Alomone Labs
Immunodetection Protocols

Immunocytochemistry
Immunohistochemistry
Flow Cytometry
Western Blot
Blocking Peptides
Sample Preparation

alomone labs

Welcome to The Protocol Book: Immunodetection.

This is the beginning of a whole series of protocol guidebooks. You'll find the same protocols and troubleshooting guides that we use here at Alomone Labs. As membrane protein specialists, we know you often need to adjust the standard protocols a little to get the best out of them. And that's what you'll find here. We want you to benefit from our years of trial and error to get your assays working as quickly, and as efficiently, as possible.

But who are we? We're the experts in venom toxins, ion channels, and membrane proteins. From labeled toxins and antibodies to recombinant proteins and growth factors. We're not a large company, but we are a focused one who put the science we do above all else.

Unlike many suppliers, we make and test everything in-house. No OEMs. No third parties. It's all us. Everything comes from our scientists in our labs, straight to yours.

We hope these protocols get you the results you're after. Good luck in the lab!

Contents

Immunocytochemistry

1

Immunocytochemistry for Fixed or Live Cells: Indirect and Direct Methods

Detailed cell preparation and direct and indirect immunocytochemistry methods

Immunocytochemistry (ICC) allows you to locate a specific protein within or on a cell with the aid of an antibody that recognizes a particular epitope.

Indirect ICC uses a secondary antibody conjugated to a reporter. This secondary antibody binds the primary antibody. Even though this indirect method requires more steps and materials compared to the direct method, it benefits from signal amplification as multiple secondary antibodies – and their reporters – bind to the primary antibody. However, indirect ICC methods can also produce more background than direct ICC methods.

Direct ICC uses a single primary antibody to target your protein of interest. Here, the primary antibody is conjugated to a reporter such as an ATTO Fluor. Since the direct method doesn't require a secondary antibody, it is quicker, cheaper, and may result in less non-specific binding. However, the signal may appear weaker than the signal from indirect ICC methods, especially with proteins that have low expression levels.

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1. First, determine if you need to fix your cells.
2. Next, chose either indirect (Option A) or direct (Option B) ICC methods.

Immunocytochemistry for Fixed Cells

Cell Preparation

1 Plate the cells in chamber slides and allow them to grow for 1–2 days in an appropriate medium. Cells need to attach strongly to the plate.

Note
Some cell lines will need a special coating, e.g., polylysine on the chamber slides, to aid in cell attachment. The specific type of coating needs to be determined empirically as it varies between chamber types and cell lines.

2 Wash the cells 3 times with ice-cold ICC phosphate-buffered saline (ICC-PBS).

ICC-PBS (pH 7.4)

Reagent	Concentration
Na_2HPO_4	0.016 M
KH_2PO_4	0.003 M
NaCl	0.14 M

Fixation

3 Fix the cells by adding 1–4% paraformaldehyde (PFA) in ICC-PBS and incubate for 10 minutes at room temperature.

Note
The optimal PFA concentration depends on the cell type, and you need to establish this experimentally.

4 Wash the cells 3 times with ice-cold ICC-PBS.

Permeabilization and Blocking

5 Permeabilize the cell membranes by adding Saponin Assay Buffer and incubate for 10 minutes at room temperature.

Saponin Assay Buffer

Reagent	% of final volume
ICC-PBS	97.9
Bovine serum albumin (BSA)	2
Saponin	0.1

6 Block the non-specific binding sites with 5% normal serum (NS)* in Saponin Assay Buffer for 15 minutes at room temperature.

*Use a serum based on the species that your secondary antibody was raised in. For example, if your secondary antibody was raised in donkeys, use normal donkey serum (NDS). Likewise, if your secondary antibody was raised in goats, use normal goat serum (NGS).

Proceed to EITHER

A **Indirect labeling methods**
B **Direct labeling methods**

A

Indirect Labeling of Fixed Cells

1 Add the primary antibody at the appropriate dilution in Saponin Assay Buffer and incubate for 1–2 hours at 4°C.

2 Wash the cells 3 times with Saponin Assay Buffer.

3 Add the fluorophore-conjugated secondary antibody at the appropriate dilution in Saponin Assay Buffer and incubate for 1 hour at 4°C, protected from light.

4 Add DAPI (5 mg/ml stock solution in deionized water or dimethylformamide (DMF); dilute to 500 nM in ICC-PBS for final use) and incubate for 5 minutes at room temperature.

5 Wash 3 times with Saponin Assay Buffer and drain well.

6 Add ICC-PBS to cover the cells and proceed with microscopy.

B

Direct Labeling of Fixed Cells Using ATTO-Conjugated Primary Antibodies

1 Add the ATTO Fluor-conjugated primary antibody at the appropriate dilution in Saponin Assay Buffer.

2 Incubate for 1 hour at 4°C, protected from light.

3 Add DAPI (5 mg/ml stock solution in deionized water or dimethylformamide (DMF); dilute to 500 nM in ICC-PBS for final use) and incubate for 5 minutes at room temperature.

4 Wash 3 times with Saponin Assay Buffer and drain well.

5 Add ICC-PBS to cover the cells and proceed with microscopy.

Notes

Immunocytochemistry for Live Cells

Cell Preparation

1 Plate the cells in chamber slides and allow them to grow for 1–2 days in an appropriate medium. Cells need to attach strongly to the plate.

Note
Some cell lines will require a special coating, e.g., polylysine on the chamber slides, to aid in cell attachment. The specific type of coating needs to be determined empirically as it varies between chamber types and cell lines.

2 Wash the cells 3 times with ice-cold Assay Buffer.

ICC-PBS (pH 7.4)

Reagent	Concentration
Na_2HPO_4	0.016 M
KH_2PO_4	0.003 M
NaCl	0.14 M

Assay Buffer

Reagent	% of final volume
ICC-PBS	97.95
Bovine serum albumin	2
NAN_3	0.1

Proceed to EITHER

A Indirect labeling methods
B Direct labeling methods

A

Indirect Labeling of Live Cells

1 Add the primary antibody at the appropriate dilution in ice-cold Assay Buffer.

2 Incubate for 1–2 hours at 4°C.

3 Wash the cells 3 times with ice-cold Assay Buffer.

4 Add the fluorophore-conjugated secondary antibody at the appropriate dilution in ice-cold Assay Buffer and incubate for 1 hour at 4°C protected from light.

5 Add DAPI (5 mg/ml stock solution in deionized water or dimethylformamide (DMF); dilute to 500 nM in ICC-PBS for final use) and incubate for 5 minutes at room temperature.

6 Wash 3 times with ice-cold Assay Buffer and drain well.

7 Add ICC-PBS to cover the cells and proceed with microscopy.

B

Direct Labeling of Live Cells Using ATTO Fluor-conjugated Primary Antibodies

1 Add the ATTO Fluor-conjugated primary antibody at the appropriate dilution in ice-cold Assay Buffer.

2 Incubate for 1 hour at 4°C.

3 Add DAPI (5 mg/ml stock solution in deionized water or dimethylformamide (DMF); dilute to 500 nM in ICC-PBS for final use) and incubate for 5 minutes at room temperature.

4 Wash 3 times with ice-cold Assay Buffer and drain well.

5 Add ICC-PBS to cover the cells and proceed with microscopy.

Example data

Figure 1
Expression of TRPC3 in microglial cells.
TRPC3 (red) (Anti-TRPC3 Antibody (#ACC-016)) is markedly up-regulated in the BDNF-treated (20 ng/ml, 10 min) HAPI cells (B) compared with control cells (A). Reproduced from Mizoguchi Y., et al. (2014). J Biol Chem. 27;289(26):18549-55.

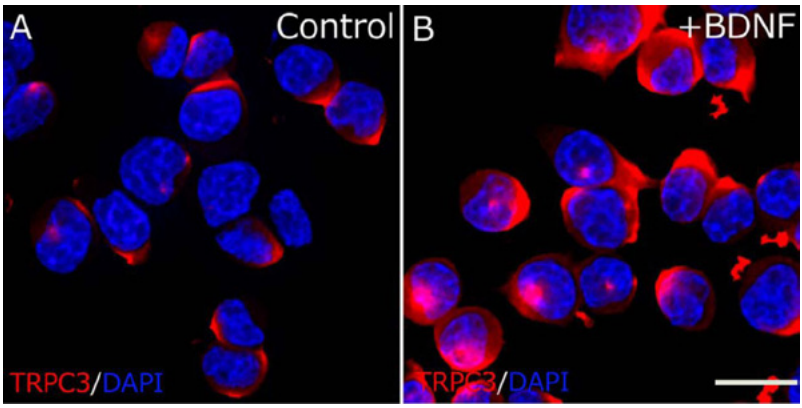


Figure 2
Expression of TRPV2 in HeLa cells.
HeLa cells transiently expressing TRPV2-1D4 immunolabeled with indicated TRPV2 antibodies (green) and 1D4 antibody (red). Scale bar represents 10 µm. Reproduced from Cohen, M. R. et al. (2013). *PloS* one, 8(12), e85392.

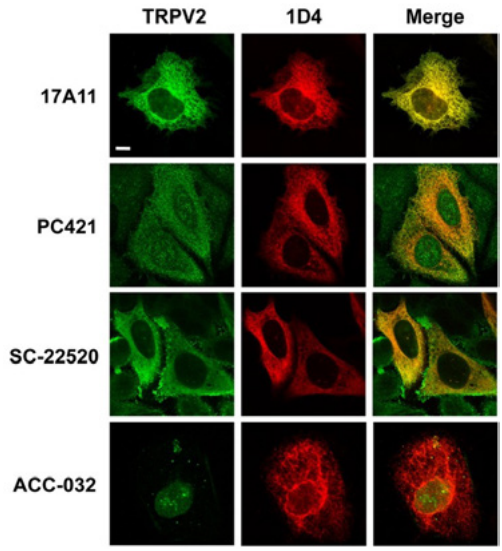


Figure 3
Expression of Kv7.1 in kidney cells.
Madin-Darby canine kidney (MDCK) cells stably expressing Kv7.1 (Anti-KCNQ1 Antibody (#APC-022)) were subjected to a calcium switch for 24 h followed by treatment with 20 µM H-89 for 3-5 h. A: confocal scans of MDCK-Kv7.1 cells before (0 h) and 3 (3 h H-89) and 5 (5 h H-89) h after addition of H-89. Untreated cells are presented as a control (5 h). Reproduced from Andersen, M. N. et al. (2015). Cell physiology, 309(10), C693–C706.

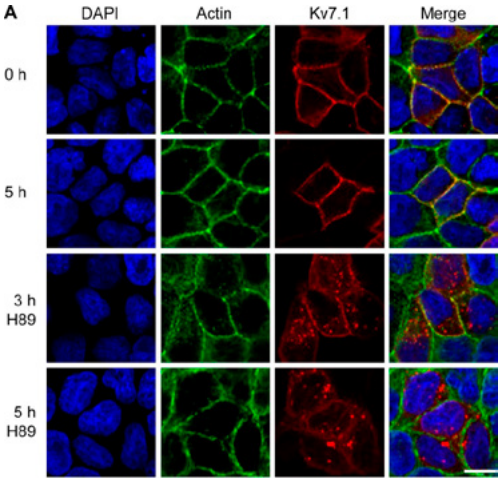
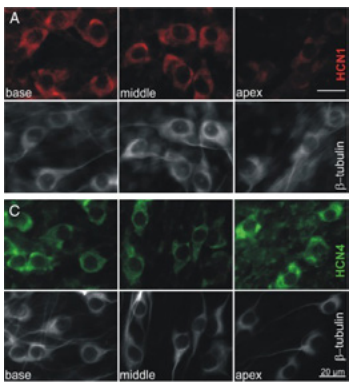


Figure 4
Expression of HCN1 and HCN 4 in neurons.
Immunocytochemical labeling of two HCN α-subunits, HCN1 (Anti-HCN1 Antibody (#APC-056) and HCN4 (Anti-HCN4 Antibody (#APC-052)), revealed different tonotopic distributions in murine spiral ganglion neurons. Reproduced from Liu, Q., et al. (2014) JARO, 15(4), 585–599.



Notes

Troubleshooting Immunocytochemistry

I have no signal at all.

All our antibodies undergo strict QC analysis by western blotting. We purify each lot with an affinity column, and we use the same protocol and conditions throughout the analysis. Also, we only release our antibodies after we obtain satisfactory results in comparison with the previous lot. If you get no signal, there could be a fundamental issue with your antibody or technique.

- You may not have used enough primary or secondary antibody. Please follow the recommended antibody dilutions or test different antibody dilutions to determine the optimal antibody concentrations.

- If you're using a fluorescent detection system, you may not have kept your conjugated primary or secondary antibody in the dark. Ensure that these antibodies are not exposed to light whenever possible.

- There's the possibility that your primary and secondary antibodies do not work together. Ensure that the secondary antibody was raised against the animal that the primary antibody was raised in.

- The NaN_3, present in the antibody solution, can sometimes cause problems.

- Your fixation method could be damaging the epitope and preventing the primary antibody from recognizing it. Either reduce the fixation time or try an antigen retrieval method.

- Your protein of interest may not be present or may be present at very low levels, in the sample that you're testing. Alternatively, the primary antibody may not recognize the protein of interest in the species that you're testing. If you

are certain the protein is expressed, you can try an enrichment step to improve the signal. You should also check the antibody datasheet to ensure that it cross-reacts with the species that you're testing.

– Your washing step may be too harsh, which can cause cells to detach from the slide. Use polylysine to adhere your cells to the slide and wash your cells more gently.

I'm getting a lot of background.

High background levels may be due to a sub-optimal primary antibody concentration, an insufficient blocking step, or there could be non-specific binding between the antibodies and the blocking reagent.

– Titrate the primary antibody to obtain the optimal concentration. Check the datasheet for the optimal dilution, but we recommend 1:100 as a starting point for most primary antibodies. For antibodies directly conjugated to a fluorophore, we suggest a 1:50–1:60 dilution.

– Your sample incubation temperature may be too high. Incubate the tissue sections at 4°C.

– Excessive fixation times can affect the epitope and cause high background levels. Try a fixation protocol with a lower exposure time, lower temperature, and/or reduced concentration of fixative. In general, we incubate the sample for 24–72 hours in 4% paraformaldehyde (PFA). When the samples consist of bloody organs, such as spleen and liver, we recommend you replace the PFA after 24 hours.

– If you grew your cells on a coated chamber slide, the antibody may stick to the surface. In this case, try using another coating solution (e.g., polyethylene glycol, polyethyleneimine, etc.) since it may affect the background levels.

Notes

Immunohistochemistry

Immunohistochemistry for Frozen Sections: Indirect Methods

Detailed protocols to take you through tissue processing and on to indirect immunohistochemistry methods for floating sections.

Immunohistochemistry (IHC) allows you to detect a specific protein in tissue sections. In our experience, the optimal type of section is from frozen tissue fixed by transcardial perfusion with a buffered paraformaldehyde solution. This tissue is cut frozen in a cryostat and sections collected either by thaw mounting on slides (10–12 µm thick sections) or floating (30–36 µm thick sections).

These protocols describe IHC with floating sections. Typically, floating sections are from tissues like adult brain and so require a thin brush to transfer them from one well to the next in a multi-well plate.

With indirect IHC methods, the primary antibody binds your protein of interest, and a secondary antibody conjugated to a reporter binds the primary antibody. There are more steps compared to the direct IHC method, but it is benefits from signal amplification since multiple secondary antibodies can bind the primary antibody. On the other hand, the indirect IHC methods can also produce more background than direct IHC methods in some situations.

This is the IHC protocol we use with rabbit-raised polyclonal primary antibodies on rat floating tissue sections.

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Sacrifice and Tissue Processing

Cell Preparation

1 Anesthetize the rats with pentobarbital sodium (Pental).

2 Perform the transcardial perfusion, first with 50 ml/rat of IHC phosphate-buffered saline (IHC-PBS), then with 220 ml/rat of ice-cold Fixative Buffer.

IHC-PBS (pH 7.4)

Reagent	Concentration	Volume/Weight
Na_2HPO_4	0.2 M	80 ml
NaH_2PO_4	0.2 M	16 ml
NaCl		8 g
Double distilled water		860 ml

Fixative Buffer (pH 7.4)

Reagent	Concentration	Volume/Weight
Na_2HPO_4	0.2 M	120 ml
NaH_2PO_4	0.2 M	24 ml
NaCl		1.31 g
Sucrose		11.5 g
Paraformaldehyde	8%	144 ml

3 Divide the tissue into coronal blocks and further fix by immersion in the fixative described above.

4 Incubate at 4–8°C overnight.

5 Transfer the tissue blocks to Sucrose Buffer.

Sucrose Buffer (pH 7.4)

Reagent	Concentration	Volume/Weight
Na_2HPO_4	0.2 M	220 ml
NaH_2PO_4	0.2 M	50 ml
NaCl		2.45 g
Sucrose		81 g
Double distilled water		270 ml

6 Cut the tissue in a cryostat within 21 days.

7 Float the tissue sections, 30 µm thick, in Cryopreservation Buffer and preserve at -20°C.

Cryopreservation Buffer (pH 6.5)

Reagent	% of final volume
Ethylene glycol	40%
Polyvinylpyrrolidone	1%
Potassium acetate buffer	0.1 M

Indirect Immunohistochemistry

To detect indirect IHC signals, use a bright-field or fluorescent microscope. It's important to know which type of microscope you plan to use before beginning the experiment.

Proceed to EITHER

A Detection by Bright-Field Microscopy
B Detection by Fluorescent Microscopy

A

Detection by Bright-Field Microscopy

1 Rinse the floating sections wit IHC-PBS for 2 x 5 minutes.

2 Quench endogenous peroxidase activity by incubating the sections with 0.2% hydrogen peroxide in IHC-PBS with 0.2% Triton X-100* and 20% methanol for 25 minutes at room temperature.

3 Rinse the sections with IHC-PBS for 2 x 5 minutes.

4 If antigen retrieval is necessary, proceed to the protocol on enzymatic retrieval in the troubleshooting section. Please note that treatment with hydrogen peroxide (step 2) induces moderate antigen retrieval.

5 Rinse the sections with IHC-PBS for 2 x 5 minutes.

6 Incubate the sections with the rabbit primary antibody in Antibody Solution for 1 hour at room temperature.

Antibody Solution (pH 7.4)

Reagent	Concentration
IHC-PBS	97.65
Triton X-100*	0.3
Tween-20	0.05
Normal serum**	2

7 Transfer to 4°C overnight.

8 Rinse the sections with IHC-PBS containing 2% NS** for 2 x 5 minutes.

*If your primary antibody targets an extracellular protein, reduce the Triton X-100 to 0.05% in both the primary and secondary antibody solutions.

** Use a serum based on the species that your secondary antibody was raised in. For example, if your secondary antibody was raised in donkeys, use normal donkey serum (NDS). Likewise, if your secondary antibody was raised in goats, use normal goat serum (NGS).

9 **Two options for indirect bright-field IHC with secondary antibodies are available here:**

A1

Secondary Antibody Conjugated to Biotin

1 Incubate the sections with biotinylated donkey anti-rabbit antibody (Merck, catalog no. AP182B) diluted 1:400 in Antibody Solution, for 1 hour at room temperature.

2 Transfer to 4°C overnight.

3 Rinse the sections with IHC-PBS containing 2% NDS** for 2 x 5 minutes.

4 Incubate the sections with extravidin-peroxidase (Merck, catalog no. E2886) diluted 1:200 in IHC-PBS, for 1 hour at room temperature.

A2

Secondary Antibody Conjugated to HRP

1 Incubate the sections with horseradish peroxidase (HRP)-conjugated donkey anti-rabbit antibody (Merck, catalog no. AP182P) diluted 1:400 in Antibody Solution for 1 hour at room temperature.

2 Transfer to 4°C overnight.

3 Rinse the sections with IHC-PBS containing 2% NDS** for 2 x 5 minutes.

**Use a serum based on the species that your secondary antibody was raised in. For example, if your secondary antibody was raised in donkeys, use NDS. Likewise, if your secondary antibody was raised in goats, use NGS.

10 **Proceed to Detection with a Bright-Field Microscope.**

Detection with a Bright-Field Microscope

1 Incubate the sections with a solution containing 0.0125% diaminobenzidine (DAB; Merck, catalog no. D5637) and 0.05% nickel ammonium sulfate for 10 minutes at room temperature.

2 Transfer the sections to the same DAB solution described above, which has been supplemented with hydrogen peroxide at a final concentration of 0.0015%. This is necessary to monitor the color reaction.

3 Rinse the sections with IHC-PBS for 4 x 10 minutes.

4 Mount the sections on glass slides (gelatinized or coated with any other type of adhesive material) and allow them to dry.

5 Dehydrate the sections by incubation with increasing ethanol concentrations (70%, 90%, and 100%; 5 minutes at each concertation). Delipidate in xylene for 10 minutes and apply a coverslip with Permount (or any other xylene diluted adhesive).

6 Detect with a bright-field microscope.

B

Detection by Fluorescent Microscopy

1 Rinse the floating sections with IHC-PBS for 2 x 5 minutes.

2 If antigen retrieval is necessary, proceed to the protocol on enzymatic retrieval in the troubleshooting section.

3 Rinse the sections with IHC-PBS for 2 x 5 minutes.

4 Incubate the sections with the rabbit primary antibody in Antibody Solution for 1 hour at room temperature.

Antibody Solution (pH 7.4)

Reagent	Concentration
IHC-PBS	97.65
Triton X-100*	0.3
Tween-20	0.05
Normal serum**	2

5 Transfer to 4°C overnight.

6 Rinse the sections with IHC-PBS containing 2% NDS for 2 x 5 minutes.

7 **Two options for indirect fluorescent IHC with secondary antibodies are available here:**

B1

Secondary Antibody Conjugated to Biotin

1 Incubate the sections with biotinylated donkey anti-rabbit antibody (Merck, catalog no. AP182B) diluted 1:400 in Antibody Solution for 1 hour at room temperature.

2 Transfer to 4°C overnight.

3 Rinse the sections with IHC-PBS containing 2% NDS** for 2 x 5 minutes.

4 Incubate the sections with streptavidin-Cy3 (Sigma, catalog no. S6402) diluted 1:200 in IHC-PBS, for 1 hour at room temperature, protected from light.

B2

Secondary Antibody Conjugated to a Fluorophore

1 Incubate the sections with a fluorophore-conjugated goat anti-rabbit antibody diluted 1:200 in Antibody Solution for 1 hour at room temperature.

2 Transfer to 4°C overnight.

3 Rinse the sections with IHC-PBS containing 2% NGS** for 2 x 5 minutes.

** Use a serum based on the species that your secondary antibody was raised in. For example, if your secondary antibody was raised in donkeys, use NDS. Likewise, if your secondary antibody was raised in goats, use NGS.

8 **Proceed to Detection with a Fluorescent Microscope.**

Detection with a Fluorescent Microscope

1 Mount the sections on glass slides in IHC-PBS (pH 7.4).

2 Dry the glass slides in a fume hood for 1 hour.

3 Stain the sections on the slides with DAPI by placing DAPI solution (5 mg/ml stock solution in deionized water or dimethylformamide (DMF); dilute to 500 nM in IHC-PBS for final use) on each slide. Next, cover each slide with a piece of parafilm to spread the solution evenly on each slide.

4 After 2 minutes, remove the parafilm and rinse the slide with 0.5 ml IHC-PBS using a pipette (repeat twice).

5 Dry the slides in a fume hood for 30 minutes.

6 Apply coverslips using the adhesive Immu-Mount™ (Shandon™)

7 Dry the slides overnight, protected from light.

8 Store at -18°C until they ready to view under the microscope.

Notes

Example Data

Figure 5
Expression of IP3 receptor 1 in mouse cerebellum.
Immunohistochemical staining of mouse cerebellum with Anti-IP3 Receptor-1 (ITPR1) Antibody (#ACC-019). A) Immunoreactivity appeared in Purkinje cells (indicated with arrows) and their dendritic trees. B) Axonal processes coursing through cerebellar white matter were visualized (shown by the arrow).

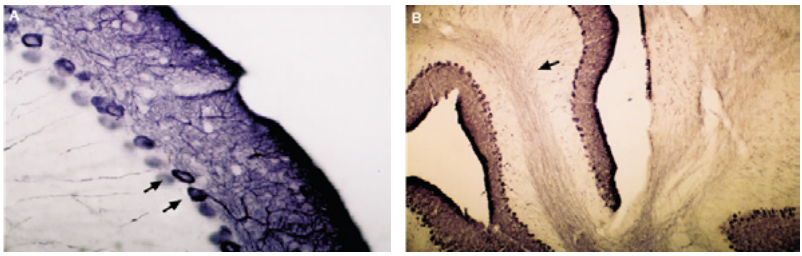


Figure 6
Glucose transporter 3 (GLUT3) expression in the murine hippocampus and cerebellum.
Immunohistochemical staining of perfusion-fixed frozen mouse brain sections with Anti-GLUT3 (extracellular) Antibody (#AGT-023) (1:200) and goat anti-rabbit Alexa Fluor 488. A) GLUT3 staining (green) in the mouse hippocampal dentate gyrus is detected in interneurons (arrows pointing up) in the hilus and granule layer arrow pointing down). The cell nuclei are stained with DAPI (blue). B) GLUT3 staining (green) in the mouse cerebellum is observed in Purkinje cells (vertical arrows) and dendrites (horizontal arrows) in the molecular layer (Mol). The cell nuclei are stained with DAPI (blue).

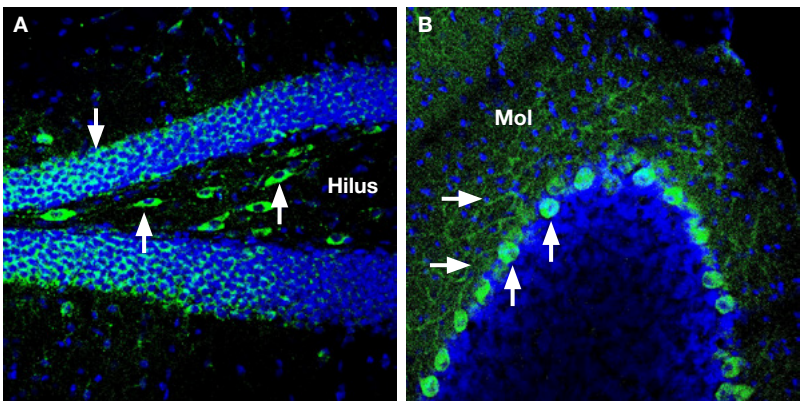


Figure 7

P2RY6 expression in the rat parietal cortex.

Immunohistochemical staining of perfusion-fixed frozen rat brain sections with Anti-P2Y6 Receptor (extracellular) Antibody (#APR-106) (1:1,000), donkey anti-rabbit biotin-conjugated antibody, and streptavidin-Cy3. P2RY6 immunoreactivity (red) appeared in the pyramidal neurons (arrows). The cell nuclei are stained with DAPI (blue).

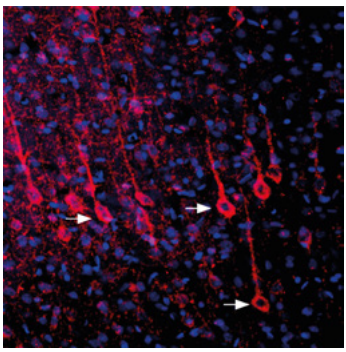
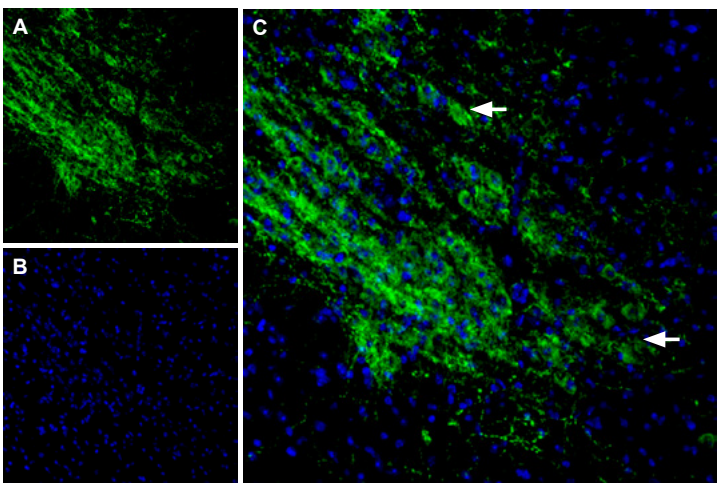


Figure 8

Expression of GIRK2 (Kir3.2) in mouse brain.

Immunohistochemical staining of mouse substantia nigra pars compacta (SNC) using Guinea pig Anti-GIRK2 (Kir3.2) Antibody (#APC-006-GP), (1:400). A. GIRK2 staining (green) appears in soma (horizontal arrows) and dendrites of dopaminergic neurons (vertical arrow). B. Nuclei staining using DAPI as the counterstain (blue). C. Merged image of panels A and B.



Immunohistochemistry for Frozen Sections: Direct Methods

A detailed protocol to take you through tissue processing and onto direct immunohistochemistry for floating sections using a fluorescent microscope.

Immunohistochemistry (IHC) allows you to detect a specific protein in tissue sections. We find the optimal section is from frozen tissue fixed by transcardial perfusion with a buffered paraformaldehyde solution. Sections are cut on cryostat at 10–12 µm thick sections for slides or 30–36 µm thick for floating sections.

For direct IHC, you use a primary antibody conjugated directly to a reporter, like an ATTO Fluor. Since the direct IHC method doesn't require a secondary antibody, it is quicker, cheaper, and may reduce non-specific binding when compared to an indirect IHC protocol. However, you may get a weaker signal than if you were using indirect IHC, especially if the protein of interest is present in low amounts.

Here we describe our IHC protocol that uses rabbit-raised polyclonal primary antibodies on rat floating tissue sections.

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Sacrifice and Tissue Processing

1 Anesthetize the rats with pentobarbital sodium (Pental).

2 Perform the transcardial perfusion, first with 50 ml/rat of IHC phosphate-buffered saline (IHC-PBS), then with 220 ml/rat of ice-cold Fixative Buffer.

IHC-PBS (pH 7.4)

Reagent	Concentration	Volume/Weight
Na_2HPO_4	0.2 M	80 ml
NaH_2PO_4	0.2 M	16 ml
NaCl		8 g
Double distilled water		860 ml

Fixative Buffer (pH 7.4)

Reagent	Concentration	Volume/Weight
Na_2HPO_4	0.2 M	120 ml
NaH_2PO_4	0.2 M	24 ml
NaCl		1.31 g
Sucrose		11.5 g
Paraformaldehyde	8%	144 ml

3 Divide the tissue into coronal blocks and further fix by immersion in the fixative described above.

4 Incubate at 4–8°C overnight.

5 Transfer the tissue blocks to Sucrose Buffer.

Sucrose Buffer (pH 7.4)

Reagent	Concentration	Volume/Weight
Na_2HPO_4	0.2 M	220 ml
NaH_2PO_4	0.2 M	50 ml
NaCl		2.45 g
Sucrose		81 g
Double distilled water		270 ml

6 Cut the tissue in a cryostat within 21 days.

7 Float the tissue sections, 30 µm thick, in Cryopreservation Buffer and preserve at -20°C.

Cryopreservation Buffer (pH 6.5)

Reagent	% of final volume
Ethylene glycol	40%
Polyvinylpyrrolidone	1%
Potassium acetate buffer	0.1 M

Direct Immunohistochemistry

1 Rinse the floating sections in IHC-PBS for 2 x 5 minutes.

2 If antigen retrieval is necessary, please consult the antigen retrieval protocol in the troubleshooting section.

3 Rinse the sections in IHC-PBS for 2 x 5 minutes.

4 Incubate the sections with the primary antibody diluted in Direct Antibody Solution for 1 hour at room temperature.

Direct Antibody Solution

Reagent	% of final volume
IHC-PBS	97.9
Triton X-100	0.05
Tween-20	0.05
Normal serum	2

Note
When using ATTO Fluor-conjugated antibodies, the optimal dilution should be initially tested with a relatively low dilution (e.g., 1:60). The dilution should then be increased to optimize the signal-to-background ratio.

5 Incubate the sections at 4–8°C overnight.

6 Rinse the sections in IHC-PBS, containing 2% normal serum for 2 x 5 minutes.

Detection

1 Mount the sections on glass slides in IHC-PBS (pH 7.4).

2 Dry the slides in a fume hood for 1 hour.

3 Stain the sections on the slides with DAPI by placing DAPI solution (5 mg/ml stock solution in deionized water or dimethylformamide (DMF); dilute to 500 nM in IHC-PBS for final use) on each slide. Next, cover each slide with a piece of parafilm to spread the solution evenly on each slide.

4 After 2 minutes, remove the parafilm and rinse the slide with 0.5 ml of distilled deionized water using a pipette (repeat twice).

5 Dry the slides in a fume hood for 30 minutes.

6 Apply coverslips using the adhesive Immu-Mount™ (Shandon™)

7 Dry the slides overnight, protected from light.

8 Store at -18°C until they are viewed under the microscope.

Notes

Example Data

Figure 9
CaVβ2 expression in the rat brain.
Immunohistochemical staining of the rat hippocampus using Anti-CACNB2-ATTO Fluor-594 Antibody (#ACC-105-AR). CaVβ2 staining (red) appeared in the CA3 pyramidal layer (P; arrows). The cell nuclei are stained with DAPI (blue).

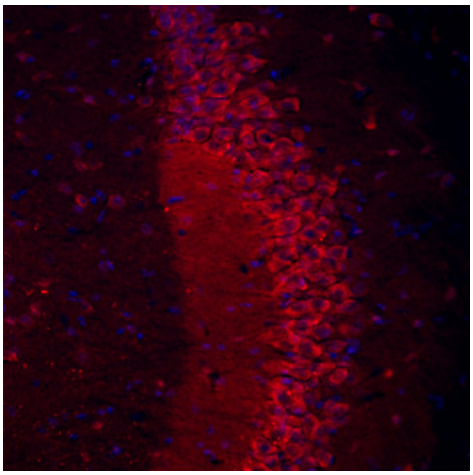
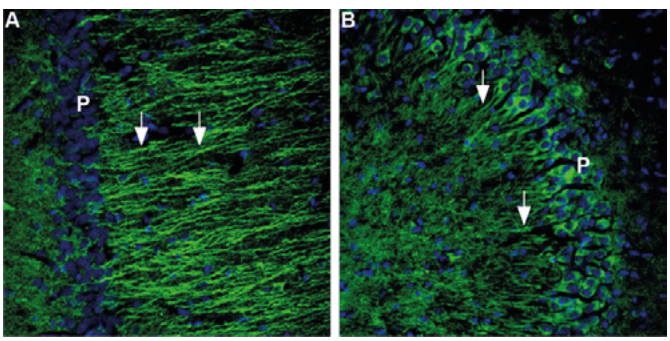


Figure 10
5-Hydroxytryptamine receptor 1B (HTR1B) expression in rat and mouse brains.
Immunohistochemical staining of rat and mouse brain sections using Anti-5HT1B Receptor (HTR1B) (extracellular)-ATTO Fluor-488 Antibody (#ASR-022-AG) (1:80). A) 5-HT1B receptor staining (green) in the rat hippocampal CA1 region was detected near the pyramidal layer (P) and in apical dendrites (arrows). B) In the mouse hippocampal CA1 region, 5-HT1B receptor staining (green) appeared in the pyramidal layer (P) and in apical dendrites. The cell nuclei are stained with DAPI (blue).



Notes

Immunohistochemistry for Frozen Heart Sections

Clear and detailed immunohistochemistry steps for working with heart sections, from tissue preparation to staining.

Immunohistochemistry (IHC) for fresh frozen mouse heart sections requires some adjustments compared to working with other tissues. Here you can find out how to do this where immunodetection is by fluorescent microscopy.

Tissue Preparation

1 Remove heart from euthanized mouse.

2 Blot out blood and wrap heart in aluminum foil.

3 Freeze organ by dipping in liquid nitrogen or by placing on dry ice.

4 Store the heart in a 1.5 ml Eppendorf tube, tightly closed and keep at -80°C until sectioning on a cryostat.

Cryostat Sectioning

1 The cryostat chamber should be set to -25°C to -27°C.

2 Pre-arrange in a holder and chill on crushed ice super-frost slides.

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3 Thaw mount 10 µm thick transverse sections, 5–6 per slide.

4 Arrange slides in a box and keep at -80°C until fixation for immunohistochemistry.

Fixation

1 Place a volume of acetone and a Coplin jar in advance in a -18°C freezer.

2 Remove slides from -80°C and place leaning in a flat tray in a fume hood to dry at room temperature for 10 minutes.

3 Place slides in the pre-chilled Coplin jar and pour pre-chilled acetone into the jar to cover the slides.

4 Place Coplin jar in -18°C freezer for 10 min.

5 Pour out acetone and allow slides to dry in a fume hood for 10 minutes.

6 Fill jar with IHC-phosphate-buffered saline (IHC-PBS) and put in refrigerator (2–8°C) until immunohistochemical processing.

IHC-PBS (pH 7.4)

Reagent	Concentration	Volume/Weight
Na_2HPO_4	0.2 M	80 ml
NaH_2PO_4	0.2 M	16 ml
NaCl		8 g
Double distilled water		860 ml

Antigen Retrieval

1 Remove slides from Coplin jar and put on a flat tray and add 200 µl of Antigen Retrieval Solution on top of each slide.

Antigen Retrieval Solution

Reagent	% of final volume
0.2 M Na_2HPO_4	37.4
0.2 M NaH_2PO_4	12.5
Methanol	20
Triton 30%*	0.2
Hydrogen peroxide 30%	5
Distilled deionized water	25

2 Cover slide with a rectangle of parafilm and leave at room temperature for 25 minutes.

3 Rinse off antigen retrieval solution with IHC-PBS and arrange slides leaning in a box to dry for 5 minutes.

Staining

1 Arrange slides on a flat tray.

2 Add your primary antibody in 200 µl Antibody Solution on each slide and cover with parafilm.

Antibody Solution

Reagent	% of final volume
IHC-PBS	97.65
Triton X-100*	0.3
Tween-20	0.05
Normal serum**	2

*If your primary antibody targets an extracellular protein, reduce the Triton X-100 to 0.05% in both the primary and secondary antibody solutions.

** Use a serum based on the species that your secondary antibody was raised in. For example, if your secondary antibody was raised in donkeys, use normal donkey serum (NDS). Likewise, if your secondary antibody was raised in goats, use normal goat serum (NGS).

3 Incubate at room temperature for 1 hour and then refrigerate overnight.

4 Wash for 3 x 5 minutes in IHC-PBS

5 Arrange slides on a flat tray and incubate with your secondary antibody (conjugated to a fluorophore or biotin†) in Antibody Solution for 2 hours at room temperature.

†If the secondary antibody is conjugated to biotin, incubate with a solution of streptavidin conjugated to a fluorophore for 1 hour at room temperature.

6 Wash for 3 x 5 minutes in IHC-PBS

Detection by fluorescent microscopy

1 Stain the sections on the slides with DAPI by placing DAPI solution (5 mg/ml stock solution in deionized water or dimethylformamide (DMF); dilute to 500 nM in IHC-PBS for final use) on each slide. Next, cover each slide with a piece of parafilm to spread the solution evenly on each slide.

2 After 2 minutes, remove the parafilm and rinse the slide with 0.5 ml distilled deionized water using a pipette (repeat twice).

3 Allow slides to dry in a fume hood for 30 minutes

4 Apply coverslips using the adhesive Immu-Mount™ (Shandon™).

5 Dry the slides overnight, protected from light.

6 Store at -18°C until they're ready to view under the microscope.

Notes

Example Data

Figure 11
Expression of KCNQ1 in mouse heart.
Immunohistochemical staining of mouse heart frozen section using Guinea pig Anti-KCNQ1 Antibody (#APC-022-GP), (1:200). A. KCNQ1 staining (red) appears in muscle fiber membranes (arrows). B. Nuclei are stained using DAPI counterstain (blue). C. Merged image of panels A and B.

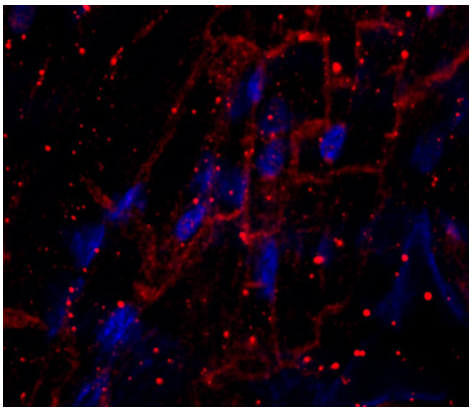
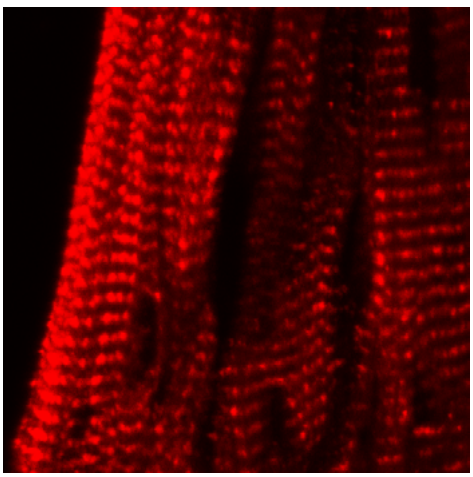


Figure 12
Expression of KCNE2 in mouse heart.
Immunohistochemical staining of mouse heart frozen sections using Anti-KCNE2 (MiRP1) Antibody (#APC-054), (red). Immunoreactivity appears as a striated pattern in heart muscle.



Immunohistochemistry for Frozen Sections: Multiplex Staining

Target multiple proteins simultaneously with these multiplex immunohistochemistry protocols using antibodies from the same or different host species.

Most immunohistochemistry (IHC) protocols describe experiments using only one antibody. In practice, however, you often need multiple antibodies against multiple targets. As the number of targets increases, so does the complexity of your multiplex experiment. However, with the right protocol, you can easily resolve the issues of managing antibodies from different host species.

Here, we describe our multiplex IHC protocol using both rabbit and guinea pig polyclonal primary antibodies on the same floating tissue sections.

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Preparation and Antigen Retrieval

1 Rinse the floating sections with IHC phosphate-buffered saline (IHC-PBS) for 2 x 5 minutes.

IHC-PBS (pH 7.4)

Reagent	Concentration	Volume/Weight
Na_2HPO_4	0.2 M	80 ml
NaH_2PO_4	0.2 M	16 ml
NaCl		8 g
Double distilled water		860 ml

2 For antigen retrieval and to quench endogenous peroxidase activity, incubate the floating sections with IHC-PBS with 0.2% hydrogen peroxide, 0.2% Triton X-100*, and 20% methanol, for 25 minutes at room temperature.

3 Rinse the sections with IHC-PBS for 2 x 5 minutes.

*If your primary antibody targets an extracellular protein, reduce the Triton X-100 to 0.05% in both the primary and secondary antibody solutions.

Two options for multiplex IHC are available here:

A Primary Antibodies Raised in Different Hosts
B Primary Antibodies Raised in the Same Host

A

Primary Antibodies Raised in Different Hosts

1 Incubate the sections with a cocktail containing two primary antibodies:

   a. The first raised in rabbit (1:200 to 1:400)
   b. The second raised in guinea pig (1:200 to 1:400)

   Dilute both antibodies in Multiplex Antibody Solution for 1 hour at room temperature.

Multiplex Antibody Solution

Reagent	% of final volume
IHC-PBS	95.65
Triton X-100*	0.3
Tween-20	0.05
Normal goat serum (NGS)	2
Normal donkey serum (NDS)	2

*If your primary antibody targets an extracellular protein, reduce the Triton X-100 to 0.05% in both the primary and secondary antibody solutions.

2 Incubate the sections at 4°C overnight.

3 Rinse the sections with IHC-PBS containing 2% NGS and 2% NDS for 2 x 5 minutes.

4 Incubate the sections with a cocktail of diluted secondary antibodies:

   a. Anti-rabbit conjugated to a fluorescent dye
   b. Anti-guinea pig conjugated to a different fluorescent dye

   Dilute both antibodies in Multiplex Antibody Solution for 1 hour at room temperature.

5 Incubate the sections at 4°C overnight.

6 Proceed to **Mounting and Detection**.

B

Primary Antibodies Raised in the Same Host

1 Incubate the sections with rabbit primary antibody (un-conjugated) diluted in Muliplex Antibody Solution for 1 hour at room temperature.

Multiplex Antibody Solution

Reagent	% of final volume
IHC-PBS	97.65
Triton X-100*	0.3
Tween-20	0.05
Normal goat serum (NGS)	2

*If your primary antibody targets an extracellular protein, reduce the Triton X-100 to 0.05% in both the primary and secondary antibody solutions.

2 Incubate the sections at 4°C overnight.

3 Rinse the sections with IHC-PBS containing 2% NGS for 2 x 5 minutes.

4 Incubate the sections with the secondary antibody (goat anti-rabbit conjugated to a fluorescent dye) in Muliplex Antibody Solution for 1 hour at room temperature.

5 Incubate the sections at 4°C overnight.

6 Rinse the sections with IHC-PBS containing 2% NGS for 2 x 5 minutes.

7 Incubate the sections with 2% normal rabbit serum (NRS) (to saturate residual binding ability of the secondary goat anti-rabbit antibody), for 1 hour at room temperature.

8 Rinse the sections with IHC-PBS containing 2% NGS for 2 x 5 minutes.

9 Incubate the sections with rabbit primary antibody conjugated to a different fluorescent dye, diluted 1:50–1:60 in Muliplex Antibody Solution for 1 hour at room temperature.

10 Incubate the sections at 4°C overnight.

11 Proceed to **Mounting and Detection**.

Mounting and Detection

1 Rinse the sections with IHC-PBS (containing 2% NGS) for 2 x 5 minutes.

2 Mount sections on slides and dry for 2 hours to overnight.

3 Stain the mounted sections with DAPI stain (5 mg/ml stock solution in deionized water or dimethylformamide (DMF); dilute to 500 nM in IHC-PBS for final use) to label all cells in the field. Incubate in DAPI at room temperature for 5 minutes.

4 Apply coverslips using the adhesive Immu-Mount™ (Shandon™)

5 Detect with a microscope.

Notes

Example Data

Figure 13
Multiplex staining of calnexin and presenilin-1 in the mouse cortex, using two rabbit primary antibodies.
The sections were incubated with a cocktail of rabbit Anti-Presenilin-1-ATTO Fluor-488 Antibody (#AIP-011-AG) and rabbit Anti-Presenilin-1-ATTO Fluor-488 Antibody (#AIP-011-AG) antibodies. A) Calnexin staining (red) appeared in the neuronal profiles. B) Presenilin-1 staining (green) in the same section appeared in the neuronal profiles and apical dendrites (arrows). C) A merged image of A and B demonstrated the colocalization of calnexin and presenilin-1 in several neurons (arrows). The cell nuclei are stained with DAPI (blue) as the counterstain.

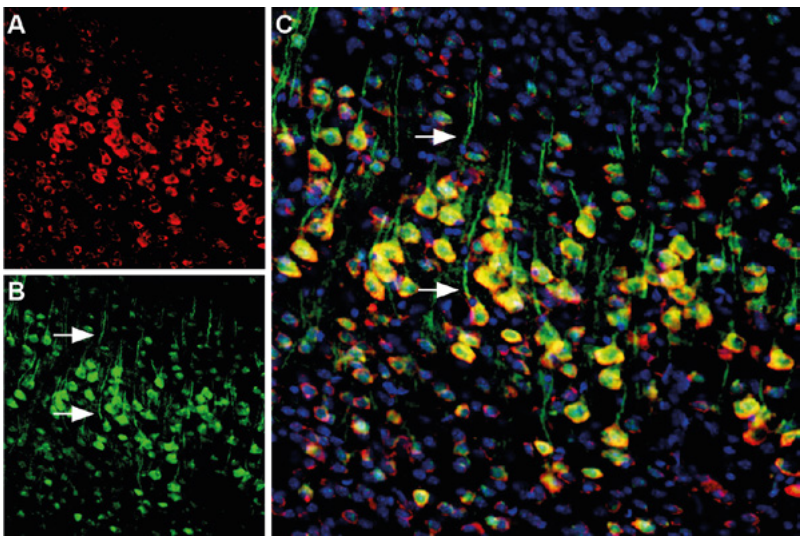


Figure 14

Multiplex staining of synapsin II (SYN2) and GABA(A) α1 receptor in the rat striatum using primary antibodies from different hosts

The sections were incubated with a cocktail of rabbit Anti-Synapsin II (#ANR-015) and guinea pig Anti-GABA(A) α1 receptor (#AGA-001-GP) antibodies, followed by incubation with donkey anti-rabbit Cy3 (red) and goat anti-guinea pig Alexa 488 (green). A) Synapsin II (red) appeared in the Islands of Calleja (arrow). B) The GABA(A) α1 receptor (green) was detected in the Islands of Calleja. C) A merged image of A and B demonstrated partial colocalization of synapsin II and GABA(A) α1 receptor. The cell nuclei are stained with DAPI (blue).

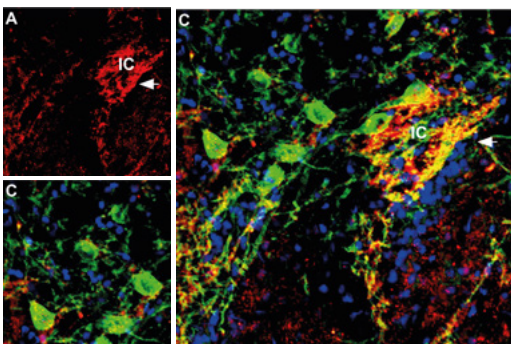
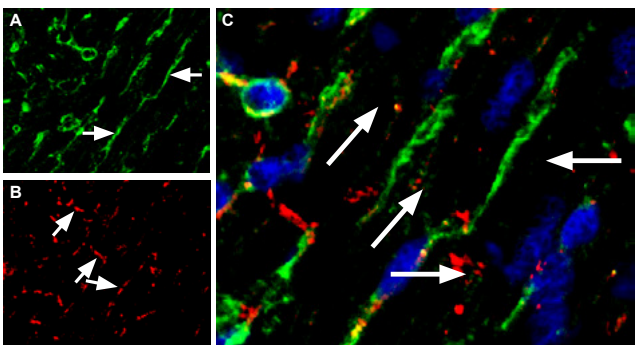


Figure 15

Multiplex staining of TRPV2 and SERCA1 in mouse heart.

Immunohistochemical staining of mouse heart immersion-fixed, free floating frozen sections, using Guinea pig Anti-TRPV2 (VRL1) (extracellular) Antibody (#ACC-039-GP), (1:200) and rabbit Anti-SERCA1 Antibody (#ACP-011) (1:200). A. SERCA1 staining (green) appears in T tubules (arrows). B. TRPV2 staining (red) in same section is detected in intercalated discs (diagonal arrows) and T tubules (horizontal arrow). C. Merge of panels A and B demonstrates co-localization of SERCA1 and TRPV2. Nuclei are stained using DAPI (blue).



Immunohistochemistry for Paraffin-embedded Sections

A clear protocol for using paraffin-embedded sections with either DAB or fluorescence.

Immunohistochemistry (IHC) protocols allow you to detect a specific protein in tissue sections. Here, we show you how to perform IHC using paraffin-embedded sections. Typically, these will be formalin-fixed paraffin-embedded (FFPE) section. Unlike using a cryostat on frozen section, paraffin-embedded sections can sometimes better preserve tissue morphology. Paraffin sections also let you work with larger tissues and storage is more convenient.

Here you'll find protocols for staining with diaminobenzidine (DAB) or a secondary antibody conjugated to a fluorophore, such as an ATTO Fluor.

De-paraffinization and clear with alcohol

1 Immerse your slides in the following solutions – make sure there is enough liquid.

Note
The ethanol solution should be replaced once a week.

a. 10–15 minutes in xylene (work in the hood)
b. 5–30 minutes in hood (to dry the xylene)
c. 10 minutes in 100% ethanol
d. 6 minutes in 90% ethanol
e. 6 minutes in 70% ethanol
f. 3 minutes in IHC-PBS

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IHC-PBS (pH 7.4)

Reagent	Concentration	Volume/Weight
Na_2HPO_4	0.2 M	80 ml
NaH_2PO_4	0.2 M	16 ml
NaCl		8 g
Double distilled water		860 ml

Staining with DAB

1 Prepare Quenching Solution. For every 4 slides use 1 ml of solution.

Quenching Solution

Reagent	Volume required						
	1,000µl	2ml	3ml	4ml	8ml	10ml	40ml
0.2M Na_2HPO_4	375	750	1,125	1,500	3,000	3,750	15,000
0.2M NaH_2PO_4	125	250	375	500	1,000	1,250	5,000
Methanol	200	400	600	800	1,600	2,000	8,000
30% Triton X-100	7	14	21	28	56	70	270
30% H_2O_2	50	100	150	200	400	500	2,000
Double distilled water	250	500	750	1000	2,000	2,500	10,000

2 Pipette 240 µl of Quenching Solution onto each slide and smear with parafilm. Avoid creating air bubbles.

3 Incubate for 25 minutes at room temperature (RT).

4 Wash slides twice with IHC-PBS.

Antigen Retrieval

1 Place the slides in a cup containing the Antigen Retrieval Solution. This solution will depend on the antigen retrieval method you choose. We recommend you try both heat-induced epitope retrieval (HIER) methods: TRIS-EDTA (10mM Tris + 20mM EDTA in double distilled water (DDW), pH 9) and citrate buffer (x10, pH 6) to see what works best for you. Alternatively, you can use an enzymatic approach like trypsin, but be advised these is more aggressive than HIER.

2 For HIER approaches add 0.5 L DDW to the pressure cooker and place the cup with slides in it – do not cover. Close the lid of the pressure cooker set your program and press start.

The default program: 125°C for 30 seconds, 95°C for 10 seconds.

Note
If you choose to use trypsin to retrieve the epitope, then immediately after quenching add 190 µl of 0.1% Trypsin + $CaCl_2$ (add to 1 ml of 0.1% trypsin 1 µl of 10% $CaCl_2$) to each slide, smear with parafilm, cover the box and leave it for 25 minutes at 37°C. After that, wash twice with IHC-PBS.

Blocking

1 Prepare a Blocking Solution.

Blocking Solution

Reagent	Volume (µl)					
IHC-PBS	804	1,607	2,410	3,215	6,430	8,034
0.1% Trypsin inhibitor*	**10**	**20**	**30**	**40**	**80**	**100**
30% Triton-X100	10	20	30	40	80	100
5% Tween-20	10	20	30	40	80	100
Goat serum**	40	80	120	160	320	400
30% BSA**	1	333	500	666	1,330	1,666
Total	**1 ml**	**2 ml**	**3 ml**	**4 ml**	**8 ml**	**10 ml**

*ONLY add if you use trypsin.
** Use goat serum or/and BSA.

2 Remove slides from the pressure cooker wash twice with IHC-PBS, wipe, and place on plastic cover.

3 Add 240 µl of Blocking Solution to each slide, cover with parafilm.

4 Leave for 1 hour at RT or at 4°C overnight. If incubating overnight, use a wet tissue to keep the slides moist.

Primary Antibody

1 Take the slides out the fridge and let them warm up for 30 minutes.

2 Wash the slides twice with IHC-PBS. Dry and place on plastic cover.

3 Add 240 µl of primary antibody diluted in blocking solution 1:50–1:1000, +/- the blocking peptide control in blocking buffer to each slide. Cover with parafilm.

4 Leave for 2 hours at RT or at 4°C overnight. If incubating overnight, use a wet tissue to keep the slides moist.

5 Rinse the slides with IHC-PBS twice.

Staining

Here you can decide to use either DAB or a secondary conjugated to a fluorescent probe.

Staining with DAB
We use SuperPicTure™ Polymer Detection Kit (Invitrogen Cat# 87-8963). The manufacturer's instructions are as follows:

1 Pipette 200 µl of HRP Polymer to each slide smear and incubate for 10min.

2 Wash with IHC-PBS for 5 minutes.

3 Prepare DAB substrate (Invitrogen 00-2104). For each 1 ml of DDW add 1 drop of reagent A, mix well. Add 1 drop of reagent B and C, mix well.

4 Pipette 190 µl of DAB solution to each slide smear and leave for 5 minutes or till brown color appears. Neutralize the remaining solution with dish washer powder detergent.

5 Wash with DDW twice.

6 Immerse in hematoxylin for 1–2 minutes.

7 Wash with tap water for 2 minutes.

8 Optional: Contrast stain with eosin for 1 minute and wash with tap water.

9 Immerse in the following solution:

   a. 2 minutes in IHC-PBS
   b. 3 minutes in 70% ethanol
   c. 3 minutes in 90% ethanol
   d. 3 minutes in 100% ethanol
   e. 6 minutes in xylene (work in the hood)

10 Leave to dry in hood for at least 10 minutes.

11 Use DPX Mountant for histology (Sigma-Aldrich) to close the slides – one drop for each slide and cover with coverslip.

12 Leave to dry in the hood overnight.

13 Detect by microscope.

Staining with a secondary antibody conjugated to a fluorophore

1 De-paraffinize and clear with alcohol

2 Immerse your slides in the following solutions – make sure there is enough liquid.

Note
The ethanol solution should be replaced once a week.

a. 10–15 minutes in xylene (work in the hood)
b. 5–30 minutes in hood (to dry the xylene)
c. 10 minutes in 100% ethanol
d. 6 minutes in 90% ethanol
e. 6 minutes in 70% ethanol
f. 3 minutes in IHC-PBS

3 **Do not apply Quenching Solution!**

4 Choose and apply your antigen retrieval technique (trypsin, Tris-EDTA, or citrate).

5 Wash twice with IHC-PBS.

6 Block with 240 µl Blocking Solution to each slide, cover with parafilm and leave 1 hour at RT or at 4°C overnight. If incubating overnight, use a wet tissue to keep the slides moist.

7 Take the slide out the fridge and let them warm up for 30 minutes.

8 Wash the slides twice with IHC-PBS. Dry and place on plastic cover.

9 Incubate with the primary antibody for 2 hours at RT or at 4°C overnight in Blocking Solution.

10 Remove the parafilm and wash with IHC-PBS twice.

11 Prepare your fluorophore conjugated secondary antibody in Blocking Solution and add Hoechst (1:1000) – work in a dark room.

12 Add 240 µl per slide smear, cover with parafilm, and leave for 1 hour at RT in the dark

13 Remove the parafilm and wash with IHC-PBS twice.

14 Wipe the slides and mount with Immu-Mount™ (Shandon™).

15 Detect with a fluorescent microscope.

Notes

Example Data

Figure 16
Expression of RyR2 in rat cardiac muscle
Immunohistochemical staining of paraffin-embedded sections of rat myocardium using Anti-Ryanodine Receptor 2 Antibody (#ARR-002), (1:50). Staining is specific for cardiomyocytes while smooth muscles cells in the artery walls are negative (red arrows). Hematoxylin is used as the counterstain.

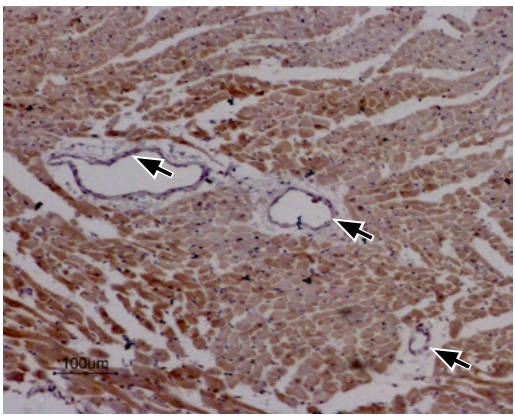


Figure 17
Expression of OX1R in rat colon
Immunohistochemical staining of paraffin-embedded longitudinal section of rat colon showing mucosa (M), submucosa (SM), and muscularis externa (ME) using Anti-Orexin Receptor 1 Antibody (#AOR-001), (1:100). Note that the stain (red-brown color) is highly specific for absorptive cells in the superior third of the intestinal glands. Immunolabeling was detected using DAB as the chromogen and hematoxylin as the counterstain.

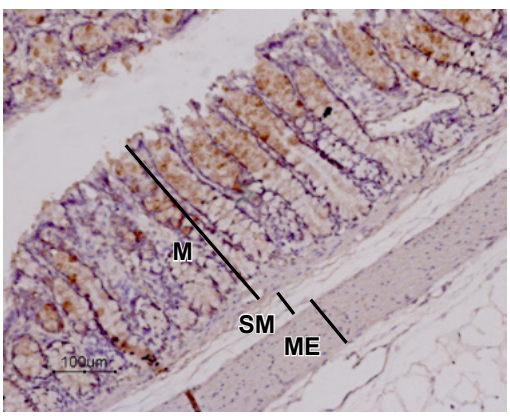


Figure 18

Expression of SERCA1 in rat skeletal muscle

Immunohistochemical staining of rat skeletal muscle paraffin-embedded sections using Anti-SERCA1 Antibody (#ACP-011), followed by goat anti-rabbit-AlexaFluor-594 secondary antibody. A. SERCA1 labeling appears in the muscle fibers, in a pattern that could indicate the location of the sarcoplasmic reticulum. The endomysium, surrounding muscle fibers, is not stained. B. Nuclear staining using DAPI as the counterstain. C. Merged image of A and B.

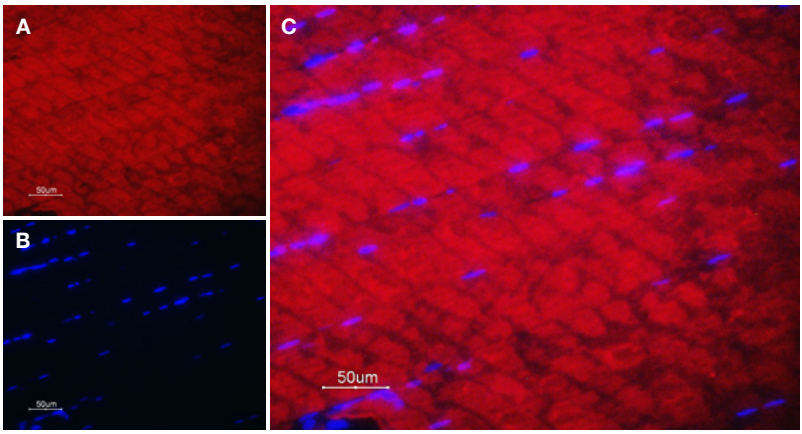
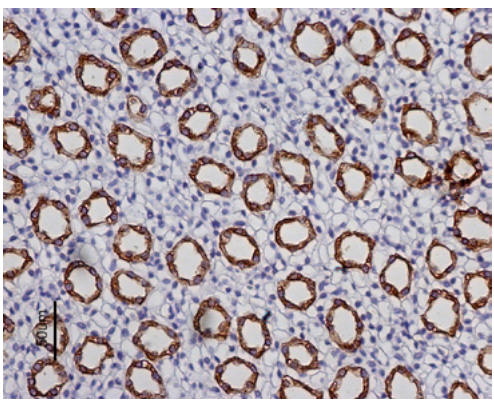


Figure 19

Expression of AQP2 in rat kidney

Immunohistochemical staining of rat kidney paraffin embedded section showing the inner medulla using Anti-Aquaporin 2 Antibody (#AQP-002), (1:100). Intense stain (brown color) is present in collecting ducts but not in thin segments of the loop of Henle. Hematoxylin is used as the counterstain.



Troubleshooting Immunohistochemistry

I have no signal at all.

All our antibodies undergo strict QC analysis by western blotting. We purify each lot with an affinity column, and we use the same protocol and conditions throughout the analysis. Also, we only release our antibodies after we obtain satisfactory results in comparison with the

Antigen Retrieval Protocol

If there is no observed staining after your IHC experiment, then we recommend that one of the following antigen retrieval protocols. You can use both methods, but you will need to determine the optimal conditions with careful testing.

Hydrogen peroxide treatment (moderate treatment)

1 Incubate the sections with 0.2% hydrogen peroxide in IHC-PBS (pH 7.4), 0.2% Triton X-100*, and 20% methanol, for 25 minutes at room temperature.

*If your primary antibody targets an extracellular protein, reduce the Triton X-100 to 0.05%.

Enzymatic retrieval (aggressive treatment)

Stock solutions required for trypsin treatment:

- Trypsin type II-S (Sigma, catalog no. T-8128): 0.1% trypsin dissolved in IHC-PBS. Store as frozen aliquots.
- Trypsin type II-S inhibitor (Sigma, catalog no. T-9128): 0.1% trypsin inhibitor diluted in IHC-PBS. Store as frozen aliquots.

1 Dilute the trypsin stock solution 1:100 to obtain a final trypsin concentration of 0.001%.
2 Add $CaCl_2$ to the trypsin stock solution for a final concentration of 0.001%.
3 Incubate the sections in this solution for 5–7 minutes at 37°C.
4 Rinse the sections with IHC-PBS for 2 x 5 minutes.
5 Dilute the trypsin inhibitor stock solution 1:100 into the primary antibody solution*.

*It's incredibly important to remember that if you use trypsin in this step, then you must also add 0.001% trypsin inhibitor to the primary antibody solution.

previous lot. If you do not obtain a signal, there could be a fundamental issue with your antibody or technique.

- You may not have used enough primary or secondary antibody. Please follow the recommended antibody dilutions or test different antibody dilutions to determine the optimal antibody concentrations.

- If you're using a fluorescent detection system, you may not have kept your conjugated primary or secondary antibody in the dark. Ensure that these antibodies are not exposed to light whenever possible.

- There's the possibility that your primary and secondary antibodies do not work together. Ensure that the secondary antibody was raised against the animal that the primary antibody was raised in.

- The NaN_3, present in the antibody solution, can sometimes cause problems.

- Your fixation method could be damaging the epitope and preventing the primary antibody from recognizing it. Either reduce the fixation time or try an antigen retrieval method.

- Your protein of interest may not be present or may be present at very low levels, in the sample that you're testing. Alternatively, the primary antibody may not recognize the protein of interest in the species that you're testing. If you are certain the protein is expressed, you can try an enrichment step to improve the signal. You should also check the antibody datasheet to ensure that it cross-reacts with the species that you're testing.

I'm getting a lot of background.

High background levels may be due to a sub-optimal primary antibody concentration, an insufficient blocking step, or there could be non-specific binding between the antibodies and the blocking reagent.

- Titrate the primary antibody to obtain the optimal concentration. Check the datasheet for the optimal dilution, but we recommend 1:100 as a starting point for most primary antibodies. For antibodies directly conjugated to a fluorophore, we suggest a 1:50-1:60 dilution.

- Try adding 0.1%–0.5% of Tween-20 to the antibody solution and washing buffer since this strengthens the signal and reduces non-specific staining. Tween-20 is more gentle than other detergents such as Triton X-100, which can damage the membrane during permeabilization.

- Your sample incubation temperature may be too high. Incubate the tissue sections at 4°C.

- Excessive fixation times can affect the epitope and cause high background levels. Try a fixation protocol with a lower exposure time, lower temperature, and/or reduced concentration of fixative. In general, we incubate the sample for 24–72 hours in 4% paraformaldehyde (PFA). When the samples consist of bloody organs, such as spleen and liver, we recommend you replace the PFA after 24 hours.

- If you grew your cells on a coated chamber slide, the antibody may stick to the surface. In this case, try using another coating solution (e.g., polyethylene glycol, polyethyleneimine, etc.) since it may affect the background levels.

I have high background with biotin-avidin amplification (for Indirect IHC OPTION 2A)

High background in this case is likely due to endogenous avidin-binding sites in tissues. The biotin-conjugated secondary antibody will bind streptavidin or extravidin conjugated to peroxidase or a fluorophore. However, the streptavidin or extravidin may bind not only to the biotin on the secondary antibody, but also to additional binding sites in the tissue.

To overcome this problem, the endogenous avidin binding sites need to be blocked before adding the primary antibody. You can achieve this by incubating the tissue sections in a solution containing non-conjugated streptavidin.

To block non-specific avidin binding sites in tissues, we use a kit from Vector Laboratories (catalog no. SP-2002). If you require this treatment for high background levels, perform this step after antigen retrieval (if required) and after rinsing the slides for 2 x 5 minutes in IHC-PBS.

Pre-saturation of endogenous avidin binding ability

1 Add 4 drops from the “streptavidin” bottle to 5 ml of IHC-PBS.
2 Incubate the sections in this solution for 30 minutes at room temperature.
3 Rinse the sections for 2 x 5 minutes in IHC-PBS.

Saturation of residual biotin binding potential

1 Add 4 drops from the “biotin” bottle to 5 ml of IHC-PBS.
2 Incubate the sections in this solution for 30 minutes at room temperature.
3 Rinse the sections for 2 x 5 minutes in IHC-PBS.
4 Start you IHC experiment by incubating the sections with the primary antibody.

My signal is messy or lumpy.

This is likely due to damaged tissue from sectioning or other similar procedures.

- Aggregates may form in the antibody solution following reconstitution and especially after thawing of the reconstituted antibody solution. Thus, we recommend centrifuging all the antibody preparations before use (10000 x g for 5 min).

- Sectioning with a dull blade can cause folding or air bubbles. Moreover, cutting sections too thick can make them difficult to resolve. Ensure your section equipment is well-maintained, and your protocol is optimized.

Can I use sections adhered to slides?

We find that floating sections are stained more effectively compared to sections adhered to slides. When sections are adhered to slides, there are several potential problems since the access and penetration of the antibody beyond the surface of the sections is limited. You can overcome this to some degree by increasing the Triton X-100 content in the antibody solution. However, we don't recommend this in many cases where the antibody targets an extracellular epitope because Triton X-100 is relatively harsh detergent and too much can deteriorate the antigens.

Western Blot

3

Western Blot

A straightforward western blot protocol from sample denaturation to blotting.

A western blot (WB) lets you detect and evaluate the size of specific proteins in cell or tissue extracts. An important and widely used tool in biology, western blotting involves separating proteins according to their size via gel electrophoresis and then transferring them to a membrane to detect with specific antibodies.

Sample Denaturation

1 Heat the samples in Laemmli buffer at 70–100°C for 10 minutes.

2 Load the samples into the gel*.

*Load 80–100 µg tissue lysate/lane or lysate from 2–5 x 105 cells/lane

SDS-PAGE

1 Run the gel according to the manufacturer's instructions.

Transfer

1 Transfer to a nitrocellulose membrane at 200 mA for 2.5 hours at 4°C for wet transfer. For dry transfer, follow the manufacturer's instructions.

See the latest online version

Note
High molecular weight proteins may need a longer transfer time (see Western Blot Protocol for High Molecular Weight (HMW) Proteins)

Western Blotting

1 Block the membrane with Blocking Solution (PBS with 3% BSA and 0.05% NaN_3) for 2–5 hours at room temperature with gentle agitation.

2 Add the primary antibody diluted in PBS 1% BSA, 0.1% Tween-20, and 0.05% NaN_3.

Note
If you use a blocking peptide as a negative control, refer to our Peptide Blocking Protocol for Western Blot.

3 Incubate for 2–3 hours at room temperature or overnight at 4°C with gentle agitation.

4 Wash the membrane with Washing Buffer (PBS and 0.1% Tween-20) for 3 x 10 minutes at room temperature.

Note
If you are using a secondary antibody conjugated to HRP, do not use solutions containing NaN3 from this point on.

5 Incubate the secondary antibody in PBS with 1% BSA and 0.1% Tween-20 for 1 hour at room temperature with gentle agitation.

6 Wash the membrane with Washing Buffer for 3 x 10 minutes at room temperature.

7 Proceed to detection using an enhanced chemiluminescence (ECL) system.

Note
If using a commercial kit, follow the manufacturer's instructions.

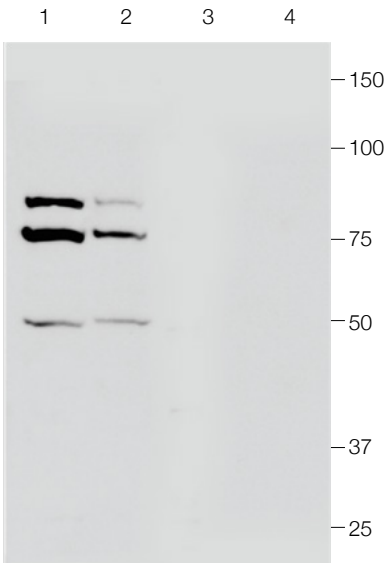
8 The membrane exposure time to the film/imager depends on the abundance of the protein and the detection system.

Example data

Figure 20
WB analysis of mouse brain membranes (lanes 1 and 3) and rat brain membranes (lanes 2 and 4).

1-2. Anti-OCTN1/SLC22A4 Antibody (#ACT-014), (1:600).

3-4. Anti-OCTN1/SLC22A4 Antibody, preincubated with OCTN1/SLC22A4 Antibody Blocking Peptide (#BLP-CT014).



Notes

Western Blot for High Molecular Weight (HMW) Proteins

Easily run your western blot with large proteins like membrane channels.

Western blotting high molecular weight (HMW) proteins, like sodium and calcium channels, can be challenging.

Here, we take you through the steps to successfully transfer HMW proteins from your gel to the membrane, all the way through to detection.

Sample Denaturation

1 Heat the samples in Laemmli buffer at 70–100°C for 10 minutes.

2 Load the samples into the gel*.

*Load 80–100 µg tissue lysate/lane or lysate from 2–5 x 10^5 cells/lane

SDS-PAGE

1 Run the gel according to the manufacturer's instructions.

Note
For HMW proteins, Tris-Glycine 4–6% or Tris-Acetate 3–8% gels are the best options, but the gels should be chosen empirically.

See the latest online version

Wet Transfer

1 Transfer the proteins from the gel to the membrane at 100 mA for 20 hours at 4°C.

Note
This step is crucial since it determines the effectiveness of the transfer.
A longer transfer time will increase the efficiency of HMW protein transfer.

Western Blotting for HMW proteins

1 Block the membrane with Blocking Solution (PBS with 3% BSA and 0.05% NaN_3) for 2–5 hours at room temperature with gentle agitation.

2 Add primary antibody diluted in PBS with 1% BSA, 0.1% Tween-20, and 0.05% NaN_3. Incubate overnight at 4°C with gentle agitation.

Note
If you use a blocking peptide as a negative control, refer to our Peptide Blocking Protocol for Western Blot.

3 Wash the membrane with Washing Buffer for 3 x 10 minutes at room temperature.

Note
If you are using a secondary antibody conjugated to HRP, do not use solutions containing NaN3 from this point on.

4 Incubate the secondary antibody in PBS with 1% BSA, and 0.1% Tween-20, for 1 hour at room temperature with gentle agitation.

5 Wash the membrane with Washing Buffer for 3 x 10 minutes at room temperature.

6 Proceed to detection using an enhanced chemiluminescence (ECL) system.

Note
If using a commercial kit, follow the manufacturer's instructions.

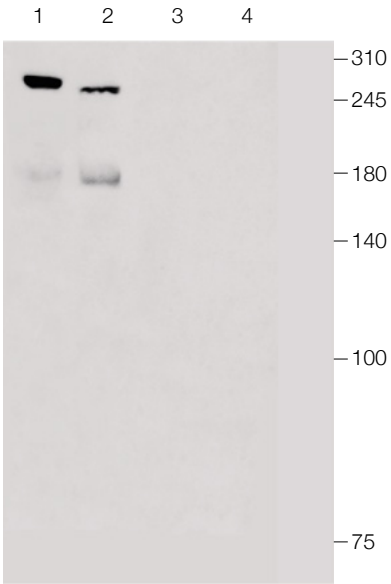
7 The membrane exposure time to the film/imager will depend on the abundance of the protein and the detection system.

Example Data

Figure 21
Western blot analysis of rat dorsal root ganglion lysate (lanes 1 and 3) and mouse lung lysate (lanes 2 and 4):

1, 2: Anti-Piezo2 Antibody (#APC-090), (1:200).

3, 4: Anti-Piezo2 Antibody, preincubated with Piezo2 Blocking Peptide (#BLP-PC090).



Notes

Troubleshooting Western Blot

I have no signal at all

All our antibodies undergo strict QC analysis by western blotting. We purify each lot with an affinity column, and we use the same protocol and conditions throughout the analysis. In addition, we only release our antibodies after we obtain satisfactory results in comparison with the previous lot. If you do not obtain a signal, there could be a fundamental issue with your antibody or technique.

- You may not have used enough primary or secondary antibody. Please follow the recommended dilutions for the antibodies or test different antibody dilutions to determine the optimal antibody concentrations.

- There's the possibility that your primary and secondary antibodies do not work together. Ensure that the secondary antibody was raised against the animal that the primary antibody was raised in.

- The NaN_3, present in the washing buffer and secondary antibody solution, can sometimes cause problems.

- Your protein of interest may not be present or could be present at very low levels in the sample. Alternatively, the primary antibody may not recognize the protein from the species that you're testing. If you are certain the protein is expressed, you can try an enrichment step to improve the signal. You should also check the antibody datasheet to ensure that the antibody cross-reacts with the species you're using.

- Try loading more protein into your gels or use an alternative protein extraction method for sample preparation. Remember, the optimal sample preparation method for your target protein may not necessarily be identical to the one used for a control housekeeping protein (e.g., GAPDH).

My band is at an unexpected position

The expected molecular weight (MW) listed in the product datasheet is based solely on the size of the target protein's amino acid sequence. It's important to remember that many factors can affect the banding pattern of your western blot including: (1) the existence of a splice variant, (2) the quality of the loaded sample, (3) the protein extraction method, and (4) the protein transfer conditions. To achieve accurate results, you may need to systematically adjust the protein extraction method or the protein transfer conditions.

- If the band's MW is below the expected MW, it could be due to a splice variant with a slightly different MW, or the target protein may have undergone proteolytic cleavage, generating a lower MW band. Changing your protein extraction method or transfer conditions may resolve this issue. You can also try the following steps:
  - Heat the samples at 70°C for 10 min (instead of 100°C for 5 min) as this reduces potential degradation.
  - Increase the transfer time. This will increase the transfer efficiency of high molecular weight (HMW) proteins. We recommend 100 mA for 20 h at 4°C.
- If the band's MW is above the expected MW, it could be due to post-translational modifications (e.g., glycosylation or phosphorylation) or the existence of a different splice variant.
- In either event, you should use a blocking peptide as a negative control. This peptide is the antigen used for immunization and specifically blocks the antibody. So, in the presence of the blocking peptide, specific staining will disappear while non-specific staining will remain. You should use the blocking peptide at the ratio indicated in the datasheet, or 1 µg of peptide per µg of antibody.

I'm getting a lot of background

High background levels may be due to the following: (1) a sub-optimal primary antibody concentration, (2) the type of membrane, (3) the blocking conditions, or (4) non-specific binding between the antibodies and the blocking reagent.

- Titrate the primary antibody to obtain the optimal concentration. Check the datasheet for the optimal dilution, but we recommend 1:200 in most cases.
- You may consider changing to a nitrocellulose membrane. Compared with polyvinylidene difluoride (PVDF) membranes, a nitrocellulose membrane tends to give you less background noise but does lack some of the detection sensitivity of a PVDF membrane.
- In addition, ensure that the membrane doesn't dry out during the western blot.
- If you haven't blocked for long enough, you can end up with a lot of background in your WB. We recommend 2–5 hours with 3% bovine serum albumin (BSA), but overnight at 4°C can also work.
- Try adding 0.1%–0.5% of Tween-20 to the primary antibody solution and washing buffer since this strengthens the signal and reduces non-specific staining.

There are too many bands on my membrane

Usually, too many bands indicate that the sample preparation method was not optimal and/or that the target protein was degraded during sample preparation.

- Multi-transmembrane proteins like such as the calcium channels are usually vulnerable to degradation during sample preparation. The optimal sample preparation method can vary depending on the tissue (e.g., brain, adrenal gland, etc.) and/or species (e.g., mouse, human, etc.). For this reason, we normally recommend running different samples prepared by different protocols in parallel, to establish the optimal sample preparation method. Remember, the optimal sample preparation method for your target protein may not necessarily be identical to the one used for a control housekeeping protein (e.g., GAPDH).

- If you are analyzing HMW proteins, you may need to adjust the SDS-PAGE conditions and the transfer conditions.

- Additional bands may indicate that there is a splice variant or a truncated version of your target protein.

- You can try a more diluted primary antibody concentration and/or add up to 0.5% Tween-20 to the primary antibody solution.

What's the best way to use my blocking peptide for western blotting?

To make sure you get optimal blocking of the primary antibody, incubate the antibody, in parallel, with and without the antigen (the antibody/antigen ratio is available in the certificate of analysis delivered with the antibody) in a small volume (500 µl of 1% BSA in PBS) for 1 hour at room temperature with rotation. After the incubation, dilute each vial to the appropriate working concentration in the desired buffer and apply the contents of each vial to each membrane for parallel experiments.

- We recommend that you use the blocking peptide at the ratio specified in the datasheet, or 1 µg of peptide per µg of antibody.

- If you're using a fusion protein to block the antibody, be aware that fusion proteins are sometimes more delicate to handle than peptides. If the fusion protein was correctly reconstituted (by adding 100 µl of PBS) and handled (avoidance of repeat freezing and thawing), a more diluted antibody together with an increased amount of fusion protein may be necessary for complete blocking of the antibody. We normally recommend adding 3 µg of fusion protein for each µg of antibody, but sometimes you need a larger ratio depending on the sample.

Why are there curved bands in my gel?

These curved bands (sometimes called "smiling bands") are likely due to excessively high voltage, which causes excessively rapid migration of the proteins. Smiling bands can also occur when the temperature is too high.

- Reduce the voltage and run the gel in a cooler room.

Flow Cytometry

4

Flow Cytometry for Live Cells: Indirect and Direct Methods

Flow cytometry allows you to detect molecules on the cell surface by targeting them with specific antibodies. You can use this to characterize and define cell populations or measure various properties you're interested in. Indirect flow cytometry used a secondary antibody, conjugated to a reporter, to bind the primary antibody. Direct flow cytometry uses a primary antibody directly conjugated to a reporter.

We highly recommend an isotype control antibody for your flow cytometry work. This control antibody should be the same isotype as your primary antibody (e.g., IgG) and conjugated to the same fluorophore (e.g., FITC) if you're using the direct method. You can find isotype control antibodies to use as negative controls on our catalog.

See the latest online version

Indirect Flow Cytometry

Cell Preparation

1 Transfer 1 x 10^6 cells into a microtube. Centrifuge for 5 minutes at 200 x *g* at 4°C. Discard the supernatant.

2 Wash the cell pellet by adding 500 µl of ice-cold Labeling Buffer (PBS with 2% BSA and 0.05% NaN_3) and resuspend the cell pellet by gentle pipetting.

3 Next, centrifuge for 5 min at 200 x *g* at 4°C.

4 Discard the supernatant.

5 Perform steps 2–3 twice in total.

Labeling

1 Add primary antibody at the appropriate dilution in 50 µl of Labeling Buffer for each tube.

2 Add the primary antibody solution to the cells and resuspend the pellet.

3 Incubate on ice for 1 hour.

4 Wash away the unbound antibody by adding 500 µl of Labeling Buffer and resuspend the cells by gentle pipetting. Next, centrifuge for 5 minutes at 200 x *g* at 4°C. Discard the supernatant. Repeat twice.

5 Add fluorescently conjugated secondary antibody at the appropriate dilution in 50 µl of ice-cold Labeling Buffer for each tube.

6 Incubate on ice for 1 hour, protected from light.

7 Wash away the unbound antibody by adding 500 µl of Labeling Buffer and resuspend the cells by gentle pipetting. Next, centrifuge for 5 minutes at 200 x *g* at 4°C. Discard the supernatant. Repeat twice.

8 Resuspend the cells in 1 ml of ice-cold Labeling Buffer. Filter the cell solution into FACS tubes and analyze by flow cytometry.

Notes

Direct Live Cell Flow Cytometry Using FITC/PE/APC-Conjugated Primary Antibodies

Cell Preparation

1 Transfer 1 x 10^6 cells into a microtube. Centrifuge for 5 minutes at 200 x *g* at 4°C. Discard the supernatant.

2 Wash the cell pellet by adding 500 µl of ice-cold Labeling Buffer (PBS, 2% BSA, and 0.05% NaN_3) and resuspend the cell pellet by gentle pipetting. Next, centrifuge for 5 min at 200 x *g* at 4°C. Discard the supernatant. Repeat twice.

Blocking

1 Add 50 µl of filtered 2% inactivated normal rabbit serum in PBS and pipette gently.

2 Incubate on ice for 10 minutes.

Note
inactivate the NRS by heating at 56°C for 30 minutes.

Labeling

1 Add FITC, PE or, APC-conjugated primary antibody at the appropriate dilution in 50 µl of Labeling Buffer for each tube.

2 Add the antibody solution to the cells and resuspend the cells.

3 Incubate on ice for 1 hour, protected from light.

4 Wash away the unbound antibody by adding 500 µl of Labeling Buffer and resuspend the cells by gentle pipetting. Next, centrifuge for 5 minutes at 200 x *g* at 4°C. Discard the supernatant. Repeat twice.

5 Resuspend the cells in 1 ml of ice-cold Labeling Buffer. Filter the cell solution into FACS tubes and analyze by flow cytometry.

Notes

Example Data

Figure 22
Cell surface detection of Tetraspanin-3 by indirect flow cytometry in live intact human THP-1 monocytic leukemia cell line.

- Cells.
- Cells + goat-anti-rabbit-FITC.
- Cells + Anti-Tetraspanin-3 (extracellular) Antibody (#ANR-185), (2.5µg) + goat-anti-rabbit-FITC.

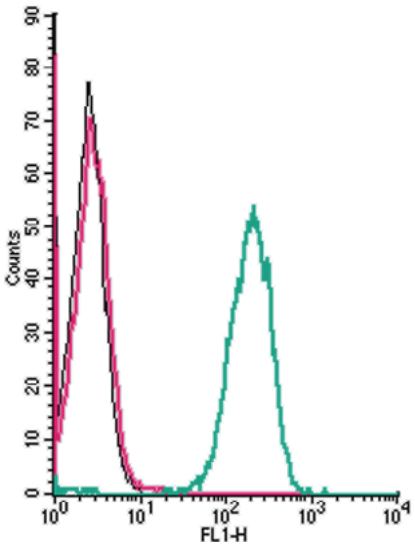
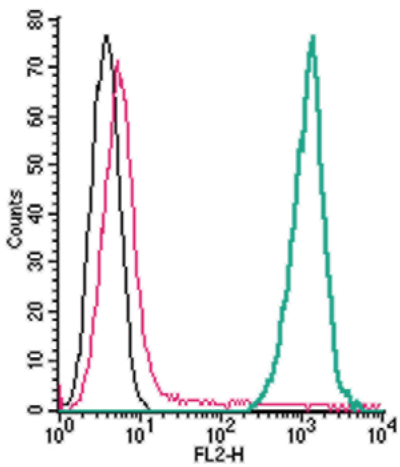


Figure 23
Cell surface detection of TREM2 by direct flow cytometry in live intact mouse J774 macrophage cells.

- Cells.
- Cells + Rabbit IgG isotype control-PE.
- Cells + Anti-TREM2 (extracellular)-PE Antibody (#ANR-018-PE), (2.5µg).



Troubleshooting Flow Cytometry

I have little or no signal.

Little or no signal may be due to: (1) the primary antibody concentration, (2) the cell collection method, or (3) the flow cytometry assay itself.

- Please check that your secondary antibody recognizes the primary antibody: most of our antibodies are generated in rabbits.
- If you have previously used your secondary antibody with successful results, we suggest trying longer incubation times with the primary antibody (up to 2 hours at 4°C) in a smaller reaction volume.
- Your primary antibody concentration may be too low. Try using more primary antibody or set up a series of dilutions to determine the optimal concentration for your system.
- If you are trying to stain an intracellular protein, you'll need to permeabilize your cells first.
- If you are attempting to detect cell surface proteins, they may have become internalized. Trypsin can induce the internalization of cell surface proteins so you may need to alter the cell detachment method. You can reduce the internalization of cell surface proteins by adding NaN_3 or by keeping your assay reagents on ice.

I'm getting a lot of background.

High background levels are most likely due to too much primary antibody concentration or insufficient blocking. Both cases can independently lead to a high signal in what should be negative cell populations.

- If you are using direct flow cytometry, use a suitable conjugated IgG isotype control.
- If you are using indirect flow cytometry, use your secondary antibody alone as a negative control.
- Too much antibody will produce a lot of background. Test different antibody dilutions to determine the optimal concentration.
- Your antibody may be binding off-targets such as the Fc receptors. You can try an Fc receptor blocking reagent to minimize the off-target binding.
- There may be doublets in your cell population, i.e., dividing cells. Try adding EDTA to your buffer or filtering the cells through a 30 µm filter.
- It's possible that the flow cytometer equipment settings may need adjusting: if the offset is too low, or the gain too high, you will generate background signal.
- If you are not using a conjugated primary antibody, you may need to simply increase the wash times, or add extra wash steps to your protocol.
- When running multiple fluorochromes, spillover can occur. Ensure that you have used a multicolor panel building to confirm that you have a workable combination of fluorochromes.
- When choosing the fluorochromes, the brightest fluorochrome for the target with the lowest expression or density should be selected. Conversely, choose the dimmest fluorochrome for those targets with the highest expression or density.
- If you have high side scatter background it could be due to bacterial contamination, which will autofluoresce and give high event rates.

My event rate doesn't look right.

If your flow cytometer cannot consistently distinguish between individual cells, your event rate will appear abnormal. Remember, you may need to obtain as many as 107 events for significant detection.

- If the event rate is too low, ensure that the cells are properly mixed and that the population is between 1 x 10^5 and 1 x 10^6 cells/ml.

- If the event rate is too low, your cells could be clumping together. Make sure you sieve the cells before they are acquired and sorted to remove any debris.

Blocking Peptides

5

Blocking Peptides for Western Blot

A blocking peptide is the original antigen we use for immunization during antibody generation. This makes blocking peptides good controls to help validate antibody specificity. Sometimes called “immunizing peptides” or “negative control antigens,” a blocking peptide works as a control by competing with, or blocking, the primary antibody.

You can easily use a blocking peptide control alongside your western blot (WB) to show that your antibody binds the target it is supposed to. It’s important to remember that a blocking peptide is just one of the tools you should use when setting up proper controls for your WB.

An antibody blocked with a blocking peptide should produce no signal when added to your membrane. Any positive results with the blocking peptide control means the antibody is binding to a protein besides the intended target.

See the latest online version

Method

1 Reconstitute your lyophilized blocking peptide in 100µl sterile phosphate-buffered saline (PBS) or double-distilled water (DDW) according to the instructions in the datasheet.

2 Optimize your WB protocol. Refer to our WB protocol for more information. Follow the product guidelines or previous experimental optimizations for a recommended dilution for your primary antibody. Determine the quantity of antibody required for two experiments.

3 If your optimal antibody concentration is a 1:200 dilution, add 20 µg of antibody to a 1.5 ml Eppendorf tube containing 500 µl of PBS with 1% bovine serum albumin (BSA). Label the tube “antibody alone”.

4 To a second identical tube, add 20 µg of antibody and 20 µg of blocking peptide to 500 µl of 1% BSA in PBS. Label the tube “+peptide”.

Note
We recommend beginning with a 1:1 ratio between the antibody and the blocking peptide. You will need to test a series of dilutions to obtain full inhibition.

5 Rotate both tubes for 1 hour at room temperature.

6 Transfer the contents of each Eppendorf tube to larger tubes and add 4.5 ml of PBS with 1% BSA, 0.1% Tween-20, and 0.05% NaN_3 to each tube to get a final antibody dilution of 1:200.

7 Add the contents of each tube to its respective membrane test strip for parallel experiments.

8 Incubate both membrane strips for 2–3 hours at room temperature or overnight at 4°C with gentle agitation.

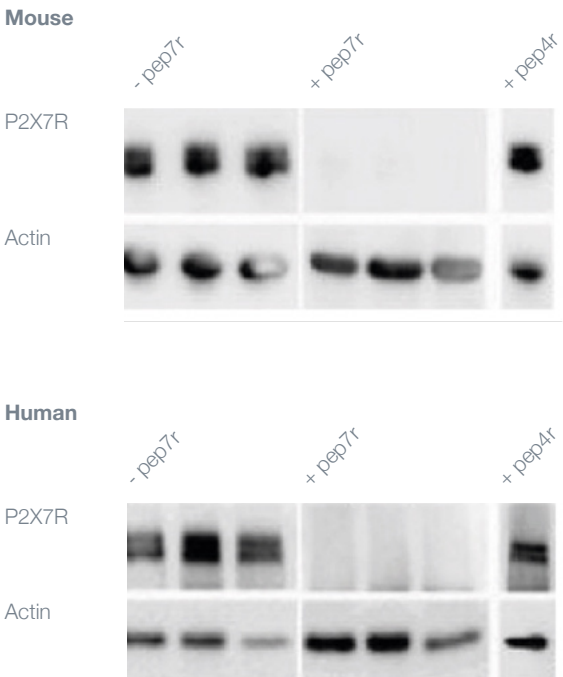
9 Proceed with the WB protocol, ensuring that you handle both the unblocked and blocked samples in the same way.

10 Develop your blots and compare the signal obtained in the two test strips. The band that disappears when using the blocking peptide is specifically recognized by the antibody. Other visible bands represent non-specific antibody binding.

Example data

Figure 24
WB using the Anti-P2X7 Receptor Antibody (APR-004) in the presence or absence of blocking peptides.
Western blots show a P2X7 receptor band (left panels) in epileptic mouse and human hippocampal samples blotted in the absence of the P2X7 Receptor Blocking Peptide (#BLP-PR004) (-pep7r). This band was eliminated (middle panels) when the Anti-P2X7 Receptor Antibody (#APR-004) was preincubated with the P2X7 Receptor Blocking Peptide (#BLP-PR004) (+pep7r). In a separate experiment, the anti-P2X7 receptor antibody was preincubated with the P2X4 Receptor Blocking Peptide (#BLP-PR002) (+pep4r; right panels). As expected, the P2X4 receptor blocking peptide did not block the anti-P2X7 receptor antibody. The blots were also probed with an antibody directed against actin, which functioned as a loading control.

Adapted from Jimenez-Pacheco, A. et al. (2016) J. Neurosci. 36, 5920 with permission from the Society for Neuroscience.



Notes

Blocking Peptides for Immunohistochemistry and Immunocytochemistry

A blocking peptide is the original antigen we use for immunization during antibody generation. As such, a blocking peptide is a great control to help validate the specificity of your antibody. Sometimes called “immunizing peptides” or “negative control antigens,” a blocking peptide works as a control by competing with, or blocking, the primary antibody.

You can easily use a blocking peptide control alongside your immunohistochemistry (IHC) immunocytochemistry (ICC) experiments to help show that your antibody binds the intended target. It’s important to remember that a blocking peptide is just one of the many tools you should use for proper immunoassay controls.

An antibody blocked with a blocking peptide should produce no signal when added to your tissue sections. Any positive results in the blocking peptide control mean the antibody is binding to a protein besides the target.

Method

1 Complete the preparation, fixation, and permeabilization (for intracellular epitopes) of your tissue section or cells according to the immunostaining protocol. Refer to our IHC, ICC, or IF protocols for further information.

See the latest online version

2 Reconstitute your lyophilized blocking peptide in 40 µl of sterile IHC/ICC phosphate-buffered saline (IHC/ICC-PBS) according to the instructions in the datasheet.

IHC-PBS (pH 7.4)

Reagent	Concentration	Volume/Weight
Na_2HPO_4	0.2 M	80 ml
NaH_2PO_4	0.2 M	16 ml
NaCl		8 g
Double distilled water		860 ml

3 Optimize your protocol in advance. Follow the product guidelines or previous experimental optimizations for a recommended dilution for your primary antibody.

4 If your optimal antibody concentration is a 1:200 dilution, add 6 µl of antibody to an Eppendorf tube that contains 1.2 ml of Antibody Solution.

Antibody Solution

Reagent	% of final volume
IHC/ICC-PBS	97.65
Triton X-100*	0.3
Tween-20	0.05
Normal serum**	2

*If your primary antibody targets an extracellular protein, reduce the Triton X-100 to 0.05% in both the primary and secondary antibody solutions.

** Use a serum based on the species that your secondary antibody was raised in. For example, if your secondary antibody was raised in donkeys, use normal donkey serum (NDS). Likewise, if your secondary antibody was raised in goats, use normal goat serum (NGS).

5 Divide the solution into two different identical Eppendorf tubes. The first one contains 600 µl of Antibody Solution with antibody. Label the tube “antibody alone”.

6 To the second identical tube, add 40 µl of blocking peptide. Label the tube “+peptide”.

Note
we recommend that the concentration (mg/ml) of the blocking peptide be at least 10x the concentration of the antibody in the working dilution.

7 Rotate both tubes for 1 hour at room temperature.

8 Add the contents of each tube to its respective tissue section well for parallel staining experiments.

9 Incubate at room temperature for 1 hour with occasional gentle shaking of the multi-well plate.

10 Incubate the multi-well plate overnight at 4°C.

11 Proceed with the IHC, ICC, or IF protocol making sure you handle the unblocked and blocked tissues in the same way.

12 Observe and compare the staining pattern obtained in the two test slides. The staining that disappears when using the blocking peptide is specific to the antibody. Any other staining that is visible represents non-specific binding.

Example Data

Figure 25
Expression of GPR109A in rat cingulate cortex.
Immunohistochemical staining of perfusion-fixed frozen rat brain sections with Anti-GPR109A/HCAR2 (extracellular) Antibody (#AHR-012), (1:200), followed by goat anti-rabbit-AlexaFluor-488. A. GPR109A immunoreactivity (green) appears in outlines of cortical neurons (arrows). B. Pre-incubation of the antibody with GPR109A/HCAR2 (extracellular) Blocking Peptide (#BLP-HR012), suppressed staining. Cell nuclei are stained with DAPI (blue).

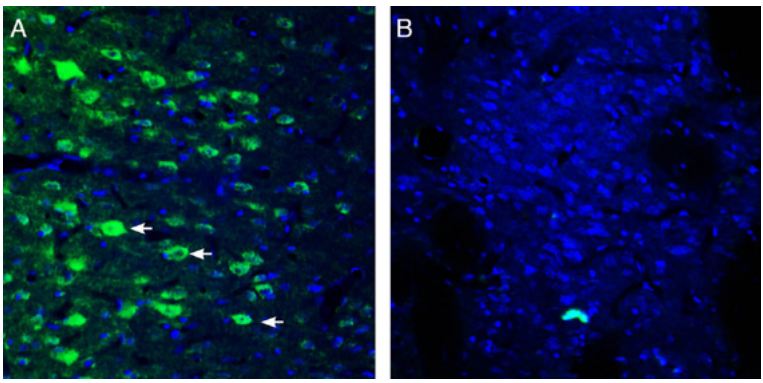
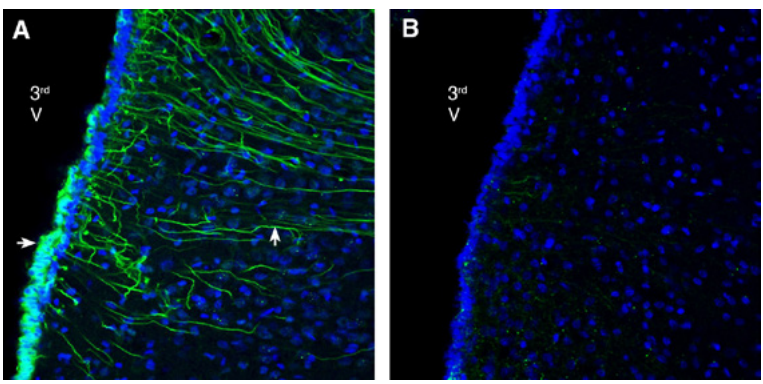
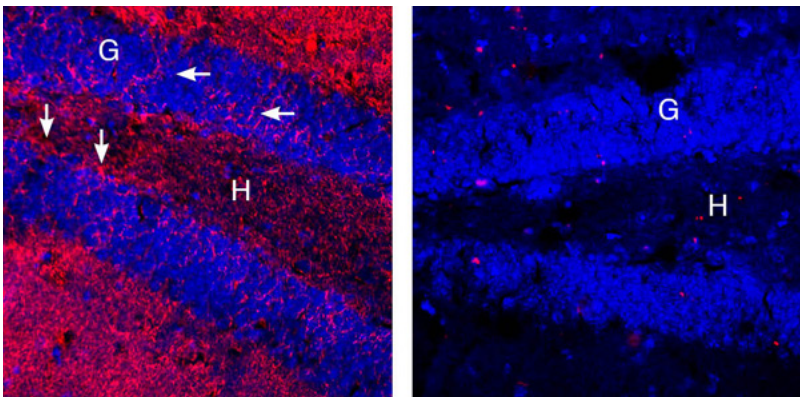


Figure 26
Expression of GPR142 in rat ventromedial hypothalamus.
Immunohistochemical staining of perfusion-fixed frozen rat brain sections with Anti-GPR142 (extracellular) Antibody (#AGR-082), (1:1000), followed by goat anti-rabbit-AlexaFluor-488. A. GPR142 immunoreactivity (green) appears in glial processes (vertical arrow) and in cells lining the wall of 3rd ventricle (horizontal arrow). B. Pre-incubation of the antibody with GPR142 (extracellular) Blocking Peptide (#BLP-GR082), suppressed staining. Cell nuclei are stained with DAPI (blue). 3rd V= 3rd ventricle.



Example Data

Figure 27
Expression of EAAT2 in mouse hippocampus.
Immunohistochemical staining of perfusion-fixed frozen mouse brain sections with Guinea Pig Anti-EAAT2 (GLT-1) (extracellular) Antibody (#AGC-022-GP), (1:100), followed by goat anti-guinea pig-AlexaFluor-594. A. Staining in the hippocampal dentate gyrus region, showed immunoreactivity (red) in the hilus (H) subgranular layer (vertical arrows) and between cells in the granule layer (G) (horizontal arrows). B. Pre-incubation of the antibody with EAAT2 (GLT-1) (extracellular) Blocking Peptide (#BLP-GC022), suppressed staining. Cell nuclei are stained with DAPI (blue).



Notes

Sample Preperation

6

Sample Preparation for Tissues

Essential sample protocols to prepare tissues and brain synaptosomes for western blot.

Sample preparation is a crucial first step in any western blot (WB). The preparation protocol you use depends on the type of sample you plan to use. For example, the optimal method to extract the same protein from heart versus adipose tissue, will not necessarily be the same one. You also need to consider the subcellular localization of your protein (plasma membrane proteins and nuclear proteins, for example, would need different sample preparation methods) and whether the target protein is enriched in specific microdomains like lipid rafts.

Beginning your WB with the proper sample preparation protocol, that considers the nature and location of your protein, is critical if you want to produce robust results.

Below you can find detailed steps for the following three sample preparation protocols:

– Enriched Membrane Fraction Preparation
– Lysate Preparation
– Brain Synaptosome Preparation

Sample Preparation for Cell Lines

See the latest online version

Enriched Membrane Fraction Preparation

1 Remove the tissue of interest from the animal and flash freeze it in liquid nitrogen. Store the tissue at -80°C until further use.

2 Place the frozen tissue in 5 volumes of ice-cold Lysis Buffer A

Lysis Buffer A

Reagent	Concentration
HEPES (pH 7.4)	4 mM
Sucrose	320 mM
EDTA (pH 8)	5 mM
Complete EDTA-free protease inhibitor cocktail (Roche)	

3 Homogenize the tissue with a polytron homogenizer.

4 Centrifuge the homogenate for 10 minutes at 2,000 x *g* at 4°C. Discard the large debris.

5 Transfer the supernatant to a clean tube and resuspend the pellet in 2 volumes of Lysis Buffer A and rehomogenize.

6 Centrifuge the homogenate for 10 minutes at 2,000 x *g* at 4°C. Combine the supernatant with that from step 5.

7 Centrifuge the supernatants (from steps 5 and 6) for 1 hour at 100,000 x *g* at 4°C.

8 Discard the supernatant. Resuspend the pellet (that contains tissue membranes) in 2 volumes of Lysis Buffer A and briefly homogenize with a polytron homogenizer.

9 Measure the protein concentration using the Bradford method. Adjust the protein concentration to 4 mg/ml with Lysis Buffer A.

10 Store protein samples at -80°C until further use.

Lysate Preparation

1 Remove the tissue/organ of interest from the animal and flash freeze it in liquid nitrogen. Store the tissue/organ at -80°C until further use.

2 Place the frozen tissue in 5 volumes of ice-cold Lysis Buffer B.

Lysis Buffer B

Reagent	Concentration
Tris (pH 7.4)	50 mM
Triton X-100	1%
EDTA (pH 8)	5 mM
Complete EDTA-free protease inhibitor cocktail (Roche)	

3 Homogenize the tissue with a polytron homogenizer.

4 Rotate the sample for 30 minutes at 4°C.

5 Centrifuge the homogenate for 1 hour at 100,000 x *g* at 4°C.

6 Transfer the supernatant to a clean tube and measure the protein concentration using the Bradford method. Adjust the protein concentration to 4 mg/ml with Lysis Buffer B. Store the lysate at -80°C until further use.

Sample Preparation for Brain Synaptosomes

This brain synaptosomal preparation is commonly called the “P2” protocol. This protocol enriches the synaptosomal fraction of the brain and generally enables easy detection of synapse-enriched proteins for WB.

1 Remove brains and flash-freeze in liquid nitrogen. Keep brains at -80°C until further use.

2 Resuspend the frozen brains in 5 volumes of ice-cold Lysis Buffer C. Work on ice.

Lysis Buffer C

Reagent	Concentration
HEPES (pH 7.4)	4 mM
Sucrose	320 mM
EDTA (pH 8)	5 mM
Phenylmethylsulfonyl fluoride (PMSF)	1 mM
Complete EDTA-free protease inhibitor cocktail (Roche)	

3 Homogenize brains with a Polytron homogenizer.

4 Centrifuge homogenates at 700 x *g* for 10 minutes at 4°C.

5 Transfer resulting supernatant to a clean tube.

6 Centrifuge at 37,000 x *g* for 40 minutes at 4°C.

7 Discard the supernatant.

8 Resuspend the pellet (P2) in half the original volume (volume added in step 2) with Extraction Buffer.

Extraction Buffer

Reagent	Concentration
Tris (pH 9)	50 mM
NaCl	150 mM
NP-40	1%
Sodium deoxycholate	0.5%
Phenylmethylsulfonyl fluoride (PMSF)	1 mM
Complete EDTA-free protease inhibitor cocktail (Roche)	

9 Incubate at 37°C for 30 minutes.

10 Centrifuge the solution at 100,000 x *g* for 60 minutes.

11 Transfer the resulting supernatant (enriched brain synaptosome) to a clean tube.

12 Determine protein concentration using the Bradford method and adjust to 4 mg/ml with Extraction Buffer.

13 Store samples at -80°C until further use.

Notes

Sample Preparation for Cell Lines

Clear protocols for preparing cells for western blot with Laemmli buffer or mild detergents.

Your sample preparation is critical for any western blot (WB) as it can affect the quality of your results. When it comes to using cell culture for WB analysis of your protein, the simplest method is to lyse them directly with electrophoresis (Laemmli) sample buffer. This method releases almost all cellular proteins into the buffer, making them readily available for separation by standard SDS/PAGE methods. However, for some downstream applications (notably immunoprecipitation) you should refer to the Cell Line Preparation Using Mild Detergents protocol.

It's worth paying attention to your sample preparation method, and choosing the right one based on your sample type, if you plan on producing reproducible and robust result

Below you can find detailed steps for the following two sample preparation protocols:

– Lysate Preparation Using Laemmli Sample Buffer
– Cell Line Sample Preparation Using Mild Detergents

See the latest online version

Lysate Preparation Using Laemmli Sample Buffer

This protocol refers to adherent cells but can be easily adapted to cells growing in suspension.

1 Wash the cell plate with ice-cold PBS. Repeat 3 times.

2 Place the plate on ice and add cold Sample Buffer (62.5 mM Tris (pH 6.8), 2% SDS, 10% glycerol, and 100 mM DTT) at a ratio of 5 × 106 cells/ml Sample Buffer.

Sample Buffer (Laemmli)

Reagent	Concentration
Tris (pH 6.8)	62.5 mM
SDS	2%
Glycerol	10%
Dithiothreitol (DTT)	100 mM

3 Scrape the dish with a cell scraper and collect the lysate into a microtube.

4 Boil the sample at 100°C for 5 minutes.

5 Sonicate the boiled sample for 5 seconds.

6 Centrifuge the sample at 14,000 rpm for 5 minutes at 4°C.

7 Transfer the supernatant to a clean tube and store at -80°C until further use.

Note
You can count cells can from a parallel plate by dislodging the cells with trypsin and counting them.

Cell Line Sample Preparation Using Mild Detergents

We recommend cell lysis with a mild detergent such as Triton X-100 or NP-40 for downstream applications like immunoprecipitation. The protocol refers to lysis of adherent cells but can be easily adapted to cells growing in suspension.

1 Wash the cell plate with ice-cold PBS. Repeat 3 times.

2 Place the plate on ice and add ice-cold Lysis Buffer D at a ratio of 5×10^6 cells/ml Lysis Buffer D.

Lysis Buffer D

Reagent	Concentration
Tris (pH 7.6)	50 mM
Triton X-100	1%
EDTA (pH 8)	5 mM
NaCl	150 mM
Complete EDTA-free protease inhibitor cocktail (Roche)	

3 Scrape the dish with a cell scraper and collect the lysate into a microtube.

4 Rock the sample in the microtube for 30 minutes at 4°C.

5 Centrifuge the sample at 14,000 rpm for 10 minutes at 4°C.

6 Carefully transfer the clear supernatant to a clean microtube. You can store your sample at -20°C for several months or immediately mixed with Laemmli sample buffer.

Note
You can count cells from a parallel plate by dislodging the cells with trypsin and counting them.

Notes

Get in Touch

As we said at the start of this book, if anything's unclear, or you just want to discuss what might be best for your experiments, get in touch.

Our technical support team are all scientists; it's likely you'll end up speaking with someone who runs the same assay right here at Alomone Labs that you might be struggling with.

We're here to make sure you research goes as smoothly as possible.

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