AXOL

Immunocytochemistry (ICC) guide

Materials

Material	Supplier	Catalog number	Volume/ size
Phosphate Buffered Saline (PBS)	ThermoFisher Scientific	14190-094 or equivalent	500mL
4% Paraformaldehyde	ThermoFisher Scientific	J19943.K2	1L
16% Paraformaldehyde	Alfa Aesar	043368.9M	10x10mL (or equivalent)
Triton X-100	Sigma	T8787	100mL
Normal Donkey serum (NDS) 100%	Sigma	D9663	100mL
Prolong ™ Gold antifade reagent with DAPI	ThermoFisher Scientific	P36935	5x2mL
Primary and secondary antibodies	See Antibody Guide	See Antibody Guide	N/A

5% Donkey serum: Add 5mL donkey serum to 95mL PBS. Expiry 3 months.

Procedure

General

Specific antibody details can be found in Axol's antibody guide.

When deciding on serum for blocking or antibody dilution, choose serum from the species in which the secondary antibody was raised. For most purposes, donkey serum is used.

For dual staining, the species in which the primary antibody for each protein target was raised must be exclusive (e.g. Rabbit with Mouse) and include species specific secondary antibodies, with exclusive wavelengths, for each primary antibody (e.g. 488nm with 595 nm).

Table 1. Staining volumes:

Format	Fix	Wash	Permeabilisation/Block	Diluted Antibody
24-well plate	300µL	300µL	300µL	300µL
96-well plate	100µL	100µL	100µL	100µL

Fixation

Grow cells to be stained on coated tissue culture plastics as per their normal culture conditions.

As standard, wash cells with one volume (Table 1) PBS and fix in 4% PFA for 20 mins at room temperature.

However, for cells which are prone to peeling or rapid morphology changes, e.g. motor neurons, sensory neurons and astrocytes fixation can also be accomplished as such:-

- Remove half of the culture media from the wells and replace with a half volume of 8% PFA. This will give an overall concentration of 4% PFA.
- Leave cells for 10 mins at room temperature before removing the media/PFA and replacing with one volume 4% PFA.
- Leave for a further 10 mins at room temperature.
- This is preferrable for cells which are prone to peeling or rapid morphology changes, e.g. motor neurons, sensory neurons and astrocytes.

Commercially available 4% PFA or 16% PFA diluted to 8% or 4% may be used. Do not fix for any longer than 20 mins total as this can dry out the cells.

After fixation, remove the PFA and discard separately as chemical waste.

Wash wells once using double volume PBS and once using single volume PBS (Table

1).

If required, plates can be stored in the fridge for up to one week. If storing, add double volume PBS to all wells and Parafilm.

Preparation of 3% Triton X-100

Prepare 3% Triton X-100 and aliquot, e.g. take 900uL Triton-X100 and add to 30mL of PBS. Store aliquots for up to 6 months in the fridge. Be careful during pipetting as undiluted Triton X-100 is very viscous. Vigorous vortexing may be required until it is completely in solution.

Preparation of 5% donkey serum

Donkey serum is supplied as 100% and must be diluted down to 5%. Perform a 1:20 dilution, e.g. by adding 10mL of donkey serum to 190mL of PBS under sterile conditions. Freeze 5% solution as aliquots and keep for 3 months. Once thawed, use within 2 days of preparation.

Permeabilisation and block

Permeabilisation is performed using 0.1% Triton in 5% donkey serum. Add 66.7uL 3% Triton X-100 to 2mL 5% donkey serum. Scale up or down as required. Use within 2 days of preparation (i.e. for overnight staining).

Decant the PBS and add the appropriate volume of 0.1% Triton X-100 in 5% donkey serum (or other serum if applicable) to each well. Incubate for 1 hour at room temperature (do not over permeabilise). Wells which are designated as "spare" (if applicable) should not be permeabilised or blocked and kept under PBS until needed (if required).

Wash the cells twice with one volume PBS per well.

The serum to be used depends on the species in which the primary and secondary antibody has been raised in, i.e. if a Goat anti-target antibody is used as a primary antibody then goat serum can not be used, if a 488 Goat anti-rabbit antibody is used for the secondary stain then goat serum can be used but not rabbit serum.

Primary antibody staining

Make up primary antibody solution(s) in 5% serum as per dilutions in Antibody Guide. For dual stains, two antibodies raised in different species can be used in the same solution, e.g. Mouse anti-X and Goat anti-Y. Flick and centrifuge the antibody working solution to mix.

Remove the wash and add the primary antibody solution to the wells according to cell type-specific plate plans outlined in the QC SOPs.

For control wells, add 1x volume PBS alone.

Incubate overnight at 5±3°C. Alternatively, plates can be incubated for 2 hours (internal markers) OR 1 hour (surface markers) at room temperature, although best results obtained with overnight incubation.

Secondary antibody staining

Remove the primary antibody solution/PBS.

Wash once with double volume PBS and once with single volume PBS (Table 1).

Prepare secondary antibody solution(s) in 5% serum as per dilutions in Antibody Guide. For dual stains, two antibodies specific to the primary markers conjugated to separate fluorophores can be used, e.g. Anti-mouse-594 (red) and Anti-goat-488 (green). Flick and centrifuge the antibody working solution to mix.

Remove the wash and add the secondary antibody solution and incubate for 1 hour at room temperature in the dark.

Wash once with double volume PBS and once with single volume PBS (Table 1).

Add enough drops of Prolong Gold antifade reagent with DAPI to cover the base of the well (2 drops for 24-well plates, 1 drop for 96-well plates).

Leave the plate at room temperature for 5-10min in the dark. Plates can be stored in the fridge and should ideally be imaged within 1 week of staining to prevent fading of the fluorescence signal and to keep products moving through QC.

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