



User Manual

**GeneChip® 3' IVT PLUS Reagent
Kit**

Manual Target Preparation for
GeneChip® 3' Expression Arrays

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3' IVT PLUS Reagent Kit


Product Information


Purpose of the Product

The 3' IVT PLUS Reagent Kit enables you to prepare RNA samples for gene expression profiling analysis with GeneChip® 3' Expression Arrays. The kit generates amplified and biotinylated complementary RNA (cRNA) from poly(A) RNA in a total RNA sample. cRNA is also known as amplified RNA or aRNA. The kit does not need an up-front removal of ribosomal RNA and is optimized for use with GeneChip® 3' Expression Arrays.

The 3' IVT PLUS Reagent Kit uses a reverse transcription priming method that primes the poly(A) tail junction of RNA to provide gene expression profiles from mRNA. RNA amplification is based upon linear amplification and employs T7 *in vitro* transcription (IVT) technology. The kit is comprised of reagents and a protocol for preparing hybridization-ready targets from 50 to 500 ng of total RNA (Figure 1.1). 3' IVT PLUS Reagent is optimized to work with total RNA from a wide range of samples including tissues, cells, and cell lines. The total RNA from whole blood samples should be processed for globin reduction prior to target preparation with 3' IVT PLUS Reagent.

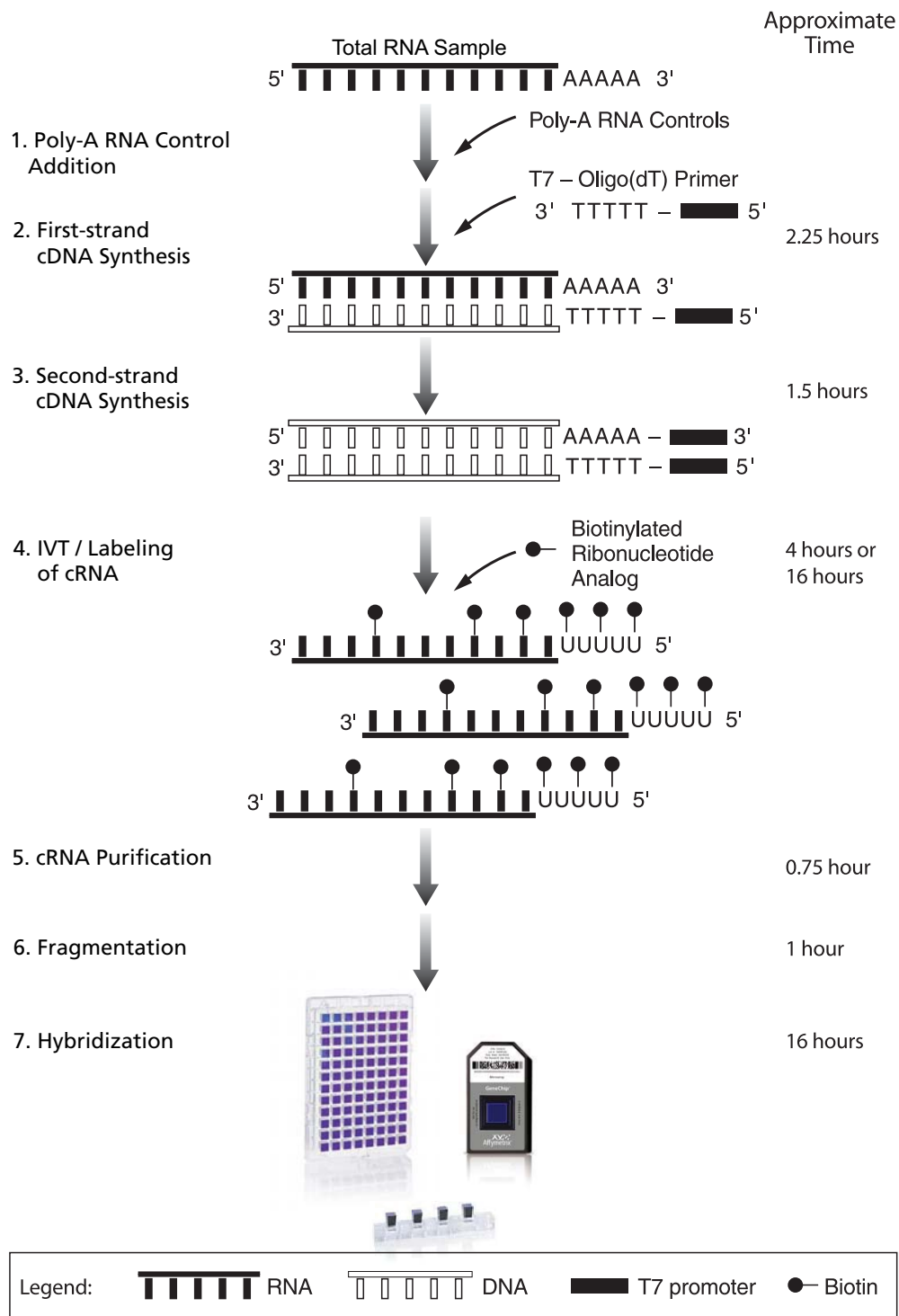
Safety

 **WARNING:** For research use only. Not recommended or intended for diagnosis of disease in humans or animals. Do not use internally or externally in humans or animals.

 **CAUTION:** All chemicals should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing, such as lab coat, safety glasses and gloves. Care should be taken to avoid contact with skin and eyes. In case of contact with skin or eyes, wash immediately with water. See MSDS (Material Safety Data Sheet) for specific advice.


Assay Workflow

Figure 1.1 3' IVT PLUS Amplification and Labeling Process



Kit Contents and Storage

Table 1.1 GeneChip® 3' IVT PLUS Reagent Kit Contents and Storage

Component	10-Reaction Kit for manual use (P/N 902415)	30-Reaction Kit for manual use (P/N 902416)	Storage
3' IVT Amplification Kit Module 1			
3' First-Strand Enzyme	11 µL	50 µL	-20°C
3' First-Strand Buffer	44 µL	160 µL	-20°C
3' Second-Strand Enzyme	22 µL	70 µL	-20°C
3' Second-Strand Buffer	55 µL	180 µL	-20°C
3' IVT Enzyme	66 µL	210 µL	-20°C
3' IVT Buffer	220 µL	660 µL	-20°C
3' IVT Biotin Label	44 µL	140 µL	-20°C
Control RNA (1 mg/mL HeLa total RNA)	5 µL	5 µL	-20°C
Nuclease-free Water	1 x 1.0 mL	2 x 1.0 mL	any temp *
3' IVT Amplification Kit Module 2			
3' Fragmentation Buffer	1 mL	1 mL	room temp
Purification Beads	1.1 mL	3.3 mL	4°C †
GeneChip® Poly-A RNA Control Kit			
Poly-A Control Stock	16 µL	16 µL	-20°C
Poly-A Control Dil Buffer	3.8 mL	3.8 mL	-20°C
GeneChip® Hybridization Control Kit			
20X Hybridization Controls	450 µL	450 µL	-20°C
3 nM Control Oligo B2	150 µL	150 µL	-20°C
	Tubes Organizer: Plastic vinyl template for organization and storage of components in 9 x 9 array, 81-places square wells, 5 1/4 in. x 5 1/4 in (e.g., Nalgene CryoBox P/N 5026-0909, or equivalent).		

* Store the Nuclease-free Water at -20°C, 4°C, or room temp.

†Do not freeze.

Required Materials

Instruments

Table 1.2 Instruments Required for Target Preparation

Item	Supplier
Heat block or oven for incubation of Nuclease-free Water during Purification	Major Laboratory Supplier
Magnetic Stand-96	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
Microcentrifuge	Major Laboratory Supplier
NanoDrop® UV-Vis Spectrophotometer	Thermo Scientific, or equivalent quantitation instrument
Optional: 2100 Bioanalyzer	Agilent Technologies, Inc., or equivalent DNA and RNA sizing instrument
Pipette	Major Laboratory Supplier
Thermal Cycler	Various
Vortex Mixer	Major Laboratory Supplier

Table 1.3 Instruments Required for Array Processing

Instruments	Supplier	Part Number
GeneChip® System for Cartridge Arrays		
GeneChip® Hybridization Oven 645	Affymetrix	P/N 00-0331 (110/220V)
GeneChip® Fluidics Station 450	Affymetrix	P/N 00-0079
GeneChip® Scanner 3000 7G	Affymetrix	P/N 00-0212 (North America) P/N 00-0213 (International)
GeneChip® AutoLoader with External Barcode Reader	Affymetrix	P/N 00-0090 (GCS 3000 7G S/N 501) P/N 00-0129 (GCS 3000 7G S/N 502)
GeneAtlas® System for Array Strips		
GeneAtlas® Workstation	Affymetrix	P/N 90-0894
GeneAtlas® Hybridization Station	Affymetrix	P/N 00-0380 (115VAC) P/N 00-0381 (230VAC)
GeneAtlas® Fluidics Station	Affymetrix	P/N 00-0377
GeneAtlas® Imaging Station	Affymetrix	P/N 00-0376
GeneAtlas® Barcode Scanner	Affymetrix	P/N 74-0015

Table 1.3 Instruments Required for Array Processing (Continued)

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

Reagents and Supplies

Table 1.4 Additional Reagents and Supplies Required

Item	Supplier
100% Ethanol (Molecular Biology grade or equivalent)	Major Laboratory Supplier*
96-well round bottom microtiter plate	Costar, P/N 3795 or equivalent
GeneChip® Hybridization, Wash, and Stain Kit	Affymetrix (P/N 900720, 30 rxns)
GeneAtlas® Hybridization, Wash, and Stain Kit for 3' IVT Array Strips	Affymetrix (P/N 901531, 60 rxns)
GeneTitan® Hybridization, Wash and Stain Kit for 3' IVT Array Plates	Affymetrix (P/N 901530, 96 rxns)
Nuclease-free aerosol-barrier tips	Major Laboratory Supplier
Nuclease-free 1.5, and 0.2 mL tubes or plates	Major Laboratory Supplier
Nuclease-free 15 mL tubes or containers	Major Laboratory Supplier
Nuclease-free water (for preparing 80% ethanol wash solution)	Affymetrix (P/N 71786) or Major Laboratory Supplier
Optional: 96-well plate sealing film	Major Laboratory Supplier
Optional: Reagent reservoir for multichannel pipette	Major Laboratory Supplier
Optional: RNA 6000 Nano Kit	Agilent Technologies, Inc. P/N 5067-1511; or equivalent DNA and RNA sizing reagents
Tough-Spots®	Major Laboratory Supplier

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

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 the:

- Method of RNA isolation
- Amount of input RNA that is used for each tissue type
- RNA purity and integrity
- *Equipment Preparation*
- Workflow stopping points
- *Reagent Preparation*

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. This is not the preferred method. Yields of cRNA may be greatly reduced if a heated lid is used during the Second-Strand cDNA Synthesis or during the In Vitro Transcription cRNA Synthesis steps. We recommend leaving the heated lid open during Second-Strand cDNA Synthesis. A small amount of condensation will form during the incubation. This is expected, and should not significantly decrease cRNA yields. For In Vitro Transcription cRNA Synthesis, we recommend incubating the reaction in a 40°C hybridization oven 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

Program	Heated Lid Temp	Alternate Protocol*	Step 1	Step 2	Step 3	Volume
First-Strand cDNA Synthesis	42°C	105°C	42°C, 120 min	4°C, 2 min		10 µL
Second-Strand cDNA Synthesis	RT or disable	Lid open	16°C, 60 min	65°C, 10 min	4°C, 2 min	30 µL
In Vitro Transcription cRNA Synthesis	40°C	40°C oven	40°C, 4-16 hr [†]	4°C, hold		60 µL
Fragmentation	94°C	105°C	94°C, 35 min	4°C, hold		Variable
Hybridization Control	65°C	105°C	65°C, 5 min			Variable
Hybridization Cocktail	99°C	105°C	95°C or 99°C, 5 min	45°C, 5 min		Variable

*For thermal cyclers that lack a programmable heated lid.

[†]4 hr for 250 - 500 ng RNA input, and 16 hr for 50 - 250 ng RNA input.

Reagent Preparation

Handling kit components as follows:

- Enzymes: Mix by gently vortexing the tube followed by a brief centrifuge to collect contents of the tube, then keep on ice.
- Buffers: 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 $\leq 37^{\circ}\text{C}$ for 1 to 2 min, or until the precipitate is fully dissolved, then keep on ice.
- Purification Beads: Allow to equilibrate at room temperature before use.
- Prepare master mixes for each step of the procedure to save time, improve reproducibility, and minimize pipetting error.
- Prepare Master Mixes as follows:
 - Prepare only the amount needed for all samples in the experiment plus ~5% overage to correct for pipetting losses when preparing the master mixes.
 - Use non-stick nuclease-free tubes to prepare the master mix.
 - 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.

Prepare Control RNA

Prepare Control RNA

To verify that the reagents are working as expected, a Control RNA sample (1 mg/mL 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 78 μL of Nuclease-free Water for a total volume of 80 μL (25 ng/ μL).
2. Follow the *Prepare Total RNA/Poly-A RNA Control Mixture* on page 15, but use 2 μL of the diluted Control RNA (50 ng) in the control reaction.



NOTE: The positive control reaction should produce > 15 μg of cRNA from 50 ng Control RNA using 16 hour incubation for the IVT reaction.

Prepare Poly-A RNA Controls



NOTE:

- To include premixed controls from the GeneChip® Poly-A RNA Control Kit, add the reagents to the total RNA samples. Follow the *Prepare Total RNA/Poly-A RNA Control Mixture* on page 15. Affymetrix strongly recommends the use of Poly-A RNA Controls for all reactions that will be hybridized to GeneChip® arrays.
- If frozen, the Poly-A Control Dil Buffer may take 15 to 20 min to thaw at room temperature.

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 Poly-A Control Dil Buffer and spiked directly into RNA samples to achieve the final concentrations (referred to as a ratio of copy number) summarized in Table 2.2.

Table 2.2 Final concentrations of Poly-A RNA Controls when added to total RNA samples

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

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

The Poly-A RNA Control Stock and Poly-A Control Dil Buffer are provided in the GeneChip® Poly-A RNA Control Kit to prepare the appropriate serial dilutions based on [Table 2.3](#). This is a guideline when 50, 100, 250, or 500 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 2.3 Serial Dilution of Poly-A RNA Control Stock

Total RNA Input Amount	Serial Dilutions				Volume of 4 th Dilution to Add to Total RNA
	1 st Dilution	2 nd Dilution	3 rd Dilution	4 th Dilution	
50 ng	1:20	1:50	1:50	1:20	2 µL
100 ng	1:20	1:50	1:50	1:10	2 µL
250 ng	1:20	1:50	1:50	1:4	2 µL
500 ng	1:20	1:50	1:50	1:2	2 µL

! **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 100 ng of total RNA:

1. Add 2 µL of the Poly-A Control Stock to 38 µL of Poly-A Control Dil Buffer for the 1st Dilution (1:20).
2. Add 2 µL of the 1st Dilution to 98 µL of Poly-A Control Dil Buffer to prepare the 2nd Dilution (1:50).
3. Add 2 µL of the 2nd Dilution to 98 µL of Poly-A Control Dil Buffer to prepare the 3rd Dilution (1:50).
4. Add 2 µL of the 3rd Dilution to 18 µL of Poly-A Control Dil Buffer to prepare the 4th Dilution (1:10).
5. Add 2 µL of this 4th Dilution to 100 ng of total RNA. The final volume of total RNA with the diluted Poly-A controls should not exceed 5 µL.



TIP: The first dilution of the Poly-A RNA controls can be stored up to 6 weeks in a non-frost-free freezer at -20°C and frozen/thawed up to eight times. Label the storage tube with the expiration date for future reference.

Prepare Total RNA

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} ratio. RNA of acceptable quality is in the range of 1.7 to 2.1.

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 partially-degraded mRNA may generate cDNA that lacks parts of the coding region.

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 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.



NOTE: Total RNA samples with lower RIN values may require increased input amounts to generate enough labeled cRNA for hybridization to an array.

Determine 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 2.4](#) are based on total RNA from HeLa cells. Use these values as reference points for determining your optimal RNA input.


 **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.

Table 2.4 Input RNA Limits

RNA Input	Total RNA
Recommended	100 ng
Minimum	50 ng
Maximum	500 ng

Table 2.5 Recommended IVT Incubation Time

Recommendations	RNA Amount	IVT Incubation Time
Recommended	50 - 250 ng	16 hours
Optional	250 - 500 ng	4 hours

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 the RNA must be 3 μL or less ([Table 2.6](#)). See *Prepare Poly-A RNA Controls* [on page 12](#) for more information. For example, when performing the Control RNA reaction, combine 2 μL of RNA (25 ng/ 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 2.6 Total RNA/Poly-A RNA Control Mixture

Component	Volume for One Reaction
Total RNA Sample (50 - 500 ng)	variable
Diluted Poly-A RNA Controls (4 th Dilution)	2 μL
Nuclease-free Water	variable
Total Volume	5 μL

Synthesize First-Strand cDNA

In this reverse transcription procedure, total RNA is primed with T7 oligo(dT) primer. The reaction synthesizes single-stranded cDNA with the 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.

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 ~5% excess volume to correct for pipetting losses.

Table 2.7 First-Strand Master Mix

Component	Volume for One Reaction
3' First-Strand Buffer	4 μL
3' First-Strand Enzyme	1 μL
Total Volume	5 μL

- 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 2.6) 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 15 for more information.
 - B. 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 2 hr at 42°C, 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 11.
 - 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 Second-Strand cDNA* on page 17.



IMPORTANT: Transferring Second-Strand 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 Second-Strand cDNA Synthesis.

Synthesize Second-Strand 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 DNA polymerase and RNase H to simultaneously degrade the RNA and synthesize second-strand cDNA.

! **IMPORTANT:** Pre-cool thermal cycler block to 16°C.

1. Prepare Second-Strand Master Mix.
 - A. On ice, prepare the Second-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 first-strand cDNA samples in the experiment. Include ~5% excess volume to correct for pipetting losses.

Table 2.8 Second-Strand Master Mix

Component	Volume for One Reaction
Nuclease-free Water	13 μ L
3' Second-Strand Buffer	5 μ L
3' Second-Strand Enzyme	2 μ L
Total Volume	20 μL

- 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 20 μ L of the Second-Strand Master Mix to each (10 μ L) first-strand cDNA sample for a final reaction volume of 30 μ L.
 - 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 1 hr at 16°C, then for 10 min at 65°C, then for at least 2 min at 4°C.
 - A. Incubate the second-strand synthesis reaction in a thermal cycler using the Second-Strand cDNA Synthesis program that is shown in [Table 2.1 on page 11](#).

! **IMPORTANT:** Disable the heated lid of the thermal cycler or keep the lid off during the Second-Strand cDNA Synthesis.

- B. Immediately after the incubation, centrifuge briefly to collect the second-strand cDNA at the bottom of the tube or well.
 - C. Place the sample on ice, then proceed immediately to *Synthesize Labeled cRNA by In Vitro Transcription* [on page 18](#).

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

Synthesize Labeled cRNA by In Vitro Transcription

In this procedure, labeled complementary RNA (cRNA) is synthesized and amplified by *in vitro* transcription (IVT) of the second-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).



IMPORTANT:

- Transfer the second-strand 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.

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 second-strand cDNA samples in the experiment. Include $\sim 5\%$ excess volume to correct for pipetting losses.

Table 2.9 IVT Master Mix

Component	Volume for One Reaction
3' IVT Biotin Label	4 μ L
3' IVT Buffer	20 μ L
3' IVT Enzyme	6 μ L
Total Volume	30 μL

- 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 μ L of the IVT Master Mix to each (30 μ L) second-strand cDNA sample for a final reaction volume of 60 μ L.
- 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 4 - 16 hr at 40°C, then at 4°C.
When incubating at 40°C, incubate 4 hr for 250 - 500 ng RNA input, and 16 hr for 50 - 250 ng RNA input.
- 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 11](#).
- B. After the incubation, centrifuge briefly to collect the labeled cRNA at the bottom of the tube or well.
- C. Place the reaction on ice, then proceed to *Purify Labeled cRNA* [on page 19](#), or immediately freeze the samples at -20°C for storage.



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

Purify Labeled cRNA

In this procedure, enzymes, salts, inorganic phosphates, and unincorporated nucleotides are removed to prepare the labeled cRNA for fragmentation. See [Appendix A, cRNA Purification Photos](#).

Beginning the cRNA Purification



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, 100 μ L 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 (Not Supplied) in a nuclease-free tube or container. For each reaction, 600 μ L 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.
- 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.
- B. Add 100 μ L of the Purification Beads to each (60 μ L) cRNA sample, mix by pipetting up and down, and transfer to a well of a U-bottom plate.



TIP:

- Any unused wells should be covered with a plate sealer so that the plate can safely be reused.
- Use multichannel pipette when processing multiple samples.

- 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 μ L 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 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 \sim 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 and Size Distribution](#), 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 - 30 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.

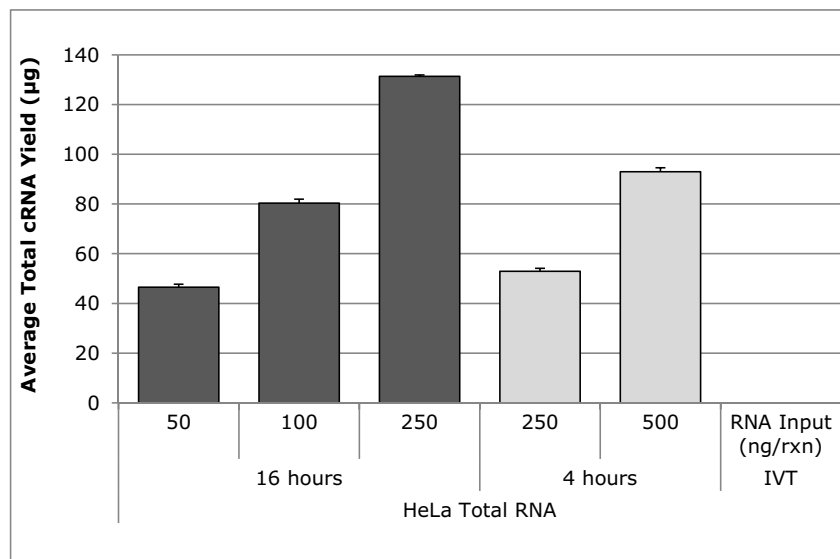
Assess cRNA Yield and Size Distribution

Expected cRNA Yield

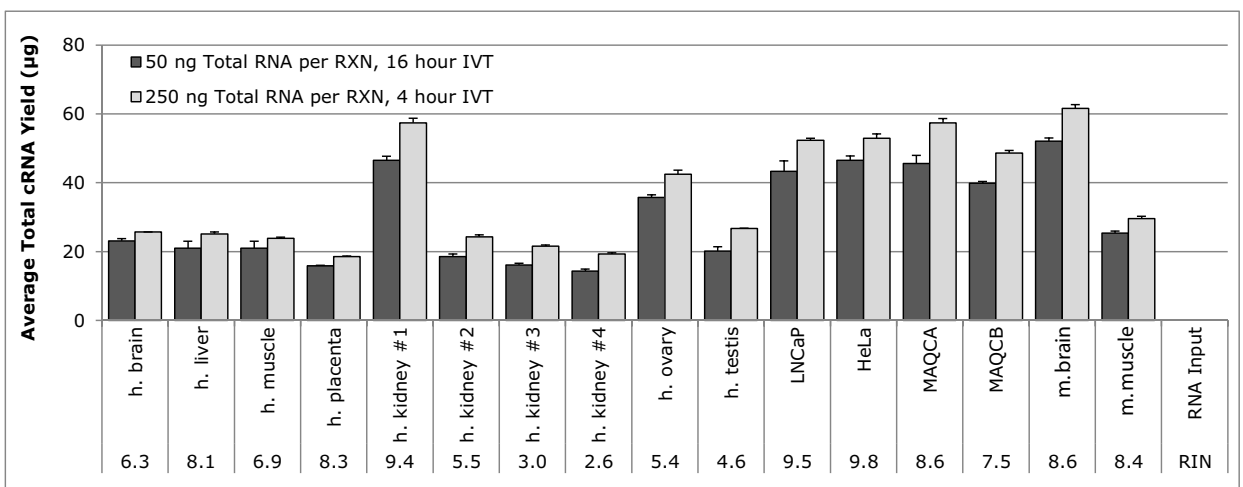
The cRNA yield depends on the amount and quality of poly(A) in the input total RNA. Because the proportion of poly(A) in total RNA is affected by factors such as the health of the organism and the organ from which it is isolated, cRNA yield from equal amounts of total RNA may vary considerably.

During development of this kit, using a wide variety of tissue types, 50 ng of input total RNA yielded 15 to 40 μg of cRNA. For most tissue types, the recommended 100 ng of input total RNA should provide $> 20 \mu\text{g}$ 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 HeLa (A) and a Variety of Total RNA Samples (B)



Example A



h. is human and m. is mouse

Example B

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 1.5 μ L of the cRNA sample directly. Samples with cRNA concentrations greater than 3,000 ng/ μ L should be diluted with Nuclease-free Water before measurement and reaction setup. Use the diluted cRNA as the input to prepare fragmentation 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 μ g/mL using the equation shown below ($1 A_{260} = 40 \mu$ g RNA/mL).

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

The amounts of labeled cRNA required for fragmentation are listed in [Table 2.10 on page 22](#).

Table 2.10 Labeled cRNA Amounts Required for Fragmentation by Array Format

Component	49 or 64-Format		100 or 81/4-Format	169, 400-Format, or Array Plate	Array Strip
Labeled cRNA*	15 μ g (in 1 to 32 μ L)	12 μ g † (in 1 to 25.6 μ L)	12 μ g (in 1 to 25.6 μ L)	7.5 μ g (in 1 to 16 μ L)	9.4 μ g (in 1 to 20 μ L)

* Minimum concentration of the eluate is 468.75 ng/ μ L.

† Alternative protocol for samples with cRNA yields below 15 μ g.

NOTE: High-concentration labeled cRNA samples (> 3000 ng/ μ L) should be diluted with Nuclease free Water before measurement and reaction setup. Use the diluted cRNA as the input to prepare required amount of labeled cRNA.

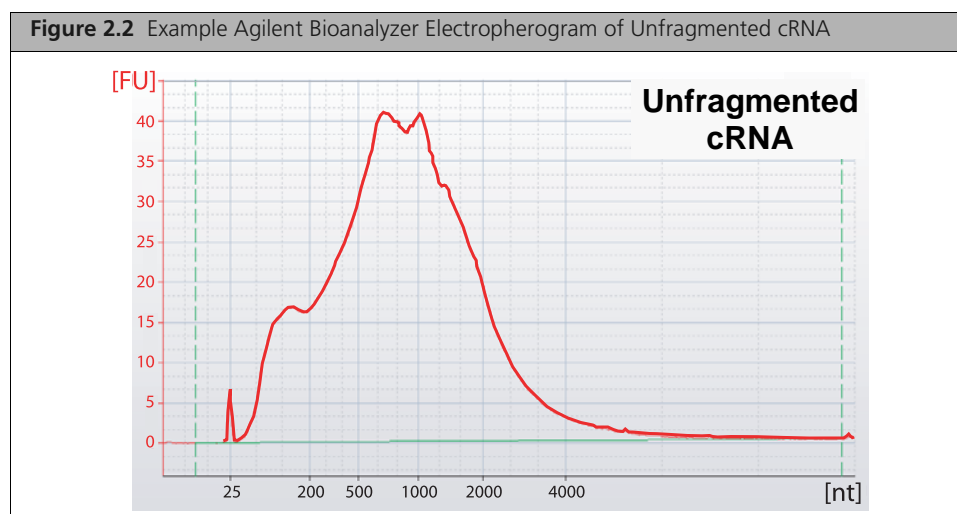
NOTE: Please refer to [Table 2.11](#) and [Table 3.3](#) for the amount of labeled cRNA required for one fragmentation reaction and one array hybridization experiment, respectively. The amount varies depending on the array format. Please refer to the specific array package insert for information on the array format.

(Optional) Expected cRNA Size Distribution

The expected cRNA profile is a distribution of sizes from 250 to 5,500 nt with most of the cRNA sizes in the 600 to 1,200 nt range. Average cRNA size may vary slightly depending on RNA quality and total RNA input amount. This step is optional.

Determine cRNA size distribution using a Bioanalyzer.

Affymetrix recommends analyzing cRNA size distribution 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 300 ng of cRNA per well on the Bioanalyzer. If there is insufficient yield, then use as little as 200 ng of cRNA per well. 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 . 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.

Fragment Labeled cRNA

In this procedure, the purified cRNA is fragmented by divalent cations and elevated temperature. The amounts of labeled cRNA required for fragmentation depends on the array formats (Table 2.10).

1. Prepare the required amount of labeled cRNA.

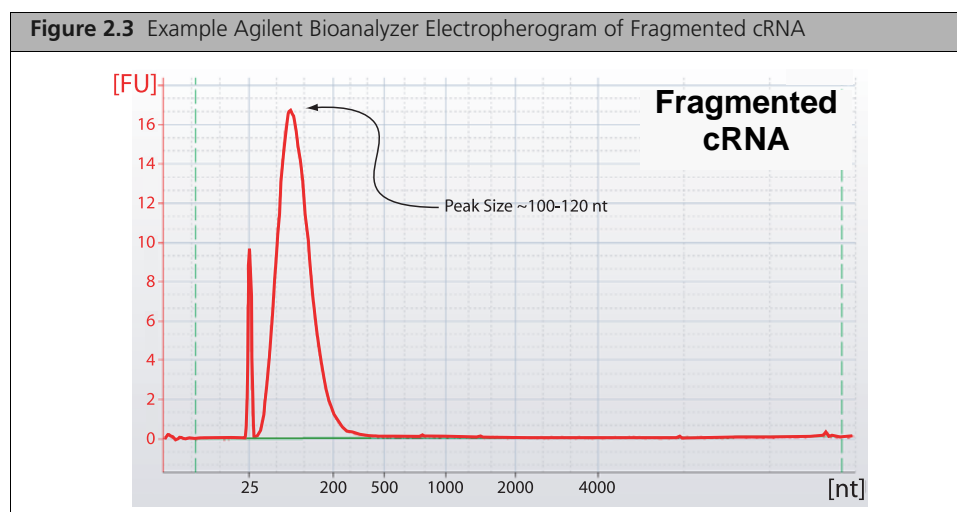
On ice, prepare 468.75 ng/ μ L labeled cRNA. If necessary, use Nuclease-free Water to bring the labeled cRNA sample to the required volume.
2. Prepare Fragmentation Reaction.
 - A. On ice, prepare the Fragmentation Reaction in a nuclease-free tube. Combine the components in the sequence shown in the table below.

Table 2.11 One Labeled cRNA Fragmentation Reaction by Array Format

Component	49 or 64-Format	100 or 81/4-Format	169, 400-Format, or Array Plate	Array Strip
Labeled cRNA	15 μ g (in 32 μ L)	12 μ g * (in 25.6 μ L)	12 μ g (in 25.6 μ L)	9.4 μ g (in 20 μ L)
3' Fragmentation Buffer	8 μ L	6.4 μ L	6.4 μ L	5 μ L
Total Volume	40 μL	32 μL	32 μL	25 μL

*Alternative protocol for samples with cRNA yields below 15 μ g.

- 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.
3. Incubate for 35 min at 94°C, then for at least 2 min at 4°C.
 - A. Incubate the fragmentation reaction in a thermal cycler using the Fragmentation program that is shown in Table 2.1 on page 11.
 - B. Immediately after the incubation, centrifuge briefly to collect the fragmented and labeled cRNA at the bottom of the tube or well.
 - C. Place the sample on ice, then proceed immediately to the next step.



TIP: STOPPING POINT. The fragmented and labeled 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.

3' 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's Guide AGCC* (P/N 08-0295), the *GeneChip® Expression Wash, Stain, and Scan User Manual for Cartridge Arrays* (PN 702731), and the *Affymetrix® GeneChip® Command Console® User Manual* (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 1.4 on page 9](#) or the Affymetrix website.
 - 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® 3' Cartridge Array(s). (Not supplied)

Procedure

1. In preparation of the hybridization step, prepare the following:
 - A. Pull the array from storage at 4°C so that it can begin to equilibrate to room temperature.
 - B. Turn on the Hybridization Oven and set the temperature to 45°C.
 - C. Warm the pre-hybridization buffer to room temperature.



NOTE: Aliquot ~220 µL of pre-hybridization buffer per array to be hybridized into a 1.5 mL tube to accelerate the equilibration to room temperature.

2. Prepare Hybridization Master Mix.

- A. At room temperature, thaw the components listed in [Table 3.1](#).



NOTE: DMSO will solidify when stored at 2 - 8°C. Ensure 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 11](#).
- 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 cRNA samples in the experiment. Include ~10% overage to correct for pipetting losses.

Table 3.1 Hybridization Master Mix for a Single Reaction

Component	49 or 64-Format	100 or 81/4-Format	169 or 400-Format	Final Concentration
Control Oligo B2 (3 nM)	3.7 µL	3.3 µL	1.7 µL	50 pM
20X Hybridization Controls (<i>bioB</i> , <i>bioC</i> , <i>bioD</i> , <i>cre</i>)	11 µL	10 µL	5 µL	1.5, 5, 25, and 100 pM respectively
2X Hybridization Mix	110 µL	100 µL	50 µL	1X
DMSO	22 µL	20 µL	10 µL	10%
Nuclease-free Water	43.9 µL	40 µL	20 µL	
Total Volume	190.6 µL	173.3 µL	86.7 µL	

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

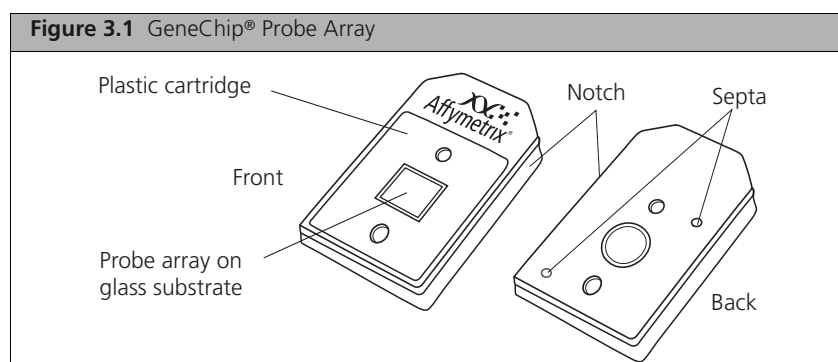
3. Pre-hybridize array.

- A. Insert a pipette tip into the upper right septum to allow for venting.
- B. Wet the array with an appropriate volume of Pre-Hybridization Mix ([Table 3.2](#)) by filling it through the bottom left septa.

Table 3.2 Array Cartridge Volumes for Pre-Hybridization Mix

	49 or 64-Format	100 or 81/4-Format	169 or 400-Format
Volume to Load on Array	200 µL	130 µL	80 µL

- C. Remove the pipette tip from the upper right septum of the array.
- D. Incubate the array filled with Pre-Hybridization Mix with 60 rpm rotation for 10 - 30 min at 45°C.



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.

4. Prepare Hybridization Cocktail.

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

Table 3.3 Hybridization Cocktail for a Single Array

Component	49 or 64-Format	100 or 81/4-Format	169 or 400-Format	Final Concentration
Hybridization Master Mix	190.6 μ L	173.3 μ L	86.7 μ L	
Fragmented and labeled cRNA	29.4 μ L (11 μ g)	26.7 μ L (10 μ g)	13.3 μ L (5 μ g)	50 ng/ μ L
Total Volume	220 μL	200 μL	100 μL	

- 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), 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 11](#).
- D. After the incubation, centrifuge briefly to collect contents of the tube and proceed to Hybridization ([Step 5](#)).

5. Hybridize array.

NOTE: It is important to allow the arrays to equilibrate to room temperature completely. Specifically, if the rubber septa are not equilibrated to room temperature, they may be prone to cracking, which can lead to leaks.

- A. Remove the array from the hybridization oven. Vent the array with a clean pipette tip and extract the Pre-Hybridization Mix from the array with a micropipettor.
- B. Refill the array with the appropriate volume ([Table 3.4](#)) of the hybridization cocktail ([Step 4D](#)), avoiding any insoluble matter at the bottom of the tube.

Table 3.4 Array Cartridge Volumes for Hybridization Cocktail

	49 or 64-Format	100 or 81/4-Format	169 or 400-Format
Volume to Load on Array	200 μ L	130 μ L	80 μ L

- C. Remove the pipette 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's Guide AGCC (P/N 08-0295)*, the *GeneChip® Expression Wash, Stain, and Scan User Manual for Cartridge Arrays (PN 702731)*, and the *Affymetrix® GeneChip® Command Console® User Manual (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 3.5 Fluidics Protocol

	49 or 64-Format	100 or 81/4-Format	169 or 400-Format
Fluidics Protocol	FS450_0001	FS450_0002	FS450_0003

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 Manual (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 29*
- *GeneAtlas® Software Setup on page 35*



NOTE: If you are using a hybridization-ready sample, or re-hybridizing previously made hybridization cocktail continue the protocol from [Step 4 on page 30](#).



IMPORTANT: Before preparing hybridization ready samples, register samples as described in *GeneAtlas® Software Setup on page 35*.

Please refer to *GeneAtlas® System User's 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 3' IVT Array Strips. (Not supplied) For ordering information please refer to [Table 1.4 on page 9](#) or the Affymetrix website.
 - 1X Pre-Hybridization Mix
 - 1.3X Hybridization Mix Solution A
 - 1.3X Hybridization Mix Solution B
 - 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® Array Strip and consumables (Not supplied)
 - Affymetrix 3' Array Strip(s)
 - 1 hybridization tray per array strip

Procedure

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 two (2) hybridization trays per array strip.
 - C. Set the temperature of the GeneAtlas Hybridization Station to 45°C. Push the start button to begin heating.
 - D. Warm the pre-hybridization buffer to room temperature.



NOTE: Aliquot ~500 µL of pre-hybridization buffer per array strip to be hybridized into an Eppendorf tube to accelerate the equilibration to room temperature.

2. In preparation of the hybridization master mix, prepare the following:
 - A. Warm the following vials to room temperature on the bench:
 - 1.3X Hybridization Solution A
 - 1.3X Hybridization Solution B
 - 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 Hybridization Master Mix.
 - 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 11](#).
 - 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 cRNA samples in the experiment.

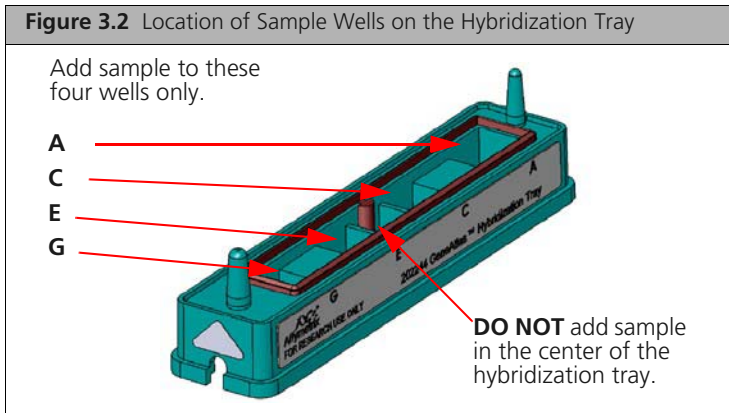
Table 3.6 Hybridization Master Mix

Component	Volume for One Array	Volume for Four Arrays (Includes 10% Overage)	Final Concentration
Control Oligonucleotide B2 (3 nM)	2.5 µL	11 µL	50 pM
20X Hybridization Controls (<i>bioB</i> , <i>bioC</i> , <i>bioD</i> , <i>cre</i>)	7.5 µL	33 µL	1.5, 5, 25 and 100 pM, respectively
1.3X Hybridization Solution A	40 µL	176 µL	
1.3X Hybridization Solution B	75 µL	330 µL	
Nuclease-free Water	5 µL	22 µL	
Total Volume	130 µL	572 µL	

- C. Mix thoroughly by gently vortexing. Centrifuge briefly to collect the mix and proceed to the next step.
4. Pre-hybridize Array Strip.
 - A. Gently pipette **120 µL** of Pre-Hybridization Buffer into the appropriate wells of the hybridization tray ([Figure 3.2](#)). Avoid generating air bubbles.



CAUTION: The center of the hybridization tray is not a sample well. It is important that you do not add anything to this area ([Figure 3.2](#)).



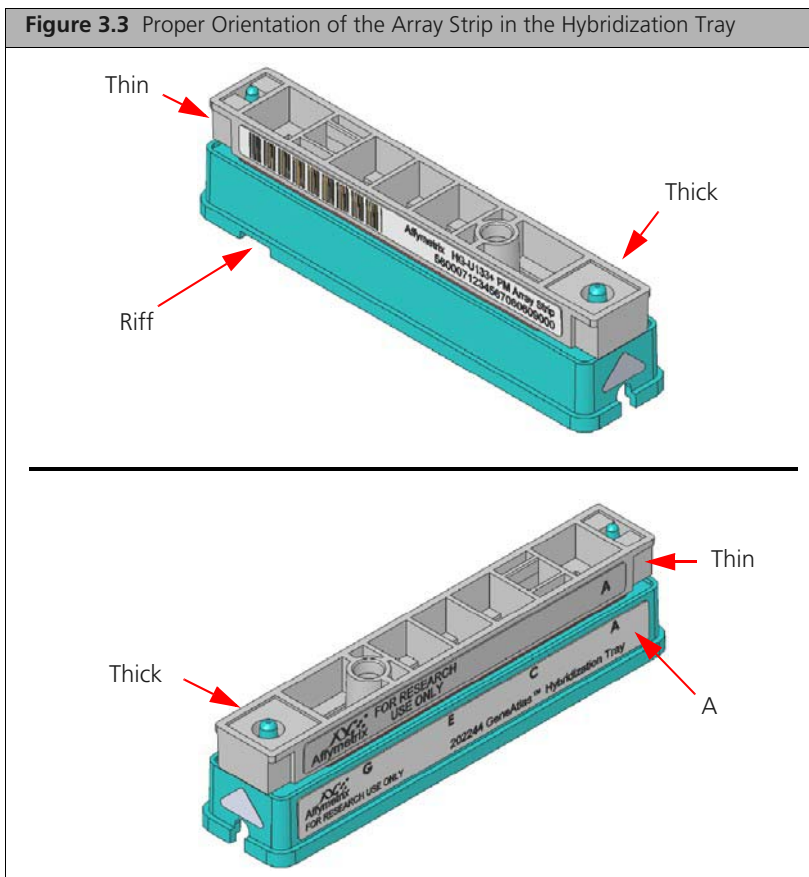
- B. Remove the array strip from its foil pouch and carefully place it into the hybridization tray (Figure 3.3) making sure that there are no bubbles beneath the array strip.



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



TIP: To avoid any possible mixups, the hybridization tray and array strips should be labeled on the white label if more than 1 array strip is processed overnight.



- C. 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.

- D. 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 airbubbles.
- E. Place the array strip/hybridization tray into the Hybridization Station for 10 - 15 min. at 45°C.

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 3.4](#).

Figure 3.4 Opening the Clamps on the GeneAtlas™ Hybridization Station



5. Prepare Hybridization Cocktail.

- A. At room temperature, prepare the Hybridization Cocktail in the order as shown in [Table 3.7](#) for all samples.

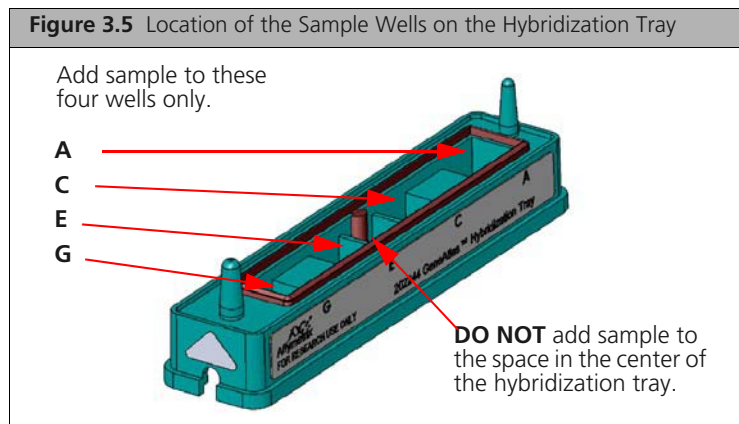
Table 3.7 Hybridization Cocktail for a Single Array

Component	Volume for One Array	Final Concentration
Hybridization Master Mix	130 μ L	
Fragmented and labeled cRNA	20 μ L (7.5 μ g)	50 ng/ μ L
Total Volume	150 μL	

- 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 10 min at 96°C, then for 2 min at 45°C in a thermal cycler.
- D. After the incubation, centrifuge briefly to collect contents of the tube or well and proceed to Hybridization ([Step 6](#)).

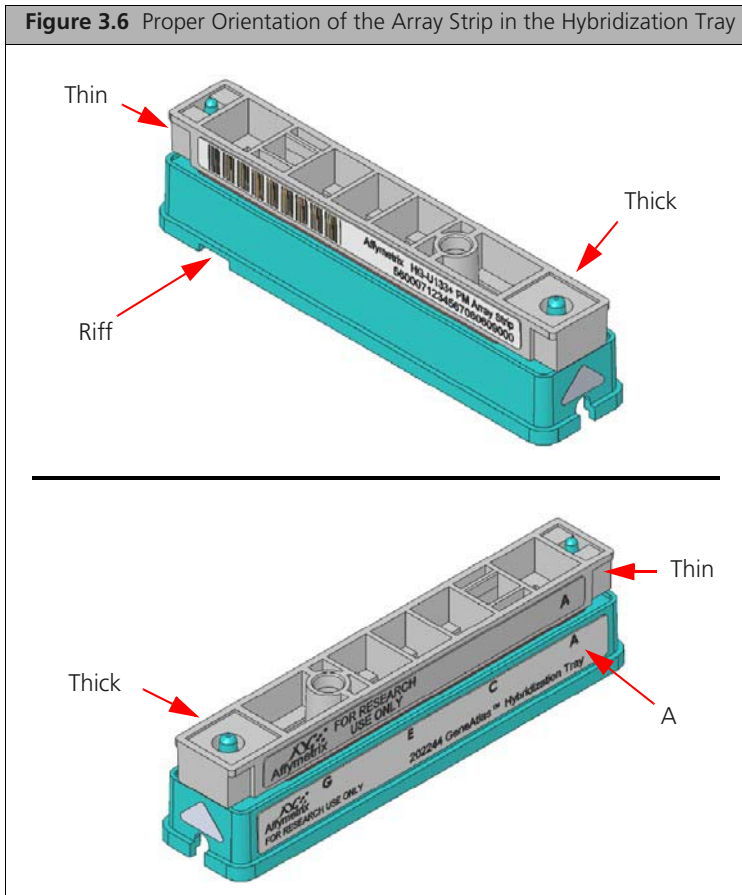
- E. Optional: the remainder of the hybridization cocktail can be stored at -20°C to supplement Hybridization Cocktail volume should a rehybridization be necessary. (Refer to *Rehybridizing Used Cocktails* on page 39 for additional information.)
6. Hybridize Array Strip.
- A. After 10-15 min of pre-hybridization, remove the array strip from the Hybridization Station and place on bench top keeping the arrays immersed in the pre-hybridization solution.
- B. Apply the **120 μL** of pre-heated hybridization cocktail (Step 5D) to the middle of the appropriate wells of a new clean hybridization tray (Figure 3.5).

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

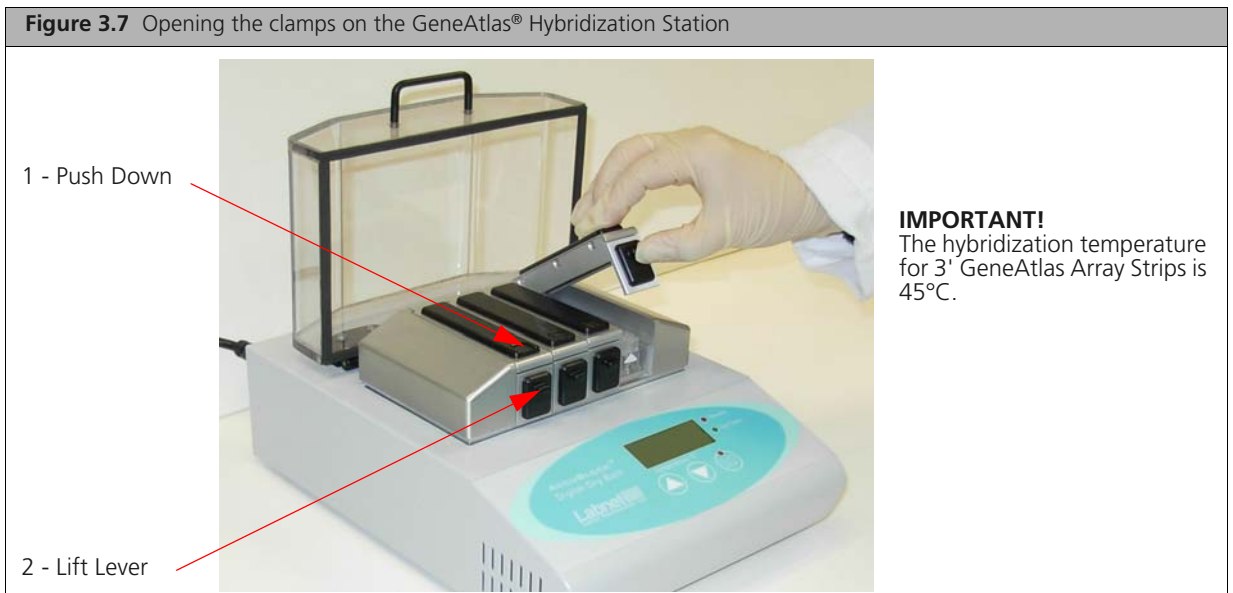


- C. Carefully remove the array strip from the hybridization tray containing the pre-hybridization buffer and place it into the hybridization tray containing the hybridization cocktail samples.
- D. Check for and remove any bubbles that were introduced. Refer to Figure 3.6 for proper orientation of the array strip in the hybridization tray.

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



- E. 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](#).



7. Proceed to *Hybridization Software Setup* on [page 37](#).

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's Guide* (P/N 08-0306).

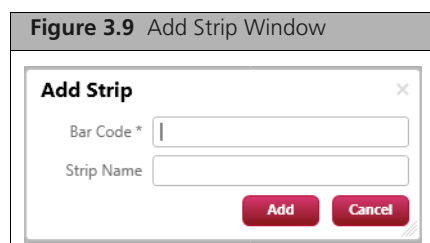
Sample Registration

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's Guide* (P/N 08-0246).

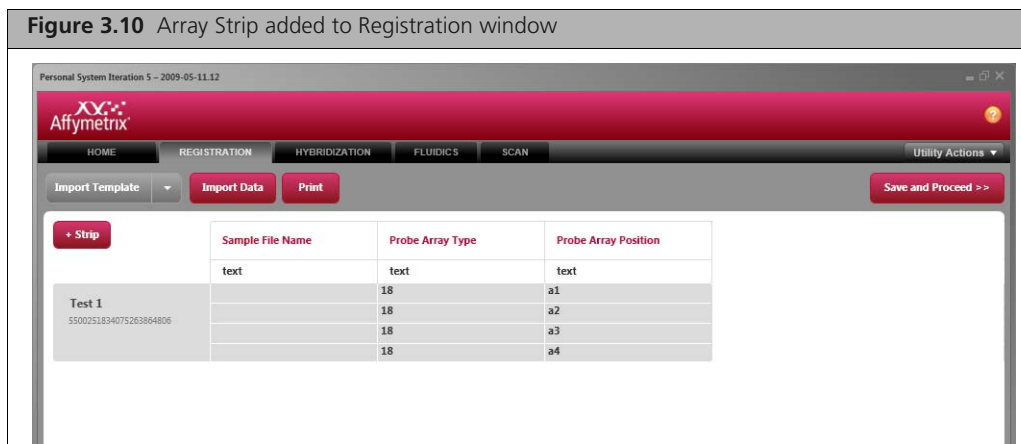
1. Click **Start** → **Programs** → **Affymetrix** → **GeneAtlas** to launch the GeneAtlas Software.
2. Click the **Registration** tab.
Figure 3.8 appears.




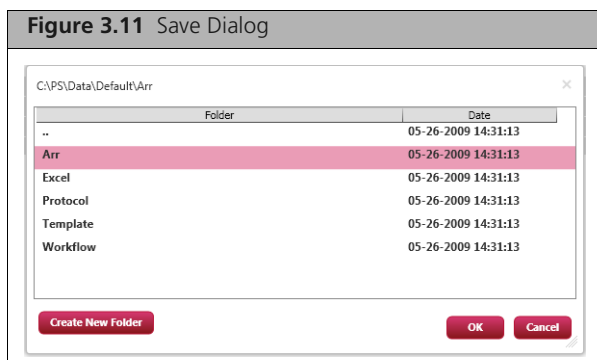
3. Click the **+ Strip** button: .
The Add Strip Window appears (Figure 3.9).



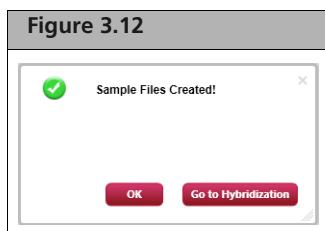
4. Enter or scan the array strip **Bar Code** and enter a **Strip Name**, then click **Add**.
The array strip is added and appears in the Registration window (Figure 3.10)



- 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.
- When complete click the **Save and Proceed** button: . The Save dialog box appears ([Figure 3.11](#)).



- 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](#)).



- 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](#).

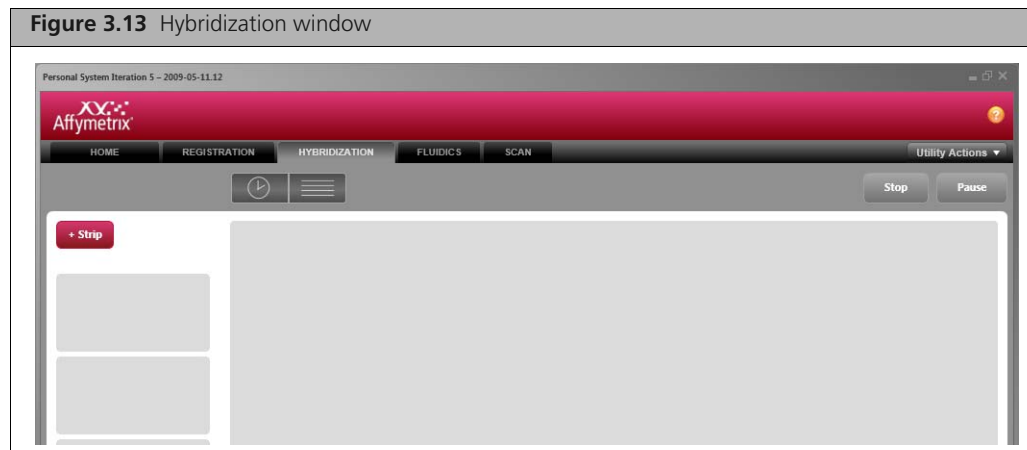
- Proceed to *Hybridization Software Setup* on [page 37](#).

Hybridization Software Setup

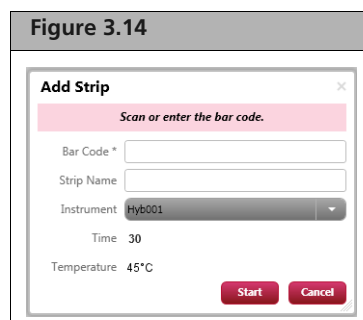
All Affymetrix Array Strips to be processed must first be registered prior to setting up the hybridizations in the GeneAtlas Software. Refer to *Sample Registration on page 35* for instruction on registering array strips.

! **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.

1. Navigate to the **Hybridization** tab on the GeneAtlas Software interface.

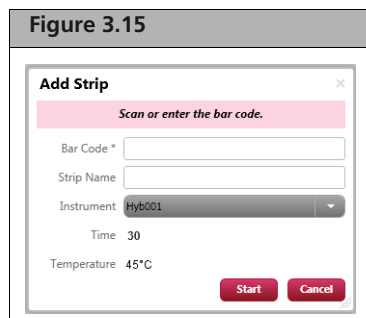


2. Click the **+ Strip** button: . The Add Strip Window appears ([Figure 3.14](#)).

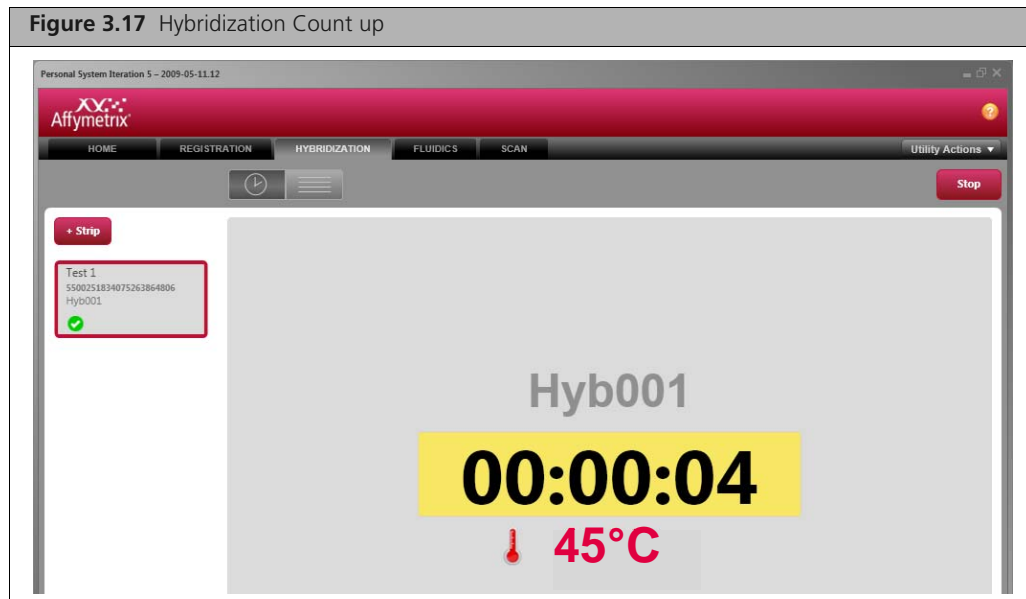


3. Scan or enter the **Bar Code** (required) of the array strip you registered. The **Strip Name** field is automatically populated.
4. From the **Instrument** drop-down box, select the correct hybridization station.
5. The **Time** and **Temperature** settings are automatically populated and are read from the installed library files.
6. DO NOT click **Start**. Proceed to *Target Hybridization Setup for Affymetrix® Array Strips on page 29*.

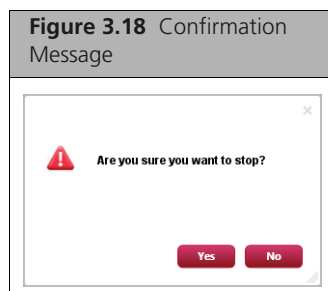
7. With the hybridization tray and array strip already in the GeneAtlas Hybridization Station, click **Start** in [Figure 3.15](#).



NOTE: The software displays the hybridization time countdown. This time is displayed with a white background ([Figure 3.16](#)). When the countdown has completed the display turns yellow and the time begins to count up.



8. When hybridization has completed, click the **Stop** button in the upper right corner. A confirmation message box appears (Figure 3.18).



9. Click **Yes** to complete hybridization.
10. Be sure 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.
11. Save the remaining hybridization cocktail in -20°C for future use (refer to *Rehybridizing Used Cocktails* on page 39 for additional information).
12. Immediately proceed to the GeneAtlas Wash, Stain and Scan protocol. Please refer to *GeneAtlas[®] System User's Guide* (P/N 08-0306) for further detail.

Rehybridizing Used Cocktails

A used hybridization cocktail can be rehybridized to a new array if necessary. Collect the used hybridization cocktail immediately after the Fluidics run is completed, add to the remainder of the hybridization cocktail master mix from [Step E on page 33](#) and store at -20°C .

For rehybridization, continue the protocol from [Step 4 on page 30](#). The hybridization cocktail needs to be denatured again prior to reapplication to a new array.

! **IMPORTANT:** Rehybridization of hybridization cocktails should only be necessary in case of serious array problems. The performance of rehybridized samples has not been thoroughly tested and is recommended only when absolutely necessary.

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 40*
- *Processing 3' Array Plates on the GeneTitan® Instrument on page 42*

Please refer to *GeneTitan® Instrument User Guide for Expression Arrays Plates* (P/N 702933) and *Affymetrix® GeneChip® Command Console™ User's Guide* (P/N 702569) for further detail.

Target Hybridization Setup for Affymetrix® Array Plates

Reagents and Materials Required

- GeneTitan® Hybridization, Wash and Stain Kit for 3' IVT Array Plates. (Not supplied) For ordering information please refer to [Table 1.4 on page 9](#) or the Affymetrix website.
 - 1.3X Hybridization Mix Solution A
 - 1.3X Hybridization Mix Solution B
 - 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® 3' Array Plate(s)

Procedure

1. In preparation of the hybridization step, prepare the following:
 - A. Warm the following vials to room temperature on the bench:
 - 1.3X Hybridization Mix Solution A
 - 1.3X Hybridization Mix Solution B
 - 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 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 11](#).
 - 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 cRNA samples in the experiment.

Table 3.8 Hybridization Master Mix

Component	Volume for One Array	16-Array Plate*	24-Array Plate*	96-Array Plate*	Final Concentration
Control Oligo B2 (3 nM)	2 µL	35.2 µL	52.8 µL	211.2 µL	50 pM
20X Eukaryotic Hybridization Controls (<i>bioB</i> , <i>bioC</i> , <i>bioD</i> , <i>cre</i>)	6 µL	105.6 µL	158.4 µL	633.6 µL	1.5, 5, 25 and 100 pM, respectively
1.3X Hybridization Solution A	32.3 µL	568.5 µL	852.7 µL	3,411 µL	
1.3X Hybridization Solution B	60 µL	1,056 µL	1,584 µL	6,336 µL	
Nuclease-free Water	3.7 µL	65.1 µL	97.7 µL	390.6 µL	
Total Volume	104 µL	1,830.4 µL	2,745.6 µL	10,982.4 µL	

*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.
3. Prepare Hybridization Cocktail.
 - A. At room temperature, prepare the Hybridization Cocktail in the order as shown in [Table 3.9](#) for all samples.

Table 3.9 Hybridization Cocktail for a Single Array

Component	Volume for One Array	Final Concentration
Hybridization Master Mix	104 µL	
Fragmented and labeled cRNA	16 µL (6 µg)	50 ng/µL
Total Volume	120 µL	

- 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), 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 11](#).
- 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. Save the remaining hybridization cocktail in -20°C for future use.
- G. Proceed to *Hybridization Setup* [on page 42](#).

Hybridization Setup

This section describes the GeneTitan Setup protocol for 3' Array Plates. The reagent consumption per process on the GeneTitan® Instrument for processing 3' Array Plates is shown in Table 3.11.

Table 3.10 The Minimum Volumes of Buffer and Rinse Required to Process on the GeneTitan Instrument

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

Table 3.11 Volumes Required to Process 3' Array Plates per Run

Reagent	Amount Required for One Array Plate	Number of Plates that can be Processed using the GeneTitan Hybridization, Wash and Stain Kit for 3' Array Plates (P/N 901530)		
		16-Format	24-Format	96-Format
Wash A*	~920 mL	1	1	1
Wash B*	300 mL	1	1	1
Stain 1 and 3	105 µL/well	6	4	1
Stain 2	105 µL/well	6	4	1
Array Holding Buffer	150 µL/well	6	4	1

* Please use GeneTitan® Wash Buffers A and B Module (PN 901583) for ordering Wash A and B buffers for additional plates.

! **IMPORTANT:** The instrument must have a minimum of 450 mL of Wash B in the Wash B reservoir of the instrument for each 3' Array Plate prior to starting Hyb, Wash, Stain and Scan process. The waste bottle should be empty.

Processing 3' Array Plates on the GeneTitan® Instrument

Please follow the instructions provided in the *GeneTitan® Instrument User Guide for Expression Array Plates* (P/N 702933) to process HT array plates on the GeneTitan Instrument.

cRNA Purification Photos

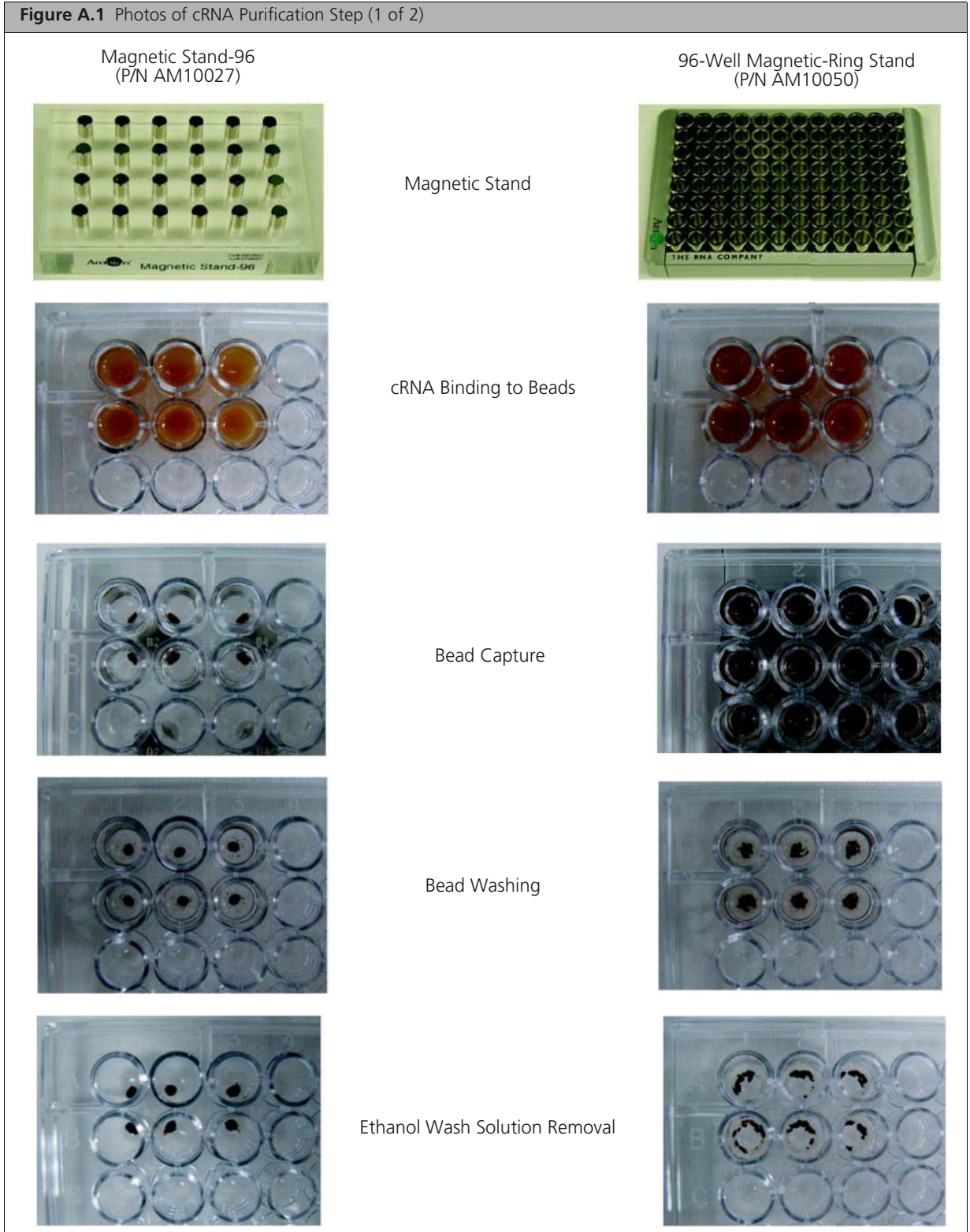
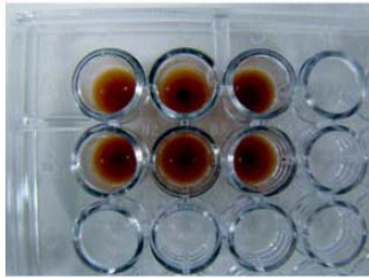
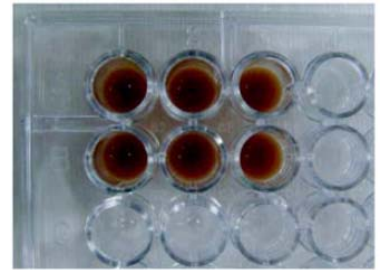


Figure A.2 Photos of cRNA Purification Step (2 of 2)

Magnetic Stand-96
(P/N AM10027)



96-Well Magnetic-Ring Stand
(P/N AM10050)



cRNA Elution



Elution Step

References

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Van Gelder, R.N., von Xastrow, M.E., Yool, E. *et al.* 1990. *Proc Natl Acad Sci USA* 87:1663-1667.