Products and Services for **Cancer Research.**

Human Topoisomerase I Assay Kit (Includes Topo I) USER MANUAL

Shipping and Storage of Reagents

The kit is shipped on dry ice. The DNAs should be stored at 4° C and the enzyme + buffers 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

Human Topoisomerase I is a type Ib topoisomerase that relaxes supercoiled DNA substrate (pHOT1 plasmid) in the absence of ATP or MgCl2. This is a prototypical eukaryotic enzyme mechanism and the topo I will relax (change DNA linking number in steps of one) either positively or negative supercoiled plasmids. The assay is relatively simple and measures loss of supercoiled DNA or appearance of relaxed DNA products. There should be no nicked or cleaved DNA intermediates in the final products. The kit contains all the reagents for routine detection of type I topoisomerase (topo I). DNA markers are included to allow unequivocal detection of activity. The assay is highly specific for topo I (topo II requires ATP). Note that contamination by nucleases can interfere by causing DNA substrate nicking and an accumulation of nicked, open circular DNA; however, this is generally not a problem since there is no magnesium in the topo I reaction mixtures. Nucleases can still complicate matters; therefore, always monitor for degradation products (nicked or linear DNA) or low MW smears derived from the plasmid.

B. Kit Contents (100 assay kit size)

1. 100 Units Human Topoisomerase I (see attached data sheet for unit definition, quality control, and activity assay parameters). Store at -20° C.

2. pHOT1 DNA, supercoiled substrate. Concentration of 0.25 ug/ul (25 ug pHOT1 DNA in 100 ul TE buffer, 10mM Tris-HCl, pH7.5, 1 mM EDTA). DNA concentration may in some cases vary; therefore refer to the DNA Label on the tube.

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

4. 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.

5. 5x gel loading "stop" buffer (600 ul): 0.125% bromophenol blue, 25% glycerol, 5% Sarkosyl.

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** 1 ul (250-500 ng is ideal for most assays).
- ✓ **Test extract: 1 ul** (vary as needed)

1. Incubate 30 minutes at 37°C.

2. Stop by addition of 4 ul 5x Stop Buffer.

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

   Optional Step: the samples can be cleaned up by proteinase K digestion (50 ug/ml for 15 min at 37°C) followed by CIA extraction. For CIA extractions add an equal volume (20 ul) of Chloroform: isoamyl Alcohol or CIA (24:1), vortex briefly; spin in a microfuge for 5 sec. Withdraw blue colored (upper) 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.

4. 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 (we use 2-2.5V/cm). 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, below).

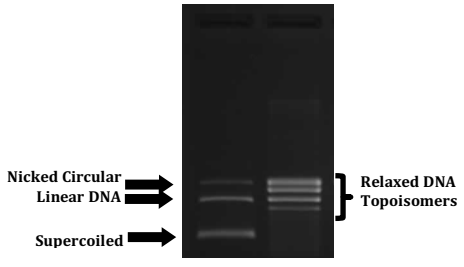

Fig. 1. Typical Topo I reaction products with marker DNAs. A 1% agarose was run until the dye front of bromophenol blue is about 70-80% down the gel. Left lane contains a mix of Nicked, Linear and supercoiled DNA Markers. Right Lane shows Topo I was incubated with supercoiled plasmid (200ng).

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 (with known topo I activity) and negative control (no extract) are also very critical for data interpretation. For details on how to make a nuclear salt extract from cells, follow our protocol on the website: http://www.topogen.com/p/methods-protocols.html. See "Methods for Extracting Topoisomerases for Enzyme Activity" PDF.

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 NOT CONTAIN 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.

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.

E. Data Interpretation and additional helpful hints.

Look carefully at the gels for evidence of plasmid DNA breakdown, indicating the presence of a nuclease activity (usually NOT a problem).

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?

- Marker DNAs (supercoiled, linear DNAs) (see Fig. 1) are extremely important.
- Include a negative control (no extract).
- Be sure to check solvent effects (if included) or effects of salt (used to extract topo from nuclei). More than 200mM NaCl from the crude extract will impact negatively on the results.

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.

Why should I run Non-EB gels?

In general, 1% gels in the absence of Ethidium Bromide (EB) are ideal for testing enzyme activity; however, the DNA nicking of plasmid substrate can sometimes be masked by topoisomers. 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 almost all cases, you should run gels without EB to garner maximum resolution of products.

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°C 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.

Is proteinase K required?

- Usually it is not necessary; however, if the nuclear extract is concentrated with protein, it may be a good idea to degrade these proteins to improve the cosmetics of your gel.

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.

Any further questions or comments, please feel free to contact us:
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