Automated Protein Purification using Hamilton® Microlab STAR with Various Wet Resins to Achieve Miniaturized Laboratory Workflow with High Consistencies

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INTRODUCTION

Dispersive pipette extraction is a patented micro-extraction platform that leverages turbulent mixing within the pipette tip to increase interaction time between the loose resin and the liquid sample. Coupling this core technology to the highly flexible, automated liquid handling system – Hamilton Robotics – enables rapid enrichment of antibodies, recombinant proteins and various other tagged proteins in a consistent manner. Unlike the traditional chromatography columns which rely on fixed beds, dispersive pipettes contain loose resin with a large void between the two porous filters. Here, we demonstrate the flexibility of the micro-extraction platform on Hamilton STAR to purify milligram scale antibodies from 1 mL aliquots, as well as purification of his-tagged recombinant proteins from micro-cultures in 96 well format. The "ready to go" workflows provide eluted proteins that are processed through high throughput buffer exchange on the liquid handling platform to ensure a fully automated laboratory process starting from crude samples to purified and buffered samples.

MATERIALS & METHODS

The automated workflows are generic templates for several different INtip chemistries to purify different types of targets, ranging from tagged recombinant proteins to immunoglobulins followed by buffer exchange using SizeX IMCStips™. IMCStips are available with different affinity resins (Ni-IMAC for his-tagged proteins, Protein A/G for immunoglobulins, streptavidin for biotinylated targets) to target the specific protein of interest. The deck layout shown here is one of the generic templates as "ready to go" workflows for affinity purification on Hamilton Microlab STAR. The options in the graphical user interface can provide optimized workflow along with flexibility for maximum recoveries versus a rapid purification workflow for quick qualitative analysis. Ultimately, this automated workflow provides consistency, but still some added flexibility to alter the workflows (aspirate/dispense cycles) and wash steps to customize each workflow for specific targets.

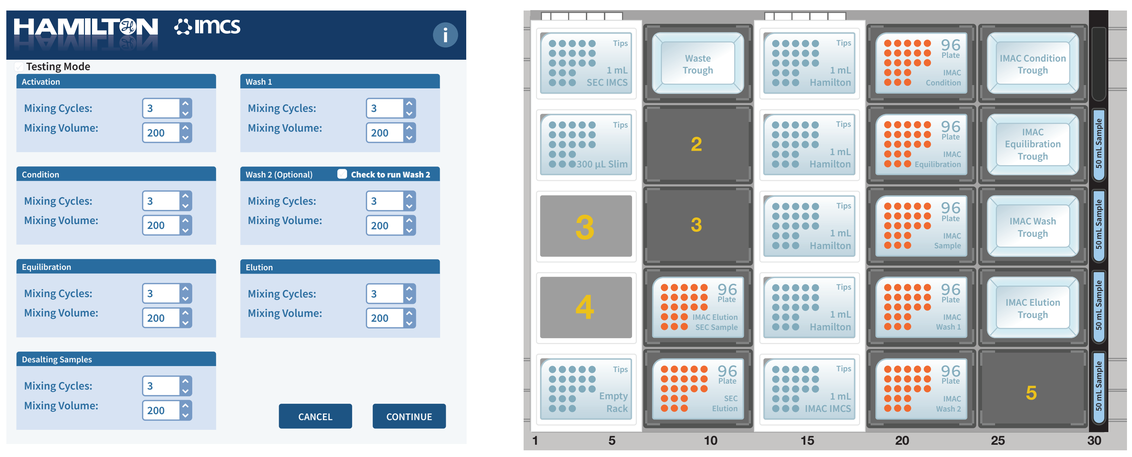


Figure 1. *Graphical user interface to control aspirate and dispense cycles and the typical deck layout for affinity purification followed by buffer exchange.*

Affinity Purification Method (Protein A, G, A/, L, and Ni-IMAC)

EQUILIBRATE — *1x PBS pH 7.4*
SAMPLE BINDING — *Cell Media 1x PBS pH 7.4 500mM NaCl*
WASH 1 — *1x PBS pH 7.4 500mM NaCl*
WASH 2 — *1x PBS pH 7.4*
ELUTE — *0.1 M Sodium Acetate pH 3.5*

Size Exclusion Chromatography (SEC) SizeX

EQUILIBRATE — *Tris Buffer*
SAMPLE LOADING — *6M GnHCl + Denatured Protein*
FLOW THROUGH COLLECTION

Streptavidin

EQUILIBRATE — *PBS 0.1% NP40*
BIND PRIMARY BAIT — *PBS 0.1% NP40*
WASH 1 — *PBS 0.1% NP40*
WASH 2 — *PBS*
BIND TARGET — *PBS 0.1% NP40*
WASH 3 — *PBS 0.1% NP40*
WASH 4 — *PBS*
ELUTION — *0.1% Formic Acid*

Table I. INtip chemistry workflow using MabSelect SuRe LX 50 µL resin

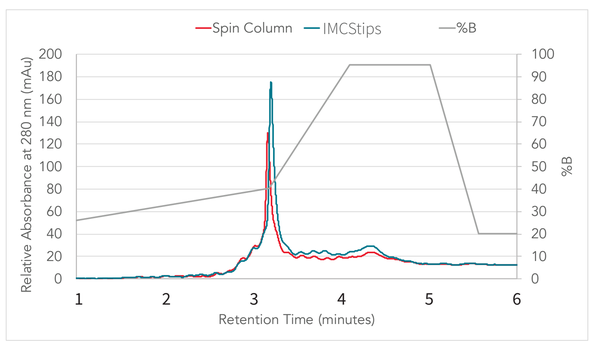
Steps	Buffer	Spin columns			IMCStips		
		Volume (µL)	Cycle	Spin Speed	Volume µL	Aspiration µL	Aspiration Dispense Cycle
Equilibration	Conditioning Solution	500	1	100	1000	800	1
Sample	Cell Supernatant	1000	5	100	1000	800	5
Wash 1	1X PBS, 500mM NaCl	500	1	100	1000	800	3
Wash 2	1X PBS	500	1	100	1000	800	3
Elution	Sodium Acetate, pH 3.5	400	1	100	400	350	3

RESULTS

Protein A

Figure 2. *Purification of a single lot of CHO cell fermentation using MabSelect SuRe LX resin (50 µL resin bed) by centrifugation (spin) and IMCStips™. For consistency, the spin method utilized 100 x g RCF for 5 minutes and flow through samples were re-applied to the column either 3x or 5x, similar to the tip format. Spin format consistency yielded less mAb than tip based format, as indicated by both UV-Vis measurements (Abs@ 280 nm) and by SDS-PAGE.*

Purified mAbs on RP-HPLC



IEX column for analyzing acidic variants and truncations

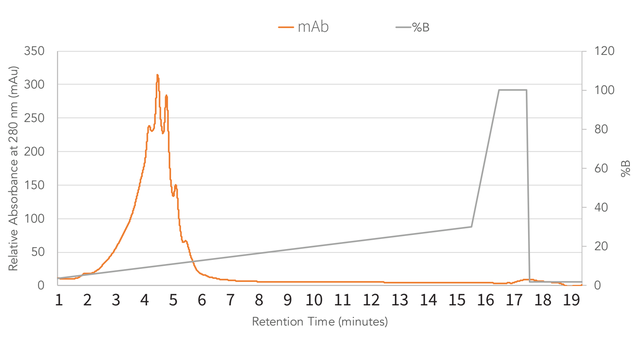
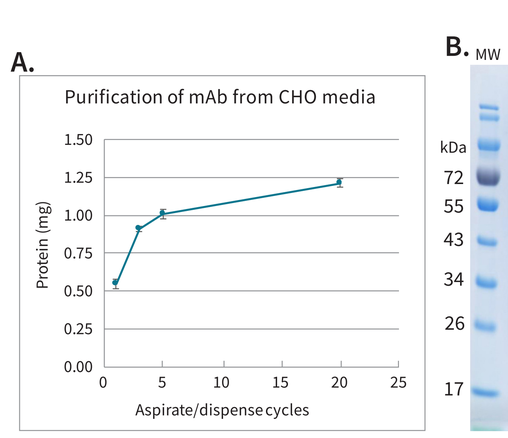


Figure 3. *Acidic variants and truncations of the purified antibody were analyzed on ion exchange resin, and reverse phase HPLC was used for detection of IgG isoform integrity*



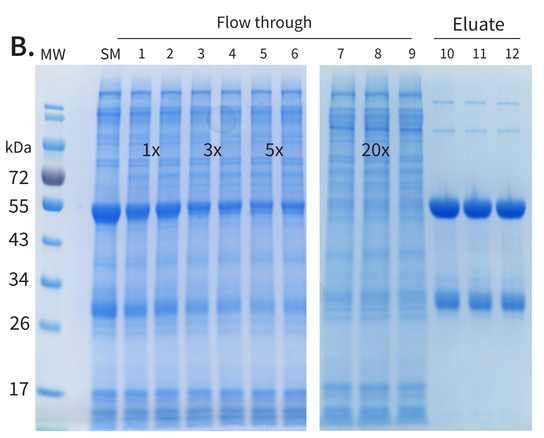


Figure 4. *Increasing aspirate/dispense cycles improve the final yield of IgG as indicated by protein quantification by UV-Vis (Abs @280 nm, **A**) and also indicated by the depletion of the heavy and light chains in the flow through media (**B**). The eluants are after 20x aspirate/dispense cycles followed by a final elution volume of 400 µL in NaOAc pH 3.5. The sample was neutralized with 1/10 volume of 1 M Tris pH 8. Each cycle is a duplicate run with the exception of the final 20x flow through and the eluants in triplicates.*

Interday Reproducibility

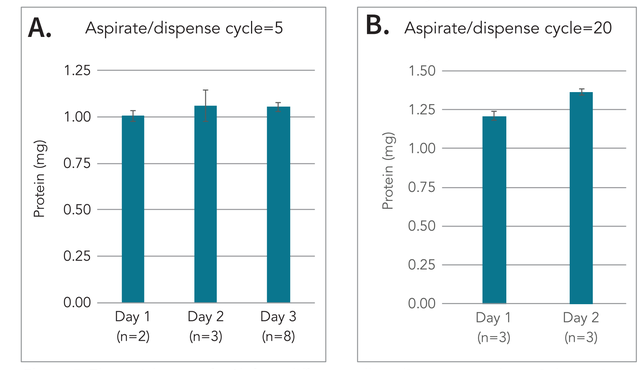


Figure 5. *The enrichment of mAb from different cell media were tested over 3 days using different number of tips and two difference aspirate/dispense cycles.*

Recovery across varying concentration

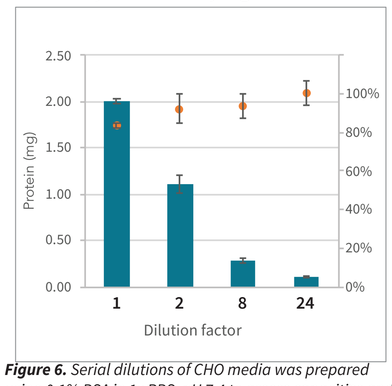
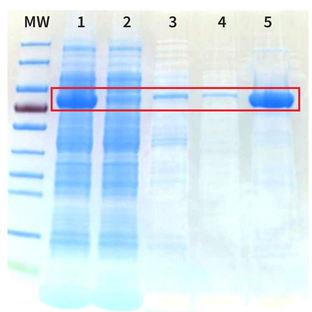


Figure 6. *Serial dilutions of CHO media was prepared using 0.1% BSA in 1x PBS, pH 7.4 to assess capacities and recoveries across the different concentrations.*

Ni-IMAC



1: Starting Material
2: Flow Through
3: Wash 1
4: Wash 2
5: Eluant
Red box highlights the target protein

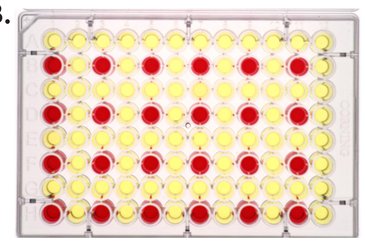


Figure 7. *Enzyme activity assay of his-tagged sulfatase measured using p-nitrocatechol sulfate. Every fourth quadrant contains purified sulfatase, which converts the substrate to a red solution. The cell lysates were purified and eluted on 96 well plate format, followed by buffer exchange. The activity assay indicates no cross contamination during the workflow. Yellow solution indicates no sulfatase activity.*

Table II. Protein Quantification

	Average	Stdev	% CV
β-GUS (n=8)	*0.397*	*0.052*	*13.3*
SULF (n=24)	*0.123*	*0.017*	*14.5*
Blank (n=24)	*0.024*	*0.006*	*27.9*

Protein quantification (Bradford Assay) of his-tagged β-glucuronidase (β-GUS) and sulfatase (SULF) purified using IMCStips Ni-IMAC. Recombinant proteins were expressed in *E. coli.* The protein quantification shows reproducibility and consistency of IMCStips Ni-IMAC high throughput purification.

Size Exclusion

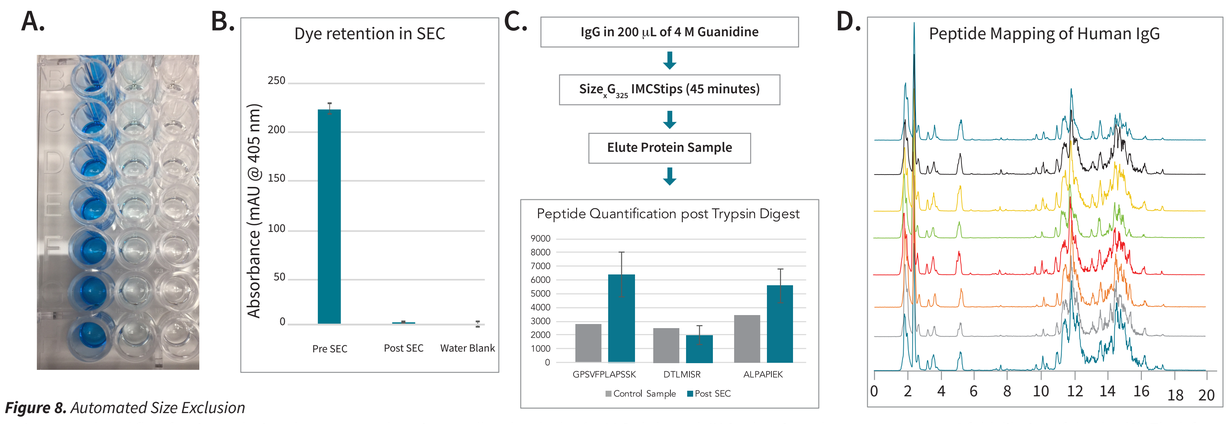
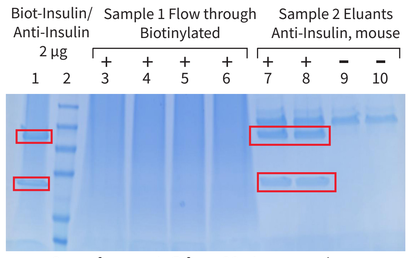


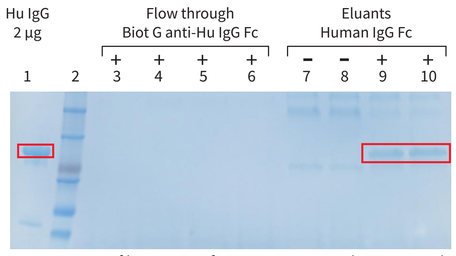
Figure 8. *Automated Size Exclusion*

*Automated workflow for size exclusion chromatography using small molecule dye (~500 Da) through INtip chemistry demonstrates over 18 fold dilution of the dye (**A**). The left most column contain dye solution prior to SEC and the center column contain the samples post SEC and the right most column are water blank samples. (**B**). Trypsin digestion workflow for the immunoglobulin after treating with 4 M GnHCl, DTT and IAA followed by SEC to desalt prior to trypsin/LysC digestion. Three peptides were monitored by Thermo LC-MS/MS using 0.5 M GnHCl treated as control sample and post-SEC samples. The total peak area counts were higher or similar to the control samples, which indicates that trypsin digestion was effective using post SEC samples (**C**). Eight post-SEC human IgG samples were trypsin/LysC treated and analyzed by UPLC-ESI-qTOF MS. The overlaid chromatograms and monitoring several critical quality attributes indicate that the results are highly consistent for all of the samples (**D**).*

Streptavidin



Capture 2 µg of mouse IgG from 30 µL mouse plasma sample



Capture 5 µg of human IgG from 30 µL mouse plasma sample

CONCLUSION

Affinity enrichment of various target analytes is a routine process that is implemented throughout the early to late research phases. The work shown here are based on automation and high throughput purification, but still providing room for flexibility to adjust the process for varied number of samples, varying affinities and coupling multiple workflow processes such as buffer exchange after affinity purification. The high consistency and flexibility are key points when implementing these workflows for purification of tagged proteins with Ni-IMAC, immunoglobulins from cell media using Protein A immobilized resin, pulling down biotinylated targets with streptavidin beads or automating the buffer exchange process with size exclusion chromatography. Ultimately, IMCStips coupled to the Hamilton microlab Star automated liquid handling system has multiple modalities that can provide flexibility, consistency and evolutionary compatibility as the needs of the scientist change over time.

ACKNOWLEDGEMENTS

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