Fastback Protein G Sepharose® FF Resin

Technical Datasheet

Affinity purification of monoclonal antibodies has been largely confined to the use of Protein A and Protein G chromatography. The Fastback Protein G Sepharose® FF Resin is designed for simple, one-step and rapid antibody purification from serum, ascites and tissue culture supernatant such as those derived from static cultures and bioreactors. Antibody samples purified using this affinity resin may be used in a wide range of laboratory procedures such as 1D or 2D polyacrylamide gel electrophoresis, Western blotting, ELISA etc. The antibodies are sufficiently pure for radiolabelling, conjugations (for example fluorescein) or preparation of immuno-affinity columns.

Specification:

Specificity:	Protein G affinity antibodies
Source:	Recombinant Protein G expressed in *E. coli* (N.B. The recombinant Protein G lacks the albumin-binding domain found in native Protein G)
Matrix:	Sepharose®
Ligand density:	2 mg Protein G / ml resin
Binding capacity (human IgG):	20 mg/ml (Note that the Protein G resin can have different binding capacities to subtypes of immunoglobulins derived from the same species).
Bead size:	45-165 µm
Maximum pressure:	120-140 psi
Buffer compatibility:	Common aqueous buffers from pH 2.0-9.0
Chemical stability:	High
Endotoxin levels	Unknown
Toxin levels:	Free of Staphyloccocus enterotoxins and hemolysins
Solubility in water:	Insoluble
Shipping/delivery:	50% (v/v)resin suspension in 20% ethanol
Storage:	20% ethanol at 2-8°C for up to 2 years from manufacture

Chemical compatibility of the Protein G Sepharose® FF Resin

- All resins are susceptible to oxidative agents. Avoid high temperatures. Protein G is resistant to limited exposure to 8 M urea pH 10.5 and extremes of pH (eg. pH 1.0 and pH 11). It lacks the robustness of Protein A with respect to 1 M NaOH and it is denatured under these aggressive conditions. However, Protein G is stable to treatment with 0.1 M NaOH.

Storage conditions:

- It is transported in 20% ethanol. The Protein G resin is stable for up to 2 year at 2-8 ˚C from the date of manufacture. Do not freeze the resin or store it at room temperature. Freezing the suspension will damage the agarose beads. The resin is pre-swollen and de-fined.

Technical support:

Contact the Protein Ark technical support and sales centre for assistance:

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Disclaimer:

- This product is for research use only and is not intended for use in clinical diagnosis. No claims beyond replacement of unacceptable material or refund of purchase price shall be allowed.

Ordering Information:

Product	Volume	Order Code
Fastback Protein G Sepharose® FF Resin (1 ml)	1 ml	Fastback-PG-1
Fastback Protein G Sepharose® FF Resin (5 ml)	5 ml	Fastback-PG-5
Fastback Protein G Sepharose® FF Resin (25 ml)	25 ml	Fastback-PG-25

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Principles of Protein A and G chromatography:

All modes of chromatography can be used effectively for the separation of antibodies. Although ion exchange chromatography can resolve different polyclonal antibodies and different subclasses, a degree of customization of the protocol is required. Affinity techniques include protein A or G, immobilized anti-antibodies and immobilized antigens. The simplicity of Protein A is extremely attractive as it lends itself to the bind, wash and elute mode of operation if the appropriate buffer formulations are selected. The use of proteins A and G is widespread, and has largely superseded the use of anti-antibodies.

Applications of Protein A and G chromatography include:

Monoclonal antibody purification
Antibody concentration
Purification of polyclonal antibodies
Salt removal from antibodies
Removal of endotoxins from an antibody solution

Affinity chromatography:

The essence of affinity chromatography utilizes the concept of bio-specificity, implying an interaction between a natural binding site and the natural ligand, whether it be enzyme-substrate, enzyme-inhibitor or Protein A/G antibody interactions.

Often a researcher needs to choose the correct base matrix, select the optimal activation chemistry and couple a suitable ligand to develop an affinity column to purify the target protein. The Protein G resin from Protein Ark removes the guesswork associated with optimizing the resin chemistry by offering ready-to-use resin that has satisfied stringent quality control to guarantee reproducible purification performance.

General considerations for selecting optimal binding conditions for recombinant Protein G resin.

Any sample, such as a crude biological extract, a cell culture supernatant, serum, ascites or an artificial standard can be used with the Protein A resin. It is important that the sample is first filtered through a 0.45-1.2 μm syringe-end filter to remove particulates that could clog the resin flow channels. All samples should be filtered just prior to loading even if they have been filtered several days before the chromatographic run.

Aggregation/precipitation of proteins is common during storage and repeated freeze/thaw cycles in sera, ascites and tissue culture supernatants. Lipids, which can be found at high levels in serum or ascites should also be removed. Of equal importance is the ability to process the samples rapidly and, if the need arises, to be able to purify the target protein at 4 ˚C.

Optimal conditions for binding the target molecule to a resin are critical for successful separation of the protein. If the binding conditions are not optimal with respect to pH, salt concentration, presence or absence of metal ions etc, the interaction could be weak or non-existent. In many instances, the sample needs to be dialysed or diafiltered by ultrafiltration before it is applied to an affinity or ion exchange chromatographic support. In Protein A or G separations, the sample should simply be diluted 1:1 (v/v) in 1 x binding buffer.

The interaction of immobilized Protein A or Protein G with immunoglobulins (Igs) is pH-dependent. The binding capacity for Protein A is optimal at pH 8-9, whereas the binding capacity of Protein G is high over a broader pH range. Salt concentration can significantly affect the binding of mouse Igs to protein A by reducing severe ionic interactions and enhancing hydrophobic interactions. Mouse IgG1, rat IgG1 and rat IgG2b bind well to immobilized protein A when the salt concentration is higher than 1 M, but bind poorly at low salt concentrations. Note, however, that their binding capacities can be substantially lower than those antibodies that bind strongly to Protein A or G resin.

Typical binding buffers employed in Protein G affinity separations are:

I. 10-100 mM Sodium phosphate, 2.7 mM potassium chloride, 0.137 M NaCl pH 7.4 (PBS)
II. 50 mM Tris, 2.7 mM potassium chloride,0.137 M NaCl pH 8.0 (TBS)
III. 20-100 mM Sodium phosphate pH 7.0-7.2
IV. 20 mM Sodium acetate pH 7.4

Typical elution buffers employed in Protein G affinity separations are:

I. 0.1 M Sodium citrate pH 3.0-6.0
II. 0.1-0.2 M Glycine/HCl pH 2.5-3.0
III. 0.1 M Sodium acetate pH 3.5

The typical neutralization buffer used for Protein G affinity separations is:

1M Tris/HCl pH 9.0

Eluting the antibody from the recombinant Protein G Sepharose® resin:

The most common elution conditions for Protein A or G affinity and immuno-affinity separations involve a reduction in pH to between pH 2.5 and 5.0. It is important to appreciate that a few proteins (e.g. some monoclonal antibodies) are acid-labile and they can lose their activity at very low pH values. Above all, the elution conditions must preserve the integrity and activity of the target protein. Most observed denaturation is caused by harsh elution conditions. Acidic pH is known to reduce the antibody titre, decrease immuno-reactivity and distort the antibody structure. It is therefore critical that the pH is restored to neutrality after elution. Flow rate through an affinity chromatography support is important in achieving optimal separation. Flow rate

through the column support is inextricably related to the efficiency of the separation; too fast a flow will cause the mobile phase to move past the beads faster than the diffusion time necessary to reach the internal bead volume. The Protein G resin chemistries have sufficiently rapid association kinetics between the protein molecule and the immobilized ligand to allow for optimal diffusional flow through the internal bead structure.

Immuno-affinity separations:

Three types of immunoaffinity ligands are commonly employed:

I. Protein G.
II. Antibody directed against the monoclonal species (usually anti-mouse IgG).
III. Antigen to which the monoclonal antibody was raised.

The important criteria to consider when purifying antibodies are:

I. Time and cost of the purification
II. Downstream application e.g. immunoassay, immunotags etc
III. Yield and purity required
IV. Host species and isotype
V. Antibody source

Protein G affinity resin:

Protein G is a cell wall protein from group C and G Streptococci that binds strongly to the Fc region of IgG. It is specific for IgG. Protein G has some benefits over Protein A in that it binds to a wider range of immunoglobulins such as rat IgG2a, IgG2b, human IgG3, bovine IgG1 and sheep IgG1 and the binding buffer does not need to have such a high ionic strength. Typically, a researcher will use 10 mM phosphate pH 7.4 with 0.138 M NaCl and 2.7 mM KCl (PBS). Protein G also tends to have a higher antibody binding affinity than Protein A.

However, a lower elution pH is required to desorb the immunoglobulin from a Protein G agarose column. In addition, native Protein G has an albumin binding site which is removed in recombinant protein G. Consequently, most researchers currently use recombinant Protein A or G for purifying immunoglobulins. Recombinant Protein G is produced in E. coli and has a molecular weight of approximately 22 kDa.

Choosing between Protein A and Protein G resin:

Immunoglobulin G from most species consists of several subclasses with different biological properties. Four subclasses of IgG have been identified in human (IgG1, IgG2, IgG3, and IgG4) and in mouse (IgG1, IgG2a, IgG2b and IgG3). For immunological studies, it is often necessary to isolate one particular subclass of IgG from the other subclasses.

Protein G binds to all major Ig classes except IgM and therefore has a wider reactivity profile than Protein A. However, the binding of Igs to Protein G is often stronger, making elution

and complete recovery of the immunoglobulin more difficult. Interestingly, due to the lower cost of Protein A compared to Protein G, researchers tend to experiment first with Protein A then Protein G. Protein A withstands harsher conditions used in cleaning and regeneration. The affinity of interaction of Protein A with mouse IgG subclasses varies. The most common subclass of mouse monoclonal antibodies is IgG1. Customization of the purification strategy may be required for the affinity separation as mouse IgG1 does not generally bind well to Protein A. However, as the affinity interaction is pH- and salt-dependent, under high salt regimes (2-3 M NaCl) and high pH (pH 8-9), the antibodies will bind to Protein A.

The needs of the researcher dictate that the speed of sample processing, the cost and the reproducibility are key criteria for selecting purification tools. By selecting immunoglobulin-binding proteins with the appropriate cross-reactivities coupled to careful design of the strategic protocol, objectives such as the selective recovery of therapeutic antibodies from complex mixtures is assured.

Table 2. Binding Affinities of Protein A and Protein G.

Isoform	Protein A	Protein G	Isoform	Protein A	Protein G
Human IgG1	✓✓✓✓	✓✓✓✓	Rabbit IgG	✓✓✓✓	✓✓✓
Human IgG2	✓✓✓✓	✓✓✓✓	Hamster IgG	✓	✓✓
Human IgG3	×	✓✓✓✓	Guinea Pig IgG	✓✓✓✓	✓✓
Human IgG4	✓✓✓✓	✓✓✓✓	Bovine IgG	✓✓	✓✓✓✓
Human IgA	✓✓	×	Sheep IgG	✓/×	✓✓
Human IgD	✓✓	×	Goat IgG	✓/×	✓✓
Human IgE	✓✓	×	Pig IgG	✓✓✓	✓✓✓
Human IgM	✓✓	×	Chicken IgG	×	✓
Mouse IgG1	✓	✓✓			
Mouse IgG2a	✓✓✓✓	✓✓✓✓			
Mouse IgG2b	✓✓✓	✓✓✓			
Mouse IgG3	✓✓	✓✓✓	**Fragments**	**Protein A**	**Protein G**
Mouse IgM	✓/×	×	Human Fab	✓	✓
Rat IgG1	×	✓	Human F(ab')2	✓	✓
Rat IgG2a	×	✓✓✓✓	Human scfv	✓	×
Rat IgG2b	×	✓✓	Hunam Fc	✓✓	✓✓
Rat IgG2c	✓	✓✓	Human κ	×	×
Rat IgM	✓/×	×	Human λ	×	×

Key code for relative affinity of Protein A & G for respective antibodies:

Strong Affinity	✓✓✓✓
Moderate affinity	✓✓✓
Weak affinity	✓✓
Slight affinity	✓
No Affinity	×

Protocol for purifying monoclonal antibodies using Protein G kits

Immunoglobulin binding proteins have been used extensively for the purification of IgG molecules from serum, ascites, or cell culture supernatants such as those derived from static cultures and bioreactors. The affinity of Protein G for the Fc region of immunoglobulins makes it the natural ligand choice for many researchers involved in IgG isolation.

Delipidation procedure:

All protein A & G affinity columns are affected by the presence of lipids and lipoproteins, especially in antibody samples derived from ascites fluid. For end users who have antibody solutions which they need to delipidate, the following protocol is a gentle and easy method for removing lipids and lipoproteins.

1. Add 0.04 ml 10% dextran sulphate solution and 1 ml 1 M calcium chloride per ml sample.
2. Mix for 15 min.
3. Centrifuge at 10,000 g for 10 min.
4. Discard the precipitate.
5. Exchange the sample into TBS (Tris Buffered Saline) using dialysis, ultrafiltration or a desalting column. Do not buffer exchange into a phosphate-containing buffer such as PBS.

Procedure for Immobilized Protein G Separation:

Gel Preparation:

As the gel is shipped in 20% ethanol, decant off the ethanol and add distilled water to a final slurry concentration of approximately 50 %.

Resin packing:

Gently shake the bottle to form a gel slurry. It is often preferable to de-gas the resin slurry. Pour or pipette the resin slurry into a glass or plastic column with the column outlet slightly open. Add 5-10 column volumes of distilled water to wash the resin and to ensure that the resin is packed well. Close the column outlet valve. The column is now ready for pre-equilibration with binding buffer.

PROTOCOL USING GRAVITY FLOW OR A PERISTALTIC PUMP:

Pre-equilibration:

Equilibrate the immobilized Protein G column with 3-5 column volumes of binding buffer.

Sample loading:

Load an appropriate amount of 0.45 µm filtered cleared lysate on to the column. Please note that the binding capacity of the resin is approximately 20-30 mg IgG/ml sedimented resin. Collect the sample wash for further analyses.

Washing:

Wash the column with 5 x 3 column volumes of wash buffer. The washes should be collected for further analyses to ensure that all unbound protein is removed.

Elution:
Elute the bound IgG into fresh tubes with a minimum 5 x 2 column volumes (e.g. 10-15 CVs) of low pH elution buffer. The eluate must be neutralized rapidly. You can either elute the purified IgG directly into a high pH neutralization buffer eg 1M Tris/HCl pH 9.0 or dialyze the purified IgG in 100 x volume TBS overnight with a minimum of 2 changes of dialysate. The eluate should be collected for further analyses. Check the protein content of each eluted fraction before pooling them. Otherwise, you risk diluting a concentrated, purified sample.

DESALTING AND CONCENTRATING THE PURIFIED PROTEIN

The purified IgG can be buffer exchanged or concentrated further using ultrafiltration concentrators for your downstream application.

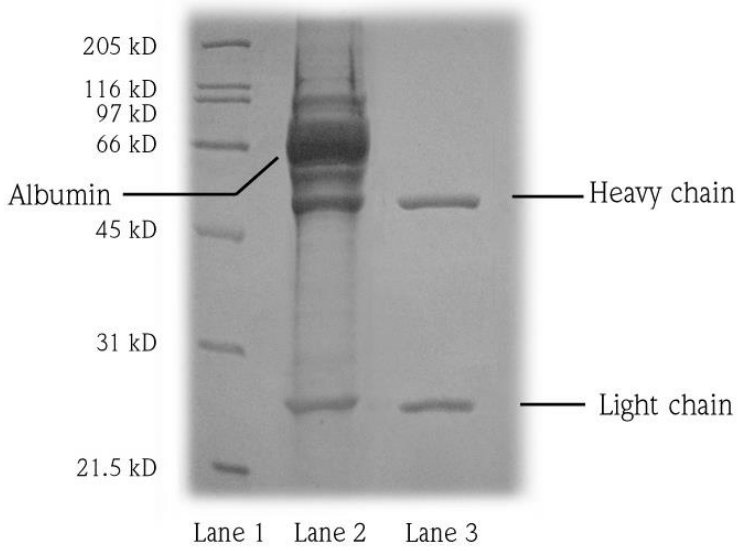
REGENERATION OF THE Fastback PROTEIN G SEPHAROSE® RESIN

Wash the column with 10 CVs of elution buffer followed by 10 CVs of binding buffer. Proceed to the pre-equilibration step of another bind-wash-elute cycle if the column is to be re-used immediately. After regeneration, the resin can also be stored in a screw capped bottle in 0.1 % sodium azide (made up in distilled water) at 2-8 ˚C until further use.

By using the Beer-Lambert law, A = ε.c.l, the concentration of IgG (mg/ml) in the sample can be measured by multiplying the absorbance at 280 nm by 0.72. If IgM or IgA are purified, multiply the absorbance at 280 nm by 0.84 or 0.94, respectively. These antibody concentrations are only estimates as other contaminating proteins can also contribute to the absorbance reading. However, they can provide a reliable and quantitative method for determining the concentrations of pure antibody solutions. Most researchers use a sandwich ELISA assay to accurately measure antibody concentrations within a range of 1 mg/ml to 20 mg/ml sample.

The antibodies can also be monitored for purity by SDS-PAGE under reducing or non-reducing conditions. Note that IgG appears in a reducing SDS-PAGE as 25 kDa and 50-55 kDa bands and IgM appears as 25 kDa and 70-80 kDa bands. Recovery of immunoglobulins can be quantified by a standard protein assay, scanning densitometry of reducing or non-reducing SDS-polyacrylamide gels or ELISA. Antigen binding parameters can be measured for both affinity and avidity.

Figure 1. Reducing SDS-Polyacrylamide gel of mouse IgG1 purified from cell culture supernatant (+ foetal calf serum) with Protein G resin.



Lane 1: Molecular weight markers.
Lane 2: Hybridoma cell culture supernatant before loading on to the Proteus spin column.
Lane 3: Pure mouse IgG1 eluted from the Proteus Protein G spin column

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Recommended Protein G buffer formulation

Use the following recipes to prepare the buffers for purification of all IgGs. All buffers contain 0.1 % sodium azide as a preservative and can be stored at room temperature:

Binding Buffer A (0.1 M sodium phosphate buffer, 0.15 M NaCl, pH 7.4)

Add 12.3 g sodium phosphate (dibasic; Na_2HPO_4; anhydrous Mr 142.0), 1.565 g sodium phosphate (monobasic; NaH_2PO_4; anhydrous Mr 120.0), 8.77 g NaCl (Mr 58.44), 1.0 g NaN_3 to 900 ml distilled water.
Make up final volume to 1 L with distilled water.

Elution Buffer B (0.2 M Glycine/HCl buffer pH 2.5)

Add 15.0 g glycine (free base; Mr 75.07), 1.0 g NaN_3 to 900 ml distilled water.
Titrate with HCl to pH 2.5.
Make up final volume to 1 L with distilled water.

Neutralization Buffer C (1 M Tris/HCl buffer pH 9.0)

Add 103.72 g Tris base (Mr 121.1), 22.72 g Tris hydrochloride (Mr 157.6),1.0 g NaN_3 to 800 ml distilled water.
Make up final volume to 1 L with distilled water.

Questions and Answers:

1. *What is the shelf-life of the resin?*

The resin is guaranteed for 2 year after manufacture, provided it is stored at 2-8 ˚C.

2. *Do I need to degas the buffers?*

Although most buffers are aqueous, it is good laboratory practice to filter and degas all buffers.

3. *Do I need to pre-filter my sample before loading it on to the Fastback Protein G agarose resin?*

All samples should be filtered through a 0.45-1.2 µm syringe filter or centrifuged to remove particulates.

4. *What is the binding capacity ofFastback Protein G resin?*

We have been able to bind up to 20 mg human IgG from serum.

5. *How should I prepare my sample for the resin?*

Many chromatographic procedures demand that the sample is pre-conditioned prior to loading. Protein G affinity separations usually require the sample to be diluted 1:1 (v/v) in 1 x binding buffer. We recommend that all samples are diluted 1:1 (v/v) in the binding buffer.

6. *Do I need to be careful with the type of chaotropic ion I use when eluting antibodies from affinity columns?*

It is recommended to use the mildest chaotropic agents at the lowest possible concentration that will ensure rapid elution and high recovery of activity. Iodination reactions employing either chloramine T or IODO-GEN (1,3,4,6-tetrachloro-3-6-diphenylglycouril) are particularly sensitive to inhibition by low concentrations of thiocyanate ions and, if antibodies are eluted from affinity columns or Protein G, using this chaotropic ion, it is essential that they are dialyzed thoroughly after elution to remove thiocyanate ions.

7. How do I monitor purity of the isolated antibodies?

Purity is best measured by gel electrophoresis. When analyzed by SDS-PAGE under non-reducing conditions, IgG antibodies should give a single protein band of about 160-170 kDa. On reduction with DTT or 2-mercaptoethanol, two or more bands will be seen corresponding to the individual heavy chains (50-55 kDa) or light chains (25-30 kDa). Other protein bands that are visible only on reduction may point to proteolytic action. This can often be prevented by careful use of protease inhibitors in culture supernatants before storage.

8. *What are the critical starting conditions for Protein A and G resin?*

Sample pH and salt concentration are usually not critical, except that the pH should be equal to or above 5.0. The salt concentration is normally quite high (> 0.1 M) to prevent non-specific binding.

9. *Do I need to control the salt concentrations during Protein A and G chromatography?*
Use 0.1-0.5 M salt to reduce non-specific adsorption. When working with Protein A, use high salt (2-3 M NaCl) with high pH to promote the binding of mouse IgG1.

10. *Is pH an important parameter to control during Protein A and G chromatography?*
The elution pH is the most critical variable. Protein G usually requires more acidic pH conditions to desorb the target immunoglobulins. For Protein A, elution by pH steps (starting at pH 6) may fractionate different species (weaker binding bovine IgG from target antibodies) or subclasses. High pH (pH 8-9), in conjunction with high salt may promote binding of mouse IgG1 to Protein A. The binding buffer pH should normally be higher than pH 6.0-7.0.

11. *Can I elute antibodies from a Protein A and G column using divalent cations?*
Concentrations of divalent cations (particularly Mg^{2+}) up to 1 M can sometimes replace acidic pH if there is concern about loss of activity of acid-labile immunoglobulins.

Troubleshooting Assistance:

Bubbles or cracks appear in the resin bed

- The resin has been stored at a cool temperature and then rapidly warmed up. The resin should be warmed slowly to room temperature before use.

The sample does not flow easily through the column

- The resin is clogged with particulates. Pre-filter the sample just before loading it on to the resin. . Ascites must be delipidated before use (see earlier procedure).
- If the resin is not stored at 2-8 ˚C, or they have been used more than once and stored in the absence of a bacteriostat, microbial growth may restrict flow through the resin.

No elution of the target protein is observed from the column

- The pH of the elution buffer may be incorrect. It is advisable to prepare new solutions.
- The elution conditions are too mild to desorb the target protein.

The recovery of target protein is low

- The binding of antibodies to Protein A or G is attributed in part to hydrophobic forces. Use chaotropic salts to reduce the strength of all hydrophobic interactions.

Poor resolution of the target protein

- The sample volume or concentration may be too large for the capacity of the resin. In this case, reduce the sample load or sample volume.
- The sample may also need to be filtered carefully.

The target protein elutes at an unexpected position

- There may be an ionic interaction between the protein and resin. You should maintain the ionic strength above 50 mM.
- There may be hydrophobic interactions between the sample and resin. In this instance, reduce the salt concentration and add suitable detergents or organic solvents.
- The column may be dirty.

The elution profile cannot be reproduced

- The nature of the sample may have altered and so it may be important to prepare a fresh sample.
- The sample load may be different from the original sample load. It is advisable to keep all these parameters constant.
- Proteins or lipids may have precipitated in the resin bed. Use elution conditions which stabilize the sample.
- The buffer pH and ionic strength is incorrect and new buffers will need to be prepared.

Glossary:

affinity chromatography - chromatographic separation based on a specific interaction between an immobilized ligand and a binding site on a macromolecule.

antibody - an immunoglobulin produced by the immune system of vertebrates in response to exposure to a foreign substance.

antigen - a molecule which can bind specifically to an antibody.

antiserum - the serum fraction from an animal that has been immunized or exposed to an immunogen and contains antibodies to a particular antigen.

ascites - a liquid tumour formed by injection of a hybridoma cell line into the peritoneal cavity. It is a common source of monoclonal antibodies from mice.

bed volume - the total volume occupied by the chromatographic packed bed. It is also referred to as the column volume or CV.

cell culture supernatant - the fluid made during cell culture (either roller bottle, suspension or perfusion) containing tissue media components and the secreted target.

chaotropic agent - a molecule which interferes with hydro-phobic interactions by disrupting the ordered structure of water molecules. Examples include urea and guanidine.

hybridoma - a hybrid cell line produced by fusing antibody producing cells with myeloma cells to generate immortal reproducing cells that produce specific monoclonal antibodies indefinitely in cell culture.

immobilized - bound to a surface, usually through covalent linkages.

immunoglobulin (Ig) - comprising 5 distinct classes in most higher animals. Classes called IgG (the most common), IgM, IgA, lgD and IgE. They differ from each other in size, charge, amino acid composition and carbohydrate content.

ion exchange chromatography - chromatographic separation based on different charge properties of macromolecules.

isoelectric point - the pH at which the protein has no net charge.

monoclonal antibody - an antibody derived from a single clone of immune cells. They are usually formed from a hybridoma cell line.

polyclonal antibodies - antibodies produced to the same immunogen by different cell types. Antibodies from antiserum are almost always polyclonal.

protein A/protein G - cell wall proteins of certain pathogenic bacteria which specifically bind to the Fc region of immunoglobulins.

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