User Guide

CaptivA™ PriMAB

Protein A Affinity Resin

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These user instructions provide general guidance on the basic practices associated with the use of agarose bead based Protein A affinity resins for the purification of monoclonal antibodies. As CaptivA™ PriMAB resin is based on GE's Sepharose 4FF bead and Repligen's recombinant protein the well established user practices apply. These instructions are based on those well established practices as detailed in instructions for other Protein A Sepharose affinity resins. For the full original text refer to the references[4].

Included sections are:

- Column Packing
    - Slurry Preparation
    - Packing Evaluation
- Method design, optimization and screening
- Scale Up
- Optimization
- Processing and Maintenance
    - Regeneration
    - Cleaning-in-place (CIP)
    - Sanitization
    - Storage

Column Packing

Slurry preparation for and packing of smaller scale developmental columns is typically a simple manual process; although any bed height can be created the accepted default standard is 20cms. A generic protocol for manually packing a small column is as follows:

Materials

- Appropriate volume of CaptivA™ PriMAB resin depending on column diameter and bed height. For small columns this typically in the 10's of milliliter range and can be accurately calculated using the following formula:

  $v=(r^2 x \pi)b$ *where:*

  v= volume of IPA in milliliters

  r= radius of the column in centimeters

  b= bed height in centimeters

- Glass filter funnel
- Scoop
- Erlenmeyer flask (of appropriate volume)
- Beaker (of appropriate volume)
- 20% EtOH in water
- 250 ml 20% EtOH + 0.25 M NaCl

Step 1 – Resin Washing

Equilibrate the resin slurry required to room temperature. Pour the CaptivA™ PriMAB resin into the funnel and wash into the flask as follows:

- First with a 2x volume of 20% EtOH
- Followed by a 4x volume of 20% EtOH + 0.25 M NaCl

Step 2 - Slurry Preparation

The washed resin is now suspended in 20% EtOH + 0.25 M NaCl. Pour the washed CaptivA™ PriMAB resin into a beaker and add an equivalent volume of 20% EtOH + 0.25 M NaCl (i.e. for 50mls resin add 50mls 20% EtOH + 0.25 M NaCl)

Step 3 - Column Packing

Materials

- Column (various)
- Pump
- Packing reservoir
- Syringe, tubing and glass rod

Assemble the column - following the column manufacturers instructions, ensure that all parts are clean and intact.

1. Connect the packing reservoir to the column.
2. Inject 0.25 M NaCl, 20% ethanol into the bottom of the column with a syringe; ensure that there are no trapped air bubbles. Close the tubing with a stopper
3. Flush the column and reservoir with 0.25 M NaCl, 20% EtOH, leaving a few ml at the bottom and mount the column vertically on a laboratory stand.

Pack CaptivA™ PriMAB resin **–** As the CaptivA™ PriMAB resin is based on the GE Sepharose 4FF base bead these agarose bead packing instructions are well established[(5)].

CaptivA™ PriMAB resin is supplied pre-swollen in 18.5±1% Ethanol preservative solution. Prepare the CaptivA™ PriMAB resin by removing the storage solution and replace it with starting buffer in a ratio of 75% settled CaptivA™ PriMAB resin to 25% buffer. The starting buffer should not contain agents which significantly increase the viscosity. The column may be equilibrated with viscous buffers at reduced flow after packing has been completed.

Pour the slurry into the column, for best results the column should be filled in one time at a smooth steady rate. Typically this is accomplished by pouring down a glass rod held against the wall of the column that helps prevent the introduction of air bubbles.

1. Fill the remaining column space and reservoir with 0.25 M NaCl in 20% EtOH. Place the lid on the packing reservoir and connect it to the pump.
2. Open the column outlet and start the packing by pumping 0.25 M NaCl, 20% EtOH through the column at a flow rate of 1.5 X the maximum process flow rate until the bed height stabilizes, this should take no more than 5 minutes.

3. Switch off and disconnect the pump. Close the column outlet.
4. Remove the packing reservoir (this is best done over a sink or drain). Refill the column to the top with 0.25 M NaCl, 20% EtOH.
5. Wet the column adaptor by submerging the plunger end in 0.25 M NaCl, 20% ethanol, and drawing through with a syringe. Ensure that all bubbles have been removed.
6. Insert the adaptor into the top of the column, taking care not to introduce air bubbles.
7. With the adaptor outlet open, push the adaptor into the column and down onto the resin bed, allowing the 0.25 M NaCl, 20% EtOH to displace any air remaining in the tubing.
8. Lock the adaptor in place, connect it to the pump, open the column outlet and continue packing at a flow rate equivalent to the process flow rate **(≤ 3 bar)** for 3 minutes.
9. Mark the position of the top of the bed height on the column cylinder wall. Stop the pump, close the column outlet and reposition the adaptor to approximately 1 mm below the marked bed height position.

Use of an adaptor

Adaptors should be fitted as follows:

1. After the gel has been packed as described above, close the column outlet and remove the top piece from the column.
2. Carefully fill the rest of the column with buffer to form an upward meniscus at the top.
3. Insert the adaptor at an angle into the column, ensuring that no air is trapped under the net.
4. Make all tubing connections at this stage. There must be a bubble-free liquid connection between the column and the pump and column and the sample application system (LV-3 or LV-4).
5. Slide the plunger slowly down the column so that the air above the net and in the capillary tubing is displaced by eluent. Valves on the inlet side of the column should be regularly turned to ensure that all air is removed.
6. Lock the adaptor in position, open the column outlet and start the eluent flow. Pass eluent through the column at the packing flow rate until the bed is stable. Re-position the adaptor on the gel surface as necessary.

Packing large-scale columns - General recommendations

Columns can be packed in different ways depending on the type of column and equipment used. Please refer to the relevant column instruction manual carefully.

CaptivA™ PriMAB resin is easy to pack since its rigidity allows the use of moderately high flow rates. CaptivA™ PriMAB resin is typically packed at up to a maximum velocity of $300 cmhr^{-1}$ or pressure < 3 bar.

Note: As the columns increase in diameter the packing flow rate decreases, at packing flow rates below $150 cmhr^{-1}$ there is generally little impact, at higher flow rates a 3 fold increase in column diameter can increased packing pressure approximately 2 fold.

In general there are three suitable types of packing methods:

- Pressure packing (for columns with moveable adaptors).

- Combined pressure/suction packing (for medium sized columns with fixed bed heights).
- Suction packing (for large columns with fixed bed heights).
- Hydraulic pressure packing.

How well the column is packed will have a major effect on the performance of the resin and the purity and yield of the purification process. Guidelines are given for determining the optimal packing flow rates for different column designs columns with specific design features like adaptors and fixed bed heights.

Determining the optimal packing pressure

The optimal packing pressure/flow rate is dependent on column size, type, desired bed height, packing solution and temperature. The optimal packing flow rate must therefore be determined empirically for each individual system. Generically this is done as follows:

1. Calculate the exact amount of CaptivA™ PriMAB resin needed for the slurry (this is especially important for columns with fixed bed heights). Extra resin is required to allow for settling of the bed allow approximately 1.15L of resin slurry per 1 liter of packed bed.
2. Prepare the column per the column instructions.
3. Begin packing the column at a low flow rate (e.g. 30% of the expected max process flow rate), record the flow rate and back pressure when the bed is completely packed and the pressure has stabilized.
4. Increase flow rate recording both flow rate and pressure drop in a stepwise manner always allowing the pressure to stabilize at each step.
5. Continue recording flow and pressure until the maximum process flow rate has been reached. This reached when the pressure flow curve levels off or the maximum column pressure is reached.
6. Plot pressure against flow rate.

The optimal packing pressure is about 70% of the maximum pressure. From the packing pressure point on the curve, draw a straight line to zero. The maximum operational pressure should be <70% of the packing pressure. From the straight line, the maximum operational flow rate can be found.

Pressure Packing **–** typically for columns supplied with a movable top flow plate (e.g., GE BPG™; Millipore Vantage™ and Quickscale™) are packed by conventional pressure packing where packing solution is pumped through the settling chromatographic bed at a constant back pressure. Specific packing instructions and pressure flow curves are generally provided by the column manufacturers and can be matched with each resins pressure flow properties to develop a robust packing protocol for each column/resin combination. Generically the steps are as follows:

1. Make sure no air is trapped under the bottom bed support by pumping packing buffer through it from below. Excess liquid in the column can be removed by connecting tubing to the suction side of a pump. Leave about 2 cm of liquid in the column.
2. Mix the packing buffer with the medium to form a 50% slurry (settled bed volume/slurry volume = 0.5). Pour the slurry into the column. Insert top distributor plate the adaptor and lower to the surface of the slurry, making sure no air is trapped under the plate and secure in place.
3. Fill the adaptor inlet with packing solution.

4. Connect a pump and a pressure meter; apply a flow that gives the proscribed back pressure (typically about 0.1 bar). When the bed has settled, run for a few minutes, close the valve and stop the pump. Lower the plate down to the top of the bed.
5. Start the pump and apply a flow that gives the desired packing pressure. Keep the pressure constant during packing and check the pressure at the column inlet. **Never** exceed the pressure limit for column or medium. Run for at least 15 min.
6. When the bed has stabilized, mark the bed height, close the valve and stop the pump.
7. Disconnect the column inlet tubing and replace it with tubing leading to waste, push the top plate adaptor down to approximately 3 mm below the mark on the column tube. The packing solution will flush the adaptor inlet. Remove any trapped air by pumping liquid from the bottom (after the inlet tubing and the bottom valve have been properly filled).

Combined pressure/suction packing **–** typically these columns have a fixed bed height of 15 cm. It is packed by a combined pressure/suction technique. Follow the column manufacturer's instructions, which generically include:

1. Fitting an extra column section on top of the column tube as a packing reservoir.
2. Pour water or packing buffer into the column making sure that there is no air trapped under the bottom bed support. Leave about 2 cm of liquid in the column.
3. Pour the slurry into the column. Stir gently to give an homogeneous slurry. Add buffer until level with the upper rim and secure the lid in place.
4. Connect a pump and a pressure meter and start packing at the predetermined packing flow rate (or pressure). Keep the flow rate (or pressure) constant during packing and check the pressure at the column inlet. Never exceed the pressure limit for the column or medium.
5. When the bed has stabilized, the top of the bed should be exactly level with the top of the column tube. Switching the valve takes the buffer tank off line the inlet pump is now connected to the outflow side of the column. The packing buffer is recirculated in the system. If, when stabilized, the packed bed is not exactly level with the top of the column, add or remove slurry.
6. Keep the pump running, disconnect the column inlet and direct it to waste. The packing solution in the packing section is removed by suction through the bed.
7. Remove the packing reservoir section.
8. When the packing solution is within 5–8 mm of the bed surface stop the pump. This final operation should be completed as quickly to prevent bed expansion.
9. Start pumping buffer with upward flow through the column to remove any air bubbles.

Suction Packing **–** typically for columns with fixed end pieces. These columns are packed by suction, i.e. by sucking packing solution through the chromatographic bed at a constant flow rate.

1. Fit a packing device on top of the column tube.
2. Pour water or packing buffer into the column making sure that there is no air trapped under the bottom bed support. Leave about 2 cm of liquid in the column.
3. Mix the packing buffer with the medium to form a 50% slurry (settled bed volume/slurry = 0.5). Pour the slurry into the column.

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4. Connect the column outlet valve to the suction side of a pump and start packing the bed by suction through the bed at the predetermined flow rate. Keep the flow rate constant during packing.
5. When the bed has stabilized, the top of the bed should be just below the junction between the column and the packing device.
6. Just before the last of the packing solution enters the packed bed, stop the pump and quickly remove the packing device and replace it with the lid. This final operation should be completed quickly to prevent bed expansion when the flow stops.
7. Start pumping buffer with upward flow through the column to remove any air bubbles trapped under the lid.

Hydraulic Packing **-** is for columns supplied with a hydraulic function GE INdEX™ and FineLine™; Novasep Prochrom® DAC. In these systems an automated hydraulic systems controls packing as the adaptor is lowered into position at the correct pressure. The adaptor is pushed down by a constant hydraulic pressure, forcing packing buffer through the slurry and compressing it so that a packed bed is gradually built up.

The quantity of medium required when packing CaptivA™ PriMAB resin by hydraulic pressure is approximately 1.15L of resin slurry per 1 liter of packed bed. Generically packing is completed as follows:

1. Make sure that there is no air trapped under the bottom bed support, by pumping packing buffer through it from below. Leave about 2 cm of liquid in the column.
2. Pour the slurry into the column. Fill the column with packing solution up to the top of the tube allowing the medium bed to settle just below the top of the column tube.
3. Put the adaptor in a resting position in the column tube and lower the lid and secure it in place.
4. Connect a pump to the inlet, to start the packing, applying a predefined constant hydraulic packing pressure. When packing CaptivA™ PriMAB resin in this type of column pack the bed to less than the recommended operational pressure.
8. When the adaptor has reached the surface of the settled bed, continue to run the pump until the adaptor has been lowered fractionally into the packed bed (depending on the column manufacturer's instructions)

Packing Efficiency Assessment

To check the quality of the packing and to monitor this during the working life of the column, column efficiency should be tested directly after packing, prior to re-use and if there is an observed deterioration in separation performance. The efficiency of a packed column is expressed in terms of the height equivalent to a theoretical plate (HETP) and the asymmetry factor (As). These values are easily determined by applying a sample such as 1% acetone solution to the column and using water as eluent. Sodium chloride can also be used as a test substance. Use a concentration of 0.8 M NaCl in water with 0.4 M NaCl in water as eluent. It is important that conditions and equipment are kept constant so that results are comparable. Changes in solute, solvent, eluent, sample volume, flow rate, liquid pathway, temperature, etc., will influence the results. A sample volume of less than 2.5% of the column volume and the flow velocity between 15 and 30 cm/h will give the most optimal results.

Method for measuring HETP and Asymmetry

Important: For best results avoid sample dilution by applying it as close to the column inlet as possible, and placing the UV meter as close to the column outlet as possible.

Conditions

- Sample volume: 1.0% of the bed volume
- Sample conc.: 1.0% v/v acetone
- Flow velocity: 20 cm/h
- UV: 280 nm, 1 cm, 0.1 AU

Calculate HETP and A_s from the UV curve (or conductivity curve if NaCl is used as sample) as follows:

$HETP = L/N$

$N = 5.54(Ve/Wh)^2$

where L = Bed height (cm)

N = Number of theoretical plates

Ve = Peak elution distance

Wh = Peak width at half peak height

Ve and Wh are in the same units.

To facilitate comparison of column performance the concept of reduced plate height is often used.

Reduced plate height is calculated:

HETP/d where

d is the mean diameter of the bead. As a guideline, a value of <3 is normally acceptable.

For a well-packed efficient column the peak should be symmetrical, and the asymmetry factor as close as possible to 1 (values between 0.8–1.5 are usually acceptable). A change in the shape of the peak is usually the first indication of bed deterioration due to use.

Peak asymmetry factor calculation:

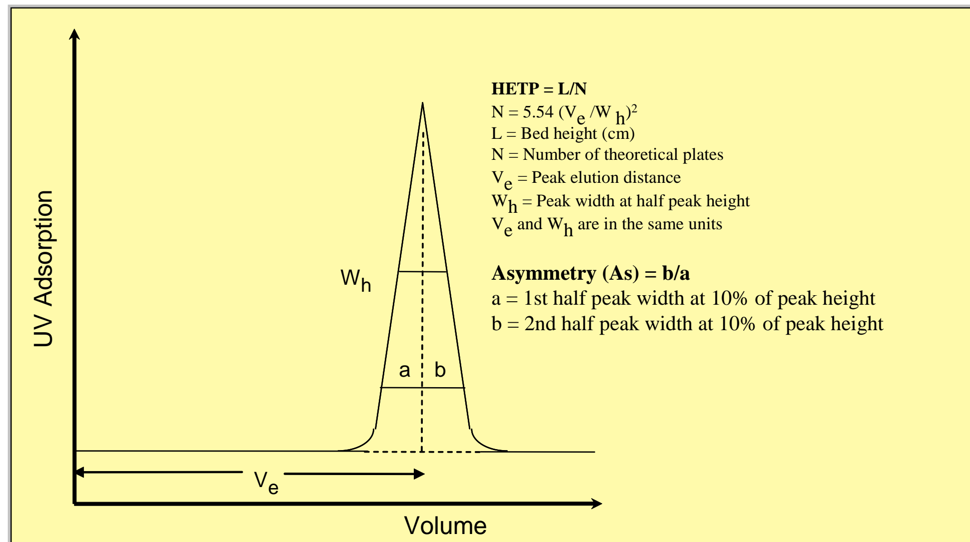
$A_s = b/a$ *where*

a = 1st half peak width at 10% of peak height

b = 2nd half peak width at 10% of peak height.

Figure 5, shows a UV trace for acetone in a typical test chromatogram in which the HETP and As values are calculated.

Figure 5. HETP and A_s Calculations.



Performance testing of packed CaptivA™ PriMAB columns.

To check the quality of the column packing users should test according to the specification of the manufacturer [6] of the beads, in the case of CaptivA™ PriMAB resin this is the GE Sepharose 4FF agarose. An efficiency test should be performed to determine the theoretical plate number and peak asymmetry factor.

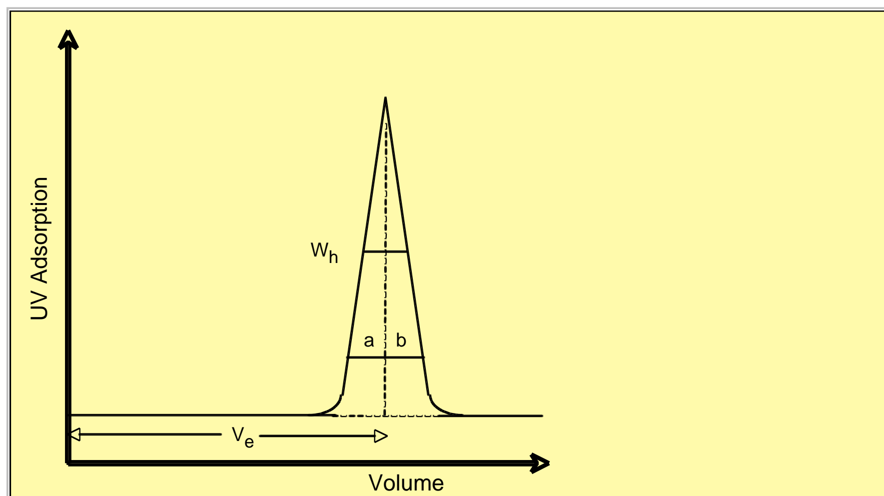
Using distilled water as an eluent and 1% (v/v) acetone in distilled water as the sample. The column is tested by pumping 200 µl of acetone (20mg/ml) through the column at a flow typically between 20 and 100cmhr^{-1}. Calculations for both plate number using the formula: $N/m = 5.54\ (VR/Wh)^2 \times 1000/L$ and peak asymmetry factor (A_s) by the formula: $A_s = b/a$ are shown in Figure 6.

If the column is packed according to the instructions described above typical values obtained for CaptivA™ PriMAB resin should be:

Number of theoretical plates > 3,000

Peak asymmetry 0.7-1.3

Figure 6. Calculation of Packing Performance



Method Design and Optimization

As with most affinity chromatography media, CaptivA™ PriMAB resin offers high selectivity for Monoclonal antibodies, which reduces the impact of process related parameters such as sample load, flow rate, bead size and bed height on resolution. The primary aim of method optimization is to establish the conditions that bind the highest amount of target molecule, in the shortest time and with the highest product recovery.

Specificity and affinity

The degree to which Protein A binds to IgG varies with respect to both the origin and antibody subclass [(7)].

There might even be a substantial diversity in binding characteristics within a single subclass. This is an important consideration when developing the purification protocol.

To achieve efficient capture of the target antibody it is often necessary to enhance the binding strength by formulation of the binding buffer in one of the following ways.

- By increasing pH, which reduces electrostatic repulsion between Protein A and IgG, allowing an uninhibited affinity interaction.
- By increasing salt concentration to reduce electrostatic repulsion and increase hydrophobic interactions.
- By reducing the temperature to improve binding.

Method screening

Because the affinity of Protein A affinity resins like CaptivA™ PriMAB resin varies for antibodies of different species, classes and subclasses varies, initial screening should be conducted under a broader range of conditions that will bind the largest diversity of antibodies and highlight potential interference between the target antibody and possible contaminating antibodies. A good general approach to evaluating primary mAb binding to CaptivA™ PriMAB resin is to start with high pH and high salt conditions, then elute them in a reducing linear salt/pH gradient. It is important to make certain that the antibody is stable under the elution conditions in order not to lose biological activity.

General Screening Recommendations

Example of suitable buffers:

- Buffer A: 0.05 M boric acid, 1.0 to 2.0 M NaCl, pH 9.0
- Buffer B: 0.05 M sodium citrate, 0.3 M NaCl, pH 3.0

Experimental conditions:

- Equilibrate the column with 10 column volumes of buffer A
- Apply a small sample of antibody
- Wash the column with 5 column volumes of buffer A
- Elute the column with a linear gradient of 10 column volumes to 100% buffer B
- Collect fractions into titrating diluent (e.g. 1.0 M Tris-HCl, pH 8.0 so that the diluent volume equals 5% of the programmed fraction volume)

- Regenerate the column with 5–10 column volumes of 100% buffer B
- Re-equilibrate the column with buffer A

Conditions can be subsequently modified to provide the best purification performance. High salt concentration and high pH will often increase dynamic binding capacity, even for antibodies, decreasing salt concentration and/or pH during binding may change contaminant mAb binding. This may also increase the dynamic binding capacity since more binding sites will be available for the target antibody. It may also increase selectivity in the system. The balance between selectivity and capacity must be defined with respect to the nature of the feed, i.e. presence of contaminating antibodies and the purity requirement in the eluted product. When optimizing elution conditions, determine the highest pH that allows efficient desorption of antibody from the column. Low pH values tend to encourage denaturation and aggregation of antibodies.

General Usage Instructions

Flow rate, and operating pressures (charts & language explaining limitations)

Scale Up

After optimizing the antibody fractionation at laboratory scale, the process can be scaled up. For this, some parameters will change while others remain constant.

- Bed volume changes according to required binding capacity and process load.
- Column diameter changes as a function of bed volume to obtain a bed height of approximately 20 cm so that high flow rates and high dynamic capacity can be used.
- Linear flow rate remains constant during sample application to ensure that residence time is not shorter than that established in the small-scale experiments. The residence time is equal to the bed height (cm) divided by the mobile phase velocity (cm/h) applied during sample loading.
- Keep sample concentration and gradient slope constant.
- The larger equipment needed when scaling up may cause some deviations from the optimized method at small scale. Different lengths and diameters of outlet pipes can cause zone spreading check the buffer delivery system and monitoring system for time delays or volume changes.

Optimization of Throughput

The optimal flow rate is that which gives the highest throughput in terms of amount of antibody processed per time unit and volume of medium.

This is achieved by defining the highest sample load over the shortest sample application time with the least amount of product loss. Frontal curve analysis provides this information. Since the dynamic binding capacity is a function of the linear flow rate applied during sample application, the breakthrough capacity must be defined over a range of different flow rates. The optimal flow rate is that which gives the highest throughput in terms of amount of antibody processed per time unit and volume of medium.

Removal of leached Protein A from final product

Leakage of Protein A from CaptivA™ PriMAB resin is generally very low. However, in many monoclonal applications it is a requirement that leached Protein A is eliminated from the final product. In a multi-

step purification process this is usually achieved through the use of a 2nd and/or 3rd chromatographic step.

Size exclusion chromatography can be applied for removal of Protein A-IgG aggregates by conducting the separation under moderate pH conditions. The large IgG-Protein A complexes that are formed will elute early from the column.

Cation exchange chromatography is an effective tool for removing residual Protein A, especially when the particular monoclonal has strong cation exchange binding characteristics. The run is conducted at a pH in which the antibody is known to dissociate from Protein A. Protein A binds poorly to cation exchangers and will elute early in the gradient.

Anion exchange chromatography can also be used to reduce leached Protein A contamination. It is best suited to antibodies that are weakly retained on anion exchangers. Because of the strong anion exchange binding characteristics of Protein A, Protein A-IgG complexes tend to be more strongly retained than noncomplex antibodies.

Processing and Maintenance

Cleaning-in-place (CIP)

Cleaning-in-place (CIP) is the removal of very tightly bound, precipitated or denatured substances from the resin and hardware. The accumulation of these contaminants may affect subsequent performance of the purification system or allow unwanted, potentially immunogenic, contaminants into the bulk API. If the fouling is severe, it may block the column, increase back pressure and reduce flow rate. Regular CIP prevents the build up of these contaminants in the packed bed, and helps to maintain the capacity, flow properties and general performance of CaptivA™ PriMAB resin.

CIP protocols

The following CIP protocols are intended as a starting point cleaning protocols specific for a given feed material. Typically, CIP is conducted every 5 cycles but this will ultimately depend on the nature of the feed material. Different contaminants require different or even combine CIP protocols. Severe fouling will require specific protocol development.

Precipitated or denatured substances:

- Wash with 2 column volumes of 6 M guanidine hydrochloride[1] 10 mM NaOH[2], 0.1 M H_3 PO_4 or 50 mM NaOH in 1.0 M NaCl or 50 mM NaOH in 1.0 M Na_2SO_4[(3)].
- Wash immediately with at least 5 column volumes of 0.2µm filtered binding buffer at pH 7–8.
- Reverse flow direction.

Hydrophobically bound substances

- Wash the column with 2 column volumes of a non ionic detergent[1] (e.g. conc. 0.1%).
- Wash immediately with at least 5 column volumes of sterile filtered binding buffer at pH 7–8.
- Reverse flow direction.

OR

- Wash the columns with 3–4 columns volumes of 70% ethanol[1] or 30% isopropanol[1].
- Wash immediately with at least 5 columns volumes of sterile filtered binding buffer at pH 7–8.

- Reverse flow direction.
- Apply increasing gradients to avoid air bubble formation when using high concentrations of organic solvents.

Notes:

1. Apply for an approximate contact time of 10 minutes on the column.

2. Apply for an approximate contact time of 30 minutes on the column.

3. Apply for an approximate time of 16 minutes on the column.

Sanitization

Sanitization protocols are used to reduce microbial contamination of the resin bed, often prior to storage. Effective sanitization prevents the build up of microorganisms that can lead to endotoxin contamination or a fouled resin bed. There are 3 common approaches to sanitization protocols:

Equilibrate the column with a solution consisting of 2% hibitane digluconate and 20% ethanol. Allow to stand for 6 hours, and then wash with at least 5 column volumes of sterile binding buffer.

OR

Equilibrate the column with a solution consisting of 0.1 M acetic acid and 20% ethanol. Allow to stand for 1 hour, and then wash with at least 5 column volumes of sterile binding buffer.

OR

Equilibrate the column with 70% ethanol[1]. Allow to stand for 12 hours, then wash with at least 5 column volumes of sterile binding buffer.

Note: Specific regulations may apply when using 70% ethanol since it can require the use of explosion proof areas and equipment.

Storage

Unused media can be stored in the container at a temperature of +2 to +8 °C. Ensure that the screw top is fully tightened. Packed columns should be equilibrated in binding buffer containing 20% ethanol to prevent microbial growth. After storage, equilibrate with at least 5 bed volumes of starting buffer before use.

Further information

Please read these instructions carefully before using CaptivA™ PriMAB media. For further information using this product please visit www.repligen.com, OR contact our customer service specialists for more information or to schedule and appointment with Repligen's Applied Customer Engineering (ACE) Team for on site technical support and problem solving.

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Important Information

CaptivA™ PriMAB Protein A Affinity Resin is a trademark of Repligen Corporation.

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