GeneChip Expression Analysis Sample Preparation and Hybridization Procedures

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(Adapted from the GeneChip Expression Analysis Technical Manual, Affymetrix, 2004)

1. Materials

1.1 cDNA Synthesis from total RNA

1. RNaseZAP (Ambion).
2. Heat block (VWR Scientific)
3. PCR Machine (MJ Research)
4. DEPC Treated Water (invitrogen)
5. T7-Oligo(dT) Promoter Primer (Affymetrix)
6. SuperScript Choice Reagents (Invitrogen)
   - 0.1 M DTT
   - 10 mM dNTP mix
   - 5X First Strand Buffer
   - SuperScript II RT (200 U/µl)
   - 5X Second Strand Buffer
   - *E. coli* DNA Ligase
   - *E. coli* DNA Polymerase
   - *E. coli* RNAse H
   - T4 DNA Polymerase
7. 0.5 M EDTA (Ambion)
8. ETOH 96-100% (Fisher)
9. Microfuge (VWR Scientific)
10. cDNA Cleanup Module (Affymetrix)
    - cDNA Spin columns
    - Collection Tubes
    - cDNA Binding Buffer

cDNA Wash Buffer

cDNA Elution Buffer

1.2 Synthesis and Clean-Up of Biotin-Labeled cRNA

1. RNaseZap (Ambion)
2. Microfuge (VWR Scientific)
3. Oven Incubator or Thermocycler (MJ Research)
4. ETOH 96-100% (Fisher)
5. cRNA Cleanup Module (Affymetrix)

    cRNA Spin columns

    Collection Tubes

    cRNA Binding Buffer

    cRNA Wash Buffer

    RNase free water

1.3 Fragmenting the cRNA for Target Preparation

1. RNaseZap (Ambion)
2. Fragmentation Buffer (Affymetrix)
3. Heatblock (VWR Scientific)
4. RNase-free Water (Affymetrix)

1.4 Target Hybridization

1. Acetylated BSA (50 mg/ml) (Invitrogen)
2. Herring Sperm DNA (Promega Corp)
3. GeneChip Eucaryotic Hybridiztion Control Kit (Affymetrix)
4. Control Oligo B2 3 nM (Affymetrix)
5. 5M NaCl (Ambion)
6. MES free acid monohydrate sigmaultra (Sigma)
7. MES sodium salt (Sigma)
8. 0.5 M EDTA (Ambion)
9. Heatblock (VWR Scientific)

10. Hybridization Oven 610 (Affymetrix)
11. Arabidopsis ATH1 GeneChips

1.5 Washing, Staining, and Scanning

1. Acetylated BSA (50 mg/ml) (Invitrogen)
2. R-Phycoerythrin Stepavidin (Molecular Probes)
3. 5 M NaCl (Ambion)
4. PBS, pH 7.2 (invitrogen)
5. 20X SSPE (3 M NaCl, 0.2 M NaH_2PO_4, 0.02 M EDTA) (BioWhittaker Molecular Applications)
6. Goat IgG (Sigma Aldrich)
7. Anti-streptavidin antibody (goat), biotinylated (Vector Laboratories)
8. 10% surfact-Amps20 (Tween20) (Pierce Chemical)
9. Bleach (5.25% Sodium Hypochlorite) (VWR Scientific)

2. Methods

Note: Pipets and countertops are routinely cleaned suing RNaseZAP and only nuclease-free water, buffers, and pipette tips are used.

2.1 cDNA Synthesis from Total RNA

Prior to cDNA synthesis the concentration and purity of RNA is determined spectrophotometrically, with acceptable $A_{260/280}$ ratios between 1.9-2.1. The integrity of the RNA is verified by using the Agilent 2100 Bioanalyzer or performing agarose gel electrophoresis. Intact high quality RNA is indicated by the presence of distinct rRNA bands without any obvious signs of degradation or DNA contamination.

Performing the reactions

A. Primer Hybridization

1. Primer annealing reaction are set up in a 12 µl reaction by combining the following on ice:

Table 1. Primer Hybridization

Reagent	Volume	Final Conc. Or Amt.
Total RNA (5 µg)	X µl	5 µg
T7-Oligo(dT) Primer (50 µM)	2 µl	100 pmol
DEPC water	add to 12 µl total	add to 12 µl total

For primer hybridization the reaction mixture is incubated at 70°C for 10 minutes and put on ice.

2. To the hybridized primer reaction from above the following components are added:

Table 2. Temperature Adjustment

Reagent	Volume	Final Concentration
5X First Strand Buffer	4 µl	1X
0.1 M DTT	2 µl	10 mM
10 mM dNTP mix	1 µl	500 µM ea

The sample is mixed well and incubated at 42°C for 2 minutes.

3. First-Strand cDNA synthesis is initiated by adding SuperScript RT as indicated below and incubate at 42°C for 1 hour.

Table 3. First-Strand cDNA Synthesis

Reagent	Volume	Final Concentration
SuperScript II RT (200 U/µl)	1 µl	200 U

4. First Strand synthesis reactions are placed on ice. To collect any condensation that may have accumulated on the sides of the tube, the sample is briefly centrifuged.

5. Second Strand Synthesis reactions are set up by adding the following reagents to the First Strand Synthesis reaction tube:

Table 4. Second-Strand cDNA Synthesis

Reagent	Volume	Final Concentration
DEPC Treated Water	91 µl	
5X Second Strand Reaction Buffer	30 µl	1X
10 mM dNTP mix	3 µl	200 µM ea
10 U/µl *E coli* DNA Ligase	1 µl	10 U
10 U/µl *E coli* DNA Polymerase I	4 µl	40 U
10 U/µl *E coli* RNase H	1 µl	2 U
Final Volume	150 µl	

The contents of the tube are mixed by gentle tapping. The contents are collect by a brief centrifugation and the sample is incubated at 16°C for 2 hours.

5. 2 µl (10 U) T4 DNA Polymerase is added and the sample is incubated for an additional 5 minutes at 16°C.
6. The reaction is stopped by the addition of 10 µl 0.5 M EDTA.

B. Cleanup of Double-Stranded cDNA

After cDNA synthesis and before the in-vitro transcription reaction, the remaining dNTPs, enzymes, salts and other reaction components are removed from the cDNA synthesis products using the Affymetrix GeneChip Cleanup Module.

Note: The cDNA Wash Buffer is supplied as a concentrate. To obtain a working solution, 24 ml of ETOH (96-100%) is added. All steps of the purification protocol are performed at room temperature without interruption.

Performing the reactions

1. cDNA synthesis reaction products are transferred to a 1.5-2.0 ml microfuge tube and 600 µl cDNA Binding Buffer is added. The sample is mixed by vortexing for 3 seconds.

2. The color of the sample should be yellow. If the color of the mixture is orange or violet, 10 µl 3 M sodium acetate, pH 5.0 is added and the color of the sample should turn yellow.
3. 500 µl of the sample is applied onto the cDNA Cleanup Spin Column sitting in a 2 ml collection tube. The Spin Column is centrifuged for 1 minute at ≥ 8,000 xg or 10,000rpm.
4. The flow-through is discarded and the remainder of the sample is loaded onto the column. The column is centrifuged again for 1 minute at ≥ 8,000 xg or 10,000rpm.
5. The Spin Column is transferred to a clean Collection Tube and 750 µl of the cDNA Wash Buffer is pipeted onto the spin column. The column is centrifuged for 1 minute at ≥ 8,000 xg or 10,000 rpm.
6. The flow-through is discarded. The spin column is centrifuged with the cap open for 5 minutes at max speed to completely dry the column.
7. The spin column is placed into a 1.5 ml Collection Tube and 14 µl of the cDNA Elution Buffer is pipeted directly onto the center of the column membrane. After incubating for 1 minute at room temperature, cDNA synthesis products are collected by centrifugation at max speed for 1 minute.

2.2 Synthesis of Biotin-Labeled cRNA

Note: The reaction is assembled on ice to avoid precipitation of the template DNA by spermidine in the 10X IVT Labeling Buffer.

Performing the Reactions

1. The following IVT reaction components are added to the template cDNA in the order indicated below:

Table 5. IVT Reaction

Reagent	Volume
Template cDNA	12 µl
RNase-free Water	30 µl
10X IVT Labeling Buffer	4 µl
IVT Labeling NTP Mix	12 µl
IVT Labeling Enzyme Mix	4 µl
Final Volume	40 µl

2. The reagents are mixed by pipeting and the contents of the reaction are collected by briefly centrifuging the sample (5 seconds).
3. The IVT reaction is incubated at 37°C for 16 hours in a thermocycler to prevent condensation.
4. The labeled cRNA product is either stored at -20°C or -70°C or immediately purified.

A. Cleanup and Quantification of Biotin Labeled cRNA

Note: All purification steps are performed without interruption at room temperature. The cRNA Wash Buffer is supplied as a concentrate and requires the addition of 20 ml of ETOH (96-100%) to obtain a working solution. The cRNA Binding Buffer occasionally forms a precipitate upon storage. If necessary, it is redissolved by warming in a 37°C water bath, and cooled to room temperature before using.

Performing the Reactions

1. IVT reaction products are transferred to a 1.5 ml microfuge tube and 60 µl of RNase-free Water is added. The sample is mixed vortexing for 3 seconds.
2. 350 µl of IVT cRNA Binding Buffer is added to the sample which is then mixed by vortexing for 3 seconds.
3. 250 µl of ETOH (96-100%) is added to the lysate. The lysate is mixed well by pipeting.

4. The sample (700 µl) is applied to the IVT cRNA Cleanup Spin Column sitting in a 2 ml Collection Tube. Centrifuge for 15 seconds at ≥ 8,000 xg or 10,000rpm.
5. The flow through and the Collection Tube are discarded. The spin column is placed into a new 2 ml Collection Tube and 500 µl of IVT cRNA Wash Buffer is pipeted onto the column. The column is centrifuged for 15 seconds at ≥ 8,000 xg or 10,000rpm.
6. The flow through is discarded and 500 µl of 80% ETOH added to the spin column. The column is centrifuged for 15 seconds at ≥ 8,000 xg or 10,000rpm.
7. After discarding the flow through, the column is centrifuged with the cap open for 5 minutes at maximum speed (≤ 25,000 xg).
8. The flow through and the Collection Tube are discarded, and the spin column is transferred onto a new 1.5 ml Collection Tube. 11 µl of RNase-free Water is pipeted directly onto the spin column membrane. The cRNA is eluted by centrifuging the column for 1 minute at maximum speed (≤ 25,000 xg).
9. The cRNA yield and purity are determined by reading the absorbance of the sample at 260 and 280 nm using a dilution between 1:100 and 1:200 of the eluate. By convention, 1 absorbance unit at 260 nm is equivalent to 40 µg/ml of RNA, and a pure RNA sample will exhibit an A_{260}/A_{280} ratio of 2.0 (ratios between 1.9 and 2.1 are acceptable).
   The RNA yield is adjusted to obtain the cRNA yield, accounting for the carryover of unlabeled total RNA. We estimate a 100% carryover and use the formula below to determine the adjusted cRNA yield:

   Adjusted cRNA yield= RNA_m – (total RNA_i)(y)

   RNA_m= amount of cRNA measured after IVT (µg)
   total RNA_i=starting amount of total RNA (µg)
   y= fraction of cDNA reaction used in IVT

2.3 Fragmenting the cRNA for Target Preparation

The cRNA target is fragmented prior to hybridization onto GeneChip probe arrays to obtain optimal assay sensitivity. The fragmentation reaction is accomplished by metal induced hydrolysis facilitated by magnesium, and is intended to break down full-length cRNA sequences to 35-200 base pieces.

Note: The cRNA used in the fragmentation procedures is sufficiently concentrated to maintain a small volume during the procedure. This is important in order to minimize the amount of magnesium in the final hybridization cocktail.

Performing the Reactions

1. The fragmentation reaction mix is set up as indicated below and is intended to keep the cRNA samples at a final concentration of 0.5 μg/μl:

Table 6. Fragmentation Reaction for 64 Array Format

Reagent	Volume
cRNA	20 μg (1-21 μl)
5X Fragmentation Buffer	6 μl
RNAse-free Water	variable
Final Volume	30 μl

2. Samples are incubated at 94°C for 35 minutes and then placed on ice.
3. An aliquot (1 ug) is saved for analysis using agarose gel electrophoresis or on the Agilent 2100 Bioanalyzer. The standard fragmentation procedure yields a distribution of RNA fragment sizes from ~35-200 bases.
4. Fragmented sample RNA are stored a -20°C until the hybridization is to be performed.

2.4 Target Hybridization

A. Reagent Preparation

12X MES Stock (1.22 M MES, 0.89 M [Na^{+}])

For 1,000 ml:

70.4 g MES-free acid monohydrate

193.3 g MES sodium salt

800 ml Molecular Biology Grade Water

Mix and bring to 1,000 ml

pH should be between 6.5-6.7

Filter sterilize using a 0.2 μm filter

Store at 2-8˚C protected from the light

2X Hybridization Buffer

For 50 ml:

8.3 ml 12X MES Stock

17.7 ml 5 M NaCl

4.0 ml 0.5 M EDTA

0.1 ml 10% Tween 20

19.9 ml water

Store at 2-8˚C protected from the light

B. Target Hybridization

Note: Frozen stocks of 20X GeneChip Eukaryotic Hybridization Control Mix is heated to 65˚C for 5 minutes to resuspend the cRNA.

1. Hybridization Cocktail is prepared for each target by assembling the following:

Component	
Fragmented cRNA	15 µg
Control Oligo B2 (3 nM)	5 µl
20X Eukaryotic Hybridization Controls	15 µl
Herring Sperm DNA (10 mg/ml)	3 µl
Acetylated BSA (50 mg/ml)	3 µl
2X Hybridization Buffer	150 µl
Water	µl
Final Volume	300 µl

2. The probe array is equilibrated to room temperature.
3. The Hybridization Cocktail is heated to 99˚C for 5 minutes in a heatblock or thermocycler.
4. The array is primed by filling it with 200 µl of 1X Hybridization Buffer. When filling the probe array cartridge one pipette tip is used to fill the chamber and a second pipette tip is inserted as a vent to allow air to pass from the chamber as it is being filled.
5. The probe array filled with the 1X Hybridization Buffer is incubated at 45˚C for 10 minutes with rotation.
6. The Hybridization Cocktail that has been heated to 99˚C in step 3 is placed at 45˚C for 5 minutes.
7. The Hybridization Cocktail is centrifuged at maximum speed for 5 minutes to remove any insoluble material.
8. The Hybridization Buffer is removed from the probe array cartridge and it is filled with 200 µl of cleared Hybridization Cocktail (care is taken to avoid insoluble material at the bottom of the tube).
9. Probes are placed in the Hybridization Oven at 45˚C and hybridized for 16 hours at 60 rpm.

2.5 Washing, Staining, and Scanning

A. Reagent Preparation

Wash A: Non-Stringent Wash Buffer (6X SSPE, 0.01% Tween 20)

For 1,000 ml

300 ml 20X SSPE

1.0 ml of 10% Tween-20

669 ml water

Filter sterilize using a 0.2 μm filter

Wash B: Stringent Wash Buffer (100 mM MES, 0.1 M [Na^+], 0.01% Tween 20

For 1,000 ml

83.3 ml 12X MES Stock Buffer

5.2 ml 5M NaCl

1.0 ml 10% Tween 20

910.5 ml water

Filter sterilize using a 0.02 μm filter

Store at 2-8˚C protected from the light

2X Stain Buffer (200 mM MES, 2M [Na^+], 0.1% Tween 20)

For 250 ml :

41.7 ml 12X MES Stock Buffer

92.5 ml 5 M NaCl

2.5 ml 10% Tween 20

113.3 ml water

Filter sterilize using a 0.02 μm filter

Store at 2-8˚C protected from the light

10 mg/ml Goat IgG Stock

50 mg is resuspended in 5 ml 150 mM NaCl

Store at 4˚ C

B. Washing and Staining the Probe Array

1. After the 16 hour hybridization, the Hybridization Cocktail is removed from the probe array and saved in a microfuge tube and stored at -80˚C
2. The probe array is filled with 200 μl of Wash Buffer A: Non-Stringent Wash Buffer.

3. The Streptavidin Phycoerythrin (SAPE) Stain Solution is prepared by mixing the following in an amber colored tube:

Component	**Volume**	**Final Conc.**
2X MES Stain Buffer	300.0 µl	1X
50 mg/ml acetylated BSA	24.0 µl	2 mg/ml
1 mg/ml Streptavidin Phycoerythrin (SAPE)	6.0 µl	10 mg/ml
water	270 µl	-
Total	600 µl	

4. The Affymetrix Fluidics Station is set up to perform the following:

Post Hybridization Wash 1	10 cycles of 2 mixes/cycle Wash Buffer A at 25˚C
Post Hybridization Wash 2	4 cycles of 15 mixes/cycle Wash Buffer B at 50˚C
Stain	Stain probe array for 30 minutes SAPE Solution at 24˚C
Final Wash	10 cycles of 4 mixes/cycle Wash Buffer A at 25˚C

5. Excess fluid is cleaned from around the septa of the array cartridge and tough spots are placed over the two septa.
6. Arrays are scanned with a GeneArray Scanner at a pixel value of 3 µm at 570 nm.

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