Protein Kinase Filter Binding Assay Protocol (Manual Assay)

Standard Procedure for all kinases

Materials:

ATP (e.g. Sigma, # A-7699)
$CaCl_2$ (e.g. VWR, # 1.02382)
Calmodulin (e.g. Millipore, # 14-368)
cGMP (e.g. Sigma, # G-6129)
1,2-Dioleoyl-glycerol (e.g. Sigma, # D-8394)
DNA (e.g. Sigma, # D-4522)
DMSO (e.g. Sigma, # 154938)
DTT (e.g. Sigma, # D-0632)
EDTA (e.g. Roth, # 8040.1)
H_2O p.a. (e.g. VWR, # 1.16754.9010)
HEPES (e.g. Sigma, # H-3375)
$MgCl_2$ (e.g. Sigma, # M-3634)
$MnCl_2$ (e.g. VWR, # 1.05927.1000)
NaCl (e.g. Merck, # 1.06404)
Na-orthovanadate (e.g. Sigma, # S-6508)
Ortho-phosphoric acid (e.g. VWR, # 1.00563.1000)
$PEG_{20.000}$ (e.g. SERVA, # 33138)
Phosphatidyl-serine (e.g. Fluka, # 79405)
(10 mg/ml in chloroform/methanol)
^{33}P-γ-ATP (e.g. PerkinElmer, # NEG 302 H)
96-well V-bottom MTP
(Polypropylen) (e.g. Nunc, # 442587)
96-well Multiscreen Vacuum Manifold
(e.g. Millipore, # MAVM096OR)
96-well Multiscreen Filterplates
(depending on used substrate):
- Phosphocellulose Membrane
(e.g. Millipore, # MSPHN6B10)
- GlassFiber Membrane
(e.g. Millipore, # MSFCN6B10)
Scintillation Cocktail (e.g. Fluka, # 03999)

Prepare in advance (store at 4° C):

Standard-Assay-Buffer (2.5x stock)
- 125 mM HEPES-NaOH, pH 7.5
- 7.5 mM $MgCl_2$
- 7.5 mM $MnCl_2$
- 7.5 µM Na-orthovanadate
- 2.5 mM DTT

Kinase-Dilution-Buffer (10x stock)
- 500 mM HEPES-NaOH, pH7.5
- 2.5 mg/ml $PEG_{20.000}$
- 10 mM DTT

Final Assay Conc. (50 µl reaction cocktail):

- 70 mM HEPES-NaOH, pH 7.5
- 3 mM $MgCl_2$
- 3 mM $MnCl_2$
- 3 µM Na-orthovanadate
- 1.2 mM DTT
- 50 µg/ml $PEG_{20.000}$
- ATP [var. conc. (dep. on K_M) + ^{33}P-γ-ATP]
- 1 % (v/v) DMSO
- Substrate (variable)
- Recombinant Protein Kinase (variable)

All PKC assays (except the PKC-mu and the PKC-nu assay) additionally contain 1 mM $CaCl_2$, 4 mM EDTA, 5 µg/ml Phosphatidylserine and 1 µg/ml 1,2-Dioleyl-glycerol.

The CAMK1D, CAMK2A, CAMK2B, CAMK2D, CAMK4, CAMKK1, CAMKK2, DAPK2, EEF2K, MYLK, MYLK2 and MYLK3 assays additionally contain 1 µg/ml Calmodulin and 0.5 mM $CaCl_2$.

The PRKG1 and PRKG2 assays additionally contain 1 µM cGMP.

The DNA-PK assay additionally contains 2.5 µg/ml DNA.

Manual Assay Procedure:

In PP V-bottom plate:
1. Add 20 µl Standard-Assay-Buffer (2.5x stock)
2. Add 5 µl 10% DMSO *(w, w/o test cpd)*
3. Add 10 µl substrate (in 50mM HEPES pH 7.5)
4. Add 10 µl recombinant protein kinase (in 1x Kinase-Dilution-Buffer)
5. Add 5 µl ATP (in H_2O)
6. Mix on shaker
7. Incubate for 40 min at 30°C
8. Stop reaction with 20 µl 10% H_3PO_4
9. Prewet filterplate with 200 µl 150 mM H_3PO_4
(wash MSPH-filterplate with 20 µl 100% EtOH first)
10. **Transfer reaction mix to filterplate**
11. Incubate for at least 10, but no longer than 20 min
12. Filtrate reaction mix by applying vacuum
13. Wash 3 times with 200 µl 150 mM H_3PO_4
14. Wash once with 20 µl 100% EtOH
15. Dry for 30 min at 40°C
16. Add 50 µl scintillator per well
17. Count with Scintillation Counter