Secondary Antibodies - Optimize your Detection System

Experimental Design - a How-to Reference Guide

Experimental design is a critical step to obtaining precise results. To ensure success it is important to consider:

1

Clonality of the secondary antibody

- Polyclonal — population of antibodies usually raised in goats, rabbits, sheep or donkeys which recognize many different epitopes on the antibody they are raised against. The signal is then amplified as multiple secondary antibodies bind to the same primary antibody
- Monoclonal — these antibodies are usually raised in mice or rats. They recognize a single epitope on the antibody they are raised against, therefore they will not give amplification of signal

2

Target of the secondary antibody

- Class — IgG, IgA, IgD, IgE and IgM
- Isotypes — polyclonal secondaries raised against, for example, mouse IgG will have a bias towards reactivity with the most common isotype, in this case IgG1. To get a stronger signal for a rarer isotype, such as IgG2b, it can be advantageous to select a secondary specifically raised against this isotype. Example isotypes are IgG1, IgG2a, IgG2b
- Chain — while it is often unimportant which part(s) of the primary antibody are epitopes for the secondary antibody, it is possible to choose secondaries raised against specific regions of the heavy chain or against the light chain should this be necessary
  - Heavy for example, Fc domain or hinge region
  - Light for example, kappa or lambda
  - Binding location – you should consider where on the primary antibody you want the secondary antibody to bind. Visit **bio-rad-antibodies.com/bindinglocations** to find out more.

3

Secondary antibody host

Ideally the secondary antibody should be from a host species that is phylogenetically distant from the target species of the primary antibody. This will minimize direct binding of the secondary antibody by Fc receptors on the target tissue or cells, otherwise Fc blocking can reduce this binding.

4

Target species

In this context it is defined as the host species of your primary antibody. Note some closely related species have very antigenically similar antibodies, which can be exploited, for instance anti-goat IgG antibodies will often cross-react with sheep antibodies.

5

Cross-absorbed antibodies

Cross-reactions are often seen in secondary antibodies. For example, anti-mouse IgG may cross-react to some degree with rat IgG, or an anti-rat IgG may show some cross-reaction with IgM, and an anti-mouse IgG2a with mouse IgG2b. In few cases these problems are not significant, but in others a high degree of specificity is required for accurate data.

Here the use of a cross-adsorbed secondary is recommended. Unwanted cross-reactivity is removed by pre-adsorbtion of the secondary antibody with the cross-reacting antigen, to yield a more specific secondary and therefore reducing nonspecific background staining. View our guide on cross-adsorbed antibodies: **bio-rad-antibodies.com/cross-adsorbed**

6

Controls

Include a secondary only control to ensure that there is no nonspecific staining from the secondary.

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7

Detection reagents

The choice of the detection reagents will depend on the application and the specifics of that particular experiment.

- ELISA — enzyme-linked secondaries are the most popular choice
- Flow cytometry — choose a secondary conjugated with a fluorophore
- Cell or tissue staining — HRP, alkaline phosphatase or a fluorescent-linked secondary antibody
- Western blotting — enzyme-linked and fluorescently labeled secondaries are best

Example detection reagents:

- Fluorescent dyes for example, PE and FITC
- Chemiluminescent for example, HRP
- Colorimetric for example, alkaline phosphatase
- Conjugated streptavidin — for use with biotin-labeled primary antibodies

8

Applications

This is a key component to experimental design and will affect many of the choices to be made in secondary antibody selection. Refer to the antibody selection step-by-step flow chart — **bio-rad-antibodies.com/ab-selector**

Indirect staining would involve careful optimization of your experiment, to avoid nonspecific binding. To help with this Bio-Rad has created a how-to guide so you can achieve the best specific flow staining using secondary detection reagents. **bio-rad-antibodies.com/secondaries-in-flow**

9

Multiplexing unconjugated primary antibodies using secondary antibodies

Multiplexing using differentially labeled primary antibodies is not always possible, but by a careful choice of unconjugated primary antibodies in combination with selected secondary antibodies, multiplexing can be achieved.

- Different species — a combination of different host species is often the simplest way to achieve multiplexing. A primary antibody raised in mouse together with a primary raised in rat plus a primary raised in rabbit can be detected with goat anti-mouse, goat anti-rat and goat anti-rabbit labeled as required
- Different classes — IgG and IgM classes can be independently detected even if they are from the same species with the use of class specific secondary antibodies
- Different isotypes — using different isotypes of antibody, such as IgG1, IgG2a and IgG2b, in combination with isotype specific secondaries is an additional way to multiplex with primaries from the same species

Mix and match these different methods to increase options or simplify staining. There are however two key issues to consider:

1. When mixing multiple secondaries, ensure they are cross-adsorbed against unwanted targets to ensure specificity of your staining; for example, when using mouse and rat primaries together ensure that the anti-mouse secondary cannot cross-react with the rat primary and vice versa. This also applies to class-specific and isotype-specific secondaries.
2. It is also important not to use a secondary antibody raised in the same host species as any of your primary antibodies, as this will result in false positives.

10

Secondary antibody detection of HuCAL® generated antibodies

Human Combinatorial Antibody Library (HuCAL) generated recombinant antibodies, for custom development of novel antibody specificities, can be used in a wide range of immunoassays such as western blotting, immunohistochemistry, ELISA and flow cytometry. Anti-human and anti-tag secondary antibodies of various specificities are ideal for detection of these antibodies; for full details download our resource on Reagents to Support HuCAL Assay Development.

Tips

- When using more than one secondary antibody ensure that they don't cross-react
- When working with some immune tissues or cells that contain a lot of Fc receptors, it helps to choose a F(ab) or F(ab')2 fragment to eliminate nonspecific binding. Alternatively, block Fc receptors via an absorption step, using purified IgG from the host species of your secondary antibody or serum from target cells
- View our IHC tips when using a secondary antibody — **bio-rad-antibodies.com/IHC-tips**

Any questions? Let us help, contact our technical support team for advice

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