TopoGEN

Products and Services for **Cancer Research.**

Human TDP-2 (Tyrosyl-DNA-phosphodiesterase 2) Assay Kit
Catalog TG1005-1 (100 Assays)
Catalog TG1005-2 (250 Assays)

Overview:

TDP-2 is a relatively recently discovered repair protein. TDP2 (unlike TDP1) is specifically directed to act on phosphotyrosyl bonds that form when topoisomerase II is covalently trapped on the DNA helix. This Kit is designed to assay Human TDP-2 activity using a novel, non-radiolabeled substrate constructed as a huTop2/DNA covalent complex (CC) intermediate.

Shipping and Storage of Reagents

The kit may be shipped at ambient temperature or on ice (dry ice or wet ice). The DNAs should be stored at 4°C and the buffers stored at -20°C upon receipt. Avoid frequent freeze/thaw cycles with the plasmid as this may contribute to DNA breakage.

TopoGEN, Inc.
27960 CR 319
UNIT B
BUENA VISTA, CO
81211 USA
Tel: 614-451-5810
Fax: 614-559-3932
Orders sales@topogen.com
Support: support@topogen.com
Website: www.topogen.com

Introduction

A. Background:

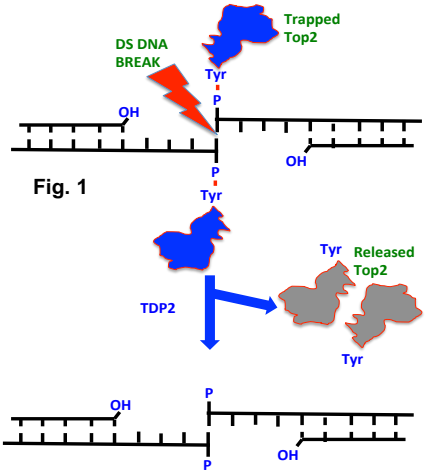


Fig. 1

Tyrosyl-DNA phosphodiesterase-2 (TDP2) functions to remove trapped topoisomerase II/DNA complexes by processing 5′- blocking protein (topo II) at the site of the DNA break (Fig 1). In terms of physiological relevance, there appear to be multiple roles for TDP2, well beyond the repair of type II topoisomerase damage and subsequent DNA repair. This is quite different from the Top1/DNA repair protein, TDP1. There are a number of mechanistic differences between TDP1 and 2 as well (see reviews from Pommier, PMID 24856239). In humans, TDP2 should be considered a multi-functional enzyme with roles in TNF and MAPK/JNK/p38 pathways. There is some similarity with APE1 and the superfamily of Mg^{++}/Mn^{++} dependent phosphodiesterases and there is evidence that picorna viruses pirate TDP2 as a VPg un-linkase function. Given the key role for topo II in anticancer therapy, drugs that inhibit TDP2 might prove useful in combination therapy with established Interfacial Poisons (IFP) such as doxorubicin and etoposide (VP16). As a result of this thinking, there have been a few TDP2 candidate inhibitors. TDP2 specifically recognizes 5'-phosphotyrosyl bonds and synthetic substrates have been useful for HTS and drug discovery (example, 4-nitrophehyl phenylphosonate or radioactively tagged oligos). While these synthetic or semi-synthetic substrates are useful for high throughput screening, inhibitors need to be tested and validated on bona fide Top2/DNA covalent adducts. For this reason, TopoGEN has developed a true Top2/DNA substrate. We provide trapped, unprocessed Top2/kDNA covalent complexes that can be effectively used as TDP2 substrates (see Fig. 2 for the assay outline). Due to the presence of a covalently bound protein, the linear CC complexes cannot be detected (Fig. 2, left lane) unless either proteinase K is added (to degrade and release the bulk of the denatured Top2a) or TDP2 is reacted to release the trapped protein.

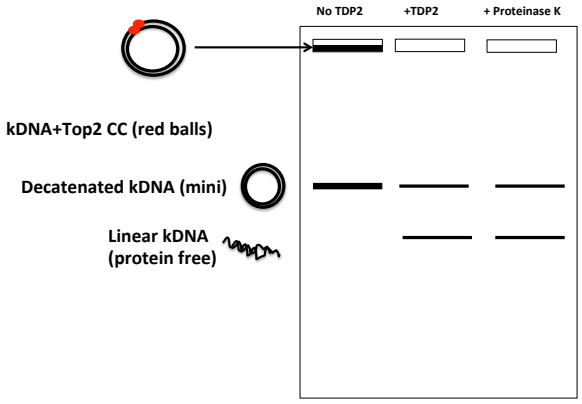


Fig. 2. TDP2 Assay Scheme. kDNA is reacted with huTop2a under conditions where the covalent complex (cc) is favored. This DNA is then used to assay for TDP2. Left most lane shows the substrate containing decatenated mini-circular DNA plus trapped Top2/DNA cc. These Top2/DNA cc contain linear DNA that cannot be resolved due to the trapped protein. The assay measures the release of the protein, which releases linear DNA into the gel.

The Human TDP2 Assay Kit contains reagents necessary to quantify TDP2 activity in easy to prepare 1% agarose gels. Since kDNA is such a large substrate, the gels can be run quickly, (<10 min at 100-250v) thereby improving throughput. Markers are included to allow unambiguous interpretation of the results. By scanning the gels using shareware such as ImageJ, the results can easily be quantified.

B. Kit Contents (100 assay kit size; for 250 assay kit, all reagents multiplied by 2.5 fold)

1. 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.

2. Decatenated kDNA marker (25 ul) in gel loading buffer. Run 2 ul of decatenated DNA marker per lane for each gel as a ref marker.

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

*4. 10x TDP2 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.

*5. 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.***

6. 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 reactants in the following order.

- ✓ **H_20**: 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 or Purified TDP2: 1 ul or** vary as needed (adjust water in reaction to compensate total volume

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.**

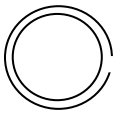
Withdraw blue colored (upper) aqueous phase and load onto agarose gel. CIA extraction will usually improve the cosmetic quality of the agarose gel results. In addition, CIA extraction will extract non-polar compounds that may interfere with the gel staining, with some test drugs.

4. Run a 1% agarose containing 0.5 ug/ml ethidium bromide until the dye front (bromophenol blue) is about 3-5 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 (run at 100-200v). Destain for 15 min in water and photo-document results. You may also run non- EB but we find EB gels are ideal (you can run these gels at relatively high voltage for short times and still resolve linear kDNA (as protein free mini-circular kDNA).

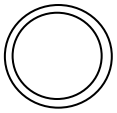
D. Interpretation of Data.

The kDNA substrate provided in this kit is an admixture of three kDNA forms:

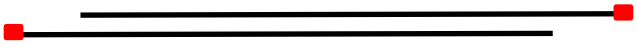
i) Nicked mini-circular (decatenated) kDNA that is protein free



ii) Fully Circular decatenated kDNA that is protein free.



iii) Linear kDNA/Top2a DNA complexes with covalently bound Top2a



Note that the Linear kDNA/top2a complex will not be detectable on the gel because it is shifted by the bound protein. However, in the presence of TDP2, the protein is removed and the linear kDNA can be detected on the gel:

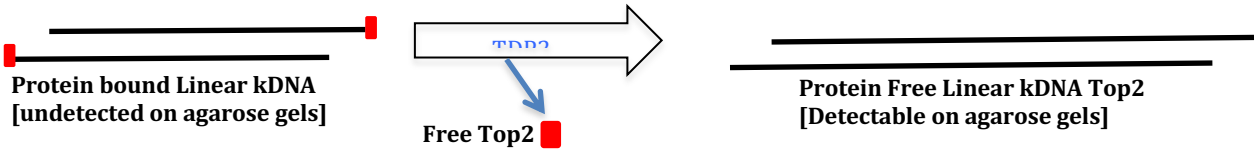
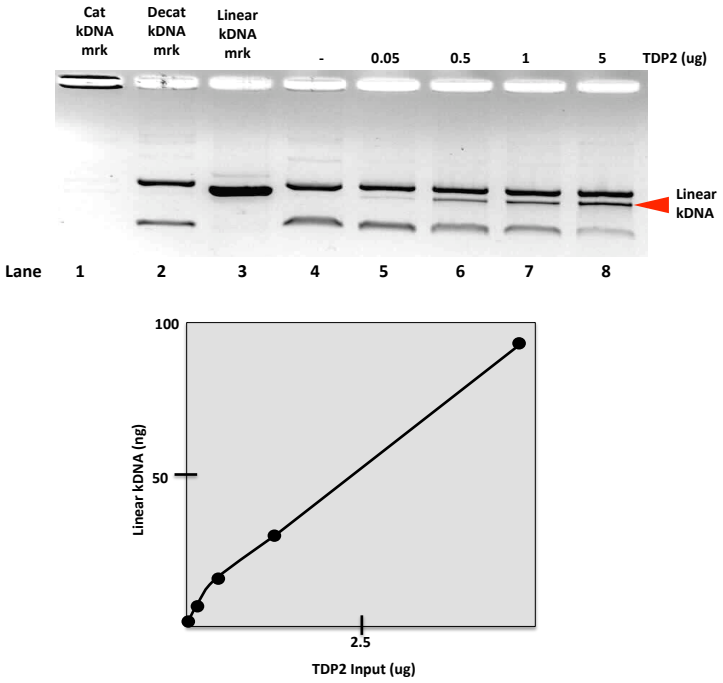


Fig. 3. Typical TDP2 reaction products with marker DNAs. A 1% EB containing agarose gel was run, de-stained in water and photographed. Lanes are labeled.



E. 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 (with known TDP2 activity) and negative control (no TDP2) 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. Alternatively, purified human TDP2 is available for purchase (TG2038-1).

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) are ideal for resolution of nicked open circular kDNA and circular kDNA and linear kDNA forms; see Fig. 3).

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.

F. 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 appearance of linear kDNA (see Fig. 3) and using gel imaging and quantitation (such as Image J shareware).

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 nuclei). More than 200mM NaCl from the crude extract will impact negatively on the results.
- You can include a Proteinase K control which will also release free Linear kDNA (as does TDP2).

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 you are assaying from a crude nuclear extract (especially if its concentrated with protein), it may be a good idea to degrade these proteins to improve the cosmetics of your gel.

I see three 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 in addition to linearized kDNA. The latter will appear when TDP2 removes the covalently bound TDP2 protein.

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
Telephone: 614-451-5810