applied biosystems

GeneChip™ WT PLUS Reagent Kit

USER GUIDE

Manual Target Preparation for GeneChip™ Whole Transcript (WT) Expression Arrays

Catalog Numbers 902280 and 902281

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ThermoFisher SCIENTIFIC

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Products:
GeneChip™ WT PLUS Reagent Kit

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Products:
GeneChip™ Whole Transcript (WT) Expression Arrays

For descriptions of symbols on product labels or product documents, go to thermofisher.com/symbols-definition.

The information in this guide is subject to change without notice.

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Revision	Date	Description
B.0	26 October 2020	Changed volume to load on array for the 169/400 format to “90 µL”. Updated image of GeneChip™ cartridge array. Added reagent reservoir for multichannel pipettes.
A.0	20 December 2018	• Initial release in Thermo Fisher Scientific document control system. • Supersedes legacy Affymetrix™ publication number 703174. • Updated to the current document template, with associated updates to trademarks, logos, licensing, and warranty.

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Product information

Purpose

The GeneChip™ WT PLUS Reagent Kit enables you to prepare RNA samples for whole transcriptome expression analysis with GeneChip™ Whole Transcript (WT) Expression Arrays. The kit generates amplified and biotinylated sense-strand DNA targets from total RNA without the need for an up-front selection or enrichment step for mRNA. The kit is optimized for use with GeneChip™ Sense Target (ST) Arrays.

The GeneChip™ WT PLUS Reagent Kit uses a reverse-transcription priming method that primes the entire length of RNA, including both poly(A) and non-poly(A) mRNA, to provide complete and unbiased coverage of the transcriptome. See Figure 1.

The GeneChip™ WT PLUS Reagent Kit is comprised of reagents and a protocol for preparing hybridization-ready targets from 50 to 500 ng of total RNA. The kit is optimized to work with a wide range of samples including tissues, cells, cell lines, and whole blood. The total RNA samples can be used directly without removal of ribosomal or globin RNA prior to target preparation with the kit.

Assay workflow

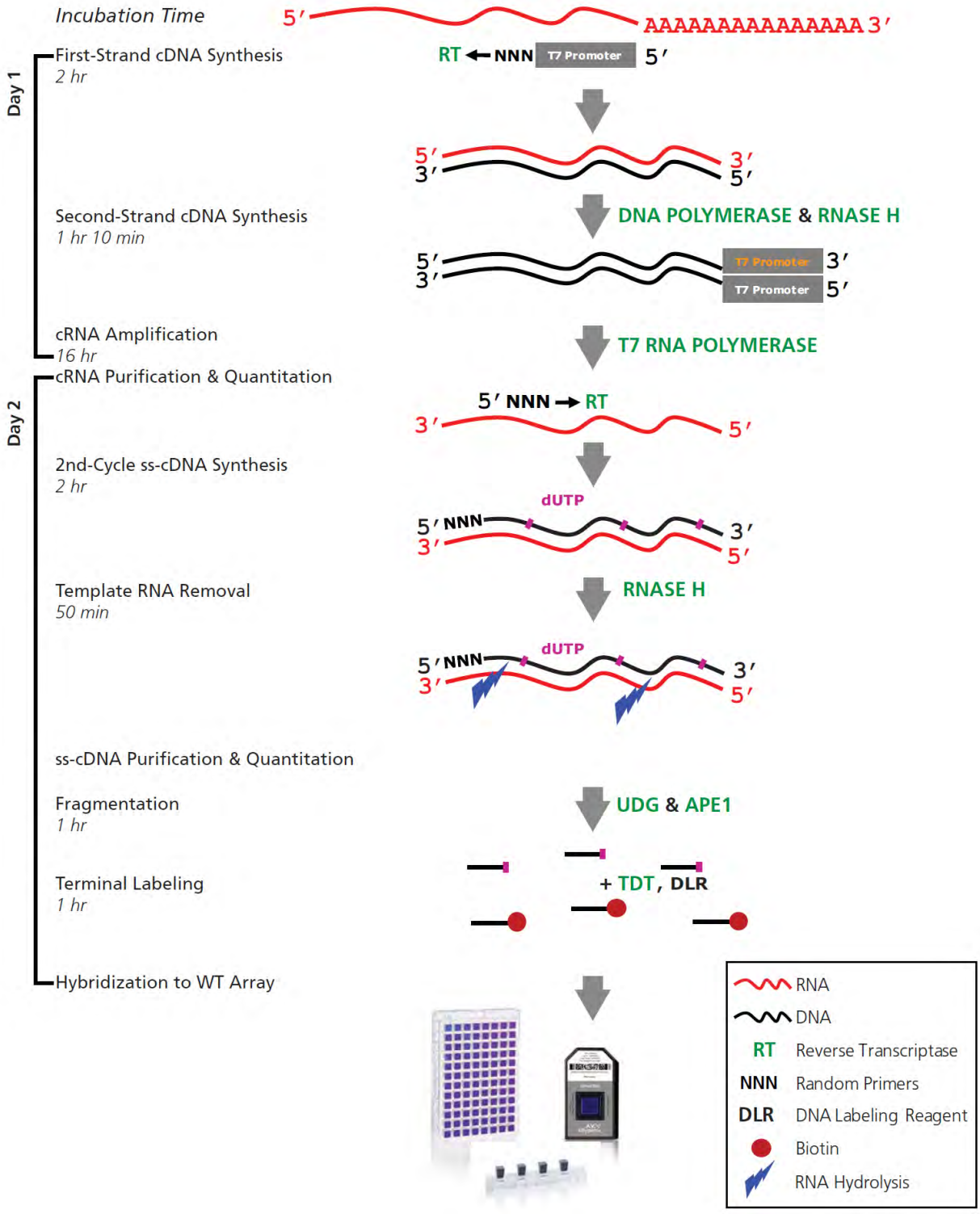


Figure 1 The WT PLUS amplification and labeling process.

Kit contents and storage

Table 1 GeneChip™ WT PLUS Reagent Kit contents and storage.

Component	10-reaction kit for manual use (902280)	30-reaction kit for manual use (902281)	Storage
WT Amplification Kit Module 1			
First-Strand Enzyme	11 µL	50 µL	–20°C
First-Strand Buffer	500 µL	500 µL	
Second-Strand Enzyme	22 µL	70 µL	
Second-Strand Buffer	198 µL	600 µL	
IVT Enzyme	66 µL	210 µL	
IVT Buffer	500 µL	1,580 µL	
Control RNA (1 mg/mL HeLa total RNA)	5 µL	5 µL	
2nd-Cycle Primers	44 µL	180 µL	
2nd-Cycle ss-cDNA Enzyme	44 µL	140 µL	
2nd-Cycle ss-cDNA Buffer	500 µL	500 µL	
RNase H	44 µL	180 µL	
Nuclease-free Water	2 x 1.0 mL	4 x 1.0 mL	Any temperature
WT Amplification Kit Module 2			
Purification Beads	22 mL	6.6 mL	4°C (Do not freeze.)
GeneChip™ Eukaryotic Poly-A RNA Control Kit (Cat. No. 900433)			
Poly-A Control Stock	16 µL	16 µL	–20°C
Poly-A Control Dil Buffer	3.8 mL	3.8 mL	
GeneChip™ WT Terminal Labeling Kit (900670)			
10x cDNA Fragmentation Buffer	48 µL	213 µL	–20°C
UDG, 10 U/µL	10 µL	49 µL	
APE 1, 1,000 U/µL	10 µL	49 µL	
5X TdT Buffer	120 µL	475 µL	
TdT, 30 U/µL	20 µL	99 µL	
DNA Labeling Reagent, 5 mM	10 µL	49 µL	
RNase-free Water	825 µL	2 x 825 µL	Any temperature

Table 1 GeneChip WT PLUS Reagent Kit contents and storage. *(continued)*

Component	10-reaction kit for manual use (902280)	30-reaction kit for manual use (902281)	Storage
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) in (for example, Nalgene™ Polycarbonate 9 x 9 CryoBox™, 5026-0909 , or equivalent).			

Required equipment and 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	Thermo Fisher Scientific
(*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 for cartridge arrays	
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	
GeneAtlas™ Workstation	90-0894
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 Instrument, NA/Japan includes 110v UPS	00-0372
GeneTitan™ Multi-Channel Instrument, Int'l includes 220v UPS	00-0373
GeneTitan™ Multi-Channel Instrument, NA/Japan includes 110v UPS	00-0360
GeneTitan™ Multi-Channel Instrument, Int'l Includes 220v UPS	00-0363
GeneTitan™ ZeroStat AntiStatic Gun (for processing WT array plates)	74-0014

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	07-200-103
GeneChip™ Hybridization, Wash, and Stain Kit, 30 reactions	900720
GeneAtlas™ Hybridization, Wash, and Stain Kit for WT Array Strips , 60 reactions	901667
GeneTitan™ Hybridization, Wash, and Stain Kit for WT Array Plates, 96 reactions	901622
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 cartridge array processing	**MLS**
(*Optional*) Agilent™ RNA 6000 Nano Kit or equivalent DNA and RNA sizing reagents For those intending to use the gel-shift assay methodology, see additional required reagents in Appendix A, “Gel-shift assays”.	Agilent™ Technologies, Inc., 50671511
Tough-Spots™ labels	**MLS**
Nuclease-free Water (for preparing 80% ethanol wash solution)	**MLS**
(*Optional*) 96-well plate-sealing film	**MLS**
(*Optional*) Reagent reservoir for multichannel pipette	**MLS**
100% ethanol (molecular-biology grade)[1]	**MLS**

[1] Before handling any chemicals, see the SDS provided by the manufacturer, and observe all relevant precautions.

2 Protocol

Procedural notes

Implement a plan to maintain procedural consistency

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

Recommended thermal cycler

Make sure that the heated cover of the 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 that you 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.

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

Thermal cycler protocols

Table 5 Thermal cycler protocols.

Protocol	Heated lid temperature	Alternate protocol[1]	Step 1	Step 2	Step 3	Step 4	Volume
First-Strand cDNA Synthesis	42°C	105°C	25°C for 1 hour	42°C for 1 hour	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 16 hours	4°C, hold			60 µL
2nd-Cycle Primers-cRNA Annealing	70°C	105°C	70°C for 5 minutes	25°C for 5 minutes	4°C for 2 minutes		28 µL
2nd-Cycle ss-cDNA Synthesis	70°C	105°C	25°C for 10 minutes	42°C for 90 minutes	70°C for 10 minutes	4°C, hold	40 µL
RNA Hydrolysis	70°C	105°C	37°C for 45 minutes	95°C for 5 minutes	4°C, hold		44 µL
Fragmentation	93°C	105°C	37°C for 60 minutes	93°C for 2 minutes	4°C, hold		48 µL
Labeling	70°C	105°C	37°C for 60 minutes	70°C for 10 minutes	4°C, hold		60 µL

Table 5 Thermal cycler protocols. *(continued)*

Protocol	Heated lid temperature	Alternate protocol[1]	Step 1	Step 2	Step 3	Step 4	Volume
Hybridization Control	65°C	105°C	65°C for 5 minutes				Variable
Hybridization Cocktail	99°C	105°C	95°C or 99°C for 5 minutes	45°C for 5 minutes			Variable

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

Reagent preparation

IMPORTANT! You can freeze and thaw the reagents in the 12- and 30-reaction kits ≤ 3 times.

Handle kit components as follows

- Enzymes: Mix by gently vortexing the tube, followed by a brief centrifuge to collect contents at the bottom of the tube, then keep on ice.
- Buffers and primers: Thaw on ice, thoroughly vortex to dissolve precipitates, followed by a brief centrifuge 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 when preparing the master mixes.
  - 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.

To prepare the Control RNA for positive control reaction, follow these steps.

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 using a NanoDrop™ 2000/2000c Spectrophotometer, then use the measured concentration for calculation and preparing the 25 ng/µL working stock.
- The positive control reaction should produce >15 µg of cRNA and >5.5 µg of 2nd-cycle ss-cDNA from 50 ng of Control RNA.

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 is designed specifically to provide exogenous positive controls to monitor the entire target preparation. The control should be added to the RNA samples 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. 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 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, 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 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 solutions of less than 2 µL to maintain precision and consistency when preparing the dilutions.
- Use nonstick nuclease-free tubes (not included) to prepare dilutions.
- 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 first dilution (1:20).

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

The 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, 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, 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 2 discrete bands with no significant smearing below each band.
- 28S rRNA band intensity is approximately twice that of the 18S rRNA band.

Determine RNA quantity

Consider the type and amount of sample RNA that are available when planning the 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 are based on total RNA from HeLa cells. See Table 8. 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. High-concentration RNA samples should be prediluted 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

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 RNA must be 3 µL or less (Table 9). See "Prepare poly-A RNA controls" on page 14. 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: If you are adding Poly-A Control Stock to the 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 9 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 primers containing a T7 promoter sequence. The reaction synthesizes single-stranded cDNA with T7 promoter sequence at the 5' end.

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 10 First-Strand Master Mix.

Component	Volume for 1 reaction
First-Strand Buffer	4 µL
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, transfer 5 µL of the total RNA from Table 9 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 1 hour at 25°C, then for 1 hour at 42°C, then for at least 2 minutes at 4°C.
   a. Incubate the first-strand synthesis reaction in a thermal cycler.

      The First-Strand cDNA Synthesis protocol that is shown in Table 5 can be used as a reference.

   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 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 to correct for pipetting losses.

      Table 11 Second-Strand Master Mix.

Component	Volume for 1 reaction
Second-Strand Buffer	18 µL
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 sample of first-strand cDNA, 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.

      The Second-Strand cDNA Synthesis protocol that is shown in Table 5 can be used as a reference.

      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 well.

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

      Tip: When the thermal cycler has approximately 15 minutes left to run, start reagent preparation for *in vitro* transcription.

Synthesize cRNA by *in vitro* transcription

In this procedure, antisense RNA (complimentary RNA or cRNA) is synthesized and amplified by *in vitro* transcription (IVT) of the 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 IVT Master Mix.
- After the IVT Buffer is thawed 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 12 IVT Master Mix.

Component	Volume for 1 reaction
IVT Buffer	24 µL
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 (30 µL) 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 16 hours at 40°C, then hold at 4°C.

   a. Incubate the IVT reaction in a thermal cycler.

   The *In Vitro* Transcription cRNA Synthesis protocol that is shown in Table 5 can be used as a reference.

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

   c. Place the reaction on ice, then proceed to “Purify the cRNA” on page 21 or immediately freeze the samples at –20°C for storage.

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

Purify the cRNA

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

IMPORTANT!

- Preheat the Nuclease-free Water in a heat block or thermal cycler to 65°C for at least 10 minutes.
- Mix the Purification Beads thoroughly by vortexing before use to ensure that the beads 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. Each reaction requires 100 µL plus ~10% overage.
- Prepare fresh dilutions of 80% ethanol wash solution each time from molecular-biology grade 100% ethanol and Nuclease-free Water in a nuclease-free tube or container. Each reaction requires 600 µL plus ~10% overage.
- Transfer the cRNA sample to room temperature while preparing the Purification Beads.

Note:

- Occasionally, the mixture of beads and sample may be brownish in color and not completely clear when placed on a magnet. If that occurs, 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 the 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 in use and the amount of cRNA generated by *in vitro* transcription.
   f. Carefully aspirate and discard the supernatant without disturbing the Purification Beads. Keep the plate on the magnetic stand.

2. Wash the Purification Beads.
   a. While on the magnetic stand, add 200 µL of 80% ethanol wash solution to each well and incubate for 30 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 to each sample 27 µL of the 65°C Nuclease-free Water 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 the sample with high concentration cRNA, add an additional 10–30 µL of the preheated Nuclease-free Water to the well and incubate for 1 minute before proceeding to Step 3B on page 22.

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 non-rRNA in the input total RNA. Because the proportion of non-rRNA in total RNA is affected by factors such as the health of the organism and the organ from which it is isolated, cRNA yield from equal amounts of total RNA can vary considerably.

During development of the GeneChip™ WT PLUS Reagent Kit, using a 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 µg of cRNA.

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; just use 1.5 µL of the cRNA sample directly. Samples with cRNA concentrations greater than 3,000 ng/µL should be diluted with Nuclease-free Water before measurement and reaction setup. Use the diluted cRNA as the input to prepare 15 µg of cRNA in the 2nd-cycle cDNA synthesis reaction.

Alternatively, determine the cRNA concentration by diluting an aliquot of the preparation in Nuclease-free Water and reading the absorbance in a traditional spectrophotometer at 260 nm. Calculate the concentration in µg/mL using the following equation:

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

where 1 A_{260} = 40 µg RNA/mL.

(*Optional*) Expected cRNA size distribution

The expected cRNA profile is a distribution of sizes from 50 nt to 4,500 nt, with most of the cRNA sizes in the 200-nt to 2,000-nt range. The distribution is quite jagged and does not resemble the profile observed when using a traditional dT-based amplification kit such as the GeneChip™ 3' IVT Express Kit. This step is optional.

Determine 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, 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 per well.

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

Synthesize 2nd-cycle single-stranded cDNA

In this procedure, sense-strand cDNA is synthesized by the reverse transcription of cRNA using 2nd-cycle primers. The sense-strand cDNA contains dUTP at a fixed ratio relative to dTTP. 15 µg of cRNA is required for 2nd-cycle single-stranded cDNA synthesis.

1. Prepare 15 µg of cRNA.
   a. On ice, prepare 625 ng/µL cRNA. This is equal to 15 µg cRNA in a volume of 24 µL.
   b. If necessary, use Nuclease-free Water to bring the cRNA sample to the required volume of 24 µL.

Note: Dilute high-concentration cRNA samples (>3,000 ng/µL) with Nuclease-free Water before measurement and reaction setup. Use the diluted cRNA as the input to prepare 15 µg of cRNA.

---

2. Prepare the cRNA and 2nd-cycle primers mix.
   a. On ice, combine these components:
      - 24 µL of cRNA (15 µg)
      - 4 µL of 2nd-Cycle Primers
   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 5 minutes at 70°C, then for 5 minutes at 25°C, then for 2 minutes at 4°C.
   a. Incubate the cRNA/primers mix in a thermal cycler.
      The 2nd-Cycle Primers-cRNA Annealing protocol that is shown in Table 5 can be used as a reference.
   b. Immediately after the incubation, centrifuge briefly to collect the cRNA/primers mix at the bottom of the tube or well.
   c. Place the mix on ice, then proceed immediately to the next step.
4. Prepare 2nd-Cycle ss-cDNA Master Mix.
   a. On ice, prepare the 2nd-Cycle ss-cDNA Master Mix in a nuclease-free tube. Combine the components in the sequence shown in the following table. Prepare the master mix for all the cRNA/primers samples in the experiment. Include ~5% overage for pipetting losses.

      Table 13 2nd-Cycle ss-cDNA Master Mix.

Component	Volume for 1 reaction
2nd-Cycle ss-cDNA Buffer	8 µL
2nd-Cycle ss-cDNA Enzyme	4 µL
Total volume	**12 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 12 µL of the 2nd-Cycle ss-cDNA Master Mix to each 28-µL sample of the cRNA/2nd-cycle primer mix for a final reaction volume of 40 µL.

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

5. Incubate for 10 minutes at 25°C, then for 90 minutes at 42°C, then for 10 minutes at 70°C, then for at least 2 minutes at 4°C.
   a. Incubate the 2nd-cycle synthesis reaction in a thermal cycler.

      The 2nd-Cycle ss-cDNA Synthesis protocol that is shown in Table 5 can be used as a reference.

   b. Immediately after the incubation, centrifuge briefly to collect the 2nd-cycle ss-cDNA at the bottom of the tube or wells.

   c. Place the sample on ice and proceed immediately to “Hydrolyze RNA using RNase H” on page 25.

Hydrolyze RNA using RNase H

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

1. Add RNase H to each 2nd-cycle ss-cDNA sample.
   a. On ice, add 4 µL of the RNase H to each 40-µL sample of 2nd-cycle ss-cDNA, for a final reaction volume of 44 µL.

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

2. Incubate for 45 minutes at 37°C, then for 5 minutes at 95°C, then for at least 2 minutes at 4°C.
   a. Incubate the RNA hydrolysis reaction in a thermal cycler.

      The RNA Hydrolysis protocol that is shown in Table 5 can be used as a reference.

   b. Immediately after the incubation, centrifuge briefly to collect the hydrolyzed 2nd-cycle ss-cDNA at the bottom of the tube or well.

   c. Place the samples on ice and proceed immediately to the next step.

3. Add Nuclease-free Water to each hydrolyzed 2nd-cycle ss-cDNA sample.
   a. On ice, add 11 µL of the Nuclease-free Water to each (44 µL) hydrolyzed 2nd-cycle ss-cDNA sample for a final reaction volume of 55 µL.

   b. Mix thoroughly by gently vortexing. Centrifuge briefly to collect the reaction at the bottom of the tube or well.

c. Place the sample on ice, then proceed to "Purify the 2nd-cycle single-stranded cDNA" on page 26, or immediately freeze the samples at –20°C for storage.

STOPPING POINT The hydrolyzed ss-cDNA samples can be stored overnight at –20°C.

Purify the 2nd-cycle single-stranded cDNA

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

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 the beads 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. Each reaction requires 100 µL plus ~10% overage.
- Prepare fresh dilutions of 80% ethanol wash solution each time from molecular-biology grade 100% ethanol and Nuclease-free Water in a nuclease-free tube or container. Each reaction requires 600 µL plus ~10% overage.
- 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 a magnet. If that occurs, 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 the ss-cDNA to Purification Beads.
   a. Mix the Purification Beads container by vortexing to resuspend the magnetic particles that may have settled.
   b. Add 100 µL of the Purification Beads to each (55 µL) 2nd-cycle ss-cDNA sample, mix by pipetting up and down, and 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. Add 150 µL of 100% ethanol to each (155 µL) ss-cDNA/beads sample. Mix well by pipetting up and down 10 times.
   d. Incubate for 20 minutes.

      The ss-cDNA 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 in use and the amount of ss-cDNA generated by 2nd-cycle ss-cDNA synthesis.

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 Purification Beads, as this will reduce the elution efficiency. The bead surface will appear dull, and may have surface cracks when it is over-dry.

3. Elute ss-cDNA.
   a. Remove the plate from the magnetic stand. Add to each sample 30 µL of the preheated (65°C) Nuclease-free Water, then 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 ss-cDNA, to a nuclease-free tube.

   e. Place the purified ss-cDNA samples on ice, then proceed to “Assess single-stranded cDNA yield and size distribution” on page 28, or immediately freeze the samples at –20°C for storage.

Note: Minimal bead carryover will not inhibit subsequent enzymatic reactions.

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

Assess single-stranded cDNA yield and size distribution

Expected single-stranded cDNA yield

During development of the GeneChip™ WT PLUS Reagent Kit, using a wide variety of tissue types, 15 µg of input cRNA yielded 5.5 to 15 µg of ss-cDNA. For most tissue types, the recommended 15 µg of input cRNA should yield >5.5 µg of ss-cDNA.

Determine single-stranded DNA yield by UV absorbance

Determine the concentration of a ss-cDNA solution by measuring its absorbance at 260 nm. Use Nuclease-free Water as blank. We recommend using NanoDrop™ spectrophotometers for convenience. No dilutions or cuvettes are needed; just use 1.5 µL of the cDNA sample directly.

Alternatively, determine the ss-cDNA concentration by diluting an aliquot of the preparation in Nuclease-free Water and reading the absorbance in a traditional spectrophotometer at 260 nm. Calculate the concentration in µg/mL using the following equation, where 1 A_{260} = 33 µg DNA/mL:

A_{260} × dilution factor × 33 = µg DNA/mL

Note: This equation applies only to single-stranded cDNA.

(*Optional*) Expected single-stranded cDNA size distribution

The expected cDNA profile does not resemble the cRNA profile. The median cDNA size is approximately 400 nt. This step is optional.

Determine single-stranded cDNA size distribution using a Bioanalyzer™ Instrument

We recommend analyzing cDNA size distribution using an Agilent™ 2100 Bioanalyzer™ Instrument, an Agilent™ RNA 6000 Nano Kit, and mRNA Nano Series II assay. To analyze cDNA size using an Agilent™ 2100 Bioanalyzer™ Instrument, follow the manufacturer's instructions.

1. If there is sufficient yield, load approximately 250 ng of cDNA per well.
2. If there is insufficient yield, use as little as 200 ng of cDNA per well.

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

Fragment and label single-stranded cDNA

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

1. Prepare 5.5 µg of ss-cDNA.
   a. On ice, prepare 176 ng/µL ss-cDNA. This is equal to 5.5 µg ss-cDNA in a volume of 31.2 µL.
   b. If necessary, add Nuclease-free Water to bring the ss-cDNA sample volume to 31.2 µL.
2. Prepare Fragmentation Master Mix.
   a. On ice, prepare the Fragmentation 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 ss-cDNA samples in the experiment. Include ~5% overage for pipetting losses.

      Table 14 Fragmentation Master Mix.

Component	Volume for 1 reaction
Nuclease-free Water	10 µL
10x cDNA Fragmentation Buffer	4.8 µL
UDG, 10 U/µL	1 µL
APE 1, 1,000 U/µL	1 µL
Total volume	**16.8 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. On ice, transfer 16.8 µL of the Fragmentation Master Mix to each 31.2-µL sample of purified ss-cDNA, for a final reaction volume of 48 µL.
   d. Mix thoroughly by gently vortexing the tube. Centrifuge briefly to collect the reaction at the bottom of the tube or wells, then proceed immediately to the next step.
3. Incubate for 1 hour at 37°C, then for 2 minutes at 93°C, then for at least 2 minutes at 4°C.
   a. Incubate the fragmentation reaction in a thermal cycler.
      The Fragmentation protocol that is shown in Table 5 can be used as a reference.
   b. Immediately after the incubation, centrifuge briefly to collect the fragmented ss-cDNA at the bottom of the tube or well.
   c. Place the sample on ice, then proceed immediately to the next step.

4. (*Optional*) The fragmented ss-cDNA sample can be used for size analysis using an Agilent™ 2100 Bioanalyzer™ Instrument.

   For detailed instructions, see the reagent kit guide that comes with the Agilent™ RNA 6000 Nano Kit. The range in peak size of the fragmented samples should be approximately 40 nt to 70 nt.

5. On ice, transfer 45 µL of the fragmented ss-cDNA sample to each tube or well.

6. Prepare the Labeling Master Mix.

   a. On ice, prepare the Labeling 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 fragmented ss-cDNA samples in the experiment. Include ~5% excess volume to correct for pipetting losses.

   Table 15 Labeling Master Mix.

Component	Volume for 1 reaction
5X TdT Buffer	12 µL
DNA Labeling Reagent, 5 mM	1 µL
TdT, 30 U/µL	2 µL
Total volume	**15 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. On ice, transfer 15 µL of the Labeling Master Mix to each 45-µL fragmented ss-cDNA sample for a final reaction volume of 60 µL.

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

7. Incubate for 1 hour at 37°C, then for 10 minutes at 70°C, then for at least 2 minutes at 4°C.

   a. Incubate the labeling reaction in a thermal cycler.

   The Labeling protocol that is shown in Table 5 can be used as a reference.

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

   c. Place the sample on ice, then proceed to Chapter 3, “WT array hybridization”, or immediately freeze the samples at –20°C for storage.

8. (*Optional*) Remove 2 µL of each fragmented and labeled ss-cDNA sample for gel-shift analysis to assess the fragmentation and labeling efficiency as described in Appendix A, “Gel-shift assays”.

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

3 WT array hybridization

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 GeneChip™ Hybridization Oven 645 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 into the GeneChip™ Command Console™ Software.

Target hybridization setup for cartridge arrays

Reagents and materials required

- GeneChip™ Hybridization, Wash, and Stain Kit (Not supplied. For ordering information, see Table 4).
  - 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
- WT cartridge arrays (not supplied)

Procedure

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

   Note: DMSO solidifies at 2–8°C. Ensure that the reagent is completely thawed before use. We recommend that DMSO be stored at room temperature after the first use.

   b. Heat the 20X Hybridization Controls for 5 minutes at 65°C in a thermal cycler.
   The Hybridization Control protocol that is shown in Table 5 can be used as a reference.

   c. At room temperature, prepare the Hybridization Master Mix in a nuclease-free tube. Combine the components in the amounts and sequence shown in Table 16. Prepare the master mix for all the fragmented and biotin-labeled ss-cDNA samples in the experiment. Include ~10% overage to correct for pipetting losses.

   Table 16 Hybridization Master Mix for a single reaction.

Component	49- or 64-format[1]	100- or 81/4-format[1]	169- or 400-format[1]	Final concentration
Fragmented and labeled ss-DNA	5.2 µg	3.5 µg	2.3 µg	23 ng/µL
3 nM Control Oligo™ B2	3.7 µL	2.5 µL	1.7 µL	50 pM
20X Hybridization Controls (*bioB*, *bioC*, *bioD*, *cre*)	11 µL	7.5 µL	5 µL	1.5, 5, 25, and 100 pM, respectively
2X Hybridization Mix	110 µL	75 µL	50 µL	1X
DMSO	15.4 µL	10.5 µL	7 µL	7%
Nuclease-free Water	19.9 µL	13.5 µL	9.3 µL	
Total volume	**160 µL**	**109 µL**	**73 µL**	

[1] See specific probe array package insert for information on array format.

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

2. Prepare the Hybridization Cocktail.

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

   Table 17 Hybridization Cocktail for a single array.

Component	49- or 64-format	100- or 81/4-format	169- or 400-format
Hybridization Master Mix	160 µL	109 µL	73 µL
Fragmented and labeled ss-cDNA	~60 µL[1] (5.2 µg)	41 µL (3.5 µg)	27 µL (2.3 µg)
Total volume	**220 µL**	**150 µL**	**100 µL**

[1] 58 µL if a portion of the sample was set aside for gel-shift analysis.

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

   c. Incubate the Hybridization Cocktail reaction for 5 minutes at 99°C in tubes or 95°C in plates, then for 5 minutes at 45°C in a thermal cycler.

   The Hybridization Cocktail protocol that is shown in Table 5 can be used as a reference.

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

3. Inject and hybridize the array.

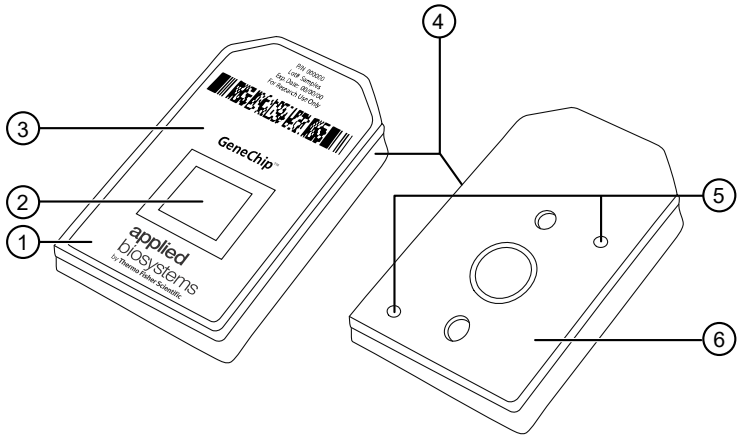


Figure 2 GeneChip™ cartridge array.

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 the second to allow venting of air from the hybridization chamber.

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

b. Inject the appropriate amount (see Table 18) of the specific sample into the cartridge array through 1 of the septa.

See Figure 2 for the location of the septa on the cartridge array.

Table 18 Probe array cartridge volumes for the 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 cartridge array. Cover both septa with 1/2-inch Tough-Spots™ label to minimize evaporation and prevent leaks.

d. Place the array or 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 contact all portions of the array.

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

Note: 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.
2. Remove the Tough-Spots™ label from the arrays.
3. Extract the Hybridization Cocktail mix from each array.
4. (*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.
5. Fill each array completely with Wash Buffer A.
6. 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 hours before proceeding with washing and staining. Equilibrate arrays to room temperature before washing and staining.

7. 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.

8. Wash the arrays according to array type and components used for hybridization, wash and stain. For HWS kits the fluidics protocols are:

	49- or 64-format	100- or 81/4-format	169- or 400-format
Fluidics protocol	FS450_0001	FS450_0002	FS450_0007

9. 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.

10. 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

Instructions for using the scanner and scanning arrays can be found 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 36.
- "GeneAtlas™ software setup" on page 42.

IMPORTANT! Before preparing hybridization-ready samples, register samples as described in "GeneAtlas™ software setup" on page 42.

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

Target hybridization setup for array strips

Reagents and materials required

Note: The WT Hyb Add reagent names were created to match the order in which reagents are added. For example, WT Hyb Add 1 is the first component added during preparation of the hybridization mix. WT Hyb Add 2, 3, and 5 are not used and are not part of the hybridization module.

- GeneAtlas™ Hybridization, Wash, and Stain Kit for WT Array Strips. (Not supplied, available separately. For ordering information, see Table 4.)
  - 5X WT Hyb Add 1
  - 15X WT Hyb Add 4
  - 2.5X WT Hyb Add 6
  - Stain Cocktail 1
  - Stain Cocktail 2
  - Array Holding Buffer
  - Wash Buffer A
  - Wash Buffer B
- GeneChip™ Hybridization Control Kit
  - 20X Hybridization Controls
  - 3 nM Control Oligo™ B2 (3 nM)
- Array strip and consumables (not supplied)
  - WT array strips
  - 1 hybridization tray per array strip

Procedure

Note: If you are using a hybridization-ready sample, or rehybridizing a previously made Hybridization Cocktail, start the procedure with step 1, skip step 2–substep 8b, then continue the procedure starting with substep 8c.

1. Prepare the following (in preparation of the hybridization step):
   a. Pull the array strip from storage at 4°C so that it can begin to equilibrate to room temperature.

b. Gather 1 hybridization tray per array strip.

c. Set the temperature of the GeneAtlas™ Hybridization Station to 48°C. Press the start button to begin heating.

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

3. Vortex and centrifuge the tubes briefly (~5 seconds) to collect contents of the tubes.

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

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

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

7. Prepare the Hybridization Master Mix.
   a. Heat the 20X Hybridization Controls for 5 minutes at 65°C in a thermal cycler.
   The Hybridization Control protocol that is shown in Table 5 can be used as a reference.

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

   Note: The 5X WT Hyb Add 1 solution is very viscous. Pipet slowly to ensure addition of the correct volume. Mix well. Vortex and centrifuge briefly (~5 seconds) to collect liquid at the bottom of the tube.

Table 19 Hybridization Master Mix for strip arrays.

Component	Volume for 1 array	Volume for 4 arrays (includes 10% overage)	Final concentration
5X WT Hyb Add 1	30 µL	132 µL	1X
3 nM Control Oligo™ B2	1.5 µL	6.6 µL	30 pM
20X Hybridization Controls (*bioB*, *bioC*, *bioD*, *cre*)	7.5 µL	33 µL	1.5, 5, 25 and 100 pM, respectively
15X WT Hyb Add 4	10 µL	44 µL	1X
Total volume	**49 µL**	**215.6 µL**	

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

8. Prepare the Hybridization Cocktail.

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

Table 20 Hybridization Cocktail for a single array.

Component	Volume for 1 array	Final concentration
Hybridization Master Mix	49 µL	
Fragmented and labeled ss-cDNA	41 µL	23 ng/µL
2.5X WT Hyb Add 6	60 µL	1X
Total volume	**150 µL**	

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

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.

The Hybridization Cocktail protocol that is shown in Table 5 can be used as a reference.

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

9. Hybridize the array strip.

a. Apply 120 µL of Hybridization Cocktail to the middle of the appropriate wells of a new clean hybridization tray (Figure 3).

IMPORTANT! Do not add more than 120 µL of Hybridization Cocktail to the wells, as this can result in cross-contamination of the samples.

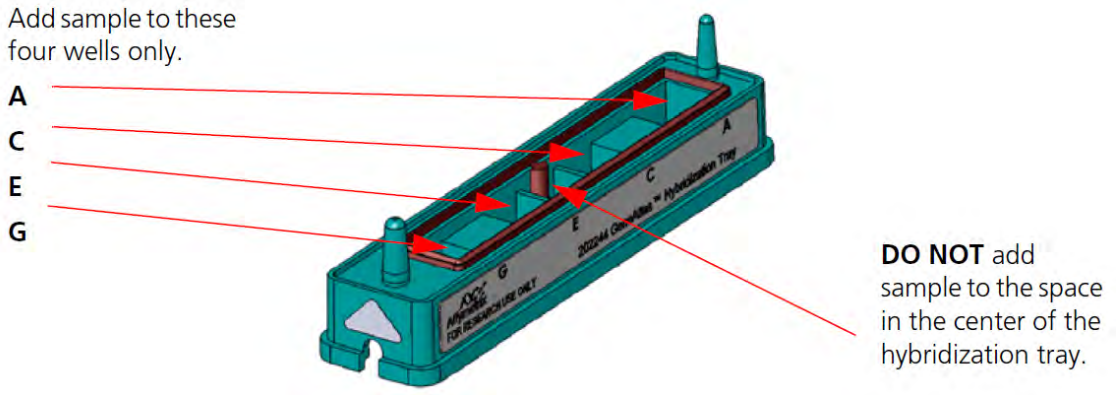


Figure 3 Location of sample wells (A, C. E, and G) on the hybridization tray.

b. Carefully remove the array strip and protective tray from its foil pouch and place it on the bench (Figure 4).

IMPORTANT! Leave the array strip in its protective tray.

Figure 4 Array strip in its protective tray.

c. Place the array strip into the hybridization tray containing the Hybridization Cocktail samples (Figure 5). See Figure 6 for proper orientation of the array strip in the hybridization tray.

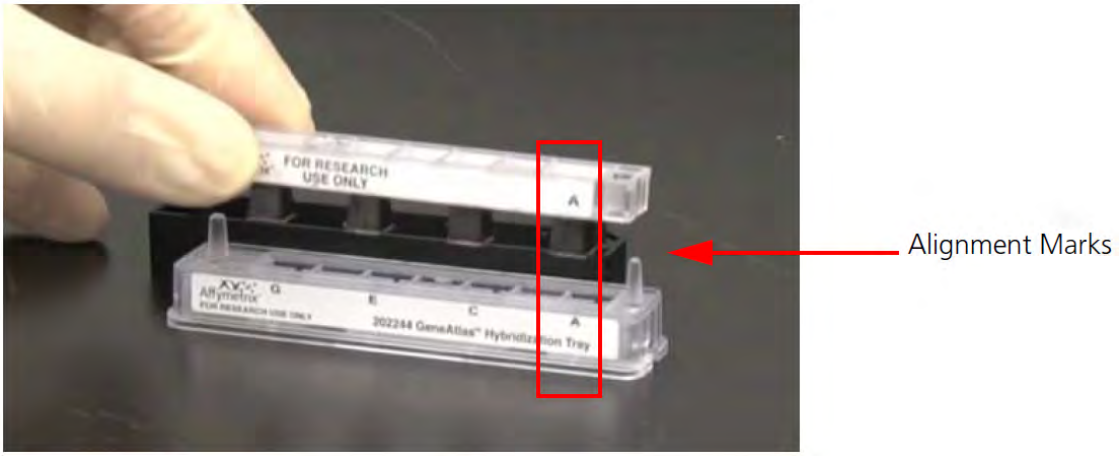


Figure 5 Placing the array strip into the hybridization tray.

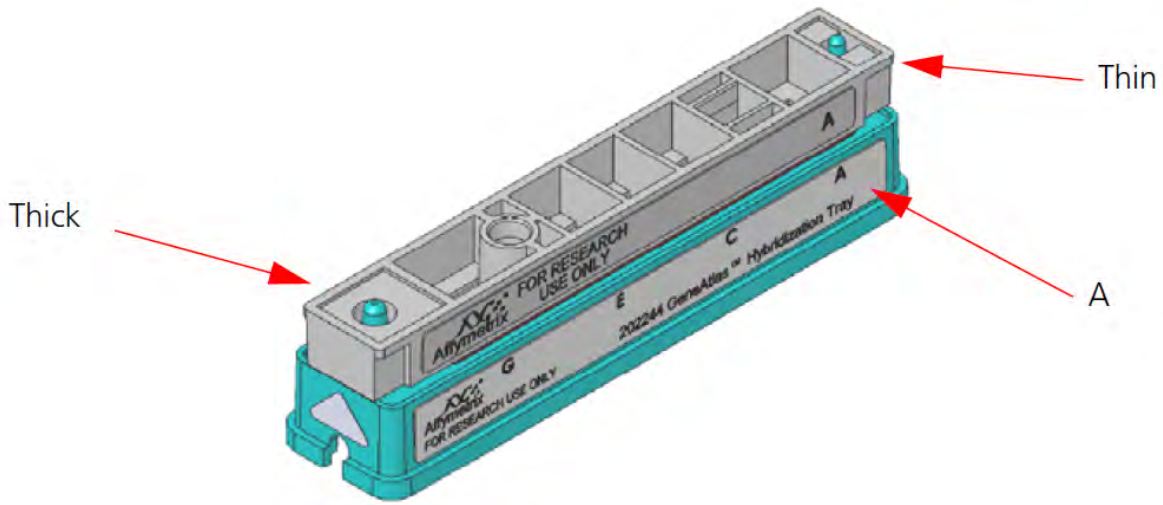


Figure 6 Proper orientation of the array strip in the hybridization tray.

d. (*Optional*) The remainder of the Hybridization Master Mix can be stored at –20°C to supplement the Hybridization Cocktail volume if a rehybridization is necessary.

CAUTION! Be very careful not to scratch or damage array surfaces.

Tip: To avoid any possible mix-ups, the hybridization tray and array strip should be labeled on the white label if more than 1 array strip is processed overnight.

e. Bring the hybridization tray to just above eye level and look at the underside of the hybridization tray to check for bubbles.

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

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

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

g. Open a hybridization station clamp by applying pressure to the top of the clamp while gently squeezing inward. While squeezing, lift the clamp to open (Figure 7).

WARNING! Do not force the 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.

IMPORTANT! The hybridization temperature for GeneAtlas™ WT array strips is 48°C.

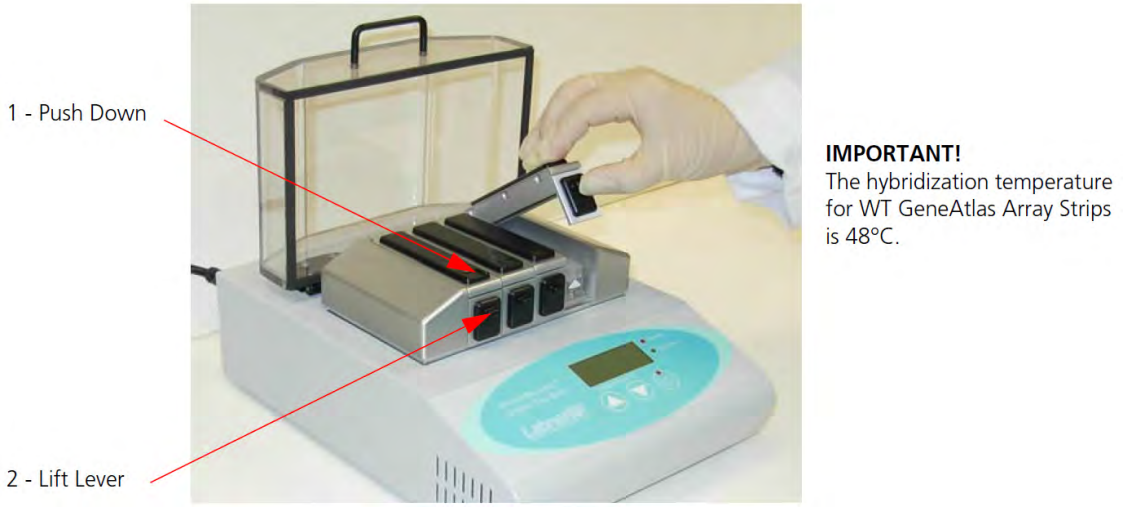


Figure 7 Opening the clamps on the GeneAtlas™ Hybridization Station.

h. Place the hybridization tray with the array strip into a clamp in the GeneAtlas™ Hybridization Station and close the clamp as shown in Figure 8.

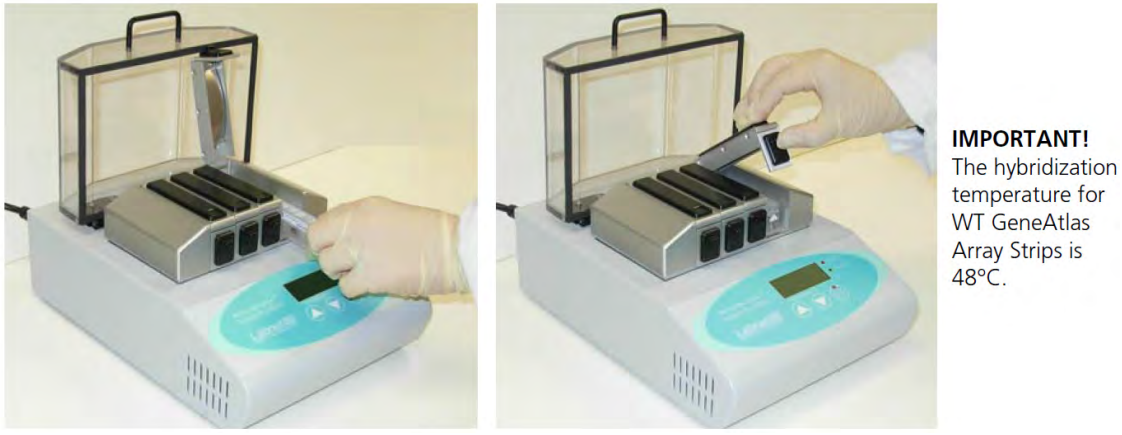


Figure 8 Inserting an array strip and closing the clamp on the GeneAtlas™ Hybridization Station.

10. Proceed to "Hybridization software setup" on page 44.

GeneAtlas™ software setup

Before 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™ Instrument Control 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 appears.

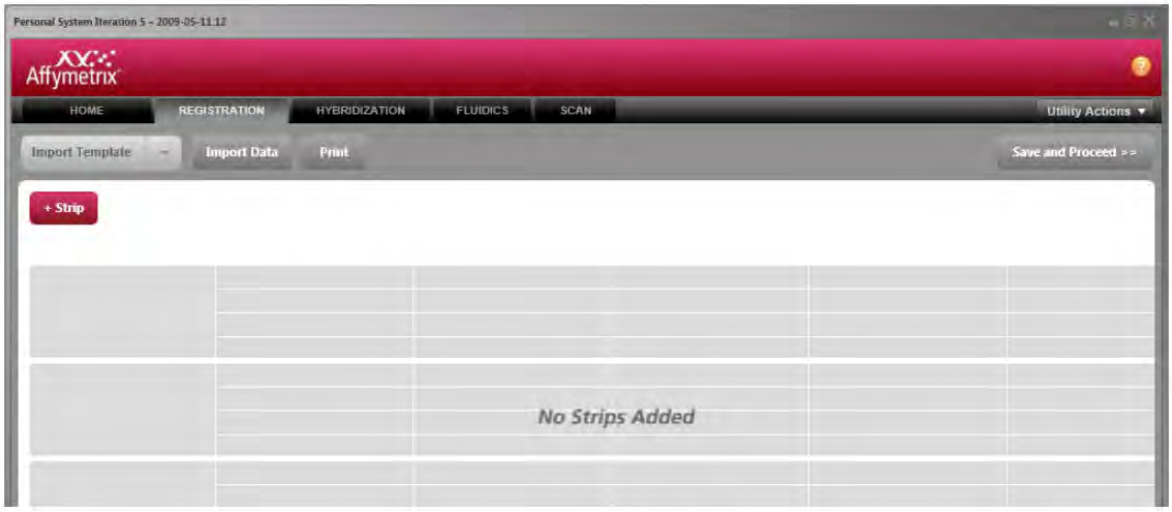
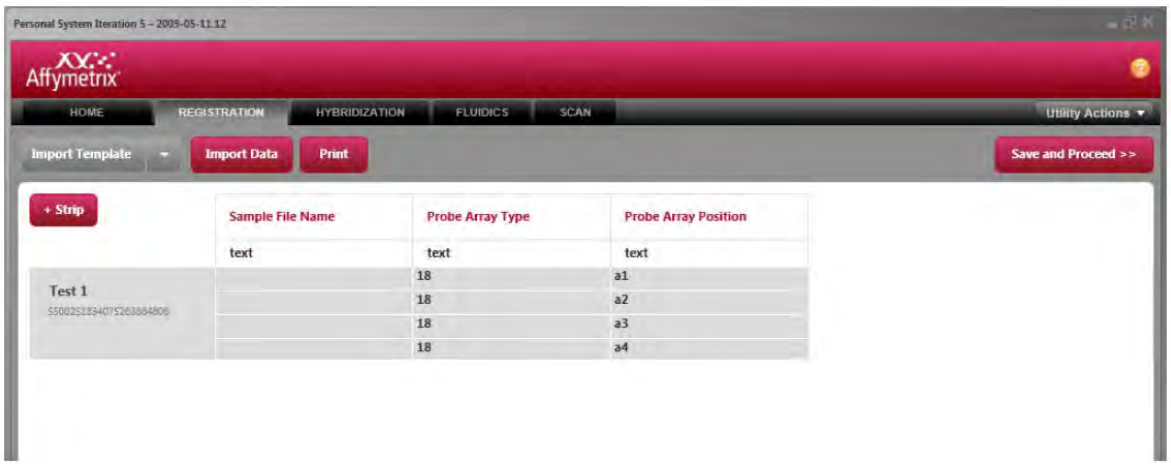


Figure 9 Registration tab of GeneAtlas™ software.

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

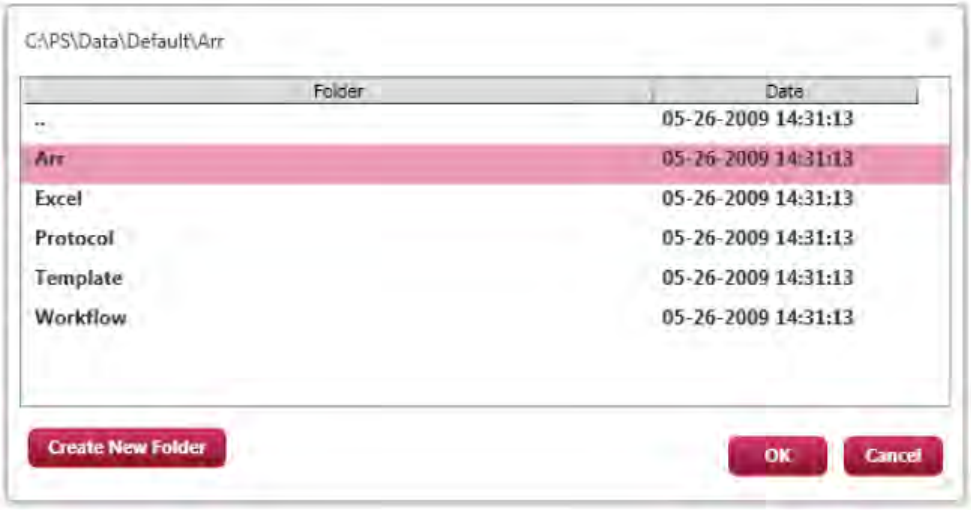
4. Enter or scan the array strip **Bar Code** and enter a **Strip Name**, then click **Add.**
   The array strip is added and appears in the **Registration** window.



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 the data, then click **OK.**
   The 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 42 through Step 8 on page 44.**

9. Proceed to "Hybridization software setup" on page 44.

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 42 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. Recommended hybridization time is 20 ±1 hour.

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

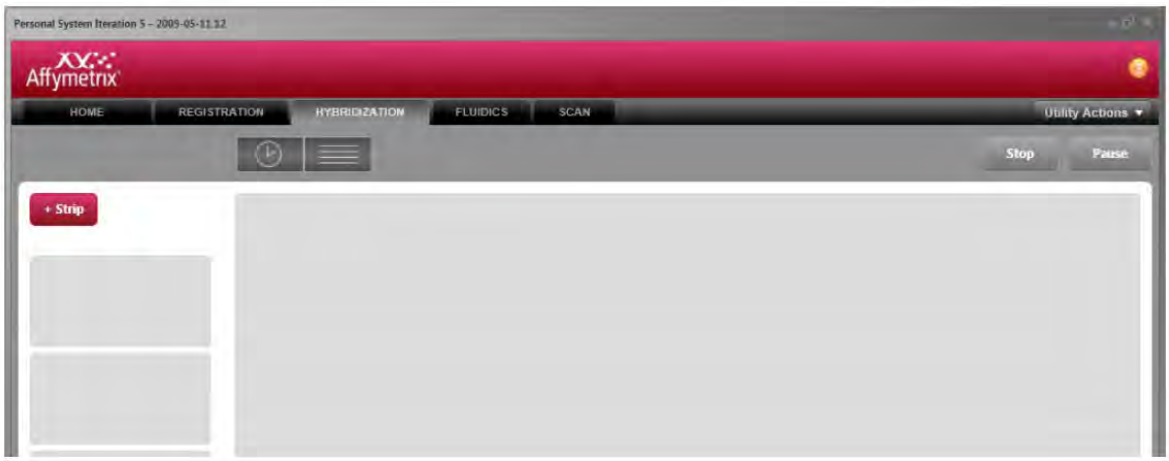


Figure 10 Hybridization tab.

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. When the hybridization tray and array strip are in the GeneAtlas™ GeneAtlas™ Hybridization Station, click **Start**.

   The software displays the hybridization time countdown. This time is displayed with a white background (Figure 11). When the countdown has completed the display turns yellow and the time begins to count up.

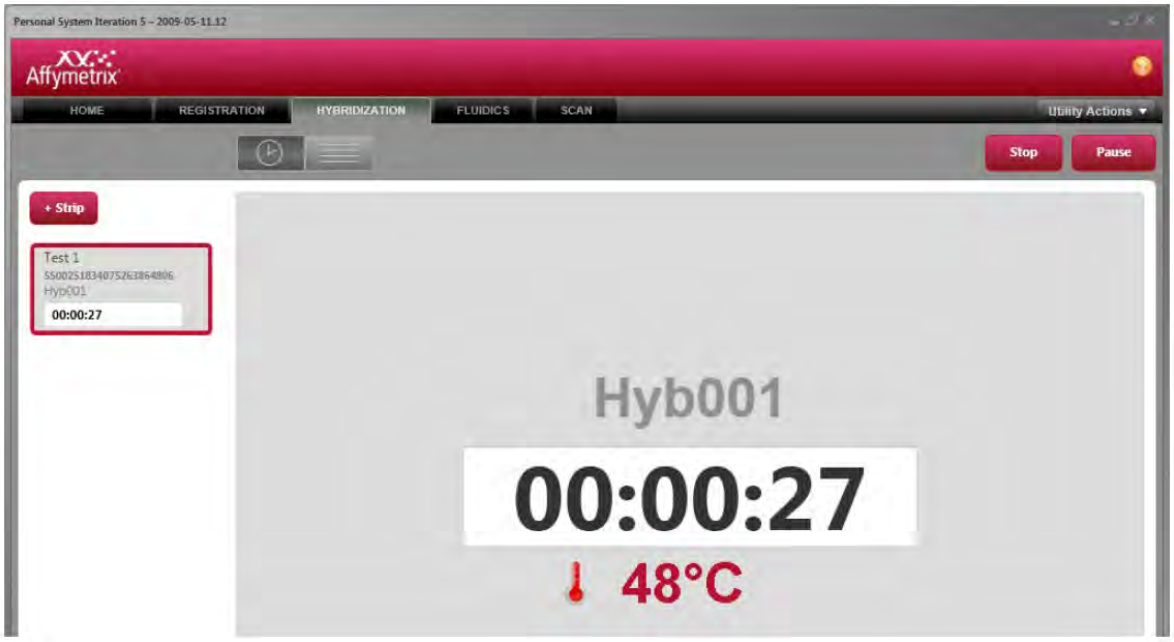


Figure 11 Hybridization countdown.

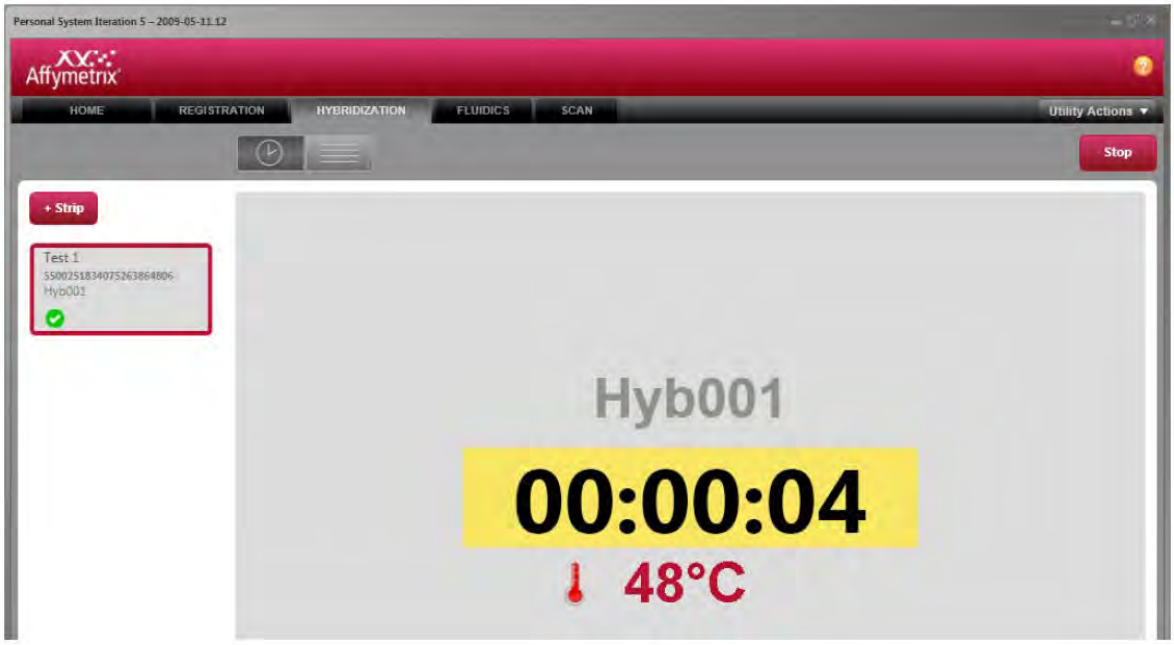


Figure 12 Hybridization count up.

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

6. 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.

7. Save the remaining Hybridization Cocktail in –20°C for future use.

8. Immediately proceed to the GeneAtlas™ Wash, Stain and Scan protocol.
   For more information, see the *GeneAtlas™ System User Guide*.

Array plate hybridization on the GeneTitan™ MC Instrument

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

- "Target hybridization setup for array plates" on page 47.
- "Hybridization setup" on page 49.
- "Process WT array plates on the GeneTitan™ MC Instrument" on page 50.

For more information, see:

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

Target hybridization setup for array plates

Reagents and materials required

Note: The WT Hyb Add reagent names were created to match the order in which reagents are added. For example, WT Hyb Add 1 is the first component added during preparation of the hybridization mix. WT Hyb Add 2, 3, and 5 are not used and are not part of the hybridization module.

- GeneTitan™ Hybridization, Wash, and Stain Kit for WT Array Plates. (Not supplied. For ordering information, see Table 4).
  - 5X WT Hyb Add 1
  - 15X WT Hyb Add 4
  - 2.5X WT Hyb Add 6
  - Stain Cocktail 1 & 3
  - Stain Cocktail 2
  - Array Holding Buffer
  - Wash Buffer A
  - Wash Buffer B
- GeneChip™ Hybridization Control Kit
  - 20X Hybridization Controls (*bioB*, *bioC*, *bioD*, *cre*)
  - 3 nM Control Oligo™ B2 (3 nM)
- Array plate and consumables (not supplied)
  - WT array plates

Procedure

1. In preparation of the hybridization step, prepare the following:
   a. Warm the following vials to room temperature on the bench.
      - 5X WT Hyb Add 1
      - 15X WT Hyb Add 4
      - 2.5X WT Hyb Add 6

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

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

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 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.
   The Hybridization Control protocol that is shown in Table 5 can be used as a reference.

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

Note: The 5X WT Hyb Add 1 solution is very viscous. Pipet slowly to ensure addition of the correct volume. Mix well. Vortex and centrifuge briefly (~5 seconds) to collect liquid at the contents of the tube.

Table 21 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
5X WT Hyb Add 1	24 µL	422.4 µL	633.6 µL	2,534.4 µL	1X
3 nM Control Oligo™ B2	1.2 µL	21.1 µL	31.7 µL	126.7 µL	30 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
15X WT Hyb Add 4	8 µL	140.8 µL	211.2 µL	844.8 µL	1X
Total volume	**39.2 µL**	**689.9 µL**	**1,034.9 µL**	**4,139.5 µL**	

[1] Includes 10% overage for pipetting loss.

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 in the order shown in the following table for all samples.

Table 22 Hybridization Cocktail for a single array.

Component	Volume for 1 array	Final concentration
Hybridization Master Mix	39.2 µL	
Fragmented and labeled ss-cDNA	32.8 µL	23 ng/µL
2.5X WT Hyb Add 6	48 µL	1X
Total volume	**120 µL**	

   b. If you are using a plate, seal, vortex, and centrifuge briefly (~5 seconds) to collect the liquid at the bottoms of the well. If you are using 1.5-mL tubes, vortex and centrifuge briefly (~5 seconds) to collect the 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.
   The Hybridization Cocktail protocol that is shown in Table 5 can be used as a reference.

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

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

   f. Proceed to "Hybridization setup" on page 49.

Hybridization setup

This section describes the GeneTitan™ MC Instrument setup protocol for WT array plates. The reagent consumption per process on the GeneTitan™ MC Instrument for processing WT array plates is shown in Table 24.

Table 23 Minimum volumes of buffer and rinse required to process on the GeneTitan™ MC Instrument.

Fluid type	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 24 Reagent volumes required to process WT array plates per run.

Reagent	Amount required for 1 array plate	Number of plates that can be processed with 1 GeneTitan™ Hybridization, Wash, and Stain Kit for WT Array Plates		
		16-format	24-format	96-format
Wash Buffer A	~920 mL	1	1	1
Wash Buffer B	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

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

Process WT 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 P/N 501025".
   a. Place a stain tray on the tabletop.
   b. Hold the antistatic gun within 12 inches (30.5 cm) of the surface of the 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 this procedure 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 gun within 12 inches (30.5 cm) of the surface or the 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 this procedure at several points across the surface of the tray cover.
4. After removing the static electricity, place the cover on top of stain tray 1.
5. Repeat step 1, then aliquot 105 µL of Stain Cocktail 2 into the GeneTitan™ 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 GeneTitan™ 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 the GeneTitan™ scan tray identified with the label "HT Scan Tray P/N 500860" on the tray.

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.

The remaining hybridization-ready samples can be stored at –20°C after covering the sample plate with plate sealing film.

A Gel-shift assays

The efficiency of the labeling procedure can be assessed using the following procedure. This quality-control protocol prevents hybridizing a poorly labeled target onto the probe array. The addition of biotin residues is monitored in a gel-shift assay, where the fragments are incubated with avidin prior to electrophoresis. The nucleic acids are then detected by staining. The procedure takes approximately 90 minutes to complete.

Gel-shift assay reagents required

Unless otherwise indicated, all materials are available through **thermofisher.com**.

Item	Source
XCell™ SureLock™ Mini-Cell™[1]	EI0001
Novex™ TBE Gels, 4-20%, 12 well1.0 mm[1]	EC62252BOX
Novex™ Hi-Density TBE Sample Buffer (5X)	LC6678
TBE Buffer, 5X Solution	75891
SYBR™ Gold Nucleic Acid Gel Stain	S11494
Invitrogen™ 10 bp DNA ladder and Invitrogen™ 100 bp DNA ladder100 bp DNA ladder	10-821-015 and 15-628-019
NeutrAvidin™ Protein	31000
PBS (1X), pH 7.2	20012027

[1] Or equivalent.

Gel-shift assay procedure

Place a 4–20% TBE gel into the gel holder and add 1X TBE Buffer to the gel system and equilibrate to room temperature.

1. Prepare NeutrAvidin™ and biotin-labeled cDNA sample mix.
   a. On ice, prepare a NeutrAvidin™ solution of 2 mg/mL in PBS.
   b. For each sample to be tested, prepare 2 aliquots of 1 µL fragmented and biotin-labeled ss-cDNA sample in a tube or well.
   c. Heat the samples for 2 minutes at 70°C. Centrifuge briefly to collect the reaction at the bottom of the tube or well, then proceed immediately to the next step.

d. At room temperature, add 5 µL of the 2 mg/mL NeutrAvidin™ solution to 1 tube or well and add 5 µL of PBS to the other tube or well.

e. Mix thoroughly by gently vortexing the tube. Centrifuge briefly to collect contents of the tube, and incubate for 5 minutes at room temperature.

2. Separate the fragmented and labeled ss-cDNA by size and stain.

a. Prepare 10 bp and 100 bp DNA ladders by combining 1 µL of ladder and 7 µL of Nuclease-free Water.

b. At room temperature, add loading dye to all samples and DNA ladders to a final concentration of 1X loading dye.

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

d. Carefully load samples and ladders on gel. Each well can hold a maximum of 20 µL.

e. Run the gel at 150 volts until the front dye almost reaches the bottom, ~1 hour.

f. While the gel is running, prepare 100 mL of a 1X solution of SYBR™ Gold for staining. SYBR™ Gold may be diluted in 1X TBE running buffer or water.

Note: SYBR™ Gold is light sensitive. Use caution and shield the staining solution from light. Prepare a new batch of stain at least once a week.

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g. After the gel is complete, break open cartridge and stain the gel in 1X SYBR™ Gold for 10 minutes at room temperature.

3. Place the gel on a UV light box and image using the appropriate filter for SYBR™ Gold.

4. The absence of a shift pattern indicates poor biotin labeling. The problem should be addressed before proceeding to the hybridization step.

B

Troubleshooting

Troubleshooting

Table 25 Troubleshooting GeneChip™ WT PLUS Reagent Kit procedures.

Observation	Possible cause	Solution
The positive control sample and the total RNA sample yield low levels of amplified cRNA product or low levels of appropriately sized cRNA product.	Incubation temperatures are incorrect or inaccurate.	Calibrate the thermal cycler.
	Condensation formed in the tubes during the incubations.	Ensure that the heated lid is working correctly and is set to the appropriate temperature.
	cRNA purification is not performed properly.	Perform the purification as described in this user guide.
	Pipettes, tubes, or equipment are contaminated with nucleases.	Use an RNase decontamination solution to remove RNases and DNases from surfaces.
The positive control sample produces expected results, but the total RNA sample results in low levels of amplified cRNA/cDNA product.	The input total RNA concentration is lower than expected.	Repeat the A_{260} reading of the RNA sample.
		Use 100–200 ng of total RNA in the first-strand cDNA synthesis procedure.
	The input RNA contains contaminating DNA, protein, phenol, ethanol, or salts, causing inefficient reverse transcription.	Phenol extract and ethanol precipitate the total RNA.
The positive control sample produces expected results but the total RNA sample results in low levels of appropriately sized cRNA/cDNA product.	The total RNA is partially degraded, thereby generating short cDNA fragments.	Assess the integrity of the total RNA sample by determining the size of the 18S and 28S rRNA bands and the relative abundance of 28S to 18S rRNA.
	The mRNA content of the total RNA sample is lower than expected.	Verify the mRNA content of the total RNA. **Note:** In healthy cells, mRNA constitutes 1–10% of total cellular RNA.

C

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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  - Safety Data Sheets (SDSs; also known as MSDSs)

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

Limited product warranty

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