Antibody Purification

Overview

Antibodies are proteins; therefore, methods of purification from biological samples (serum, ascites fluid or culture supernatant) are really specialized forms of general protein purification methods (see the Protein Purification section of the Pierce Technical Handbook and Catalog). Antibody purification can be rather crude (as in precipitation of a fraction containing immunoglobulins and other proteins), particular to immunoglobulins as a group or highly specific to only those antibodies in a sample that bind to a given antigen.

Crude purification of antibodies can be accomplished by methods such as ammonium sulfate precipitation, thiophilic adsorption or simple ionic exchange chromatography. Such methods are useful in many circumstances and often yield antibodies of sufficient purity to use as unlabeled probes in immunoassays and immunoblotting experiments. In the pages that follow, ammonium sulfate precipitation is mentioned briefly and thiophilic adsorption is described in some detail. A unique precipitation method for purification of chicken antibodies (IgY) is also described.

In large commercial applications, resources may be available to develop and optimize specific ion exchange or other chromatographic systems to purify particular immunoglobulins. However, most researchers in academic laboratories and small-scale production facilities affinity-purify antibodies using one of several immobilized proteins and lectins that are known to bind specifically to immunoglobulins of interest.

In affinity chromatography (i.e., affinity purification), a ligand is covalently coupled to a solid support material such as cross-linked beaded agarose gel. Sample fluids are passed through the support material, allowing immunoglobulins to bind to the immobilized ligand. After non-bound sample components are washed from the support, washing buffer conditions are altered so that the immunoglobulins are dissociated (i.e., eluted) from the immobilized ligand and recovered from the support in a purified form.

Protein A and Protein G, and recombinant forms thereof, are bacterial cell wall components that bind primarily to the Fc region of immunoglobulins and are by far the most popular choices for affinity purification of IgG. Protein L is a third protein of bacterial origin that has been developed for use in affinity purification of antibodies; it binds to several different classes of immunoglobulin (e.g., IgG, IgM, IgA) if they have particular kappa light chains. Jacalin, an α-D-galactose binding lectin extracted from jackfruit seeds, binds quite specifically to human IgA. Mannan binding protein (MBP) binds to and allows purification of mouse or human IgM.

As with any affinity purification method, binding and elution buffers are an important component in antibody purification using the ligands listed previously. Generally, ionic strength and pH are the most important factors affecting efficient binding and subsequent elution of immunoglobulin from these ligands. However, temperature and other components are also critical in particular cases. In addition to offering the immobilized ligands mentioned above, Pierce offers a full line of binding and elution buffer products.

The “alphabet proteins” (Protein A, G and L), as well as Jacalin and MBP, purify antibodies in a general way. They bind to particular Fc or Fv portions common to one or multiple immunoglobulin classes, regardless of antigen specificity. For example, Protein A will purify all IgG from rabbit serum, but only 2-5% of that purified IgG will comprise antibody that is specific to the antigen of interest.

When IgG is to be purified from serum, Melon™ Gel provides an excellent alternative to the “alphabet proteins.” Melon™ Gel purifies antibodies based on a negative selection, wherein most proteins bind to the support, but IgG remains in solution and is collected in the flow-through. This eliminates the need to choose an elution buffer and subject antibodies to harsh elution conditions, and it allows the entire procedure to be completed in approximately 15 minutes. Melon™ Gel purifies antibodies from many different host species and generally yields higher recovery and purity of polyclonal antibodies than either Protein A or Protein G.

To purify antigen-specific antibodies, the antigen itself must be immobilized to a support and then used to affinity-purify immunoglobulins that bind it. Descriptions of activated affinity supports for immobilizing proteins and other kinds of antigens, as well as a general description of affinity purification, are given in the Pierce catalog, or in the Affinity Purification Handbook shown on page 41. Many of the same issues that were described with regard to hapten-carrier protein conjugations are relevant to immobilization of haptens and other antigens for affinity purification of antibodies.

Table 4. Properties of antibody-binding proteins.

	Recombinant Protein L	Native Protein A	Recombinant Protein A	Recombinant Protein G	Recombinant Protein A/G
Source	*Peptostreptococci*	*Staphylococcus aureus*	*Bacillus*	*Streptococci*	*Bacillus*
Molecular Weight	35,800	42,000	44,600	22,000	50,449
Number of Binding Sites for IgG	4	4	5	2	4
Albumin-Binding Site	No	No	No	No	No
Optimal Binding pH	7.5	8.2	8.2	5	5-8.2
Binds to	V_LK	Fc	Fc	Fc	Fc

Table 5. Binding characteristics of immunoglobulin-binding proteins.

Species	Antibody Class	Protein A	Protein G	Protein A/G	Protein L**	T-Gel™ Adsorbent
Human	Total IgG	S	S	S	S	M
	IgG_1	S	S	S	S	M
	IgG_2	S	S	S	S	M
	IgG_3	W	S	S	S	M
	IgG_4	S	S	S	S	M
	IgM	W	NB	W	S	M
	IgD	NB	NB	NB	S	–
	IgE	M	NB	M	S	–
	IgA	W	NB	W	S	M
	IgA_1	W	NB	W	S	M
	IgA_2	W	NB	W	S	M
	Fab	W	W	W	S	M
	ScFv	W	NB	W	S	M
Mouse	Total IgG	S	S	S	S	S
	IgM	NB	NB	NB	S	M
	IgG_1	W	M	M	S	S
	IgG_{2a}	S	S	S	S	S
	IgG_{2b}	S	S	S	S	S
	IgG_3	S	S	S	S	S
Rat	Total IgG	W	M	M	S	S
	IgG_1	W	M	M	S	S
	IgG_{2a}	NB	S	S	S	S
	IgG_{2b}	NB	W	W	S	S
	IgG_{2c}	S	S	S	S	S
Cow	Total IgG	W	S	S	NB	S
	IgG_1	W	S	S	NB	S
	IgG_2	S	S	S	NB	S
Goat	Total IgG	W	S	S	NB	S
	IgG_1	W	S	S	NB	S
	IgG_2	S	S	S	NB	S
Sheep	Total IgG	W	S	S	NB	S
	IgG_1	W	S	S	NB	S
	IgG_2	S	S	S	NB	S
Horse	Total IgG	W	S	S	–	S
	IgG(ab)	W	NB	W	–	S
	IgG(c)	W	NB	W	–	S
	IgG(T)	NB	S	S	–	S
Rabbit	Total IgG	S	S	S	W	M
Guinea Pig	Total IgG	S	W	S	W	S
Pig	Total IgG	S	W	S	S	S
Dog	Total IgG	S	W	S	–	S
Chicken	Total IgY	NB	NB	NB	M	M
Hamster	Total IgG	M	M	M	S	–
Donkey	Total IgG	M	S	S	–	–
Cat	Total IgG	S	W	S	–	S
Monkey (Rhesus)	Total IgG	S	S	S	–	S

** Data represent a summary of binding properties reported in the literature. Inevitably some discrepancies exist among reported values as a result of differences in binding buffer conditions and form of the proteins used.*

***Binding will occur only if the appropriate kappa light chains are present. Lambda light chains will not bind, regardless of their class and subclass.*

Legend:
W = weak binding S = strong binding M = medium binding NB = no binding – = information not available

Protein A

Protein A Characteristics and IgG Binding Properties

Protein A is a cell wall component produced by several strains of *Staphylococcus aureus*. It consists of a single polypeptide chain (MW 42,000) and contains little or no carbohydrate.[1] Protein A binds specifically to the Fc region of immunoglobulin molecules, especially IgG. It has four high-affinity ($K_{\alpha} = 10^8$ l/mole) binding sites that are capable of interacting with the Fc region of IgGs of several species.[2] The molecule is heat-stable and retains its native conformation even after exposure to denaturing reagents such as 4 M urea, 4 M thiocyanate and 6 M guanidine hydrochloride.[3]

In its immobilized form (e.g., covalently coupled to beaded agarose gel), Protein A has been used extensively for isolation of a wide variety of immunoglobulins from several species of mammals. However, the interaction between Protein A and IgG is not equivalent for all animal sources and subclasses of IgG. For example, human IgG_1, IgG_2 and IgG_4 bind strongly to Protein A, while IgG_3 does not bind.[2] In mice, IgG_{2a}, IgG_{2b} and IgG_3 bind strongly to Protein A, but IgG_1 (the dominant subclass in serum) binds only weakly using standard buffer conditions. Most rat IgG subclasses bind weakly or not at all to Protein A. Despite this variability, Protein A is very effective for routine affinity purification of IgG from the serum of many species. It is especially suited for purification of polyclonal antibodies from rabbits.

Weak binding of Protein A to mouse IgG_1 using traditional Tris•HCl or sodium phosphate buffer systems is of particular concern and is one reason to choose Protein G when purifying mouse antibodies. However, Pierce has developed a binding buffer that allows Protein A to bind mouse IgG_1 nearly as well as other subclasses (see subsequent discussion of IgG Binding and Elution Buffers on page 32).

The variable binding properties of Protein A for different subclasses of IgG can be used advantageously to separate one IgG type from another. Antibodies that do not bind to immobilized Protein A may be recovered by collecting the non-bound ("flow-through") fractions during binding and wash steps in an affinity purification procedure. In this way, human IgG_3 and other immunoglobulin subclasses may be isolated from those that do bind to Protein A; however, other IgGs and serum proteins, such as albumin, will also be present in the non-bound fraction. Certain IgM, IgD and IgA molecules also do not bind to Protein A and may be separated from Protein A-binding proteins in the same manner.

Immobilized Protein A Products

Pierce offers Protein A immobilized to several different solid supports and made available in different binding capacity formats, package sizes and kit formats. ImmunoPure® Immobilized Protein A generally denotes those products using highly purified Protein A that is covalently coupled to 6% cross-linked beaded agarose gel. ImmunoPure® Immobilized Protein A has a binding capacity of 12-19 mg of human IgG per ml of gel. It exhibits excellent elution properties when used with Pierce buffer systems (Figure 10), which generally enable the gel to be regenerated and used for at least 10 rounds of purification. Supplied as a 50% gel slurry in storage buffer, ImmunoPure® Immobilized Protein A is the usual choice either for small-scale batch method purification procedures or for packing gravity-flow columns.

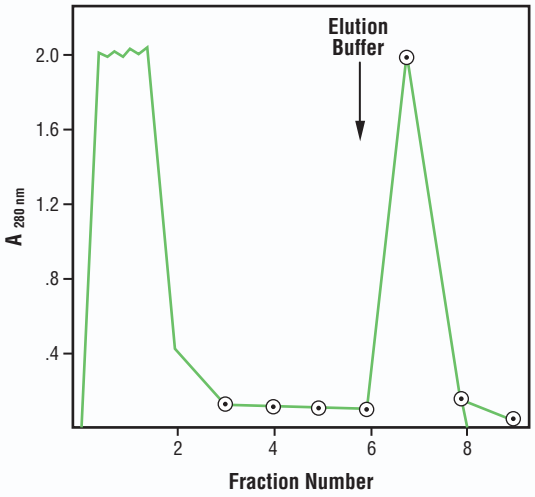


Figure 10. Affinity chromatographic purification of mouse IgG from mouse ascites fluid using Pierce Immobilized Protein A and the ImmunoPure® Buffer System. From 1 ml of mouse ascites fluid, 5.5 mg of mouse IgG was recovered.

Protein A AffinityPak™ Columns (Product # 20356) are 5 x 1 ml pre-packed plastic columns of ImmunoPure® Immobilized Protein A. The stop-flow action of AffinityPak™ Columns prevents the gel bed from drying out when a column is left unattended for short periods of time.

ImmunoPure® Immobilized Protein A is also available on Trisacryl® GF-2000, rather than agarose gel. This stable affinity support can withstand the high-throughput volumes required in large-scale purification procedures. In addition, because Trisacryl® GF-2000 is a hydrophilic matrix, nonspecific binding of proteins is minimized.

UltraLink® Immobilized Protein A is another alternative for large-scale, high-throughput applications. UltraLink® Biosupport Medium is composed of a hydrophilic, cross-linked *bis*-acrylamide/azlactone copolymer. It has an average bead diameter of 60 µm, can withstand pressures exceeding 100 psi, retains good chromatographic properties using flow rates up to 3,000 cm/hour and displays extremely low nonspecific binding. UltraLink® Immobilized Protein A is the ideal choice for medium-pressure liquid chromatographic systems.

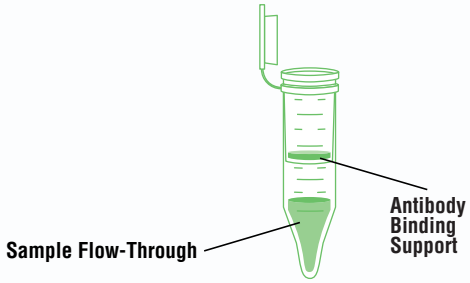
Immobilized Recomb® Protein A (Product # 20365, 20366) uses a genetically engineered form of Protein A that is produced recombinantly in a nonpathogenic form of *Bacillus*. Nonessential regions have been removed, and five IgG-binding sites are included, resulting in a mass of 44.6 kDa.Some researchers believe that the recombinant form should be used if the antibody preparation has strict requirements for being enterotoxin-free. Otherwise, the native form serves as a highly efficient means for purifying antibodies. Immobilized Recomb® Protein A is also compatible with ImmunoPure® Binding and Elution Buffers.

For the greatest convenience, choose the ImmunoPure® (A) IgG Purification Kit (Product # 44667). This kit contains everything needed to isolate IgG from rabbit or mouse serum or ascites fluid, as well as other sample types. The included ImmunoPure® Buffer System provides optimal binding and elution of IgG with Immobilized Protein A. The columns in the kit can be regenerated at least 10 times without a significant loss of binding capacity.

To quickly purify small batches of antibody, use NAb™ Spin Purification Kits. These kits are designed to purify up to 1 mg of antibody in about one hour using a simple, benchtop protocol. NAb™ Kits are available with Proteins A, G or L and all of the reagents needed for antibody purification. The resin in each kit can be reused up to 10 times without loss of activity.

Simplified Bench-top Purification Protocol

1. Wash the gel.
2. Incubate antibody-containing sample with Immobilized Protein A, Protein G or Protein L support.
3. Wash away unbound material.
4. Elute the antibody.



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2. Hjelm, H., *et al.* (1975). *Eur. J. Biochem.* **57**, 395-403.
3. Sjoholm, I., *et al.* (1975). *Eur. J. Biochem.* **51**, 55-61.

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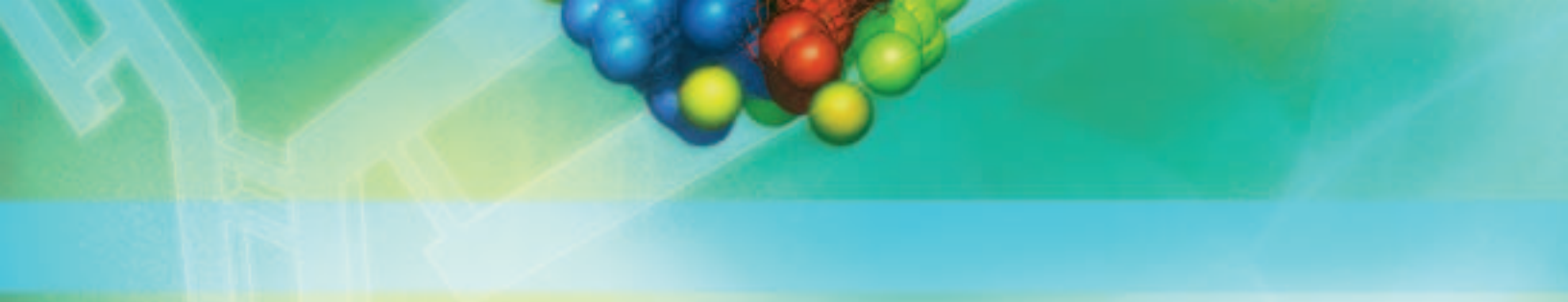

Immobilized Protein A Products

Ordering Information

Product #	Description	Pkg. Size
20333	**ImmunoPure® Immobilized Protein A** Support: Cross-linked 6% beaded agarose Capacity: 12-19 mg human IgG/ml of gel	5 ml
20356	**AffinityPak™ Protein A Columns**	5 x 1 ml
20334	**ImmunoPure® Immobilized Protein A**	25 ml
44667	**ImmunoPure® (A) IgG Purification Kit** *Contains everything needed to isolate IgG from mouse ascites or other serum.* Includes: Immobilized Protein A Columns (A) IgG Binding Buffer IgG Elution Buffer Excellulose™ Desalting Columns	Kit 5 x 1 ml 1,000 ml 500 ml 5 x 5 ml
20338	**ImmunoPure® Immobilized Protein A** Support: Trisacryl® GF 2000 Capacity: >15 mg human IgG/ml of gel	5 ml
53139	**UltraLink® Immobilized Protein A** Support: UltraLink® Biosupport Medium Capacity: ≥16 mg of human IgG/ml of gel	5 ml
21348	**MagnaBind™ Protein A Beads** Support: Superparamagnetic iron oxide beads Capacity: ≥200 µg rabbit IgG/ml of beads	5 ml
15130	**Reacti-Bind™ Protein A Coated 96-Well Plates** Support: 96-well polystyrene plate Capacity: ~1-3 µg rabbit IgG/well	5 plates
15132	**Reacti-Bind™ Protein A Coated Strip Plates** Support: 96-well polystyrene plate (8-well strips) Capacity: ~1-3 µg rabbit IgG/well	5 plates

Immobilized Protein A Plus … with twice the amount of Protein A coupled per ml of gel.

Product #	Description	Pkg. Size
22811	**ImmunoPure® Immobilized Protein A Plus** Support: Cross-linked 6% beaded agarose Capacity: ≥35 mg of human IgG/ml of gel; 16-17 mg mouse IgG/ml of gel	5 ml
22814	**AffinityPak™ Immobilized Protein A Plus Columns**	5 x 1 ml
22812	**ImmunoPure® Protein A Plus**	25 ml
44679	**ImmunoPure® (A) Plus IgG Purification Kit** *Sufficient for isolating 800 mg of mouse IgG.* Includes: Immobilized Protein A Columns Binding Buffer Elution Buffer Excellulose™ Desalting Columns	Kit 5 x 1 ml 1,000 ml 500 ml 5 x 5 ml
53142	**UltraLink® Immobilized Protein A Plus** Support: UltraLink® Biosupport Medium Capacity: ≥30 mg of human IgG/ml of gel	5 ml
45200	**NAb™ Protein A Spin Purification Kit** Includes: Immobilized Protein A Plus Binding Buffer Elution Buffer Spin X Tubes Microcentifuge Tubes	Kit 1 ml 500 ml 50 ml 12 72

References

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Immobilized Recomb® Protein A

Our recombinant form of Protein A, immobilized with a leach-resistant linkage.

Highlights:

- Support: 6% cross-linked agarose beads

References

Murrell, M.T., *et al.* (2001). *J. Virol.* **75**, 6310-6320.

Wang, C.Y., *et al.* (1999). *Proc. Nat. Acad. Sci. USA* **96**, 10367-10372.

Ordering Information

Product #	Description	Pkg. Size
20365	**Immobilized Recomb® Protein A** Capacity: ≥12 mg human IgG/ml of gel using the ImmunoPure® (A) IgG Buffer System	5 ml
20366	**Immobilized Recomb® Protein A** Capacity: ≥12 mg human IgG/ml of gel using the ImmunoPure® (A) IgG Buffer System	25 ml

Protein G

Protein G Characteristics and IgG Binding Properties

Protein G is a bacterial cell wall protein isolated from group G streptococci.[1] Like Protein A from *Staphylococcus aureus*, Protein G binds to most mammalian immunoglobulins primarily through their Fc regions. Protein G binds weakly to Fab fragments.[1] Native Protein G contains two immunoglobulin-binding sites, as well as albumin and cell surface binding sites.[2] In the recombinant form of Protein G, these albumin and cell surface binding sites have been eliminated to reduce nonspecific binding when purifying immunoglobulins. With the albumin site removed, recombinant Protein G can be used to separate albumin from crude human immunoglobulin samples. Recombinant Protein G has a mass of approximately 22 kDa. However, its apparent mass by SDS-PAGE is nearly 34 kDa.

Immobilized Protein G is most commonly used for the purification of mammalian monoclonal and polyclonal antibodies that do not bind well to Protein A. It has been reported that most mammalian immunoglobulins bind with greater affinity to Protein G than Protein A.[1] Protein G binds with significantly greater affinity to several immunoglobulin subclasses including human IgG_3 and rat IgG_{2a}. Unlike Protein A, Protein G does not bind to human IgM, IgD or IgA.[1]

Differences in binding characteristics between Protein A and Protein G are explained by differences in the immunoglobulin-binding sites of each protein. Although the tertiary structures of these proteins are similar, their amino acid compositions differ significantly.

Inconsistency in reporting of Protein G binding characteristics occurs in the literature. One cause for this inconsistency likely results from differences in the particular source and isolation method used for the native Protein G characterized in each study. In addition, several methods have been used to assess relative binding affinity including radiolabeling experiments and ELISA techniques, the results of which are not directly comparable. Finally, significant binding differences result from different binding buffers used with Protein G. Optimal binding for most immunoglobulins to Protein G occurs at pH 5.0,[3] although many studies have used more neutral Tris or phosphate buffers for binding. Approximately 44% more IgG from rat serum bound to Protein G using acetate buffer, pH 5.0 [e.g., ImmunoPure® (G) Binding Buffer, Product # 21011] compared to Tris•HCl pH 7.5 buffer.

Immobilized Protein G Products

Pierce Immobilized Protein G Products incorporate the recombinant form of Protein G immobilized to either 6% cross-linked beaded agarose or UltraLink® Biosupport Medium. For a more detailed description of supports, see the previous pages about Immobilized Protein A Products. Both types of immobilized Protein G utilize coupling chemistries that are leach-resistant and provide a matrix with minimal nonspecific binding. Both supports can be regenerated and reused multiple times when stored properly.

Like Immobilized Protein A already discussed, Immobilized Protein G is offered in several package sizes and kit formats. The ImmunoPure® (G) IgG Purification Kit includes a 2 ml pre-packed column of ImmunoPure® Immobilized Protein G, as well as binding and elution buffers and desalting columns. The 2 ml affinity column will bind 20-30 mg of human IgG when using the included ImmunoPure® Buffers.

References

1. Bjorck, L. and Kronvall, G. (1984). *J. Immunol.* **133,** 969-974.
2. Guss, B., *et al.* (1986). *EMBO J.* **5,** 1567-1575.
3. Åkerström, B. and Bjorck, L. (1986). *J. Biol. Chem.* **261,** 10240-10247.

Immobilized Protein G Products

Gives better selectivity for IgG isotype than Protein A.

Ordering Information

Product #	Description	Pkg. Size
20398	**ImmunoPure® Immobilized Protein G** Support: Cross-linked 6% beaded agarose Capacity: 11-15 mg human IgG/ml of gel	2 ml
20399	**ImmunoPure® Immobilized Protein G**	10 ml
44441	**ImmunoPure® (G) IgG Purification Kit** Includes: Immobilized Protein G Column (G) Binding Buffer IgG Elution Buffer Excellulose™ Desalting Columns	Kit 1 x 2 ml 240 ml 120 ml 5 x 5 ml
53125	**UltraLink® Immobilized Protein G** Support: UltraLink® Biosupport Medium Capacity: ≥20 mg of human IgG/ml of gel	2 ml
53127	**UltraLink® AffinityPak™ Immobilized Protein G Columns**	2 x 2 ml
53126	**UltraLink® Immobilized Protein G**	10 ml
21349	**MagnaBind™ Protein G Beads** Support: Superparamagnetic iron oxide beads Capacity: ≥200 µg rabbit IgG/ml beads	5 ml

References

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Immobilized Protein G Plus … with twice the amount of Protein G immobilized per ml of gel.

Product #	Description	Pkg. Size
22851	**ImmunoPure® Immobilized Protein G Plus** Support: Cross-linked 6% beaded agarose Capacity: ≥20 mg human IgG/ml of gel	2 ml
22852	**ImmunoPure® Immobilized Protein G Plus**	10 ml
53128	**UltraLink® Immobilized Protein G Plus** Support: UltraLink® Biosupport Medium Capacity: ≥25 mg of human IgG/ml of gel	2 ml
45201	**NAb™ Protein G Spin Purification Kit** Includes: Immobilized Protein G Plus Binding Buffer Elution Buffer Spin X Tubes Microcentrifuge Tubes	Kit 1 ml 500 ml 50 ml 12 72
15131	**Reacti-Bind™ Protein G Coated 96-Well Plates** Support: 96-well polystyrene plate Capacity: ~1-3 µg rabbit IgG/well	5 plates
15133	**Reacti-Bind™ Protein G Coated Strip Plates** Support: 96-well polystyrene plate (8-well strips) Capacity: ~1-3 µg rabbit IgG/well	5 plates

Protein A/G

Protein A/G is a genetically engineered protein that combines the IgG binding profiles of both Protein A and Protein G. Protein A/G is a gene fusion product secreted from a nonpathogenic form of *Bacillus*. Protein A/G (MW 50,449) is designed to contain four Fc binding domains from Protein A and two from Protein G. The secreted protein is readily isolated in a pure form from fermentation medium. Protein A/G is not as pH-dependent as Protein A (Figure 11), but otherwise has the additive properties of Protein A and G.

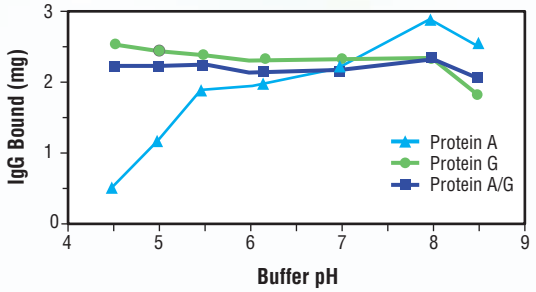

Figure 11. Comparison of the binding characteristics of mouse IgG at various buffer pH levels.

Protein A/G binds to all human IgG subclasses. In addition, it binds to IgA, IgE, IgM and, to a lesser extent, IgD. Protein A/G also binds well to all mouse IgG subclasses but does not bind mouse IgA, IgM or serum albumin.[1] This makes Protein A/G an excellent tool for purification and detection of mouse monoclonal antibodies from IgG subclasses, without interference from IgA, IgM and murine serum albumin. Individual subclasses of mouse monoclonals are more likely to have a stronger affinity to the chimeric Protein A/G than to either Protein A or Protein G.[2]

Immobilized Protein A/G is an ideal choice for purification of polyclonal or monoclonal IgG antibodies whose subclasses have not been determined. Overall binding capacity is greater when pH 8.0 buffer (optimal for Protein A) is used rather than pH 5.0 buffer, which is optimal for Protein G used alone. Furthermore, ImmunoPure® (A) Binding Buffer provides for greater binding than Tris•HCl, pH 8.0 (see description of IgG Binding and Elution Buffers on page 32).

Immobilized Protein A/G is offered in similar package sizes and kit formats as Immobilized Protein A and Protein G. ImmunoPure® (A/G) IgG Purification Kit, like the Protein G Purification Kit, includes a single 2 ml affinity column and accessories.

References

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ImmunoPure® Immobilized Protein A/G and (A/G) IgG Purification Kit

Binds all IgG species that bind both Protein A and Protein G, taking the guesswork out of isolating your antibody.

Ordering Information

Product #	Description	Pkg. Size
20421	**ImmunoPure® Immobilized Protein A/G** Support: Cross-linked 6% beaded agarose Capacity: ≥7 mg of human IgG/ml gel	3 ml
20422	**ImmunoPure® Immobilized Protein A/G**	15 ml
44902	**ImmunoPure® (A/G) IgG Purification Kit** Includes: Immobilized Protein A/G Column IgG Binding Buffer IgG Elution Buffer Desalting Columns	Kit 1 x 2 ml 240 ml 120 ml 5 x 5 ml

Product #	Description	Pkg. Size
53132	**UltraLink® Immobilized Protein A/G** Support: UltraLink® Biosupport Medium Capacity: ≥20 mg of human IgG/ml gel	2 ml
53133	**UltraLink® Immobilized Protein A/G**	10 ml

Immobilized Protein A/G Plus… with twice the amount of Protein A/G per ml of gel.

Product #	Description	Pkg. Size
53135	**UltraLink® Immobilized Protein A/G Plus** Support: UltraLink® Biosupport Medium Capacity: ≥28 mg human IgG/ml gel	2 ml

Product #	Description	Pkg. Size
15138	**Reacti-Bind™ Protein A/G Coated Strip Plates** Support: 96-well polystyrene plate (8-well strips) Capacity: ~1-3 µg rabbit IgG/well	5 plate

Reference

1. Preston, G.A., *et al.* (2004). *J. Biol. Chem.* **279**, 4260-4268.

Protein L

Protein L is an immunoglobulin-binding protein (MW 35,800) that originates from the bacteria *Peptostreptococcus magnus*, but is now produced recombinantly. Unlike Protein A and Protein G, which bind primarily through Fc regions (i.e., heavy chain) of immunoglobilins, Protein L binds immunoglobulins through interactions with their light chains. Since no part of the heavy chain is involved in the binding interaction, Protein L binds a wider range of Ig classes than Protein A or G. Protein L will bind to representatives of all classes of Ig including IgG, IgM, IgA, IgE, IgD and IgY. Single-chain variable fragments (ScFv) and Fab fragments can also be bound by Protein L.

Despite this wide-ranging binding capability with respect to Ig classes (which are defined by heavy chain type), Protein L is not a universal immunoglobilin-binding protein. Binding of Protein L to immunoglobulins is restricted to those containing kappa light chains (i.e., κ chain of the V_L domain).[1] In humans and mice, kappa (κ) light chains predominate. The remaining immunoglobulins have lambda (λ) light chains. Furthermore, Protein L is effective in binding only certain subtypes of kappa light chains. For example, it binds human VκI, VκIII and VκIV subtypes but does not bind the VκII subtype. Binding of mouse immunoglobulins is restricted to those having VκI light chains.[1]

Given these specific requirements for effective binding, immobilized Protein L is not appropriate for general polyclonal antibody purification from serum, which contains a mixture of immunoglobulins having different types of light chains. The main application for immobilized Protein L is purification of monoclonal antibodies from ascites or culture supernatant that are known to have the kappa light chain.

Protein L is extremely useful for purification of V_Lκ-containing monoclonal antibodies from culture supernatant because it does not bind bovine immunoglobilins, which are present in the media serum supplement. Also, in contrast to Protein A and G, Protein L is very effective at binding IgM. Although it binds to the Fab portion of the immunoglobulin monomer, Protein L does not interfere with the antigen-binding site of the antibody. Therefore, Protein L potentially can be used in immunoprecipitation (IP) procedures.

ImmunoPure® Immobilized Protein L is offered in several formats including gel slurries, pre-packed gravity-flow columns and as column and spin cup kits.

Reference

1. Nilson, B., *et al.* (1992). *J. Biol. Chem.* **267**, 2234-2238.
Åkerström, B. and Björck, L. (1989). *J. Biol. Chem.* **264,** 19740-19746.
Björck, L., *et al.* (1988). *J. Immunol.* **140,** 1194-1197.
Kastern, W., *et al.* (1992). *J. Biol. Chem.* **267,** 12820-12825.
Nilson, B.H., *et al.* (1993). *J. Immunol. Method* **164,** 33-40.

ImmunoPure® Protein L Products

Purify ScFv or Fab fragments that have kappa light chains.

Highlights:

- Binds to the VL region of kappa light chains (human I, III and IV and Mouse I) without interfering with antigen-binding sites
- Binds to all classes of IgG (e.g., IgG, IgM, IgA, IgE, IgD and IgY)
- Does not bind bovine, goat or sheep immunoglobulins
- Binds single-chain variable fragments (ScFv)
- Binds chicken IgY

Applications:

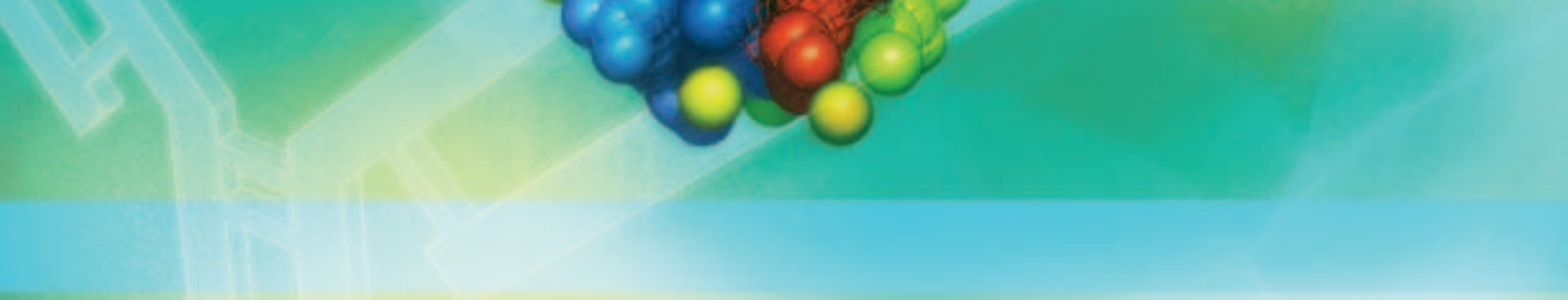
- Purification/detection of ScFv and Fab fragments containing kappa light chains
- Purification/detection of IgG, IgM, IgA, IgE, IgD and IgY
- Purification of monoclonal antibodies from BSA- or FCS-supplemented media because Protein L does not bind bovine antibodies
- Purification/detection of recombinantly produced or engineered antibodies

References

Drykova, D., *et al.* (2003). *Plant Cell.* **15**, 465-480.
Nozawa, K., *et al.* (2001). *J. Immunol.* **167**, 4981-4986.

Ordering Information

Product #	Description	Pkg. Size
20510	**ImmunoPure® Immobilized Protein L** Support: Cross-linked 6% beaded agarose Capacity: ≥5 mg human IgG/ml of gel	2 ml
20540	**AffinityPak™ Protein L Columns** Capacity: ≥5 mg human IgG/ml of gel	2 x 2 ml
20520	**ImmunoPure® Immobilized Protein L Plus** Capacity: 8-10 mg human IgG/ml of gel	2 ml
20550	**ImmunoPure® (L) Immunoglobulin Purification Kit**	Kit
	Includes: Immobilized Protein L Column	1 x 2 ml
	Binding Buffer	500 ml
	Elution Buffer	120 ml
	Excellulose™ Desalting Columns	5 x 5 ml
20530	**NAb™ Protein L Spin Purification Kit** Capacity: 8-10 mg human IgG/ml of gel	Kit
	Includes: Immobilized Protein L Plus	1 ml
	Binding Buffer	500 ml
	Elution Buffer	50 ml
	Spin X Tubes	12
	Microcentrifuge Tubes	72
15190	**Reacti-Bind™ Protein L Coated 96-Well Plates** Support: 96-well polystyrene plate (8-well strips) Capacity: ~1-3 µg IgG/well	5 plates



IgG Binding and Elution Buffers for Protein A, G, A/G and L

Binding and Elution Steps in Affinity Purification

Affinity purification procedures involving interaction of an antibody with its antigen generally use binding buffers at physiologic pH and ionic strength. However, many antibody purification methods do not use the antibody-antigen interaction; rather, they involve binding of antibodies by immobilized ligands that are not the antigen. In such cases, optimal binding conditions are determined by the unique properties of the antibody-ligand interaction, which may be different from physiologic pH and ionic strength.

Once the binding interaction occurs (i.e., the antibody is "captured" by the immobilized ligand), the support is washed with additional buffer to remove nonbound components of the sample. Finally, elution buffer is added to break the binding interaction and release the target molecule, which is then collected in its purified form. Elution buffer can dissociate binding partners by extremes of pH (low or high), high salt (ionic strength), the use of detergents or chaotropic agents that denature one or both of the molecules, removal of a binding factor, or competition with a counter ligand. In most cases, subsequent dialysis or desalting is required to exchange the purified protein from elution buffer into a more suitable buffer for storage or use.

ImmunoPure® IgG Binding and Elution Buffers have been optimized to provide the highest possible efficiency of IgG binding and elution using immobilized Protein A, Protein G and Protein A/G. Use of other buffer formulations may significantly alter not only the binding capacity but also the volumes of wash buffer required to ensure good purification.

General Binding and Elution Buffers for Protein A, G, A/G and L

Although Protein A, G and A/G bind immunoglobulins adequately at physiologic pH and ionic strength (as with phosphate buffered saline, pH 7.2), optimal binding conditions are different for each protein. For this reason, Pierce offers separate ImmunoPure® IgG Binding Buffers for use with the immobilized "alphabet protein" products. All ImmunoPure® Buffers have long shelf lives and are premixed for maximum ease of use.

The ImmunoPure® (A) IgG Binding Buffer is a unique, phosphate-based formulation (pH 8.0) developed by Pierce scientists to achieve maximum binding capacity of IgG to immobilized Protein A. Overall IgG binding capacity is increased with this buffer relative to traditional binding buffers (Table 6). Most notably, the otherwise weak binding of mouse IgG_1 is greatly improved.

Table 6. Binding capacities with different buffers expressed as mg of IgG bound per 2 ml of gel.

	Immobilized Protein A		Immobilized Protein G		Immobilized Protein A/G	
Serum Sample	0.1 M Tris•HCl pH 8.0	ImmunoPure® (A) Binding Buffer	0.1 M Tris•HCl pH 8.0	ImmunoPure® (G) Binding Buffer	0.1 M Tris•HCl pH 8.0	ImmunoPure® (A/G) Binding Buffer
Rabbit	17.81	33.19	21.51	27.75	13.89	19.61
Sheep	2.15	10.64	25.53	33.33	9.83	15.71
Bovine	6.16	22.76	31.72	48.10	15.13	22.06
Mouse	5.25	7.15	5.65	15.05	4.32	11.49
Rat	4.99	8.30	8.43	11.80	5.20	6.66
Horse	6.25	16.50	36.19	21.46	14.88	17.12
Dog	35.77	22.27	13.38	20.55	21.96	24.60
Chicken	0.91	1.21	1.63	7.27	1.21	4.10
Pig	29.61	24.83	21.25	27.51	19.24	29.48
Human	19.88	25.53	11.68	23.59	9.92	17.67

ImmunoPure® (G) IgG Binding Buffer uses sodium acetate (pH 5.0) to obtain the highest possible binding capacity of IgG to immobilized Protein G. The binding buffer for Protein A/G is similar to the ImmunoPure® IgG Binding Buffer for Protein A. The optimal binding with Protein L occurs at pH 7.5; ImmunoPure® Protein L Kits use phosphate buffered saline (PBS) as the binding buffer.

Generally, an ImmunoPure® Binding Buffer is used by combining it 1:1 (v/v) with clarified serum or ascites fluid. For a dilute sample, or to minimize its total volume, a sample can be dialyzed into the recommended buffer. Purity of the immunoglobulin samples will affect the total binding capacity of Protein A, G and A/G; total immunoglobulin binding capacities are higher for purified and concentrated immunoglobulins than for crude serum or dilute samples.

Elution of immunoglobulins that are bound to immobilized alphabet proteins, regardless of the binding buffer used, is most often accomplished using 0.1 M glycine•HCl (pH 2-3) or other low pH buffer. In the vast majority of cases, this condition breaks affinity interactions without damaging either the immobilized protein (allowing the affinity column to be re-used) or the antibody. ImmunoPure® IgG Elution Buffer uses this acidic (pH 2.8) condition. With this buffer, elution of IgG is usually sharp and complete. For example, nearly all bound IgG will elute in 3 ml of buffer from a 1 ml column of Protein A.

Although brief exposure of antibody to acidic elution buffer usually is not harmful, it is advisable to neutralize the eluate as soon as possible after its recovery to minimize the possibility of degradation. ImmunoPure® IgG Elution Buffer can be neutralized easily by adding 1/10th volume of 1 M Tris•HCl, pH 7.5-9.0. Although long-term storage of the purified antibody in the neutralized buffer may be possible in certain cases, it is common practice to dialyze or desalt into a buffer that is known to be suitable for storage.

Gentle Ag/Ab Elution Buffer

Some antibodies are extremely labile and irreversibly denature in the acidic conditions of the default ImmunoPure® IgG Elution Buffer. For such situations, Pierce offers ImmunoPure® Gentle Ag/Ab Elution Buffer. This near-neutral (pH 6.55) buffer dissociates affinity-bound immunoglobulins by ionic strength rather than by low pH. While being much less likely to degrade an antibody, it still retains excellent elution properties.

Pierce researchers have tested the effect of exposure to Gentle Elution Buffer on monoclonal antibody activity. In one experiment, three mouse monoclonals were incubated overnight in the Gentle Elution Buffer and then desalted. When analyzed in an ELISA system, all three monoclonals retained full antigen-binding capability as compared to untreated controls.

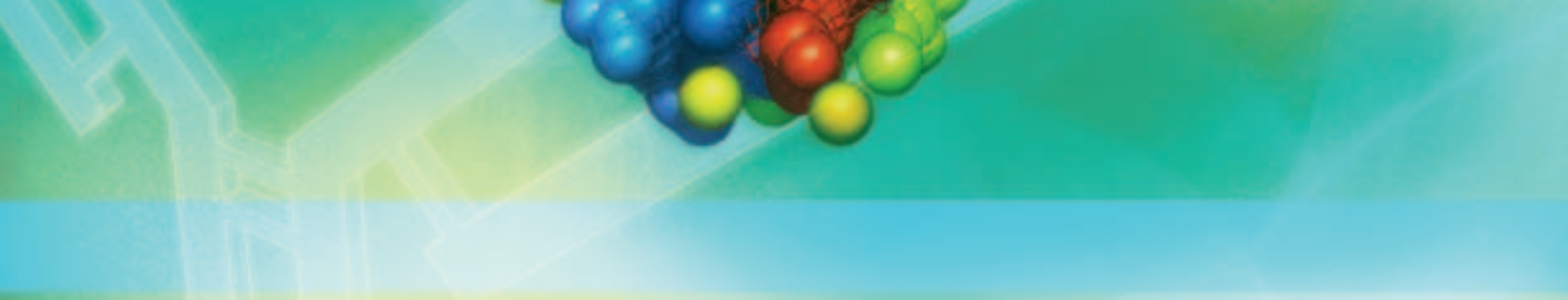
The Gentle Elution Buffer does not require neutralization and is directly compatible with borate, citrate and acetate buffers including ImmunoPure® (G) IgG Binding Buffer. However, Gentle Elution Buffer is not directly compatible with phosphate-containing buffers including ImmunoPure® (A) IgG Binding Buffer, with which it will form an insoluble precipitate. For this reason, ImmunoPure® Gentle Ag/Ab Binding Buffer, pH 8.0 is offered as a substitute for use with Protein A.

Mouse IgG_1 Mild Elution Buffer

A unique opportunity exists in Protein A with its weaker binding affinity to mouse IgG_1 compared to other mouse IgG subclasses. After binding total mouse IgG to immobilized Protein A using ImmunoPure® (A) IgG Binding Buffer, ImmunoPure® Mouse IgG_1 Mild Elution Buffer can be used to selectively elute IgG_1 without affecting the bound state of other IgG subclasses.

The buffer has a mild pH (6.0-6.1) to retain better biological activity in both the recovered antibody and the immobilized Protein A. Neutralization or desalting of the collected IgG_1 is not necessary to retain activity. This advantage is especially important when isolating potentially fragile monoclonal IgG_1 antibodies. Because the majority of mouse monoclonals are of the IgG_1 subclass, this buffer has many applications in the production of monoclonal antibodies.

After eluting the IgG_1, other bound IgGs can be eluted using standard IgG Elution Buffer. ImmunoPure® (A) Binding Buffer and both IgG and IgG_1 Mild Elution Buffers are available as a kit. The system enables quick, clean and mild isolation of mouse IgG_1 from serum, ascites or hybridoma culture supernatant.



Ordering Information

Product #	Description	Highlights	Pkg. Size
54200	ImmunoPure® (A/G) IgG Binding Buffer	• Assures maximum recovery of IgG from immobilized Protein A/G	240 ml
21001	ImmunoPure® (A) IgG Binding Buffer	• High-yield isolation of Mouse IgG_1 using Protein A columns	1 L
21007		• Premixed and easy to use	3.75 L
21011	ImmunoPure® (G) IgG Binding Buffer	• Assures maximum recovery of IgG from immobilized Protein G	3.75 L
21004	ImmunoPure® IgG Elution Buffer	• High-yield isolation of IgG from immobilized Protein A and Protein G	1 L
21009			3.75 L
21020	ImmunoPure® Gentle Ag/Ab Binding Buffer, pH 8.0	• Specially formulated and prefiltered	1 L
21012		• Eliminates use of harsh acidic elution conditions	3.75 L
21030	ImmunoPure® Gentle Ag/Ab Elution Buffer, pH 6.6	• Specially formulated for neutral pH elutions	100 ml
21027		• Not compatible with phosphate buffers	500 ml
21013			3.75 L
21016	ImmunoPure® IgM Binding Buffer	• Specially formulated for optimal binding of mouse IgM	800 ml
21017	ImmunoPure® IgM Elution Buffer	• Specially formulated for optimal recovery of mouse IgM	500 ml
21018	ImmunoPure® MBP Column Preparation Buffer	• Specially formulated for use with immobilized MBP and IgM Purification Kit	50 ml
21034	Mouse IgG_1 Mild Elution Buffer	• Separate IgG_1 from other IgG subclasses	500 ml
21033	Mouse IgG_1 Mild Binding and Elution Buffer Kit Includes: ImmunoPure® (A) IgG Binding Buffer Mouse IgG_1 Mild Elution Buffer ImmunoPure® IgG Elution Buffer	• Complete kit to allow mouse IgG_1 to be separated from other mouse IgG subclasses	Kit 1 L 500 ml 1 L

References

Al-Hallaq, R.A., *et al.* (2002). *Mol. Pharmacol.* **62**, 1119-1127.

Harsay, E. and Schekman, R. (2002). *J. Cell Biol.* **156**, 271-286.

Kierszenbaum, A.L., *et al.* (2003). *Mol. Biol. Cell.* **14**, 4628-4640.

Thiophilic Antibody Purification

Thiophilic Adsorption

Thiophilic adsorption is a low-cost, efficient alternative to ammonium sulfate precipitation for immunoglobulin purification from crude samples. Ammonium sulfate precipitation must be followed by several additional steps to completely remove contaminants in crude samples. Thiophilic adsorption is a simple, rapid, one-step method for antibody purification from serum, ascites or tissue culture supernatant.

Thiophilic adsorption is a highly selective type of lyotropic salt-promoted protein:ligand interaction phenomenon that has been studied extensively by Porath and co-workers and other researchers.[1] This interaction is termed thiophilic because it is distinguished by proteins that recognize a sulfone group in close proximity to a thioether. Thiophilic adsorption incorporates properties of both hydrophobic and hydrophilic adsorption. However, in contrast to strictly hydrophobic systems, thiophilic adsorption is not strongly promoted by high concentrations of sodium chloride. Instead, thiophilic adsorption is promoted by increased concentrations of water-interacting, non-chaotropic salts such as potassium and ammonium sulfate.

T-Gel™ Adsorbent

T-Gel™ Adsorbent is 6% beaded agarose gel modified to contain simple sulfone/thioether groups (Figure 12). T-Gel™ Adsorbent has a high-binding capacity (20 mg of immunoglobulin per ml of gel) and broad specificity toward immunoglobulins derived from various animal species. Notably, thiophilic adsorption is one of few methods available for purification of IgY from chicken (see also subsequent discussion of IgY purification, page 40). Among human serum proteins, immunoglobulins and α_2-macroglobulins are preferentially bound by T-Gel™ Adsorbent.[2]

Purification using T-Gel™ Adsorbent results in good protein recovery with excellent preservation of antibody activity. Sample preparation requires the addition of 0.5 M potassium sulfate to the serum, ascites or culture fluid. Greater specificity for immunoglobulins is obtained if the sample is buffered at pH 8.0. The gentle elution conditions (e.g., 50 mM sodium phosphate, pH 7-8) yield concentrated, essentially salt-free, highly purified immunoglobulins at near neutral pH.

After use, T-Gel™ Adsorbent can be regenerated by treatment with guanidine•HCl. Pierce data indicate that the T-Gel™ Adsorbent column can be used at least 10 times without significant loss of binding capacity.

T-Gel™ Purification Kit includes 4 x 3 ml prepacked columns of T-Gel™ Adsorbent, binding and elution buffers, column storage buffer, and guanidine•HCl for use in column regeneration. This simple, one-step method eliminates the need for post-treatment of the sample before storage or subsequent conjugation to enzymes for use in immunoassays.

Suggested applications for T-Gel™ Adsorbent:

- Efficient and selective isolation of immunoglobulins from human serum under mild conditions[1]
- Convenient and fast method for purification of mouse monoclonals from the culture media of cloned cells or from ascites fluid[2]
- Selective removal of immunoglobulins from fetal calf serum – useful for cell culture in monoclonal antibody production[3]
- Rapid, straightforward procedure yielding essentially pure immunoglobulins from crude rabbit serum[4]
- Purification of IgY from chicken[5]
- Large-scale purification for biotechnology applications

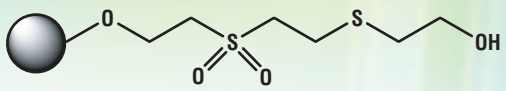


Figure 12. Structure of T-Gel™ Adsorbent.

References

1. Porath, J., *et al.* (1985). *FEBS Lett.* **185**, 306-310.
2. Belew, M., *et al.* (1987). *J. Immunol. Method* **102**, 173-182.
3. Hutchens, T.W. and Porath, J. (1987). *Biochemistry* **26**, 7199-7204.
4. Lihme, A. and Heegaard, P.M.H. (1990). *Anal. Biochem.* **192**, 64-69.
5. Unpublished internal Pierce documents.

T-Gel™ Adsorbent and T-Gel™ Purification Kit

Economical purification of mouse antibodies from ascites fluid.

Table 7. Binding characteristics of T-Gel™ Adsorbent.

Species	Total Ab. bound from 1 ml serum	% Purity by HPLC
Human	4.8	70
Mouse	8.6	63
Mouse IgG_1	11.6	92
Mouse IgG_{2a}	9.3	88
Mouse IgG_{2b}	9.8	97
Mouse IgG_3	10.7	94
Rat	13.0	79
Bovine	17.9	90
Calf	11.1	89
Chicken	5.2	76
Dog	12.2	91
Goat	17.3	92
Guinea Pig	11.1	71
Horse	13.0	93
Pig	21.1	90
Rabbit	6.7	84
Sheep	12.3	89

Highlights:

- Binds to Fab and $F(ab)_2$ fragments
- Binds to ScFv[1]
- High-capacity (20 mg/ml), good protein recovery and retention of antibody function
- Broad specificity toward immunoglobulins derived from various animal species (Table 7)
- Binds chicken IgY (also called IgG)
- Simple, rapid, one-step purification for monoclonal antibodies from ascites; easy to scale up
- Used to enrich the immunoglobulin fraction from serum or tissue culture supernatant
- Efficient alternative to ammonium sulfate precipitation for enriching antibodies from crude samples
- Gentle elution conditions yield concentrated, salt-free immunoglobulin at near neutral pH
- High degree of purity

References

1. Schulze, R.A., *et al.* (1994). *Anal. Biochem.* **220**, 212-214.
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Koustova, E. *et al.* (2001). *J. Clin. Invest.* **107(6)**, 737-44.
Montesano, M.A., *et al.* (2002). *J. Exp. Med.* **195**, 1223-1228.
Palmer, D.A., *et al.* (1994). *Anal. Biochem.* **222**, 281-283.
Suh, J.S., *et al.* (1998). *Blood.* **91(3)**, 916-22.
Turpin, E.A., *et al.* (2003). *J. Clin. Microbiol.* **41**, 3579-3583.

Ordering Information

Product #	Description	Pkg. Size
20500	T-Gel™ Adsorbent	10 ml
44916	T-Gel™ Purification Kit*	Kit
	Includes: T-Gel™ Adsorbent Prepacked Columns	4 x 3 ml
	T-Gel™ Binding Buffer	1,000 ml
	T-Gel™ Elution Buffer	1,000 ml
	T-Gel™ Column Storage Buffer (2X)	100 ml
	Guanidine•HCl Crystals	230 g
	Column Extenders	

** U.S. patent # 4,696,980.*

Melon™ Gel

New Melon™ Gel from Pierce produces purified antibodies from serum in about 15 minutes without exposing them to harsh elution conditions. The same gentle conditions are also used to isolate monoclonal IgG from ascites fluid or tissue culture supernatant. Antibodies are recovered ready-to-use for downstream assays or modification; there is no need to desalt. Melon™ Gel saves time compared to classical Protein A or Protein G purifications without compromising the quality of the IgG recovered.

How does it work?

Melon™ Gel retains the component proteins typically found in serum while allowing the IgG to pass through. The resulting recoveries and purity of the IgG isolated rivals that obtained from the same samples from bind-and-release supports such as Protein A or Protein G.

The unique Melon™ Gel Resin is offered in three kit formats: a spin column kit for 25 small-volume rapid serum purifications of up to 1 mg IgG from each 100 µl of Melon™ Gel, a kit that allows you to use up to 25 ml total bed volume for larger scale serum purifications of 250 mg up to 2 g of IgG, and a monoclonal antibody purification kit to purify up to 1 L of culture supernatant or 200 ml of ascites fluid.

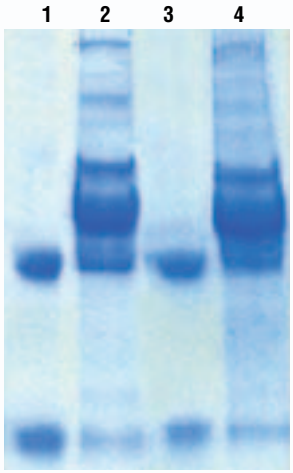


Figure 13. Melon™ Gel provides excellent separation of antibodies from other serum proteins. The purification of IgG from serum was evaluated by electrophoresing the purified IgG (lanes 1 and 3) and the contaminants retained on the column (lanes 2 and 4) from rabbit and sheep serum, respectively, on a polyacrylamide gel stained with GelCode™ Blue Stain Reagent.

Melon™ Gel support can be regenerated up to three times, allowing four separate purifications on a given volume of support. When preceded by an ammonium sulfate precipitation, seven regenerations or eight uses are routinely possible. For small-scale purifications, you may prefer to discard the support after each purification.

Table 8. Purification of IgG from serum of various species.

Source	Melon™ Gel	Gel Protein A	Protein G
Human	**H**	**H**	**H**
Mouse	**H**	**H**	**H**
Rabbit	**H**	**H**	**H**
Rat	**H**	L	M
Goat	**H**	L	**H**
Cow	M	L	**H**
Sheep	M	L	**H**
Horse	**H**	L	**H**
Guinea Pig	**H**	**H**	L
Pig	**H**	**H**	L
Chicken	N	N	N
Hamster	**H**	M	M
Donkey	**H**	M	**H**

Legend: H = high recovery, M = medium recovery, L = low recovery, N = no recovery

Table 9. Melon™ Gel outperforms Protein A and Protein G.

	% Recovery		
	Melon Gel	**Protein A**	**Protein G**
Human	96	82	89
Rabbit	97	81	96
Goat	92	61	94

Melon Gel Monoclonal IgG Purification Kit Protocol	**Immobilized Protein A or Protein G Protocol**
1. Buffer-exchange sample. 2. Add MAb-containing supernatant to the Melon Gel Support. 3. Incubate for 5 minutes. 4. Vacuum filter to isolate purified IgG from the Melon Gel Support.	1. Dilute sample in Binding Buffer. 2. Pour and equilibrate column. 3. Add MAb-containing solution to the resin. 4. Wash column with 10-20 column volumes of buffer. 5. Elute with three column-volumes (minimum) of Elution Buffer. 6. Neutralize MAb-containing sample.
Total time to perform a single isolation: 2.1 hours	**Total time to perform a single isolation: 4 hours (without subsequent dialysis)**

The Melon Gel Monoclonal IgG Purification Kit isolates much faster than immobilized Protein A or Protein G columns. Cell culture supernatant sample is 500 ml to 1 L.

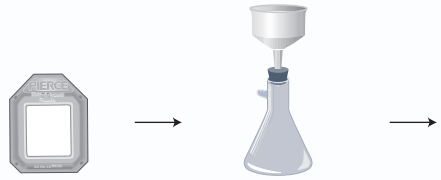
Melon™ Gel IgG Purification Kits

The quickest and easiest way to isolate IgG.

You supply the sample – the Melon™ Gel IgG Purification Kits contain everything else you need.

Highlights:

- **One-step, easy protocol** – IgG is isolated in the flow-through
- **Purifies IgG from serum in 15 minutes** – up to six times faster than Protein A or G methods
- **Provides better recovery and purity than Protein A- or Protein G-based purification methods** – greater than 90% yield and greater than 80% purity
- **Purifies antibodies or subclasses that do not bind well to Protein A or Protein G**
- **Purifies polyclonal IgG from most animal sources** – 12 host animals have been verified (Note: Melon™ Gel is not recommended for chicken IgY purification)
- **Maintains antibody activity** – extreme pH or high salt elutions are not necessary



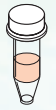
1. Buffer exchange 2 x 1 hour.
2. In a funnel, drain storage buffer from resin and then transfer funnel to a new filter flask. Add buffer-exchanged sample and stir for 5 minutes.

3. Apply vacuum to collect the purified sample in the new flask.

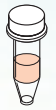
Figure 14. Protocol for purification of MAbs from cell culture supernatant and ascites.

Melon™ Gel IgG Spin Kit Protocol for Serum

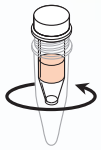
Dilute serum in purification buffer.



Add serum to Melon™ Gel.



Incubate for 5 minutes.



Centrifuge to collect antibodies.

Ordering Information

Product #	Description	Pkg. Size
45206	**Melon™ Gel IgG Spin Purification Kit*** *Sufficient material to purify up to 25 mg of IgG from serum. One-time-use, disposable format kit for small-volume antibody purification.*	Kit
	Includes: Melon™ Gel IgG Purification Support	3 ml settled gel (supplied as a 20% slurry)
	Melon™ Gel Purification Buffer	100 ml
	Handee™ Mini Spin Column Accessory Pack	27 columns/pkg.
	Handee™ Microcentrifuge Tubes	30 tubes/pkg.
45212	**Melon™ Gel IgG Purification Kit*** *Sufficient material to purify up to 2 g of IgG from serum. Flexible and regeneratable format for large-scale antibody purification.*	Kit
	Includes: Melon™ Gel IgG Purification Support	25 ml settled gel (supplied as a 20% slurry)
	Melon™ Gel Purification Buffer	100X, (dry mix; reconstitute to 1 L)
	Melon™ Gel Regenerant	(dry mix; reconstitute to 1 L)
45214	**Melon™ Gel Monoclonal IgG Purification Kit*** *Sufficient reagents to purify IgG from up to 1 L of cell culture supernatant or up to 200 ml of ascites fluid.*	Kit
	Includes: Melon™ Gel IgG Purification Support	200 ml settled gel
	Melon™ Gel Purification Buffer	100X, dry mix
	Melon™ Gel Regenerant	5X, dry mix
45216	**Saturated Ammonium Sulfate Solution** *For use with the Melon Gel Monoclonal IgG Purification Kit and Melon Gel IgG Purification Kits for Sera or as a general-purpose salting-out agent for protein-preparation applications.*	1 L
45219	**Ascites Conditioning Reagent** *For use with the Melon Gel Monoclonal IgG Purification Kit.* Instructions not included. Use of this reagent is described in the instructions provided with Product # 45214.	5 ml

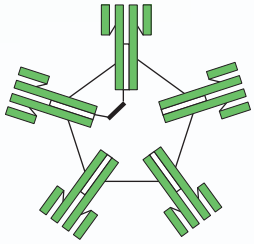
** U.S. patent pending on Melon™ Gel Technology.*

IgM Purification

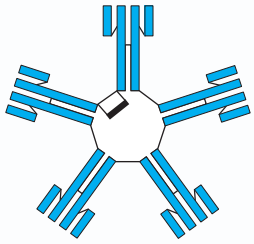
Structure of IgM

IgM is a high molecular weight glycoprotein (MW 900,000-950,000) with a carbohydrate content of approximately 12%. This antibody is found at concentrations of 0.5-2 mg/ml in serum.[1] *In vivo*, IgM has a half life of five days, and its catabolism is two- to three-fold greater than that of IgG.

In the sera of mammals, birds and reptiles, IgM has a pentameric structure. However, mouse and human IgM structure differs in the location of disulfide bridges that link monomers together to form the pentamer (Figure 15).[2] Disulfides are arranged in series in mouse IgM and in parallel in human IgM.



Human IgM



Mouse IgM

Figure 15. Structure of IgM, adapted from Matthew and Reichardt.[9]

Challenges to IgM Purification

Protein A binds IgM poorly, in part because binding sites on the Fc region of the monomers are sterically hindered by the pentameric structure of IgM. Until recently, no readily available affinity chromatography product existed for one-step IgM purification. Standard methods for IgM purification generally are multistep, tedious processes or they are not effective for removing all of the major impurities present in IgM samples.[3]

Traditionally, IgM was purified by ammonium sulfate precipitation followed by gel filtration chromatography, ion exchange chromatography or zone electrophoresis.[4] Other methods that have been used include use of DEAE cellulose,[5] immobilized DNA[6] and a combination of ammonium sulfate precipitation and subsequent removal of IgG with Protein A or G.[3]

Nethery, *et al.* developed an IgM affinity purification method using C1q, a 439 kDa complement component that recognizes carbohydrate on cell surfaces.[7] This temperature-dependent binding method yielded relatively pure IgM. However, co-purification of IgG was a problem, and C1q is expensive and difficult to purify.

Immobilized Mannan Binding Protein

To develop an effective affinity matrix, Pierce scientists examined C1q and another similarly structured protein, mannan binding protein (MBP). Serum MBP, like C1q, is capable of initiating carbohydrate-mediated complement activation. MBP is a mannose and *N*-acetylglucosamine specific lectin found in mammalian sera, and it has considerable structural homology to C1q.[8] MBP subunits are identical, each with molecular mass of approximately 31 kDa (C1q has six each of three different polypeptide subunits of molecular mass 24-28 kDa). Studies in Pierce labs show that MBP does not bind $F(ab')_2$ and Fab.

Pierce has developed an easy-to-use ImmunoPure® Immobilized Mannan Binding Protein and buffer system to purify IgM. It is most effective for purifying mouse IgM from ascites. Purified IgM can be obtained from a single pass over the affinity column. Human IgM will bind to the support, albeit with slightly lower capacity, and yield a product at least 88% pure as assessed by HPLC. The purification of IgM from other species and mouse serum has not yet been optimized.

IgM purification with ImmunoPure® Immobilized Mannan Binding Protein is temperature- and calcium-dependent. Binding and washing steps are performed at 4°C in 10 mM Tris•HCl (pH 7.4) buffer containing sodium chloride and 20 mM calcium chloride. Elution is made at room temperature in a similar Tris buffer, except that it contains EDTA and is devoid of calcium chloride. An Immobilized MBP Column can be regenerated at least 10 times with no apparent loss of binding capacity.

Immobilized MBP is available in both beaded agarose and UltraLink® Biosupport Medium formats. Binding, elution and column preparation buffers are also available. The ImmunoPure® IgM Purification Kit contains sufficient buffers to perform 10 purifications using a 5 ml column of ImmunoPure® Immobilized MBP. The kit is easy to use and yields 90% pure mouse IgM (from ascites) with a very simple protocol.

References

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2. Milstein, C., *et al.* (1975). *Biochem. J.* **151**, 615-624.
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8. Ohta, M., *et al.* (1990). *J. Biol. Chem.* **264**, 1980-1984.
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ImmunoPure® Immobilized MBP and IgM Purification Kit

Easy IgM purification … with guaranteed 88% pure mouse IgM!

Demonstration of the High Purity of MBP-purified IgM From Mouse Ascites

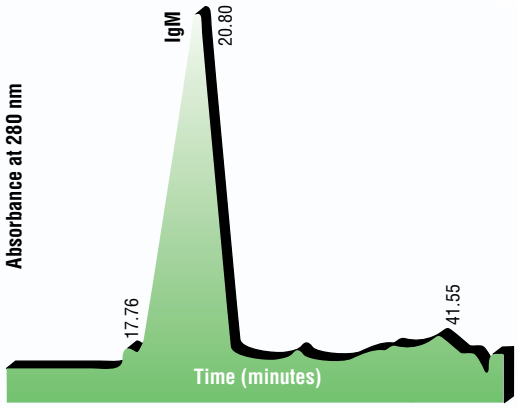

Figure 16. The bound material from mouse ascites was eluted from the 5 ml MBP column as described in the Standard Protocol. The highest 280 nm absorbing fraction from the elution was chromatographed using the conditions described in the instruction booklet.

References

Beger, E., *et al.* (2002). *J. Immunol.* **168**, 3617-3626.
Feldmesser, M., *et al.* (2000). *Infect. Immun.* **68**, 3642-3650.
Nevens, J.R., *et al.* (1992). *J. Chromatogr.* **597**, 247-256.
Zhou, B., *et al.* (2000). *J. Biol. Chem.* **275**, 37733-37741.

Ordering Information

Product #	Description	Pkg. Size
22212	ImmunoPure® Immobilized Mannan Binding Protein Capacity: ~ 1 mg IgM/ml of gel	10 ml
44897	ImmunoPure® IgM Purification Kit Includes: Immobilized MBP Column IgM Binding Buffer IgM Elution Buffer MBP Column Preparation Buffer Column Extender	Kit 5 ml 800 ml 500 ml 50 ml
21016	ImmunoPure® IgM Binding Buffer	800 ml
21017	ImmunoPure® IgM Elution Buffer	500 ml
21018	ImmunoPure® MBP Column Preparation Buffer	50 ml
53123	UltraLink® Immobilized Mannan Binding Protein Capacity: >0.75 mg IgM/ ml of gel	5 ml

Human IgA Purification

Jacalin is an α-D-galactose binding lectin extracted from jack fruit seeds *(Artocarpus integrifolia)*. The lectin is a glycoprotein of approximately 40,000 MW composed of four identical subunits. Jacalin immobilized on supports such as agarose has been useful for the purification of human serum or secretory IgA_1. IgA can be separated from human IgG and IgM in human serum or colostrum.[1] IgD is reported to bind to jacalin.[2] Immobilized jacalin is also useful for removing contaminating IgA from IgG samples.

Binding of IgA to immobilized jacalin occurs at physiologic pH and ionic strength, as in phosphate buffered saline (PBS). Elution of bound IgA occurs with competitor ligand (e.g., 0.1 M melibiose or 0.1 M α-D-galactose) in PBS. Pierce offers immobilized jacalin on cross-linked 6% agarose.

References

1. Roque-Barreira, M.C. and Campos-Neto, A. (1985). *J. Immunol. Method* **134(30)**, 1740-1743.
2. Aucouturier, P., *et al.* (1987). *Mol. Immunol.* **24(5)**, 503-511.
Kondoh, H., *et al.* (1986). *J. Immunol. Method* **88**, 171-173.
Kumar, G.S., *et al.* (1982). *J. Biosci.* **4**, 257-261.
Mestecky, J., *et al.* (1971). *J. Immunol.* **107**, 605-607.
Van Kamp, G.J. (1979). *J. Immunol. Method* **27**, 301-305.

Immobilized Jacalin

Ideal for human IgA purification.

Highlights:

- Ideal for preparing human IgA that is free of contaminating IgG
- Found to bind human IgA_1, but **not** human IgA_2 – useful for separating the two subclasses

Reference

Kaider, B.D., *et al.* (1999). *Human Reprod.* **14**, 2556-2561.

Ordering Information

Product #	Description	Pkg. Size
20395	Immobilized Jacalin Capacity: 1-3 mg human IgA/ml of gel Support: Cross-linked 6% beaded agarose Loading: 4.5 mg jacalin/ml of gel	5 ml

Chicken IgY Purification

Properties of IgY

Chickens produce a unique immunoglobulin molecule called IgY. There are several advantages to production and use of IgY over mammalian immunoglobulins. With regard to production, raising and immunizing chickens is relatively simple, chickens are more likely to produce an immune response to conserved mammalian protein antigens, and chickens produce 15-20 times more antibody than rabbits.

Most importantly, IgY is naturally packaged at high concentrations in egg yolks, making repeated collection of antibody from immunized hens noninvasive. A single egg yolk from an immunized chicken contains approximately 300 mg of IgY. Whole eggs or separated egg yolks can be collected and stored frozen for later extraction of antibody.

Other advantages of IgY for use in immunoassays are that it does not bind rheumatoid factor or other anti-mammalian IgGs, does not activate complement, and generally has much lower probability of nonspecific binding to mammalian tissues and extracts.

IgY Purification Methods

One challenge with regard to IgY is that it can be difficult to purify. Protein A, Protein G and other Fc-binding proteins do not bind IgY. However, IgY from chicken possesses light chains that can be bound with high affinity by Protein L[1-3] (see page 31).

T-Gel™ Adsorbent (see page 34) enables moderate yields of fairly pure IgY from serum and other fluids. However, complete procedures for T-Gel™ Adsorbent have not been developed for use with egg yolks, which have very high lipid concentrations.

Eggcellent® Chicken IgY Purification Kits were specifically developed for efficient purification of IgY from egg yolks. After separating an intact yolk from egg white using an Eggcellent® Egg Separator, Eggcellent® Delipidation Reagent is added to separate the proteins from lipid. The delipidation reagent can also be used to store an egg yolk in the freezer for up to one year. After delipidation, the protein-containing sample fraction is mixed with Eggcellent® IgY Precipitation Reagent to create a relatively pure IgY precipitate that is recovered by centrifugation.

Routinely, 80-120 mg of high purity (>85%), intact IgY can be obtained per egg using the Eggcellent® Kit.

References
1. Bjorch, L. (1988). *J. Immunol.* **140**, 1194-1197.
2. Nilson, B.H.K., *et al.* (1993). *J. Immunol. Method* **164**, 33-40.
3. Akerstrom, B., *et al.* (1989). *J. Biol. Chem.* **264**, 19740-19746.

Eggcellent® Chicken IgY Purification Kit

Purifies 100 mg of chicken IgY with higher purity than ever before!

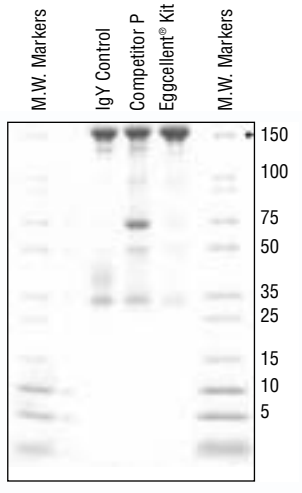

Figure 17. SDS-PAGE analysis of Eggcellent® Chicken IgY Purification. Chicken IgY was purified according to each manufacturer's instructions. The gel shows the analysis of 2 µg of protein applied per well. The Eggcellent® Kit purified the chicken IgY to a purity level of >85% using GelCode™ Blue Stain Reagent (Product # 24590). The competitor's product achieved only a 53% purity level. The arrow indicates intact IgY.

References
Da Costa, L., *et al.* (2003). *Blood* **101**, 318-324.
Rao, J., *et al.* (2003). *Biol. Reprod.* **68**, 290-301.

Highlights:

- More for your money – purifies twice the amount of IgY as the leading competitor's kit with a lower cost-per-mg of IgY purified
- Higher purity – 85-95% by SDS-PAGE analysis (Figure 17)
- Ease-of-use – the simple precipitation method works without affinity columns
- Flexibility – eggs can be stored in buffer and purified at a later date
- Convenience – use eggs directly out of the refrigerator; no need to wait for them to warm up

Ordering Information

Product #	Description	Pkg. Size
44918	Eggcellent® Chicken IgY Purification Kit *Sufficient reagents to purify five egg yolks.* Includes: Eggcellent® Delipidation Reagent Eggcellent® IgY Precipitation Reagent Eggcellent® Egg Separator	Kit 500 ml 500 ml 1
44922	Eggcellent® Chicken IgY Purification Kit *Sufficient reagents to purify 25 egg yolks.*	Kit
21055	Eggcellent® Delipidation Reagent	500 ml
21057	Eggcellent® IgY Precipitation Reagent	500 ml
21060	Eggcellent® Egg Separator	1
44916	T-Gel™ Purification Kit	Kit

Affinity Purification of Specific Antibodies

Although Proteins A, G, A/G and L are excellent ligands for purification of total IgG from a sample, purification of an antibody specific for a particular antigen and free of contamination from other immunoglobulins is often required. This can be accomplished by immobilizing the particular antigen used for immunization so that only those antibodies that bind specifically to the antigen are purified in the procedure. Activated affinity supports that can be used to immobilize peptides or other antigens for use in affinity purification are described in the Affinity Purification Handbook (see below to request your free copy).

Successful affinity purification of antibody depends on effective presentation of the relevant epitopes on the antigen to binding sites of the antibody. If the antigen is small and immobilized directly to a solid support surface by multiple chemical bonds, important epitopes may be blocked or sterically hindered, prohibiting effective antibody binding. Therefore, it is best to immobilize antigens using a unique functional group (e.g., sulfhydryl on a single terminal cysteine in a peptide) and to use an activated support whose reactive groups occur on spacer arms that are several atoms long. For larger antigens, especially those with multiple sites of immobilization, the spacer arm length becomes less important since the antigen itself serves as an effective spacer between the support matrix and the epitope. Generally, if the antigen was cross-linked to a carrier protein to facilitate antibody production, best results are obtained when the antigen is immobilized for affinity purification using the same chemistry (e.g., reaction to primary amines, sulfhydryls, carboxylic acids or aldehydes). In this way, all epitopes will be available for antibody binding, allowing greater efficiency in purification and recovery of the specific immunoglobulin.

Little variation exists among typical binding and elution conditions for affinity purification of antibodies because at the core of each procedure is the affinity of an antibody for its respective antigen. Since antibodies are designed to recognize and bind antigens tightly under physiologic conditions, most affinity purification procedures use binding conditions that mimic physiologic pH and ionic strength. The most common binding buffers are phosphate buffered saline (PBS) and Tris buffered saline (TBS) at pH 7.2 and 1.5 M NaCl. Once the antibody has been bound to an immobilized antigen, additional binding buffer is used to wash unbound material from the support. To minimize nonspecific binding, the wash buffer may contain additional salt or detergent to disrupt any weak interactions.

Specific, purified antibodies are eluted from an affinity resin by altering the pH or ionic strength of the buffer. Antibodies in general are resilient proteins that tolerate a range of pH from 2.5 to 11.5 with minimal loss of activity, and this is by far the most common elution strategy. In some cases an antibody-antigen interaction is not efficiently disrupted by pH changes or is damaged by the pH, requiring that an alternate strategy be employed.

Anti-peptide antibodies are often generated against sulfhydryl-(cysteine-)labeled peptides. A terminal cysteine is added to the peptide sequence and the free sulfhydryl is used as a coupling handle to attach the peptide to a carrier protein such as KLH for immunization. Following immunization, the resultant anti-peptide antibodies can be specifically purified on an affinity resin made by attaching the same sulfhydryl-labeled peptide to a solid support such as the MicroLink™ Peptide Coupling Kit (Product # 20485).

For more information on immobilizing antigens and affinity purification of specific antibodies, request your free copy of our Affinity Purification Handbook . Log on to the Pierce web site or contact your local Perbio office or distributor.

Structure courtesy of: Title: Fv Fragment Of Mouse Monoclonal Antibody D1.3 (Balb/C, Igg1, K) Variant For Chain L Glu81->Asp and Chain H Leu312->Val; PDB: 1A7N; Citation: Marks, C., Henrick, K., Winter, G.: X-Ray Structures of D1.3 Fv *Mutants To be Published.*

Antibody Fragmentation

Overview

Often it is useful to study or make use of the activity of one portion of an immunoglobulin without interference from other portions of the molecule. It is possible to selectively cleave the immunoglobulin molecule into fragments that have discrete characteristics. Antibody fragmentation is accomplished using proteases that digest or cleave certain portions of the immunoglobulin protein structure. Although fragmentation of all immunoglobulin classes is possible, only procedures for fragmentation of mouse, rabbit and human IgG and IgM have been well-characterized.

The two groups of antibody fragments of primary interest are antigen-binding fragments such as Fab and nonantigen-binding, class-defining fragments such as Fc. More than one type of antigen-binding fragment is possible, but each contains at least the variable regions of both heavy and light immunoglobulin chains (V_H and V_L, respectively) held together (usually by disulfide bridges) so as to preserve the antibody-binding site. Fc fragments consist of the heavy chain constant region (Fc region) of an immunoglobulin and mediate cellular effector functions.

Antibody fragmentation is somewhat laborious, requires optimization of enzyme-mediated digestion of the protein and necessitates an ample supply (e.g., 10 mg) of antibody to make it reasonably efficient. For these reasons, fragmentation is usually performed only when the antibody of interest is available in large quantity and the particular application demands it.

Advantages of Antibody Fragments

Antibody fragments offer several advantages over intact antibody as reagents in an immunochemical technique:

- Using antigen-binding regions that have been separated from the Fc region reduces nonspecific binding that results from Fc interactions (many cells have receptors for binding to the Fc portion of antibodies).
- Small antigen-binding fragments generally provide higher sensitivity in antigen detection for solid-phase applications as a result of reduced steric hindrance from large protein epitopes.
- Because they are smaller, antibody fragments more readily penetrate tissue sections, resulting in improved staining for immunohistochemical applications.
- Antibody fragments are the best choice for antigen-antibody binding studies in the absence of Fc-associated effector functions (e.g., complement fixation, cell membrane receptor interaction).
- Antibody fragments offer a simple system by which to study the structural basis for immune recognition using X-ray crystallography or nuclear magnetic resonance.
- Antibody fragments have lower immunogenicity than intact antibody.

Types of Antibody Fragments

$F(ab')_2$, Fab, Fab′ and Fv are antigen-binding fragments that can be generated from the variable region of IgG and IgM. These antigen-binding fragments vary in size (MW), valency and Fc content. Fc fragments are generated entirely from the heavy chain constant region of an immunoglobulin. The structures of these antibody fragments are illustrated in schematic form in Figure 19 and summarized below. In addition, several unique fragment structures can be generated from pentameric IgMs, including an “IgG”-type fragment, an inverted “IgG”-type fragment and a pentameric Fc fragment. IgM fragmentation is discussed in detail on pages 48-49.

$F(ab')_2$

$F(ab')_2$ (110,000 dalton IgG fragment, 150,000 dalton IgM fragment) fragments contain two antigen-binding regions joined at the hinge through disulfides. This fragment is void of most, but not all, of the Fc region.

Fab′

Fab′ (55,000 dalton IgG, 75,000 dalton IgM) fragments can be formed by the reduction of $F(ab')_2$ fragments. The Fab′ fragment contains a free sulfhydryl group that may be alkylated or utilized in conjugation with an enzyme, toxin or other protein of interest. Fab′ is derived from $F(ab')_2$; therefore, it may contain a small portion of Fc.

Fab

Fab (50,000 daltons) is a monovalent fragment that is produced from IgG and IgM, consisting of the V_H, C_{H1} and V_L, C_L regions, linked by an intrachain disulfide bond.

Fv

Fv (25,000 daltons) is the smallest fragment produced from IgG and IgM that contains a complete antigen-binding site. Fv fragments have the same binding properties and similar three-dimensional binding characteristics as Fab. The V_H and V_L chains of the Fv fragments are held together by noncovalent interactions. These chains tend to dissociate upon dilution, so methods have been developed to cross-link the chains through glutaraldehyde, intermolecular disulfides or a peptide linker.

"r IgG"

"r IgG" (80,000 daltons) is a reduced form of IgG composed of one complete light chain and one complete heavy chain. It is essentially one-half of an intact IgG molecule and it contains a single antigen-binding site. "r IgG" fragments are formed by the selective reduction of disulfide bonds in the hinge region of an antibody.

Fc

Fc (50,000 daltons) fragments contain the C_{H2} and C_{H3} region and part of the hinge region held together by one or more disulfides and noncovalent interactions (Figure 18). Fc and Fc5µ fragments are produced from fragmentation of IgG and IgM, respectively. The term Fc is derived from the ability of these antibody fragments to crystallize. Fc fragments are generated entirely from the heavy-chain constant region of an immunoglobulin. The Fc fragment cannot bind antigen, but it is responsible for the effector functions of antibodies, such as complement fixation.

$F(ab')_2$, Fab′, Fab and Fv fragments produced from IgM function in much the same way as $F(ab')_2$, Fab′, Fab and Fv fragments from IgG. However, compared to those in IgG, individual antigen-binding sites in IgM generally have lower binding affinities, which are compensated in the complete IgM by its pentameric form. The increased binding valency of $F(ab')_2$ may make it preferable to Fv and Fab fragments.

$F(ab')_2$ fragments are divalent, and they may be a superior alternative to Fab fragments for antibodies with low affinity. The $F(ab')_2$ fragments have higher avidity than the Fab and Fab′ fragments. $F(ab')_2$ fragments can precipitate antigen. Fab and Fab′ are univalent molecules that cannot precipitate antigen. Fab and Fab′ fragments have a decreased binding strength, and normally stable antigen-antibody complexes may dissociate during washes in certain applications.

Fragmentation of IgG

The hinge region of an immunoglobulin monomer (IgG) is readily accessible to proteolytic attack by enzymes. Cleavage at this point produces $F(ab')_2$ or Fab fragments and the Fc fragment. The Fc fragment may remain intact or become further degraded, depending upon the enzyme and conditions used. Proteolytic IgG fragmentation using three different enzymes is discussed below and summarized in Figure 19. Traditionally, proteolysis was accomplished in solution using free enzyme. Pierce has developed immobilized enzyme products that enable better control of the digestion and separation of reaction products from the enzyme.

Immobilized Papain

Papain is a nonspecific, thiol-endopeptidase that has a sulfhydryl group in the active site, which must be in the reduced form for activity. When IgG molecules are incubated with papain in the presence of a reducing agent, one or more peptide bonds in the hinge region are split, producing three fragments of similar size: two Fab fragments and one Fc fragment.[1] When Fc fragments are of interest, papain is the enzyme of choice because it yields a 50,000 dalton Fc fragment.

Papain is primarily used to generate Fab fragments, but it also can be used to generate $F(ab')_2$ fragments.[2] To prepare $F(ab')_2$ fragments, the papain is first activated with 10 mM cysteine. The excess cysteine is then removed by gel filtration. If no cysteine is present during papain digestion, $F(ab')_2$ fragments can be generated. These fragments are often inconsistent, and reproducibility can be a problem. If the cysteine is not completely removed, overdigestion can be a problem.[2]

Figure 18. Structure of Fab, Fab′, $F(ab')_2$ and Fv Fragments.

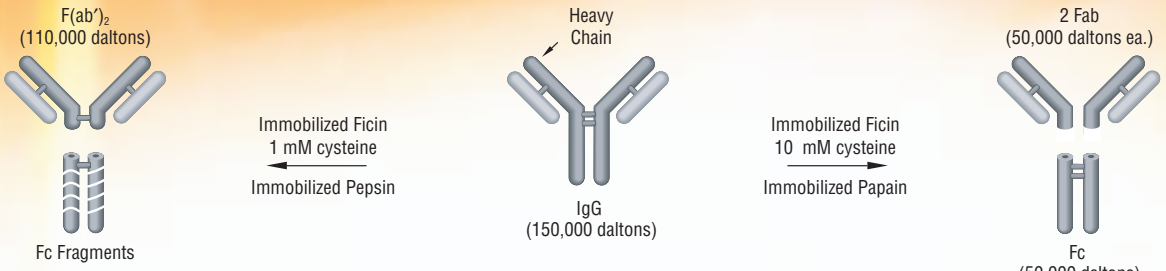

Figure 19. Use of papain, pepsin and ficin for IgG fragmentation.

Preparation and Isolation of Fab and Fc Fragments

1. Equilibration of Immobilized Papain

a) Add 0.5 ml of Immobilized Papain slurry + 4 ml of digestion buffer to the tube as in A.
b) Mix the tube contents as in B.
c) Compress the gel as in C and discard the buffer.
d) Repeat steps a, b and c.
e) Resuspend the gel in 0.5 ml buffer.

2. Addition of IgG sample to Immobilized Papain

Add IgG in 1.0 ml digestion buffer to tube containing Immobilized Papain.

3. Digestion of IgG

Incubate in a shaker water bath at 37°C for the appropriate amount of time.

4. Separation of crude digest

Compress gel with separator and pour crude digest into clean tube.

5. Washing of Immobilized Papain

a) Add 1.5 ml of Binding Buffer to gel as in A. Mix as in B.
b) Add wash to rest of crude digest.

6. Addition of crude digest to Immobilized Protein A column

Add crude digest to an equilibrated Immobilized Protein A Column. Collect column eluate.

7. Collection of Fab fragments

a) Add 6 ml ImmunoPure® IgG Binding Buffer to Protein A column and collect eluate. Dialyze against PBS, pH 7.4 overnight.
b) Wash column with additional 3 ml binding buffer. Discard wash.

8. Collection of Fc and undigested IgG

a) Transfer Protein A column to another tube.
b) Elute Fc and undigested IgG from column with 6 ml ImmunoPure® IgG Elution Buffer.

Figure 20. Preparation and isolation of Fab and Fc fragments with Immobilized Papain.

Crystalline papain is often used for the digestion of IgG; however, it is prone to autodigestion. Mercuripapain, which is less prone to autodigestion than crystalline papain, can be used; however, both of these non-immobilized enzymes require an oxidant to terminate digestion. Immobilized papain is the preferred reagent because it allows for easy control of the digestion reaction, as well as separation of enzyme from the crude digest. There is no need to develop an ion exchange method for separating the fragments from the enzyme. The use of immobilized papain will also prevent formation of antibody-enzyme adducts, which can occur when using the soluble form of sulfhydryl proteases (such as papain). These adducts can be detrimental to fragments in the presence of reductants.

Immobilization also increases stability of the enzyme against heat denaturation and autolysis and results in longer maintenance of activity. Regeneration of the papain is often possible after immobilization, resulting in decreased costs. Cleavage can be regulated by digestion time or flow rate through a column, yielding reproducible digests. Pierce Immobilized Papain (Product # 20341) offers all the advantages of immobilized enzyme supports (Figure 20). Pierce ImmunoPure® Fab Preparation Kit (Product # 44885) has been optimized for human IgG digestions. It also has been used successfully for mouse and rabbit digestions, and suggestions on how to vary the protocols for other species' IgG are provided with the kit. The procedure required that the IgG is able to be bound by Protein A, which is used to separate Fc from Fab fragments.

Immobilized Pepsin

Pepsin is a nonspecific endopeptidase that is active only at acid pH. It is irreversibly denatured at neutral or alkaline pH. Digestion by the enzyme pepsin normally produces one $F(ab')_2$ fragment and numerous small peptides of the Fc portion (Figure 19). The resulting $F(ab')_2$ fragment is composed of two disulfide-connected Fab units. The Fc fragment is extensively degraded, and its small fragments can be separated from $F(ab')_2$ by dialysis, gel filtration or ion exchange chromatography.

$F(ab')_2$ can be separated by mild reduction into two sulfhydryl-containing, univalent Fab′ fragments. The advantage of Fab′ fragments is that they can be conjugated to detectable labels directly through their sulfhydryl groups, ensuring that the active binding site remain unhindered and active. Pierce offers 2-Mercaptoethylamine•HCl (2-MEA, Product # 20408) for mild reduction of $F(ab')_2$ fragments. For alternative labeling protocols, the free sulfhydryl may be blocked with an alkylating reagent, such as *N*-Ethylmaleimide (NEM, Product # 23030).

Immobilized Pepsin (Product # 20343) can be substituted for free pepsin in any application. Immobilized pepsin is advantageous because of its ability to immediately stop the digestion process, yielding reproducible digests. Immobilization of the enzyme allows for easy separation of the enzyme from the crude digest, eliminating the need to develop an ion exchange method for separating the fragments from the enzyme. Also, immobilization increases the stability of the pepsin against heat denaturation and autolysis, resulting in longer maintenance of activity. ImmunoPure® $F(ab')_2$ Preparation Kit (Product # 44888) has been optimized for human IgG digestions. The kit also has been used successfully for rabbit and mouse IgG digestions. Suggestions on how to vary protocols for other species' IgG (IgG must bind to Protein A) are provided.

Immobilized Ficin

Ficin is a thiol protease that can digest mouse monoclonal IgG_1 into either $F(ab')_2$ or Fab fragments, depending on the concentration of cysteine used. Ficin will generate $F(ab')_2$ in the presence of 1 mM cysteine. Fab fragments will be generated with ficin in the presence of 10 mM cysteine (Figure 19).

Ficin cleavage produces $F(ab')_2$ fragments of nearly identical size to those obtained from IgG by pepsin but with immunoreactivities and affinities comparable to those of intact IgG_1 antibody.[3] By increasing the concentration of cysteine activator, Fab antigen-binding fragments can be generated.[4] The integrity of the resultant antigen-binding fragments is aided by the neutral pH conditions of the ficin digestion. The difficulties of using pepsin in this application makes ficin digestion the preferred method for producing $F(ab')_2$ fragments from murine monoclonal IgG_1. Although $F(ab')_2$ fragments have been generated from an IgG_1 antibody using preactivated papain,[5] stable, consistent product by papain is often difficult to obtain.[6]

Immobilized Ficin (Product # 44881) enables better control of the digestion reaction than free ficin, resulting in antibody fragments that are free of autodigestion products (Figure 21). In addition, the use of Immobilized Ficin eliminates the incorporation of ficin into antibody fragments. The ImmunoPure® IgG_1 Fab and $F(ab')_2$ Preparation Kit (Product # 44880) was developed to allow gentle production and purification of both Fab and $F(ab')_2$ fragments from intact murine IgG_1 antibodies. Immobilized Ficin can be used repeatedly to cleave an IgG_1 subclass antibody, yielding either Fab or $F(ab')_2$ fragments. The type of fragment produced is controlled by the specific concentration of cysteine activator used during the digestion.

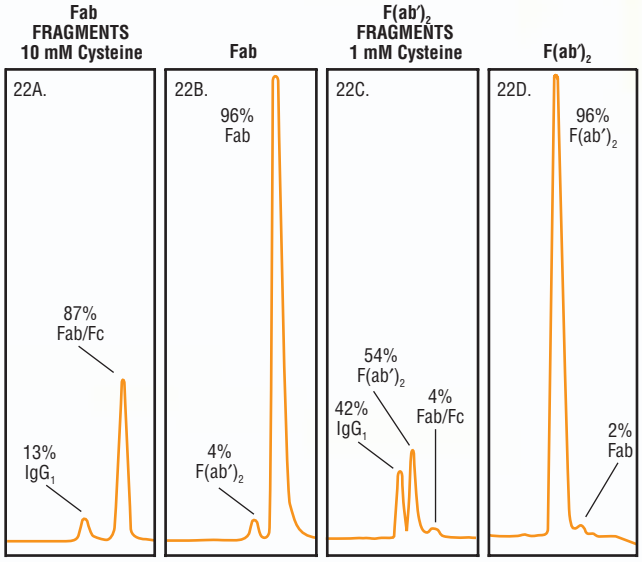

Figure 21. Selective preparation of monovalent or bivalent antigen-binding fragments from IgG_1. Figure 21A. Digestion products of mouse anti-avidin IgG_1 monoclonal on immobilized ficin. 5 hours, 37°C, 10 mM cysteine. Ficin: Mab = 11:1 (mole:mole). **Figure 21B.** Size exclusion profile of fraction not bound to Protein A column. Component composition: 4% $F(ab')_2$, 96% Fab. Total Recovery: 47% Fab. **Figure 21C.** Digestion products of mouse anti-avidin IgG_1 monoclonal on immobilized ficin. 20 hours, 37°C, 1 mM cysteine. Ficin: MAB = 11:1 (mole:mole). **Figure 21D.** Size exclusion profile of fraction not bound to Protein A column. Component composition: 98% $F(ab')_2$, 2% Fab. Total Recovery: 35% $F(ab')_2$.

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Table 10. Enzymes used for antibody digestion.

Enzyme	Molecular Weight	Ext. Coefficient A_{280} of 1%	Type	Specificity	pH Optimum	Activators (Enhancers)	Inhibitors	Imm. Enzyme (mg/ml BCA)	pI
Pepsin	35,000	14.7	Acid	Broad – prefer Phe, Met Leu, Tryp bonds	1 (1-5)		pH >6, epoxides	1	11
Papain	23,000	25	Thiol	Broad – prefer Arg, Lys, His, Gly, Tyr bonds	6.5 (4-9.5)	cysteine, sulfide, sulfite, cyanide, (EDTA) (NBS*) (acridine dye)	heavy metals, carbonyls, *N*-ethyl maleimide (NEM), *p*-chloromercuro-benzoate	9.6	1.5
Ficin	26,000	21	Thiol	Uncharged or aromatic amino acids	6.5 (4-9.5)	cysteine, sulfide, sulfite, cyanide, (EDTA) (NBS) (acridine dye)	heavy metals, carbonyls, NEM, *p*-chloromercuro-benzoate		1.5
Trypsin	24,000	14.3	Serine	Arg, Lys	8	Ca^{2+} acts as a stabilizer	organophosphorous compounds, DFP**, benzimidine	10.5	1.5

**NBS = N-bromosuccinimide **DFP = diisopropyl fluorophosphate*

ImmunoPure® Fab Preparation Kit

The easiest, most convenient way to generate Fab fragments from IgG.

Pierce has developed a complete kit to digest human or mouse IgG molecules into Fab fragments and Fc fragments by using immobilized papain. After digestion, the fragments are purified on an immobilized Protein A column provided in the kit. Detailed instructions allow for flexibility in the protocol for hard-to-digest antibodies.

Highlights of Fab Fragments:

- Will not be affected by Fc receptors on cells such as macrophages, B cells, T cells, neutrophils and mast cells
- Will not precipitate antigen
- Easier to make and purify than $F(ab')_2$ fragments
- More rapid clearance of radiolabeled fragments from normal tissue than whole IgG conjugates
- Reduced immunogenicity (as a result of Fc region absence), minimizes human anti-mouse immunoglobulin (HAMA) response
- Fragments are less susceptible to phagocytosis

Antibody Fragment Applications:

- Immunohistochemistry
- Immunoassays, including *in vitro* diagnostic assays
- Radioimmunolocalization for tumor detection and radiotherapy
- Immunotargeting through the use of fragment conjugates as immunotoxins
- Crystallographic study of antibody-binding sites
- Study of Fc-binding proteins and effector functions

Highlights of Fc Fragments:

- Useful for studying effector functions of IgG without interference from antigen-binding sites
- Fc fragments can be used as blocking agents for histochemical staining

References

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Ordering Information

Product #	Description	Pkg. Size
44885	**ImmunoPure® Fab Preparation Kit** *Isolates and purifies Fab fragments from up to 200 mg of mouse IgG.* Includes: Immobilized Papain Cysteine•HCl AffinityPak™ Immobilized Protein A Columns ImmunoPure® IgG Binding Buffer ImmunoPure® IgG Elution Buffer	Kit 5 ml 1 g 2 x 2.5 ml 1,000 ml 500 ml
20341	**Immobilized Papain** Support: Cross-linked 6% beaded agarose Activity: 7 BAEE units per ml of settled gel Loading: 250 µg/ml of gel	5 ml

ImmunoPure® F(ab′)$_2$ Preparation Kit

The easiest, most convenient way to generate F(ab′)$_2$ fragments from IgG.

Pierce offers a complete kit for digesting antibodies into F(ab′)$_2$ fragments that retain antigen-binding activity. Using immobilized pepsin allows the antibody digest to be free of any enzyme contaminants. Purifying F(ab′)$_2$ fragments is as easy as passing the solution over an immobilized protein A column. Detailed instructions allow for flexibility in the protocol for hard-to-digest antibodies.

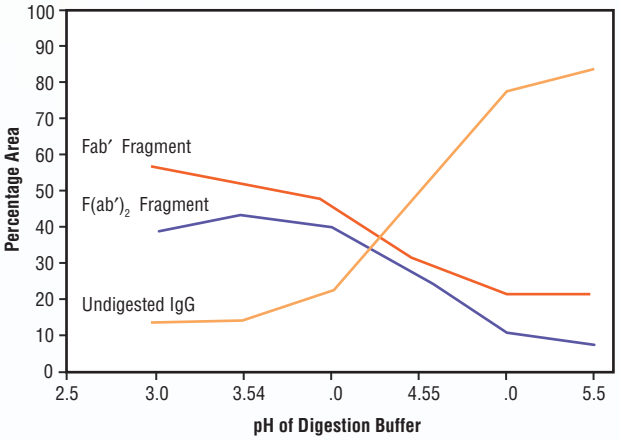

Figure 22. High-performance liquid chromatographic studies indicate the optimal pH for generation of F(ab′)$_2$ fragments from immobilized pepsin for human IgG.

Highlights of F(ab′)$_2$ Fragments:

- Will not be affected by Fc receptors on cells such as macrophages, B cells, T cells, neutrophils and mast cells
- They are divalent, which is recommended for retaining antigen-binding capabilities of low affinity antibodies
- Will precipitate antigen

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Ordering Information

Product #	Description	Pkg. Size
44888	**ImmunoPure® F(ab′)$_2$ Preparation Kit** *Digests up to 200 mg of mouse IgG.*	Kit
	Includes: AffinityPak™ Immobilized Protein A Columns	2 x 2.5 ml
	ImmunoPure® IgG Binding Buffer	1,000 ml
	ImmunoPure® IgG Elution Buffer	500 ml
	Immobilized Pepsin	5 ml
20343	**Immobilized Pepsin** Support: Cross-linked 6% beaded agarose Activity: ≥ 2,000 units per ml of settled gel Loading: 2-3 mg/ml of gel	5 ml

ImmunoPure® IgG$_1$ Fab and F(ab′)$_2$ Preparation Kit

Generate both Fab and F(ab′)$_2$ fragments from monoclonal IgG$_1$ antibodies.

Problems with digesting mouse monoclonal IgG$_1$ can now be overcome by using immobilized Ficin. Ficin cleavage produces F(ab′)$_2$ fragments of nearly identical size to those obtained from IgG by pepsin, but with immunoreactivities and affinities comparable to those of the intact IgG$_1$ antibody. Similarly, by increasing the concentration of cysteine activator in the digestion buffer, Fab fragments can be created from the original IgG.

Highlights:

- Can generate both Fab and F(ab′)$_2$ fragments from mouse IgG$_1$
- Reaction can be easily controlled
- Antibody fragments are free of autodigestion products
- Ficin contamination into antibody fragments is eliminated

References

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Ordering Information

Product #	Description	Pkg. Size
44880	**ImmunoPure® IgG$_1$ Fab and F(ab′)$_2$ Preparation Kit** *For digesting up to 250 mg of mouse IgG$_1$.*	Kit
	Includes: Immobilized Ficin Columns (Activity = 1.2 mg/ml gel)	5 x 2 ml
	Cysteine•HCl	1 g
	ImmunoPure® IgG$_1$ Digestion Buffer (10X)	50 ml
	AffinityPak™ Immobilized Protein A Columns	2 x 2.5 ml
	ImmunoPure® IgG Binding Buffer	1,000 ml
	ImmunoPure® IgG$_1$ Mild Elution Buffer	2 x 500 ml
	Immobilized Ficin Storage Buffer	100 ml
	Column Extenders	2
44881	**Immobilized Ficin**	5 ml
44889	**Cysteine•HCl**	5 g

Fragmentation of IgM

IgM is an extremely large molecule that has a tendency to interact with other molecules and matrices besides the antigen. The large size of IgM creates difficulties in applications in which IgM is used for *in vitro* experiments. Intact IgM does not effectively penetrate tissues for immunohistochemical studies; it is necessary to produce smaller, active fragments for these studies. Also, because IgM molecules have difficulty permeating cell membranes, they are not ideal for use *in vivo*. Fragments are cleared more rapidly than intact IgM.

Each species of IgM reacts differently to enzymatic cleavage and reduction. For example, the relative structure of mouse and human IgM differ in the manner in which the monomers are linked to give the pentameric form, primarily as a result of differences in the location of disulfides between the monomers.[1] Oligosaccharides components, which may hinder enzymatic cleavage, also vary between species. Therefore, optimal digestion and reduction conditions for one species may prove ineffective for another.

Fragmentation of IgM by proteolyic enzymes proceeds differently from IgG fragmentation. These changes are related to differences in structure. The heavy (μ) chains are folded into multiple globular domains, and IgM has an actual domain (Cμ2) in place of a hinge region. Cμ2 lacks the proline-rich sequence that is found in the hinge region of IgG. This proline sequence makes the hinge more susceptible to cleavage. Also, IgM has a large carbohydrate portion in the Cμ2, which may sterically interfere with the action of proteolytic enzymes.

Enzymes and Reagents for IgM Fragmentation

Papain is a nonspecific, thiol-endopeptidase that has a sulfhydryl group in the active site, which must be in the reduced form for activity. Papain has been shown to produce heterogeneous fragments from IgM. Oligosaccharides in the hinge region of IgM interfere with papain digestion, causing a cleavage shift of 3-5 amino acids in either direction.

Pepsin is a nonspecific endopeptidase that is active only at an acid pH, and it is irreversibly denatured at neutral or alkaline pH. It is possible to produce F(ab′)$_2$, Fab and Fv fragments using pepsin to digest IgM (Figure 23). Many methods have been developed that use pepsin to produce different IgM fragments from different species.[2]

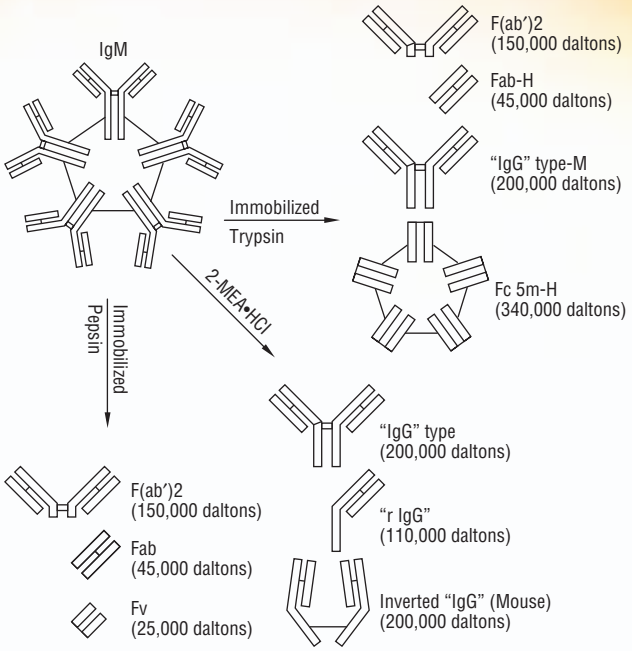

Figure 23. Fragmentation of IgM.

Trypsin is a serine protease that reacts optimally at pH 8.0. In general, increasing the enzyme/substrate ratio and/or temperature will increase the rate of digestion. Trypsin can generate F(ab′)$_2$, Fab, "IgG"-type and Fc5μ fragments from IgM (Figure 23). Trypsin digestion of several species of IgM was studied using trypsin with and without urea pretreatment.[2] Urea alters the susceptibility of the domains to digestion and produces different fragments than those digested in aqueous buffer. Many other procedures have been developed to digest IgM using trypsin.[3]

Mild reduction can be achieved using 2-Mercaptoethylamine•HCl (2-MEA, Product # 20408). Reduction will vary among IgM species, but an "IgG"-type and/or reduced IgG ("rIgG") should be formed in varied proportions, depending upon reduction time and/or temperature (Figure 23).[4] Fragmentation of mouse IgM also produces an inverted "IgG"-type fragment.

IgM Fragmentation Kit

Pierce has developed the ImmunoPure® IgM Fragmentation Kit (Product # 44887) to allow quick, easy production of fragments from mouse and human IgM. This kit can be used for species other than human and mouse; however, the protocols have not been optimized for all species. The kit contains everything needed to produce IgM fragments using trypsin, pepsin and 2-MEA protocols. The trypsin and pepsin are supplied in immobilized forms, eliminating the need to separate enzyme from the IgM fragments. The IgM is digested as it passes through the prepacked immobilized enzyme columns. The enzyme remains bound to the support matrix, ensuring there is no autodigested enzyme to contaminate the IgM fragments. Also, the immobilized enzymes offer increased stability against heat denaturation and autolysis. Regeneration of the immobilized enzymes makes the process more cost-efficient. The cleavage of IgM is easily regulated by adjustment of incubation times. Sample concentrators are included in the kit for easy fragment separation and concentration. Complete instructions and protocols are also included.

References

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ImmunoPure® IgM Fragmentation Kit

Makes IgM fragmentation easy!

The large size of IgM creates difficulties in applications in which IgM is used for *in vitro* experiments. Intact IgM does not effectively penetrate tissues for immunohistochemical studies, therefore it is necessary to produce smaller, active fragments for *in vitro* or *in vivo* studies. Pierce created a kit combining immobilized trypsin and pepsin to easily generate a variety of IgM fragments (Figure 23).

Highlights:

- Immobilized trypsin can generate F(ab′)$_2$, Fab, "IgG"-type and Fc(5µ) fragments from IgM
- Immobilized pepsin can produce F(ab′)$_2$, Fab and Fv fragments from IgM
- Complete kit, including detailed instructions, to digest and purify IgM fragments
- Immobilized enzymes prevent enzyme contaminants in final fragment preparation

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Ordering Information

Product #	Description	Pkg. Size
44887	**ImmunoPure® IgM Fragmentation Kit**	Kit
	Fragments up to 26 mg of human or mouse IgM.	
	Includes: Immobilized Trypsin Columns	2 x 2 ml
	Immobilized Pepsin Columns	2 x 2 ml
	2-Mercaptoethylamine	6 mg
	IgM Digestion Buffer	400 ml
	IgM F(ab′)$_2$ Digestion Buffer	200 ml
	Iodoacetamide	
	Sample Concentrators	
	Desalting Columns	2 x 5 ml
20343	**Immobilized Pepsin**	5 ml
20408	**2-Mercaptoethylamine•HCl**	6 x 6 mg

Structure courtesy of: Title: Chicken Egg White Cystatin Wildtype, NMR, 16 Structures; PDB: 1A67; Citation: Dieckmann, T., Mitschang, L., Hofmann, M., Kos, J., Turk, V., Auerswald, E. A., Jaenicke, R., Oschkinat, H.: The structures of native phosphorylated chicken cystatin and of a recombinant unphosphorylated variant in solution. *J Mol Biol* 234 pp. 1048 (1993).