Acinetobacter baumannii Topoisomerase IV Relaxation Assay

Introduction

A.baumannii topo IV is a type II topoisomerase that is able to relax supercoiled DNA. In this assay, the substrate is supercoiled pBR322 which is relaxed by the enzyme. The two forms of the plasmid can be separated by agarose gel electrophoresis. It can be used to determine the activity of compounds as inhibitors of topo IV.

Materials

***A.baumannii* Topo IV Assay Buffer :** 50 mM HEPES. KOH (pH 7.9), 6 mM magnesium acetate, 4 mM DTT, 1 mM ATP, 100 mM potassium glutamate, 2 mM spermidine, 0.05 mg/mL albumin (supplied as 5X). Store at -20°C or below.

Dilution Buffer : 100 mM potassium phosphate (pH 7.6), 1 mM EDTA 1 mM DTT , 30 % (w/v) glycerol (supplied as 1X). Store at -20°C or below.

Enzyme : *A.baumannii* Topo IV (supplied at a minimum 5 U/µL) (NOTE 1). Store at -80°C.

Plasmid : Supercoiled pBR322 (supplied at 1 µg/µL). Store at -20°C or below.

GSTEB: 40% (w/v) Glycerol, 100 mM Tris-HCl pH 8, 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 (6 µL of 5X buffer per assay) (NOTE 2), supercoiled pBR322 (0.5 µL per assay) and water (17.5 µL). 26.7 µL of MIX are needed 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 (see NOTE 6).

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

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.

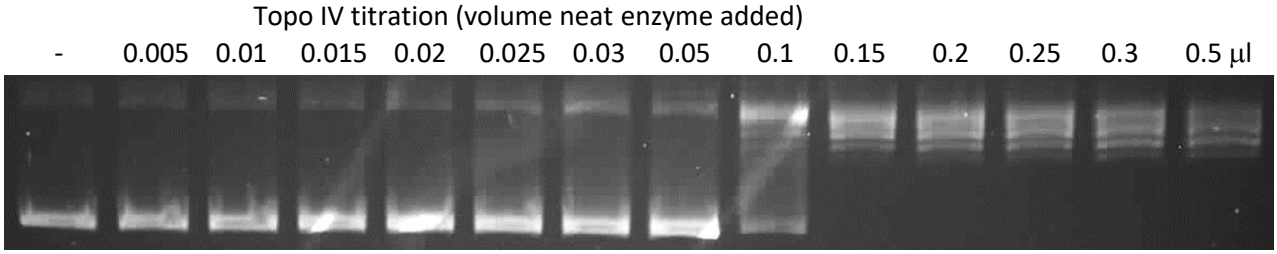
Example Results

1. Determination of enzyme activity in the presence of 1% (v/v) DMSO.

The gel below shows the results for gel-based relaxed experiment to determine the amount of enzyme to use in a subsequent assay. This is performed with supercoiled pBR322 and set up as follows: a MIX was prepared for 14 reactions with 84 µL of Assay Buffer (5X), 7 µL supercoiled pBR322, 4.2 µL DMSO and 282.8 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. 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

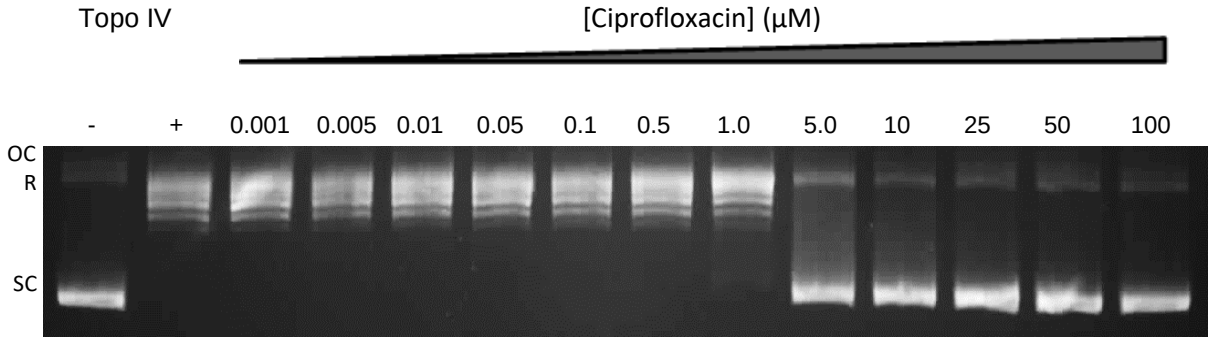


In the example above, 0.15 µl of enzyme are needed to just get full relaxation in the presence of 1% (v/v) DMSO. It is not recommended to store the diluted enzyme as it can lose activity

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

The gel below shows the results for the inhibition of relaxation activity by ciprofloxacin. A MIX was prepared of 84 µL of Assay Buffer (5X), 7 µL of supercoiled pBR322 and 282.8 µL of water. 26.7 µL was added to each tube followed by 0.3 uL of each ciprofloxacin concentration. Finally the dilution buffer (sample 1) or enzyme (samples 2-14) were added.

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 Cmp (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	0.01	0.05	0.1	0.5	1	5	10	50	100	250	500	1000
Final [Cpd] µM	0	0	0.001	0.005	0.01	0.05	0.1	0.5	1	5	10	25	50	100
Dil. Buffer (µL)	3													
Enzyme (µL)		3	3	3	3	3	3	3	3	3	3	3	3	3



OC= nicked, open circular R= relaxed topoisomers SC= supercoiled topoisomers

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 relax 0.5 µg of relaxed pBR322 in 30 minutes, 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 relaxation (e.g. 90% relaxation or just full relaxation) 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) 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 **substantial** 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 DMSO then twice as much (i.e. 2U) will need to be added to get full relaxation. In this case more inhibitor will probably need to be added to get 50% inhibition. Other solvents (e.g. ethanol) may also have an inhibitory affect on the enzyme.

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 IV.

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 should be run at about 4 V/cm. Gels are usually run until the bromophenol blue dye has run at least 6-7 cm. This will resolve the relaxed and supercoiled bands. Longer runs will give greater separation if necessary.

7) Gel tanks, gel formers and combs should be free of intercalators such as ethidium bromide and chloroquine which will affect the mobility of the various forms of the plasmid (relaxed, supercoiled etc.). If the apparatus has been used with intercalators then they should be thoroughly cleaned before use. This can be done by soaking for several hours or preferable overnight in a detergent solution (e.g. washing up liquid) and then thoroughly rinsing with water before rinsing with distilled water.

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.