

Nov 09, 2022

Immunocytochemistry

DOI

dx.doi.org/10.17504/protocols.io.eq2ly779w1x9/v1

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Protocol Citation: Addgene The Nonprofit Plasmid Repository 2022. Immunocytochemistry. **protocols.io**
<https://dx.doi.org/10.17504/protocols.io.eq2ly779w1x9/v1>

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Protocol status: Working

We use this protocol and it's working

Created: November 04, 2022

Last Modified: November 09, 2022

Protocol Integer ID: 72295

Keywords: labeling hela cell, formaldehyde fixation method, antigens in cell, antibody, fluorescent secondary antibody, antigen, primary antibody, immunocytochemistry immunocytochemistry, labeling cell, cells in culture, cell, target protein, different cell, protein

Disclaimer

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Abstract

Immunocytochemistry is a technique that uses antibodies to detect antigens in cells. Here we describe the basic steps for fixing and labeling cells in culture with a primary antibody against a target protein and a fluorescent secondary antibody. This protocol outlines the steps for fixing and labeling HeLa cells for a target protein using the formaldehyde fixation method. The protocol may need to be optimized for different cells, target proteins, etc.



Guidelines

Workflow Timeline:

Day 1: Seed cells

Day 3-4: Fix and label cells

Tips and Troubleshooting:

We recommend wiping down all pipettes and equipment with 10% bleach prior to use.

The optimal fixation method will vary depending on the sample type and the protein of interest. You may need to try a variety of fixation methods to find the best conditions for your target.

The optimal antibody concentration will vary between antibodies. Review the manufacturer's instructions before starting your experiment and consider titrating your antibody to determine the optimal dose.

To ensure that your antibody is both functioning as expected and specific, include a positive control sample that you know expresses the protein, such as cells transfected with a plasmid to express the protein of interest, and a negative control sample such as cells that do not express the protein of interest.



Materials

Equipment:

- Pipette controller
- Pipette tips and pipettes
- Rocking platform
- Tweezers
- Fluorescent microscope
- 0.5–10 μ L single channel pipette
- 2–20 μ L single channel pipette
- 20–200 μ L single channel pipette
- 200–1000 μ L single channel pipette

Reagents and Consumables:

- 1X PBS
- Microcentrifuge tubes
- Sterile Poly-D-lysine coated coverslips
- HeLa cells
- 24-well plate
- 4% Paraformaldehyde
- 5 mg/mL 4',6-diamidino-2-phenylindole (DAPI)
- Bovine serum albumin (BSA)
- Triton X-100
- Primary antibody
- Secondary antibody
- Deionized water
- Microscope slide
- Anti-fade mounting medium
- Laboratory wipes
- 15 mL conical tubes
- 50 mL conical tubes

Reagent preparation:

Permeabilization buffer:

Dilute  20 μ L of Triton X-100 in  10 mL PBS.

Blocking buffer:

Dilute  0.5 g BSA and  30 μ L Triton X-100 in  10 mL PBS.

**Antibody dilution buffer:**

Dilute  0.5 g BSA and  150 μL Triton X-100 in  50 mL PBS.

300 nM DAPI working solution:

Prepare a 300 μM DAPI stock solution by diluting  2.1 μL of the 5 mg/mL DAPI solution to  100 μL PBS.

Protect from light.

Prepare a 300 nM DAPI working solution by diluting  5 μL of the 300 μM DAPI stock solution into  5 mL

PBS. Protect from light.

Troubleshooting

Before start

See the Materials section for preparation of necessary stock solutions.

Refer to the manufacturer's instructions for additional information specific to your antibodies, such as antibody concentrations, incubation times, and recommended compatible reagents.

Secondary antibodies must match the host species of the primary antibody. For example, use an anti-mouse secondary antibody for primary antibodies raised in a mouse.



Seeding cells

- 1 Place a sterile poly-D-lysine coated coverslip in each well of a 24-well cell culture treated plate.
- 2 Seed 5×10^3 HeLa cells per well.
- 3 Allow the HeLa cells to grow to the desired density before labeling.

Fixing and permeabilizing cells

- 4 Gently aspirate the media from the 24-well plate.
- 5 Wash each well with  500 μ L of PBS, remove the wash, and dispose of it in an appropriate waste container.
- 6 Fix each well with  500 μ L of cold 4% paraformaldehyde in PBS on ice for  00:15:00 .

15m

Note

While 4% Paraformaldehyde fixation works well for many target proteins, it may not be the best fixation method for all. Alternative fixation methods such as methanol or acetone may be better for some applications.

- 7 Remove the paraformaldehyde and follow your institution's laboratory safety guidelines for disposing of waste in the appropriate container.
- 8 Wash 3x for  00:05:00 in  500 μ L PBS on a rocking platform.

5m



9 Permeabilize cells for  00:10:00 at  Room temperature on a rocking platform in  500 μL permeabilization buffer. 10m

10 Remove the permeabilization buffer and dispose of it in an appropriate waste container.

11 Wash 3x for  00:05:00 in  500 μL PBS on a rocking platform. 5m

Labeling with antibody

3h 15m

12 Block for  00:20:00 at  Room temperature on a rocking platform in  500 μL blocking buffer. 20m

13 Remove the blocking buffer and dispose of it in an appropriate waste container.

14 Dilute the primary antibody to the desired concentration in antibody dilution buffer.

Note

The optimal antibody concentration will vary but generally ranges from 1-10 $\mu\text{g/mL}$.

15 Add  500 μL of the diluted antibody to the wells and incubate  02:00:00 at  Room temperature . 2h

16 Remove the primary antibody and dispose of it in an appropriate waste container.

17 Wash 3x for  00:05:00 in  500 μL PBS on a rocking platform. 5m

18 Dilute the fluorescently-labeled secondary antibody to the desired concentration in antibody dilution buffer.

**Note**

The optimal antibody concentration will vary but generally ranges from 1-10 µg/mL.

- 19 Add  500 µL fluorescently-labeled secondary antibody to the wells and incubate  00:30:00 at  Room temperature in the dark.

30m

Note

The plate can be wrapped in foil to block light.

- 20 Remove the secondary antibody and dispose of it in an appropriate waste container.

- 21 Wash 3x for  00:05:00 in  500 µL PBS on a rocking platform.

5m

- 22 (Optional) Counterstain nuclei with  500 µL of 300 nM DAPI working solution for  00:10:00 at  Room temperature in the dark.

10m

- 23 Remove the DAPI and dispose of it in an appropriate waste container.

- 24 Wash 3x for  00:05:00 in  500 µL PBS on a rocking platform.

5m

- 25 Use tweezers to gently remove the coverslip.

- 26 Blot the coverslip with a laboratory wipe to remove excess liquid.

- 27 Add 1 drop of anti-fade mounting medium to the microscope slide.



- 28 Gently place the coverslip on the microscope slide with the cell side facing down.
- 29 Observe the cell labeling on a microscope with appropriate fluorescent filters.