Extracellular Immunofluorescence Kit

Extracellular Immunofluorescence Labeling Kit (EIF-1)
FIVEphoton Biochemicals 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 eye and skin contact.
Kit contents: (Trial kits do not provide PBS).

1. Instruction Manual
2. PBS pH 7.4 (PBS-1: 10X, 32 ml)
3. Quenching Buffer (Q-1: 10X, 26 ml)
4. Extracellular Blocking Buffer (EB-1: 10X, 10 ml)
5. Extracellular Detection Buffer (E-1; 10X, 80 ml)

Materials not provided:

1. Fixing Solution (4% Paraformaldehyde)
2. Anti-fade solution
3. Six-well cell culture dish
4. Glass cover slips
5. Glass Microscope slides

OVERVIEW: Antigens expressed in cells (and tissues), such as cell surface and intracellular receptors, cell signaling proteins, ion channels, cytoskeletal proteins, cytosolic peptides, nuclear transcription factors, and chaperone proteins are all examples of molecules that can be detected by immunofluorescence cell labeling. Cellular proteins are first cross-linked and immobilized by "fixing" *in situ* by either paraformaldehyde or methanol (used only for intracellular labeling) cross-linking dependent on the antibody tolerance, followed by quenching of the crosslinking solution. If the researcher wishes to detect extracellular epitopes, the cell membrane is often not permeabilized and an antibody that recognizes an extracellular epitope is added in a non-detergent buffer to cells instead. The FIVEphoton Biochemicals Extracellular Immunofluorescence Labeling Kit, EIF-1, is designed for extracellular immunofluorescence. To detect intracellular antigens, the cell surface membrane is permeabilized with a buffer that contains either saponin or detergent, a method that is provided in the FIVEphoton Biochemicals Intracellular Immunofluorescence Labeling Kit, INF-1.

"Primary" antibody that is specific to the antigen is added and incubated with the fixed cells for 1hour. Unbound antibody is then washed and the "secondary" antibody which is conjugated to a fluorescent tag is added. The slide or cover slip with a second antibody is kept in the dark for another 1 hour period. The glass slide or glass cover slip is finally mounted in an anti-fade solution that preserves the fluorescence. This entire procedure for immunofluorescence labeling requires approximately 3 hours to complete. The immuno-stained slides should first be stored in the dark for 24 hours at room temperature to harden the anti-fade solution, and then at room temperature or 4°C for longer term storage.

Multiple Labeling of Cellular Antigens

The investigator can employ the "Immunofluorescence Labeling-Staining Kits" to label up to three different cellular antigens with three different fluorescent colored tags. The use of three distinct primary antibodies is an example of triple immunofluorescent labeling. To perform multiple labeling, antibodies targeting each antigen are added in the "primary" antibody exposure step, followed by the addition of three "secondary" antibodies (which bind to the primary antibodies) that are conjugated with reporter molecules that have distinct fluorescence excitation and emission profiles.

An important requirement for multiple labeling immunofluorescence is that each "primary" antibody must be derived from a different animal host. An appropriate combination of three different antibody hosts may, for example, 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 distinctly to the three "primary" antibodies and conjugate to distinct color tags as well. 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 and organelles. In addition, multiple labeling immunofluorescence can detect interactions between two cellular components by Fluorescent Resonance Energy Transfer (FRET) through energy transfer from donor to acceptor.

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 into ethanol and flaming with a Bunsen burner, then cooled prior to the addition of culture media. Glass cover slips can also be coated with substances such as sterile polylysine or other matrices to enhance adhesion and growth characteristics. 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, confluent cover slips will leave more 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. This can be performed directly in a six-well cell culture dish. Be careful not to make bubbles in the solution. The cover slip should be kept moist at all times. Use a rocker table with gentle shaking to dispense liquids equally over the cover slip.

Extracellular Immunofluorescent Labeling Protocol

A. Dilutions:

On the day of immunofluorescent staining, thaw and dilute solutions in de-ionized water. All solutions are provided as 10X concentrated solutions. For a six-well dish with six cover slips, dilute the provided solutions to make the final volumes of 1X reagent as follows.

Diluted Volumes of 1X Reagents to label 6 cover slips

1. PBS (PBS-1): 30 ml
2. Quenching Buffer (Q-1): 25 ml
3. Extracellular Blocking Buffer (EB-1): 12 ml
4. Extracellular Detection Buffer (E-1): 110 ml

Scale up or down to corresponding volumes "as needed." These are recommended volumes: (However, the researcher might find that other volumes are best suited for their hands-on procedures.) *Caution: Paraformaldehyde procedures should be performed in a fume hood to limit inhalation. Wear gloves and eye protection.*

B. Fix Step:

(Paraformaldehyde is not included with the kits and is provided by the experimenter. Methanol permeabilizes membranes, and therefore cannot be used for selective extracellular epitope labeling).

Paraformaldehyde: For each cover slip, prepare 1.5 ml of 4% paraformaldehyde solution in the provided diluted PBS-1 solution. *Paraformaldehyde fix procedures should be performed in a fume hood to limit inhalation. Use caution when handling paraformaldehyde. Avoid skin and eye contact and inhalation.* Paraformaldehyde solution can either 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 use in this procedure.

Gently remove media and wash cells on the cover slip twice in 1.5 ml of PBS-1 to remove media. Gently remove the PBS-1 wash and add 1.5 ml of 4% paraformaldehyde solution in PBS-1 for 15 minutes at room temperature. This procedure can also be performed with ice-cold paraformaldehyde-PBS to limit endocytosis, with the cell culture dish-slide container placed on a bed of ice.

C. Quenching the Paraformaldehyde Fix:

Rinse each cover slip twice with 1.0 ml Quenching Buffer (Q-1) and then submerse each cover slip in 1.5 ml Quenching Buffer (Q-1) for 10 minutes at room temperature. Use a rocker table to dispense liquid equally over the cover slip. Use gentle rotating to limit cell detachment.

D. Extracellular Blocking Step:

Remove Quenching Buffer and submerse each cover slip in 2.0 ml of Extracellular Blocking Buffer (EB-1) for 15 minutes. Rock gently on a rocker table. This step blocks non-specific antibody binding sites.

E. "Primary Antibody" Incubation:

Dilute "primary antibody" (or combination of primary antibodies for multiple labeling) in Extracellular Detection Buffer (E-1) at the antibody titer recommended by the manufacturer for each antibody, or empirically identify the antibody dilution that provides the highest signal to noise ratio. Prepare 1.5 ml of antibody solution for each cover slip. Remove Blocking Buffer and submerse cover slips with 1.5 ml of antibody diluted in Extracellular Detection Buffer (E-1). Rotate gently at room temperature for 1 hour.

Alternative methods can also be used to conserve antibody such as flipping over the cover slip on 100 μl of antibody solution placed on parafilm in a humidified chamber. After the one hour period, wash unbound primary antibody as follows: Remove the antibody solution and use 1ml Extracellular Detection Buffer (E-1) without antibody to first rinse the cover slip quickly one time. Then submerse in 1.5 ml of Extracellular Detection Buffer (E-1) for 15 minutes. Remove buffer and then submerse in 1.5 ml of Extracellular Detection Buffer (E-1) two more times for 5 minutes each time. Use a rocking table to gently swirl solutions during the wash steps.

F. Secondary Antibody" Incubation:

Use Extracellular Detection Buffer (E-1) to dilute secondary antibodies, using the dilutions recommended by the antibody manufacturer. Prepare 1.5 ml of Extracellular Detection Buffer (E-1) with secondary antibody for each cover slip.

Remove the Extracellular Detection Buffer (E-1) used for washes of the primary antibody and submerse the cover slips with the secondary antibody solution. For multiple labeling, add all antibodies simultaneously in the solution. Cover slips should now be kept in the dark for 1 hour in room temperature. Light exposure should be minimized in all subsequent steps and during slide storage. To limit light exposure, the six-well dish can be wrapped with aluminum foil.

After the 1 hour period, wash with unbound secondary antibody. Remove secondary antibody solution and wash cover slips as described in step Eadding two additional 5 minute 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 μl) of anti-fade solution (we recommend Fluoromount-GT, Electron Microscopy Sciences) in the middle of a glass microscope slide. Using the provided forceps, carefully hold the cover slip by an edge and partially drip dry the cover slip by wicking against a Kimwipe paper. Be careful not to break or completely dry the cover slip during this step.

Place the cover slip with the side containing the stained cell surface downward on top of the anti-fade solution on the glass slide. *Be careful not to move the cover slip after this step.* Place the cover slip-slide combination in the dark at room temperature overnight to allow the anti-fade solution to harden. Afterwards, the cover slip-slide can be stored in the dark at room temperature, or at 4^{o}C. The immuno-stained cover slip is now ready for microscopy.

Troubleshooting

1. Cells detached from the cover slip. Resolution: Grow cells to confluency, coat cover slip with polylysine and perform liquid handling steps gently. Make sure that cells are healthy, elongated, and attached during the cell culture procedures.

2. Staining was faint. Resolution: Increase the antibody titers for primary antibody. Attempt to use another antibody targeting the same antigen. Often the researcher must purchase several different antibodies targeting the same antigen to identify the antibody that maximizes the fluorescent signal to noise ratio.

3. Fluorescence becomes photo-bleached. Resolution: Intense 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.