

AMPK/pAMPK Western Blot Analysis

We've performed this assay on the following cell types:

- Dissociated Primary Cortical Neurons Plated at $0.2 - 1.0 \times 10^6$ cells/ml (4 ml/well of 6 well plate).
- Mouse hypothalamic tissue
- Mouse hippocampal tissue

Dissociated Primary Cortical Neuron Cultures:

- **Cell harvesting**
 - Remove cells from incubator and immediately place plate on a bed of ice.
 - Rinse cells once with ice-cold 1X DPBS (containing Mg and CaCl_2) - 2 ml/well of a 6 well plate.
 - To each well add 350 μl of lysis buffer (**if different size plates are used, adjust lysis volume according to the relative area of the dish).
 - We have had the best results by performing the lysis directly in 1X-Protein Sample Buffer (1X PSBF).
 - **1X PSBF:** 62.5 mM Tris-Cl pH 6.8; 10% glycerol; 2% SDS; 1% β -Mercaptoethanol; trace of bromophenol blue.
 - If performing a protein assay do not add dye to the PSBF until after an aliquot has been put aside for protein determination.
 - Scrape and pool like-samples.
 - Boil for 10 min.
 - Spin lysates at 14,000 rpm for 5 min @ 4°C (10,000 in eppendorf centrifuge) to remove cell debris.
 - Aliquot and immediately freeze at -80°C (may snap freeze with liquid N_2 then store at -80°C).

Mouse Tissue:

- **Tissue harvesting/homogenizing**
 - Tissue is dissected and immediately frozen in liquid nitrogen.
 - Tissue is homogenized in lysis buffer with the following final concentrations:
 - 50 mM Tris-HCl, pH 7.5, 250 mM sucrose, 5 mM sodium pyrophosphate (NaPPi), 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM benzamidine, 50 $\mu\text{g}/\text{ml}$, leupeptin, 50 $\mu\text{g}/\text{ml}$ soybean trypsin inhibitor.
 - Following lysis SDS is added to a final of 0.2%.
 - Boil lysates for 5 min.
 - **The following are made *fresh* and added to the buffer immediately before use.
 - 1M DTT made in H_2O
 - 1M NaF made in H_2O
 - 0.25 M NaPPi made in H_2O
 - Stocks of the following can be made up ahead of time, aliquoted and stored at -20°C .
 - 50 mM Benzamidine made in H_2O
 - 13 mg/ml Trypsin Inhibitor made in PBS
 - 1 mg/ml Leupeptin made in H_2O
 - 100 mM PMSF made in isopropanol or methanol
- **AMPK/pAMPK Western Blot Procedure**
 - Run 30-40 μg of protein/lane on a 10% polyacrylamide gel (or 5-15% gradient).
 - Transfer to PVDF membrane.

- **Block** for 1 hr shaking @ RT.
 - For pAMPK α use in 3% milk made up in 1X TBST + 50 mM NaF.
 - For AMPK α use in 3% milk made up in 1X TBST – NaF is not necessary if you will not re-probe with a phospho-antibody in the future.
 - For Actin anti AMPK α antibody 1 use 3-5% milk made up in 1X TBST – again, NaF is not necessary if you will not re-probe with a phospho-antibody in the future.
- Incubate ON shaking @ 4°C with **primary antibody**.
 - For pAMPK α use 5% BSA (protease free, Sigma A-3059) made in TBST + 50 mM NaF.
 - Dilute the rabbit anti pAMPK α antibody 1:1000.
 - For AMPK α use 5% BSA made in TBST no NaF
 - Dilute the rabbit anti AMPK α antibody 1:1000.
 - For Actin use 5% BSA made in TBST no NaF.
 - Dilute the mouse anti-actin antibody 1:1000.
- Wash with TBST 1x15' and 4x5'.
- Incubate with **secondary antibody** for 1 hr shaking @ RT.
 - For pAMPK and AMPK use goat anti-rabbit HRP @ 1:7500 dilution in 5% milk in 1X TBST (+ 50 mM NaF for pAMPK).
 - For Actin use goat anti-mouse HRP @ 1:5000 dilution in 5% milk in 1X TBST.
- Wash with TBST 1x15' and 4x5'.
- **Signal Detection**
 - Incubate in ECL reagent for 1-3 min, remove excess reagent, seal in bag and develop.
 - For AMPK or actin use the pico ECL kit with a 1:1 mixture of the two solutions
 - For pAMPK use the femto ECL kit: Mix the two solutions 1:1 then dilute 1:10 using water.
 - Incubate with ECL reagent for 1-3 min, remove excess reagent, seal in bag and develop.
 - If the western has worked well signal (pAMPK/AMPK = 62 kDa; actin = 45 kDa) will be evident in < 30 sec.
- **Reagents and Solutions for Western Blot Analysis**
 - For 1 L of **Protein Gel Running Buffer (Electrode Buffer)**: 14.4 g Glycine, 3.0 g Tris base, 20 % final SDS, up to 1L with ddH₂O.
 - For 1 L of **Transfer Buffer**: 14.4 g Glycine, 3.0 g Tris base, 20 % (200 ml) methanol, up to 1 L with ddH₂O. *Store @ 4°C so that it is cold when needed.*
 - For 1 L of **20X TBS**: 160 g NaCl, 4 g KCl, 60 g Tris base, up to 1 L with ddH₂O
 - pH to 7.4
 - To make 1X TBST add 0.1% Tween-20 to 1X TBS.

Materials/Ordering Information:

- Protease free BSA; Sigma A-3059.
- **Antibodies:**
 - Anti-phospho-AMPK-alpha (Thr172) antibody; Cell Signaling #2531 **OR** Anti-phospho-AMPK-alpha (Thr172) (40H9) Rabbit mAB; Cell Signalling #2535.
 - Anti-AMPK-alpha antibody; Cell Signaling #2532.
 - Anti-actin antibody; JLA20 from the hybridoma bank.

- **ECL kits:**
 - SuperSignal West Pico Chemiluminescent kit; Pierce # 650211.
 - SuperSignal West Femto maximum Sensitivity Substrate: Pierce #34095.