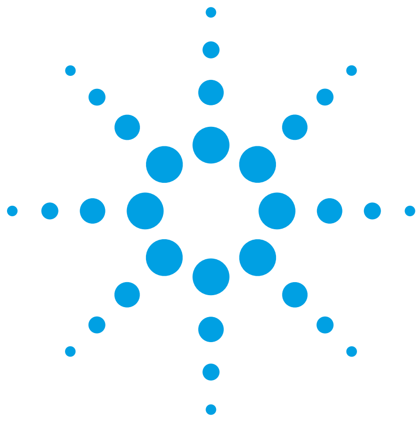

An Introduction to High Throughput Microchromatography

White Paper

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Introduction

The growing importance of biotherapeutics to the pharmaceutical industry has created an increasing demand for protein analysis techniques with high precision, high sensitivity, and high throughput. These techniques are used throughout the development process, starting with the discovery phase and continuing into lead optimization and characterization, preclinical and clinical trials (for both the biotherapeutic itself and related protein biomarkers), and manufacturing process development and control. Complex samples to be analyzed range from research samples from protein expression systems to biological samples such as serum to production cell lysates and cell culture supernatants.

There are two general objectives for protein analysis. One is to quantify the amount of a specific protein present in the sample accurately and precisely. Several techniques, including HPLC and immunoassays, are widely used for specific protein quantitation. All of these methods involve using either physical or, more commonly, affinity methods to selectively separate the target protein from the complex sample, followed by a detection method usually based on optical absorbance, fluorescence or chemiluminescence.

The other objective of protein analysis is to determine the features of a target protein, such as its overall structure, specific structural features, and activity. A wide range of analytical techniques, such as LC/MS and NMR, are used for structural analyses of proteins to examine characteristics such as the peptide map or post-translational modification. In all cases, an intact target protein must first be selectively purified from a complex sample matrix. Many structural analysis methods also involve other complex preparation methods (such as enzymatic treatment or labeling) prior to instrumental analysis.

Technical Requirements

Over the last 50 years, liquid chromatography has proven to be a highly robust and versatile general technique for protein purification from complex samples, for both analytical and preparative applications. Two general chromatographic approaches are used: physical modes based primarily on molecular size, charge, and hydrophobicity, and affinity modes based on selective binding interactions between the target protein antibodies or other ligands immobilized on resins. When used singly or in combination, these approaches are capable of separating virtually any protein from any sample matrix with good yield. A well-characterized, related technique using immobilized enzymes has also been developed to operate in a packed bed format to drive specific chemical reactions on substrates including proteins.

A significant challenge has been to adapt liquid chromatography to the requirements of high throughput analysis. To accomplish this, a number of critical technical requirements for scale, throughput, quantitative binding and elution, controlled flow rate, air entrainment, and microplate format must be met.

Scale

Analytical samples in biopharmaceutical development are often found in low abundance in a complex matrix, are quite precious, and may be needed for several different purposes. Typical sample volumes are as low as tens to hundreds of microliters, with target proteins in amounts ranging from femtograms to micrograms. Typical protein chromatography systems are designed for samples that are orders of magnitude larger, and while HPLC systems can accommodate these sample ranges, they are not usually designed to enable recovery of the purified target protein.

Throughput

The throughput requirements for protein analysis vary widely, depending upon the application. However, a common theme across the biopharmaceutical industry is that sample capacity requirements continue to increase, driving a need to consider new technical approaches. For many applications, batches of hundreds to thousands of samples at a time are common.

Traditional liquid chromatography is relatively low throughput, as systems are designed to run one sample at a time in serial fashion. Even with modern UHPLC or enhanced mass transport media, runs require at least several minutes between sample injections. More widely available preparative media or enzyme reactions can require much longer run times. To reach the throughputs required for many applications, significant parallel processing is clearly required.

Quantitative binding and elution

If chromatography is the method used to quantitate the target protein across a range of sample concentrations and matrices, it is critical that both the binding and elution of the analytical target are nearly complete *and* highly reproducible. Even if the application involves only qualitative structural analysis, quantitative binding and elution can still be highly desirable to prevent biasing the results between variant forms of the protein. In many cases, precious sample can be conserved if both quantitation and qualitative structural analysis can be accomplished at the same time.

Two distinct technical requirements must be met to obtain quantitative recovery of the target protein. One requirement is to obtain complete binding, which requires sufficient exposure time of the sample to the resin to 1) allow the target protein to reach the binding surface (mass transport) and 2) interact with the selective binding groups on the surface (binding kinetics). The former is often limiting due to the slow diffusion rates of large protein molecules, but the latter can also be slow, particularly in certain antibody/antigen interactions.

The other requirement is to obtain efficient washing away of non-binding impurities and complete elution of the target. These steps require sufficient time for mass transport, although the need is usually much less stringent than for quantitative binding. It is essential to completely separate the “free” washed or eluted molecules from the resin. This is very difficult to do quantitatively when the resin is used in a batch or equilibrium adsorption mode, where a volume of the wash or elution buffer is allowed to come to equilibrium with a volume of resin. Multiple steps are always required to reach equilibrium, resulting in a relatively large dilution factor. If a packed bed is used in flow-through chromatography mode, quantitative washing and elution can be accomplished instead in a single step with as little as five bed volumes.

Control of flow rate

One key to obtaining quantitative binding and elution is to precisely control the flow rate through the chromatography column. Dynamic binding capacity (the threshold where significant amounts of target flow through the resin bed during loading) is determined by a combination of the media characteristics and the residence time (bed volume divided by flow rate). Reproducibly driving enzyme reactions to completion also requires precise control of residence time and therefore flow rate.

When the scale and throughput requirements for protein analysis are taken into account, obtaining the necessary flow rate control can be a major challenge. For example, with agarose resins frequently used for protein purification, approximately 5-minute residence times are required to produce good dynamic binding characteristics. With a 5 µL packed bed of resin, this translates to a flow rate of only 1 µL/min. Even with modern “high speed” resins, residence times of 0.5 to 1 minute are needed, necessitating flow rates of 5 to 10 µL/min. Many microbore HPLC systems can easily achieve these flow rates with very precise control, but can run only one sample at a time. Achieving these controlled flow rates across an entire 96-well plate at once is a major challenge.

Air entrainment

Trapping of air bubbles in a chromatography bed is a problem for conventional methods, but the effects are greatly magnified as the scale of operation is reduced. With a 5 µL packed bed, a seemingly miniscule 1 µL air bubble can cause a major loss of capacity or recovery. It is critical that the system can minimize air entrainment in routine operation and allow management of it reliably if it occurs.

Format

The SBS/ANSI microplate format has become the standard for handling protein analysis samples, and most users have a substantial investment in liquid handling, automation, and detection systems designed to handle microplates. A high throughput microchromatography system should be fully compatible with this standard. However, because of the wide variation in the number of samples to be processed in different applications, systems with 96 columns fixed in a single unit are less desirable.

Previous Approaches

A number of approaches have been attempted for high throughput microchromatography of proteins, but each has suffered from significant drawbacks. One approach has been to form a column with a packed resin bed in the distal end of a pipet tip using a number of methods. Pipet tip columns with a number of common protein resins are available, with bed volumes ranging upward from the 5 to 10 µL range. Air displacement is used to aspirate and dispense liquid into and out of the tip and thus through the bed, producing a highly variable and essentially uncontrolled flow rate with air entrainment issues. Because there is a single entry and exit port through the packed bed, washing and elution operate in batch mode, where multiple steps and high dilution factors are needed to reach equilibrium. Pipet tip columns are useful for qualitative extraction but have never been successful for quantitative chromatography.

Another common approach is to use packed bed cartridges with a vacuum manifold that uses air pressure to drive liquids through the bed. This method is used frequently and successfully for solid phase extraction of small molecules, and often can provide quantitative results. However, because proteins have much lower diffusion rates, the residence times required for their quantitative binding are much higher than for small molecules, making it difficult or impossible to adjust the vacuum low enough to achieve the controlled flow rates needed. In addition, vacuum manifold systems often suffer from foaming issues when high protein content samples are used.

Centrifugal force is another method used for driving liquids through small, multiple-packed beds. So-called "spin columns" are widely used for protein separations and have bed volumes in the 50 to 1000 µL range that are substantially oversized for many protein analysis applications. Smaller bed volumes create significant challenges with air entrainment and bed drying issues. There are limits to the residence times that can be achieved with spin columns and the variability of flow rates between columns makes it difficult to achieve quantitative results in some applications.

A High Throughput Microchromatography Platform

The Agilent Bravo for Protein Purification with the AssayMAP[1] high throughput microchromatography platform is designed specifically to meet all of the technical requirements for quantitative sample preparation for protein analysis. The AssayMAP system is comprised of a microchromatography cartridge, special associated labware, and a liquid handling device designed to interface with the cartridge and provide precise flow control in a 96-channel microplate format.

Cartridge

The AssayMAP cartridge is designed around a 5 µL packed bed of resin. The bed is contained between two non-woven support filters that are molded into the cartridge housing. Any resin with a particle size of roughly 20 to 100 µm can be used in the cartridge, including polymeric, agarose, or silica-based materials in all of the common chromatographic modes, as well as immobilized enzyme supports. The resin is packed into the bed under highly controlled conditions and slight compression to ensure stability in both flow directions, and each cartridge is tested for pressure drop to ensure proper packing.

The resin bed is contained within a housing that has a geometry that enables small volumes of liquid to be precisely aspirated and dispensed through the bed from a variety of common sample containers, including 96- and 384-well microplates. Immediately above the upper bed support is a small conical inlet seal that can function as a miniature Luer-type fitting to connect the bed directly to the liquid handler as described below. Above the inlet seal is a sample cup with a volume capacity of 200 µL. The inside of the sample cup incorporates some small ribs that enable the cartridge to be picked up by standard laboratory pipets but prevent formation of an airtight seal (so that liquid cannot be accidently displaced through the bed by air pressure, which can cause air entrainment).

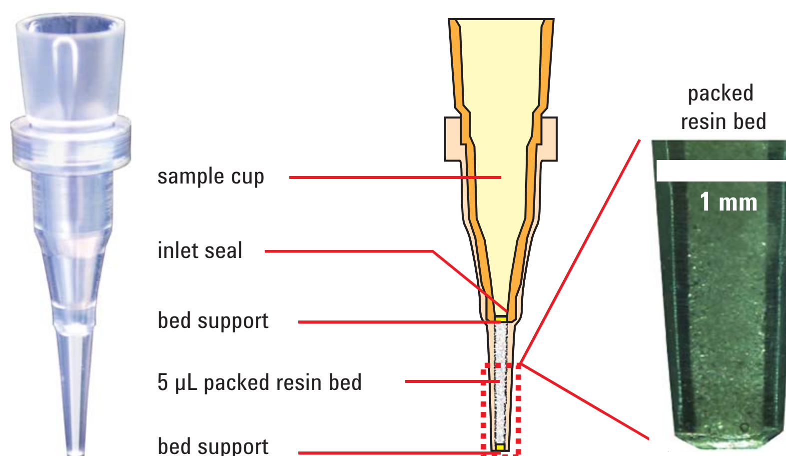


Figure 1. The AssayMAP cartridge

1. AssayMAP is a registered US trademark of BioSystem Development.

Labware

AssayMAP cartridges are supplied in a special molded rack that holds 96 units in an 8 by 12 array that can mount on virtually any standard 96-well microplate, with the cartridges held precisely centered with their outlet tips just below the entrances of the wells. The cartridges fit loosely in the rack so that the liquid handling system can pick them up easily without disturbing the rack, regardless of whether all or fewer than 96 cartridges are used.

The cartridges are also supplied with a special molded "receiver plate" that fits below the rack. The cartridges hang into the wells of the receiver plate with their tips just above the well bottom so that the packed beds are immersed in liquid contained within the wells. The receiver plate thus protects the cartridges and can keep them wet during shipping. During operation on an automated system, the receiver plate provides support to the rack to prevent flexing, ensuring reliable cartridge pickup from any position.

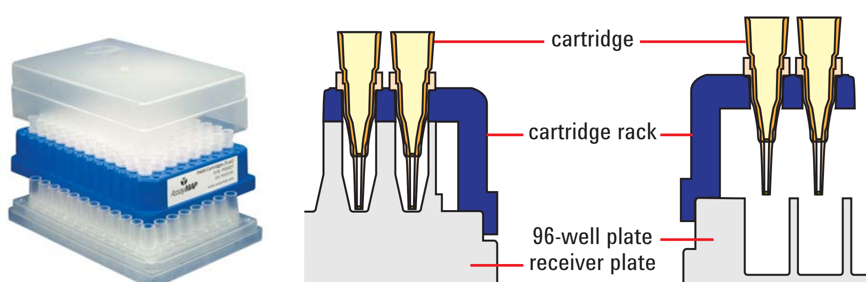


Figure 2. AssayMAP Labware

Probe syringe and liquid handler

The other key element of the AssayMAP system is a liquid handling system – the Agilent Bravo with 96 AM liquid handling head – designed to provide precise, positive-displacement control over the flow rate through the cartridges. The heart of the system is a set of syringes, each coupled directly to a liquid handling probe and containing a specially designed plunger. The tip of the probe is designed to mate with the inlet seal above the packed bed in the cartridge to form a direct liquid connection between the inside of the syringe and the packed bed. The seal taper is small enough so that even though only a kilogram or so force is required to form the seal, it can hold a liquid pressure of at least 15 bar.

The syringe plunger also has a tapered shape that tightly fits the inside of the probe. This design provides an ultra-low dead volume in the syringe, which effectively ejects air bubbles that could cause erratic flow control and enables very efficient washout between samples and reagents. With an appropriate syringe drive system, a very broad range of flow rates through the cartridge can be achieved, from well under 1 μL/min to nearly 100 μL/s.

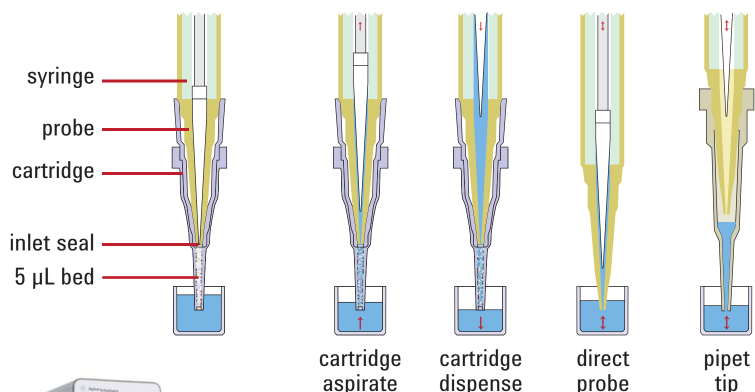


Figure 3. Probe syringe and Agilent Bravo for Protein Purification

On its own, the probe is able to precisely aspirate and dispense fluids from various containers, so that they can be pumped through or recovered from the packed bed in either upward or downward flow. It is also designed so that a standard pipet tip can be attached, enabling the same liquid handling system to handle both cartridge operation and conventional liquid handling. The probe syringes are mounted in a modified version of a standard liquid handling head, so that up to as many as 96 microplate samples can be processed in parallel.

Operating modes

With the AssayMAP cartridges mounted on the probe syringes, samples, buffers and reagents can be moved through the bed (aspirated upward or dispensed downward) at a precisely controlled flow rate. Upward aspiration has the advantage that a series of different liquids can be pumped through the bed in sequence without disconnecting the cartridges and washing out the syringe in between, which can make protocols very rapid and efficient. Downward dispensing is usually used for steps where the eluate is to be recovered into a microplate well, and can also be used to apply high pressures to the bed (for example when dealing with highly viscous or particulate-laden samples). Samples can also be aspirated and dispensed in a rapid cycle to distribute binding evenly throughout the bed, which can be useful in immobilization or coating steps.

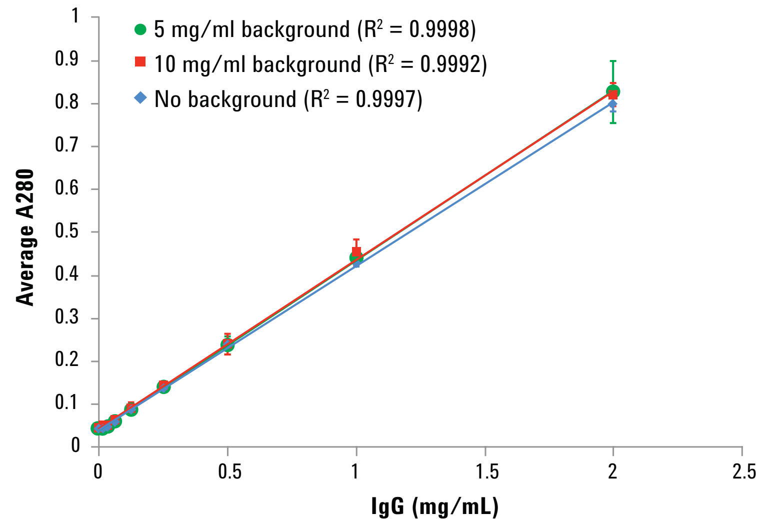
Software

The Agilent VWorks Automation Control software enables easy operation and precise control liquid handling operations for rapid deployment of AssayMAP-enabled applications. Users can make use of preconfigured forms, protocols, and macros to run default applications out-of-the-box, while being able to edit liquid handling and flow-rate parameters to optimize for their particular analyte separation method. Basic equilibration, elution, priming, and washing steps are provided as drag-and-drop macro icons, and specific 96AM tasks are editable within these macros. Flow rate is adjusted within 96AM aspirate and dispense tasks to provide the precision control necessary for true chromatographic separations.

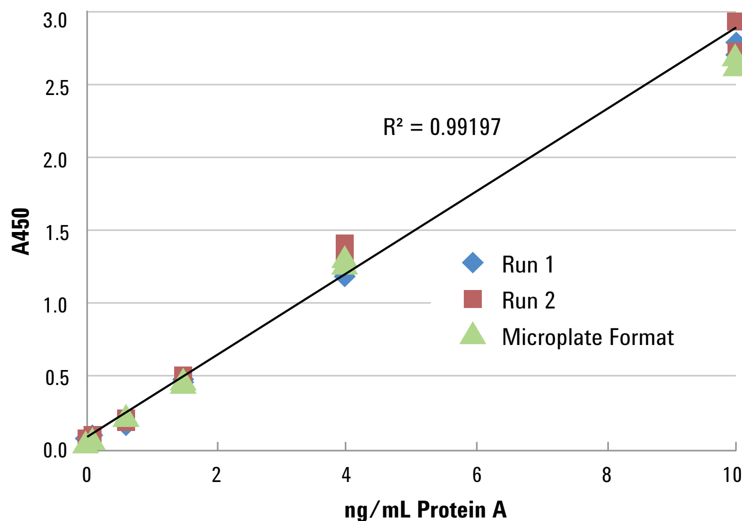
Applications

In general, applications for high throughput microchromatography for protein analysis are designed to *purify, quantitate* and *react*. In purification, the goal is to separate the target protein from all or most of the impurities present in the sample, isolating the target for further operations. In quantitation, the goal is to measure the amount of the target protein present in the sample. In reaction, the goal is to modify the target protein chemically by either enzymes or other reactants. Sample preparation protocols may involve any or all of these types of steps. Some example applications follow.

Figure 4. Application examples



Human IgC-containing samples purified on AssayMAP protein A cartridges, eluted into a microplate and quantitated using A280nm. Results using different sample volumes are shown. Quantitative binding capacity is >100 µg hIgG.



Samples containing protein A quantitated on AssayMAP ELISA cartridges, using reagents from a commercial microplate ELISA kit, read in a standard plate reader. Results are essentially identical to the standard microplate format, but the run time is <30 minutes.

Affinity purification and quantitation

Perhaps the most basic application is to use an affinity ligand immobilized on a resin in the cartridge to selectively bind the target protein. After washing away the unbound impurities, the purified target protein is eluted off and recovered in a microplate well. The affinity ligands can include protein ligands (such as protein A to target antibodies), antibodies and antigens, cell surface receptors or synthetic chemical ligands (such as those used in immobilized metal affinity chromatography to target proteins with histidine tags). The ligands can be immobilized on the resin before it is packed or immobilized *in situ*, using either hydrophobic adsorption or methods such as biotin/streptavidin binding. Alternately, non-affinity resins including ion exchange, hydrophobic interaction, reversed-phase, and others can be used.

The amount of target protein recovered from the packed bed cartridge can be precisely and accurately quantitated in the microplate well, using methods such as UV absorbance at 280 nm or protein assay reagents (such as Coomassie blue) on standard plate readers. Because the binding and elution to the cartridge is itself quantitative, this simple method can be used to accurately quantitate the target protein in the original sample. The sensitivity range and analytical precision of this method are generally similar to protein HPLC, but because many samples can be run in parallel, the throughput is much higher.

In addition to quantitation, the recovered target protein is also available in purified form for additional analytical methods. The amount recovered for an individual sample typically can range from 1 to 100 µg for cartridges with a 5 µL packed bed, which is sufficient for many analytical applications.

Immunoassay

AssayMAP cartridges can also be used to perform a number of different types of immunoassays, including ELISAs. For these applications, the cartridge is packed with large-particle, non-porous beads with a hydrophobic surface. Both the chemical nature of the surface and the total surface area in the cartridge are similar to a standard microplate well, used as the solid phase in many immunoassay procedures. However, because of the packed bed format, the diffusion path is much shorter for the molecules in the sample to reach the binding ligands on the solid phase surface – just the micron-scale space between the beads versus the millimeter-scale size of the microplate well. As a result, binding reactions go to completion much faster, eliminating the need for long incubation steps. A standard sandwich ELISA can be completed (from coating to readout) in 30 minutes, versus the 4 to 24 hours required for standard microplate methods, using the same reagents, buffers, and standards as plate-based methods.

Immunoassays on the AssayMAP system start with the coating step, which typically takes just a few minutes; pre-coating and subsequent storage of cartridges is not required. After blocking, the sample is loaded at a low enough flow rate to enable complete binding (typically around 5 µL/min). For maximum sensitivity, it is often advantageous to pre-incubate the sample with the labeled conjugate antibody, but in other cases, the conjugate antibody is loaded on the cartridge after the sample. After washing (which is very rapid and efficient due to the packed bed format), the syringe is filled with enzyme substrate solution, which is dispensed through the cartridge, and the resulting product is collected in a microplate for readout on a standard plate reader. Because the amount of product formed (and thus the signal) is inversely proportional to the residence time of the substrate solution in the packed bed, the precise control of flow rate enabled by the Agilent Bravo 96AM is critical for this step.

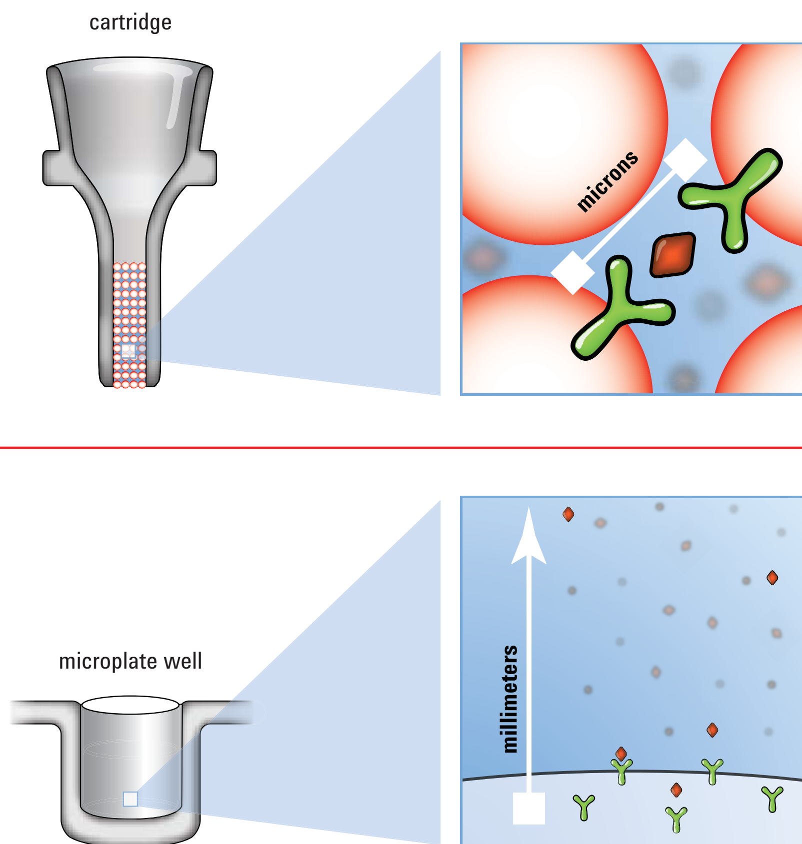


Figure 5. Comparison of diffusion paths in cartridge and plate-based assays

Glycan analysis

Glycan analysis is an example of sample preparation for more complex structural analysis of proteins. The profile of glycoforms found in a biotherapeutic protein can be quite complex, and is controlled in a poorly understood way by the expression cell culture conditions, but can significantly affect the safety, availability, and efficacy of the drug. The demand for glycan analysis is increasing dramatically, but sample preparation for glycan profile analysis is typically a long, manual, labor-intensive method. Only tens of samples are usually processed at a time, and the entire procedure can take up to 3 days from start to finish.

High throughput microchromatography can be used to dramatically speed up and automate this task. The target glycoprotein is first purified from complex samples, using the affinity purification methods described above, and then immobilized on resin in a cartridge. The glycoprotein is then exposed to an enzyme – such as PNGase F, which selectively cleaves off the glycan groups. The released glycans are eluted from the cartridge and collected in a well, where they are reacted with fluorescent labels for sensitive analysis. In some methods, the labeled glycans are then bound to a cleanup cartridge, excess label washed away and the cleaned-up, labeled glycans are eluted for analysis by HPLC, LC/MS, or capillary electrophoresis.

Summary

The biotherapeutic industry is driving an ever-increasing demand for protein analysis. Chromatography is a well-proven and effective general method to purify, quantitate, and react protein samples, but meeting the technical challenges of scale, throughput, quantitative binding and elution, and others has been difficult. The Agilent Bravo for Protein Purification with AssayMAP high throughput microchromatography is a highly versatile workflow solution for meeting this growing need in biotherapeutic discovery and development.

Biography

Scott Fulton, the founder and CEO of BioSystem Development, has over 30 years of experience in the life science and biopharmaceutical industry. He has B.S. degrees from MIT in physics and applied biology, and an M.S. from MIT in biomedical engineering. He has been responsible for the development, marketing, and technical and sales support of over 15 separation media, instrumentation, and process system product lines at Amicon and PerSeptive Biosystems. He was Chief Technical Officer at SolmeteX, a start-up company developing advanced wastewater treatment systems. He then became Vice President of Program Management at Genzyme Transgenics (now GTC Biotherapeutics), directing programs with major biotechnology and pharmaceutical companies to develop manufacturing of recombinant proteins in the milk of transgenic dairy animals. Prior to founding BioSystem Development, he worked as an independent consultant in the fields of biopharmaceutical development and life science tools.

BioSystem Development, established in 2002, creates, manufactures, and markets tools and products to meet the critical and growing analytical needs of biopharmaceutical development and life science research. The company's patented AssayMAP platform, based on disposable microchromatography cartridges and automation, enables high throughput solutions for bioprocess development, biomarkers, and general life science research that reduce development time and increase efficiency. For more information about AssayMAP technology, visit www.AssayMAP.com.

www.agilent.com/lifesciences/automation

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