Mycobacterium tuberculosis Gyrase Supercoiling 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. In this assay, the substrate is relaxed pBR322 which is supercoiled by the gyrase. These 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 gyrase.

Materials

***M. tuberculosis* Gyrase 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^{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 (supplied at a minimum 5 U/µL). (NOTE 1) Store at -80^{o}C.

Plasmid : Relaxed pBR322 (supplied at 1 µg/µL).

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 (6 µL of 5X buffer per assay) (NOTE 2), relaxed pBR322 (0.5 µL per assay) and water 20.2 µL). 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.

Add 0.3 µL of the appropriate solvent (e.g. water, 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^{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 (NOTES 6 to 9).

Run at ~75V for approximately 2 hours or until the blue dye front has run about 5cm or more.

Stain with 1 µg/mL ethidium bromide in water (15 mins), destain (5-10 mins) in water (NOTE 8) and visualise with a transilluminator or gel documentation system.

Example Results

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

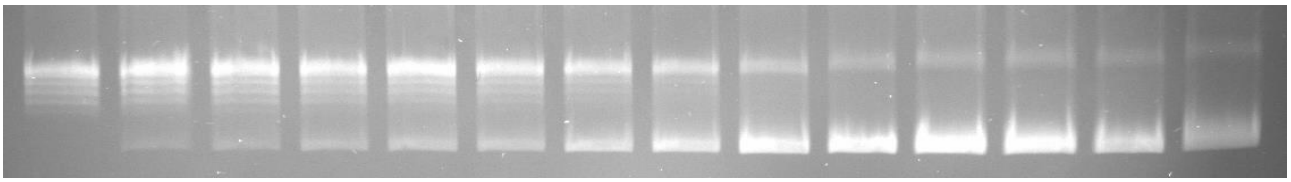
The gel below shows the results for a gel-based supercoiling experiment to determine the amount of enzyme to use in a subsequent inhibition assay. This is performed with relaxed pBR322 set up as follows: a MIX was prepared of 84 µL of Assay Buffer (5X), 7 µL relaxed pBR322, 4.2 µL DMSO, 282.8 µL of water.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

M. tuberculosis gyrase titration (volume neat enzyme added)

- 0.005 0.01 0.015 0.02 0.025 0.03 0.05 0.1 0.15 0.2 0.25 0.3 0.5 ul



In this case approximately 0.1 µL of the neat enzyme is required to give full supercoiling. It is not recommended to store the diluted enzyme as it can lose activity.

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

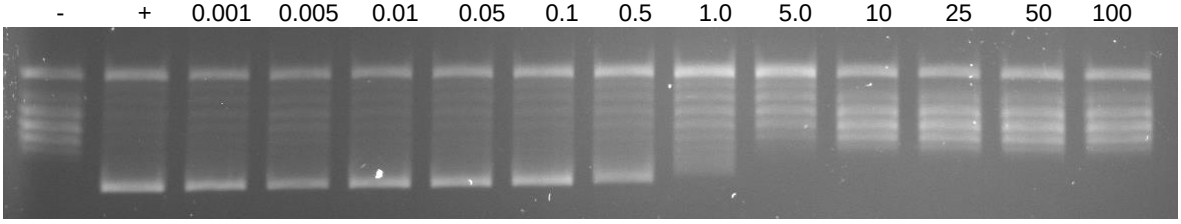
The gel below shows the results for a supercoiling inhibition experiment with increasing amounts of the inhibitor novobiocin (dissolved in DMSO). The assay was set up as follows: a MIX was made consisting of 84 µL of Assay Buffer (5X), 7 µL relaxed pBR322, 4.2 µL DMSO and 282.8 µL of water. 26.7 µL of the MIX was added to each tube then 0.3 µL DMSO or novobiocin and finally the dilution buffer (DB) /enzyme (using a dilution determined 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	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

[Novobiocin] µM

Mtb gyrase

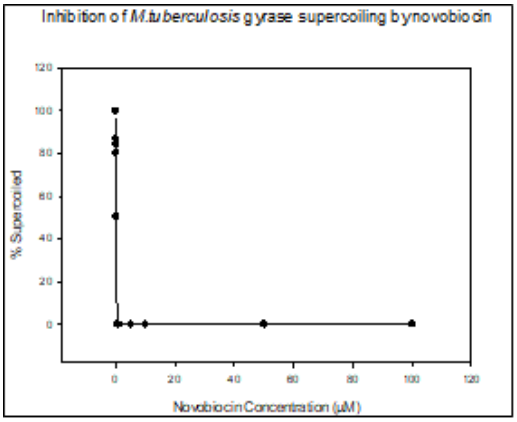
- + 0.001 0.005 0.01 0.05 0.1 0.5 1.0 5.0 10 25 50 100



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

The supercoiled bands for each sample in the gel were scanned and the percentage of supercoiling activity determined as compared to the positive (gyrase, no inhibitor) control. These were plotted against the inhibitor concentration and the IC_{50} calculated from a fitted curve.

Calculated IC_{50} 0.11 µM



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 DNA gyrase required to just supercoil 0.5 µg of relaxed pBR322 in 30 minutes at 37°C. 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 supercoiling (e.g. 90% supercoiling or just full supercoiling) 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.

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 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 **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 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 DNA gyrase.

6) Due to the high potassium glutamate content of the assay buffer, it is advised that samples are left in the wells for about 20 minutes before running the gel in order to improve gel resolution. The gels should then be run at a slower rate than samples with lower salt concentrations (4 V/cm).

7) 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.

8) 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.

9) 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.