Immunofluorescence (Intracellular) Labeling Kit

Intracellular Immunofluorescence Labeling Kit (INF-1)
FIVEphoton Biochemical's website link: http://fivephoton.com/index.php?route=product/product&path=20&product_id=51

Storage: Store all solutions at -20°C upon arrival.
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; 16 ml, 10X)
3. Permeabilization Buffer (P-1; 8 ml, 10X)
4. Quenching Buffer (Q-1; 25 ml, 10X)
5. Intracellular Detection Buffer (A-1; 60 ml, 10X)
6. Forceps set

Materials not provided:

1. Fixing Solution (4% Paraformaldehyde or Methanol)
2. Anti-fade solution
3. Six well cell culture dish
4. Glass cover slips (clean before using)
5. Glass Microscope slides (clean before using)
6. Pointed disposable plastic Pasteur pipettes to remove and dispense solutions

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OVERVIEW: Antigens expressed in cells (and tissues), which include cell surface and intracellular receptors, cell signaling proteins, ion channels, cytoskeletal proteins, cytosolic peptides, nuclear transcription factors, and chaperone proteins are examples of molecules that can be detected by immunofluorescence labeling. Cellular organelles such as Golgi, mitochondria, nuclei, endoplasmic reticulum and lysosomes are likewise detected by intracellular staining techniques by labeling a resident protein marker.

Cellular proteins are first cross-linked and immobilized by "fixing" *in situ*. To detect intracellular and cell surface antigens, membranes are permeabilized, providing access to antibodies to all cellular regions. "Primary" antibody that is specific to the antigen of interest is added and incubated with the fixed cells for 1 hour. Unbound antibody is washed and the "secondary" antibody with a conjugated fluorescent tag is then applied. The slide or cover slip with the secondary antibody is kept in the dark for another 1 hour period. After washes, the slide or glass cover slip is mounted in an anti-fade solution that preserves fluorescence. The entire immunofluorescence labeling procedure requires approximately 3 hours to complete.

Multiple Immunofluorescent Labeling
The investigator can label up to three cellular antigens with three different fluorescent colored tags. Antibodies targeting each antigen are added together in the "primary" antibody exposure step, This is followed by the simultaneous addition of three "secondary-fluorophore conjugated" antibodies that selectively bind to the primary antibodies.

An important requirement for multiple labeling is that each "primary" antibody must be derived from a different animal host. An example of an appropriate combination of three different antibody hosts may include one antibody generated in mouse directed to actin, a second antibody raised in goat directed to tubulin, and a third antibody raised in rabbit that targets beta-galactosidase. Likewise, each of the three corresponding secondary antibodies must bind to the three "primary" antibodies. For example, the three corresponding secondary antibodies could include an anti-mouse-FITC (green tag), anti-goat-Rhodamine (red tag), and an anti-rabbit-Cyan (blue-tag). Multiple labeling immunofluorescence is a powerful tool to identify the cellular distribution and co-localization of proteins in cellular organelles.

Protocol: Intracellular Immunofluorescence Labeling

Cell Culture Tips:

A recommended method to culture cells for immunofluorescent staining is on sterilized glass cover slips placed in six well culture dishes. Cover slips can be sterilized by dipping in ethanol,flaming with a Bunsen burner, and cooled prior to the addition of culture media with cells. Alternatively, cover slips can be sterilized by UV light in a cell culture hood. Glass cover slips can also be coated with substances such as sterile polylysine or other matrices to enhance growth characteristics and adhesion. Cells should be grown to approximately 90 percent confluency prior to staining. A large number of cells typically detach during the immunofluorescent staining procedures: therefore, nearly confluent cell populations on cover slips should leave a larger number of stained cells at the end of the labeling procedures**.**

All cell labeling procedures and liquid handling steps should be performed as gently as possible to minimize cell detachment. Be careful not to make bubbles in the solutions. During the labeling steps, the cover slips should be covered by a continuous film of liquid. Use a rocker table with gentle rotating to dispense liquids equally over the cover slip. The rocker table should be set at the slowest speed that enables full coverage of the cover slip with a film of labeling solution. The cover slip should be kept moist at all times.

Intracellular Immunofluorescent Labeling Protocol

A. Dilutions:

On the day of immunofluorescent labeling, thaw and dilute the provided 10X solutions in de-ionized water to make 1X solutions. The 1X volumes listed below are recommended for six cover slips in six well dishes. Scale volumes for other numbers of cover slips. (These are recommended volumes: the researcher may find that other volumes are optimal for their hands-on procedure).

1X reagent volumes required to label 6 cover slips

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

B. Fix Step:

(Paraformaldehyde or methanol are not included with the kits and are provided by the experimenter. The selection of fix solution is determined by the antibody tolerance. Consult the antibody manufacturer to determine which fix reagent is suitable).

1. **Transfer cover slips to a new six-well dish:** Prepare a separate six-well dish with 1.5 ml 1X PBS per well for each cover slip. Gently transfer each cover slip to a separate well. The surface with cells should face upward during the labeling procedures.

2. **Wash the cover slips with PBS**. Use disposable plastic Pasteur pipettes to dispense and remove solutions in all labeling steps. Use fresh pipettes for each distinct solution: do not cross-contaminate solutions during the labeling procedures. Remove the PBS solution in the wells and gently dispense another 1.5 ml of PBS for washes.

3. **Paraformaldehyde Fix:** For each cover slip, prepare 1.5 ml of 4% paraformaldehyde solution in 1X PBS-1 solution. *Use caution when handling paraformaldehyde. Avoid skin and eye contact and inhalation. Paraformaldehyde fix procedures should be performed in a fume hood to limit inhalation.* Paraformaldehyde solutions 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 cell fixing.

4. Gently remove the PBS wash, and add 1.5 ml of 4% paraformaldehyde solution to each well. Allow 15 minutes for paraformaldehyde fixing. This procedure can also be performed with ice-cold paraformaldehyde to limit endocytosis with the cover slip container placed on a bed of ice to maintain cooling.

Methanol Fix: Cool a sufficient volume of 100% methanol to -20^{o}C to submerse cells. (For a 6-well dish, 2 mls methanol/well is recommended). Wash cover slips twice with 1.5 ml of 1X PBS and then add ice-cold 100% methanol. Place cover slips in a -20^{o}C freezer for 10 minutes. Subsequently wash cover slips 3 times in 1.5 ml of 1X PBS. The quenching step described below is not required with methanol fix: proceed to step D.

C. Quenching the Paraformaldehyde Fix:

1. (Reiterating, quenching is not required for methanol fix). Rinse each well twice with 1.0 ml 1X Quenching Buffer (Q-1) and then submerse the cover slips in 1.5 ml 1X Quenching Buffer (Q-1) for 10 minutes at room temperature. Use a rocker table to gently disperse liquid equally over the cover slip.

D. Permeabilization-Blocking Step:

1. Remove Quenching Buffer and submerse each cover slip in 1.5 ml 1X Permeabilization Buffer (P-1) for 15 minutes. Rotate the solution gently with the rocker table, setting it to the slowest speed that submerses the cover slips with a liquid film. This step permeabilizes membranes and blocks non-specific antibody binding.

E. "Primary Antibody" Incubation:

1. Dilute "primary antibody" (or combination of primary antibodies for multiple labeling) in 1X Intracellular Detection Buffer (A-1) at the antibody titer recommended by the manufacturer. Prepare 1.5 ml of antibody solution for each cover slip. (For multiple labeling, all antibodies should be dispensed together in this solution). Remove Permeabilization Buffer and submerse cover slips with 1.5 ml of antibody solution. With a rocker table, gently rotate the six-well dish at room temperature for 1 hour. 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 evaporation.

   Alternative methods can also be employed to conserve antibody such as flipping over the cover slip on 100 µl of antibody solution placed on parafilm in a humidified chamber.

2. After the one hour period, wash unbound primary antibody from the cover slips in the six-well dish as follows: Remove the antibody solution and use 1 ml 1X Detection Buffer (A-1) without antibody to first rinse cover slips once quickly , then submerse cover slips in 1.5 ml 1X Detection Buffer (A-1) for 15 minutes with gentle rotating. Remove the solution and then submerse the cover slips in 1.5 ml 1X Detection Buffer two more times for 5 minutes with gentle rotating.

F. "Secondary Antibody" Incubation:

1. Use 1X Intracellular Detection Buffer (A-1) to dilute secondary-fluorophore conjugated antibody (or multiple secondary antibodies in the case of multiple labeling) into the dilution recommended by the manufacturer. Prepare 1.5 ml Intracellular Detection Buffer-secondary antibody solution for each cover slip in the six-well dish.

Remove the Intracellular Detection Buffer used for washes of the primary antibody, and submerse the cover slips in the secondary antibody solution. Using a rocker table, gently rotate the six-well dish so that a film of liquid entirely submerses the cover slips. Cover slips should now be kept in the dark for 1 hour in room temperature. To limit light exposure, the six-well dish can be wrapped with aluminum foil. Light exposure should be minimized in this and all subsequent steps.

After the 1 hour period, wash the unbound secondary antibody. Remove the secondary antibody solution and wash cover slips as described in step E, except perform two additional 5 minute washes.

G. Mount Cover Slip or Slide in Anti-Fade Solution:

1. If cells were grown on cover slips, add a large drop (50-100 μl) of anti-fade solution (for example, Fluoromount-GT, Electron Microscopy Sciences) in the middle of a clean,dust-free glass microscope slide. Using the provided forceps, carefully hold the cover slip by an edge and drip dry the edge of the cover slip by wicking the edge against a kimwipe paper. Be careful not to break or completely dry the cover slip at this step.

2. Position the cover slip surface with stained cells facing downward over the anti-fade solution on the glass microscope slide. Let 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 combination in the dark, overnight at room temperature to allow for solidification of the anti-fade solution. Afterwards, the cover slip-slide combination can be stored in the dark at room temperature, or at 4^{o}C. The microscope slide is now ready for microscopy.

Troubleshooting

1. Cells detached from cover slip. Resolution: Grow cells to approximately 90 percent 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 may have been moved after being attached to the microscope slide. Do not move the cover slip.