Mycobacterium tuberculosis Gyrase Cleavage Assay

Introduction

DNA gyrase is a type II topoisomerase that can introduce negative supercoils into DNA at the expense of ATP hydrolysis. It is essential in all bacteria but absent from higher eukaryotes. The enzyme acts by creating a double-strand break in one segment of DNA (forming a 'cleavage-complex'), passing another DNA segment through it and resealing the break. Some compounds such as the quinolones inhibit the enzyme by stablilising the cleavage complex. In this assay, the substrate, supercoiled pBR322, is incubated under supercoiling conditions but in the absence of ATP. It is then treated with SDS and proteinase K to trap any cleavage complexes and the products can then be analysed by agarose gel electrophoresis.

Materials

***M.tuberculosis* Gyrase Assay Buffer:** 50 mM HEPES.KOH (pH 7.9), 100 mM potassium glutamate, 6 mM magnesium acetate, 4 mM DTT, 2 mM spermidine, 0.1 mg/mL albumin (supplied as 5X). Store at -20^{o}C or below.

Dilution Buffer: 50 mM Tris.HCl (pH 7.9), 5 mM DTT, 30 % (w/v) glycerol (supplied as 1X). Store at -20^{o}C or below.

Enzyme: *M.tuberculosis* Gyrase (see data sheet for concentration). Store at -80^{o}C. (NOTE 1)

Plasmid: Supercoiled pBR322 (supplied at 1 µg/µL). Store at -20^{o}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)

Other materials not included in the kit

SDS: 2% (w/v) sodium dodecyl sulphate in water

Proteinase K: 10mg/ml in water

Method

On ice, set up a MIX of (per assay): Assay buffer (6 µL of 5X buffer) (NOTE 2), supercoiled pBR322 (0.5 µL) and water (20.2 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 (but see NOTE 3).

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

Mix briefly.

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

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 for 60 minutes at 37 °C.

After incubation add 3 µL of 2% (w/v) SDS and 1.5 µL of 10mg/mL Proteinase K. (see NOTE 5)

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

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

Vortex vigorously ~5 secs and centrifuge for 2 minutes.

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

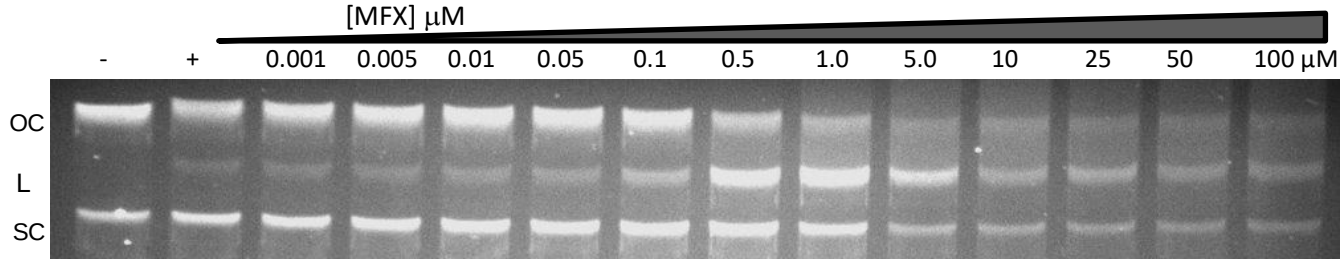
Run at 80V for approximately 2 hours

Stain with ethidium bromide (15 mins) , destain (5 – 10 mins) in water and visualise with a transilluminator or gel documentation system.

Example results

The gel below shows the results for a cleavage experiment with increasing amounts of the inhibitor moxifloxacin (MFX). The assay was set up as follows: a MIX was made consisting of 84 µL of Assay Buffer (5X), 7 µL supercoiled pBR322, 282.8 µL of water. 26.7 µL of the MIX was added to each tube then 0.3 µL of 100% DMSO or moxifloxacin (made up in 100% DMSO) and finally the dilution buffer or enzyme. In this case the enzyme was diluted 1 in 9 and 3 µL added per reaction (equivalent of 0.33µL of neat enzyme).

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	0.1	0.5	1	5	10	50	100	500	1000	2500	5000	10000
Final [Cpd] µM	0	0	0.001	0.005	0.01	0.05	0.1	0.5	1	5	10	25	50	100
DB (µL)	3													
Enzyme(µL)		3	3	3	3	3	3	3	3	3	3	3		



OC= nicked, open circular L=linear plasmid SC= supercoiled topoisomers

Notes

1) Enzyme is usually supplied at a particular number of cleavage units per microliter which is specified on the accompanying datasheet. This should give optimal cleavage with moxifloxacin.

2) 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. It is possible to use 5-10% (v/v) DMSO but this may lead to a loss in activity..

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) It is advisable to make up the diluted 2% (w/v) SDS fresh each day. However, this can be made by diluting from a stock of 10% (w/v) SDS which can be stored at room temperature.

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 should be run at about 4-5 V/cm. Gels are usually run until the bromophenol blue dye has run at least 6-7 cm. The gels shown were run in the

absence of intercalators but the inclusion of an intercalator (e.g. 5 μg/ml chloroquine) may help to resolve any topoisomers away from the linear band as the relaxed and supercoiled topoisomers will migrate together below any linear with a separate nicked, open-circular band above.

7) Once the reactions have been stopped with the GSTEB and mixed, it is possible to store them overnight at $4^{o}C$ or $-20^{o}C$ although it is preferable to load them on a gel as soon as possible.