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High-Throughput Sample Processing and Affinity Characterization of Antibodies and Recombinant Proteins

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Abstract

The rapidly evolving field of proteomics continues to create the demand for technologies that increase productivity and throughput of protein interaction analysis. We present the use of novel sample purification technology for preparation of proteins from complex samples and subsequent high-throughput kinetic analysis.

Introduction

The Applied Biosystems 8500 Affinity Chip Analyzer, developed in collaboration with HTS Biosystems, is a highly flexible detection platform for parallel kinetic analysis 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 biomolecular interactions can be monitored simultaneously in real-time without the requirement for a label.

When the sensor is illuminated by light of the appropriate wavelength, polarization, and angle of incidence, energy from the light couples into the electrons of the metal creating a resonance condition manifested by a large fall in the reflectance of the incident beam **(Figure 1A, 1B)**. The angle of minimum reflectance (the SPR angle) changes as material is adsorbed at the gold surface. The condition under which this resonant coupling occurs is dependent on the optical properties of the region very close to the sensor surface. Therefore, a change in the index of refraction at the surface of the sensor (due for example to protein binding) may be monitored as a shift in the resonance angle **(Figure 1C)**. Binding events at the surface of the gold-coated optical grating cause a shift in the SPR angle allowing for simultaneous real-time measurement of up to 400 binding events on the surface of the chip.

The grating-coupled SPR imaging technology is well suited for kinetic analysis of a wide range of biomolecular interactions of interest in the field of proteomics (antibody-antigen, protein-protein interactions, epitope mapping, etc.).

High-Throughput Kinetic Analysis

Up to 400 unique targets can be immobilized on the Affinity Chip. Target molecules are spotted onto the chips using a robotic spotter **(Figure 2)**. 5-20 microliters of each target is required for spotting from a 96 or 384-well source plate.

The significant increase in biomolecular interaction analysis throughput creates the need for novel sample purification and enrichment methods that allow for screening and characterization of proteins from complex sources such as cell culture (hybridomas) and bacterial lysate (phage-display).

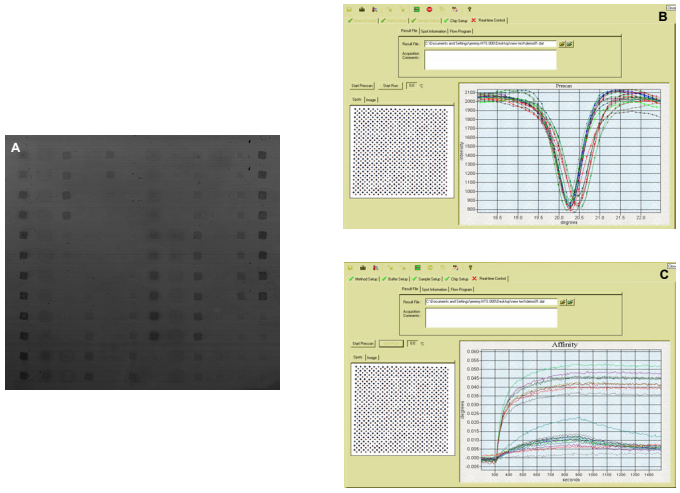

Figure 1. SPR image of 144 antibodies spotted onto a Protein A/G Affinity Chip. The intensity of the spots is proportional to the mass of protein bound to the surface of the chip **(A)**. Reflected intensity as a function of the angle of incident light. The angle of minimal reflectivity (SPR Angle) is independently measured for each spot on the chip **(B)**. The change in the position of SPR curve minimum as a function of time allows for label-free characterization of biomolecular interactions (Affinity Trace) **(C)**.

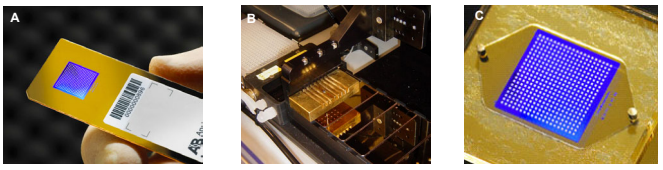

Figure 2. 8500 Affinity Chip containing 400 spots of protein depositing using conventional contact spotting robotic technology **(A,B)**. Each spot is in the range of 150-250 microns in diameter, created by dispensing nanoliter volumes of protein onto the surface of the chip. The photograph shows the fully assembled chip and flow cell **(C)**. The Affinity Chips are designed as single-use, disposable parts allowing affinity characterization of hundreds to thousands of interactions per day.

Sample Preparation

To address the sample processing bottlenecks created within ultra-high throughput affinity characterization, PhyNexus, Inc. (www.phynexus.com) has developed technologies and products for processing small starting samples for the enrichment and purification of target proteins such as antibodies.

PhyTip™ Columns
The PhyTip™ products are designed for highly efficient application of micro-volume quantities of traditional separation media, such as agarose resins **(Figure 3)**. By maintaining the separation bed in such a manner that introduces virtually no "dead" volumes, separations can be performed at micro-scales in a highly efficient and robust manner. This approach allows for high enrichment factors (up to 20-fold) from moderately sized sample volumes (0.2-1 mL) that may require higher protein capacities (10s to 100s of micrograms).

MicroExtractor 100
Processing samples with the PhyTip™ and products is easily automated with a single instrument, thus providing a universal hardware interface. Software control of aspiration, wash and elution steps provides rapid and reproducible results.

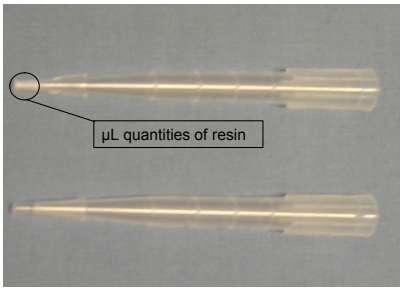

Figure 3. PhyTip™ columns are designed to encapsulate a small amount of resin within the end of a pipette tip. The dead volume if the column is kept to a minimum by suspending the resin between two screens, allowing for high enrichment at small volumes.

Antibody Characterization from Hybridoma Media

Enrichment of MAbs

PhyTips containing ProteinG resin were employed to demonstrate the integration of rapid sample preparation and affinity characterization of monoclonal antibodies from hybridoma growth media.

Two mouse monoclonal anti-FITC antibodies were added to growth media in concentrations ranging from 2 to 20 ug/ml. 500 ul aliquots of each sample were processed using the extraction tips to a final volume of 20 ul. Buffer neutralized samples were spotted onto a Protein A/G Affinity Chip containing a coating of rabbit anti-mouse IgG-Fc. Unprocessed samples were spotted along with purified antibody in PBS at 250 ug/ml.

100 nM FITC-labeled BSA was introduced under flow and the resulting Affinity Trace curves for the enriched and non-enriched samples for each clone are shown in **Figure 5**.

Kinetic Analysis

Monoclonal anti-leptin was added to hybridoma growth media to final concentrations ranging from 2 ug/ml to 20 ug/ml. The mouse antibody was purified and spotted as described above. 100 nM leptin protein was introduced to the chip and the kinetics of the interactions were calculated from the resulting Affinity Trace curves using the 8500 Affinity Chip Analyzer Data Analysis Software **(Figure 6 and Table 1)**.

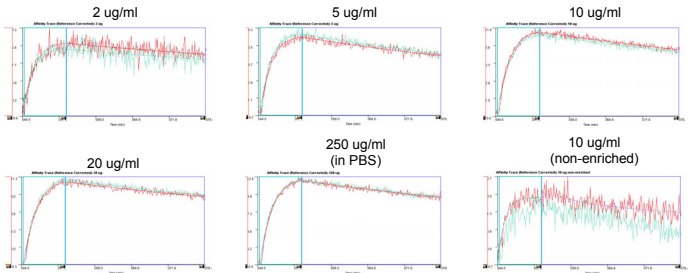

Figure 6. Affinity Trace and fit results for leptin/anti-leptin interaction. The initial concentration of monoclonal anti-leptin in hybridoma media is listed at the top of each trace.

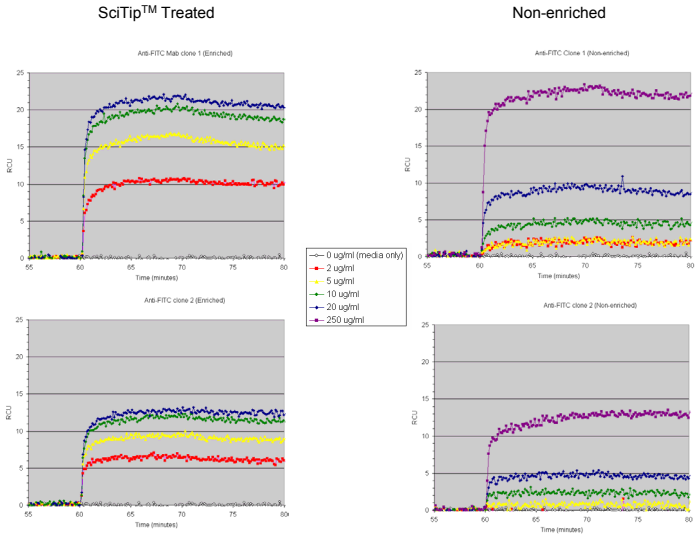

Figure 5. Affinity Trace of FITC-labeled BSA binding to two immobilized monoclonal anti-FITC antibodies in hybridoma growth media. Panel A is the response from enriched samples of clone 1. The non-enriched samples are shown in B. Results from clone 2 are shown in panels C and D (enriched and non-enriched, respectively).

Starting MAb Concentration (ug/ml)	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})
2	7.33×10^4	1.73×10^{-4}
5	7.02×10^4	1.9×10^{-4}
10	6.51×10^4	1.81×10^{-4}
20	6.91×10^4	1.74×10^{-4}
250 (in PBS)	6.85×10^4	1.62×10^{-4}
10 (non-enriched)	not detected	not detected

Table 1. Kinetics of leptin binding to immobilized anti-leptin with and without sample processing using Protein G PhyTip™. The results demonstrate the ability to accurately measure rate constants for antibody-antigen interactions with the 8500 Affinity Chip Analyzer after enrichment from hybridoma media with expression levels in the low ug/ml range.

Summary

We have demonstrated the use of two novel technologies for high-throughput sample purification and interaction analysis. The complementary technologies provide the capability and capacity to perform detailed kinetic analysis on purified and enriched materials in the range of hundreds to thousands of analyses per day.