Immunoblots of Markers of the Unfolded Protein Response:
Method for Preparing Cytoplasmic and Nuclear Extracts

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Solutions:

Harvest buffer:	For 50ml:
10 mM HEPES pH 7.9	0.5 ml 1 M HEPES
50 mM NaCl	0.5 ml 5 M NaCl
0.5 M Sucrose	8.56 g sucrose
0.1 mM EDTA	10 μl 0.5 M EDTA
0.5% Triton-X100	2.5 ml 10% Triton-X100

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

Buffer A:	For 50ml:
10 mM HEPES pH 7.9	0.5 ml 1 M HEPES
10 mM KCL	250 μl 2 M KCL
0.1 mM EDTA	10 μl 0.5 M EDTA
0.1 mM EGTA	10 μl 0.5 M EGTA

Freshly add to buffer A just before use:
1 mM DTT
1 mM PMSF
4 μg/ml Aprotinin
2 μg/ml Pepstatin A

Buffer C:	For 50ml 1X:	for 50ml 2X
10 mM HEPES pH 7.9	0.5ml 1 M HEPES	1 ml 1 M HEPES
500 mM NaCl	5 ml 5 M NaCl	10 ml 5 M NaCl
0.1 mM EDTA	10 μl 0.5 M EDTA	20 μl 0.5 M EDTA
0.1 mM EGTA	10 μl 0.5 M EGTA	20 μl 0.5 M EGTA
0.1% IGEPAL(NP40)	500 μl 10% IGEPAL	1 ml 10% IGEPAL

Freshly add to buffer C just before use:
1 mM DTT
1 mM PMSF
4 μg/ml Aprotinin
2 μg/ml Pepstatin A

Ice cold PBS and PBS-1mM EDTA

Method:

•Place plates on ice.
•Wash 2X with cold PBS.
•Add 1 ml PBS-EDTA and scrape cells. Transfer to 1.5ml tube.
•Pellet at 3000 rpm for 5 min.
•Resuspend in 250-500 μl (at least 6 vol) of Harvest buffer.
•Incubate on ice for 5 min.
•Pellet at 1000 rpm in a table top swinging bucket rotor (for example by resting microfuge tubes in 15ml tubes while spinning) for 10 min to pellet nuclei @ 4°C (preferable). Alternatively spin in a fixed angle rotor at 2000 rpm. Note that the nuclei sometimes smear up the side of the tube in the fixed angle rotor.
•Transfer supernate to new tube. For best results, clear at 14,000 rpm for 15 min. and transfer supernate to new tube = **"Cytoplasmic/membrane extract"**. Save for PERK or IRE IP or eIF2α western.
•Wash/resuspend nuclear pellet in 500 μl buffer A (optional).
•Pellet at 1000 rpm in swinging bucket rotor 5 min 4°C.
•Remove and discard sup.
•Add 4X vol buffer C. (*For a more concentrated extract use 2 vol buffer C made with 1M NaCl.)*
•Vortex 15 min at 4°C. (in cold room) Start on high speed vortex to loosen pellet, then turn to medium.
•Pellet at 14,000 rpm 10 min 4°C.
•Transfer sup to new tube = **"Nuclear extract"**.
If nuclear extract will be used for functional assays such as gel shift (EMSA), then 10% glycerol should be added prior to freezing.

Measure protein concentrations of cytoplasmic and nuclear extracts.

Load ~40-50 μg of nuclear extract for XBP1, ATF4 and CHOP Western (20 μg is enough for many cell types, but loading more is better.)
Load 25-40 μg of cytoplasmic extract for Phospho-eIF2α Western.
Use 500-1500 μg of cytoplasmic extract for PERK or IRE immunoprecipitation followed by Western (the more the better).

Antibodies for Immunoblots:

Antibody	MW of protein	Source	Dilution
CHOP	29 kD	rabbit	1:5000
XBP-1	54 kD	rabbit	1:2000
PERK	125 kD*	rabbit	1:1000-3000
IRE1	110 kD*	rabbit	1:1000-3000
ATF4	42 kD	rabbit	1:3000

*phosphorylated forms migrate slightly slower on gel