TopoGEN

Products and Services for **Cancer Research.**

Topoisomerase II Drug Screening Kit (plasmid based).

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 stored at -20° C upon receipt. The VP-16 can be stored at ambient temperature. Avoid frequent freeze/thaw cycles with the plasmid as this may contribute to DNA breakage.

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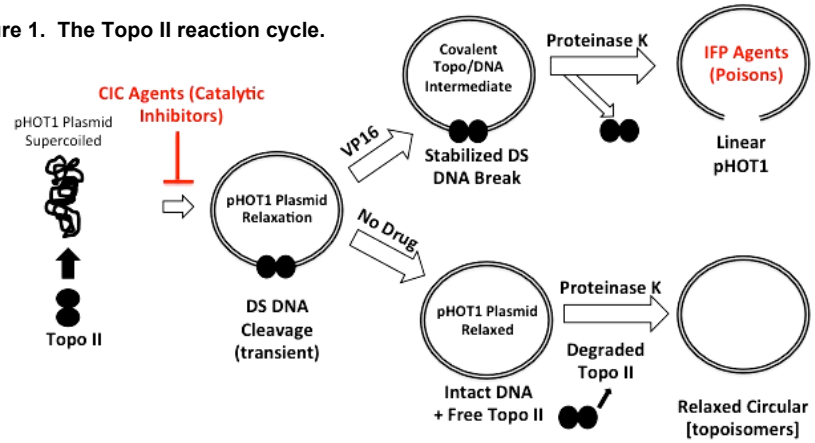
I. Introduction

A. Summary

This Kit will unambiguously identify compounds that target topoisomerase II action (topo II). The kit detects two kinds of Topo II effectors: 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 or altering ATPase action and enzyme turnover. IFPs are another type of inhibitor that blocks the resealing step of the reaction on DNA such that DS DNA breaks tend to accumulate. Plasmid based kits, such as this Kit, are ideal for detecting IFP (poisons like VP16). A kDNA based Kit (TG1019) is also available from TopoGEN.

Under normal circumstances, Topo II enters into a breakage/resealing cycle that favors the resealed product (Fig 1). The cleavage intermediate has an extremely short lifetime and cannot be identified. A known Topo II poison (etoposide or VP16) is included as a control IFP since a positive drug is required to ensure that the assay is working properly and is capable of resolving unknown IFP mechanistic drugs. The DNA substrate (pHOT1) included in this assay is ideal for these studies because it is small and easy to handle and has a large number of Topo II recognition elements. The assay system is based upon evaluating the formation of DNA cleavage products, primarily linearized DNA (linear DNA). Note that most IFPs will generate linears; however, since the DS cleavage/resealing reaction involves separate subunits (Topo II is a homodimer) it is possible to also detect nicked cleavage products. See the following video for more information (http://www.youtube.com/watch?v=B4s_pi2KXVw&feature=plcp).

Figure 1. The Topo II reaction cycle.



B. Kit Contents (100 assay kit size)

1. pHOT1 DNA, supercoiled (refer to tube for accurate DNA concentration). 25 ug total pHOT1 DNA

2. Marker DNA, Linear pHOT1; 0.05 ug/ul in 1x gel loading buffer (25 ul, load 2 ul as marker).

3. **Buffer A: 0.5 M Tris-HCl (pH 8), 1.50 M NaCl, 100 mM Mg_2Cl, 5 mM Dithiothreitol, 300 ug BSA/ml.

4. **Buffer B: 20mM ATP in sterile distilled water.

****IMPORTANT**: You must mix Buffers A and B together prior to make a 5x working Assay Buffer. To prepare a fresh stock of the 5x Assay buffer: Add equal volumes Buffer A and B to give the Complete 5x Assay Buffer (example, if you need 50 ul of 5x Complete Buffer for a single experiment, mix 25 ul of Buffer B with 25 ul of Buffer A). The Complete 5x Buffer MUST be made fresh for each experiment. Prepare only the amount needed fresh each day. DO NOT SAVE THE 5X COMPLETE ASSAY BUFFER, IT IS NOT STABLE.

5. VP-16 (etoposide) control drug (TG4140) in lyophilized state. Add 250 ul of methanol or DMSO to give a 10 mM stock, then dilute this stock to 1mM in Tris buffer (10 mM Tris-HCl, pH 7.5). This will reduce the solvent concentration to 10%. The 1 mM stock may be added to the assay mix to yield a final concentration of 0.05 to 0.1mM in the reaction. For example, in a 20 ul reaction 1 or 2 ul of 1mM stock will work as a control for cleavage. Note that solvent can inhibit at concentrations above 1%.

6. 10% sodium dodecyl sulfate (SDS) (300 ul): to terminate reactions, use 1/10 volume.

7. 10x loading buffer (300 ul): 0.25% bromophenol blue, 50% glycerol: Use 0.1 vol.

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

Purified Topoisomerase II is not included in this kit but is available for purchase on our website, www.topogen.com. Catalog number TG2000H-1 (250 units) or TG2000H-2 (500 units). Contact us to order.

C. Protocol for a typical reaction mixture (final volume of 20 ul)

1. Assemble all reactants in the following order.
   - ✓ H_20 Variable: to make up to volume (20 ul in this case)
   - ✓ 5x Complete Buffer 4 ul (prepared fresh as described above)
   - ✓ DNA 1 ul (100 to 150 ng is sufficient, depends on concentration of DNA in kit).
   - ✓ 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.
   - ✓ Topoisomerase II. Enzyme (sold separately, TG2000H-2) is typically at 2 to 10 units/ul. 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 linear DNA (see comments below).

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

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

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

5. Load Gel. Samples may be loaded directly onto a 1% agarose gel[a].

[a]**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 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.

6. Run a 1% agarose until the dye front is about 75% down the gel. Stain with 0.5 ug/ml ethidium bromide, destain with water 15 min 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 using non-EB gels to verify enzyme activity, PRIOR to testing new compounds. Once enzyme activity is demonstrated, the researcher may wish to run both non-EB and EB gels as described below. The latter gels are perfect to

resolve nicked circular from linear DNA, but less than ideal to resolve substrate (supercoiled DNA) from product (relaxed DNA).

D. Frequently asked questions.

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

*Marker DNAs (supercoiled, linear DNAs) (see Fig. 2) are extremely important.

*Be sure to run a positive control (VP16) to demonstrate good cleavage activity.

*Include a negative control (either no drug or a topo I drug such as CPT). CPT 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 and linear DNA formation). 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 linear DNA (a topo II cleavage product); however, EB gels remove this complication.

*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.

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.

*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 microcentrifuge 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 II 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 II 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 p. 8).

How come the control inhibitor, VP16, is not showing inhibition of enzyme activity. Topo II appears to relax the DNA fully even when VP16 is included. What is wrong?

Actually, nothing is wrong. This is a poison and many people refer to it as an inhibitor. Topo II must be able to relax DNA through its normal cycle of breakage/resealing in order to observe a Vp16 effect. In this case, the positive result is linear DNA, generated by the poisoning effects of catalytically active topo II. Topo II can be catalytically inhibited only at very high concentrations of VP16, while its activity as a poison is manifest at much lower levels of drug (low uM).

Will this kit detect CIC (catalytic inhibitors)?

Yes but the plasmid based kit is really optimized for detection of topo II poisons. The reason is that linear DNA cleavage products are easier to detect using a plasmid based system and you can run EB gels to monitor DNA nicking as well as DS DNA breaks. A kinetoplast or kDNA based system for screening topo II CICs (Cat #1019) actually is optimized for this purpose. The reason is that kDNA is really a very good substrate for detecting topo II enzymatic activity; thus, it is more sensitive than plasmid based assays.

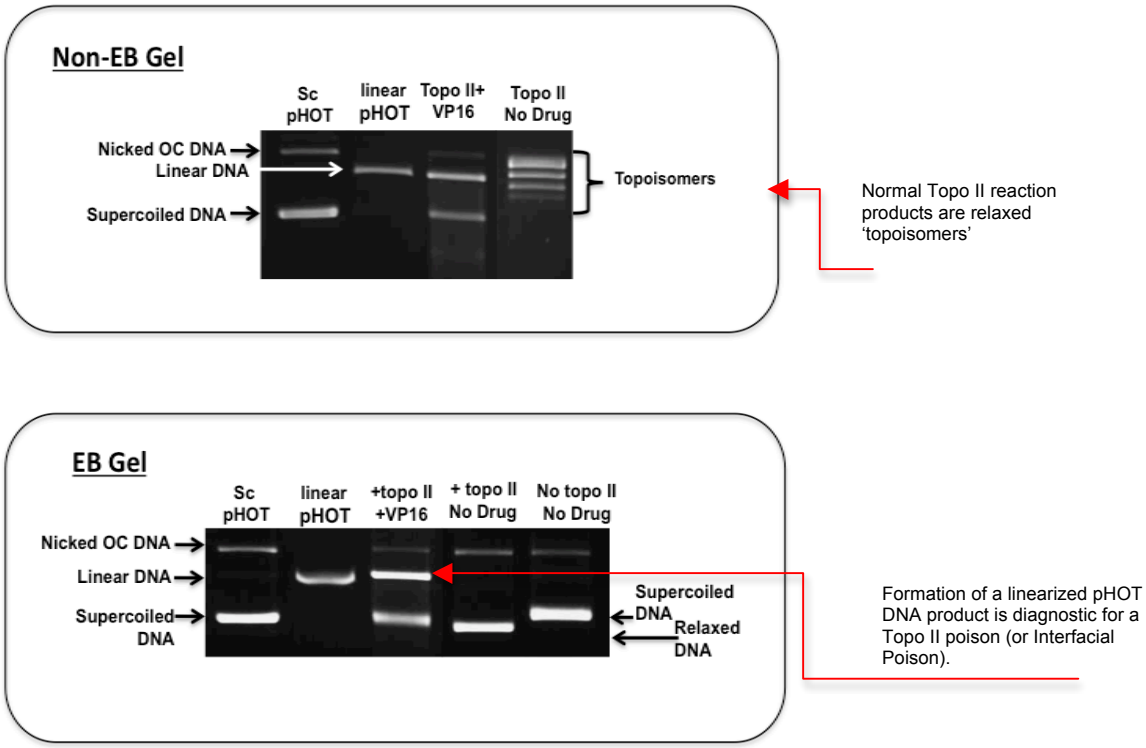


Figure 2. Topo II Reaction Products Resolved on Non-EB and EB Gels.

Topo II reactions were carried out in a final volume of 20 ul (see above protocol). Reactions 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 using a minigel unit. 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 a gel 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. Topoisomers are relaxed DNA forms that resolve after incubating with topo II in the absence of any drugs; these topoisomers are fully circular and contain no single stranded interruptions. These topoisomers are diagnostic for strong topo II catalytic activity and demonstrate that the enzyme is showing excellent activity. To see this result, a non-EB gel must be used in the analysis. The EB gel (lower) is ideal for detecting topo II cleavage products, including linear DNA (see VP16 reaction where a prominent linear band can be seen). 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 II catalytic activity in this gel system as a result (depending on conditions of electrophoresis).