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Phospho-p53 (Ser15) Translocation Assay Kit (Cell-Based)

(Catalog # K966-50; 50 assays; Store at -20°C) *10/16*

I. Introduction:

Tumor protein p53 (TP53, p53) is a widely conserved transcription factor and tumor suppressor commonly referred to as the "guardian of the genome". Due to the presence of transcriptional activation, DNA binding, and oligomerization domains, p53 responds to diverse cellular stresses to regulate expression of target genes, thereby inducing cell cycle arrest, apoptosis, senescence, DNA repair, or changes in metabolism. Mutations in p53 gene are associated with numerous human cancers and about 50% of those mutations result in loss of function. Multiple genotoxic and non-genotoxic stimuli lead to stabilization and activation of p53 that are mediated by numerous post-translational modifications. The N-terminus of p53 is heavily phosphorylated, whereas the C-terminus may be phosphorylated, acetylated neddylated, ubiquitinated or sumoylated. In unstressed cells, the level of p53 protein is downregulated via binding to MDM2, COP1, PIRH2 or JNK that promote p53 degradation. Cellular stress and Nutlin-3, a potent inhibitor of Mdm2-p53 interaction, activate and increase level of p53 in the cell due to lack of negative regulation. Presence of three putative nuclear localization signals (NLSs) on its C-terminus, promotes translocation of some of the activated p53 into the nucleus thus activating genes that induce cell cycle arrest, senescence, or apoptosis. BioVision's Phospho-p53 (Ser15) Translocation Assay Kit provides a simple and complete assay in a ready to use format to visualize the nuclear translocation of activated p53 in mammalian cells.

II. Applications:

- Detection of nuclear translocation of phosphorylated p53 in mammalian cells
- Characterize/Screen/Study for effectors of p53 cellular levels
- Detection of cellular stress or DNA damage caused by genotoxic and non-genotoxic stimuli

III. Sample Type:

- Adherent or suspension mammalian cell cultures

IV. Kit Contents:

Components	K966-50	Cap Code	Part Number
Fixative Solution	10 ml	WM	K966-50-1
Blocking Buffer	10 ml	NM	K966-50-2
Wash Buffer	75 ml	NM	K966-50-3
Phospho-p53 Primary Antibody (100X)	50 µl	Violet	K966-50-4
Secondary Antibody (100X)	50 µl	Red	K966-50-5
Nutlin-3	30 µl	Yellow	K966-50-6
DAPI (1000X)	20 µl	Blue	K966-50-7

V. User Supplied Reagents and Equipment:

- A 96-, 48, or 24-well clear tissue culture plate 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.
- **Nutlin-3 and DAPI:** Store at –20°C after opening. Completely thaw before each use. Avoid freeze and thaw cycle.

VII. Assay Protocol:

Notes:

The protocol below refers to a 96-well tissue culture plate format and it should be adjusted accordingly for other plate formats. Cell number per well should be optimized based on cell line specifications. Cells should be grown, treated, fixed and stained directly in multi-well plates. Assay conditions optimization is strongly recommended. 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 3 x 10^5 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.

b. The next day, apply desired treatments to the experimental wells omitting the negative control wells. Add 1 µl of Nutlin-3 stock directly into the positive control wells to induce the activation and nuclear translocation of phosphorylated p53. Incubate the plate for 4 hours, or for the period of time required by your experimental protocol.

c. Upon completion, gently aspirate off the culture medium from all wells with adherent cells and rinse cells briefly with 200 µl of PBS.

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For suspension cells; centrifuge the plate at 300 rpm (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 300 rpm for 3 minutes followed by gentle aspiration.

b. Wash cells two times with 100 µl of Wash Buffer 5 min each. Remove the Wash Buffer. Centrifuge the plate between each wash at 300 rpm for 3 minutes.

c. Incubate cells with 100 µl of Blocking Buffer for 60 minutes. Remove the Blocking buffer by aspiration after centrifugation at 300 rpm 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. During the incubation the plate should always be covered and protected from light to prevent drying and photobleaching.

a. Primary Antibody Incubation: Dilute the Phospho-p53 Primary Antibody 1:100 in Wash Buffer and add 100 µl into each well. Incubate the plate for 2 hours at room temperature, or for best results overnight at 4°C. Centrifuge the plate at 300 rpm for 3 minutes to remove the antibody and rinse wells briefly with 100 µl of Wash Buffer, centrifuge again to remove the wash.

b. Secondary Antibody Incubation: Dilute the Secondary Antibody 1:100 in Wash Buffer and add 100 µl 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-p53 Secondary Antibody and DAPI respectively.

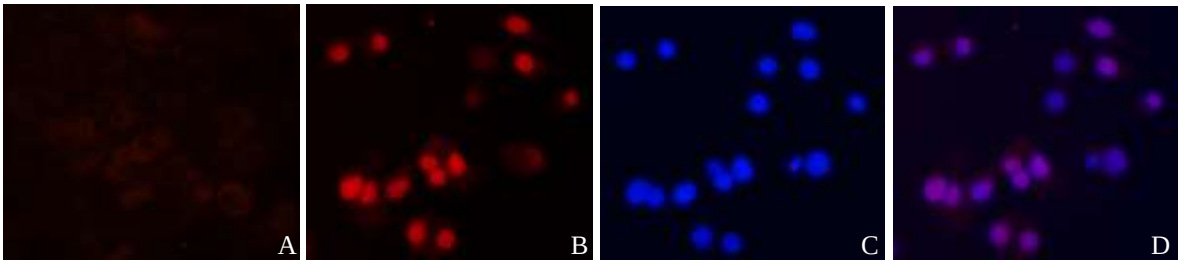

Figure: Nutlin-3-induced activation and translocation of phosphorylated p53 in J774 cells. J774 cells were grown, fixed and stained according to the provided protocol. Panel A represents untreated cells whereas cells in panel B were treated for 4 hours with Nutlin-3. In the untreated cells, phosphorylated p53 is present in very low levels and localizes in the cytoplasm (A). Upon stimulation with Nutlin-3, phosphorylated p53 translocates into the nucleus which is reflected by strong nuclear fluorescence (B). Panel C shows activated cells in B counterstained with DAPI for DNA content. Panel D, a merger of B and C, demonstrates co-localization of phospho-53 and nuclear staining (magenta).

VIII. RELATED PRODUCTS:

p53 Nuclear Translocation Assay Kit (Cell-Based) (K961-100)
p53 Transcription Factor Activity Assay Kit (K923-100)
p53 (human) ELISA Kit (K4829-100)
p53 Antibody (Clone C6A5) (3036-100)
p53 Antibody (3036R-100)
Nutlin-3 (1842-1, -5)
Phospho-p38 MAPK (Thr180+Tyr182) Translocation Assay Kit (Cell-Based) (K965-100)
NSC66811 (2009-5)
RG7388 (2577-1, -5)