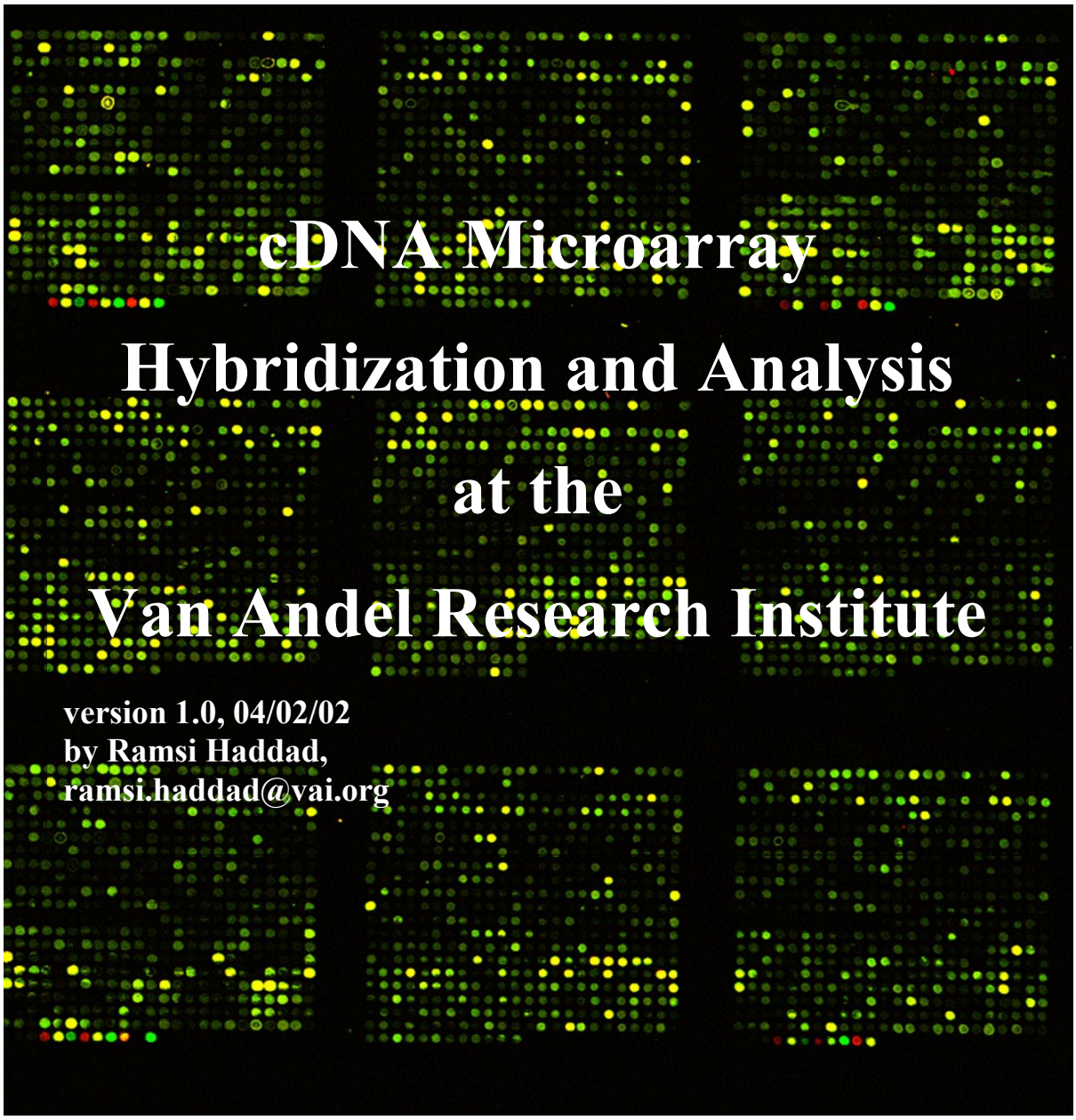

cDNA Microarray Hybridization and Analysis at the Van Andel Research Institute

version 1.0, 04/02/02
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Introduction

The hybridization and subsequent interpretation of cDNA microarray experiments is an involved process that requires many steps and a considerable amount of time to master. A typical experiment at this time requires the following discrete steps:

a) Design of experiment
b) Isolation and purification of total RNA
c) Generation of fluorescent labeled cDNA and microarray hybridization
d) Scanning microarrays
e) Image analysis using GenePix software
f) Experiment quality assessment
g) Submitting and extracting data using AMAD database
h) Interpretation of resulting expression profiles

While steps a) to g) are relatively easy to execute, it is the last step that can take up the largest amount of time. For complex profiling experiments involving multiple tumor samples, the interpretation of the data is not static, but more aptly thought of as an iterative refinement. This manual will cover steps a) to f). Since we are in the process of replacing the AMAD database and updating analysis tools, please consult with me directly for the most recent set of tools and instruction on their use. There may also be an analysis document that comes after this current document.

The next page shows a schematic review of the procedure for microarray hybridization. The Laboratory of Microarray Technology will provide slides that are pre-processed and ready to use in the protocols described in this manual.

I am intimately familiar with all the protocols in this manual and will provide you with all the knowledge I am able to give. But since I am not familiar with all methods of doing things, I may not be able to assist users with alternate protocols.

For the most up to date information about microarrays, I invite VAI users to please visit the Microarray Users Resources web site: http://elm.vai.org/uarray/index.htm

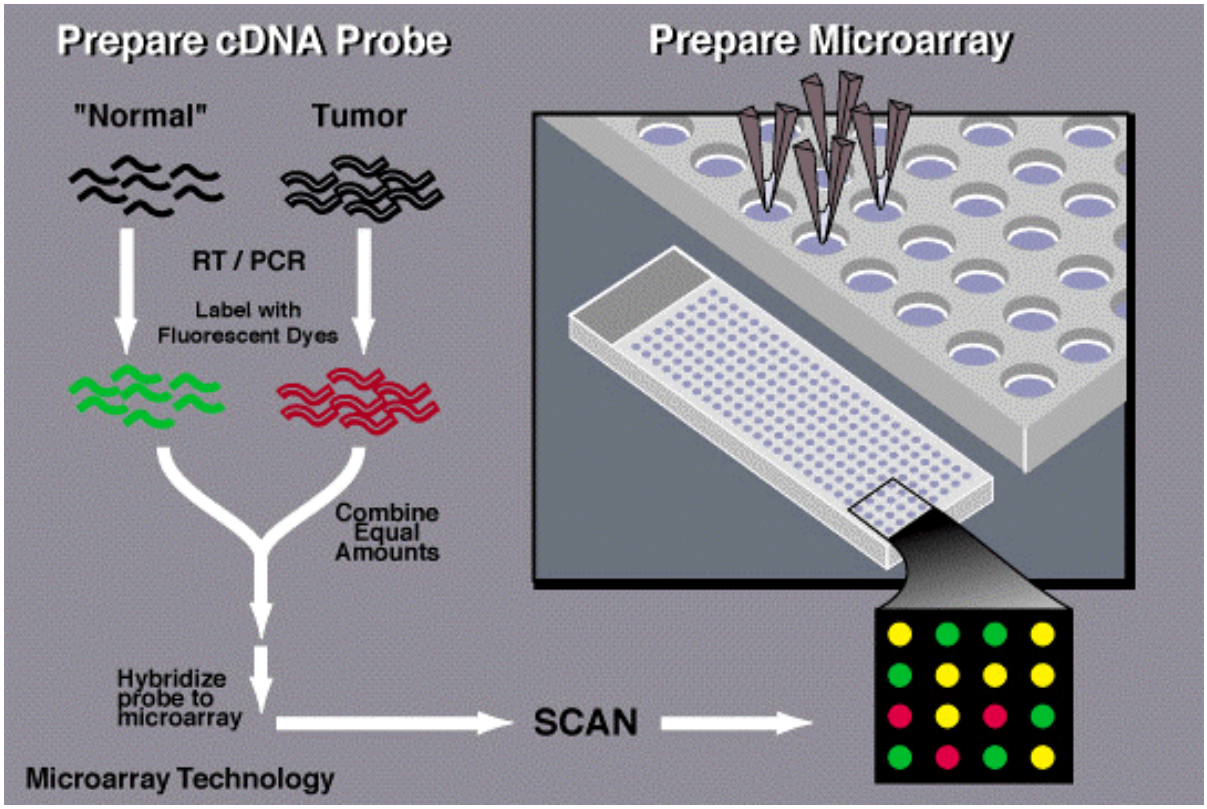


Figure 1: Overview of cDNA microarray experiment. cDNA microarrays are spotted with either ~21 000 PCR products amplified from the Research Genetics Human Clone Set or ~15 000 PCR products amplified from the National Institute Of Aging Mouse Clone Set. cDNA microarray hybridizations always compare two RNA pools or samples. One pool of RNA is reverse transcribed in the presence of Cy3-dCTP which generates cDNA that fluoresces green and the other RNA pool is labeled with Cy5-dCTP which fluoresces red. If a given mRNA species is equally represented in each pool of RNA, both red and green labels will bind to the appropriate spot on the array equally. In this case, the combined red and green fluorescence will be yellow. If a given mRNA species is more abundant in the Cy3 labeled RNA pool relative to the Cy5 labeled RNA pool, the spot on the array that it binds to will have more green than red label bound to it and will therefore appear 'greener'. The color intensity of a given spot on the array is quantitative. Consequently, cDNA microarray experiments yield quantitative results that are ratios of red/green intensities.

A) Design of Experiment

Microarray experiments performed with spotted arrays generate red to green ratios of expression which are not absolute expression levels. Consequently, one cannot legitimately compare expression profiles from multiple experiments without a common reference. The common reference serves to provide a baseline comparison of the experimental RNA sample. The best common reference is a "biologically matched" one that is related to all the experimental samples. Such a reference allows multiple experiments to be compared AND the actual expression values have biological meaning. For a transcriptional profiling experiment of multiple human hepatocellular carcinoma samples, a good common reference would be normal human liver RNA.

The use of a common reference has frequently been used by the microarray groups at Stanford in their large studies of transcription profiles of many human tumors or cell lines. They have commercialized a product called "Universal Human Reference RNA" sold by Stratagene (catalog 740000 gives 400 ug). Its a mixture of 10 human cell lines isolated from diverse tissues. For mouse arrays, one can use 16 day old embryo RNA that has been prepared in the Microarray Lab by Paul Norton. I have used this RNA for experiments and it labels beautifully! Both of these references are not "biologically matched", but they are nevertheless useful since they allow multiple experiments to be compared.

Since cDNA microarray experiments compare two RNA pools, it is important that we remove systematic variation inherent in the generation of fluorescently labeled cDNA that is hybridized to the array. One element of this variation is caused by the different quantum energy released by each dye. The mathematical correction of systematic variation in cDNA microarray results is generally referred to as normalization. Many methods exist to normalize microarray experiments. The normalization method used presently at the Van Andel Research Institute is to assume that the median signal intensity of the green channel is equivalent to the median signal intensity of the red channel.

Despite the use of normalization, an important point to consider in experimental design is color bias. The incorporation efficiency of Cy3 and Cy5 dyes into cDNA probes is not identical and can depend on signal intensity. In addition, there can be color

bias in an array that is spatially dependent. For instance, some arrays look 'greener' near the bottom of the array compared to the top of the array. For these two reasons, gene expression artifacts, such as false positives, can appear if color bias is not taken into account. Fortunately, there is a stunningly simple way to deal with this problem of color bias. All arrays should be performed at least in duplicate and replicate arrays should be reciprocally labeled. This is sometimes referred to as 'color swapping.' Specifically, if your experimental sample is red and your control sample is green in the first array, the replicate array should be labeled with a red control and a green experimental. (When the array data is processed downstream, a simple switch will be made so that all the ratios of gene expression are in the same direction). If a given spot on the array changes in expression in reciprocally labeled replicate experiments, a very high level of confidence can be ascribed to this change.

The above discussion on color bias in microarray experiments is based on the following paper:

Yang YH, Dudoit S, Luu P, Lin DM, Peng V, Ngai J, Speed TP. Related Articles Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation.
Nucleic Acids Res. 2002 Feb 15;30(4):e15.

B) Isolation and Purification of Total RNA

The generation of fluorescently labeled cDNA from total RNA is critically dependent on the integrity and cleanliness of the RNA. RNA is easily degraded by the ubiquitous presence of RNases. It is therefore essential that care is exercised at each step to inactivate RNases in the sample and to avoid their introduction through carelessness. All items used in the preparation of RNA should be touched only with gloved hands. Water should be DepC treated. There are other precautions to take and these are listed in Appendix A: Precautions to take while working with RNA. Included are Technical Bulletins 159 and 178, both from Ambion (The RNA Company).

It is also important to minimize the level of contaminating DNA in total RNA preparations. In my experience, the most efficient and rapid method for the isolation of intact total RNA is TRIzol (Invitrogen, formerly GIBCO). This method is based on the use of acid phenol to inactivate RNase in the sample and requires a subsequent extraction step. I customarily use two to three fold more TRIzol than suggested by the manufacturer's protocol to help minimize the co-isolation of contaminating DNA. Subsequent precipitation of total RNA with lithium chloride also removes much of the contaminating DNA. I also use phase lock gel heavy tubes (Brinkmann-Eppendorf) which locks away the phenol/chloroform interphase and allows complete recovery of the RNA containing aqueous phase. Once the total RNA is isolated with TRIzol, I have found that an additional purification step with lithium chloride greatly enhances the efficiency with which the RNA is able to generate good quality hybridization probes. The following protocols are presented below:

i) Isolation of total RNA from tissue and tissue culture cells using TRIzol.
ii) Purification of RNA by lithium chloride precipitation

B. i) Isolation of total RNA using TRIzol and Phase Lock Gel

1. Homogenization.

i. Tissues samples: (It is imperative that your tissue remain frozen before you homogenize in TRIzol). Wrap tissue sample in heavy duty aluminum foil and dip into liquid nitrogen using a set of locking forceps. With locking forceps, place the sample wrapped in foil on a steel block that has been sitting on dry ice for at least one hour and gently pound with hammer that has also been cooled on dry ice. This will pulverize the tissue into a powder. Quickly dip into the liquid nitrogen again. The pulverized tissue is then put into a waiting 50 mL Falcon tube filled with the appropriate amount of TRIzol. Use 10 mL of Trizol for each 200 mg of tissue. By my estimation, 200 mg of tissue is about the size of a pea or kernel of corn. I use about 20-30 mL of TRIzol for a piece of tissue the size of a kidney bean. Once the powdered tissue is in the TRIzol, homogenize on full speed for at least 30 seconds using a homogenizer such as the "Tissue Tearor" from Fisher (catalog 15-338-55). Continue to homogenize until you see no bits of tissue!

ii. Cells Grown In Monolayer: Aspirate media and immediately add 10 mL TRIzol to each 100 mm dish or 25 mL trizol for each 150 mm dish. Pipette goopy suspension up and down several times to make sure that all the cells have been dissolved.

2. While the tissue sits in TRIzol for 2-3 minutes, spin down Phase Lock Gel Heavy tubes (Brinkmann-Eppendorf catalog 0032 005.330) for one minute to collect the gel at the bottom of the tube.

3. Transfer sample into the Phase Lock Gel tube and add 0.2 mL chloroform for each 1 mL of TRIzol added at the homogenization step. For example, if you homogenized your sample in 10 mL TRIzol, add 2 mL chloroform to the tube. Shake tubes vigorously for 15 seconds. Do not vortex.

4. Centrifuge tubes in an Eppendorf 5710 centrifuge for 25 minutes at full speed.

5. Precipitate RNA by first transferring the aqueous phase to a Corex tube and subsequently adding 0.5 mL isopropyl alcohol per 1 mL TRIzol used initially. Cover with a piece of parafilm and invert several times to mix.

6. Centrifuge in an SS34 rotor in the Sorval Super Speed Centrifuge at 9000 g for 15 minutes.

(Steps 5 and 6 can be performed in an Eppendorf 5710 centrifuge using 15 mL or 50 mL Falcon tubes. Centrifuge samples at the maximum speed for 50 minutes. This will not give the most efficient recovery of RNA. I have found it acceptable for RNA from tissue culture cells since I usually have excess RNA at the end of the day).

7. Decant supernatant and rinse RNA pellet with 10-20 mL of 75% ethanol depending on the size of the tube being used. Give the tube a quick vortex (covering with parafilm for Corex tubes....) and centrifuge for 5 minutes using previous conditions.

8. Decant supernatant and centrifuge tube for 1 minute. Remove residual ethanol with a pipettor and immediately re-suspend RNA in DepC treated water (Ambion catalog 9915G). Use 800 uL water if you intend to further purify your RNA using LiCl precipitation. Pipette up and down to completely dissolve the RNA pellet. Never let the RNA pellet dry since it will be very difficult to dissolve!

B. ii) Purification of RNA by LiCl precipitation

1. Add 0.5 volumes of 7.5 M LiCl solution (Ambion catalog 9480, 100 mL bottle) to RNA sample and vortex. For RNA in 800 uL water, add 400 uL 7.5 M LiCl solution.

2. Place tube at -20 C for at least 1 hour (overnight works well) and subsequently centrifuge for 15 minutes at full speed in a table top Eppendorf centrifuge (at room temperature is fine).

3. Decant supernatant and wash pellet with 1 mL of 75% ethanol. Add ethanol, vortex quickly and spin for 1 minute.

4. Decant ethanol and repeat with another 1 mL of 75% ethanol.

5. Decant ethanol and spin tube quickly to collect residual ethanol. Remove residual ethanol with a pipettor and immediately add DepC treated water to dissolve the RNA. Try to re-suspend RNA so that it is at a final concentration of between 2 and 5 ug/uL. This is usually between 20 and 200 uL of water. For a tissue fragment the size of a pea or kernel of corn, use 50 uL water. For each 100 mm dish of 80% confluent cells, use about 100 uL water. These are rough guidelines.

C) Generation of Fluorescently Labeled cDNA and Microarray Hybridization

The following protocol describes the generation of cDNA probes using 50 ug of total RNA for each color channel and suitable conditions for microarray hybridization.

C. i) Ramsi's MicroArray Protocol For Total RNA, 3/14/02

This protocol requires 50 µg of each type of RNA to be compared for each microarray. The word probe will be used to describe the labeled cDNA that binds to the DNA on the microarray.

Reverse transcription of probes.

1. Start with 50 µg RNA of each type in 9 µL water.

2. Have the following temperatures available:
   a) 37°C, 50°C water baths
   b) 42°C, 68°C and 95°C heating blocks
   c) Thaw 5X 1st strand buffer, 0.1M DTT, oligo $d(T)_{20}VN$, Cy3-dCTP, Cy5-dCTP, 10X Low dC/dNTP, microarray pre-hybridization and hybridization solution.

3. Anneal oligo dT primers to RNA.
   a) Add 1 µL dTVN primer (0.5 µg/µL) and incubate 5 minutes at 68°C
   b) Give a quick spin to collect condensation. Keep at room temperature or solution will get too viscous.

4. Prepare 2 separate tubes of each fluorescent mix.

	(X4.5)	(X 5.5 reactions)
a) 4 µL 5X 1st strand buffer	18	(22 µL)
b) 2 µL 0.1 M DTT	9	(11 µL)
c) 2 µL 10X low dC dNTP	9	(11 µL)
d) 2 µL Cy3 or Cy5 dCTP	9	(11 µL)
e) 0.5 µL RNAsin	2.2	(2.7 µL)

   f) This should give a total of 10.5 µL.

5. Mix annealed RNA with appropriate fluorescent mix.

6. Add 1 µL SuperScript II RT per RNA reaction and incubate at 42°C for 45 minutes.
   Using another pipettor at ~15 µL, pipette up and down to mix. This mixing step is VERY important. Since Cy5 and Cy3 are light sensitive, cover tops of tubes with foil during reaction. Do not let the temperature exceed 44°C.

7. Add another 1 mL SuperScript II RT per RNA reaction, mix with 15 mL pipette, and incubate at 42°C for another 45 minutes. Subsequently place at 94°C for 2 minutes, quench on ice and then combine reactions in one tube.

GO TO STEP 10 FOR MICROARRAY PREHYBRIDIZATION, THEN COMPLETE STEPS 8 AND 9.

8. Clean up cDNA.

a) Get rid of RNA
   i) Add 50 µL water
   ii) 10 µL 10X RNAse One buffer
   iii) 2 µL RNAse One
   iv) Incubate at 37°C for 30 minutes.
   v) This should give a total of 99 µL

b) Remove unincorporated label and small cDNA fragments using the QIAquick PCR Purification Kit.
   i) Add 500 µL Qiagen Buffer PB to probe and mix.
   ii) Apply the sample to QIAquick spin column and centrifuge at Room Temperature 45 seconds, 10 000 g.
   iii) Discard flow through, add 750 µL Buffer PE (wash buffer) to spin column and centrifuge for 45 seconds, 10 000 g.
   iv) Discard flow through and spin again for an additional minute to remove residual Buffer PE.
   v) Elute probe by adding 50 µL Buffer EB (elution buffer) to the center of the QIAquick membrane, let sit one minute and then centrifuge for one minute.
   vi) Place probe eluted from QIAquick column on a MICRON YM 50 column and centrifuge at Room Temperature for 1.5 minutes, 12400g. This should concentrate the probe to less than 15 µL.
   vii) To collect purified probe, invert column into new tube. Set centrifuge to 3000 RPM and spin for 15 seconds.
   viii) Check the volume and bring up to 15 µL.

9. Probe pre-hybridization.

a) To purified probe, add 28.8 µL probe hybridization and 2.3_µL 20X Blocking solution to probe. This gives a total of 46 µL (extra is due to evaporation during prehybridizaiton).
b) Place in 94°C heating block for 2 minutes.
c) To remove any particulate matter, centrifuge at 14K g for 5 minutes and transfer to a clean tube with a rubber o-ring cap to prevent excessive evaporation.
d) Place probe at 50°C for one hour (minimum 45 minutes) to pre-hybridize. Do not exceed 1.5 hours due to evaporation.
e) Once probe has pre-hybridized, centrifuge 5 minutes at14K g and use 32-36 µL per microarray for hybridization in step 11.

10. Prehybridize microarrays.

a) Add 34 µL of microarray prehybridization buffer solution (frozen aliquots at -20°) over microarray. This is accomplished by slowly ejecting the solution to form a drop on the pipettor tip and gently touching the drop (NOT the tip) to the middle of the microarray.

b) Place coverslip over the prehybe. Use Plastic Coverslips or you'll have a difficult time removing it later! Coverslips are sandwiched between two plastic films. Break one film by bending coverslip to one side and remove coverslip with forceps. This gives a coverslip with the plastic film on one side. Using 90° forceps, lay so that tips are on line defining arrray. Coverslips will use this as a fulcrum to prevent sliding. While holding the corner of the coverslip, place the edge of the coverslip at the border of the micro array at ~45° angle, against the forcep. A line of prehybridization solution will form at the contact. Gently lower the slide until its almost down and then let go with the forceps. Do not drop the slide from too high a height or it will move. Once the coverslip is positioned, do NOT move it.

c) Place the microarray with coverslip into hybridization chamber. Add 10 µL of 50% formamide/50% water to each dimple at the end of the CMT hybridization chamber.

d) Place cover on chamber and place in 50°C water bath. Allow prehybridization of microarray to proceed for at least two hours.

11. Remove prehybridization and add hybridization solution.

a) Rinse hybridization chamber and dry the outside of the chamber well. Make sure to keep chamber level at all times. Remove microarray from chamber and place on paper towel.

b) Remove coverslip and microarray prehybridization solution. Using the left hand, tilt the slide so that its long edge remains in contact with paper towel and an acute angle forms between the coverslip side of the slide and the paper towel. Grasp the top corner of the coverslip with forceps and while holding coverslip in place, gently tilt the slide away. The prehybe solution will be seen to separate from the coverslip just like a acrlyamide gel pulling away from a glass plate. Make sure the slide and coverslip maintain contact at the bottom edge where all the solution will gather. Once the coverslip touches the paper towel, the prehybe will blot all over.

c) Add 32-36 µL hybridization solution to each microarray and hybridize overnight. Add 10 µL of 50% formamide/50% water to each dimple at the end of the CMT hybridization chamber.

The next day, wash and scan the microarrays and scan them.

12. Prepare 500 mL of the following wash solutions:
    a) 1X SSC/0.1% SDS (25 mL 20X SSC/2.5 mL 20% SDS)
    b) 0.2X SSC/0.1% SDS (5 mL 20X SSC/2.5 mL 20% SDS)
    c) 0.2X SSC (5 mL 20X SSC)
    d) 0.1X SSC (2.5 mL 20X SSC)

13. Rinse hybridization chambers making sure to keep chambers level so that the glass coverslip does not shift. Remove microarray from the chamber and place slides in a histology staining dish filled with ~200 mL 1X SSC/0.1% SDS and shake gently so that the cover slip falls off. Prewarm the dish in tissue culture incubator and place 1X SSC/0.1% SDS in 50C water bath.

14. Place slides in a slide holder and put holder into slide dish with ~200 mL of 0.2X SSC/0.1% SDS. Shake on orbital shaker 5-45 minutes. Shake with sufficient vigor that slides are moving in their holder. Make sure to cover the slide dish with a cardboard box because the dyes are light sensitive and the ceiling is glass.

15. Transfer slides only (not the entire slide rack) to dish with 0.2X SSC, then 0.1X SSC. Each wash is for 5-45 minutes.

16. Place slides in 50 mL Corning tube and centrifuge in Eppendorf 5819r at 550 g for 7 minutes. Make certain that the back of the slide is dry and spot free.

17. Scan the microarray and analyze data.

Solutions

Low dCTP/dNTP Solution

25 µL dGTP (100 mM)
25 µL dATP (100 mM)
25 µL dTTP (100 mM)
10 µL dCTP (100 mM)
415 µL DepC water

MicroArray Prehybridization Buffer Solution

5 mL deionized formamide
3 mL 20X SSPE
500 µL 10% SDS
1 mL 50X Denhardt's
200 µL ss Salmon sperm DNA (10 mg/mL)
930 µL dd water

Assemble solution and keep 100 µL aliquots at -20C.

Probe Hybridization Solution

	X 7
14 µL formamide	98 µL
1 µL 20% SDS	7 µL
2 µL 50X Denhardt's	14 µL
8 µL 20X SSPE	56 µL

Assemble solution and keep 100 µL aliquots at -20C.

20X Hybridization Blocking Solution

40 µL poly dA (1 µg/ µL)
8 µL tRNA (10 µg/ µL)
200 µL Human (or mouse) CoT 1 DNA (1 µg/ µL)
28 µL 3M Sodium Acetate (pH 5.2)

Ethanol precipitate with 700 µL ethanol.
Wash 2 X 1mL X 75% Ethanol and dissolve in 20 µL water.

C. ii) cDNA Microarray Parts List

The following page has a list of supplies required to perform cDNA microarray hybridizations. Most items are available in the Invitrogen Freezer Program. We also have Cy-dye dCTP which is purchased using a bulk discount.

Company	Cat. Number	Description
Amersham Pharmacia Biotech	27-7836-01	poly d(A) sodium salt or use Sigma poly d(A)
	PA-53021	FluoroLink Cy3-dCTP
	PA-55021	FluoroLink Cy5-dCTP
Ambion	9915G	DepC treated water
	9480	7.5 M LiCl solution
Brinkmann/Eppendorf	0032 005.330	Phase Lock Gel-Heavy, 50 mL tubes
Qiagen	28104	QIAquick PCR purification Kit (50 columns)
Roche	799-017	Rnasin (2000 U)
	1467140	ss Salmon Sperm DNA
PGC Scientifics	62-6504-04	22 X 40 mm hybrislip, pack of 100
Fisher	42416	Microcon-50 microconcentrators (Amicon no. 42416)
	2551	CMT Hybridization Chamber (Corning no. 2551), 5 chambers
	08-812	Histology staining dish (horizontal)
	08-953-E	Jewelers Microforceps, 10.8 cm (straight)
	13-812-14	Kelly Forceps, curved, 140 mm
	12-548-5J	24 X 40 mm glass coverslips, 1 oz pack
	05-669-39	1.5 mL microfuge tubes with screw cap O-ring seal, 500 tubes
Gibco/BRL Invitrogen	15279-011	Human Cot-1 DNA, 500 ug
	18064-014	SuperScript II RT.
	18440-016	Mouse Cot-1 DNA (if required)
	10297-018	100 mM dNTP Set
	15596-026	TRIzol Reagent, 100 mL (200 mL bottle is also available)
	15634-017	Herring Sperm DNA, 5 X 1 mL or below
	15632-011	Salmon Sperm DNA, 5 X 1 mL or above
Promega	PRM-4261	Rnase One
Sigma	D-2532	Denhardt's Solution 50X concentrate, 5 mL
	S-2015	20X SSPE, 1 L
	S-6639	20X SSC, 1 L
	F-9037	Formamide, 100 mL
	P-9403	Poly d(A) or use Pharmacia Amersham.
	R-7876	2500 U tRNA (type V wheat germ)
Ambion	9906	10 X 50mL DepC treated water
	9480	100 mL 7.5 M LiCl solution for RNA purification, 100 mL

D) Scanning Microarrays

D. i) File Organization and cDNA Microarray Image Analysis

The analysis of cDNA microarray data results in an impressive deluge of image and data files. Without proper care and organization, much time will be lost looking for specific files at a later time.

It is necessary to save the scanned TIFF files of array experiments on the VAI network drive because of the security policy of the scanning computer. Please do NOT save image files on the scanning computer. I suggest that you create a subfolder in the "General Lab Information" folder. In this folder, create a subfolder for each set of microarray experiments. If you are treating five cell lines with the same drug, for instance, then all of these arrays would be kept together along with their reciprocally labeled replicates. When you save microarray TIFF files from the scanner, save them as the microarray number appended with _Cy3 or _Cy5, as required. Your lab book is where you have a record of which RNA sample was labeled with which Cy dye.

After scanning the images, downstream analysis will involve the generation of GenePix grid files (.GPS) and GenePix results files (.GPR). Once your arrays have been submitted to a microarray database, multiple experiments will be extracted together, usually as tab delimited text files (.TXT). It is best to save each of these files under the name of the array that they originated from. In addition, each type of file should be stored together. That is, the grid files should go together in a folder called 'grids' and the GenePix results files should go into another folder called 'GenePix Results.'

To continue the example of five cell lines treated with the same drug, I would create a folder in 'General Lab Information' called 'Ramsi Microarray.' There would be a sub-folder in here called 'Drug X-Melanoma cell lines'. In this sub-folder would be four sub-folders with the following names: 'SCANNED IMAGES', 'GRIDS', 'RAW DATA' and 'AMAD EXTRACTED DATA.' The contents of the first sub-folder would be the original scans from the scanner, the next sub-folder would hold the aligned grid files (.gps) from GenePix, the next sub-folder would hold the GenePix results files (.gpr) and the last sub-folder would hold the data extracted from the AMAD database (.txt). When

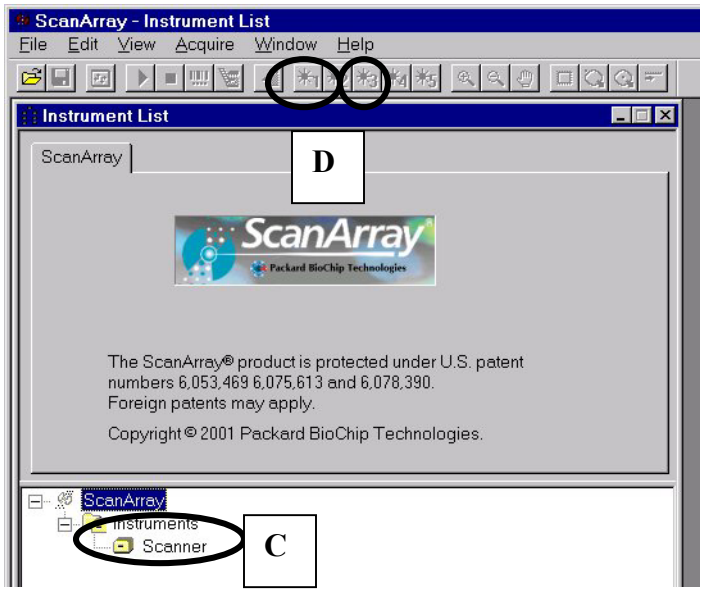
this analysis extends into the generation of clustered image maps, I would create another sub-folder called 'CLUSTERED IMAGE MAPS.'

I can only suggest this system because it is the one I have used for all my experiments and it has served me very well. If you do not want to use it, by all means devise your own system. But please make sure you have a system!

D. ii) Scanning Microarrays With Scanarray Lite

Once the microarray has been hybridized and washed, the fluorescence must be measured with the scanner. Before using the scanner, it is necessary to sign it out by marking your name and the time you want to use it on the white board in the room that houses the scanner. If you have any trouble, anyone in the Microarray Lab would be pleased to assist you.a) Make sure the scanner is on and ready as evidenced by the green LED lights on the front labeled "Power" and "Ready". Otherwise, switch the device on. The on/off switch is on the right hand side of the instrument.

b) Make sure the scanner software is running. If you do not see the screen below, then start the software. There is a shortcut on the desktop labeled "ScanArray". Once the program is on, you will see the following screen:

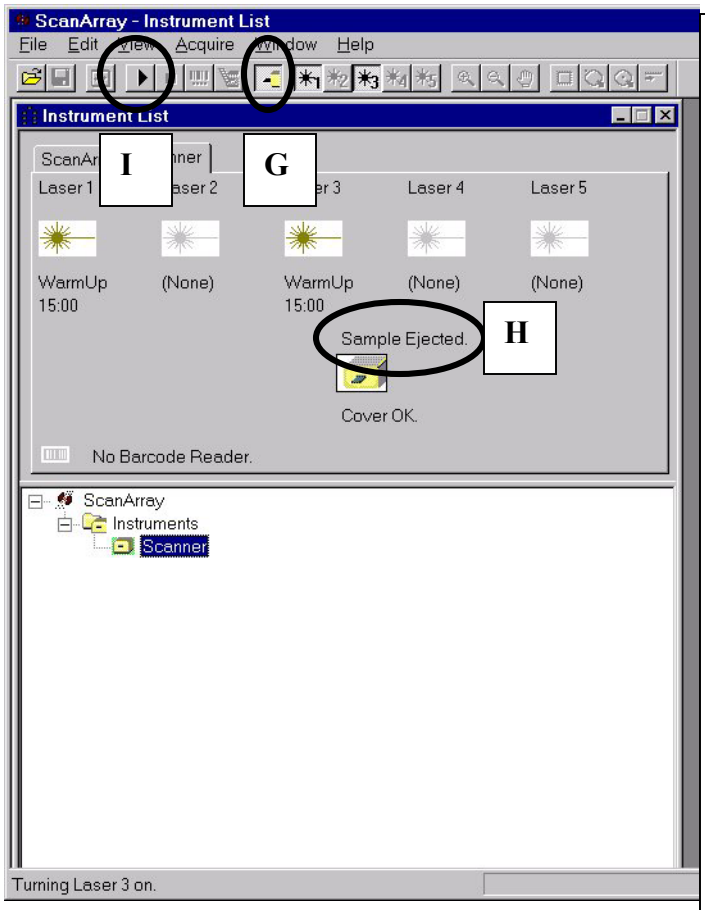


ScanArray Start up Screen Section:

c) Double click on Scanner which is close to the bottom of this picture on left. After a few seconds, several of the buttons on the toolbar will no longer be inactive.

d) Turn on lasers 1 and 3 and wait 15 minutes for them to warm up. This is performed by clicking on the 9th and 11th button on the toolbar starting from the left. The buttons have the following symbols in them: "*1" and "*3". Do NOT use the instrument while the lasers are warming up as inaccurate results will be obtained.

e) Once the lasers have been turned on, the following screen will change slightly to show the progress of the lasers warming up.

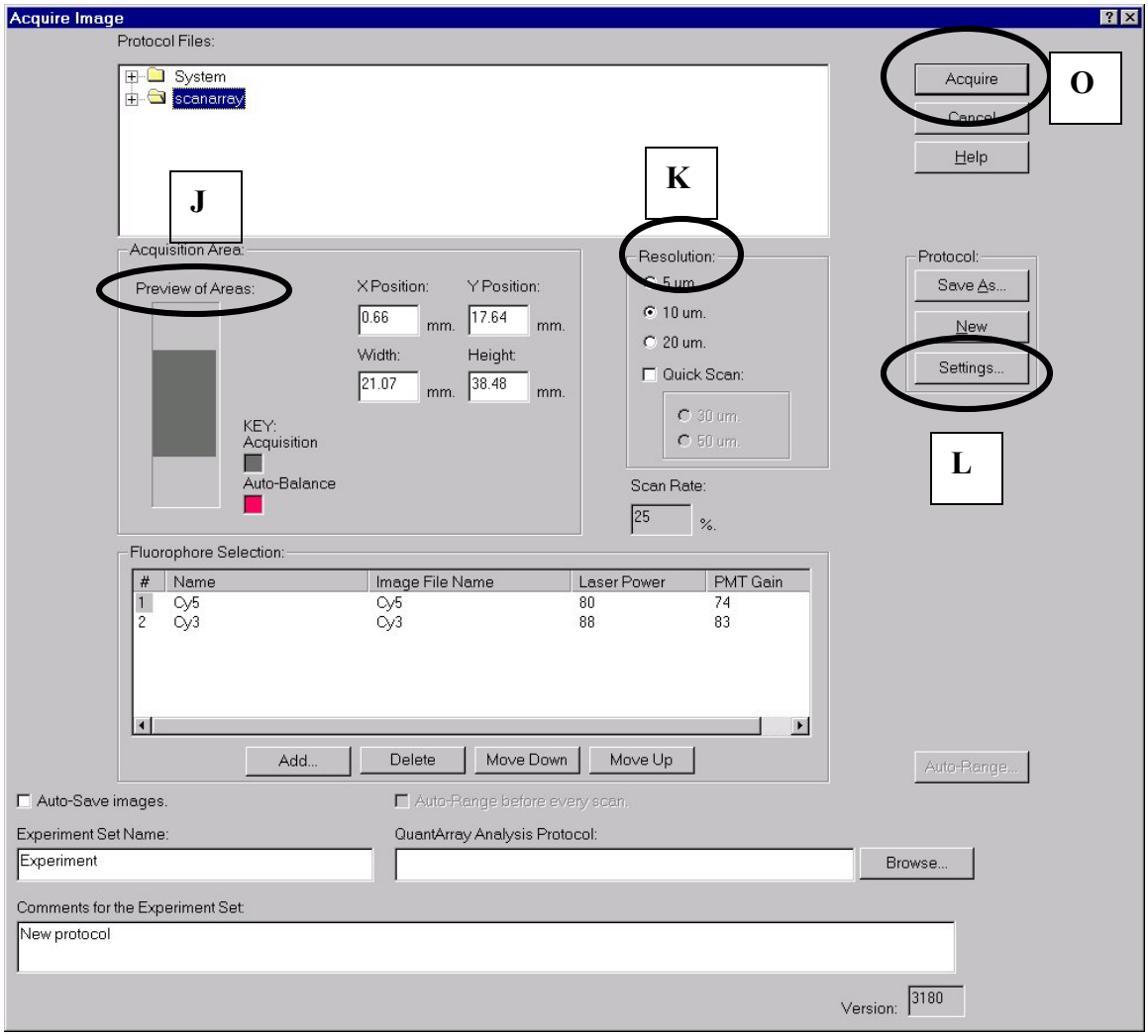


f) Once the lasers have warmed up, insert the slide into the scanner with the array side up and the label out.

g) Next, press the button immediately to the left of the laser 1 button "*1" and this will take the slide into the machine.

h) Once the slide is ready to be scanned, the screen will say "Sample Loaded". This will appear in the same place where it is written "Sample Ejected" on the screen to the left of this text box.

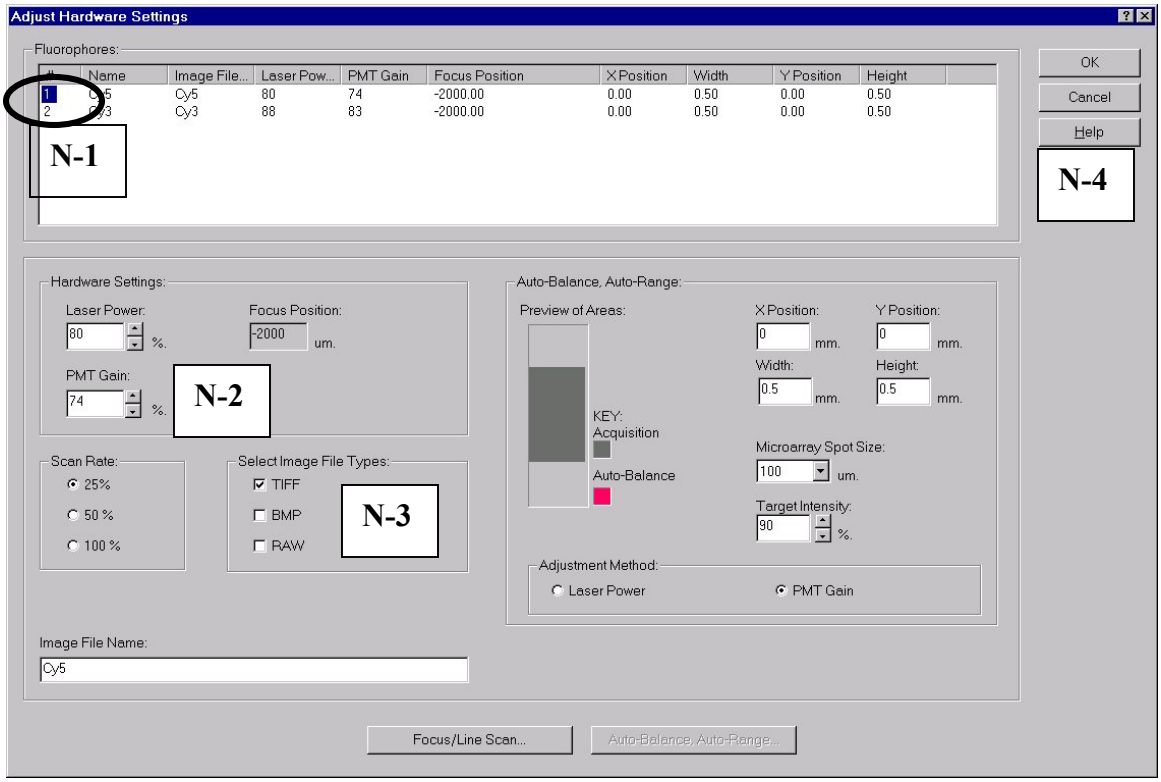
i) When the sample has been loaded, press the "play" button. This is the 4th button from the left. The screen will then look quite different and is shown on the next page.



j) Set the "Preview of Areas" by dragging a box that will be certain to include the entire array. Don't worry about drawing a box that is too big, it will be resized. It is important that it is not too small since such a box cannot be resized. What is shown above should include the entire array.

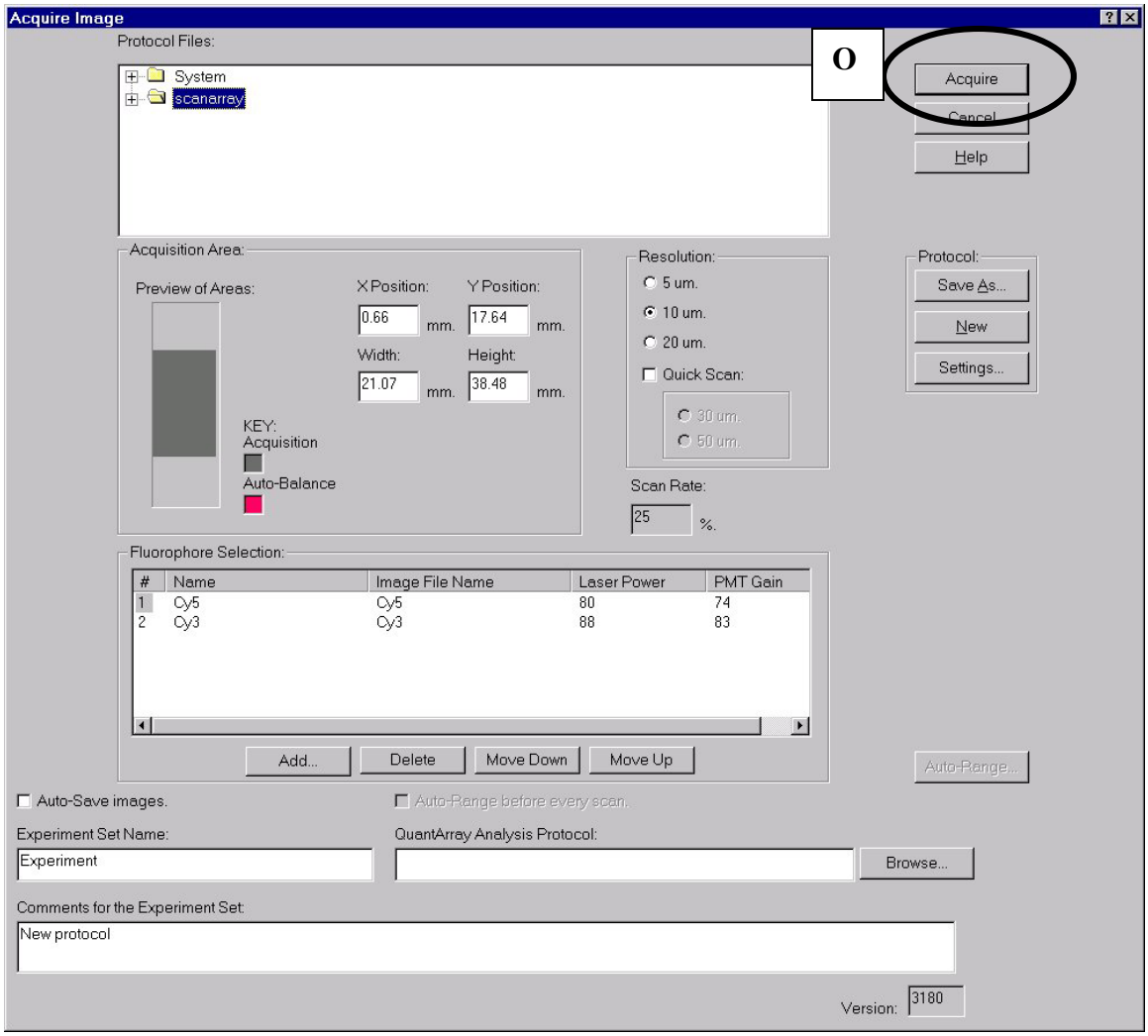
k) Next, make sure that the resolution is "Quick Scan". Directly below this button is an option to use 30 um or 50 um. I usually use 50 um.

l) To set the laser power, press the "Settings" button which will reveal a new screen shown below.

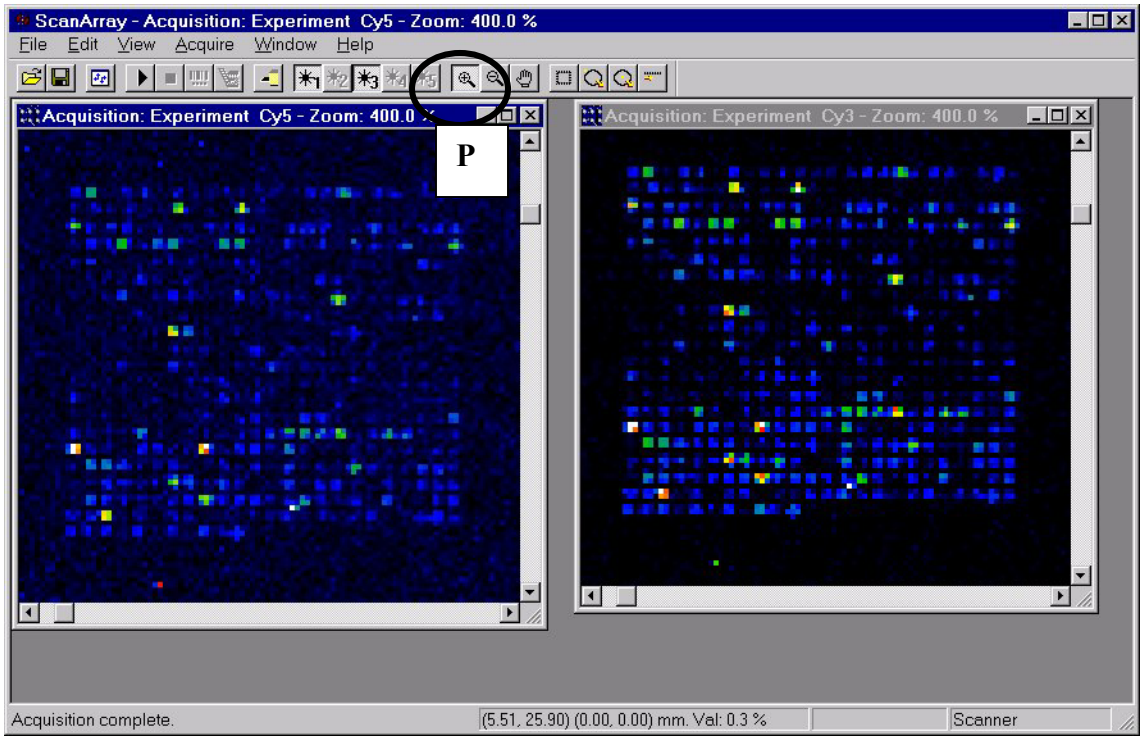


m) There are two settings that control the image acquisition: laser power and PMT gain (photomultiplier tube gain). These settings can be altered for the Cy5 and Cy3 channels separately. Several test scans will be performed after changing these settings so that the Cy5 and Cy3 images appear similar in intensity while minimizing the number of saturated spots. In addition, it is benificial to minimize PMT gain before reducing laser power. PMT gain controls the detector. It has been my experience that the lower the PMT gain, the lower the noise and the better the images appear. For these reasons, the aim is to reduce the PMT gain to about 80% while making the two images relatively equal.

n) Click on the number 1 or 2 to adjust the laser power and PMT gain for each channel. It is best to start at 90% laser and 90% PMT gain for each channel. After clicking on the number in the "Fluorophores" window (N-1), the laser and PMT gain settings can be changed in the "Hardware Settings" window (N-2). Make sure the "Selected File Type" is TIFF (N-3). Once these settings have been changed, press the OK button (N-4) to return to the previous screen, part of which is shown below.



o) Press the "Acquire" button and the scan will start (The acquire button is labeled O in the image above step j). The program will usually give you a warning reminding you that the settings have changed and will inquire whether you want to keep these changes. The comment is reproduced below. Just answer yes. The program will then scan the image in the Cy3 and Cy5 channel. Once the scan is complete in both channels, the two images can be compared.



p) Pictured above is a sample scan where each channel has been magnified on one of the 32 grids that make up a typical array at Van Andel Institute. The easiest way to tell whether the two channels have similar intensity is to look at one grid from both channels using the magnification tool. This particular grid is representative of the others on the array. The color shown in these images is a gradient where blue represents the weakest intensity followed by green, red and white. White represents saturating spots. If the Cy5 channel is dimmer (it usually is), make note of this and also determine the frequency of white, saturated spots. We do not want more than 2 or 3 white spots per grid, on average. If there are more than 4 or 5 white spots per grid, then turn down the laser power (2%) or PMT gain (8%) for the next test scan. Generally, turning down the laser power 1% is about the same as turning down the PMT gain by about 4%. This may sound a bit confusing, but is worth understanding since less mathematical manipulation of your data will be required at future steps. Generally, one must alter the one channel relative to the other to make the two channels similar. As well, it is usually necessary to change both channels to either make the total intensity weaker or stronger.

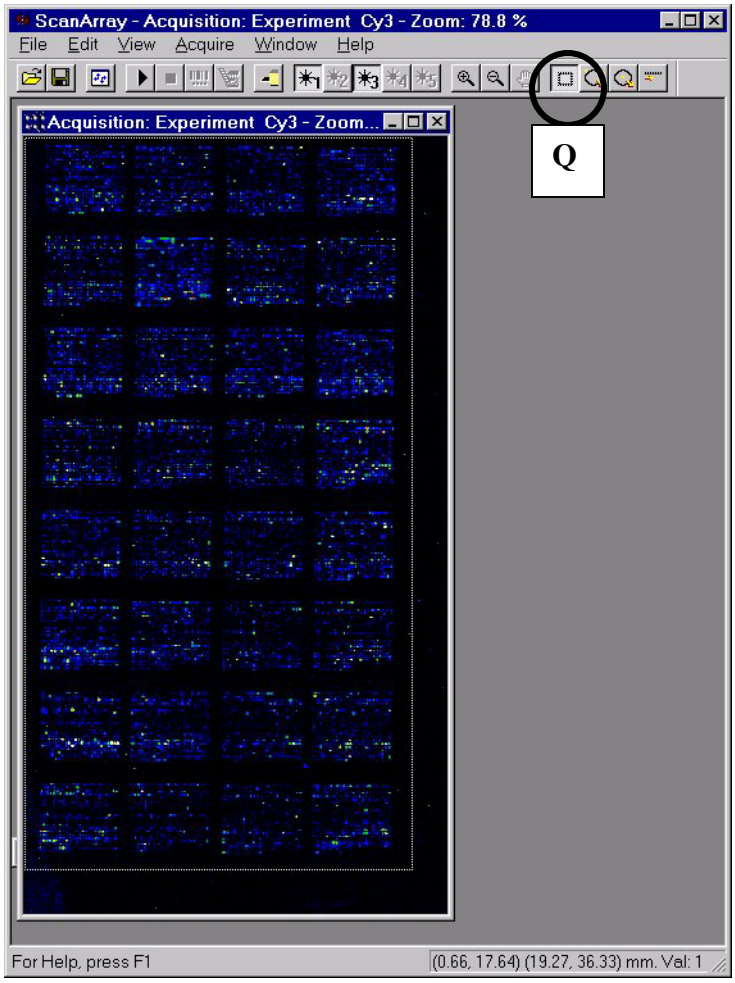
It is important to remember that some spots may be of different intensity in the two channels since different samples of RNA are usually being compared. These are

usually obvious and present themselves on a background of spots that are of similar intensity in both channels.

In the image above, the intensity of spots is relatively similar between the two channels. The Cy3 spots do appear a bit brighter as evidenced by the “hotter colors” they show. The Cy5 channel has about 2 spots with white in them. This indicates that only two spots are approaching saturation. For all of these reasons, I would leave the Cy5 channel alone and turn down the laser power on the Cy3 channel by 1%. Then I would do one more test scan to verify that such a change would make the two images relatively equal and not have too many saturating spots.

Notice that the background is higher on the Cy5 channel. That is, the background is not as black. I scanned an old slide and the Cy5 dye has begun to break down. In order to get good signal strength, the scan above in the Cy5 channel was performed using 97% laser power and 90% PMT gain. The Cy3 channel is at 85% laser power and 80% PMT gain. To decrease the background, it would be better during the next test scan to also change the Cy5 channel to 99% laser power and 84% PMT gain. The basic rule is that a change of 1% laser power gives a similar change in signal intensity as a 4% change in PMT gain. This is a rough guide gleaned from scanning hundreds of slides.

q) Before changing the settings as described above and subsequently re-scanning, close either the Cy5 or Cy3 image and do not save when prompted by the computer. With the one remaining image, press the 17th button from the left (its a square). Then drag a rectangle over the array making sure to include the entire array and not too much non-array area. Once the rectangle is of the appropriate size, right click the mouse to reveal a menu. One of the options will be "Use area as acquisition area". Select this option and the program will revert to the normal screen. I cannot take a screen snap-shot of this event, but I can show the screen just before you do this:



r) At this point, press the "Settings" button to alter the laser power and PMT gain according to your educated guess (described in step n). It is important to write down the numbers at each step to build an appropriate feeling of what changes are required for a given array. Re-scan the array and determine what new changes to laser power and PMT gain are required.

s) Once you are satisfied that the Cy5 and Cy3 channels are of similar intensity and that the number of saturating spots in each image are less than 2 or 3 per grid, you can change the scan "Resolution" to 10 um and scan (changing resolution is described in step k). Such a scan will take close to 10 minutes per channel.

t) After the scan is complete, use the "File" menu to save each image. I find that it is best to save images under the slide name followed by an underscore character and the color channel. For instance, microarray M6-117 would be saved as M6-117_Cy5 and M6-117_Cy3.

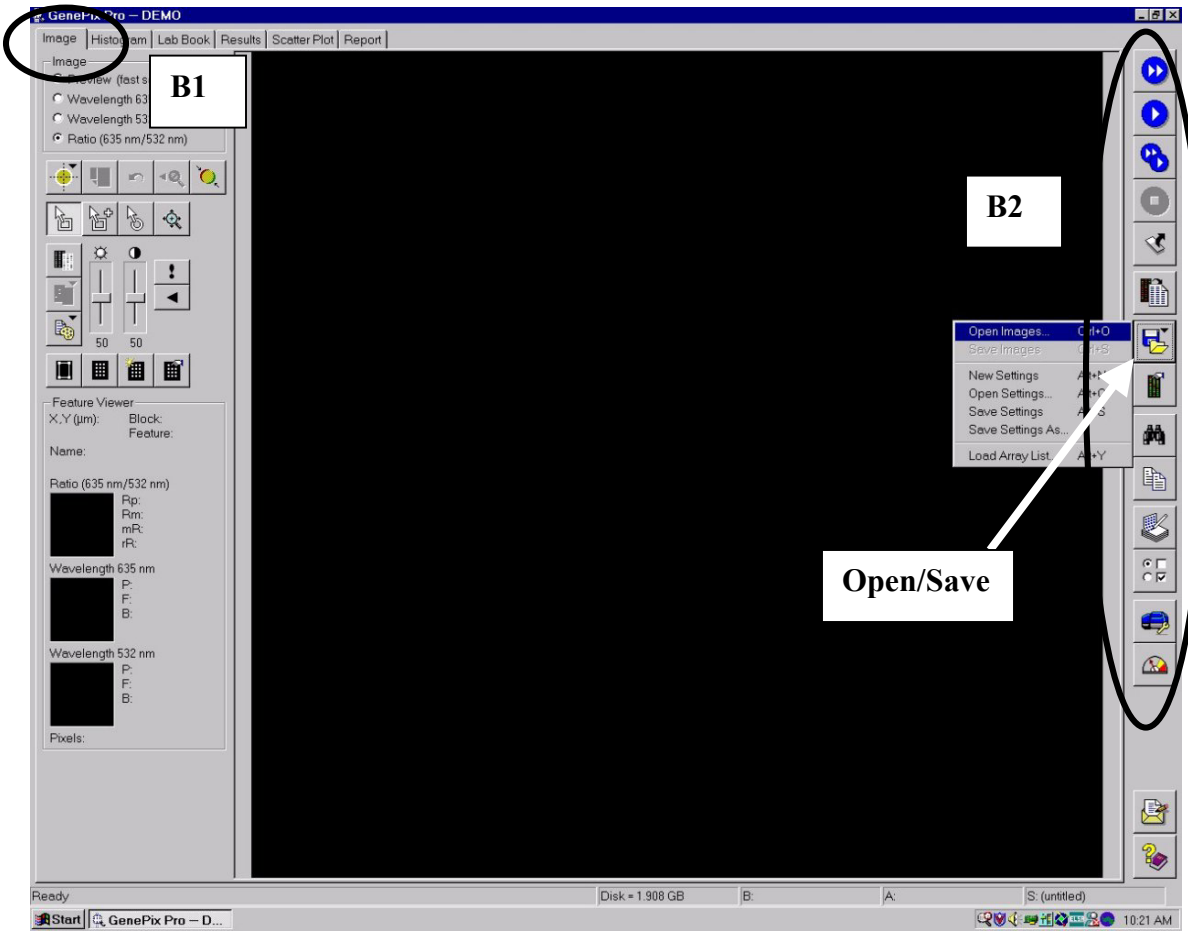
E) Image Analysis Using GenePix Software

Once the microarray has been scanned, the information in the two color images must be extracted. This step and subsequent steps are strictly computer based. We use a program called GenePix 3 Pro to analyze the microarray images. The program loads in the Cy3 and Cy5 fluorescence scans and presents a combined image. GenePix allows you to easily draw a little circle around each microarray spot and gives numerical values for the intensity of each spot in both color channels. The entire process is interactive and is not completely automated. It will take about 20 or 30 minutes for an experienced user.

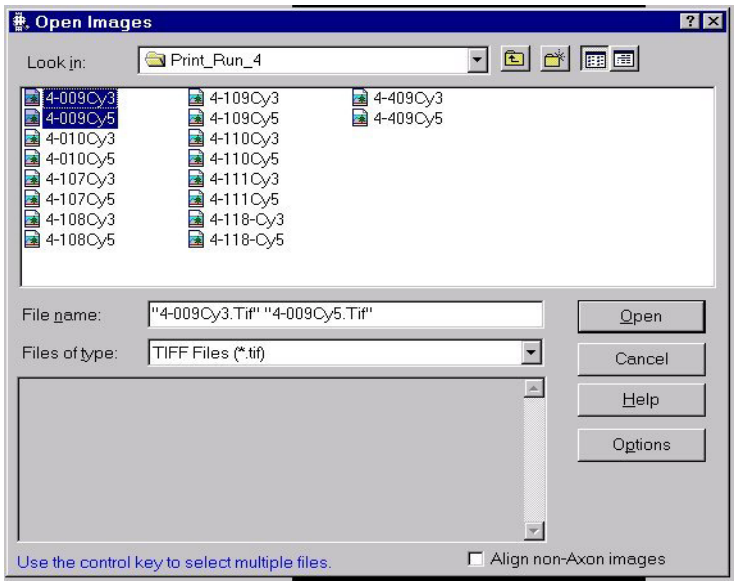
E. i) Instructions on how to use GenePix Pro 3.0

a) Start GenePix Pro 3. If you do not have it up and running, it is available in the MICROARRAY folder in "General Lab Information" on the network. There is a folder called "Software" in MICROARRAY and it always has the most recent installer version in it. GenePix will not save data unless you have a software key (the so-called 'dongle'). We have a total of 10 software keys for both parallel and USB ports. See Paul Norton in the Microarray Lab to sign one out. Make sure you have the software key plugged in before you attempt to install the software.

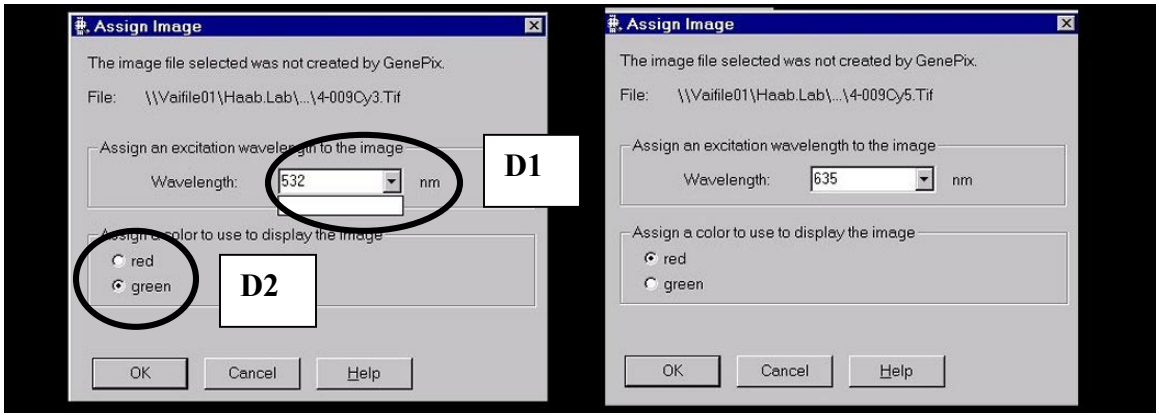
b) Open Images. The start-up screen is shown below. On the very top of the screen, there are a series of tabs of which "Image" is selected (B1). Once the cDNA spots have been overlaid with a grid of circles around each feature and the numerical data has been extracted, other tabs will be invoked to quickly analyze the quality of the data. On the right hand side there is a vertical tool bar that has buttons for file operations (B2) (open, close, etc.) and analysis functions. The "Open/Save" button has been pressed and reveals a small menu of which the first option is "Open Images...".



c) Chose the files to open. It is necessary to specify the two image files to open. One file holds the data from the Cy3 channel and the other file is required for the Cy5 channel. As shown below, chose the files to open by selecting the appropriate file names while depressing the shift or control key in the usual Microsoft Windows manner and then click on Open.



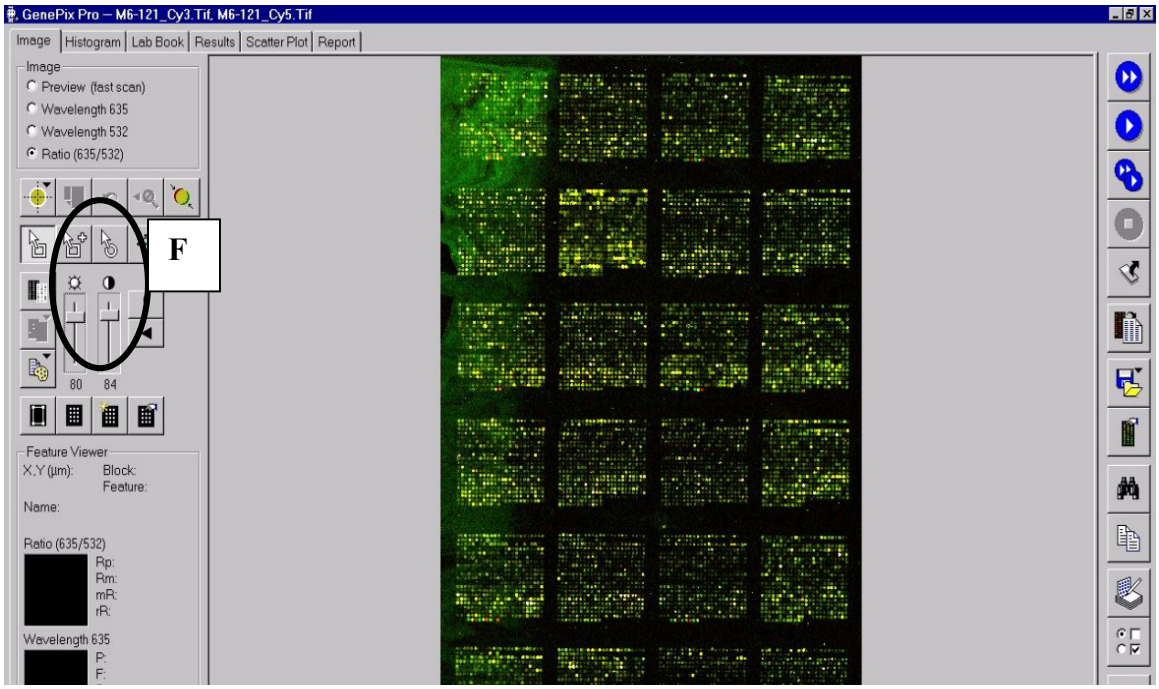
d) Assign color and wavelength to the images. After clicking on the Open button, a window will appear asking for the wavelength (D1) and color (D2) to be assigned to the first image file. Once the wavelength and color are assigned, click OK and the next window will appear. GenePix will make a random assignment. Cy3 should be assigned the color green and wavelength of 532 nm (which you need to enter the first time, but will be part of the drop down menu in the future). Press the OK button and the next Assign Image window will open. Cy5 is red and assigned a wavelength of 635 nm.



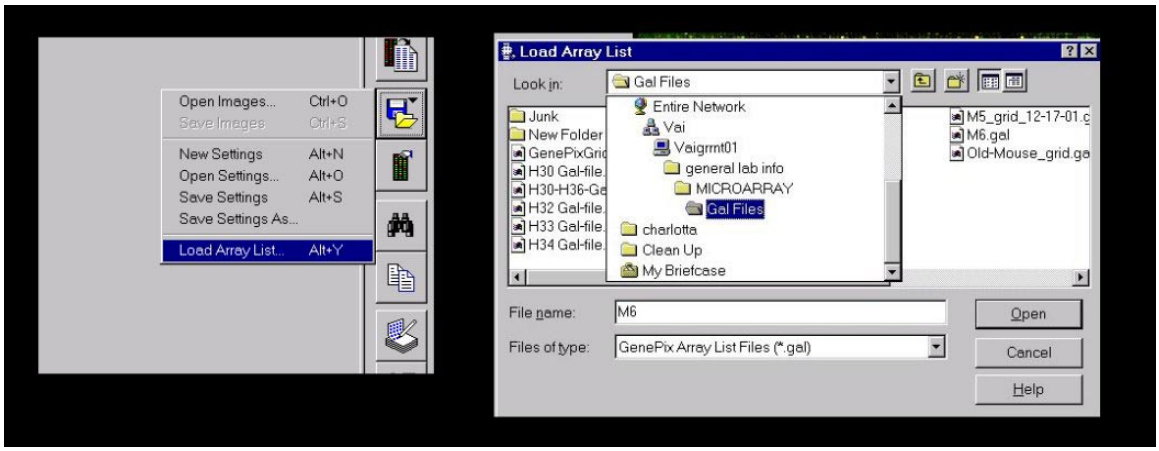
e) **Color swapping experiments.** For experiments involving reciprocal labeling of replicates, this color assignment is useful for simplifying data analysis. The images from the first experiment are loaded into GenePix as described above. That is, the Cy3 image is assigned to the green color with a wavelength of 532 nm and Cy5 is assigned red with a wavelength of 635 nm. The data is extracted and saved in the usual manner.

The reciprocally labeled replicate experiment is loaded into GenePix backwards: the Cy3 channel is assigned to the RED color with a wavelength of 635 nm and the Cy5 channel is assigned to the GREEN channel with a wavelength of 532 nm. Once the data is extracted, the results file is saved as the name appended with "_R" (for reverse). In this manner, the ratios of the original experiment and the reciprocally labeled replicate experiment will be in the same direction and have the same biological meaning.

f) Adjust brightness and contrast. After the images are opened, the contrast and brightness controls can be altered to visualize the cDNA spots more easily. The top half of the screen is shown below. It is best to have the contrast slider (under the half filled circle) and the brightness slider (under the white circle) at similar levels to get the best picture quality. These settings are strictly for your viewing pleasure and have no effect on the final extracted data. Contrast and brightness settings can be altered at any time.

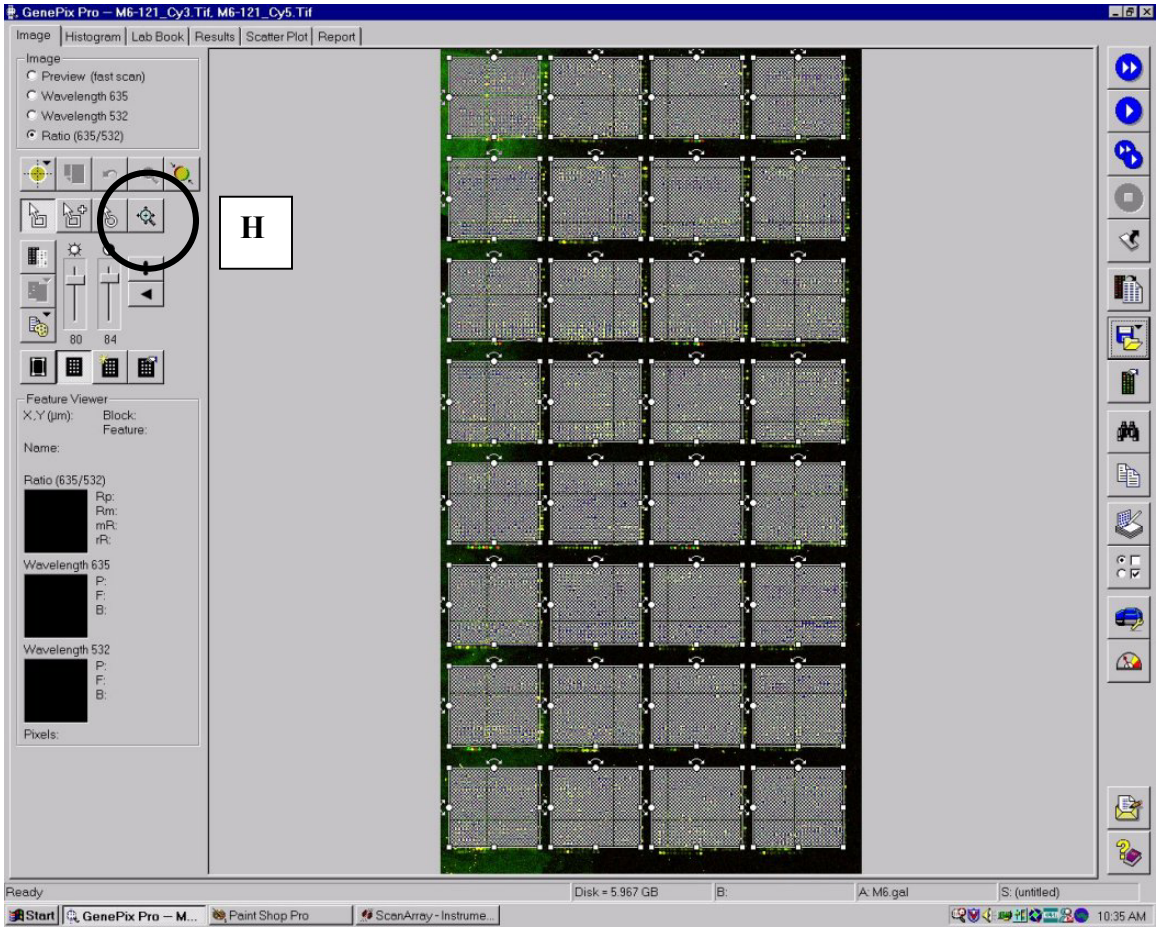


g) Load the grids. After the image contrast and brightness has been adjusted, the grid must be loaded. The grid (referred to as the .GAL file) has the correct number of circles to associate with each cDNA spot on the array. The .GAL file is specific to the print run and can be found in "General Lab Information", MICROARRAY, "Gal Files". Click on the "Open/Save" button (the one used to load the images) and choose the last item on the menu, "Load Array List". A Windows "Open File" type window will open and the appropriate .GAL file can be opened. This process is shown below. Since the image loaded for this discussion is from print run M6, it is necessary to load the M6.GAL file.



h) Blunt alignment of grids. Once the .GAL file is loaded, the grids will appear superimposed on the microarray image as shown below. Each group of spots are referred to as a block and each spot itself is referred to as a feature in GenePix. Each block has several 'handles' for stretching and tilting. Each block is also "selected" when the .GAL file is opened. To "de-select" all the blocks, just click anywhere on the array without a block. To select one specific block, click on it. To select several block, drag a box around them.

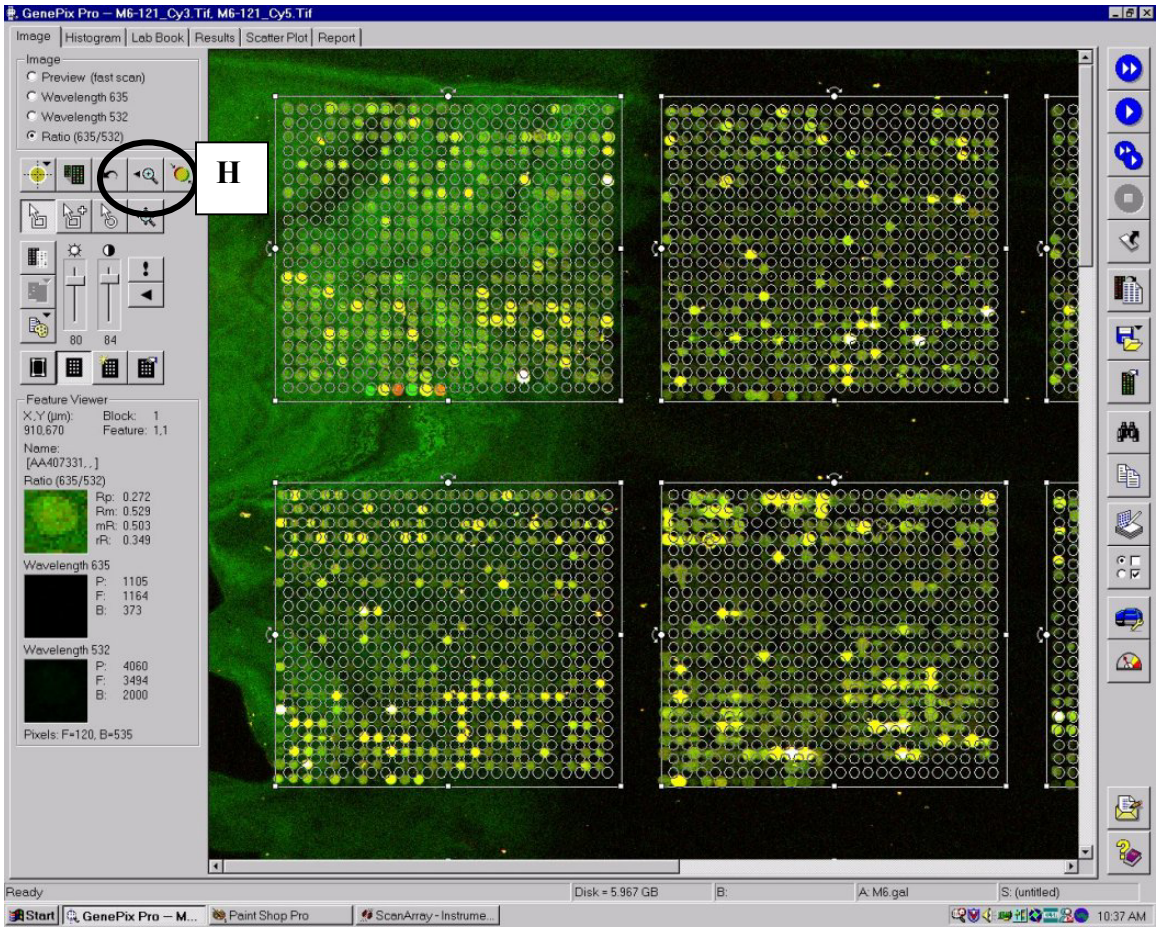
For now however, it is best not to de-select all the blocks since they can all be bluntly aligned en masse before each block is individually aligned. If they have been de-selected, just select them all by dragging a box around all the blocks. Next, press the Zoom Mode button (labeled H in the screen shot shown below). The cursor will change to a magnifying glass on the array. Drag a rectangle to zoom in on a given region. Once you have clicked on the zoom button, you can only drag one rectangle before the cursor reverts to the regular mode (actually called the block mode...).



h) Blunt alignment continued

The zoom-in operation has been performed on the top left four blocks and is shown in the next screen shot. Clicking on a block and holding down the button will allow you to move all the selected blocks, including the blocks that are not visible in the current view. Thus, since all the blocks were selected before zooming in, we can click on a block and hold the mouse button down while we move all the blocks so that they are closely aligned with the array. (You could have also just moved the blocks for blunt alignment without zooming in.).

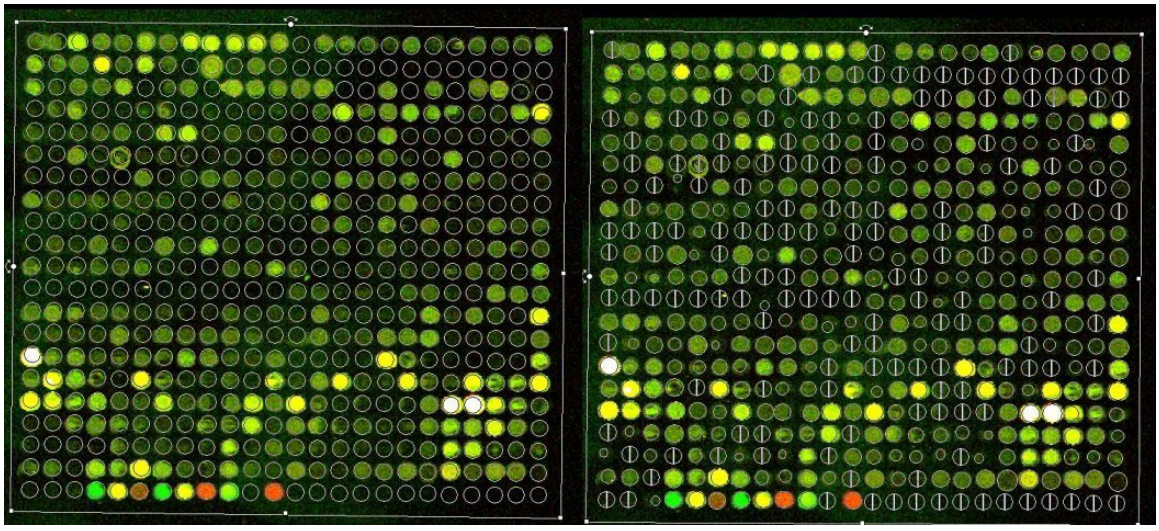
As an aside, to "un-zoom", simply press the "un-zoom" button just above the zoom mode button (labeled H below).



i) Align each block onto the array. We now need to align each block onto the array individually. Block alignment is most easily accomplished by zooming in on an area slightly larger than a given block. I usually start with the block at the upper-left and work towards the right and then align the next row of blocks until the entire array is complete.

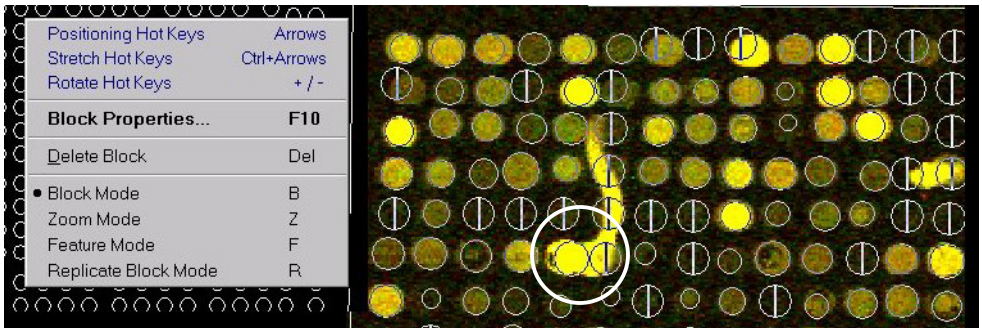
De-select all the blocks and then select the block in question. Next, click on the block and drag the block to align the top left features of the block as closely as possible to the spots on the array. It may help to alter the image contrast and brightness at this point. Frequently, the top left features will align nicely, but the rest of the block will not. Usually you will need to tilt the entire block. This can be performed by clicking and dragging the block "handle" on the top middle of the block and then moving the mouse (its the handle with a curved arrow around it). It is much more efficient to use the keys on the keyboard that have the "+" and "-" keys (without pressing the shift key). The "+" key tilts up and the "-" key tilts down. Moving the block and tilting is usually enough to get most of the spots on the array to match the features on the block. Occasionally, you may need to stretch the block by clicking and dragging any handle on the block that doesn't have an arrow on it.

The block on the left-hand side below has been aligned by hand. Next, the F5 function key is depressed and GenePix Pro will magically align each feature to each spot on the array and will "flag" suspicious or non-existent spots. This is the block on the left.

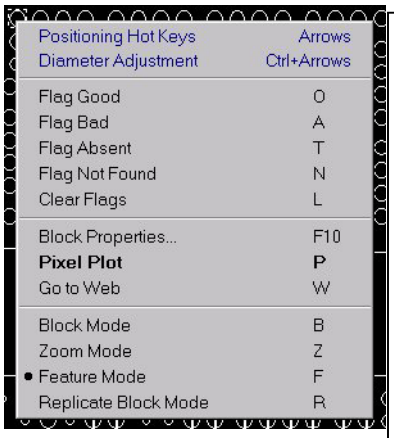


j) Verify alignment validity. GenePix Pro aligns the block after the F5 function key has been depressed. This results in the features being aligned to the cDNA spots on the array. Some features have been resized and "flagged". Flags are used to indicate spots on the array that would provide erroneous data. The most common one (as observed in the right side image above) is a circle with a vertical line through it (spot not found flag).

k) Manually flag spots. Quickly scan the array to verify that no streaks or blemishes have been aligned. Sometimes a bright blemish or scratch in the array will be inappropriately aligned as a feature as shown below. Most of the blemish was flagged. If nothing was done about such a situation, the spot would be analyzed. To prevent this type of error, you can manually flag the feature yourself. This is done by right clicking on the grid to reveal a pop up menu shown below:



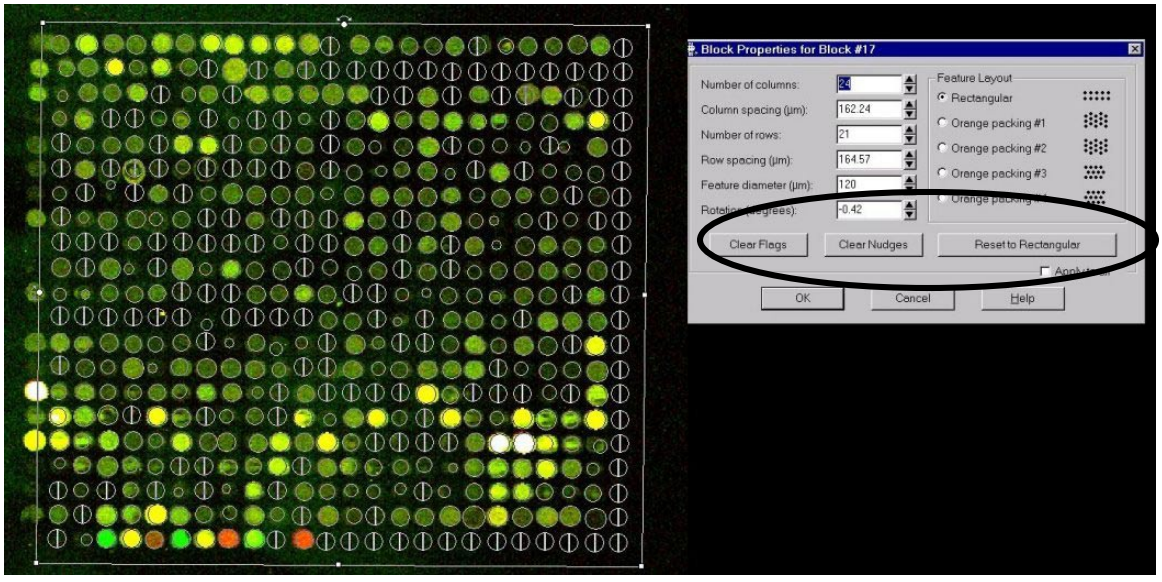
First click on the second from last menu item, "Feature Mode". In feature mode, you can click on a given feature and alter its flag status. If you right click on a spot when in feature mode (the pointer will have an arrow with a circle; block mode was an arrow with a square), a pop-up menu will appear and is shown below:



For a blemish on the array, I usually use the second option, "Flag Bad". As you can see from this list, you can change the diameter of a feature as well as take spots that GenePix has flagged as "Flag Not Found" and manually "Flag Good". My advice is, with the exception of blemishes on the array, let GenePix find the spots in its objective way. If your arrays are good, GenePix will do a great job. If your arrays are not so good, you may want to consider re-doing them.

Before continuing onto the next block to align, don't forget to right click on the block and invoke the block mode again.

l) Changing your mind. Sometimes the block is aligned incorrectly (as shown below). This will happen if you didn't align the block well manually or if the array is dim. To start over with this block, double click the block and a window will appear as shown below on the right. There is a row of 3 buttons that need to be sequentially pressed to revert the block to its original character. The three buttons are circled in the screen image below. Click on the button "Clear Flags". The window will disappear and all the flags will also disappear on the block. Double click the block again and press the next button, "Clear Nudges". Last, double click the block and then click on "Reset to Rectangle". Once all three of these buttons have been pressed, the block reverts to its original form.

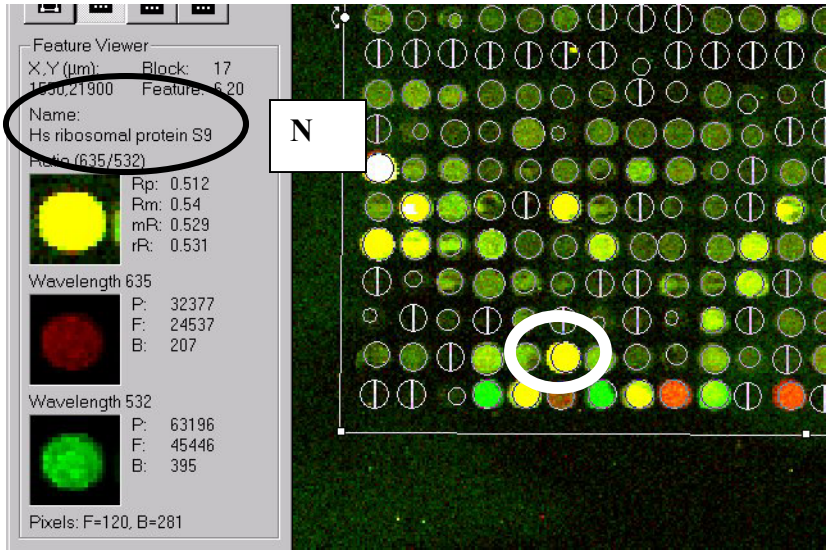


m) Save the grid. After aligning each block to the microarray and checking to verify that blemishes are appropriately flagged, save the grid file. Press the "Open/Save" button on the tool bar and the second last menu item is "Save Settings As". Select this option and you will be prompted for a name. I usually name everything by the array number. It is very important that you save your settings. It is possible to easily recreate all your analysis with the original TIFF images and the saved settings.

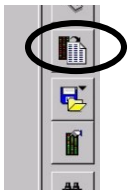
n) Peruse your microarray. One of the major benefits of using the .GAL file for aligning grids on the microarray is that individual features have names associated with them. When the pointer is placed over a feature of a grid, its name is shown in the "Feature Viewer" located on the bottom left of the screen. This makes it possible to point to an interesting spot and see the name of the clone in that spot. In the example below, the circled yellow spot is believed to be similar to "homo sapiens ribosomal protein S9" (labeled N).

The Feature Viewer also includes ratios for the composite image and separate pixel intensities for the two color channels. For the individual color channels, the values adjacent to F and B represent the average foreground and background pixels, respectively. (The value P: represents the actual number of pixels at the precise spot where the mouse is pointing. This P value is NOT an average, which makes it quite meaningless at this level). Adjacent to the composite picture are the following ratios: Rp is ratio of pixel intensities for red/green, Rm is ratio of median pixel intensity, mR is median of ratios and Rr is regression ratio. <u>The important one is Rm because it is less sensitive to extreme values in the intensity distribution across a cDNA spot.</u>

Thus, in the example below, ribosomal protein S9 has an average red channel intensity of 24537 pixels and an average green channel intensity of 45446 pixels. It is therefore a high expression gene that has changed in expression close to two fold (Rp = 0.512). It is best to analyze replicate data properly, but this function is good to whet the appetite for analysis and also to verify expression changes detected at a later time point.

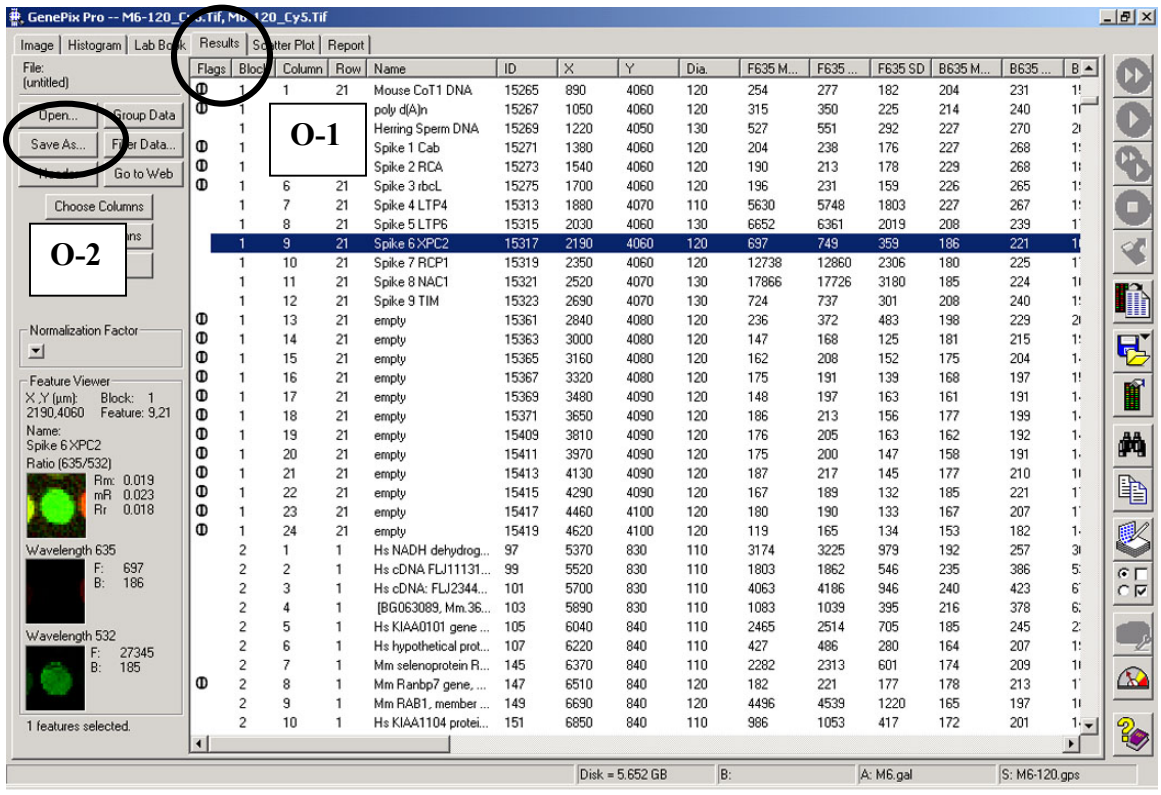


o) Extract the expression values from the images. The time has come to ask GenePix to calculate the expression values for each feature on the microarray. The "Analyze" button is on the right hand tool bar just above the "Open/Save" button. If you have a slow computer, this analysis could take several minutes.



Analyze Button

In order to analyze more than the first 100 values, you must have a software security key (the so-called 'dongle'). After GenePix has calculated the expression values, the screen will change and you will be mesmerized by a seemingly incomprehensible table of numbers. (GenePix makes many calculations that are relatively easy to understand for those who are interested). Whereas GenePix was in the "Images" tab earlier (refer to step B), GenePix changes to the "Results" tab (O-1) after pressing the "Analyze" button. A screen shot is shown below. Press the "Save As" button to save the GenePix results file (.GPR) (O-2).



p) End of data extraction process. The last step in extracting expression data from a microarray is saving the .GPR file. Nevertheless, GenePix has several features that can be used to determine the quality of the array and these will be explored in the next section.

F) Experiment Quality Control

F. i) False Discovery Rates

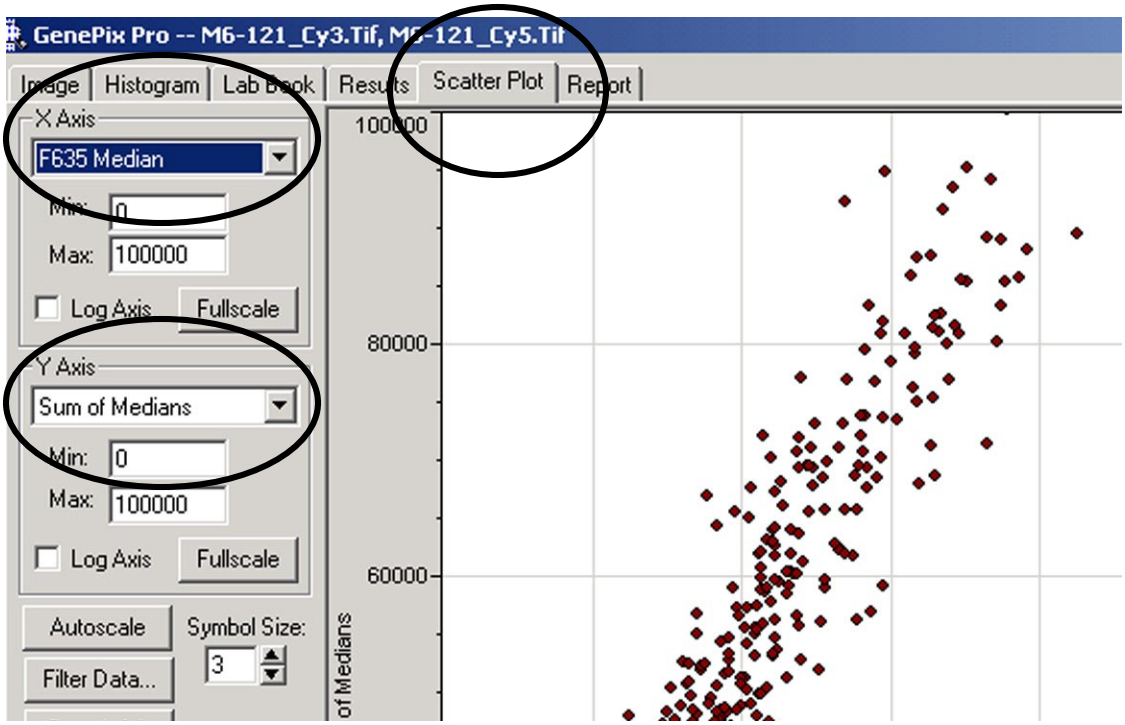
cDNA microarray data is inherently noisy. False positive and false negative signals can occur at a detectable rate. In addition, the statistical significance of your results will take on a new relevance because of the huge number of data points generated in even the simplest microarray experiment. The problem stems from the fact that large data sets introduce a certain level of false discovery.

The concept of false discovery rate applies to microarray data and is best illustrated by an example. Suppose you have analyzed your data consisting of 3000 genes that are detectably expressed in your arrays and produce a list of 100 genes that change between your two RNA samples. Further suppose that this list of genes has been demonstrated be changed at the 95% confidence interval ($p = 0.05$). But since you chose your list of 100 genes from 3000 genes that were measured, you would expect 5 genes to change out of every 100 by chance alone. This would mean that 150 genes out of 3000 would be expected to change randomly, greater than your list of 100. It would be far more meaningful to accept fewer changes at the 99% confidence interval ($p = 0.01$) and have a smaller list of genes.

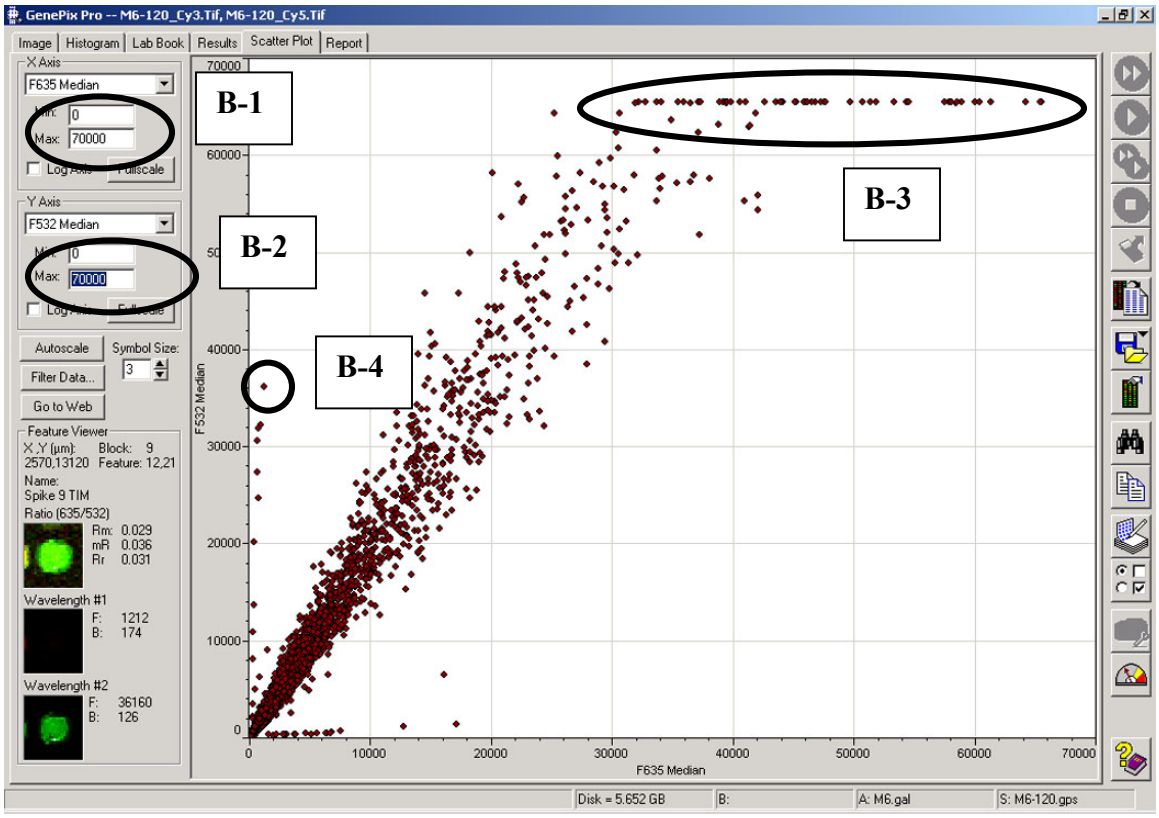
The above discussion is intended to emphasize the importance of generating good quality array hybridizations. Good quality cDNA microarrays can be noisy and bad quality cDNA microarrays can be very noisy. GenePix Pro can plot the values of your array to generate several scatter plots which give a good, but indirect, indication of the quality of your arrays. GenePix also has a java-script based 'Array Quality Control Report' that it can generate for a given array. These features of GenePix are covered below.

F. ii) Using GenePix Scatter Plot for Quality Control

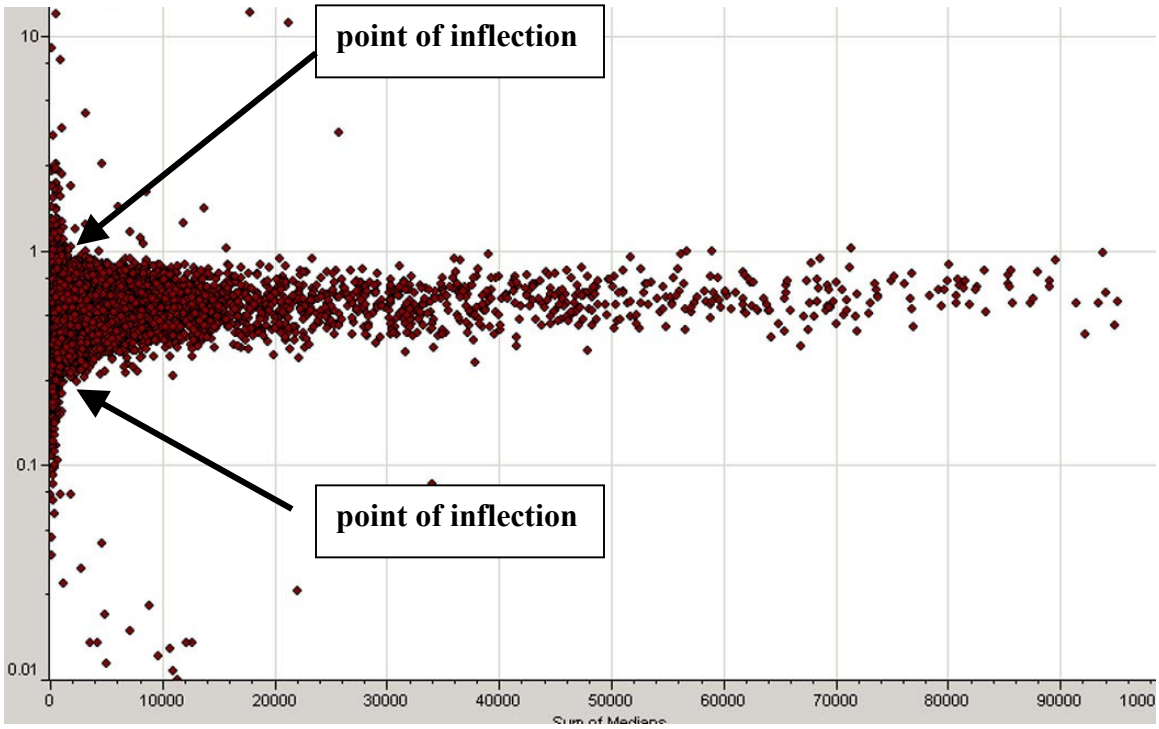
a) After you have saved your GenePix results file, click on the ‘Scatter Plot' tab adjacent to the 'Results' tab at the top o the GenePix window. A zoomed screen image of this tab is shown below. The ‘Scatter Plot’ will automatically plot F635 Median in the X axis and F532 Median in the Y axis. (In the event that you have changed these settings, simply click on the X and Y axis drop down menus and choose these values to plot).



b) The computer will think for a few moments and show you a plot of the median signal intensity for every spot on the array that was not flagged. One such plot is shown below. The Y axis represents the green signal intensity and the X axis represents the red signal intensity. I usually change the maximum value of each axis to 70 000 by clicking in the box entering 70000 (labeled B-1 and B-2). The maximum signal intensity possible is about 65000. The series of spots labeled B-3 are all saturating. There are about 30 or 40 such spots which is about the maximum number you want. The green dye labels more efficiently and is usually the dye with the saturating spots. The mouse pointer was placed over the spot labeled B-4 (it does not show in screen capture mode). The bottom left corner shows the Feature Viewer which shows the actual spot and its associated name. Rm describes the Ratio of medians of red/green intensity (this is the best one to use). In this way, the Scatter Plot can be used to look at interesting features. Notice that the spots all cluster along a straight line and that not many spots deviate from the line. This experiment is a normal-normal comparison with some unique mRNA spiked into only one RNA pool (e.g. B-4). Most cell lines treated with drug should look like this graph: i.e. most genes do not change, but a few will change to a large degree.

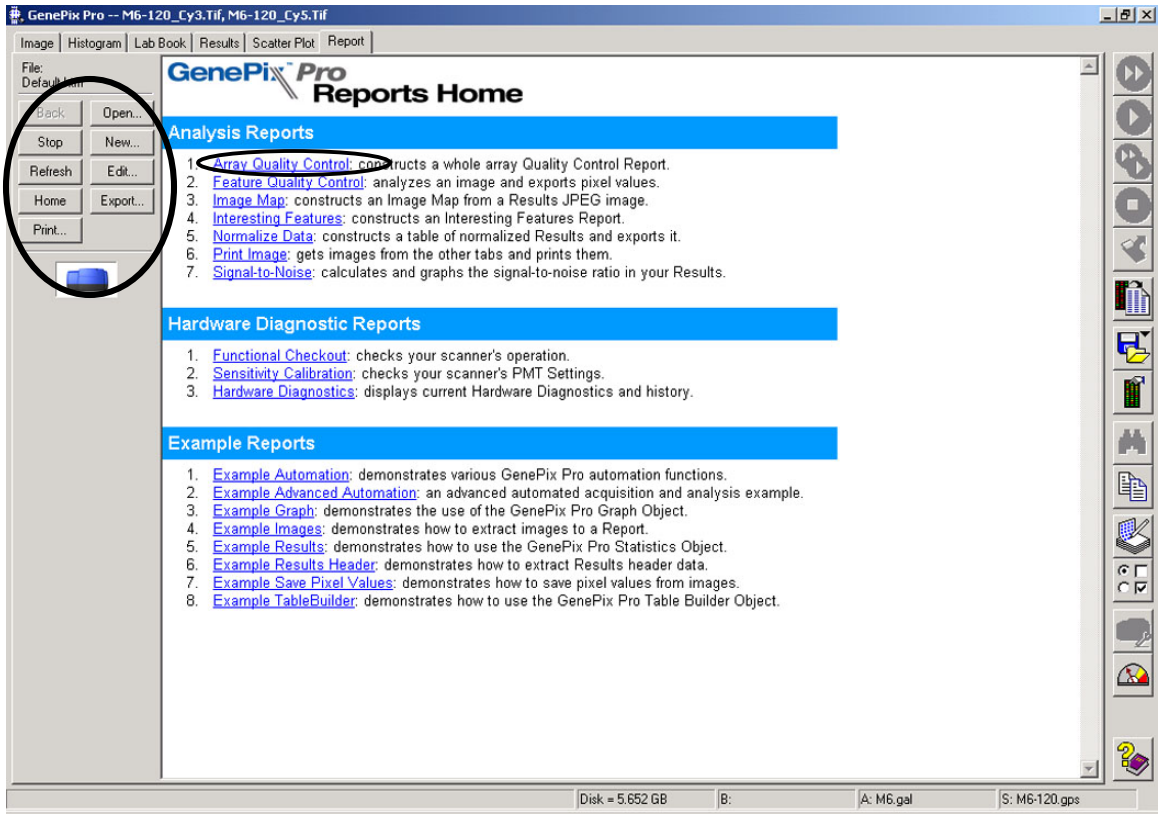


c) Another useful plot is “Sum of Medians” versus “Median of Ratios”. Simply change the X axis value to ‘Sum of Medians’ and change the Y axis value to ‘Median of Ratios’. It is necessary to check on the ‘Log Axis’ box on the Y axis and to also change the Y axis min and max values to 0.01 and 10, respectively. Such a plot is shown below with these specific alterations. Notice that as the X axis approaches 0 there is an increase in spots that show altered log ratios. This phenomenon is noticeable on all cDNA microarrays including those that compare the same sample to itself (the so-called normal-normal experiment) such as the plot shown below. As spot signals get weaker, they are noisier and therefore more likely to show changes in the ratio of medians. In good arrays, the point at which the ratio of medians stops being high (the point of inflection) is generally when the sum of medians of the red and green channel intensity is about 1500-2000 (pixel units). This number is consistent with the fact that negative control spots that should have no binding generally have intensities of 500-1000 pixels in each channel. For this reason, spots that show changes in expression where the signal intensity of the red or green channel is below 1000 should be regarded with extreme skepticism and suspicion. As verification that these spots are uninformative, simply mouse over one and you will see in the ‘Feature Viewer’ how undefined and pathetic most of these spots are. Control spots with hybridization to mRNA spiked into the RNA pools are an exception.



F. iii) Using the GenePix Array Quality Control Report

a) GenePix Pro has an integrated browser (Microsoft Internet Explorer) as part of the software package. When the 'Report' tab is invoked, there is a choice of Reports that GenePix will generate based on the data viewed or loaded in the 'Results' tab. The buttons in the image below that are enclosed in the large oval are the navigator buttons. After choosing a report to produce and viewing that report, it is necessary to press the 'back' button to return to the previous screen, just as you would do when navigating an internet site. The most useful of these reports is the first one that is circled and is named "Array Quality Control." Simply press the hyperlink and another page will appear. This page will give a series of thresholds after which is a "Start" button. Press the start button and an Array Quality Control Report will be generated.



b) Following this discussion is a sample Quality Control Report that I will be referring to. First, there is an Array Summary which is a nice little record keeping feature. When a report is generated, you can use the 'Print' button in the navigator tool bar. When the print window opens, click on the "Print to File" option to save a copy of the report.

Under the 'Vital Statistics' section, there is a breakdown of various parameters for each color channel. The 'Median signal-to-background' numbers are good if they are between 4 and 10. Don't worry about failing the threshold set by GenePix Pro. I don't. The 'Features with saturated pixels' should not be more than 50 to 75. In the example below, there are a considerable number. If there are this many, it may suggest that the arrays should be re-scanned at a slightly lower laser power or decreased PMT gain. Notice in this section that the red channel (635 nm) is much dimmer than the green channel (532 nm).

After the 'Vital Statistics' are two graphs that are similar to the ones plotted in the previous section. They are F635 Mean vs. F532 Mean. and Log Ratio vs. Sum of Medians. In the previous section we plotted the medians of F635 and F532.

The next set of graphs show the ratio of medians vs X position and ratio of medians vs. Y position for every block on the array. These graphs are able to show if there is spatial bias in the array. Sometimes the edges of the graphs will 'frown' or 'smile' if there are problems at the edge of the array.

The next section shows a Histogram of Normalized Log Ratios. If the procedure for scanning the arrays was executed well and enough patience was exercised to make the two channels relatively similar, the graphs should be almost superimposed.

Last, there is a section on statistics for each color channel.

In general, I find it is useful to have a record of the two first graphs, the graphs that describe the spatial distribution of ratios and the graph showing the histogram of normalized log ratios.

Axon Array Quality Control Report

Help

This Quality Control Report constructs a summary information table, a vital statistics table that determines whether the array data passes or fails based on user-entered values; it draws five different graphs and imports the ratio image from the Results file; and constructs a full statistical analysis of the data.

QC elements:

- [x] Array Summary
- [x] Vital Statistics
- [x] Intensity Graphs
- [x] Log Ratio Graphs
- [x] Position Graphs
- [x] Normalization Graphs
- [x] Ratio Images
- [x] Statistics Tables

Vital Statistics thresholds:

Median signal-to-background	>	15	(ratio)
Mean of median background	<	500	(intensity)
Median signal-to-noise ratio	>	10	(ratio)
Features with saturated pixels	<	3	(number)
Not Found features	<	7	%
Bad features	<	7	%

Press the Start button to generate the Report.

Start

Array Summary

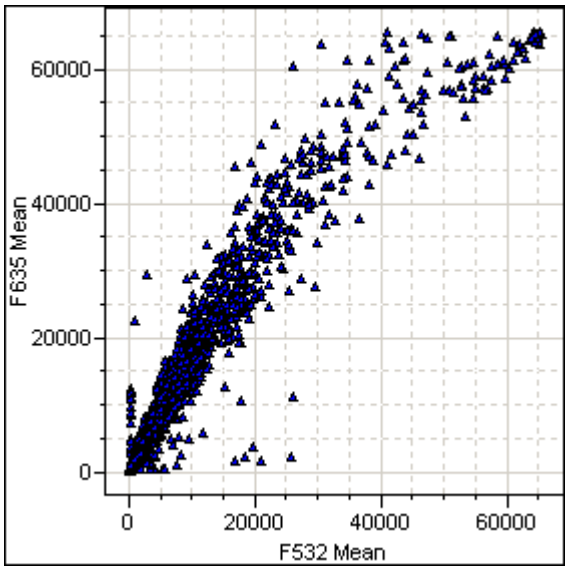
Scanned by:
When scanned: 3/1/2002 4:33:24 PM
Image wavelengths: 635, 532
PMT: 0, 0 V
Laser Power: 0, 0 V
Scan Power: 100, 100 %
Comment: ScanArray 5000
Results file name: (untitled)

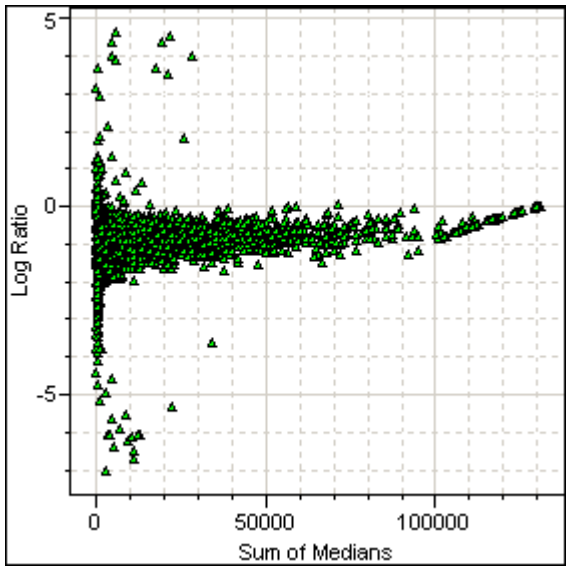
Analyzed by: GenePix Pro 3.0.6.89
GPS file: M6-121.gps
GAL file: M6.gal
Temperature: 0 V
Laser On-time: 0, 0
Barcode: none

Vital Statistics

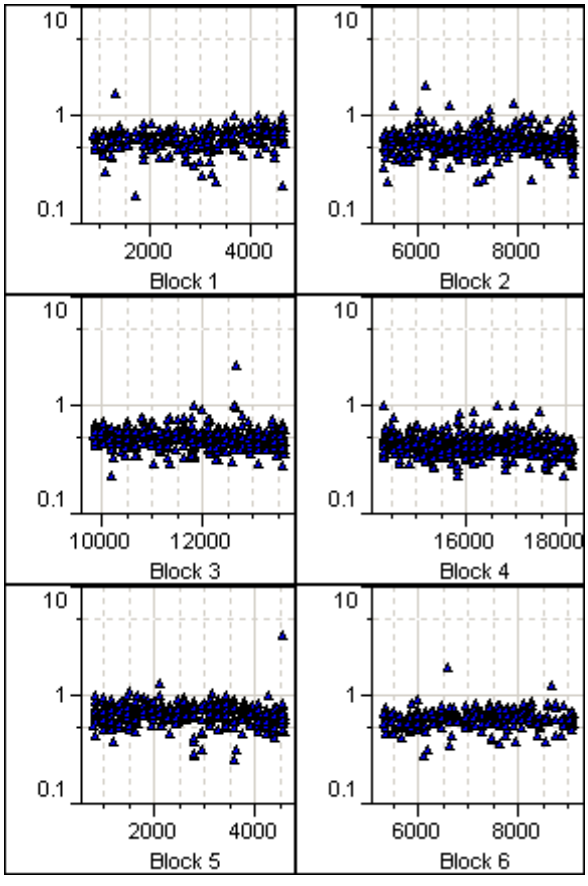
	635	532	Threshold	Results	
				635	532
Median signal-to-background	5.235	9.764	> 15	**Fail**	**Fail**
Mean of median background	176.323	224.686	< 500	**Pass**	**Pass**
Median signal-to-noise	4.007	6.412	> 10	**Fail**	**Fail**
Features with saturated pixels	108	152	<= 3	**Fail**	**Fail**
Not Found features	4188/16128 (26%).		< 7 %	**Fail**	
Bad features	0/16128 (0%).		< 7 %	**Pass**	

F635 Mean v F532 Mean **Log Ratio v Sum of Medians**

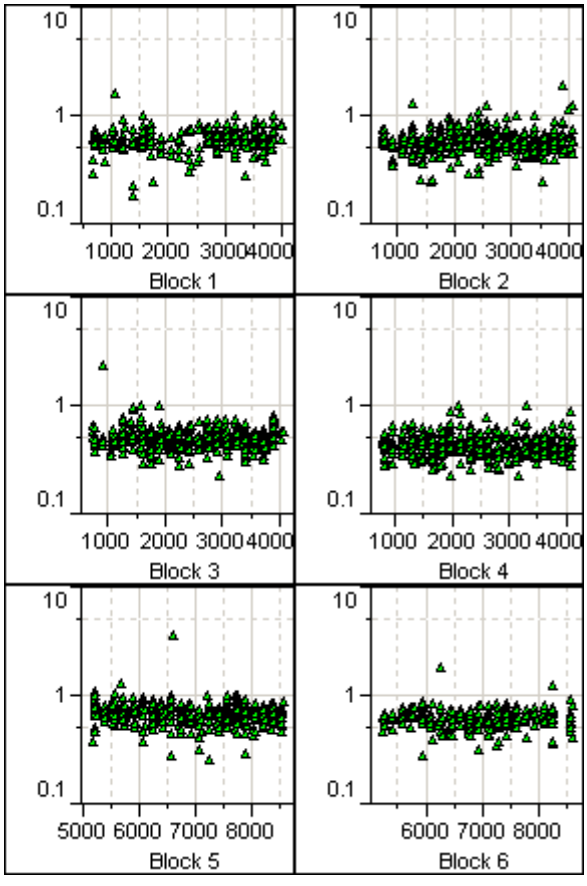


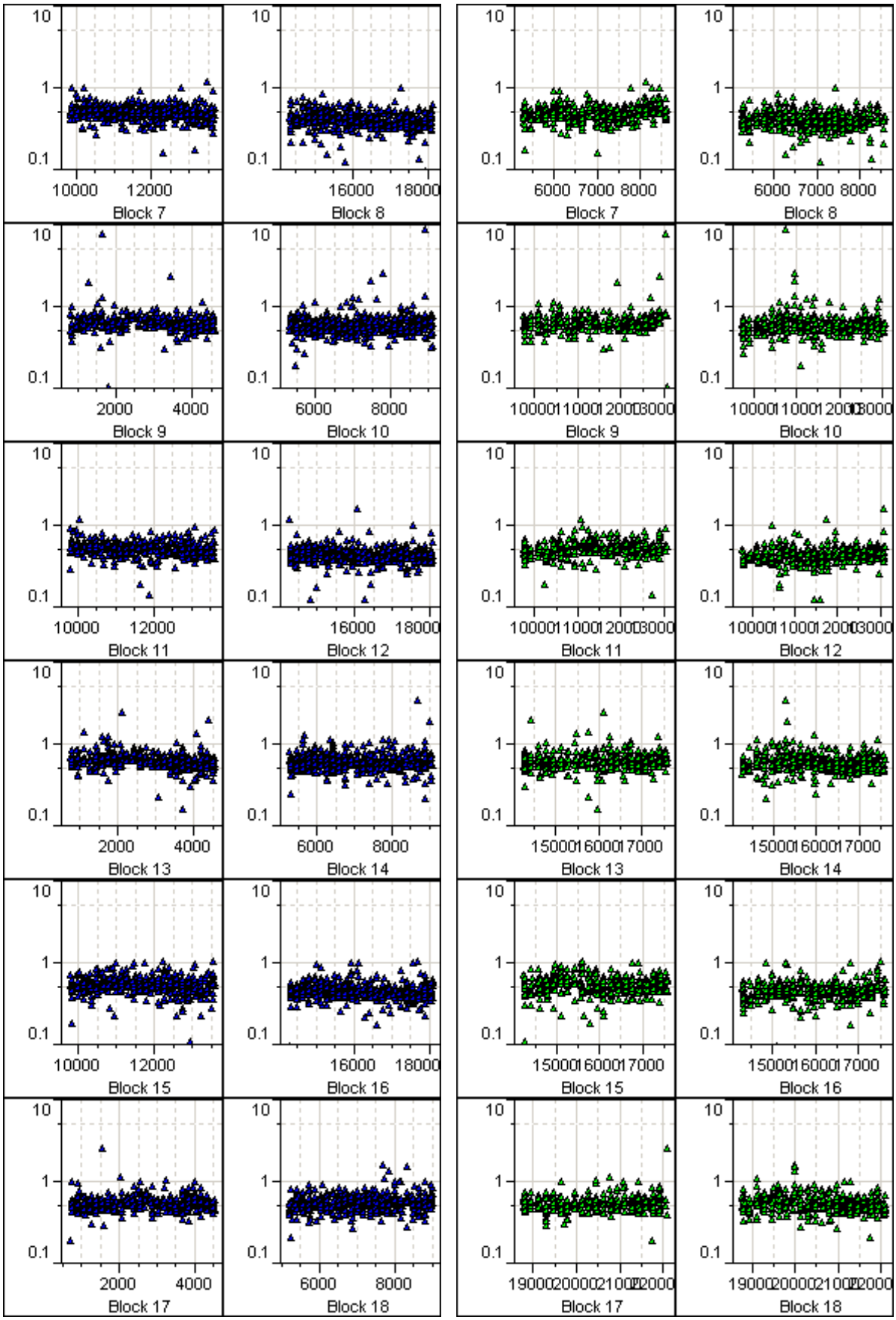


Ratio of Medians v X Position



Ratio of Medians v Y Position

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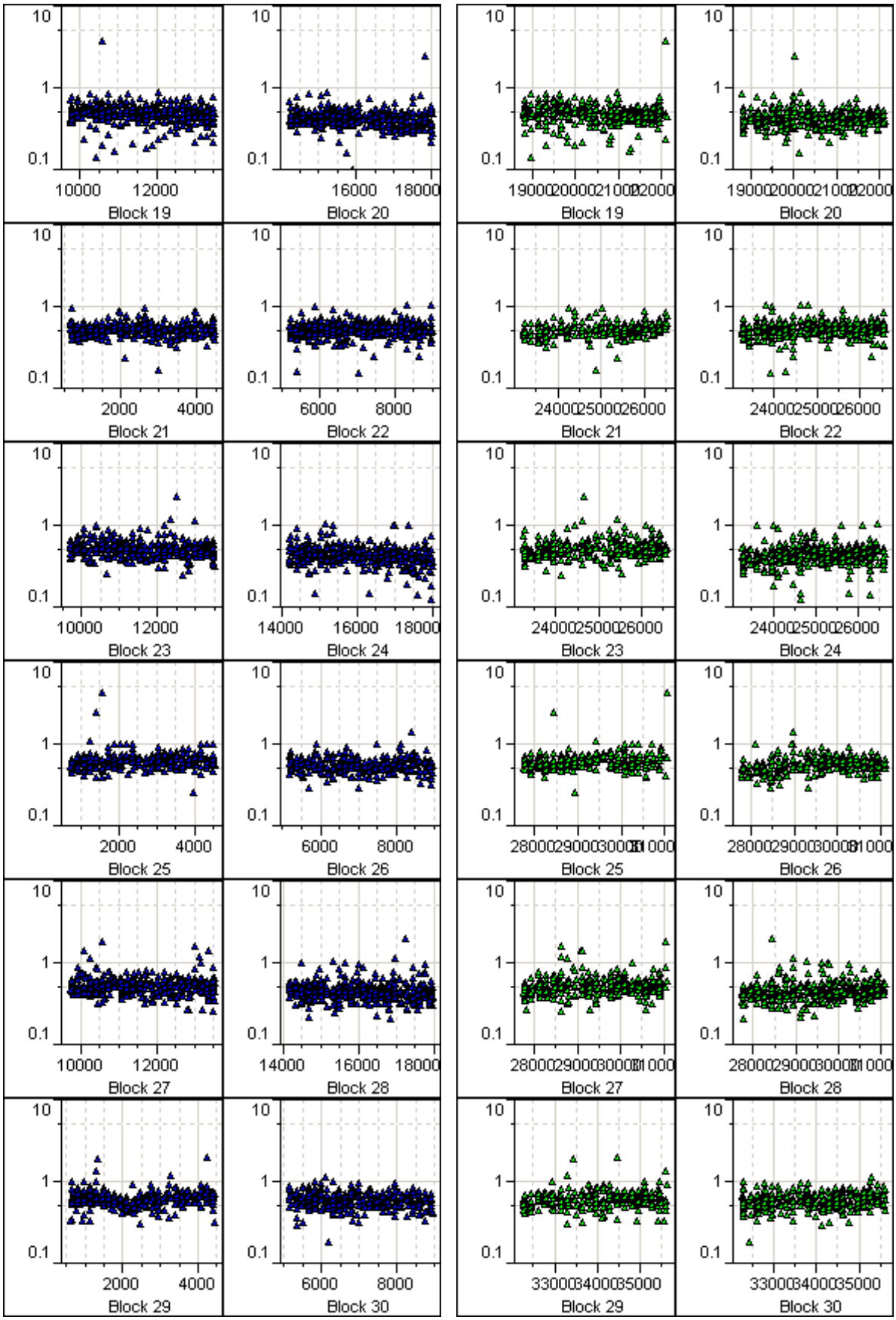
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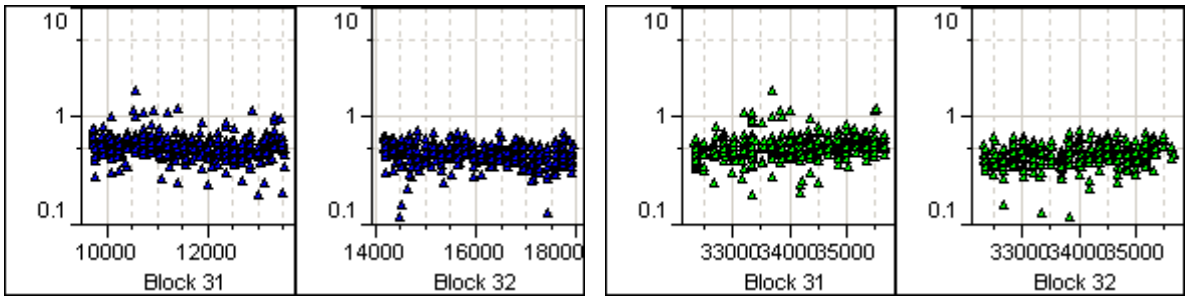
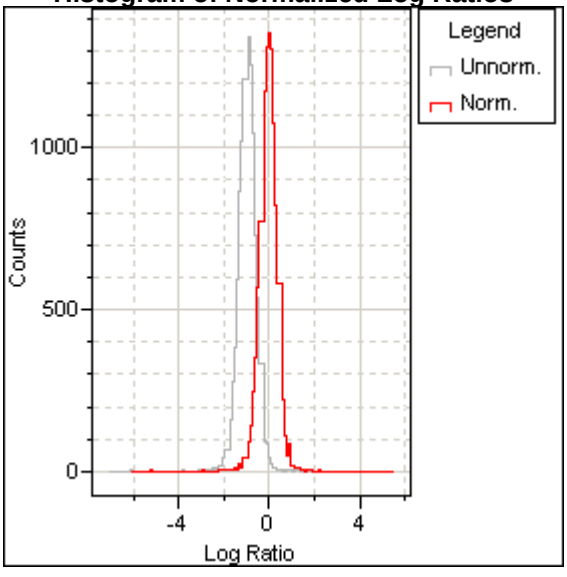
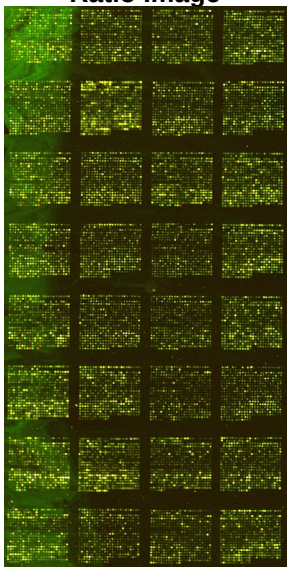


Histogram of Normalized Log Ratios



Ratio Image



635 Statistics

	F635 Median	F635 Mean	F635 SD	B635 Median	B635 Mean	B635 SD
Mean	2413.002	2424.34	889.782	176.323	226.588	245.887
Median	899	954	419	166	197	156
SD	6087.82	5744.084	1919.713	45.766	127.263	522.145
Max	65535	65419	25394	1811	4352	14365
Min	121	181	114	78	121	103

532 Statistics

	F532 Median	F532 Mean	F532 SD	B532 Median	B532 Mean	B532 SD
Mean	3933.24	3894.261	1269.257	224.686	295.369	315.213
Median	1613	1679	636	124	171	173
SD	8218.062	7710.874	2280.552	276.476	320.093	586.497
Max	65535	65535	26341	2859	5202	16133
Min	153	201	0	52	92	87

Ratio Statistics

	Ratio of Medians	Ratio of Means	Median of Ratios	Mean of Ratios	Rgn Ratio
Mean	0.561	0.573	0.585	0.575	0.617

Median	0.53	0.545	0.554	0.542	0.474
SD	0.539	0.453	0.436	0.452	3.787
Max	24.781	22.417	21.228	21.604	341.973
Min	-0.63	0.013	0.023	0.026	0.003

Comments:

QC Report Generated: 3/28/2002 12:39:09 PM

G) Interpretation of Resulting Expression Profiles

The Michigan Life Sciences Corridor is tending toward the use of an integrated software analysis package for interpretation of microarray data and transcriptional profiles. Gene Spring appears to be a favorite at this time and we have access to a 6 month trial version presently. Until Gene Spring or some other commercial or home-brew software package is available, cDNA microarray analysis at Van Andel Institute will rely on using the AMAD database in conjunction with Cluster, Tree View and Cluster Identification Tool (CIT).

Instructional material is on the intranet that describes the use of AMAD, Cluster and Tree View. This can be found at http://elm.vai.org/uarray/index.htm under the "Instructional Materials" menu. After reading this material, I would be pleased to give a demonstration and assist in the appropriate use of these programs.

H) How to Acknowledge the cDNA Microarray Facility

As a core facility, it is important that the facility is acknowledged in any research reports that make use of cDNA microarrays. We request that papers using microarrays include the following sentence in the Acknowledgements (or some variation of your choosing):

"We thank the Van Andel Institute cDNA Microarray Core Facility for providing us with printed microarrays and instruction on their use."

Appendix A: Precautions to Take While Working With RNA

Appendix A. i) Ambion Technical Bulletin 159: Working with RNA

Ambion Technical Bulletin 159

Working with RNA

Living with RNase

Most researchers are acutely aware of the risk of RNase contamination, and we do not want to belabor this point or cause undue worry. We do not routinely find it necessary to treat the microcentrifuge tubes used with RNA if they are from unopened bags or from bags in which care was taken to avoid contaminating the tubes. Yet we do consistently find a small percentage of tubes (even those marketted as being RNase-free), the use of which results in RNA degradation. We do recommend that gloves be worn when handling any reagents or reaction vessels. (Note: Gloves which have touched refrigerator handles, door knobs, or pipettors are not RNase-free.) When performing procedures that use RNases (eg. ribonuclease protection assays and plasmid purifications), care should be taken that pipettors are not contaminated by accident. One potential source of contamination is the metal tip ejector mechanism on the side of the pipettor. Removing the metal ejector bar when it is necessary to insert the pipettor into a larger vessel where the ejector could come into contact with the walls or contents of the vessel will eliminate this concern.

A. Detecting RNase

While contaminating RNase can result in a failed experiment, it is often difficult an time-consuming to determine which solution or piece of equipment is responsible. In Ambion's Quality Control Department, we use an extremely sensitive RNA probe stability assay to detect RNase contamination. This assay can be performed in your own lab to detect RNases and a protocol is provided in Ambion TechNotes 2(4) 1995 to facilitate this. However this assay is much more sensitive than is generally necessary for most RNA work, is time consuming and requires working with radioactivity. As an alternative, Ambion's RNaseAlert™ Kit (Cat. #1960) allows researchers to identify contaminated reagents and equipment quickly, and non-isotopically. The kit uses a detectable RNA substrate which is immobilized onto a test strip, submerged into a test solution, and then subjected to a series of detection steps. A blue dot of comparable intensity to an untreated control (on the same dipstick) appears if there is no RNase contamination. [Note: We have recently improved the format of our RNaseAlert™ Kit. Due to this improvement, we have discontinued Cat. #1960. Please see the online catalog for details about the new RNaseAlert Lab Test Kit and QC System (Cat #1964 and 1966, respectively).]

B. Getting rid of RNase

If RNase contamination of reagents or equipment is suspected to be a problem, extra precautions may be necessary. Autoclaving tips, tubes and solutions is not sufficient to inactivate RNases. Glassware can be baked at 300°C for four hours and plasticware, tubes and most solutions can be DEPC-treated (see below). However, both procedures are time-consuming, and DEPC is both expensive and possibly carcinogenic. As an alternative, Ambion's RNase*Zap*™ (Catalog #9780) can be used to eliminate RNase from glassware, plastic surfaces, countertops, and pipettors. RNase*Zap*™ has been shown to effectively

inactivate 5µg of RNase dried onto the bottom of eppendorf tubes without inhibiting subsequent enzymatic reactions performed in the same tube. The solution contains three ingredients known to be active against RNase. RNase*Zap*™ can be poured onto or wiped over surfaces and works immediately upon contact. Treated labware is simply rinsed twice with distilled water and is ready for use.

Treating Solutions with DEPC to Remove RNase

To ensure that solutions are free of RNase contamination, they can be treated with diethylpyrocarbonate (DEPC) [WARNING: DEPC is a suspected carcinogen: Take appropriate precautions when handling; e.g., always wear gloves and handle under an approved fume hood]. DEPC reacts with histidine residues of proteins and will inactivate RNases. However, it can also react with RNA, so it needs to be removed by heat treatment before the solution is used (DEPC breaks down to CO_2 and ethanol). Add DEPC to solutions at a concentration of 0.05 - 0.1% (e.g., add 0.5 - 1 ml DEPC per liter); stir or shake into solution, incubate for several hours; autoclave at least 45 minutes, or until DEPC scent is gone. Please be aware that compounds containing primary amine groups, such as Tris (2-Amino-2-hydroxymethyl-1,3-propanediol), will also react with DEPC, and thus should be added only after DEPC treatment is complete. Note: We have observed that distilled water, treated with DEPC and thoroughly autoclaved, caused a 20% inhibition of translation in a reticulocyte lysate. We find that distilled water is generally already RNase-free, and so does not need to be treated.

How to Store RNA

RNA may be stored in a number of ways. For short-term storage, RNase-free H_2O (with 0.1mM EDTA) or TE buffer (10 mM Tris, 1mM EDTA) may be used. RNA is generally stable at -80° C for up to a year without degradation. Magnesium and other metals catalyze non-specific cleavages in RNA, and so should be chelated by the addition of EDTA if RNA is to be stored and retrieved intact. It is important to use an EDTA solution known to be RNase-free for this purpose (older EDTA solutions may have microbial growth which could contaminate the RNA sample with nucleases). It has been suggested that RNA solubilized in formamide may be stored at -20°C without degradation for at least one year (Chomczynski, 1992).

For long term storage, RNA samples may also be stored at -20°C as ethanol precipitates. Accessing these samples on a routine basis can be a nuisance, however, since the precipitates must be pelleted and dissolved in an aqueous buffer before pipetting, if accurate quantitation is important. An alternative is to pipet directly out of an ethanol precipitate that has been vortexed to create an even suspension. We have found, however, that while this method is suitable for qualitative work, it is too imprecise for use in quantitative experiments. RNA does not disperse uniformly in ethanol, probably because it forms aggregates; non-uniform suspension, in turn, leads to inconsistency in the amount of RNA removed when equal volumes are pipetted.

How to Precipitate RNA

A. Precipitating with alcohol

Precipitating RNA with alcohol (ethanol or isopropanol) requires a minimum concentration of monovalent cations (for example: 0.2 M Na+, K+; 0.5 M NH_4+) (Wallace, 1987). After the salt concentration has been adjusted, the RNA may be precipitated by adding 2.5 volumes of ethanol or 1 volume of isopropanol and mixing thoroughly, followed by chilling for at least 15 minutes at -20° C. While isopropanol is somewhat less efficient at precipitating RNA, isopropanol in the presence of NH_4+ is better than ethanol at keeping free nucleotides in solution, and so separating them from precipitated RNA. RNA precipitation is faster and more

complete at higher RNA concentrations. A general rule of thumb is that RNA concentrations of 10 µg/ml can usually be precipitated in several hours to overnight with no difficulty, but at ower concentrations a carrier nucleic acid or glycogen should be added to facilitate precipitation and maximize recovery.

B. Precipitating with lithium chloride

Lithium Chloride may also be used to precipitate RNA, and has the advantage of not precipitating carbohydrate, protein or DNA. LiCl is frequently used to remove inhibitors of translation which copurify with RNA prepared by other methods. A final LiCl concentration of 2-3 M is needed to precipitate RNA (adding an equal volume of 4 M LiCl, 20 mM Tris-HCl, pH 7.4, and 10 mM EDTA works well). Note that no alcohol is needed for LiCl precipitation. RNA should be allowed to precipitate at -20°C; precipitation time depends on RNA concentration. It is generally safe to allow the RNA to precipitate for several hours to overnight. After centrifugation to collect the RNA, pellets can be rinsed with 70% ethanol to remove traces of LiCl. LiCl efficiently precipitates RNA greater than 300 nt in length. While LiCl can effectively precipitate RNA from more dilute solutions, for best results, the RNA concentration should exceed 200 µg/ml.

Incorporation and Yield

"Percent incorporation" is calculated by comparing the amount of radioactivity incorporated into synthesized RNA with the total amount of radioactivity in the reaction. This is often done by TCA precipitation (see below) but can also be done by simply counting an aliquot of the transcription reaction before and after removal of unincorporated nucleotides. Note that the counts used for comparison must be adjusted to represent equivalent aliquots.
Unincorporated nucleotides may be removed by precipitation using LiCl or NH_4OAc and EtOH (see above), by passing the transcription reaction over a Sephadex column (must be RNase-free), or by gel purification.

The amount of radioactivity incorporated into RNA may also be determined by precipitation with trichloroacetic acid (TCA), filtration, and counting in a liquid scintillation counter. Add a 2 µl aliquot of an RNA labeling reaction to 98 µl of water containing 10 µg of carrier DNA or RNA. To this add 2 ml of cold 10% TCA, vortex and incubate on ice 5 minutes. Collect the precipitate by filtering under vacuum through GF/C glass fiber filters. Wash the sample tube twice with 2 ml 10% TCA and once with 2 ml of 95% ethanol, passing the washes through the filter. After drying, these filters may be placed in vials with liquid scintillation cocktail and counted. Note: Both RNA and DNA may be precipitated using this method.

Since percent incorporation of a radiolabeled nucleotide is directly proportional to yield, the actual yield of a transcription reaction is equivalent to that proportion of the theoretical maximal yield. For example, Ambion's MAXIscript™ kit reactions have a theoretical 100% yield of 77 ng when the transcription reaction contains a limiting nucleotide concentration of 3 uM. Therefore, if for a given reaction the percent incorporation was 80%, then 0.80 X 77 ng or 62 ng of labeled RNA were synthesized.
Some ribosomal subunit size relationships within the eukaryotes are illustrated in Table 1. Both 18S and 28S rRNA contain modified nucleotides, including methylated ribose and pseudouridine (46 and 37 for 18S; 71 and 60 for 28S, respectively) .

	Avg. # of bases	
Organism	**18S**	**28S**
Drosophila	1976	3898
Rat	1874	4718
Human	1868	5025

Table 1. Ribosomal Subunit Sizes in Representative Eukaryotes.

RNA Size Markers

Ambion offers several different ranges of RNA size markers that can be obtained unlabeled for staining with EtBr or biotinylated for subsequent secondary detection. The RNA Century Marker Set (Cat. 7140 - unlabeled, 7175 - biotinylated) contains 5 transcripts evenly spaced between 100 -500 nt, which are ideal for ribonuclease protection assays and gel purification of RNA probes. The RNA Century Markers can also be obtained as DNA templates (Cat. # 7780 and 7782) for the synthesis of radiolabled RNA markers in an in vitro transcription reaction. Ambion's RNA Millennium Marker Set (Cat. 7150 - unlabeled, 7170 - biotinylated) contains 10 transcripts ranging from 0.5-9.0 kb for use with Northern analysis.
RNA transcripts and double-stranded DNA markers (e.g. pUC 19/Hpa II, Cat. 7760 and 7770) can also be end-labeled with polynucleotide kinase (5 prime end-labeling reaction) or Klenow Fragment (3 prime filling reaction) and denatured, for use as labeled size markers.

Other guides to RNA size and migration position are the xylene cyanol and bromophenol blue dyes present in most loading buffers, and rRNA species present during electrophoresis of total RNA for Northern analysis. The migration position of the dyes included in loading buffers is affected both by gel percentage and composition (denaturing vs. nondenaturing). Ribosomal RNA comprises 80% of total RNA samples. Both the 18S and 28S species are strongly visible in Northern gels stained with EtBr or UV-shadowed. The table above gives their sizes in several different vertebrate species.

References

- Chomczynski, P. (1992) Solubilization in formamide protects RNA from degradation. *Nuc. Acids Res.* **20**:3791-3792.
- Wallace, D.M. (1987) Precipitation of Nucleic Acids. *Methods of Enzymology* **152**:41-46.

Ordering Information

For prices and availability, please contact our Customer Service Department.

Cat#	Product Name	Size
1964	RNaseAlert™ Lab Test Kit	25 rxns
1966	RNaseAlert™ QC System	5 x 96 assays
7000	THE RNA Storage Solution	10 x 1.0 ml
7001	THE RNA Storage Solution	50 ml
7140	RNA Century™ Markers	50 µg
7145	RNA Century™-Plus Markers	50 µg
7150	RNA Millennium Markers™	25 lanes
7170	BrightStar™ Biotinylated RNA Millennium Markers™	25 lanes
7175	BrightStar™ Biotinylated RNA Century™ Markers	50 lanes
7180	BrightStar™ Biotinylated RNA Century™-Plus Markers	50 lanes
7760	pUC19 DNA - Sau3A I digested	50 µg
7770	pUC19 DNA - Hpa II digested	50 µg
7780	Century™ Marker Templates	5 µg
7782	Century™-Plus Marker Templates	5 µg
7785	Millennium™ Marker Probe Template	10 rxns
9480	7.5 M LiCl Precipitation Solution	100 ml
9780	RNase*Zap*®	250 ml
9782	RNase*Zap*®	6 x 250 ml
9784	RNase*Zap*®	4 L
9786	RNase*Zap*® Wipes	1 container (100 sheets)
9788	RNase*Zap*® Wipes Refill	3 x 100 sheets
9860	TE Buffer	10 x 1.0 ml
9861	TE Buffer	50 ml
9911	0.1 mM EDTA	10 x 1.0 ml
9912	0.1 mM EDTA	50 ml

Appendix A. ii) Ambion Technical Bulletin 178: RNase and DepC Treatment: Fact or Laboratory Myth.

Ambion Technical Bulletin 178

RNase and DEPC Treatment: Fact or Laboratory Myth

Researchers are usually trained in RNA isolation and analysis methods by one another or by technical manuals. Experimental procedures are often not questioned and quickly become dogma. Furthermore, it is difficult to find literature to document the "facts" taught by mentors and technical manuals. One of these potential myths is the use of DEPC treatment to make solutions RNase-free. At Ambion, we have systematically investigated some of the DEPC fables, and the results are discussed below. Watch upcoming issues of TechNotes in which Ambion will put other RNA "truths" to the test.

1. Autoclaving is not effective at eliminating RNase in solution because the RNases simply renature as the solution cools.

FALSE, but... Autoclaving alone does indeed inactivate a substantial amount of RNase A (Figure 1). Various concentrations of RNase A were added to PBS and autoclaved. Aliquots of each solution were mixed with a 304 base ^{32}P-labeled RNA probe and incubated at 37°C for one hour, followed by electrophoresis and exposure to film. Without autoclaving, the probe begins to degrade at an RNase concentration of 100 pg/ml. Autoclaving inactivates enough of the RNase A to protect the probe from degradation up to a concentration of 1 µg/ml. Note that only a portion of the RNase is inactivated by autoclaving, otherwise the RNA probe would remain intact at any RNase concentration. Autoclaving alone may be sufficient to eliminate enough RNase for some applications. However, since neither the extent of RNase contamination nor at what RNase concentration the assay is sensitive is known, DEPC should be used as an added precaution. Also note that these experiments were only performed on RNase A and may not hold true for other RNases.

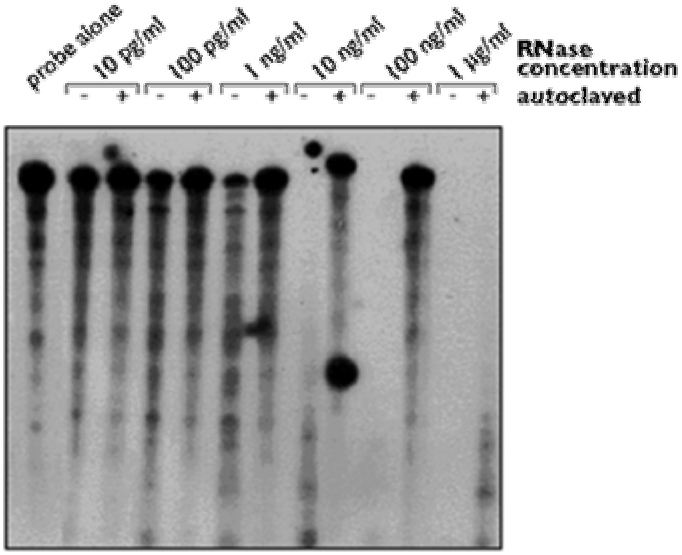


Figure 1. Effect of Autoclaving on RNase Activity. Various concentrations of RNase A were added to PBS and autoclaved for 25 minutes. 1 µl of each solution was mixed with 1 ng of a 5 x 10^4 cpm RNA probe which was 304 bases long and incubated at 37°C for one hour. 5 µl of the reaction was assessed on a 5% acrylamide/8 M urea gel and exposed to film for 5 hours with an intensifying screen.

2. Autoclaving inactivates DEPC.

TRUE. Autoclaving does inactivate DEPC by causing hydrolysis of diethylpyrocarbonate. CO_2 and EtOH are released as reaction by-products. DEPC has a half-life of approximately 30 minutes in water, and at a DEPC concentration of 0.1%, solutions autoclaved for 15 minutes/liter can be assumed to be DEPC-free.

3. Autoclaving DEPC-containing solutions should be of sufficient duration to rid the solution of any smell.

FALSE. A faint EtOH smell may linger after autoclaving, but more commonly a sweet, fruity smell is observed. This is caused by the EtOH by-product combining with trace carboxylic acid contaminates and forming volatile esters. It does not mean that trace DEPC remains in the solution.

4. Solutions containing Tris cannot be treated with DEPC.

TRUE. Tris contains an amino group which "sops up" DEPC and makes it unavailable to inactivate RNase (Figure 2). 1 M solutions of Tris, MOPS, HEPES and PBS were prepared, and 0.1% or 1% DEPC was added to each. One µg/ml RNase A was also added to each solution. The solutions were autoclaved and aliquots of each solution were mixed with a 304 base ^{32}P-labeled RNA probe and incubated at 37°C for one hour. Probe integrity was assessed by electrophoresis and exposure to film. Tris and HEPES do indeed make DEPC unavailable to inactivate RNase at a DEPC concentration of 0.1% (recommended by most protocols). However, 1% DEPC is sufficient to overcome this effect. When 1M MOPS and PBS are treated with DEPC, the DEPC remains available to inactive RNase at both concentrations (0.1% and 1%). It would be impossible to predict the different interactions of DEPC with all molecular biology reagents. The most cautious approach for making

RNase-free solutions would be to mix molecular biology grade powdered reagents up in DEPC-treated water. Alternatively, many pre-made nuclease-free solutions can be purchased from Ambion and other companies.

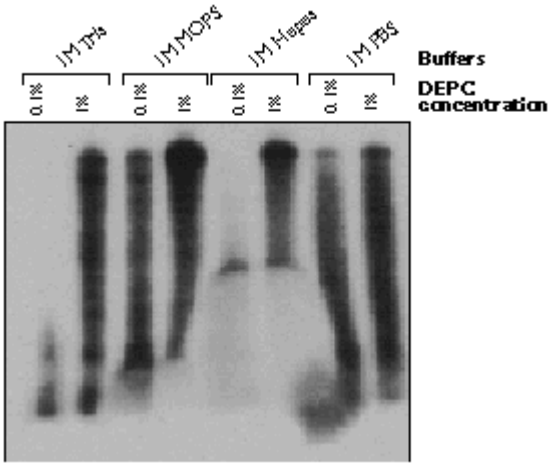
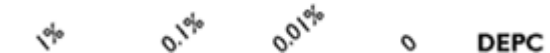


Figure 2. Effects of DEPC Treatment of Various Buffers. One µg/ml RNase A was added to various buffers along with 0.1% or 1% DEPC. Solutions were vigorously shaken for 1 minute, incubated for 1 hour at room temperature and autoclaved for 25 minutes. 1 µl of each solution was mixed with 1 ng of a 5 x 10^4 cpm 304 nt RNA probe and incubated at 37°C for one hour. 5 µl of the reaction was assessed on a 5% acrylamide/8 M urea gel and exposed overnight to film.

5. 0.1% DEPC is sufficient to inhibit any amount of RNase in a solution.

FALSE, but... The amount of DEPC required to inactivate RNase increases as the amount of contaminating RNase in a solution increases (Figure 3). 100, 500, and 1000 ng/ml RNase A was added to water followed by various amounts of DEPC. The solutions were autoclaved, and aliquots of each solution were mixed with a 304 base 32P labeled RNA probe and incubated at 37°C for one hour followed by electrophoresis and exposure to film. Untreated solutions or those treated with 0.01% DEPC could inactivate 100 ng/ml RNase A. When the RNase concentration was increased to 500 ng/ml, the DEPC concentration was insufficient to inactivate the RNase, and the probe degraded. Increasing the DEPC concentration to 0.1% protects the probe from RNase A up to 500 µg/ml and a 1% DEPC solution inactivates RNase A at a concentration of 1000 µg/ml. 0.1% DEPC is probably adequate to inactivate most RNase contamination from environmental sources and from laboratory procedures such as ribonuclease protection assays and plasmid preparations in which larger amounts of RNase are used.



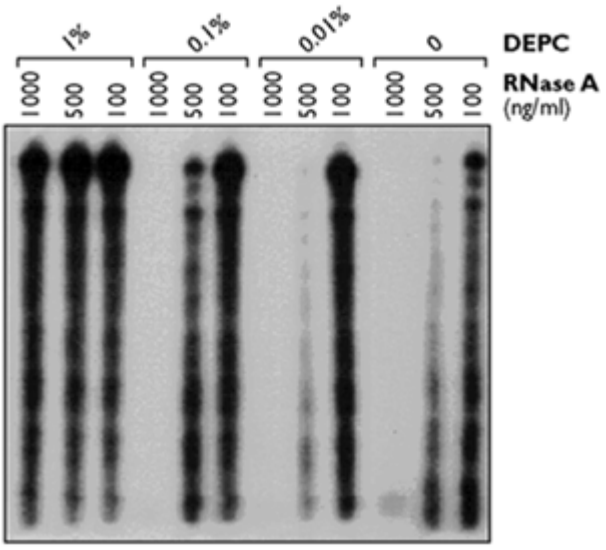


Figure 3. Effects of Varying Percentages of DEPC on Increasing Concentrations of RNase. Various concentrations of RNase A were added to aliquots of water, shaken vigorously for one minute, incubated for one hour at room temperature and autoclaved for 25 minutes. 1 µl of each solution was mixed with 1 ng of a 5 x 10^4 cpm RNA 304 nt probe and incubated at 37°C for one hour. 5 µl of the reaction was assessed on a 5% acrylamide/8 M urea gel and exposed overnight to film.

6. If 0.1% DEPC works well to inhibit RNase, 1% should work even better.

TRUE, but... Increasing DEPC concentrations inactivate increasing amounts of RNase A contamination (Figure 3). However, it is also true that high levels of residual DEPC or DEPC by-products in a solution can inhibit some enzymatic reactions or chemically alter (carboxymethylate) RNA. It has been documented that DEPC byproducts in RNA samples can inhibit in vitro translation reactions (Winkler, unpublished results). For this study, we tested transcription reactions for DEPC inhibition effects. Template DNA was dried to completion in a vacuum centrifuge and resuspended in 0.01%, 0.1% or 1% DEPC-treated water. Duplicate MAXIscript™ transcription reactions were assembled using ^{32}P-UTP, and the same concentration of DEPC treated water bought up the volume. The reactions were incubated and percent incorporation was assessed via TCA precipitation. The average percent incorporation were as follows:

% DEPC in reaction	% incorporation
0.01	64
0.10	59
1.00	53

The above data indicate that increasing amounts of DEPC increasingly inhibit transcription. Again, 0.1% DEPC is probably sufficient to inhibit most RNases with minimal effect on reactions. If DEPC is suspect in inhibiting reactions, high quality (MilliQ™) or autoclaved water can probably be substituted into the reaction. Water can be tested using Ambion's

RNaseAlert™ Kit or see Ambion's Technical Bulletin #166, Nuclease and Protease Testing: Laboratory and Commercial Considerations, which describes an RNase testing protocol similar to that used in this study.

Ordering Information

For prices and availability, please contact our Customer Service Department.

Cat#	Product Name	Size
1964	RNaseAlert™ Lab Test Kit	25 rxns
1966	RNaseAlert™ QC System	5 x 96 assays
9780	RNase*Zap*®	250 ml
9782	RNase*Zap*®	6 x 250 ml
9784	RNase*Zap*®	4 L
9786	RNase*Zap*® Wipes	1 container (100 sheets)
9788	RNase*Zap*® Wipes Refill	3 x 100 sheets
9906	DEPC-treated Water	10 x 50 ml
9915G	DEPC Treated Water	100 ml
9916	DEPC-treated Water	5 x 100 ml
9920	DEPC-treated Water	500 ml
9922	DEPC-treated Water	1 L
9924	DEPC-treated Water	4 x 1 L
9930	Nuclease-free Water (not DEPC-treated)	500 ml
9932	Nuclease-free Water (not DEPC-treated)	1 L
9934	Nuclease-free Water (not DEPC-treated)	4 x 1 L
9937	Nuclease-free Water (not DEPC-treated)	10 x 50 ml
9938	Nuclease-free Water (not DEPC-treated)	100 ml
9939	Nuclease-free Water (not DEPC-treated)	5 x 100 ml

Appendix B: Manufacturer's Protocols

Invitrogen TRIzol Reagent Protocol (formerly from GIBCO/BRL Life Sciences)

Brinkman protocol for RNA isolation using TRIzol with Phase Lock Gel-Heavy

Ambion Technical Bulletin 160-The use of LiCl precipitation for RNA purification

Qiagen protocol for QIAquick purification could not be copied but is available at www.qiagen.com

GIBCOBRL

LIFE TECHNOLOGIES™

TRIZOL® Reagent

Total RNA Isolation Reagent

WARNING: Toxic in contact with skin and if swallowed. Causes burns. After contact with skin, wash immediately with plenty of detergent and water. If you feel unwell, seek medical advice (show label where possible). Phenol (108-95-2) and Other Components (NJTSRN 80100437-5000p).

Cat. No. 15596 100 mL
200 mL

Storage Conditions: 2 to 8°C
TRIZOL has demonstrated stability of 12 months when stored at room temperature. However, we recommend storage at 2 to 8°C for optimal performance.

Description

1. TRIZOL Reagent (U.S.Patent No.5,346,994) is a ready-to-use reagent for the isolation of total RNA from cells and tissues. The reagent, a mono-phasic solution of phenol and guanidine isothiocyanate, is an improvement to the single-step RNA isolation method developed by Chomczynski and Sacchi[1]. During sample homogenization or lysis, TRIZOL Reagent maintains the integrity of the RNA, while disrupting cells and dissolving cell components. Addition of chloroform followed by centrifugation, separates the solution into an aqueous phase and an organic phase. RNA remains exclusively in the aqueous phase. After transfer of the aqueous phase, the RNA is recovered by precipitation with isopropyl alcohol. After removal of the aqueous phase, the DNA and proteins in the sample can be recovered by sequential precipitation[2]. Precipitation with ethanol yields DNA from the interphase, and an additional precipitation with isopropyl alcohol yields proteins from the organic phase[2]. Copurification of the DNA may be useful for normalizing RNA yields from sample to sample.
2. This technique performs well with small quantities of tissue (50-100 mg) and cells (5 X 10^6), and large quantities of tissue (≥1 g) and cells (>10^7), of human, animal, plant, or bacterial origin. The simplicity of the TRIZOL Reagent method allows simultaneous processing of a large number of samples. The entire procedure can be completed in one hour. Total RNA isolated by TRIZOL Reagent is free of protein and DNA contamination. It can be used for Northern blot analysis, dot blot hybridization, poly $(A)^+$ selection, in vitro translation, RNase protection assay, and molecular cloning. For use in the polymerase chain reaction (PCR*), treatment of the isolated RNA with amplification grade DNase I (GIBCO BRL® Cat. No. 18068) is recommended when the two primers lie within a single exon.
3. TRIZOL Reagent facilitates isolation of a variety of RNA species of large or small molecular size. For example, RNA isolated from rat liver, electrophoresed on an agarose gel, and stained with ethidium bromide, shows discrete bands of high molecular weight RNA between 7 kb and 15 kb in size, (composed of mRNA's and hnRNA's) two predominant ribosomal RNA bands at ~5 kb (28S) and at ~2 kb (18S), and low molecular weight RNA between 0.1 and 0.3 kb (tRNA, 5S). The isolated RNA has an $A_{260/280}$ ratio of 1.6-1.8 when diluted into distilled water. The expected yield of RNA per mg of tissue is: liver and spleen, 6-10 μg; kidney, 3-4 μg; skeletal muscles and brain, 1-5 μg; placenta, 1-4 μg. The expected yield of RNA from 1 x 10^6 cultured cells is: epithelial cells, 8-15 μg; fibroblasts, 5-7 μg.

Reagents required, but not supplied:
•Chloroform
•Isopropyl alcohol
•75% Ethanol (in DEPC-treated water)
•RNase-free water or 0.5% SDS solution [To prepare RNase-free water, draw water into RNase-free glass bottles. Add diethylpyrocarbonate (DEPC) to 0.01% (v/v). Let stand overnight and autoclave. The SDS solution must be prepared using DEPC-treated, autoclaved water.]

Precautions for Preventing RNase Contamination:
RNases can be introduced accidentally into the RNA preparation at any point in the isolation procedure through improper technique. Because RNase activity is difficult to inhibit, it is essential to prevent its introduction. The following guidelines should be observed when working with RNA.
•Always wear disposable gloves. Skin often contains bacteria and molds that can contaminate an RNA preparation and be a source of RNases. Practice good microbiological technique to prevent microbial contamination.
•Use sterile, disposable plasticware and automatic pipettes reserved for RNA work to prevent cross-contamination with RNases from shared equipment. For example, a laboratory that is using RNA probes will likely be using RNase A or T1 to reduce background on filters, and any nondisposable items (such as automatic pipettes) can be rich sources of RNases.
•In the presence of TRIZOL Reagent, RNA is protected from RNase contamination. Downstream sample handling requires that nondisposable glassware or plasticware be RNase-free. Glass items can be baked at 150°C for 4 hours, and plastic items can be soaked for 10 minutes in 0.5 M NaOH, rinsed thoroughly with water, and autoclaved.

Other Precautions
•Use of disposable tubes made of clear polypropylene is recommended when working with less than 2-mL volumes of TRIZOL Reagent.
•For larger volumes, use glass (Corex) or polypropylene tubes, and test to be sure that the tubes can withstand 12,000 x g with TRIZOL Reagent and chloroform. Do not use tubes that leak or crack.
•Carefully equilibrate the weights of the tubes prior to centrifugation.
•Glass tubes must be sealed with parafilm topped with a layer of foil, and polypropylene tubes must be capped before centrifugation.

RNA ISOLATION NOTES:

1. Isolation of RNA from small quantities of tissue (1 to 10 mg) or Cell (10^2 to 10^4) Samples: Add 800 μL of TRIZOL to the tissue or cells. Add 200μg glycogen (Cat. No 10814) directly to the TRIZOL (final glycogen concentration in TRIZOL is 250 μg /mL). To reduce viscosity, shear the genomic DNA with 2 passes through a 26 guage needle prior to chloroform addition. The glycogen remains in the aqueous phase and is co-precipitated with the RNA. It does not inhibit first-strand synthesis at concentrations up to 4 mg/mL and does not inhibit PCR.
2. After homogenization and before addition of chloroform, samples can be stored at -60 to -70°C for at least one month. The RNA precipitate (step 4, RNA WASH) can be stored in 75% ethanol at 2 to 8°C for at least one week, or at least one year at –5 to -20°C.
3. Table-top centrifuges that can attain a maximum of 2,600 x g are suitable for use in these protocols if the centrifugation time is increased to 30-60 minutes in steps 2 and 3.

INSTRUCTIONS FOR RNA ISOLATION:

CAUTION: When working with TRIZOL Reagent use gloves and eye protection (shield, safety goggles). Avoid contact with skin or clothing. Use in a chemical fume hood. Avoid breathing vapor.

Note: Unless otherwise stated, the procedure is carried out at 15 to 30°C, and reagents are at 15 to 30°C. See page 2 for reagents required but not supplied, and for precautions.

1. HOMOGENIZATION (see notes 1-3)
   a. *Tissues*
      Homogenize tissue samples in 1 mL of TRIZOL Reagent per 50-100 mg of tissue using a glass-Teflon® or power homogenizer (Polytron, or Tekmar's TISSUMIZER® or equivalent). The sample volume should not exceed 10% of the volume of TRIZOL Reagent used for homogenization.
   b. *Cells Grown in Monolayer*
      Lyse cells directly in a culture dish by adding 1 mL of TRIZOL Reagent to a 3.5 cm diameter dish, and passing the cell lysate several times through a pipette. The amount of TRIZOL Reagent added is based on the area of the culture dish (1 mL per 10 cm^2) and not on the number of cells present. An insufficient amount of TRIZOL Reagent may result in contamination of the isolated RNA with DNA.
   c. *Cells Grown in Suspension*
      Pellet cells by centrifugation. Lyse cells in TRIZOL Reagent by repetitive pipetting. Use 1 mL of the reagent per 5-10 x 10^6 of animal, plant or yeast cells, or per 1 x 10^7 bacterial cells. Washing cells before addition of TRIZOL Reagent should be avoided as this increases the possibility of mRNA degradation. Disruption of some yeast and bacterial cells may require the use of a homogenizer.
2. OPTIONAL: An additional isolation step may be required for samples with high content of proteins, fat, polysaccharides or extracellular material such as muscles, fat tissue, and tuberous parts of plants. Following homogenization, remove insoluble material from the homogenate by centrifugation at 12,000 x g for 10 minutes at 2 to 8°C. The resulting pellet contains extracellular membranes, polysaccharides, and high molecular weight DNA, while the supernate contains RNA. In samples from fat tissue, an excess of fat collects as a top layer which should be removed. In each case, transfer the cleared homogenate solution to a fresh tube and proceed with chloroform addition and phase separation as described.
3. PHASE SEPARATION
   Incubate the homogenized samples for 5 minutes at 15 to 30°C to permit the complete dissociation of nucleoprotein complexes. Add 0.2 mL of chloroform per 1 mL of TRIZOL Reagent. Cap sample tubes securely. Shake tubes vigorously by hand for 15 seconds and incubate them at 15 to 30°C for 2 to 3 minutes. Centrifuge the samples at no more than 12,000 x g for 15 minutes at 2 to 8°C. Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. The volume of the aqueous phase is about 60% of the volume of TRIZOL Reagent used for homogenization.
4. RNA PRECIPITATION
   Transfer the aqueous phase to a fresh tube, and save the organic phase if isolation of DNA or protein is desired. Precipitate the RNA from the aqueous phase by mixing with isopropyl alcohol. Use 0.5 mL of isopropyl alcohol per 1 mL of TRIZOL Reagent used for the initial homogenization. Incubate samples at 15 to 30°C for 10 minutes and centrifuge at no more than 12,000 x g for 10 minutes at 2 to 8°C. The RNA precipitate, often invisible before centrifugation, forms a gel-like pellet on the side and bottom of the tube.
5. RNA WASH
   Remove the supernate. Wash the RNA pellet once with 75% ethanol, adding at least 1 mL of 75% ethanol per 1 mL of TRIZOL Reagent used for the initial homogenization. Mix the sample by vortexing and centrifuge at no more than 7,500 x g for 5 minutes at 2 to 8°C.
6. REDISSOLVING THE RNA
   At the end of the procedure, briefly dry the RNA pellet (air-dry or vacuum-dry for 5-10 minutes). Do not dry the RNA by centrifugation under vacuum. It is important not to let the RNA pellet dry completely as this will greatly decrease its solubility. Partially dissolved RNA samples have an $A_{260/280}$ ratio < 1.6. Dissolve RNA in RNase-free water or 0.5% SDS solution by passing the solution a few times through a pipette tip, and incubating for 10 minutes at 55 to 60°C. (Avoid SDS when RNA will be used in subsequent enzymatic reactions.) RNA can also be redissolved in 100% formamide (deionized) and stored at -70°C. (See Bracete, et.al. (1998) Focus 20:3 p 82).

INSTRUCTIONS FOR DNA ISOLATION:

After complete removal of the aqueous phase, as described in the RNA isolation protocol, the DNA in the interphase and phenol phase from the initial homogenate may be isolated. Following precipitation and a series of washes, the DNA is solubilized in 8 mM NaOH. Full recovery of DNA from tissues and culture cells permits the use of TRIZOL Reagent for the determination of the DNA content in analyzed samples[2]. Simultaneous extraction of genomic DNA allows for normalization of the results of Northern analysis per genomic DNA instead of the more variable total RNA or tissue weight. (Depending on the source, the DNA pellet obtained may require additional purification (e.g., phenol extraction) prior to other applications.

Reagents required, but not supplied:
•Ethanol
•0.1 M Sodium citrate in 10% ethanol
•75% Ethanol
•8 mM NaOH

Unless otherwise stated, the procedure is carried out at 15 to 30°C.

1. DNA PRECIPITATION
   Remove the remaining aqueous phase overlying the interphase, and precipitate the DNA from the interphase and organic phase with ethanol. Add 0.3 mL of 100% ethanol per 1 mL of TRIZOL Reagent used for the initial homogenization, and mix samples by inversion. Next, store the samples at 15 to 30°C for 2-3 minutes and sediment DNA by centrifugation at no more than 2,000 x g for 5 minutes at 2 to 8°C.
   Careful removal of the aqueous phase is critical for the quality of the isolated DNA.

2. DNA WASH
Remove the phenol-ethanol supernate, and if desired, save it for protein isolation. Wash the DNA pellet twice in a solution containing 0.1 M sodium citrate in 10% ethanol. Use 1 mL of the solution per 1 mL of TRIZOL Reagent used for the initial homogenization. At each wash, store the DNA pellet in the washing solution for 30 minutes at 15 to 30°C (with periodic mixing) and centrifuge at 2,000 x g for 5 minutes at 2 to 8°C. Following these two washes, suspend the DNA pellet in 75% ethanol (1.5-2 mL of 75% ethanol per 1 mL TRIZOL Reagent), store for 10-20 minutes at 15 to 30°C (with periodic mixing) and centrifuge at 2,000 x g for 5 minutes at 2 to 8°C.

An additional wash in 0.1 M sodium citrate-10% ethanol solution is required for large pellets, containing > 200 mg DNA or large amounts of a non-DNA material.

3. REDISSOLVING THE DNA
Air dry the DNA 5 to 15 minutes in an open tube.(***DO NOT DRY UNDER CENTRIFUGATION; it will be more difficult to dissolve.) Dissolve DNA in 8 mM NaOH such that the concentration of DNA is 0.2 – 0.3 µg/µL. Typically add 300 – 600 µL of 8mM NaOH to DNA isolated from 10^7 cells or 50 – 70mg of tissue. Resuspending in weak base is HIGHLY recommended since isolated DNA does not resuspend well in water or in Tris buffers. The pH of the 8 mM NaOH is only ~9 and should be easily adjusted with TE or HEPES once the DNA is in solution. At this stage, the DNA preparations (especially from tissues) may contain insoluble gel-like material (fragments of membranes, etc.) Remove the insoluble material by centrifugation at >12,000 g for 10 minutes. Transfer the supernate containing the DNA to a new tube. Stability of DNA in 8 mM NaOH is several months at 4°C; greater than one year at -20°C; indefinitely at –70°C.

QUANTITATION AND EXPECTED YIELDS OF DNA

Take an aliquot of the DNA preparation solubilized in 8 mM NaOH, mix it with water and measure the A_{260} of the resulting solution. Calculate the DNA content using the A_{260} value for double-stranded DNA. One A_{260} unit equals 50 µg of double-stranded DNA/mL. For calculation of cell number in analyzed samples, assume that the amount of DNA per 1 x 10^6 diploid cells of human, rat, and mouse origin equals: 7.1 µg, 6.5 µg, and 5.8 µg, respectively [3]. The expected yield of DNA per mg of tissue is: 3-4 µg from liver and kidney; and 2-3 µg from skeletal muscles, brain and placenta. The expected yield of DNA per 1 x 10^6 cultured human, rat and mouse cells is 5-7 µg.

APPLICATIONS

Amplification of DNA by PCR.

After redissolving the DNA in 8 mM NaOH, adjust the pH to 8.4 with 0.1 M HEPES (see table). Add 0.1 to 1.0 µg of the DNA sample to your PCR reaction mixture and perform the standard PCR protocol.

Restriction endonuclease reactions.

Adjust the pH of the DNA solution to a required value using HEPES (see table). Alternatively, samples may be dialyzed against 1 mM EDTA, pH 7 to pH 8.0. Use 3-5 units of enzyme per microgram of DNA. Use the conditions recommended by the manufacturer for the particular enzyme, and allow the reaction to proceed for 3 to 24 h. In a typical assay, 80-90% of the DNA is digestible.

pH Adjustment of DNA Samples Dissolved in 8 mM NaOH

(For 1 mL of 8 mM NaOH use the following amounts of 0.1 M or 1 M HEPES, free acid.)

Final pH	0.1 M HEPES (µL)	Final pH	1 M HEPES (µL)
8.4	66	7.2	30
8.2	90	7.0	42
8.0	115		
7.8	135		
7.5	180		

Notes:
1. The phenol phase and interphase can be stored at 2 to 8°C overnight.
2. Samples suspended in 75% ethanol can be stored at 2 to 8°C for months.
3. Samples dissolved in 8 mM NaOH can be stored overnight at 2 to 8°C. For long-term storage, adjust the pH to 7-8, and adjust the EDTA concentration to 1 mM.

INSTRUCTIONS FOR PROTEIN ISOLATION:

Proteins are isolated from the phenol-ethanol supernate obtained after precipitation of DNA with ethanol (step 1, DNA PRECIPITATION). The resulting preparation can be analyzed for the presence of specific proteins by Western blotting[2].

Reagents required, but not supplied:
- Isopropyl alcohol
- 0.3 M Guanidine hydrochloride in 95% ethanol
- Ethanol
- 1% SDS

1. PROTEIN PRECIPITATION
Precipitate proteins from the phenol-ethanol supernate (approximate volume 0.8 mL per 1 mL of TRIZOL Reagent) with isopropyl alcohol. Add 1.5 mL of isopropanol per 1 mL of TRIZOL Reagent used for the initial homogenization. Store samples for 10 minutes at 15 to 30°C, and sediment the protein precipitate at 12,000 x g for 10 minutes at 2 to 8°C.

2. PROTEIN WASH
Remove the supernate and wash the protein pellet 3 times in a solution containing 0.3 M guanidine hydrochloride in 95% ethanol. Add 2 mL of wash solution per1 mL of TRIZOL Reagent used for the initial homogenization. During each wash cycle, store the protein pellet in the wash solution for 20 minutes at 15 to 30°C and centrifuge at 7,500 x g for 5 minutes at 2 to 8°C. After the final wash, vortex the protein pellet in 2 mL of ethanol. Store the protein pellet in ethanol for 20 minutes at 15 to 30°C and centrifuge at 7,500 x g for 5 minutes at 2 to 8°C.

3. REDISSOLVING THE PROTEIN PELLET
Vacuum dry the protein pellet for 5-10 minutes. Dissolve it in 1% SDS by pipetting. Complete dissolution of the protein pellet may require incubating the sample at 50°C. Sediment any insoluble material by centrifugation at 10,000 x g for 10 minutes at 2 to 8°C, and transfer the supernate to a fresh tube. The sample is ready for use in Western blotting or may be stored at -5 to -20°C for future use.

Notes:
1. The protein pellet suspended in 0.3 M guanidine hydrochloride-95% ethanol or in ethanol can be stored for at least one month at 2 to 8°C, or for at least one year at -5 to -20°C.
2. The following protocol is an alternative approach that allows for more efficient recovery of proteins. Dialyze the phenol-ethanol supernate against three changes of 0.1% SDS at 2 to 8°C. Centrifuge the dialyzed material at 10,000 x g for 10 minutes. Use the clear supernate for Western blotting.
3. Proteins may be quantified by the Bradford method as long as the concentration of SDS is low enough (<0.1%) so that it will not interfere. Methods that do not have detergent-interface problems, and that do not rely on A_{260}/A_{280} measurements may be used (traces of phenol may cause overestimation of protein concentrations).

References:

1. Chomczynski, P., and Sacchi, N. *Anal. Biochem*. **162**, 156 (1987).
2. Chomczynski, P. *Biotechniques* **15**, 532 (1993).
3. Ausubel, F.M., et.al, eds. *Current Protocols in Molecular Biology*, Vol.2, Greene Publishing Assoc. and Wiley-Interscience, New York, p.A.1.5 (1990).
4. Simms, D., Cizdziel, P.E., Chomczynski, P. *Focus* **15**.4, 99 (1993).

TROUBLESHOOTING GUIDE

RNA ISOLATION

- Expected yields of RNA per mg of tissue or 1 X 10^6 cultured cells
  - Liver and spleen, 6-10 µg
  - Kidney, 3-4 µg
  - Skeletal muscles and brain, 1-1.5 µg
  - Placenta, 1-4 µg
  - Epithelial cells, 8-15 µg
  - Fibroblasts, 5-7 µg
- Low yield
  - Incomplete homogenization or lysis of samples.
  - Final RNA pellet incompletely redissolved.
- $A_{260/280}$ ratio <1.65
  - RNA sample was diluted in water instead of TE prior to spectrophotometric analysis. Low ionic strength and low pH solutions increase absorbance at 280nm. See Wilfinger, W. et.al, Biotechniques 22: 474-481. and Fox, D.K. (1998) Focus 20:2 p.37).
  - Sample homogenized in too small a reagent volume.
  - Following homogenization, samples were not stored at room temperature for 5 minutes.
  - The aqueous phase was contaminated with the phenol phase.
  - Incomplete dissolution of the final RNA pellet.
- RNA degradation
  - Tissues were not immediately processed or frozen after removal from the animal.
  - Samples used for isolation, or the isolated RNA preparations were stored at -5 to-20°C, instead of -60 to -70°C.
  - Cells were dispersed by trypsin digestion.
  - Aqueous solutions or tubes were not RNase-free.
  - Formaldehyde used for agarose-gel electrophoresis had a pH below 3.5.
- DNA contamination
  - Sample homogenized in too small a reagent volume.
  - Samples used for the isolation contained organic solvents (e.g., ethanol, DMSO), strong buffers, or alkaline solution.
- Proteoglycan and polysaccharide contamination
  - The following modification of the RNA precipitation (step 3) removes these contaminating compounds from the isolated RNA. Add to the aqueous phase 0.25 mL of isopropanol followed by 0.25 mL of a high salt precipitation solution (0.8 M sodium citrate and 1.2 M NaCl) per 1 mL of TRIZOL Reagent used for the homogenization. Mix the resulting solution, centrifuge and proceed with the isolation as described in the protocol. The modified precipitation effectively precipitates RNA while maintaining polysaccharides and proteoglycans in a soluble form. A combination of the modified precipitation with an additional centrifugation of the initial homogenate (note #2, RNA isolation protocol) is required to isolate pure RNA from plant material containing a very high level of polysaccharides.

DNA ISOLATION

- Expected yields of DNA per mg of tissue or 1 x 10^6 cultured cells
  - Liver and kidney, 3-4 µg
  - Skeletal muscles, brain, and placenta 2-3 µg
  - Cultured human, rat, and mouse cells, 5-7 µg
  - Fibroblasts, 5-7 µg
- Low yield
  - Incomplete homogenization or lysis of samples.
  - Final DNA pellet incompletely redissolved.
- $A_{260/280}$ ratio <1.70
  - DNA sample was diluted in water instead of TE prior to spectrophotometric analysis.
  - Phenol was not sufficiently removed from the DNA preparation. Wash the DNA pellet an additional time with 0.1 M sodium citrate in 10% ethanol.
- DNA degradation
  - Tissues were not immediately processed or frozen after removal from the animal.
  - Samples used for isolation, or the isolated RNA preparations were stored at -5 to-20°C, instead of -60 to -70°C.
  - Samples were homogenized with a Polytron or other high speed homogenizer.
- RNA contamination
  - Incomplete removal of aqueous phase.
  - DNA pellet insufficiently washed with 0.1 M sodium citrate in 10% ethanol.
- Other applications
  - Prior to use in PCR amplification, adjust the pH to 8.4.
  - For digestion of the DNA with restriction endonucleases, adjust the pH to the desired value, use 3-5 units of enzyme per µg of DNA, and allow the reaction to go for 3-24 hours under optimal conditions for the particular enzyme.
  - Typically 80-90% of the DNA is digested.

PROTEIN ISOLATION

- Low yield
  - Incomplete homogenization or lysis of samples.
  - Final DNA pellet incompletely redissolved.
- Protein degradation
  - Tissues were not immediately processed or frozen after removing from the animal.
- Band deformation in PAGE
  - Protein pellet insufficiently washed.

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For laboratory research use only.
CAUTION: Not for diagnostic use. The safety and efficacy of this product in diagnostic or other clinical uses has not been established.

April 1999 **Form No. 3796**

Applications On-line

Protocol for RNA Isolation using TRIzol® Reagent with Phase Lock Gel-Heavy

Phase Lock Gel may be used in conjunction with TRIzol Reagent for the isolation of total RNA from cell and tissue samples. Increased yields are observed using this method, because the entire aqueous phase can be recovered without interphase contamination. Below is a protocol outlining the steps involved in RNA isolation with TRIzol Reagent and Phase Lock Gel-Heavy. For further details about TRIzol Reagent, please refer to TRIzol Reagent protocol.

IMPORTANT: When working with TRIzol Reagent, use appropriate protective clothing and work under a chemical fume hood.

1. Homogenization:
    1. Tissues: Add 1 ml TRIzol Reagent per 50–100 mg tissue and homogenize with a POLYTRON® homogenizer. For 1–10 mg quantities of tissue, add 0.8 ml TRIzol Reagent and 0.2 ml 1 mg/ml mussel glycogen and homogenize.
    2. Cells grown in a Monolayer: Lyse cells by adding 1 ml TRIzol Reagent per 10 cm^2 area of culture dish. Pipette suspension several times to disrupt cells. For 10^2–10^4 cells, add 0.8 ml TRIzol Reagent and 0.2 ml 1 mg/ml mussel glycogen and homogenize.
    3. Cells grown in Suspension: Pellet cells by centrifugation. Do not wash cell pellet. Add 1 ml TRIzol Reagent per 5–10 x 10^6 animal, plant, or yeast cells or 1 x 10^7 bacterial cells. Resuspend pellet by pipetting to lyse cells. For 10^2–10^4 cells, add 0.8 ml TRIzol Reagent and 0.2 ml 1 mg/ml mussel glycogen and homogenize.
2. Pre-spin the appropriate sized Phase Lock Gel-Heavy tubes briefly to collect gel on tube bottoms (1500 x g for 30 seconds is sufficient to collect gel at tube bottoms).
3. Add cell lysate to the tubes containing pre-spun Phase Lock Gel-Heavy and incubate 5 minutes at 15–30 °C.
4. Add 0.2 ml chloroform (or chloroform-isoamyl alcohol) per 1 ml TRIzol Reagent initially used. Cap tubes and shake vigorously for 15 seconds. **DO NOT VORTEX!**
5. Centrifuge samples at no more than 12,000 x g for 10 minutes at 2–8 °C.
   NOTE: 15 ml and 50 ml PLG-H screw-cap tubes should be centrifuged at or below 2000 x g.
6. Examine phasing. Clear, aqueous phase should be entirely atop Phase Lock Gel. The phenol-chloroform phase and cloudy interphase should be below Phase Lock Gel layer. If this is not the case, add another 0.2 ml chloroform (or chloroform-isoamyl alcohol) per 1 ml TRIzol Reagent used initally and shake vigorously. Repeat centrifugation and re-examine phasing.
7. Transfer aqueous phase containing RNA to a fresh tube (aqueous phase may be decanted).
8. Precipitate RNA by adding 0.5 ml Isopropyl alcohol per 1 ml TRIzol Reagent used initially. Mix samples by repeated inversion. Allow samples to incubate at 15–30 °C for 10 minutes. Centrifuge samples for 10 minutes at no more than 12,000 x g, 2–8 °C. RNA pellet should be visible on side and bottom of tube.
9. Decant supernatant. Add 1 ml 75% ethanol per 1 ml TRIzol Reagent used initially to wash the RNA pellet. Mix samples to dislodge pellet, using a vortex if necessary. Centrifuge samples at no more than 7,500 x g for 5 minutes at 2–8 °C.
10. Carefully decant supernatant. Briefly air-dry or vacuum-dry the RNA pellet to remove residual ethanol (5–10 minutes). Do not overdry pellet by centrifugation under vacuum as this will make resuspension more difficult. Dissolve RNA pellet in Molecular Biology Grade water, incubating at 55–60 °C for 10 minutes to facilitate dissolution.

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15. Wilfinger, W.W. *et al.* (1997) Effect of pH and ionic strength on the spectrophotometric assessment of nucleic acid purity. Biotechniques **22**:474-481.

12 Ordering information

Product	***Package size***	***Product number***
Phase Lock Gel 0.5 ml Light	300 preparations	0032 005.004
Phase Lock Gel 0.5 ml Heavy	300 preparations	0032 005.055
Phase Lock Gel 1.5 ml Light	200 preparations	0032 007.961
Phase Lock Gel 1.5 ml Heavy	200 preparations	0032 007.953
Phase Lock Gel 2 ml Light	200 preparations	0032 005.101
Phase Lock Gel 2 ml Heavy	200 preparations	0032 005.152
Phase Lock Gel 15 ml Light	100 preparations	0032 005.209
Phase Lock Gel 15 ml Heavy	100 preparations	0032 005.250
Phase Lock Gel 50 ml Light	25 preparations	0032 005.306
Phase Lock Gel 50 ml Heavy	25 preparations	0032 005.330
Phase Lock Gel 3 ml Syringe Light	10 syringes	0032 007.988
Phase Lock Gel 3 ml Syringe Heavy	10 syringes	0032 007.970

Ambion Technical Bulletin 160

The Use of LiCl Precipitation for RNA Purification

LiCl has been frequently used to precipitate RNA, although precipitation with alcohol and a monovalent cation such as sodium or ammonium ion is much more widely used. LiCl precipitation offers major advantages over other RNA precipitation methods in that it does not efficiently precipitate DNA, protein or carbohydrate (Barlow et al., 1963). It is the method of choice for removing inhibitors of translation or cDNA synthesis from RNA preparations (Cathala et al., 1983). It also provides a simple rapid method for recovering RNA from in vitro transcription reactions.

Ambion provides LiCl as an RNA recovery agent in its MEGAscript™ and mMESSAGE mMACHINE™ large scale in vitro transcription kits. However, while providing telephone technical service, we have noticed that many users are reluctant to use LiCl, presumably because there is not good data in the literature describing its properties. We have conducted a systematic study of the use of LiCl and find that it is a very effective method for precipitating RNA, especially from in vitro transcription reactions.

The three key variables we studied were: (a) the temperature at which the precipitate is allowed to form, (b) the concentration of the RNA and the lithium chloride used and, (c) the time and speed of centrifugation used to collect the precipitated RNA. All of these variables have been explored and are discussed below. We find that LiCl precipitated RNA samples prepared in this way require no further purification for use in hybridization and in vitro translation reactions. It has been reported that lithium chloride is unsuitable for cell free translations due to the inhibition of chloride ions (Maniatis, et al., 1989); However, we have not been able to document any deleterious effect in either translation or microinjection experiments. Another advantage is that lithium precipitation efficiently removes unincorporated NTPs, which allows for more accurate quantitation by UV spectrophotometry.

Experimental Procedures

Unlabeled RNA transcripts with the lengths of 100, 300, and 500 bases were synthesized in large amounts using the MEGAscript™ in vitro Transcription Kit. Lithium chloride was used to precipitate the RNA followed by resuspension in water. The concentration of each RNA was determined by spectrophotometry. An additional set of labeled transcripts were synthesized in the presence of 50 µCi of alpha-[^{32}P] UTP (800 Ci/mmol) to produce the three RNA transcripts with a specific activity of 3.3 x 10^6 cpm/µg.

Comparison of Lithium Chloride and Ammonium Acetate/Ethanol

In preliminary experiments, we compared the precipitation efficiency of 2.5 M lithium chloride with 0.5 M ammonium acetate and 2.5 volumes of ethanol with RNA transcripts of 100 and 300 bases in length. The average recovery with the lithium chloride was 74%

compared to 85% with the ethanol. Gel analysis of the precipitated products suggested that the lithium chloride may not precipitate the smallest RNA fragments as efficiently as the ethanol. This can be an advantage when preparing labeled probe for ribonuclease protection assays in that the lithium chloride precipitated product will give a cleaner band on gel analysis, especially with non-gel purified probe.

Precipitation Parameters of Lithium Chloride RNA Concentration

Decreasing amounts of each size of RNA were precipitated using a constant concentration of 2.5 M LiCl to determine if there is a threshold of precipitation for a given size and concentration of RNA. The three stocks of non-radioactive RNA mixed with tracer labeled RNA (5×10^4 cpm) were aliquotted in tubes. Water and then lithium chloride were added to a final volume of 50 µl, with a constant concentration of 2.5 M lithium chloride. Each size transcript was tested separately to observe possible size effects on precipitation efficiency. All samples were chilled 30 minutes at -20°C then centrifuged for 15 minutes at 16,000 x g at 4°C. The supernatant was removed by aspiration and dried for 10 minutes. The pellets were resuspended in 10 µl of gel loading buffer (80% formamide, 0.1% bromophenol blue, 0.1% xylene cyanol, and 1mM EDTA) and heated for 5 minutes at 95° C. A portion of each sample was run on a 4% PAGE-urea gel. The gel was dried and exposed directly to film for 30 minutes. Figure 1 shows the effect of RNA concentration on lithium chloride precipitation of the 100 base transcript. It appears that RNA as small as 100 nucleotides and as dilute as 5 µg/ml can be efficiently precipitated by lithium chloride. This was a surprising result since it is generally thought that RNA must be at relatively high concentrations in order to be efficiently precipitated with lithium chloride.

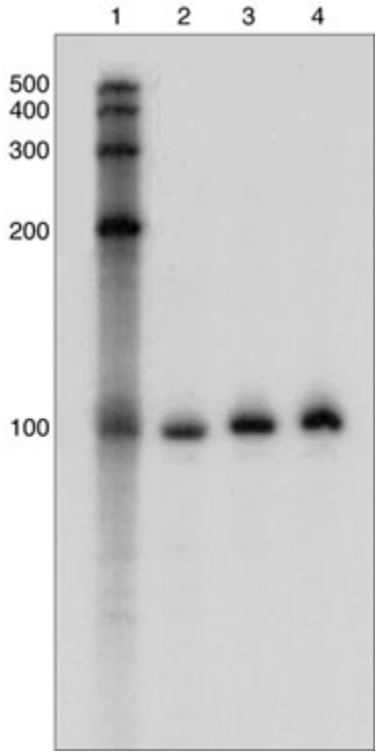

Figure 1. The Effect of RNA Concentration on Lithium Chloride Precipitation. Lane 1, RNA size standards. Lane 2, 500 µg/ml RNA, Lane 3, 50 µg/ml RNA, and Lane 5, 5 µg/ml of RNA.

Lithium Chloride Concentration

The effect of lithium chloride concentration on precipitation efficiency was tested on three different sized transcripts. Each size transcript was kept at a constant concentration of 1 µg/ml while the lithium chloride was tested at 2.5, 1.0, and 0.5 molar concentrations. Labeled RNA (5 x 10^4 cpm) was also added as a tracer. The samples were centrifuged 10 minutes at 4°C, aspirated and dried. The pellets were resuspended in 10 µl of gel loading buffer, heated for 10 minutes at 95°C, and a portion of each was run on a 4% PAGE-urea gel. The gel was dried and exposed for 30 minutes without intensifying screens. Figure 2 shows the effect of lithium chloride on precipitation of the 300 base transcript. It appears that lithium chloride is effectively precipitating RNA at a 0.5 molar concentration and recovery was similar at all concentrations of lithium chloride. Lane 5 is a zero lithium chloride control to analyze the effect of centrifugation.

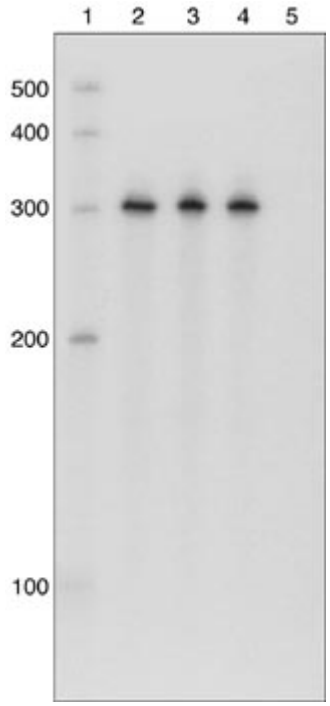

Figure 2. Effect of Lithium Chloride Concentration on Precipitating RNA. Lane 1, RNA size standards. Lane 2, 2.5 M LiCl. Lane 3, 1.0 M LiCl. Lane 4, 0.5 M LiCl, and lane 5, no LiCl.

Chilling Time

The RNA was kept at a constant concentration of 1 µg/ml, with 1.0 M lithium chloride. The length of time for precipitation was tested at 0, 0.5, and 1.0 hour. The 0.5 and 1.0 hour time points were incubated at -20°C and 25°C to test precipitation time and temperature independently. Samples were prepared as before, and visualized on a 4% PAGE-urea gel. In Figure 3, it appears that allowing precipitation to occur for a 30 minute period is more efficient than immediate centrifugation; compare Lane 2 to Lane 3. Although it appears there is no difference in precipitating 30 or 60 minutes at -20°C and 25°C, as seen in Lanes 3-6, it is advisable to precipitate at -20°C for 30 minutes to lower the activity of any possible RNases that might be present.

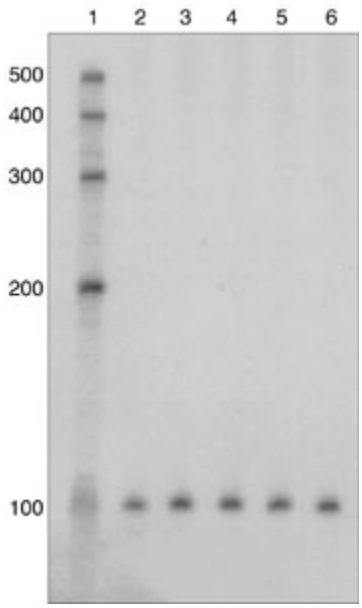

Figure 3. Effect of Precipitation Temperature Using Lithium Chloride. Lane 1, RNA size standards. Lane 2, RNA centrifutged immediately without chilling. Lane 3, RNA chilled at -20°C for 30 minutes before centrifugation. Lane 4, RNA incubated at 25°C for 30 minutes to test precipitation time independently of chilling. Lane 5, RNA chilled at -20°C for 1 hour. Lane 6, RNA incubated at 25°C for 1 hour.

Centrifugation Time

Using a constant concentration of 1 µg/ml RNA, in a volume of 50 µl with 1.0 M lithium chloride, samples were centrifuged for 0.5, 1, 2, 5, 10, and 20 minutes at 4°C at 16,000 x g. The different sized transcripts, with radioactive RNA, were tested independently. Figure 4 shows that centrifugation time is a major factor in recovery of RNA. As little as 50 ng of RNA can be quantitatively recovered by centrifugation at 16,000 x g for 20 minutes at 4°C. Lanes 2-7 show decreasing recovery as spin time is lowered.

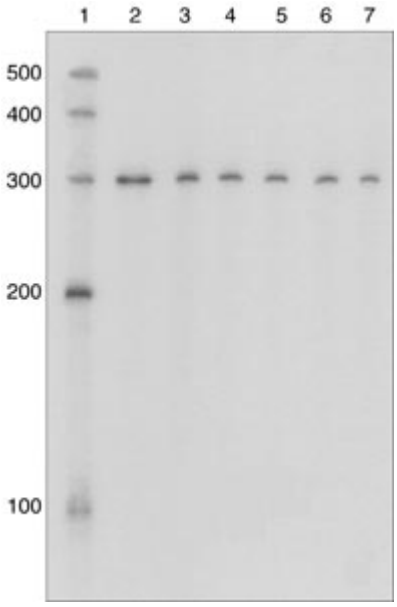

Figure 4. Effects of Centrifugation Time in Precipitating RNA. Lane 1, RNA size standards. Lane 2, RNA centrifuged for 20 minutes, Lane 3, 10 minutes, Lane 4, 5 minutes, Lane 5, 2 minutes, Lane 6, 1 minutes, and Lane 7, 30 seconds.

Discussion

The use of lithium chloride in RNA precipitation is a fast, convenient method of isolating transcripts from in vitro transcription reactions with very low carry over of unincorporated nucleotides. A major advantage of lithium chloride is that it does not efficiently precipitate either protein or DNA. For some applications, gel purification may be necessary, as in a ribonuclease protection assay. For in vitro or in vivo translation, the lithium chloride method may be preferable to ethanol precipitation since full-length transcripts are often preferentially recovered. Moreover, RNAs precipitated by this method give more accurate values when quantitated by UV spectroscopy since lithium chloride is so effective at removing free nucleotides. This strategy is similar to the use of isopropanol rather than ethanol to precipitate nucleic acids. Isopropanol is less efficient than ethanol at precipitating nucleotides and thus, gives more accurate values when RNA concentration is quantitated by UV spectrophotometry.

Contrary to previously published reports, we find that lithium chloride does not appear to preferentially precipitate higher molecular weight RNA rather than smaller RNA. Lithium chloride precipitations using mixtures of equal amounts of RNA of lengths 100, 200, 300, 400, and 500 bases (RNA Century™ Size Standards) showed that all sizes were precipitated equally well (data not shown). Since it was thought that the larger sizes might aid in the precipitation of smaller size transcripts, the experiments in this paper were performed using each size of transcript separately. No differences in precipitating a single size of RNA (e.g. 100 bases) as compared to a mix of all sizes of the RNA markers was seen. It should be noted, however, that some small RNAs such as tRNAs are not efficiently precipitated by lithium chloride. This is likely due to the high degree of secondary structure in tRNA. While we recommend the routine use of lithium chloride for precipitating RNA from solutions containing at least 400 µg/ml RNA, we are cautious about recommending its use with lower concentrations of RNA until we have tested its use with a wider range of

RNAs.

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Ordering Information

For prices and availability, please contact our **Customer Service Department**.

Cat#	Product Name	Size
9480	7.5 M LiCl Precipitation Solution	100 ml

Appendix C: Clone Naming Schemes

Appendix C. i) Gene Naming Conventions for Human Arrays

December 6, 2001.

Preamble:

The human clone set has been re-annotated. This was performed by Kyle Furge. The gene names were annotated in the original clone list from Research Genetics. This clone list was subsequently manipulated to give the Print_List in AMAD and to generate a GAL file that can be used in GenePix. The GAL file and the Print_List were generated independently and have been verified to be perfectly consistent.

Introduction:

The human clone set from Research Genetics has annotation associated with it, but this annotation is old and was performed before the draft sequence of the human genome was complete. The annotation is based on Unigene Build 106, whereas Unigene Build 143 came out a month ago I think. The annotation was performed by looking at Unigene, and another database, REFSEQ. REFSEQ is described below in the appendix to this document.

The basic scheme used to re-annotate the clones was to compare the sequence of each Research Genetics clone to Unigene and REFSEQ. If there was a match to a REVIEWED or PROVISIONAL REFSEQ record, the name provided was thought to be reliable and was used to name the clone. If no REFSEQ record matched, the Unigene name was used instead. In instances where there was no Unigene match, the clone remained un-named.

The second element of the name was based on the protein family annotation. The protein family name is appended in CAPITAL letters.

The last element of the name is a set of accession numbers to the various databases. These are enclosed in [] square parentheses. The first number is the accession number, followed by the REFSEQ number if available. The last number is the Unigene cluster identifier.

Example 1: The following gene has accession number AA411440, has a REFSEQ identifier NM_003379 and belongs to Unigene cluster Hs. 155191. Since it has a REFSEQ identifier, the name given is from the REFSEQ database. This protein does not have a known protein family association.

villin 2 (ezrin) (VIL2), mRNA. [AA411440,NM_003379,Hs.155191]

Example 2: This record is accession number H65066, has a REFSEQ identifier of NM_003385 and belongs to Celera family CALMODULIN-RELATED.

visinin-like 1 (VSNL1), mRNA. CALMODULIN-RELATED [H65066, NM_003385, Hs.2288]

Appendix to Gene Naming Conventions for Human Arrays.

REFSEQ:

A new database has also come out since Unigene. As part of the Locus Link at NCBI, there is a database called REFSEQ (short for reference sequence). This is a database that contains all known human genes. They have three categories of genes. PREDICTED genes are just that and have no real certainty associated with them and have no evidence for their existence other than an EST. PROVISIONAL genes are those for which there is some other line of supporting evidence for its existence beyond the presence of an EST. The PROVISIONAL genes are therefore more likely to describe real genes as opposed to an EST artifact. REVIEWED genes are PROVISIONAL genes that have been reviewed and inspected and researched by a human being. These records are therefore of the highest level of confidence.

REFSEQ for human genes has about 4000 REVIEWED and about 7000 PROVISIONAL. The REVIEWED records are a great starting point for following up microarray data. The following is an example of the wealth of information provided by a REVIEWED REFSEQ record:

```
//
LOCUS       NM_004861    1791 bp    mRNA            PRI       01-NOV-
2000
DEFINITION  Homo sapiens cerebroside
            (3'-phosphoadenylylsulfate:galactosylceramide 3')
sulfotransferase
            (CST), mRNA.
ACCESSION   NM_004861
VERSION     NM_004861.1  GI:4758087
KEYWORDS    .
SOURCE      human.
  ORGANISM  Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata;
Euteleostomi;
            Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.
REFERENCE   1  (sites)
  AUTHORS   Honke,K., Tsuda,M., Hirahara,Y., Ishii,A., Makita,A. and
Wada,Y.
  TITLE     Molecular cloning and expression of cDNA encoding human
            3'-phosphoadenylylsulfate:galactosylceramide 3'-
sulfotransferase
  JOURNAL   J. Biol. Chem. 272 (8), 4864-4868 (1997)
  MEDLINE   97184132
REFERENCE   2  (bases 1 to 1791)
  AUTHORS   The Sanger Centre and The Washington University Genome
Sequencing
            Center.
  TITLE     Toward a complete human genome sequence
  JOURNAL   Genome Res. 8 (11), 1097-1108 (1998)
  MEDLINE   99063792
   PUBMED   9847074
```

```
COMMENT     REVIEWED REFSEQ: This record has been curated by NCBI
staff. The
            reference sequence was derived from D88667.1.
            Summary:  Sulfonation, an important step in the metabolism
of many
            drugs, xenobiotics, hormones, and neurotransmitters, is
catalyzed
            by sulfotransferases. The CST cDNA encodes a 423-amino acid
protein
            with a predicted type II transmembrane topology and 2
potential
            N-linked glycosylation sites; it is not homologous to
either the
            cytosolic sulfotransferases or the Golgi sulfotransferases.
            Galactosylceramide sulfotransferase catalyzes the
conversion
            between 3'-phosphoadenylylsulfate +  a galactosylceramide
<=>
            adenosine 3',5'-bisphosphate + galactosylceramide sulfate.
            Activity is enhanced in renal cell carcinoma by the action
of
            epidermal growth factor, transforming growth factor, and
hepatocyte
            growth factor.  The role of this enzyme in renal cell
carcinoma is
            currently unknown.
            COMPLETENESS: complete on the 3' end.
FEATURES             Location/Qualifiers
     source          1..1791
                     /organism="Homo sapiens"
                     /db_xref="taxon:9606"
                     /chromosome="22"
                     /map="22q12.2"
     gene            1..1791
                     /gene="CST"
                     /db_xref="LocusID:9514"
                     /db_xref="MIM:602300"
     CDS             204..1475
                     /gene="CST"
                     /EC_number="2.8.2.11"
                     /note="GalCer sulfotransferase"
                     /codon_start=1
                     /db_xref="LocusID:9514"
                     /db_xref="MIM:602300"
                     /product="galactosylceramide sulfotransferase"
                     /protein_id="NP_004852.1"
                     /db_xref="GI:4758088"
```

```
/translation="MLPPQKKPWESMAKGLVLGALFTSFLLLVYSYAVPPLHAGLAST

TPEAAASCSPPALEPEAVIRANGSAGECQPRRNIVFLKTHKTASSTLLNILFRFGQKH

RLKFAFPNGRNDFDYPTFFARSLVQDYRPGACFNIICNHMRFHYDEVRGLVPTNAIFI

TVLRDPARLFESSFHYFGPVVPLTWKLSAGDKLTEFLQDPDRYYDPNGFNAHYLRNLL

FFDLGYDNSLDPSSPQVQEHILEVERRFHLVLLQEYFDESLVLLKDLLCWELEDVLYF

KLNARRDSPVPRLSGELYGRATAWNMLDSHLYRHFNASFWRKVEAFGRERMAREVAAL

RHANERMRTICIDGGHAVDAAAIQDEAMQPWQPLGTKSILGYNLKKSIGQRHAQLCRR
                    MLTPEIQYLMDLGANLWVTKLWKFIRDFLRW"
     polyA_site      1791
BASE COUNT      324 a    615 c    523 g    329 t
ORIGIN
        1 ggcagcctgg gagttggacg tggctcaggc agtgggtaga aaggggcagc
cagccacagc ********** cut by Ramsi **********
     1741 ccttaaaggg gagacctcag aagtaaagga atttgatgtt gtgtttttgt t
```

Appendix C. ii) Gene Naming Conventions in Effect for Mouse Arrays

By Ramsi Haddad, December 20, 2001.

Like all annotations, these names are fluid and can change. No annotation is 100% reliable here or anywhere else.

The Mouse cDNA microarrays use a clone set provided by the National Institutes of Aging. The clone set is frequently referred to as the NIA 15k cDNA cDNA clone set. This clone set has been re-annotated by the NIA (nature genetics 28:17-18, 2001). I have taken the re-annotated clone list from their web site and used it to generate an AMAD print list (available in AMAD) and to generate a GenePix .gal file. The gal file can be found in GeneralLabInformation/MICROARRY/Gal Files as Mouse_grid_12-17-01.

I am constrained in naming these clones by the MTA. The MTA states that we cannot rename the clones. For this reason, the name of the clone is as they've provided. Appended to the name is a [] square set of parentheses. The numbers in parentheses are [accession, unigene cluster ID, locus link identifier]. The best source of information is the locus link identifier. If there is no unigene cluster ID for a given clone, the parentheses will look like this: [12421, , 123342]. The commas are still there, but the missing value is not. Caviat emptor. Not all clones have been assigned a name.

That's it.