UserGuide

SensationPlus™ FFPE Amplification and WT Labeling Kit

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P/N 703089 Rev. 4

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Chapter 1

SensationPlus™ FFPE Amplification and WT Labeling Kit

Introduction

SensationPlus™ FFPE Amplification and WT Labeling Kit uses a unique RNA amplification method that produces thousands of nearly identical copies of each original RNA molecule[1-5]. Based on a linear amplification method using T7 RNA polymerase, SensationPlus is simple to use and generates high quality amplified RNA from degraded RNA samples for further use in expression analysis experiments such as microarrays, quantitative RT-PCR, or other relevant RNA-based applications. (Figure 1.1).

The SensationPlus™ FFPE Amplification Kit is designed for one round of whole transcriptome amplification using both random and dT primers in the first cDNA Synthesis step allowing researchers to perform global gene expression analysis on archives of degraded RNA derived from FFPE samples. The system is comprised of reagents and a protocol for amplification of 20 to 200 ng of total RNA derived from FFPE samples.

The SensationPlus™ WT Labeling Kit contains reagents designed to convert the senseRNA generated from the SensationPlus FFPE Amplification Module into labeled double-stranded cDNA. The cDNA can then be analyzed on Affymetrix® Whole-Transcript Expression Arrays (Figure 1.2).

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 SDS (Safety Data Sheet) for specific advice.

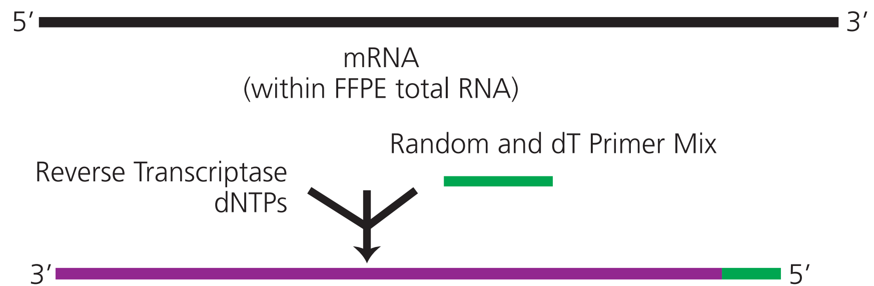
Amplification Step Overview

Figure 1.1 Amplification Step Overview

First Strand cDNA Synthesis

1.5 hours, then 1 hour for purification

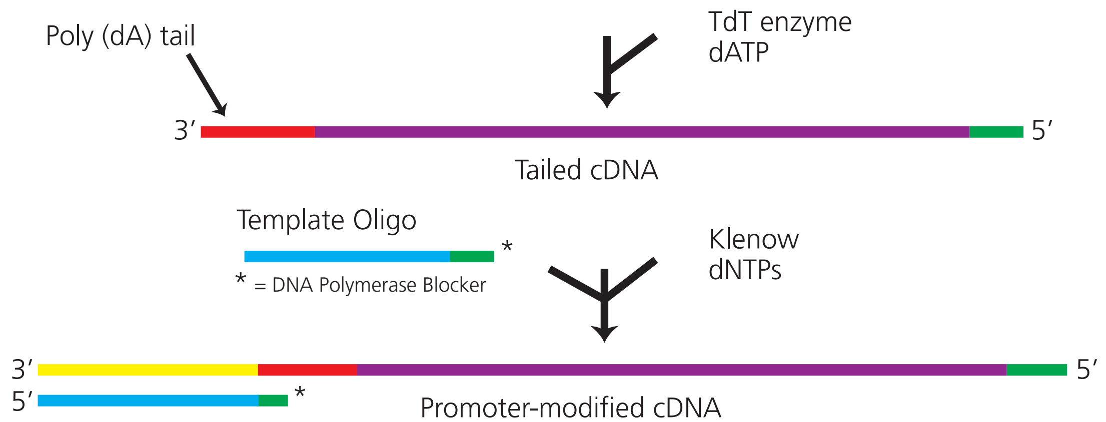
Total RNA is reverse transcribed using random primer and dT primer, dNTP mix, and Reverse Transcriptase. The cDNA is purified with Purification Beads.



Promoter Synthesis

1.5 hours

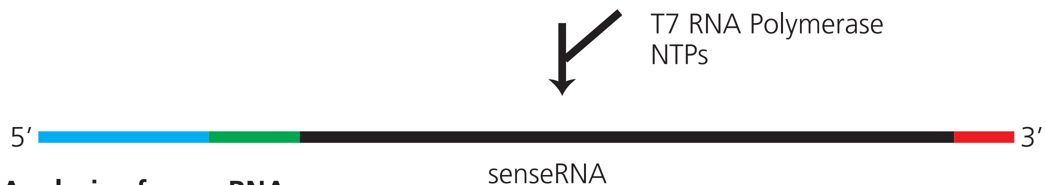
Total cDNA is poly (dA) tailed on the 3′ end using dATP and Terminal Deoxynucleotidyl Transferase. A T7 dT oligo with a 3′ blocking group is hybridized to the 3′ (dA) tail of the cDNA. Klenow and dNTP mix generate a double stranded T7 promoter region on the single stranded cDNA.



In Vitro Transcription

Overnight (16-18 hours), then 1 hour for purification

The promoter-modified cDNA is in vitro transcribed using T7 RNA Polymerase and NTP Mix. The senseRNA is purified with Purification Beads.



Analysis of senseRNA

The senseRNA is ready for gene expression applications.

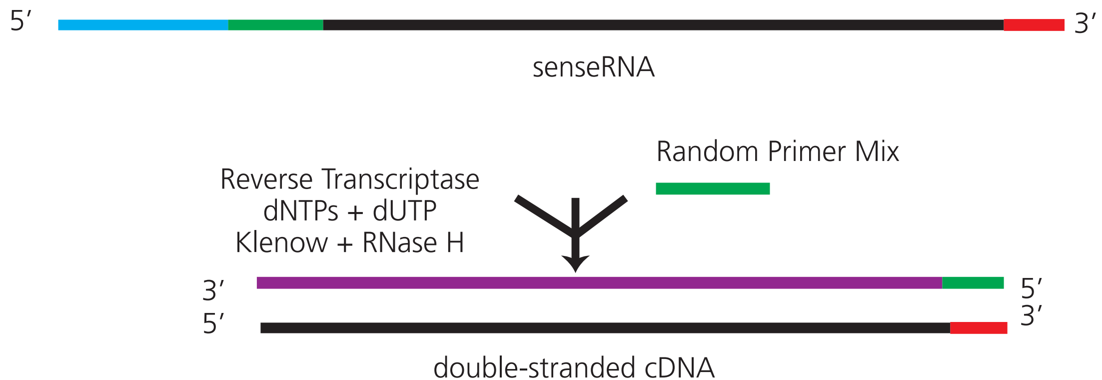
Biotin Labeling for Whole-Transcript Expression Arrays Overview

Figure 1.2 Biotin Labeling for Whole-Transcript Expression Arrays

Double-Stranded cDNA Synthesis

4 hours, then 1 hour for purification

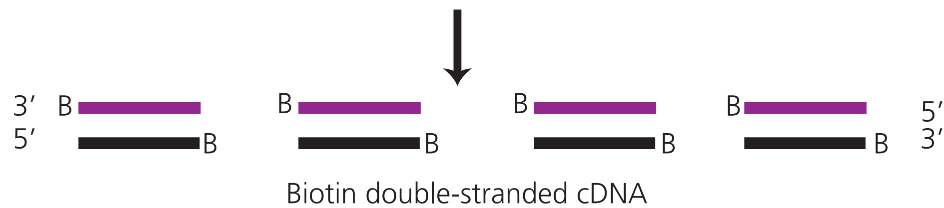
SenseRNA is reverse transcribed using random primer mix, dNTP mix + dUTP, and Reverse Transcriptase. Second Strand cDNA is made with Klenow and RNase H. The double-stranded cDNA is purified with Purification Beads.



Terminal Labeling of Double-Stranded cDNA

1 hour

Double-stranded cDNA is fragmented and end-labeled with Fragment and Label Buffer and Enzyme.



Analysis of Biotin Double-Stranded cDNA

The biotin-double-stranded cDNA is ready for hybridization to Affymetrix® Whole-Transcript Expression Arrays.

Kit Specifications

Table 1.1 SensationPlus™ FFPE Amplification Kit

Vial	Component	Cap Color	Storage	Handling Kit Components
1	RT Primer Mix	Red	–20°C	Thaw at room temperature, briefly vortex and centrifuge. Keep at room temperature until use.
2	RT Buffer Mix	Red	–20°C	Thaw on ice, briefly vortex and centrifuge. Keep on ice.
3	RT Enzyme Mix	Red	–20°C	Gently tap tube, briefly centrifuge, then keep on ice at all times. Do not vortex.
4	RNase Inhibitor	Red	–20°C	Gently tap tube, briefly centrifuge, then keep on ice at all times. Do not vortex.
5	DTT	Red	–20°C	Thaw on ice, briefly vortex and centrifuge. Keep on ice.
6	dNTP Mix	Red	–20°C	Thaw on ice, briefly vortex and centrifuge. Keep on ice.
7	Tailing Buffer Mix	Blue	–20°C	Thaw on ice, briefly vortex and centrifuge. Keep on ice.
8	Tailing Enzyme Mix	Blue	–20°C	Gently tap tube, briefly centrifuge, then keep on ice at all times. Do not vortex.
9	Promoter Synthesis Buffer Mix	Green	–20°C	Thaw on ice, briefly vortex and centrifuge. Keep on ice.
10	Promoter Synthesis Enzyme Mix	Green	–20°C	Gently tap tube, briefly centrifuge, then keep on ice at all times. Do not vortex.
11	T7 Nucleotide Mix	Yellow	–20°C	Thaw at room temperature, briefly vortex and centrifuge. Keep at room temperature until use.
12	T7 Buffer Mix	Yellow	–20°C	Thaw at room temperature, briefly vortex and centrifuge. Keep at room temperature until use.
13	T7 Enzyme Mix	Yellow	–20°C	Gently tap tube, briefly centrifuge, keep on ice until added to Master Mix, then keep Master Mix at room temperature. Refer to page 18.
14	Nuclease-free Water	White	–20°C	Thaw at room temperature, and briefly centrifuge. Keep at room temperature until use.
15	Control DNA	Clear	–20°C	Thaw at room temperature, briefly vortex and centrifuge. Keep at room temperature until use.
16	Purification Beads - Amplification		4°C	Keep at room temperature before use.

Table 1.2 SensationPlus™ WT Labeling Kit

Vial	Component	Cap Color	Storage	Handling Kit Components
1	WT Labeling Primer Mix	Red	–20°C	Thaw at room temperature, briefly vortex, and centrifuge. Keep at room temperature until use.
2	RT Buffer Mix	Red	–20°C	Thaw on ice, briefly vortex, and centrifuge. Keep on ice.
3	RT Enzyme Mix	Red	–20°C	Gently tap tube, briefly centrifuge, then keep on ice at all times. Do not vortex.
4	RNase Inhibitor	Red	–20°C	Gently tap tube, briefly centrifuge, then keep on ice at all times. Do not vortex.
5	DTT	Red	–20°C	Thaw on ice, briefly vortex and centrifuge. Keep on ice.
6	Labeling dNTP Mix	Red	–20°C	Thaw on ice, briefly vortex, and centrifuge. Keep on ice.
7	$MgCl_2$	Purple	–20°C	Thaw at room temperature, briefly vortex and centrifuge. Keep at room temperature until use.
8	Klenow	Purple	–20°C	Gently tap tube, briefly centrifuge, then keep on ice at all times. Do not vortex.
9	RNase H	Purple	–20°C	Gently tap tube, briefly centrifuge, then keep on ice at all times. Do not vortex.
10	WT Stop Solution	Brown	–20°C	Thaw at room temperature, briefly vortex and centrifuge. Keep at room temperature until use.
11	WT Neutralization Mix	Brown	–20°C	Thaw at room temperature, briefly vortex and centrifuge. Keep at room temperature until use.
12	Fragment and Label Buffer Mix	Purple	–20°C	Thaw on ice, briefly vortex, and centrifuge. Keep on ice.
13	Fragment and Label Enzyme Mix	Purple	–20°C	Gently tap tube, briefly centrifuge, then keep on ice at all times. Do not vortex.
14	Nuclease-free Water	White	–20°C	Thaw at room temperature, and briefly centrifuge. Keep at room temperature until use.
15	Purification Beads - Labeling		4°C	Keep at room temperature before use.

Additional Materials/Equipment Required

- Total RNA Sample (see page 11)
- Thermal Cycler
- Microcentrifuge
- Nuclease-free 1 mL, 0.5 mL and 0.2 mL tubes
- Nuclease-free aerosol-barrier tips
- Magnetic Stand [Agencourt SPRI®Plate Super Magnet Plate (Beckman Coulter Genomics, P/N A32782), Ambion Magnetic Stand-96 (Life Technologies, P/N AM10027), 96-well Magnetic-Ring Stand (Life Technologies, P/N AM10050) or equivalent magnetic stand]
- 96-well round bottom microtiter plate (Costar, P/N 3795 or equivalent)
- 70% Ethanol (Prepare fresh dilutions each time Purification Beads are used)
- 100% Ethanol (Molecular Biology Grade)
- 65°C heat block or oven for Nuclease-free Water during Purification
- NanoDrop™ for RNA Quantitation (or equivalent quantitation instrument)
- Optional: materials for Gel Shift assay, refer to Appendix B, *Gel-Shift Assay* on page 29
- Optional: GeneChip® Poly-A RNA Control Kit
- GeneChip® Whole-Transcript Expression Arrays
- Hybridization oven
- Affymetrix GeneChip Command Console® Software (AGCC)
- GeneChip® Hybridization, Wash and Stain Kit (Affymetrix, P/N 900720)
- GeneChip® Eukaryotic Hybridization Control Kit (Affymetrix, P/N 900454)
- Fluidics station
- Scanner

Important Parameters

Procedural Notes

- The following kits may be used for isolation of FFPE total RNA:
  - ReliaPrep™ FFPE Total RNA Miniprep System (Promega)
  - RNeasy® FFPE Kit (QIAGEN)
  - RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE (Life Technologies)
  - Agencourt FormaPure® Kit (Beckman Coulter Genomics)
- Carriers composed of nucleic acid should not be used when purifying RNA samples for amplification, since these carriers will also be amplified by SensationPlus. Choose a carrier that does not reverse transcribe or have a functional 3' hydroxyl, like linear acrylamide.
- DNase treatment of the RNA sample is recommended, as contaminating genomic DNA will also be amplified by SensationPlus. After treatment, completely inactivate the DNase by the recommended method, heating, or phenol-chloroform extraction.
- If any stored RNA samples are suspected to be contaminated with RNases, treat the samples with RNase inhibitor.
- Prepare master mixes for all samples in the experiment plus ~5 to 10% overage to correct for pipetting losses when preparing the master mixes.
- When preparing master mixes, the enzyme should be added last and just prior to adding the master mix to the reaction. After the master mix or other reagent is added to the reaction, gently vortex the tube and briefly centrifuge.
- A thermal cycler is recommended to prevent evaporation and condensation of the sample. If a thermal cycler is not available, heat blocks or water baths may be used. Centrifuge the reactions after incubations, if condensation is observed.
- 25 µg senseRNA (in a volume of 20 µL) is recommended for the labeling procedure. If 25 µg senseRNA is unavailable, proceed with 10-25 µg senseRNA. For each sample, use as much senseRNA as possible, up to 25 µg, but not less than 10 µg.

RNA Input Recommendations

The SensationPlus™ Amplification Kit is designed for one round of whole transcriptome amplification using 20-200 ng of FFPE total RNA. Use 50 ng to ensure optimal results for most FFPE RNA samples of varying quality, especially when sample RNA is not limiting. RNA samples must be free of contaminating DNA, protein, cellular material, organic solvents and salts. RNA purity may be measured as the ratio of absorbance readings at 260 and 280 nm (≥ 1.8).

Table 1.3

Recommendations	Amount
Recommended	50 ng
Minimum	20 ng
Maximum	200 ng

Protocol Timelines

The SensationPlus™ FFPE Amplification and WT Labeling protocol is designed to reduce the amount of hands-on time required for one round of RNA amplification.

Table 1.4 Protocol Timelines (total time including incubation)

Stage	Time
First Strand cDNA Synthesis	90 minutes
Purification of cDNA	60 minutes
Promoter Synthesis	90 minutes
In Vitro Transcription	overnight
Total Time to IVT	**~4 hours**
Purification of senseRNA	60 minutes
Labeling cDNA Synthesis	230 minutes
Purification of cDNA	60 minutes
Terminal Labeling of cDNA	60 minutes
Total Time to Hybridization	**~7 hours**

Thermal Cycler Programs

NOTE: The thermal cycler lid temperature has been tested set at the same temperature as the highest temperature in each program (e.g., Eppendorf Thermal Cycler), set to a temperature that is 5°C above the well and is tracking (e.g., MJ Thermal Cycler), and set at the default temperature (e.g., Applied Biosystems of 103°C) without a significant change in results.

Table 1.5 Thermal Cycler Programs for Amplification

Stage	Program	Setting	Volume
First Strand cDNA Synthesis			
	1	80°C - 10 min, 4°C - 2 min	11 µL
	2	42°C - 60 min, 25°C - 2 min	20 µL
Promoter Synthesis			
	3	80°C - 10 min, 4°C - 2 min	12 µL
	4	37°C - 2 min, 80°C - 10 min, 4°C - 2 min	20 µL
	5	25°C - 30 min	25 µL
In Vitro Transcription			
	6	37°C - overnight (16-18 hours), 4°C - hold	55 µL

Table 1.6 Thermal Cycler Programs for Labeling

Stage	Program	Setting	Volume
Labeling Double-Stranded cDNA Synthesis			
	7	80°C - 5 min, 4°C - 2 min	23 µL
	8	42°C - 120 min, 25°C - 2 min	40 µL
	9	37°C - 40 min, 75°C - 10 min, 4°C - 2 min	60 µL
	10	65°C - 30 min, 25°C - 2 min	62 µL
Terminal Labeling of Double-Stranded cDNA			
	11	37°C - 60 min, 93°C - 2 min, 4°C - 2 min	30 µL
Hybridization Cocktail			
	12	65°C - 5 min	
	13	99°C - 5 min, 47°C - 5 min	90 µL, 130 µL or 200 µL

Chapter 2

Amplification Procedure

Prepare the Poly-A RNA Controls

NOTE: To include premixed controls from the Affymetrix® GeneChip® Poly-A RNA Control Kit (PN 900433), add the reagents to the total RNA samples. Follow the instructions below. Affymetrix strongly recommends the use of poly-A controls for all reactions that will be hybridized to GeneChip® arrays.

A set of poly-A RNA controls supplied by Affymetrix, designed specifically to provide exogenous positive controls to monitor the entire target labeling process, should be added to the RNA prior to the First Strand cDNA Synthesis step.

Each eukaryotic GeneChip® probe array contains probe sets for several *B. subtilis* genes that are absent in eukaryotic samples (*lys, phe, thr*, and *dap*). These poly-A RNA controls are *in vitro* synthesized, and the polyadenylated transcripts for the *B. subtilis* genes are premixed at staggered concentrations. The concentrated Poly-A Control Stock can be diluted with the Poly-A Control Dil Buffer and spiked directly into RNA samples to achieve the final concentrations (referred to as a ratio of copy number) summarized in Table 2.1.

Table 2.1 Final concentrations of Poly-A RNA Controls when added to total RNA samples

Poly-A RNA Spike	Final concentration (ratio of copy number)
lys	1:100,000
phe	1:50,000
thr	1:25,000
dap	1:6,667

The controls are then amplified and labeled together with the total RNA samples. Examining the hybridization intensities of these controls on GeneChip® arrays helps to monitor the labeling process independently from the quality of the starting RNA samples.

The Poly-A RNA Control Stock and Poly-A Control Dil Buffer are provided in the Affymetrix® GeneChip® Poly-A RNA Control Kit (P/N 900433) to prepare the appropriate serial dilutions based on Table 2.2. This is a guideline when 20, 50, 100, or 200 ng of total RNA is used as starting material. For starting sample amounts other than those listed here, calculations are needed in order to perform the appropriate dilutions to arrive at the same proportionate final concentration of the spike-in controls in the samples.

Table 2.2 Serial Dilution of Poly-A RNA Control Stock

Total RNA input amount	Serial Dilutions				Volume of 4th dilution to add to total RNA
	1st dilution	2nd dilution	3rd dilution	4th dilution	
20 ng	1:20	1:50	1:50	1:50	2 µL
50 ng	1:20	1:50	1:50	1:20	2 µL
100 ng	1:20	1:50	1:50	1:10	2 µL
200 ng	1:20	1:50	1:50	1:5	2 µL

NOTE: Avoid pipetting solutions less than 2 µL in volume to maintain precision and consistency when preparing the dilutions.

IMPORTANT: Use non-stick, RNase-free centrifuge tubes to prepare all of the dilutions (not included).

For example, to prepare the Poly-A RNA dilutions for 100 ng of total RNA:

1. Add 2 µL of the Poly-A Control Stock to 38 µL of Poly-A Control Dil Buffer for the first dilution (1:20).
2. Mix thoroughly and spin down to collect the liquid at the bottom of the tube.
3. Add 2 µL of the first dilution to 98 µL of Poly-A Control Dil Buffer to prepare the second dilution (1:50).
4. Mix thoroughly and spin down to collect the liquid at the bottom of the tube.
5. Add 2 µL of the second dilution to 98 µL of Poly-A Control Dil Buffer to prepare the third dilution (1:50).
6. Mix thoroughly and spin down to collect the liquid at the bottom of the tube.
7. Add 2 µL of the third dilution to 18 µL of Poly-A Control Dil Buffer to prepare the fourth dilution (1:10).
8. Mix thoroughly and spin down to collect the liquid at the bottom of the tube.
9. Add 2 µL of this fourth dilution to 100 ng of total RNA.

NOTE: The first dilution of the Poly-A RNA controls can be stored up to 6 weeks in a non-frost-free freezer at –20°C and frozen/thawed up to eight times.

First Strand cDNA Synthesis

The SensationPlus FFPE Amplification Kit is designed for one round of whole transcriptome amplification using 20-200 ng FFPE total RNA. Use 50 ng to ensure optimal results for most FFPE RNA samples of varying quality, especially when sample RNA is not limiting. Spikes required for microarrays should also be added to the total RNA sample.

1. Adjust the volume of total RNA (including spikes) to 7 µL with Nuclease-free Water.
2. On ice, add 4 µL RT Primer Mix (red cap) to the 7 µL total RNA to make an 11 µL RNA-Primer Mix. Gently vortex the tube to mix, followed by a brief centrifugation.
3. Incubate the 11 µL RNA-Primer Mix (Thermal Cycler Program 1):
   - 80ºC for 10 minutes
   - 4ºC for 2 minutes
   - Transfer to ice
4. Prepare an Amplification RT Master Mix in a separate tube on ice:

Table 2.3 Amplification RT Master Mix

Component	Cap Color	Volume for One Reaction
RT Buffer Mix	Red	4 µL
DTT	Red	2 µL
dNTP Mix	Red	1 µL
RT Enzyme Mix	Red	1 µL
RNase Inhibitor	Red	1 µL
Total		**9 µL**

5. Gently vortex the tube to mix, briefly centrifuge, and add the 9 µL Amplification RT Master Mix to the 11 µL RNA-Primer Mix for a volume of 20 µL. Gently vortex the tube to mix, followed by a brief centrifugation.
6. Incubate the 20 µL RT reaction (Thermal Cycler Program 2):
   - 42ºC for 60 minutes
   - 25ºC for 2 minutes

NOTE: During this time, shake or vortex the Purification Beads bottle to resuspend the magnetic particles that may have settled. Aliquot the appropriate amount of Purification Beads, and keep at room temperature. For each reaction, 36 µL will be needed.

7. Immediately proceed to *Purification of cDNA with Purification Beads*.

Purification of cDNA with Purification Beads

NOTE:
- **Use Purification Beads - Amplification.**
- **Prepare fresh dilutions of 70% ethanol each time Purification Beads are used.**
- **Preheat the Nuclease-free Water at 65°C for elution.**
- **Occasionally, the bead/sample mixture may be brownish in color, and not completely clear when placed on the magnet. In those situations, switch to a different position on the magnetic stand, switch to a new magnetic stand, or pellet beads by centrifugation.**

1. If not already done, shake or vortex the Purification Beads bottle to resuspend the magnetic particles that may have settled. Aliquot the appropriate amount of Purification Beads, and bring to room temperature. Ensure that the samples are at room temperature.
2. Place the Nuclease-free Water in a 65˚C heat block or oven.
3. For each reaction, add 36 µL of Purification Beads to a well of a 96-well round bottom plate. Transfer the cDNA sample to a designated well in the plate, and mix well by pipetting up and down 10-20 times.
4. For FFPE samples, add 30 µL of 100% ethanol and mix well by pipetting up and down 10-20 times.

NOTE: Do not add ethanol to non-FFPE samples.

5. Incubate for 10 minutes at room temperature (20-25˚C).
6. Place the plate onto a Magnetic Stand for 10 minutes to separate the beads from the solution.
7. Slowly aspirate and discard the cleared solution, being careful not to disturb the magnetic beads.
8. While on the Magnetic Stand, add 180 µL of 70% ethanol to each well and incubate for 30 seconds at room temperature. With a pipette set to 200 µL slowly aspirate the ethanol wash solution, being careful not to disturb the magnetic beads. Repeat for a total of 3 washes. Completely remove the final wash solution.
9. Air-dry on the magnetic stand for 5 minutes until no liquid is visible, yet the pellet appears shiny. Additional time may be required. Do not over-dry the beads as this will reduce the elution efficiency. The bead surface will appear dull, and may have surface cracks when it is over-dry.
10. Remove the plate from the Magnetic Stand.
11. Add 14 µL of the 65˚C Nuclease-free Water to each well. Once water is added to all wells, incubate at room temperature (20-25˚C) for 2-3 minutes to elute the sample from the magnetic beads. During this time, beads can be mixed by gentle shaking of the plate, or by pipetting up and down until resuspended.
12. Place the plate onto the Magnetic Stand for 3 minutes to separate the beads from the solution.

13. Slowly aspirate 12 µL of purified cDNA and transfer to a new tube, being careful not to disturb the magnetic beads. Record the volume recovered. If necessary, adjust to 12 µL with Nuclease-free Water. Minimal bead carryover will not inhibit subsequent enzymatic reactions.
14. Immediately proceed to *Promoter Synthesis*.

Promoter Synthesis

1. Heat the 12 µL purified cDNA (Thermal Cycler Program 3):
   - 80ºC for 10 minutes
   - 4ºC for 2 minutes
   - **Transfer to ice**
2. Prepare a Tailing Master Mix in a separate tube on ice:

Table 2.4 Tailing Master Mix

Component	Cap Color	Volume for One Reaction
Tailing Buffer Mix	Blue	6 µL
Tailing Enzyme Mix	Blue	2 µL
Total		**8 µL**

3. Gently vortex the tube to mix, briefly centrifuge, and add the 8 µL Tailing Master Mix to the 12 µL purified cDNA for a volume of 20 µL. Gently vortex the tube to mix, followed by a brief centrifugation.
4. Incubate the 20 µL Tailing Reaction (Thermal Cycler Program 4):
   - 37°C for 2 minutes
   - 80°C for 10 minutes
   - 4°C for 2 minutes
   - **Transfer to ice**
5. Prepare a Promoter Synthesis Master Mix in a separate tube on ice:

Table 2.5 Promoter Synthesis Master Mix

Component	Cap Color	Volume for One Reaction
Promoter Synthesis Buffer Mix	Green	4 µL
Promoter Synthesis Enzyme Mix	Green	1 µL
Total		**5 µL**

6. Gently vortex the tube to mix, briefly centrifuge, and add the 5 µL Promoter Synthesis Master Mix to the 20 µL Tailed cDNA for a volume of 25 µL. Gently vortex the tube to mix, followed by a brief centrifugation.
7. Incubate the 25 µL Promoter Synthesis Reaction (Thermal Cycler Program 5):
   - 25°C for 30 minutes
8. Immediately proceed to *In Vitro Transcription*.

In Vitro Transcription

NOTE: This step is overnight incubation.

1. Thaw the T7 Nucleotide Mix (yellow cap) and T7 Buffer Mix (yellow cap) at room temperature, and **keep at room temperature (20-25°C) until use**. Thoroughly vortex the T7 Buffer Mix to resuspend and then spin down to collect tube contents.
2. Prepare an IVT Master Mix in a separate tube **at room temperature and keep at room temperature**:

Table 2.6 IVT Master Mix

Component	Cap Color	Volume for One Reaction
T7 Nucleotide Mix	Yellow	16 µL
T7 Buffer Mix	Yellow	5 µL
T7 Enzyme Mix	Yellow	9 µL
Total		**30 µL**

3. Transfer the Promoter-modified cDNA to room-temperature.
4. Gently vortex the tube to mix, briefly centrifuge, and add the 30 µL IVT Master Mix to the 25 µL Promoter-modified cDNA for a volume of 55 µL. Gently vortex the tube to mix, followed by a brief centrifugation.
5. Incubate the 55 µL IVT reaction in a thermal cycler (Program 6) or in an air hybridization oven:
   - 37ºC for 16-18 hours
   - 4ºC - hold
6. Prior to purification, shake or vortex the Purification Beads bottle to resuspend the magnetic particles that may have settled. Aliquot the appropriate amount of Purification Beads, and keep at room temperature. For each reaction, 99 µL will be needed. .

NOTE: Proceed directly to Purification. Alternatively, the reactions can be stored at –20°C for up to 1 week.

Purification of senseRNA with Purification Beads

NOTE:

- **Use Purification Beads - Amplification.**
- **Prepare fresh dilutions of 70% ethanol each time Purification Beads are used.**
- **Preheat the Nuclease-free Water at 65°C for elution.**
- **Occasionally, the bead/sample mixture may be brownish in color, and not completely clear when placed on the magnet. In those situations, switch to a different position on the magnetic stand, switch to a new magnetic stand, or pellet beads by centrifugation.**

1. If not already done, shake or vortex the Purification Beads bottle to resuspend the magnetic particles that may have settled. Aliquot the appropriate amount of Purification Beads, and bring to room temperature. Bring the samples to room temperature.
2. Place the Nuclease-free Water in a 65°C heat block or oven.
3. For each reaction, add 99 µL of Purification Beads to a well of a 96-well round bottom plate. Transfer the senseRNA sample to a designated well in the plate, and mix well by pipetting up and down 10-20 times.
4. Incubate for 10 minutes at room temperature (20-25°C).
5. Place the plate onto a Magnetic Stand for 10 minutes to separate the beads from the solution.
6. Slowly aspirate and discard the cleared solution, being careful not to disturb the magnetic beads.
7. While on the Magnetic Stand, add 180 µL of 70% ethanol to each well and incubate for 30 seconds at room temperature. With a pipette set to 200 µL, slowly aspirate the ethanol wash solution, being careful not to disturb the magnetic beads. Repeat for a total of 3 washes. Completely remove the final wash solution.
8. Air-dry on the magnetic stand for 5 minutes until no liquid is visible, yet the pellet appears shiny. Additional time may be required. Do not over-dry the beads as this will reduce the elution efficiency. The bead surface will appear dull, and may have surface cracks when it is over-dry.
9. Remove the plate from the Magnetic Stand.
10. Add 23 µL of the 65°C Nuclease-free Water to each well. Once water is added to all wells, incubate at room temperature (20-25°C) for 2 to 3 minutes to elute the sample from the magnetic beads. During this time, beads can be mixed by gentle shaking of the plate, or by pipetting up and down until resuspended.
11. Place the plate onto the Magnetic Stand for 3 minutes to separate the beads from the solution.
12. Slowly aspirate the purified senseRNA and transfer to a new tube, being careful not to disturb the magnetic beads. Record the volume recovered, as it may vary.

NOTE: When senseRNA is very concentrated, it may be difficult to aspirate the purified senseRNA because the beads quickly aspirate as well. When it is difficult to aspirate the purified senseRNA, remove the plate from the magnet and add an additional 10-20 µL of 65°C Nuclease-free Water to each well. Mix by pipetting up and down until resuspended. Incubate at room temperature (20-25°C) for 1 minute to elute the sample from the magnetic beads. Proceed to Step 11, above. If it is still difficult to aspirate, transfer the sample (with beads) to a new tube, briefly centrifuge, and aspirate the senseRNA away from the beads. Minimal bead carryover will not inhibit subsequent enzymatic reactions.

Quantitation of senseRNA

1. Quantify 1 to 2 µL of senseRNA by using a NanoDrop or similar instrument. In general, measurement of sample from FFPE RNA does not require dilution.
2. From the OD, determine the nucleic acid concentration of each sample:

   A_{260} nm x 40 (RNA extinction coefficient) x dilution factor = concentration of RNA in ng/µL

   Calculate the $A_{260/280}$ ratio to determine RNA purity. A ratio of 2.0-2.6 is most desirable.

NOTE: Samples can be stored at –20°C at this point.

Chapter 3

Labeling Procedure

Double-Stranded cDNA Synthesis

25 µg senseRNA (in a volume of 20 µL) is recommended for this labeling procedure. If 25 µg senseRNA is unavailable, proceed with 10-25 µg senseRNA. For each sample, use as much senseRNA as possible, up to 25 µg, but not less than 10 µg.

1. Adjust the volume of senseRNA to 20 µL with Nuclease-free water.
2. On ice, add 3 µL WT Labeling Primer Mix (red cap) to the 20 µL senseRNA to make a 23 µL senseRNA-Primer Mix. Gently vortex the tube to mix, followed by a brief centrifugation.
3. Incubate the 23 µL senseRNA-Primer Mix (Thermal Cycler Program 7):
   - 80°C for 5 minutes
   - 4°C for 2 minutes
   - **Transfer to ice**
4. Prepare a Labeling RT Master Mix in a separate tube on ice:

Table 3.1 Labeling RT Master Mix

Component	Cap Color	Volume for One Reaction
RT Buffer Mix	Red	8 µL
DTT	Red	4 µL
Labeling dNTP Mix	Red	2 µL
RT Enzyme Mix	Red	2 µL
RNase Inhibitor	Red	1 µL
Total		**17 µL**

5. Gently vortex the tube to mix, briefly centrifuge, and add the 17 µL Labeling RT Master Mix to the 23 µL senseRNA-primer mix for a volume of 40 µL. Gently vortex the tube to mix, followed by a brief centrifugation.
6. Incubate the 40 µL reactions for first strand synthesis (Thermal Cycler Program 8):
   - 42°C for 120 minutes
   - 25°C for 2 minutes
   - **Transfer to ice**
7. Prepare a Second Strand Master Mix in a separate tube on ice:

Table 3.2 Second Strand Master Mix

Component	Cap Color	Volume for One Reaction
$MgCl_2$	Purple	13 µL
Labeling dNTP Mix	Red	2 µL
Klenow	Purple	3 µL
RNase H	Purple	2 µL
Total		**20 µL**

8. Gently vortex the tube to mix, briefly centrifuge, and add 20 µL Second Strand Master Mix to each 40 µL sample for a volume of 60 µL. Gently vortex the tube to mix, followed by a brief centrifugation.
9. Incubate the 60 µL reactions for second strand synthesis (Thermal Cycler Program 9):
   - 37°C for 40 minutes
   - 75°C for 10 minutes
   - 4°C for 2 minutes
10. At room temperature, immediately add 2 µL of WT Stop Solution (brown cap) to each sample. Gently vortex the tube to mix, followed by a brief centrifugation.
11. Incubate the 62 µL samples (Thermal Cycler Program 10).
   - 65°C for 30 minutes
   - 25°C for 2 minutes

NOTE: During this time, shake or vortex the Purification Beads bottle to resuspend the magnetic particles that may have settled. Aliquot the appropriate amount of Purification Beads, and keep at room temperature. For each reaction, 126 µL will be needed.

12. At room temperature, immediately add 8 µL of WT Neutralization Mix (brown cap) to each sample for a volume of 70 µL. Gently vortex the tube to mix, followed by a brief centrifugation.

NOTE: Samples can be stored at –20°C at this point.

Purification of Double-Stranded cDNA for Labeling with Purification Beads

NOTE:

- **Use Purification Beads - Labeling.**
- **Prepare fresh dilutions of 70% ethanol each time Purification Beads are used.**
- **Preheat the Nuclease-free Water at 65°C for elution.**
- **Occasionally, the bead/sample mixture may be brownish in color, and not completely clear when placed on the magnet. In those situations, switch to a different position on the magnetic stand, switch to a new magnetic stand, or pellet beads by centrifugation.**

1. If not already done, shake or vortex the Purification Beads bottle to resuspend the magnetic particles that may have settled. Allow samples and Purification Beads to reach room temperature. Ensure that the samples are at room temperature.
2. Place Nuclease-free Water in a 65°C heat block or oven.
3. For each reaction, add 126 µL of Purification Beads to a well of a 96 well round bottom plate. Transfer each 70 µL double-stranded cDNA sample to a well containing Purification Beads and mix well by pipetting up and down 10-20 times.
4. **Add 65 µL of 100% Ethanol to the double-stranded cDNA sample Purification Beads mixture and mix well by pipetting up and down 10-20 times. Incubate for 10 minutes at room temperature (20-25°C).**
5. Place the plate onto a Magnetic Stand (or equivalent) for 10 minutes to separate the beads from the solution.
6. Slowly aspirate and discard the cleared solution, being careful not to disturb the magnetic beads.
7. While on the Magnetic Stand, add **170 µL** of 70% ethanol to each well and incubate for 30 seconds at room temperature. With a pipette set to 200 µL, slowly aspirate the ethanol wash solution, being careful not to disturb the magnetic beads. Repeat for a total of 3 washes. Completely remove the final wash solution.
8. Air-dry on the magnetic stand for 5 minutes until no liquid is visible, yet the pellet appears shiny. Additional time may be required. Do not over-dry the beads as this will reduce the elution efficiency. The bead surface will appear dull, and may have surface cracks when it is over-dry.

9. Remove the plate from the Magnetic Stand.
10. Add 25 µL of the 65°C Nuclease-free Water to each well. Once water is added to all wells, incubate at room temperature (20-25°C) for 2 to 3 minutes to elute the sample from the magnetic beads. During this time, beads can be mixed by gentle shaking of the plate, or by pipetting up and down until resuspended.
11. Place the plate onto the Magnetic Stand for 3 minutes to separate the beads from the solution.
12. Slowly aspirate the purified double-stranded cDNA sample and transfer to a new tube, being careful not to disturb the magnetic beads. Record the volume recovered. Minimal bead carryover will not inhibit subsequent enzymatic reactions.

Quantitation of Double-Stranded cDNA

Quantify 1 to 2 ?L of double-stranded cDNA using a NanoDrop or other instrument. From the OD, determine the nucleic acid concentration of each labeled sample:

A_{260} nm x 50 (double-stranded DNA extinction coefficient) x dilution factor
= concentration of double-stranded cDNA in ng/µL

Calculate the $A_{260/280}$ ratio to determine purity. A ratio of 1.9-2.1 is desirable.

Optional: Unlabeled double-stranded cDNA may be analyzed by Quantitative PCR (do not use PCR master mixes containing Uracil N-Glycosylase).

The minimum amounts of double-stranded cDNA required for terminal labeling are listed in Table 3.3. Store remaining double-stranded cDNA at –20°C.

Table 3.3 Double-stranded cDNA Amounts Required for Terminal Labeling

	169-Format Array	100-Format Array	49-Format Array
ds-cDNA per Array	3.5 µg	5 µg	6 µg
Minimum Concentration of Eluate	≥ 156 ng/µL	≥ 225 ng/µL	≥ 270 ng/µL

NOTE: Samples can be stored at –20°C at this point.

Terminal Labeling of Double-Stranded cDNA

1. Adjust the volume of required double-stranded cDNA given in Table 3.3 to 22.5 µL with Nuclease-free Water.
2. Prepare a Terminal Labeling Reaction Master Mix in a separate tube on ice:

Table 3.4 Terminal Labeling Reaction Master Mix

Component	Cap Color	Volume for One Reaction
Fragment and Label Buffer Mix	Purple	6.0 µL
Fragment and Label Enzyme Mix	Purple	1.5 µL
Total		**7.5 µL**

3. Gently vortex the tube to mix, briefly centrifuge, and add the 7.5 µL Terminal Labeling Reaction Master Mix to the 22.5 µL double-stranded cDNA for a final reaction volume of 30 µL. Gently vortex the tube to mix, followed by a brief centrifugation.

4. Incubate samples for terminal labeling (Thermal Cycler Program 11):
   - 37°C for 60 minutes
   - 93°C for 2 minutes
   - 4°C for 2 minutes
5. Optional: Run the gel shift assay as indicated in Appendix B, *Gel-Shift Assay* on page 29.
6. Immediately proceed to Chapter 4, *Affymetrix® Whole-Transcript Expression Array Hybridization* on page 25.

Chapter 4

Affymetrix® Whole-Transcript Expression Array Hybridization

Preparation of Ovens, Arrays, and Sample Registration Files

1. Turn Affymetrix® Hybridization Oven on and set the temperature to 47°C. Set the RPM to 60. Turn the rotation on and allow the oven to preheat.
2. Equilibrate the arrays to room temperature immediately before use. Label the array with the name of the sample that will be hybridized.
3. Register the sample and array information in AGCC.

Hybridization

1. Bring the reagents listed in Table 4.1, below, to room temperature. The reagents may be found in the GeneChip® Hybridization, Wash and Stain Kit, and the GeneChip® Hybridization Control Kit.

NOTE: DMSO will solidify when stored at 2-8°C. Please ensure that the reagent is completely thawed prior to use. After the first use, it is recommended to store DMSO at room temperature.

2. Heat the 20X Hybridization Controls at 65°C for 5 minutes (Thermal Cycler Program 12).
3. For each sample, prepare a Hybridization Master Mix:

Table 4.1 Hybridization Master Mix for a Single Array

Component	169-Format	100-Format	49-Format
Control Oligo B2 (3 nM)	1.5 µL	2.2 µL	3.3 µL
20X Hybridization Controls (*bioB, bioC, bioD, cre*)	4.5 µL	6.5 µL	10 µL
2X Hybridization Mix	45 µL	65 µL	100 µL
DMSO	9 µL	13 µL	20 µL
Nuclease-free Water	0 µL	13.3 µL	36.7 µL
Volume of Master Mix	**60 µL**	**100 µL**	**170 µL**

4. Gently vortex the tube to mix, briefly centrifuge, and add the Hybridization Master Mix to the biotin-labeled double-stranded cDNA to prepare the Hybridization Cocktail:

Table 4.2 Hybridization Cocktail Mix for a Single Array

Component	169-Format	100-Format	49-Format
Volume of Master Mix	60 µL	100 µL	170 µL
Biotin-labeled ds-cDNA	30 µL	30 µL	30 µL
Total	**90 µL**	**130 µL**	**200 µL**

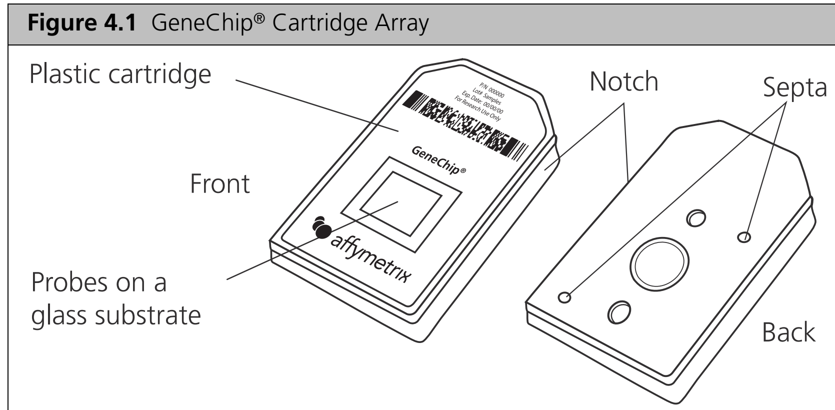
5. Incubate the Hybridization Cocktail Mix (Thermal Cycler Program 13):
   - 99°C for 5 minutes
   - 47°C for 5 minutes
6. Briefly spin the hybridization cocktail at maximum speed to collect the hybridization mixture.

7. Insert a pipet tip into the upper right septum to allow for venting. Inject the appropriate amount of Hybridization Cocktail into each array:

Table 4.3 Array Volumes for Hybridization Cocktail Mix

Component	169-Format	100-Format	49-Format
Volume to load on Array	90 µL	130 µL	200 µL

Figure 4.1 GeneChip® Cartridge Array



8. Remove the pipet tip from the upper right septum of the array. Cover both septa with 1/2" Tough-Spots to minimize evaporation and/or prevent leaks.
9. Place the arrays into hybridization oven trays. Load the trays into the hybridization oven.
10. Hybridize with rotation at 60 rpm for 16 to 18 hours at 47°C.

Washing and Staining

For additional information about washing, staining, and scanning, refer to the *Genechip® Expression Wash, Stain, and Scan User Guide for Cartridge Arrays* (PN 702731) and the *Affymetrix® Command Console® User Guide* (P/N 702569).

1. Remove the arrays from the oven. Remove the Tough-Spots from the arrays.
2. Extract the hybridization cocktail from each array and transfer it to a new tube or well of a 96-well plate in order to save the hybridization cocktail. Store on ice during the procedure, or at –80°C for long-term storage.
3. Fill each array completely with Wash Buffer A.
4. Allow the arrays to equilibrate to room temperature before washing and staining.

NOTE: Arrays can be stored in Wash Buffer A at 4°C for up to 3 hours before proceeding with washing and staining. Equilibrate arrays to room temperature before washing and staining.

5. Place vials into sample holders on the fluidics station:
   - A. Place one (amber) vial containing 600 µL Stain Cocktail 1 in sample holder 1.
   - B. Place one (clear) vial containing 600 µL Stain Cocktail 2 in sample holder 2.
   - C. Place one (clear) vial containing 800 µL Array Holding Buffer in sample holder 3.

6. Wash the arrays according to array type and components used for Hybridization, Wash and Stain. For HWS kits the protocols are:

Table 4.4 Fluidics Protocol

	169-Format	100-Format	49-Format
Fluidics Protocol	FS450_0007	FS450_0002	FS450_0001

7. Check for air bubbles. If there are air bubbles, manually fill the array with Array Holding Buffer. If there are no air bubbles, cover both septa with 3/8" Tough-Spots. Inspect the array glass surface for dust and/or other particulates and, if necessary, carefully wipe the surface with a clean lab wipe before scanning.

Scanning

The instructions for using the scanner and scanning arrays can be found in the *Affymetrix® Command Console® User Guide* (P/N 702569).

Troubleshooting

For troubleshooting purposes, we recommend following Appendix A, *Use of Control DNA* on page 28, to ensure certain kit components are working appropriately.

References

1. Roberts L, *et al*. Identification of methods for use of formalin-fixed, paraffin-embedded tissue samples in RNA expression profiling. Genomics 2009, 94(5):341-8.
2. Pillai R, *et al*. Validation and Reproducibility of a Microarray-Based Gene Expression Test for Tumor Identification in Formalin-Fixed, Paraffin-Embedded Specimens. Journal of Molecular Diagnostics January 2011, Vol. 13, No. 1.
3. Koh SS, *et al*. Molecular classification of melanomas and nevi using gene expression microarray signatures and formalin-fixed and paraffin-embedded tissue. Mod Pathol. 2009 Apr, 22(4):538-46.
4. Goff LA, *et al*. Evaluation of sense-strand mRNA amplification by comparative quantitative PCR. BMC Genomics 2004, 5:76.
5. LeMessurier KS, *et al*. Differential Expression of Key Pneumococcal Virulence Genes In Vivo. Microbiology 2006, 152:305-311.

Appendix A

Use of Control DNA

The Control DNA can be used to test reactions after First Strand cDNA Synthesis and Purification. This procedure can be followed for troubleshooting purposes, to ensure the following kit components for Promoter Synthesis, In Vitro Transcription and Purification of senseRNA with Purification Beads are working appropriately. After following this procedure, at least 25 µg senseRNA should be obtained.

Use of Control DNA

1. Prepare 20 ng of Control DNA for tailing:

Table A.1 Dilution of Control DNA

	Cap Color	Volume for One Reaction
Control DNA	Clear	4 µL
Nuclease-free Water	White	8 µL
Total		**12 µL**

2. Follow the procedures for *Promoter Synthesis* on page 17, *In Vitro Transcription* on page 18, and *Purification of senseRNA with Purification Beads* on page 19.
3. At least 25 µg of purified senseRNA should be obtained.

Appendix B

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. The procedure takes approximately 90 minutes to complete.

Materials Required (Optional)

- NeutrAvidin®
- 4% to 20% TBE gel
- Electrophoresis system with power supply
- 1X TBE running buffer
- DNA Ladder
- TBE Gel loading dye
- SYBR® Gold Nucleic Acid Gel Stain
- Transilluminator

1. Dilute 1 µL of each terminal labeling reaction: 3 µL Nuclease-free water and 1 µL terminal labeling reaction.

NOTE: 2 µL of this dilution will be used for the gel shift. Save the remaining 2 µL for repeat gel shift analysis if necessary.

2. Prepare a NeutrAvidin solution of 2 mg/mL following the manufacturer's recommendation.
3. Place a 4% to 20% TBE gel into the gel holder and load system with 1X TBE Buffer.
4. 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 70ºC for 2 minutes.
5. Add 5 µL of 2 mg/mL NeutrAvidin to one of the two tubes for each sample tested.
6. Bring the volume of the other tube to 6 µL with water.
7. Mix and incubate at room temperature for 5 minutes.
8. Bring the volume of DNA ladders to 6 µL with water.
9. Add 4 µL loading dye to all samples and DNA ladders.
10. Carefully load 10 µL samples and ladders on gel.
11. Run the gel at 150 volts until the front dye almost reaches the bottom, approximately 1 hour.
12. While the gel is running, prepare 100 mL of a 1X solution of SYBR Gold for staining. SYBR Gold may be diluted in 1X TBE running buffer.
13. Break open cartridge and stain the gel in 1X SYBR Gold for 30 minutes.
14. Place the gel on a UV light box and image using the appropriate filter for SYBR Gold.
15. The absence of a shift pattern indicates poor biotin labeling. The problem should be addressed before proceeding to the hybridization step.

Appendix C

Revision History

Table C.1

Description	Section
GeneChip® Poly-A RNA Control Kit is added to Additional Materials/Equipment Required.	Additional Materials/Equipment Required
Specify volume for one reaction in Master Mix Tables.	Amplification Procedure Labeling Procedure
More detailed guidance is given in regard to mixing Master Mixes.	Amplification Procedure Labeling Procedure
The term "RNA Purification Beads" has been changed to "Purification Beads".	Kit Specifications Purification
More detailed guidance is given in regard to bead drying and yields.	Purification
Stop Solution and Neutralization Mix reaction set up temperature is specified for room temperature.	Labeling Procedure
The cDNA hybridization mass amounts have been changed.	Labeling Procedure

Appendix D

Contact Information

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