

AMPK Activity Assay Protocol - SAMS Peptide Assay

This procedure was adapted from Witters, L. A., and Kemp, B. E. (1992) J. Biol. Chem. 267, 2864-2867 and a protocol provided by Dr. Lee Witters.

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).

Cell Harvest:

- Remove culture dish from 37°C incubator and place on ice immediately. Rinse cells 1x with 2 ml cold 1x PBS.
- Add 350 μ L of Triton X-100 Lysis Buffer per well.
- Scrape cells from dish and transfer to 1.5 ml eppi. tube. Pool samples from three wells of a 6 well plate.
- Vortex tubes for 15 sec each.
- Spin lysates at 13,000 rpm for 5 min at 4°C.
- Take 10 μ l aliquot for protein assay. Remove remainder of supernatant to fresh tube.

Protein Pull-Down:

- Add 40 μ l of 50% Protein A/G bead slurry to 10 μ l of an anti-AMPK α antibody.
 - Incubate on ice for 1 hr mixing frequently by gently flicking the tube.
- Rinse 4x with 500 μ l of triton X-100 lysis buffer: spin at 13,000 rpm for 15 sec, remove supernatant with Pasteur pipette (flame and pull tip) and re-suspend in buffer.
 - On last rinse, remove as much buffer as possible.
- Add 900 μ l of cell lysate to bead/antibody solution.
 - Incubate on rotator for 3 hrs in cold room.
- Rinse 2x with 500 μ l triton X-100 lysis buffer.
- Rinse 2x with 500 μ l 4x Assay Buffer (containing DTT and PI's)
- Remove supernatant and add 60 μ l of 4x Assay Buffer.

Kinase Activity Assay:

- Set up 2 reactions for each sample. 1 with and 1 without SAMS peptide see mixes below.
 - Add **15 μ l** (+) OR (-) *peptide mix* to tubes.
 - Add **10 μ l** sample to tube. Make sure to tap tube to mix sample before pipetting, beads will settle quickly.
 - Add **5 μ l** initiation mix** to each tube.
 - **Mixes:**
 - **(+) peptide mix/reaction:** 2.5 μ l of 200 μ M SAMS peptide, 5 μ l of 1.2 mM AMP, 5 μ l of 5 mM DTT, 2.5 μ l H₂O
 - **(-) peptide mix/reaction:** 5 μ l of 1.2 mM AMP, 5 μ l of 5 mM DTT, 5 μ l H₂O
 - **initiation mix/reaction:** 0.3 μ l 500 mM MgCl₂, 0.3 μ l 20 mM ATP, 1 to 4.4 μ l hot ATP (depending on the freshness of the lable), 0 to 3.4 μ l H₂O (to bring the final volume to 5 μ l/rxn)
- Incubate tubes in 30°C water bath for 20 min, tapping tubes to mix every few minutes.
- Spot 15 μ l of sample on a disc of P81 Whatman phosphocellulose paper and let absorb (1-3 sec).
- Place discs in stirring bath containing ~200 ml 1% phosphoric acid. Stir in hood 20 min.
- Wash 3 more times 20 minutes each with 200 ml 1% phosphoric acid.
- Wash 5 min with 200 ml of acetone.
- Wash 5 min with 200 ml petroleum ether.

- Remove pet. ether and let filters dry in hood.
- Place filter in plastic vial containing 3 ml H₂O for Cerenkov counting. Count each vial 2 times for 10 min each.

Buffers and Solutions:

- **Triton X-100 Lysis Buffer (4°C):**
 - 20 mM Tris-HCl pH 7.4, 50 mM NaCl, 250 mM sucrose, 1% Triton X-100, 50 µg/ml leupeptin, 0.1 mM Benzamidine, 50 µg/ml Trypsin Inhibitor, 1.0 mM PMSF, 50 mM **NaF, 5 mM **NaPPi, 1 mM **DTT.
 - Bring up to volume with H₂O.
 - **The following are made fresh and added to the buffer immediately before use.
 - 1M DTT made in H₂O
 - 1M NaF made in H₂O
 - 0.25 M NaPPi made in H₂O
 - Stocks of the following can be made up ahead of time, aliquoted and stored at -20°C.
 - 50 mM Benzamidine made in H₂O
 - 13 mg/ml Trypsin Inhibitor made in PBS
 - 1 mg/ml Leupeptin made in H₂O
 - 100 mM PMSF made in isopropanol or methanol
- **4x Assay Buffer:**
 - 240 mM NaHEPES, 480 mM NaCl, 50 µg/ml Leupeptin, 0.1 mM Benzamidine, 50 µg/ml Trypsin Inhibitor, 1.0 mM PMSF, 1.0 mM **DTT.
 - Bring up to volume with ddH₂O.

Materials/Ordering Information:

- **Stock Solutions:**
 - 1.2 mM AMP 5' in HPLC H₂O (Powder stored @ -20°C; Sigma #A1752, 5g)
 - 20 mM ATP in HPLC H₂O (Powder stored @ -20°C; Sigma #, 5g)
 - 5 mM DTT (2.5 µl of 1 M DTT into 500 HPLC H₂O)
 - 200 µM SAMS Peptide in HPLC H₂O (2.43 mg/ml; aliquots kept @ -80°C; Princeton Biomolecules, #251-891-0415, with 95% purity)
 - 500 mM MgCl₂ in HPLC H₂O
 - 4 mCi/mL Hot ATP (Adenosine-5'-Triphosphate [γ -³²P], ICN Biochemicals, # 35020)
- **Other Reagents:**
 - P81 Cellulose Phosphate paper – Whatman #3698321, size = 2.1 cm, 100 count
 - Petroleum ether – Sigma #173-4, 1 or 2 L
 - Protein A/G bead slurry - Santa Cruz # sc-2003