Staphylococcus aureus Topo IV BisCat Decatenation Assay Kit

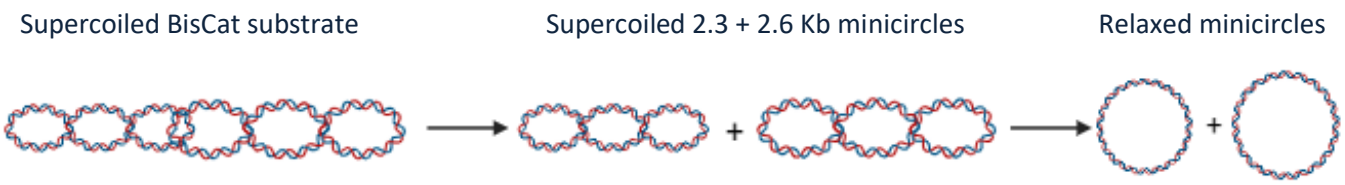
Introduction

S. aureus topo IV is a type II topoisomerase that can relax negatively or positively supercoiled DNA, and decatenate interlinked double-stranded DNA molecules. It functions as a hetero dimer and acts by generating DNA double- strand breaks with strand passage events changing the topology of the DNA. ATP is hydrolysed during the reactions.

The BisCat substrate consists of two supercoiled, singly linked minicircles of 2.3 and 2.6 kb which are decatenated to produce two supercoiled minicircle products. It provides greater sensitivity than the standard decatenation assay using kDNA as the substrate. In addition, each decatenation event leads to the conversion of one substrate molecule to products which is not the case with kDNA which consists of a network of minicircles. Since the activity is much higher with the singly-catenated substrate BisCat compared to DNA it provides a practical assay for examining decatenation activity of gyrase as well as determining the activity of compounds as inhibitors of this enzyme.

BisCat Decatenation Assay

The substrate consists of two supercoiled, singly-linked minicircles of 2.3 and 2.6 Kb. Type II topoisomerases such as human topo II can decatenate the substrate to produce two supercoiled minicircle products. As more enzyme is added the minicircles are then progressively relaxed.



Materials

***S. aureus* Topo IV Assay Buffer:** 50 mM Tris-HCl (pH 7.5), 350 mM potassium glutamate, 5 mM magnesium chloride, 5 mM DTT, 1.5 mM ATP, and 50 µg/mL albumin (supplied as 5X). Store at -20 °C or below.

Dilution Buffer: 50 mM Tris.HCl (pH 7.5), 1 mM DTT, 1 mM EDTA, 40% (w/v) glycerol (supplied as 1X). Store at -20 °C or below.

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

Substrate: BisCat DNA (supplied at 200 ng/µL) in 10 mM Tris-HCl, 1mM EDTA pH8. 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)

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), BisCat substrate (1 µL per assay) and water (19.7 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 °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 phase onto a 1% (w/v) agarose gel containing 0.5 µg/mL ethidium bromide. (NOTE 6-7)

Run at 90 V for approximately 90 minutes.

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 a gel-based decatenation experiment to determine the amount of enzyme to use in a subsequent inhibition assay. It is performed with BisCat substrate and set up as follows:

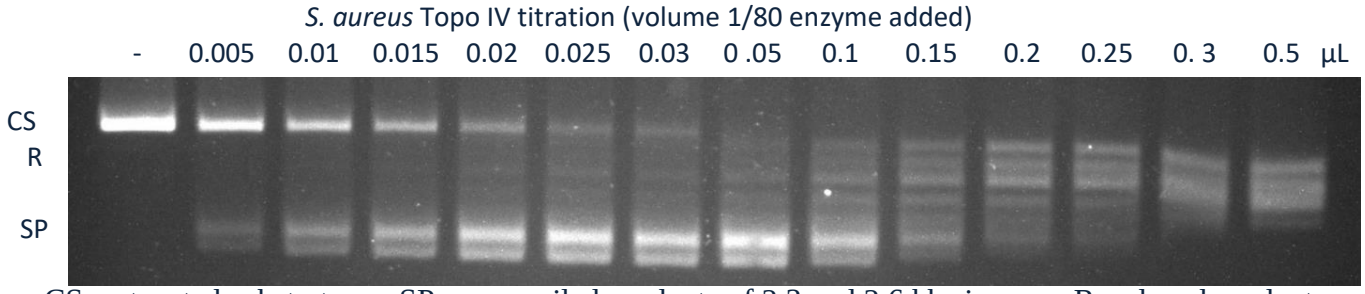
A MIX was prepared for 14 reactions with 84 µL of Assay Buffer (5X), 14 µL BisCat, 4.2 µL DMSO, 275.8 µL of water. 27 µL of this MIX was added to each tube.

The stock enzyme was diluted 1/80 in Dilution Buffer and serial dilutions of this were made. 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

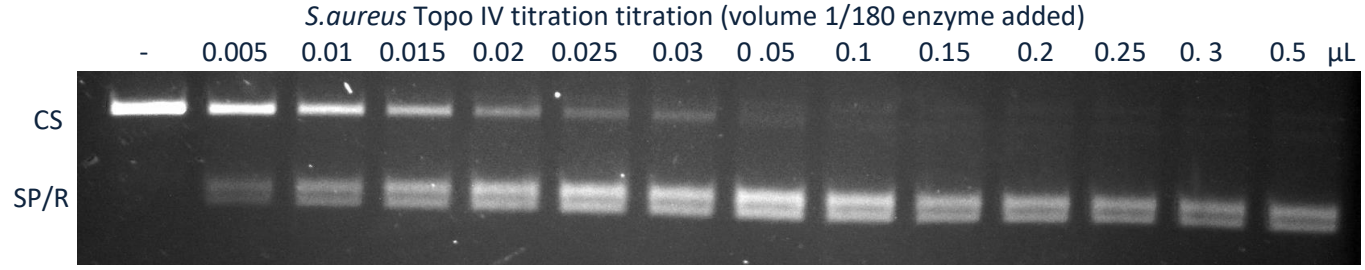
The gels below show the results of the decatenation reaction with the equivalent amount of neat enzyme added (e.g. reaction 7 contains 3 µL of a 1/100 dilution of the 1/80 diluted enzyme, equivalent to 0.000125 µL of neat enzyme). Identical samples were run on gels in the absence or presence of 0.5µg/mL ethidium bromide.

i) Samples run in the absence of intercalators



CS=catenated substrate SP=supercoiled products of 2.3 and 2.6 kb size R=relaxed products

ii) Samples run in the presence of ethidium bromide



In this case approximately 0.1 µL of the 1/80 diluted enzyme is required to give full decatenation in the presence of 1% (v/v) DMSO equivalent to 0.00125 µL of the original enzyme. It is not recommended to store the diluted enzyme as it can lose activity.

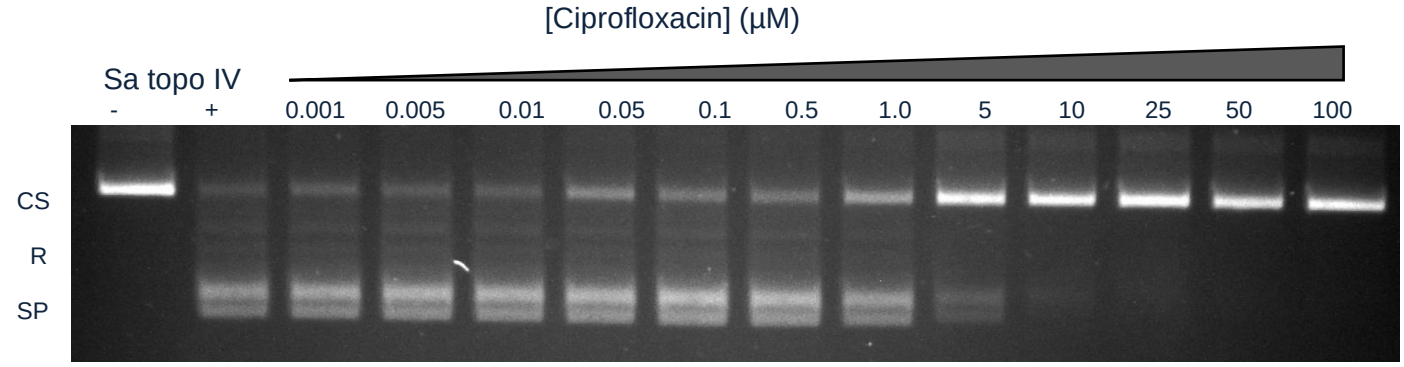
2. Determination of the inhibitory activity of a compound

The assay was set up as follows: a MIX was made consisting of 84 µL of (5X) Assay Buffer, 14 µL of BisCat DNA, 275.8 µL of water. 26.7 µL of the MIX was added to each tube then 0.3 µL of DMSO or ciprofloxacin and finally the dilution buffer (DB)/enzyme (using a dilution determined as 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	DMS0	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													
ENZ (µL)		3	3	3	3	3	3	3	3	3	3	3	3	3

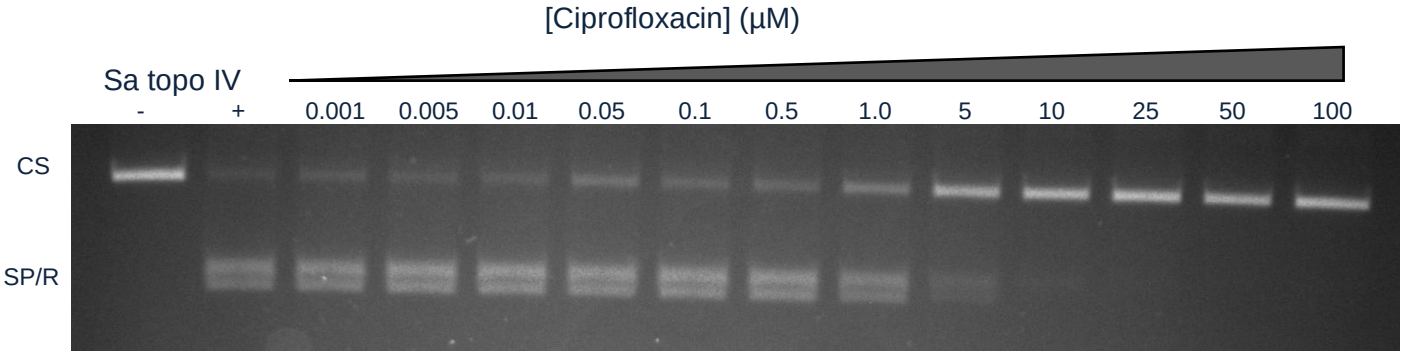
The gel below shows the results for a decatenation inhibition experiment with increasing amounts of the inhibitor ciprofloxacin (dissolved in DMSO).

i. Samples run in the absence of intercalators

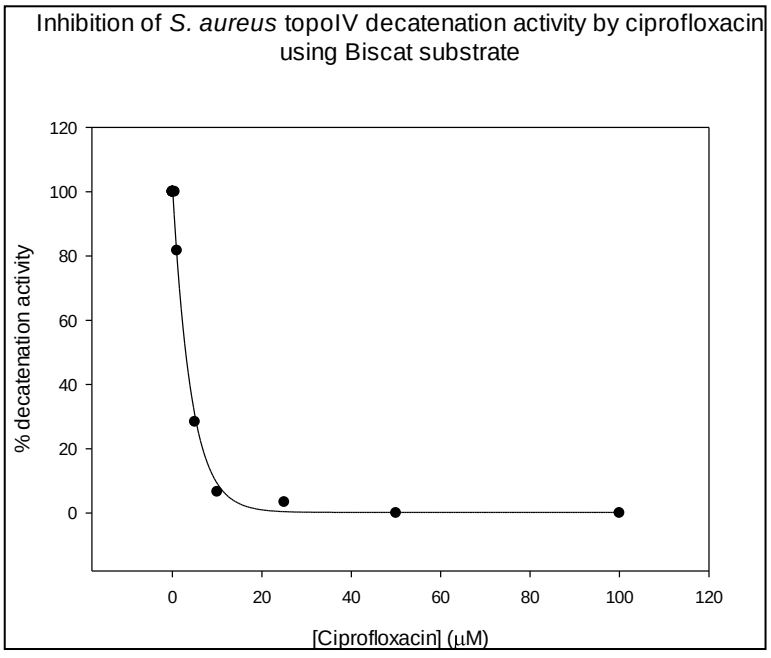


CS=catenated substrate SP=supercoiled products of 2.3 and 2.6 kb size R=relaxed products

ii. Samples run in the presence of ethidium bromide



The intensity of the two decatenated product bands were determined from the gel run in the presence of ethidium bromide and the intensity calculated as a percentage of the positive (enzyme only) control after subtraction of the negative (no enzyme) control. These percentages were plotted against the concentration of etoposide and the IC_{50} determined after curve fitting.
In this case an IC_{50} value of 2.95 μM was determined.



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 decatenate 200 ng of BisCat substrate 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 decatenation (e.g. 90% decatenation or 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) 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 5% 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.

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) 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 substrate and product bands. Longer runs will give greater separation. The gel shown was run in the presence of 0.5 µg/mL ethidium bromide but gels can be run in the absence of intercalators. In this case then the gel will need to be stained with ethidium bromide (0.5 µg/mL for ~10-15minutes) before detaining and visualisation.

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