WorkBeads Protein A

WorkBeads™ Protein A resin is designed for the purification of monoclonal and polyclonal antibodies using affinity chromatography. For small scale purification and initial screening in process development we recommend BabyBio A 1 ml and 5 ml columns prepacked with WorkBeads Protein A resin. WorkBeads Protein A resin can also be used for applications in other formats, such as test tube batch adsorption, spin columns, gravity columns or multi-well filter plates. The resin can be used for immunoprecipitation experiments.

- High dynamic binding capacity for monoclonal and polyclonal antibodies, with excellent recovery and purity
- Improved coupling chemistry results in high pH stability and low protein A leakage
- Reliable, reproducible and efficient

Short protocol

This short protocol is for column packing of WorkBeads Protein A and protein purification. Detailed instructions and recommendations for optimization are given later in this instruction. Recommended buffers are listed in Table 1.

1. Make a slurry of the desired resin concentration.
2. Pour the slurry into the column.
3. Pack the resin with an appropriate flow rate.
4. Apply an axial compression of less than 2%.
5. Equilibrate the column with binding buffer.
6. Apply sample.
7. Elute the target protein with elution buffer.
8. Wash the column with deionized water.
9. Equilibrate the column with 20% ethanol for storage.

Principle

Affinity chromatography is a useful technique for the separation of proteins by exploiting the reversible interaction between the target protein and the immobilized ligand. The interaction can be bio-specific, for example antibodies binding to protein A, or not bio-specific, for example histidine-tagged proteins binding to metal ions.

This chromatography technique provides high selectivity, resolution and capacity. High purity is often achieved in a single step. Large sample volumes can be processed and samples applied under conditions that favour specific binding to the ligand. Elution is often carried out under gentle conditions which helps to preserve bioactivity. The target protein is eluted, in a purified and concentrated form, by modification of pH, ionic strength, or by introducing a competitive agent.

Column packing

WorkBeads resins are cross-linked using a proprietary method that results in a very rigid resin, which tolerate pressures of several bars and consequently can run at high flow rates. Follow both this general advice when packing a column and the column manufacturer's specific instructions. Preferably, use a column with an adjustable adaptor. In some instances, a packing reservoir or column extension may be needed.

Note: Always make sure that the maximum pressure of the column hardware is not exceeded. Backpressure caused by the chromatography system components connected downstream of the column may reduce the maximum flow that can be used. Wear eye protection.

1. Wash the resin

The resin is provided in 20% ethanol. To avoid undue backpressure when packing, wash the desired amount of resin with several column volumes of deionized water before packing.

2. Make a slurry

Add deionized water to the washed resin to obtain a 40% to 60% slurry concentration. Make approximately 15% extra slurry to compensate for the compression during packing. The amount of slurry to be prepared can be calculated according to:

$$\text{Slurry volume} = \frac{\text{bed volume x } 100}{\text{\% slurry}} \text{ x } 1.15$$

3. Pour the slurry into the column

Pour the slurry slowly down the side of the column to avoid formation of air bubbles. Preferably, use a packing reservoir or an extra column tube to extend the column volume to accommodate the entire slurry volume during packing. If no packing adaptor is available packing can be done by stepwise additions and packing. Although not recommended this will give acceptable results for most applications.

4. Pack the bed

Pack the resin with a downward flow higher than the intended operational flow. We recommend 300 cm/h for columns up to 25 mm i.d. and with 200 mm bed height. Make sure the packing flow rate does not exceed the maximum pressure of the column hardware or the resin. The operational flow should not be more than 75% of the packing flow rate.

5. Close the column

When the bed height is constant mark the bed height on the column. Stop the flow and remove the packing reservoir or extra tube. Note that the bed height will increase temporarily when the flow is stopped. If needed, adjust the bed height by removing excess resin. Be careful not to remove too much resin. Gently fill the column with packing solution to its rim without disrupting the packed bed. Insert the adjustable adapter on top of the packed bed. Apply a small axial compression of less than 2% of the final bed height by lowering the adapter into the packed bed.

6. Apply a flow

Apply a flow of 225 cm/h (taking account of section 4) and check for any gap formation above the surface of the resin bed. If a gap is observed, stop the flow and adjust the adaptor to eliminate the gap.

Purification

The following brief instruction gives general conditions for purification using a column packed with WorkBeads Protein A. Recommended buffers are listed in Table 1 and recommendations for optimization are mentioned below.

Table 1. Recommended buffers for purification. Other buffers can possible be used.

Buffer	Composition
Binding buffer	PBS; 20 mM Na-phosphate, 150 mM NaCl, pH 7.4
Elution buffer	100 mM Na-citrate, pH 3.0

Sample preparation

Clarify the sample by centrifugation at 10 000 - 20 000 × *g* for 15 - 30 minutes. It is recommended to also pass the sample through a 0.22 - 0.45 µm filter to remove any remaining particles. If the sample contains only small amounts of particles, it may be enough to only carry out filtration. Make sure that the sample has a pH between 5 and 8. Preferably, the sample should have the same pH and ionic strength as the binding buffer.

Standard purification

1. Equilibrate the column using 10 column volumes (CV) binding buffer.
2. Apply a clarified sample under neutral conditions.
3. Wash using 10-20 CV binding buffer.
4. Elute with 5 CV elution buffer. Include 100 µl 1 M Tris-HCl, pH 9 per 1 ml collected fraction, to prevent degradation of eluted target protein.
5. Re-equilibrate with 10 CV binding buffer.
6. Equilibrate with 10 CV 20% ethanol for storage.

Before starting a purification run, it is recommended to make a blank run (with no sample applied) to remove any loosely bound ligands or impurities on the resin. Do this also for a newly packed resin. Although the above standard conditions usually give excellent results it may be worthwhile to optimize the purification protocol for highest purity of the target protein, see *Optimization.*

Optimization

Selection of column size

The column size should be selected based on estimated amount of target protein in each run, and the dynamic binding capacity (DBC) of the resin. DBC is the capacity obtained under the chosen run conditions and is usually lower than the static binding capacity (total binding capacity). Figure 1 shows an example of the DBC at different flow rates (or residence times). At a low flow rate, the capacity is high. At increasing flow rates the binding capacity decreases.

To obtain the highest possible recovery of the target protein we recommend the application of no more sample than 80% of the capacity of the packed column at the selected flow rate. Consider using a larger column or dividing the sample into repeated purification runs if needed. If the amount of the antibody in the sample is not known, it can be determined by running a small sample on a BabyBio A 1 ml and determine the amount of eluted antibody. Alternatively, make the purification and collect the flow through material for subsequent analysis to determine whether the column was "over-saturated". If desired the collected flow through material can be reapplied on the packed column after proper regeneration, in a new purification run.

Binding capacity

Antibody binding capacity depends on the flow rate used for binding and may differ between different antibody species. For WorkBeads Protein A, the binding capacity is more than 40 mg human serum IgG/ml resin at 2.5 minutes residence time in a 6.6 x 100 mm column, which corresponds to a linear flow rate of 240 cm/min. The binding capacity decreases with increased flow rate, see Figure 1.

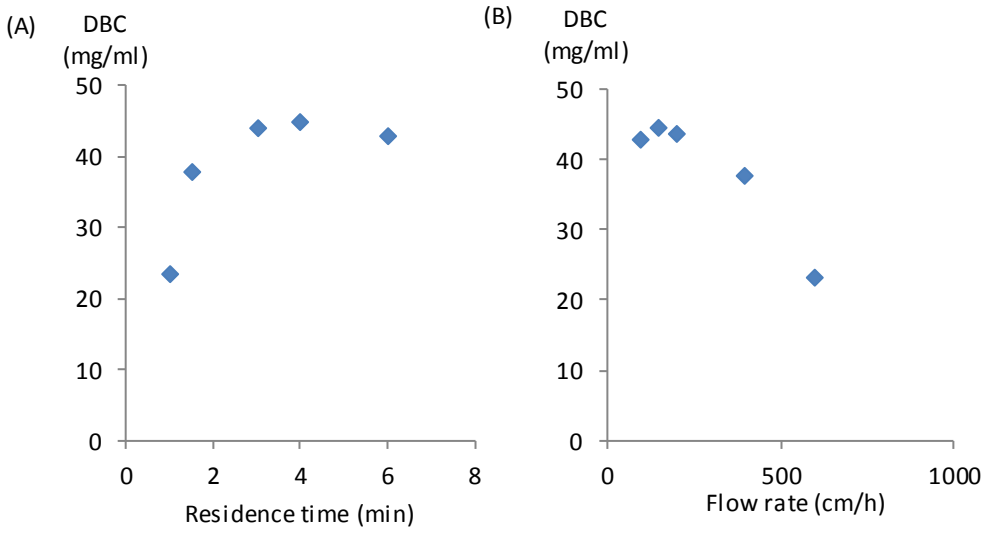

Figure 1. DBC at 10% ($QB_{10\%}$) for human serum IgG at different flow rates determined by frontal analysis. Sample: 1 mg/ml IgG in PBS, pH 7.4 in a 6.6 x 100 mm packed bed with WorkBeads Protein A. (A) DBC for WorkBeads Protein A versus residence time in minutes. (B) DBC for WorkBeads Protein A versus flow rate in cm/h.

Optimization of binding

Human IgG and IgG from several other species bind to WorkBeads Protein A under neutral pH at moderate salt concentrations. Apart from the recommended binding buffer in Table 1, other buffers can be used. For example, 50 mM Na-phosphate, pH 7.4 or 50 mM Na-borate, pH 9. However, IgG with weaker affinity (e.g., mouse IgG1) may need a binding buffer with a combination of high pH and ionic strength to be able to bind. For example, 50 mM Na-borate, 4 M NaCl, pH 9.

Extra wash step

To remove weakly adsorbed impurities, it may be useful to add an extra washing step, with the binding buffer, after the standard wash. This can be done using a buffer with slightly increased ionic strength compared to the binding buffer or by a small decrease in pH that do not elute the target protein, see *Optimization of elution*.

Optimization of elution

Apart from the recommended elution buffer in Table 1, other buffers can be used. For example, 100 mM glycin-HCl, pH 2.7. IgG can be sensitive to low pH. In order to avoid denaturation after elution with low pH, the pH can be neutralized by adding 100 µl of 1 M Tris-HCl, pH 9 per ml collected fraction to each fractionation tube before starting the purification or immediately after completed elution. Collect the target protein and perform buffer exchange using a BabyBio Dsalt column equilibrated with a neutral buffer, see *Related products*.

Perform gradient elution using a gradient from 100 mM Na-citrate, pH 6.0 to 100 mM Na-citrate, pH 3.0 over 10 - 20 CV. Desorption will occur when the pH is low enough, while avoiding too low pH. Run a test gradient elution (as above) with a small amount of sample to determine at what pH the target protein is eluting. The pH measured at the tail of the peak can be selected for elution. Prepare a 100 mM Na-citrate buffer with the selected elution pH and make the scale-up using this elution buffer.

Scale-up

After developing a chromatographic procedure in a small scale column, e.g., 10 (i.d.) × 100 mm (bed height), WorkBeads resins can be packed into larger columns for scale-up. Large scale purification is often carried out in columns with bed heights of 200 - 300 mm.

Scale-up principles

During scale-up the ratio between sample volume and column volume should be kept constant. The column volume is scaled up by increasing the column diameter while keeping the bed height the same (e.g., 200 mm). The linear flow rate should remain the same while the volumetric flow rate increases. The volumetric flow rate for each column can be calculated according to:

$$\text{Volumetric flow rate (ml/min)} = \frac{\text{Linear flow rate (cm/h)} \times \text{Column cross sectional area (cm}^2\text{)}}{60}$$

Flow

The concepts of volumetric flow, linear flow rate and residence time is important when doing scale-up in chromatography. Volumetric flow is measured in ml/min or l/min, linear flow in cm/h and residence time in minutes. The relationship between these metrics are:

$$\text{Linear flow rate (cm/h)} = \frac{\text{Volumetric flow (ml/min)} \times 60}{\text{Column cross sectional area (cm}^2\text{)}}$$

$$\text{Residence time (minutes)} = \frac{\text{Column bed height (cm)} \times 60}{\text{Linear flow rate(cm/h)}}$$

In the initial process development phase it is common to use a small column, e.g., 10 × 100 mm, to save sample, buffers and time. This column has a shorter bed height than the final column, which may have a bed height of 200 mm or more. The flow rate for the larger column can be calculated from the flow established on the small column, using the equation above by keeping the residence time of the small column the same for the larger column. This will allow an increase of the linear flow in proportion to the increase in bed height between the columns see Table 3 for examples. If the column bed heights are constant during scale-up the linear flow rate should be also constant (as well as the residence time).

Table 3. Example of scale-up parameters.

Column dimension	Residence time (minutes)	Linear flow rate (cm/h)	Volumetric flow rate (ml/min)
10 × 100	4	150	1.96
25 × 200	4	300	24.5
50 × 200	4	300	98.2

Additional purification

Antibody purification on WorkBeads Protein A frequently gives high purity in a single step. For very high purity requirements, it may thought be necessary to add a second purification step. The additional purification step is used to remove traces of leaked protein A ligand, DNA, aggregates and other remaining impurities from the sample. In research-scale purification, size exclusion chromatography (gel filtration) is often a good polishing step since it removes impurities, the imidazole used for elution and potential aggregates of the target protein. Size exclusion chromatography can be done using WorkBeads 40/1000 SEC, WorkBeads 40/100 SEC and WorkBeads 40/10 000 SEC resins having different separation ranges. Ion exchange chromatography is suitable for both research scale purification and process scale. WorkBeads 40S, WorkBeads 40Q and WorkBeads 40 DEAE resins provide different selectivities for ion exchange chromatography. These resins are also available as ready-to-use 1 ml and 5 ml BabyBio S, BabyBio Q and BabyBio DEAE columns, see *Related products*.

The polishing purification step can be based on several chromatographic techniques depending on the target molecule and the contaminants:

Size exclusion chromatography

Size exclusion chromatography (SEC) can be used for the separation of monomeric antibodies from dimeric antibodies, antibody aggregates as well as complexes of leaked protein A and antibody. SEC technique separates proteins and other biomolecules according to size, hence the monomeric antibodies will elute after antibody dimer, aggregates and complexes of leaked protein A and the antibody. This technique is simple to run. It is carried out under neutral conditions, and is recommended for high purity demands in lab scale purification, (e.g. using WorkBeads 40/1000 SEC). Optimization is often not required for significant purification, but may sometimes be worthwhile. The technique is not recommended for bioprocess scale applications due to dilution effects, low capacity and that it is time consuming.

Cation exchange chromatography

Cation exchange chromatography is commonly used as a polishing step in antibody purification strategies. Many antibodies are weakly basic at neutral pH and will hence bind to a cation exchange chromatography resin, (e.g., WorkBeads 40S). Conversely, protein A does not bind to a cation exchange resin under the same conditions. Dissociation between antibodies and potential leakage of protein A can therefore be carried out by cation exchange chromatography technique under neutral pH. This technique usually requires optimization for each specific antibody to be purified.

Anion exchange chromatography

Anion exchange chromatography technique is often used in a negative chromatography mode, during the polishing antibody purification. Potential leakage of protein A as well as complexes between protein A and the antibody tend to bind to an anion exchange chromatography resin (e.g., WorkBeads 40Q) at neutral pH, whereas the antibody itself usually does not bind. Also the use of this technique as a polishing step, usually require optimization for optimal antibody purification.

To find out more about Bio-Works chromatography resins for additional purification, please visit www.bio-works.com

Desalting and buffer exchange

Protein samples collected after purification using Protein A using the conditions describe will contain low pH and sometimes a relatively high concentration of salt which usually needs to be removed. Buffer exchange or desalting of the sample can be done, using BabyBio Dsalt 1 ml or BabyBio Dsalt 5 ml (see *Related products*). Chromatographic desalting is especially useful when the sample needs to be processed rapidly to avoid degradation. For process-scale, diafiltration should be considered.

Maintenance of the resin

Unpacking and inspection

Unpack the shipment as soon as it arrives and inspect it for damage. Promptly report any damage or discrepancies to your local supplier.

Cleaning using NaOH

During purification impurities such as cell debris, lipids, nucleic acids and protein precipitates from the samples may gradually build up in the resin. The severity of this process depends on the type of sample applied to the packed column, and the pre-treatment of the sample. The bound impurities may reduce the performance of the column over time. Regular cleaning (Cleaning-in-place, CIP) keeps the resin clean, reduces the rate of further contamination, and prolongs the capacity, resolution and flow properties of the column. Cleaning of a column using 1 M NaOH applied by a low reversed flow for 2 hours or overnight is often sufficient.

Sanitization (reduction of microorganisms) can be done using combinations of NaOH and ethanol, e.g., incubation with a mixture of 0.5 M NaOH and 40% ethanol for 3 hours. The sanitization procedure and its effectiveness will depend on the microorganisms to be removed, and needs to be evaluated for each case. Prolonged wash with alkaline conditions will reduce the functionality of the column and must therefore be kept to a minimum (see Figure 2).

Regeneration of WorkBeads Protein A

After purification using WorkBeads Protein A perform the following steps:

1. Unless elution was carried out at very low pH there may be a need for regeneration by cleaning the column with, for example, 10 CV 100 mM glycin-HCl, pH 2.7 or 100 mM Na-citrate, pH 3.

2. Wash the column with 5 CV deionized water.

3. Cleaning-in-place by passing 15 CV 15 mM NaOH over 15 minutes, or for harsher conditions 50 mM NaOH. For increased efficiency, before the NaOH wash, include a passage of 15 CV 100 mM 1-thioglycerol, pH 8.5, over 15 minutes to reduce any oxidized aggregates adsorbed to the column.

4. Wash with 10 CV neutral buffer. Make sure that neutral pH is restored in the column. Prolonged exposure to extreme pH may harm the resin.

5. Wash with 10 CV deionized water.

6. Wash with 10 CV 20% ethanol before storage.

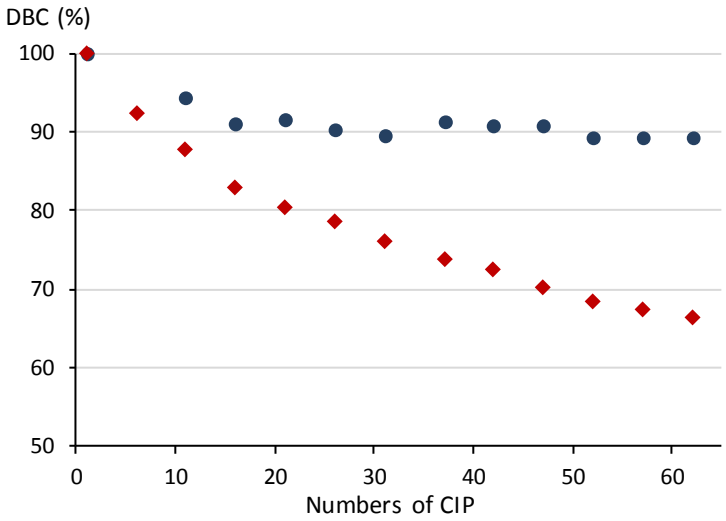

Figure 2. Alkaline stability of WorkBeads Protein A determined by frontal analysis using 1 mg/ml IgG in the presence of PBS, pH 7.4. CIP cycle: 100 mM 1-thioglycerol, pH 8.5, 15 minutes' incubation; followed by 15 mM NaOH (blue circles) or 100 mM NaOH (red diamonds), for 15 minutes.

Storage

Store at 2 to 8°C in 20% ethanol.

Additional information

Product description

	WorkBeads Protein A
Target substance	Antibodies (IgG), bound via the F_c-region
Matrix	Rigid, highly cross-linked agarose
Average particle size[1] (D_{v50})	45 µm
Ligand	Recombinant protein A expressed in *E. coli* using animal-free medium
Dynamic Binding Capacity[2] (DBC)	> 40 mg human IgG/ml resin
Maximum recommended flow rate[3]	300 cm/h
Chemical stability	Compatible with all standard aqueous buffers used for protein purification, 10 mM HCl (pH 2), 10 mM NaOH (pH 12), 0.1 M sodium citrate-HCl (pH 3), 6 M guanidine-HCl, 20% ethanol Should not be stored at low pH for prolonged time
pH stability	3 - 10 (short term) 2 - 12 (cleaning)
Storage	2 to 8 °C in 20 % ethanol

1. The median particle size of the cumulative volume distribution.
2. DBC was determined at 10% breakthrough ($QB_{10\%}$) by frontal analysis with 1 mg/ml human polyclonal IgG in PBS, pH 7.4 at 1.4 ml/min (240 cm/h) in a column packed with WorkBeads Protein A resin, column bed 6.6 x 100 mm and 2.5 minutes residence time.
3. Maximum recommended flow rate at 20 °C using aqueous buffers. Decrease the maximum flow rate if the liquid has a higher viscosity. Higher viscosities can be caused by low temperature (use half of the maximum flow rate when operating at 4 °C), or by additives (e.g., use half of the maximum flow rate for 20% ethanol).

Intended use

WorkBeads Protein A resin is intended for research, process development and industrial use only. The resin shall not be used for preparation of material for clinical or diagnostic purposes

Safety

Please read the associated Safety Data Sheets (SDS) for WorkBeads Protein A resin, and the safety instructions for any equipment to be used.

Related products

Product name	Pack size[1]	Article number
Prepacked columns		
BabyBio A 1 ml	1 ml x 1	45 605 101
BabyBio A 5 ml	5 ml x 1	45 605 105
BabyBio Dsalt 5 ml	5 ml x 1	45 360 105
BabyBio S 1 ml	1 ml x 1	45 200 105
BabyBio Q 1 ml	1 ml x 1	45 100 105
Bulk resins		
WorkBeads 40/1000 SEC	25 ml	40 300 001
WorkBeads 40S	25 ml	40 200 001
WorkBeads 40Q	25 ml	40 100 001

1. Other pack sizes can be found in the complete product list on www.bio-works.com

Ordering information

Product name	Pack size	Article number
WorkBeads Protein A	1.5 ml	40 605 001
	5 ml	40 605 002
	10 ml	40 605 003
	100 ml	40 605 004
	1 L	40 605 005

Orders: sales@bio-works.com or contact your local distributor.

For more information about local distributor and products please visit www.bio-works.com or contact us at info@bio-works.com

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