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Operating Instructions

Eshmuno® A Affinity Chromatography Media

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Labscale Column Packing

User Supplied Materials

- Eshmuno® A resin
- A lab scale chromatography column and extension tube
- Packing buffer: deionized water
- 25 mL syringe
- Tracer solution to check symmetry (e.g., 1 M NaCl in running buffer)

Compression and Resin Calculations

Eshmuno® A resin should be packed to a compression factor of 1.08 to 1.11 in lab scale columns (inner diameter (ID) ≤ 1.6 cm). Compression factor (CF) is defined as:

$$CF = SV/CV$$

where: SV is the settled bed volume and CV is the packed bed volume.

Calculate the settled bed volume required at a given percent compression for a target packed column bed volume:

$$SV = CF \times CV$$

The slurry volume required for a target bed height = (SV/slurry concentration) x 100

Transfer the required slurry volume to a graduated cylinder before packing the column.

Example

To pack Eshmuno® A resin to a target bed height of 50 mm in a 10 mm ID column (CV = 3.927 mL) using a compression factor of 1.11, an aliquot of 8.72 mL resin suspension (aqueous solution of 20% v/v ethanol + 150 mM NaCl, 50% slurry concentration) is needed. This is equivalent to 4.36 mL settled bed volume.

Resin Slurry Preparation

Eshmuno® A resin is rigid and does not swell in common buffers or solvents used in the biopharmaceutical setting. Storage buffer or equilibration buffer (EQ buffer) can be used for packing Eshmuno® A resin.

New Resin Slurry

Resin from a new container can be used for packing without exchanging the storage solution (aqueous solution of 20% ethanol + 150 mM NaCl, 70% slurry). Mix the resin slurry thoroughly into a homogeneous suspension before transferring the required amount of slurry.

Prepare a new resin slurry for packing as follows:

1. Mix the resin thoroughly into a homogeneous suspension and transfer the slurry to a graduated cylinder.
2. Let the resin settle under gravity for ≥ 4 hours and determine the slurry concentration.
3. Add additional storage buffer (aqueous solution of 20 % ethanol) or packing buffer to make a 50% slurry concentration.
4. Refer to *Compression and Resin Calculations* to determine the required slurry volume.

Used Resin Slurry

Used resin slurry may be stored in buffers other than the original storage solution (aqueous solution of 20% ethanol + 150 mM NaCl) and may have a different slurry concentration.

Prepare a resin slurry for packing as follows:

1. Mix the resin slurry into a homogeneous suspension.
2. Take an aliquot of resin slurry. The aliquot volume should contain enough resin to achieve the target column volume (CV).
3. Pour the resin slurry into a funnel with a sintered frit.
4. Wash the resin five times with one to two CV of water.
5. Equilibrate the resin into storage buffer (aqueous solution of 20% ethanol) or packing buffer (deionized water) by washing five times with one to two CV of storage buffer or packing buffer.
6. Resuspend the resin in storage or packing buffer into a homogeneous suspension and transfer to a graduated cylinder.
7. Let the resin settle under gravity for ≥ 4 hours.
8. Adjust the total volume to 50% slurry concentration with storage or packing buffer.
9. Refer to *Compression and Resin Calculations* to determine the required slurry volume.

The buffer exchanges may also be performed by repeated settling and decanting.

1. Let the resin settle for at least four hours in a graduated cylinder to accurately determine the settled volume.
2. Remove the supernatant and add storage or packing buffer.
3. Mix the resin into a homogeneous suspension and let the resin settle for at least four hours.
4. Adjust the total volume to 50% slurry concentration.
5. Repeat these steps two to three more times.
6. Refer to *Compression and Resin Calculations* to determine the required slurry volume.

Packing Procedure

1. Mark the target bed height on the column tube.
2. Install and mount the column vertically. Connect an extension tube or place a funnel with a large enough capacity on top of the column.
3. Connect the bottom of the column to the chromatography system.
4. Pump liquid through the bottom to wet the bottom bed support and fill the column with one to two cm of packing buffer.
5. Mix the slurry in the graduated cylinder into a homogeneous suspension. Ensure there are no clumps of media at the bottom of the container.
6. Add the slurry to the column assembly. Avoid air entrapment by pouring the slurry down the column wall using a funnel or a glass rod.
7. Rinse the graduated cylinder with a few mL of packing buffer, mix with the leftover resin in the cylinder and add this slurry to the column. Rinse with packing buffer any leftover resin from the column tube wall.

8. Ensure the column outlet is closed and connect the top flow adapter while venting air out of the inlet tube. Only lower the top adapter as much as needed to remove the air, i.e. a few millimeters into the slurry.
9. Connect the column to the chromatography system.
10. Start flow with the pump at a low flow rate (2 mL/min) and prime the column inlet line.
11. Open the bottom outlet and make a liquid to liquid connection to the column inlet. Ensure there are no leaks or air inside the column near the top adapter.
12. Immediately pump packing buffer in the downward direction at 500 cm/h until all the resin has settled onto the packed bed and all the liquid above the packed bed is clear.

NOTE
Reduce the linear velocity of this step if the system pressure exceeds the pressure rating of the column, particularly when packing long bed heights and/or if using columns rated to < 5 bar.

13. Stop the flow and close the bottom outlet of the column.
14. If an extension tube was used, remove the top adapter and the extension tube and then reconnect the top adapter into the column as described above
15. If an extension tube was not used, open the bottom outlet of the column and move to next step.
16. Lower the top adapter to the target bed height.
17. Apply downward flow at 500 cm/h for 1 CV.

NOTE
Reduce the linear velocity of this step if the system pressure exceeds the pressure rating of the column, particularly when packing long bed heights and/or if using columns rated to < 5 bar.

18. Check the quality of the packed bed.

Pilot scale column packing

Introduction

The recommended compression factor for packing Eshmuno® A in pilot scale columns is 1.11 to 1.14.

Materials

- Eshmuno® A resin
- Graduated cylinder
- Recommended packing buffer: Purified water or equilibration buffer
- Recommended tracer solution: 1 M NaCl

Resin Slurry Preparation

Eshmuno® A resin is supplied as a nominal 70% resin suspension in 20% aqueous ethanol + 150 mM NaCl.

NOTE
Mix the sedimented slurry with a paddle, rod or stirrer. If mixing a settled bed, start the mixing on top of the bed.

The bottled resin can be hand shaken.

DO NOT USE permanent/intensive agitation within the settled bed or magnetic stirrers (the bar will crush the beads).

Buffer exchanges

Prior to packing, ethanol in the storage solution should be removed and disposed of according to local regulations.

1. After allowing resin to settle in the shipping container, decant the storage solution in 20% EtOH once. Resuspend the resin using packing buffer.
2. Pour the desired amount of resin into the column or another appropriate container.

3. Perform at least two additional buffer exchanges in the column removing the supernatant by syphoning or pumping. If another container is used, perform the buffer exchanges by decanting the supernatant into waste. These steps will remove the ethanol prior to packing, and clear the potential fines created during shipment, resulting from base bead abrasion. Between each buffer exchange, allow the resin to settle for at least four hours.
4. Once the buffer exchanges have been performed, allow the resin to settle for four hours, to have an accurate measure of the settled bed height/volume (settling for less than four hours will result in an overestimation of the amount of resin available for packing).

Packing Procedure

Different column designs can have slightly different packing options. Consult the column manual for specifications. The mean particle size for Eshmuno® A resin is 50 µm, and a 10 µm bed support is recommended.

1. Add the appropriate volume of resin slurry to achieve the desired packed bed height at the recommended compression factor.
2. Reslurry the resin bed by mixing with a paddle to achieve a homogeneous suspension.
3. Rinse down the walls of the column with water to ensure resin particles are not trapped between the top adapter seal and the column wall.
4. Secure the column top, engage the seal and lower the top adapter to the surface of the liquid slurry, allowing excess liquid to escape through the inlet line.
5. Make sure the column inlet line is full of liquid before connecting the column inlet to the pump.
6. Open the column outlet and pack the column with the packing buffer at a starting flow rate $\geq$ 300 cm/h until the packed bed height is stable. Do not recirculate the packing buffer during this step. Turn off the pump.

NOTE
Use a packing flow rate at least 20% higher than the maximum process flow rate.

7. Lower the top adapter to the target packed bed height (this will generally be below the bed height achieved during flow packing). It is recommended to exhaust the liquid through the top of the column. If the resistance of the bed is too high to lower the adjuster manually to the targeted bed height, re-apply a flow at 300 cm/h in downflow mode, to recompress the bed. Once the bed is stable again, stop the flow and lower the adapter to the target bed height.
8. Condition of the packed bed by running the column for 1 CV in the upward flow direction at the highest processing flow rate or at 2 bar net pressure drop, followed by running the column for 1 CV in the downward flow direction at the highest process flow rate or at 2 bar gross pressure .

Packed Column Evaluation

The quality of the packing can be checked by measuring the packed column efficiency as follows:

1. Run the column at a flow rate of 100-200 cm/h and inject 1-2% of the packed bed volume of one of the recommended tracer solutions listed below.
2. Monitor the conductivity (1M NaCl or water as tracer) or the UV absorption (acetone as tracer) of the column effluent, respectively.

The qualification parameters, e.g. Asymmetry, depend on the specific test conditions sample concentration and volume, flow rate and system hold-up volume. These values should only be used as references and these conditions maintained constant when directly comparing specific values.

Recommended test sample/buffer systems as tracer solution.

Sample	Mobile Phase
1 M NaCl	water
water	200 mM NaCl
2% v/v acetone in running buffer	50 mM NaCl or running buffer

The acceptance range for asymmetry is 0.7 to 1.8 and HETP < 0.1 cm.

Operating Procedure

Ensure all buffers and sample feedstock are sterile (0.22 µm) filtered prior to use.

Before applying sample to a newly packed column or one that is being reused after storage, run a blank cycle of the equilibration, elution and regeneration buffers, then reequilibrate the column before applying sample.

Column volumes are provided for guidance and may be adjusted for system hold up volumes.

Modifying the wash buffer may increase purity by reducing nonspecific binding, for example, use of an intermediate pH wash (pH 5.5) and/or the inclusion of salt (0.5 to 1.0M NaCl) or detergent (0.1 to 1% Tween) to nullify ionic or hydrophobic interactions.

The optimum elution pH will depend on the specific antibody and its stability.

Materials

Equilibration Buffers (EQ)

- 50 mM Tris, 25 mM NaCl, pH 7.2-7.4, or,
- Phosphate Buffered Saline (PBS) pH 7.4

Elution Buffers

- 0.1 M acetic acid pH 3.0
- 0.1 M citric acid pH 3.0

Method

Always replace the column stop ends when not attached to a chromatography system.

Step		Buffer		Column Volume (CV)	Residence Time (min)
No.	Description	Solution	pH		
1	Equilibration	PBS or Tris buffered saline (TBS)	7.4	5 - 10	3
2	Load	Direct clarified feed 0.22 µm filtered	N/A	Dependent on sample volume and concen-tration	4-6
3	Wash	PBS, Tris buffered saline (TBS) or intermediate wash buffer	7.4	5 – 10 Wash to UV baseline	3
4	Elute	0.1M acetate or citrate	3.0	5-10	4
5	Post-elution Wash (optional)	PBS or Tris buffered saline (TBS)	7.4	2	3
6	Clean in Place	0.1-0.3M NaOH or	12	4	4
		150 mM Phosphoric Acid	1.7	4	4
7	Return to Step 1 or				
	Transfer the column into storage buffer 20% ethanol with 150 mM NaCl or 0.1M sodium acetate buffer, pH 5.2 + 0.5, containing 2% benzyl alcohol.				

Intermediate wash

The purpose of the intermediate wash step is to remove unbound or weakly bound process impurities from the column. All chromatography media may exhibit nonspecific binding under certain process conditions. Such nonspecific binding is characterized as the binding of any nonimmunoglobulin species present in the feed which then coelutes with the IgG. In general, the levels of non-specific binding to Eshmuno® A resin are very low and the purity of the eluate can routinely exceed 99%.

Nonspecific binding is generally due to either ionic or hydrophobic interactions of impurities with the base matrix, the immobilized ligand, or the Fc-containing protein product. Selecting an intermediate wash buffer with suitable pH and conductivity is usually sufficient to reduce any non-specific binding that may occur.

Select a pH condition between the loading pH and the elution pH such that contaminating species are eluted while the Fc containing protein remains bound. In the case of nonspecific binding due to ionic interactions, adding salt (up to 0.5 M NaC) to the intermediate wash has been found to be effective.

If the nonspecific binding is not addressed by pH and salt based intermediate wash, it may be due to hydrophobic interactions. Lowering the ionic strength of the intermediate buffer, or alternatively, adding a chaotropic agent or an organic solvent may be effective.

Certain host cell proteins (HCP) tend to bind to Fc containing proteins and do not dissociate under Protein A loading and intermediate wash conditions. These are often cleared in the subsequent purification steps, such as ion exchange chromatography.

We have been granted a nonexclusive license under US Patent 6,870,034 (and its counterparts) by Genentech, Inc., relating to certain intermediate wash conditions. We have a right to grant a sublicense to the users of Eshmuno® A resin who wish to practice the methods claimed in the licensed patent.

Cleaning In Place

Cleaning in place (CIP) results in the removal of residual proteins and contaminants from the column. The use of a low pH (pH 1.5 to 1.7) regeneration with 150 mM phosphoric acid solution for 10 to 15 min after every cycle is very effective at removing strongly bound material from the Eshmuno® A resin. Alternatively, the use of high pH, 0.1 to 0.3 M NaOH for 15 minutes, has been proven to effectively remove undesirable impurities from the Eshmuno® A resin.

Sanitization

Sanitization reduces bioburden (i.e., microorganisms and spores) in the column. All columns should be sanitized on a regular basis.

2% benzyl alcohol in 0.1M acetate pH 5.2 (+ 0.5) may be used as a sanitant and storage solution. At least 24 hours exposure is recommended to achieve the desired reduction in microorganisms (LRV).

When a faster sanitization time is required, use PAB (120 mM phosphoric acid, 167 mM acetic acid, 2.2% benzyl alcohol). The acidified benzyl alcohol provides faster microbial kill kinetics than the benzyl alcohol alone, enabling sanitization times of around three hours. Static binding capacity of Eshmuno® A resin is well maintained after 24 hour exposure to PAB solution at room temperature with no increase in leached Protein A level. PAB is not recommended for continuous long term storage. Sanitization with alkaline solution (e.g., > 0.3 M NaOH) is an alternative to PAB sanitization for Eshmuno® A resin. PAB sanitization is more effective in microbial kill while causing less detrimental impact on resin performance than alkaline conditions.

Storage and Handling

Eshmuno® A resin is supplied in 20% ethanol with 150 mM NaCl. An alternate storage solution is 0.1M sodium acetate buffer, pH 5.2 + 0.5, containing 1 to 2% benzyl alcohol.

When using acrylic columns, check compatibility with benzyl alcohol.

Store Eshmuno® A resin between 2 to 8 °C. DO NOT FREEZE.

If used under the recommended conditions, the product will be reusable over many cycles without significant loss of performance.

DO NOT store resin for prolonged periods without sanitizing solution.

Ordering Information

Eshmuno® A Media Quantity	Catalog Number
10 mL	1200890010
100 mL	1200890100
500 mL	1200890500
5 L	1200895000
10 L	1200899010

Eshmuno® A media is also available in convenient pre-packed columns, which are compatible with HPLC, FPLCTM or AKTA® systems.

Column Bed Volume	Columns Dimensions	Catalog Number
1 mL	8mm (i.d.) x 20mm (bed length)	1251600001
5 mL	8mm (i.d.) x 100mm (bed length)	1251610001

ELISA Kit – Available for purchase from Cygnus Technologies, Inc.

Catalog Number	Description	Contact Information
F400	Protein A ELISA kit	Cygnus Technologies, Inc 4332 Southport Supply Rd., SE Southport, NC 28461 Phone: 910-454-9442 www.cygnustechnologies.com

Standard Product Warranty

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