TopoGEN

Products and Services for **Cancer Research.**

Topoisomerase II Drug Screening Kit (kDNA based, includes Topo IIa).

Overview:

This Kit is designed to identify agents that target type II topoisomerases acting as catalytic inhibitors, primarily; however, poisons can also be detected. For a video description see:
http://www.youtube.com/watch?v=B4s_pi2KXVw&feature=plcp

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 TG1009, are ideal for detecting IFP (poisons like VP16). This kDNA based Drug Kit is optimized for detecting topo II catalytic inhibitors (CIC) type of drugs. 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 (kDNA) included in this assay is ideal because it is a very attractive substrate for topo II catalysis. This kDNA assay system is based upon evaluating either the formation of DNA cleavage products (linear kDNA) for IFPs. For CIC detection, the assay simply measures inhibition of the decatenase activity of topo II. In this case, there is a clear loss of decatenated kDNA products. The following video has more information (http://www.youtube.com/watch?v=B4s_pi2KXVw&feature=plcp).

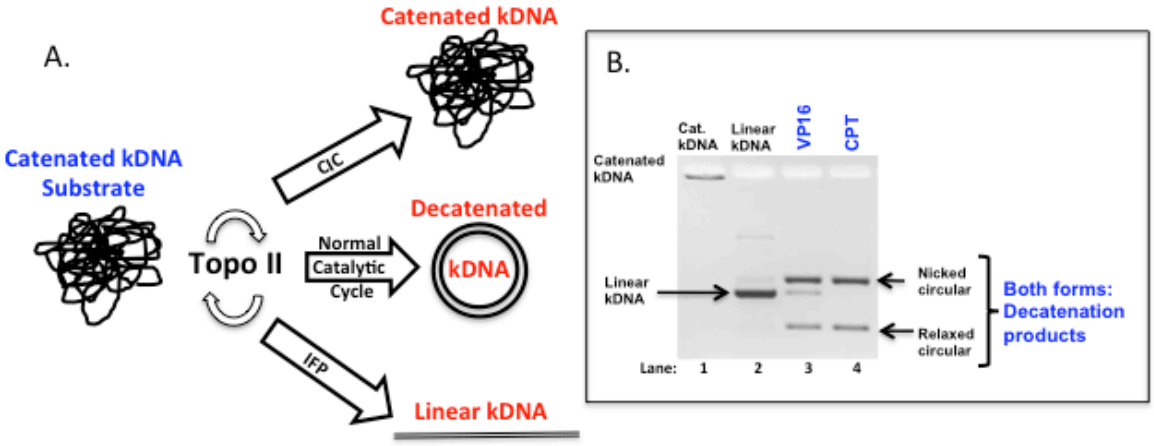

Figure 1. Reaction mechanisms with a kDNA substrate to detect IFP or CIC.
Panel A shows a schematic outline of reaction products derived from catenated kDNA networks. The normal topo II reaction products are decatenated minicircular rings (2.5 KB nominal size). Since kDNA is a mix of circular and nicked circular minicircles, the decatenation products may resolve into two species revealed by an EB containing gel shown in panel B. The EB gel shows the relative positions of linear DNA, nicked circular and fully circular kDNA rings. Notice that VP16 induces linear DNA while CPT (camptothecin) has no influence on the topo II reaction.

B. Kit Contents (100 assay kit size)

1. 100 Units Human Topoisomerase II (see attached data sheet for unit definition, quality control, and activity assay parameters). Store at -70° C.

2. kDNA (20 ug total) substrate at the concentration specified on the tube provided. Typically one should use 0.1 to 0.2 ug per reaction.

3. Decatenated kDNA marker (25 ul) in gel loading buffer. Run 2 ul of decatenated DNA marker per gel. Refer to Fig. 2, lane 4.

4. Linearized kDNA marker (25 ul) in gel loading buffer; run 2 ul of linear marker per gel. Refer to Fig. 2, lanes 2,3.

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

6. **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.*

7. 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%. A typical cleavage is shown in Lane 3 (Fig. 2). Linear DNAs are not detected in the absence of etoposide.

8. 10% sodium dodecyl sulfate (SDS) (300 ul): to terminate reactions, use 1/10 volume.

9. 10x loading buffer (300 ul): 0.25% bromophenol blue, 50% glycerol: Use 0.1 vol.

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

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 containing 0.5 ug/ml ethidium bromide in gel and buffer.

6. Run the gel at a relatively high voltage (100-250 v) until the dye front has migrated about 4-6 cm down the gel. These gels can be run fast (in as little as 15 min) because the separation between kDNA and products is easily achieved.

7. De-stain the gel in water for 15 min or so.

8. Photo-document the results.

9. IMPORTANT TIP: Prior to testing your compounds, we recommend that you perform two simple experiments:
FIRST, run the markers (kDNA, linear kDNA, Decatenated kDNA) to demonstrate that your gels are working as expected (see Fig. 2).
SECOND, perform a quick topo II titration over a range of enzyme from 1 unit to 6 units or so. Include markers to demonstrate activity. Use an input concentration of drug that gives >80% full decatenation, in your drug testing regimen.

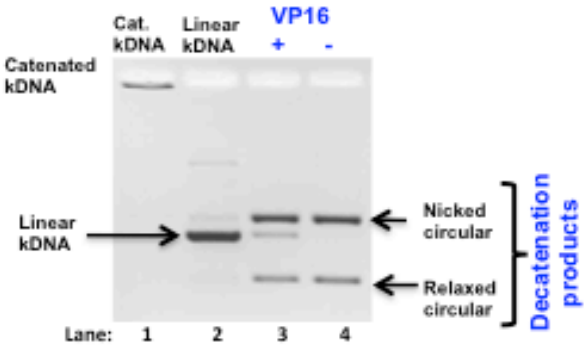

Figure 2

D. Frequently asked questions.

What are the critical controls to allow me to clearly identify a topo II targeting agent?
*Marker DNAs (see Fig. 2) are extremely important.
*Be sure to run a positive control (VP16) to demonstrate good cleavage activity
*If you plan to produce a publication quality result, we suggest that you include a negative control, such as camptothecin. CPT is not supplied but we have it available (TG4110).
*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 with Ethidium Bromide (EB), can be used (these gels are ideal for testing enzyme activity and linear DNA formation). EB gels 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.

What are the running conditions in terms of time and voltage?
*Gel separations with kDNA should be run rather fast (higher voltage). For example, we typically run gels at 200 v for 15-20 min.
*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 as short as possible to minimize diffusion which reduces gel quality.

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.

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 act catalytically on 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) and IFP (poisons) equally well?

- Yes in theory; however, kDNA based assays are better for detecting CICs, while plasmid based kits work well for detecting IFPs. Both kits are available from TopoGEN (plasmid kit is TG1009, kDNA kit is TG1019).