Human tyrosyl-DNA phosphatase 2 microtitre plate assay kits

Introduction:

TDP2 is a tyrosyl-DNA phosphatase that safeguards cellular genomes against the abortive activity of DNA topoisomerase II (Top2). In the case where Top2 is unable to reseal the DNA double-stranded break (DSB) causing a cleavage complexes to be stabilised, cellular repair enzymes are required for their removal. TDP2 is one such enzyme, capable of hydrolysing the covalent phosphotyrosyl bond between the active site of Top2 and the 5'-terminal phosphate at the DSB, in a process termed DNA end processing. In this assay, we replicate this process with the use of a fluorescently-tagged phosphotyrosine oligo. It can be used to determine the activity of compounds as inhibitors of TDP2.

Materials

Assay Buffer (supplied at 10X concentration): Final concentrations in assay are 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 80 mM KCl, 0.05% (v/v) Tween-20, 1 mM DTT.

Dilution Buffer: 25 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM DTT, 50% Glycerol.

Substrate: 4 µM Fluorescently-tagged Phosphotyrosine Oligo – (5'-(6-FAM NHS)(5'-pTyr)GATCT(3'-BHQ-1)-3' Mw = 2634.8

Unit Definition: 1 unit is defined as the amount of enzyme required to convert 20 pmoles of substrate in 30 minutes at 37 °C.

Plate: TDP2 assays are performed in a black 384-well plate. Ex = 485 Emm = 520

Assay Protocol: (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 20 µL.

1. On ice, set up a MIX of (per assay): Assay Buffer (2 µL of 10X) and H_2O (12 µL). 14 µL of MIX is required per assay.
2. Dilute the TDP2 enzyme in Dilution Buffer (Note 1).
3. Aliquot 14 µL of MIX into the appropriate wells.
4. Pipette either 1 µL Dilution Buffer (Control) or diluted TDP2 into the 384-well black plate and mix gently by pipetting.
5. Place the plate into the plate reader at 37 °C. Monitor the fluoresecence (Ex = 485 Emm = 520).
6. After 5 minutes, the assay run is paused and 5 µL of 4 µM substrate is added to each well, the run is then continued at 37 °C for a further 30 minutes.

Example Results

1. TDP2 Serial Dilution

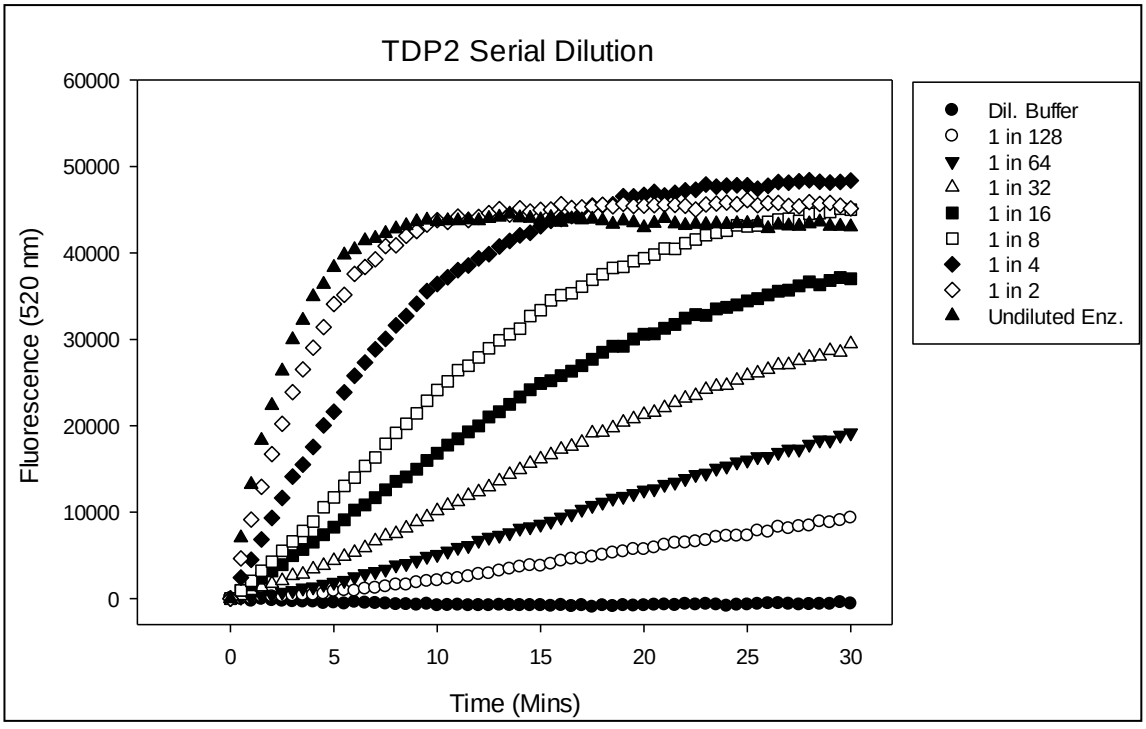
Example TDP2 Serial Dilution Setup:

	Mastermix (µL)	Dilution Buffer (µL)	TDP2 (µL)	Substrate (µL)
1	14	1		5
2	14		1 (1/128)	5
3	14		1 (1/64)	5
4	14		1 (1/32)	5
5	14		1 (1/16)	5
6	14		1 (1/8)	5
7	14		1 (1/4)	5
8	14		1 (1/2)	5
9	14		1 (Neat)	5

The graph below shows the results for the plate-based assay with a serial dilution of TDP2. It was set up as follows: a MIX was prepared of 32 µL Assay Buffer (10X) and 192 µL H_2O. 14 µL of MIX was added to each well.

Serial dilutions of the enzyme were made in dilution buffer. Then 1 µL added to the reactions.

The plate was incubated at 37 °C to allow the plate to equilibrate. The assay was initiation with the addition of 5 µL of substrate.



Result. In the experiment above the stock enzyme was fully active at an approximately 1 in 8 dilution, equivalent to 0.125 µL per assay.

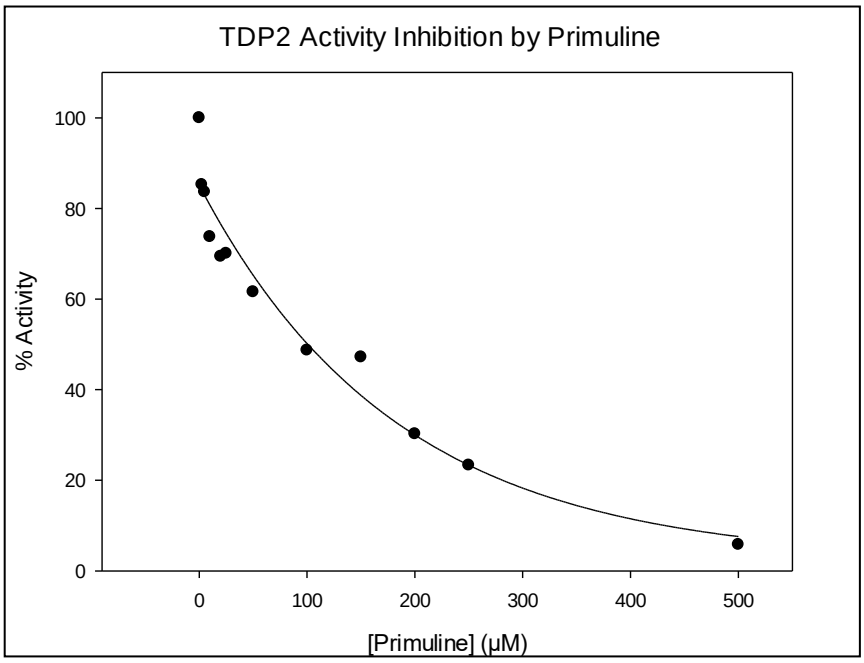
2. Determination of the inhibitory activity of a compound

The assay below shows the results for an TDP2 inhibition experiment with increasing amounts of inhibitor. The assay was set up as follows: a MIX was prepared of 32 µL Assay Buffer (10X) and 176 µL dH_2O. 13 µL of MIX was added to each well, then 1 µL DMSO/Inhibitor and 1 µL Dilution buffer or enzyme (in this case at a 1 in 8 dilution as in (1) above) was added. The plate was incubated at 37 °C to allow the plate to acclimatise. The assay was initiated with the addition of 5 µL of substrate.

3.

Sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
MIX (µL)	13	13	13	13	13	13	13	13	13	13	13	13	13	13	13	13
Vol cpd (µL)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Initial [Cpd] µM	H_2O	H2O	H2O	H2O	10000	5000	4000	3000	2000	1000	500	400	200	100	50	10
Final [Cpd] µM	0	0	0	0	500	250	200	150	100	50	25	20	10	5	2.5	0.5
DB (µL)	1	1														
Enzyme (µL)			1	1	1	1	1	1	1	1	1	1	1	1	1	1

4.



IC_{50} - 100 µM.

Notes

1) The enzyme is usually supplied at a minimum number of 5 U/µL (see data sheet or certificate of analysis), where 1 U is the amount of TDP2 required to convert 20 pmoles of substrate in 30 minutes at 37 °C. 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 if solvents are to be included.

2) The TDP2 enzyme may show sensitivity to solvents at higher concentrations, for example we do not recommend using more than 2 % (v/v). An enzyme titration must be done in the actual assay conditions to determine the effect of the solvent.