Topoisomerase I Hi-Loading Inhibition Kit.

Catalog TG1017-1 500 units
Catalog TG1017-2 1000units

Overview:

This kit is designed to allow the customer to screen for agents (drugs, natural products, small molecules, synthetics) that block the catalytic activity of human topoisomerase I (topo I). The use of high stoichiometric ratios of topo I:DNA induces cleavage complexes without the need to use topo I poisons (camptothecin) which facilitates detection of catalytic inhibitors of topo I. The assay (see ref 1) is based on the finding that topo I acts in a cooperative manner on DNA template, whereby it tends to display clustered activity (at neighboring sites, see ref 3); thus, at high ratios of enzyme:DNA, it is relatively easy to detect nicking by topo I in the absence of topo I poisons like camptothecin and its various congeners.

Shipping and Storage of Reagents

The kit will be shipped on dry ice. Upon receipt, the DNA and buffers should be stored at 4° C, and enzyme stored at -20° C. Avoid frequent freeze/thaw cycles with the plasmid as this may contribute to DNA breakage and enzyme inactivation.

TopoGEN, Inc.
27960 County Rd. 319 Unit B
Buena Vista, CO 81211 USA
Tel: 614-451-5810
Fax: 614-559-3932
Orders sales@topogen.com,
Support: support@topogen.com
Website: www.topogen.com

I. Introduction

A. Summary

This High Load Kit contains reagents specifically designed to identify catalytic inhibitors of topoisomerase I (topo I). Catalytic Inhibitory Compounds or CICs may affect enzyme in different ways (including blocking access to DNA substrate, DNA intercalation, high salt or polyamines). One major complication with traditional CIC topo I assays is that some small molecules may alter DNA topology but otherwise do not influence the catalytic cycle of nicking/resealing by the enzyme. In this case, it is difficult to distinguish between a DNA unwinding action and a true CIC. In other words, compounds that cause alterations in DNA topology will interfere with assays that measures loss or retention of DNA supercoiling. To avoid this problem, we have developed the Hi Loading Kit. This Kit uses very high topo I inputs and relatively low DNA levels in a specialized buffer that promotes formation of topo I nicks on the DNA. Therefore, the assay uses an EB gel system to measure conversion of superhelical DNA to Nicked, Open Circular DNA product. A true catalytic inhibitor (CIC) will block the conversion from supercoiled to nicked DNA. Thus, this assay does not depend on DNA topology to identify a potential CIC effector. A positive CIC effector will block the enzyme activity of topo I, in which case no nicked open circular DNA will be detected (see Fig. 1)

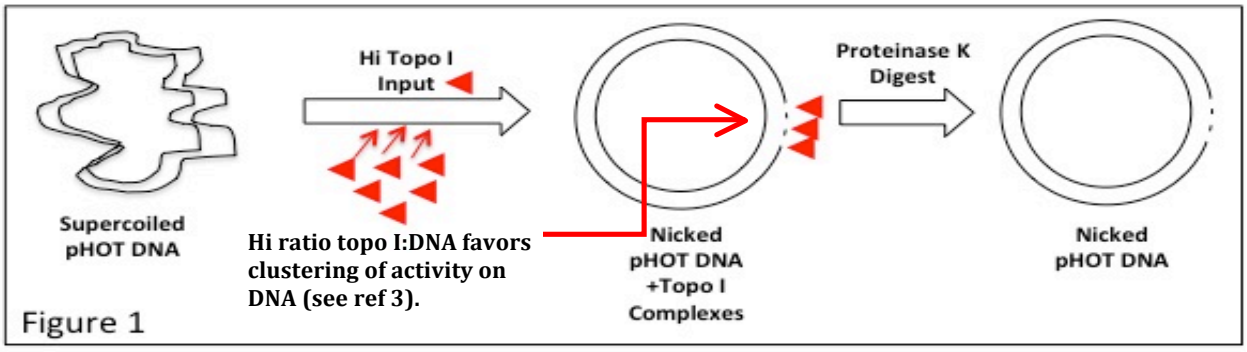

Figure 1. Hi Load Assay Outline. With high inputs of topo I, the enzyme will overload the DNA template and induce single strand nicks due to topo I crowding at cleavage sites (when topo I cut sites are adjacent, it tends to nick and not reseal, thereby emulating a topo I poison such as Camptothecin, see ref 3). The protein is removed by proteinase K and a nicked pHOT plasmid is measured by running an EB containing agarose gel. If the yield of nicked pHOT DNA is reduced by a given test compound, it is reasonable to conclude that a CIC has been detected (see data in Fig. 3 below).

B. Kit Contents

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 TE; 300 ul) TE Buffer (1x) is 10 mM Tris-HCl pH 7.9, 1 mM EDTA
4. 10% Sodium Dodecyl Sulfate (300 ul): To terminate reactions, use 0.1 volume (final 1%).
5. 10x gel loading buffer (300 ul): 0.25% bromophenol blue, 50% glycerol, use 0.1 volume in reactions.
6. Proteinase K (500 ul) at 0.5 mg/ml. This is a 10x stock of proteinase K.
7. Purified human topoisomerase I provided at 25 units/ul (20ul). A total 500 units is included for Cat TG1017-1 and 1000 units for Cat TG1017-2.**.

***** Additional purified Topoisomerase I 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

1. Assemble all reactants in the following order.
   - ✓ **H_2O**: to make up to final volume (20 ul in this case)
   - ✓ **10x TE 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.
   - ✓ **Topoisomerase I.** Enzyme is supplied at a high concentration (typically 25 units/ul but refer to the Lot Number of Enzyme). Usually, 25-50 units of stock enzyme is enough to detect cleavages. You should not expect to see 100% conversion of substrate to nicked open circular DNA (see additional comments below).

2. Procedure.

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

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

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

- Samples may be loaded directly onto the agarose gel at this point or you may perform an optional clean up step.

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

- Run a 1% agarose containing 0.5ug/ml ethidium bromide in the gel and running buffer. Electrophorese until the dye front of bromophenol blue is about 75% 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. De-stain with water for 15-30 min (change water several times, wear gloves). 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 negative control (no drug) is extremely 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.

A simple positive control using pHOT and a small amount (1-5 units) of topo I is important to show that the enzyme is fully active. 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.).

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.
- You may wish to include a positive DRUG control (like camptothecin or CPT) to demonstrate elevated cleavage activity. In the "Hi Load Topo I" assay format, CPT will typically produce significantly higher nicked and even some linear DNAs products.
- Include a negative control (either no drug or a topo II drug such as etoposide). Neither CPT nor VP16 (etoposide) are supplied but we have it available on our site.
- 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.
- 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. See Fig 2 for aid in interpreting EB and non EB gel data.

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).

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

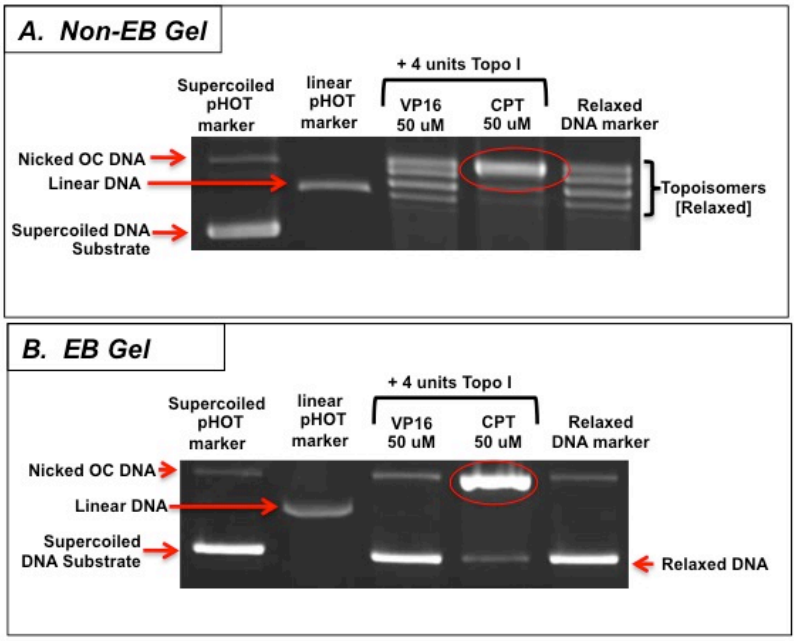

Figure 2. Topo I Reaction Products Resolved on Non-EB and EB Gels.
Reactions (20 ul) were terminated with 1% SDS, digested with proteinase K and extracted with CIA. The final volume after addition of Loading Dye was approximately 26 ul. Two agarose gels (1%) were prepared. The top gel was cast and run 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.

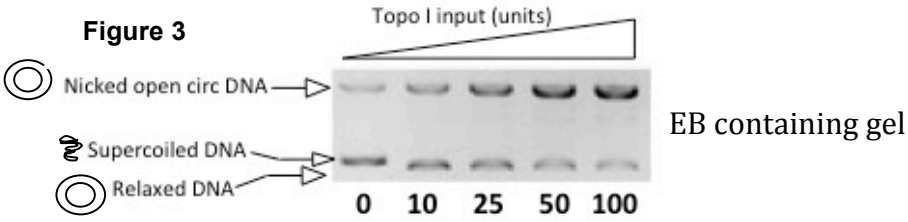

Figure 3. Hi Loading topo I results
Reactions (20 ul) contained the indicated amount of topo I input (units/reaction) and were incubated for 30 min at 37oC, terminated with 1% SDS and run on an EB gel (0.5ug/ml). The positions of nicked, supercoiled and relaxed DNA forms are marked on the left of the gel image. Note that 25 units of topo I induced a substantial increase in nicked DNA compared to the control. The cleavage was elevated by increasing amounts of topo I. Reaction products were digested with proteinase K and extracted with CIA. In the EB gel system, the nicked pHOT1DNA is well resolved. Note that in this gel system the separation of the circular forms (supercoiled and relaxed DNA) is not that great unless the gel is run longer (dye front to end of gel) or unless you run a non-EB gel (see Fig. 2).

References

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