In-Vitro Kinase Assay

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Protocol Information

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Category
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Title
In-Vitro Kinase Assay

Overview
Kinase Assays: Protocol for Src-family protein tyrosine kinase assays utilizing enolase as an exogenous substrate.

Material
Kinase Lysis Buffer (200mL)

1.5% Nonidet P-40
0.45% sodium deoxycholate
10% glycerol
25mM Tris, pH 8
150mM NaCl
5mM EGTA (0.38g)
10mM NaF (0.084g/200ml)
10mM Na2MoO4 (0.484g/200ml)
50uM phenylarsine oxide (lab stock in fume hood is 2000x)

Add just before use:
Protease inhibitors - 0.2mg/ml aprotinin, 2mM leupeptin, 5mM iodoacetamide, 1mM PMSF
Phophatase inhibitors - 200uM Na3VO4, 10mM Na4P2O7
1mg/mL chicken ovalbumin
0.1% Pansorbin (Calbiochem, La Jolla CA)

Kinase Buffer (200mL)

25mM HEPES, pH 7.4
150mM NaCl
5mM MgCl2
5mM MnCl2
1mM CaCl2
10mM NaF (0.084g/200ml)
10mM Na2MoO4 (0.484g/200ml)

Add just before use:
Protease inhibitors - 0.05mg/ml aprotinin, 0.5mM leupeptin
Phosphatase inhibitor - 200uM pervanadate
1mM DTT (10uL 1M/10mL)
20mM ATP (2 uL 100mM/10mL)

Procedure
Lyse Cells

Mechanically disrupt lymphoid tissues with frosted glass slides, lyse red blood cells, and filter. Wash cells in PBS and lyse at no more than 1x10^8 cells/ml lysis buffer. Incubate on ice 15 minutes and centrifuge at 12000 rpm for 15 minutes at 4 C.

Immunoprecipitations

Add antibodies (1-2 ug purified, 1-2 ul ascites, or 2-3 ul of polyclonal antisera) and rotate at 4 C for 1 hour. Add up to 25 ul of a 50% slurry of protein A-sepharose (or whatever as appropriate) in lysis buffer, and rotate at 4 C for 2 hours to o/n. Wash (5000 rpm, 3 minutes, 4 C) immune complexes 2-3 times with lysis buffer (optional - with one change of tubes), once with 10mM NaHPO4, pH 7.4, 150mM NaCl (optional) or 50mM Tris pH 7.4, 500mM LiCl (also optional, more stringent), and once with kinase reaction buffer.

Kinase assays

Resuspend immunoprecipitates in ~15 ul of kinase buffer containing 0.6mM g-[32P]-ATP (3000 Ci/mmol, Amersham) and 10mg acid-denatured enolase, and incubate 15 minutes at room temperature or 5-15 minutes at 30 C. Terminate reactions by the adding ~15 ul 6x SDS-PAGE sample buffer containing 15% B-mercaptoethanol and boil 5 minutes. Freeze on
or off beads, as desired, if needed.

Blot proteins

Resolve on 12% SDS-polyacrylamide gel run with 0.192mM glycine, 0.025mM Tris pH 8.3, and 0.1% SDS running buffer. You will be able to visualize unincorporated label; it's easy to manage waste if you don't run it off the gel. Transfer to NitroPlus 2000 (MSI) using an ice-bath cooled BioRad transblot apparatus at 90V for 2-3 hr in transfer buffer (20mM glycine, 2.5 mM Tris-OH, 20% methanol). Wrap in saran wrap and expose or perform westerns as indicated.

Preparation of Acid-Denatured Enolase

We use BM enolase #104647

Place 500uL of each of two tubes, spin 30 minutes at 4 C. Resuspend in 50uL of 50mM HEPES pH 7.0, 1mM DTT, 1mM MgCl2.
Place on ice for 30 minutes without shaking.
Add one volume of glycerol and store in 20uL aliquots at -70 C.

Mix equal volumes of prepared enolase and 100mM acetic acid (dilute glacial 174:1). Place at 30 C for 10 minutes. A white precipitate will form.
Hold on ice until use, then use at 1uL per reaction.
This works out to about 10ug/reaction. Enolase runs at about 40kD.

Troubleshooting

Reference
Thomas Lab
(http://pathbox.wustl.edu/~thomlab/thomlab.html)

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