Microscale Analysis and DoE

Putting Microplates and Microplate Readers to (More Efficient) Work

by Cheryl Scott

Traditional biochemistry assays use multigram and -liter quantities of reagents; microscale analysis uses milli-, micro-, and even nanograms and –liters of valuable substances such as recombinant proteins. It allows for process optimization and product characterization work to be performed relatively early in a project lifecycle — which is an important part of quality by design (QbD).

Microplates (or microwell plates) are flat labware that incorporate multiple nanoliter- to milliliter-volume depressions (wells) that take the place of traditional test tubes and culture plates. Typically made of molded polystyrene, polypropylene, cycloolefin, and/or polycarbonate, modern plates with 6, 24, 96, 384, 1,536, 3,456, and 9,600 wells arranged in columns and rows in a 2:3 ratio have become a standard tool in analytical research and diagnostic testing. Both disposable and reusable versions are available. Composite designs incorporate filters and membranes for solid-phase extraction (SPE) and other applications or conical wells for such processes as polymerase chain reaction (PCR). Some plates are assembled from separate strips of eight wells each to facilitate partial use. And "array" products are continuous strips of microwells. For cell-based assays, microplate surfaces are modified to provide a surface on which cells can adhere.

Microplate assays can be performed manually or robotically, the latter involving (for example) liquid handlers, plate movers and stackers, and storage incubators, as well as plate readers that detect biological, chemical, and/or physical activity. Widely used in research, drug discovery, bioassay validation, quality control, and manufacturing processes, microplate readers involve detection modes such as absorbance, fluorescence intensity, luminescence, time-resolved fluorescence, and fluorescence polarization. The most common microplate format has 96 wells arranged in an 8 × 12 matrix with typical volumes of 100–200 µL each. Higher-density plates are used in screening applications, in which throughput and assay cost become critical parameters, with typical working volumes of 5–50 µL per well.

Design of experiments (DoE) incorporates statistical methods and multivariate analysis into microscale chemistry. Controlled experiments help analysts evaluate processes with that involve several variables, such as temperature and osmolality in cell culture processes. Often three variables are studied together, with the results expressed in a three-dimensional response-surface graph.

A Little History

Microscale chemistry has been popular for decades in academic settings thanks to its cost-saving benefits. The approach allows students to gain laboratory experience even in developing countries. Life-science companies where many of those students end up working are also keen to save money, of course, especially when using expensive reagents. Throughout the second half

of the 20th century, successful scale-down of processes and experiments showed that even microscale assays could accurately represent many aspects of full-size operations.

The first microplate was invented in Hungary by Gyula Takátsy, who in 1951 machined six rows of 12 wells using the transparent thermoplastic polymethyl methacrylate (PMMA). Before the end of the decade, a molded version was introduced in the United States, and by 1990 more than 15 companies were making and selling a broad range of microplate styles and formats. Millions are used in life science every year now. Because the word *Microtiter* is a registered trademark, we should use the generic term *microplate* rather than the colloquial "microtiter plate." Standardization around the turn of the 21st century has made microplates interoperable and allows scientists to use instruments and equipment from different suppliers interchangeably.

Statistical design of experiments traces back to the 1700s, with scurvy research by English physician James Lind. About a century later, American philosopher, logician, mathematician, and scientist Charles S. Peirce

Multiplate Analysis and DoE in the BPI Archives at www.bioprocessintl.com

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published papers on randomized experiments and probability. He also produced the first English-language publication on an optimal design for regression models (after pioneering work suggested by French mathematician Joseph Diaz Gergonne in 1815). In 1918, Danish mathematician Kirstine Smith continued this progress by publishing optimal designs for sixth-degree polynomials. The related field of sequential analysis built on that work in the 20th century, beginning with Hungarian Abraham Wald working at Columbia University and American Herman Chernoff working at the Massachusetts Institute of Technology (and now Harvard University), followed by Israeli mathematician Shelemyahu Zacks at Binghamton University and American Herbert Robbins at Columbia University (and later Rutgers University). Factorial design helped speed industrial development for the allied forces in World War II, after which a statistician at Imperial Chemical named George Box published a method for generating response surfaces for process optimization. His work partly formed the basis for the first DoE software: Design-Ease from Stat-Ease.

The many suppliers of microplates now include Advanced Microplates, BD Biosciences, Bio-Rad Laboratories, Cole-Parmer, Corning Life Sciences, Eppendorf International, Molecular Devices, Pierce/Thermo Scientific, Perkin Elmer, Qiagen, Seahorse Bioscience, and Whatman. Many of them also offer microplate handlers and readers, as do Beckman Instruments, Berthold Technologies, Biochrom, Bio-Tek Instruments, Dynatech (home of the Microtiter plate), Dynex, BMG LabTech, Promega, Tecan Group, and VMAX — among others.

Software plays an important role in controlling many of those instruments — and an even more important role in DoE statistical analysis. Some programs are available free for downloading in full or trial form. More capable and/or user-friendly software is sold by companies such as Camo, JMP, Mathworks, Minitab, MoreSteam, Noesis Solutions, Productivity-Quality Systems, ReliaSoft, SAS Institute, SigmaZone, Stat-Ease, StatSoft, and Umetrics.

Applications

A perusal of the BPI archives (as sampled in the box herein) will give you a good idea of the many applications for microscale analysis in biopharmaceutical development: from fermentation and cell culture process engineering and development of cell lines and growth media to optimization of separation and purification operations, as well as formulation testing and product characteritation. Different questions are answered using different reader technologies.

Light absorbance is used for enzyme-linked immunosorbent assays (ELISAs), protein and nucleic acid quantification, and enzyme-activity assays. A light source illuminates a sample at a specific wavelength (controlled by an optical filter or monochromator), and a light detector on the other side of the clear microplate measures how much of that light makes it through the sample.

Fluorescence intensity (FI) detection offers a broader range of applications using a more expensive instrument. An optical excitation system illuminates samples at a specific wavelength, and they fluoresce, then an optical emission system collects and separates that fluorescence from the initial excitation light (using a filter or monochromator) before measuriung the signal with a detector. The latter technique is the more sensitive of the two and requires fluorescent labeling.

Time-resolved fluorescence (TRF) is similar to FI measurement, but with simultaneous excitation and emission (light emitted by a sample is measured during excitation). This came about in response to background signal interference in FI results. TRF requires lanthanide fluorescent molecules, which emit over milliseconds after excitation; most other fluorescent dyes emit for just a few nanoseconds. So a TRF instrument uses a pulsed light source and measures after each excitation pulse to prevent background interference. Both the instrumentation and reagents are even more expensive, however, and are only compatible with certain applications: e.g., drug screening.

Fluorescence polarization (FP) is also similar to FI, with an optical system that includes polarizing filters along a light path so that samples are excited by polarized light. Because large molecules such as proteins rotate slowly because of their size, they will emit polarized light when excited with polarized light. But fast-rotating small molecules depolarize that signal. The plate reader measures the polarity of emitted light: lower levels indicates freer movement of molecules in a sample. So FP detection is good for molecular binding assays, which show whether small fluorescent molecules have bound to larger, nonfluorescent molecules.

In **luminescence detection**, light emitted by samples is the result of chemical reactions rather than light excitation of fluorescent labels, so luminescence plate readers require only a light detector (no light source). Common applications include luciferase-based gene-expression assays as well as cell viability and cytotoxicity assays based luminescent detection of adenosine triphosphate (ATP). Finally, companies such as Alliance Protein Laboratories, Avid Nano, Beckman Coulter, Brookhaven Instruments, Harbinger Biotechnology and Engineering, HORIBA, Intertek, LightIntegra Technology, Malvern Instruments, Particle Technology Labs, Retsch Technology, and Wyatt Technology specialize in static and dynamic **light-scattering detection**. The latter is of particular utility in particulate detection and impurity testing.

Many **modern instruments** combine two or more of those detection methods into one multimode plate reader. Such systems can be used for ELISA; protein/cell-growth assays; nucleic-acid quantitation; molecular interaction and enzyme activity studies, testing for cytotoxity and cell viability/proliferation; ATP quantification; immunoassays; and high-throughput screening. Other recent variations are label-free analysis, high-throughput imaging, and high-content screening. And the enzyme-linked immunosorbent spot (ELISPOT) colorimetric assay requires its own detection technology.

Getting Together

Because microscale analysis and DoE have become so integral to bioprocess and product development, you'll find applications of both discussed at most any conference that covers analytical and bioanalytical topics, from the Cambridge Healthtech Institute's annual "Protein Engineering Summit" (PEGS) to the CASSS analytical meetings to IBC Life Sciences' annual conferences for oligonucleotides and peptides, biological assays, cell line engineering, vaccine development, enzyme technology, formulation and delivery strategies, well-characterized biologics, process and product variants, and antibody engineering — and of course the BPI conference and exhibitions in Europe, Asia, and the United States.

If you're looking for something more specific, you might like to check out the annual International Symposium on Microscale Bioseparations (the 29th was in Charlottesville, VA, this past March) and the "Asia Pacific Symposium on Microscale Separation and Analysis" (www.apce2013.org, 3–6 November 2013). And "Bioprocess Miniaturization: Development and Optimisation 2013" will be held in London, UK, on 26 November 2013 (www.regonline.co.uk). Also, instrument/software companies such as Beckman Coulter often hold their own events specific to the techniques in which they specialize. Finally, a number of mathematics/statistics departments of well-known academic institutions present conferences on experimental design and results analysis every year.

Cheryl Scott *is cofounder and senior technical editor of* BioProcess International, *1574 Coburg Road #242, Eugene, OR 97401; 1-646-957-8879; cscott@bioprocessintl.com.*