Evaluation of microarray hybridization data using Universal ScoreCard with oligonucleotide probes

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key words: oligonucleotide probes • amine-modified control oligonucleotide probes • oligonucleotide spotting controls • mRNA spike controls • cDNA targets • amplified or complementary RNA targets • Eberwine amplification • CodeLink Activated slides • dynamic range of oligo-array data • ratio controls for gene expression analysis • mRNA level estimation in pg

Universal ScoreCard™ consists of a set of 23 artificial genes that serve as analytical controls to validate and normalize microarray data obtained from array hybridization. The genes have been selected from yeast intergenic regions and show no cross-hybridization to human, mouse, rat, and E. coli, making the Universal ScoreCard system species independent. The Universal ScoreCard system comes complete with DNA for spotting (PCR products of genes) and spike mRNA for labeling. The DNA is generated by PCR and the RNA labeled with Cy™3 and Cy5 dyes for single or two-color microarray hybridization experiments.

The system consists of 10 calibration controls for dynamic range determination, 8 ratio controls for calculation of gene expression ratios, 2 negative controls and 3 utility controls. The calibration and ratio controls span array data covering 4.5 and 2.5 orders of magnitude, respectively. However, Universal ScoreCard PCR probes do not carry any amine modification and can only be used for PCR-spotted slides that utilize a non-covalent method for DNA attachment.

We have therefore designed oligonucleotide probes, 50- and 70-mers in length, corresponding to each of the genes in the Universal ScoreCard system to serve as spotting and hybridization controls on oligonucleotide-based microarray slides. The oligonucleotide probes have been synthesized both as amine-modified (5'-end) 50-mers and as unmodified 70-mers for spotting on slides that utilize covalent or non-covalent methods for probe attachment, respectively. Both 50- and 70-mer oligonucleotide probes have been functionally tested using microarray hybridization assays to compare performance to PCR probes. The hybridization performance of unmodified 70-mers were tested by spotting on Corning UltraGAPS™ slides, whereas, the amine-modified 50-mers have been functionally validated on CodeLink™ Activated slides, which require 5'-amino modification on oligonucleotides for coupling to the slide surface.

Here, we present microarray hybridization data comparing the performance characteristics of the oligonucleotide probes relative to PCR probes and demonstrate that the dynamic range of oligonucleotide probes is equivalent (3 logs) to PCR probes and that the accuracy of the oligonucleotide version of ratio controls remains high. The oligonucleotide probe performance in microarray hybridization has been validated using Cy3- and Cy5- labeled targets prepared from both standard first-strand cDNA synthesis techniques and Eberwine RNA amplification technology (1) on two slide types that utilize different chemistries for probe attachment. When used with Universal ScoreCard spike mix, these oligonucleotide probes provide controls for microarray gene expression data analysis on oligonucleotide-based slides.

Amersham Biosciences

Products used

Amersham Biosciences products used:

Universal ScoreCard	63-0042-85
SlidePro	18-1162-01
CyScribe™ First-Strand cDNA Labelling Kit	RPN6200
Cy3-dCTP	PA53021
Cy5-dCTP	PA55021
CyScribe GFX™ Purification Kit	27-9606-01
Microarray Hybridization Solution, Version 2	RPK0325
CodeLink Expression Assay Reagent Kit, 24 reactions	320012
Cy3-UTP	PA53026
Cy5-UTP	PA55026
CodeLink Activated Slides	300011
Water, RNase-Free	US70783
SOURCE 15Q packed in an HR 16/10 column	
NAP™ 10 Columns	
17-0854-01	
GEN III Spotter	

Other materials required

Oligonucleotide probes (Tib MolBiol)	
UltraGAPS slides (Corning Life Sciences)	40015
RNeasy Protect MiniKit (Qiagen)	74124
QIAquick™ PCR Purification Kit (Qiagen)	28104
Biotin-11-UTP (PerkinElmer)	NEL543001
Stratalinker™ UV Cross-linker (Stratagene)	
SUPERase•In™ (Ambion)	2696
GenePix™ Pro (Axon Instruments)	
Axon 4000B laser scanner	
PCR tubes, RNase-free	
Thermal cycler	
DMSO	
384-well plates	
Oligo R3™ medium (Applied Biosystems) packed in an HR 16/10 column	

Protocol

1. Spotting 70-mer oligonucleotide probes on Corning UltraGAPS slides

We recommend the 70-mer oligonucleotide probes for spotting on glass slides such as Corning UltraGAPS that utilize a non-covalent method for DNA attachment (via backbone of DNA). Concentrations of oligonucleotide probes in the range of 500 ng/µl to 1000 ng/µl will generally yield high signal from array features. For Corning UltraGAPS slides, spotting in 50% DMSO spotting buffer has been shown to yield high signals. Other spotting buffers such as 3× SSC may also be used.

For other slide types, follow slide manufacturers' recommendations for spotting solution and spotting concentration. In practice, the optimal spotting concentration may need to be determined by titration depending on the slide chemistry used for spotting.

Oligonucleotide probes (1 nmol) were dissolved in 14 µl deionized water and 14 µl DMSO and transferred into separate wells of a 384-well plate for a final spotting concentration of 800 ng/µl (~35 µM). The 70-mer probes were then spotted on Corning UltraGAPS slides using Amersham Biosciences Gen III spotter under relative humidity of 50%.

After spotting, the oligonucleotides were immobilized onto slides by UV cross-linking (600 mJ). The slides were stored at room temperature in a desiccator until needed.

2. Spotting and coupling of the 50-mer oligonucleotide probes on CodeLink Activated Slides

The 50-mer probes contain an amino linker at the 5' end of each oligonucleotide. This modification is necessary for attachment on slides such as CodeLink Activated Slides and poly-L-lysine coated glass slides. The oligomer concentration required for spotting CodeLink Activated Slides is 15–25 µM.

The 50-mer probes (1 nmol) were dissolved in 40 µl of spotting buffer (150 mM sodium phosphate, pH 8.5) for a final spotting concentration of 25 µM. The probes were then spotted on CodeLink Activated Slides using Amersham Biosciences Gen III spotter under relative humidity of 40%.

To facilitate binding of the oligonucleotides to the active groups on the CodeLink slides, the spotted slides were placed in a sealed storage box with a solution of saturated NaCl, which provides a relative humidity level of approximately 75%, and left overnight.

Prior to hybridization, the slides were placed in a slide rack and residual reactive groups were blocked using pre-warmed blocking solution (for recipe, see CodeLink Activated Slide protocol booklet) at 50 °C for 30 min, followed by washing the slides with 4× SSC, 0.1% SDS (pre-warmed to 50 °C) for 30 min on the shaker. The slides were rinsed several times in water, dried using centrifugation, and stored at room temperature in a desiccator until needed.

3. Target preparation

cDNA targets were prepared using CyScribe First-Strand cDNA Synthesis Kit using Cy3-dCTP and Cy5-dCTP according to the recommended protocol. The specific activities of the labeled cDNA were calculated from the UV absorbances at 260 nm, 550 nm and 650 nm as described in the CyScribe protocol booklet.

Cy3 was used with the Universal Scorecard reference mRNA spike mix, and Cy5 with the Universal Scorecard test mRNA spike mix. The utility mRNA spikes were used for both reactions.

4. Eberwine amplified RNA targets (cRNA)

The targets from Eberwine RNA amplification reactions were prepared using the CodeLink Expression Assay Reagent Kit with the following protocol modifications: Double-stranded cDNA was synthesized starting from 5–10 µg of human skeletal muscle total RNA. Universal ScoreCard mRNA spike mix was diluted with RNAse-free water in the range of 1:6–1:12 to give high signal without saturation of high copy spots.

Two separate cDNA synthesis reactions were set up corresponding to reference (control) and test samples. Necessary components were mixed and incubated at 70 °C for 10 min and cooled at 4 °C for 5 min. Reactions were then incubated for 2 hours at 42 °C for cDNA synthesis.

Second-strand cDNA synthesis was performed according to the recommended protocol.

Labeled cRNA was made by *in vitro* transcription (IVT) using Cy3-UTP for the reference sample and Cy5-UTP for the test sample. The IVT reactions were incubated overnight at 37 °C, targets purified, and quantitated by UV absorbance.

cRNA targets were fragmented according to the recommended protocol. Fragmentation resulted in fragment sizes of 50–200 bases.

5. Hybridization

The hybridization step is the same for both UltraGAPS and CodeLink Activated Slides. The arrays were hybridized using a SlidePro automated hybridization station. Detailed protocols can be obtained from Amersham Biosciences web site at www.amershambiosciences.com/microarray.

Note: PCR hybridization protocols should not be used on oligonucleotide printed slides. The hybridization

conditions for these oligonucleotides are less stringent than PCR protocols. Lower formamide concentration and temperatures should be used for oligonucleotide slides, otherwise appreciable signal loss will occur.

For cDNA targets, 30–35 pmol of Cy3- and Cy5-labeled targets were hybridized to both UltraGAPS and CodeLink slides.

For cRNA targets, 5 µg of fragmented Cy3- and Cy5-labeled targets were hybridized to UltraGAPS slides. 10 µg of fragmented Cy3- and Cy5-labeled cRNA targets were hybridized to CodeLink Activated Slides.

UltraGAPS slide hybridizations were performed in 25–30% formamide using Microarray Hybridization Solution, Version 2 in a final volume of 200 µl.

CodeLink slide hybridizations were performed in 200 µl solution of 25% formamide, 5× SSC and 5× Denhardt's solution.

Prior to hybridization of either slide type, the mix was heated at 95 °C for 2 min and centrifuged briefly. After injection of labeled targets into SlidePro chambers (loaded with spotted slides), hybridizations were performed for 16 h at 42 °C.

6. Post-hybridization washing of slides

After hybridization, slides were washed on the SlidePro with 1× SSC, 0.2% SDS for 10 min at 42 °C, 0.1× SSC, 0.2% SDS for 10 min at room temperature, and finally in 0.1× SSC for 3 min. The slides were then dried and removed.

7. Scanning of hybridized slides, data quantification and analysis

Hybridized arrays were scanned using an Axon 4000B laser scanner. Spots were quantitated using GenePix Pro 3.1 and the raw data (minus background) was analyzed using Microsoft™ Excel.

Results

Hybridization data using cDNA targets

The PCR and 70-mer probes were spotted on Corning UltraGAPS slides. The performance of oligonucleotide probes were compared with PCR probes by microarray hybridization to Cy3- and Cy5-labeled cDNA targets made from CyScribe First-Strand cDNA Synthesis Kit. For two-color hybridization assays, it is recommended to add Universal ScoreCard reference mRNA spike to Cy3 labeling reactions and add Universal ScoreCard test mRNA spike to reactions labeled with Cy5. The results from a typical microarray slide hybridization are shown in Figure 1.

Qualitative data from the image shows that the overall performance of the oligonucleotide probes are comparable to PCR probes in terms of signal levels, spot morphology, spot diameter, and background.

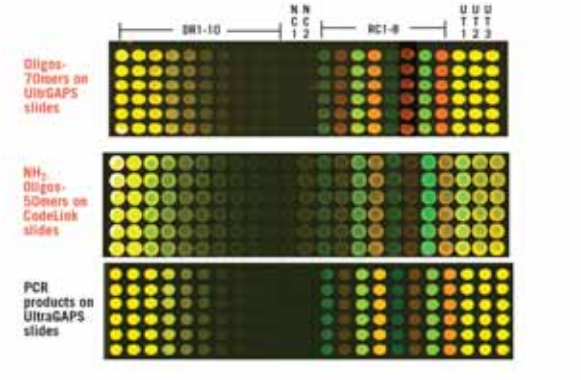

Fig 1. Slide image of oligonucleotide probes compared against PCR probes. A section of combined Cy3 and Cy5 image containing the probe sets hybridized with cDNA targets. The top image shows the image from UltraGAPS slides spotted with 70-mer oligonucleotide probes. The middle image is from CodeLink Activated Slide spotted with 50-mer probes. For comparison, image of PCR probes spotted on same UltraGAPS slide as the 70-mers is shown (bottom).

The raw signal was compared by quantification of the array feature intensities using GenePix Pro 3.1 software and results are shown in Table 1. The signal intensities of the 70-mer oligonucleotides were around 30–40% lower than PCR products on UltraGAPS slides. This is consistent with generally lower signals obtained for oligonucleotide arrays compared with cDNA arrays. However, the lower signal levels do not impact sensitivity. The dynamic range of the calibration control oligonucleotide probes was linear up to 3 logs with R^2 values for both Cy3 and Cy5 to be near identical to PCR probes (R^2 values calculated from log transformation of signal values). The limit of detection was observed to be at 3–5 pg on UltraGAPS. As expected, the relative Cy3 and Cy5 signal intensities for each calibration control were close to 1:1 for oligonucleotide probes.

The amine-modified 50-mer oligonucleotide probes were spotted on CodeLink Activated Slides and coupled to the slide surface from the 5'-amino end. The image from a typical hybridization experiment with Cy3- and Cy5-labeled cDNA targets is shown in Figure 1. The oligo probes showed excellent spot morphology. CodeLink slides were scanned at higher PMT setting (50 V gain over UltraGAPS) as recommended in the protocol. The Cy3 signals from calibration controls were roughly 35% higher than the Cy5 signal. CodeLink slides had limit of detection at 10 pg from labeled cDNA targets.

Ratio controls can be extremely useful for evaluating gene expression levels from test samples. Data can be normalized and compared within and across slides.

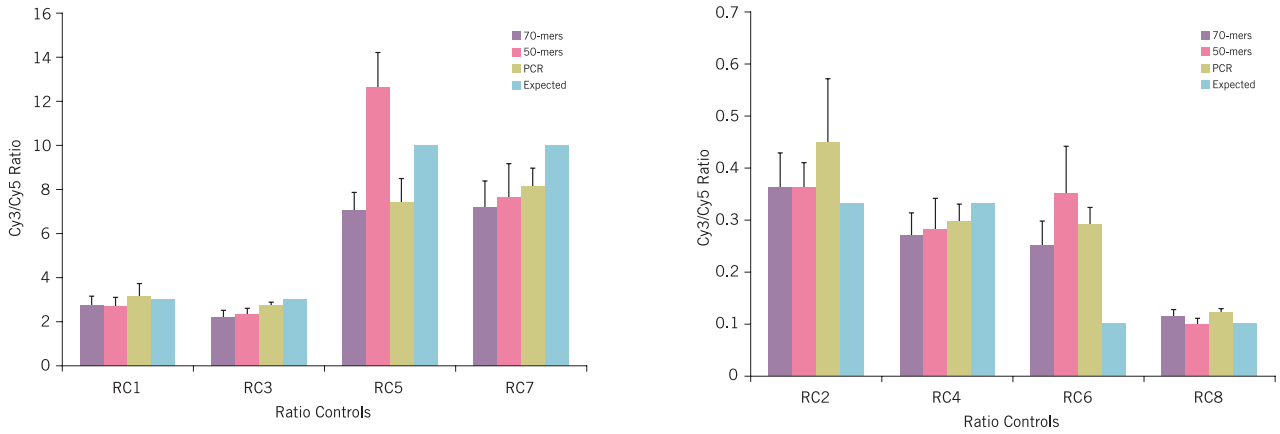

Fig 2. The hybridization performance of ratio controls. Discrimination of ratio controls at the level of 1:3, 3:1, 1:10, 10:1 was observed for both 70- and 50-mer oligonucleotide probes similar to values obtained with PCR probes using cDNA targets. Data obtained from 3 or more slides.

Table 1. Signal levels of calibration controls from oligonucleotide probes and PCR probes comparing dynamic range with cDNA targets (all signal values are in pixels, with local background subtracted)

Control Type	Spike (pg)	Cy3 Signal Oligo UltraGAPS	Cy3 Signal Oligo CodeLink	Cy3 Signal PCR UltraGAPS	Cy5 Signal Oligo UltraGAPS	Cy5 Signal Oligo CodeLink	Cy5 Signal PCR UltraGAPS
Calib 01	30 000	25 450	43 745	22 690	51 136	65 467	33 664
Calib 02	10 000	14 363	23 323	13 322	21 838	22 096	14 764
Calib 03	3 000	2 768	8 617	5 613	3 662	6 079	5 493
Calib 04	1 000	1 174	3 638	2 441	1 229	2 334	2 312
Calib 05	300	606	1 129	848	765	986	915
Calib 06	100	203	567	345	238	403	354
Calib 07	30	88	236	106	86	148	109
Calib 08	10	41	121	41	27	75	46
Calib 09	3	34	96	21	15	46	18
Calib 10	1	29	60	13	10	36	17
NC1	n/a	29	63	5	3	62	1

Table 2. Performance summary of oligonucleotide probes for both CodeLink and UltraGAPS slides

Universal Oligonucleotide ScoreCard Probe Feature	Corning UltraGAPS slides	CodeLink Activated slides
Oligo probe length	70mers	50mers (amine modified)
Coupling chemistry on microarray slide	Non-covalent attachment	Covalent attachment via 5-amino group on probes
System dynamic range from calibration controls	4.5 logs	4.5 logs
Limit of detection	3–5 pg of mRNA (cDNA targets) <1 pg of mRNA (cRNA targets)	10 pg of mRNA (cDNA targets) <1 pg of mRNA (cRNA targets)
R^2 value of calibration controls	0.96 Cy3, 0.99 Cy5	0.98 Cy3, 0.98 Cy5
Ratio controls	1:3, 3:1, 1:10, 10:1	1:3, 3:1, 1:10, 10:1
Spot diameter	260 µm	270 µm
Target labeling	Cy3 and Cy5	Cy3 and Cy5
Target preparation	cDNA and cRNA	cDNA and cRNA
Hybridization stringency	25–30% Formamide	25% Formamide
Wash conditions	1×SSC/0.2%SDS, 42 °C, 10 min, 0.1×SSC/0.2%SDS, RT, 10 min, 0.1×SSC, 3 min	1×SSC/0.2%SDS, 42 °C, 10 min, 0.1×SSC/0.2%SDS, RT, 10 min, 0.1×SSC, 3 min

The performances of oligonucleotide probes were compared against PCR probes for both slide types and are shown in Figure 2. The oligonucleotide probes accurately discriminate between the 1:3, 3:1, 1:10 and 10:1 ratio controls ranging from low copy to high copy mRNA levels with ratio values close to targets. The precision of ratio controls to expected values requires data normalization and depends on relative Cy3 versus Cy5 signal on a slide and the data normalization method chosen (3). Table 2 summarizes the performance metrics of oligos for both CodeLink and UltraGAPS slides.

Hybridization data using Eberwine amplification method

The performances of the oligonucleotide probes were validated by microarray hybridization with Cy3- and Cy5-labeled targets prepared using Eberwine RNA amplification (cRNA) method. Since small quantities of starting total RNA were utilized in the cRNA reaction, it is necessary to dilute the Universal ScoreCard mRNA spike mix to prevent saturation of high copy spots. It is recommended to optimize the cRNA reaction to obtain roughly 600-fold amplification (starting from total RNA) prior to setting up hybridization experiments. Typically 6–12 fold dilutions of Universal ScoreCard spike mixes (reference and test) can be added at the beginning of Eberwine reactions to prevent saturation under optimal amplification conditions. It is extremely important to get efficient incorporation of Cy3 and Cy5 dyes with high cRNA yields to obtain good signals on oligonucleotide arrays.

Results from typical labeling experiments starting from 5–10 µg of total RNA are shown in Table 3. When incorporation of Cy3 and Cy5 dyes is measured using the unlabeled nucleotide/dye-labeled nucleotide ratio, the average incorporation with Cy3-UTP was 62 ± 6, whereas, with Cy5-UTP the incorporation was 107 ± 8 unlabeled nucleotides/dye-labeled nucleotide when the

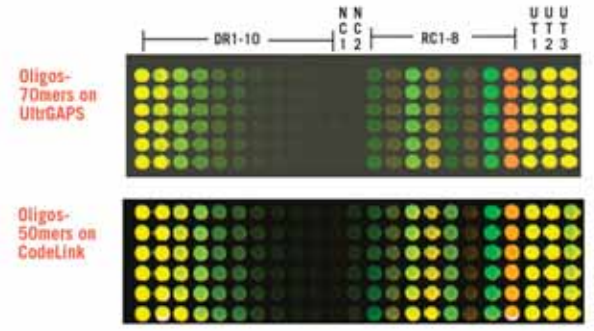

Fig 3. Slide image of oligonucleotide probes hybridized with cRNA targets.

Table 3. Yield of amplified RNA from total RNA using CodeLink Expression Array Kit with diluted Universal ScoreCard spike mRNA added to reaction

Complementary RNA	cRNA yield (µg)	Unlabeled nucleotide: dye ratio (unlabeled/Cy3 labeled)	Unlabeled nucleotide: dye ratio (unlabeled/Cy5 labeled)
cRNA-undiluted test spike mRNA	113 680	52	93
cRNA 1:4 dilution of test spike mRNA	50 176	69	109
cRNA 1:6 dilution of test spike mRNA	80 752	64	104
cRNA 1:8 dilution of test spike mRNA	34 496	68	113
cRNA 1:10 dilution of test spike mRNA	56 448	59	114
cRNA 1:12 dilution of test spike mRNA	72 677	59	108

average length of cRNA is assumed to be 1000 bases. Typically, the incorporation of Cy3-UTP is roughly twice as efficient as that of Cy5-UTP into cRNA.

High signal intensities and low background were seen from hybridization with Cy3- and Cy5-labeled cRNA targets demonstrating that mRNA spikes and oligonucleotide probes are fully compatible with the Eberwine amplification method (Fig 3).

The signal intensities from array features were quantitated by GenePix Pro 3.1 software and data compared to validate performance of calibration and ratio controls. The signal intensity values from CodeLink and UltraGAPS were high for both Cy3 and Cy5 hybridized array features (Table 4). The dynamic range of calibration controls was linear up to 3 logarithmic values with R^2 values at or higher than 0.98.

The ratio of Cy3:Cy5 signal from calibration controls ranged between 1.2–4 and 1.1–1.8 for UltraGAPS and CodeLink slides, respectively, with an increase in values from high to low copy RNA spikes as expected.

Table 4. Performance of oligonucleotide calibration controls for CodeLink and UltraGAPS slides.

CodeLink slides			
Calibration Controls	**Cy5 Signal**	**Cy3 Signal**	**Cy3/Cy5 signal ratio**
Calib 01	49 990 ± 1 123	56 960 ± 5 880	1.14
Calib 02	39 612 ± 12 796	43 156 ± 8 086	1.10
Calib 03	12 865 ± 3 232	16 461 ± 4 123	1.28
Calib 04	5 231 ± 2 085	7 094 ± 3 267	1.36
Calib 05	1 420 ± 578	2 087 ± 1 049	1.47
Calib 06	730 ± 330	1 117 ± 578	1.53
Calib 07	244 ± 81	386 ± 156	1.58
Calib 08	141 ± 23	207 ± 55	1.47
Calib 09	80 ± 8	135 ± 21	1.69
Calib 10	58 ± 11	103 ± 15	1.79
UltraGAPS slides			
Calibration Controls	**Cy5 Signal**	**Cy3 Signal**	**Cy3/Cy5 signal ratio**
Calib 01	47 813 ± 17 154	49 646 ± 9 006	1.15
Calib 02	33 106 ± 15 483	37 338 ± 9 798	1.28
Calib 03	10 188 ± 5 011	14 075 ± 7 053	1.43
Calib 04	3 055 ± 1 447	4 996 ± 2 947	1.67
Calib 05	909 ± 431	1 606 ± 459	1.80
Calib 06	429 ± 187	762 ± 354	1.87
Calib 07	115 ± 49	235 ± 72	2.21
Calib 08	61 ± 19	133 ± 32	2.25
Calib 09	21 ± 7	80 ± 14	4.10
Calib 10	20 ± 7	68 ± 21	3.58

Since signals from low copy spots have higher standard deviation and greater deviation from linearity, it is recommended to consider an exponential curve to fit the data for normalization. Since calibration controls are present at a 1:1 ratio, the raw signals from the calibration curve can be plotted to estimate the normalization factor for correcting raw data for array features prior to estimating differences in expression levels (3).

Signals from calibration controls can be used to generate a calibration curve. This curve can be used to estimate the amount of mRNA in each spot and limit of detection on a hybridized slide. An example of a calibration curve using data from CodeLink slide hybridization with 10-fold dilution of Universal ScoreCard mRNA spike mix is shown in Figure 4. Due to amplification of RNA, the Eberwine method can be more sensitive than standard cDNA targets. The limit of detection was observed to be less than 1 pg for UltraGAPS and CodeLink slides. The hybridization data from calibration controls fits an exponential or a 4-order polynomial function. For accurate determination of copy number of genes, it is important to only work with feature data that falls in the linear range of the calibration curve. Signals from array spots either above (saturation levels) or below (near background) the linear range should be excluded from data analysis.

The results from ratio control performance from cRNA hybridization are shown in Figure 5. The discrimination of 1:3, 3:1, 1:10 and 10:1 ratios is observed from both 70- and 50-mer oligonucleotide probes as expected and is similar to PCR probes. Because the raw signal from Cy3 is generally higher than Cy5 signal, the raw values of ratios are higher. Data normalization is required to reduce the bias created by differences in spectral properties and incorporation levels of Cy3 and Cy5 dyes and depends on the observed signal differences from calibration controls and other array features on the slide (see above). When working with gene expression ratios,

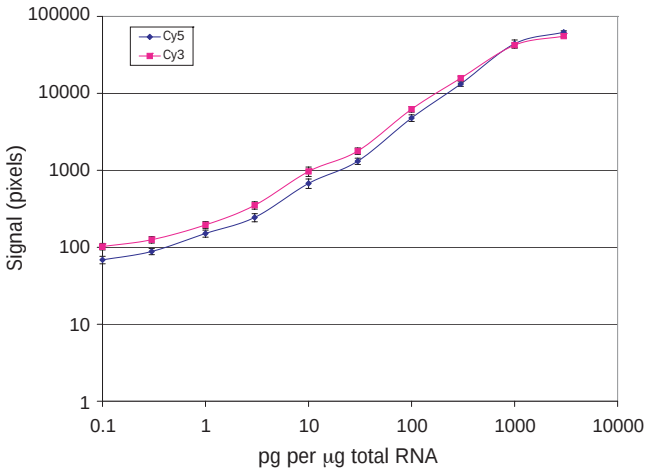

Fig 4. Calibration curve from ten calibration controls on CodeLink slides. The calibration curve can be used to estimate the amount of mRNA in each spot. Data is from CodeLink slide spotted with 50-mer oligonucleotide probes and hybridized with cRNA targets containing 10-fold dilution of Universal ScoreCard mRNA spike mixes.

it is also recommended to analyze data in log scale because distribution of ratios becomes normal in logarithmic scale (3).

Of the eight ratio controls, the value of low copy RC6 was sometimes compressed, in which case RC5 and RC8 can be substituted for data analysis. Due to direct incorporation of CyDye fluors during amplification of RNA, there was greater variation observed in ratio controls than seen from cDNA targets. Consequently, for low copy messages, the performance of ratio controls showed higher variance and were farther apart from target values. It is strongly recommended to perform data correction (normalization) prior to determining expression levels of individual genes from array spots (3). The variation in data may be less

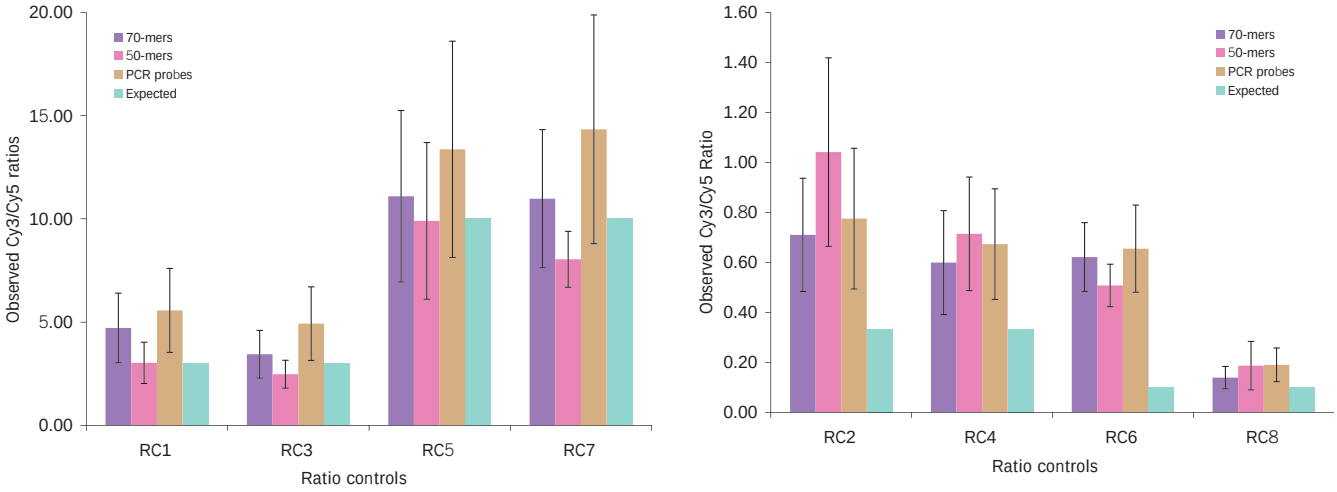

Fig 5. Performance of ratio controls from hybridization with cRNA targets. The raw data values from eight ratio controls from 70- and 50-mer oligonucleotide probes are shown demonstrating the discrimination and trends of 1:3, 3:1, 1:10, and 10:1 ratios.

when cRNA and post-labeling methods are used for detection (but only one dye can be hybridized per slide).

Conclusions

Oligonucleotide probes corresponding to each of the exogenous genes for Universal ScoreCard have been developed to create a new set of controls for microarrays. Functional testing using microarray hybridization assay on two different slide types (CodeLink and UltraGAPS) has been used to validate each oligonucleotide probe performance. The length of the oligonucleotide probes was found to strongly correlate to the observed signal strength from microarray hybridization.

For Corning UltraGAPS slides, 70-mer probes yield higher signal than 50-mers. The 70-mers do not carry any modification (at 5'-end) and are recommended for all substrates involving non-covalent methods of slide attachment. Since signals from 70-mer probes are similar to PCR probes, these long oligonucleotide probes are suitable for use with both unamplified cDNA and cRNA targets (4). The long oligonucleotides (70-mers) are also ideal for hybridization against targets made from post-labeling methods.

Since spotting is done under non-denaturing conditions on CodeLink Activated Slides, shorter 50-mer oligonucleotide probes (5'-amino modified) have been developed and recommended for spotting. While longer oligonucleotides do generate higher signals, probes longer than 50-mers were found to have higher propensity to form sequence-dependent secondary structures and yield nonspecific hybridization signals.

All hybridizations were performed under complex conditions using Cy3- and Cy5-labeled cDNA and cRNA targets made from reference and test mRNA spikes added to human skeletal muscle RNA. The hybridization kinetics of oligonucleotide-based probes

differs from PCR probes because of the smaller length of oligonucleotides. This impacts the stringency at which hybridization and washing parameters can be set. During hybridization, the oligonucleotide-based slides require lower formamide concentration and reduced temperatures for wash buffers (Table 2). The single biggest factor to distortion of ratios from oligonucleotide probes comes from signals from nonspecific hybridization. It has been shown that oligonucleotide probes with greater than 15 contiguous bases of homology to labeled targets show significant cross hybridization signals and should be avoided when designing oligonucleotide probes for genes (5).

The Universal ScoreCard oligonucleotide system allows quantitative evaluation of hybridization metrics such as dynamic range, limit of detection and ratio analysis for gene expression on multiple slide chemistries. The utility controls in the oligonucleotide probe set can also be used to monitor sources of inconsistency in target labeling and sample preparation to ensure reproducibility between array experiments.

The performance matrices of the Universal ScoreCard oligonucleotides were functionally validated on two different slide chemistries using targets made from labeled cDNA and labeled cRNA and are summarized in Table 2. The hybridized slides generated high signal, excellent spot morphology and low background. The dynamic range and sensitivity of the oligonucleotide probes was found to be equivalent to PCR probes with excellent correlation coefficients from calibration controls for both Cy3 and Cy5 hybridized spots. Using microarray hybridization assays, discrimination of ratio controls have been demonstrated for both cDNA and cRNA targets under competitive and complex dual (Cy3 and Cy5) hybridization conditions.

In summary, the Universal ScoreCard oligonucleotide probes offer a complete set of exogenous controls for evaluating microarray data and allow comparison of results conducted in different laboratories on oligonucleotide-spotted slides.

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Troubleshooting

Problem: Signals from Calibration Controls are low.

Solution: Oligonucleotide printing concentration is too low. The oligonucleotide probes should be spotted at the recommended concentration for a given slide type. See protocol above for details on optimal spotting conditions.

Problem: Ratio controls are not discriminating between Cy3 and Cy5 signals as expected.

Solutions:

A. Optimize target labeling methods. If cRNA is used, make sure amplification is in the range of 600-fold. Otherwise, optimize Eberwine method to improve amplification efficiency prior to setting up hybridization experiments.

B. It is recommended to evaluate ratio control performance by hybridization of labeled cDNA targets. Once this has been shown, then proceed to cRNA target hybridization. When using cDNA targets, the frequency of CyDye labeling should be in the range of one dye per every 50 unlabeled nucleotides. The CyDye incorporation should be verified using a spectrophotometer and known quantities on slides.

C. Optimize hybridization and wash protocol to reduce non-specific hybridization signal. Nonspecific hybridization signal will confound the true signal and alter the ratio control data.

D. Apply normalization method prior to calculating ratio control values (see text).

Problem: There is very low Cy5 signal versus Cy3 signal.

Solution: Make sure incorporation of Cy5 into cRNA is at roughly one dye per every 100–120 unlabeled nucleotides and at least 5–10 µg of cRNA is used for hybridization. If the Cy5 incorporation is poor, then optimize the labeling reaction prior to hybridization.

Avoid exposure of slide to light during hybridization and washing steps. This will reduce Cy5 signal. Perform hybridization on automated hybridization stations such as SlidePro. The slides must be completely dried and scanned immediately after hybridization to acquire image.

Problem: Negative control is giving high signal.

Solution: Increase washing time and temperature in hybridization protocol to reduce nonspecific hybridization signal. Reducing the UV cross-linking energy on UltraGAPS slides will lower signal from negative controls.

Problem: I am observing nonspecific signal and high background.

Solution: Optimize hybridization and wash buffer settings. Perform additional wash buffer steps and/or increase time. Increase hybridization stringency to reduce signal from cross-hybridization.

Problem: The signal from Universal ScoreCard spike mix has gone down on my hybridization compared to when I used it previously.

Solution: Reduced signal from array features can be due to degradation of Universal ScoreCard spike mix. It is recommended to store the Universal ScoreCard mRNA spike mixes in small amounts (~10–20 µl) when conducting the first target labeling reactions. Repeated freeze thawing of old spike mRNA spike mixes can degrade mRNA and result in lower signals from spots on arrays.

Amersham Biosciences