

***Arabidopsis* Functional Genomics Consortium**

DNA Microarray Protocols

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I – Preparation of DNA Clone Stocks

A - Plasmid Bacterial Transformation and Storage in 96-Well Plates

This is a modification of the protocol described by Seidman CE (1989) Introduction of Plasmid into Cells. *In: Current Protocols in Molecular Biology* (Ausubel FM, Brent R, Kingston RE, Moore D, Seidman JG, Smith JA, Struhl K, eds) p. 1.8.1 – 1.8.3. John Wiley & Sons, Inc., New York, NY.

➤ *Materials and Reagents*

- LB medium - see Appendix A
 - 1 mL 96-well plates polypropylene, non-sterile - E&K Cat. 662000
 - 96-Well polypropylene, V-bottom PCR plate - MJ Research Cat. MLP-9601
 - 3M Scotch Aluminum Tape
 - 50% Glycerol Solution
 - Competent *Escherichia coli* cells
-

➤ *Prepare beforehand:*

- 1mL 96-well plate containing 250 µL of LB media, covered with aluminum foil and autoclaved for 15 min (Allow plates to cool before use)
 - Sterile 96-well PCR plates and lids
 - Chemically competent (heat-shock competent) *E. coli* cells, such as DH5α, XL-1 Blue or JM101
 - Chilled pipet tips
 - Sterile 50% glycerol solution
 - Sterile 1 mL 96-well plates for freezing and storage of transformed bacterial stocks
 - Multi-channel pipet
-

- Place approximately 10 ng of plasmid DNA (3-5 µL solution) into each well of a 96-well PCR plate and let it rest on ice.
- Add 20 µL of properly thawed chemically (heat-shock) competent cells (10^6). Keep the plate on ice until next step.
- Place the plate in the PCR machine and run program: 4°C for 20min, 42°C for 2min and 4°C for 10min.
- Pipet 20 µL of the transformed cells into 1mL 96-well plate containing 250 µL LB media.
- Let the cells incubate at 37°C for 2 hrs.

- Add selective antibiotics. (e.g. ampicillin to a final concentration of 50 µg/mL, i.e., 6 µL /well of a 50X (2.5mg/mL) ampicillin stock).
- Incubate overnight at 37°C on a shaker.
- Pipet 100 µL of the transformed cells in LB media into wells of a sterile 96-well plate. Add 50 µL of 50% glycerol. Pipet – mix (Avoid vortexing because it may cause cross-contamination)
- Store at – 80°C.

Note: Keep plates covered during incubation steps to avoid evaporation.

B – Plasmid DNA Preparation in 96-Well Plates

This is a modification of the protocols described by Engebrecht JA and Bent R. Alkaline Lysis in 96-well Microtiter Dishes and by Heilig JS, Lech K and Bent R. Plasmid Purification by PEG precipitation. *In: Current Protocols in Molecular Biology* edited by Ausubel *et al.* (1989) p. 1.6.4 – 1.6.6. and p. 1.7.9. John Wiley & Sons, Inc, New York, NY.

➤ *Materials and Reagents*

- 2 mL 96-well plates polypropylene, non-sterile - E&K Cat. 662000
- 3M Scotch aluminum tape
- Multichannel pipet (the larger the volume the better)
- Selective antibiotics as needed
- Aluminum foil
- Isopropanol, -20°C
- 70% ethanol, -20°C
- 95% ethanol, -20°C (optional)
- 5M potassium acetate, pH 4.8
- TE pH 8.0 *
- TYGPN Medium*
- GTE Medium*
- Base-SDS Medium*
- Salt-PEG medium* (for optional extra clean-up)

* See Appendix A

-
- Add 800 µL TYGPN media to each 2 mL well. Cap lightly (if using caps) or cover wells with aluminum foil and autoclave. Autoclave separately in a flask, 200 µL TYGPN media per well to be used.

- Mix enough selective antibiotics for 1 mL of media per well in the (cooled) flask media (i.e. make a 5X selective media) then carefully (sterile) pipet the remaining 200 μ L into each well.
- Inoculate the media with the bacteria of choice and grow at preferred growing conditions with agitation for 12-24 hrs.
- Pellet cells by centrifugation at 600 x g for 10 min. Flick decant.
- Resuspend cells in 100 μ L GTE and vortex briefly. (About 20 sec)
- Add 200 μ L Base-SDS and **wait** 2 min.
- Add 100 μ L potassium acetate, **cover**, and mix.
- Spin 10 min at maximum table top setting (at least 600 x g).
- Insert tip of pipet at edge of U-bottom and remove 300 μ L of “clean” solution. Avoid precipitate. Transfer to new 1 mL (or 2 mL) plate.
- Add 300 μ L cold isopropanol, cover, agitate and chill for 60 min to O/N at -20°C .
- Spin down at maximum speed (4°C if possible) for 25 min. and flick decant, being careful not to dislodge pellet.
- Wash pellet with approximately 50 μ L 70% cold ethanol (re-spin for 5-10 min if pellet becomes dislodged (it is always a good idea just in case), flick decant.
- An additional washing of the pellet using chilled 95% ethanol can be done but not always necessary.
- Air dry for 30 min (do not allow pellet to become fully **dry**) and resuspend in 50 μ L TE pH 8.0 (overnight at 4°C is recommended). Spin to pellet debris and transfer DNA solution to standard 96-well plate (e.g. approx. 200 μ L volume).

For higher quality DNA (e.g. for sequencing) the following can be used to further purify the plasmids.

- Add 50 μ L salt-PEG media. Precipitate on ice for 1 hr at 4°C or overnight.
- Spin at maximum speed for plate for 20 min at 4°C .
- Rinse pellet with 70% chilled ethanol.
- Resuspend in 40 μ L TE pH 8.0 or ultra pure water (overnight at 4°C is recommended) and adjust to desired final concentration.

Note: Vortexing of the 96-well plate should be avoided during the whole procedure because it may lead to cross-contamination between wells, unless each well is individually sealed.

II - Preparation of DNA Microarray Printing Solution

The DNA microarray printing solution consists of PCR products at a final concentration of greater than 100 ng/ μ L. For quality control, all PCR reactions are evaluated on gels for size and number of bands. The concentration of the PCR products is determined using the PicoGreen Assay.

A₁- PCR Amplification of DNA – EST amplification

➤ *Materials and Reagents*

- 96-Well polypropylene, V-bottom Microplate - MJ Research Cat. MLP-9601
- 5 mM dNTPs – dilute from 100 mM stock – Amersham Cat. 27-2035-01
- 25 mM MgCl₂
- Taq DNA Polymerase*
- Pfu DNA Polymerase*
- DNA Primers*
- PCR Mix *
- TE pH 7.5 (filtered)*

* See Appendix A

-
- Amplify DNA using appropriate primer combination.
 - Set up PCR reactions in 200 μ L 96-well plates by adding 96 μ L of PCR Mix to 4.8 μ L DNA template (Approximately 20 ng DNA template).
 - Perform PCR under the following conditions:

Cycles	Temp / time
	94°C 2'
35 cycles	94°C 30"; 65°C 30"; 72°C 2'
1 cycle	72°C 10'
1 cycle	4°C hold to end

Note: PCR conditions optimized for GeneAmp PCR System 9700,
PE Applied Biosystems

A₂- PCR Amplification of DNA from genomic DNA using Gene Specific Primers – Hot Start Method

➤ Materials and Reagents

- 96-Well polypropylene, V-bottom Microplate - MJ Research Cat. MLP-9601
- 5 mM dNTPs – dilute from 100 mM stock – Amersham Cat. 27-2035-01
- 25 mM MgCl₂
- Taq DNA Polymerase*
- Pfu DNA Polymerase*
- Gene specific primers pairs designed by Fredrik Sterky (see page on AFGC)
- Genomic DNA*

PCR Mix – Add 86 µL per well containing 4 µL primers (20 µM each)

Reagent	For 1 reaction, µL
10 X PCR buffer*	8
25 mM MgCl₂	8
dNTPs 5mM each	4
Genomic DNA 10 ng/µL	2
Ultra pure water	68
Total	90

Put plate in PCR machine and heat to 94°C then pause program. Add 10 µL of the Hot start enzyme mix to each well and pipette up and down once.

Hot start enzyme mix	For 1 reaction, µL
10 X PCR buffer*	2
Ultra pure water	7.34
Taq Polymerase*	0.6
Pfu*	0.06
Total	10

Seal plate with foil and resume program.

Cycles	Temp / time
	94°C 5' (Pause when T=94°C to add enzyme, resume)
35 cycles	94°C 15"; 65°C 30"; 72°C 2'
1 cycle	72°C 7'
1 cycle	4°C hold to end

B - PCR Products Clean up

The PCR products can be purified in different ways. At the AFGC we have used the ethanol precipitation method for the 11.5K. AFGC Y2000 arrays). For the 15K, AFGC Y2001 array we have used the Millipore Multiscreen method.

B₁ - Ethanol Precipitation Method

➤ *Materials and Reagents*

- 3M sodium acetate pH 5.2
 - 95% ethanol
 - 80% ethanol
 - 3X SSC, dilute from 20X SSC - Life Technologies Cat. 15557-036
 - 3M Scotch aluminum tape
 - Plastic wrap
 - Multichannel pipet
-
- Dry down the PCR products to approximately 50 µL in a Speed Vac at low temperature setting (approximately 1 hour and 20 minutes).
 - At the conclusion of the PCR reaction, add 5 µL 3M sodium acetate pH 5.2 and 150µL of 95% ethanol per well (or 155µL of a mixture of both);
 - Precipitate DNA at -20°C overnight;
 - Centrifuge for 60 min at 2750 x g at 4°C;
 - Remove supernatant by rapidly inverting plates, then blotting on paper towel. Be careful otherwise pellets may be lost;
 - Wash pellet with 100 µl 80% ethanol;
 - Centrifuge 2750 x g for 45 min at 4°C;
 - Remove supernatant by carefully inverting plates. Tap plates gently on paper towel to remove ethanol;
 - Let plate sit open in a laminar flow hood to allow remaining ethanol to evaporate. This takes anywhere from 30 min to 2 hrs. Use speed vac if needed, but **do not overdry!**
 - Add 10 µl of filtered 3X SSC. Cover plate with 3M Scotch aluminum tape;
 - Wrap plate in wet paper towels, then plastic wrap. Store at 4°C for 48 hrs to allow DNA to go into solution;
 - Store plates with DNA solutions at -20°C in a non-defrosting freezer.

B₂ - Millipore Multiscreen Method

➤ *Materials and Reagents*

- Multiscreen Vacuum Manifold – Millipore Cat. MAVM 096 OR
 - Multiscreen PCR plates – Millipore Cat. MANU 030
 - 3X SSC, dilute from 20X SSC - Life Technologies Cat. 15557-036
 - TE pH 7.5 – see Appendix A
 - Multichannel pipet
 - 96-Well polypropylene, V-bottom Microplate - MJ Research Cat. MLP-9601
 - 3M Scotch aluminum tape
 - Plastic wrap
 - Ziploc plastic bags
-
- At the conclusion of the PCR reaction, pipet the reaction products (100 µL) into the 96 well Millipore Multiscreen PCR plate.
 - Place the Multiscreen plate on top of the Multiscreen Vacuum Manifold and apply vacuum at 20 inches of Hg for 10 min until no liquid remains in the wells.
 - Wash by adding 120 µL water to each well and apply vacuum again for 13 min.
 - To resuspend PCR products, add 35 µL of 3X SSC to each well. Cover plate with cover provided and shake vigorously on a plate shaker for 10 min.
 - Remove as much as possible of the resuspended products (recovery about 30 µL) from each well using a multichannel pipet and place in a new, 96 well plate for storage. Cover plate with 3 M Scotch aluminum tape, wrap in moist paper towels and plastic wrap, then place it in a Ziploc plastic bag. Store at -20°C (non-defrosting freezer).

C - Quality Control (QC) of PCR Products

Quality Control (QC) analysis of the PCR products is carried out to assure the quality of the DNA microarray spots. QC involves two parts: Agarose gel analysis is used to examine the number of bands produced by each PCR. Ideally each reaction will give rise to only one band on a gel. The presence of multiple bands is an indication of the presence of contaminating DNA. The PicoGreen assay is used to determine the concentration of the DNA printing solution. For a PCR solution to be used for array printing our target concentration is 100 ng/ µL for the AFGC Y2001 array (average yield per plate = 434 ng/µL, >95% of all PCR products are greater than 100 ng/ µL). We aim for 10% or less of DNA classified as bad (blank, multiple bands, etc) in the gel QC analysis (actual % of Y2001 array that does not have a single band is 10.7%) . Quality control is carried out with PCR products resuspended in the printing solution (3X SSC).

➤ **Materials and Reagents**

- TE pH 7.5 – see Appendix A
 - Ultra pure water
 - 3M Scotch Aluminum tape
 - 96-Well polypropylene, V-bottom Microplate - MJ Research Cat. MLP-9601
 - Rapid Analysis Gel Electrophoresis System - Cascade Biologics Cat. RGX-100
 - 1 Kb DNA Ladder – Life Technologies Cat. 15615-016
 - DNA Loading dye – See Appendix A
-

◆ **Gel Electrophoresis**

- **Preparation of “Temporary Plates”** - Take 2 µL of the printing solution and add 38µL of TE pH 7.5. Cover with aluminum tape and freeze if plate is not to be used the same day. **Do not discard** temporary plates while performing the QC as they may be needed for reexamination.
- **Preparation of the “Gel Plate”** - Aliquot 5 µL from the “Temporary Plate” into a new 96-well plate and add 5 µL of 2X DNA loading dye per well. Mix by pipeting. Cover plates with 3M Scotch Aluminum tape to prevent evaporation. “Gel Plates” should be kept at -20°C if gels are not to be run the same day.
- **Gel electrophoresis** – An aliquot of 10 µL of the DNA solution from the “Gel Plate” is run on a 1.2% agarose gel in 1X TAE buffer. The gels, running buffer and cooling water contained 0.5 µg/mL ethidium bromide. The gels were run on the Rapid Analysis Gel Electrophoresis system of Cascade Biologics (RGX-100) and photographed using a BioRad Gel Doc 2000.

Note: We use 300 ng 1 Kb DNA Ladder as molecular weight marker. The marker is also used to help quantify the PCR products.

◆ **Scoring QC gels**

PCR products are screened for their quality and scored as follows:

Code 0 - Acceptable - The sample contains only one band of a substantial intensity.

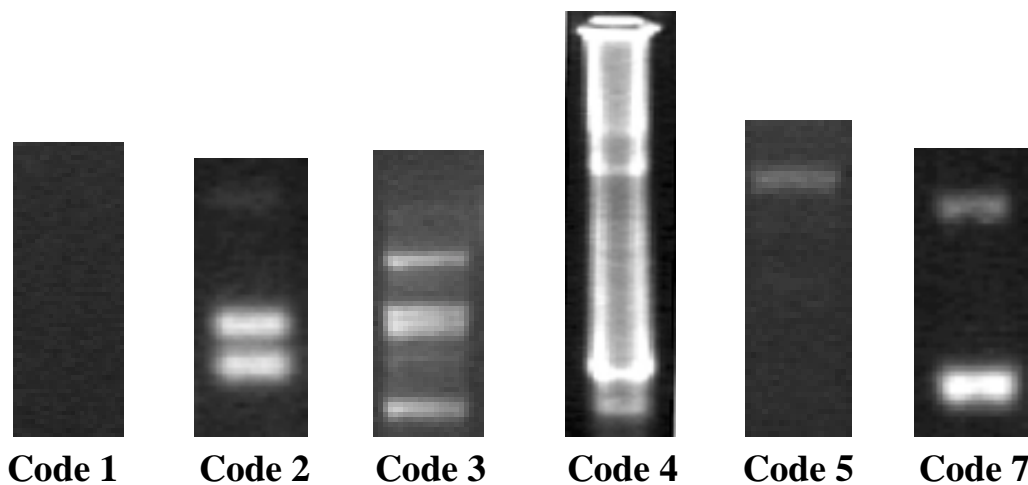
Code 1 - Blank - No PCR product is visible on the gel.

Code 2 - Double bands - Two bands of roughly equal intensity are visible in the same sample.

Code 3 - Multiple bands - Three or more bands of roughly the same intensity are visible in the same sample.

- Code 4 - Smear** - The PCR fails to produce a distinct product, or chemical contaminants in the sample prevented the sample from running properly. Smears are a rare occurrence.
- Code 5 - Faint band** - The PCR product is of “low” intensity when compared to the DNA marker
- Code 7 - Extra band(s)** - The PCR product contains 2 or more bands, but a single dominant PCR product is visible. Extra bands may or may not interfere with array hybridization. Extra bands were not marked in the array analysis of Fall/Winter1999-2000.

Examples of QC Scores



Note: Images have been manipulated to more clearly illustrate examples of QC scores.

- **Priority of Quality Control Codes:** Some PCR products have two or more different quality concerns, but only one code can be used per sample. Doubles and Multiples have precedence over Faints. When, for example, a sample contains 2 faint bands of equal strength, the sample is marked as a Double as opposed to a Faint sample.
- **Comparing gels:** The gels are not directly comparable to each other. The concentration of ethidium bromide may vary slightly between gels. Furthermore, the exposure time and lens aperture may vary between photographs. However, each section of the gel contains a standard DNA molecular weight marker to which the PCR products can be compared.

D - DNA Quantification by PicoGreen Assay

DNA quantification by the PicoGreen Assay method is carried out according to supplier's (Molecular Probe, Inc.) recommendations.

➤ *Materials and Reagents*

- TE pH 7.5 – see Appendix A
- PicoGreen dsDNA Quantitation kit - Molecular Probes Cat. P-7589
- Aluminum foil -
- 3X SSC, dilute from 20X SSC - Life Technologies Cat. 15557-036
- 96-Black well, clear bottom plates -E&K Scientific 25096

-
- Prepare DNA Standards for assay (see Table below). Prepare duplicates of each standard. Stock solutions of DNA of 10 ng/μL were prepared and 100 μL aliquots frozen for assays.

Suggested DNA Standards for PicoGreen Assay

Standard #	μL DNA Standard Solution in Assay	Total ng DNA per well
1	100.00	200.00
2	100.00	100.00
3	100.00	50.00
4	100.00	25.00
5	100.00	12.50
6	100.00	6.25
7	100.00	3.125
8	100.00	0.00

- Pipet 5 μL of DNA solution from “Temporary Plate” to the Black wall, 96 well plate and add 95 μL TE pH 7.5 to each well for a total of 100 μL per well.
- Prepare PicoGreen working solution by diluting 1:200 PicoGreen dye stock in TE pH 7.5 just before use;
- Add 100μL of PicoGreen working solution to each well of the black well, clear bottom 96 well plate and mix thoroughly on a plate shaker for 5 minutes (spin plate for 1 minute following shaking) or use the plate shaking feature of the Tecan Plate Reader to shake the plate just prior to measuring.
- Cover plates with aluminum foil to protect from light.

- Read all plates in the plate reader (Tecan Spectra Fluor) and export data to Excel or SigmaPlot. Create a standard curve and calculate the concentration of the DNA Array Printing Solution.

III - DNA Microarray Slides Preparation

We currently use Superaldehyde slides (Telechem, San Jose, CA). Other slides from other sources (ex. Corning and poly-lysine coated slides) also give good results. For more information see Hegde, P.;Qi, R.;Abernathy, K.;Gay, C.;Dharap, S.;Gaspard, R.;Hughes, J.E.;Snesrud, E.;Lee, N.;Quackenbush, J.(2000) A Concise Guide to cDNA Microarray Analysis. BioFeature, BioTechniques 29:548-56. For more information on home-made poly-lysine; seehttp://cmgm.stanford.edu/pbrown/protocols/1_slides.html)

A - DNA Microarray Slides Printing

Printing of the DNA microarray slides should be carried out according to the recommendations of the manufacturer of choice. After printing, a reference slide should be prepared with the array boundaries, marked with a diamond pen.

B - DNA Microarray Staining

B1- Imaging spotted cDNA (before post-processing of arrays)

Materials and Reagents

- Dissecting microscope or microarray scanner with 633 nm laser and 633 nm filter (eg. Packard BioScience, Scan Array Lite 4000 or 5000)

Note1: These methods do not work with DMSO or other non-salt printing buffer; neither do the conditions when the deposited salt crystals on the spots are washed off.

- Check the shape of spots under microscope.

The size and shape of the spots on an array can be directly examined by dissecting microscope. Usually, bigger spots contain more DNA. However, since the size of a spot depends on the coating of the slide, one should not compare the spots on a slide with one coating to that on a slide with a different coating.

- Check the shape of spots by using Red Reflection scanning.

Alternatively, the size and shape of the spots on an array can be examined by means of a microarray scanner with 633 nm laser and 633 nm filter.

Note2: The technical staff at Packard Biochip (<http://www.packardbiochip.com>) have developed a protocol called “Reflective Imaging”, which allows one to view slides printed with salt-containing DNA solutions in a simple, non-destructive manner using a scanner. The method is based on capturing the laser light that is reflected from the salt crystals that remain in each spot after the DNA solution has dried onto the slide. The reflected light is filtered through a neutral density filter prior to collection by the PMT. The Reflective Imaging does not provide a quantitative estimate of the amount of DNA printed to the slide although spots with very small amounts of DNA can be identified (compare the spots printed at 200 ng/μl presented in Fig. 1, A3 versus those printed at 50 ng/μl displayed in Fig. 1, A4). We also noticed that the size of the spots derived from Red Reflection scanning cannot be directly compared to those derived from hybridization (compare Fig. 1, A4 to D4). Thus, we use this method primarily to make present/absent calls for each printed spot.

B2- Imaging spotted cDNA (after post-processing of arrays)

➤ *Materials and Reagents*

- 1 mM ToTo-3 iodide in dimethylformamide (DMSO) (Molecular Probes, Cat. P-3604) or 1 mM PoPo-3 iodide in dimethylformamide (DMSO) (Molecular Probes, Cat. T3604)
 - 10X PBS (Life Technologies, Cat. 70013-032)
 - A plastic surface (e.g. plastic lip which can hold up to 4 slides from E&K, Cat. EK26161) or plastic cover slip (e.g. Hybrislip from Research Products)
 - Slide rack (Wheaton, Cat. 900234)
 - glass jar
 - SpeedVac
 - Microarray scanner
 - Wash solution
0.1%SDS, 10 mM Tris, 1mM EDTA, pH 7.5
-

- Thaw the frozen dye (ToTo-3 iodide or PoPo-3 iodide) and then dilute the dye to final concentration of 1 μ M in 1X PBS at room temperature (Note: we prefer ToTo-3 iodide for DNA staining)
- Apply 50 μ L of the diluted dye to a plastic lip. Place a slide (array faces down) to the plastic lip. Make sure that the diluted dye immediately covers the whole slide. Alternatively, add appropriate volume of the dye to the center of the array (face up) and immediately cover the array with plastic cover slip. Note: it is critical to quickly apply diluted dye on the array.
- Incubate the array with the diluted dye for 30 minutes at room temperature in the dark.
- While for the incubation, place a slide rack in a glass jar which is filled it with 350mL 1X PBS
- After the completion of 30 minutes incubation, use forceps to place the slide into a slide rack. Plunge slide rack up and down for two minutes.
- Dry the slides with SpeedVac for 5 minutes.
- If the DNA is stained by ToTo-3 iodide, scan the array by using the red laser and Cy5 filter. (ToTo-3 exhibits absorption/emission maximum of 642/660 nm when bound to double stranded DNA.) If the DNA is stained by PoPo-3 iodide, scan the slide by using the green laser and Cy3 filter. (PoPo-3 iodide exhibits absorption/emission maximum of 534/570 nm when bound to double stranded DNA.)

Note 1: Although we do not recommend using the stained slide for hybridization experiment, if necessary, the slides could be used for hybridization when the dye is removed. To remove the dye, incubate the slide at room temperature for one hour in wash solution. After being dried, the slides can be used for hybridization. (For more information, please check the web site, <https://www.probes.com>).

Note 2: Some scanners have more than two lasers. With these scanners, a third laser can be used to assess the quality of the slide by staining with a general DNA stain appropriate for the wavelength of light emitted by the third laser.

Note 3: The ToTo-3 iodide staining can be used to roughly estimate the amount of spotted DNA based on the intensity of the spot. We have been able to do such estimation when the concentrations of DNA solution spotted on the slide are between 800 ng/ μ L (Fig.1 A1) and 50 ng/ μ L (Fig. 1 A4). We also noticed that the size of the spots derived from ToTo-3 iodide staining correlate well to those derived from hybridization as demonstrated by the comparison between Fig. 1 A3 and Fig. 1 D3. The property of PoPo-3 iodide staining is basically the same as that of ToTo-3 iodide staining. However, we prefer ToTo-3 iodide staining because, for unknown reason, PoPo-3 iodide staining can some time give high background.

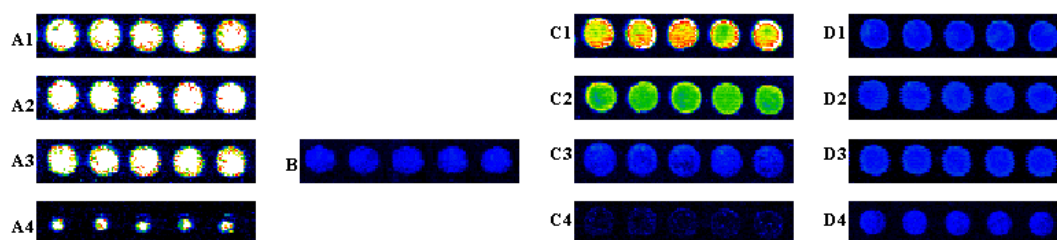


Fig. 1 Examples of good DNA spots on arrays.

cDNA at 800 ng/μl, 600 ng/μl, 200 ng/μl, 50 ng/μl were spotted on glass slides as 5 duplicates and then examined by Red Reflection scanning (A1 to A4), ToTo-3 iodide staining (C1 to C4), or hybridization (D1 to D4). In panel B, cDNA at 200 ng/μl were also spotted in a similar manner and examined by PoPo-3 iodide staining. In panel B, C1 to C4, and D1 to D4, the intensity of each spot was represented by a color. The strength of spot intensity is arbitrarily ordered as white, red, yellow, green, and blue with white being the strongest in intensity.

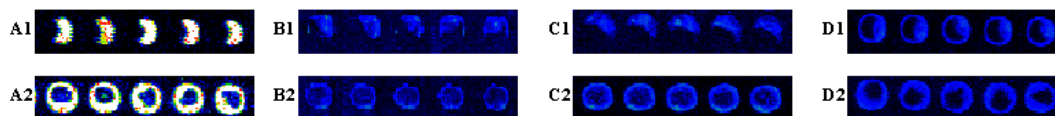


Fig. 2 Examples of imperfect DNA spots on arrays.

cDNA 200 ng/μl were spotted on glass slides as 5 duplicates and then examined by Red Reflection scanning (A1, A2), PoPo-3 iodide staining (B1, B2), ToTo-3 iodide staining (C1, C2), or hybridization (D1, D2). Imperfect spots can emerge as moon shape (A1, B1, C1, D1) or donut shape (A2, B2, C2, D2). In panel B, C, and D the intensity of each spot was represented by a color. The strength of spot intensity is arbitrarily ordered as white, red, yellow, green, and blue with white being the strongest in intensity.

C – Postprocessing of Superaldehyde Slides

Processing of the slides after printing consists of drying the slides, cross-linking of the DNA in the slide matrix, DNA denaturation, prewashing and subsequent blocking of the slide.

➤ Materials and Reagents

- ♦ 10X PBS - Life Technologies Cat. 70013-032

- Superaldehyde Slides – TeleChem Cat. SMA-25
 - 10% SDS solution - Life Technologies Cat. 15553-035
 - NaBH₄ - Sigma Cat. S-9125
 - Wheaton slide rack – Wheaton Cat. 900234
 - 95% ethanol
 - Ultra pure water
 - UV Stratalinker 1800- Stratagene
-

➤ ***Prepare beforehand***

- Five glass square jars (~500 mL size), to hold 0.2% SDS solution, ultra pure water and blocking solution
- One 2 L beaker, with about 1 L of ultra pure water, brought to boiling. One 500 mL beaker for preparing blocking solution
- 2 L of 0.2% SDS solution (dilute from 10% SDS stock solution)
- 300 mL 1X PBS (dilute from 10X PBS stock solution)

Note: This protocol is optimized for a Wheaton slide rack that can hold 30 slides maximum. The horizontal dimension of the slide holder is 8 x10cm. **We usually process 15 slides at a time, slides are placed in the holder with space in between to ensure that each slide is exposed to the washing and blocking solutions properly.** Glass jars should be just big enough to fit the slide holder, and the level of the liquid in the glass jar should be high enough so that the slides are always submerged under the liquid, even when plunging the slide holder up and down in the washing process.

- **Drying** - Dry printed slides for at least 12 hrs at room temperature;
- **DNA fixation** – Place slides with printed DNA side up in the UV Stratalinker, UV fixation settings: 650 x 100 microjoules
- **Prewash** – Prepare 4 glass containers, two with 0.2% SDS and two with ultra pure water, about 350-400 mL each. Place the cross-linked slides into a slide rack and carefully and quickly submerge the slide rack under the first 0.2% SDS solution. **Start shaking vigorously using the wrist IMMEDIATELY!** Prewash slides in 0.2% SDS solution for 2 min each and subsequently in ultra pure water for 2 min each, **always using vigorous manual shaking.**

Note: If this step is done sluggishly, the DNA tends to stick to the unblocked surface of the slide, which creates "comet-tails" on the DNA spots. This should be avoided.

- **Denaturation** – Immerse slide rack with slides in boiling ultra pure water (95-100°C) for 2 min. Allow to cool at room temperature for 5 min in a clean glass jar.

- **Blocking** - During the 5 min rest above, dissolve 1g NaBH₄ into 300 mL 1X PBS. After everything has dissolved, add 90mL 95% ethanol and stir. Put a glass jar with the solution on the shaker. Incubate slides in slide rack in the solution for 5 min with gentle shaking on a shaker. Watch the slides for air bubbles. **Once every minute, manually shake slides gently to get rid of the air bubbles on the slide surface.** Wash out the original four glass jars and prepare for the next step.
- **Postwash** – Wash three times in 0.2% SDS for 1 min each, **with continuous manual shaking** and once with ultra pure water for 1 min, **with continuous manual shaking.** Spin dry in plate-holding speed vac (with vacuum, no heat) for 5-7 min.
- **Store** slides in a slide box in a dry place.

IV - RNA Isolation and Purification

We usually recommend one of the two following methods: the TRIzol and the Pine Tree methods. The TRIzol is a fast and easy method that will give good quality and yield of RNA for samples from various types of tissues. The Pine Tree method is very effective for isolation of RNA from tissue containing a high amount of polysaccharides and/ or phenolic compounds. For a comparison of RNA isolation procedures see Appendix B.

A - Total RNA Isolation: TRIzol Method

This is a modification of the procedure originally described by Chomczynski P and Sacchi N. 1987. Signal-Step Method of RNA Isolation by Acid Guanidinium Thiocyanate-Phenol-Chloroform Extraction. *Analytical Biochemistry* 162: 156-159

➤ *Materials and Reagents*

- TRIzol Reagent - Life Technologies Cat. 15596 or Alternative Solution for TRIzol as described in Appendix A.
 - 0.8 M sodium citrate / 1.2 M NaCl
 - Isopropanol (2-Propanol)
 - Chloroform
 - DEPC-Water - See Appendix A
 - 75% ethanol prepared with DEPC-Water
 - RNase Inhibitor aseERASETM – BIO 101 – Cat. 2601-104
 - 50 mL sterile plastic screw-cap centrifuge tubes
-

- Grind 1g tissue in liquid nitrogen in a mortar and pestle.
- Transfer powdered tissue to a 50 mL sterile plastic screw-cap centrifuge tube containing 15 ml TRIzol reagent. Incubate samples at room temperature or at 60°C for 5 min.

Note: Centrifuge tubes containing TRIzol are kept in liquid nitrogen prior to grinding step and while processing other samples.

- Homogenize tissue with homogenizer for 15 seconds. Repeat once.
- Centrifuge samples at 12,000 x g at 4°C for 10 min.
- Transfer supernatant into new sterile 50 mL sterile plastic screw-cap centrifuge tube. Discard pellet.
- Add 3 mL chloroform to each tube in hood.
- Shake tubes vigorously with vortex for 15 sec.
- Let tubes sit at room temp 2-3 min.
- Centrifuge tubes at 10,000 x g at 4°C for 15 min.
- Carefully pipet aqueous phase into a clean screw-cap centrifuge tube; discard interphase and lower phase into waste tube.
- Precipitate RNA by adding isopropanol and 0.8 M sodium citrate/1.2 M NaCl, half volume of the aqueous phase each. Cover tube and mix by gentle inversion.
- Let sit at room temperature for 10 min.
- Centrifuge tubes at 10,000 x g at 4°C for 10 min. Discard supernatant.
- Wash pellet with 20 ml of 75% ethanol. Vortex briefly.
- Centrifuge at 10,000 x g at 4°C for 10 min. Discard supernatant; briefly dry pellet (5-10 min; not longer).
- Add 250 µL DEPC-Water, to pellet. Resuspend RNA by pipetting up and down a few times.
- Add 1 µL RNase inhibitor aseERASE to a 250 µL RNA sample. If having problems resuspending the RNA pellet, we suggest incubation at 55 - 60°C for 10 min.
- Transfer sample to microcentrifuge tube at room temperature.
- Spin samples at high speed in microcentrifuge tube for 5 min at room temperature (to pellet the material that would not resuspend).
- Transfer RNA solution (supernatant) to a new tube. Determine RNA concentration and quality by spectrophotometry.

Note: For optimal spectrophotometric measurements, RNA aliquots should be diluted with water or buffer with a pH 7.5. Water with pH < 7.5 falsely decreases the 260/280 ratio.

B - Total RNA Isolation: Pine Tree Method

This method was originally described by Chang S., Puryear J., Cairney J. (1993) A Simple and Efficient Method for Isolating RNA from Pine Trees. Plant Molecular Biology Reporter 11: 113-116.

➤ ***Materials and Reagents****

* Use DEPC-treated water for all solutions – See Appendix A

- Extraction Buffer – See Appendix A
- Chloroform:isoamyl alcohol (24:1)
- 10 M Lithium chloride
- SSTE – See Appendix A

-
- Warm 5 mL extraction buffer to 65°C in a water bath, quickly add 1g ground tissue and mix by inverting the tube and vortexing.
 - Extract two times with an equal volume of chloroform:isoamyl alcohol, separating phases at room temperature by centrifugation for 10 min at 12,000 x g. Centrifuge longer if phases are not well separated.
 - Add 1/4 volume 10 M LiCl to the supernatant and mix. The RNA is precipitated overnight at 4°C and harvested by centrifugation at 12,000 x g for 20 min. Shorter precipitations time may also be used with lower yield.
 - Dissolve pellet into 500 µL SSTE. When poly (A)+ RNA is to be extracted, dissolve total RNA in 0.5% SDS instead of SSTE and proceed with selection directly.
 - Extract once with an equal volume of chloroform:IAA.
 - Add two volumes ethanol to the supernatant, precipitate at -70°C for 30 min or 2 hrs at -20°C.
 - Spin 20 min in microfuge to pellet the RNA. Wash pellet with 75% ethanol. Dry RNA and resuspend in DEPC treated ultra pure water.

C -Poly (A)+ RNA Purification

Poly (A)+ RNA can be purified in many different ways. At the AFGC we normally use the Oligotex mRNA Mini Kit – Batch Protocol – Qiagen Cat. 70022.

D - Quality Control of Poly (A)+ RNA

The quality of the poly (A)+ RNA is done by evaluation of the quality of the 1st strand cDNA. That is done by gel electrophoresis analysis of labeled 1st strand cDNAs.

➤ ***Materials and Reagents***

- 10X Klenow reaction buffer – Amersham Cat. E2141Y
- Klenow enzyme (5 U/µL) – Amersham Cat. E2141Y

- Cy3 dUTP or Cy5-dUTP (25 nmole) – Amersham Cat. PA53022 / PA55022
- Oligo dT-V (2µg/µL) – Sigma custom ordered
- 5X Superscript II reaction buffer - Life Technologies Cat. 10864014
- Superscript II reverse transcriptase (200 U/µL) - Life Technologies Cat. 10864014
- 50X dNTPs (25mM each except for dTTP at 10 mM) - Amersham Cat. 27-2035-01
- 10X dNTPs (0.25 mM each except dTTP at 0.09 mM – Amersham Cat. 27-2035-01
- 0.1M DTT - Life Technologies Cat. 10864014
- PCR clean up kit – Qiagen Cat. 28106
- TE pH 8.0 – see Appendix A
- 2X loading buffer – see Appendix A
- 1X TAE buffer – see Appendix A
- Lambda *Hind* III

◆ Labeling 1st strand cDNA with Cy-dye

- Mix 2 µL RNA (80 ng mRNA) with 0.5 µL oligo dT-V (2 µg/µL)
- Heat 10 min at 70°C and quickly chill on ice.
- Add:

5X Superscript II buffer	1.0 µL
0.1 M DTT	0.5 µL
50X dNTPs (25 mM dNTPs except dTTP at 10 mM)	0.1 µL
Cy3- or Cy5-dUTP	0.5 µL
Superscript II reverse transcriptase (200 U/µL)	0.4 µL

- Incubate at 42°C for 2 hrs.
- Clean up reaction and elute with 50 µL EB buffer from Qiagen PCR clean up kit.
- Dry the eluted DNA down and resuspend in 3 µL TE pH 8.0.
- Mix 3 µL of labeled 1st strand cDNA (labeled 1st strand cDNA from 80 ng mRNA) with 3 µL of 2X loading buffer.
- Load 6 µL into each well.
- Run a 1.2% agarose gel in 1X TAE buffer at 100mA, 30 min. A labeled DNA molecular weight marker is used for comparison. DNA molecular weight marker is labeled as follows:

◆ Labeling Lambda *Hind* III with Cy-dye

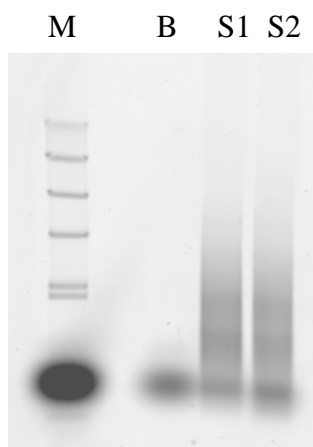
- Mix the following components and incubate at 37°C for 1hr:

Klenow 10X buffer	4 μ L
10X dNTP (0.25 mM each except dTTP at 0.09 mM)	4 μ L
λ /Hind III (0.5 μ g / μ L) – 250 ng	4 μ L
Cy3-dUTP/or Cy5-dUTP	2 μ L
Klenow enzyme	2 μ L
Ultra pure water	24 μ L

- Clean up reaction with Qiagen PCR clean up kit and elute with 50 μ L EB buffer.
- Dry the eluted DNA down and resuspend in 10 μ L TE.
- Mix 3 μ L of the labeled DNA with 3 μ L of 2X loading buffer.
- Load 6 μ L into each well. Run together with your labeled sample on a 1.2% agarose gel in 1X TAE buffer at 100mA, 30 min.

◆ QC gel analysis

An example of a gel scan is shown below. Generally we look for a fairly bright smear higher than 500 bp in size. For two alternative ways to do the gel scanning, please see Chapter V, page 22.



M – Lambda *Hind* III
 B – Water blank (Plus Cy 3 dye)
 S1 & S2 – Cy 3 labeled cDNA

V – Preparation of Labeled cDNA

A - cDNA Synthesis and Labeling

This protocol is a modification of the one originally developed by Michael B. Eisen and Patrick O. Brown. 1999. DNA Arrays for Analysis of Gene Expression. *In* Methods in Enzymology (Weissman SM, ed.) 303: pp179-205. Academic Press, San Diego, CA

➤ *Materials and Reagents*

- Oligo dT-V (2 µg/µL) – Sigma custom ordered – See Appendix A
- 5X Superscript II reaction buffer - Life Technologies Cat. 10864014
- 0.1M DTT - Life Technologies Cat. 10864014
- Superscript II reverse transcriptase (200 U/µL) - Life Technologies Cat. 10864014
- 10 mM dNTP (dilute from 100mM stock) - Amersham Cat. 27-2035-03
- RNaseH - Amersham Cat. E70054Y
- TE pH 8.0 - see Appendix A
- Centrifugal filter device Microcon YM-30 - Millipore Cat. 42410
- PCR Clean up kit – Qiagen Cat. 28106
- 10X Klenow reaction buffer – Amersham Cat. E2141Y
- Klenow (5 U/µL) - Amersham, Cat. E2141Y
- Random primer (3mg/mL) - Life Technologies Cat. 48190-011
- 10X dNTP (0.25mM each except for dTTP at 0.09 mM) - Amersham Cat. 27-2035-01
- Cy3 dUTP (25 nmole) – Amersham Cat. PA53022
- Cy5 dUTP (25 nmole) – Amersham Cat. PA55022
- 10X SSC, dilute from 20X SSC - Life Technologies Cat.15557-036
- DEPC-Water - see Appendix A

◆ **First Strand cDNA Synthesis**

- Mix the following in 0.2 mL PCR tubes:

RNA sample (~1µg of PolyA RNA)	X µL
DEPC-Water	24 - X µL
Oligo-dT-V	0.5 µL
Total Volume	24.5 µL

- Incubate for 10 min at 70°C in a PCR machine. Immediately transfer to ice.

- Briefly after, add:

5X Superscript Buffer	8 μ L
0.1 M DTT	4 μ L
10 mM dNTPs	2 μ L
Superscript II RT	1.5 μ L
Total Volume	40 μ L

- Mix thoroughly upon adding each reagent.
- Incubate for 1 hr at 42°C (in a PCR machine)
- Add: 0.25 μ L RNase H, mix thoroughly.
- Incubate at 37°C for 30 min (in the PCR machine).

◆First Strand cDNA Clean up:

- Label Microcon columns. Insert into receiving tubes.
- Label 1.5 mL tubes for the elutants and set aside.
- Add 160 μ L TE to first strand synthesis reaction. Mix thoroughly. Transfer to column.
- Spin at maximum speed for 5 min and discard flow-thru.
- Add 200 μ L TE to column. Spin again at maximum speed for 5 min. Discard receiving tube.
- **Invert and insert the column into a new 1.5ml tube** for recovery of first strand cDNA product.
- Spin at 960 x g for 1 min. (Recovery volume of about 2 μ L).
- Add 40 μ L ultra pure water to column. Invert and insert the column into original 1.5 mL recovery tube.
- Spin at 960 x g for 1 min. Adjust recovered volume to 56 μ L with ultra pure water.
- Take out 28 μ L into new 0.2 mL tube for 2nd Strand cDNA reaction.
- Label the original 1.5 mL tubes (which has the remaining 28 μ L). **Save as backup** by storing at - 20°C.

Second Strand cDNA Synthesis and Labeling

- Mix:

First-strand cDNA product	28 μ L
Klenow buffer	4 μ L
Random primer	1 μ L

- Incubate at 100°C for 2 min. Leave at room temperature for 5 min.
- Add:

10X dNTPs	4 µL
Cy3-dUTP or Cy5-dUTP	1 µL
Klenow	2 µL
Final volume	40 µL

- Incubate at 37°C for 3 hrs in the PCR machine.

Note: The two different dyes are used to label different samples for comparison.

♦ Second Strand cDNA Clean up:

The two second strand cDNA samples are combined in the same tube before clean up. Removal of unincorporated nucleotides is carried out using Qiagen PCR clean up Kit, as follows:

- Insert QiaQuick columns into the receiving tubes.
- Label lid of the column (**not** the side of the columns!).
- Combine the two cDNA samples for each slide and adjust volume to 100 µL by adding TE
- Add 500 µL PB Qiagen buffer. Mix thoroughly.
- Pipet the solution into the column.
- Spin at maximum speed for 1 min.
- Discard flow-through and reinsert column into same receiving tube.
- Wash: Add 750 µL PE Qiagen buffer to column.
- Spin at maximum speed for 1 min.
- Discard flow-through and reinsert column into same receiving tube.
- Repeat wash and spin.
- Spin additional 1 min at maximum speed.
- Discard receiving tube and insert the column into a new 1.5 mL microcentrifuge tube.
- Apply 50 µL EB Qiagen buffer to **center** of column. Let sit for 1 min.
- Spin at maximum speed for 1 min to elute labeled cDNA*.

- Dry eluate in a speed-vac for 15 min.
- Adjust final combined volume to 18 μ L with ultra pure water. This is the labeled cDNA to be used for hybridization.

* From this eluate an aliquot can be taken to run Quality Control of the cDNA labeling, as described below.

Proceed to Quality Control of the cDNA synthesis and labeling and hybridization steps.

B - Quality Control of 2nd Strand cDNA Labeling

Quality Control of the 2nd strand cDNA synthesis and labeling with Cy-dye is routinely performed by the AFGC group using one of the two procedures described below:

Gel Analysis 1:

- Take out 1 μ L from 50 μ L Qiagen column eluted solution (this would give you labeled 2nd strand from 10 ng mRNA) and mix with 1 μ L ultra pure water. Mix diluted cDNA with 2 μ L of 2X loading buffer and load 4 μ L into each well and run on a small (10 cm) horizontal 1.2 % agarose gel at 100 mA, 30 min. Scan gel using a Fluorescence Imager (we use Typhoon 8600, Variable Mode Imager, Molecular Dynamics, NJ).
- Generally we look for a fairly bright smear higher than 500 bp in size (as exemplified in page 18 for First strand cDNA labeling)

Note: Alternatively, an aliquot may be taken from the hybridization solution described below, which is the approach we are using currently, for convenience.

Gel Analysis 2:

- Prepare the samples combining 2 μ L of labeling reaction with 2 μ L of glycerol 30% (you can mix Cy3 and Cy5 labeling reactions in one tube; if so, add the same total volume of glycerol 30%)
- Pour 1% TAE agarose gel in a vertical gel apparatus (ex. BioRad Miniprotean II) using 0.75 mm spacers.
- Remove the comb carefully and clean the wells from residual agarose.
- Load the Cy3/Cy5 ladder and the samples side by side; load 6-8 μ L BPB dye in a well by itself.
- Cover the apparatus or put in a dark room and run the gel at 100 V (about 2 mA) for 15 – 20 min or until the blue dye is 2 cm from the bottom gel.

- Cut the gel vertically every 2 lanes, discarding about 1.5 cm of the top.
- Gently slide the gel slices on a slide and dry it in the dark, putting it on top of a heating block at 70°C.
- Scan the slide with the gel using the following settings:
 - Laser power: 70%
 - PMT gain: 50
 - Resolution quick scan: 30 μ m
- Generally we look for a fairly significant (i.e. fairly blue or brighter) smear higher than 500 bp in size. (as exemplified in page 18 for First strand cDNA labeling).

VI - DNA Microarray Hybridization

➤ *Materials and Reagents*

- 2% SDS - dilute from 10% SDS stock - Life Technologies Cat. 15553-027
- yeast tRNA (2 μ g/ μ L) - Life Technologies Cat. 16051-039
- Printed and processed array slides (see section III)
- Hybrislip TM - Research Products Cat. 247456, 22x40mm
- 3X SSC, dilute from 20X SSC - Life Technologies Cat. 15557-036
- Hybridization chambers
- Wheaton slide rack – Wheaton Cat. 900234
- Wash solutions (listed in the protocol below)
- DEPC-Water - see Appendix A

A - *Hybridization Reaction:*

➤ *Prepare beforehand:*

- Set up water bath to 65°C.
- Acquire necessary parts to the hybridization chambers, make sure that the screw lengths are even, and that the chamber can be sealed tightly.
- Clean the bench top.
- Using a reference slide, as described in page 11, mark the boundaries of the array on the side of the slide with permanent marker. Only mark on the side of the slide, never

on top or bottom as ink from the marker is fluorescent. If a reference slide is not available, expose the slide briefly to vapor to visualize the array.

- Have the pack of Hybrislip within reach.
- Find a pair of forceps (with flat ends) - to handle the slides – avoid using fingers as much as possible.

-
- Add to the 18 μL labeled cDNAs:

yeast tRNA (2 $\mu\text{g}/\mu\text{L}$)	2.5 μL
20X SSC	5.1 μL
2% SDS	4.8 μL

Note: a small aliquot from this solution may be used to run the Quality Control of the labeled second strand cDNA.

- Heat denature for 90 sec at 100°C. (We use the thermal cycler for this step).
- Spin at maximum speed for 2 min to get rid of any dust particles.

Note: At this point, 2 μL of hybridization solution may be taken to run Quality Control of second strand cDNA labeling, as describe in Section V-B.

- Pipet up about 28 μL of hybridization solution, carefully avoiding any dust particles at the bottom.
- Apply **hybridization solution** to the center of the array.
- Peel the Hybrislip off the green and clear backings. Do NOT bend the Hybrislip!
- Use the edge of the Hybrislip to break any bubbles in the hybridization solution.
- Gently drop the Hybrislip onto the slide. Get rid of air bubbles as well as you can.
- Place the slide in the hybridization chamber.
- Pipet 3 drops of 20 μL 3X SSC each onto the lower edge of the slide.
- Assemble the hybridization chamber. Make sure to tighten the screws.
- Gently lower the entire apparatus into the water bath (65°C).
- Let hybridize for 16 hrs.

B - Washing Conditions

➤ Prepare beforehand:

- Turn Scanner on to let it warm up while the slides are being washed.
- Set timer to 2 min.

- Wipe off dust from the speed vac for 96-well plates, use ethanol and Kimwipe.
- Prepare a balance with metal slide-rack and blank glass slides.
- Get wash solutions:

• I	• 2X SSC, 0.03% SDS
• II	• 1X SSC
• III	• 0.05X SSC

- Get 3 glass wash-dishes: make sure they are clean!
- Get 2 glass slide-holders with wire handles and put into Dishes I and II. Pour enough of the solutions into appropriate dishes.

Wash slides as follows:

- Retrieve the hybridization chamber from water bath and disassemble quickly. Be sure to wipe off excess water from the chamber before opening it up. **Avoid dust** by minimizing exposure of the slides to the air.
- Carefully place the slides into the slide holder and into the washing solution in Dish I as fast as possible. **Work fast** to avoid indiscriminate hybridization.
- Let the Hybrislips fall away by themselves. Remove the Hybrislips from the solution and discard. Make sure corners of the slips do not scratch the surface of the slides.
- Start the timer for 2 min. Dip the slides up and down in a continuous motion.
- Transfer **just the slides** to Dish II. Wash for 2 min.
- Transfer **slides and slide rack** to Dish III. Wash for 2 min.
- Transfer the slide rack with the slides to the metal spin-rack. Place rack in speed vac.
- Spin (with vacuum, no heat) 5 - 10 min.
- Retrieve slides and put into a light-proof slide box.
- Scan slides as soon as possible. Otherwise, slides may be stored at - 80°C for 1-2 weeks.

Appendix A: Primers and Solutions

Note: All solutions should be prepared with ultra pure water.

LB medium

10 g tryptone
5 g yeast extract
5 g NaCl
1 mL 1 N NaOH
15 g agar

TE pH 8.0

10 mM Tris-HCl (dilute from 1 M Tris-HCl pH 8.0 – Sigma Cat. T 3038)
1 mM EDTA

5M potassium acetate, pH 4.8

To 29.5 mL glacial acetic acid add KOH pellets to pH 4.8 (several). Add ultra pure water to 100 mL final volume. **Do not autoclave.**

TYGPN Medium*

* This media allows for higher bacterial densities where oxygen can be limiting.

Per **100 mL** add:

2 g tryptone
1 g yeast extract
1 mL 80% glycerol
0.5 g Na₂HPO₄
1 g KNO₃

Prepare 100 mL media per plate; dispense into the plate wells. **Autoclave the plates with media in the wells. Store at 4°C.**

GTE Medium

50 mM glucose
25 mM Tris-HCl pH 8.0
10 mM EDTA
Autoclave and store at 4°C

Base-SDS Medium*

*Prepare immediately before use.

0.2N NaOH

1% w/v SDS.

Salt-PEG (polyethylene glycol) medium*

*for optional extra clean-up plasmid DNA

1 M NaCl

25% PEG 8000

PCR Primers:

Three combinations of primers are used for PCR amplification of the EST's in the AFGC DNA Microarray. Primers are diluted to 20 μ M in sterile ultra pure water (not TE).

ZL Primers:	T7ZL1	CGACTCACTATAGGGAAAGCTGG
	SP6ZL1	ATTGAATTTAGGTGACACTATAGAAGAGC

BS Primers:	T7BSK	CGACTCACTATAGGGCGAATTGG
	M13Rbsk	GGAAACAGCTATGACCATGATTACG

M13 Primers:	M13-20	GTAAAACGACGGCCAGT
	M13-R	GGAAACAGCTATGACCATG

Oligo dT-V

This is a 23-mer oligo dT with a combination of any of the other 3 bases at the end. Custom ordered from Sigma.

TE pH 7.5

10 mM Tris-HCl pH 7.5

1 mM EDTA

10 X PCR buffer

300 mM Tricine pH 8.4

500 mM KCl

PCR Mix

PCR Mix is prepared according to the number of reactions to be carried out. Use 96µL per reaction with 4µL template. For PCR product clean-up with Multiscreen plates, we recommend 100 µL total volume reaction with 4.8 µL template. Numbers in the table indicate volumes in microliters.

Reagent	1 X	1 Plate	2 Plates	3 Plates	4 plates	4 plates*
10 X PCR buffer	10	1020	2040	3060	4080	3818.9
25 mM MgCl ₂	8	816	1632	2448	3264	3055.1
dNTPs 5mM each	4	408	816	1224	1632	1527.6
Primer A	2	204	408	612	816	763.8
Primer B	2	204	408	612	816	763.8
Ultra pure water	69.34	7072	14144	21216	28288	26477.6
Taq 5U/µL	0.6	61.2	122.4	183.6	244.8	229.1
Pfu 1U/µL	0.06	6.12	12.24	18.36	24.48	22.9
Total	96	9791.32	19582.64	29373.96	39165.28	36658.7

* volumes adjusted so less volume leftover

Note: dNTPs – diluted from 100 mM stocks - Amersham Cat. 27-2035-01

Purification of Taq DNA Polymerase

Taq DNA Polymerase at a final concentration of 5 U/µL may be prepared as described in: FG Pluthero 1993 Rapid purification of high activity Taq DNA Polymerase Nucleic Acid Research 21: 4850-51.

Purification of Pfu DNA Polymerase

Pfu DNA Polymerase at a final concentration of 1 U/µL was prepared as described in CL Lu and HP Erickson 1997 Expression in Escherichia coli of the thermostable DNA polymerase from Pyrococcus furiosus. Protein Expression and Purification 11: 179-184.

Purification of Genomic DNA from dark-grown Arabidopsis Col 0 leaves

Beginning with 1 g of axenically and dark-grown Arabidopsis Col 0 leaves, we isolated genomic DNA using the Qiagen DNeasy Plant Maxi Kit Cat. 68161

1X TAE buffer

Dilute from 50X TAE (Research Organics Cat. 9651 T)

6X DNA loading buffer

0.25% bromophenol blue
40% (w/v) sucrose in ultra pure water

2X DNA loading buffer

0.08% bromophenol blue
14% (w/v) sucrose in ultra pure water

DEPC-Water

Dissolve 200 μ L diethylpirocarbonate (DEPC) in 100 mL ultra pure water and stir overnight. **Autoclave for 20 min.**

Note: When working with RNA always use DEPC-treated water

Alternative TRIzol Reagent:

Reagents	For 1 L TRIzol reagent	Final Concentration
Phenol in saturated buffer	380 mL	38%
Guanidine thiocyanate	118.16 g	0.8 M
Ammonium thiocyanate	76.12 g	0.4 M
Sodium acetate, pH 5.0	33.4 mL of 3 M stock	0.1 M
Glycerol	50 mL	5%
DEPC-Water	Adjust the final volume to 1 L	

Extraction buffer (For RNA extraction Pine Tree Method)

2% CTAB (hexadecyltrimethylammonium bromide)
2% PVP (polyvinylpyrrolidone K 30)
100 mM Tris-HCl pH 8.0
25 mM EDTA
2.0 M NaCl
0.5 g/L spermidine
Mix and autoclave
2% beta-mercaptoethanol (**add just before use**)

SSTE

1.0 M NaCl
0.5% SDS
10 mM Tris-HCl pH 8.0
1 mM EDTA pH 8.0

Appendix B: Comparison of RNA Isolation Protocols

METHOD	TRIzol	IMPROVED METHOD	SMALL-SCALE	SINGLE-STEP	Rneasy MINI KIT	RNA ISOLATOR
EXTRACTION BUFFER	.8 M GTC .4 M ATC .1 M NaOAC Glycerol Phenol	8M GHC .02 M MES .02 M EDTA .05 M β M-EtOH	PHENOL .1 M LiCl .1 M Tris 8.0 .01 M EDTA 1% SDS	4 M GTC .25 M NaCitrate .5% Sarkosyl .1 M β M-EtOH (Soln D) 2 M NaOAC	N / A	RNA ISOLATOR
PHASE SEPARATION	BCP OR C	P:C:IAA	C:IAA	P:C:IAA	N/A	C
PRECIPITATION	ISO-PROPANOL	EtOH 1 M AA	4 M LiCl	ISO-PROPANOL	EtOH	ISO-PROPANOL
RE-PRECIPITATION			NaOAC EtOH	SOLN D ISOPROPANOL		
WASH	75% EtOH	3 M NaOAC 70% EtOH	70% EtOH	75% EtOH	Wash Buffers	75% EtOH
SOLUBILIZATION	FORMAzol, WATER or 0.5% SDS	WATER	WATER	0.5% SDS	WATER	TE or 0.5% SDS
YIELD	N / A	1.71 μ g/ mg	.2 – .5 μ g/ mg	1.76 μ g/ mg	.35 μ g/ mg	N / A
A260 / A280	1.6 - 1.9	2.0 +/-	N / A	1.85 +/- 0.04	1.9 – 2.1	1.7 - 2.1
TISSUE WEIGHT	N / A		N / A			
TISSUE TESTED	Roots Leaves	Flowerbuds Roots Leaves Stems Siliques	Flowerbuds Roots Leaves Stems Seedlings	Leaves Cell Culture	Leaves Roots Plantlets Floral Stems Flowerbuds Siliques Seedlings	Leaves
PREPARATION TIME	1 hr	4 hrs	Overnight	4 hrs	1 hr	1 hr
REFERENCE	Molecular Res Center, Inc.	Logemann, Anal. Biochem. 163: 16-20	Verwoerd, NAR 17(6): 2362	Chomzynski, Anal. Biochem. 162: 156-159	Qiagen	Genosys
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GTC: Guanidium thiocyanate; **P:** Phenol; **MES:** 4-morpholinethanesulfonic acid; **IAA:** isoamyl alcohol; **ATC:** Ammonium thiocyanate; **BCP:** 1-bromo-3-chloropropane; **C:** Chloroform; **AA:** Ammonium acetate; **NaOAC:** Sodium acetate; **β M-EtOH:** β -mercaptoethanol; **N/A** – Not Applicable