KDR Phosphorylation Assay

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Lee, Sunyoung

Materials required:

Cells
PBS
HAM F-12 media
Growth factor (VEGF)

Lysis Buffer mix (w/o PMSF and Aprotinin, keep at 4°C, add PMSF and Aprotinin right before use):

1% Triton-X100
10mM Tris
1mM EDTA
150mM NaCl
30mM PPiNa
50mM NaFi
2.1mM Na_3VO_4

Lysis Buffer (Final):

	µl
PMSF	30
Aprotinin	6
Buffer	5964
Total	6000

Preincubation buffer: Serum-free media (30ml) + Na3VO4 (15µl)
Wash buffer: 1x PBS (15ml) + Na3VO4 (15µl)

Method:

1- plate cells in 12-well (or 240well) plate
2- cells in serum-free media for o/n
3- cells in preincubation media, 37°C, 5min
4- aspirate media
5- treat cells with growth factor, 37°C, 5min
6- aspirate growth factor
7- wash cells with cold wash buffer, 2x
8- add 200-300µl lysis buffer to each well, rocking plate, 4°C, 15min
9- collect cells in eppendorf tubes, rocking, 4°C, 15min

10-spin, 14,000rpm, 4°C, 1h
11- collect supernatant, store at -80°C or go ahead with next step
12-for phophotyrosine western analysis, use 15-60µl of lysate
13-10 or 12 % SDS-PAGE gel
14-Blocking, 3% BSA/TBST, RT, 1h
15-1° Ab, anti-pTyr Ab (4G10, Upstate) in blocking buffer, 4C, ON
16-wash with TBST
17-2°Ab, RT, 1h
18-wash with TBST, at least 2h, change buffer as often as possible
19-rinse blot with dH2O, 5x
20-ECL, 2min, RT
21-Develop

mRIPA Lysis Buffer (this also works well, recipe from Tom Graeber):

	Stock		50 mls
50 mM Tris pH 7.4	1M	1:20	2.5 ml
1% NP-40	10%	1:10	5 ml
0.25% Na deoxycolate	2.5%	1:10	5 ml
1 mM EDTA	0.5 M	1:500	0.1 ml (100 ul)
0.15 M NaCl	5 M	1:33	1.5 ml
1 mM Na vanadate	100 mM	1:100	0.5 ml (500 ul)
10 mM ß-glycerophosphate	1 M	1:100	0.5 ml (500 ul)
1 mM NaF (optional)			
H2O			34.9 ml
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			50 ml

<u>add fresh</u> (5-10 min. prior is okay, PMSF has short half-life in aqueous solution):

1 mM PMSF	100 mM	1:100
20 ug/ml leupeptin	10 mg/ml	1:500
20 ug/ml aprotinin	2 mg/ml	1:100