Satoru Mochida

Protein phosphatase assay

This protocol describes the standard strategy for measuring Ser/Thr protein phosphatase (PPase) activity in our laboratory using an artificial substrate (ex. Fzy-S50), a recombinant protein kinase (ex. Cdk) and [γ-^{32}P]-ATP. This protocol can be modified/utilized to measure various PPase activity of your interest by changing substrate and kinase.

This protocol consists from 3 parts.

1, Purification of substrate protein

2, Phosphorylation of substrate

3, Phosphatase assay in Xenopus egg extract

Reference

1, Mochida and Hunt, 2007, Nature, 449(7160), 336-340, PMID 17882219

2, Mackintosh C. & Moorhead G. *Protein phosphorylation: A practical approach* (ed. Hardie D.G.) pp 153-181 (Oxford University Press.1999)

Satoru Mochida

Purification of substrate protein

This protocol uses an artificial substrate, which is composed of tag protein (such as maltose binding protein) and a short peptide sequence derived from native phospho-protein.

Artificial substrate

Fuse 25a.a.-length phospho-peptide sequence to MBP or GST expression vector. Phosphorylation site should be at the center of this peptide. I strongly recommend you to start with 5 or more different peptide sequences. Because some sequences are not expressed in bacteria or are not phosphorylated efficiently *in vitro*. And I do NOT recommend putting more than 1 phosphorylation site in each substrate, as different sites could be dephosphorylated by different PPases.

Materials

BL21(DE3) strain + pLysS +pMALc2-FzyS50)
Amylose resin (New England Biolab)

Protein Expression

1 L of LB media supplemented with ampicillin & chloramphenicol
Add 1 mM IPTG to your culture and incubate it for another 3 hrs @37 °C

Protein purification

Collect bacterial cells & wash cell pellet once in PBS
Suspend them in 25 ml of Extraction buffer +lysozyme
Freeze this solution using liquid nitrogen
Thaw cell pellet and incubate it in 37°C water bath for 15 minutes
Cool it down on ice
Sonicate it for 20-30 seconds 3-4 times (until no viscosity)
Centrifuge @10 krpm for 15 minutes @4 °C
Apply the supernatant to 5 ml of amylose resin
Rotate the mixture for 0.5 hr @4°C
Wash the beads 3 times with the Extraction buffer
Add 5 ml of Elution buffer to the beads and rotate for 10 minutes @R.T.
Retrieve eluate
Repeat elution twice more
Concentrate 15 ml of eluate (5 ml x3) into 1-2 ml using a centrifugal filter unit
Dialyze it 3 times against Elution buffer
Measure protein concentration
Stock it in 1-mg aliquots @-20°C
You would get 10-20 mg substrate from 1L bacterial culture

Extraction buffer : 20 mM Tris-Cl pH7.5, 150 mM NaCl, 1 mM DTT, 1 mM PMSF, 2 mM Benzamidine, 1 mM EDTA, 1 mM EGTA, 0.1% Tween-20

Elution buffer : 20 mM Tris-HCl pH7.5, 150 mM NaCl, 10 mM maltose

Satoru Mochida

Phosphorylation of substrate

Materials

1 mg of substrate protein
Active recombinant kinase
[γ-^{32}P]-ATP

Phosphorylation reaction

[Reaction mix]

1 mg	Substrate
1-5 micro-g	Kinase (depends on how active your kinase is)
5 micro-L	[γ-^{32}P]-ATP
40 micro-L	5x kinase buffer
To 200 micro-L	Distilled/deionized water

Incubate your reaction mixture at 37°C overnight (longer than 12 hrs)
Add 200 µl of amylose resin (New England Biolab) to the reaction mix
Rotate for 15 minutes
Wash beads 5 times in Wash buffer
Add 200 micro-L of Elution buffer to your beads
Rotate for 10 minutes
Retrieve 1st eluate to a new tube
Repeat elution twice more, resulting in 600 micro-L of eluate
Remove free ATP by using a filter unit and Wash buffer
(until radio activities of flowthrough does not change)
Concentrate substrate solution into 100 micro-L
Measure its radioactivity using a liquid scintillation counter
If successful, your preparation would have 100 ~200 kcpm/micro-L
Freeze in 10-20 micro-L aliquots @-20°C

5x CDK kinase buffer : 0.1 M Hepes-Na pH7.8, 50 mM $MgCl_2$, 75 mM KCl, 5 mM EGTA, 25 mM NaF, 0.1 M β-glycero PO_4, 50 micro-M ATP, 5 mM DTT (Very low concentration of cold ATP is to get highly radio-active substrate)

[γ-^{32}P]-ATP : we use PerkinElmer NEG502A, 3000 Ci/mmol, 10 mCi/mL

Wash buffer : 20 mM Tris-Cl pH7.5, 150 mM NaCl, 0.05% Tween-20

Elution buffer : Wash buffer +10 mM maltose

If your [γ-^{32}P]-ATP is old, do increase its amount used accordingly.

Satoru Mochida

Phosphatase assay in *Xenopus* egg extract

Materials

Xenopus egg extract (or your PPase source)
Phosphorylated substrate

Reaction pre-mix for 10 assays

200 kcpm	PPase substrate
1 micro-L	Okadaic acid* (100 micro-M in DMSO) or DMSO
To 10 micro-L	Buffer (20 mM Tris-Cl pH7.5, 150 mM NaCl)

PPase reaction

Mix egg extract (3 micro-L) and reaction pre-mix (1 micro-L)
Incubate it @23°C for 4 minutes
Add 20 micro-L of 10 % trichloroacetic acid to your reaction
Vortex it for 5 seconds (you see white protein precipitate)
Incubate it on ice for longer than 10 minutes
(You can leave them on ice until all samples become ready)
Centrifuge it for 5 minutes @13 krpm @4°C
Retrieve all supernatant into a new tube, while trying not to take precipitate
Centrifuge it for another 10 minutes @13 krpm @4°C
Retrieve 15 micro-L of its supernatant into a new tube
Add 20 micro-L of 5% (w/v) ammonium molybdate** (in 0.5M H_2SO_4)
Add 80 micro-L of organic solvent mix***
(2-methyl-1-propanol : heptane = 50 : 50, water-saturated)
Vortex it vigorously for 30 seconds
Centrifuge it for 1 minute @13 krpm
Take 50 micro-L of organic (upper) phase to a scintillation tube with appropriate scintillant (we use Ecoscint A)
Shake it well by hand
Measure radioactivity using a scintillation counter

* 2.5 micro-M of okadaic acid can suppress most of PP2A activity in *Xenopus* egg extract. You need very high concentration of this kind of inhibitor, because, in crude cell extracts, PPases are expressed at concentrations much higher than their reported IC_{50} values.

** I use SIGMA #A7302, but products from other suppliers would work too.

*** Molybdate/organic solvent extraction method is introduced in detail in Mackintosh C. & Moorhead G. *Protein phosphorylation: A practical approach* (ed. Hardie D. G.) pp 153-181 (Oxford University Press.1999).

IMPORTANT !!! It is crucial to keep protein concentration as high as *in vivo*. Because simple dilution of cell extract disrupt physiological regulation of PPases by unknown mechanism.