Immunofluorescence，IF

I. Preparation of Solutions and Reagents

1. PBS Buffer: Prepared by dissolving PBS powder in ddH_2O.
2. 4% Paraformaldehyde: Store at room temperature; pre-warm to 37 °C before use.
3. 0.5% Triton X-100 Solution: Prepared by mixing Triton X-100 with PBS; store at 4 °C.
4. Blocking Buffer: 10% goat serum, prepared by diluting goat serum in PBS; store at –20 °C.
5. 0.5 μg/mL DAPI Staining Solution: Prepared by diluting DAPI in PBS; store at –20 °C, protected from light.

II. Experimental Procedure

A. Preparation of Cell Samples

1. Placing Coverslips

Take a 12-well plate and add 20 μL of PBS solution into each well. Using forceps, place a 20 mm diameter cell coverslip into each well. Gently press the coverslip down to ensure it adheres tightly to the bottom of the well due to atmospheric pressure, preventing it from floating during subsequent steps.

2.Washing the Coverslips

Add 1 mL of PBS solution to each well. Gently shake the plate, then use a vacuum pump to completely remove the PBS.

3.Cell Seeding

Depending on experimental needs, add the cell suspension to each well and culture the cells.

B. Fixation

1. Cell Collection and Washing

Remove the culture medium. Add 1 mL of PBS solution to each well to wash the cells. Repeat the wash 3 times, each for 3 minutes.

2.Fixation

Add 700 μL of 4% paraformaldehyde to each well to fix the cells for 10 minutes.

3.Post-Fixation Washing

Wash with PBS 3 times, each for 3 minutes.

C. Permeabilization

Add 700 μL of 0.5% Triton X-100 solution to each well and incubate for 30 minutes to permeabilize the cell membranes. Wash with PBS 3 times, 3 minutes each time.

D. Blocking

Add 700 μL of blocking buffer to each well and incubate at room temperature for 45 minutes.

E. Primary Antibody Incubation

1. Prepare the primary antibody dilution using blocking buffer (refer to the product website for recommended dilution ratios).
2. Remove the blocking buffer, then add 700 μL of the primary antibody dilution to each well. Incubate either overnight at 4 °C or for 2.5 hours at room temperature. If incubated at 4 °C, allow the cells to return to room temperature for 30 minutes before proceeding to the next step.
3. Wash with PBS 3 times, 3 minutes each time.

F. Secondary Antibody Incubation

1. Select a fluorophore-conjugated secondary antibody that recognizes the species of the primary antibody. Prepare the secondary antibody dilution using blocking buffer (refer to the product website for recommended dilution; protect from light).
2. Add 700 µL of the secondary antibody dilution to the surface of the cells and incubate at room temperature for 1 hour (protect from light).
3. Wash with PBS 3 times, 3 minutes each (protect from light).

G. Nuclear Staining

1. Add 0.5 µg/mL DAPI staining solution and incubate at room temperature for 5 minutes (protect from light).
2. Wash with PBS 3 times, 3 minutes each (protect from light).

H. Mounting

1. Use forceps to carefully pick up the cell-covered coverslip, and use absorbent paper to remove any moisture around the coverslip. Add an appropriate amount of anti-fade mounting medium onto a microscope slide, then gently place the coverslip (cell side facing down) onto the mounting medium. Try to avoid the formation of air bubbles during the process. (Protect from light.)
2. Add mounting sealant around the coverslip to seal it. Once the sealant has dried, immediately observe and image the sample under a fluorescence microscope. (Protect from light.) If immediate observation is not possible, store the slide protected from light at 4 °C and observe within three days to prevent fluorescence quenching.

III. Experimental Precautions

1. The volumes of 4% paraformaldehyde, 0.5% Triton X-100, blocking solution, primary antibody dilution, secondary antibody dilution, and DAPI staining solution added to each well are not fixed and can be adjusted according to the specific experimental conditions. However, the added liquids should at least cover the coverslip to prevent cell drying.
2. Throughout the experimental procedures before mounting, the cells should always be kept in a moist state.
3. Triton X-100 solution tends to separate after standing for a long time; invert the solution several times before use to resuspend it evenly.
4. The coverslips used in this procedure must be sterile, surface-treated, and suitable for cell culture.

IV. Recommended Expermental Products

1. Antibody: https://www.targetmol.com/all-antibodies
2. TritonX-100, T64297
3. DAPI Dihydrochloride, T19827; DAPI dilactate, T86160
4. Tween 20, T9526

Immunocytochemistry, ICC

I. Preparation of Solutions and Reagents

1. PBS Buffer: Prepare using PBS powder and ddH_2O.
2. 4% Paraformaldehyde: Store at room temperature; preheat to 37°C before use.
3. 0.5% Triton X-100 Solution: Prepare by diluting Triton X-100 in PBS; store at 4°C.
4. Blocking Buffer: 10% goat serum prepared with goat serum and PBS; store at -20°C.

II. Experimental Procedure

A. Preparation of Cell Samples

1. Placing Coverslips

Take a 12-well plate and add 20 µL of PBS to the center of each well. Use forceps to place a 20 mm diameter cell coverslip into the well. Gently press the coverslip down so that atmospheric pressure adheres it tightly to the well bottom, preventing it from floating during subsequent steps.

2.Washing Coverslips

Add 1 mL of PBS to each well, gently shake the plate to rinse, then use a vacuum pump to completely remove the PBS.

3.Seeding Cells

Add the cell suspension to the wells according to experimental needs and culture the cells.

B. Fixation

1. Collect the cells and aspirate the culture medium. Add 1 mL of PBS to each well to wash the cells. Wash three times, 3 minutes each.
2. Add 700 µL of 4% paraformaldehyde to each well and fix the cells for 10 minutes.
3. Wash with PBS three times, 3 minutes each.

C. Permeabilization

1. Add 700 µL of 0.5% Triton X-100 solution to permeabilize the cells for 30 minutes.
2. Wash with PBS three times, 3 minutes each.

D. Incubation with 3% Hydrogen Peroxide

Add 700 µL of 3% hydrogen peroxide solution to each well and incubate for 10 minutes to block endogenous peroxidase activity.

Wash with PBS three times, 3 minutes each.

E. Blocking

Add 700 µL of blocking buffer to each well and incubate at room temperature for 45 minutes.

F. Primary Antibody Incubation

1. Prepare the primary antibody dilution using the blocking buffer (refer to the product website for recommended dilution ratios).
2. Remove the old blocking buffer. Add 700 µL of primary antibody dilution to each well, and incubate either overnight at 4°C or for 2.5 hours at room temperature. If incubated at 4°C, allow the cells to return to room temperature for 30 minutes before proceeding to the next step.
3. Wash with PBS three times, 3 minutes each time.

G. Secondary Antibody Incubation:

1. Select an HRP-conjugated secondary antibody that matches the host species of the primary antibody. Prepare the secondary antibody dilution using the blocking buffer (refer to the product website for recommended dilution ratios).
2. Add 700 µL of the secondary antibody dilution to the cell surface, and incubate at room temperature for 30 minutes.

3. Wash with PBS three times, 3 minutes each time.

H. Color Development

Prepare the DAB working solution and add 400 μL onto the cells. Develop the color for 1–3 minutes. During DAB staining, continuously monitor the color change. Once the cells exhibit a brown-yellow coloration with moderate intensity, immediately add distilled water to stop the color development.

I. Hematoxylin Counterstaining of Nuclei

Add an appropriate amount of hematoxylin staining solution. Staining time varies depending on the type of hematoxylin, ranging from a few seconds to several minutes. It is recommended to start with a few seconds and adjust based on the staining result.

After staining, immediately rinse with distilled water. Alternatively, the 12-well plate can be placed under a gentle stream of running water to remove residual dye (be careful not to let the water flow directly onto the cells).

J. Dehydration

Immerse the cell coverslips sequentially in 85% ethanol and absolute ethanol for 3 minutes each.

K. Mounting

Use tweezers to carefully retrieve the coverslip, placing it cell-side up and allowing it to air-dry naturally. Add an appropriate amount of neutral balsam onto a microscope slide, then place the cell-side of the coverslip onto the balsam. Avoid creating air bubbles during this process. Once the balsam has dried, observe and capture images under a microscope.

III. Experimental Precautions

1. The volume of 4% paraformaldehyde, 0.5% Triton X-100, 3% hydrogen peroxide, blocking solution, antibody diluent, DAB working solution, and hematoxylin staining solution added to each well is not fixed and can be adjusted according to the specific experimental conditions. However, the liquid must at least cover the coverslip to prevent the cells from drying out.
2. During the entire experimental process prior to mounting, cells should be kept in a moist state at all times.
3. Triton X-100 solution may exhibit phase separation after standing for a long time. Shake or invert the container before use to ensure the solution is resuspended properly.
4. Coverslips used in this procedure must be sterile, surface-treated, and suitable for cell culture.

IV. Recommended Experimental Products

1、Antibody: https://www.targetmol.com/all-antibodies
2、TritonX-100, T64297
3、Tween 20, T9526