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APPLICATION NOTE

Semi-automated affinity purification at microgram and milligram scales using IMCStips on INTEGRA's ASSIST pipetting robot

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IMCStips® PRODUCT

Protein A

1250 µL

Protein A (MabSelect SuRe™ LX)

Binding Capacity (per tip)	Tip Quantity	Catalog Number
1 mg IgG	8	02-TK-I1(R80-25-8)
1 mg IgG	96	02-TK-I1(R80-25-96)

Description:

IMCStips® with INTEGRA's ASSIST pipetting robot for semi-automated and small-scale purification of immunoglobulins.

METHOD BENEFITS

- High reproducibility with error-free pipetting
- Reduced hands-on time and unmatched ergonomics
- Dispersive solid phase extraction (dSPE) pipette tips facilitate maximum contact between resin and analyte leading to higher recoveries in less time

INTEGRA's ASSIST pipetting robot

INTRODUCTION

Protein A immobilized resins are utilized to purify immunoglobulins (IgGs) by both academic and industry laboratories. Small-scale purification is typically performed in spin columns, or magnetic beads in 96-well plate formats. As an alternative, IMCStips use a patented purification technique that allows multiple samples to be purified in parallel using multi-channel pipettes. By integrating INtip purification with INTEGRA's ASSIST pipetting robot, any scientist can easily incorporate this workflow into their laboratory.

The key factor driving IMCStips' advantages over other purification techniques is the chromatography media contained loosely within each pipette tip **(Figure 1)**. Each tip contains both bottom and top porous filters. The top filter is a long distance from the resin, with the same placement as an aerosol barrier. A disperser ring is placed within the tip, constricting liquid flow to introduce turbulent mixing between the liquid sample and the solid resin.

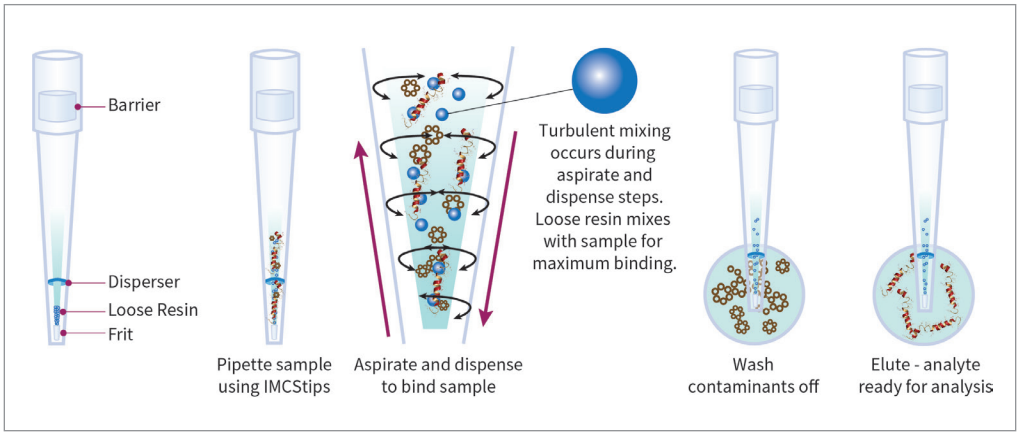

Figure 1. *IMCStips use a patented dispersive solid-phase extraction (dSPE) technology. Unlike fixed-bed SPE (fbSPE) devices, IMCStips contain loosely packed resins that mix with sample solutions during aspirate and dispense cycles (mixing cycles), ensuring maximum contact between the resin and analytes of interest. Turbulent mixing within the tip is further enhanced by the addition of a patented disperser. Purification follows a typical bind, wash, elute workflow.*

Unlike traditional chromatographic techniques with fixed bed columns, samples in pipette solutions are exposed to each resin during aspiration and dispense steps **(Figure 2)**. Subsequent wash steps are proceeded by moving each pipette across well plates. Loose resins eliminate backpressure concerns during aspiration and resets the column bed to address potential channeling or voiding issues. The contact time between each sample and resin is adjusted by modifying aspiration and dispense cycles, which are also known as mixing cycles.

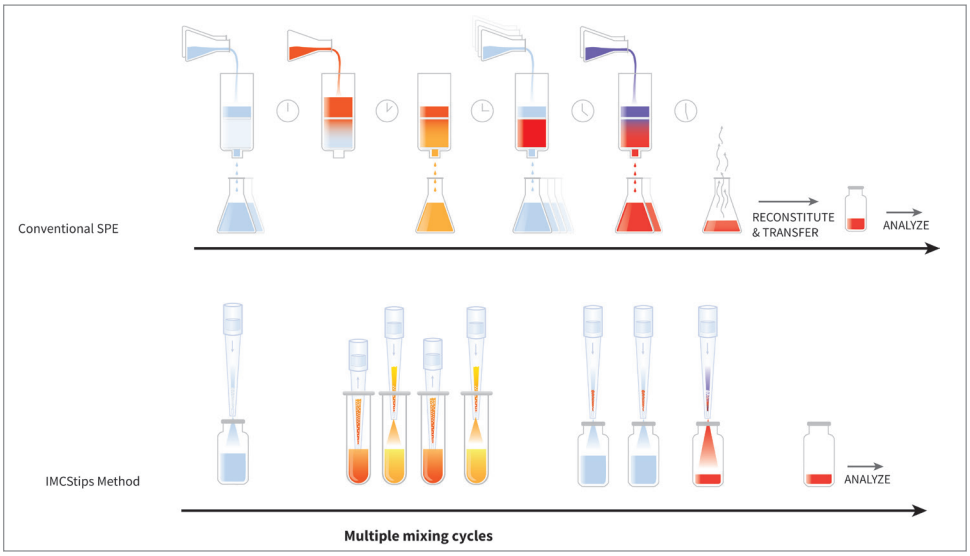

Figure 2. The conventional fixed bed solid phase extraction (fbSPE) and the tip based dispersive SPE (dSPE) methods are two possible workflows for extracting analytes from a mixture. IMCStips are unique because of the patented disperser ring within the pipette used in conjunction with loosely contained resin that mixes during aspiration and dispense cycles (mixing cycle). The dSPE approach is similar to batch extraction, where resins and liquid mixtures containing the analyte of interest are mixed end-over-end for a fixed time period. In this case, the pipette contains filters to retain the resin.

Here, we demonstrate IgG capture using Protein A IMCStips on the ASSIST pipetting robot by increasing the mixing cycles at various antibody concentrations and evaluating elution parameters. In conjunction with INTEGRA's ASSIST, small-scale purification can be performed on up to 12 samples in 90 minutes without any manual intervention. This provides an affordable, accessible entry point for any scientist interested in automating sample preparation, increasing throughput, and testing proof-of-concept workflows by adjusting buffers, mixing cycles, or resins.

BINDING KINETICS

Binding kinetics for IMCStips on the ASSIST pipetting robot were obtained by using a VIAFLO 8 channel 1250 µl electronic pipette. A volume of 800 µL was set on the VIAFLO electronic pipette for both equilibration and sample binding. Prior to initiating sample binding, IMCStips were equilibrated with 1x PBS using three mixing cycles. Different concentrations of polyclonal human IgG (huIgG) were prepared by diluting a stock antibody solution (10 mg/mL) with 1x PBS, and 0.1% IgG-depleted BSA (bovine serum albumin).

The fraction of bound material was calculated based on the differences in the absorbance value of flow-through relative to the starting material. The protein was bound in a series of aspirate (100 µL/s) and dispense (30 µL/s) steps with an additional 25 seconds of hold time ensuring all liquid was expelled from the tip during the mixing cycle. Flow-through was measured using NanoDrop at 280 nm after the following mixing cycles: 1, 2, 3, 4, 5, 10, 15, 20, 25, and 30. Five different concentrations, used at a fixed sample volume of 1 mL, ranged from 0.2 mg/mL to 1 mg/mL **(Figure 3)**.

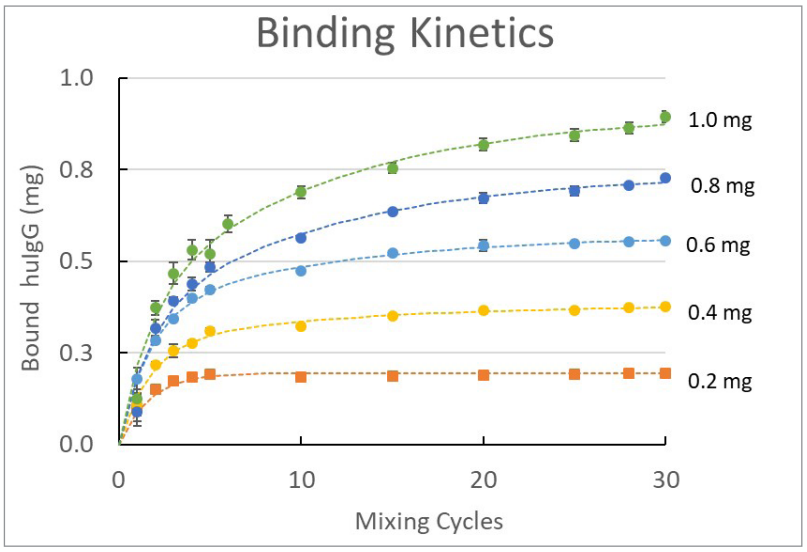

Figure 3. These binding curves were generated by using a fixed pipetting program on INTEGRA's ASSIST with VIAFLO 1250 µl electronic multichannel pipettes; each sample aspiration and dispense volume is set to 800 µL. The sample solution is then fortified with polyclonal human IgG (huIgG) in 1x PBS, 0.1% IgG-depleted BSA. IMCStips (P/N 04T-IR80-1-25-8) will bind up to 90% of the antibody in 30 mixing cycles at a 1 mg level. Increasing mixing cycles allow for increased binding as overall residence time is increased (results not shown). The error bars denote a single standard deviation from the mean from triplicate samples.

Antibody capture is dependent on antibody concentrations and affinity ligand density on each resin. Two binding kinetics, one fast and one slow, are seen with IMCStips on an ASSIST pipetting robot. Based on this specific resin amount, fast binding at lower sample quantities (0.2 and 0.4 mg) is likely a result of binding to surface exposed ligands; it is not limited by diffusion through the resin's pores. As the amount of IgG increases, more surface ligands are occupied, and the subsequent antibody binding requires accessing ligands within the pores. This second, slower capture rate retains higher antibody amounts and requires increased mixing cycles to capture these antibodies. This product line is designed to capture at least 90% of the human polyclonal antibody in a 1 mL solution with just 30 mixing cycles. Increasing the number of mixing cycles to 40 will increase the overall capture of IgG, but it also requires additional processing time. Larger resin beds can lead to faster workflows by leveraging higher amounts of external ligands, but this approach also produces larger dead volumes.

BINDING CAPACITY

To determine the binding capacity of IMCStips, huIgG samples were subjected to 25 sample mixing cycles and two separate elutions (300 µL each for total of 600 µL). The parameters used to identify antibody recoveries start with huIgG concentrations of 0.2 mg/mL and increase to 6 mg/mL in 1 mL of 1x PBS, 0.1% IgG-depleted BSA **(Figure 4).**

Recoveries above 80% at 25 mixing cycles were obtained with IMCStips loaded with lower starting samples (0.2, 0.4, and 0.6 mg); higher starting samples (0.8, 1.0 mg) resulted in 75% recovery. Recovery percentages can be improved to 80% for 1 mg/mL of huIgG by increasing mixing cycles to 30 or higher (although this requires longer binding times). As antibody concentrations in each sample increase beyond 1 mg/mL, binding rates shift to slow binding kinetics. As a result, longer residence times are required, leading to an increased number of mixing cycles.

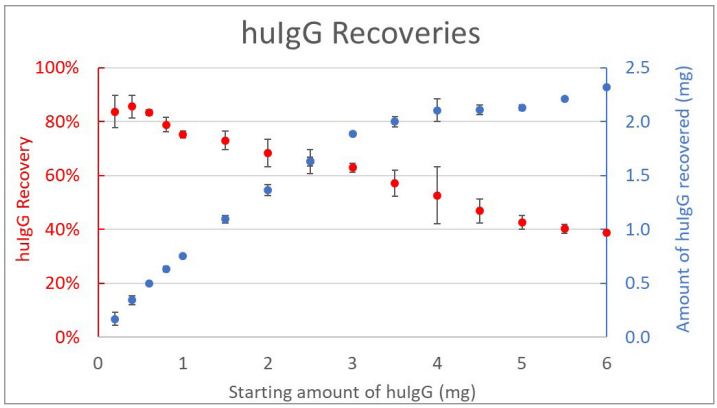

Figure 4. *The apparent static binding capacity, ignoring the slight increase with a 6 mg sample load, is close to 2 mg for this product line. If the target recovery is > 80% with 25 mixing cycles, the cut-off for sample load is near 1 mg, whereas if the acceptable recovery percentage is 60%, the sample load is close to 2 mg.*

ELUTION KINETICS

Using IMCStips to bind samples at 25 mixing cycles resulted in the capture of a smaller amount of huIgG (400 µg). According to prior results, this condition was enough to capture 100% of huIgG in under 90 minutes. The elution buffer was fixed at 100 mM sodium acetate with a pH of 3.4, and both the first and second elution used 300 µL **(Figure 5)**. Recoveries were based on absorbance values after each mixing cycle.

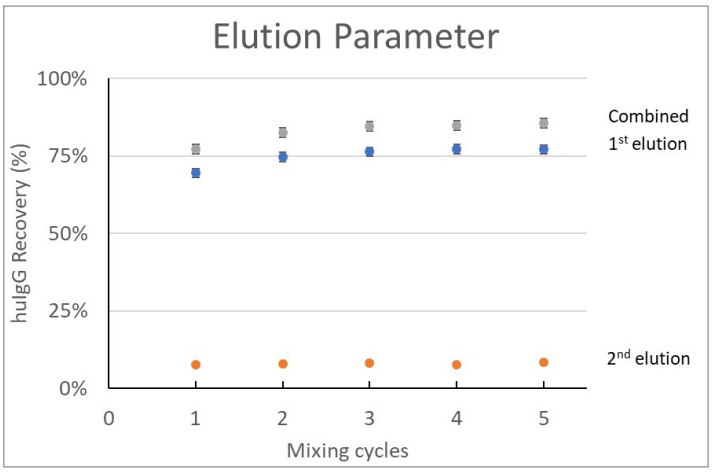

Figure 5. *The number of mixing cycles is plotted against analyte recoveries. To achieve consistent recoveries in the first elution, this step requires 3 mixing cycles. Due to the dead volume created by the resin and filter, the second elution provides an additional 10% analyte recovery. Increasing beyond three cycles does not alter recoveries.*

Low pH buffers are generally used to elute antibodies from protein A resin. Three different elution buffers (0.1 M glycine pH 3.0, 0.1 M citric acid pH 3.0, and 0.1 M sodium acetate pH 3.4) were tested using a 1.05 mg/mL huIgG sample with 25 sample mixing cycles and two 300 µL elution steps with 2 mixing cycles each. The three buffers (n = 3 for glycine and citrate buffers, n = 5 for acetate buffer) recovered an average of 80% **(Figure 6)**, showing no significant difference among the three buffers.

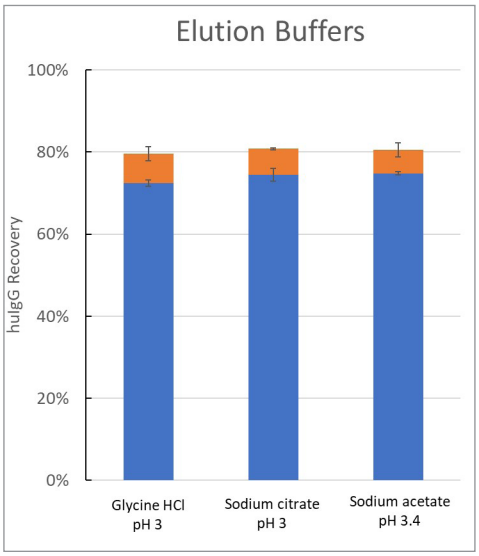

Figure 6. *Low pH elution regardless of the buffer type was enough to recover huIgG.*

CONCLUSION

The work shown here utilizes IMCStips containing MabSelect SuRe LX resin in an INTEGRA 1250 µl wide bore GripTip, making this tip-based purification approach compatible with INTEGRA's pipetting robots. INTEGRA's ASSIST and ASSIST PLUS utilize VIAFLO electronic multichannel pipettes with programmable aspiration and dispense speeds and precise X-axis and Z-axis positioning. This affinity purification protocol was programmed to purify 8 samples in parallel in less than 90 minutes. These demonstrated kinetics and capacities can be used to customize ideal workflows for purifying immunoglobulins. Elution parameters suggest that 3 mixing cycles is sufficient for consistent recoveries, and a second elution step affords slight improvements in recoveries. Overall, this workflow demonstrates the consistent performance of IMCStips for IgG purification using INTEGRA's pipetting robots.

MATERIALS AND METHODS

Chemicals and Reagents

Protein A affinity purified polyclonal huIgG was purchased from ImmunoReagents. Immunoglobulin and protease free bovine serum albumin (BSA) was purchased from Rockland. Glycine, sodium hydroxide, acetic acid, and hydrochloric acid were purchased from Fisher. Sodium acetate was purchased from Millipore Sigma. Sodium citrate buffers were purchased from Boston Bioproducts. Tris buffer and phosphate buffered saline were purchased from Alfa Aesar. Eppendorf™ 96 deep-well plates were purchased from Fisher Scientific.

IMCStips (P/N: 04T-I1R80-1-25-8/12) was manufactured with a fixed amount of MabSelect SuRe LX resin with binding capacities defined using this build specification. Silicone seals were removed, and the storage solution was removed by engaging the tips with a pre-aspirated multi-channel system to dispense 1000 µL of air through the tips.

Instruments

All experiments were carried out using INTEGRA's ASSIST pipetting robot (INTEGRA Biosciences P/N: 4500) and VIAFLO 8 channel 1250 µL electronic pipette (INTEGRA Biosciences P/N: 4624). Absorbance values at 280 nm were measured with a NanoDrop 2000 from Thermo Fisher Scientific.

BINDING KINETICS

Effective Binding Capacity

The effective binding capacity was measured by running purification methods at different antibody concentrations from 0.2-6 mg/mL in 1x PBS containing 1 mg/mL BSA. The purification methods contained equilibration steps (2 cycles), sample mixing steps (25 cycles), three wash steps (3 cycles each), and two elution steps (2 cycles each). The eluted protein was measured at 280 nm wavelength on a NanoDrop, and the results were combined to determine the total protein eluted. Protein recovery in both percentages and milligrams were plotted as functions of protein concentration.

Elution Parameters

Protein A IMCStips were equilibrated with 1x PBS prior to their use in a sample well. Protein A IMCStips performed 25 sample mixing cycles (aspiration 100 µL/s and dispense 30 µL/s, followed by 25 seconds for both aspiration and dispense) in the sample well. After 25 sample mixing cycles, an additional 350 µL of dispense volume was used for the blow-out and tip-touch steps to ensure liquid was expelled prior to elution. Different volumes of elution buffer (0.1 M sodium acetate, pH 3.4) were tested (aspiration 100 µL/s and dispense 60 µL/s with an additional 15 seconds hold time for both aspiration and dispense steps). The resulting eluate were measured with a NanoDrop after each elution mixing cycle. Protein recovery and un-eluted proteins were plotted as functions of the elution mixing cycle. After the optimal elution mixing cycle was found, different types of elution buffers (0.1 M glycine pH 3.0, 0.1 M sodium citrate pH 3.0, and 0.1 M sodium acetate pH 3.4) were screened.

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