Pure but not simple

Protein purification is stepping into the limelight, as proteomics researchers demand faster ways to purify more proteins. Tim Chapman looks at what is around to help them.

Protein isolation is one of the oldest 'biotechnologies', but the demands from proteomics for the purification of potentially vast numbers of proteins is driving new developments in long-established techniques. "Researchers working in genomics are looking for the next step to fully understand the results of their sequence analysis — the proteins that the genome expresses — in the context of systems biology," says Anke Cassing, associate director for corporate strategy at Qiagen in Hilden, Germany. "The delicate interplay of proteins is, of course, also of extreme interest to pharmaceutical companies, which are always on the lookout for new drug targets."

There is increasing demand from researchers producing biopharmaceuticals — antibodies and proteins used as drugs. "There's a driving force towards protein purification, separation and analysis," says Carsten Buhlmann, product manager at Agilent, based in Palo Alto, California. "Especially for the biopharmaceuticals, there's a high demand for purity of these proteins that are used for drugs and have to get through all the regulations."

For virtually all applications, researchers need to maintain a protein's biological activity, which can rule out some purification processes. Proteins can be fragile and easily denatured, and many of the most important are insoluble in the most common media.

"If you look at the average protein, it's quite complex, it's a buzzing, vibrating molecule — it's not a fixed structure," says Allan Simpson, vice-president for product development at the protein separations division of GE Healthcare Biosciences in Uppsala, Sweden. "They're very hard to handle, they're difficult to purify, they can aggregate easily — these are very hard things to manipulate."

Anke Cassing: systems biology is the next step.

QIAGEN

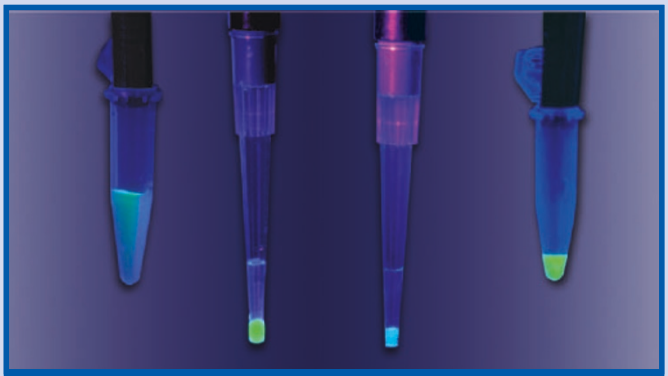
Proteomics workhorse

With proteins taking centre stage in many laboratories, equipment developers are rolling out a new generation of automated systems to take the grind out of separating and purifying proteins of interest. Chromatographic separation is one of the basic protein-purification techniques and one platform that is emerging as a workhorse of the large proteomics lab is ÄKTAxpress. Made by GE Healthcare, this is a dedicated high-throughput multistep chromatography system for purifying histidine (His)- and glutathione-*S*-transferase (GST)-tagged recombinant proteins.

GE began developing the platform in the late 1990s after realizing that there were not enough trained chromatographers to produce proteins in the quantities and varieties demanded by post-genomic researchers. "We decided to see if we could automate a system that would be better than the current technologies at solving that problem," Simpson says. "Instead of taking a robot and automating the current system, we set out to develop a

SMALL-SCALE SEPARATION

Sometimes you can do more with less. Proprietary pipette tips developed by PhyNexus in San Jose, California, promise high performance in tiny volumes with minimum fuss. The key lies in encapsulating very small quantities — just 5–10 microlitres — of protein separation resin between hydrophilic screens in the very end of the pipette tip. "The whole sample is obliged to make highly intimate contact with that resin," says Chris Hanna, vice-president of business development. "That results in high trapping efficiency for the sample. We can get a 10–20-fold increase in the target protein sample from just a few hundred microlitres of sample, and get purities that are often over 95% with a single separation step."



Thinking small: PhyNexus protein separation tips.

As well as the technical collaboration with Caliper of Hopkinton, Massachusetts, PhyNexus has designed its PhyTips to be compatible with liquid-handling robots from Tecan of Männedorf, Switzerland, Beckman Coulter of Fullerton, California, and PerkinElmer of Boston, Massachusetts. The firm has also agreed licences to use some of the most advanced resins in its tips, including Qiagen's Ni-NTA resin for purifying histidine-tagged proteins. The combination of tips, resins and platforms makes for exquisite control of the separation process, Hanna says. "You can control the number of cycles that go back and forth through the bed, the rate they do so, the composition of the washes. We can really make that microvolume of material dance and perform at its best," he says. "To be able to do that and maintain fully functional proteins at these very small scales gives you ÄKTA-type purification off a very small amount of starting sample. People can avoid scaling up."

The tips are initially being deployed for rapid purification and enrichment of antibodies from small cultures of *Escherichia coli* for use in high-throughput cell-based assays, giving significant savings in time and money. "People are wanting to get real biological information much earlier in their screening process, and to do that they have to have the stuff properly prepped," notes Hanna. Interest is also coming from protein engineering and biopharmaceutical companies looking to miniaturize their protein-expression systems.

T.C.

new one. A robot represents nothing more than a mechanical technician — it reproduces all the successes but also all the errors, so you don't move forward in your science."

The company produced a high-throughput system that needs no specialized knowledge to operate and can be programmed to carry out up to four common purification steps starting with affinity purification. "The skill of chromatography is sitting within the system," says Simpson. The software is written as a series of wizards, each representing a single step. GE will shortly be launching a software package for the purification of monoclonal antibodies.

The basic four-module set-up can purify up to 2,500 proteins a year, each module producing up to 50 mg of protein per run. A twin-pack version is aimed at smaller labs wanting to purify up to 1,000 proteins a year.

The new protein-purification facility at Monash University in Melbourne, Australia, is deploying a 12-module ÄKTAxpress set-up for its ambitious development programme. "I'm a structural biologist, so my interest is in producing large amounts of recombinant protein for structural and functional studies," says James Whisstock, scientific director of the facility. "What's really important is that the cost of equipment is within reach of a normal university laboratory set-up. There are some very big structural biology institutes with between US$50 million and $100 million's worth of industrial-scale protein preparation equipment. From our point of view that's not achievable, but we're bringing in this technology, which is going to make a huge difference to our research."

The ability to deal with many more proteins simultaneously will allow the lab to approach problems differently. "If you have a very challenging protein target and want to try 50 different constructs, at the moment it's really not feasible to do that manually one after the other," Whisstock points out. "Now, you can try the same molecule from 50 different species. With parallel advances in expression technology, the whole process is simplified and really streamlined, and provides the capacity to perform that experiment. You're trying so many different things simultaneously, you're likely to get a result."

Automating innovation

Several companies are developing equipment and product ranges that can be used at different points in the proteomics pipeline, from raw cell extracts to mass spectrometry and beyond. Beckman Coulter in Fullerton, California, is rolling out its ProteomeLab family to help with everything from initial purification of cell extracts, through protein fractionation and characterization, to the ultimate steps of disease diagnosis.

"We try to link technologies together to simplify the job for what takes place at the end, which is typically mass spectrometry," says John Hobbs, group product manager for ProteomeLab. "To get to that point, a lot of people have realized that it's garbage in, garbage out. If you put crap into a mass spectrometer, the results you get out will be the same."

Scaling-up: ÄKTAxpress at Monash University's protein purification facility.

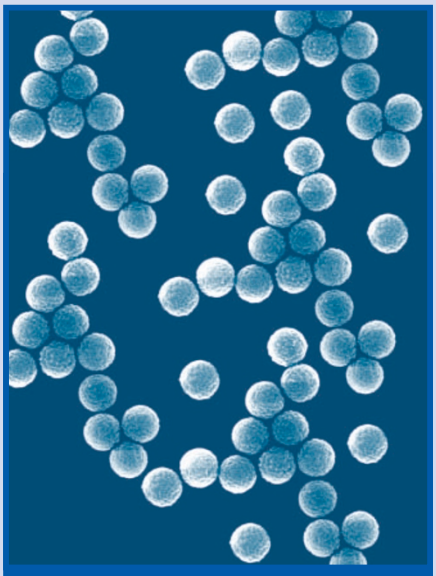
The ProteomeLab PF 2D Protein Fractionation System automates two-dimensional chromatographic fractionation, resolving proteins by isoelectric point and hydrophobicity. The emphasis is on standardizing the protocols and techniques used by researchers. "Biologists want to be able to look at their results and see if they relate to someone else's,"

ATTRACTING ATTENTION

Magnetic beads have been used for protein separation since the 1980s, but the technology is now being adapted for new proteomic applications and use with automated platforms. The market leader in paramagnetic beads is Dynal Biotech based in Oslo, Norway, and recently acquired by life-sciences giant Invitrogen in Carlsbad, California.

Dynal has just signed a co-marketing agreement for its Dynabead kits and Tecan's Freedom EVO automated platform, and has developed protocols for other platforms such as Beckman Coulter's Biomek FX and the KingFisher magnetic separation platform from Thermo Electron of Waltham, Massachusetts. "The main purpose of having magnetic beads is that you can automate the whole process," says Lars Korsnes, director of research and development at Dynal. "The bead technology has some advantages compared with standard chromatography systems — while it's not so easy to put a whole-blood sample into a chromatography column, with magnetic beads you can put the whole sample in."

Dynabeads, like those from some other suppliers, are superparamagnetic, with no residual magnetism outside an applied magnetic field. They are also uniform in size, shape and surface properties. This all helps to prevent the beads clogging up an automated device, Korsnes notes.


DYNAL BIOTECH
Magnetic beads are branching out.

Bead technology is also more scaleable than chromatography columns, although Dynal is concentrating on more analytical or small-scale protein isolation and protein fractionation for different applications in proteomics. The firm is currently launching a new range of beads with functionalities such as ion-exchange groups, reverse-phase chromatography and hydrophobic chemistries. New owner Invitrogen plans to apply Dynal's surface technologies to a wider range of products.

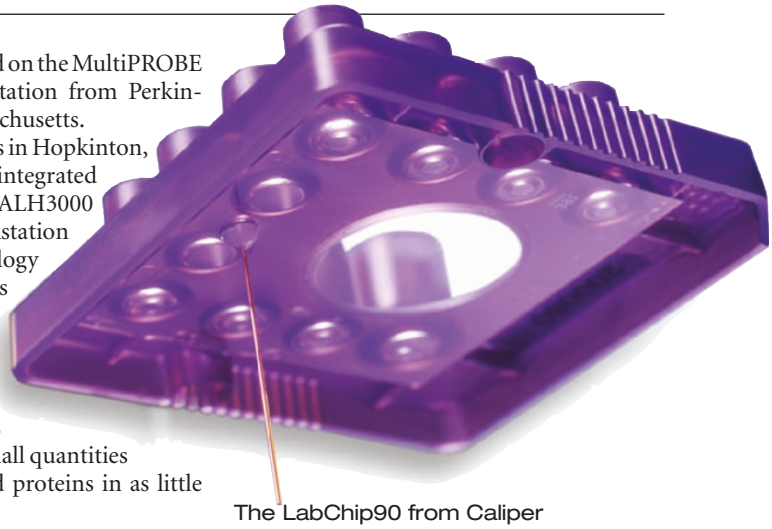
The beads are also showing promise in the challenging separation of membrane proteins. "There have been some publications where one can bind membrane proteins either before or after lysing the cells," Korsnes says. "Whether we will develop a special protocol is a question for the future, but the technology is there already." T.C.

Hobbs notes. "As well as providing the instrument, we provide the methodology and buffers, but we do specify you have to use that method to get the full support. It's a little bit different from the normal research instrument approach, but we thought there was a need for that and it seems to be accepted."

Beckman is also currently commercializing an innovative system of protein partitioning using affinity fractionation to decrease the unwanted complexity of protein mixtures before analysis. The aim is to remove not just very abundant proteins, for example serum albumin from blood plasma, but also other proteins that are already well characterized. The firm claims that up to 95% of the proteins in a cell lysate can be removed before the full fractionation stage with less risk than other purification procedures of losing the proteins you're interested in. "There's a growing interest in what might be going away with these large-abundance proteins — albumin is a binding protein, and possibly some interesting proteins go with it," Hobbs notes.

Several big equipment producers have teamed up with smaller specialist firms to include cutting-edge reagents or media in application kits for their automated systems. Tecan in Männedorf, Switzerland, recently signed a licensing agreement to deploy the paramagnetic beads developed by Dynal Biotech in Oslo, Norway, on its Freedom EVO liquid-handling platform (see 'Attracting attention', page 796). The Robopop protein purification kits from Novagen in Madison, Wisconsin, can also be used on Tecan's workstation and on the MultiPROBE liquid-handling workstation from Perkin-Elmer in Boston, Massachusetts.

Caliper Life Sciences in Hopkinton, Massachusetts, has integrated into its Sciclone ALH3000 liquid-handling workstation a new column technology developed by PhyNexus based in San Jose, California (see 'Small-scale separation', page 795). The combination allows researchers to purify and enrich small quantities of up to 96 engineered proteins in as little as 15 minutes.



The LabChip90 from Caliper Life Sciences.

The market for protein purification systems has changed in the past six months, notes Mark Roskey, vice-president of marketing at Caliper, with more groups getting involved in larger-scale protein purification. "It's not at the industrial scale, but regular pharma and biotech R&D people are now trying to purify proteins in a more parallel situation. A lot of this stems from having all the genes and working with huge numbers of them to develop new drugs," he says.

Analysis and optimization

To maintain the benefits of high-throughput separation and purification, the proteins of interest must be able to pass smoothly into the next stage of the process. "Once you've got a relatively pure protein you need to determine whether it is pure, so there's issues with analysis as well," says Roskey. A common analysis method is SDS-polyacrylamide gel electrophoresis "but we feel that that is a bottleneck", Roskey adds.

Although the established protocols of macroscale gel electrophoresis are being successfully automated (see 'Automation in two dimensions', below), many users are turning instead to microfluidic and lab-on-a-chip solutions. In January, Caliper launched the Protein Express Assay for its LabChip 90 automated electrophoresis system.

"It's a microfluidic replacement for SDS-PAGE that automates the whole process," Roskey says. "Rather than putting samples on a gel, you get them off a multiwell plate, and it does integrated separation, but we can help them out by automating key aspects of the process."

The heart of the a2DEoptimizer is automated gel casting, allowing researchers to create customized gradient gel profiles with a minimum of fuss. "Everyone knows casting a gradient gel gives you far superior spot resolution, separation and definition, but the problem is that these gradients can be quite tricky to pour, especially the more exotic ones," Orange says. "There's a very small number of people who can get good reproducible gels when they're pouring gradients. As we see 2D going forward, people are dealing with very small amounts of sample. They've got one or two gels they can run so they have to get the best data they can out of there."

The system also has integrated power packs that can focus samples at high voltages and reduce the time for a separation run. And it has real-time monitoring of the electrical profile. "That's of particular importance, because when you're dealing with new samples, it's very important to look at the electrical profile and tell whether you've got some kind of issue with salt content or protein content," Orange notes. "What we're doing is enabling people to get better data out, and also analyse what's going on in their system." T.C.

AUTOMATION IN TWO DIMENSIONS

Microfluidic systems have taken over from two-dimensional (2D) gel electrophoresis techniques in some areas of protein separation and analysis, but the established methods are far from dead. Even though they can be slower and messier, the tried and tested 2D protocols still offer some advantages, especially if automation can take out most of the hassle.

"Some people will be claiming otherwise, but I think 2D still has the best levels of sensitivity when you're looking at complex samples," says Paul Orange, senior product manager at NextGen Sciences in Cambridge, UK. "With 2D you're seeing resolution of 2,000–3,000 spots on a gel — that's quite a lot of information, but people understand it. Also 2D is a very accessible technology — you can go and buy equipment relatively cheaply and get started if you're looking for a proteomics approach."

In 2003, NextGen launched the first fully automated 2D electrophoresis platform, called a2DE. The firm has now brought out a spin-off system called the a2DEoptimizer which can improve 2D separations on a variety of commercial platforms. "We've not tried to reinvent the wheel," says Orange. "We know what people are using, and know that they have lots of data and experience,

2D gel electrophoresis gets a facelift.

NEXTGEN

detection and analysis. It can do an individual protein in around 30 seconds, so you can do 96 proteins in an hour with the same quality as SDS — in fact better, because the data are digital."

Automated electrophoresis using chips developed with Caliper is the basis of two new systems — the bench-top Experion from Bio-Rad in Hercules, California, which analyses a single Pro260 chip carrying a maximum of 10 samples in 30 minutes for the medium-scale user, and the ultra-high-throughput 5100 Automated Lab-on-a-Chip system from Agilent in Palo Alto, California. The latter is aimed at pharmaceutical companies and other laboratories needing to separate, purify and analyse thousands of proteins a day. The fully automated 5100ALP using the Protein 200 Plus LabChip kit can take up to twelve 96- or 384-well plates for overnight analysis, with each chip capable of up to 6,000 sample runs. "The real innovation with the 5100 is that you have a complete unattended solution ending up with digital data," says Carsten Buhlmann, product manager for Agilent's microfluidics group.

The 5100ALP has been deployed at the centralized protein-production facility for AstraZeneca in Alderley Park, UK. "We're developing a high-throughput protein-production platform and wanted a quantitative and qualitative data-analysis method for that process," says Paul Hawtin, senior research chemist at AstraZeneca's UK protein group. "Previously we used SDS-PAGE gels and although they get you the information you need, they're very cumbersome, especially when you're using the numbers that we're using."

With high-throughput protein production, an integrated analysis capability is invaluable. "We were in the situation where we could do high-throughput molecular biology, high-throughput expression and also high-throughput production — once you've gone through that process and you're testing a number of variables, the numbers you've got coming out the back end are incredibly large," Hawtin notes. The AstraZeneca team also uses the ÄKTAxpress to follow up with larger-scale protein production.

ASTRAZENECA

Large-scale protein production needs equally high-throughput analysis.

Allan Simpson: membrane proteins will be "the pot of gold".

Kits and columns

Researchers who don't need automation can choose from an increasing selection of specialized purification and fractionation kits. Kits for affinity purification of recombinant proteins tagged with His_6, GST, streptactin-binding Strep-tag, streptavidin-binding peptide and other tags abound, and many incorporate magnetic bead technology.

On the protein fractionation and preparation front, in January Qiagen launched a new line of protein fractionation kits under the Qproteome brand. The range features kits, including reagents, buffers and columns, for such common tasks as phosphoprotein purification, glycoprotein fractionation and albumin depletion.

"The simplicity of the kits and procedures offers a gentle introduction to the world of protein science for molecular biologists who might be wary of having to learn new techniques or understand complex technologies," says Cassing.

Many protein purification kits based on filtration are available in spin-column format, allowing faster processing. Vivascience, a subsidiary of Sartorius, based in Hanover, Germany, has developed a range of specialized spin-column purification kits, based on the firm's membrane matrix of stabilized regenerated cellulose. Because of the porous structure, the surface available for contact and binding is about 100 times that of the same volume of traditional bead-based resins, allowing parallel separation of proteins with high yields in less than 20 minutes.

The new ProteoSpin line of spin column kits for protein clean up from Norgen Biotek in St Catharines, Ontario, is based on a patent-pending technology using modified silicon carbide (SiC) as the matrix rather than the usual silica (SiO_2). SiC has all the benefits of silica resin and more, says Yousef Haj-Ahmad, president and chief executive of Norgen Biotek. The hydrophobic and hydrophilic surfaces of SiC can be exploited directly rather than having to chemically add active sites, as with a silica matrix. "The lack of porosity is an advantage because it enables the purification of a wide size range of proteins," Haj-Ahmad notes. "With silica-based resins, even if they are ion-exchange type, one finds size restrictions for proteins because of the micropores." Also, the unique way that SiC's surface charges allow it to function as an ion exchanger means that salts are not needed to elute proteins in most cases. The first ProteoSpin kits are focused on protein preparation for downstream applications such as mass spectrometry.

GE HEALTHCARE

The next challenge

Many of the large equipment providers are now concentrating on streamlining and speeding up the overall protein-processing workflow, from improving initial sample clarification through to a smoother transition to mass spectrometry, microarray technology or X-ray crystallography. Informatics at the back end also remains a challenge, in terms of analysing the results of large-scale analysis and feeding them back into the production process.

More sophisticated successors to the His_6 and GST tags commonly used in protein purification are also being sought. "Most people are still using the classic protein purification tags that have been around for many years," says Roskey. "There probably need to be some new advances in that area, better ways to grab hold of proteins as you express them. That's something that a lot of people are working on."

But perhaps the biggest challenge is in applying the skills learned with soluble proteins to membrane proteins. "There's not a soluble protein that we can't purify for you," says Simpson. "But once you get to membrane proteins, which are particularly interesting for the pharma industry, they're a nightmare. They're probably key receptors for most drugs but they're designed to be insoluble in physiological structures. How to address membrane proteins is one of the critical bottlenecks. Everyone's putting so much effort into it that it will be solved, and whoever solves it will really hit the pot of gold because it will change the face of our industry and the way medicines are designed." ■

Tim Chapman is a freelance journalist based in Halifax, UK.