Supplemental Material: Multiple Lab Comparison of Microarray Platforms

February 23, 2005

Accuracy Plots

Figure 1: Observed log fold change versus RT-PCR log fold change for the four altered genes and 12 selected genes. The solid line is the identity function and represents perfect accuracy.

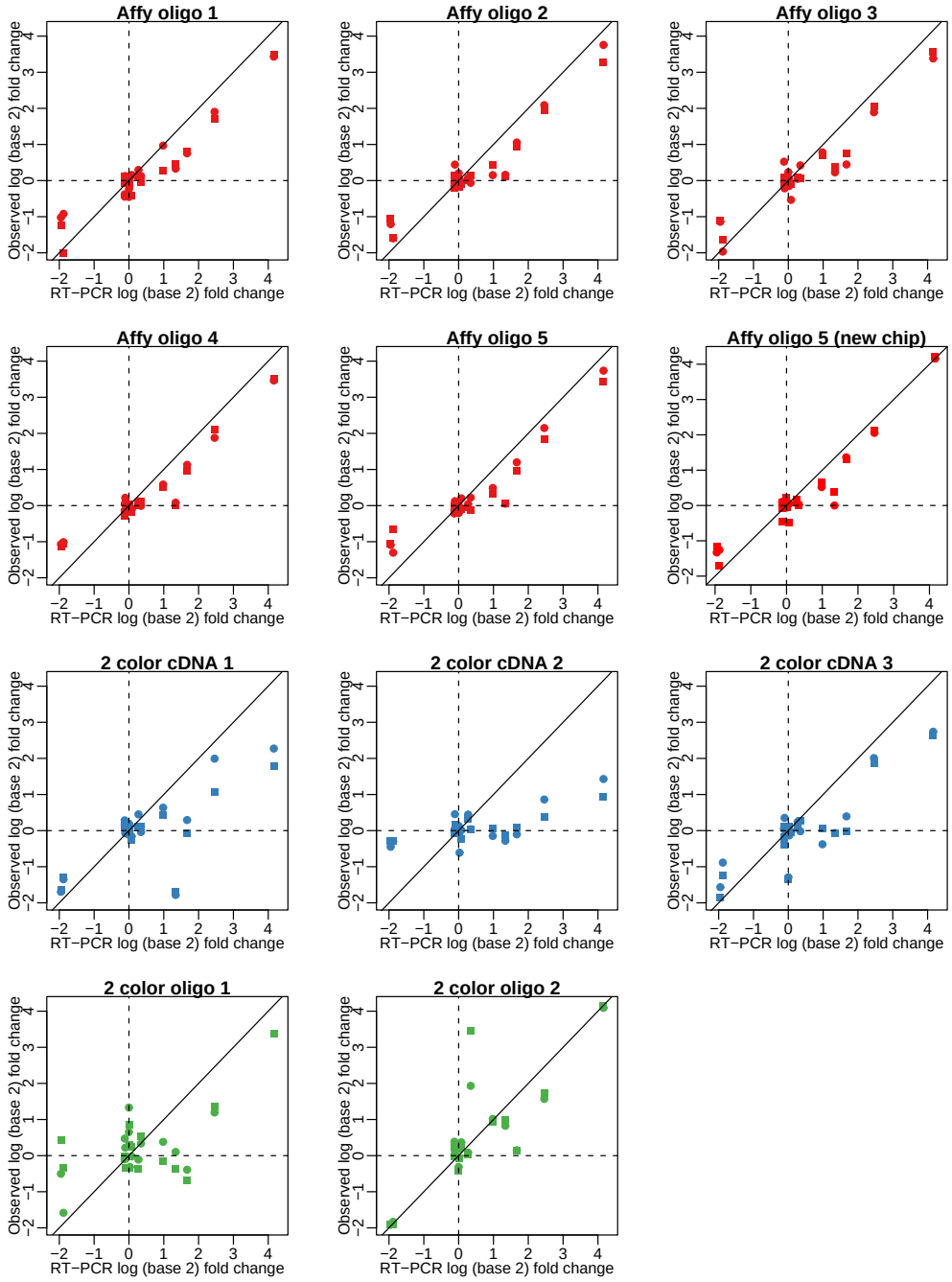

Figure and Tables Related to Pre-processing

For illustrative purposes, in Figure 2 and Tables 1 and 2, we show the best performing labs: Affy oligo lab 4, two-color cDNA lab 1, and two-color oligo lab 2.

Apart from the pre-preprocessing algorithms described in the paper, we can also pre-filter genes. Affymetrix MAS 5.0 also provides algorithms for pre-filtering genes by the so-called present/marginal/absent calls. Filtering genes that were consistently absent (absent in all 4 arrays) for at least one lab improved the results from the default algorithms but were still inferior to the results obtained with the Bioconductor procedures. Filtering made a rather small improvement on the precision of Bioconductor algorithms. However, the results presented in our paper are obtained without the pre-filtering to avoid the possibility of incorrectly removing genes of interests.

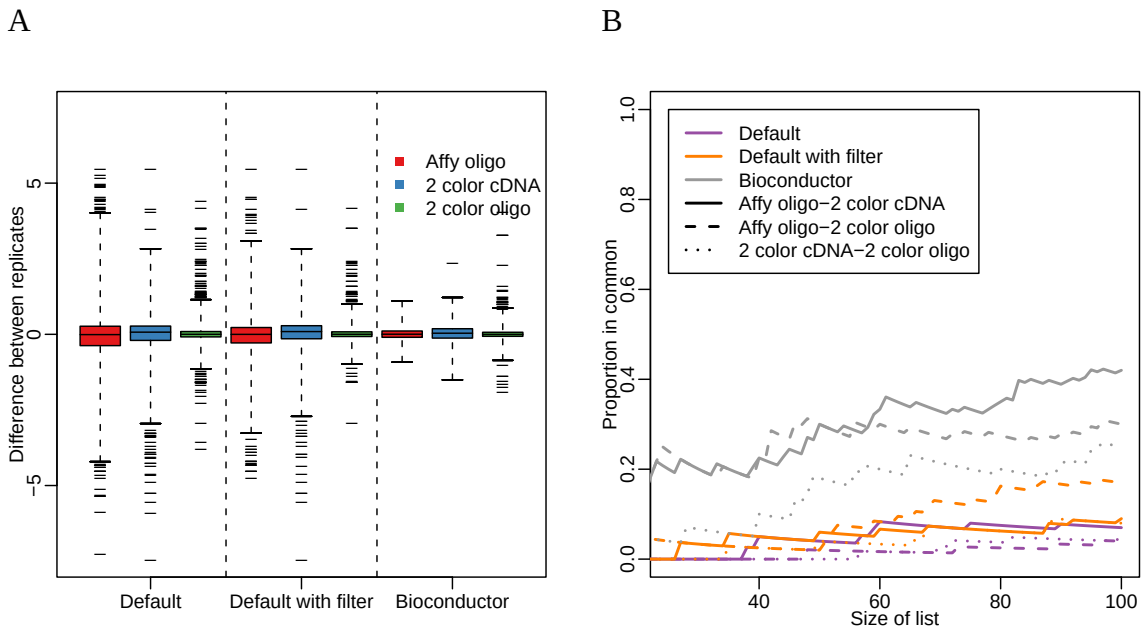

Figure 2: A) Box-plots of differences between log fold changes from replicate arrays for Affy oligo lab 4, two-color cDNA lab 1, and two-color oligo lab 2 using three different pre-processing procedures. B) CAT plot comparing the agreement between Affy oligo lab 4, two-color cDNA lab 1, and two-color oligo lab 2 using three different pre-processing procedures.

Table 1: Assessment measures comparing four different pre-processing procedures for three labs representing three different platforms: Affy oligo lab 4, two-color cDNA lab 1, and two-color oligo lab 2 using.

		Precision		Accuracy	Proportion of Agreement		
Platform	Pre-processing	Correlation	SD	slope (SE)	25	50	100
Affy oligo	Default	0.22	0.46	0.82 (0.12)	0.04	0.10	0.20
Affy oligo	Default w/filter	0.37	0.37	0.82 (0.12)	0.16	0.24	0.33
Affy oligo	Bioconductor	0.79	0.15	0.59 (0.04)	0.80	0.70	0.65
Affy oligo	Bioconductor w/filter	0.82	0.16	0.59 (0.04)	0.80	0.70	0.65
two-color cDNA	Default	0.31	0.34	0.44 (0.22)	0.08	0.18	0.23
two-color cDNA	Default w/filter	0.36	0.32	0.44 (0.22)	0.24	0.20	0.31
two-color cDNA	Bioconductor	0.65	0.23	0.41 (0.12)	0.68	0.64	0.65
two-color cDNA	Bioconductor w/filter	0.67	0.23	0.41 (0.12)	0.68	0.74	0.61
two-color oligo	Default	0.85	0.13	0.71 (0.15)	0.24	0.48	0.56
two-color oligo	Default w/filter	0.88	0.12	0.71 (0.15)	0.56	0.62	0.77
two-color oligo	Bioconductor	0.90	0.10	0.76 (0.13)	0.44	0.72	0.81
two-color oligo	Bioconductor w/filter	0.90	0.09	0.76 (0.13)	0.44	0.78	0.88

Table 2: Assessment measures comparing four different pre-processing procedures for across platform agreement between three labs representing three different platforms: Affy oligo lab 4, two-color cDNA lab 1, and two-color oligo lab 2 using.

				Proportion of Agreement		
Platform	Pre-processing	Correlation	SD	25	50	100
Affy oligo-two-color cDNA	Default	0.13	0.53	0.00	0.02	0.07
Affy oligo-two-color cDNA	Default w/filter	0.23	0.45	0.02	0.09	0.12
Affy oligo-two-color cDNA	Bioconductor	0.44	0.25	0.24	0.36	0.42
Affy oligo-two-color cDNA	Bioconductor w/filter	0.50	0.25	0.26	0.39	0.45
Affy oligo-two-color oligo	Default	0.24	0.51	0.00	0.05	0.07
Affy oligo-two-color oligo	Default w/filter	0.34	0.43	0.06	0.08	0.18
Affy oligo-two-color oligo	Bioconductor	0.48	0.31	0.30	0.32	0.30
Affy oligo-two-color oligo	Bioconductor w/filter	0.55	0.30	0.32	0.35	0.36
two-color cDNA-two-color oligo	Default	0.23	0.46	0.00	0.01	0.05
two-color cDNA-two-color oligo	Default w/filter	0.29	0.42	0.02	0.07	0.16
two-color cDNA-two-color oligo	Bioconductor	0.35	0.35	0.16	0.27	0.27
two-color cDNA-two-color oligo	Bioconductor w/filter	0.41	0.34	0.20	0.30	0.34

Tables Related to Annotation

The annotation one uses has an effect on the across platform agreement. Table 2 shows the assessment measures obtained when comparing Affy oligo lab 4 and two-color cDNA lab 1 using different annotations. Similar results are obtained when comparing different pairs of laboratories (data not shown). Notice that the intersection of all four annotation mappings provides the best agreement.

Table 3: Assessment measures obtained from comparing measurements from Affy oligo lab 4 and two-color cDNA lab 1.

			Proportion of Agreement			
Platform	Correlation	SD	25	50	100	Subset Size
UNIGENE	0.39	0.26	0.2	0.26	0.33	11989
LOCUSLINK	0.40	0.25	0.2	0.22	0.32	12004
REFSEQ	0.43	0.26	0.18	0.33	0.37	5756
EGO	0.44	0.26	0.18	0.33	0.34	6105
INTERSECTION	0.44	0.25	0.24	0.36	0.42	4675

Experimental Design

Different experimental designs were used among the two-color labs. We decided not to use a fixed design for all labs because this would not yield realistic results. In practice, each lab chooses the design they feel more comfortable with. The chosen designs are illustrated in Figure 3.

Figure 3: Possible designs for the two channel platforms. The arrows point to the sample represented with the red (Cy5) dye.

	Hybridization			
Design	1	2	3	4
I	$B_1 \longrightarrow A_1$	$B_2 \longrightarrow A_2$		
II	$B_1 \longrightarrow A_1$	$A_2 \longrightarrow B_2$		
III	$A_1 \longrightarrow Ref$	$Ref \longrightarrow A_2$	$B_1 \longrightarrow Ref$	$Ref \longrightarrow B_2$
IV	$A_1 \longrightarrow B_1$	$A_2 \longrightarrow B_1$	$A_2 \longrightarrow B_1$	$A_2 \longrightarrow B_2$
V	$A_1 \longrightarrow B_1$	$B_1 \longrightarrow A_1$	$A_2 \longrightarrow B_2$	$B_2 \longrightarrow A_2$
VI	$A_1 \longrightarrow B_1$	$B_2 \longrightarrow A_1$	$B_1 \longrightarrow A_2$	$A_2 \longrightarrow B_2$

To explore the effect of the chosen designs, one of the labs ran three experimental designs. Two designs they would typically consider (designs V and VI in Figure 3 and the design used by most labs in our comparison (design II in Figure 3). These designs deal with dye swaps in different ways.

The three compared designs gave very similar results. The correlations final estimates of relative expression between II and V, II and VI and V and VI were 0.90, 0.89, and 0.98. The CAT curves, Figure 4, demonstrate that these give very similar bottom line results as well.

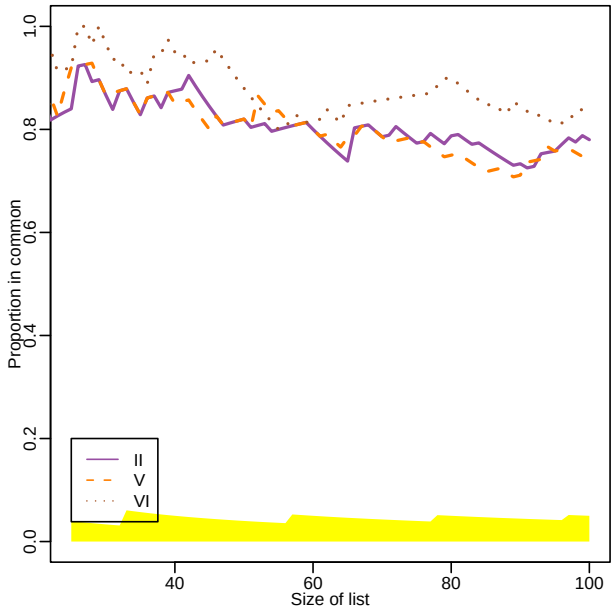

Figure 4: CAT plot comparing results using three different experimental designs.

Two-color oligo lab 1 was the only one to chose a design that uses *indirect comparisons* of the samples, i.e. samples A and B are compared to a reference sample instead of to each other. This has been shown, theoretically and empirically, to yield less precise results[1, 2]. Our results are in agreement with this finding.

Experience Effect

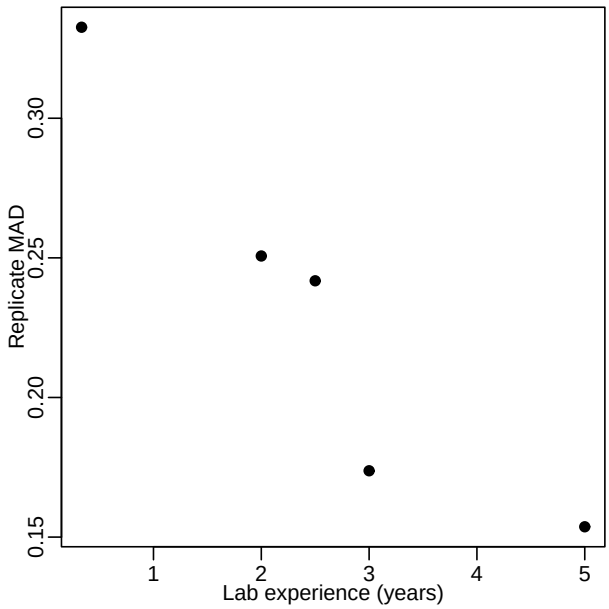

Figure 5: SD (measure of precision) plotted against experience of technician for each of the five Affymetrix labs.

Tables of Platform Agreement

Table 4 and 5 show all pairwise correlations and agreement proportions (in a list of size 100) between the different laboratories.

Table 4: Correlations of log fold changes for each pairwise comparison of the 10 labs.

		Affy oligo					two-color cDNA			two-color oligo	
	Lab ID	1	2	3	4	5	1	2	3	1	2
Affy oligo	1	0.48	0.68	0.71	0.67	0.68	0.39	0.19	0.43	0.16	0.49
	2		0.76	0.7	0.74	0.73	0.43	0.2	0.47	0.16	0.54
	3			0.67	0.64	0.7	0.38	0.13	0.43	0.22	0.57
	4				0.79	0.74	0.44	0.22	0.48	0.13	0.48
	5					0.59	0.41	0.17	0.46	0.17	0.5
two-color cDNA	1						0.65	0.23	0.39	0.08	0.35
	2							0.68	0.14	0.03	0.14
	3								0.46	0.12	0.35
two-color oligo	1									0.68	0.2
	2										0.9

Table 5: Agreement proportions (in a list of size 100) for each pairwise comparison of the 10 labs.

		Affy oligo					two-color cDNA			two-color oligo	
	Lab ID	1	2	3	4	5	1	2	3	1	2
Affy oligo	1	0.54	0.57	0.61	0.52	0.56	0.34	0.13	0.34	0.1	0.32
	2		0.7	0.64	0.58	0.6	0.40	0.13	0.38	0.1	0.38
	3			0.6	0.52	0.55	0.36	0.08	0.34	0.11	0.38
	4				0.65	0.6	0.42	0.14	0.36	0.08	0.3
	5					0.55	0.41	0.14	0.37	0.12	0.32
two-color cDNA	1						0.65	0.2	0.32	0.04	0.27
	2							0.38	0.13	0.04	0.08
	3								0.5	0.06	0.24
two-color oligo	1									0.33	0.05
	2										0.81

RT-PCR Result Table

Table 6: For 16 genes we show log fold change estimates obtained using RT-PCR and microarray data from the labs that performed well. Because the Affy labs gave similar results we only present results from one lab.

GeneBank Accession #	Gene Symbol	RT-PCR	Affy oligo lab 4	two-color cDNA lab 1	two-color cDNA lab 3	two-color oligo lab 2
NM_000466	PEX1	0.27	0.06	0.28	0.18	0.05
NM_000287	PEX6	0.98	0.55	0.54	-0.16	0.97
NM_000288	PEX7	0.08	-0.14	-0.21	-0.05	0.33
NM_000286	PEX12	-0.12	-0.15	0.21	-0.06	0.00
M60316	BMP7	0.00	-0.04	-0.08	-0.02	0.25
AF062537	CUL3	-0.11	0.15	0.04	-0.02	0.06
NM_007051	FAF1	0.03	-0.02	0.07	-0.01	0.05
NM_002405	MFNG	-0.12	-0.12	0.12	0.07	0.30
NM_002422	MMP3	4.16	3.49	2.03	2.69	4.12
NM_002658	PLAU	2.46	1.99	1.53	1.94	1.65
NM_004105	EFEMP1	-1.88	-1.04	-1.32	-1.06	-1.87
NM_000393	COL5A2	-1.95	-1.11	-1.67	-1.71	-1.90
NM_000584	IL8	1.67	1.05	0.11	0.19	0.14
NM_004186	SEMA3F	1.34	0.04	-1.74	-0.07	0.91
NM_000698	ALOX5	0.00	-0.04	0.06	-1.32	-0.36
AK025329	RNF167	0.35	0.06	0.03	0.13	2.69

Assessment of New Affymetrix Chip

Since the start of this project, Affymetrix has upgraded their human expression chip. These are now scanned with entirely different instrumentation. To make sure that our results are still relevant, Affy oligo lab 5 re-ran the entire experiment using the new chips. The correlations between measurements from the old and new chips were over 0.94 for all comparisons. The table below demonstrates that results improved, but not enough to change our conclusions. See Table 7 and Figure 6 below.

Table 7: Assessments for new chip compared to select lab results.

					Proportion of Agreement		
Platform	Lab ID	Correlation	SD	Signal (SE)	25	50	100
Affy oligo (old)	4	0.79	0.15	0.59 (0.04)	0.80	0.70	0.65
Affy oligo (old)	5	0.59	0.25	0.58 (0.05)	0.64	0.68	0.55
Affy oligo (new)	5	0.71	0.22	0.70 (0.05)	0.76	0.70	0.73
two-color oligo	2	0.90	0.10	0.76 (0.13)	0.44	0.72	0.81

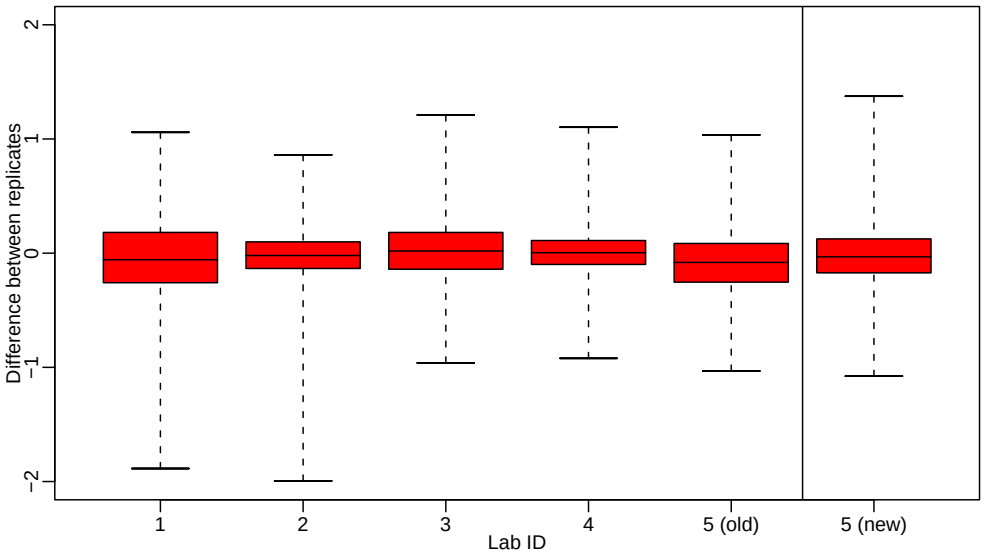

Figure 6: Box-plot of the difference in log fold change between replicate measurements for the Affy labs including results from the new chip.

Microarray Hybridization Methods

Affy oligo lab 1 Prior to hybridizing the chips, 13 μg of each sample were prepared by creating cDNA and then biotin-labeled cRNA. The concentration of cRNA was then measured so that 12.5 μg of cRNA was fragmented into 25-mers in 40 μl volume. A hybridization cocktail containing 2$\times$ Hybridization Buffer, BSA, Herring Sperm DNA, 20$\times$ Eukaryotic Hybridization Control, and Control Oligo B2 was made and added to the 40 μl of fragmented cRNA for a 250 μl volume. This cocktail was then heated at 99° C for 5 minutes. Meanwhile, the array chips that were equilibrated to room temperature were filled with 200 μl of 1$\times$ hybridization buffer and rotated at 60 rpm in the hybridization oven for 10 minutes. The cocktail was then placed at 45° C for 5 minutes and centrifuged at max speed for an additional 5 minutes. Once the cocktail and chips were ready, 200 μl (containing 10 μg of fragmented cRNA) of cocktail was placed in the chip. These were hybridized in the oven for 18 hours. The chips were removed from the oven, and the cocktail was removed from the chips. The chips were then filled with 200 μl of non-stringent buffer and washed and stained with the Fluidics Station 400 (Affymetrix). There were three staining steps, two streptavidin and one IgG stain. Once complete, the chips were scanned using the GeneChip High Resolution Scanner (Affymetrix).

Affy oligo lab 2 Double-stranded cDNA was synthesized from 6μg of total RNA using the SuperScript Choice system (Invitrogen Life Technologies, Carlsbad, CA) and the T7-Oligo(dT) promoter primer kit (Affymetrix, Santa Clara, CA). cDNA was purified using Phase Lock Gels (Eppendorf 5-prime). Biotin-labeled cRNA was then synthesized from double-stranded cDNA using the ENZO BioArray High Yield RNA transcript labeling kit. cRNA was purified using an RNeasy Mini kit (Qiagen, Chatsworth, CA) and fragmented into 35-200 base pair fragments by metal-induced hydrolysis (200mM Tris-acetate, pH 8.2, 500 mM KOAc, 150 mM MgOAc). Fifteen micrograms of biotin-labeled cRNA was hybridized onto Human Genome U133A and U133B GeneChips (Affymetrix) for 16 hours at 45°C and 60rpm. GeneChips were then washed and stained as per the Affymetrix GeneChip Expression Analysis Manual. This procedure includes the removal of non-hybridized material, staining with phycoerythrin-streptavidin followed by a laser scan to detect bound cRNA. Fluorescence was detected using the Hewlett-Packard GS2500 Gene Array Scanner. Strict quality control measures were followed to ensure high quality data. Such measures included the requirement that scaling factors, percent of genes called present, average intensity, background values, housekeeping 3'/5' ratios and measured intensities of spiked-in controls all fell within predefined limits. Additionally, only samples with 'acceptable' amplification at the T7 RNA polymerase cRNA step were hybridized onto chips.

Affy oligo Lab 3 Double-stranded cDNAs were synthesized from 5 μg total RNA of each sample using the SuperScript Double-Stranded cDNA Synthesis Kit (Cat. No. 11917-010, Invitrogen, Carlsbad, CA). Each double-stranded cDNA was subsequently used as a template to prepare the biotin-labeled cRNA using the BioArray HighYield RNA Transcript Kit (cat. no. 42655-10, Enzo Life Sci., Inc., Farmingdale, NY) and 15 μg of fragmented, biotin-labeled cRNA from each sample were hybridized to a Human Genome U133A Array (cat. no. 900366, Affymetrix, Inc., Santa

Clara, CA) at 45° C for 16 hours. The arrays were washed and stained in the Affymetrix GeneChip Fluidics Station 450 using the supplier's reagents and scanned using the Affymetrix GeneChip Scanner 3000.

Affy oligo lab 4 5 μg of total RNA are used to synthesize first strand cDNA using oligonucleotide probes with 24 oligo-dT plus T7 promoter as primer (Proligo LLC, Boulder, Colorado) and the SuperScript Choice System (Invitrogen, Carlsbad, California). Following the double stranded cDNA synthesis, the product is purified by phenol-chloroform extraction, and biotinilated anti-sense cRNA is generated through in vitro transcription using the BioArray RNA High Yield Transcript Labelling kit (ENZO Life Sciences Inc., Farmingdale, New York). 15 μg of the biotinilated cRNA are fragmented at 94 C for 35 minutes (100mM Tris-acetate, pH 8.2, 500mM KOAc, 150mM MgOAC), and 10μg of total fragmented cRNA are hybridized to the Affymetrix GeneChip arrays for 16 hours at 45° with constant rotation (60 rpm). Affymetrix Fluidics Station 450 is then used to wash and stain the Chips, removing the non-hybridized target and incubating with a streptavidin-phycoerythrin conjugate to stain the biotinilated cRNA. The staining is then amplified using goat IgG as blocking reagent and biotinilated anti-streptavidin antibody (goat), followed by a second staining step with a streptavidin-phycoerythrin conjugate. Fluorescence is detected using the Affymetrix GC3000 GeneArray Scanner and image analysis of each GeneChip was done through the GeneChip Operating System 1.1.1 (GCOS) software from Affymetrix, using the standard default settings.

Affy oligo lab 5 For each experimental sample, RNA quality was assessed by RNA Nano LabChip analysis on an Agilent Bioanalyzer 2100. Processing of RNAs for GeneChip Analysis was in accordance with methods described in the Affymetrix GeneChip Expression Analysis Technical Manual, revision three, as subsequently detailed.

For each experimental sample, 10 μg of total RNA was used as a template for cDNA synthesis. For first strand synthesis, RNA was incubated at 70°C for ten minutes with a T7-(dT)24 oligomer. Buffer, DTT, and dNTPs were added and incubated at 42°C for 2 minutes. SuperScript II RT enzyme was subsequently added, and the reaction was incubated for one hour at 42°C. For Second strand synthesis, the following reagents and enzymes were added: second strand buffer, dNTPs, E. coli DNA Ligase, E. coli DNA polymerase I, and E. coli RNase H. Incubation was performed at 16°C for two hours. Ten units of T4 DNA polymerase were added to ensure completion of synthesis to blunt ends. Following a 5-minute incubation, the reaction was terminated by the addition of EDTA. cDNA was purified by Phenol/Chloroform extraction followed by Ethanol precipitation. Phase Lock Gels (Eppendorf) were used in conjunction with the extraction protocol, and glycogen was utilized during the precipitation. Precipitated and washed cDNAs were resuspended in RNase-free water.

cRNA synthesis and fragmentation: cRNA was synthesized from one half of the resultant cDNA by in vitro transcription (IVT) using the BioArray High Yield RNA Transcript Labeling Kit (ENZO), according to the manufacturer's recommended protocol. cRNA products were purified with the GeneChip Sample Cleanup Module (Affymetrix), and quantified. Fifteen micrograms of cRNA was fragmented by metal-induced hydrolysis in fragmentation buffer (250mM Tris acetate

pH 8.1, 150 mM MgOAc, 500mM KOAc) at 94o for 35 minutes. Aliquots of pre- and post-fragmentation cRNAs were assessed for quality by RNA Nano LabChip analysis on an Agilent Bioanalyzer 2100. A hybridization cocktail was prepared for each sample consisting of the 15μg fragmented cRNA, hybridization buffer (100mM MES, 1M Na[+], 20mM EDTA, 0.1% Tween 20), 0.1 mg/mL Herring sperm DNA, 0.5mg/mL acetylated BSA, 50pM control Oligonucleotide B2, and 1x Eukaryotic Hybridization controls (Affymetrix) in a final volume of 300μl. Samples were heated to $94°$C for 5 minutes, and then $45°$C for 5 minutes, in a pre-programmed thermal cycler. Following centrifugation at maximum speed for 5 minutes, samples were pipetted into the GeneChips and hybridized at $45°$C for 16 hours at 60 rpm in the Affymetrix rotisserie hybridization oven.

Washing, Staining and Scanning: The signal amplification protocol for washing and staining of eukaryotic targets was performed in an automated fluidics station (Affymetrix FS450) as described in the Affymetrix Technical Manual, revision three. Briefly, upon completion of hybridization, the solutions were removed from the GeneChip arrays, and subsequent washes with Non-stringent wash buffer A (6$\times$ SSPE, 0.01% Tween 20) and stringent wash buffer B (100mM MES, 0.1 M [Na+], 0.01% Tween 20) were performed, at 25oC and 50oC, respectively. Arrays were then stained with R-Phycoerythrin Streptavidin in 1$\times$ staining buffer (100mM MES, 1M [Na+], 0.05% Tween 20) with 2 mg/mL acetylated BSA for 10 minutes at 25oC, followed by rinses with wash A. The signal was amplified with a biotinylated antistreptavidin antibody (Vector Laboratories) in 1$\times$ staining buffer with 2 mg/mL acetylated BSA, followed by a second streptavidin-phycoerythrin staining, and final washing. The arrays were then transferred to the GCS3000 laser scanner (Affymetrix) and scanned at an emission wavelength of 570nm at 2.5 μm resolution. Intensity of hybridization for each probe pair was computed by GCOS 1.1 software.

two-color cDNA lab 1 Array design Human 20K cDNA microarrays were obtained from a local Microarray Core. 20,000 genes and ESTs, printed on-site, from the Research Genetics "Image Consortium" cDNA set (Invitrogen, Carlsbad, CA).

Experimental design II was used. Indirect labeling of the RNA samples was performed using the SuperScript Indirect cDNA Labeling System (Invitrogen, Carlsbad, CA) per manufacturer's instructions. Briefly, per dye-swap, 20 μg RNA of each sample was reverse transcribed using 4 μl of 2.5μg/μl anchored oligo (dT)20 primer. The resulting cDNAs were divided between two tubes and labeled with either Cy3- or Cy5-monoreactive dye (Amersham, Piscataway, NJ). Following purification, the labeled cDNAs to be compared were combined and concentrated to 50 μl using Micron-30 filter units (Millipore, Bedford, MA) in preparation for hybridization.

Slides were prehybridized at $42°$C for one hour with blocking solution (5_SSC, 0.1% SDS, 1% BSA) applied under Lifterslips (Erie Scientific, Portsmouth, NH). Prehybridization solution was washed off by vigorously plunging the slides in double-distilled water for 2 minutes, followed by 100% isopropanol for 2 minutes. Slides were used within an hour to ensure optimal hybridization efficiency. While the slides air-dried, the concentrated Cy3- and Cy5-labeled cDNA pairs were combined with an equal volume of 2$\times$ hybridization buffer (50% formamide, 10xSSC, 0.2%SDS) and, to prevent nonspecific hybridization, 1 μl each of 10μg/μl poly(dA) (Amersham, Piscataway, NJ) and 10μg/μl species-specific COT-1 DNA (Invitrogen, Carlsbad, CA). Combined

cDNA/hybridization solution was denaturated at $100°$C for 3 minutes, quickly chilled on ice for 30 seconds, preheated to $42°$C and hybridized to an array at $42°$C for 18hr. After the incubation, the hybridized slides were washed sequentially in: 2xSSC, 0.1% SDS for 2 min at $42°$C, 1xSSC, 0.1% SDS for 2 min at 42C, twice in 0.2xSSC for 2 min at room temperature, and 0.05xSSC for 1 min at room temperature. The slides were immediately dried by centrifugation at 600 rpm for 3 minutes.

Slides were scanned with a GenePix 4000B scanner (Axon Instruments, Union City, CA) at 10 μM pixel resolution, and gene annotations and intensity data were extracted using GenePix Pro 3.0 software (Axon).

two-color cDNA lab 2 Human 20K clone set (Research Genetics) was spotted onto Corning UltraGAPS coated slides. Experimental design I was used. 10 μg of total RNA of each sample was labeled by using Atlas Glass Fluorescent Labeling Kit (Clontech) except using PowerScript reverse transcriptase in RT reaction. Labeled samples were purified by using Qiagen's MinElute PCR Purification Kit. Combined Cy3- and Cy5-samples were denatured at $95°$C for 2 minutes, mixed with equal volume of hybridization buffer (5x SSC, 25% formamide and 0.1% SDS in final concentration) and hybridized in dark with pre-hybridized microarrays at $42°$C for 16 hours. Microarrays were scanned with Axon's GenePix 4000B scanner under 10 um resolution. Microarray data were extracted with GenePix Pro 4.0 software.

two-color cDNA lab 3 Custom spotted cDNA microarrays containing 32,448 elements were used. These comprise ten exogenous positive controls and four negative controls printed 48 times each and 31,782 human cDNAs representing 30,849 distinct transcripts. Clone inserts were amplified by PCR, purified, and printed in a 50% DMSO buffer on UltraGAPs aminosilane-coated slides (Corning, Corning, NY) using an Intelligent Automation Systems arrayer (Cambridge, MA). After printing, the DNA was cross-linked to the slides by UV irradiation with a Stratalinker UV Crosslinker (Stratagene, La Jolla, CA) and stored under desiccation until used.

Experimental designs II, V and VI were used. However, for the comparisons in this paper we used design II. Data from the other designs were used to compare experimental designs.

Using total RNA as starting material, target cDNA was synthesized by random-primed reverse transcription in the presence of aminoallyl dUTP using 10μg of total RNA. Reaction products were purified and coupled to Cy5 and Cy3 NHS-esters (Amersham, Piscataway, NJ). The coupled cDNAs were purified and combined as appropriate for each hybridization and lyophilized. Slides were prehybridized in 1% bovine serum albumin in 5SSC, 0.1% SDS for 45 minutes at $42°$C, after which the slides were washed and dried. The combined Cy5- and Cy3-labeled cDNA was resuspended in 30μl of 50% formamide, 5$\times$ SSC, 0.1% SDS containing 0.5μg human CotI-DNA and 1μg poly-dA and hybridized at $42°$C overnight under glass coverslips. Following hybridization, the slides were washed for 4 minutes at $42°$C in a solution containing 1$\times$ SSC and 0.2% SDS, followed by 4 minute wash of 0.1$\times$ SSC and 0.2% SDS at ambient temperature, and finally two 2 minute washes of 0.1$\times$ SSC at ambient temperature. Slides were dried by centrifugation and scanned immediately at 10μm resolution using an Axon 4000B scanner with PMT values set to 740 for 635nm laser and 600 for the 532nm laser.

two-color oligo lab 1 Microarrays were manufactured at a local Microarray facility, Human Genome Oligo Set Version 2.0 containing approximately 22,000 oligonucleotides of 70 bases in length were obtained from Operon, Inc. (Alameda, CA). Arrays were printed by standard protocols on Corning Ultra-GAPS II slides (Corning, NY) using a GeneMachine (San Carlos, CA) OmniGrid 100 instrument. Oligonucleotides were suspended at 25 M in 3XSSC buffer, and the arrays printed using SMP3 pins from Telechem International (Sunnyvale, CA). The spotted nucleic acids were fixed to the slides and blocked with protocols supplied by the manufacturer.

Experimental design III was used. Labeled cDNA for the long oligonucleotide arrays were synthesized and labeled by the indirect amino-allyl method using reagents and protocols supplied with the Stratagene FairPlaył Microarray Labeling Kit. For cDNA synthesis, Stratascript reagents (Stratagene, La Jolla, CA) were used, and Cy3/Cy5 fluorophore amino-allyl reagents were obtained from Amersham (Piscataway, NJ). Twenty micrograms of total RNA were used for each synthesis. Labeled cDNA targets were purified using MINElute purification kits (Qiagen, Valencia, CA).

The long oligonucleotide microarrays were prehybridized in 40 μl of 5XSSC, 0.1% SDS and 1% BSA at 42°C for 30 minutes. The prehybridization solution was removed and arrays were hybridized for 16 hours at 42°C in 5XSSC buffer containing Cy3/Cy5 labeled targets, 25% formamide, 0.1% SDS, 1 μg Cot-1 DNA, and 1 μg poly A RNA. The long oligonucleotide arrays were washed at room temperature in 2XSSC, 0.1% SDS for 2 minutes, 1XSSC for 2 minutes, and 0.2XSSC for 2 minutes. The slides were dried by spinning at 650 rpm for 3 minutes and scanned immediately.

Array scanning and image processing: Long oligonucleotide arrays were scanned using Axon 4000B scanner at 10 micron resolution. Image processing and quantification of signal values of spotted arrays were performed using Genepix 3.0 software (Axon Instruments, Union City, CA). The array was first evaluated in GenePix's Preview mode and then scanned at high resolution. The PMT settings were adjusted to ensure proper overlap of the red and green signal intensities as illustrated by histograms. A 16-bit TIF image was saved for further analysis.

two-color oligo lab 2 Fluorescently labeled cDNA was prepared from 30μg of total RNA by reverse transcription and direct incorporation of either Cy3-dUTP or Cy5-dUTP. Experimental design IV was used. For the purposes of this comparison we used only a subset equivalent to experimental design I. The labeled cDNAs to be hybridized on the same slide were co-purified by filtering the samples through six successive washes in TE, pH8.0 (10K MWCO Vivaspin 500, Vivascience AG, Hannover, Germany). The microarrays were produced by printing a library of 70mer oligonucleotide mouse probes (Mouse Genome Oligo Set Version 2.0, Qiagen, Valencia, CA) onto epoxy-activate glass slides (MWG Biotech, High Point, NC). The microarrays were prehybridized as described (Hedge), and the cDNA's were mixed with a $2\times$ hybridization cocktail so that the final hybridization cocktail consisted of $5\times$ SSC, 0.2% SDS, 25% formamide with 1μg Mouse Cot-1 DNA (Invitrogen), 4μg yeast tRNA (Sigma), 1μg poly dA(40-60) (Amersham Pharmacia Biotech Inc). Hybridizations were performed overnight at 45°C on the MAUI (BioMicro Systems, Salt Lake City) hybridization system according to the manufacturers' instructions. Fluorescence images were captured with a GenePix 4000B scanner and the spot intensities were extracted using GenePix Pro 4.0 software (Axon Instruments, Union City, CA).

References

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