Troubleshooting Forum

Molecular Biology Techniques Q&A

Non-specific antibody interactions

This month's questions from the Molecular Biology Forums (online at molecularbiology.forums.biotechniques.com) come from the "Immunology and Immunochimistry Methods" section. Entries have been edited for concision and clarity. Mentions of specific products and manufacturers have been retained from the original posts, but do not represent endorsements by, or the opinions of, *BioTechniques*.

What is the best way to reduce background staining when using a mouse antibody on mouse tissue sections? (Thread 22108)

Q I am using a mouse primary antibody on paraffin-embedded mouse sections and experiencing high background. I have altered the staining protocol by using TBS-Tween (0.05%) instead of PBS-Tween, adding 1% BSA to my blocking buffer (10% serum from the secondary antibody species), and increasing both antibody incubation steps by 30 minutes.

I also read that incubating sections in unconjugated AffiniPure Fab fragment anti-Mouse IgG (H+L) for 1 hour after the blocking step may help, and using F(ab) monomeric secondary antibodies is another option. I do have a product in the lab that is a Rat Anti-Mouse CD16/CD32 (Mouse BD Fc Block). I believe this contains purified Fc receptors of mouse origin. Can I use this product to help decrease background in mouse on mouse staining?

A Is your primary antibody a mouse-anti-mouse-protein? The background staining may increase if your secondary antibody isn't specifically anti-antibody, but instead is anti-mouse protein. Since everything in the tissue section is mouse protein, this will lead to high background.

Q That is correct. My primary antibody is a mouse monoclonal and the secondary antibody is goat anti-mouse. I know specific kits are available to bypass the problem, but I don't have them. I would like to know if I can decrease the non-specific background staining using the tools I already have in the lab.

A First, you need to determine if the primary or secondary antibody is causing the background staining. Once you know the antibody responsible, you can preabsorb it with mouse protein. If the primary antibody is at fault, then you should preabsorb with mouse protein depleted of the protein of interest. If the secondary antibody is leading to problems, then preabsorb with mouse protein depleted of immunoglobulins.

How specific to the protein of interest is the epitope against which the primary antibody was made? If the epitope is a common sequence or structure, then that can also lead to high background staining. If this is the case, you would need to produce a new and more specific antibody.

A I use the Fc block (antiCD16/CD32) in my flow cytometry staining protocol. I stain for mouse IgG, which is nonspecific, and Fc block helps reduce background. I do not think the reagent is actually made up of purified Fc receptors, rather antibodies against CD16 and CD32 receptors on the cell surface that readily

bind the Fc part of the antibody. Blocking those receptors will reduce nonspecific background staining. I don't know if you can use it for purposes other than flow cytometry, but you might call the company's technical support line for more complete information.

A If you do the tests suggested above and determine that the anti-mouse secondary antibody is causing the problem, you can just label the primary antibody directly and avoid the second antibody incubation step.

A I use a procedure based on pre-labeling the primary antibody with the secondary antibody fragments and then exposing this antibody complex directly to the tissues. To do this, add biotin or enzyme-labeled anti-mouse secondary Fab fragments to your primary antibody, with the concentration of the secondary antibody about 0.5-1x the concentration of the primary antibody. Incubate this mixture 30 min or up to overnight.

You can then use this mixture in your IHC procedure after the protein blocking step. Obviously you will then skip the secondary antibody step. If you try this, you will need to use Fab fragments instead of full antibodies since large antibody complexes often precipitate. If you still get background staining, reduce the Fab concentration since excess Fab not bound by your primary antibody will bind to tissue.

A Although not ideal, mouse primary antibodies can be used for detecting antigens in mouse tissues. I frequently do mouse on mouse staining for tissues from the central nervous system. In our lab, we use 0.3% Triton X-100 to increase permeability. For blocking, we use 1% BSA and 5% normal serum from the species in which the secondary antibody is raised. Of course, you should omit detergent when staining for surface proteins since detergents may damage the antigens.

In common tissues, high background staining is usually the result of poor quality primary antibodies or the tissue preparation method chosen, especially for frozen sections. In addition, for blood, immune, or infected tissues where non-specific mouse primary antibodies are present, you will see high background staining. In those cases, it is often better to use pre-conjugated primary antibodies.

You can use Fc blocker raised in rat, but anti-mouse secondary antibodies may bind rat antibody because of the high cross-reactivity between mouse and rat. Because of this, a fluorescent dye- or biotin-conjugated primary antibody is the best candidate for these tissues.

Cut the Cord from the Nano

Unmatched performance meets superior design

NanoPhotometer™ Pearl

- Built-in computer allows mobile operation
- Only 0.3µl sample volume needed
- 3.5 seconds per measurement
- Sealed optics without moving parts eliminate the need for costly recalibration or service

The Superior NANO
for your DNA, RNA,
and Protein samples

Call 800.453.0385
for a FREE DEMO.

DENVILLE
SCIENTIFIC INC.

800.453.0385 | www.densci.com

How can I avoid background staining in serum samples? (Thread 30958)

Q I am trying to develop an assay to measure total IgG as well as different IgG subtypes against the Fab fragment of a therapeutic antibody. I want to incubate the biotinylated Fab fragment with the test sera and bind the complexes to plate-immobilized Neutr-Avidin. To validate a bioassay, it is customary to test a number of normal human sera as negative controls to detect any non-specific binding, determine the appropriate dilution of the test sera, and find the cutoff value for positivity.

In a different experiment, I immobilized a recombinant protein, diluted the sera 1/20, and saw no more than OD₄₅₀ of 0.1 for negative sera. Therefore, I diluted the test sera 1/20 and determined the cutoff for positivity as the OD₄₅₀ that exceeds the mean +3 SD of the negative sera. I want to do the same here, but the ODs I observe are well above 0.5 for some 1/100 diluted negative sera, which is not expected. I read in one paper that anti-avidin antibodies are present in normal human sera with a log-normal distribution, however I was not expecting such a high response. Perhaps it could be something used to prepare the NeutrAvidin? I am going to compare the results with streptavidin coated plates, although I fear the background signal will be even higher.

A Your detection problem sounds similar to the one described by Cressey et al. in BMC Biotechnology 8:16 (2008).

Q This seems to be a common phenomenon. However, unlike the authors of the paper, I cannot express my Fab fragment with a His tag.

A What about labeling the Fab fragment with another haptene, such as digoxigenin (DIG), and then using an anti-DIG antibody?

Q Thanks for the suggestion. Unfortunately, the human anti-mouse antibody response in normal sera is even worse. I tried substituting avidin with a biotin binding monoclonal antibody, but the background was worse. Has anyone tried to conjugate a His-tagged peptide to a protein rather than expressing the His tag?

A Anti-human IgG should recognize all IgGs in human serum since they account for a large fraction of the proteins present.

If a serum donor takes a regular vitamin supplement, they likely will have elevated, variable, free biotin levels in their serum. You might check the serum for soluble vitamin levels.

Q I am not sure why free biotin levels would influence anti-avidin antibodies. I read that circulating IgGs are biotinylated to a certain extent in older individuals, which could mean that I am actually detecting biotinylated IgGs binding to avidin. But I don't know how to check this.

A You could pre-treat with a biotin solution. At saturation, all avidin fixed to the plate will be bound to biotin. Then you can wash away the biotin and add the serum. If the background staining was caused by endogenous biotinylated antibody, it should not produce a signal.

Selected and edited by Kristie Nybo, PhD

BioTechniques 52:305-306 (May 2012) doi 10.2144/000113849

To purchase reprints of this article, contact: biotechniques@fosterprinting.com