Proteomic Applications of Specialty High Capacity Streptavidin-Coated Multiwell Plates

By John Dapron, Jodi Zobrist, Kelly Foster, Layle Barbacci, Tom Hassell and William Kappel
Sigma-Aldrich Biotechnology, St. Louis, MO

Introduction

The binding of biotin to avidin (or streptavidin) is the highest known affinity of a ligand for a protein ($K_d = 10^{-15}$). This attribute, along with the relative ease by which many molecules may be specifically biotinylated, make it uniquely suited for a variety of detection and purification methods.[1,2] In recent years, variations of the system have been extensively employed in areas of proteomics.

This discussion will focus on two proteomics applications utilizing a specialty high capacity (HC) streptavidin-coated 96-well plate to capture biotinylated molecules for subsequent analysis. By comparison, most commercially available streptavidin-coated plates do not possess the binding capacity required for analysis by typical proteomics methods. In the first example, using the unique HC plate, biotinylated peptides from a tryptic digest of proteins are captured and released for analysis by mass spectrometry (MS). The second method demonstrates how a captured biotinylated molecule may be used to study interactions with a binding partner.

Capture and Release of Biotinylated Peptides

In proteomics, a major goal is simplification of complex protein mixtures to allow identification and quantitation of individual proteins. This may be accomplished by targeting specific protein sites for modification, thereby, allowing for capture and subsequent identification of signature peptides by peptide-mass fingerprinting.[3] One method gaining prominence involves biotinylation of cysteine residues in proteins, followed by trypsin digestion. Commonly, the tryptic digest is fractionated by ion-exchange HPLC followed by affinity purification with an avidin/streptavidin matrix. There is a potential ten-fold simplification with greater than 90 % total coverage by this method.[4] Analogous methods biotinylate at serine/threonine phosphorylation sites via controlled β-elimination and nucleophilic insertion, thereby allowing for analysis of phosphopeptides.[5] To expand the analytical capabilities the biotin linker may incorporate stable isotopes to provide differentiation of sample populations. This variation is termed isotope coded affinity tags (ICAT) and has been pioneered by Aebersold, et al.[6]

The systems previously mentioned generally necessitate capture and release of the biotinylated molecule for subsequent MS analysis. Common methods utilize a modified biotin or monomeric avidin matrix, which demonstrate reduced affinity. In other instances, biotinylation reagents may be used which incorporate a cleavable spacer. Finally, relatively good recoveries may be attained in systems using standard streptavidin matrices and unmodified biotin by selecting optimized elution conditions with sufficiently stringent reagents.

Capture of a Biotinylated Protein for Preparation of New Affinity Surface

In addition to "capture and release" methods, a streptavidin matrix may be considered a base for a universal affinity platform. Following biotinylation, most molecules of interest can be conveniently and firmly attached to a streptavidin support. For example, immobilized primary antibodies have been utilized in a variety of "pull-down" experiments for the capture of target proteins and their associated complexes.[7] Alternatively, immobilization of biotinylated target proteins (baits) can be used to capture binding partners/complexes.[8] A streptavidin surface would, therefore, be a useful tool in constructing a protein array to monitor various binding interactions within a system.

The unique potential of the specialty high capacity streptavidin-coated plate will be illustrated in both major types of applications using simple model systems. To demonstrate capture and release, a protein was biotinylated at cysteine residues and digested with trypsin. The biotinylated peptides were captured, released, and then analyzed by matrix-assisted laser desorption ionization (MALDI) time-of-flight (TOF) MS. In the second example, the potential to prepare high-density affinity surfaces is shown. Capture of biotinylated mouse antibody at saturating levels followed by binding of an antibody to mouse IgG is demonstrated. The bound antibodies were then quantitated by a simple protein assay to demonstrate protein-protein complex formation.

Materials and Methods

All materials were supplied by Sigma-Aldrich Corporation (St. Louis, MO) unless otherwise stated.

Biotin Binding Capacity

To a SigmaScreen™ Streptavidin HC coated 96-well clear plate (Product Code S 6940), 1000 pmol/well of biotin-fluorescein (Product Code B 8889) in 200 µl of 0.05 M Tris-buffered saline, pH 8.0 (TBS, Product Code T 6664) was added to triplicate wells. This represents a 2-fold excess over the expected binding capacity of the plates. For comparison, commercially available high capacity streptavidin-coated plates were obtained from leading competitors along with representative regular binding capacity or high sensitivity (HS) plates. These comparison plates were loaded similarly with a 2-fold excess of biotin-fluorescein relative to the manufacturer claims. To show specificity, triplicate wells were incubated with 100,000 pmol/well of free biotin in 200 µl of TBS in addition to the 2-fold excess of biotin-fluorescein. After a one-hour incubation, 100 µl of the supernatant was transferred into a black non-binding surface 96-well plate. Fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 535 nm using a Wallac Victor plate fluorometer (Perkin Elmer, Boston, MA). The fluorescence of these wells was compared to that of 100 µl of the biotin-FITC loading solution measured at the same time. The total biotin-fluorescein captured per well was based on the difference in fluorescence units between the two samples.

Capture and Release Application

Lysozyme (Product Code L 6876) was dissolved at 2 mg/ml in 6 M guanidine-HCl, pH 7.5. The sample was allowed to denature for 30 minutes at room temperature, then was reduced with 5 mM tributyl phosphine (Product Code T 7567) by incubation for one hour at 37 °C. A 4 molar excess (to cysteine) of biotin polyethylene oxide (PEO) iodoacetamide (Product Code B 2059) was added for labeling from a 20 mg/ml fresh stock in dimethyl sulfoxide (DMSO) and allowed to react one hour at 37 °C (protected from light). Excess reagents were removed by dialysis against 50 mM ammonium bicarbonate, pH 8.0. Proteomics grade trypsin (Product Code T 6567) was added at a 1:50 ratio (w/w) of trypsin:lysozyme and incubated overnight at 37 °C. The resulting protein digest was lyophilized. The biotinylated lysozyme peptides were dissolved at 2 nmol/ml in 150 mM ammonium chloride with 10 mM ammonium phosphate, pH 7.0. Two hundred microliters of this solution was added to the wells of a SigmaScreen Streptavidin HC coated 96-well plate. After timed incubations at room temperature, the wells were washed three times with 0.25 ml/well of 50 mM ammonium phosphate, pH 7.0, followed by three washes with 0.25 ml/well of water. The wells were eluted by a 30-minute incubation at room temperature with 0.2 ml/well of 70 % acetonitrile (ACN) with 5 % formic acid and 1 mM biotin. The eluted samples were then mixed 1:1 with the MALDI matrix (10 mg/ml α-cyano-4-hydroxycinnamic acid [ACCA] in 70 % ACN with 0.1 % formic acid). In some cases, a solution containing ACCA with 70 % ACN, 2.5 % formic acid and 1 mM biotin, was used directly as the eluant. The samples were then applied onto a 96-well MALDI target (Shimadzu Biotech, UK) and analyzed using a Axima-CFR mass spectrometer (Shimadzu Biotech, UK).

Protein-Protein Interaction Study

To six wells of a SigmaScreen Streptavidin HC coated plate or two competitors' HC plates was added 200 µl/well of a 0.25 mg/ml solution of ANTI-FLAG® biotinylated M2 antibody (Product Code F 9291) in 0.01 M phosphate buffered saline, pH 7.4, containing 0.05 % Tween® 20 (PBST, Product Code P 3563, ICI Americas, Inc.). This solution was allowed to incubate at room temperature for 4 hours. The plate was then washed three times with 300 µl/well of PBST. Then 200 µl of a 0.25 mg/ml solution of Goat Anti-Mouse IgG (Product Code M 8642) in PBST was added to three of the washed wells. This was allowed to incubate overnight at 2-8 °C. The plate was then washed three times with 300 µl/well of PBST and three times with 300 µl/well of water. After aspirating the final wash, the total protein captured per well was determined directly in the wells using a bicinchoninic acid (BCA) kit for protein determination (Product Code BCA-1). The values obtained in triplicate wells were averaged.

Results and Discussion

The SigmaScreen Streptavidin HC coated 96-well plates were developed to address a need for a multi-sample affinity capture platform, based on the versatile streptavidin/biotin system, with sufficient capacity to provide for subsequent analysis by a variety of methods. The wells of the plates were coated with streptavidin by a proprietary process (patent pending) resulting in an exceptionally high coating density with subsequent high binding capacity. The biotin binding capacity is greater than 400 pmol biotin/well, which is significantly greater than any other commercially available plate (Figure 1). This capacity provides for ample MS signal using typical elution conditions. In contrast, the most common type of commercial streptavidin plates typically have relatively low biotin binding capacity (≤ 25 pmol/well) and are suitable for highly sensitive screening of analytes using detection by procedures which require signal amplification such as enzyme immunoassay (EIA).

Table 1. Peptide profile of trypsin digested lysozyme-biotin. *Theoretical peptide fragments from trypsin digestion of lysozyme. The calculated mass values include the mass of the biotinylation reagent for the cysteine containing residues. The biotinylated peptides are designated in red.*

Designation	From-To	Sequence	Mass (M+H)+
T_{18}	129-129	L	132.1809
T_{1}	1-1	K	147.1956
T_{12}	97-97	K	147.1956
T_{4}	14-14	R	175.2091
T_{14}	113-114	NR	289.3118
T_{2}	2-5	VFGR	478.5654
T_{10}	69-73	TPGSR	517.5569
T_{15}	115-116	CK	663.8523
T_{17}	126-128	GCR	748.9172
T_{5}	15-21	HGLDNYR	874.9210
T_{16}	117-125	GTDVQAWIR	1046-1578
T_{3}	6-13	CELAAAMK	1250.5550
T_{9}	62-68	WWCNDGR	1350.5273
T_{7}	34-45	FESNFNTQATNR	1429.4715
T_{13}	98-112	IVSDGNGMNAWVAWR	1676.8748
T_{6}	22-33	GYSLGNWVCAAK	1682.9631
T_{8}	46-61	NTDGSTDYGILQINSR	1754.8303
T_{11}	74-96	NLCNIPCSALLSSDITASVNCAK	3579.2433

To assess the capabilities of the SigmaScreen Streptavidin HC coated plate for capture and release protocols, lysozyme was biotinylated specifically at reduced cysteine residues then digested with proteomics grade trypsin. The expected peptide profile is presented in Table 1. The peptide mixture was incubated in a streptavidin-coated plate for timed intervals to monitor kinetics of binding. As depicted in Figure 2, greater than 70 % of binding occurs in the first 30 minutes, and greater than 90 % saturation is attained within 2 hours. The amount of biotinylated peptide loaded into the wells of the HC streptavidin-coated plate was 400 pmol/well. Therefore, the capacity for biotinylated tryptic peptides is close to the total biotin binding capacity as determined previously. (Figure 1).

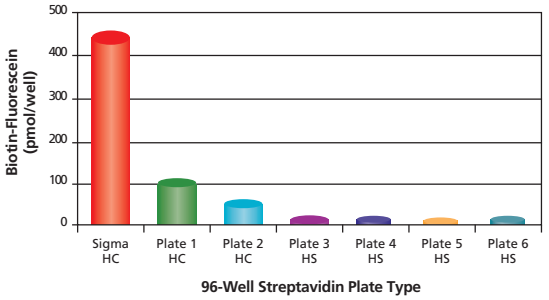

Figure 1. Binding capacity based on biotin-fluorescein. *SigmaScreen Streptavidin HC coated plate binding capacity comparison with commercially available HC and high sensitivity (HS) streptavidin-coated plates.*

Elution of the biotinylated peptides is illustrated in Figure 3. It was determined that optimal recovery of biotinylated compounds was significantly enhanced by inclusion of 1 mM biotin in the elution solution. A solution of 70 % acetonitrile with 5 % formic acid and 1 mM biotin was found to be an efficient elution mix. In addition, it was determined that the ACCA MALDI matrix solution supplemented with 1 mM biotin was similarly efficient in recovery and allowed direct MALDI analysis. The elution mix without ACCA was utilized when the matrix would interfere with subsequent analysis such as by LC-electrospray ionization (ESI) MS. The Streptavidin HC coated plate demonstrated excellent specificity for capture of the biotinylated peptides. Each theoretical biotinylated peptide was readily detected and clearly identified in the eluant by MALDI MS analysis.

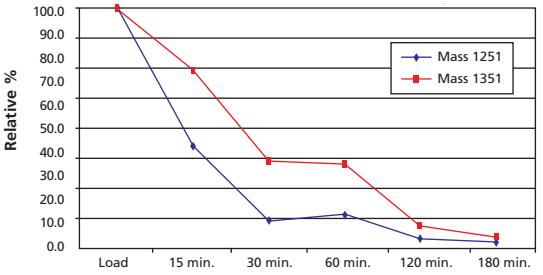

Figure 2. Binding kinetics of Streptavidin HC coated plate. *Tryptic digested lysozyme-biotin peptides were incubated in the wells of a Streptavidin HC coated plate. The supernatants were withdrawn at timed intervals, concentrated 10X and analyzed by Reverse Phase (RP) HPLC-MS. Kinetics of binding was calculated by plotting reconstructed ion chromatogram (RIC) of two representative biotinylated peptides over time relative to time 0 (zero) loading values.*

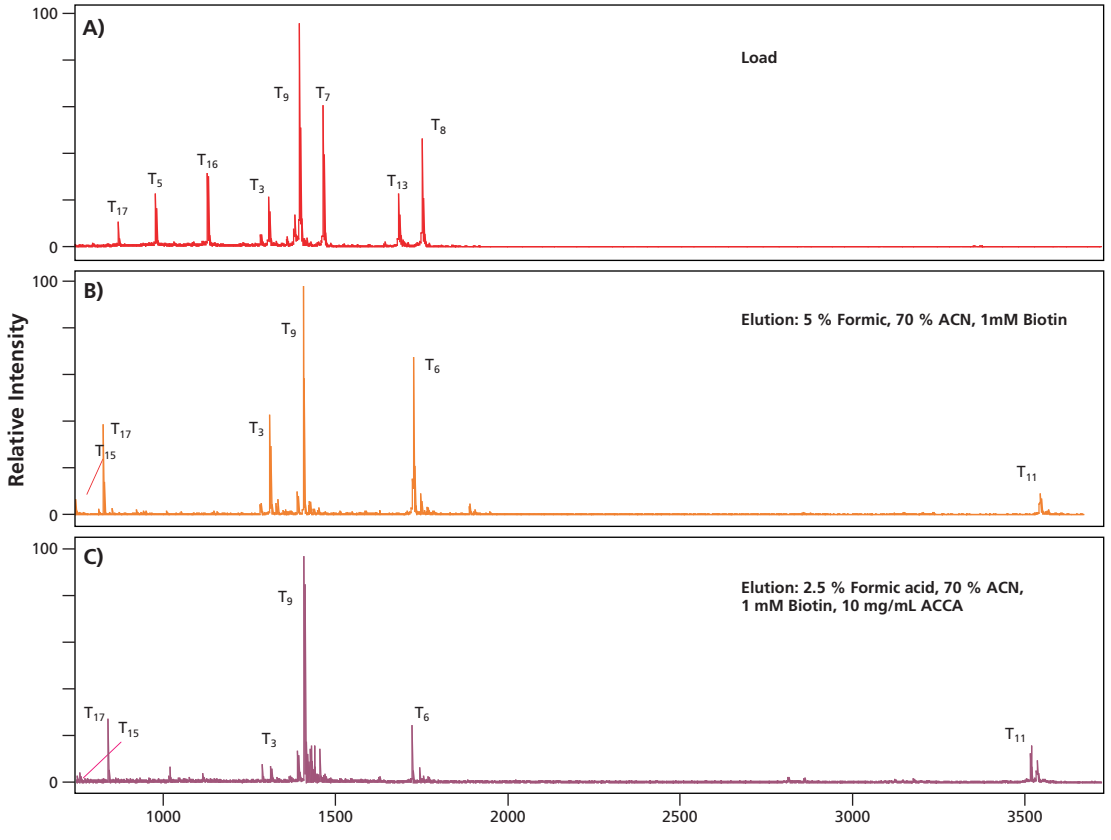

Figure 3. MALDI-MS spectra plate load and recovery. *Spectrum* ***(A)*** *shows MALDI-MS analysis of trypsin-digested biotinylated lysozyme prior to loading on Streptavidin HC coated plates. Spectra* ***(B)*** *and* ***(C)*** *show purified biotinylated peptides eluted from the plates with various reagents.*

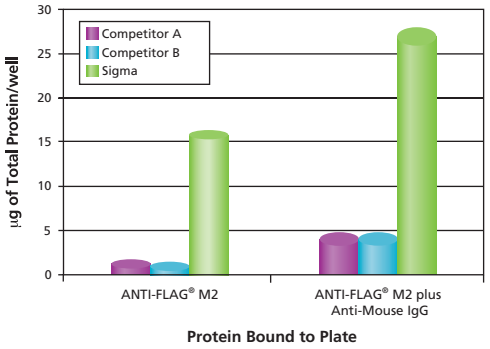

Figure 4. Protein-Protein complexes. *SigmaScreen Streptavidin HC coated 96-well plate compared to two competitor HC plates for binding of a biotinylated monoclonal mouse antibody (ANTI-FLAG M2) followed by incubation with anti-mouse antibody (ANTI-FLAG M2 plus anti-mouse IgG). The total protein bound per well was determined by BCA. The values shown are uncorrected for the streptavidin protein coated in the well.*

To demonstrate the utility of the SigmaScreen Streptavidin HC coated plates for preparation of a new affinity surface for protein-protein interactions, a biotinylated primary antibody was loaded at saturating levels, followed by similar loading of a secondary antibody. Following each protein incubation, total bound protein was quantitated directly in the wells by BCA (see Figure 4). The SigmaScreen Streptavidin HC coated plate demonstrated binding of multi-microgram quantities of both a biotinylated protein and its binding partner. In contrast, two competitors' HC plates bound nearly undetectable amounts of the biotinylated protein using this assay. The bound biotinylated antibody was determined to be at least 9 µg/well for the SigmaScreen Streptavidin HC coated plate after correcting for the contribution of the streptavidin coated on the plate. By a similar calculation, the total bound secondary antibody was 11.5 µg/well. The captured proteins represent a high-density complex that enables facile analysis by a variety of methods including SDS-PAGE, MS, and direct protein quantitation assay.

Conclusions

The unique SigmaScreen Streptavidin HC coated 96-well plate was developed to provide for multi-sample analysis of affinity captured molecules or complexes by a variety of techniques. In the field of proteomics, this is a useful tool for parallel processing of samples. Sub-populations of peptides, biotinylated at specific residues, may be captured and recovered in sufficient amounts for analysis by MS. In addition, this specialty plate may serve as a universal platform for easy preparation of new high-density affinity surfaces for the study of specific protein-protein interactions.

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About the Authors

John Dapron, B.S., Jodi Zobrist, M.S., Kelly Foster, B.S., Layle Barbacci, Ph.D. are Scientists in the Biotechnology R&D Department at Sigma-Aldrich, St. Louis, MO. Tom Hassell, M.S. and William Kappel, Ph.D. are Managers in the Biotechnology R&D Department at Sigma-Aldrich, St. Louis, MO.

ORDERING INFORMATION

Product Code	Product Description	Unit
S 6940	SigmaScreen™ Streptavidin HC coated 96-Well Plate	5 x 1 each and 1 each
S 8815	SigmaScreen™ Streptavidin HC coated 384-Well Plate	5 x 1 each and 1 each