MICROPLATE MIXING

bioassay panacea or of unproven distraction?

Despite general awareness of the significance of microplate mixing, it is still a relatively low priority in most laboratories. Microplate mixing has largely been ignored as a serious problem, although potentially it could undermine the generation of meaningful data. In September 2007, HTStec undertook a market survey on microplate mixing to gain a better understanding of the current and future market requirements for mixing technologies that can be applied to compounds or bioassays in microplates. This article reviews some of the findings of that study, such as how big a problem microplate mixing is perceived to be today and where the greatest need for improved mixing exists. It also discusses the microplate formats and applications where there is greatest concern about achieving improved mixing. The drivers for the adoption of mixing technology are presented, together with a review of those mixing technologies (magnetic mixing, orbital shaking, sonication and acoustics) that are available or have recently emerged to address microplate mixing.

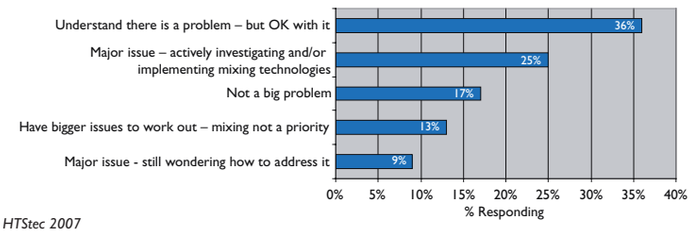
By Dr John Comley

There have been several recent reports in the literature about microplate mixing and mixing technologies[1-3]. One demonstrated that by incorporating a microplate orbital mixing step into high throughput cell-based reporter assays readouts were improved[1]. They suggested that mixing facilitated maximal luciferase signal induction from high cell density containing samples as well as minimising the effects of common sources of variation and thereby led to improved assay precision. What is increasingly evident is that, as assay volumes decrease and miniaturised plate formats are more widely adopted, end users' expectations of the equipment they use for sample preparation and assay assembly increase. Although many methods exist for mixing small volumes, sufficiently rapid mixing in microplates still represents an unsolved and often underestimated or neglected problem[2]. In September 2007, HTStec undertook an end user survey on microplate mixing with respect to compounds and bioassays[4]. One of the main purposes was to understand how big a problem microplate mixing is perceived to be today and where the greatest need for improved microplate mixing exists.

How big a problem is microplate mixing today?

In the survey the majority (36%) of respondents indicated they understood there is a problem with

Figure 1: How big a problem is microplate mixing today?



microplate mixing – but are OK with it. A further 34% of respondents believed microplate mixing was a major issue, of these 25% are actively investigating and/or implementing mixing technologies and the remaining 9% are still wondering how to address the problem. In comparison, only 17% of respondents do not see microplate mixing as a problem today and a further 13% have bigger issues to work out, ie mixing is not currently a priority for them (**Figure 1**).

Most interest in applying microplate mixing

Of various bioassay and compound screening applications survey respondents were asked to rank if they considered mixing assay components (reagents, cells, beads, etc) prior to or at the start of an assay as the one they were most interested in applying improved microplate mixing to. This was closely followed by re-solubilising compounds prior to dispensing into assay plates. The respondents sampled in this study were least interested in mixing of isolated single wells within a microplate and DNA shearing (**Figure 2**). The aqueous media

Figure 2: Where does greatest interest in applying microplate mixing reside?

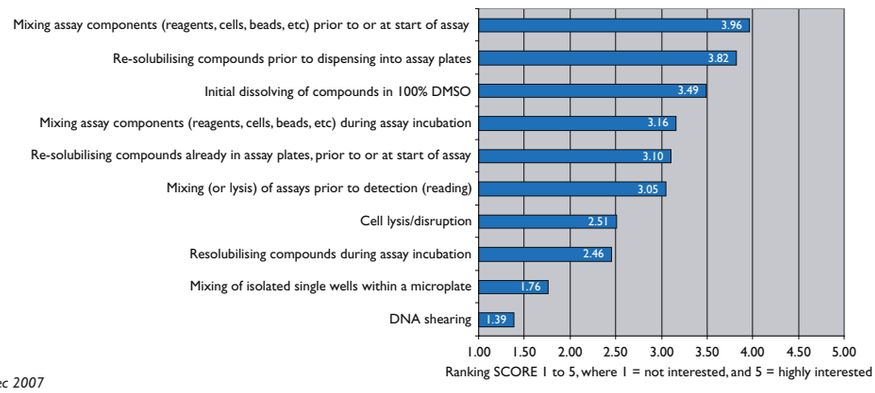
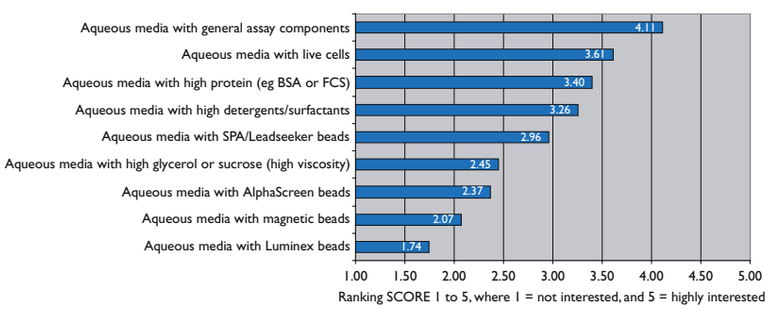


Figure 3: Aqueous media types respondents are most interested in mixing



survey respondents were most interested in applying microplate mixing was general assay components, followed by live cells and then high protein (eg BSA or FCS). Respondents were least interested in mixing aqueous media with some types of assay beads (**Figure 3**).

Microplate formats requiring mixing

The microplate format that survey respondents ranked they were most concerned about achieving improved or adequate microplate mixing in (ie where greatest improvement was needed) was low volume 384-well plates, followed closely by standard 384-well and 1536-well, with least improvement needed for 96-well (**Figure 4**). The main microplate format that the majority (75%) of survey respondents actually wanted to apply microplate mixing to was standard 384-well plates. This is followed by 384-well low volume, 96-well, and then 1536-well plates (**Figure 5**). However, interest in 1536-well plates was slightly higher from those survey respondents that came from Large Pharma.

Microplate mixing technologies

Current use (in 2007) by survey respondents of available microplate mixing technologies was greatest for orbital shaking/vortexing (89% use) and liquid handler pipetting (87% use) and least for magnetic stirring (23% use) and acoustics (12% use). Future plans to purchase microplate mixing devices show greatest interest in technologies based on acoustics (39% purchasing) and sonication (19% purchasing) (**Figure 6**). Interest in purchasing acoustics was particularly high in survey respondents that had compound mixing focus and/or came from Large Pharma.

Concerns about available microplate mixing technologies

Survey respondents ranked 'technology invasive, requires the use of components that must enter the fluid in the well' as their biggest concern about some of the available microplate mixing technologies. The next most important concerns were 'plates must be sealed as mixing causes liquid to move, jump from wells or wick up sides'. Most of the other concerns ranked by respondents were rated of moderate importance, with little difference in the ranking score (**Figure 7**). Of least concern to survey respondents was that the mixing 'technology requires a liquid interface between transducer and the microplate bottom', which is the case for some acoustic methods.

Figure 4: Microplate formats where improved mixing is the biggest concern

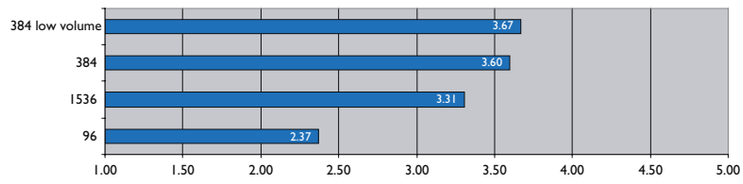


Figure 5: Microplate formats where respondents most want to apply mixing

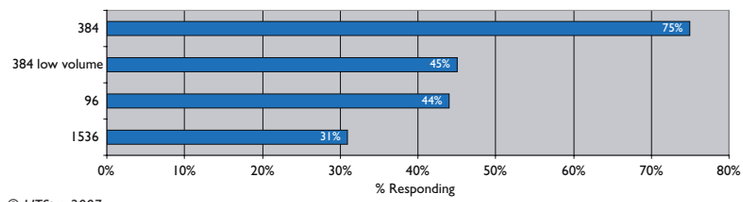
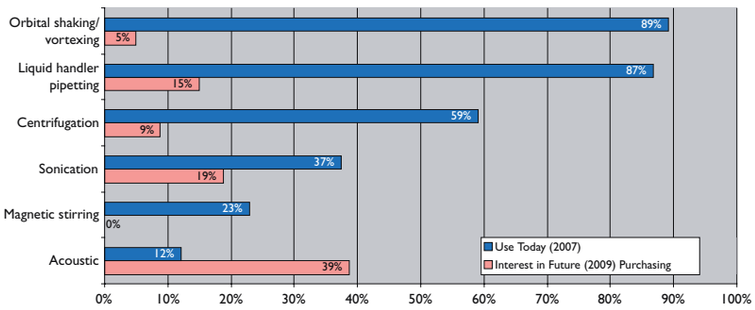


Figure 6: Current use and future interest in purchasing microplate mixing technologies



Drivers influencing the purchase of microplate mixers

'Lower variability – better assay statistics (%CV, Z')' was ranked the driver that most influenced survey respondents' decision to purchase a microplate mixing technology. This was followed by 'non-invasive technology' and then 'promotes enhanced aqueous solubility'. The 'ability to randomly access and mix isolated (single) wells within a microplate' was ranked the least important driver in purchasing decisions (**Figure 8**).

Figure 7: Biggest concerns about available microplate mixing technologies

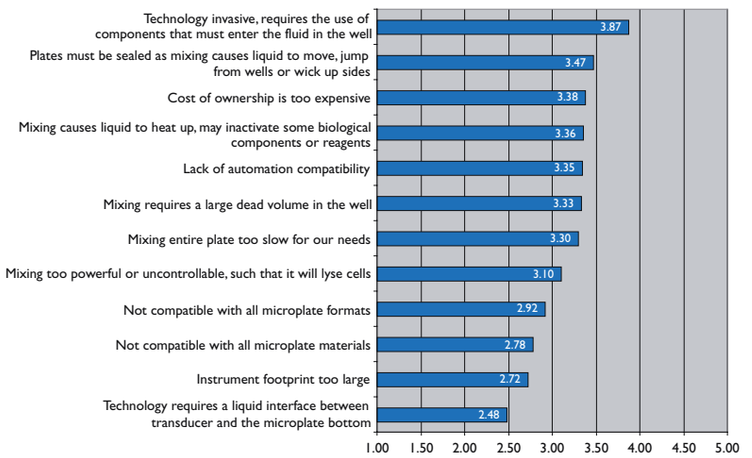
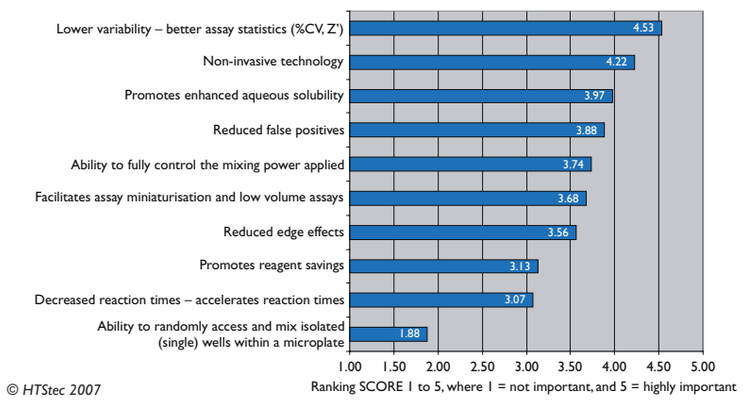


Figure 8: Drivers most influencing decision to purchase microplate mixing



Vendor updates

In the following updates we review some of the mixing technologies and solutions vendors are developing and applying to microplate mixing. **Table 1** summarises the products reviewed, the underlying technology and its suitability for a range of different microplate formats.

Advalytix (www.advalytix.de) introduced the PlateBooster384, a non-invasive acoustic mixer for 384-well microplates, at MipTec 2007. The instrument uses a unique mixing technology based on surface acoustic waves (SAW). The PlateBooster384 is especially well suited for cell-based assays, where minute amounts of compound are dissolved in DMSO and must be mixed with the relatively large volume of cell medium. The advantages of the PlateBooster384 for this application are two-fold: (1) there is no creation of foam or spill since the PlateBooster384 technology is absolutely vibration free. This feature even allows the user to mix the sample while the robot is dispensing the compound; and (2) the cells are not exposed to a high local DMSO concentration, which increases assay stability and reproducibility as the compound is immediately diluted during the pipetting process. The PlateBooster384 can also be used for 1536-well micro plates using an x-y indexing stage. Together with the PlateBooster96 which was introduced last year, Advalytix now offers mixing solutions for all standard microtiter plates formats (**Figure 9**).

The **Covaris** (www.covarisinc.com and www.kbioscience.co.uk) L series (L8 and L800) acoustic plate mixers utilise Covaris's patented Adaptive Focused Acoustic (AFA) technology. To date all of its systems have utilised single point transducers which allow scientists to process one

Figure 9
The Advalytix PlateBooster384, a non-invasive acoustic mixer for 384-well microplates

Table 1: Microplate mixing technologies. *(This table relates only to those technologies discussed in this article)*

VENDOR	WEBSITE	PRODUCT NAME(S)	MIXING TECHNOLOGY	PLATE FORMATS
Advalytix	www.advalytix.de	PlateBooster 96	Surface acoustic waves (SAW), based on 96 transducers, one per well	96-wells, plus 384-well plates via indexer
		PlateBooster384	Surface acoustic waves (SAW), based on 384 transducers, one per well	38-wells, plus 1536-well plates via indexer
Covaris	www.covarisinc.com	L8 Acoustic Plate Mixer	Adaptive focused acoustics (AFA), based on an 80mm linear transducer	All plate formats, density independent
	www.kbioscience.co.uk	L800 Acoustic Plate Mixer	Adaptive focused acoustics (AFA), based on an 80mm linear transducer	All plate formats, density independent
Eppendorf	www.eppendorf.com/platemixing	MixMate®	Orbital shaker, up to 3,000 rpm in controlled in 2-D axis	All plate formats and types
Matrical	www.matrical.com	SonicMan™	Variable sonication, via an interchangeable disposable pinned lid, matched to plate density	48, 96, 384 and 1536-well or custom-formatted lid
Scientific Industries	www.scientificindustries.com/microplate.html	Microplate Genie™	Orbital shaker, up to 3,200 rpm, with small vortexing orbit of 1.0mm	All plate formats and types
Tecan	www.tecan.com	Te-Shake	Software-controlled orbital shaker, with heating options	All plate formats and types
Thermo Fisher Scientific	www.thermo.com	iEMS® Incubator/Shaker	Highly uniform microplate incubator (up to 69°C) and orbital shaker up to 1,400rpm	96-well plates
V&P Scientific	www.vp-scientific.com	Burt Lancaster Trapeze Stirrers	Magnetic stirrer based on 'U' shaped trapeze bar wire	96-well plates
		Sandwich Stir Bars	Magnetic stirrer based on encased neodymium iron boron with protective layers of PTFE and Parylene	96-well plates
		Magnetic Levitation Stirrers	Magnetic mixing by raising and lowering of stainless steel balls in microplate wells	96 and 384-well plates

tube/well at a time. The L8 is a first of its kind linear transducer-based system. Rather then focusing the energy to a single point, a linear transducer provides an 80mm line of acoustic energy. The energy is tunable for providing low energy doses for mixing reagents/cells, or high energy applications such as cell lysis, DNA shearing and compound dissolution. By using a line of acoustic energy that scans along the length of the plate, the L8 provides a non-contact mix that is plate density independent. Plates that meet SBS specifications can be effectively processed in densities ranging from 96 to 3456. The L8 has shown to dramatically reduce incubation times of a cAMP HTRF

Figure 10 (right)
Schematic representation of the linear transducer in the Covaris L Series acoustic mixers. Rather then focusing the energy to a single point, the linear transducer provides an 80mm line of focused acoustic energy (shown here in purple), that scans along the plate

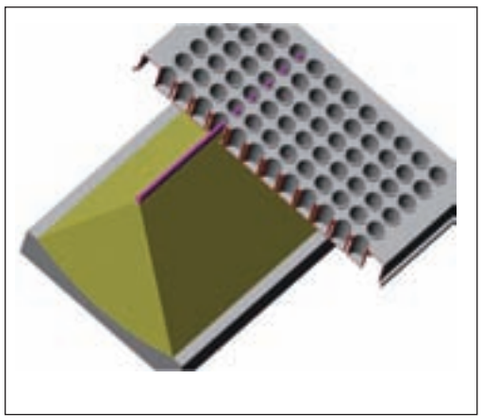


Figure 11 (far right)
The Covaris L8 acoustic mixer provides a non-contact mix that is microplate density independent

assays and Z' values where improved by a factor of 0.2. This phenomenon is due to the acoustic energy accelerating the collision frequencies between both substrate and compound. The use of the L8 for cell lysis applications has also proven to be very effective, especially in higher density formats. Using the L8 for cell lysis eliminates the need for adding cell lysis buffers and thus reduces total assay volumes and liquid addition steps (**Figures 10 and 11**).

All laboratory personnel are aware of the significance of mixing. Complete and effective mixing of all reagents, particularly in small volumes, is a prerequisite for obtaining meaningful data. Due to the need for an effective and reliable plate mixer, **Eppendorf** (www.eppendorf.com) launched in June 2006 MixMate®, an orbital mixer that can accommodate all variations of PCR-plates, microplates and deepwell plates as well as micro test tubes. MixMate provides a mixing frequency up to 3,000rpm and an optimal setting of all mixing parameter leads to a thorough mixing in shortest time. Additionally, the controlled mixing movement, just in a 2-dimensional axis (2D Mix-Control), prevents spilling and lid wetting and thereby cross contamination from well to well. MixMate mixes all kinds of molecular biological samples within one minute, independent of sample properties or plate geometry. Beside aqueous solutions, latest data from Eppendorf prove that MixMate also enables efficient mixing of highly viscous samples. A further important step is the resuspension of solid cell pellets, a labour intensive and time-consuming process. The time required for this step can be shortened considerably. For example, with the help of MixMate bacterial pellets in 96 well, deepwell plates can be resuspended in as little as 30 seconds. Owing to the fact that most of the time complete mixing is largely invisible to the naked eye, Eppendorf has put a lot of effort into proofing the outstanding mixing performance of MixMate. These results are published in several 'Application Notes' that can be downloaded from Eppendorf's animated MixMate microsite (www.eppendorf.com/platemixing) (**Figure 12**).

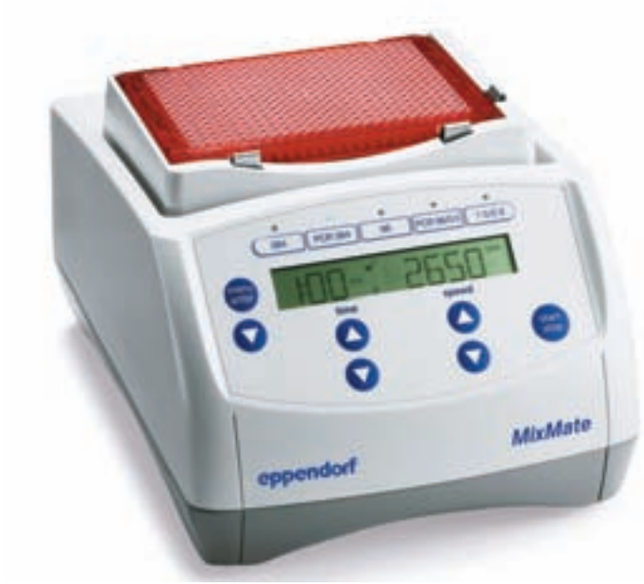


Figure 12: Eppendorf's MixMate can effortlessly fulfill your highest expectations in effective plate mixing. More detailed information is available at www.eppendorf.com/platemixing

The **MatriCal** (www.matrical.com) SonicMan™ is a bench-top instrument that employs sonication on a massively parallel scale to enable processing in microplate formats (48, 96, 384, 1536, or custom formatted). The unit is routinely used to ensure low volume homogeneous mixtures are achieved and removal of air bubbles introduced by pipetting steps (eliminating the need for centrifugation),

Figure 13
The MatriCal SonicMan™ is a bench-top microplate sonication device

with the added advantage of fast parallel processing. Shock waves and cavitation induced 'solvent flow' are the likely mechanisms that allow sonication to significantly increase the rate of mixing to achieve homogeneous mixtures and thereby ensure optimal testing conditions in drug screening. To integrate sonic energy with high-density microplate processing a multi-probe sonicator has been developed. This technology relies on a patented cost-effective disposable pinned lid multi-probe technology. The inert customisable length pinned lids are placed on top of a microplate providing a gasketed silicone aerosol tight seal and allowing for simultaneous sonication of all wells of a microplate. The instrument itself is fully automatable and includes an extendable plate shuttle enabling easy integration with stacking devices or pick and place robotics. With these attributes microplate sonication can now be performed in a controllable uniform manner allowing for precise energy input and uniform mixing and dissolution. Additional benefits of sonication include re-solubilising DMSO stored or aqueous diluted compound precipitation prior to screening campaigns, generating reliable results comparable to shake-flask aqueous solubility values but achieved in only 10 to 20 seconds. Other benefits include the mixing of small volumes in high surface tension 384 and 1536 format plates. Additional applications include the lyses of mammalian or bacterial cell membranes for the extraction of target proteins, shearing/sizing of RNA/DNA, and many more (**Figure 13**).

The MicroPlate Genie™ from **Scientific Industries** (www.scientificindustries.com) provides high speed action for increased mixing efficiency in every well of the microplate. The mixer has an aggressive mixing speed from 750 to 3,200rpm for complete and uniform mixing in any microplate format. Its small vortexing orbit of 1.0mm provides thorough mixing regardless of sample viscosity. Combination of high speed and small orbit combine to offer true vortexing. The Genie accepts most microplate types within the recommendations of the Society for Biomolecular Screening (SBS), even 384-well plates. The mixer also features a built-in timer which operates from 1-60 minutes or continuous for hands-free use (**Figure 14**).

Figure 14: Scientific Industries Microplate Genie™ Micro (left) and Multi (right) are orbital shakers with a unique vortexing action

The Te-Shake from **Tecan** (www.tecan.com) is an orbital mixer for microplates, deep-well plates and microfuge tubes, with software-controlled shaking and heating options. The module is available in a variety of configurations to address specific requirements, such as multiple parallel microplate shaking, and is suitable for wide-ranging applications, including cell lysis processes, bead-based extraction methods, cell metabolism assays and solubilisation and homogenisation tasks. The Te-Shake's fully adjustable shaking speed and rotation settings ensure that optimal mixing results will always be achieved, even for delicate resuspension tasks. The module is fully compatible with Tecan's automated liquid handling platforms where straightforward operation of the device is provided by pipetting software packages such as Freedom EVOware®. Plates can be automatically transported to and from the shaker module, and the liquid handling arms can pipette into plates while they are on the Te-Shake module (**Figure 15**).

Figure 15: The Te-Shake from Tecan is an orbital mixer for microplates

The iEMS® Incubator/Shaker from **Thermo Fisher Scientific** (www.thermo.com) has been designed with microplate mixing in mind. The iEMS Incubator/Shaker is a microplate incubator with orbital shaking. It can be used for any microplate based assay requiring optimal incubation conditions up to 40°C and can incubate/mix up to nine 96-well plates at once. The iEMS Incubator/Shaker has an individual thermal microplate holder for each microplate. To eliminate temperature gradients and edge effects, microplates are heated evenly from all sides. This ensures reproducibility of the assay wherever the sample is positioned on the plate. The iEMS Incubator/Shaker offers a temperature uniformity of less than 0.3°C across the entire microplate. For effective mixing, the iEMS Incubator/Shaker incorporates a powerful variable-speed orbital shaker. With an orbit of 1.0mm and speeds from 400 to 1,400rpm in 250rpm increments, the shaker motion ensures efficient mixing of even very viscous liquids. Superior temperature control and efficient orbital shaking dramatically increase the sensitivity of EIA assays, as well as reducing incubation times. A high-performance version (the iEMS Incubator/Shaker HT for up to three microplates) is ideal for assays requiring high temperatures up to 69°C, such as DNA hybridisation (**Figure 16**).

V&P Scientific (www.vp-scientific.com) has been providing innovative solutions for microplate mixing for many years. The following are some of V&P's recent mixing developments: (a) The Burt Lancaster Trapeze Stirrers (VP 791) were designed to stir the contents of wells without touching the bottom of the vessel. They consist of a 'U' shaped trapeze bar with a magnetic wire with a tight coil and two arms ('spinner') over the bar. When placed over a VP 710 series Rotary Magnetic Tumble Stirrer the 'spinner' is coupled with the rotating magnetic field and spins thus mixing the contents of the well. This allows for the liquid to be thoroughly mixed without disturbing cells, crystals or

Figure 16: Thermo Fisher Scientific iEMS Incubator/Shaker is a high-performance microplate incubator and shaker offering excellent temperature uniformity across the plate

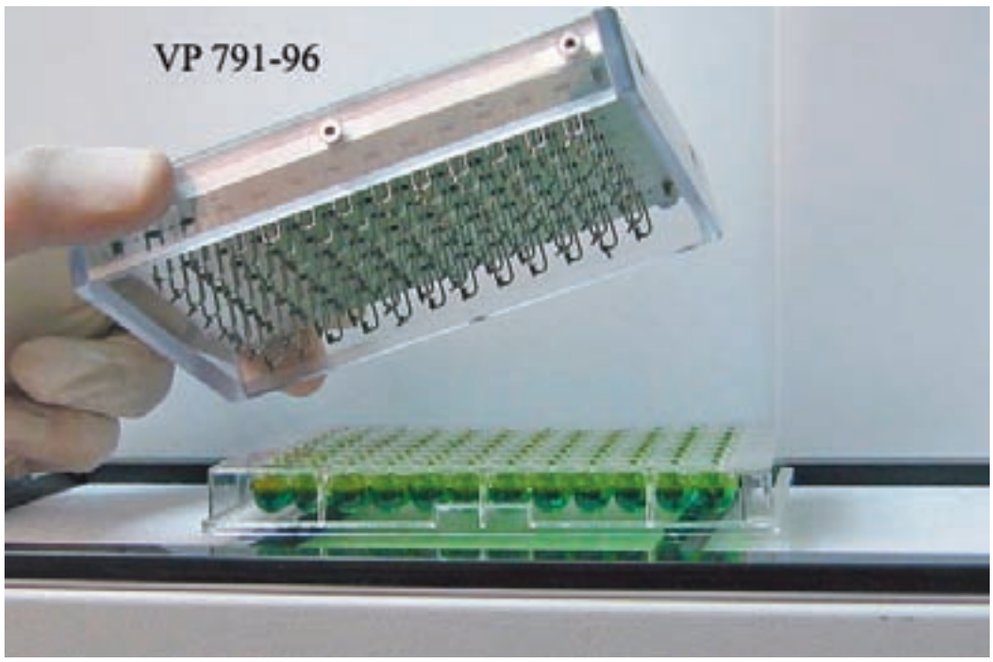
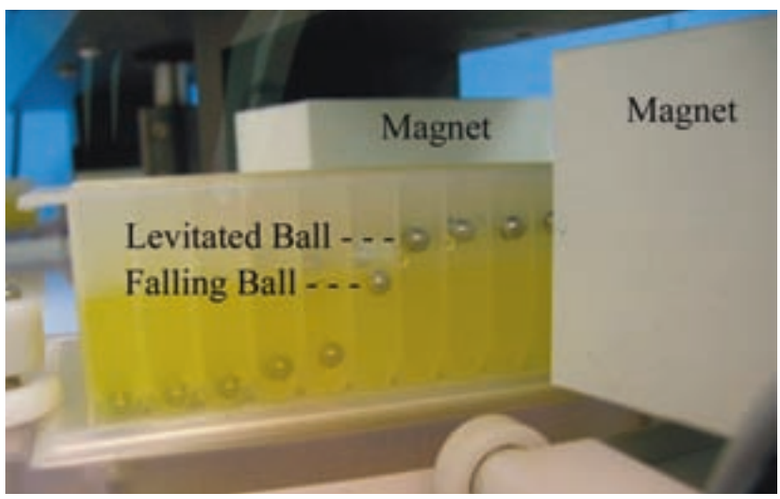


Figure 17: The V&P Scientific Burt Lancaster Trapeze Stirrers (VP 791-96) for 96-well plates

membranes on the bottom of a well; (b) V&P has developed a new Sandwich technique for encasing neodymium in protective layers of PTFE and parylene. This ability allows for the production of very powerful neodymium iron boron (NdFeB 48 MGO) stir bars and other shapes that are now capable of greater coupling to the drive magnet and thereby achieving higher stirring speeds for a more rapid mixing action and the stirring of extremely viscous solutions. When coupled with the VP 710 Series of Alligator Tumble Stirrers or VP 708 Series Vortex Stirrers you get better, faster, stronger and uniform mixing throughout the entire plate; and (c) V&P's Magnetic Levitation Stirrers are as an efficient and inexpensive method to aerate, resuspend DNA released from lysed cells for sequencing, and mix the contents of deep well 96 and 384 microplates. This is done by simply raising and lowering stainless steel balls in all the wells using a very strong horizontal magnetic field. This method produces a vigorous stirring action which stimulates growth of micro-organisms, mixes with ease two or more liquids, and keeps particulates in suspension. Furthermore, if the media level in the wells is adjusted so the stir balls pass through the meniscus, aeration of a microbial culture is



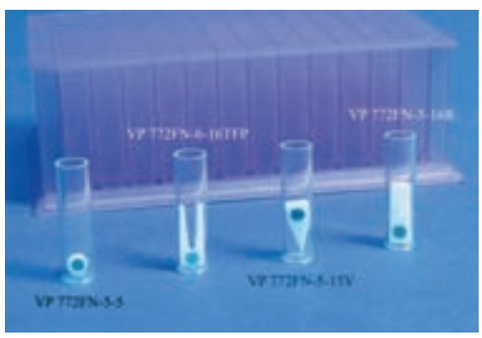


Figure 18 (above): The V&P Scientific Sandwich Stir Bars for microplate wells, made by encasing neodymium iron boron in protective layers of PTFE and Parylene
Figure 19 (left): The V&P Scientific Magnetic Levitation Stirrers promote mixing by raising and lowering stainless steel balls in microplate wells

increased and results in greater microbial yield as well as DNA and protein production (**Figures 17-19**).

Beyond the microplate world, microfluidic technologies are now emerging to facilitate mixing. For example **Dolomite** (www.dolomite-microfluidics.com) is developing technology that allows the rapid mixing and analysis of chemicals, helping instrument manufacturers worldwide to create faster systems that will accelerate drug discovery. Dolomite's Micro Mixer chip is a glass device that enables the rapid and real-time analysis of fluids. Originally developed for dilution of samples prior to UV analysis, it has also been supplied to customers for nanoparticle synthesis where rapid mixing is important to achieve uniform size or surface properties of the nanoparticles. Key to the development of the Micro Mixer is the use of microfabrication techniques capable of creating microchannels and complex structures in the glass. The main fabrication processes are photolithography, wet etching of microchannel structures, micro-drilling of fluid ports, thermal bonding and surface modification. Dolomite's capability to undertake traditional precision glass processing techniques such as mechanical and optical polishing, grinding, cutting and dicing also enables them to extend the range of geometries that are possible. The Micro Mixer is a standard Dolomite product, which is currently manufactured in glass. As demand grows, the engineers at Dolomite anticipate that future manufacture will also be in Polymer depending on the application and the type of solvents and fluids used (**Figure 20**).

Summary

The survey findings suggest that, overall, achieving adequate microplate mixing is still a relatively low priority and continues to be ignored as a serious problem by a significant proportion of laboratories. This situation is not helped by the fact that in most everyday applications complete mixing in microplates is largely invisible to the naked eye and not readily measured. Until more case studies (such as [1]) demonstrating tangible benefits from using microplate mixing are published, this looks destined to remain the status quo. However, user expectations on the benefits they hope to derive from improved mixing are very high, ie mainly related to increasing assay quality and robustness (more accurate, consistent and reproducible results); reducing reaction times (faster kinetics); improving compound solubilisation (avoiding compound precipitation and aggregation); and

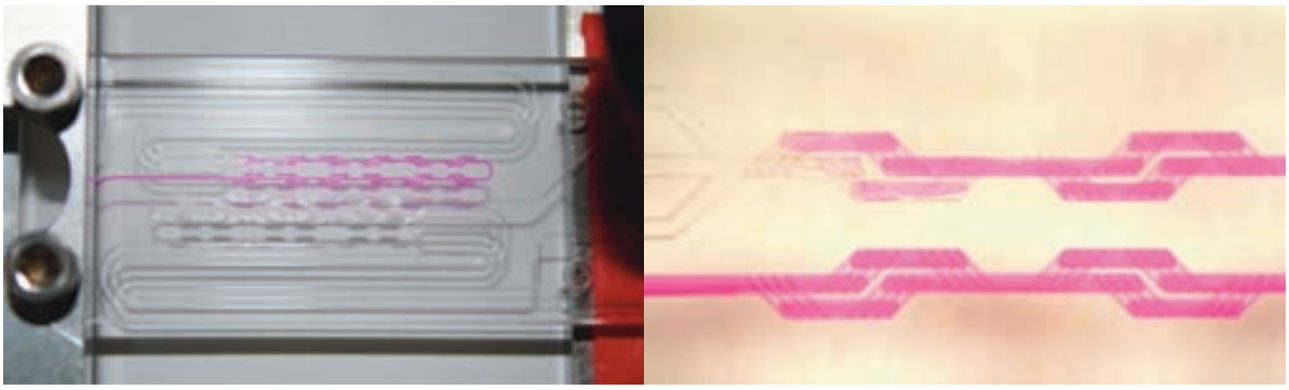


Figure 20: Dolomite Micro Mixer and holder within microfluidic system (left image). Close up of the Micro Mixer with phenolphthalien and base solution mixing in 5 milliseconds (right image)

References

1 Hancock, MA et al (2007). Microplate orbital mixing improves high-throughput cell-based reporter assay readouts. J. Biomol. Screen. 12(1):140-144.

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3 Kirby, S et al (2007). Benefits of Covaris AFA ultra sonication on the HTS process from compound master to HTS assay plate. Poster presented at Joint ELRIG-SBS Meeting Nottingham, UK, 2-3 October 2007.

4 Microplate Mixing Trends 2007 Report, published by HTStec Limited, Cambridge, UK, September 2007.

reducing target or substrate adsorption on to plastics. Many labs would give active consideration to the wider implementation of mixing if user testimonials were more prevalent, if the technology available performed and if the downsides (eg liquid wicking up the walls of microplate well, spilling or jumping or splashing, lid wetting, requirement to seal plates, heat transfer, invasive method etc) of the mixing process were minimised. In this review we have highlighted a number of different types of mixing technology; however they all have their pros and cons. In some lab situations invasive methods like those provided by V&P Scientific (magnetic mixing) and Matrical (sonication with pinned lid) are to be avoided (eg due to concerns related to contamination and sterility), although pricing the invasive mixing components as very cheap disposable items does negate this downside to some extent. Other approaches based around orbital shaking (eg Eppendorf, Scientific Industries, Tecan and Thermo Fisher Scientific) undoubtedly are both simple and highly cost-effective non-invasive ways to address microplate mixing, but they are all prone to some extent to cross-contamination issues. Perhaps the most promising approaches are the recent developments in acoustics technologies (eg Advalytix SAW and Covaris AFA). For not only are these non-contact methods, but they are less liable to generate vortexes with the resulting possibility of reagent creep or ensuing foam, they are very rapid, give more effective mixing and the energy delivered to the wells is tunable (controlled) so that is possible not to damage cells or at the other extreme to effect complete cell lysis. However, low energy acoustic waves are absorbed by some plates type (eg polypropylene), rendering them unsuitable for use with some type of acoustic technology, and the high energy acoustics needed for the re-solubilisation of compounds in high density storage formats is not possible without splashing[3]. In conclusion, more effort needs to be put in by the manufacturers of these technologies, in collaboration with end users, to understand the true value of microplate mixing and how best to utilise and exploit the potential benefits that microplate mixing technologies could yield. DDW

***Dr John Comley** is Managing Director of HTStec Limited, an independent market research consultancy whose focus is on assisting clients delivering novel enabling platform technologies (liquid handling, laboratory automation, detection instrumentation and assay reagent technologies) to drug discovery. Since it formation in 2003 HTStec has published 30 market reports on drug discovery technologies and Dr Comley has authored 21 review articles in* **Drug Discovery World**. *Further information on accessing the market report 'Microplate Mixing Trends 2007' can be obtained by visiting www.htstec.com or by emailing john.comley@htstec.com to receive a free copy of the Report's Executive Summary and Table of Contents.*