



Protocol

GeneChip® Eukaryotic Double Strand Whole Transcript Protocol

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GeneChip® Eukaryotic Double Strand Whole Transcript Protocol

The GeneChip® Eukaryotic Double Strand Whole Transcript Protocol is to be used in preparation of biotin-labeled ds cDNA whole transcript target for hybridization to Affymetrix Human and Mouse Tiling arrays. This protocol replaces the former GeneChip® Whole Transcript (WT) Amplified Double-Stranded Target Assay. For questions, please contact Affymetrix Technical Support at 1-888-362-2447 (1-888-DNA-CHIP).

Safety Information

Warning: For research use only. Not recommended or intended for diagnosis of disease in humans or animals. Do not use internally or externally in humans or animals.

Caution: All chemicals should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing, such as lab coat, safety glasses and gloves. Care should be taken to avoid contact with skin and eyes. In case of contact with skin or eyes, wash immediately with water. See MSDS (Material Safety Data Sheet) for specific advice.

Materials Required

Reagents Required

Table 1.1 Reagents Required

Material	Source	P/N
cRNA Synthesis		
Ambion WT Expression Kit	Ambion	4411973 (10Rxns) 4411974 (30 Rxns)
cDNA Synthesis		
Random Primers, 3 µg/µL, 300 µg	Invitrogen	48190-011
dNTP's (dATP, dCTP, dGTP, dTTP), 100 mM	Promega	U1240
dUTP, 100 mM	Promega	U1191
Nuclease-free Water	USB	71786
RNase Inhibitor (Recombinant)	USB	10777-019
(MgCl ₂), 1M	USB	78641
SuperScript® II, 200 U/µL, 40,000 U (5X First Strand buffer and 0.1 M DTT included)	Invitrogen	18064-071
Exonuclease-Free Klenow, 10 U/µL	USB	70057
RNase H, 5 U/µL	USB	70054
Poly-A RNA Control Kit		
GeneChip® Eukaryotic Poly-A RNA Control Kit	Affymetrix	900433

Table 1.1 Reagents Required (Continued)

Material	Source	P/N
Fragmentation and Labeling		
Uracil-DNA Glycosylase (UDG) (2 U/μL)	USB	71960
Human Apurinic/Apyrimidinic Endonuclease 1 (APE 1) (Includes 10X APE 1 Reaction Buffer)	USB	78454
RNA 6000 Nano LabChip Kit	Agilent	5065-4476
Terminal Deoxynucleotidyl Transferase (rTdT), Recombinant, (30 U/μL) (5X TdT buffer included)	USB	72033
EDTA, 0.5M Solution (Ethylenediamine Tetraacetic Acid)	USB	15694
Microcon columns YM-3	Millipore	42404
DNA Labeling Reagent, DLR, 10 mM	USB	79015
cDNA Clean-up		
PrepEase DNA Clean-Up Kit	USB	78758
Hybridization, Wash and Stain		
GeneChip® Expression Hybridization Control Kit	Affymetrix	900454 or 900457
GeneChip® Hybridization, Wash, and Stain Kit	Affymetrix	900720

Instruments Required

Table 1.2 Instruments Required

Instrument	Manufacturer	P/N
NanoDrop® ND-1000*	Ambion	N/A
GeneChip® Hybridization Oven 640	Affymetrix	8001318
Eppendorf Centrifuge	Eppendorf	5417C
GeneChip® Fluidics Station 450	Affymetrix	00-0079
GeneChip® Scanner 3000 upgraded for 5 μm Arrays Autoloader (Optional)	Affymetrix	00-0073 90-0351
ABI GeneAmp® PCR System 2400	Applied Biosystems	N/A
Bioanalyzer 2100	Agilent	G2940CA
Heating block	VWR	13259-030
Pipette for 0.1 to 2 μL	Rainin	L-2
Pipette for 2 to 20 μL	Rainin	L-20
Pipette for 20 to 200 μL	Rainin	L-200
Pipette for 100 to 1000 μL	Rainin	L-1000

*Or equivalent instrument.

Eukaryotic Double Strand Whole Transcript Protocol

Generate purified cRNA from at least 50 ng total RNA using the Ambion WT Expression Kit (P/N 4411973 or 4411974). Check by OD and start the next step with 15 µg cRNA split into three tubes of 5 µg each.

Preparation of RNA-Random Primer Mix

1. Mix cRNA with the Random Primers in a strip tube, as listed in [Table 1.3](#) below.

Table 1.3 cRNA-Primer Mix

Component	Volume in 1 Rxn
cRNA (5 µg)	variable
Random Primers (3 µg/µL)	1.0 µL
Nuclease-free Water	up to 8 µL
Total Volume	8.0 µL

2. Flick-mix, and spin down the tubes.
3. Incubate for 5 minutes at 70°C, 5 minutes at 25°C, and then cool the samples at 4°C for at least 2 minutes.
4. Prepare dNTP mix as described in [Table 1.4](#).

Table 1.4 dNTP Mix

Component	Volume in 1 Rxn
dATP, 100 mM	5 µL
dCTP, 100 mM	5 µL
dGTP, 100 mM	5 µL
dTTP, 100 mM	4 µL
dUTP, 100 mM	1 µL
RNase-free Water	30 µL
Total Volume	50 µL

5. In a separate tube, prepare a master mix as described in [Table 1.5](#).

Table 1.5 First Strand cDNA Synthesis

Component	Volume in 1 Rxn	Master Mix for 3.5 Rxns
5X 1st Strand Buffer	4.0 µL	14.0 µL
DTT, 0.1 M	2.0 µL	7.0 µL
dNTP, 10 mM (4dTTP:1dUTP)	1.0 µL	3.5 µL
RNase Inhibitor (Recombinant)	1.0 µL	3.5 µL
SuperScript II, 200 U/µL	4.0 µL	14.0 µL
Total Volume	12.0 µL	42.0 µL

6. Transfer 12 μL of the 1st Strand Master Mix to the cRNA samples (the total reaction volume is 20 μL). Mix thoroughly by gently flicking the tubes a few times. Centrifuge briefly to collect the reactions at the bottom of the tube.
7. Incubate the reactions at 25°C for 5 minutes, at 42°C for 1 hour, and 4°C for at least 2 minutes.



TIP: POTENTIAL STOPPING POINT. Store products at –20°C if desired.

Second-Cycle, Second Strand cDNA Synthesis and Cleanup

1. In a separate tube, assemble a master mix as listed in [Table 1.6](#).

Table 1.6 Second Strand cDNA Synthesis

Component	Volume in 1 Rxn	Master Mix for 3.5 Rxns
MgCl ₂ [*] , 17.5 mM	8.0 μL	28 μL
dNTP, 10 mM (4 dTTP:1dUTP) (From Step 4 on page 6)	0.6 μL	2.1 μL
Exonuclease-Free Klenow, 10 U/ μL	2.7 μL	9.5 μL
Nuclease-free Water	8.5 μL	29.7 μL
RNAse H, 5 U/ μL [†]	0.2 μL	0.7 μL
Total Volume	20 μL	70 μL

^{*}Make a fresh dilution of the MgCl₂ each time. Mix 2 μL of 1M MgCl₂ with 112 μL of RNase-free water.

[†]If needed dilute RNAse H in the appropriate storage buffer: 20mM Tris-HCl (pH 7.9), 100mM KCl, 10mM MgCl₂, 0.1mM EDTA, 0.1mM DTT, 50% glycerol.

2. Transfer 20 μL of the 2nd Strand Master Mix to the 1st strand reaction tubes (the total reaction volume is 40 μL). Mix thoroughly by gently flicking the tubes a few times. Centrifuge briefly to collect the reactions at the bottom of the tubes.
3. Incubate the reactions at 37°C for 40 minutes, at 75°C 10 minutes, and 4°C for at least 2 minutes.



TIP: POTENTIAL STOPPING POINT. Store products at –20°C if desired.

4. Proceed to ds cDNA clean-up using the USB PrepEase DNA Clean-Up Kit:
 - A. Adjust DNA binding conditions.
 - 1) Add 5 volumes of N2P Buffer to 1 volume of sample (e.g. 200 μL N2P Buffer and 40 μL sample). Mix well.
 - B. Bind DNA sample to column.
 - 1) Place PrepEase® Clean-Up Column into a 2 ml PrepEase® Collecting Tube.
 - 2) Pipet the sample directly into the center of the column.
 - 3) Centrifuge 1 minute at 11,000 x g.
 - 4) Discard flow-through.
 - C. Wash column.
 - 1) Add 600 μL NT3 Buffer to column.
 - 2) Centrifuge 1 minute at 11,000 x g.
 - 3) Discard flow-through. Place column back into collecting tube.
 - D. Dry column
 - 1) Centrifuge 2 minutes at 11,000 x g.

- E. Elute DNA
 - 1) Place the column into a clean 1.5 mL microcentrifuge tube.
 - 2) Add 50 μ L NE Buffer to column.
 - 3) Incubate at room temperature for 1 minute.
 - 4) Centrifuge 1 minute at 11,000 x g.
5. Take 1 μ L from each sample to determine the ds cDNA yield by spectrophotometric UV measurement at 260 nm, 280 nm and 320 nm.

Fragmentation, Labeling, and Hybridization

1. Fragmentation of ds cDNA.
 - A. Combine all three tubes of ds cDNA into one tube.
 - B. Take 1 μ L to determine the ds cDNA yield by spectrophotometric UV measurement at 260nm, 280 nm and 320 nm
 - C. Take 20 μ g of ds DNA from the tube containing the pooled samples and split it into three tubes.
 - D. Fragment each of the samples as listed in [Table 1.7](#) below:

Table 1.7 Fragmentation of ds cDNA

Component	Volume/Amount in 1 Rxn
ds cDNA	6.67 μ g
Nuclease-free Water	up to 32.2 μ L
10X APE 1 Reaction Buffer	4.8 μ L
Uracil-DNA Glycosylase (2U/ μ L)	4.0 μ L
Human Apurinic/Apyrimidinic Endonuclease 1 (APE 1) 10 units/ μ L	7.0 μ L
Total Volume	48 μL

- 1) Flick-mix, and spin down the tubes.
 - 2) Incubate the reactions at 37°C for 1 hour, 93°C for 1 minute, and at 4°C for at least 2 minutes.
 - 3) Flick-mix, spin down the tubes, and remove 3 μ L for fragmentation analysis using a Bioanalyzer. Please see the Reagent Kit Guide that comes with the RNA 6000 Nano LabChip Kit. The average peak size of the fragmented samples should be approximately 50 bp.
 - 4) If not labeling the samples immediately, store the fragmented ds cDNA at -20°C.
2. Labeling of fragmented ds cDNA:
 - A. Prepare the labeling reactions for the samples as listed in [Table 1.8](#) below.

Table 1.8 Labeling of Fragmented ds cDNA

Component	Volume in 1 Rxn
Fragmented ds cDNA	45.0 μ L
5x TdT Reaction Buffer	12.0 μ L
Terminal Deoxynucleotidyl Transferase (rTdT), Recombinant, (30 U/ μ L)	2.0 μ L
DNA Labeling Reagent, DLR, 10 mM	1.0 μ L
Total Volume	60.0 μL

- B. Flick-mix, and spin down the tubes.
- C. Incubate the reactions at 37°C for 60 minutes, and cool at 4°C for at least 2 minutes.
- D. Stop the reactions by adding 2 µL of 0.5 M EDTA (pH 8.0), flick-mix and spin down.
- E. Remove 4 µL of each sample for Gel-shift analysis (optional), and combine samples into one tube (the total volume is 174 µL).

Sample Concentration

1. Pipet the sample into a YM-3 Microcon column already inserted into a 1.5 mL tube (tubes supplied with the Microcon columns).
2. Centrifuge at 14,000 x g for 40-50 minutes. After 30 minutes, start measuring flow-through until the volume is close to 100 µL.
3. In a new tube, place column upside down and centrifuge at 1000 x g for 3 minutes. The sample should be 68.7 µL.



NOTE: It is difficult to get exactly 68.7 µL. If the volume is less than this, add RNase-free water to bring the total volume up to 68.7 µL.

NOTE: Debris from the column membrane might result if spinning at maximum speed in Step 3 above.

4. Hybridization of Fragmented and labeled ds cDNA on the arrays (Std. arrays).
 - A. Prepare the hybridization mix in a 1.5 mL RNase-free microfuge tube as shown in Table 1.9 below.

Table 1.9 Hybridization Mix

Component	Volume in 1 Rxn	Final Concentration or Amount
Fragmented-labeled ds DNA	68.7 µL	~20 µg
Control Oligonucleotide B2	3.7 µL	50 pM
20X Eukaryotic Hybridization Controls (<i>bioB</i> , <i>bioC</i> , <i>bioD</i> , <i>cre</i>)	11 µL	1.5, 5, 25 and 100 pM, respectively
2X Hybridization Mix	110 µL	1X
DMSO	15.4 µL	7%
Nuclease-free Water	Up to 220.0 µL	
Total Volume	220.0 µL	

- B. Flick-mix, and centrifuge the tube.
- C. Heat the hybridization cocktail mix at 99°C for 5 minutes. Cool to 45°C for 5 minutes, and centrifuge at maximum speed for 1 minute.
- D. Inject ~200 µL of the specific sample into the array.
- E. Place array in 45°C hybridization oven, at 60 rpm, and incubate for 16 (+/- 1.0) hours.
5. Wash/Stain arrays with Affymetrix® Hybridization, Wash and Stain Kit using the FS450_0001 fluidics protocol.