

DNA Microarrays: Optimization of Fabrication Parameters and Control Plate Design

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Introduction

While microarray technology has dramatically increased the throughput of gene expression studies by allowing changes in the level of expression of thousands of genes to be monitored simultaneously (Schena et al. 1995), the technology has drawn significant criticism because of problems with reproducibility. Natural biological variation (Pritchard et al. 2001), technical difficulties preparing samples for hybridization (Bammler et al. 2005), and poorly fabricated arrays (Yue et al. 2001) are the primary sources of variability. Fabrication problems can include mistracked samples, poor precision of printing, poor glass slide quality, and inconsistent spot volume (Wang et al. 2003). If too little material is arrayed, sensitivity is reduced, and in two-color experiments, the ratio of signal intensities is compressed (Yue et al. 2001). If the concentration of arrayed material is too high, there may be insufficient room for labeled cDNA to hybridize, or labeled cDNA may hybridize at densities that cause quenching and inaccurate signal intensity values (Shchepinov et al. 1997, Peterson et al. 2001, Dorris et al. 2003).

Bio-Rad arrayers include two high-precision robotic arrayers, the VersArray ChipWriter™ Pro system and the BioOdyssey™ Calligrapher™ miniarrayer. The actuators in these instruments execute 1 µm motion increments, and print repeatability is within 5 µm along the x-axis and 8 µm along the y-axis. Thus, these instruments are capable of producing very precisely printed arrays.

To ensure consistency of data from microarrays printed with either of these systems, it is critical to understand the key factors for printing — or fabricating — arrays, and to design proper controls. The key fabrication factors include DNA immobilization and retention, and the consistent and accurate deposition of DNA. Controls are an important component of any gene expression experiment. The selection of appropriate controls can facilitate data interpretation and help identify fabrication problems as well as other experimental issues. Understanding the arraying parameters is key when designing a control plate that will detect possible artifacts introduced by differences in pen performance during arraying.

Here we discuss: 1) key factors for optimizing and troubleshooting array fabrication; 2) the types of controls commonly used for gene expression experiments and proper control plate design; and 3) the optimization process used to fabricate an array designed to monitor expression of approximately 2,000 human genes. We describe this process using either the BioOdyssey Calligrapher or the VersArray ChipWriter Pro system.

Methods

Plate Preparation

A set of seven 384-well plates for arraying, containing oligonucleotides representing approximately 2,000 human genes, was assembled using primarily ready-to-array synthetic 70-mer oligonucleotides from multiple independent vendors (Qiagen, MWG-Biotech, and Illumina; Table 1). The oligonucleotides were resuspended in a proprietary print buffer at 25 µM (MWG-Biotech and Illumina) or 50 µM (Qiagen).

Two 96-well control plates were assembled, in which each row would be printed by a different pen. Each plate contained oligonucleotides for 18S rRNA and β-actin as well as either PCR cDNA (GE Healthcare) or 70-mer oligonucleotides (TIB MOLBIOL) for 18 exogenous spike-in RNAs from the *Lucidea* Universal ScoreCard controls (GE Healthcare). Quadruplicate dilutions of a 70-mer oligonucleotide (1–50 µM) for detection of the *Arabidopsis XCP1* gene described by Wang et al. (2003) were placed in the oligonucleotide control plate to generate a DNA concentration curve. Prior to arraying, the three 96-well source plates were each compressed into four consecutive rows of a 384-well plate using a 12-channel pipet to transfer the contents of one row of the 96-well plate into the odd or even wells of a row of a 384-well plate as shown in Figure 1.

Array Fabrication and DNA Crosslinking

Printing of the array was performed at 55% humidity and 22°C using a VersArray ChipWriter Pro equipped with eight SMP3 pens (TeleChem International). Pen contact force was controlled by adjusting the approach speed to 11 mm/sec, the travel time up and down to 1 sec each, and the wait time to 1 sec. To minimize print carryover, for each dip the pens were washed for 5 cycles, with each of the first 4 wash cycles consisting of 6 sec in the wash bath, 1 sec in the vacuum

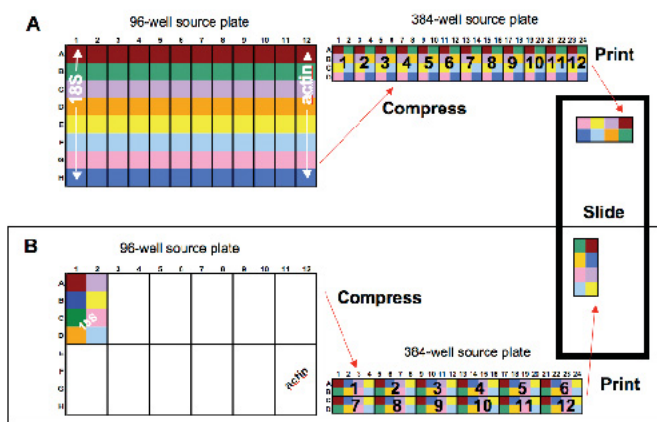


Fig. 1. A 96-well source plate layout that ensures different pen array replicates. A, the VersArray ChipWriter Pro, with a 2 x 4 array of pens; B, the BioOdyssey Calligrapher, with a 4 x 2 array of pens. Wells printed by the same pen are shown in the same color, and this color-coding is used throughout. Samples picked up simultaneously are shown in the same numbered box in the 384-well source plate. For clarity, color-coding is shown for only the first box of the 96-well plate used with the Calligrapher. In both examples, material is removed from the source plate from left to right and top to bottom.

station, and 10 sec in the sonication bath. For the last wash cycle, the time in the vacuum station was increased to 4 sec. Pens were blotted 10 times to reduce misfiring. Each pen printed a 12 x 16 grid of 120 μm spots spaced 250 μm apart, center to center. The array consisted of two supergrids, each composed of eight grids. Oligonucleotides were arrayed on UltraGAPS slides (Corning) and allowed to dry on the platen for 30 min. Marks to indicate the location of the printed area and a unique identifier for each slide were etched on the printed side. Oligonucleotides were immobilized by exposure to UV irradiation using a GS Gene Linker™ UV chamber set at 254 nm with an energy output of 450 mJ/cm^2 . Before hybridization, arrays were incubated in 0.1% BSA, 5x SSC, 0.1% SDS at 55°C for 30 min, rinsed twice in 0.2x SSC, and dried by centrifugation.

Hybridization

A Cy5 end-labeled random 9-mer oligonucleotide (TriLink Biotechnologies) was used to indirectly visualize spot morphology and estimate DNA concentration. The labeled 9-mer was solubilized to a final concentration of 7.5 μM in 4x SSC, 0.2% SDS, 50 mM HEPES (pH 7.4) with 1 mg/ml poly(dA) applied to the array, and incubated for 5–30 min. The array was washed for 10 min in 2x SSC, 0.1% SDS and then for 3 min in 0.2x SSC, dried by centrifugation, and scanned immediately. Exposure to light was minimized, and all incubations and washes were performed at room temperature.

Results and Discussion

DNA Retention and Immobilization

Maximizing DNA retention on the array surface is a critical first step for fabricating high-quality DNA microarrays and is highly dependent on surface chemistry. The quality of the glass slides commonly used for arraying can have a significant impact on array performance (Hessner et al. 2004). Ideally, glass slides should be flat and have a uniform surface that yields spots of consistent shape and size, generate low background fluorescence, and possess high DNA retention capacity. Typically, low-autofluorescence slides are ground flat and coated with a molecule that has a surface chemistry that allows electrostatic interaction with and subsequent immobilization of DNA. Various surface chemistries are available for microarray production, including poly-L-lysine, aminosilane, aldehyde, epoxide, and gel coatings. After spotting and DNA immobilization, free DNA is removed and unreacted surface groups are blocked.

UV irradiation is another critical parameter for immobilizing DNA to the chip surface. Wang et al. (2003) reported that DNA oligonucleotides require more UV irradiation than PCR products to immobilize them to the same surface. In addition, not only did the crosslinking optima differ for different slide chemistries, but also different optima were observed for slides of similar chemistries from different vendors. Arrays were irradiated as described in Methods after determining the optimal amount of energy required, as described in BioRadiations 115 (2005).

Array and Control Plate Design

For transcriptional profiling experiments, exogenous RNA transcripts are often added to the labeling reaction as controls, and oligonucleotides with complementary sequences are included in the array to detect these transcripts (Wang et al. 2003). These controls are used to correlate signal intensity with transcript abundance, facilitate data normalization, allow direct comparison of different arrays, and determine the sensitivity with which transcripts can be detected. Poly(dA), poly(dT), COT-1 DNA, and salmon sperm DNA can also be used as controls to evaluate hybridization specificity, and are more useful when arraying PCR products of EST clones, which may include large poly(dA) stretches, regions of low sequence complexity, or highly conserved domains that share conservation between members of a gene family (Wilson et al. 1987). In addition, repetitive DNA, such as COT-1, may hybridize to genomic DNA contaminating an RNA preparation (Weiner et al. 1986).

The control plate for the array examined here included probes for 18S rRNA, β -actin, 18 exogenous spike-in RNAs, and a DNA concentration curve consisting of an exogenous oligonucleotide arrayed at different concentrations, which was used to assess fabrication quality. Poly(dA), poly(dT), human COT-1 DNA, and salmon sperm DNA were not used because their signal intensities in initial hybridization experiments were consistently either low or undetectable.

The primary objective in assigning controls to source plate wells was to have replicates printed by different pens. By assigning replicates to different pens, it is possible to determine variation in signal intensity, assess pen variability, and ensure that a sticking pen will not cause loss of key control data. Here, each exogenous spike-in control was replicated in each control plate a minimum of three times. 18S rRNA and β -actin oligonucleotides served as positive controls, and were arrayed by all eight pens at the start and end of the row, respectively, to facilitate gridding. β -Actin was chosen as a marker of mRNA quality because it is a housekeeping gene expressed at an invariant level in most cells (Kreuzer et al. 1999). 18S rRNA was used to assess levels of rRNA contamination of mRNA preparations. In two-color experiments, the signal intensities from the 18S rRNA spots can also be used to determine optimal photomultiplier tube (PMT) gain and laser power settings for the microarray scanner. Because the bulk of any total RNA sample is rRNA, if equivalent amounts of total RNA are added to both labeling reactions, the observed signal intensity from the 18S rRNA spots should be equivalent at both wavelengths.

Since it is much easier to work with 96-well plates by hand, two 96-well control plates were prepared initially and then transferred into a 384-well plate for arraying (Figure 1). Although both plates included sequences to detect the same 18 exogenous spiked-in RNA controls, one control plate consisted of PCR products while the other was composed of oligonucleotides. This allowed direct comparison of any difference between the performance of PCR products and long oligonucleotides. The 60–70-mer control oligonucleotide sequences had minimal secondary structure and a similar G:C content, melting temperature, and length to those in the sample plates. Thus, the ability to detect the controls should be comparable to the ability to detect the transcript for a given arrayed gene.

Six 384-well gene plates from different vendors, which contained long oligonucleotides (60–70-mers) designed to detect approximately 2,000 different human genes, were printed using eight pens in a 2 x 4 array. A 12 x 16 array was chosen for each grid to facilitate correlation of source plate location with array location. Since the source plate has 24 wells in the x-direction, each row of a grid corresponds to the printhead traveling once across the source plate in the x-direction. Since eight pens are used in printing, four rows of the array correspond to one 384-well source plate. The 384-well plate containing the Illumina oligonucleotides and the controls was arrayed at the start and end of the print run.

Assessing Array Quality and Spot Morphology

After arraying, the slides were irradiated and blocked. One of the arrays was hybridized with a Cy5-labeled random 9-mer to assess spot morphology and identify pen misfires (Figure 2). Other fluorescent dyes could have been substituted, such as SYTO-61 (Yue et al. 2001), Vistra Green (Wang et al. 2003), or POPO-3 or TOTO-3 (Bowtell and Sambrook 2003). Overall,

the misfiring rate for this print run was 2%; however, significant variations in the rate of misfiring were observed. While most pens misfired <1% of the time, two of the pens misfired 4–10% of the time. A grid generated by a pen with a high misfiring rate is shown in Figure 2C. Generally, misfired spots would be flagged and removed from experimental analysis. Loss of approximately 40 data points out of 2,000 is acceptable and has minimal impact on the utility of this array to detect changes in gene expression.

To facilitate assessment of array fabrication quality, a DNA concentration curve was included in the control plate of oligonucleotides. The *Arabidopsis XCP1* gene was chosen because it has low homology with human sequences and serves as a negative control for hybridization (Wang et al. 2003). The DNA concentration curve was printed by four pens and arrayed twice. Figure 2A shows two blocks from this 3,072-element array hybridized with the Cy5-labeled random 9-mer oligonucleotide. The signals for plates 2–4 were visibly brighter than those of plates 5–8. Part of the array containing one DNA concentration series is shown at higher magnification in Figure 2B, where it can be clearly seen that the signal intensity increased as the DNA concentration increased. The signal intensity obtained from all eight prints of the DNA concentration curve is plotted as a function of DNA concentration in Figure 3. A linear relationship was observed.

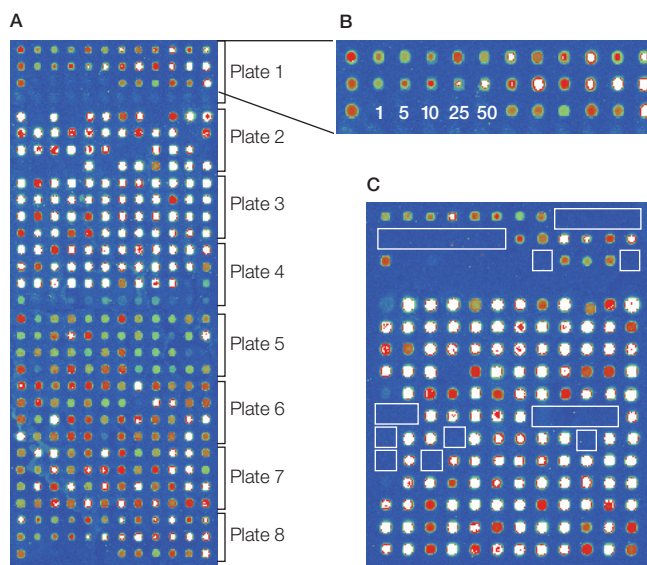


Fig. 2. Visualization of an array with a random 9-mer shows differences in DNA concentration and identifies pen misfires. A, two 12 x 16 blocks (plates 1–4 and 5–8) from a 3,072-element array, printed with eight pens on the VersArray ChipWriter Pro system; B, an enlarged view of the subarray containing a DNA concentration series (with the concentration in μ M indicated below each spot), generated with a 70-mer oligonucleotide from the *Arabidopsis XCP1* gene, which should not crosshybridize with any human gene sequence; C, a third subarray with a large number of pen misfires; misfires are boxed in white. Increasing relative fluorescent signal is color coded from blue to yellow to red to white.

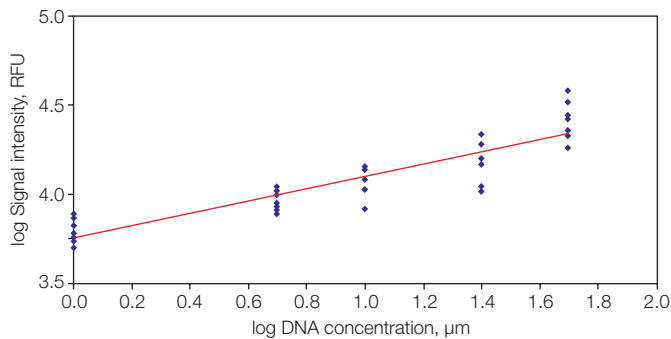


Fig. 3. Correlation between signal intensity and DNA concentration. The concentration curve was generated from the control plate containing a 70-mer complementary to the *Arabidopsis XCP1* gene. This plate was arrayed at the start and end of the run. Evaporation during arraying accounts for the apparent increase in DNA concentration that was observed.

The concentration of each plate was estimated based on the average spot intensity of the plate as a whole after staining with the labeled random 9-mer (Table 1). In general, the estimated DNA concentration for oligonucleotides from Qiagen was about twice that for those from MWG-Biotech, as expected based on their initial resuspension at 50 and 25 µM, respectively (see Methods). Taken together, these observations support the conclusion that the labeled random 9-mer binds to DNA in a concentration-dependent manner.

Table 1. Actual and estimated DNA concentration of arrayed oligonucleotides.

Sources were either vendor-supplied 384-well plates of oligonucleotides representing human genes or an in-house-assembled 384-well plate containing controls and the oligonucleotides purchased from Illumina (see Methods). Estimates were based on average spot intensity of an entire plate.

Plate	Concentration (µM)		Vendor and Plate*
	Estimated	Actual	
1	25 (start) 30 (end)	25	Controls and Illumina trial Oligator RefSet
2	100	50	Qiagen: Apoptosis
3	95	50	Qiagen: Stress and aging 1
4	90	50	Qiagen: Stress and aging 2
5	20	25	MWG-Biotech: Metabolism
6	50	25	MWG-Biotech: Tumor antigen + suppressor + apoptosis inhibitor + DNA repair protein + receptor signaling protein
7	50	25	MWG-Biotech: Diverse functions (plate b)

* The MWG-Biotech and Qiagen oligonucleotides were supplied lyophilized in 384-well plates; the Illumina oligonucleotides were supplied in a 96-well plate. Plate 1 was arrayed at both the start and end of the run.

Conclusions

We have demonstrated that following optimization of array fabrication, a high-quality medium-density microarray can be made using Bio-Rad arrayers. In addition, we have provided information on plate design and controls to ensure proper experimental design, so that the resulting data can be reliably interpreted. Arrays designed following these recommendations will provide high-quality differential gene expression data.

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