TopoGEN

Products and Services for **Cancer Research.**

In Vivo Link Kit
USER MANUAL

Overview:

TopoGEN has extensive experience with assays for topoisomerase inhibition in vivo. This analysis allows the investigator to ascertain whether a novel agent is active against endogenous topo in a chromosomal setting in nuclei. An important benefit to this analysis is that one can use any tumor cell or tissue in order to establish clinical efficacy against a specific tumor cell line. We use essentially the same basic approach for Topo I as for Topo II. The methods are based upon physically separating the topo/DNA adducts from free DNA and using antibodies to mea- sure bound topo I or II.

Shipping and Storage of Reagents

Store this kit at -20° C upon receipt. Keep the CsCl tightly capped at all times to prevent evaporation and changes in density.

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In Vivo Topoisomerase Cross Linking Kit

Detection of topo covalently bound to DNA in vivo. In this analysis, tissue culture cells are treated with a test compound along with negative controls (no drug) and positive controls (with known poisons or inhibitors). The cells are drug treated and rapidly lysed with SDS which traps some fraction of the endogenous topo on DNA in a covalent cleavage complex. Following detergent lysis, the lysate is diluted to fully dissociate non-covalent DNA/protein complexes. Formation of covalent topo/DNA complexes is measured on preformed step CsCl gradient (ionic DNA/protein interactions are dissociated by 5 M salt in CsCl). The gradient resolves DNA, chromatin aggregates, and protein, respectively. Gradients are centrifuged and fractions are taken. The amount of topo coincident with the DNA peak is a measure of covalent DNA/topo complexes. The amount of topo in the DNA peak is determined by immunoblotting using antibody to topo I or II as probe. In the absence of agents (etoposide, camptothecin) that stabilize the cleavable complex, very low levels of topo are found in the DNA peak; this is particularly obvious with topo II since the type II enzyme is not trapped by this method unless an inhibitor is used. In contrast, topo I is trapped to a small but detectable extent even in the absence of camptothecin.

References

Ebert et al., J. Virology 64: *4069-4066, 1990*
Muller and Mehta, Mol. Cell. Biol. 8: *3661-3669, 1988*
Trask and Muller, Proc. Natl. Acad. Sci. 85:1417-1421

Kit Contents *Sufficient material is included for 6 SW41 gradients or >12 SW50.1 gradients. Sufficient antibody is included for a large number of analyses. Once the technology is established, the investigator should prepare his or her own reagents (CsCl stock, sarkosyl etc.) and using TopoGEN's antibody supplied in the kit, a large number of experi- ments can be carried out. The following reagents are supplied in microfuge tubes:*

•**Camptothecin** (Supplied with Topo I Kits): Lyophilized; resuspend in 0.25m1 DMSO to give 10 mM Stock

•**Etoposide** (Supplied with Topo II Kits): Lyophilized; resuspend in 0.25ml DMSO to give 10 mM Stock

•**Sarkosyl (20%)** 1 ml

-Antibody to Topo I (Supplied with Topo I Kits): This is a rabbit polyclonal antibody. This AB is supplied at 2.5 units/ ul where one unit corresponds to a 1:1000 dilution required to make a working stock of antibody as a Western probe. For example 1 ul of the above stock solution (2.5 u/ul) will make 2.5 ml of diluted antibody solution for probing blots. *To replenish this reagent, order Cat# TG2012-2 (see www.topogen.com).*

-Antibody to Topo II (170 kDa form, supplied with Topo II Kits): This is a rabbit polyclonal antibody that was pre- pared against a peptide derived from the C-terminal of the 170 kDa sequence. Incuded are 250 units of antibody at 2.5 u/ul (see unit definition for antibody to topo I above). This antibody cross reacts with calf and rodent (mouse, rat, hamster) but not with yeast or *Xenopus laevis.* To replenish this antibody, order Cat TG2011-1 *(see www.topogen.com).*

-The Following reagent is packaged separately from the blister package in a 60 ml bottle:
CsCl Stock in TE (=50 mM Tris, pH 7.5, 1 mM EDTA) at 1.86 g/cc (45 ml). To prepare additional CsCl, dissolve 120 g of CsCl in 70 ml of 1X TE. The final density should be 1.86 g/cc.

*Store **all** reagents at -20° C. The CsCl Stock must be kept tightly capped at all times to prevent evaporation and possible density changes. After thawing, vortex the CsCl stock well to ensure complete mixing.*

Not Supplied with this kit:
•1 x TE (10 mM Tris-Cl, pH 7.5, 1 mM EDTA)
-Beckman SW41 Tubes and rotor or equivalent; SW50.1 tubes or equivalent.
•25 mM sodium phosphate buffer (pH 6.8 prepared with equal volumes of mono and dibasic sodium phosphate stocks at 25 mM each).
•TBS-T (20 mM Tris-Cl, pH 7.6, 137 mM NaCl, 0.1% Tween 20)

•5% Blotto (5 g of Carnation dried milk or equivalent per 100 ml of TBS-T). Make fresh each time.
•[125l)-protein A (Amersham) or for Topo II, ECL Detection Kit (Amersham)
•Nitrocellulose membrane
-Slot blot or dot blot device.

IN VIVO CROSSLINKING OF TOPOISOMERASES AND WESTERN BLOTTING

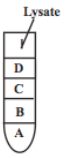
The following procedure was designed for tissue culture cells growing as monolayers (HeLa cells for example). The method can easily be scaled for suspension cultures or other tissue or cell systems.

A. Cell growth and lysis

1) Cells should healthy and in exponential phase of growth. Note that successful detection of the cleavable com- plexes in vivo depends upon: the presence of topoisomerase as an endogenous activity and the ability of the topo poison to access the topo active site. Cells (approximately 1 x 107 cells or a single 100 mm petri dish) are treated with 100 uM Camptothecin or 100 uM etoposide (diluted from a 10 mM stock) for topo I or II, respectively. Incubate the cells with the appropriate drug in 1 ml of serum free medium for 30 min at 37° C. A negative control (no drug or solvent alone) should be run in parallel.
2) Following drug treatment, the medium is completely removed (tilt the cultures to drain and use pasteur pipette to remove all traces of medium) and the cells are lysed by the rapid addition of lysis buffer (1% sarkosyl in lx TE buffer). Make appropriate dilutions of the 20% sarkosyl and 10X TE stocks to make the lysis buffer. For the SW41 gradients, use 3 ml of lysis buffer and use 1 ml for the SW50.1 tubes.

B. CsCI step gradient centrifugation and fractionation

1) The gradients are formed by successive overlay of 4 different CsCI solutions prepared from the 1.86 g/cc stock according to the following table (for six SW41 gradients).



Solution:	ml of Stock CsCl	ml of 1xTE	Final Density (g/cc)
A	13.013	0.98	1.82
B	11.452	2.548	1.72
C	7.756	6.244	1.50
D	6.02	7.98	1.37

Step gradients are prepared by successive overlaying of 2 ml (SW41) or 1 ml (SW50.1) aliquots of solutions A through D to clean polyallomer tubes. Use a pipette to prepare each step by slow and careful layering of each solution (start with A at the bottom of the tube, see illustration above).

2) Carefully overlay the lysate over step D in the gradient. Centrifuge the gradients at 31,000 RPM for 12- 18 hrs. at 25° C (CAUTION: do not run at 4° C or CsCl may precipitate and lead to rotor failure!!).

3) Fractionate the gradients using any one of several methods. Ideally, a tube perforator should he used; however, you may simply collect fractions from the top using a pipettman p200 or p1000. It is a good idea to enlarge the pipette tip (slice off 2-3 mm to create a larger orifice) since the DNA containing fractions may be viscous. Collect about 30 fractions. If you are using the SW41 configuration, collect 0.4 ml fractions. For the SW50.1 collect 0.2 ml per fraction.

4) Locate the DNA peak by reading the absorbance at 260 of each fraction. Read an aliquot of the fraction (50-100 ul) diluted to give a reading in the 0.1 to 1 OD range. As a start, dilute 50-100 ul of each fraction into 0.5 ml of water. The DNA peak will be near the bottom of the gradient typically distributed over about 3 fractions (see sample data that follow). The UV absorbing material near the top of the gradient represents cell debris, proteins and insoluble complexes. *Because the top part of the gradient contains largely debris, any signal picked up by Western Slot blots is not due to Topo (I or II). This admixture of membranes, unlysed cells, etc. gives high backgrounds on slot blots that is unrelated to topo.*

NOTE: Cleavage complexes will be located with the main DNA peak; thus, topo I or II cross linked to DNA will be found in the DNA fractions near the bottom of the gradient. Free topo will be located at the top of the gradient; however, because these fractions are very crude, high backgrounds will be detected that do *not* reflect true topo levels.

C. Immunodetection of Topo I and II Complexes.

1) Detection of free and bound topo I or II is carried out by Western blotting of fractions on either a slot blot or dot blot device.
2) Remove 50 ul of each fraction and mix with 100 ul of 25 mM sodium phosphate buffer (pH 6.5). Leave diluted fractions at ambient temperature on bench top.
3) Soak the nitrocellulose filter (cut to the appropriate size) in 28 mM sodium phosphate buffer (pH 6.5) for at least 30 min at room temperature.
4) Assemble the blotting device and apply the diluted CsCl fractions (vol. 150 ul) blot using a vacuum.
5) Remove the membrane (handle with gloves) and rinse briefly in 30 ml of 25 mM phosphate buffer.
6) Transfer membrane (wear gloves) to 30 ml of TBS-T for 10 min (gentle rotation is ideal).
7) Incubate membrane with 50 ml of 5% blotto (dry milk) in TBS-T for 2 hours.
8) Wash 3 x 10 min each with TBS-T (30 ml per wash).
9) Dilute the antibody 1:2500 in TBS-T to give sufficient solution to just cover the blot completely (generally about 30-40 ml for a Schleicher and Schuell “Slot Blot” sized filter).
10} Incubate at room temperature for 3 hours with gentle agitation (or 4°C overnight).
11) Wash the membrane 3 x 10 min each with TBS-T.
12) Incubate blot with 4 uCi [125I]- protein A per 10 ml of TBS-T for 2 hours. Make sure you cover the blot fully with the isotope solution. This may require more than 10 ml, depending upon the size of the blot.
13) Remove isotope and discard appropriately (USE CAUTION WITH ISOTOPE .... wear gloves).
14) Wash blot 10 min with 20 ml of TBS-T. Repeat wash a total of 3 times for 10 min each.
15) Drain the membrane completely and encase with saran wrap or equivalent and expose for autoradiography (8- 24 hours).

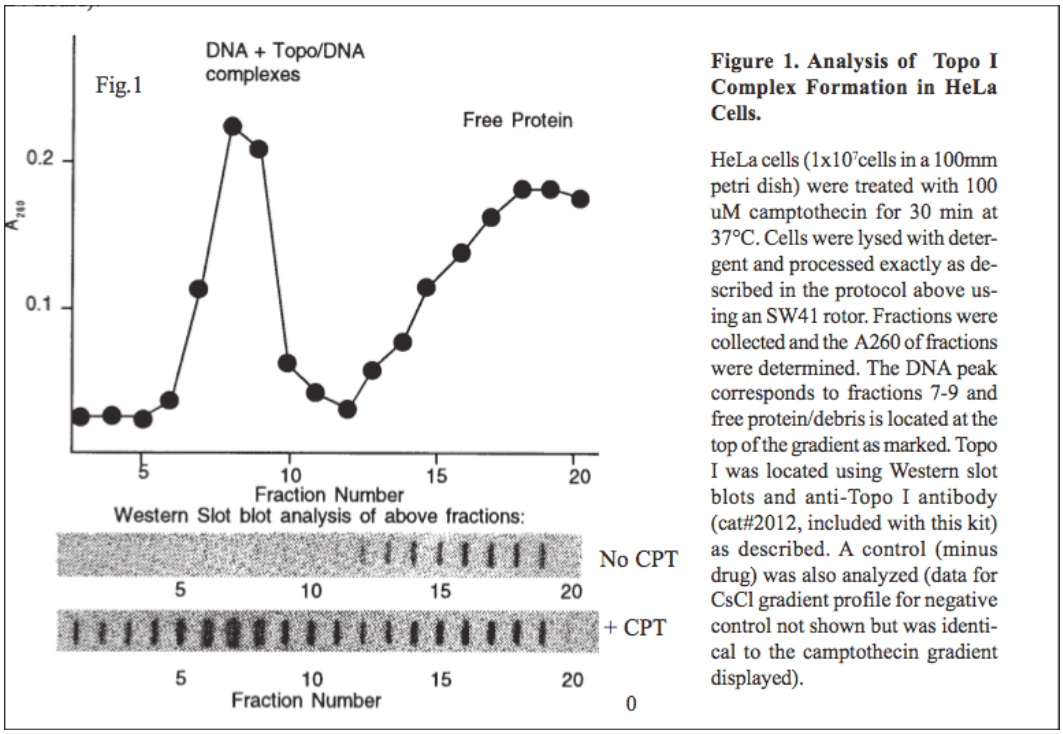


Figure 1. Analysis of Topo I Complex Formation in HeLa Cells.

HeLa cells ($1x10^7$cells in a 100mm petri dish) were treated with 100 uM camptothecin for 30 min at 37°C. Cells were lysed with detergent and processed exactly as described in the protocol above using an SW41 rotor. Fractions were collected and the A260 of fractions were determined. The DNA peak corresponds to fractions 7-9 and free protein/debris is located at the top of the gradient as marked. Topo I was located using Western slot blots and anti-Topo I antibody (cat#2012, included with this kit) as described. A control (minus drug) was also analyzed (data for CsCl gradient profile for negative control not shown but was identical to the camptothecin gradient displayed).

D. Critical Parameters and Considerations

The procedure is straight forward. Two controls are critical however. First, you should run a minus drug (or solvent only) negative control (Fig. 1 upper blot). In the absence of poisons, there should be no topo in the DNA peak fraction; all of the endogenous enzyme is released as free topo. Second, a positive control (100 uM VP16 or camptothecin) is necessary to ensure that in your particular cells (or system), cross linking of the endogenous enzyme is detectable. Finally, you should be able to detect a strong signal at the top of the gradient with free protein (see Fig. 1); however, as noted above, the signal here is largely if not totally non-specific since these fractions are very dirty. Failure to see any signal in the top part of the gradient is clearly indicative of a detection problem of some sort.

TopoGEN has designed this kit to facilitate replenishment of reagents. The kit comes with suffi- cient antibody for a large number of experiments, but only sufficient amounts of the more common re- agents (CsCl, detergent, etc.) to allow technology transfer and successful

reconstruction of the method. In this way, each lab can establish the method and adapt it to their particular cell system using reagents that have been quality controlled. Once established, the host lab can then make its own reagents for future applications. Follow carefully the recipes given for the various reagents, in particular, the CsCl must be made exactly as specified above.

E. Modifications

Immunodetection with topo II antibody can be adapted to Amersham's ECL detection system (a rabbit polyclonal antibody is used in TopoGEN's kit). (NOTE: The ECL system *will not* work with the human topo I antibody.) The advantage is that the ECL system is very fast and does not require use of isotope. Here is the method we use with Amersham's ECL System:

1. From Step 10 (previous page): Incubate with primary antibody to Topo II for 1 hr. at room tempera- ture.
2. Wash 3 x 10 min with TBS-T
3. Incubate with 1:1000 dilution of goat anti-rabbit conjugated to horseradish peroxidase in TBST for 30 min.
4. Wash 4 x 10 min with TBS-T 5. Detect using ECL reagents A and B from the Amersham Kit. Vary exposures of blot.

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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