Human Topoisomerase I Relaxation High Throughput Plate Assay

Introduction

Human topo I is a type I topoisomerase that is able to relax supercoiled DNA. This assay is based upon the fact that negatively-supercoiled plasmids form intermolecular triplex DNA more readily than do relaxed plasmids under some conditions. This assay overcomes some of the problems of gel-based assays, which are time consuming and are therefore inherently low-throughput. In this assay the substrate is supercoiled pNO1, a modified form of pBR322 which contains a 'triplex-forming sequence'. The assay can be used to determine the activity of compounds as inhibitors of topo I either as an initial screen or in determination of IC_{50} values.

Materials (supplied with the kit)

Human Topo I Assay buffer : 20 mM Tris.HCl (pH 7.5), 200 mM NaCl, 0.25 mM EDTA and 5 %(v/v) (Supplied as 10X).

Dilution Buffer : 10 mM Tris.HCl (pH 7.5), 1 mM DTT, 1 mM EDTA, 50 % (v/v) glycerol, and 100 µg/ml albumin (Supplied as 1X).

Plate Assay Wash Buffer: 20 mM Tris·HCl (pH 7.6), 137 mM NaCl, 0.005% (w/v) bovine serum albumin (acetylated), 0.05% (v/v) Tween-20 (supplied as 20X).

Plate Assay TF Buffer: 50mM sodium acetate (pH 4.7), 50mM sodium chloride, 50mM magnesium chloride (supplied as 20X).

T_{10} Buffer: 10 mM Tris-HCl pH8, 1 mM EDTA (supplied as 20X) (NOTE 1).

Enzyme : Human Topoisomerase I (see data sheet for concentration). Store at -80°C. (NOTE 2)

Plasmid: Supercoiled pNO1 (supplied at 1 µg/µL).

Oligo: TFO1 (supplied at 10 µM).

Plate: 96 well streptavidin coated black walled plates (store at 4 °C).

The enzyme must be stored at -80 °C. The other components can be stored at either -20 or -80 °C apart from the plate which should be kept at 4 °C.

DNA-detection dye (Promega Diamond dye supplied in 100% DMSO at 1,000x concentration). (see NOTE 11)

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).

Rehydrate the wells by adding 200 µl of 1X Wash Buffer.

Remove the Wash Buffer (NOTE 3) and immobilize 100 µl of 500nM TFO1 oligo in each well (5 µl of 10 µM TFO1 in 95 µl Wash Buffer (1X)), incubate for 5 minutes at room temperature and remove. Wash off excess oligo with 3 x 200 µl wash buffer then remove all the remaining wash buffer in the wells (NOTE 4).

Set up a MIX of assay buffer (3 µL of 10X buffer per assay) (NOTE 5), supercoiled pNO1 (0.75 µL per assay) and water (22.95 µL per assay). You will need 26.7 µL of MIX per assay.

Aliquot 26.7 µL of MIX into each well (NOTE 6).

Add 0.3 µL of the appropriate solvent to wells 1 and 2 and mix thoroughly by pipetting. Add 0.3 µL of the inhibitor. (NOTE 7)

Dilute the enzyme in dilution buffer to the appropriate concentration; add 3 µL of dilution buffer to well 1 and 3 µL of enzyme to well 2 and the inhibitor wells (NOTE 2).

Mix by pipetting, cover and incubate for 30 minutes at 37 °C.

Add 100 µL of TF Buffer to each well, mix thoroughly by pipetting and incubate for a further 30 minutes at room temperature to allow triplex formation (NOTE 8).

Remove all liquid from the wells and wash each well with 3 x 200 µL TF Buffer (1X) to remove unbound plasmid. Again remove all remaining liquid from the wells.

Add 200 µl of 1X DNA stain (diluted to 1X with T_{10} buffer) per well. Incubate for 10 - 20 minutes, **mix** and read in fluorescence plate reader (Ex: 495 nm; Em: 537 nm) (NOTE 11).

Example results

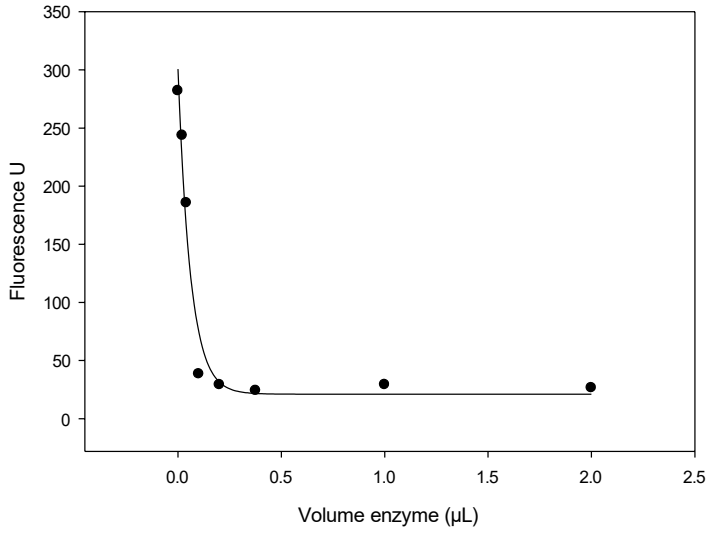
1. Determination of enzyme activity.

   In the experiment below the amount of enzyme needed to just fully relax the plasmid is determined in the presence of 1% DMSO, set up as follows: a MIX was prepared of 24 µL of Assay Buffer (10X), 6 µL supercoiled pNO1, 2.4 µL DMSO, 183.6 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
Dilution	DB	1/150	1/75	1/30	1/15	1/8	1/3	1/1.5
Vol. neat enzyme added (µL)	0	0.02	0.04	0.1	0.2	0.38	1	2.0
Reading	282	244	186	39	29	24	29	27

The table above and the plot below show the results of the relaxation reaction with the equivalent amount of neat enzyme added (e.g. reaction 8 contains 3 µL of a 1/1.5 dilution equivalent to 2 µL of neat enzyme)



In this case approximately 0.2 µL of undiluted enzyme (3 µL of a 1/15 dilution) would just give full relaxation.

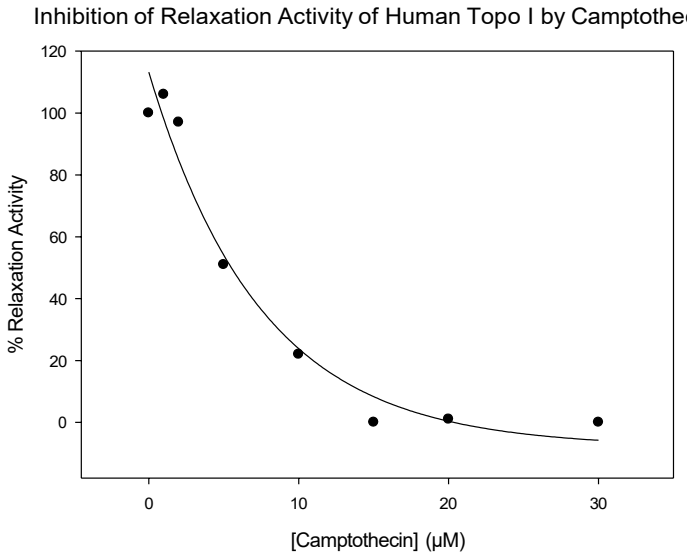
2. Determination of an IC_{50} value for camptothecin with human topo I

The results below show the inhibition of human topo I relaxation activity by increasing amounts of the inhibitor camptothecin (dissolved in 100% DMSO). The assay was set up as follows: a MIX was made consisting of 27 µL of (10X) Assay Buffer, 6.75 µL of sc pNO1, 206.55 µL of water. 26.7 µL of the MIX was added to each well then 0.3µL of camptothecin (or 100% DMSO for the negative (well 1) and positive (well 2) controls) added and finally the dilution buffer or enzyme (using a dilution determined as in example 1 above- 3 µL of a 1/15 dilution).

DM10= 10% (v/v) DMSO

Reaction #	1	2	3	4	5	6	7	8	9
MIX (µL)	26.7	26.7	26.7	26.7	26.7	26.7	26.7	26.7	26.7
Initial [Cpd] µM	DMSO	DMSO	100	200	500	1000	1500	2000	3000
Final [Cpd] µM	0	0	1	2	5	10	15	20	30
Dil. Buffer (µL)	3								
Enzyme (1/15 dil) (µL)		3	3	3	3	3	3	3	3
Reading	322.6	37.2	20.1	45.8	177.0	259.8	332.8	319.7	328.9
% activity		100	106	97	51	22	0	1	0

The % relaxation activity was calculated from the readings, plotted and an IC_{50} value calculated from the fitted curve.



The IC_{50} value was calculated as 5.4 µM

Notes

1) When diluting to 1X all buffers should be diluted in DNase-free (autoclaved) ultra pure water.

2) Enzyme is supplied at a minimum number of U/µL (see data sheet and certificate of analysis), where 1U is the amount of human topo I required to just relax 0.5 µg of supercoiled pBR322 in a standard gel-base relaxation reaction) but it can be significantly more. Thus if you require a known amount of relaxation (e.g. 90% relaxation) then an enzyme dilution series, where the enzyme is titrated into the assay, is recommended. The results of this assay will allow the determination of the amount of enzyme to put in the test assays. The plate assays may require more enzyme than in a regular gel based assay.

3) When removing the wash buffers, in order to save time, it is possible to tip the buffer out then tap the plate gently on absorbent tissue before pipetting off any excess buffer remaining in the wells.

4) It is important to remove all remaining wash buffer as it can give a high background signal and interfere with the enzyme reaction.

5) The volume of the assay mix can be varied but the final concentration of assay buffer should be 1X. For example, if no inhibitor/solvent (e.g. DMSO) is being added then the total volume of assay mix added to each well would be increased to 27 µl with water. Also see NOTES 8 and 10.

6) The assay buffer, plasmid and oligo should be kept on ice. It is recommended that the MIX is also kept on ice until aliquoted into the plates. Enzyme and dilutions must be kept on ice until added to the wells.

7) 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. 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 e.g. adding 3 µL of 10% solvent in a total volume of 30 µL (24 µL of MIX plus 3 µL of enzyme).

8) The formation of triplexes relies partly on the pH being lowered by the addition of TF buffer. 100 µl of TF buffer is required for a 30 µl reaction volume. If the reaction volume is doubled (i.e. 60 µl) then the volume of TF buffer must be increased. However, it is possible to use 140 µl of TF buffer in this case. (see NOTE 10)

9) While the readings produced may differ from those in the table, the positive control values should be at least 3-4X the negative control.

10) The method described above uses a reaction volume of 30 µL. However, it is possible to increase the volume which uses larger amounts of plasmid and so gives higher signals. It can also be useful if pipetting of larger volumes is necessary or convenient. In this case, the oligo TFO1 is immobilised as above, the assay performed using a final volume of 60 µL and 140 µL of TF buffer are added to stop the reaction and form the triplexes. Otherwise, the assay is performed as above.

11) It is important to mix the samples after the incubation with the DNA-detection dye or there will be a much lower difference between positive and negative readings. Dilute only enough dye for the assays being run as it cannot be stored for long periods diluted. It is important to protect it from light. This applies both to the 1000X stock and to the reactions containing the dye as much as is possible.