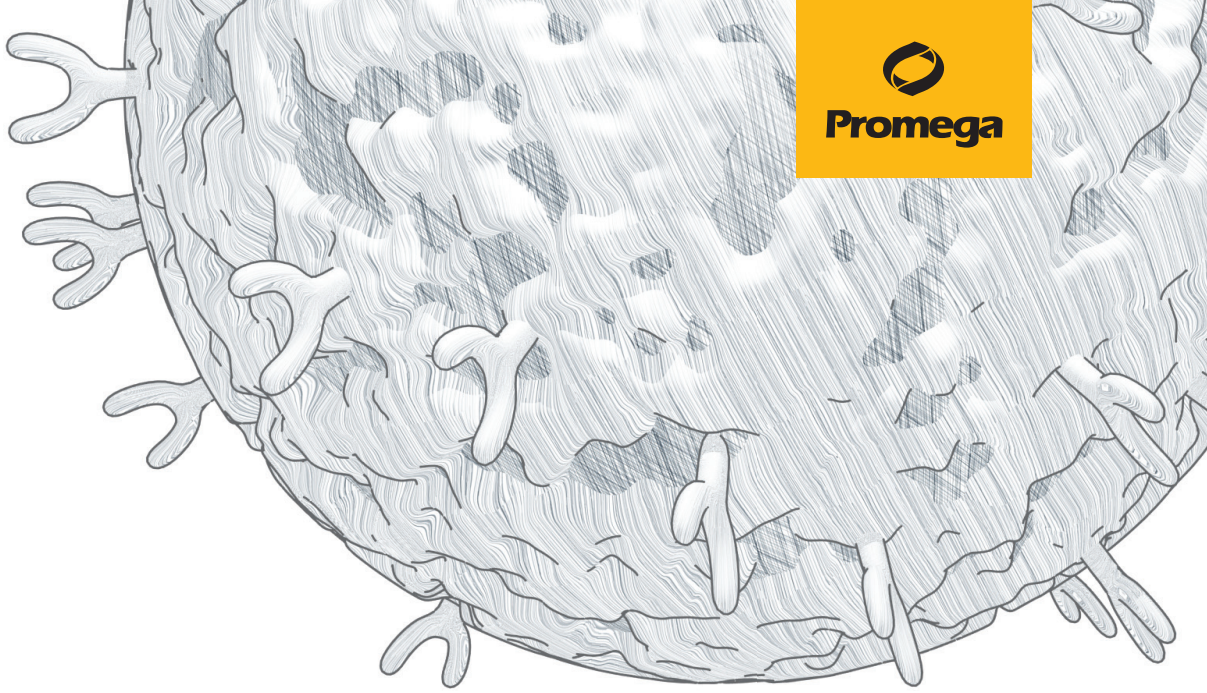
5 Antibody Purification and Labeling



Antibody Purification and Labeling

Antibodies are an indispensable tool for bioresearch, diagnosis and therapy, thus there is a great demand for antibody purification strategies. These strategies include antibody purification from various sources, such as serum (polyclonal antibodies), ascites (monoclonal antibodies) and cell culture supernatant of a hybridoma cell line (monoclonal antibodies). Polyclonal antibodies are a mixture of different antibodies produced by a diverse population of B cells. They may be of different isotypes, have different antigen specificities or recognize a different antigen epitope.

In contrast, moncolonal antibodies are produced by specific B cells that are clonally related; these antibodies are the same isotype, and have identical specificity for the antigen epitope. Depending on the starting material used, various antibody purification strategies exist, including classical chromatography (e.g., ion exchange), affinity-purification (e.g., on immobilized protein A and protein G beads) and antigen-specific affinity purification.

This chapter introduces the use of magnetic protein A and G beads for antibody purification as well as for on-bead antibody labeling.

5.1 Antibody Purification

OVERVIEW

The choice of the antibody purification method depends on the starting material, the intended application, and the manufacturing scale. However, most antibody purification strategies include the use of immobilized Protein A or Protein G **(Table 5.1)**. These bacterial proteins have different affinities for various antibody species and isotypes **(Table 5.2)**. Immobilized Protein A and Protein G can be used to purify monoclonal antibodies from ascites fluid or cell culture supernatant. However, in the case of polyclonal antibodies (serum samples) the use of Protein A or Protein G will enrich mainly the IgG fraction **(Figure 5.1)**. IgG is the main antibody isotype found in blood. For target-specific antibody isolation from serum, antigen-specific affinity-purification is required.

Table 5.1. Features of Native Ig-binding Proteins: Protein A and Protein G.

	Protein A	Protein G
Species	*Staphylococcus aureus*	*Streptococcus* spp. (group C and G)
Native Size	40-60 kDa	40-65 kDa
Ig-Binding Target	Heavy chain constant region (F_c) of IgG (CH2-CH3 region)	Heavy chain constant region (F_c) of IgG (CH2-CH3 region)

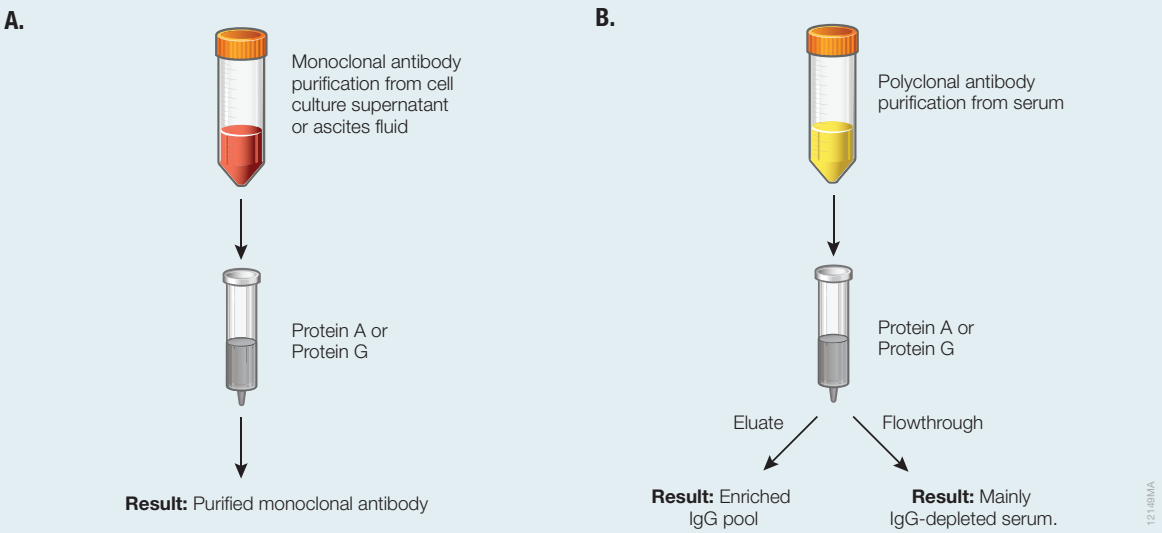


Figure 5.1. Different workflows for antibody purification using Protein A and Protein G. Monoclonal antibodies can be purified using Protein A or Protein G **(Panel A)**. The IgG fraction of polyclonal antibodies from serum can be enriched using Protein A or Protein G. Furthermore, Protein A or Protein G can be used to deplete the IgG fraction from serum. Note: For target-specific antibody purification from serum antigen-specific affinity-purification is required **(Panel B)**.

Table 5.2. Relative Recovery of Different Antibody Species and Isotypes by Magne™ Protein A Beads and Protein G Beads.

Species	Isotype	Magne™ Protein A Beads (µg)	Magne™ Protein G Beads (µg)
Bovine	IgG	36.2	33
Goat	IgG	26.2	26
Human	IgA	4.8	0.4
	IgG1	35.2	39.4
	IgG2	34.4	32.6
	IgG3	0	37.6
	IgG4	28.8	38
	IgM	8.2	0
Mouse	IgG1	18.6	30.4
	IgG2a	33.2	31.6
	IgG2b	29.6	31.4
	IgG3	16	9.2
Porcine	IgG	35.2	27.6
Rat	IgG1	29.4	34.2
	IgG2a	0	33
	IgG2b	0	31.2
Sheep	IgG	26.6	25.6

Note: Starting material for the recovery experiment was comprised of 50µg of purified antibody diluted in 1ml of buffer and captured with 50µl of bead slurry (20%). Experiment performed in triplicate (CV ≤10%).

Magne™ Protein A Beads and Magne™ Protein G Beads

Manual or automated antibody purification from different sample types; antibody enrichment or antibody depletion from serum samples; antibody labeling with small molecules or fluorophores.

Description

Magne™ Protein A Beads and Magne™ Protein G Beads are magnetic affinity beads with high specificity and high capacity for binding antibodies from cell culture supernatant, ascites fluid and serum samples. Antibody purification can be performed easily from a single sample or multiple samples in parallel, or in a high-throughput automated fashion. The magnetic beads allow for superior purification and recovery of concentrated antibodies from small input volumes (20µl) by decreasing losses normally associated with handling of small volumes and nonmagnetic resins.

Principle

Recombinant Protein A from *Staphylococcus* and recombinant Protein G from *Streptococcus* are covalently attached in an oriented fashion to magnetic beads. Biological samples are added, and antibodies are captured by the beads. Using magnetic devices, beads are attached and unbound material is washed away. Finally, antibodies are eluted using lower pH buffer and the solution is neutralized **(Figure 5.2)**.

Features and Benefits

- **High Binding Capacity:** Binding capacities of 25mg of antibody per ml of settled beads are observed, depending on antibody species and isotype.
- **Simple:** Easy to handle beads with fast magnetic response.
- **High Purity:** Due to low nonspecific binding.
- **Flexible:** Handles sample volumes from 20µl–50ml.

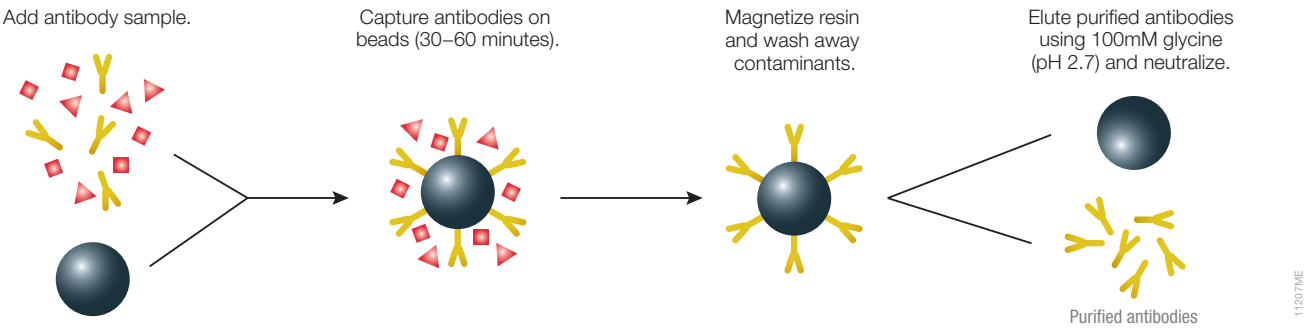
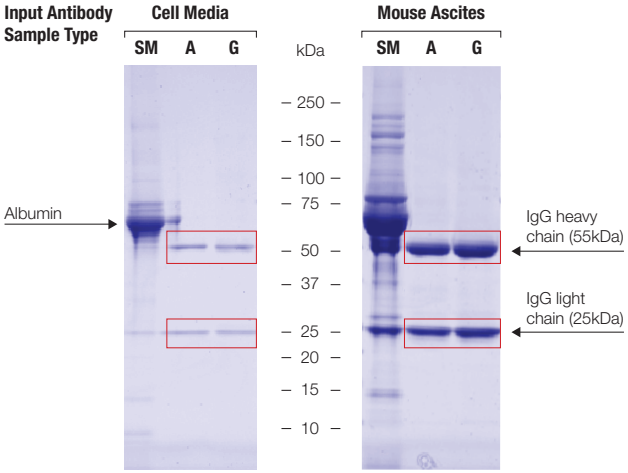


Figure 5.2. Antibody purification using Magne™ Protein A Beads or Magne™ Protein G Beads.

Antibody Purification



SM = Starting Material
A = Antibody purified with Magne™ Protein A Beads
G = Antibody purified with Magne™ Protein G Beads

Figure 5.3. Antibody purified from various sample types using Magne™ Protein A and Magne™ Protein G Beads. Antibody was purified from 50µl of cell culture media (mouse IgG1), mouse ascites (IgG2a) using 50µl of Magne™ Protein A Beads (A) and Magne™ Protein G Beads (G). Samples were analyzed by adding 1µl of starting material (SM) or 5µl of purified sample to SDS buffer, and heating at 80°C for ten minutes before loading onto a 4–20% Tris-glycine gel. The gel was stained with SimplyBlue® Safe Stain.

Additional Information:

- Magnetic stands and spacer for the use with this product are listed in Chapter 4.
- High-throughput antibody purification platforms such as the ReliaPrep™ LV 32 HSM Instrument (Cat.# **A1715**) or a robotic liquid handler platform such as the Beckman Coulter Biomek® FX can be used.
- Custom solutions for using the beads with a Maxwell® Instrument are possible upon request.
- Note: Magne™ Protein A Beads and Magne™ Protein G Beads are not recommended for use in immunoprecipitation (IP) or Co-IP applications.

References

Godat, B. and Nath, N. (2012) High-Capacity and High-Purity Antibody Purification Using Magnetic Beads. [Promega Corporation web site. November 2012. Accessed 1-20-2014. http://www.promega.com/resources/pubhub/high-capacity-and-high-purity-antibody-purification/

Ordering Information

Magne™ Protein A Beads
(Cat.# **G8781, G8782, G8783**)

Magne™ Protein G Beads
(Cat.#. **G7471, G7472, G7473**)

5.2 Antibody Labeling

OVERVIEW

Antibodies can be labeled with a diverse number of small molecules including biotin, fluorophores (e.g., FITC, Alexa Dyes, Cy3) and other large proteins (e.g., horseradish peroxidase). These antibody conjugates are useful reagents in bioresearch mainly for analyte/antigen detection in ELISA, fluorescent microscopy and for antibody-based assays. A specific class of antibody conjugates, Antibody Drug Conjugates (ADCs), is comprised of antibodies labeled with cytotoxic compounds intended for human therapy to target and kill specific cells (e.g., solid tumors). An ADC binds to target cells via a specific antibody-cell surface antigen interaction, the antibody and drug conjugate are internalized into endosomes. In the endosomes the linker between the cytotoxic drug and antibody are cleaved causing the release of the cytotoxic molecule and subsequent killing of the target cell. There are three aspects of ADC production that require optimization: the targeting antibody, the labile linker and the cytotoxic agent.

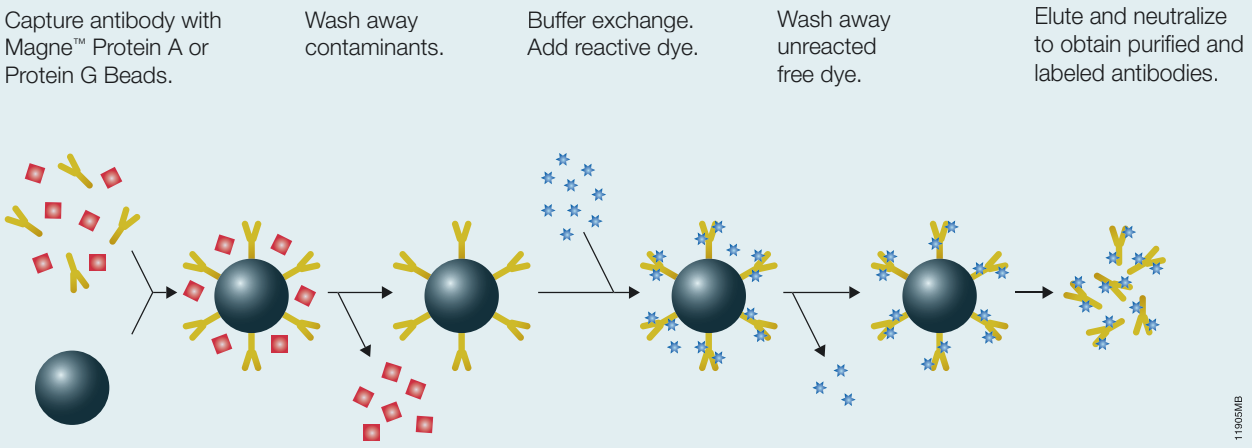


Figure 5.4. Schematic diagram of on-bead antibody conjugation/labeling.

Conjugation and Labeling Strategies

Antibodies are labeled with small molecules using two common chemistries: 1) a small molecule that reacts with primary amines on the lysines amino acids; and 2) reducing interchain disulfide bonds in antibodies with a reducing agent (DTT or TCEP) followed by reaction with thiol-reactive small molecules.

Antibody labeling is typically performed in solution and consists of several steps. For example, labeling antibody with a thiol-reactive small molecule starts with a purified antibody preparation that is buffer exchanged or dialyzed into an appropriate buffer for reduction of interchain disulfide bonds using reducing agents. After reduction, the antibody solution is buffer exchanged to remove the reducing agent. Antibody is then reacted with a small molecule followed by another dialysis step to remove any nonreacted small molecule. The method is tedious and time-consuming because of the several dialysis steps involved. In addition, the method has a very limited throughput because of the need for purified antibody.

On-Bead Antibody Labeling

To simplify the workflow for labeling antibody and to increase throughput, we have developed an on-bead antibody conjugation method using high-capacity magnetic Protein G and Protein A beads **(Figure 5.4)**. The method involves binding antibody to Protein G or Protein A beads. Instead of dialysis, simple wash steps are performed to remove unreacted small molecules. In addition, antibody can be directly captured from cell media, serum or ascites fluid without the need for pre-purification. Magnetic beads allow 1–96 samples to be labeled in-parallel (for sample volumes of 100µl–1ml), significantly improving the throughput.

Advantages of On-Bead vs. In-Solution Labeling

- Avoid pre-purification, dialysis and concentration steps required for in-solution labeling.
- Resulting antibody is labeled and highly concentrated.
- Conjugate/Label from 20µl to 50ml sample size.
- Automatable for 1–96 samples.
- Eluted, labeled antibody is compatible with downstream applications such as cell internalization and Antibody-dependent Cell-mediated Cytotoxicity (ADCC assay).

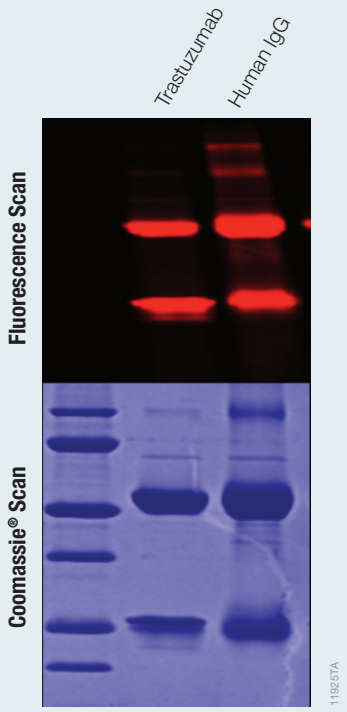


Figure 5.5. Gel images showing purification and labeling of anti-Her2 antibody (Trastuzumab) and control human IgG with Alexa Fluor® 647.

pHAb Amine and Thiol Reactive Dyes

pH Sensor Dyes for Screening Antibody Internalization

Description

pHAb Reactive Dyes are pH sensor dyes that have very low fluorescence at pH > 7 and a dramatic increase in fluorescence as the pH of the solution becomes acidic. pHAb Dyes have excitation maxima (Ex) at 532nm and emission maxima (Em) at 560nm. pHAb Dyes are designed specifically for antibody labeling and are available in two reactive forms suitable for antibody conjugations.

Principle

pHAb Amine Reactive Dyes have a succinimidyl ester group that reacts with primary amines on lysine amino acids of antibodies. pHAb Thiol Reactive Dyes have a maleimide group that reacts with thiols. This maleimide group is conjugated to the antibody after the cysteine disulfide bonds in the hinge region of the antibody are reduced to thiols using a reducing agent.

A key feature of pHAb Dyes is the two sulfonate groups per dye, which increase solubility in water and reduce the aggregation often seen with other non-sulfonated dyes. pHAb Dyes maintain their fluorescence response to pH change even after conjugation to antibody, as shown in Figure 5.6.

pHAb Reactive Dyes can be conjugated to antibodies using two different workflows. One is the traditional solution-based (or in-solution) chemistry workflow. The other workflow, shown in Figure 5.7, is on-bead conjugation, which uses magnetic protein A and protein G affinity beads to selectively capture antibodies from solutions containing purified antibody or directly from biological samples expressing antibodies (i.e., from cell culture media).

Features and Benefits

- **Accurately Determine Antibody Internalization:** Fluorescence increases upon internalization, with lower pH inside the cell.
- **Use with On-bead Conjugation:** Conjugate directly from biological samples expressing antibodies.
- **Compatible with Plate-based Assays:** Measure internalization in 96-well plates, real time.
- **Minimal Background Fluorescence:** Only conjugated dyes are internalized and thus fluorescent. Free dye outside the cell has negligible fluorescence.

Antibody Labeling

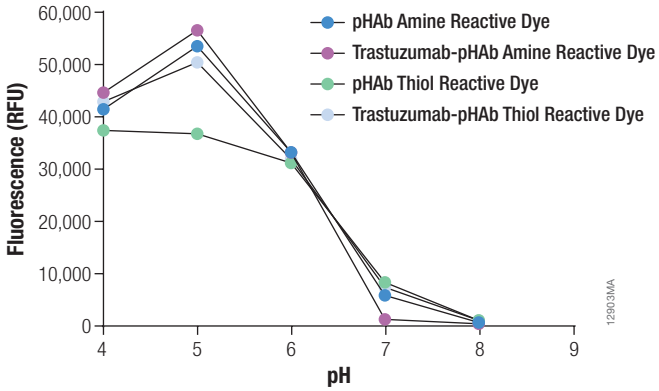


Figure 5.6. Fluorescence is shown as a function of pH for Trastuzumab labeled with pHAb Amine Reactive Dye or pHAb Thiol Reactive Dye compared to pHAb Amine Reactive Dye or pHAb Thiol Reactive Dye alone.

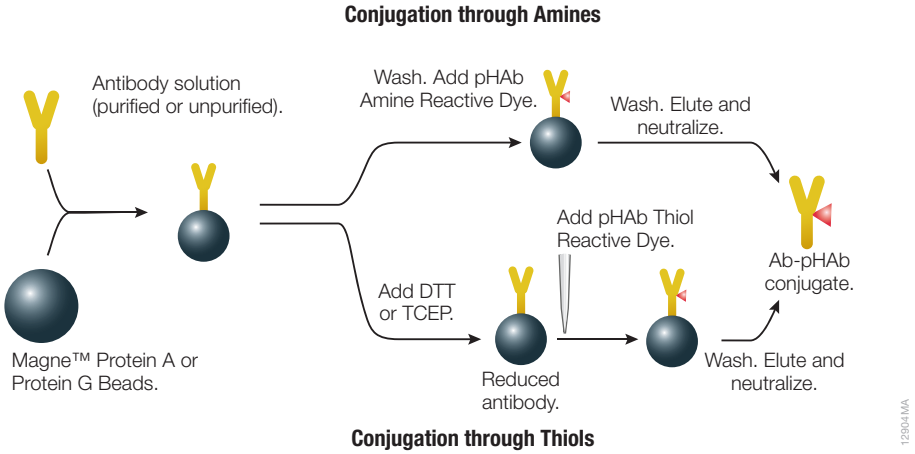


Figure 5.7. On-bead conjugation of antibody using Magne™ Protein A Beads or Magne™ Protein G Beads.

Ordering Information

pHAb Amine Reactive Dye
(Cat.# G9841, G9845)

pHAb Thiol Reactive Dye
(Cat.#. G9831, G9835)