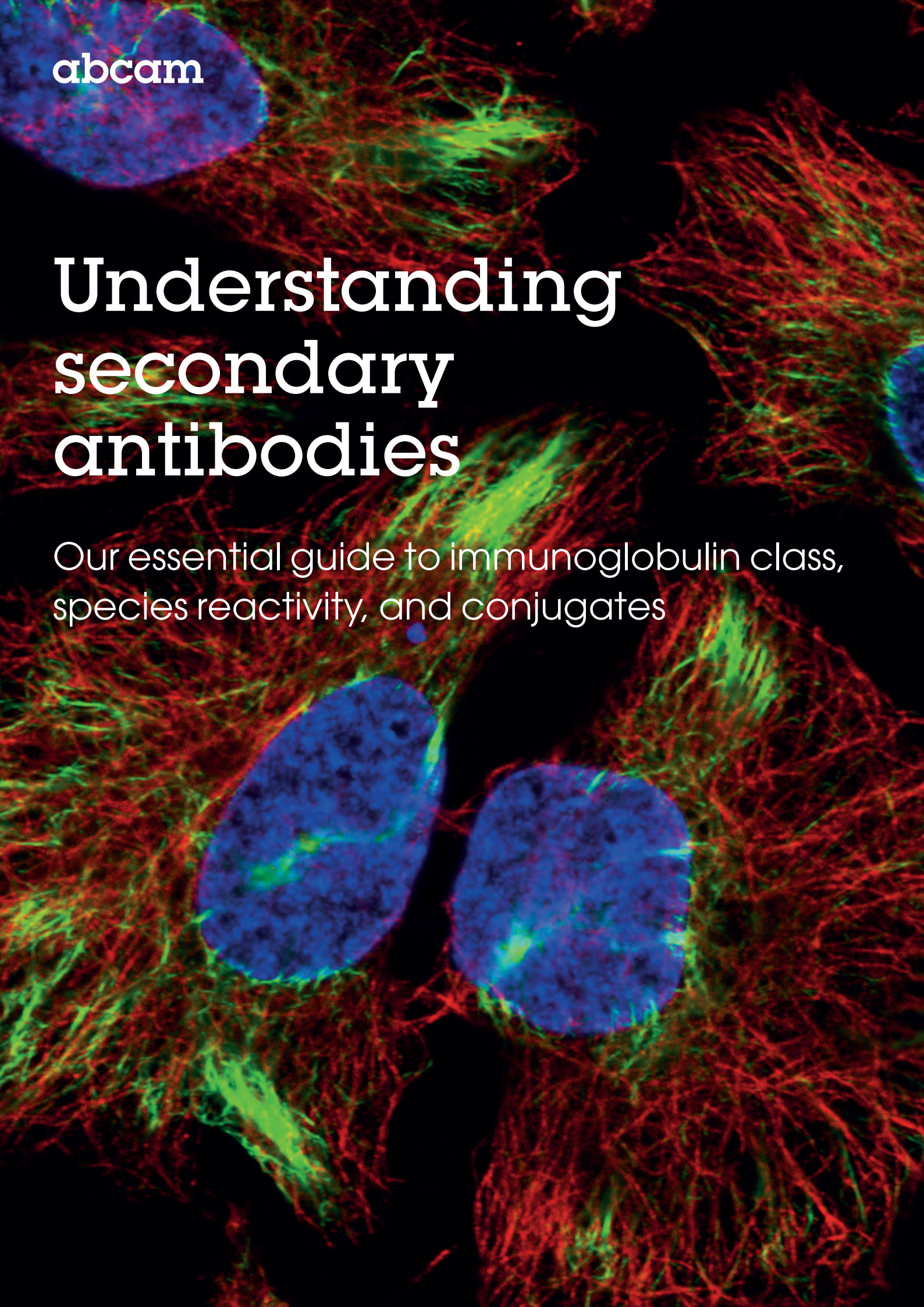


Understanding secondary antibodies

Our essential guide to immunoglobulin class, species reactivity, and conjugates

Introduction to secondary antibodies

What are secondary antibodies?

Secondary antibodies are raised to bind to primary antibodies to allow detection, sorting, and purification of target antigens. They allow the detection of your protein of interest due to their specificity for the primary antibody species and isotype. Different dyes or enzyme conjugates bound to the secondary antibody can then be chosen based on your needs for your specific application.

What determines secondary antibody performance?

- Specificity, which refers to the degree with which the antibody detects its target antigen and structurally dissimilar antigens.
- Sensitivity, which refers to the amount of antigen that the antibody is able to detect. Sensitivity not only depends on the binding affinity of the secondary antibody but also the label and degree of labeling of the antibody.
- Consistency, which refers to the ability of the antibody to perform with little variability when using different batches. Consistency will be mainly influenced by the source of the antibody and its clonality.

Antibody structure

Antibodies exist as one or more copies of a Y-shaped unit, composed of four polypeptide chains (Figure 1). Each Y contains two identical copies of a heavy chain (H), and two identical copies of a light chain (L), which are different in their sequence and length. The top of the Y shape contains the variable domain (V) also known as the fragment, antigen-binding (F(ab)) region. This region binds tightly and specifically to an epitope on a given antigen. The base of the antibody consists of constant domains (C) and forms what is known as the fragment, crystallizable region (Fc). This region is important for the function of the antibody during an immune response.

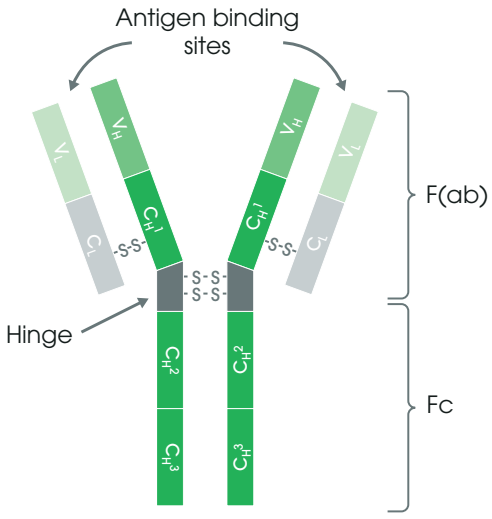


Figure 1: antibody structure. The Y shaped antibody is joined in the middle by a flexible hinge region. Antigen binding occurs at the variable domain (V) consisting of immunoglobulin heavy (H) and light chains (L). The base of the antibody consists of constant domains (C).

Choosing a secondary antibody

When you are choosing a secondary antibody for your experiment, here are some things you should take into consideration.

Host species

The secondary antibody binds specifically to the IgG of the host species used to generate the primary antibody. For example, if you use a primary antibody raised in rabbit, you will need an anti-rabbit secondary antibody raised in a host species other than rabbit (eg donkey anti-rabbit secondary).

Choosing a conjugate

Secondary antibodies tend to come in conjugated forms. For applications such as ELISA or western blotting, enzyme-linked secondary antibodies are the most popular. For flow cytometry or immunofluorescence, secondary antibodies will be conjugated to fluorescent proteins or dyes such as Alexa Fluor®. See the conjugate selection diagram below (Figure 2) for help with choosing the right conjugate for your experiment.

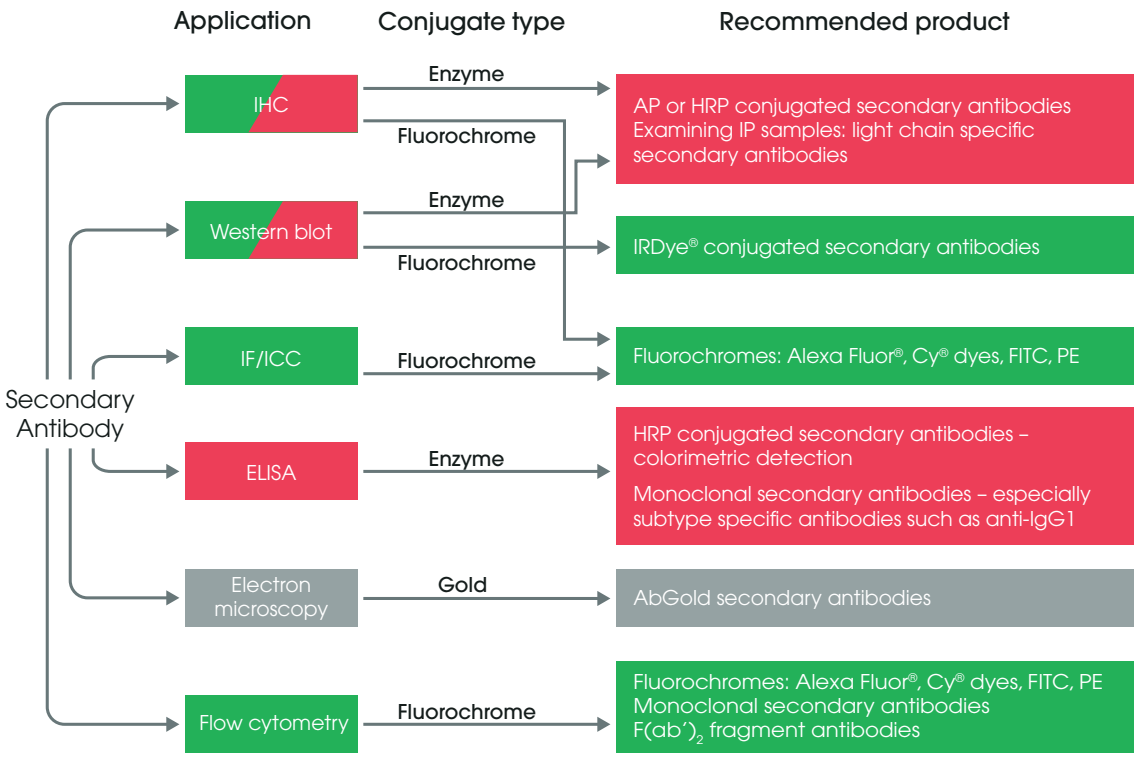


Figure 2: choosing a conjugate. Based on the application you are using (left panels) you can choose enzymatic, fluorescent, or gold-conjugated secondary antibodies. Suggested secondary antibodies are found in the panels on the right.

Enzymatic detection methods

Enzymatic detection methods using horseradish peroxidase (HRP) or alkaline phosphatase (AP) conjugated secondary antibodies allow colorimetric and chemiluminescent detection of primary antibodies in a wide range of applications, such as immunohistochemistry (IHC), ELISA, southern blot, and western blot. The advantages and disadvantages of using HRP or AP in different applications are outlined in the table below (Table 1).

Enzyme	Substrates	Applications	Advantages	Disadvantages
Horseradish Peroxidase (HRP)	Chromogenic, soluble (TMB, ABTS, OPD...)	ELISA	Easy to use	Light sensitive coloration
	Chromogenic, precipitating (CN, AEC, DAB...)	WB, SB, IHC	Easy to use	Background in blood samples and some other tissues. Staining stability lower than AP
	Fluorogenic (ADHP/ resotufin)	ELISA	High sensitivity	Need fluorescence equipment
	Luminol	WB, SB, IHC	High sensitivity	Need radiographic equipment or light scanner
Alkaline Phosphatase (AP)	Chromogenic, soluble (pNNP)	ELISA	Linear kinetic. Often more sensitive than HRP	Unstable
	Chromogenic, precipitating (BCIP/NBT...)	WB, SB, IHC	Staining stability higher than HRP	Interference with nuclear counterstain
	Fluorogenic (4-MUP)	ELISA, IHC	Sensitivity	Need fluorescence equipment

Table 1: Enzymatic detection methods. HRP and AP can be visualized using different substrates as listed here. This table shows the advantages and disadvantages of using HRP or AP conjugated secondary antibodies with these different substrates for a range of applications.

Fluorescent detection methods

The use of fluorescent conjugated secondary antibodies allows detection of primary antibodies in a wide range of applications, such as cell imaging, flow cytometry, and western blot.

Alexa Fluor® dyes

Secondary antibodies conjugated to Alexa Fluor® dyes offer the brightest, most stable staining for your cells. See below (Table 2) to see the available Alexa Fluor® conjugated secondary antibodies and their characteristics.

Alexa Fluor@	Color in vial*	Absorption Max (nm)	Emission Max (nm)	Emission color*	Matching dyes
405	Colorless	402	421	Blue	Cascade Blue
488	Yellow	495	519	Green	Cy2, TRITC (fluoresceine)
555	Pink	555	565	Orange	Cy3, TRITC (Rhodamine)
568	Violet	578	603	Orange/Red	Rhodamine Red
594	Purple	590	617	Red	Texas Red
647	Blue	650	668	Far Red	APC,Cy5
680	Blue	679	702	Near-IR***	Cy5.5, IR680
750	Cyan	749	775	Near-IR***	CY7
790	Cyan	784	814	Near-IR***	IR800

Table 2: Alexa Fluor® conjugates. This shows the range of Alexa Fluor® dyes available as secondary antibody conjugates. * Appearance of the solution in the vial. ** Typical emission color seen through a conventional microscope with appropriate filters. *** It is not possible to view the light emitted by near-IF fluorescent dyes since human vision is insensitive to light beyond 650 nm approximately

Secondary antibodies conjugated to fluorophores such as Alexa Fluor® are commonly used for immunostaining and flow cytometry experiments, including double or even triple stainings. These experiments will use a combination of secondary antibodies and this will require careful planning.

Choosing a set of compatible fluorescent secondary antibodies for your imaging experiment will reduce bleed-through (also known as crossover or crosstalk) between channels and prevent inappropriate excitation of the fluorophores you are using. Use fluorophores with narrow emission spectra to avoid bleed-through. See the table below for help with choosing compatible secondary antibodies for your multiplex experiments (Table 3).

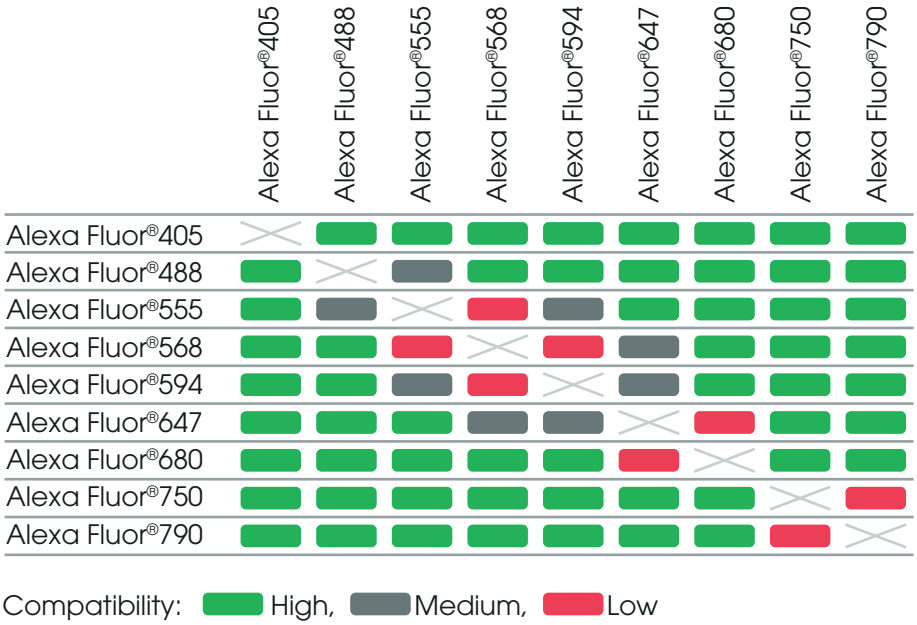


Figure 3: Alexa Fluor® compatibility. For optimal results use Alexa Fluor® dyes with high compatibility (green). If this is not possible use those with medium compatibility (orange). Using Alexa Fluor® combinations with low compatibility (red) is not recommended and can give false positive results.

Important note if using any antibody conjugated to Alexa Fluor® 405:
We recommend the use of Alexa Fluor® 405 with a higher expressing protein for best results, but also the use of a dedicated 405 filter since the DAPI filter may not excite the higher peak of Alexa Fluor® 405.

IRDye®-conjugated secondary antibodies

Secondary antibodies conjugated with IRDye® are used for fluorescent western blot. The advantages of infrared fluorescent western blot are

1. The ability to detect more than one protein in the same blot at the same time.

2. IRDye® signal is directly proportional to the amount of target protein, while in chemiluminescence enzyme/substrate kinetics may affect performance.

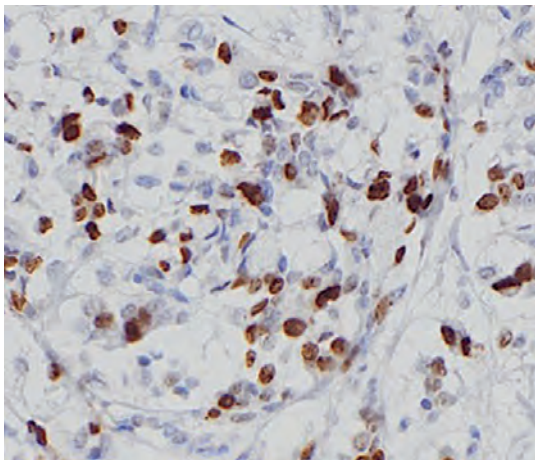
3. IRDye® fluorescent signal is highly stable, so you can store blots and re-image later.

Gold conjugated secondary antibodies (AbGold)

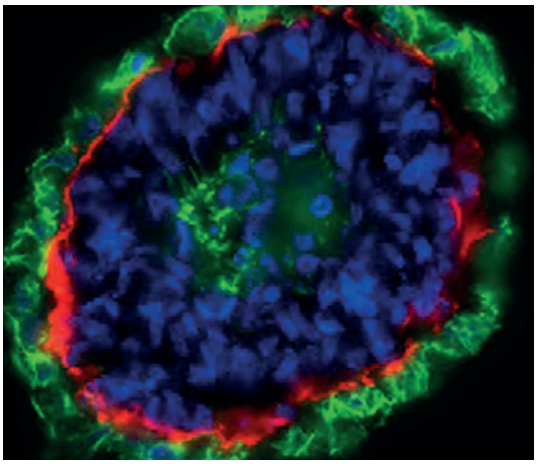
Secondary antibodies can be conjugated to gold nanoparticles for use in applications such as electron microscopy. AbGold antibodies are available with gold nanoparticle conjugates varying in size from 1.4–40 nm. The gold nanoparticle size will differ based on the requirement of your application. These are also available in anti-mouse, rabbit and goat variants.

Secondary antibody output images

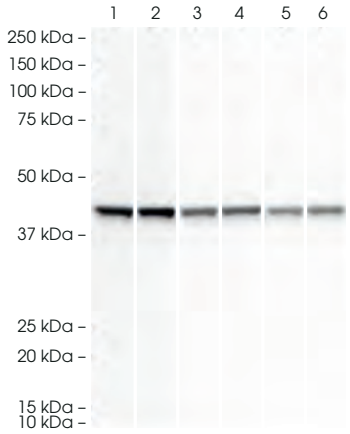
Below are a few examples of end results using the secondary antibodies described in this guide. We've included fluorescent and enzymatic secondary conjugates for both IHC and western blot.



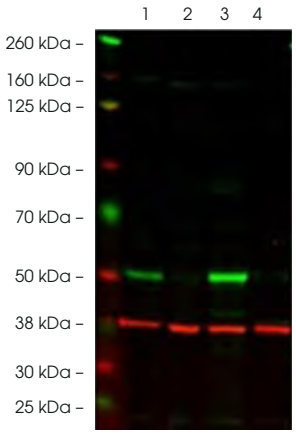
Immunohistochemical analysis of triple positive breast cancer tissue stained with Ki-67 and detected with anti-mouse IgG HRP-polymer secondary (ab214879).



Immunohistochemical analysis of embryoid bodies (EBs) stained with cytokeratin 8 and detected with anti-rabbit IgG Alexa Fluor® 488 pre-adsorbed secondary (ab150081).



Western blot analysis of HeLa whole cell lysate stained with beta actin and detected with anti-rabbit IgG HRP secondary (ab97051).



Western blot analysis of Hap1 whole cell lysate stained with beta actin (red) detected with anti-rabbit IgG IRDye® 680RD (ab216777) and p53 (green) detected with anti-mouse IgG IRDye® 800CW preabsorbed (ab216772).

Class/subclass of antibody

The secondary antibody should be directed against the isotype of the primary antibody. Polyclonal primary antibodies are mostly raised in rabbit, goat, sheep or donkey and are generally IgG isotypes. The secondary antibody will typically be an anti-IgG H&L (heavy & light chains) antibody.

Monoclonal primary antibodies are commonly raised in mouse, rabbit, and rat. If the primary monoclonal antibody is a mouse IgG1, you will need an anti-mouse IgG or a less specific F(ab) fragment anti-mouse IgG secondary antibody.

It is worth noting that although IgG is the most common isotype used for primary and secondary antibodies, occasionally other isotypes are used. See below for details of the different antibody isotypes (Figure 4).

Human immunoglobulin isotypes, subclasses, types, and subtypes

- Isotypes or classes: IgG (γ heavy chains), IgM (μ), IgA (α), IgE (ε), IgD (δ)
- Subclasses: IgG1 (γ1 heavy chains), IgG2 (γ2), IgG3 (γ3), IgG4 (γ4); IgA1 (α1), IgA2 (α2)
- Types: κ light chain, λ light chain
- Subtypes: λ1, λ2, λ3, λ4

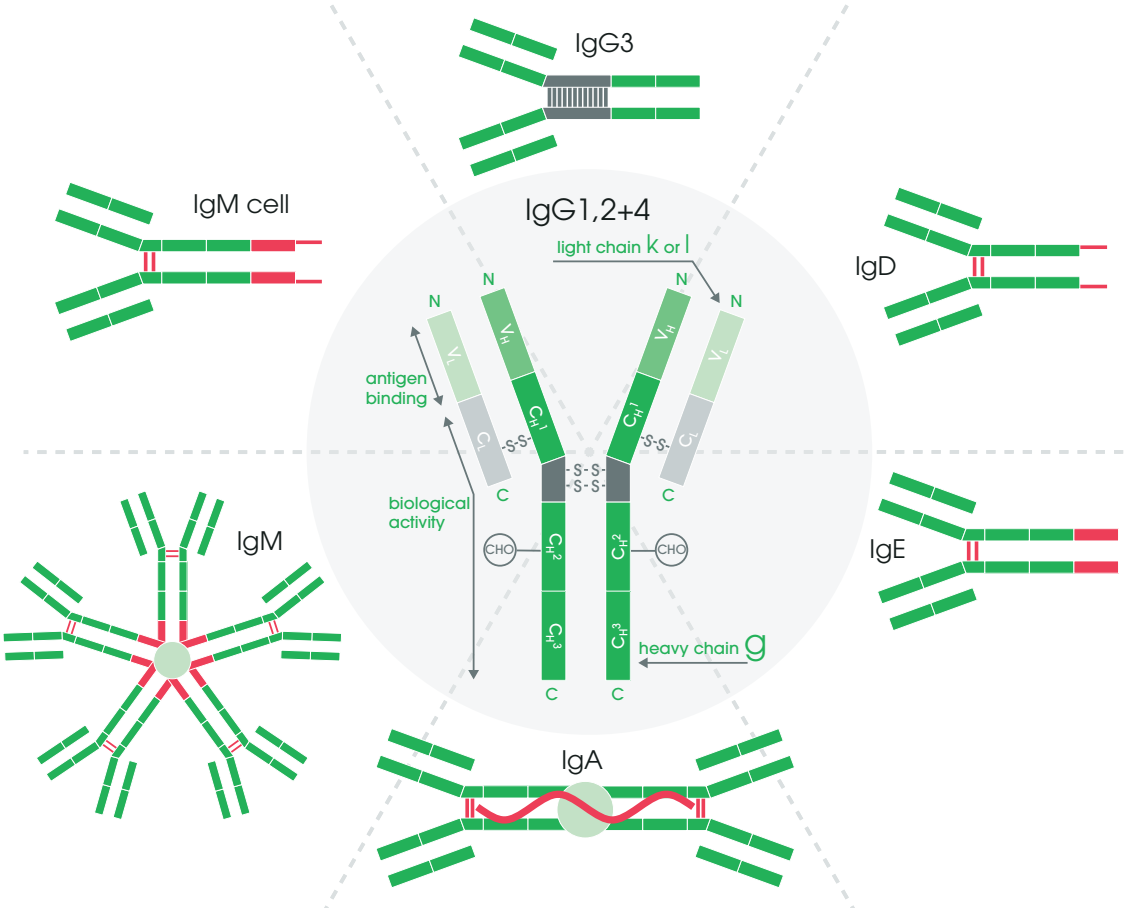


Figure 4: antibody isotypes. The five main antibody isotypes – IgG, IgD, IgE, IgA, and IgM. Each has a unique structure as depicted here. They vary based on the number of Y units and the types of heavy chain present. They will also differ in their biological properties, functional locations and ability to deal with different antigens.

Pre-adsorbed secondary antibodies

Pre-adsorption (or cross-adsorption) is an extra purification step to increase the specificity of an antibody. The solution containing secondary antibodies is passed through a column matrix containing immobilized serum proteins from potentially cross-reactive species. Non-specific secondary antibodies are retained in the column, while highly specific secondary antibodies flow through.

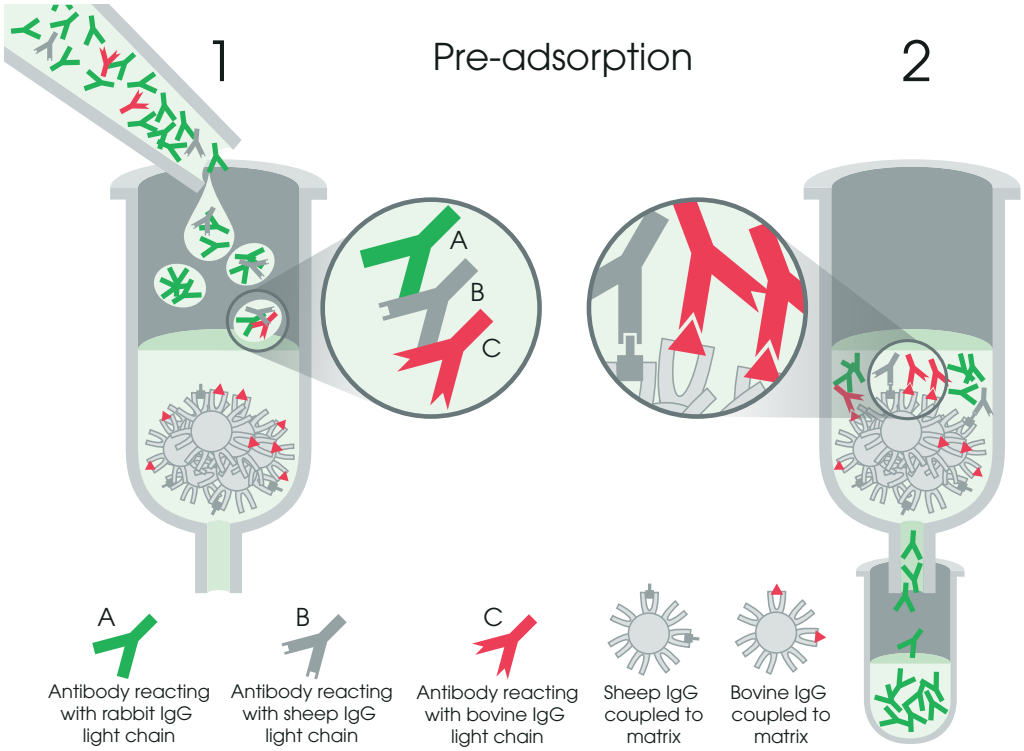


Figure 5: antibody pre-adsorption. A solution of secondary antibodies recognizing rabbit IgG light chains is passed through a matrix containing immobilized serum proteins from potentially cross-reactive species such as sheep and bovine IgGs (1). Only antibodies highly specific to rabbit IgG light chain will flow through the column, while secondary antibodies cross-reacting with sheep or bovine IgG light chains will remain bound to the proteins in the column (2). The result of the procedure is a secondary antibody that recognizes rabbit IgG light chains with high specificity.

When to use pre-adsorbed secondary antibodies

Pre-adsorbed secondary antibodies are recommended to reduce non-specific background in immunostainings. If you are carrying out an IHC experiment on samples with abundant amounts of endogenous immunoglobulins (Igs), using secondary antibodies pre-adsorbed against the same species the sample originated from reduces the risk of cross-reactivity between the secondary antibody and endogenous Igs.

F(ab) or (Fab')2 fragment secondary antibodies

F(ab) antibodies are generated from whole IgG antibodies (see Figure 1). The IgG antibody will undergo papain digestion to give rise to individual F(ab) regions. F(ab')2 fragment antibodies are generated by pepsin digestion of whole IgG antibodies. This removes most of the Fc region while leaving intact some of the hinge region and two antigen-binding F(ab) regions linked together by disulfide bonds.

F(ab) and (Fab')2 fragment antibodies eliminate non-specific binding between Fc regions of antibodies and Fc receptors on cells (such as macrophages, dendritic cells, neutrophils, NK cells, and B cells). These fragments also penetrate tissues more efficiently due to their smaller size.

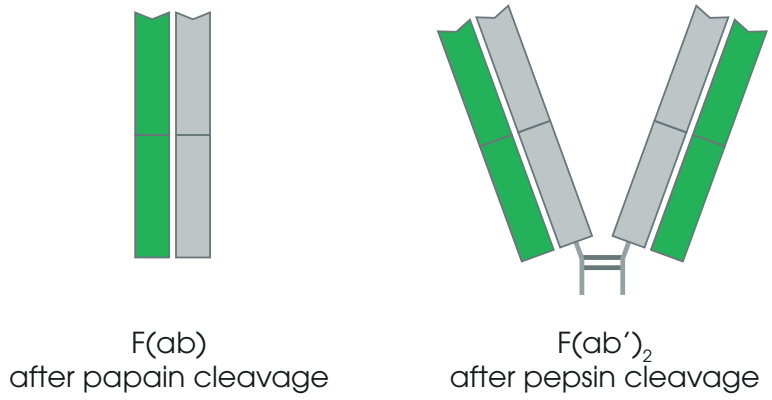


Figure 6. F(ab) and F(ab')2 antibodies. Whole IgG antibodies (see figure 1) undergo papain cleavage to generate F(ab) antibodies (left) and pepsin cleavage to form F(ab')2 antibodies (right).

When to use F(ab) or (Fab')2 fragment secondary antibodies

F(ab) fragment antibodies are used to block endogenous immunoglobulins on cells, tissues, and exposed immunoglobulins. This can be a powerful tool for use in multiple labeling experiments using primary antibodies from the same species. After the normal serum blocking step in your IHC/ICC protocol, incubate your sample with F(ab) fragments in excess to block endogenous immunoglobulins.

F(ab')2 are smaller than whole IgG molecules and enable a better penetration into tissue thus allowing better antigen recognition and increased IHC signals. F(ab')2 antibodies are mainly used in double labeling experiments or when the sample is a tissue/cells containing high levels of endogenous Fc receptors (eg thymus and spleen). Since IgG F(ab')2 fragment antibodies react only with light chains, these antibodies do not exclusively bind to IgG. Other immunoglobulins sharing the same light chain as IgG are also recognized, which can be advantageous if your primary antibody is not an IgG isotype.

Secondary selection checklist

Choose secondary antibodies raised in a different species

1) Do you know which host species your primary antibody was raised in? Choose a secondary antibody raised against the host species used to generate the primary antibody.

2) Do you know which conjugate best suits your application? See figure 2 for help with choosing a conjugate.

3) Are you using enzymatic detection methods? Choose between HRP and AP using the information in table 1.

4) Are you using fluorescent detection methods? You can pick a fluorescent conjugate from a range of emission colors. See our available Alexa Fluor dyes® in table 2.

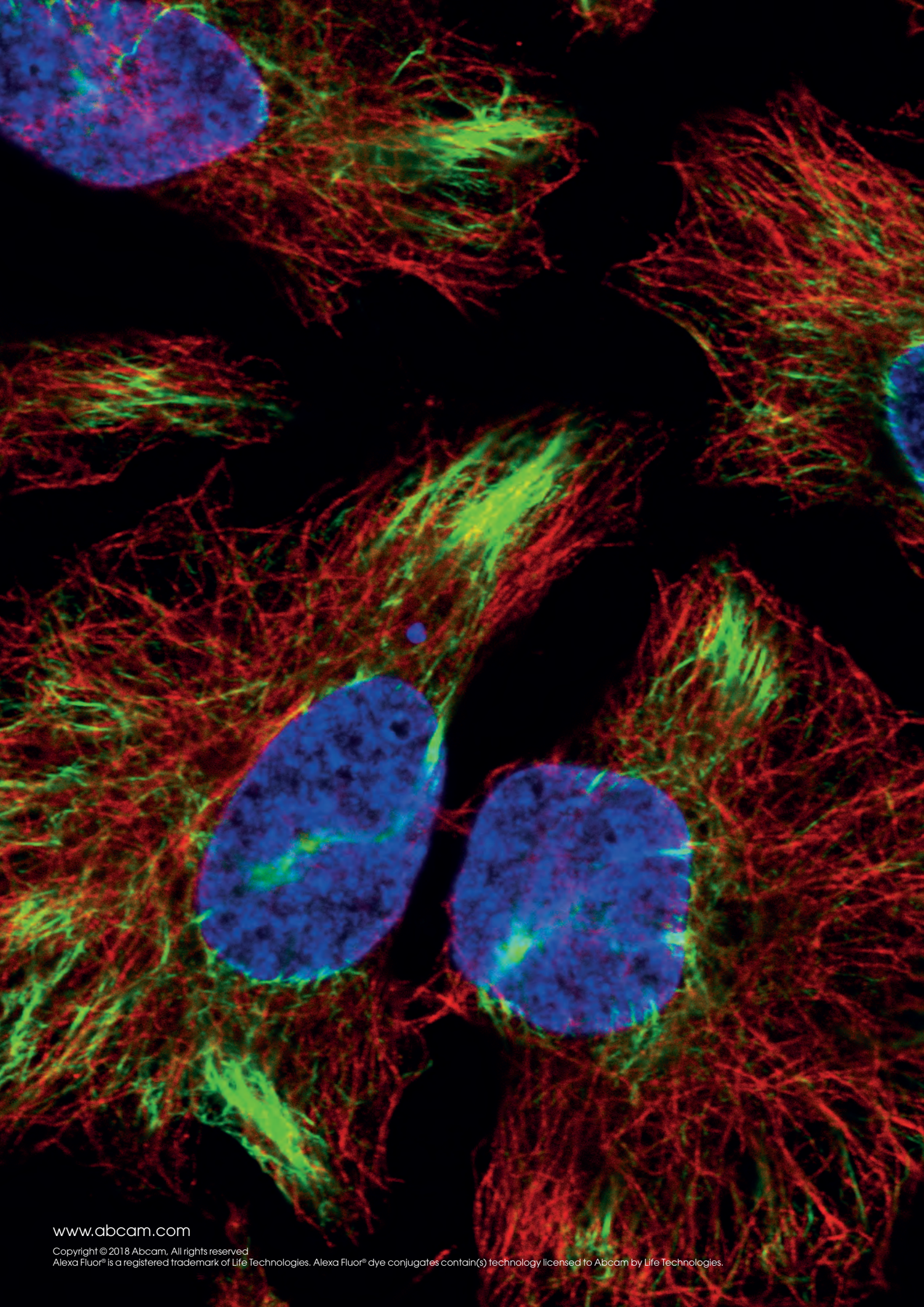
5) Are you carrying out a multiplex experiment? Make sure you minimize crosstalk in your experiments using our compatibility table in figure 3.

6) Do you want to carry out fluorescent western blot? See the advantages of using IRDyes® on page 7.

7) Do you know the class or isotype of your antibody? Learn more about this from figure 4.

8) Would your experiments benefit from pre-adsorbed secondary antibodies? Find out more on page 10.

9) Could you benefit from better secondary antibody penetration and increased IHC signals? Perhaps you could try F(ab) and (Fab′)2 fragment secondary antibodies. See page 11 for details.



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