S.aureus Topoisomerase IV ATPase Assay

Introduction

Hydrolysis of ATP is essential for the activity of topo IV and inhibition of the ATPase activity is the mechanism by which compounds such as coumarins (e.g. novobiocin) inhibit the enzyme. The activity is stimulated by binding DNA. This assay links the hydrolysis of ATP by gyrase to the conversion of NADH to NAD^+ which can be measured by a change in absorbance at 340nm.

Materials

***S. aureus* topoIV Assay Buffer** (supplied as 5X)**:** 50 mM Tris.HCl (pH 7.5), 5 mM magnesium chloride , 5 mM DTT, 350 mM potassium glutamate, 0.05 mg/mL albumin. Store at -20°C or below.

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

Enzyme: *S. aureus* topoIV (500 nM concentration: final concentration in assay will be 50 nM). Store at -80°C. It is recommended to avoid repeated freeze-thaw cycles so if this is likely to be the case then the enzyme should be aliquoted.

Plasmid: Linear pBR322 (supplied at 1 µg/µL: 350nM). Store at -20°C or below.

ATP: 30 mM. Store at -20°C or below.

Linked assay reagents

Phosphoenol pyruvate (PEP): 80 mM in water. Store at -20°C or below.

Pyruvate kinase/lactate dehydrogenase (PK/LDH): Stock. Store at -20°C or below.

NADH: 20 mM in water. Store at -20°C or below and protect from light.

All the materials above are supplied with the kits. Other materials/equipment NOT supplied with the kits but required are:-

Flat-bottomed, clear 96-well microtitre plates.

Spectrophotometer plate reader capable of continuous reading at OD 340nM

Ultra pure water

Method

Set up Assay Mix of Assay Buffer (20 µL of 5X buffer per assay), linear pBR322 (3 µL per assay), 1 µL PEP, 1.5 µL PK/LDH, 2 µL NADH and 45.8 µL water. 73.3 µL of Assay Mix are required per assay.

Add 73.3 µL of Assay Mix into the wells of the microtitre plate. (NOTE 1)

Add 10 µL of water or DMSO to the positive and negative control wells (e.g. wells 1 and 2) and mix. (NOTE 2; NOTE 3)

Add 10 µL of inhibitors to the test wells and mix.

Add 10 µL of Dilution buffer to well 1 (negative control) and mix.

Add 10 µL of enzyme to well 2 (positive control) and to the test wells. Mix

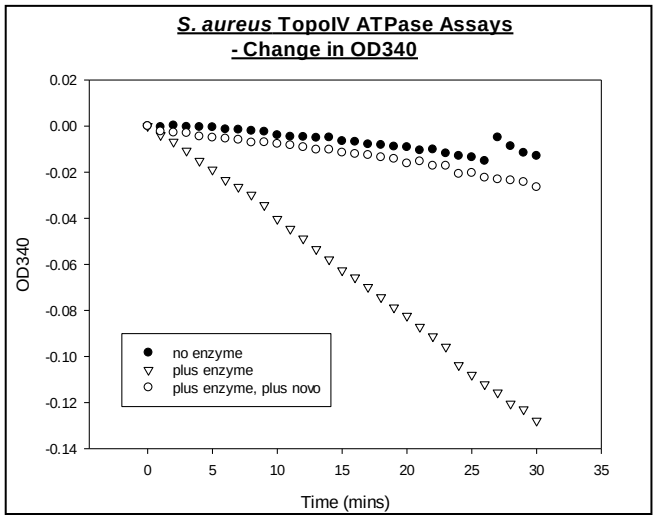
Put plate in plate reader and monitor absorbance at OD 340 nm for 10 mins at 37°C.

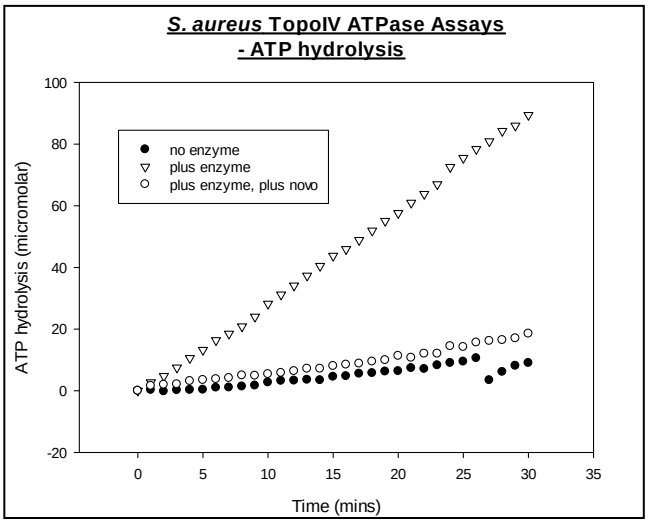
Stop the plate reader, remove the plate and add 6.7 µL of ATP to each well. This starts the reaction.

Return to plate reader and monitor absorbance at OD340 nm for up to 60 minutes at 37°C.

Results

The output from the plate reader is usually a change (decrease) in OD340 with time (see NOTE 4). This can be converted to ATP hydrolysis rates using an extinction coefficient of 6.22 mM^{-1}. cm^{-1} for NADH (and assuming 1 NADH mol. equiv. to 1 ATP mol). The path length may vary but a 100 µL reaction in a 96-well microtitre plate in our system was 0.23 cm (e.g. a change in OD of 0.005/min equates to an ATP hydrolysis rate of 0.0035 nM/min). Inhibitors of the topoisomerase ATPase activity will result in a reduced (or no) rate of ATP hydrolysis and therefore in a reduced drop in OD340.





Notes

1) The components of the Assay Mix are moderately stable at room temperature and the plate itself can be set up at room temperature. However, it is better to keep the Assay Mix on ice prior to aliquoting into the wells.

   Example of Assay Mix

Reagent	Volume per assay	Example for 16 assays	Initial concentration of reagent	Final concentration of reagent
Assay Buffer	20 µL	320 µL	5X	1X
Linear pBR	3 µL	48 µL	350 nM (1 mg/mL)	10.5 nM
PEP	1 µL	16 µL	80 mM	0.8 mM
PK/LDH	1.5 µL	24 µL		
NADH	2 µL	32 µL	20 mM	0.4 mM
Ultra pure water	45.8 µL	732.8 µL		
TOTAL	73.3 µL	1172.8 µL		

2) The negative control consists of dilution buffer in place of enzyme. The positive control contains enzyme but no inhibitor. Both controls have either water or DMSO in place of inhibitors depending on the solvent used for the inhibitors.

3) The DMSO/inhibitors and the dilution buffer/enzyme should be mixed with the pipette tips on adding to the wells either by stirring, pipetting up and down or a combination of stirring/pipetting.

4) The OD at 340 nM will drop during the reaction as NADH which absorbs at 340 nM is converted to NAD.

Schematic of linked assay