A Novel Approach to High-Throughput Monoclonal and Recombinant Antibody Enrichment and Characterization

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Introduction

The rapidly growing field of antibody engineering continues to create the demand for technologies that increase the productivity and throughput of protein interaction analysis. Here we present a novel sample preparation technology that can rapidly purify and enrich IgG and Fab samples at micro-volume scale, where the final sample is ready for sensitive kinetic analysis using high throughput SPR.

Background

As the interest in characterizing protein interactions has increased, so has the number of high-throughput analytical technologies (microarrays, protein chips, mass spec) that offer the ability to study large numbers of samples in fine detail. However, as proteins are inherently complex molecules, elucidation of protein interactions requires that the samples for study are of sufficient purity and activity to produce reliable, high-quality data. Access to suitable expression systems for generating large collections of native and recombinant antibodies has enabled researchers to begin embarking on large-scale studies but a significant bottleneck exists for isolating and purifying proteins in scale and quantity that are required for such studies.

PhyNexus PhyTip™ Columns

PhyNexus has developed a unique, yet simple process to purify and enrich antibodies or recombinant proteins containing affinity tags. PhyTip™ columns from PhyNexus can be used in applications ranging from simple low throughput to fully automated 96-well processes. These unique devices are capable of preparing concentrations of up to 5mg/ml of purified proteins in small final volumes e.g. 10-20 ul, in less than 20 minutes.

To demonstrate the effectiveness of this new sample preparation system, antibodies (IgGs and Fabs) were processed with PhyTip™ columns. These columns are specifically designed to contain micro-volume quantities of conventional separation media in a manner that introduces virtually zero dead volume, and either Protein A, Protein G, or IMAC resin was used for processing of the samples (see Figure 1). PhyTip™ columns are available as either 200+ columns (for use with 200 µl pipettors) or 1000+ columns (for use with 1 ml pipettors).

Sample enrichment steps were automated through the use of a computer-controlled mutichannel electronic pipettor or robotic liquid handling system.

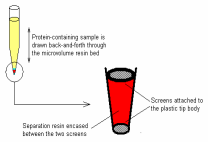

Figure 1. PhyTip™ columns are designed to encapsulate a small amount of resin within the end of a pipette tip. The dead volume if the column is kept to a minimum by suspending the resin between two screens, allowing for high enrichment at small volumes.

Table 1. PhyTip™ Rapid Enrichment Protocol

	200+ PhyTip ™	*1000+ PhyTip ™*
Capture	*2.:12*	*11:00*
Purify 1	*2:12*	*2:10*
Purify 2	*2:12*	*2:10*
Enrich	*2:24*	*2:20*
Total time	*9:00*	*17:30*

Protein A PhyTip™ Binding Capacity

The PhyTip™ columns were characterized with respect to their maximum binding capacity. A titration of monoclonal murine IgG2a in 500 µl PBS was processed, demonstrating IgG binding capacities exceeding 100 µg. It was determined at low levels of IgG (10 µg) that recoveries on the order of 70% of initial antibody is possible in final volumes of 20 µl (15 µl low pH elution buffer + 5 µl 200 mM phosphate neutralization buffer). Accurate quantitation of IgG was performed by offline reduction of the IgG sample with TCEP (17 mM TCEP, room temperature for 16 hours), followed by HPLC analysis and absorbance detection at 214 nm.

Table 2. Protein A PhyTip™ column IgG binding capacity.

Initial IgG (µg)	Breakthrough IgG (µg)	Bound IgG (µg)	% Bound
10	<2	>8	>80
50	4	46	92
100	16.5	83.5	84
150	47.5	102.5	68
200	71	129	65

Selectivity of Protein A PhyTip™ Columns

Data shown below from these experiments indicates that IgG purification using the Protein A PhyTip™ column is highly selective. A 333-fold excess of BSA can quantitatively be removed by using Protein A columns in less than 20 minutes. Similarly, the same IgG2a can be selectively purified from serum free hybridoma medium containing BSA or FBS (purity data not shown here).

Recoveries from selectivity assay (determined by HPLC method)

Sample	Recovery
15 µg IgG2a /0.5 ml PBS (2 cycles of loading)	**43%**
15 µg IgG2a /0.5 ml PBS+5 mg BSA (2 cycles of loading)	**56%**
15 µg IgG2a /0.5 ml PBS+5 mg BSA (5 cycles of loading)	**62 %**

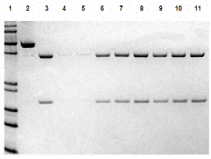

Figure 2. SDS-PAGE image of PhyTip selectivity. Lane 1: marker, 2: 2 µg BSA, 3: 2 µg IgG2a, 4, 5: Protein A resin only, respectively; 6, 7, 8: 2 µl each of Protein A purified IgG2a from PBS, PBS containing 5 mg BSA (2 and 5 cycles capture), respectively; 9, 10, 11: 2 µl each of Protein A purified IgG2a from PBS, PBS containing 5 mg BSA (2 and 5 cycles capture), respectively.

Reproducibility of IgG Recovery

In order to verify the role of carrier protein for maximum IgG_{2a} recovery, and to demonstrate reproducibility, IgG_{2a} was purified from PBS (and also PBS containing 5 mg BSA) from multiple samples (n=4). Results suggest increased recovery (at least 20% or more) of purified IgG in the presence of 500-fold excess of BSA concentration and that the procedure is also highly reproducible.

Recovery in PBS = 42.25% (SD = 4.66%)
Recovery in PBS + 5 mg BSA = 66.5% (SD = 7.5%)

PhyTip Enrichment of Recombinant Fab Antibodies from E. coli Periplasmic Extracts

Recombinant anti-FITC Fabs from a human antibody scaffold library were selected by phage display. Positive samples were subsequently sub-cloned in an bacterial expression vector containing an N-terminal 6X-His tag.

E. coli culture pellets were obtained by centrifugation following overnight induction with IPTG. Periplasmic extracts were prepared and the resulting material was enriched using IMAC PhyTip™ columns.

Figure 3 shows the SDS-PAGE analysis of a periplasmic extract before and after enrichment with IMAC PhyTip™ columns. 200 µl samples were processed using an eight-channel electronic pipettor and enriched with 250 mM imidazole to elute the protein from the IMAC column.

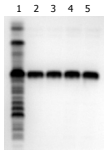

Figure 3. SDS-PAGE image of His-tagged Fabs in periplasmic extracts prior to enrichment (lane 1) and replicate Fabs after enrichment on IMAC PhyTip™ (lanes 2-5).

96-well robotics

Periplasmic extracts were processed using 200 µl PhyTip™ columns on a MiniTrak™ 96-channel liquid handling system (Perkin-Elmer, Inc.). The robotic platform provides simultaneous enrichment of up to 96 samples in less than 20 minutes.

Figure 4. Parallel enrichment of 96 samples using PhyTip™ technology in combination with integrated robotic workstations.

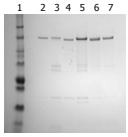

Figure 5. SDS-PAGE image of His-tagged Fabs enriched from periplasmic extracts using a 96-channel robot. Lane 1: MW marker; 2-7: Fab clones A-F after enrichment.

Application of PhyTip™ Columns to High-Throughput Surface Plasmon Resonance (SPR)

The Applied Biosystems 8500 Affinity Chip Analyzer (Applied Biosystems, Inc.) utilizes SpotMatrix SPR technology in a highly flexible detection platform for parallel label-free kinetic analysis of biomolecular interactions. The technology is based on the phenomenon of grating-coupled surface plasmon resonance. The Affinity Chip sensor consists of a plastic chip containing an optical grating coated with a thin (~80 nm) layer of gold onto which up to 400 interactions can be monitored simultaneously in real-time.

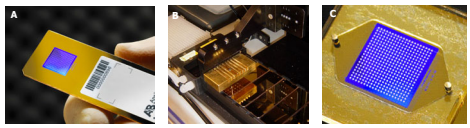

Figure 6. 8500 Affinity Chip containing 400 spots of protein depositing using conventional contact spotting robotic technology **(A,B)**. Each spot is in the range of 150-250 microns in diameter, created by dispensing nanoliter volumes of protein onto the surface of the chip. The photograph shows the fully assembled chip and flow cell **(C)**.

Demonstration of enrichment while maintaining IgG function

A murine monoclonal anti-FITC antibody was added to DMEM + 10% FBS in concentrations ranging from 1 to 5 µg/ml. Aliquots of each sample (200 µl) were processed using 200+ Protein G PhyTip™ columns and eluted to a final volume of 10 µl (8 µl elution volume + 2 µl neutralization buffer). Approximately 1 nl of each buffer-neutralized sample was spotted onto an 8500 Affinity Chip coated with goat anti-mouse IgG-Fc. Nanoliter deposition was performed with the OmniGrid Micro™ (Genomic Solutions, Inc.).

Unprocessed samples were spotted along with purified mAbs. FITC-labeled BSA (100 nM) was introduced under flow and the resulting real-time SPR signals were collected for the processed and unprocessed samples for each clone (Figure 7).

Fab Enrichment for SpotMatrix SPR Analysis

Recombinant anti-FITC Fab was enriched from initial concentrations of 1 µg/ml and 5 µg/ml in 200 µl PBST with 1 mg/ml BSA. Selective enrichment was performed using 200+ Protein A PhyTip™ columns. Unprocessed samples were spotted along with the enriched Fabs onto an 8500 Gold Affinity Chip for SPR analysis. 100nM FITC-labeled NeutrAvidin™ (Pierce Chemical) was introduced under flow and the resulting real-time SPR signals were collected for the processed and unprocessed samples (Figure 7).

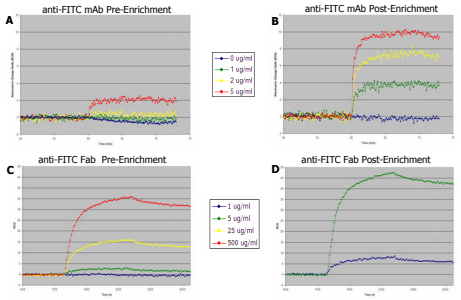

Figure 7. Antibody characterization via SpotMatrix SPR. The top two panels show the affinity traces resulting from the binding of FITC-labeled BSA to immobilized monoclonal anti-FITC spotted at varying initial concentrations. Panel **A** is the response from samples prior to enrichment on Protein G PhyTip™. The enriched samples are shown in **B**. The lower panels show the affinity traces resulting from the binding of FITC-labeled NeutrAvidin™ to immobilized anti-FITC Fab before (**C**) and after (**D**) Protein A PhyTip™ enrichment prior to immobilization. Results demonstrate that PhyTip enrichment enables high-throughout SPR analysis of protein interaction kinetics from starting volumes and expression levels consistent with hybridoma and recombinant antibody screening.

Conclusions

PhyTip™ columns are highly effective, simple tools that can be used to purify and enrich antibody preparations at a micro volume scale with unprecedented speed and the resulting protein, eluted from the process, has been shown to retain functional activity as measured by SPR. With the exponential improvements in enrichment capability and simple adoption to variable formats, i.e., adaptable to various fluid handling formats, application of this technology to the process of antibody purification allows for truly high-throughput capabilities for antibody affinity screening. Coupled with the need for smaller volumes of starting material and the value of obtaining kinetic information earlier in the screening process, this will lead to overall reductions in screening costs.

References

S. Wiltshire, *et al*, "High-Throughput Sample Processing and Affinity Characterization of Antibodies and Recombinant Proteins", Presentation 284 in *Protein Chips; New Techniques in Protein Detection*, 17th Symposium of the Protein Society, 26-30 July 2003, Boston MA