TROUBLESHOOTING IMMUNOHISTOCHEMISTRY

Troubleshooting *This troubleshooting document gives the guidelines to the problem, possible cause and suggested solution for problems during the immunohistochemistry application:*

Problem: Weak or no staining

Inadequate deparaffinization	Deparaffinize sections longer or change fresh xylene.
Inactive primary antibodies	Replace with a new batch of antibodies.
The primary/secondary antibody may have lost its activity due to improper storage	Aliquot antibodies into smaller volumes and store in freezer (-20 ºC to -70 ºC) and avoid repeated freeze and thaw cycles. Or store antibodies according to manufacturer's instructions.
Improper dilution or extensive freezing/thawing	Run positive controls to ensure that the primary/secondary antibody is working properly.
The protein is not present in the tissue of interest.	Run an appropriate positive control.
The protein of interest is not abundantly present in the tissue.	Use an amplification step to maximize the signal.
Antibody concentration was too low	Increase the concentration of primary and/or secondary antibodies. Run a serial dilution test to determine the optimal dilution that gives the best signal to noise ratio.
Inadequate antibody incubation time	Increase antibody incubation time, incubate longer (e.g. overnight) at 4ºC.
Inadequate or improper tissue fixation	Increase duration of post-fixation or try different fixatives.
Fixation procedures (using formalin and paraformaldehyde fixatives) may be modifying the epitope the antibody recognizes.	Reduce the duration of post-fixation. If the tissue has already been overfixed, perform an appropriate or recommended antigen retrieval procedure.
The antibody may not be suitable for IHC procedures which reveal the protein in its native (3D) form	Test the antibody in a native (non-denatured) Western Blotting to make sure it is not damaged.
The primary antibody and the secondary antibody are not compatible.	Use secondary antibody that will interact with primary antibody, antibodies that are raised against the species in which the primary was raised. For example, if primary antibodies are raised from rabbits, use anti-rabbit secondary antibodies.
The secondary antibody is inactive	Use a new batch of antibodies.
The enzyme substrate system is defect or incompatible	Use a new batch of reagents.
The enzyme substrate system is defect or incompatible	Use a new batch of reagents.
The substrate incubation time is inadequate	Increase the substrate incubation time.
The protein is located in the nucleus (nuclear protein) and the antibody cannot penetrate the nucleus	Add a permeabilizing agent to the blocking buffer and antibody dilution buffer.
Incorrect mounting medium	Choose a correct mounting medium.
The reagents are applied in wrong order or steps omitted	Check protocol used.
The conjugate was not stored in the dark.	Always prevent the conjugate from exposure to light.

Problem: Non-specific staining

The concentration of primary and/or secondary antibodies was too high	Reduce antibody concentration or perform a titration to determine the optimal dilution for primary and secondary antibodies.
Incubation time was too long	Reduce incubation time.

Incubation temperature was too high	Reduce incubation temperature.
Substrate incubation time was too long	Reduce substrate incubation time.
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 secondary antibody that will interact with primary antibody, antibodies that are raised against the species in which the primary was raised. For example, if primary antibodies are raised from rabbits, use anti-rabbit secondary antibodies. Treat sample with e.g. Mouse-On-Mouse blocking reagent prior to the primary antibody incubation.
Sections dried out	Avoid sections being dried out.

Problem: High Background

Tissue not washed enough, fixative still present.	Wash at least 3 times in PBS between all steps.
Tissue contains endogenous enzyme such as peroxidase or alkaline phosphatase	Block endogenous enzyme activities using 3% H_2O_2 in methanol or levamisole solution prior to incubation of primary antibodies.
Tissue contains endogenous biotin activity	Block endogenous biotin activity using the avidin/biotin blocking reagent prior to incubation of primary antibodies.
Blocking of non specific binding might be absent or insufficient.	Increase the blocking incubation period and consider changing blocking agent.
Non-specific binding of primary antibodies to tissue or antibody concentration was too high	Non-specific binding may be reduced by using higher dilution of primary antibodies. Titrate the antibody to the optimal concentration, incubate for longer but in more dilute antibody (a slow but targeted binding is best).
Incubation temperature may be too high.	Incubate sections at 4℃.
Fixation procedures (using formalin and paraformaldehyde fixatives) are too strong and modified the epitope the antibody recognizes.	Change antigen retrieval method, decrease the incubation time with the antigen unmasking solution.
Non-specific binding of secondary antibodies to tissue	Treat tissue with normal serum from the same species as secondary antibodies, or use pre-adsorbed second antibody. Run a secondary control without primary antibody.
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.
Diffusion of tissue antigen due to inadequate fixation	Increase duration of post-fixation.
Permeabilization has damaged the membrane and removed the membrane protein (membrane protein).	Remove permeabilizing agent from your buffers.
Mouse antibodies used on mouse tissues	Use secondary antibody that will interact with primary antibody, antibodies that are raised against the species in which the primary was raised. For example, if primary antibodies are raised from rabbits, use anti-rabbit secondary antibodies. Treat sample with e.g. Mouse-On-Mouse blocking reagent prior to the primary antibody incubation.
Sections have dried out	Avoid sections being dried out.

Helpful links / references

www.ihcworld.com
www.protocol-online.org