PERK or GCN2 IP Western

In our experience, the detection of PERK phosphorylation (or IRE1 phosphorylation) as markers of ER stress is fraught with great difficulties. None of the anti-phosphoPERK antisera we have tested are able to detect the protein in straight immunoblots. Therefore, to detect PERK activation we are forced to resort to the laborious procedure of immunoprecipitation of PERK from detergent lysates followed by immunoblot. This procedure is difficult and consumes large amount of sample and antiserum. See below.

Solutions

Cell Lysis buffer: 500ml	
1% Triton x 100	5ml
150mM NaCl	15ml 5M
20mM Hepes pH 7.5	10ml 1M
10% glycerol	50ml
1mM EDTA	1ml 0.5M

*freshly add to lysis buffer just before use:
10mM tetrasodium pyrophosphate
100mM NaF
17.5 mM β-glycerophosphate
1mM PMSF
4 µg/ml Aprotinin
2 µg/ml Pepstatin A

Ripa buffer (optional more stringent wash for IP)
10mM Tris pH 7.5
100mM NaCl (up to 500mM for more stringent wash)
1mM EDTA
0.5% Na-deoxycholate
0.1% SDS
1% Triton x100

2X laemmli
100mM Tris pH 6.8
20% glycerol
4% SDS
0.2% bromophenol blue
200mM DTT

50X Methanol-free transfer buffer: 1L	
1.9M glycine	**144.1g**
250mM Tris base	**30.3g**

• dilute to 1X for methanol free transfer

1X Methanol-transfer buffer: 1L	
50X "methanol-free" transfer buffer *(this is not a mistake, if methanol is added the tris-glycine buffer must be used at 5 times the strength as buffer with no methanol)*	**100ml**
methanol	**200ml**
dH2O	**700ml**

Sample treatment and harvest

For Cells:

1. Treat cells e.g. Thapsigargin 0.1 µM, 15-30 min.
   Tunicamycin 0.5 - 2.5µg/ml, 3hrs
   Amino acid deprivation (dMEM –leucine,
   10% dialized FCS, Pen-strep, glutamine)
2. Wash cells 2x ice cold PBS
   1x ice cold PBS – EDTA (1mM)
3. Lyse cells in 500µl lysis buffer + phosphatase + protease inhibitors.
4. Scrape to 1.5ml tube, 5 min ice.
5. Clear extract by centrifugation at 14,000rpm 10 min, 4°.

For Tissues:

1. Harvest fresh tissue as quickly as possible.
2. Homogenize in 4ml/g tissue weight of lysis buffer using a motorized teflon and glass dounce.
3. When the tissue is completely homogenized, transfer to 1.5ml tubes and clear by centifugation at 14,000rpm at 4C for 30 min.
4. Repeat centrifugation of supernatant for the best results. (keep repeating until extract is completely clear.)
5. Preclear extract with non-specific antibody by incubating for 1hr with 1µl nonspecific (or preimmune) antisera and 20µl washed protein A beads for 1hr at 4C with end-over-end rotation. Centrifuge at 14,000rpm and discard beads for 10 minutes. Use supernatant for IP.

Immunoprecipitation for both cells and tissues:

1. For each sample:

   Prebind1µl Ab

   15µl Protein A beads
   50-100µl lysis buffer + phosphatase +
   protease inhibitors
   15min-1hr RT on end-over-end
   rotator

3. Preclear extract with non-specific antibody by incubating with 1µl nonspecific (or preimmune) antisera and 15µl washed protein A beads for 1hr at 4C with end-over-end rotation. Centrifuge at 3000rpm and discard beads. Use supernatant for IP.
4. 1-2x wash Ab bound beads in lysis buffer or Ripa, resuspend in 50 – 100µl/sample aliquot to 1.5ml tubes.
5. Add tissue or cell extract to tubes with washed antibody bound beads.
6. End-over-end rotation @ 4°, 3 hours-Overnight.
7. Wash 3x 1ml lysis buffer + protease/phosphatase inhibitors. (or RIPA)
8. Wash 1x 1ml with PBS.
9. Remove all solution with 27g needle + syringe.
10. Add 15-20µl 1x laemmli buffer ® boil and load on gel (6–7% SDS PAGE).

Western

1. Transfer in methanol-free transfer buffer diluted to 1X. (for PERK either + or - methanol is okay) *****For phospho-GCN2 use methanol transfer buffer.**
2. Block 30 min in PBS, 5% Non Fat Dry (NFD)-milk (no tween).

   ***For Phospho-GCN2 or Phospho-Perk specific westerns use 5% BSA (ICN cat #160069) in place of NFD milk in block and antibody incubations. BSA solution can be sterile filtered and reused to reduce costs.
3. Primary antibody: in PBS- 5% NFD milk (or 5% BSA for phospho-specific antibodies), no tween. (see below for times and temp)
4. Wash 3X PBS -0.02% tween 20.
5. Secondary antibody 1/3000 proteinA-HRP (best) or 1/5000 anti-rabbit-HRP 1hr RT. Dilute antibody in PBS- 5% NFD milk (or 5% BSA for phospho-specific westerns), no tween.
6. Wash 3X PBS-0.02% tween 20.
7. ECL development.

Antibody dilutions		
PERK	1/3000-1/5000	Incubate 1-2 hrs RT or 4C ON.
GCN2	1/1000	Incubate 1-2 hrs RT or 4C ON.
P-GCN2	1/500-1/1000	Incubate 4C ON.