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Phospho-ERK1/2 (Thr202/Tyr204) Translocation Assay Kit (Cell-Based)

(Catalog # K696-50; 50 assays; Store at -20°C) *11/16*

I. Introduction:

Mitogen-activated protein kinases (MAPKs) are proline-directed serine and threonine protein kinases that regulate numerous physiological cell responses including: embryogenesis, cell differentiation, proliferation, migration, apoptosis and death. Extracellular signal-regulated kinases (ERKs) 1 and 2 (ERK1/2), also known as p44 MAPK and p42 MAPK respectively, belong to one of the five major groups of MAPKs. Closely-related ERK1/2 isoforms are uniquely activated by several extracellular signals including growth factors, cytokines, hormones, and neuro-transmitters. Activation of ERK1/2 by the upstream kinases MEK1 and MEK2 occurs via dual phosphorylation on specific threonine (Thr202) and tyrosine (Tyr204) residues on the T*EY* motif. MEK1 and MEK2 are activated through receptors (tyrosine kinases or integrins) via pathways involving adaptor proteins, guanine nucleotide exchange factors, small GTP binding proteins, and MAPKKs. Activated ERK1/2 phosphorylates both, cytosolic (SOS, MNK1/2, RSKs) and nuclear targets. In the nucleus, it affects gene expression and DNA replication by the phosphorylation of MSK 1 and 2 and the transcription factors Elk-1, Sap1, and Sap2. In cultured cells, growth factors or mitogens induce rapid and transient translocation of activated ERK1/2 to nucleus. Different cell lines exhibit various duration, magnitude, and subcellular localization of activated/phosphorylated ERK1/2. The response of the protein may differ even within the same cell line depending on the dose and cell density. BioVision's Phospho-ERK1/2 (Thr202/Tyr204) Translocation Assay Kit provides a simple and complete assay in a ready-to-use format to visualize the translocation of activated ERK1/2 between cytoplasmic and nuclear compartments in mammalian cells.

II. Applications:

- Detection of subcellular localization of activated ERK1/2 in mammalian cells
- Screening and characterizing effectors of ERK1/2 kinases
- Studying of cell signaling, cell division and cell proliferation mechanism

III. Sample Type:

- Adherent or suspension mammalian cell cultures

IV. Kit Contents:

Components	K696-50	Cap Code	Part Number
Fixative Solution	10 ml	WM	K696-50-1
Blocking Buffer	10 ml	NM	K696-50-2
Wash Buffer	75 ml	NM	K696-50-3
Phospho-ERK1/2 Primary Antibody (100X)	50 µl	Violet	K696-50-4
Secondary Antibody (100X)	50 µl	Red	K696-50-5
Tamoxifen (1000X)	30 µl	Yellow	K696-50-6
DAPI (1000X)	20 µl	Blue	K696-50-7

V. User Supplied Reagents and Equipment:

- Tissue culture plates and appropriate culturing media
- Phosphate Buffered Saline (PBS) (pH 7.4)
- 0.1% Gelatin Solution (optional, only required for suspension cells)
- Fluorescence microscope (570 nm excitation and UV filter)

VI. Storage Conditions and Reagent Preparation:

Upon arrival, store the kit at -20°C protected from light. Briefly centrifuge small vials prior to opening. Read the entire protocol before performing the assay.

- **Fixative Solution, Blocking Buffer, Wash Buffer:** Ready to use. After opening, store at 4°C and protected from light.
- **Primary and Secondary Antibodies (100X):** After opening, aliquot and store at –20°C in the dark. Keep on ice while in use. Avoid freeze and thaw cycle.
- **Tamoxifen and DAPI:** Store at –20°C after opening. Completely thaw before each use. Avoid multiple freeze and thaw cycles.

VII. Assay Protocol:

Notes:

This assay was developed with MCF-7 cells and can be modified for any suspension or adherent cell line. The protocol below refers to a 96-well tissue culture plate format and the assay volume is 100 µl. Adjust the volumes accordingly for other plate formats. Cell number per well and assay conditions should be optimized based on cell line specifications. Cells should be grown, treated, fixed and stained directly in multi-well plates. Bring all buffers to room temperature prior to the experiment. All steps should be carried out at room temperature unless otherwise specified.

1. Preparation of control and experimental wells:

a. Subculture cells of interest in appropriate medium to desired confluency. The day before the experiment, seed a 96-well plate with 1 X10^4 viable cells in 100 µl volume per well and incubate overnight at 37°C, 5% CO_2 to allow cell attachment. For suspension cells: add 125 µl of 0.1% gelatin solution into each well, tilt the plate to cover the entire well surface and place it in a tissue culture hood for 1 hour. Gently remove the 0.1% gelatin solution and seed your cells with 100 µl medium. Your experiment should always consist of parallel negative, positive and experimental wells respectively.

a. The next day, apply desired treatments to the experimental wells omitting the negative and positive control wells. To use Tamoxifen as positive control, dilute Tamoxifen (1000X) directly into the culture medium of positive control wells to obtain 1:1,000 - 1:10,000 dilution. Incubate the plate for the period of time required by your experimental protocol.

b. Upon completion, gently aspirate off the culture medium from all wells and rinse cells briefly with 200 µl of 1X PBS. For suspension cells: centrifuge the plate at 200 g (or the lowest centrifuge setting) for 3 minutes to gently deposit the cells onto the surface. Tilt the plate and gently remove the media by aspirating with a pipette tip. Rinse cells briefly with 200 µl of PBS and spin again. It is important to avoid excessive centrifugation speeds, which can damage the cells. Make note of the place that is used, and perform subsequent aspirations from the same place.

2. Permeabilization and Blocking:

a. Remove PBS and incubate the cells with 100 µl of Fixative Solution for 15 min in the dark. Remove Fixative Solution by gentle aspiration for adherent cells, or centrifuge the plate at 200 g for 3 minutes followed by gentle aspiration for suspension cells.

b. Wash cells three times with 100 µl of Wash Buffer 5 min each. Remove the Wash Buffer. For suspension cells: centrifuge the plate between each wash at 200 g for 3 minutes.

c. Incubate cells with 100 µl of Blocking Buffer for 30 minutes. Remove the Blocking buffer by aspiration after centrifugation at 200 g for 3 minutes. While blocking, prepare the primary antibody and proceed to Immunofluorescence Staining.

3. Immunofluorescence Staining:

Notes:
The recommended dilution for primary and secondary antibodies is 1:100 but it may vary for different cell lines. To prevent cells from drying and photobleaching, the plate should be always covered and protected from light during the incubation periods.

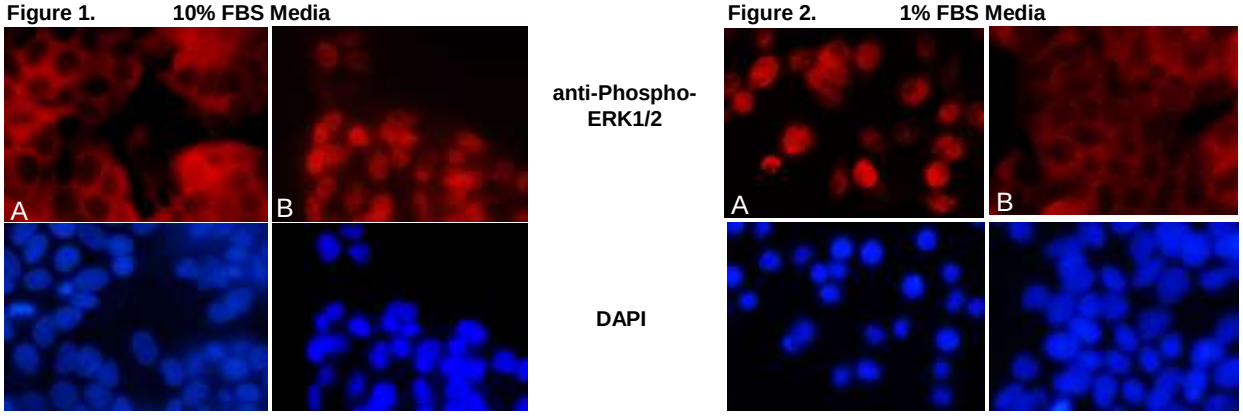
a. Primary Antibody Incubation: Dilute the Phospho-ERK1/2 Primary Antibody 1:100 in Wash Buffer. Add 100 µl of antibody dilution into each well. Incubate the plate for 2 hours at room temperature, or for best results overnight at 4°C. Remove the antibody by aspiration or centrifugation for suspension cells. Rinse cells briefly three times with 100 µl of Wash Buffer and remove the washes.

b. Secondary Antibody Incubation: Dilute the Secondary Antibody 1:100 in Wash Buffer. Add 100 µl of antibody dilution into each well. Incubate the plate for 2 hours at room temperature in the dark, or overnight at 4°C fridge protected from light. Repeat the removal of antibody and wash from step 3a.

c. DAPI Staining: Dilute DAPI stain 1:1000 in Wash Buffer, aliquot 100 µl to each well and incubate for 10 minutes in the dark. Remove the stain and rinse wells with 100 µl of Wash Buffer. For removal of DAPI stain and wash follow the steps from paragraph 3a. Add 100 µl of PBS into each well. Cells are ready to be imaged. For later analysis, store the plate at 4°C in the dark.

d. Examine the staining under fluorescence microscope with 570 nm excitation and UV laser for Phospho-ERK1/2 Secondary Antibody and DAPI respectively.

Figure 1. **10% FBS Media** **Figure 2.** **1% FBS Media**



Figures: Tamoxifen-induced translocation of phosphorylated ERK1/2 in MCF-7 cells. MCF-7 cells (1X10^5 cells per well) were grown, fixed and stained according to the included protocol. **Figure 1:** Cells grown in media supplemented with 10% FBS and treated with a vehicle (A) or 1X Tamoxifen for 20 min (B). Immunofluorescent staining revealed translocation of phosphorylated ERK1/2 from the cytoplasm (A) to nuclei (B). **Figure 2:** Cells grown in presence of 1% FBS in absence (A) or presence (B) of 1X Tamoxifen for 20 min exhibited translocation of phosphorylated ERK1/2 from nuclei (A) to cytoplasm (B). Bottom panels in Figures 1 and 2 show nuclear staining with DAPI.

VIII. RELATED PRODUCTS:

Phospho-p38 MAPK (Thr180+Tyr182) Translocation Assay Kit (Cell-Based) (K965-100)
Phospho-p53 (Ser15) Translocation Assay Kit (Cell-Based) (K966-100)
Tamoxifen Citrate (1551-1000)
Erk2 Antibody (3442-100)
p53 Nuclear Translocation Assay Kit (Cell-Based) (K961-100)
p53 (human) ELISA Kit (K4829-100)
Phospho-Mek1/2 Antibody (3519-100)
p44/42 MAPK (Erk1/2) Antibody (3085R-100)