ROCKLAND antibodies & assays

Choosing the best secondary antibody for your research

Secondary antibodies play an important role in many research applications, including immunofluorescence (IF), immunohistochemistry (IHC), Western blot (WB), enzyme-linked immunosorbent assay (ELISA), flow cytometry (FC), gel shift assay or electrophoretic mobility shift assay (EMSA), immunoprecipitation (IP), and chromatin immunoprecipitation (ChIP). Choosing the right secondary antibody will improve your results by increasing signal detection while reducing background and nonspecific staining.

Important selection tips:

1. Match the host of the primary antibody

If your primary antibody is raised in rabbit, for example, use an α-rabbit secondary antibody.

2. Match the class/subclass of the primary antibody

Polyclonal antibodies are usually IgG isotype; therefore, use an α-IgG antibody that recognizes either the heavy chain or the heavy and light chains of the primary antibody (α-IgG Fc or α-IgG H+L).

Monoclonal antibodies are subclass specific; therefore, use a secondary antibody directed against that specific subclass: e.g., if the primary antibody is mouse IgM, choose a secondary antibody that reacts with mouse IgM (α-mouse IgM); if the primary antibody is mouse IgG1, choose a secondary antibody that recognizes all IgG subclasses, or, when working in multiplex assays, use a subclass-specific antibody (α-mouse IgG1).

3. Select the correct reporter for your application

Fluorescence: secondary antibody is conjugated to a fluorophore, e.g., FITC, Texas Red, Alexa Fluor®, Cy™, or DyLight™. A light source excites the fluorophore, and its brightness is measured.

Chemiluminescence: secondary antibody is enzyme linked, e.g., HRP (horseradish peroxidase). An enzymatic reaction produces light when chemiluminescent substrate is applied.

Chromogenic: secondary antibody is enzyme linked, e.g., alkaline phosphatase or HRP. An enzymatic reaction converts a chemical substrate to a colored precipitate.

Did you know?

Pre-adsorbed antibodies improve specificity by eliminating cross-reactivity with immunoglobulins of undesired species, antibody fragments, or cell tissue samples.

F(ab')2 and Fab fragment secondary antibodies eliminate nonspecific binding to Fc receptors.

Affinity-purified antibodies increase specificity and lower background, leading to greater sensitivity and lot-to-lot consistency.

IHC — Chromogenic, Fluorescence

ELISA — Chromogenic, Chemiluminescence, Fluorescence

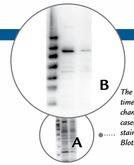
FC — Fluorescence

WB — Chromogenic, Chemiluminescence, Fluorescence

EMSA — Chromogenic, Chemiluminescence, Fluorescence

IF — Fluorescence

Western Blotting



The blocking agent influences outcome and exposure time. Unhindered detection of Notch1 is possible by changing the blocking buffer from BSA (blot A) to casein (blot B), which reduced nonspecific background staining. Detection: gt α-rabbit IgG HRP
Blots were exposed for the same time.

Gel electrophoresis

Transfer → Block membrane

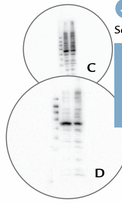
- Reduce nonspecific secondary antibody binding → use appropriate blocking agent (BSA, nonfat dry milk, casein or FBS)
- Try several blocking agents if necessary
- For interference with the reporter system or auto-fluorescence → change blocking agents
- For chromogenic detection → block endogenous enzyme activity

Primary antibody incubation → Wash

Control: omit primary antibody to detect nonspecific binding of the secondary antibody

- Remove residual primary antibody → agitate appropriately
- Reduce nonspecific bands → use a mild detergent in wash steps

Secondary antibody incubation



- Reduce nonspecific antibody binding → titrate your secondary antibody
- Block binding of secondary antibody to Fc receptor → add normal serum to antibody
- Reduce cross-reactivity → use pre-adsorbed secondary antibodies
- Reduce nonspecific binding in fluorescent WB → add 0.01% SDS to secondary antibody

D → Wash → Detect

- For single protein detection → use HRP or alkaline phosphatase–conjugated secondary antibody
- Chemiluminescence detection → use X-ray films or imaging systems
- Avoid protein loss due to stripping → multiprotein detection in a single blot (multiplex)
- Save time from multiple primary antibody incubations → multiplex WB
- Multiplex WB → important to detect each antigen using primary antibodies raised in different hosts, each matched with a specific secondary antibody conjugated to fluorescent dyes that can be imaged at different wavelengths
- For simultaneous detection of modified (phosphorylation or methylation) and unmodified protein → multiplex WB

The background on a WB can be improved by using the right antibody dilution and incubation time.

Blot C with high background was incubated overnight at 4 °C at 1:1,000 dilution. Blot D was incubated for 1 hr at room temperature with a primary antibody dilution of 1:2,000.

Do not use sodium azide with HRP-conjugated secondary antibodies.

Immunofluorescence

Sample Preparation

- Tissue autofluorescence can be impacted by the fixation method
- Compare fixation methods for samples with high autofluorescence → sample treatment with autofluorescence reducing chemicals (sodium borohydride)[1]

Wash

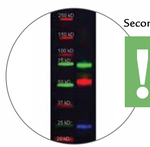
- Improve membrane permeabilization for antibody staining → add detergents
- For membrane proteins → avoid detergent usage[2]

Block

- Reduce high background → block with serum from the same host species as the secondary antibody

Primary antibody incubation

Control: omit primary antibody to block nonspecific binding of the secondary antibody. Alternatively, use an isotype control that corresponds to the isotype of the primary antibody.



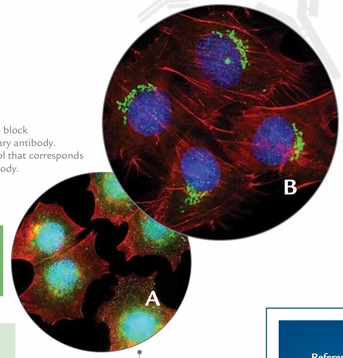
Secondary antibody incubation → Wash

- Minimize nonspecific background → use pre-adsorbed secondary antibody
- Reduce nonspecific binding → titrate the secondary antibody
- Perform the secondary antibody incubation in the dark

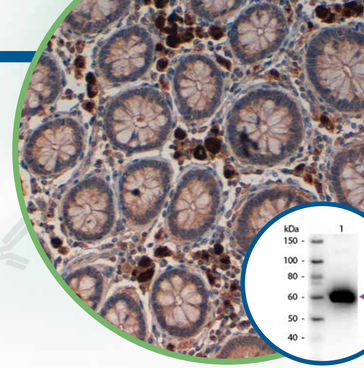
Wash → Mount

Use antifade mounting medium to preserve the fluorescent signal

Multiplex fluorescent WB of GST-spiked albumin-depleted human serum samples. Simultaneous detection of transferrin (green), α1-trypsin (red) and GST (blue). Here, α-GST (mouse host), α1-transferrin (rabbit host), and α1-trypsin (goat host) were detected using secondary antibodies: α-mouse IgG DyLight™-488, α-rabbit IgG DyLight™-549 and α-goat IgG DyLight™-649 (all raised in donkey).



Using too much primary or secondary antibody can cause nonspecific staining. Cells were stained with Tubulin (red), Akt (green), and DAPI (blue). Image A used a high concentration of primary and secondary antibodies, resulting in nonspecific staining. Titrating the antibodies reduces nonspecific staining (Image B) so that localization of Akt in the Golgi is visible.



IP and **ChIP** rely on biotin-labeled, enzyme-conjugated, epitope-tagged secondary antibodies or IgG-binding proteins to detect the protein complex or protein/DNA complex.

IP Tip: Use of a secondary antibody that recognizes the native, nonreduced form of IgG can increase specificity by avoiding interference from denatured antibody fragments and nonspecific binding to contaminants.

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References:

1. Beisker, W., Dolbeare, F., & Gray, J.W. (1987). An improved immunocytochemical procedure for high-sensitivity detection of incorporated bromodeoxyuridine. Cytometry. *8(2):235–39.*

2. Goldenthal, K.L., Hedman, K., Chen, J.W., August, J.T., & Willingham, M.C. (1985). Postfixation detergent treatment for immunofluorescence suppresses localization of some integral membrane proteins. J Histochem Cytochem, *33(8):813–20.*

3. All images provided by Rockland Immunochemicals, Inc.

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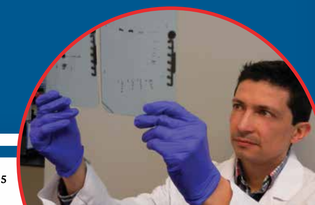

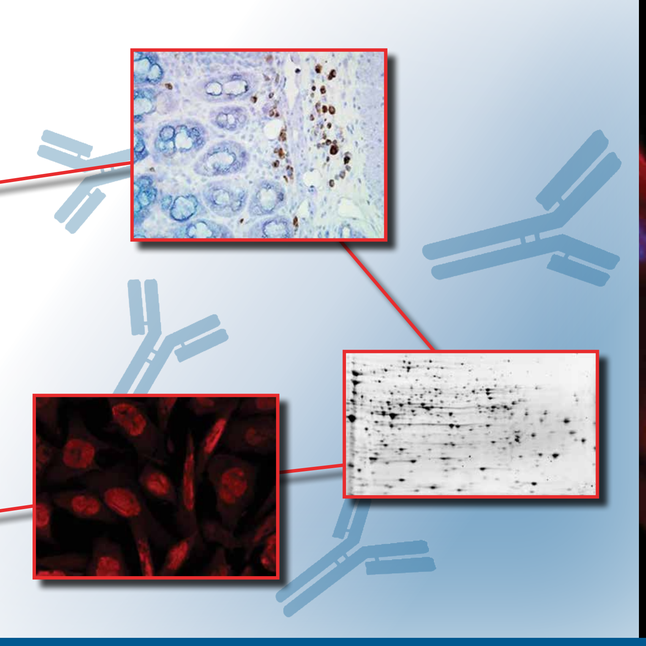

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For over fifty years now, Rockland has assembled an outstanding team of scientists and technicians with a singular dedication to making great antibodies fit for the exacting needs of scientific discovery. From thought to finish, we think, innovate, refine, troubleshoot, deliberate, hone, solve, synthesize, purify, conjugate, digest, quantify, qualify, test, package, ship and guarantee. As we manufacture and validate ***your antibody***, whether selected from our catalog or custom-made, we are keen and intent to deliver ***reproducible and reliable results*** in your assay. It's why we exist.

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James Fendrick
President & CEO

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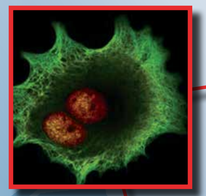

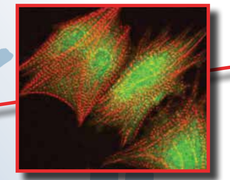

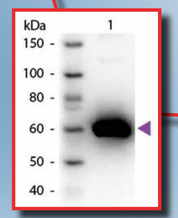

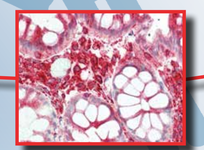

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