Date:

AFFYMETRIX GENECHIP HYBRIDIZATION ANALYSIS

(Updated: April 19, 2007)
Experimental Organs:

***Note:** This protocol is slightly modified from the general protocol for the biotin-labeled cDNA generated with the NuGen Ovation kit.*

A. SETTING UP INSTRUMENTS FOR HYBRIDIZATION

(Must be done at least 30 minutes before hybridization. Normally, I used instruments in the Microarray Facility or Stan Nelson lab - 5th Floor in Gonda building)

1. Turn on a **heat block** and set it to **99ºC**. or
2. Check to make sure that a **thermocycler PE480** is available.
3. Turn on the **Hybridization oven** and make sure that the temperature setting is **45ºC**.

B. HYBRIDIZATION

Preparations

1. Equilibrate **Affymetrix probe** arrays to room temperature before use by removing them from a refrigerator. *Note: Do NOT open the bags containing the arrays.*
2. Thaw out tubes of **Acetylated BSA**, **Herring sperm DNA**, **Affymetrix B2 Oligo** and **20X Hybridization stocks**, and **sheared cDNA solutions** that are stored in the **-20ºC RNA Freezer**.
3. Vortex to mix the contents.
4. Heat the **20x hybridization stock** to **65ºC** for **5 minutes** to resuspend cDNAs completely.
5. Spin tubes for 15 seconds to bring down liquid on the lids and side of tubes.
6. Set tubes on ice.

7. Prepare the **hybridization (targets) cocktails** in either **1.5-mL microfuge** tubes (if using a **heat block** to denature sheared cDNA) or **0.5-mL microfuge** tubes (if using a **thermocycler PE480**).
8. Determine the volume of sheared cDNA solutions to be added to microfuge tubes based on the concentration of cDNA solutions.

	Sample #1	**Sample #2**	**Sample #3**	**Sample #4**
Concentration (µg/µL)				
Volume of 5 µg fragmented cDNA (µL)				
Volume of RNase-free water (µL) (= 69 µL - vol. of sheared cDNA)				

9. Label on the lids and sides of microfuge tubes with either **sample name** or **number 1, 2, 3, etc**. Put labeled tubes on a rack at room temperature.
10. Pipet the appropriate volume of RNase-free water into appropriate tubes (see Table in step 7).
11. Prepare a **master mix** for the hybridization cocktail in an RNase-free **1.5-mL microfuge tube** sitting **on ice** (see below).

	Midi Array	**Master Mix**
5 µg of fragmented cDNA	X µL	----------
RNase-free water to the final volume	Y µL	----------
Control oligonucleotide B2 (3 nM)	3.7 µL	µL
20X control cDNA cocktail (Affy)	11.0 µL	µL
Herring sperm DNA (Promega, 10 mg/mL)	2.2 µL	µL
Acetylated BSA (Invitrogen, 50 mg/mL)	2.2 µL	µL
100% DMSO	22.0 µL	
2X MES Hybridization buffer	110.0 µL	µL
Total volume	**220.0 µL**	----------

a. Prepare a **master mix** for **4.5x** (4 samples + 0.5 extra) in an RNase-free microfuge tube. Vortex briefly to mix the contents. Spin the tube briefly at full speed. Put the tube back on ice.
b. Pipet **151 µL** of **master mix** to each tube of samples in step 7.
c. Pipet the volume of fragmented (or sheared) cDNA solutions (see Table in step 7) to appropriate tubes. Mix well by vortexing briefly. Spin briefly. Put tubes on a microfuge-tube rack at room temperature.

12. Put in a **small white carton box** the following things:
    - a **P-200 pipetman** wrapped in a piece of aluminum foil
    - the **microfuge-tube rack** containing **tubes of hybridization cocktail solutions**
    - a glass beaker with a 50-mL tube of **1X MES hybridization buffer**
    - a box of **PCR tips** for P-200 pipetman
    - 1 or 2 pairs of **gloves** wrapped in a piece of aluminum foil
    - equilibrated microarrays
    - a roll of scotch tape for sealing the septa of the microarrays during hybridization
    - a timer
    - this note on hybridization.
13. Bring the box in step 11 to the **Microarray Facility** on **5th floor** in the **Gonda building**.

Clean up targets and set up hybridization

*Note: **If** you **hybridize more than eight arrays**, it is recommended that you **pre-hybridize** the arrays **starting with step 2 before heating up the samples**. So, the arrays are prehybridized for >10 minutes.*

1. Denature cDNA targets in the hybridization cocktails by incubating the tubes either in
   (a) the **99ºC heat block** for **2 minutes** or
   (b) (Mostly preferable) the Perkin/Elmer **P480 thermocycler** with **file #10** (**99ºC, 5 min./ 45ºC, 5 min.**).

<u>Attention:</u> Wait until ~2 min. 15 sec. <u>remaining</u> at 99°C and put the tubes in for incubation.

2. At the meantime, get a rack for arrays from a **second** drawer below the fluidisc station #1.
3. Open the bags of arrays and **save one bag** with its sticker for pasting the sticker on this note in step 9 below.
4. Label each array with **sample name** or **number** according to the **number on the microfuge tubes of hybridization cocktails**. Place the microarrays in the rack.
5. Equilibrate the array with **200 µL** of **1X MES Hybridization buffer** using a P-200 pipetman by
   (a) inserting a PCR pipet tip into an **upper right septum** on the back of the array. (This tip vents the air as the **1X MES Hybridization buffer** is injected in) and
   (b) injecting **200 µL** of **1X MES Hybridization buffer**.
6. Carefully, remove both pipet tips and gently **invert the array two times** to coat the entire array glass surface.
7. Put the equilibrated array on the rack for arrays.
8. Repeat **steps 5-7** for other arrays.
9. Incubate the arrays in the hybridization oven at **45°C** with **60 rpm** rotation for ~**10 minutes**. <u>Note:</u> *it is okay to incubate more than 10 minutes.*
   - (Paste the Sticker on the Array bag below)

   <u>Lot #:</u>
   <u>Expiration Date:</u>

10. If using the **heat block**, after 2 min at 99°C, transfer the tubes of cocktail to either a **45˚C heat block** or put them on a microfuge-tube rack and then incubate the tubes in the **45˚C hybridization oven** for **5 minutes**.
11. Spin tubes of hybridization cocktails at **13,000 rpm** for **5 minutes** at **room temperature**.
12. Replace the **1X MES hybridization buffer** with **200 µL** of **hybridization cocktail**.
    <u>Note:</u> *AVOID PIPETTING THE DEBRIS PELLET!*

13. Put **a piece of Scotch tape** (~1.5-2 in) to **cover both septa** on the back of each array. Apply some pressure over the septum area.
14. Place the arrays in the **45˚C Rotisserie oven**.
15. Incubate the arrays at **45˚C** for **18 hours**.

    Starting time:

    Ending time:

Date:

WASHING, STAINING, SCANNING PROBE ARRAYS AND COPYING MICROARRAY DATA

Staining Method (circle one):

(1) Simple staining

(2) Antibody amplification staining (Goldberg lab uses this method)

PREPARATION OF BUFFERS AND SOLUTIONS IN THE LAB BEFORE GOING TO THE MICROARRAY FACILITY IN GONDA BUILDING (~30 minutes)

1. Warm a bottle of **Wash buffer B** wrapped in aluminum foil (stored in the refrigerator in room 2826) in a **55ºC water bath** for at least 15 minutes.
2. Thaw a tube of **50 mg/mL Acetylated BSA** (stored in the -20ºC RNA freezer in room 2918 or in a box containing SAPE, normal IgB, and anti-streptavidin biotinylated Ab in the refrigerator in room 2826) at room temperature.
3. Bring out **2x Staining buffer** in a 50-mL tube wrapped with aluminum foil from the refrigerator in room 2826 to the bench.
4. Remove one tube of **1 mg/mL SAPE**, **10 mg/mL normal goat IgG**, and **0.5 mg/mL Anti-streptavidin biotinylated Ab** from a Revco box labeled as **"Light Sensitive GeneChip Stainin 4ºC"** in the refrigerator in room 2826 and put the tubes on ice.
5. Spin tubes briefly to bring down water condensation on the lids of the tubes.

6. Prepare a master mix of **Streptavidin Phycoerythrin (SAPE) staining solution** for either **1 set** for **SIMPLE staining** or **2 sets** for **ANTIBODY AMPLIFICATION staining** in a 50-mL Falcon tube wrapped with aluminum foil at room temperature. *Note: SAPE is light sensitive; therefore, we want to avoid photobleaching of the dye.* **Master mix** for number of samples = **2x [number of microarrays] + 1 extra array.** *For example, I hybridized 5 microarrays, then the number of samples for the master mix is **11** [or 2x (5) + 1].*

	Per Sample	**Master mix (x)**
2X Staining buffer	**300 µL**	
RNase-free (DEPC'd) water	**270 µL**	
50 mg/mL Acetylated BSA (Invitrogen)	**24 µL**	
1 mg/mL SAPE (Molecular Probes)	**6 µL**	
Total Volume	**600 µL**	

Vortex briefly to mix the contents. *Note: Do NOT vortex the tube vigorously.*

7. Label TWO sets of **1.5-mL microfuge tubes wrapped with aluminum foil** or **amber tubes** as **"1"** and **"3"**. Put tubes on a microfuge tube rack. *The number of tubes equals **2x** the number of hybridized microarrays.*
8. Aliquot **600 µL** of **SAFE master mix** into each tube.
9. Prepare a master mix for ONE set of **anti-streptavidin biotinylated antibody solution** in a 14-mL disposable centrifuge tube at room temperature.

	Per Sample	**Master Mix (x)**
2X Staining buffer	**300.0 µL**	µL
RNase-free (DEPC'd) water	**266.4 µL**	µL
50 mg/mL Acetylated BSA (Invitrogen)	**24.0 µL**	µL
10 mg/mL normal goat IgG (Sigma)	**6.0 µL**	µL
0.5 mg/mL Anti-streptavidin biotinylated Ab	**3.6 µL**	µL
Total Volume	**600.0 µL**	µL

Vortex briefly and gently to mix the contents.

10. Label a set of 1.5-mL microfuge tubes as "**2**". Put tubes on a microfuge tube rack. *The number of tubes equals the number of hybridized microarrays.*
11. Aliquot **600.0 µL** of the master mix into each of the tubes "**2**".
12. Put in a **brown carton box** the following things:
    - a bottle of 500 mL of **Wash buffer B** (~150 mL for washing 8 microarrays)
    - a bottle of 500 mL of **Wash buffer A** (~400 mL for washing 8 microarrays)
    - a bottle of 500 mL of **DEPC'd water** (~450 mL for washing 8 microarrays)
    - a glass beaker with a tube of ~5-10 mL **Wash buffer A**
    - a rack full of the **original 0.5-mL hybridization tubes** for saving the hybridization cocktails
    - a Revco box with **aliquots of staining solutions**
    - a box of **PCR pipet tips for the P-200 pipetman**
    - the **P-200 pipetman** wrapped in a piece of aluminum foil
    - **2 pairs of gloves** wrapped in a piece of aluminum foil
    - a piece of several or many **microtube tough-spots for sealing the lower septa** of arrays after washing and staining and before scanning in a scanner.
    - a blank **CD** (for data without analysis (CHP) of 8 Arabidopsis arrays, ~550 MB) or a blank **DVD** (for data without analysis (CHP) of at least 8 soybean arrays, >800 MB). ***Note: To burn the file onto the Apple DVD, you need to change the setting to burn ONE session in the OPTIONS selection of the SONIC program.***
13. Bring the box to the Microarray Facility in the Gonda building.
14. After **18 hours of incubation**, remove the microarrays from the Rotisserie oven.
15. Pipet off and **save the hybridization cocktails** in 1.5 mL labeled microfuge tubes. **Store** the **cocktail solutions** in the **–20˚C RNA freezer** (room 2911) or a **–70˚C freezer** (room 2911).
16. Fill each array with ~**300 µL** of **Wash buffer A**.

    Note: If the Fluidisc station is not available, you can store the arrays at room temperature (or at 4˚C for up to 18 hours before proceeding with washing and staining. The probe arrays need to be equilibrated to room temperature before washing).

17. **Prime** the Fluidisc station via Affymetrix Microarray Suite program on the computer by placing correct tubings from the DEPC'd water bottle into the bottles of Wash buffer A and Wash buffer B. *It would take about 10-15 minutes. Caution: Make sure that **correct tubings** go into **correct buffer bottles** by checking the area (on the right side of the station) where the tubings coming out into the DEPC'd water bottle.*
18. Enter in the **Experiments** field **Name** of **each sample** and **other information**.
19. Scan the barcode on the array using a scanner.
20. Select the correct **Probe Array Type**, e.g. Arabidopsis.
21. Save the entry.
22. Repeat for other arrays.
23. Choose either **fluidisc station 1** or **2 (each has four modules)**, then select each **module** and the protocol ***EukGE-WS2V4_450*** to run. Then, select "Run".
24. Follow the instruction on the LCD of the fluidisc module.

Station	Module	Experimental Name	Probe Array Type	Protocol
1	1		ATH1/Soybean	EukGE-WS2V**4**_450
	2		ATH1/Soybean	EukGE-WS2V**4**_450
	3		ATH1/Soybean	EukGE-WS2V**4**_450
	4		ATH1/Soybean	EukGE-WS2V**4**_450
2	1		ATH1/Soybean	EukGE-WS2V**4**_450
	2		ATH1/Soybean	EukGE-WS2V**4**_450
	3		ATH1/Soybean	EukGE-WS2V**4**_450
	4		ATH1/Soybean	EukGE-WS2V**4**_450

*<u>Comment:</u> It will take about **1 hr 22 min** to process each array with **Antibody amplification staining** protocol for each perfect run, i.e. no bubble errors or leaking of tubing.*

25. During either the **3rd staining step** or **washing after 3rd staining**, **turn on** the **Scanner model 7G** by pressing a **silver button** at the center and near the base of the scanner. *It would take about 10-15 minutes for the scanner to warm up.*
26. After each array is filled with buffer A, the message on the LCD of each module tell you to Reject the Genechip. Remove the array and follow the instruction on the LCD.
27. Inspect the glass area of the array to make sure that there is **no bubble**. ***What would you do if there is a bubble or airspace observed?*** *You remove a small volume (~50-100 uL) of the wash buffer A in the array and then inject ~100-200 uL wash buffer A into the array the same way as you injecting the hybridization cocktail. You gently pipet the buffer back and forth to help pushing the bubble out of the array chamber.*
28. **Before scanning the arrays**, fold a piece of kimwipes and moisten it with a little bit of distilled water. Then, wipe the glass of the array to remove any dirts or smudge. Dry the glass surface with another piece of dry kimwipes.
29. Start scanning the arrays by opening the hatch of the scanner and placing the first array into the slot with **Red mark** (at a 9 o'clock position) of the carousel holding 48 arrays. (**Orientation of the arrays in the carousel** - The **glass of the array** faces **away from you**. Actually, there is **only one direction to load the array on the carousel**). Load the **next arrays** into **Clockwise direction relative to the first array**.
30. Close the hatch of the scanner.
31. Press the **laser start symbol** in the task bar. *The scanning starts. <u>Note:</u> it takes about 10 minutes to scan one array.*

BACKING UP MICROARRAY DATA TO STAN NELSON'S MICROARRAY SERVER AND COPYING DATA FILE ON A CD OR DVD

1. Microarray data **cannot** be opened if the **.CEL** and **.DAT files** are **not converted** into a **CAB file** because these data were generated from a very high density microarray scan with a scanner (model 7G). Therefore, the .CEL and .DAT data in drive D (D:\Program Files\Affymetrix\Genechip\Affy Data\Data\) must be converted into a single CAB file using **Data Transfer Tools (DTT)**. **This CAB file is saved in the drive C as (C:\Backup\ _ADTT.CAB)**. Use DTT to do **Transfer Out the .CAB file**.
2. **Copy** the files generated from microarrays on either:

   a. **CD** (~400-550 MB, depending on type of Arrays and number of probe sets on array) for data analysis generated from 4 microarrays of soybean and human and from 8 arrays of Arabidopsis **or**

   b. **DVD** (>700-800 MB, depending on type of Arrays and number of probe sets on array) for data analysis generated from 5 or more microarrays. ***Note:*** ***To burn the file onto the Apple DVD, you need to change the setting to burn ONE session in the OPTIONS selection of the SONIC program. Otherwise, the program will burn the file into a multiple session format, and you cannot open the file with our PC computer.***