Automating Multi-Attribute Monitoring Process for At-Line Monitoring of Biotherapeutics

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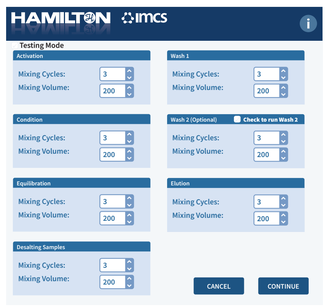
INTRODUCTION

Protein purification is a multi-step process that incorporates various resins and consumables to achieve the desired outcome. For instance, recombinant protein purification is typically achieved using an affinity resin such as Ni-NTA followed by buffer exchange to remove the excess salts. Other affinity-based purification methods are also similar in that the IgG purified with Protein A or G utilize basic buffer to neutralize the acidic elution, but the final storage buffer may require alternative formulations. Here, we integrated Hamilton Microlab STAR automated liquid handling system with IMCStips to achieve high throughput protein purification followed by multiple workflow steps to alleviate switching equipment or consumables for each step. The first workflow is the purification of the recombinant protein by Ni-NTA, followed by buffer exchange on SizeX 150 to remove the excess imidazole salts. The purified protein is then aliquoted for protein quantification. The workflow demonstrates turn-key approach from cell lysate to quantified, purified proteins within less than 2 hours per batch. The second workflow is the characterization of IgG after Protein A purification followed by denaturation, reduction, alkylation, buffer exchange on SizeX followed by trypsin digestion for multi-attribute monitoring. The third is the reverse phase peptide desalting and phosphopeptide enrichment on a single workflow. And the last workflow is the use of streptavidin beads inside the pipette tip to capture biotinylated anti-Fc antibody, followed by pull-down of Avastin in mouse serum. The eluted sample is then reduced, alkylated, trypsin digested for quantifying biotherapeutic antibody in mouse sera. These integrated workflows with IMCStips demonstrate purification of different proteins using different INtip™ chemistries and the flexibility of the Hamilton Microlab STAR to achieve a variety of processes on a single platform to achieve high throughput workflows with minimal human intervention.

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.



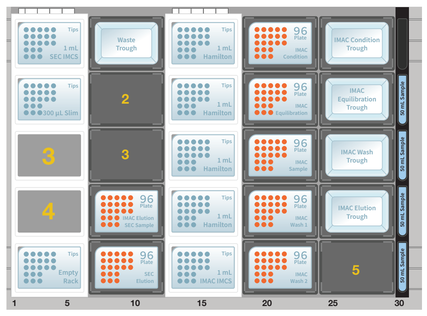


Figure 1. *Graphical user interface to control aspirate and dispense cycles and the typical deck layout for affinity purification followed by buffer exchange.*

RESULTS

Recombinant Protein Purification

A. Affinity Purification using IMCStips

EQUILIBRATE *50 mM sodium phosphate, 300 mM NaCl, pH 8* → SAMPLE BINDING *Cell Lysate* → WASH 1 *50 mM sodium phosphate, 300 mM NaCl, 10 mM imidazole, pH 8* → WASH 2 *50 mM sodium phosphate, 300 mM NaCl, 10 mM imidazole, pH 8* → ELUTE *50 mM sodium phosphate, 300 mM NaCl, 500 mM imidazole, pH 8*

Buffer Exchange using SizeX

EQUILIBRATE → LOAD SAMPLE → COLLECT FLOW THROUGH

Table I. 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

Table I. 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.

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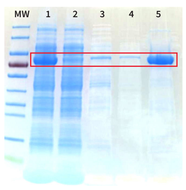
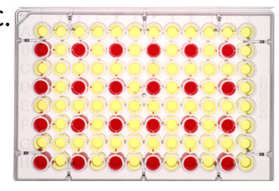


1: Starting Material
2: Flow Through
3: Wash 1
4: Wash 2
5: Eluant
Red box highlights the target protein

C.



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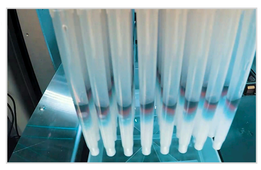

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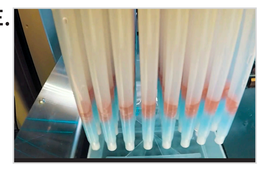

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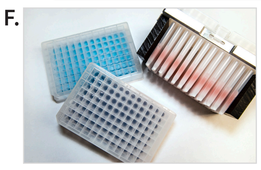


Figure 2. ***(A)*** *INTip chemistry workflow using Ni-IMAC affinity resin for purifying recombinant protein* ***(B)*** *SDS PAGE of crude lysate containing the recombinant protein, the flow through, two wash solutions, and the final eluate.* ***(C)*** *Enzyme activity assay of his-tagged sulfatase measured 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.* ***(D)*** *Side view of SizeX tips containing the dextran blue and cobalt chloride salt solution, when combined has a purple hue. This solution is to exemplify a sample solution. Dextran blue is approximately 20 kDa, whereas the cobalt chloride is a salt and exhibits a pink hue.* ***(E)*** *The separation of the two compounds are based on size, where the larger molecule (dextran blue) elutes prior to the salts as indicated with the color separations.* ***(F)*** *The eluates of blue dextran in 96 well plate and the retention of the cobalt chloride salts in the tips are shown.*

At Line Multi-Attribute Monitoring

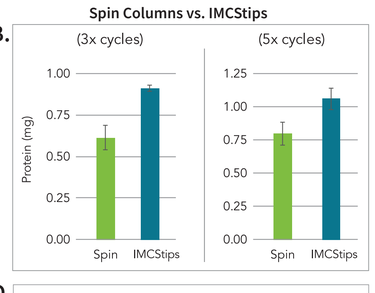
A. Protein A IMCStips

EQUILIBRATE → SAMPLE BINDING → WASH 1 → WASH 2 → ELUTE *6 M GnHCl, 10 mM Tris pH 8*

REDUCE → ALKYLATE → EQUILIBRATE *Tris Buffer* → SAMPLE LOADING *6 M GnHCl + Denatured Protein* → FLOW THROUGH COLLECTION

TRYPSIN DIGESTION → LC-MS ANALYSIS

B.



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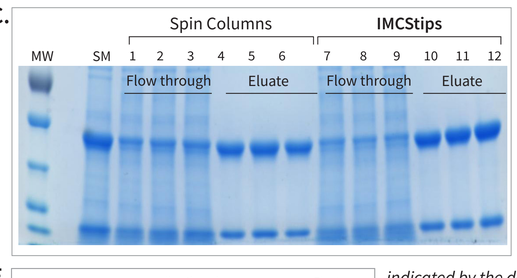

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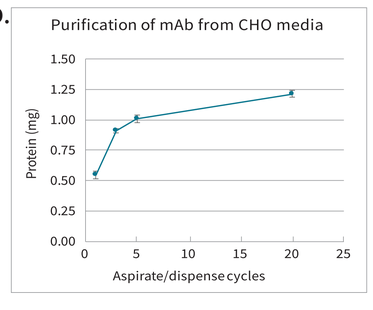

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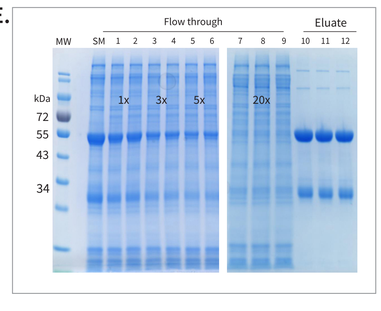
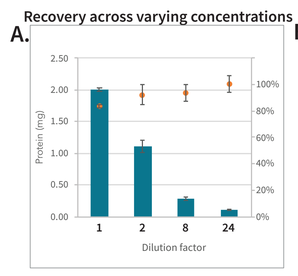
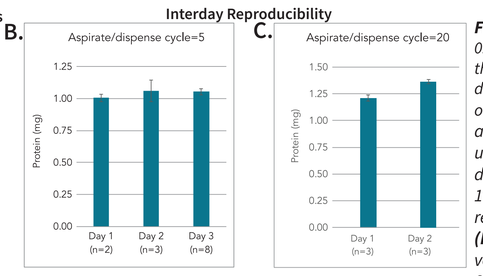


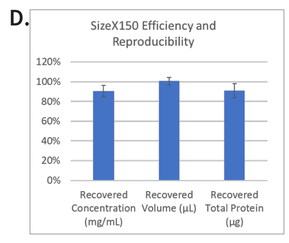
Figure 3. ***(A)*** *The workflow scheme of at-line multi-attribute monitoring on Hamilton platform using a combination of Protein A IMCStips for purifying the antibody from cell supernatant, followed by subsequent reagent additions such as reducing and alkylating agents. The denatured antibody is then desalted using SizeX, a size exclusion chromatography tip, followed by trypsin digestion for final LC-MS injection and analysis.* ***(B, C)*** *Purification of a single lot of CHO cell fermentation using MabSelect SuRe LX resin by centrifugation (spin) and IMCStips™. For comparative purpose, 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. Increasing aspirate/dispense cycles increase the yield of IgG as indicated by protein quantification by UV-Vis,* ***(D, E)*** *and indicated by the depletion of the heavy and light chains in the flow through media. 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.*

Recovery across varying concentrations



Interday Reproducibility





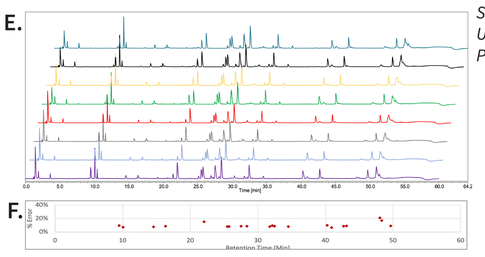


Figure 4. ***(A)*** *Serial dilutions of CHO media was prepared using 0.1% BSA in 1x PBS, pH 7.4 to assess capacities and recoveries across the different concentrations.* ***(B,C)*** *The enrichment of mAb from different cell media were tested over 3 days using different number of tips and two difference aspirate/dispense cycles. Monoclonal antibody at a final concentration of 1 mg/mL was denatured using 6 M guanidium hydrochloride. Next, it was reduced with dithiothreitol and alkylated with iodoacetic acid, respectively. 100 µL of the antibody was loaded onto IMCStips SizeX100 to remove denaturing agents for subsequent tryptic digestion.* ***(D)*** *Average percentages of recovered antibody concentration, volume, and total antibody amount (n=20). Error bar shows ± 1 S.D.* ***(E)*** *After the digestion, the sample was analyzed on HPLC-UV. Eight representative A280 chromatograms are overlaid.* ***(F)*** *Percent error of area integrations of 8 replicates.*

Reverse Phase and Phosphopeptide

The associated deck layout for the method couples both the desalting and the phosphopeptide enrichment method with multiple wash steps **(Figure 5)**. The program starts with a trypsin digested sample set after the proteins from cell lysates are precipitated using acetone.

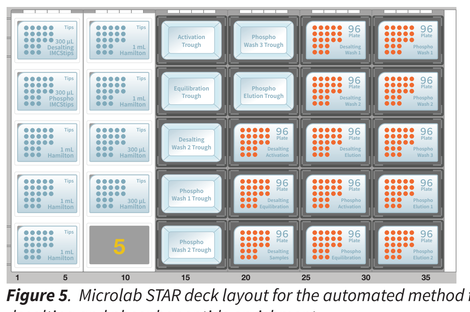


Figure 5*. Microlab STAR deck layout for the automated method for desalting and phosphopeptide enrichment.*

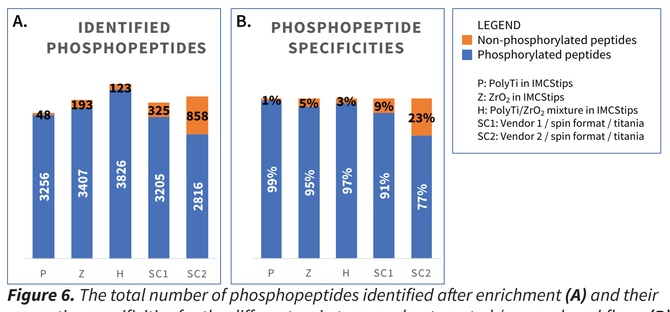


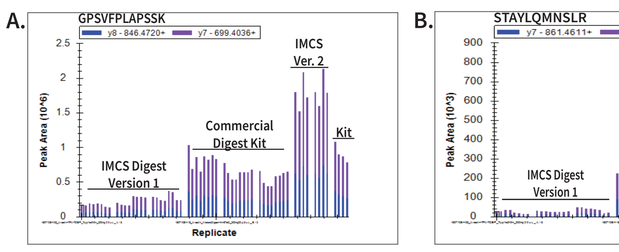
Figure 6. *The total number of phosphopeptides identified after enrichment* ***(A)*** *and their respective specificities for the different resin type, and automated / manual workflows* ***(B)****. P, Z, and H were processed using the IMCStip using automated phosphopeptide enrichment method. SC1 and 2 were processed follow the vendors' protocol. All samples were processed in duplicates. The numbers shown are averages from duplicate runs.*

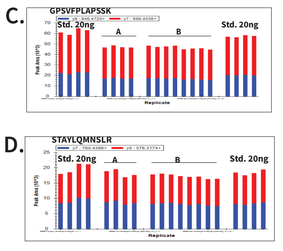
Streptavidin

DILUTE *20 µL mouse serum with 220 uL of 1x PBS/0.5% NP-40* → ADD *0.2 µg SILuMab and 0.2 µg of therapeutic antibody* → ADD *1 µg of anti-human Fc, biotinylated goat antibody* → MIX AND INCUBATE *1 Hour* → CAPTURE *Using Streptavidin IMCStips*

WASH 1 → WASH 2 → ELUTE 1 AND 2 *Combine both eluates* → HEAT *Denaturation at 95° C* → REDUCE *with DTT (20 min., 56° C)*

ALKYLATE *with IAA at room temperature in dark* → DIGEST *1 µg of trypsin and incubate for 2 hours at 45° C* → ANALYZE *5% FA to inactivate and centrifuge prior to analysis by LC-MS/MS*





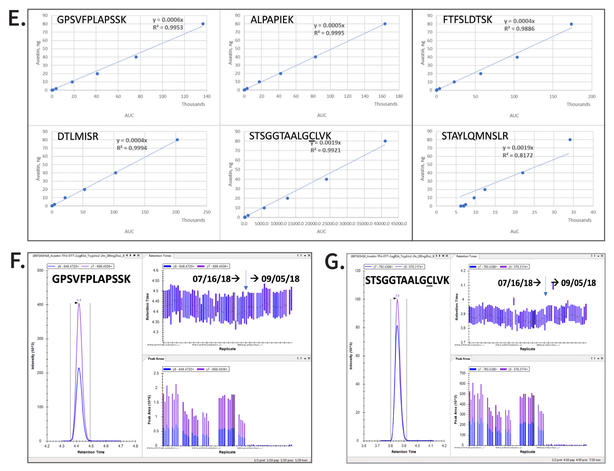


Figure 7. *The workflow scheme for automating antibody quantification in pharmacokinetics study. The target therapeutic antibody is a humanized IgG and the sample matrix is mouse serum. The therapeutic antibody was enriched by using anti-human Fc from goat with a biotin tag. The captured antibody was further enriched using streptavidin IMCStips on Hamilton STAR platform, followed by elution, denaturation, reduction/alkylation and digestion.* ***(A, B)*** *The modifications of the workflow and digestion times further increased the sensitivity by nearly 10-fold for the generic antibody peptides.* ***(C, D)*** *The recovery and consistency of the workflows were monitored with repeat injections using multiple IMCStips. Recovery was normalized against neat standards supplemented with trypsin digested BSA.* ***(E)*** *The four generic peptides and four unique signature peptides show linearity across a range from 10 pg to 80 ng.* ***(F)*** *The retention times and peak areas for the two generic peptides were continuously monitored to assess the robustness of the automated workflow.*

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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