APPLICATION NOTE

RPPA - Transition from contact pin spotting of cell lysates and protein extracts to high-speed, contactless inkjet printing

Modification of Laemmli buffer formulation for Reverse-Phase Protein Array (RPPA) using Arrayjet inkjet microarrayers.

INTRODUCTION

The Reverse-Phase Protein Array (RPPA) is a high-throughput, antibody-based proteomic technology that allows targeted, sensitive measurement of proteins along with their post-translational modifications in complex protein matrices [1]. Diverse laboratories and core facilities worldwide have implemented RPPA, adopting substantially different technical protocols. Staining, signal amplification, imaging and data analysis techniques depend upon a research group's expertise or preferences [2–4], but all RPPA workflows involve sample miniaturization and immobilization onto a substrate by a robotic arrayer.

Historically, contact pin printing has been the mainstay of the RPPA procedure, and for a long time it had several advantages over non-contact printing in terms of speed, precision and range of usable sample buffers and substrates [5]. In the last decade, inkjet technology has entered the spotlight for developing biosensors and for 3D bioprinting of cells, tissues and organs, fueling technical developments in the field of inkjet printing [6,7].

For RPPA, inkjet arrayers dramatically increase the available surface for sample immobilization, since the size of the features is not limited by the physical size of the pin tip. In addition, contact pin printing takes significantly longer, because pins must dip into the sample source plate with each deposition cycle. Conversely, inkjet printing allows fast successive depositions, thus limiting sample evaporation and increasing the overall printing consistency. The speed of inkjet printing requires specific physical properties, so the formulation of resuspension buffers as well as the concentration of samples need to be adjusted accordingly.

This application note is intended as a basic guide for researchers and technicians considering moving their RPPA printing procedures from contact pin (e.g. Aushon) to inkjet microarray technology from Arrayjet. Practical work and analysis was carried out independently by an Arrayjet customer using a Marathon Argus microarrayer. Findings and recommendations remain applicable to Arrayjet's newer Mercury series of instruments.

EXPERIMENTAL DESIGN

Prior to deposition of regular RPPA samples, a print quality check (QC) is recommended for fine evaluation of printing robustness in terms of spot size, shape and intensity. The QC printing should be run as close as possible to the regular RPPA printing session, using the same resuspension buffer and a material as close as possible to regular RPPA samples. Inspection of these QC results is critical to a successful print run ensuring there is no dust or debris on pins (for contact printing) and that all nozzles are firing optimally (for inkjet printing).

We will compare a 10-point, twenty percent fold-decrease dilution curve of bovine serum albumin (BSA) printed with an Aushon 2470 contact pin printer and a 12-point, ten percent fold-decrease BSA dilution curve printed with an Arrayjet Marathon inkjet arrayer.

The BSA curve printed with the Aushon 2470 arrayer is prepared using 1X sample buffer as per the Laemmli recipe:

SDS 2X (20 mL)

- 5 mL 0.5 M Tris-HCl, pH 6.8
- 4 mL Glycerol
- 8 mL 10% (w/v) SDS
- 2 mL 0.4% Bromophenol blue
- Distilled water to 20 mL

The BSA curve printed with Arrayjet's arrayer is prepared by using a custom version of the Laemmli sample buffer with features of Arrayjet's Protein Printing Buffer C (PPBC) to create a buffer with correct physical properties for inkjet printing, immobilization and reactivity for RPPA assays:

PPBC/SDS 2X (20 mL)

- 1.25 mL Tris/Hcl 2M pH6.8
- 2 mL Glycerol
- 0.8g SDS (powder)
- 0.8 mL Bromophenol Blue 1%
- 16.13 mL Ethylene Glycol
- 0.02 mL Tween-20

Prepare a 1 % (w/v) BSA solution (equivalent to 10 mg/mL) by resuspending 1g of BSA powder in ultrapure water (or dH2O) with 0.9 % NaCl (equivalent to 150 mM). Prepare a working RPPA sample by diluting BSA stock solution 1:10 (1 mg/mL final) in either SDS or PPBC/SDS buffer (1X final) along with TCEP at a final concentration of 2.5 % (v/v). Use either TPER (Thermo-Fisher Scientific) or standard RIPA buffer to bring the 1 mg/mL BSA dilution to volume. The total volume required ultimately depends on the number of wells that must be filled with each dilution step. A volume of 20 µL is suitable for both arrayer types and this results in a minimum required volume of 0.4 mL per dilution step if using the Aushon 2470 arrayer with a 5x4 pin head configuration. The volume required for each dilution step printed with the Arrayjet arrayer at 20 µL/well is at least 0.25 mL.

Due to physical constraints, i.e. the available space between pins and the size of the features, a maximum of 10 dilution points can be printed by each individual pin using Aushon's 2470 arrayer, with the last point at 0.134 mg/mL. Arrayjet printing allows for a broader dilution curve of 12 points down to a concentration of 0.086 mg/mL (or even lower by adding further dilution points). The procedures and deriving results described in this application note apply to printing onto nitrocellulose substrate, specifically GRACE Bio-Labs ONCYTE® AVID 20 x 51 mm nitrocellulose-pad glass slides (part. no. 305170).

The total printing time for the Aushon 2470 arrayer is strictly dependent on the number of i) slides, ii) replicates, and iii) depositions per feature. An acceptable compromise between turnaround time, amount of starting volume and ultimately the overall assay sensitivity, is three depositions per spot. These settings, coupled to using pins with a tip size of 185 µm, result in features with an average diameter of 300 µm and an average spot area of 70,685 μm^2. Depending on the required sensitivity and the starting concentration of RPPA samples, an acceptable number of drops per spot for the Arrayjet arrayer is either four or six. With these settings, the feature diameter produced by the Arrayjet arrayer is 175 µm on average, resulting in a three times smaller spot area of 24,053 μm^2 compared to the Aushon 2470.

DATA ANALYSIS

The use of a modified Laemmli buffer to modulate the physical properties of RPPA samples may lead to differences in terms of spot size and shape. This, combined with the different modes of liquid deposition may alter the total protein content of printed material, putatively due to the volumes deposited. Likewise, the different buffer composition may result in differences in downstream antibody detection.

The shape of the spots produced by inkjet printing has comparable (if not improved) roundness and the size is dependent on the amount of material deposited by the instrument's specific print head (Figure 1). The amount of material deposited by contact pin printing at three depositions is higher than that released by 4 drops (400 pL) using the inkjet printer (Figure 2).

Despite a three-times smaller area, spotting at 600 pL/spot with the inkjet arrayer allows for faster deposition of an amount of material, using about 2/3 total protein content compared to contact pin printing (Figure 2).

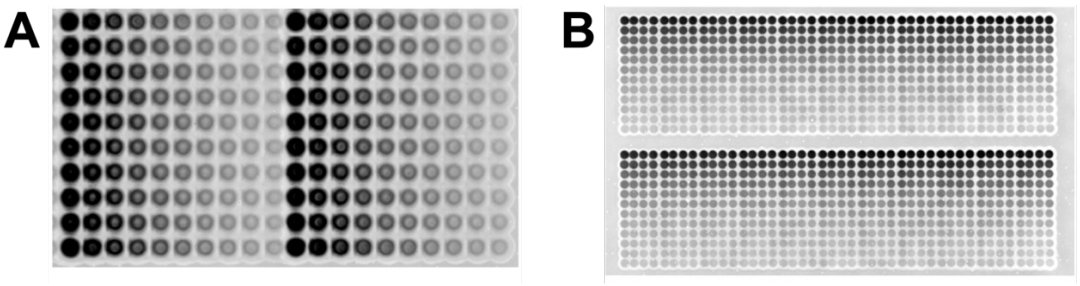

Figure 1: BSA quality check. Staining for total protein content using SYPRO[a] Ruby Protein Blot Stain (Thermo-Fisher Scientific) of (A) 10-point BSA dilution curves printed using Aushon's 2470 arrayer equipped with 20 x 185 µm pins and (B) 12-point BSA dilution curves using the Arrayjet Marathon arrayer. The spot-to-spot distance has been set to 450 µm in the horizontal axis and 500 µm in the vertical axis for the 2470 arrayer and the number of drops/spot has been set to 4 for the Marathon arrayer. The images in (A) and (B) show areas of the nitrocellulose with comparable physical sizes and the contrast has been adjusted to better show the intensity differences of all available dilution points.

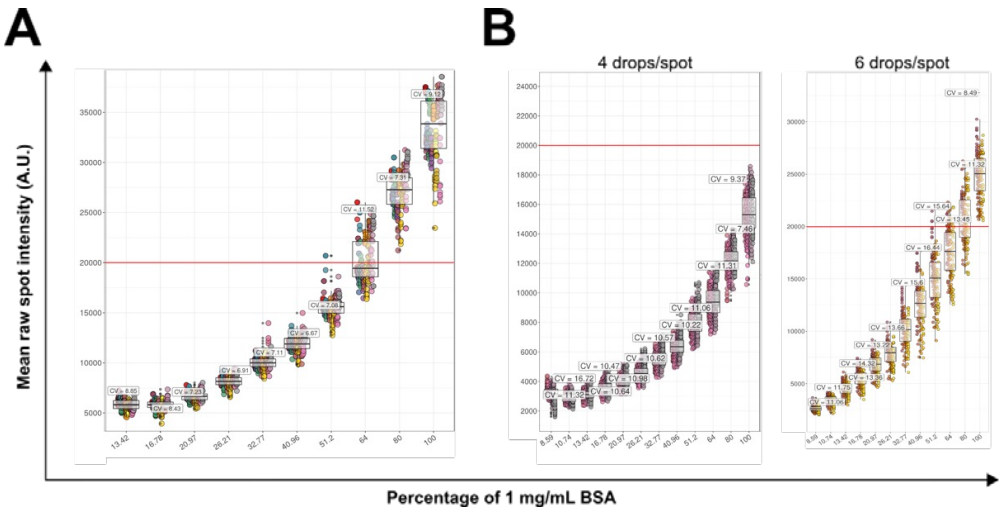

Figure 2: BSA curve data analysis. Spot detection, local background subtraction and quantification of total protein content from (A) 9 replicates of a 10-point BSA dilution curve for each of the 20 pins used to print with Aushon's 2470 and (B) 308 replicates of a 12-point BSA dilution curve printed with Arrayjet's Marathon. Number of drops/spot has been set to either 4 or 6 (i.e. 400 or 600 pL) for the Arrayjet Marathon arrayer and the red line in each plot marks an intensity level of 20.000 to show the relative difference between arrayers when adjusting the printing settings. The coefficient of variation (CV) is reported as a text box in the proximity of the summary boxplots at each dilution percentage.

Indeed, even if using 400 pL/spot the lower detection limits of the assay can be increased by i) starting from higher amounts of material and use a higher number of drops/spot; ii) using protein extracts with a higher starting concentration; iii) pushing the sensitivity limits of the image acquisition instrument. An example of the latter option is shown in Figure 3, whereby antibody staining of the same original RPPA samples printed on the same slide using either Aushon's 2470 at three depositions/spot or Arrayjet's arrayer at 400 pL/spot, results in comparable endpoint intensity levels along the dilution curve.

Overall, these data demonstrate the feasibility of RPPA printing using an inkjet technology, by increasing the viscosity of the Laemmli sample buffer without noticeably affecting the overall concentration and quality of the protein samples.

SUMMARY - THE INKJET ADVANTAGE

The use of an Arrayjet inkjet microarrayer provides several improvements over standard contact pin printing. One advantage is the amount of available printing area, which allows twice the number of RPPA samples that to be arrayed at once. Since i) the staining reagents are expensive and ii) cross comparison of RPPA samples assayed in different experimental sets may introduce undesired biases, printing a higher number of samples on the same slide is a major improvement in accuracy and budget.

Another critical point is the time required to print a defined number of slides. Contact pin printing is very demanding in terms of time when the number of slides to be printed is increased, ultimately leading to sample evaporation issues. Given a comparable number of RPPA samples and slides, using the Arrayjet inkjet printer significantly reduced printing times and sample evaporation, ultimately leading to improved consistency in the printed slides. A real-world example of printing 550 RPPA samples on 210 slides using the Aushon 2470 arrayer takes a full week of consecutive printing, with samples sitting in the microplates for up to three days per slide half. Filling a comparable portion of the nitrocellulose slide with twice the RPPA samples using the Arrayjet Marathon would take about 24h at 600 pL/spot.

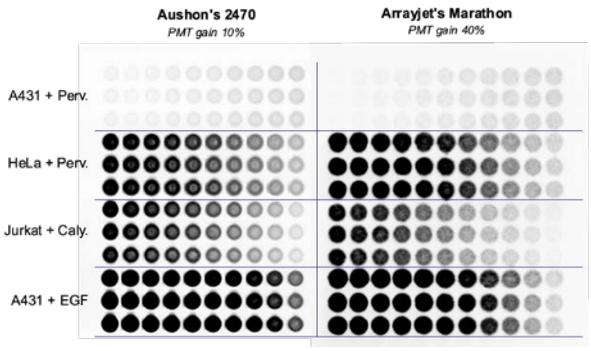

Figure 3: Antibody staining comparison test. Commercially available cellular lysates from cancer cell lines either left untreated or treated with agents that increase the overall phosphorylation levels, have been mixed to build dilution curves of 10 % fold-change at each consecutive point. Starting from identical batches of cell extracts at 1 mg/mL concentration, dilution curves have been prepared using either 2X SDS or 2X PPBC/SDS sample buffers, for printing on Aushon's 2470 and Arrayjet's Marathon, respectively. The number of useful dilution points in the Aushon's 2470 sample set was 10 while the Arrayjet Marathon printer allowed for at least one additional dilution point. The cellular lysates used comprise A431 + Pervanadate, A431 + EGF (Becton-Dickinson), HeLa + Pervandate and Jurkat + Calyculin (Santa Cruz Biotechnology). Printed slides have been subjected to antibody staining using anti-ERK1 phospho-threonine 202 / ERK2 phospho-tyrosine 204 (Cell Signaling Technology) as per the procedures described in [8]. Stained slides have been digitalized by using a laser scanner (Tecan Power Scanner) at 1 % power and varying photomultiplier tube (PMT) gain. The image from the Marathon arrayer slide has been magnified on purpose to match the size of the sister dilution curves printed by Aushon's 2470.

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