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Detection of Phospho-eIF2alpha:

1. For most cell types use dishes which are less than 95% confluent .
   For cells with a high metabolism such as ES cells or 293T cells use 50-60% confluent dishes.
2. Change the media 1-2hrs before treatment. (Use pre-warmed media)
3. Treat cells for 30min to 1hr with one of the following
   400-1000nM Thapsigargin (4ul/10ml media 1mM in DMSO)
   2mM DTT (20ul/10ml media of 1MDTT stock)
   100uM sodium arsenite (10ul 100mM stock in PBS)
   media missing one or more amino acids (see below)

4. Wash cells in ice cold PBS-1mM EDTA prior to scraping into the same buffer and collection by centrifugation.
5. Lyse cells in 4 packed cell volumes (50-70ul/10cm dish of cells) of lysis buffer (20mM HEPES, pH7.5, 150mM NaCl, 1%triton-X100, 10% glycerol, 1mM EDTA 10mM tetrasodium pyrophosphate, 100mM NaF, 17.5mM beta-glycerophosphate, 1mM phenylmethysulfonyl fluoride, 4mg/ml aprotonin, and 2mg/ml pepstatin A) on ice for 5-10 min.

****Note that the use of serine-threonine phosphatase inhibitors in the lysis buffer such as beta-glycerophosphate are critical to the retention of eIF2a phosphorylation.

6. Clear by centrifugation at 14,000rpm 15min at 4C.
7. Determine the protein concentration of a diluted aliquot by Bradford assay.
8. Denature equal amounts (10-50ug, depending on the cell type) of protein are by the addition of 2X SDS-PAGE buffer (100mM Tris pH 6.8, 20% glycerol, 4% SDS, 0.2% bromophenol blue,200mM DTT) followed by heating at 95C for 5min.
9. Separated on a 10%gel and transfer to nitocellulose membrane in 0.19 M glycine, 25mM tris base, 20% methanol.
10. Block the membrane in PBS-5%BSA-0.02% tween 20, for 1-2hr. (use BSA from ICN cat #160069)
11. Block the membrane in PBS-5%milk. (always do after BSA block)
12. Wash in PBS 3X
13. Incubate with phospho-eIF2a antibody (research genetics)1/400 in PBS-5%
    BSA (no tween) for exactly 1hour at room temp. (save antibody at 4C
    for 1-3 more uses within 1week).
14. Wash in PBS 0.02% tween 20 3X

15.Secondary antibody 1/3000 proteinA-HRP (best) or 1/5000 anti-rabbit-HRP 1hr RT. Dilute antibody in PBS-or 5%BSA , no tween.
16.Wash 3X PBS-0.02% tween 20.
17.ECL development.

Additional precautions for cell culture conditions and harvesting including alternative phosphatase inhibitors are described in "Savinova O, Jagus R. Use of vertical slab isoelectric focusing and immunoblotting to evaluate steady-state phosphorylation of eIF2 alpha in cultured cells. Methods. 1997 Apr;11(4):419-25."

A. Media lacking a single amino acid:

DMEM without leucine or another amino acid (available from cellgro)
10% <u>dialized</u> fetal calf serum (available from gibco),
1X glutamine (gibco)
1X pen/strep (gibco)

For the control plate add back l-leucine or the appropriate amino acid from a sterile 100X solution (gibco 12406-013).

B. Media lacking most amino acids:

	to make >500ml	
Dulbeccos PBS (Gibco Cat #14040133)	500ml	1x
MEM Vitamin Solution (gibco cat #11120052	5ml	100X
pen strep	5ml	100X
1M glucose (to make 25mM final)	12.5ml	1M
7.5% Sodium Bicarb.(gibco cat #25080094)	2.33ml	7.5%
l-glutamine 100X (gibco 25030081)	5ml	
l-methionine 100X (gibco 12408-019)	5ml	
0.5% BSA or 5-10% dialized FCS	25-50ml dialized serum or 2.5g BSA	

Adjust pH to ~7.2, sterile filter

Add back to + aa control:	to make 10ml
MEM essential amino acids (50X) (Gibco #11130-051)	200ul
MEM non-essential amino acids (100X) (gibco#11140050)	100ul

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