TopoGEN
Products and Services for Cancer Research.

Topoisomerase Type IIα,β Nuclear Extraction/Assay Kit

Application Manual
TopII-NucEx Kit™

Overview:
This Kit is designed to allow a researcher to prepare a nuclear extract enriched for type II topoisomerase (topo II) activity and topo II polypeptide. The appropriate assay buffer specific for topo II is included. This kit is suitable for most animal cell lines growing in vitro but is optimized for human and rodent species. This Kit also allows the investigator to quantify topo II activity in any given cell line. This Kit also provides the end user a source of enzyme for preliminary drug screening or HTS.

Shipping and Storage of Reagents
The kit may be shipped at ambient temperature or on ice (dry ice or wet ice) and should be stored at -20°C upon receipt. Avoid frequent freeze/thaw cycles which may cause DNA nicking. The kit may be store for up to 1 month at 4°C.

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A. Summary

Human Topoisomerase II (Topo II) exists as two isoforms: p170 (alpha) and p180 (beta). Both isoforms act as type II topoisomerases and will relax superhelical DNA and decatenate kineotoplast DNA (kDNA) from the insect trypanosome, *Crithidia fasciculata*. The type II enzymes require $MgCL_2$/ATP and will relax DNA in discrete linking number 'steps' of two. This is a prototypical eukaryotic enzyme mechanism that will relax either positively or negative supercoiled plasmids. Topo IIα is important as a decatenase activity *in vivo*, it has an acute preference for binding to DNA 'nodes' or duplex/duplex crossovers, where it will promote one duplex passage through another intact duplex. In this way, these

enzymes are highly proficient at decatenating interlocked DNA rings. This Kit will allow the researcher to prepare a crude nuclear extract enriched in topo IIa for quantifying enzyme activity in a given cell line. In addition, the crude extract is suitable for topo II drug screening experiments or HTS (although we recommend validation with the purified topo II available from TopoGEN). The kDNA assay measures the release of minicircular DNAs by decatenation of high molecular weight kDNA. Topo II is especially good at performing this reaction *in vitro*. The enzyme binds robustly to kDNA networks and releases intact 2.5 KB monomeric rings. Since kDNA networks are extremely large, they fail to enter a 1% agarose gel. In contrast, the minicircular 2.5 KB rings rapidly migrate into the gel. The released (decatenated) products are somewhat heterogeneous but are predominantly in the form of nicked open circular minicircles and fully closed circular rings. Both are considered decatenation products. Topo II also does not induce formation of linear DNA products under the conditions of this assay; therefore, linear kDNA should not be detected in the gels. This Kit contains reagents necessary to quantify topo II activity in crude cell extracts. Markers are included to allow unambiguous detection of topo II even in the presence of contaminating topoisomerase I. The assay is kDNA based and is highly specific for topo II. Nuclease activity may however cause some degradation of the kDNA substrate. Such degradation will be ATP independent. In addition, nucleases will generate a smear of degradation products in addition to linear kDNA.

In summary, this kit contains everything needed to assay topo II in a crude extract (including kDNA, buffers and markers) and everything needed to make the crude nuclear extract from adherent cell lines (HeLa). The extraction method can be adopted to tissues or other cell lines with some optimization. The kit is suitable for drug screening experiments as noted. We recommend that drug controls (+/- Etoposide) be included for all drug screening experiments. Topo II drugs are available on our website.

B. Kit Contents (100 assay kit size)

1. 10x TEMP Buffer. (1X TEMP Buffer is: 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 4 mM $MgCl_2$, 0.5 mM PMSF). The kit includes 10 mL (in a 15 mL conical tube). Dilute to 1x before use (add tube contents to 90 mL of Sterile milli-Q grade H_2O). Store at 4^oC for up to 1 month or -20^oC for longer term storage.

2. 10x TEP Buffer. (1x TEP is 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.5 mM PMSF). A microfuge tube with 1 mL is provided; dilute to 1x before use (add tube contents to 9 mL of Sterile milli-Q grade H_2O). Store at 4°C for up to 1 month or -20°C for longer term storage.

3. 1 M NaCl for extraction (0.5 mL in microfuge tube).

4. kDNA (20 ug total) substrate at the concentration specified on the tube provided. Typically one should use 0.1 ug (100 ng) per reaction.

5. Decatenated kDNA marker (25 ul) in gel loading buffer. Run 2 ul of decatenated DNA marker per gel.

6. Linearized kDNA marker (25 ul) in gel loading buffer; run 2 ul of linear marker per gel.

7. 10x gel loading buffer (300 ul): 0.25% bromophenol blue, 50% glycerol, use 0.1 volume in reactions.

8. 10x Proteinase K (500 ul) at 0.5 mg/ml (10x stock of proteinase K should be diluted to a final 1x to degrade proteins after reaction termination; i.e. use at 50 ug/mL final).

9. 10% SDS (300 ul). To be added to reaction mix to give a final concentration of 1% to terminate the relaxation reactions.

*10. 10x Topo II Incomplete Assay Buffer A: 0.5 M Tris-HCl (pH 8), 1.50 M NaCl, 100 mM $MgCl_2$, 5 mM Dithiothreitol, 300 ug BSA/ml.

*11. 10x ATP Buffer B: 20mM ATP.

*Mix Buffers A and B (1:1 ratio) together make a FINAL 5x Complete Assay Buffer. Add equal volumes Buffer A and B (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 should be made fresh for each experiment. Prepare only the amount as needed each day. THE 5x COMPLETE ASSAY BUFFER IS NOT STABLE.

C. Materials needed. The following items are required but are not included in the kit.

- Tight fitting dounce (B pestle) homogenizer.
- Rubber scrapper for physical release of cells off plate (only for adherent monolayers).
- Agarose gel unit (mini or maxi gel) with comb/tray. Use a comb that casts long narrow wells like this ▭ as opposed to a more 'squared' well ▭. This will improve gel resolution to display topoisomers and provide more cosmetic results.
- Materials and solutions for agarose gel analysis (agarose, ethidium bromide, running buffer, UV imaging source, camera, power supply)
- Microwave
- Centrifuges (ultra and microfuge), tubes and rotors
- Ice and ice bucket
- Micropipettors (p20, p200, p1000) and sterile tips
- Tissue culture related materials and cell line source

D. Protocol: Preparing the nuclear extract.

1. Perform all operations on ice (ice bucket or equivalent). Nuclear proteins are subject to degradation and activity loss unless kept on ice.

2. Usually 1-2 large (100 mm) petri dishes of cells is sufficient to obtain a rich topo I source. ***Cells should be freshly confluent and/or in exponential growth.*** While topo I is not dependent on cell cycle phase, topo IIa is strongly dependent upon cell cycling. Healthy, exponentially growing monolayers tend to have the least amount of contaminating nuclease and protease and the highest amount of Top2. The more cells, the better and we recommend a minimum of 10^7 but 10^8 or more is ideal.

3. Scrape up the cells into the culture medium using a cell scraper, rubber policeman or equivalent item. DO NOT use trypsin. Physical release of the monolayer is ideal. Wear gloves and avoid contaminating the cells.

4. Transfer the cells to a pre-chilled 15 mL centrifuge tube on ice and pellet the cells (centrifuge for 3-4 min at 800g in a clinical centrifuge in the cold). Discard the supernatant.

5. Resuspend the cell pellet in ice cold 1X TEMP (3-5 mL) with gentle pipetting.

6. Spin again (800 g, 3 min); Discard the supernatant and suspend in 3 mL of cold 1x TEMP.

7. Leave the solution on ice for 10 min to allow the cells to swell up in hypotonic medium.

8. To release nuclei, dounce in a tight fitting glass homogenizer (pestle "B") 6-8 strokes (1 stroke is down then up). Keep homogenizer on ice. Check that nuclei are present by phase microscopy if possible.

9. Pellet nuclei by centrifugation in cold (1500g x 10 min at 4^{o}C).

10. Suspend the nuclear pellet in 1 mL of cold TEMP in a pre-chilled microfuge tube and spin in microfuge in cold room for 2 min at max speed.

11. Estimate the volume of the nuclear pellet (example 25 ul) and resuspend in 2 pellet volumes of cold TEP buffer (50 ul in this example), on ice.

12. Add an equal volume of 1M NaCl (50 ul in this example) to give a final concentration of 0.5M NaCl. Vortex briefly and place on ice for 30 min to extract chromosomal proteins.

13. Centrifuge in a microfuge at 13,000 RPM in the cold for 15-20 min.

14. Recover the supernatant (contains active topo enzyme). Typically, 1-2 ul will give plenty of detectable activity. One unit corresponds to volume required to decatenate 100 ng of kDNA in 30 min at 37^{o}C. If the activity is very high, you should perform serial two fold dilutions of the extract down to 1:16 or lower (see Section E below).

15. If you are using this extract for western marker material for the 170 kDa form of topo IIa you should use it fresh and immediately. For assays, the extract may be frozen in aliquots at -20^{o}C. To test for westerns, try a dilution scheme to identify the best conditions to see the 170 kDa band (test a wide range of extract from 0.1 ug up to 50 ug of extract, see Fig.2)

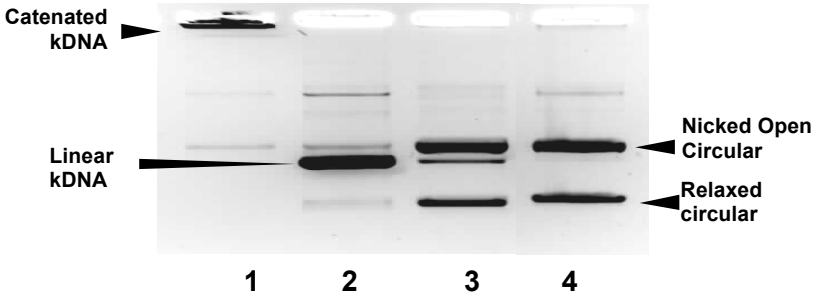
E. Topoisomerase II Assay: Protocol for a typical Reaction Mixture of 20 ul.

We recommend performing a quick titration for topo II activity (2ul, 1ul, 1:2, 1:4, 1:8, 1:16 diluted into 1x TGS buffer) as follows:

1. Label 1.5mL microfuge reactions sequentially and place on Ice.
2. Assemble all reactants in the following order on ice.
3. **H_20**: to make up to final volume (20 ul in this case)
4. **5x Complete Reaction Buffer (made 1:1 of A:B)** 4 ul
5. **kDNA** 100 ng (refer to tube for concentration in ng/ul)
6. **Topoisomerase II containing nuclear extract.** Add extract last (ie to start the reaction on ice). Do not assay more than 2-3 ul of extract due to the amount of salt in the reaction (which can poison the assay). You should see activity with 1-2 ul of undiluted extract and dilute 2 fold down from there (e.g. 1 ul of 1:2, 1:4, 1:8 dilutions). Dilute into a 1x Topo II reaction buffer.
7. If you are performing an effector assay (testing a compound or another protein addition on topo I activity) simply adjust the amount of water to compensate.
8. Move the tubes from the ice into a heat block set for 37°C.
9. Incubate 30 minutes at 37°C; stop by addition of 2 ul 10% SDS.
10. Add proteinase K to 50 ug/ml, (incubate 37°C for 15 min.).
11. Add 0.1 vol. loading buffer (blue juice).
12. Samples may be loaded directly onto the agarose gel**.
13. Run a 1% agarose containing 0.5 ug/mL ethidium bromide in gel and running buffer; run until the dye front of bromophenol blue is about 25% (typically, the run should take about well under 1h at 50-75V). Do not run overnight as this will cause the DNA bands to diffuse. Destain for 15 min in water and photodocument results. Do not let the gels sit too long before photography as this will cause band diffusion and create non-cosmetic gel data. (See Fig. 1 for typical results).

** Optional (but recommended) Step: the samples can be cleaned up by extraction and then loaded: Add 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 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.

Fig. 1. Typical Topo II reaction products with marker DNAs. A 1% EB containing agarose gel was run. Lane 1: kDNA Catenated DNA marker. Lane 2, kDNA digested with Xho1. Lane 3, kDNA + 4 units topo II with 50 uM Etoposide. Lane 4, same as lane 3 without etoposide. Red star marks the position of a topo II cleavage product (linear DNA). This band is not seen in the absence of a topo II poison.



F. FAQ

Can I use a crude extract for drug screening, say to identify a topo II inhibitor or] poison?

YES but you will need to include key controls (see next point).

What are the critical controls to allow me to clearly identify a topo II targeting agent?

- Marker DNAs (decat kDNA, Linear, fully catenated kDNA) (see Fig. 1) are extremely important.
- Be sure to run a positive DRUG control (like etoposide) to demonstrate good cleavage activity. You should see an increase in linear kDNA. Drugs are not included in this kit but are available from TopoGEN.
- Include a negative control (either no drug or a topo I drug such as camptothecin).
- 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?

- In general, 1% gels with Ethidium Bromide (EB), should be used. EB gels (0.5 ug/ml, EB in gel and buffer) work best for kDNA based assays. Be sure to destain with water for 15 min prior to photodocumenting your data. Refer to Fig. 1 data.

What are the running conditions in terms of time and voltage?

- These gels can be run at relatively high voltage (50-200 v) for short times. Don't let the dye run too far down the gel ca. 25% or less is ok).
- 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 1h. 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/extract, 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.

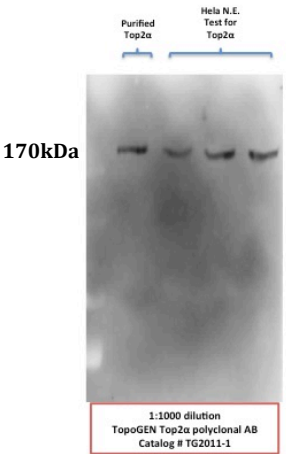
Why is proteinase K required?

- 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 we use this extraction kit to detect topo IIa on Western Blots?

Yes. We have excellent mono and polyclonal AB for probes (see Cat#TG2011-1). Note that we can also detect topo IIβ using our AB (Cat# 2010-3) (see Fig. 2).

Fig. 2. Crude HeLa nuclear extracts were prepared using the kit protocol and 5,10 and 20 ug of total nuclear extract was loaded onto an SDS-PAGE and processed for Western blotting using the indicated anti-topo IIa polyclonal antibody.



Y7Can 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:
support@topogen.com