

# *Escherichia coli* Topo IV BisCat Decatenation Inhibition Assay

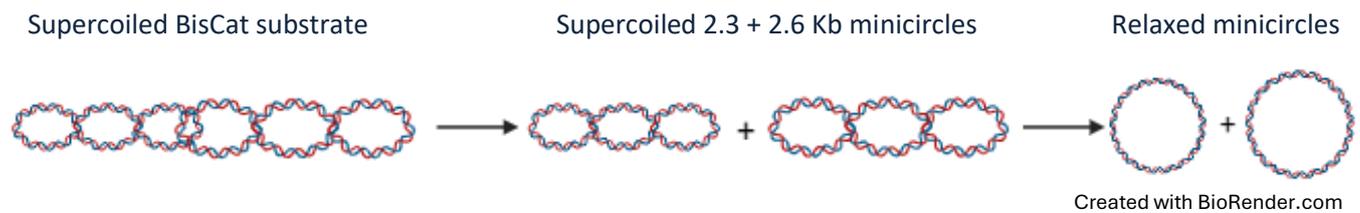
## Introduction

*E. coli* 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.

In this assay, the substrate consists of two interlinked supercoiled mini-circles of 2.3 and 2.6 Kb. The enzyme will release the two mini-circles from this bis-catenated substrate. The substrate will migrate more slowly than the 2 products during agarose gel electrophoresis. The advantages over the commonly used decatenation substrate kDNA, are that both substrate and products are visible on the gel and that each reaction will release one pair of products per reaction cycle whereas in reactions with kDNA the response is not linear as the mini-circles are part of a network. This BisCat substrate will be decatenated at lower enzyme concentrations and the products are then relaxed as more enzyme is added.

## **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

***E. coli* Topo IV Assay Buffer:** 40 mM HEPES.KOH (pH 7.6), 100 mM potassium glutamate, 10 mM magnesium acetate, 10 mM DTT, 1 mM ATP, and 50 µg/mL albumin (supplied as 5X). Store at -20 °C or below.

**Dilution Buffer:** 40 mM HEPES.KOH (pH 7.6), 100 mM potassium glutamate, 1 mM DTT, 1 mM EDTA, and 40% (v/v) glycerol (supplied as 1X). Store at -20 °C or below

**Enzyme:** *E. coli* 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 pH 8.0, 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  $\mu$ L)

On ice, set up a MIX of Assay Buffer (6  $\mu$ L of 5x buffer per assay) (NOTE 2), BisCat substrate (1  $\mu$ L per assay) and 19.7  $\mu$ L water. 26.7  $\mu$ L of MIX are required per assay.

Set up the appropriate number of 1.5 mL tubes and aliquot 26.7  $\mu$ L of MIX into each tube.

Add 0.3  $\mu$ L of the appropriate solvent (e.g. water, DMSO) to tubes 1 and 2 (NOTE 3).

Add 0.3  $\mu$ L of the test compounds to the other tubes as appropriate.

Mix briefly (gentle vortexing or pipetting).

Add 3  $\mu$ L of dilution buffer (NOTE 4) to tube 1.

Dilute the enzyme in dilution buffer then add 3  $\mu$ L of this to the remaining tubes (see NOTE 1).

Mix by gentle vortexing and incubate 30 minutes at 37  $^{\circ}$ C.

Stop reaction by adding 30  $\mu$ L of 2X GSTEBS and 30  $\mu$ L of chloroform/isoamyl alcohol (v:v, 24:1) (NOTE 5).

Vortex briefly ~5 secs and centrifuge for 1 minute.

Load 20  $\mu$ L of aqueous phase onto a 1% (w/v) agarose gel containing 0.5  $\mu$ g/mL ethidium bromide. (NOTE 6)

Run at 90 V for approximately 90 minutes.

Destain (5-10 mins) in water (NOTE 7) 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. This is performed with BisCat substrate and set up as follows:

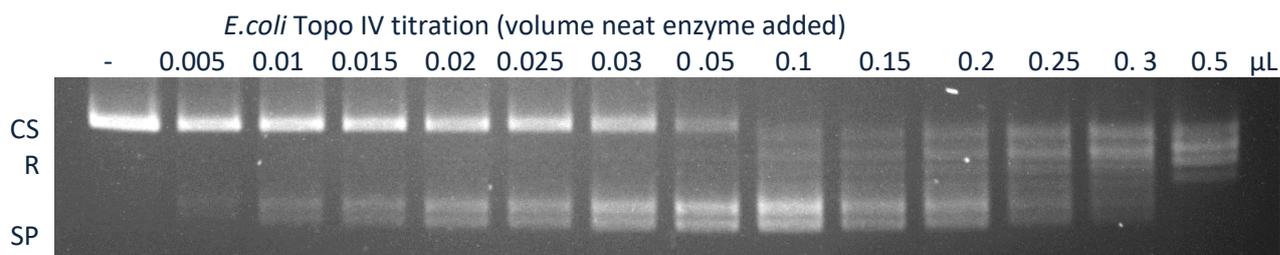
A MIX was prepared for 14 reactions with 84  $\mu$ L of Assay Buffer (5X), 14  $\mu$ L BisCat, 4.2  $\mu$ L DMSO, and 275.8  $\mu$ L of water. 27  $\mu$ L of this MIX was added to each tube.

The stock enzyme was diluted 1/10 with Dilution Buffer and then serial dilutions of this enzyme were made in Dilution Buffer (DB). Then 3.0  $\mu$ L added to the reactions.

Sample	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

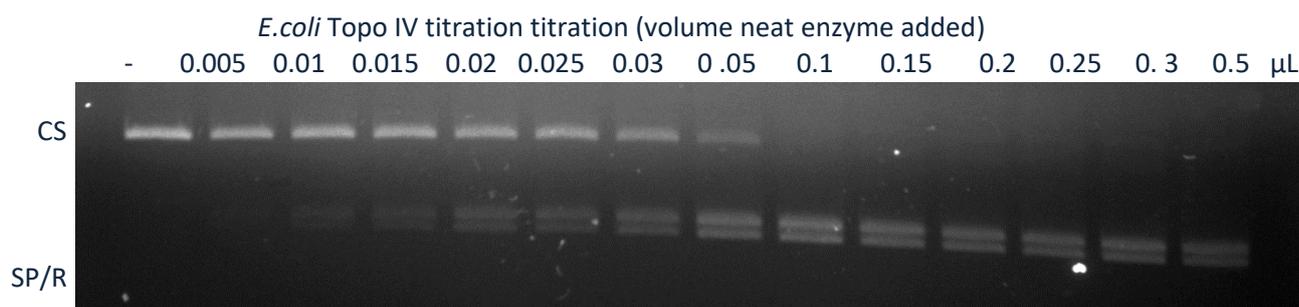
The gels show the results of the decatenation reaction with the equivalent amount of neat enzyme added (e.g. reaction 2 contains 3  $\mu$ L of a 1/600 dilution of the 1/10 diluted enzyme, equivalent to 0.0005  $\mu$ L of neat enzyme). Identical samples were run on gels in the absence or presence of 0.5  $\mu$ g/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 1/10 diluted enzyme is required to give full decatenation in the presence of 1% (v/v) DMSO equivalent to 0.01 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

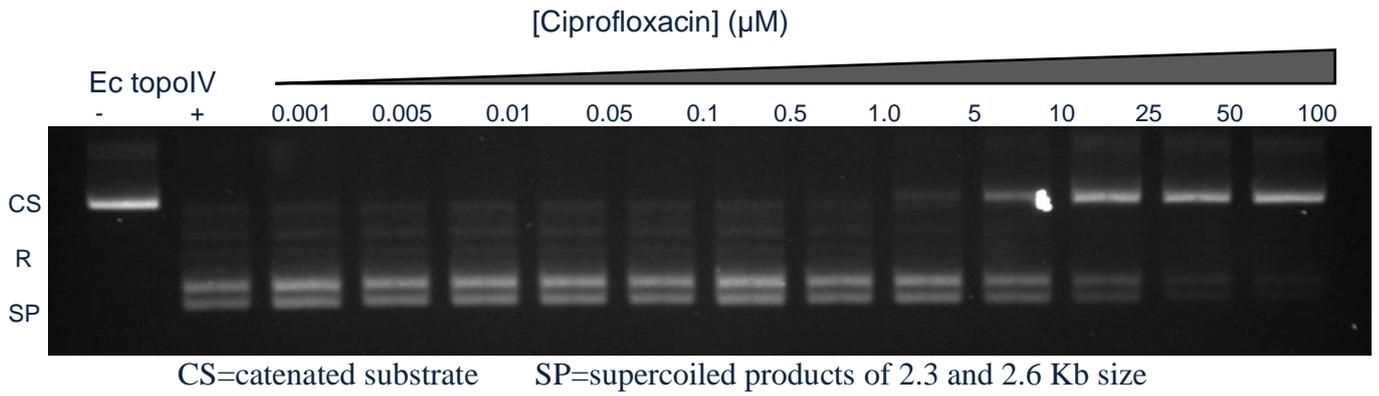
The assay was set up as follows: a MIX was made consisting of 84 μL of (5X) Assay Buffer, 14 μL of BisCat DNA, 275.8 μL of water. 26.7 μL of the MIX was added to each tube then 0.3 μL of DMSO or ciprofloxacin and finally the dilution buffer (DB)/enzyme (using a dilution determined as in example 1 above).

**Example table** (Note: This is an example table that can be used as a template for the testing of DNA Gyrase with inhibitors)

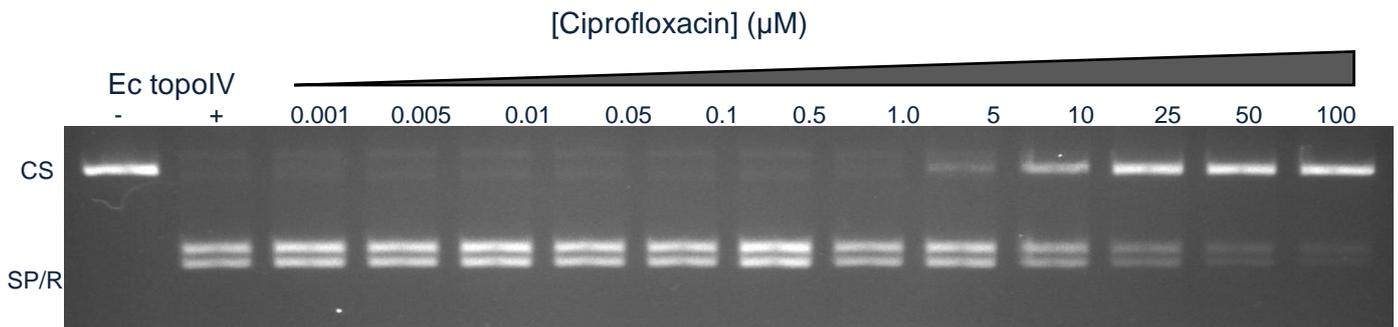
Sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14
MIX (μL)	26.7	26.7	26.7	26.7	26.7	26.7	26.7	26.7	26.7	26.7	26.7	26.7	26.7	26.7
Vol cpd (μL)	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Initial [Cpd] μM	DMSO	DMSO	0.1	0.5	1	5	10	50	100	500	1000	2500	5000	10000
Final [Cpd] μM	0	0	0.001	0.005	0.01	0.05	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	3	3

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

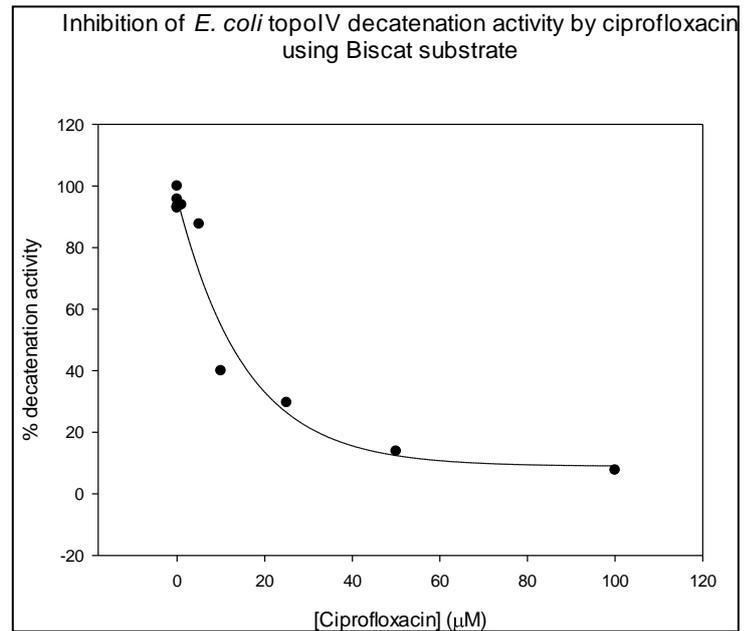
i. Samples run in the absence of intercalators



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  $\text{IC}_{50}$  determined after curve fitting. In this case an  $\text{IC}_{50}$  value of  $11.7 \mu\text{M}$  was determined.



## Notes

- 1) Enzyme is supplied in U/ $\mu$ L (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/ $\mu$ L) 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 pH7.8) or TBE (89 mM Tris, 89 mM borate, 2 mM EDTA pH 7.6) buffers can also be used. Gels can be run faster than relaxation assays, at 4-5 V/cm. Gels are usually run until the bromophenol blue dye has run at least 4-5 cm. This will be enough to see the decatenated mini-circles.
- 7) Gels can be run in the presence or absence of intercalators such as chloroquine or ethidium bromide. In the absence of intercalators, the decatenated mini-circles will usually run as two bands consisting of negatively supercoiled 2.3 and 2.6 Kb mini circles. As more enzyme is present a series of relaxed topoisomers are produced which migrate more slowly. When gels are run in the presence of ethidium, all the products, supercoiled and relaxed, will run as two supercoiled bands. The relaxed mini-circles will become positively supercoiled in the presence of the ethidium bromide and the positively and negatively supercoiled forms will co-migrate.
- 8) Once the reactions have been stopped with the GSTEB and chloroform and mixed, it is possible to store them overnight at 4°C or -20°C although it is preferable to load them on a gel as soon as possible. After thawing the samples should be mixed and centrifuged before loading.