Striving for purity: advances in protein purification

A substantial bottleneck in working with proteins, both native and recombinant, is purifying the protein of interest efficiently, with a minimum of labor and cost. Recent advances in purification technology from many companies are making the protein scientist's job easier. Caitlin Smith reports.

With the human genome sequenced, increasing numbers of researchers are turning their attention to proteins—with the hurdle that they must be purified first. A pure sample of protein is required to generate antibodies, conduct binding assays and study structure. Yet the target protein must first be isolated; the debris, salts and reagents washed away; the amount of protein quantified; and, often, the sample concentrated. A major challenge, according to Joern Kirchhuebel, project manager of Proteome Analysis at Eppendorf, is to retain proteins' native conformations while bringing them into solution. Yet the difficulties, and the importance, of protein work cannot be overstated when it comes to understanding the mechanisms of many, if not all, physiological pathways. Studying the complexities of protein-protein interactions is no easy feat, yet it is vital to unraveling the mysteries of conditions such as Alzheimer disease, diabetes and cancer.

The Vivaspin product family from Vivascience. (Courtesy of Vivascience.)

Chromatography, protein purification's workhorse

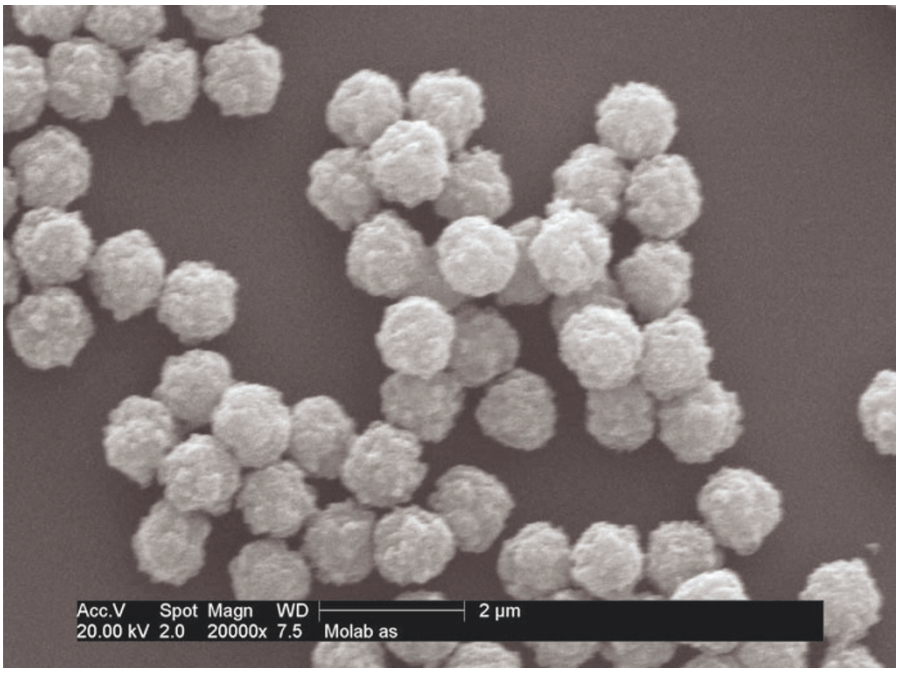
Chromatography has long been the trusted method for purifying both native and recombinant proteins. Many companies now offer improvements on this tried-and-true technique, both to increase performance and to alleviate the tedium for the researcher.

Companies such as Qiagen, Applied Biosystems, BioChrom and Amersham Biosciences (now part of GE Healthcare) have introduced new tweaks to improve purity, efficiency and ease of use. According to Qiagen, their Two-Step Affinity Purification System, which combines sequential affinity purification steps, yields ultrapure (>98%) protein. Doubly tagged recombinant proteins carrying His and *Strep* tags are purified using Qiagen's patented nickel-nitrilotriacetic acid (Ni-NTA) resin and *Strep*-Tactin (a modified streptavidin). As Achim Ribbe, executive director of Corporate Business Development at Qiagen, explains, "This system is especially useful when purifying proteins in the presence of high concentrations of chelating compounds or from eukaryotic cell lysates." If you need to separate phosphorylated and unphosphorylated forms of a protein, Qiagen offers the PhosphoProtein Purification Kit. Focusing on time efficiency, Applied Biosystems offers a perfusion medium, the POROS Perfusion Chromatography particles, which contain pores of two sizes. The larger pores allow flow through the particles, giving molecules quicker access to the smaller pores. This design was optimized to reduce the time necessary for the sample to interact with the matrix, speeding flow rates without affecting separation quality. Applied Biosystems claims flow rates of up to 1,000–5,000 cm/h, compared to 50–360 cm/h for conventional media.

BioChrom has also focused on improving column matrices for chromatography. According to BioChrom's presi-

dent, Michael Lu, "the improvement of recoveries of proteins and the speed of protein purification are still the major issue for most separation media companies." To tackle this issue, BioChrom has developed a proprietary polymeric material, Hydrocell. The high level of cross-linking in the polymer beads is intended to give better and faster protein separation compared to conventional silica-based HPLC columns. Biochrom also claims that their Hydrocell columns are especially durable, tolerating a broader pH range and harsher cleaning regimens.

Combining small-bead technology with affinity purification, Amersham Biosciences (now part of GE Healthcare) recently launched a new chromatography matrix for the purification of glutathione-S-transferase (GST)- and His-tagged proteins. One matrix, called Glutathione Sepharose High Performance, is made of highly cross-linked 34 μm agarose beads to which glutathione has been immobilized. According to GE Healthcare, the relatively small bead size results in distinctly separated protein elution peaks. For His-tagged proteins, they also offer a small bead matrix called Ni Sepharose High Performance. GE Healthcare claims that their new nickel beads give the highest binding capacity on the market, and with their negligible nickel leakage, provide better purity and yield while minimizing protein precipitation.



Magnetic Dynabeads from Dynal Biotech. (Courtesy of Dynal Biotech.)

Magnetic beads

Magnetic beads, with their speed, ease of use and affordability, have become a popular choice for protein purification. With an iron core surrounded by agarose or inert polymer material, the beads behave like magnets when subjected to a magnetic field, yet retain no residual magnetism when the magnetic field is removed. This simplifies purification procedures, as no columns or centrifugation are required. Beads can be purchased for use with specific molecular tags suitable for affinity purification. Most magnetic beads are also ideal for use in automated liquid-handling systems. Realizing the appeal of these many advantages, some companies such as Dynal Biotech, Polysciences and Bioscience Beads have capitalized on magnetic beads as one of their flagship products.

Dynal Biotech offers Dynabeads for isolating proteins tagged with HAT or HN6 histidine-containing tags (BD Biosciences Clontech). The use of nickel in immobilized metal affinity chromatography, as during isolation of His-tagged proteins, can hinder the purification of recombinant proteins. To circumnavigate this limitation, Dynal Biotech has partnered with BD Biosciences Clontech to offer cobalt-based products called Dynabeads MyOne TALON, which minimize non-specific binding of histidine-rich bacterial proteins during isolation of the intended protein. The cobalt-containing TALON core binds only adjacent histidines, or histidines in certain arrangements. Bead cores containing nickel have less rigorous binding requirements, so they may bind to histidines in areas other than the histidine tag—for example, those found in various other proteins in cell lysates. Lars Korsnes, director of R&D and Business Development, Molecular Systems, of Dynal Biotech, says that the Dynabeads MyOne TALON have a range of automated applications as well: "With this fully automatable Dynabead technology, recombinant proteins can be purified, and used directly into biopanning or drug discovery applications, as well as magnetic isolation of protein complexes from cell lysates or blood which have binding activity for the recombinant protein sitting on the magnetic beads." Dynal Biotech has designed protocols for automation of Dynabeads MyOne TALON use with platforms such as Thermo KingFisher, Tecan Genesis, Tecan Freedom EVO and Beckman Biomek FX.

Although more companies continue to increase the variety of molecular tags available on magnetic beads surface, the support itself has also been the object of interesting developments. For example, the particular characteristic of BioMagPlus particles from Polysciences is their irregular shape, intended to give them 20 to 30 times the surface area of a spherical particle with a comparable diameter, thus making them more efficient at binding target protein. BioMagPlus particles are distinguished from their recent BioMag predecessors by being more uniform in size and more magnetically responsive. This latter quality is especially useful for faster magnetic separations when using an automated high-throughput platform. Kits are available that include the tagged particles along with buffers, quenching solution, centrifuge tubes and other reagents.

Another example is the company BioScience Beads, which strives to offer flexibility and customization. "We make the product to your specifications," says its president, Richard Cook. "Our technology allows any research scientist to cost-effectively design their own chromatography media. As a result, there is a multitude of new products possible from the permutations and combinations of such

parameters as matrix type, matrix concentration, bead size distribution and functionalization." Cook adds that the magnetic agarose beads are their most popular product among their chromatography reagents, a testimony to the beads' popularity.

Mini-scale and preparatory enrichment

For purifying small amounts of protein, concentrating protein samples or preparing protein samples for mass spectroscopy, Vivascience and several other companies offer user-friendly products such as mini spin columns and microcolumn pipette tips.

Vivascience's Vivaspin devices use a vertically-oriented ultrafiltration membrane that gives high protein recovery during sample concentration or re-buffering. The vertical membrane design increases flow rate by increasing the filtration area and reducing membrane fouling. Their newest ultrafiltration product has a cutoff of less than 3 kDa, suitable for the enrichment of small peptides. In an effort to showcase their versatility, Vivapure devices are available in spin column format for various sample volumes and capacities as well as in 96-well format for high-throughput analyses. These efforts at downscaling to small membrane areas are justified by Robert Zeidler of Vivascience as "strictly necessary for handling tiny sample amounts used in mass spectrometry or clinical studies."

The increasing use of high-throughput mass spectroscopy in protein analysis has driven several companies to develop purification products specifically adapted for the job. Vivascience sells Vivapure MALDI-Prep Micro spin columns, which containin the C18 membrane, for de-salting and concentrating protein digests for mass spectroscopy. C18 refers to the number of carbons in the alkyl chains attached to silica particles in conventional column chromatography, such as reverse-phase HPLC; the length of the carbon chains in the matrix can vary, but C18 chains are the most popular and usually the most effective for small-molecule separation.

The Amicon Ultra centrifugal concentrator from Millipore. (Courtesy of Millipore.)

Eppendorf has recently introduced two products that should make sample preparation more efficient without sacrificing quality. PerfectPure C18 Tips are

microcolumn pipette tips that can purify, desalt and concentrate femtomole and picomole quantities of peptides from proteolytic digests. To reduce sample loss due to proteins adhering to tube sides, Eppendorf also offers Protein LoBind Tubes, especially useful for working with small volumes and/or low concentration protein solutions. "A special plastics formulation with no surface coating ensures high recovery rates (>99%), protection [from] enzyme activities and prevention of denaturing effects," says Eppendorf's Kirchhuebel.

Like Eppendorf, PhyNexus is meeting the growing demand for quick and convenient filter devices by incorporating chromatography matrices into the common 200 µl and 1,000 µl pipette tips. PhyNexus claims that their PhyTips are capable of high purification (>95%) and enrichment (up to 5 mg/ml) of samples less than a milliliter, with a choice of manual, automated or semi-automated use. PhyTips are sold with various affinity-based matrices, including protein A and G products for the purification of immunoglobulins, and products for the purification of His- and GST-tagged proteins. PhyNexus has recently teamed up with Qiagen to offer Qiagen's Ni-NTA affinity resin in PhyTips for purification of His-tagged proteins on a micro-volume scale.

Although many recent advances in purification technology are aimed at smaller volumes, researchers still require methods for the quick and convenient purification of larger volumes of protein samples. Companies like Millipore are filling this need with centrifugal concentration devices. The Amicon concentrators remain Millipore's most popular products, including their new Amicon Ultra and Centricon Plus 70 devices. Mark Kavonian, Group Product Manager of the Life Sciences Division at Millipore, says that "the Ultra devices are used to concentrate and desalt from 4 to 15 ml of solution. The Centricon Plus 70 device can purify up to 70 ml and is a convenient alternative to stirred cells and dialysis tubing." In addition, the Amicon Ultra "combines a low-binding ultrafiltration filter with a new vertical design to yield >90% recovery with quick centrifugations."

One of the main uses of the small- and large-scale membrane filtering devices is to change the buffer during or in the final stages of protein purification, because the reagents necessary for purification interfere with the next steps. "Following purification," according to Robert Zeidler of Vivascience, "protein quantification is frequently troublesome, especially when verifying protein amounts for gel loading, activity analysis or comparative studies." The alternative approach currently taken at Vivascience to tackle these problems is to investigate protein quantification methods that are less affected by high salt concentrations, detergents or protein sequences.

Protein detection and quantification

In effect, efficient protein purification requires accurate detection, size analysis and quantification at multiple stages in the purification pathway. In addition to being dependent on the buffer system at each step, these intermediate detection and quantification steps often involve a large number of samples. Some of the latest innovations in detection and quantification include the use of microfluidics, which involves the manipulation of liquid samples in miniaturized systems,

BOX 1 NO TIME OR HANDS? TRY OUTSOURCING YOUR PROTEIN PURIFICATION

To an overworked bench scientist struggling to purify a protein for experiments that have nothing to do with purification, it sounds like heaven. You provide a sample containing your protein, and someone else does the rest and returns a pure sample of your target. Several groups offer a range of purification services; some use their own protocols, and some will follow yours.

The Protein Purification Group at the National Cancer Institute (NCI) at Frederick is the NCI's sole contractor for purification. They purify using Amersham Biosciences (now part of GE Healthcare) FPLC workstations, with most of their targets being His-tagged recombinant proteins, although they also purify GST-tagged and biotinylated proteins. The FPLC product is subsequently purified using chromatography—the type depends on the protein but may include ion exchange, hydrophobic interaction or size exchange chromatography.

Trenzyme will synthesize or purify your protein of interest. According to Reinhold Horlacher, Trenzyme's CEO, the most popular procedures for purification include cloning the target into expression vectors, followed by recombinant expression and affinity purification, mostly with His tags. Using a variety of expression systems, the people at Trenzyme optimize the expression of your protein for you. Along with your purified protein, you receive a detailed description of the purification procedure.

Paragon Bioservices strives to be an extension of the client's laboratory. The client provides Paragon with starting material, in the form of cell pellets, bacterial paste or conditioned medium. Paragon then purifies the protein using its own protocols or protocols detailed by the client. Paragon delivers the purified protein to the client, along with batch records and specifications about yield and purity. Paragon can produce up to 100–200 g of purified protein.

GenScript offers a range of services including small- and large-scale protein purification. They use affinity, gel filtration, ion exchange, hydrophobic column chromatography or a combination of these for the purification steps. They deliver your purified protein to you, with or without an affinity tag, from a few milligrams to a few kilograms. They can also establish high-expression stable cell lines for you, including yeast, CHO or HEK293 cells. GenScript has developed a proprietary recombinant peptide technology that enables them to synthesize peptides of any length—even complicated peptides containing multiple disulfide bonds. Compared to conventional chemical synthesis methods, GenScript's method is cheaper, allows easy scale-up in bacteria and is more reproducible from batch to batch.

If you need quick protein identification, turn to Protana Analytical Services, a division of Protana Inc. They offer proteomic analysis services such as quantitation, sequencing and peptide coverage maps for isoform or variant determination. With more than 30 spectrometers, Protana promises results within five business days of receiving your protein sample.

BOX 2 STEPPING IT UP: HIGH-THROUGHPUT PROTEIN PURIFICATION

The Freedom EVO automated workstation from Tecan. (Courtesy of Tecan.)

High-throughput protein purification is necessary for some applications, including target discovery and validation studies in drug development. Many companies are striving to meet this increasing need by developing automated platforms that allow researchers to walk away from the routine purification steps.

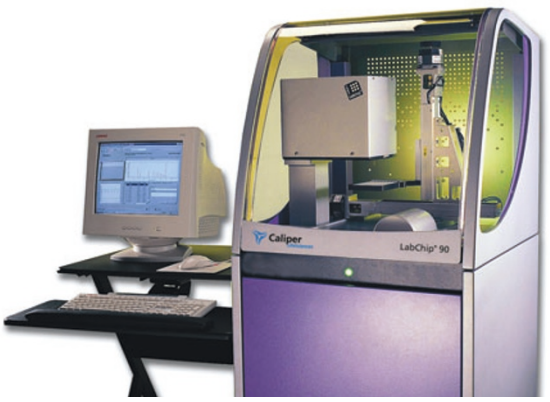
GE healthcare's newest addition, the ÄKTAxpress, is designed for the automated high-throughput purification of GST- and His-tagged proteins, delivering >95% purity. The system uses a two- to four-step protocol, depending on the types of column chromatography desired, and includes His or GST affinity chromatography, desalting or gel filtration and ion exchange chromatography. Following these protocols is an optional tag removal step. With a capacity of up to 50 mg of purified target protein, the ÄKTAxpress can purify 16 samples in 11 h using a two-step protocol, or 8 samples in 8 h using a four-step protocol.

Another popular device for high-throughput protein purification is Tecan's Freedom EVO platform. Tecan is partnering with Dynal Biotech to offer Dynabeads TALON for the automated purification of His-tagged proteins using magnetic beads. In another collaboration with EMD Biosciences/Novagen, Tecan has developed a series of automated high throughput protocols for the purification of recombinant proteins, says Josef Syfrig, Tecan's Content Specialist in Marketing Communications. Novagen's Robopop purification kits for His- or GST-tagged proteins, using either column chromatography or magnetic beads, are fully compatible with Tecan's Genesis, Genesis Freedom and Freedom EVO automated workstations.

often on a chip. Inside the chip, minute amounts (picoliters to nanoliters) of liquid containing the protein of interest are moved through circuits of tiny channels. For example, common inlet channels may lead to individual chambers (of which there may be hundreds), each of which contains a tiny chromatography column designed to purify several picoliters of sample. Separate chambers are delineated by small hydrophobic barriers. Each column is washed and eluted in parallel, during which the flow rates can be precisely controlled. Thus the use of microfluidics is beneficial in protein detection and quantification for several reasons. Because movements of the liquid samples can be so finely controlled, the results are more reproducible. Factors such as precise engineering, surface chemistry and small volumes reduce sample loss dramatically compared to conventional methods. Consequently, low-abundance proteins are relatively easier to detect and quantify using the fine machinery of microfluidics. Additionally, the ability to process up to hundreds of samples in parallel on one chip enables automation of analysis and higher throughput.

For example, Caliper Life Sciences' LabChip90 is an automated electrophoresis system, an alternative to the conventional slab gel electrophoresis system such as SDS-PAGE. Caliper's new protein assay for the LabChip90 is less expensive than traditional gel electrophoresis and increases sample throughput by two- to threefold. Because the LabChip90 can sample from 96-well plates, it also saves time. "The LabChip 90 integrates all of the steps of the process—from sampling to separation to detection to quantification via the software that comes with the instrument," says Caliper's product manager Jackie Richards. "It generates high-quality digital sizing and concentration data in seconds per sample." Mark Roskey, vice president of marketing at Caliper Life Sciences, predicts "continued advancement of microfluidics-based techniques for quantification. Caliper will launch a new high-speed, high-sensitivity protein assay at the end of 2004 for the LabChip 90 System. In the past months Bio-Rad launched their Experion electrophoresis system, which uses Caliper's LabChip technology and makes these more advanced approaches to protein separations more accessible for the academic market. Agilent launched their 5100 system, which also uses Caliper microfluidics, to accomplish high-throughput protein separations."

Gyros builds their microfluidics-based Gyrolab microlaboratory on a compact disc. Spinning the disc creates centrifugal force that drives fluids through nanoliter-scale channels in the disc; sample movement is also controlled by capillary forces and surface chemistry. For protein mass mapping or sequence analysis in mass spectroscopy using a Gyros Workstation, one disc can process 480 samples in parallel in about an hour. Gyros recently introduced another disc, Gyrolab Bioaffy, along with an updated Gyrolab Workstation equipped with laser-induced fluorescence detection, which allows researchers to run immunoassays for protein quantification using nanoliter sample volumes.



The LabChip90 automated electrophoresis system from Caliper Life Sciences. (Courtesy of Caliper Life Sciences.)

Another increasingly popular microfluidic device is the Agilent 2100 bioanalyzer, initially commercialized in 1999 as a 'Lab-on-a-Chip' to perform size separation of proteins and nucleic acids. Now increasing the throughput one step further, Agilent has recently unveiled its new Agilent 5100 Automated Lab-on-a-Chip Platform, along with its protein purification assay, the Protein 200 HT-2 kit. This combination allows hands-free protein analysis by combining the microfluidic instrument with complete automation. Running ten plates (96- or 384-well) simultaneously, the Agilent 5100 yields more reproducible digital data for a larger number of samples than in gel electrophoresis. Analysis time is reduced by a factor of 10, with unattended analysis of 3,840 samples per run. In addition to the digital data, the Agilent 5100 generates a gel-like image for visual display.

Striking a balance between greater purity and faster purification is a theme that protein researchers will wrestle with in the near future. Says Mark Kavonian: "Scientists want faster methods and higher purity, but they also want to retain the functionality and structure of their protein." With scientists increasingly turning to proteomics research, "new and improved depletion and fractionation methods will be needed [to reduce] the complexity of starting material," says Achim Ribbe. "They will be based on specific properties such as post-translational modifications and glycosylation patterns." Qiagen plans to launch easy-to-use protein depletion and fractionation kits next year. Ribbe also comments that "we look forward to a time when a protein preparation becomes as simple and routine as plasmid preps are now. Genomics researchers now moving into proteomics are used to high-throughput procedures and the automation of protein purification and analysis will be a growth area."

Although many researchers see the current high-throughput methods as a boon to proteomics, others are cautious about this new direction. William Gillette voices this concern succinctly: "The problem, to paraphrase a former US president, is 'the protein, stupid'." Gillette says that high-throughput purification, "the 'one-size-fits-all' approach that was so successful in genomics, will not work for proteins. A predictive algorithm which might advise as to more likely purification methods would be incredibly useful but seems a long way off."

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SUPPLIERS GUIDE: COMPANIES OFFERING PROTEIN PURIFICATION PRODUCTS

Company	Web Address
Agarose Bead Technologies	www.abtbeads.com
Agilent	www.agilent.com
Amersham Biosciences (now part of GE Healthcare)	www.amershambiosciences.com
Applied Biosystems	www.appliedbiosystems.com
Beckman Coulter	www.beckman.com
BD Biosciences Clontech	www.bd.com
Biochrom Labs Inc.	www.biochrom.com
Bio-Rad	www.bio-rad.com
BioScience Beads	www.bioscience-beads.com
Brinkmann	www.brinkmann.com
Calbiochem	www.calbiochem.com
Caliper Life Sciences	www.caliperls.com
Center for Integrated Biosystems	www.biosystems.usu.edu
Chemicon	www.chemicon.com
Ciphergen	www.ciphergen.com
Dynal Biotech	www.dynalbiotech.com
EMD Biosciences	www.emdbiosciences.com
Eppendorf	www.eppendorf.com
Fluidigm	www.fluidigm.com
GenScript	www.genscript.com
Gyros	www.gyros.com
Invitrogen	www.invitrogen.com
J.T. Baker	www.jtbaker.com
MDS Inc.	www.mdsintl.com
Millipore	www.millipore.com
Molecular Probes	www.probes.com
NCI/SAIC Protein Purification Group	www.ncifcrf.gov/rtp/PEL/PP/
New England BioLabs	www.neb.com
Novagen	www.novagen.com
Paragon Bioservices	www.paragonbioservices.com
PhyNexus	www.phynexus.com
Pierce Biotechnology	www.piercenet.com
Polysciences, Inc.	www.polysciences.com
Protana	www.protana.com
QBIOgene	www.qbiogene.com
QIAGEN	www.qiagen.com
SEPIAtec	www.sepiatec.com
Sigma-Aldrich	www.sigmaaldrich.com
Stratagene	www.stratagene.com
SuNyx	www.sunyx.de
Tecan	www.tecan.com
Tosoh Biosep	www.tosohbiosep.com
Trenzyme	www.trenzyme.com
Vivascience	www.vivascience.com
Whatman	www.whatman.com