This protocol is a general protocol, users should optimize the best condition depending on the samples, target protein and the used antibodies. Please refer the antibody datasheet or references for the suitable conditions for sample preparation, concentration or incubation condition of the primary antibodies and the secondary antibodies.

Materials and Reagents used in this protocol:

Coverslip / chamber slides

Coverslip: In general, coverslip #1.5 is recommend to be used in Immunofluorescence assay for culture cell on coverslip, since the thickness of #1.5 coverslip (0.17mm) provides the proper working distance for most microscope objectives.

Chamber slides: poly-L-lysine or collagen coated chamber slides

Solutions:

10 × PBS:

NaCl ---------- 80 g

KCl ---------- 2.0 g

Na_2HPO_4 ---------- 14.4 g

KH_2PO_4 ---------- 2.4 g

Distilled Water---------- 800 mL

Adjust pH to 7.2-7.4 with HCl.

Adjust volume to 1 L with additional H_2O

Sterilize by autoclaving.

1 x PBS

Dilute 100 ml of the 10 x PBS in a total 1L volume of ddH_2O.

Washing Buffer:

1 X PBS

(or) PBST: add 0.5 ml of Tween-20 in 1L 1 x PBS and mix well.

Fixatives:

4% Paraformaldehyde (PFA) (pH 7.4)

(Please prepare PFA in a fume hood and wear gloves to avoid fumes and air-borne powder)

- Add 4 g of paraformaldehyde to 88 ml of water.
- Heat at 55-60˚C in water bath or hot-plate (not over 60˚C) to dissolve the powder.
- Slowly add 5N NaOH (approx. 10-15µl) until the paraformaldehyde is dissolved.

- Remove from heat, and add 10 ml of 10 x PBS and mix.
- Adjust pH of the solution to 7.2 by adding 5N HCl (approx. 5-10µl).
- Add ddH_2O to make a final volume of 100 ml.
- Filter the solution through a filter.
- Cool on ice, store at 4°C up to 1 month or aliquots (5-20ml) and store at -20°C up to 1 year.

(or) 100% ice-cold methanol

(or) Ice-cold 1:1 methanol/Acetone

Blocking Buffer (chose one of below):

5-10% serum from host species of secondary antibody (blocking)

(or) 1-3% BSA (stabilizer) in 1 x PBS

(or) 1-3% BSA (stabilizer) in 1 x PBST

Permeabilization Buffer

0.05-0.3% Triton X-100 (or 100 µM digitonin or 0.5% saponin) in PBS

Antibody Dilution Buffer:

1% BSA (for stabilizer and blocking) in 1 x PBST

Coverslip preparation:

Sterile coverslip by washing in ethanol or exposing to UV in cell-culture hood for at least 60 min.

Coverslip coating:

- Coating the sterilized coverslips by 50-100 µg/ml poly-lysine for 1h at RT,
- Wash the coverslips by H_2O for 3 times,
- Dry the coverslips completely in the cell-culture hood overnight and expose the coverslips under UV light at least 4h to sterilize the coverslips.

Cell Preparation:

- Put the coated coverslips in the culture plate (round 18 mm coverslips for 12-well plate, round 12 mm coverslips for 24-well plate)
- Transfer the cells on the coverslip in plates and culture them for overnight or 2-3 days until the cells reach 50-70% confluence.

Process:

Fixation:

- Aspirate culture medium and rinse the cells with PBS twice.
- Fix the cells with 2-4% paraformaldehyde (PFA) for 10-20 min at RT or with cold methanol, acetone (1-10 min) at -20°C.
- Wash the cells by PBS 3 times for 5 min each.

Permeabilization:

- Aspirate PBS, add 0.05-0.25% Triton X-100 (or 100 µM digitonin or 0.5% saponin) in PBS and incubate the samples for 10-15 min at RT.
- Wash the cells by PBS 3 times for 5 min each.

  Note:

  1. If the sample fixed with methanol or acetone do not need permeabilization.
  2. Epitopes of primary antibodies located at extracellular region of proteins do not require much/any permeabilization.
  3. If the target epitopes of primary antibodies is located at intracellular region of protein, it is very important to permeabilize the cells to help the antibodies entry the cells.
  4. Triton X-100 is the popular detergent to improve the antibody penetration in this step. However, it might destroy the cell membrane so it is not appropriate for the use with membrane-associated protein staining. (It can be insteaded by 100 µM digitonin or 0.5% saponin or Tween-20 or NP-40)

Blocking:

- Aspirate PBS and incubate the cells with 1-3% BSA in PBS for 30-60min at RT to block unspecific

Primary Ab staining:

- Aspirate blocking buffer and incubate cells with the primary antibody in a proper dilution with dilution buffer (keep samples in a humid chamber and avoid the samples to dry out) for 1-4 hr at RT (or 37°C), or overnight at 4°C.
- Wash the cells by PBS 3 times for 5 min each.

Secondary Ab staining:

- Aspirate washing buffer and incubate cells with the secondary antibody in a proper dilution with dilution buffer (keep samples in dark in a humid chamber and avoid the samples to dry out) for 30-60 min at RT.
- Wash the cells by PBS 3 times for 5 min each.

Counterstain staining:

- Stain nuclear with DAPI (0.5 µg/ml) or Hoechst (0.1-12 µg/ml) in PBS for 5-10 min.
- Wash the cells by PBS 2 times for 5 min each.
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Mounting:

- Aspirate the wash buffer on the coverslip by fiber-free paper or paper towel and try to remove the washing buffer as much as possible.
- Mount the coverslips up site down on slides with a small drop of mounting medium.
- Put the coverslips on the slide slowly to avoid bubbles.
- Seal the coverslip to the slide with nail polish and allow to air dry.
- Store slides in a covered box to away from light at 4°C.