIALGTSKINYMDPRITVAFCKKFEIPIEKVFNRSLRLKFPWAMFATKNFTF          839
At  ............EKKIAQQSAKIE....KMERDMHTKEDLKTVALGTSKINYLDPRITVAWCKRHEVPIEKIFTKSLLEKFAWAMDVEPE.YRFSRR  916
Sc  ............VEKIKAQVEKLEQRIQTSSIQLKDKEENSQVSLGTSKINYIDPRLSVVFCKKYDVPIEKIFTKTLREKFKWAIESVDENWRF  769
Sp  ............MEQLEKRLNKLNERINVMRTQMIDKDENKTTALGTSKINYIDPRLTYSFSKREDVPIEKLFSKTIRDKFNWAADTPPD.WKW  812
Um  RKTNKSQATSCNFVSSAKKILSKYEMIKKQEAELVNKNNTSDVALSTSKLNYIDPRITLAWLKEWDDRLSDLGQGKAAPKKKVKKEEEENDIKPKKKDAK  870
Fv  ..........................................SISKRAYIANTVLEYLTHDSE.LINTIRDISFDEFIRLITDYITNTQTV          314
Vr  ..........................................SISKRAYMATTILEMV.KDKN.FLDVVSKTTFDEFLSIVVDHVKSSTDG          314
Vc  ..........................................SISKRAYMATTILEMV.KDKN.FLDVVSKTTFDEFLSIVVDHVKSSTDG          314
Or  ..........................................SISRSAYMATAVLELV.RDGA.FLDRVAATDTLDDFVDIVVDYVNNSEQVNG        318

Um  GAASKKRAAKTGLANSTGDSEKMELGLQVMNISQFFANALQKKFKWAASGDDGRDISAKWVFVKDAQSKMRKLDSAERKGQKGGSMAAMTDAADSKEAQP  970

Um  KVNCVLKKQTSADRKMSKPIKAVDKTEESDDDLSSDSSDDGDKPLASVV                                                  1019
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Fig. 1. Part 2, page 2.

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HsTop2a                                                    MEVSPLQPVNENMQVNKIKKNEDAKKRLSVERIYQKKTQLEHILLRPDTYIGSVE.   55
CgTop2a                                                    MELSPLQPVNENMQMNK.KKNEDAKKRLSIERIYQKKTQLEHILLRPDTYIGSVE.   54
MmTop2a                                                    MELSPLQPVNENMLMNK.KKNEDGKKRLSIERIYQKKTQLEHILLRPDTYIGSVE.   54
RnTop2a                                                    MEVSPLQPVNENMLLNK.KKNEDGKKRLSVERIYQKKTQLEHILLRPDTYIGSVE.   54
HsTop2b                                          MAKSGGCGAGAGVGGGNGALTWVNNAAKKEESETANKNDSSKKLSVERVYQKKTQLEHILLRPDTYIGSVE.   71
ClTop2b                                                  MAKSSLAGADGALTWVNNAAKKEELETSNKNDSSKKLSVERVYQKKTQLEHILLRPDTYIGSVE.   64
MmTop2b                                                  MAKSSLAGSDGALTWANNATKKEELETANKNDSTKKLSVERVYQKKTQLEHILLRPDTYIGSVE.   64
DmTop2                                                                                  MENGNKALSIEQMYQKKSQLEHILLRPDSYIGSVE.   35
CeTop2b                    MSDSDSEFSIEDSPKKKTAPKKEKASPKKKKDDANESMVMTEEDRNVFTSIDKKGGGSKQMAIEDIYQKKSQLEHILLRPDTYIGSVE.   88
CeTop2  FLNHSCDPNVHVQHVMYDTHDLRLPWVAFFTRKYVKAGDELTWDYQYTQDQTATTQLTCHCGAENCTGRLLKKAAAKYEKKSPTEHVLLRPDTYIGGVA.   99
AtTop2                                                                  MATKLPLQNSNAANVAKAPAKSRAAAGGKTIEEMYQKKSQLEHILLRPDTYIGSIE.   56
ScTop2                                                                                  MSTEPVSASDKYQKISQLEHILKRPDTYIGSVE.   33
SpTop2  MSIDADFSDYEDEASGDENVLPNTTTKRKASTTSSKSRAKKASTPDLRQTSLTSMTASEQIPLVTNNGNGNSNVSTQYQRLTPREHVLRRPDTYIGSIE.   99
TbTop2                                                                                  MAEAHKYKKLTPIEHVLTRPEMYIGSLD.   28
TcTop2                                                                                  MAEASKYKKLTPIDHVLIRPEMYVGSVD.   28
CfTop2                                                                                  MTDASKYQKLTPIDHVLLRPEMYVGSIE.   28
ASFVTop                                                                                   MEAFEISDFKEHAKKKS.MWAGALNK   25
EcGyrB                                                                                MSNSYDSSSIKVLKGLDAVRKRPGMYIGDTDD   32
HiGyrB                                                                                MSETTNDNYGASSIKVLKGLDAVRKRPGMYIGDTDD   36
PmGyrB                                                                                MSNTYDSSSIKVLKGLDAVRKRPGMYIGDTDD   32
BaGyrB                                                                                MIDTYDSSKIKILRGLDAVRKRPGMYIGDTDD   32
PpGyrB                                                                                MSENQTYDSSSIKVLKGLDAVRKRPGMYIGDTDD   34
NgGyrB                                                                                MTEQKHEEYGADSIQVLEGLEAVPKRPGMYIGDTQD   36
CcGyrB                                                          MTENTEDQVPDLSTPEMTTEEAAAQYGADSIKVLKGLDAVRKRPGMYIGDTDD   53
BsGyrB                                                                                MEQQQNSYDENQIQVLEGLEAVRKRPGMYIGSTNS   35
SpGyrB                                                                               MTEEIKNLQAQDYDASQIQVLEGLEAVRMRPGMYIGSTSK   40
SaGyrB                                                                               MVTALSDVNNTDNYGAGQIQVLEGLEAVRKRPGMYIGSTSE   41
HfGyrB                                                                                MSQDNEYGAGQIQVLEGLEAVRKRPAMYIGSTDS   34
ScGyrB                                                                                MGDNYNSESIQILEGLEAIRKRPGMYIGATNA   32
MgGyrB2                                                                               MKSNYSATNIKILKGLDAVKKRPGMYIGSTDS   32
BsGyrB2                                                                             VRLARKQQFDYNEDAIQVLEGLEAVRKRPGMYIGSTDA   38
BbGyrB                                                                                MEGLLNYVASNIQVLKGLEAVRKRPGMYIGSVSI   34
BbGyrB2                                                                               MKTQNYDESKIITLSSLEHIRLRSGMYIGRLGD   33
MpGyrB                                                                               MEDNNKTQAYDSSSIKILEGLEAVRKRPGMYIGSTGE   37
MaGyrB                                                                               MNNTKKDQYSSQSIKVLEGLSAVRKRPGMYIGSTDQ   36
MgGyrB                                                                               MEENNKANIYDSSSIKVLEGLEAVRKRPGMYIGSTGE   37
MhGyrB                                                                               MDKIEEIHKYNADNIQILEGLEAVRKRPGMYIGSIGF   37
MlGyrB                                                          MTAAVTGPLTCNLKESIQTVAAQRKAQDEYGAASITILEGLEAVRKRPGMYVGSTGE   57
MtGyrB                                                                MHATPEESIRIVAAQKKKAQDEYGAASITILEGLEAVRKRPGMYIGSTGE   50
MsGyrB                                                                               MAAQKNNAPKEYGADSITILEGLEAVRKRPGMYIGSTGE   39
SoGyrB                                                        VADSGNPNENNPSTDTGVNDAVSTSHGDASASYDASAITVLEGLDAVRKRPGMYIGSTGE   60
SsGyrBs                                                       VADSGNPNENTPSVATGENGEV.......TGSYNASAITVLEGLDAVRKRPGMYIGSTGE   53
SsGyrBr                                                             VTTYDTRTATDTRGSEQPGHVGTASYDANAITVLDGLDAVRKRPGMYIGSTGE   53
EcParE                                                                                MTQTYNADAIEVLTGLEPVRRRPGMYTDTT..   30
StParE                                                                                MTQTYNADAIEVLTGLEPVRRRPGMYTDTT..   30
HiParE                                                                                MTTNYSAQEITVLKDLEPVQIRPGMYTDTT..   30
T4Gn39                                                                                    MIKNEIKILSDIEHIKKRSGMYIGSSA.   27
T2Gn39                                                                                    MIKNEIKILSDIEHIKKRSGMYIGSSA.   27
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Fig. 2. Part 1, page 1.

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HsTop2a .LVTQQMWVY.DEDVG.INYREVTFVPGLYKIFDEILVNAAD...NKQRDPKMSCIRVTIDPENNLISIWNNGKGIPVVEHKV......EKMYV.PALIF  142
CgTop2a .LVTQQMWVY.DEDVG.INYREVTFVPGLYKIFDEILVNAAD...NKQRDPKMSCIRVTIDPENNLISIWNNGKGIPVVEHKV......EKMYV.PALIF  141
MmTop2a .LVTQQMWVY.DEDVG.INYREVTFVPGLYKIFDEILVNAAD...NKQRDPKMSCIRVTIDPENNVISIWNNGKGIPVVEHKV......EKIYV.PALIF  141
RnTop2a .LVTQQMWVY.DEDVG.INYREVTFVPGLYKIFDEILVNAAD...NKQRDPKMSCIRVTMM.RNNLISIWNNGKGIPVVEHKV......EKMYV.PALIF  140
HsTop2b .PLTQFMWVY.DEDVG.MNCREVTFVPGLYKIFDEILVNAAD...NKQRDKNMTCIKVSIDPESNIISIWNNGKGIPVVEHKV......EKVYV.PALIF  158
ClTop2b .PLTQLMWVY.DEDVG.MNCREVTFVPGLYKIFDEILVNAAD...NKQRDKNMTCIKVSIDPESNIISIWNNGKGIPVVEHKV......EKVYV.PALIF  151
MmTop2b .PLTQLMWVY.DEDVG.MNCREVTFVPGLYKIFDEILVNAAD...NKQRDKNMTCIKVSIDPESNIISIWNNGKGIPVVEHKV......EKVYV.PALIF  151
DmTop2  .FTKELMWVY.DNSQNRMVQKEISFVPGLYKIFDEILVNAAD...NKQRDKSMNTIKIDIDPERNMVSVWNNGQGIPVTMHKE......QKMYV.PTMIF  123
CeTop2b HTEKTPMWVY.NMEESKLEQRDISYVPGLYKIYDEILVNAAD...NKQRDPKMNTIKITINKEKNEISVYNNGKGIPVTQHKV......EKVYV.PELIF  177
CeTop2  MREDQIIWLR.DSENRKMIAKEVTYPPGLLKIFDEILVNAAD...NKARDSSMNRLEVWLDRETARISVWNNGSGLPVEIHPT......EGIYV.PTLVF  188
AtTop2  .KHTQTLWVY.EKDEM..VQRPVTYVPGLYKIFDEILVNAAD...NKQRDAKMDSVQVVIDVEQNLISVCNSGAGVPVEIHQE......EGIYV.PEMIF  142
ScTop2  .TQEQLQWIY.DEETDCMIEKNVTIVPGLFKIFDEILVNAAD...NKVRDPSMKRIDVNIHAEEHTIEVKNDGKGIPIEIHNK......ENIYI.PEMIF  121
SpTop2  .PTTSEMWVF.DSEKNKLDYKAVTYVPGLYKIFDEIIVNAAD...NKVRDPNMNTLKVTLDPEANVISIYNNGKGIPIEIHDK......EKIYI.PELIF  187
TbTop2  .TTATPMFIY.DEQKGHMVWETVKLNHGLLKIVDEILLNASDNI..SNRSARMTYIRVTIT.DTGEITIENDGAGIPIVRSRE......HKLYI.PEMVF  116
TcTop2  .TSSSSMFVF.DHEKGRMVWESLKVNHGLLKIVDEILLNASDNI..ANKGGRMTYIRVHIT.EAGEITIENDGAGIPIVRSKE......HKLYI.PEMVF  116
CfTop2  .TQSIPMFVF.DPAKGKMVWESMQVNQGLLKIVDEILLNAADNINNSVRGARMTYISIKIS.DSGEIMVENDGAGLPIVKSKE......HKMYI.PEMVF  118
ASFVTop VTISGLMGVFTEDEDLMALPIHRDHCPALLKIFDELIVNATDHE.RACHSKTKKVTYIKISFDKGVFACENDGPGIPIAKHEQASLIAKRDVYV.PEVAS  123
EcGyrB  GT.....................GLHHMVFEVVDNAIDE....ALAGHCKEIIVTIHADNS.VSVQDDGRGIPTGIHPE......EGVSA.AEVIM   95
HiGyrB  GT.....................GLHHMVFEVVDNAIDE....ALAGHCSDIIVTIHDDNS.VSVQDDGGGIPVDIHPE......EGVSA.AEVIM   99
PmGyrB  GT.....................GLHHMVFEVVDNAIDE....ALAGYCDEIIVTIHSDNS.VSVRDDGRGIPTGIHEE......EGVSA.AEVIM   95
BaGyrB  GS.....................GLHHMVFEIVDNSIDE....ALAGFCKEIKVVIHSDNS.VSIKDDGRGIPTDIHPE......EKISAA-       91
PpGyrB  GS.....................GLHHMVFEVVDNSIDE....ALAGHCDDITVIIHTDES.ISVRDNGRGIPVDVHKE......EGVSA.AEVIM   97
NgGyrB  GS.....................GLHHMVFEVLDNAIDE....ALAGHCDKITVTIHADHS.VSVADNGRGMPTGIHPK......EGRSA.AEVIM   99
CcGyrB  GS.....................GLHHMVYEVVDNAIDE....ALAGHATKVQVILNADGS.VTVTDDGRGIPVDMHEG......EGVSA.AEVIM  116
BsGyrB  K......................GLHHLVWEIVDNSIDE....ALAGYCTDINIQIEKDNS.ITVVDNGRGIPVGIHEK......MGRPA.VEVIM   97
SpGyrB  E......................GLHHLVWEIVDNSIDE....ALAGFASHIQVFIEPDDS.ITVVDDGRGIPVGIQEK......TGRPA.VETVF  102
SaGyrB  R......................GLHHLVWEIVDNSIDE....ALAGYANQIEVVIEKDNW.IKVTDNGRGIPVDIQEK......MGRPA.VEVIL  103
HfGyrB  R......................GLHHLVYEVVDNSIDE....ALAGHCDAIEVALHEDGS.VSVTDNGRGIPVGTHEQ......YDRPA.LEVIM   96
ScGyrB  R......................GLHHLVWEIVDNSIDE....VLANFANKIKIILNKDES.ITVIDNGRGIPIEIHPK......TKVST.LETVF   94
MgGyrB2 K......................GLHHMLWEILANSVDE....VLAGYATNITVTLDLNNT.ITVSDDGRGIPYEIHQD......SNIST.IDTVF   94
BsGyrB2 R......................GLHHLVYEIVDNSVDE....VLAGHGDHIIVKIHKDNS.ISVQDRGRGMPTGMHKL.......GKPT.PEVIL   99
BbGyrB  N......................GLHHLVYEVVDNSIDE....ALAAFCDRIDVIINLDNT.ITVIDNGRGIPTDIHEE......EGISVTLELVL   97
BbGyrB2 G.....................SNIDDGIYVLIKEIIDNSIDE....FIMGYGNEIFIKK.ENNL.ISIRDYGRGIP-                     83
MpGyrB  E......................GLHHMIWEIIDNSIDE....AMGGFASTVKLTL.KDNFVTIVEDDGRGIPVDIHPK......TNRST.VETVF   99
MaGyrB  K......................GLHHMIWEIIDNSVDE....MMAGYGTTVKLTL.KDNYLVEVEDDGRGIPVDIHEK......TNKST.VETVL   98
MgGyrB  E......................GLHHMIWEIVDNSIDE....AMGGFASFVKLTL.EDNFVTRVEDDGRGIPVDIHPK......TNRST.VETVF   99
MhGyrB  K......................GLHHLLWEIVDNSVDE....AMAGFATEIKIKL.YPNNVIEVEDNGRGMPTGIHSG......TKKSA.VETIL   99
MlGyrB  R......................GLHHLIWEVVDNSVDE....AMAGYATQVDVRL.FDDGSVEVADNGRGIPVAVHAT.......GVPT.VDVVM  118
MtGyrB  R......................GLHHLIWEVVDNAVDE....AMAGYATTVNVVL.LEDGGVEVADDGRGIPVATHAS.......GIPT.VDVVM  111
MsGyrB  R......................GLHHLIWEVVDNAVDE....AMAGFATRVDVKI.HADGSVEVRDDGRGIPVEMHAT.......GMPT.IDVVM  100
SoGyrB  R......................GLHHLVQEVVDNSVDE....ALAGHADTIDVTILPDGG.VRVVDNGRGIPVGIVPS......EGKPA.VEVVL  122
SsGyrBs R......................GLHHLVTEVVDNSVDE....ALAGHADTIDVTILADGG.VRVVDNGRGIPVGIVPS......EGKPA.VEVVL  115
SsGyrBr R......................GLHHLVQELVDNSVDE....ALAGVADRIDVTVLADGG.VRVVDNGRGIPVGMHPV......EKRPA.VEVVL  115
EcParE  .......................RPNHLGQEVIDNSVDE....ALAGHAKRVDVILHADQS.LEVIDDGRGMPVDIHPE......EGVPA.VELIL   91
StParE  .......................RPNHLGQEVIDNSVDE....ALAGHAKRVDVILHADQS.LEVIDDGRGMPVDIHPE......EGVPA.VELIL   91
HiParE  .......................RPNHLAQEVIDNSVDE....ALAGFATKIEVILHPDQS.IEVTDNGRGMPVDIHPT......EGVSG.VEVIL   91
T4Gn39  .NETHERFMF.......GKWESVQYVPGLVKLIDEIIDNSVDE.GIRTKFKFANKINVTI.KNNQ.VTVEDNGRGIPQAMVKTPTG...EEIPG.PVAAW  112
T2Gn39  .NEMHERFLF.......GKYESVQYVPGLVKLIDEIIDNSVDE.GIRTKFKLANKINVTI.KNNQ.VTVEDNGRGIPQAMVKTPTG...EEIPG.PVAAW  112
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Fig. 2. Part 2, page 1.

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HsTop2a GQLLTSSNYDDDEKKVTGGRNGYGAKLCNIFSTKFTV.ETASREYKKMFKQTWMDNMGRAGE.MELKPFN..GEDY.TCITFQPDLSKFKM.......QS  230
CgTop2a GQLLTSSNYDDDEKKVTGGRNGYGAKLCNIFSTRFTV.ETASKEYKKMFKQTWMDNMGRAGD.MELKPFN..GEDY.TCITFQPDLSKFKM.......QS  229
MmTop2a GQLLTSSNYDDDEKKVTGGRNGYGAKLCNIFSTKFTV.ETASRAYKKMFKQTWMDNMGRAGD.MELKPFS..GEDY.TCITFQPDLSKFKM.......QS  229
RnTop2a GQLLTSSNYDDDEKKVTGGRNGYGAKLCNIFSTKFTV.ETASREYKKMFKQTWMDNMGRAGD.MELKPFS..GEDY.TCITFQPDLSKFKM.......QS  228
HsTop2b GQLLTSSNYDDDEKKVTGGRNGYGAKLCNIFSTKFTV.ETACKEYKHSFKQTWMNNMMKTSE.AKIKHFD..GEDY.TCITFQPDLSKFKM.......EK  246
ClTop2b GQLLTSSNYDDDEKKVTGGRNGYGAKLCNIFSTKFTV.ETACKEYKHSFKQTWMNNMMKTSE.AKIKHFD..GEDY.TCITFQPDLAKFKM.......EK  239
MmTop2b GQLLTSSNYDDDEKKVTGGRNGYGAKLCNIFSTKFTV.ETACKEYKHSFKQTWMNNMMKTSE.AKIKHFD..GEDY.TCITFQPDLSKFKM.......EK  239
DmTop2  GHLLTSSNYNDDEKKVTGGRNGYGAKLCNIFSTSFTV.ETATREYKKSFKQTWGNNMGKASD.VQIKDFN..GTDY.TRITFSPDLAKFKM.......DR  211
CeTop2b GTLLTSSNYNDDEKKVTGGRNGYGAKLCNIFSTKFTL.ETSSRDYKSAFKQTWIKNMTRDEE.PKIVKST..DEDF.TKITFSPDLAKFKM.......KE  265
CeTop2  GNLFTSSNYDDSEIKTVGGRNGYGAKLCNIFSKEFIV.ETVDTRIKRRFRQKWYDNMKKCNEAEVVEILDETVKDY.TKVEFVPDLERFQI.......DK  279
AtTop2  GHLLTSSNYDDNVKKTTGGRNGYGAKLTNIFSTEFII.ETADGKRLKKYKQVFENNMGKKSE.PVITKCNK.SENW.TKVTFKPDLKKFNM.......TE  231
ScTop2  GHLLTSSNYDDDEKKVTGGRNGYGAKLCNIFSTEFIL.ETADLNVGQKYVQKWENNMSICHP.PKITSYKK.GPSY.TKVTFKPDLTRFGM.......KE  210
SpTop2  GNLLTSSNYDDNQKKVTGGRNGYGAKLCNIFSTEFVV.ETADKERMKKYKQTWYDNMSRKSE.PVITSLKK.PDEY.TKITFKPDLAKFGM.......DK  276
TbTop2  GHLLTSSNYDDDNQNAVAGRHGYGAKLTNILSLSFSV...CCRTNGREFHMSWQDHMRKATA.PRVSNVGT.KEKNVTRVKFLPDYERFGM.....KEKK  206
TcTop2  GHLLTSSNYDDTSQNAVAGRHGYGAKLTNILSHRFSV...CCRTKGKEFHMSWHDHMRRATA.PRVSNVDP.KEKNLTRVKFLPDYERFGL.....DANK  206
CfTop2  GHLLTSSNYNNDASSTTAGRHGYGAKLTNILSTKFSV...VCRTAGREFHMSWTDHMRMATT.PRVSNVDP.KEKNVTRVTFMPDYAHFGF.....PTAA  208
ASFVTop CHFLAGTNINKAKDCIKGGTNGVGLKLAMVHSQWAIL...TTADGAQKYVQHINQRLDIIEP.PTITPS...REMF.TRIELMPVYQELGYAQPLSETEQ  215
EcGyrB  TVLHAGGKFDDNSYKVSGGLHGVGVSVVNALSQKLEL..VIQREG.KIHRQIYEHGVPQAP....LAVTGE.TEKTGTMVRFWPSLETFT......NVTE  181
HiGyrB  TVLHAGGKFDDNSYKVSGGLHGVGVSVVNALSDKLQL..TIRRQG.HVHEQFYHLGEPQSP....LTVIGE.TEATGTTVRFWPSSDIFA.......ITT  184
PmGyrB  TVLHAGGKFDDNSYKVSGGLHGVGVSVVNALSEKLEL..TIHRDG.KIHQQIYRSGVPDDR....LKVIGE.TDKSGTFVRFWPSLDTFKG.....ETEF  182
PpGyrB  TVLHAGGKFDDNSYKVSGGLHGVGVSVVNALSEKLVL..TVRRSG.KIWEQTYVHGVPQAP....MAVVGE.SETTGTHIHFKPSAETF.......KNIH  182
NgGyrB  TVLHAGGKFDNNSYKISGGLHGVGVSVVNALSDWVTL..TIYRDG.KEHFVRFVRGETEEP....LKIVGD.SDKKGTTVRFLAGTETF.......GNIE  184
CcGyrB  TQLHAGGKFDQNSYKVSGGLHGVGVSDVNALSKWLEL..LIHRNG.KVHQMRFERGDAVTSLK..VTGDSP.VR...TEGPKAGETLTGT......EVTF  201
BsGyrB  TVLHAGGKFDGSGYKVSGGLHGVGASVVNALSTELDV..TVHRDG.KIHRQTYKRGVPVTD....LEIIGE.TDHTGTTHFVPDPEIFS......ETTE  183
SpGyrB  TVLHSGGKFGGGGYKVSGGLHGVGLSVVNALSTQLDV..HVHKNG.KIHYQEYRRGHVVAD....LEIVGD.TDKTGTTVHFTPDPKIFT......ETTI  188
SaGyrB  TVLHAGGKFGGGGYKVSGGLHGVGSSVVNALSQDLEV..YVHRNE.TIYHQAYKKGVPQFD....LKEVGT.TDKTGTVIRFKADGEIFT......ETTV  189
HfGyrB  TVLHAGGKFDNKSYQVSGGLHGVGVTVVNALSSELEV..EVKHDG.AVWTHRFEVGEPQVEEFERVRDLEP.GEDTGTTIRFWPDDGIF.......ETTE  185
ScGyrB  TILHAGGKFDSNTYKISGGLHGVGASVVNALSKYLKV..EVRKNN.KKYVMEFHNGGQILTP...IKEVGS.TSETGTTVTFLPDEKIFK......ETTI  181
MgGyrB2 TFLHAGGKFDDQSYKLAGGLHGVGASVVNALSDHLEV..TVKRNG.QIYQSVYQAGGKIIQK...AKKIGD.TTSHGTTVSFHADPKVFK.......KAQ  180
BsGyrB2 TVLHAGGKFGQGGYKTSGGLHGVGASVVNALSEWLTV..TIERDG.FVYQQRFENGGKPVTS...LEKIGK.TKKTGTLTHFKPDPTMFS.......TTT  185
BbGyrB  TKLHSGGKFNKGTYKVSGGLHGVGISVVNALSSFLEV..YVNRDG.KIFRQTFSKGIPTSK....VEVVGE.SSVTGTKVTFLADSEIF.......ETLD  182
MpGyrB  TVLHAGGKFDNDSYKVSGGLHGVGASVVNALSSSFKV..WVAREH.QQYFLAFHNGGEVIGDL..VNEGKC.DKEHGTKVEFVPDFTVM.......EKSD  186
MaGyrB  TILHAGGKFDSDTYSMSGGLHGVGASVVNALSSSFKV..WVNRDY.KIHYIEFKDGGVSLKPL..EIIGTD.NKKQGTRIQFVPDFSIM.......EQFE  185
MgGyrB  TVLHAGGKFDNDSYKVSGGLHGVGASVVNALSSSFKV..WVFRQN.KKYFLSFSDGGKVIGDL..VQEGNS.EKEHGTIVEFVPDFSVM.......EKSD  186
MhGyrB  TVLHAGGKFDGSNYKVSGGLHGVGASVVNALSSEFEV..WVKRDG.KLHYQQFRNGGIPVKPLE.VIGNFS.EVETGTTIKFHPDYTIM.......EKEN  187
MlGyrB  TQLHAGGKFGGKDSGYNVSGGLHGVGVSVVNALSTRVEVDIKRDG.YEWSQFYDKAVPGI.....LKQGEA.TEATGTTIRFWADPDIF.......ETTK  204
MtGyrB  TQLHAGGKFDSDAYAISGGLHGVGVSVVNALSTRLEV..EIKRDG.YEWSQVYEKSEPLG.....LKQGAP.TKKTGSTVRFWADPAVF.......ETTE  195
MsGyrB  TQLHAGGKFDGETYAVSGGLHGVGVSVVNALSTRLEA..TVLRDG.YEWFQYYDRSVPGK.....LKQGGE.TKETGTTIRFWADPEIF.......ETTD  184
SoGyrB  TVLHAGGKFGGGGYAVSGGLHGVGVSVVNALSTRVAV..EVKTDG.YRWTQEYKLGVPTAS....LARHEA.TEETGTTVTFWADGDIF.......ETTD  207
SsGyrBs TVLHAGGKFGGGGYSVSGGLHGVGVSVVNALSTKVAV..EVKTDG.YRWTQDYKLGVPTRR....CAQNEA.TDETGTTVTFWADPDVF.......ETTE  200
SsGyrBr TVLHAGGKFGGGGYGVSGGLHGVGLSVVNALSTRLSA..EIWTDG.HRWTQDYRDGAPTAP....LARHEA.TSRTGTSLTFWADGDIF.......ETTE  200
EcParE  CRLHAGGKFSNKNYQFSGGLHGVGISVVNALSKRVEV..NVRRDG.QVYNIAFENGEKVQDLQ..VVGTCG.KRNTGTSVHFWPDETFF.......DSPR  178
StParE  CRLHAGGKFSNKNYQFSGGLHGVGISVVNALSKRVEV..TVRRDG.QVYNIAFENGEKVQDLQ..VVGTCG.KRNTGTSVHFWPDESFF.......DSPR  178
HiParE  TKLHAGGKFSNKNYEFAGGLHGVGISVVNALSERVDI..QVKRNG.EIYKIAFENGSKVBELE..IIGTCG.RRTTGTIVHFKPNPKYF.......DSAK  178
T4Gn39  TIPKAGGNFGDDKERVTGGMNGVGSSLTNIFSVMFVG...ETGDGQNNIVVRCSNGMENKS....WEDIPG.KWK.GTRVTFIPDFMSF.......ETNE  196
T2Gn39  TIPKAGGNFGDDKERVTGGMNGVGSSLTNIFSVMFVG...ETGDGQNNIVVRCSNGMENKS....WEDIPG.KWK.GTRVTFIPDFMSF.......ETNE  196
McGyrB          -STAYKSSGGLHGVGSSVTNALSKRFKA..IIYRDK.KIHEIEFKNGGKLEKPL...TFINT.TYKTGTTINFLPDDTIF.......SNAK   76
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HsTop2a LDKDIVALMVRRAY....DIAGS.TKDVKVFLN.............................GNKLPVKGFRSYVDMYLKDKLDETG........  283
CgTop2a LDKDIVALMVRRAY....DIAGS.TKDVKVFLN.............................GNKLPVKGFRSYVDMYLKDKLDETG........  282
MmTop2a LDKDIVALMVRRAY....DIAGS.TKDVKVFLN.............................GNMLPVKGFRSYVDLYLKDKVDETG........  282
RnTop2a LDKDIVALMVRRAY....DIAGS.TKDVKVFLN.............................GNRLPVKGFRSYVDMYLKDKVDETG........  281
HsTop2b LDKDIVALMTRRAY....DLAGS.CRGVKVMFN.............................GKKLPVNGFRSYVDLYVKDKLDETG........  299
ClTop2b LDKDIVALMTRRAY....DLAGS.CKGVKVMFN.............................GKKLPVNGFRSYVDLYVKDKLDETG........  292
MmTop2b LDKDIVALMTRRAY....DLAGS.CKGVKVMFN.............................GKKLPVNGFRSYVDLYVKDKLDETG........  292
DmTop2  LDEDIVALMSRRAY....DVAAS.SKGVSVFLN.............................GNKLGVRNFKDYIDLHIKNTDDDSG........  264
CeTop2b LDDDICHLMARRAY....DVAGS.SKGVAVFLN.............................GKRIPIKGFEDYVQMYTSQFNNEGE........  318
CeTop2  LSDDVIDLIGRRVF....EVAATLPRDVDVYLN.............................GQKCDVDGFEDYVKMFNDSSSLLFL........  333
AtTop2  LEDDVVALMSKRVF....DIAGCLGKSVKVELN.............................GKQIPVKSFTDYVDLYLSAANKSRTE........  286
ScTop2  LDNDILGVMRRRVY....DINGS.VRDINVYLN.............................GKSLKIRNFKNYVELYLKSLEKKRQLDNGEDGA  271
SpTop2  IDDDMVSIIKRRIY....DMAGT.VRETKVYLN.............................NERISISGFKKYVEMYLASDTKPDE........  329
TbTop2  ISNDMKRVLYKRIM....DLSAM.FPNIQITLN.............................GSSFGFKSFKDYATLYSAMTPKGEK........  259
TcTop2  ISHDMKRVLHKRIM....DLAAM.FPSIEISLN.............................GVPFAFKSFADYAMLYSSPSSSGEM........  259
CfTop2  ISLDMKRVLHKRIM....DLAAM.FSKIEVRLN.............................NVPFGFQTFNDYARLYSLPGADGAM........  261
ASFVTop ADLSAWIY.LRACQ....CAAYV.GKGTTIYYN.............................DKPCNTGSVMALAKMYTLLSAPNST....IHTT  271
EcGyrB  FEYEILAKRLRELSFLNSGVSIR.LRDKRDGKE.............................DHFHYEGGIKAFVEYLNKNKTPIHP........  238
HiGyrB  FDYKILAKRLRELSFLNSGVSIR.LIDKRDGSE.............................DHFHYEGGIQAFVEYLNKNKNPIHP........  241
PmGyrB  -                                                                                               182
PpGyrB  FSWDILAKRIRELSFLNSGVGIL.LKDERSGKE.............................EFFKYEGGLRAFVEYLNTNKTPVNS........  239
NgGyrB  YSFDILAKRIRELSFLNNGVDIE.LTDERDGKH.............................ESFALSGGVAGFVQYMNRKKTPLHE........  241
CcGyrB  F-                                                                                              202
BsGyrB  YDYDLLANRVRELAFLTKGVNIT.IEDKREGQE.............................RKNEYHYEGGIKSYVEYLNRSKEVVHE........  242
SpGyrB  FDFDKLNKRIQELAFLNRGLQIS.ITDKRQGLE.............................QTKHYHYEGGIASYVEYINENKDVIFD........  247
SaGyrB  YNYETLQQRIRELAFLNKGIQIT.LRDERDEEN.............................VREDSYHYEGGIKSYVELLNENKEPIHD........  249
HfGyrB  FDFKTLENRLRELAFLNSGVEIS.LSDERTDES.............................STFLFEGGIREFVEYLNETKTALHD........  242
ScGyrB  FSFSTIQNRIKQLVFLNKGLEIS.LVDLREEDE.............................EKTVLYQFNNGIKDYVLELNKTIGTP........  239
MgGyrB2 FDSNIIKSRLKELSFLFAKLKLT.FTDQKTNKT.............................TVFFSTSGLVQFLDEINNTVETLGQK........  238
BsGyrB2 YNFETLSERLRESAFLLKGLKIE.LIDERNDQR.............................EVFYYENGIEAFVAYLNEEKDVLSE........  242
BbGyrB  YNFDVLEKRLKELAFLNDKIYIS.IEDKRIGKE.............................KSSKFYFEGGIKSFVDYLTNDSKAFQS........  241
MpGyrB  YKQTVIASRLQQLAFLNKGIQID.FVDERRQNP.............................QSFSWKYDGGLVQYIHHLNNEKEPLFEDIIFGE.K  252
MaGyrB  YDETIISDRIEQLAFLNKGIKFI.FNDERTDKK.............................TKQEWLYEGGIKQYVENLNASKEPIIPQIIYGE.K  251
MgGyrB  YKQTVIVSRLQQLAFLNKGIRID.FVDNRKQNP.............................QSFSWKYDGGLVEYIHHLNNEKEPLFNEVIADE.K  252
MhGyrB  FFFDTIIDHSKQIAYLNKGLKIT.VENVEKNII.............................KVFCFEGGLIDYVKELNKGKKLIVPEVIYAE.G  251
MlGyrB  YDFGTVARRIQEVAFLNKGLTIN.LVDERVKQDEVVDDVVSDTAEAPVAMTVEEKSTESSAPHKVRHRTFHYPGGLVDFVKHINRTKTPIQQ........  295
MtGyrB  YDFETVARRLQEMAFLNKGLTIN.LTDERVTQDEVVDEVVSDVAEAPKSAS..ERAAESTAPHKVKSRTFHYPGGLVDFVKHINRTKNAIHS........  284
MsGyrB  YNFETVARRLQEMAFLNKGLTIE.LTDERVTAEEVVDDVVKDTAEAPKTAD..EKAAEATGPSKVKHRVFHYPGGLVDYVKHINRTKTPIQQ........  273
SoGyrB  YSFETLSRRFQEMAFLNKGLKIN.LTDERESAKATAGADEAGEDEK.............HEVKSVSYHYEGGIVDFVTYLNSRKGELVHP........  283
SsGyrBs YSFETLSRRFQEMAFLNKGLTLK.LTDERESAKAVVGADVAGTDSA...........ETPGEEPVRSVTYYYEGGIVDFVKYLNSRKGDLIHP........  281
SsGyrBr YSFETLARRHQEMAFLNGGLTLT.LTDERSSARATAAVDEA.................DSDPTAKTVSYRYDGGITDFVVHLNARKGEPAHP........  274
EcParE  FSVSRLTHVLKAKAVLCPGVEIT.FKDEINNTE.............................QRWCYQDGLNDYLAEAVNGLPTLPE........  235
StParE  FSVSRLMHVLKAKAVLCPGVEIT.FKDEVNNSE.............................QRWCYQDGLNDYLGEAVNGLPTLPE........  235
HiParE  FSVSRLRHLLRAKAVLCSGLEIK.FIDKVNNTQ.............................DIWLYEDGLSDYLIEAVNGFETLPK........  235
T4Gn39  LSQVYLDITLDRL....QTLAVV.YPDIQFTFN.............................GKKVQGNFKKYARQYDE.................  241
T2Gn39  LSQVYLDITLDRL....QTLAVV.YPDIQFTFN.............................GKKVQGNFKKYARQYDE.................  241
McGyrB  FNFSLISERLKESALLNSGLKIT.LSDLISNRY.............................VEYQFQDGLVEFVKELVDDKTPVTD........  133
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HsTop2a ...NSLKVIHEQVNHRWEVCLTMSEKGFQQI.SFVNSIATSKGGRHVDYVADQIVTKLVDVVKKK..NKGGVAVK..AHQVKNHMWIF....VNALIENP  371
CgTop2a ...NALKVVHEQVNPRWEVCLTMSERGFQQI.SFVNSIATSKGGRHVDYVADQIVSKLVDVVKKK..NKGGVAVK..AHQVKNHMWIF....VNALIENP  370
MmTop2a ...NSLKVIHEQVNPRWEVCLTMSERGFQQI.SFVNSIATSKGGRHVDYVADQIVSKLVDVVKKK..NKGGVAVK..AHQVKNHMWIF....VNALIENP  370
RnTop2a ...NALKVVHEQVNPRWEVCLTMSEKGFQQI.SFVNSIATSKGGRHVDYVADQIVSKLVDVVKKK..NKGGVAVK..ADQVKNHMWIF....GNAVIENP  369
HsTop2b ...VALKVIHELANERWDVCLTLSEKGFQQI.SFVNSIATTKGGRHVDYVVDQVVGKLIEVVKKK..NKAGVSVK..PFQVKNHIWVF....INCLIENP  387
ClTop2b ...VALKVIHELANERWDVCLTLSEKGFQQI.SFVNSIATTKGGRHVDYVVDQVVGKLIEVVKKK..NKAGVSVK..PFQVKNHIWVF....INCLIENP  380
MmTop2b ...VALKVIHELANERWDVCLTLSEKGFQQI.SFVNSIATTKGGRHVDYVVDQVVSKLIEVVKKK..NKAGVSVK..PFQVKNHIWVF....INCLIENP  380
DmTop2  ...PPIKIVHEVANERWEVACCPSDRGFQQV.SFVNSIATYKGGRHVDHVVDNLIKQLLEVLKKK..NKGGINIK..PFQVRNHLWVF....VNCLIENP  352
CeTop2b ....PLKIAYEQVGDRWQVALALSEKGFQQV.SFVNSIATTKGGRHVDYVADQMVAKFIDSIKRK.LTKTSMNIK..PFQIKNHMWVF....VNCLIENP  406
CeTop2  ...HPTPRWHVGVAKRNNFFGESHVVLPKIV.SFVNNINTEKGGSHVDYVMDKIVNIIKPIVDSK.LGDPTKSVK..PAVIKNNLSIF....INCLIENP  422
AtTop2  ...DPLPRLTEKVNDRWEVCVSLSEGQFQQV.SFVNSIATIKGGTHVDYVTSQITNHIVAAVNKK..NKNA.NVK..AHNVKNHLWVF....VNALIDNP  373
ScTop2  AKSDIPTILYERINNRWEVAFAVSDISFQQI.SFVNSIATTMGGTHVNYITDQIVKKISEILKKK..KKK..SVK..SFQIKNNMFIF....INCLIENP  360
SpTop2  ...EPPRVIYEHVNDRWDVAFAVSDGQFKQV.SFVNNISTIRGGTHVNYVANKIVDAIDEVVKKE..NKK.APVK..AFQIKNYVQVF....VNCQIENP  416
TbTop2  ...PPPPYVYESKSGCVAFIPSVVPGVRRMF.GVVNGVVTYNGGTHCNAAQDILTGCLDGVERELK..KKENKVM.DTNRVLRHFTIL....VFLVQVQP  347
TcTop2  ...PPAPFVYESRNGAIAFIPSLTAGTRRIF.GVVNGVVTHNGGTHCNAAQEVLQSSLESVEKAL...KKDNKVI.DTNRVLRHFMIL....VFLVQVQP  347
CfTop2  ...PPEPFVHTGPNGSIAFVPQLTQSPKRIV.GVVNGVVTYNGGTHCTSAMEILETGLDSLSRSL...KKDGKVI.DTNRVARHFTVL....VFLIQSQP  349
ASFVTop AIKADAKPYSLHP.LQVAAVVSPKFKKFEHV.SIINGVNC.VKGEHVTFLKKTINEMVVKKFQQT..IKDKNRKTTLRDSCSNIFVVI....VGS.IPGI  361
EcGyrB  ...NIFYFSTEKDGIGVEVALQWNDGFQENIYCFTNNIPQRDGGTHLAGFRAAMTRTLNAYMDKEGYSKKAKVSA.TGDDAREGLIAV....VSVKVPDP  330
HiGyrB  ...KPFYFTAEKDGIGVEVALQWNDGVNENVYCFTNNIPQRDGGTHLAGFRGALTRSLNSYMENEGMLKKEKVAT.SGDDAREGLVAI....ISVKVPDP  333
PpGyrB  QVFHFSVQREDGVG..VEVALQWNDSFNENLLCFTNNIPQRDGGTHLVGFRSSLTRSLNSYIEQEGLAKKNKVAT.TGDDAREGLTAI....ISVKVPDP  332
NgGyrB  ...KIFYAFGEKDGMSVECAMQWNDSYQESVQCFTNNIPQRDGGTHLTALRQVMTRTINSYIEANEVAKKAKVET.AGDDMREGLTCV....LSVKLPDP  333
BsGyrB  ...EPIYIEGEKDGITVEVALQYNDSYTSNIYSFTNNINTYEGGTHEAGFKTGLTRVINDYARKKGLIKENDPNL.SGDDVREGLTAI....ISIKHPDP  334
SpGyrB  ...TPIYTDGEMDDITVEVAMQYTTGYHENVMSFANNIHTHEGGTHEQGFRTALTRVINSYARKNKLLKDNEDNL.TGEDVREGLTAV....ISVKHPNP  339
SaGyrB  ...EPIYIHQSKDDIEVEIAIQYNSGYATNLLTYANNIHTYEGGTHEDGFKRALTRVLNSYGLSS..RYEEEKIA.SGEDTREGMTAI....ISIKHGDP  339
HfGyrB  ...DVIYYDDESEGIEVEIAMQATDELQGSIHAFANNINTREGGTHLTGFKTALTRVVNDYANSHDMLDDLDGDNLRGEDVREGLTAV....ISVKHPDP  335
ScGyrB  .LNDVFYVEGIEDNIVVEFGLQYNDNYSENIFSFCNNINTHEGGTHEEGARLAIVREINNYFKNQ.INKNNKGNE..DKFTWDDIKEGMTIIISIRHPEP  335
MgGyrB2 .....TLIKGEKDGIEVEVVFQFNQSDQETILSFANSIKTFEGGSHENGFCLAISDVINSYCRKYNLLKEKDKNF.QLSEIRQGLNAI..IKVNLPEKNI  330
BsGyrB2 ....VVSFEGEHHSIEVDFAFQFNDGYSENILSFVNNVRTKDGGTHESGAKTAMTRAFNEYARKVALLKEKDKNL.EGTDIREGLSAI..ISVRIPEELL  335
BbGyrB  ...EPYYIDGFINDVIVNVGLKWTESYSDNILSFVNNINTREGGTHVMGFRSGLTKAMNEAFKNSKISKKDIPNL.TGDDFKEGLTAV....ISVKVPEP  333
MpGyrB  TDTVKSVSRDESYTIKVEVAFQYNKTYNQSIFSFCNNINTTEGGTHVEGFRNALVKIINRFAVENKFLKETDEKI.TRDDICEGLTAI....ISIKHPNP  347
MaGyrB  KTKVTLPKRNLEVTMLLEVAFQYTNGYYNSTYSFCNNIHTNQGGTHEEGLR.MLFIRSYRYALEKKFIKETDGKI.SKEDLSEGLTAI....ISIKHSEP  345
MgGyrB  TETVKAVNRDENYTVKVEVAFQYNKTYNQSIFSFCNNINTTEGGTHVEGFRNALVKIINRFAVENKFLKDSDEKI.NRDDVCEGLTAI....ISIKHPNP  347
MhGyrB  VFNDKNFTNGQDV..IVEVAMQYNEAYTNSIVSYANNIQTIDGGTHEQGFYDALVRIYNNYAETNKLFKTSSEKI.TREDVKEGLVAI....ISIKHTDP  344
MlGyrB  ..SIIDFDGKGAGH.EVEVAMQWNGGYSESVHTFANTINTHEGGTHEEGFRSALTSVVNKYAKDKKLLKDKDPNL.TGDDIREGLAAV....ISVKVSEP  387
MtGyrB  ..SIVDFSGKGTGH.EVEIAIEWNAGYSESVHTFANTINTHEGGTHEEGFRSALTSVVNKYAKDRKLLKDKDPNL.TGDDIREGLAAV....ISVKVSEP  376
MsGyrB  ..SIIDFDGKGPGH.EVEIAMQWNAGYSESVHTFANTINTHEGGTHEEGFRAALTSVVNRYAKDKKLLKDKDPNL.TGDDIREGLAAV....ISVKVAEP  365
SoGyrB  TVIDLEAEDKDKS.LSLEVAMQWNGGYTEGVYSFANIIHTHEGGTHEEGFRAALTSLINKYARDKKLLREKDDNL.TGDDIREGLTAI....ISVKLAEP  377
SsGyrBs TVIDIDAEDKERM.LSVEIAMQWNSQYSEGVYSFANTIHTHEGGTHEEGFRAAMTGLVNRYAREKKFLREKDDNL.AGEDIREGLTAI....ISVKLGEP  375
SsGyrBr SVITIAAEDTERL.LSAEIALQWNGQYTDSVYSYANAIHTHEGGTHEEGFRTALTTVVNRYAREKRLLRDKDANL.SGEDIREGLTAI....ISVNVGEP  368
EcParE  .KPFIGNFAGDTEAVDWALL.WLPEGGELLTESYVNLIPTMQGGTHVNGLRQGLLDAMREFCEYR.NILPRGVKL.SAEDIWDRCAYV....LSVKMQDP  327
StParE  .KPFIGNFNGETEAVDWALL.WLPEGGELLTESYVNLIPTMQGGTHVNGLRQGLLDAMREFCEYR.NILPRGQKL.SAEDIWDRCAYV....LSVKMQDP  327
HiParE  .KPFVGEFKGANEAVSWALL.WLPEGGELIGESYVNLIPTIQGGTHVNGLRQGLLDAIREFCEFR.NLLPRGVKL.TADDIWDRCSYI....LSLKMQDA  327
T4Gn39  .....HAIVQEQENCSIAVG.RSPDG.FRQL.TYVNNIHTKNGGHHIDCAMDDICEDLIPQIKRK..FKIDVTK...ARVKECLTIVM....FVRDMKNM  324
T2Gn39  .....HAIVQEQENCSIAVG.RSPDG.FRQL.TYVNNIHTKNGGHHIDCVMDDICEDLIPQIKRK..FKIDVTK...RRCQRMFDYVI....VVRDMKNM  324
McGyrB  ....IITINNESKNIIVEIALQYTEDDNEIILGFANNVKTIDGGTHLVGFKSGLIRAINDYAKDQKILKDKTKL..DSNDLREGLVAI..VTVKIPENLI  225
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HsTop2a TFDSQTKENMTLQPKSFGSTCQLSEKFI.........KAAIGCGIVESILNW..VKFKAQVQL.NKKCSAVKHNRIKGIPKLDDANDAGGRNSTE.CTLI  458
CgTop2a TFDSQTKENMTLQAKSFGSTCQLSEKFI.........KAAIGCGIVESILNW..VKFKAQIQL.NKKCSAVKHNRIKGIPKLDDANDAGSRNSTE.CTLI  457
MmTop2a TFDSQTKENMTLQAKSFGSTCQLSEKFI.........KAAIGCGIVESILNW..VKFKAQIQL.NKKCSAVKHTKIKGIPKLDDANDAGSRNSTE.CTLI  457
RnTop2a TFDSQTKENMTLQAKSFGSTCQLSEKFI.........KAAIGCGIVESILNW..VKFKAQIQL.NKKCSAVKHNRIKGIPKLDDANDAGSRNSAE.CTLI  456
HsTop2b TFDSQTKENMTLQPKSFGSKCQLSEKFF.........KAASNCGIVESILNW..VKFKAQTQL.NKKCSSVKYSKIKGIPKLDDANDAGGKHSLE.CTLI  474
ClTop2b TFDSQTKENMTLQPKSFGSKCQLSEKFF.........KAASNCGIVESILNW..VKFKAQTQL.NKKCSSVKYSKIKGIPKLDDANDAGGKHSLE.CTLI  467
MmTop2b TFDSQTKENMTLQPKSFGSKCQLSEKFF.........KAASNCGIVESILNW..VKFKAQTQL.NKKCSSVKYSKIKGIPKLDDANDAGGKHSLE.CTLI  467
DmTop2  TFDSQTKENMTLQQKGFGSKCTLSEKFI.........NNMSKSGIVESVLAW..AKFKAQNDI.AKTGG.RKSSKIKGIPKLEDANEAGGKNSIK.CTLI  438
CeTop2b TFDSQTKETMTLQQKQFGSTCVLSEKFS.........KAASSVGITDAVMS..WVRFKQMDDLNKKCSKTKTSKLKGIPKLEDANDAGTKNSQQ.CTLI  493
CeTop2  SFESQTKETLTTKAKNFGSIFECDAKKT.........AEWAEQSGLIEDIVEE..VLNMKKKKL.PGKRV..SVSSVRDIVKLEDAEWAGITGTAEKCTLI  509
AtTop2  AFDSQTKETLTLRQSSFGSKCELSEDFL.........KKVGKSGVVENLLSW..ADFKQNKDL..KKSD.GAKTGRVLVEKLDDAAEAGGKNSRL.CTLI  458
ScTop2  AFTSQTKEQLTTRVKDFGSRCEIPLEYI.........NKIMKTDLATRMFEI..ADANEENAL.KKSDG.TRKSRITNYPKLEDANKAGTKEGYK.CTLV  446
SpTop2  SFDSQTKETLTTKVSAFGSQCTLSDKFL.........KAIKKSSVVEEVLKF..ATAKADQQL.SKGDG.GLRSRITGLTKLEDANKAGTKESHK.CVLI  502
TbTop2  KFDSQNKARLVSTPTMPRVPRQDVMKYL.........LRMPFLEAHVSTITGQ.LAQELNKEIGTGRRMS..SKTLLTSITKLVDATSTRRDPKHT.RTLI  435
TcTop2  KFDSQNKARLVSVPTMPRVPRQELMDFL.........LRMPFLEAHVNTVTGQ.LADELNKEMGAGRRMS..SKSLISSITKLVDATTTRRDPRFV.RTLI  435
CfTop2  KFDSQSKARLVSTVTMPRVPRTALDQYL.........AAMPFLEAHMNSMDDQ.LAAELNKEIGTGKRLS..SRSLISSITKLVDATSSRSDGKNI.RTLI  437
ASFVTop EWTGQRKDELSIAENVFKTHYSIPSSFL.........TNMTRSIVDILLQS.............ISKKD...NHKQVDVDKYTRAN.AGGKKAQD.CMLL  434
EcGyrB  KFSSQTKDKLVSSEVKSAVEQQMNELLAEYLLENPTDAKIVVGKIIDAARAR.EAARRAREMT..RRKG..ALDLAGLPGKLADCQ...ERDPAL.SELY  421
HiGyrB  KFSSQTKDKLVSSEVKSAVESAMNEKMQEYLLENPADAKIIVNQIIMAARAR.EAARKAREMT..RRKG..ALDIAGLPGKLADCQ...EKDPAL.SELY  424
PpGyrB  KFSSQTKDKLVSSEVKTAVEQEMNKYFSDFLLENPNEAKAVVGKMIDAARAR.EAARKAREMT..RRKG..ALDIAGLPGKLADCQ...EKDPAL.SELY  423
NgGyrB  KFSSQTKDKLVSGEIGPVVNEVINQALTDFLEENPNEAKIITGKIVDAARAR.QAARKAREIT..RRKG..LMDGLGLPGKLADCQ...EKDPAL.SELY  424
BsGyrB  QFEGQTKTKLGNSEARTITDTLFSTAMETFMLENPDAAKKIVDKGLMAARAR.MAAKKARELT..RRKS..ALEISNLPGKLADCS...SKDPSI.SELY  425
SpGyrB  QFEGQTKTKLGNSEVVKITNRLFSEAFSDFLMENPQIAKRIVEKGILAAKAR.VAAKRAREVT..RKKS..GLEISNLPGKLADCS...SNNPAE.TELF  430
SaGyrB  QFEGQTKTKLGNSEVRQVVDKLFSEHFERFLYENPQVARTVVEKGIMAARAR.VAAKKAREVT..RRKS..ALDVASLPGKLADCS...SQSPEE.CEIF  430
HfGyrB  QFEGQTKTKLGNSEVRGIVESVTHQQLGTFFEENPDTATAIISKAVEAARAR.KAAKQAEELT..RRKS..ALESTSLPGKLADCQ...SRDPSE.SELF  426
ScGyrB  QYEGQTNQKLLNSEVKKIVSNIVGKGLSSYLLENPEDAKKIIEKISLSLKAT.IVAQRAKEIT..RRKI..VMDSFSLPGKLSDCE...TKDAKI.AELY  426
MgGyrB2 AFEGQTKSKLFSKEVKNVVYELVQQHYFQFLERNNNDAKLIIDKLLNARKIK.EQIKQQREL...KKSLSSPQKEKILFGKLAPCQ...TKKTSE.KELF  422
BsGyrB2 QFEGQTKGKLGTSEARSAVDAIVSEQLAYFLEENRDTATLLVKKAIKASQAR.EAARKAREEARSGKKR..KKSEATLSGKLTPAG...SRNPAK.NELY  428
BbGyrB  QFEGQTKSKLGNSEIRKIVEVVVYEHLLEIINLNPLEIDTILGKAIKAARAR-                                              385
MpGyrB  QYEGQTKKKLGNTEVRPLVNSIVSEIFERFMLENPQEANAIIRKTLLAQEAR.RRSQEARELT..RRKS..PFDSGSLPGKLADCT...TRDPSI.SELY  438
MaGyrB  QYQGQTKDRLGNTEVREFTNSVVSELLERFFLENPEEAAKITAKAVSAMFSR.KRSEAALESA..RKS...PFESASLPGKLADCT...TKDGI..SELY  434
MgGyrB  QYEGQTKKKLGNTEVRPLVNSVVSEIFERFMLENPQEANAIIRKTLLAQEAR.RRSQEARELT..RRKS..PFDSGSLPGKLADCT...TRDPSI.SELY  438
MhGyrB  IFEGQTKGKLENKDARIATNKILSDSLERYLNENPEIARAIIEKCLLSQHTR.LLEIKAREAS..RKGN..GLDLGNLPGKLADCS...SKNAEI.RELF  435
MlGyrB  QFEGQTKTKLGNTEVKSFVQRVCNEQLIHWFEANPVDAKAVVNKAISSAQAR.IAARKARELV..RRKS..ATDLGGLPGKLADCR...STDPRS.SELY  478
MtGyrB  QFEGQTKTKLGNTEVKSFVQKVCNEQLTHWFEANPTDAKVVVNKAVSSAQAR.IAARKARELV..RRKS..ATDIGGLPGKLADCR...STDPRK.SELY  467
MsGyrB  QFEGQTKTKLGNTEVKSFVQKICNEQLQHWFEANPAEAKTVVNKAVSSAQAR.IAARKARELV..RRKS..ATDIGGLPGKSADCR...STDPSK.SELY  456
SoGyrB  QFEGQTKTKLGNTEVKTFVQKVVYEHLTDWLDRNPNEAADIIRKGIQAAHAR.VAARKARDLT..RRKG..LLESASLPGKLSDCQ...SNDPTK.CEIF  468
SsGyrBs QFEGQTKTKLGNTEAKTFVQKIVHEHLTDWFDRHPNEAADIIRKSIQAATAR.VAARKARDLT..RRKG..LLESASLPGKLSDCQ...SNDPSK.CEIF  466
SsGyrBr QFEGQTKTKLGNTEVRTLLQKIVHEHLADWFDRNPNEAVDIVRKAVQAATAR.VAARKARDLT..RRKG..LLETAALPGKLSDCQ...SNDPAT.SEIF  459
EcParE  QFAGQTKERLSSRQCAAFVSGVVKDAFILWLNQNVQAAELLA....EMAISSAQRRMRAAKKV.VRKKLL..TSGPALPGKLADCT...AQDLNR.TELF  415
StParE  QFAGQPKERLSSRQCAAFVSGVVKDAFSLWLNQNVQAAEQLA....EMAIASAQRRLRAAKKV.VRKKLL..TSGPALPGKLADCT...AQDLNR.TELF  415
HiParE  QFAGQTKERLSSRQSAVFVSGVLKDAFSLWLNQNVQDAEKLA....EIAISSAQRRLRAAKKV.VRKKLL..VSGPALPGKLADCG...SQDLEK.TELF  415
T4Gn39  RLIRQTKERLTSPFGEIRSHIQLDAKKISRDILNNEAILMPII...EAALARKLAAEKAAETKAAKKA...SKAKVHKHIKANLC....GKDADT..TLF  412
T2Gn39  RFDSQTKERLTSPFGEIRSHIQLDAKKISRAILNNEAILMPII...EAALARKLAAEKAAETKAAKKA...LKAKVHKHIKANLC....GKDADT..TLF  412
McGyrB  EYEGQTKSKLGTSDAKTVVEQIVYEFMSYWLIENKVLANKVIENALNAQKAR.IAAKQARQAVKSVKGK..KNVNKLMLGKLTPAQ...GKKREL.NELY  318
CeTop2c                    MMVANESLV.........MSEEDRNVFTSIDKK..GGGSKQMDDLNQKCPKRKTSKLKGIPKLEDANDAGTKNSQQ.CTLI   69
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Fig. 2. Part 2, page 3.

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HsTop2a LTEGDSA.......KTLAVSGLGVVGRDKYGVFPLRGKILNVREASHK.............QIMENAEINNIIKIVGLQYKKNYEDEDSLKTLRYGKIM  537
CgTop2a LTEGDSA.......KTLAVSGLGVVGRDKYGVFPLRGKILNVREASHK.............QIMENAEINNIIKIVGLQYKKNYEDEDSLKTLRYGKIM  536
MmTop2a LTEGDSA.......KTLAVSGLGVVGRDKYGVFPLRGKILNVREASHK.............QIMENAEINNIIKIVGLQYKKNYEDEDSLKTLRYGKIM  536
RnTop2a LTEGDSA.......KTLAVSGLGVVGRDKYGVFPLRGKILNVREASHK.............QIMENAEINNIIKIVGLQYKKNYEDEDSLKTLRYGKIM  535
HsTop2b LTEGDSA.......KSLAVSGLGVIGRDRYGVFPLRGKILNVREASHK.............QIMENAEINNIIKIVGLQYKKSYDDAESLKTLRYGKIM  553
ClTop2b LTEGDSA.......KSLAVSGLGVIGRDRYGVFPLRGKILNVREASHK.............QIMENAEINNIIKIVGLQYKKSYDDAESLKTLRYGKIM  546
MmTop2b LTEGDSA.......KSLAVSGLGVIGRDRYGVFPLRGKILNVREASHK.............QIMENAEINNIIKIVGLQYKKSYDDAESLKTLRYGKIM  546
DmTop2  LTEGDSA.......KSLAVSGLGVIGRDLYGVFPLRGKLLNVREANFK.............QLSENAEINNLCKIIGLQYKKKYLTEDDLKTLRYGKVM  517
CeTop2b LTEGDSA.......KTLAVSGLSVVGRDKYGVFPLRGKLLNVREGNMK.............QIADNAEVNAMIKILGLQYKKKYETEDDFKTLRYGKLM  572
CeTop2  LTEGDSA.......KALALAGLEVLGRETYGVFPLKGKLLNVSNLDDA.............RASKNEEISNLLRILGLKFEDSNSIT..RESLRYGRLL  586
AtTop2  LTEGDSA.......KSLALAGRSVLGNNYCGVFPLRGKLLNVREASTT.............QITNNKEIENLKKILGLKQNMKYENV...NSLRYGQMM  534
ScTop2  LTEGDSA.......LSLAVAGLAVVGRDYYGCYPLRGKMLNVREASAD.............QILKNAEIQAIKKIMGLQHRKKYEDT...KSLRYGHLM  522
SpTop2  LTEGDSA.......KSLAVSGLSVVGRDYYGVFPLRGKLLNVREASHS.............QILNNKEIQAIKKIMGFTHKKTYTDV...KGLRYGHLM  578
TbTop2  VTEGDSA.......KALAQNSLSSDQKRYTGVFPLRGKLLNVRNKNLK.............RLRNCKELQELFCALGLELDKDYTDA...DELRYQRIL  511
TcTop2  VTEGDSA.......KALAQNSLSSDQKRYTGVFPLRGKLLNVRNKNLK.............RLKNCKELQELFCALGLELGKIYKDA...EELRYQRLL  511
CfTop2  VTEGDSA.......KALALNSLSSEQKKFCGVFPLRGKLLNVRNKNLK.............RLKTCKELQDLFLALGLELGKTYKSP...AELRYQRLL  513
ASFVTop AAEGDSALSLVRAGLTLGKSNPSGPSFDFCGMISLGGGVIMNACKKVTNITTTDSGETIMVRNEQLTNNKVLQGIVQVLGLDFNCHYKTQEERAKLRYGCIV  534
EcGyrB  LVEGDSA..........GGSAKQGRNRKNQAILPLKGKILNVEKARFD.............KMLSSQEVATLITALGCGIGRDEYNP...DKLRYHSII  494
HiGyrB  LVEGDSA..........GGSAKVGRDRKTQAILPLKGKILNVEKARFD.............KMLSSQEVGTLITALGCGIGRDEYNP...DKLRYHHII  497
PpGyrB  LVEGDSA..........GGSAKQGRNRRTQAILPLKGKILNVEKARFD.............KMISSQEVGTLITALGCGIGREEYN...IDKLRYHNII  496
NgGyrB  LVEGNSA..........GGSAMQGRDRKFQAILPLKGKILNV.....................ATLITALGAGIGKEEFN...PEKLRYHRII  483
BsGyrB  IVEGDSA..........GGSAKQGRDRHFQAILPLRGKILNVEKARLD.............KILSNNEVRSMITALGTGIGEDFNL....EKARYHKVV  497
SpGyrB  IVEGDSA..........GGSAKSGRNREFQAILPIRGKILNVEKASMD.............KILANEEIRSLFTAMGTGFGAEFDV....SKARYQKLV  502
SaGyrB  LVEGDSA..........GGSTKSGRDSRTQAILPLRGKILNVEKARLD.............RILNNNEIRQMITAFGTGIGGDFDL....AKARYHKIV  502
HfGyrB  IVEGDSA..........GGSAKQGRDRKFQAILPLKGKILNVEKHRLD.............RILENDEIRALITAIGGGVGDEFDI....EKARYQRLI  498
ScGyrB  IVEGDSA..........GGSAKSGRNRKFQAILPLRGKILNVEKAKQI.............KIFENNEINSIITALGAGIKDNFND....KKLRYQKVI  498
MgGyrB2 IVEGDSA..........GGTAKMGRDRIFQAILPLRGKVLNVEKINNK.............KEAITNEEILTLIFCIGTGILTNFNI....KDLKYGKII  495
BsGyrB2 LVEGDSA..........GGSAKQGRDRRFQAVLPLRGKVINTEKAKLA.............DIFKNEEINTIIHAIGGGVGADFSI....DDINYDKII  500
MpGyrB  IVEGDSA..........GGTAKTGRDRYFQAILPLRGKILNVEKSHFE.............QIFNNVEISALVMAVGCGIKPDFEL....EKLRYNKII  510
MaGyrB  IVEGDSA..........GGSAKSGRDRFYQAILPLRGKVLNVEKANHE.............KIFKNEEIRTLITAIGAGVNPEFSL....DKIRYNKII  506
MgGyrB  IVEGDSA..........GGTAKTGRDRYFQAILPLRGKVLNVEKSNFE.............QIFNNAEISALVMAIGCGIKPDFEL....EKLRYSKIV  510
MhGyrB  IVEGNSA..........GGSAKMGRDRSIQAILPLRGKVINAEKNSFA.............SVLSNKEIATMIHALGTGINTEFDI....NKLKYHKII  507
MlGyrB  VVEGDSA..........GGSAKSGRDSMFQAILPLRGKIINVEKARID.............RVLKNTEVQAIITALGTGIHDEFDI....SRLRYHKIV  550
MtGyrB  VVEGDSA..........GGSAKSGRDSMFQAILPLRGKIINVEKARID.............RVLKNTEVQAIITALGTGIHDEFDI....GKLRYHKIV  539
MsGyrB  VVEGDSA..........GGSAKSGRDSMFQAILPLRGKIINVEKARID.............RVLKNTEVQSIIRALGTGIHDEFDI....SKLRYHKIV  528
SoGyrB  IVEGDSA..........GGSAKSGRNPQYQAILPIRGKILNVEKARID.............RILQNQEIQAMISAFGTGVHEDFDI....EKLRYHKII  540
SsGyrBs IVEGDSA..........GGSAKSGRNPQYQAILPIRGKILNVEKARID.............KILQNTEVQALISAFGTGVHEDFDI....EKLRYHKII  538
SsGyrBr IVEGDSA..........GGSAKAGRNPQYQAILPIRGKILNVEKARID.............KVLQNQENQALISAFGTGVHEDFDI....AKLRYHKII  531
EcParE  LVEGDSA..........GGSAKQARDREYQAIMPLKGKILNTWEVSSD.............EVLASQEVHDISVAIGIDPDSDDL.....SQLRYGKIC  486
StParE  LVEGDSA..........GGSAKQARDREYQAIMPLKGKILNTWEVSSD.............EVLASQEVHDISVAIGIDPDSDDL.....SQLRYGKIC  486
HiParE  LVEGDSA..........GGSAKQARDREYQAILPLRGKILNTWEVSPD.............QVLGSTEIHDIAVALGIDPDSNDL.....SQLRYGKVC  486
T4Gn39  LTEGDSA..........IGYLIDVRDKELHGGYPLRGKVLNSWGMSYA.............DMLKNKELFDICAITGLVLGEKAFEEKEDGEWFTFELN  488
T2Gn39  LTEGDSA..........IGYLIDVRDKELHGGYPLRGKVLNSWGMSYA.............DMLKNKELFDICAITGLVLGEKA......ENLNYHNIA  482
McGyrB  LVEGDSA..........GGSAKSGRDRNFQAILPLRGKVINSEKAKLV.............DLLKNEEIQSIINAIGAGVGKDFDI....ISDINYGKII  390
CeTop2c LTEGDSA.......KTLAVSGLSVVGRDKYGVFPFRRKLLNVCDLNVN.............QIADSAEVNAIIKILGLQYTKKYETEDDFKTLRYGKLL  148
T4Gn60                                                                  MKFVKIDSSSVDMKKYKLQNNVRRSIKSSSMNYANVA   37
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Fig. 2. Part 1, page 4.

Fig. 2. Part 2, page 4.

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HsTop2a IMTDQDQDG.SHIKGLLINFIHHNWPSLLR..HRFLEEFITPIVKVS...KNKQ.EMAFYSL..... 592
CgTop2a IMTDQDQDG.SHIKGLLINFIHHNWPSLLR..HRFLEEFITPIVKVS...KNKQ.ELAFYSL..... 591
MmTop2a IMTDQDQDG.SHIKGLLINFIHHNWPSLLR..HRFLEEFITPIVKVS...KNKQ.EIAFYSL..... 591
RnTop2a IMTDQDQDG.SHIKGLLINFIHHNWPSLLR..HRFLEEFITPIVKVS...KNKQ.EIAFYSL..... 590
HsTop2b IMTDQDQDG.SHIKGLLINFIHHNWPSLLK..HGFLEEFITPIVKAS...KNKQ.ELSFYSI..... 608
ClTop2b IMTDQDQDG.SHIKGLLINFIHHNWPSLLK..HGFLEEFITPIVKAS...KNKQ.ELSFYSI..... 601
MmTop2b IMTDQDQDG.SHIKGLLINFIHHNWPSLLK..HGFLEEFITPIVKAS...KNKQ.ELSFYSI..... 601
DmTop2  IMTDQDQDG.SHIKGLLINFIHTNWPELLR..LPFLEEFITPIVKAT...KKNE.ELSFYSL..... 572
CeTop2b VMADQDQDG.SHIKGLVINFIHHFWPSLIQ..RNFVEEFITPIVKAT...KGKE.EVSFYSL..... 627
CeTop2  ILADQDEDG.SHIKGLIVNFIHKFWPSLVH.TDGFIQSFRTPLLKAK...KGDK.VRSFFSM..... 642
AtTop2  IMTDQDHDG.SHIKGLLINFIHSFWPSLLQ.VPSFLVEFITPIVKAT.RKGTKK.VLSFYSM..... 592
ScTop2  IMTDQDHDG.SHIKGLIINFLESSFPGLLD.IQGFLLEFITPIIKVSITKPTKN.TIAFYNM..... 581
SpTop2  IMTDQDHDG.SHIKGLIINYLESSYPSLLQ.IPGFLIQFITPIIKCT...RGNQ.VQAFYTL..... 634
TbTop2  IMTDQDADG.SHIKGLVINAFESLWPSLLVRNPGFISIFSTPIVKARL..RDKS.VVSFFSM..... 569
TcTop2  VMTDQDADG.SHIKGLVINAFEALWPSLLNRNPGFISIFSTPIVKVRL..RDKS.THSFFSL..... 569
CfTop2  VMTDQDADG.SHIKGLVINAFESLWPSLLQHNPGYISLFSTPIVKIKVNGKAKE.VVAFHSF..... 573
ASFVTop ACVDQDLDGCGKILGLLLAYFHLFWPQLII..HGFVKRLLTPLIRVY..EKGKTVPVEFYYE..... 592
EcGyrB  IMTDADVDG.SHIRTLLLTFFYRQMPEIVE..RGHVYIAQPPLYKVK...KGKQ.EQYIKDDEAMDQYQISIALDGATLHTNASAPALAGEALEKLVSEY 587
HiGyrB  IMTDADVDG.SHIRTLLLTFFYRQMPELIE..RGYVYIAQPPLYKVK...KGKQ.ERYIKDADEMEQYELTLALDGAELHISTNAPAMNALVFEKLVAEY 590
PpGyrB  IMTDADVDG.SHIRTLLLTFFFRQLPELVE..RGYIYIAQPPLYKVK...KGKQ.EQYIKDDEAMEEYMTQSALEDASLHLDESAPAVSGVQLESLVNEF 589
NgGyrB  IMTDADVDG.AHIRTLLLTFFYRQMPDLVE..RGYIYIAQPPLYKAK...YGKQ.ERYLKDELEKDQWLLGLALEKAKIVSD..GRTIEGAELADTAKQF 574
BsGyrB  IMTDADVDG.AHIRTLLLTFFYRYMRQIIE..NGYVYIAQPPLYKVQ...QGKR.VEYAYND..... 552
SpGyrB  LMTDADVDG.AHIRTLLLTLIYRYMKPILE..AGYVYIAQPPIYGVKVGSEIKEYIQPGADQ..... 561
SaGyrB  IMTDADVDG.AHIRTLLLIFFYRFMRPLIE..AGYVYIAQPPLYKLT...QGKQ.KYYVYND..... 557
HfGyrB  LMTDADVDG.AHIRTLLLTLLYRHMRPLIE..AGYVYAAQPPLYRVR..YRGNT.YDAM.DE..... 553
ScGyrB  IMTDADVDG.AHIRILLLTFFYRYMKDLIE..NGNIYIAQPPLYKVE...NSNQ.IRYVYSD..... 553
MgGyrB2 IMTDADNDG.AHIQILLLTFFYRYMQPLIE..LGHVYLALPPLYKLE..TKDRKTVKYLWSD..... 552
BsGyrB2 IMTDADTDG.AHIQVLLLTFFYRYMKPLIE..HGKVFIALPPLYKVSKGSGKKEIIEYAWSD..... 559
MpGyrB  IMTDADVDG.AHIRTLLLTFFFRFMYPLVE..QGNIYIAQPPLYKVS...YSNK.DLYMQTD..... 565
MaGyrB  IMTDADVDG.AHIRILLLTFFFRHMFPLIE..KGHVYIAQPPLYRVS...YNKQ.NKYIYSD..... 561
MgGyrB  IMTDADVDG.AHIRTLLLTFFFRFMYPLVE..QGNIFIAQPPLYKVS...YSHK.DLYMHTD..... 565
MhGyrB  IMTDADVDG.AHITTLLLTFFYRYMKPLIE..YGFVYLAQPPLYKIT...SGKN.VEYAYND..... 562
MlGyrB  LMADADVDG.QHISTLLLTLLFRFMRPLIE..HGYVFLAQPPLYKLK...WQRMDPEFAYSDSER..... 609
MtGyrB  LMADADVDG.QHISTLLLTLLFRFMRPLIE..NGHVFLAQPPLYKLK...WQRSDPEFAYSDRER..... 598
MsGyrB  LMADADVDG.QHISTLLLTLLFRFMKPLVE..NGHIFLAQPPLYKLK...WQRSEPEFAYSDRER..... 587
SoGyrB  LMADADVDG.QHINTLLLTFLFRFMRPLVE..SGHVYLSRPPLYKIK...WGRDDFEYAYSDRER..... 599
SsGyrBs LMADADVDG.QHINTLLLTFLFRFMRPLVE..AGHVYLSRPPLYKIK...WGRDDFEYAYSDRER..... 597
SsGyrBr LMADADVDG.QHISTLLLTFLFRFMRPLVE..EGHVHLSRPPLYKIK...WSREHVEYAYSDRER..... 590
EcParE  ILADADSDG.LHIATLLCALFVKHFRALVK..HGHVYVALPPLYRID...LGKE.VYYALTE..... 541
StParE  ILADADSDG.LHIATLLCALFVRHFRALVK..NGHVYVALPPLYRID...LGKE.VYYALTE..... 541
HiParE  ILADADSDG.LHIATLLCALFLRHFPKLVQ..DGHVYVAMPPLYRID...LNKE.VFYALDE..... 541
T4Gn39  GDTIIVNENDEVQINGKWITVGELRKNL                                        516
T2Gn39  IMTDADHDGLGSIYPSLLGFF.SNWPELFE..QGRIRFVKTPVIIAQ...VGKK.QEWFYTV..... 537
McGyrB  I-                                                                  391
CeTop2c IMANHDSDG.SQFKGLLLNFFHRFWPALFK..RDFVEDFITPIAKAT...EGKE.EVSFYSL..... 203
T4Gn60  IMTDADHDGLGSIYPSLLGFF.SNWPELFE..QGRIRFVKTPVIIAQ...VGKK.QEWFYTV..... 92
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HsTop2a  ..........................................................................  592
CgTop2a  ..........................................................................  591
MmTop2a  ..........................................................................  591
RnTop2a  ..........................................................................  590
HsTop2b  ..........................................................................  608
ClTop2b  ..........................................................................  601
MmTop2b  ..........................................................................  601
DmTop2  ..........................................................................  572
CeTop2b  ..........................................................................  627
CeTop2  ..........................................................................  642
AtTop2  ..........................................................................  592
ScTop2  ..........................................................................  581
SpTop2  ..........................................................................  634
TbTop2  ..........................................................................  569
TcTop2  ..........................................................................  569
CfTop2  ..........................................................................  573
ASFVTop  ..........................................................................  592
EcGyrB  NATQKMINRMERRYPKAMLKELIYQPTLTEADLSDEQTVTRWVNALVSELNDKEQHGS...QWKFDVHTNAEQNLFEPIVRVRTHGVDTDYPLDHEFITG  684
HiGyrB  NSVQKLIGRLNRHYPAPVLQGLIYQSPISIEMMKEESAVENWGKSFVEQLTAKETEAH...QYSVRTQFNAERQVYEAVITVRKHGIDTDYFLNFDFVHG  687
PpGyrB  RSVMKTLKRLSRLYPEELTEHFVYLPEVTLEQLGDHAVMQAWLAKLQERLNSSQKSGL...AYNASLREDKERNVWLPEVEITSHGLASYITFNRDFFGS  686
NgGyrB  LLAKTVIEQESRFV.DELVLRAM..........LHASPIDLTSSENADKAVAELSGLLDEKEAALERIEGHEGHQFIKITRKLHGNVMVSYIEPKFLNS  662
BsGyrB  ..........................................................................  552
SpGyrB  ..........................................................................  561
SaGyrB  ..........................................................................  557
HfGyrB  ..........................................................................  553
ScGyrB  ..........................................................................  553
MgGyrB2  ..........................................................................  552
BsGyrB2  ..........................................................................  559
MpGyrB  ..........................................................................  565
MaGyrB  ..........................................................................  561
MgGyrB  ..........................................................................  565
MhGyrB  ..........................................................................  562
MlGyrB  ..........................................................................  609
MtGyrB  ..........................................................................  598
MsGyrB  ..........................................................................  587
SoGyrB  ..........................................................................  599
SsGyrBs  ..........................................................................  597
SsGyrBr  ..........................................................................  590
EcParE  ..........................................................................  541
StParE  ..........................................................................  541
HiParE  ..........................................................................  541
T2Gn39  ..........................................................................  537
CeTop2c  ..........................................................................  203
T4Gn60  ..........................................................................  92
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Fig. 2. Part 1, page 5.

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HsTop2a  ..................................PEF.EEWKSSTPNH.KKWKVKYYKGLGTSTSKEAKEYFADMKRHRIQFKYSGPED......DAAI  649
CgTop2a  ..................................PEF.EEWKSSTPNH.KKWKVKYYKGLGTSTSKEAKEYFADMKRHRIQFKYSGPED......DAAI  648
MmTop2a  ..................................PEF.EEWKSSTPNH.KKWKVKYYKGLGTSTSKEAKEYFADMKRHRIQFKYPGPED......DAAI  648
RnTop2a  ..................................PEF.EEWKSTNPNH.KKWKVKYYKGLGTSTSKEAKEYFANMKRHRIQFKYSGPED......DAAI  647
HsTop2b  ..................................PEF.DEWKKHIENQ.KAWKIKYYKGLGTSTAKEAKEYFADMERHRILFRYAGPED......DAAI  665
ClTop2b  ..................................PEF.DEWKKHIENQ.KAWKIKYYKGLGTSTAKEAKEYFADMERHRILFRYAGPED......DAAI  658
MmTop2b  ..................................PEF.DEWKKHIENQ.KAWKIKYYKGLGTSTAKEAKEYFADMERHRILFRYAGPED......DAAI  658
DmTop2   ..................................PEF.EEWKNDTANH.HTYNIKYYKGLGTSTSKEAKEYFQDMDRHRILFKYDGSVD......DESI  629
CeTop2b  ..................................PEY.SEWRMNTDNW.KSYKIKYYKGLGTSTSKEAKEYFLDMVRHRIRFKYNGADD......DNAV  684
CeTop2   ..................................NEY.RKWADVEEGG..KWKIKYYKGLGTSTSNEAREYFSDLDHHTVNFKYTGTTD......DDAI  698
AtTop2   ..................................PEY.EEWKESLKGNATGWDIKYYKGLGTSTAEEGKEYFSNLGLHKKDFVWEDEQD......GEAI  650
ScTop2   ..................................PDY.EKWREEESHK.FTWKQKYYKGLGTSLAQEVREYFSNLDRHLKIFHSLQGND......KDYI  638
SpTop2   ..................................PEY.EYWKEANNNG.RGWKIKYYKGLGTSDHDDMKSYFSDLDRHMKYFHAMQEKD......AELI  691
TbTop2   ..................................KEF.HKWQRSNANT..PYTCKYYKGLGTSTTAEGKEYFKDMEKHTMRLLVDRSD.......HKLL  624
TcTop2   ..................................KEF.HKWQKTHGNV..SYTAKYYKALGTSTTAEGKEYFKDMDKHTMRLVVERND.......HKLL  624
CfTop2   ..................................RDF.HRWQRANPNA..RYSAKYYKGLGTSTTAEGKEYFADMERNVMRLVVEPKD.......HRLL  628
ASFVTop  ..................................QEF.DAWAKKQTSL.ANHTVKYYKGLAAHDTHEVKSMFKHFDNM.VYTFTLDDS.......AKEL  647
EcGyrB   GEYRRICTLGEKLRGLLEEDAFIERGERRQPVASFEQA.LDWLVKESR..RGLSIQRYKGLGEMNPEQLWETTMDPESRRMLRVTVKDAIA.....ADQL  776
HiGyrB   NEYAKIVSLNKQLNGLLEEGAYVIRGEKVQPVRSFEQA.VEWLVKESR..KGLEVQRYKGLGEMNADQLWETTMDPNSRRMLKVSIKDAVA.....ADQL  779
PpGyrB   NDYRTVVNIGAKLSSLLGEGAYVQRGERRKAIVEFKEG.LDWLMNETT..KRHTIQRYKGLGEMNPDQLWETTMDPTVRRMLKVTIEDAIA.....ADQI  778
NgGyrB   KAYQTLTQTAAALKGLVGEGAKLYKGENEYDADSFETA.LDILMSVAQ..KGMSIQRYKGLGEMNPEQLWETTMDPTVRRLLKVRIEDRI......ADEV  753
BsGyrB   ..................................KEL.EELLKTLPQTP.KPGLQRYKGLGEMNATQLWETTMDPSSRTLLQVTLEDAMD.....ADET  610
SpGyrB   ..................................EIKLQEALARYSEGRTKPTIQRYKGLGEMDDHQLWETTMDPEHRLMARVSVDDAAE.....ADKI  621
SaGyrB   ..................................REL.DKLKSELNPTP.KWSIALYKGLGEMNADQLWETTMNPEHRALLQVKLEDAIE.....ADQT  615
HfGyrB   ..................................AER.DRIIEEECNGN.PTQVQRFKGLGEMNPDQLWDTTMNPENRVLKRITVEDAAA.....ADRM  611
ScGyrB   ..................................NEL.ELYKEELLKQNKNYTIQRYKGLGEMNPEQLWETTMDPERRLLLKVSVNNAFE.....ANLI  612
MgGyrB2  ..................................LEL.ESVKLKLN....NFTLQRYKGLGEMNADQLWDTTMNPTTRKLVQVKLDDLIN.....AEKQ  607
BsGyrB2  ..................................EEM.GDVLKKVG...KGYTIQRYKGLGEMNADQLWETTMNPESRTLVRVKIDDAAR.....VERR  615
MpGyrB   ..................................VQL.EEWKQQHPNL..KYNLQRYKGLGEMDAIQLWETTMDPKVRTLLKVTVEDASI.....ADKA  622
MaGyrB   ..................................AQL.EEWKNQNPNV..RYELQRYKGLGEMDDVQLWETTMDPEKRILLKVSINDAAN.....ADKT  618
MgGyrB   ..................................VQL.EQWKSQNPNV..KFGLQRYKGLGEMDALQLWETTMDPKVRTLLKVTVEDASI.....ADKA  622
MhGyrB   ..................................LQK.EQIMAKLEDK.RNVAIQRYKGLGEMDPEQLWETTMDPETRKMLQVQIDDAAI.....CDTV  620
MlGyrB   ..................................DGLLETGLKLGKKINKEDGIQRYKGLGEMDAKELWETTMDPSVRVLRQVTLDDAAA.....ADEL  669
MtGyrB   ..................................DGLLEAGLKAGKKINKEDGIQRYKGLGEMDAKELWETTMDPSVRVLRQVTLDDAAA.....ADEL  658
MsGyrB   ..................................DGLLEAGRAAGKKINVDDGIQRYKGLGEMDAKELWETTMDPSVRVLRQVTLDDAAA.....ADEL  647
SoGyrB   ..................................DALIEMGRQAGKRI.REDSVQRFKGLGEMNAEELRITTMDQEHRVLGQVTLDDAAQ.....ADDL  658
SsGyrBs  ..................................DALVELGKQNGKRI.KEDSIQRFKGLGEMNAEELRITTMDVDHRVLGQVTLDDAAQ.....ADDL  656
SsGyrBr  ..................................NTLLERGRRDGRRI.RDDSIQRFKGLGEMNAEELRVTTMDPDHRVLGQVTLDDAAF.....ADDL  649
EcParE   ..................................EEKEGVLEQLKRKK.GKPNVQRFKGLGEMNPMQLRETTLDPNTRRLVQLTIDDEDDQ..RTDAMM  603
StParE   ..................................EEKAGVLEQLKRKK.GKPNVQRFKGLGEMNPMQLRETTLDPNTRRLVQLTISDEDDQ..RTNAMM  603
HiParE   ..................................NEKEAILDRLKNKK.GKPNVQRFKGLGEMNPSQLRETTMDPNTRRLVQLTYDLGEDQGSDTLELM  605
T2Gn39   ..................................AEYESAKDAL.....PKHSIRYIKGLGS...LE.KSEYREMIQNPVYDVVKLPEN.....WKEL  587
CeTop2c  ..................................PEY.SEWRMNTDNW.KSYTIKYYNGLGTLTSKEAKKCFSDMVRHRIRFKYNGADD......DKAV  260
T4Gn60   ..................................AEYESAKDAL.....PKHSIRYIKGLGS...LE.KSEYREMIQNPVYDVVKLPEN.....WKEL  142
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Fig. 2. Part 2, page 5.

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HsTop2a  SLAFSKKQIDDRKEWLTNFMEDRRQRKLLGLPEDYLYGQTTTYLTYNDFINKELILFSNSDNERSIPSMVDGLKPGQRKVLFTCFKRNDKR...EVKVAQ  746
CgTop2a  SLAFSKKQVDDRKEWLTHFMEDRRQRKLLGLPEDYLYGQTTTYLTYNDFINKELILFSNSDNERSIPSMVDGLKPGQRKVLFTCFKRNDKR...EVKVAQ  745
MmTop2a  SLAFSKKQVDDRKEWLTNFMEDRRQRKLLGLPEDYLYGQSTSYLTYNDFINKELILFSNSDNERSIPSMVDGLKPGQRKVLFTCFKRNDKR...EVKVAQ  745
RnTop2a  SLAFSKKQVDDRKEWLTNFMEDRRQRKLLGLPEDYLYGQTTMYLTYNDFINKELILFSNSDNERSIPSMVDGLKPGQRKVLFTCFKRNDKR...EVKVAQ  744
HsTop2b  TLAFSKKKIDDRKEWLTNFMEDRRQRRLHGLPEQFLYGTATKHLTYNDFINKELILFSNSDNERSIPSLVDGFKPGQRKVLFTCFKRNDKR...EVKVAQ  762
ClTop2b  TLAFSKKKIDDRKEWLTNFMEDRRQRRLHGLPEQFLYGTATKHLTYNDFINKELILFSNSDNERSIPSLVDGFKPGQRKVLFTCFKRNDKR...EVKVAQ  755
MmTop2b  TLAFSKKKIDDRKEWLTNFMEDRRQRRLHGLPEQFLYGTATKHLTYNDFINKELILFSNSDNERSIPSLVDGFKPGQRKVLFTCFKRNDKR...EVKVAQ  755
DmTop2   VMAFSKKHIESRKVWLTNHMDEVKRRKELGLPERYLYTKGTKSITYADFINLELVLFSNADNERSIPSLVDGLKPGQRKVMFTCFKRNDKR...EVKVAQ  726
CeTop2b  DMAFSKKKIEERKDWLSKWMREKKDRKQQGLAEEYLYNKDTRFVTFKDFVNRELVLFSNLDNERSIPCLVDGFKPGQRKVLFACFKRADKR...EVKVAQ  781
CeTop2   RMAFDRDKSDERKEWIRRS...........ENEITNEDDEQKKARMSDGV.EDLQRF.ESQRIQSIPSLIDGLKPSQRKILWTLLNNMDES..TEIKVSQ  783
AtTop2   ELAFSKKKIEARKNWLSSY...........VPGNHLDQRQPKVTYSDFVNKELILFSMADLQRSIPSMVDGLKPGQRKILFVAFKKIARK...EMKVAQ  735
ScTop2   DLAFSKKKADDRKEWLRQY...........EPGTVLDPTLKEIPISDFINKELILFSLADNIRSIPNVLDGFKPGQRKVLYGCFKKNLKS...ELKVAQ  723
SpTop2   EMAFAKKKADVRKEWLRTY...........RPGIYMDYTQPQIPIDDFINRELIQFSMADNIRSIPSVVDGLKPGQRKVVYYCFKRNLVH...ETKVSR  776
TbTop2   DNVFDSQEVEWRKDWMTKA.........NAFTGEVDIDRSKKMLTVTDFVHKEMVHFALVGNARALAHSVDGLKPSQRKIIWALMRRSGNE...AAKVAQ  712
TcTop2   DSVFDSQEVEWRKDWMTKA.........NAYTGEVDIDRSKKTLTVPDFVHKEMVHFALAGNARALAHAVDGLKPSQRKILWAIMRRSGNE...SAKVAQ  712
CfTop2   DSVFDSAEVEWRKEWMSKA.........NAFQGEVDIDRSKKLLTIGDFVHKEMVHFALVGNARAIPHCVDGLKPSQRKILWAMLKRHSSE...AAKVAQ  716
ASFVTop  FHIYFGGESELRKRELCTGVV.......PLTETQTQSIHSDRQIPCSLHLQVDTKAYKLDAIERQIPNFLDGMTRARRKILAGGLKCFASN.NRERKVFQ  739
EcGyrB   FTTLMGDAVEPRRAFIEENALKAANIDI                                                                        804
HiGyrB   FTTLMGDEVEPRREFIELNALRA.NLDV                                                                        806
PpGyrB   FNTLMGDAVEPRREFIESNALSVSNLDF                                                                        806
NgGyrB   FVTLMGDEVEPRRAFIENNALIAQNIDA                                                                        781
BsGyrB   FEMLMGDKVEPRRNFIEANARYVKNLDI                                                                        638
SpGyrB   FDMLMGDRVEPRREFIEENAVYS.TLDV                                                                        648
SaGyrB   FEMLMGDVVENRRQFIEDNAVYA.NLDF                                                                        642
HfGyrB   FNILMGDAVGPRKQFIKDHANDAEWVDI                                                                        639
ScGyrB   CNELMGENVEPRKKFIRENAKYVKNLDV                                                                        640
MgGyrB2  INIFMGEKSDLRKHWIEANINFSVEN                                                                          633
BsGyrB2  VTTLMGDKVEPRRKWIEKNVAFGLDEESNILENENLSVAEEV                                                          657
MpGyrB   FSLLMGDEVPPRREFIEQNARNVKNIDI                                                                        650
MaGyrB   FSLLMGDEVSPRRDFIEKNAKSVKNIDF                                                                        646
MgGyrB   FSLLMGDEVPPRREFIEKNARSVKNIDI                                                                        650
MhGyrB   FATLMGEEIEPRHDFIQENAKYANNIDI                                                                        648
MlGyrB   FSILMGEDVDARRSFITRNAKDVRFLDV                                                                        697
MtGyrB   FSILMGEDVDARRSFITRNAKDVRFLDV                                                                        686
MsGyrB   FSILMGEDVEARRSFITRNAKDVRFLDV                                                                        675
SoGyrB   FSVLMGEDVEARRAFIQRNAKDVRFLDI                                                                        686
SsGyrBs  FSVLMGEDVEARRSFIQRNAKDVRFLDI                                                                        684
SsGyrBr  FSVLMGEDVEARRHFIQRNAQDVRFLDI                                                                        677
EcParE   DMLLAKKRSEDRRNWLQEKGDMA.EIEV                                                                        630
StParE   DMLLAKKRSEDRRNWLQEKGDLA.DLDV                                                                        630
HiParE   DMLLAKKRSEDRKNWLQAKGDQVDLSV                                                                         632
T2Gn39   FEMLMGDNADLRKEWMSQ                                                                                  605
CeTop2c  NMAFSKKKIEARTDYLMKLMQDKNQRKQQGLAEECLYNKETRFVTLKDFFNYEIVCSWNL...HSIPCLVDGLKPGQRKVLFACFKRANKR...EVKVAQ  354
T4Gn60   FEMLMGDNADLRKEWMSQ                                                                                  160
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Fig. 2. Part 1, page 6.

Fig. 2. Part 2, page 6.

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RnTop2b                                                                                -AQ    2
CpTop2                                                                    -LKKRLNN...ELKVAQ   13
EcGyrA                                  MSDLAREITPVNIEEELKSSYLDYAMSVIVGRALPDVRDGLKPVHRRVLYAMNVLGNDW...NKAYKK   65
HiGyrA                                  MTDSIQSSITPVNIEEELKSSYLDYAMSVIVGRALPDVRDGLKPVHRRVLFSMDREGNTA...NKKYVK   66
ErGyrA                                  MSDLAREITPVNIEEELKSSYLDYAMSVIVGRALPDVRDGLKPVHRRVLYAMSVLGNDW...NKPYKK   65
VsGyrA                                                          -ALPDVRDGLKPVHRRVLFAMDVLGNDW...NKPYKK   33
KpGyrA                                  MSDLAREITPVNIEEELKSSYLDYAMSVIVGRALPDVRDGLKPVHRRVLYAMNVLGNDW...NKAYKK   65
AsGyrA                                  MSDLAREITPINIEEELKSSYLDYAMSVIVGRALPDVRDGLKPVHRRVLFAMNELGNDW...NKPYKK   65
NgGyrA                               MTDATIRHDHKFALETLPVSLEDEMRKSYLDYAMSVIVGRALPDVRDGLKPVHRRVLYAMHELKNNW...NAAYKK   73
PaGyrA                                  MGELAKEILPVNIEDELKQSYLDYAMSVIVGRALPDARDGLKPVHRRVLYAMSELGNDW...NKPYKK   65
BsGyrA                                  MSEQNTPQVREINISQEMRTSFLDYAMSVIVSRALPDVRDGLKPVHRRILYAMNDLGMTS...DKPYKK   66
BsGyrA2                                   MSQPELFHDLPLEEVIGDRFGRYSKYIIQDRALPDARDGLKPVQRRILYAMHTDGNTF...DKNFRK   64
SaGyrA2                                    MSEIIQDLSLEDVLGDRFGRYSKYIIQERALPDVRDGLKPVQRRILYAMYSSGNTH...DKNFRK   62
SaGyrA                                  MAELPQSRINERNITSEMRESFLDYAMSVIVARALPDVRDGLKPVHRRILYGLNEQGMTP...DKSYKK   66
AtGyrA                                                          -RDGLKPVHRRIIHAMSEMGIRP...NSAFKK   28
CfGyrA                                MEENIFSSNQDIDAIDVEDSIKASYLDYSMSVIIGRALPDARDGLKPVHRRILYAMNDLGVGS...RSPYKK   69
CjGyrA                                 MENIFSKDSDIELVDIENSIKSSYLDYSMSVIIGRALPDARDGLKPVHRRILYAMQNDEAKS...RTDFVK   68
HpGyrA                                 MQDRLVNETKNIVEVGIDSSIEESYLAYSMSVIIGRALPDARDGLKPVHRRILYAMHELGLTS...KVAYKK   69
ScGyrA                              MMKSENDGYDYDGKIRDIDIADEMKNGFLDYAMSVIVSRAIPDVRDGLKPVHRRIIYAMWDLKMTY...EKQHKK   72
HfGyrA                            MSSDAPDSFEPGAGIAAEVKNARIEDEMEQSYIDYAMSVIAGRALPDVRDGLKPVHRRILYAMHQAGVTS...NSSHRK   76
MlGyrA                                 MTDITLPPGDGSIQRVEPVDIQQEMQRSYIDYAMSVIVGRALPEVRDGLKPVHRRVLYAMLDSGFRP...DRSHAK   73
RpGyrA                                  MIDKYSSNLVPVNIEDEMKVSYLDYAMSVIVSRAIPDVRDGLKPVHRRIIYSMYEAGNHA...SKPYRK   66
MpGyrA                            MAKQQDQIDKIRQELAQSAIKNISLSSELERSFMEYAMSVIVARALPDARDGLKPVHRRVLYGAYTGGMHH...DRPFKK   77
MgGyrA                            MAKQQDQVDKIRENLDNSTVKSISLANELERSFMEYAMSVIVARALPDARDGLKPVHRRVLYGAYIGGMHH...DRPFKK   77
MgGyrA2                                 MDQKNNNLFQKAIEEVFAVSFSKYAKYIIQDRALPDLRDGLKPVQRRILYGMFQMGLKP...TTPYKK   65
MaGyrA                                    MDKKKIFQKDLDDIMSLSFGRYAKYIIQERALPDIRDGLKPVQRRVLYGMYNLGLYY...NKSYRK   63
MtGyrA                               MTDTTLPPD.DSLDRIEPVDIEQEMQRSYIDYAMSVIVGRALPEVRDGLKPVHRRVLYAMFDSGFRP...DRSHAK   72
MsGyrA                                MTDTTLPPEGEAHDRIEPVDIQQEMQRSYIDYAMSVIVGRALPEVRDGLKPVHRRVLYAMYDSGFRP...DRSHAK   73
SoGyrA                            MTDENTPVTTPEGDALAMRVEPVGLETEMQRSYLDYAMSVIVSRALPVVRDGLKPVHRRVLYAMYDGGYRP...ERGFYK   77
SsGyrA                                 MTDSPDRLIATDLRNEMSQSYLEYAMSVIVGRALPDARDGLKPVHRRILYAMYELGLTP...DRPFRK   65
SeGyrA                                 MAELPQSRINERNITSEMRESFLDYAMSVIVSRALPDVRDGLKPVHRRILYGLNEQGMTP...DKPYKK   66
EcParC                                    MSDMAERLALHEFTENAYLNYSMYVIMDRALPFIGDGLKPVQRRIVYAMSELGLNA...SAKFKK   62
StParC                                    MSDMAERLALHEFTENAYLNYSMYVIMDRALPFIGDGLKPVQRRIVYAMSELGLNA...TAKFKK   62
HiParC                                 MTNINYEGIEQMPLRTFTEKAYLNYSMYVIMDRALPFIGDGLKPVQRRIVYAMSELGLNA...TAKYKK   66
AbParC                                                                  -MSELGLKS...SGKPKK   14
T4Gn52                                MQLNNRDLKSIIDNEALAYAMYTVENRAIPNMIDGFKPVQRFVIARALDLARGNKDKFHKLAS   63
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HsTop2a LAGSVAEMSS.YH.HGEMSLMMTIINLAQNFVGSNNLNLLQ.PIGQFGTRL.HGGKDSASPRYIFTML.SSLARLLFP.PKDDHTLKFLYDDNQRVEPEW  840
CgTop2a LAGSVAEMSS.YH.HGEMSLMMTIINLAQNFVGSNNLNLLQ.PIGQFGTRL.HGGKDSASPRYIFTML.SPLTRLLFP.PKDDHTLKFLYDDNQRVEPEW  839
MmTop2a LAGSVAEMSS.YH.HGEMSLMMTIINLAQNFVGSNNLNLLQ.PIGQFGTRL.HGGKDSASPRYIFTML.SPLARLLFP.PKDDHTLRFLYDDNQRVEPEW  839
RnTop2a LAGSVAEMSS.YH.HGEMSLMMTIINLAQNFVGSNNLNLLQ.PIGQFGTRL.HGGKDSASPRYIFTML.SPLARLLFP.SKDDHTLRFLYDDNQRVEPEW  838
HsTop2b LAGSVAEMSA.YH.HGEQALMMTIVNLAQNFVGSNNINLLQ.PIGQFGTRL.HGGKDAASPRYIFTML.STLARLLFP.AVDDNLLKFLYDDNQRVEPEW  856
ClTop2b LAGSVAEMSA.YH.HGEQALMMTIVNLAQNFVGSNNINLLQ.PIGQFGTRL.HGGKDAASPRYIFTML.SSLARLLFP.AVDDNLLKFLYDDNQRVEPEW  849
MmTop2b LAGSVAEMSA.YH.HGEQALMMTIVNLAQNFVGSNNINLLQ.PIGQFGTRL.HGGKDAASPRYIFTML.SSLARLLFP.AVDDNLLKFLYDDNQRVEPEW  849
DmTop2  LSGSVAEMSA.YH.HGEVSLQMTIVNLAQNFVGANNINLLE.PRGQFGTRL.SGGKDCASARYIFTIM.SPLTRLIYH.PLDDPLLDYQVDDGQKIEPLW  820
CeTop2b LAGAVAEISA.YH.HGEQSLMGTIVNLAQDYVGSNNINLLL.PIGQFGTRL.QGGKDSASARYIFTQL.SPVTRTLFP.AHDDNVLRFLYEENQRIEPEW  875
CeTop2  LAGAVAHRQS.YH.HGEESLVRTIIRMGQTFCGSSNLPLLQ.PIGQFGTRH.EGGNDAASARYIFTAL.APTTRLLFP.QADDDLLQKNVEEGMVVEPTL  877
AtTop2  LVGYVSLLSA.YH.HGEQSLASAIIGMAQDYVGSNNINLLL.PNGQFGTRT.SGGKDSASARYIFTKL.SPVTRILFP.KDDDLLLDYLNEDGQRIEPTW  829
ScTop2  LAPYVSECTA.YH.HGEQSLAQTIIGLAQNFVGSNNIYLLL.PNGAFGTRA.TGGKDAAAARYIYTEL.NKLTRKIFH.PADDPLYKYIQEDEKTVEPEW  817
SpTop2  LAGYVASETA.YH.HGEVSMEQTIVNLAQNFVGSNNINLLM.PNGQFGTRS.EGGKNASASRYLNTAL.SPLARVLFN.SNDDQLLNYQNDEGQWIEPEY  870
TbTop2  LSGYISEASA.FH.HGETSLQETMIKMAQSFTGGNNVNLLV.PEGQFGSRQ.QLGNDHAAPRYIFTKL.SKVARLLFP.SEDDPLLDYIVEEGQQVEPNH  806
TcTop2  LSGYISEVSA.FH.HGEMSLQETIIKMAQNFTGGNNINLLI.PEGQFGSRQ.QLGNDHAAARYIFTKL.SSLARILFP.SEDEPLLDYVTEEGQQVEPNH  806
CfTop2  LSGYISEVSS.FH.HGEASLQETIVKMAQNFTGGNNINLLV.PEGQFGSRQ.QLGNDHAAPRYIFTKL.SRFARLLFP.EDDDPLLDYIDEEGTMVEPNH  810
ASFVTop FGGYVADHMF.YH.HGDMSLNTSIIKAAQYYPGSSHLYPVFIGIGSFGSRH.LGGKDAGSPRYISVQLASEFIKTMFP.AEDSWLLPYVFEDGQRAEPEY  835
CeTop2c LAGAVAEISA.YH.HGEQSLMGTIVNLAQDYVGSHNINLLL.PIGQFGTRL.QGGKDSASARSIFAQL.SQVTRTLFP.AHDDNVLRFLYEENQRIEPEW  448
RnTop2b LAGSVAEMSA.YH.HGEQALMMTIVNLAQNFVGSNNINLLQ.PIGQFGTRL.HGGKDAASPRYIFTML.SSLARLLFP.AVDDNLLKFLYDDNQRVEPEW   96
CpTop2  LAGYVAEHSA.YH.HGESSLQSTIVNMAQNFVG-                                                                    44
EcGyrA  SARVVGDVIGKYHPHGDSAVYDTIVRMAQPF....SLRYML.VDGQGNFGS.IDGDSAAAMRYTEIRL.AKIAHELMADLEKETVDFVDNYDGTEKIPDV  158
HiGyrA  SARVVGDVIGKYHPHGDSAVYDTIVRMAQPF....SLRYML.VDGQGNFGS.IDGDAPAAMRYTEVRM.QKITQALLTDLDKETVNFSPNYDGELMIPDV  159
ErGyrA  SARVVGDVIGKYHPHGDSAVYETIVRMAQPF....SLRYML.VDGQGNFGS.IDGDSAAAMRYTEIRM.SKIAHELLADLEKETVDFVPNYDGTEQIPDV  158
VsGyrA  SARVVGDVIGKYHPHGDSAVYDTIVRIAQPF....SLRYML.VDGQGNFGS.IDGDSAAAMRYTEVRM.SKIAHELLADLDKETVDYVPNYDGTEQIPAV  126
KpGyrA  SARVVGDVIGKYHPHGDTAVYDTIVRMAQPF....SLRYML.VDGQGNFGS.VDGDSAAAMRYTEIRM.SKIAHELMADLEKETVDFVDNYDGTEKIPDV  158
AsGyrA  SARVVGDVIGKYHPHGDSAVYDTIVRLAQDF....SMRYML.VDGQGNFGS.VDGDSAAAMRYTEVRM.ARISHELLADLDKETVDWVPNYDGTEMIPAV  158
NgGyrA  SARIVGDVIGKYHPHGDSAVYDTIVRMAQNF....AMRYVL.IDGQGNFGS.VDGLAAAAMRYTEIRM.AKISHEMLADIEEETVNFGPNYDGSEHEPLV  166
PaGyrA  SARVVGDVIGKYHPHGDTAVYDTIVRMAQPF....SLRYML.VDGQGNFGS.VDGDNAAAMRYTEVRM.AKLAHELLADLEKETVDWVPNYDGTEQIPAV  158
BsGyrA  SARIVGEVIGKYHPHGDSAVYESMVRMAQDF....NYRYML.VDGHGNFGS.VDGDSAAAMRYTEARM.SKISMEILRDITKDTIDYQDNYDGSERIPVV  159
BsGyrA2 AAKTVGNVIGNYHPHGDSSVYEAMVRMSQDW....KVRNVL.IEMHGNNGS.IDGDPPAAMRYTEARL.SPIASELLRDIDKNTVEFVPNFDDTSKEPVV  157
SaGyrA2 SAKTVGDVIGQYHPHGDSSVYEAMVRLSQDW....KLRHVL.IEMHGNNGS.IDNDPPAAMRYTEAKL.SLLAEELLRDINKETVSFIPNYDDTTLEPMV  155
SaGyrA  SARIVGDVMGKYHPHGDSSIYEAMVRMAQDF....SYRYPL.VDGQGNFGS.MDGDGAAAMRYTEARM.TKITLELLRDINKDTIDFIDNYDGNERESPSV  159
AtGyrA  CARIVGDVIGKFHPHGDQSVYDALVRLAQDF....SQRYPI.VDGQGNFGN.IDGDGAAAYRYTEARM.TDVAALLLEGIGEDAVDFRATYNEEDEEPVV  121
CfGyrA  SARIVGDVIGKYHPHGDTAVYDALVRMAQNF....SMRVPA.VDGQGNFGS.VDGDGAAAMRYTEARM.TVLAEELLRDLDKDTVDFIPNYDDSLSEPDV  162
CjGyrA  SARIVGAVIGRYHPHGDTAVYDALVRMAQDF....SMRYPS.ITGQGNFGS.IDGDSAAAMRYTEAKM.SKLSHELLKDIDKDTVDFVPNYDGSESEPDV  161
HpGyrA  SARIVGDVIGKYHPHGDNAVYDALVRMAQDF....SMRLEL.VDGQGNFGS.IDGDNAAAMRYTEARM.TKASEEILRDIDKDTIDFVPNYDDTLKEPDI  162
ScGyrA  SARIVGEVIGKYHPHGDTAVYEAMVRMAQDF....SYRYPL.IDGHGNFGS.MDGDPPAAMRYTEAKM.SKIAGEIIKDIEKETTIFIDNYDGSEEEPTF  165
HfGyrA  SSSIVGETMGDYHPHGDSAIYDTLARMAEDF....SMRYPL.VDGQGNFGS.VDGDPPAAMRYTEARM.SPIAEELLDDIDKDTVDFQSNYDDRKQEPTV  169
MlGyrA  SARSVAETMGNYHPHGDASIYDTLVRMAQPW....SLRYPL.VDGQGNFGS.PGNDPPAAMRYTEARL.TPLAMEMLREIDEETVDFISNYDGRVQEPMV  166
RpGyrA  SARIVGDVMGKYHPHGDSAIYDSLVRMAQDF....SLRLPL.VDGQGNFGS.MDGDAAAAMRYTESRM.AKVAHKLVEDIDKGTVSFNINYDGSEEEPSV  159
MpGyrA  SARIVGDVMSKFHPHGDMAIYDTMSRMAQDF....SLRYLL.IDGHGNFGS.IDGDRPAAQRYTEARL.SKLAGELLRDIDKDTVD-              156
MgGyrA  SARIVGDVMSKFHPHGDMAIYDTMSRMAQDF....SLRYLL.IDGHGNFGS.IDGDRPAAQRYTEARL.SKLAAELLKDIDKDTVDFIANYDGEEKEPTV  170
MgGyrA2 SARAVGEIMGKYHPHGDSSIYDAIIRMSQSW....KNNWTT.VSIHGNNGS.VDGDNAAAMRYTETRL.SLYGFELLKDIDKKLVSFINNFDDSEKEPTV  158
MaGyrA  SAATVGEVIGKFHPHGDSSIYEA-                                                                            86
MtGyrA  SARSVAETMGNYHPHGDASIYDSLVRMAQPW....SLRYPL.VDGQGNFGS.PGNDPPAAMRYTEARL.TPLAMEMLREIDEETVDFIPNYDGRVQEPTV  165
MsGyrA  SARSVAETMGNYHPHGDASIYDTLVRMAQPW....SLRYPL.VDGQGNFGS.PGNDPPAAMRYTEARL.TPLAMEMLREIDEETVDFIPNYDGRVQEPTV  166
SoGyrA  CARVVGDVMGNYHPHGDSSIYDALVRLAQPW....SMRMPL.VDSNGNFGS.PGNDPAAAMRYTECKM.APLSMEMVRDIDEETVDFTDNYDGRSQEPTV  170
SsGyrA  CARVVGEVLGKYHPHGDTAVYDALVRMAQDF....SMREPL.IDGHGNFGS.VDNDPPAAMRYTESRLRPLSTNSLLRDIEAETVDFIDNFDGSQQEPTV  159
SeGyrA  SARIVGDVMGKYHPHGDSSIYEAMVRMA-                                                                        94
EcParC  SARTVGDVLGKYHPHGDSACYEAMVLMAQPF....SYRYPL.VDGQGNWGAPDDPKSFAAMRYTESRL.SKYSELLLSELGQGTADWVPNFDGTLQEPKM  156
StParC  SARTVGDVLGKYHPHGDSACYEAMVLMAQPF....SYRYPL.VDGQGNWGAPDDPKSFAAMRYTESRL.SKYAELLLSELGQGTADWVPNFDGTMQEPKM  156
HiParC  SARTVGDVLGKFHPHGDSACYEAMVLMAQPF....SYRYPL.VDGQGNWGAPDDPKSFAAMRYTESRL.SKISEILLNELGQGTVDYQPNFDGTLAEPQY  160
AbParC  SARTVGDVLGKYHPHGDSACYEAMVLMAQPF....SYRYPL.IEGQGNWGSPDDPKSFAAMRYTEAKL.SAYSELLLSELGQGT-              92
T4Gn52  IAGGVAD.LG.YH.HGENSAQDAGALMANTW....NNNFPL.LDGQGNFGS.RTVQKAAASRYIFARV.SKNFYNVY....KDTEYAPVHQDKEHIPPAF  149
AbGyrA     -VGDVIGKYHPHGDSAVYETIVRMAQDF....SLRYLL.VDGQGNFGS.IDGDSAAAMRYTEVRM.TKLAHELLADLEKDTVDWEDNYDGSERIPEV   89
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Fig. 2. Page 7.

300

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HsTop2a YIPIIPMVLINGAEGIGTGWSCKIPNFDVREIVNNIRRLMDGEE.........PLPMLPSYKNFKGTIEELAPNQYVISGEVAI..............  915
CgTop2a YIPIIPMVLINGAEGIGTGWSCKIPNFDIREVVNNIRRLLDGEE.........PLPMLPSYKNFKGTIEELASNQYVINGEVAI..............  914
MmTop2a YNPINTMVLINGAEGIGTGWSCKIPNFDVREVVNNIRRLLDGEE.........PLPMLPSYKNFKGTIEELASNQYVINGEVAI..............  914
RnTop2a YIPIIPMVLINGAEGIGTGWSCKIPNFDVREVVNNIRRLLDGEE.........PLPMLPSYKNYKGTIEELASNQYVINGEVAI..............  913
HsTop2b YIPIIPMVLINGAEGIGTGWACKLPNYDAREIVNNVRRMLDGLD.........PHPMLPNYKNFKGTIQELGQNQYAVSGEIFV..............  931
ClTop2b YIPIIPMVLINGAEGIGTGWACKLPNYDAREIVNNVRRMLDGLD.........PHPMLPNYKNFKGTIQELGQNQYAVSGEIFV..............  924
MmTop2b YIPIIPMVLINGAEGIGTGWACKLPNYDAREIVNNVRRMLEGLD.........PHPMLPNYKNFKGTIQELGQNQYAVSGEIFV..............  924
DmTop2  YLPIIPMVLVNGAEGIGTGWSTKISNYNPREIMKNLRKMINGQE.........PSVMHPWYKNFLGRMEYVSDGRYIQTGNIQI..............  895
CeTop2b YCPIIPMVLVNGAQGIGTGWSTNIPNYNPRELVKNIKRLIAGEP.........QKALAPWYKNFRGKIIQIDPSRFACYGEVAV..............  950
CeTop2  .CPIVPLILINGTEGIGTGWSTKIANRNPIDIIDMIRRKIDSIS........TEYEIPPFYEEFRGKLEVVTPTKFISSGKIQLIRP.............  955
AtTop2  YMPIIPTVLVNGAEGIGTGWSTFIPNYNPREIVANVRRLLNGES.........MVPMDPWYRGFKGTIEKTASKEGGCTYTITG..............  904
ScTop2  YLPILPMILVNGAEGIGTGWSTYIPPFNPLEIIKNIRHLMNDEE.........LEQMHPWFRGWTGTIEEIEPLRYRMYGRIEQ..............  892
SpTop2  YVPILPMVLVNGAEGIGTGWSTFIPNYNPKDITANLRHMLNGEP.........LEIMTPWYRGFRGSITKVAPDRYKISGIINQ..............  945
TbTop2  YVPILPLLLCNGSVGIGFGFSSNIPPFHRLDVSAAVRAMISGER.....AKSVVRRLVPWAVGFQGEIRRGPEGEFIAVGTYTY..............  885
TcTop2  YVPILPLLLCNGSVGIGFGFASNIPPFHPLDVSAAVRSMINGEA.....AKVVVRRLVPWAVGYQGEVRRGPEGEFIAAGSYQY..............  885
CfTop2  YVPILPLLLCNGAVGIGFGFATNIPSFHPLDVSAAVRAMINGES.....AKQVVRNLVPWAVGFQGTVRRGPEKEYIAVGKYTA..............  889
ASFVTop YVPVLPLAIMEYGANPSEGWKYTTWARQLEDILALVRAYVDKNN......PKHELLHYAIERKITVLPLRP...SNYNFKGHLKRFGQYYY....SYGT.  921
CeTop2c YCPIIPMVLVNGAQGTGTGWSTNIPNYNPRELVKNIKRLIAGEP.........QKALAPWYKNFRGKIIQIDPSRFACYGEVSV..............  523
RnTop2b YIPIIPMVLINGAEGIGTGWACKLPNYDAREIVDNVRRMLEGLD.........PHPMLPNYKNFKGTIQELGQNQYAVSGEIFV..............  171
EcGyrA  MPTKIPNLLVNGSSGIAVGMATNIPPHNLTEVINGCLAYIDDEDI....SIEGLMEHIPGPD.FPTAAIIN...GRRGIEEAYRTGRGKVYIRARAEVE.  249
HiGyrA  LPTRIPALLANGSSGIAVGMATNIPPHNLNEVLNGCLAYIDKNEI....TIDELMQHIPGPD.FPTAALIN...GRKGIEEAYRTGRGKVYVRARATVE.  250
ErGyrA  MPTRIPNLLVNGSSGIAVGMATNIPPHNLTEVVNGCLAYIDDENI....SLEGLMEHIKGPD.FPTAAIIN...GRRGIEEAYRTGRGKIYIRARAEVE.  249
VsGyrA  LPTRVPNLLVNGSSGIAVGMA-                                                                                147
KpGyrA  MPTKIPNLLVNGSFGIAVGMATNIPPHNLTEVINGRLAYVEDEEI....SIEGLMEHIPGPD.FPTAAIIN...GRRGIEEAYRTGRGKVYICARAEVE.  249
AsGyrA  MPTKVPNLLVNGSSGIAVGMAWNIPPHNLTEIVNGCLALIENGNL....TIDELMTYITGPD.FPTGAIIN...GRAGIVQAYRTGRGSVYVRAKAEVE.  249
NgGyrA  LPTRFPTLLVNGSSGIAVGMATNIPPHNLTDTINACLRLLDEPKT....KIDELIDIIQAPD.FPTGATIY...GLGGVREGYKTGRGRVVMRGKTHIE.  257
PaGyrA  MPTKIPNLLVNGSSGIAVGMATNIPPHNLGEVIDGCLALMDNPDL....TVDELMQYIPGPD.FPTAGIIN...GRAGIIEAYRTGRGRIYIRARAVVE.  249
BsGyrA  MPSRFPNLLVNGAAGIAVGMATNIPPHQLGEIIDGVLAVSENPDI....TIPELMEVIPGPD.FPTAGQIL...GRSGIRKAYESGRGSITIRAKAEIE.  250
BsGyrA2 LPAMFPNLLVNGSTGISAGYATDIPPHHLGEVIDAVIKRIQMPSC....SVDELMELIKGPD.FPTGGIIQ...GVDGIRKAYETGKGKIIIRGKAEIE.  248
SaGyrA2 LPSRFPNLLVNGSTGISAGYATDIPPHNLAEVIQATLKYIDNPDI....TVNQLMKYIKGPD.FPTGGIIQ...GIDGIKKAYESGKGRIIVRSKVEEE.  246
SaGyrA  LPARFPNLLANGASGIAVGMATNIPPHNLTELINGVLSLSKNPDI....SIAELMEDIEGPD.FPTAGLIL...GKSGIRRAYETGRGSIQMRSRAVIE.  250
AtGyrA  LPGAFPNLLANGASGIAVGMATNIPPH-                                                                          148
CfGyrA  LPARVPNLLLNGSSGIAVGMATNIPPHSLDELVNGLLTLLDDKEV....GLEDIMTHIKGPD.FPTGGIIF...GKKGIIEAYKTGRGRIKLRAKTHIE.  253
CjGyrA  LPSRVPNLLLNGSSGIAVGMATNIPPHSLNELIDGLLYLLDNKDA....SLEEIMQFIKGPD.FPTGGIIY...GKKGIIEAYRTGRGRVKVRAKTHIE.  252
HpGyrA  LPSRLPNLLVNGANGIAVGMATSIPPHRIDEIIDALAHVLENPNA....ELDEILEFVKGPD.FPTGGIIY...GKAGIIEAYKTGRGRVKVRAKVHVE.  253
ScGyrA  LPGYFPNLLVNGASGIAVGMATNIPPHNLNEVIDGVIAVTKNPEI....TTVELMKIIKGPD.FPTG-                                  227
HfGyrA  LPSSFPNLLVNGSSGIAVGMSTNIPPHNLGEVVDAAVELIENPDA....TVADLMEHIKGPD.FPTGANIV...ARNAVHKAYKTGRGRVRVRADYDVF.  260
MlGyrA  LPSRFPNLLANGSGGIAVGMATNIPPHNLYELADAVFWCLENHDADEETMLVAVMERVKGPD.FPTAGLIV...GSQGIADAYKTGRGSIRIRGVVEVE.  261
RpGyrA  LPAMFPNLLVNGSGGIAVGMATNIPPHNLGEIIDACCLYIDNHDI....EILDLLEVVKGPD.FPTGSMIL...GISGIRSAYLTGRGSIIMRGKAEIE.  250
MgGyrA  LPAAFPNLLANGSSGIAVGMSTSIPSHNLSELIAGLIMLIDNPQC....TFQELLTVIKGPD.FPTGANII...YTKGIESYFETGKGNVVIRSKVEIE.  261
MgGyrA2 LPTLLPNLFINGASGIAAGYATNIAPHNTNELLDSLCLRIDQPNC....ELKQILKIVKGPD.FPTGGNVY...FEKSLSDIYQAGKGKFIIQAKYEVN.  249
MtGyrA  LPSRFPNLLANGSGGIAVGMATNIPPHNLRELADAVFWALENHDADEEETLAAVMGRVKGPD.FPTAGLIV...GSQGTADAYKTGRGSIRMRGVVEVE.  260
MsGyrA  LPSRFPNLLANGSGGIAVGMATNIPPHNLGELAEAVYWCLENYEADEEATCEAVMERVKGPD.FPTSGLIV...GTQGIEDTYKTGRGSIKMRGVVEIE.  261
SoGyrA  LPARFPNLLINGSAGIAVGMATNIPPHNLREVAAGAQWYLENYEA....SHEELLDALIERIKGPDFPTGALVVGRKGIEEAYRTGRGSITMRAVVEVE.  265
SsGyrA  LPARIPQLLINGSSGIAVGMATNIPPHNLGEVIDGAIALIRNPEI....TEQELMQIIPGPDFPTGAQIL....GRSGIREAYLTGRGSITMRGVASIET  251
EcParC  LPARLPNILLNGTTGIAVGMATDIPPHNLREVAQAAIALIDQPKT....TLDQLLDIVQGPD.YPTEAEII..TSRAEIRKIYENGRGSVRMRAV.....  244
StParC  LPARLPNILLNGTTGIAVGMATDIPPHNLREVAKAAITLIEQPKT....TLDQLLDIVQGPD.YPTEAEII..TPRAEIRKIYENGRGSVRIGAV.....  244
HiParC  LPARLPHILLNGTTGIAVGMATDIPPHNINEIADAAVMLLDNPKA....GLDDVLEIVQGPD.FPTEAEII..SPKSEIRKIYEQGRGSIKMRAT.....  248
T4Gn52  YLPIIPTVLLNGVSGIATGYATYILPHSVSSVKKAVLQALQGKK........VTKPKVEFPE.FRGEVVEI..DGQYEIRGTYKF..............  223
AbGyrA  LPTRVP-                                                                                             95
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HsTop2a .....LNSTTI.EISELPVRTWTQTYKEQVLEPMLNGTEKTPPLITDYREYHTDTTVKFVVKMTEEKLA...EAERVGLHKVFKLQTSLTCNSMVLFDHV 1006
CgTop2a .....LNSTTI.EISELPIRTWTQTYKEQVLEPMLNGTEKTPPLITDYREYHTDTTVKFVIKMTEEKLA...EAERVGLHKVFKLQTSLTCNSMVLFDHV 1005
MmTop2a .....LDSTTI.EISELPIRTWTQTYKEQVLEPMLNGTEKTPSLITDYREYHTDTTVKFVIKMTEEKLA...EAERVGLHKVFKLQSSLTCNSMVLFDHV 1005
RnTop2a .....LNSTTI.EITELPIRTWTQTYKEQVLEPMLNGTEKTPPLITDYREYHTDTTVKFVIKMTEEKLA...EAERVGLHKVFKLQTSLTCNSMVLFDHV 1004
HsTop2b .....VDRNTV.EITELPVRTWTQVYKEQVLEPMLNGTDKTPALISDYKEYHTDTTVKFVVKMTEEKLA...QAEAAGLHKVFKLQTTLTCNSMVLFDHM 1022
ClTop2b .....VDRNTV.EITELPVRTWTQVYKEQVLEPMLNGTDKTPALISDYKEYHTDTTVKFVVKMTEEKLA...QAEAAGLHKVFKLQTTLTCNSMVLFDHM 1015
MmTop2b .....VDRNTV.EITELPVRTWTQVYKEQVLEPMLNGTDKTPALISDYKEYHTDTTVKFVVKMTEEKLA...QAEAAGLHKVFKLQTTLTCNSMVLFDHM 1015
DmTop2  .....LSGNRL.EISELPVGVWTQNYKENVLEPLSNGTEKVKGIISEYREYHTDTTVRFVISFAPGEFER.IHAEEGGFYRVFKLTTTLSTNQMHAFDQN  988
CeTop2b .....LDDNTI.EITELPIKQWTQDYKEKVLEGLMESSDKKSPVIVDYKEYHTDTTVKFVVKLSPGKLRE..LERGQDLHQVFKLQAVINTTCMVLFDAA 1042
CeTop2  .ERKNASTFSI.EIVELPIGIWTSKYKEKLSKIVETL......PVLEFSERHTEKRVHFRITLDRKKSSRFLQKSNSDLLNYFKLRTSLTENR.VLFDRN 1046
AtTop2  .LYEEVDETTI.RITELPIRRWNDDYKNFLQSLKTDNG...APFFQDVKAYNDEKSVDFDLILSEENML...AARQEGFLKKFKLTTTIATSNMHLFDKK  996
ScTop2  .....IGDNVL.EITELPARTWTSTIKEYLL.LGLSGNDKIKPWIKDMEEQH.DDNIKFIITLSPEEMA...KTRKIGFYERFKLISPISLMNMVAFDPH  981
SpTop2  .....IGENKV.EITELPIRFWTQDMKEYL.EAGLVGTEKIRKFIVDYESHHGEGIVHFNVTLTEAGMK...EALNESLEVKFKLSRTQATSNMIAFDAS 1035
TbTop2  .....CKGGRV.HVTELPWTCSVEAFREHI..SYLATKDIVNRIADYSGANHVDIDVEVAQGAVN.........TYAECESELGL.TQRIHINGTVFSPN  967
TcTop2  .....YVDGRV.HVTEIPWTLSIEAFRDHI..SVLASKDVVQRIADYSGANHVDIDLELTNGAMT.........TYAECESELSL.TQRIYINGTVFSPT  967
CfTop2  .....HRNGRL.HVSELPWMTSIEAFRSHI..SSLASSDVVQRIADYSGANHIDIDLIVREGSMT.........TWAECETDLAL.SQRIYINGTVFSPD  971
ASFVTop .YVVSEQRNII.TITELPLRVPTVAYIESI.KKSSNRMAFIEEIIDYSSSETIEILVKLKPNSLNRIVEE.FKETEEQDSIENFLRLRNCLHSHLNFVKP 1017
CeTop2c .....LDDNTI.EITELPIKQWTQDYKEKVLEGLMESSDKKSPVIVDYKEYHTDTTVKFVVKLSPGKLRE..LERGQDLHQVFKLQAVINTTCMVLFDAA  615
RnTop2b .....VDRNTV.ETTELPVRTWTQVYKEQVLEPMLNGTDKTPALISDYK-                                                  214
EcGyrA  .VDAKTGRETI.IVHEIPYQVNKARLIEKIAELVKEKRVEGISALRDE.SDKDGMRIVIEVKRDAV.........GEVVLNNLYS.QTQLQVSFGINMVA  336
HiGyrA  .TNEK.GREQI.IVSELPYQVNKAKLVEKIAELIREKKIEGISNITDL.SNKEGIRIEIDIKRDAV.........GEVVLNHLYS.LTQMQVTFGINMVA  336
ErGyrA  .ADAKTGRETI.IVHEIPYQVNKARLIEKIAELVKDKRIEGISALRDE.SDKDGMRIVIEIKRDAV.........GEVVLNNLYS.QTQLQTSFGINMVA  336
KpGyrA  .ADAKTGRETI.IVHEIPYQVNKARLIEKIAELVKEKRVEGISALRDE.SDKDGMRIVIEVKRDAV.........GRVVLNNLYS.QTQLQVSFGINMVA  336
AsGyrA  .VDDKTSRETIIIVHELPYQVNKARLIEKIAELVKEKKVEGISALRDE.SDKDACRIVIEIKRGES.........GEIVLNNLYK.HTQLQTTFGINMVA  337
NgGyrA  PIGKNGERERI.VIDEIPYQVNKAKLVEKIGDLVREKTLEGISELRDE.SDKSGMRVVIELKRNEN.........AEVVLNQLYK.LTPLQDSFGINMVV  345
PaGyrA  EMEKGGGREQI.IITELPYQVNKARLIEKIAELVKEKKIEGISELRDE.SDKDGMRVVIELKRGEV.........GEVVLNNLYA.QTQLQSVFGINVVA  337
BsGyrA  ..QTSSGKERI.IVTELPYQVNKAKLIEKIADLVRDKKIEGITDLRDE.SDRTGMRIVIEIRRDAN.........ANVILNNLYK.QTALQTSFGINLLA  336
BsGyrA2 ..TIRGGREQI.VITEIPFEVNKANLVKKMDEFRIDKKVEGISEVRDE.TDRTGLRVVIELKKEAD.........AKGILNFLYK.NTDLQITYNFNMVA  334
SaGyrA2 ..TLRNGRKQL.IITEIPYEVNKGSLVKRIDELRADKKVDGIVEVRDE.TDRTGLRIAIELKKDVN.........SESIKNYLYK.NSDLQISYNFNMVA  332
SaGyrA  ..ERGGGRQRI.VVTEIPFQVNKARMIEKIAELVRDKKIDGITDLRDETSLRTGVRVVIDVRKDAN.........ASVILNNLYK.QTPLQTSFGVNMIA  337
CfGyrA  ...KKPNKDVI.VVDELPYQVNKAKLHADIADLVKEKLIDGISEVRDE.SDRDGIRLVIELKRDAM.........SEIVLNNLFK.STQMEVTFGVIMLA  338
CjGyrA  ...KKTNKDVI.VIDELPYQTNKARLIEQIAELVKERQIEGISEVRDE.SNKEGIRVVIELKREAM.........SEIVLNNLFK.STTMESTFGVIMLA  337
HpGyrA  ...KTKNKEII.VLDEMPFQTNKAKLVEQISDLAREKQIEGISEVRDE.SDREGIRVVIELKRDAM.........SEIVLNHLYK.LTTMETTFSIILLA  338
HfGyrA  .....EEEGRI.VINELPYQENKARLIERIADDVNEGKIEGIRDIRDE.SERDGIRVVIELKRGAM.........AEVVKNQLL.DNHLESTFGVINLA  342
MlGyrA  ..EDSRGRTSL.VITELPYQVNHDNFITSIAEQVRTGRLAGISNVEDQGSDRVGVRIVIEIKRDAV.........AKVVLNNLYK.HTQLQTSFGANMLS  348
RpGyrA  ..NVGNSRQAI.IITEIPYMVNKARLVEKIAEMVKEKRIEGISDLRDE.SNKNGIRIFIELKKDVV.........AEVVLNQIYACTQLQTNFGVIMLA  336
MgGyrA  ...QLQTRSAL.VVTEIPYMVNKTTLIEKIVELVKAEEISGIADIRDE.SSREGIRLVIEVKRDTV.........PEVLLNQLFKSTRLQVRFPVNMLA  346
MgGyrA2 .....KNLNQI.EITQIPYETLKANIVKQIEEIIFDNKLSAIESVIDS.SDRNGIRIIIKHKDFLP.........AEKIMAFLFKHTQLQVNFNLNNTV  332
MtGyrA  ..EDSRGRTSL.VITELPYQVNHDNFITSIAEQVRDGKLAGISNIEDQSSDRVGLRIVIEIKRDAV......AKVVINNLYKHTQLQTSFGANMLAIVD.  350
MsGyrA  ..EDSRGRTSI.VITELPYQVNHDNFITSIAEQVRDGKLAGISNIEDQSSDRVGLRIVVEIKRDAV......AKVVLNNLYKHTQLQTSFGANMLSIVD.  351
SoGyrA  ...EIQNRQCL.VVTELPYQTNPDNLAQKIADLVKDGKVGGIADVRDETSSRTGQRLVIVLKRDAV......AKVVLNNLYKHTDLQSNFGANMLALVD.  354
SsGyrA  MEHPGRPDRDAIIVTELPYQTNKAALIERIADLVNDKKIDGIADIRDE.SDRDGMRIVIELKRDAV......ARVVLNNLYKQTPIQSNFGANLLALVN.  343
EcParC  ...WKKEDGAV.VISALPHQVSGARVLEQIAAQMRNKKLPMVDDLRDE.SDHENPTRLVIVPRSNR.......VDMDQVMNHLFATTDLEKSYRINLNMI  332
StParC  ...WTKEDGAV.VISALPHQVSGAKVLEQIAAQMRNKKLPMVDDLRDE.SDHENPTRLVIVPRSNR.......VDMEQVMNHLFATTDLEKSYRINLNMI  332
HiParC  ...WKKEDGEI.IISALPHQSSPSKVIAQIAEQMTAKKLPMLEDIRDE.ADHENPIRIVLVPRSNR.......VDTDALMAHLFATTDLEKSYRVNMNMI  336
T4Gn52  .....TSRTQM.HITEIPYKYDRETYVSKILDPLENK...GFITWDDACGEHGFGFKVKFRKEYSL...SDNEEERHAKIMKDF.GLIERRSQNITV.IN  309
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HsTop2a GCLKKYDT.VLDILRDFFELRLKYYGLRKEWLLGMLGAESAKLNNQARFILEKIDGKIIIENKPKKELIKVLIQRGYDSDPVKAWKEAQQKVPDEEENEE 1105
CgTop2a GCLKKYDT.VLDILKDFFELRLKYYGLRKEWLLGMLGAESAKLNNQARFILEKIDGKIIIENKPKKELIKVLIQRGYDSDPVKAWKEAQQKVPDEEENEE 1104
MmTop2a GCLKKYDT.VLDILRDFFELRLKYYGLRKEWLLGMLGAESSKLNNQARFILEKIDGKIVIENKPKKELIKVLIQRGYDSDPVKAWKEAQQKVPDEEENEE 1104
RnTop2a GCLKKYDT.VLDILRDFFELRLKYYGLRKEWLLGMLGAESSKLNNQARFILEKIDGKIVIENKPKKELIKVLIQRGYDSDPVKAWKEAQQKVPEEEENEE 1103
HsTop2b GCLKKYET.VQDILKEFFDLRLSYYGLRKEWLVGMLGAESTKLNNQARFILEKIQGKITIENRSKKDLIQMLVQRGYESDPVKAWKEAQEKAAEEDETQN 1121
ClTop2b GCLKKYET.VQDILKEFFDLRLSYYGLRKEWLVGMLGAESTKLNNQARFILEKIQGKITIENRSKKDLIQMLVQRGYESDPVRAWKEAQEKAAEEEDTQN 1114
MmTop2b GCLKKYET.VQDILKEFFDLRLSYYGLRKEWLVGMLGAESTKLNNQARFILEKIQGKITIENRSKKDLIQMLVQRGYESDPVKAWKEAQEKAAEEEDSQN 1114
DmTop2  NCLRRFPT.AIDILKEYYKLRREYYARRRDFLVGQLTAQADRLSDQARFILEKCEKKLVVENKQRKAMCDELLKRGYRPDPVKEWQRRIKMEDAEQADEE 1087
CeTop2b GCLRTYTS.PEAITQEFYDSRQEKYVQRKEYLLGVLQAQSKRLTNQARFILAKINNEIVLENKKKAAIVDVLIKMKFDADPVKKWKEEQKLKELRESGEI 1141
CeTop2  GELKEFGN.ISEIAAEFFEVRRDLYEKRLKIQKEECEAKLIYVENQLNFIEMVTNGTIEIRSMGRNQLEEKLQEMGFRVDPMATIAKNSKKVNLINYEDF 1145
AtTop2  GVIKKYVT.PEQILEEFFDLRFEYYEKRKETVVKNMEIELLKLENKARFILAVLSGEIIVNKRKKADIVEDLRQKGFTPFPRKAESVEAAIAGAVDDDAA 1095
ScTop2  GKIKKYNS.VNEILSEFYYVRLEYYQKRKDHMSERLQWEVEKYSFQVKFIKMIIEKELTVTNKPRNAIIQELENLGFPRFNKEGKPYYGSPNDEIAEQIN 1080
SpTop2  GRIKKYDS.VEDILTEFYEVRLRTYQRRKEHMVNELEKRFDRFSNQARFIHMIIEGELVVSKKKKKDLIVELKEKKFQPISKPKKGHLVDLEVENALAEE 1134
TbTop2  GTLSPLESDLTPVLQWHYDRRLDLYKKRRQRNLTLLEQELAREKSTLKFVQHFGAGHIDFANATEATLEKVCSKLGLVRVD..................... 1048
TcTop2  GVLTPLEGDLAPVLQWHYDRRLDLYKKRRQRNLGLLEAELAREKSTLKFVTHFREGKIDIVNATDDSLAKTCSKLGMVRVD..................... 1048
CfTop2  GTLSPIDADLSPVLQWHYDRRLDLYKRRRTRQIGLLEMDLARLQSTRKFVEHFRQGHIDFLAATDDTLTKTCVKLGLVRVD..................... 1052
ASFVTop KGGIIEFNSYYEILYAWLPYRRDVYQKRLMRERAVLKLRIIMETAIVRYINESADLNLSHYEDEKEAS.RILSEHGFPPLNQSLITSPEFASIEELNQKA 1116
CeTop2c GWLRTYTS.PEAITQEFYDSRQEKYVQRKEYLLGVLQAQSKRLTNQARFILATINNKIVLENKKKTAIVDVLIKMKFDADPVKKWKEDQKLKELRESGEI 714
EcGyrA  LHHGQPKIMNLKDIIAAFVRHRREVVTRRTIFE.....................LRKARDRAHILEALAVALANIDPIIELIRHAPTPAEAKTAL 410
HiGyrA  LDHGQPRLFNLKEIIEAFVLHRREVVTRRSIFE.....................LRKARERTHILEGLAVARSNIDEMIAIIRNSKNREEAATSI 410
ErGyrA  LHQGQPKIMPLKDILVAFVRHRREVVTRRTIFE.....................LRKARDRAHILEGLAIALANIDPIIELIRRAASPAEAKASL 410
KpGyrA  LHHGQPKIMNLKEIIAAFVRHRREVVTRRTILA.....................LRKARDRADILEALSIALANIDPIIELIRRAPTPAEAKAGL 410
AsGyrA  LDNNQPKVMNLKDILDAFLLHRREVVTRRTVFE.....................LRKARDRAHILEGLAVALANIDPIIELIRHSDTPADAKAKL 411
NgGyrA  LVDGQPRLLNLKQILSEFLRHRREVVTRRTLFR.....................LKKARHEGHIAERKAVALSNIDEIIKLIKESPNAAEAKEKL 419
PaGyrA  LVDGQPRTLNLKDMLEVFVRHRREVVTRRTVYE.....................LRKARERGHILEGQAVALSNIDPVIELIKSSPTPAEAKERL 411
BsGyrA  LVDGQPKVLTLKQCLEHYLDHQKVVIRRRTAYE.....................LRKAEARAHILEGLRVALDHLDAVISLIRNSQTAEIARTGL 410
BsGyrA2 IHNRRPMLMSLPSILDAYIGHQKEVVTNRSVYE.....................LQKAKDRHHIVEGLMKALSILDEVIATIRSSSDKRDAKNNL 408
SaGyrA2 ISDGRPKLMGIRQIIDSYLNHQIEVVANRTKFE.....................LDNAEKRMHIVEGLIKALSILDKVIELIRSSKNKRDAKENL 406
SaGyrA  LVNGRPKLINLKEALVHYLEHQKTVVRRRTQYN.....................LRKAKDRAHILEGLRIALDHIDEIISTIRESDTDKVAMESL 411
CfGyrA  INNKEPKVFSLLELLKLFLNHRKTVIIRRTIFE.....................LQKARARAHILEGLKIALDNIDAVINLIKTSADTNSARDGL 412
CjGyrA  IHNKEPKIFSLLELLNLFLTHRKTVIIRRTIFE.....................LQKARARAHILEGLKIALDNIDEVIALIKNSSDNNTARDSL 411
HpGyrA  IYNKEPKIFTLLELLRLFLNHRKTIIIRRTIFE.....................LEKAKARAHILEGYLIALDNIDEIVQLIKTSPSPEAAKNAL 412
HfGyrA  LVDGQPQVLTLKETLEHYLDHRRDVVRRRSEYE.....................LAEAEDRAHILDGRLKALDNIDDVVETIRNSESRDDAKAAL 416
MlGyrA  IVDGVPRTLRLDQMICYYVEHQLDVIVRRTTYR.....................LRKANERAHILRGLVKALDALDEVITLIRASQTVDIARVGV 422
RpGyrA  LKDGLPKVMNLKEVIAAFVSFREVVITNRTIYL.....................LNKARDRAHILLGLTIAISNIDEIIYIIKASNDTNLAKQEL 410
MgGyrA  LVKGAPVLLNMKQALEVYLDHQIDVLVRRTKFV.....................LNKQQERYHILSGLLIAALNIDEVVAIIKKSANNQEAINTL 420
MgGyrA2 IANRFPIQIGLLSYLDHFLKFCHELIINKAKYE.....................LELASKRLEIILGLIKAISIIDKIIKLIRSAVDKSDAREKL 406
MtGyrA  ...GVPRTLRLDQLIRYYVDHQLDVIVRRTTYR.....................LRKANERAHILRGLVKALDALDEVIALIRASETVDIARAGL 421
MsGyrA  ...GVPRTLRLDQLIRLYVDHQLDVIVRRTRYR.....................LRKANERAHILRGLVKALDALDEVIALIRASQTVDIARAGL 422
SoGyrA  ...GVPRTLSLDAFIRHWVNHQIEVIVRRTRFR.....................LRKAEERAHILRGLLKALDAIDEVIALIRRSDTVEIARGGL 425
SsGyrA  ...GTPEVLTIKKFLTVFWEFRIETITRRTRYE.....................LRKAEERDHLLQGLLIALDNLDAVIRLIRGAADTASAKTEL 414
EcParC  GLDGRPAVKNLLEILSEWLVFRRDTVRRRLNYR.....................LEKVLKRLHILEGLLVAFLNIDEVIEIIRNEDEPKPALMS. 405
StParC  GLDGRPAVKNLLEILTEWLAFRRDTVRRRLNYR.....................LEKVLKRLHILEGLLVAFLNIDEVIEIIRSEDEPKPALMS. 405
HiParC  GLDHKPAVKGLLEILNEWLDFRRTTVTRRLQYR.....................LDKVLSRLHILEGLMIAFLNIDEVIEIIRHEDDPKAELMA. 409
T4Gn52  EKGKLQVYDNVVDLIKDFVEVRKTYVQKRIDNKIKETESA.....................FRLAFAKAHFIKKVISGEIVVQGKTRKELTEELSKIDMYS. 389
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HsTop2a SDNEKETEKSDSVTDSG.....PTFNYLLDMPLWYLTKEKKDELCRLRNEKEQELDTLKRKSP.....SDLWKEDLA 1172
CgTop2a SDNE....NSDSVAESG.....PTFNYLLDMPLWYLTKEKKDELCKQRNEKEQELNTLKNKSP.....SDLWKEDLA 1167
MmTop2a SDTET..STSDSAAEAG.....PTFNYLLDMPLWYLTKEKKDELCKQRNEKEQELNTLKQKSP.....SDLWKEDLA 1169
RnTop2a NEESE.SESTSPAAESG.....PTFNYLLDMPLWYLTKEKKDELCKQRDEKEQELNTLKKKTP.....SDLWKEDLA 1169
HsTop2b QHDD...SSSDSGTPSG.....PDFNYILNMSLWSLTKEKVEELIKQRDAKGREVNDLKRKSP.....SDLWKEDLA 1185
ClTop2b QHDD...SSSDSGTPSG.....PDFNYILNMSLWSLTKEKVEELIKQRDTKGREVNDLKRKSP.....SDLWKEDLA 1178
MmTop2b QHDD...SSSDSGTPSG.....PDFNYILNMSLWSLTKEKVEELIKQRDTKGREVNDLKRKSP.....SDLWKEDLA 1178
DmTop2  DEEEEEAAPSVSSKAKKEKEVDPEKAFKKLTDV.....KKFDYLLGMSMWMLTEEKKNELLKQRDTKLSELESLRKKTP.....EMLWLDDLD 1170
CeTop2b ELDEDDLAAVAVEEDEAISSA.AKAVETKLSDY.....DYLVGMALIKLSEEEKNKLIKESEEKMAEVRVIEKKTW.....QDLWHEDLD 1220
CeTop2  QYLNFQ.....ANYGYLLEMPVSRLTSDEMKRLEERKSRRRTELEAAESADW.....KSVWRSELD 1201
AtTop2  EEPEEILVDPESSSSYIPG.....SEYDYLLAMAIASLTIEKVEELLADRDKMIIAVADMKKTTP.....KSLWLSDLE 1164
ScTop2  DVKGATSDEEDEESSHEDTENVINGPEELY.....GTYEYLLGMRIWSLTKERYQKLLKQKQEKETELENLLKLSA.....KDIWNTDLK 1160
SpTop2  EQSGDVSQDEDS.....DAYNYLLSMPLWSLTYERYVELLKKKDEVMAELDALIKKTP.....KELWLHDLD 1196
TbTop2  .....DSFDYILRKPITFYTKTSFENLLKKIAETERRIEALKKTTP.....VQLWLGELD 1098
TcTop2  .....DSYDYVLRKPITFYTKTSLENLNRKISETEKRIDKLKKTAP.....VQMWLDELD 1098
CfTop2  .....DGYDYILKKPITFYTKTSTEKLQADIKKTQDSIAVLKQTTP.....VKMWLTDLD 1102
ASFVTop LQ.....GCYTYILSLQARELLIAAKTRRVEKIKKMQARLDKVEQLLQESPFPGASVWLEEID 1174
CeTop2c ELDEDDLAAVAVEEGEDISSA.AKAVETKLSDY.....DYLVGLALIKLSEEEKNKLIKESEEKMAEEGIDFNSDDDGVERENVVSKLRRS 799
EcGyrA  VANPWQLGNVAAMLERAGDDAA..RPEWLEPEFGVRDGLYYLTEQQAQAILDLRLQKLTGLEHEKLLDEYKELLDQIAELLRILG.....SADRLMEVI 502
HiGyrA  SSRSWTLHSDIINLL...DASA..RPDELEENLGIQGEQYYLSPAQVNAILELRLHRLTGIAFEEVIKEYBELLVKIADLLHILS.....SAERLMEVI 499
ErGyrA  IAQAWELGSVATMLERAGDDAA..RPEWLEPEFGIRDGRYYLTEQQAQAILDLRLQKLTGMEHEKLLDEYKELLAEIAELLYILN.....SPERLMEVI 502
KpGyrA  IARSWDLGNVSAMLE.AGDDAA..RPEWLEPEFGVRDGQYYLTEQQAQAILDLRLQKLTGLEHEKLLDEYKELLEQIAELLHILG.....SADRLMEVI 501
AsGyrA  VARGWELGNVAAMLEKAGDDAA..RPEWLEPEFGIREGQYFLTEQQAQAILDLRLHKLTGLEHGKILEEYQSLLDLIAELLFILA.....SPERLMEVI 503
NgGyrA  LARPWASSLVEEMLTRSGLDLEMMRPEGLVANIGLKKQGYYLSEIQADAILRMSLRNLTGLDQKEIIESYKNLMGKIIDFVDILS.....KPERITQII 513
PaGyrA  IATAWESSAVEAMVERAGADAC..RPEDLDPQYGLRDGKYYLSPEQAQAILELRLHRLTGLEHEKLLSEYQEILNLIGELIRILT.....NPARLMEVI 503
BsGyrA  IE.....QFSLTEKQAQAILDMRLQRLTGLEREKIEEEYQSLVKLIAELKDILA.....NEYKVLEII 468
BsGyrA2 IA.....KYEFTEPQAEAIVSLQLYRLTNTDITALKEEAEELGKKIEELESILS.....NDKKLLKVI 466
SaGyrA2 IE.....VYEFTEEQAEAIVMLQLYRLTNTDIVALEGEHKELEALIKQLRHILD.....NHDALLNVI 464
SaGyrA  QQ.....RFKLSEKQAQAILDMRLRRLTGLERNKIEAEYNELLNYISELEAILA.....DEEVLLQLV 469
CfGyrA  MA.....KFGLSELQSNAILDMRLSKLTGLEREKLEAELKEILELIEKLDAILK.....SETLIENII 470
CjGyrA  VA.....KFGLSELQANAILDMKLGRLTGLEREKIENELAELMKEIARLEEILK.....SETLLENLI 469
HpGyrA  ME.....RFTLSEIQSKAILEMRLQRLTGLERDKIKEEYQNLLELIDDLNGILK.....SEDRLNGVV 470
HfGyrA  RGEVEVEVDGEPLP.....TFDFSEEQANHIVSMQLGSLTSMEAAEIEAEYEDVQATIERLETILG.....DQSELDAVI 486
MlGyrA  VE.....LLDIDDIQAQAILDMQLRRLAALERQRIIDDLAKIEVEIADLGDILA.....KPERRRGII 480
RpGyrA  MARQWEVLDILPLI.....KLVDDKVILNERGTLSFTEVQAKAILEMKLQRLTAMEKEKLEQDLKHLATDIAEYLNILA.....SRTRLLEIL 493
MgGyrA  NT.....KFKLDEIQAKAVLDMRLRSLSVLEVNKLQTEQKELKDSIEFCKKVLA.....DQKLQLKII 478
MgGyrA2 ID.....NFKFTFNQAEAIVSLRLYQLTNTDIFELNQEQNELEKTVISSEQLIA.....SEKARNKLL 464
MtGyrA  IE.....LLDIDEIQAQAILDMQLRRLAALERQRIIDDLAKIEAEIADLEDILA.....KPERQRGIV 479
MsGyrA  IE.....LLDIDDIQAQAILDMQLRRLAALERQKIVDDLAKIEAEIADLEDILA.....KPERQRGIV 480
SoGyrA  MD.....LLEIDEIQANAILEMQLRRLAALERQKIVREHDELQAKITEYNEILA.....SPVRQRGIV 483
SsGyrA  VE.....GFSLSEVQADAILQMQLRRLTALEADKITAEHDELQTKIADFQDILA.....RRERVNAII 472
EcParC  .....RFGLTETQAEAILELKLRHLAKLEEMKIRGEQSELEKERDQLQGILA.....SERKMNNLL 461
StParC  .....RFGISETQAEAILELKLRHLAKLEEMKIRGEQDELEKERDQLQGILA.....SERKMNTLL 461
HiParC  .....RFNLSDEQADAILNLRLRHLAKLEENQLKAEQDELEKERLNLEAILG.....SERRLNTLI 465
T4Gn52  .....SYVDKLVGMNIFHMTSDEAKKLAEEAKAKKEENEYWKTTDV.....VTEYTKDLE 439
McGyrA                                                -MRLYRLTSTDVNKLLEKTELIDKIKKYQEILN.....DNLVLDNEI  42
BbGyrA                                                -SLIKDYEDILL.....NPVRIINIYKRRTINLGLK  30
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HsTop2a TFIEELEAV.......EAKEKQDEQVGL.............................PGKGGKAKG.................KKTQMA.......EV 1210
CgTop2a VFIEELEVV.......EAKEKQDEQVGL.............................PGKGGKAKG.................KKAQMS.......EV 1205
MmTop2a VFIEELEVV.......EAKEKQDEQVGL.............................PGKAGKAKG.................KKAQMCA......DV 1208
RnTop2a AFVEELEVV.......EAKEKQDEQVGL.............................PGKGVKAKG.................KKAQIS.......EV 1207
HsTop2b AFVEELDKV.......ESQEREDVLAGM.............................SGKAIKGKVGKPKV.............KKLQLE.......ET 1228
ClTop2b AFVEELDKV.......EAQEREDILAGM.............................SGKAIKGKVGKPKV.............KKLQLE.......ET 1221
MmTop2b AFVEELDKV.......EAQEREDILAGM.............................SGKAIKGKVGKPKV.............KKLQLE.......ET 1221
DmTop2  ALESKLN.........EVEEKERAEEQGINLK.........................TAKALKGQKSASAKG.............RKVKSMGGGAGAGDV 1223
CeTop2b NFVSELD.........KQEAREKADQDASIKN.........................AAKKLAADAKTGRGP.............KKNVCTEVLPSKDGQ 1273
CeTop2  KLAEAVGN........NRKS                                                                              1213
AtTop2  SLDKELEKL.......DLKDAQVQQAIEAAQKKI.......................RAKSGAAVK.................VKRQAP.......KK 1208
ScTop2  AFEVGYQ.........EFLQRDAEARGGNVPN.........................KGSKTKGKG.................KRKLVDDE.....DY 1202
SpTop2  AFEHAWNKV......MDDIQREMLEEEQSSRDFVNRTKKKP...............RGKSTGTRKPRAIAG.....SSSSTAVKKEASS.......ES 1261
TbTop2  QFDRFFQDHEKKMVEAILKERRQRSPP.SDLLPGLQQPRLEVEE.....................AKGGKKFE.................MRVQVRKY.....VP 1159
TcTop2  RFDRAFEEHENTAVATILKERRVNPPT.GDVSRNLQQPRLELEEVKV...........SSSGGKSVP.................MRVRVRKYV....PP 1164
CfTop2  KFDKTFQEYERVLIHSIQKEQRPASITGGEEVPALRQPPLMLEA.............PAKGAASSS.................YRVHICRYE....EP 1166
ASFVTop AVEKAIIKGRSTQWKFH                                                                                 1191
CeTop2c RPQAKTPTRTAGWPPQD                                                                                  816
EcGyrA  REELELVREQFGDKRRTEITANSAD..INLEDLITQEDVVVTLSHQGYVKYQ.....................................................  552
HiGyrA  REELEEVKAQFGDDRLTEITAASGD..IDLEDLIAQEDVVVTLSHEGYVKYQ.....................................................  549
ErGyrA  REELEAFKTQYSDERRTEITANTAD..INIEDLINQEDVVVTLSHQGYVKYQ.....................................................  552
KpGyrA  REELELVREQFGDARRTDITANSVD..INIEDLITQEDVVVTLSHEGYVKYQ.....................................................  551
AsGyrA  RDELLAVREQYGDERPPEISASSAE..INIEDLITPEDVVVTLSHQGYVKYQ.....................................................  553
NgGyrA  RDELEEIKTNYGDERRSEINPFGGD..IADEDLIPQREMVVTLTHGGYIKTQ.....................................................  563
PaGyrA  REELEAVKAEFGDARRTEIVASQVD..LTIADLITEEDRVVTISHGGYAKSQ.....................................................  553
BsGyrA  REELTEIKERFNDERRTEIVTSGLET.IEDEDLIERENIVVTLTHNGYVKRL.....................................................  519
BsGyrA2 TNSLKALKKKYADTRRSVIEEKIEEIKINLEVMVASEDVYVTVTKDGYLKRT.....................................................  518
SaGyrA2 KEELNEIKKKFKSERLSLIEAEIEEIKIDKEVMVPSEEVILSMTRHGYIKRT.....................................................  516
SaGyrA  RDELTEIRDRFGDERRTEIQLGGFED.LEDEDLIPEEQIVITLSHNNYIKRL.....................................................  520
CfGyrA  RDELLEIKSKFKCPRITDIVDDYDD..IDVEDLIPNENMVVTITHRGYIKRV.....................................................  520
CjGyrA  RDELKEIRSKFDVPRITQIEDDYDD..IDIEDLIPNENMVVTITHRGYIKRV.....................................................  519
HpGyrA  KTELLEVKEQFSSPRRTEIQESYES..IDIEDLIANEPMVVSMSYKGYVKRV.....................................................  520
HfGyrA  ESELLDIKDEYADDRRTSFVANTGE..VTRADLIPEEDVVVVVSEDDYIKRM.....................................................  536
MlGyrA  RNELTEIAEKYGDDRRTRIIAVDGD..VNDEDLIAREEVVVTITETGYAKRT.....................................................  530
RpGyrA  KEELIKVKEEFASPRLTSIEFGEFD..QDIEDLIQREEMVVTVTLGGYIKRV.....................................................  543
MgGyrA  KEELQKINDQFGDERRSEILYDISEE.IDDESLIKVENVVITMSTNGYLKRI.....................................................  529
MgGyrA2 KKQFEGYKKQFHQQRRSQICGFINQKKVEESELIENKTYGVLITKAGNYHKF.....................................................  516
MtGyrA  RDELAEIVDRHGDDRRTRIIAADGD..VSDEDLIAREDVVVTITETGYAKRT.....................................................  529
MsGyrA  RDELKEIVDKHGDARRTRIVPADGE..VSDEDLIAREDVVVTITETGYAKRT.....................................................  530
SoGyrA  SEELTALVEKYGDDRKTKLIPYEGD..MSIEDLIAEEDIVVTVTRGGYIKRT.....................................................  533
SsGyrA  EEELEQIKAIHATPRRTVIVQEDGE..LIDTDLIANDQALILLTEQGYIKRM.....................................................  522
EcParC  KKELQADAQAYGDDRRSPLQEREEAKAMSEHDMLPSEPVTIVLSQMGWVRSAKGHDIDAPGLNYKAGDSFKAAVKGKSNQPVVFVDSTGRSYAID...PI  558
StParC  KKELQADADAYGDDRRSPLREREEAKAMSEHDMLPSEPVTIVLSQMGWVRSAKGHDIDAPGLNYKAGDSFKAAVKGKSNQPVVFIDTTGRSYAID...PI  558
HiParC  KKEIQEDAKKYANPRMSQLVEREEAKMISESDMTPAEPVTVILSEMGWVRCAKGHDIDPKSLSYKAGDSYLAHACGKSNQAVVFIDSTGRSYALD...PL  562
T4Gn52  EIK                                                                                                442
McGyrA  ISRLEEVKKQFGIKRKSQVEDLVEDLDVDQKEVIIEKEINLWISKDGYIKVI.....................................................   94
BbGyrA  FGDERRTKIIYDEEVLKTSMSDLMQKENIVVMLTKKGFLKRL.....................................................   72
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HsTop2a LPSPRGQRVIPRIT.............IEMKAEAEKKNK.KKIKNENTEGSPQ.....................EDGVELEGLKQRLEKKQ..KREPGTK 1273
CgTop2a LPSPHGKRVIPQVT.............MEMKAEAEKKIR.KKIKSENVEGTPT.....................ENGLELGSLKQRIEKKQ..KKEPGAM 1268
MmTop2a LPSPRGKRVIPQVT.............VEMKAEAEKKIR.KKIKSENVEGTPA.....................EDGAEPGSLRQRIEKKQ..KKEPGA. 1270
RnTop2a LPSPVGKRVIPQVT.............MEMRAEAEKKIR.RKIKSENVEGTPA.....................EDGAE.PGLRQRLEKRQ..KREPGTR 1269
HsTop2b MPSPYGRRIIPEI..............TAMKADASKKLL.KKKKGDLDTAAV.....KVEFDEEFSGAPVEGAGEEALTPSVPINKGPKPKREKKEPGTR 1308
ClTop2b MPSPYGRRIVPEI..............TAMKADASRKLL.KKKKGDPDTPVV.....KVEFDEEFSGTPVEGTGEETLTPSAPVNKGPKPKREKKEPGTR 1301
MmTop2b MPSPYGRRIVPEI..............TAMKADASRKLL.KKKKGDPDTTVV.....KVEFDEEFSGTPAEGTGEETLTPSAPVNKGPKPKREKKEPGTR 1301
DmTop2  FPDPDGEPVEFKITE............EIIKKMAAAAKVAQAAKEPKKPKEPK.................................EPK.VKKEPKGK 1275
CeTop2b RIEPMLDAATKAKYEKMSQPKKERVKKEPKEPKEPKKVKKEGQDIKKFMSPAAPKTAKKEKSDGFNSDMSEESDVEFDEGIDFDSDDDGVEREDVVSKPK 1373
AtTop2  PAPKKTTKKASESETTEASYSAMDTDNNVAEVVKPKARQGAKKKASESETTEASHSAMDTDNNVAEVVKPKGRQGAKKKAPAAAKEVEEDEMLDLAQRLA 1308
ScTop2  DPSKKNKKSTARKG.........................KKIKLE............................DK..........NFERI.LLEQKLVT 1237
SpTop2  KPSTTNRKQQTLLEF.............AASK.EPEKSSDININVKTE.............................DN....SHGLSVEENRI..SKSPGLD 1314
TbTop2  PPTKRGAGGRSDGDGG....ATAAGAAAAVGGRGEKKGPGRAGGVRRM......VLDALAKRV.TRL...LPRLLF                            1221
TcTop2  PPSKRPHVGQSVGGGGGGGSVRSSAAAVVAHVKAEKKAA.RARSMQKM...LLDVVARQVARVLPRL....PWFLF                            1232
CfTop2  PASKRKPEDTYGGALSSGGSTRNVGKRLTGARGAKKKKV.VRRTRTKM..SLGTRVAEFAGAQLGRLLPQLPRLLF                            1239
EcGyrA  ..................................................PLSEYEAQRRGGKGKSAARIKEEDFIDRLLVANTHDHILCFSSRGRVYS  601
HiGyrA  ..................................................PLTDYEAQRRGGKGKSATKMKEEDFIEKLLVANTHDTILCFSSRGRLYW  598
ErGyrA  ..................................................PLSDYEAQRRGGKGKSAARIKEEDFIDRLLVANTHDTILCFSSRGRLYW  601
KpGyrA  ..................................................PVNDYEAQRRGGKGKSAPRIKEEDFIDRLLVANTHDTILCFSSRGRLYW  600
AsGyrA  ..................................................PITDYEAQRRGGRGKSATRIKEENFVERLLVANTHDTILCFSTRGKVYW  602
NgGyrA  ..................................................PTTDYQAQRRGGRGKQAAATKDEDFIETLFVANTHDYLMCFTNLGKCHW  612
PaGyrA  ..................................................PLAAYQAQRRGGKGKSATGMKDEDYIEHLLVANSHATLLLFSSKGKVYW  602
BsGyrA  ..................................................PASTYRSQKRGGKGVQGMGTNEDDFVEHLISTSTHDTILFFSNKGKVYR  568
BsGyrA2 ..................................................SQRSFAASNGQDFGMKDTDRMLHQFEMNTT.DVLLLFTNKGSYIY  562
SaGyrA2 ..................................................SIRSFNASGVEDIGLKDGDSLLKHQEVNTQ.DTVLVFTNKGRYLF  560
SaGyrA  ..................................................PVSTYRAQNRGGRGVQGMNTLEEDFVSQLVTLSTHDHVLFFTNKGRVYK  569
CfGyrA  ..................................................PSKSYEKQKRGGKGKVAVTTYDDDFIESFFTCMSHDTLMFVTDRGQLYW  569
CjGyrA  ..................................................PSKQYEKQKRGGKGKLAVTTYDDDFIESFFTANTHDTLMFVTDRGQLYW  568
HpGyrA  ..................................................DLKVYEKQNRGGKGKLSGSTYEDDFIENFFVANTHDILLFITNKGQLYH  569
HfGyrA  ..................................................PVSRFRAQHRGGKGIIGTDLKEGDNVSSVFVTNTHDDLLCFTNHGQVYQ  585
MlGyrA  ..................................................KTDLYRSQKRGGKGVQGAGLKQDDIVRHFFVCSTHDWILFFTTQGRVYR  579
RpGyrA  ..................................................PLSSYRSQKRGGKGRSGLSMRDEDITTQVFVGSTHTPMLFFSNIGKVYS  592
MgGyrA  ..................................................GVDAYNLQHRGGVGVKGLTTYVDDSISQLLVCSTHSDLLFFTDKGKVYR  578
MgGyrA2 ..................................................ESNQLLKSTTDFKSESDTIIFAQTIANTDQIFIVTSLGNII  557
MtGyrA  ..................................................KTDLYRSQKRGGKGVQGAGLKQDDIVAHFFVCSTHDLILFFTTQGRVYR  578
MsGyrA  ..................................................KTDLYRSQKRGGKGVQGAGLKQDDMVNHFFVCSTHDWILFFTTQGRVYR  579
SoGyrA  ..................................................KTDDYRAQKRGGKGVRGTKLKEDDIVNHFFVSTTHHWLLFFTNKGRVYR  582
SsGyrA  ..................................................PASTFGTQNRATRGKAAAKIKDDDGVEHFLSCCDHDKVLFFSDRGVVYS  571
EcParC  TLPSARGQGEPLYGKLTLPPGATV...DHMLMESDDQKLLMASDAGYGFVCTFNDLVARNRAGKALITLPE.NAHVMPPVVIEDASDMLLAITQAGRMLM  654
StParC  TLPSARGQGEPLTGKLTLPPGATV...EHMLMEGDDQKLLMASDAGYGFVCTFNDLVARNRAGKTLITLPE.NAHVMPPLVIEDEHDMLLAITQAGRMLM  654
HiParC  SLPSARSQGEPLTGKLNLPTGATI...EYVVMASEQQELLMASDAGYGFICKFEDLIARNKAGKALISLPE.NAKVLKPKTLINSTALVVAITSAGRMLI  658
McGyrA  ..................................................DNNILNKNELSSFGKKPNDMWISQGVCSNL.DHLILISDQANYYS  138
BbGyrA  ..................................SQNEYKLQGTGGKGLSSYDL.NDGD-                                            96
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Fig. 2. Part 2, page 10.

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HsTop2a TKKQTTLAFKP.IKKGKKRNPWPDSESDRSSD.....................................ESNFDVPPRETE.PRRAATKTK. 1325
CgTop2a TKKQTTLAFKP.IKKGKKRNPWSDSESDMSSN.....................................ESNVDVPPREKD.PRRAATKAK. 1320
MmTop2a .KKQTTLPFKP.VKKGRKKNPWSDSESDVSSN.....................................ESNVDVPPRQKE.QRSRAAKAK. 1321
RnTop2a AKKQTTLPFKP.IKKAQKQNPWSDSESDMSSN.....................................ESNFDVPPREKE.PRIAATKAK. 1321
HsTop2b VRKTPTSSGKPSAKKVKKRNPWSDDESKSESDL.....................................EETEPVVIPRDSLLRRAAAERPK. 1364
ClTop2b VRKTPTSAGKPNAKKVKKRNPWSDDESKSESDL.....................................EETEPVVIPRDSLLRRAAAERPK. 1357
MmTop2b VRKTPTSTGKTNAKKVKKRNPWSDDESKSESDL.....................................EEAEPVVIPRDSLLRRAAAERPK. 1357
DmTop2  QIKAEPDASGD...EVDEFDAMVEGGSK.TSPK.....................................AKKAVVKKEPGEKKPRQKKENGG. 1327
CeTop2b PRTGKGAAKAEVIDLSDDDEVPAKKPAPAKKAAPKKKKSEFSDLSGGDSDEEAEKKPSTSKKPSPKKAAPKTAEPKSKAVTDFFGASKKNGKKAAGSDDE 1473
AtTop2  QYNFGSAPADSSKTAETSKAIAVDDDDDDVVVEVAPVKKGGRKPAATKAAKPPAAPRKRGKQTVASTEVLAIGVSPEKKVRKMRSSPFNKKSSSVMSRLA 1408
ScTop2  K....SKAPTK.IKKEKTPSV.SETKTEEEENA.....................................................PSSTSSS.. 1271
SpTop2  S....SDSG...KSRKRSQSVDSEDAGSKKPVK.....................................................KIAASA... 1346
EcGyrA  MKVYQLPEATRGARGRPIVNLLPLEQDERITAIL.....................................PVTEFEEGVKVFMATANGTVKKT 658
HiGyrA  LKVYQLPQASRGARGRPIVNILPLQENERITAIL.....................................PVSAYEEDKFVVMATAGGIVKKI 655
ErGyrA  MKVYQLPEASRGARGRPIVNLLPLEADERITAIL.....................................PVREYEEGRHIFMATASGTVKKT 658
KpGyrA  MKVYQVPEASRGARGRPIVNLLPLEANERYTAIL.....................................PVREYEEGVNVFMATASGTVKKT 657
AsGyrA  LKVYQLPEASRGARGRPIINLLPLEEGERITAIL.....................................PVKEYADDKYVFFATADGTVKKT 659
NgGyrA  IKVYKLPEGGRNSRGRPINNVIQLEEGEKVSAIL.....................................AVREFPEDQYVFFATAQGMVKKV 669
PaGyrA  LRTFEIPEASRTARGRPLVNLLPLDEGERITAMLQIDLEALQQNGGADDDLDEAEGAVLEGEVVEAAEVEEVEGETAELVAEPTGAYIFMATAFGTVKKT 702
BsGyrA  AKGYEIPEYGRTAKGIPIINLLEVEKGEWINAII.....................................PVTEFNAELYLFFTTKHGVSKRT 625
BsGyrA2 CPVHQLPDIRWKDMGQHFSNLITIDRDETIVKAI.....................................PIKEFDPSAYLLFFTKNGMVKKT 619
SaGyrA2 IPVHKLRDIRWKELGQHVSQIVPIEEDEVVINVY.....................................NEKDFNTDAFYVFATQNGMIKKS 617
SaGyrA  LKGYEVPELSRQSKGIPVVNAIELGNDEVISTMI.....................................AVKDLESEDNFLVFATKRGVVKRS 627
CfGyrA  LKVYKIPEGSRTAKGKAVVNLISLQADEKIKAII.....................................PTTDFDESKSLAFFTKNGIVKRT 626
CjGyrA  LKVYKIPEGSRTAKGKAVVNLINLQAEEKIMAII.....................................PTTDFDESKSLCFFTKNGIVKRT 625
HpGyrA  LKVYKIPEASRIAMGKAIVNLISLAPDEKIMATL.....................................STKDFSNERSLAFFTKNGVVKRT 626
HfGyrA  LKAYQVPEMSRTARGKSAVNLLDFDDGEEITAVV.....................................NCDDLEDIEGYLTMVTRNGYIKRT 643
MlGyrA  AKAYELPEASRTARGQHVANLLAFQPEERIAQVI.....................................QIRSYEDAPYLVLATRAGLVKKS 636
RpGyrA  LKLYKLPLSNPQGKGRPMVNILSLQENEHITNIM.....................................PLPENQDEWDHLNIMFATAKGNIRRS 652
MgGyrA  IRAHQIPYGFRTNKGIPAVNLIKIEKDERICSL.....................................LSVNNYDDGYFFFCTKNGIVKRT 634
MgGyrA2 NIPVYKLAFNSKNKLASLVSKKPILLEYETIVFV.....................................GTMNSVNQPILVLTSKLGMVKRI 614
MtGyrA  AKAYDLPEASRTARGQHVANLLAFQPEERIAQV.....................................IQIRGYTDAPYLVLATRNGLVKKS 635
MsGyrA  AKAYELPEASRTARGQHVANLLAFQPEERIAQV.....................................IQIKSYEDAPYLVLATRNGLVKKS 636
SoGyrA  AKAYELPDAGRDARGQHVANLLAFQPDETIAQI.....................................RAIRDYEAVPYLVLATKAGLVKKT 639
SsGyrA  LNAYQIPIASRTARGVPIVQMLPIPKDEKITSL.....................................VSVSEFDDDTYFIMLTKQGYIKKT 628
EcParC  FPVSDLPQLSK.GKGNKIINIPSA.....................................................EAATGEDGLAQLY 690
StParC  FPVDSLPQLSK.GKGNKIINIPSA.....................................................EAAKGDDGLAHLY 690
HiParC  FPAQDLPVLSK.GKGNKMITIPAA.....................................................NAKDRSELLTKLL 694
McGyrA  IPLYKISTSKWKEQGVHINSVATTQPNETIINAL.....................................VIKEFINSTQHLLLVTKNGLIKRT 196
McGyrA2                                                                          -MIKRT   5
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HsTop2a .FTMDLDSDEDFSDFDEKTD.....DEDFV.................................PSDASPPKTKTSPKLSNKELKPQKSV.VS....DL 1379
CgTop2a .FTMDLDSDEDFSGSDGKDE.....DEDFF.................................PLDTTPPKTKIPQKNTKKALKPQKSA.MS...GDP 1375
MmTop2a .FTVDLDSDEDFSGLDEKDE.....DEDFL.................................PLDATPPKAKIPPKNTKKALKTQGSS.MS..VVDL 1377
RnTop2a .FTADLDSDDDFSGLDEKDE.....DEDFF.................................PLDDTPPKTKMPPKNTKKALKPQKSS.TS...VDL 1376
HsTop2b .YTFDFSEEEDDDADDDDDD.....NNDLE.............ELKVKASPITNDGEDEFVPSDGLDKDEYTFSPGKSKAT.PEKSL.HDKKSQDF 1439
ClTop2b .YTFDFSEEEEEDADDD.DD.....NNDLE.............ELKVKASPITNDGEDEFVPSDGLDKDEYAFSPGKSKAT.PEKSS.HDKKSQDF 1431
MmTop2b .YTFDFSEEEDDDAAAA.DD.....SNDLE.............ELKVKASPITNDGEDEFVPSDGLDKDEYAFSSGKSKAT.PEKSS.NDKKSQDF 1431
DmTop2  GLKQSKIDFSKAKAKKSDDD.....VEEVT.................................PRAERPGRRQASKKIDYSSLFSDEEE.DGG...NV 1383
CeTop2b DDESFVVAPREKSGRARKAPPTYDVDSGSDSDQPKKKRGRVVDSDSD                                                  1520
AtTop2  DNKEEESSENVAGNSSSEKSGGDVSAISRPQRANRRKMTYVLSDSESESANDSEFDDIEDDEDDE                                  1473
ScTop2  SI.FDIKKEDKDEGELSKIS.....NKFKK.....................................ISTIFDKMGSTSATSKENTPEQDDVA..... 1321
SpTop2  SGRGRKTNKPVATTIFSSDD.....EDDLL.................................PSSLKPSTITSTKASAKN.................. 1389
EcGyrA  VLTEFNRLRTA.GKVAIKLVD.....GDELI.....................................GVDLTSGEDEVMLFSAEG.KVVRFK..ESSVRA 713
HiGyrA  ALTEFSRPRSN.GIIALNLRD.....EDELI.....................................GVDITDGSNEIMLFSSQG.RVVRFA..ENAVRA 710
ErGyrA  ALTEFSRHVS..GIIAVNLNE.....GDELI.....................................GVDLTDGSDEAMLFSAEG.KVVRFS..EQAVRS 712
KpGyrA  PADEFSRPRSA.GIIAVNLNE.....GDELI.....................................GVDLTSGQDEVMLFSAAG.KVVRFK..EDDVRA 712
AsGyrA  SLSAFSRPLSS.GIRAINLKE.....GDELI.....................................GVDITDGSNEIMLFSDAG.KVVRFN..EGSAAP 714
NgGyrA  QLSAFKNVRAQ.GIKAIALKE.....GDYLV.....................................GAAQTGGADDIMLFSNLG.KAIRFN..EYWEKS 724
PaGyrA  PLVQFSRPRSS.GLIALKLEE.....GDTLI.....................................AAAITDGAKEVMLFSSAG.KVIRFA..ESVVRI 757
BsGyrA  SLSQFANIRNN.GLIALSLRE.....DDELM.....................................GVRLTDGTKQIIIGTKNG.LLIRFP..ETDVRE 680
BsGyrA2 ELTHYKAQRYSKALVALNLKG.....EDELI.....................................DVHVTNGESQIFMATHLG.YGLWFG..EDEVNV 675
SaGyrA2 TVPLFKTTRFNKPLIATKVKE.....NDDLI.....................................SVMRFEKDQLITVITNKG.MSLTYN..TSELSD 673
SaGyrA  ALSNFSRINRN.GKIAISFRE.....DDELI.....................................AVRLTSGQEDILIGTSHA.SLIRFP..ESTLRP 682
CfGyrA  NLSEFKNIRSI.GVKAINLDD.....NDELVTVVIANSEPDE...SYDDSFEDGEGVSNLQTISEDNSENSLESGKMLFAVTKKG.MCIKFA..LNKVRQ 714
CjGyrA  NLSEYQNIRSV.GVRAINLDE.....NDELVTAIIVQRDEDEIFATGGEENLENQEIENLDDENLENEESVSTQGKMLFAVTKKG.MCIKFP..LAKVRE 716
HpGyrA  NLSEFGSNRSC.GIRAIVLDE.....GDELVS.....................................AKVVDKNAKHLLIASHLG.IFIKFP..LEDVRE 682
HfGyrA  GTDRFQNILST.GIIATKLDE.....GDELV.....................................DVEVTDGETDLVIGTERG.MSIRFD..EDEVRA 698
MlGyrA  KLTDFDSNRSG.GIVAINLRD.....NDELV.....................................GAVLCAADGDLLLVSANG.QSIRFSATDEALRP 693
RpGyrA  DLLDFKKIQSN.GKIAIRLDE.....DDKLI.....................................DVKPCKEDEHILLATKAG.KALRFPVESLRIIK 709
MgGyrA  SLNEFINILSN.GKRAISFDD.....NDTLY.....................................SVIKTHGNDEIFIGSTNG.FVVRFH..ENQLRV 689
MgGyrA2 DLTKLNIKPLK.ATLCISLRD.....KDHLV.....................................SAFLQQDDKLICLVSDHN.YYTVFH..TNEIPL 669
MtGyrA  KLTDFDSNRSG.GIVAVNLRD.....NDELV.....................................GAVLCSAGDDLLLVSANG.QSIRFSATDEALRP 692
MsGyrA  KLSDFDSNRSG.GIVAINLRE.....GDELV.....................................GAVLCSAEDDLLLVSANGQSIRFSA.TDEALRP 693
SoGyrA  PLKDYDSPRSG.GVIAINLREQADGSDDELI.....................................GAELVSAEDDLLLISKKAQSIRFTA.SDDTLRP 701
SsGyrA  ALSAFSNIRAN.GLIAISLVE.....GDQLR.....................................WVRLAKAEDSVIIGSQKGMAIHFKA.DQDELRA 685
EcParC  VLPPQSTLTIHVGKRKIKLR......PEELQKVTGERGRRG.TLMRGLQRIDRVEIDSPRRASSGDSEE                            752
StParC  VLPPQSTLTIHVGKRKIKLR......PEELQKVVGERGRRG.TLMRGLQRIDRIEIDSPHRVSHGDSEE                            752
HiParC  LISDQASLEFYSGKRKIVLK......PEDLQKFRAERGRKGSTLPRGLHTNLEIMVIEP                                       747
McGyrA  QISDLETKIFN.KSFKIMKIS.....DDDSL.....................................VYADLISSKTSYC.CIVTKN..GYAVRY 246
McGyrA2 KISEFENINRN.GKKAINLRE.....NDQLV.....................................SVFATTGQDTIFIANESG.KVIRIK..ESVVNP  60
FsGyrA                                                                      -DLLMIATKNG.QAVTFP..ISCFRA  22
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Fig. 2. Part 2, page 11.

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HsTop2a EADD.....VKGSVPLSSSPPATHFPD 1401
CgTop2a ESDE.....KDSVPASPGPPAADLPA 1396
MmTop2a ESDV.....KDSVPASPGVPAADFPA 1398
RnTop2a ESDG.....KDSVPASPGASAADVPA 1397
HsTop2b GNLFSFP.....SYSQKSEDDSAKFDSNEEDS 1466
ClTop2b GNLFSFP.....SYSQKSEDDSAKFDSNEEDT 1458
MmTop2b GNPFSFP.....SYSQKSEDDSAKFDSNEEDT 1458
DmTop2  GSDD..... 1387
ScTop2  ..... 1321
SpTop2  ..... 1389
EcGyrA  M.....GCNTTGVRGIRLGEG.....DKVVS  734
HiGyrA  M.....GRLATGVRGIKLALTNDISDDESAVEIEDISDDNAEASLDLNI...DKVVS  759
ErGyrA  M.....GRTATGVRGINLQGE.....DRVVS  733
KpGyrA  M.....GRTATGVRGIKLAGE.....DKVVS  733
AsGyrA  MQPMLMSSDDVDGDDESVIDAGNDDDGSDNGEGSESTESKGTFKGVRPMGRTAGGVRGIRLLNG.....DKVVS  783
NgGyrA  G.....NDEAEDADIETEISDDLEDETADNENTLPSGKNGVRPSGRGSGGLRGMRLPADGKIVS.....LITFA  788
PaGyrA  M.....GRNARGVRGMRLGKG.....QQLIS  778
BsGyrA  M.....GRTAAGVKGITLTDD.....DVVVG  701
BsGyrA2 V.....GARAAGVKGINLKED.....DFVVS  696
SaGyrA2 T.....GLRAAGVKSINLKVE.....DFVVM  694
SaGyrA  L.....GRTATGVKGITLREG.....DEVVG  703
CfGyrA  I.....GRVSRGVTAIRFKENL.....DEVVG  736
CjGyrA  I.....GRVSRGVTAIKFKEKN.....DELVG  738
HpGyrA  M.....GRNARGVIGIRLNEND.....FVVGA  704
HfGyrA  M.....GRSARGVRGIKLEGD.....DVVAG  719
MlGyrA  M.....GRATSGVQGMRFNAD.....DRLLS  714
RpGyrA  SRISDGVRGMKLAKEDSVISMTVLKGINSTKEDR...DAYLTVPWEKRLEIAKGEEFNLEELGV.....NLNAD  775
MgGyrA  L.....SRTARGVFGISLNKG.....EFVNG  710
MgGyrA2 I.....SSKGMGVKGMKLKLEDQIKFVVAFEANEPLVMICSDGSVINLKQTELVVVS  721
MtGyrA  M.....GRATSGVQGMRFNID.....DRLVS  713
MsGyrA  M.....GRATSGVQGMRFNED.....DRLLS  714
SoGyrA  M.....GRATSGVKGMSFREG.....DELLS  722
SsGyrA  L.....GRATRGVKSMRLRSGDALISMDILPSQVVANIAVGSEDEPDEDLGGDTDAI  737
McGyrA  NI-  248
McGyrA2 Q.....SRVGSGVRALKLETN.....DVVVG   81
FsGyrA  M.....GRGTHGVKGITLAEG.....DEVIS   43
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Fig. 2. Part 1, page 12.

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HsTop2a ETEITNPVPK...KNVTVKKTAAKSQSSTSTTG.AKKRAAPKG........TKRDPALNSGVSQKP.DPAKTKNRRKRKPSTSDDSDSNFEKIVSKAVTS 1488
CgTop2a DTEQLKPSSK...QTVAVKKTATKSQSSTSTAG.TKKRAVPKG........SKSDSALNAHGPEKP.VPAKAKNSRKRKQSSSDDSDSDFEKVVSKVAAS 1483
MmTop2a ETEQSKPSKK....TVGVKKTATKSQSSVSTAG.TKKRAAPKG........TKSDSALSARVSEKP.APAKAKNSRKRKPSSSDSSDSDFERAISKGATS 1484
RnTop2a ETEPSKPSSK...QTVGVKRTITKGQSLTSTAG.TKKRAVPKE........TKSDSALNAHVSKKP.APAKAKNSRKRMPSSSDSSDSEFEKAISKGATS 1484
HsTop2b AS.VFSPSFGLKQTDKVPSKTVAAKKGKPSSDTVPKPKRAPKQKKVVEAVNSDSDSEFGIPKKTTT.PKGKGRGAKKRKASGSENEGDYNPGRKTSKTTS 1564
ClTop2b AS.VFTPSFGLKQTDKVPSKTVAAKKGKPPSDTAPKAKRAPKQKKVVETVNSDSDSEFGIPKKTTT.PKGKGRGAKKRKASGSENEGDYNPGRKPSKTAS 1556
MmTop2b AS.VFAPSFGLKQTDKLPSKTVAAKKGKPPSDTAPKAKRAPKQKKIVETINSDSDSEFGIPKKTTT.PKGKGRGAKKRKASGSENEGDYNPGRKPSKTAS 1556
DmTop2  .........................DGNASDD.DSPKRPAKR..............GREDESSGGAKKKAPPKKRRAVIESDDDDIEIDEDDDDDSDF 1445
ScTop2  ...............................TKKNQTTAKK..................TAVKPKLAKKPVRKQQKVVELSGESDLEILDSYTDREDS 1370
SpTop2  ................................KGKKASSVKKQ.................SPEDDDDDFIIPGSSSTPKASSTNAEPPEDSDSPIRKRPT 1440
EcGyrA  LIV..PRGDGAILTATQNGYGKRTAVAEYPTKSRATKGVISIKVTERNGLVVGAVQVDD.CDQIMMITDAGTLVRTRVSEISIVGRNTQGVILIRTAEDE  831
HiGyrA  LVV..PKGEGAILTATQNGYGKRTQLSEYPTKSRNTKGVISIKVSERNGKVVAATQVEE.TDQIMLITDAGTLVRTRVSEVSIVGRNTQGVRLIRTADDE  856
ErGyrA  LII..PRGEGDILTVTQNGFGKRTAVSEYPTKSRATKGVISIKVSERNGKVVGAVQVDA.ADQIMMITDAGTLVRTRVSEVSIVGRNTQGVTLIRTAEDE  830
KpGyrA  LIV..PRGEGRILTATENGYRKRTAVAEYPTKSRATQGVISIKVTERNGSVVGAVQVDD.CDQIMMITDAGTLVRIRVSEVSIVGRNTQGVILIRTAEDE  830
AsGyrA  LIV..PRGEGAILTATENGYGKGTALTEYPTKSRGTQGVRSIKVDED..GKVSIDQVDD.TDQIMLITNGGTLVRTRVSEVSIIGRNTGGVRLIRTGEDE  878
NgGyrA  PET..EESGLQVLTATANGYGKRTPIADYSRKNKGGQGSIAINTGERNGDLVAATLVGE.TDDLMLITSGGVLIRTKVEQIRETGRAAAGVKLINLDEGE  885
PaGyrA  MLI..PESGAQILTASERGFGKRTPLSKFPRRGRGGQGVIAMVTNERNGALIAAVQVQE.GEEIMLISDQGTLVRTRVDEVSLSGRNTQGVTLIKLASDE  875
BsGyrA  MEI..LEEESHVLIVTEKGYGKRTPAEEYRTQSRGGKGLKTAKITENNGQLVAVKATKG.EEDLMIITASGVLIRMDINDISITGRVTQGVRLIRMAEEE  798
BsGyrA2 GEI..LQQSDSIVLFTQRGAVKRMSLSEFEKTSRAKRGVVMLRELKKNPHRVVALFACGLEQRLMAETEKGDRKELQTKELRTNDRYSNGSFFFDEEESG  794
SaGyrA2 TEG..VSENDTILMATQRGSLKRISFKILQVAKRAQRGITLLKELKKNPHRIVAAHVVTGEHSQYTLYSKSNEEHGLINDIHKSEQYTNGSFIVDTDDFG  792
SaGyrA  LDVAHANSVDEVLVVTENGYGKRTPVNDYRLSNRGGKGIKTATITERNGNVVCITTVTG.EEDLMIVTNAGVIIRLDVADISQNGRAAQGVRLIRLGDDQ  802
CfGyrA  AVV.IENDSQEILSVSQKGIGKRTTADEYRLQSRGGKGVICMKLTPKTKDLVGVVMVDE.EMDLMALTSSGKMIRVDMQSIRKAGRNTSGVIVVNVDGDE  834
CjGyrA  AVV.IENDEQEILSISAKGIGKRTNAGEYRLQSRGGKGVICMKLTEKTKDLISVVIVDE.TMDLMALTSSGKMIRVDMQSIRKAGRNTSGVIVVNVENDE  836
HpGyrA  VVI..SDDGNKLLSVSENGLGKQTLAEAYREQSRGGKGVIGMKLTQKTGNLVGVISVDDENLDLMILTASAKMIRVSIKDIRETGRNASGVKLINTADKV  802
HfGyrA  VAAIDEAHHSWILTVTENGYGKRTDLDAYRTQSRNGKGLIDIKANERNGPVCAINTVGE.GDHLVVMSDEGQILRTPVEDISTVGRNTMGIIVMDLDEGD  818
MlGyrA  LNV..VREDTYLLVATSGGYAKRTSIEEYPMQGRGGKGVLTVMYDRRRGSLVGAIVVDE.DSELYAITSGGGVIRTTARQVRQAGRQTKGVRLMNLGEGD  811
RpGyrA  SILEMANSEEFILTVTENGFGKRSSAYGYRITDRGGSGIINMDINDKTGLVVGVMPVKM.DDELMLITNSGKLIRCKLESVRITGRNTSGVILFKLDDDE  874
MgGyrA  LST..SSNGSLLLSVGQNGIGKLTSIDKYRLTKRNAKGVKTLRVTDRTGPVVTTTTVFG.NEDLLMISSAGKIVRTSLQELSEQGKNTSGVKLIRLKDNE  807
MgGyrA2 RMATAKKLPVKKAINYCFSDATNTQLINFQGKNGSKLITTSELNQMSKTAISQTRFNKLN                                          781
MtGyrA  LNV..VREGTYLLVATSGGYAKRTAIEEYPVQGRGGKGVLTVMYDRRRGRLVGALIVDD.DSELYAVTSGGGVIRTAARQVRKAGRQTKGVRLMNLGEGD  810
MsGyrA  LNV..VRPDTYLLVATSGGYAKRTSIDEYSVQGRGGKGILTIQYDRKRGSLVGALIVDD.DTELYAITSTGGVIRTAARQVRKAGRQTKGVRLMNLAEGD  811
SoGyrA  MNV..VRAGTFVFTATDGGYAKRTSVDEYRVQGRGGLGIKAAKIVEDRGSLVGALVVEE.HDEILAITLSGGVIRTRVNGVRETGRDTMGVQLINLGKRD  819
SsGyrA  LEES.DNPGPWLLGVTMKGFGKRVPIGQFRLQHRAGLGVKAIRFKSKDDQLVALHVVNA.DDELMIVTNRGIIIRQSVNDISPQSRSATGVRVQRLDADD  835
McGyrA2 AIS..SFKLTHITTVSNKGLFKKTPIDDYRISGRNGKGIKVMNLNQRTGKFKAIIDARE.TDLILIISSDGNLIKTKVSNIPSLSRNASGVKAIRLADXQ  178
FsGyrA  LLW..LKAGNKILTITEKGYGKRSEPGSYRVTRRGSKGVRNLNVTDKIGAAVFVESVAD.DYDLIITSKDGQVIRIKAADIRLTGRNAQGVKAITLRDGD  140
StGyrA                                                          -TVDD.CDQIHDDHDAGTLVRTRVSEISVVGRNTQGVILIRTAEDE   44
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Fig. 2. Part 2, page 12.

Fig. 2. Page 13.

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HsTop2a KKSKGESDDFHMDFDSA......VAP.RAKSVRAKKPIKYLEESD..EDDLF                                                1531
CgTop2a KKSKGENQDFRVDLDET......MVP.RAKSGRAKKPIKYLEESD..DDDLF                                               1526
MmTop2a KKAKGEEQDFPVDLEDT......IAP.RAKSDRARKPIKYLEESD.DDDDLF                                               1528
RnTop2a KKLKGEERDFHVDLDDT......VAP.RAKSGRARKPIKYLEESD...DDLF                                               1526
HsTop2b KKPKKTSFDQDSDVDIFPSDFPTEPPSLPRTGRARKEVKYFTESDEEEDDVDFAMFN                                           1621
ClTop2b KKPKKTSFDQDSDVDIFPSDFTSEPPALPRTGRARKEVKYFAESDE.EEDVDFAMFN                                           1612
MmTop2b KKPKKTSFDQDSDVDIFPSDFTSEPPALPRTGRARKEVKYFAESDE.EEDVDFAMFN                                           1612
DmTop2  NC                                                                                                  1447
ScTop2  NKD...EDDAIPQRSRRQ......RSSRAASVPKKSYVETLELSDDSFIEDDEEENQGSDVSFNEED                                1428
SpTop2  RRA...AATVKTPIYVD........PS.FDSMDEPSMQDDSFIVDNDEDVDDYDESD                                           1485
EcGyrA  NVVGLQRVAEPVDEEDLDTIDGSAAEGDDEIAPEVDVDDEPEEE                                                        875
HiGyrA  HVVSLERVCDADEDDSLEESSSEE                                                                            880
ErGyrA  HVVGLQRVAEPVEDEELDGVVKVEGEVAEDDDAIDDIDGDDDIAEDDE                                                    878
KpGyrA  NVVALQRVAEPVDDEELDAIDGSAAEGDEDIAPEADTDDDIAEDEE                                                      876
AsGyrA  TVVGLQRIAESYEEENDVMAIDGEVSEGTDTAPDAGSAAADPEE                                                        922
NgGyrA  TLVSLERVAEDESELSGASVISNVTEPEAEN                                                                     916
PaGyrA  VLVGLERVQEPSGGDDEDLPEGEEAAESLGESAESESEPAAEAEGNEE                                                    923
BsGyrA  HVATVALVEKNEEDENEEEQEEV                                                                             821
BsGyrA2 KVTAVWRLHTEQ                                                                                        806
SaGyrA2 EVIDMYIS                                                                                            800
SaGyrA  FVSTVAKVKEDADEVNEDEQSTVSEDGTEQQREAVVNDETPGNAIHTEVIDSEENDEDGRIEVRQDFMDRVEEDIQQSSDEDEE              886
CfGyrA  VVSIARCPKEESDDDDIVADDTQEQDME                                                                        862
CjGyrA  VVSIAKCPKEENDEDELSDENFGLDLQ                                                                         863
HpGyrA  MYVN.SCPKEEEPENLENSPTQLFE                                                                           826
HfGyrA  AVASVDVIPAAMTTEAEELDDADSVEEDAETDAKADADDE                                                            858
MlGyrA  TLLAIARNAEESADGVSVKVMISRSRVLSFFGSDSNTSPDRT                                                          853
RpGyrA  KVVSVSLIAETSESEEASELAEEGLENDVKV                                                                     905
MgGyrA  RLERVTIFKEELEDKEMQLEDVGSKQITQ                                                                       836
MtGyrA  TLLAIARNAEESGDDNAVDANGADQTGN                                                                        838
MsGyrA  TLIAIARNADEDEAAESISESDADTAESPEA                                                                     842
SoGyrA  AVVGIARNAEAGREAEEVDGDVAVDETAEGAATTGTDEGEAPSAE                                                       864
SsGyrA  AIAAVALVPPSGEEELAEMSESEES                                                                           860
McGyrA2 EINAXTLEYRKHGLENEDFEED                                                                              200
FsGyrA  VVKDATALPSVEDIEQDSADAKETFDKVKGVEVDDDSVVKDDAEKQEIGPTETEE                                             195
StGyrA  NVVGLQRVAEPVDDEELDAIDGSVTEGDEDIAPEAESDDDVADDADE                                                      91
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EcTop1                          MGKALVIVESPAKAKTINKYLGSDY.............................VVKSSVGHIRDLPTSGSAAKKS   47
KaTop1                          MGKALVIVESPAKAKTINKYLGNDY.............................VVKSSVGHIRDLPTSGSASKKS   47
HiTop1                          MSKSLVIVESPAKAKTINKYLGSQY.............................VVKSSVGHIRDLPTVGSSTGEK   47
SsTopA                            MPKLVIVESPTKARTIRNYLPKDY.............................RVEASMGHVRDLPQSAS.....   41
MtTopI  MERGAQLADPKTKGRGSGGNGSGRRLVIVESPTKARKLASYLGSGY.............................IVESSRGHIRDLRGPRRCTRKY   68
BaTop1                          MGKTLFIAEKPKVANEIMKSPRFRHSQKYIGSKPYYGYYENDHY.............IVSWCRGHLLELKNPEEMDPKY   66
BsTop1                          MSDYLVIVESPAKAKTIERYLGKKY.............................KVKASMGHVRDLPKSQMGVDIE   47
TmTop1                      MSKKVKKYIVVESPAKAKTIKSILGNEY.............................EVFASMGHIIDLPKSKFGVDLE   50
MgTop1                          MIKNLVVIESPNKVKTLKQYLPSDEF.............................EIVSTVGHIREMVYKNFGFDEN   48
RP4TraE                       MQFERLVIAEKPELAKAIVEGLGGGSRKDGYYECGSD.....................RVTWCYGHMLALLDPEDYDERY   59
BfTop1                                                    -IYVAPPSSLLPEGA.............LICAAVGHILEFLEPGELNEKY   36
SaTrsI                            MNTLILCEKPSQAMDLSTVFAKKKKQNGYMEISDEQLNVSG.............FLTWAVGHLVELKEPQEYDEKY   63
pAMb1                             MSTVILAEKPSQALAYASALKQSTKKDCYFEIKDPIFADET.............FITFGFGHLVELAEPGHYDEKW   63
BT223g                            MSTVILAEKPSQALAYAQAFNQSDKKDGYFEIKDPLFTDET.............FITFGFGHLVELAEPGNYDEKW   63
EcTopB                              MRLFIAEKPSLARAIADVLPKPHRKGDGFIECGNGQ.................VVTWCIGHLLEQAQPDAYDSRY   58
HiTop3                              MRLFIAEKPSLARAIADVLPKPHQRGDGFIKCGDND.................VVTWCVGHLLEQAEPDAYDPKF   58
ScTop3                            MKVLCVAEKNSIAKAVSQILGGGRSTSRDSGYMYVKNYDFMFSGFPPARNGANCEVTMTSVAGHLTGIDFSHDS.HGW   77
HsTop3                      MEMALRGVRKVLCVAEKNDAAKGIADLLSNGRMRRREGLSKFNKIYEFDYHLY.....GQNVTMVMTSVSGHLLAHDFQMQF.RKW   80
SaRevG        NEISKLKNEGNVAPALQKVKTVLLVVESPNKAKTISSFFSRPSIRQIGNMRVYETVLGDKVL.............MVTASGGHVYDLTTQDMGIYGV   84
MkRevGB   RERVRKVLAGELKPEETGRLARSALMIVESPNKARMIASLFSQRPSRRRLNGGVAYEAAADGLH.............LTVVATQGHVADLVEEPGVHGVL   87

EcTop1  ADSTSTKT......AKKPKKDERGALVNRMGVDPWHNWEAHYEVLPGKEKVVSELKQLAEKA..DHIYLATDLDREGEAIAWHLREVIGG.....DDARY  134
KaTop1  ADSTATKG......AKKPKKDERSALVNRMGVDPWHDWNAHYEVLPGKEKVVSELKQLAEKA..DHIYLATDLDREGEAIAWHLREVIGG.....DEQRY  134
HiTop1  AKPISTKGMDAEEKAKIKAEKERNALVKRMGIDPYHDWKANYQILPGKEKVVSELKSLAKKA..DHIYLATDLDREGEAIAWHLREVIGG.....NDDRF  140
SsTopA  ..............DIPT.ELKGEKWSNLGVDVENNFAPLYIVPKDKKKIVKTLKDALKDA..DELILATDEDREGKVISWHLLQLLQP......RCPR  117
MtTopI  KSQPWARL.....................GVNVDADFEPLYIISPEKRSTVSELRGLLKDV..DELYLATDGDREGEAIAWHLLETLKP......RIPV  138
BaTop1  KLFQL.........EHLPLIFQP.................SYKVIQENAEQLQILVKLLQRPD.VDHAVNICDADREGELIYREVYEYAGVN......KKQ  134
BsTop1  Q.................................NFEPKYITIRGKGPVLKELKTAAKKA..KKVYLAADPDREGEAIAWHLAHSLDL.....DLNSD  105
TmTop1  K.................................DFEPEFAVIKGKEKVVEKLKDLAKKG...ELLIASDMDREGEAIAWHIARVTNT......LGRK  106
MgTop1  ..............TYTPIWEDW.........TKNKQKNPKQKHLLSKFEIIKSIKAKASDA..QNIFLASDPDREGEAISWHVYDLLDQK....DKAKC  119
RP4TraE ANWNM.........ADLPIVHIP.............WRKKPSGDAGAK.AQFKTILSLLKQA..KSVVHAGDPDDEGQLLVDEILEYANC......RLPV  128
BfTop1  KSYSL.........DSLPIIIDL.............FQYKVVSDK.K.EVLQRIKDTIFDKRVKTIILATDAAAEGEYIGRNILYRLHC......KKTI  105
SaTrsI  KNF...........STYPILLEK.............DDFQFKV.SDKTK.DQFNNIKKIFKENKIDEVIIATDPAREGENIAYKILNQLKVTD....KVTI  134
pAMb1   QNWKL.........ESLPIFPDR..............YDFEVATDKKKQFKIVAELLKQA..NTIIVATDSDREGENIAWSIIHKANAFSK...DKTY  133
BT223g  QNWKL.........ESLPIFPDR..............YDFEVAKDKGKQFKIVAELLKKA..NTIIVATDSDREGENIAWSIIHKANAFSK...DKTF  133
EcTopB  ARWNL.........ADLPIVPEK...............WQLQPRPSVTKQLNVIKRFLHEA..SEIVHAGDPDREGQLLVDEVLDYLQLAPE..KRQQV  129
HiTop3  KQWRL.........EHLPIIPEK...............WQLLPRKEVKKQLSVVEKLIHQA..DTLVNAGDPDREGQLLVDEVFSYANLSAE..KRDKI  129
ScTop3  GKCAIQEL......FDAPL.NEI.....................MNNNQKKIASNIKREARNA..DYLMIWTDCDREGEYIGWEIWQEAKRGNRLIQNDQV  148
HsTop3  QSCNPLVL......FEAEI.EKY.....................CPENFVDIKKTLERETRQC..QALVIWTDCDREGENIGFEIIHVCKAVKP...NLQV  148
SaRevG  DIMKQNSSLVFIPIYNSIKKCENNHQFTDFFESNKCPRCMTTKVRYDSLKSINVLRNLAVEA..DEVLIGTDPDTEGEKIAWDLYLALRPYNS.....NI  177
MkRevGB RIDE.........RWVPMYDVLGRCSECGEQVVGSEECPNCGGEVELKTPLLESIRELASEA..DVILIGTDPDTEGEKIGWDVFNYLGWTTA.....QV  171
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Fig. 3. Part 1, page 1.

Fig. 3. Part 2, page 1.

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EcTop1   SRVVFNEITKNAIRQAFNKP...GELNIDRVNAQQARRFMDRVVGYMVSPLLWKKIARGL.....................SAGRVQSVAVRLVVER  207
KaTop1   SRVVFNEITKNAIRQAFEKP...GELNIDRVNAQQARRFMDRVVGYMVSPLLWKKIARGL.....................SAGSVQSVAVRLVVER  207
HiTop1   SRVVFNEITKNAIKQAFEKP...EQLNMDRVNAQQTRRFLDRVVGFMVSPLLWKKVARGL.....................SAGRVQSVAVKLLVER  213
SsTopA   SAWSFDEITQEAIQAAHEKLS..GCRSAALVHAQETSQDSRSLGGLHPVGPALEKNCLGP.....................IAGRVQSVAVRLLVQR  191
MtTopI   KRMVFHEITEPAIRAAAEHP...RDLDIDLVDAQETRRILDRLYGYEVSPVLWKKVAPKL.....................SAGRVQSVATRIIVAR  211
BaTop1   SRVYKSSFEAAELEAALNRLESASKYDGLAYSAK.ARQYLDYLLGMNITRGCTTKLAQNKFL.....................LSSGRVQMCLLHEIRQR  212
BsTop1   CRVVFNEITKDAIKESFKHP...RMINMDLVDAQQARRILDRLVGYKISPILWKKVKKGL.....................SAGRVQSVALRLIIDR  178
TmTop1   NRIVFSEITPRVIREAVKNP...REIDMKKVRAQLARRILDRIVGYSLSPVLWRNFKSNL.....................SAGRVQSATLKLVCDR  179
MgTop1   KRITFNEITKKAVVDALKQP...RNIDLNWVESQFARQILDRMIGFRLSRLLNSYLQAK.....................SAGRVQSVALRFLEER  191
RP4TraE  QRLLINDNNVKIVRRQLAAMRDNREFAGLSAAAE.ARSVGDQLYGFNITRLYTLAARAKGYQG.................LLSVGRVQTPILGLVVRR  208
BfTop1   KRLWTSSMTAFSIQKAFSQLKADAETLPLYYQAK.ARAESDYMIGLTLSRAYGILLKEQGIVP.................HNTTISLGRVQTPLLAEIVKR  188
SaTrsI   KRLWLTSKVESSIRKAFKNILPKEKTYGFYKEGR.ARELSDWLVGINLSRHFTKISRELGNDG.................VIHIGRVSSPTLNMVYNR  214
pAMb1    KRLWINSLEKDVIRSGFQNLQPGMNYYPFYQEAQ.TRQIADWLIGMNASPLYTLNLQQKGVQG.................TFSLGRVQTPTLYLIFQR  213
BT223g   KRLWINSLEKDVIRSGFQNLQPGMNYYPFYQEAQ.TRQIADWLIGMNASPLYTLNLQQKGVQG.................TFSLGRVQTPTLYLIFQR  213
EcTopB   QRCLINDLNPQAVERAIDRLRSNSEFVPLCVSAL.ARARADWLYGINMTRAYTILGRNAGYQG.................VLSVGRVQTPVLGLVVRR  209
HiTop3   LRCLISDLNPSAVEKAVKKLQPNRNFIPLATSAL.ARARADWLYGINMTRAYTIRGRQTGYDG.................VLSVGRVQTPVLGLIVRR  209
ScTop3   YRAVFSHLERQHILNAARNPS...RLDMKSVHAVGTRIEIDLRAGVTFTRLLTETLRNKLRNQATMTKDGAKHRGGNKNDSQVVSYGTCQFPTLGFVVDR  245
HsTop3   LRARFSEITPHAVRTACENLT...EPDQRVSDAVDVRQELDLRIGAAFTRFQTLRLQRIFPEV.....................LAEQLISYGSCQFPTLGFVVER  230
SaRevG   RRAEFHEVTRKAILQAINQPR...EFNVNLVKSQLVRRIEDRWIGFKLSSILQTRFWPEYCKSLSSNKQ......LNCNENKNLSAGRVQTPVLSWIVDR  268
MkRevGB  YRTEFHEVTRRGISEALKEES.WKNVDAGRVSAQILRRVADRWIGFSLSQDLWDVFKHLEIKLGELPSGSRIEVRLDIPSGVEVVDFRRTFDEDSSVRSR  270

EcTop1   EREIKAFVPEEFWEVDASTTTPSGEALALQVTHQNDKPFRPVNKEQTQAAVSLLEK.....................ARYSVLEREDKPTTSKPGA  282
KaTop1   EREIKGFVQEEYWEVDASTTTP.GGDLPLQVTHKDDKPFRPVSRDETMAAVSLLEK.....................ASYSVLEREDKPTSSKPGS  281
HiTop1   EREIKAFQPEEYWEVAVLTNNQNKQAIRLDVTDYKGKKFDPKNQKEAQSAVDFLNV.....................SDYVVTDLETKPTSSRPRA  288
SsTopA   ERARRAFRQGSYWDLKAQLTVEAGQFEAKLWTLAGQRLATGNDFDESTGQIIAGRQVCLLDQQEAEALPIRRP.....DPALASKVAEEKPTHCANQRP.  285
MtTopI   ERDRMAFRSAAYWDILAKLDASVSDPDAAPPTFSA.RLTAVAGRRVATGAISTRWARCAKATKSLCSTRGARPRWPAGLDGTQLTVASAEEKPYARRPYP  310
BaTop1   ELAIENYREQSYYHLQLITDLGLKPVMKTEDQVLNPSPLKSLGEN.....................................LKDQYLTVEDFKEGTRKQNPK  278
BsTop1   EKEINDFKPEEYWTIDGTFLKGQETFEASFFG.KNGKKLPLNSEADVKEILSQLKG.....................NQYTVEKVTKKERKRNPAL  252
TmTop1   EREILRFVPKKYHRITVNFDGLTAEIDVKEKKFFDAETLKEIQSI.....................DELVVEEKKVSVKKFAPPE  243
MgTop1   EKEIAKFVPRFWWTVDVLLNKENNQKVVCANKSIPLVLREINPELSASLKLDFEAAENVSGIDFLNEASATRFAN...QLTGEYEVYFIDEPKIYYSSPN  288
RP4TraE  CRENAAHQKTYYYLVNGQFEVE.GIQFPARYQVADGDPVDEKGRLSNKEHAEGIAAAVSG.....................QPARIVSVTTKAKEAAAPL  286
BfTop1   ERLLEQFTAENFWFTVKATFNNQ.GNVYEGEWFHEKENRIFTEEQDEQLCELVR.....................NQSSTIMEMKEEMRTYQPP  259
SaTrsI   ENNIKGFKGKKFYKVSATINKDEQEVKTELKNKFDSEDELHEFLFENDITDLT.....................QKGLVTDIEKEIGYTMPPK  286
pAMb1    QEAIENFRKEPFFEVEASIKVNQGSFK.GVISPTQRFKT.QEELLAFVSSEQ.....................AKIGNQEGIIADVQTKEKKTNSPS  287
BT223g   QEAIENFKKEPFFEVEASIKVNQGSFK.GVLSPTQRFKT.QEELLAFVSSKQ.....................AKIGNQEGRITDVQTKEKKTNSPS  287
EcTopB   DEEIENFVAKDFFEVKAHIVTPADERFTAIWQPSEACEPYQDEEGRLLHRPLAEHVVNRISG.....................QPAIVTSYNDKRESESAPL  290
HiTop3   DLEIEHFQPKDFFEVQAWVNPESKEEKTPEKSTALFSALWQPSKACEDYQDDDGRVLSKGLAEKVVKRITN.....................QPAEVTEYKDVREKETAPL  299
ScTop3   FERIRNFVPEEFWYIQLVVENKDNGGTTTFQWDRGHLFDRLSVLTFYETCIETA.....................GNVAQVVDLKSKPTTKYRPL  319
HsTop3   FKAIQAFVPEIFHRIKVTHDHKDGIVEFNWKRHRLFNHTACLVLY.....................QLCVEDPMATVVEVRSKPKSKWRPQ  300
SaRevG   YTEYQRNKSRVYYGKIDQLQDIVIYVPKQDGVRK.....................NSKIVVVFNEINQLEEEFGPLP.  324
MkRevGB  SVRLRREGDEYVVRTRISRGGDVTYTATLLDPNRKLGDRNGVRPELVRVRASVNGEPVDPNVKLEPMTWLSAGRVQTPVLGWIIDRAREYRETEFYACRA  370
MkrevGA                                                                MNATLRIRNRPVAESTYTSLRGAKAEVVRVEREEREIHPKP   41
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EcTop1  .PFIT.STLQQAASTRLGFGVKKTMMMAQRLYEA.........GYITYMRTDSTNLSQDA....VNMVRGYISDNFGKKYL.................PES  351
KaTop1  .PFIT.STLQQAASTRLGFGVKKTMMMAQRLYEA.........GHITYMRTDSTNLSQDA....LNMVRGYISDKFGKKYL.................PDS  350
HiTop1  .PFIT.STLQQTASTRLGFGVKKTMMLAQRLYEA.........GYITYMRTDSTNLSQDA....LNMARSYIENHFGAQYL.................PEK  357
SsTopA  .PFTT.STLQQESNRKLRLSARETMRVAQSLYER.........GFITYMRTDSVHLSQQA....IEAARSCVEQMYGKNYL.................SPQ  354
MtTopI  .PFMT.STLQQEASRKLRFSAERTMSIAQRLYEN.........GYITYMRTDSTTLSESA....INAARTQARQLYGDGVR.................RPA  379
BaTop1  LLYNL.TDLYKDAHAQLQINAETAKKHIQNLYEE.........GFITYPRSSSRHLPTEQ....VDRVKGVMQALAKSRYS........LLVQSVDIDAI  356
BsTop1  .PFTT.STLQQEAARKLNFRAKKTMMIAQQLYEGIDLGREGTVGLITYMRTDSTRISNTA....VDEAAAFIDQTYGKEFL.................GGK  330
TmTop1  .PFKT.STLQQEAYSKLGFSVSKTMMIAQQLYEGVET.KDGHIAFITYMRTDSTRVSDYA....KEEARNLITEVFGEEYV.................GSK  320
MgTop1  PVYTT.ASLQKDAINKLGWSSKKVTMVAQRLYEGISVNGKQT.ALISYPRTDSIRISNQFQSECEKYIEKEFGSHYLADKN.................K  368
RP4TraE .PYNL.LKLQMDASRKFGFKPDQVKDITQALREKH.........KLITYNRSDCEYLSEEQ....HGDAPGVLAAIAQTAPM.........LAAAAQRANP  363
BfTop1  .LLYL.STLQMDAGNAFGFKPAETLKYAQSLYDK.........GYLSYPRTQDERITESD....ARELENNIQFLSGHDTS..........GALFPLPVS  334
SaTrsI  .FYDL.SALQEDMNDKYKISAKRTLEIAQTLYEK.........KLITYPRTDSRYITEDE....KEMLLENIDYLKEITKI..............NLNNE  357
pAMb1   .LFSL.SSLQSKVNQLYKATASQTLKAMQGLYEA.........KLLSYPRTDTPFITENE....FAYLKANFGKYSGFLGL............DLEMVQT  360
BT223g  .LFSL.SSLQSKVNQLYKATASQTLKAMQGLYEA.........KLLSYPRTDTPFITENE....FAYLKANFGKYSGFLGL............DLEMVQT  360
EcTopB  .PFSL.SALQIEAAKRFGLSAQNVLDICQKLYETH.........KLITYPRSDCRYLPEEH....FAGRHAVMNAISVHAPD..........LLPQPVVDP  366
HiTop3  .PYSL.SALQIDAAKRFGMSAQAVLDTCQRLYETH.........RLITYPRSDCRYLPEEH....FAERHNVLNAISTHCEA..........YQVLPNVILT  376
ScTop3  .PLTT.VELQKNCARYLRLNAKQSLDAAEKLYQK.........GFISYPRTETDTFPHAMDLKSLVEKQAQLDQLAAGGRTAWASYAASLLQPENTSNNN  408
HsTop3  .ALDT.VELEKLASRKLRINAKETMRIAEKLYTQ.........GYISYPRTETNIFPRDLNLTVLVEQQTPDPRWGAFAQS..............ILER  374
SaRevG  .PYTT.DTLLSDSNNFFGLSAPETMRIAQDLFEL.........GLITYHRTDSNRISNTG....ISVAENYLKDVLGDKYT..............NIFK  394
MkRevGB EVPADDVTIRALIEELKVPRALTEKLDEATIRVLSKIAEEGPDAEFSEEEVGRFTETELFERKDGRYRLSEEGRKVLESEGVIGLMLHLAGVSGR      465
MkrevGA .PFETGTMLQAATRRLRL.SSERVMQLAQDLFEG.........GLITYHRTDSTRVSEEGKRVRRDYIRANFDPEDYNPRT.................  111

EcTop1  PNQYASKE.NSQEAHEAIRPSDVNVMAE...........SLKDMEADAQKLYQLIWRQFVACQMTPAKYDSTTLTVGAGDFR.................L  422
KaTop1  ANQYASKE.NSQEAHEAIRPSDVNVLAE...........TLKDMEADAQKLYQLIWRQFVACQMTPAQYDSTTLTVAAGDFK.................L  421
HiTop1  PNFYSSKE.NAQEAHEAIRPSDIRALPE...........SLEGMEKDAVRLYDLIWCQFLACQMPPAQYDSSTLTVTAGDYT.................L  428
SsTopA  PRQFTTKSKNAQEAHEAIRPAGNTFRLP...........QETGLSGAEFAVYDLIWKRTIASQMAEARQTMLSVLLEVDNAE.................F  426
MtTopI  PRQYTRKVKNAQEAHEAIRPAGETFATP......DAVRRELDGPNIDDFRLYELIWQRTVASQMADARGMTLSLRITGMSGH.............QEVVF  460
BaTop1  DIKHKTFDDDLVSSHFAIIPTTKQYQE.............EGRPEIEKQLYSLVVKRFVGNFMRPAVYLVRDVSLIDAMGN.................TY  426
BsTop1  RKPAKKNE.NAQDAHEAIRPTSVLRKPS...........ELKAVLGRDQMRLYKLIWERFVASQMAPAVLDTMSVDLTNNGLT................F  402
TmTop1  RERRKSNA.KIQDAHEAIRPTNVFMTPE...........EAGKYLNSDQKRLYELIWKRFLASQMKPSQYEETRFVLRTKDGK................YRF  394
MgTop1  LKRHKKDEKIIQDAHEGIHPTYITI..........TPNDLKNGVKRDEFLLYRLIWIRTVASLMADAKTSRTIVRFINQKNKF.................  441
RP4TraE TIKSRAFNSSKVSAHHAIIPTESTAD.............LSKLTDAEQKIYLLIARAYVAQFWPKHLYDQTDVLAQVGDHR.................F  432
BfTop1  TLMNNKRYIGEVTDHHALLITDKIPKD.............KDLSEDEKSIYHLVVKRILAAHYPDVAMSHKEIITKVMDRF................TF  404
SaTrsI  LTNNSLINPSKIEDHYAILITGNDFNK.............VDLKEEEINVYKSILQNVAMNFMDKEQYETTTIEIAVKKLM.................F  426
pAMb1   EPRKRYVDGSKVQEHHAIIPTKQVPTE...........SALAKMDDLQRKIYALVVKTTVAMFLPDYLYEETKIQTKVADLL................F  432
BT223g  EPRKRYVDGSKVQEHHAIIPTKQVPTE...........SALAKMDDLQRKIYALVVKTTVAMFLPDYLYEETKIQTKVADLL................F  432
EcTopB  DIRNRCWDDKKVDAHHAIIPTARS..............SAINLTENEAKVYNLIARQYLMQFCPDAVFRKCVIELDIAKGK................F  434
HiTop3  EQRNRCWNDKKVEAHHAIIPTAKN..............RPVNLTQEERNIYSLIARQYLMQFCPDAEYRKSKITLNIAGGT................F  444
ScTop3  KFKFPRSGSHDDKAHPPIHPIVSLG.............PEANVSPVERRVYEYVARHFLACCSEDAKGQSMTLVLDWAVER................F  477
HsTop3  GGPTPRNGNKSDQAHPPIHPTKYTN..............NLQGDEQRLYEFIVRHFLACCSQDAQGQETTVEIDIAQER................F  440
SaRevG  PRS.WGDG....GAHEGIRPTKPIDVEQLRLLIEEGELELAKRLTNNHFKVYDIIFRRFISSQIIPLKVRKEIVKIELYGENKKEKINSNQNIIEVITGI  489
MkrevGA ...WEPEAEHVEGAHECIRPTRPADAEELRTMVREGAIQTTVTLTSHHLRLYDLVFRRFVASQMKPAKVLYQEAVLEVEVKG..........VPVAELE  197
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EcTop1   KARGRILRFDGWTKVMPAL.....RKGDEDRILPAVNKGDALTLVELTPAQHFTKPPARFSEASLVKEL.....EKR  489
KaTop1   KARGRTLRFDGQTKVMPAL.....RKGDEDRTLPLVKQGDRLSLVELTPAQHFTKPPARFSEASLVKEL.....EKR  488
HiTop1   KAKGRILRFDGWTKVLPQI.....GKNPEDQELPSVTVSEKLALKEVQPTQHFTKPPARFTEAALVKEL.....EKR  495
SsTopA   APVASGSISRASSALSVEGSDDPD.AALEDPRNSAAGVEGCGSPHLSGTGRESVMKPQPPARYTEASLVKML.....ENE  500
MtTopI   SATGRTLTFPGFLKAYVETVDELV.GGEADDAERRLPHLTPGQRLLDIVELTPDGHATNPPARYTEASLVKAL.....EEL  534
BaTop1   QIKESVLREKGFLEVFQEEVK.....EESVETFKVPILQKGQELQIYDFELQESKTKKPALHTESSILTFMETAGRKIDDEHLKEL.....MKGK  511
BsTop1   RANGSKVKFSGFMKVYVEG.....KDDQMEEKDRMLPDLQEGDTVLSKDIEPEQHFTQPPPRYTEARLVKTL.....EER  472
TmTop1   KGTVLKKIFDGYEKVWKTE.....RNTGEFP.FEEGESVKPVVVKIEEQETKPKPRYTEGSLVKEM.....ERL  457
MgTop1   YTSSKSLLFDGYQRLYEEIKPNTKDELYIDLSKLKIGDKFSFEKISV.....NEHKTNPPPRYTQASLIEEL.....EKS  511
RP4TraE  GVRSNVTTSPGWKILYKNDAGNEDLEGNADDIEQDLRKLRDGQAGTCTDAKAEQQETKPQPLYTMESLLSDLTRVAKYIRDDRLRKILIEKDKGKQGEHG  532
BfTop1   RSKGKELLSKGRHHIIPP.....TNENDIMLPTLLKGSEGVVTDTLTTKSKTKPPNRYTSSSLIGFMKNAAQAIEDED.RKSI.....SNL  484
SaTrsI   EVKGKIIQDNGFKALLNK.....QKTSEETIPNFEKNEEVDI.ELDLLEKETTPPKRYTEKTLLKAMANPIETLEDEGLKSTL.....KEVK  507
pAMb1    QSIGKTPKQEGWKILFKQQTK.....EEEEDVQTLPLVIIGEHAEV.DVKSAEKETQPPKA-                          487
BT223g   QSIGKTPKQEGWKILFKQQNK.....EEKEDVQTLPLVIIGEHAEV.DVKSAEKETQPPKAFTEGTLLTAMKTAGKTIDDEEAQKIL.....KDTE  517
EcTopB   VAKARFLAEAGWRTLLGSKER.....DEENDGTPLPVVAKGDELLCEKGEVVERQTQPPRHFTDATLLSAMTGIARFVQDKDLKKIL.....RATD  520
HiTop3   IAQARNLQTAGWKELLGKED.....DTENQEPLLPIVKKGQILHCERGEVMSKKTQPPKPFTDATLLSAMTGIARFVQDKELKKIL.....RETD  529
ScTop3   SASGLVVLERNFLDVYPW.....ARWETTKQLPRLEMNALVDIAKAEMKAGTTAPPKPMTESELILLM.....DTN  543
HsTop3   VAHGLMILARNYLDVYPY.....DHWSDKILPVYEQGSHFQPSTVEMVDGETSPPKLLTEADLIALM.....EKH  505
SaRevG   TLPGIDTEISKFAYVPVRNVSR.....SVAERLKELGRSIPTDFSIE...ISNSFIKSTVNLYTQADLVMEM.....KNK  556
MkrevGA  LSGVLEIVEPGFTKVLTEYDLP.....AYGIRETPELEEGDRLEI..GAVEVLERHEEYPYDQSELVEDM.....RER  263

EcTop1   GIGRPSTYASIISTIQD.RGYV....RVENRRFYAEKMGEIVTDRLEENFREL..MNYDFTAQMENSLDQVANHEAEWKAVLDHFFSDFTQQLDKAEKDP  582
KaTop1   GIGRPSTYASIISTIQD.RGYV....RVENRRFYAEKMGEIVTDRLEENFRDL..MNYDFTAQMEDRLDQVANHQAEWKEVLNHFFGDFTTQLATAEKDP  581
HiTop1   GIGRPSTYAAIISTIQE.RGYV....RTENRRFYAEKMGEIVTDRLNESFGEL..MNYDFTANMEDTLDKIASGSVNWKTELNQFFKDFSSQLSKAELDE  588
SsTopA   GIGRPSTYASIIGTIVD.RGYA....QLVSNTLTPTFTAFAVTALLEQHFPDL..VDTSFSARMEQSLDDISNGEVDWLPYLSQFYRGDRGWKNRSNFAK  593
MtTopI   GIGRPSTYSSIIKTIQD.RGYV....HKKGSALVPSWVAFAVTGLLEQHFGRL..VDYDFTAAMEDELDEIAAGNERRTNWLNNFYFGGDHGVPDSVARS  627
BaTop1   RIGTVATEAAFIPVLHE.KNFI....DIEKGKIITTPIGRAFIEQFPVQQI....KDPLYTAEMEGMIHRIEKNEMSYENFIAQTNAFVQKITQEIIRIP  602
BsTop1   GIGRPSTYAPTLDTIQR.RGYV....ALDNKRFVPTELGQIVLDLIMEFFPEI..INVEFTAKMERDLDHVEEGNTEWVKIIDNFYTDFEKRVKKAESEM  565
TmTop1   GIGRPSTYASTIKLLLN.RGYI....KKIRGYLYPTIVGSVVMDYLEKKYSDV..VSVSFTAEMEKDLDEVEQGKKTDKIVLREFYESFSSVFDRNDRIV  550
MgTop1   NIGRPSTYNTMASVNLE.RGYA....NLVNRFFYITELGEKVNNELSKHFGNV..INKEFTKKMEKSLDEIAENKVNYQEFLKQFWTNFKSDVKLAENSI  604
RP4TraE  GIGTPATRDSIIATLFE.RGYL....VEKGKHIVSTPTGEELYDALPDTAR.....FPDMTALWHEQQKAIQAGERDTLSFVNELMEYIGAEVANIKDNG  622
BfTop1   PLGTEATRAGLTHLVRIEKIYE.....WKKNKVYPTLLGITAVDSIKRGSVI...KSPILTAKWDVKLNEIGASLYNHKDFIAHSKKLSS.VLFEEVKTY  575
SaTrsI   GLGTPATRADIIENLKK.NKYI....QVQKNKIYITKNGILACLLLEGHLL....SKPDLTGQWEKYLNGISKGEKDDDSFINTINEMIKKTINEEVKNK  598
BT223g   GIGTEATRASIIEALKQ.KEYI....QVIKNKLVVTEKGKLLCQAVESQHLL....TSAEMTAKWETYLKKIGKREGNQENFITNIKKFIVHLLEAVPTDI  609
EcTopB   GLGTEATRAGIIELLFK.RGFL....TKKGRYIHSTDAGKALFHSLPEMA.....TRPDMTAHWESVLTQISEKQCRYQDFMQPLVGTLYQLIDQAKRTP  610
HiTop3   GLGTEATRAGIIELLFK.RGFL....TKKGRNIHSTETGRILIQALPNIA.....TQPDMTAHWESQLTDISQKQATYQQFMHNLNQILPDLVRFVDLNA  619
ScTop3   GIGTDATIAEHIDKIQV.RNYVRSEKVGKETYLQPTTLGVSLVHGFEAIGLEDSFAKPFQRREMEQDLKKICEGHASKTDVVKDIVEKYRKYWHKTNACK  642
HsTop3   GIGTDATHAEHIETIKA.RMYV...GLTPDKRFLPGHLGMGLVEGYDSMGYEM..SKPDLRAELEADLKLICDGKKDKFVVLRQQVQKYKQVFIEAVAKA  599
SaRevG   KIGRPSTYATIIGTILR.RGYV..LESLKTKKLIPTRLGVEVNKYLNENYGRF..VSEDRTRKLLQLMDMVEAGQEKYEEVLKQVYEEINEIR        644
MkrevGA  GLGRPSTYAQIVEKLFR.RGYV..YEVPQRRWIFPTTRGEAVYEYLSTHYERF..VSEETTRDLEERMDAVALGKAEYQEEMEKLYLELERVVEMPDPEP  358
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Fig. 3. Page 3.

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EcTop1  EE.................GGMRPNQMVLTSIDCPTCGRKMGIRTASTG..VFLGCSGYALPPKERCKTTINLVPENEVLNVLEGEDAETNALRAKRRCP  663
KaTop1  EE.................GGMQPNPMVLTSIDCPTCGRKMGIRTASTG..VFLGCSGYALPPKERCKTTINLVPENEVLNVLEGDDAETN.LRAKRRCQ  661
HiTop1  LE.................GGMRPNSLVETDIKCPTCGRNMAIRTASTG..VFLGCTGYALPPKERCKTTINLIPEAELLNVLDESS.ETKALMDRKRCT  668
SsTopA  VK.................LIRLPLA.QSRWRDCPPKS.....................................VLVVLALTRAEADGEPIKANLP  635
MtTopI  GG.................LKKLVGINLEGIDAREVNSIKLFDDTHGRPIYVRVGKNGPYLERLVAGDTGEPTPQRANLSDSI.TPDELTLQVAEELFAT  709
BaTop1  DT.............VSYNLIETWKKQIEVCQCP.CGNGIILDRGKFFGCSNHPNCNKGLPKRVKEKTI.PTAQVKKLFEENKTDIIKGFKSNGKEFSA  686
BsTop1  KEVE....IEPEYAGEDCELCSSPMVYKMGRYGKFLACSNFPDCRNTKPIVKQIGVKCPSCGEGNIVERKSK.KKRVFYGCDRYPDCEFVSWDKPIERKC  660
TmTop1  VD.....................FPTNQKCSCGKEMRLSFGKYG.....FYLKCECGKTRSVKNDEIAVIDDGKIFLGRKDSESGSPDGRSVEGKGNL  622
MgTop1  QK.....................VKKEKELVERDCPKCNQPLVYRYTKRGNEKFVGCSDF.....PKCKYSEFSNPKPKLTLETLDELCPECNNKLVKRRT  679
RP4TraE LN.....................MKIDTHPCPSCGKPLRRLKKKDKNEYFWGCTGF....ADGCKFA.......CDDKGGKPVPREAPKVSELHKCM  687
BfTop1  SS.............TWNQNGVERIKSESIGACLLCGSNVVLRKGKHG..EFYGCSNY...KDSGCTFNLPFKVLNKKLSKKQLMELLKNEKTDIIKGF  656
SaTrsI  ESIQ..............KVAKEKVSTNNIAKCPACDNGYLIDRKG.....FYGCTEY....NNGCEFTIPKKLLEKSIPPTVVKALCESKTTKKLKGF  674
BT223g  EKLN..........FSDYQEQKEKEAEKSIVGKCPKCGNNIVLKKS......FYGCSNY.....PECKFTLAEHFRKKKLTKTNVKELLEGKET.LVKGI  687
EcTopB  VRQFRGIVAPGSGGSADKKKAAPRKRSAKKSPPADEVGSGAIA                                                        653
HiTop3  LRQLSRIKMIKSDRAKPKSAVKKSSKSNGETD                                                                   651
ScTop3  NTLLQVYDRVKASM                                                                                    656
HsTop3  KKLDEALAQYFGNGTELAQQEDIYPAMPEPIRKCPQCNKDMVLKTKKNG.GFYLSCMGFPE.....CRSAVWL.PDSVLEASRDSSV..........CP  681

EcTop1  KCGTAMD....SYLIDPKRKLHVCGNNP.............TCDGYEI.............EEGEFRIKGY.................DGPIVE  710
KaTop1  KCGTAMD....SYLIDPKRKLHVCGNNP.............TCDGYEI.............EEGEFRIKGY.................DGPVVE  708
HiTop1  KCGTAMD....SYVIDAHRKIHICGNNP.............NCDGYLI.............EEGSFKIKGY.................DGPVVE  715
SsTopA  KELTPAD.......LDVQRVEHYCGKKPKGRTNWEHILKPTSRFTCLRVPTVPTSNLGQPPRKNPSEAGFFA.KGM..SLETISLEQAVGLLSLPRTLGE  725
MtTopI  PQQGRTLGLDPETGHEIVAREGRFGPYVTEILPEPAADAAA...AAQGVKK......RQKAAGPKPRTGSLLRSMD...LQTVTLEDALRLLSLPRVVGV  797
BaTop1  YLAFVNGEVSFNLPSVEELSLGQCPKCQKGKILNRKTFFGCSEYQNGCDFMLPAKIKGKKLSDSQIKKLVNNHVTDFINGFSGEKGEFTAAIRLKTDLSI  786
BsTop1  PKCGKML.VEKKLKKGIQVQCVECDYKEEPQK                                                                    691
TmTop1  SEKRRKGKKGS                                                                                       633
MgTop1  KFNAKKTFIGCSNFPNCRFIKKDNAAEFKQ                                                                     709
RP4TraE ACGHGLS...RRPGKKRGMFWWGCSNFP.............TCKQTYP...........DLKGRPDYS.KGRNGTNQE                     737
BfTop1  KWK.DKTFNAPLVWNREDQKVQFGK                                                                         680
SaTrsI  KSKKSGKSFDCKLILTKENKLQFSFD                                                                       700
BT223g  KNKEKKSYNAVVKIGEKGYIDFISFSK                                                                      714
HsTop3  VCQPHPV....YRLKLKFKRGSLPPTMP...........LEFVCCIGGCDDTLREILDLRFSGGPPRASQPSGRLQANQSLNRMDNSQHPQPADSRQTG  765

EcTop1  CEKCGSEMHLKMGRFGKYMACTNEECKNTRKILRNGEVAPPKEDPVPLPELPCEKSDAYFVLRDGAAGVFLAANTFPKSRETRAPLVEELYRFRDRLPEK  810
KaTop1  CEKCGSEMHLKMGRFGKYMACTNDECKNTRKILRNGEVAPPKEDPVPLPELPCEKSDAYFVLRDGAAGVFLAANRFPKSRETRAPLVEELY.FRDRLPEK  807
HiTop1  CDKCGADMHLKLGRFGKYMGCTN..CDNTRKILKNGEVAPPKEEPVHFPELKCEKSDAYFVLRDGASGVFMSAHNFPKSRETRPVKIAELVQYRERLPEK  813
SsTopA  HPETGRRIQAGLGRFGPYVVCD.....................................................LGGGEKDYRSLKADDDVLTIDLDRALELLAQP  779
MtTopI  DPASVEEITAQNGRYGPYLKRGNDSRSLVTEDQIFTITLDEALKIYAEPKRRGRQSASAPACASWEQIRRRASQWSSRTADSGRTSPTVRPMPACVRATT  897
BaTop1  CFEFPTTDDRTVGKCPLCQSRVIIGKTNYLCEQYKRGCDFIVSGMILEKRITASQIKKLLEKNMTDTVKGFVSKKTGKSFDAKLTYDSTQKRVTFIYEKK  886
HsTop3  SSKALAQTLPPPTAAGESNSVTCNCGQEAVLLTVRKEGPNRGRQFFKCNGGSCNFFLWADSPNPGAGGPPALAYRPLGASLGCPPGPGIHLGGFGNPGDG  865

EcTop1  LRYLADAPQQDPEGNKTMVRFSRKTKQQYVSSEKDGKATGWSAFYVDGKWVEGKK                                             865
KaTop1  LRYLADAPQQDPEGNKTLVRFSRKTKQQYVASEKEGKATGWSAFFIDGKWTEAKK                                             862
HiTop1  LAYLADAPQKDPEENEAIVRFSRKEKKQYVTSEKEGKATKWIVDFTNGKWVERKK                                             868
SsTopA  KKSRGRGKEPIRRLASILMTKPPFKFLRVLRSLPQTWQGQCRCPRMRSRRRSAWKQQLQP                                      839
MtTopI  WLP                                                                                               900
HsTop3  SGSGTSCLCSQPSVTRTVQKDGPNKGRQFHTCAKPREQQCGFFQWVDENTAPGTSGAPSWTGDRGRTLESEARSKRPRASSSDMGSTAKKPRKCSLCHQP  965

HsTop3  GHTRPFCPQNR                                                                                       976
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