EnzyFluo™ AMPK Phosphorylation Assay Kit (EAMPK-100)

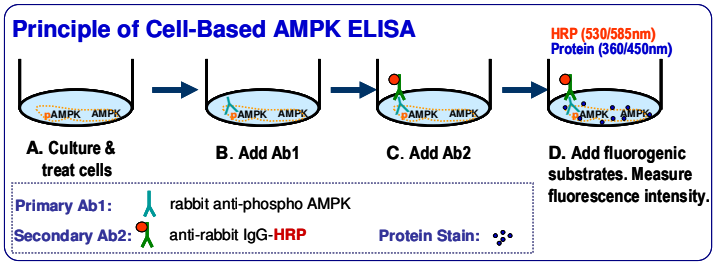
Fluorimetric Cell-Based Assay for AMPK Phosphorylation Status

DESCRIPTION

The 5-AMP-activated protein kinase (AMPK) is a key sensor of intracellular energy balance. AMPK is activated in response to an increase in the AMP/ATP ratio which can be caused by a number of factors such as muscle contraction, starvation, or hypoxia. AMPK is a heterotrimeric protein complex comprising of α- (63 kDa), β- (38 kDa) and γ- (38 kDa) subunits. For each subunit, isoforms have been identified (α1, α2, β1, β2, γ1, γ2, γ3) which theoretically allow the formation of 12 different proteins. The α-subunit contains a serine/threonine kinase domain and the regulatory subunits contain binding sites for AMP and ATP (γ-subunit) and for glycogen (β-subunit).

AMPK is activated by phosphorylation on Thr-172 within the catalytic domain. AMP binding results in a 2 to 5-fold increase in AMPK activity compared to the basal level. Binding of AMP to the γ-subunit causes allosteric activation of the kinase and induces a conformational change in the kinase domain that protects AMPK from dephosphorylation of Thr-172.

BioAssay Systems' cell-based ELISA measures phosphorylated AMPK in whole cells and normalizes the signal to the total protein content. The antibody recognizes both α-subunits and thus can be used for cells from all tissues (human, mouse, rat). This simple and efficient assay eliminates the need for cell lysate preparation and can be used to study AMPK regulation in short-term and long-term assays. In this assay, cells grown in 96-well plates are fixed and permeabilized in the wells. AMPK phosphorylation (pAMPK) is measured using a fluorescent ELISA followed by total protein measurement in each well.



KEY FEATURES

Sensitive. Can measure pAMPK modulation in as little as 500 cells/well.
Simple and convenient. No cell lysis necessary, cells can be cultured for several days.

APPLICATIONS

Determination of AMPK phosphorylation status in whole cells.
Evaluation of direct and indirect modulation of AMPK phosphorylation.
Species tested: human, mouse, rat.

KIT CONTENTS

10x Wash Buffer:	25 mL	**Blocking Buffer:**	25 mL
Protein Stain:	6 mL	**HRP Substrate:**	6 mL
pAMPK-Ab1	10 µL	**HRP-Ab2**	10 µL

Storage conditions: this kit is shipped on ice. Upon delivery, store all reagents at -20°C. Shelf life of 6 months after receipt.

Precautions: reagents are for research use only. Normal precautions for laboratory reagents should be exercised while using the reagents. Please refer to Material Safety Data Sheet for detailed information.

ASSAY PROCEDURE

Important:

1. To avoid cross-contamination, change pipette tips between additions of each reagent or sample. Use separate reservoirs for each reagent. Prior to Assay, dilute 10x Wash Buffer in dH_2O to prepare 250 mL 1x Wash Buffer.
2. It is recommended that assays be run in duplicate. Include in every experiment two "Sample Blank" wells with no cells but with the same volume of culture medium. The Sample Blank wells are treated the same way as with the "Sample Wells" for background fluorescence determination.

A. Culture and Treat Cells

1. Seed 100 µL of up to 10,000 adherent cells into each well of a black clear flat-bottom 96-well plate. Incubate for at least 4 h at 37°C in a cell culture incubator to allow cells to adhere.

   Note: The cell number to be used depends on the cell line and AMPK phosphorylation status.

2. Treat the cells as desired (e.g. drugs or starvation).
3. Prepare formaldehyde solutions (*warning*: formaldehyde is toxic. Use chemical hood and wear appropriate gloves and eye protection):

   For adherent cells, prepare 4% formaldehyde by mixing 1.3 mL of 37% formaldehyde and 10.7 mL of 1x Wash buffer. Fix cells in each well by replacing the medium with 100 µL of 4% formaldehyde.
   For suspension cells, prepare 8% formaldehyde by mixing 2.6 mL of 37% formaldehyde and 9.4 mL of 1x Wash buffer. Centrifuge the plate at 500*g* for 15 min at 4°C and carefully remove as much media as possible without disturbing the cell pellet. Fix the cells in each well by adding 100 µL of 8% formaldehyde to cell pellet.

   Cover the plate and incubate for 20 min at room temperature. Alternatively, the plate containing the fixed cells can be sealed and stored for up to 2 weeks at 2-8°C.
4. Remove the formaldehyde solution and wash the cells 3 times with 150 µL of 1× Wash Buffer. Each wash step should be performed for 5 min with gentle shaking. For suspension cells centrifuge the plate at 500*g* for 15 min at 4°C before removing the formaldehyde solution. *Note, for all following wash and treatment steps, suspension cells need to be centrifuged before removal of any liquid.*
5. Prepare Quench Buffer by mixing 2.2 mL of 3% H_2O_2 and 8.8 mL of 1x Wash Buffer.

   Remove the Wash Buffer and add 100 µL of Quench Buffer to each assay well. Cover plate and incubate for 20 min at room temperature.
6. Remove the Quench Buffer and wash the cells 3 times with 150 µL of 1x Wash Buffer. Each wash step should be performed for 5 min with gentle shaking.
7. Remove the Wash Buffer, and add 100 µL of Blocking Buffer. Cover plate and incubate for 1 hr at room temperature.

B. Add Primary Antibody (pAMPK-Ab1)

1. Add 100 µL of PBS to the pAMPK-Ab1 tube and mix well. Prepare enough primary antibody for each well by mixing 1 µL diluted pAMPK-Ab1 and 55 µL Blocking Buffer. Unused pAMPK-Ab1 can be stored at -20°C for up to 45 days.
2. Remove the Blocking Buffer from all assay wells. Add 50 µL of Ab1 to all Sample and Sample Blank wells. Cover plate and incubate for 3 hrs at room temperature or overnight at 2-8°C with gentle shaking.
3. Remove the Ab1 and wash the cells 3 times with 150 µL of 1x Wash Buffer. Each wash step should be performed for 5 min with gentle shaking.

C. Add Secondary Antibody (HRP-Ab2)

1. Immediately before use, add 100 µL of PBS to the HRP-Ab2 and mix well. Prepare enough secondary antibody for each well, by mixing 1 µL diluted HRP-Ab2 and 55 µL Blocking Buffer. Unused HRP-Ab2 can be stored at -20°C for up to 45 days.
2. Remove Wash Buffer and add 50 µL of the HRP-Ab2 Mixture to all Sample and Sample Blank wells. Cover plate and incubate for 1 hr at room temperature with gentle shaking.

3. Remove the HRP-Ab2 from each well and wash the cells 5 times with 150 µL of 1x Wash Buffer. Each wash step should be performed for 5 min with gentle shaking.

D. Detection

1. Immediately before use, add 6 µL 3% H_2O_2 to the provided 6 mL HRP Substrate (for partial plate assay, adjust the volumes accordingly). Remove the Wash Buffer from the plate and add 50 µL of reconstituted HRP Substrate to each well. Incubate for 20 min at room temperature in the dark.
2. Add 50 µL of Protein Stain to each well and incubate for an additional 3 min at room temperature in the dark.
3. Read the plate at $\lambda_{ex/em}$ = 530/585nm for phosphorylated AMPK (*p*AMPK) and at $\lambda_{ex/em}$ =360/450nm for total protein.

CALCULATION

Calculate the mean fluorescence intensities for the Sample Blank ("BLK") wells and "SAMPLE" wells. Subtract the mean fluorescence of the Sample Blank wells from the fluorescence value of the Sample well to yield ΔF values for the phosphorylated AMPK (ΔF_{pAMPK}) at 530/585nm and for the total Protein (ΔF_{PROT}) at 360/450nm.

$$\Delta\bar{F}_{pAMPK} = \bar{F}^{SAMPLE}_{pAMPK} - \bar{F}^{BLK}_{pAMPK}; \quad \Delta\bar{F}_{Prot} = \bar{F}^{SAMPLE}_{Prot} - \bar{F}^{BLK}_{Prot}$$

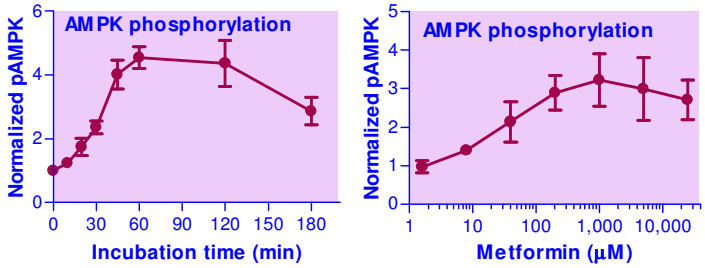
Normalized phosphorylated AMPK (pAMPK) is calculated as,

$$\text{Normalized p}\mathbf{AMPK} = \frac{\Delta\bar{F}_{pAMPK} / \Delta\bar{F}_{Prot}}{(\Delta\bar{F}_{pAMPK} / \Delta\bar{F}_{Prot})_o}$$

where $(\Delta F_{pERK} / \Delta F_{PROT})_o$ is the control reference value (e.g. time zero in kinetic studies or untreated wells in drug potency studies.)

MATERIALS REQUIRED BUT NOT PROVIDED

37% formaldehyde (Sigma, cat # F8775); 3% H_2O_2 (Sigma, cat # H1009); black (clear bottom) cell culture 96-well plate: available separately at BioAssay Systems (cat# P96BCC) or at Sigma (cat# CLS3603); plate sealers: available separately at BioAssay Systems (cat# AB96SL) or at Sigma (cat# A5596); deionized or distilled water; pipetting devices; cell culture incubators; centrifuge tubes; fluorescence plate reader capable of reading at $\lambda_{ex/em}$ = 530/585nm and at $\lambda_{ex/em}$ =360/450nm.



Left: Time course of the induction of AMPK phosphorylation in PANC-1 cells by metformin.

Right: Dose response curve of AMPK phosphorylation in PANC-1 cells following 3 hour treatment with metformin. 100% was defined as the basal AMPK phosphorylation in untreated PANC-1 cells.

LITERATURE

1. Mihaylova MM, Shaw RJ (2011). The AMPK signalling pathway coordinates cell growth, autophagy and metabolism. Nat Cell Biol. 13:1016-23.
2. Viollet B, et al (2010). AMPK inhibition in health and disease. Crit Rev Biochem Mol Biol 45:276-95.
3. Kisfalvi K et al (2009). Metformin disrupts crosstalk between G protein-coupled receptor and insulin receptor signaling systems and inhibits pancreatic cancer growth. Cancer Res. 69:6539-45

EXAMPLE OF A 96-WELL ASSAY PLATE LAY-OUT