User Manual

GeneChip® WT Terminal Labeling and Hybridization User Manual

for use with the Ambion® WT Expression Kit

P/N 702808 Rev. 1

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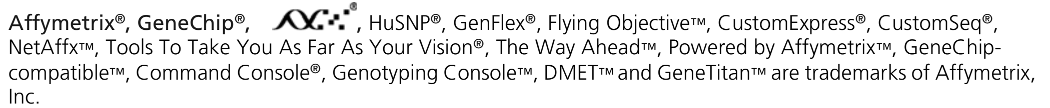
Affymetrix®, GeneChip®, , HuSNP®, GenFlex®, Flying Objective™, CustomExpress®, CustomSeq®, NetAffx™, Tools To Take You As Far As Your Vision®, The Way Ahead™, Powered by Affymetrix™, GeneChip-compatible™, Command Console®, Genotyping Console™, DMET™ and GeneTitan™ are trademarks of Affymetrix, Inc.

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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.

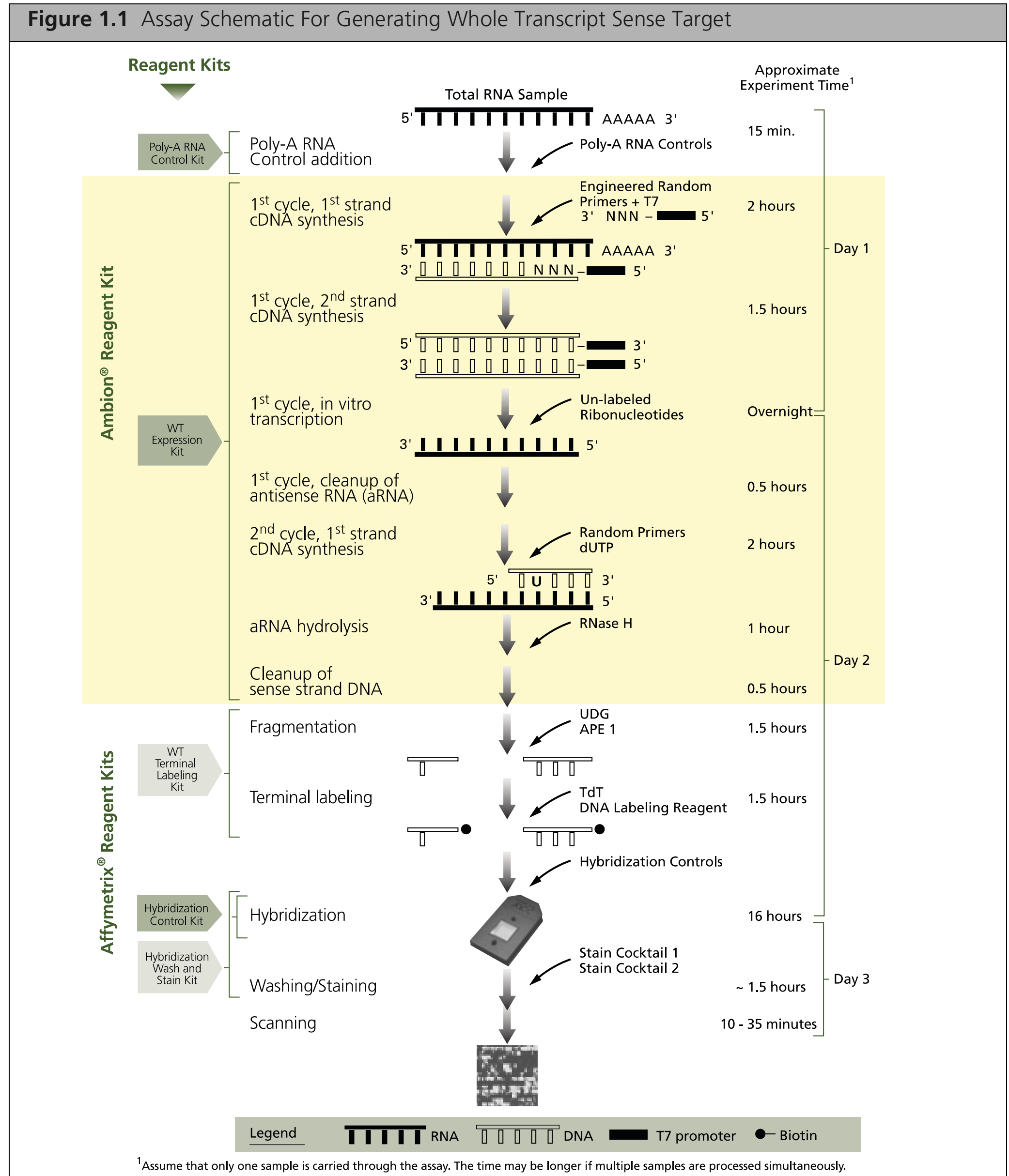
In order to reproducibly fragment the single-stranded DNA, the Affymetrix GeneChip WT Terminal Labeling Kit employs a novel approach where dUTP is incorporated in the DNA during the second-cycle, first-strand reverse transcription reaction. This single-stranded DNA sample is then treated with a combination 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	411973 (30 Rxn) or 411974 (10 Rxn)
GeneChip® Poly-A RNA Control Kit Contains: ▫ Poly-A Control Stock ▫ Poly-A Control Dil Buffer	Affymetrix	900433 (100 Rxn)
Fragmentation and Labeling		
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)

** 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
Hybridization, Stain and Wash		
GeneChip® Hybridization Control Kit Contains: ▫ 20X Hybridization Controls ▫ 3 nM Control Oligo B2	Affymetrix	900454 (30 Rxn) or 900457 (150 Rxn)
GeneChip® Hybridization, Wash, and Stain Kit (30 reactions) 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)

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

* *Or equivalent.*

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 9
  - *Labeling of Fragmented Single-Stranded DNA* on page 11
- Start Hybridization – 17 hours – Start on Day 2, finish on Day 3
  - *Hybridization* on page 12

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.

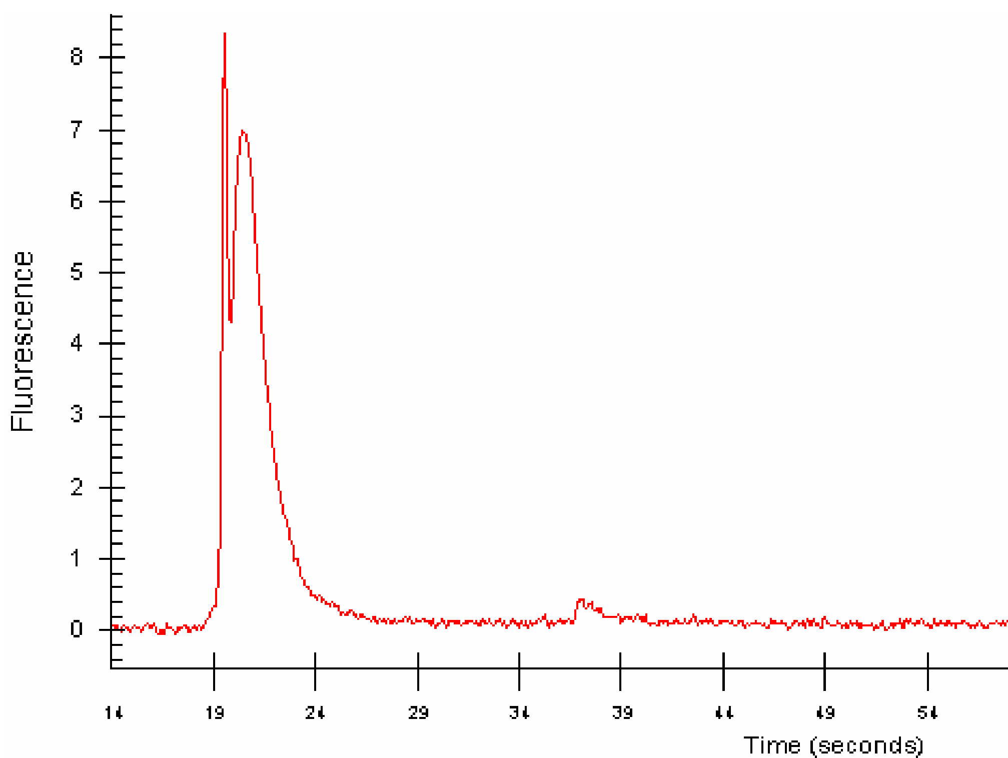
Table 2.2 Fragmentation Master Mix

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.

Hybridization

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

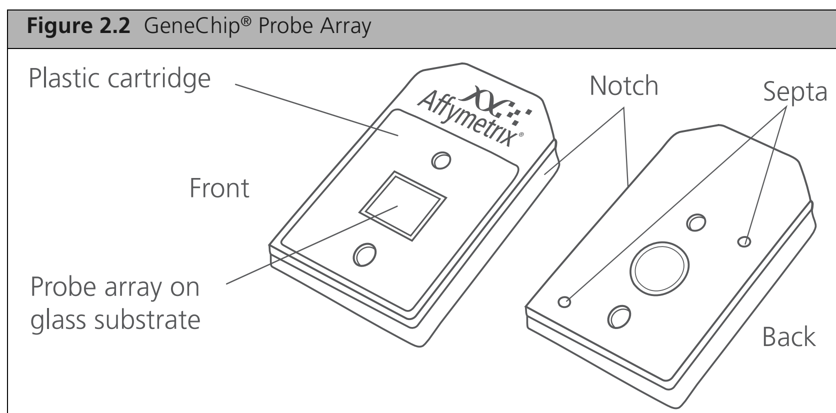
Table 2.5 Probe Array Cartridge Volumes for Hybridization Cocktail

Array Format	Volume
49 (Standard)	200 µL
64	200 µL
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. During the latter part of the array hybridization, commence preparation of the reagents required immediately after completion of hybridization.

Wash, Stain and Scan

Please refer to the *GeneChip® Expression Wash, Stain and Scan User Manual* (P/N 702731) 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.

FAQ

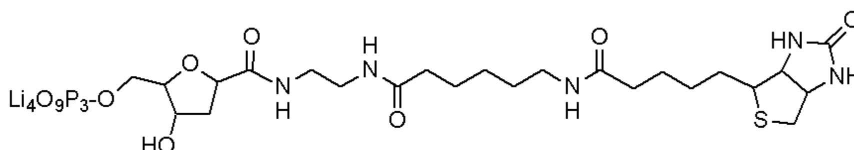
WT Terminal Labeling Assay

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 ST arrays.

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 Arrays?

New Fluidics Protocols have been developed for this assay, FS450_0001 for Exon Arrays and FS450_0007 for Gene 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 19 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 an array?

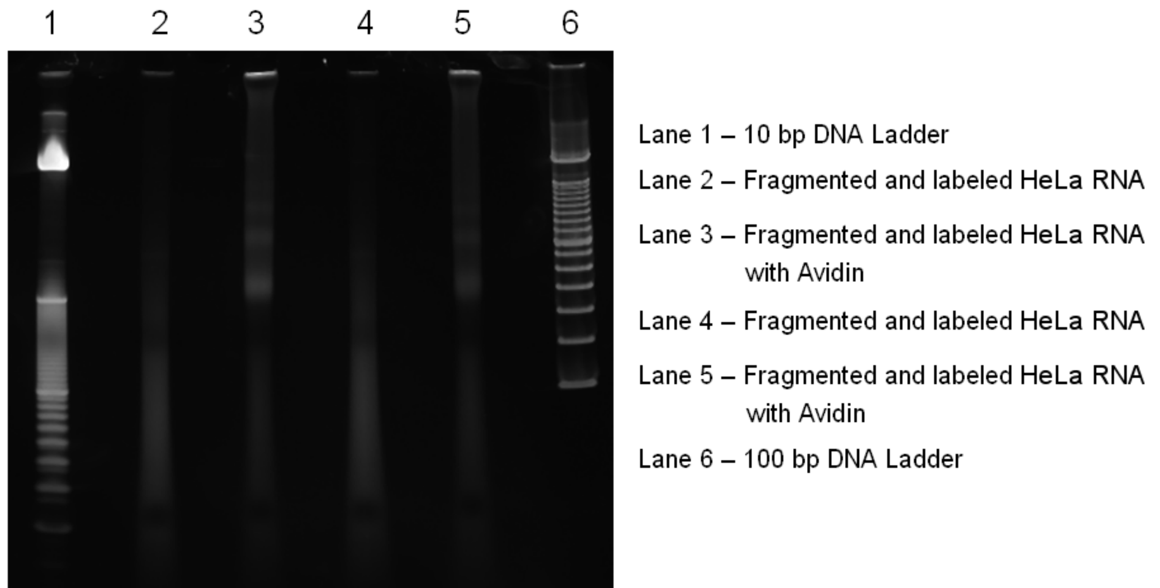
It takes approximately 35 minutes to scan each Exon Array and approximately 10 minutes to scan each Gene Array.

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 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 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 Arrays

	Fluidics Station 450 FS450_0001 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 Array Types

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