Human Topoisomerase II Assay Kit Cat. # TG-1001-1A (Includes Topo II) User Manual

Overview:

This Kit is designed to assay any type II topoisomerase, either eu- or prokaryotic. For a video description see TopoGEN's Youtube Channel, search for "Topoisomerase II Assay Kit".

Shipping and Storage of Reagents

The kit is shipped on dry ice. The DNAs should be stored at 4°C and the buffers stored at -20°C upon receipt. Store enzyme at -70°C. Avoid frequent freeze/thaw cycles with all products.

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Introduction

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, changing DNA linking number in discrete steps of two. Because Topo II 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. The Assay Kit measures the release of minicircular DNAs by decatenation of an intertangled mass of 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 does not induce formation of linear DNA products under the conditions of this assay; therefore, linear kDNA should not be detected in the gels. The Human Topoisomerase II Assay 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.

B. Kit Contents (100 assay kit size)

1. 100 Units Human Topoisomerase II (see attached data sheet for unit definition, quality control, and activity assay parameters). Store at -70° C.

2. kDNA (20 ug total) substrate at the concentration specified on the tube provided. Typically one should use 0.1 to 0.2 ug per reaction.

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

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

*5. 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.

*6. 10x ATP Buffer B contains 20mM ATP in water.

*You must mix Buffers A and B together prior to make a 5x Complete Assay Buffer. To prepare a fresh stock of the 5x 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. DO NOT STORE THE 10x COMPLETE ASSAY BUFFER, IT IS NOT STABLE.

7. 5x Stop Buffer/gel loading dye (600 ul): 5x buffer is 5% Sarkosyl, 0.125% bromophenol blue, 25% glycerol.

C. Protocol for a typical Reaction Mixture of 20 ul

Assemble all reactants in the following order.

- ✓ **$H_2$0**: to make up to final volume (20 ul in this case)
- ✓ **5x Complete Reaction Buffer (made 1:1 of A:B)** 4 ul
- ✓ **DNA** 1 ul (100-200 ng is idea for most assays).
- ✓ **Test extract: 1 ul** (vary as needed)

1. Incubate 30 minutes at 37°C.

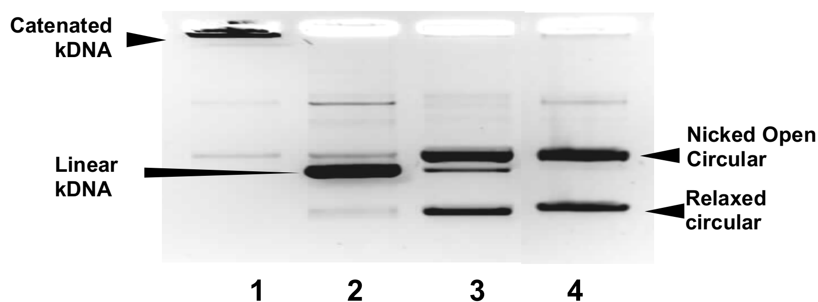
2. Stop by addition of 4 ul 5x Stop Buffer.

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 37ºC) 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.

5. Run a 1% agarose until the dye front (bromophenol blue) is about 4-6 cm down the gel. Do not run overnight as this will cause the DNA bands to diffuse. Usually a gel gives good separation after 30 min or less (we use 4-5V/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 that works well (see gel data, below). You may also run EB containing gels (0.5ug EB/ml) with EB in Gel and Buffer (beware, it is a mutagen, so wear gloves at all times; do not inhale powder). Run gels as above and destain with water for 15 min prior to photo-documenting the results (see Fig. 1 for typical gel result).

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.



D. Important Considerations about this kit.

Marker DNAs are extremely important. You should always run decatenated and linear kDNA markers.

Any nuclease-free agarose of reasonable quality can be used (from Sigma).

A positive control (such as included enzyme) and negative control (no extract) are also very 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.

EB agarose gels (EB at 0.5 ug/ml in gel and buffer) will improve the resolution of cleavage products (nicked open circular kDNA and circular kDNA, see Fig. 1 above).

Run gels at a relatively high voltage (100-150 v) to facilitate rapid run times. Even if the bromophenol blue goes less than a few cm, you should have sufficient resolution to detect decatenated kDNA products.

After running, EB gel should be 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 kDNA breakdown or degradation, indicating the presence of a nuclease activity (usually not a problem).

Activity can be measured by the disappearance of kDNA networks (catenanes) or the formation of decatenated DNA (see Fig. 1).

F. Frequently asked questions.

What are the critical controls?

- Marker DNAs (linear kDNA, decatenated and catenated kDNA) (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 EB gels?

In general, Ethidium Bromide (EB) gels are ideal for testing enzyme activity in this system because it is much easier and faster than the non-EB system. Be sure to destain with water for 15 min prior to photodocumenting your data. If the DNA products are poorly resolved, you can simply re-electrophorese until resolution improves.

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

Run the gels at a relatively high voltage so that the dye front moves 4-5 cm in about 20-30 min. Thus, the gels run rather fast which accelerates the pace of the assay.

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.

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.

I see two decatenated bands in the gel. Why is that?

This is normal when you include EB in the gel system. In the presence of EB, the minicircles resolve out as nicked (ss nick) and intact circular. Both of these are decatenation products and you can quantify either of these to measure topo II.

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:
E-mail: info@topogen.com
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Purified Human DNA Topoisomerase IIa (see tube label for unit concentration)

Contains purified DNA Topoisomerase II (p170, over- expressed and purified). Included with the enzyme are two buffers (Incomplete Buffer A and ATP Buffer B) that are used to make a final working stock of 5x topo II assay buffer, as described below.

Storage and Shipping Conditions

The enzyme is shipped on dry ice and should be stored at -70°C. We also recommend that the enzyme be aliquoted after the first thaw (repeated rounds of freeze/thaw will lead to loss of activity); the enzyme activity is generally stable for 1-2 days (not longer) at 4°C or on ice. The 10x Incomplete Buffer A and the 10x ATP Buffer B should be stored frozen at -20°C.

IMPORTANT NOTE REGARDING ACTIVITY: The enzyme is provided at a unit/ul concentration that represents a certified minimum. For example, we certify that the product will have X units/ul under conditions of our assay. In some cases, activity may be greater than X units to take into account that freezing/thawing may lead to some loss over time. The enzyme will retain the certified minimum unit concentration (see label) for 6 months after receipt.

Unit Definition

One unit will decatenate 0.2 ug of kDNA in 30 min at 37°C. We do NOT recommend using a plasmid DNA substrate with this product for several reasons, the most important of of which is our unit definition is based on kDNA. TopoGEN's Topoisomerase II is validated and is pre-screened based on kDNA units (a natural and optimal substrate). We do not recommend using 'relaxation' with plasmid DNA because supercoiled DNA substrates (especially from other suppliers or made in-house) may carry over contaminants (heavy metals in the ion atmosphere of some plasmid preps) which may inhibit enzyme activity. Finally, most plasmids are a mix of supercoiled (circular DNA) and nicked DNA. This contaminating nicked plasmid DNA may significantly affect quantifying enzyme action. For these reasons, we cannot certify our Topoisomerase IIa based on supercoiling/relaxation assays using plasmids.

Topo IIα Quality Control Tests

1. Nuclease contamination was assayed by testing for the formation of linear kDNA. Incubation of 1 µg of catenated kDNA is peformed for 4 hrs. at 37°C (under topo II assay conditions and with or without ATP). Linear DNA is not generated under these conditions.
2. A major band is seen when loaded on an SDS--PAGE gel (170kDa). Protein concentrations typically vary from lot to lot between 25-- and 60 ug/ml.
3. The final fraction of topoisomerase II comes off the purification column in the

following buffer: 10% glycerol, 50 mM Tris (pH 7.7), 1 mM EDTA and EGTA, 650 mM NaCl.

Dilution Buffer

The enzyme should not be diluted for cleavage assays since larger amounts of enyzme activity are required (typically 2--6 units per reaction). For catalytic assays (decatenation or relaxation assays), dilutions should be performed in a dilution buffer (10 mM Tris--Cl (pH 7.5), 500 mM NaCl or KCl, 1 mM PMSF and 2 mM DTT and 50 ug BSA/ ml). Note that very large dilutions of the enzyme (>5-10 fold) may accelerate the decay of enzyme activity

Assay Conditions

Catalytic assays (decatenation) are carried out using kinetoplast DNA substrate in a final volume of 20--30 µl in topo II reaction buffer (1x topo II buffer = 50 mM Tris--Cl, pH 8.0, 150 mM NaCl, 10 mM $MgCl_2$. 5 mM ATP 0.5 mM dithiothreitol and 30 ug BSA/ml) with 0.2 µg KDNA. Reactions are terminated with 5x stop buffer (5 µl per 20 µl reaction volume). Stop buffer contains 5% sarkosyl, 0.0025% bromophenol blue, 25% glycerol. Reaction products are analyzed on a 1% agarose gel containing 0.5 ug/ml ethidium bromide or other DNA stain. Resolution of a 2.5 kb kDNA minicircle decatenated product can be easily monitored with a hand held UV light source while the gel is running. Gels can be at a very high voltage since decatenation products are easily resolved.

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TopoGEN provides two buffers that *must be mixed in equal proportions to yield a final 5x working stock of assay buffer. The 5x Complete Buffer should be made fresh daily!

1. Incomplete **Buffer A** contains the following: 0.5M Tris--HCl (pH8.0), 1.5 M NaCl, 0.1M $MgCl_2$, 5 mM dithiothreitol, 300 ug/ml BSA). [0.25 mL provided]
2. Incomplete ATP **Buffer B** contains 20 mM ATP in water [0.25 mL provided].

These two buffers must be made fresh and mixed in equal volumes to make the final 5x Complete Topo II Assay Buffer (A+B) as follows. Add equal volume of Buffer A and Buffer B to give a final 5x Topo II assay buffer fresh and ready to use. Prepare only as much of the Complete 5x Topo II Assay Buffer (A+B) as needed for each day or set of experiments. For example: If you are performing 10 assays (each with a final volume of say 20 ul per assay) in a single day, you will need 40 ul of the 5x Complete Buffer (4 ul/reaction x 10 reactions). Thus, you should prepare slightly more than needed as follows: 25 ul of Buffer A plus 25 ul of B to give 50 ul total of Complete Buffer (5x stock). Note that this buffer should be kept on ice and used the same day, then discarded.

References

Muller et al., Biochemistry 27: 8369--8379 (1988)
Spitzner and Muller, Nuc. Acid. Res. 16: 5533--5556 (1988)