GeneChip® Whole Transcript (WT) Double-Stranded Target Assay Manual

P/N 702179 Rev. 3

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Use of the GeneChip® WT Amplified Double-Stranded cDNA Synthesis and Amplification Kit in accordance with the instructions provided is accompanied by a limited license to U.S. Patent Nos. 5,716,785; 5,891,636; 6,291,170; and 5,545,522. Users who do not purchase this Kit may be required to obtain a license under these patents or to purchase another licensed kit.

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Chapter 1

Overview

Chapter 1

Introduction

GeneChip® Whole Transcript (WT) Double-Stranded Target Assays are designed to be used in conjunction with Affymetrix GeneChip® Tiling Arrays for unbiased genome-wide transcript mapping studies. Both the amplified and unamplified versions of the assay produce double-stranded, labeled DNA targets which interrogate the genome for all regions of expression. The specific protocol used is dependent on the sample and array type.

For users of multi array sets, such as the GeneChip® Human Tiling 1.0R Array Set (P/N 900774) and GeneChip® Mouse Tiling 1.1R Array Set (P/N 900853), Affymetrix recommends using the WT Amplified Double-Stranded Target Assay. This assay incorporates both a ribosomal RNA (rRNA) depletion step and an *in vitro* transcription (IVT) amplification to efficiently enrich mRNA pools and create sufficient target to hybridize multi-array sets.

For users of single tiling array designs, such as the model organism tiling arrays[1], the protocol without an amplification step is recommended and has the advantage of quicker target preparation time when sample enrichment is not required.

[1] *Such as Arabidopsis Tiling 1.0R Array P/N 900594, S. pombe Tiling 1.0FR Array P/N 900647, and S. cerevisiae 1.0R Array P/N 900645. For the full product line of Affymetrix model organism and other tiling arrays, please visit www.affymetrix.com.*

Materials

NECESSARY REAGENTS

Table 1.1
Necessary Reagents

Material	Source	P/N
GeneChip® WT Amplified Double-Stranded Target Assay (For use with the multi tiling array sets (e.g., GeneChip® Human Tiling 1.0R Array Set and GeneChip® Mouse Tiling 1.1R Array Set)		
rRNA Reduction		
RiboMinus™ Human/Mouse Transcriptome Isolation Kit	Invitrogen	K1550-02
Magna-Sep™ Magnetic Particle Separator	Invitrogen	K1585-01
Betaine, 5M	Sigma-Aldrich	B-0300
cDNA and cRNA Synthesis, and Amplification		
GeneChip® WT Amplified Double-Stranded cDNA Synthesis Kit	Affymetrix	900811
GeneChip® WT Double-Stranded cDNA Synthesis Module Contains: • T7-$(N)_6$ Primers, 2.5 µg/µL • 5X 1st Strand Buffer • DTT, 0.1M • dNTP, 10 mM • RNase Inhibitor • SuperScript™ II • $MgCl_2$, 1M • DNA Polymerase I • RNase H • Random Primers, 3 µg/µL • dNTP+dUTP, 10 mM • RNase-free Water		
GeneChip® WT cDNA Amplification Kit Contains: • 10X IVT Buffer • IVT NTP Mix • IVT Enzyme Mix • IVT Control		

Table 1.1 (Continued)
Necessary Reagents

Material	Source	P/N
GeneChip® WT Double-Stranded Target Assay (For use with individual tiling arrays (e.g., GeneChip® Arabidopsis Tiling 1.0R Array)		
cDNA Synthesis		
GeneChip® WT Double-Stranded cDNA Synthesis Kit Contains: • T7-$(N)_6$ Primers, 2.5 µg/µL • 5X 1st Strand Buffer • DTT, 0.1M • dNTP, 10 mM • RNase Inhibitor • SuperScript™ II • $MgCl_2$, 1M • DNA Polymerase I • RNase H • Random Primers, 3 µg/µL • dNTP+dUTP, 10 mM • RNase-free Water	Affymetrix	900813
Fragmentation and Labeling		

Table 1.1 (Continued)
Necessary Reagents

Material	Source	P/N
GeneChip® WT Double-Stranded DNA Terminal Labeling Kit Contains: • 10X cDNA Fragmentation Buffer • UDG, 10 U/µL • APE 1, 100 U/µL • 5X TdT Buffer • TdT, 30 U/µL • GeneChip® DNA Labeling Reagent, 5 mM • RNase-free Water	Affymetrix	900812
cDNA, cRNA Clean-up		
GeneChip® Sample Cleanup Module Contains: • cDNA Cleanup Spin Columns • cDNA Binding Buffer • cDNA Wash Buffer, 6 mL concentrate • cDNA Elution Buffer • IVT cRNA Cleanup Spin Columns • IVT cRNA Binding Buffer • IVT cRNA Wash Buffer, 5 mL concentrate • RNase-free Water • 1.5 mL Collection Tubes (for elution) • 2 mL Collection Tubes • 5X Fragmentation Buffer	Affymetrix	900371
Hybridization, Wash, and Stain		

Table 1.1 (Continued)
Necessary Reagents

Material	Source	P/N
GeneChip® Hybridization, Wash, and Stain Kit Contains: Box 1 of 2: Hybridization Module • Pre-Hybridization Mix • 2X Hybridization Mix • DMSO • Nuclease-free Water Stain Module • Stain Cocktail 1 • Stain Cocktail 2 • Array Holding Buffer Box 2 of 2 • Wash Buffer A, 3 bottles, 800 mL/bottle • Wash Buffer B, 1 bottle, 600 mL/bottle	Affymetrix	900720
Wash Buffer A, 800 mL*	Affymetrix	900721
Wash Buffer B, 600 mL*	Affymetrix	900722
Control Oligonucleotide B2, 3 nM	Affymetrix	900301

**Optional if additional buffer needed.*

MISCELLANEOUS REAGENTS

Table 1.2
Miscellaneous Reagents

Materials	Source	P/N
Miscellaneous Reagents		
Absolute ethanol	Various	Various
RNA 6000 Nano LabChip® Reagents and Supplies	Agilent	5065-4476
Gel-Shift Assay (Optional)		
Novex XCell SureLock™ Mini-Cell*	Invitrogen	EI0001
TBE Gel, 4-20%,1.0 mm, 12 well*	Invitrogen	EC62252
5X Sucrose Gel Loading Dye	Amresco	E-274
10X TBE Buffer	Cambrex	50843
SYBR® Gold	Invitrogen	S-11494
10 bp DNA ladder and 100 bp DNA ladder	Invitrogen	10821-015 15628-019
ImmunoPure NeutrAvidin	Pierce	31000
PBS, pH 7.2	Invitrogen	20012-027

**Or equivalent.*

MISCELLANEOUS SUPPLIES

Table 1.3
Miscellaneous Supplies

Materials	Source	P/N
Miscellaneous Supplies		
1.5 mL RNase-free Microfuge Tubes*	Ambion	12400
1.5 mL Non-stick RNase-free Microfuge Tubes*	Ambion	12450
0.2 mL MicroAmp reaction tubes (8 tubes/strip)*	Applied Biosystems	N801-0580
MicroAmp caps for 8 strip tubes	Applied Biosystems	N801-0535
Pipette for 25 mL*	VWR	53283-710
Pipet-aid*	VWR	53498-103

**Or equivalent.*

INSTRUMENTS

Table 1.4
Instruments

Instruments	Manufacturer	P/N
GeneChip® Hybridization Oven 640	Affymetrix	800138
GeneChip® Fluidics Station 450 or 400	Affymetrix	00-0079
GeneChip® Scanner 3000 7G	Affymetrix	00-0073
GeneChip® AutoLoader (Optional)	Affymetrix	90-0351
NanoDrop® ND-1000*	Ambion	58922-612
Eppendorf Centrifuge*	Eppendorf	5417C
Tube-Strip Picofuge*	Stratagene	400540
ABI GeneAmp® PCR System 9700*	Applied Biosystems	4314878
Bioanalyzer 2100	Agilent	G2940CA
Heating block*	VWR	13259-030
SpeedVac®	Thermo Electron Corporation	Various
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.*

Chapter 2

Target Preparation for Samples Requiring Amplification

Chapter 2

Introduction

The GeneChip® Whole Transcript (WT) Amplified Double-Stranded Target Assay is designed to generate double-stranded labeled DNA targets from the entire expressed genome. This assay has been designed specifically for use with Human Tiling 1.0R Array Set or Mouse Tiling 1.1R Array Set for RNA mapping applications in order to probe the genome for all regions of expression.

The procedure described in this Manual recommends as little as 3 µg of total RNA or 300 ng of poly-A^+ RNA as starting material from human or mouse samples. Affymetrix currently recommends the use of the RiboMinus™ Transcriptome Isolation Kit from Invitrogen to remove 18S and 28S ribosomal RNAs from total RNA samples thereby maximizing sensitivity and specificity of hybridizations. Following the recommended protocol, the amount of labeled target produced (starting from 3 µg total RNA) is sufficient to hybridize up to nine arrays, allowing for re-hybridizations of saved hybridization cocktails.

The quality of the RNA is essential to the overall success of the analysis. Since the most appropriate protocol for the isolation of RNA can be source-dependent, we recommend using a protocol that has been established for the tissues or cells being used. In the absence of an established protocol, using one of the commercially available kits designed for RNA isolation is suggested.

GeneChip® Whole Transcript (WT) Amplified Double-Stranded Target Assay Flow Diagram

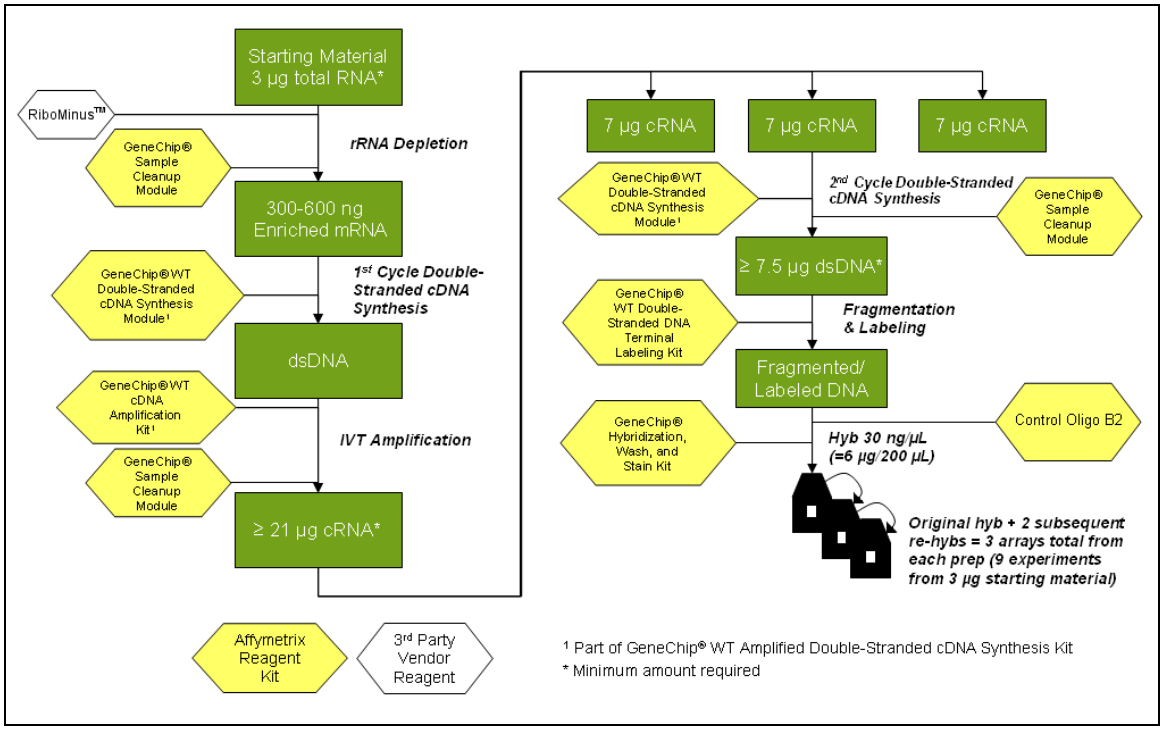


Figure 2.1
Flow Diagram of the GeneChip® WT Amplified Double-Stranded Target Assay: Schematic for Human and Mouse 14 Array Sets

Figure 2.1 outlines the workflow of the GeneChip® WT Amplified Double-Stranded Target Assay. The mRNA fraction from total RNA is first purified and enriched by rRNA reduction using the RiboMinus™ Human/Mouse Transcriptome Isolation Kit (Invitrogen P/N K1550-02). Isolated mRNA is copied into double-stranded (ds) DNA using the GeneChip® WT Amplified Double-Stranded cDNA Synthesis Kit and amplified by *in vitro* transcription into cRNA using the same kit.

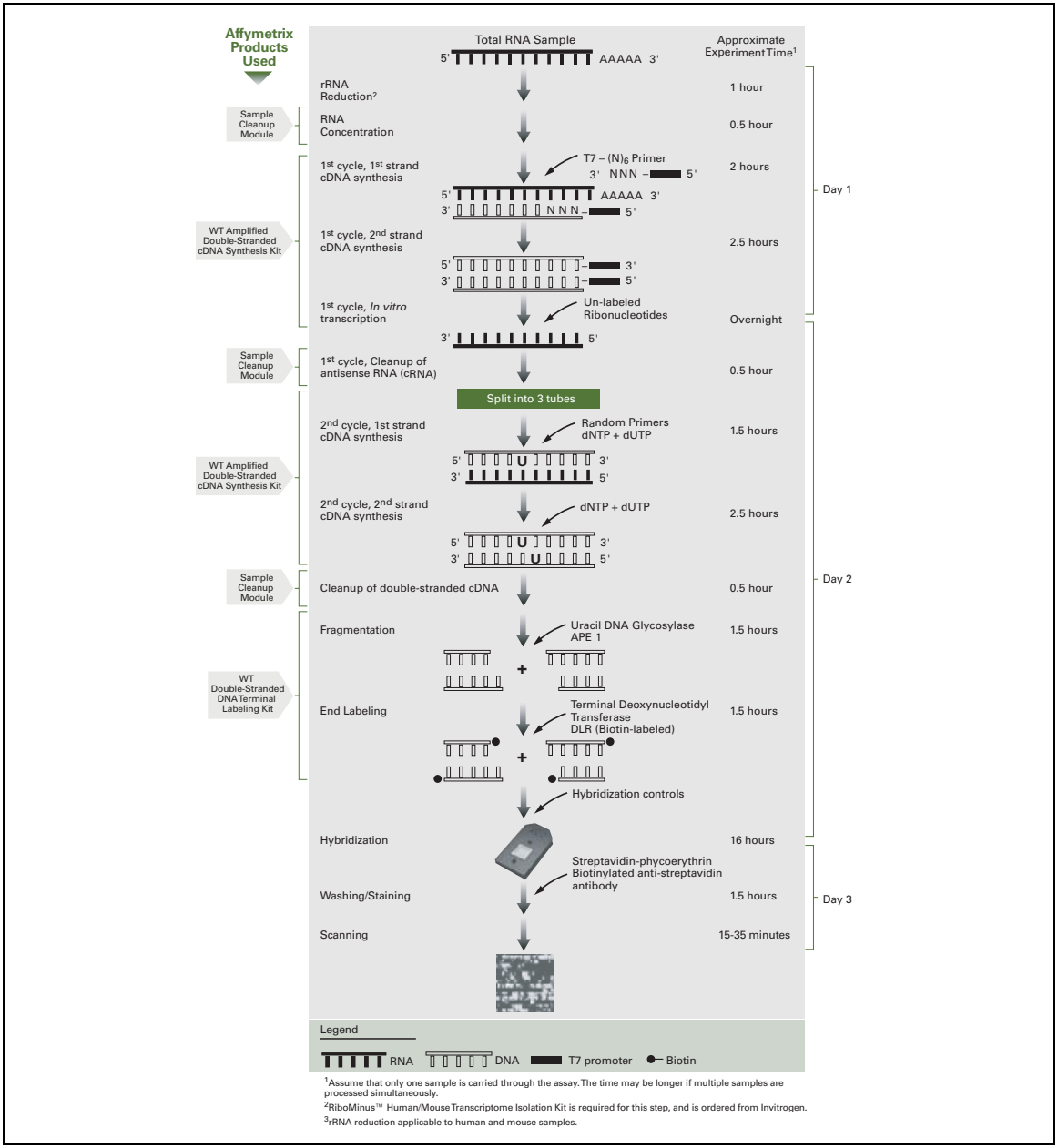
The cRNA is split into three subsequent 2nd cycle double-stranded cDNA synthesis reactions, which incorporate deoxyuridine into each cDNA strand at predefined ratios. The dU-incorporated dsDNA is fragmented using apurinic/apyrimidinic endonuclease (APE 1) and uracil DNA glycosylase (UDG) enzymes and then labeled using terminal deoxynucleotidyl transferase (TdT) and Affymetrix' proprietary DNA Labeling Reagent supplied in the GeneChip® WT Double-Stranded DNA Terminal Labeling Kit.

The labeled double-stranded target is then ready to add to the hybridization cocktail and hybridize onto GeneChip® Tiling Arrays. For array sets with multiple tiling arrays, Affymetrix suggests saving the original hybridization cocktail and re-hybridizing up to two more times for a total of three hybridizations from a single 2nd cycle dsDNA target. Studies during development of these kit reagents showed negligible loss of sensitivity and specificity following two rehybridizations of saved hybridization cocktail when stored properly at –20°C.

IMPORTANT ! **When using a commercial kit, follow the manufacturer's instructions for RNA isolation.**

If starting from poly-A+ RNA, skip the RiboMinus steps for rRNA reduction and proceed directly with *Procedure G: Preparation of RiboMinus™-Enriched RNA /T7-(N)6 Primer Mix* on page 24.

The following protocol requires a minimum of 3 µg of total RNA as starting material, and the concentration may not be below 0.24 µg/µL.



[1]Assume that only one sample is carried through the assay. The time may be longer if multiple samples are processed simultaneously.

[2]RiboMinus™ Human/Mouse Transcriptome Isolation Kit is required for this step, and is ordered from Invitrogen.

[3]rRNA reduction applicable to human and mouse samples.

Figure 2.2
GeneChip® Whole Transcript Amplified Double-Stranded Target Assay Schematic

Procedure A: Preparation of Hybridization Buffer with Betaine

Procedures A through D require the use of the RiboMinus™ Human/Mouse Transcriptome Isolation Kit (Invitrogen).

1. Prepare the buffer by mixing the components in Table 2.1.

Table 2.1
Hybridization Buffer with Betaine

Component	Volume for 1 Reaction
Betaine, 5M	120 µL
RiboMinus™ Hybridization Buffer	280 µL
Total Volume	400 µL

A 50 µL overfill is included in the Total Volume.

Procedure B: RiboMinus™ Probe Hybridization

1. In a 0.2 mL strip tube, mix the following components in Table 2.2 if the total RNA is in ≥ 0.40 µg/µL of concentration. If the total RNA sample is at a lower concentration (between 0.24 µg/µL to 0.40 µg/µL) follow the protocol listed in Table 2.3.

Table 2.2
RiboMinus™ Probe Hybridization Mix for Samples ≥ 0.40 µg/µL

Component	Volume or Amount for 1 Reaction
Total RNA	3 µg
RiboMinus™ Probe, 100 pmol/µL	2.4 µL
Hybridization Buffer with Betaine (from Procedure A)	60 µL
RNase-free Water	up to 70 µL
Total Volume	70 µL

Table 2.3
RiboMinus™ Probe Hybridization Mix for Samples between 0.24 µg/µL to 0.40 µg/µL

Component	Volume or Amount for 1 Reaction
Total RNA	3 µg
RiboMinus™ Probe, 100 pmol/µL	2.4 µL
Hybridization Buffer with Betaine (from Procedure A)	90 µL
RNase-free Water	up to 105 µL
Total Volume	105 µL

2. Flick the tube gently to mix, spin briefly and incubate at 70ºC for 5 minutes in a thermal cycler
3. Quench the reaction immediately by placing the tube on ice while preparing the magnetic beads.

Procedure C: Bead Preparation

NOTE **Procedures C and D require the use of a 37°C heat block and a 50°C heat block, respectively. Pre-heat the heat blocks prior to initiating these steps.**

1. Completely re-suspend the RiboMinus™ Magnetic Beads bottle by flicking it until no deposit is observed at the bottom of the bottle.
2. Pipette 150 µL of beads suspension into a 1.5 mL non-stick RNase-free tube.
3. Briefly spin and place the tube with the beads suspension on the magnetic stand for ~1 minute with the tube remaining in the stand, gently aspirate, and discard the supernatant.

NOTE **Drying of the beads reduces the recovery of RNA. Resuspend the beads promptly after each of the following aspiration steps.**

4. 1st **Wash**: Add 150 µL of RNase-free water to the beads and re-suspend them by flicking the tube. Spin briefly. Place the tube on the magnetic stand for ~1 minute. With the tube remaining in the stand, gently aspirate, and discard the supernatant.
5. 2nd **Wash**: Add 150 µL of RNase-free water to the beads; re-suspend them by flicking the tube. Spin briefly. Place the tube on the magnetic stand for ~1 minute. With the tube remaining in the stand, gently aspirate, and discard the supernatant.
6. 3rd **Wash**: Add 150 µL of the Hybridization Buffer with Betaine (from Procedure A) to the beads and re-suspend them by flicking the tube. Spin briefly. Place the tube on the magnetic stand for ~1 minute. With the tube remaining in the stand, gently aspirate, and discard the supernatant.

7. If the concentration of sample used in Procedure B was ≥ 0.40 µg/µL, add 90 µL of Hybridization Buffer with Betaine. If the concentration of sample used in Procedure B was 0.24 µg/µL, but less than 0.40 µg/µL, add 60 µL of Hybridization Buffer with Betaine. Keep the tubes at 37°C in a heating block for 1 to 2 minutes.
8. Proceed to *Procedure D: rRNA reduction* on page 21.

Procedure D: rRNA reduction

1. Transfer the ice-cooled hybridized sample prepared in Procedure B to the beads prepared in Procedure C, mix well, and briefly spin.
2. Incubate the tube with the mixture at 37°C for 10 minutes in a heating block. After 5 minutes of incubation, gently flick-mix the tube. Spin briefly. Replace in heating block for the remaining 5 minutes
3. Briefly spin and place the tube in the magnetic stand for 1 to 2 minutes to obtain the rRNA-probe pellet.

NOTE **The supernatant contains the RiboMinus™-Purified RNA.**

4. With the tube remaining in the stand, transfer the supernatant to a 1.5 mL non-stick RNase-free tube, and leave on ice.
5. Wash the beads by re-suspending them in 50 µL of Hybridization Buffer with Betaine. Spin briefly. Incubate at 50°C for 5 minutes.
6. Briefly spin and place the tube in the magnetic stand for 1 to 2 minutes to obtain the rRNA-probe pellet. With the tube remaining in the stand, carefully aspirate the supernatant.
7. Transfer and combine the wash-supernatant with the supernatant in the tube from Procedure D Step 4. The total volume of the RiboMinus sample is approximately 210 µL.

Procedure E: Cleanup

This Procedure requires the use of the GeneChip® IVT cRNA Cleanup Columns, provided in the GeneChip® Sample Cleanup Module (P/N 900371).

1. Proceed to the cleanup procedure using the cRNA Cleanup Spin Columns from the GeneChip IVT cRNA Cleanup Kit following the protocol described below.
2. If not already done, add 20 mL of ethanol (100%) to the cRNA Wash Buffer supplied in the GeneChip IVT cRNA Cleanup kit.
3. Add 735 µL of cRNA Binding Buffer to each sample and vortex for 3 seconds.
4. Add 525 µL of 100% ethanol to each reaction and flick the tube to mix.
5. Apply 700 µL of sample to the IVT cRNA Column in a 2 mL Collection Tube.
6. Centrifuge for 15 seconds at ≥ 8,000 x g. Discard the flow-through.
7. Apply 700 µL of sample to the column and centrifuge for 15 seconds at ≥ 8,000 x g. Discard the flow-through. If any sample remains, apply remaining sample to the column and centrifuge for 15 seconds at ≥ 8,000 x g.
8. Transfer the IVT cRNA Column to a new 2 mL Collection Tube and add 500 µL of cRNA Wash Buffer and centrifuge for 15 seconds at ≥ 8,000 x g. Discard the flow-through.
9. Wash again with 500 µL of 80% (v/v) ethanol. Centrifuge for 15 seconds at ≥ 8,000 x g and discard the flow-through.
10. Open column cap and spin at ≤ 25,000 x g (maximum speed) for 5 minutes with the cap left open.
11. Transfer the IVT cRNA Column to a new 1.5 mL Collection Tube and add 11 µL of RNase-free water directly to the membrane. Spin at ≤ 25,000 x g (maximum speed) for 1 minute.
12. The eluted RiboMinus™-Purified RNA is ~9.8 µL.

Procedure F: Analysis with Bioanalyzer

1. Use 1 µL of the purified sample to check rRNA depletion by running the Eukaryotic Total RNA Nano Assay in the Bioanalyzer. Please see the Reagent Kit Guide provided with the RNA 6000 Nano LabChip® Kit for instructions. Based on the Bioanalyzer results, an average 10% (≥ 300 ng) recovery can be obtained depending on the tissue. See Figure 2.3.

Recommendation: Analyze 300 ng (1 µL) of total RNA sample without RiboMinus™ treatment as a control.

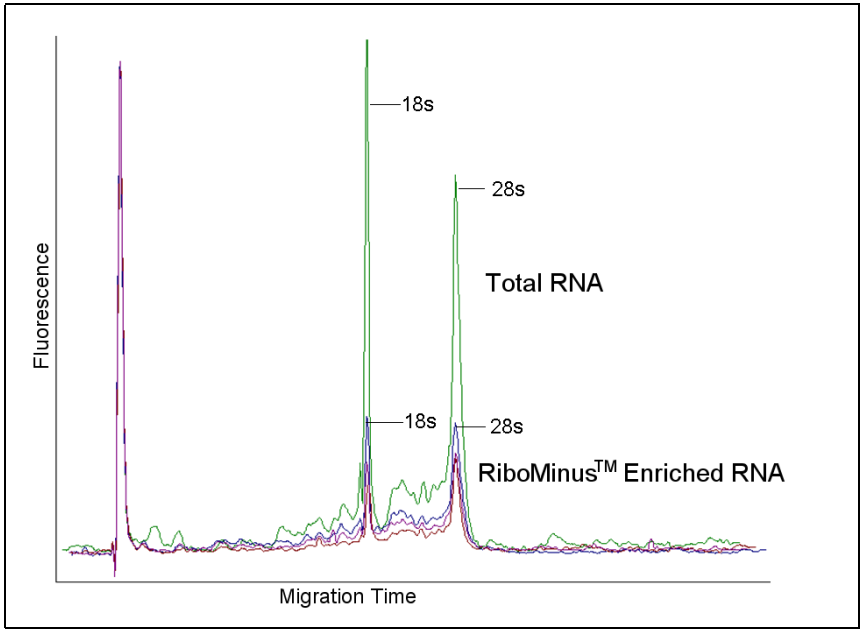


Figure 2.3
Bioanalyzer profile of rRNA reduced human brain (triplicates)

Procedure G: Preparation of RiboMinus™-Enriched RNA /T7-$(N)_6$ Primer Mix

Procedures G through K require the use of the GeneChip® WT Amplified Double-Stranded cDNA Synthesis Kit (900811).

1. Dilute the T7-$(N)_6$ Primers, 2.5 µg/µL stock 1:5 with RNase-free water to make a 500 ng/µL working solution.
2. Mix the diluted T7-$(N)_6$ Primers with the RNA sample as indicated in Table 2.4 below.

Table 2.4
First-cycle, Preparation of RiboMinus™-Enriched RNA/T7-$(N)_6$ Primers

Component	Volume in 1 Reaction
RiboMinus™-Enriched RNA or 300 ng poly-A^+ RNA	4 µL
Diluted T7-$(N)_6$ Primers, 500 ng/µL	1 µL
Total Volume	5 µL

NOTE

Maximum cRNA yields can be achieved by concentrating the entire elution volume of the RiboMinus-enriched RNA (from the previous procedure) by SpeedVac® down to 4 µL and using all of the eluted enriched RNA in the subsequent cDNA synthesis

3. Flick the tube to mix, spin down the tube, and incubate for 5 minutes at 70°C.
4. Cool the sample for at least 2 minutes at 4°C, and spin briefly.
5. Place on ice for use in *Procedure H: First-Cycle, First-Strand cDNA Synthesis* on page 25.

Procedure H: First-Cycle, First-Strand cDNA Synthesis

1. Prepare the First-Cycle, First-Strand Master Mix as shown in Table 2.5.

Table 2.5
First-Cycle, First-Strand Master Mix

Component	Volume in 1 Reaction
5X 1st Strand Buffer	2 µL
DTT, 0.1M	1 µL
dNTP Mix, 10 mM	0.5 µL
RNase Inhibitor	0.5 µL
SuperScript™ II	1 µL
Total Volume	5 µL

2. Add 5 µL of the First-Cycle, First-Strand Master Mix to the tube containing the RiboMinus™ Purified RNA/ T7-$(N)_6$ Primers Mix, flick-mix, and spin briefly. The total reaction volume is 10 µL.
3. Incubate the reactions in a thermal cycler (with heated lid) using the following protocol:
   - 25°C for 10 minutes
   - 42°C for 1 hour
   - 70°C for 10 minutes
   - 4°C for 2 to 10 minutes
4. Immediately continue to *Procedure I: First-Cycle, Second-Strand cDNA Synthesis*.

Procedure I: First-Cycle, Second-Strand cDNA Synthesis

1. Prepare a First-Cycle, Second-Strand Master Mix as described in Table 2.6.

Table 2.6
First-Cycle, Second-Strand Master Mix

Component	Volume in 1 Reaction
$MgCl_2$*,17.5 mM	4.0 µL
dNTP Mix, 10 mM	0.4 µL
DNA Polymerase I	0.6 µL
RNase H	0.2 µL
RNase-free Water	4.8 µL
Total Volume	10.0 µL

**Make a fresh dilution of the $MgCl_2$ each time. Mix 2 µL of 1M $MgCl_2$ with 112 µL of Nuclease-free water.*

2. Add 10 µL of the First-Cycle, Second-Strand Master Mix to the reaction tube from the First-Strand cDNA Synthesis Reaction for a total reaction volume of 20 µL. Flick-mix the solution, and spin briefly.
3. Incubate the reactions in a thermal cycler using the following protocol:
   - 16°C for 2 hours **without heated lid**
   - 75°C for 10 minutes **with heated lid**
   - 4°C for 2 to 10 minutes
4. Immediately continue to *Procedure J: First-Cycle, cRNA Synthesis and Cleanup*.

Procedure J: First-Cycle, cRNA Synthesis and Cleanup

1. In a separate tube, assemble the IVT Master Mix at room temperature as listed in Table 2.7.

Table 2.7
First-cycle, cRNA Synthesis

Component	Volume in 1 Reaction
10X IVT Buffer	5.0 µL
IVT NTP Mix	20.0 µL
IVT Enzyme Mix	5.0 µL
Total Volume	30.0 µL

2. Transfer 30 µL of the IVT Master Mix to each First-Cycle cDNA Synthesis Reaction sample to a final volume of 50 µL. Flick-mix the solution, and briefly spin in a microfuge.
3. Incubate the reaction in a thermal cycler for
   - 16 hours at 37°C
   - 4°C Hold
4. Proceed to the clean-up procedure for cRNA using the cRNA Cleanup Spin Columns from the GeneChip Sample Cleanup Module following the protocol described below. Store the sample at –80°C if not purifying the cRNA immediately.
5. If not already done, add 20 mL of ethanol (100%) to the cRNA Wash Buffer supplied in the GeneChip Sample Cleanup Module.
6. Add 50 µL of RNase-free water to each IVT reaction to a final volume of 100 µL.
7. Add 350 µL of cRNA Binding Buffer to each sample and vortex for 3 seconds.
8. Add 250 µL of 100% ethanol to each reaction and flick-mix.
9. Apply the sample to the IVT cRNA Column sitting in a 2 mL Collection Tube.

10. Centrifuge for 15 seconds at ≥ 8,000 x g. Discard the flow-through.

11. Transfer the IVT cRNA Column to a new 2 mL Collection Tube and add 500 µL of cRNA Wash Buffer and centrifuge for 15 seconds at ≥ 8,000 x g. Discard the flow-through.

12. Wash again with 500 µL of 80% (v/v) ethanol. Centrifuge for 15 seconds at ≥ 8,000 x g and discard the flow-through.

13. Open the column cap and spin at ≤ 25,000 x g (maximum speed) for 5 minutes with the caps open.

14. Transfer the IVT cRNA Column to a new 1.5 mL Collection Tube and add 12 µL of RNase-free water directly to the membrane. Spin at ≤ 25,000 x g (maximum speed) for 1 minute.

15. Repeat Step 14 above. The eluted cRNA is ~21 µL. Determine the cRNA yield by spectrophotometric UV measurement at 260nm, 280 nm and 320 nm:

Concentration of cRNA (µg/µL) = $[A_{260} - A_{320}]$ x 0.04 x dilution factor

µg cRNA = eluate in µL x cRNA in µg/µL

Each tube should have ≥ 21 µg of cRNA remaining.

NOTE **This average yield range may vary depending on the type of tissue used.**

16. If not continuing to Procedure K, place samples in –80°C until ready for use.

Procedure K: Second-Cycle, Double-Stranded cDNA Synthesis

NOTE **In order to generate sufficient target for hybridization across a multiple array tiling set, the cRNA sample generated in Procedure J Step 15 is split into three (3) second-cycle double-stranded cDNA synthesis reactions.**

1. Mix cRNA with the Random Primers in a strip tube, as listed in Table 2.8. For hybridization to multiple designs in a GeneChip® Tiling Array Set, prepare three replicate reactions from the cRNA obtained in Procedure J Step 15 using the amounts indicated below. Each reaction produces sufficient dsDNA to hybridize to an array with up to two rehybridizations.

Table 2.8
Second-cycle, cRNA/Random Primer Mix

Component	Volume in 1 Reaction
cRNA, 7 µg	variable
Random Primers (3 µg/µL)	1.0 µL
RNase-free Water	up to 8 µL
Total Volume	8.0 µL

2. Flick-mix and spin down the tubes.
3. Incubate the Second-Cycle, cRNA/Random Primers Mix in a thermal cycler (with heated lid) using the following protocol:
   - 70°C for 5 minutes
   - 25°C for 5 minutes
   - 4°C for 2 to 10 minutes
4. In a separate tube, prepare the Second-Cycle, First-Strand cDNA Synthesis Master Mix as described in Table 2.9.

Table 2.9
Second-Cycle, First-Strand cDNA Synthesis Master Mix

Component	Volume in 1 Reaction
5X 1st Strand Buffer	4.0 µL
DTT, 0.1M	2.0 µL
dNTP+dUTP, 10 mM	1.25 µL
SuperScript™ II	4.75 µL
Total Volume	12.0 µL

5. Transfer 12 µL of the Second-Cycle, First-Strand cDNA Synthesis Master Mix to the Second-Cycle, cRNA/Random Primers Mix for a total reaction volume of 20 µL. Mix thoroughly by gently flicking the tubes a few times and centrifuge briefly.
6. Incubate the reactions in a thermal cycler (with heated lid) using the following protocol:
   - 25°C for 10 minutes
   - 42°C for 90 minutes
   - 70°C for 10 minutes
   - 4°C for 2 to 10 minutes
7. In a separate tube, prepare the Second-Cycle, Second-Strand cDNA Synthesis Master Mix as described in Table 2.10.

Table 2.10
Second-Cycle, Second-Strand cDNA Synthesis Master Mix

Component	Volume in 1 Reaction
$MgCl_2$*,17.5 mM	8.0 µL
dNTP+dUTP, 10 mM	1.0 µL
DNA Polymerase I	1.2 µL
RNase H	0.5 µL
RNase-free Water	9.3 µL
Total Volume	20.0 µL

**Make a fresh dilution of the $MgCl_2$ each time. Mix 2 µL of 1M $MgCl_2$ with 112 µL of Nuclease-free water.*

8. Transfer 20 µL of the Second-Cycle, Second-Strand cDNA Synthesis Master Mix to the reaction tube from the Second-Cycle, First-Strand cDNA Synthesis Reaction for a total volume of 40 µL. Flick-mix the solution, and spin briefly.
9. Incubate the reactions at:
   - 16°C for 2 hours **without heated lid**
   - 75°C for 10 minutes **with heated lid**
   - 4°C Hold
10. Store the sample at –20°C if not immediately purifying the Double-Stranded DNA.

Procedure L: Cleanup of Double-Stranded DNA

This Procedure requires the use of the GeneChip® Sample Cleanup Module (P/N 900371).

1. Proceed to the Double-Stranded DNA cleanup using the cDNA Cleanup Spin Columns from the GeneChip Sample Cleanup Module following the protocol as described below.
2. If not already done, add 24 mL of ethanol (100%) to the cDNA Wash Buffer supplied in the GeneChip Sample Cleanup Module.
3. Add 60 µL of RNase-free water to each sample, 370 µL of cDNA Binding Buffer, and vortex for 3 seconds.
4. Apply the entire sample (the total volume is 471 µL) to a cDNA Spin Column sitting in a 2 mL Collection Tube.
5. Spin at ≥ 8,000 x g for 1 minute. Discard the flow-through.
6. Transfer the cDNA Spin Column to a new 2 mL Collection Tube and add 750 µL of cDNA Wash Buffer to the column. Spin at ≥ 8,000 x g for 1 minute and discard the flow-through.
7. Open cap of the cDNA Spin Column, and spin at ≤ 25,000 x g for 5 minutes with the caps open. Discard the flow-through, and place the column in a 1.5 mL collection tube.
8. Pipette 15 µL of the cDNA Elution Buffer directly to the column membrane and incubate at room temperature for 1 minute. Then, spin at ≤ 25,000 x g for 1 minute.
9. Pipette again 15 µL of the cDNA Elution Buffer directly to the column membrane and incubate at room temperature for 1 minute. Then, spin at ≤ 25,000 x g for 1 minute.

10. The eluted Double-Stranded DNA is ~28 µL total. Take 2 µL from each sample to determine the yield by spectrophotometric UV measurement at 260nm, 280 nm and 320 nm:

Concentration of Double-Stranded cDNA (µg/µL) = $[A_{260} - A_{320}]$ x 0.05 x dilution factor

µg DNA = eluate in µL x DNA in µg/µL

Each tube should have ≥ 7.5 µg of Double-Stranded DNA remaining.

11. For samples containing ≥ 7.5 µg, it is advisable to pool the double-stranded DNA samples to minimize variability across reactions.

12. If not continuing to Procedure M, place samples in –20°C until ready for use.

Procedure M: Fragmentation of Double-Stranded DNA

NOTE **The pooled sample generated in Procedure L Step 11 is split into three (3) fragmentation reactions.**

Procedures M and N require the use of the GeneChip® WT Double-Stranded DNA Terminal Labeling Kit (P/N 900812).

1. Fragment the samples using the reactions described in Table 2.11.

Table 2.11
Fragmentation of Double-Stranded DNA

Component	Volume or Amount in 1 Reaction
Double-Stranded DNA	7.5 µg
10X Fragmentation Buffer	4.8 µL
UDG, 10 U/µL	1.5 µL
APE 1, 100 U/µL	2.25 µL
RNase-free Water	up to 48 µL
Total Volume	48.0 µL

2. Combine the reagents above to the double-stranded DNA sample, flick-mix, and spin down the tubes. When preparing multiple reactions, a master mix is recommended.
3. Incubate the reactions in a thermal cycler (with heated lid) using the following protocol:
   - 37°C for 1 hour
   - 93°C for 2 minutes
   - 4°C for 2 to 10 minutes

4. Flick-mix, spin down the tubes, and transfer 45 µL of the sample to a new tube. The remainder of the sample is to be used for fragmentation analysis using a Bioanalyzer. Please see the Reagent Kit Guide that accompanies the RNA 6000 Nano LabChip® Kit for instructions.

NOTE

Results of the Bioanalyzer analysis of fragmented DNA should show that the majority of fragmented DNA is between 25 to 200 bases, with the peak of the distribution between 25 to 100 bases.

5. If not labeling the samples immediately, store the fragmented Double-Stranded DNA at –20°C.

Procedure N: Labeling of Fragmented Double-Stranded DNA

1. Prepare the labeling reactions as listed in Table 2.12.

Table 2.12
Double-Stranded DNA Labeling

Component	Volume in 1 Reaction
Fragmented Double-Stranded DNA	45 µL
5X TdT Buffer	12 µL
TdT, 30 U/µL	2 µL
DNA Labeling Reagent, 5 mM	1 µL
Total Volume	60 µL

2. Combine the reagents above to the fragmented double-stranded DNA, flick-mix, and spin down the tubes. When preparing multiple reactions, a master mix is recommended.
3. Incubate the reactions in a thermal cycler (with heated lid) using the following protocol:
   - 37°C for 1 hour
   - 70°C for 10 minutes
   - 4°C for 2 to 10 minutes
4. Remove 4 µL of each sample for Gel-shift analysis (optional, Appendix A).
5. Store at –20°C or go to Chapter 4, *Hybridization* on page 49 for hybridization setup.

Chapter 3

Target Preparation for Model Organisms, Single Tiling Arrays (No Amplification)

Chapter 3

Introduction

The Affymetrix GeneChip® WT Double-Stranded Target Assay is designed to generate double-stranded labeled DNA targets from the entire expressed genome. This assay has been designed specifically for use with single-array design tiling arrays for RNA mapping applications in order to probe the genome for all regions of expression. Random-prime cDNA is generated from total RNA without enrichment or amplification of the mRNA pool. The procedure described in this manual recommends 7 µg of total RNA as starting material. Following the recommended protocol, the amount of labeled target produced by this procedure is sufficient to hybridize to a single array.

GeneChip® Whole Transcript (WT) Double-Stranded Target Assay Flow Diagram (No Amplification)

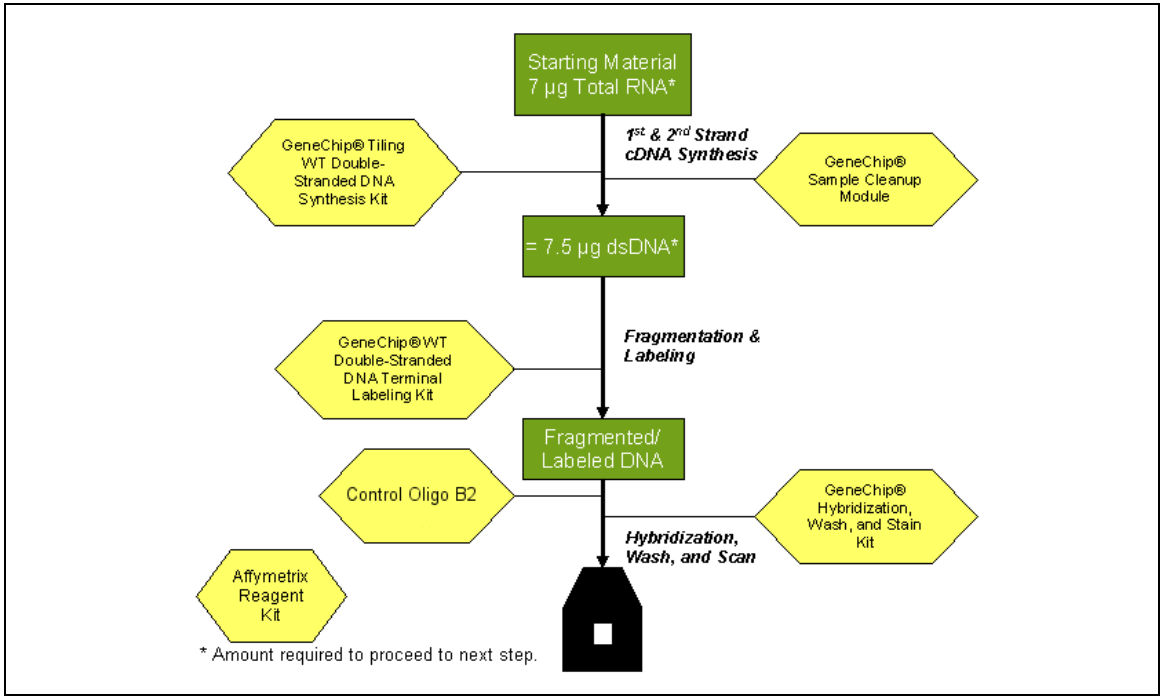


Figure 3.1
Flow Diagram of the GeneChip® Whole Transcript (WT) Double-Stranded Target Assay

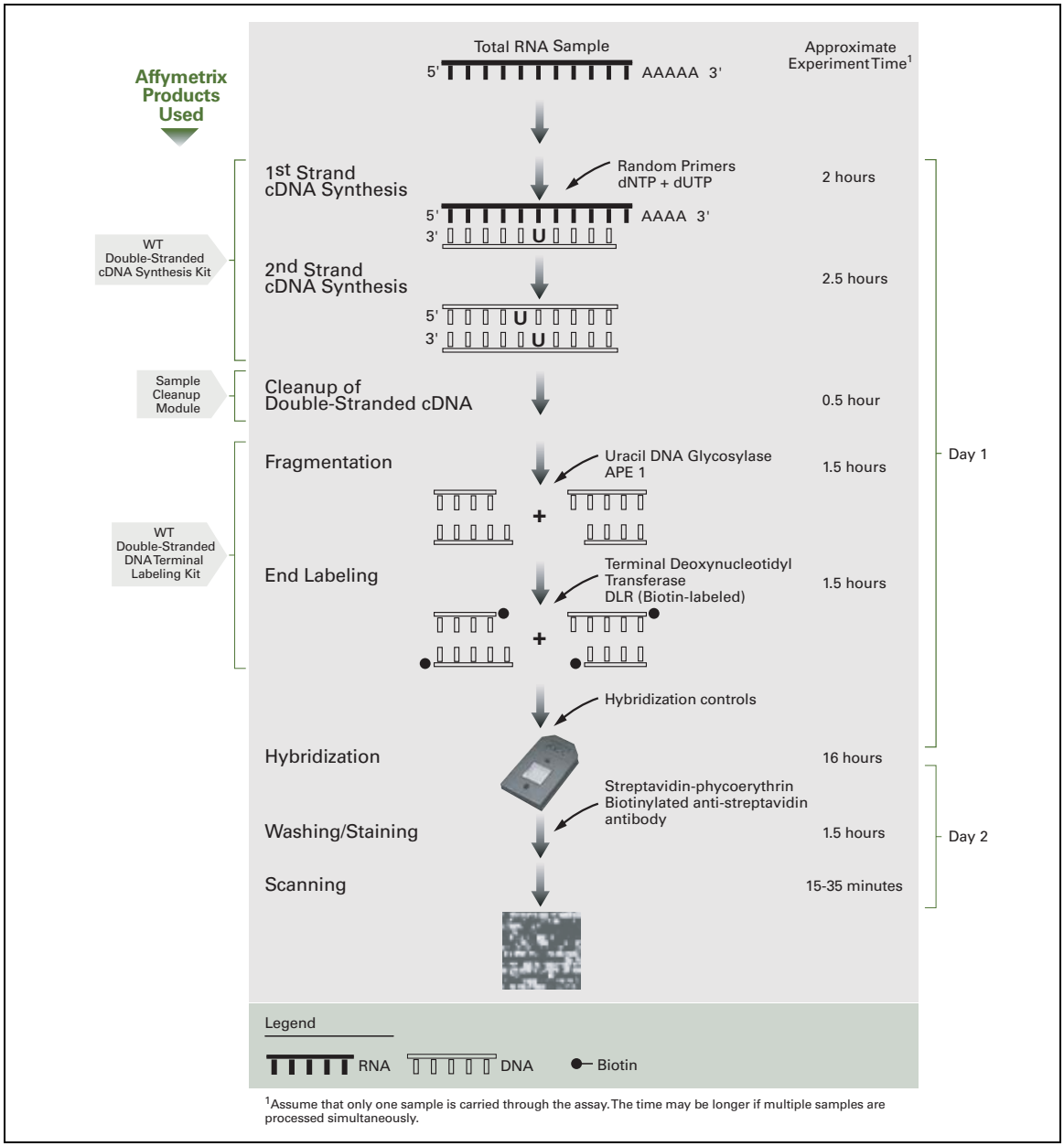


Figure 3.2
GeneChip® Whole Transcript Double-Stranded Target Assay Schematic

IMPORTANT !

When using a commercial kit for RNA isolation, carefully follow the manufacturer's instructions. The quality of the RNA is essential to the overall success of the analysis. Since the most appropriate protocol for the isolation of RNA can be source dependent, we recommend using a protocol that has been established for the tissues or cells being used. In the absence of an established protocol, using one of the commercially available kits designed for RNA isolation is suggested.

Procedure A: First-Strand cDNA Synthesis

This procedure requires the use of the GeneChip® WT Double-Stranded cDNA Synthesis Kit (PN 900813).

1. Prepare the total RNA/Random Primer mix in a strip tube, as listed in the Table 3.1.

Table 3.1
Total RNA/Random Primer Mix

Component	Volume in 1 Reaction
Total RNA, 7 µg	variable
Random Primers (3 µg/µL)	1.0 µL
RNase-free Water	up to 8 µL
Total Volume	8 µL

2. Flick-mix and briefly spin down the tubes.
3. Incubate the total RNA/Random Primers Mix in a thermal cycler (with heated lid) using the following protocol:
   - 70°C for 5 minutes
   - 25°C for 5 minutes
   - 4°C for 2 to 10 minutes

4. In a separate tube, prepare the First-Strand cDNA Synthesis Master Mix as described in Table 3.2.

Table 3.2
First-Strand cDNA Synthesis Master Mix

Component	Volume in 1 Reaction
5X 1st Strand Buffer	4 µL
DTT, 0.1M	2 µL
dNTP+dUTP, 10 mM	1 µL
RNase Inhibitor	1 µL
SuperScript™ II	4 µL
Total Volume	12 µL

5. Transfer 12 µL of the First-Strand cDNA Synthesis Master Mix to the Total RNA/Random Primers Mix for a total reaction volume of 20 µL. Mix thoroughly by gently flicking the tubes a few times and centrifuge briefly.
6. Incubate the reactions in a thermal cycler (with heated lid) using the following protocol:
   - 25°C for 10 minutes
   - 42°C for 90 minutes
   - 70°C for 10 minutes
   - 4°C for 2 to 10 minutes

7. In a separate tube, prepare the Second-Strand cDNA Synthesis Master Mix as described in Table 3.3.

Table 3.3
Second-Strand cDNA Synthesis Master Mix

Component	Volume in 1 Reaction
$MgCl_2$*,17.5 mM	8 µL
dNTP+dUTP, 10 mM	1 µL
DNA Polymerase I	1.2 µL
RNase H	0.5 µL
RNase-free Water	9.3 µL
Total Volume	20 µL

**Make a fresh dilution of the $MgCl_2$ each time. Mix 2 µL of 1M $MgCl_2$ with 112 µL of Nuclease-free water.*

8. Add 20 µL of the Second-Strand cDNA Synthesis Master Mix to the First-Strand cDNA Synthesis Reaction for a total volume of 40 µL. Flick-mix the solution, and centrifuge briefly.
9. Incubate the reactions in a thermal cycler using the following protocol:
   - 16°C for 2 hours **without heated lid**
   - 75°C for 10 minutes **with heated lid**
   - 4°C hold
10. Store the sample at –20°C if not immediately purifying the Double-Stranded DNA.

Procedure B: Cleanup of Double-Stranded DNA

This Procedure requires the use of the GeneChip® Sample Cleanup Module (PN 900371).

1. Proceed to the Double-Stranded DNA clean-up using the cDNA Cleanup Spin Columns from the GeneChip Sample Cleanup Module following the protocol as described below.
2. If not already done, add 24 mL of ethanol (100%) to the cDNA Wash Buffer supplied in the GeneChip Sample Cleanup Module.
3. Add 60 µL of RNase-free water to each sample, 370 µL of cDNA Binding Buffer, and vortex for 3 seconds.
4. Apply the entire sample (the total volume is 470 µL) to a cDNA Spin Column in a 2 mL Collection Tube.
5. Spin at ≥ 8,000 x g for 1 minute. Discard the flow-through.
6. Transfer the cDNA Spin Column to a new 2 mL Collection Tube and add 750 µL of cDNA Wash Buffer to the column. Spin at ≥ 8,000 x g for 1 minute and discard the flow-through.
7. Open cap of the cDNA Spin Column, and spin at ≤ 25,000 x g for 5 minutes with the caps open. Discard the flow-through, and place the column in a 1.5 mL collection tube.
8. Pipette 15 µL of the cDNA Elution Buffer directly to the column membrane and incubate at room temperature for 1 minute. Then, spin at ≤ 25,000 x g for 1 minute.
9. Pipette 15 µL of the cDNA Elution Buffer directly to the column membrane and incubate at room temperature for 1 minute. Then, spin at ≤ 25,000 x g for 1 minute.

10. Quantify the collected DNA (approximately 28 µL in each sample). Take 2 µL from each sample to determine the yield by spectrophotometric UV measurement at 260 nm, 280 nm and 320 nm:

Concentration of Double-Stranded cDNA (µg/µL) = $\{A_{260} - A_{320}\}$ x 0.05 x dilution factor

µg DNA = eluate in µL x DNA in µg/µL

Each tube should have ≥7.5 µg of Double-Stranded DNA remaining.

Procedure C: Fragmentation of Double-Stranded DNA

Procedures C and D require the use of the GeneChip® WT Double Stranded DNA Terminal Labeling Kit (PN 900812).

1. Fragment the samples using the reagents in Table 3.4.

Table 3.4
Fragmentation of Double-Stranded DNA

Component	Volume/Amount in 1 Reaction
Double-Stranded DNA	7.5 µg
10X Fragmentation Buffer	4.8 µL
UDG, 10 U/µL	1.5 µL
APE 1, 100 U/µL	2.25 µL
RNase-free Water	up to 48 µL
Total Volume	48 µL

2. Add the above mix to the samples, flick-mix, and briefly centrifuge the tubes.
3. Incubate the reactions in a thermal cycler (with heated lid) using the following protocol:
   - 37°C for 1 hour
   - 93°C for 2 minutes
   - 4°C for 2 to 10 minutes
4. Flick-mix, briefly centrifuge the tubes, and transfer 45 µL of the sample to a new tube. Reserve the remaining samples as it is to be used for fragmentation analysis using a Bioanalyzer. Please see the Reagent Kit Guide that comes with the RNA 6000 LabChip® Kit for instructions.

NOTE **Results of the Bioanalyzer analysis of fragmented DNA should show that the majority of fragmented DNA is between 25 to 200 bases, with the peak of the distribution between 25 to 100 bases.**

5. If not labeling the samples immediately, store the fragmented Double-Stranded DNA at –20°C.

Procedure D: Labeling of Fragmented Double-Stranded DNA

1. Prepare the labeling reactions as described in Table 3.5.

Table 3.5
Single-Stranded DNA Fragmentation Master Mix

Component	Volume in 1 Reaction
5X TdT Buffer	12 µL
TdT, 30 U/µL	2 µL
DNA Labeling Reagent, 5 mM	1 µL
Total Volume	15 µL

2. Add 15 µL of the Double-Stranded DNA Fragmentation Master Mix to 45 µL of the fragmented double-stranded DNA samples, flick-mix, and centrifuge briefly.
3. Incubate the reactions in a thermal cycler (with heated lid) using the following protocol:
   - 37°C for 60 minutes
   - 70°C for 10 minutes
   - 4°C for 2 to 10 minutes
4. Remove 4 µL of each sample for Gel-shift analysis (optional) as described in Appendix A.
5. Store at –20°C or go to Chapter 4, *Hybridization* on page 49.

Chapter 4

Hybridization

Chapter 4

Hybridization of Labeled Target on the Arrays

This procedure requires the use of the GeneChip® Hybridization, Wash, and Stain Kit (P/N 900720). Alternatively, users may prepare their own hybridization mix using Table B.2 in Appendix B.

1. Depending on the array type used, prepare the Hybridization Cocktail in a 1.5 mL RNase-free microfuge tube as shown in Table 4.1 or Table 4.2.

Table 4.1
Hybridization Cocktail for use with serial hybridizations (e.g., GeneChip® Human Tiling 1.0R Array Set and GeneChip® Mouse Tiling 1.1R Array Set)

Component	Volume in 1 Reaction	Final Concentration or Amount
Fragmented and Labeled DNA Target	~60.0* µL	~7.5 µg
Control Oligonucleotide B2	4.17 µL	50 pM
2X Hybridization Mix	125 µL	1X
DMSO	17.5 µL	7%
RNase-free Water	up to 250.0 µL	
Total Volume	250.0 µL	

**This volume is 56 µL if a portion of the sample was set aside for gel-shift analysis.*

Table 4.2
Hybridization Cocktail for single tiling arrays (e.g., GeneChip® Arabidopsis Tiling 1.0R Array)

Component	Volume in 1 Reaction	Final Concentration or Amount
Fragmented and Labeled DNA Target	~60.0* µL	~37.5 ng/µL
Control Oligonucleotide B2	3.3 µL	50 pM
2X Hybridization Mix	100 µL	1X
DMSO	14 µL	7%
RNase-free Water	up to 200.0 µL	
Total Volume	200.0 µL	

**This volume is 56 µL if a portion of the sample was set aside for gel-shift analysis.*

2. Flick-mix, and centrifuge the tube.

NOTE

If samples will not be hybridized immediately, store the hybridization cocktails at –20°C.

3. Heat the Hybridization Cocktail at 99°C for 5 minutes. Cool to 45°C for 5 minutes, and centrifuge at maximum speed for 1 minute.
4. Inject the appropriate amount of the specific sample (200 µL for 49-format arrays and 130 µL for 100-format arrays) into the array through one of the septa (see Figure 4.1 for location of the septa on the array).

IMPORTANT

For hybridization to multiple-design tiling array sets, it is required to save the remaining 50 µL of Hybridization Cocktail to be reused for subsequent hybridizations. Place the saved Hybridization Cocktail in –20°C storage until needed.

5. Place array in 45°C hybridization oven, at 60 rpm, and incubate for 16 hours.

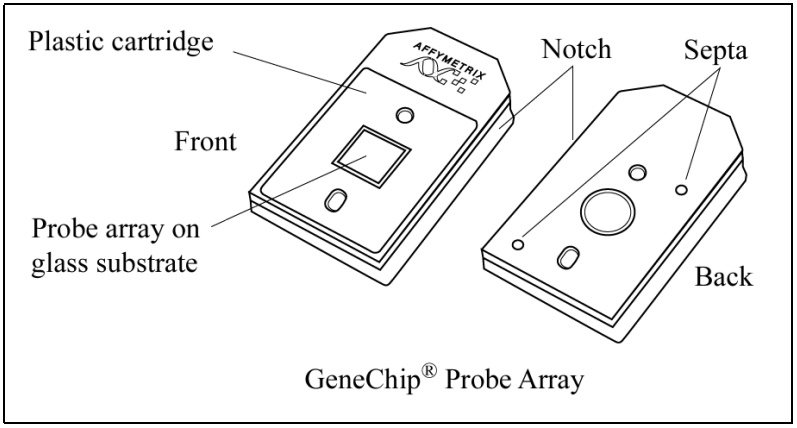


Figure 4.1
GeneChip® Probe Array

Chapter 5

Array Washing and Staining

Chapter 5

Procedure A: Enter Experiment Information

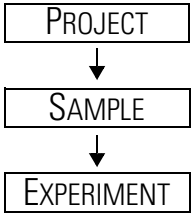
To wash, stain, and scan a probe array, an experiment must first be registered in GeneChip® Operating Software (GCOS). Please follow the instructions detailed in the "Setting Up an Experiment" section of the GCOS User's Guide.

The fields of information required are:

- Experiment Name
- Probe Array Type (For multi-array sets, please refer to the design number indicated on the array cartridge label.)
- Sample Name
- Sample Type
- Project

Sample templates, experiment templates, and array barcodes can also be employed in GCOS to standardize and simplify the registration process. Please see the *Affymetrix GeneChip® Operating Software User's Guide* (P/N 701439) for more information.

The Project, Sample Name, and Experiment Name fields establish a sample hierarchy that organizes GeneChip data in GCOS. In terms of the organizational structure, the Project is at the top of the hierarchy, followed by Sample Name and then Experiment Name.



Procedure B: Prepare the Fluidics Station

The GeneChip® Fluidics Station 450/250 or 400 is used to wash and stain GeneChip® Tiling Arrays. It is operated using GCOS.

Use the GeneChip® Hybridization, Wash, and Stain Kit (P/N 900720) or prepare buffers as indicated in Appendix B.

SET UP THE FLUIDICS STATION

1. Turn on the Fluidics Station using the toggle switch on the lower left side of the machine.
2. Select **Run → Fluidics** from the menu bar.

   The Fluidics Station dialog box appears with a drop-down list for selecting the experiment name for each of the fluidics station modules. A second drop-down list is accessed for choosing the Protocol for each of the fluidics station modules.

NOTE **Refer to the *Fluidics Station User's Guide* for instructions on connecting and addressing multiple fluidics stations.**

PRIME THE FLUIDICS STATION

Priming ensures that the lines of the GeneChip® Fluidics Station are filled with the appropriate buffers and the Fluidics Station is ready for running fluidics station protocols.

Priming should be done:

- when the Fluidics Station is first started.
- when wash solutions are changed.
- before washing, if a shutdown has been performed.
- if the LCD window instructs the user to prime.

1. To prime the fluidics station, select **Protocol** in the Fluidics Station dialog box.
2. Choose **Prime_450** (for FS450) or **Prime** (for FS400) for the

respective modules in the **Protocol** drop-down list.

3. Change the intake buffer reservoir A to **Wash Buffer A** (non-stringent wash buffer), and intake buffer reservoir B to **Wash Buffer B** (stringent wash buffer).
4. Select the **All Modules** check box, then click **Run**.

Procedure C: Wash and Stain Probe Arrays

After 16 hours of hybridization, remove the hybridization cocktail from the probe array and add to any remaining Hybridization Cocktail that was saved in Chapter 4, Procedure A. For rehybridizations onto additional tiling array designs, repeat steps 3 through 5 in Chapter 4 on page 52. Fill the probe array completely with the appropriate volume of Non-Stringent Wash Buffer (Wash Buffer A), as described in Table 5.1 below.

Table 5.1

Format	Total Fill Volume
49 e.g., Human Tiling 1.0R Array Set	250 µL
64 e.g., Human Promoter 1.0R Array	250 µL
100 e.g., *S. pombe* Tiling 1.0 FR Array	160 µL

NOTE **If necessary, at this point, the probe array can be stored at 4°C for up to 3 hours before proceeding with washing and staining. Equilibrate the probe array to room temperature before washing and staining.**

This procedure takes approximately 90 minutes to complete.

FLUIDICS PROTOCOLS

Table 5.2
Fluidics Protocols for GeneChip® Tiling Arrays

	Fluidics Station 400 EukGE-WS2v5 / Midi_euk2v3	Fluidics Station 450 FS450_0001 / FS450_0002
Post Hyb Wash #1	10 cycles of 2 mixes/cycle with Wash Buffer A at 30°C	10 cycles of 2 mixes/cycle with Wash Buffer A at 30°C
Post Hyb Wash #2	6 cycles of 15 mixes/cycle with Wash Buffer B at 50°C	6 cycles of 15 mixes/cycle with Wash Buffer B at 50°C
Stain	Stain the probe array for 10 minutes in Stain Cocktail 1 at 35°C	Stain the probe array for 5 minutes in Stain Cocktail 1 at 35°C
Post Stain Wash	10 cycles of 4 mixes/cycle with Wash Buffer A at 30°C	10 cycles of 4 mixes/cycle with Wash Buffer A at 30°C
2nd Stain	Stain the probe array for 5 minutes in Stain Cocktail 2 at 35°C	Stain the probe array for 5 minutes in Stain Cocktail 2 at 35°C
3rd Stain	Stain the probe array for 5 minutes in Stain Cocktail 1 at 35°C	Stain the probe array for 5 minutes in Stain Cocktail 1 at 35°C
Final Wash	15 cycles of 4 mixes/cycle with Wash Buffer A at 35°C. The holding temperature is 25°C	15 cycles of 4 mixes/cycle with Wash Buffer A at 35°C.
Holding Buffer	N/A - manual process	Fill the probe array with Array Holding Buffer.

- Wash Buffer A = non-stringent wash buffer
- Wash Buffer B = stringent wash buffer

Table 5.3

Format	FS450 Fluidics Protocol	FS400 Fluidics Protocol
49 e.g., Human Tiling 1.0R Array Set	FS450_0001	EukGE-WS2v5 and add Array Holding Buffer
64 e.g., Human Promoter 1.0R Array	FS450_0001	EukGE-WS2v5 and add Array Holding Buffer
100 e.g., *S. pombe* Tiling 1.0 FR Array	FS450_0002	Midi_euk2v3 and add Array Holding Buffer

WASH AND STAIN THE PROBE ARRAY ON FLUIDICS STATION 450

NOTE

If a Fluidics Station 450 instrument is unavailable, proceed with washing and staining with the appropriate FS400 fluidics protocol and save first vial of Stain Cocktail 1 to be reused in the 3rd Stain step. Add Holding Buffer to the cartridge manually prior to scanning.

1. In the Fluidics Station dialog box on the workstation, select the correct experiment name from the drop-down **Experiment** list.

   The **Probe Array Type** appears automatically.

2. In the **Protocol** drop-down list, select FS450_0001 or FS450_0002 to control the washing and staining of the probe array.

3. Choose **Run** in the Fluidics Station dialog box to begin the washing and staining.

   Follow the instructions in the LCD window on the fluidics station.

   If you are unfamiliar with inserting and removing probe arrays from the fluidics station modules, please refer to the appropriate *Fluidics Station User's Guide* or *Quick Reference Card* (P/N 08-0093 for the FS-450/250 fluidics station).

4. Insert the appropriate probe array into the designated module of the fluidics station while the cartridge lever is in the down, or

eject, position. When finished, verify that the cartridge lever is returned to the up, or engaged position.

5. Remove any microcentrifuge vials remaining in the sample holders of the fluidics station module(s) being used.
6. Follow the instructions on the LCD window. Place the following three vials (the microcentrifuge vials) into the sample holders 1, 2, and 3 on the fluidics station.
   - Place one vial containing 600 µL of Stain Cocktail 1 in sample holder 1.
   - Place one vial containing 600 µL of Stain Cocktail 2 in sample holder 2.
   - Place one vial containing 800 µL Array Holding Buffer in sample holder 3.
   - Press down on the needle lever to snap needles into position and to start the run.

   The run begins. The Fluidics Station dialog boxes at the workstation terminal and the LCD window display the status of the washing and staining as they progress.
7. At the end of the run, or at the appropriate prompt, remove the microcentrifuge vials containing the stain solutions and replace with three empty microcentrifuge vials.
8. Remove the probe arrays from the fluidics station modules by first pressing down the cartridge lever to the eject position.
9. Check the probe array window for large bubbles or air pockets.
   - If bubbles are present, proceed to Table 5.4.
   - If the probe array has no large bubbles, it is ready to be scanned on GeneChip® Scanner 3000 7G. Pull up on the cartridge lever to engage washblock and proceed to Chapter 6, *Scanning* on page 63.

   If you do not scan the arrays right away, keep the probe arrays at 4°C and in the dark until ready for scanning.

   If there are no more samples to hybridize, shut down the fluidics station following the procedure outlined in the section, *Shut Down the Fluidics Station* on page 62.

Table 5.4

If Bubbles are Present
Return the probe array to the probe array holder. Engage the washblock by gently pushing up on the cartridge lever to the engaged, or closed, position. Follow the instructions on the LCD window. The fluidics station will drain the probe array and then fill it with a fresh volume of Array Holding Buffer. When finished, the LCD window displays 'EJECT CARTRIDGE.' Again, remove the probe array and inspect for bubbles. If no bubbles are present it is ready to be scanned. Proceed to Chapter 6, *Scanning* on page 63. If your attempt to fill the probe array without bubbles is unsuccessful manually drain the 1x Array Holding Buffer from the array, using a micropipette, and fill the array completely with a fresh aliquot of 1x Array Holding Buffer. Inspect the array and ensure that no bubbles are present. Excessive washing will result in a loss of signal intensity.

SHUT DOWN THE FLUIDICS STATION

1. After removing a probe array from the probe array holder, the LCD window displays the message **ENGAGE WASHBLOCK**.
2. If you are using the Fluidics Station 400, latch the probe array holder by gently pushing up until a light click is heard. Engage the washblock by firmly pushing up on the cartridge lever to the **ENGAGE** position.

   If you are using the Fluidics Station 450, gently lift up the cartridge lever to engage, or close, the washblock.

   - The fluidics station automatically performs a Cleanout procedure. The LCD window indicates the progress of the Cleanout procedure.
3. When the fluidics station LCD window indicates **REMOVE VIALS**, the Cleanout procedure is complete.
4. Remove the sample microcentrifuge vial(s) from the sample holder(s).
5. If no other arrays are to be processed, place wash lines into a bottle filled with deionized water.
6. Choose **Shutdown** (FS400) or **Shutdown_450 (FS450)** for all modules from the drop-down **Protocol** list in the Fluidics Station dialog box. Click the **Run** button for all modules.

   The Shutdown protocol is critical to instrument reliability. Refer to the appropriate Fluidics Station User's Guide for more information.
7. After Shutdown protocol is complete, flip the ON/OFF switch of the fluidics station to the OFF position.

IMPORTANT !

To maintain the cleanliness of the fluidics station and obtain the highest quality image and data possible, the bleach protocol is highly recommended. Please refer to the *GeneChip® Fluidics Station 450/250 User's Guide* (P/N 08-0092) available at www.affymetrix.com.

Chapter 6

Scanning

Chapter 6

Scanning

The GeneChip® Scanner 3000 7G is also controlled by GeneChip® Operating Software (GCOS). The probe array is scanned after the wash protocols are complete. Make sure the laser is warmed up prior to scanning by turning it on at least 10 minutes prior to use. If probe array was stored at 4°C, warm to room temperature before scanning. Refer to the GCOS online help and the appropriate scanner user's manual for more information on scanning.

WARNING ▲

The scanner uses a laser and is equipped with a safety interlock system. Defeating the interlock system may result in exposure to hazardous laser light.

You must have read, and be familiar with, the operation of the scanner before attempting to scan a probe array. Please refer to the the *GeneChip® Scanner 3000 Quick Reference Card* or user's manual.

HANDLING THE PROBE ARRAY

Before you scan the probe array, follow the directions in this section on handling the probe array. If necessary, clean the glass surface of the probe array with a non-abrasive towel or tissue before scanning. Do not use alcohol to clean glass.

Before scanning the probe array cartridge, apply Tough-Spots® label dots to each of the two septa on the probe array cartridge to prevent the leaking of fluids from the cartridge during scanning.

IMPORTANT !

Apply the spots just before scanning.

1. On the back of the probe array cartridge, clean excess fluid from around septa.
2. Carefully apply one Tough-Spots to each of the two septa. Press to ensure that the spots remain flat. If the Tough-Spots do not apply smoothly, that is, if you observe bumps, bubbles, tears, or curled edges, do not attempt to smooth out the spot. Remove the spot and apply a new spot. See Figure 6.1.

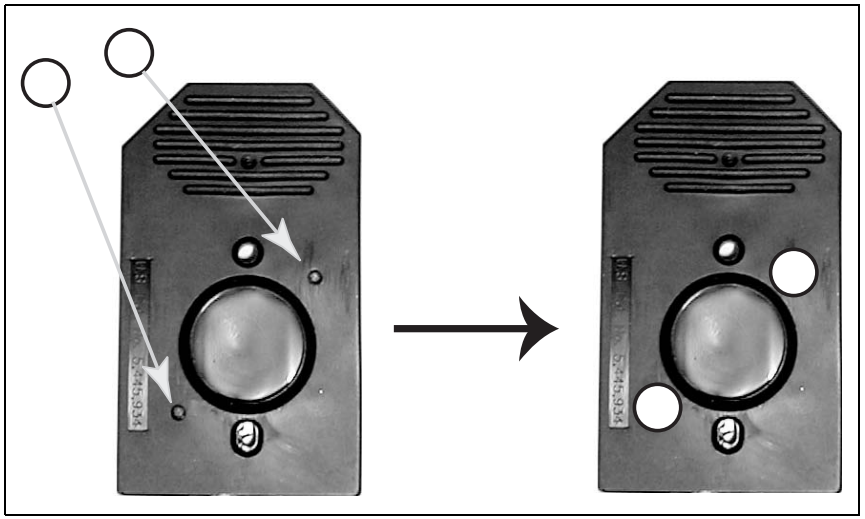


Figure 6.1
Applying Tough-Spots® to the probe array cartridge

3. Insert the cartridge into the scanner and test the autofocus to ensure that the Tough-Spots do not interfere with the focus. If you observe a focus error message, remove the spot and apply a new spot. Ensure that the spots lie flat.

SCANNING THE PROBE ARRAY

1. Select **Run → Scanner** from the menu bar. Alternatively, click the **Start Scan** icon in the tool bar.
   - The Scanner dialog box appears with a drop-down list of experiments that have not been run.
2. Select the experiment name that corresponds to the probe array to be scanned.

   A previously run experiment can also be selected by using the **Include Scanned Experiments** option box. After selecting this option, previously scanned experiments appear in the drop-down list.
3. Once the experiment has been selected, click the **Start** button.
   - A dialog box prompts you to load an array into the scanner.
4. Open the sample door on the scanner and insert the probe array into the holder. Do not force the probe array into the holder. Close the sample door of the scanner.
5. Click **OK** in the Start Scanner dialog box.
   - The scanner begins scanning the probe array and acquiring data. When **Scan in Progress** is selected from the **View** menu, the probe array image appears on the screen as the scan progresses.

Appendix A

Gel-Shift Assay

Appendix A

Gel-Shift Assay

The efficiency of the labeling procedure can be assessed using the following procedure. This quality control protocol prevents hybridizing poorly labeled target onto the probe array. The addition of biotin residues is monitored in a gel-shift assay, where the fragments are incubated with avidin prior to electrophoresis. The nucleic acids are then detected by staining, as shown in the gel photograph Figure A.1. The procedure takes approximately 90 minutes to complete.

NOTE

The absence of a shift pattern indicates poor biotin labeling. The problem should be addressed before proceeding to the hybridization step.

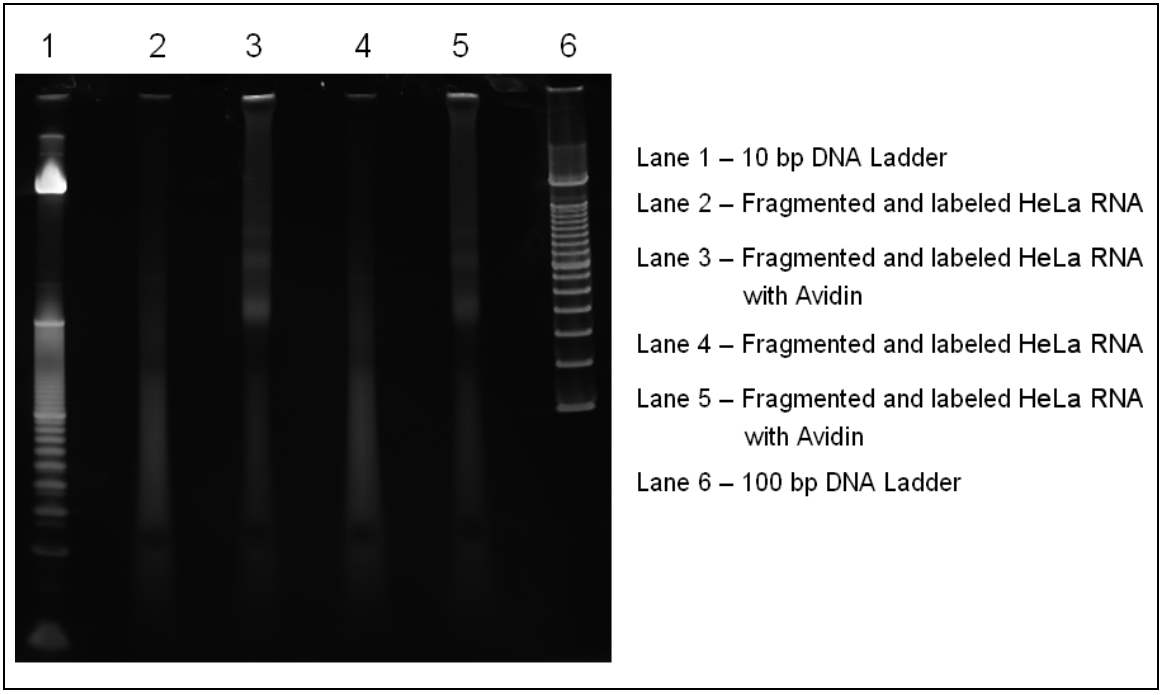


Figure A.1
Gel-Shift

1. Prepare a NeutrAvidin solution of 2 mg/mL. Following the manufacturer's recommendation, reconstitute the NeutrAvidin with molecular biology grade water and then dilute to 2 mg/mL with PBS.
2. Place a 4% to 20% TBE gel into the gel holder and load system with 1X TBE Buffer.
3. For each sample to be tested, remove two 1 µL aliquots of fragmented and biotinylated sample to fresh tubes. Heat the aliquots of samples at 70°C 2 minutes.
4. Add 5 µL of 2 mg/mL NeutrAvidin to one of the two tubes for each sample tested.
5. Mix and incubate at room temperature for 5 minutes.

6. Add loading dye to all samples to a final concentration of 1X loading dye.
7. Prepare 10 bp and 100 bp DNA ladders (1 µL ladder + 7 µL water + 2 µL loading dye for each lane).
8. Carefully load samples and two ladders on gel. Each well can hold a maximum of 20 µL.
9. Run the gel at 150 volts until the front dye (red) almost reaches the bottom. The electrophoresis takes approximately 1 hour.
10. While the gel is running, prepare at least 100 mL of a 1X solution of SYBR Gold for staining.

NOTE

SYBR Gold is light sensitive. Therefore, use caution and shield the staining solution from light. Prepare a new batch of stain at least once a week.

11. After the gel is complete, break open cartridge and stain the gel in 1X SYBR Gold for 10 minutes.
12. Place the gel on the UV light box and produce an image following standard procedure. Be sure to use the appropriate filter for SYBR Gold.

Appendix B

User-Prepared Buffers and Solutions for Array Hybridization, Washing, and Staining

Appendix B

Buffers and Solutions for Array Hybridization, Washing, and Staining

Table B.1
Reagents for Hybridization, Wash, and Satin

Material	Source	P/N
GeneChip® Control Oligo B2, 3 nM	Affymetrix	900301
Acetylated Bovine Serum Albumin (BSA) solution (50 mg/mL)*	Invitrogen	15561-020
Herring Sperm DNA*	Promega	D1811
R-Phycoerythrin Streptavidin*	Molecular Probes	S-866
Goat IgG, Reagent Grade*	Sigma-Aldrich	I 5256
Anti-streptavidin antibody (goat), biotinylated *	Vector Laboratories	BA-0500
Water, Molecular Biology Grade*	Cambrex	51200
5M NaCl, RNase-free, DNase-free*	Ambion	9760G
MES hydrate SigmaUltra*	Sigma-Aldrich	M5287
MES Sodium Salt*	Sigma-Aldrich	M5057
EDTA Disodium Salt, 0.5M solution (100 mL)*	Sigma-Aldrich	E7889
DMSO*	Sigma-Aldrich	D5879
Surfact-Amps® 20 (Tween-20), 10%*	Pierce	28320
PBS, pH 7.2*	Invitrogen	20012-027
20X SSPE (3M NaCl, 0.2M NaH_2PO_4, 0.02M EDTA)*	Cambrex	51214
Tough-Spots®, Label Dots	USA Scientific	9185-0000

** Available in the GeneChip® Hybridization, Wash, and Stain Kit (P/N 900720).*

Table B.2
Buffers Required to be Prepared

12X MES Stock Buffer (1.22M MES, 0.89M [Na^+])
For 1,000 mL: 64.61g of MES hydrate 193.3g of MES Sodium Salt 800 mL of Molecular Biology Grade water Mix and adjust volume to 1,000 mL The pH should be between 6.5 and 6.7. Filter through a 0.2 µm filter. **IMPORTANT:** Do not autoclave. Store at 2°C to 8°C and shield from light. Discard solution if yellow.
2X Hybridization Buffer (Final 1X concentration: 100 mM MES, 1M [Na^+], 20 mM EDTA, 0.01% Tween-20)
For 50 mL: 8.3 mL of 12X MES Stock Buffer 17.7 mL of 5M NaCl 4.0 mL of 0.5M EDTA 0.1 mL of 10% Tween-20 19.9 mL of water **IMPORTANT:** Store at 2°C to 8°C and shield from light.
Wash Buffer A: Non-Stringent Wash Buffer (included in P/N 900720) (6X SSPE, 0.01% Tween-20)
For 1,000 mL: 300 mL of 20X SSPE 1.0 mL of 10% Tween-20 699 mL of water Filter through a 0.2 µm filter.

Table B.2 (Continued)
Buffers Required to be Prepared

Wash Buffer B: Stringent Wash Buffer (included in P/N 900720) (100 mM MES, 0.1M [Na^+], 0.01% Tween-20) **For 1,000 mL:** 83.3 mL of 12X MES Stock Buffer 5.2 mL of 5M NaCl 1.0 mL of 10% Tween-20 910.5 mL of water Filter through a 0.2 µm filter. **IMPORTANT:** Store at 2°C to 8°C and shield from light.
2X Stain Buffer (Final 1X concentration: 100 mM MES, 1M [Na^+], 0.05% Tween-20) **For 250 mL:** 41.7 mL of 12X MES Stock Buffer 92.5 mL of 5M NaCl 2.5 mL of 10% Tween-20 113.3 mL of water Filter through a 0.2 µm filter. **IMPORTANT:** Store at 2°C to 8°C and shield from light.
10 mg/mL Goat IgG Stock Resuspend 50 mg in 5 mL of 150 mM NaCl. Store at 4°C **IMPORTANT:** If a larger volume of the 10 mg/mL IgG stock is prepared, aliquot and store at –20°C until use. After the solution has been thawed it should be stored at 4°C. Avoid additional freezing and thawing.
1X Array Holding Buffer (100 mM MES, 1M [Na^+], 0.01% Tween-20) **For 100 mL:** 8.3 mL of 12X MES Stock Buffer 18.5 mL of 5M NaCl 0.1 mL of 10% Tween-20 73.1 mL of water **IMPORTANT:** Store at 2°C to 8°C and shield from light.

Table B.3
Hybridization Cocktail for Human 1.0R and Mouse 1.1R Tiling Sets (if not using the GeneChip® Hybridization, Wash, and Stain Kit)

Component	Volume in 1 Rection	Final Concentration or Amount
Fragmented and Labeled DNA Target	~60 µL	~ 7.5 µg
Control Oligonucleotide B2	4.17 µL	50 pM
Herring Sperm DNA (10 mg/mL)	2.0 µL	0.1 mg/mL
Acetylated BSA (50 mg/mL)	2.5 µL	0.5 mg/mL
2X Hybridization Buffer	125 µL	1X
DMSO	17.5 µL	7%
RNase-free Water	up to 250.0 µL	
Total Volume	250.0 µL	

Table B.4
Hybridization Cocktail for arrays other than Human 1.0R and Mouse 1.1R Tiling Sets (if not using the GeneChip® Hybridization, Wash, and Stain Kit)

Component	Volume in 1 Rection	Final Concentration or Amount
Fragmented and Labeled DNA Target	~60 µL	~ 37.5 µg
Control Oligonucleotide B2	3.33 µL	50 pM
Herring Sperm DNA (10 mg/mL)	2.0 µL	0.1 mg/mL
Acetylated BSA (50 mg/mL)	2.0 µL	0.5 mg/mL
2X Hybridization Buffer	100 µL	1X
DMSO	14 µL	7%
RNase-free Water	up to 200.0 µL	
Total Volume	200.0 µL	

Preparing the Staining Reagents

Prepare the following reagents. Volumes given are sufficient for one probe array.

SAPE Stain Solution

Streptavidin Phycoerythrin (SAPE) should be stored in the dark at 4°C, either foil-wrapped or kept in an amber tube. Remove SAPE from the refrigerator and tap the tube to mix well before preparing stain solution. Do not freeze SAPE. Always prepare the SAPE stain solution fresh, on the day of use.

Table B.5
SAPE Solution Mix

Components	Volume	Final Concentration
2X Stain Buffer	300 µL	1X
50 mg/mL BSA	24 µL	2 mg/mL
1 mg/mL Streptavidin Phycoerythrin (SAPE)	6 µL	10 µg/mL
Molecular Biology Grade Water	270 µL	—
Total Volume	600 µL	

Mix well. The 600 µL of SAPE Solution Mix will be used for the 1st and 3rd stain.

NOTE **If using the Fluidics Station 400, after the first stain is done save the tube with the SAPE stain solution. Reuse the saved tube for the third stain.**

Antibody Solution

Table B.6
Antibody Solution Mix

Components	Volume	Final Concentration
2X Stain Buffer	300.0 µL	1X
50 mg/mL BSA	24.0 µL	2 mg/mL
10 mg/mL Goat IgG Stock	6.0 µL	0.1 mg/mL
0.5 mg/mL biotinylated antibody	3.6 µL	3 µg/mL
Molecular Biology Grade Water	266.4 µL	—
Total Volume	600 µL	

Mix well. The 600 µL of Antibody Solution Mix will be used for the 2nd Stain.

Array Holding Buffer

To prepare the Array Holding Buffer, refer to Table B.2 on page 79.

Appendix C

Contact Information

Appendix C

Contact Information

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