生物芯片实验手册（NIH）

OVERVIEW

Fluorescent cDNA microarray technology is useful for making estimates of the abundance of particular messages relative to a designated source of mRNA that serves as a reference point. Commercial support of this technology has recently reached a level where it is reasonable for departments or large laboratories to consider setting up their own cDNA array facility. This set of protocols is intended to serve as a basic introduction to making and using cDNA microarrays for those embarking on this path. There are three fundamental types of operations required in a cDNA microarray experiment. The first operation, BASIC PROTOCOL 1, cDNA AMPLIFICATION AND PRINTING, deals with making the cDNA microarray itself. It is necessary to collect an inventory of cDNA bacterial clones that represent the genes whose message abundance you wish to survey. Plasmid templates are made from these clones and used as PCR substrates to produce DNA representations of the EST inserts. The PCR products are then purified and spotted onto poly-L-lysine coated microscope slides. In the second operation, BASIC PROTOCOL 2, RNA EXTRACTION AND LABELING, RNA is extracted from the cell samples to be examined, purified, and used as the substrate for reverse transcription in the presence of fluor-derivatized nucleotides. This procedure provides the tagged representations of the mRNA pools of the samples that will be hybridized to the gene-specific cDNA detectors immobilized on the microarray. The third fundamental operation, BASIC PROTOCOL 3, HYBRIDIZATION AND DATA EXTRACTION, covers the steps in which fluor-labeled cDNAs hybridize to their complements on the microarray, and the resulting localized concentrations of fluorescent molecules are detected and quantitated.

FABRICATION

This protocol describes the steps required to produce a cDNA microarray. Gene-specific DNA is produced by PCR amplification of purified template plasmid DNAs from cloned ESTs. The PCR product is purified by ethanol precipitation, thoroughly resuspended in 3XSSC, and printed onto a poly-L-lysine coated slide.

Materials, Reagents & Solutions

96 well alkaline lysis miniprep kit (Edge BioSystems, Gaithersburg, MD)

LB Broth (Biofluids, Rockville, MD)

Superbroth (Biofluids, Rockville, MD)

.dATP, dCTP, dGTP, dTTP, 100 mM each #27-2035-02, store frozen, -20°C (Pharmacia, Peapack, NJ)

PCR primer AEK M13F (5'-GTTGTAAAACGACGGCCAGTG-3') and AEK M13R (5'-CACACAGGAAACAGCTATG-3') at 1 mM concentration, store frozen, -20°C

10X PCR Buffer, # N808-0189, and Ampli-Taq DNA polymerase, # N808-4015 store frozen, -20°C (Perkin Elmer, Norwalk, CT)

Carbenicillin (Gibco-BRL, Rockville, MD)

Ethanol (200 Proof USP Ethyl Alcohol)

1M Tris-HCl (pH 8)

0.5M NaEDTA (pH 8)

T Low E Buffer

20X SSC

Glycerol (enzyme grade)

Sodium Acetate (tri-hydrate)

Boric Acid

Sodium Hydroxide (1M)

Glacial Acetic Acid

Succinic anhydride, #23969-0 and 1-methyl-2-pyrrolidinone, # 32863-4 (Aldrich Chemical Co., St. Louis, MO)

Diethyl Pyrocarbonate (DEPC) treated H2O

Master set of clone-purified, sequence verified human ESTs (e.g. gf211 release, Research Genetics, Huntsville, AL)

96 pin inoculating block (#VP 4088, V&P Scientific, Inc, San Diego, CA)

Airpore Tape Sheets, (# 19571, QIAGEN Inc., Valencia, CA)

Sterile 96-well plate seals, (e.g. # SEAL-THN-STR (Elkay Products, Inc., Shrewsbury, MA)

96-well U-Bottom Microtiter Plates, #3799 and 96-well V-Bottom Microtitre Plates, #3894 (Corning Inc., Corning, NY)

Thin wall PCR plate and Cylcleseal PCR plate sealer (e.g. #1038-50-0 and #1044-39-4, Robbins Scientific Corp. Sunnyvale, CA)

household one-gallon sealable storage bags (e.g. Glad Lock)

heat sealable storage bags and heat sealer

0.2mm Sterile Filtration unit

Diamond scribe for writing on slides

Pyrex baking dish (~ 24x34x5 cm)

UV transparent plastic wrap (e.g. Glad Cling Wrap)

30 slide rack (stainless steel) #113 and 30 slide glass tank, #122 (Shandon Lipshaw, Pittsburgh, PA)

1L glass tank

1L glass beaker

1L graduated cylinder

Stir bar

Slide Box (plastic with no paper or cork liners), (e.g. #60-6306-02, PGC Scientific, Gaithersburg, MD)

PCR heat cycler (e.g. DNA Engine Tetrad, MJ Research, Waltham, MA)

Centrifuge with a horizontal ("swinging bucket") rotor with a depth capacity of 6.2 cm for spinning microtiter plates and filtration plates (e.g. Sorvall Super T 21, Sorvall Inc., Newtown, CT)

37°C Shaker incubator with holders for deep-well plates

37°C Waterbath

65°C Incubator

Vortex mixer

Immunowash microtiter plate washer, #1575 (BioRad, Hercules, CA)

pH Meter

Platform Shaker

UV Stratalinker 2400, (Stratagene La Jolla, CA)

Stirrer/Hotplate

Robotic slide printer

- 80°C Freezer

- 20°C Freezer

45% (w/v) Sterile Glycerol

450 grams enzyme grade glycerol per liter

Autoclave and store at room temperature.

T low E Buffer

1M Tris-HCl (pH 8.0) 10 mls

0.5 M EDTA (pH 8.0) 0.2 mls

DEPC treated H2O 990 mls

1000

Autoclave and store at room temperature.

Carbenicillin stock solution

1 gram of carbenicillin in 10 mls of sterile water.

Sterile filter with a 0.2 micron filter.

Store frozen at -20°C.

LB with 100 µg/ml carbenicillin

Add 1 ml of carbenicillin stock solution to 1 liter of LB.

Make fresh.

3M Sodium Acetate pH=6.0

Prepare 3M sodium acetate

408.24 grams sodium acetate (tri-hydrate) per liter

Prepare 3M acetic acid

172.4 ml per liter

Titrate the pH of the 3M sodium acetate solution to pH 6.0 with the 3M acetic acid solution.

Filter sterilize using a 0.2 micron filter.

Store at room temperature.

Ethanol/acetate mix

Ethanol (100%) 950 ml

Sodium acetate pH=6.0 50 ml

1000 ml 3X SSC

DEPC H2O 42.5ml

20X SSC 7.5ml

50ml 70% Ethanol

Ethanol (100%) 350 ml

DEPC H2O 150 ml

500 ml

2.1 EST clone growth

1. Incubate sealed master plates over night at 37°C.

Most suppliers provide low density bacterial cultures. Replicating directly from these dilute stocks frequently results in non-growth in the secondary culture. If making template from a plate that has previously been cultured to high density before freezing, this initial growth step should not be used, as it will reduce the viability of the cultures.

2. Prepare sets of standard 96 well round (U) bottom plates by labeling all plates and placing 100 µl of LB broth containing 100ug/ml carbenicillin in each well. These plates will be used as working copies.

To preserve the master set of plates, it is useful to make replicate copies of the master plate to serve as working copies when the master plate is first replicated. Check to insure that the EST clones are in a vector conferring ampicillin resistance, as is common with human IMAGE clones.,

4. Spin the master plates briefly, two minutes, at 1000 rpm in a horizontal microtiter plate rotor to remove condensation and droplets from the seals before opening.

Bacterial culture fluid on the sealers can easily be transferred from one well of the plate to others, cross-contaminating the stocks.

5. Partially fill a container with 100% alcohol. Dip the 96 pin-replicating tool in the alcohol. Remove from the alcohol bath and then fla me the pins.

6. Allow the inoculation block to cool briefly, then dip the replicating tool in the

master plate, and then into the daughter plate. Repeat as necessary for each plate that you need to inoculate.

It is useful to color the plate corner near the A-1 well of all master and daughter plates with a marker pen before beginning the replication process, to reduce mistakes in relative orientation of the plates. The suggested plates have a notch at this corner as well.

7. Place the inoculated LB plates with lids on into a one gallon "zip-lock" bag containing a moistened paper towel and grow overnight at 37°C.

Many 37°C incubators tend to dry out microtiter plate cultures. Placing the plates in a highly humidified bag avoids this problem.

3. Fill deep well plates with 1ml of Superbroth (100ug/ml carbenicillin) per well. These plates will serve as the source of culture for template preparation.

8. Using the replicating tool, inoculate the deep well plates directly from the freshly grown LB plates.

9. Cover the openings of the deep well plates with Qiagen Airpore Tape Sheets and place the plastic lid over the sheet. Place the plates in a 37°C shaker incubator at 200 RPM for twenty-four hours.

10. Add 50 µl of 45% (w/v) sterile glycerol to each well of any working plates that are to be frozen (-80°C) and subsequently used as culture sources.

2.2 Isolate plasmid templates

1. Warm the lysis buffer (Edge Biosystems Kit) to 37°C to dissolve the SDS. This buffer can be stored at room temperature.

2. Add the RNAse solution to the resuspension buffer (Edge Biosystems Kit), 1ml/100ml. Store at 4°C. The remaining reagents from the kit, neutralization buffer and precipitation buffers, are ready to use and should be stored at 4°C.

3. Prepare the receiving plates from the Edge Biosystems Kit by adding 350 µl of ethyl alcohol to each well of the receiving plates. Place the filter plate on top and secure in place with tape. Handle with care as the wells will be very full.

4. Centrifuge the bacterial cultures in the deep well plates at 1500 x g for seven minutes in a centrifuge equipped with a horizontal rotor for 96-well plates.

5. Invert briefly and tap out excess media on a clean paper towel. Do not delay or the pellets will loosen and may be lost when pouring off excess media.

6. Resuspend the pellet in 100 µl of Resuspension Buffer. Vortex until entire pellet is re-suspended. This step is critical. Poor resuspension of the cells results in clumps of cells that do not lyse in subsequent steps. This reduces the yield and decreases the purity of the product.

7. Add 100 µl of Lysis Buffer. Mix gently by rocking the plates from side, avoid shearing the bacterial chromosomal DNA.

8. Add 100 µl Precipitation buffer to each well. Mix briefly.

9. Add 100 µl Neutralization buffer to each well. Vortex.

10. Transfer the contents of the deep wells to the waiting filter plates/receiving plate stacks using the wide bore pipette tips provided in the kits.

11. Centrifuge the stacked plates at 1500 x g for twelve minutes in a centrifuge equipped with a horizontal rotor for 96-well plates.

12. Remove the stacked plates from the centrifuge. Remove and discard the filter plates . Decant the alcohol and filtrate from the receiver plate. Touch off excess alcohol on clean paper towels.

12. Add 500 µl of 70% ethanol to each well. Decant immediately. Touch off excess alcohol on clean paper towels.

14. Place plates in a clean drawer without their lids, cover with a clean paper towel and allow to dry overnight.

15. Re-suspend DNA in 200 µl of T Low E Buffer. Seal top with plate sealer. Rehydrate at 4°C for at least two days before using. Store at -20°C.

2.3 Amplify EST inserts

1. For each 96 well plate to be amplified, prepare a PCR reaction mixture containing the following ingredients:

1000 µl 10X PCR Buffer

20 µl dATP (100 mM)

20 µl	dGTP (100 mM)
20 µl	dCTP (100 mM)
20 µl	dTTP (100 mM)
5 µl	AEK M13F primer (1 mM)
5 µl	AEK M13R primer (1 mM)
100 µl	Ampli-Taq polymerase (5 U/µl)
8800 µl	H2O

2. Label 96-well PCR plates and aliquot 100 µl of PCR reaction mix to each well. Gently tap plates to insure that no air bubbles are trapped at the bottom of the wells.

3. Add 1 µl of purified EST plasmid template to each well.

Mark the donor and recipient plates at the corner near the A1 well to facilitate correct orientation during transfer of the template. It is important to watch that the pipette tips are all submerged in the PCR reaction mix when delivering the template. Missing the liquid is easier when multi-channel pipettes are used.

4. Perform the following thermal cycle series: 1 initial cycle of heating to 96°C and holding for 30 sec; 25 cycles of denaturing at 94° C for 30 sec, reannealing at 55°C for 30 sec, and extending at 72°C for 150 sec; one final cycle of holding at 72°C for 5 minutes, then cooling to ambient temperature.

After PCR, plates made be held at 4°C while quality controls are performed.

2.4 Check PCR products by agarose gel electrophoresis of ESTs

If this is the first time the template for these ESTs is being amplified, analyze 2 µl of each PCR product on a 2% agarose gel. If amplified products from this template have been previously tested, then analyze one row of wells from each plate amplified. Gel imaging allows a rough quantitation of product while giving an excellent characterization of the product. Band size, as well as the number of bands observed in the PCR products, contribute to understanding the final results of the hybridization. The use of gel well formats suitable for loading from 96 well plates and

programmable pipetters makes this form of analysis feasible on a large scale.

Materials, Reagents and Solutions

Electrophoresis apparatus with capacity for four 50 well combs, (e.g. #D3, Owl Scientific, Woburn, MA)

50 X Tris-Acetate Electrophoresis BufferM

Agarose

Dye Solution (Xylene Cyanol/Bromophenol Blue) (e.g. #351-081-030, Quality Biological Inc., Gaithersburg MD)

Glycerol (enzyme grade)

Ethidium Bromide solution (10 mg/ml)

100 base-pair ladder size standard

Programmable, 12-channel pipetter (e.g. #2019, Matrix Technologies, Lowell, MA)

Disposable microtiter mixing trays (e.g. Falcon #353911, Becton Dickinson, Franklin Lake, NJ)

Electrophoresis power supply

1X TAE Buffer

50X TAE Buffer 40 ml

Ethidium Bromide (10 mg/ml) 0.1 ml

Water 960 ml

1000 ml

Loading Buffer

Glycerol (enzyme grade) 4.0 ml

DEPC Water 0.9 ml

Dye Solution* 0.1 ml

5.0 ml

(*This solution is 0.25% (w/v) Xylene Cyanol and 0.25% (w/v) Bromophenol Blue)

100 bp Size Standards

DNA ladder (1 mg/ml) 50 µl

1 M Tris-HCl (pH 8.0) 5 µl

0.5 M EDTA (pH 8.0) 5 µl

Loading Buffer 440 µl

500 µl

Method

1. Cast a 2% agarose gel (1X TAE) with four combs (50 tooth) and submerge in an electrophoresis apparatus with sufficient 1X TAE buffer to just cover the surface of the gel.

2. Prepare a reservoir of Loading Buffer, using 12 wells of a microtiter plate.

2. Program pipetter to sequentially carry out the following steps:

fill with 2 µl

fill with 1 µl

fill with 2 µl

mix a volume of 5 µl five times

expel 5 µl

3. Place 12 disposable tips on the pipetter.

4. Load 2 µl of PCR product from wells A1-A12 of the PCR plate.

5. Load 1 µl of air.

6. Load 2 µl of Loading Buffer from the reservoir.

7. Place tips in clean wells of disposable mixing tray and allow pipette to mix the sample and loading dye.

8. Place the pipette tip in a 50 well row so that the tip containing the PCR product from well A1 is in the second well of the row, and the other tips are in every other succeeding well.

9. Repeat the process (changing tips each time), loading PCR plate row B starting in the 3rd well, interleaved with the A row, the C row starting at well 26, and the D row at well 27, interleaved with the C row.

10. Place 5 µl of 100 bp Size Standards, in wells 1 and 50.

11. Repeat this process, loading samples from rows E, F, G, and H in the second, 50 well row of gel wells, loading samples from two 96 well PCR plates per gel, or single row samples from 16 PCR plates.

To reduce diffusion and mixing, apply voltage to the gel for a minute between loading

each well strip. This will cause the DNA to enter the gel, and reduce band spreading and sample loss.

12. Apply voltage to the gel and run until the bromophenol blue (faster band) has nearly migrated to the next set of wells.

For a gel that is 14 cm in the running dimension, and 3 cm between each row of wells, apply 200 volts for 15 minutes.

13. Take digital photo of gel and store image for future reference.

The gels should show bands of fairly uniform brightness distributed in size between 600 to 2000 base-pairs as in Figure 2. Further computer analysis of such images can be carried out with image analysis packages to provide a list of the number and size of bands. Ideally this information can be made available during analysis of the data from hybridizations involving these PCR products.

2.5 Purify PCR products

1. Fill 96 well V-bottom plates with 200ul per well of ethanol/acetate mix.

The ethanol acetate solution used for precipitation is less acidic (pH 6) than is typically used. In this instance, more acidic solutions produce precipitates which are harder to resuspend without improving yield.

2. Transfer 100ul per well of PCR product into V-bottom plates and mix by pipetting a volume of 75 µl per well four times.

3. Place the plates in -80°C freezer for one hour or store overnight at -20°C.

Place plates at -20°C if they are to be left for more than one hour, aggressive precipitation produces precipitates which are hard to resuspend.

4. Thaw the plates to reduce brittleness and melt any ice, which may have formed in the wells.

5. Load the plates into a centrifuge with a horizontal microtiter plate rotor and spin at 2600 x g for 40 minutes at 4°C.

6. Aspirate the supernatant from each well using the Immunowash plate washer.

Settings for the depth of aspiration by the plate washer will need to be adjusted to suit the microtiter plates used. It is advisable to leave approximately 10-20ml in the bottom of the well to avoid disturbing the pellet.

7. Deliver 200 µl of 70% ethanol to each well in the plate using the Immunowash plate washer.

8. Centrifuge plates at 2600 x g for 40 minutes.

9. Aspirate the supernatant from each well using the Immunowash plate washer.

10. Allow the plates to dry overnight in a closed drawer.

Do not dry in a speed-vac. Desiccated PCR products are hard to resuspend.

2.6 Resuspend the PCR products

1. Add 40 µl of 3X SSC per well. Seal plates with a foil sealer, taking care to achieve a tight seal over each well.

2. Place the plates in heat sealable bags with paper towels moistened with 3X SSC and seal the bag with a heat sealer.

The high external humidity within the sealed bag helps keep the volumes in the individual wells from varying.

3. Place the bags in a 65°C incubator for 2 hours, then turn off the heat in the incubator.

Allowing the plates to cool down gradually in the incubator avoids condensation on the sealers.

2.7 Check PCR resuspension for yield by fluorometric determination of DNA concentration

Analyze 1 µl of resuspended PCR product from one row of wells from each plate on a 2% agarose gel as previously described. Adequate precipitation and resuspension will produce very intense bands, with no material failing to leave the loading well, and no smear of material from the band towards the loading well.

While it would be ideal to be able to exactingly quantify each EST PCR product and spot each DNA species at equivalent concentrations, it is impractical for most labs to do so when thousands of ESTs must be prepared. Fortunately, it is possible to use a strategy where excess DNA is spotted, so that the exact quantities used do not produce much variation in the observed results. When using this strategy, it is necessary to track the average productivity of the PCR reactions. Fluorometry provides a simple way to obtain an approximate concentration of the double-stranded PCR product in

the PCR reaction mix.

Store the plates at -20°C after resuspension.

Materials, Reagents and Solutions

Reference double-stranded DNA (0.5 mg/ml) (e.g. #15612-013 Gibco/BRL, Bethesda, MD)

96 well plates for fluorescent detection (e.g. #7105, Dynex, Chantilly, VA)

Fluorometer (e.g. #LS50B, Perkin Elmer, Norwalk, CT)

FluoReporter Blue dsDNA Quantitation Kit (#F-2962, Molecular Probes, Eugene, OR)

TE

12 channel multi-pipettors

Computer equipped with Microsoft Excel software

Ds-DNA Standards

50 µg/ml 100 µg/ml 250 µg/ml 500 µg/ml µl TE 90 80 50 0 µl ds-DNA (0.5 mg/ml) 10 20 50 100

It is good practice to check both the integrity (agarose gel) and the concentration (absorbance) of the standard before use.

Fluor Buffer

Hoechst 33258 solution* (from kit) 25 µl TNE Buffer** (from kit) 10 ml

* Hoechst 33258 solution contains the dye at an unspecified concentration in a 1:4 mixture of DMSO:H2O

** TNE Buffer is 10 mM Tris-HCl (pH 7.4), 2 M NaCl, 1 mM EDTA

Quantitating ds-DNA

1. Label 96 well plates for fluorescence assay.

2. Add 200 µl of Fluor Buffer to each well.

3. Add 1 µl of PCR product from each well in a row of a PCR plate to a row of the fluorometry plate. Samples can be added to rows A through G of the fluorometry plate.

4. In the final row of the fluorometry plate add 1 µl of each of the series of ds-DNA standards 0 µg/ml (TE only), 50, 100, 250 and 500 µg/ml ds-DNA. Repeat this series twice in the final row.

5. Set the fluorometer for excitation at 346 nm and emission at 460 nm. Adjust as necessary to read the plate.

6. If the fluorometer does not support automated analysis, export the data table to Excel.

7. Test to see that the response for the standards is linear and reproducible from the range of 0 to 500 µg/ml of ds-DNA.

8. Calculate the concentration of ds-DNA in the PCR reactions using the following equation after subtracting the average 0 µg/ml value from all other sample and control values:

[ds-DNA(µg/ml)] = ((PCR sample value)/(average 100 µg/ml value))*100

Constantly tracking the yields of the PCRs makes it possible to rapidly detect many of ways in which PCR can fail or perform poorly. This assay can also be applied after precipitation and resuspension of the PCR products to monitor overall recovery of product.

31. Analyze 1 µl of amplified products from one row of wells from each amplified plate by fluorometry (Supplementary Protocol 2).

2.8 SLIDE COATING

Slides coated with poly-L-lysine have a surface that is both hydrophobic and positively charged. The hydrophobic character of the surface minimizes spreading of the printed spots, and the charge appears to help position the DNA on the surface in a way that makes cross-linking more efficient.

Materials, Reagents and Solutions

Gold Seal Microscope Slides (#3011, Becton Dickinson, Franklin Lake, NJ)

Ethanol (100%)

Poly-L-lysine (#P8920, Sigma, St. Louis, MO.)

50 Slide Stainless Steel Rack, #900401, and 50 Slide Glass Tank, #900401, (Wheaton Science Products, Millville, NJ)

Sodium Hydroxide

Stir Plate

Stir Bar

Platform Shaker

30 Slide Rack, #196, plastic, and 30 slide Box, #195, plastic, (Shandon Lipshaw, Pittsburgh, PA)

Sodium Chloride

Potassium Chloride

Sodium Phosphate Dibasic Heptahydrate

Potassium Phosphate Monobasic

Autoclave

0.2mm Filter : Nalgene

Centrifuge : Sorvall Super 20

Slide Box (plastic with no paper or cork liners), (e.g. #60-6306-02, PGC Scientific, Gaithersburg, MD)

1L Glass Beaker

1L Graduated Cylinder

1M Sodium Borate (pH 8.0)

Dissolve 61.83g of Boric acid in 900 ml of DEPC H2O. Adjust the pH to 8.0 with 1N NaOH. Bring volume up to one liter. Sterilize with a 0.2 micron filter and store at room temperature.

Cleaning Solution

H2O 400 ml

Ethanol 600 ml

NaOH 100 g

Dissolve NaOH in H2O. Add ethanol and stir until the solution clears. If the solution does not clear, add H2O until it does.

Poly-L-lysine Solution

poly-L-lysine (0.1% w/v) 35 ml PBS 35 ml H2O 280 ml 350 ml

Method

1. Place slides into 50 slide racks and place racks in glass tanks with 500 ml of cleaning solution. Gold Seal Slides are highly recommended, as they have been found to have consistently low levels of autofluorescence.

It is important to wear powder free gloves when handling the slides. Change gloves frequently, as random contact with skin and surfaces transfers grease to the gloves.

2. Place tanks on platform shaker for two hours at 60 rpm.

3. Pour out cleaning solution and wash in H2O for three minutes. Repeat wash four times.

4. Transfer slides to 30 slide plastic racks and place into small plastic boxes for coating.

5. Submerge slides in 200 ml poly-L-lysine solution per box.

6. Place slide boxes on platform shaker for one hour at 60 rpm.

7. Rinse slides three times with H2O.

8. Submerge slides in H2O for one minute.

9. Spin slides in centrifuge for two minutes at 400 xg and dry slide boxes used for coating.

10. Place slides back into slide box used for coating and let stand overnight before transferring to new slide box for storage.

This allows the coating to dry before handling.

11. Allow slides to age for two weeks on the bench, in a new slide box, before printing on them. The coating dries slowly, becoming more hydrophobic with time.

Slide boxes used for long term storage should be plastic and free of cork lining. The glue used to affix the cork will leach out over time and give slides stored in these types of boxes a greasy film that has a high degree of autofluorescence. Clean all glassware and racks used for slide cleaning and coating with highly purified H2O only. Do not use detergent.

2.9. SLIDE BLOCKING

At the end of the print, slides are removed from the printer, labeled with the print identifier and the slide number by writing on the edge of the slide with a diamond scribe and placed in a dust free slide box to age for one week.

It is useful to etch a line, which outlines the printed area of the slide, onto the first slide. This serves as a guide to locate the area after the slides have been processed, and the salt spots are washed off.

1. Place slides, printed face up, in casserole dish and cover with cling wrap. Expose slides to a 450mJ dose of ultraviolet irradiation in the Stratalinker.

Slides should have been aged at ambient temperature in a closed slide box for one week prior to blocking.

2. Transfer slides to a 30 slide stainless steel rack and place rack into a small glass tank.

3. Dissolve 6.0g succinic anhydride in 325ml 1-methyl-2-pyrrolidinone in a glass beaker by stirring with a stir bar.

Nitrile gloves should be worn and work carried out in a chemical fume hood while handling 1-methyl-2-pyrrolidinone (a teratogen).

4. Add 25ml 1M sodium borate buffer (pH 8.0) to the beaker. Allow the solution to mix for a few seconds, then pour rapidly into glass tank with slides.

Succinic anhydride hydrolyzes quite rapidly once the aqueous buffer solution is added. To obtain quantitative passivation of the poly-L-lysine coating, it is critical that the reactive solution be brought in contact with the slides as quickly as possible.

5. Place the glass tank on a platform shaker in a fume hood for 20 minutes.

Small particulates resulting from precipitation of reaction products will be visible in the fluid.

6. While the slides are incubating on the shaker, prepare a boiling H2O bath to denature the DNA on the slides.

7. After the slides have incubated for 20 minutes, transfer them into the boiling H2O bath. Immediately turn off the heating element after submerging the slides in the bath. Allow slides to stand in the H2O bath for 2minutes.

8. Transfer the slides into a glass tank filled with 100% ethanol and incubate for 4 minutes.

9. Remove the slides and centrifuge at 400 rpm for 3 minutes in a horizontal microtiter plate rotor to dry the slides.

10. Transfer slides to a clean, dust free slide box and let stand overnight before hybridizing.

2.10. PRINTING

The variety of printers and pens for transferring PCR products from titer plates to slides precludes highly detailed descriptions of the process. The following steps provide a general description of the processing.

1. Pre-clean the print pens according to the manufacturer's specification.
2. Load the printer slide deck with poly-L-lysine coated slides (Supplemental Protocol 3).
3. Thaw the plates containing the purified EST PCR products and centrifuge briefly, two minutes, at 1000 rpm in a horizontal microtiter plate rotor to remove condensation and droplets from the seals before opening.
4. Transfer 5 to 10 µl of the purified EST PCR products to a plate that will serve as the source of solution for the printer.

Printing with quill-type pens usually requires that the volume of fluid in the print source is sufficiently low, that when the pen is lowered to the bottom of the well, it is submerged in the solution to a depth of less than a millimeter. This keeps the pen from carrying a large amount of fluid on the outside of the pen shaft and producing variable, large spots on the first few slides printed.

5. Run a repetitive test print on the first slide. In this operation, the pens are loaded with the DNA solution, and then the pens serially deposit this solution on the first slide in the spotting pattern specified for the print.

This test is run to check the size and shape of the specified spotting pattern, and its placement on the slide. It also serves to verify that the pens are loading and spotting, and that a single loading will produce as many spots as are required to deliver material to every slide in the printer.

6. If one or more of the pens is not performing at the desired level, re-clean or substitute another pen and test again.
7. If all pens are performing, carry out the full print.

RNA EXTRACTION

This protocol details the methods used to extract RNA from cells, purify the RNA by a combination of phase extraction and chromatography, and prepare a labeled cDNA copy of the message fraction of the purified RNA. The protocol also describes the

process of making fluorescent cDNA representations of the message pools within the isolated total RNA pools. This is accomplished by using the pure total RNA as a substrate for reverse transcription in the presence of nucleotides derivatized with either a Cy3 or a Cy5 fluorescent tag.

Materials

Trizol Reagent (#15596-018, Life Technologies, Rockville, MD)

RNeasy Maxi Kit (# 75162, Qiagen, Valencia, CA)

Chloroform

Ethanol (200 Proof USP Ethyl Alcohol)

DPBS (Dulbecco's phosphate buffered saline)

3M sodium acetate (pH 5.2)

dATP, dCTP, dGTP, dTTP, 100 mM each, store frozen, -20°C (#27-2035-02, Pharmacia, Peapack, NJ)

pd(T)12-18 resuspend at 1mg/ml, and store frozen -20°C (#27-7858, Amersham Pharmacia Biotech)

Anchored oligo primer (anchored;5'-TTT TTT TTT TTT TTT TTT TTV N-3') resuspend at 2mg/ml, store frozen -20°C (e.g. # 3597-006, Genosys)

CyTM3-dUTP, 1 mM, and CyTM5-dUTP, 1 mM, store -20°C, light sensitive

RNasinâ Rnase inhibitor, store -20°C (#N211A, Promega)

SUPERSCRIPTTM II Rnase H` Reverse Transcriptase Kit, store -20°C, (#18064-014, Life Technologies, Rockville, MD)

C0t-1 DNA, 1mg/ml, store frozen -20°C (#15279-011, Life Technologies, Rockville, MD)

0.5M EDTA(pH 8.0)

1 N NaOH

1M TRIS-HCL (pH7.5)

TE pH 7.4

DEPC water 50 X Tris Acetate Buffer

15 ml round bottom polypropylene centrifuge tubes

50 ml conical polypropylene centrifuge tubes

1.5 ml Eppendorf tubes

0.2 ml thinwall PCR tube

MicroCon 100 (Amicon Cat No. 42412)

High speed centrifuge for 15 ml tubes

Clinical centrifuge with horizontal rotor for 50 ml conical tubes

Tissue homogenizer (e.g. Polytron PT1200 with Polytron-Aggregate-Dispergier-und-Mischtechnik 147a Ch6014 #027-30-520-0, Brinkmann Instruments Inc., Westbury, NY)

Reagents and Solutions

RPE Buffer

Add 4 volumes of ethanol per volume of RPE concentrate supplied in Quiagen Kit.

RW1 Buffer

Supplied in Qiagen Kit

75% EtOH

Ethanol (100%) 375 ml

DEPC H2O 125 ml

500 ml

10x low T dNTP Mix

Component	Vol.(ul)	mM final(1/10) concentration
dGTP (100 mM)	25	0.5
dATP (100 mM)	25	0.5
dCTP (100 mM)	25	0.5
dTTP (100 mM)	10	0.5
DEPC H2O	415	0.2
Total volume	500	

5x First Strand Buffer

Provided with Superscript II

TAE Buffer

50X Tris Acetate Electrophoresis Buffer 20 ml

DEPC H2O 980 ml

1000 ml

Method

1. If starting with cells harvested from tissue culture, wash the cell pellet twice in DPBS.
2. If starting with cells from tissue culture, add 1 ml of Trizol per 2x107 cells and mix by shaking. If starting with tissue, add 100 mg of frozen tissue directly to 4 ml of Trizol, and dissociate by homogenization with a rotating blade tissue homogenizer.
3. Add 2/10 volume of chloroform and shake for 15 seconds.
4. Let stand for 3 minutes. Centrifuge at 12,000 x g for 15 minutes at 4°C.
5. Take off the supernatant and add it to a polypropylene tube, recording the volume of the supernatant.
6. Add 0.53 volumes of ethanol to the supernatant slowly while vortexing, this will produce a final ethanol concentration of 35%.

The ethanol should be added drop by drop and allowed to mix completely with the supernatant before more ethanol is added. If a high local concentration of ethanol is produced, the RNA in that vicinity will precipitate.

7. Add the supernatant from an extraction of 2x107 to 1x108 cells to an RNeasy maxi column, which is seated in a 50 ml centrifuge tube.
8. Centrifuge at 2880 x g in a clinical centrifuge with a horizontal rotor at room temperature for 5 minutes.
9. Pour the flow-through back onto the top of the column and centrifuge again.

A significant amount of RNA is not captured by the column matrix in the first pass of the RNA containing solution through the column.

10. Discard the flow-through and add 15 ml of RW1 buffer to the column.
11. Centrifuge at 2880 x g for 5 minutes.
12. Discard flow-through then add 10 ml of RPE buffer.

13. Centrifuge at 2880 x g for 5 minutes.

14. Discard flow-through and add another 10 ml of RPE buffer.

15. Centrifuge at 2880 x g for 10 minutes.

16. Put the column in a fresh 50 ml tube and add 1 ml of DEPC treated water from the kit to the column.

17. Let stand for 1 minute.

18. Centrifuge at 2880 x g for 5 minutes.

19. Add another 1 ml of water to the column.

20. Let stand for 1 minute.

21. Centrifuge at 2880 x g for 10 minutes.

22. Aliquot out 400 µl portions of the column eluate to 1.5 ml Eppendorf tubes.

23. Add 1/10 volume of 3M sodium acetate (pH 5.2).

24. Add 1 ml of ethanol to each tube.

25. Let stand for 15 minutes.

26. Centrifuge at 12000 x g at 4C for 15 minutes.

27. Wash pellet 2 times in 75% EtOH then store at -80°C

RNA Cleanup

28. Resuspend RNA at approximately 1 mg/ml in DEPC H2O.

29. Concentrate to greater than 7 mg/ml by centrifugation on a MicroCon 100 filter unit, centrifuge at 500 x g, checking as necessary to determine the rate of concentration.

This step removes many residual, small to medium sized, molecules that inhibit the reverse transcription reaction in the presence of fluorescently derivatized nucleotides.

30. Determine the concentration of RNA in the concentrated sample by spectrophotometry. Store at -80°C.

Sample LABELING by Reverse Transcription

1. If using an anchored oligo dT primer, anneal the primer to the RNA in the following 17 µl reaction (use a 0.2 ml thinwall PCR tube so that incubations can be carried out in a PCR cycler):

Component	addition for Cy5 labeling	addition for Cy3 labelling
Total RNA (>7 mg/ml)	150-200 µg	50-80 µg
Anchored primer (2 µg/µl)	1 µl	1 µl
DEPC H2O	to 17 µl	to 17 µl

If using an oligo dT(12-18) primer, anneal the primer to the RNA in the following 17 µl reaction:

Component	addition for Cy5 labeling	addition for Cy3 labelling
Total RNA (>7 mg/ml)	150-200 µg	50-80 µg
dT(12-18) primer (1 µg/µl)	1 µl	1 µl
DEPC H2O	to 17 µl	to 17 µl

The incorporation rate for Cy5-dUTP is less than that of Cy3-dUTP, so more RNA is labeled to achieve more equivalent signal from each species.

2. Heat to 65°C for 10 minutes and cool on ice for 2 minutes.

3. Add 23 µl of reaction mixture containing either Cy5-dUTP or Cy3-dUTP nucleotides, mix well by pipetting and use a brief centrifuge spin to concentrate in the bottom of the tube:

reaction mixture	ul
5x first strand buffer	8
10x low T dNTPs mix	4
Cy5 or Cy3 dUTP (1mM)	4
0.1 M DTT	4
Rnasin (30u/µl)	1
Superscript II (200u/ul)	2

Total volume 23

Superscript polymerase is very sensitive to denaturation at air/liquid interfaces, so be very careful to suppress foaming in all handling of this reaction.

4. Incubate at 42°C for 30 min. then add 2 µl Superscript II. Make sure the enzyme is well mixed in the reaction volume and incubate at 42O C for 30-60 min.

5. Add 5 µl of 0.5M EDTA.

Be sure you have stopped your reaction with EDTA before adding NaOH, since nucleic acids precipitate in alkaline magnesium solutions.

6. Add 10 µl 1N NaOH, incubate at 65O C for 60 minutes to hydrolyze residual RNA. Cool to room temperature.

The purity of the sodium hydroxide solution used in this step is crucial. Slight contamination or long storage in a glass vessel can produce a solution that will degrade the Cy5 dye molecule, turning the solution yellow. Some researchers achieve better results by reducing the time of hydrolysis to 30 minutes.

7. Neutralize by adding 25 µl of 1M Tris-HCl (pH 7.5).

8. Desalt the labeled cDNA by adding the neutralized reaction, 400 µl of TE pH 7.5 and 20 µg of human C0t-1 DNA to a MicroCon 100 cartridge. Pipette to mix, spin for 10 minutes at 500 x g.

9. Wash again by adding 200 µl TE pH 7.5 and concentrating to about 20-30 µl (approximately 8-10 min at 500 x g).

Alternatively, a smaller pore MicroCon 30 can be used to speed the concentration step. In this case, centrifuge the first wash for approximately 4.5 minutes at 16,000 xg and the second (200 µl wash) for about 2.5 minutes at 16,000 xg.

10. Recover by inverting the concentrator over a clean collection tube and spinning for 3 min at 500 x g.

In some cases, the cy5 labeled cDNA will form a gelatinous blue precipitate that is recovered in the concentrated volume. The presence of this material signals the presence of contaminants. The more extreme the contamination, the greater the fraction of cDNA which will be captured in this gel. Even if heat solubilized, this

material tends to produce uniform, non-specific binding to the DNA targets.

When concentrating by centrifugal filtration, the times required to achieve the desired final volume are variable. Overly long spins can remove nearly all the water from the solution being filtered. When fluor-tagged nucleic acids are concentrated onto the filter in this fashion, they are very hard to remove, so it is necessary to approach the desired volume by conservative approximations of the required spin times. If control of volumes proves difficult, the final concentration can be achieved by evaporating liquid in the speed-vac. Vacuum evaporation, if not to dryness, does not degrade the performance of the labeled cDNA.

11. Take a 2-3 µl aliquot of the Cy5 labeled cDNA for analysis, leaving 18-28 µl for hybridization.

12. Run this probe on a 2% agarose gel (6cm wide x 8.5 cm long, 2 mm wide teeth) in Tris Acetate Electrophoresis Buffer (TAE).

For maximal sensitivity when running samples on a gel for fluor analysis, use loading buffer with minimal dye and do not add ethidium bromide to the gel or running buffer.

13. Scan the gel on a Molecular Dynamics Storm fluorescence scanner (setting: red fluorescence, 200 micron resolution, 1000 volts on PMT)

Successful labeling produces a dense smear of probe from 400 bp to >1000 bp, with little pile-up of low molecular weight transcripts (as in Figure 1, Lane A). Weak labeling and significant levels of low molecular weight material indicates a poor labeling (as in Figure 1, Lane B). A fraction of the observed low molecular weight material is unincorporated fluor nucleotide.

Hybridization

5.1 Hybridize fluorescent cDNA to slide

1. Determine the volume of hybridization solution required. The rule of thumb is to use 0.033 µl for each mm2 of slide surface area covered by the cover slip used to cover the array. An array covered by a 24 mm by 50 mm coverslip will require 40 µl of hybridization solution. The volume of the hybridization solution is critical. When too little solution is used, it is difficult to seat the coverslip without introducing air

bubbles over some portion of the arrayed ESTs, and the coverslip will not sit at a uniform distance from the slide. If the coverslip is bowed toward the slide in the center, there will be less labeled cDNA in that area and hybridization will be non-uniform. When too much volume is applied, the coverslip will move easily during handling, leading to misplacement relative to the arrayed ESTs, and non-hybridization in some areas of the array.

2. For a 40 µl hybridization, pool the Cy3 and Cy5 labeled cDNAs into a single 0.2 ml thinwall PCR tube and adjust the volume to 30 µl by either adding DEPC H2O, or removing water in a SpeedVac. If using a vacuum device to remove water, do not use high heat or heat lamps to accelerate evaporation. The fluorescent dyes could be degraded.

3. For a 40 µl hybridization combine the following components:

	High Sample Blocking	High Array Blocking
Cy5+Cy3 probe	30 µl	28 µl
Poly d(A) (8mg/ml)	1 µl	2 µl
Yeast tRNA (4mg/ml)	1 µl	2 µl
Human C0t-1 DNA (10mg/ml)	1 µl	0 µl
20x SSC	6 µl	6 µl
50x Denhardt's blocking solution	1 µl (optional)	2 µl
Total volume	40ul	40ul

Arrays and samples can vary somewhat, making it necessary to vary the composition of the hybridization cocktail. In cases where there is residual hybridization to control repeat DNA samples on the array, more C0t-1 DNA can be used, as in the High Sample Blocking formulation. When there is diffuse background or a general haze on all of the array elements, more of the non-specific blocker components can be used, as in the High Array Blocking formulation.

4. Mix the components well by pipetting, heat at 98°C for 2 minutes in a PCR cycler,

cool quickly to 25°C and add 0.6ul of 10% SDS.

5. Centrifuge for 5 min at 14,000 x g. The fluor labeled cDNAs have a tendency to form small, very fluorescent, aggregates which result in bright, punctate background on the array slide. Hard centrifugation will pellet these aggregates, allowing you to avoid introducing them to the array.

6. Apply the labeled cDNA to a 24 mm x 50 mm glass coverslip and then touch with the inverted microarray.

Applying the hybridization mix to the array and coverslipping it is an operation which requires some dexterity to get the positioning of the coverslip and the exclusion of air bubbles just right. It is helpful to practice this operation with buffer and plain slides before attempting actual samples. The hybridization solution is added to the coverslip first, since some aggregates of fluor remain in the solution and will bind to the first surface they touch.

7. Place the slide in a microarray hybridization chamber, add 5 µl of 3x SSC in the reservoir, if the chamber provides one, or at the scribed end of the slide and seal the chamber. Submerge the chamber in a 65°C water bath and allow the slide to hybridize for 16-20 hours.

There are a wide variety of commercial hybridization chambers. It is worthwhile to prepare a mock hybridization with a blank slide, load it in the chamber and incubate it to test for leaks, or drying of the hybridization fluid, either of which cause severe fluorescent noise on the array.

5.2 Wash off unbound fluorescent cDNA

8. Remove the hybridization chamber from the water bath, cool and carefully dry off. Unseal the chamber and remove the slide.

As there may be negative pressure in the chamber after cooling, it is necessary to remove water from around the seals so that it is not pulled into the chamber and onto the slide when the seals are loosened.

9. Place the slide, with the coverslip still affixed, into a Coplin jar filled with 0.5X SSC/0.01% SDS wash buffer. Allow the coverslip to fall from the slide and then remove the coverslip from the jar with a forceps. Allow the slide to wash for 2-5

minutes.

10. Transfer the slide to a fresh Coplin jar filled with 0.06X SSC. Allow the slide to wash for 2-5 minutes.

The sequence of washes may need to be adjusted to allow for more aggressive noise removal, depending on the source of the sample RNA. Useful variations are to add a first wash which is 0.5X SSC/0.1% SDS or to repeat the normal first wash twice.

11. Transfer the slide to a slide rack and centrifuge at low rpm (700-1000) for 3 minutes in a clinical centrifuge equipped with a horizontal rotor for microtiter plates.

If the slide is simply air dried, it frequently acquires a fluorescent haze. Centrifuging off the liquids results in a lower fluorescent background. As the rate of drying can be quite rapid, it is suggested that the slide be placed in the centrifuge immediately upon removal from the Coplin jar.