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Topoisomerase Assays

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Abstract

Topoisomerases are nuclear enzymes that play essential roles in DNA replication, transcription, chromosome segregation, and recombination. All cells have two major forms of topoisomerases: type I, which makes single-stranded cuts in DNA, and type II enzymes, which cut and pass double-stranded DNA. DNA topoisomerases are important targets of approved and experimental anti-cancer agents. The protocols described in this unit are of assays used to assess new chemical entities for their ability to inhibit both forms of DNA topoisomerase. Included are an *in vitro* assay for topoisomerase I activity based on relaxation of supercoiled DNA and an assay for topoisomerase II based on the decatenation of double-stranded DNA. The preparation of mammalian cell extracts for assaying topoisomerase activity is described, along with a protocol for an ICE assay for examining topoisomerase covalent complexes *in vivo* and an assay for measuring DNA cleavage *in vitro*.

Keywords

Topoisomerase; Topoisomerase I; Topoisomerase II; camptothecin; etoposide; topoisomerase poison

INTRODUCTION

Topoisomerases (Table 3.3.1) are nuclear enzymes that play essential roles in DNA replication, transcription, chromosome segregation, and recombination. All cells contain two major forms of topoisomerase, type I: EC 5.99.1.2, which makes single-stranded cuts in DNA, and type II: EC 5.99.1.3, which cuts and passes double-stranded DNA (Leppard and Champoux, 2005; Pommier et al., 1998; Schoeffler and Berger, 2008; Wang, 1998). Type I topoisomerases are further subdivided into two mechanistically distinct subgroups; type IA enzymes, which are homologous to *E. coli* topoisomerase I, and type IB enzymes, which are

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Introduction to drugs targeting DNA topoisomerases

homologous to human topoisomerase I. Topoisomerases play critical roles in DNA replication, transcription, and chromosome structure by altering the topological state of DNA. These enzymes are capable of relaxing supercoiled DNA and of decatenating interlocked DNA (Fig. 3.3.1). While bacterial DNA gyrase, a type II topoisomerase, can introduce negative supercoils into DNA (Schoeffler and Berger, 2008), all known eukaryotic topoisomerases can only relax DNA. The decatenation of interlocked DNA is a critical topoisomerase function, since semi-conservative DNA replication results in catenated sister chromatids (Nitiss, 2009a; Postow et al., 2001). Topoisomerases are important targets for many chemotherapeutic agents and antibiotics. Compounds active against eukaryotic topoisomerases are clinically useful anticancer agents. Fluoroquinolones are potent inhibitors of prokaryotic type II topoisomerases, and are commonly employed broad-spectrum antibiotics (Drlica and Malik, 2003).

The DNA topoisomerase drugs in current clinical use influence these enzymes in a very selective manner. These agents—including the eukaryotic DNA topoisomerase I drugs, camptothecin, irinotecan, and topotecan, and the eukaryotic DNA topoisomerase II drugs, doxorubicin and etoposide—convert their target topoisomerases to DNA-damaging agents. Normally, topoisomerases bind to and cleave DNA by forming an enzyme:DNA covalent intermediate (see Background Information). The DNA is cut in one or both strands depending upon whether DNA topoisomerase I or II is involved. By forming a drug-enzyme-DNA complex, these chemotherapeutic agents prevent the subsequent DNA-resealing step normally catalyzed by topoisomerases. Such drugs are referred to as “topoisomerase poisons,” and are mechanistically similar to the bactericidal quinolones, which act on DNA gyrase and DNA topoisomerase IV, the bacterial counterparts of eukaryotic DNA topoisomerase II (Vos et al., 2011). Since the covalent complex plays a key role in the mechanism of action of topoisomerase poisons, measurement of complex formation *in vivo* and *in vitro* is critical in characterizing compounds targeting topoisomerases and also for understanding potential mechanisms of drug resistance.

Described in this unit is an *in vitro* assay for topoisomerase I activity based on relaxation of supercoiled DNA (Basic Protocol 1). This is followed by an assay for topoisomerase II based on the decatenation of double-stranded DNA (Basic Protocol 2). The preparation of mammalian cell extracts for assaying topoisomerase activity is described (Support Protocol), as are procedures for the assaying topoisomerase covalent complexes *in vivo* (Basic Protocol 3), for measuring DNA cleavage caused by topoisomerase I (Basic Protocol 4) and for studying topoisomerase II cleavage *in vitro* (Basic Protocol 5). An alternative electrophoretic method for examining levels of cleavage and for mapping topoisomerase cleavage sites is included as well (Alternate Protocol).

BASIC PROTOCOL 1

ASSAY OF TOPOISOMERASE I ACTIVITY—A principal reaction of topoisomerase I is the relaxation of supercoiled DNA, which has a different electrophoretic mobility than DNA that is completely relaxed (not supercoiled). Because plasmid DNA isolated from most natural sources is negatively supercoiled, any plasmid isolated from *E. coli* can be used to assay topoisomerase I activity. Topoisomerase I from eukaryotic cells is an ATP-independent enzyme, and it does not require a divalent cation (e.g., Mg^{2+}), for activity, although Mg^{2+} stimulates activity ~3- to 5-fold. These enzymatic properties allow for a clear distinction between topoisomerase I and other cellular topoisomerases. The assay detailed below is designed for eukaryotic topoisomerase I, neither topoisomerase II nor topoisomerase III is active under these conditions (see Background Information).

Materials:

10× topoisomerase I reaction buffer (see recipe)

Substrate: plasmid DNA

Purified topoisomerase I or cell extract (see Support Protocol)

5× loading dye (see recipe)

Apparatus for gel electrophoresis (Gel box, comb, power supply)

0.8% agarose gel (Voytas, 2001)

1.5-ml microcentrifuge tubes

UV transilluminator

DMSO minimum of 95 % GC (Sigma-Aldrich, USA)

Additional reagents and equipment for agarose gel electrophoresis, ethidium bromide staining, and gel photography (Voytas, 2001)

1. Add 2 μ l of 10× topoisomerase I reaction buffer and 200 ng plasmid DNA (e.g., 10 μ l of a 20 μ g/ml stock) to each of a series of 1.5-ml microcentrifuge tubes on ice. Adjust volumes with distilled water so that the final reaction volume in each tube, including that of the protein or extract added in step 2, is 20 μ l.
2. Add various amounts of purified topoisomerase I protein or cell extract to the tubes, then incubate 30 min at 37°C.

For crude extracts, it is important to use a range of concentrations. A reasonable starting point for cell extracts is 0.1 μ g protein, up to ~5 μ g protein. If necessary, the extract may be diluted with high-salt extraction buffer (see Support Protocol), but the diluted extract should be used promptly.

This assay can also be used for studying inhibition of relaxation catalyzed by topoisomerase I. The test agent(s) under examination should normally be added before the enzyme is added. If the test agent is dissolved in a solvent (commonly DMSO) other than water, it is critical to include a solvent control. If the solvent interferes with the reaction, it may be useful to increase the reaction volume. The amount of enzyme added need not be changed if the reaction volume is increased slightly (up to 50 μ l).

3. Add 5 μ l of 5× loading dye to each tube and load contents on 0.8% agarose gel. Run gel 2 - 3 hr at 5 - 10 V/cm (Voytas, 2001).
4. Stain gel with ethidium bromide, destain briefly with water, and photograph the gel illuminated with a UV transilluminator (Voytas, 2001).

CAUTION: Ethidium bromide is a mutagen and potential carcinogen. It should be handled with gloves, and solutions should be disposed of according to institutional guidelines.

5. Determine the amount of topoisomerase I needed to completely relax a supercoiled plasmid under standard conditions.

See Figure 3.3.2 for an example of a topoisomerase I assay performed with purified protein. Also shown on Figure 3.3.2 are data demonstrating how an intercalating agent can interfere with topoisomerase I assays performed using the conditions described here. See Anticipated Results for interpretation.

BASIC PROTOCOL 2

ASSAY OF TOPOISOMERASE II ACTIVITY—Like topoisomerase I, topoisomerase II catalyzes relaxation of supercoiled DNA. However, topoisomerase II is unique in its ability to catalyze the decatenation of intact double-stranded DNA, a property that allows the enzyme to separate replicated DNA molecules at mitosis (Nitiss, 2009a; Wang, 2002). This assay utilizes the kinetoplast DNA from *Crithidia fasciculata*, since it forms a large network of interlocked (catenated) circles (Marini et al., 1980). Topoisomerase II decatenates the circles from the network. While the catenated circles are unable to enter an agarose gel, upon decatenation the free circles are detected as a discrete band on the gel. Because type I topoisomerases cannot catalyze this particular reaction, this assay is selective for topoisomerase II. Note that relaxation of supercoiled DNA in cell extracts is a less appropriate assay for topoisomerase II, since topoisomerase I is fully active in topoisomerase II reaction buffer. The assay described in Basic Protocol 1 can be used with purified topoisomerase II provided there is no topoisomerase I activity present. Note: Because relaxation by topoisomerase II requires ATP and a divalent cation, topoisomerase II reaction conditions as described in this protocol must be used.

Materials:

10× topoisomerase II reaction buffer (see recipe)

Substrate: kinetoplast DNA (Topogen)

Purified topoisomerase II (see Critical Parameters) or cell extract (see Support Protocol)

5× loading dye (see recipe)

0.8% agarose gel (Voytas, 2001)

Additional reagents and equipment for agarose gel electrophoresis, ethidium bromide staining, and gel photography

1. Add 2 μ l of 10× topoisomerase II reaction buffer and 200 ng kinetoplast DNA (e.g., 10 μ l of a 20 μ g/ml stock) to each of a series of 1.5-ml microcentrifuge tubes. Adjust volumes with distilled water so that the final reaction volume in each tube, including that of the protein or extract added in step 2, is 20 μ l.
2. Add various amounts of purified topoisomerase II protein or cell extract to the tubes, then incubate 30 min at 37°C.

For crude extracts it is especially important to use a range of enzyme concentrations. At high enzyme concentrations, topoisomerase II will catalyze the catenation of circular DNA. A reasonable starting point for cell extracts is 0.1 μ g protein, up to ~5 μ g protein.

This assay can also be applied to studying inhibition of decatenation by experimental agents. The agents under examination should normally be added before the enzyme is added. If the agent is dissolved in a solvent (commonly DMSO) other than water, it is critical to include a solvent control. If the solvent interferes with the reaction, it may be useful to increase the reaction volume. The amount of enzyme added need not be changed if the reaction volume is increased slightly (up to 50 μ l).

3. Add 5 μ l of 5× loading dye to each tube and load contents on a 0.8% agarose gel. Run gel 2 to 3 hr at 5 to 10 V/cm (Voytas, 2001).

4. Stain gel with ethidium bromide, destain briefly with water, and photograph the gel illuminated with a UV transilluminator (Voytas, 2001).
5. Determine the amount of topoisomerase II needed to fully decatenate kinetoplast DNA under standard conditions.

See Figure 3.3.3. for an example of a decatenation reaction using kinetoplast DNA and purified topoisomerase II. See Anticipated Results for interpretation.

SUPPORT PROTOCOL

PREPARATION OF MAMMALIAN CELL EXTRACTS FOR ASSAYING TOPOISOMERASE ACTIVITY

—Described in this protocol is the isolation of nuclear extracts suitable for assaying topoisomerase I and topoisomerase II activity. The extracts can also be used to measure levels of topoisomerase proteins using western blotting (Gallagher et al., 2008), which is a useful adjunct to measurement of topoisomerase activity. While both topoisomerase I and topoisomerase II are nuclear enzymes, it is common to find enzyme activity in cytosolic fractions. Therefore, accurate determination of total topoisomerase activity in a cell requires assaying both the nuclear and cytosolic fractions. Because these assays can be performed rapidly, they are suitable for monitoring topoisomerase activity during purification of the proteins. Two parallel procedures are described that are appropriate with either suspension cultures or cells growing as monolayers. Other methods have been described for isolation of extracts from tissues (Halligan et al., 1985), with the approach depending very much on the tissue being studied and the amount of available material.

Materials:

Cells of interest growing in tissue culture: e.g., HeLa (ATCC #CCL-2.2) or K562 (ATCC #HB-84)

Phosphate-buffered saline (PBS; see recipe), room temperature and ice cold

PBS containing 5 mM EDTA Low-salt extraction buffer (see recipe), ice cold

High-salt extraction buffer: low-salt extraction buffer containing 0.35 M KCl 15-ml centrifuge tubes

Dounce homogenizer with loose pestle

Additional reagents and equipment for measuring protein concentration (*APPENDIX 3A*)

1. Grow cells in appropriate medium.

Cells should generally be harvested in mid- to late log phase of growth.

2a. For cells grown as monolayer cultures: Count cells using any standard method. Remove medium by aspiration and wash cells with 10 ml room temperature PBS. Aspirate wash, and then add 10 ml PBS/EDTA. Incubate 5 min at 37°C, then transfer cells ($\sim 1 \times 10^7$) to a 15-ml centrifuge tube.

The EDTA treatment removes the cells from the surface without exposure to proteases, as would occur if the cells were released by trypsinization.

2b. For cells growing in suspension culture: Count cells using a hemacytometer or other standard method. Transfer $\sim 1 \times 10^7$ cells to a 15-ml centrifuge tube and proceed to step 3.

3. Collect cells by centrifuging 10 min at $200 \times g$, 4°C . Wash cells twice with ice-cold PBS.

4. Resuspend pellet ($\sim 1 \times 10^7$ cells) in 180 μl ice-cold low-salt buffer?,. Incubate 10 min at 0°C .

If desired, this protocol can be scaled up for use with a larger Dounce homogenizer by resuspending in a greater volume (here and in step 7) but the ratio must be kept at 180 $\mu\text{l}/10^7$ cells. Several pellets can be combined in one homogenizer.

5. Add the cells to a precooled Dounce homogenizer and process using ~15 to 20 strokes with a loose pestle.

6. Incubate the homogenate 30 min at 0°C . Examine the extract under a phase-contrast microscope to ensure that most of the cells are lysed and that the nuclei remain intact.

7. Centrifuge homogenate 3 min at $15,000 \times g$, 4°C . Decant supernatant (cytosol) and place on ice. Resuspend nuclear pellet in 180 μl (per 1×10^7 cells) of ice-cold high-salt buffer and incubate 80 min at 0°C .

8. Centrifuge nuclear extract 10 min at $15,000 \times g$, 4°C . Decant and save the supernatant.

9. Measure protein concentrations using the Bradford method with BSA as the standard (*APPENDIX 3A*) in both the cytosol and the nuclear extracts.

The extracts can now be used for topoisomerase assays, western blots, or other purposes. For enzyme activity assays they should be used as quickly as possible (see Critical Parameters).

The yield of protein depends on the cells used and the efficiency of cell lysis. Two dishes of cells that are 75% confluent should yield ~150 μg protein.

BASIC PROTOCOL 3

IN VIVO DETERMINATION OF TOPOISOMERASE COVALENT COMPLEXES USING THE IN-VIVO COMPLEX OF ENZYME (ICE) ASSAY—

Many drugs affecting DNA topoisomerases act by stabilizing the covalent complex, an intermediate state in the enzyme reaction (Nitiss, 2009c; Pommier, 2006). The effect of topoisomerase drugs is to increase the amount of topoisomerase covalently bound to DNA. The procedure described below allows for the quantification of covalent protein–DNA complexes in cells. The assay depends on the separation of free topoisomerase protein from topoisomerase bound to DNA in a cesium chloride gradient. Under the conditions described below, free DNA, as well as DNA:topoisomerase covalent complexes, are pelleted by the centrifugation, while the free protein remains near the top of the centrifuge tube. As detection of the topoisomerase protein relies on antibodies directed against topoisomerases, this assay can be used to assess either topoisomerases I or II or covalent complexes. Since antibodies specific for topoisomerase II isoforms α or β are available, this assay can be used to quantify trapping of either isoforms.

The experimental procedures described below can be used to determine whether a test agent stabilizes covalent complexes that include topoisomerases I and II. The results will reveal whether an agent is selective for a topoisomerase II isoforms *in vivo*. It can also be used to characterize cells treated with a topoisomerase targeting drug to demonstrate alterations in drug action, such as characterizing resistance of a cell line to a class of topoisomerase targeting agents. Its simplicity makes this assay the method of choice for assessing topoisomerase:DNA covalent complexes (see commentary for further discussion).

Materials:

- 1) Cells of interest: e.g., HeLa (ATCC #CCL-2.2) or K562 (ATCC #HB-84)
- 2) 1X TE buffer, pH7.5 (10 mM Tris HCl, pH 7.5; 1mM EDTA)
- 3) 1% Sarkosyl (w/v) in 1X TE
- 4) 1ml latex free syringe with 25G5/8 gauge needle and 3 ml latex free syringe with a 16G1/2 precision glide needle (BD syringe, USA)
- 5) CsCl solution (Add 75 g of CsCl (molecular biology grade) to 50 ml H₂O. If necessary, warm to completely dissolve the CsCl)
- 7) OptiSeal tubes for ultracentrifugation (Beckman Coulter, USA)
- 8) 70% ethanol
- 9) Ultracentrifuge
- 10) Beckman NVt90 ultracentrifuge rotor or equivalent
- 11) UV spectrophotometer or equivalent for measuring DNA concentration
- 12) 25 mM NaPO₄ (pH 6.5)
- 13) BioRad Nitrocellulose membrane Trans Blot # 162-0115
- 14) BioRad Slot format filter paper # 162-0161
- 15) Slot blotting apparatus (BioRad Bio-Dot SF Apparatus #170-6542)
- 16) Rabbit anti-human TOPII α IgG antibody (Bethyl laboratories Inc # BL 983)
- 17) Mouse anti-human TOPII β IgG antibody (BD Transduction laboratoriesTM # 611493)
- 18) 1x phosphate buffered saline with 0.01% Tween 20
- 19) Instant Non-fat dry milk (Nestle, Carnation, USA)
- 20) Amersham ECL PlusTM Western Blotting Detection Reagents (GE Healthcare # RPN2132)

Day 1. Prepare cells for assay

1. Grow cells to a confluency of $1-2 \times 10^6$ cells per culture plate for analysis. One plate is used for each condition to be examined (i.e., control, plus one plate for each test compound concentration).

If a higher volume of cells are used, or if more than one plate needs to be combined to obtain the required cell number, then the amount of sarkosyl buffer used in the later steps to collect cells can be increased from 1.5ml to 2ml. Using less than 5×10^5 cells, or more than 2×10^6 cells/ml, may compromise the reproducibility of the results. If using less than a million cells then the pellet obtained at step 7 will be small and there is a greater chance of losing the pellet while washing with 70% ethanol. The large pellet obtained when using a large number of cells can be dislodged easily and can be lost while washing with 70% ethanol.

Day 2. Test compound treatment, harvesting of samples, preparation of cell lysate and ultracentrifugation.

2. If assessing covalent complexes with a small molecule, add test compound to plates after 24 h cell growth/ attachment. Incubate in the presence of the test compound for the desired length of time.

The length of time for test compound incubation depends on experimental goals. Typically, a 1h exposure is sufficient to obtain a good signal with standard agents such as camptothecin or etoposide. Long compound exposures may reduce the signal because of degradation of topoisomerases (Nitiss, 2009c; Zhang et al., 2006) or the induction of apoptosis (Kaufmann, 1998)

3. After test compound exposure, aspirate the growth medium using suction. Then add 1.5 ml of 1% Sarkosyl solution to the plates. Gentle tapping of the plates from the sides allows the Sarkosyl to reach all the cells on the plate. Leave the plates tilted on the bench at an angle of approximately 40° to allow the cell lysate to be collected on the edge of the culture plate. Then using a 100-1000 µL pipette, collect the lysate into a 14 ml polypropylene round bottom tube.

It is important to add the Sarkosyl solution as soon as possible after removing the media.

4. The solution containing DNA is sheared to reduce its viscosity. Using a 1ml latex free syringe with 25G5/8 gauge needle shear 1 ml of lysate at a time. Pass the lysate through the syringe ten times. After shearing, increase the final volume of the lysate to 3 ml using the 1% Sarkosyl solution.

Pull the solution into the syringe slowly to minimize detergent foaming.

If necessary, the lysate can be stored at -70 °C for up to 24 hrs. (Longer periods of storage may be acceptable, but have not been attempted).

5. Add 2 ml of CsCl solution to a 4.9 ml OptiSeal tube (Beckman Coulter, USA). Then add the lysate using a 3 ml latex free syringe with a 16G1/2 precision glide needle. Wipe the top of the tube with a Kimwipe, and seal the tubes following manufacturer's instructions.
6. Tubes should be completely full, as suggested by the manufacturer.
7. Place the sealed tubes in an NVt90 rotor (Beckman coulter, USA) being sure to balance the tubes evenly if all slots are not filled. Add appropriate caps as recommended by the manufacturer. Mount the rotor in the ultracentrifuge chamber. Centrifuge the tubes at 42,000 rpm (121900 xg) for 20 h, 25 °C.

Warning, Carefully follow all of the manufacturer's recommendations for using the ultracentrifuge.

Rotors other than the NVT90 rotor can be used although centrifugation times will need to be adjusted. Use of a fixed angle rotor will likely require significantly longer centrifugation times.

Day 3. Recover DNA and apply the resuspended DNA solution to a membrane.

8. Carefully remove the tubes from the ultracentrifuge rotor. To retrieve the pellet, first remove about 1000 µl of the top layer. Using a sharp blade cut off about 1 cm from the neck of the tube. Carefully pour off the rest of the solution. Any remaining liquid can be carefully removed with a laboratory wipe.

While pouring off the solution try to avoid dislodging the pellet. The pellet is colorless and can be difficult to see. When all the solution is removed, a glossy oval viscous pellet is noticeable and can be marked easily.

9. Wash the pellet once with 500 μ l of 70 % ethanol. Gently remove the ethanol with suction being careful to avoid dislodging the pellet. Set aside the tube containing the pellet after the removal of ethanol for 15 seconds to allow any residual ethanol to evaporate.
10. Dissolve the pellet by adding 500 μ l of 1X TE buffer pH 7.5. Make sure the volume added is sufficient for the pellet to be completely immersed in TE buffer, incubate overnight at 4 °C to complete the reaction.
11. After overnight incubation in TE buffer for resuspension, incubate the solution in a water bath at 65 °C for 5 min. After cooling to room temperature, transfer the solution to 1.5 ml microcentrifuge tubes.
12. Measure the DNA concentration of the samples. A convenient method is to use an UV spectrophotometer measuring absorbance at 260nm.

A typical DNA concentration under these conditions is 100-200 ng/ μ l.

Day 4. Apply DNA solution to a nitrocellulose membrane and measure associated topoisomerase protein by immunoblotting.

13. The samples are applied to a nitrocellulose membrane using a slot blot apparatus. The amount of sample applied is based on DNA concentration. Typically 2-20 μ g of DNA will be applied per sample. Dilute the DNA solution with 25 mM NaPO₄ (pH 6.5) buffer so that the total volume to be applied is 200 μ l. Prepare the nitrocellulose membrane by equilibrating it in 25 mM NaPO₄ pH 6.5 for 15 min before placing the membrane on the slot blotting apparatus. Assemble the slot blot apparatus using manufacturer's instructions. Wash the membrane slots with 200 μ L NaPO₄ buffer pH 6.5 and apply vacuum. Remove vacuum, and apply the DNA solution to the slots. Apply vacuum until the samples have passed through the filter. Remove vacuum, and add 200 μ L NaPO₄ buffer pH 6.5. Apply vacuum until the wash has passed through the membrane. Open the slot blot apparatus and carefully remove the membrane.
14. Place the membrane in 1X PBS-T (make a 1:10 dilution of 10X PBS and add 0.1% Tween 20) and incubate for 5 min to wash the membrane. Discard the wash and block the membrane using 5 % non-fat dry milk prepared with 1X PBS-T for 1 h at room temperature on a shaker at medium speed (Hoefer Red orbital shaker or any equivalent).
15. Discard the blocking solution; add appropriate dilution of primary antibody in 5% non-fat dry milk prepared with 1X PBS-T. Incubate overnight with shaking at 4°C.

The appropriate dilution of antibody is determined empirically. Dilutions suitable for Western analysis are a good starting point for detection of topoisomerases.

Day 5. Treatment with secondary antibody and immune complex detection

16. After overnight incubation with primary antibody, discard the solution. Rinse with 1X PBS-T and pour off the solution and then wash three times with 1X PBS-T, 5 min for each wash at room temperature with shaking.
17. Proceed with treatment with appropriately diluted secondary antibody in 5% milk (typically 1 hr at room temperature with shaking).
18. Rinse with 1X PBS-T and pour off the solution and then wash three times with 1X PBS-T, 5 min for each wash at room temperature with shaking.

19. Detect immune complexes using any conventional method such as ECL (using a kit and following manufacturer's recommended conditions).

See Figure 3.3.4. For an example of an ICE assay detecting topoisomerase II α complexes in HeLa cells treated with etoposide. See Anticipated Results for interpretation of findings.

SUPPORT PROTOCOL

PREPARATION OF MOUSE TISSUE FOR IN VIVO DETERMINATION OF TOPOISOMERASE COVALENT COMPLEXES USING THE IN-VIVO COMPLEX OF ENZYME (ICE) ASSAY—Described in this protocol is the initial processing required for mouse tissue samples before conducting the ICE assay similar to mammalian cell processing as mentioned in *Basic Protocol 3*.

Materials:

Mouse Tissue sample

Phosphate-buffered saline (PBS; see recipe), ice cold, pH 7.0

1% Sarkosyl Buffer (w/v) in 1X TE buffer

Tissue grinder, 2 mL (Wheaton, USA)

Additional reagents and equipment for ICE Assay (*BASIC PROTOCOL 3*)

Day 1. Prepare mouse tissue for assay

1. Collect the dissected mouse tissue sample in ice-cold 1X PBS pH 7.0 for initial rinsing of the dissected tissue.
2. Place the rinsed mouse tissue in ice-cold tissue grinder containing 1.5 mL 1% sarkosyl buffer.
3. Dounce the tissue samples for minimum of 10 times. After douncing collect the homogenized samples in a 14 ml polypropylene round bottom tube.
Depending upon the viscosity of the solution additional douncing will be required or additional volumes of 1% Sarkosyl buffer may be required.
4. The homogenized samples are further sheared to reduce its viscosity. Using a 1ml latex free syringe with 25G5/8 gauge needle shear 1 ml of lysate at a time. Pass the lysate through the syringe ten times. After shearing, increase the final volume of the lysate to 3 ml using the 1% Sarkosyl solution.

Pull the solution into the syringe slowly to minimize detergent foaming.

If necessary, the lysate can be stored at -70°C for up to 24 hrs. (Longer periods of storage may be acceptable, but have not been attempted).

If required to get rid of cellular debris, the sheared lysate is quickly centrifuged at 4°C at 1700 rpm for 15 minutes

Note: Follow the Basic Protocol 3 from step 5 onwards wherein the Sarkosyl buffer lysate can be applied to CsCl gradient to isolate the covalent complexes and later be detected using Slot Blot assay.

BASIC PROTOCOL 4

DETERMINATION OF DNA CLEAVAGE BY PURIFIED TOPOISOMERASE I—

Topoisomerase poisons stabilize a covalent complex between the topoisomerase protein and

DNA. The level of covalent complexes may be quantified by measuring the amount of broken DNA formed in the presence of test compound and enzyme. Although the covalent complexes are reversible, denaturation traps the protein on the DNA and allows for the measurement of DNA strand breaks. The protocol detailed below allows for the measurement of topoisomerase I-mediated strand breaks in the presence of a topoisomerase I poison, taking advantage of a DNA sequence identified by Westergaard et al (Bonven et al., 1985) that is a primary site for topoisomerase cleavage. For the assay, a double-stranded oligonucleotide is synthesized that includes the primary topoisomerase I cleavage site (indicated by the arrow in Fig.3.3.5). The oligonucleotide substrate is end labeled with [^{32}P] cordycepin, and in the presence of topoisomerase I and camptothecin, the oligonucleotide is cleaved. Denaturation of the protein traps the topoisomerase on the oligonucleotide, generating a single-strand break. Separation of the DNA strands reveals a unique, labeled oligonucleotide that is shorter and therefore readily separated from the starting substrate by gel electrophoresis. This procedure is suitable for assaying test agents as topoisomerase I poisons (Tanizawa et al., 1995). It can also be used to characterize the sensitivity of topoisomerase I derived from many different sources to test agents.

Materials:

Oligonucleotides (custom-synthesized):

5'-GATCTAAAAGACTTGGAAAAATTTTAAAAAAGATC-3' (upper strand)

5'-GATCTTTTTTAAAAATTTTCCAAGTCTTTTAGATC-3' (lower strand)

[α - ^{32}P]cordycepin 5000 Ci/mmol (Perkin-Elmer Product BLU26250UC)

Terminal deoxynucleotidyl transferase (TdT) labeling kit (Stratagene)

10 $\times$ topoisomerase I cleavage buffer (see recipe)

50 U/ μl purified topoisomerase I (Topogen; also see Critical Parameters; 1 U topoisomerase I is the quantity of enzyme that will relax 200 ng pUC18 DNA in 30 min)

Compounds to be tested

5% (w/v) SDS

Formamide loading buffer (see recipe)

30- to 40-cm denaturing 16% polyacrylamide/7 M urea sequencing gel (Prepared as described in (Albright and Slatko, 2001). Electrophoresis of DNA on sequencing gels requires a large vertical gel apparatus, a high voltage power supply, and a darkroom for developing autoradiograms or a phosphorimager).

Sephadex G-25 spin columns (GE Healthcare)

95°C heating block

400- μl microcentrifuge tubes

25°C water bath

Whatman 3MM filter paper

Additional reagents and equipment for sequencing by denaturing polyacrylamide gel electrophoresis (Slatko and Albright, 1992)

CAUTION: Radioactive materials require special handling; all supernatants must be considered radioactive waste and disposed of appropriately.

Prepare substrate:

1. End label 10 pmol of the upper strand of the oligonucleotide in a total volume of 40 μ l with [α -³²P]cordycepin using a terminal deoxynucleotidyl transferase labeling kit according to the manufacturer's instructions.
2. Remove unincorporated [α -³²P]cordycepin using a Sephadex G-25 spin column. Transfer a 1- μ l portion of the purified oligonucleotide to a scintillation vial and determine radioactivity. Adjust volume to yield ~100,000 cpm/ μ l.
3. Anneal the labeled upper strand with an equimolar amount of unlabeled lower strand by heating the mixture 5 min at 95°C, allowing the samples to cool to room temperature, and letting them stand overnight.

Perform topoisomerase I reactions:

Assemble reactions in 400 μ l microcentrifuge tubes, adding ingredients to each tube in the following order:

~50 fmol (25,000 - 50,000 cpm) labeled oligonucleotide substrate (from step 3)

1 μ l 10 $\times$ topoisomerase I cleavage buffer

H₂O for final volume of 10 μ l (taking into account volumes of protein and test compound to be added)

1 μ l (50 U) purified topoisomerase I protein

1 μ l test compound solution (or solvent alone for controls)

It is also possible to use a crude extract containing topoisomerase I for the cleavage reaction. The amount of topoisomerase I activity will vary depending on the cell line from which the extract was obtained. A reasonable range of protein concentrations for testing is 1 to 20 ng protein per reaction.

Incubate reactions 15 min at 25°C, then stop the reaction by adding 1 μ l of 5% SDS.

Analyze cleavage products by gel electrophoresis:

Prepare samples for gel electrophoresis by adding 4 vol (44 μ l) formamide loading buffer to each reaction mixture. Load a 5 μ l aliquot from each reaction onto a 30- to 40-cm denaturing 16% polyacrylamide/7 M urea DNA sequencing gel.

Slatko and Albright (2001) contains a detailed protocol for pouring, running, and processing sequencing gels, including autoradiography.

An alternative procedure is to precipitate the oligonucleotide by adding 3 vol of 100% ethanol and incubating in the microcentrifuge tube for 30 min on dry ice. The sample is then microcentrifuged 15 min at maximum speed, at 4°C. The ethanol is then removed, and the pellet resuspended in 5 μ l formamide loading buffer. This additional step increases the sensitivity of the assay.

Run the gel 2-3 hr at 40 V/cm at 50°C. After electrophoresis, transfer the gel to 3MM Whatman paper and dry in a gel dryer. Appose the dried gel to film for autoradiography (Albright and Slatko, 2001).

Alternately, visualize the radioactive bands using a phosphorimager, which allows quantitative determination of the cleaved band.

BASIC PROTOCOL 5

DETERMINATION OF INHIBITOR EFFECTS ON DNA CLEAVAGE BY TOPOISOMERASE II USING A PLASMID LINEARIZATION ASSAY—

The protocol detailed below uses a plasmid linearization assay. Simple and rapid, this assay is somewhat less sensitive than that described in the Alternate Protocol and uses relatively large amounts of purified enzyme. The assay is derived in large part from one described by Osheroff et al., (Anderson et al., 1999; Burden et al., 2001).

CAUTION: Ethidium bromide is a mutagen. Wear gloves when handling, and properly dispose of all solutions containing ethidium bromide.

Materials:

10× topoisomerase II reaction buffer

Supercoiled plasmid DNA (see Critical Parameters) positive control (e.g., etoposide, mAMSA)

20 mM ATP (diluted with H₂O from purchased stock solution; store up to 6 months in small aliquots at –20°C)

4–20 U/μl purified topoisomerase II (see Critical Parameters)

10% (w/v) SDS

250 mM EDTA

4 mg/ml stock solution of proteinase K: dilute to 0.8 mg/ml before use

0.8% agarose gel (Voytas, 2001)

Restriction enzyme appropriate for cutting plasmid once

10 mg/ml ethidium bromide stock solution: store at room temperature, protected from light

Additional reagents and equipment for performing agarose gel electrophoresis, ethidium bromide staining, and gel photography (Voytas, 2001)

1. Add to 1.5 ml microcentrifuge tubes (on ice) the following ingredients in the order listed:

2 μl 10× topoisomerase II reaction buffer

5 nM supercoiled plasmid DNA

1 to 2 μl of test agent or positive control

H₂O was added to bring the final reaction volume to 20 μl (including the volume of enzyme to be added in step 2).

2. Add 1 to 2 μl of 4 to 20 U/μl topoisomerase II (0.1 to 1 μg purified protein). Incubate the reaction mixture 15 to 30 min at 37°C.

The optimal incubation temperature depends on how rapidly enzyme/DNA cleavage occurs. Incubation times as short as 5 min are often sufficient.

3. Terminate the reaction by adding 2 μl of 10% SDS.

It is important to add the SDS stop solution to tubes in the 37°C water bath. Do not remove the tubes to ice before adding SDS. It is especially critical to add SDS

before adding the EDTA solution. Addition of EDTA prior to SDS will result in complete reversal of DNA cleavage.

4. Add 1.5 μ l of 250 mM EDTA and 2 μ l of 0.8 mg/ml proteinase K solution. Incubate 1 to 2 hr at 30°C.

The proteinase K incubation time is not critical, although sufficient time must be allowed for complete digestion of the enzyme covalently bound to DNA. Some prefer to incubate the proteinase K reaction at 50°C.

5. Add 2 μ l of 5 $\times$ loading dye to each sample.
6. Load the sample on a 0.8% agarose gel, and carry out electrophoresis for 2 to 4 hr at 5 V/cm. Include a lane of linearized plasmid (cut with a restriction enzyme that will only cut the plasmid one time) as a control.

The concentration of agarose is not critical. Agarose gels from 0.8% to 1% agarose can be used, with 1% agarose preferable for small plasmids such as pUC18.

7. Stain the gel for 30–60 min in 1 μ g/ml ethidium bromide (diluted from 1 mg/ml stock), followed by destaining in water for 5 to 10 min.

CAUTION: Ethidium bromide is a mutagen. Wear gloves when handling, and properly dispose of all solutions containing ethidium bromide.

8. Visualize lanes using a UV transilluminator and photograph the gel.
9. Using densitometry, quantify the concentration of linear DNA formed, typically by comparing to quantities of linear DNA concentrations formed in the presence of specific concentrations of the positive control (e.g., ciprofloxacin).

See Figure 3.3.6. for an example of a cleavage with purified topoisomerase II in the presence of etoposide. See Anticipated Results for interpretation.

ALTERNATE PROTOCOL

GEL ELECTROPHORESIS DETERMINATION OF TOPOISOMERASE II

CLEAVAGE—This protocol is conceptually similar to Basic protocol 5. In this case, radioactively labeled DNA is used as a substrate for DNA cleavage. After cleavage, the covalently bound protein is removed by Proteinase K digestion, and the DNA samples separated by electrophoresis. While the protocol detailed below uses agarose gel electrophoresis, higher resolution of smaller fragments can be obtained using polyacrylamide gel electrophoresis.

The first part of the protocol describes preparation of a linear end-labeled substrate for DNA cleavage. Other approaches to generating the substrate include PCR amplification of a specific DNA followed by labeling (Dexheimer and Pommier, 2008).

Additional Materials:

Substrate: plasmid DNA (e.g., pUC18 or pBluescript)

*Eco*RI restriction endonuclease

Additional restriction endonuclease: e.g., *Bam*HI (optional)

0.8% agarose gel

TE buffer, pH 8.0: 10 mM Tris-Cl, pH 8.0/1 mM EDTA, pH 8.0 (see *APPENDIX 2A* for both ingredients)

5 mM dCTP, dGTP, and dTTP

10 mCi/ml [α - 32 P] dATP (800 Ci/mmol; Perkin-Elmer)

5 U/ μ l Klenow fragment of DNA polymerase I and 10 $\times$ Klenow buffer

10 $\times$ topoisomerase II reaction buffer (see recipe)

Compounds to be tested

20 to 40 U/ μ l purified topoisomerase II

5% (w/v) SDS containing 1 mg/ml proteinase K

5 $\times$ loading dye (see recipe)

Additional reagents and equipment for agarose gel electrophoresis (Voytas, 2001) and autoradiography (Voytas and Ke, 2001)

Electrophoresis apparatus?

CAUTION: Radioactive materials require special handling; all supernatants must be considered radioactive waste and disposed of appropriately.

Prepare substrate:

1. Digest DNA substrate (e.g., plasmid pUC18) with *Eco*RI or other enzyme as appropriate
2. Add an aliquot of the restriction enzyme digestion to formamide loading buffer in a microcentrifuge tube, electrophorese the sample on a small 0.8% agarose gel, and visualize with ethidium bromide (Voytas, 2001).

Include a lane of uncut plasmid to use as a comparison. The electrophoresis product will depend upon the substrate; for pUC18 a single band will be seen.

3. Purify digested plasmid from the restriction digest reaction by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation (*APPENDIX 3C*). Resuspend DNA at 0.35 μ g/ μ l in TE buffer, pH 8.0.
4. Prepare the following labeling mixture (for 20 reactions; may be scaled appropriately), adding ingredients in the indicated order:

20 μ l (7 μ g) digested DNA

15 μ l H₂O

1 μ l 5 mM dCTP

1 μ l 5 mM dGTP

1 μ l 5 mM dTTP

5 μ l 10 $\times$ Klenow buffer

5 μ l 10 mCi/ml [α - 32 P]dATP

2 μ l 5 U/ μ l Klenow fragment of DNA polymerase I.

5. Incubate reaction mixture for 30 min at 37°C..
 6. Purify the labeled substrate from unincorporated nucleotides using a Sephadex G-25 spin column or other appropriate method.
- For detailed mapping of cleavage sites, the DNA should be uniquely end labeled. For pUC 18, this can be accomplished by digestion with another enzyme such as

BamHI. It is convenient to do the second digestion after labeling and before the spin column. Note that this will remove 50% of the incorporated label. The digestion can also be done after the cleavage reaction if desired.

Perform cleavage assay:

Assemble the following reactants (add ingredients in the order listed):

2 μ l 10 $\times$ topoisomerase II reaction buffer

350 ng labeled DNA substrate (step 6)

1 to 2 μ l test compound solution

H₂O for final volume of 20 μ l

20 U topoisomerase II (0.5 to 1 μ g purified protein)

Terminate the reaction by adding 0.5 ml of 5% SDS containing 1 mg/ml proteinase K and incubating 1 hr at 37°C

Add 5 $\times$ loading dye to each reaction and load on an 0.8% agarose gel. Electrophorese 2 to 4 hr at 5 V/cm (Voytas, 2001).

It is also possible to use polyacrylamide gel electrophoresis (APPENDIX 3B) to separate the cleavage products.

Dry the gel, wrap in plastic wrap, and appose to film for 24-48 hr at -80°C (Voytas and Ke, 2001).

See Figure 3.3.7. for an example of a cleavage with purified topoisomerase II in the presence of the intercalating agent mAMSA. See Anticipated Results for interpretation.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; *for suppliers, see SUPPLIERS APPENDIX.*

Formamide loading buffer—

80% (v/v) formamide

1 mM EDTA

10 mM NaOH

1 mg/ml xylene cyanol

1 mg/ml bromphenol blue

Store up to 1 year at -20°C

Loading dye, 5 $\times$ —

30% (v/v) glycerol

0.25 mg/ml bromphenol blue

Store up to 1 year at 4°C

As an alternative to the dye described here, almost any commonly used loading dye can be used for topoisomerase I and topoisomerase II assays.

Low-salt extraction buffer—

20 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)

5 mM KCl

1 mM MgCl₂

10% (v/v) glycerol

Store indefinitely at room temperature

Immediately before use, add dithiothreitol (DTT) to a final concentration of 1 mM and phenylmethylsulfonyl fluoride (PMSF) from a 0.1 M solution in isopropyl alcohol (store up to 6 months at –20°C) to a final concentration of 1 mM.

PMSF should be added just before the buffer is used as it is very unstable in aqueous solution.

Phosphate-buffered saline (PBS)—

8 g NaCl

0.2 g KCl

1.44 g Na₂HPO₄

0.24 g KH₂PO₄

H₂O to 1 liter

Adjust pH to 7.4 with 1 M HCl

Store indefinitely at room temperature

Topoisomerase I cleavage buffer, 10×—

100 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)

500 mM KCl

50 mM MgCl₂

1 mM EDTA

150 µg/ml BSA

Store up to 1 year at room temperature

Topoisomerase I reaction buffer, 10×—

500 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)

1 M KCl

10 mM dithiothreitol

100 mM EDTA

50 µg/ml acetylated bovine serum albumin (Life Technologies)

Store up to 1 year at –20°C

Topoisomerase II reaction buffer, 10×—

200 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)

100 mM MgCl₂

10 mM ATP
10 mM EDTA
10 mM dithiothreitol (DTT)
1.5 M KCl
300 µg/ml acetylated bovine serum albumin (Life Technologies)
Store up to 2 months at –20°C

CsCl solution—

Add 75 g of CsCl to 50 ml H₂O. If necessary warm gently to completely dissolve the CsCl.

CsCl solutions are stable indefinitely at room temperature.

COMMENTARY

Background Information

The procedures detailed in this unit deal with the two principal eukaryotic topoisomerases: topoisomerase I (EC 5.99.1.2) and topoisomerase II (EC 5.99.1.3). Topoisomerase I is a type IB topoisomerase and topoisomerase II is a type II topoisomerase. The full complement of human topoisomerases, along with their genes, is shown in Table 3.3.1. Mammalian cells contain two type IB topoisomerases, a nuclear enzyme and a mitochondrial enzyme (Zhang et al., 2001). The assays described for topoisomerase I in Basic protocols 1, 3, and 4 should be applicable to both enzymes, although there have been no reports of application of the ICE protocol to mitochondria-specific enzymes.

In mammalian cells there are two isozymes of topoisomerase II—a 170-kD form (termed p170 or α), and a 180-kD form (termed p180 or β) (Austin et al., 1993; Drake et al., 1987). These two proteins are the products of different genes, located in human cells on chromosomes 17q and 3q respectively (Wang, 1996). These isoforms are expressed differently through the cell cycle, with topoisomerase II α being preferentially expressed in proliferating cells and topoisomerase II β expressed at all points in the cell cycle. Studies suggest that the two proteins have similar biochemical properties and no major differences in their sensitivities to current antitopoisomerase agents (Austin et al., 1995). The assays described for topoisomerase II in Basic protocols 2, 3, and 5 is applicable to both isozymes.

Both yeast and human cells have an additional type I nuclear topoisomerase, termed topoisomerase III that is homologous to the bacterial type I enzyme but not to other eukaryotic topoisomerases (Hanai et al., 1996; Kawasaki et al., 1997; Wallis et al., 1989). Topoisomerase III is a type I topoisomerase that is active only on negatively supercoiled DNA. Its ability to relax supercoiled DNA to a completely relaxed form is considerably less than topoisomerase I (Kim and Wang, 1992). As topoisomerase III absolutely requires a divalent cation for activity, its presence does not interfere with the topoisomerase I assay described in Basic Protocol 1. Since there are as yet no small molecules reported that selectively inhibit eukaryotic type IA topoisomerases, detailed protocols for studying inhibitors of these enzymes have not been developed.

Topoisomerases are crucial targets for antitumor agents. Topoisomerase I is specifically inhibited by camptothecin and its analogs topotecan and irinotecan, as well as a series of non-camptothecin analogs such as certain indenoisoquinolines (Nitiss and Beck, 1996; Pommier, 2006; Pommier et al., 2010). A wide range of agents have been identified that

inhibit topoisomerase II, including the epipodophyllotoxins, etoposide and teniposide, the anthracyclines doxorubicin and daunorubicin, and other chemically diverse compounds (Nitiss, 2009c; Walker and Nitiss, 2002). Determining the activity of novel anticancer agents against DNA topoisomerases is an important component of an anticancer drug discovery program.

An understanding of the action of topoisomerase poisons requires consideration of the catalytic mechanisms of DNA topoisomerases. Both type I and type II enzymes bind to and cleave DNA by forming a covalent complex with it. A key tyrosine residue attacks the phosphodiester backbone of DNA forming a phosphotyrosine bond. The enzyme then passes DNA strands through the break, resulting in relaxation of DNA supercoiling or decatenation of catenated DNA. After strand passage, the phosphotyrosine bond reverses, and the energy of the phosphotyrosine bond is used to reform the phosphodiester backbone of DNA. Topoisomerase poisons stabilize the covalent enzyme–DNA complex. There are several key characteristics of this complex: it includes protein covalently bound to DNA as well as a strand break in the DNA substrate, and it is also freely reversible. Accordingly, if the drug is removed the enzyme rapidly reseals the DNA. Covalent complexes are quantified in two ways: by measuring the levels of protein covalently bound to DNA or by directly assaying for DNA strand breaks in the presence of topoisomerase and test agent or known drug. The assay described in Basic Protocol 4 directly measures DNA strand breaks induced by topoisomerase I in a substrate that carries a strong DNA cleavage site. Similarly, the plasmid linearization assay measures double strand breaks induced in plasmid DNA by topoisomerase II. The Alternate Protocol allows for the visualization of breaks induced on a larger substrate. The procedures in Basic Protocol 3 are used to measure the amount of the cleavage complex by determining the levels of topoisomerases that are covalently associated with DNA. Since the covalent complex is a normal step in the topoisomerase reaction, it can be detected (using very sensitive assays) even in the absence of a topoisomerase poison. However, addition of a topoisomerase poison greatly increases the levels of covalent complexes.

There is a large body of evidence indicating that stabilization of covalent complexes is a critical factor in the action of topoisomerase poisons, and that most clinically active anti-topoisomerase drugs do not kill cells merely by inhibiting enzyme activity. As a complete discussion of the evidence supporting statement is beyond the scope of this commentary, interested readers are referred to other sources ((Li and Liu, 2001; McClendon and Osheroff, 2007; Nitiss, 2009c; Pommier et al., 2006). In general, determination of the ability of a drug or test compound to trap topoisomerase–DNA covalent complexes is a key step in characterizing the action of an antitopoisomerase agent.

While most drugs targeting these enzymes are topoisomerase poisons, there are small molecules that inhibit the catalytic activity of eukaryotic DNA topoisomerases without generating elevated levels of DNA cleavage. These agents, termed catalytic inhibitors include agents such as merbarone and ICRF-187. These agents clearly have different effects on cells compared to topoisomerase poisons (Andoh and Ishida, 1998; Drake et al., 1989; Jensen et al., 2000). The biochemical effects of catalytic inhibitors are discussed in detail in several recent reviews (Nitiss, 2009b; Vos et al., 2011)

There has been renewed interest in identifying topoisomerase inhibitors that are not poisons, and it remains an open question whether inhibiting topoisomerases without stimulating cleavage represents a viable anticancer strategy.

Critical Parameters and Troubleshooting

Detailed on Table 3.3.2 are some problems commonly encountered using the assays described in this unit, along with possible reasons for the problems and suggestions for overcoming or avoiding them.

Sources of purified enzymes for Basic Protocols 1, 2, 4,5 and the Alternate Protocol—Many of the protocols described in this chapter require purified topoisomerases, although the assays can be conducted with crude lysates with reduced sensitivity. Most investigators will choose to carry out experiments with human topoisomerases, although there is an extensive literature with the yeast enzymes (Reid et al., 1998; Rogojina et al., 2007). The success of the experiments depends on reliable enzyme preparations. Two specialized popular commercial sources of purified topoisomerase I and topoisomerase II are TopoGen (www.topogen.com) and Inspiralis (<http://www.inspiralis.com>). The assay buffers recommended by the manufacturer may differ from those described in this unit. In this case it is best to use the manufacturer's assay buffers with the enzymes provided by them.

Many purification schemes for topoisomerases have been described. In general, topoisomerases are overexpressed in eukaryotic expression systems. Expression of both topoisomerase I and topoisomerase II in *E. coli* has proven to be disappointing with relatively low activity compared to eukaryotic expression systems. Human topoisomerase I may be overexpressed and purified from *E. coli* or baculovirus systems (Fujimori et al., 1995; Knab et al., 1995; Rubin et al., 1994; Stewart et al., 1996; Zhelkovsky and Moore, 1994). Yeast topoisomerase II, as well as human topoisomerase II α and topoisomerase II β (Austin et al., 1995; Burden and Osherooff, 1999; Jannatipour et al., 1993; Wasserman et al., 1993; Worland and Wang, 1989) have been overexpressed and purified using yeast.

Regardless of the source of the enzymes, they must be handled appropriately for the assay to work properly. In general, purified enzymes are stored in small portions at -80°C , and multiple freeze-thaw cycles should be avoided. Enzymes maintain activity best when stored at relatively high concentrations, as recommended by the manufacturer. Enzyme dilution buffers are usually provided by the supplier. For the sake of consistency, it is important to be wary of results obtained with preparations that have markedly different enzyme activities.

A large number of compounds are available to use as positive controls for the experiments described in this unit. The standard control for topoisomerase I poisons is camptothecin. Topotecan is currently used clinically, but it is substantially less potent than camptothecin. Irinotecan is also used clinically but is a prodrug with minimal activity until it is activated by carboxylesterases. The active compound is termed SN38, and is quite potent but poorly soluble (Guichard et al., 1999). Easily available topoisomerase II poisons include etoposide, teniposide, mAMSA, mitoxantrone, ellipticine, amonafide, and doxorubicin. Etoposide is also commonly used as a control compound. If an intercalating topoisomerase II poison is required, mAMSA is frequently used since it is relatively potent, and selective. Doxorubicin and mitoxantrone are more potent, but have non-topoisomerase II targets as well.

Supercoiled plasmid DNA for Basic Protocols 1 and 5 and the Alternate Protocol—Basic Protocols 1 and 5 require purified supercoiled plasmid DNA. The plasmid can be chosen for the convenience of the investigator. High-copy plasmids, such as pUC18, are typically a good choice. Standard plasmid preparation assays, or kits that provide good quality DNA suitable for subcloning, are also appropriate for the assays described here. However, care must be taken to use plasmid DNA with low levels of nicked circles, especially when assaying relaxation of supercoiling. Typically, plasmid preparations that are >90% supercoiled will yield reliable results

Preparation of extracts and assaying topoisomerase activity—There are a number of ways to prepare extracts for assaying nuclear enzymes. To avoid loss of enzyme activity, it is important to maintain the extracts at a low temperature throughout the extraction procedure and to use protease inhibitors such as phenylmethylsulfonyl fluoride (PMSF). Because the activity in cell extracts is relatively unstable, assays should be performed as soon as possible after the extract is prepared. Because freezing and thawing of extracts generally causes a substantial loss of enzyme activity, this should be avoided. If the assays cannot be performed immediately after preparing the extract, it is better to freeze cells than to prepare then freeze the extract. If storage for more than ~2 days is required, the extracts should be stored as single-use aliquots in a -80°C freezer or in liquid nitrogen.

In vitro cleavage assays—The importance of high quality preparations of topoisomerases is discussed above. It is crucial that a known amount of topoisomerase activity be used in any of the *in vitro* cleavage assays, making it advisable to assay topoisomerase activity (see Basic Protocols 1 and 2) shortly before an enzyme preparation is used in this way.

Most topoisomerase II, and some topoisomerase I, poisons bind to DNA and intercalate in the DNA helix. Such agents yield an unusual dose-response curve. At lower concentrations, the compound will increase DNA cleavage in a concentration-dependent manner, whereas there is a decrease in cleavage at higher concentrations of test agent. At very high concentrations of compound, the level of cleavage may be below that observed in the absence of drug. The most likely explanation for this pattern of responses is that high levels of compound intercalated in DNA completely block cleavage (Tewey et al., 1984). Thus, it is important that a wide range of compound concentrations be tested when examining an unknown agent for its ability to increase the levels of cleavage complexes. For example, mitoxantrone, a strong intercalating agent, yields a six-fold increase in precipitated counts at a concentration of $0.1\text{ }\mu\text{g/ml}$ using Basic Protocol 5. At $2\text{ }\mu\text{g/ml}$, the counts precipitated are 20% of those found in the absence of this agent.

Anticipated Results

Topoisomerase assays—Figure 3.3.2A shows a sample topoisomerase I reaction, with lane S representing the DNA substrate alone. Under the electrophoresis conditions used here (i.e., absence of ethidium bromide), the supercoiled form of the DNA migrates most rapidly. Addition of topoisomerase I produces a ladder of more slowly migrating DNA bands. A potential issue is agents that intercalate in DNA. As shown in figure 3.3.2B, it would appear that ethidium bromide inhibits topoisomerase I. In fact, the intercalation leads to supercoils that are relaxed by topoisomerase I. When the compound is removed, the net result is a positively supercoiled plasmid that migrates in a manner similar to negatively supercoiled DNA. There are a variety of ways of distinguishing the effects of intercalation (Bailly, 2001).

Shown in Figure 3.3.3 is a gel from a sample topoisomerase II reaction. Although no bands are present in the substrate lane, there is fluorescence just below the well. Shown in lane 1 is a single DNA band, which represents the decatenated rings from the kinetoplast network. Because faint fluorescence is still seen in the well, the sample in lane 1 has incomplete decatenation. Care should be taken in interpreting the fluorescence in the well as catenated substrate when a crude extract is used, since DNA binding proteins and other cellular components may also contribute to fluorescence in the wells. The proteins in the extract can bind DNA and prevent it from entering the gel. In Figure 3.3.3, it is clear that a maximum amount of decatenated product is formed in the sample in lane 2, indicating it is a concentration that completely decatenates the substrate. In lane 6, there is an almost

complete disappearance of the decatenated product, with the DNA remaining catenated as a result of the high concentration of enzyme in this sample.

The In-vivo Complex of Enzyme (ICE) assay is a simple, specific and flexible assay for demonstrating that agent stimulates topoisomerase:DNA covalent complexes *in vivo*. The experiment shown in Figure 3.3.4 shows sensitive detection of topoisomerase II α covalent complexes by etoposide. Similar results can be obtained when an antibody directed specifically against topoisomerase II β is used. The assay is also applicable to studying topoisomerase I targeting agents, using an antibody directed against topoisomerase I. In proliferating cells, the levels of topoisomerase II α typically are greater than the levels of topoisomerase II β , therefore the signal with topoisomerase II β complexes will often be lower than that illustrated in Figure 3.3.4. However, non-proliferating cells usually express little topoisomerase II α , and topoisomerase II β will be the predominant species in covalent complexes. This assay is particularly useful for laboratories examining aspects of agents targeting topoisomerases, but which do not have the expertise or need to examine purified proteins. This assay is available in kit form from Topogen (www.topogen.com).

The greatest shortcoming of the ICE assay (Basic Protocol 3) is the relatively small number of samples that can be easily processed.

Figures 3.3.6 and 3.3.7 show results from two different types of cleavage assays. Figure 3.3.6 shows results from a plasmid linearization assay. A high concentration of purified protein was used for this experiment. Similar results are obtained if lower amounts of protein are added. However, it is important to remember that DNA cleavage is stoichiometric. It is therefore desirable to add (at least) approximately equimolar concentrations of plasmid and enzyme. Assays similar to that shown in Figure 3.3.7 are frequently used because the detection of cleavage is quite sensitive. The total amount of cleavage can be estimated from the intensities of all of the cleaved DNA bands (Jannatipour et al., 1993). Frequently, the most important aspect of this analysis is visualization of cleavage, rather than the location of specific cleavage sites. However, drugs of different classes frequently give differences in the specific cleavage sites, and the demonstration of differences in cleavage patterns can be used to suggest that test agents may belong to different classes.

Time Considerations

Preparation of cell extracts requires 2-4 hr once the cells are grown. The preparation of cell extracts and completion of the assays for both topoisomerase I and topoisomerase II can be accomplished in 1 day (using 20 samples and 2 gels).

The ICE assay (Basic Protocol 3) requires a total of approximately 5 days, although much of that time is taken up by overnight spins or incubation.

The preparation of the substrates for Basic Protocols 4 and the alternate protocol can be performed in ~3 to 4 hr and can be done the day before the cleavage reactions are performed. The reactions with topoisomerase take <1 hr to perform. For the topoisomerase I cleavage reaction, ~4 hr are required to run the sequencing gel and to prepare the gel for autoradiography. The topoisomerase II cleavage reaction can also be performed quickly. The gel analysis of topoisomerase cleavage products requires ~4 to 6 hr after the reactions are complete. Most of this time is consumed during the incubation with proteinase K and during electrophoresis of the samples.

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Figure 3.3.1.

Reactions of DNA topoisomerases. DNA topoisomerases catalyze the interconversion of different topological forms of DNA, such as the knotting and unknotting of DNA and catenation and decatenation of DNA rings. Type I topoisomerases are able to unknot or decatenate single-stranded knots and catenanes, but are unable to carry out these reactions on intact double-stranded DNA. (A) Both type I and type II topoisomerases can relax supercoiled DNA. Type II topoisomerases are able to carry out (B) the knotting/unknotting of intact double stranded DNA and (C) the catenation/decatenation of intact double-stranded DNA.

Figure 3.3.2.

Gels obtained from topoisomerase I activity assays. Yeast topoisomerase I was purified from yeast cells expressing wild type yeast Top1 from the Gal1 promoter. **A.** Lane λ, λ *Hind*III molecular weight markers; lane S, 0.1 μg pUC18 plasmid with no added protein, other lanes contain decreasing amounts of purified protein. Samples were electrophoresed on a 1.0% agarose gel. **B.** The same conditions as in panel A, lane 4 were used, but increasing concentrations of ethidium bromide were added (from 100 ng/ml to 2 μg/ml ethidium bromide). Ethidium bromide is not an inhibitor of Top1, but because it intercalates and unwinds DNA, carrying out the reaction in the presence of ethidium bromide introduces positive supercoils in the DNA, which can be relaxed by Top1. Therefore one might erroneously conclude that ethidium bromide inhibits Top1. Approaches to distinguish effects of intercalation are discussed in detail by Bailly (Bailly, 2001).

Figure 3.3.3. Topoisomerase II decatenation assay

Different amounts of purified yeast topoisomerase II were added to reactions that contained 0.2 μg kinetoplast DNA as a substrate. Lane M, λ *Hind*III molecular weight markers; lane S, substrate DNA; lane 1, substrate and 5 ng purified yeast topoisomerase II; lane 2, substrate and 50 ng purified yeast topoisomerase II; lane 3, substrate and 100 ng purified yeast topoisomerase II; lane 4, substrate and 200 ng purified yeast topoisomerase II; lane 5, substrate and 500 ng purified yeast topoisomerase II; lane 6, substrate and 1 μg purified yeast topoisomerase II. The fluorescence just below the well in lane S is due to the catenated kinetoplast DNA that fails to enter the gel; the decatenated product is clearly seen in lanes 1 to 5. Samples were electrophoresed on a 0.8% agarose gel.

Figure 3.3.4. ICE assay of Top2 covalent complexes

HeLa cells were treated with various etoposide concentrations for 1 hr. The ICE assay was performed as described in Basic protocol 3, and the slot blots of DNA recovered from the pellet after CsCl centrifugation, probed with an antibody directed against Top2 α is shown in panel A. The signal arises from Top2 that covalently associates with DNA. Panel B shows a quantitation of the signal from the blot shown in panel A.

Figure 3.3.5. Schematic representation of in vitro cleavage of a double-stranded oligonucleotide by topoisomerase I

The annealed oligonucleotides are shown; “*L” indicates the cordycepin label. Note that only the top strand is labeled. Upon addition of topoisomerase I and camptothecin, a specific cleavage by topoisomerase I occurs. The oligonucleotide is designed so that cleavage occurs specifically at the site indicated by the arrow. The topoisomerase I protein is trapped on the DNA upon addition of SDS, and the strands are separated by the addition of formamide. If no topoisomerase I cleavage occurs, the only product observed on a sequencing gel is the 37-nucleotide labeled substrate (the top strand). Topoisomerase I cleavage results in a smaller oligonucleotide. Note that the oligonucleotide that has protein covalently bound is unlabeled, so it will not be detected.

Figure 3.3.6. Plasmid DNA cleavage with purified Top2

The plasmid cleavage assay was carried out with 2 μg of purified yeast Top2 (a very high concentration). λ *Hind*III markers, Sc, substrate with no added protein, L, pUC18 linearized with a restriction enzyme, and increasing concentrations of etoposide as indicated on the figure. Note that a band that has the same electrophoretic mobility as linear DNA is absent without etoposide, but clearly seen when 1 $\mu\text{g/mL}$ of etoposide is added. The linear DNA is seen with 3 $\mu\text{g/mL}$ etoposide, and decreases in intensity as more etoposide is added. Samples containing 3 $\mu\text{g/mL}$ of etoposide show smearing of DNA that arises from cleaving the DNA at multiple sites. This smearing is readily interpretable when purified Top2 is used, however care must be taken with crude enzyme preparations that may contain contaminating nuclease activity.

Figure 3.3.7. DNA cleavage with purified Top2

DNA cleavage assays were carried out using ^{32}P end labeled pUC18 DNA. Cleavage was carried out in the absence of inhibitor (lane marked 0), in the presence of 0.2 $\mu\text{g/ml}$ mAMSA or 1.0 $\mu\text{g/ml}$ mAMSA. Enzyme concentrations added ranged from 2 units (50 ng) to 25 units (1.25 μg).

Table 3.3.1 Eukaryotic Topoisomerases

Enzyme	Type	GenBank accession no.	Inhibitors
Topoisomerase I ^a	IB	K03077 (<i>S. cerevisiae</i>) L20632 (mouse) J03250 (<i>H. sapiens</i>) AF349017 (<i>H. sapiens</i> Mitochondrial)	Camptothecin, topotecan, irinotecan, actinomycin D ^b , aclarubicin ^b
Topoisomerase II ^{c,d}	II	M13814 (<i>S. cerevisiae</i>) D12513 (mouse II α) D38046 (mouse II β) J04088 (<i>H. sapiens</i> II α) X68060 (<i>H. sapiens</i> II β)	Doxorubicin, daunomycin, etoposide, mitoxantrone
Topoisomerase III ^a	IA	M24939 (<i>S. cerevisiae</i>) U43431 (<i>H. sapiens</i> III α) AF017146 (<i>H. sapiens</i> III β)	No known inhibitors

^aThe International Enzyme Commission functional designation EC 5.99.1.2 includes all bacterial and eukaryotic type I topoisomerases—e.g., eukaryotic topoisomerase I and topoisomerase III.

^bActinomycin D and aclarubicin are examples of compounds that inhibit both topoisomerase I and topoisomerase II.

^cLower eukaryotes such as yeast and *Drosophila* appear to have only a single type II topoisomerase; mammals have at least two type II isozymes termed topoisomerase II α and topoisomerase II β .

^dThe International Enzyme Commission functional designation EC 5.99.1.3 includes all type II topoisomerases—e.g., bacterial DNA gyrase, yeast topoisomerase II, and mammalian topoisomerase II α and topoisomerase II β .

Table 3.3.2**Troubleshooting for Topoisomerase Assays**

Problem	Possible cause	Solution
<i>Basic Protocol 1</i>		
No detected relaxation of supercoiled DNA in samples lacking drug	Loss of enzyme activity	Use a fresh aliquot of enzyme
<i>Basic Protocol 2</i>		
No detected decatenation in samples lacking drug	Loss of enzyme activity	Use a fresh aliquot of enzyme
No detected decatenation in samples lacking drug	Degradation of ATP (a common issue with these assays)	Use a new ATP aliquot
<i>Basic Protocol 3</i>		
No detected enzyme covalent complexes in positive control	Antibody issues	Use a new aliquot of antibody. Verify antibody activity using Western analysis.
No detected enzyme covalent complexes in positive control	Lack of expression of target enzyme	Verify that the target enzyme (e.g., Top2 α) is expressed under the growth conditions used (e.g., by Western analysis)
<i>Basic Protocol 4</i>		
No detected cleavage of DNA in samples with positive control	Loss of enzyme activity	Use a fresh aliquot of enzyme
Low levels of cleavage in samples with positive control	Inaccurate determination of enzyme activity	Measure enzyme activity using Basic protocol 1
<i>Basic Protocol 5</i>		
No detected cleavage of DNA in samples with positive control	Loss of enzyme activity	Use a fresh aliquot of enzyme
Low levels of cleavage in samples with positive control	Insufficient enzyme activity	Increase added enzyme quantity
Low levels of cleavage in samples with positive control	Degradation of ATP	Use a new ATP aliquot