Cellufine™ MAX IB

Mixed mode chromatography resins are well-known to have **u**nique selectivity differences from traditional IEX or HIC resins. JNC Corporation has developed a new mix-mode resin, Cellufine™ MAX IB, for monoclonal antibody (Mab) purification after initial rProtein A capture. This resin has a salt tolerant polyallylamine surface modification that has been partially modified with butyl groups. The resulting ligand structure exhibits a mixed mode functionality with a hybrid primary amine + butyl surface chemistry. In addition to the Mab polishing application this resin can be used in other downstream processes, such as plasma fractionation exploiting the unique selectivity of this mixed-mode ligand. This mixed mode resin is built on the cross-linked Cellufine cellulose base bead that is very stable, resistant to base CIP and can be operated under high flow modes with minimal back pressure.

Resin properties are summarized in Table 1 below.

Table 1, Performance Characteristics of Cellufine MAX IB

Property	Characteristic
Ligand	Polyallyl amine partially modified with a butyl group
Matrix	Highly cross-linked cellulose beads
Particle size	90 µM average (40 – 130 µm)
Microscopic test (%)[1]	< 5
Flow velocity	≧500 cm/h (0.3 MPa) I.D.10 cm-L 13 cm, pure water at 24 ºC
BSA binding capacity (mg/mL)	64 (low salt)[2] 59 (high salt)[3]
Recommend CIP solution	0.5 M NaOH
Recommended cleaning conditions	Ethanol (70 %), Isopropanol (30%), Guanidine HCL (6 M) and Urea (6 M)
Storage	2-8 °C n 20 % (v/v) ethanol

[1] % of broken particles under microscopic evaluation

[2] 50 mM Tris-HCL, pH 8.5,

[3] 50 mM Tris-HCL, pH 8.5 + 0.2 M NaCl.

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Column Flow Packing Procedure with flow adapters

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)

   Cf = [gravity settled /flow packed] bed heights for Cellufine MAX IB = 1.35–1.40 with a water mobile phase.

   For example, for a 100 ml CV you will need (100 x 2) x 1.35 = 270 mL of 50% slurry, for a resin compression factor of 1.35 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

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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.
***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 6 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

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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.

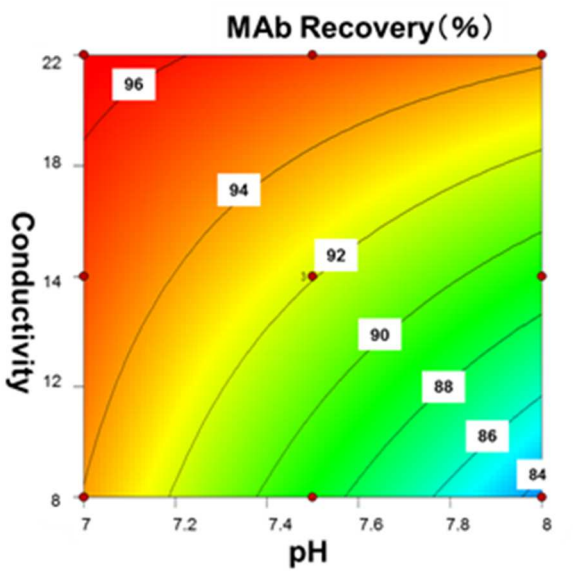
21) Check and evaluate the status of packing by measuring HETP and peak symmetry (As) as described in Appendix 1.

Operating Guidelines

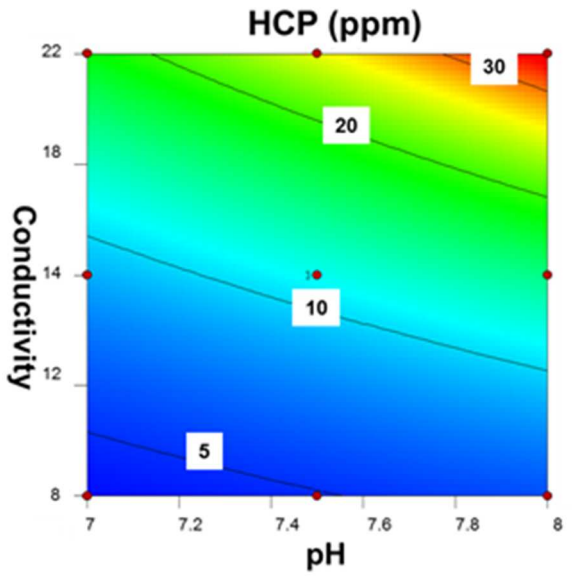
Cellufine MAX IB has mixed mode properties based on a primary amine anion exchange mode and a butyl HIC modification on the ligand. Because of these properties, the adsorption/desorption performance of the resin is strongly affected by pH and conductivity (ionic strength). Operation conditions should be carefully optimized. An example of a design of experiment (DOE) study is summarized in contour plots in Figure 1 below. In panel A, the recovery of a target Mab was screened against pH and conductivity variables. In panel B, the removal of CHO-HCP was screened against the same two variables.

Figure 1, Screening of Mab Recovery and CHO-HCP removal for Cellufine MAX IB

Panel A, % Mab recovery in the step



Panel B, HCP removal in the step



The above contour plots can be used to define the optimal loading pH and ionic strength conditions to achieve maximum recovery of the target Mab and removal of CHO-HCP contaminants using this mixed mode resin.

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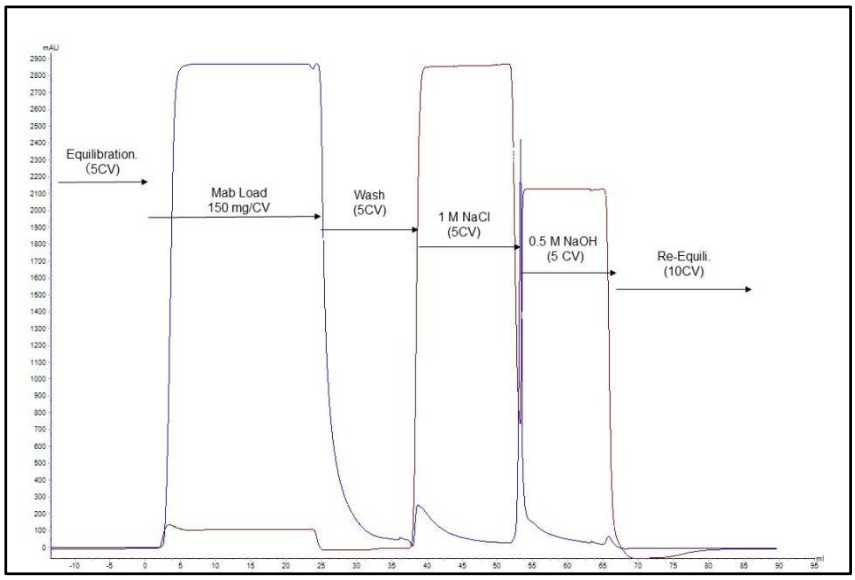
Flow-Through Polishing of a Mab after rProtein A Capture

Cellufine MAX IB can be used as negative (flow-through) mode for Mab polishing applications. In Figure 2 below, a typical chromatogram illustrates the flow-through polishing of a Mab with Cellufine MAX IB after initial rProtein A capture from CHO cell culture

Figure 2, Flow-through Polishing of rProtein Captured Mab by Cellufine MAX IB



Cellufine MAX IB was packed into a 5 mmID x 13.5 cmL (2.65 mL) column and equilibrated in 20 mM Tris-HCL buffer at pH 7.0 at a flow rate of 1.325 mL/min (407 cm/h). After initial capture by a rProtein A column, the resulting elution fraction in 60 mM Acetic Acid pH 3.5 was then held for 60 min at pH 3.4 for viral inactivation. After this step the sample (at 18.0 mg/mL Mab) was adjusted to pH 7.0 with Tris base and a conductivity of 5 mS/cm with NaCl or dilution with column loading buffer. After 0.22 µM filtration, the clarified sample was pumped onto the MAX IB column at 150 mg Mab / mL of resin and the flow-through + wash fraction in equilibration buffer were collected. After wash down to baseline the column was then regenerated with 5 CV of 1.0 M NaCL followed by 5 CV of 0.5M NaOH. The column was then re-equilibrated with 10 CV of equilibration buffer.

The above chromatogram clearly shows high recovery of Mab in the flow-through fraction with minimal adjustment required after elution from rProtein A and viral inactivation.

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A summary of the removal of CHO-HCP, dsDNA (HCD), leached rProtein A and aggregated material is shown in Table 2, below for Cellufine MAX IB, MAX Q-h and a polymer modified agarose Q competitive media in a flow-through polishing format.

Table 2, Two-step Mab purification with Cellufine MAX IB and two different polymer modified Q IEX resins

	Elution Buffer from ProA	HCP (ng/mg mAb)	Leak ProA (ng/mg_mAb)	Aggregate (%)	HCD (pg/mg mAb)	Recovery (%)
Loading solution	60 mM Acetate Buffer (pH 3.5)	72	3.0	1.7	10	100
Cellufine MAX Q-h		22	2.1	1.9	＜10	97
Polymer modified Agarose Q		27	2.1	1.8		96
Cellufine MAX IB		3	0.0	1.0		95

Cellufine MAX IB was packed in a 5 mmID x 3 cmL (0.29mL) column and equilibrated in 20 mM Tris-HCL buffer pH 7.0. Mab was eluted from rProtein A with 60 mM Acetic acid pH 3.5 and after viral inactivation at pH 3.4 was adjusted to pH 7.0 with Tris base and a conductivity of 6 mS/cm with NaCl or dilution with column loading buffer. The sample was then filtered (0.22 µM) to remove any insoluble material and then loaded onto the Cellufine MAX IB column at 190-200 mg Mab /mL of resin at a flow rate of 0.075 mL/min (4 min residence time). The flow through fraction was collected and tested for the above contaminants in the Protein A elution fraction.

In the above table, the excellent clearance of CHO-HCP leached rProtein A, dsDNA and protein aggregates is demonstrated for the Cellufine MAX IB mixed mode resin in a very efficient flow-through polishing format.

Sample Preparation and Load

Clarify sample by centrifugation or microfiltration with a 0.22 µm low protein binding filter to remove aggregated particulate material. Samples should be adjusted to a concentration in the range 1 to 20 mg/mL, in equilibration buffer or comparable conditions of ionic strength and pH to achieve the required environment to maximize the amount of Mab in the non-retained flow-through fraction. If necessary, exchange sample buffer by dialysis, diafiltration with a UF membrane (centrifugal, hollow fiber or TFF device format) or buffer exchange by SEC chromatography.

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Recommended Buffers

Equilibration buffer: 10 - 50 mM sodium phosphate, 0 - 0.15 M NaCl, pH 6.0 to 9.0
10 – 50 mM Tris-HCl, 0 - 0.15 M NaCl, pH 7.5 – 9.0

Elution buffer: 0.1 – 2.0 M NaCl in the above equilibration buffer

Regeneration and equilibration

After separation, wash the Cellufine MAX IB packed column with over 5 CV (column volumes) of high ionic strength elution buffer (1 - 2 M NaCl) followed by 0.5 M NaOH to regenerate the resin. After this step, re-equilibrate with 10 CV of equilibration buffer until the column eluate reaches a stable pH and conductivity values matching the buffer.

Depyrogenation

Wash the column with 5 CV of 0.2 M NaOH stop the flow and let stand for 16 hours, followed by a 5-10 CV flush with endotoxin-free water and then equilibration buffer as described above. 0.2 M NaOH-20 % (v/v) ethanol is also effective for endotoxin removal. For a more rapid de-pyrogenation, 0.2 M NaOH in 90% (v/v) ethanol in contact with the resin for 2 hours will yield an equivalent removal of the endotoxin.

Clean in Place (CIP) Recommendations

0.5 M sodium hydroxide 3 CV for 15 min or overnight in 0.1 M NaOH.

Flow Rate Recommendations

Cellufine Max IB is based on highly cross linked cellulose resin, and is stable at high flow velocities. For example:

- 1,000 cm/h in a 2.2 cm diameter column with 20 cm bed height at <0.3 MPa.
- > 500 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**.

Short-term storage for bulk and packed columns - room temperature for up to 4 weeks. 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).

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

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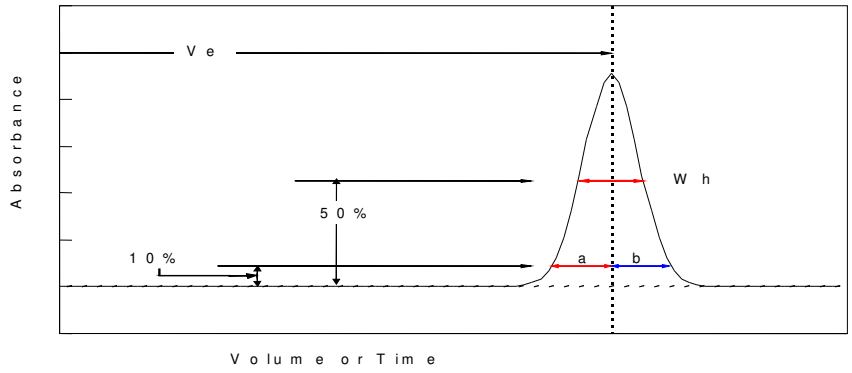
Appendix 1: Evaluation of column packing of Cellufine resins

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 volume (CV)
Sample concentration	1-2 %(v/v) 1 M NaCl
Flow rate	30 cm/h
Detector	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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