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Immunofluorecence assay (IFA)

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Protocol status: Working
We use this protocol and it's working

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Abstract

The protocol describes the immunofluorescence assay

Materials

REAGENT	SOURCE	IDENTIFIER
Antibodies		
See Antibodies sheet on Drive for specifications for each antibody you use		
Chemicals and Recombinant proteins		
32% Paraformaldehyde	Fisher Scientific	Cat#50-980-495
Triton X-100	Sigma Aldrich	Cat#CAS 9002-931-1
Hoechst	Invitrogen	Cat#H-3570
Fetal Bovine Serum	Serum Source International	Cat#FB22-500
Enzymes and other reagents		
ProLong Diamond Antifade mounting media	ThermoFisher	Cat#P36961
Critical Commercial Assays		
Experimental Model: Cell lines and viruses		
Sequence-Based Reagents		
Software and Algorithms		

Material:

1) Fixative: 4% PFA in PBS (prepared 1:8 dilution from 32% paraformaldehyde) - stored in 4 °C fridge in TC

2) Permeabilization: 0.2% Triton X-100 (200 µL 100% TritonX-100+ 99 mL PBS) - stored in 4 °C fridge

3) Blocking buffer: 3% FBS in PBS (1st and 2nd Abs) - can be stored in 4 °C fridge - always check that there is no contamination (easy to grow stuff in FBS)

4) Wash buffer : PBS 1X

4) Cover slips (#1.5 very important!, VWR Cat # 16004-336 - squared for 6 well plates, Fisher Cat # NC1129240 - circular for 24 well plates)

5) Slides

5) **Abs/Dilution** - location should be listed on antibody sheet in drive.

Immunofluorescence assay (IFA)

1d

1 **Set up**

1) Cells should be seeded onto #1.5 coverslips in well. Can use square coverslips in 6-well plate or round coverslips in 24 well plates.

2) Proceed with infection/treatment as normal.

2 **IFA** 1d

(specifications are for square coverslips in 6-well plate, adjust volumes if other size is used):

1) Prepare a new plate with 1 mL of PBS in wells for each slide.

2) Using tweezers, move slide from original plate into new plate.

3) Remove PBS. (This counts as a “wash” so swirl around PBS before removing)

4) Add 1 mL of 4% PFA. Fix for 10-20min.
*At this step, you can move outside the TC hood to lab bench to continue the protocol

5) Wash cell 3x with PBS.
*At this stage, slides can be stored indefinitely (as long as they don't dry out) but very reasonably up to a week. If you want to store for longer periods, add an additional 1 mL PBS/well, seal plate with parafilm to delay evaporation, and store in 4 °C degree fridge.

6) Permeabilize cells with 1 mL of 0.2% Triton X-100 for 7-10min.

8) Wash cell 3x with PBS.

9) Dilute 1ry Antibody (Ab) in 3% FBS/PBS for a total volume of 300 µL /sample.
Dilution factor varies based on Abs. Check google sheet or company website for specifications per antibody.
* **If you are staining for more than one molecule in your samples, you should include single stained slides as controls.** This will help you assess if bleed through of channels occurred.

10) Take out coverslips with tweezers, remove extra PBS with a vacuum system or by tapping the coverslip on Kimwipes, and place coverslip on a parafilm covered dish, cell side up. Put 300 µL Ab mix on each coverslip. *Make sure you do this process quickly to prevent the slide drying up for too long.

11) Incubate for 1-2 hrs at room temp. Cover the plate to prevent evaporation, and put in a dark place if your primary antibody is conjugated with a fluorophore.

12) Return coverslips to plate containing PBS. Wash 3X with PBS.

13) Dilute 2ry Abs into 3% FBS PBS to 500 µL /sample, repeat steps 10-12.

14) Nuclear staining: Dilute Hoechst (1:100,000) in PBS. Add 1 mL to each well. Stain 5-10 min

15) Wash 2X with PBS.

16) Mount coverslips on slide:

1. Add 7-9 µL of Prolong Diamond antifade (Cat # P36961) to the slide.
2. Take the coverslip and remove excess PBS with vacuum or tapping on kimwipes.
3. Place the coverslip on the slide, on top of the mounting media, cell side down.
4. Smoosh down (gently) with tweezers to remove bubbles.

17) Dry slides at room temperature for 24:00:00. You can image 24 hrs after or store the slides in 4 °C fridge.

	6 well	24 well
PBS for wash	1mL	500mL
Formaldehyde 4%	1mL	500uL
Triton 0.2%	1mL	500uL
Abs dilution 1ry	300uL	100uL
Abs dilution 2ry	500uL	300uL
Hoechst dilution	1mL	500uL
ProLong mounting	7-9uL	5-7uL

Total volumes of the reagents to add based on the size of the coverslip and plate size.

Further specifications/things to consider:

3 **Step 6**: Over-permeabilizing can cause damage to epitopes, preventing 1ry antibodies from binding properly to proteins. Be strict with the 10 min limit to prevent this from happening. If you are interested in staining proteins in the outer-membrane of the cell, no permeabilization is required.
Step 11: overnight 1ry ab incubation can be done, but you risk drying out the slides. To prevent this, make sure the chamber is kept humid by putting a small piece of wet paper in the chamber and incubating at 4 °C. Also, increase the volume from 300 µL to 500 µL.
Step 16: The mounting media will be at -20 °C and frozen. Take it out a few minutes before to thaw it.
Step 17: Drying the slides for 24hrs prior to imaging is important because the mounting media needs to "cure" to correct refractive index over night at room temp (in the dark). Imaging after mounting can affect the quality of the images.

Protocol references

Updated by: Lavinia Gonzalez 03/31/23