Supplementary Material

Beyond Affymetrix arrays: expanding the set of known hybridization isotherms and observing pre-wash signal intensities

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In order to ensure that isopropanol did not adversely affect the quality of signal, we sequentially treated the microarrays with isopropanol. Two sets of experiments were conducted using microarrays that were hybridized to the same target for 4 or 24 h. Each set consisted of at least two independent microarray experiments. The combined data revealed that the first and subsequent isopropanol treatments had a slope of approximately one (Figure S2), and that the experimental data closely followed the Line of Equality, indicating that the repeated isopropanol treatments did not substantially change the fluorescence density of the probes.

Supplementary Tables

Table S1. Relationship between *K* and Nearest Neighbor counts using an overdefined systems of equations. R^2 values represent how well the model fits the numeric solution. NA, not applicable.

Microarray	Model	Probe Conc. (μM)	Number of probes	R^2
Erie	Linear	6.25	95	0.50
		9.38	95	0.52
		12.5	95	0.43
		18.75	95	0.49
		25	95	0.45
		37.5	95	0.50
		50	95	0.53
		All	665	0.36
Erie[a]	Linear	6.25	95	0.47
		12.5	95	0.52
		25	95	0.46
		50	95	0.52
		All	380	0.42
VWR	Langmuir	6.25	95	0.12
		12.5	95	0.11
		25	95	0.25
		50	95	0.13
		All	380	0.06

[a], Replicated experiment.

Table S2. Distribution of fluorescence density (*Fd*, fluorophores/µm^2) at equilibrium for rRNA target hybridized for 4h to perfect match probes on an Erie microarray.

Probe Conc (µM)	Mean Fd	± Std	Number of probes
6.25	65	50	96
8.75	79	67	96
12.5	88	77	96
17.5	94	79	96
25	94	86	96
35	100	86	96
50	117	110	96

Table S3. Distribution of fluorescence density (*Fd*, fluorophores/µm^2) at equilibrium for rRNA target hybridized for 24 h to perfect match probes on an Erie microarray.

Probe Conc (µM)	Mean Fd	± Std	Number of probes
6.25	68	79	17
8.75	109	76	16
12.5	120	83	17
17.5	139	129	28
25	173	151	29
35	230	231	35
50	199	164	32

Supplementary Figures

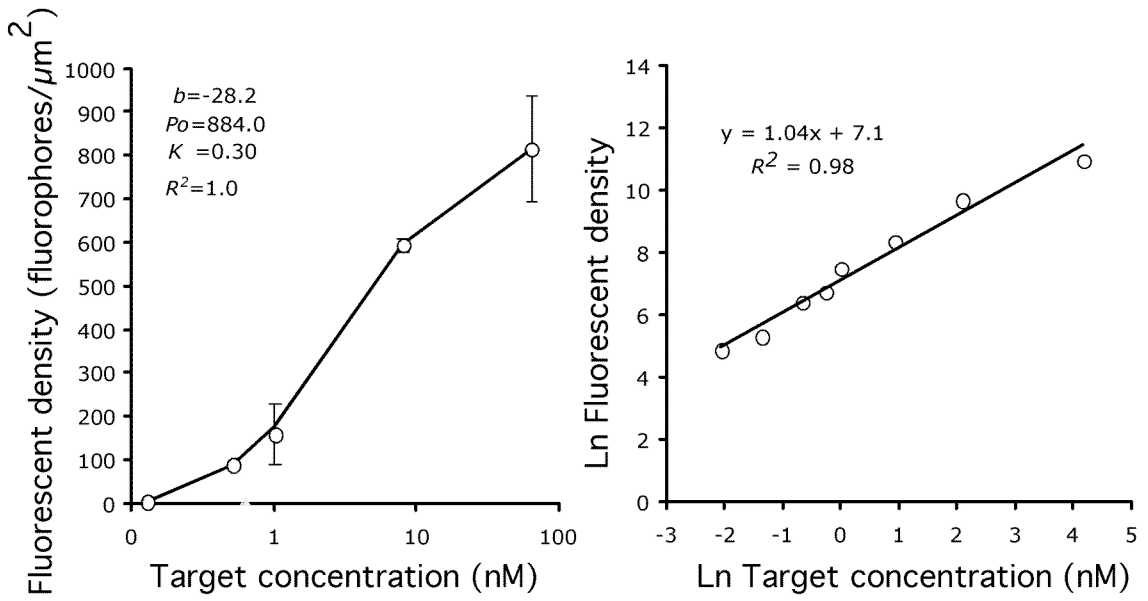

Figure S1. Representative hybridization isotherms obtained using a 70-mer target to the same probe on different microarrays. This example is based on Probe 18. Left panel, NimbleGen; Right panel, CombiMatrix. Probes from the NimbleGen microarray followed Langmuir isotherms, while CombiMatrix microarray followed the power-law ($y = 7.1x^{1.04}$). Details of the Methods and Materials are presented below.

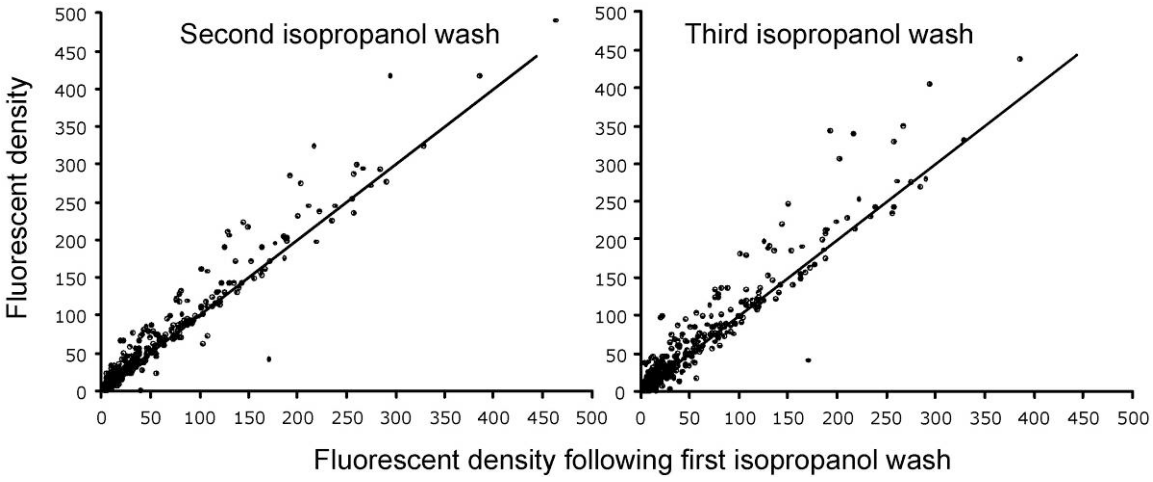

Figure S2. Probe fluorescent densities (Fd, fluorophores/μm^2) following isopropanol washes (n=445 points). Shown is the Line of Equality. Samples were hybridized for 4 or 24 h with active mixing. Note that fluorescent densities did not significantly change with successive isopropanol washes. Left panel, $Fd_2 = 1.1 \times Fd_1$, R^2=0.94; right panel, $Fd_3 = 1.1 \times Fd_1$, R^2=0.92.

Design of target and microarray probes for the high density microarrays

The DNA target used for NimbleGen and CombiMatrix microarray experiments was based on the order of single numbers in the number Pi (http://zenwerx.com/pi.php) and therefore represents a truly random target. For example, the number Pi starts with: 3.14159265.... Each time a "1" was encountered, the letter A is assigned to the sequence. Similarly, when a "2" was encountered, the letter "G" was assigned, a "3" the letter "T" was assigned, and a "4" the letter "C" was assigned until a 70-mer was generated. The other numbers in Pi were not assigned to any nucleotides. Thus, the order of the first six nucleotides of an oligonucleotide target based on Pi is TACAGT. We generated several thousand 70-mer targets *in silico*, and their Gibbs free energy will be calculated using *mfold* (http://frontend.bioinfo.rpi.edu/ applications/hybrid/quikfold.php). The target with the highest folding energies for DNA was then synthesized. The specific sequence was: 5'-CGAACCACCTGCACCCGA CCAAACTCTTTCTATCACTGTAATCGTTTCAAGCCCTATAACTGTAATTTCG-3'. The 5'-end of the molecule was labeled with AlexaFluor 546 (spectral equivalent of cy3). The probes (25-nt) were generated *in silico* by tiling along the targets using a one base-pair shift. The actual number of oligonucleotides produced and the number of replicates for each probe were determined by the amount of available space on the microarrays.

Hybridization, microarray image acquisition and processing

For high density arrays, the same hybridization conditions and reagents were used as those stated for low-density arrays. Hybridization and all washing steps for NimbleGen arrays were performed in a Maui Hybridization Systems. For CombiMatrix arrays, hybridization was performed in a Fisher Scientific Isotemp hybridization oven with array rotisserie clamps and washing steps were completed by hand.

Two different Axon Instruments GenPix 4000B scanners were used to scan the NimbleGen and CombiMatrix microarrays. These scanners were also calibrated and optimal PMT settings were used. Images were stored as 16-bit TIFF files and processed

using ImaGene software (BioDiscovery, Inc., USA). Local background of each spot was not subtracted.