

## GeneChip Protocol

### Preparation of Labeled Targets for Affymetrix GeneChip Analysis

**NOTE:**

Keep in mind: use “**RNA-only**” pipet tips and stock solutions.

When isolating RNA from cells or tissue, resuspend in **nuclease-free H<sub>2</sub>O; DEPC H<sub>2</sub>O is OK at this stage.** For nuclease-free water, use sterile Milli-Q H<sub>2</sub>O. Then:

- Purify RNA using Qiagen RNeasy Mini Clean-up kit (samples should be dissolved in RNase free water, elute in nuclease-free water, **not DEPC**)
- Determine [RNA]: should be at least 2 µg/ul (If instructions in clean-up kit are followed, the [RNA] will be < 2 µg/ul. Elute final step in 30 ul nuclease-free water, and reuse those 30 ul to elute column a second time).
- Run 5ug on a formaldehyde gel to exclude RNA degradation

#### **I. cDNA SYNTHESIS (Affymetrix Chapter 2 Protocol) – 4 HOURS**

- Pre-adjust all heat blocks/baths before adding reagents
- Thaw reagents on ice (Note: label tube with dot each time thawed; try not to use tube that has been thawed >5x or past expiration date for enzymes, make sure there is enough of each reagent prior to starting experiment).
- Reagents from **Affymetrix, Part #900493**, “GeneChip One Cycle Target Labeling and Control Reagents”
  - There is a new polyA Control Spike (obtained from gene chip facility), need to make serial dilutions:
    - Dilution 1: 1 ul polyA spike + 19 ul polyA spike buffer
    - Dilution 2: 1 ul dilution 1 + 49 ul polyA spike buffer
    - Dilution 3: 1 ul dilution 2 + 4 ul polyA spike buffer
  - OK to store and reuse Dilution 1, -20°C, Dilutions 2 and 3 should be discarded after each use.
- Add the following reagents for first strand synthesis:

<b>REAGENTS</b>	<b>Sample</b>
<b>Add following Reagents</b>	
Nuclease Free Water	
10-15 µg RNA	7ul (water +RNA)
1X PolyA Control Spikes Dilution 3	2 ul
Genset T7T <sub>24</sub> Primer	2 ul
<b>Quick spin</b> <b>Incubate 70° 10 min.</b> <b>Add Following Reagents</b>	
5x First strand buffer	4 ul

0.1 M DTT	2 ul
10 mM dNTP mix	1 ul
<b>Flick tube, Quick spin Incubate 42° 2 min.</b>	
Superscript II RT	2 ul
<b>Add Reagent; Flick tube, Quick spin, Incubate 42° 1 hour</b>	<b>20 ul</b>

- Quick spin reactions and place on ice.
- Add the following reagents for second strand synthesis: (keep reagents on ice)

<u>Added</u>	<u>Component</u>	<u>Volume</u>
<input type="checkbox"/>	<b>Nuclease free water</b>	<b>91 ul</b>
<input type="checkbox"/>	<b>5x Second Strand Buffer</b>	<b>30 ul</b>
<input type="checkbox"/>	<b>10 mM dNTPs</b>	<b>3 ul</b>
<input type="checkbox"/>	<b>10 U/ul DNA Ligase</b>	<b>1 ul</b>
<input type="checkbox"/>	<b>10 U/ul <u>E. COLI</u> DNA Polymerase I</b>	<b>4 ul</b>
<input type="checkbox"/>	<b>2 U/ul RNase H</b>	<b>1 ul</b>
	<b>TOTAL VOLUME</b>	<b>150 ul</b>

- Flick tube to mix; Quick spin; Incubate at 16°C for 2 hours
- Add 2 µl [10U] **T4** DNA Polymerase
- Incubate for 5 minutes at 16°C
- Add 10 µl 0.5 M EDTA (pH 8, “clean” RNA stock)
- Proceed to clean-up or store at –20°C

## **II. cDNA CLEAN UP (Affymetrix Chapter 2 Protocol) – 1 HOUR**

- Add 162 ul of Phenol:Chloroform:Isoamyl alcohol (50:48:2) to cDNA reaction (make 1 ml fresh from “clean” RNA stocks) and vortex well.
- Spin in microfuge at full speed for 5 min.
- Transfer top (aqueous) phase to new 1.5 ml tube.
- Add 0.5 ul of glycogen (5 ug/ul: “home-made”); Flick tube to mix.
- Add 0.5 volumes (162 ul) of nuclease-free 7.5 M NH<sub>4</sub>OAC (stored at 4°C common shelf 824); Flick tube to mix.

(Note: prepare 7.5 M NH<sub>4</sub>OAC in nuclease-free H<sub>2</sub>O ahead of time and filter into 15 mL conical tube, or buy commercially available solution, then aliquot into 1mL epi-tubes)

- Add 2.5 volumes (810 ul) of cold (-20°C) 100% ethanol (“clean” RNA-only stock); mix by inverting tube
- Spin **immediately** in microfuge at full speed for 30 min at **room temperature**.
- Pour off supernatant; Wash pellet with 0.5 ml cold (-20°C) 80% ethanol (“clean” RNA-only stock) (squirt in, do not pipet up and down).
- Spin in microfuge at full speed for 5 min at **room temperature**.
- Pour off supernatant; Wash pellet again with 0.5 ml cold (-20°C) 80% ethanol (“clean” RNA-only stock) (squirt in, do not pipet up and down).
- Spin in microfuge at full speed for 5 min at room temperature.
- Pour off supernatant **completely**. Air dry pellet **completely** (on ice, keep lids open, cover with Kimwipe).
- Resuspend the dry pellet in 10 µl nuclease-free water. Store at -20°C until IVT reaction.

**III. IN VITRO TRANSCRIPTION (Enzo Bioarray High Yield Protocol, Catalog #42655-10) – This takes approximately 5 HOURS.**

- Add components to template DNA at room temperature to prevent the DTT from precipitating.

<u>Added</u>	<u>Component</u>	<u>Volume</u>
<input type="checkbox"/>	<b>Template DNA (room temp)</b>	<b>10 µl</b>
<input type="checkbox"/>	<b>Nuclease-free water (room temp)</b>	<b>12 µl</b>
<input type="checkbox"/>	<b>10X HY Reaction Buffer (on ice)</b>	<b>4 µl</b>
<input type="checkbox"/>	<b>10X Biotin Labeled Ribonucleotides (on ice)</b>	<b>4 µl</b>
<input type="checkbox"/>	<b>10X DTT (room temp)</b>	<b>4 µl</b>
<input type="checkbox"/>	<b>10X RNase Inhibitor Mix (on ice)</b>	<b>4 µl</b>
<input type="checkbox"/>	<b>20X T7 RNA Polymerase (on ice)</b>	<b>2 µl</b>
	<b>TOTAL VOLUME</b>	<b>40 µl</b>

**Note: Label tubes with date each time new tube opened; Label # of reactions used up or left**

- Flick tube to mix the components; Quick spin
- Incubate 5 hours at 37°C; Mix + Quick spin tube every 30 minutes  
START TIME: \_\_\_\_\_ END TIME: \_\_\_\_\_
- Store at -20°C if not purifying RNA immediately

#### **IV. PURIFICATION OF IN VITRO TRANSCRIPTION**

Qiagen RNeasy Mini Clean-Up Protocol, Catalog # 74-104 – 3 HOURS

- Add 22  $\mu$ l of  $\beta$ -Mercaptoethanol to 2.2 ml of Buffer RLT; This is sufficient for 6 purifications.
- Add 60  $\mu$ l nuclease-free water to 40  $\mu$ l cRNA reaction; Add 350  $\mu$ l Buffer RLT; Mix thoroughly
- Add 250  $\mu$ l 100% ethanol (room temperature, “clean” RNA-only stock); Mix well by pipetting and use same tip to apply sample (700  $\mu$ l) to RNeasy mini spin column.
- Centrifuge 15 sec at full speed (room temp); Reapply sample (700  $\mu$ l) to spin column; Recentrifuge
- Transfer RNeasy column into a new 2 ml collection tube; Add 500  $\mu$ l Buffer RPE; Centrifuge 15 sec at full speed (room temp); Dump out eluate
- Pipet 500  $\mu$ l Buffer RPE onto the RNeasy column; Centrifuge 2 min at full speed (room temp). Check that there are no ethanol droplets on bottom of column.
- Transfer column to new 1.5 ml collection tube (autoclaved); Pipet 30  $\mu$ l ice cold nuclease-free water onto the membrane; Let stand for 1 min
- Centrifuge 1 min at full speed (room temp); Pipette an additional 30  $\mu$ l RNase-free water onto the membrane; Let stand for 1 min
- Centrifuge 1 min at full speed (room temp).  
Final volume of purified cRNA is 60  $\mu$ l.
- Add 30  $\mu$ l RNase-free 7.5M  $\text{NH}_4\text{Ac}$  and 150  $\mu$ l 100% ethanol (stored at  $-20^\circ\text{C}$ , “clean” RNA-only stock); Vortex; Put on ice then transfer to  $-20^\circ$  freezer.
- Incubate at  $-20^\circ$  for 1 hour; Centrifuge 30 min at full speed (in cold room).
- Remove supernatant with pipet; Wash pellet with 0.5 ml 80% ethanol (“clean” RNA only stock, stored  $-20^\circ\text{C}$ ); Centrifuge 5 min at full speed (in cold room)
- Repeat 80% ethanol wash and centrifuge
- Draw off 80% ethanol carefully and let pellet air dry, on ice, for ~30 minutes
- Resuspend the dry pellet in 20  $\mu$ l nuclease-free water (ice cold); Let solubilize for ~1hr on ice; Read O.D. 1  $\mu$ l (in duplicate or triplicate) to check for concentration and purity. Can remain frozen if you are not proceeding immediately to fragmentation.

#	Sample Name	OD260	260/280 Ratio	Concentration (ug/ul)	Yield (ug)
1					
2					

#### **V. FRAGMENTATION OF cRNA (Affymetrix Chapter 2 Protocol and Agilent RNA LabChip) – 2 HOURS**

- Set heat block to 94°
- Add the following components:

<b>Component:</b>	<b>Sample</b>
<b>20 ug cRNA</b>	
<b>Nuclease-free water</b>	<b>32ul (H2O+cRNA)</b>
<b>5X Fragmentation Buffer</b>	<b>8 ul</b>
<b>TOTAL VOLUME</b>	<b>40 ul</b>

- Incubate at 94° for 35 min (secure lid) ; Place on ice; Store fragmented cRNA at – 20° until GeneChip hybridization
- Fill out Analysis Request Form. Get form from GeneChip Facility website: [www.pathology.wustl.edu/~mgacore/](http://www.pathology.wustl.edu/~mgacore/) : Click on “Gene Chip Facility”, then “Submitting Samples”, then “Analysis Request Form.” **Optional: Select “Test chip” to assess quality of cRNA.**
- Drop off samples (transfer on ice or in cooler) to GeneChip Lab: (it’s a good idea to call before you go to drop off samples, just to make sure someone is there)

**Siteman Cancer Center**  
**GeneChip Facility**  
**Room 2417**  
**Barnes-Jewish Hospital North**  
**Kingshighway Bldg.**  
**216 S. Kingshighway**  
**St. Louis, MO. 63110**  
**(314) 454-8520**  
**(314) 454-5525 - FAX**  
[genechip@labmed.wustl.edu](mailto:genechip@labmed.wustl.edu)

- You will be notified by email when data is ready. Follow instructions in email to download data.
- Data analysis:
  - For starters, you will need to unzip the file that you downloaded off of the GeneChip facility server. You can use WinZip Wizard to do this.
  - Open the unzipped file with Microsoft Excel, and save the file as an Excel Workbook.
  - Data will be grouped in columns, with a header at the top of each column labeling it as “Sample #, Sample Detection, Sample Present/Absent/Missing, Unigene ID, Ascension numbers”, etc.
  - In most cases, you will want to normalize your experimental sample detection values to your control sample detection values. I.E. Divide experimental sample detection values by control sample detection values. This will give you the fold change (i.e. fold induction value) for each gene target on the chip.
  - Then you can sort the data (Click on “Data” Menu, then “Sort”) according to whatever method you choose, i.e. by detection, by fold changes, in ascending/descending order etc.

- There are many computer programs available for GeneChip analysis, but see GeneChip website ([www.pathology.wustl.edu/~mgacore/](http://www.pathology.wustl.edu/~mgacore/)) and click on “Analyzing Data” for full list of software, plus description of software program. Some examples:
  - Function Express
  - Cluster and TreeView
  - GeneCluster