

# NF-kB p65 Immunofluorescence Labeling Kit

## NF-kB p65 Immunofluorescence Labeling Kit

Part No. NFKB-3

**Storage:** Storage: p65 Ab; aliquot, -20°C. Antirabbit Rhodamine; 4°C. Store other reagents at -20°C.

**Precautions:** Contains irritants: use gloves and eye protection. Avoid ingestion, eye and skin contact.

### Kit contents:

1. Instruction Manual
2. PBS pH 7.4 (PBS-1; 10X; 16ml)
3. Permeabilization Buffer (PNFKB; 10X; 8ml)
4. Quenching Buffer (Q-1; 10X; 25ml)
5. Intracellular Detection Buffer (ANFKB-1; 10X; 60ml)
6. Rabbit IgG to p65 (P65Ab; 120 µl: Use at 1:500 dilution)
7. Anti-rabbit IgG-Rhodamine (rRhod; 50µl. Use at 1:1000 dilution)
8. Forceps set to handle glass cover slips

### Materials not provided:

1. Fixing Solution (4% Paraformaldehyde in PBS pH 7.4)
2. Anti-fade solution
3. Six-well cell culture dishes
4. Glass cover slips (clean surface before use)
5. Glass Microscope slides (clear surface before use)
6. Disposable plastic Pasteur pipettes for siphoning and dispensing solutions

**Background:** The NF-kB complex resides in the cytoplasm in its inactive state consisting of the inhibitor subunit IκB assembled with p65-p50 through ankyrin repeat structures. Upon exposure to stimuli, the IκB inhibitory subunits become ubiquitinated and degraded, exposing nuclear localization signals on the p65 subunit, which then translocates into the nucleus. Quantitative differences of p65 in the cytoplasm and nucleus can be detected following NF-kB activation using fluorescence microscopy, digital image capture and pixel quantification. Antibody selective to p65 as well as a secondary antibody with a fluorescent tag to bind to the anti-p65 antibody are included with the kit. The kit provides sufficient reagent to label 40 microscope slides.

**Methods Overview:** Cellular proteins are first cross-linked by “fixing” *in situ* with paraformaldehyde. The cross-linking reaction is subsequently quenched with a provided solution. Cells on the cover slip or slide are then permeabilized with a provided permeabilization buffer that contains a surplus of fish gelatin and BSA protein to inhibit non-specific binding of antibody to cells. The provided p65 antibody is diluted into a detection solution, which is then incubated with cells for one hour. Unbound antibody is washed away and the secondary antibody-fluorophore tagged antibody is applied to cells for 1 hr. Unbound secondary antibody is then washed away, and a sandwich is configured with slide and cover slip and antifade solution in-between. The antifade solution is allowed to solidify overnight. The microscope slide-cover slip configuration is now ready for microscopy.

### Protocol: Labeling cells with p65 antibody

#### Cell Culture Tips:

A recommended method to culture cells is on sterilized glass cover slips placed in six-well culture dishes. Cover slips can be sterilized by dipping in ethanol and flaming with a bunsen burner, and cooled prior to the addition of culture media with cells. Glass cover slips can also be coated with substances such as sterile polylysine or other matrices to enhance adhesion and growth characteristics. However, note that certain coating solutions may be toxic to cells or activate NF-kappa B. The researcher may opt to perform control experiments to determine whether the coating solution is a NF-kappa B activator.

Cells should be grown to approximately 90 per cent confluency prior to staining. A large number of cells typically detach during the immunofluorescent staining procedures: Therefore, confluent cover slips will leave a greater number of stained cells at the end of the procedure.

All cell labeling procedures and liquid handling steps should be performed as gently as possible to minimize cell detachment, and can be performed in six-well cell culture dishes. Be careful not to make bubbles in the solution. All regions of the cover slip should be kept moist at all times. Use a rocker table, with gentle rotating, to dispense liquids equally over the cover slip.

#### A. Dilutions:

On the day of immunofluorescent staining, thaw and dilute solutions in de-ionized water as follows. All solutions are provided as 10X concentrated solutions. For a six-well dish with six cover slips, make the final volumes of 1X reagent as indicated below. Scale volumes for other numbers of wells or vessel volumes. You can transfer the cover slips from the 6-well dish used to culture cells to a new 6-well dish to perform the immunofluorescence labeling procedures.

**Diluted Volumes of 1X Reagents to label 6 cover slips in a 6-well culture dish. Dilute the provided 10X solutions in dH-20 to make 1 X solutions.**

1. PBS (PBS-1): 36 ml (1X)
2. Quenching Buffer (Q-1): 30 ml (1X)
3. Permeabilization Buffer (P-1): 12 ml (1X)
4. Intracellular Detection Buffer (A-1): 80 ml(1X)

Scale up or down to corresponding volumes “as needed.” These are recommended volumes: However the researcher may find that other volumes are best suited for their “hands-on” procedures.

**Caution:** Paraformaldehyde fix procedures should be performed in a fume hood to limit inhalation. Wear gloves, lab coat and eye protection.

**B. Fix Step:**

**Paraformaldehyde:** For each cover slip, prepare 1.5 ml 4% paraformaldehyde solution in 1X PBS-1 solution. (Paraformaldehyde is not included with the kit). Paraformaldehyde can be purchased already solubilized in water as a concentrated solution (Electron Microscopy Sciences Cat. No. 15710), or suspended from a crystalline solid. Microscopy grade paraformaldehyde is recommended for this application).

Gently remove media from the wells, and wash the cover slips twice in 1.5 ml 1X PBS-1. Alternatively, place the cover slips in a new six-well dish, with the cell surface facing upward, with 1.5 ml PBS in each well. Wash the cover slips 2X in PBS

Gently remove the PBS wash, and add 1.5 ml 4% paraformaldehyde solution in PBS to each well. Submerge the cover slip in the paraformaldehyde solution for 15 min at room temperature.

**C. Quenching the Paraformaldehyde Fix:**

Remove the paraformaldehyde solution and quickly rinse each well twice with 1.0 ml Quenching Buffer (Q-1) and then submerge in 1.5 ml Quenching Buffer (Q-1) for 10 min at room temperature. Use a rocker table to dispense liquid equally over the cover slip.

**D. Permeabilization-Blocking Step:**

Remove Quenching Buffer and submerge each cover slip in 2.0 ml Permeabilization Buffer (P-1) for 15 min. Rotate the slides in the dish gently on a rocker table. This step both permeabilizes cellular membranes, including the nuclear membrane, and blocks non-specific antibody binding.

**E. "Primary Antibody" Incubation:**

Dilute the provided anti-p65 antibody (P65Ab) in 1X Detection Buffer (A-1) to a 1:500 dilution.

Prepare 1.5 ml Detection Buffer-antibody solution for each cover slip. Remove the Permeabilization Buffer and submerge cover slips with 1.5 ml antibody solution in each well. With a rocker table, gently rotate the six-well dish at room temperature for 1 hr: Set the rocker table at the slowest speed that enables a liquid film to entirely cover the cover slips. Place the lid on the six-well dish to prevent solution evaporation.

Alternative methods can also be used to conserve antibody such as flipping over the cover slip on 100  $\mu$ l of antibody solution placed on parafilm in a humidified chamber.

After the one hour period, wash unbound primary antibody from the cover slips in the six-well dish as follows: Remove antibody solution and use 1 ml 1X Detection Buffer (A-1) without antibody to first rinse the cover slip quickly one time, then submerge the cover slips in 1.5 ml 1X Detection Buffer (A-1) for 15 min. Rotate the six-well dish gently to submerge the cover slips with a liquid film. Remove the wash solution and submerge the cover slips in 1.5 ml Detection Buffer two more times for 5 min, each time.

**F. Secondary Antibody Incubation:**

Use 1X Detection Buffer (A-1) to prepare the secondary antibody (rRhod) solution at a 1:1000 dilution. Prepare 1.5 ml Detection Buffer-secondary antibody solution for each cover slip in the six-well dish.

Remove the Detection Buffer used for washes and submerge the cover slips in 1.5 mls secondary antibody solution. Using a rocker table, gently rotate the six-well dish so that liquid covers the cover slip surface. Cover slips should now be kept away from light for 1 hr in room temperature. To limit light exposure, the six-well dish can be wrapped with aluminum foil. Light exposure should be minimized in all subsequent steps and during slide storage.

After the 1 hr period, remove the secondary antibody solution and wash cover slips as described in step E, except add two additional 5 min washes. Cover slips should be kept in the dark as much as possible during this step.

**G. Mount Cover Slip or Slide in Anti-Fade Solution:** If cells were grown on cover slips, add a large drop (50-100  $\mu$ l) of anti-fade solution (we recommend Fluoromount-GT, Electron Microscopy Sciences) in the middle of a dust-free, cleaned glass microscope slide. Using the provided forceps, carefully hold the cover slip by an edge and partially drip dry the edge of the cover slip by wicking against kimwipe paper. Be careful not to break or completely dry the cover slip at this step.

Position the cover slip surface with stained cells facing downward over the anti-fade solution on the glass microscope slide. Let the flat surface of the cover slip settle by gravity on the microscope slide. *Be careful not to move the cover slip after it is*

*positioned on the microscope slide.* Place the cover slip-microscope slide sandwich in the dark, overnight, at room temperature to allow for solidification of the anti-fade solution. Afterwards, the cover slip-slide can be stored in the dark at room temperature, or at 4°C for longer term storage. The immuno-labeled microscope slide is now ready for microscopy. A Rhodamine conjugated anti-rabbit IgG is the provided secondary antibody. Therefore, use the appropriate excitation and emission setting (Ex/Em: 541/572) on the fluorescent microscope.

### Troubleshooting

1. Cells detached from cover slip. Resolution: Grow cells to approximately 90 per cent confluency. Make sure that cells are healthy, elongated and attached during the cell culture procedures. Perform wash procedures gently.
2. Staining was faint. Resolution: Determine the optimal antibody titers empirically.
3. Fluorescence becomes photo-bleached. Resolution: Intense UV emission from the fluorescent microscope may photo-bleach a spot in the microscope slide viewing field. Simply move the slide to another location for viewing.
4. Cells become distorted. Resolution: Cover slip was likely moved after being mounted in anti-fade. Do not move the cover slip.