Supplementary Methods
Standardizing Global Gene Expression Analysis Between Laboratories and Across Platforms

I. STANDARD PROTOCOLS

A. Standard Protocol for Tissue Extraction

Tissue extraction was performed by the NIEHS National Toxicology Program (NTP). Twenty-five male C57 black mice (10-11 weeks) were purchased from Taconic Farms and housed five in a cage in pathogen-free rooms determined by sentinel animal survey. They acclimated for 7-10 days until they reached 12 weeks of age. They were housed in shoebox cages on sawdust and fed NTP 2000 chow. Between 9:00-10:00 Am EST, they were removed to a necropsy room. One mouse at a time was euthanized by CO_2 from a regulated source and removed from view of the remaining animals. The fur was wet with 70% alcohol and a midline excision made to expose the body cavity. The diaphragm was nicked and the following organs removed and trimmed of fat: liver, kidneys, spleen, heart, brain and lungs. Time for removal was less than two minutes. Average organ weights were: liver (1260-1660 mg), kidneys (400 mg), lung (220 mg), brain (390 mg) and spleen (75 mg). Liver samples were placed in 50 ml polypropylene conical centrifuge tubes with 20 ml of RNAlater®. Other organs were placed in RNAlater® (10 µl per mg tissue) at room temperature, mincing 4-5 times, into 60 individual cryovials (no tissue pooling). Vials were stored at 4°C and RNA extracted within four weeks of necropsy.

B. Standard Protocol for RNA Sample Preparation. RNA isolation was performed by the NIEHS Microarray Group. Total RNA was isolated from entire mouse organs (except the liver) utilizing QIAGEN RNeasy® Maxi Kits following the standard Maxi Protocol for animal tissues. The Maxi Protocol was modified slightly for the liver sample. Tissue sections (100-300 mg for tissues except liver, 1000 mg for liver) were placed in an appropriate volume of QIAGEN RLT buffer (7.5 ml for 150-300 mg tissue and 15 ml for >300-1000 mg tissue) and homogenized using an Omni® International TH hand-held homogenizer, Omni A1000Tip Adapter and Omni-Tips plastic disposable Rotor Stator Generator Probes. Homogenates were centrifuged to remove particulate debris, debris-free lysates were mixed with equal volumes of 70% ethanol (50% ethanol for liver) and loaded on RNeasy silica gel columns. The samples were washed three times on the column and RNA was extracted in an appropriate volume of RNAse-free water (0.8 ml for $\leq$ 1 mg total RNA, 1.2 ml for 1-6 mg total RNA yield). The RNA samples were concentrated to a 10-fold concentration and total RNA was quantified using spectrophotometric determination (Beckman DU520 UV/Vis Spectrophotometer) at 260 nm. Total RNA quality was determined by running 1µl at 100 ng/µl on the Agilent® BioAnalyzer. Individual samples that showed degradation on the BioAnalyzer® were omitted from the pool. Total RNA samples were snap frozen and stored at –80°C. For each tissue, all RNA from each animal (n = 22) that passed quality assessment was pooled to generate a single pool for each organ. Total RNA samples were quantified by measuring absorbance at 260 nm in the spectrophotometer. For the five-tissue reference sample, equal amounts of RNA were mixed from each of the single organ RNA pools (3 mg RNA from each of the kidney, spleen, lung, brain and liver single organ pools). RNA quality was rechecked on the BioAnalyzer® and the concentration rechecked on the spectrophotometer. Test hybridizations were performed on the liver RNA and five-tissue

RNA pool to ensure that each RNA pooled sample was acceptable for gene expression analysis.

C. Standard Protocol for Quality Control Genes (in RNA Samples and on Microarrays)

Arabidopsis transcripts provided by The Institute for Genomics Research (http:pga.tigr.org) and were added to each of the standard RNA samples (liver and pooled) as a means of assessing experimental variability for individual RNA analyses (labeling and hybridizations). A total of 10 Arabidopsis genes were chosen for this spike in design, with fixed concentrations of each transcript in two distinct sets, A3 and A5. Three of these genes would have a 1:3 ratio, another 3 would have a 3:1 ratio, and the remaining 4 would be at unity (1:1) with intensities throughout the dynamic range of the microarray. The appropriate amount of Arabidopsis cRNA transcript was added to the following samples: two liver RNAs (LA3 and LA5) and one pooled tissue RNA (PA3). After the addition of the transcripts, RNA quality of each sample was reanalyzed using the BioAnalyzer® and the concentration rechecked using the spectrophotometer. The three spiked RNAs were run on test hybridizations with the Standard Spotted Array (an 18K mouse oligonucleotide array manufactured by Laboratory 1), and the resident oligonucleotide spotted array for Laboratory 4 which contained the same 18K gene set (Operon). Seven aliquots of each standard (0.57 ml PA3/aliquot, 0.65 ml LA3/aliquot and 0.65 ml LA5/aliquot) were prepared and stored at -80°C and then shipped on dry ice to each of the laboratories. Arabidopsis 70-mer probes corresponding to the cRNA Arabidopsis control *in vitro* transcripts were spotted randomly onto the Standard Spotted Array slides. Several of the laboratories using cDNA resident microarrays either printed 70-mer olgonucleotide Arabidopsis probes (Invitrogen) onto their arrays or developed corresponding cDNA probes using a clone set provided by TIGR (http:pga.tigr.org).

D. Standard Protocol for Production of Labeled cRNA from Total RNA

1.0 Purpose

This Standard Operating Procedure (SOP) discusses the amplification and fluorescent labeling of cRNA from total RNA.

Term Definition

cRNA	Complimentary Ribonucleic Acid
MMLV-RT	Moloney Murine Leukemia Virus Reverse Transcriptase
DTT	Dithiothreitol
dNTP	Deoxynucleotide Triphosphate
NTP	Nucleotide Triphosphate

2.0 MATERIALS

Nuclease-free (non-DEPC-treated) water, Ambion cat. No. 9932 TE buffer (10mM Tris; 1mM EDTA; pH 8.0, nuclease-free), Amresco cat. no. E112 70% ethanol, diluted from Amresco cat. no. E193 10% Triton X-100, proteomics grade, diluted from Amresco cat. no. M143 1mM EDTA, RNase-free sterile solution, diluted from Amresco cat. no. E522-100mL Cyanine 3-CTP (10.0mM), PerkinElmer/NEN Life Sciences cat. no. NEL580 Cyanine 5-CTP (10.0mM), PerkinElmer/NEN Life Sciences cat. no. NEL581 Agilent Fluorescent Linear Amplification Kit (product number G2556A): includes T7 Promotor Primer, 5x First Strand Reaction Buffer, 0.1M DTT, 10mM dNTP mix, Random

Hexamers, MMLV-RT, RNase OUT, 4x Transcription Buffer, NTP mix, Inorganic Pyrophosphatase, T7 RNA Polymerase, 4M Lithium Chloride.

3.0 PROCEDURES

3.1 General Procedure Notes:

1) When possible, prepare amplified cRNA in batches of no less than 6 reactions. This is intended to minimize errors associated with pipetting small volumes. This protocol specifies reagent volumes for 1 reaction and 6.5 reactions. An additional one-half reaction has been added to ensure enough master mix for 6 cDNA synthesis reactions. To specify reagent volumes for **n** reactions, multiply the volumes for 1 reaction by **n+1.0.**
2) Always wear gloves, use dedicated nuclease-free solutions, and pipettors with nuclease-free aerosol-resistant tips to prevent contamination of reagents by ribonucleases.
3) Cyanine dyes are photolabile: take care to minimize exposure to light.
4) Frozen stock reagent solutions should be thawed as quickly as possible without heating above room temperature; vortex briefly to mix, spin to collect; store on ice in a cold block until use.
5) Prepare a separate transcription master mix for each dye.
6) LiCl is harmful by inhalation, by contact with skin, and if swallowed. Wear suitable protective equipment. MSDS for this compound is available at www.chem.agilent.com)

3.2 Reagent Preparation

Cyanine 3-CTP (6.0mM, enough for 4 labeling reactions)
1) Thaw 10mM cyanine 3-CTP, vortex briefly, spin to collect, place on ice.
2) Add 6.7uL nuclease-free water and mix gently.
4) Store frozen in a light-tight box at –80^{o}C.

Cyanine 5-CTP (4.0 mM, enough for 6 labeling reactions)
1) Thaw 10mM cyanine 5-CTP, vortex briefly, spin to collect, place on ice.
2) Add 15uL nuclease-free water and mix gently.
4) Store frozen in a light-tight box at –80^{o}C.

70% ethanol
1) Measure 35 mL 100% Ethanol into a 50 mL conical tube.
2) Add nuclease-free water to 50 mL.

0.3% Triton X-100
1) Combine 970uL of 1mM EDTA with 30uL of 10% Triton X-100 in a nuclease-free microcentrifuge tube.

3.3 Procedure

1) Pipet 5.0µg of the total RNA to be labeled in a volume of 4.5µL or less into a nuclease-free microcentrifuge tube. The total RNA concentration should be between **1.11 µg/uL** and **5 µg/uL.**
2) Add 5.0µL of T7 Promotor Primer (from kit).
3) Using nuclease-free water, bring the total volume of RNA and primer to 9.5µL.
4) Denature the mixture by incubating at 65oC in a heat block for 10 minutes.
5) Place the tube on ice and incubate for 5 minutes (maximum of 30 minutes).
6) Prepare a cDNA master mix by combining the following reagents in the order indicated and maintaining on ice:

Component	Volume (µL)/reaction	Master Mix for 6 Reactions Volume (µL)/ (n+1) reactions
5X First Strand Buffer	4.0	26
0.1M DTT	2.0	14
10 mM dNTP mix	1.0	7
Random hexamers	1.0	7
MMLV-RT	1.0	7
RNase OUT	0.5	3.5
0.3% Triton X-100	1.0	7
Total	10.5	73.5

7) Briefly spin the contents of the RNA-containing tubes from step 5.
8) Add 10.5 µL cDNA master mix to each tube containing RNA. Mix by pipetting.
9) Incubate reactions at 40°C in a water bath for **4** hours.
10) Incubate at 65°C for 15 minutes to inactivate the MMLV-RT.
11) Incubate the reactions on ice for 5 minutes (maximum of 30 minutes). Spin briefly before proceeding.
12) Add EITHER 4.0 uL cyanine 3-CTP (6 mM) or cyanine 5-CTP (4 mM) to the appropriate reactions.
13) Immediately prior to use, gently mix the following reagents by pipetting, in the order indicated, at room temperature. Add enzyme just before the master mix will be combined with cDNA.

Component	Volume (µL)/reaction	Master Mix for 6 Reactions Volume (µL)/ (n+1) reactions
Nuclease-free water	20.1	140.7
4X Transcription Buffer	20	140
0.1M DTT	6.0	42
NTP mix	8.0	56
RNase OUT	0.5	3.5
Inorganic Pyrophosphatase	0.6	4.2
T7 RNA Polymerase	0.8	5.6
Total	56	392

14) Add 56 µL of transcription master mix to each reaction tube. Mix gently by pipetting.
15) Incubate reactions in a light protected waterbath at 40°C for **1** hour.
16) Following transcription, add 80uL of 4M LiCl to each labeling reaction. Mix gently by pipetting.
17) Precipitate reactions overnight (or 1 hour minimum) at –20°C. DO NOT precipitate at –80°C or on dry ice because this will cause the LiCL to precipitate.
18) Spin the tubes in a 4°C microcentrifuge at maximum speed for 20 minutes.
19) Remove supernatants by carefully aspirating by pipet. Isolate waste LiCl and pipet tips for proper disposal.
20) Rinse each pellet once with 500uL of 70% room temperature ethanol. Repellet in a 4°C microcentrifuge for 10 minutes. Aspirate supernatant by pipetting.
21) Air dry pellet at room temperature uncapped and protected from light for 10 minutes.
22) Add 100 µL water, allow 5 minutes for pellet to absorb water, then gently pipette several times to resuspend pellet.

Quantification of fluorescent-labeled cRNA products

23) Determine the concentration of each labeled cRNA by measuring 1 ul sample using SOP GEPT 042-00. Calculate the amplification yield by multiplying cRNA concentration (ug/mL) by the sample volume (0.1mL) and dividing by the amount of poly A+ RNA initially present:

[cRNA conc (ug/mL) x 0.1mL] / [(5 ug total RNA) x (1 ug poly (A)+ RNA/100 ug total RNA)]

24) The acceptable fold amplification range is between 100 and 900. The typical fold amplification range is between 200 and 450. All fold amplification values (including those above and below the typical range) are recorded in LIMS.

Quality analysis of fluorescent-labeled cRNA products

25) Determine the quality of each labeled cRNA using the Agilent 2100 Bioanalyzer. Please refer to SOP number GEPT-013-00 for detailed procedures. Dilute cRNA to a range of 5-500 ng/uL. If not using the cRNA immediately, aliquot into smaller volumes, freeze on dry ice and store in a light-tight box at – 80^{o}C. This is intended to reduce excessive freeze-thaw cycles that could compromise RNA integrity. Record information in LIMS.

26) Evaluate the Bioanalyzer results. Typical size distribution of labeled cRNA is in the range of 200bp to 2000bp. Cyanine 3 labeled cRNA typically produces a time-resolved fluorescence profile showing a relatively broad distribution between 24 and 50 seconds. Cyanine 5 labeled cRNA typically produces a time-resolved fluorescence profile showing a relatively intense, 5-second duration peak between 24 and 29 seconds, corresponding to unincorporated Cy5-dNTP, followed by a relatively broad distribution between 29 and 50 seconds corresponding to the labeled cRNA. Significant deviation from these patterns should be considered labeling reaction failures.

E. Standard Protocol for Scanning With the Agilent G2565AA Scanner for Standard Commercial Arrays

1.0 Purpose

This Standard Operating Procedure (SOP) discusses the proper use of the Agilent G2565AA Microarray Scanner

2.0 MATERIALS

Microarray Scanner (Agilent Technologies, G2565AA)
Slide holders for G2565AA scanner (Agilent Technologies, G2505-60500)
Microarrays (Agilent Technologies, hybridized and washed)

3.0 PROCEDURES

PRECAUTIONS: Wear powder-free gloves, a lab coat, and safety glasses at all times during this procedure. Make certain all solutions, containers, and pipettors are RNAse-free.

1. Make sure the PC and Microarray Scanner are both turned on. If either is off, turn them on.
2. Allow 20 minutes for the scanner to warm-up. If the scanner is already on

and warmed up, continue to the next step.
3. Launch the scanner control software by double clicking on its icon. This will start the initialization and stabilization of the scanner.
4. When the scanner initialization is complete, indicated by a “Scanner Ready” message in the “Scanner Status” section of the interface. Carefully insert the microarray slide into a dust-free slide holder placed on a stable surface. Hold the microarray by the edges of the barcode end of the microarray, barcode facing up.
5. While simultaneously depressing the two inset buttons with one hand, gently insert the microarray slide with the other hand along the slide holder tracks and underneath the four tabs.
6. Slide the microarray completely into the slide holder while depressing the inset buttons until the microarray is flush against the back of the slide holder.
7. Carefully insert the slide holder into the slide carousel, beginning with slot 1. The slide holder only fits one way in the carousel with the barcode facing the center of the carousel. DO NOT place a slide holder in the slot labeled “H”.
8. Continue loading microarrays into slide holders and placing them in the carousel as listed above into slot 2, 3, etc., until all microarrays are loaded.
9. Install the carousel cover making sure the two side tabs lock into position.
10. Close the scanner lid.
11. In the “Operator” box, enter your initials.
12. In the “Start slot” box enter the slot position for the first slide to be scanned (default is 1).
13. In the “End slot” box enter the slot position for the last slide to be scanned (default is 48).
14. From the Settings menu of the Scanner Control interface choose “Modify Default Settings.”
15. Verify the default settings for the following fields: Scan region (60x21.6mm), Red PMT (100%), Green PMT (100%), Scan resolution (10um), Dye channel (Red&Green), Output path (c:\).
16. Within the Scan Configuration and Options area select the “attempt to retrieve from XML (GEML) files” box.
17. This option directs the software to access and retrieve the specific microarray design from the designated directory of \\Filer03\gep\GEMLPatternFiles\.
18. Populate the “Description” dialog box with the following information about the scan event (project, experiment, your initials).
19. Within the Scan image file handling area, designate the output file path to the D drive inside a folder with the date in the format YYYYMMDD (i.e. 20020101).
20. Within the Automatic file naming area, choose “Instrument serial #” for Prefix 1 and “Barcode” for Prefix 2.
21. To execute click the “OK” box at the bottom of the interface.
22. Using the Scan Control interface, click the “Edit Slot Values” button.
23. In the “Current slot settings” section, select all the slides to be scanned by clicking on the starting slot number, and simultaneously hold down the “Shift” key and click on the ending slot number.
24. Click on the “Set Values” button.
25. Select “Scan Slot 1-48” to initiate scanning. A progress window will appear to track the scan process. When scanning is complete, a “Scan Report” box will appear that displays information each individual scan. Click the “OK” box, to deactivate the Scanner lid lock allowing access to the carousel.
26. Remove slide holders from the carousel

27. Remove the slides form the holders, and place them in a light tight storage box in a dessicator.
28. Record the scan in the scanner log book. Include the date, your initials, the number of scans that were performed, and any comments about the scan.
29. Upon completion, move the images to the designated space on the network.
30. Once the images are on the network after 2 nightly, and one full network backup, you can delete them from the local hard drive.

F. Standard Protocol for Image Analysis of Standard Spotted Arrays

Introduction:
To address the issue of variability in results between laboratories due to application of different image analysis approaches, all of the scanned images from the microarray hybridizations to the Standard Spotted Array were re-analyzed using a common image analysis program (Axon GenePix Pro v 4.1.1.28). Paradigm Genetics provided these analyses which resulted in the generation of Dataset C (see paper).

Inputs:
Each of seven laboratories provided images in TIFF format corresponding to 8 hybridizations using the Standard Spotted Arrays (LvsL and LvsP). Six laboratories provided separate TIFF images for each of the two channels (96 files), while one lab (Laboratory 2) provided composite TIFF images (8 files) for a total of 104 image files. Two gene alignment (gal) files were provided to account for the two separate print runs that produced the microarrays that were hybridized.

Procedure:
1. Standard array images from all laboratories were imported into GenePix Pro version 4.1.1.28 in pairs, excepting those from Laboratory 2, which provided one composite tiff image per hybridization. The images from Laboratory 4 were flipped and rotated prior to import so that spot locations would align properly with the gal file. Composite TIFF images were split into two images representing, each representing a single channel, and then flipped and rotated using <Tiff Splitter A.6.1.1>, a Tiff Image Channel Splitter Utility from Agilent Technologies.
2. The gal file was imported. For all images except those from Laboratory 2, the gal file <MO17K Set3.gal> was used.
    a. Laboratory 2 images were from a separate microarray print run and were analyzed using <EPAMOP17K_r.gal>. The "_r" in the filename indicated a revised version of <EPAMOP17K.gal>, which was present on the TRC shared data site. One line of the original file was corrupt and required separation of a single data field into two different rows in order to be recognized by GenePix as a valid gal file.
    b. Images submitted by laboratory 3 were smaller in overall dimensions compared to other images. To compensate, column and row spacing were decreased from 180 μm to 140μm and feature diameter was decreased from 100 μm to 75 μm.
    3. The <find all blocks> command was used. Following automatic alignment proper placement of all 32 blocks was confirmed by visual inspection.
    4. <Align features in all blocks> was used with visual inspection to confirm that feature indicators were properly placed.
    5. The analysis portion of GenePix was launched using default values except for the <resizing features during alignment> parameter. In order to constrain the size variation of the features the default minimum diameter value was increased

from 50% to 75% and the maximum diameter was decreased from 150% to 125%.

Outputs:

1. Data output from the analysis was saved in GenePix Results (gpr) format. Results files were named using a systematic convention with the following items separated by underscores: An abbreviated identifier for each laboratory, a one letter code for the Cy5 sample followed by a one letter code for the Cy3 sample, the Standard Spotted Array number, the word results, and the suffix <.gpr>. For example, <Laboratory 1_PL_29_results.gpr> indicates a hybridization performed by Laboratory 1, wherein the tissue pool RNA was labeled with Cy5 and the liver RNA was labeled with Cy3, and these samples were hybridized to array number 29. If the Laboratory 1 array number could not be ascertained, a single letter abbreviation for the participating laboratory followed by a single digit number was used, *e.g.* <Laboratory 2_LL_F1_results.gpr>.
2. A Quality Control analysis was performed using default parameters in GenePix Pro. In this script, several statistics are calculated from the extracted data and compared to a user-definable threshold level – in this case default settings. For example, the median signal-to-background value must equal or exceed 10 in order to "pass". Values below 10 are designated "fail". A total of 16 quality metrics are evaluated on the pass/fail system (NOTE: the “bad features” metric requires the user to manually define features as “bad”. We did not flag any features in our analyses and therefore all images received a “pass” for this metric). The quality report also includes three different graphs, a miniature representation of the two-color image, summary statistics, and a space for user comments in which we detailed some characteristics of the image. The nomenclature for the quality control reports is the same as the Results files, except for “results.gpr” is replaced with “QC_report.pdf”.

Validation:

An individual scientist performed this protocol in order to provide consistency in the evaluation of the images and data extraction. As a means of validating results generated by these analyses, a different scientist replicated this process. Calculated log ratio values from the same images were compared using Pearson’s correlation (r). As detailed in the table below 30 of 55 images had an r-value in excess of 0.99, suggesting that individual users did not greatly impact the image extraction process. In instances in which the r-value was less than 0.95, the original images were re-evaluated. These images were found either to have low feature intensity levels or high background levels (or both). Low feature intensity and/or high background levels frequently generate problems in the automatic gridding algorithm in GenePix Pro and require hand-editing the block placement. The decreased correlation in these images could therefore be attributed to differences in the user defined block placement.

r-value	Number of Arrays
>0.99	30
>0.95	13
>0.90	8
>0.80	4

G. Standard Protocol for Image Analysis of Standard Commercial Arrays

1.0 Purpose

This Standard Operating Procedure (SOP) discusses the processing of microarray image files for data capture for the Standard Commercial Arrays.

Term Definition

Primary Image File	Image produced by Agilent Scanner
Cropped Image File	Image produced from a Primary Image File using Agilent Technologies Image Analysis Software
Design File	A file written in Extensible Mark-up Language used to map locations of an array and provide specific information associated with each location. Design files are specific to each array type.

2.0 MATERIALS

-Agilent Technologies Feature Extraction and Image Analysis software (Version A.6.1.1)
-Computer (minimum requirements: Pentium III with 500mHz processor, 512MB RAM)
-Primary Image File
-Microarray Design File
-Agilent Technologies Image Analysis and Feature Extraction User's Guide (for in-depth reference)

3.0 PROCEDURES

1. Launch the Agilent Image Analysis software and open the Primary Image file.
2. Select the cropping tool. Crop the Primary Image such that 2-5 feature diameters of space remain around the perimeter of the array.
3. A second active image window will appear containing the cropped image. In the upper left hand corner of the tool bar for this window is a diskette icon. Select this icon. A "Save Cropped View" dialog box will appear and the cropped image will be named by appending a suffix to the primary image file name. Select both "Open file after saving" and "Close original view." Select the appropriate array and designate an output filepath. Execute your selections by choosing "Save Cropped Image."
4. Select the Feature Extraction menu and select "FeatureExtractor." Executing this step will activate the software to search and obtain the correct design file from a specific directory located on the network.
A dialog box will appear with the text "Parsing design file\\…\" followed by another dialog box with the text "Calculating Unique ProbeList." No action is necessary.
5. A "Feature Extraction Configuration" dialog box will appear. Verify that the correct design file has been obtained by visually inspecting the Input file section at the top of the dialog box. The design file identifier is contained within the 14 digit barcode. Digits 3-8 specify the design identifier.
6. Within the Output files section, activate the browse button to designate a location for the output file ("Base filename:"). For convenience, designate the space that contains the Primary Image and cropped image. Select the XML file output, Text file output, and Save visual results boxes.

7. Within the Modules to run section, select all choices except Deletion Ctrl.
8. Within the FindSpots tab and Grid Initialization section, choose the Autofind corners box.
9. Each time the Feature Extraction Configuration window is displayed, the default settings or parameter values contained in the design file for the specific type of array used is loaded. Verify that the default settings have been chosen for the individual algorithm tabs.
10. Execute by selecting the "Run" box at the bottom of the Configuration window.

During the course of the Feature Extraction event, several progress dialog boxes will appear which require no input from the user. Following successful Feature Extraction of a .tif image, a log file dialog box appears listing several summary statistics.

H. Identifying Common Gene Sequences Represented On Microarray Platforms

The purpose of this effort was to provide a list of gene sequences that are common to all standard and resident arrays using a combination of approaches including direct sequence and ID-based mapping. The list of common sequences were used to compare gene expression results across platforms using the results of the TRC Standardization Experiment.

Inputs:

Arrays used are:

- 2 common arrays: Standard Spotted Array (spotted oligo array manufactured by Laboratory 1), Standard Commercial Array (Agilent ToxArray), as presented in Fig. 1 of the paper.
- 7 resident non-commercial arrays (Laboratories 1-7), as presented in Figs.1 and 4 of the paper.
- 3 resident commercial arrays (Affymetrix U74Av2 Mouse GeneChip, Agilent Mouse Development Microarray, Codelink Array), as presented in Figs. 1 and 4 of the paper.

Data used:

1) All gene information from the TRC shared data site was downloaded. Entries had information about Genbank Identifier, Source, and Unigene ID. The actual contents included a mixture of valid identifiers and other entries such as "n/a", null, empty, alien, etc.
2) Annotation from the TRC standardization experiment: twelve array description files representing all microarray platforms, common and resident, across all laboratories (refer to Figure 1 of the paper).
3) Affy_U74Av2 target sequences were downloaded from the Affymetrix website. It contained the 12,488 target sequences on the mouseU74Av2 array.
4) Agilent 60mer sequences on the Agilent Mouse Development Microarray – a total of 20281 probe sequences -- were obtained from Agilent.
5) A complete set of NAP sequences from NIA mouse gene index were used. NAP sequences are consensus sequences for each mouse transcript. There were a total of 116,880 sequences in this set (http://lgsun.grc.nia.nih.gov/geneindex/). From a utility standpoint, the NAP cluster sequences are analogous to Unigene. However, unlike Unigene, each NAP cluster is represented by a consensus

sequence and is mapped to a U-cluster (see below), which is further mapped to the genome sequence when possible.

6) A map file which maps NAP name to U-cluster number was generated. There were a total of 39,678 U-cluster numbers. Approximately 65% of NAP transcripts are mapped to a U-cluster number. From a utility standpoint, U-clusters can be regarded as transcription units.

Procedure:

Two approaches were employed: mapping to NAP clusters by direct sequence comparison and mapping to U-clusters via NAP cluster association. In the first approach, we used a highly stringent sequence-based criteria in order to reduce the chances of inappropriate mapping. The resulting data are most useful for follow-up comparisons including TaqMan. In the second approach, the stringency was relaxed to produce a more inclusive set. As a result, the probability that mapped sequences in fact identify different transcripts is increased. Details of the procedure are as follows:

1) Obtain the mouse sequences represented on each array according to the Genbank identifiers or NIA Clone-ID. Not all sequences on a given array were retrievable from Genbank. The control sequences present on the arrays are not included in the sequence sets.
2) Blast the sequences against NAP clusters (116,880), using a threshold cut-off 1e-10. A large proportion of sequences on each array met this cut-off.
3) In order to increase stringency, additional criteria were used to identify an array sequence to map to an NAP cluster sequence. Basically, at least 80% of the query sequence had to match a NAP and the percent identity must be greater than 95%. A common sequence is defined as a NAP that is present across all the arrays tested.
4) Using a less stringent approach, common genes are defined by matching disparate NAP cluster IDs to the same U-cluster ID.

Results:

In the first iteration of this process, we used the gene information for all standard and resident arrays available on the TRC shared data site. Based on the array description files from each laboratory, the array contents of Laboratory 4 resident array and Standard Spotted Array were the same. They had different control elements and array layout, but the sequence content was the same. The array content from Laboratory 2, 3 and 7 resident arrays were very similar, and were all based on the same 15K NIA clone set. Laboratory 7 printed duplicate clones on their arrays. Laboratory 5 resident array was printed from ~17K NIA clones, with each clone printed twice. In all cases, except for the Affymetrix (mouse U74Av2) platform, a high proportion of the input sequences were mapped to NAP cluster sequences using the stringent criteria. In the case of the Affymetrix platform, a large number of sequences had unknown base content (NNNs), thus penalizing their score.

The results indicate that 502 common genes were identified by NAP cluster and 1005 common genes were identified by U cluster. The sequences that are mapped to a NAP cluster in each of the 12 microarray platforms (**Supplementary Table 3**). In the gene list file, the Genbank ID, Agilent probe ID, and Affymetrix probe ID are listed for each common sequence. If more than one sequence present on any given array mapped to the same NAP sequence, both IDs were listed and separated by a comma.

I. Standard Protocol for Gene Expression Data Preprocessing

Non-normalized intensity measurements obtained from the standardized image processing protocol (Dataset C) were used to generate four normalized datasets by applying: (1) Global Intensity Normalization, (2) Global Intensity Normalization with background adjustment, (3) Lowess Normalization with background adjustment applied to a $\log_2$-ratio versus $\log_2$-geometric-mean-intensity (R-I or M-A plot), and (4) Lowess Normalization without background subtraction applied to an R-I plot. Lowess and global gene expression data normalization were conducted on the two fluorescence intensity channels, both with and without background adjustment, for all dual-channel microarrays. For background subtraction, local median background values were subtracted from spot intensity values prior to normalization. Global median normalization (GMN) was performed for each microarray by subtracting the median log ratio across the microarray from the log ratio for each spot. Rank Invariant LOWESS (RIL) normalization was performed on each microarray using the lcDNA program (version 0.03) (Hyduke et al. Omics 7, 227-234, 2003). RIL uses a rank invariant method to identify genes with low likelihood of differential expression, and uses these genes to construct a normalization curve with LOWESS (locally weighted least-squares regression (Tseng et al., Nucleic Acids Res. 29, 2549-2557, 2001).

J. Standard Protocol for Statistical Analysis of Variance

To assess the contributions of different potential sources of variability, an ANOVA mixed model (Kerr was fit for each of the 502 genes represented on all the platforms. For each laboratory, a list of statistically significant up- or down-regulated genes was generated based on a pre-specified false discovery rate (FDR=0.05). The FDR is calculated in a step-up fashion for the mixed model[22] (Supplementary Information, Standard Protocols).

The specific ANOVA model has the form:

$$Y_{ijkltd} = \mu + P_i + C_j + A_{k(ij)} + T_t + D_d + TP_{ti} + TC_{tj} + e_{ijkltd}$$

where *Y* represents the observed $\log_2$ intensity measurements, and μ is the intercept (overall mean). *P,C,A, T, D* represent random effects for platform (*i=1,…,11*), laboratory (j=1,..,7), microarray (*k=1,…,4*, nested within laboratory and platform), tissue (*t*=1,2 for liver, pool), and dye (*d*=Cy3,Cy5), respectively. Concatenation of these symbols represents interactions of the constituent effects. Except for the intercept μ, all random effects are assumed to follow a normal distribution with mean 0. The unknown variance parameters of these distributions are the parameters of interest in the model, which we estimate. Across genes, the average of the variability estimates from each source was used to assess relative contribution from each source. To assess differential expression between the standard Liver and Pooled RNA samples, we fit a mixed model, as described under 'Sources of Variability,' but took the tissue effect T (liver or pooled) as a fixed effect. For each laboratory, a list of statistically significant up- or down-regulated genes was generated based on a pre-specified false discovery rate (FDR=0.05). The FDR is calculated in a step-up fashion[23] for the mixed model. Specifically, suppose the *n* p-values are sorted from the largest $p_{(n)}$ to the smallest $p_{(1)}$. *Then* FDR is calculated as

$FDR_{(n)}=p_{(n)}$

$FDR_{(n-i)} =min[FDR_{(n-i+1)}, np_{(n-i)}/(n-i)]$ $\quad i=1,\ldots n$

K. Standard Protocol for Scoring and Evaluating Gene Ontology Categories

For each array, the feature intensity values were rank ordered for each channel (Cy3 and Cy5) to identify the lowest 2% of values, which were used to calculate a mean and standard deviation. The mean of the lowest 2% of values plus one standard deviation was used to set a threshold background value, and features with intensity values below threshold in both channels were omitted from further analyses. Mean log ratio and standard error were then calculated for each laboratory/platform combination. For two-channel arrays, mean log ratios derived from at least two arrays with at least one dye-flip replicate pair were included in subsequent analyses. Significantly induced transcripts were selected based on a mean log ratio minus one standard error being greater than or equal to 1.0. Significantly repressed transcripts were selected based on a mean log ratio plus one standard error being less than or equal to -1.0. Array features were mapped to GenBank accessions for analysis with Expression Analysis Systematic Explorer (EASE) which was used essentially as described to determine enriched Gene Ontology nodes (Hosack et al., *Genome Biol.* 4, R60, 2003). Two-way hierarchical clustering of the laboratory/platforms and the common GO nodes was based on the calculated EASE scores and performed using Spotfire DecisionSite (v7.2, Sommerville, MA). Common GO nodes were defined as those nodes for which an EASE score was calculated for 20 of the 24 laboratory/platform combinations; R – 5- cDNA, C - 5, R - 3 - cDNA, and R – 2 – C2 were omitted from the identification of common GO nodes due to the small number of enriched categories, but were included in the hierarchical clustering of these data (Fig. 6 of paper). Furthermore, the GO node had to be scored as significant (EASE score < 0.05) for at least one of the laboratory/platform combinations.

Gene Ontology EASE scores for the 502 common genes are presented below.

Gene Category	C - 1	C - 2	C - 3	C - 4	C - 5	C - 6	D - 1	D - 2	D - 3
D - 4	D - 5	D - 6	D - 7	D - 8	R - 1 - cDNA	R - 2 - C2	R - 3 - cDNA		
R - 3 - C1	R - 3 - C2	R - 4 - oligo	R - 5 - cDNA	R - 6 - oligo	R - 7 – cDNA	R - 8 - C2			
organic acid metabolism	4.40248E-18	3.057E-15	0.00067499	1.90962E-09	0.547120235	1.64354E-10	0.000644859	0.000192719	4.31845E-06
5.0425E-06	3.07103E-11	0.033891153	0.001187236	8.26409E-07	5.05781E-07	1.6105E-05	0.000176761	0.025890987	2.608E-07
9.90793E-06	0.458511412	0.005146303	0.001479238	0.002817825					
carboxylic acid metabolism	1.11389E-17	9.17611E-15	0.00065245	5.61167E-09	0.545322449	1.46799E-10	0.000615563	0.000181282	3.93157E-06
4.73765E-06	2.73892E-11	0.032929127	0.001144384	7.53572E-07	1.26655E-06	1.6105E-05	0.00016973	0.035237676	2.608E-07
9.43655E-06	0.456375081	0.004875514	0.001932564	0.002817825					
lipid metabolism	3.70005E-12	7.75923E-09	0.009313315	1.36197E-05	0.064624742	3.70631E-07	0.001904806	0.007371335	0.000273132
5.71956E-05	0.002396641	0.022951506	0.01604868	0.00197092	3.24454E-05	0.000106809	0.021830358	0.007208962	9.679E-06
0.053828999	0.121330801	0.008480364	1.35268E-05	0.000664978					
organismal physiological process	1.76732E-07	3.3556E-10	0.000496488	8.35619E-08	0.013352981	1.80919E-11	7.78E-08	8.51473E-12	
1.77706E-10	3.09999E-10	1.85481E-08	5.06621E-08	4.08552E-06	9.31297E-09	0.000150461	0.078930373	0.606527448	7.08975E-08
0.000255473	2.36809E-06	1	3.9547E-07	0.234818374	1.13454E-06				

response to biotic stimulus 0.012278672 0.038972823 0.011854969 0.000678719
0.207919188 0.000151314 0.001679668 8.43331E-06 1.07045E-05
0.000458336 0.000214207 0.001314424 0.021501392 9.32242E-06
0.003931752 0.005136693 0.169183604 0.002096585 0.004674923
0.011372094 #N/A 0.005315402 0.14414949 0.006136019
amine catabolism 5.92761E-09 4.1445E-08 0.005077078 0.000409752
0.19873044 0.000118973 0.021938969 0.000407799 3.40656E-06
0.000667784 6.31256E-06 0.006532935 0.023911251 0.000405024
0.017577595 0.168079345 0.011077559 0.018823694 0.00027579
0.045772869 #N/A 0.125853686 0.001722616 0.045728507
"response to pest\, pathogen or parasite" 0.002867389 0.000504474 0.024812558
1.60246E-05 0.7373848 0.000368949 0.0000289 2.91021E-07
4.24568E-08 2.85273E-05 1.2095E-05 0.000450106 0.005683422
2.41678E-07 0.009596787 0.187567931 0.423621275 7.76699E-06
0.008758372 0.022337196 #N/A 0.000488052 0.400234682 0.036161542
response to external biotic stimulus 0.003138431 0.000546612 0.025453534
1.74055E-05 0.738988648 0.000395222 0.0000333 3.60664E-07
5.61989E-08 3.27635E-05 1.43723E-05 0.000507239 0.006126435
3.06688E-07 0.010854559 0.193891871 0.429202992 1.09604E-05
0.010159039 0.022963276 #N/A 0.0005236 0.444874238 0.041460124
lipid transport 1.5907E-07 2.69834E-06 0.0001458 0.019690213 0.002085167
7.54269E-05 0.000400661 0.004260762 0.000347638 0.000252539
0.000630286 0.002778198 0.128201629 0.001128921 1.73931E-05
0.024326537 0.198652241 0.006327846 0.001457942 3.26448E-06 1
0.079542531 0.010025021 0.034062323
amino acid and derivative metabolism 5.364E-12 9.94439E-12 0.007074941
2.44101E-06 0.361434101 3.22807E-08 0.029787667 0.000267276
1.17904E-05 0.000606925 4.66982E-09 0.04420884 0.013551895
2.88033E-05 0.00176966 0.010159508 0.000858792 0.006061017
5.81469E-06 0.006501917 1 0.089029938 0.147412661 0.000396813
steroid metabolism 1.07522E-12 2.47802E-08 0.004035387 8.6223E-06
0.011892673 4.63536E-06 0.002259417 0.003886281 0.002489279
0.000348183 0.002187551 0.003268821 0.08646001 0.002840431
0.000316871 0.320461982 1 0.000175615 0.000771425 0.251971988
#N/A 0.004327926 0.000464313 0.0237455
humoral immune response 0.002348703 2.6845E-06 0.001002025 4.16995E-05
1 8.54179E-05 0.003236596 9.35861E-06 2.81693E-06 0.000472982
0.000102607 0.015394968 0.006850113 9.07478E-05 0.00628378
0.19679573 1 6.63558E-07 0.005009554 0.002009161 #N/A
0.036627615 0.315753245 0.078738182
alcohol metabolism 5.37395E-09 2.20139E-06 0.011513541 5.38023E-05
0.249056153 1.24853E-07 0.005132556 0.01465716 0.009593655
0.006826291 0.000585132 0.00049772 0.719346878 0.007477034
0.004661415 0.26477882 1 0.000847918 1.72434E-05 0.061430707
#N/A 0.007919589 0.032272745 0.003388097
amino acid catabolism 6.30127E-08 2.22182E-08 0.002641749 0.000109247
1 0.000182726 0.01107543 0.00300189 0.000427453 0.000226807
8.23595E-06 0.013599801 0.013570967 0.000479626 0.007695223
0.138357534 0.007547776 0.053738928 0.00078991 0.029088573 #N/A
0.300573397 0.002511418 0.097482081

transport 2.12414E-05 6.86512E-08 0.015030623 0.004667104 0.034667516
0.000743724 0.063342899 0.029121111 0.013992435 0.029121231
0.047622484 0.026112175 0.185107069 0.006972402 0.004764894
0.141080636 0.00032698 0.001910087 0.007677373 0.007720579
0.208076216 0.000201388 0.122463006 0.032968395
immune response 0.014295208 0.014465555 0.142169196 0.001022253
0.431656789 0.000532343 0.000407828 3.5342E-06 5.28522E-06
0.000115639 0.000118604 0.000991193 0.009529742 3.25567E-06
0.069425682 0.219659541 0.636078427 0.000653183 0.04773115
0.014156449 #N/A 0.012897856 0.372764564 0.020166442
cation homeostasis 3.39227E-05 2.93596E-07 0.07905835 1.47996E-06 1
0.023314018 0.002908258 0.000684313 0.000551821 0.001900855
0.001494583 0.000982067 0.006311563 0.003529617 0.00177489 1
1 0.004633535 7.91456E-05 0.107774675 #N/A 0.557204174
0.00040186 7.88835E-06
cell ion homeostasis 0.00010628 9.4123E-07 0.093593645 3.58014E-06 1
0.033283561 0.004396407 0.001209833 0.001107965 0.002901094
0.002485937 0.001584779 0.008665245 0.005935463 0.003408976 1
1 0.007460716 2.28327E-05 0.126645602 #N/A 0.58612675
0.001542195 1.75694E-05
steroid biosynthesis 1.96964E-11 3.9764E-07 0.003466075 0.001344068
0.040939683 0.000120754 0.028206073 0.018170578 0.003390608
0.00571754 0.036183711 0.034987171 0.211682328 0.007262019
0.002304223 0.264404093 1 0.001570931 0.000911115 1 #N/A
0.079542531 0.001104938 0.006931504
coagulation 3.15403E-07 2.83667E-10 0.000917673 3.28864E-09 1
1.46223E-05 0.000189139 9.76345E-09 8.12916E-10 4.60629E-07
2.30713E-08 1.77243E-06 0.000303534 3.91421E-08 0.87185888 #N/A
#N/A 1.41594E-08 0.322047799 5.11636E-06 #N/A 0.000141193
0.551987815 0.02164015
blood coagulation 3.15403E-07 2.83667E-10 0.000917673 3.28864E-09 1
1.46223E-05 0.000189139 9.76345E-09 8.12916E-10 4.60629E-07
2.30713E-08 1.77243E-06 0.000303534 3.91421E-08 0.87185888 #N/A
#N/A 1.41594E-08 0.322047799 5.11636E-06 #N/A 0.000141193
0.551987815 0.02164015
hemostasis 1.27437E-06 1.78197E-10 0.001291168 9.71546E-09 1
3.04666E-05 0.000323624 2.95185E-08 3.46942E-09 9.99798E-07
6.50154E-08 3.77591E-06 0.00047171 1.22411E-07 0.883313963 #N/A
#N/A 1.0327E-07 0.373148527 8.84273E-06 #N/A 0.000234649
0.623667838 0.034062323
regulation of body fluids 3.29402E-06 5.13819E-10 0.00164015 2.06372E-08
1 5.06877E-05 0.000595786 1.03453E-07 1.77066E-08 2.42124E-06
2.10708E-07 8.93377E-06 0.000780734 4.43087E-07 0.893747377 #N/A
#N/A 2.52668E-07 0.389850334 1.29778E-05 #N/A 0.000322145
0.688605871 0.039011086
defense response 0.014532333 0.058707456 0.079991526 0.000414586
0.562216806 0.000545559 0.00132256 2.63032E-06 1.0196E-05
0.000370196 0.00018515 0.001775959 0.015491907 3.91246E-06
0.048696801 0.297573129 0.712800476 0.001284839 0.08020616
0.019404169 #N/A 0.005594073 0.24729593 0.009235172

amine metabolism 1.67025E-11 1.6143E-10 0.014367735 1.93759E-05
0.4125303 4.32634E-08 0.07260652 0.001762979 4.42813E-05
0.002588341 3.86316E-08 0.102134899 0.032322148 0.000153796
0.004245208 0.014111759 0.00125472 0.003943439 2.10415E-05
0.013889599 1 0.098161462 0.305275739 0.001223397
"di-\, tri-valent inorganic cation homeostasis" 0.001284835 2.67594E-05
0.168763272 0.000597675 1 0.057717653 0.000287012 0.000148276
0.000884953 0.000180022 0.000438272 0.000405001 0.001097552
0.000765861 0.000178817 1 1 0.004804657 4.47031E-05
0.207692198 #N/A 0.462177528 0.00590789 1.56622E-06
physiological process 8.32489E-07 3.84831E-05 0.002243478 0.000614036
0.027043526 0.029583432 3.48E-09 8.28137E-07 4.27806E-05
2.01558E-08 1.6511E-10 1.39918E-09 3.76989E-05 4.20731E-10
0.20882733 0.305367359 0.014593804 0.144477159 0.014256284
0.002255529 0.23795432 0.033128727 0.752608211 0.073248352
fatty acid metabolism 6.74322E-06 0.000470863 0.002404043 0.009257507
0.441658471 0.000688254 0.0105192 0.045283791 0.020193686
0.002150143 0.012531439 0.296931902 0.026964859 0.008430547
0.000554244 0.000211568 0.079117606 0.539829167 0.002455579
0.005089917 0.154020301 0.222109681 0.0051844 0.245676194
response to external stimulus 0.000788883 0.001655525 0.08736648
1.55634E-05 0.706171701 8.57937E-05 0.0003298 1.48374E-05
2.38367E-06 0.000173514 0.001569142 0.005352664 0.054226422
0.000101938 9.28293E-05 0.465383426 0.721201873 7.48576E-05
0.017794206 0.060840846 #N/A 0.050117764 0.200609425 0.026156028
amino acid metabolism 5.60347E-10 5.26311E-09 0.048925011 6.69951E-06
1 2.36319E-06 0.052150653 0.009892931 0.002746194 0.002851974
2.05432E-07 0.126426283 0.018810715 0.000687322 0.003373491
0.027214842 0.00274221 0.109768905 0.000104479 0.014050352 #N/A
0.109676567 0.177279845 0.002426218
transition metal ion homeostasis 0.123315552 0.00340283 0.054409089
0.016117217 1 0.295723874 0.001788661 0.001513246 0.002299379
0.000146256 0.001050905 0.002239955 0.023327553 0.000745408
0.023059242 1 1 0.049101626 0.00078991 0.355960741 #N/A
0.152065822 0.02145439 0.003894737
amino acid derivative metabolism 6.8938E-05 4.98636E-06 0.015658964
0.013247308 0.263339531 1.27723E-05 0.073106732 0.000307854
5.17426E-05 0.017806411 0.000732633 0.029917671 0.196978302
0.001691277 0.028234209 0.031225814 0.002025306 0.002074638
0.00051352 0.098788579 1 0.557204174 0.172833252 0.004116023
one-carbon compound metabolism 0.000294573 0.007324816 0.018730035
0.003321155 #N/A 0.004783818 0.007363466 0.103399882 0.014628636
0.005534411 0.001112517 0.001657645 0.578620831 0.005297444
0.002911806 0.168079345 1 0.014969836 0.252571365 0.026689942
#N/A 0.074607961 0.015885591 0.157663885
lipid biosynthesis 4.56522E-10 4.11698E-07 0.001493218 0.000261373
0.052339276 2.37609E-05 0.173447011 0.036490217 0.00167343
0.013984466 0.221720627 0.06513145 0.421595662 0.007111345
0.025994427 0.044231985 0.492203021 0.006717586 0.008758372
0.372483118 #N/A 0.001893543 0.001011262 0.036161542

catabolism 0.012123006 0.000108431 0.002149199 0.000776847 0.985177851
0.046025624 0.003701029 0.00774036 0.005784935 0.000277108
0.00058567 0.000435189 0.039267184 0.010545797 0.105153862
0.48937065 0.627497404 0.340555693 0.000590695 0.219833153 #N/A
0.319666303 0.797376741 0.102256868
aromatic compound metabolism 0.000390764 7.4658E-06 1 0.009696775
1 0.00157942 0.005380244 0.002557575 0.000506058 0.000224048
2.75054E-05 0.057405359 0.073342154 0.029988217 0.180153143 1
1 0.041185398 0.001607365 0.019933692 #N/A 0.033581957
0.92787817 0.407115071
electron transport 0.000258796 1.97178E-05 0.000384893 0.000351775
0.011642302 0.002399315 0.011282632 0.019733119 0.0005724
0.002476586 1.33018E-05 0.005935285 0.041011202 0.00370442
0.800400036 0.828392249 1 0.053978583 0.125565164 0.047223675
#N/A 0.120130245 0.702164099 0.956075672
organelle organization and biogenesis 0.002244749 0.054607273 0.721855845
0.326637989 0.853708654 0.090635643 0.033619008 0.01835371
0.000896459 0.13298227 0.012611067 0.002825915 0.004827774
0.000151747 0.027468547 0.138874846 0.213040002 0.00020328
0.263530163 0.020314149 0.449918795 0.416247326 0.032963471
0.001085414
acute-phase response 0.026561033 0.010611188 0.291934677 0.011020024
#N/A 0.070374714 0.0000284 3.61523E-05 1.20961E-05 0.001633756
2.17658E-05 0.000365626 0.484360193 1.98011E-07 0.011392826 1
1 9.19086E-05 0.172283531 0.326960853 #N/A 2.13807E-05
0.795851259 1
amine biosynthesis 0.000556598 2.64082E-05 0.05334435 0.001606998 #N/A
0.002561029 0.05201226 0.000565369 0.000268444 0.040748444
1.94886E-06 0.062138446 0.161463031 0.000175025 0.001089032
0.264404093 1 0.097480346 0.000911115 0.256059634 #N/A
0.785107827 0.348228976 0.001623245
cell growth and/or maintenance 0.011044933 0.078681331 0.44772617
0.419971627 0.315329331 0.221261242 0.000664519 0.005152099
0.002340802 0.021680667 0.011288901 0.000106071 0.033715528
2.90955E-05 0.185339077 0.664745279 0.00437927 0.015498998
0.391910001 0.124080869 0.075018335 0.098482098 0.047467555
0.00278307
endocytosis 0.000390764 0.009643123 0.398232871 0.003055059 1
0.005163439 0.390372392 0.038221906 0.016480419 0.074772983
0.025926613 0.005784633 0.365951761 0.023184132 0.037551144 #N/A
1 0.001446072 0.128033566 0.466452631 #N/A 0.02417421
0.112083812 0.085475279
cytoskeleton organization and biogenesis 0.013385253 0.162832429 0.729567144
0.231939747 0.792025776 0.024032803 0.053047441 0.013460386
0.002897608 0.147565295 0.031676393 0.00364481 0.003230083
0.000568995 0.045141404 0.474099978 0.347360325 4.56792E-06
0.218714603 0.025735824 1 0.518129084 0.070107305 0.000797851
"complement activation\, alternative pathway" 0.006298198 0.000529049
0.000730741 0.011648012 1 0.014249401 0.006910601 0.000152464
0.00097313 0.000392559 0.000109173 0.068292447 0.250085886

0.000400185 0.243876132 1 #N/A 0.094504903 0.132205902
0.00108442 #N/A 1 0.653235577 0.185292447
sulfur amino acid metabolism 0.000942494 0.000111679 0.006947715
0.00328218 #N/A 0.024417287 0.232594977 0.008715616 0.010771611
0.210088115 0.006504854 0.250590347 0.127819218 0.004264863
0.000235295 0.011565693 0.008656476 0.417190823 0.000149106 1
#N/A 0.535978374 0.110773743 0.000130062
amino acid biosynthesis 9.2798E-05 8.04827E-05 0.116519517 0.003946756
#N/A 0.005666331 0.156730345 0.000598902 0.000427453 0.135448727
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0.210787349 1 0.08885262 0.00119089 0.494603863 #N/A
0.64091743 0.10206921 0.001445663
cellular physiological process 0.012661539 0.162112686 0.492385551
0.671591253 0.264431171 0.224965171 0.002433923 0.008014506
0.011439246 0.023495687 0.021766131 0.000155053 0.104997355
4.55276E-05 0.074897861 0.576008979 0.008364018 0.011542715
0.391278645 0.19774569 0.132476289 0.153231991 0.049242717
0.000885693
humoral defense mechanism (sensu Vertebrata) 0.000631337 3.98667E-06
0.00016022 5.5982E-06 1 0.000573901 0.310766319 0.000854835
0.000375121 0.085763621 0.003281402 0.332751994 0.540532693
0.002805018 0.052275568 #N/A 1 1.4876E-05 0.103307377
0.000301676 #N/A 0.300573397 0.394849159 0.559731348
carbohydrate catabolism 0.002881105 0.012274113 0.022688648 0.007279679
#N/A 0.011141806 0.035313684 0.062853867 0.077351883 0.078553248
0.016630481 0.043575539 0.81768482 0.028086237 0.085317883 #N/A
#N/A 0.234723329 0.08228236 1 #N/A 0.016811197 0.694337889
0.028679538
energy pathways 0.00691012 0.000404162 0.444746214 0.012828169 #N/A
0.16112436 0.001459117 0.003008264 0.008489207 0.002269932
0.003641662 0.00623057 0.74168128 0.00087355 0.04863038 1
#N/A 0.360081182 0.064272208 0.775142605 #N/A 0.672158901
0.419154395 0.158815622
cholesterol metabolism 1.69039E-06 0.00041061 0.032566899 0.002154681
0.19873044 0.000671203 0.077601803 0.0828968 0.055070365
0.063600914 0.173588706 0.089736928 0.675015164 0.064506862
0.05619972 0.203821659 1 0.006327846 0.00096296 0.19178116
#N/A 0.015181597 0.009321905 0.025386085
sulfur metabolism 0.000407038 0.000169309 0.01782334 0.016190373 1
0.322975084 0.195075969 0.04580313 0.114472232 0.16492642
0.033201915 0.220153289 0.07007962 0.01844928 8.39943E-05
0.005251295 0.002025306 0.758149841 0.000468986 0.689056417 1
0.110324559 0.233031432 0.004655242
alcohol catabolism 0.003522919 0.003514952 0.012736509 0.002465474 #N/A
0.014024056 0.015465387 0.072358595 0.144133327 0.040748444
0.018715321 0.019421833 0.756553445 0.066841434 0.076109208 #N/A
#N/A 0.300516363 0.069503971 1 #N/A 0.102184906 0.589158859
0.02211341
hexose catabolism 0.003522919 0.003514952 0.012736509 0.002465474 #N/A
0.014024056 0.015465387 0.072358595 0.144133327 0.040748444
0.018715321 0.019421833 0.756553445 0.066841434 0.076109208 #N/A

#N/A 0.300516363 0.069503971 1 #N/A 0.102184906 0.589158859 0.02211341
monosaccharide catabolism 0.003522919 0.003514952 0.012736509 0.002465474 #N/A 0.014024056 0.015465387 0.072358595 0.144133327 0.040748444 0.018715321 0.019421833 0.756553445 0.066841434 0.076109208 #N/A #N/A 0.300516363 0.069503971 1 #N/A 0.102184906 0.589158859 0.02211341
cytoplasm organization and biogenesis 0.002217662 0.132355454 0.75832582 0.597529629 0.90824086 0.29067647 0.00000752 0.000313953 0.000147221 0.070871425 1.4277E-05 2.56722E-07 0.004999708 1.29024E-08 0.139392363 0.258170834 0.386397158 0.02251709 0.397023436 0.083898255 0.568116079 0.45078663 0.481894363 4.82257E-05
energy derivation by oxidation of organic compounds 0.001273135 3.53954E-05 0.347353385 0.003416158 #N/A 0.074220132 0.001046201 0.001537799 0.006919684 0.001809057 0.002058162 0.001658567 0.643617605 0.00029543 0.088800939 #N/A #N/A 0.300656403 0.169763154 0.694788944 #N/A 0.507937213 0.542012568 0.084487193
cell adhesion 0.017894328 0.55834645 0.289938159 0.024094343 0.926291963 0.930711327 0.552754345 0.019331618 0.001126444 0.430403725 0.12416715 0.075613058 0.105465192 0.002265288 0.000917155 1 1 6.39211E-05 0.027916076 0.447903881 #N/A 7.58843E-06 0.084887394 0.036635875
glycolysis 0.005220223 0.011177519 0.039751036 0.003426068 #N/A 0.0196096 0.026699125 0.098019259 0.155718463 0.073790912 0.025700145 0.032331879 1 0.078649 0.097384148 #N/A #N/A 0.237689195 0.136793444 1 #N/A 0.225510748 0.67883861 0.008644277
biosynthesis 0.000123017 0.001898858 0.426862824 0.092654657 0.860100253 0.009314034 0.0000472 0.023884154 0.012601439 0.053061134 4.2476E-05 0.000200972 0.321757829 7.44662E-06 0.503197894 0.590136796 0.523595243 0.966131829 0.376638189 0.885077752 1 0.127040302 0.979579138 0.372626833
cell organization and biogenesis 0.04917068 0.518297765 0.888524663 0.752181702 0.975208376 0.482709416 0.001076629 0.007128513 0.003316961 0.25805749 0.000325828 3.93589E-05 0.032021893 3.99617E-06 0.222087871 0.573243217 0.428661762 0.060930597 0.638753788 0.228050984 0.344650434 0.784508723 0.306178822 0.000719352
biogenic amine metabolism 0.002547016 0.001800346 0.50853766 0.284788306 0.19873044 0.00327049 0.220447045 0.001927919 0.001792566 0.063600914 0.020483378 0.089736928 0.303735829 0.022489145 0.454466675 0.168079345 0.150897484 0.014672669 0.06998688 0.19178116 1 0.427945322 0.589364055 0.157663885
main pathways of carbohydrate metabolism 0.005940977 0.002380712 0.179621011 0.004999685 #N/A 0.055948844 0.006341602 0.053902184 0.055499594 0.037755582 0.005375043 0.002728137 0.67388873 0.015320357 0.226873679 #N/A #N/A 0.587145263 0.128033566 0.817207154 #N/A 0.483917678 0.463428051 0.085475279
aromatic amino acid family metabolism 0.006684714 0.001866074 1 0.05884326 #N/A 0.014188853 0.036731015 0.295989258 0.018092133

0.004466943 0.016025931 0.553877406 0.091036937 0.143381997
0.372509207 1 1 0.087982078 0.028148512 0.326960853 #N/A
0.188194011 0.95838381 0.054601027
response to stress 0.322885148 0.233892411 0.137420204 0.050968204
0.563633452 0.07860755 0.013240282 0.003721494 0.001284622
0.025522679 0.064357304 0.060866373 0.199422674 0.004357569
0.031527453 0.011201008 0.34388601 0.097397196 0.022608625
0.177563478 1 0.085939372 0.687689467 0.524359449
nitrogen metabolism 0.003952889 0.000447674 0.381004998 0.007260598 1
0.15096692 0.27954377 0.060204562 0.180072039 0.253699949
0.002160205 0.300048088 0.513253383 0.008372975 0.023059242 #N/A
#N/A 0.261216846 0.024246083 0.423135699 #N/A 0.602128066
0.338402744 0.060300836
carboxylic acid transport 0.000318286 0.017136286 1 0.019690213 #N/A
0.20238837 0.870741375 0.160591802 0.008849055 0.851632547
0.050610248 0.351854164 0.74941502 0.023114918 0.067275948 #N/A
1 0.00344246 0.096401942 0.612177865 #N/A 0.125853686
0.315753245 0.018273269
organic acid transport 0.000318286 0.017136286 1 0.019690213 #N/A
0.20238837 0.870741375 0.160591802 0.008849055 0.851632547
0.050610248 0.351854164 0.74941502 0.023114918 0.067275948 #N/A
1 0.00344246 0.096401942 0.612177865 #N/A 0.125853686
0.315753245 0.018273269
hexose metabolism 0.01193906 0.025495447 0.070184054 0.021533493 #N/A
0.012747922 0.159903918 0.27886851 0.091794268 0.257207555
0.02524679 0.038633784 0.921304453 0.058710888 0.240491895 #N/A
#N/A 0.168397115 0.331460742 0.263723264 #N/A 0.384983805
0.313221507 0.234561308
monosaccharide metabolism 0.017383402 0.03273277 0.077860067
0.026541009 #N/A 0.016156066 0.178135753 0.307850559 0.11096642
0.279642139 0.031001428 0.04568752 0.927864986 0.071335969
0.240491895 #N/A #N/A 0.197093128 0.342097394 0.281472058 #N/A
0.396450017 0.35615737 0.245676194
vesicle-mediated transport 4.43681E-05 0.010672183 0.772599947 0.06655369
1 0.055309973 0.2231028 0.031484933 0.078363623 0.170972978
0.065753444 0.048471726 0.477142354 0.02673566 0.252212384 #N/A
1 0.02494796 0.200854429 0.833648881 #N/A 0.100969879
0.744438741 0.106068625
glucose catabolism 0.007896855 0.009758968 0.059329629 0.008544383 #N/A
0.040021385 0.048282988 0.160591802 0.256076028 0.115352001
0.050610248 0.057779608 1 0.140407275 0.164852564 #N/A #N/A
0.422236931 0.063529938 1 #N/A 0.252492143 0.672165083
0.0192469
response to xenobiotic stimulus 0.005963518 0.030971999 1 0.068040922
#N/A 0.234731641 0.014157144 0.056706729 0.03276189 0.002074353
0.044168479 0.065313507 0.261396762 0.112458551 0.154571204 #N/A
#N/A 0.580552211 0.11850693 1 #N/A 0.707550671 0.431729194
0.588857168
xenobiotic metabolism 0.004585547 0.026687617 1 0.061602163 #N/A
0.220174341 0.012552384 0.05102524 0.028318946 0.001775978
0.039616485 0.059828881 0.25079934 0.10225993 0.154571204 #N/A

#N/A 0.544843299 0.103307377 1 #N/A 0.692147792 0.431729194
1
glutamine family amino acid metabolism 0.054555285 0.004452925 0.331569492
0.285522706 1 0.689946581 0.624372139 0.046286262 0.049058109
0.598750645 0.001355169 0.267090882 0.484360193 0.005415769
0.083987212 1 #N/A 0.593964055 0.029296261 0.369991011 #N/A
1 0.895454468 0.641469153
urea cycle intermediate metabolism 0.056819155 0.013240824 0.125558149
0.00380441 1 1 1 0.062135378 0.124098432 1
0.000300469 0.269282652 1 0.000863601 0.311181328 #N/A #N/A
0.719153381 0.011973015 0.142630839 #N/A 1 0.543908972
0.023855973
neurogenesis 0.033968432 0.029410346 0.506962238 0.341042699 0.838486848
0.020895451 0.923418433 0.565999339 0.352717148 0.936231308
0.86968706 0.546665096 0.695173817 0.507061116 0.459750753
0.20373002 #N/A 0.03436457 0.069305978 0.786413204 1
0.769963677 0.004912329 0.026156028
ion transport 0.002262559 0.005665745 0.962970669 0.082212987 0.725100806
0.001386432 0.984970841 0.941541226 0.684821282 0.992855385
0.999490913 0.968620755 0.93900067 0.911019285 0.047107906 1
0.169183604 0.003354089 0.236997661 0.903617324 #N/A 0.023886573
0.15032353 0.377415631
aromatic compound catabolism 0.003995128 0.00803106 1 0.03310825
#N/A 0.037989875 0.225255194 1 0.094060423 0.020391209
0.040593769 1 1 0.369112682 1 #N/A #N/A 0.261196561
1 1 #N/A 1 1 1
aromatic amino acid family catabolism 0.003995128 0.00803106 1
0.03310825 #N/A 0.037989875 0.225255194 1 0.094060423
0.020391209 0.040593769 1 1 0.369112682 1 #N/A #N/A
0.261196561 1 1 #N/A 1 1 1
transmission of nerve impulse 0.00276931 0.001829457 0.762633961
0.109499901 0.579975725 0.004828265 0.202606467 0.146478487
0.230054191 0.154011705 0.260462001 0.146894845 0.154954029
0.367286049 0.038995377 1 #N/A 0.318886492 0.034050092
0.09369253 #N/A 0.108799605 0.36910967 0.057772207
neuromuscular physiological process 0.00276931 0.001829457 0.762633961
0.109499901 0.579975725 0.004828265 0.202606467 0.146478487
0.230054191 0.154011705 0.260462001 0.146894845 0.154954029
0.367286049 0.038995377 1 #N/A 0.318886492 0.034050092
0.09369253 #N/A 0.108799605 0.36910967 0.057772207
glucose metabolism 0.005703228 0.012918207 0.129855131 0.017108271 #N/A
0.00879203 0.166131187 0.244838454 0.170754603 0.289246468
0.041590298 0.085572165 1 0.071404326 0.371527178 #N/A #N/A
0.069721559 0.17928296 0.422212103 #N/A 0.470965874 0.345980109
0.093928314
phenylalanine catabolism 0.00074752 0.013240824 1 0.00380441 #N/A
0.322961123 0.288463411 1 0.156013164 0.036152913 0.007973303
1 1 0.458943331 0.170012653 #N/A #N/A 0.392772327
0.132205902 1 #N/A 1 0.653235577 1
tyrosine catabolism 0.008901472 0.033144476 1 0.014163994 #N/A
0.199752629 0.191580643 1 0.066574357 0.013950567 0.028095659

1 1 0.318758049 1 #N/A #N/A 0.194598565 1 1
#N/A 1 1 1
cation transport 0.030515632 0.0150286 0.911432091 0.189222741 1
0.006246469 0.995451743 0.946775635 0.898720309 0.997111684
0.99989045 0.961387236 0.987152259 0.911907325 0.35306125 1
0.272692799 0.012956041 0.417931846 0.872247793 #N/A 0.027377008
0.268380117 0.696885901
coenzyme metabolism 0.002543497 0.002186539 0.259051995 0.018103027
0.428006328 0.238624934 0.251187494 0.271411091 0.567915674
0.364337262 0.118636681 0.464451324 0.163498297 0.220188506
0.051448644 0.024343644 0.096431387 0.398645272 0.04719228
0.329198077 0.170046229 0.137423788 0.444874238 0.338895157
coenzyme and prosthetic group metabolism 0.001273135 0.002137153
0.347353385 0.049811861 0.483856122 0.141439863 0.383284391
0.436209153 0.51244106 0.502254258 0.136499652 0.609409189
0.254054912 0.393981877 0.070156806 0.03839626 0.140345207
0.335270907 0.04394022 0.429665782 0.2073598 0.058441766
0.620106828 0.339699286
cell-cell signaling 0.08530092 0.044719173 0.848892768 0.251865275
0.773334557 0.043478438 0.409333043 0.410993316 0.570085121
0.321599026 0.615604862 0.479754114 0.326197404 0.820834356
0.182173529 0.222666691 #N/A 0.702463403 0.045005162 0.284131652
#N/A 0.084006698 0.178802963 0.035376308
heterocycle metabolism 0.039698642 0.008462348 1 0.907522697 #N/A
0.101590054 0.345901063 0.355255376 0.287537814 0.307832082
0.0062491 0.376037895 0.416702518 0.510493291 0.295629303 1
1 0.099372086 0.23020825 0.264163804 #N/A 0.022399437
0.92787817 0.42776772
cell motility 0.217337813 0.700617747 0.771550684 0.629463059 1
0.038851521 0.569953248 0.251975417 0.244624338 0.627489917
0.583917986 0.05970254 0.875162376 0.135476642 0.002377214 1
#N/A 0.003509743 0.560040261 0.681581517 #N/A 0.631818182
0.090473269 0.023535967
pregnancy 0.410029732 0.14385881 0.040406442 0.584557153 1
0.234240567 0.048284967 0.115761159 0.263343032 0.040711048
0.09871951 0.217716468 0.109010323 0.180161596 1 #N/A #N/A
0.553042024 1 1 #N/A 0.04237452 0.783742983 1
metal ion transport 0.080962039 0.027881656 0.802800329 0.237743072 1
0.009883526 0.984158468 0.912537104 0.929489753 0.974131769
0.996852177 0.93796848 0.937006458 0.971374904 0.255227093 1
1 0.007519012 0.406346421 0.86994912 #N/A 0.022916654
0.084873772 0.310306579
organic acid biosynthesis 0.049878867 0.043050101 0.044949095 0.158990192
1 0.191111259 0.539764237 0.258635794 0.085018428 0.233448528
0.437309629 0.291143662 0.710469809 0.094391567 0.795406059
0.182561106 #N/A 0.928882776 0.663475371 0.231793858 #N/A
0.072616095 0.226178679 0.792772883
carboxylic acid biosynthesis 0.049878867 0.043050101 0.044949095 0.158990192
1 0.191111259 0.539764237 0.258635794 0.085018428 0.233448528
0.437309629 0.291143662 0.710469809 0.094391567 0.795406059

0.182561106 #N/A 0.928882776 0.663475371 0.231793858 #N/A
0.072616095 0.226178679 0.792772883
amine transport 0.007930291 0.065921856 1 0.036440677 #N/A
0.138174585 1 0.231968275 0.155718463 1 0.196520985
0.541879777 1 0.078649 0.221381109 #N/A 1 0.034235475
0.287399534 0.557257128 #N/A 0.31928835 0.548425319 0.061047625
group transfer coenzyme metabolism 0.041607096 0.026687617 1
0.061602163 #N/A 0.021186576 0.774827604 0.631659929 0.617092421
1 0.313481868 0.792116599 1 0.257096431 0.361364644 1
1 0.106580208 1 1 #N/A 0.262935778 0.619736344
0.819335538
macromolecule metabolism 0.733185934 0.875311474 0.486245923 0.847167633
0.977826398 0.94013788 0.005880827 0.077580639 0.372346515
0.311058613 0.067249746 0.00088844 0.611538379 0.004548479
0.999698142 0.966097958 0.998830809 0.994344201 0.970231684
0.968631868 0.991870824 0.983564865 0.996344017 0.69861392
regulation of neurotransmitter levels 0.049671521 0.002143518 1 0.798724343
1 0.234731641 0.402079949 0.319682069 0.576317155 0.369328814
0.282226626 0.427533756 0.240214491 0.711919129 0.567962061 #N/A
#N/A 0.507620111 0.530998566 0.494603863 #N/A 0.337881574
0.991528786 1
respiratory gaseous exchange 0.143262375 0.558428178 1 0.086988298
1 0.427232696 0.099675444 0.182415841 0.326455184 0.088682276
0.03302182 0.108671007 0.050875388 0.248912204 0.47930467 #N/A
#N/A 0.270494443 1 1 #N/A 0.335859513 0.904944315
0.421152381
proteolysis and peptidolysis 0.751266306 0.38744472 0.208411719 0.185845221
#N/A 0.77543148 0.21150947 0.323834323 0.355312808 0.08671843
0.241329077 0.040694417 0.217111531 0.254755317 0.915189906 1
1 0.788098299 0.277274758 0.361380081 #N/A 0.792572422
0.998380529 0.822421636
angiogenesis 0.913748143 0.750780004 0.23813746 0.714245976 #N/A
0.752367993 0.664088844 0.224021431 0.019926791 0.883188863
0.360039869 0.423767489 0.789317524 0.100955571 0.84547492 1
#N/A 0.779966566 0.780514436 0.660316141 1 0.457870276
0.807968891 0.157280897
prostaglandin metabolism 1 0.558428178 0.014579511 1 1 1
0.346525517 0.462813042 1 0.32751904 0.439030336 0.361254327
0.250085886 0.535997256 1 1 1 1 1 0.197380842
1 1 0.368004212 1
prostanoid metabolism 1 0.558428178 0.014579511 1 1 1
0.346525517 0.462813042 1 0.32751904 0.439030336 0.361254327
0.250085886 0.535997256 1 1 1 1 1 0.197380842
1 1 0.368004212 1
learning and/or memory 0.066752019 0.025141514 0.344284604 0.304478059
0.12333252 0.113538321 1 1 0.904156828 1 1 1
1 1 0.102265603 1 #N/A 0.429490404 1 1 #N/A
1 0.113286499 1
intracellular signaling cascade 0.996280098 0.960326734 0.999936141
0.752187663 #N/A 0.868020714 0.935189819 0.980457386 0.773771513
0.966934091 0.997328516 0.942233481 0.718904968 0.940002254

0.490660584 #N/A 1 0.363573837 0.974961997 0.99950703
0.632257343 0.992585451 0.001462154 0.499032888

II. RESIDNT (IN-HOUSE) PROTOCOLS

Summary of Resident Labeling and Hybridization Protocols *

Laboratory	1	2	3	4	5	6	7
Array Type	oligo	cDNA	cDNA	oligo	cDNA	oligo	cDNA
RNA quantity	10 µg total RNA or 2 µg mRNA	10 µg total RNA	10 µg total RNA	35 ug total RNA	10 µg total RNA	40 µg total mRNA	2 µg mRNA
Labeling method	Indirect Aminoallyl	Indirect Aminoallyl	Direct	Direct	Indirect aminoallyl	Direct	Direct
Priming	Oligo dT	Random hexamer	Oligo dT	Oligo dT	Oligo dT	Oligo dT	Random 9mers
Primer Source	Life technology Cat#18064-018	In house	in house	Amersham Pharmacia cat#27-7858-01	not stated	Qiagen Operon custom set	multiple sources stated
Nucleotide Labeling	dUTP	dUTP	dUTP	dUTP	dUTP	dUTP	dCTP
Label ratio	2nM aa-dUTP: 3nM dUTP	200 uM aa-dUTP; 300 uM dUTP	1.33nM Cy*-dUTP: 0.2 nM dTTP	1 Cy-dUTP:4 dTTP	1nM aa-dUTP: 1 nM dUTP	1:1 or 2.46 nM each	1nM Cy*-dCTP: 1 nM dCTP
Labels Used	Cy3, Cy 5 ester	NHS-Cy3, NHS-Cy5	Cy3-dUTP Cy5-dUTP	Cy3-dUTP Cy5-dUTP	Cy3 Cy5 ester	Cy3-dUTP Cy5-dUTP	Cy3-dCTP, Cy5-dCTP(?)
Cy3	Amersham Pharmacia Cat#PA23001	Amersham Pharmacia Ca#PA23001	NEN	Amersham Pharmacia Cat#PA23001	Amersham Pharmacia	Amersham Pharmacia Cat#PA53023	Amersham Pharmacia Cat#PA53023
Cy5	Amersham Pharmacia Cat#PA25001	Amersham Pharmacia Ca#PA25001	NEN	Amersham Pharmacia Cat#PA25001	Amersham Pharmacia	Amersham Pharmacia cat#PA55023	Amersham Pharmacia Cat#PA55023
RT temp	42^0 C	42^0 C	42^0 C	42^0 C	37^0 C*	42^0 C	42^0 C
RT time	2 hr- overnight	2 hours	2 hr	90 min	2 hr to overnight	2 hr	2 - 3 hours

* Array type refers to resident non-commercial arrays used across the seven laboratories, as described in Figs. 1 and Methods of the paper; RT temp = reaction temperature; RT time = reaction time.

Summary of Resident Scanning and Image Analysis Protocols*

Lab	Array Type*	Probe Source	Scanner	Image Acquisition and Analysis Software
1	Spotted Oligo	TIGR	Axon 4000B	GenePix 3.0
2	Spotted cDNA	NIA	Axon 4000B	GenePix 3.0
	Commercial Oligo	Agilent	Axon 4000B	GenePix 3.0
	Commercial Oligo	Affymetrix	2500 Microarray Scanner	MAS 5.0
3	Spotted cDNA	NIA	API “e” CCD/white light scanner	API ArrayWoRx “e”, Molecularware, GenePix 3.0
	Commercial Oligo	Agilent	API “e” CCD/white light scanner	API ArrayWoRx “e”, Molecularware, GenePix 3.0
	Commercial Oligo	Affymetrix	2500 Microarray Scanner	MAS 5.0
4	Spotted Oligo	Operon	Axon 4000, Agilent G2505B	GenePix 3.0, Agilent feature extraction software, Iplab's ArraySuite 2.0
5	Spotted cDNA	NIA	ScanArray 5000, ScanArray "Lite"	ScanArray 3.1,QuantArray
6	Spotted Oligo	Compugen	Axon 4000, Agilent G2505B	GenePix 3.0 & 4.0
7	Spotted cDNA	NIA	ScanArray 5000XL	Spot-On (In-house version), Spot 2.0, and QuantArray 3.0
	Commercial Oligo	Amersham	ScanArray 5000XL	Code Link Expression Analysis

* Array type refers to resident arrays (non-commercial and commercial) used across the seven laboratories, as depicted in Figs. 1 and 4 of the paper.

Toxicogenomics Research Consortium – Laboratory 1
Resident (In-House) Protocol for:
RNA LABELING and HYBRIDIZATION FOR MICROARRAYS

Aminoallyl RNA Labeling

1. PURPOSE
This protocol describes the labeling of eukaryotic RNA with aminoallyl-labeled nucleotides via first strand cDNA synthesis followed by a coupling of the aminoallyl groups to either Cyanine 3 or 5 (Cy 3/Cy5) fluorescent molecules.

2. SCOPE
This procedural format is utilized by Laboratory 1 for oligonucleotide spotted microarrays.

3. MATERIALS
3.1 5-(3-aminoallyl)-2'deoxyuridine-5'-triphosphate (AA-dUTP) (Sigma; Cat # A0410)
3.2 100 mM dNTP Set PCR grade (Life Technologies; Cat # 10297-018)
3.3 Random Hexamer primers (3mg/mL) (Life Technologies; Cat # 48190-011)
3.4 SuperScript II RT (200U/□L) (Life Technologies; Cat # 18064-014)
3.5 Cy-3 ester (AmershamPharmacia; Cat # PA23001)
3.6 Cy-5 ester (AmershamPharmacia; Cat # PA25001)
3.7 QIAquick PCR Purification Kit (Qiagen; Cat # 28106)
3.8 RNeasy® Mini Kit (Qiagen; Cat # 74106)

4. REAGENT PREPARATION
4.1 Phosphate Buffers (make or purchase as 1M solution, pre-made)
4.1.1 Prepare 2 solutions: 1M K_2HPO_4 and 1M KH_2PO_4

4.1.2 To make a 1M Phosphate buffer (KPO_4, pH 8.5-8.7) combine:
1M K_2HPO_4……..9.5 mL
1M KH_2PO_4……..0.5 mL

4.1.3 For 100 mL Phosphate wash buffer (5 mM KPO_4, pH 8.0, 80% EtOH) mix:
1 M KPO_4 pH 8.5…. 0.5 mL
MilliQ water…….. 15.25 mL
95% ethanol…….. 84.25 mL
Note: Wash buffer will be slightly cloudy.
4.1.4 Phosphate elution buffer is made by diluting 1 M KPO_4, pH 8.5 to 4 mM with MilliQ water.

4.2 Aminoallyl dUTP
4.2.1 For a final concentration of 100mM add 19.1 µL of 0.1 M KPO_4 buffer (pH 7.5) to a stock vial containing 1 mg of aa-dUTP. Gently vortex to mix and transfer the aa-dUTP solution into a new microfuge tube. Store at –20°C.

4.2.2 Measure the concentration of the aa-dUTP solution by diluting an aliquot 1:5000 in 0.1 M KPO_4 (pH 7.5) and measuring the OD_{289}.
(Stock concentration in mM = OD_{289} x 704)

4.3 Labeling Mix (50X) with 2:3 aa-dUTP: dTTP ratio

4.3.1 Mix the following reagents:
Final concentration
dATP (100 mM)……5µL….. (25 mM)
dCTP (100 mM)……5µL….. (25 mM)
dGTP (100 mM)……5µL….. (25 mM)
dTTP (100 mM)……3µL……(15 mM)
aa-dUTP (100 mM)…..2µL……(10 mM)
Total: 20µL
Store unused solution at –20°C.

4.4 Sodium Carbonate Buffer (Na_2CO_3): 1M, pH 9.0
4.4.1 Dissolve 10.8 g Na_2CO_3 in 80 mL of MilliQ water and adjust pH to 9.0 with 12 N HCl; bring volume up to 100 mL with MilliQ water.

4.4.2 To make a 0.1 M solution for the dye coupling reaction dilute 1:10 with water. Note: Carbonate buffer changes composition over time; make it fresh every couple of weeks to a month.

4.5 Cy-dye esters
4.5.1 Cy3-ester and Cy5-ester are provided as a dried product in 5 tubes.
Resuspend a tube of dye ester in 73 µL of DMSO before use.

4.5.2 Wrap all reaction tubes with foil and keep covered as much as possible in order to prevent photobleaching of the dyes.
Note: Dye esters must either be used immediately or aliquoted and stored at –80°C. Any introduced water to the dye esters will result in a lower coupling efficiency due to the hydrolysis of the dye esters. Since DMSO is hygroscopic (absorbs water from the atmosphere) store it well sealed in desiccant.

5. PROCEDURE
5.1 Aminoallyl Labeling
5.1.1 To 10 µg of total RNA (or 2 µg poly(A+) RNA) which has been DNase I-treated and Qiagen RNeasy purified, add 2 µL Random Hexamer primers (3mg/mL) and bring the final volume up to 18.5 µL with RNase-free water.

5.1.2 Mix well and incubate at 70°C for 10 minutes.

5.1.3 Primer annealing is done at 0.1 degree/sec from 70 degrees to 25 degrees. There is a 5 minute pause at room temperature during which the items listed below in 5.1.4 are added.
5.1.4 Add:
5X First Strand buffer…….. 6 µL
0.1 M DTT…….. 3 µL
50X aminoallyl-dNTP mix…….. 0.6 µL
SuperScript II RT (200U/µL)….. 2 µL

5.1.5 Mix and incubate at 42°C for 2 hours to overnight, and cooled to 4°C.

5.1.6 To hydrolyze RNA, add:
1 M NaOH 10 µL
0.5 M EDTA 10 µL

mix and incubate at 70^{o}C for 15 minutes.

5.1.7 Add 10 µL of 1 M HCl to neutralize pH. (Alternatively, one can add 25 µL 1 M HEPES pH 7.0 or 25 µL 1 M Tris pH 7.4)

5.2 Reaction Purification I: Removal of unincorporated aa-dUTP and free amines (Qiagen PCR Purification Kit)
Note: This purification protocol is modified from the Qiagen QIAquick PCR purification kit protocol. The phosphate wash and elution buffers (prepared in 4.1.3 & 4.1.4) are substituted for the Qiagen supplied buffers because the Qiagen buffers contain free amines which compete with the Cye dye coupling reaction.

5.2.1 Mix cDNA hydrolyzed reaction with 300 µL (5X reaction volume) buffer PB (Qiagen supplied) and transfer to QIAquick column.

5.2.2 Place the column in a 2 ml collection tube (Qiagen supplied) and centrifuge at ~14,000 rpm for 1 minute. Empty collection tube and reuse.

5.2.3 To wash, add 750 µL phosphate wash buffer to the column and centrifuge at ~14,000 rpm for 1 minute.

5.2.4 Empty the collection tube and repeat the wash and centrifugation step (5.2.3).

5.2.5 Empty the collection tube and centrifuge column an additional 1 minute at maximum speed to remove residual ethanol.

5.2.6 Transfer column to a new 1.5 mL microfuge tube and carefully add 30 µL phosphate elution buffer (see 4.1.4) to the center of the column membrane.

5.2.7 Incubate for 2 minute at room temperature.

5.2.8 Elute by centrifugation at ~13,000 rpm for 1 minute.

5.2.9 Elute a second time into the same tube by repeating steps 5.2.6-5.2.8.
The final elution volume should be ~60 µL.

5.2.10 Dry sample in a speed vac.

5.3 Coupling aa-cDNA to Cy Dye Ester.
5.3.1 Resuspend aminoallyl-labeled cDNA in 4.5 µL 0.1 M sodium carbonate buffer (Na_2CO_3), pH 9.0.
Note: Carbonate buffer changes composition over time so make sure you make it fresh every couple of weeks to a month.

5.3.2 Add 4.5 µL of the appropriate NHS-ester Cy dye (prepared in DMSO: see 4.5)
Note: To prevent photobleaching of the Cy dyes wrap all reaction tubes in foil and keep them sequestered from light as much as possible.

5.3.3 Incubate the reaction for 1 hour in the dark at room temperature.

5.4 Reaction Purification II: Removal of uncoupled dye (Qiagen PCR Purification Kit)

5.4.1 To the reaction add 35 µL 100 mM NaOAc pH 5.2 to quench reaction. Incubate for 5 min.

5.4.2 Add 250 µL (5X reaction volume) Buffer PB (Qiagen supplied).

5.4.3 Place a QIAquick spin column in a 2 mL collection tube (Qiagen supplied), apply the sample to the column, and centrifuge at ~14,000 for 1 minute. Empty collection tube.

5.4.4 To wash, add 0.75 mL Buffer PE (Qiagen supplied) to the column and centrifuge at ~14,000 for 1 minute.
Note: Make sure Buffer PE has added ethanol before using (see label for correct volume).

5.4.5 Empty collection tube and centrifuge column for an additional 1 minute at maximum speed.

5.4.6 Place column in a clean 1.5 mL microfuge tube and carefully add 30 µL Buffer EB (Qiagen supplied) to the center of the column membrane.
5.4.7 Allow buffer to incubate on membrane for 2 minute at room temperature.

5.4.8 Elute by centrifugation at ~13,000 rpm for 1 minute.

5.4.9 Elute a second time into the same tube by repeating steps 5.4.6-5.4.8. The final elution volume should be ~60 µL.
Note: This protocol is modified from the Qiagen QIAquick Spin Handbook (04/2000, pg. 18).

5.5 Analysis of Labeling Reaction

5.5.1 Use a 50 µL Beckman quartz MicroCuvette to analyze the entire undiluted sample in a spectrophotometer.

5.5.2 Wash the cuvette with water and blow dry with compressed air duster.

5.5.3 Pipette sample into cuvette and place cuvette in spectrophotometer.

5.5.4 For each sample measure absorbance at 260 nm and either 550 nm for Cy3 or 650 nm for Cy5, as appropriate.

5.5.5 Pipette sample from cuvette back into the original sample tube.

5.5.6 For each sample calculate the total picomoles of cDNA synthesized using:
pmol nucleotides = [OD_{260} * volume (µL) * 37 ng/µL * 1000 pg/ng] 324.5 pg/pmol
(Note: 1 OD_{260} = 37 ng/µL for cDNA; 324.5 pg/pmol average molecular weight of a dNTP)

5.5.7 For each sample calculate the total picomoles of dye incorporation (Cy3 or Cy5 accordingly) using:
pmol Cy3 = OD_{550} * volume (µL) 0.15
pmol Cy5 = OD_{650} * volume (µL) 0.25

nucleotides/dye ratio = pmol cDNA pmol Cy dye
Note: >200 pmol of dye incorporation per sample and a ratio of less than 50 nucleotides/dye molecules is optimal for hybridizations (see Microarray Cookbook II)

5.5.8 After analysis mix together the two differentially labeled probes (Cy3 vs. Cy5) which will be hybridized to the same microarray slide for study of relative gene expression.

5.5.9 Dry the Cy3/Cy5 probe mixture to completion in a speed vac and continue with the hybridization of the probe to a microarray.

Microarray Labeled Probe Hybridization

Modified 06/24/02

1. PURPOSE

This protocol describes the hybridization of a Cy labeled cDNA probe (mix of Cy3 and Cy5) onto coated slide spotted with oligonucleotides (70mers).

2. SCOPE

This procedural format is currently utilized by Laboratory 1 for hybridization of oligonucleotide arrays

3. MATERIALS

3.1 20X Saline-Sodium Citrate (SSC) (Sigma; Cat # S-6639)
3.2 10% Sodium Dodecyl Sulfate (SDS)(Life Technologies; Cat # 15553-035)
3.3 Bovine Serum Albumin (BSA) 30%, Sigma
3.4 Formamide, redistilled (Life Technologies; Cat # 15515-081)
3.5 Isopropanol (Fisher Scientific; Cat # A451-1)
3.6 Coplin jar (VWR; Cat # 25457-200)
3.7 Human COT1-DNA (Life Technologies; Cat # 15279-011)
3.8 Mouse COT1-DNA (Life Technologies; Cat # 18440-016)
3.9 Poly(A)-DNA, 25U
3.10 Microscope Cover Glass (Fisher Scientific; Cat # 12-545J)
3.11 Hybridization chamber (Corning Costar; Cat #2551)
3.12 1 L .22 □m CA (cellulose acetate) Filter System (Corning; Cat #430517)
3.13 Pressurized air duster (Fellowes; Cat # 99790)

4. PROCEDURE

Slide processing:

1. Bake slides for 80 minutes at 80^{o}C
2. UV-Cross link slides with 200mJ of energy
3. Outline array and number slide with diamond tip pencil
4. Continue with 4.1

4.1 Prehybridizaton

4.1.1 Prepare prehybridization buffer (5X SSC, 0.1% SDS, 1% BSA) and sterilize by filtration using a CA filter. Preheat at 42^{o}C for ~30 minutes before use.

4.1.2 Place the printed slide(s) which will be used for the hybridization in a Coplin jar containing preheated prehybridization buffer and incubate at 42^{o}C for 1 hour.

4.1.3 Washing Slides
- Fill two Coplin jars with MilliQ water and another with isopropanol.
- With forceps carefully grasp slide by the labeled end and vertically dip slide into the first Coplin jar (water) so that the slide is completely submerged. Dip slide four or five times.
- Dip the slide again in the water five times but only submerging the slide enough to wash the printed array itself.
- Using the same technique dip slide into the second Coplin jar (water).
This procedure can be done in glass slide boxes on a nutator mixer.

4.1.4 Drying Slides
Spin dry the slide for 1 minute at 1200rpm.
- Make sure back of slide is dry as well.
- Note the general appearance of the slide. Streaking or mottling on the slide surface indicates further washing is necessary.

4.1.5 Use slides immediately following prehybridization to ensure optimal hybridization efficiency.

4.2 Hybridization
4.2.1 Prepare 1X hybridization buffer (50% formamide, 5X SSC, and 0.1% SDS).

4.2.2 Prepare Poly(A)-DNA by dissolving stock Poly(A)-DNA in a neutral buffer (i.e. 10 mM Tris, pH 7) to a final concentration of 20 µg/µL.

4.2.3 Prepare COT1-DNA (stock conc.1 µg/µL) by ethanol precipitation:
- Add 2 to 3 volumes of ethanol and 0.1 volumes of 3 M Sodium Acetate (NaOAc) to the stock tube.
- Mix well and place on dry ice for 20-30 minutes or in –20^{o}C freezer overnight.
- Centrifuge for 20-30 minutes in a cold room microfuge at maximum angular velocity.
- Remove supernatant and allow excess ethanol to dry off.
- Dissolve precipitated COT1 in a neutral buffer (i.e. 10 mM Tris, pH 7) to the final concentration of 20 µg/µL.

4.2.4 Resuspend labeled probe (Cy3/Cy5 probe mixture: see SOP-labeling)
in 30 µL of 1X hybridization buffer.
Note: Expose Cy labeled probe to light as little as possible during the hybridization process.

4.2.5 To block nonspecific hybridization add:
COT1-DNA (20 µg/µL)……. 1 µL
Poly(A)-DNA (20µg/µL)….. 1 µL
Note: The COT1–DNA is organism specific: add mouse COT1 to labeled mouse probes and human COT1-DNA to labeled human probes.
4.2.6 To denature, heat the probe mixture at 95^{o}C for 2 minutes and snap cool on ice for 30 sec.

4.2.7 Centrifuge the probe mixture at maximum angular velocity for 1 minute. Keep at room temperature and use immediately on baked and blocked array

4.2.8 To Apply Labeled Probe Mixture
- Place a prehybridized microarray slide (array side up) in hybridization chamber.

- Pipette the labeled probe mixture (~32 µL) to the slide surface near one end of the array print area keeping bubbles to a minimum.
- Take a 22mm x 60mm microscope glass coverslip, dust it with compressed air, and grasp one end with forceps.
- Holding the coverslip over the array print area, lower the end nearest the pool of cDNA probe until solution wicks to the surface of the coverslip.
- Gradually lower the opposite end of the coverslip (held by the forceps) onto the slide. The solution may take a minute or two to wick across the entire length of the slide.
- After probe has wicked across the slide carefully adjust the coverslip's position with the tip of the forceps so that there is an even margin between the edge of the coverslip and the edge of the slide.
- Work any large bubbles toward the edge by gently tapping the coverslip surface; small bubbles will absolve themselves during hybridization.

4.2.9 To the small wells at each end of the chamber add 10 µL of water (20 µL total), cover, and seal the chamber.

4.2.10 Incubate in a 42^{o}C water bath for 16-20 hours. To ensure chamber remains level and does not float to the surface place a small weight upon it.
Note: Do not flip hybridization chamber upside down during hybridization; this may cause the coverslip to shift from the slide and adversely affect the hybridization.

4.2.11 Wash slides in the following sequence: (wash is performed on nutator mixer)

1. 1xSSC, 0.2%SDS at 42, let sit until cover slip slides off easily (no more than 2-3 minutes)
2. 1xSSC, 0.2%SDS at 42 for 1 minute
3. 0.2xSSC, 0.01%SDS at room temperature for 1 minute
4. 0.2xSSC at room temperature for 2 minutes
5. 0.05xSSC at room temperature for 1 minute
6. Spin dry 2 minutes at 1200rpm

4.2.12 Place slides in a light tight slide box until they can be scanned.

Toxicogenomics Research Consortium – Laboratory 2
Resident (In-House) Protocol for:
RNA LABELING, HYBRIDIZATION, SCANNING AND ANALYSIS

Aminoallyl RNA Labeling Protocol

I. RT REACTION

Oligo-dT/Random Prime RNA

Reagent	Final Quantity in Rxn	Volume
Oligo-dT*	5ug	
Random Primers**	5ug	
RNA	Recommended quantities: 30ug Total RNA, 2ug PolyA or 4ug aRNA(not modified)	
Water (RNAse free)		

Final Volume = 18.5ul

* Use Oligo-dT when labeling Total RNA
** Use Random primers when labeling amplified RNA that is not modified or poly-A selected RNA

- **Incubate** RNA/Oligo Mixture **@ 70˚C** - **10 min.**
- **Chill** on ice (immediately) – **10 min.**

cDNA Synthesis

Reagent	Volume for 1 Rxn
5X First Strand Buffer	6ul
0.1M DTT	3ul
50X aa-dUTP/dNTPs	0.6ul
SuperScriptII *	1.9ul

Final Volume = 11.5

- **Add 11.5ul** Rxn mix to Oligo/RNA mixture.
- **Incubate @ 42°C – 2 hrs.**

II. HYDROLYSIS

- **Add:**
  10ul 1N NaOH
  10ul 0.5M EDTA
- **Incubate @ 65˚C –15 min.**
- **Neutralize**
  Add: **25ul** 1M Tris (pH 7.4)

III. CLEANUP

To continue with the Amino-Allyl dye coupling procedure, all Tris must be removed from the

reaction to prevent monofunctional NHS-ester Cye dyes from coupling with free amine groups in solution.

- **Add 450ul** H_2O to a Microcon-30 concentrator.
- **Add** neutralized reaction (rinse out tube).
- **Spin @ 10,000 x g – 8 min.**
- **Decant** flo-thru.
- **Wash 2X: Refill** original container with **500ul** H_2O.
- **Elute** cDNA – Turn column upside down, **Spin @ 10,000 x g – 2 min.** (in clean tube).
- **Dry** eluate in Speed-Vac.

IV. DYE COUPLING

Aliquoting Cy3 and Cy5 Monoreactive Dyes

- ***Resuspend*** fresh tube of dye in 36 ul of DMSO that has been treated with Molecular Sieves.
  Technical Note: DMSO should be dried using 100g/L of 4 Å molecular sieve pellets from Sigma (cat. #M-0133).
  Prior to use, the pellets should be baked @ 300-350°C for 3 hrs, followed by cooling and storage with desiccant until needed. Pellets should be introduced directly into the DMSO container and followed with occasional shaking.
- **Aliquot 2.25 ul** x 16 tubes and store the aliquots in the dark @ 4°C.

Coupling Dye to cDNA

- **Resuspend** cDNA pellet in **4.5 ul** H_20.
- **Add 2.25 ul** of 0.2M Sodium Bicarbonate to dye aliquot…
- **Immediately! combine** the dye and cDNA.
- **Incubate @ RT** - **1 hr** in the dark.

V. QUENCHING AND CLEANUP

Before combining Cy3 and Cy5 labeled samples for hybridization, the reactions must be quenched to prevent cross-coupling.

- **Add 4.5 ul** 4M Hydroxylamine to each reaction.
- **Incubate @ RT** – **15 min.** in the dark.

To remove unincorporated/quenched Cye dye, proceed with Qia-quick PCR Purification Kit.

- **Combine** Cy3 and Cy5 reactions.
- **Add 70 ul** H_2O.
- **Add 500 ul** Buffer PB – **Mix.**
- **Apply** to Qia-quick column **– Spin @ 13,000 x g – 2 min.**
- **Discard** flo-thru.
- **Add 750 ul** Buffer PE **– Spin @ 13,000 x g – 2 min.**
- **Discard** flo-thru – Repeat wash.
- **Discard** flo-thru **– Spin @ max rpm** to dry column.
- **Transfer** to clean/new Eppendorf tube.
- **Add 30 ul** Buffer EB to center of filter **– Allow to stand 1 min.** @ **RT.**
- **Spin @ 13,000 x g – 2 min.**

- **Repeat** elution step again.

Final volume should be ~ 60 ul.

VI. SAMPLE PREPARATION FOR HYBRIDIZATION

- **Dry d**own eluate (Qiaquick) in speed-vac.

Standard Format 16 block Arrays:	**Large Format 32 block Arrays:**
Resuspend in **20 ul** H_2O	**Resuspend** in **27.8 ul** H_2O
Add 4 ul 20X SSC	**Add 5.4 ul** 20X SSC
Add 2 ul Poly A (10mg/ml stock)	**Add 2.8 ul** Poly A (10 mg/ml stock)

- **Filter** in Millipore 0.45 um spin column.
- **Prewet** filter with 20 ul H_20
- **Spin @ 10,000 x g – 2 min.** and Decant completely.
- **Apply** sample to side of column avoiding probe contact with filter.
- **Spin @ 10,000 x g – 2 min.**
- **Store** sample @ **-20°C** until ready for hybridization.

Array Prehybridization with Milk

Updated 09.29.2003

- In 500mL graduated cylinder, measure the following:
  - 370mL Nano-H_2O
  - 125mL 20X SSC
  - 5mL 10% SDS
- Weigh out 15 grams of powdered non-fat milk and place in beaker (at least 500mL beaker).
- Pour prehybridization solution from beaker into slide dish. Cover with plastic wrap and place in 42°C water bath. Water level should be the same level as solution in dish…no higher.
- Allow solution to heat for about 45 minutes.
- Place rack of slides to be prehybridized into preheated solution. Dip up and down a few times to get slides wet. Cover the dish with a metal lid or plastic wrap and let sit (still in the 42°C bath) for approximately 1 hour.
- Remove slides from prehybridization solution and immediately place in a dish of Nano-H_2O. Dip up and down 15-20 times vigorously.
- Transfer rack of slides to a dish of 100% isopropanol. Dip up and down 15-20 times vigorously.
- Immediately transfer to centrifuge and spin dry at 500xg for 5 minutes.

Hybridization and Wash Protocol

Updated 09.10.2003

Array (Slide) Examination

- **Prescan** Array slide with Axon scanner at low resolution.
- **Prehybridize** Arrays in 3% Milk solution for 1 hour (see MILK PREHYBRIDIZATION PROTOCOL).

Sample Preparation

- *Allow* Labeled RNA sample(s) *to thaw.*
- *Add* 0.6ul 10% SDS (Do not ice after adding SDS).
- *Heat* @ 99.9°C – 2 min. on heatblock.
- *Centrifuge* @ 14,000 RPM – 3 min.
- *Cool* to RT.

Hybridization Set-up

- *Place* array slide in Hybridization chamber.
- *Add* 10ul 3X SSC to slide (well away from spotted array).
- *Add* probe sample onto array area (Do NOT touch surface with pipette)
- *Apply* coverslip *PROMPTLY* over array (Position carefully and Avoid bubbles)
- *Seal* hybridization chamber.
- *Incubate* @ 63°C ~ 16 hrs by submerging in water bath *immediately*.

Post-Hybridization Washes

Wash 1A (with SDS): 1X SSC/ 0.03% SDS

- *Remove* array slide from Hybridization chamber.
- *Place* slide in Slide rack submerged in wash.

(If coverslip begins to come off, allow it to fall free in wash to avoid scratching array)

- *Soak* in solution – 2 min.
- *Dip* slide rack until coverslip(s) falls free.

Wash 1B (NO SDS): 1X SSC

- *Dip* slide (rack) 15 times to remove excess SDS.

Wash 2: 0.2X SSC

- *Soak* and *Shake* @ 60RPM protected from light – 20 min.

Wash 3: 0.05X SSC

- *Soak* and *Shake* @ 60RPM protected from light – 10 min.

Note: Immediate transfer of slide(s) through wash steps will avoid drying effects.

Drying

- *Centrifuge* slide(s) @ 500RPM – 5 min.

<u>Array Scanning</u>

Use GenePix® 4000B
532 nm (17 mW) and 635 nm (10 mW) lasers
Scanned at 10 microns

Toxicogenomics Research Consortium – Laboratory 3
Resident (In-House) Protocol for:
RNA EXTRACTION, LABELING, HYBRIDIZATION, SCANNING AND ANALYSIS

Expression profiling considerations

Experiment Design:

Laboratory 3 is developing various protocols for RNA extraction, labeling, hybridization, scanning and analysis. Current protocols include direct total RNA labeling with Cy3 and Cy5 labeled cDNA targets. More protocols for labeling will become available as they are developed and optimized. The protocols will allow researchers to label and hybridize arrays in their own laboratories, then scan using one of the scanners in the facility.

The first production batches of mouse, human and yeast chips are available. The yeast oligonucleotide arrays were printed in the facility and contain 70mers purchased from Operon. Information on the yeast Array-Ready™ can be found at the following website: http://www.operon.com/arrays/arraysets. Mouse and human arrays were fabricated using amplified cDNA inserts from libraries created at the NIA and Research Genetics, respectively. Information on the mouse cDNA library is available at http://lgsun.grc.nia.nih.gov/cDNA/cDNA.html. Information on the human sequence verified library could be found at the following website: http://www.resgen.com/products/.

How to proceed:

List of items needed to carry out experiments:

- -Waterbath
- -Hybridization chambers
  - CMT - Hybridization Chamber, Corning, cat# 2551
- -Coverslips
  - Corning (through VWR)

Analysis:

In addition to providing infrastructure for the fabrication and supply of arrays, Laboratory 3 is evaluating three platforms for the rudimentary analysis of microarrays. These platforms are MolecularWare, Spotfire and GenoMax, which contain overlapping data visualization and data mining algorithms. We are also in the process of developing an understanding of the statistical underpinnings of the analysis of microarray data. It is difficult for us as a core facility to configure a single methodology suitable for the statistical analysis of microarray data due to the varied nature of the biological questions being explored. We highly recommend that researchers read *COMPUTATIONAL ANALYSIS OF MICROARRAY DATA* (Quackenbush) in the June 2001 edition of Nature (http://www.nature.com/reviews/genetics).

RNA Extraction with TRIzol and RNeasy

This method can be used for both tissue and cultured cells. We recommend that each experiment be carried out in triplicate with dye swapping. This means that every experiment will use six microarrays each so 600μg total RNA for both the reference RNA and the analyte RNA will be needed.

For mouse and human culture or tissue samples extract the RNA with TRIzol. For yeast cultures we recommend hot acid phenol extraction. The RNA is then cleaned using

Qiagen's RNeasy columns. RNeasy midi columns are recommended for this amount of RNA as RNAeasy mini columns have a maximum binding capacity of 100μg.

1.1 TRIzol extraction of mammalian cells

1.1.1 Materials:

Tissue
Chloroform ($CHCl_3$)
TRIzol reagent
100% Isopropanol
100% Ethanol
75% Ethanol
70% Ethanol
DEPC ddH_2O (pH 7)

1.1.2 Procedure:

1. Homogenize up to 30-50mg of tissue or contents of one 150mm tissue culture dish in 2ml TRIzol reagent.
2. Transfer 1ml to each of two 1.7ml eppendorf snap cap tubes, or screw cap. freezing vials. At this point the sample can be stored at -80°C.
3. Incubate at 25°C for 5 min.
4. Add 300μl chloroform ($CHCl_3$) to each tube.
5. Shake vigorously for 15 sec.
6. Incubate at RT for 2-3 min.
7. Spin at 12,000G for 15 min in the cold room
8. Transfer combined aqueous phases to a new 1.7ml eppendorf tube
9. Precipitate with 0.5ml isopropanol
10. Incubate at RT for 10min.
11. Spin at 12,000G for 15 min. in the cold room and immediately decant supernatant.
12. Disrupt pellet in 1ml 75% ethanol and spin down in cold at 15k for 10 minutes, immediately decant supernatant and carefully remove excess with pipette tip.
13. Dry and resuspend in 100μl DEPC ddH_2O (pH 7)
14. Quantitate yield of RNA on spectrophotometer. RNAeasy mini columns have a maximum binding capacity of 100μg total RNA. If total RNA exceeds 100μg, consider using RNAeasy Midi kits. Run the samples out on a gel once to compare against the post-cleanup RNA.

1.2 Yeast hot acid phenol RNA extraction

1.2.1 Materials

Yeast cell culture grown to desired density
TES solution:
- 10mM Tris•Cl pH7.5
- 10mM EDTA
- 0.5% (w/v) SDS

Acid phenol – saturate solid phenol with water. Store in the dark at 4°C.

Chloroform
3M Sodium Acetate pH 5.2
100% Ethanol, ice cold
70% Ethanol, ice cold
DEPC ddH_2O pH7, ice cold

1.1.2 Procedure

1. Spin down culture for 3min at 1500xg and 4°C in culturing tube.
2. Discard supernatant then resuspend cell pellet in 1ml ice cold DEPC ddH_2O pH7.
3. Transfer cells to 1.7ml microfuge tube. Spin down for 10sec (max speed unless otherwise noted) at 4°C and discard supernatant. At this point the pellet can be frozen by liquid nitrogen and stored at -80°C for later extraction.
4. For greater yield, split large (volume greater than 400µl) pellets into two tubes. Resuspend pellet in 400µl TES.
5. Add 400µl acid phenol and vortex for 10sec.
6. Incubate for 60min at 65°C with occasional, brief vortexing.
7. 5min on ice then spin down for 5min in 4°C.
8. Transfer the top aqueous phase into new tube. Add 400µl acid phenol and vortex.
9. 5min on ice then spin down for 5min in 4°C.
10. Transfer the top aqueous phase into new tube. Add 400µl chloroform and vortex. Spin down 5min at 4°C.
11. Transfer the top aqueous phase into new tube then add 40µl 3M sodium acetate pH5.2 and 1ml ice cold 100% ethanol. Precipitate by gently mixing and standing in -20°C for 20min. Spin down 15min in 4°C. Wash pellet by vortexing in 1ml ice cold 70% ethanol. Spin down 10min in 4°C.
12. Dry pellet by air or SpeedVac then resuspend in 200µl DEPC ddH_2O pH7. Quantitate the RNA by UV spectrophotometer and run it out on a gel to check for quality. Proceed with the RNA cleanup.

1.3 Ethanol Precipitation

This method adjusts RNA or DNA solutions to a desired concentration. First find the total amount of RNA in the sample with a UV spectrophotometer reading then ethanol precipitate and resuspend in amount of DEPC water to get desired concentration.

1.3.1 Materials

100% Ethanol
3M sodium acetate pH5.2
70% Ethanol
DEPC ddH_2O pH7

1.3.2 Procedure

1. Add 0.1vol 3M sodium acetate pH5.2
2. Mix by flicking then add 2.5vol for RNA or 2vol for DNA ice cold 100% ethanol.
3. Incubate in -20°C for 20min
4. Spin down for 15min at max micro-centrifuge speed.
5. Decant the supernatant and wash by adding 1ml 70% ethanol and vortex.
6. Spin for 10min in 4°C, decant the supernatant and dry pellet by air or SpeedVac.

7. Resuspend in DEPC ddH_2O pH7.

1.4 RNeasy RNA Cleanup

Refer to Qiagen RNAeasy handbook for details on RNA cleanup protocol.

1.4.1 Materials

RNeasy midi kit
β-mercaptoethanol
100% Ethanol
DEPC ddH_2O (pH 7)

Before beginning cleanup, label and set up the columns in collection tubes, add 10μl β-mercaptoethanol per 1ml of Buffer RLT, and add 4vol of 100% ethanol to Buffer RPE.

1.4.2 Procedure

If RNA concentration is	≤ 500μg	500μg - 1000μg
Add DEPC ddH_2O pH7 to♦	0.5 ml	1.0 ml
Buffer RLT to add	2.0 ml	4.0 ml
100% Ethanol	1.4 ml	2.8 ml
Total	3.9 ml	7.8 ml

15. Quickly load the solution into columns. Cap the columns gently and spin down for 5min (3000-5000 x g). Discard the flow-through. For larger volumes load the remaining 3.8ml, spin again like before and discard flow-through.
16. Add 2.5ml Buffer RPE then spin down for 2min and discard flow-through.
17. Add another 2.5ml Buffer RPE, spin down for 5min and discard flow through.
18. Spin for 1min and discard flow-through.

If starting RNA conc. was	≤ 500μg	500μg - 1000μg
DEPC ddH_2O pH 7 to elute in	150 μl	250 μl

19. Add the water to the center of the column matrix. Let the column stand for 2min and spin 3min. You may wish to add an additional amount of DEPC ddH_2O (pH 7) to column and pool elutions.
20. Quantitate yield of RNA on an UV spectrophotometer and run 5μl on formaldehyde gel(see below). For simplicity's sake, our labeling protocol calls for the total RNA template concentration to be 10μg/μl. We recommend you target this concentration for total RNA by ethanol precipitating (see footnote).

1.5 Checking Extracted RNA

1.5.1 Formaldehyde gel for RNA

1% agarose, 6% formaldehyde in 1X MOPS

♦ May need to ethanol precipitate if volume exceeds desired volume.

1.5.1.1 Materials:

Agarose
10mg/ml Ethidium bromide
Ambion RNA marker
Ambion NorthernMax Formaldehyde Load Dye

10X MEN:
200mM MOPS
50mM Sodium acetate (NaOAc)
10mM EDTA

For 500ml solution:
20.9g MOPS
2.05g NaOAc or 3.4g NaOAc•$3H_2O$
1.86g EDTA•$2H_2O$

FA gel running buffer (1L):
880ml DEPC H_2O
100ml 10X MEN
20ml formaldehyde

Keep both 10X MEN and FA gel running buffer in the dark and at 4°C

1.5.1.2 Procedure

1. Microwave 0.5g agarose in 36.6ml DEPC dH_2O
2. Cool to ~60°C
3. Add 5ml 10X MEN
4. Add 8.4ml formaldehyde (37% stock)
5. Add 2μl ethidium bromide
6. Mix gently and pour gel in fume hood
7. Fill gel box with FA gel running buffer and equilibrate for 60min
8. Combine samples and gel loading buffer (1:1)
9. Place in 65°C for 15 min; quick chill on ice
10. Spin down and load
11. Run at 70-90V for 3-5hrs depending on size of gel

1.5.2 DEPC 1% Agarose gel

If this method is chosen, EVERYTHING must be RNase free. Treat everything with RNase Away and use only DEPC dH_2O in diluting solutions.

1.5.2.1 Materials (all RNase free)

Agarose
10mg/ml Ethidium bromide
1X TAE
10X loading buffer
DEPC dH_2O

1.5.2.2 Procedure

21. Microwave 0.5g agarose in 50ml 1X TAE
22. Cool to ~60°C
23. Add 2μl ethidium bromide
24. Mix gently and pour gel
25. Fill gel box with 1X TAE
26. Combine samples with 1/10 volume of loading buffer
27. Place in 65°C for 15 min; quick chill on ice
28. Spin down and load
29. Run at 70-90V for 3-5hrs depending on size of gel

2. Total RNA Labeling Protocol

This direct labeling protocol utilizes reverse transcriptase to incorporate Cy3 and Cy5 conjugated dUTPs into the cDNA target. The 1X volume covers smaller arrays (24mmx24mm) while the 2X volume covers larger arrays like the mouse and human cDNA arrays (24mmx40mm). We supply Arabidopsis thailiana control mRNA spikes for the mouse and yeast arrays for $15 per pair of tubes or the spikes can be purchased from Stratagene directly. These controls can be used to normalize intensities or to construct standard curves and are to be spiked into the labeling reactions. We also stock Cy3 and Cy5 dyes from NEN for $182 per tube of 25nmol in 25μl.

Use aluminum foil to cover Cy dyes and subsequent labeling reactions as nucleotides labeled with fluorophores should be protected from extended exposure to light. We recommend working quickly through steps in which the dyes are exposed to light and/or turning off overhead lights and working in the semi-darkness.

2.1 Materials

42°C water bath

65°C water bath

80°C block

DEPC treated dH_2O pH 7.0(we prefer to use Ambion DEPC treated water)

Cy3 and Cy5 conjugated dUTPs at 1mM (1 nmol/μl)

Total RNA at 10μg/μl – amount is dependent on type of experiment

Oligo $d(T)_{18}$ (4μg/μl)-we recommend having the oligo dT synthesized by an oligo house as opposed to buying pre-made aliquots.

SpotReport mRNA spikes in isopropanol

The spikes are supplied in isopropanol and sodium acetate. Spin down each of the control tubes for each array in a cold room for 15 minutes,

- Spin down for 15 min, rinse pellet once in 75% EtOH, decant super, spin down briefly and draw off remainder with pipette. Dry the pellet in a speedvac for 5-10 minutes and resuspend in 6.63μl DEPC treated dH_2O (pH 7).

0.2M DTT

dNTPs	dATP	25mM	25μl of 100mM stock
	dGTP	25mM	25μl of 100mM stock
	dCTP	25mM	25μl of 100mM stock
	dTTP	10mM	10μl of 100mM stock

5X 1st Strand Buffer

Super Script II Reverse Transcriptase (200U/μl)

0.5M EDTA

1N NaOH (equivalent to 1M NaOH)

1 M Tris, pH 7.5

cold isopropanol

5M ammonium acetate

75% ethanol, ice cold

Hybridization Buffer:	Final conc.	1X	4X	8X
Formamide	25%	3.75µl	15µl	30µl
20xSSC	5X	3.75µl	15µl	30µl
10% SDS	0.1%	0.15µl	0.6µl	1.2µl
Herring Sperm (10mg/ml)	0.13%	2µl	8µl	16µl
H_2O		5.35µl	21.4µl	42.8µl
Total		15µl	60µl	120µl

Hybridization chambers

cover slips

Ziploc bags

3X SSC

20°C freezer

2.2 Procedure

-Establish "RNA ONLY" benchtop, eppendorf tubes and pipettes. Wipe pipettes and surfaces down with RNase away before proceeding.
- Spin down control RNA spikes and begin thawing reagents on ice.

	1x Volume (30µl)♣		**2x Volume (60µl)**	
	Cy3	**Cy5**	**Cy3**	**Cy5**
1. Total RNA (10µg/µl)	5µl	5µl	10µl	10µl
2. Oligod$(T)_{18}$ (4µg/µl)	1µl	1µl	2µl	2µl
3. mRNA spikes♣	6.63µl	6.63µl	6.63µl	6.63µl
4. DEPC dH_2O	2.77µl	2.77µl	12.17µl	12.17µl
5. Incubate 65°C for 10 min				
6. Incubate 25°C for 5 min				
7. Add Cy dyes	4µl	4µl	8µl	8µl
Total	19.4µl	19.4µl	38.8µl	38.8µl
8. Incubate 42°C for 2 min				
9. Add Master Mix:				
DEPC dH_20	0.5µl	0.5µl	1µl	1µl
DTT (0.2 M)	1.5µl	1.5µl	3µl	3µl
DNTPs	0.6µl	0.6µl	1.2µl	1.2µl
5X 1st Strand Buffer	6µl	6µl	12µl	12µl
10. RT Super Script II♠	2µl	2µl	4µl	4µl
Total	10.6µl	10.6µl	21.2µl	21.2µl

♣ Substitute DEPC dH_2O if not using mRNA spikes.

♠ There are indications that the addition of more Super Script RT II than is indicated in this protocol to the labeling reaction will yield a higher level of Cy Dye incorporation.

For the following steps 12 to 23 the volumes of all solutions should be divided by two for 1X volume experiments; 24mm x 24mm arrays.

11. Incubate 42°C for 60 min. (NOTE: proceed with Array Processing now)
12. Spike 2μl RT Superscript II♠ to reactions and incubate for 1 hour at 42°C
13. Cool on ice for 5 min
14. Add 6μl 0.5 M EDTA pH 8, followed by 6μl 1N NaOH
15. Incubate at 65°C, 30 min
16. Cool on ice for 5 min
17. Add 15μl 1 M Tris, pH 7.5
18. Combine into one Eppendorf tube
19. Add 12μl of 5M ammonium acetate and 240μl cold isopropanol
20. Vortex and place in –20°C for ~20 minutes.
21. Spin down in 4°C at 15k for 15 minutes, immediately decant supernatant. **Disrupt** pellet in 500μl 75% EtOH and spin down in 4°C at 15k for another 10 minutes, immediately decant supernatant and carefully remove excess with pipette tip. We believe the pellet must be fragmented to remove all excess dyes and therefore reduce background.
22. Dry down for 5 to 10 minutes in speed vac.
23. Resuspend in 50μl for Lifter slips and 25μl for cover slips of pre-warmed (42°C) hybe buffer, may need to pipette vigorously.
24. Place array in Corning hybridization chamber. Place lifter slip/cover slip on top of array. Then place the whole chamber on a 42°C heat block to pre-warm the cassette; this helps the hybridization solution to spread more evenly and reduces chance of air bubbles.
25. Denature labeled cDNA by placing tubes in 80°C heat block for 2min.
26. Chill on ice for 2min.
27. Spin briefly.
28. Slowly apply labeled cDNA in hybe solution to edge of slip. Capillary action will draw solution over the array.
29. Add 8-10μl 3xSSC to both reservoirs in chamber, cover and incubate for 16 hours at 42°C in light proof water bath. Free-floating the Corning hybe chamber in waterbath has been stressful so we place our chambers in Ziploc bags and then weigh it down with a tube rack.

3. Array processing

3.1 Materials:

Arrays
Diamond tip scriber
UV crosslinker
65°C water bath
95°C heat block or hot plate
Succinic anhydride
1-methyl-2-pyrrolidinone
1M Sodium Borate

Dissolve 3.09g of boric acid in 50mL filtered water by heating then bring room temperature solution to pH 8.0 with 10N NaOH.

2 Glass and 1 stainless steel assay dishes with wire racks

Prehybe solution:	Final conc.	30μl	90μl
Formamide	50%	15μl	45μl
20X SSC	5X	7.5μl	22.5μl
10% SDS	0.1%	0.3μl	0.9μl
10% BSA	1%	3.0μl	9.0μl
dH_2O		4.2μl	12.6μl

Filtered dH_2O pH 7.0
100% isopropanol

3.2 Procedure

1. Dissolve 6g succinic anhydride in 325ml 1-methyl-2-pyrrolidinone with agitation.
2. At this point, trace over the black writing on the array with a diamond tip scriber as the writing will disappear in the succinic anhydride/pyrrolidinone solution.
3. Rehydrate arrays by holding over a 65°C water bath until water vapor coats surface of array
4. Snap dry array on 95°C heat block
5. UV cross link DNA to the slide by UV crosslinker set at 65mJ (650x100μJ)
6. Immediately before treating arrays, add 15mL of 1M sodium borate to succinic anhydride, 1-methyl-2-pyrrilidinone solution
7. Arrays are soaked in succinic anhydride/sodium borate solution for 15 minutes with gentle agitation
8. Boil 400ml water in microwave in the 5 min before step 7 is done
9. Transfer arrays to 95°C (just boiling) water for 2min then rinse in 100% ethanol for several seconds
10. Place in 50ml conical and spin dry in centrifuge at 100rpm for ~2 minutes. Air drying in hood or blow drying with nitrogen gas is also an option but may introduce more dust than centrifuging.
11. OPTIONAL BUT RECOMMENDED. Several researchers also prehybe their slides, slides must be used within 1-2 hours of pre-hybing. If pre-hybing is preferred:
    - Place array in hybe chamber with lifter slip or cover slip on top.
    - Add prehybe solution, same volume as hybe solution, to edge of coverslip on top of array.
    - Add 10μl 3XSSC to reservoirs and close up hybe chamber.

- Incubate at room temp for 45minutes to 1 hour.
- Wash slide in filtered dH_20 for 2 minutes then 100% isopropanol for 2 minutes.
- Dry slide with nitrogen gas or place in 50ml conical and spin dry in centrifuge at 500rpm for ~2 minutes.

The arrays can either be treated in glass rectangular slide staining assay dishes with removable trays or in 50mL conical tubes. We recommend staining dishes for better washing. If using staining dish, then small stir bars should be used so that wire racks will fit on top during 15min agitation. Also, water should be microwaved for approximately 3-4min during end of agitation period and then poured into stainless steel dish. If conical tubes are to be used then only two arrays can be treated per tube (back to back) and amount of solutions used should similarly be scaled down. If using 50mL conical, agitate by gently shaking conical, and 95°C water should be in a beaker.

4. Array Washing and Scanning

4.1 Materials

20X SSC
10% SDS
Filtered dH_2O pH 7
Glass assay dishes or 50ml conical tubes

Wash	Composition	Recipe for 400ml:		
1	1XSSC, 0.03%SDS	378.8ml dH_2O	20ml 20XSSC	1.2ml 10%SDS
2	0.1XSSC, 0.01%SDS	397.6ml dH_2O	2ml 20XSSC	0.4ml 10%SDS
3	0.1X SSC	398ml dH_2O	2ml 20XSSC	-----

Note: all washing solutions should be filtered using 0.22 micron filter.

4.2 Procedure

-Washing hybridized arrays can be done in glass staining dishes like those used in blocking or 50ml conical tubes; depending on how many arrays you wish to process at once. We recommend glass dishes because the arrays are usually cleaner afterwards but we have seen good results with both methods.
-Start with Wash 1 and go to Wash 3.
-Each wash should be 5min long and the slides should be agitated during the washes.
-The timing of the washes should commence AFTER the lifter slip falls off in the first wash. If the lifter slip does not fall off after ten seconds or so then pick the slide out of the metal tray and remove the lifter slip by individually dunking the slides repeatedly. Wash at room temperature but dry by spinning down in centrifuge tubes or by blowing with nitrogen immediately after the last wash. Also clean the non-array side of the slide with 100% ethanol.
-Scan slides as soon as possible.

Vendors and Catalog numbers

Ambion:

- Thin-walled, RNase-free 0.2 ml PCR Tubes (1000), cat# 12225
- RNase free 10X TAE, cat# 9869
- RNase free Gel Loading buffer, cat# 8552

- RNA Millennium Marker, cat# 7150
- NorthernMax Formaldehyde Load Dye, cat# 8552
- 5 x 100ml DEPC treated dH_2O pH 7.0, cat# 9916

Corning (VWR):

- CMT - Hybridization Chamber, cat# 2551
- Corning 0.22 micron CA filter units, cat# 28199-788
- Corning 50ml conical tubes, cat# 21008-690
- Coverslips
  -test chip: 22mm sq #48371-045
  -yeast, mouse, human: 22x40mm #48396-046

Invitrogen:

- TRIzol reagent, 100ml, cat# 15596026
- Super Script II Reverse Transcriptase (200U/μl), cat# 18064022
  - Comes with 5X 1st strand buffer and can also be obtained from VWR stockroom

Promega:

- Set of 200μmol (2 x 100μmol each, 100mM) dATP, dCTP, dGTP, dTTP, cat# U1410

Qiagen:

- RNeasy Mini kit (50), cat# 74104
- RNeasy Midi kit (50), cat# 75144
- RNeasy Maxi kit, cat# 75162

Shandon Lipshaw:

- Glass slide assay dish 121, cat#122-123-113
- Stainless steel staining dish, cat# 112

Sigma:

- Silica gel desiccant Sigma Aldrich, S7625, Type III, 500g
- Sodium bicarbonate, molecular grade, DNase, RNase, protease free, S7277
- Succinic anhydride, 500g, S 7626
- 2-mercaptoethanol, 25 ml, cat# M 3148

Stratagene:

- 100ng each of SpotReport *A. thailiana* mRNA spikes, cat# 252201, 252202, 252203, 252204, 252205, 252206, 252207, 252208, 252209, 252210

VWR:

- 20X SSC, 1L, cat# PAV4261
- 10% SDS from building 68 media room
- Ammonium acetate, 125g, cat# MK327202
- Boric Acid, 500g, cat# JT4035-1
- BSA, 10g, cat# 80055-694
- Chloroform, 500ml, cat# IB05040
- Desiccator's cabinet, medium, cat# 24983-331
- Diamond tip scriber, cat# 52865-005
- 1,4-Dithiothreitol (DTT), 5g, cat# IB21040
- EDTA, 125g, cat# MK493102
- Ethanol, 500ml, cat# IB15720
- Ethidium bromide, 1g, cat# MK773812
- Formaldehyde (37-40%HCH), 1L, cat# VW3408-1
- Formamide, 100ml, cat# EM-4610
- Isopropanol, 4L bottle, cat# EM-PX1834-1

- 1-methyl 2-pyrrolidinone, 4L bottle, cat# EM-MX1392-1
- MOPS, 100g, cat# EM-6310
- dNTPs from Promega set of 40μmol dATP, dCTP, dGTP, dTTP at 100mM, cat# PAU1240
- RNASE AWAY, 250ml, cat# 72830-022
- Salmon sperm DNA, cat# 62111-454
- Sodium hydroxide, 500g, cat# JT3722-11
- Sodium acetate, 500g, cat# MK737212
- Tris, 500g, cat# MK773204

mRNA Control Spikes

Control	Reference (nanograms)	Analyte (nanograms)
1	0.01ng	1ng
2	0.02ng	1ng
3	0.1ng	1ng
4	0.5ng	1ng
5	1ng	1ng
6	1ng	1ng
7	1ng	0.5ng
8	1ng	0.1ng
9	1ng	0.02ng
10	1ng	0.01ng
Total	6.63ng	6.63ng

*Please note that the control spikes are supplied in sodium acetate and isopropanol and must be spun down and resuspended in DEPC water before use.

Toxicogenomics Research Consortium – Laboratory 4
Resident (In-House) Protocol for:
RNA LABELING and POST-HYBRIDIZATION WASHING

RT Labeling from total RNA source & Qiagen Clean-Up

I. Labeling

Tube#	Sample	PI/Project ID/Exp ID	Location	Conc µg/µl	Label	µg RNA	µl RNA	µl H2O	Chip
1					Cy3				
2					Cy5				
3					Cy3				
4					Cy5				
5					Cy3				
6					Cy5				
7					Cy3				
8					Cy5				
9					Cy3				
10					Cy5				
11					Cy3				
12					Cy5				
13					Cy3				
14					Cy5				
15					Cy3				
16					Cy5				

Master mix (1 tube/probe)		**2 pairs**	**3pairs**	**4pairs**	**5pairs**	**6pairs**	**7pairs**	**8pairs**
5X buffer	9µl	18	27	36	45	54	63	72
DTT	5µl	10	15	20	25	30	35	40
Cy3 or Cy5	***4µl***	***8***	***12***	***16***	***20***	***24***	***28***	***32***
dNTP mix	1.2µl	2.4	3.6	4.8	6	7.2	8.4	9.6
RNAsin	1µl	2	3	4	5	6	7	8
Super script	2µl (add last)	4	6	8	10	12	14	16

First add water to each tube, then add:
2 µl oligo dT and 1 µl RNAsin to each tube,
place tubes on ice and add RNA to each tube.
Incubate @ 65°C for 10 min.
Chill on ice 2 min.

Add 22 µl master mix/tube.
Incubate @ 42°C, 1 hr 15 min.
Add 2µl Superscript.
Incubate @ 42 C for 1.5 hours.
Add 30 µl 0.1M NaOH, incubate @ 70°C, 30 min.
Add 30µl 0.1M HCl

II. Probe Clean-Up

-Turn on 100°C heating block and 65°C water bath, check water level, turn on speed vac.
-Add 500 µl Buffer PB to each100 µl reaction mix. Into QIAquick column and tube, combine 300 µl each probe. Spin* 1 min, discard flow through.
-Add remaining probes, spin 1 min, discard flow through.
-Wash with 750 µl 35% guanidine HCl** and spin 1 min, discard flow through.
-Add 750 µl Buffer PE*** to column and spin 1 min. Discard flow through and spin again for 1 min.
-Place column into clean 1.5 ml tube. Add 30µl Buffer EB to membrane, let stand for 2 min at RT and spin 1 min. There will be ~ 30 µl left, so speed vac10 min to concentrate (final volume of < 10 µl)
Add:
1ul/10ugRNA Cot human DNA (35Cy3 +75Cy5 = 110ug = 11 ul Cot)
5 ul t RNA yeast (4ug/ul)
Add TE for final volume of 26 µl for 2x4 array or 14.8 µl for 2x2 array.
* spin at 12K

** 35 g guanidine HCl, water to 100 mL
*** be sure 100% ethanol has been added as directed to PE buffer provided in kit.

III. Hybridization

For 26 µl add:	For 14.8 µl add:
5.2 µl 20X SSC (3X final) 1:6.7	3 µl 20X SSC
2.7 µl 25X Denhardt's (2X final) 1:12.5	1.6µl 25X Denhart's
1.1 µl 10% SDS (0.3X final) 1:33	0.6 µl 10% SDS
Total 35 µl	Total 20 µl

Heat for 2 min @ 100°C.
Incubate @ RT for 10 min.
Filter through prewashed (with 10 µl H2O) millipore filter, spin @ 12K for 3 minutes.

Add entire volume of probe to slide, carefully apply coverslip, insert into chamber, add 10 µl of 3X SSC along inside bottom edge of chamber, to provide humidity and incubate 16-24 hrs @ 65°C.

Post-Hybridization Washing

1. Remove chip from chamber and place upside-down in buffer* until the coverslip falls off.
2. Transfer to a Wash Station containing 500ml 1x Wash Buffer A. (50ml Buffer A + 450 ml dH2O). Once all slides have been transferred to buffer, wash for 5 minutes with *Vigorous Agitation* on a stir plate (stir plate on highest setting). (Suggest no more than 8 slides per batch, leaving room between every slide.)
3. Transfer slide rack to a Wash Station with 500ml 1x Wash Buffer B. (5ml 20xSSC + 495ml dH2O). Wash for 5 minutes with *Vigorous Agitation.*
4. Transfer slide rack to a Wash Station with 500ml 1x Wash Buffer C. (0.5ml 20xSSC + 500ml dH2O). Wash for 5 minutes with *Vigorous Agitation.*
5. Remove slide rack and spin dry at 1000-1500K for 3-5 minutes until dry.

Note: Save all Buffers for reuse. Buffers A+B can be reused 4x, and Buffer C can be used 2x.
*Once your buffers have been used their maximum time, you can use them once more as the buffer to let the coverslips fall off into. All buffers, or a mixture of them, will work for this purpose.

Buffer (500ml,1x)	**Amount of Ingredient**	**Amount of dH2O**
A	50 ml Buffer A	450ml
B	5ml 20x SSC	495ml
C	0.5ml 20x SSC	500ml

Toxicogenomics Research Consortium – Laboratory 5
Resident (In-House) Protocol for:
SLIDE PREPARATION, RNA LABELING AND REVERSE TRANSCRIPTION

I. Slide Preparation for Hybridization and Scanning

Silylated slides will be used for printing microarray (TeleChem. Intl. Superaldehyde CSS-100, CEL Associates, Inc.).

Processing of silylated slides after printing:

Schiff Base Attachment:

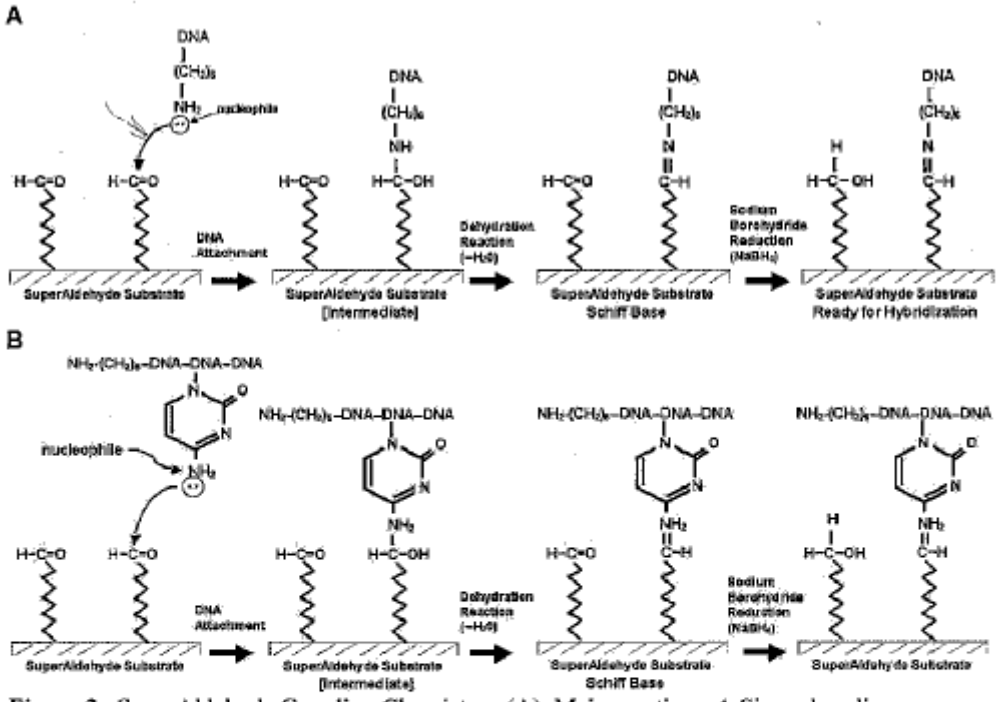

Figure 2. SuperAldehyde Coupling Chemistry. (A) Main reaction. A Six carbon linear (aliphatic) amine is added to each DNA molecule via a synthetic primer. Amino-modified DNA can be double-stranded PCR products or single-stranded oligonucleotides. The aliphatic amine on the amino-modified DNA acts as a nucleophile, attacking the carbon atom on reactive aldehydes covalently attached to the surface of the substrate. DNA attachment proceeds via an unstable intermediate that converts quickly to a Schiff based as a dehydration reaction ($-H_2O$). Schiff base formation occurs naturally at neutral pH and room termperature as the printed DNA dries on the surface of the substrate. To minimize fluorescent background, the unreacted aldehyde groups are reduced to non-reactive primary alcohols by treatment with sodium borohydride ($NaBH_4$). (B) Side reaction. Aromatic amines on the G, C, and A bases of naturally occuring DNA react with aldehyde groups, resulting in surface coupling. The efficiency of the side reactions is ~0.01% for short oligonucleotides and ~10% for double-stranded PCR products.

1. Slides are washed once in a 0.2% SDS immersion for 1'
2. Rinse twice in ddH_2O for 1' each time

3. Treat in sodium *borohydride* solution, make fresh each time (1.0g **$NaBH_4$** in 300mL of PBS and 100mL of 100% EtOH) for 5'.

4. Rinse slides once in ddH_2O for 2' at 95°C.
5. Rinse once in 0.2% SDS for 1'.
6. Rinse twice in ddH_2O, air dry, and store in the dark at RT.

Slide Washing and Processing

- Take out TNB Buffer from –20 to thaw (Place in 50 ^oC water bath)
- DO NOT let slides dry out during this protocol

1. Carefully remove Slide Cover
2. Quickly rinse in 2 X SSC
3. Wash two times in 2 X SSC for 5 min each wash at 50 ^oC
4. Wash two times in .2 X SSC for 5 min each wash at 50 ^oC
   -Washes are done in Yellow Tip Box Lid, on the Rocker

-Block the Slides in ~4ml TNB Buffer at room temp for 30 min slowly on rocker in appropriate container.

-Dilute Streptavidin-HRP 1-600 (Perkin Elmer Cat. # NEL750) in TNB Buffer (4uL Strep to 2400uL TNB for each slide)
-Place the slides in 2.4ml of Dilute Streptavidin-HRP in appropriate container
-Incubate for 10 min at room temp rocking gently
[Make sure to thoroughly mix Streptavidin before use as it is very viscous]

-Quickly rinse in TNT Buffer to remove excess Streptavidin
-Wash two times in TNT Buffer for 10 min each to remove non-specific Streptavidin binding. Washes are done in Yellow Tip Box Lid, on Rocker**.**

-Dilute Cy5 Tyramide (Perkin Elmer Cat. # SAT705A)1:150 in Amplification Dilutant (Perkin Elmer Cat. # FP1152)
-Add 2.4mL dilute Cy5 Tyramide in appropriate container on rocker
-Incubate for 10 min at room temp in Dark (Cover slides to avoid photo-bleaching)
[Note- Dilute Cy5 Tyramide can be used up to 3 times]

-Turn on the Lasers on the Scanner

-Remove Cy5 Tyramide from slide
-Quickly rinse in TNT Buffer to remove excess Tyramide
-Wash two times in TNT Buffer for 10 min each to remove non-specific Tyramide Binding
-Wash one time in 0.02 X SSC for 3 min at 50 degrees
-Quickly rinse slide in MilliQ H_2O. Washes are done in Yellow Tip Box Lid, on Rocker.

-Spin Slides for 5 min at 2000 rpm to dry
-Scan

TNB Buffer: 500ml

Blocking Reagent (Perkin Elmer Cat. # FP329)	2.5grams
2M Tris pH 7.4	25ml
5M NaCl	15ml
H^2O	to vol.

500ml

*Heat to 65 degrees if needed to make blocking reagent soluble
Store at –20 degrees

TNT Buffer: 1 Liter

5M NaCl	30ml
2M Tris pH 7.4	50ml
Tween 20	500 microliters
H^2O	to vol
	1 liter

* Filter Steri

II. Amino Allyl Labeling of RNA

RNA	- (10 □g, max volume 13.9 □l)
Oligo dT primer (1mg/ml)	- 2 □l
H_2O	-to vol
Total volume	**15.9 l**

70° C for 10 min, 2 min on ice and quick spin and mixed with 14.1 □l of the labeling mix.

Labeling reaction mix

	(1 tube)	(4 tube)	(6 tube)	(8 tube)
5X buffer	- 6 µl	27 µl	39 µl	51 µl
0.1 M DTT	- 3 µl	13.5 µl	19.5 µl	25.5 µl
10 mM dA,G,C	- 0.6 µl	2.7 µl	3.9 µl	5.1 µl
2 mM dTTP	- 1.5 µl	6.7 µl	9.7 µl	12.7 µl
2 mM AA dUTP	- 1.5 µl	6.8 µl	9.8 µl	12.8 µl
Superscript RTase	- 1.5µl	6.8 µl	9.8 µl	12.8 µl
Total volume	**14.1 µl**			

-Incubate at 37 °C for 2 hrs to overnight.
-Add 5 µl of 2 M NaOH, incubate for 5 min at 37°C (RNA Hydrolysis)
-Add 5 µl of 2 M Tris, pH 7.4 incubate 10 min at 37°C (Neutralization)

Qiagen PCR purification kit

-Mix the cDNA with 300 µl of buffer PB and transfer to QIA quick column
-Centrifuge at 14K rpm for 1 min and empty the collection tube
-Wash the column with 750 µl phosphate buffer (5 mM, pH 8.0, 80%EtOH) at 14K rpm for 1 min
-Repeat the wash and empty the collection tube
-Spin the column for additional 1 min at 14 K rpm to remove the residual ethanol
-Transfer the column to a fresh 1.5 ml microfuge tube and add 30 µl H_2O to the center of the column membrane and incubate at RT for 2 min.
-Elute by centrifugation at 13 K rpm for 1 min
-Elute the column for second time with 30 µl of H_2O and pool the eluted volumes
-Dry the sample (60 µl) in a speed vac. (60 °C for ~25 min)

Coupling amino allyl cDNA to Cy Dye ester (Monofunctional reactive dye)

-Resuspend the aa-labelled cDNA in 4.5 µl of 0.1 M sodium carbonate buffer, pH 9.0 (freshly dilute from 1 M stock each time and do not use the stock older than one week)

-Add 4.5 µl of appropriate NHS-ester Cy dye prepared in DMSO (1 mg/73 □l of DMSO)
-Mix the sample and incubate in dark at RT for 1 h.
-Add 250 µl buffer PB (**Qiagen PCR purification kit**) to the reaction mix
-Transfer to the QIAquick spin column and centrifuge at 14 K rpm for 1 min
-Empty the collection tube
-Wash with 0.75 ml of buffer PE (with ethanol added) and centrifuge at 14K rpm for 1 min. Empty the collection tube.
-Centrifuge the column for additional 1 min at max rpm.
-Place the column in a fresh 1.5 ml microfuge tube, add 30 µl H_2O to the center of the column and incubate for 2 min at RT.
-Elute by centrifugation at 13K rpm for 1 min.
-Elute a second time with 30 □l H_2O in the same tube.
-Dry the 60 µl of eluted sample in SpeedVac.
-Resuspend the cDNA in 70 µl of ribohybe (40% Formamide, 4X SSC, 1% SDS), vortex, boil for 2 min, quick spin.
-Pre-warm the slides and ribohybe to 65^{o} C before applying the ribohybe to the slide.

Pre treatment of super aldehyde slides
-Cross link the slide (UV Stratalinker 2400) at 400 milliJoules.
-Wash in 2X SSC at RT for 5 min (two times)
-Keep in Boiling water for 5 min
-Dry the slide by centrifuging at 1600 rpm for 5 min.
-Pre-warm to 65 ^{o}C before applying hybridization mix

Washing
-Cover the slides from light during the washing steps as the dyes are highly photosensitive!!
-After overnight hybridization remove the coverslip by dipping in 2X SSC
-Wash twice in 2X SSC at RT for 10 min each
-Wash twice in 0.2X SSC at 50 ^{o} C for 10 min each

III. Reverse Transription (RT) Reaction

1.	Total RNA	2 micrograms
2.	Oligo dT Primer	2 micrograms
3.	RT cocktail	8 microliters
4.	H_2O	To Vol.
		20 microliters

*RT Cocktail: Per Reaction:

5X Transcription Buffer	4 microliters
0.1 mM DTT	2 microliters
10mM dNTPs	1 microliter
Superscript II enzyme (Invitrogen)	1 microliter

*Combine H2O, RNA, and oligo dT primer. Heat to 65 degrees Celsius for 10 min to denature secondary RNA structures. Place on Ice for 2 minutes. Spin down to bottom of tube.

*Add RT Cocktail

*Incubate at 37 degrees Celsius for 90 minutes, 45 degrees Celsius for 15 minutes, 4 degrees Celsius until ready for down stream application.

Toxicogenomics Research Consortium – Laboratory 6
Resident (In-House) Protocol for:
RNA LABELING, HYBRIDIZATION AND WASHING

1. Reverse Transcription Setup

Anchored Oligo dT primer	2 microliters	(2.5 micrograms/microliter)
Controls	2 microliters	(Optional)
mRNA	whatever volume is needed	(1.5-3 micrograms)
DEPC-dH2O	bring up to 16 microliters	

FINAL VOLUME = 16 MICROLITERS

2. Heat at 70 C for 10 minutes
3. Chill on ice for 1-2 minutes, quick spin.

4. RT Reaction for each individual tube

5X RT 1st strand Buffer	6 microliters	(comes with RT setup)
50X dNTPs	0.7 microliters	(25mM A,C,G, 10mM T) (100mM Pharmacia stocks)
Cy Dyes dUTP	3 microliters	(either Cy3 or Cy5, 25nmol stock)
DTT Stock	3 microliters	(comes with RT setup)
Superscript II RT	1.7 microliters	(cat# 18064-014 Gibco-BRL)

5. Mix well
6. 42C for 1 hour
7. Add another 1 microliter of Superscript II RT and mix
8. 42 C for 1 more hour, quick spin down
9. Degrade mRNA with 1.5 microliters of 1M NaOH / 2mM EDTA
10. 65 C for 8 minutes (do NOT go TOO long here)
11. Add 15 microliters of 0.1M HCL
12. Combine both samples and add 450 microliters of T.E (10mM Tris/0.1mM EDTA 7.4) and place combined sample into a microcon-30 filter
13. Add 30-35 microliters of Human COT1 DNA (Gibco-BRL = 1 microgram/microliter) to sample in the microcon filter
14. Spin in Eppendorf centrifuge until volume equals about 50 microliters (8 min at 9000 RPM in our IEC Micromax)
15. Remove and discard flow through
16. Repeat washing 2 more times for a total of 3 X 450 microliter T.E ashes.
17. Spin final washed sample down to about 10-20 microliters, being very careful to NOT spin the sample dry!! (usually one more 9000RPM for 3-4 minutes).
18. Invert microcon and recover the labeled sample in the bottom of the provided tube by spinning 9000 RPM for 2 minutes (sample should have a purple color).
19. Carefully remove the sample/supernatant to a fresh and clean new Eppendorf tube.

20. **To the Cy3+Cy5 combined sample, add the following reagents in this order for a large 24K-44K array (note, cut all volumes in half for 2 x 2 cm array)**

Labeled Sample	15-25 microliters	
DEPC-dH2O	(bring probe+DEPC-dH2O volume up to 25 microliters)	
Yeast tRNA	2 microliter	(5 micrograms/microliter)
PolyA DNA	2 microliters	(10 micrograms/microliter)
20XSSC	5.3 microliters	(FINAL SSC concentration=3X)
10% SDS	0.7 microliters	(add this last and mix well)

FINAL TOTAL VOLUME = 35 MICROLITERS (note that yeast tRNA, PolyA, SSC and SDS equal 10 microliters)

21. Heat sample at 100 C for 2 minutes with small hole in lid, quick spin
22. Place samples at 42 C for 20-30 minutes
23. During Step 23, get and set up the necessary number of Hyb chambers and get 22mm X 44mm, or 22mm X 50-60mm coverslips ready, and get arrays ready.
24. Add the 35 microliters of probe onto the center of the array while being careful to NOT touch the array face with the pipette tip (pop any bubbles using the end of the coverslip if necessary).
25. Quickly and gently place the #1 glass coverslip onto the array face using fine tweezers (if any air bubbles are present, gently press the coverslip with the tweezers to force out the bubbles).
26. Add about 15 microliters of 3XSSC in each of two drops onto the end of the array slide away from the actual array location for hydration purposes.
27. Assemble the Hyb chamber with the array slide in it and coverslip side up, and place into a 65 C water bath overnight.
28. Pull out the hyb chamber and dry off the excess water using a paper towel.
29. Disassemble the hyb chamber quickly, and quickly place the slides into a slide washing chamber that contains 2XSSC/0.025%SDS and jiggle the slide holder up and down until the coverslip falls off. Repeat this individually for each array, one at a time, until all are done.
30. Wash slides in 1XSSC for 3-5 minutes with occasional shaking
31. Wash slides at 50-65 C in 0.2XSSC for 3-5 minutes, twice (4 total washes)
32. Spin slides down in centrifuge at 500 RPM for 4 minutes
33. SCAN ASAP!

Toxicogenomics Research Consortium – Laboratory 7
Resident (In-House) Protocol for:
RNA LABELING, HYBRIDIZATION AND WASHING

I. Direct labeling: Probe Synthesis and Hybridization Protocol

Probe Synthesis and Hybridization for Microarrays adapted from 05-01-00

Recommended reaction conditions:

- 2ug mRNA (1-3ug range), at concentration 1ug/ul
- If using Total RNA, do not use Random 9-mers in 1st Strand Synthesis (sub with dH_2O)
- Proper experiments require 2 slides, flipping the Cy3 & Cy5 labeled RNAs.

	1	2	3	4	5	6	7	8	9	10
mRNA										
dye										
Slide Barcode										
Prescan Notes										
Prewash Method										
Notes										

A. 1st Strand Synthesis

Premix 1	Per rxn	x rxns		Premix 2	Per rxn	x rxns
mRNA, 2ug	(2.0ul)	each		5x Superscript Bf	4.0ul	
(2.5ng GFP)*	Varies			0.1M DTT	2.0ul	
Random 9-mers @ 1ug/ul	2.0ul			dNTP mix @ 5mM d(AGT)TP, 1mM dCTP	1.0ul	
dT primer @ 8uM	2.0ul			RNasin	0.5ul	
H_2O				Cy3 or Cy5 @ 1nmol/ul	(1.0ul)	each
Total **premix1** vol	8.5ul			Superscript RT (200u)	(1.0ul)	each
Total **reaction** vol	10.5ul			Total **premix2** vol	7.5ul	

*GFP = RNA spike for use with Control Plate on Human arrays, **completely optional**, may be substituted for other control RNA (referred to as 'spikes').

1. Make Premix1, flick to mix, spin down.
2. Add 8.5ul of premix to reaction tubes.
3. Add 2.0ul of mRNA directly into premix in reaction tubes.
4. Flick to mix, spin down.
5. Incubate at 70C for 10 min. (can start making Premix 2 at this time).
6. Ice 30", spin down, leave tubes at room temp.
7. Add 7.5ul of Premix2 to each reaction tube, pipetting up and down to mix.
8. Add 1.0ul of either Cy3 or Cy5 to each reaction tube.
9. Flick to mix, spin down. ***take care to keep reaction tubes in the dark, out of light, from here on out***
10. Incubate at RT for 10 min – in the dark.
11. Add 1.0ul of Superscript II to each reaction tube, flick to mix, spin down.
12. Incubate 2-3 hours at 42C – in the dark (heat block or water bath OK).

B. Denature cDNA/mRNA hybrid

1. Add 1.0ul 5M NaOH to each reaction tube, flick to mix, spin down.
2. Incubate at 37C for 10 min – in the dark.
3. Add 10ul 2M MOPS.
4. Add 19ul dH2O to bring final volume to 50ul.

C. Purify probe, plate purify.

1. Use 96-well Millipore MAFNNOB DNA Purification plate. Make sure to note which wells to use for your probes.
2. Prewet the filter with 100ul of Binding Buffer (150mM KOAc/5.3M guanidine HCL, pH 4.8), apply vacuum or spin at 3500rpm 1 min.
3. Add 200ul of Binding Buffer to each reaction tube, mix well by pipetting, and apply to filter. Apply vacuum or spin at 3500rpm 1 min.
4. Wash 5x with 200ul 80% EtOH (vacuum or spin as above).
5. Spin dry at 3500rpm 1 min.

6. Put filter plate onto elution plate and elute cDNA probe with 50ul 10mM Tris pH 8.0 (spin at 3500rpm 1 min).
7. Elute with another 50ul 10mM Tris pH 8.0, spin at 3500rpm 1 min. Probe volume is 100ul. Probes should be either pink or blue in color.
8. Probes may be stored at –20C at this point.

D. Scan the Probe

1. Zero UV spec with 10mM Tris pH 8.0.
2. Take a full spectrum (210-700nm) reading, using –0.1A – 0.5A range.
3. Measure each probe for pmol quantification:
    a. Cy3: ab550 x probe volume / 0.15 = pmol Cy3 probe
    b. Cy5: ab650 x probe volume / 0.25 = pmol Cy5 probe
    c. Usual amounts to expect are 100-200 pmol of Cy3 probe and 40-120 pmol of Cy5 probe, using SuperscriptII and 2.0ug of mRNA.
    d. Can use as little as 50 pmol of either probe for acceptable data.

E. Purify Probe, G50 column.

1. Use Amersham G50 Probe Quant spin columns, 27-5335-01.
2. Vortex upside down for 10", snap off bottom and crack the lid.
3. Spin at 3000rpm for 1 min into catch epi tubes.
4. Toss catch tubes, apply probes to middle of column, taking care that no volume goes down the side of the tube.
5. Spin at 3000rpm for 2 min into labeled epi tubes.

F. Dry the probe

1. Turn on vacuum pump attached to Speed Vac.
2. Set to 50C.
3. Cover lid with foil.
4. Dry for 1-3 hours, until probes are crystallized.
5. Probes may be stored at –20C.

G. Pretreat the slides

1. Record the barcode numbers on the worksheet provided on page 1.
2. If available, look at prescans and make notes.
3. Dip slides (holding onto barcode end) in sterile dH2O 10x (fairly quickly) and immediately blow dry with either wall air or nitrogren **towards the barcode**. The barcode has adhesive that will spread and fluoresce, so always blow towards the barcode when drying. Very important for water to be dried immediately. The spots will disappear after this pre-treatment.
4. Keep slides in dust-free slide box until ready to hybridize.

H. Hybridization

1. Resuspend probes in 25ul of hyb solution (50% formamide, 5xSSC, 5xDenhardt's, 0.1% SDS, 100ug/ml CotI DNA, 20ug/ml polyA72 primer) by breaking up pellet with pipette tip and pipetting up and down until all crystals in solution.

2. Boil 3 min, ice 30", spin down.
3. Combine appropriate reactions, pipette gently to mix (without introducing air bubbles), and apply along the edge of slide towards the barcode.
4. Steadily lower cover slip so that probe is wicked across the slide's surface.
5. Incubate in humid chamber (80-90%) at 42C for 14-16 hours. Not recommended to go beyond 16 hours.

I. Washing the hybridized slides

Wash 1: 1xSSC/0.2% SDS
Wash 2: 0.1xSSC/0.2%SDS
Wash 3: 0.1xSSC

1. Preheat Wash 1 & Wash 2 solutions to 54C in water bath.
2. Put glass containers in hyb oven to help maintain temperature during the washes.
3. Dip slides from oven into warm Wash 1 to release cover slip. Immediately put into glass carriage in glass container with 54C Wash 1 solution.
4. Washes 1-3 should be put on shaker or rocker during the incubation period.
5. Incubate in Wash 1 (@54C) for 10 min.
6. Incubate in Wash 2 (@54C) for 10 min. Repeat in fresh Wash 2 (@54C) for 10 min.
7. Incubate in Wash 3 (@RT) for 1 min. Repeat in fresh Wash 3 (@RT) for 1 min.
8. Do 2 quick dips in dH_2O (@RT) and immediately dry with either wall air or nitrogen.
9. Scan slides.

Reagents and Supply information

Reagents:

- **GFP**: spike RNA used for Human Control Plate, completely optional (other spike/control RNA may be used instead).
- **Random 9-mer primers:** 5' NNN NNN NNN 3' (Gibco, Operon, ect) OR Amersham RPK-0158 $134.40, quote 7G-3709
- **Anchored dT primer:** 5' T(25)V 3' (V = A, c, or g) (Gibco, Operon, ect) OR Amersham RPK-0145 $110 each, quote 7G-3709
- **dNTPs:** Promega U1330 (10umol each) $75 for the set
- **SuperscriptII (with 0.1M DTT and 5x buffer):** Gibco 18064-014 (10,000 units) $177 each.
- **Rnasin**: Promega N2511 (2500 units) $82 each.
- **Cy3-dCTP dye:** Amersham PA53023 (25nmol) $145 each, quote 7G-3709
- **Cy5-dCTP dye**: Amersham PA55023 (25nmol) $145 each, quote 7G-3709
- **5M NaOH**
- **2M MOPS**
- **10mM Tris pH 8.0**
- **80% EtOH**
- **Hyb solution**: 50% deionized formamide, 5xSSC, 5xDenhardt's, 0.1% SDS, 100ug/ml CotI DNA, 20ug/ml polyA72 primer
    - **Deionized formamide**: Sigma F9037, 100ml, $23.60

- **20xSSC**: Ambion 9763, 1L, $30
- **50xDenhardt's**: Fisher BP515-5, 500g, $39.18
- **10%SDS**: Ambion 9822, 500ml, $40
- **Human CotI DNA**: Gibco 15279-011, 500u, $75 each (enough for 5ml hyb soln)
- **PolyA72 primer**: 5' A(72) 3' (Gibco, Operon, ect)

Supplies:

- **Millipore DNA purification 96 well filter plates**: Millipore MAFBNOB50 (50/pk) $659
- **Probe Quant G50 spin columns**: Amersham 27-5335-01 (50/pk) $145
- **Coverslips, No.1 24x60**: VWR 48393-106 (1 ounce) $15.92
- **Rectangular staining dish w/ glass cover, carriage**: VWR 25461-003 (6 each) $271.85

II Amino-allyl labeling and hybridization protocol

A. Probe Labeling (1)

First Strand cDNA synthesis

1. Use 10µg total RNA
2. Add 2µl Random Hexamer primers V= 18.5µl H2O
3. Mix well and incubate @ 70°C for 10 minutes
4. Snap-freeze in ice for 30 seconds
5. Mircofuge for 1 minute

B. Probe labeling (2)

1. Add:

5X Superscript II buffer	6 µl
0.1 M DTT	3 µl
50X amino-allyl-dNTP mix	0.6 µl
Superscript II RT (200U/µl)	2µl

2. Mix and incubate @ 42C for 3 hours
3. To hydrolze the RNA, add:

1 M NaOH	10 µl
0.5 M EDTA	10 µl

4. Incubate @ 65C for 15 minutes
5. Add 10µl 1 M HCL to neutralize

C. Probe labeling (3)

Cleanup using QiaQuick PCR Columns

1. Mix cDNA rxn w/ 300µl buffer PB, transfer to column
2. Spin for 1 minute @ 14,000 rpm/dump supernatant
3. Add 750µl (5mM) Phosphate wash buffer, spin 1 min.
4. Repeat
5. Transfer column to fresh tube, add 30µl 4mM KPO_4 pH 8.5, incubate for 1 minute, spin for 1 minute to elute
6. Repeat, for total elution volume of 60µl
7. Dry in speed vac (40-50 min.)

D. Probe labeling (4)

Secondary Coupling and Cleanup

1. Resuspend the aminoally-labeled cDNA in 4.5µl 0.1 M (9.0µl)* carbonate buffer, pH 9.0
2. Add 4.5µl of appropriate NHS-Cy suspended in DMSO**
3. Incubate for 1 hour (in dark) - RT
4. To the reaction, add 35µl 100mM NaOAc pH 5.2
5. Add 250µl PB buffer
6. Wash w/PE buffer, and elute w/EB elution buffer(1" incubation) 2x to = Vf of 60µl
7. Dry in speed vac (40-50 min.)

*Add 9.0µl .1M carbonate buffer/then add to dye tubes

**Dye + 73µl DMSO, aliquot in 4.5µl increments

E. Hybridization (1)

Prehybridization

Place slide in Coplin jar, fill w/prehyb buffer:

final:	use:	
5X SSC	20XSSC	12.5ML
0.1% SDS	20% SDS	250µl
1% BSA	10% BSA	5ML
	H_2O	32.25ML

incubate @ 42C for 45 minutes

1. Wash slide by dipping 10X in RT MilliQ H2O
2. Dip the slides in RT isopropanol and blow dry

Hybridization (2)

Hybridization

1. Resuspend each labeled probe in 12µl of 1x buffer:

final:	use:	
50% formamide	500µl	
5X SSC	20XSSC	250µl
0.1% SDS	20% SDS	5µl
	H_2O	245µl

2. Combine 12µl of each of purified Cy3 and Cy5 labeled probe mix well and add:

COT1-DNA	(20µg/µl)	1µl
Poly(A)-DNA	(20µg/µl)	1µl

3. Heat the probe mixture @95C for 3 min. to denature, spin 1 min.

4. Apply labeled probe to prehyb. Slide/cover w/ glass coverslip Put 15µl H2O top of slide
5. Place slide in chamber
6. Place sealed chamber in 42C water bath, incubate 16-20 hours

Hybridization (3)

		Washes	Vf=500
1. Place slide in low-stringency wash buffer:		dH20	470ml
	final:	use:	
	1X SSC	20X	25ml
	0.2% SDS	20%	5ml

gently remove coverslip by agitating-42C, 4 min.

2. Wash slide in high-stringency wash buffer:		dH_20	492.5ml
	final:	use:	
	0.1X SSC	20X	2.5ml
	0.2% SDS	20%	5ml

agitating for 4 min. @ RT

3. Wash slide in 0.1X SSC, agitating for 4 min.

dH20	497.5ml
20X	2.5ml

4. Blow dry
5. Scan