Topic Introduction

Antibody Purification and Storage

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Antibodies have become a common and necessary tool in biochemistry, cell biology, and immunology laboratories. There are many different types of antibodies and antibody fragments being used for a myriad of applications. As a result, many different purification protocols have been developed to obtain antibodies of the desired specificity and sensitivity. Here, we introduce the options for small- to large-scale antibody purification and isolation of polyclonal and monoclonal antibodies (and fragments generated from these) that target-specific proteins, as well as methods to properly purify antibodies that recognize posttranslational modifications. Optimal conditions for the long-term storage of antibodies are also discussed.

ANTIBODY TYPES

Antibodies (a.k.a. immunoglobulins [IgGs]) account for ~20% of the plasma proteins in humans. Human immunoglobulins consist of several different types of proteins, such as IgD, which functions as an antigen receptor and is found on the surface of B cells; IgE, which binds allergens, subsequently triggering histamine release from mast cells (the “allergic” reaction); IgG, which has four different forms (subclasses IgG1, IgG2, IgG3, and IgG4) (Schur 1987) that provide immunity against foreign pathogens and can also cross the placenta and provide fetal immunity in utero; and IgM, which is unique in that it can exist as a monomer when expressed on the surface of B cells or as a pentamer when it is secreted. IgM represents the initial immune reaction to an infection and, because of its high avidity, can detect and bind even small amounts of antigen. Polyclonal IgG and/or fragments of IgG from various mammalian species such as goats, sheep, or rabbits are used most often for research purposes. Owing to their polyclonal nature, it is not practical in most cases to use polyclonal antibodies for therapeutic applications or for critical applications where large quantities of antibodies will be required for large screens or analyses requiring consistency over a long period of time. Monoclonal antibodies (mAbs) from rodents or rabbits as well as from recombinant sources are the source of most large-scale antibody preparations. Dependent on the source of the antibody, purification and analysis will differ in part based on the final application, requirement for cGMP manufacture, and/or storage conditions required for long-term storage.

Unlike mammalian species, the major immunoglobulin class in birds, reptiles, amphibia, and lungfish is called IgY (Larsson et al. 1993). Structurally related to IgG, this immunoglobulin has a slightly higher molecular weight owing to an extra constant domain within the IgY heavy chain. IgY purification cannot be performed with Protein A or G because IgY does not bind to these proteins, and thus more traditional biochemical methods must be used, as detailed below. The isolation of each of the antibody types and subclasses requires different methods, affinity ligands (target-specific peptides or proteins, Protein A, or Protein G), or basic biochemical techniques (IgY). With proper planning,

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antibodies can be isolated and stored for many years, providing a consistent and inexpensive source of reagents for a variety of biomedical research applications.

ISOLATION OF A TOTAL IgG FRACTION

During the generation of an immune response to an external stimuli such as a bacterial or viral infection or in the case of a vaccination with a protein or peptide (in some cases, conjugated to a carrier or hapten), the antibody produced will only represent a small portion of the total IgG present. For many applications, however, the isolation of a total IgG fraction is still sufficient for the experiments planned. When the antibody to be isolated is a mAb from a hybridoma grown in vitro (cell culture or other artificial apparatus) or it is a recombinant antibody that has been expressed in *Escherichia coli* or other organism, the mAb could be the only IgG present, and thus a total IgG isolation procedure can be used to obtain a single antibody species. If, on the other hand, the hybridoma will be isolated from ascites, the host animal's immune system may also introduce into the ascites other IgGs that would have to be considered and if necessary removed. The final application for the antibody thus will often dictate not just the purification method(s) to be used but also the very nature of the antibody scale-up.

For smaller-scale work, the use of Protein A or G can generate a highly purified antibody preparation; however, the high cost of Protein A or G may preclude its use for large-scale applications. There are several well-documented and robust methods for the isolation of antibodies that can be used alone or in tandem to generate antibody of sufficient amount, purity, specificity, and sensitivity for most applications.

Ammonium Sulfate Precipitation

Before the widespread availability and use of Protein A (rabbit) or Protein G (rodent) for the purification of IgG, the use of an ammonium sulfate “cut” was the standard method to isolate IgG and other serum proteins. To isolate the antibody with a minimum of contamination from other serum proteins, a three-step procedure is often used. The antibody source (serum, supernatant, ascites) is first centrifuged and then saturated and neutralized (with HCl), then ammonium sulfate is slowly added to a final concentration of 25% (Protocol: **Ammonium Sulfate Fractionation of Antibodies** [Fishman and Berg 2018a]). This will precipitate many plasma proteins over 5–15 h at 4°C. A second centrifugation removes the precipitated proteins, after which time additional ammonium sulfate is added to the supernatant to a final concentration of 50%. After a second incubation for 5–15 h with stirring at 4°C, the antibody fraction can be isolated by centrifugation. The pellet is resuspended in PBS and subjected to dialysis (24–48 h, three to four changes of dialysate), and the protein concentration of the final product is determined by measuring the absorbance at 280 nm (a reading of 1.35 corresponds to an antibody concentration of 1 mg/mL). This crude antibody fraction can be further purified by a variety of methods, including Protein A/G purification, anion-exchange chromatography using diethylaminoethyl (DEAE)–Sephacrose (see below), or other methods (affinity chromatography, size exclusion, or other chromatographic methods). If the IgG fraction is to be stored for any length of time without further workup, it should be filtered through a 0.45-μm filter and stored at 4°C. Alternative long-term storage methods include the addition of 35%–50% glycerol (discussed in detail below) and freezing the solution in reasonable-sized aliquots or lyophilization of the IgG and reconstitution when required.

Caprylic Acid IgG Isolation

In contrast to ammonium sulfate, which causes antibodies to precipitate, the addition of short-chain fatty acids such as octanoic acid, also known as caprylic acid, will precipitate most serum proteins with the exception of IgG (Protocol: **Preparation of Antibody Using Caprylic Acid** [Fishman and Berg 2018b]; dos Santos et al. 1989). By precipitating all of the unwanted serum proteins rather than the

antibodies, the tendency of antibodies to aggregate when precipitated is avoided. This method should not be used with antibody sources that contain low concentrations of antibody, such as many cell culture supernatants, owing to the potential loss of high-affinity antibodies, which may be bound by the caprylic acid. Following centrifugation to remove precipitated serum proteins, the caprylic acid can be removed from the supernatant by desalting or further isolation of the antibody. Many laboratories perform ammonium sulfate precipitation in tandem with caprylic acid treatment, generating a relatively pure IgG preparation at very low cost.

ANION-EXCHANGE CHROMATOGRAPHY

Anion-exchange chromatography is the most commonly used method for the isolation of large amounts of purified IgG. Unlike Protein A and Protein G chromatography or affinity-based methods, anion-exchange chromatography is far more affordable, offers the ability to isolate a wide variety of IgG subclasses, and uses milder conditions. With the continuing development of therapeutic antibodies for the treatment of a variety of pathophysiological conditions, large-scale purification of antibodies (e.g., mAbs expressed in bacteria) is one of the major applications of chromatography in the pharmaceutical arena. It is therefore not surprising that anion-exchange chromatography media suitable for the isolation of antibodies is offered by many manufacturers.

Chromatography using DEAE-agarose (or other DEAE-modified resins or membranes) can be performed in a variety of ways. For small-scale work, the chromatographic separations should be performed based on the equipment available and the size of the preparations and the purity required. Options include the use of open columns, where the buffers are flowing through the column using simple gravity feed; and bulk separations, where centrifugation is used to pellet the resin with bound protein followed by washing and elution using a sintered glass funnel, multiple centrifugation steps, or the use of small vessels for rapid washing and elution of bound antibodies. For applications requiring large amounts of highly purified antibody or those requiring multiple lots with consistent lot-to-lot activity, automated chromatographic systems such as fast protein liquid chromatography (FPLC) or high-pressure liquid chromatography (HPLC) should be used. These use large columns packed with appropriate anion-exchange media, membrane- or ceramic-based media, or other types of low-to-high capacity and slow-to-fast flow-based anion supports.

Although it is beyond the scope of this introduction to discuss all of the possible types of anion-exchange media with respect to stability, suitability, and so on, it is important that the working parameters chosen are compatible with the type of media selected. For example, DEAE or other anion-exchange moieties are attached to a variety of media including beaded cellulose (Sephacel; GE Healthcare), cross-linked allyl dextran/*N,N'*-methylenebisacrylamide copolymer (Sephacryl; GE Healthcare), cross-linked agarose (available from Bio-Rad, GE Healthcare, and others), ceramic-based media (AcroSep; Pall Corporation), or as disks suitable for specific applications (CIM Disk; BIA Separations). Each of these has a specific working range for pH, flow rate (so-called fast-flow or slow-flow media), capacity, and chemical and physical stability, as well as whether they can withstand autoclaving. A typical DEAE resin can bind the antibody contained in 0.2–2 mL of serum per 1 mL of resin. The chemical and physical properties of the resin will determine its suitability for batch versus column applications, FPLC versus HPLC, and whether to use aqueous or organic solvents and buffers. It is important to choose the proper media suitable for your intended application.

Because IgG from most species (other than rodents) tends to have an isoelectric point around neutral, two approaches can be used when separating IgG using DEAE resins (see Protocol: **Purification of Antibodies: Diethylaminoethyl (DEAE) Chromatography** [Fishman and Berg 2019a]). If the pH is adjusted to pH 6.3, then the immunoglobulins will not bind to the DEAE, whereas the other serum proteins will. Once the serum is applied to a DEAE column, washing the column with two to three columns of 5–7 mM sodium phosphate (pH 6.3) will elute the unbound IgG while other serum proteins bind to the column. Once the IgG fraction is cleared from the column bed and the

absorbance (A_{280}) baseline returns to zero, the column can be regenerated and reused. It is critical that if serum is to be applied to a DEAE column without a prepurification step, it must be dialyzed against 5–7 mM sodium phosphate (pH 6.3) at least three times with a ratio of one part serum to 100 parts dialysate.

Alternatively, if the serum or IgG fraction from an ammonium sulfate isolation is adjusted to a pH of 8–8.5, the IgG will bind to the DEAE but will elute as the first peak when a gradient of phosphate buffer and salt is applied. Stepwise elution can also be performed because the IgG fraction elutes very early in the gradient. As a general rule, a high-binding-capacity DEAE can be used for this purpose.

OTHER ION-EXCHANGE METHODS FOR THE ISOLATION OF ANTIBODIES

There are other ion-exchange media that have become popular in recent years for the purification of antibodies. Hydroxyapatite chromatography is being used more frequently for purifying antibodies now that better quality hydroxyapatite media have become available. Hydroxyapatite $[\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]$ is useful for fractionating immunoglobulins regardless of the species, class, or subclass of the antibody. The isolation of high-purity antibodies (>90%) from serum or ascites can be accomplished in a single step. The nature of the interactions between antibodies and hydroxyapatite are quite complex and occur for the most part because of the attraction of the amino and carboxyl groups of the antibody molecules to the phosphate and calcium moieties of hydroxyapatite. Because the phosphate groups are involved in the binding of the antibodies, the samples are usually added to the column in low ionic strength (5–50 mM) phosphate buffer (pH 6.8). Antibodies are typically eluted using a gradient of phosphate buffer of 50–500 mM at pH 6.8. For some IgG and IgM species, solubility in such low ionic strength can be an issue, but the addition of 1 M NaCl usually improves the solubility and the purification. The NaCl may also improve selectivity that can lead to higher yields and purities of the isolated antibodies.

Ceramic-based hydroxyapatite chromatography has been used to isolate F(ab')_2 fragments in high yield (see below) as well as to separate various IgG subclasses. There are relatively small differences in composition among IgG subclasses; thus, their separation has always been challenging. Using this method, Moro et al. (2008) have shown the ability to isolate different IgG subclasses from mouse ascites and also to isolate pepsin-generated F(ab')_2 fragments free from serum protein contamination. In situations where the use of high-purity IgG is critical, such as for therapeutic applications, dual-mode affinity gels can be used. One example is DEAE Affi-Gel Blue (Bio-Rad), which combines the power of DEAE to bind IgG with the utility of Cibacron Blue F3GA (Bio-Rad) to bind albumin. Because the gel binds albumin, IgG fractions can be obtained with minimal albumin contamination. The downside of using this kind of dual-mode gel is that careful optimization of both the ionic strength and the pH of the application buffer is necessary. Although DEAE Affi-Gel Blue has not been used widely in the small-scale laboratory environment, for larger-scale applications requiring large batches of a single antibody, this resin could be used as one of the two orthogonal methods required by the FDA for the purification of mAbs for therapeutic applications (USFDA 1997, 2001; <http://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/OtherRecommendationsforManufacturers/UCM153182.pdf>). Immobilized metal affinity chromatography (IMAC) can also be used for the purification of antibodies (for review, see Block et al. 2009). Antibodies are unique among proteins due to the presence of multiple adjacent histidine residues within their primary amino acid sequence, making them suitable targets for IMAC. IMAC resins are composed of cross-linked agarose or other supports derivatized with chelating groups, typically nitrilotriacetic acid (NTA) or iminodiacetic acid (IDA) to immobilize metal ions. Once a specific divalent metal ion is added to the resin, it can be packed into columns and used to purify, in this case, antibodies or other His-tagged proteins. Typically these kinds of supports have a capacity of 1–10 mg of His-tagged protein/mL of resin. Binding to these resins is performed at or near neutral pH in PBS or TBS. One application for IMAC is to separate excess reactants from antibody that has been labeled with fluorophores, proteins such as horseradish peroxidase, or other modifications. Following

extensive washing of the column, elution of the antibody can be performed with 20–200 mM imidazole and a chelator-like EDTA, to capture the Ni^{2+} or 0.1 M glycine-HCl (pH 2.5–3) (with rapid neutralization as detailed in Protocol: **Peptide Affinity Purification of Antibodies** [Fishman and Berg 2019b]). IMAC is also used as a second purification step following thio-Sepharose purification of recombinant antibody from a cellular supernatant (Nadkarni et al. 2007).

PROTEIN A AND PROTEIN G CHROMATOGRAPHY

Protein A and Protein G are immunoglobulin-binding proteins expressed in *Staphylococcus aureus* and *Streptococcus* sp., respectively, that have been adapted for use in purifying large amounts of IgG. They are available covalently attached to affinity resins such as 4% cross-linked agarose, making them suitable for low-pressure antibody isolation. Protein A and Protein G contain binding sites for the Fc portion of mammalian IgG. Naturally occurring Protein G also has an additional binding site for albumin that is absent from recombinant Protein G. Protein A is not recommended for the isolation of mouse mAbs because it lacks affinity for mouse IgG1, or for the isolation of antibodies from sheep, goat, chicken, hamster, or rat. Protein G, on the other hand, has a higher affinity than Protein A for IgG from many species and is the method of choice for isolating immunoglobulins from most mammalian species. Neither Protein A nor Protein G can be used for the isolation of chicken antibodies. Protocol: **Protein A and Protein G Purification of Antibodies** (Fishman and Berg 2019c) describes antibody purification using Protein A and Protein G.

Another antibody-binding protein is Protein L, first isolated from *Peptostreptococcus magnus*. Protein L binds immunoglobulins through κ light chain interactions. Although it can bind a wider range of Ig classes and subclasses than Protein A or Protein G, its utility is limited because it cannot bind some κ light chains (such as VkII in humans) and in mouse its binding is restricted to only VkI light chains. Once an antibody has been characterized and is determined to have appropriate κ chains, Protein L can be a useful tool for antibody purification from cell culture supernatants, because Protein L's inability to bind bovine immunoglobulins is of particular advantage when bovine serum has been added to the cell culture medium.

PURIFICATION OF CHICKEN (IgY) ANTIBODIES

The yolk of eggs from immunized chickens provides an abundant source of antibody, and the fact that IgY has other advantages over antibodies produced in mammalian species makes it an attractive choice for antibody production in some cases. The avian immune system is dramatically different from that of mammals, and given their phylogenetic divergence, it is possible to generate antibody responses to conserved proteins that do not elicit robust immune responses in mammalian species. In addition, chicken immunoglobulins differ from mammalian antibodies in other ways that can be exploited for certain immunological techniques. Chicken IgG does not activate the mammalian complement systems, nor does it react with rheumatoid factors. Because IgY does not interact with either Protein A or Protein G, more traditional methods must be used to isolate IgY. Although eggs can be accumulated for short periods of time, IgY must be isolated from the yolk within 2–3 wk to avoid fouling of the material. Two methods are described for isolating IgY. In Protocol: **Isolation of IgY from Chicken Eggs** (Fishman and Berg 2018c), sodium sulfate is used to differentially precipitate protein fractions from chicken egg yolk. This protocol also includes a differential precipitation technique, but in this case with Polyethylene Glycol 6000.

PURIFICATION OF Fab AND F(ab')₂ FRAGMENTS

Intact IgG molecules can be enzymatically cleaved into a number of different structures, dependent on the enzyme used. The heavy chain can be cleaved at the hinge region by papain. This generates two Fab

fragments and one Fc region. The Fc region can then be bound by Protein A and/or Protein G and removed, leaving behind a purified Fab fraction. If the IgG is instead treated with pepsin, the cleavage site is below the hinge region, and the resultant fragments consist of a disulfide-bonded F(ab')₂ and the pFc' portion of the IgG molecule. In this case, the F(ab')₂ fragment consists of two disulfide-bonded regions that can be reduced to obtain individual Fab' fragments.

Improvements in recombinant protein expression and purification have led to antibody fragments being produced recombinantly rather than from isolated antibodies (Hayden et al. 1997; Dimitrova et al. 2009). In particular, phage-display libraries encoding human antibodies have been used to select specific antibodies that can then be expressed as His-tagged proteins or with other easy-to-use modifications. In the case of His-tagged proteins, Ni-NTA columns can be used to purify the Fab. This method can be scaled to the desired quantity of antibody. Other researchers have used *E. coli* to express Fab fragments with a His-tag to facilitate purification (Kwong and Rader 2009).

PURIFICATION OF IgM

Owing to their promise as effective therapeutic reagents (e.g., anticancer activity [Azuma et al. 2007] and the treatment of neurodegenerative diseases and CNS diseases [Wright et al. 2009; Paul et al. 2010; Sarkar et al. 2011]), IgM purification has become very important in both research and industrial settings. Given the large mass of IgM (960 kDa) molecules, their inherent instability in the harsh conditions used for IgG purification, and their lack of binding to Protein A and Protein G, purifying IgM tends to be more difficult than purifying IgG.

When designing purification methods for IgM, the properties of IgM that must be taken into consideration include (1) the narrow range of pH under which IgMs are typically soluble (pH 6–8 for many IgMs); (2) their large mass and thus slow diffusion, which affects column washing, capacity, and discrimination when using media such as cross-linked agarose and similar porous particles; (3) the potential to destabilize or denature the IgM upon exposure to various conditions that are normally used to purify IgGs or other large proteins; and (4) the tendency for IgM to aggregate, necessitating additional steps to provide stable, monomeric IgM such as the addition of low percentages of polyethylene glycol.

On the positive side, IgMs are highly charged, and most bind strongly to anion- and cation-exchange materials (Jacobin et al. 2004), hydroxyapatite (for review, see Gagnon 2009), and weak hydrophobic interaction chromatography media. It is critical to avoid strong hydrophobic surfaces because those have been shown to denature IgM. In the research setting, small-scale isolation of IgM can be affected using affinity resins such as C1q bound to cross-linked agarose (Nethery et al. 1990) or mannan-binding protein (Neuens et al. 1992), as well as hydroxyapatite. Scale-up, however, is often challenging, and several methods are reviewed by Gagnon et al. (2008).

The methods that have been developed for the purification of IgM can be incorporated into any number of multistep, orthogonal strategies for the manufacturing of IgM for therapeutic applications. Although, for example, only weak hydrophobic interaction chromatography can be used for the purification of IgM, the differential binding of IgM (good) versus DNA (poor) to such chromatographic media can be exploited as one step of a validated, multistep method for IgM isolation along with, for example, an ion-exchange step.

AFFINITY PURIFICATION OF ANTIBODIES

The purification of antigen-specific antibodies has become a widely used and critical tool for the production of antibodies against posttranslational modifications (PTMs), mutations, and neo-epitopes, and for other applications. The antigen is covalently bound to a support made from agarose, 4% cross-linked agarose, polyacrylamide, or a polyacrylic. The most commonly used support is 4%

cross-linked agarose because of its relative stability, moderate price, and adaptability through the addition of a variety of linkers. Preactivated beads are available from a variety of manufacturers, allowing for the straightforward and specific addition of peptides or proteins. The most common type of linkage used for the addition of proteins is a cyanogen bromide- or *N*-hydroxysuccinimidyl ester-activated support. Both react with free amines (amino-terminal amines and the ϵ amine of lysines) on peptides or proteins. Given the facts that lysines are critical residues for epigenetic modification, are part of consensus motifs used by many kinases, and are an immunogenic amino acid, the use of amine- (lysine-) reactive supports should be avoided for peptides that contain lysine. In such cases, other chemistry can be used to provide a specific means of covalently attaching peptides to supports, thus making the entire primary sequence of the peptide available to bind antibody. The most commonly used reactive site is the thiol side chain found in cysteine. Peptides can be designed that use naturally occurring cysteines within the protein target's primary sequence, or a cysteine can be added to either end of the peptide to provide free thiols for attachment. The peptides can then be covalently attached to resins bearing thiol-reactive linkers. The most commonly used thiol-reactive moieties are iodoacetyl and maleimide, both of which react selectively with peptides containing cysteine thiols.

As more PTMs have been discovered, it is not surprising that a growing number have been found within regions of proteins that contain one or more lysines and cysteine. If a lysine or cysteine in close proximity to the modification (e.g., within an epitope length, which for class II would be eight to nine amino acids) is used for conjugation of the peptide immunogen, the modification of the lysine and/or cysteine due to the cross-linking of the peptide to carrier or resin would negatively impact the ability to produce antibodies that can detect the native protein. There are a few alternatives to avoid using amine and thiol-reactive reagents, most notably the use of hydrazine and aldehyde. Hydrazine and aldehyde building blocks suitable for peptide synthesis and resin modification have been developed, allowing the production of peptides bearing either a hydrazine or aldehyde moiety. Although there is additional cost to using this orthogonal method, the reactivity of hydrazine and aldehyde is specific and highly efficient. These reagents are readily available, and one company (Solulink) has produced a hydrazine building block containing a nicotinic acid group that can be followed spectrophotometrically to verify conjugation to an aromatic aldehyde. Alternatively, specific peptide resins can be used to generate peptides bearing carboxy-terminal hydrazines (Mellor et al. 2000). Resins as well as carrier proteins (for immunization) can then be modified with an aromatic aldehyde such as benzaldehyde, resulting in a hydrazine-aldehyde conjugation system adaptable to many different types of peptides. Finally, “click” chemistry—the covalent attachment of an azide with an alkyne in a copper-catalyzed reaction—could also be used, although its utility is somewhat limited.

Affinity Purification of Protein-Specific Antibodies

Once the type of solid support and manner of attachment of the protein have been determined, the amount of immunogen support to be used for purification of the specific IgG needs to be calculated. Because mAbs are typically not ligand-affinity-purified (given their mAb nature), the following discussion is directed toward the isolation of antigen-specific polyclonal antibodies. A reasonable estimate of the amount of antibody in serum is 10–20 mg/mL for the species most commonly used for antibody production. Rabbits tend to be at the low end of this range, whereas goats tend to be close to 20 mg/mL split between IgG1 and IgG2. Given that at most 10% of the IgG will be specific to the immunogen (but in many cases far less), it is necessary to provide sufficient binding sites to recover the maximum amount of antibody.

Because protein antigens are proteolytically processed by the immune system into small peptides (Goldberg and Rock 1992), even a small protein will result in a myriad of overlapping and unique epitopes that could result in the generation of a large number of antibodies to different regions of the protein. It is therefore not easy to estimate accurately the amount of affinity resin required for protein affinity purification without previous experience. Qualitatively, one can estimate that when using

rabbit serum and a protein with a mass of 50 kDa, conjugation of 3 mg of protein (0.06 μ mol of protein) could theoretically bind as much as 9 mg of IgG per epitope.

Several factors, however, typically reduce the amount of antibody that will bind to a protein affinity column. Theoretically, one can estimate that between 0.05 and 0.5 mg of specific antibody is present per milliliter of serum. This wide range is due to the variability in the immune response and is governed by many factors. The actual recovery of the specific antibody could be much lower. First, because proteins are bound to the support in a random fashion typically via free amines, its orientation is random. For smaller proteins this could interfere with antibody binding if the exposed surface of the protein is small. Second, IgG is bivalent, and therefore one molecule of IgG can bind two of the antigen sites, thus reducing the capacity of the column by up to 50%. Third, the epitopes could overlap; thus, limited antibody binding or the binding of an antibody to one site could sterically hinder the binding to other sites. Finally, in practice, a rule of thumb that we use is to expect 10% of the theoretical maximum binding. Starting with 25 mL of serum containing an expected 2.5–25 mg of antigen-specific antibody, a column containing 3 mg of protein bound to 3 mL of solid support should be sufficient for purification of the antibody. The size of the column that can be produced is most often limited by the amount of protein that can be isolated. Through the use of His6, GST, or other tagged proteins, larger amounts of recombinant protein can be isolated and used for immunization and purification of the resulting protein-specific antibody. To obtain antigen-specific antibodies, the “tag” can be removed from either the protein fraction to be used for immunization or that to be used for production of the affinity support. Given that it is advisable to introduce the immunogen free from extraneous sequence and that typically less protein is used for immunization compared with producing the affinity column, removal of the “tag” from at least the portion used for immunization would be prudent if it cannot be done for all of the protein to be used.

Affinity Purification of Peptide-Specific Antibodies

The purification of antibodies specific for unmodified peptide immunogens is relatively straightforward. Because synthetic peptides can be designed with specific points of covalent attachment to the solid support, it is easy to generate columns of any size without concern for epitope inactivation. Considering that the average peptide immunogen has a mass of $\sim$ 2 kDa and an antibody a mass of 150 kDa, 1 mL of a peptide support has the capacity to bind 37.5 mg of antibody (keeping in mind that IgGs are bivalent and have the capacity therefore to bind two peptide molecules). As previously discussed, antigen-specific antibody is estimated to be 0.05–0.5 mg per milliliter of serum, and it is advisable to use a column that has 10 $\times$ the capacity of the highest amount of antibody that could theoretically be bound. For example, if 25 mL of serum is going to be used for the purification of antibody, it would be estimated that this serum contains 1.25–12.5 mg of specific antibody requiring a column of $\sim$ 3.5–4 mL.

Affinity Purification of Modification State-Specific Antibodies

When the goal is the development of an antibody directed against a specific PTM—a modification state-specific antibody, such as the site of a specific phosphorylation, methylation, acetylation, or other modification—isolation of these antibodies is far more complicated. Several critical issues need to be addressed in the planning stages for any modification state-specific antibody. First, the use of high-quality peptides is paramount to the success of any project for the production of modification state-specific antibodies. This is most critical when the modified target can exist as one of several different, related amino acid derivatives. Examples of this include lysine or arginine, which can exist in several different but closely related methylation states: monomethyl (Me1), dimethyl (Me2), or trimethyl (Me3) lysine, or monomethyl (MMA), symmetrical dimethyl (SDMA), or asymmetric dimethyl (ADMA) arginine. As an example, if an antibody is being produced to a monomethylated lysine (KMe1), it is critical that the KMe1 building block be of as high purity as possible. If the peptide immunogen contains more than trace amounts of one or more of the other forms of lysine (unmethylated—KMe0, KMe2, KMe3), it may not be possible to isolate an anti-KMe1 antibody, because the

FIGURE 1. Immunodepletion of serum using an unmodified peptide column. Two rabbits were immunized with a phosphorylated peptide for the purpose of generating an antibody to a phosphorylated protein target. Serum was harvested after multiple immunizations with KLH-conjugated phosphopeptide. Approximately 50 mL of serum was subjected to multiple immunodepletion steps (labeled 1–5) as shown. For each immunodepletion step, the serum was cycled over the column for 45 min at room temperature, and then the column was washed with PBS (10 column volumes) followed by PBS plus 0.5 M NaCl until the eluent showed no detectable protein being eluted. Bound protein was then eluted with 0.1 M glycine (pH 2.5), and the protein was determined by measuring the absorbance at 280 nm. The dashed line represents the amount of protein eluted from the column using control rabbit serum and indicates nonspecific adsorption.

contaminating forms of the peptide will interfere with every stage of anti-KMe1 antibody production: peptide purification, immunization, and antibody purification. Although it might appear from analysis of the amino acid building blocks that small amounts of the above contaminants are present in the lysine used to manufacture the peptide, given that an excess of building block is used during the peptide synthesis process (typically fourfold to eightfold), the final peptide product could contain a higher percentage of unwanted contaminating building blocks than was observed during the QC of the raw material. It is therefore critical to use the highest-quality building blocks available, to ensure that the final peptide and the resulting antibody are specific for the intended target modification.

Second, several immunodepletion steps should be performed, because a single immunodepletion step may not be sufficient to clear the serum of antibodies that are not specific for the modified amino acid, as shown in Figure 1. Diluted serum is applied to a column that contains resin with covalently attached unmodified peptide. After cycling the serum over the column for 30–45 min at room temperature, the serum is recovered and the column is washed with buffer and then buffer containing 0.5 M NaCl. Once no protein is seen eluting from the column during the salt wash (by measurement at A_{280} or other protein detection method), the bound material is eluted with 0.1 M glycine (pH 2.5). The column is regenerated by washing with 10 column volumes of phosphate buffer, and the process is repeated as shown in Figure 1, or until the protein that is being eluted from the column has an OD reading similar to that observed for control serum when passed over the same type of column. It is at this stage (immunodepletion) that the activity of the final antibody can be compromised by over- or underimmunodepletion. If the antibody is not sufficiently immunodepleted, antibody that can recognize the protein target in the unphosphorylated state might remain. If too many immunodepletion steps are performed, then high-affinity antibodies could be lost, and the final antibody might have reduced sensitivity. It is recommended, therefore, that before performing more than three or four immunodepletion steps, you should test the serum for specificity. For example, perform a trial immunodepletion with a small amount of serum. It is important to carefully match the resin volume to the serum volume so that scale-up can be performed without loss of performance. If it was calculated that 5 mL of peptide resin would be used to process 50 mL of serum and if a small-scale immunodepletion series is to be performed with 10 mL of serum, then only 1 mL of peptide resin should be used. The serum can then be immunodepleted and a portion of the serum is reserved for analysis after, for example, two, four, and six immunodepletions. Analysis by western blot, dot blot, or ELISA can be used to compare the effect on specificity to the modified target of varying the number of immunodepletion steps. In some cases the serum can be immunodepleted two or three times before affinity purification, and if QC still indicates detection of the unmodified protein, immunodepletion after purification can be used to improve the antibody specificity.

For the purification of more complicated modification state-specific antibodies, such as antibodies targeting more than one modification within the same epitope or methylated lysine and/or arginine targets, it is often necessary to perform the immunodepletions a number of different ways to optimize the recovery of antibody to the target site and modification. Immunodepletions can be performed either before or after affinity purification of the antibody or a combination of both. We typically immunodeplete serum before affinity purification using the unmodified peptide conjugated to resin (see Protocol: **Conjugation of Peptides to Thiol-Reactive Gel for Affinity Purification of Antibodies** [Fishman and Berg 2019d] for conjugation of a peptide to resin using an amino- or carboxy-terminal cysteine), following the guidelines detailed above. For a phospho-specific antibody with a single phosphorylation site or two sites that are either juxtaposed or with few intervening amino acids, immunodepletion of the serum before affinity purification may be sufficient to remove cross-reactivity to the unmodified peptide/protein. Dot blots are often a good way to determine potential cross-reactivity, and that information can then be used to determine which, if any, further immunodepletion needs to be included to generate an antibody specific for the intended target. In the case of antibodies to methylated targets, the situation can be far more complicated, and several modified peptides may need to be used in an immunodepletion strategy. Figure 2 shows the dot-blot analysis of serum and purified antibody produced to a single monomethylated lysine residue. In Figure 2A, dot-blot analysis of the serum indicated detection of the desired modified peptide with some detection of the unmodified peptide and some slight detection of the dimethylated form. No detection of the trimethylated peptide was observed (data not shown). Following three rounds of immunodepletion using the unmodified peptide followed by affinity purification (Fig. 2B), no detection of the unmodified peptide or the dimethylated peptides was observed.

Unlike the antibody detailed in Figure 2, the purification of an antibody targeting a peptide containing more than one modification is typically a multistep process involving immunodepletion to remove antibodies that are not specific for the desired combination of modified amino acids followed by affinity purification using the immunogen peptide. Dependent on the target, some of the immunodepletions may best be performed after affinity purification. In the case of a dual phospho-specific antibody, immunodepletion (as described above) might require only the unmodified peptide, but often one or more of the singly phosphorylated peptides has to be used to ensure dual phospho-specificity. Whether this is required depends on the relative location of each of the phosphorylation sites within the peptide epitope and on the nature of the intervening amino acids. Although the peptide bond itself is planar owing to two possible resonance structures that can be adopted by the peptide bond, the bonds between the amino group and the α -carbon atom and between the α -carbon atom and the carbonyl group are single bonds and may rotate around these bonds, taking on a variety of orientations. The freedom of rotation around these two bonds of each amino acid allows proteins to fold in many different ways (with the exception of proline where the peptide bond uses the pyrrolidine ring, nitrogen thus constraining rotation). Thus, specific amino acid side chains within a peptide or protein can rotate “out of plane” relative to one another, creating antibodies that can detect both of the target-modified amino acids, one of them, or neither, depending on whether the antibody determinants are in or out of plane with the specific modified amino acid side

FIGURE 2. Immunodepletion of serum to generate an antibody to histone H3 K4Me1. Rabbits were immunized with a peptide conjugated to KLH containing the epigenetic modification monomethyl lysine (KMe1) at amino acid 4 of histone H3. After multiple immunizations, serum was obtained and analyzed by dot blots (A) against peptides that had been applied to a PVDF membrane (0.5–10 pmol of peptide). (Lane 1) Unmodified H3 1-11 peptide; (lane 2) H3K4Me1, 1-11; (lane 3) H3K4Me2, 1-11. The serum was subjected to three rounds of immunodepletion using an unmodified peptide attached to cross-linked agarose followed by affinity purification using the immunogen peptide and reanalyzed on dot blots (B).

chains. In each case, the antibodies that do not detect the specific dual-modified target must be removed to generate a specific antibody for the intended target.

In the case of antibodies being produced to target epigenetic modifications, the extent of immunodepletion required to produce specific antibodies to a lysine methylation state could require many different peptides—unmodified lysine as well as K-acetyl, KMe1, KMe2, and/or KMe3, with the exception, of course, of the modified site that is being targeted. In some cases, additional peptides containing the same modification but within a different primary sequence might also be required. For example, histone H3 is modified at both Lys-9 and Lys-27, and these sequences share four amino acids including the modified lysine, ARKS. To obtain an antibody specific for the K9Me3 (trimethyl) modification, additional immunodepletion using the K27Me3 peptide is required. Given that the amino-terminal tail of histones H3 and H4 are known to contain a large number of modified amino acids (and many with multiple potential modified states), the number of possible peptides that could be considered for immunodepletion rises geometrically. The use of a peptide array containing dozens of epigenetically modified peptides is often used to determine the combination of immunodepletion steps required to obtain the required specificity.

As shown in Figure 3 for the production of an antibody specific for the dual modification of phosphorylated threonine 6 and trimethyl lysine 9 of histone H3 (H3T6pK9Me3), the use of a large number of peptides during immunodepletion is sometimes not necessary. In the case of this dual modification, rather than be concerned with preaffinity purification immunodepletion steps with multiple peptides, only the unmodified peptide was used for initial immunodepletion. Following affinity purification of the antibody and subsequent dot-blot analysis (Fig. 3A), we observed cross-reactivity at a low level to a few undesired sites, of which most contained phosphorylated threonine (pT6 only, pT6/K9Me1, and pT6/K9Me2). The only other cross-reactivity that was observed was against the K9Me3 site only. Given that most of the cross-reactivity was to peptides containing pT6, the affinity-purified antibody (after initial immunodepletion with the unmodified peptide) was further immunodepleted with just two peptide resins: peptide containing phosphorylated threonine 6 only or peptide containing the single K9Me3 modification (Fig. 3B). When the resultant antibody was reanalyzed by dot blots, the above additional immunodepletion steps had removed the cross-reactive antibodies.

FIGURE 3. Generation of an antibody that specifically detects a dual modification of histone H3: Affinity purification and immunodepletion. A peptide immunogen was manufactured to the amino-terminal tail of histone H3 (amino acids 1–13) containing phosphorylated threonine 6 (T6p) and trimethyl lysine 9 (K9Me3). A variety of other peptides were also manufactured containing various combinations (or lack thereof) of modifications at K9Me3 and/or T6p, and these peptides along with the immunogen were spotted on PVDF for dot blotting. (A) Dot-blot analysis of immunodepleted (unmodified peptide only) and affinity-purified H3T6pK9Me3 antibody. (B) The antibody prepared in A was further immunodepleted with two peptides: peptide resin containing T6p as the only modification and K9Me3 as the only modification. Ten or 1 pmol of the following peptides were spotted for dot-blot analysis as indicated on the y-axis: (lane 1) unmodified peptide; (lane 2) T6p-modified peptide; (lane 3) T6pK9Me1-modified peptide; (lane 4) T6pK9Me2-modified peptide; (lane 5) T6pK9Me3-modified peptide; (lane 6) unmodified peptide; (lane 7) K9Me1-modified peptide; (lane 8) K9Me2-modified peptide; (lane 9) K9Me3-modified peptide; (lane 10) K9-acetyl-modified peptide.

A straightforward immunodepletion method may not be the best scenario for the purification of all antibodies produced to multiply modified targets. The above example, however, shows the importance of careful analysis during the purification process to minimize cost, effort, and exposure of the antibody and/or serum to multiple peptide columns. The utility of an antibody is only as good as the specificity and sensitivity of the final product. When care and planning are an integral part of an overall antibody production and purification program, the final product is often a high-quality immunological reagent with many useful applications. This is of paramount importance for the production of modification state-specific antibodies.

LARGE-SCALE ANTIBODY PRODUCTION

With the advent of therapeutic antibodies and their growth as treatments for cancer and autoimmune diseases, robust methods have been developed to produce large quantities of highly purified antibodies prepared under cGMP guidelines (Kelley 2007). Because a large number of the therapeutic antibodies are recombinant, the challenges in obtaining purified antibody preparations are a bit different from the purification of antibodies from serum. The final antibody preparations must be free from numerous contaminants, including bacteria, viruses, mold, prions, endotoxins, glycans, other proteins, and DNA (USFDA 2001; <http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Blood/ucm076753.htm>). Filter sterilization can remove infectious agents; however, the chromatography medium used to purify the antibody requires extensive cleaning in place (CIP) to ensure that the chromatographic medium does not introduce contaminants during the purification process. CIP can be performed with 0.5 N NaOH, or, if the material is compatible, autoclaving can be used.

Owing to its specificity and the availability of a low-cost recombinant form, Protein A is often used as a primary purification step for large-scale purification. Additional steps are also necessary to obtain the purity required. Although column chromatography is appropriate for the isolation of small-scale antibody preparations, it is not scalable because of back-pressure issues, nonuniform bead geometry, nonuniform packing, lack of optimization, and capacity. As an alternative to resin-based purification methods, various manufacturers have developed membrane technologies for the high-throughput purification of proteins including antibodies.

There are several obvious advantages with using membranes for antibody purification. Unlike resins, the membranes can be constructed with large pore sizes to facilitate high flow rates. Instead of flowing through a column, feed streams can move across the membrane (and through a multistage stacked membrane device) via convective flow at very high flow rates. Membranes can be stacked, or porous material with large pore sizes can be constructed with more traditional filter-capsule-based flow, enabling reasonable scale-up for large-scale applications. High resolution can be achieved through a balance of overall membrane capacity, flow rate, and pore size coupled with ionic strength and pH. Currently, there are several options on the market for membrane-based products. Pall Corporation provides a single-use format membrane called the Mustang that is the largest single-use format available. The XT5000 is a single-use 5-L capsule that eliminates the need for CIP/sanitization steps, and it can accept flow rates of up to 50 L/min. Sartorius has developed Sartobind, which uses single-use, ion-exchange capsules based on their 3- μ m pore size macroporous membrane, which can be used with flow rates up to 15 L/min. Multiple chemistries are available for both of these membrane platforms, including anion, cation, and Protein A. They are particularly useful for the removal of viruses, DNA, unwanted proteins, and endotoxins.

ANTIBODY STORAGE

As with any biochemical reagent, the ultimate application or any further downstream methods (e.g., labeling, fragmentation) that will be used must be taken into consideration during the

planning stages of purification of an antibody. Counterions, inhibitors (such as sodium azide), conjugation to/with other moieties, the loss of activity or the tendency to aggregate during freeze–thaw cycles, and the nature of the antibody itself must all be carefully weighed to avoid the loss of product or product activity as well as provide a reagent with the widest possible number of applications.

The first consideration is whether to add sodium azide for the inhibition of bacterial growth. Please note that not only is sodium azide toxic to cells, but it also inhibits the labeling of antibody with reagents (e.g., dyes, biotin) that target the free amine of the lysine side chain (or the amino-terminal amine if present). If preparations are to be labeled or modified in any way, the inclusion of other proteins to aid in stability (i.e., BSA) must also be avoided. Whereas sodium azide can be removed via dialysis given the difference in mass between an antibody (150,000 Da) and sodium azide (65 Da), the presence of other proteins would require much more involved methods of separation such as Protein A or Protein G repurification.

The second consideration is the temperature at which the antibody is to be stored. Freeze–thaw cycles are known to cause a loss of activity over time; however, the actual temperature that should be used for storage will depend a bit on the conditions under which the antibody will be formulated. Glycerol (typically 50% [v/v]; 53% by weight) is often used to improve antibody activity during long-term freezing. Acting as a cryoprotectant, glycerol remains in a fluid-like state when added at a final concentration of 50% (the freezing point for a 50% solution of water:glycerol is -26.0°C) (Lane 1925). Thus, the storage of antibodies containing 50% glycerol at -20°C keeps the antibody solution in a desirable fluid-like state. Below the freezing point of any storage solution, proteins can be damaged by ice crystals. One note of caution: Because glycerol can be contaminated with bacteria, antibody solutions should be filter-sterilized using low protein-binding filters such as a hydrophilic polyethersulfone (PES) or PVDF membrane. The viscosity of 100% glycerol precludes its filtration before dilution.

Antibodies can vary with respect to their stability, and therefore storage conditions for large antibody preparations have to be worked out for each antibody. Antibodies have evolved to be stable to breakdown over a wide variety of conditions. Many can be stored at 4°C for days, weeks, or even months without a loss of activity. Before freezing, the antibody solutions should be broken down into smaller, working-sized aliquots to minimize freeze–thaw cycles. Once an aliquot has been thawed, it should be kept at 4°C and not refrozen. The conjugation of antibodies with other proteins (enzymes, fluorescent proteins) or fluorophores can alter their stability. Antibodies that have been conjugated with any type of molecule or protein should be stored in the dark, and exposure to light should be minimized. In the case of antibodies that have been modified with proteins (e.g., HRP), these should not be stored frozen. Storage at 4°C is typically recommended to ensure the stability of the enzyme activity.

Large-scale antibody preparations, particularly those destined for therapeutic application, present a wider range of issues with respect to long-term storage. In many cases, lyophilization (freeze-drying) is the accepted manner for long-term storage. There are several parameters that must be taken into account to properly freeze-dry protein preparations in general and antibody preparations specifically, including the concentration of the protein solution that will be lyophilized, and the inclusion of excipients such as sucrose, trehalose, Polysorbate 80, or other molecules that can form a crystalline matrix and stabilize the protein. Exercise caution, however, when using some excipients, such as Polysorbate 80, because they have been linked to nonimmunological anaphylactic shock (Coors et al. 2005). Although discussions of all the issues that need to be considered during lyophilization are beyond the scope of this introduction, specific issues include protein stability during the lyophilization process due to pH changes, the effects of protein concentration that determine the exposure of the protein to the ice/water interface, and changes in protein conformation (resulting in loss of activity) during the freeze-drying process. The *Handbook of Therapeutic Antibodies* (Dubel 2007) is a good resource for the issues related to the production, purification, formulation, and long-term storage of therapeutic antibodies.

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