Escherichia coli 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

***E. coli* Gyrase Assay Buffer:** 35 mM Tris.HCl (pH 7.5), 24 mM KCl, 4 mM $MgCl_2$, 2 mM DTT, 1.8 mM spermidine, 6.5% (w/v) glycerol, 0.1 mg/mL albumin (supplied as 5X). Store at -20°C or below.

Dilution Buffer: 50 mM Tris·HCl (pH 7.5), 100 mM KCl, 2 mM DTT, 1 mM EDTA, and 50% (w/v) glycerol (supplied as 1X). Store at -20°C or below.

Enzyme: *E. coli* gyrase (see data sheet for cleavage activity). (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 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: 10 mg/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 (17.5 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 (but see NOTE 3).

Add 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 30 minutes at 37°C.

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

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). (NOTE 5)

Vortex vigorously ~5 secs and centrifuge for 2 minutes.

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

Run at 90 V for approximately 90 minutes.

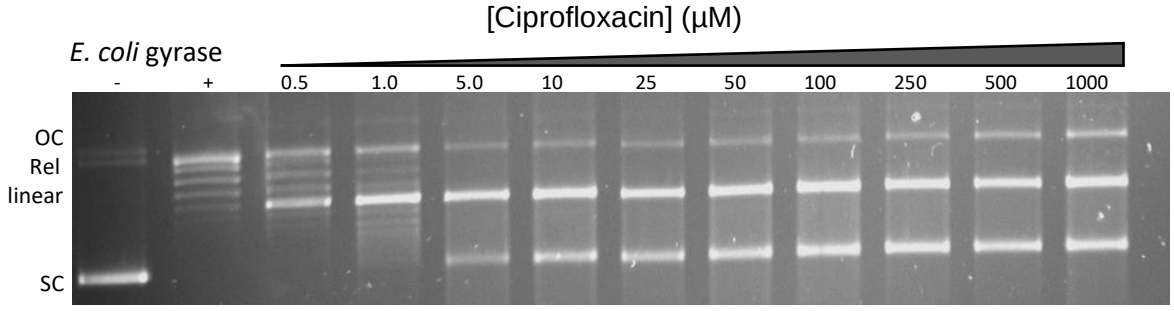
Stain in ethidium bromide (0.5 µg/mL for ~10-15minutes), destain (5 – 10 mins) in water and visualise with a transilluminator or gel documentation system. (see Note 6).

Example results

The gel below shows the results for a cleavage experiment with increasing amounts of the inhibitor ciprofloxacin (CFX). The assay was set up as follows: a MIX was made consisting of 72 µL of Assay Buffer (5X), 6 µL supercoiled pBR322, 210 µL of water. 24 µL of the MIX was added to each tube then 3 µL of 10% (v/v) DMSO or ciprofloxacin (made up in 10% DMSO) and finally the dilution buffer or enzyme.

DM10 = 10% DMSO

Sample	1	2	3	4	5	6	7	8	9	10	11	12
MIX (µL)	24	24	24	24	24	24	24	24	24	24	24	24
Initial [CFX] µM	DM10	DM10	5	10	50	100	250	500	1000	2500	5000	10000
Final [CFX] µM	0	0	0.5	1	5	10	25	50	100	250	500	1000
Dil. Buffer (µL)	3											
Enzyme (µL)		3	3	3	3	3	3	3	3	3	3	3



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

Notes

1) Enzyme is usually supplied at a particular number of cleavage units per microliter (see Certificate of Analysis for each lot number). This should give optimal cleavage with the stated compound (e.g. ciprofloxacin or another quinolone).

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 higher concentrations (e.g. up to 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. The CoA will state under what conditions the activity was ascertained.

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 addition of GSTEB and chloroform) 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 gyrase.

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 nicked, linear and supercoiled bands. Longer runs will give greater separation. The gel shown was run in the absence of intercalators so any relaxation activity will be shown by the presence of relaxed topoisomers. However, gels can be run in the presence of ethidium bromide (0.5 □g/mL) which helps to resolve any topoisomers away from the linear band which can make analysis of this band easier. Under these conditions, any relaxed topoisomers will migrate near to the supercoiled plasmid.

7) Once the reactions have been stopped with the GSTEB/chloroform and mixed, it is possible to store them overnight at -20^{o}C or 4^{o}C although it is preferable to load them on a gel as soon as possible. After storage they should be remixed, centrifuged briefly before loading.