

# MicroCaster™

## A Tool For Building Protein Microarrays

Tom Owen, Debbie English, Lara Dowland and Breck Parker

### INTRODUCTION

Much of today's high visibility, cutting-edge microarray technology entails the use of high-throughput systems such as sophisticated robotics that require large capital expenditures. There is a large population of researchers who do not have access to robotic arrayers and who want to screen or study a small collection of molecules. As an alternative to robotic arrayers, the MicroCaster™ can be used as a tool for hand-arraying molecules onto FAST® Slides in a microarray format.

The use of nitrocellulose as a matrix to immobilize nucleic acids targeting the hybridization of their complementary sequences has been well documented in the literature.<sup>1</sup> It has served as a key tool for molecular biologists who have employed it in Southern, Northern, and Western blotting procedures. These procedures, which have been modified and miniaturized to create microarrays, continue to form the backbone of scientific discovery.<sup>2-11</sup> Microarray technology allows researchers to screen large libraries of molecules such as DNA and proteins in time-spans that previously would not have been feasible.<sup>12,13</sup> This technology provides the tools necessary to begin the process of deciphering the functional aspects of sequences buried within the human genome.<sup>14</sup>

Construction of a microarray requires that biomolecules be immobilized on a matrix in an ordered fashion. The molecules must be available for hybridization or binding to their labeled target molecules in order to elicit the detection of target elements. DNA microarrays have engendered tremendous interest since the first experiments were published in 1995. This is due in part to the ease of printing microarrays using the vast amount of available sequence data as well as DNA's uniquely homogenous nature and chemical stability. Protein microarrays, on the other hand, have only recently been introduced into the researcher's toolbox. They present many challenges to the researcher because proteins are heterogeneous in chemical composition and present a wide range of molecular stabilities and binding affinities. The methods used to prepare protein microarrays are constantly evolving to provide scientists with improved tools for protein discovery. Whatman Schleicher & Schuell's nitrocellulose-based FAST Slide is being used extensively to produce several different types of protein arrays, e.g., arrays for enzyme-substrate, protein-protein, antibody-antigen and drug-protein interaction studies.<sup>15,16</sup>

The work presented in this application note demonstrates the ability of the Whatman Schleicher & Schuell MicroCaster to successfully array proteins onto FAST Slides. For the purpose of demonstration,

we prepared a titration series of antibodies for use in a cytokine expression analysis assay. Using a cytokine stimulation protocol from Moody *et al.*,<sup>17</sup> human THP-1 leukemia cells were activated with a cytokine-stimulating agent. Complementary binding of expressed cytokines to monoclonal capture antibodies arrayed onto FAST Slides was demonstrated.<sup>16</sup> We show that protein arrays produced with the MicroCaster can be processed using fluorescent, chemiluminescent and colorimetric detection methods.

### METHODS

#### MicroCaster Preparation and Cleaning Protocol

Prior to use, the MicroCaster hand tool and indexing unit/slide holder were cleaned with a 5% bleach solution. The entire unit was subsequently dried before using. The 8 solid replicator pins, which are 0.457 mm in diameter and anchored in the hand tool, must be cleaned and conditioned before printing an array. To clean and condition the pins, 3 consecutive shallow petri dishes were filled 3/4 full with sterile water, followed by a single dish 3/4 full with 100% ethanol. The replicator pins were dipped into the first water bath and gently swirled for a few seconds (without letting the pins scrape the bottom of the dish), then transferred to the second and third water baths, each time swirling the pins in a similar motion. Finally, the pins were dipped into the ethanol bath, gently swirled, removed and dried completely with a hot air drier (hair dryer). A 1:5 dilution of MicroCaster Pin-Conditioner in sterile water was prepared and poured into a shallow petri dish until 3/4 full. The replicator pins were dipped into the conditioner up to the halfway point of the pins and transferred to Whatman Schleicher & Schuell GB002 blotting paper where the tips were gently blotted dry. This conditioning step was repeated a second time followed by hot air drying the replicator pins. A final wash series of 3 water bath swirls and an ethanol bath swirl was performed in a similar manner as previously stated. Prior to arraying, the hand tool was maintained in a stationary, upright position on the indexing unit to prevent pin damage.

#### Source Plate Preparation

This work utilized 4 monoclonal capture antibodies to detect cytokines (IL-4, IL-8, IL-1 $\beta$  and ICAM-1) in untreated and lipopolysaccharide (LPS)-treated THP-1 cell lysates. The capture antibodies were diluted in Protein Array Buffer to 0.5 mg/ml, 0.25 mg/ml and 0.125 mg/ml. Donkey anti-goat (DAG) antibody diluted to 0.125 mg/ml, and buffer (no protein) were used as positive and negative controls respectively. The capture antibody locations in the array are shown in Figures 1 and 2a.

## Arraying Methods

Two FAST Slides (single pad, 20 mm x 51 mm) were placed on the stage of the slide holder and the indexing unit was lowered into position above the slides. The entire unit was oriented with the X-axis motion pin distally located and set in the first position (farthest right), and the Y-axis motion pin distally located in the first position as well (farthest left).

Proteins were transferred onto FAST Slides following the MicroCaster protocol. Briefly, the pin tool was lowered into the protein sample making sure the pin was centered within the source plate well and did not touch the well's bottom. With a steady motion, the pin tool was removed from the well and placed in the indexing unit. The pin tool was depressed with an even, steady motion until the second stop position was reached on the indexing unit. The hand tool was then placed back into the same set of source plate wells and a duplicate array was made on the second FAST Slide. The Y-axis motion pin was moved two places every cycle until a total of six replicates were transferred.

The preceding steps were repeated for each subsequent concentration set of capture antibodies. Between each concentration set, the x-axis motion pin was moved two places to the left. The pins were washed and dried between concentration sets by dipping them into the three consecutive water baths with a swirling motion followed by an ethanol wash as described in the "MicroCaster Preparation and Cleaning Protocol" section of this application note. Immediately after the ethanol wash step, the pins were put through the same series of washes a second time and hot air-dried using a hair dryer. The arrayed FAST Slides were removed from the holder, air-dried for 5 minutes, and stored at room temperature prior to processing.

## Cell Lysate Preparation

Human THP-1 leukemia cells (ATCC, Manassas, VA) were cultured to log phase in flasks containing 50 ml of RPMI 1640/10% fetal bovine serum. One flask was treated with 5 µg/ml lipopolysaccharide (LPS; *Sigma-Aldrich*) in the media to induce a cytokine response, while the other flask only contained media. Both flasks were incubated for an additional 6 hours. The cells were pelleted by centrifugation. The cell pellet was washed 2 times with ice cold 1X PBS, pH 7.4 and spun in a centrifuge to pellet the cells. The cells were resuspended in 1 ml of lysis buffer (1X PBS pH 7.4, 0.2% NP-40, 1mM EDTA, protease inhibitor cocktail) and subsequently vortexed. After a 15-minute incubation on ice, the lysed cells were centrifuged and the supernatants, containing the targeted cytokines, were removed and stored at -70°C until use.

## Detection Protocol

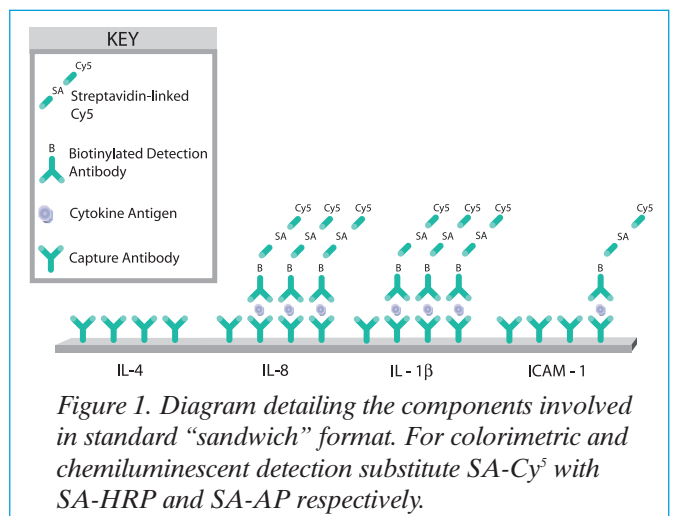
**Fluorescence:** The arrayed FAST Slides were placed in sufficient Protein Array Blocking Solution cover the slides and gently agitated for 15 minutes. Following the blocking step, Incubation Chambers were attached to the slides and 725 µl of a 1:10 dilution of treated or untreated cell lysates with Protein Array Blocking Solution as the diluent was added to the appropriate slide. After completion of a 4-hour incubation at room temperature, the chambers were removed and the cell lysate solutions were discarded. The slides were washed 3 times with Protein Array Wash/Block Buffer for 5 minutes each and new chambers were added to the slides. A cocktail solution containing 200 ng/ml each of biotinylated IL-4, IL-8, IL-1β and ICAM-1 polyclonal antibodies in Protein Array Wash/Block Buffer was prepared and 725 µl of the cocktail was added to each of the slides. The slides were mixed and incubated for one hour at room temperature. The slides were washed three times with Protein Array

Wash/Block Buffer for 5 minutes each. From this point on, the slides were protected from light to prevent photobleaching. Streptavidin-Cy<sup>5</sup> (*Amersham Biosciences*) was diluted 1:5000 in Protein Array Wash/Block Buffer and added to the slides, which were gently agitated for 1 hour at room temperature. The slides were subsequently washed 3 times at room temperature with Protein Array Wash/Block Buffer for 5 minutes each, and then rinsed for 2 seconds in dH<sub>2</sub>O, dried and scanned with the Perkin Elmer Scanarray 4000. ScanArray and QuantArray software from Perkin Elmer were used to capture images and to calculate specific intensities and spot morphologies.

**Chemiluminescence and Colorimetric Detection:** The arrayed slides were placed into a sufficient volume of 1X TBS/1% casein to cover the slides and gently agitated for 15 minutes. Following the blocking step, Incubation Chambers were attached to the slides and 725 µl of a 1:10 dilution of treated or untreated cell lysates with 1X TBS/1% casein as the diluent was added to the appropriate slide. After completion of a 4 hour incubation at room temperature, the chambers were removed and the cell lysate solutions were discarded. The slides were washed 3 times with 1X TBS for 5 minutes each and new chambers were added to the slides. A cocktail solution containing 100 ng/ml each of biotinylated IL-4, IL-8, IL-1β and ICAM-1 polyclonal antibodies in 1X TBS/1% casein was prepared. The cocktail solution (725 µl) was added to each of the slide chambers, mixed and incubated for 1 hour at room temperature. They were washed 3 times with 1X TBS for 5 minutes each.

**Chemiluminescence:** Streptavidin-HRP (horse radish peroxidase) was diluted 1:5000 in 1X TBS/1% casein and added to the Incubation Chamber. The slides were gently agitated for 1 hour at room temperature. The slides were subsequently washed 3 times at room temperature with 1X TBS for 5 minutes each. Detection utilized Super Signal<sup>®</sup> Substrate (*Pierce Endogen*) treatment for 5 minutes followed by a 5 minute exposure to Kodak's BioMax film.

**Colorimetric:** Streptavidin-AP (alkaline phosphatase) was diluted 1:1650 in 1X TBS/1% casein and added to the Incubation Chamber. The slides were gently agitated for 1 hour at room temperature, and subsequently washed 3 times at room temperature with 1X TBS for 5 minutes each. The slides were placed into 25 mls of BCIP substrate (*Sigma-Aldrich*) and gently rocked to generate a colorimetric reaction.



## RESULTS

The cytokine antibody microarrays used in this work were printed on FAST Slides using the MicroCaster manual arrayer, screened with cell lysates, and detected with a labeled antibody in a standard “sandwich” format (Figure 1). The titration of each cytokine antibody was printed as a row of 12 replicates, while the control rows consisted of a combination of six positive and six negative replicates (Figure 2a). The immobilized monoclonal antibodies to IL-4, IL-8, IL-1 $\beta$  and ICAM-1 were used to screen untreated and LPS-stimulated THP-1 cell lysates. IL-4 cytokine was not LPS-induced and therefore, was not detected, while IL-8, IL-1 $\beta$  and ICAM-1 cytokines generated signals representing the induction of each cytokine (Figure 2a).

The cytokine antibodies arrayed on the *FAST Slide* supported linear binding of antibody to its cytokine target as shown for IL-8 cytokine in Figure 3 (IL-1 $\beta$  and ICAM-1 also display similar linearity [data not shown]).

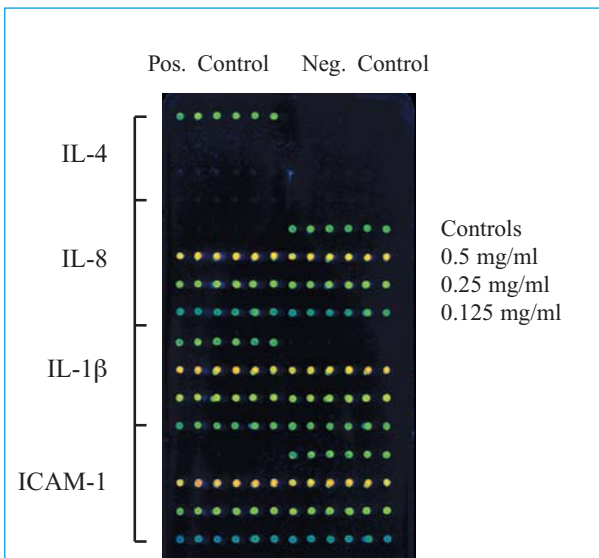


Figure 2a. Image generated from the LPS-stimulated cell lysate. Image was scanned at Laser Power 85 and PMT 41.

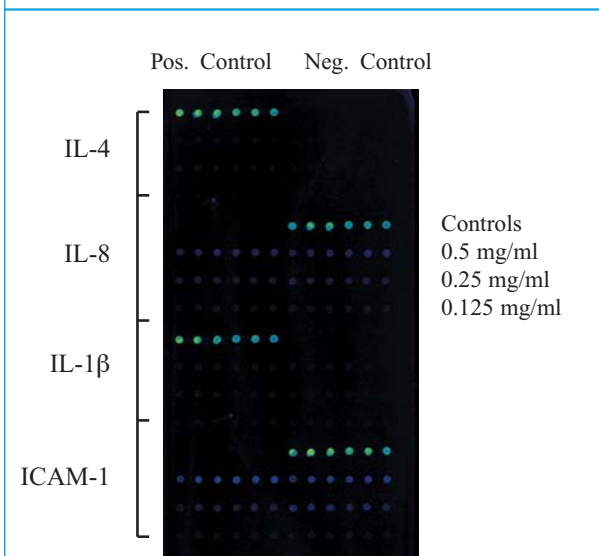


Figure 2b. Image generated from the untreated cell lysate. Image was scanned at Laser Power 85 and PMT 41.

The spot-to-spot CV's ranged from 3.66-10.78%. The non-treated cell lysates resulted in low to non-detectable levels of all specific targeted cytokines (Figure 2b). The level of induction of cytokines in LPS-treated cells was determined by calculating the average specific intensities of the 0.5 mg/ml antibody rows in the LPS stimulated THP-1 cells and dividing it by the corresponding average specific intensities of the untreated cells. Figure 4 shows the fold-induction of cytokine protein expression when THP-1 cells are stimulated with LPS as judged by a protein microarray experiment (Figure 4).

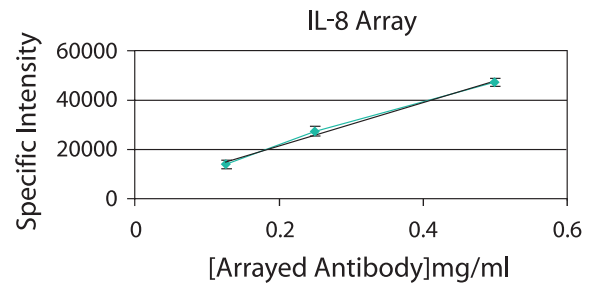


Figure 3. The filled diamonds represent the average specific intensity of the array per concentration of IL-8 capture antibody spotted. The calculated  $R^2=0.9936$  for the

The MicroCaster pins are 457  $\mu\text{m}$  in diameter, and each solid pin contains a small slot to assist with the sample transfer. These pins produce an average spot diameter of 973  $\mu\text{m}$  at a center-to-center pitch of 2.5 x 1.5 mm resulting in 192 spots on the FAST Slide surface (20 mm x 51 mm). The evaluation of spot uniformity was based on a normality of 1.0 being perfect, and the mean uniformity was calculated to be 0.922. The fluorescent signal intensity was very consistent throughout the spot itself, which resulted in near perfect uniformity across an individual spot of the array. Spot morphology was not expected to be as robust and circular as robotically controlled deposition, however the spots produced by the solid pins of the MicroCaster proved to be consistently circular and of excellent morphology (Figure 5).

In addition to fluorescent detection, arrays produced with the MicroCaster may be processed using chemiluminescent, colorimetric, and isotopic detection techniques. Examples of chemiluminescent and colorimetric detection of a cytokine array are shown in Figure 6. The arrays were incubated with LPS-stimulated THP-1 cell extracts and detected as described above. Only the treated slides are presented; the arrays probed with untreated extracts did not indicate stimulation of cytokines (data not shown) and are in agreement with the data collected using fluorescent detection methods. We used a pitch of 2.5 mm by 1.5 mm to provide extra room for chemiluminescent detec-

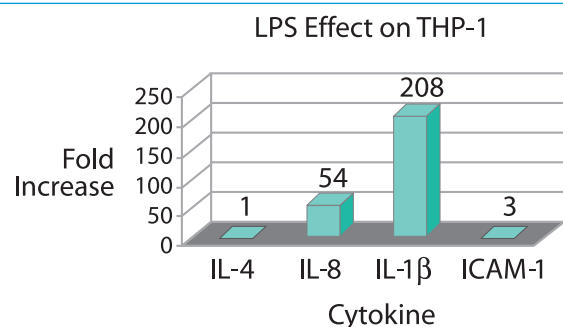


Figure 4. Shows the fold-increase of four cytokines in LPS-treated versus untreated THP-1 cells.

tion since this method may produce spot images on film of 1.5 mm in diameter as observed in Figure 6. The MicroCaster can place up to 768 spots on the FAST Slide by using a pitch of 1.25 mm by 0.75 mm. Each pin delivers approximately 40 nl of fluid to the surface. Fluid delivery will vary depending on composition of buffer system, salt, detergent, viscosity, etc.

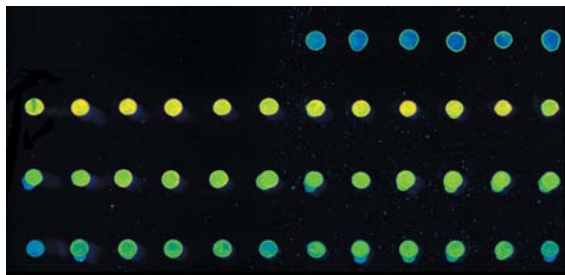


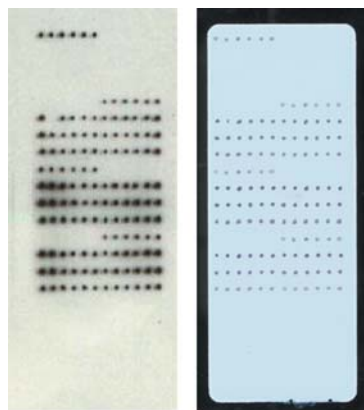
Figure 5. Typical fluorescent image of cytokine array (actual image is IL-8 array from figure 2a, LPS-treated cell lysate detected.)

## DISCUSSION

The reproducibility of data points per antibody concentration set is illustrated by low spot to spot CV values and the strong positive correlation of linearity. We have found that one of the most consistent methods for producing arrays is through piezo-electric deposition, which requires expensive robotics. Our data show that the MicroCaster is capable of arraying a titration of capture antibodies with linearity that is close to that generated using piezo-electric deposition technologies.

Other methods of detection were demonstrated successfully using the MicroCaster as a deposition tool. Chemiluminescent and colorimetric detection assays were performed as described above using treated and non-treated cell lysates bound to complementary biotinylated primary antibodies and appropriate secondary streptavidin labeled antibodies. Each method utilized their recommended substrates for detection. Both of these methods resulted in easily detectable signals, with titration gradients that display linearity of binding, similar to that described by the fluorescence assay reported in this application note.

Figure 6. Digitally scanned images of the chemiluminescent (left) and colorimetric (right) detection systems for the targeted cytokines. The layout of the detected cytokines is the same as in figure 2.



The concept of protein screening is an invaluable tool for researchers and clinicians. Miniaturization reduces the amount of precious sample required to build an array, and it also opens the door to the use of multiple probes, i.e., multiplexing. The system allows researchers to do comparative expression studies for drug target analysis as well as providing a platform for clinical applications, where complex solutions can be screened for specific antibodies and proteins.<sup>18</sup> There are protein arrays currently on the market that can be purchased "off-the-shelf", containing sub-sets of easily distinguishable capture antibodies commonly screened in the field (i.e., cytokines).<sup>18,19</sup> Frequent applications of protein screening technologies may require the researcher to personalize the array to his or her needs.

In conclusion, it has been found that current protein microarraying protocols for high throughput objectives have conveniently conformed to the equipment used in generating DNA microarrays, specifically robotic arrayers. These arraying robots are very expensive, though necessary nonetheless for high-throughput processes. For low-throughput experiments, the need for this type of technology is not absolutely required since the use of manual type array tools will provide adequate performance. The MicroCaster generates reproducible, consistent arrays for low throughput research at a fraction of the cost of an automated arrayer. Similarly to high-throughput technologies, this nucleic acid-designed arrayer conforms to protein applications with little manipulation of the current protocol for ease in producing protein arrays.

- Gillespie, D. and Spiegelman, S.; *J. Mol. Biol.* 12; 829-842 (1965).
- Schleicher & Schuell BioScience, Inc., Application Note #710, Methods for Preparing PCR Based Arrays on Nytran N and SuPerCharge Nylon Membranes. Debbie English and Breck Parker.
- Stillman, B.A., and Tonkinson, J.L., FAST™ Slides: A novel surface for microarrays., *BioTechniques* (2000), 29: 630-35
- De Wildt, R. et. al; Antibody arrays for high-throughput screening of antibody-antigen interactions, *Nature Biotechnology* 18:989-994 (2000).
- Stillman, B.A. and Tonkinson, J.L., Expression microarray hybridization kinetics are dependant on the length of immobilized species, but independent of surface chemistry., *Analytical Biochemistry* (2001), 295: 149-157.
- Knezevic, V., et. al; Proteomic profiling of the cancer microenvironment by antibody arrays, *Proteomics* 1:1271-1278 (2001).
- Pawelcz, C. P., et. al; Reverse phase protein microarrays which capture disease progression show activation of pro-survival pathways at the cancer invasion front, *Oncogene* 20:1981-1989 (2001).
- Tonkinson, J.L., Osborne, D.S., Zhao, W.W. and Stillman, B.A., Expression microarray hybridization kinetics are dependant on the length of immobilized species, but independent of surface chemistry., *Analytical Biochemistry* (2001), 295:149-157
- Tonkinson, J.L., Parker, B.O., and Harvey, M.A., Chemiluminescent detection of immobilized DNA from Southern blots to microarrays., *Luminescence Biotechnology*, (2002) CRC Press, pp189-201
- Wang, D., et. al; Carbohydrate microarrays for the recognition of cross-reactive molecular markers of microbes and host cells, *Nature Biotechnology* 20:275-281 (2002)
- Tonkinson, J.L., Osborne, D.S., Zhao, W.W. and Stillman, B.A., Development of micro scale immunoassays for parallel analysis of multiple analytes.
- Schena, M., Shalen, D., Davis, R.W., and Brown, P.O.; *Science* 270; 467-469 (1995).
- Duggan, D.J., Bittner, M., Chen, Y., Meltzer, P., and Trent, J. M.; *Nature Genetics* 21;10-14 (1999).
- Venter, J. C. et.al; *Science* 291:1304-1351 (2001).
- MacBeath, G. and Schreiber, S.L.; *Science* 289;1760-1763 (2000)
- Walter, G. et. al; *Current Opinion in Microbiology* 2000; 3:298-302
- Moody, M.D.; S.W. Van Arsdell, K.P. Murphy, S.F. Orencole, and C. Burns, *BioTechniques* 31: 186-194 (July 2001)
- Schleicher & Schuell BioScience *ProVision Microarray Kit*, request ordering information from S&S.
- Haab, B.B., Dunham, M.J. and Brown, P.O.; *Genome Biology* 2(2);RESEARCH0004.1 (2001).

**Note: MicroCaster protocol can be found on [www.arraying.com](http://www.arraying.com)**

*Cy* is a trademark of Amersham Biosciences. *BioMax* is a trademark of the Eastman Kodak Company. *Super Signal* is a registered trademark of Pierce Chemical Company. *FAST*™, *MicroCaster*™ and *Whatman*® are trademarks of the Whatman Group

## Further Information

Whatman Inc., 200 Park Avenue, Suite 210, Florham Park, NJ 07932 USA, Tel: 1-800-Whatman (US and Canada), Fax: 1-973-245-8329, Email: [info@whatman.com](mailto:info@whatman.com)  
 Whatman International Ltd., Springfield Mill, James Whatman Way, Maidstone, Kent ME14 2LE UK, Tel: +44 (0) 1622 676670, Fax: +44 (0) 1622 677011, Email: [information@whatman.com](mailto:information@whatman.com)  
 Whatman GmbH, Hahnstrasse 3, D-37586 Dassel, Germany, Tel: +49 (0) 5564 204 100, Fax: +49 80) 5564 204 533, Email: [information@whatman.com](mailto:information@whatman.com)