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ICC Methanol Fixed Cells—Indirect Method

Staining using a purified/biotinylated antibody

Research Use Only

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Materials

- Phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4)
- Fixation reagent: 100% methanol
- Blocking reagent: IHC/ICC Blocking Buffer-Low Protein (cat. no. 00-4953) or High Protein (cat. no. 00-4952) or 10% Normal Serum (from the species from which the secondary antibody was made) in PBS
- Primary antibody: purified or biotinylated format
- Secondary antibody/visualization reagent: biotinylated and streptavidin-conjugated fluorophore (3-step protocol) or fluorophore-conjugated secondary (2-step protocol)
- Nuclear counterstain: DAPI or DRAQ5 (cat. no. 65-0880)
- Mounting medium: Fluoromount-G (cat. no. 00-4958) or Fluoromount-G with DAPI (cat. no. 00-4959)

Accessories

- Humidified container in which to place the samples during incubations
- Parafilm
- Glass coverslips
- Clear nail polish

Methods

1. Cells are plated at an appropriate density and allowed to attach to the slide or dish (ex. 30,000 cells/chamber in an 8-chamber slide). Cells are usually plated one day prior to staining in order to achieve 60-80% confluency.
2. Fix the cells with 100% methanol for 10 minutes at -20°C.
   Note: Optimal fixation time and reagent depends on the antigen of interest and must be optimized. The times and methods are suggested starting points for optimization.
3. Gently wash the cells 3 times in PBS (5 min/wash) using a dropper to add PBS to the chamber followed by aspiration to remove the buffer.
   Note: It is critical from this point on that the cells do not dry out, as this will cause increased levels of background staining and difficulty interpreting staining results. It is best not to aspirate more than 2 wells at a time in order to reduce the possibility of the cells within the wells drying.
4. Cover the cells with blocking solution (100 µL/chamber in an 8-chamber slide). To limit evaporation, use a piece of parafilm to tightly cover the opening of the chamber slide. Incubate in a humidified chamber for 1 hour at room temperature.

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5. Remove the parafilm cover and gently wash the cells 3 times in PBS (5 min/wash) as described in step 3.
6. Dilute purified or biotinylated antibody, at manufacturer's recommended dilution, in blocking reagent and overlay onto cells. Tightly cover the opening of the chamber slide with Parafilm. Incubate in a humidified chamber overnight at 4°C.
7. Remove the Parafilm cover and gently wash the cells 3 times in PBS (5 min/wash) as described in step 3.
   If using an unconjugated primary antibody, continue to step 8 (3-step protocol).
   If using a biotinylated primary antibody, continue to step 10 (2-step protocol).
8. Dilute the fluorophore-conjugated secondary antibody, at manufacturer's recommended dilution, in blocking reagent, protecting from light. Overlay the secondary antibody solution on the cells and cover with parafilm as described in step 4. Incubate in a humidified chamber for 1 hour at room temperature.
9. Gently wash the tissue 3 times in PBS (5 min/wash) as described in step 3. Proceed to step 12.
10. Dilute streptavidin-conjugated fluorophore, at manufacturer's recommended dilution, in blocking reagent, protecting from light. Overlay the visualization reagent on the tissue and cover with Parafilm as described in Step 4. Incubate in a humidified chamber for 30 minutes at room temperature.
11. Gently wash the cells 3 times in PBS (5 min/wash) as described in step 3.
12. Optional: Nuclei can be counterstained using DAPI or DRAQ5. It is necessary to select a counterstaining agent with a fluorescent emission spectra that does not overlap with the emission spectra of the other fluorophores used in the experiment.
13. Mount and coverslip using Fluoromount-G or Fluoromount-G with DAPI. Seal the edge of the coverglass with clear nail polish.
14. Allow slides to dry for 1-2 hours before visualizing.
15. Slides can be stored at 4°C protected from light, if needed.

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