IHC and ICC FAQs

Detailed troubleshooting techniques for flow cytometry.

1. No staining

The primary antibody and the secondary antibody are not compatible

Use a secondary antibody that was raised against the species in which the primary was raised (e.g. primary is raised in rabbit, use anti-rabbit secondary). Be sure that the isotypes of the primary and secondary are compatible (e.g. IgY vs. IgG).

Not enough primary antibody is bound to the protein of interest

Concentrate the antibody more, incubate longer (e.g. overnight) at 4°C.

The antibody may not be suitable for IHC procedures which reveal the protein in its native (3D form)

Check the antibody datasheet to see if it has been validated in IHC, and what type of IHC (formalin/PFA fixation, fresh frozen, etc.).

Test the antibody in a native (non-denatured) WB to make sure it is not damaged.

The primary/secondary antibody/amplification kit may have lost its activity due to improper storage, improper dilution or extensive freezing/thawing

Run positive controls to ensure that the primary/secondary antibody is working properly (see section 4 for more information about controls).

The protein is not present in the tissue of interest

Run a positive control recommended by the supplier of the antibody (see section 4 for more information about controls).

The protein of interest is not abundantly present in the tissue

Use an amplification step to maximize the signal. For example, use a biotin conjugated secondary antibody and a conjugated streptavidin.

The secondary antibody was not stored in the dark (if your detection system is immunofluorescence)

Always prevent the secondary antibody from exposure to light.

Deparaffinization may be insufficient

Deparaffinize sections longer and use fresh xylene.

Fixation procedures (using formalin and paraformaldehyde fixatives) may be modifying the epitope the antibody recognizes

Use different antigen retrieval methods to unmask the epitope (heat mediated with pH 6 or pH 9 buffer, enzymatic, etc.).

Fix the sections for less time.

The protein is located in the nucleus and the antibody (nuclear protein) cannot penetrate the nucleus

Add a strong permeabilizing agent like Triton X to the blocking buffer and antibody dilution buffer. See our protocol about permeabilization techniques.

The PBS buffer is contaminated with bacteria that damage the phosphate groups on the protein of interest

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Add 0.01% azide in the PBS antibody storage buffer or use fresh sterile PBS.

2. High background

Blocking of non-specific binding might be absent or insufficient

Increase the blocking incubation period and consider changing blocking agent. Abcam recommends 10% normal serum of the species of the secondary antibody for 1 hr, or 1-5% BSA for 30 min for cells in culture.

Another option is to try a secondary antibody that has been pre-adsorbed against the lg of the species of your samples.

The primary antibody concentration may be too high

Titrate the antibody to the optimal concentration, dilute the antibody further and incubate at 4°C (a slow but targeted binding is best).

Incubation temperature may be too high

Incubate sections or cells at 4°C.

The secondary antibody may be binding non-specifically

Run a secondary control without primary antibody.

If you see staining with your secondary only, change your secondary or use a secondary antibody that has been pre-adsorbed against the lg of the species of your samples.

Tissue not washed enough, fixative still present

Wash extensively in PBS between all steps.

Endogenous peroxidases are active

Use enzyme inhibitors i.e. Levamisol (2mM) for alkaline phosphatase or H2O2 (0.3% v/v) for peroxidase.

Fixation procedures (using formalin or paraformaldehyde fixatives) are causing autofluorescence (if your detection system is immunofluorescence)

Formalin/PFA usually autofluorescence in the green spectrum, so try a fluorophore in the red range.

Use a fluorophore in the infrared range if you have an infrared detection system.

Too much amplification (amplification technique)

Reduce amplification incubation time and dilute the secondary antibody or amplification kit.

Too much substrate was applied (enzymatic detection)

Dilute substrate more and reduce substrate incubation time.

The chromogen reacts with the PBS present in the cells/tissue (enzymatic detection)

Use Tris buffer to wash sections prior to incubating with the substrate, then wash sections/cells in Tris buffer.

Permeabilization has damaged the membrane and removed the membrane protein (membrane protein)

Use a less stringent detergent (e.g.) Tween 20 instead of Triton X). Or simply remove permeabilizing agent from your buffers. See our protocol about permeabilization techniques.

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3. Non-specific staining

Primary/secondary antibody concentration may be too high

Try decreasing the antibody concentration and/or the incubation period. Compare signal intensity against cells or tissue that do not express the target.

Endogenous peroxidases are active

Use enzyme inhibitors i.e. Levamisol (2 mM) for alkaline phosphatase or H2O2 (0.3% v/v) for peroxidase.

The primary antibody is raised against the same species as the tissue stained (e.g. mouse primary antibody tested on mouse tissue). When the secondary antibody is applied it binds to all the tissue as it is raised against that species

Use a primary antibody raised against a different species than your tissue. Use a biotinylated primary antibody and a conjugated streptavidin for the detection system.

The sections/cells have dried out

Keep sections/cells at high humidity and do not let them dry out.

4. Using controls

Positive controls

Why positive controls are necessary

To validate the staining in your sample, use a positive control. This is a section from a cell line or tissue known to express the protein you are detecting. A positive result from the positive control, even if the samples are negative, will indicate the procedure is optimized and working. It will verify that any negative results are valid.

How to know what tissue type or cell line is a suitable positive control

First check the antibody datasheet. This will often provide a suggested positive control. Always ensure the tissue or cell line you use is from a tested species.

Negative contols

Why negative controls are necessary

Use a section from a cell line or tissue sample known not to express the protein you are detecting. This is to check for non-specific binding and false positive results. Recommended negative control tissues are knock down (KD) or knock out (KO) tissue samples.

No primary controls

This is when the primary antibody is not added to the sample. This indicates if any non-specific binding or false positives may be due to non-specific binding of the secondary antibody.

Antibody dilution buffer containing no antibody is incubated on the same sample in the same way as usual.

Isotype controls

An isotype control is an antibody of the same isotype (IgG2a, IgY, etc.), clonality, conjugate, and host species as the primary antibody that is raised

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against a molecule that is non-existent in the sample you are using. Usually this is raised against a chemical or a non-mammalian protein.

Use the same concentration (µg/ml) for the isotype control antibody and the primary antibody. This will determine the level of background in your sample.

Much like the no-primary control step, you would use this on your sample instead of the specific primary antibody. You would then use your secondary antibody as usual.

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