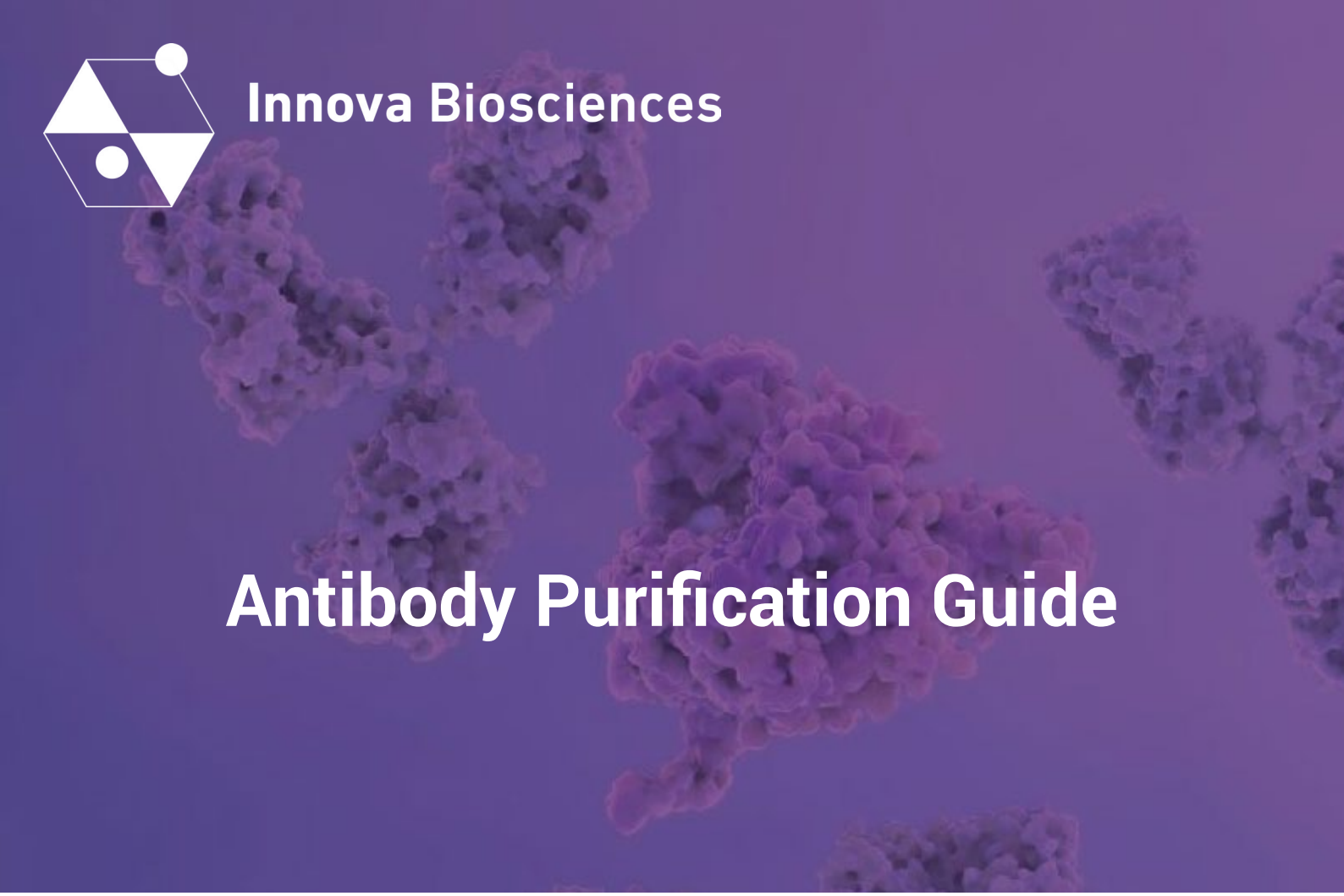
Innova Biosciences



Antibody Purification Guide

Innova Biosciences Guide

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Innova Biosciences specializes in easy to use bioconjugation kits which enable the direct labeling of antibodies or proteins with enzymes, fluorescent labels, biotin, streptavidin, gold nanoparticles, latex beads or oligonucleotides. Unfortunately, many antibodies are provided in buffers which contain additives that are incompatible with labeling technologies, making purification a key consideration prior to carrying out any conjugation reaction. With this in mind, we have developed a range of purification kits that complement our labeling technologies. The aim of this guide is to provide an overview of these products.

Antibody Structure

Antibodies, or immunoglobulins, are host proteins which are produced in response to the presence of foreign molecules in the body. They are found on the surface of B lymphocytes, in extravascular fluids, and in exocrine secretions such as saliva and tears. Antibodies were first referenced in the literature well over a century ago, and they have since become a hugely powerful tool, used extensively in a wide variety of settings.
All antibodies share a common structure. They are made up of one or more units, with each unit consisting of two identical heavy polypeptide chains and two identical light chains. A disulfide bond joins a heavy chain to a light chain, and disulfide bonds are also found at the flexible hinge region of the heavy chains to join these to one another. The hinge region is accessible to enzymatic cleavage. Each globular region of the antibody which is formed as a result of protein folding is referred to as a domain. Light chains have a single variable domain and a single constant domain, while heavy chains have a single variable domain and three constant domains. Broadly speaking, the variable region of the antibody confers specificity, while the constant region determines which immune mechanism will be used to destroy the antigen. The basic structure of an antibody is illustrated in figure 1.

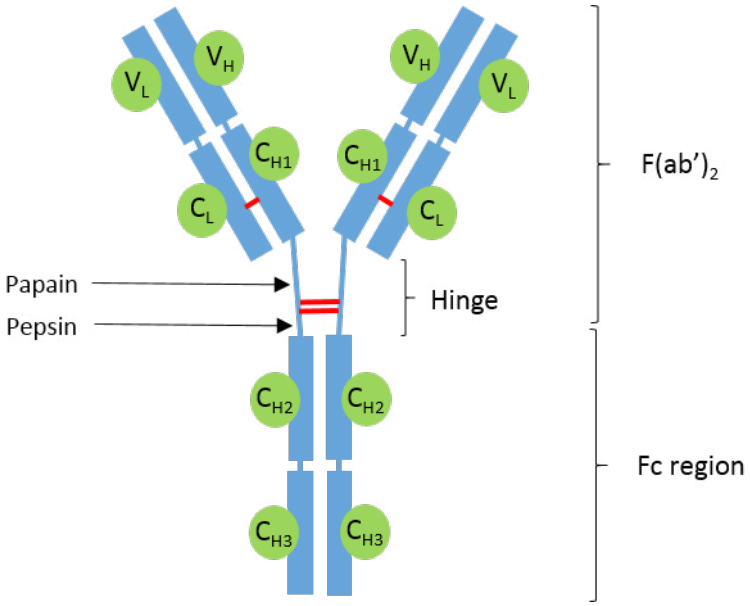

Figure 1. Basic structure of an antibody. H – heavy chain, L – light chain, V - variable region, C- constant region. Disulfide bonds are shown in red.

Immunodetection Techniques

The term immunodetection describes the process of using antibodies to detect specific antigens in a sample, and is applicable to an extensive range of techniques. During a typical immunodetection process the sample is first blocked with an agent such as milk, Bovine Serum Albumin (BSA) or serum to reduce non-specific binding, before being incubated with the primary antibody, which binds specifically to the target antigen. Following incubation, any unbound antibody is removed by washing, and a labeled secondary antibody is then used for detection.

The detection method which is used depends largely on the sample type, and user preference.

Colorimetric detection relies on the generation of a colored product, which is usually formed following the conversion of a chromogenic substrate by an appropriate enzyme. This method requires the use of an antibody that has been labeled with an enzyme such as HRP, Alkaline Phosphatase or Glucose Oxidase, and paired with an appropriate substrate.

Fluorometric detection utilizes an antibody which has been labeled with a fluorophore. A light source is used to excite the fluorophore, which then produces a transient light emission as it returns to its ground state. The light is emitted at a higher wavelength than that used for excitation, and is detected with a specialized reader. Fluorescent reagents provide the unique advantage of allowing multiplexing to be performed.

Chemiluminescence occurs when a substrate is catalyzed by an enzyme and produces light as a by-product of the reaction. This detection method again relies on an enzyme-labeled antibody.

Gold nanoparticles or colored **latex beads** can be conjugated to antibodies and used for antigen detection. Labels of this kind are particularly popular for lateral flow immunoassays.

The use of an antibody which has been labeled with an **oligonucleotide** enables the detection of small amounts of the target antigen via a process known as immuno-PCR. Immuno-PCR combines the sensitivity of antibody binding with the signal amplification of PCR to produce an extremely powerful technique that is highly amenable to multiplexing.

Antibodies which have been conjugated to **europium chelate microspheres** can be used in either an immunochromatographic lateral flow assay or a microwell-based assay.

Direct Labeling of Primary Antibodies

Although the majority of immunodetection staining methods were developed using labeled secondary antibodies, it is becoming increasingly popular to use directly labeled primary antibodies for detection.These provide a number of significant advantages:

- ✓ Non-specific binding is avoided since secondary antibodies are not used
- ✓ Multiplexing is possible using antibodies from the same species
- ✓ The staining process is significantly faster since there is no secondary antibody incubation step and therefore fewer wash steps
- ✓ Data quality is improved through assay simplification

Innova Biosciences offers a wide range of easy to use antibody-labeling kits which circumvent many of the issues that have beset traditional labeling procedures for years, such as loss of material, sample dilution during column chromatography, batch-to-batch variation and difficulties in scaling up. Our bioconjugation kits have been carefully designed to save time, money and effort required within the laboratory without compromising results.

The main bioconjugation kit ranges are outlined here:

- **Lightning-Link® kits**
  For conjugating antibodies, proteins and peptides to enzymes, fluorescent labels, biotin or streptavidin
- **InnovaCoat® GOLD kits**
  For conjugating antibodies, proteins and peptides to gold nanoparticles ranging from 10nm-80nm in size, using a variety of surface chemistries
- **Latex conjugation kits**
  For conjugating antibodies, proteins and peptides to blue, red or black latex beads
- **ThunderLink® PLUS kits**
  For conjugating antibodies and proteins to oligonucleotides
- **Europium conjugation kits**
  For conjugating antibodies and proteins to europium chelate microspheres

These kits allow conjugations to be performed easily in the lab, and are compatible with any antibody species and sub-type, meaning that antibodies can be obtained from a wide range of sources.

Key Considerations for Antibody Labeling

1) Buffers and Additives

It is important to remember that every antibody storage buffer will contain substances other than the antibody itself, and therefore may be incompatible with the labeling technology. For instance, many antibodies are supplied in a buffer which contains a stabilizing protein such as BSA or gelatin, or a preservative to prevent microbial growth, such as sodium azide or Thimerosal. Other antibody storage buffers may contain residual glycine following elution of the antibody from an immunogen affinity purification column, or may include Tris to provide pH buffering. Glycerol is often added to minimize damage to the antibody during freeze-thaw cycles.

Many of our labeling technologies target lysine residues on the antibody, therefore buffers which contain substances which have primary amines, for example BSA, gelatin or glycine, should be avoided.

2) Antibody Purity

Some commercially available antibodies are provided in a form that is unsuitable for labeling, for example as hybridoma tissue culture supernatant (TCS), ascites fluid or crude serum. All of these formulations contain a multitude of substances that can interfere with the labeling reaction. Although ascites fluid and crude serum generally contain higher concentrations of antibody than TCS, antibodies supplied in any of these formats must be purified before the labeling reaction can proceed.

For most labeling reactions it is preferable for the antibody to have a purity of >95%. It is worth highlighting here the difference between class specific affinity purification and immunogen specific affinity purification. Class specific affinity purification takes advantage of the differential binding capacity of the Fc region of particular antibody classes to an immobilized capture protein such as Protein A or Protein G; although this type of purification eliminates the majority of serum proteins, it does not exclude non-specific antibodies hence some cross-reactivity may remain. Immunogen specific affinity utilizes the immunizing antigen as the capture protein, therefore resulting in a product which primarily contains the specific antibody.

Figure 2. shows the relative affinities for Protein A and Protein G of the most commonly used antibody subtypes.

Species	Subtype	Protein A Affinity	Protein G Affinity
Rabbit	IgG	High	High
Human	IgG	High	High
Mouse	IgG_1	Low	High
	IgG_{2a}	High	High
	IgG_{2b}	High	Medium
	IgG_3	Medium	Medium
Goat	IgG	Low	Medium
Sheep	IgG	Low	Medium
Rat	IgG_1	None	Low
	IgG_{2a}	None	Medium
	IgG_{2b}	None	Medium
	IgG_{2c}	Low	Medium

Figure 2. Relative antibody affinities for Protein A and Protein G.

3) Antibody Concentration

The antibody to be labeled should be at a suitable concentration, ideally of at least 1mg/ml. The protocols which are provided with our antibody conjugation kits provide information regarding the recommended starting concentration of antibody which is required to produce optimal labeling.

Antibody Purification Kits from Innova Biosciences

We have developed a range of purification kits which can be used to separate antibodies from unfavorable starting points. These are extremely easy and convenient to use, and have been carefully designed to complement our bioconjugation kits. The AbSelect™ purification kit range is compatible with our Lightning-Link® and Thunder-Link® PLUS conjugation kits, while the AbPure™ purification kit range is compatible with our InnovaCoat® GOLD, Latex and Europium conjugation kits. The AbPure™ Magnetic Purification System is compatible with all our bioconjugation kits. This is summarized in figure 3.

Purification Kit Range	Conjugation Kit Range
AbPure™ Magnetic Purification System	Applicable to all our bioconjugation kits
AbSelect™	Lightning-Link® kits ThunderLink® PLUS kits
AbPure™	InnovaCoat® GOLD kits Latex conjugation kits Europium conjugation kits
Antibody Concentration and Clean Up Kit for Latex and Europium	Latex conjugation kits Europium conjugation kits

Figure 3. Selection of the appropriate antibody purification kit to complement our conjugation kits.

These purification kits share a number of key advantages:

- ✓ Compatibility with our bioconjugation kits
- ✓ Quick and easy to use
- ✓ High levels of antibody recovery
- ✓ No specialist equipment required
- ✓ Fully scalable

By combining the appropriate antibody purification kit with the chosen conjugation kit, it is possible to directly label an antibody quickly and easily irrespective of its starting formulation.

When selecting the most appropriate antibody purification kit, it is important to consider the following points:

- Which conjugation kit will be used?
  Each of our conjugation kits is provided with a detailed protocol that provides information regarding the compatibility of various buffer components with the labeling technology. The conjugation kit protocols should always be referred to prior to initiating the conjugation reaction.
- Is the antibody purified?
- If the antibody is purified, what is the composition of the antibody storage buffer and what is the concentration of the antibody?
- If the antibody is not purified, what is the nature of the starting material, what volume of starting material is available, and which species is the antibody host?

To aid in this selection, we have created decision diagrams based on the most common buffer formulations which are found in the commercial marketplace:

AbSelect™ antibody purification kits decision diagram

AbPure™ antibody purification kits decision diagram

Purification kits compatible with Latex and Europium decision diagram

Each of the purification kit product ranges is now described.

Antibody Concentration and Clean Up Kits

- ✓ Concentration of antibodies
- ✓ Removal of low molecular weight (<10kDa) contaminants
- ✓ Utilize a simple spin column

The Antibody Concentration and Clean Up Kits can be used to remove any undesirable low molecular weight additives such as Tris, glycine and sodium azide from the antibody storage buffer. The kits utilize a simple spin column which allows low molecular weight molecules to pass through a filter while trapping the antibody which is then recovered in a conjugation-friendly buffer. This is illustrated in figure 4.

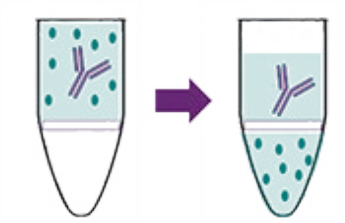

Figure 4. Schematic representation of the Antibody Concentration and Clean Up Kit. Low molecular weight contaminants are shown in green.

Since the antibody can be recovered in any volume, the Antibody Concentration and Clean Up Kits also allow for quick and easy concentration of the antibody. It is however important to note that any buffer components with a molecular weight >10kDa, for example BSA, will also become concentrated during this process. If, following concentration and resuspension, these additives will be at a concentration which is incompatible with the subsequent conjugation reaction, the relevant purification kit for the additive should be chosen instead of the Antibody Concentration and Clean Up Kit.

The removal of sodium azide is of particular importance if the antibody is next to be conjugated to Horseradish Peroxidase (HRP), because sodium azide rapidly reduces the enzyme activity of HRP. Traditional methods of sodium azide removal rely on dialysis, which is a notoriously time-consuming process, yet the Antibody Concentration and Clean Up Kit enables a simple buffer exchange to be performed in just minutes.

BSA Removal Kits

- ✓ Concentration of antibodies
- ✓ Removal of BSA
- ✓ Complete buffer exchange
- ✓ Utilize a proprietary BSA Removal Buffer

The BSA Removal Kits utilize a simple, one-step method which separates BSA from the antibody, leaving the antibody in a suitable position for transfer to a conjugation-friendly buffer. The antibody is simply incubated with the BSA Removal Buffer, which specifically induces antibody precipitation; other proteins such as BSA are unaffected and remain in solution. The antibody is then pelleted by centrifugation, prior to resuspension in the buffer which is provided with the kit. The removal of BSA from an antibody using the AbPure™ BSA Removal Kit is shown in figure 5.

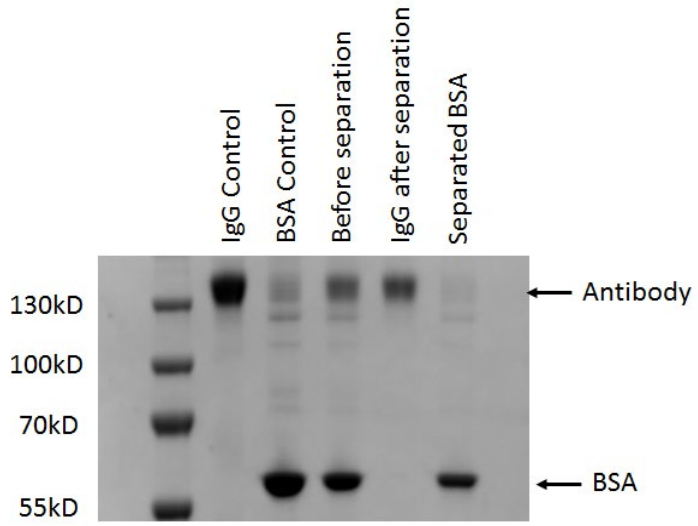

Figure 5. Non-reducing gel illustrating BSA removal from an antibody using the AbPure™ BSA Removal Kit.

Since the antibody can be resuspended in any volume, the BSA Removal Kit also allows for quick and easy concentration of the antibody.

Traditional methods of BSA removal rely on column-based chromatography, which involves multiple steps and can result in significant loss of antibody. The BSA Removal kit requires no specialist knowledge, and allows BSA removal to be achieved easily in just a matter of minutes.

AbPure™ Magnetic Purification System

- ✓ Concentration of antibodies
- ✓ Complete buffer exchange
- ✓ Compatible with all of our labeling technologies
- ✓ Can be used for the purification of very low amounts (20-200ug) of antibody
- ✓ Utilizes magnetic beads

The AbPure™ Magnetic Purification System is a protein A-based antibody purification system with a unique set of elution and neutralization buffers, making it compatible with all of our labeling technologies. The antibody becomes bound via its Fc region to Protein A magnetic beads, which are then captured with a magnetic stand (available as product code 265-1006). Figure 6 shows a schematic representation of this purification method.

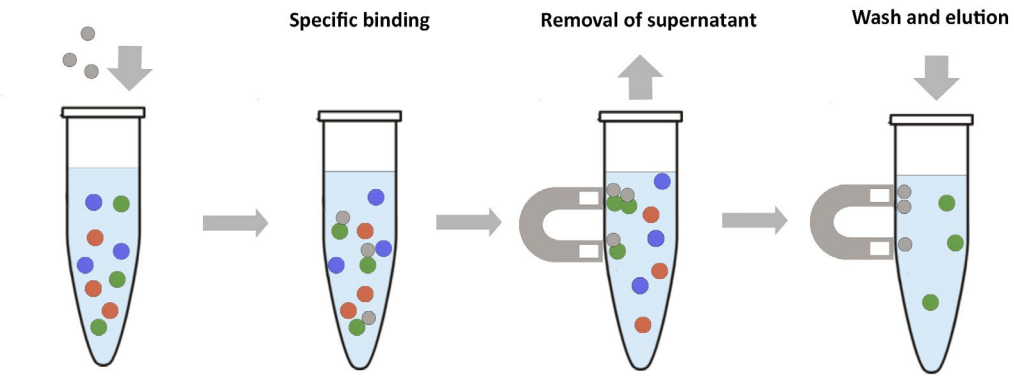

Figure 6. Schematic representation of the AbPure™ Magnetic Purification System. Grey - Protein A magnetic beads, Green – antibody, Red and Blue – undesirable buffer components which are not compatible with the antibody labeling process.

Since the antibody can be recovered in any volume, the AbPure™ Magnetic Purification System also allows for quick and easy concentration of the antibody.

	AbPure™ Magnetic Purification System
Amount of antibody which can be purified in each run	20 - 200ug
Volume of antibody required	20ul - 1ml

The AbPure™ Magnetic Purification System can directly replace any existing protein A purification kit on the market. It is suitable for use with small amounts of antibody, but is also fully scalable upon request.

Tissue Culture Supernatant (TCS) Purification Systems

- ✓ Purification of antibodies from large volumes (>10ml) of Tissue Culture Supernatant
- ✓ Utilize resin, and purification columns (862-, 832-, 264-) or spin cartridges (842-)

Antibodies are often generated from hybridoma cell lines and supplied in Tissue Culture Supernatant (TCS). The concentration of antibodies in TCS is usually very low, furthermore TCS contains a wide variety of components that can interfere with the conjugation chemistry. For these reasons, if the antibody is supplied in TCS it is essential to purify it before going ahead with the conjugation reaction.

The TCS Antibody Purification Systems rely on the use of resins. Following incubation of the TCS with the appropriate resin, the resin is loaded in to a column, and unwanted substances are removed by a simple wash procedure. The antibody is then eluted and neutralized.

The AbSelect™ TCS Antibody Purification System and AbPure™ TCS Antibody Purification System rely on the use of Protein A which has been coupled to agarose beads. The antibody is captured by virtue of the interaction between its Fc region and the Protein A. The AbSelect™ Mouse TCS and AbSelect™ Rat TCS Purification Systems rely on the use of a resin which has a high affinity and specificity for either mouse or rat IgG molecules, while the binding strength of the resin for bovine IgG molecules is minimal. Tissue culture supernatant contains bovine IgG molecules from the Fetal Bovine Serum (FBS) which is used to supplement the cell growth media, and these must be removed prior to carrying out the conjugation reaction since they will compete for labeling.

The maximum amount of antibody which can be purified in each run is shown in the following table.

	AbSelect™ TCS Antibody Purification System	AbSelect™ Mouse TCS Antibody Purification System	AbSelect™ Rat TCS Antibody Purification System
Maximum amount of antibody which can be purified in each run	5mg	1.5mg	0.6mg
Volume of TCS required	10 - 50ml	10 - 25ml	10 – 50ml

Serum Purification Systems

- ✓ Purification of antibodies from large volumes of serum or ascites fluid
- ✓ Utilize resin and purification columns

Antibodies generated from ascites fluid or serum are often supplied as crude formulations which contain numerous components that can interfere with the conjugation chemistry.
The AbSelect™ Serum Antibody Purification System and the AbSelect™ G Serum Antibody Purification System rely on the use of Protein A or Protein G which has been coupled to agarose beads. The antibody is captured as a result of the interaction between its Fc region and the resin. Following incubation of the antibody with the resin, the resin is loaded in to a column, and unwanted substances are removed by a simple wash procedure. The antibody is then eluted and neutralized.
The AbSelect™ Serum Antibody Purification System can be used to purify up to 20mg of antibody in each run, while the AbSelect™ G Serum Antibody Purification System can be used to purify up to 10mg of antibody. The volume of sample which is required varies depending on the host species, as detailed in figure 7.

Species	Normal IgG range (mg/ml)	Suggested suitable volume for AbSelect™ Serum Antibody Purification System (ml)	Suggested suitable volume for AbSelect™ G Serum Antibody Purification System (ml)
Rabbit	12 - 15	1.3 - 1.7	0.7 – 0.8
Human	7 - 23	0.9 – 2.9	0.4 – 1.4
Mouse	2 - 5	4 - 10	2 - 5
Goat	18 - 24	0.8 – 1.1	0.4 – 0.6
Sheep	18 - 24	0.8 – 1.1	0.4 – 0.6
Rat	5 - 7	2.9 - 4	1.4 - 2
Ascites fluid	0.5 - 5	4 - 40	2 - 20

Figure 7. Approximation of IgG levels in serum and ascites fluid, and suggested volumes for use with the AbSelect™ and AbSelect™ G Serum Antibody Purification Systems.

AbSelect™ and AbPure™ Antibody Purification Systems

- ✓ Species-optimized purification of antibodies from buffers containing protein additives other than, or in addition to, BSA
- ✓ Species-optimized purification of antibodies from small volumes of serum (<0.1ml) or ascites fluid (<0.5ml)
- ✓ Utilize resin and spin cartridges

The AbSelect™ and AbPure™ Antibody Purification Systems are designed for the removal of protein additives from the antibody storage buffer. If the only protein additive which is present is BSA, then the BSA Removal Kit should be used for antibody purification. If the antibody storage buffer contains protein additives other than, or in addition to, BSA the appropriate AbSelect™ or AbPure™ Antibody Purification System should be used. These purification kits are also suitable for the purification of antibodies from small volumes of serum or ascites fluid.

These kits rely on the use of Protein A (AbSelect™ and AbPure™) or Protein G (AbSelect™ G) which has been coupled to agarose beads, or on the use of a resin which has a high affinity and specificity for mouse IgG molecules (AbSelect™ Mouse and AbPure™ Mouse). In all cases the antibody is incubated with the resin and captured by virtue of the interaction between its Fc region and the resin. The resin is loaded in to a column, and unwanted substances are removed by a simple wash procedure. The antibody is then eluted and neutralized. The maximum amount of antibody which can be purified in each run is shown in the following table.

	AbSelect™ Antibody Purification System	AbSelect™ G Antibody Purification System	AbSelect™ Mouse Antibody Purification System	AbPure™ Antibody Purification System	AbPure™ Mouse Antibody Purification System
Amount of antibody which can be purified in each run	20 - 500ug	20 - 300ug	20 - 150ug	20 - 500ug	20 - 150ug
Volume of antibody required	0.1 – 0.5ml	0.1 – 0.5ml	0.1 – 0.5ml	0.1 – 0.5ml	0.1 – 0.5ml

	Lightning-Link®	InnovaCoat® GOLD	Latex conjugation kits	Thunder-Link® PLUS
Antibody Concentration and Clean Up Kits	AbSelect™ Antibody Concentration and Clean Up Kit 861-0010	AbPure™ Antibody Concentration and Clean Up Kit 262-0010	Antibody Concentration and Clean Up Kit for Latex and Europium 1020-0040	AbSelect™ Antibody Concentration and Clean Up Kit 861-0010
BSA Removal Kits	AbSelect™ BSA Removal Kit 820-0100	AbPure™ BSA Removal Kit 263-0100	AbPure™ BSA Removal Kit 263-0100	AbSelect™ BSA Removal Kit 820-0100
AbPure™ Magnetic Purification Kit	AbPure™ Magnetic Purification Kit 265-0200			
Tissue Culture Supernatant (TCS) Purification Systems	AbSelect™ TCS Antibody Purification System 862- AbSelect™ Mouse TCS Antibody Purification System 832- AbSelect™ Rat TCS Antibody Purification System 842-	AbPure™ TCS Antibody Purification System 264-	AbPure™ TCS Antibody Purification System 264-	AbSelect™ TCS Antibody Purification System 862- AbSelect™ Mouse TCS Antibody Purification System 832- AbSelect™ Rat TCS Antibody Purification System 842-
Serum Purification Systems	AbSelect™ Serum Antibody Purification System 863- AbSelect™ G Serum Antibody Purification System 893-	N/A	N/A	AbSelect™ Serum Antibody Purification System 863- AbSelect™ G Serum Antibody Purification System 893-
AbSelect™ and AbPure™ Antibody Purification Systems	AbSelect™ Antibody Purification System 860- AbSelect™ G Antibody Purification System 890- AbSelect™ Mouse Antibody Purification System 830-	AbPure™ Antibody Purification System 260- AbPure™ Mouse Antibody Purification System 261-	AbPure™ Antibody Purification System 260- AbPure™ Mouse Antibody Purification System 261-	AbSelect™ Antibody Purification System 860- AbSelect™ G Antibody Purification System 890- AbSelect™ Mouse Antibody Purification System 830-

Figure 8. Antibody Purification Kits overview. Where more than one pack size is available, only the product code prefix is given.

For any questions regarding our products please contact our technical support team:
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