Luminex®

Whitepaper

Automation of xMAP® Technology-Based Multiplex Assays

Tomasz Zborowski, M.Sc

Introduction

Automation provides a number of benefits to clinical, industrial, and research laboratories, including elimination of human errors, improvement in consistency, minimization of contamination, increased throughput, decreased costs, and reduced hands-on time. At the same time, multiplex assays offer the benefits of more data per sample, faster reaction times, reduced sample consumption, enhanced scalability, and a number of additional benefits due to elimination of repetitive steps.

Integrating laboratory automation with multiplex assays provides significant efficiencies to workflow processes, especially when a large number of samples are being analyzed, or when a highly multiplexed assay is being developed. Automation can be partially or fully implemented. For example, a low test volume lab may automate only the washing steps of an assay, whereas a core facility or high volume testing lab may want to automate all assay steps—including incubation, microplate washing, thermocycling, and microplate shaking—into a single, walkaway system. Multiple automation criteria should be considered for each lab, such as plate format, speed of liquid handling, deck capacities, and system and software upgradability for future considerations.

Luminex® xMAP® Technology is designed for multiplex assays in clinical, industrial, and life science research labs that require fast and repeated assays of multiple targets. The platform allows multiplexing of up to 500 unique assays within a single sample. High sample throughput is needed in diagnostics reference labs or during clinical trials. High throughput is also needed during drug screening stages, although multiplexing is also an advantage during such processes. A clinical reference lab may test thousands of samples per day in low to moderate multiplex, while higher multiplexing may be needed in a research lab testing dozens of targets per sample. Flexibility to customize assay steps for both the throughput and multiplexing needs of each individual lab is a distinct benefit of xMAP Technology.

Microsphere or bead-based arrays offer advantages such as reduction of sample consumption and hands-on time, optimizing efficiency during assay development and performance. Having the capability for both protein and nucleic based applications on a single platform is another advantage for labs with limited bench space for multiple equipment footprints. Unlike traditional immunoassays or nucleic acid arrays, xMAP bead-based assays occur entirely in a liquid medium without having to interact with solid-phase substrates. This makes them ideal for rapid reaction times and liquid handling automation.

As evidenced by the more than 27,000 publications to date, xMAP Technology has become the most widely used multiplexing technology. Concerns such as uniform dispensing of beads, well-to-well carryover, and minimization of bead loss during transfers are easily mitigated with a robust automation protocol. The aim of this white paper is to provide examples of automation solutions and how these have been implemented into labs using xMAP Technology.

Semi-Automated Liquid Handling Workstations

Multiplex assays offer a number of advantages over singleplex assays, but require a number of upfront preparation steps before a fully automated protocol can be implemented. Liquid handling robots not only have the advantage of improving output efficiency during sample processing, but also during disconnected preparatory steps. Those individual processes can each be carried out equally well by appropriate semi-automated equipment that fits quickly and easily into an existing routine. xMAP Technology itself is a compilation of various stages of work, including bead coupling, coupling confirmation, plate preparation, reaction incubation, and washing steps, all of which can be partially or fully automated.

Standardizing an assay to microplate size (96- or 384-well format) is an important first step in reducing both reaction volume and total assay costs. High throughput single or multichannel pipette dispensers, currently available on the market, are designed to dispense very precisely measured quantities of any suspension into different microplate formats. The core elements of these robotic systems are a liquid handling arm that is moveable along the x- and y-axes, a pipetting head mounted on the forefront of the arm moveable in the z-direction, and pipetting channels that transfer the liquids from tubes, reservoirs, and plates into the pipetting tips.

Automated Liquid Dispensing Systems

Automated dispensers range from single-channel instruments (one volume at a time) to multichannel dispensers capable of dispensing up to 384 aliquots simultaneously (Table 1). Software-controlled sample dilution and multiple plate preparations enable scale up of xMAP protocols for routine operation with greater reproducibility than manual procedures. Liquid handling robots may be complemented with innovative options such as tip loading for serial 96/384-well dilutions (including 96/384 interchangeable pipetting heads), plate heating and cooling, orbital shaking, and barcode reading. Some automated dispensers are easily operated under a laminar flow hood without requiring connection to a computer (e.g. VIAFLO 96/384 from Integra Biosciences Corp.). These single and multichannel dispensers can be easy implemented into a workflow for a series of sample dilution steps and a multitude of plate preparations, prior to the addition of coupled microspheres to an xMAP Technology assay.

Examples of Commercially Available Single and Multichannel Pipette Dispensers for Semi-automated Workflows

Table 1a. Single semi-automated pipette dispensers

Company	Product Name	Plate Format
BioTek®	MultiFlo™ Microplate Dispenser	96-384
Formulatrix	Mantis	96-384
Hudson Robotics	SOLO™	96-384
Thermo Scientific	Multidrop™	96-384

Table 1b. Multichannel semi-automated pipette dispensers

Company	Product Name	Plate Format
Agilent Technologies	Agilent™ Bravo™ Automated Liquid Handling Station	96-384
Apricot Designs	i-Pipette/Pro	96-384
Aurora Biomed	VERSA 10/110/1100	96-384
BioTek®	MicroFill™ Microplate Dispenser	24-96-384
CyBio	Cybi®-FeliX	96-384
Formulatrix	Tempest	96-384
Gilson®	Quad Z-215 Liquid Handler	96-384
Hudson Robotics	Micro10x™	96-384
INTEGRA Biosciences	VIAFLO 96/384	96-384
Rainin	Liquidator™ Manual Pipetting System	96-384
Labcyte™	Echo® Liquid Handler	96-384
Thermo Scientific	Thermo Scientific™ Versette™ automated liquid handler	96-384
TOMTEC®	Quadra Tower™	96-384

Automated dispensers have become more widely adopted as they incorporate advanced, multifunction robotic features. It is common practice to continue all washing steps on multichannel dispensers equipped with an integrated plate vacuum filtration station and/or magnetic bead separation deck (e.g. Agilent™ Bravo™ Automated Liquid Handling Platform, Agilent Technologies). Depending on the types of the microspheres used in the multiplexing protocol (MicroPlex® or MagPlex®), laboratories can now perform various stages of xMAP assays under one partially automated, unattended workflow, minimizing human-induced variability.

Some liquid handlers are fundamentally simple dispensers, but also have a built-in robotic functionality that allows multiple microplates to be moved automatically (e.g., Thermo Scientific™ Versette™ automated liquid handler/Thermo Scientific®; Precision™ XS/BioTek®, Quadra4®/TOMTEC®). A robotic arm can shuttle 96- and 384-well plates to and from a plate stacker, which can be configured to hold different numbers of microplates. These multiplate stacker systems are an ideal solution for robust walkaway capabilities, providing an extra microplate workspace and increased laboratory efficiency (Figure 1).

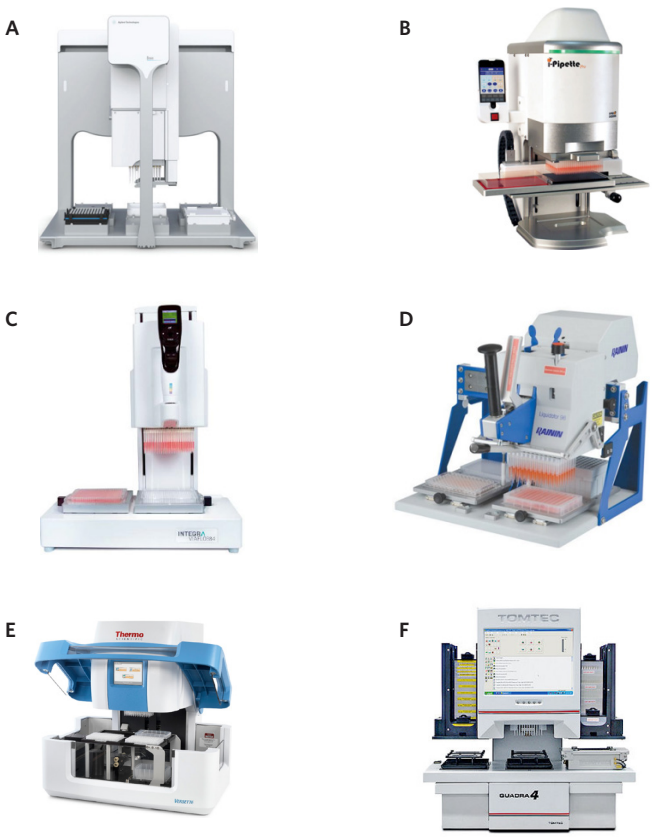


Figure 1
A: Agilent™ Bravo™ Automated Liquid Handling Platform.
B: i –Pipette. **C:** VIAFLO (Integra Bioscience Corp). **D:** Liquidator™ 96 (Rainin®)
Advanced robotic liquid handlers with hotels and plate transport functionality.
E: Thermo Scientific™ Versette™ automated liquid handle. **F:** Quadra4™ (Tomtec®).

A: © Agilent Technologies, Inc. 02/2015. Reproduced with Permission, Courtesy of Agilent Technologies, Inc.
B: i-Pippette is manufactured by Apricot Designs Inc®. For more information, contact them at info@apricotdesigns.com.
E: Thermo Scientific and Versette are trademarks of Thermo Fisher Scientific. Photograph used by permission from Thermo Fisher Scientific

Plate Washing Systems

Instruments dedicated to a single function, such as buffer dispensing, serial dilution, or plate preparation, provide an easy way to incorporate automation for multiplex microsphere-based screening protocols. Patented xMAP Technology uses microspheres as the solid phase for a binding reaction with different types of antigens, antibodies, or oligonucleotides. Compared to ELISA, coupling of capture molecules to xMAP microspheres provides greater surface area and improved distribution of capture molecules throughout the clinical sample. Although the assay itself is carried on the surface of the microsphere rather than on the surface of the microplate (as with ELISA), microplate washing is used to remove sample matrix and unbound materials from the microsphere suspensions between incubations to ensure accurate and reliable data.

Plate washing can have a substantial impact on immunoassay results; therefore, standardizing this process can improve a method's performance across multiple batches. Usually several washing steps are required during an assay run. xMAP polystyrene microspheres (MicroPlex® with a diameter of 5.6µm) are typically separated from the rest of the sample by vacuum-based bead filtration. Likewise, LumAvidin® Microspheres are not magnetic and require vacuum filtration, such as MultiScreen® Filter Plates (Millipore®, Cat. no. MABV N12) used with a vacuum pump system manifold, such as the MultiScreen™ Resist Vacuum Manifold from Millipore (MAVM0960R) dedicated for 96- and 384-well plate formats. The high throughput compatible MultiScreen® HTS filter plates, also available from Millipore, have been specifically designed for use with automated equipment. The plate dimensions are standardized to meet ANSI/SBS 2004 1-4 standard compliance for multiwell plates and are fully integrated with automated gripper arms and further filtration to waste. Rigid sidewalls with moving grippers can also provide ample surfaces for bar codes and plate readers. All of these features are important and should be taken into consideration before the scaling up of any assay.

Automated vacuum filtration systems (96/384-well microplate washers) process either individual rows or full assay microplates much more quickly than manual filtration procedures using separate vacuum manifolds. Unfortunately, sticky and undiluted biological fluids (serum, plasma, saliva, etc.) might easily clog filter plates, and even a single clogged well can cause failure and sample loss for a complete microtiter plate. Leaving the plate on the vacuum apparatus too long between wash steps and the addition of subsequent reagents may allow the plate to dry, and microspheres may adhere to the filter bottom wells or clump.

Magnetic Separation

In order to keep costs low while achieving the highest possible throughput, microplate washers may use the principle of magnetic separation as an alternative to vacuum filtration and plate centrifugation. Second generation xMAP microspheres (superparamagnetic MagPlex with a diameter of 6.5µm) contain magnetic particles, allowing easier separation of the microspheres from the reaction sample and easy automation of the washing steps. These semi-automated devices come in the form of strip washers, full plate washers, and combination washer-dispensers (Figure 2).

Figure 2
A (from left to right): ELx50™ Microplate Strip Washer (low throughput), ELx405™ Microplate Washer (high throughput), EL406™ Microplate Washer Dispenser (high throughput) (Tecan).
B (from left to right): HydroFlex™ (low throughput), HydroSpeed™ (high throughput) (Tecan).

Each washing device has to be specifically pre-programmed for any xMAP-based assay. There are specific requirements for handling protein versus genomic applications that are dependent on the type of assay chemistry used (e.g., washing with use of disposable tips due to contamination risk). Therefore, washing protocols must be optimized for bead separation method (magnetic or vacuum filtration), combination of washer, magnetic carrier, and plate type used. Automation serves to simplify and speed processing, but also tends to improve bead recovery and precision leading to better assay performance.[1]

The **3rd Edition xMAP Cookbook** (Appendix C) provides an example of programming instructions for BioTek® ELx405 and ELx406 microplate washer (magnet P/N 7103016, Dexter LifeSep® 96F technology) for round bottom 96-well plate and MagPlex beads. The wash programs described below were optimized for Tecan washers to achieve optimal bead recovery for multiplex xMAP assays in 96-well plate format with low residual volume per well (Tecan HydroFlex™ washer with Smart-2 MBS magnetic plate carrier and Hydrospeed™ washer with Smart-2 MBS carrier with 96HT wash head; recommended plate: Greiner Bio-one 96-well low bottom thickness of the wells of 0.3mm only, Cat. no. 655096) (Tecan, personal communication).

BioTek achieved high throughput for handling multiple plate batches by simple integration of a microplate washer with the BioStack™ Microplate Stacker (Figure 3). The sensitive gripper arm with rotational wrist exchanges plates between storage stacks and an attached microplate washer in less than ten seconds. The robot allows hands-free processing of up to 50 plates. An efficient alternative for a large screening laboratory might be the PerkinElmer Twister II Microplate Handler for 96-well or 384-well capacity with the Luminex FLEXMAP 3D® high throughput analyzer, which can handle deep well and low profile microplates.

Table 2. Optimized Tecan programs for HydroFlex™ and HydroSpeed™ washers

	HydroFlex™ with Smart-2 MBS Magnetic Plate Carrier	HydroSpeed™ with Smart-2 MBS Carrier and 96HT Wash Head
Wash Program:	Parameters	Parameters
96-well µ-clear plate	Low bottom thickness	Low bottom thickness
Plate Definition File:	[GRE96fb_magbeads]	[MAG_GRE96ft]
Name:	MAG	MAG_96
Cycle 1:	# of cycles: 1	# of cycles: 1
Soak (bead settling)	Time: 90 sec	Time: 90 sec
Aspirate	Mode: normal (one asp. point/well) • z-pos: custom 6mm • Asp. time: 1 sec • Aspirate rate: 1 • Head speed: 8mm/s	Mode: normal (one asp. point/well) • z-pos: custom 6mm • Asp. time: 1 sec • Aspirate rate: 1 • Head speed: 2mm/s
Dispense	z-pos.: overflow • disp. volume: 200µl/well • channel: 1 • disp. rate: 300µl/sec	z-pos.: overflow • disp. volume: 200µl/well • channel: 1 • disp. rate: 350µl/sec
Cycle 2:	# of cycles: 1	# of cycles: 1
Soak	Time: 60 sec	Time: 60 sec
Aspirate	Mode: normal (one asp. point/well) • z-pos: custom 6mm • Asp. time: 1 sec • Aspirate rate: 1 • Head speed: 8mm/s	Mode: normal (one asp. point/well) • z-pos: custom 6mm • Asp. time: 1 sec • Aspirate rate: 1 • Head speed: 2mm/s
Dispense	z-pos.: overflow • disp. volume: 200µl/well • channel: 1 • disp. rate: 300µl/sec	z-pos.: overflow • disp. volume: 200µl/well • channel: 1 • disp. rate: 350µl/sec
Cycle 3:	# of cycles: 1	# of cycles: 1
Soak	Time: 60 sec	Time: 60 sec
Aspirate	Mode: normal (one asp. point / well) • z-pos: cust. 5.5mm • Asp. time: 1 sec • Aspirate rate: 1 • Head speed: 5mm/s	Mode: normal (one asp. point / well) • z-pos: cust. 5.5mm • Asp. time: 1 sec • Aspirate rate: 1 • Head speed: 1mm/s

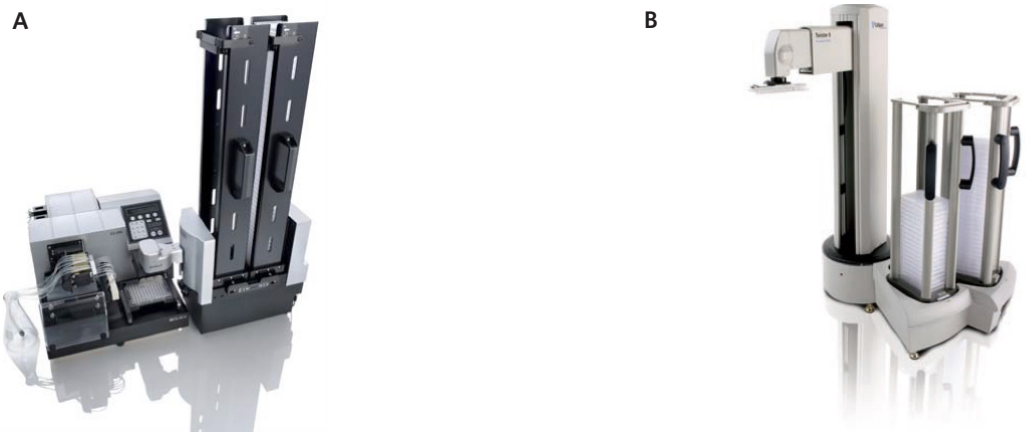
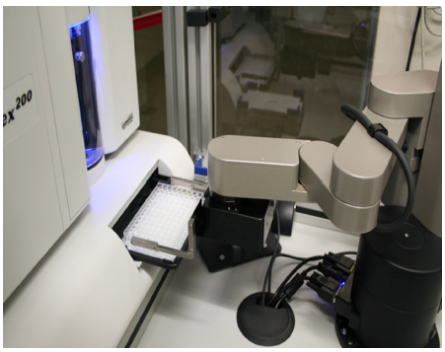


Figure 3
A: EL406™ Washer Dispenser integrated with Bio-Stak™ Microplate Stacker. (BioTek)
B: PerkinElmer (formerly Caliper) Twister II Microplate Handler. (©Q3-2016 PerkinElmer, Inc. All rights reserved. Printed with permission)

A

B



C

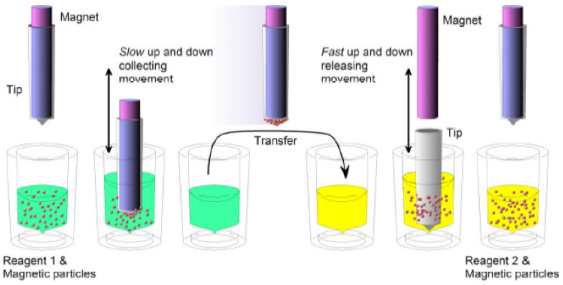
Figure 4
A. Orbitor™ Microplate Mover attached to KingFisher Flex (Thermo Scientific, Orbitor, and KingFisher are trademarks of Thermo Fisher Scientific. Photographs used by permission from Thermo Fisher Scientific.)
B. KiNEDx robot (PAA Inc.) arm integrated with Luminex® 200™ **C.** Caliper Twister II robot (PAA Inc.) loading microplate into Luminex® 200™ system. (Photographs used by permission from Peak Analysis and Automation Ltd.)

Several devices such as the KiNEDx robot arm, Caliper Twister II robot (PAA Inc.), or Orbitor™ RS Microplate Mover (Thermo Scientific) can simplify the automation of xMAP instruments. These automated robots are able to move microplates rapidly in and out of the xMAP instrument and between microplate devices such as liquid handlers, dispensers, or washers located on the laboratory bench (Figure 4). Their extensive vertical reach allows multiple stacked or high density instruments to be loaded in a small footprint, and a bidirectional telescoping arm provides superior reach with unlimited base rotations (360°).

Coupling Microspheres with a Semi-automated Device

xMAP microspheres can be covalently coupled with different reagents for specific bioassays such as antigens, antibodies, or

A



B

Figure 5
A. Inverse magnetic particle processing
B. KingFisher Flex—front view with see-through lid and plates. KingFisher is a trademark of Thermo Fisher Scientific. (Photographs used by permission from Thermo Fisher Scientific.)

oligonucleotides. Although the coupling protocol typically involves some level of manual work, automation of the bead coupling process can improve lot to lot variation (CV%). In addition to protocols listed in the literature, the xMAP Cookbook provides the general guidelines for the coupling of biomolecules to microspheres. An efficient, semi-automated protocol for bead coupling uses MagPlex Microspheres on the KingFisher™ Flex instrument (Thermo Fisher Scientific), originally designed for nucleic acid isolation and purification but adapted for the multiplexing needs of xMAP Technology.[2] The device features an innovative inverse magnetic separation technique, which eliminates the aspiration and other liquid handling steps that can lead to waste or contamination in the assay. The KingFisher coupling protocol results in almost 100% bead recovery.

In principle, the KingFisher approach involves a mechanical arm composed of a row of disposable plastic sleeves that is lowered into the plate wells. The sleeves move like a plunger slowly up and down, mixing the liquid reagents and the suspended magnetic beads. After the mixing step, a second arm composed of a row of magnets slides into the sleeves to collect all beads (along with any coupled molecules) and transfers them to new solution (Figure 5).

By adapting a standard 96-well format plate for experimental throughput needs, individual assay stages in the KingFisher Flex pipeline can be automated independent of each other by the end user. The following procedure describes how to perform protein coupling to MagPlex beads in 96-well format plate. Using the KingFisher Flex as a semi-automatic device enables automated activation and conjugation steps (up to 1mL when using Thermo Scientific Deep Well 96 Plates) with efficient magnetic bead transfer.[2]

Materials for automated protein coupling:

- Carboxylated MagPlex microspheres (Luminex Corp., Austin, TX, USA) Local distributors are listed on the Luminex web page: **www.luminexcorp.com**
- Activation buffer (100mM Na_2HPO_4 + 0.005% (v/v) Triton X-100), pH 6.2
- Sulfo-NHS (Pierce, Cat. No. 24510 or 24520 - no weigh format)
- EDC (Pierce, Cat. No. 77149)
- Coupling buffer (50mM MES + 0.005% (v/v) Triton X-100), pH 5.0
- Storing buffer (PBS + 1% (w/v) BSA) containing 0.05% NaN_3)
- Antibody/protein to be coupled (any suitable source)
- KingFisher plate 96-well Thermo (Thermo Fisher Scientific, Cat. No. 97002540)
- KingFisher Flex (Thermo Fisher Scientific)

Method:

1. Sonicate and vortex the selected MagPlex beads stocks thoroughly for at least 10 sec and transfer 300µL (1.25 x10^7 beads) of each bead stock solution to the respective wells in the 96-well plate (KingFisher plate 96-well Thermo).
2. Magnetic beads of the first plate are transferred by the KingFisher Flex particle handler into second plate for washing with 250µL activation buffer (100mM Na_2HPO_4 + 0.005% (v/v) Triton X-100).
3. For carboxyl group activation, MagPlex beads are moved into wells containing 150µL activation buffer with 5mg/mL EDC and 5mg/mL sulfo-NHS. Activation time: 20 min with slow agitation at room temperature.
4. Activated magnetic beads are transferred by magnetic particle handler to wash plates (two times wash with 250µL coupling buffer (50 mM MES + 0.005% (v/v) Triton X-100).
5. It is important to determine optimal amounts of antigens or antibodies in separate preliminary experiments. Antigens or antibodies diluted to an applicable protein concentration in coupling buffer and distributed on a 96-well assay plate, are incubated with the activated beads and agitated for 2h at room temperature.
6. Coupled beads are transferred by KingFisher Flex for further washing with 250µL wash buffer and resuspended in 200µL blocking/storage buffer (PBS + 1% (w/v) BSA) containing 0.05% NaN_3 and stored in the dark at 2–8°C until further use.

Following coupling confirmation, the microsphere population will be pooled by KingFisher Flex to form an array on a 96-well format plate. To perform a multiplex sandwich immunoassay, all assay reagents including wash buffer, detection antibody mixture, streptavidin-R-phycoerythrin (SAPE), and prediluted samples can be placed simultaneously on the KingFisher Flex for further magnetic particle processing. It takes approximately 3–4 hours to process an assay. Standard settings for the xMAP instrument (96-well/2500 beads per well, sample volume 100µL, minimum bead count per region 100) enables plate reading within 30 minutes (FLEXMAP 3D®). An example of a multiplexed sandwich immunoassay protocol using KingFisher Flex can be found in Table 3.

The use of a magnetic particle handler such as the KingFisher Flex device enables the quantitative transfer of magnetic beads from the sample well into the wells containing washing solutions or other assay reagents (e.g., activation buffer, capture/detection

Table 3. Sandwich immunoassay protocol for magnetic bead handler

The key settings, such as incubation time, position, speed of motion, strength of shaking movements, and number of washing steps can be programmed accordingly.

Step	Plate content	Volume µl	Mixing parameters		
			Time	Temp	Speed
1. Transfer	Magnetic bead array	50			
2. Analyte capture—6 cycles	Sample	100	10 min, pause 2min	RT	Very slow
3. Wash + transfer	Wash buffer	100	20 sec	RT	Slow
4. Wash + transfer	Wash buffer	100	20 sec	RT	Slow
5. Detection antibody—5 cycles	Detection antibody mix	100	10 min, pause 2 min	RT	Very slow
6. Wash + transfer	Wash buffer	100	20 sec	RT	Slow
7. Wash + transfer	Wash buffer	100	20 sec	RT	Slow
8. Detection reagent—4 cycles	SAPE solution	100	10 min, pause 2 min	RT	Very slow
9. Wash + transfer	Wash buffer	100	20 sec	RT	Slow
10. Wash + transfer	Wash buffer	100	20 sec	RT	Slow
11. Bead release to assay plate	Assay buffer	100	20 sec	RT	Slow

Adapted from [2] Poetz O, Henzler T, Hartmann M, Kazmaier C, Templin MF, Herget T, Joos TO. Sequential Multiplex Analyte Capturing for Phosphoprotein Profiling. Mol Cell Proteomics 2010 9(11):2474-2481.

A

B

C

Figure 6
A. Custom MagPlex microspheres in MTP tubes (Luminex Photograph) **B.** A permanently bonded, unique 2D barcode is laser-etched onto the base of every storage tube to securely identify and track the content of every tube (Luminex Photograph) **C.** 2D barcode tube reader (e.g. Thermo Scientific VisionMate 2D barcode) (Photograph used by permission from Thermo Fisher Scientific.)

antibody mixture, SAPE reporter solution). The device does not require vacuum filtration or advanced liquid transfer steps, which are often the basis for fully automated workstations. Such workstations (e.g., Tecan or Hamilton) trade these more flexible options to ensure high throughput.

KingFisher Flex is also well-suited to run commercially available bead-based assay kits, delivering a highly practical laboratory solution for a more robust workflow. A number of laboratories have published protocols and results for such approaches. Using this instrument, Poetz et al[2] used three WideScreen® profiling panels (EMD Millipore) to analyze multiple receptor tyrosine kinases and their degree of phosphorylation within the same sample. Setting up a workflow that employs magnetic bead arrays and a KingFisher Flex processor, Heubach et al[3] demonstrated a faster and more cost-efficient approach for generating bead assays with up to 500 different peptides for further protein-peptide interaction studies. Several studies have shown that a semi-automated Thermo Fisher concept adopted for different xMAP protocols might be used for efficient screening of multiple analytes.[4,5,6]

To simplify the complete semi-automated workflow, routine procedures such as pipetting immunoassay reagents into multiple plates can easily be performed with multichannel pipette dispensers to reduce handling errors. KingFisher Flex can accommodate several 96-well microtiter plates in the same amount of time filled with individual buffers for washing and incubation, but some steps still require human intervention (e.g. final transfer of 96-well plates into an xMAP instrument). Luminex produces custom format MagPlex microspheres (RUO) in MTP tubes that also can be used for automated bead coupling, using all kinds of multichannel liquid handling options that help to simplify laboratory workflow (Figure 6).

Fully Automated Immunoassay Platforms

Ideally, a fully automated workflow for xMAP-based assays would perform hands-free steps such as bead coupling, sample pre-processing (plate preparation with required dilution), washing (multiple steps, including bead separation), plate transferring (for incubation or thermal cycling), and loading into the xMAP instrument. At this point, there are few instruments or kit manufacturers that have developed fully automated solutions dedicated for a large variety of tasks and suited to protein/genomic applications and specific market segments.

Tecan, a Swiss company specializing in the development,

Figure 7
Top: Luminex® 200™ integrated with fully automated Freedom EVO liquid handling workstation. (Tecan)
Bottom from left to right: FLEXMAP 3D with swivel base and Tecan washer located under xMAP instrument with rotation for ease of maintenance; Freedom EVO robust solution for MAGPIX (Tecan).

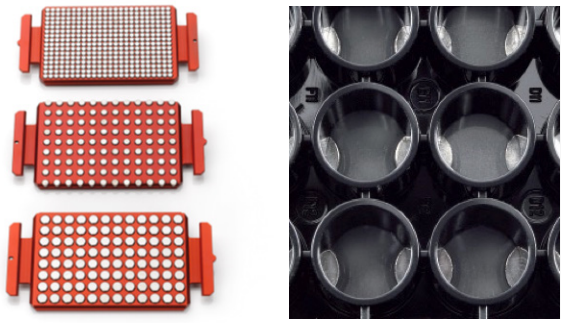


Figure 8: Magnetic carriers for the HydroSpeed plate washer.
Left, top to bottom: 384-well (MBS 384 carrier), 96-well two magnets (smart-2 MBS 96) and 96-well one magnet (MBS 96 carrier) for optimized magnetic bead washing.
Right: smart-2 MBS carrier for automated washing with Tecan's HydroFlex™.

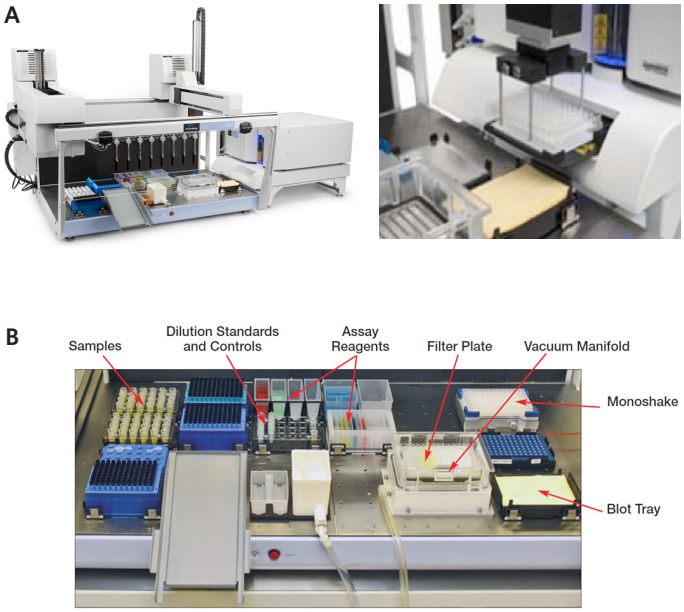


Figure 9
A. Deck layout design from JANUS ® CS Autoplex Workstation On.-board gripper moves plate into integrated Luminex® 200™ analyzer.
B. Additionally, pipetting station, vacuum filtration for plate washing (option for magnetic bead separation), blotting and mixing station. (©Q3-2016 PerkinElmer, Inc. All rights reserved. Printed with permission).

production, and distribution of fully automated workflow solutions, conducts different assay chemistries with advanced robotics.As shown in Figure 7, all xMAP instruments (Luminex 100/200, MAGPIX, and FLEXMAP 3D) can be integrated with the Tecan Freedom EVO® liquid handling platform, which has been actively adopted by many molecular diagnostic, genomic, proteomic, and drug discovery laboratories.[7] The Freedom EVO® series offers four different worktable capacities (width: 75, 100, 150, and 200 cm), each with similar tip configuration (1, 2, 4, 8, 96, and 384) for the 100µL–5000µL volume range. Tecan created a user-friendly automated workstation for performing all pipetting/liquid transfer tasks (from both reservoirs and 1.5 mL centrifuge tubes into all wells), plate movements, separation of microspheres from assay suspensions (vacuum filtration or magnetic bead separation), and mixing steps. The highly effective shaker (Te-Shake™) handles standard 96–384 microplates, deep-well plates, and PCR tubes, with the added option of sample heating if necessary. For the robotic aspect any plate which corresponds to the Society of Biomolecular Screening (SBS) standards can be used with Freedom EVO for plate logistics and carriers. Common plate brands integrated with the EVO device are: Greiner®, Nunc®, Corning®, Macherey-Nagel®, and Eppendorf®.

Due to an automation module available in the Luminex xPonent® software, Tecan drivers enable communication between the xMAP instrument and Freedom EVO, which allows the robotic manipulator arm to transfer the assay plate to the xMAP instrument. The typical, fully automated process includes the pipetting steps for all reagents, PosID™ barcode tube scanning, an incubator tower for dark refrigeration/incubation of up to six plates (5°C to 60°C), and plate storage (hotel option). The Freedom EVOware® software controls for any errors during the performance, also providing automatic xMAP system maintenance, mid-run calibration routines, and dynamic assay scheduling for parallel processing of multiple plates.

Two types of washers can be integrated into a Freedom EVO® device: HydroSpeed™or HydroFlex™. HydroSpeed is an advanced microplate washer optimized for washing cells or xMAP beads in both 96- and 384-well plates, while HydroFlex is designed for automated microplate strip washing and vacuum filtration performance for 96-well microplate formats only. The HydroSpeed washer provides two standard magnetic carriers (MBS 96- and 384-well carrier), and one an innovative patent-pending design that uses two magnets per well for optimized washing results with xMAP microspheres (Smart-2 MBS 96 carrier), as shown in Figure 8. This configuration provides a more robust assay performance and guarantees higher bead recovery (typically ≥90%) in comparison to polystyrene microspheres filtration. Moreover, HydroFlex washers with optional magnetic bead separation are required to use a 96-well flat bottom plates, such as Greiner Bio-One, Germany (Cat. no. 655096).

Software drivers for Research Use Only (RUO) applications with several third party washers including Embla®, BioTek405™ TS/LS, Biotek EL406, and Biotek ELx405™, are also available from Tecan. xMAP platforms (Luminex 100/200, MAGPIX, and FLEXMAP 3D) are not restricted to a specific manufacturer for kits or reagents, which gives the end user more flexibility in terms of running different tests under one deck.

The JANUS® CS Autoplex Workstation from PerkinElmer has been developed to automate a number of proteomic applications, such as multiplexed cytokine immunoassays (e.g., Millipore Human Cytokine LINCOplex™ Kit, cat. no #HCYTO-60K) to improve biomarker screening and to more easily process commercially available kits (Figure 9). This provides a tremendous advantage over manual processing and guarantees efficient management

of xMAP bead-based protocols, which are suitable for setting up multi-batches in large-scale studies.[8] The JANUS liquid handling system and the Luminex 100 analyzer were evaluated by testing multiplex flow immunoassays for the detection of IgG type-specific IgG antibodies to Herpes Simplex Virus (HSV) types 1 and 2.[9] Intra- and interassay reproducibility studies showed excellent precision and resulted in a 50% reduction in turnaround time compared to routine testing by EIA. Together, both instruments can create a prospective solution for improving laboratory medicine, and offering physicians more rapid and accurate clinical information.

Nucleic Acid High Throughput Solutions

There are different requirements for handling protein versus genomic applications that are dependent on the type of assay chemistry used. Therefore, each automation platform has to be programmed for a specific xMAP application and chemistry. Over the past few years, nucleic acid-based assays have become accepted in clinical diagnostics owing to their high specificity, low sample requirement, and ease of automation. In addition, recent advances allow such assays to be configured in xMAP multiplex format, enabling effective screening for multiple assay targets.

Luminex xTAG® Technology provides a method for the simultaneous detection of 150 different nucleotide sequences in a single reaction. Proprietary sets of 24 base oligonucleotides (also known as anti-TAG sequences) are covalently coupled to MagPlex beads. Each MagPlex-TAG bead region's anti-TAG is complementary to a specific TAG sequence incorporated into reporter molecules. This approach has been validated and xTAG Technology has become an industry standard in genetic disease screening, gene profiling, microbial detection, and characterization of antibiotic resistance associated with bacterial SNPs.[10]

Song et al[11] utilized xTAG with Allele Specific Primer Extension (ASPE) chemistry to develop a reliable and cost-effective multiplexed genotyping assay for simultaneous detection of 11 mutations in *gyrA*, *gyrB*, and *parE* of *S. enterica* serovars, Typhi, and Paratyphi A that result in nalidixic acid resistance (NalR) and/or decreased susceptibility to fluoroquinolones. The assay is capable of rapidly screening and identifying the underlying genetic changes in quinolone-resistant isolates and demonstrates that the xMAP platform is ideal for molecular epidemiological analysis. This assay might be adapted to full automation workstations and used as a screening tool more easily than techniques such as sequencing, which are more suited for biomarker discovery. It is important to note that a potential limitation for such automation implementation is the requirement of sample preprocessing to extract and purify the nucleic acid of interest. However, there have been significant advances in addressing these limitations over the past few years, and several semi- or fully-automated systems that perform rapid nucleic acid extraction are now available. Currently, the Freedom EVO 150 is capable of extracting DNA from 96 samples including controls and subsequent PCR set up in less than 3.5 hours.[12] High throughput performance of up to three 384-well PCR plates in less than eight hours is achieved with the dynamic scheduling of the Freedom EVOware software. In order to keep the complexity and cost low while reaching the highest possible throughput, the thermocycling, plate sealing, and plate peeling steps might be performed offline.

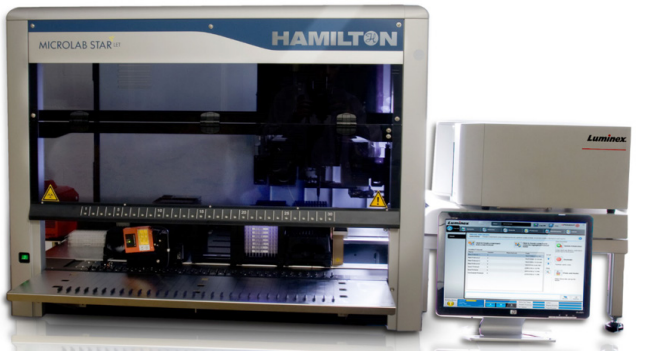


Figure 10
Luminex® 200™ integrated with MICROLAB® STAR –HLA solution (Microlab is a registered trademark of Hamilton Company in the U.S. and/or other countries.)

A number of commercially available standalone workstations as well as "homemade" integrated systems have been developed for nucleic acid preparation during the last few years. Today, fully automated devices cover critical steps of genomic applications from nucleic acid extraction (DNA/RNA) to PCR set up with thermocycler integration (e.g., using automated extraction platforms from QIAGEN, bioMérieux, Roche, Chemagen, Tecan, and Hamilton). Instruments such as Biomek® 4000 (Beckman Coulter), Zephyr® Molecular Biology Workstation (PerkinElmer) or VERSA™ GENE (Aurora Biomed Inc.) have been designed to perform nucleic acid purification or real-time PCR with high precision, throughput, and accuracy. Precise pipetting of reagents, preparation of master mix, creation of dilution series, and sample addition guarantees hands-free preparation of the PCR plate for amplification. Also, popular chemistries used in genomic applications are available from companies such as Promega, Applied Biosystems™, Invitrogen™, and QIAGEN.

IVD Automation

As the IVD market continues to grow globally, it must adapt to new testing environments, innovative technologies, and new types of clinical markers. Key assay components, such as buffers, antibodies, and detection reagents used in multiplex must be optimized to work together to produce a sensitive result that is consistent across all measurement channels and assay parameters. Therefore, it is clear that there is a demand for technologically advanced instruments with greater automation that offer reproducible results independent from the environment in which they are performed.

In February 2010, Hamilton Robotics announced the launch of solutions for HLA antibody screening and identification and HLA DNA typing methods based on Gen-Probe's (now Immucor's)

Figure 11
From left to right: LABXpress™ Pipettor (aspirate/dispense into 96-well reaction plate, mixing, shaking, centrifuging, reading on xMAP instrument). The arm with grid automatically transfers plates to the integrated reader and acquired data are analyzed with HLA Fusion software. (One Lambda photographs used by permission from Thermo Fisher Scientific.)

LifeCodes HLA reagents (Press release BoxID 324512, Martinsried, 22.02.2010). Filling the market gap for histocompatibility and transplant immunology labs that require a high degree of multiplexing and throughput, Hamilton finalized workflow solutions for blood banks and long term storage systems (-20°C to 80°C) under one liquid handling deck. Hamilton Robotics developed fully automated solutions for IVD sample preparation that reduce hands on time, minimize operational variation, and deliver consistent results. The Microlab® STAR™ liquid handling workstation is based on patented Monitored Air Displacement pipetting technology (MAD), where the risk of contamination of critical assays is reduced to an absolute minimum (Figure 10). The system allows for real-time detection of pipetting errors when an insufficient amount of liquid has been aspirated for a single, multiple, or partial dispense, or due to introduction of air and blocked tips (TADM—Total Aspirate and Dispense Monitoring). The combination of MAD pipetting with compressed CO-RE tip attachment technology meets IVD requirements for precision, even in critical situations when the STAR channels must pipette liquids with extremely low viscosity and high vapor pressure (e.g., methanol/acetone).Depending on the complexity of the xMAP application, three unique workstations are currently available: STARlet, STAR, and STARplus (deck size: 1, 1.5, and 2 meters). Up to 16 independent 1000µL channels can be combined with any of Hamilton's multiprobe heads (96,

384 or nanopipetting) on a single instrument with a pipette volume range of 0.5 to 1000µL. This results in a doubling of throughput by simultaneous preparation of two microplates. The 96 channel head is able to pipette liquids in range of 1 to 1000 µL and the 384 probe head from 0.5 to 50µL. All of these features are important for integration of any xMAP instrument with the Microlab® STAR™ device, especially for the FLEXMAP 3D which can analyze 384 samples within 1.5 to 2 hours. Similar to other fully automated liquid handling systems, the Microlab STAR is controlled by advanced software (Venus One), which allows flexible robot arms mounted on the decks to transfer the plates between individual system components (e.g., pick a plate from a microplate stacker and place it into an adjacent xMAP instrument or pass microplates from a plate stacker to a pressured control vacuum system or to magnetic washers). Hamilton has integration experience with the following washers: Biotek ELx405R, Tecan Power Washer 384, Thermo Wellwash Ascent, MD Embla 96/384, and MD Embla Skan Wash 300/400 (Molecular Devices).

xMAP Technology, together with Hamilton Robotics, provides an appropriate platform to study the impact of different HLA antibodies isotypes of on transplantation outcomes. The xMAP screening approach offers high sensitivity, which is comparable to the high sensitivity of cross-match by flow cytometry.[13] An ideal fit for the transplant community is One Lambda's LABType® SSO and LABScreen® IVD labeled products; especially LABScan 3D (Luminex FLEXMAP 3D), which can detect up to 500 HLA specificities in a single test, eliminating the need to run multiple tests. A sample preparation device called LABXpress™ Pipettor (One Lambda) can accommodate eight plates under one deck, and guarantees hands-free capabilities suited for high-volume solid organ and bone marrow transplant centers (Figure 11).

Demand for excellent quality and reproducible tests in healthcare continues to be driven by IVD kit providers together with automation manufacturers. There are other fully automated platforms that come to the forefront, which are more critically developed around very specific methodologies. Theradiag (formerly Biomedical Diagnostics) has launched a comprehensive range of tests based on xMAP Technology for the diagnosis of autoimmune pathologies, allergies, and early detection of heart failure. The commercially available FIDIS™ kits are processed with a liquid handling platform called Theralis, which fills the laboratory IVD gap for critical public health tests.

Summary and Conclusions

Multiplex bead-based assays have become established in high-throughput laboratories, including combinations of multiple semi-automated workstations to carry out the traditionally manual parts of xMAP protocols. The immediately obvious benefits of labor savings, better quality data, and cost reduction are key drivers in integrating independent liquid handling devices (liquid dispensers, plate washers, plate handlers, etc.) into small-scale, benchtop or walkaway automation applications for xMAP Technology.

In addition, large robotic systems require dedicated, highly trained personnel and have steeper learning curves to become fully familiar with these devices. Nevertheless, automation holds the promise of increasing sample throughput and improving data output. Given the rise of molecular testing, it is also likely that there will be multiplex testing combinations that include both traditional immunoassays as well as nucleic acid-based molecular assays, all of which must be optimized to work together under one automation deck.

The examples of xMAP-based assays mentioned in this review have been used to illustrate the various means of multiplexing presently in practice and to open possibilities of further integration of multiplexing and laboratory automation. The author does not attempt to provide a listing of every assay, application, or

platform that is currently available, but rather to guide readers toward looking for novel liquid handling techniques for integration with xMAP instruments. Since not all screening assay chemistries are compatible with automated platforms, new robotic innovations will continue to contribute to the development of cost-effective xMAP-based applications.

Acknowledgements

All images are used at the courtesy of the companies listed in the references. The instruments are used as examples in this article and represent those that the author has either used or has learned of during the course of his career.

REFERENCES

1. Paul H, Banks P. Automated workflows for Luminex xMAP® assays. Biotek (Internet). Cited 2015 September. Available from: www.biotek.com.
2. Poetz O, Henzler T, Hartmann M, Kazmaier C, Templin MF, Herget T, Joos TO. Sequential Multiplex Analyte Capturing for Phosphoprotein Profiling. Mol Cell Proteomics 2010 9(11):2474–2481.
3. Heubach Y, Planatscher H, Sommersdorf C, Maisch D, Maier J, Joos TO, Templin MF, Poetz O. From spots to beads—PTM-peptide bead arrays for the characterization of anti-histone antibodies. Proteomics 2013 Mar;13(6):1010-5.
4. Bigalke B, Pötz O, Kremmer E, Geisler T, Seizer P, Puntmann VO, Phinikaridou A, Chiribiri A, Nagel E, Botnar RM, Joos T, Gawaz M. Sandwich immunoassay for soluble glycoprotein VI in patients with symptomatic coronary artery disease. Clin Chem 2011 Jun;57(6):898-904.
5. Planatscher H, Rimmele S, Michel G, Pötz O, Joos T, Schneiderhan-Marra N. Systematic reference sample generation for multiplexed serological assays. Sci Rep 2013;3,3259.
6. Luckert K, Götschel F, Sorger PK, Hecht A, Joos TO, Pötz O. Snapshots of protein dynamics and post-translational modifications in one experiment--beta-catenin and its functions. Mol Cell Proteomics 2011 May;10(5):M110.007377.
7. Allinson JL, Automated immunoassay equipment platforms for analytical support of pharmaceutical and biopharmaceutical development. Bioanalysis 2011 Dec;3(24):2803-16.
8. Bourdon D, Tack L. Fully Automated High Throughput Biomarker Screening using xMAP Polystyrene Bead-based Multiplex Cytokine Detection Assay. Perkin Elmer (Internet). Cited 2015 September. Available from: www.perkinelmer.com.
9. Binnicker MJ, Jespersen DJ, Harring JA. Evaluation of three multiplex flow immunoassays compared to an enzyme immunoassay for the detection and differentiation of IgG class antibodies to herpes simplex virus types 1 and 2. Clin Vaccine Immunol 2010 Feb;17(2):253–257.
10. Deshpande A, White PS. Multiplexed nucleic acid-based assays for molecular diagnostics of human disease. Expert Rev Mol Diagn 2012 Jul;12(6):645-59.
11. Song Y, Roumagnac P, Weill FX, Wain J, Dolecek C, Mazzoni CJ, Holt KE, Achtman M. A multiplex single nucleotide polymorphism typing assay for detecting mutations that result in decreased fluoroquinolone susceptibility in Salmonella enterica serovars Typhi and Paratyphi A. J Antimicrob Chemother 2010 Aug;65(8):1631-41.
12. Stangegaard M, Frøslev TG, Frank-Hansen R, Laursen SS, Jørgensen M, Hansen AJ, Morling N. Automated extraction of DNA and PCR setup using a Tecan Freedom EVO® liquid handler. Forensic Science International: Genetics Supplement Series 2 2009;2(1):74-76.
13. Heinemann FM. HLA genotyping and antibody characterization using the Luminex multiplex technology. Transfus Med Hemother 2009;36(4):273-278.

Automated Liquid System Providers

Company	Site Address
Agilent	www.agilent.com
Apricot Designs	www.apricotdesigns.com
Aurora Biomed	www.aurorabiomed.com
Beckman Coulter	www.beckmancoulter.com
BioMicroLab	www.biomicrolab.com
Biosero	www.bioseroinc.com
BioTek Instruments	www.biotek.com
Cetac	www.cetac.com
CyBio	www.cybio-ag.com
Eppendorf	www.eppendorfna.com
Gilson	www.gilson.com
Hamilton Robotics	www.hamiltonrobotics.com
Integra Biosciences	www.integra-biosciences.com
Labnet International	www.labnetlink.com
Molecular Devices	www.moleculardevices.com
Peak Analysis and Automation	www.paa-automation.com
PerkinElmer	www.perkinelmer.com
Rainin	www.rainin.com
Tecan	www.tecan.com
Thermo Fisher Scientific	www.thermoscientific.com
Titerek-Berthold	www.titertek.com
Tomtec	www.tomtec.com

Note:This table provides some examples of automation vendors and does not indicate that these platforms have been tested with xMAP Technology or are compatible with all xMAP applications. Consult your vendor for more information.

Luminex®

To learn more, please visit: **www.luminexcorp.com**

WP1149.0215