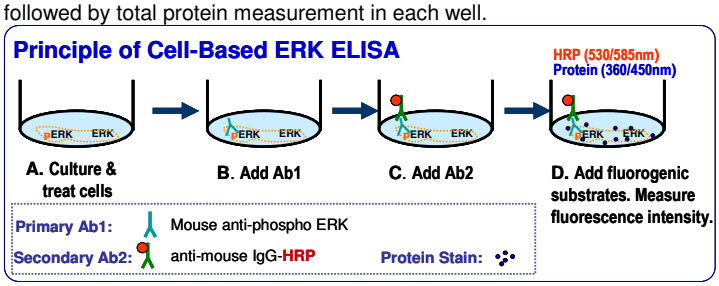
EnzyFluo™ ERK Phosphorylation Assay Kit (EERK-100)

Fluorimetric Cell-Based Assay for ERK Phosphorylation Status

DESCRIPTION

The mitogen-activated protein kinase (MAPK/ERK) pathway plays a key role in cell proliferation, differentiation and migration. Stimulation by mitogens eventually leads to phosphorylation of ERK1 (T202/Y204) and ERK2 (T185/Y187). The MAPK/ERK cascade presents many interesting drug targets for the development of cancer therapies.

BioAssay Systems' cell-based ELISA measures dually phosphorylated ERK1/2 in whole cells and normalizes the signal to the total protein content. This simple and efficient assay eliminates the need for cell lysate preparation and can be used to study kinase signaling and the effects of kinase inhibitors on cells. In this assay, cells are grown in 96-well plates and treated with ligands or drugs. Cells are then fixed and permeabilized in the wells. ERK1/2 phosphorylation (pERK) using a fluorescent ELISA followed by total protein measurement in each well.



KEY FEATURES

New and improved. Total assay time reduced from the standard 21 hours to 6.5 hours (hands-on time 2.5 hrs).

Simple and convenient. Cells are directly cultured in 96-well plates. No cell lysis necessary.

Accurate and high-throughput. Protein phosphorylation is normalized to total cellular protein in the same well, greatly minimizing well-to-well variations. Can be readily automated as a high-throughput 96-well plate assay for thousands of samples per day.

APPLICATIONS

For quantitative fluorescent immunoenzymatic assay of ERK1/2 phosphorylation status in cultured cells.
Evaluation of effects of ligands or drugs on ERK phosphorylation.
Species tested: human, mouse, rat.

KIT CONTENTS

Stock Wash Buffer:	25 mL	**Blocking Buffer:**	25 mL
Protein Stain:	6 mL	**Dye Reagent:**	120 µL
Ab1:	10 µL	**Ab2 (gM-HRP):**	10 µL

Storage conditions: 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. We recommend the use of a multi-channel pipette. Use separate reservoirs for each reagent. Prior to the Assay, prepare *1x Wash Buffer* by diluting Stock Wash Buffer 20-fold with dH_2O, e.g. mix 15 mL Stock Wash Buffer and 285 mL dH_2O.

   Important: reserve 6 mL *1x Wash Buffer* for Detection Step 2 below.
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 pERK respectively.

A. Culture and Treat Cells

1. Seed 100 µL of 2-4 × 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 ERK1/2 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 500*g* 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 Ab1 into Blocking Buffer in a 1:625 dilution.
2. Remove the Wash 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.

C. Add Secondary Antibodies (Ab2)

1. Prepare 55 µL of secondary antibody Ab2 Mixture for each well by mixing 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.

D. 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, prepare HRP Substrate by mixing 60 µL Dye Reagent with 6 mL 1x Wash Buffer and 6 µL 3% H_2O_2 (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 ERK (*p*ERK) and at $\lambda_{ex/em}$ =360/450 nm for total protein (Pr).

CALCULATION

Calculate the mean pERK fluorescence intensities at 530/585nm for the Sample Blank wells ($F_{BLK\ pERK}$) and Sample wells ($F_{SAMPLE\ pERK}$). Also calculate the mean protein fluorescence intensities at 360/450nm for the Protein Blank (no cells) well ($F_{BLK\ Prot}$) and Sample wells ($F_{SAMPLE\ Prot}$). Subtract the mean pERK fluorescence of the Sample Blank wells from the pERK fluorescence of the Sample wells to yield ΔF values for the pERK (ΔF_{pERK}). Subtract the mean protein fluorescence of the Protein Blank (no cells) well from the protein fluorescence value of the Sample wells to yield ΔF values for the total protein (ΔF_{PROT}).

$$\Delta\bar{F}_{pERK} = \bar{F}^{SAMPLE}_{pERK} - \bar{F}^{BLK}_{pERK} \;;\; \Delta\bar{F}_{Pr} = \bar{F}^{SAMPLE}_{Pr} - \bar{F}^{BLK}_{Pr}$$

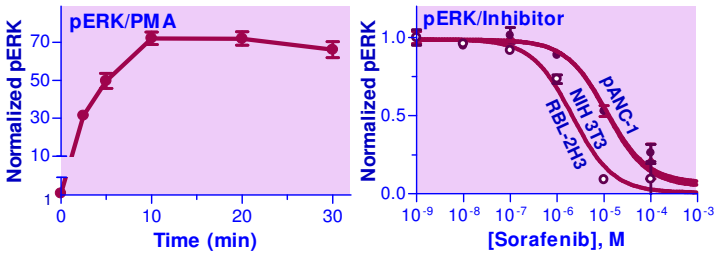
Normalized phosphorylated ERK (pERK) is calculated as,

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

where $(\Delta F_{pERK} / \Delta F_{Pr})_o$ 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 # 323381); black cell culture 96-well plate: available separately at BioAssay System (cat# P96BCC) or at Sigma (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 $\lambda_{ex/em}$ = 530/585 nm and at $\lambda_{ex/em}$ =360/450 nm.



***Left*:** Kinetics of ERK1/2 phosphorylation in PANC-1 cells on treatment with phorbol myristate acetate (PMA). ***Right*:** inhibition of ERK1/2 phosphorylation by the kinase inhibitor Sorafenib. Cells were treated with drug for 3 hours and then 5 min with PMA. IC_{50} values were 2.1, 11.4 and 11.5 µM respectively, for RBL-2H3, NIH 3T3 and PANC-1 cell lines.

LITERATURE

1. Cobb MH, et al (1994). The mitogen-activated protein kinases, ERK1 and ERK2. Semin Cancer Biol. 5(4):261-268.
2. Daniluk J, Dabrowski A. (2007). The effect of concomitant stimulation with cholecystokinin and epidermal growth factor on extracellular signal-regulated kinase (ERK) activity in pancreatic acinar cells. J Physiol Pharmacol. 58(3):441-53.
3. Iqbal J, et al (2007). Rapid in vivo effects of estradiol-17beta in ovine pituitary gonadotropes are displayed by phosphorylation of extracellularly regulated kinase, serine/threonine kinase, and 3',5'-cyclic adenosine 5'-monophosphate-responsive element-binding protein. Endocrinology 148 (12): 5794-802.