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# ICC Formaldehyde Fixed, Permeabilized Cells—Direct Method

# Staining using a fluorophore—conjugated antibody

**Research Use Only**

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### Materials

Tris buffered saline (TBS) (50 mM Tris, 150 mM NaCl, pH 7.4) or 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) (NOTE: for maximum signal intensity, we recommend using TBS with eFluor Nanocrystals)

- Fixation reagent: 4% formaldehyde in PBS
- Permeabilization: 0.1% Triton X-100 in PBS (w:v)
  Blocking reagent: IHC/ICC Blocking Buffer-Low Protein (cat. no. 00-4953) or High Protein (cat. no. 00-4952) (Note: for nuclear staining using eFluor Nanocrystals, IHC/ICC Blocking Buffer-High Protein must be used)
- Primary antibody: fluorophore-conjugated format
- 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 4% formaldehyde for 15 min at room temperature.
   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.


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4. Overlay the cells with permeabilization reagent. To limit evaporation, use a piece of parafilm to tightly cover the opening of the chamber slide. Incubate at 37°C for 30 min followed by 10 min at room temperature.
5. Remove the parafilm cover and gently wash the cells 3 times in PBS or TBS (5 min/wash) as described in step 3.
6. Cover the cells with blocking solution (100 µL/chamber in an 8-chamber slide). Tightly cover the opening of the chamber slide with parafilm. Incubate in a humidified chamber for 1 hour at room temperature.
7. Remove the parafilm cover and gently wash the cells 3 times in PBS or TBS (5 min/wash) as described in step 3.
8. Dilute fluorophore-conjugated antibody, at manufacturer's recommended dilution, in blocking reagent and overlay onto cells, protecting from light. Tightly cover the opening of the chamber slide with parafilm. Incubate in a humidified chamber overnight at 4°C.
9. Remove the parafilm cover and gently wash the cells 3 times in PBS or TBS (5 min/wash) as described in step 3.
   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.
10. Mount and coverslip using Fluoromount-G or Fluoromount-G with DAPI. Seal the edge of the coverglass with clear nail polish.
11. Allow slides to dry for 1-2 hours before visualizing.
12. Slides can be stored at 4°C protected from light if needed.


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