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Patents
This product may be covered by one or more of the following patents: U.S. Patent Nos. 6,864,059; 6,965,020; 7,282,327; 7,291,463 and 7,468,243.

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Chapter 1

WT Pico Reagent Kit

Product Information

Purpose of the Product

The WT Pico Reagent Kit protocol prepares hybridization ready targets from picogram to nanogram quantities of total RNA samples for whole transcriptome analysis with GeneChip® Whole-Transcript (WT) Expression Arrays (Figure 1.1). Reverse transcription is initiated at the poly-A tail as well as throughout the entire length of RNA to capture both coding and multiple forms of non-coding RNA, making the WT Pico Reagent Kit ideal for amplification of intact, partially degraded, and compromised RNA samples. RNA amplification is achieved using low-cycle PCR followed by linear amplification using T7 \textit{in vitro} transcription (IVT) technology. The cRNA is then converted to biotinylated sense-strand DNA hybridization targets for unbiased coverage of the transcriptome. The kit is optimized to work with a wide range of samples including tissues, cell lines, whole blood, and formalin-fixed paraffin-embedded (FFPE) tissues.

Sample Requirements

The WT Pico Reagent Kit is comprised of reagents and a protocol for producing hybridization-ready DNA from 100 pg to 10 ng of purified total RNA from cells or tissues and 500 pg to 50 ng of purified total RNA from FFPE tissues. The total RNA samples can be used directly without removal of ribosomal or globin RNA prior to target preparation with WT Pico Reagent Kit. The recommended total RNA inputs in Table 1.1 are based on total RNA from HeLa cells and 1 to 9 years old FFPE tissues.

To ensure high reproducibility in whole transcriptome amplification, we recommend using a starting amount of 500 pg RNA template of purified total RNA from cells or tissues and 2 ng of purified total RNA from FFPE tissues. Depending on the copy number of the transcripts, it may be possible to use smaller amounts of RNA template (Table 1.1). Input amounts that are lower than the recommended amounts can result in insufficient yields, poor conversion to cDNA, and reduction in array signals. The starting RNA template should not be less than the minimum input amounts listed in Table 1.1. If your RNA sample is not limiting, we recommend that you start with more total RNA.

<table>
<thead>
<tr>
<th>RNA Input</th>
<th>Total RNA from Fresh-Frozen Cells or Tissues</th>
<th>Total RNA from Formalin-Fixed, Paraffin-Embedded Tissues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimum</td>
<td>100 pg</td>
<td>500 pg</td>
</tr>
<tr>
<td>Recommended</td>
<td>500 pg – 10 ng</td>
<td>2 ng – 50 ng</td>
</tr>
<tr>
<td>Maximum</td>
<td>10 ng</td>
<td>50 ng</td>
</tr>
</tbody>
</table>

Table 1.1 Input RNA Limits

Performance Specifications

The WT Pico Reagent Kit and protocol has been validated to generate greater than 20 μg cRNA and greater than 5.5 μg of single-stranded cDNA from 500 pg of HeLa total RNA and 2 ng of 1 to 9 years old FFPE tissues.
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.
Assay Workflow

**Figure 1.1** WT Pico Amplification and Labeling Process

<table>
<thead>
<tr>
<th>Day 1</th>
<th>First-Strand cDNA Synthesis</th>
<th>Clean-up Excess Primers</th>
<th>3’ Adaptor Synthesis</th>
<th>Pre-IVT Amplification</th>
<th>cRNA Amplification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 hr 5 min</td>
<td>40 min</td>
<td>40 min</td>
<td>&lt; 1.5 hr</td>
<td>14 hr</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Day 2</th>
<th>cRNA Purification &amp; Quantitation</th>
<th>2nd-Cycle ss-cDNA Synthesis</th>
<th>Template RNA Removal</th>
<th>ss-cDNA Purification &amp; Quantification</th>
<th>Hybridization to WT Array</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2 hr</td>
<td>50 min</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Incubation Time**

- Reverse Transcriptase
- Exonuclease
- Exo-free Klenow
- Taq
- T7 RNA Polymerase
- UDG & APE1
- TdT & DLR

**Symbols**

- RNA
- DNA
- NNN Random Primers
- TTT dT Primers
- DLR DNA Labeling Reagent
- Biotin
# Kit Contents and Storage

## Table 1.2 GeneChip® WT Pico Reagent Kit Contents and Storage

<table>
<thead>
<tr>
<th>Component</th>
<th>12-Reaction Kit for manual use (P/N 902622)</th>
<th>30-Reaction Kit for manual use (P/N 902623)</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WT Pico Amplification Kit, Module 1, –20°C: cRNA Step</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT Pico First-Strand Enzyme</td>
<td>14 μL</td>
<td>45 μL</td>
<td>–20°C</td>
</tr>
<tr>
<td>WT Pico First-Strand Buffer</td>
<td>56 μL</td>
<td>160 μL</td>
<td>–20°C</td>
</tr>
<tr>
<td>WT Pico Cleanup Reagent</td>
<td>28 μL</td>
<td>90 μL</td>
<td>–20°C</td>
</tr>
<tr>
<td>WT Pico 3’ Adaptor Enzyme</td>
<td>14 μL</td>
<td>45 μL</td>
<td>–20°C</td>
</tr>
<tr>
<td>WT Pico 3’ Adaptor Buffer</td>
<td>98 μL</td>
<td>245 μL</td>
<td>–20°C</td>
</tr>
<tr>
<td>WT Pico PCR Enzyme</td>
<td>14 μL</td>
<td>45 μL</td>
<td>–20°C</td>
</tr>
<tr>
<td>WT Pico PCR Buffer</td>
<td>407 μL</td>
<td>1015 μL</td>
<td>–20°C</td>
</tr>
<tr>
<td>WT Pico IVT Enzyme</td>
<td>84 μL</td>
<td>210 μL</td>
<td>–20°C</td>
</tr>
<tr>
<td>WT Pico IVT Buffer</td>
<td>337 μL</td>
<td>840 μL</td>
<td>–20°C</td>
</tr>
<tr>
<td>Poly-A Control Stock</td>
<td>16 μL</td>
<td>16 μL</td>
<td>–20°C</td>
</tr>
<tr>
<td>Nuclease-free Water</td>
<td>4 x 1 mL</td>
<td>10 x 1 mL</td>
<td>any temp*</td>
</tr>
<tr>
<td><strong>WT Pico Amplification Kit, Module 2, –20°C: cDNA Step</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT Pico 2nd-Cycle Primers</td>
<td>56 μL</td>
<td>140 μL</td>
<td>–20°C</td>
</tr>
<tr>
<td>WT Pico 2nd-Cycle ss-cDNA Enzyme</td>
<td>56 μL</td>
<td>140 μL</td>
<td>–20°C</td>
</tr>
<tr>
<td>WT Pico 2nd-Cycle ss-cDNA Buffer</td>
<td>112 μL</td>
<td>280 μL</td>
<td>–20°C</td>
</tr>
<tr>
<td>WT Pico RNase H</td>
<td>56 μL</td>
<td>180 μL</td>
<td>–20°C</td>
</tr>
<tr>
<td>WT Pico Frag. &amp; Label Enzyme</td>
<td>28 μL</td>
<td>70 μL</td>
<td>–20°C</td>
</tr>
<tr>
<td>WT Pico Frag. &amp; Label Buffer</td>
<td>168 μL</td>
<td>420 μL</td>
<td>–20°C</td>
</tr>
<tr>
<td><strong>WT Pico Amplification Kit, Module 3, 4°C: Purification Beads</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Purification Beads</td>
<td>3 mL</td>
<td>8 mL</td>
<td>4°C†</td>
</tr>
<tr>
<td><strong>Control RNA, –20°C: HeLa Total RNA</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control RNA (100 ng/μL HeLa total RNA)</td>
<td>6 μL</td>
<td>6 μL</td>
<td>–20°C</td>
</tr>
<tr>
<td><strong>GeneChip® Hybridization Control Kit</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20X Hybridization Controls</td>
<td>450 μL</td>
<td>450 μL</td>
<td>–20°C</td>
</tr>
<tr>
<td>3 nM Control Oligo B2</td>
<td>150 μL</td>
<td>150 μL</td>
<td>–20°C</td>
</tr>
</tbody>
</table>

* Store the Nuclease-free Water at –20°C, 4° C, or room temperature.
† Do not freeze.
Required Materials

Instruments

Table 1.3 Instruments Required for Target Preparation

<table>
<thead>
<tr>
<th>Item</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Magnetic Stand-96</td>
<td>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</td>
</tr>
<tr>
<td>Microcentrifuge</td>
<td>Major Laboratory Supplier</td>
</tr>
<tr>
<td>Spectrophotometer and Fluorometer</td>
<td>Thermo Fisher Scientific, or equivalent quantitation instrument</td>
</tr>
<tr>
<td>• NanoDrop® UV-Vis Spectrophotometer</td>
<td>Thermo Fisher Scientific, or equivalent quantitation instrument</td>
</tr>
<tr>
<td>• Optional: NanoDrop® Fluorospectrometer</td>
<td>Thermo Fisher Scientific, or equivalent quantitation instrument</td>
</tr>
<tr>
<td>• Optional: Qubit® 2.0 Fluorometer</td>
<td>Thermo Fisher Scientific, or equivalent quantitation instrument</td>
</tr>
<tr>
<td>Optional: 2100 Bioanalyzer</td>
<td>Agilent Technologies, Inc., or equivalent DNA and RNA sizing instrument</td>
</tr>
<tr>
<td>Pipette</td>
<td>Major Laboratory Supplier</td>
</tr>
<tr>
<td>Thermal Cycler</td>
<td>Various</td>
</tr>
<tr>
<td>Vortex Mixer</td>
<td>Major Laboratory Supplier</td>
</tr>
<tr>
<td>65°C heat block or oven for incubation of Nuclease-free Water during Purification</td>
<td>Major Laboratory Supplier</td>
</tr>
</tbody>
</table>

Table 1.4 GeneChip® Instrument Systems Required for Array Processing

<table>
<thead>
<tr>
<th>Instruments</th>
<th>Supplier</th>
<th>Part Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>GeneChip® Hybridization Oven 645</td>
<td>Affymetrix</td>
<td>P/N 00-0331 (110/220V)</td>
</tr>
<tr>
<td>GeneChip® Fluidics Station 450</td>
<td>Affymetrix</td>
<td>P/N 00-0079</td>
</tr>
<tr>
<td>GeneChip® Scanner 3000 7G</td>
<td>Affymetrix</td>
<td>P/N 00-0212 (North America)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P/N 00-0213 (International)</td>
</tr>
<tr>
<td>GeneChip® AutoLoader with External Barcode Reader</td>
<td>Affymetrix</td>
<td>P/N 00-0090 (GCS 3000 7G S/N 501)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P/N 00-0129 (GCS 3000 7G S/N 502)</td>
</tr>
<tr>
<td>GeneAtlas® System for Array Strips</td>
<td>Affymetrix</td>
<td>P/N 90-0894</td>
</tr>
<tr>
<td>GeneAtlas® Workstation</td>
<td>Affymetrix</td>
<td>P/N 00-0380 (115VAC)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P/N 00-0381 (230VAC)</td>
</tr>
<tr>
<td>GeneAtlas® Hybridization Station</td>
<td>Affymetrix</td>
<td>P/N 00-0377</td>
</tr>
<tr>
<td>GeneAtlas® Fluidics Station</td>
<td>Affymetrix</td>
<td>P/N 00-0376</td>
</tr>
<tr>
<td>GeneAtlas® Imaging Station</td>
<td>Affymetrix</td>
<td>P/N 74-0015</td>
</tr>
</tbody>
</table>
Table 1.4  GeneChip® Instrument Systems Required for Array Processing  (Continued)

<table>
<thead>
<tr>
<th>Instruments</th>
<th>Supplier</th>
<th>Part Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>GeneTitan® System for Array Plates</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GeneTitan® MC Instrument, NA/Japan includes 110v UPS</td>
<td>Affymetrix</td>
<td>P/N 00-0372</td>
</tr>
<tr>
<td>GeneTitan® MC Instrument, Int'l includes 220v UPS</td>
<td>Affymetrix</td>
<td>P/N 00-0373</td>
</tr>
<tr>
<td>GeneTitan® Instrument, NA/Japan includes 110v UPS</td>
<td>Affymetrix</td>
<td>P/N 00-0360</td>
</tr>
<tr>
<td>GeneTitan® Instrument, Int'l Includes 220v UPS</td>
<td>Affymetrix</td>
<td>P/N 00-0363</td>
</tr>
</tbody>
</table>

Reagents and Supplies

Table 1.5  Additional Reagents and Supplies Required

<table>
<thead>
<tr>
<th>Item</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>96-well round bottom microtiter plate</td>
<td>Costar, P/N 3795 or equivalent</td>
</tr>
<tr>
<td>GeneChip® Hybridization, Wash, and Stain Kit</td>
<td>Affymetrix (P/N 900720, 30 rxns)</td>
</tr>
<tr>
<td>GeneAtlas® Hybridization, Wash, and Stain Kit for WT Array Strips</td>
<td>Affymetrix (P/N 901667, 60 rxns)</td>
</tr>
<tr>
<td>GeneTitan® Hybridization, Wash, and Stain Kit for WT Array Plates</td>
<td>Affymetrix (P/N 901622, 96 rxns)</td>
</tr>
<tr>
<td>Nuclease-free aerosol-barrier tips</td>
<td>Major Laboratory Supplier</td>
</tr>
<tr>
<td>Nuclease-free 1.5 and 0.2 mL tubes or plates</td>
<td>Major Laboratory Supplier</td>
</tr>
<tr>
<td>Nuclease-free 15 mL tubes or containers</td>
<td>Major Laboratory Supplier</td>
</tr>
<tr>
<td>Nuclease-free Water (for preparing 80%ethanol wash solution)</td>
<td>Affymetrix (P/N 71786) or major laboratory supplier</td>
</tr>
<tr>
<td>Optional:</td>
<td></td>
</tr>
<tr>
<td>RNA Quantification Kit For SYBR Green I and ROX™ Passive Reference Dye</td>
<td>Affymetrix (P/N 902905; or equivalent reagents</td>
</tr>
<tr>
<td>RNA Quantification Kit For SYBR Green I and Fluorescein Passive Reference Dye</td>
<td>Affymetrix (P/N 902906; or equivalent reagents</td>
</tr>
<tr>
<td>Optional:</td>
<td></td>
</tr>
<tr>
<td>Quant-i™® RiboGreen® RNA Reagent Kit</td>
<td>Thermo Fisher Scientific</td>
</tr>
<tr>
<td>RNA HS Assay Kit</td>
<td>P/N R11490; or equivalent reagents</td>
</tr>
<tr>
<td>P/N Q32852; or equivalent reagents</td>
<td></td>
</tr>
<tr>
<td>Optional:</td>
<td></td>
</tr>
<tr>
<td>RNA 6000 Nano Kit</td>
<td>Agilent Technologies, Inc.</td>
</tr>
<tr>
<td>RNA 6000 Pico Kit</td>
<td>P/N 5067-1511; or equivalent DNA and RNA sizing reagents</td>
</tr>
<tr>
<td>Optional:</td>
<td></td>
</tr>
<tr>
<td>96-well plate sealing film</td>
<td>Major Laboratory Supplier</td>
</tr>
<tr>
<td>Tough-Spots®</td>
<td>Major Laboratory Supplier</td>
</tr>
<tr>
<td>100% Ethanol (Molecular Biology grade or equivalent)</td>
<td>Major Laboratory Supplier</td>
</tr>
</tbody>
</table>

* Before handling any chemicals, refer to the MSDS provided by the manufacturer and observe all relevant precautions.
Chapter 2
Protocol

Procedural Notes

Implement a Plan to Maintain Procedural Consistency

To minimize sample-to-sample variation that is caused by subtle procedural differences in gene expression assays, consider implementing a detailed procedural plan. The plan standardizes the variables in the procedure and should include:

- Method of RNA purification (Purify Total RNA)
- RNA quality and integrity (Evaluate RNA Quality and Evaluate RNA Integrity)
- Method of RNA quantitation (Evaluate RNA Quantity on page 12)
- Equipment preparation (Equipment Preparation on page 13)
- Reagent preparation (Reagent Preparation on page 14)
- RNase contamination prevention (RNase Contamination Prevention on page 15)
- DNA contamination prevention (DNA Contamination Prevention on page 15)
- Workflow stopping points

Sample Preparation

Purify Total RNA

Total RNA samples should be free of genomic DNA and we recommend including a DNase treatment with the RNA purification method. The contaminating genomic DNA may be amplified along with the RNA, which will lead to inaccurate measurement of whole transcriptome expression. In addition, the contaminating genomic DNA could cause over-estimation of the RNA amount.

We strongly recommend against the use of nucleic acid based carriers during RNA purification because many have been shown to produce cDNA product in first-strand synthesis reaction.

Choose a purification method or commercially available kit that is appropriate for your sample amount. For limiting cell numbers, choose purification methods that enable purification of total RNA preparations from small amounts.

Evaluate RNA Quality

RNA quality affects how efficiently an RNA sample is amplified using this kit. High-quality RNA is free of contaminating proteins, DNA, phenol, ethanol, and salts. To evaluate RNA quality, determine its \( A_{260}/A_{280} \) and \( A_{260}/A_{230} \) ratios. High quality total RNA samples should have an \( A_{260}/A_{280} \) ratio of 1.8 to 2.0, which indicates the absence of contaminating proteins. They should also have an \( A_{260}/A_{230} \) ratio of \( >2.0 \), which indicates the absence of other organic compounds, such as guanidium isothiocyanate, alcohol and phenol as well as cellular contaminants such as carbohydrates. An \( A_{260}/A_{230} \) ratio of \( <2.0 \) indicates the presence of contaminants, which may interfere with quantitation of total RNA.

The quality of RNA from FFPE samples can impact the success of gene expression analyses due to chemical modifications of RNA, cross-links of RNA with other molecules, degradation of RNA, and the limited amounts of sample usually available. Using real-time RT-PCR, quality of RNA from FFPE samples can be reliably and reproducibly assessed by measuring levels of abundance gene such as 18S ribosomal RNA prior to performing microarray experiments.
Evaluate RNA Integrity

The integrity of the RNA sample, or the proportion that is full length, is an important component of RNA quality. Reverse transcribing degraded input RNA may generate cDNA that lacks exonic regions. While it is impossible to guarantee satisfactory results with all degraded samples, the WT Pico Reagent Kit can work with samples that are moderately to severely degraded.

Two methods to evaluate RNA integrity are:

- Microfluidic analysis, using the Agilent 2100 Bioanalyzer with an RNA LabChip Kit or equivalent instrument.
- Denaturing agarose gel electrophoresis.

With microfluidic analysis, you use the RNA Integrity Number (RIN) to evaluate RNA integrity. For high concentration samples of 25 to 500 ng/μL, use the Agilent RNA 6000 Nano Kit and for low concentration samples of 0.05 to 5 ng/μL, use the Agilent RNA 6000 Pico Kit. For more information on how to calculate RIN, go to www.genomics.agilent.com.

With denaturing agarose gel electrophoresis and nucleic acid staining, you separate and make visible the 28S and 18S rRNA bands. The mRNA is likely to be full length if the:

- 28S and 18S rRNA bands are resolved into two discrete bands that have no significant smearing below each band.
- 28S rRNA band intensity is approximately twice that of the 18S rRNA band.

Evaluate RNA Quantity

Consider both the type and amount of sample RNA that are available when planning your experiment. Because mRNA content varies significantly with tissue type, determine the total RNA input empirically for each tissue type or experimental condition. The recommended total RNA inputs in Table 1.1 are based on total RNA from HeLa cells and 1 to 9 year old FFPE tissues. Use these values as reference points for determining your optimal RNA input. If your RNA sample is not limiting, we recommend that you start with more total RNA.

Determine RNA Quantity by UV Absorbance

Determine the concentration of total RNA by measuring its absorbance at 260 nm. Use Nuclease-free Water as a blank. Affymetrix recommends using NanoDrop Spectrophotometers for convenience. No dilutions or cuvettes are needed; just use 1 to 1.5 μL of the RNA sample directly. The detection limit is 5 to 500 ng/μL with ND-1000 Spectrophotometer (Aranda; 2009). Affymetrix recommends that samples with high concentrations should be diluted with Nuclease-free Water before measurement and reaction setup.

Determine RNA Quantity by Fluorescence-Based Quantitation

Determine the concentration of total RNA by fluorescence-based quantitation using an RNA RiboGreen® dye assay (e.g., Quant-iT™ RiboGreen® RNA Reagent and Kit) and the NanoDrop Fluorospectrometer for initial RNA concentration of 5 pg/μL to 1 ng/μL (www.nanodrop.com). Fluorescence-based RNA quantitation can also be performed using the Qubit® RNA HS Assay Kit and the Qubit® 2.0 Fluorometer for initial RNA concentration of 250 pg/μL to 100 ng/μL (www.lifetechnologies.com).

Determine RNA Quantity by Bioanalyzer

Affymetrix does not recommend RNA concentration determination using a Bioanalyzer as it is not accurate, especially at RNA concentrations less than 25 ng/μL. The RNA 6000 Nano Kit may be used for total RNA quantity determination of high concentration samples (25 to 500 ng/μL) but the RNA 6000 Pico Kit should not be used for total RNA quantitation.
**Determine RNA Quantity by Quantitative, Real-Time RT-PCR**

The amounts of RNA that are too small for quantitation by UV absorbance or fluorometric assays, real-time RT-PCR should be used for quantitation. Using real-time RT-qPCR such as RNA Quantification Kit from Affymetrix, small amount of RNA samples can be reliably and reproducibly quantified by measuring levels of abundance gene such as 18S ribosomal RNA or beta actin prior to performing target preparation for microarray experiments.

**Equipment Preparation**

**Recommended Thermal Cycler**

Make sure that the heated cover of your thermal cycler either tracks the temperature of the thermal cycling block or supports specific temperature programming.

**Program the Thermal Cycler**

Set the temperature for the heated lid to or near the required temperature for each step. An alternate protocol may be used for thermal cyclers that lack a programmable heated lid, although this is not the preferred method. Yields of cRNA may be greatly reduced if a heated lid is used during the 3’ Adaptor cDNA Synthesis or during the *In Vitro* Transcription cRNA Synthesis steps. We recommend leaving the heated lid open during the 3’ Adaptor cDNA Synthesis. A small amount of condensation will form during the incubation. This is expected and should not significantly decrease cRNA yields. For the *In Vitro* Transcription cRNA Synthesis, we recommend leaving the heated lid open or incubating the reaction in a hybridization oven at 40°C if a programmable heated lid thermal cycler is unavailable. Incubation temperatures and times are critical for effective RNA amplification. Use properly calibrated thermal cyclers and adhere closely to the incubation times.

**NOTE:** Concentration fluctuations that are caused by condensation can affect yield. Ensure that the heated lid feature of the thermal cycler is working properly.

**Table 2.1 Thermal Cycler Programs**

<table>
<thead>
<tr>
<th>Program</th>
<th>Heated Lid Temp</th>
<th>Step 1</th>
<th>Step 2</th>
<th>Step 3</th>
<th>Step 4</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>First-Strand cDNA Synthesis</td>
<td>42°C or 105°C</td>
<td>25°C for 5 min</td>
<td>42°C for 60 min</td>
<td>4°C for 2 min</td>
<td>10 μL</td>
<td></td>
</tr>
<tr>
<td>Cleanup</td>
<td>80°C or 105°C</td>
<td>37°C for 30 min</td>
<td>80°C for 10 min</td>
<td>4°C for 2 min</td>
<td>12 μL</td>
<td></td>
</tr>
<tr>
<td>3’ Adaptor Synthesis</td>
<td>RT, disable, or left open</td>
<td>15°C for 15 min</td>
<td>35°C for 15 min</td>
<td>70°C for 10 min</td>
<td>4°C for 2 min</td>
<td>20 μL</td>
</tr>
<tr>
<td>Pre-IVT Amplification</td>
<td>105°C</td>
<td>95°C for 2 min</td>
<td>6, 9 or 12 cycles of 94°C for 30 sec, 70°C for 5 min</td>
<td>4°C for 2 min</td>
<td>50 μL</td>
<td></td>
</tr>
<tr>
<td><em>In Vitro</em> Transcription cRNA Synthesis</td>
<td>40°C or 50°C</td>
<td>40°C for 14 hr</td>
<td>4°C hold</td>
<td>80 μL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2nd-Cycle ss-cDNA Synthesis</td>
<td>70°C or 105°C</td>
<td>25°C for 10 min</td>
<td>42°C for 90 min</td>
<td>70°C for 10 min</td>
<td>4°C hold</td>
<td>40 μL</td>
</tr>
<tr>
<td>RNA Hydrolysis</td>
<td>70°C or 105°C</td>
<td>37°C for 45 min</td>
<td>95°C for 5 min</td>
<td>4°C hold</td>
<td>44 μL</td>
<td></td>
</tr>
<tr>
<td>Fragmentation and Labeling</td>
<td>93°C or 105°C</td>
<td>37°C for 60 min</td>
<td>93°C for 2 min</td>
<td>4°C hold</td>
<td>60 μL</td>
<td></td>
</tr>
</tbody>
</table>
Reagent Preparation

**IMPORTANT:** You can freeze/thaw the reagents in the 12 and 30 reaction kits ≤3 times.

Handle kit components as follows:

- Properly chill essential equipment such as cooling blocks and reagent coolers before use.
- Enzymes and Reagents: Mix by gently vortexing the tube followed by a brief centrifuge to collect contents of the tube and then keep on ice.
- Buffers and Primers: Thaw on ice, thoroughly vortex to dissolve precipitates followed by a brief centrifuge to collect contents of the tube. If necessary, warm the buffer(s) at ≤37°C for 1 to 2 min, or until the precipitate is fully dissolved and then keep on ice.
- Purification Beads: Allow to equilibrate to room temperature before use.
- Prepare master mixes for each step of the procedure to save time, improve reproducibility, and minimize pipetting errors.
- Prepare Master Mixes as follows:
  - Prepare only the amount needed for all samples in the experiment plus ~10% overage to correct for pipetting losses when preparing the master mixes.
  - Use non-stick nuclease-free tubes to prepare the master mixes.
  - Enzyme should be added last and just before adding the master mix to the reaction.
- Return the components to the recommended storage temperature immediately after use.
- Ensure that all temperature transitions to incubation temperatures are rapid and/or well-controlled to help maintain consistency across samples.

**IMPORTANT:** Master Mixes and samples should be mixed thoroughly by gently vortexing followed by a quick spin to remove air bubbles and collect contents of tube or well.
RNase Contamination Prevention

RNase contamination in reagents and the work environment will result in failure to generate amplified targets. Follow these guidelines to minimize contamination:

- Wear disposable gloves and change them frequently.
- Clean and decontaminate work areas and instruments, including pipettes, with commercially available decontamination reagents.
- Avoid touching surfaces or materials that could introduce RNases.
- Use RNase-free filter tips and microcentrifuge tubes.
- Use a work area specifically designated for RNA work.

DNA Contamination Prevention

The most likely potential source of contamination for the WT Pico Assay is previously amplified DNA. Follow these guidelines to minimize possible sources of contamination:

- Before you set up the experiment, make sure you have two physically separated work areas with dedicated supplies and equipment in each area:
  - A Pre-Amplification Clean Area for performing all pre-amplification reaction setup:
    - Prepare total RNA sample
    - Prepare Poly-A RNA Controls
    - Prepare total RNA/Poly-A RNA Control mixture
    - Synthesize First-Strand cDNA
    - Synthesize 3' Adaptor cDNA
    - Synthesize Double-Stranded cDNA (reaction setup)
  - A Post-Amplification Area for performing all post-amplification reaction setup and concentration measurement:
    - Synthesize Double-Stranded cDNA (reaction incubation)
    - Synthesize cRNA by In Vitro Transcription
    - Purify cRNA
    - Synthesize 2nd-Cycle Single-Stranded cDNA
    - Hydrolyze RNA using RNase H
    - Purify 2nd-Cycle Single-Stranded cDNA
    - Fragment and Label Single-Stranded cDNA
  - Maintain a single direction workflow. Do not bring amplified products into the Pre-Amplification Clean Area.
  - Keep dedicated equipment in each of the areas used for this protocol, including pipettes, ice buckets, coolers, etc. Do not move equipment back and forth between the areas.
  - Concentration measurements can be performed in Post-Amplification Area.
  - Clean and decontaminate work areas and instruments, including pipettes, with commercially available decontamination reagents.
  - Always use filter tips for pipetting to reduce sample contamination.
Prepare Control RNA

HeLa Control RNA Preparation

To verify that the reagents are working as expected, a Control RNA sample (100 ng/μL total RNA from HeLa cells) is included with the kit.

To prepare the Control RNA for positive control reaction:

1. On ice, dispense 2 μL of the Control RNA in 38 μL of Nuclease-free Water for a total volume of 40 μL (5 ng/μL).
2. Add 2 μL of the 1st Dilution (5 ng/μL) to 38 μL of Nuclease-free Water for a total volume of 40 μL (250 pg/μL).
3. Follow the Prepare Total RNA/Poly-A RNA Control Mixture on page 18, but use 2 μL of the 2nd Dilution (500 pg) in the control reaction.

Note: The positive control reaction should produce >20 μg of cRNA and >5.5 μg of 2nd-cycle ss-cDNA from 500 pg Control RNA.

Poly-A RNA Control Preparation

Note:
- Affymetrix strongly recommends the use of Poly-A RNA Controls for all reactions that will be hybridized to GeneChip® arrays. To include the premixed controls from the Poly-A RNA Control Stock, prepare appropriate dilution of the Poly-A RNA Controls and add to the total RNA samples. Follow the Prepare Total RNA/Poly-A RNA Control Mixture on page 18.
- Do not use the Poly-A Control Dil Buffer to prepare serial dilution of Poly-A RNA Controls because it may cause non-target amplification.
- Prepare serial dilution of Poly-A RNA Control Stock with Nuclease-free Water.

A set of poly-A RNA controls supplied by Affymetrix is designed specifically to provide exogenous positive controls to monitor the entire target preparation. It 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 Nuclease-free Water and spiked directly into RNA samples to achieve the final concentrations (referred to as a ratio of copy number) summarized in Table 2.3.

Table 2.3 Final concentrations of Poly-A RNA Controls when added to total RNA samples

<table>
<thead>
<tr>
<th>Poly-A RNA Spike</th>
<th>Final Concentration (ratio of copy number)</th>
</tr>
</thead>
<tbody>
<tr>
<td>lys</td>
<td>1:100,000</td>
</tr>
<tr>
<td>phe</td>
<td>1:50,000</td>
</tr>
<tr>
<td>thr</td>
<td>1:25,000</td>
</tr>
<tr>
<td>dap</td>
<td>1:6,667</td>
</tr>
</tbody>
</table>

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 Nuclease-free Water are provided in the WT Pico Amplification Kit, Module 1 to prepare the appropriate serial dilutions based on Table 2.4. This is a guideline when ≤1, 2, 5, 10, 20, or 50 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 2.4 Serial Dilution of Poly-A RNA Control Stock with Nuclease-free Water

<table>
<thead>
<tr>
<th>Total RNA Input Amount</th>
<th>Serial Dilutions</th>
<th>Volume of Fourth Dilution to Add to Total RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>First Dilution</td>
<td>Second Dilution</td>
</tr>
<tr>
<td>≤1 ng</td>
<td>1:50</td>
<td>1:100</td>
</tr>
<tr>
<td>2 ng</td>
<td>1:50</td>
<td>1:100</td>
</tr>
<tr>
<td>5 ng</td>
<td>1:50</td>
<td>1:100</td>
</tr>
<tr>
<td>10 ng</td>
<td>1:50</td>
<td>1:100</td>
</tr>
<tr>
<td>20 ng</td>
<td>1:50</td>
<td>1:100</td>
</tr>
<tr>
<td>50 ng</td>
<td>1:50</td>
<td>1:100</td>
</tr>
</tbody>
</table>

IMPORTANT:
- Avoid pipetting solutions less than 2 μL in volume to maintain precision and consistency when preparing the dilutions.
- Use non-stick nuclease-free tubes to prepare all of the dilutions (not included).
- After each step, mix the Poly-A Control dilutions thoroughly by gently vortexing followed by a quick centrifuge to collect contents of the tube.

For example, to prepare the Poly-A RNA dilutions for 500 pg of total RNA:
1. Add 2 μL of the Poly-A Control Stock to 98 μL of Nuclease-free Water for the 1st Dilution (1:50).
2. Add 2 μL of the 1st Dilution to 198 μL of Nuclease-free Water to prepare the 2nd Dilution (1:100).
3. Add 2 μL of the 2nd Dilution to 198 μL of Nuclease-free Water to prepare the 3rd Dilution (1:100).
4. Add 2 μL of the 3rd Dilution to 198 μL of Nuclease-free Water to prepare the 4th Dilution (1:100).
5. Add 2 μL of this 4th Dilution to 500 pg of total RNA. The final volume of total RNA with the diluted Poly-A controls should not exceed 5 μL.

NOTE: Always prepare fresh-dilution of Poly-A Controls from Poly-A Controls provided in the WT Pico Reagent Kit.
Prepare Total RNA/Poly-A RNA Control Mixture

Prepare total RNA according to your laboratory’s procedure. A maximum of 5 μL total RNA can be added to first-strand synthesis reaction. If you are adding Poly-A Spike Controls to your RNA, the volume of RNA must be 3 μL or less (Table 2.5). See Poly-A RNA Control Preparation on page 16 for more information. For example, when performing the Control RNA reaction, combine 2 μL of RNA (250 pg/μL), 2 μL of diluted Poly-A Spike Controls, and 1 μL of Nuclease-free Water.

NOTE: If you are adding Poly-A Spike Controls to your RNA, the volume of RNA must be 3 μL or less. If necessary, use a SpeedVac or ethanol precipitation to concentrate the RNA samples.

Table 2.5 Total RNA/Poly-A RNA Control Mixture

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume for One Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total RNA Sample (100 pg – 50 ng)</td>
<td>variable</td>
</tr>
<tr>
<td>Diluted Poly-A RNA Controls (4th Dilution)</td>
<td>2 μL</td>
</tr>
<tr>
<td>Nuclease-free Water</td>
<td>variable</td>
</tr>
<tr>
<td>Total Volume</td>
<td>5 μL</td>
</tr>
</tbody>
</table>

Synthesize First-Strand cDNA

In this reverse transcription procedure, total RNA is primed with primers containing a T7 promoter sequence. The reaction synthesizes single-stranded cDNA with T7 promoter sequence at the 5’ end.

NOTE: Avoid pipetting solutions less than 2 μL in volume to maintain precision and consistency. High-concentration RNA samples should be pre-diluted with Nuclease-free Water before adding to first-strand cDNA synthesis reaction.

IMPORTANT: Master Mixes and samples should be mixed thoroughly by gently vortexing followed by a quick spin to remove air bubbles and collect contents of tube or well.

1. Prepare First-Strand Master Mix.
   A. On ice, prepare the First-Strand Master Mix in a nuclease-free tube. Combine the components in the sequence shown in the table below. Prepare the master mix for all the total RNA samples in the experiment. Include ~10% excess volume to correct for pipetting losses.

Table 2.6 First-Strand Master Mix

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume for One Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT Pico First-Strand Buffer</td>
<td>4 μL</td>
</tr>
<tr>
<td>WT Pico First-Strand Enzyme</td>
<td>1 μL</td>
</tr>
<tr>
<td>Total Volume</td>
<td>5 μL</td>
</tr>
</tbody>
</table>

   B. Mix thoroughly by gently vortexing the tube. Centrifuge briefly to collect the mix at the bottom of the tube. Proceed immediately to the next step.
   C. On ice, transfer 5 μL of the First-Strand Master Mix to each tube or well.

2. Add total RNA to each First-Strand Master Mix aliquot.
   A. On ice, add 5 μL of the total RNA (Table 2.5) to each (5 μL) tube or well containing the First-Strand Master Mix for a final reaction volume of 10 μL.

See Prepare Total RNA/Poly-A RNA Control Mixture on page 18 for more information.
B. Mix thoroughly by gently vortexing the tube. Centrifuge briefly to remove air bubbles and collect the reaction at the bottom of the tube or well, then proceed immediately to the next step.

3. Incubate for 5 min at 25°C, for 60 min at 42°C, and then for at least 2 min at 4°C.
   A. Incubate the first-strand synthesis reaction in a thermal cycler using the First-Strand cDNA Synthesis program that is shown in Table 2.1 on page 13.
   B. Immediately after the incubation, centrifuge briefly to collect the first-strand cDNA at the bottom of the tube or well.
   C. Place the sample on ice for 2 min to cool the plastic, then proceed immediately to the next step.

4. Add Cleanup Reagent to each cDNA sample.
   A. On ice, transfer 2 μL of WT Pico Cleanup Reagent to each (10 μL) cDNA sample for a final reaction volume of 12 μL. Pipet up and down twice and carefully eject all liquid from pipette tip to ensure complete transfer of the Cleanup Reagent.
   B. Mix thoroughly by gently vortexing the tube. Centrifuge briefly to remove air bubbles and collect the reaction at the bottom of the tube or well, then proceed immediately to the next step.

NOTE: Air bubbles that may form during mixing should be removed by a quick spin.

5. Incubate for 30 min at 37°C, for 10 min at 80°C, and then for at least 2 min at 4°C.
   A. Incubate the first-strand cleanup reaction in a thermal cycler using the Cleanup program shown in Table 2.1 on page 13.
   B. Immediately after the incubation, centrifuge briefly to collect the first-strand cDNA at the bottom of the tube or well.
   C. Place the sample on ice for 2 min to cool the plastic, then proceed immediately to Synthesize 3’ Adaptor cDNA on page 20.

IMPORTANT: Transferring 3’ Adaptor Master Mix to hot plastics may significantly reduce cRNA yields. Holding the First-Strand cDNA Synthesis reaction at 4°C for longer than 10 min may significantly reduce cRNA yields.

TIP: When there is approximately 15 min left on the thermal cycler you may start reagent preparation for 3’ Adaptor cDNA Synthesis.
Synthesize 3' Adaptor cDNA

In this procedure, 3' Adaptor is added to single-stranded cDNA, which acts as a template for double-stranded cDNA synthesis in pre-IVT amplification reaction. The reaction uses DNA polymerase and RNase H to simultaneously degrade the RNA and synthesize single-stranded cDNA with 3' Adaptor.

**IMPORTANT:** Pre-cool thermal cycler block to 15°C while preparing the 3' Adaptor Master Mix.

1. Prepare 3' Adaptor Master Mix.
   a. On ice, prepare the 3' Adaptor Master Mix in a nuclease-free tube. Combine the components in the sequence shown in the table below. Prepare the master mix for all the first-strand cDNA samples in the experiment. Include ~10% excess volume to correct for pipetting losses.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume for One Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT Pico 3' Adaptor Buffer</td>
<td>7 μL</td>
</tr>
<tr>
<td>WT Pico 3' Adaptor Enzyme</td>
<td>1 μL</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td><strong>8 μL</strong></td>
</tr>
</tbody>
</table>

b. Mix thoroughly by gently vortexing the tube. Centrifuge briefly to collect the mix at the bottom of the tube and proceed immediately to the next step.

c. On ice, transfer 8 μL of the 3' Adaptor Master Mix to each (12 μL) first-strand cDNA sample for a final reaction volume of 20 μL.

d. Mix thoroughly by gently vortexing the tube. Centrifuge briefly to collect the reaction at the bottom of the tube or well, then proceed immediately to the next step.

2. Incubate for 15 min at 15°C, for 15 min at 35°C, for 10 min at 70°C, and then for at least 2 min at 4°C.
   a. Incubate the 3' Adaptor cDNA synthesis reaction in a thermal cycler using the 3' Adaptor cDNA Synthesis program that is shown in Table 2.1 on page 13.

   **IMPORTANT:** Disable the heated lid of the thermal cycler or keep the lid off during the 3' Adaptor cDNA Synthesis.

b. Immediately after the incubation, centrifuge briefly to collect the 3' Adaptor cDNA at the bottom of the tube or well.

c. Place the sample on ice, then proceed immediately to Synthesize Double-Stranded cDNA.

   **TIP:** When there is approximately 15 min left on the thermal cycler you may start reagent preparation for Pre-IVT Amplification.
Synthesize Double-Stranded cDNA

In this procedure, single-stranded cDNA is converted to double-stranded cDNA, which acts as a template for *in vitro* transcription. The reaction uses Taq DNA polymerase and Adaptor-specific primers to synthesize and pre-amplify double-stranded cDNA.

1. Prepare Pre-IVT Amplification Master Mix.
   A. On ice, prepare the Pre-IVT Master Mix in a nuclease-free tube. Combine the components in the sequence shown in the table below. Prepare the master mix for all the cDNA samples in the experiment. Include ~10% excess volume to correct for pipetting losses.

   ![Table 2.8 Pre-IVT Amplification Master Mix](image)

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume for 1 Rxn</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT Pico PCR Buffer</td>
<td>29 μL</td>
</tr>
<tr>
<td>WT Pico PCR Enzyme</td>
<td>1 μL</td>
</tr>
<tr>
<td>Total Volume</td>
<td>30 μL</td>
</tr>
</tbody>
</table>

   B. Mix thoroughly by gently vortexing the tube. Centrifuge briefly to collect the mix at the bottom of the tube and proceed immediately to the next step.

   C. On ice, transfer 30 μL of the Pre-IVT Amplification Master Mix to each (20 μL) 3’ Adaptor cDNA sample for a final reaction volume of 50 μL.

   D. Mix thoroughly by gently vortexing the tube. Centrifuge briefly to collect the reaction at the bottom of the tube or well, then proceed immediately to the next step.

   **NOTE:** The following steps should be performed in Post-Amplification Area using dedicated supplies and equipment.

2. Incubate for 2 min at 95°C, for 6, 9 or 12 cycles of 30 sec at 94°C and 5 min at 70°C, and then for at least 2 min at 4°C.
   A. Incubate the pre-IVT amplification reaction in a thermal cycler using the Pre-IVT Amplification program that is shown in Table 2.1 on page 13. See Table 2.2 on page 14 for Pre-IVT Amplification cycling guidelines based on sample type and the amount of starting total RNA input.

   B. Immediately after the incubation, centrifuge briefly to collect the double-stranded cDNA at the bottom of the tube or well.

   C. Place the sample on ice, then proceed immediately to *Synthesize cRNA by In Vitro Transcription* on page 22.

   **NOTE:** One or two PCR cycles may be added to the cycling guidelines for Pre-IVT Amplification program to improve cRNA yield of poor quality RNA sample.

   **TIP:** When there is approximately 15 min left on the thermal cycler you may start reagent preparation for *In Vitro Transcription.*
Synthesize cRNA by *In Vitro* Transcription

In this procedure, antisense RNA (complimentary RNA or cRNA) is synthesized and amplified by *in vitro* transcription (IVT) of the double-stranded cDNA template using T7 RNA polymerase. This method of RNA sample preparation is based on the original T7 *in vitro* transcription technology known as the Eberwine or RT-IVT method (Van Gelder *et al*., 1990).

1. Prepare IVT Master Mix.

   **NOTE:** This step is performed at room temperature.

   A. At room temperature, prepare the IVT Master Mix in a nuclease-free tube. Combine the components in the sequence shown in the table below. Prepare the master mix for all the double-stranded cDNA samples in the experiment. Include ~10% excess volume to correct for pipetting losses.

   Table 2.9 IVT Master Mix

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume for One Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT Pico IVT Buffer</td>
<td>24 μL</td>
</tr>
<tr>
<td>WT Pico IVT Enzyme</td>
<td>6 μL</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td><strong>30 μL</strong></td>
</tr>
</tbody>
</table>

   B. Mix thoroughly by gently vortexing the tube. Centrifuge briefly to collect the mix at the bottom of the tube, then proceed immediately to the next step.

   C. At room temperature, transfer 30 μL of the IVT Master Mix to each (50 μL) double-stranded cDNA sample for a final reaction volume of 80 μL.

   D. Mix thoroughly by gently vortexing the tube. Centrifuge briefly to collect the reaction at the bottom of the tube or well, then proceed immediately to the next step.

2. Incubate for 14 hr at 40°C, and then at 4°C.

   A. Incubate the IVT reaction in a thermal cycler using the *In Vitro* Transcription cRNA Synthesis program that is shown in Table 2.1 on page 13.

   B. After the incubation, centrifuge briefly to collect the cRNA at the bottom of the tube or well.

   C. Place the reaction on ice, then proceed to Purify cRNA on page 23, or immediately freeze the samples at −20°C for storage.

   **NOTE:** The IVT incubation time may be extended up to 16 hr.

   **TIP:** STOPPING POINT. The cRNA samples can be stored overnight at −20°C.

---

**IMPORTANT:**

- Transfer the double-stranded cDNA samples to room temperature for ≥5 min while preparing IVT Master Mix.
- After the IVT Buffer is thawed completely, leave the IVT Buffer at room temperature for ≥10 min before preparing the IVT Master Mix.

---

**Table 2.9** IVT Master Mix

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume for One Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT Pico IVT Buffer</td>
<td>24 μL</td>
</tr>
<tr>
<td>WT Pico IVT Enzyme</td>
<td>6 μL</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td><strong>30 μL</strong></td>
</tr>
</tbody>
</table>
Purify cRNA

In this procedure, enzymes, salts, inorganic phosphates, and unincorporated nucleotides are removed to prepare the cRNA for 2nd-cycle single-stranded cDNA synthesis.

**IMPORTANT:**
- Preheat the Nuclease-free Water in a heat block or thermal cycler to 65°C for at least 10 min.
- Mix the Purification Beads thoroughly by vortexing before use to ensure that they are fully dispersed. Transfer the appropriate amount of Purification Beads to a nuclease-free tube or container and allow the Purification Beads to equilibrate at room temperature. For each reaction, 140 μL plus ~10% overage will be needed.
- Prepare fresh dilutions of 80% ethanol wash solution each time from 100% ethanol (Molecular Biology Grade or equivalent) and Nuclease-free Water in a nuclease-free tube or container. For each reaction, 600 μL plus ~10% overage will be needed.
- Transfer the cRNA sample to room temperature while preparing the Purification Beads.

**NOTE:**
- Occasionally, the bead/sample mixture may be brownish in color and not completely clear when placed on magnet. In those situations, switch to a different position of magnet on the magnetic stand, a new magnetic stand, or spin out pellets.
- The beads/sample mixture may form a loose pellet for samples with a high concentration of cRNA. The supernatant should be aspirated carefully with minimum disturbance to the beads.
- This entire procedure is performed at room temperature.

1. Bind cRNA to Purification Beads.
   **A.** Mix the Purification Beads container by vortexing to resuspend the magnetic particles that may have settled. Transfer 140 μL of the Purification Beads to a clean well of a round bottom plate.
   **B.** Add 80 μL of cRNA sample to each (140 μL) Purification Beads, and mix by pipetting up and down.
   **C.** Mix well by pipetting up and down 10 times.
   **D.** Incubate for 10 min. The cRNA in the sample binds to the Purification Beads during this incubation.
   **E.** Move the plate to a magnetic stand to capture the Purification Beads. When capture is complete (after ~5 min), the mixture is transparent, and the Purification Beads form pellets against the magnets in the magnetic stand. The exact capture time depends on the magnetic stand that you use and the amount of cRNA generated by in vitro transcription.
   **F.** Carefully aspirate and discard the supernatant without disturbing the Purification Beads. Keep the plate on the magnetic stand.

2. Wash the Purification Beads.
   **A.** While on the magnetic stand, add 200 μL of 80% ethanol wash solution to each well and incubate for 30 sec.
   **B.** Slowly aspirate and discard the 80% ethanol wash solution without disturbing the Purification Beads.
C. Repeat Step A and Step B twice for a total of 3 washes with 200 μL of 80% ethanol wash solution. Completely remove the final wash solution.

D. Air-dry on the magnetic stand for 5 min 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-dried.

3. Elute cRNA.

A. Remove the plate from the magnetic stand. Add to each sample 27 μL of the preheated (65°C) Nuclease-free Water and incubate for 1 min.

B. Mix well by pipetting up and down 10 times.

C. Move the plate to the magnetic stand for ~5 min to capture the Purification Beads.

D. Transfer the supernatant, which contains the eluted cRNA, to a nuclease-free tube.

E. Place the purified cRNA samples on ice, then proceed to Assess cRNA Yield, or immediately freeze the samples at –20°C for storage.

**NOTE:**

- Minimal bead carryover will not inhibit subsequent enzymatic reactions.
- It may be difficult to resuspend magnetic particles and aspirate purified cRNA when the cRNA is very concentrated. To elute the sample with high concentration cRNA, add an additional 10 to 70 μL of the preheated Nuclease-free Water to the well, incubate for 1 min and proceed to Step 3B.

**TIP:** STOPPING POINT. The purified cRNA samples can be stored overnight at –20°C. For long-term storage, store samples at –80°C and keep the number of freeze-thaw cycles to 3 or less to ensure cRNA integrity.

**Assess cRNA Yield**

**Expected cRNA Yield**

The cRNA yield depends on the amount and quality of non-rRNA in the input total RNA. Because the proportion of non-rRNA in total RNA is affected by factors such as the health of the organism and the organ from which it is isolated, cRNA yield from equal amounts of total RNA may vary considerably. During development of this kit, using a wide variety of tissue types, 500 pg of input total RNA from fresh-frozen samples and 2 ng of input total RNA from FFPE samples yielded >20 μg of cRNA. Figure 2.1 shows yield data for cRNA produced with the kit from several different types of input RNA.
Figure 2.1 Average cRNA Yield from MicroArray Quality Control (MAQC) (A), a Variety of Fresh-Frozen Tissues (B), and a 9 Years Old FFPE Tissue (C) Total RNA Samples.
Determine cRNA Yield by UV Absorbance
Determine the concentration of a cRNA solution by measuring its absorbance at 260 nm. Use Nuclease-free Water as blank. Affymetrix recommends using NanoDrop Spectrophotometers for convenience. No dilutions or cuvettes are needed; just use 1 to 1.5 μL of the cRNA sample directly. Samples with cRNA concentrations greater than 3,000 ng/μL should be diluted with Nuclease-free Water before measurement and reaction setup. Use the diluted cRNA as the input to prepare 20 μg cRNA in 2nd-cycle cDNA synthesis reaction.

Alternatively, determine the cRNA concentration by diluting an aliquot of the preparation in Nuclease-free Water and reading the absorbance in a traditional spectrophotometer at 260 nm. Calculate the concentration in μg/mL using the equation shown below (1 A_{260} = 40 μg RNA/mL).

\[ A_{260} \times \text{dilution factor} \times 40 = \mu g \text{ RNA/mL} \]

(Optional) Expected cRNA Size Distribution
The expected cRNA profile is a distribution of sizes from 200 to 1000 nt. This step is optional.

Determine cRNA size distribution using a Bioanalyzer.
cRNA size distribution can be analyzed using an Agilent 2100 Bioanalyzer, a RNA 6000 Nano Kit (PN5067-1511), and mRNA Nano Series II assay. If there is sufficient yield, then load approximately 500 ng of cRNA per well on the Bioanalyzer. To analyze cRNA size using a Bioanalyzer, follow the manufacturer’s instructions.

TIP: STOPPING POINT. The purified cRNA samples can be stored overnight at –20°C.

Synthesize 2nd-Cycle Single-Stranded cDNA
In this procedure, sense-strand cDNA is synthesized by the reverse transcription of cRNA using 2nd-Cycle Primers. The sense-strand cDNA contains dUTP at a fixed ratio relative to dTTP. 20 μg of cRNA is required for 2nd-cycle single-stranded cDNA synthesis.

1. Prepare 20 μg of cRNA.
   On ice, prepare 833 ng/μL cRNA. This is equal to 20 μg cRNA in a volume of 24 μL. If necessary, use Nuclease-free Water to bring the cRNA sample to 24 μL.

   NOTE: High-concentration cRNA samples (>3,000 ng/μL) should be diluted with Nuclease-free Water before measurement and reaction setup. Use the diluted cRNA as the input to prepare 20 μg of cRNA.

2. Prepare 2nd-Cycle ss-cDNA Master Mix.
   A. On ice, prepare the 2nd-Cycle ss-cDNA Master Mix in a nuclease-free tube. Combine the components in the sequence shown in the table below. Prepare the master mix for all the cRNA samples in the experiment. Include ~10% excess volume to correct for pipetting losses.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume for One Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT Pico 2nd-Cycle ss-cDNA Primers</td>
<td>4 μL</td>
</tr>
<tr>
<td>WT Pico 2nd-Cycle ss-cDNA Buffer</td>
<td>8 μL</td>
</tr>
<tr>
<td>WT Pico 2nd-Cycle ss-cDNA Enzyme</td>
<td>4 μL</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td><strong>16 μL</strong></td>
</tr>
</tbody>
</table>
B. Mix thoroughly by gently vortexing the tube. Centrifuge briefly to collect the mix at the bottom of the tube and proceed immediately to the next step.

C. On ice, transfer 16 μL of the 2nd-Cycle ss-cDNA Master Mix to each (24 μL) cRNA sample for a final reaction volume of 40 μL.

D. Mix thoroughly by gently vortexing the tube. Centrifuge briefly to collect the reaction at the bottom of the tube or well, then proceed immediately to the next step.

3. Incubate for 10 min at 25°C, for 90 min at 42°C, for 10 min at 70°C, and then for at least 2 min at 4°C.
   A. Incubate the 2nd-cycle synthesis reaction in a thermal cycler using the 2nd-Cycle ss-cDNA Synthesis program that is shown in Table 2.1 on page 13.
   B. Immediately after the incubation, centrifuge briefly to collect the 2nd-cycle ss-cDNA at the bottom of the tube or well.
   C. Place the sample on ice and proceed immediately to Hydrolyze RNA Using RNase H.

Hydrolyze RNA Using RNase H

In this procedure, RNase H hydrolyzes the cRNA template leaving single-stranded cDNA.

1. Add RNase H to each 2nd-cycle ss-cDNA sample.
   A. On ice, add 4 μL of the RNase H to each (40 μL) 2nd-cycle ss-cDNA sample for a final reaction volume of 44 μL.
   B. Mix thoroughly by gently vortexing. Centrifuge briefly to collect the reaction at the bottom of the tube or well, then proceed immediately to the next step.

2. Incubate for 45 min at 37°C, for 5 min at 95°C, and then for at least 2 min at 4°C.
   A. Incubate the RNA hydrolysis reaction in a thermal cycler using the RNA Hydrolysis program that is shown in Table 2.1 on page 13.
   B. Immediately after the incubation, centrifuge briefly to collect the hydrolyzed 2nd-cycle ss-cDNA at the bottom of the tube or well.
   C. Place the samples on ice and proceed immediately to the next step.

3. Add Nuclease-free Water to each hydrolyzed 2nd-cycle ss-cDNA sample.
   A. On ice, add 11 μL of the Nuclease-free Water to each (44 μL) hydrolyzed 2nd-cycle ss-cDNA sample for a final reaction volume of 55 μL.
   B. Mix thoroughly by gently vortexing. Centrifuge briefly to collect the reaction at the bottom of the tube or well.
   C. Place the sample on ice, then proceed to Purify 2nd-Cycle Single-Stranded cDNA on page 28, or immediately freeze the samples at −20°C for storage.

TIP: STOPPING POINT. The hydrolyzed ss-cDNA samples can be stored overnight at −20°C.
Chapter 2 | Protocol

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Purify 2nd-Cycle Single-Stranded cDNA

After hydrolysis, the 2nd-cycle single-stranded cDNA is purified to remove enzymes, salts, and unincorporated dNTPs. This step prepares the cDNA for fragmentation and labeling.

1. Bind ss-cDNA to Purification Beads.
   A. Mix the Purification Beads container by vortexing to resuspend the magnetic particles that may have settled. Transfer 100 μL of the Purification Beads to a clean well of a round bottom plate.
   A. Add 55 μL of 2nd-cycle ss-cDNA sample to each (100 μL) Purification Beads, and mix by pipetting up and down.
   B. Add 150 μL of 100% ethanol to each (155 μL) ss-cDNA/Beads sample. Mix well by pipetting up and down 10 times.
   C. Incubate for 20 min. The ss-cDNA in the sample binds to the Purification Beads during this incubation.
   D. Move the plate to a magnetic stand to capture the Purification Beads. When capture is complete (after ~5 min), the mixture is transparent and the Purification Beads form pellets against the magnets in the magnetic stand. The exact capture time depends on the magnetic stand that you use and the amount of ss-cDNA generated by 2nd-Cycle ss-cDNA Synthesis.
   E. Carefully aspirate and discard the supernatant without disturbing the Purification Beads. Keep the plate on the magnetic stand.

2. Wash the Purification Beads.
   A. While on the magnetic stand, add 200 μL of 80% ethanol wash solution to each well and incubate for 30 sec.
   B. Slowly aspirate and discard the 80% ethanol wash solution without disturbing the Purification Beads.
   C. Repeat Step A and Step B twice for a total of 3 washes with 200 μL of 80% ethanol wash solution. Completely remove the final wash solution.
D. Air-dry on the magnetic stand for 5 min 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.

3. Elute ss-cDNA.
   A. Remove the plate from the magnetic stand. Add to each sample 30 μL of the preheated (65°C) Nuclease-free Water and incubate for 1 min.
   B. Mix well by pipetting up and down 10 times.
   C. Move the plate to the magnetic stand for ~5 min to capture the Purification Beads.
   D. Transfer the supernatant, which contains the eluted ss-cDNA, to a nuclease-free tube.
   E. Place the purified ss-cDNA samples on ice, then proceed to Assess Single-Stranded cDNA Yield, or immediately freeze the samples at –20°C for storage.

**NOTE:** Minimal bead carryover will not inhibit subsequent enzymatic reactions.

**TIP:** STOPPING POINT. The purified ss-cDNA samples can be stored overnight at –20°C. For long-term storage at –20°C, we recommend not to proceed to the fragmentation and labeling reaction, and store the samples as ss-cDNA.

### Assess Single-Stranded cDNA Yield

#### Expected Single-Stranded cDNA Yield

During development of this kit, using a wide variety of tissue types, 20 μg of input cRNA yielded 6 to 20 μg of ss-cDNA. For most tissue types, the recommended 20 μg of input cRNA should yield >5.5 μg of ss-cDNA. Figure 2.2 shows yield data for ss-cDNA produced with the kit from several different types of input RNA.
Figure 2.2 Average ss-cDNA Yield from MicroArray Quality Control (MAQC) (A), a Variety of Fresh-Frozen Tissues (B), and a 9 Years Old FFPE Tissue (C) Total RNA Samples.
Determine Single-Stranded DNA Yield by UV Absorbance

Determine the concentration of a ss-cDNA solution by measuring its absorbance at 260 nm. Use Nuclease-free Water as blank. Affymetrix recommends using NanoDrop Spectrophotometers for convenience. No dilutions or cuvettes are needed; just use 1 to 1.5 μL of the cDNA sample directly. Alternatively, determine the ss-cDNA concentration by diluting an aliquot of the preparation in Nuclease-free Water and reading the absorbance in a traditional spectrophotometer at 260 nm. Calculate the concentration in μg/mL using the equation below (1 A_{260} = 33 μg DNA/mL).

A_{260} \times \text{dilution factor} \times 33 = \mu g \text{ DNA/mL}

NOTE: The equation above applies only to single-stranded cDNA.

(Optional) Expected Single-Stranded cDNA Size Distribution

The expected cDNA profile does not resemble the cRNA profile. The expected cDNA profile is a distribution of sizes from 50 to 1000 nt with most of the cDNA sizes in the 25 to 500 nt range. This step is optional.

Determine Single-Stranded cDNA Size Distribution Using a Bioanalyzer

cDNA size distribution may be analyzed using an Agilent 2100 Bioanalyzer, a RNA 6000 Nano Kit (PN5067-1511), and mRNA Nano Series II assay. If there is sufficient yield, load approximately 400 ng of cDNA per well. To analyze cDNA size using a bioanalyzer, follow the manufacturer’s instructions.

TIP: STOPPING POINT. The purified ss-cDNA samples can be stored overnight at –20°C. For long-term storage at –20°C, we recommend not to proceed to the fragmentation and labeling reaction, and store the samples as ss-cDNA.

NOTE: Although 100 or 81/4-Format and 169 or 400-Format arrays use less than 5.5 μg of fragmented and labeled ss-cDNA in hybridization, the fragmentation and labeling reaction should be performed with 5.5 μg of purified ss-cDNA.
Fragment and Label Single-Stranded cDNA

In this procedure, the purified, sense-strand cDNA is fragmented by uracil-DNA glycosylase (UDG) and apurinic/apyrimidinic endonuclease 1 (APE 1) at the unnatural dUTP residues and breaks the DNA strand. The fragmented cDNA is labeled by terminal deoxynucleotidyl transferase (TdT) using the Affymetrix proprietary DNA Labeling Reagent that is covalently linked to biotin. 5.5 μg of single-stranded cDNA is required for fragmentation and labeling.

1. Prepare 5.5 μg of ss-cDNA.
   On ice, prepare 120 ng/μL ss-cDNA. This is equal to 5.5 μg ss-cDNA in a volume of 46 μL. If necessary, use Nuclease-free Water to bring the ss-cDNA sample to 46 μL.

2. Prepare Fragmentation and Labeling Master Mix.
   A. On ice, prepare the Fragmentation and Labeling Master Mix in a nuclease-free tube. Combine the components in the sequence shown in the table below. Prepare the master mix for all the ss-cDNA samples in the experiment. Include ~10% excess volume to correct for pipetting losses.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume for One Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT Pico Frag. &amp; Label Buffer</td>
<td>12 μL</td>
</tr>
<tr>
<td>WT Pico Frag. &amp; Label Enzyme</td>
<td>2 μL</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td><strong>14 μL</strong></td>
</tr>
</tbody>
</table>

   B. Mix thoroughly by gently vortexing the tube. Centrifuge briefly to collect the mix at the bottom of the tube, then proceed immediately to the next step.

   C. On ice, transfer 14 μL of the Fragmentation and Labeling Master Mix to each (46 μL) purified ss-cDNA sample for a final reaction volume of 60 μL.

   D. Mix thoroughly by gently vortexing the tube. Centrifuge briefly to collect the reaction at the bottom of the tube or well, then proceed immediately to the next step.

3. Incubate for 1 hr at 37°C, for 2 min at 93°C, and then for at least 2 min at 4°C.
   A. Incubate the fragmentation reaction in a thermal cycler using the Fragmentation and Labeling program shown in Table 2.1 on page 13.

   B. Immediately after the incubation, centrifuge briefly to collect the fragmented and labeled ss-cDNA at the bottom of the tube or well.

   C. Place the sample on ice, then proceed immediately to the next step.

4. (Optional) The fragmented and labeled ss-cDNA sample can be used for size analysis using an Agilent 2100 Bioanalyzer, a RNA 6000 Nano Kit (PN5067-1511), and mRNA Nano Series II assay. 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.

   **TIP:** STOPPING POINT. The fragmented and labeled ss-cDNA samples can be stored overnight at –20°C. For long-term storage at –20°C, we recommend to store the samples as unfragmented and unlabeled ss-cDNA.
Chapter 3
WT Array Hybridization

Cartridge Array Hybridization on the GeneChip® Instrument

This section provides instruction for setting up hybridizations for cartridge arrays.
Please refer to Affymetrix® GeneChip® Fluidics Station 450 User Guide AGCC (P/N 08-0295), the GeneChip® Expression Wash, Stain, and Scan User Guide for Cartridge Arrays (PN 702731), and the Affymetrix® GeneChip® Command Console® User Guide (P/N 702569) for further detail.

Prepare Ovens, Arrays, and Sample Registration Files

1. Turn Affymetrix® Hybridization Oven on, set the temperature to 45°C and 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 into AGCC.

Target Hybridization Setup for Cartridge Arrays

Reagents and Materials Required
- GeneChip® Hybridization, Wash, and Stain Kit. (Not supplied) For ordering information please refer to Table 1.5 on page 10 or the Affymetrix website.
  (Not required for WT arrays)
  □ Pre-Hybridization Mix
  □ 2X Hybridization Mix
  □ DMSO
  □ Nuclease-free Water
  □ Stain Cocktail 1
  □ Stain Cocktail 2
  □ Array Holding Buffer
  □ Wash Buffer A
  □ Wash Buffer B
- GeneChip® Hybridization Control Kit
  □ 20X Eukaryotic Hybridization Controls (bioB, bioC, bioD, cre)
  □ Control Oligonucleotide B2 (3 nM)
- Affymetrix® WT Cartridge Array(s). (Not supplied)
Procedure
1. Prepare Hybridization Master Mix.
   A. At room temperature, thaw the components listed in Table 3.1.

   **NOTE:** DMSO will solidify when stored at 2 to 8°C. Ensure that the reagent is completely thawed before use. We recommend to store DMSO at room temperature after the first use.

   B. Heat the 20X Hybridization Controls for 5 min at 65°C in a thermal cycler using the Hybridization Control program that is shown in Table 2.1 on page 13.

   C. At room temperature, prepare the Hybridization Master Mix in a nuclease-free tube. Combine the appropriate amount of components in the sequence shown in the table below. Prepare the master mix for all the fragmented and biotin-labeled ss-cDNA samples in the experiment. Include ~10% overage to correct for pipetting losses.

   **Table 3.1** Hybridization Master Mix for a Single Reaction

<table>
<thead>
<tr>
<th>Component</th>
<th>49 or 64-Format*</th>
<th>100 or 81/4-Format*</th>
<th>169 or 400-Format*</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fragmented and Labeled ss-DNA</td>
<td>5.5 μg</td>
<td>3.8 μg</td>
<td>2.5 μg</td>
<td>25 ng/μL</td>
</tr>
<tr>
<td>Control Oligo B2 (3 nM)</td>
<td>3.7 μL</td>
<td>2.5 μL</td>
<td>1.7 μL</td>
<td>50 pM</td>
</tr>
<tr>
<td>20X Hybridization Controls (bioB, bioC, bioD, cre)</td>
<td>11 μL</td>
<td>7.5 μL</td>
<td>5 μL</td>
<td>1.5, 5, 25, and 100 pM respectively</td>
</tr>
<tr>
<td>2X Hybridization Mix</td>
<td>110 μL</td>
<td>75 μL</td>
<td>50 μL</td>
<td>1X</td>
</tr>
<tr>
<td>DMSO</td>
<td>15.4 μL</td>
<td>10.5 μL</td>
<td>7 μL</td>
<td>7%</td>
</tr>
<tr>
<td>Nuclease-free Water</td>
<td>19.9 μL</td>
<td>13.5 μL</td>
<td>9.3 μL</td>
<td></td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td><strong>160 μL</strong></td>
<td><strong>109 μL</strong></td>
<td><strong>73 μL</strong></td>
<td></td>
</tr>
</tbody>
</table>

   * Please refer to specific probe array package insert for information on array format.

   D. Mix thoroughly by gently vortexing. Centrifuge briefly to collect the mix and proceed immediately to the next step.

2. Prepare Hybridization Cocktail.
   A. At room temperature, add the appropriate amount of Hybridization Master Mix to each fragmented and biotin-labeled ss-cDNA sample to prepare Hybridization Cocktail.

   **Table 3.2** Hybridization Cocktail for a Single Array

<table>
<thead>
<tr>
<th>Component</th>
<th>49 or 64-Format</th>
<th>100 or 81/4-Format</th>
<th>169 or 400-Format</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hybridization Master Mix</td>
<td>160 μL</td>
<td>109 μL</td>
<td>73 μL</td>
<td></td>
</tr>
<tr>
<td>Fragmented and labeled ss-cDNA</td>
<td>60 μL (5.5 μg)</td>
<td>41 μL (3.8 μg)</td>
<td>27 μL (2.5 μg)</td>
<td>25 ng/μL</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td><strong>220 μL</strong></td>
<td><strong>150 μL</strong></td>
<td><strong>100 μL</strong></td>
<td></td>
</tr>
</tbody>
</table>

   B. Mix thoroughly by gently vortexing. Centrifuge briefly to collect contents of the tube and proceed immediately to the next step.

   C. Incubate the hybridization cocktail reaction for 5 min at 99°C (tubes) or 95°C (plates), and then for 5 min at 45°C in a thermal cycler using the Hybridization Cocktail program that is shown in Table 2.1 on page 13.
D. After the incubation, centrifuge briefly to collect contents of the tube and proceed immediately to the next step.

3. Inject and hybridize array.

![Figure 3.1 GeneChip® Probe Array Cartridge](image)

**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.

A. Insert a pipet tip into the upper right septum to allow for venting.

B. Inject the appropriate amount (see Table 3.3) of the specific sample into the array through one of the septa (see Figure 3.1 for location of the septa on the array).

**Table 3.3** Probe Array Cartridge Volumes for Hybridization Cocktail

<table>
<thead>
<tr>
<th>Format</th>
<th>Volume to Load on Array</th>
</tr>
</thead>
<tbody>
<tr>
<td>49 or 64-Format</td>
<td>200 μL</td>
</tr>
<tr>
<td>100 or 81/4-Format</td>
<td>130 μL</td>
</tr>
<tr>
<td>169 or 400-Format</td>
<td>80 μL</td>
</tr>
</tbody>
</table>

C. 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.

D. Place the arrays into hybridization oven trays. Load the trays into the hybridization oven.

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

E. Incubate with rotation at 60 rpm for 16 hr at 45°C.

**NOTE:** During the latter part of the 16-hr hybridization prepare reagents for the washing and staining steps required immediately after completion of hybridization.
Wash and Stain

For additional information about washing, staining, and scanning, please refer to the *Affymetrix® GeneChip® Fluidics Station 450 User Guide* AGCC (P/N 08-0295), the *GeneChip® Expression Wash, Stain, and Scan User Guide for Cartridge Arrays* (PN 702731), and the *Affymetrix® GeneChip® 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 mix from each array. (Optional) Transfer it to a new tube or well of a 96-well plate in order to save the hybridization cocktail mix. Store on ice during the procedure, or at –20°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 the Wash Buffer A at 4°C for up to 3 hr 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>
<thead>
<tr>
<th></th>
<th>49 or 64-Format</th>
<th>100 or 81/4-Format</th>
<th>169 or 400-Format</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluidics Protocol</td>
<td>FS450_0001</td>
<td>FS450_0002</td>
<td>FS450_0007</td>
</tr>
</tbody>
</table>

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.

Scan

The instructions for using the scanner and scanning arrays can be found in the *Affymetrix® GeneChip® Command Console® User Guide* (P/N 702569).
Array Strips Hybridization on the GeneAtlas® Instrument

This section outlines the basic steps involved in hybridizing array strip(s) on the GeneAtlas® System. The two major steps involved in array strip hybridization are:

- Target Hybridization Setup for Affymetrix® Array Strips on page 37
- GeneAtlas® Software Setup on page 42

NOTE: If you are using a hybridization-ready sample, or re-hybridizing previously made hybridization cocktail, continue the protocol from Step 5 on page 39.

IMPORTANT: Before preparing hybridization ready samples, register samples as described in GeneAtlas® Software Setup on page 42.

Please refer to GeneAtlas® System User Guide (P/N 08-0306) for further detail.

Target Hybridization Setup for Affymetrix® Array Strips

Reagents and Materials Required

- GeneAtlas Hybridization, Wash, and Stain Kit for WT Array Strips. (Not supplied) For ordering information please refer to Table 1.5 on page 10 or the Affymetrix website.
  - 5X WT Hyb Add 1
  - 15X WT Hyb Add 4
  - 2.5X WT Hyb Add 6
  - Stain Cocktail 1
  - Stain Cocktail 2
  - Array Holding Buffer
  - Wash Buffer A
  - Wash Buffer B
- GeneChip® Hybridization Control Kit
  - 20X Eukaryotic Hybridization Controls (bioB, bioC, bioD, crc)
  - Control Oligonucleotide B2 (3 nM)
- Affymetrix® Array Strip and consumables (Not supplied)
  - Affymetrix WT Array Strip(s)
  - 1 hybridization tray per array strip

Procedure

NOTE: The “WT Hyb Add” reagent names were created to match the order in which reagents are added. For example, WT Hyb Add 1 is the first 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.

1. In preparation of the hybridization step, prepare the following:
   A. Pull the array strip from storage at 4°C so that it can begin to equilibrate to room temperature.
   B. Gather one (1) hybridization tray per array strip.
   C. Set the temperature of the GeneAtlas Hybridization Station to 48°C. Press the Start button.
2. In preparation of the hybridization master mix, prepare the following:

A. Warm the following vials to room temperature on the bench:
   - 5X WT Hyb Add 1
   - 15X WT Hyb Add 4
   - 2.5X WT Hyb Add 6

B. Vortex and centrifuge briefly (~5 sec) to collect contents of the tube.

C. Remove the following tubes from the GeneChip Hybridization Control Kit and thaw at room temperature:
   - Control Oligonucleotide B2 (3 nM)
   - 20X Eukaryotic Hybridization Controls

D. Vortex and centrifuge briefly (~5 sec) to collect contents of the tube.

E. Keep the tubes of Control Oligonucleotide B2 (3 nM) and 20X Eukaryotic Hybridization Controls on ice.

3. Prepare the Hybridization Master Mix & Cocktail.

A. Heat the 20X Hybridization Controls for 5 min at 65°C in a thermal cycler using the Hybridization Control program that is shown in Table 2.1 on page 13.

B. At room temperature, prepare the Hybridization Master Mix in a nuclease-free tube. Combine the appropriate amount of components in the sequence shown in the table below. Prepare the master mix for all the fragmented and biotin-labeled ss-cDNA samples in the experiment.

   **NOTE:** The 5X WT Hyb Add 1 solution is very viscous; pipet slowly to ensure addition of the correct volume. Mix well. Vortex and centrifuge briefly (~5 sec) to collect liquid contents at the bottom of the tube.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume for One Array</th>
<th>Volume for Four Arrays (Includes 10% Overage)</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>5X WT Hyb Add 1</td>
<td>30 μL</td>
<td>132 μL</td>
<td>1X</td>
</tr>
<tr>
<td>Control Oligonucleotide B2 (3 nM)</td>
<td>1.5 μL</td>
<td>6.6 μL</td>
<td>30 pM</td>
</tr>
<tr>
<td>20X Hybridization Controls (bioB, bioC, bioD, cre)</td>
<td>7.5 μL</td>
<td>33 μL</td>
<td>1.5, 5, 25, and 100 pM, respectively</td>
</tr>
<tr>
<td>15X WT Add 4</td>
<td>10 μL</td>
<td>44 μL</td>
<td>1X</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td><strong>49 μL</strong></td>
<td><strong>215.6 μL</strong></td>
<td></td>
</tr>
</tbody>
</table>

C. Mix thoroughly by gently vortexing. Centrifuge briefly to collect the mix and proceed immediately to the next step.
   A. At room temperature, prepare the Hybridization Cocktail in the order as shown in Table 3.6 for all samples.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume for One Array</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hybridization Master Mix</td>
<td>49 μL</td>
<td></td>
</tr>
<tr>
<td>Fragmented and labeled ss-cDNA</td>
<td>41 μL</td>
<td>25 ng/μL</td>
</tr>
<tr>
<td>2.5X WT Hyb Add 6</td>
<td>60 μL</td>
<td>1X</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td><strong>150 μL</strong></td>
<td></td>
</tr>
</tbody>
</table>

B. If you are using a plate; seal, vortex, and centrifuge briefly (~5 sec) to collect liquid at the bottom of the well. If you are using tubes; vortex and centrifuge briefly (~5 sec) to collect contents of the tube.

C. Incubate the hybridization cocktail reaction for 5 min at 99°C (tubes) or 95°C (plates), and then for 5 min at 45°C in a thermal cycler using the Hybridization Cocktail program that is shown in Table 2.1 on page 13.

D. After the incubation, centrifuge briefly to collect contents of the tube or well and proceed immediately to the next step.

5. Array Strip Sample Hybridization.
   A. Apply 120 μL of hybridization cocktail to the middle of the appropriate wells of a new clean hybridization tray (see Figure 3.2).

   **IMPORTANT:** Do not add more than 120 μL of hybridization cocktail to the wells as that could result in cross-contamination of the samples.

   B. Carefully remove the array strip and protective cover from its foil pouch and place on bench (Figure 3.3).

   **IMPORTANT:** Leave array strip in protective cover.
C. Place the array strip into the hybridization tray containing the hybridization cocktail samples (Figure 3.4). Refer to Figure 3.5 for proper orientation of the array strip in the hybridization tray.

D. Optional: the remainder of the hybridization cocktail Master Mix can be stored at −20°C to supplement Hybridization Cocktail volume should a rehybridization be necessary.

**CAUTION:** Be very careful not to scratch/damage the array surface.

**TIP:** To avoid any possible mix-ups, the hybridization tray and array strip should be labeled on the white label if more than 1 array strip is processed overnight.
E. Bring the hybridization tray to just above eye level and look at the underside of the hybridization tray to check for bubbles.

**CAUTION:** Be careful not to tip the hybridization tray to avoid spilling.

**IMPORTANT:** Insertion of the array strip and air bubble removal should be performed quickly to avoid drying of the array surface.

F. If an air bubble is observed, separate the array strip from the hybridization tray and remove air bubbles. Place array strip back into hybridization tray and recheck for air bubbles.

G. Open a Hybridization Station clamp by applying pressure to the top of the clamp while gently squeezing inward. While squeezing lift the clamp to open (Figure 3.6).

**WARNING:** Do not force the GeneAtlas Hybridization clamps up. To open, press down on the top of the clamp and simultaneously slightly lift the protruding lever to unlock. The clamp should open effortlessly. Refer to Figure 3.6.

**IMPORTANT:** The hybridization temperature for WT GeneAtlas Array Strips is 48°C.

H. Place the hybridization tray with the array strip into a clamp inside the Hybridization Station and close the clamp as shown in Figure 3.7.
6. Proceed to *Hybridization Software Setup* on page 44.

**GeneAtlas® Software Setup**

Prior to setting up the target hybridization and processing the Affymetrix Array Strips on the GeneAtlas System, each array strip must be registered and hybridizations setup in the GeneAtlas Software.

- **Sample Registration**: Sample registration enters array strip data into the GeneAtlas Software, and saves and stores the Sample File on your computer. The array strip barcode is scanned, or entered, and a Sample Name is input for each of the four samples on the array strip. Additional information includes Probe Array Type and Probe Array position.

- **Hybridization Software Setup**: During the Hybridization Software Setup the array strip to be processed is scanned, and the GeneAtlas Hybridization Station is identified with hybridization time and temperature settings determined from installed library files.

For additional information, please refer to *GeneAtlas® System User Guide* (P/N 08-0306).
Sample Registration
The following information provides general instructions for registering Affymetrix Array Strips in the GeneAtlas Software. For detailed information on Sample Registration, importing data from Excel and information on the wash, stain, and scan steps, please refer to the GeneAtlas® System User Guide (P/N 08-0246).

1. Click Start → Programs → Affymetrix → GeneAtlas to launch the GeneAtlas Software.
2. Click the Registration tab. Figure 3.8 appears.

3. Click the + Strip button: + Strip. The Add Strip Window appears (Figure 3.9).

4. Enter or scan the array strip Bar Code and enter a Strip Name, then click Add. The array strip is added and appears in the Registration window (Figure 3.10).
5. Under the **Sample File Name** column, click in the box and enter a sample name, and press **Enter**. Enter a unique name for each of the four samples on the array strip.

6. When complete click the **Save and Proceed** button: 📜. The Save dialog box appears *(Figure 3.11)*.

![Figure 3.11 Save Dialog](image)

7. In the Save dialog box, click to select a folder in which to save your data. Click **OK**. Your files are saved to the selected folder and a confirmation message appears *(Figure 3.12)*.

![Figure 3.12](image)

8. Click **OK** to register additional array strips, or click **Go to Hybridization**.

**NOTE:** You may enter a total of four array strips during the registration process. To add additional strips please repeat Step 3 through Step 8.

9. Proceed to *Hybridization Software Setup* on page 44.

**Hybridization Software Setup**

All Affymetrix Array Strips to be processed must first be registered prior to setting up the hybridizations in the GeneAtlas Software. Refer to *Sample Registration* on page 43 for instruction on registering array strips.

**IMPORTANT:** When hybridizing more than one array strip per day, it is recommended to keep the hybridization time consistent. Setup hybridizations for one array strip at a time, staggered by 1.5 hours so that washing and staining can occur immediately after completion of hybridization for each array strip the next day. Recommended hybridization time is 20 ± 1 hour.

1. Navigate to the **Hybridization** tab on the GeneAtlas Software interface.
2. Click the + Strip button: + Strip. The Add Strip Window appears (Figure 3.14).

3. Scan or enter the Bar Code (required) of the array strip you registered. The Strip Name field is automatically populated.

4. With the hybridization tray and array strip already in the GeneAtlas Hybridization Station, click Start in Figure 3.15.
5. When hybridization has completed, click the Stop button in the upper right corner. A confirmation message box appears (Figure 3.18).
6. Click Yes to complete hybridization.
7. It is important to remove the hybridization tray from the Hybridization Station after the timer has completed the countdown as the Hybridization Station does not shut down when the hybridization is complete.
8. Save the remaining hybridization cocktail in –20°C for future use.
Array Plates Hybridization on the GeneTitan® Instrument

This chapter outlines the basic steps involved in hybridizing array plate(s) on the GeneTitan® Instrument. The two major steps involved in array plate hybridization are:

- **Target Hybridization Setup for Affymetrix® Array Plates** on page 48
- **Processing WT Array Plates on the GeneTitan® Instrument** on page 50


Target Hybridization Setup for Affymetrix® Array Plates

**Reagents and Materials Required**

- GeneTitan® Hybridization, Wash, and Stain Kit for WT Array Plates. (Not supplied) For ordering information please refer to **Table 1.5 on page 10** or the Affymetrix website.
  - 5X WT Hyb Add 1
  - 15X WT Hyb Add 4
  - 2.5X WT Hyb Add 6
  - Stain Cocktail 1 & 3
  - Stain Cocktail 2
  - Array Holding Buffer
  - Wash Buffer A
  - Wash Buffer B
- GeneChip® Hybridization Control Kit
  - 20X Eukaryotic Hybridization Controls (*bioB, bioC, bioD, cre*)
  - Control Oligonucleotide B2 (3 nM)
- Affymetrix® Array Plate and consumables (Not supplied)
  - Affymetrix® WT Array Plate(s) and Trays

**Procedure**

**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.

1. In preparation of the hybridization step, prepare the following:
   A. Warm the following vials to room temperature on the bench:
      - 5X WT Hyb Add 1
      - 15X WT Hyb Add 4
      - 2.5X WT Hyb Add 6.
   B. Vortex and centrifuge briefly (~5 sec) to collect contents of the tube.
   C. Remove the following tubes from the GeneChip Hybridization Control Kit and thaw at room temperature:
      - Control Oligonucleotide B2 (3 nM)
      - 20X Eukaryotic Hybridization Controls
   D. Vortex and centrifuge briefly (~5 sec) to collect liquid at the bottom of the tube.
   E. Keep the tubes of Control Oligonucleotide B2 (3 nM) and the tube of 20X Eukaryotic Hybridization Controls on ice.
2. Prepare the WT Hybridization Master Mix & Cocktail.
   A. Heat the 20X Hybridization Controls for 5 min at 65°C in a thermal cycler using the
      Hybridization Control program that is shown in Table 2.1 on page 13.
   B. At room temperature, prepare the Hybridization Master Mix in a nuclease-free tube. Combine
      the appropriate amount of components in the sequence shown in the table below. Prepare the
      master mix for all the fragmented and biotin-labeled ss-cDNA samples in the experiment.

   **NOTE:** The 5X WT Hyb Add 1 solution is very viscous; pipet slowly to ensure addition
   of the correct volume. Mix well. Vortex and centrifuge briefly (~5 sec) to collect liquid
   contents at the bottom of the tube.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume for One Array</th>
<th>16-Array Plate*</th>
<th>24-Array Plate*</th>
<th>96-Array Plate*</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>5X WT Hyb Add 1</td>
<td>24 μL</td>
<td>422.4 μL</td>
<td>633.6 μL</td>
<td>2,534.4 μL</td>
<td>1X</td>
</tr>
<tr>
<td>Control Oligo B2 (3 nM)</td>
<td>1.2 μL</td>
<td>21.1 μL</td>
<td>31.7 μL</td>
<td>126.7 μL</td>
<td>30 pM</td>
</tr>
<tr>
<td>20X Eukaryotic Hybridization Controls (bioB, bioC, bioD, cre)</td>
<td>6 μL</td>
<td>105.6 μL</td>
<td>158.4 μL</td>
<td>633.6 μL</td>
<td>1.5, 5, 25, and 100 pM, respectively</td>
</tr>
<tr>
<td>15X WT Hyb Add 4</td>
<td>8 μL</td>
<td>140.8 μL</td>
<td>211.2 μL</td>
<td>844.8 μL</td>
<td>1X</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td><strong>39.2 μL</strong></td>
<td><strong>689.9 μL</strong></td>
<td><strong>1,034.9 μL</strong></td>
<td><strong>4,139.5 μL</strong></td>
<td></td>
</tr>
</tbody>
</table>

   * Includes ~10% overage to cover pipetting error.

   C. Mix thoroughly by gently vortexing. Centrifuge briefly to collect the mix and proceed immediately
to the next step.

   A. At room temperature, prepare the Hybridization Cocktail in the order as shown in Table 3.8 for
      all samples.

   **Table 3.8** Hybridization Cocktail for a Single Array

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume for One Array</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hybridization Master Mix</td>
<td>39.2 μL</td>
<td></td>
</tr>
<tr>
<td>Fragmented and labeled ss-cDNA</td>
<td>32.8 μL</td>
<td>25 ng/μL</td>
</tr>
<tr>
<td>2.5X WT Hyb Add 6</td>
<td>48 μL</td>
<td>1X</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td><strong>120 μL</strong></td>
<td></td>
</tr>
</tbody>
</table>

   B. If you are using a plate; seal, vortex, and centrifuge briefly (~5 sec) to collect liquid at the bottom
   of the well. If you are using 1.5 mL tubes; vortex and centrifuge briefly (~5 sec) to collect contents
   of the tube.

   C. Incubate the hybridization cocktail reaction for 5 min at 99°C (tubes) or 95°C (plates), and then
   for 5 min at 45°C in a thermal cycler using the Hybridization Cocktail program that is shown in
   Table 2.1 on page 13.

   D. After the incubation, centrifuge briefly to collect contents of the tube or well and proceed
   immediately to the next step.

   E. Place 90 μL of the centrifuged supernatant hybridization cocktail as indicated into the appropriate
   well of the hybridization tray.

   F. Proceed to Hybridization Setup on page 50.
Hybridization Setup

This section describes the GeneTitan Setup protocol for WT Array Plates. The reagent consumption per process on the GeneTitan® Instrument for processing WT Array Plates is shown in Table 3.10.

Table 3.9 The Minimum Volumes of Buffer and Rinse Required to Process on the GeneTitan Instrument

<table>
<thead>
<tr>
<th>Fluid Type</th>
<th>Amount Required for One Array Plate</th>
<th>Minimum Level in Bottle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rinse</td>
<td>300 mL</td>
<td>450 mL</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2,000 mL</td>
</tr>
<tr>
<td>Wash A</td>
<td>~920 mL</td>
<td>1,040 mL +</td>
</tr>
<tr>
<td>Wash B</td>
<td>300 mL</td>
<td>450 mL</td>
</tr>
</tbody>
</table>

Table 3.10 Volumes Required to Process WT Array Plates per Run

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

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

Processing WT Array Plates on the GeneTitan® Instrument

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 all wells of the GeneTitan Scan Tray identified with the label \textit{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 \textit{GeneTitan® Instrument User Guide for Expression Arrays Plates} (P/N 702933).

\textbf{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 in the hybridization tray using aluminum foil.
## Troubleshooting

### Table A.1 Troubleshooting Possible Problems

<table>
<thead>
<tr>
<th>Observation</th>
<th>Possible Cause</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>The positive control sample and your total RNA sample yield low levels of amplified cRNA product.</td>
<td>Incubation conditions are incorrect or inaccurate.</td>
<td>Calibrate your thermal cycler.</td>
</tr>
<tr>
<td></td>
<td>Condensation formed in the tubes during the incubations.</td>
<td>Check that the heated lid is working correctly and is set to the appropriate temperature.</td>
</tr>
<tr>
<td></td>
<td>cRNA purification is not performed properly.</td>
<td>Perform the purification as described in this manual.</td>
</tr>
<tr>
<td></td>
<td>Pipettes, tubes, and/or equipment are contaminated with nucleases.</td>
<td>Remove RNases and DNases from surfaces using RNase decontamination solution.</td>
</tr>
<tr>
<td>The positive control sample produces expected results, but your total RNA sample results in low levels of amplified cRNA product.</td>
<td>The input total RNA concentration is lower than expected.</td>
<td>Repeat the concentration measurement of your RNA sample.</td>
</tr>
<tr>
<td></td>
<td>Increase amount of total RNA in the First-Strand cDNA Synthesis procedure.</td>
<td>Run an extra 1 or 2 amplification cycles during Pre-IVT Amplification.</td>
</tr>
<tr>
<td></td>
<td>Phenol extract and ethanol precipitate your total RNA.</td>
<td></td>
</tr>
<tr>
<td>The positive control sample produces expected results, but your total RNA sample results in high levels of amplified cRNA product.</td>
<td>The input total RNA concentration is higher than expected.</td>
<td>Repeat the concentration measurement of your RNA sample.</td>
</tr>
<tr>
<td></td>
<td>Decrease amount of total RNA in the First-Strand cDNA Synthesis procedure.</td>
<td>Reduce amplification cycles by 1 or 2 cycles during Pre-IVT Amplification.</td>
</tr>
<tr>
<td>The positive control sample produces expected results but your total RNA sample results in low levels of cRNA/cDNA product.</td>
<td>The total RNA integrity is partially degraded, thereby generating short cDNA fragments.</td>
<td>Assess the integrity of your total RNA sample by determining the size of the 18S and 28S rRNA bands and the relative abundance of 28S to 18S rRNA. Refer to Evaluate RNA Integrity on page 12.</td>
</tr>
<tr>
<td></td>
<td>The mRNA content of your total RNA sample is lower than expected.</td>
<td>Verify the mRNA content of your total RNA. Note: In healthy cells, mRNA constitutes 1 to 10% of total cellular RNA (Johnson, 1974; Sambrook and Russel, 2001).</td>
</tr>
</tbody>
</table>
References


## Appendix B

### cRNA Purification Photos

**Figure B.1** Photos of cRNA Purification Step (1 of 2)

<table>
<thead>
<tr>
<th>Magnetic Stand-96</th>
<th>96-Well Magnetic-Ring Stand</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="Magnetic Stand" /></td>
<td><img src="image" alt="96-Well Magnetic-Ring Stand" /></td>
</tr>
<tr>
<td>cRNA Binding to Beads</td>
<td>cRNA Binding to Beads</td>
</tr>
<tr>
<td><img src="image" alt="Bead Capture" /></td>
<td><img src="image" alt="Bead Capture" /></td>
</tr>
<tr>
<td>Bead Washing</td>
<td>Bead Washing</td>
</tr>
<tr>
<td><img src="image" alt="Ethanol Wash Solution Removal" /></td>
<td><img src="image" alt="Ethanol Wash Solution Removal" /></td>
</tr>
</tbody>
</table>
Figure B.2 Photos of cRNA Purification Step (2 of 2)

Magnetic Stand-96

96-Well Magnetic-Ring Stand

cRNA Elution

Elution Step
### Revision History

<table>
<thead>
<tr>
<th>Description</th>
<th>Section</th>
</tr>
</thead>
<tbody>
<tr>
<td>Revision 4:</td>
<td></td>
</tr>
<tr>
<td>Revision 3:</td>
<td></td>
</tr>
<tr>
<td>Update to indicate appropriate for 400-format arrays</td>
<td></td>
</tr>
<tr>
<td>Revision 2:</td>
<td></td>
</tr>
<tr>
<td>Increase maximum input RNA limits</td>
<td>Product Information on page 5.</td>
</tr>
<tr>
<td>Update pre-IVT amplification cycling guidelines</td>
<td>Protocol on page 13 and 14.</td>
</tr>
<tr>
<td>Update serial dilution of Poly-A RNA Control Stock for ≤1 ng input RNA samples</td>
<td>Protocol on page 17.</td>
</tr>
<tr>
<td>Reduce the recommended elution volume for purified ss-cDNA</td>
<td>Protocol on page 29.</td>
</tr>
</tbody>
</table>
Appendix D
Technical Support

Affymetrix, Inc.
3420 Central Expressway
Santa Clara, CA 95051
USA
www.affymetrix.com
Email: support@affymetrix.com
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Fax: 1-408-731-5441

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High Wycombe HP10 0HH
United Kingdom
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