Pseudomonas aeruginosa Topoisomerase IV Decatenation Assay

Introduction

Topoisomerase IV 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

***P. aeruginosa* Topo IV Assay Buffer :** 50 mM HEPES.KOH (pH 7.9), 100 mM potassium glutamate, 6 mM magnesium acetate, 4 mM DTT, 2 mM spermidine, 1 mM ATP, and 50 µg/mL albumin (supplied as 5X). Store at -20^{o}C or below.

Dilution Buffer : 50 mM Tris.HCl (pH 7.6), 50 mM NaCl, 2 mM DTT, 1 mM EDTA, and 50% (v/v) glycerol (supplied as 1X). Store at -20^{o}C or below.

Enzyme : *P. aeruginosa* Topo IV (see data sheet for concentration). Store at -80^{o}C. (NOTE 1)

Substrate : kDNA (supplied at 100 ng/µL). Store at -20^{o}C or below.

GSTEB: 40% (w/v) Glycerol, 100 mM Tris-HCl pH8, 1 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 (6 µL of 5X buffer) (NOTE 2), kDNA (2 µL) and water (16 µL).

24 µL of MIX are needed per assay.

Set up the appropriate number of 1.5 mL tubes and aliquot 24 µL of MIX into each tube.

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

Add 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^{o}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 1 minute.

Load 20 µL of aqueous (upper blue) phase onto a 1% (w/v) agarose gel (NOTE 6).

Run at 85V for approximately 2 hours (or 15 V overnight).

Stain with 1 µg/mL ethidium bromide in water (15 mins), destain (5-10 mins) in water (NOTE 7) and visualise with a transilluminator or gel documentation system.

Example Results

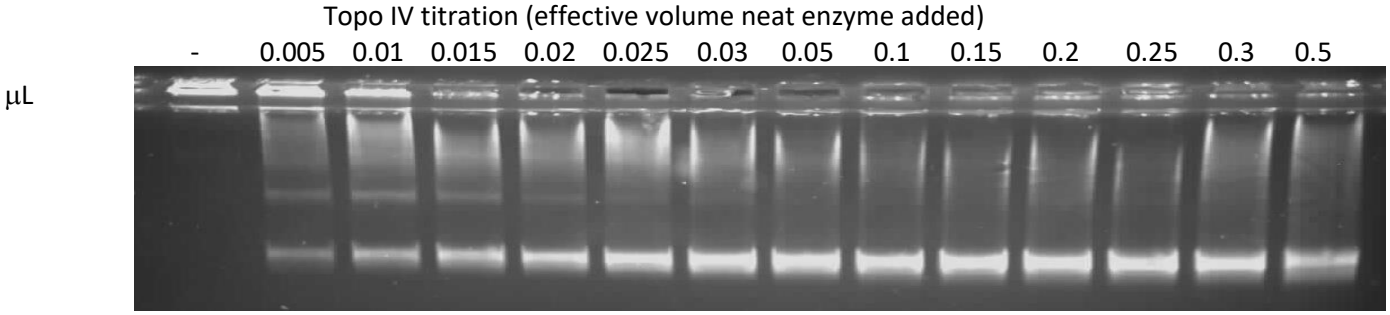
1. Determination of enzyme activity.

The gel below shows the results for a gel-based decatenation experiment with 200 ng kDNA, set up as follows: a MIX was prepared of 84 µL of Assay Buffer (5X), 28 µL of kDNA and 266 µL of water. 27 µL was added to each tube followed by dilution buffer or enzyme.

Serial dilutions of the enzyme were made in dilution buffer as in the table below. Then 3.0 µL of each added to the appropriate 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)

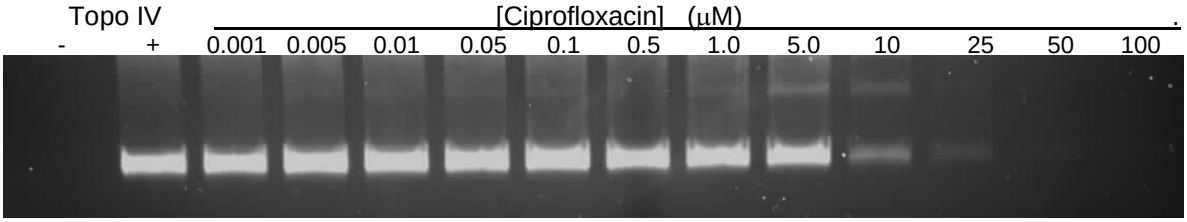


In this example 3 µL of 1/120 dilution of enzyme (equivalent to 0.025 µL) are needed to just get full decatenation.

2. Determination of the inhibitory activity of a compound

The gel below shows the results for the inhibition of decatenation activity by ciprofloxacin. A MIX was prepared of 84 µL of Assay Buffer (5X), 28 µL of kDNA and 224 µL of water. 24 µL was added to each tube followed by 3uL of each ciprofloxacin concentration. Finally 3 µL dilution buffer (Sample 1) or enzyme (samples 2-14) was added.

Sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14
MIX (µL)	24	24	24	24	24	24	24	24	24	24	24	24	24	24
Initial [Cipro]	H_2O	H_2O	0.01	0.05	0.1	0.5	1	5	10	50	100	250	500	1000
Final [Cipro]	0	0	0.001	0.005	0.01	0.05	0.1	0.5	1	5	10	25	50	100
DB (µL)	3													
Topo IV (µL)		3	3	3	3	3	3	3	3	3	3	3	3	3



(Note, only the decatenated mini circles are shown in this case)

Notes

1) Enzyme is supplied in U/µL (see data sheet and certificate of analysis for lot specific concentrations), where 1U is the amount of topo IV required to just decatenate 200 ng of kDNA but it can be significantly more. It is recommended that you run an enzyme dilution series when you receive the kit, where the

enzyme is titrated into the assay, to ascertain the activity in your hands or if a known amount of decatenation (e.g. 90% decatenation or just full decatenation) is required. 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) Final concentration of assay buffer should be 1X. The assays can be set up by adding the components individually but a MIX is preferred when doing large numbers of reactions. In general, the order of addition should be water/assay buffer/kDNA. The MIX is then aliquoted into the tubes to which the inhibitors (if used) and finally enzyme are added.

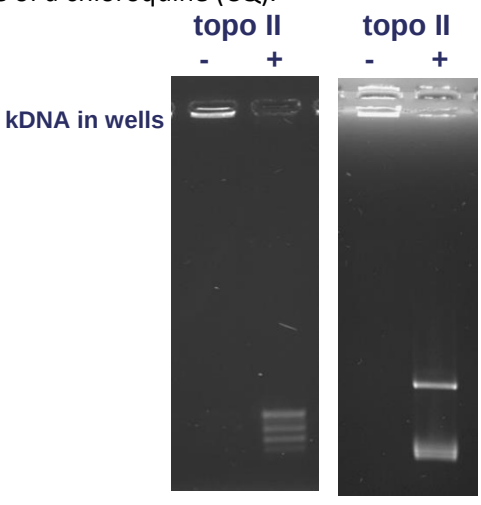
3) DMSO has an inhibitory effect on the enzyme and we do not recommend using more than 1-2% (v/v) final concentration with this enzyme. The initial titration of the enzyme should be done in the presence of the final concentration of DMSO that will be used (see NOTE 1 above). It is possible to use 5-10% (v/v) DMSO (final concentration) but this will lead to a **substantial** loss of 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 DMSO then twice as much (i.e. 2U) will need to be added to get full decatenation. In this case more inhibitor will probably need to be added to get 50% inhibition. A similar effect is seen with ethanol.

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

5) Compounds can be removed from the reactions by extracting with water-saturated butanol before this step (GSTEB/chloroform addition) 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 enzyme.

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 pH 7.8) 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 those of 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.