New Developments in Microplates for Biological Assays and Automated Sample Preparation

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This month's "Sample Preparation Perspectives" reviews some key considerations in the manufacture and use of microplates, particularly the 96-well plate, which has revolutionized laboratory automation and permits reduction of labour and sample, reagent and solvent requirements.

A microplate is a flat plate, tray, panel or dish containing multiple test tubes arranged in rows. The number of test tubes, commonly known as "wells," has gone from the standard 96 to as many as 9600, which now brings the microplate into the nanoplate range. The first microplates, sometimes called micro-well plates, were attributed to G. Takatsy of the National Institute of Public Health (NIPH), Budapest, Hungary, who was looking for a way to speed up diagnostic testing of hundreds of patients during a severe outbreak of influenza during the mid-1950s. His handmade plates had 72 wells (6 × 12 wells) that he later machined into 96-well "V" bottomed acrylic plates with 8 × 12 wells arranged in a rectangular format. Several years later, after improved designs were made at the National Institute of Health in the United States, the microplates were commercialized by Dynatech Laboratories (now Dynex Technologies, Chantilly, Virginia, USA) as microtitre plates. Although 100- and 120-well versions were introduced, with the development of plastic moulding techniques, the 96-well microtitre plates quickly became the standard format. With the development of microdiluters and later multichannel pipetters, this dodecimal format became a standard in serological testing for a wide range of assays such as enzyme-linked immunosorbent assays (ELISAs) and kinetic measurements, DNA sequencing, polymerase chain reaction (PCR) testing and more recently, high-throughput screening.

High-throughput screening is an approach to drug discovery that has been gaining in popularity. The goal of high-throughput screening is to accelerate drug discovery by screening large libraries composed of hundreds of thousands of compounds that are potential drug candidates. Hence, approaches to handling large numbers of samples are needed on a daily basis.

Although a number of variations of the 96-well plate came to the market, unlike high performance liquid chromatography (HPLC) column endfittings and solid-phase extraction (SPE) cartridges, there has been a gradual acceptance of a fixed dimension format for not only 96-well plates but for other uniform well arrangements such as the 384-well (16 × 24 wells) microplates. Driven by the Society of Biomolecular Screening (SBS, Danbury, Connecticut, USA), standards have been established for the design of microplates and have been approved as universal industry standards by the American National Standards Institute (ANSI, Washington DC, USA). The four standards — footprint, (5.030 in. lengthwise and 3.365 in. across), height, bottom outside flange dimensions and well positions — should result in more efficient, cost-effective automated use of microplates. Previously, scientists would have to programme a liquid-handling instrument for every microplate on the market. Now, if plates meet the ANSI–SBS standard, results should be more consistent across platforms and laboratory costs reduced. Typical 96- and 384-well microplates are depicted in Figure 1.

This month's "Sample Preparation Perspectives" will review some of the more recent developments in microplates with emphasis on the 96-well plate, which, by far, has the most usage in automated sample preparation. A large number of 96-well microplates are available for filtration, SPE, liquid handling and other similar uses.

Microplate Instrumentation

With the development of standard microplates being used for a wide range of assays, instrumentation to handle plate manipulation continues to grow. Microplate liquid dispensers, incubators, robotic handlers, shakers, washers, centrifuges, vacuum manifolds, evaporators and readers are just a few of the instruments that have been developed specifically to handle the 96-well plates. In their 1998 article on automated sample preparation, Smith and Lloyd[2] covered a wide variety of peripheral devices suitable for use with the microplates available at that time, and Wells[3] updated this article with SPE automation devices. A recent book by Wells[4] further updates the available instrumentation and therefore, this topic will not be covered here. This book is highly recommended for those who are involved with automated sample

preparation in a pharmaceutical environment.

The successful adaptation of the 96-well microplate serves as a practical example of the power of miniaturization. Needless to say, for a high-throughput laboratory, the convenience, the labour savings, the savings of chemicals and valuable sample, and the turnaround time of performing automated assays in such a compact format has paid big dividends. The purchase costs of robotic plate handling systems can often be recouped in a matter of weeks or months when compared with manual handling of large numbers of samples. As the format density continues to increase, even bigger savings should result. Already, many laboratories are turning to the 384-well plates, but as will be pointed out later, sometimes method protocols must be changed when upgrading from 96- to the 384-well environment.

Microplates for Liquid Handling

The original 96-well microplates started out having flat bottom wells that quickly gave way to other changes within the wells themselves. The "U" bottom wells emerged so it was easier to to facilitate mixing within the well and to retrieve smaller amounts of material. To better concentrate the sample, "V"-shaped wells were designed so that small amounts of material could be retrieved or solvent more easily evaporated for reconstitution. It was found that optical measurements of absorbance, fluorescence, turbidity and so on could be performed directly at the base of the well, so the "C" design became a compromise between the "U" bottom (better mixing) and the flat bottom (better optical reading). Today, there are a wide variety of well designs with many more shapes than the test tubes they replaced. In addition to the shape of the base, there are other design features that improve the performance such as spaces or cavities between the wells to prevent cross contamination, raised rims to aid in sealing, barcoding of plates for chain of custody tracking, microplate construction materials for better sample–solvent and temperature compatibility and coloured plates (e.g. black) to cut down on stray light for better fluorescence–luminescence reading.

Most microplates are constructed from plastic because it can be easily moulded into the variety of well shapes discussed earlier. The first plates were constructed of acrylics but as the technology developed, polystyrene, polypropylene, polycarbonate, polyvinyl chloride, nitrocellulose, glass fibre, polyvinylidene fluoride and even reusable glass microplates were made available commercially. The latter plates were more expensive but provided an interior surface that could be made more sample-friendly, and the glass base gave superior optical characteristics compared with clear plastics. In addition, composite plates such as polypropylene with a UV-transparent glass base were developed. With such UV-transparent plates, UV/vis-capable microplate readers sometimes allow direct measurement of UV spectra of the well contents at wavelengths as short as 190 nm.

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With various construction materials, it was found that some plastics worked better than others for specific applications. For example, the binding and wettability characteristics of various plastics gave rise to speciality microplates, some with surface modifications. For example, polycarbonate plates were found to keep dilutions in suspension, ideal for serology and antibiotic-susceptibility assays.[5] Tissue culture applications required modified surfaces that encourage attachment and growth of anchorage-dependent cells. Untreated polystyrene plates provide a hydrophobic surface that binds biomolecules through passive interactions, and immunoassays sometimes require high-binding surfaces that are ionic or hydrophobic in character. Non-binding surfaces such as polystryrene plates with a hydrophilic surface (e.g. with a polyethyleneoxide-like coating) minimize protein and nucleic acid binding at low concentrations, providing increased recovery and a higher S/N in a typical assay.

A big concern with biochemists was that cell growth on a microplate might be different than in a natural environment. The nature of the culture substrate is thought to have a major effect on cell growth and the requirements for serum proteins. Sometimes treatments such as irradiation or vacuum-gas plasma treatment are performed or coatings such as poly-D-lysine, dextran or streptavidin are applied to the plastic surfaces to enhance cell attachment and for better protein binding. For antibody immobilization, coatings such as protein A or G can be used.

For PCR applications, the temperature characteristic of the microplates was deemed to be important because temperature cycling using ovens (Peltier heating–cooling, resistance heating and passive air or water heating) is essential for amplification experiments. The most popular Peltier devices, also called thermoelectric coolers, can produce rapid temperature changes. Microplates must be capable of responding to these changes in a short period of time. Hence, thin-wall PCR microplates were developed to meet these needs. However, with plastic materials, a certain amount of swelling or shrinking might occur during this temperature cycling. Hence, prevention of warping and the ability to maintain well-to-well uniformity is a design challenge. Evaporation can be a problem when using thermal cycling, so the ability to seal the microplate wells is important. Analysts seal wells with silicone elastomer mats, PTFE-coated film or PTFE film. It is important that the sealing devices be chemically pure, show no adherence and allow resealing after penetration. For certain applications, another important requirement is the ability to sterilize or autoclave microplates. Thermal (steam or dry heat), chemical (cold or gas autoclaves) and UV autoclaves require microplates that

Figure 1: Typical 96- and 384-well microplates. (Courtesy of Thermo.)

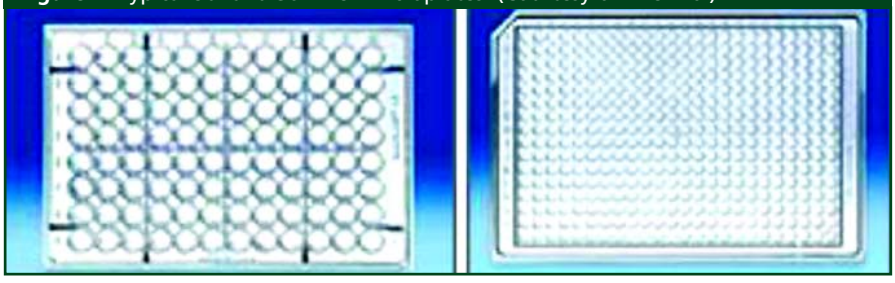
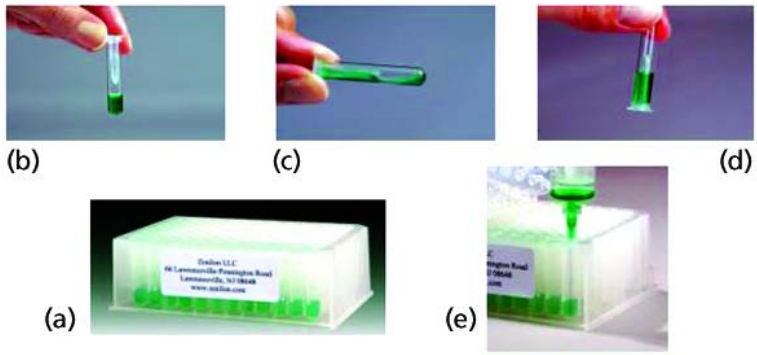


Figure 2: Design concept of the Zenilon Omniplate. (a) Microplate, (b) individual well, (c) well sideways liquid protected, (d) well upside-down liquid protected and (e) depicts well access by needle. (Courtesy of Zenilon).



can stand up to the conditions imposed by the sterilization process.

Although, as already discussed, there are many design features of modern microplates, a particularly novel design is the Omniplate, available from Zenilon (Lawrenceville, New Jersey, USA). The OmniPlate is a spill-proof resealable microplate (Figure 2). The core technology is a sealed insert that creates a liquid barrier within a well or container while allowing full access to the contents by automation needles and tips. Using the "lobster-trap" concept, the insert opening is located near the centroid of the well that protects contents from spilling even if the well is inverted. The liquid-protected insert can be sealed reliably multiple times with a cap plug mat. Compared to open-well plates, this design eliminates cross contamination, allows more vigorous agitation for better mixing, suppresses evaporation and reduces DMSO water adsorption.

One important use of 96-well microplates is to serve as collection vessels for the flow-through SPE and filtration microplates described later. The collection plates are placed underneath the flow-through plates and by vacuum, centrifugation or pressure, samples are eluted into them for subsequent use, for further clean-up or for concentration by solvent evaporation.

384-Well Plates

The 96-well plate has been established firmly as the universal format for high-throughput screening in drug discovery. However, there is a growing interest in reducing assay volumes, increasing sample throughput and increasing sample density, all of which result in lower costs. Hence, the increased interest in the next logical format, the 384-well microplate. With the standardization of the microplate format and with the increased speed, precision and accuracy of liquid-handling workstations, the use of this format is readily adaptable, as has been proven already in many laboratories throughout the world. Most robotic workstations can be adapted to fit this format, eliminating the need to replace existing expensive equipment. Because the footprint of the 384-well microplate is exactly the same as the 96-well microplate, four times the number of samples can be analysed without the need for additional space. Typical square well volumes in this format are in the 100–120 µL range, the surface area inside the well is around 60% that of the 96-well plate, and the bottom area is about a quarter of the 96-well plate.

There are some practical hurdles in preventing simple migration to the 384-well format: lack of assay sensitivity in the smaller volumes, surface tension problems resulting in mixing issues, and format incompatibilities between library compounds stored in 96-well plates and their adaptation to 384-well plates. In downsizing 96-well plate assays to 384-well plate assays, there are problems encountered for which adjustments usually can be made. Liquid delivery rates sometimes must be slowed. For example, polystryene plates are hydrophobic, and the flow of water over the surface at a high flow-rate can trap air bubbles on the surface of the well — bubbles that are hard to dislodge. Sometimes trapped bubbles are difficult to see. Larger trapped bubbles can result in an overfill situation if too large a volume is pipetted into the well. The outcome of overfilled wells is cross-well contamination. Trapped air in a well will distort optical-based measurements. The optimum delivery rates seem to be microplate-dependent, probably because of different internal well dimensions from one manufacturer to the next. Mixing of liquids sometimes takes longer in the smaller well volumes. The mixing volume should be about one half the volume of the well so that adequate mixing occurs. With some 384-well microplates, capillary action (wicking effect) can cause cross contamination. Square wells seem to produce the wicking effect, in which fluid rises up at the corners of the well; round wells do not seem to exhibit this effect.

SPE in the 96-Well Plate Format

In the past, we have given a great deal of coverage to developments in 96-well flow through SPE microplates. Needless to say, a large number of such plates are on the market with just about every stationary-phase chemistry found in SPE cartridges now available in this format. Often, both the SPE cartridges and the SPE well plates are produced by the same manufacturer. In the SPE microplate, packing masses vary from as little as 5 mg per well up to 100 mg per well. The most popular sizes are 10, 25 and 50 mg of packing per well. In each well, the stationary phase can exist as loose packing, usually sandwiched between two small frits or embedded in PTFE or fibreglass discs.

Because 96-well SPE plates are rather expensive (as much as several hundred dollars per plate), many users shy away from using a single plate with a single phase to develop an SPE automated method. In some instances, only a few of the 96 wells can be used. As long as the user keeps track of the used wells, these plates can be used later for analysis or saved for future method development experiments. In the simplest situation, regular syringe barrel SPE cartridges can be used to perform the initial method development followed by transfer of the method to the 96-well SPE microplate. If the user prefers to perform method development using an actual SPE plate, some manufacturers have fabricated method development plates that can contain different stationary phases for a given mode (e.g. reversed-phase plates with CN, C4, C8 and C18 phases).

Another method development approach uses flexible SPE arrays in which individual cartridges that contain a specific stationary phase with a specific mass of material are used (see Figure 3). The user places

individual cartridges in a reusable base plate so that a self-assembled 96-well SPE plate can be used for method development. Users can select as many of the 96 holes in the base plates that they care to use, and the remainder can be blocked off using a sealing strip. A nice feature of the SPE array is that the individual cartridges can be used manually in a regular SPE manifold with the help of a simple cartridge adapter. In the 96-well SPE microplate with the flexible configuration, rows of the same phase with different masses, different phases with the same masses, or any combination can be constructed. Although self assembly of 96-well flexible-array plates is time consuming, users are offered the most flexibility in configuring a method development plate. Once the method is developed, it makes sense to convert the final method to fixed configuration plates with the proper packing and optimum mass per well.

A recent twist on the 96-well plate format was introduced by the 3M Corporation (St. Paul, Minnesota, USA). The Empore SPE card (Figure 4) is a packing-embedded PTFE sheet similar in composition to the company's Empore SPE disc products. The card construction is a multilayer design with an Empore PTFE membrane fused between two porous polypropylene non-woven layers. The card features 96 discrete elution zones. It is designed to work with a Tomtec SPExpress system (Tomtec, Hamden, Connecticut, USA) consisting of two parts: the Harvex module, used for activating, loading and rinsing the card, and the Elutrix module, which elutes analytes from each position of the 96-position card into a mass spectrometer or other measurement device. The Elutrix system is designed for running single cards or unattended operation with as many as 30 cards using stacking cassettes.

Although the 96-well SPE plates are the most popular format, some research groups have investigated the potential for 384-well SPE plates.[6,7] The study by Biddlecomb and colleagues[6] used a packed bed with 5 mg of Oasis HLB phase (Waters, Milford, Massachusetts, USA) per well. They applied their homemade SPE polypropylene plates to an existing LC–tandem mass spectrometry (MS-MS) method for 5-HT antagonist sumatriptan in human plasma. Some practical difficulties were encountered such as the attempted use of vacuum to pull solutions through the tiny packed bed, which resulted in an uneven liquid spray exiting the outlet well tip, potentially causing cross contamination. They elected to use centrifugation as a more gentle liquid transfer method. Also, the absence of 384-well drying systems precluded the dry-down of collected eluents. Although the potential of the plate method was demonstrated, peripheral accessories to support 384-well SPE will be needed to make the technique routine. Rule and colleagues[7] also found that centrifugation was a better alternative to vacuum for these experimental plates. At the current time, to the authors' knowledge, no 384-well SPE plates are commercially available.

96-Well Filtration Plates

Another popular configuration for the 96-well microplate is the filtration plate. The plates contain a membrane filter fixed at the bottom of each well in the plate. The filters are often the same material as that found in disposable syringe filters. These filtration plates now find widespread use in the application of protein precipitation to investigate drugs and drug metabolites in biological fluids. Diluted plasma containing the drug is pipetted directly into the filtration plate well. Acetonitrile, sometimes containing a small concentration of trifluoroacetic acid, is added to the well and with agitation, proteinaceous material precipitates, usually as a bead. The supernatant is filtered through the membrane filter and further analysed by HPLC or HPLC–MS. Although the filtrate obtained from biological samples is not as clean as that obtained from a SPE, the selectivity of MS frequently allows the determination of the drugs and their metabolites at trace levels. Protein precipitation requires almost no method development, while SPE frequently requires several experiments to optimize the conditioning, loading, rinsing and elution steps. It is no wonder that many pharmaceutical laboratories now favour protein precipitation over SPE for analysing drugs in biological fluids.

Supported Liquid Extraction in a 96-Well Plate

Traditional liquid–liquid extraction has been performed in separatory funnels and more recently in vials. This technique has been difficult to automate because one must cleanly separate the immiscible layers, and the technique is plagued by the age-old problem of emulsion formation. The use of supported liquid extraction overcomes most of the problems. Extraction is performed using a cartridge or a well in a 96-well plate that is packed with specially conditioned diatomaceous earth. The aqueous portion of the sample, such as a biological fluid, is applied to this solid support and the aqueous phase is absorbed, leaving the drugs of interest spread out on the surface of the packing in a very thin layer. When a water-immiscible solvent is applied, the analytes, if in their non-ionized form by proper pH control, are eluted efficiently and collected in a test tube or collection plate. The extraction efficiency is very high because of the high surface area at the interface between the organic and aqueous phases. Because the results are similar to traditional liquid–liquid extraction, existing methods can be transferred with minimal method development. Examples of commercial 96-well products that support liquid extraction include Varian's (Palo Alto, California, USA) Hydromatrix (250 mg/well) and Argonaut's (Redwood City, California, USA) Array HM-N plates (200 mg/well).

Other Measurements Performed in 96-Well Plates

The popularity of the 96-well format and instrumentation that goes with it has given rise to the miniaturization of other measurement techniques. I shall describe briefly three of these techniques that might warrant consideration if the number of samples requiring measurement increases beyond your current capability.

Ultrafiltration: A variation of the filtration

Figure 3: The Argonaut EVOLUTE Array SPE plate. (Courtesy of Argonaut).

Figure 4: The 3M Empore SPE card. (Courtesy of 3M).

plate is the use of ultrafiltration membranes in the 96-well configuration. A cut off filter is a porous membrane that will exclude molecules over a certain molecular weight while letting smaller proteins and other molecules pass through. Such a plate can be used for sample purification, concentration and desalting of biological solutions. Centrifugation or vacuum can be used to move the liquid samples through the cut off membrane filter. An example of such an ultrafiltration membrane would be the Millipore (Billerica, Massachusetts, USA) MultiScreen filter plate with a 10 000 nominal molecular weight limit regenerated cellulose membrane. Applications of this plate include parallel protein purification, protein concentration and buffer exchange in cell lysates for subsequent separation or assay.

Nephelometry: In modern drug discovery, the use of laser nephelometry to measure aqueous drug solubility of hundreds of new drug candidates offers many advantages over the traditional equilibrium measurements. Drug samples are routinely supplied in DMSO solutions, and laser nephelometry can use these solutions directly without the need to use the classic methods. Bevan and Lloyd[8] used this technique with 96-well plates to measure the scattered light in each well as the solute precipitates out of solution. This high-throughput technique rivals the HPLC method and, when automated, can potentially, be faster, thereby improving physicochemical property screens of combinatorial compounds.

Surface tension: Surface tension is generally measured by tensiometers, devices that measure the forces at the surface of a liquid. Surface tension measurements are very important in the study of biomembranes and lipids. In these studies, lipid monolayers have paved the way to breakthroughs in the eludication of the molecular mechanisms of the action of lung surfactants, lipid signaling, blood coagulation and cell adhesion, just to name a few examples. Tensiometers detect the penetration of different molecules such as drugs, peptides and proteins into lipid monolayers of specified compositions and lateral packing density. They also allow the rapid and accurate determination of surface activity and the critical micelle concentration of drugs, surfactants and other molecules of interest. An automated instrument developed by Kibron (Helsinki, Finland) has eight small microbalances that can measure the surface tension in liquids placed in 96-well plates in about 2 min. Such a rapid turnaround will aid drug discovery by automating the tedious job of measuring surface tension on large numbers of samples.

Conclusions

The use of 96-well microplate technology has revolutionized high-throughput drug discovery and laboratory automation and has allowed cost savings by reduction in labour, a reduction in sample requirements, reagents and solvent, and has improved overall laboratory productivity. The ongoing interest in the 384-well microplate is being driven by a desire for even more cost savings and higher sample throughput. In order for some of the sample preparation techniques such as SPE and filtration to make better use of this higher density technology, new plate designs and new accessories are needed that can conveniently perform the same tasks that have provided user acceptance of the 96-well plate technologies.

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