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Cleavage of Plasmid DNA by Eukaryotic Topoisomerase II

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Abstract

Topoisomerase II is an essential enzyme that is required for a number of critical nuclear processes. All of the catalytic functions of topoisomerase II require the enzyme to generate a transient double-stranded break in the backbone of the double helix. To maintain genomic integrity during the cleavage event, topoisomerase II forms covalent bonds between active site tyrosyl residues and the newly generated 5'-DNA termini. In addition to the critical cellular functions of the type II enzyme, several important anticancer drugs kill cells by increasing levels of covalent topoisomerase II-DNA cleavage complexes. Due to the physiological importance of topoisomerase II and its role in cancer chemotherapy, several methods have been developed to monitor the *in vitro* DNA cleavage activity of the type II enzyme. The plasmid-based system described in this chapter quantifies enzyme-mediated double-stranded DNA cleavage by monitoring the conversion of covalently-closed supercoiled DNA to linear molecules. The assay is simple, straightforward, and does not require the use of radiolabeled substrates.

Keywords

Topoisomerase II; plasmid DNA; supercoiled DNA; DNA cleavage; agarose gel electrophoresis; topoisomerase II-targeted agents

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²In addition to Mg²⁺, the divalent cation requirement of topoisomerase IIα may be fulfilled in DNA cleavage assays by Ca²⁺, or Co²⁺, Mn²⁺ (12,17). Although these alternative divalent metal ions do not support the ATPase activity of the enzyme, and hence the DNA strand passage activity of topoisomerase II (12,17,18), they promote higher levels of pre-strand passage DNA cleavage (*see Note 5*) than Mg²⁺ and do not significantly alter the site specificity of the type II enzyme.

¹²Since topoisomerase II-mediated DNA cleavage is the target reaction for a number of important chemotherapeutic agents, the plasmid-based assay represents a straightforward technique for analyzing the effects of drugs on the enzyme. An example of such a drug-induced DNA cleavage titration is shown in Fig. 1.

¹³While most topoisomerase II-targeted drugs interact with the enzyme in a non-covalent manner (2,5,25), compounds such as 1,4-benzoquinone (a major metabolite of benzene) and (–)-epigallocatechin gallate (EGCG; the most abundant and biologically active polyphenol in green tea) require redox cycling and interact covalently with topoisomerase II (5,26,27). The most straightforward method for distinguishing between “traditional” enzyme-targeted agents and “redox-dependent” agents is to incubate the compound with a reducing agent such as dithiothreitol (DTT) prior to its addition to DNA cleavage assay mixtures. The activity of redox-dependent agents will be greatly diminished following exposure to the reducing agent (*see Fig. 2*).

¹⁴Dimethyl sulfoxide, which often is used as a solvent for drugs, alters the DNA cleavage/ligation equilibrium of topoisomerase II and tends to increase levels of cleavage. Therefore, care must be taken to minimize the final concentration of dimethyl sulfoxide in reaction mixtures.

1. Introduction

Topoisomerase II is a ubiquitous enzyme that remove knots and tangles from the genetic material and is required for a number of critical nuclear processes (1-3). The enzyme modulates the topological state of DNA by generating transient double-stranded breaks in the backbone of the genetic material (1-3). The points of cleavage on the opposite strands of the double helix are staggered by 4 bases, such that scission results in 5'-overhanging cohesive ends (1-3). To maintain genomic integrity during the cleavage event, topoisomerase II forms covalent bonds between active site tyrosyl residues (one on each subunit of the homodimeric enzyme) and the 5'-terminal phosphates created by scission of the DNA (1-3). These covalent topoisomerase II-cleaved DNA intermediates are known as *cleavage complexes*.

Under normal conditions, topoisomerase II-cleaved DNA complexes are fleeting intermediates in the catalytic cycle of the enzyme and are tolerated by the cell (2,4,5). However, conditions that significantly increase the concentration of cleavage complexes generate permanent DNA strand breaks that trigger illegitimate recombination, chromosomal aberrations, sister chromatid exchange, and cell death pathways (2,5-7).

A variety of important anticancer drugs, such as etoposide and doxorubicin, kill cells by increasing levels of topoisomerase II-DNA cleavage complexes (2,5,8). Since the DNA cleavage/ligation equilibrium of topoisomerase II was found to be the primary target for these drugs (9), there has been intense interest in the DNA cleavage activity of the type II enzyme.

All of the essential catalytic functions of topoisomerase II require the enzyme to generate a transient double-stranded break in the backbone of DNA (1-3,5). Therefore, several methods have been developed to monitor the *in vitro* DNA cleavage activity of the type II enzyme. They all take advantage of the fact that cleavage complexes can be trapped by rapidly denaturing the complexed enzyme with SDS. The plasmid-based system was first utilized by Sander and Hsieh (10) and Liu *et al.* (11). The assay that is described in this chapter is based on the method of Osherooff and Zechiedrich (12). Although the method discussed below has been optimized for human topoisomerase II α , it can be adapted for virtually any type II topoisomerase.

The plasmid-based assay quantifies enzyme-mediated double-stranded DNA cleavage by monitoring the conversion of covalently-closed supercoiled DNA to linear molecules. It also can quantify topoisomerase II-generated single-stranded breaks by monitoring the production of nicked plasmids. The assay is simple, straightforward, and does not require the use of radiolabeled substrates. However, it requires relatively high concentrations of topoisomerase II and is not as sensitive as methods that utilize radiolabeled DNA.

2. Materials

2.1. Assay Components

1. Human topoisomerase II α (*see Note* ¹).
2. 5X assay buffer stock: 50 mM Tris-HCl, pH 7.9, 500 mM KCl, 0.5 mM Na₂EDTA, 25 mM MgCl₂, 12.5% glycerol (stored at 4 °C) (*see Note* ²).

¹Reaction conditions described have been optimized for human topoisomerase II α . However, enzyme and DNA concentrations as well as the specific buffer components, reaction volume, assay temperature, and reaction time can be altered to optimize individual systems. Topoisomerase II from a variety of species can be obtained from commercial sources. However, these enzymes generally are diluted for use in DNA relaxation/catenation/decatenation assays and are sold as activity "units." Consequently, commercial enzymes are often too dilute (or prohibitively expensive) to use routinely for DNA cleavage assays. If an extensive number of DNA cleavage assays are required, it is recommended that the type II enzyme be prepared "in house." Systems for the overexpression and purification of recombinant human topoisomerase II α and β , as well as yeast, *Drosophila*, and chloroella virus topoisomerase II have been described (13-16).

3. Stock solution of 20 mM ATP or a non-hydrolyzable ATP analog such as adenylyl-5'-yl imidodiphosphate (optional) (stored at -20 °C).
4. Supercoiled plasmid DNA such as pBR322 (*see Notes* ³ and ⁴).
5. Stock solution of 5% (w/v) SDS (stored at room temperature).
6. Stock solution of 250 mM Na₂EDTA (stored at 4 °C).
7. Stock solution of 0.8 mg/mL proteinase K in 50 mM Tris-HCl, pH 7.9, 1 mM CaCl₂ (stored at 4 °C).

2.2. Gel Electrophoresis

1. Agarose gel loading buffer: 10 mM Tris-HCl, pH 7.9, 60% w/v sucrose, 0.5% (w/v) bromophenol blue, 0.5% (w/v) xylene cyanole FF (stored at room temperature).
2. Stock solution of 10 mg/mL ethidium bromide in H₂O (stored in the dark at 4 °C).
3. 50X TAE electrophoresis running buffer: 2 M Tris, 5.4% (v/v) glacial acetic acid, 100 mM Na₂EDTA (Stored at 4 °C).
4. Agarose (SeaKem LE agarose, FMC BioProducts or equivalent).
5. Agarose gel electrophoresis apparatus.
6. Microwave oven.
7. 50 °C water bath.
8. Digital imaging system with medium wave (~300 nm) ultraviolet light box.

3. Methods

1. Prepare samples on ice for 20 µL reactions as follows: mix 4 µL of 5X assay buffer, 1 µL of ATP or APP(NH)₄P if desired (*see Notes* ⁵ and ⁶), supercoiled plasmid DNA to a final concentration of 5-10 nM (this is equivalent to ~20–40 µM base pairs when pBR322 plasmid DNA is used), and H₂O to a final volume that will result in a 20 µL reaction after the addition of topoisomerase II.
2. Initiate the reaction by the addition of topoisomerase II α to a final concentration of 200 nM (*see Note* ⁷) and incubate the sample at 37 °C for 6 min (*see Note* ⁸). Terminate the reaction by the addition of 2 µL of 5% SDS (*see Note* ⁹).

³Negatively supercoiled plasmid DNA can be prepared from bacteria by a number of protocols. The Plasmid Mega Kit by Qiagen routinely works well.

⁴The affinity of topoisomerase II for its DNA substrate varies with sequence (19). Therefore, the cleavage activity of the enzyme may depend on the plasmid utilized. Levels of cleavage also depend on the topological state of the plasmid, with negatively supercoiled substrates being preferred over positively supercoiled, relaxed, nicked, or linear molecules (20,21).

⁵Topoisomerase II establishes two distinct DNA cleavage/ligation equilibria: one prior to its double-stranded DNA passage event (which corresponds to the ATP-free form of the enzyme) and one following its DNA passage event (which corresponds to the ATP-bound form) (1-3,22). Hence, assays carried out in the absence of a nucleotide triphosphate monitor pre-strand passage DNA cleavage, those in the presence of a non-hydrolyzable ATP analog monitor post-strand passage DNA cleavage, and those in the presence of ATP monitor a mixture of the two. Levels of DNA cleavage observed following strand passage generally are higher than those observed prior to strand passage (22).

⁶Due to the high concentration of topoisomerase II employed in cleavage assays, DNA catenation may be observed in the presence of ATP or nonhydrolyzable ATP analogs (23). If uncleaved (*i.e.*, non-linear) plasmid molecules become part of catenated networks, they remain at the origin in agarose gels (23).

⁷The DNA cleavage/ligation equilibrium of topoisomerase II greatly favors the ligation event. Therefore, at any given time, only a small proportion (<1%) of the topoisomerase IIDNA complex exists with DNA in the cleaved state. Consequently, in order to visualize DNA cleavage products, the topoisomerase II:plasmid ratio is generally in excess of 10:1.

⁸In the absence of cleavage-enhancing drugs, topoisomerase II establishes its DNA cleavage/ligation equilibrium rapidly (within 5-10 s) (24). In contrast, equilibrium often is reached more slowly in the presence of drugs (several minutes may be required to reach maximal levels of cleavage) (24).

3. Add 1 μL of 250 mM Na_2EDTA followed by 2 μL of 0.8 mg/mL proteinase K, and incubate the samples at 45 $^{\circ}\text{C}$ for 30 min to digest topoisomerase II α .
4. Prepare the agarose gel by making a 1% agarose solution (w/v) in 1X TAE buffer containing 0.5 $\mu\text{g/mL}$ ethidium bromide (*see Note* ¹⁰). Melt the agarose by heating the solution in a microwave oven (~2 min) and let to cool to ~50 $^{\circ}\text{C}$ in a water bath. Pour the gel and allow it to solidify (this should take 15-30 min).
5. Add 2 μL of agarose gel loading buffer to assay samples and heat at 45 $^{\circ}\text{C}$ for 2 min immediately prior to electrophoresis.
6. Load the samples on the agarose gel and resolve reaction products by electrophoresis in 1X TAE buffer at ~5 V/cm for ~3 h (until the bromophenol blue has run approximately to the end of the gel and the xylene cyanole FF has run approximately half as far) (*see Note* ¹¹).
7. The gel may be destained in H_2O for 30-60 min, but generally it is not necessary.
8. Visualize DNA reaction products by transillumination with medium wave (~300 nm) ultraviolet light. DNA cleavage products may be quantified by digital imaging based on the fluorescence of DNA-bound ethidium bromide.
9. Topoisomerase II-mediated double-stranded or single-stranded DNA cleavage results in the conversion of covalently closed supercoiled plasmids (form I DNA, FI) to linear (form III, FIII) or nicked (form II, FII) molecules, respectively (Fig. 1).

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⁹It is critical to terminate DNA cleavage reactions with SDS prior to the addition of EDTA, as chelation of the Mg^{2+} in the assay buffer inhibits topoisomerase II-mediated DNA scission and leads to the rapid loss of cleavage complexes (10,12). The ability of SDS to trap cleavage complexes diminishes considerably when the final concentration of the detergent drops below ~0.5%.

¹⁰The inclusion of ethidium bromide in the gel and running buffer alters the electrophoretic mobility of all covalently closed plasmid molecules and causes them to co-migrate with the supercoiled band. Since the relative electrophoretic mobilities of nicked and linear DNA molecules (in TAE buffer) are not affected by the presence of ethidium bromide, the presence of the intercalating dye ensures that cleaved DNA bands are separated from any relaxed plasmid topoisomers (generated in the presence of ATP or nonhydrolyzable ATP analogs). If a nucleotide triphosphate is not included in assays, gels can be stained with ethidium bromide following electrophoresis. In this case, the gel can be stained in 1 $\mu\text{g/mL}$ ethidium bromide (in H_2O or 1X TAE buffer) for ~30 min with continuous agitation.

¹¹The mobility of linear DNA molecules in agarose gels changes relative to those of nicked and supercoiled circular plasmids depending on the running buffer that is used. In TAE buffer, linear pBR322 molecules migrate intermediate to nicked and supercoiled DNA (supercoiled molecules have the fastest electrophoretic mobility; *see Fig. 1*). However, in TBE (Tris/borate/EDTA) buffers, the mobility of linear DNA is often greater than that of supercoiled plasmid. The optimal concentration of agarose and the running buffer may have to be determined if plasmids other than pBR322 are employed.

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Fig. 1. DNA cleavage mediated by human topoisomerase II α . The effects of the anticancer drug etoposide on enzyme-mediated DNA cleavage are shown. Double-stranded DNA cleavage by topoisomerase II converts negatively supercoiled plasmid (Form I, FI) to linear DNA (Form III, FIII), and single-stranded cleavage converts it to nicked circular DNA (Form II, FII).

Fig 2. Effects of a reducing agent (DTT) on the ability of traditional and redox-dependent topoisomerase II-targeted agents to enhance DNA cleavage mediated by human topoisomerase II α . EGCG (500 μ M), 1,4-benzoquinone (BQ) (25 μ M), or etoposide (Etop) (50 μ M) was incubated without DTT (-DTT; closed bars) or with 1 mM DTT (+DTT; open bars) prior to its addition to topoisomerase II α -DNA complexes. Control reactions contained DNA and enzyme in the absence of compounds (hTII). Error bars represent standard deviations for three independent experiments.