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APPLICATION NOTE

Affinity Purification of TST-Proteins using Strep-Tactin®XT 4Flow® High Capacity in IMCStips®

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IMCStips® XT 4Flow® HC

Strep-Tactin®XT 4Flow® high capacity

Application using 300 µL

Product formats available in 1 mL and 300 µL tip sizes

Binding Capacity (per tip)	Tip Quantity	Catalog Number
50 µg	8	04T-H3R88F-1-5-8
50 µg	96	04T-H3R88F-1-5-96

Description:

Automated affinity purification with IMCStips® XT 4Flow® HC on a Hamilton Microlab® STAR™.

METHOD BENEFITS

- High reproducibility and reduced hands-on time without the back-pressure issues found in fixed-bed micro-purification pipette tips or the tediousness of manual sample preparation methods.
- Patented dispersive solid-phase extraction (dSPE) technology uses loose resin within the tip, facilitating maximum contact between resins and analytes, leading to higher recoveries in less time.
- IMCStips come with fully customizable scripts, confidential method development, and ongoing technical support for seamless implementation.

INTRODUCTION

The Strep-tag®II peptide, with its complementary engineered streptavidin called Strep-Tactin®XT by IBA Lifesciences, is designed for affinity-based purifications of target proteins, cells, or exosomes from crude cell lysates. The basis for the Strep-tag® technology derives from the highly selective, non-covalent interaction between streptavidin and biotin, but instead of biotinylating the target, a Strep-tag® sequence (WSHPQFEK) is engineered and fused to the protein. The Twin-Strep-tag® (TST) contains two Strep-tag®II moieties and has been designed specifically to fit into the biotin binding pocket of a genetically modified streptavidin, known as Strep-Tactin®. Recombinant expression of the TST-tagged protein and subsequent immobilization onto Strep-Tactin®XT allows the target analyte to be isolated from cell lysate, washed, and then eluted with a biotin solution. In pursuit of maximizing the purification performance, the ongoing development of Strep-Tactin® has led to the creation of several generations of Strep-Tactin®, with the latest version brought to market in July of 2021: Strep-Tactin®XT 4Flow® high capacity (STXT4FHC).

The design of the STXT4FHC is distinct from previous Strep-Tactin® resins. The 4Flow® consists of a 4% agarose highly crosslinked base matrix, providing a larger pore size than the traditional 6% agarose crosslinked base matrix of earlier versions of Strep-Tactin® high capacity resins. This increase in pore size equates to an increase in surface area and allows for greater accessibility to internal binding sites that otherwise would be blocked due to steric effects or physical congestion of the pore by large proteins. Increasing the pore size of the resin should thus result in greater purification yields and improved purification of larger proteins (> 90 kDA).

Both affinity and accessibility govern the binding kinetics of a given protein. TST, which has previously been shown to exhibit picomolar affinity for Strep-Tactin®XT, can interact with either surface-immobilized ligands or ligands immobilized to the pore interior of a bead. The resulting kinetics of this is biphasic and include a fast-binding component (surface-bound ligands) and a slow binding component (pore interior-bound ligands). Large proteins suffer from lower effective diffusivities while also occluding binding sites on the interior of porous resins due to their larger hydrodynamic radius. As a result, larger proteins often require more time to reach a lower maximum binding capacity resulting in decreased purification yields. The newly designed STXT4FHC aims to mitigate this effect by improving the slow-binding component for proteins of greater mass.

To test the performance of IMCStips® with STXT4FHC, proteins of variable size and concentration were purified. Affinity purification performed in IMCStips® utilizes dispersive solid-phase extraction (dSPE) technology to distribute the resin in solution, allowing for maximum interaction between surface-bound ligands and target proteins. This approach has been previously shown to be successful with Strep-Tactin® resins and has the added benefit of workflow automation and high-throughput purification capabilities. Proteins tested included recombinantly TST-tagged mCherry, TST-tagged secreted embryonic alkaline phosphatase (SEAP), and a TST-tagged CD34 antibody provided by IBA. Initial protein extraction was performed out of pure protein samples spiked into 1X PBS, while later experiments involved purification from either bacterial cell lysate or cell supernatant. From these methods, we compiled a comprehensive profile detailing the purification efficacy of IMCStips with STXT4FHC.

RESULTS

Binding Kinetics for Variable Size Proteins

To determine the binding capacity, 300 µL IMCStips containing either 5 µL or 10 µL of STXT4FHC resin were built for automation on a Hamilton Microlab® STAR™ with CO-RE 96-MPH. Binding kinetics were tested over a range of concentrations for each tagged protein and scaled according to the amount of resin used. In order of molecular weight, the proteins tested were TST-mCherry (29.8 kDa), TST-SEAP (58.0 kDa), and TST-CD34 mAb (150.2 kDa). A range of 10 µg to 130 µg was used for 5 µL of resin, and 25 µg to 330 µg for 10 µL of resin. Each concentration was created in triplicate, with sample binding occurring in 250 µL of solution over 30 binding cycles. Specific flowrates for the aspiration and dispense function, plus programmed wait times amounted to each cycle lasting 52.2 seconds. Quantification of protein bound to the resin was calculated by measuring the fluorescence (TST-mCherry) or absorbance at 280 nm (TST-SEAP, TST-CD34 mAb) of free protein in solution after each binding cycle and subtracting this value from the initial fluorescence or absorbance. Determination of the concentration was dependent on a standard curve for fluorescence measurements and theoretical extinction coefficients for absorbance measurements. Data were modeled for each binding curve using a bi-phasic exponential associative function. These models provide a means for understanding the intersection of concentration relative to the number of binding cycles and their combined effect on the rate of binding.

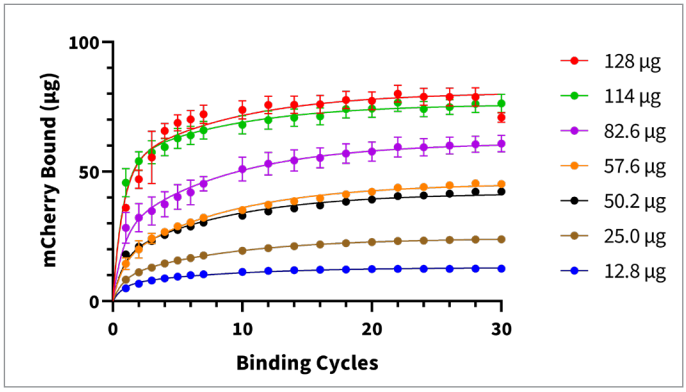

Figure 1. *Binding kinetics for various amounts of mCherry (shown on chart) extracted out of 250 µL of solution. A sample size of n=3 was created for each concentration of TST-mCherry. IMCStips® containing 5 µL of STXT4FHC resin were used on a Hamilton STAR® for sample binding. Kinetic data was fit using a biphasic exponential function. Error bars show SD.*

Differences regarding the rate of binding and binding capacity for proteins of different masses were observed. In **Figure 1,** the binding curve for TST-mCherry displays a rapid binding event occurring over the first 5 binding cycles, followed by a plateau forming around the 10th binding cycle across all concentrations. 97% of the lowest amount of initial protein (12.8 µg) was bound, while 54% of the highest amount of initial protein (128 µg). In the case of TST-SEAP (data not shown), the percent bound from all aliquots of starting material fell between a range of 41 % (from 73 µg TST-SEAP) and 36% (from 316 µg TST-SEAP). These results suggest that TST-SEAP may require additional cycles to reach binding capacity.

The largest protein tested, TST-CD34 mAb (150kDa), demonstrated slower kinetics and reduced values in the percentage of protein bound relative to TST-mCherry. **Figure 2** shows the binding curves for TST-CD34 mAb over 30 binding cycles, with the range for percent binding falling between 50% (27.4 µg) and 69% (109 µg). All proteins showed a rapid initial binding followed by a slow-binding stage. In the case of TST-mCherry, binding was nearly complete at cycle 10 across all concentrations tested. In the mAb sample, initial binding was slower, with an additional ~20% of binding occurring after cycle 10. In addition to a more gradual binding, larger proteins showed a negative correlation to moles of protein bound, suggesting there is still a steric impact on binding.

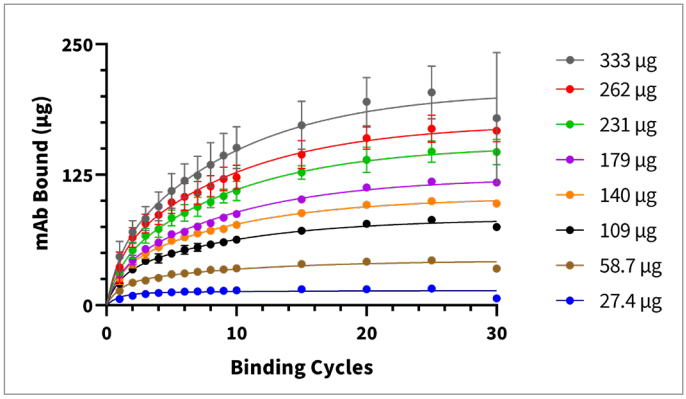

Figure 2. *Binding kinetics for various amounts of CD34 mAb purified from 250 µL of solution over 30 binding cycles. Each concentration of TST-CD3 mAb was tested in triplicate using IMCStips® filled with 10 µL of STXT4FHC resin on a Hamilton Microlab STAR. Data was fit using a biphasic exponential function. Error bars show SD.*

Full Method Purification

Full method affinity purification entailed complete automation on a Hamilton Microlab® STAR™ for equilibrate, bind, wash, and elute steps. Variable concentrations of TST proteins were purified from 250 µL solutions over the course of 30 binding cycles, followed by two wash steps and two elution steps using buffers provided by IBA. **Figure 3a** shows a gel of mCherry purified from bacterial cell lysate, with **Figure 3b** showing a gel run for CD34 mAb purified from CHO-S cell supernatant side-by-side. In addition, a comparison for reduced (left) and non-reduced (right) samples of the CD34 mAb demonstrates the effectiveness of the purification. Each purification process resulted in protein samples of >95% purity. Further confirmation was obtained by quantitative analysis in which standard curves were created to measure the molecular weight of sample bands. From this, the TST-mCherry eluate came out to ~33 kDa, which matches the standard TST-mCherry sample at ~32 kDa. The bands for the eluate and the standard are proximal to the true molecular weight of TST-mCherry at 29.8 kDa. Similar results were observed for CD34 purification wherein the estimated mass corresponding to the heavy chain and light chain for the reduced eluate came out to 64 kDa and 25 kDa, and 67 kDa and 23 kDa for the reduced standard. Diminished band intensity in the flowthrough (lane 8) versus the sample (lane 7) corroborates partial binding of the starting sample. **Figure 4** depicts a range of proportional yields for the purification of CD34 mAb falling between 68% recovery for a 240 µg load to a 100% recovery for a 20 µg load. A gradual downward trend in the percent yield correlates to an increasing amount of protein per well, with recoveries dropping below 80% in amounts greater than 120 µg per 10 µL of STXT4FHC resin.

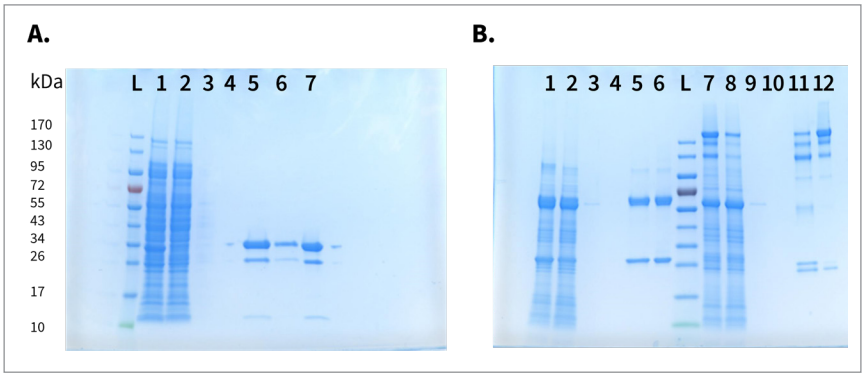

Figure 3. *SDS-PAGE gels showing purification of (A) TST-mCherry from bacterial cell lysate and (B) TST-CD34 from CHO-S supernatant. Lanes are as follows: (A) L- Ladder, 1- Sample, 2- Flowthrough, 3- Wash 1, 4- Wash 2, 5- Elution 1, 6- Elution 2, 7- Standard. (B) 1- Sample, 2- FT, 3- W1, 4- W2, 5- Elution 1, 6- Standard, L- Ladder, (lanes 7-12 are non-reduced) 7- sample, 8- FT, 9- W1, 10- W2, 11- Elution, 12- Standard.*

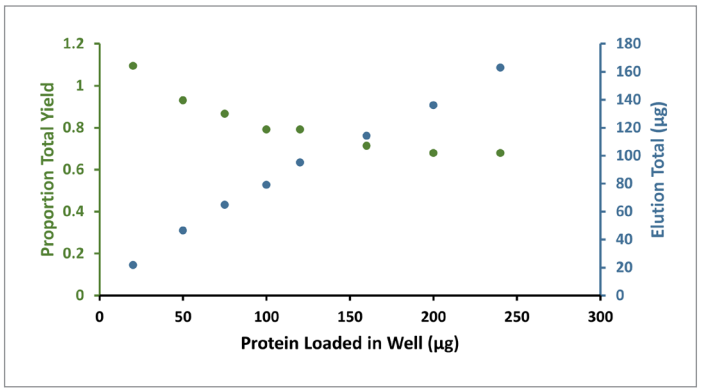

Figure 4. *Comparison of protein load to protein yield for purification of CD34 mAb on a Hamilton STAR®. Blue dots corresponds to the right y-axis depicting the amount of protein eluted (µg). Green dots corresponds to the left y-axis depicting the fractional yield of protein after elution.*

CONCLUSIONS

TST-tagged mCherry, SEAP, and CD34 mAb were successfully purified using STXT4FHC resin in IMCStips via a fully automated Hamilton STAR® method. The molar amount of purified protein was observed to be inversely proportional to the molecular weight of the protein. Yields of over 80% were obtained for the largest protein tested (CD34) at capacities < 12 mg/mL resin, demonstrating an improved profile for large molecular weight proteins over earlier iterations of the Strep-Tactin® resin series. Binding and purification profiles for TST-mCherry were consistent with previous studies on Strep-Tactin®XT HC. Consequently, the new STXT4FHC resin enables IMCStips to be used for a wider gamut of proteins while offering increased yields and more time-efficient workflows.

MATERIALS AND METHODS

Chemicals and Reagents

Twin Strep-Tag® fused proteins, mCherry, SEAP, and CD34 mAb were provided by IBA Lifesciences. Strep-Tactin®XT 4Flow® high capacity resin (STXT4FHC; P/N: 22-5027-001), 10X Strep-Tactin®/Strep-Tactin®XT Wash Buffer (Buffer W; P/N: 2-1003-100), and 10X Strep-Tactin®XT Elution Buffer with Biotin, pH 8.0 (Buffer BXT; P/N: 2-1042-025) were also provided by IBA Lifesciences. 10X phosphate-buffered saline (PBS), pH 7.4 was purchased from Alfa Aesar (P/N: AAJ62036K2). Bacterial cell lysate used for purification of mCherry was derived from *E. coli* cultures. CHO-S supernatant was used for purification of CD34 mAb samples. Spectramax M5 was used at 280 nm to measure absorbance of TST-CD34 mAb and TST-SEAP.

Instrumentation

Hamilton Microlab® STAR™ with a CO-RE 96-channel multi-probe head was used for extraction and purification experiments. SpectraMax M5 was used to quantify absorbance (SEAP and CD34 mAb) and fluorescence (mCherry excitation at 589 λ at Emission at 610 λ).

Binding Kinetics

Varying amounts of protein diluted in 1X PBS were loaded in triplicate onto a 300 µL 96-well plate. The starting amounts for each TST-tagged protein are as follows: mCherry at 12.8 µg, 25.0 µg, 50.2 µg, 57.6 µg, 82.6 µg, 114 µg, 128 µg. SEAP at 42 µg, 73 µg, 119 µg, 169 µg, 225 µg, 265 µg, 316 µg. CD34 mAb at 27.4 µg, 58.7 µg, 109 µg, 140 µg, 179 µg, 231 µg, 262 µg, 333 µg. The absorbance or fluorescence of samples was measured preceding experimentation and after binding cycles (ranging from 1-30) in SpectraMax M5. IMCStips® with 10 µL of STXT4FHC resin were used to test SEAP and CD34 mAb samples, and IMCStips® with 5 µL of STXT4FHC resin were used to test mCherry samples. All IMCStips® were equilibrated in 1X PBS for 5 cycles prior to protein extraction. Each well contained 250 µL of solution with 225 µL of solution aspirated and dispensed per cycle.

Full Method Purification

Full method purification was performed for several concentrations of TST-mCherry and TST-CD34 mAb, with samples of known concentrations spiked into bacterial cell lysate or CHO-S cell supernatant, respectively. Extraction parameters resembled those performed for binding kinetic experiments, using 10 µL of STXT4FHC resin in IMCStips® on a Hamilton® Star®, 225 µL sample aspiration and dispense volumes, and 30 total binding cycles. The method was adjusted to include two wash steps and two elution steps. The wash steps were performed in 250 µL of 1X Strep-Tactin®XT Wash Buffer, while the elution steps were performed in 60 µL of 1X Strep-Tactin®XT BXT. Each eluate was loaded onto a 4-20% Mini-PROTEAN®TGX™ protein gel (Bio-Rad, Cat# 4561096) to garner 3 µg of protein per lane.

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