Qualification of Microarrayers for Clinical Research: Results From Testing of the BioOdyssey™ Calligrapher™ MiniArrayer

Amy VanMeter, Valerie S Calvert, Julia D Wulfkuhle, Emanuel F Petricoin III, Lance A Liotta, Virginia Espina, George Mason University, Manassas, VA 20110 USA

Introduction

While both genomics and proteomics play a crucial role in unraveling the mysteries of molecular interactions, the information garnered from each area is vastly different. Gene arrays provide information describing changes in mRNA expression, while proteomics provides insights into deranged cellular signaling mechanisms. This proteomic cellular signaling is a complex interplay between kinases and phosphatases, resulting in specific posttranslational modifications of proteins such as phosphorylation, lipidation, glycosylation, or cleavage.

Advances in protein microarray technology have been made possible by gleaning information from genomics technologies such as cDNA arrays. Although different information is derived from genomics and proteomics, a common bond is shared: the microarray. In the simplest sense, a microarray is a series of samples immobilized on a substratum. The immobilized sample can be an antibody, cellular lysate, nucleic acid, serum, or aptamer.

Gene expression can be evaluated via cDNA or oligonucleotide microarrays. The arrays are constructed with immobilized nucleic acid strands as a capture molecule on a glass surface. A fluorescence labeled cDNA probe is applied to the array and the complementary strands are allowed to hybridize, thus permitting analysis of changes in mRNA expression levels.

Protein microarrays can be constructed in two formats: forward phase (also referred to as an antibody array) or reverse phase. A forward-phase array consists of different immobilized capture molecules, usually antibodies. The array is probed with serum or a cellular lysate sample as the bait molecule. A second labeled antibody probe provides the detection molecule. The disadvantages of a forward-phase array are (1) requirement for two distinct antibodies directed against the same epitope, and (2) inability to match the antibody affinities to the sample protein concentration.

In contrast, a reverse-phase array is constructed by immobilizing the bait molecule on a substratum and probing with a single antibody. The bait molecule may be a serum protein, a cellular lysate, or subcellular protein fraction. By immobilizing the bait molecule in a dilution series, it is possible to effectively match the protein concentration of the sample with the antibody probe affinity, facilitating measurement within the linear dynamic range of the array. The advantages of the reverse-phase array format are (1) ability to match the antibody affinity to the protein concentration, and (2) need for only one antibody directed against the epitope.

Widespread adoption of microarray technology to clinical research and clinical trials emphasizes the need to assess the technical and quality aspects of robotic devices used to construct the arrays. Instruments approved for use in human clinical diagnostic laboratories must undergo extensive quality assessments, including documentation of accuracy, precision, linearity, and sensitivity for a given assay. As in a clinical setting, it is necessary to understand both the capabilities and limitations of a robotic microarray printing device. Criteria to be considered when selecting a robotic printing device include (1) the type of material to be deposited, (2) the number of samples and number of microarrays to be printed (throughput), (3) the pin type/style, and (4) the pin-washing capabilities of the arraying device.

The ability to print a plethora of materials, including but not limited to cell lysates, microdissected material, whole tissue lysates, and serum, makes robotic printing technology exceptionally desirable. To determine the reliability, throughput, and reproducibility of the BioOdyssey Calligrapher for reverse-phase protein microarrays (RPPAs), we printed epidermal growth factor (EGF)-treated and untreated A549 human carcinoma cell lysates. The arrays were subsequently stained to evaluate multiplexed protein expression changes over time for selected phosphorylated protein endpoints. The results presented here represent a series of experiments typically used for evaluation of robotic printing devices.

Methods

Cell Line and Treatments

A549 cells (American Type Culture Collection, ATCC) were cultured in F-12K medium (ATCC) supplemented with 10% fetal bovine serum (ATCC). Approximately 2.75 x 10^5 cells were plated per well in 6-well microplates. The following day, growth medium was replaced with serum-free medium. After 24 hr, medium supplemented with EGF peptide (Cell Signaling Technology, Inc.) or medium alone was added to the treated and untreated cells, respectively, at different time points. At each time point, the cells were washed twice with Dulbecco's phosphate buffered saline and then lysed in a 2.5% solution of β-mercaptoethanol in T-PER (Pierce)/2x SDS Tris/glycine/SDS buffer (Invitrogen).

Arraying

The BackTracker™ program provided with the BioOdyssey Calligrapher miniarrayer, which assumes all slides will be read vertically, was used to determine sample placement on the arrays. All samples were plated into a 384-well microplate in a four-point, 2-fold dilution curve (neat, 1:2, 1:4, 1:8), and arrayed onto FAST nitrocellulose slides (Whatman, Inc.) using the miniarrayer equipped with Stealth SNS15 solid pins (TeleChem International, Inc.). Samples were printed at three depositions per feature to ensure ample protein concentration. Assuming that the pins deposited 7.0 nl, the specified delivery volume, each spot in the dilution curve contained approximately 24, 12, 6, and 3 cells, respectively. All spots were replicated four times on the arrays (Figure 1). To limit potential carryover, the washes were carried out as shown in Table 1.

Table 1. Wash conditions for arraying. Humidity was set at 57% to help prevent evaporation of samples.

Wash	Flow-Through Bath	Passive Bath	Vacuum
Prewash	2.5 sec	2.5 sec (10% ethanol)	2.0 sec
Cycle wash (5 cycles)	3.0 sec	5.0 sec	0.5 sec
Final wash	5.0 sec	5.0 sec	2.0 sec

Two batches of eight slides were printed from the same sample microplate in order to determine interprinting variability for total protein. Another set of samples was printed onto a batch of eight slides to establish inter- and intraslide variability for phosphorylated protein detection.

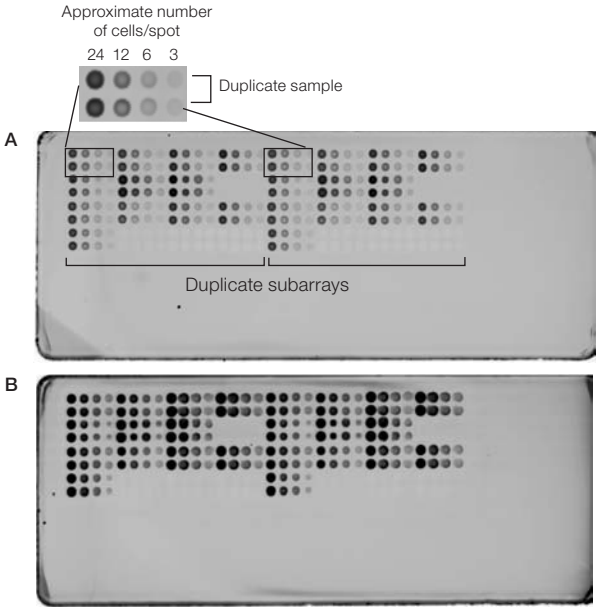

Fig. 1. Comparison of slide printing batches. Two batches of arrays were printed with identical samples from the same source microplate. Samples were printed in duplicate and the entire subarray was also replicated. Approximate number of cells per spot is shown. **A**, SYPRO Ruby-stained slide from first batch of eight printed slides; **B**, SYPRO Ruby-stained slide from second batch of eight printed slides. The more intense staining in slide B illustrates the effect of significant sample evaporation due to the elevated temperature of the platen after a 4 hr print run.

Staining

Arrays were blocked (I-Block, Applied Biosystems) and subsequently stained for Akt (Ser^{473}) and p90RSK (Ser^{380}) (Cell Signaling Technology, Inc.) in triplicate using a Dako autostainer with a catalyzed signal amplification system (CSA) (Dako) according to manufacturers' recommendations. Negative control slides were stained with secondary antibody alone (goat anti-rabbit IgG (H+L), Vector Laboratories). Chromogenic detection was achieved with diaminobenzadine (DAB) (Dako) and arrays were imaged on a flatbed scanner (UMAX PowerLook 1120) (Umax Technologies, Inc.). Arrays were also stained for total protein using SYPRO Ruby (Invitrogen Corporation) and visualized with a Molecular Imager® PharosFX™ Plus system (Bio-Rad Laboratories, Inc.).

Spot Analysis

Spot intensity was determined with MicroVigene image analysis software, version 2.5 (VigeneTech, Inc.). Local spot background was calculated for each spot. Additionally, negative control spot intensities (secondary antibody only) were calculated. Each antibody background-corrected spot intensity value was normalized to total protein. Coefficient of variation (CV) was determined for both raw background-corrected spot intensities and normalized data in order to compare inter- and intraslide variation.

Sample Carryover Experiment

Spot carryover was investigated by arraying streptavidin-conjugated Qdot 655 quantum dots (Invitrogen Corporation) at full strength and diluted 2-fold in 5% bovine serum albumin. A series of spots was printed in duplicate at two, three, and four hits per spot. An additional three empty wells were printed following the spotted samples to measure carryover. Default wash settings were used (Table 2). Arrays were imaged with a Kodak image station 4000MM digital imaging system at 385 nm excitation and 670 nm emission.

Table 2. Wash conditions for sample carryover experiment.

Wash	Flow-Through Bath	Passive Bath	Vacuum
Prewash	—	2.5 sec (10% ethanol)	2.0 sec
Cycle wash (5 cycles)	3.0 sec	5.0 sec	0.5 sec
Final wash	3.0 sec	5.0 sec	2.0 sec

Results and Discussion

Evaluation of instrumentation performance is a necessary process for analytical instruments used in clinical settings. As clinical translational research bridges the gap between basic research and the clinic, researchers should be increasingly aware of the quality assessment processes required for clinical testing. This is particularly important if these technologies are being applied to "home-brew" clinical testing. Instrument quality assessments provide the initial qualification studies needed to develop diagnostic assays. As such, it is imperative to evaluate new instrumentation for precision, accuracy, and throughput as applicable for the particular instrument and testing volume (Lasky 2005). The results presented here represent a series of experiments typically used for evaluation of robotic printing devices.

RPPAs are an innovative technology that applies the principles of conventional antigen-antibody immunoassays to quantitatively identify changes in multiple protein expression patterns (Charboneau et al. 2002, Espina et al. 2003). RPPAs are constructed using whole cellular lysates prepared in a dilution series and subsequently deposited on nitrocellulose substratum by a robotic printing device. The array is constructed with multiple samples, including positive and negative controls, as well as reference standards. Each array is probed with a single primary antibody directed against the antigen of interest and the signal is amplified via horseradish peroxidase-mediated deposition of biotinyl tyramide (Bobrow et al. 1989, King et al. 1997). DAB, which provides a relatively stable visual endpoint, is then employed for colorimetric detection. These features make RPPAs a promising tool in clinical translational research, such as individualized patient therapy (Petricoin et al. 2005). Therefore, all parameters of the experiment from sample collection, manipulation, array printing, and staining require tremendous precision and accuracy to ensure reliable results.

RPPAs may be printed in a variety of formats using various printing devices. In this evaluation, arrays were printed with whole cell lysates, prepared from cell lines, in a denaturing buffer. The initial precision experiment included intra- and interslide reproducibility as well as evaluation of the effects of the printing chamber temperature and humidity conditions on the spot quality. This is crucial because small sample volumes (10–20 µl), as used for the RPPA, are subject to evaporation and consequently increase in concentration in low-humidity and high-temperature conditions.

The Calligrapher was equipped with four out of eight possible flat-tip solid pins. We were able to print eight slides with 256 spots (16 samples, each in a four-point dilution curve spotted in quadruplicate) at three depositions per spot in 4 hr. We printed two sets of eight slides without a cooled platen at 57% humidity.

Visual inspection of the slides for spot consistency, size, and morphology proved that the results were satisfactory. Spot morphology was consistent with an average diameter of 658.3 µm for spots with ample protein concentrations. For less concentrated samples, spots in the dilution curve were smaller, with an average diameter of 442.1 µm.

Abundant evaporation of the samples in the microplate was observed between the first print run (eight slides) (Figure 1A) and the second set of eight slides (Figure 1B). This was also evident by quantitatively and qualitatively analyzing the slides. The intraslide CV (n = 12) was acceptable with an average of 2.7% (van Hijum et al. 2005). However, significant evaporation did lead to increased interslide variation, with CVs as high as 32% (n = 12).

Although we did not evaluate this feature, the Calligrapher is available with a cooled platen. The cooled platen can be set at 22°C to maintain samples at room temperature, thus limiting the undesirable side effect of sample precipitation at lower platen temperatures. Without a cooled platen, the platform temperature increased from room temperature to 35°C after 4 hr of printing. In our experience, with humidity levels set to 57% and a cooled platen set at 22°C, undesirable evaporation of samples should be limited.

For the eight slides that were printed in the same run, inter- and intraslide variability were within acceptable limits. As shown in Figure 2, the first three dilution points (neat, 1:2, and 1:4) had excellent intraslide CVs ranging from 2.5 to 5.6% and interslide CVs ranging from 4.1 to 8.2%. The 1:8 dilution spot had higher variability due to lower protein concentration. These results illustrate the necessity of printing reverse-phase arrays in dilution curves in order to match the sample protein concentration with antibody probe affinity that is within the linear dynamic range of the assay.

Contamination of sample due to sample transfer from pin to pin or spot to spot is commonly termed carryover. Carryover using the Calligrapher equipped with TeleChem solid pins was evaluated by printing streptavidin-conjugated quantum dots, 655 nm, directly on the FAST slide. A fluorescent molecule, such as a quantum dot, is an ideal molecule for evaluating carryover because the quantum dot is not photobleached or quenched during long exposure times. Long exposure times permit larger dynamic range analysis and the ability to detect very low-abundance signal that may be present if contamination or carryover is occurring.

The most common source of carryover contamination is inadequate pin washing between sample depositions. The Calligrapher was programmed to the default setting as listed above with a combination of water and 70% ethanol solutions to maximize the dissolution of sample on the pins during the wash cycle. In general, longer wash cycle times and multiple pin immersions provide enhanced pin cleaning and less contamination. The optimum pin washing steps are sample dependent, as highly viscous samples require longer wash times and/or a series of various wash solutions for effective pin cleaning.

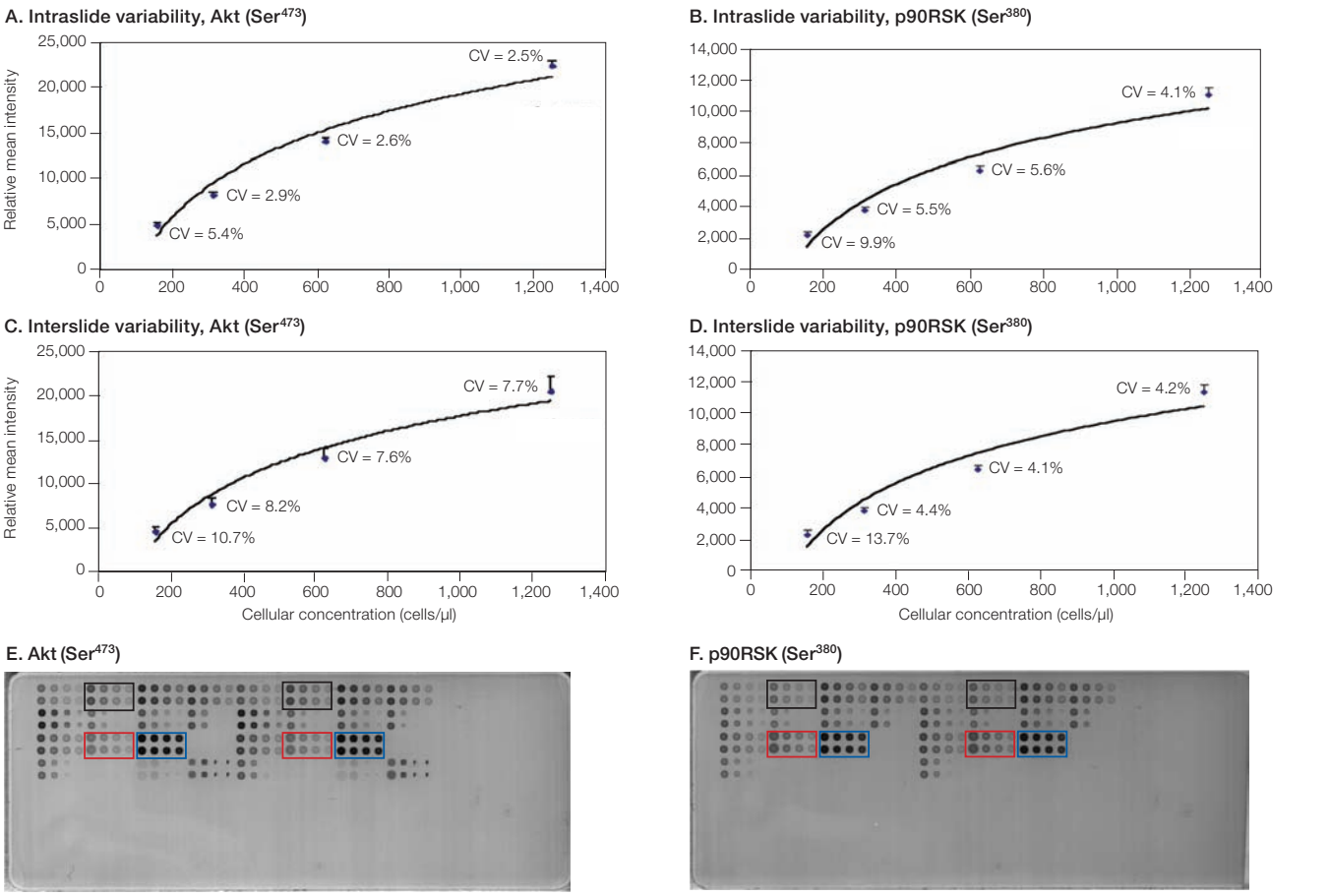

Fig. 2. Precision and linearity studies. The linear dynamic range for two different antibody probes was assessed for arrays printed with cellular concentrations of 156–1,250 cells/µl. **A** and **B**, intraslide values obtained from one sample printed four times on one array. Arrays probed with A, Akt (Ser^{473}) and B, p90RSK (Ser^{380}). Ser^{473} showed good intraslide linearity ($r^2 = 0.9657$ and $r^2 = 0.94$, respectively). **C** and **D**, interslide values obtained from one sample printed four times on three separate arrays. Interslide r^2 values for C, Akt (Ser^{473}) and D, p90RSK (Ser^{380}) were $r^2 = 0.9656$ and $r^2 = 0.9388$, respectively. Intraslide CVs were ≤5.4% for Akt (Ser^{473}) and ≤9.9% for p90RSK (Ser^{380}). Interslide CVs were ≤10.7% for Akt (Ser^{473}) and ≤13.7% for p90RSK (Ser^{380}). **E**, Akt (Ser^{473})-stained array. **F**, p90RSK (Ser^{380})-stained array. The dilution curve inside the black boxes represents samples whose raw background-subtracted intensities were used for analysis above. The dilution curves of A431 and A431 + EGF control samples printed on the arrays are indicated in red and blue boxes, respectively.

The experiments performed by printing Qdot 655 showed no evident carryover when visualized with the Kodak image station 4000MM imager (data not shown). To emulate actual studies, two, three, and four hits per spot were all tested to ensure that carryover did not occur when greater concentrations of protein were printed on arrays. Using the default wash setting to determine carryover gave assurance that the minimal wash cycles were efficient. A more stringent wash cycle was then used when printing the lysates due to their increased viscosity.

Additionally, empty wells were printed on the array in quadruplicate for evaluation of carryover. Evaluations were performed colorimetrically with DAB and fluorescently with SYPRO Ruby. After staining with primary and secondary antibodies, the arrays were visualized with DAB. Total protein slides were visualized with SYPRO Ruby. Both revealed no apparent contamination resulting from carryover of the previous samples (Figure 3).

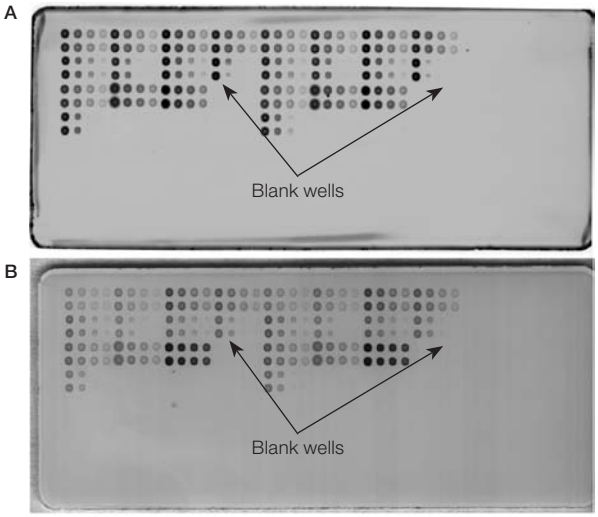

Fig. 3. Assessment of sample carryover. The blank wells represent areas on the array in which carryover was assessed by intentionally leaving microplate wells empty directly adjacent to sample-containing wells for assessment of sample carryover. Arrays stained with **A**, SYPRO Ruby, and **B**, p90RSK (Ser^{380}), did not show any evidence of sample carryover with fluorescence and colorimetric detection (DAB), respectively, in the indicated areas.

Conclusions

This evaluation confirms that the BioOdyssey Calligrapher is technically effective for printing reverse-phase microarrays in batches up to 16 slides. For spots with ample protein concentrations, the average spot diameter was 658.3 µm, with variation of 7.3%. Inter- and intraslide CVs were also <10% for samples with adequate protein concentrations. Therefore, based on our precision and carryover experiments, the BioOdyssey Calligrapher meets the technical quality characteristics that are desirable for printing small sample sets of RPPAs.

References

Bobrow MN et al., Catalyzed reporter deposition, a novel method of signal amplification. Application to immunoassays, J Immunol Methods 125, 279–285 (1989)

Charboneau L et al., Utility of reverse phase protein arrays: applications to signaling pathways and human body arrays, Brief Funct Genomic Proteomic 1, 305–315 (2002)

Espina V et al., Protein microarrays: molecular profiling technologies for clinical specimens, Proteomics 3, 2091–2100 (2003)

King G et al., A highly sensitive detection method for immunohistochemistry using biotinylated tyramine, J Pathol 183, 237–241 (1997)

Lasky FD, Technology variations: strategies for assuring quality results, Lab Med 36, 617–620 (2005)

Petricoin EF III et al., Mapping molecular networks using proteomics: a vision for patient-tailored combination therapy, J Clin Oncol 23, 3614–3621 (2005)

van Hijum SA et al., A generally applicable validation scheme for the assessment of factors involved in reproducibility and quality of DNA-microarray data, BMC Genomics 6, 77 (2005)

Information in this tech note was current as of the date of writing (2006) and not necessarily the date this version (rev A, 2007) was published.

BIO-RAD

Bio-Rad Laboratories, Inc.

Life Science Group

Web site *www.bio-rad.com* ***USA*** *800 4BIORAD* ***Australia*** *61 02 9914 2800* ***Austria*** *01 877 89 01* ***Belgium*** *09 385 55 11* ***Brazil*** *55 21 3237 9400* ***Canada*** *905 712 2771* ***China*** *86 21 6426 0808* ***Czech Republic*** *420 241 430 532* ***Denmark*** *44 52 10 00* ***Finland*** *09 804 22 00* ***France*** *01 47 95 69 65* ***Germany*** *089 318 84 0* ***Greece*** *30 210 777 4396* ***Hong Kong*** *852 2789 3300* ***Hungary*** *36 1 455 8800* ***India*** *91 124 4029300* ***Israel*** *03 963 6050* ***Italy*** *39 02 216091* ***Japan*** *03 5811 6270* ***Korea*** *82 2 3473 4460* ***Mexico*** *52 555 488 7670* ***The Netherlands*** *0318 540666* ***New Zealand*** *0508 805 500* ***Norway*** *23 38 41 30* ***Poland*** *48 22 331 99 99* ***Portugal*** *351 21 472 7700* ***Russia*** *7 495 721 14 04* ***Singapore*** *65 6415 3188* ***South Africa*** *27 861 246 723* ***Spain*** *34 91 590 5200* ***Sweden*** *08 555 12700* ***Switzerland*** *061 717 95 55* ***Taiwan*** *886 2 2578 7189* ***United Kingdom*** *020 8328 2000*

Bulletin 5534 US/EG Rev A *06-0724 0407 Sig 1106*