

Phosphatase Assays (using 32P-Phosphorylase a as substrate)

Buffer A:

50 mM Tris, pH 7.5
0.1 mM EGTA
0.1% 2-mercaptoethanol
1 mg/ml BSA
2 mM MnCl₂

Buffer B:

50 mM Tris, pH 7.5
0.1 mM EGTA
0.03% Brij-35

Phos. a

Dilute aliquot in 0.1 ml 75 mM caffeine + 0.4 ml Buffer B

Note: caffeine can be stored at room temp. covered in foil (light-sensitive), but Buffer A and Buffer B must be made up fresh each day.

PP1: Check several dilutions to find the one that releases 5-10% of the total counts (usually somewhere between 1:50,000 and 1:200,000)

Phosphatase Assay:

10 ul PP1 (diluted in Buffer A)
10 ul Buffer B (containing desired inhibitors/peptides)
10 ul 32P-Phos. A to start reaction (10 min at 30C)

Stop reaction by adding 100 ul of ice-cold 20% TCA and vortex tubes.
Centrifuge for 5 min and remove 120 ul for counting.

Inhibitors:

When checking a new protein for inhibitory activity against PP1, test the following concentrations:

10, 5, 1, 0.5, 0.1, 0.05 nM protein

Since the protein will be diluted in Buffer B and then added as 1/3 the total volume of the assay, make up the following serial dilutions of the protein in Buffer B:

30 nM
5 nM
1 nM
0.5 nM
0.1 nM
0.05 nM

When diluted by 1/3 they give the final concentrations listed above.

To set up the assay, pipette 10 ul of PP1 into each tube, then add 10 ul of the appropriate stock in Buffer B (control = Buffer B alone) and place tubes in the 30C water bath. When they're all in there, start adding 10 ul of ³²P-Phos a at 10 second intervals and start the timer. After 10 min start adding 20% TCA to the tubes at 10 second intervals to stop the reaction. Centrifuge and count 120 ul.

Calculations:

Count 10 ul of ³²P-Phos a in 3 tubes for your TOTAL

Assay 10 ul Buffer A (no PP1) + 10 ul Buffer B (no inhibitor) + 10 ul Phos a for your BLANK

Assay 10 ul PP1 + 10 ul Buffer B (no inhibitor) + 10 ul Phos a for your MAX PP1 ACTIVITY

Do assays in duplicate or triplicate and average the counts (throwing out obvious deviations). For final data, average at least three of these experiments.

$ACTIVITY = (COUNTS - BLANK)/TOTAL.$

$\% \text{ Inhibition of PP1} = 1 - ACTIVITY/MAX \text{ PP1 ACTIVITY}$