Cellufine™ SPA-HC

Cellufine SPA-HC is an affinity chromatography resin designed for the isolation of immunoglobulins, including monoclonal antibodies (mAb) from complex matrices, such as cell culture supernatant and serum/plasma. This resin is based on a spherical highly cross-linked cellulose base bead functionalized with an alkali stable rProtein A affinity ligand. Cellufine SPA-HC shows excellent flow properties, low ligand leachate levels, high dynamic binding capacity and good retention of binding after multiple cycles of base cleaning in place (CIP) and re-use. The highly cross-linked cellulose base beads were developed to accommodate the higher flow rates required to meet the demands of future high productivity purification workflows. This new high-performance affinity resin enables development of efficient purification processes for downstream purification of therapeutic monoclonal antibodies. Resin properties are summarized in Table 1 below.

Table 1, Performance Characteristics of Cellufine SPA-HC

Property	Characteristic
Ligand	Alkali-stable rProtein A produced in *E.coli*
Matrix	Highly cross-linked cellulose beads
Particle size	Average 70 μm
Ligand coupling method	Coupling via formyl groups
Flow velocity	≧600 cm/h (0.3 MPa) I.D.30 cm-L 20 cm, pure water at 24 ºC
Dynamic binding capacity (DBC)	≧ 70 mg /ml (C_{10} with Polyclonal IgG) [1]
Recommend CIP solution	0.1 M NaOH
Working temperature	4 ºC ~ 40 ºC
Storage	2～8 ºC in 20 % ethanol

[1] C_{10} DBC measured at 10% break through point at a residence time of 6 min using human polyclonal gamma globulin (Human IgG for infusion).

Column Flow Packing Procedure with flow adapters

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1) ***For column volumes < 1 L***; transfer sufficient slurry for the target column volume (CV) into a filter funnel (glass fitted) and wash with at least 5 volumes of water for a total of 3 x to remove the storage solution. If necessary, repeat with packing buffer if different from water.
2) ***For column volumes > 1 L*;** decant the storage buffer from above the settled resin in the shipping container and replace with water. Then re-suspend the resin and allow to settle again to wash away the storage buffer. Repeat 2-3x or consider packing in the storage buffer and washing the column on-line.
3) After the final wash, add sufficient packing buffer to suspend the resin into a 50-60% (v/v) slurry.
4) Transfer some of the slurry into a 50-mL measuring cylinder and allow to settle overnight or a minimum of 4h.
5) Measure bed height (volume) of a gravity settled bed and calculate the slurry% from;

   % = (Gravity settled bed volume/Total slurry volume) x 100
6) Adjust to a 50 % (v/v) slurry concentration of resin with packing buffer or water.
7) Calculate the volume of slurry required to pack the column using the following equation;

   Volume 50% slurry required = (Target column volume [CV] x 2) x (Cf)

   Where Cf is the resin compression factor derived from:

   Cf = [gravity settled /flow packed] bed heights

   For example, for a 100 ml CV you will need (100 x 2) x 1.15 = 230 mL of 50% slurry, for a resin compression factor of 1.15 to achieve a final CV of 100 mL.

   Note: *resin compression factor Cf can impact the column packing efficiency (see Appendix 1 for details) and may need to be optimized by adjusting the axial compression on the column.*
8) Calculate the expected final bed height to achieve the desired CV.

   For example, for a 100-mL target CV in a 2.5 cm diameter column the target bed height will be calculated using the following equation;

   Final target bed height = CV (mL) / column cross section area (cm^2)

   = 100 / π x $radius^2$

   = 100 / 4.91

   = 20.4 cm
9) Assemble the column hardware with the bottom flow adapter in place. Prime the bottom frit assembly to remove air with packing buffer from a syringe or pump for a large diameter column. Leave about 1 cm in the bottom of the column.
10) If necessary add a bed height adapter to the top of the column to accommodate the full volume of the slurry.

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***Note**: the full volume of slurry will be poured into the column in one step to ensure a uniform packed bed.*

11) Close the bottom outlet of the column.
12) Pour the volume of slurry into column in one operation and avoid trapping air in the resin slurry.
13) Open the bottom outlet and allow the bed to start to settle until 2-3 cm of clear liquid is seen above the resin bed.
14) Stop the outlet flow and carefully fill the column with packing buffer up to the top without disturbing the settling resin bed.
15) Prime the upper flow adapter as described in step 9 above.
16) Assemble the top flow adapter on to the column minimizing any trapped air bubbles in the head of the column.
17) Initiate flow with the packing buffer at 200 cm/h for 5 min and check for leaks. Then increase the flow rate in steps up to 600 cm/h or until the maximum 0.3MPa pressure is reached to flow pack the resin bed for 30 min.

    ***Note**: the column back pressure* should be in the range 0.25 to 0.30 MPa at this flow rate. Which is higher than normal operation of the column to ensure a stable bed packing.*

    ** This is the pressure drop across the column when the column is filled with resin. Allowance should be made for the system back pressure where an empty buffer filled column of the same size is placed in-line. Backpressure is best measured with a gauge on the inlet side of the column.*
18) After the bed height, has stabilized, close the outlet and start flow from the top of the column (DO not remove the flow adapter) and slowly move the top flow adapter down displacing packing buffer from the top of the column. Bring the top adapter down to contact the settled resin bed.
19) Re-start flow at 600 cm/h. If the bed settles and shrinks away from the top adapter, adjust the top adapter down to accommodate the new bed height.
20) At the target bed height, the CV should be at the target volume as expected based on the compression factor used in the calculation in step 7 above. If the bed height is higher than expected, axial compression can be applied by lowering the top adapter. If the bed height is lower than expected, the original volume of slurry may have been lower, or the resin may have packed down more on flow since its compression factor may have been higher than expected. In this case you may have to re-pack or accept a smaller CV. If the latter is the case, re-calculate the operating flow rate based on the reduced CV.

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21) Check and evaluate the status of packing by measuring HETP and peak symmetry (As) as described in Appendix 1.

Operating Guidelines

Cellufine SPA-HC will bind immunoglobulins with the same specificity as the wild type Protein A ligand. The cellulose base bead shows very low non-specific binding of host cell protein and the retained IgG elutes readily between pH 3-4. The ligand immobilization via reductive amination through a formyl group on the resin surface is very stable and low levels of leachate are seen with multiple cycles of re-use. Cellufine SPA-HC features a re-engineered version of the wild type protein A ligand with increase alkali resistance and higher binding capacity. Thus, up to 160 cycles of CIP with 0.1M NaOH have been shown to retain full binding activity. If necessary, 0.5 M Sodium hydroxide has been shown to retain 80% of binding activity with up to 50 cycles of CIP.

Recommended Buffers

Equilibration buffer: 50 mM sodium phosphate, 0.15 M NaCl, pH 7.4
Elution buffer: 60 mM acetate buffer, pH3.0
CIP: 0.1M NaOH
Water

Measurement of DBC with Gamma Globulin

Dynamic binding capacity (DBC) may differ for each mAb. Ideally, DBC at 10 % breakthrough should be measured for a purified sample of the mAb. If this is not possible you can substitute a sample of Gamma globulin or purified polyclonal antibody as described in this example to establish a baseline of performance of the resin.

1. Prepare a packed column as described above and qualify per Appendix 1. From this data, you will be able to measure the void volume V_0 of the column and attached hardware.
2. If the column has been stored in 20% (v/v) Ethanol, wash with at least 5 CV of the above equilibration buffer.
3. Prepare a 1mg/ml of human gamma globulin solution in equilibration buffer. Check the concentration by reading the absorbance at 280 nm of 1 mL in a 1 cm path length

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cuvette in a UV spectrophotometer. Using an extinction coefficient of $E_{1\%}$ of 14.4 estimate the protein concentration. Adjust if necessary to within 5% CV of the target 1 mg/mL concentration.

4. Start flow to the column to establish a residence time of 6 min. The flow rate to achieve this condition is calculated as follows;

   Flow rate (ml/min) = CV (mL) / residence time (min)

5. Prime all lines on the chromatography instrument with the above buffers including the 1 mg/mL gamma globulin solution.
6. With the column, out of line, purge the protein solution through the UV flow cell and record the plateau value of the Absorbance trace at 280 nm. This will be termed A_{max} for the estimation of the 10% breakthrough point
7. Wash protein solution out of the system and with the column back in-line equilibrate for 5CV.
8. Initiate loading of the 1 mg/mL gamma globulin solution at the flow rate to achieve the 6-min residence time. Keep loading until you start to see breakthrough of the protein load indicating that the DBC has been exceeded.
9. On breakthrough stop protein loading when the absorbance exceeds 50% of A_{max} and switch over to equilibration buffer to wash out with 5 CV any un-bound protein load.
10. When baseline absorbance is reached, initiate elution with 5 CV of the acetate buffer at pH 3.0
11. Collect the elution peak and estimate the amount of antibody recovered.
12. After elution wash out any residual low pH buffer in the column with 10 CV of water.
13. Then load the column with 3 CV of the 0.1 M NaOH and stop flow for 15 min to carry out CIP.
14. Wash out the base CIP with equilibration buffer for 5CV until the target conductivity and pH are achieved.
15. To store the column, exchange the equilibration buffer with 20% (v/v) ethanol and seal the inlet and outlets of the column and place at 2-8 ^{o}C.

Purification Protocol

Prepare sample by centrifugation followed by clarification through a 0.22 µm filter to remove insoluble material before loading to the column. Using the reported or measured DBC at 10 % breakthrough point and the mAb titer in the sample estimate how much

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culture supernatant can be loaded. To avoid exceeding the capacity of the resin and losing product in the flow through, load only 80% of the DBC at 10% breakthrough. For example, with an antibody titer of 1 g/L with a 100 mL Cellufine SPA-HC column and a C_{10} of 65 mg/mL at 4 min residence time the volume of culture to load is calculated below;

Volume of culture fluid to load (mL) = [{C_{10} X 0.80} x CV] / Mab Titer
= [{70 x 0.80} x 100] / 1
= 5600 mL

Flow rate to achieve 6 min residence time = CV/ residence time
= 100/4 = 25.0 mL/min

Time to load the above Protein column = Load volume (mL) / Flow rate (mL/min)
= 5600/ 25.0 = 224 min

Follow the above workflow for measuring DBC with the flowing differences;

- Check the pH and conductivity of the sample before loading and if necessary adjust to bring into the range of the equilibration buffer. Most cell culture samples are buffered close to pH 7.4 and do not have high salt levels present.
- To cut down on large load volumes if the mAb titer is low, consider concentrating the sample by TFF with a 30-50 kDa molecular weight cut off UF membrane.
- An early breakthrough of absorbance will be seen at about 1-2 column void volumes (V_0) as the non-retained components of the culture sample elute.
- Collect the last 50 mL of the sample load and confirm that no Mab is seen in the flow through fraction.
- After loading, extend the wash with equilibration buffer to 15 CV to remove any non-specific retained host cell protein (HCP) and dsDNA from the load sample.
- After elution, raise the pH to 3.5 with 1M Tris base and hold for 30 min for viral inactivation.
- After viral inactivation raise the pH > 4.0 to stabilize the mAb or equilibrate to the pH and conductivity conditions for the next polishing step.

Recommended Polishing Steps after rProtein A Capture

After Protein A capture the following contaminants need to be removed in the subsequent “polishing” steps; a) host cell protein (HCP), b) leached Protein A ligand, c) residual dsDNA and d) mAb aggregates > 300 kDa molecular weight. In some cases, removal of

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mAb dimers is also required. Cellufine resins are available to remove these contaminants and are listed below. More details on these resins can be found on the web site at http://www.jnc-corp.co.jp/fine/en/cellufine/

Cellufine MAX IB, a new mixed mode primary amine based hydrophobic modified resin for flow through removal of HCP, leached Protein A and mAb aggregates.

- Adjust to pH 7.0 with Tris base,
- Tolerant of a wide conductivity range up to 0.2 M NaCl.

Cellufine MAX GS, a dextran coated cation exchange resin with high binding capacity for bind and elute removal of dimers and aggregated mAb's.

- Adjust to pH 4.5 with Tris base and lower conductivity < 5 mS/cm.

Cellufine MAX Q-h, a high capacity anion exchange resin that can be used to remove HCP, leached Protein A and residual dsDNA in a flow through format.

- Adjust to pH 8.5 with Tris base and conductivity ~12 mS/cm.

Clean in Place (CIP) Recommendations

Cellufine SPA-HC remains more than >85% of dynamic binding capacity after 160 CIP operating cycles employing 0.1 M sodium hydroxide (3 CV for 15 min). 0.5 M sodium hydroxide (3 CV for 15 min) is possible for up to 50 cycles to retain >80% activity.

Flow Rate Recommendations

Cellufine SPA-HC is based on highly cross-linked cellulose resin and is stable at high flow velocities. For example:

- 2,800 cm/h in a 2.2 cm diameter column with 20cm bed height at <0.3 MPa.
- > 600 cm/h in a 30-cm diameter column with 20 cm bed height at <0.3 MPa.

Storage Recommendations

Store unopened container at 2 – 8 °C. **Do not freeze**.

Adsorption capacity did not change with a month storage at an elevated temperature of 40 °C which corresponds to 6-month storage at room temperature (25°C) and 2.5-year storage at 10°C.

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Longer storage should be in 20 % ethanol, at 2 – 8 °C. **Do not freeze.**

Ordering Information

Description	Quantity	Catalogue No.
Cellufine SPA-HC	1 x 1 mL (mini-column)	21900-11
	5 x 1 mL (mini-column)	21900-51
	1 x 5 mL (mini-column)	21900-15
	10 mL	21900
	50 mL	21901
	500 mL	21902
	5 L	21903
	10 L	21904

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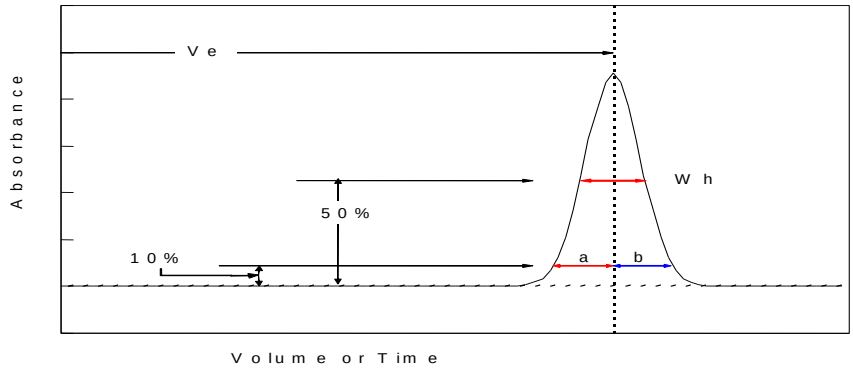
Appendix 1: Evaluation of column packing of Cellufine resins

Evaluation methods for the packing status of a column can employ indices, such as the number of (theoretical) plates (N), the height equivalents to a theoretical plate (HETP), and a symmetry factor (As). The selected evaluation indices are affected by the measurement condition. For example, they vary with changes in the difference between the diameter/height of a column, the difference in piping, solvent sample volume, the flow velocity, temperature, etc. Therefore, it is necessary to use the same measurement conditions each time.

Parameter	Condition
Sample volume	1 -2.5% of column bed volume
Sample concentration	1-2 % (V/V) acetone (mobile phase：water)
	1M NaCl（mobile phase： 0.1-0.4M NaCl aq）
Flow rate (cm/h)	30 cm/h
Detector	UV or conductivity



Calculation Formulae

HETP = L/N

N = 5.54 x (Ve/Wh)²

As = b/a

L	Column length [cm or m]
V_e	Elution time or volume
W_h	Half of width of peak
a, b	Peak width of 10% peak height (a) front, (b) rear
Note	V_e, W_h and a, b should have same dimensional units

Generally, a larger value of N is good. (Likewise, a smaller value of HETP is good.) The asymmetry factor value (As) should be close to 1. Generally, acceptable symmetry values range from 0.8-1.6.

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