TopoGEN

Products and Services for **Cancer Research.**

ICE ASSAY KIT
CATALOG TG1020-

See QC Data Sticker for Specific Antibody
Sufficient for 24 Assays

Overview:

TopoGEN has extensive experience with cell-based screens for topoisomerase targeting. Our staff scientists developed and published a method for detecting covalent topo/genomic DNA complexes in live cells (1,2). The method, called the ICE Assay (**I**n vivo **C**omplex of **E**nzyme) originally required a CsCl gradient to purify genomic DNA and specific topo antibodies to detect trapped topo protein in the DNA fraction. This method, while powerful, is slow, labor intense and requires large expensive equipment. **We have modified the technique to allow direct isolation of DNA/topo complexes in a single step, omitting the CsCl gradient step and rapidly increasing the throughput of the method.** The key to specificity is the use of TopoGEN's highly directed antibody reagents to detect complexes. The ICE Assay can be carried out in about 1-2 days. The analysis can be conducted on virtually any cell or tissue system as long as proper controls are performed and mono-specific antibody reagents are used. Finally, the assay is no longer limited to 6 samples per run (for the ultracentrifuge rotor), so larger throughput is possible. Our new ICE method is rapid and convenient and can be used with any topoisomerase target (I, II, III). Each kit is designed for a specific target (see labeling above). In addition, we offer a "base kit" that does not contain antibody probe. TopoGEN also offers convenient internal controls to efficiently assess and validate the results (sold separately as TG1020-1REF for Top1 or TG1020-2aREF for Top2a).

1. Subramanian, D., Furbee, C. and Muller, M. (2000) ICT Bioassay: Isolation of In Vivo Complexes of Enzyme to DNA. DNA Topoisomerase Protocols Vol. II: Enzymology and Drugs. Humana Press.
2. Subramainian, D., Kraut, E., Staubus, A., Young, D., and Muller, M.T. (1995) Analysis of Topoisomerase I DNA Complexes in Patients Administered Topotecan. Cancer Research 55:2097-2103.

Shipping and Storage of Reagents

This kit may be shipped ambient, wet ice packs or dry ice. Upon receipt you should store this kit at -20° C for long term or keep at 4°C for short term.

Introduction.

In this analysis, adherent tissue culture cells or suspension 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 (typically 30' or longer) and rapidly lysed with Buffer A to arrest or trap the endogenous topo on DNA in a covalent cleavage complex. Following Buffer A, the lysate is processed to release and spool genomic DNA, which is then sonicated and probed on a slot or dot blot device using a specific antibody probe (top1, top2a, top2b or others as specified in the kit). Including a 30'-60' drug treatment *in vivo*, the ICE Assay can be completed in one day from start to finish using a 2h primary AB incubation time. If the primary AB step goes overnight, the assay can be completed the next morning. The key is to include a no-drug negative and a plus-drug positive control to demonstrate that the ICE Assay is working as predicted. In drug treated cells, the signal should be significantly stronger compared to the negative control. An important parameter is the ICE-Delta (ICEΔ) which is the ratio of plus control Drug signal to minus Drug signal control. The ICEΔ ratio should be 2-6 fold or more for a valid result. In other words if the ICEΔ ratio was 1.0, it would indicate that covalent complexes are NOT being detected by the assay.

Product Application and Disclaimer. This product is not licensed or approved for administration to humans or animals. It may be used with experimental animals only. The product is for *in vitro* research diagnostic studies only. The product in non-infectious and non-hazardous to human health. This information is based on present knowledge and does not constitute a guarantee for any specific product features and shall not establish a legally valid contractual relationship. TopoGEN, Inc. shall not be held liable for product failure due to mishandling and incorrect storage by end user. TopoGEN's liability is limited to credit or product replacement.

Kit Contents are as follows: *(Note that Sufficient material is included for 24 assays using a 60mm petri plate format). Specific antibodies define the target enzyme for each kit as specific by product labeling.*

- **Camptothecin** (Supplied with Topo I Kits): Lyophilized; re-suspend in 0.25mL DMSO to give 10 mM Stock
- **Etoposide** (Supplied with Topo IIα and IIβ Kits): Lyophilized; re-suspend in 0.25ml DMSO to give 10 mM Stock
- **Buffer A: Lysis buffer: 9 mL in a 15 mL conical centrifuge tube.**
- **Buffer B: 5mL Total, 2.5mL/tube in two 5mL microfuge tubes**
- **Buffer C: 20mL in a 50mL conical tube.**
- **Buffer D: 7mL Total, 3.5mL/tube in two 5mL microfuge tubes**
- **Buffer E: 1mL in 1.5mL microfuge tube**
- **Antibody to human Top1 (for Cat TG1020-1). A total of 100 units included in kit.** This is a mouse monoclonal antibody to human Top1. This AB is supplied at 2 u/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 will make 2 ml of diluted antibody solution for probing blots.
- **Antibody to Topo IIα (Cat TG1020-2a, 170 kDa form, supplied with Topo IIα Kits). A total of 100ul included in kit.** This is a rabbit polyclonal antibody against a peptide derived from the C-terminal of the 170 kDa sequence. Included are 250 units of antibody at 2.5 u/ul (see unit definition for antibody to topo I above).
- **Antibody to Topo IIβ (Cat TG1020-2b, 180 kDa form, supplied with Topo IIβ Kits). A total of 100u included in kit.** This is a rabbit polyclonal antibody labeled at 2.5 u/ul (see unit definition for antibody to topo I above).
- ***Antibody to Topo IIIα (Cat TG1020-3a). A total of 50ul included in kit.** This is a mouse monoclonal antibody AB. We also include CPT and a mouse monoclonal Top1 AB, as internal controls (A total of 20ul of Top1 MCAB is Included in kit.)
- ***Antibody to Topo IIIβ (Cat TG1020-3b). A total of 100ul included in kit.** This is a rabbit polyclonal antibody. Included are 250 units of antibody at 2.5 u/ul (see unit definition for antibody to topo I above). We also include CPT and a mouse monoclonal Top1 AB, as an internal control (a total of 20ul of Top1 MCAB is Included in kit).

We include Top1 as a control since there are no validated Top3α or β poisons. By including a Top1 AB and Camptothecin, you can be assured that the ICE assay is working as expected.

Not Supplied with this kit but required.

- PBS (137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 2mM KH_2PO_4 (adjust pH to 7.4 with HCl)
- 25mM Phosphate Buffer: equal volumes of 25mM mono and dibasic phosphate buffer; add monobasic as need to pH 6.5.
- 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 before use.
- Nitrocellulose membrane (HyBond-C)
- Whatman 3M Filter Paper (sheets)
- Slot blot or dot blot device (optimized for Slot Blot device)
- Secondary antisera and chemiluminescent detection reagents.
- Instrumentation for immune-detection of signals on a Western blot
- Sonicator

Protocol

The following procedure was designed for tissue culture cells growing as monolayers (HeLa cells for example). The method can easily be scaled and modified for suspension cultures or other tissue or cell systems; however, the customer should reconstruct his or her own cell system for optimal performance.

A. Cell growth and Lysis

1. Cells should be healthy and in exponential phase of growth. Note that successful detection of the covalent complexes (CC) may be different in cycling vs resting cells. For adherent cells (HeLa for example) use 60mm petri dishes either 80-90% confluent or freshly confluent. Monolayers should be healthy and not 'over-grown'. Treat cells with positive control drugs for a short durations (30-60 min) to minimize toxicity and maximize CC formation. We recommend 50uM camptothecin or 50uM etoposide. Always include a negative or (or solvent control) as this is a critical control.
   - While cells are incubating:
     - *pre-warm the PBS to 37°C.*
     - *Pre-soak 2 sheets of Whatman 3mm filter paper and nitrocellulose membrane in 25mM Phosphate Buffer for at least 15' ambient temperature.*

2. Following drug treatment, the medium is completely removed (tilt the cultures to drain and use a micro-pipette to remove all traces of medium). Rinse the plates briefly 1-2x with pre-warmed PBS buffer (1-2mL per rinse) being sure to remove all traces of the PBS wash. The cells are lysed with 330ul of room temperature **Buffer A.**
   - Technical note: Pipette Buffer A in drops around the entire plate and gently swirl the plate to effect complete lysis. (Optional step: you may wish to use a rubber scraper to ensure full recovery of lysate). Use a p1000 or equivalent to transfer lysate to a sterile 1.5mL microfuge tube (Lysate Tube). If too viscous, enlarge the plastic pipette tip to facilitate transfer. It is acceptable to lightly shear the high MW genomic DNA at this step, but do not overdo it since sheared DNA is harder to spool.

B. DNA Release and Recovery

1. Add 115ul of Buffer B (which is ethanol) to Lysate Tube and gently mix by inversion 4-6 times until DNA forms a loose precipitate and leave at room temperature for about one minute.
2. Using a sterile p200 tip and p200 pipette or equivalent, insert the tip into the tube and into the clumped DNA precipitate. Gently 'spool' the DNA onto the tip by clockwise rotation. The DNA precipitate will be visible as it 'wraps' or spools around the tip. Next, carefully withdraw the tip+DNA from remaining lysate solution and slide across the side of the same microfuge tube (away from the residual lysate). Gently dislodge the DNA from the tip to place it onto the side of same microfuge tube. The remaining lysate should be removed and discarded.
3. Add 200ul of Buffer C (75% ethanol) at room temperature and mix by tapping on the side of the tube several times; let sit for one minute at room temperature. Spin in a microfuge (ca. 10-14,000 rpm) for 2 minutes at room temperature. Gently aspirate away the Buffer C.
4. Repeat the Buffer C wash in the same way two more times.
5. Decant or draw off the final wash (Buffer C supernatant) and let the pellet dry (tube upright) for one minute at room temperature.
6. Add 100uL of Buffer D. Gently pipette up and down several times and let stand at 37°C (room temperature also works) for at least 5 minutes. This step will help dissolve the DNA in aqueous.
7. Add 16ul of Buffer E and gently mix.
8. Typically, from HeLa cells, recovery of DNA should be approximately 50-70ug total (results will vary depending on cells or tissue used).
9. High MW Genomic DNA may be viscous and hard to pipette.
10. Sonicate for 5-10 seconds to reduce viscosity (low setting on sonicator will suffice).
11. Quantify the DNA concentration by Nano-Drop or UV (absorbance at 260nm). One A_{260} corresponds to 50ug/mL DNA. It is important to know precisely how much DNA is recovered at this step (in terms of ug/mL DNA). The ICE assay requires equivalent DNA loadings to compare different cultures (since DNA content per cell is identical, this normalizes the Top/DNA signals on a per cell basis).

C. Immunodetection of Topo Covalent Complexes: *Optimized for Slot blot device (optionally you can also use Dot Blot Device).*

1. Dilute DNA to desired concentrations and load precisely equivalent micrograms of DNA per slot. We recommend using a minimum of three concentrations (0.5, 5 and 10ug DNA per slot). For example, if you dilute your DNA to 2.5ng/ul, 25ng/ul and 50ng/ul, spotting 200ul/slot will put a final DNA concentration of 0.5, 5, and 10ug in 3 different wells, respectively. A typical layout for testing both positive/negative control and an unknown is shown in Fig. 1. In this example, Top2a targeting is being evaluated. Each horizontal row (Fig.1) contains different genomic DNA inputs as illustrated. Genomic DNA is prepared from 3 different cell treatments: (i) *Negative or no drug vehicle control (matched solvent); (ii) Positive control drug (VP16 for a Top2 test for poisoning); (iii) The unknown (or experimental).*

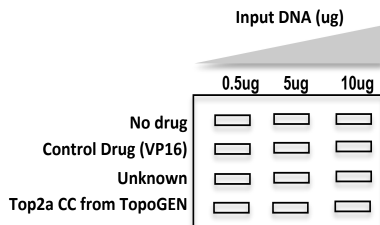

Figure 1. Optimal arrangement for testing (a Top2a test case). Purified DNA at 3 concentrations are placed on slot blot as shown. The cell treatment controls (no drug, control VP16 for Top2a in this example) are in the first two horizontal rows. An unknown test article is placed in the row three. Row four shows signals from pre-tested Top2a/genomic DNA CC (sold separately, see TopoGEN.com Cat TG1020-2aREF). This reference marker is an internal validation control to demonstrate that the ICE Blot is capable of detecting Top2a/DNA complexes.

2. Soak the nitrocellulose filter (cut to the appropriate size) in the 25 mM sodium phosphate buffer for at least 30 min at room temperature.
3. Assemble slot blot apparatus according to manufacturer's directions and load samples into slots. Attach to a very low vacuum until all samples are completely aspirated through membrane. You may also use gravity but it may take additional time; thus, a light vacuum will expedite sample filtration; however, do not apply samples with vacuum pump on (ie, don't apply DNA under vacuum). Add samples to slots and then apply light vacuum. If you apply vacuum without the sample in the slot, the membrane may dry out which can lead to a higher background.
[***IMPORTANT: never allow the membrane dry out under vacuum or else you may see high backgrounds. Keep membrane slots moist at all times especially just prior to adding DNA samples.]***
4. Remove the membrane (handle with gloves) and place in a container large enough to fit and wash for 15' in TBS-T at room temperature. Add sufficient volume to just cover nitrocellulose membrane.
5. Block membrane with a suitable volume (50 ml for 8 x 24 cm membrane) of 5% blotto (dry milk) in TBS-T for 30 minutes.
6. Wash 1x 10 min with TBS-T (suitable volume for coverage).
7. Dilute the primary antibody of choice appropriately (typically 1:1000 in TBS-T/5% dry milk) to give sufficient volume of solution to just cover the blot completely). For example, 30-40 ml will be required for a Schleicher and Schuell "Slot Blot" sized filter (membrane size is 8x24cm).
8. Incubate with the diluted primary antibody at room temperature for 2-4 hours with gentle agitation (or 4°C overnight).
9. Wash the membrane 3 x 10 min each with TBS-T.
10. Incubate with appropriate secondary antibody (mouse, rabbit or human) in TBST in 5% Blotto solution for 1 hr at room temperature.
12. Wash the membrane 3 x 10 min each with TBS-T at room temp.
13. Develop blot with appropriate detection reagents (for sample data, see Fig. 2).

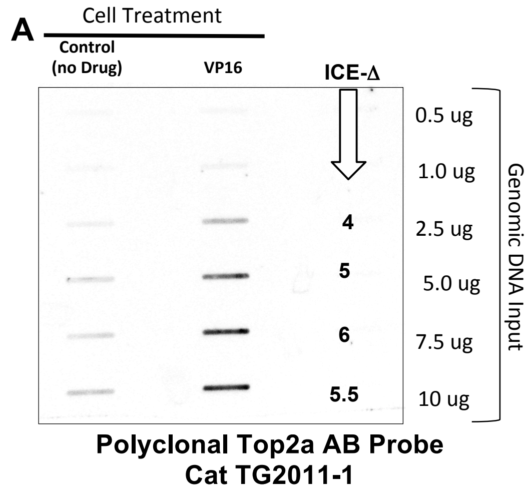

Figure 2A. ICE Blot testing for Top2a. HeLa cells were treated with 50uM VP-16 for 30' and lysed in Buffer A. DNA from negative controls (no drug) and from drug treated cells was isolated and quantified on a NanoDrop. Indicated amounts of DNA were probed with TopoGEN polyclonal Top2a AB. The signals in the VP16 slots should be significantly higher than controls a shown. The signal ratio between the +DRUG:No drug control is called the "ICE DELTA" (or **ICEΔ**). The **ICEΔ** should be 2-6 fold or more for a valid result.

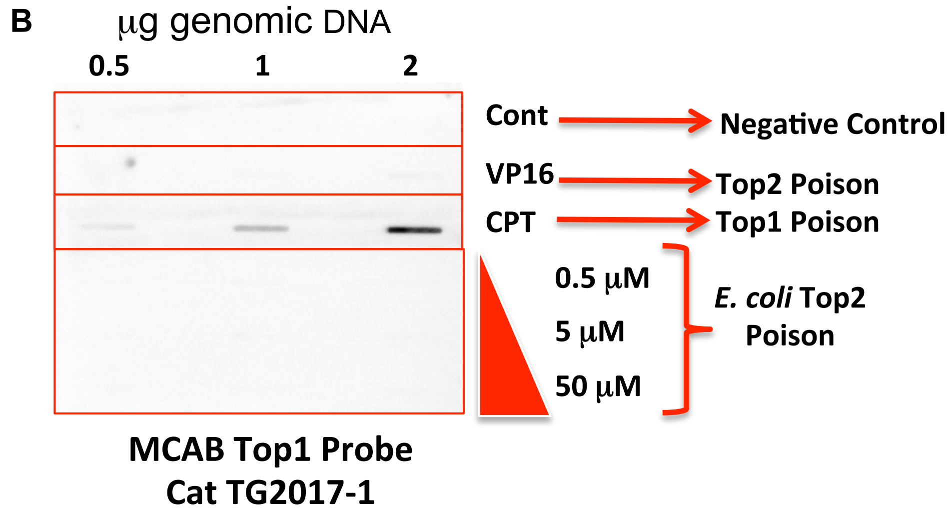

Figure 2B. ICE Blot testing for Top1. HeLa cells were untreated (control) or treated with 50uM VP-16 or 50uM Camptothecin for 30' while in exponential growth and lysed in Buffer A. DNA from negative controls (no drug) and from drug treated cells was isolated and quantified on a NanoDrop. Indicated amounts of DNA were probed with TopoGEN polyclonal Top1 Monoclonal (Cat TG2017-1). To show specificity HeLa cells were treated with an E. coli Top2 poison at three different concentrations shown (30 min at 37°C and lysed with Buffer A). Indicated amounts of DNA (0.5 to 2ug) were slot blotted.

D. Time Required

The ICE assay can be completed in 1-2 days (Fig. 3) depending on whether you perform the primary antibody step overnight (at 4°C). We designed the assay for completion in a single day (9am-5pm); however, breaking the method up into 2 partial days is easier, especially if many samples are being processed concurrently. This also gives a longer primary antibody incubation (may improve sensitivity and detection).

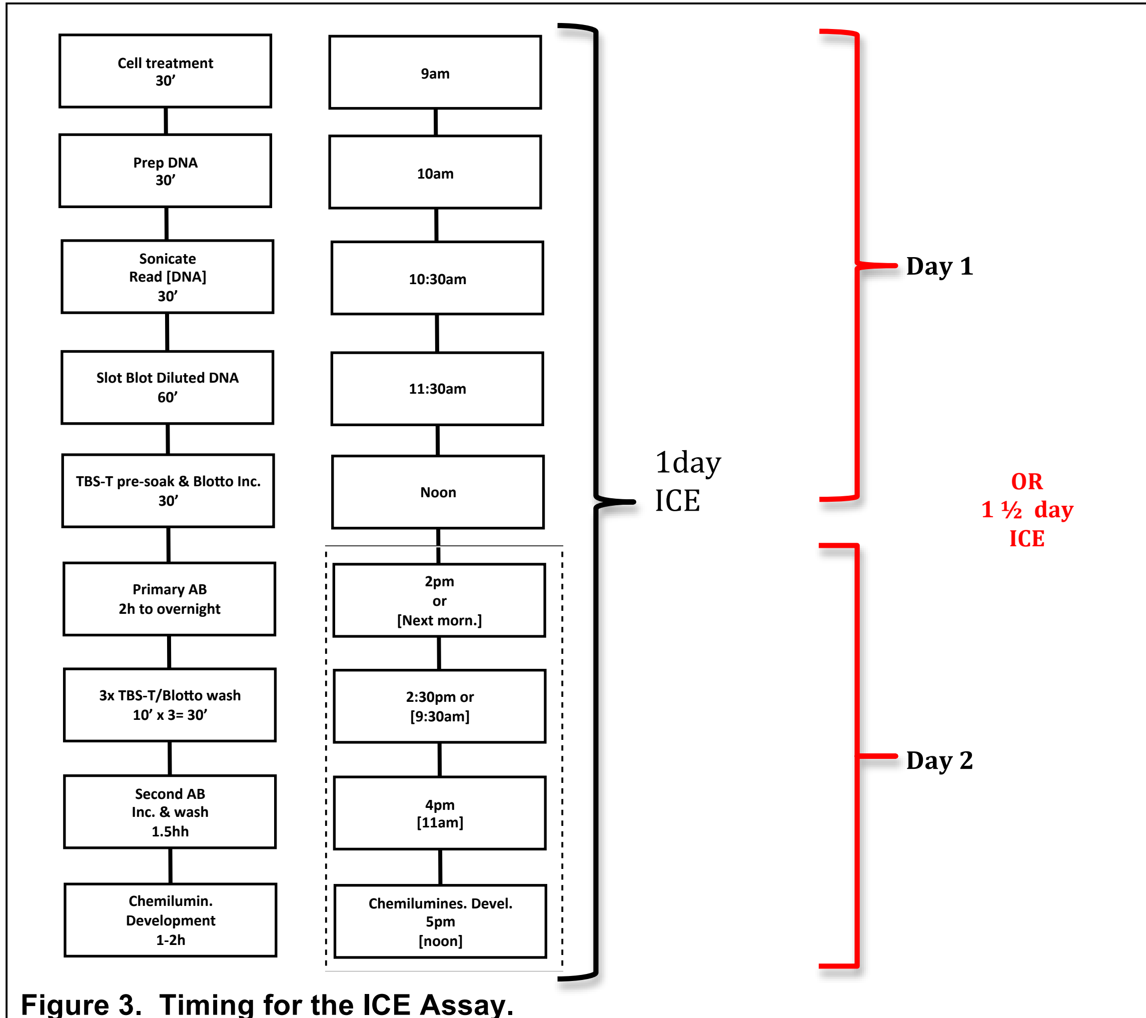

Figure 3. Timing for the ICE Assay.
The ICE assay can be completed in a single day; however, it is generally easier using 1½ days from start to finish to allow the primary AB step to go overnight (dotted lines on right flow chart). The left flow chart shows each step of the ICE method and the time required to complete (assumes that 4-6 DNA samples are being processed in parallel). The right flow chart showing actual time to complete assuming a 9am start time (including drug treatment interval).

FAQ

What are the most Critical Parameters?
Two controls are pivotal. First, you should run a minus drug (or solvent only) negative control (see Fig. 2). In the absence of poisons, there should be low levels of topo in the DNA peak fraction; all of the endogenous enzyme is released as free topo. Second, a positive control (Interfacial poison for example, like VP16 or camptothecin, matched with an appropriate antibody) is necessary to ensure that in your particular cells (or system), cross linking of the endogenous enzyme is detectable.

What is this parameter: The ICE-Δ and why is it important?
As noted, a negative control (no drug) will allow you to demonstrate background noise on the Western Slot. There may be some signal even in the absence of a poison. This is due to non-specific stickiness to residual DNA bound to the membrane. There should be a clear elevation in signals when matching the targeting control drug and specific AB probe. This is referred to as the ICE Delta or **ICE-Δ.** For example, VP16 and Top2a antibody should display an **ICE-Δ** (ratio of +VP16:no drug) of at least 2-5 fold, typically (refer to Fig. 2). The **ICE-Δ** with Camptothecin and Top1 antibody should be of similar magnitude. Furthermore, the **ICE-Δ** with VP16 and Top1 antibody should be near 1.0 or less. AB dilution is critical in these experiments and not everyone will be testing the HeLa cell system (since VP16 does not trap Top1, you should see a background AB signal in both plus drug, VP16 and no drug). If the **ICE-Δ** is low (<2), you may need to titrate the AB probe to optimize (ie, increase or decrease the AB probe dilution). Alternatively, if you are testing a very weak poison, you may need to prolong the drug exposure in cells, or test higher inputs of drug.

Why do you need to place increasing amounts of DNA on the Slot or Dot blot?
When using slot or dot blots, the signals can easily saturate if the signal strength is large. For this reason, one should test at least three different DNA inputs to identify which DNA concentration gives the optimal **ICE-Δ**. Note that a valid assay result is critically dependent on the ICE-Δ which can only be derived by running the positive and negative controls.

Can the ICE Assay be used ONLY for identifying poisons or can it be used to track catalytic inhibitors?
There are two sorts of anti-topo drug mechanisms: Interfacial Poisons (or IFP) which trap covalent complexes, and Catalytic Inhibitory Compounds or CIC, which simply block topo action. To test for CIC, you can use an IFP type of Drug (VP16 for Topo II, for example). In this situation, you could first pre-treat with a putative CIC (test short to long interval exposures plus and minus drug) and then compare how efficiently the IFP pulse (30'-60') is inhibited. The idea is that a bona fide CIC should inhibit the trapping ability of an IFP. Details would have to be worked out with stringent controls, however, this approach can and will work for most CICs.

For the Topo III kits, why did you include a topo I drug and topo I AB?
This is because there are (at this time) no known topo III IFPs. Thus, in order to validate the analysis, it is important to test another IFP and enzyme in the same system to be sure that the ICE assay is yielding the expected results.

What is the Topo reference DNA that you sell and why is it important?
We provide pre-tested Topo/GENOMIC DNA complexes to include in your ICE Blots as internal controls to show that you know for certain that your blotting technique is validated. We actually prepare and pre-screen topo/DNA complexes with matched drug+AB (ie, Top2a with VP16). We can also provide known negative genomic DNA. When working out the methodology for the first time, these control complexes are valuable (but still optional). The ref DNA complexes are only available for topo I and IIa complexes. Order: TG1020-1REF for Top1 and TG1020-2a for Top2a control complexes.

Can we use these complexes for ChIP-Seq Analsysis?
Yes, you can but keep in mind: you should NOT be doing a formaldehyde cross-linking step. This is because these drugs are all interfacial poisons; ie, they will trap Top/DNA covalent adducts, so there is no need for other crosslinking agents. There are important controls for this sort of analysis as well. Negative (no drug), Camptothecin and Etoposide. This will give you confidence for specificity (data for Top1 should be different than Top2 for example.

What can cause high backgrounds in the no drug control?
There can be many reasons but chiefly it is due to the DNA prep. Make sure you carefully prepare the genomic DNA to ensure that there are no contaminating RNA or protein.