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GeneChip™ 3' IVT PLUS Reagent Kit

USER GUIDE

Manual Target Preparation for GeneChip™ 3' Expression Arrays

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Thermo Fisher Scientific Baltics UAB |
V.A. Graiciuno 8, LT-02241 |
Vilnius, Lithuania

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GeneChip™ 3' IVT PLUS Reagent Kit

Affymetrix Pte Ltd |
7 Gul Circle #2M-01 |
Keppel Logistics Building |
Singapore 629563

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B.0	28 October 2020	Changed volume to load on array for the 169/400 format to 90 µL. Updated image of GeneChip™ cartridge array.
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1 The GeneChip™ 3' IVT PLUS Reagent Kit

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Purpose

The GeneChip™ 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 GeneChip™ 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 on 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. See “Assay workflow” on page 6.

The 3′ IVT PLUS reagent is optimized to work with total RNA from a wide range of samples including tissues, cells, and cell lines. Total RNA from whole blood samples should be processed for globin reduction prior to target preparation with the GeneChip™ 3' IVT PLUS Reagent Kit.

Assay workflow

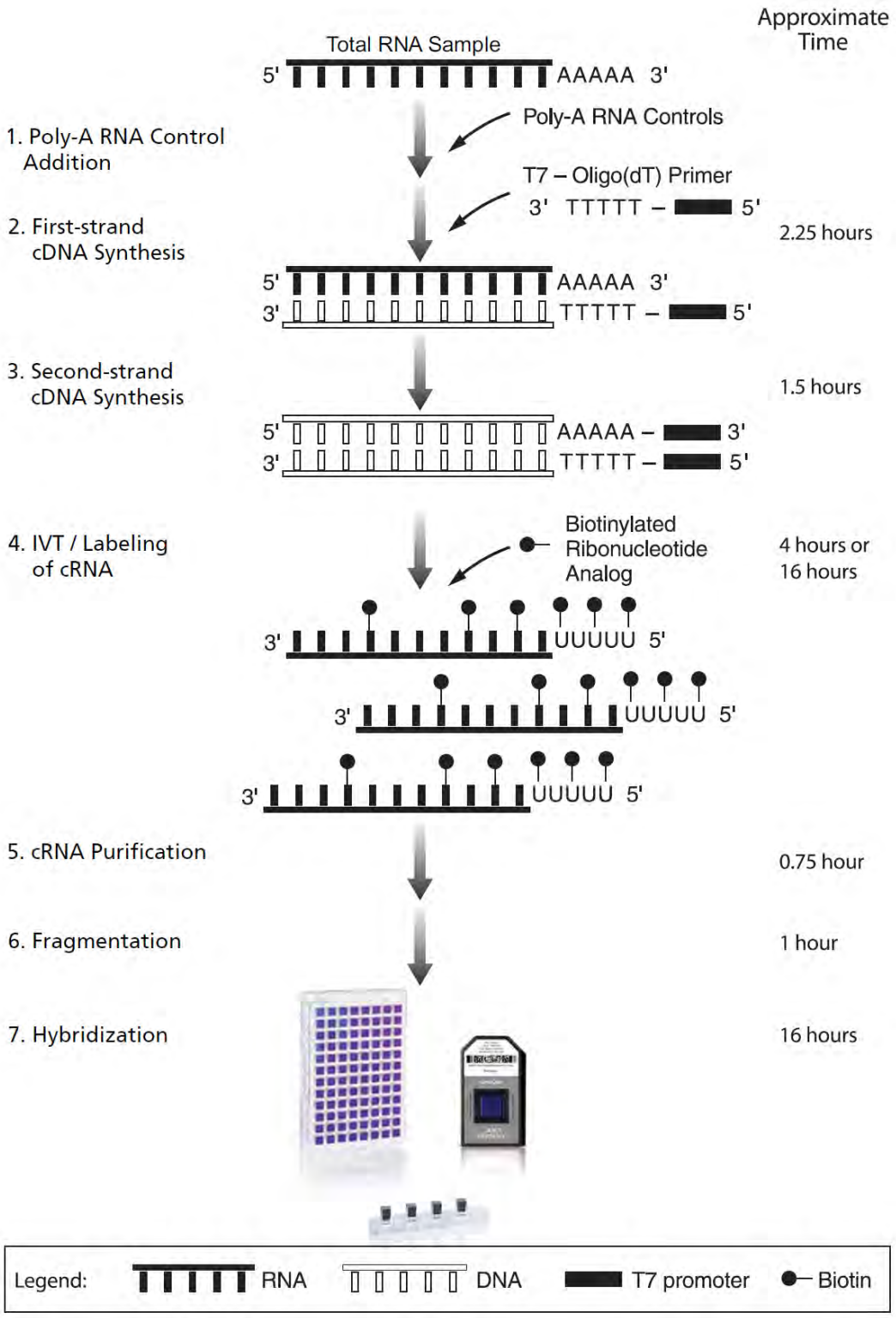

Figure 1 The 3′ IVT PLUS amplification and labeling process.

Kit contents and storage

Table 1 GeneChip™ 3' IVT PLUS Reagent Kit contents and storage.

Component	Amount, 10-reaction kit (902415)	Amount, 30-reaction kit (902416)	Storage
3′ IVT PLUS Amplification Kit Module 1			
3′ First-Strand Enzyme	11 µL	50 µL	–20°C
3′ First-Strand Buffer	500 µL	500 µL	
3′ Second-Strand Enzyme	22 µL	70 µL	
3′ Second-Strand Buffer	55 µL	180 µL	
3′ IVT Enzyme	66 µL	210 µL	
3′ IVT Buffer	500 µL	1,580 µL	
3′ IVT Biotin Label	44 µL	140 µL	
Control RNA (1 mg/mL HeLa total RNA)	5 µL	5 µL	
Nuclease-free Water	1 x 1.0 mL	2 x 1.0 mL	From –20°C to room temperature
3′ IVT PLUS Amplification Kit Module 2			
3′ Fragmentation Buffer	1 mL	1 mL	Room temperature
Purification Beads	1.1 mL	3.3 mL	4°C (Do not freeze.)
GeneChip™ Eukaryotic Poly-A RNA Control Kit (900433)			
Poly-A Control Stock	16 µL	16 µL	–20°C
Poly-A Control Dil Buffer	3.8 mL	3.8 mL	
GeneChip™ Hybridization Control Kit (900454)			
20X Hybridization Controls	450 µL	450 µL	–20°C
3 nM Control Oligo™ B2	150 µL	150 µL	
Tubes Organizer: Plastic vinyl template for organization and storage of components in 9 x 9 array, 81-places square wells, 5.25 x 5.25 x 2 in. (133 x 133 x 52 mm) (for example, Nalgene™ Polycarbonate 9 x 9 CryoBox™ 5026-0909 , or equivalent).			

Required materials

Instruments

Unless otherwise indicated, all materials are available through **thermofisher.com**. "MLS" indicates that the material is available from **fisherscientific.com** or another major laboratory supplier.

Table 2 Instruments required for target preparation

Item	Source
• Agencourt™ SPRI™ Plate Super Magnet Plate or • Magnetic Stand-96 or • Magnetic-Ring Stand (96 well) • or equivalent	• A32782 or • AM10027 or • AM10050 • or equivalent
Microcentrifuge	**MLS**
NanoDrop™ UV-Vix Spectrophotometer	Or equivalent quantitation instrument
(*Optional*) Agilent™ 2100 Bioanalyzer™ Instrument or equivalent DNA and RNA sizing instrument	**MLS**
Pipettes	**MLS**
Thermal cycler	**MLS**
Vortex mixer	**MLS**
65°C heat block or oven for incubation of Nuclease-free Water during purification	**MLS**

Table 3 Instruments required for array processing

Instruments	Source
GeneChip™ System 3000Dx v.2 for cartridge arrays (Cat. No. 00-0334)	
GeneChip™ Hybridization Oven 645	00-0331 (110/220V)
GeneChip™ Fluidics Station 450	00-0079
GeneChip™ Scanner 3000 7G System	00-0213
GeneChip™ AutoLoader with External Barcode Reader	00-0090 (GCS 3000 7G S/N 501) 00-0129 (GCS 3000 7G S/N 502)
GeneAtlas™ System for array strips (Cat. Nos. 00-0394, 00-0375)	
GeneAtlas™ Workstation	90-0894

Table 3 Instruments required for array processing *(continued)*

Instruments	Source
GeneAtlas™ Hybridization Station	00-0380 (115VAC) 00-0381 (230VAC)
GeneAtlas™ Fluidics Station	00-0377
GeneAtlas™ Imaging Station	00-0376
GeneAtlas™ Barcode Scanner	74-0015
GeneTitan™ system for array plates	
GeneTitan™ Multi-Channel (MC) Instrument, NA/Japan includes 110v UPS	00-0372
GeneTitan™ Multi-Channel (MC) Instrument, Int'l includes 220v UPS	00-0373
GeneTitan™ Multi-Channel (MC) Instrument, NA/Japan includes 110v UPS	00-0360
GeneTitan™ Multi-Channel (MC) Instrument, Int'l Includes 220v UPS	00-0363

Reagents and supplies

Unless otherwise indicated, all materials are available through **thermofisher.com**. "MLS" indicates that the material is available from **fisherscientific.com** or another major laboratory supplier.

Table 4 Additional reagents and supplies required

Item	Source
Corning™ Clear Polystyrene 96-Well Microplate	Fisher Scientific™, 07-200-103
GeneChip™ Hybridization, Wash, and Stain Kit (30 reactions)	900720
GeneAtlas™ Hybridization, Wash, and Stain Kit for 3' IVT Arrays (60 reactions)	901531
GeneTitan™ Hybridization, Wash, and Stain Kit for 3' IVT Arrays (96 reactions)	901530
100% Ethanol (molecular biology grade or equivalent)	**MLS**
Nuclease-free Water for preparing 80% ethanol wash solution	**MLS**
Nuclease-free aerosol-barrier tips	**MLS**
Nuclease-free 1.5, and 0.2 mL tubes or plates	**MLS**
Nuclease-free 15 mL tubes or containers	**MLS**
Amber 1.5 mL tubes (for processing cartridge arrays only)	**MLS**
(*Optional*) 96-well plate sealing film	**MLS**

Table 4 Additional reagents and supplies required *(continued)*

Item	Source
(*Optional*) Reagent reservoir for multichannel pipette	**MLS**
(*Optional*) Agilent™ RNA 6000 Nano Kit or equivalent DNA and RNA sizing reagents	50671511
Tough-Spots™ labels	**MLS**

2 Protocol

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Procedural notes

Implement a plan to maintain procedural consistency

Subtle procedural differences in gene-expression assays can cause sample-to-sample variation. To minimize this variation, implement a detailed procedural plan that standardizes the variables in the procedure. The plan should address the following topics.

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

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. These protocols are described in Table 5. However, 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. Leave 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, incubate 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.

Ensure that the heated lid of your thermal cycler tracks the temperature of the thermal cycling block or supports specific temperature programming.

IMPORTANT! Concentration fluctuations caused by condensation can affect yield. Ensure that the heated-lid feature of the thermal cycler is working properly.

Table 5 Thermal cycler protocols.

Protocol	Heated lid temperature	Alternate protocol[1]	Step 1	Step 2	Step 3	Volume
First-Strand cDNA Synthesis	42°C	105°C	42°C for 2 hours	4°C for 2 minutes		10 µL
Second-Strand cDNA Synthesis	Room temperature or disable	Lid open	16°C for 1 hour	65°C for 10 minutes	4°C for 2 minutes	30 µL
In Vitro Transcription cRNA Synthesis	40°C	40°C oven	40°C for 4 hours or 16 hours[2]	4°C, hold		60 µL
Fragmentation	94°C	105°C	94°C for 35 minutes	4°C, hold		Varies
Hybridization Control	65°C	105°C	65°C for 5 minutes			Varies
Hybridization Cocktail	99°C	105°C	95°C or 99°C for 5 minutes	45°C for 5 minutes		Varies

[1] *For thermal cyclers that lack a programmable heated lid.*

[2] *Four hours for 250—500 ng RNA input, or 16 hours for 50—250 ng RNA input.*

How to handle kit components

IMPORTANT! Reagents in the GeneChip™ 3' IVT PLUS Reagent Kit can be thawed and frozen a maximum of 3 times.

- Enzymes: Mix by gently vortexing the tube, centrifuge briefly to collect contents at the bottom of the tube, then keep on ice.
- Buffers: Thaw on ice, thoroughly vortex to dissolve precipitates, then centrifuge briefly to collect contents at the bottom of the tube. If necessary, warm buffers at ≤37°C for 1–2 minutes 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 account for pipetting loss.
  - Use nonstick nuclease-free tubes to prepare the master mix.
  - Add enzymes last and just before adding the master mix to the reaction.
- Return all 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 (1 mg/mL HeLa total RNA) sample is included with the kit. Use this procedure to prepare the Control RNA for a 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 procedure in "Prepare total RNA/poly-A RNA control mixture" on page 17, but use 2 µL of the diluted Control RNA (50 ng) in the control reaction.

Note:

- Measure the concentration of HeLa Control RNA with a NanoDrop™ UV-Vix Spectrophotometer. Use the measured concentration for calculation and for preparing the 25 ng/µL working stock.
- The positive control reaction should produce >15 µg of cRNA from 50 ng of Control RNA, using a 16-hour incubation for the IVT reaction.

Prepare poly-A RNA controls

Note:

- To include premixed controls from the GeneChip™ Eukaryotic Poly-A RNA Control Kit, add the reagents to the total RNA samples. Follow the procedure described in "Prepare total RNA/poly-A RNA control mixture" on page 17. We strongly recommend the use of poly-A RNA controls for all reactions that will be hybridized to GeneChip™ arrays.
- If the Poly-A Control Dil Buffer is frozen, allow 15–20 minutes to thaw at room temperature.

A supplied set of poly-A RNA controls provides exogenous positive controls to monitor the entire target preparation. The control 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 6.

Table 6 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. The hybridization intensities of these controls on GeneChip™ arrays help the operator monitor the labeling process independently from the quality of the starting RNA samples.

The Poly-A Control Stock and Poly-A Control Dil Buffer are provided in the GeneChip™ Eukaryotic Poly-A RNA Control Kit to prepare the appropriate serial dilutions based on Table 7. 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, calculate the appropriate dilutions to arrive at the same proportionate final concentration of the spike-in controls in the samples.

Table 7 Serial dilutions of poly-A RNA control stock.

Total RNA input amount	Serial dilutions				Volume of fourth dilution to add to total RNA
	First dilution	Second dilution	Third dilution	Fourth 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 less than 2 µL of any solution to maintain precision and consistency when preparing the dilutions.
- Use nonstick nuclease-free tubes to prepare all of the dilutions. Nonstick tubes are not included in the kit.
- After each step, mix the poly-A control dilutions thoroughly. Vortex them gently, then centrifuge them quickly to collect contents at the bottoms of the tubes.

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 the Poly-A Control Dil Buffer to prepare the first dilution (1:20).

   Tip: The first dilution of the poly-A RNA controls can be stored for up to 6 weeks in a non-frost-free freezer at –20°C. It can be frozen and thawed up to 8 times. Label the storage tube with its expiration date.

2. Add 2 µL of the first dilution to 98 µL of Poly-A Control Dil Buffer to prepare the second dilution (1:50).

3. Add 2 µL of the second dilution to 98 µL of Poly-A Control Dil Buffer to prepare the third dilution (1:50).

4. Add 2 µL of the third dilution to 18 µL of Poly-A Control Dil Buffer to prepare the fourth dilution (1:10).

5. Add 2 µL of this fourth dilution to 100 ng of total RNA. The final volume of total RNA with the diluted poly-A controls should not exceed 5 µL.

Prepare total RNA

Evaluate RNA quality

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

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.

Methods to evaluate RNA integrity include the following:

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

With microfluidic analysis, use the RNA Integrity Number (RIN) to evaluate RNA integrity. For more information on how to calculate RIN, see **www.genomics.agilent.com**.

Denaturing agarose gel electrophoresis and nucleic acid staining separate and make visible the 28S and 18S rRNA bands. The mRNA is likely to be full length if the 2 bands have these characteristics:

- The 28S and 18S rRNA bands resolve into 2 discrete bands with 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 the type and the amount of sample RNA 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 8 are based on total RNA from HeLa cells. Use these values as reference points for determining the optimal RNA input.

Note: Avoid pipetting less than 2 µL of any solution to maintain precision and consistency. Predilute high-concentration RNA samples with Nuclease-free Water before adding RNA to the first-strand cDNA synthesis reaction.

Table 8 Input RNA limits.

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

Table 9 Recommended IVT incubation time.

Recommendation	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 the first-strand synthesis reaction. If you are adding Poly-A Control Stock to your RNA, the volume of the RNA must be 3 µL or less (Table 10). See "Prepare poly-A RNA controls" on page 14 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 Control Stock, and 1 µL of Nuclease-free Water.

Note: When adding Poly-A Control Stock to RNA, the volume of RNA must be 3 µL or less. If necessary, use a SpeedVac™ Vacuum Concentrator or ethanol precipitation to concentrate the RNA samples.

Table 10 Total RNA/poly-A RNA control mixture.

Component	Volume for 1 reaction
Total RNA sample (50—500 ng)	Variable
Diluted Poly-A Control Stock (fourth 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 less than 2 µL of any solution to maintain precision and consistency. Predilute high-concentration RNA samples with Nuclease-free Water before adding the RNA to the first-strand cDNA synthesis reaction.

1. Prepare the 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 following table. Prepare the master mix for all the total RNA samples in the experiment. Include ~5% overage for pipetting losses.

   Table 11 First-Strand Master Mix.

Component	Volume for 1 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 the total RNA to each First-Strand Master Mix aliquot.
   a. On ice, add 5 µL of the total RNA from Table 10 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 17.

   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 hours at 42°C, then for at least 2 minutes at 4°C.
   a. Incubate the first-strand synthesis reaction in a thermal cycler using the First-Strand cDNA Synthesis protocol that is shown in Table 5.

   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 minutes to cool the plastic, then proceed immediately to "Synthesize second-strand cDNA" on page 19.

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 minutes may significantly reduce cRNA yields.

Tip: When the thermal cycler has approximately 15 minutes left to run, start reagent preparation for the 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! Precool the thermal cycler block to 16°C.

1. Prepare the 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 following table. Prepare the master mix for all the first-strand cDNA samples in the experiment. Include ~5% overage for pipetting losses.

Table 12 Second-Strand Master Mix.

Component	Volume for 1 reaction
Nuclease-free Water	13 µL
3′ Second-Strand Buffer	5 µL
3′ Second-Strand Enzyme	2 µL
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 µL of the Second-Strand Master Mix to each (10 µL) first-strand cDNA sample, for a final reaction volume of 30 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 1 hour at 16°C, then for 10 minutes at 65°C, then for at least 2 minutes at 4°C.
   a. Incubate the second-strand synthesis reaction in a thermal cycler using the Second-Strand cDNA Synthesis protocol that is shown in Table 5.

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

b. Immediately after the incubation, centrifuge briefly to collect the second-strand cDNA at the bottom of the tube or wells.

c. Place the sample on ice, then proceed immediately to "Synthesize labeled cRNA by in vitro transcription" on page 20.

Tip: When the thermal cycler has approximately 15 minutes left to run, start preparing reagents 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.

IMPORTANT!

- Transfer the second-strand cDNA samples to room temperature for ≥5 minutes while preparing the IVT Master Mix.
- After the 3′ IVT Buffer thaws completely, leave it at room temperature for ≥10 minutes before preparing the IVT Master Mix.

1. Prepare the IVT Master Mix.

Note: Perform this step at room temperature.

a. In a nuclease-free tube, combine the components in the sequence shown in the following table. Prepare the master mix for all the second-strand cDNA samples in the experiment. Include ~5% overage for pipetting losses.

Table 13 IVT Master Mix.

Component	Volume for 1 reaction
3′ IVT Biotin Label	4 µL
3′ IVT Buffer	20 µL
3′ IVT Enzyme	6 µL
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 µL of the IVT Master Mix to each tube or well containing 30 µL of second-strand 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.

2. Incubate for 4—16 hours at 40°C, then at 4°C.

   When incubating at 40°C, incubate 4 hours for 250—500 ng of RNA input, or 16 hours for 50—250 ng of RNA input.

   a. Incubate the IVT reaction in a thermal cycler using the *In Vitro* Transcription cRNA Synthesis protocol that is shown in Table 5.

   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 21, or immediately freeze the samples at –20°C for storage.

   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”.

IMPORTANT!

- Preheat the Nuclease-free Water in a heat block or thermal cycler to 65°C for at least 10 minutes.
- 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 µL plus ~10% overage will be needed.
- Prepare fresh dilutions of 80% ethanol wash solution each time from molecular-biology grade 100% ethanol 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 beads/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 centrifuge out pellets.
- Perform this entire procedure at room temperature.

1. Bind cRNA to Purification Beads.

   a. Mix the Purification Beads container by vortexing to resuspend the magnetic particles.

   b. Add 100 µL of the Purification Beads to each (60 µL) cRNA sample, mix by pipetting up and down, then transfer to a well of a U-bottom plate.

   Tip:

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

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

d. Incubate for 10 minutes. 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 minutes), 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 used 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 seconds.

b. Slowly aspirate and discard the 80% ethanol wash solution without disturbing the Purification Beads.

c. Repeat substep 2a and substep 2b 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 minutes until no liquid is visible, yet the pellet appears shiny. Additional time may be required. Do not over-dry the beads, as this will reduce the elution efficiency. If it is too dry, the bead surface will appear dull and may have surface cracks.

3. Elute the cRNA.

a. Remove the plate from the magnetic stand. Add 27 µL of the 65°C Nuclease-free Water to each sample and incubate for 1 minute.

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

c. Move the plate to the magnetic stand for ~5 minutes 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" on page 23, 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 a sample with high-concentration cRNA, add an additional 10–30 µL of the 65°C Nuclease-free Water to the well and incubate for 1 minute before proceeding to substep 3b.

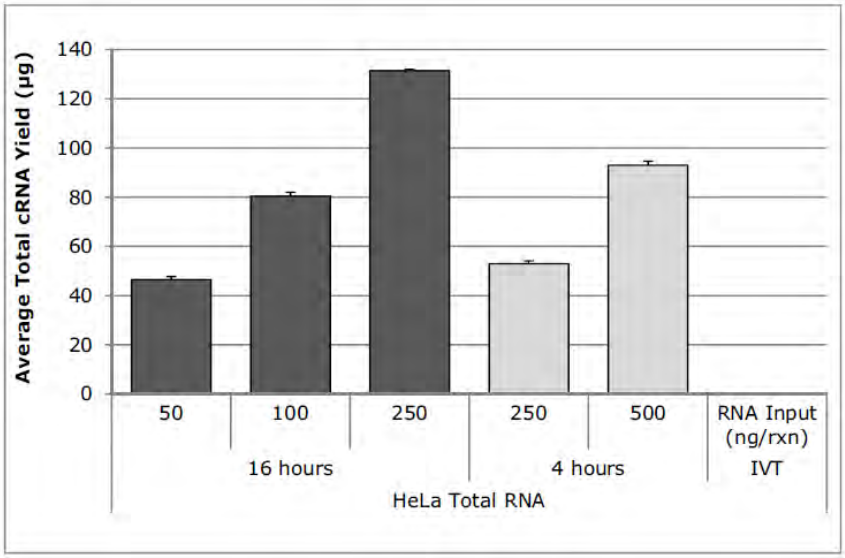
STOPPING POINT The purified cRNA samples can be stored overnight at –20°C. For long-term storage, store samples at –80°C. Keep the number of freeze-thaw cycles to 3 or less to ensure cRNA integrity.

Assess cRNA yield and size distribution

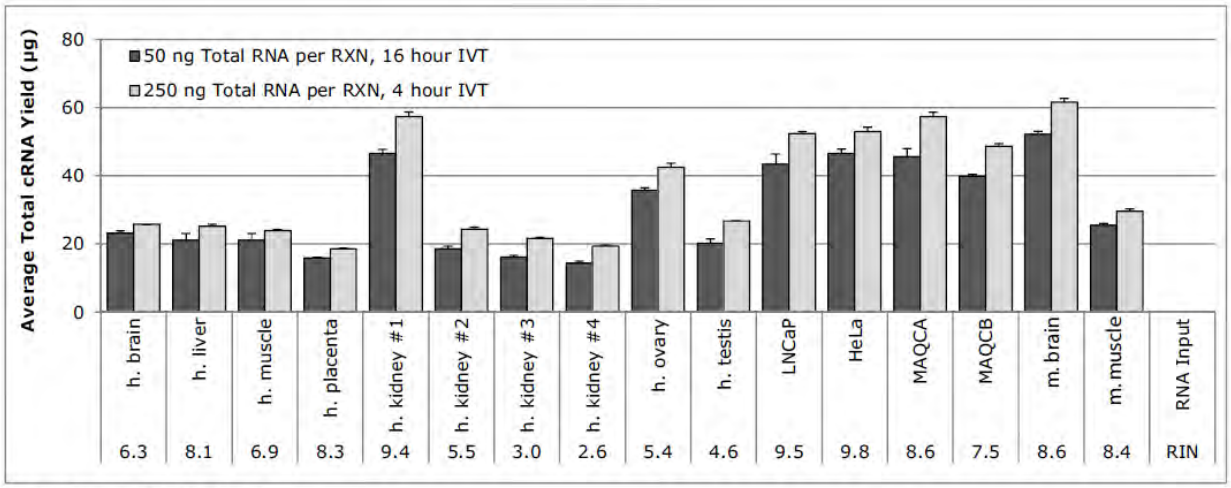
Expected cRNA yield

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 can vary considerably.

During development of the GeneChip™ 3' IVT PLUS Reagent Kit, using a wide variety of tissue types, 50 ng of input total RNA yielded 15—40 µg of cRNA. For most tissue types, the recommended 100 ng of input total RNA should provide >20 µg of cRNA. Figure 2 shows yield data for cRNA produced with the kit from several different types of input RNA.



Example A



h. is human and m. is mouse

Example B

Figure 2 Average cRNA yield from HeLa (Example A) and a variety of total RNA samples (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. We recommend using NanoDrop™ spectrophotometers for convenience. No dilutions or cuvettes are needed; use 1.5 µL of the cRNA sample directly.

Note: High-concentration labeled cRNA samples (>3000 ng/µL) 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.

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 following equation:

A_{260} × dilution factor × 40 = µg RNA/mL

where 1 A_{260} = 40 µg RNA/mL.

The amounts of labeled cRNA required for fragmentation are listed in the following table.

Table 14 Labeled cRNA amounts required for fragmentation by array format.

Component	49- or 64-format		100- or 81/4-format	169- or 400-format, or array plate	Array strip
Labeled cRNA[1]	15 µg (in 1—32 µL)	12 µg[2] (in 1—25.6 µL)	12 µg (in 1—25.6 µL)	7.5 µg (in 1—16 µL)	9.4 µg (in 1—20 µL)

[1] *Minimum concentration of the eluate is 468.75 ng/µL.*
[2] *Alternative protocol for samples with cRNA yields below 15 µg.*

Note: See Table 15 and Table 18 for the amount of labeled cRNA required for 1 fragmentation reaction and 1 array hybridization experiment, respectively. The amount varies depending on the array format. See the package insert for information on the array format.

(Optional) Expected cRNA size distribution

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

(*Optional*) Expected cRNA size distribution using a Bioanalyzer™ Instrument

We recommend analyzing cRNA size distribution using the Agilent™ 2100 Bioanalyzer™ Instrument, an Agilent™ RNA 6000 Nano Kit (Cat. No. 50671511), and mRNA Nano Series II assay. To analyze cRNA size using a Bioanalyzer™ Instrument, follow the manufacturer's instructions.

1. If there is sufficient yield, load approximately 300 ng of cRNA per well on the Bioanalyzer™ Instrument.
2. If there is insufficient yield, use as little as 200 ng of cRNA per well.

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.

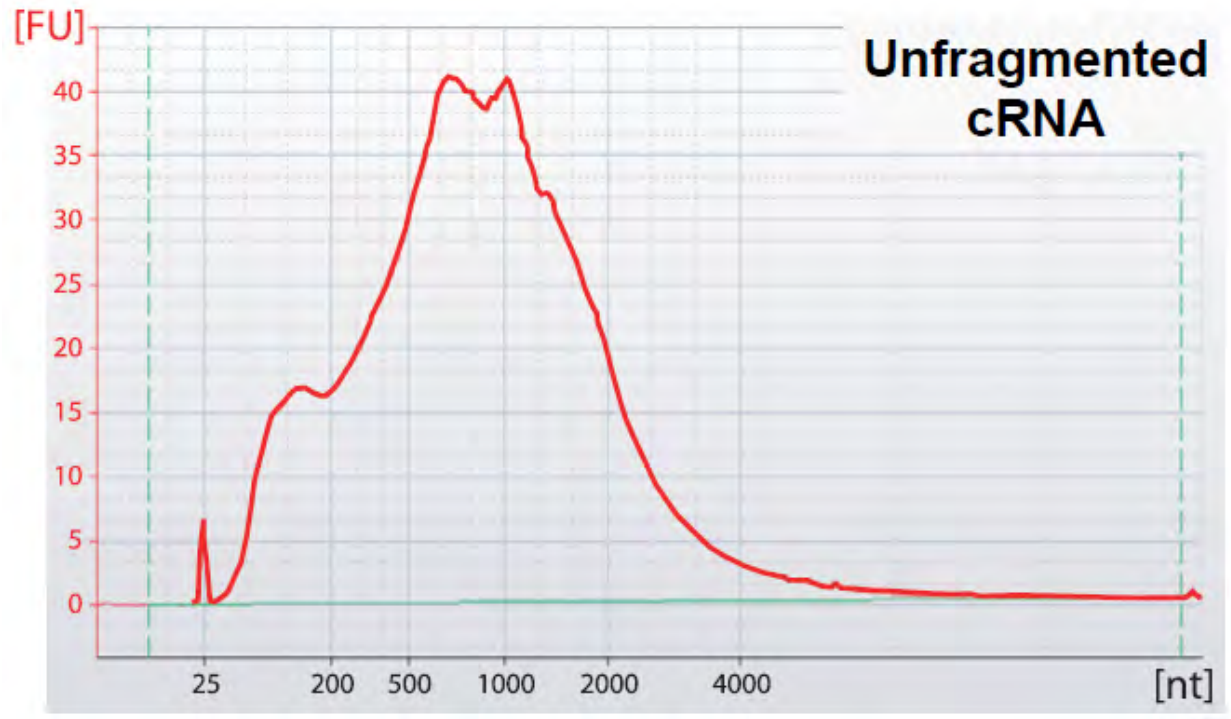


Figure 3 Example Agilent™ 2100 Bioanalyzer™ Instrument electropherogram of unfragmented cRNA.

Fragment labeled cRNA

In this procedure, the purified cRNA is fragmented by divalent cations and elevated temperature. The amount of labeled cRNA required for fragmentation depends on the array formats. See Table 14.

1. Prepare the required amount of labeled cRNA.

   On ice, prepare 468.75 ng/µL of labeled cRNA. If necessary, add Nuclease-free Water to bring the labeled cRNA sample to the required volume.

2. Prepare the fragmentation reaction.

   a. On ice, prepare the fragmentation reaction in a nuclease-free tube. Combine the components in the sequence shown in the following table.

   Table 15 One labeled cRNA fragmentation reaction by array format.

Component	49- or 64-format		100- or 81/4-format	169- or 400-format, or array plate	Array strip
Labeled cRNA	15 µg (in 32 µL)	12 µg[1] (in 25.6 µL)	12 µg (in 25.6 µL)	7.5 µg (in 16 µL)	9.4 µg (in 20 µL)
3′ Fragmentation Buffer	8 µL	6.4 µL	6.4 µL	4 µL	5 µL
Total volume	**40 µL**	**32 µL**	**32 µL**	**20 µL**	**25 µL**

[1] *Alternative protocol for samples with cRNA yields below 15 µg.*

   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 minutes at 94°C, then for at least 2 minutes at 4°C.

   a. Incubate the fragmentation reaction in a thermal cycler using the Fragmentation protocol that is shown in Table 5.

   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.

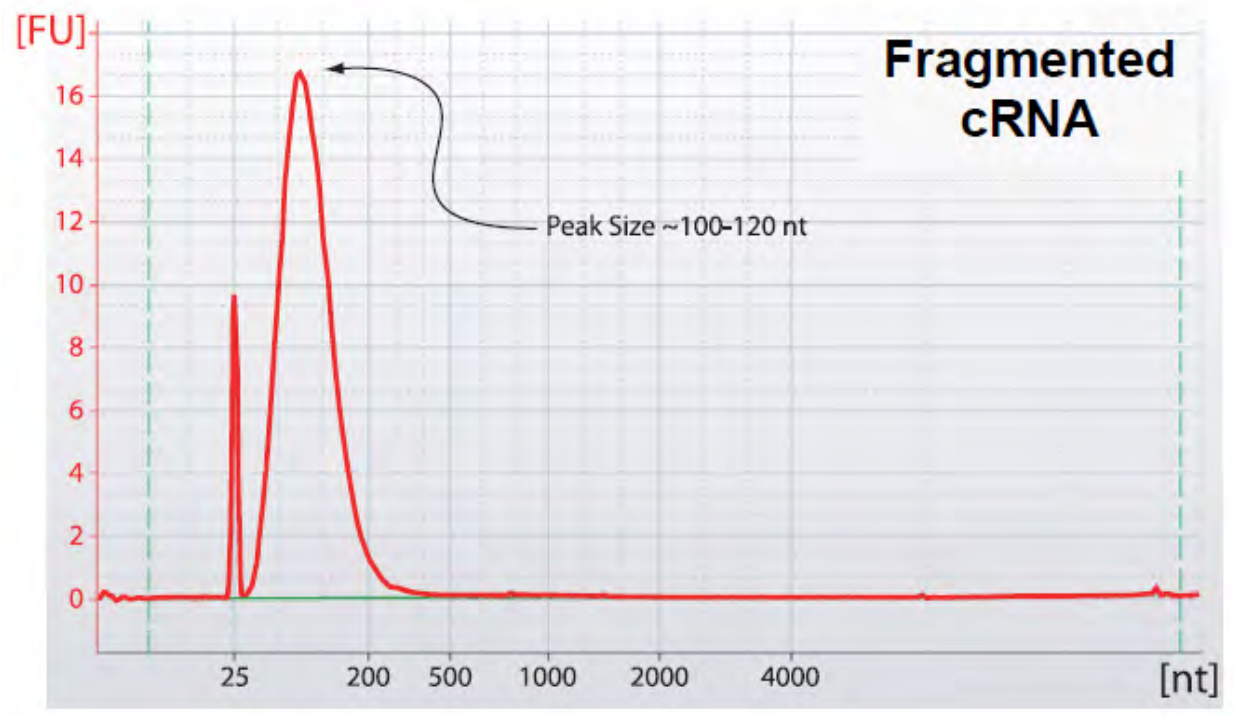


Figure 4 Example Agilent™ 2100 Bioanalyzer™ Instrument electropherogram of fragmented cRNA.

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 3′ array hybridization

- Cartridge array hybridization on the GeneChip™ Instrument 29
- Array strip hybridization on the GeneAtlas™ Instrument 34
- Array plate hybridization on the GeneTitan™ MC Instrument 46

Cartridge array hybridization on the GeneChip™ Instrument

This section provides instruction on setting up hybridizations for cartridge arrays.

For related information, see:

- *GeneChip™ Fluidics Station 450 User Guide*.
- *GeneChip™ Expression Wash, Stain, and Scan for Expression Cartridge Arrays User Guide*.
- *GeneChip™ Command Console™ (GCC) User Guide*.

Prepare ovens, arrays, and sample registration files

1. Turn on the hybridization oven and set the temperature to 45°C.
2. Set the RPM control to 60.
3. Turn the rotation on and allow the oven to preheat.
4. Equilibrate the arrays to room temperature immediately before use.
5. Label each array with the name of the sample that will be hybridized.
6. Register the sample and array information in the GeneChip™ Command Console™ software.

Target hybridization setup for cartridge arrays

Cartridge arrays: reagents and materials required

- GeneChip™ Hybridization, Wash, and Stain Kit (Not supplied. For ordering information, see Table 1).
  - 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 Hybridization Controls (*bioB*, *bioC*, *bioD*, *cre*)
  - 3 nM Control Oligo™ B2
- 3' cartridge arrays (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 Mix to room temperature.

   Note: Aliquot ~220 µL of Pre-Hybridization Mix per array to be hybridized into a 1.5 mL tube to accelerate the equilibration to room temperature.

2. Prepare the Hybridization Master Mix.
   a. At room temperature, thaw the components listed in Table 16.

   DMSO solidifies at 2—8°C. Ensure that the reagent is completely thawed before use. Store DMSO at room temperature after the first use.

   b. Heat the 20X Hybridization Controls for 5 minutes at 65°C in a thermal cycler using the Hybridization Control protocol that is shown in Table 5.

   c. At room temperature, prepare the Hybridization Master Mix in a nuclease-free tube. Combine the components in the amounts and the sequence shown in the following table. 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 16 Hybridization Master Mix for 1 cartridge array reaction.

Component	49- or 64-format	100- or 81/4-format	169- or 400-format	Final concentration
3 nM Control Oligo™ B2	3.7 µL	3.3 µL	1.7 µL	50 pM
20X Hybridization Controls (*bioB*, *bioC*, *bioD*, *cre*)	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 master mix at the bottom of the tube. Proceed to the next step.

3. Prehybridize the 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 17) by filling it through the bottom left septa.

Table 17 Array cartridge volumes for the 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	90 µL

c. Remove the pipette tip from the upper right septum.

d. Incubate the array filled with Pre-Hybridization Mix rotating at 60 rpm for 10–30 minutes at 45°C.

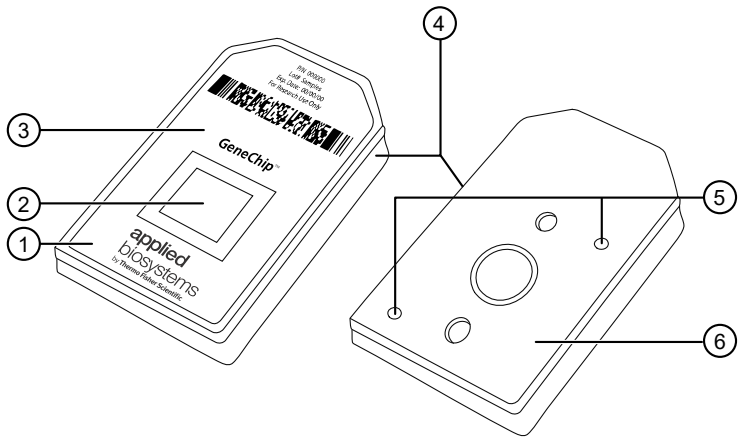


Figure 5 GeneChip™ cartridge array.

1. Front of the cartridge array
2. Probes on a glass substrate
3. Plastic cartridge
4. Notch
5. Septa
6. Back of the cartridge array

Note: It is necessary to use 2 pipette tips when filling the probe array cartridge: one for filling and one to allow venting of air from the hybridization chamber.

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

Table 18 Hybridization cocktail for 1 cartridge array.

Component	49- or 64-format	100- or 81/4-format	169- or 400-format	Final concentration
Hybridization Master Mix	190.6 µL	173.3 µL	86.7 µL	
Fragmented and labeled cRNA	29.4 µL (11 µg)	26.7 µL (10 µg)	13.3 µL (5 µg)	50 ng/µL
Total volume	**220 µL**	**200 µL**	**100 µL**	

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

   c. Incubate the hybridization cocktail reaction for 5 minutes at 99°C (tubes) or 95°C (plates), then for 5 minutes at 45°C in a thermal cycler using the Hybridization Cocktail protocol that is shown in Table 5.

   d. After the incubation, centrifuge briefly to collect the contents at the bottom of the tube, then proceed to the next step.

5. Hybridize the array.
   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 19) of the Hybridization Cocktail. Avoid any insoluble matter at the bottom of the tube.

Table 19 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 µL	130 µL	90 µL

   c. Remove the pipette tip from the upper right septum of the array. Cover both septa with 1/2-inch Tough-Spots™ label to minimize evaporation and prevent leaks.

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

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

   e. Incubate with rotation at 60 rpm for 16 hours at 45°C.

   During the latter part of the 16 hour hybridization, prepare reagents for the washing and staining steps required immediately after completion of hybridization.

Wash and stain the cartridge arrays

For additional information about washing, staining, and scanning, see:

- *GeneChip™ Fluidics Station 450 User Guide*
- *GeneChip™ Expression Wash, Stain, and Scan for Expression Cartridge Arrays User Guide*
- *GeneChip™ Command Console™ User Guide*

1. Remove the arrays from the oven. Remove the Tough-Spots™ label from the arrays.
2. Extract the Hybridization Cocktail mix from each array.
   a. (*Optional*) To save the Hybridization Cocktail mix, transfer it to a new tube or well of a 96-well plate. 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.
   Arrays can be held in the Wash Buffer A at 4°C for up to 3 hours before proceeding with washing and staining.
5. Place vials into sample holders on the fluidics station.
   a. Place 1 (amber) vial containing 600 µL of Stain Cocktail 1 in sample holder 1.
   b. Place 1 (clear) vial containing 600 µL of Stain Cocktail 2 in sample holder 2.
   c. Place 1 (clear) vial containing 800 µL of 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 use the following protocol.

   Table 20 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-inch Tough-Spots™ label.
8. Inspect the array glass surface for dust or other particulates and, if necessary, carefully wipe the surface with a clean laboratory wipe before scanning.

Scan the cartridge arrays

See instructions for using the scanner and scanning arrays in the *GeneChip™ Command Console™ User Guide*.

Array strip hybridization on the GeneAtlas™ Instrument

This section outlines the basic steps involved in hybridizing array strips on the GeneAtlas™ System. The major steps involved in array strip hybridization are:

- "Target hybridization setup for array strips" on page 34.
- "GeneAtlas™ software setup" on page 39.

Note: To use a hybridization-ready sample, or to rehybridize a previously made hybridization cocktail, continue the protocol from Step 4 on page 36 in the procedure.

IMPORTANT! Before preparing hybridization-ready samples, register samples as described in "GeneAtlas™ software setup" on page 39.

For more information, see the *GeneAtlas™ System User Guide*.

Target hybridization setup for array strips

Reagents and materials required

- GeneAtlas™ Hybridization, Wash, and Stain Kit for 3' IVT Arrays. (Not supplied, available separately. For ordering information, see Table 4.)
  - 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 Hybridization Controls
  - 3 nM Control Oligo™ B2
- Array strip and consumables (not supplied)
  - 3' array strips
  - 2 hybridization trays per array strip

Procedure

1. In preparation for hybridization, 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 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 Mix to room temperature.

Note: Aliquot ~500 µL of Pre-Hybridization Mix per array strip to be hybridized into a nuclease-free 1.5 mL tube to accelerate the equilibration to room temperature.

2. Prepare the following (In preparation of the Hybridization Master Mix):
   a. Warm the 1.3X Hybridization Mix Solution A and 1.3X Hybridization Mix Solution B vials to room temperature on the bench.

   b. Vortex and centrifuge briefly (~5 seconds) to collect the contents of the tube.

   c. Remove the 20X Hybridization Controls and 3 nM Control Oligo™ B2 tubes from the GeneChip™ Hybridization Control Kit and thaw at room temperature.

   d. Vortex and centrifuge ~5 seconds to collect the contents of the tube.

   e. Keep the tubes of 3 nM Control Oligo™ B2 and 20X Hybridization Controls on ice.

3. Prepare the Hybridization Master Mix.
   a. Heat the 20X Hybridization Controls for 5 minutes at 65°C in a thermal cycler using the Hybridization Control protocol that is shown in Table 5.

   b. At room temperature, prepare the Hybridization Master Mix in a nuclease-free tube. Combine the components in the amounts and the sequence shown in the following table. Prepare the master mix for all the fragmented and biotin-labeled cRNA samples in the experiment.

Table 21 Hybridization Master Mix for strip arrays.

Component	Volume for 1 array	Volume for 4 arrays (includes 10% overage)	Final concentration
3 nM Control Oligo™ B2	2.5 µL	11 µL	50 pM
20X Hybridization Controls (*bioB*, *bioC*, *bioD*, *cre*)	7.5 µL	33 µL	1.5, 5, 25 and 100 pM, respectively
1.3X Hybridization Mix Solution A	40 µL	176 µL	
1.3X Hybridization Mix 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. Prehybridize the array strip.
   a. Gently pipet 120 µL of 1X Pre-Hybridization Mix into the appropriate wells of the hybridization tray. See Figure 6. Avoid generating air bubbles.

CAUTION! The center of the hybridization tray is not a sample well. Do not add anything to this area (Figure 6).

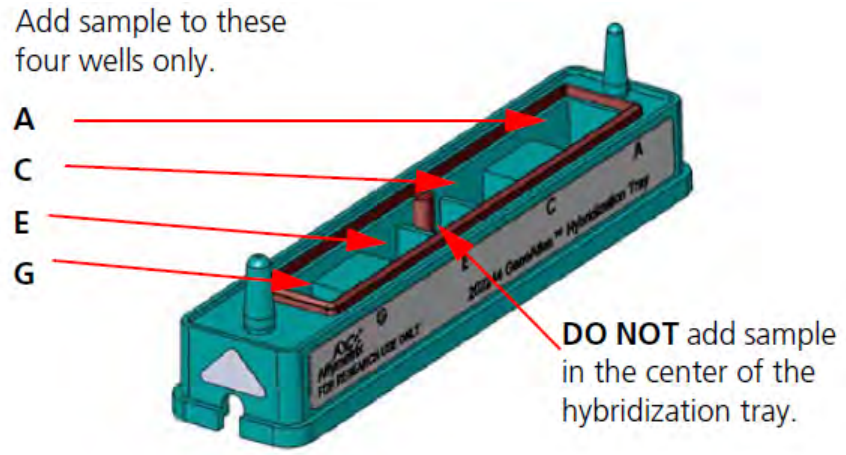


Figure 6 Location of sample wells (A, C, E, and G) on the hybridization tray.

b. Remove the array strip from its foil pouch and carefully place it into the hybridization tray (Figure 7). Ensure that there are no bubbles beneath the array strip.

CAUTION! Be very careful not to scratch or 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.

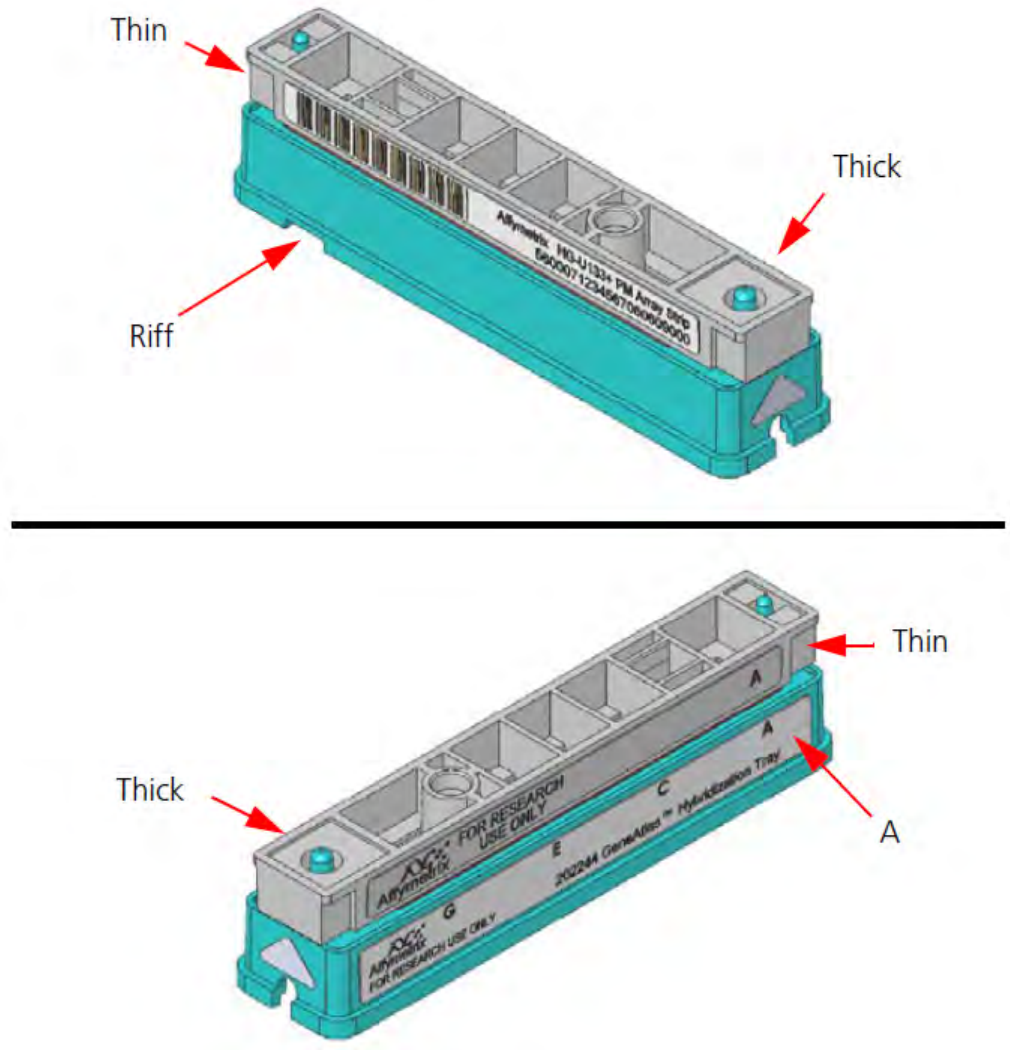


Figure 7 Proper orientation of the array strip in the hybridization tray.

c. Bring the hybridization tray to just above eye level. 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 the array strip back into the hybridization tray and recheck for air bubbles.

e. Place the array strip hybridization tray stack into the GeneAtlas™ Hybridization Station for 10—15 minutes at 45°C.

WARNING! Do not force the GeneAtlas™ Hybridization Station 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. See Figure 8.

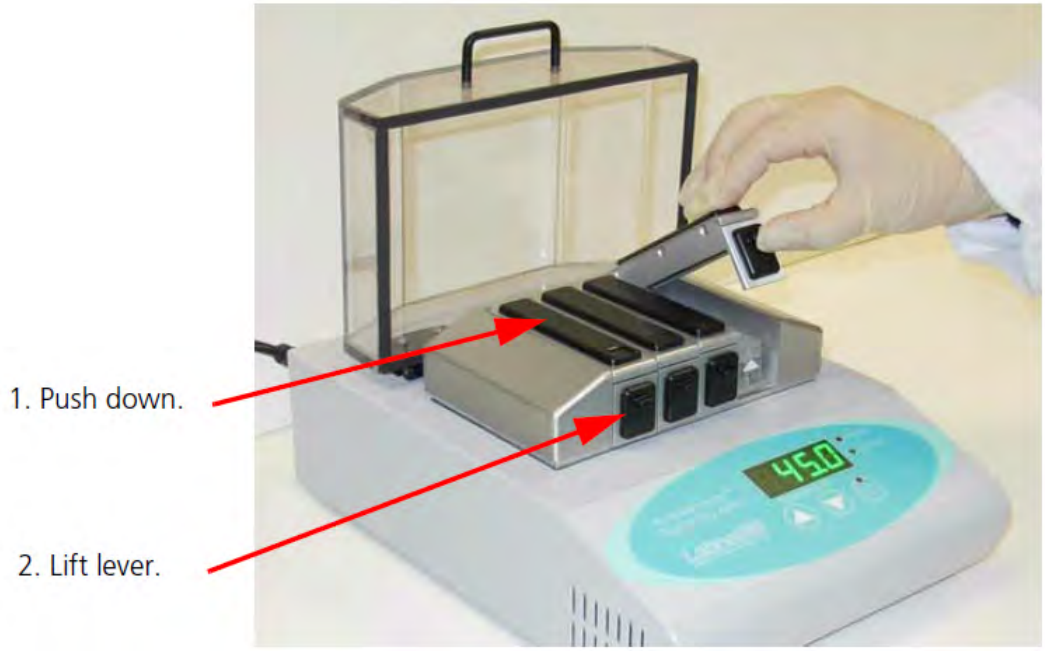


Figure 8 Opening the clamps on the GeneAtlas™ Hybridization Station.

5. Prepare the Hybridization Cocktail.

a. At room temperature, prepare the Hybridization Cocktail in the order as shown in Table 22 for all samples.

Table 22 Hybridization cocktail for a single array strip.

Component	Volume for 1 array	Final concentration
Hybridization Master Mix	130 µL	
Fragmented and labeled cRNA	20 µL (7.5 µg)	50 ng/µL
Total volume	**150 µL**	

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

c. Incubate the hybridization cocktail reaction for 10 minutes at 96°C, then for 2 minutes at 45°C in a thermal cycler.

d. After the incubation, centrifuge briefly to collect the contents at the bottom of the tube or well and proceed to the next step.

(*Optional*) The remainder of the hybridization cocktail can be stored at –20°C to supplement hybridization cocktail volume should a rehybridization be necessary. See “Rehybridizing used cocktails” on page 45.

6. Hybridize the array strip.

a. After 10—15 minutes of prehybridization, remove the array strip from the GeneAtlas™ Hybridization Station and place it on the bench top, keeping the arrays immersed in the prehybridization solution.

b. Apply 120 µL of preheated Hybridization Cocktail to the middle of the appropriate wells of a new clean hybridization tray.

See Figure 6.

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 Mix and place it into the hybridization tray containing the hybridization cocktail samples.

d. Check for and remove any bubbles that were introduced.

See Figure 7 for proper orientation of the array strip in the hybridization tray.

IMPORTANT! **Insert the array strip and remove air bubbles quickly to prevent drying of the array surface.**

e. Place the hybridization tray with the array strip into a clamp inside the GeneAtlas™ Hybridization Station and close the clamp.

See Figure 8.

IMPORTANT! The hybridization temperature for 3′ GeneAtlas™ array strips is 45°C.

7. Proceed to “Hybridization software setup” on page 42.

GeneAtlas™ software setup

Prior to setting up the target hybridization and processing the 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 4 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 more information, see the *GeneAtlas™ System User Guide*.

Sample registration

The following information provides general instructions for registering array strips in the GeneAtlas™ Software. For detailed information on sample registration, importing data from Microsoft™ Excel™ and information on the wash, stain, and scan steps, see the *GeneAtlas™ System User Guide*.

1. Click **Start** ▸ **Programs** ▸ **Affymetrix™** ▸ **GeneAtlas™** to launch the GeneAtlas™ Software.
2. Click the **Registration** tab.

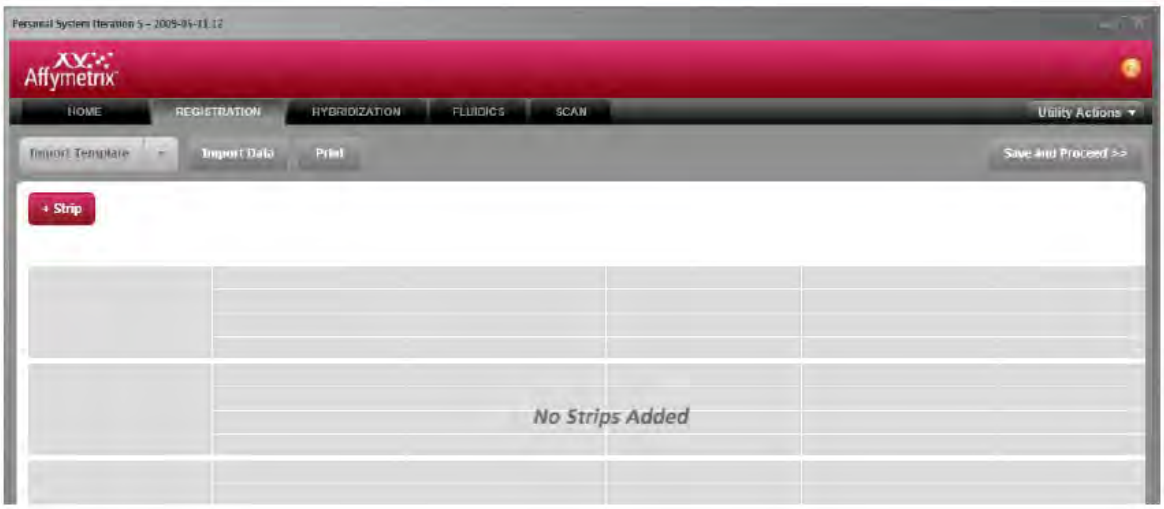
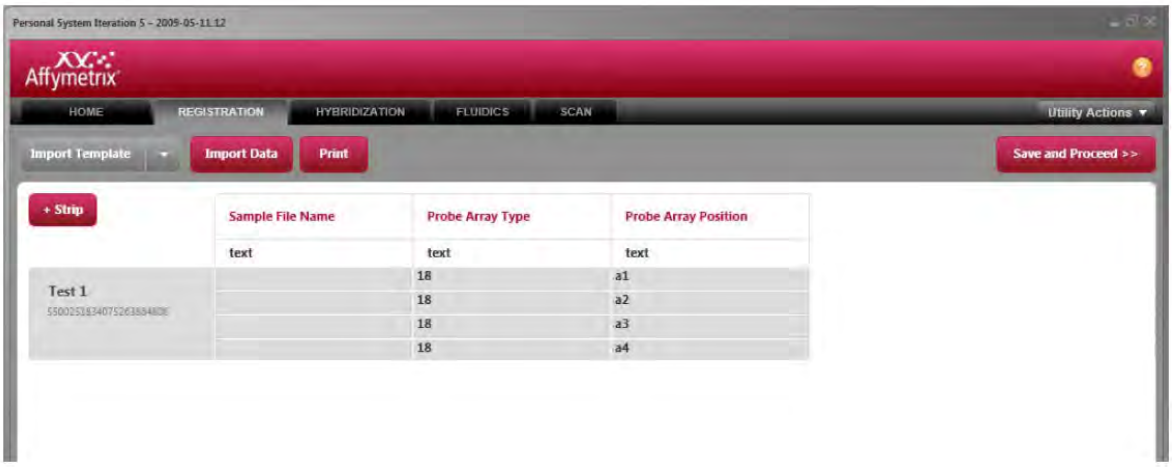


Figure 9 Registration tab of GeneAtlas™ Software.

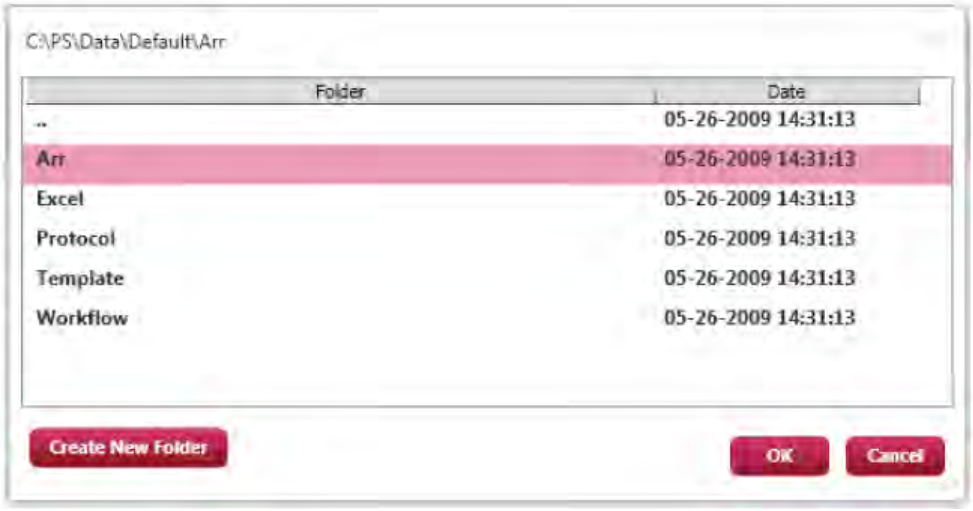
3. Click **+ Strip** (+ Strip).
   The **Add Strip** dialog box appears.

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



5. In the **Sample File Name** column, click in the text box, enter a sample name, then press **Enter.**
   Enter a unique name for each of the 4 samples on the array strip.

6. When complete, click **Save and Proceed**  .
   The **Save** dialog box appears.



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

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

   Note: You may enter a total of 4 array strips during the registration process. To add additional strips, repeat Step 3 on page 40 through Step 8 on page 42.

9. Proceed to "Hybridization software setup" on page 42.

Hybridization software setup

All array strips to be processed must first be registered prior to setting up the hybridizations in the GeneAtlas™ Software. See "Sample registration" on page 40 for instruction on registering array strips.

IMPORTANT! When hybridizing more than 1 array strip per day, it is recommended to keep the hybridization time consistent. Set up hybridizations for 1 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. In the GeneAtlas™ Software, navigate to the **Hybridization** tab.

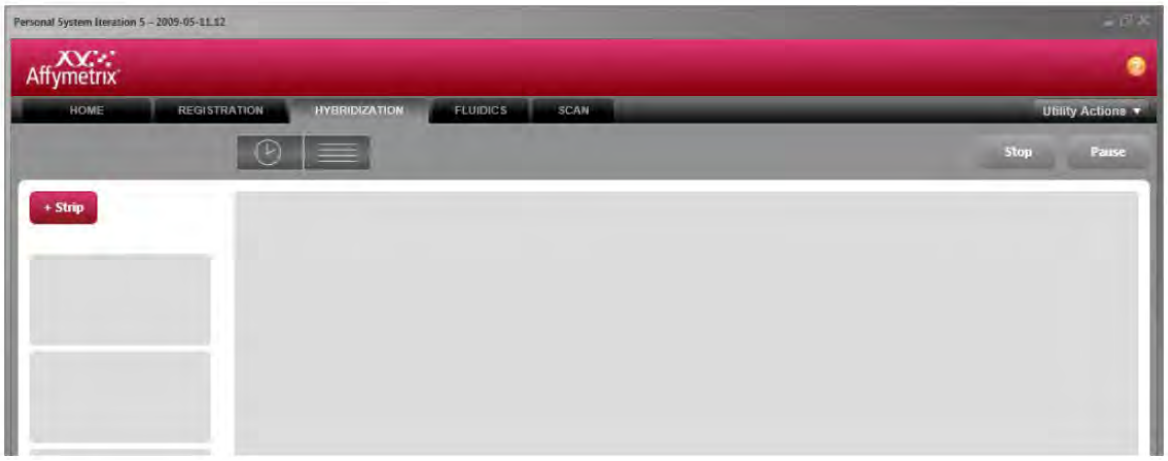


Figure 10 Hybridization tab.

2. Click **+ Strip** (+ Strip).
   The **Add Strip** dialog box appears.

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** list, 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 array strips" on page 34.
7. With the hybridization tray and array strip already in the GeneAtlas™ Hybridization Station, in the **Add Strip** dialog box, click **Start**.

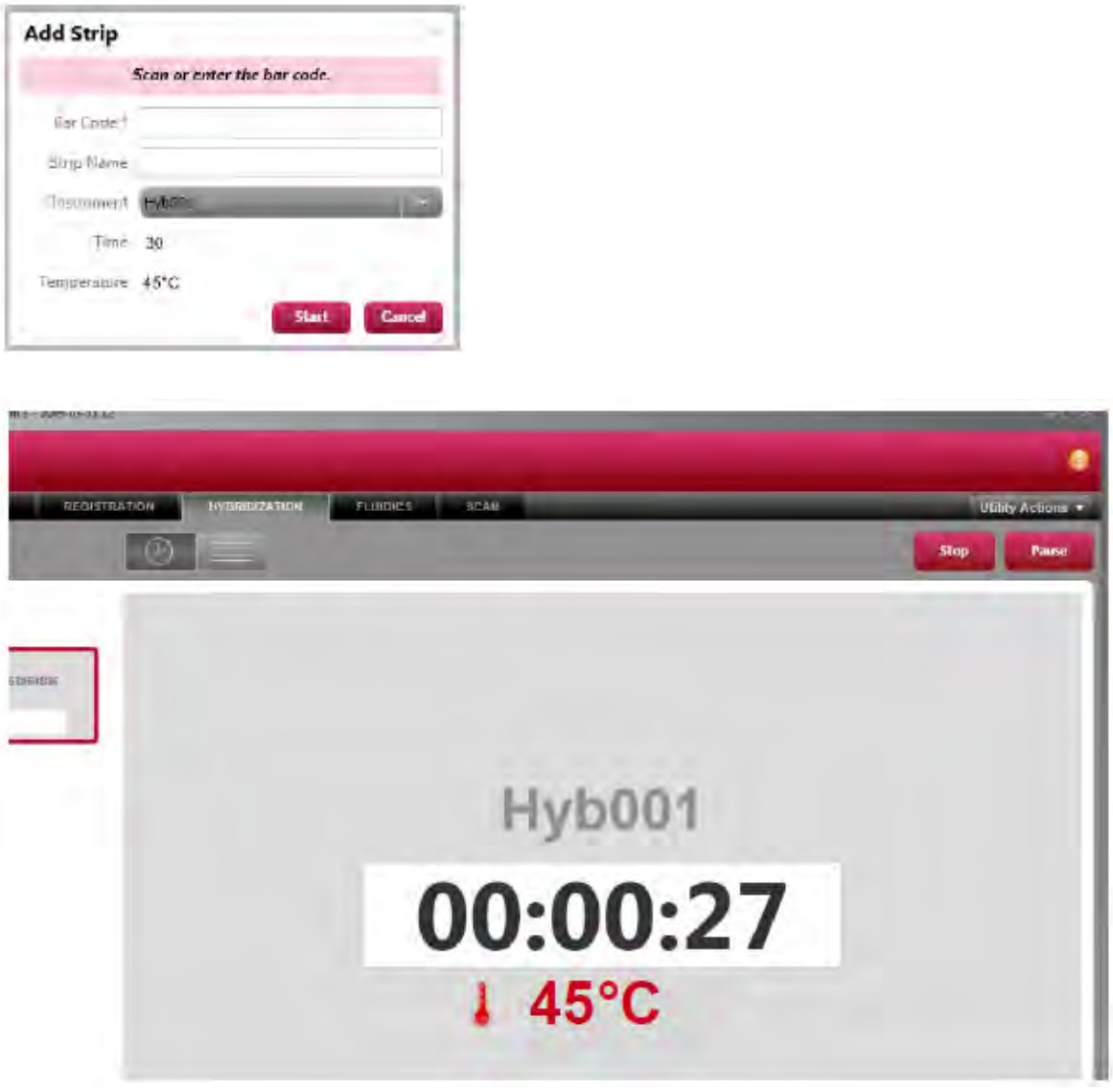


Figure 11 Hybridization countdown.

Note: The software displays the hybridization time countdown. This time is displayed with a white background (Figure 3.16 on page 43). When the countdown has completed the display turns yellow and the time begins to count up.

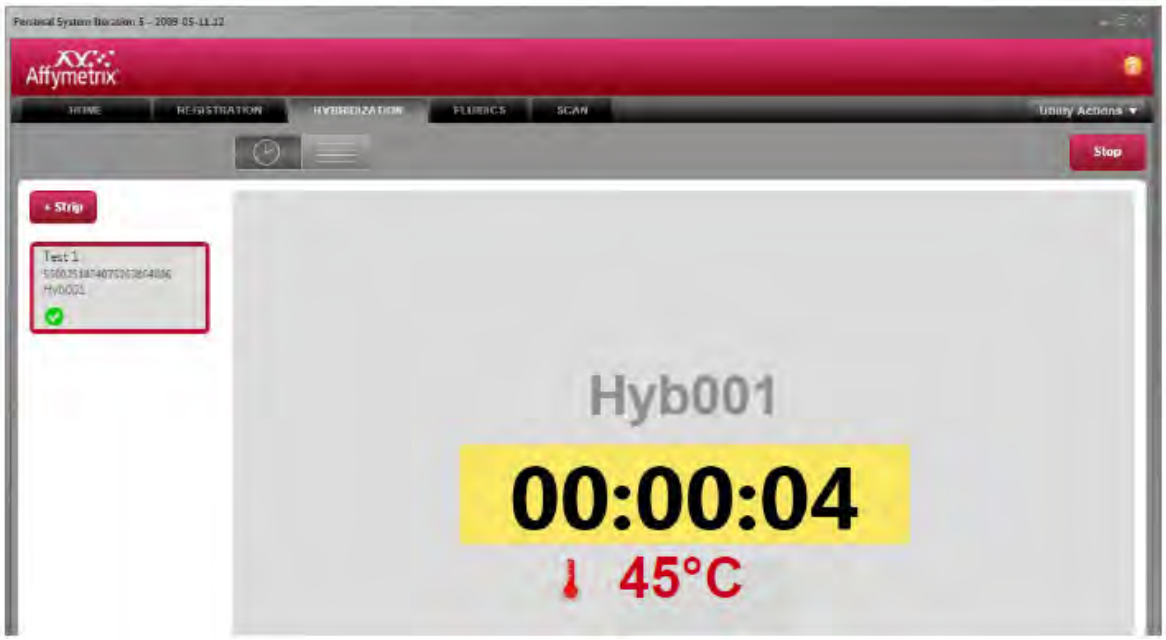


Figure 12 Hybridization count up.

8. When hybridization is complete, click **Stop** in the upper right corner.

9. In the confirmation message box, click **Yes** to complete hybridization.

IMPORTANT! Remove the hybridization tray from the GeneAtlas™ Hybridization Station after the timer has completed the countdown, because the GeneAtlas™ Hybridization Station does not shut down when the hybridization is complete.

10. Save the remaining hybridization cocktail in –20°C for future use.
See “Rehybridizing used cocktails” on page 45.

11. Immediately proceed to the GeneAtlas™ Wash, Stain, and Scan protocol.
See the *GeneAtlas™ System User Guide* 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 complete. Add it to the remainder of the Hybridization Cocktail master mix and store it at –20°C.

For rehybridization, continue the protocol from step 5 on page 38. The hybridization cocktail must be denatured again before applying it to a new array.

IMPORTANT! Rehybridization of Hybridization Cocktails should be necessary only in case of serious array problems. The performance of rehybridized samples has not been thoroughly tested. We recommend it only when absolutely necessary.

Array plate hybridization on the GeneTitan™ MC Instrument

This section outlines the basic steps involved in hybridizing array plates on a GeneTitan™ MC Instrument. The major steps involved in array plate hybridization are:

- "Target hybridization setup for array plates" on page 46.
- "Hybridization setup" on page 48.
- "Process 3' array plates on the GeneTitan™ MC Instrument" on page 49.

For more information, see:

- *GeneTitan™ Instrument User Guide for Expression Array Plates*.
- *GeneChip™ Command Console™ User Guide*.

Target hybridization setup for array plates

Arrays plates: reagents and materials required

- GeneTitan™ Hybridization, Wash, and Stain Kit for 3' IVT Arrays
  - 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 Hybridization Controls
  - 3 nM Control Oligo™ B2
- Array plate and consumables (not supplied)
  - 3′ array plate

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 seconds) to collect contents of the tube.

   c. Remove the following tubes from the GeneChip™ Hybridization Control Kit and thaw at room temperature:
      - 3 nM Control Oligo™ B2
      - 20X Hybridization Controls

   d. Vortex and centrifuge briefly (~5 seconds) to collect liquid at the bottom of the tube.

e. Keep the tubes of 3 nM Control Oligo™ B2 and the tube of 20X Hybridization Controls on ice.

2. Prepare the Hybridization Master Mix.

a. Heat the 20X Hybridization Controls for 5 minutes at 65°C in a thermal cycler using the Hybridization Control protocol that is shown in Table 5.

b. At room temperature, prepare the Hybridization Master Mix in a nuclease-free tube. Combine the approximate amount of components in the sequence shown in the following table. Prepare the Hybridization Master Mix for all the fragmented and biotin-labeled cRNA samples in the experiment.

Table 23 Hybridization Master Mix for array plates.

Component	Volume for 1 array	16-array plate[1]	24-array plate[1]	96-array plate[1]	Final concentration
3 nM Control Oligo™ B2	2 µL	35.2 µL	52.8 µL	211.2 µL	50 pM
20X Hybridization Controls (*bioB*, *bioC*, *bioD*, *cre*)	6 µL	105.6 µL	158.4 µL	633.6 µL	1.5, 5, 25 and 100 pM, respectively
1.3X Hybridization Mix Solution A	32.3 µL	568.5 µL	852.7 µL	3,411 µL	
1.3X Hybridization Mix 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**	

[1] *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 the Hybridization Cocktail.

a. At room temperature, prepare the Hybridization Cocktail for all the samples in the order shown in the following table.

Table 24 Hybridization Cocktail for a single array plate.

Component	Volume for 1 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 seconds) to collect liquid at the bottom of the well. If you are using 1.5 mL tubes; vortex and centrifuge briefly (~5 seconds) to collect contents of the tube.

IMPORTANT! Complete the above steps and prepare all stain trays and scan trays following the hybridization setup for array plates before you start the Hybridization Cocktail reaction in the next step.

c. Incubate the Hybridization Cocktail reaction for 5 minutes at 99°C (tubes) or 95°C (plates), then for 5 minutes at 45°C in a thermal cycler using the Hybridization Cocktail protocol that is shown in Table 5.

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

4. Place 90 µL of the centrifuged supernatant Hybridization Cocktail as indicated into the appropriate well of the hybridization tray.

5. Save the remaining hybridization cocktail at –20°C for future use.

6. Proceed to “Hybridization setup” on page 48.

Hybridization setup

This section describes the GeneTitan™ MC Instrument setup protocol for 3' array plates. The reagent consumption per process on the GeneTitan™ MC Instrument for processing 3' array plates is listed and described in the following tables.

Table 25 Minimum volumes of buffer and rinse required to process on the GeneTitan™ MC Instrument.

Fluid	Amount required for 1 array plate	Minimum level in bottle	
		1 array plate	2 array plates
Rinse	300 mL	450 mL	900 mL
Wash Buffer A	~920 mL	1,040 mL +	2,000 mL
Wash Buffer B	300 mL	450 mL	600 mL

Table 26 Volumes required per run to process 3′ array plates.

Reagent	Amount required for 1 array plate	Number of plates that can be processed with 1 GeneTitan™ Hybridization, Wash, and Stain Kit for 3' IVT Arrays		
		16-format	24-format	96-format
Wash Buffer A[1]	~920 mL	1	1	1
Wash Buffer B[1]	300 mL	1	1	1
Stain Cocktail 1 & 3	105 µL/well	6	4	1
Stain Cocktail 2	105 µL/well	6	4	1
Array Holding Buffer	150 µL/well	6	4	1

[1] Additional Wash Buffer A and Wash Buffer B for additional plates are available in Wash Buffers A and B Module (Cat. No. 901583).

IMPORTANT! The GeneTitan™ MC Instrument must have a minimum of 450 mL of Wash Buffer B in the Wash B reservoir for each 3′ array plate prior to starting the hybridization, wash, stain, and scan process. The waste bottle should be empty.

Process 3' array plates on the GeneTitan™ MC Instrument

1. Use the GeneTitan™ ZeroStat AntiStatic Gun on the wells of the stain tray labeled “GeneTitan™ Stain Tray (Part. No. 501025)”.
   a. Place a stain tray on the tabletop.
   b. Hold the antistatic gun within 12” (30.5 cm) of the surface or object to be treated.
   c. Squeeze the trigger slowly for about 2 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.
   d. Repeat these substeps at several points across the surface of the stain tray.
2. Aliquot 105 µL of Stain Cocktail 1 & 3 into the GeneTitan™ stain tray.
3. Use the antistatic gun on the stain tray cover.
   a. Place a stain tray cover on the tabletop with the flat surface facing upward.
   b. Hold the antistatic fun within 12” (30.5 cm) of the surface or object to be treated.
   c. Squeeze the trigger slowly for about 2 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.
   d. Repeat these substeps at several points across the surface, covering the entire stain tray cover.

4. Place the cover on top stain tray 1 after removing the static electricity.
5. Repeat step 1, then aliquot 105 µL of Stain Cocktail 2 into the stain tray.
6. Repeat step 3, then place the cover on top of stain tray 2.
7. Repeat step 1, then aliquot 105 µL of the Stain Cocktail 1 & 3 into the stain tray.
8. Repeat step 3, then place the cover on top of stain tray 3.
9. Aliquot 150 µL of the Array Holding Buffer into all wells of the GeneTitan™ scan tray labeled “HT Scan Tray (Part No. 500860).”
10. Use the fourth scan tray cover provided with the GeneTitan™ Consumables Kit to cover the scan tray.
11. Load all the consumables including the HT array plate into the GeneTitan™ MC Instrument according to instructions provided in the *GeneTitan™ Instrument User Guide for Expression Array Plates*.

IMPORTANT! It is important not to bump the trays while loading them into the GeneTitan™ MC 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.

Store any remaining hybridization-ready sample at –20C° in the original tube or plate.

A cRNA purification photos

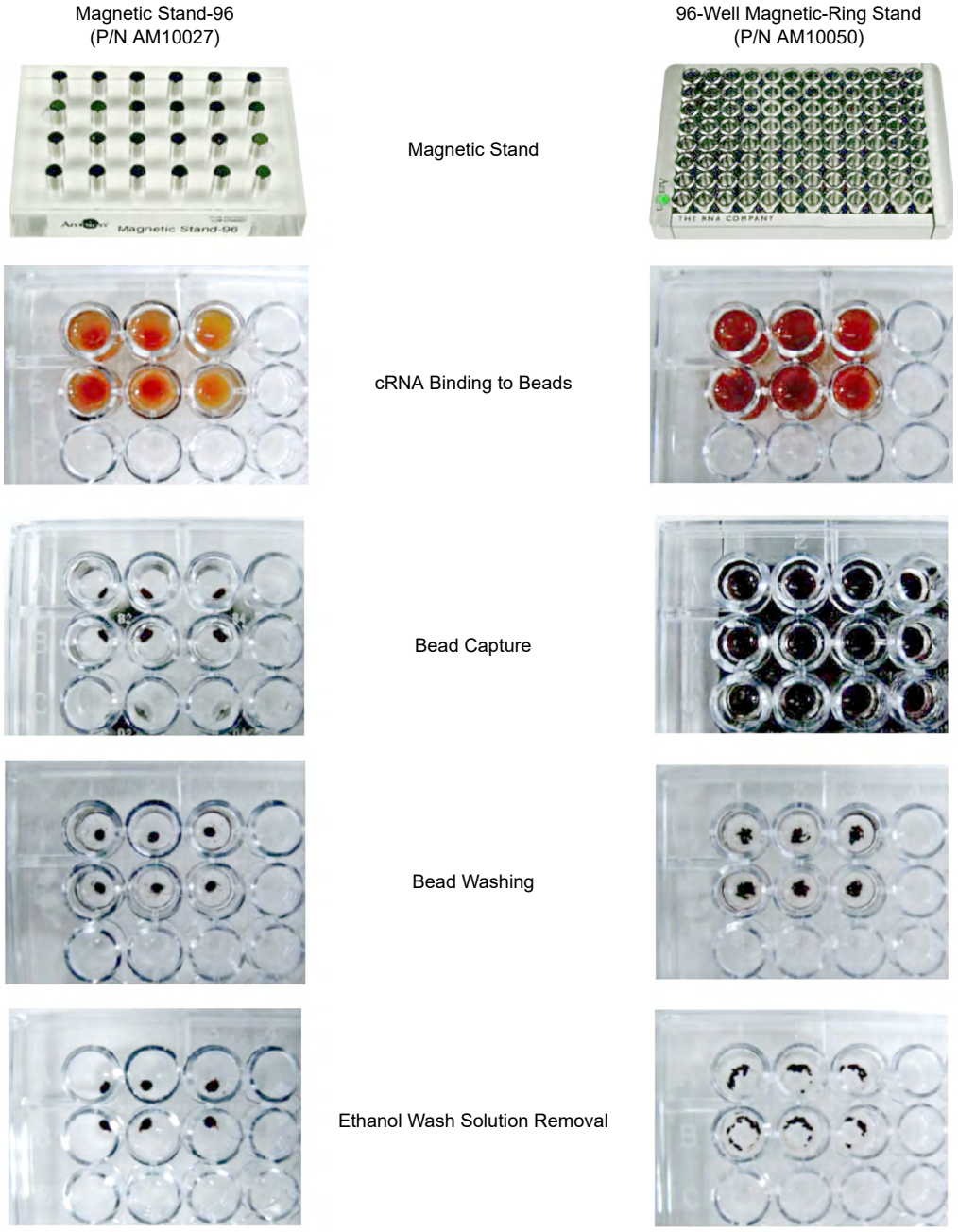


Figure 13 Photos of the cRNA purification step (1 of 2).

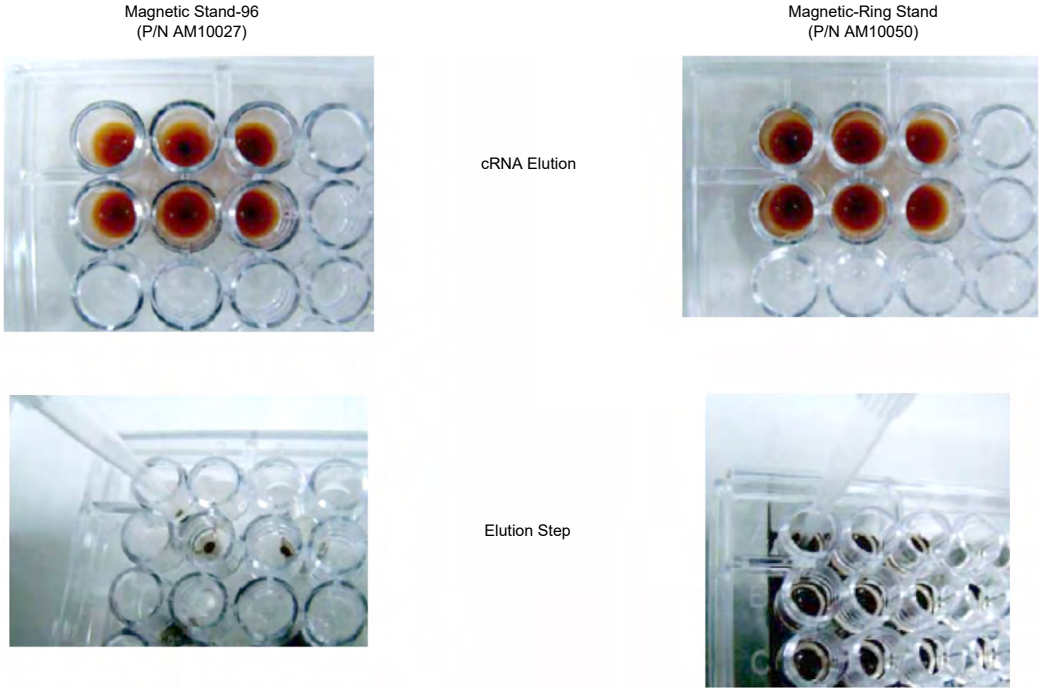


Figure 14 Photos of the cRNA purification step (2 of 2).

B

Safety

WARNING! GENERAL SAFETY. Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
- Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, and so on). To obtain SDSs, see the “Documentation and Support” section in this document.

Chemical safety

WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below. Consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with sufficient ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if needed) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

AVERTISSEMENT ! **PRÉCAUTIONS GÉNÉRALES EN CAS DE MANIPULATION DE PRODUITS CHIMIQUES.** Pour minimiser les risques, veiller à ce que le personnel du laboratoire lise attentivement et mette en œuvre les consignes de sécurité générales relatives à l'utilisation et au stockage des produits chimiques et à la gestion des déchets qui en découlent, décrites ci-dessous. Consulter également la FDS appropriée pour connaître les précautions et instructions particulières à respecter :

- Lire et comprendre les fiches de données de sécurité (FDS) fournies par le fabricant avant de stocker, de manipuler ou d'utiliser les matériaux dangereux ou les produits chimiques. Pour obtenir les FDS, se reporter à la section « Documentation et support » du présent document.
- Limiter les contacts avec les produits chimiques. Porter des équipements de protection appropriés lors de la manipulation des produits chimiques (par exemple : lunettes de sûreté, gants ou vêtements de protection).
- Limiter l'inhalation des produits chimiques. Ne pas laisser les récipients de produits chimiques ouverts. Ils ne doivent être utilisés qu'avec une ventilation adéquate (par exemple, sorbonne).
- Vérifier régulièrement l'absence de fuite ou d'écoulement des produits chimiques. En cas de fuite ou d'écoulement d'un produit, respecter les directives de nettoyage du fabricant recommandées dans la FDS.
- Manipuler les déchets chimiques dans une sorbonne.

- Veiller à utiliser des récipients à déchets primaire et secondaire. (Le récipient primaire contient les déchets immédiats, le récipient secondaire contient les fuites et les écoulements du récipient primaire. Les deux récipients doivent être compatibles avec les matériaux mis au rebut et conformes aux exigences locales, nationales et communautaires en matière de confinement des récipients.)
- Une fois le récipient à déchets vidé, il doit être refermé hermétiquement avec le couvercle fourni.
- Caractériser (par une analyse si nécessaire) les déchets générés par les applications, les réactifs et les substrats particuliers utilisés dans le laboratoire.
- Vérifier que les déchets sont convenablement stockés, transférés, transportés et éliminés en respectant toutes les réglementations locales, nationales et/ou communautaires en vigueur.
- **IMPORTANT !** Les matériaux représentant un danger biologique ou radioactif exigent parfois une manipulation spéciale, et des limitations peuvent s'appliquer à leur élimination.

WARNING! **HAZARDOUS WASTE (from instruments).** Waste produced by the instrument is potentially hazardous. Follow the guidelines noted in the preceding General Chemical Handling warning.

WARNING! **4L Reagent and Waste Bottle Safety.** Four-liter reagent and waste bottles can crack and leak. Each 4-liter bottle should be secured in a low-density polyethylene safety container with the cover fastened and the handles locked in the upright position.

Biological hazard safety

WARNING! Potential Biohazard. Depending on the samples used on this instrument, the surface may be considered a biohazard. Use appropriate decontamination methods when working with biohazards.

WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Conduct all work in properly equipped facilities with the appropriate safety equipment (for example, physical containment devices). Safety equipment can also include items for personal protection, such as gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles. Individuals should be trained according to applicable regulatory and company/ institution requirements before working with potentially biohazardous materials. Follow all applicable local, state/provincial, and/or national regulations. The following references provide general guidelines when handling biological samples in laboratory environment.

- U.S. Department of Health and Human Services, *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*, 5th Edition, HHS Publication No. (CDC) 21-1112, Revised December 2009; found at:
  https://www.cdc.gov/labs/pdf/CDC-BiosafetymicrobiologicalBiomedicalLaboratories-2009-P.pdf
- World Health Organization, *Laboratory Biosafety Manual*, 3rd Edition, WHO/CDS/CSR/LYO/2004.11; found at:
  www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf

Documentation and support

Related documentation

Document	Publication number
GeneChip™ Fluidics Station 450 User Guide	08-0295
GeneChip™ Expression Wash, Stain, and Scan for Expression Cartridge Arrays User Guide	MAN0018114
GeneChip™ Command Console™ User Guide	702569
GeneAtlas™ System User Guide	08-0306
GeneTitan™ Instrument User Guide for Expression Array Plates	MAN0017794

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  - User guides, manuals, and protocols
  - Certificates of Analysis
  - Safety Data Sheets (SDSs; also known as MSDSs)

Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale at **www.thermofisher.com/us/en/home/global/terms-and-conditions.html**. If you have any questions, please contact Life Technologies at **www.thermofisher.com/support**.