Improved Print and QC Methods for Oligonucleotide Arrays

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Microarray technology offers the best approach today for gene expression analysis on a genome-wide scale. Data quality depends directly on the quality of the starting reagents, especially the printed microarray slides. Many researchers use microarrays that are printed with pre-made oligonucleotides or PCR products using contact spotters by local core facilities or in their own laboratories, where the most advanced manufacturing and QC methods might not be available. Use of the most robust and reliable combination of nucleic acid probes and slide surface chemistry is important to ensure optimal results. Spots that have uniform morphology and probe density show improved reproducibility and sensitivity; quality control is essential. We employed a model system of 8 different probe sequences (GC range 25% - 60%) and compared the use of unmodified, amino-modified, and I-linker (hydrazide) modified 70mer oligonucleotides on 5 commercially available surfaces (aldehyde, epoxide, and 3 different amine variants) using different spotting solutions. Slides were hybridized with synthetic Cy3™-labeled targets to simulate actual sample hybridization. Unexpectedly, we found that the modified oligos performed better (at least 2X signal intensity) on the amine surface slides, with rank order I-linker > Amino-mod > unmodified oligos. The use of our own recipe spotting buffer improved spot morphology, which in combination with optimal oligo chemistry, improved data quality and overall reproducibility.

Slides were also hybridized with 5 different test reagents intended for use to QC the quality of printed arrays, and these QC results were compared with actual target-specific hybridization images. We originally planned to compare 4 commercially available reagents sold for this application. However, the spot images obtained using all four of these reagents poorly reflected actual signals seen from the target-specific hybridizations, so we developed our own QC method, including a new modified Cy3™-nonamer, hyb solutions, and protocols. The new QC kit is available from IDT and allows for hybridization, wash, and visualization in under an hour and will detect PCR products as well as oligos of a variety of lengths and GC composition.

INTRODUCTION

The development and use of microarrays for expression profiling has enabled researchers to investigate gene expression across entire genomes in a single experiment. Several competing microarray platforms are currently available. These platforms differ in both type and length of immobilized probes as well as in manufacturing technologies, from light-mediated *in situ* synthesis of oligonucleotides to spotting pre-made oligos or cDNA/PCR products via contact and non-contact methods. Common to all platforms is the fact that the accuracy and reliability of the conclusions derived from microarray experiments are dependent on the initial quality of the arrays. For arrays manufactured using spotting technologies, much attention has been given to the development of slide surfaces, attachment chemistries, and spotting solutions. The overall goal is to produce arrays with high sensitivity and low variability by optimizing immobilized probe-density, probe availability for hybridization, and spot-signal homogeneity. Consequently, assessing array quality and comparing the performance of the competing manufacturing options have become crucial to ensure the use of the most robust and reliable reagents. Many reagents and methods are available for array spot QC analysis, from ssDNA binding stains (such as POPO™-3 or SYBR®-Green II) to fluorescently labeled random-sequence oligomers. The ssDNA binding stains can assess spot presence and morphology but do not assess the functional ability of the immobilized probes to hybridize. The current consensus is that better assessment of microarray quality can be achieved through hybridization of a fluorescent-labeled oligo.

To determine the most reliable and robust combination of slide-surface chemistry and nucleic acid probe design, we spotted a test set of eight oligo-probes with different GC-contents using different print buffer compositions on commercially available epoxy, aldehyde, and amine surfaces. Each probe was synthesized with a hydroxyl (unmodified), amino-modifier, or hydrazide-modifier group on the 5'-end. Hybridization with complementary oligomers showed highest signal intensity for the combination of hydrazide-modified oligonucleotide printed on an amine-derivatized surface. We optimized the print buffer composition to exploit this attachment strategy and tested the method on three different amine-slides. Our overall best results were achieved by printing hydrazide-modified oligos on Corning GAPSII slides in a buffer of our own formulation (OSB, or Oligonucleotide Spotting Buffer). In addition, we synthesized a new modified Cy3™-labeled random 9-mer oligo for array QC and developed an optimized hybridization method that results in improved detection of spotted oligo probes over a broad range of GC-content.

METHODS

Oligo Arrays

Eight 70mer probe-oligo sequences with various 5'-modifications and two with different post-synthesis purifications **(see Table 1)** were printed in replicates using the GMS-417 Arrayer (Affymetrix). The oligo probes were spotted at either 25µM or 30µM in various spotting solutions **(see Table 2)** on five commercially available surfaces **(see Table 3)**. The printed arrays were placed in a humidity chamber for 3-4 hours and stored in a dark, dry (<30% humidity) environment or heat-coupled for 80 mins at 80°C before storing. Before hybridization, the arrays printed on aldehyde and amine surfaces were washed in 0.5% SDS for 5 mins with agitation, then twice in water for 1 min each, and allowed to air-dry. The epoxy surface arrays were washed in water for 3 mins with agitation, then in water for 1 min, and allowed to air-dry.

For specific-targets, cover slip hybridizations were performed with 40mer Cy-labeled synthetic targets **(denoted in bold blue, see Table 1)** at either 500nM or 200nM concentration each for 3-3.5 hrs at room temp in a humidity chamber using 1X HybIt® buffer (TeleChem). Hybridized arrays were washed at room temp in 2X SSC/0.2% SDS for 5 mins with agitation followed by 2X SSC for 1 min then rinsed in 0.2X SSC for 20 sec and allowed to air-dry.

For nonamer-QC reagents, cover slip hybridizations were performed at room temp for 30 mins in a humidity chamber and washed as specified in Table 4.

Hybridized oligo-arrays were scanned using a ScanArray® 5000 (Perkin Elmer) at either 20µm or 5µm resolution.

Table 1: 70mer Oligo-Probe Model Set Employed

Name	5'-mod.	Purification	Sequence	%GC	40mer Specific-Target 5'-dye
1-u	unmod.	desalt	AACCAATGATTCATAACAAT**GTATGTGAAAGTGTAAAATAGAATGTTACTTTGGAATGAC**TATAAACATT	25.7	Cy3™
1-a	amine	desalt			
1-h	hydrazide	desalt			
1-A	amine	HPLC			
1-H	hydrazide	HPLC			
2-u	unmod.	desalt	TTCTTAAATA**TGTATATGACATTTAAATGTAACTTATTATTTTTTTTGAG**ACCGAGTCTTGCTCTGTTAC	25.7	Cy3™
2-a	amine	desalt			
2-h	hydrazide	desalt			
3-u	unmod.	desalt	AGCACTG**AACTTTTGAGATATGACGGTGTACTTACTGCCTTGTAGCA**AAATAAAGATGTGCCCTTATTTT	37.1	Cy3™
3-a	amine	desalt			
3-h	hydrazide	desalt			
4-u	unmod.	desalt	ACACCTCTCT**TTGTGCTGAATTTCTGGGCCATTCTTTTCCTGTCTTATTT**CTAAATTTCCTTCTTCCAAG	38.6	Cy3™
4-a	amine	desalt			
4-h	hydrazide	desalt			
5-u	unmod.	desalt	CACAAAGCAG**TGAATTTATTGGAGCATGACCACGGAGGATAGTATGAGCC**CTAAAAATCCAGACTCTTTC	42.9	Cy 5™ & Cy3™
5-a	amine	desalt			
5-h	hydrazide	desalt			
5-A	amine	HPLC			
5-H	hydrazide	HPLC			
6-u	unmod.	desalt	AGGCAGGTGA**GAAGGTGAGAGGTAGGCAAAGGAGATACAAGAGGTCAAAG**GTAGCAGTTAAGTACACAAA	45.7	Cy3™
6-a	amine	desalt			
6-h	hydrazide	desalt			
7-u	unmod.	desalt	ACCGCAAAAA**GCACCGGGAGGAGTGAGATGTGGATGTTGCTTTTGCACCT**ACGGGGGCATCTGAGTCCAG	55.7	Cy3™
7-a	amine	desalt			
7-h	hydrazide	desalt			
8-u	unmod.	desalt	TCGAGATGTT**CCGAGAGCTGAATGAGGCCTTGGAACTCAAGGATGCCCAG**GCTGGGAAGGAGCCAGGGGG	60.0	Cy3™
8-a	amine	desalt			
8-h	hydrazide	desalt			

unmod., unmodified; amine, AmMC6; hydrazide, lLink1.2
40mer specific-target region is in bold blue

Table 2: Print Buffers.

Name	Composition
n	0.05N NaOH / 0.01% SDS
nb	0.05N NaOH / 1.5M Betaine
m	0.1M MES pH4.5 / 0.01% SDS
mb	0.1M MES pH4.5 / 1.5M Betaine
s	3X SSC pH7 / 0.01% SDS
sb	3X SSC pH7 / 1.5M Betaine
D	50% DMSO
OSB	IDT's Oligo Spotting Buffer

Table 3: Commercial Slide Surfaces.

Name	Vendor
SuperEpoxy	TeleChem
SuperAldehyde	TeleChem
SuperAmine	TeleChem
GAPSII	Corning
UltraGAPS™	Corning

High-Density PCR Product Arrays

PCR products representing 7,296 genes from a human placental expression library were printed in triplicate at 50-83 ng/µl in 50% DMSO on Corning UltraGAPS™ slides with a Cartesian ProSys™ printer using TeleChem Stealth pins. The printed arrays were UV cross-linked (150 mJ, Strategene Stratalinker) and stored in a desiccator until use. The slides were pre-hybridized for 1 hr and hybridized for 16 hrs with a target derived from chorion carcinoma cell line (JAR) RNA, labeled with Cy3™, and human placenta library runoff RNA, used as a reference and labeled with Cy5™. Labeling reactions and hybridizations were done using the 3DNA™ expression array detection kit (Genisphere), following manufacturer's instructions, using a Lucidea™ SlidePro Hyb Station (Amersham). The slides were scanned at 5µm resolution on a GenePix™ scanner (Axon).

For nonamer-QC reagents, slides were pre-washed in 0.5% SDS for 5 mins with agitation, twice in water for 1 min each, and air-dried. Cover slip hybridizations were performed for 1 hr at room temp in a humidity chamber using IDT's Cy3™-nonamer and TeleChem's SeeIt™. Washes were performed following manufacturer's instructions **(see Table 4)**. Arrays were scanned using a ScanArray® 5000 (Perkin Elmer) at 5µm resolution.

Image Analysis and Quantification

Analysis of the oligo-array scans was performed using OptiQuant™ (Perkin Elmer). The analysis grid was set so that each cell was of equal size and larger than the probe spots. Background was defined by setting an extra analysis-cell column of equal size and replicate number to the probe analysis-cell columns and adjacent to the array in an area contained within the cover slip hybridization area that was neither printed on nor touched by the printer pins. For each array, the average analysis-cell background was subtracted from each probe analysis-cell and the result was normalized to the area of the probe spot, which was based on the average diameter of the replicates for each probe. The replicate values were then combined to calculate an average DLU/mm2 and standard deviation for each probe. The relative percent signals were then graphed for comparisons.

Table 4: Comparison of Commercially Available Nonamers for Array QC.

Name	Vendor	Dye	Scanner (Ex/Em) Wave Lenghts	Hyb Conditions	Wash Conditions
HybChecker™	Sigma	Atto 655	633/670	[?µM] supplied buffer	1X SSC/0.03% SDS for 2 mins 0.2X SSC for 2 mins 0.05X SSC for 2 mins
SpotCheck™	Genetix	Cy3™	543/570	[10µM] supplied buffer	2X SSC/0.2% SDS for 2 mins 2X SSC for 2 mins 0.5X SSC for 20 secs
Alexa-546 Panomer™	* Molecular Probes	Alexa-546	543/570	[7.5µM] 4X SSC 1mg/ml poly(dA) 50mM HEPES pH7 0.2% SDS	2X SSC/0.2% SDS for 2 mins 2X SSC for 2 mins 0.5X SSC for 20 secs
SeeIt™	† TeleChem	Cy3™	543/570	[10µM] SuperHyb™	2X SSC/0.1% sarkosyl for 1.5 mins 2X SSC for 1 mins 0.5X SSC for 10 secs
Cy3™-Nonamer	**IDT**	Cy3™	543/570	**[2µM]** supplied buffer	10X SSC/0.2% sarkosyl for 4-5 mins 10X SSC for 2 mins 2X SSC for 20 secs

* recommended hyb buffer not available from supplier
† recommended hyb buffer available; sold separately

RESULTS AND DISCUSSION

Optimization of Attachment Chemistry & Print Buffer

Microarrays were printed using 70mer oligonucleotide probes with unmodified, amino-mod, and hydrazide-mod 5'-ends on various slide surfaces and were hybridized to complementary Cy5™-labeled oligo targets. Large variations in signal intensities were observed. Between the different slide surface chemistries examined, relative signal intensity varied as follows (**Figure 1**):

Amine Surface > Aldehyde Surface >> Epoxy Surface

Note that the same print conditions, oligo concentrations, hybridization conditions, and scanner laser settings were employed. The epoxy surface had the lowest overall probe-binding capacity of the three surfaces. Print buffer composition also significantly affected final spot morphology and image intensity. In general, the addition of betaine to the print buffer improved the sensitivity and uniformity of the hybridized array spots on both aldehyde and amine surfaces (the sole exception being the sodium hydroxide buffer where the addition of betaine appears to decrease probe attachment). In the absence of covalent-coupling with UV or heat treatment, use of betaine increased relative probe attachment (in rank order):

Hydrazide Modified > Amine Modified > Unmodified oligos

The beneficial effect of betaine was greater on the amine surface than on the aldehyde surface. Unexpectedly, the hydrazide-modification showed best attachment to the amine surface, even though we

expected this modification to offer the greatest advantage using an aldehyde surface where this chemistry is known to undergo a specific covalent attachment reaction.

In another series of experiments, the use of hydrazide attachment chemistry was seen to offer even greater improvement in signal intensity with use of shorter 30mer and 50mer oligo probes on amine slides (data not shown).

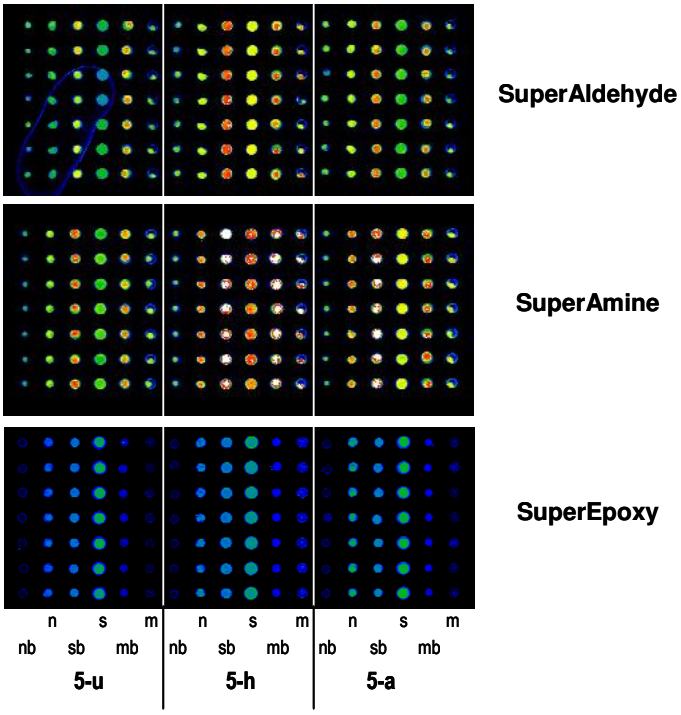

Figure 1: Initial Comparison of Print Buffers and Attachment Chemistries. 70mer oligo probes were printed @30µM with a 4-hour humidity treatment and no heat coupling. Arrays were hybridized with a Cy5™-40mer specific-target @500nM in 1X HybIt® (TeleChem). Image is 20µm resolution scan using ScanArray® 5000 (Perkin Elmer) at 60/60 laser (power/gain) settings. (Pseudo-color scale: black<blue<green<yellow<orange<red<white)

Since the amine surface appeared to be more promising than the aldehyde or epoxy surfaces, our survey was extended to study three different commercial amine surfaces in greater detail. The 70mer oligo-probe set was printed using three different spotting solutions on TeleChem SuperAmine slides, Corning GAPSII slides, and Corning UltraGAPS™ slides. On all three surfaces, the hydrazide-modified probes again showed the most intense signals relative to other oligo designs. Within this set of experiments, the best results were achieved using the Corning GAPSII surface without baking. HPLC purification further improved signal for hydrazide-modified oligos but offered little benefit for amino-modified oligos.

The IDT OSB print buffer formulation was optimized for attachment of hydrazide oligos to an amine surface and provides increased signal intensity as well as better spot morphology. This new print buffer also results in better spotting using amino-modified and unmodified oligos when compare with traditional SSC, betaine supplemented, or DMSO spotting buffers, so its application can be generalized for use in most or all oligo-based arrays.

The improved print buffer composition also eliminates the need for covalent coupling by either UV or baking, regardless of the 5'-modification employed. In particular, we observed that baking slightly decreases overall print quality by enlarging spot size and decreasing hybridization signal intensity for hydrazide-modified probes (although it does slightly improve intensity for unmodified probes in some cases). The baking step appears to be most helpful when printing unmodified or amine-modified probes in 50% DMSO.

We suspect that the hydrazide-modified probes are covalently coupling with the amine-slide surface and further that this reaction does not directly involve the primary amine group but rather is an interaction with other elements of the surface coating. Attachment of hydrazide-oligos varies significantly between amine slides from different sources. A total of four different sources were tested (data not shown) and the Corning GAPSII and UltraGAPS™ surfaces were the most reactive. Increased probe-density and greater ultimate sensitivity can be achieved using IDT's print buffer and hydrazide-modified probes with Corning GAPSII slides. This combination consistently yields more intense hybridization spot signals with greater uniformity than any other combination tested.

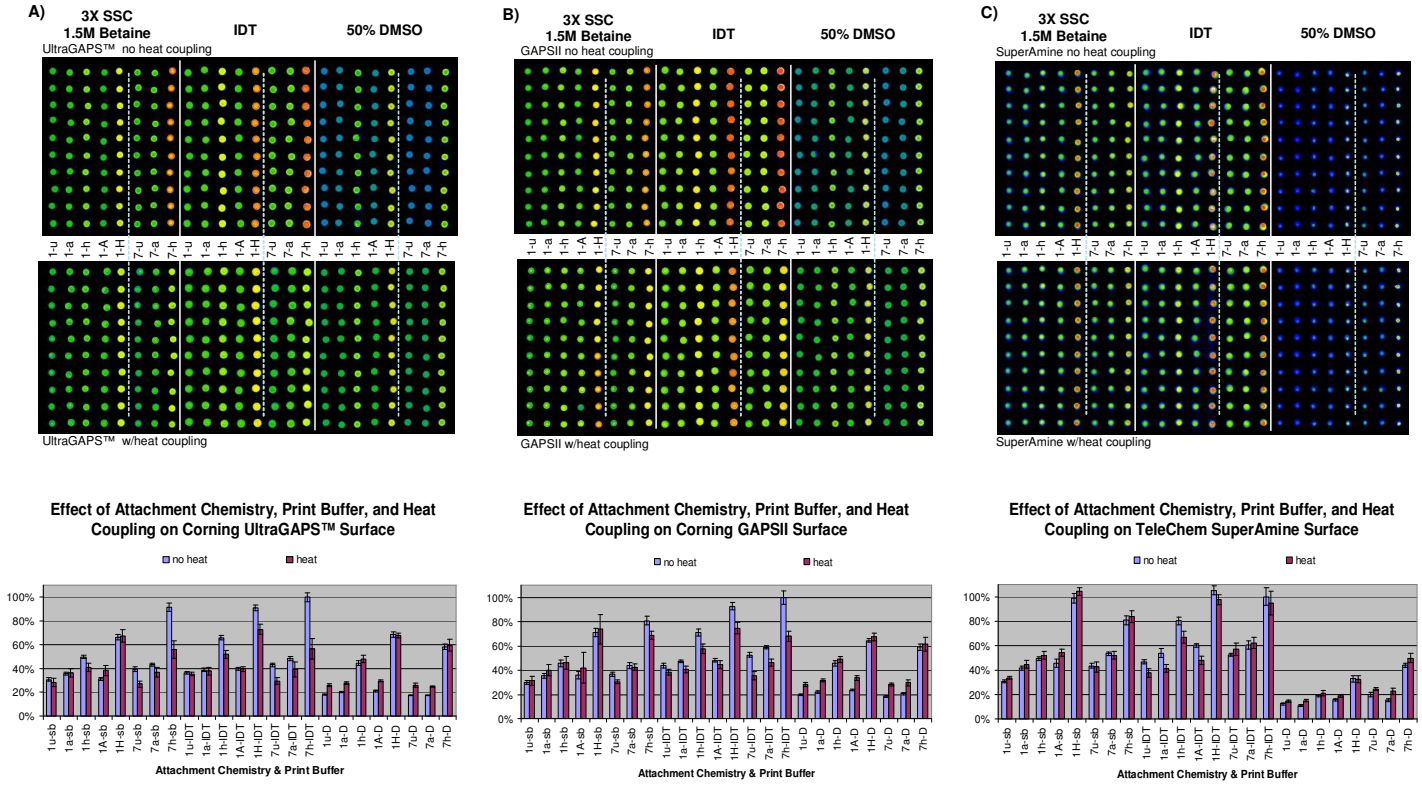

Figure 2: Specific Target Hybridizations Comparing Print Buffers and Attachment Chemistries on Different Amine Surfaces. 70mer oligo probes (1 is 25.7% GC and 7 is 55.7% GC) were printed @25µM using 3 different print buffers on UltraGAPS™ (**A**), GAPSII (**B**), and SuperAmine (**C**) slides with a 3 hour humidity treatment, followed either with (bottom array) or without (top array) heat-coupling. Arrays were hybridized with Cy3™-40mer specific-targets @200nM each in 1X HybIt® (TeleChem). Images are 5µm resolution scans using ScanArray® 5000 (Perkin Elmer) at 63/63 laser (power/gain) settings. (Pseudo-color scale: black<blue<green<yellow<orange<red<white)

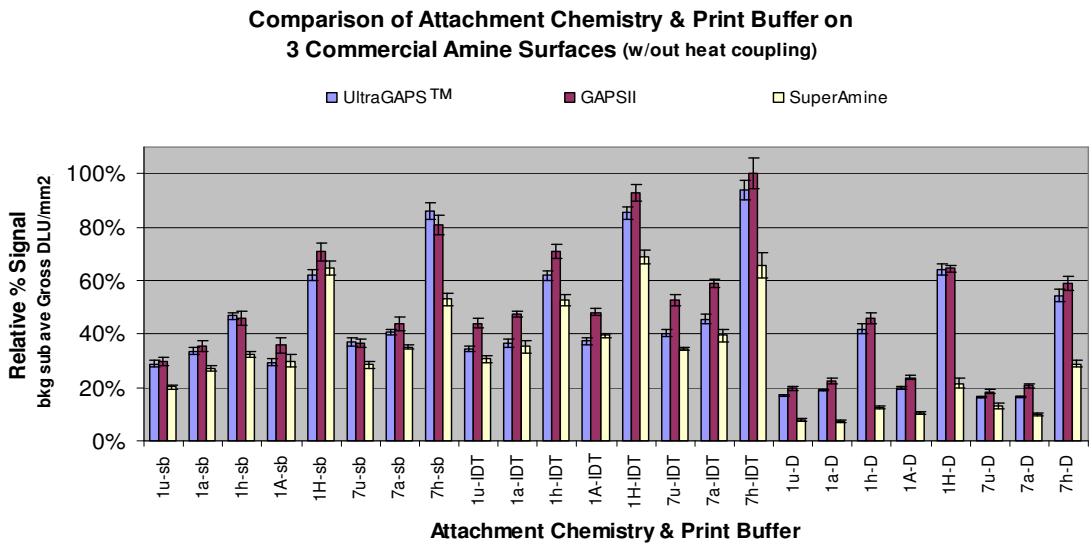

Figure 3: Hydrazide Attachment Chemistry and IDT's Print Buffer Provides Improved Performance on the Corning GAPSII Surface.

Improved Array QC Reagent and Methods

Fluorescent array QC reagents from four sources (Sigma, Genetix, Molecular Probes, and TeleChem) were tested for their ability to visualize oligonucleotide probes printed on Corning UltraGAPS™ slides. For the range of GC content oligos examined (25% - 60%), none of these four reagents reliably identified all the printed probes. We therefore developed our own reagent to QC arrays that would better meet the need for a reagent and method that could be used to detect the widest possible variety of probe sequences. A Cy3™-labeled random 9mer oligonucleotide was synthesized with base composition adjusted to enhance relative hybridization of AT-rich domains. Hybridization solutions and protocols were developed that optimized signal while keeping background low; where possible, similar optimization was attempted for the other reagents. Performance of the new IDT Cy3™-nonamer QC reagent was compared with the other four commercially available array QC reagents for detection of printed oligos and is shown in Figure 4. Using optimized protocols, the IDT Cy3™-nonamer and the TeleChem SeeIt™ reagent detected all eight of the oligo-probes printed, although the TeleChem reagent needed to be used at 5-fold higher concentration. Improved reagent design and hybridization protocols enable use of the IDT Cy3™-nonamer at much lower levels (2µM vs. 7.5-10µM) than other reagents, which helps maintain low background further improving visualization of spotted oligos with low GC content while reducing cost.

The IDT Cy3™-nonamer reagent was next tested along with the other four QC products on high-density arrays made with PCR products from a cDNA expression library (**Figure 5**). Interestingly, the new IDT Cy3™-nonamer showed similar improved performance when hybridized against the long PCR product probes as with shorter oligonucleotide probes.

To determine if array spot quality as visualized using the IDT QC reagent has utility in predicting the quality of array performance in real target hybridizations, we compared the QC image obtained from a PCR product array with the image obtained after actual hybridization with labeled cellular RNAs **(Figure 6)**. In most instances, the spot signal intensities from the two-color hybridization experiment mirror the spot signal intensities from the IDT Cy3™-nonamer QC hybridization. Note that several spots are "missing" in both images. In several cases (see arrows), the QC reagent visualized probe spots that were not seen in actual target RNA hybridization, presumably due to low representation of these specific RNA species in the target mix. Significantly, no cases were observed where PCR product probe spots were detected in the "real hybridization" and absent in the QC hybridization. Therefore the "missing spots" were successfully identified as either "print dropouts" or "target absence" using this QC method. The ability to discriminate between these two events is a critical goal of array QC analysis since it allows for meaningful interpretation of negative hybridization data. Thus, the IDT Cy3™-nonamer offers greater power for array QC, with improved sensitivity and more accurate assessment of spotted probe quality as well as functional availability for hybridization.

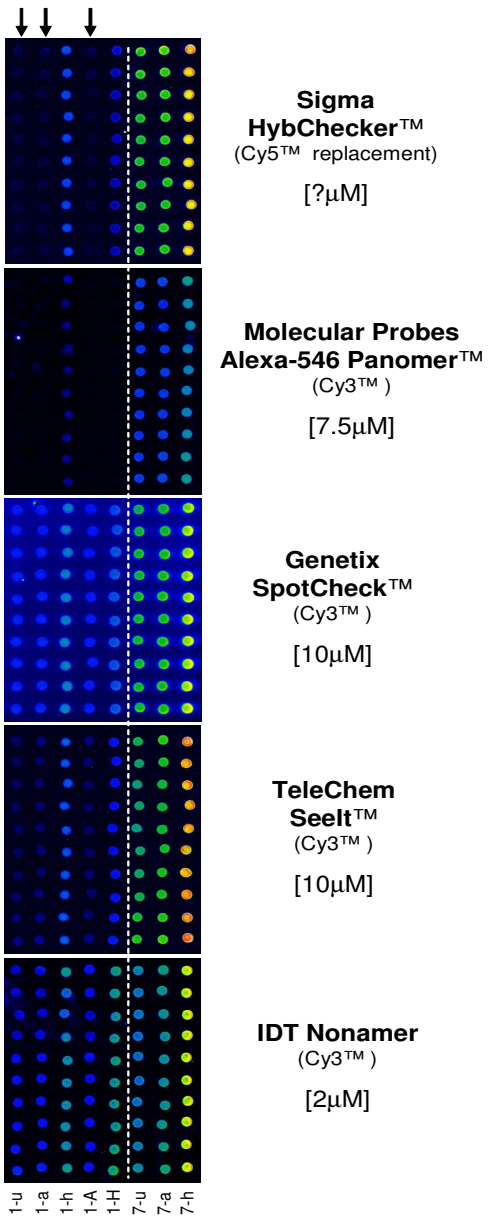

Figure 4: Comparison of Nonamer QC Products. 70mer oligo probes (1 is 25.7% GC and 7 is 55.7% GC) were printed @25µM using IDT's OSB print buffer on UltraGAPS™ slides with a 3 hour humidity treatment and no heat coupling. Images are 5µm resolution scans using a ScanArray® 5000 (Perkin Elmer) at 80/80 laser (power/gain) settings with the Cy5™-channel for the Sigma HybChecker™ and 73/73 laser (power/gain) settings with the Cy3™-channel for all others. Bold, black arrows indicate low GC-content probe-oligos immobilized on surface at low probe-density. (Pseudo-color scale: black<blue<green<yellow<orange<red<white)

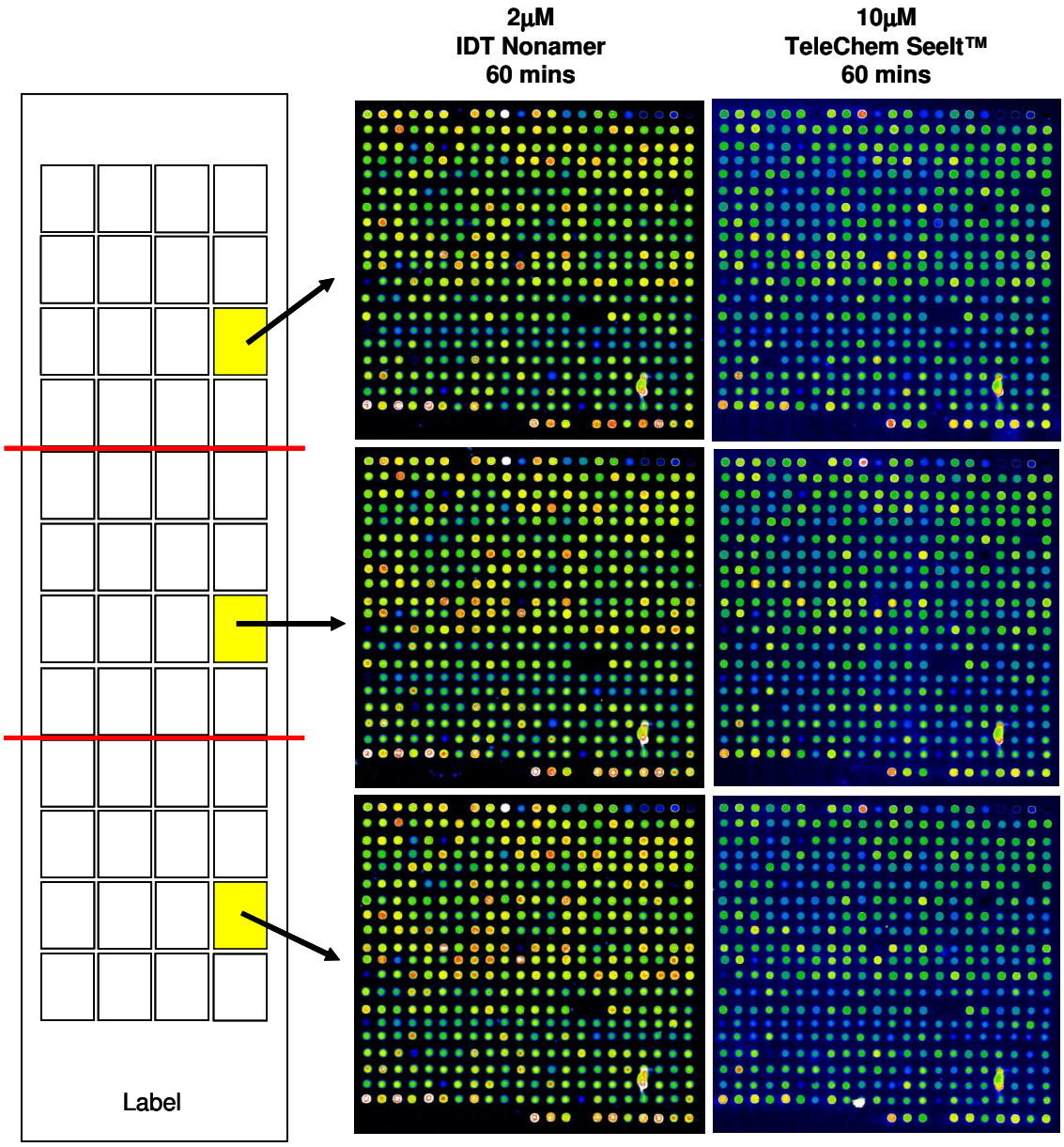

Figure 5: IDT vs. Leading Competitor QC of High-Density Array. PCR products printed in triplicate on UltraGAPS™ surface to yield an 18x54mm array with 21,888 spots. Yellow squares indicate representative replicate sub-arrays corresponding to the images on the right. QC hybridizations were performed for 1 hour at room temp following manufactures instructions. Images are 5µm resolution Cy3™ scans using a ScanArray® 5000 (Perkin Elmer) at 73/73 laser (power/gain) settings. (Pseudo-color scale: black<blue<green<yellow<orange<red<white)

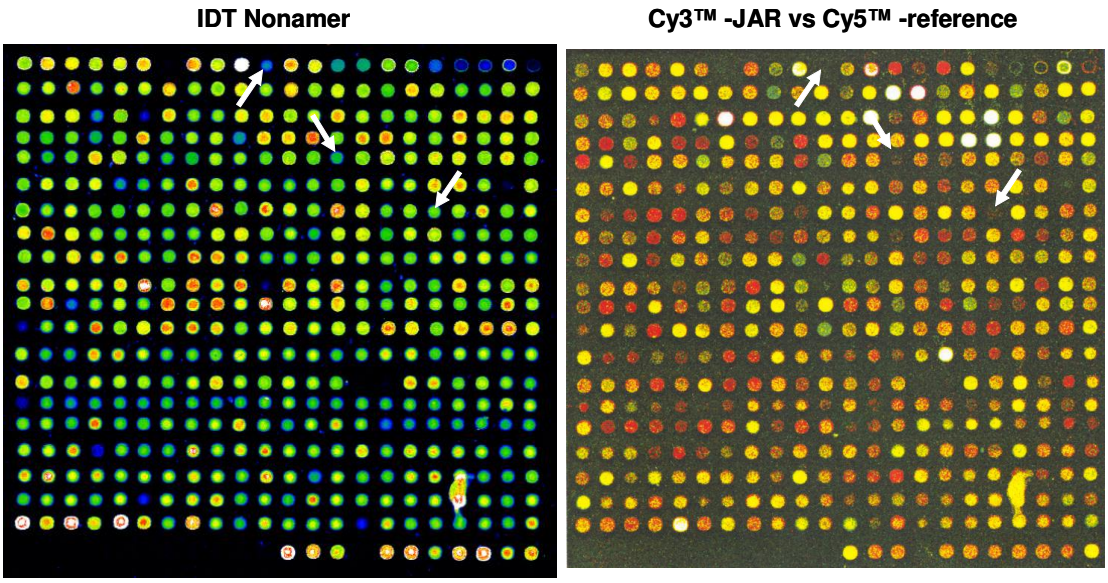

Figure 6: IDT's QC Product Results vs. Experimental Results on High-Density Array. Left image is from a 1 hour QC hybridization using the IDT Cy3™-nonamer. Right image is the corresponding replicate sub-array from an overnight experimental hybridization. White arrows indicate examples where the absence of experimental hyb signal is due to absence of mRNA target and not absence of spotted PCR probe. QC hybridization image is 5µm resolution Cy3™ scan using a ScanArray® 5000 (Perkin Elmer) at 73/73 laser (power/gain) settings. (Pseudo-color scale: black<blue<green<yellow<orange<red<white) Experimental hybridization image is 5µm resolution Cy3™/Cy5™ composite using a GenePix™ (Axon) scanner. (Cy3™, green; Cy5™, red; Cy3™/Cy5™, yellow)

CONCLUSIONS

Hydrazide-modified oligos offer a more robust attachment chemistry for manufacturing oligonucleotide-probe arrays with double the signal intensity of that achieved by using unmodified oligos on amine-surface slides. Corning GAPSII slides worked particularly well with our hydrazide-modified oligonucleotide probes.

IDT's new OSB (oligo spotting buffer) improves both spot morphology and uniformity and gives better quality arrays with higher signal and lower variance between replicates.

The IDT Cy3™-nonamer is a new reagent available for QC of printed microarrays by direct hybridization with improved detection of both spotted oligos and cDNA/PCR products over other available reagents.