Human Topoisomerase II Decatenation Assay

Introduction

Human topo II is able to decatenate interlinked double-stranded DNA molecules. In this assay, the substrate is kinetoplast DNA (kDNA) from *Crithidia fasciculata* which consists of a network of many minicircles (2.3 Kb) and a few maxicircles. The enzyme will release the minicircles from this network. The substrate will remain in the wells after gel electrophoresis (although it is not always visible) while the released minicircles will migrate into the gel where they can be seen after staining with ethidium bromide.

Materials

Human Topo II assay buffer : 50 mM Tris.HCl (pH 7.5), 125 mM NaCl, 10 mM $MgCl_2$, 5 mM DTT, and 100 µg/mL albumin (supplied at 10X). Store at -20°C or below.

Dilution Buffer : 50 mM Tris.HCl (pH 7.5), 100 mM NaCl, 1 mM DTT, 0.5 mM EDTA, 50 % (v/v) glycerol, and 50 µg/mL albumin (supplied as 1X). Store at -20°C or below.

ATP: 30 mM ATP (supplied as 30X). Store at -20°C or below.

Enzyme : Human Topo II (see data sheet for concentration). Store at -80°C. (NOTE 1)

Substrate : kDNA (supplied at 100 ng/µL). Store at 4°C or below.

GSTEB: 40% (w/v) Glycerol, 100 mM Tris-HCl pH8, 10 mM EDTA, 0.5 mg/ml Bromophenol Blue. (Supplied at 10X)

Method

(Note: This is an example method and the volume of water can be increased or decreased to allow for the addition of inhibitors although the final volume will be 30 µL)

On ice, set up a MIX of (per assay): Assay Buffer (3 µL of 10X buffer per assay) (NOTE 2), ATP (1 µL per assay), kDNA (2 µL per assay) and water (22.2 µL per assay).

26.7 µL of MIX are required per assay.

Set up the appropriate number of 1.5 ml tubes and aliquot 26.7 µL of MIX into each tube.

If the compounds are dissolved in DMSO then add 0.3 µL of DMSO to tubes 1 and 2 (NOTE 3).

Add 0.3 µL of the test compounds to the other tubes as appropriate.

Mix briefly (gentle vortexing or pipetting).

Add 3 µL of dilution buffer (NOTE 4) to tube 1.

Dilute the enzyme in dilution buffer then add 3 µL of this to the remaining tubes (see NOTE 1).

Mix by gentle vortexing and incubate 30 minutes at 37°C.

Stop reaction by adding 30 µL of 2X GSTEB and 30 µL of chloroform/isoamyl alcohol (v:v, 24:1) (NOTE 5).

Vortex briefly ~5 secs and centrifuge for 2 minutes.

Load 20 µL of aqueous (upper blue) phase onto a 1% (w/v) agarose gel. (NOTES 7 and 8)

Run at 85V for approximately 1 hour.

Stain with ethidium bromide (1 µg/mL in water) (15 mins), destain (5-10 mins) in water (NOTE 6) and visualise with a transilluminator or gel documentation system.

Example Results

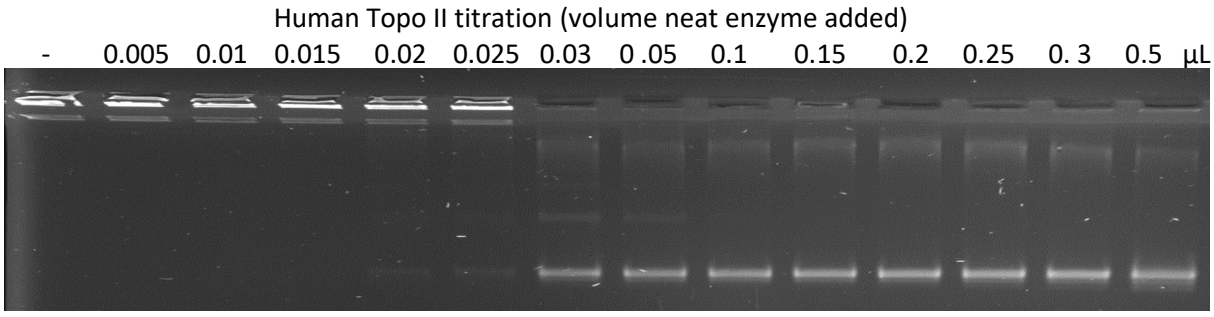
1. Determination of enzyme activity.

The gel below shows the results for a gel-based decatenation experiment with kDNA, set up as follows: a MIX was prepared of 42 µL of (10X) Assay Buffer, 14 µL of ATP, 28 µL kDNA and 317.8 µL of water. 27 µL of this MIX was added to each tube.

Serial dilutions of the enzyme were made in dilution buffer. Then 3.0 µL added to the reactions.

Reaction	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Dilution	DB	1/600	1/300	1/200	1/150	1/120	1/100	1/60	1/30	1/20	1/15	1/12	1/10	1/6

The gel shows the results of the decatenation reaction with the equivalent amount of neat enzyme added (e.g. reaction 2 contains 3 µL of a 1/600 dilution equivalent to 0.005 µL of neat enzyme)

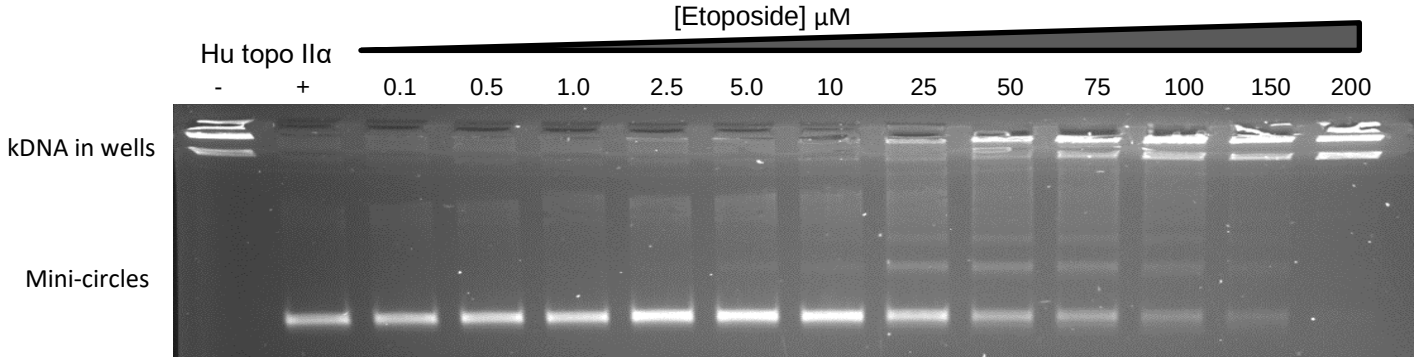


In this case approximately 0.03 µL of the enzyme is required to give full decatenation.
It is not recommended to store the diluted enzyme as it can lose activity.

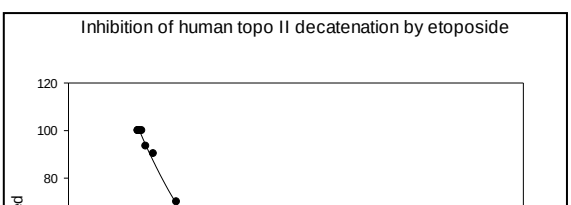
2. <u>Determination of the inhibitory activity of a compound</u>

The gel below shows the results for the inhibition of decatenation activity with increasing amounts of the inhibitor etoposide (dissolved in DMSO). The assay was set up as follows: a MIX was made consisting of 42 µL of (10X) Assay Buffer, 14 µL of ATP, 28 µL of kDNA, 317.8 µL of water. 26.7 µL of the MIX was added to each tube then 0.3 µL DMSO or etoposide and finally the dilution buffer (DB) /enzyme (using a dilution determined as in example 1 above).

Sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14
MIX (µL)	26.7	26.7	26.7	26.7	26.7	26.7	26.7	26.7	26.7	26.7	26.7	26.7	26.7	26.7
Vol Cpd (µL)	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Initial [Cpd] µM	DMSO	DMSO	10	50	100	250	500	1000	2500	5000	7500	10000	15000	20000
Final [Cpd] µM	0	0	0.1	0.5	1.0	2.5	5.0	10	25	50	75	100	150	200
Dil. Buffer (µL)	3													
Enzyme (µL)		3	3	3	3	3	3	3	3	3				



The intensity of the mini-circles (MC) in each track



was determined by scanning the gel image and the amount calculated as a percentage of that in the enzyme only track. These values were then plotted against the etoposide concentration.
The IC_{50} was calculated as 46.3 μM.

Notes

1) Enzyme is supplied at a minimum number of U/μL (see data sheet and certificate of analysis), where 1U is the amount of topo II required to just decatenate 200ng of kDNA) but it can be significantly more. Thus if you require a known amount of decatenation (e.g. 90% decatenated or just fully decatenated) then it is recommended to run an enzyme dilution series first, where the enzyme is titrated into the assay. The quoted activity (e.g. 5 U/μL) is in the absence of solvents so an enzyme titration must be done in the actual assay conditions before assaying inhibitors.

2) The final concentration of assay buffer should be 1X.

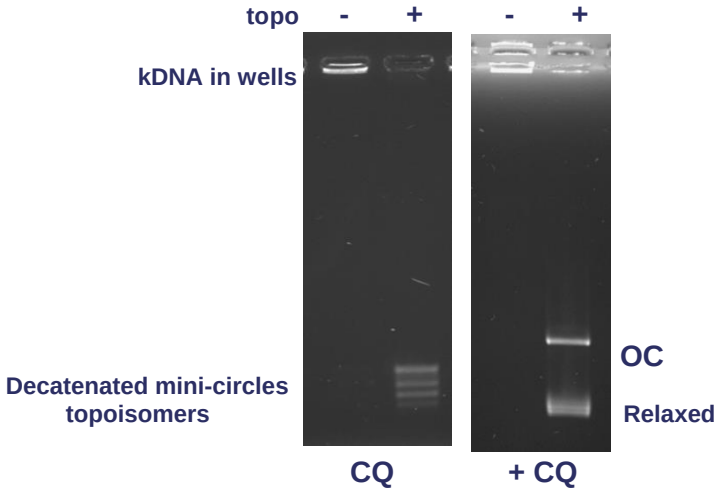
3) DMSO has an inhibitory effect on the enzyme and we do not recommend using more than 1-2% (v/v) final concentration with this enzyme. The initial titration of the enzyme should be done in the presence of the final concentration of DMSO that will be used (see NOTE 1 above). It is possible to use 5-10% (v/v) DMSO (final concentration) but this will lead to a loss in activity. More enzyme will need to be added in the presence of DMSO to allow for its inhibitory effect. For example, if the enzyme has only 50% activity in the presence of 5% DMSO then twice as much will need to be added to get full supercoiling. In this case more inhibitor will probably need to be added to get 50% inhibition.

4) The dilution buffer contains a high concentration of glycerol therefore the total dilution buffer added should not exceed 10% of the final volume.

5) Compounds can be removed from the reactions by extracting with water-saturated butanol before this step (the GSTEB/ chloroform step) if necessary. The upper (butanol) phase is then removed and discarded and the GSTEB/chloroform added as normal. Usually, however, the chloroform extraction is sufficient to remove the compounds as well as the topo II.

6) The agarose gels shown in the results are run in Tris-acetate-EDTA buffer (40 mM Tris-acetate, 1 mM EDTA) but TPE (36 mM Tris, 30 mM sodium phosphate, 1 mM EDTA pH7.8) or TBE (89 mM Tris, 89 mM borate, 2 mM EDTA pH 7.6) buffers can also be used. Gels can be run faster than relaxation assays, at 4-5 V/cm. Gels are usually run until the bromophenol blue dye has run at least 4-5 cm. This will be enough to see the decatenated mini-circles.

7) Gels can be run in the presence or absence of intercalators such as chloroquine or ethidium bromide. In the absence of ethidium, the decatenated mini-circles will usually run as a single band (or as a number of topoisomers) while in the presence of the stain, two bands are frequently present consisting of supercoiled and open-circular (relaxed) mini-circles respectively. This separation can also be achieved by including other intercalators such as chloroquine in the gel. The gels below show the typical appearance of gels run in the presence or absence of a chloroquine (CQ).



8) Once the reactions have been stopped with the GSTEB and mixed, it is possible to store them overnight at 4^{o}C although it is preferable to load them on a gel as soon as possible.