Two-Step Purification Process for Mouse Monoclonal Antibody from Hybridoma Cell Line

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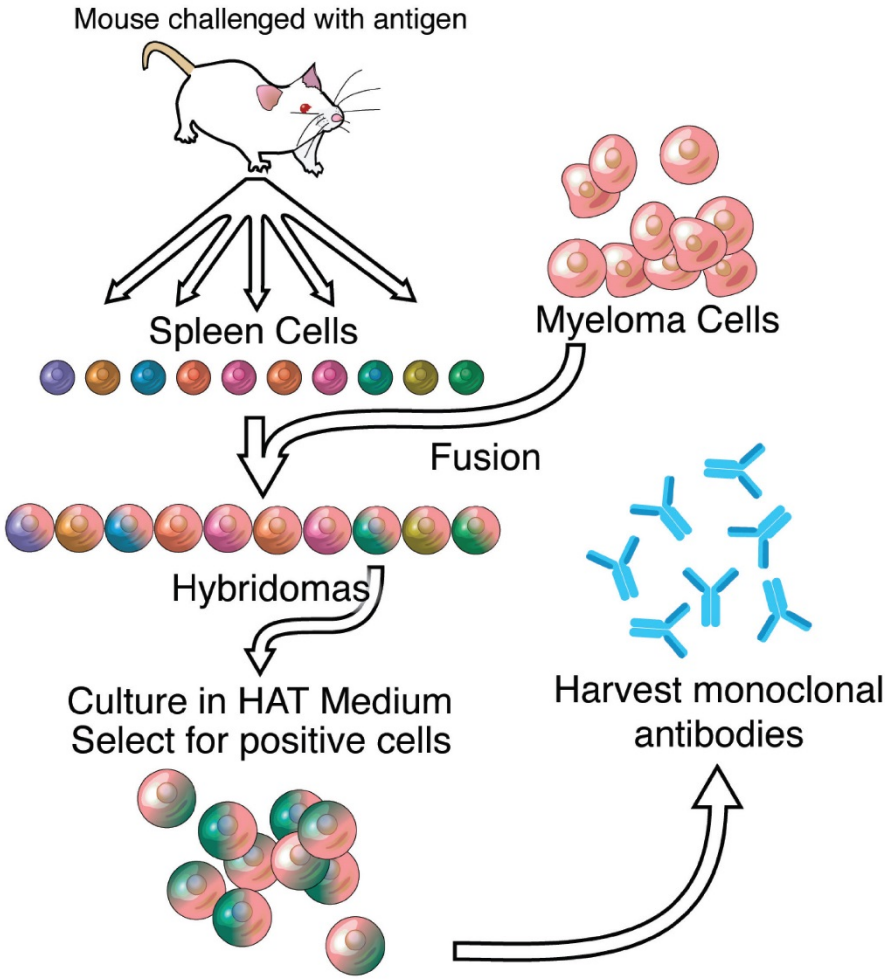
Introduction

- Hybridoma technology is an efficient method for production of monoclonal antibodies (mAbs). It uses mice for eliciting an immune response, followed by fusion of IgG-expressing B-cells with myeloma cells to allow propagation in cell culture.
- Here we present a case study where a proprietary mouse mAb, currently in preclinical development, was efficiently purified using a two-step purification process from hybridoma cell line supernatant ("feedstock").
- The challenge was to obtain a few milligrams of mAb for cell-based functional assays to evaluate the purification process in spite of very low mAb titer (~0.03 g/L) in the feedstock.
- The molecular weight of the purified mouse mAb was determined using a LenS$_3$™ multi-angle light scattering (MALS) detector.

Overview of Hybridoma Technology



https://commons.wikimedia.org/wiki/File:Monoclonals.png

Materials & Methods

Step 1 (Capture): Column Characteristics

Media	TOYOPEARL® AF-rProtein A HC-650F
Column	Tosoh prepacked
Bed size	8 mm ID × 10 cm length (5 mL)
Particle size	45 µm
Pore diameter	100 nm
DBC* (5 min)	70 g/L
DBC* (2 min)	50 g/L
Caustic stability	>200 CIP cycles (0.1 mol/L NaOH)
Max. pressure	0.3 MPa

*DBC = Dynamic Binding Capacity

TOYOPEARL AF-rProtein A HC-650F was used for mAb capture and is a polymethacrylate, high dynamic binding capacity protein A affinity media.

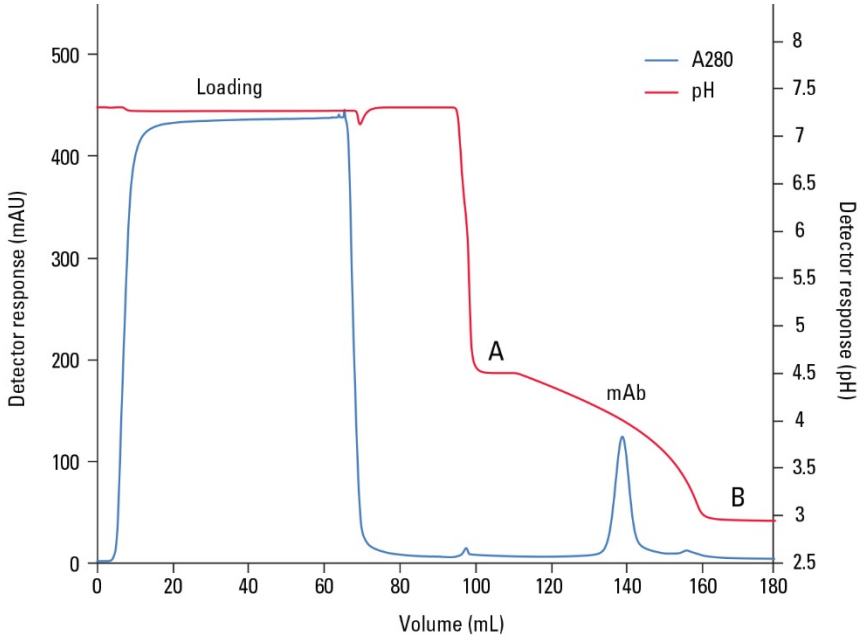
Materials & Methods

Step 2 (Polishing): Column Characteristics

Media	TOYOPEARL Sulfate-650F
Column	Tosoh prepacked
Bed size	7 mm ID × 2.5 cm length (1 mL)
Particle size	45 µm
Pore diameter	100 nm
DBC	>120 g/L of IgG
Caustic stability	0.5 mol/L NaOH
Max. pressure	0.3 MPa

TOYOPEARL Sulfate-650F was selected for mAb aggregate and fragment removal and is a high salt-tolerant cation exchange (CEX) media.

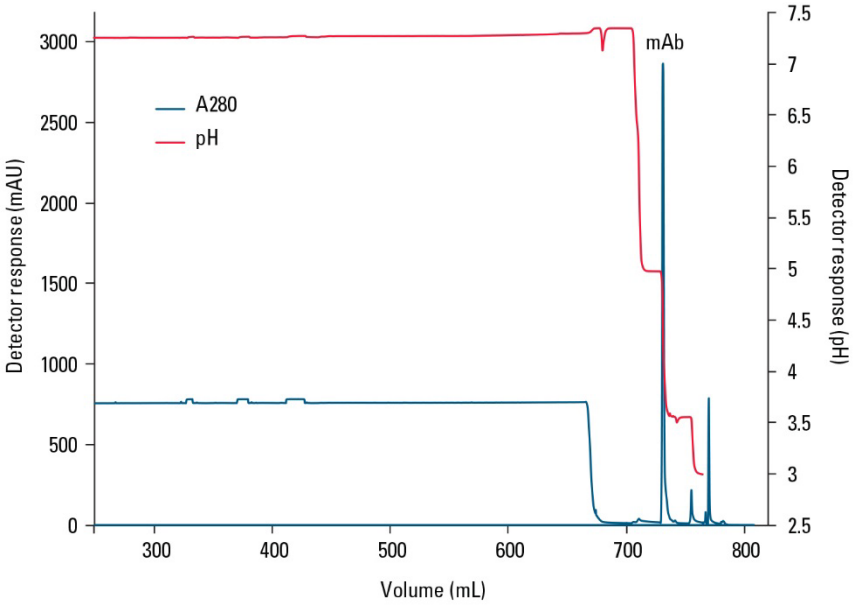
Results: Determination of mAb Elution pH on TOYOPEARL AF-rProtein A HC-650F Resin



Equilibration buffer:	0.1 mol/L Na_2HPO_4/NaH_2PO_4, 0.15 mol/L NaCl, pH 7.3
Mobile phase A (gradient):	0.1 mol/L acetate (NaOH), pH 4.5
Mobile phase B (gradient):	0.1 mol/L acetic acid, pH 2.9
Elution gradient:	linear from pH 4.5 to 2.9 over 10 CV
Flow (load):	150 cm/hr (1.25 mL/min), 4 min residence time
Flow (wash/gradient):	240 cm/hr (2.0 mL/min) (AKTA™ avant 25 instrument)
Sample:	45 mL hybridoma cell culture supernatant (buffer-adjusted)

A sharp mAb peak with elution max at pH 4.0 was obtained using a linear pH gradient.

Results: Step 1: mAb Capture on TOYOPEARL AF-rProtein A HC-650F Media

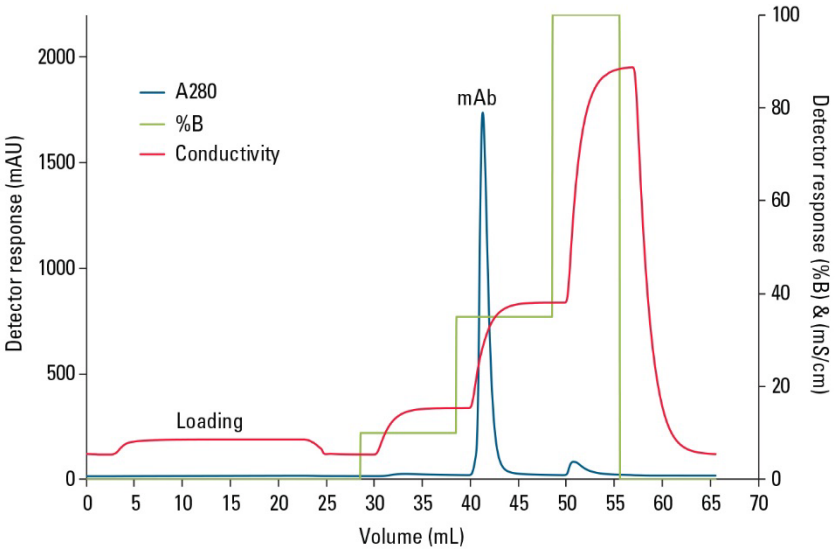


Equilibration buffer:	0.1 mol/L Na_2HPO_4/NaH_2PO_4, 0.15 mol/L NaCl, pH 7.3
Post-loading 1st wash:	equilibration buffer (5 CV)
Post-loading 2nd wash:	0.1 mol/L acetate (NaOH), pH 5.0 (5 CV)
Elution:	0.1 mol/L acetate (NaOH), pH 3.5 (5 CV)
Column strip:	0.1 mol/L acetic acid, pH 2.9 (3 CV)
Column cleaning:	0.2 mol/L NaOH (3 CV), 15 min hold
Flow (load):	150 cm/hr (1.25 mL/min), 4 min residence time
Flow (wash/elution):	240 cm/hr (2.0 mL/min) (AKTA avant 25)
Temperature:	ambient (room temperature)
Sample:	340 mL hybridoma cell culture supernatant (buffer-adjusted)

- *A sharp and efficient elution peak (4.8 mL) was obtained at the start of pH 3.5 elution*

- To ensure complete elution, pH 3.5 was selected for step elution.
- No target leak was detected during pH 5.0 wash.
- Total protein: 9.8 mg in the elution peak. Eluate was prepared for CEX by adding four eluate volumes of 0.2 mol/L acetate, pH 5.0. Diluted eluate was stored overnight at 2-8 ºC.

Results: Step 2: Polishing Chromatography on TOYOPEARL Sulfate-650F Resin

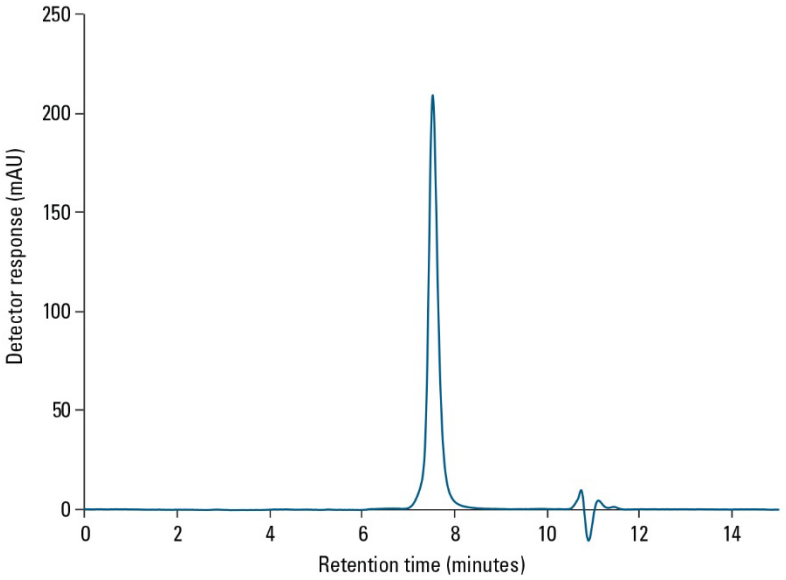


Equilibration buffer:	0.1 mol/L acetate (NaOH), pH 5.0
Post-loading 1st wash:	equilibration buffer (5 CV)
Post-loading 2nd wash:	0.1 mol/L acetate (NaOH), 0.1 mol/L NaCl, pH 5.0 (10 CV)
Elution:	0.1 mol/L acetate (NaOH), 0.35 mol/L NaCl, pH 5.0 (10 CV)
Column strip:	0.1 mol/L acetate (NaOH), 1.0 mol/L NaCl, pH 5.0 (7 CV)
Flow (all steps):	156 cm/hr (1.0 mL/min) (AKTA avant 25 instrument)
Sample:	21 mL diluted protein A eluate
Temperature:	ambient (room temperature)

- *Efficient elution of mAb occurred at 0.35 mol/L NaCl step in the equilibrium buffer.*

- Protein A eluate contained a small amount of high and low molecular weight impurities.
- High salt tolerant cation exchange resin (TOYOPEARL Sulfate-650F) was selected and step optimized to remove remaining impurities.
- Eluate was subjected to SEC and MALS analyses to confirm purity and monomeric state.

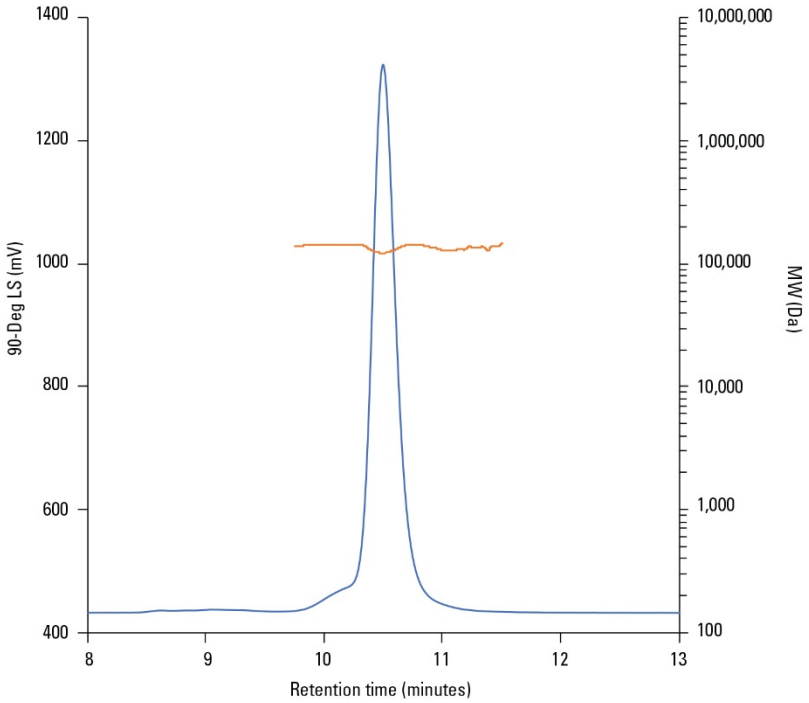
Results: Final Product: SEC Analysis (>95% Purity)



Instrument:	Agilent 1100 HPLC (Chemstation® Rev. C.01.08)
Column:	TSKgel® UP-SW3000, 2 µm, 4.6 mm ID × 30 cm
Mobile phase:	0.1 mol/L KH_2PO_4/Na_2HPO_4, 0.1 mol/L Na_2SO_4, 0.05% NaN_3, pH 6.7
Flow rate:	0.35 mL/min
Detection:	UV @ 280 nm
Temperature:	ambient (room temperature)
Injection vol:	10 µL
Sample:	mAb from hybridoma cells, final product

Elution peak at ~7.5 min indicates a largely monomeric mAb.

Results: Final Product: Molecular Weight Determination using MALS Technology



Right angle light scattering (RALS) signal of mAb is in blue and molecular weight distribution is in orange (*dn*/*dc* = 0.185, *dA/dc* = 1.4, flow = 0.25 mL/min).

LenS3 MALS instrument connected to TSKgel UP-SW3000, 2 µm, 4.6 mm ID x 30 cm column was used for analysis.

- *The molecular weight of mAb is 129 kDa, as determined using RALS signal.*
- *MALS result confirms that the final mAb product is highly monomeric.*

Conclusions & Path Forward

- TOYOPEARL AF-rProtein A HC-650F affinity media was used for capture and a novel salt-tolerant cation exchange (CEX) media, TOYOPEARL Sulfate-650F, was used as a polishing step.
- Protein A chromatography was optimized for loading at pH 7.3, followed by intermediate wash at pH 5.0 and elution at pH 3.5 in acetate buffer.
- CEX chromatography was carried out in 0.1 mol/L acetate, pH 5.0, and included a post-loading wash with 0.1 mol/L NaCl and mAb elution at 0.35 mol/L NaCl.
- Final product (total 5.7 mg) was highly monomeric, size 129 kDa, and >95% pure as analyzed by a UHPLC SEC column, TSKgel UP-SW3000, connected to UV and a LenS$_3$ multi-angle light scattering (MALS) instrument.
- In summary, the purification process presented here was effective for this novel mAb from hybridoma cell line supernatant and the process can feasibly be scaled up to a pilot or manufacturing scale.