User Manual

GeneChip® WT Terminal Labeling and Hybridization User Manual

for use with the Ambion® WT Expression Kit

P/N 702808 Rev. 6

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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.

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Overview

In conjunction with the Affymetrix® GeneChip® WT Terminal Labeling Kit, the Ambion® WT Expression Kit is designed to generate amplified and biotinylated sense-strand DNA targets from the entire expressed genome without bias. This assay and associated reagents have been optimized specifically for use with the GeneChip® ST Arrays where "ST" stands for "Sense Target," and the probes on the arrays have been selected to be distributed throughout the entire length of each transcript.

NOTE: The WT Assay is not compatible with GeneChip® brand arrays designed to focus on the 3' ends of the transcripts. For the 3' arrays, please use the Affymetrix GeneChip® 3' IVT Express Kit (visit www.affymetrix.com for more information)

This manual describes in detail how to use the Affymetrix GeneChip WT Terminal Labeling Kit in conjunction with the Ambion WT Expression Kit to generate sense-strand target for hybridization onto GeneChip ST Arrays. Figure 1.1 depicts the workflow for the complete WT assay. The Ambion WT Expression Kit generates purified sense-strand cDNA (with incorporated dUTP) that is ready for fragmentation and labeling using the Affymetrix GeneChip WT Terminal Labeling Kit.

The Ambion WT Expression Kit uses a novel method for priming the reverse transcription step of the 1st cycle, 1st strand cDNA synthesis reaction. The kit employs an engineered set of primers that exclude sequences that match Ribosomal RNA (rRNA). The result is a priming method that specifically primes non-ribosomal RNA from a total RNA sample (including both polyA and non-polyA containing mRNAs) and eliminates the need for an up-front rRNA-reduction step for optimal exon-level performance. Furthermore, the enzymatic reactions have been optimized so that lower total RNA input levels can be used to generate the same mass of target for hybridization onto Affymetrix GeneChip® Exon and Gene Arrays.

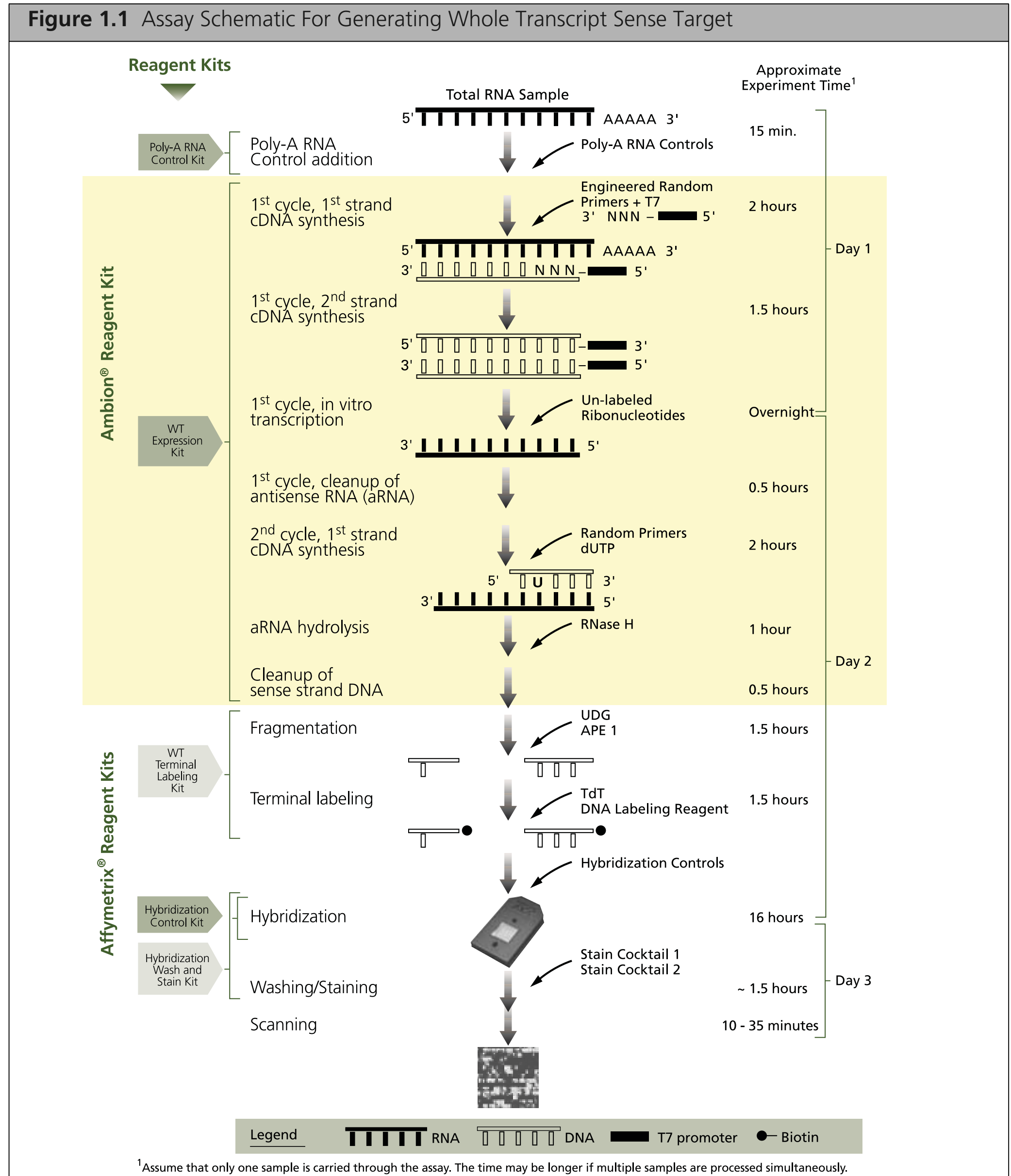
In order to reproducibly fragment the single-stranded DNA, the Affymetrix GeneChip WT Terminal Labeling Kit employs a novel approach where dUTP is incorporated in the DNA during the second-cycle, first-strand reverse transcription reaction. This single-stranded DNA sample is then treated with a combination of uracil DNA glycosylase (UDG) and apurinic/apyrimidinic endonuclease 1 (APE 1) that specifically recognizes the unnatural dUTP residues and breaks the DNA strand. DNA is labeled by terminal deoxynucleotidyl transferase (TdT) with the Affymetrix® proprietary DNA Labeling Reagent that is covalently linked to biotin.

Following the recommended procedures, sufficient target is anticipated to be generated for hybridization to a single array.

Follow the instructions closely for the most optimal results. As an Affymetrix GeneChip microarray user, your feedback is welcome. Please contact your technical support representative with any input on how we can improve this resource.

Whole Transcript Sense Target Labeling Assay Schematic

Figure 1.1 Assay Schematic For Generating Whole Transcript Sense Target



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

Materials

Necessary Reagents

Table 1.1 Necessary Reagents

Material	Source	P/N
Ambion® WT Expression Kit* Contains: ■ First-Strand Enzyme Mix ■ First-Strand Buffer Mix ■ Second-Strand Enzyme Mix ■ Second-Strand Buffer Mix ■ IVT Enzyme Mix ■ IVT Buffer Mix ■ Control RNA ■ Nuclease-free Water ■ 2nd-Cycle Buffer Mix ■ Random Primers ■ 2nd-Cycle Enzyme Mix ■ RNase H ■ Nucleic Acid Binding Buffer Concentrate ■ Nucleic Acid Binding Beads ■ Nucleic Acid Wash Solution Concentrate ■ Elution Solution ■ 8-Strip PCR Tubes & Caps ■ U-Bottom Plate ■ Reservoir	Ambion	4411973 (10 Rxn) or 4411974 (30 Rxn)
Controls		
GeneChip® Poly-A RNA Control Kit Contains: ■ Poly-A Control Stock ■ Poly-A Control Dil Buffer	Affymetrix	900433 (100 Rxn)
GeneChip® Hybridization Control Kit Contains: ■ 20X Hybridization Controls ■ 3 nM Control Oligo B2	Affymetrix	900454 (30 Rxn) or 900457 (150 Rxn)

** Please see the user manual for the Ambion® WT Expression Kit for other necessary reagents, equipment, and supplies.*

Table 1.1 (Continued) Necessary Reagents

Material	Source	P/N
Fragmentation and Labeling		

GeneChip® WT Terminal Labeling Kit and Controls Kit*
Contains:
▪ GeneChip® WT Terminal Labeling Kit (900671, 30 Rxn or 900670, 10 Rxn)
  ▫ 10X cDNA Fragmentation Buffer
  ▫ UDG, 10 U/µL
  ▫ APE 1, 1,000 U/µL
  ▫ 5X TdT Buffer
  ▫ TdT, 30 U/µL
  ▫ DNA Labeling Reagent, 5 mM
  ▫ RNase-free Water
▪ GeneChip® Poly-A RNA Control Kit (900433, 100 Rxn)
  ▫ poly-A Control Stock
  ▫ poly-A Control Dil Buffer
▪ GeneChip® Hybridization Control Kit (900454, 30 Rxn or 900457, 150 Rxn)
  ▫ 20X Hybridization Controls
  ▫ 3 nM Control Oligo B2
	Affymetrix	901524 (30 Rxn) or 901525 (10 Rxn)

GeneChip® WT Terminal Labeling Kit Contains: ▪ 10X cDNA Fragmentation Buffer ▪ UDG, 10 U/µL ▪ APE 1, 1,000 U/µL ▪ 5X TdT Buffer ▪ TdT, 30 U/µL ▪ DNA Labeling Reagent, 5 mM ▪ RNase-free Water	Affymetrix	900671 (30 Rxn) or 900670 (10 Rxn)
Hybridization, Stain and Wash - Cartridges		

GeneChip® Hybridization, Wash, and Stain Kit
Containing:
▪ Hybridization Module from Box 1
  ▫ Pre-Hybridization Mix
  ▫ 2X Hybridization Mix
  ▫ DMSO
  ▫ Nuclease-free water
▪ Stain Module from Box 1
  ▫ Stain Cocktail 1
  ▫ Stain Cocktail 2
  ▫ Array Holding Buffer
▪ Wash Buffers A and B from Box 2
  ▫ Wash Buffer A (P/N 900721)
  ▫ Wash Buffer B (P/N 900722)
	Affymetrix	900720 (30 Rxn)

** Individual components available for purchase separately.*

Table 1.1 (Continued) Necessary Reagents

Material	Source	P/N
Hybridization, Stain and Wash - Gene 1.1 ST Array Plates Processed on the GeneTitan® Instrument		
GeneTitan® Hybridization, Wash and Stain Kit for WT Array Plates Contains: ▪ GeneTitan® Hybridization Module for WT Array Plates ▪ GeneTitan® Wash Buffers A & B Module (P/N 901583)*	Affymetrix	901622 (96 Rxn)

* *Individual kit component may be ordered separately.*

Miscellaneous Reagents

Table 1.2 Miscellaneous Reagents

Materials	Source	P/N
Miscellaneous Reagents		
RNA 6000 Nano Kit	Agilent	5067-1511
Gel-Shift Assay (Optional)		
Novex XCell SureLock Mini-Cell*	Invitrogen	EI0001
TBE Gel, 4-20%,1.0 mm, 12 well*	Invitrogen	EC62252
Novex Hi-Density TBE Sample Buffer (5X)	Invitrogen	LC6678
TBE Buffer, 5x Solution	USB	75891
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
Tough-Spots®	USA Scientific	9185
GeneTitan® Consumable Upgrade Kit†	Affymetrix	901333

* *Or equivalent.*

† *For Gene 1.1 ST Array Plates Processed on the GeneTitan Instrument.*

Instruments

Table 1.4 Instruments

Instruments	Manufacturer	P/N
NanoDrop ND-1000*	NanoDrop Technologies	N/A
GeneChip® Hybridization Oven 640	Affymetrix	800138 (110 v) 800139 (220 v)
Eppendorf Centrifuge*	Eppendorf	5417C
Tube-Strip Picofuge*	Stratagene	400540
PicoFuge*	Stratagene	400550
GeneChip® Fluidics Station 450	Affymetrix	00-0079
GeneChip® Scanner 3000 7G	Affymetrix	00-0212 (North America) 00-0213 (International)
GeneChip® AutoLoader with External Barcode Reader (Optional)	Affymetrix	00-0090 (GCS 3000 7G S/N 501) 00-0129 (GCS 3000 7G S/N 502)
ABI GeneAmp PCR System 9700*	Applied Biosystems	N8050001
Bioanalyzer 2100	Agilent	G2940CA
Heating blocks*	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.*

Suggested Workflow[1]

The suggested workflow outlined below includes references to the Ambion® WT Expression Assay. For detailed information regarding the Ambion steps, please refer to the Ambion® WT Expression Kit for Affymetrix® GeneChip® Whole Transcript (WT) Expression Arrays, P/N 4425209.

Ambion

Day 1

- Synthesize first-strand cDNA
- Synthesize second-strand cDNA
- Synthesize cRNA using *in vitro* transcription

Day 2

- Purify cRNA
- Assess cRNA yield and size distribution
- Synthesize 2nd-cycle cDNA
- Hydrolyze using RNaseH
- Purify 2nd-cycle cDNA
- Assess cDNA yield and size distribution

Affymetrix

- Fragment and label the single-stranded cDNA
  - *Fragmentation of Single-Stranded DNA* on page 13
  - *Labeling of Fragmented Single-Stranded DNA* on page 15
- Start Hybridization – 17 hours – Start on Day 2, finish on Day 3
  - *Target Hybridization for Cartridge Arrays* on page 16

Day 3

- Array Washing, Staining, and Scanning – 2 hours. Please refer to the *GeneChip® Expression Wash, Stain and Scan User Manual* (P/N 702731).

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

Terminal Labeling and Hybridization

The GeneChip® WT Terminal Labeling and Hybridization User Manual is specifically designed to follow procedures detailed in the Ambion® WT Expression Kit for Affymetrix® GeneChip® Whole Transcript (WT) Expression Arrays, P/N 4425209. After completing the Ambion procedure "Assess cDNA yield and size distribution" please proceed with the steps outlined in this chapter.

Fragmentation of Single-Stranded DNA

This Procedure requires the use of the GeneChip® WT Terminal Labeling Kit.

1. Set up fragmentation reaction in 0.2 mL strip tubes using Table 2.1.

Table 2.1 Fragmentation Master Mix

Component	Volume/Amount in 1 Rxn
Single-Stranded DNA	5.5 µg
RNase-free Water	up to 31.2 µL
Total Volume	**31.2 µL**

2. Prepare the Fragmentation Master Mix using Table 2.2.

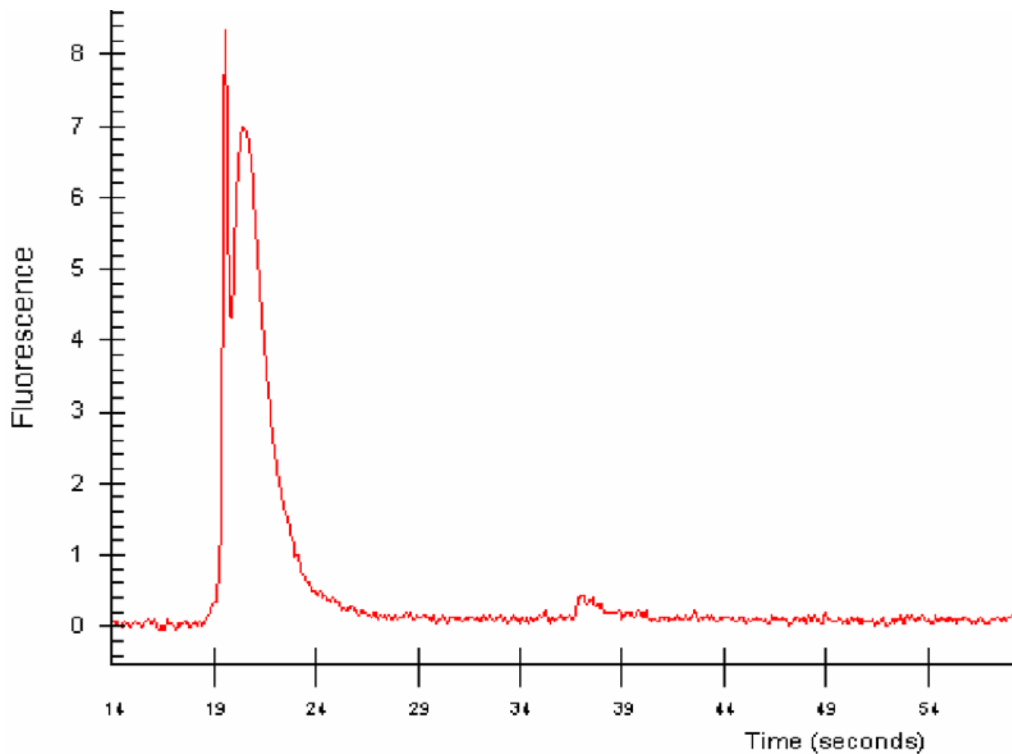
Table 2.2 Fragmentation Master Mix

Component	Volume in 1 Rxn
RNase-free Water	10 µL
10X cDNA Fragmentation Buffer	4.8 µL
UDG, 10 U/µL	1.0 µL
APE 1, 1,000 U/µL	1.0 µL
Total Volume	**16.8 µL**

3. Add 16.8 µL of the above Fragmentation Master Mix to the samples prepared in Step 1. Flick or gently vortex the tubes and spin down.
4. Incubate the reactions at:
   - 37°C for 60 minutes
   - 93°C for 2 minutes
   - 4°C for at least 2 minutes

5. Flick-mix, spin down the tubes, and transfer 45 µL of the sample to a new 0.2 mL strip tube. The remainder of the sample can be used for size analysis using a Bioanalyzer. Please see the Reagent Kit Guide that comes with the RNA 6000 Nano LabChip Kit for detailed instructions. The range in peak size of the fragmented samples should be approximately 40 to 70 nt. See Figure 2.1 as an example of typical results on fragmented samples.
6. If the samples are not labeled immediately, store the fragmented Single-Stranded DNA at –20°C.

Figure 2.1 Bioanalyzer profile of Fragmented Single-Stranded DNA from Human Brain



Labeling of Fragmented Single-Stranded DNA

This Procedure requires the use of the GeneChip® WT Terminal Labeling Kit.

1. Prepare the labeling reactions as listed in Table 2.3. A master mix using the 5X TdT Buffer, TdT and DNA Labeling reagent can be prepared just before aliquoting 15 µL into the 0.2 mL strip tubes containing the 45 µL of Fragmented Single-Stranded DNA.

Table 2.3 Labeling Reaction

Component	Volume in 1 Rxn
Fragmented Single-Stranded DNA (from Procedure G)	45 µL
5X TdT Buffer	12 µL
TdT	2 µL
DNA Labeling Reagent, 5 mM	1 µL
Total Volume	**60 µL**

2. After adding the labeling reagents to the fragmented DNA samples, flick-mix and spin them down.
3. Incubate the reactions at:
   - 37°C for 60 minutes
   - 70°C for 10 minutes
   - 4°C for at least 2 minutes
4. Remove 2 µL of each sample for Gel-shift analysis (optional) as described in Appendix B, to assess the labeling efficiency.

Target Hybridization for Cartridge Arrays

This section provides instruction for setting up hybridizations for cartridge arrays. For instructions on setting up hybridizations for Gene 1.1 ST Array Plates processed on the GeneTitan® Instrument please go to Target Hybridization for Gene 1.1 ST Array Plates Processed on the GeneTitan® Instrument on page 19.

This Procedure requires the use of the GeneChip® Hybridization, Wash and Stain Kit.

Three heating blocks are required: one at 65°C, one at 99°C, and the third one at 45°C.

1. Prepare the Hybridization Cocktail in a 1.5 mL RNase-free microfuge tube as shown in Table 2.4.

Table 2.4 Hybridization Cocktail

Component	Volume for One 49/64 Format Array	Volume for One 100 Format Array	Volume for One 169 Format Array	Final Concentration
Fragmented and Labeled DNA Target (from Ambion procedure)	~60.0* µL	41 µL	27 µL	~25 ng/µL
Control Oligonucleotide B2 (3 nM)	3.7 µL	2.5 µL	1.7 µL	50 pM
20X Eukaryotic Hybridization Controls *(bioB, bioC, bioD, cre)*	11 µL	7.5 µL	5 µL	1.5, 5, 25 and 100 pM, respectively
2X Hybridization Mix	110 µL	75 µL	50 µL	1X
DMSO	15.4 µL	10.5 µL	7 µL	7%
Nuclease-free Water	up to 220.0 µL	up to 150	up to 100	
Total Volume	**220.0 µL**	**150 µL**	**100 µL**	

**This volume is 58 µL if a portion of the sample was set aside for Gel-shift analysis.*

IMPORTANT: It is imperative that frozen stocks of 20X GeneChip® Eukaryotic Hybridization Controls are heated to 65°C for 5 minutes to completely resuspend the cRNA before aliquoting.

2. Flick or gently vortex the tubes and spin down.
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. Equilibrate the GeneChip ST Array to room temperature immediately before use. Label the array with the name of the sample that will be hybridized.

5. Inject the appropriate amount (see Table 2.5) of the specific sample into the array through one of the septa (see Figure 2.2 for location of the septa on the array).

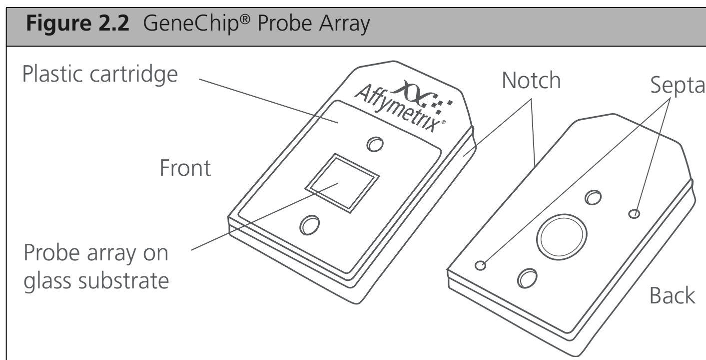
Table 2.5 Probe Array Cartridge Volumes for Hybridization Cocktail

Array Format	Volume
49 (Standard)	200 µL
64	200 µL
100	130 µL
169	80 µL

NOTE: It is necessary to use two pipette tips when filling the probe array cartridge: one for filling and the second to allow venting of air from the hybridization chamber.

NOTE: Ensure that the bubble inside the hyb chamber floats freely upon rotation to allow the hybridization cocktail to make contact with all portions of the array.

Figure 2.2 GeneChip® Probe Array



6. Place array in 45°C hybridization oven, at 60 rpm, and incubate for 17 hours ± 1 hour.

NOTE: During the latter part of the 16-hour hybridization prepare reagents for the washing and staining steps required immediately after completion of hybridization. Please refer to Appendix C for fluidics protocols and fluidics script information for GeneChip® ST Arrays. For further instruction please refer to:

- ***Affymetrix® GeneChip® Fluidics Station 450/250 User Guide* (P/N 08-0092)**
- ***GeneChip® Expression Wash, Stain and Scan Manual for Cartridge Arrays* (P/N 702731)**

Target Hybridization for Gene 1.1 ST Array Plates Processed on the GeneTitan® Instrument

This section includes Hybridization Cocktail Preparation Instructions for Processing Gene 1.1 ST Array Plates using the GeneTitan® Instrument and the WT Assay. The Hybridization Mix described below was specifically formulated for use with the Ambion® WT Expression Kit, P/N 4411973 (10 Rxn) or P/N 4411974 (30 Rxn) and the GeneChip® WT Terminal Labeling and Controls Kit, P/N 901524 (30 Rxn) or P/N 901525 (10 Rxn) (see Table 1.1 on page 7 for ordering information). For instructions on setting up hybridizations on cartridge arrays please go to Target Hybridization for Cartridge Arrays on page 16.

Reagents and Materials Required

- GeneTitan® Hybridization, Wash and Stain Kit for WT Array Plates (P/N 901622, 96 Rxn) consists of:
  - GeneTitan® Hybridization Module for WT Array Plates
  - GeneTitan® Wash Buffers A & B Module
- GeneChip® Hybridization Control Kit (P/N 900454, 30 rxns or 900457, 150 rxns)
- GeneTitan® Consumable Upgrade Kit (P/N 901333)

Prepare the Hybridization Cocktail Mix

NOTE: The "WT Hyb Add" reagent names were created to match the order in which reagents are added. For example, WT Hyb Add 4 is the fourth component added during preparation of the Hybridization Mix. WT Hyb Add 2, 3 and 5 are not used and are not part of the Hybridization Module.

Preparing the Hybridization Cocktail Master Mix

1. Remove the vials labeled 5X WT Hyb Add 1, 15X WT Hyb Add 4 and 2.5X WT Hyb Add 6 from the GeneTitan Hybridization Module for WT Array Plates.
   - A. Warm reagents to room temperature on the bench.
   - B. Vortex and spin 5X WT Hyb Add 1, 15X WT Hyb Add 4 and 2.5X WT Hyb Add 6 to mix. Centrifuge briefly (~5 sec) to collect liquid at the bottom of the tube.
2. Remove the GeneChip Hybridization Control Kit (P/N 900454, 30 rxns or 900457, 150 rxns) from –20°C freezer and thaw at room temperature.
   - A. Vortex and centrifuge briefly (~5 sec) to collect liquid at the bottom of the tube.
   - B. Keep on ice.

3. WT Hybridization Mix: A new hybridization mix has been formulated for the Ambion WT Expression Kit, P/N 4411973 (10 Rxn) or P/N 4411974 (30 Rxn). The hybridization mix is made in 3 steps, as described in Table 2.6, Table 2.7 and Table 2.8 below.

   A. Prepare the WT Hybridization Mix in the order as shown in Table 2.6. The 5X WT Hyb Add 1 solution is very viscous; pipet slowly to ensure addition of the correct volume. Mix well.

Table 2.6 WT Hybridization Mix

Order to Add Reagents	Component	Volume per Array	16-Array Plate*	24-Array Plate*	96-Array Plate*	Final Concentration
1	5X WT Hyb Add 1	24 µL	422.4 µL	633.6 µL	2,534.4 µL	1X
2	Control Oligo B2 (3 nM)	1.2 µL	21.1 µL	31.7 µL	126.7 µL	30 pM
3	20X Eukaryotic Hybridization Controls (*bioB, bioC, bioD, cre*)	6 µL	105.6 µL	158.4 µL	633.6 µL	1.5, 5, 25 and 100 pM, respectively
4	15X WT Hyb Add 4	8 µL	140.8 µL	211.2 µL	844.8 µL	1X
Total Volume		**39.2 µL**	**689.9 µL**	**1,034.9 µL**	**4,139.5 µL**	

* *Includes ~ 10% overage to cover pipetting error.*

   B. Aliquot 39.2 µL of the master mix prepared in Table 2.6 to each tube or well. Add the fragmented and labeled single-stranded DNA target generated from the Fragmentation of Single-Stranded DNA on page 13, as shown in Table 2.7.

Table 2.7

Order to Add Reagents	Component	Volume per Array	Final Concentration
5	Fragmented and Labeled DNA	32.8 µL	~25 ng/µL
Total Volume		**72 µL**	

C. Add the 2.5X WT Hyb Add 6 from the GeneTitan Hybridization Module for WT Array Plates as shown in Table 2.8.

Table 2.8

Order to Add Reagents	Component	Volume per Array	Final Concentration
6	2.5X WT Hyb Add 6	48 µL	1X
Total Volume		**120 µL**	

D. If you are using a plate; seal, vortex, and centrifuge. If you are using 1.5 mL tubes; vortex and centrifuge.

4. Denature the hybridization cocktail with target at 99°C (1.5 mL tubes) or 95°C (thermocycler plates) for 5 minutes, followed by 45°C for 5 minutes.
5. After denaturation, centrifuge hybridization cocktail with target to remove any insoluble material from the hybridization mixture. If you are using 1.5 mL tubes, use the Eppendorf 5417C centrifuge (or similar). If you are using thermocycler plates, use the Eppendorf 5804R centrifuge (or similar). Centrifuge either tubes or plates for 1 minute at 5,000 RPM at room temperature.
6. Place 90 µL of the centrifuged supernatant hybridization mix as indicated into the appropriate well of the hybridization tray.
7. Proceed to Hybridization Setup on page 22.

Hybridization Setup

This section describes the GeneTitan Setup protocol for Gene 1.1 ST Array Plates. The reagent consumption per process on the GeneTitan® Instrument for processing Gene 1.1 ST Array Plates is shown in Table 2.10.

Table 2.9 The Minimum Volumes of Buffer and Rinse Required to Process on the GeneTitan Instrument

Fluid Type	Amount Required for One Array Plate	Minimum Level in Bottle	
		One Array Plate	Two Array Plates
Rinse	300 mL	450 mL	900 mL
Wash A	~920 mL	1,040 mL +	2,000 mL
Wash B	300 mL	450 mL	600 mL

Table 2.10 Volumes Required to Process Gene 1.1 ST Array Plates per Run

Reagent	Amount Required for One Array Plate	Number of Plates that can be Processed using the GeneTitan Hybridization, Wash and Stain Kit for WT Array Plates (P/N 901622)		
		16-Format	24-Format	96-Format
Wash A	~920 mL	1	1	1
Wash B	300 mL	1	1	1
Stain 1 and 3	105 µL/well	6	4	1
Stain 2	105 µL/well	6	4	1
Array Holding Buffer	150 µL/well	6	4	1

! **IMPORTANT: The instrument must have a minimum of 450 mL of Wash B in the Wash B reservoir of the instrument for each Gene 1.1 ST Array Plate prior to starting Hyb, Wash, Stain and Scan process. The waste bottle should be empty.**

Procedure for Processing Gene 1.1 ST Array Plates on the GeneTitan® Instrument

Please use the stain trays and covers provided with the GeneTitan Consumable Upgrade Kit (P/N 901333) for the procedure described below.

1. Use the anti-static gun on the wells of the stain tray labeled GeneTitan Stain Tray P/N 501025.
   - A. Place a stain tray on the table top.
   - B. Hold the Zerostat 3 anti-static gun within 12” (30.5 cm) of the surface or object to be treated. Squeeze the trigger slowly for about two seconds, to emit a stream of positive ionized air over the surface of the object. As the trigger is slowly released, a negative flow of air ions is produced resulting in static neutralization.
   - C. Repeat this procedure at several points across the surface of the stain tray.
2. Aliquot 105 µL of the Stain 1 into the GeneTitan Stain Tray.
3. Use the anti-static gun on the stain tray cover.
   - A. Place a stain tray cover on the table top with the flat surface facing upward.
   - B. Hold the Zerostat 3 anti-static gun within 12" (30.5 cm) of the surface or object to be treated. Squeeze the trigger slowly for about two seconds, to emit a stream of positive ionized air over the surface of the object. As the trigger is slowly released, a negative flow of air ions is produced resulting in static neutralization.
   - C. Repeat this procedure at several points across the surface, covering the entire stain tray cover.
4. After removing the static electricity, place the cover on top Stain Tray 1.
5. After repeating Step 1, aliquot 105 µL of the Stain 2 into the GeneTitan Stain Tray.
6. After repeating Step 3, place cover on top of Stain Tray 2.
7. After repeating Step 1, aliquot 105 µL of the Stain 3 into the GeneTitan Stain Tray.
8. After repeating Step 3, place cover on top of Stain Tray 3.
9. Aliquot 150 µL of the Array Holding Buffer into the GeneTitan Scan Tray identified with the label HT Scan Tray P/N 500860 on the tray.
10. Use the fourth scan tray cover provided with the GeneTitan Consumable Upgrade kit to cover the Scan Tray.

11. Load all the consumables including the HT Array Plate into the GeneTitan Instrument as per instructions provided in the Affymetrix GeneChip Command Console 2.0 User Guide (P/N 702569).

IMPORTANT: It is important not to bump the trays while loading them into the GeneTitan Instrument. Droplets of the stain going onto the lid may result in a wicking action and the instrument gripper may be unable to remove the lids properly.

The remaining hybridization ready sample can be stored at –20°C after the Biorad Hardshell Plate using Aluminum Foil.

FAQ

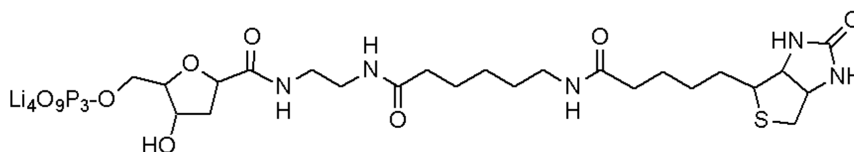
WT Terminal Labeling Assay

1. What is the basic principle of the single-stranded DNA fragmentation and labeling procedure?

Using aRNA generated from the IVT reaction at the end of the first cycle of the assay as a template, single-stranded DNA is synthesized using random primers and the dUTP + dNTP mix. The resulting single-stranded DNA (ss-DNA) containing the unnatural uracil base is then treated with Uracil DNA Glycosylase, which specifically removes the uracil residue from the ss-DNA molecules. In the same reaction, the APE 1 enzyme then cleaves the phosphodiester backbone where the base is missing, leaving a 3'-hydroxyl and a 5'-deoxyribose phosphate terminus.

2. What is the basic component in the DNA Labeling Reagent?

The key labeling molecule in the DNA Labeling Reagent is Biotin Allonamide Triphosphate. See the structure below:



3. What is the expected length of the fragmented DNA target?

On a Bioanalyzer, the fragmented single-stranded DNA target should have a peak centered around 40 to 70 bases with the majority of the fragments ranging from 20 bases to 200 bases.

4. How much single-stranded DNA target do you need to hybridize to one array?

It is recommended to hybridize approximately 5 µg or 2 µg of fragmented and labeled DNA target to each Exon or Gene Arrays respectively.

5. What is the hybridization condition?

A final concentration of 7% DMSO is included in the hybridization cocktail for hybridizing the WT sense target to cartridge ST arrays. A final concentration of 2.0 M TMAC is included in the hybridization cocktail for hybridizing the WT sense target to Gene 1.1 ST Array Plates.

6. Can I hybridize the DNA target to the HG-U133 arrays?

The Ambion® WT Expression Assay is optimized to produce targets specifically for hybridization to ST array type of design. The target is in the sense orientation and the GeneChip® Human Genome U133 Plus 2.0 Array is designed to be compatible with antisense targets. Therefore, it is not recommended to mix and match the assays and the array types.

7. Can I use this protocol for prokaryotic arrays?

This has not been tested at the moment; therefore, it is not recommended to use the protocol for any application other than on ST arrays.

Array Hybridization, Washing, Staining, and Scanning

8. Why is there no pre-hybridization step for the arrays using the targets from the WT Assay? The pre-hybridization step was required for the 3' target in the *GeneChip® 3' IVT Express Kit User Manual* (P/N 702646).

No pre-hybridization step is necessary for the WT targets. There are many differences between the WT targets and the 3' targets in terms of the nature of the molecules (DNA vs. RNA), as well as labeling molecule and hybridization cocktail makeup. It has been found that pre-hybridization is not necessary for the WT targets.

9. What Fluidics Protocol do I use for the GeneChip® ST Cartridge Arrays?

New Fluidics Protocols have been developed for this assay, FS450_0001 for 49-format and 64-format arrays, FS450_0002 for 100-format arrays and F450_0007 for 169-format arrays. In addition to tubes containing SAPE and antistreptavidin biotinylated antibody, there is a tube containing 1X Array Holding Buffer, which is added to the cartridge following the wash/stain procedure. Please refer to Appendix C on page 29 of this manual or the GeneChip® Expression Wash, Stain and Scan User Manual (P/N 702731) for further details.

10. How long does it take to scan a cartridge array?

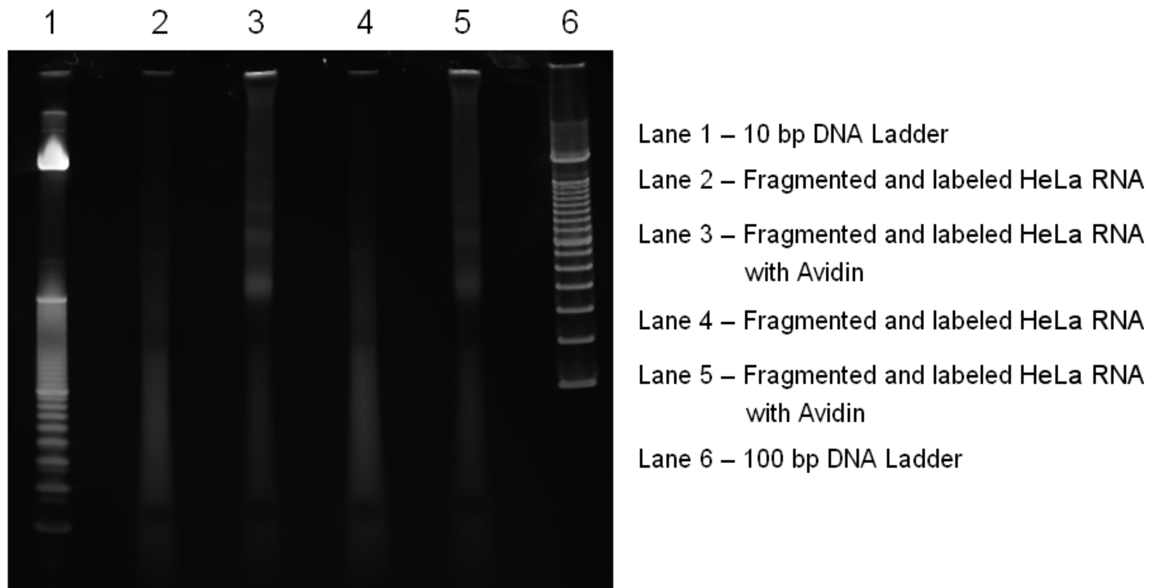
It takes approximately 35 minutes to scan each Exon Array and approximately 10 minutes to scan each Gene Array.

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 B.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 B.1 Gel-Shift



1. Prepare a NeutrAvidin solution of 2 mg/mL in 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.

Fluidics Protocols and Fluidics Scripts for GeneChip® ST Cartridge Arrays

Please refer to the GeneChip® Expression Wash, Stain and Scan User Manual (P/N 702731) for detailed information on the washing and staining steps required. Fluidics protocols and fluidics scripts for GeneChip ST Cartridge Arrays are provided below for your convenience. This information is also available online at www.affymetrix.com.

Fluidics Protocols

Table C.1 Fluidics Protocols for GeneChip® ST Cartridge Arrays

	Fluidics Station 450 **FS450_0001, FS450_0002 and FS450_0007**
Post Hyb Wash #1	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
Stain	Stain the probe array for 5 minutes in SAPE solution at 35°C
Post Stain Wash	10 cycles of 4 mixes/cycle with Wash Buffer A at 30°C
2nd Stain	Stain the probe array for 5 minutes in antibody solution at 35°C
3rd Stain	Stain the probe array for 5 minutes in SAPE solution at 35°C
Final Wash	15 cycles of 4 mixes/cycle with Wash Buffer A at 35°C.
Holding Buffer	Fill the probe array with Array Holding Buffer.

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

Fluidics Scripts

Table C.2 Fluidics Scripts for GeneChip® ST Cartridge Array Types

Array Format	Fluidics Script Protocol
49 Format	FS450_0001
64 Format	FS450_0001
100 Format	FS450_0002
169 Format	FS450_0007