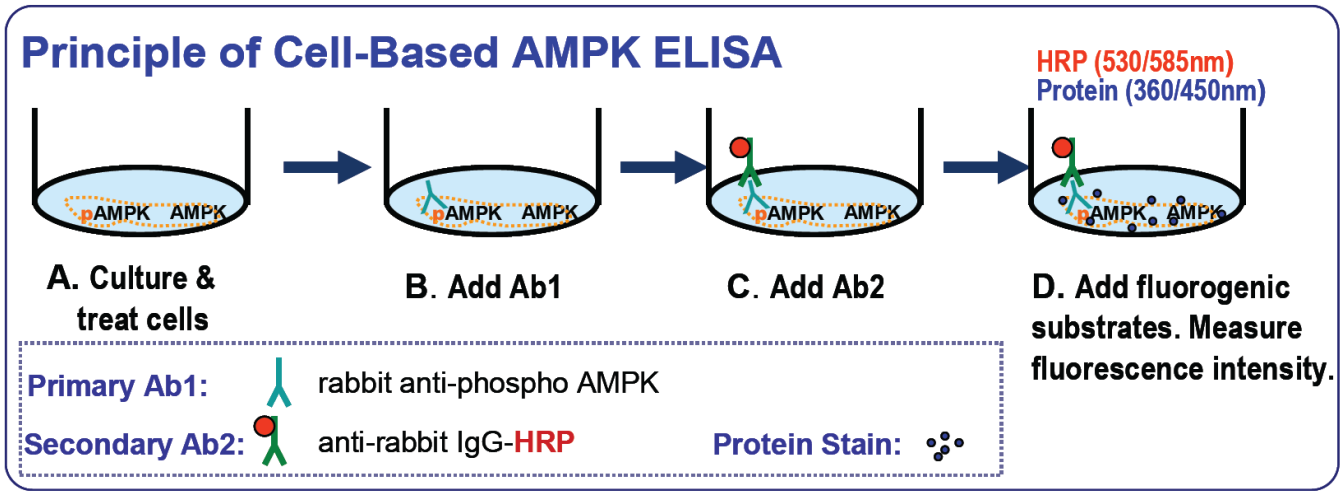
AMPK Phosphorylation Assay Kit (Fluorometric)

LS-K268-100 (100 Tests) • **Store at -20°C**

Introduction

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), ß-(38 kDa) and y-(38 kDa) subunits. For each subunit, isoforms have been identified (α-1, α-2, ß-1, ß-2, y-1, y-2, y-3) 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 and for glycogen. 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 α-subunit causes allosteric activation of the kinase and induces a conformational change in the kinase domain that protects AMPK from dephosphorylation of Thr-172.

This 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. Total protein and pAMPK can be measured in the same sample.

Applications

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

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Components

Component	K268-100
	100 Tests
10x Wash Buffer	25 mL
Protein Stain	6 mL
pAMPK-Ab1	10 µL
Blocking Buffer	25 mL
HRP Substrate	6 mL
HRP-Ab2	10 µL

Materials Not Supplied

37% formaldehyde (Sigma, cat# F8775); 3% H_2O_2 (Sigma, cat# 323381); black (clear bottom) cell culture 96-well plate (Sigma, cat# CLS3603); plate sealers (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.

Storage

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

Assay Procedure

Important

1. To avoid cross-contamination, change pipette tips between additions of each reagent or sample. We recommend the use of a multi-channel pipette. Use separate reservoirs for each reagent. Prior to Assay, dilute 10× Wash Buffer in dH_2O to prepare 250 mL 1× Wash Buffer.
2. It is recommended that samples be assayed in triplicate or higher.
3. Two different blanks are necessary. For each plate include a Protein Blank (no cells) in triplicate. For each sample include a Sample Blank (cells w/ only Ab2) in triplicate. The blanks are used to determine background fluorescence for total protein and pAMPK respectively.

Procedure

A. Culture and Treat Cells

1. Seed 100 µL of 1-3 × 10^4 adherent cells (or 4-10 × 10^4 suspension cells) into each well of a black 96-well culture plate. Add 100 µL of culture media without cells into three wells for the Protein Blank. Incubate overnight at 37°C in a cell culture incubator.

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

2. Treat the cells as desired (e.g. with ligands or drugs).
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 1× Wash buffer. Simply 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 1×Wash buffer. Centrifuge the plate at 500g for 15 min at 4°C and carefully remove as much media as possible without disturbing the cell pellet (repeat this for suspension cells with each wash step below). 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 1 min with gentle shaking.
5. Prepare Quench Buffer by mixing 2.2 mL of 3% H_2O_2 and 8.8 mL of 1×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 1×Wash Buffer.
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 Antibodies (Ab1)

1. Prepare 55 µL of primary antibody Ab1 Mixture for each well by mixing pAMPK-Ab1 into Blocking Buffer in a 1:625 dilution.
2. Remove the Blocking Buffer from all assay wells. Add 50 µL of the Blocking Buffer to the Sample Blank wells and 50 µL of Ab1 Mixture to the Sample wells. Cover plate and incubate for 90 min at room temperature or overnight at 2-8°C with gentle shaking.
3. Remove the Ab1 Mixture and wash the cells 3 times with 150 µL of 1×Wash Buffer. Each wash step should be performed for 1 min with gentle shaking.

D. Add Secondary Antibodies (Ab2)

1. Prepare 55 µL of secondary antibody Ab2 Mixture for each well by mixing HRP-Ab2 into Blocking Buffer in a 1:625 dilution.
2. Remove Wash Buffer and add 50 µL of the Ab2 Mixture to all assay wells. Cover plate and incubate for 90 min at room temperature with gentle shaking.

E. Detection

1. Remove the Ab2 Mixture from each well and thoroughly wash the cells 5 times with 150 µL of 1×Wash Buffer. Each wash step should be performed for 1 min with gentle shaking.
2. 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 mixed HRP Substrate to each well. Incubate for 30 min at room temperature in the dark.
3. Add 50 µL of Protein Stain to each well and incubate for an additional 5 min at room temperature in the dark.
4. Read the plate at $\lambda_{ex/em}$ = 530/585 nm for phosphorylated AMPK (pAMPK) and at $\lambda_{ex/em}$ =360/450 nm for total protein.

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Calculations

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.

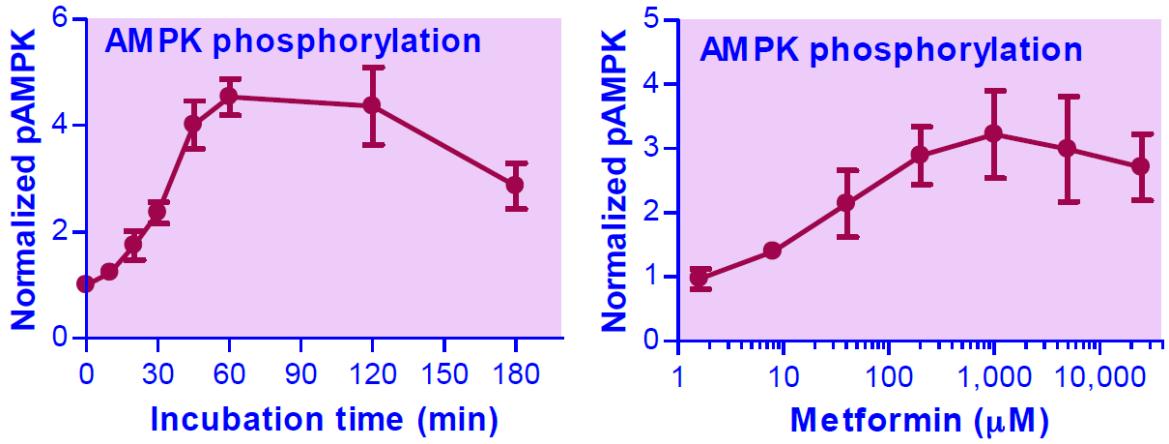
$$\Delta\bar{F}_{pAMPK} = \bar{F}^{SAMPLE}_{pAMPK} - \bar{F}^{BLK}_{pAMPK}; \quad \Delta\bar{F}_{Prot} = \bar{F}^{SAMPLE}_{Prot} - \bar{F}^{BLK}_{Prot}$$

Normalized phosphorylated AMPK (pAMPK) is calculated as,

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

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.)

Sample Data



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.

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