Human Topoisomerase I Relaxation Assay

Introduction

Human topo I is a type I topoisomerase that is able to relax supercoiled DNA. In this assay, the substrate is supercoiled pBR322 which is relaxed by the enzyme. The 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 topo I.

Materials

Human Topo I assay buffer : 20 mM Tris.HCl (pH7.5), 200 mM NaCl, 0.25 mM EDTA, 5 % (v/v) glycerol (supplied at 10X). Store at -20^{o}C or below.

Dilution Buffer : 10 mM Tris.HCl (pH 7.5), 1 mM DTT, 1 mM EDTA, 50 % (v/v) glycerol, 100 µg/ml albumin (supplied as 1X). Store at -20^{o}C or below.

Enzyme : Human Topo I (see datasheet and specific batch information for concentration). (NOTE 1) Store at -80^{o}C.

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

Other materials not included in the kit

STEB: 40 % (w/v) sucrose, 100 mM Tris-HCl pH8, 10 mM EDTA, 0.5 mg/ml Bromophenol Blue.

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 should be 30 µL). (see Note 4).

On ice, set up a MIX of (per assay): Assay Buffer (3µL of 10x buffer) (NOTE 2), supercoiled pBR322 (0.5 µL) and water (23.2 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.

If the compounds are dissolved in DMSO then add 0.3 µL of 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 5) 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 STEB and 30 µL of chloroform/isoamyl alcohol (v:v, 24:1) (NOTE 6).

Vortex briefly ~5 secs and centrifuge for 2 minutes.

Load 20 µL of aqueous (upper blue) phase onto a 1% (w/v) agarose gel. (NOTES 8 and 9)

Run at 85V for approximately 2 hours (or 15 V overnight) (see NOTE 7).

Stain with ethidium bromide (1 µg/mL in water) (15 mins), destain (5-10 mins) in water (NOTE 6) and visualise with a transilluminator or gel documentation system.

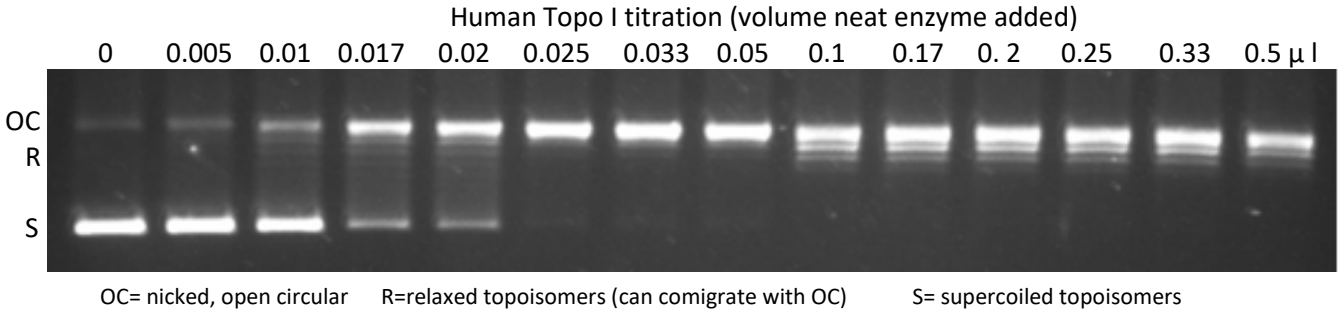
Example Results

1. Determination of enzyme activity.

The gel below shows the results for a gel-based relaxation experiment with supercoiled pBR322 to determine the amount of enzyme needed to just give full relaxation, in this case in the presence of 1% (v/v) DMSO. A mix was prepared of : 42 µL of (10X) Assay Buffer, 7 µL supercoiled pBR322, 4.2 µL of DMSO, 329 µL of water. 27 µL of this MIX was added to each tube. Serial dilutions of the enzyme were made in dilution buffer and then 3.0 µL added to the reactions. The table shows the dilutions and the equivalent amount of neat enzyme added. The table shows the dilutions and the equivalent amount of neat enzyme added.

Sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Dilution	DB	1/600	1/300	1/180	1/150	1/120	1/90	1/60	1/30	1/18	1/15	1/12	1/9	1/6
Vol (µL)	0	0.005	0.01	0.017	0.02	0.025	0.033	0.05	0.1	0.17	0.2	0.25	0.33	0.5

The gel shows the results of the relaxation reaction with the equivalent amount of neat enzyme added



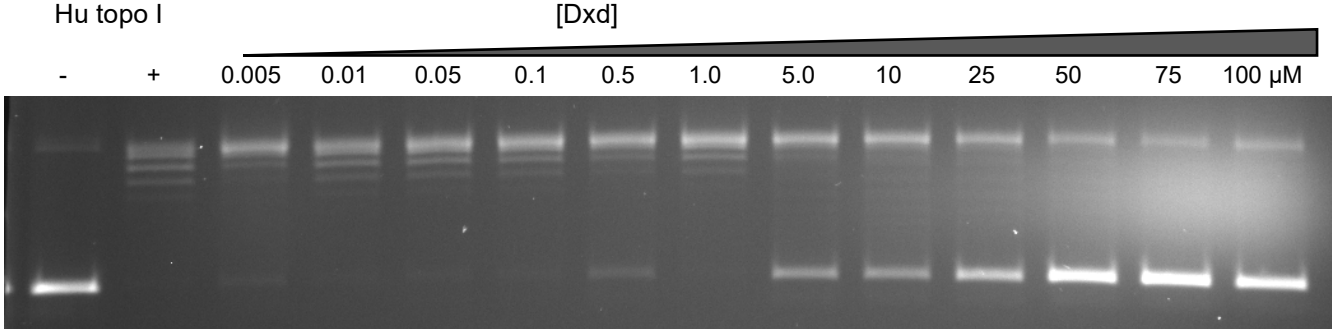
OC= nicked, open circular R=relaxed topoisomers (can comigrate with OC) S= supercoiled topoisomers

In this case approximately 0.025 µL of the neat enzyme (3 µL of 1/120 dilution) is required to give full relaxation. It is not recommended to store or freeze the diluted enzyme as it can lose activity.

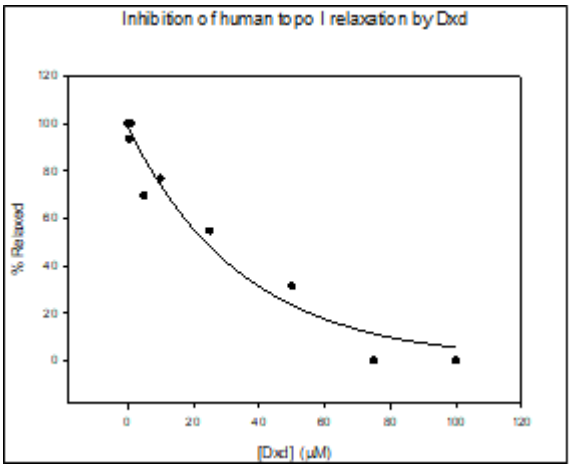
2. Determination of the inhibitory activity of a compound

The gel below shows the results for a relaxation inhibition experiment with increasing amounts of the inhibitor Dxd, a camptothecin analogue. The assay was set up as follows: a MIX was made consisting of 42 µL of (10X) Assay Buffer, 7 µL of supercoiled pBR322, 324.8 µL of water. 26.7 µL of the MIX was added to each tube then 0.3 µL DMSO or Dxd (dissolved in DMSO) and finally the dilution buffer (DB) or 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 [Dxd] µM	DMSO	DMSO	0.5	1	5	10	50	100	500	1000	2500	5000	7500	10000
Final [Dxd] µM	0	0	0.005	0.01	0.05	0.1	0.5	1.0	5.0	10	25	50	75	100
DB (µL)	3													
Enzyme (µL)		3	3	3	3	3	3	3	3	3	3	3	3	



The intensity of the supercoiled plasmid in each track was determined by scanning the gel image and the amount calculated as a percentage of that in the enzyme only track. These values were then plotted against the Dxd concentration.
The IC_{50} was calculated as 23.6 µM.



Notes

1) Enzyme is supplied at a minimum number of U/µL (see data sheet and certificate of analysis), where 1U is the amount of topo I required to just relax 0.5 µg of supercoiled pBR322, but it can be significantly more. Thus if you require a known amount of relaxation (e.g. 90% relaxed or just fully relaxed) then it is recommended to run an enzyme dilution series first, where the enzyme is titrated into the assay. The quoted activity (e.g. 5U/ 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 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 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) In this example 0.3 µL of compound in 100% DMSO has been added but the volume added can be increased and the percentage of solvent decreased if it is more practical e.g. adding 3 µL of 10% solvent in a total volume of 30 µL. In this case make the MIX of (per assay): Assay Buffer (3µL of 10x buffer), supercoiled pBR322 (0.5 µL) and water (23.2 µL). 27 µL of MIX are required per assay. Set up the appropriate number of 1.5 mL tubes and aliquot 27 µL of MIX into each tube. Then add 3 µL of 10% (v/v) DMSO to tubes 1 and 2. Add 3 µL of the test compounds to the other tubes as appropriate. Then proceed with the protocol described above adding the dilution buffer and enzyme.

5) The dilution buffer contains a high concentration of glycerol therefore the total dilution buffer added should not usually exceed 10% of the final volume.

6) Compounds can be removed from the reactions by extracting with water-saturated butanol before this step (the STEB/ chloroform step) if necessary. The upper (butanol) phase is then removed and discarded then STEB/chloroform added as normal. Usually however, the chloroform extraction is sufficient to remove the compounds as well as the enzyme.

7) The agarose gels shown in the results are run in Tris-acetate-EDTA buffer (40 mM Tris-acetate, 1 mM EDTA) but TPE (36mM Tris, 30mM sodium phosphate, 1mM EDTA pH7.8) or TBE (89mM Tris, 89mM borate, 2mM EDTA pH7.6) 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 6-7cm. This will resolve the relaxed and supercoiled bands. Longer runs will give greater separation if necessary.

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 STEB and mixed, it is possible to store them overnight at $4^{o}C$ or below.