# Topoisomerase I Drug Screening Kit User Manual

**Shipping and Storage of Reagents**
The kit may be shipped at ambient temperature or on ice (dry ice or wet ice). The DNAs should be stored at 4° C and the buffers (and camptothecin) stored at -20° C upon receipt. Avoid frequent freeze/thaw cycles with the plasmid as this may contribute to DNA breakage.

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# I. Introduction

## A. Summary

The TopoGEN Topoisomerase I Drug Screening Kit contains reagents necessary to identify compounds that target topoisomerase I (topo I). This kit detects two kinds of Topo I agents: those that inhibit the activity of the enzyme (Catalytic Inhibitory Compounds or CICs) and those that stimulate formation of the cleavage complexes (Interfacial Poisons or IFPs). CICs may affect enzyme at one of many levels, such as blocking access to DNA substrate while DNA intercalators, high salt or polyamines are potent CICs (although not specific). IFPs are another type of inhibitor that blocks the resealing step of the reaction on DNA such that SS DNA nicks tend to accumulate. Camptothecin and related chemotypes are examples of IFPs (Camptothecin (CPT) is included with this kit as a positive control for IFP action). The usual reaction sequence for Topo I is schematically shown in Fig. 1. Under normal circumstances, Topo I enters into a breakage/resealing cycle that favors the resealed product. The cleavage intermediate has an extremely short lifetime and cannot be detect. IFPs like Camptothecin stereochemically insert and mis-align the cleavage complex such that the resealing step is inhibited thereby leading to a stabilized DNA nick (ss break) with the protein covalently bound to the 5' end of the break (leaving a 3'OH). This kit is based on a circular, supercoiled plasmid DNA (pHOT1) as substrate. Topo I will efficiently relax this DNA; however, the product will be circular and devoid of supercoils, and no nicked DNA will be detected. In the presence of CPT, nicked open circular formation is formed after the denatured Topo I is degraded by proteinase K (supplied in the kit). This step is essential to see the nicked forms of plasmid. In some cases, a test drug may effectively block the enzyme activity of topo I (CICs, see above) in which case no relaxed DNA will be seen (Fig. 1 scheme)

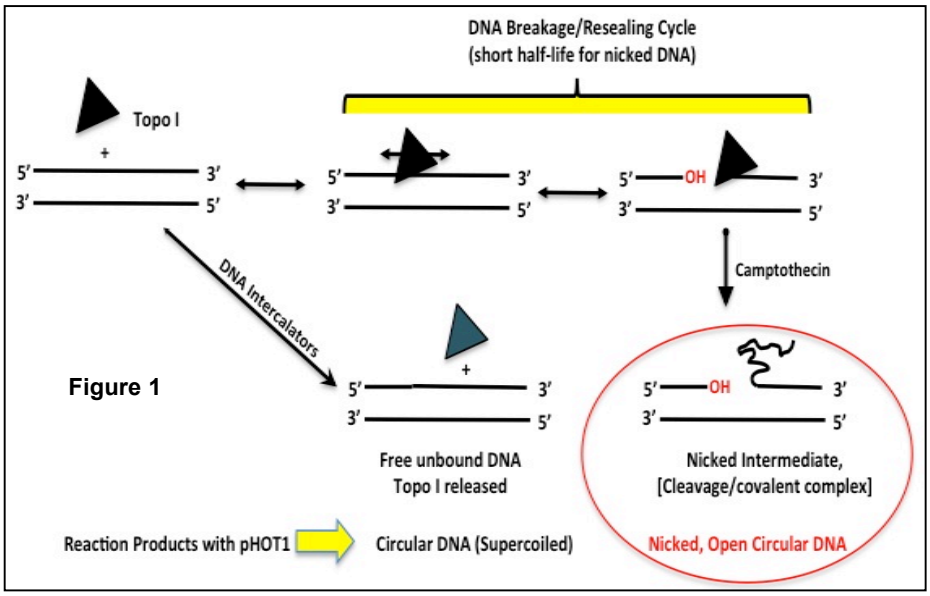


**Figure 1**

**B. Kit Contents** (100 assay kit size)

1. pHOT1 DNA, supercoiled substrate. Concentration of 0.25ug/ml (25 ug pHOT1 DNA in 100 ul TE buffer, 10mM Tris-HCl, pH7.5, 1 mM EDTA)

2. Marker DNA, Relaxed pHOT1; 0.05 ug/ul in 1x gel loading buffer (50 ul, load 2 ul as marker).

3. Topo I reaction buffer (10x TGS; 300 ul) TGS Buffer (1x) is 10 mM Tris-HCl pH 7.9, 1 mM EDTA, 0.15 M NaCl, 0.1% BSA, 0.1 mM Spermidine, 5% glycerol.

4. Control Inhibitor: Lyophilized Camptothecin (Cat. # 4110). Add 0.25ml DMSO to obtain a 10 mM stock.

5. 10% Sodium Dodecyl Sulfate (300 ul): To terminate reactions, use 0.1 volume (final 1%).

6. 10x gel loading buffer (300 ul): 0.25% bromophenol blue, 50% glycerol, use 0.1 volume in reactions.

7. Proteinase K (500 ul) at 0.5 mg/ml. This is a 10x stock of proteinase K.

***Note: purified Topoisomerase I is not included in this kit but is available for purchase on our website, www.topogen.com. Catalog number TG2005H-RC1 (500 units).***

**C. Protocol for a typical Reaction Mixture of 20 ul**

Assemble all reactants in the following order.

- ✓ **$H_20$**: to make up to final volume (20 ul in this case)
- ✓ **10x TGS Buffer** 2 ul
- ✓ **DNA** 0.5 to 1 ul (125 to 250 ng).
- ✓ **Test Compound**: Variable (0 to 100 uM titrations are usually performed with unknowns. Note that the drug solvent (DMSO for example) can cause non-specific inhibition. We recommend a matched solvent control lacking drug to control for non-specific solvent influence.
- ✓ **Positive Control with Camptothecin**: It is important that a CPT control be included. We recommend from 50-100 uM to detect cleavage complexes.
- ✓ **Topoisomerase I.** Enzyme is supplied at 2 to 10 units/ul (refer to the Lot Number of Enzyme). Usually, 2-6 units of stock enzyme is enough to detect cleavages. Note that the amount of cleavage will be low, but still detectable. You should not expect to see 100% conversion of substrate to nicked open circular DNA (see additional comments below).

1. Incubate 30 minutes at 37°C; stop by addition of 2 ul 10% SDS.

2. Add proteinase K to 50 ug/ml, (incubate 37°C for 15 min.).

3. Add 0.1 vol. loading buffer (blue juice).

4. Samples may be loaded directly onto the agarose gel at this point.

   Optional Step: the samples can be cleaned up by extraction and then loaded: Add equal volume (20 ul) of Chloroform: isoamyl Alcohol or CIA (24:1), vortex briefly; spin in a microfuge for 5 sec. Withdraw blue coloredupper aqueous phase and load onto agarose gel. CIA extraction

will usually can improve the cosmetic quality of the agarose gel results. In addition, CIA extraction will extract non-polar compounds that may interfere with the gel staining, with some test drugs.

5. Run a 1% agarose until the dye front of bromophenol blue is about 70-80% down the gel. Do not run overnight as this will cause the DNA bands to diffuse. Usually a gel gives good separation after 1-3 hrs. Stain with 0.5 ug/ml ethidium bromide, destain for 15 min in water and photodocument results. This is a "non-ethidium bromide" gel separation which is optimal for resolving relaxed and supercoiled DNAs (see gel data, Fig. 2). We recommend that each researcher use this method to verify enzyme activity, PRIOR to testing new compounds. Once enzyme activity is demonstrated, the researcher may wish to run gels containing 0.5 ug/ml ethidium bromide. In the EB gel analysis, the buffer and gel should contain ethidium bromide DURING the electrophoretic separation (followed by destaining in water for 15 min prior to photodocumentation). The ethidium bromide gel separation method allows one to clearly resolve cleavage products (open circular, linear DNA) from the closed DNA forms (relaxed circular and supercoiled circular DNA species).

**D. Important Considerations about this kit.**

Marker DNAs are extremely important. You must be able to identify supercoiled and relaxed DNAs in the gel.

Any nuclease-free agarose of reasonable quality can be used (from Sigma).

A positive control (Camptothecin) and negative control (no drug) are also very critical for data interpretation.

Agarose gels (1%) and running buffers can be any standard nondenaturing electrophoresis buffer (example, to prepare a 50x of TAE Gel Buffer: 242 g Tris base, 57.1 ml glacial acetic acid and 100 ml of 0.5 M EDTA). Dilute to 1x to use for gel separations.

Agarose gels should be run in the absence of Ethidium Bromide (EB) to demonstrate enzyme activity (maximal resolution of relaxed vs. SC DNA).

EB agarose gels (EB at 0.5 ug/ml in gel and buffer) will improve the resolution of cleavage products (nicked open circular and linear DNA). For Topo I screens, EB gels allow you see clearly an increase in nicked OC DNA. See Figure 2 for sample data.

If unsure about whether to run an EB or Non-EB gel, we suggest that you run both. Simply divide your reactions into equal parts and run two gels at the same time. In this way, you will be sure to see all reaction products and enhance your interpretation of the experiment.

Run gels at 1.5-2 V/cm (measured between electrodes) until the dye front has traveled about 80%

After running, non-EB gels should be stained with EB (0.5 ug/ml) for 15-30 min and then destained in water or buffer for 15 min prior to photodocumentation.

EB gels are run in the presence of 0.5 ug/ml (in gel and running buffer), destained with water for 15 min prior to photodocumentation.

**E. Data Interpretation and additional helpful hints.**

Solvent controls are especially important to ensure that the drug solvent (DMSO or methanol for example) are not interfering with Enzymatic activity.

As noted a quick CIA extraction prior to loading the gels is a good idea since the gels will be much more cosmetic; however, the samples must first be treated with proteinase K to digest the bound protein. CIA extractions may also be required if the test compound affects the mobility of the DNA or fluorescent detection (intercalators, strong DNA binding agents, etc.).

To confirm the formation of covalent Topo/DNA complexes, you can run the following control: Two reactions are carried out with the test drug (or with camptothecin control), both are stopped with SDS. One is digested with proteinase K and the other is undigested. The non-PK digested covalent topo I/DNA complexes will 'gel shift' and will not be detected on the gel. In contrast, the proteinase K digested sample should contain expected cleavage products (linear DNA). Figure 2 shows typical EB and

Non-EB gel results (EB) result with markers and positive drug CPT controls.

## D. Frequently asked questions.

**What are the critical controls to allow me to clearly identify a topo I targeting agent?**

- Marker DNAs (supercoiled, linear DNAs) (see Fig. 2) are extremely important.
- Be sure to run a positive DRUG control (like camptothecin or CPT) to demonstrate good cleavage activity. You should see an increase in form II (nicked) DNA.
- Include a negative control (either no drug or a topo II drug such as etoposide). VP16 or etoposide is not supplied but we have it available.
- Be sure to check solvent effects. Solvents like DMSO or methanol are used to dissolve some test drugs. Test with a control reaction lacking drug but with solvent (e.g. 1% DMSO).

**What kind of agarose should I buy?**

Any nuclease-free agarose of reasonable quality from any number of sources can be used (Sigma-Aldrich works).

**What is the best gel buffer to use?**

Agarose gels (1%) and running buffers can be any standard nondenaturing electrophoresis buffer (example, to prepare a 50x of TAE Gel Buffer: 242 g Tris base, 57.1 ml glacial acetic acid and 100 ml of 0.5 M EDTA). Dilute to 1x to use for gel separations. Be sure that the gel also has 1x TAE buffer.

**Should I run EB or Non-EB gels and how do I run the EB gels?**

- In general, 1% gels in the absence of Ethidium Bromide (EB), can be used (these gels are ideal for testing enzyme activity); however, the nicked open circular DNA product cannot be clearly identified in this gel system. As noted above, an EB containing gel (0.5 ug/ml, EB in gel and buffer) will improve the resolution of cleavage products (nicked open circular and linear DNA). Be sure to destain with water for 15 min prior to photodocumenting your data.
- In some cases, depending on how the gel is run, the topoisomer distribution can interfere with your ability to see the cleavage products; however, EB gels remove this complication.
- IMPORTANT: If unsure about whether to run an EB or Non-EB gel, we suggest that you run both. Simply divide your reactions into equal parts and run two gels at the same time. In this way, you will be sure to see all reaction products and enhance your interpretation of the experiment. With all markers in the gel system (supercoiled, relaxed, linear for example) you will obtain very clear and unambiguous results.

**What are the running conditions in terms of time and voltage?**

- Run gels at 1.5-2 V/cm (measured between electrodes) until the dye front has traveled about 80%
- After running, non-EB gels should be stained with EB (0.5 ug/ml) for 15-30 min and then destained in water or buffer for 15 min prior to photodocumentation.
- EB gels are run in the presence of 0.5 ug/ml (in gel and running buffer), then destained with water for 15 min prior to photodocumentation.

- IMPORTANT: Try not to run the gels overnight but keep your electrophoresis times to less than 1-2 hrs. Long run times cause band diffusion and degrade the quality of your gel results.

**What reaction volumes do you recommend for these assays?**
- Reaction volumes should be 20-30 ul final volume (limited by the volume that can be loaded into the wells of the agarose gel).
- The reactions should be assembled on ice in microfuge tubes (water, buffer, and DNA, test compound and enzyme, which should be added last).
- After adding enzyme, the tubes should be transferred to a heating block to initiate the reaction.

**Are the termination conditions critical for detecting cleavages?**
- Yes. Reactions should be incubated 30 min (37°C), terminated by rapid addition of 1/10 volume of 10% SDS followed by digestion with 50 ug/ml proteinase K prior to loading the gel. SDS is added to reactions at 37° to facilitate trapping the enzyme in a cleavage complex.
- Also, if the reactions are heated, cooled or treated with high salt prior to adding SDS, the topo breakage and resealing equilibrium may be altered and breaks can reseal.

**Why is proteinase K required?**
- Drugs that trap topo/DNA complexes will induce covalent complexes between DNA and protein (topo ) and this protein must be removed (degraded). Failure to do so will prevent detection of the cleavage products.
- If the reactions are heated, cooled or treated with high salt prior to SDS, the topo DNA breakage and resealing equilibrium may be altered and breaks can reseal.

**Can you help us with data interpretation?**
- Yes, we can definitely help! The best way to proceed is to send us your data (support@topogen.com) with a full description of the experiment. We will get back to you quickly with feedback.

**Can you show us some real gel data and discuss the results?**
- Yes, we can. The results and a helpful discussion is shown in Fig. 2 (see legend).

**How come the control drug, CPT, is not showing inhibition of enzyme activity. Topo I appears to relax the DNA fully even when the drug is included. What is wrong?**
- Actually, nothing is wrong. This is a poison and many people refer to it as an inhibitor. Topo I must be able to relax DNA through its normal cycle of breakage/resealing in order to observe interfacial poisoning. In this case, the positive result is nicked open circular DNA (sometimes called form II DNA), generated by the poisoning effects of catalytically active topo I enzyme.

**Any further questions or comments, please feel free to contact us:**
**support@topogen.com**

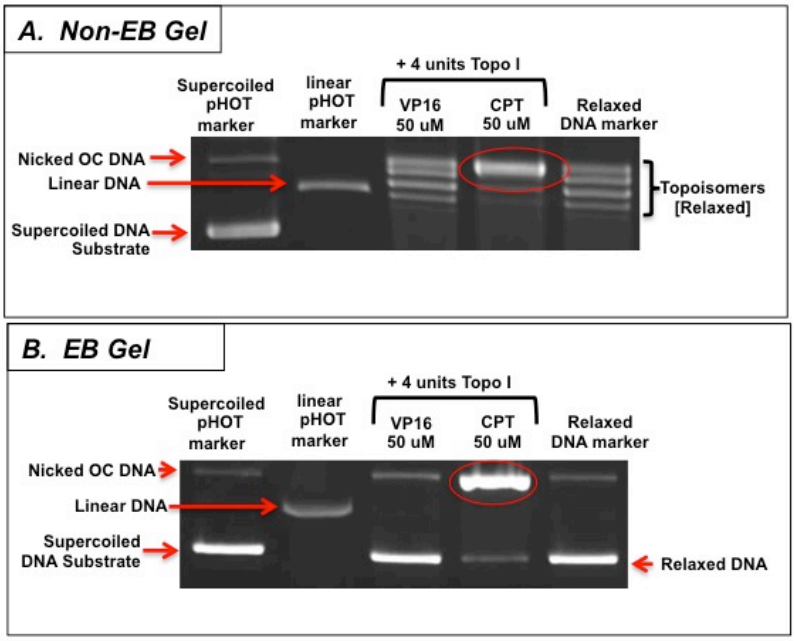


**Figure 2. Topo I Reaction Products Resolved on Non-EB and EB Gels.**
Reactions (20 ul) were terminated with 1% SDS, digested with proteinase K and extracted with CIA. The final volume after addition of Loading Dye was approximately 26 ul. Two agarose gels (1%) were prepared. The top gel was cast and run in the absence of EB and the bottom gel cast with 0.5 ug EB/ml and electrophoresed in buffer containing 0.5 ug EB/ml. Gels were run at 50v for 45-50 min and either stained with EB (non-EB gel) or destained with water (EB gel) per protocols given above. The data show the positions of nicked open circular (OC) DNA which is pHOT1 DNA containing at least one single stranded nick (all plasmids have a small amount of nicked OC DNA). Topoisomers are relaxed DNA forms that resolve after incubating with Topo I in the absence of any drugs; these topoisomers are fully circular and contain no single stranded interruptions. The formation of topoisomers is diagnostic for strong Topo I relaxation activity and demonstrate that the enzyme is showing excellent activity. To see this result, a non-EB gel should be run. The EB gel (Panel B) is ideal for detecting Topo I cleavage products, such as nicked open circular DNA. The topo II active drug, VP16, did not influence the topo I reaction, as expected. Note that supercoiled DNA substrate and relaxed DNA products are rather poorly resolved in EB gels. In some cases, it is very difficult to demonstrate topo I catalytic activity in this gel system as a result. For this reason, we recommend using a Non-EB gel separation in activity assays.