Immunocytochemistry Staining for Methanol Fixed Cells V.3

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Troubleshooting

Before start

Reagent List:

	▪ Chamber slides or cover slips ▪ Fixation solution: 100% methanol stored at -20°C for at least two hours before use	▪ Blocking soluion: 5% FBS in PBS ▪ 70% Ethanol

Sterilization

1 Transfer a single cover slip into a 12-well plate, then add 1 mL of 70% Ethanol into a well for 20 minutes at room temperature.
00:20:00

2 Wash quickly three times with PBS.

Poly-Lysine Coating for 12-Well Plates (optional; for loosely attached cells)

3 Add 1 mL of 0.1 mg/mL Poly-D-lysine solution into a well for 15 minutes at room temperature.
00:15:00

4 Wash quickly three times with PBS and let dry before plating cells.

Sample preparation

5 Grow cultured cells on cover slips or in wells overnight at 37°C. At the time of fixation, cells should be ~70-80% confluent in single layer.
00:30:00

6 Rinse cells briefly in PBS.

7 Fix and permeabilize cells by incubation with cold 100% methanol for 5-15 minutes at -20°C.
00:15:00

8 Rinse three times quickly in PBS.

Sample Blocking

9 Block samples in 1 mL of blocking buffer at room temperature for 30 minutes.
00:30:00

Sample staining

10 Dilute the primary antibody to the recommended concentration/dilution in blocking buffer.

11 For 8-well chamber slides, add 200 µL per well. For 12-well plates, add 500 µL per well. Incubate two to three hours at room temperature or overnight at 4°C. If using conjugated antibodies, perform this step in the dark.
03:00:00

12 For surface staining, rinse 3 times quickly in PBS. For intracellular staining, quickly wash once followed by incubation with wash buffer for 5-10 minutes. Then quickly wash additional two times.

Note: If using primary antibodies directly conjugated to fluorochromes, then skip to step 12.
00:10:00

13 Prepare fluorochrome-conjugated secondary antibody in blocking buffer according to the manufacturer's specification data sheet, and add 200 µl per well to the 8-well chamber slides. For 12-well plates, add 500 µL per well.

14 Incubate the samples for one hour, at room temperature, in the dark.
01:00:00

15 For surface staining, rinse three times quickly in PBS. For intracellular staining, quickly wash once followed by incubation with wash buffer for 5-10 minutes, then quickly wash additional two times.
00:10:00

16 Optional: To stain F-actin, prepare a working solution of Flash Phalloidin™ by diluting it 1:20-1:100 in PBS. Add 200 µL per well for an 8-well plate or 500 µL per well for a 12-well plate. Stain for 20 minutes at room temperature in the dark.

17 Apply anti-fade mounting medium to the cover slip.

18 Seal slides with nail polish.