

# Secondary Antibodies

Making Your Secondary Detection Work for You



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# 1 Introduction

Secondary antibodies recognize an antibody as their target antigen, usually referred to as the primary antibody which in turn is bound to its antigen or protein of interest. Unlike anti-idiotypic antibodies which bind specifically to the antigen-binding domain, secondary antibodies are specific to the conserved regions of an antibody which are constant within a particular class of antibody such as human IgG or mouse IgM. Secondary antibodies are therefore able to bind to ranges of antibody clones, making them very effective research tools.

Here we describe the applications of secondary antibodies and the considerations needed in their selection.

For information about antibody structure, refer to page 12.

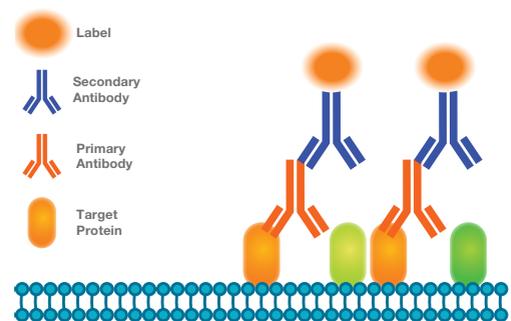
## Applications of Secondary Antibodies

### 1. Detection of a Primary Antibody

Secondary antibodies are commonly used to detect and visualize the presence of a primary antibody in applications like western blotting or immunofluorescent histology. In these applications the secondary antibody is labeled with a reporter molecule, which may be an enzyme like HRP or a fluorophore such as FITC.

Multiple secondary antibodies can bind to a single primary antibody increasing the sensitivity and amplifying the signal.

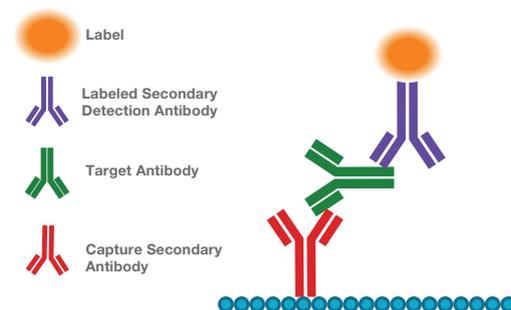
Further signal amplification can be achieved by using an unlabeled secondary antibody and a labeled tertiary antibody if required.



### 2. Antibody Capture

Unlabeled secondary antibodies can be used to capture antibodies of interest from biological solutions by quantitative ELISA. For example, an unlabeled secondary mouse anti-human IgG may be used as the capture antibody to bind human IgG from a patient serum sample. This is then detected with a labeled secondary mouse anti-human IgG which binds to the captured IgG.

The use of a calibration curve of known standards allows this signal to be quantified.



### 3. Detection and Quantification of Recombinant Proteins

Secondary antibodies can be used to detect and quantify recombinant proteins that have been engineered to contain antibody domains, for example for ease of expression, detection, stability, or increased in vivo half-life.

See Chapter 5, to find out how secondary antibodies specifically targeted to the CH2 and CH3 domains of immunoglobulins, enable the study of Fc fragments in the development of new therapeutic antibody fragments or visit [bio-rad-antibodies.com/ch2-ch3](https://www.bio-rad-antibodies.com/ch2-ch3) for more information.

# 2 Selection Process - Step-by-Step

## Select Your Secondary Antibody

Follow the steps below to find your ideal secondary antibody.

Select:

1. Target species
2. Host
3. Class and chain (found in the specificity filter)
4. Isotype
5. Format
6. Check before you select - does your antibody need to be cross-adsorbed?

Use the filters in our online tool to systematically select your requirements and find the best secondary antibody for your experiment, then download the results. See below for an example of what you can achieve using the selector tool which can be accessed from:

[bio-rad-antibodies.com/ab-selector](https://www.bio-rad-antibodies.com/ab-selector)

### Secondary Antibodies Range

DOWNLOAD

Target Species «

- Select All -

Host «

- Select All -

Specificity «

- Select All -

Isotype «

- Select All -

Format «

- Select All -

Description	Target	Host	Specificity	Format	Citations	Isotype	Code
Goat anti Human Ig:Alk. Phos.	Human	Goat	Ig	Alk. Phos.	(1)	Polyclonal IgG	201004
Goat anti Human Ig:HRP	Human	Goat	Ig	HRP	(1)	Polyclonal IgG	201005
Goat anti Human Ig	Human	Goat	Ig	Purified	(1)	Polyclonal	201001



### 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')<sub>2</sub> fragment to eliminate nonspecific binding. Alternatively, you can block Fc receptors via an absorption step, using purified IgG from the host species of your secondary antibody or serum from target cells
- Visit [bio-rad-antibodies.com/sec-tips](https://www.bio-rad-antibodies.com/sec-tips) for more tips when using a secondary antibody

## Secondary Antibodies at a Glance

Bio-Rad's secondary reagents have been carefully selected to provide optimum quality and flexibility for your experimental design. There are many options to choose from:

- Monoclonal or polyclonal
- IgG molecules or F(ab')<sub>2</sub> fragments
- Many formats available
- Suitable for a wide range of applications such as flow cytometry, western blotting, and immunocytochemistry
- Cross-adsorbed and isotype specific antibodies
- Fluorescent StarBright Secondary Antibodies. Exceptionally bright and ideal for multiplex western blotting

**Table 1. Secondary antibody product options.**

Target	Host	Class and Chain	Conjugates
Human	Donkey	Ig	Purified
Mouse	Rabbit	IgA, IgA A1, IgA A2, IgA H, IgA secretory chain	Serum
Rat	Goat	IgD, IgD H, IgD D	S/N
Goat	Mouse	IgE	FITC
Rabbit	Sheep	IgG, IgG1, IgG2, IgG2a, IgG2b, IgG2c, IgG3	Tritc
Sheep	Rat	IgG4, IgG H/L, IgG Fc, IgG Fc CH2 domain, IgG	HRP
Bovine	Human	CH2 domain, IgG CH3 domain, IgG gamma, IgG	Biotin
Guinea Pig		Fab, IgG F(ab') <sub>2</sub>	Alkaline phosphatase
Cat		IgM, IgM Mu, IgM H	Texas Red
Chicken		IgG/A/M	StarBright Blue
Monkey		Lamba light chain	DyLight 405
Fish		Kappa light chain	DyLight 488
Dog		J chain	DyLight 550
Hamster			DyLight 650
Horse			DyLight 680
Pig			DyLight 800
			Bgal

## 3

# Optimize Your Detection System

A secondary antibody is usually used to detect an unconjugated primary antibody that has bound to a target antigen.

Secondary antibodies conjugated to enzymes and fluorophores are key components of detection systems and the selection of an optimum secondary antibody can improve the positive signal in addition to reducing false positive or negative staining.

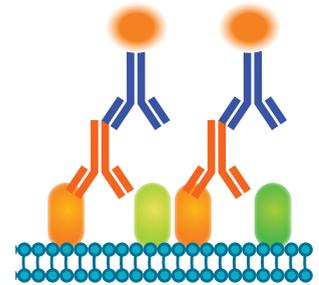
Further amplification of detection signals may be achieved by the addition of a labeled tertiary antibody which binds to the unconjugated secondary antibody.

## Experimental Design - 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 secondary antibodies 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 antibody specifically raised against this isotype. Example isotypes are IgG1, IgG2a, and IgG2b
- Chain — while it is often irrelevant which part(s) of the primary antibody are epitopes for the secondary antibody, it is possible to choose secondary antibodies raised against specific regions of the heavy chain or against the light chain, should this be necessary
- Heavy chain for example, Fc domain or hinge region
- Light chain for example, kappa or lambda
- Binding location — you should consider where on the primary antibody you want the secondary antibody to bind

See Chapter 4 for more information on binding locations.

### 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 that 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-Adsorbed 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 most 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 antibody is recommended. Unwanted cross-reactivity is removed by pre-adsorption of the secondary antibody with the cross-reacting antigen, to yield a more specific secondary antibody and therefore reducing nonspecific background staining. See Chapter 6 for more information on cross-adsorbed antibodies.

### 6. Controls

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

### 7. Detection Reagents

The choice of the detection reagents will depend on the application and conditions of a particular experiment.

- ELISA — enzyme-linked secondary antibodies are the most popular choice
- Flow cytometry — choose a secondary antibody conjugated with a fluorophore
- Cell or tissue staining — HRP, alkaline phosphatase, or a fluorescent-linked secondary antibody
- Western blotting — enzyme-linked and fluorescently labeled secondary antibodies are the 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 — see Chapter 2.

### 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 secondary antibodies, is an additional way to multiplex with primary antibodies 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 secondary antibodies, ensure they are cross-adsorbed against unwanted targets to ensure specificity of your staining; for example, when using mouse and rat primary antibodies together ensure that the anti-mouse secondary antibody cannot cross-react with the rat primary antibody and vice versa. This also applies to class specific and isotype specific secondary antibodies.
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 at [bio-rad-antibodies.com/manual](http://bio-rad-antibodies.com/manual)



#### 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')<sub>2</sub> 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
- Visit [bio-rad-antibodies.com/sec-tips](http://bio-rad-antibodies.com/sec-tips) for more tips when using a secondary antibody

## Notes

# 4 Specific Binding Locations

When choosing a secondary antibody, it is important to consider where on the primary antibody you want the secondary antibody to bind. In some experiments, binding location is not important but for others, it is beneficial or even critical that the secondary antibody binds to a specific region. To help you choose the most suitable secondary antibody, we have highlighted the different options for binding locations, their benefits, and examples of their applications. Also included is a schematic of all Ig classes, so you can easily picture the binding location.

## Contents in this section:

1. Immunoglobulin Ig Classes and Isotypes
2. Secondary Antibody Type
3. Cross-Adsorbed Antibodies
4. Secondary Antibody Specific Binding Locations
5. Detection of Light and Heavy Chains of a Mouse Monoclonal IgG1 by Western Blot Analysis
6. Detecting Fab and F(ab')<sub>2</sub> Primary Antibodies
7. Different Secondary Antibody Formats

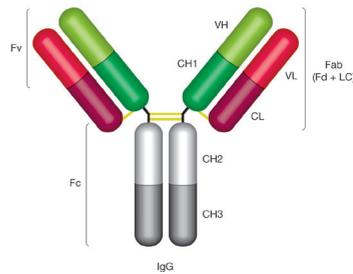
# 1. Immunoglobulin Ig Classes and Isotypes

There are five mammalian immunoglobulin (Ig) classes where the heavy chain differs in amino acid composition and number: IgA (alpha), IgD (delta), IgE (epsilon), IgG (gamma), and IgM (mu). As there are five different Ig antibody classes, it can be advantageous to select a secondary antibody raised against a specific class. Immunoglobulin classes, subclasses, and their binding valency are described fully here.

The table below shows the different Ig classes and subclasses/isotypes in human/mouse.

Ig Class	Human Ig Subclasses	Mouse Ig Subclasses
IgA	IgA1, IgA2	
IgD		
IgE		
IgG	IgG1, IgG2, IgG3, IgG4	IgG1, IgG2a, IgG2b, IgG3
IgM		

## IgA Antibody



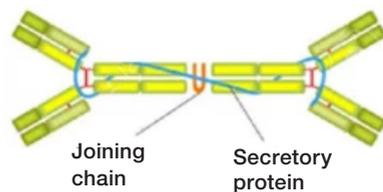
**CH1**, constant heavy chain 1; **CH2**, constant heavy chain 2; **CH3**, constant heavy chain 3; **CL**, constant light chain; **Fab**, fragment antigen binding region; **Fc**, fragment crystallisable region; **Fd**, heavy chain of the Fab; **Fv**, fragment variable region; **Lc**, light chain of the Fab; **VH**, variable heavy chain; **VL**, variable light chain.

## IgG Structure

- Two heavy chains: each chain composed of VH, CH1, hinge region, CH2, and CH3
- Two light chains: each chain composed of VL and CL. There are two types of light chain, called kappa and lambda, always identical for each antibody
- Two antigen binding sites: found at the end of the VH and VL chain, known as the paratope

Visit [bio-rad-antibodies.com/igg-antibody](http://bio-rad-antibodies.com/igg-antibody) for further information on IgG and the available antibodies to this Ig class.

## IgA Antibody

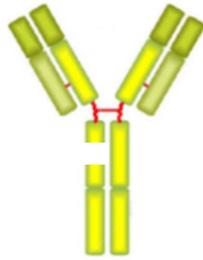


## IgA Structure

- Four alpha heavy chains
- Four light chains
- Tetravalent
- One secretory protein chain
- One joining (J) chain

Visit [bio-rad-antibodies.com/iga-antibody](http://bio-rad-antibodies.com/iga-antibody) for further information on IgA and the available antibodies to this Ig class.

### IgD Antibody

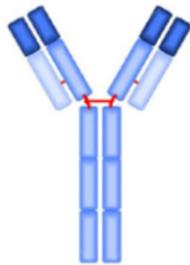


#### IgD Structure

- Two delta heavy chains
- Two light chains
- Bivalent
- Does not bind to Fc receptors

Visit [bio-rad-antibodies.com/igd-antibody](https://www.bio-rad-antibodies.com/igd-antibody) for further information on IgD and the available antibodies to this Ig class.

### IgE Antibody

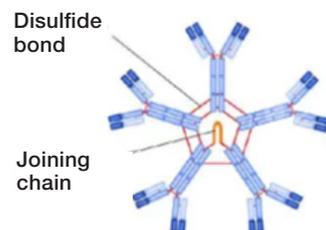


#### IgE Structure

- Two epsilon heavy chains
- Two light chains
- Bivalent
- Heavy chains contain a unique CH4 domain not found in other classes

Visit [bio-rad-antibodies.com/ige-antibody](https://www.bio-rad-antibodies.com/ige-antibody) for further information on IgE and the available antibodies to this Ig class.

### IgM Antibody



#### IgM Structure

- Pentamer of bivalent subunits
- Ten Mu heavy chains
- Ten light chains
- One joining (J) chain
- Decavalent
- Considerably higher molecular mass than other classes

Visit [bio-rad-antibodies.com/igm-antibody](https://www.bio-rad-antibodies.com/igm-antibody) for further information on IgM and the available antibodies to this Ig class.

## 2. Secondary Antibody Type

The type of secondary antibody affects where it binds on a primary antibody.

- Polyclonal antibodies: bind to multiple locations on a primary antibody
- Region specific polyclonal antibodies: bind to multiple locations within a specific region
- Monoclonal antibodies: bind to a specific location on the primary antibody — please note, this location may not be stated in the antibody specifications as it may not have been characterized

### 3. Cross-Adsorbed Antibodies

Cross reactions are often seen with 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 may show some cross reaction with mouse IgG2b. In some cases cross-reactivity is not significant, but in others a high degree of specificity is required for accurate data.

The initial way to achieve specificity is to use a cross-adsorbed polyclonal secondary antibody. Here unwanted cross-reactivity is removed by pre-adsorption of the secondary antibody with the cross reacting antigen, to yield a more specific secondary antibody and therefore reduce nonspecific background staining. As an alternative to this, a monoclonal secondary antibody may be selected that binds to an epitope unique to the chosen isotype.

See Chapter 6 to read our overview on cross-adsorbed secondary antibodies to discover why and how you should use them, how they are generated, and a list of the cross-adsorbed secondary antibodies available now.

### 4. Secondary Antibody Specific Binding Locations

The nature of the epitope recognized by the secondary antibody can have an impact on the success of an experiment, and it is worth taking the time to consider if this may affect your data. Here we have divided our secondary antibody range into four groups based on the epitope recognized.

#### Universal

H/L chain — these polyclonal secondary antibodies have a broad target binding area as they recognize both the heavy and light chains, which are found in all formats (full Ig, F(ab)<sub>2</sub>, and Fab'), and classes of secondary antibodies.

**Example of use** — due to their broad reactivity, universal polyclonal primary antibodies can be used in most applications when specificity is not an issue.

#### Light Chain

Ig lambda light chain or kappa light chain — light chain specific secondary antibodies will target 25 kDa polypeptides without detecting the heavy chain. Light chain ratios vary greatly between species, and secondary antibodies are not generally cross-reactive, so it is important to establish the specific light chain in your primary antibody when using these types of secondary antibodies.

**Example of use** — western blotting of immunoprecipitates may cause detection problems if the antigen is of a similar size to the 50 kDa heavy chain of IgG, this is because the secondary antibody may detect this component of the precipitating primary antibody. The use of an anti-light chain secondary antibody will prevent this, as only the 25 kDa light chain of the precipitating antibody will be co-detected in the blot.

### Heavy Chain

- Gamma chain — target the heavy chain found in full IgG, F(ab)<sub>2</sub>' and Fab' formats and do not cross-react with IgG light chains
- Mu chain — only recognize the heavy chain on IgM, and as a result, no cross-reactivity is seen with other Ig class heavy chains or any Ig light chains
- Alpha chain, alpha 1 chain and alpha 2 chain — recognize the alpha chain on IgA subclass, and have minimal cross-reactivity with other human immunoglobulin subclasses
- Fc — recognize the Fc region of a specific and specified Ig subclass. They can be used to capture primary antibodies with their antigen binding domains clearly presented
- CH2 domain (CH3 in IgM and IgE) — these secondary antibodies recognize the CH2 domain on the heavy chain in the Fc region of a specific and specified Ig subclass. The CH2 domain of an antibody has a role in preventing degradation in vivo, and may be added to therapeutic proteins to increase their half-life. Refer to Chapter 5, CH2 and CH3 domains for further information about how secondary antibodies specifically targeted to the CH2 and CH3 domains of immunoglobulins enable the study of the Fc fragments in the development of new therapeutic antibody fragments
- CH3 domain — recognize the CH3 domain on the heavy chain in the Fc region of a specific and specified Ig subclass. As above, this region plays a role in antibody stability
- Hinge region — recognize the hinge region of a specific and specified Ig subclass
- Fd — recognize the Fd region of human IgG. The Fd region is the heavy chain of the Fab, for example, approximately the first 220 amino acids from the N-terminus of the heavy chain comprised of the VH and CH1 regions. Typically these antibodies show minimal cross-reactivity to the Fc region of human IgG and other human immunoglobulins

**Example of use** — in an analogous situation to the one described above, the use of an anti-heavy chain secondary antibody can prevent interference with the detection of antigens of a similar size to the 25 kDa IgG light chain of the precipitating primary antibody by only detecting the larger heavy chain component.

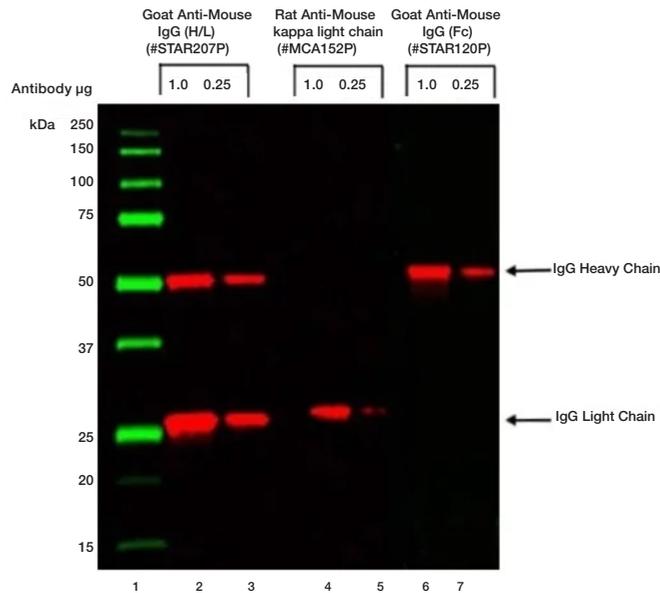
### Antibody – Associated Proteins

- Secretory chain — recognize the secretory component of IgA, thought to protect IgA antibodies from degradation and form part of the secretory immunoglobulin (Ig) A (SIgA), when two IgA molecules are covalently bound by a J chain. The secretory chain secondary antibody does not show cross-reactivity with IgM and IgG subclasses
- J chain — recognize the J chain which covalently links together two IgA molecules, and along with the secretory component, form the secretory immunoglobulin (Ig) A (SIgA)

**Example of use** — although they are not strictly antibodies, they form part of some antibody structures and potentially the basis for discriminating secretory from nonsecretory forms.

## 5. Detection of Light and Heavy Chains of a Mouse Monoclonal IgG1 by Western Blot Analysis

- Light chain detection: Rat Anti-Mouse Kappa Light Chain Antibody (#MCA152P), clone OX-20 recognizes the kappa light chain of murine immunoglobulins. OX-20 does not recognize mouse lambda light chains or bind to the immunoglobulin heavy chain
- Heavy chain detection: Goat Anti-Mouse IgG (Fc) Polyclonal Antibody (#STAR120P) reacts with mouse IgG at an epitope localized to the Fc region. Cross-reactivity with IgA and IgM is negligible
- Heavy and light chain detection: Goat Anti-Mouse IgG (H/L) Polyclonal Antibody (#STAR207P) reacts with the heavy chains of mouse IgG, and the light chains common to the majority of mouse immunoglobulins.



**Fig. 1. Light and heavy chains of a mouse monoclonal IgG1 visualized by western blot analysis.** A mouse monoclonal antibody of the IgG1 isotype was run under reducing conditions on SDS PAGE using the Bio-Rad V3 Western Workflow and transferred to a PVDF membrane. Precision Plus Protein Prestained Standards were run in lane 1. 1 µg and 0.25 µg of antibody were run in lanes 2, 4, and 6 and lanes 3, 5, and 7 respectively. Protein detection was carried out on lanes 2 and 3 using Goat Anti-Mouse IgG (H/L):HRP (#STAR207P) at a dilution of 1/10,000. Detection was carried out on lanes 4 and 5 using Rat Anti-Mouse Kappa Light Chain:HRP (#MCA152P) at a dilution of 1/1,000. Detection was carried out on lanes 6 and 7 using Goat Anti-Mouse IgG (Fc):HRP (#STAR120P) at a dilution of 1/5,000. Visualization was carried out using the ChemiDoc Touch Imaging System.

## 6. Detecting Fab and F(ab')<sub>2</sub> Primary Antibodies

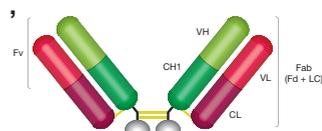
When using proteolytically derived antibody fragments as primary antibodies, it should be noted that several heavy chain specific domains are missing from their structure. This impacts the choice of secondary antibody that can be used, for instance anti-Fc, CH2, and CH3 will not work on Fab or F(ab')<sub>2</sub> fragments.

Engineered recombinant antibodies, like HuCAL technology generated antibodies, may not contain certain domains depending upon their exact structure. Please consult the HuCAL Antibody Technical Manual for further details: [bio-rad-antibodies.com/manual](http://bio-rad-antibodies.com/manual). In the case of HuCAL primary antibodies, one or more tags such as DYKDDDDK or strepavidin are incorporated into the structure to enable detection by anti-tag secondary antibodies.

## 7. Different Secondary Antibody Formats

In addition to monoclonal and polyclonal secondary antibodies with full length Ig structure, other secondary antibodies are available in different formats. Below are schematics of F(ab')<sub>2</sub> and Fab' antibody structures, and the benefits of using them as a secondary antibody.

### F(ab')<sub>2</sub>



#### Key:

**CH1**, constant heavy chain 1; **CL**, constant light chain; **Fab**, fragment antigen binding region; **Fd**, heavy chain of the Fab; **Fv**, fragment variable region; **Lc**, light chain of the Fab; **VH**, variable heavy chain; **VL**, variable light chain.

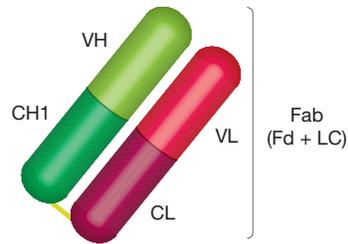
### F(ab')<sub>2</sub> Structure

- Two heavy chains: each chain composed of VH, CH1, and a hinge region
- Two light chains: each chain composed of VL and CL
- Two antigen binding sites: found at the end of the VH and VL chain, known as the paratope

#### Benefits:

- No nonspecific binding between the Fc portion of antibodies and cell Fc receptors
- Due to smaller size they can penetrate tissue more easily
- Ideal applications include flow cytometry, IHC, and IF

### Fab



### Fab Structure

- One heavy chain: composed of VH and CH1
- One light chain: composed of VL and CL
- One antigen binding site (monovalent): found at the end of the VH and VL chain, known as the paratope

### Benefits:

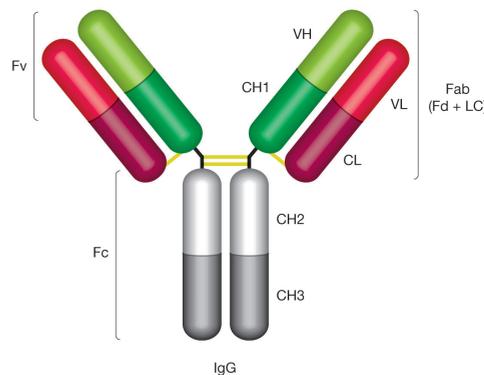
- Ideal for use in IHC and analysis of intercellular targets, as the low molecular weight does not inhibit access to the target receptor
- As a blocking agent
  - Blocking the immunoglobulin when double labeling primary antibodies from the same species
  - When staining tissue or cells expressing large amounts of Fc receptors, for example, blocking the endogenous immunoglobulin

# 5 CH2 and CH3 Domains

## The Role of Antibody Fragments

Antibody engineering has enabled the development of antibody fragments that have the same targeted specificity, but a smaller size, resolving problems with tissue penetration and target access. However, compared to full size antibodies, these fragments have a significantly reduced half-life. To overcome this, antibody fragments containing immunoglobulin (Ig) G Fc constant heavy chain 2 (CH2) regions have been developed. The CH2 in Ig classes IgG, IgA, and IgD and the constant heavy chain 3 (CH3) in Ig classes IgE and IgM, form the Fc region of an antibody. Please refer to the IgG antibody image below to visualize where these regions are located on an antibody. The CH2 and CH3 regions within the Fc region are critical for Ig effector functions to elicit an immune response. The stability conferred by the IgG Fc CH2 fragment is a result of the binding of the neonatal Fc receptor (FcRn) to CH2, contributing to the long half-life of the Ig. FcRns natural function is in the stabilization of IgG as it transitions through the placenta to the fetus, prolonging the half-life of cellular IgG antibodies after birth.

IgG Antibody



### IgG Structure

- Two heavy chains: each chain composed of VH, CH1, hinge region, CH2, and CH3
- Two light chains: each chain composed of VL and CL. There are two types of light chain, kappa and lambda, always identical for each antibody
- Two antigen binding sites: found at the end of the VH and VL chain, known as the paratope

### Key:

**CH1**, constant heavy chain 1; **CH2**, constant heavy chain 2; **CH3**, constant heavy chain 3; **CL**, constant light chain; **Fab**, fragment antigen binding region; **Fc**, fragment crystallisable region; **Fd**, heavy chain of the Fab; **Fv**, fragment variable region; **VH**, variable heavy chain; **VL**, variable light chain.

The development of antibody fragment libraries capable of binding multiple epitopes, has led to advances in antibody engineering and adaptation of the modular structure of antibodies, securing the future of antibody therapy.

The use of secondary antibodies to CH2 and CH3 domains enables Fc fragments to be studied, aiding the development of new therapeutic antibody fragments. Antibodies are available with a selection of chromogenic and fluorescent labels and suitable for many applications including ELISA, western blotting, immunohistochemistry, immunofluorescence, and flow cytometry. Each is performance guaranteed for the application listed on the product datasheet.

Visit [bio-rad-antibodies.com/ch2-ch3](https://www.bio-rad-antibodies.com/ch2-ch3) to view our CH2 and CH3 domain range.



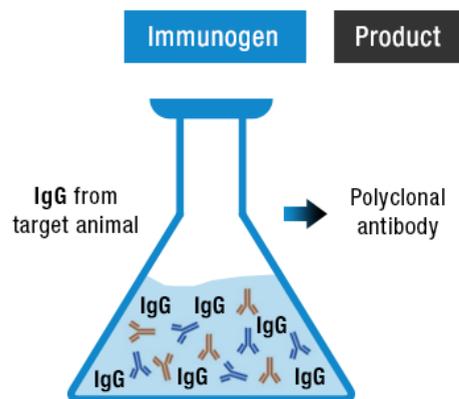
### Tips

- 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')<sub>2</sub> 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
- Visit [bio-rad-antibodies.com/sec-tips](https://www.bio-rad-antibodies.com/sec-tips) for more tips when using a secondary antibody

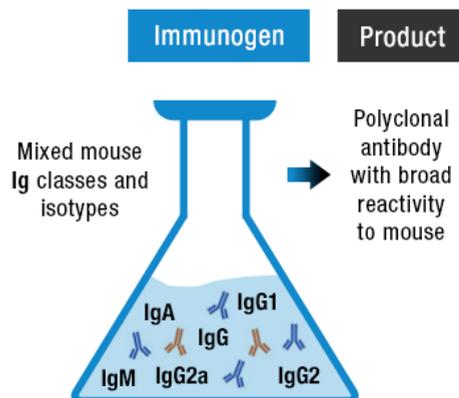
# 6 Cross-Adsorbed Polyclonal Secondary Antibodies

## Why Use Cross-Adsorbed Secondary Antibodies?

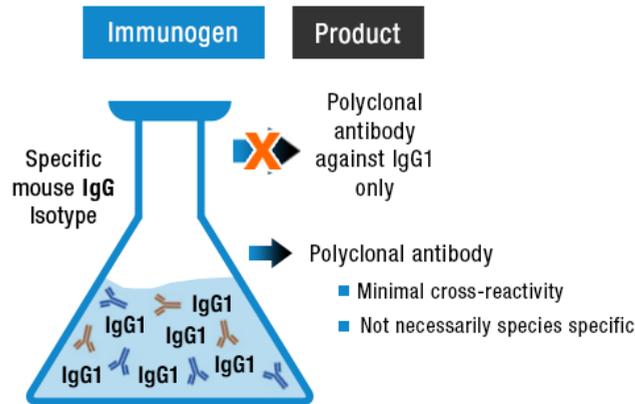
To appreciate the benefits of cross-adsorbed secondary antibodies it is important to understand the specificity of the polyclonal secondary antibody. Polyclonal secondary antibodies are raised by immunizing the host animal with immunoglobulin from the target animal.



The resulting specificity of the polyclonal secondary antibody will depend on the purity and composition of the immunoglobulin pool used for immunization. For example, immunising a goat with a pool of mixed mouse classes (for instance IgG, IgM, IgA) and isotypes (like IgG1, IgG2a, IgG2b) would be expected to give rise to a serum with a very broad reactivity towards mouse antibodies.



Immunization with a purified mouse IgG1 preparation might be expected to generate a serum with a specific reactivity towards mouse IgG1 and no cross-reactivity with other antibody isotypes or species. Such sera are indeed generated, and after testing, can be described as having minimal cross-reactivity, however, due to the polyclonal nature of the serum combined with the conservation of some epitopes between species, classes and isotypes, this is often not the case. In this example, a component of the polyclonal serum may react with an epitope on mouse IgG1 that is also found on IgG2a, confounding the isotype specificity. Likewise, an epitope on mouse IgG1 may also be shared with rat IgG, meaning the antiserum is not species specific.



In many cases, the absence of specificity of the secondary antibody, with a very broad reactivity, will not cause problems due to the specific application used or the lack of other target antibodies. However, when absolute specificity is required, for instance during multiplexing or to prevent background staining, a polyclonal secondary antibody is required where the undesired reactivity has been removed by a process known as cross-adsorption.

## How Are Cross-Adsorbed Polyclonal Secondary Antibodies Generated?

To remove an undesired reactivity from a polyclonal pool, an affinity chromatography column is used to covalently immobilize the undesired antibodies. This may consist of antibodies from the offending cross-reactive species, or antibodies of a different class or isotype. By passing the polyclonal serum through the column, clonal antibodies that also recognize the immobilized antigen(s) are removed. The unbound pool of antibodies is now cross-adsorbed against the immobilized antigen and should show no reactivity towards it. Efficient cross-adsorption may require multiple passes through a column and cross-adsorption against multiple antigens will require passes through multiple columns.

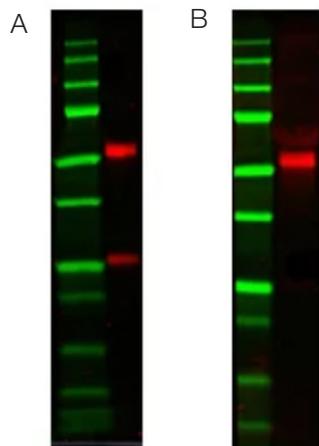
## Benefits of Cross-Adsorbed Secondary Antibodies

### Species Specific Polyclonal Secondary Antibodies

The most common type of cross-adsorbed secondary antibody is species specific, most useful for multiplexing. For example immunofluorescence might be performed using both a mouse and rabbit primary antibody. By using cross-adsorbed secondary antibodies, the user can be assured that the signal reported by each secondary antibody is specific to each primary antibody.

Species specific polyclonal secondary antibodies can also be used to generate a genuine signal in sandwich ELISAs where the capture antibody is from a different host to the detection antibody. Use of a cross-reactive antibody in this case would cause blanket detection of the capture antibody.

For western blotting of IgG rich samples, the use of a cross-reactive secondary antibody can lead to unwanted detection of heavy and light immunoglobulin chains from the sample on the blot. An example of this can be seen in Figure 2. Here Mouse Anti-Human Factor P (#MCA2617) was visualized using a noncross-adsorbed secondary antibody, Goat Anti-Mouse IgG (H/L): HRP (#STAR207P) (Image A) and the cross-adsorbed secondary antibody, Goat Anti-Mouse IgG (H/L) (multi species adsorbed) (#STAR117P) (Image B). In image A, you can clearly see that the antibody also detects the light chain, whereas in image B, only the target protein is detected. In both cases, switching to a cross-adsorbed secondary antibody can improve the situation.



**Fig. 2. Detection of Mouse Anti-Human Factor P (#MCA2617) with A, Goat Anti-Mouse IgG (H/L):HRP (#STAR207P) and B, Goat Anti-Mouse IgG (H/L) (multi species adsorbed) (#STAR117P).** Human serum was run on SDS PAGE using the primary antibody at a dilution of 1/1,000 and the secondary antibodies at a dilution of 1/10,000.

Visit [bio-rad-antibodies.com/cross-adsorbed](https://www.bio-rad-antibodies.com/cross-adsorbed) to view our full range of cross-adsorbed antibodies.

## Immunoglobulin Class and Isotype Specific Polyclonal Secondary Antibodies

In an analogous manner to species specific cross-adsorbed antibodies, secondary antibodies that have been cross-adsorbed against other classes (for example, IgG, IgM, and IgA) or isotypes (for example, IgG1, IgG2a, and IgG2b) can be used to prevent unwanted detection.

Recombinant, isotype specific antibodies have been generated against the three main mouse isotypes: IgG1 (#HCA309), IgG2a (#HCA310), and IgG2b (#HCA311). These antibodies detect individual isotypes without any species or isotype cross-reactivity, enabling more than one unlabeled mouse monoclonal antibody to be used at any one time, for multiplexing without species issues.

Visit [bio-rad-antibodies.com/multiplexing](https://www.bio-rad-antibodies.com/multiplexing) to learn more.



### Tips

- Not all polyclonal secondary antibodies are cross-adsorbed to the same standard or measured in the same way
- Check the reactivity in the application you are using; for example an antibody cross-adsorbed against rat IgG may not detect native rat IgG in an ELISA, but may detect denatured rat IgG on a western blot
- When choosing primary antibodies for use in an experiment that requires cross-adsorption, select primary species that are not closely related (for instance not mouse and rat, or goat and sheep) as secondary antibodies cross-adsorbed against similar species are likely to have a much-reduced clonality
- Likewise, if a secondary antibody that has been cross-adsorbed against your primary species of choice is not available, cross-adsorption against a closely related species may suffice (such as goat instead of sheep, human instead of chimpanzee)

# 7 Isotype Specific Monoclonal Secondary Antibodies

## Overview

Secondary antibodies are key components of the detection system; the selection of an optimum secondary antibody can improve the positive signal in addition to reducing false positive or negative staining.



Visit [bio-rad-antibodies.com/isotype-secondaries](https://www.bio-rad-antibodies.com/isotype-secondaries) to download the illustration or view the video showing the benefits of multiplexing with isotype specific antibodies in imaging.

## Cross-Adsorbed Antibodies

As discussed earlier, issues with species and isotype cross-reactivity are often overcome by the use of cross-adsorbed secondary antibodies. Here, unwanted cross-reactivity is removed by pre-adsorption of the secondary antibody with the cross-reacting antigen, to yield a more specific secondary antibody and therefore reducing nonspecific background staining.

Visit [bio-rad-antibodies.com/cross-adsorbed](https://www.bio-rad-antibodies.com/cross-adsorbed) to read our overview on cross-adsorbed secondary antibodies to discover why and how you should use them, how they are generated, and a list of the cross-adsorbed secondary antibodies available.

## Recombinant Monoclonal Antibodies - the Solution to Cross-Reactivity in Multiplexing

Recombinant monoclonal antibodies directed against the three main mouse isotypes, IgG1, IgG2a, and IgG2b are an alternative to cross-adsorbed secondary antibodies. They are capable of detecting individual isotypes without any species or isotype cross-reactivity, enabling multiple unlabeled mouse monoclonal antibodies to be used simultaneously. Multiplexing without species issues is therefore straightforward and an alternative species does not need to be sourced.

### Benefits of Isotype Specific Secondary Antibodies:

1. Advantages of recombinant monoclonal secondary antibodies over polyclonal antibodies:
  - Polyclonal isotype specific antisera are produced by depletion of reactivity to an undesired target which is not 100% efficient. Whereas recombinant human monoclonal antibodies are selected against epitopes unique to the isotype
  - Polyclonal antibodies can show variation between batches; recombinant monoclonal antibodies do not
2. High specificity: the signal detected by each secondary antibody is specific to each primary antibody.
3. Minimum nonspecific background: isotype specific secondary antibodies do not bind to non target IgG.
4. Suitable for multiplexing: no binding to non target primary antibodies in a multiplex experiment.
5. Easy-to-use: directly substitute for any secondary antibody, no special protocol or extra steps required.
6. Versatile: can be used in IHC, IF, flow cytometry, and western blotting.

## Isotype Specific Secondary Antibodies in Different Applications:

1. Western Blotting
2. Imaging IHC and IF
3. Flow Cytometry
4. ELISA

### 1. Western Blotting

In a western blot experiment, detection of multiple targets can be achieved using isotype specific secondary antibodies, as directly labeled primary antibodies may not be bright enough.

Figure 3 shows the specific detection of the target antibody by the three isotype specific antibodies in lanes 4, 6, and 8 compared to lane 2 showing the presence of all three antibodies:

- **IgG1 isotype detection**

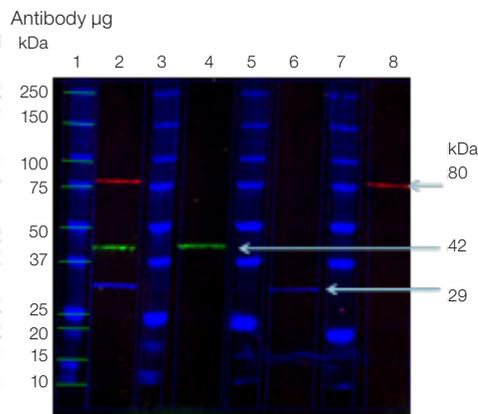
Human Anti-Mouse IgG1 DyLight 488 (#HCA309D488), clone AbD24121 specifically recognizes the IgG1 isotype of Mouse Anti-Actin Beta (#VMA00048), 42 kDa molecular weight, as seen in lane 4. AbD24121 does not recognize Mouse Anti-PCNA IgG2a or Mouse Anti-Ezrin IgG2b

- **IgG2a isotype detection**

Human Anti-Mouse IgG2a DyLight 650 (#HCA310D650), clone AbD24124 specifically recognizes the IgG2a isotype of Mouse Anti-PCNA (#VMA00018), 29 kDa molecular weight as seen in lane 6. AbD24124 does not recognize Mouse Anti-Actin Beta IgG1 or Mouse Anti-Ezrin IgG2b

- **IgG2b isotype detection**

Human Anti-Mouse IgG2b DyLight 550 (#HCA309D488), clone AbD24127 specifically recognizes the IgG2b isotype of Mouse Anti-Ezrin (#VMA00344), 80 kDa molecular weight as seen in lane 8. AbD24127 does not recognize Mouse Anti-Actin Beta IgG1 or Mouse Anti-PCNA IgG2a



**Fig. 3. Fluorescent western blotting using isotype specific secondary antibodies.** HeLa lysate (25 µg) was run in lanes 2, 4, 6, and 8. Detection was carried out as follows:

Mouse IgG1 Anti-Actin Beta (#VMA00048) at 0.5 µg/ml was used in lanes 2 and 4, followed by IgG1 specific Human Anti-Mouse Dylight 488 (#HCA309D488) at 0.3 µg/ml (green).

Mouse IgG2a Anti-PCNA (#VMA00018) at 0.5 µg/ml was used in lanes 2 and 6, followed by IgG2a specific Human Anti-Mouse Dylight 650 (#HCA310D650) 0.3 µg/ml (blue).

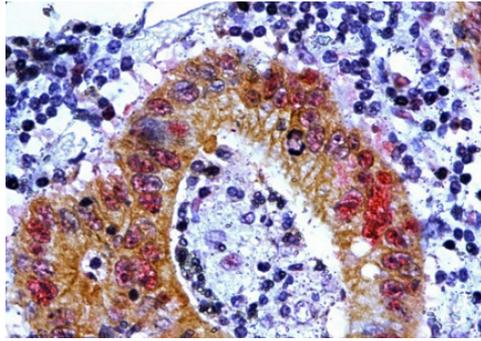
Mouse IgG2b Anti-Ezrin (#VMA00344) at 0.5 µg/ml was used in lanes 2 and 8, followed by IgG2b specific Human Anti-Mouse Dylight 550 (#HCA311D550) 0.3 µg/ml (red). Visualization with the ChemiDoc MP Imaging System.

## 2. Imaging - IHC and IF

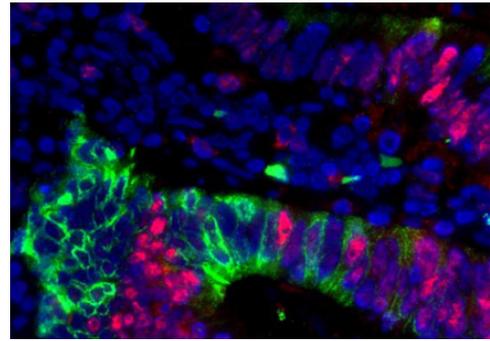
The three isotype specific secondary antibodies are designed for use in multiplex imaging:

- Highly specific: detect monoclonal antibodies from the same species and detect three different antigens/antibodies with no cross-reactivity
- Versatile: further increase your multiplexing capabilities by combining isotype specific and species specific secondary antibodies. Remove the need to use different species for subsequent antibody identification

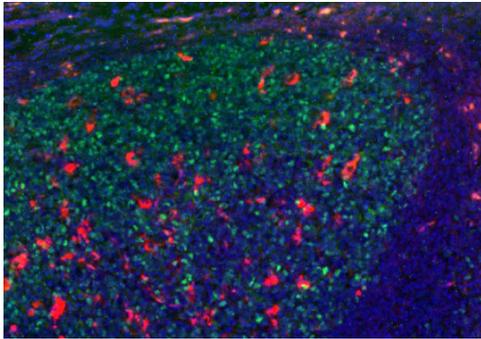
In the three images below you can see the highly specific fluorescent staining of all three isotype specific secondary antibodies.



**Fig. 4. Chromogenic staining of human colon using isotype specific secondary antibodies.** FFPE human colon adenocarcinoma stained with Mouse Anti-Cytokeratin 18 (#MCA1864) and Human Anti-Mouse IgG1 (#HCA309HRP) (brown) and Mouse Anti-PCNA (#MCA1558) and Human Anti-Mouse IgG2a (#HCA310AP) (red).



**Fig. 5. Fluorescent staining of human colon using isotype specific secondary antibodies.** FFPE human colon adenocarcinoma stained with Mouse Anti-Cytokeratin 18 (#MCA1864) and Human Anti-Mouse IgG1 (#HCA309D488) (green) and Mouse Anti-PCNA (#MCA1558) and Human Anti-Mouse IgG2a (#HCA310D550) (red).



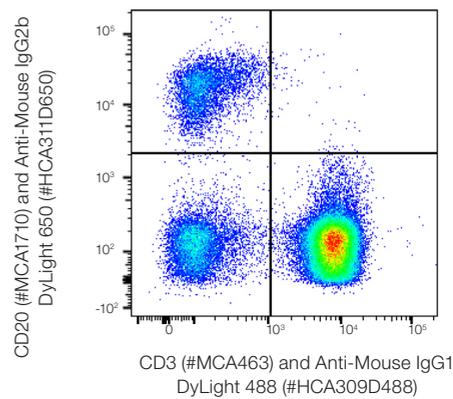
**Fig. 6. Fluorescent staining of human tonsil using isotype specific secondary antibodies.** FFPE human tonsil stained with Mouse Anti-Macrophages/Histiocytes (#MCA1478) and Human Anti-Mouse IgG2b (#HCA311D550) (red) and Mouse Anti-PCNA (#MCA1558) and Human Anti-Mouse IgG2a (#HCA310D650) (green).

### 3. Flow Cytometry

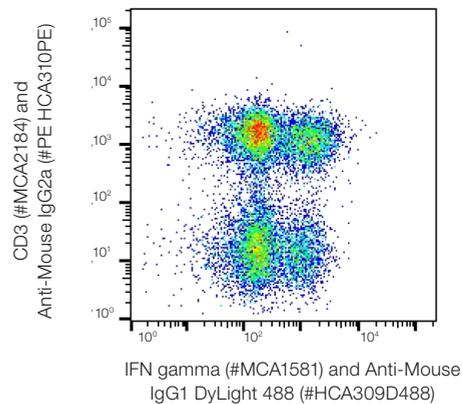
The use of secondary antibodies in flow cytometry is not recommended; it is preferable to use directly conjugated primary antibodies when multiplexing. However this may not be possible if:

- A conjugate is not available
- Or, if the antibody cannot be conjugated because:
  - It is impure or only partially pure, for example, tissue culture supernatant
  - It contains carrier protein
  - It is too dilute
  - It is insufficient in quantity

The images below show specificity of the secondary antibodies in both surface and intracellular staining.



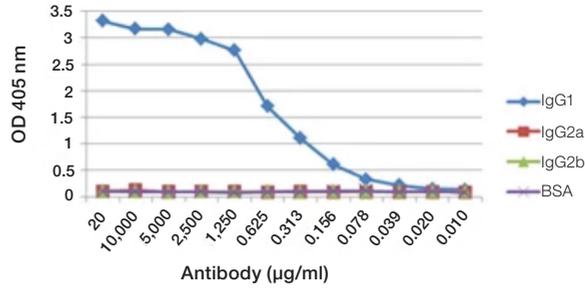
**Fig. 7. Surface staining using fluorescently conjugated secondary antibodies.** Mouse Anti-Human CD3 (#MCA463) labeled with Human Anti-Mouse DyLight 488 (#HCA309D488) specific for mouse primary IgG1 and Mouse Anti-Human CD20 (#MCA1710) labeled with HuCAL Anti-Mouse DyLight 650 (#HCA311D650) specific for mouse primary IgG2b. All experiments performed on red cell lysed human blood gated on lymphocytes in the presence of 10% human serum. Data acquired on the ZE5 Cell Analyzer.



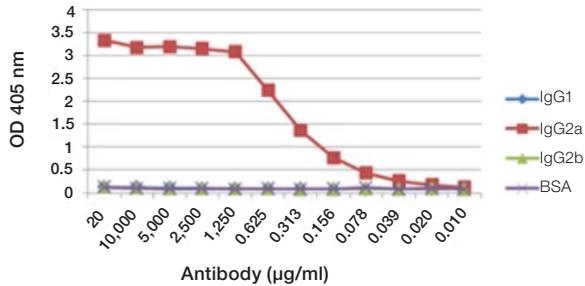
**Fig. 8. Intracellular staining using fluorescently conjugated secondary antibodies.** Mouse Anti-Human IFN Gamma (#MCA1581) labeled with Human Anti-Mouse DyLight 488 (#HCA309D488) specific for mouse primary antibody IgG1 and Mouse Anti-Human CD3 (#MCA2184) labeled with HuCAL Anti-Mouse PE (#HCA310PE) specific for mouse primary antibody IgG2a. All experiments performed on red cell lysed human blood, fixed and permeabilized with Leucoperm (#BUF09), gated on lymphocytes in the presence of 10% human serum. Data acquired on the ZE5 Cell Analyzer.

## 4. ELISA

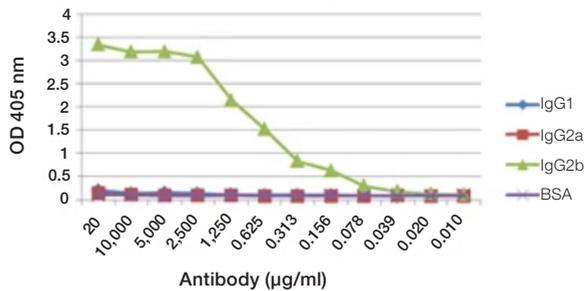
The isotype specific secondary antibodies are ideal for use in ELISA as illustrated in the images below, which clearly show the isotype specificity of all three antibodies.



**Fig. 9. ELISA detection of IgG1 using isotype specific secondary antibodies.** Mouse IgG1, IgG2a, IgG2b, and BSA were used to coat duplicated rows on an ELISA plate which was then probed with Human Anti-Mouse IgG1 AP, clone AbD24121, (#HCA309A) at doubling serial dilutions. #HCA309A only recognizes mouse IgG1, not IgG2a, or IgG2b.



**Fig. 10. ELISA detection of IgG2a using isotype specific secondary antibodies.** Mouse IgG1, IgG2a, IgG2b, and BSA were used to coat duplicated rows on an ELISA plate which was then probed with Human Anti-Mouse IgG2a AP, clone AbD24124, (#HCA310A) at doubling serial dilutions. #HCA310A only recognizes mouse IgG2a, not IgG1, or IgG2b.



**Fig. 11. ELISA detection of IgG2b using isotype specific secondary antibodies.** Mouse IgG1, IgG2a, IgG2b, and BSA were used to coat duplicated rows on an ELISA plate which was then probed with Human Anti-Mouse IgG2b AP, clone AbD24127, (#HCA311A) at doubling serial dilutions. #HCA311A only recognizes mouse IgG2b, not IgG1, or IgG2a.

## Isotype Specific Secondary Antibody Range

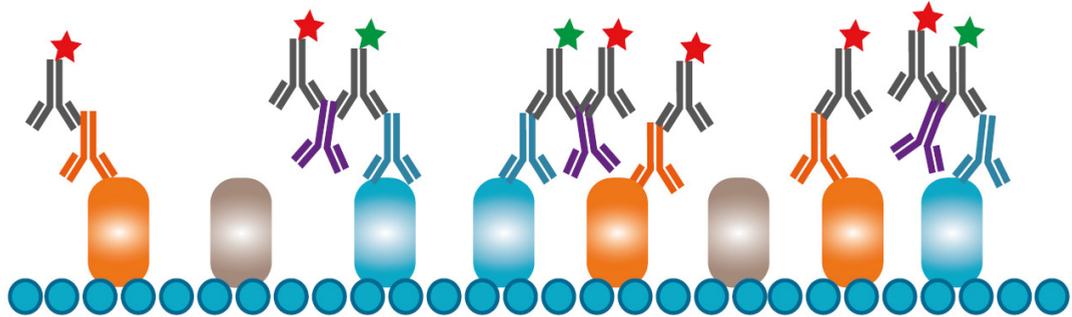
Human Anti-Mouse IgG1		Human Anti-Mouse IgG2a		Human Anti-Mouse IgG2b	
Format	Catalog #	Format	Catalog #	Format	Catalog #
DyLight 488	HCA309D488	DyLight 650	HCA310D650	DyLight 550	HCA311D550
DyLight 550	HCA309D550	PE	HCA310PE	AP	HCA311A
DyLight 650	HCA309D650	AP	HCA310A	HRP	HCA311P
PE	HCA309PE	HRP	HCA310P	DyLight 650	HCA311D650
HRP	HCA309P	DyLight 550	HCA310D550	PE	HCA311PE
Alkaline phosphatase	HCA309A				

## Notes

## 8

# Multiplexing

## Unconjugated Primary Antibodies Using Secondary Antibodies



### Overview

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 successfully achieved.

### Different Species

A combination of different host species is often the simplest way to multiplex. A primary antibody raised in mouse, together with a primary antibody raised in rat, plus a primary antibody raised in rabbit can be detected respectively 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

Another way to multiplex with primary antibodies from the same species is to use different isotypes of antibody such as IgG1, IgG2a, and IgG2b, in combination with isotype specific secondary antibodies.

Bio-Rad has generated recombinant isotype specific antibodies against the three main mouse isotypes: IgG1 (#HCA309), IgG2a (#HCA310), and IgG2b (#HCA311). These antibodies are capable of detecting individual isotypes without any species or isotype cross-reactivity, thereby allowing the use of multiple unlabeled mouse monoclonal antibodies at any one time, for multiplexing without species issues.

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

1. When mixing multiple secondary antibodies, they should be cross-adsorbed against unwanted targets to ensure specificity of your staining; for example, when using mouse and rat primary antibodies together, the anti-mouse secondary antibody should not cross-react with the rat primary antibody and vice versa. This also applies to class-specific and isotype specific secondary antibodies.
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.

Visit [bio-rad-antibodies.com/multiplexing](https://www.bio-rad-antibodies.com/multiplexing) to learn more.

## 9

# Secondary Antibodies at a Glance

The secondary reagents have been carefully selected to provide optimum quality and flexibility for your experimental design. There are many options to choose from:

- Monoclonal or polyclonal
- IgG molecules or F(ab')<sub>2</sub> fragments
- Many formats available
- Suitable for a wide range of applications such as flow cytometry, western blotting, and immunocytochemistry
- Cross-adsorbed and isotype specific antibodies
- Fluorescent StarBright Secondary Antibodies. Exceptionally bright and ideal for multiplex western blotting

**Table 1. Secondary antibody range options.**

Target	Host	Class and Chain	Conjugates
Human	Donkey	Ig	Purified
Mouse	Rabbit	IgA, IgA A1, IgA A2, IgA H, IgA secretory chain	Serum
Rat	Goat	IgD, IgD H, IgD D	S/N
Goat	Mouse	IgE	FITC
Rabbit	Sheep	IgG, IgG1, IgG2, IgG2a, IgG2b, IgG2c, IgG3	Tritc
Sheep	Rat	IgG4, IgG H/L, IgG Fc, IgG Fc CH2 domain, IgG	HRP
Bovine	Human	CH2 domain, IgG CH3 domain, IgG gamma, IgG	Biotin
Guinea Pig		Fab, IgG F(ab') <sub>2</sub>	Alkaline phosphatase
Cat		IgM, IgM Mu, IgM H	Texas Red
Chicken		IgG/A/M	StarBright Blue
Monkey		Lamba light chain	DyLight 405
Fish		Kappa light chain	DyLight 488
Dog		J chain	DyLight 550
Hamster			DyLight 650
Horse			DyLight 680
Pig			DyLight 800
			Bgal

## Notes

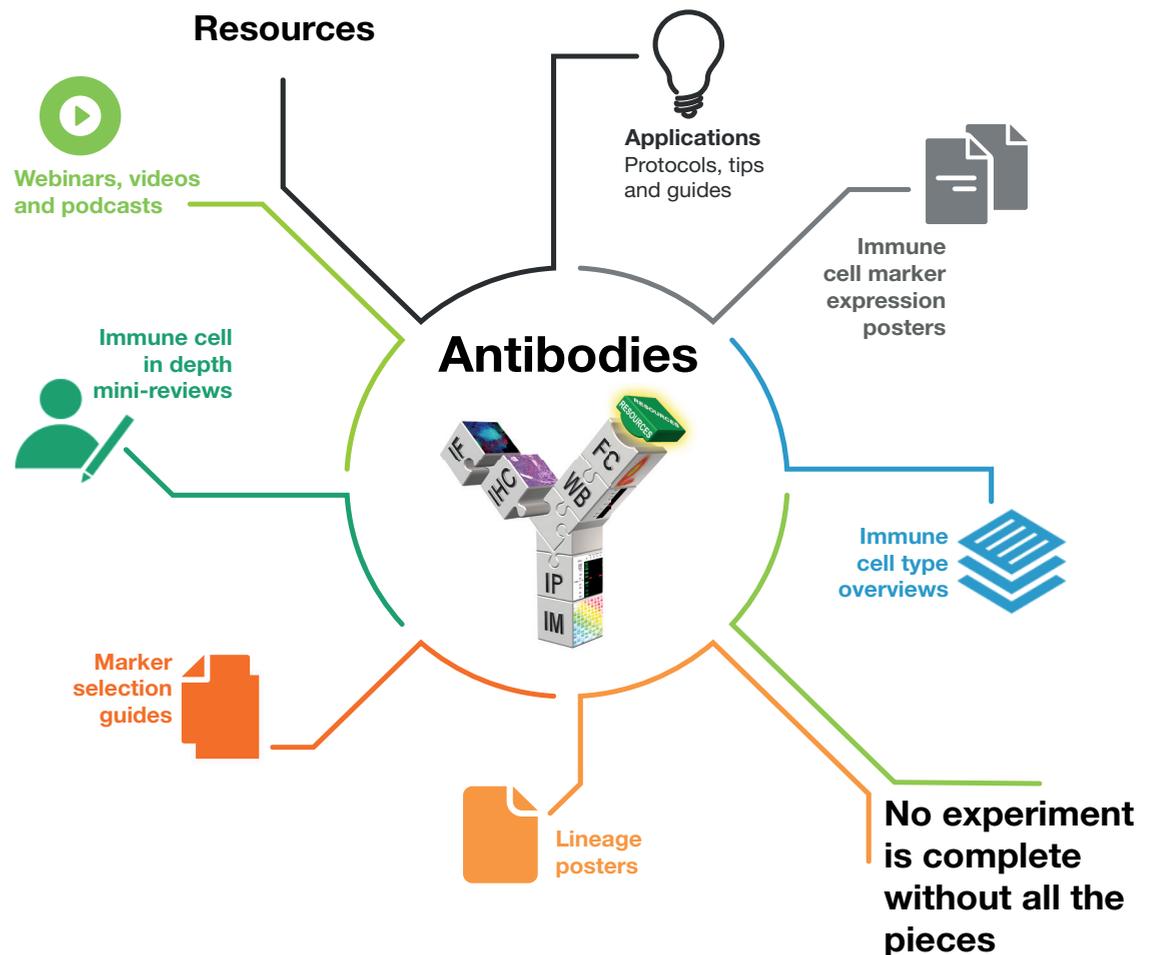
## 10

# Application Resources

To help you with your experiments, we have developed a range of useful application resources, including specific guides, protocols, tips, and webinars for:

- ELISA
- Flow cytometry
- Immunofluorescence
- Immunohistochemistry
- Immunoprecipitation
- Western blotting

Visit [bio-rad-antibodies.com/resources](https://www.bio-rad-antibodies.com/resources) for more information.



## Notes

# 11 Research Topic Resources

## Human and Mouse Posters, Guides, and Online Database

Knowing which markers to choose to identify certain cell types can often be a time consuming and confusing process. With this in mind we have developed a range of resources to help you make this choice.

- Cell marker database - interactive tools
- Biomarker expression posters
- Marker selection guides
- Marker lineage posters

### Interactive Human and Mouse Immune Cell Markers Database

Our interactive online tool is easy to use and can help you:

- Switch between human and mouse markers
- Look up immune-cell specific markers
- Choose a cell lineage
- Examine detailed marker data for over 400 markers
- Discover immune cell marker expression
- Find the antibody to your marker

Visit [bio-rad-antibodies.com/tool](https://bio-rad-antibodies.com/tool) to try the tool.



### Biomarker Expression Posters

The biomarker expression posters provide cellular expression profiles for over 400 top immune markers for human and mouse cells.

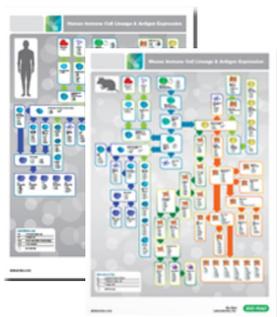
They are specifically designed to aid researchers in the quick and easy identification of more than 30 immune cell types.



## Marker Selection Guides

These guides simplify human and mouse marker selection by compiling everything in one place:

- Development of mature human and mouse immune cells from their precursors
- Over 400 signature biomarkers (both CD and non-CD) specific to each immune cell type
- Experimentally relevant information regarding each marker
- Antibodies available for each marker
- The booklets also include biomarker expression pattern grids enabling cell marker identification at a glance



## Marker Lineage Posters

These posters detail markers for each immune cell type to help you choose the right marker:

- Simplify marker selection
- Easy to follow genealogical design
- Color coded according to the exact cell types
- Include markers for myeloid cells, lymphocytes, and stem cells

With all this information at your fingertips, you decide how to use it. Download or request copies of these resources at: [bio-rad-antibodies.com/resources](https://www.bio-rad-antibodies.com/resources)

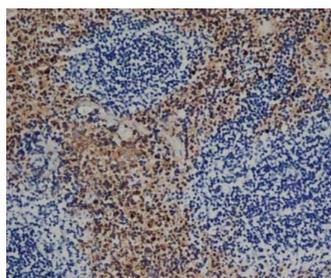
# 12 Protocols

Below you will find a selection of protocols for various applications using secondary antibodies. Visit [bio-rad-antibodies.com/protocols](https://www.bio-rad-antibodies.com/protocols) to see our full range.

## Immunohistochemistry

### Indirect Immunostaining of Frozen Tissue Sections (IHC1)

*For use with unconjugated monoclonal and polyclonal antibodies.*



This method provides a general procedure for use with the majority of Bio-Rad reagents. In some cases specific recommendations are provided on product datasheets, and these methods should always be used in conjunction with product and batch specific information provided with each vial. Please note that a certain level of technical skill and immunological knowledge is required for the successful design and implementation of these techniques - these are guidelines only and may need to be adjusted for particular applications.

#### Reagents:

0.3 ml hydrogen peroxide ( $H_2O_2$ ) 70% methanol in TBS

- 1 ml of 30%  $H_2O_2$  per 100 ml methanol/TBS

#### TBS stock solution (10x)

- Adjust pH to 7.4 using concentrated hydrochloric acid
- Distilled water, 1 L
- Sodium chloride, 87.66 g
- Tris base, 60.55 g

#### Method:

1. In most cases, Bio-Rad recommends that tissues are snap-frozen in liquid nitrogen, then prepared as 4-6  $\mu m$  sections using a cryostat.
2. Allow sections to air dry for at least 1 hr.
3. Fix sections in dry acetone for 15 min. Allow to evaporate for 10 min.
4. Block endogenous peroxidase (if necessary) by immersing slides in 0.3%  $H_2O_2$  in 70% methanol/TBS for 30 min. Bio-Rad offers Peroxide Blocking Reagent (#BUF017B). Wash once in TBS.

5. Incubate sections for 10 min in 10% normal serum from the same species in which the secondary antibody was raised. Tap excess serum off the slides before staining.
6. Incubate sections with primary antibody for at least 30 min at room temperature (RT) in a humid chamber, or overnight at 4°C. Wash 3 times (3x) in TBS.
7. Add enzyme-conjugated secondary antibody at the recommended dilution (see specific datasheet for details). Incubate for at least 30 min at RT. Wash 3x in TBS.
8. Incubate with the appropriate substrate solution for the recommended period of time (Bio-Rad suggests the use of DAB substrate with HRP-conjugated antibodies, and Fast Red/Naphthol AS-MX for alkaline phosphatase-conjugated antibodies). Wash once in water.
9. Counterstain with hematoxylin for 1-10 min. “Blue” with running water for 5 min. Then wash.
10. Mount in aqueous mounting medium, or alternatively dehydrate through a graded series of alcohols and xylene/solvent, and mount in synthetic mountant.

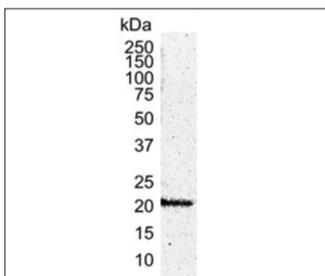
**Notes:**

- Do not allow slides to dry out after the fixation step, as drying will result in damage to the tissue structure
- Beware, certain substrates are soluble in alcohol – please refer to supplier information for details
- Appropriate controls should always be carried out. It may be useful to include a sample in which no primary antibody is used at all, in order to determine any nonspecific binding of the secondary reagent to the target tissue

Visit [bio-rad-antibodies.com/ihc-tips-tricks](https://www.bio-rad-antibodies.com/ihc-tips-tricks) for helpful tips when using a secondary antibody in IHC.

## Western Blotting

### Western Blot Protocol



This western blot protocol provides a general procedure for use with the majority of Bio-Rad reagents. In some cases specific recommendations are provided on product datasheets, and these methods should always be used in conjunction with product and batch specific information provided with each vial. Please note that a certain level of technical skill and immunological knowledge is required for the successful design and implementation of these techniques - these are guidelines only and may need to be adjusted for particular applications.

#### Reagents:

##### Blocking buffer

Block Ace #BUF029 dissolved in water, or 5% non-fat dried milk dissolved in PBS.

**\*Note:** For cleaner western blots, Block Ace is recommended over 5% non-fat dried milk dissolved in PBS. For the detection of phospho proteins, use the recommended blocking solution. Please check the datasheet for recommendations.

##### Wash buffer

Blocking buffer +0.1% Tween 20

##### Ponceau S

Acetic acid, 5 ml

Distilled water, 95 ml

Ponceau S (Sigma #P3504), 0.1 g

##### PBS

Disodium potassium phosphate, 1.15 g

Distilled water, 1 L

Potassium chloride, 0.2 g

Potassium dihydrogen phosphate, 0.2 g

Sodium chloride, 8.0 g

##### PBST

Disodium potassium phosphate, 1.15 g

Distilled water, 1 L

Potassium chloride, 0.2 g

Potassium dihydrogen phosphate, 0.2 g

Sodium chloride, 8.0 g

Tween 20, 1.0 ml

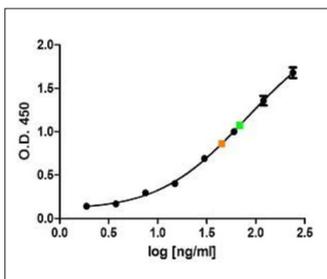
#### Method:

1. Following SDS-PAGE, transfer proteins onto blotting membrane according to the manufacturer's instructions.
2. Check protein transfer by staining the blot with Ponceau S for 1 min, then completely destain the blot by washing with distilled water.
3. Place blot into blocking solution for 2 hr at RT, or overnight at 4°C. Wash.

4. Rinse the blot briefly with wash buffer and then add a primary antibody diluted in the wash buffer (a concentration of 1-10  $\mu\text{g/ml}$  is generally acceptable, but check datasheets for precise recommendations). Incubate for 2 hr at RT, or overnight at 4°C.
5. Appropriate controls should always be carried out. It may be useful to include a sample in which no primary antibody is used at all, in order to determine any nonspecific binding of the secondary reagent to the target tissue.
6. Add appropriate enzyme-conjugated secondary antibody diluted in wash buffer and incubate for 1 hr at RT with gentle agitation.
7. Wash the membrane with gentle agitation as follows: 4x 5 min in wash buffer; 3x 5 min in PBST and 2x 5 min in PBS.
8. Add appropriate enzyme substrate solution and incubate as recommended by the manufacturer to visualize protein bands.

# ELISA

## Indirect ELISA Protocol



This method provides a general procedure for use with the majority of Bio-Rad reagents. In some cases specific recommendations are provided on product datasheets, and these methods should always be used in conjunction with product and batch specific information provided with each vial. Please note that a certain level of technical skill and immunological knowledge is required for the successful design and implementation of these techniques - these are guidelines only and may need to be adjusted for particular applications.

### Reagents:

#### Coating buffer

- Anhydrous  $\text{Na}_2\text{CO}_3$ , 1.5 g
- Anhydrous  $\text{Na}_2\text{CO}_3$ , 2.93 g
- Distilled water, 1 L, pH to 9.6

For an alternative coating buffer use ELISA coating buffer (#BUF030)

#### Blocking buffer

Phosphate Buffered Saline (PBS) containing 1% w/v BSA

For an alternative blocking buffer, use either ELISA BSA Block (#BUF032), ELISA Ultrablock (#BUF033), or ELISA Synblock (#BUF034)

#### Wash buffer

PBS containing 0.05% v/v Tween-20

For an alternative wash buffer, use ELISA Wash Buffer (#BUF031)

### Recommended Substrates and Stop Solutions

TMB Core+ (#BUF062), for use with HRP-conjugated antibodies. Stop with 0.2 M Sulfuric Acid pNPP (#BUF044), for use with alkaline phosphatase-conjugated antibodies. Stop with 1 M sodium hydroxide

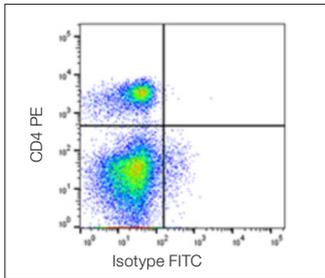
### Method:

1. Coat microtiter plate wells with 100  $\mu\text{l}$  of the antigen solution, at a concentration of between 1-10  $\mu\text{g}/\text{ml}$  in coating buffer. Cover the plate and incubate overnight at 4°C. Wash the plate 3x in wash buffer.
2. Add 150  $\mu\text{l}$  of blocking solution to each well. Incubate for 1 hr at 37°C. Wash 4x in wash buffer.
3. Add 100  $\mu\text{l}$  of unconjugated detection antibody (appropriately diluted in wash buffer) to each well. Incubate for 1 hr at 37°C. Wash 3x in wash buffer.
4. Add 100  $\mu\text{l}$  enzyme-conjugated secondary antibody (appropriately diluted in wash buffer) to each well. Incubate for 1 hr at 37°C. Wash 3x in wash buffer.
5. Add 100  $\mu\text{l}$  of the appropriate substrate solution to each well. Incubate at RT (and in the dark if required) for 30 min, or until desired color change is attained.
6. Read absorbance values immediately at the appropriate wavelength or add 50  $\mu\text{l}$  of "stop solution". Gently tap plate to ensure thorough mixing. Measure absorbance within 30 min.

Visit [bio-rad-antibodies.com/elisa-hints](https://www.bio-rad-antibodies.com/elisa-hints) for tips when using a secondary antibody in ELISA.

## Flow Cytometry

### Indirect Immunofluorescence Staining of Surface Epitopes of Cells and Blood (FC5)



This method provides a general procedure for use with the majority of Bio-Rad's reagents. In some cases, specific recommendations are provided on product datasheets, these methods should always be used in conjunction with the product and batch specific information provided with each vial. A certain level of technical skill and immunological knowledge is required for the successful design and implementation of these techniques; these are guidelines only and may need to be adjusted for particular applications. Specific methodology for blood appears in [ ] brackets.

This technique is applicable when using unconjugated or biotin-conjugated monoclonal and polyclonal antibodies recognizing cell surface antigens. A conjugated secondary reagent must be used to visualize the primary antibody, for example streptavidin in the case of biotin.

#### Reagents:

- Anticoagulant (Note: for basic staining any appropriate anticoagulant, such as heparin, EDTA, or acid citrate dextrose, may be used. In some instances specific anticoagulants may be required)
- Erythrolyse Red Blood Cell Lysing Buffer (#BUF04)
- Phosphate Buffered Saline (PBS) (#BUF036A) containing 1% Bovine Serum Albumin
- PBS
- Optional: 0.5% (w/v) paraformaldehyde in PBS (Note: dissolve on heated stirrer and cool before use)

#### Method:

1. Prepare cells appropriately; refer to protocol 'Preparation of cells for flow cytometry' available at [bio-rad-antibodies.com/fc-preparation](https://www.bio-rad-antibodies.com/fc-preparation) for further information. Adjust the cell suspension to a concentration of  $1 \times 10^7$  cells/ml with cold (4°C) PBS/BSA buffer. [Whole blood samples may be used undiluted unless the cell count is high, for example with leukemia samples. Use appropriate anticoagulant].
2. Aliquot 100  $\mu$ l of the cell suspension [or whole blood] into as many test tubes as required.
3. Add primary antibody at the vendor recommended dilution. Mix well and incubate at 4°C for at least 30 min.
4. Wash cells with 2 ml of cold (4°C) PBS/BSA, centrifuge at 300-400 g for 5 min and discard the supernatant.  
[To the blood suspension, add 2 ml freshly prepared red cell lysis buffer and mix well. Incubate for 10 min at RT. Centrifuge at 300-400 g for 5 min and discard the supernatant. Wash with 2 ml of PBS/BSA, centrifuge at 300-400 g for 5 min and discard the supernatant].
5. Add an appropriate secondary reagent at the vendor recommended dilution. Mix well and incubate at 4°C for at least 30 min, avoiding direct light.

6. Centrifuge at 300-400 g for 5 min at RT and discard the supernatant.
7. Re-suspend cells in 200  $\mu$ l of cold (4°C) PBS or with 200  $\mu$ l of 0.5% paraformaldehyde in PBS if required.
8. Acquire data by flow cytometry. Analyze fixed cells within 24 hr.

**Notes:**

- To avoid nonspecific binding, you also need to block Fc receptors on cell types such as spleen cells, with FcR blocking reagents for example, Bio-Rad's Mouse Seroblock Reagent (#BUF041)
- Appropriate controls should always be carried out, for flow cytometry the following should be considered for inclusion:
  - Isotype controls used to determine if the staining is specific
  - Unstained cells should always be included in the experimental set-up to monitor autofluorescence
- For all multicolor flow cytometry experiments it is advisable to include compensation controls and Fluorescence Minus One (FMO) controls, which assist with identifying gating boundaries

Visit [bio-rad-antibodies.com/flow](https://www.bio-rad-antibodies.com/flow) for tips, and further protocols for all your flow cytometry needs.

## Notes

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## Why Buy From Bio-Rad?

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