GeneChip™ WT Pico Reagent Kit

USER GUIDE

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

Catalog Numbers 902622 and 902623

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Revision B.0

ThermoFisher SCIENTIFIC

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Products:
GeneChip™ WT Pico 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 GeneAtlas™ system requirements for array strips. Added reagent reservoir for multichannel pipettes.
A.0	10 July 2018	Initial release in Thermo Fisher Scientific document control system. Supersedes legacy Affymetrix Publication Number 703262. Updated to the current document template, with associated updates to trademarks, logos, licensing, and warranty.

Contents

1 GeneChip™ WT Pico Reagent Kit

Product information

Purpose

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

Sample requirements

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

Table 1 Input RNA limits.

RNA input	Total RNA from fresh-frozen cells or tissues	Total RNA from formalin-fixed, paraffin-embedded tissues
Minimum	100 pg	500 pg
Recommended	500 pg—10 ng	2 ng—50 ng
Maximum	10 ng	50 ng

To ensure high reproducibility in whole transcriptome amplification, we recommend using a starting amount of 500 pg RNA template of purified total RNA from cells or tissues, or 2 ng of purified total RNA from FFPE tissues. Depending on the copy number of the transcripts, it may be possible to use smaller amounts of RNA template (Table 1). Input amounts that are lower than the recommended amounts can result in insufficient yields, poor conversion to cDNA, and reduction in array signals. The starting RNA template should not be less than the minimum input amounts listed in Table 1. If your RNA sample is not limiting, we recommend that you start with more total RNA.

Performance specifications

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

Assay workflow

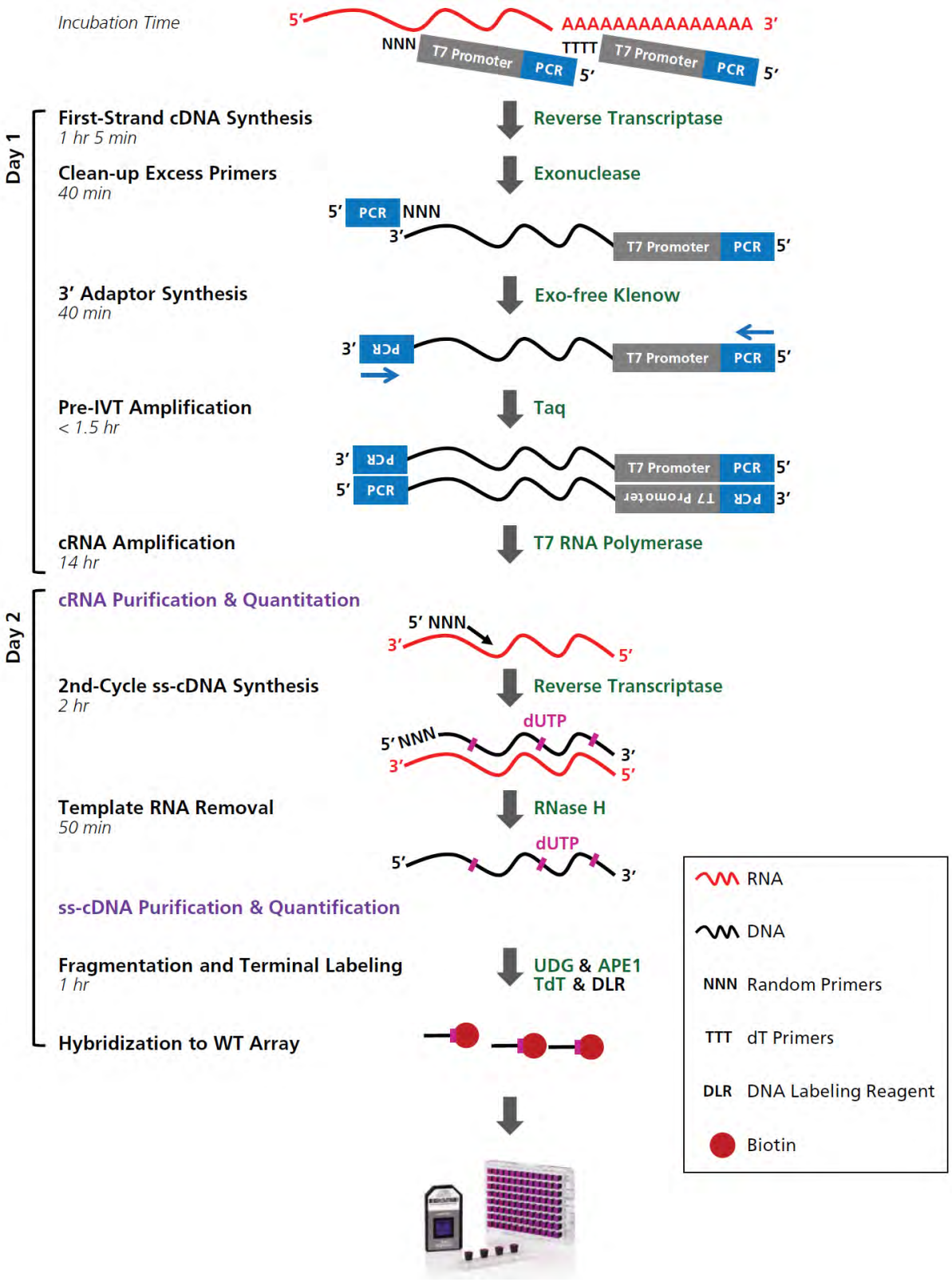

Figure 1 GeneChip™ WT Pico Reagent Kit Amplification and Labeling process.

Kit contents and storage

Table 2 GeneChip™ WT Pico Reagent Kit contents and storage.

Component	12-reaction kit for manual use (902622)	30-reaction kit for manual use (902623)	Storage
WT Pico Amplification Kit, Module 1, –20°C: cRNA Step			
WT Pico First-Strand Enzyme	14 µL	45 µL	–20°C
WT Pico First-Strand Buffer	500 µL	500 µL	–20°C
WT Pico Cleanup Reagent	28 µL	90 µL	–20°C
WT Pico 3' Adaptor Enzyme	14 µL	45 µL	–20°C
WT Pico 3' Adaptor Buffer	98 µL	245 µL	–20°C
WT Pico PCR Enzyme	14 µL	45 µL	–20°C
WT Pico PCR Buffer	407 µL	1015 µL	–20°C
WT Pico IVT Enzyme	84 µL	210 µL	–20°C
WT Pico IVT Buffer	1900 µL	1900 µL	–20°C
Poly-A Control Stock	16 µL	16 µL	–20°C
Nuclease-free Water	4 x 1 mL	10 x 1 mL	Any temperature[1]
WT Pico Amplification Kit, Module 2, –20°C: cDNA Step			
WT Pico 2nd-Cycle Primers	56 µL	140 µL	–20°C
WT Pico 2nd-Cycle ss-cDNA Buffer	500 µL	500 µL	–20°C
WT Pico 2nd-Cycle ss-cDNA Enzyme	112 µL	280 µL	–20°C
WT Pico RNase H	56 µL	180 µL	–20°C
WT Pico Frag. & Label Enzyme	28 µL	70 µL	–20°C
WT Pico Frag. & Label Buffer	168 µL	420 µL	–20°C
WT Pico Amplification Kit, Module 3 Purification Beads, 4°C:			
Purification Beads	3 mL	8 mL	4°C[2]
Control HeLa RNA, –20°C, HeLa total RNA			
Control RNA (100 ng/µL HeLa total RNA)	6 µL	6 µL	–20°C

Table 2 GeneChip WT Pico Reagent Kit contents and storage. *(continued)*

Component	12-reaction kit for manual use (902622)	30-reaction kit for manual use (902623)	Storage
GeneChip™ Hybridization Control Kit (900454)			
20X Hybridization Controls	450 µL	450 µL	–20°C
3 nM Control Oligo™ B2	150 µL	150 µL	–20°C
Tubes Organizer: Plastic vinyl template for organization and storage of components in 9 x 9 array, 81-places square wells, 5.25 x 5.25 x 2 in. (133 x 133 x 52 mm) (for example, Nalgene™ Polycarbonate 9 x 9 CryoBox™, 5026-0909 , or equivalent).			

[1] *Store the Nuclease-free Water at –20°C, 4°C, or room temperature.*
[2] *Do not freeze.*

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 3 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	• Fisher Scientific™, A32782 • or Fisher Scientific™, AM10027 • or Fisher Scientific™, AM10050 • or equivalent
Microcentrifuge	**MLS**
• NanoDrop™ UV-Vis Spectrophotometer or equivalent • Optional: NanoDrop™ Fluorospectrometer or equivalent • Optional: Qubit™ 2.0 Fluorometer or equivalent	**MLS**
Optional: Agilent™ 2100 Bioanalyzer™ Instrument or equivalent	**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 4 GeneChip™ Systems required for array processing.

Instruments	Source
GeneChip™ system for cartridge arrays	
GeneChip™ Hybridization Oven 645 (110/220V)	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 (MC) Instrument (NA/Japan, includes 110v UPS)	00-0372
GeneTitan™ Multi-Channel (MC) Instrument (Int'l Version, includes 220v UPS)	00-0373
GeneTitan™ Multi-Channel (MC) Instrument Upgrade from GeneTitan™ Single Channel	00-0360
GeneTitan™ Multi-Channel (MC) Instrument (Int'l Version, 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 5 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 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 only)	**MLS**
Nuclease-free Water (for preparing 80% ethanol wash solution)	BP248450 or **MLS**
Optional: • RNA Quantification Kit For SYBR™ Green I ROX™ Passive Ref Dye or equivalent • RNA Quantification Kit For SYBR™ Green I and Fluorescein Passive Ref Dye or equivalent	• 902905 • 902906
Optional: • Quant-iT™ RiboGreen™ RNA Assay Kit or equivalent • Qubit™ RNA HS Assay Kit or equivalent	• R11490 • Q32852
Optional: • Agilent™ RNA 6000 Nano Kit or equivalent • Agilent™ RNA 6000 Pico Kit or equivalent	• Fisher Scientific™, 50671511 • Fisher Scientific™, 50671513
Tough-Spots™ labels	**MLS**
Optional: 96-well plate sealing film	**MLS**
(*Optional*) Reagent reservoir for multichannel pipette	**MLS**
100% Ethanol (Molecular Biology Grade or equivalent)[1]	**MLS**

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

2 Protocol

Procedural notes

Implementing a plan to maintain procedural consistency

We recommend implementing a detailed plan to minimize sample-to-sample variation that is caused by subtle procedural differences in gene expression assays. A plan to standardize the variables in the procedure should include:

- Method of RNA purification (“Purifying total RNA” on page 13)
- RNA quality and integrity (“Evaluation of RNA quality” on page 13 and “Evaluation of RNA integrity” on page 13)
- Method of RNA quantitation (“Evaluation of RNA quantity” on page 14)
- Equipment preparation (“Equipment preparation” on page 15)
- Reagent preparation (“Reagent preparation” on page 16)
- RNase contamination prevention (“RNase contamination prevention” on page 17)
- DNA contamination prevention (“DNA contamination prevention” on page 17)
- Workflow stopping points

Sample preparation

Purifying total RNA

Total RNA samples should be free of genomic DNA. 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.

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

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

Evaluation of RNA quality

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

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

Evaluation of RNA integrity

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

Two methods to evaluate RNA integrity are:

- Microfluidic analysis, using the Agilent™ 2100 Bioanalyzer™ Instrument 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 high concentration samples of 25 to 500 ng/µL, use the Agilent™ RNA 6000 Nano Kit and for low concentration samples of 0.05 to 5 ng/µL, use the Agilent™ RNA 6000 Pico Kit. For more information on how to calculate RIN, go to **www.genomics.agilent.com**.

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

- 28S and 18S rRNA bands are resolved into 2 discrete bands that have no significant smearing below each band.
- 28S rRNA band intensity is approximately twice that of the 18S rRNA band.

Evaluation of RNA quantity

Both the type and amount of sample RNA available should be considered when planning your experiment.

Because mRNA content varies significantly with tissue type, the total RNA input should be empirically determined for each for each tissue type or experimental condition. The recommended total RNA inputs in Table 1 are based on total RNA from HeLa cells and 1 to 9 years old FFPE tissues. These table values serve as reference points for determining your optimal RNA input. If your RNA sample is not limiting, we recommend that you start with more total RNA.

Determining RNA quantity by UV absorbance

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

Determining RNA quantity by Fluorescence-Based quantitation

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

Determining RNA quantity using an Agilent™ 2100 Bioanalyzer™ Instrument

We do not recommend RNA concentration determination using an Agilent™ 2100 Bioanalyzer™ Instrument because it is not accurate, especially at RNA concentrations of less than 25 ng/µL. The Agilent™ Technologies, Inc. Agilent™ RNA 6000 Nano Kit may be used for total RNA quantity determination of high concentration samples (25 to 500 ng/µL), whereas the Agilent™ RNA 6000 Pico Kit should not be used for total RNA quantitation.

RNA quantitation by quantitative, Real-Time RT-PCR

For amounts of RNA that are too small for quantitation by UV absorbance or fluorometric assays, Real-Time RT-PCR should be used for quantitation. For your convenience, we offer the RNA Quantification Kit For SYBR™ Green I ROX™ Passive Ref Dye in addition to the RNA Quantification Kit For SYBR™ Green I and Fluorescein Passive Ref Dye for SYBR Green I and Fluorescein Passive Reference Dye. These kits can reliably and reproducibly quantify small amounts of RNA samples by measuring levels of abundance gene such as 18S ribosomal RNA, or beta actin prior to performing target preparation for microarray experiments.

Equipment preparation

Recommended thermal cycler

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

Program the thermal cycler

The temperature for the heated lid should be set to or near the required temperature for each step. An alternate protocol may be used for thermal cyclers that lack a programmable heated lid, although this is not the preferred method. Yields of cRNA may be reduced if a heated lid is used during the 3' Adaptor cDNA Synthesis step. We recommend leaving the heated lid open during the 3' Adaptor cDNA Synthesis. A small amount of condensation will form during the incubation. Condensation is expected and should not significantly decrease cRNA yields.

Incubation temperatures and times are critical for effective RNA amplification. Thermal cyclers should be properly calibrated and adhere closely to the incubation times.

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

Table 6 Thermal cycler protocols.

Protocol	Heated lid temperature	Step 1	Step 2	Step 3	Step 4	Volume
First-Strand cDNA Synthesis	42°C or 105°C	25°C for 5 minutes	42°C for 60 minutes	4°C for 2 minutes		10 µL
Cleanup	80°C or 105°C	37°C for 30 minutes	80°C for 10 minutes	4°C for 2 minutes		12 µL
3' Adaptor cDNA Synthesis	RT, disable, or left open	15°C for 15 minutes	35°C for 15 minutes	70°C for 10 minutes	4°C for 2 minutes	20 µL
Pre-IVT Amplification	105°C	95°C for 2 minutes	6, 9 or 12 cycles of 94°C for 30 seconds, 70°C for 5 minutes	4°C for 2 minutes		50 µL
In Vitro Transcription cRNA Synthesis	40°C or 105°C[1]	40°C for 14 hours	4°C, hold			80 µL
2nd-Cycle ss-cDNA Synthesis	70°C or 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 or 105°C	37°C for 45 minutes	95°C for 5 minutes	4°C, hold		44 µL
Fragmentation and Labeling	93°C or 105°C	37°C for 60 minutes	93°C for 2 minutes	4°C hold		60 µL

Table 6 Thermal cycler protocols. *(continued)*

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

[1] Use 0.2 mL or larger volume tubes or plates when using heated-lid setting at 105°C.

Table 7 Pre-IVT amplification cycling guidelines based on sample type and the amount of starting total RNA input.

RNA input	Typical number of PCR cycles for fresh-frozen cell or tissue samples	Typical number of PCR cycles for formalin-fixed, paraffin-embedded tissue samples
100 pg to <500 pg	12	N/A
500 pg to <2 ng	9	12
2 ng to 10 ng	6	9
>10 ng to 50 ng	N/A	6

Note: One or more PCR cycles may be added to the cycling guidelines for the Pre-IVT Amplification protocol to improve cRNA yield of poor quality RNA sample.

Reagent preparation

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

Handle kit components as follows:

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

- Return the components to the recommended storage temperature immediately after use.
- Ensure that all temperature transitions to incubation temperatures are rapid and/or well-controlled to help maintain consistency across samples.

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

RNase contamination prevention

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

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

DNA contamination prevention

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

- Before you set up the experiment, make sure you have 2 physically separated work areas with dedicated supplies and equipment in each area:
  - A Preamplification Clean Area for performing all preamplification reactions:
    - Prepare total RNA sample
    - Prepare poly-A RNA controls
    - Prepare total RNA/Poly-A RNA control mixture
    - Synthesize first-strand cDNA
    - Synthesize 3’ adaptor cDNA
    - Synthesize double-stranded cDNA (reaction setup)
  - A Post-Amplification Area for performing all post-amplification reactions and concentration measurements:
    - Synthesize double-stranded cDNA (reaction incubation)
    - Synthesize cRNA by *in vitro* transcription
    - Purify cRNA
    - Synthesize 2nd-cycle single-stranded cDNA
    - Hydrolyze RNA using RNase H
    - Purify 2nd-cycle single-stranded cDNA
    - Fragment and label single-stranded cDNA
    - Measure concentrations
- Maintain a single-direction workflow. Do not bring amplified products into the Preamplification Clean Area.

- Keep dedicated equipment in each of the areas used for this protocol, including pipettes, ice buckets, coolers, etc. Do not move equipment back and forth between the areas.
- Clean and decontaminate work areas and instruments, including pipettes, with commercially available decontamination reagents.
- Always use filter tips for pipetting to reduce sample contamination.

Prepare Control RNA

Prepare HeLa positive Control RNA

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

1. Dispense 2 µL of the Control RNA in 38 µL of Nuclease-free Water for a total volume of 40 µL (5 ng/µL), while on ice.
2. Add 2 µL of the first dilution (5 ng/µL) to 38 µL of Nuclease-free Water for a total volume of 40 µL (250 pg/µL).
3. Follow the "Prepare total RNA/poly-A RNA control mixture" on page 20, but use 2 µL of the second dilution (500 pg) in the control reaction.

Note:

- Measure concentration of HeLa positive Control RNA using a NanoDrop™ Spectrophotometer, and use the measured concentration for calculation and preparing the 250 pg/µL working stock.
- The positive control reaction should produce >20 µg of cRNA and >5.5 µg of 2nd-cycle ss-cDNA from 500 pg Control RNA.

Prepare poly-A RNA control

Note:

- We strongly recommend the use of poly-A RNA controls for all reactions that will be hybridized to GeneChip™ arrays. To include the premixed controls from the Poly-A Control Stock, prepare appropriate dilution of the poly-A RNA controls and add to the total RNA samples. Follow the "Prepare total RNA/poly-A RNA control mixture" on page 20.
- Do not use the Poly-A Control Dil Buffer to prepare a serial dilution of poly-A RNA controls because it may cause nontarget amplification.
- Prepare a serial dilution of Poly-A Control Stock with Nuclease-free Water.

A supplied set of poly-A RNA controls is designed specifically to provide exogenous positive controls to monitor the entire target preparation. The controls should be added to the total 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 Nuclease-free Water

and spiked directly into RNA samples to achieve the final concentrations (referred to as a ratio of copy number) summarized in Table 8.

Table 8 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 Nuclease-free Water are provided in the WT Pico Amplification Kit, Module 1 to prepare the appropriate serial dilutions based on Table 9. The table serves as a guideline when ≤1, 2, 5, 10, 20, or 50 ng of total RNA is used as starting material. For starting sample amounts other than those listed here, calculations are needed in order to perform the appropriate dilutions to arrive at the same proportionate final concentration of the spike-in controls in the samples.

Table 9 Serial dilutions of Poly-A Control Stock with Nuclease-free Water.

Total RNA input amount	Serial dilutions				Volume of fourth dilution to add to total RNA
	First dilution	Second dilution	Third dilution	Fourth dilution	
≤1 ng	1:50	1:100	1:100	1:100	2 µL
2 ng	1:50	1:100	1:100	1:50	2 µL
5 ng	1:50	1:100	1:100	1:20	2 µL
10 ng	1:50	1:100	1:100	1:10	2 µL
20 ng	1:50	1:100	1:100	1:5	2 µL
50 ng	1:50	1:100	1:100	1:2	2 µL

IMPORTANT!

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

The following example allows you to prepare the poly-A RNA dilutions for 500 pg of total RNA:

1. Add 2 µL of the Poly-A Control Stock to 98 µL of Nuclease-free Water for the first dilution (1:50).
2. Add 2 µL of the first dilution to 198 µL of Nuclease-free Water to prepare the second dilution (1:100).
3. Add 2 µL of the second dilution to 198 µL of Nuclease-free Water to prepare the third dilution (1:100).
4. Add 2 µL of the third dilution to 198 µL of Nuclease-free Water to prepare the fourth dilution (1:100).
5. Add 2 µL of this fourth dilution to 500 pg of total RNA. The final volume of total RNA with the diluted poly-A controls should not exceed 5 µL.

Note: Always prepare fresh-dilution of Poly-A Control Stock, which is provided in the GeneChip™ WT Pico Reagent Kit.

Prepare total RNA/poly-A RNA control mixture

Prepare total RNA according to your laboratory's procedure. A maximum of 5 µL total RNA can be added to First-Strand cDNA Synthesis reaction. If you are adding Poly-A Control Stock to your RNA, the volume of RNA must be 3 µL or less (Table 10). See "Prepare poly-A RNA control" on page 18 for more information. For example, when performing the Control RNA reaction, 2 µL of RNA (250 pg/µL) should be combined with 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 your RNA, the volume of RNA must be 3 µL or less. If necessary, a SpeedVac™ Vacuum Concentrator or ethanol precipitation should be used to concentrate the RNA samples.

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

Component	Volume for 1 reaction
Total RNA Sample (100 pg—50 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.

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

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

1. Prepare the First-Strand Master Mix in a nuclease-free tube, while on ice. Combine the components in the sequence shown in Table 11. Prepare enough master mix for all the total RNA samples in the experiment.

   Note: Include ~10% excess volume to correct for pipetting losses when performing the following steps.

   Table 11 First-Strand Master Mix.

Component	Volume for 1 reaction
WT Pico First-Strand Buffer	4 µL
WT Pico First-Strand Enzyme	1 µL
Total volume	**5 µL**

2. Mix thoroughly by gently vortexing the tube and centrifuge briefly to collect the mix at the bottom of the tube. Then, proceed immediately to the next step.
3. Transfer 5 µL of the First-Strand Master Mix to each tube or well, while on ice.
4. Add 5 µL of the total RNA to each (5 µL) tube or well containing the First-Strand Master Mix for a final reaction volume of 10 µL, while on ice.

   See "Prepare total RNA/poly-A RNA control mixture" on page 20 for more information.
5. Mix thoroughly by gently vortexing the tube and centrifuge briefly to remove air bubbles and collect the reaction at the bottom of the tube or well. Then, proceed immediately to the next step.
6. Incubate the First-Strand cDNA Synthesis reaction in a thermal cycler for 5 minutes at 25°C, 60 minutes at 42°C and then for at least 2 minutes at 4°C.

   The First-Strand cDNA Synthesis protocol that is shown in Table 6 can serve as a reference.
7. Centrifuge briefly to collect the first-strand cDNA at the bottom of the tube or well immediately after the incubation.
8. Place the sample on ice for 2 minutes to cool the plastic. Then, proceed immediately to the next step.

9. Transfer 2 µL of WT Pico Cleanup Reagent to each (10 µL) cDNA sample for a final reaction volume of 12 µL, while on ice.

10. Pipet up and down twice and carefully eject all liquid from the pipette tip to ensure complete transfer of the cleanup reagent.

11. Mix thoroughly by gently vortexing the tube, centrifuge briefly to remove air bubbles and collect the reaction at the bottom of the tube or well. Then, proceed immediately to the next step.

Note: Air bubbles that may form during mixing should be removed by a brief centrifuge.

12. Incubate the first-strand cleanup reaction in a thermal cycler for 30 minutes at 37°C, 10 minutes at 80°C, and then for at least 2 minutes at 4°C.

    The Cleanup protocol that is shown in Table 6 can serve as a reference.

13. Centrifuge briefly to collect the first-strand cDNA at the bottom of the tube or well, immediately after the incubation.

14. Place the sample on ice for 2 minutes to cool the plastic and proceed immediately to "Synthesize 3' Adaptor cDNA" on page 23.

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

Tip: When approximately 15 minutes remain on the thermal cycler, you may start reagent preparation for 3' Adaptor cDNA synthesis.

Synthesize 3' Adaptor cDNA

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

IMPORTANT! Precool the thermal cycler block to 15°C while preparing the 3' Adaptor Master Mix.

1. Prepare the 3' Adaptor Master Mix in a nuclease-free tube, while on ice. Combine the components in the sequence shown in Table 12. Prepare enough master mix for all the first-strand cDNA samples in the experiment.

   Note: Include ~10% excess volume to correct for pipetting losses.

   Table 12 3' Adaptor Master Mix.

Component	Volume for 1 reaction
WT Pico 3' Adaptor Buffer	7 µL
WT Pico 3' Adaptor Enzyme	1 µL
Total Volume	**8 µL**

2. Mix thoroughly by gently vortexing the tube and centrifuge briefly to collect the mix at the bottom of the tube. Then, proceed immediately to the next step.

3. Transfer 8 µL of the 3' Adaptor Master Mix to each (12 µL) First-Strand cDNA sample for a final reaction volume of 20 µL, while on ice.

4. Mix thoroughly by gently vortexing the tube and centrifuge briefly to collect the reaction at the bottom of the tube or well. Then proceed immediately to the next step.

5. Incubate the 3' Adaptor cDNA synthesis reaction in a thermal cycler for 15 minutes at 15°C, 15 minutes at 35°C, 10 minutes at 70°C, and then for at least 2 minutes at 4°C.

   The 3' Adaptor cDNA Synthesis protocol that is shown in Table 6 can serve as a reference.

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

6. Centrifuge briefly to collect the 3' Adaptor cDNA at the bottom of the tube or well, immediately after the incubation.

7. Place the sample on ice and proceed immediately to "Synthesize double-stranded cDNA" on page 24.

   Tip: When approximately 15 minutes remain on the thermal cycler, you may start reagent preparation for pre-IVT amplification.

Synthesize double-stranded cDNA

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

1. Prepare the Pre-IVT Amplification Master Mix in a nuclease-free tube, while on ice. Combine the components in the sequence shown in Table 13. Prepare enough master mix for all the cDNA samples in the experiment.

   Note: Include ~10% excess volume to correct for pipetting losses.

   Table 13 Pre-IVT Amplification Master Mix.

Component	Volume for 1 reaction
WT Pico PCR Buffer	29 µL
WT Pico PCR Enzyme	1 µL
Total volume	**30 µL**

2. Mix thoroughly by gently vortexing the tube and centrifuge briefly to collect the mix at the bottom of the tube. Then, proceed immediately to the next step.

3. Transfer 30 µL of the Pre-IVT Amplification Master Mix to each (20 µL) 3' Adaptor cDNA sample for a final reaction volume of 50 µL, while on ice.

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

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

5. Incubate the pre-IVT amplification reaction in a thermal cycler for 2 minutes at 95°C, for 6, 9, or 12 cycles of 30 seconds at 94°C and 5 minutes at 70°C, then for at least 2 minutes at 4°C.

   Both Table 6 and Table 7 can serve as valuable references.

6. Centrifuge briefly to collect the double-stranded cDNA at the bottom of the tube or well immediately after the incubation.

7. Place the sample on ice and proceed immediately to "Synthesize cRNA by in vitro transcription" on page 25.

   Note: One or 2 PCR cycles may be added to the cycling guidelines for the Pre-IVT Amplification protocol to improve cRNA yield of poor quality RNA sample.

   Tip: When approximately 15 minutes remain on the thermal cycler, you may start reagent preparation for *in vitro* transcription.

Synthesize cRNA by *in vitro* transcription

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

IMPORTANT!

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

1. Prepare the IVT Master Mix in a nuclease-free tube, while at room temperature. Combine the components in the sequence shown in Table 14. Prepare enough Master Mix for all the double-stranded cDNA samples in the experiment.

   Note: Include ~10% excess volume to correct for pipetting losses.

   Table 14 IVT Master Mix.

Component	Volume for 1 reaction
WT Pico IVT Buffer	24 µL
WT Pico IVT Enzyme	6 µL
Total volume	**30 µL**

2. Mix thoroughly by gently vortexing the tube and centrifuge briefly to collect the mix at the bottom of the tube. Then, proceed immediately to the next step.

3. Transfer 30 µL of the IVT Master Mix to each (50 µL) double-stranded cDNA sample for a final reaction volume of 80 µL, while at room temperature.

4. Mix thoroughly by gently vortexing the tube and centrifuge briefly to collect the reaction at the bottom of the tube or well. Then, proceed immediately to the next step.

5. Incubate the IVT reaction in a thermal cycler for 14 hours at 40°C, and then at 4°C.

   The *in vitro* Transcription cRNA Synthesis protocol that is shown in Table 6 can serve as a reference.

6. Centrifuge briefly to collect the cRNA at the bottom of the tube or well, after the incubation.

7. Place the reaction on ice and proceed to “Purify cRNA using Purification Beads” on page 26, or immediately freeze the samples at –20°C for storage.

   Note: The IVT incubation time may be extended up to 16 hours.

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

Purify cRNA using Purification Beads

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 they are fully dispersed. Transfer the appropriate amount of Purification Beadsto a nuclease-free tube or container and allow the Purification Beads to equilibrate at room temperature. For each reaction, 140 µL plus ~10% overage will be needed.
- Prepare fresh dilutions of 80% ethanol wash solution each time from 100% ethanol (Molecular Biology Grade or equivalent) and Nuclease-free Water in a nuclease-free tube or container. For each reaction, 600 µL plus ~10% overage will be needed.
- Transfer the cRNA sample to room temperature while preparing the Purification Beads.

Note:

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

1. Mix the Purification Beads container by vortexing to resuspend the magnetic particles that may have settled.
2. Transfer 140 µL of the Purification Beads to a clean well of a round bottom plate.
3. Add 80 µL of cRNA sample to each (140 µL) Purification Beads, and mix by pipetting up and down.

   Tip:
   - Cover any unused wells with a plate sealer so that the plate can safely be reused.
   - Use a multichannel pipette when processing multiple samples.
   - Set the pipette to 150 µL and pipet slowly when mixing to avoid bubble formation.
4. Mix well by pipetting up and down 10 times.
5. Incubate for 10 minutes.

   The cRNA in the sample binds to the Purification Beads during this incubation.
6. 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.

7. Carefully aspirate and discard the supernatant without disturbing the Purification Beads. Keep the plate on the magnetic stand.
8. Add 200 µL of 80% ethanol wash solution to each well and incubate for 30 seconds, while on the magnetic stand.
9. Slowly aspirate and discard the 80% ethanol wash solution without disturbing the Purification Beads.
10. Repeat step 8 and step 9 twice for a total of 3 washes with 200 µL of 80% ethanol wash solution.
11. Completely remove the final wash solution.
12. Air-dry on the magnetic stand for 5 minutes until no liquid is visible, yet the pellet appears shiny. Additional time may be required. Do not over-dry the beads as this will reduce the elution efficiency. The bead surface will appear dull and may have surface cracks when it is over-dried.
13. Remove the plate from the magnetic stand.
14. Add to each sample 27 µL of the preheated (65°C) Nuclease-free Water and incubate for 1 minute.
15. Mix well by pipetting up and down 10 times.
16. Move the plate to the magnetic stand for ~5 minutes to capture the Purification Beads.
17. Transfer the supernatant, which contains the eluted cRNA, to a nuclease-free tube.
18. Place the purified cRNA samples on ice and proceed to "Assess cRNA yield" on page 28, or immediately freeze the samples at –20°C for storage.

Note:

- Minimal bead carryover does 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 a high concentration cRNA, an additional 10 to 70 µL of the preheated Nuclease-free Water should be added to the well. Allow the mixture to incubate for 1 minute and repeat the procedure from step 15.

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

Assess cRNA yield

Expected cRNA yield

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

A wide variety of tissue types were used during development of this kit. 500 pg of input total RNA from fresh-frozen samples and 2 ng of input total RNA from FFPE samples yielded >20 µg of cRNA. Figure 2 shows yield data for cRNA produced with the kit from several different types of input RNA.

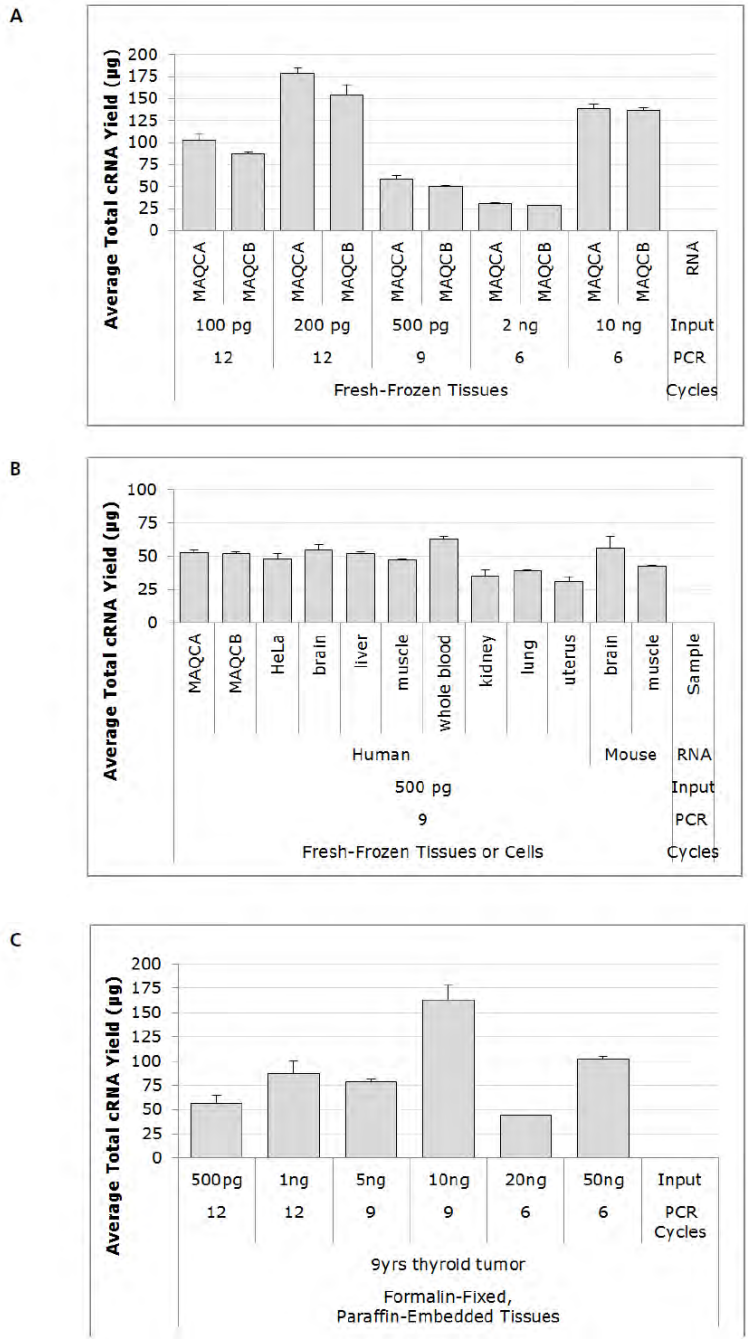

Figure 2 Average cRNA yield.

Average cRNA yield is calculated from total RNA samples from microarray quality control (MAQC) (A), a variety of Fresh-Frozen tissues (B), and a 9 year old FFPE tissue (C).

Determine cRNA yield by UV absorbance

The concentration of a cRNA solution can be determined by measuring its absorbance at 260 nm. Nuclease-free Water should be used as a blank. We recommend using NanoDrop™ spectrophotometers for convenience. No dilutions or cuvettes are needed; 1 µL to 1.5 µL of the cRNA sample should be added directly. Samples with cRNA concentrations greater than 3,000 ng/µL should be diluted with Nuclease-free Water before measurement and reaction setup. The diluted cRNA should be used as the input to prepare 20 µg cRNA in 2nd-cycle cDNA synthesis reaction.

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

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

where 1 A_{260} = 40 µg RNA/mL.

(Optional) Determining cRNA size distribution

Note: The expected cRNA profile is a distribution of sizes from 200 nt to 1,000 nt. Determining the cRNA size distribution is optional. Upon determining the size distribution, the purified cRNA samples can be stored overnight at –20°C.

cRNA size distribution can be analyzed using an Agilent™ 2100 Bioanalyzer™ Instrument, an Agilent™ RNA 6000 Nano Kit (Cat. No. 50671511), and an mRNA Nano Series II Assay. If there is sufficient yield, approximately 500 ng of cRNA per well should be loaded on the Agilent™ 2100 Bioanalyzer™ Instrument. To analyze cRNA size using an Agilent™ 2100 Bioanalyzer™ Instrument, follow the manufacturer's instructions.

Synthesize 2nd-Cycle single-stranded cDNA

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

1. Prepare 833 ng/µL cRNA, while on ice.

   This concentration is equal to 20 µg cRNA in a volume of 24 µL. If necessary, Nuclease-free Water can be used to bring the cRNA sample to 24 µL.

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

2. Prepare the 2nd-Cycle ss-cDNA Master Mix in a nuclease-free tube, while on ice. Combine the components in the sequence shown in Table 15. Prepare enough master mix for all the cRNA samples in the experiment.

   Note: Include ~10% excess volume to correct for pipetting losses.

   Table 15 2nd-Cycle ss-cDNA Master Mix.

Component	Volume for 1 reaction
WT Pico 2nd-Cycle ss-cDNA Primers	4 µL
WT Pico 2nd-Cycle ss-cDNA Buffer	8 µL
WT Pico 2nd-Cycle ss-cDNA Enzyme	4 µL
Total volume	**16 µL**

3. Mix thoroughly by gently vortexing the tube and centrifuge briefly to collect the mix at the bottom of the tube. Then, proceed immediately to the next step.

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

5. Mix thoroughly by gently vortexing the tube and centrifuge briefly to collect the reaction at the bottom of the tube or well. Then, proceed immediately to the next step.

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

   The 2nd-Cycle ss-cDNA Synthesis protocol that is shown in Table 6 can serve as a reference.

7. Centrifuge briefly to collect the 2nd-cycle ss-cDNA at the bottom of the tube or well immediately after the incubation.

8. Place the sample on ice and proceed immediately to “Hydrolyze RNA using RNase H” on page 31.

Hydrolyze RNA using RNase H

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

1. Add 4 µL of the RNase H to each (40 µL) 2nd-Cycle ss-cDNA sample for a final reaction volume of 44 µL, while on ice.

2. Mix thoroughly by gently vortexing and centrifuge briefly to collect the reaction at the bottom of the tube or well. Then, proceed immediately to the next step.

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

   The RNA Hydrolysis protocol that is shown in Table 6 can serve as a reference.

4. Centrifuge briefly to collect the hydrolyzed 2nd-Cycle ss-cDNA at the bottom of the tube or well immediately after the incubation.

5. Place the samples on ice and proceed immediately to the next step.
6. 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.
7. Mix thoroughly by gently vortexing and centrifuge briefly to collect the reaction at the bottom of the tube or well.
8. Place the sample on ice and proceed to "Purify 2nd-cycle single-stranded cDNA" on page 32, or immediately freeze the samples at –20°C for storage.

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

Purify 2nd-cycle single-stranded cDNA

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

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 100% ethanol (Molecular Biology Grade or equivalent) and Nuclease-free Water in a nuclease-free tube or container. For each reaction, 600 µL plus ~10% overage will be needed.
- Transfer the cDNA sample to room temperature while preparing the Purification Beads.

Note:

- Occasionally, the bead/sample mixture may be brownish in color and not completely clear when placed on magnet. In those situations, switch to a different position of magnet on the magnetic stand, a new magnetic stand, or centrifuge out pellets.
- This entire procedure is performed at room temperature.

1. Mix the Purification Beads container by vortexing to resuspend the magnetic particles that may have settled.
2. Transfer 100 µL of the Purification Beads to a clean well of a round-bottom plate.
3. Add 55 µL of 2nd-cycle ss-cDNA sample to each (100 µL) Purification Beads, and mix by pipetting up and down.

Tip:

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

4. Add 150 µL of 100% ethanol to each (155 µL) ss-cDNA/Beads sample. Mix well by pipetting up and down 10 times.

5. Incubate for 20 minutes. The ss-cDNA in the sample binds to the Purification Beads during this incubation.

6. 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 the 2nd-Cycle ss-cDNA Synthesis protocol.

7. Carefully aspirate and discard the supernatant without disturbing the Purification Beads. Keep the plate on the magnetic stand.

8. Add 200 µL of 80% ethanol wash solution to each well and incubate for 30 seconds, while on the magnetic stand.

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

10. Repeat step 8 and step 9 twice for a total of 3 washes with 200 µL of 80% ethanol wash solution.

11. Completely remove the final wash solution.

12. 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 because this will reduce the elution efficiency. The bead surface will appear dull and may have surface cracks when it is over-dry.

13. Remove the plate from the magnetic stand.

14. Add to each sample 30 µL of the preheated (65°C) Nuclease-free Water and incubate for 1 minute.

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

16. Move the plate to the magnetic stand for ~5 minutes to capture the Purification Beads.

17. Transfer the supernatant, which contains the eluted ss-cDNA, to a nuclease-free tube.

18. Place the purified ss-cDNA samples on ice and proceed to “Assess single-stranded cDNA yield” on page 34, or immediately freeze the samples at –20°C for storage.

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

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

Assess single-stranded cDNA yield

Expected single-stranded cDNA yield

A wide variety of tissue types were used during development of this kit. Using 20 µg of input cRNA yielded 6 µg to 20 µg of ss-cDNA. For most tissue types, the recommended 20 µg of input cRNA should yield >5.5 µg of ss-cDNA. Figure 3 shows yield data for ss-cDNA produced with the kit from several different types of input RNA.

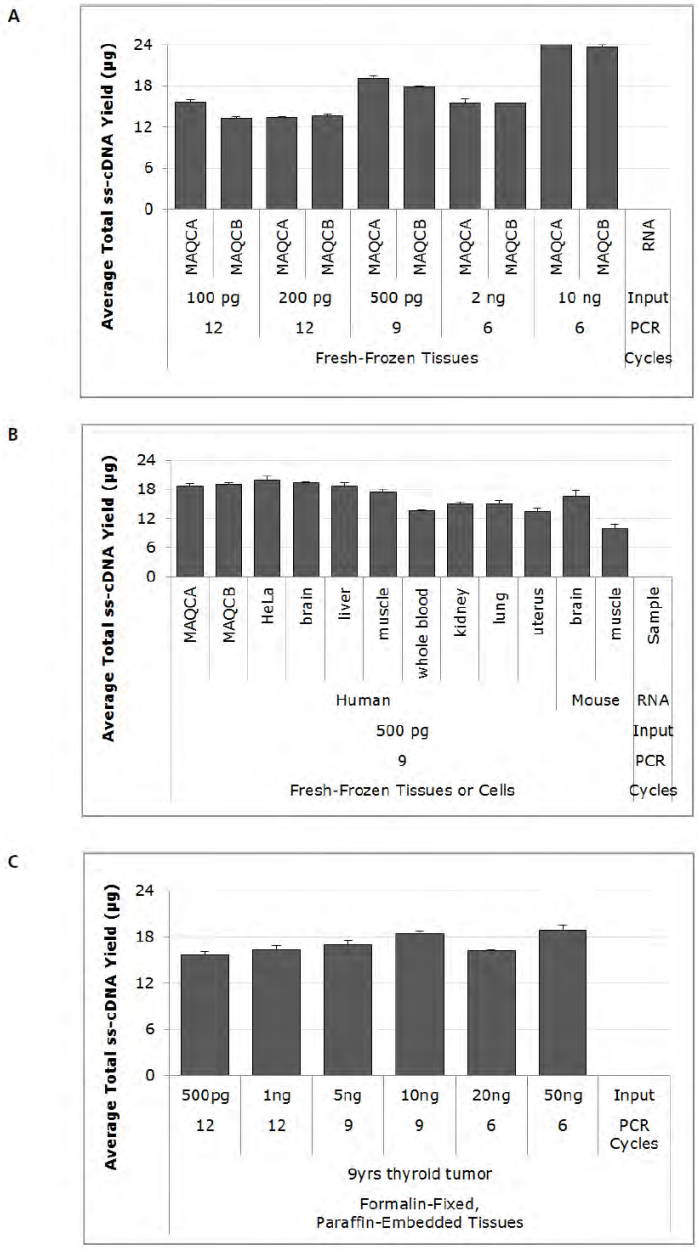

Figure 3 Average ss-cDNA yield.

Average ss-cDNA yield is calculated from total RNA samples from microarray quality control (MAQC) (A), a variety of Fresh-Frozen tissues (B), and a 9 year old FFPE tissue (C).

Determining single-stranded DNA yield by UV absorbance

The concentration of a ss-cDNA solution can be determined by measuring its absorbance at 260 nm. Nuclease-free Water should be used as a blank. We recommend using NanoDrop™ Spectrophotometers for your convenience. No dilutions or cuvettes are needed; 1 µL to 1.5 µL of the cDNA sample should be added directly.

Alternatively, the ss-cDNA concentration can be determined by diluting an aliquot of the preparation in Nuclease-free Water and reading the absorbance in a traditional spectrophotometer at 260 nm. The concentration in µg/mL can be calculated using the following equation:

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

where 1 A_{260} = 33 µg DNA/mL.

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

Determining single-stranded cDNA size distribution

Note: The expected cDNA profile does not resemble the cRNA profile. The expected cDNA profile is a distribution of sizes from 50 nt to 1,000 nt with most of the cDNA sizes in the 25-nt to 500-nt range. Determining the single-stranded cDNA size distribution is optional.

cDNA size distribution may be analyzed using an Agilent™ 2100 Bioanalyzer™ Instrument, an Agilent™ RNA 6000 Nano Kit (50671511), and an mRNA Nano Series II Assay. If there is sufficient yield, approximately 400 ng of cDNA per well should be loaded onto the Agilent™ 2100 Bioanalyzer™ Instrument. To analyze cDNA size using a Agilent™ 2100 Bioanalyzer™ Instrument, follow the manufacturer's instructions.

Note:

- The purified ss-cDNA samples can be stored overnight at –20°C. For long-term storage at –20°C, we recommend to store the samples as ss-cDNA and not to proceed to the fragmentation and labeling reaction.
- Although 100- or 81/4-format and 169- or 400-format arrays use less than 5.5 µg of fragmented and labeled ss-cDNA in hybridization, the fragmentation and labeling reaction should be performed with 5.5 µg of purified ss-cDNA.

Fragment and label single-stranded cDNA

In this procedure, the purified, sense-strand cDNA is fragmented by uracil-DNA glycosylase (UDG) and apurinic/apyrimidinic endonuclease 1 (APE 1) at the unnatural dUTP residues and breaks the DNA strand. The fragmented cDNA is labeled by terminal deoxynucleotidyl transferase (TdT) using a 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 120 ng/µL ss-cDNA, while on ice.

   This concentration is equal to 5.5 µg ss-cDNA in a volume of 46 µL. If necessary, use Nuclease-free Water to bring the ss-cDNA sample to 46 µL.

2. Prepare the Fragmentation and Labeling Master Mix in a nuclease-free tube, while on ice. Combine the components in the sequence shown in Table 16. Prepare enough master mix for all the ss-cDNA samples in the experiment.

   Note: Include ~10% excess volume to correct for pipetting losses.

   Table 16 Fragmentation and Labeling Master Mix.

Component	Volume for 1 reaction
WT Pico Fragmentation and Labeling Buffer	12 µL
WT Pico Fragmentation and Labeling Enzyme	2 µL
Total volume	**14 µL**

3. Mix thoroughly by gently vortexing the tube and centrifuge briefly to collect the mix at the bottom of the tube. Then, proceed immediately to the next step.

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

5. Mix thoroughly by gently vortexing the tube and centrifuge briefly to collect the reaction at the bottom of the tube or well. Then, proceed immediately to the next step.

6. Incubate the fragmentation reaction in a thermal cycler for 1 hour at 37°C, 2 minutes at 93°C, and then for at least 2 minutes at 4°C.

   The Fragmentation and Labeling Protocol in Table 6 can serve as a reference.

7. Centrifuge briefly to collect the fragmented and labeled ss-cDNA at the bottom of the tube or well immediately after the incubation.

8. Place the sample on ice and proceed immediately to the next step.

9. (Optional) The fragmented and labeled ss-cDNA sample can be used for size analysis using an Agilent™ 2100 Bioanalyzer™ Instrument, an Agilent™ RNA 6000 Nano Kit (Fisher Scientific™ Cat. No. 50671511), and mRNA Nano Series II Assay.

   See the reagent kit guide for the Agilent™ RNA 6000 Nano Kit for detailed instructions. The range in peak size of the fragmented samples should be approximately 40 nt to 70 nt.

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

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3 WT array hybridization

Cartridge array hybridization on the GeneChip™ Instrument

This section provides instruction for 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 into the GeneChip™ Command Console™ Software.

Target hybridization setup for cartridge arrays

Reagents and materials required

- GeneChip™ Hybridization, Wash, and Stain Kit (Not supplied, available separately. See Table 5.)
  - 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 (Cat. Nos. 900454 and 900457)
  - 20X Hybridization Controls *(bioB, bioC, bioD, cre)*
  - 3 nM Control Oligo™ B2
- WT cartridge arrays. (Not supplied, available separately)

Prepare and hybridize the array

1. Thaw the components listed in Table 17 at room temperature.

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

Table 17 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	5.5 µg	3.8 µg	2.5 µg	25 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 the specific probe array package insert for information on array format.

2. Heat the 20X Hybridization Controls for 5 minutes at 65°C in a thermal cycler.
   The Hybridization Control protocol that is shown in Table 6 can serve as a reference.

3. Prepare the Hybridization Master Mix in a nuclease-free tube, while at room temperature. Combine the appropriate volume of components in the sequence shown in Table 17. Prepare enough Hybridization Master Mix for all the fragmented and biotin-labeled ss-cDNA samples in the experiment.

   Note: Include ~10% overage to correct for pipetting losses.

4. Mix thoroughly by gently vortexing and centrifuge briefly to collect the mix, then proceed immediately to the next step.

5. Add the appropriate amount of Hybridization Master Mix to each fragmented and biotin-labeled ss-cDNA sample to prepare the Hybridization Cocktail, while at room temperature.

Table 18 Hybridization Cocktail for a single array.

Component	49- or 64-format	100- or 81/4-format	169- or 400-format	Final concentration
Hybridization Master Mix	160 µL	109 µL	73 µL	
Fragmented and labeled ss-cDNA	60 µL (5.5 µg)	41 µL (3.8 µg)	27 µL (2.5 µg)	25 ng/µL
Total volume	**220 µL**	**150 µL**	**100 µL**	

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

7. 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 in Table 6.

8. Centrifuge briefly to collect contents of the tube, then proceed immediately to the next step.

9. Inject and hybridize the 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 pipet tip into the upper right septum to allow for venting.

b. Inject the appropriate amount (see Table 19) of the specific sample through one of the septa on the back of the cartridge array see (Figure 4).

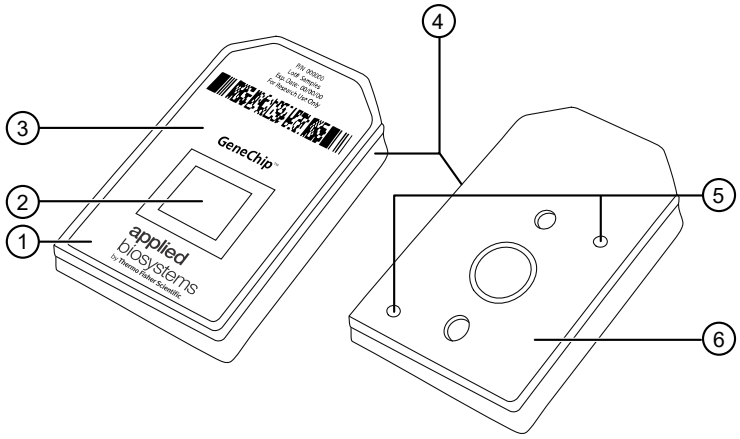


Figure 4 GeneChip™ cartridge array.

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

Table 19 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 array. Cover both septa with 1/2" Tough-Spots™ label to minimize evaporation and/or prevent leaks.

d. Place the arrays into hybridization oven trays, then load the trays into the GeneChip™ Hybridization Oven 645.

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: Prepare reagents for the washing and staining steps during the latter part of the 16-hour hybridization. These reagents are immediately required 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™ (GCC) 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.
3. (Optional) Transfer it to a new tube or well of a 96-well plate in order to save the Hybridization Cocktail mix. Then, store on ice during the procedure, or at –20°C for long-term storage.
4. Fill each array completely with Wash Buffer A.
5. 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.

6. Place vials into sample holders on the Fluidics Station:
   a. Place 1 (amber) vial containing 600 µL Stain Cocktail 1 in sample holder 1.
   b. Place 1 (clear) vial containing 600 µL Stain Cocktail 2 in sample holder 2.
   c. Place 1 (clear) vial containing 800 µL Array Holding Buffer in sample holder 3.
7. Wash the arrays according to array type and components used for hybridization, wash, and stain (Table 20).

   Table 20 Fluidics protocol.

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

8. 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.
9. Inspect the array glass surface for dust and/or other particulates and, if necessary, carefully wipe the surface with a clean lab wipe before scanning.

Scan the cartridge array

The instructions for using the GeneChip™ Scanner 3000 System can be found in the *GeneChip™ Command Console™ User Guide*.

Array strip hybridization on the GeneAtlas™ System

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 43.
- "GeneAtlas™ Instrument Control Software setup" on page 49.

IMPORTANT! Register samples as described in "GeneAtlas™ Instrument Control Software setup" on page 49 before preparing hybridization-ready samples.

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. See Table 5.)
  - 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 (*bioB*, *bioC*, *bioD*, *cre*)
  - 3 nM Control Oligo™ B2 (3 nM)
- Array strip and consumables (Not supplied.)
  - WT array strips
  - 1 hybridization tray per array strip

Prepare the Hybridization Cocktail and master mix for array strips

Note: If you are using a hybridization-ready sample, or rehybridizing a previously made Hybridization Cocktail, continue the protocol from step 1 in "Hybridize the array strip" on page 45.

1. Prepare the following (in preparation of hybridization):
   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 briefly (~5 seconds) to collect contents of the tube.

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

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

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

7. 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 6.

8. 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 enough Hybridization 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 contents at the bottom of the tube.

Table 21 Hybridization Master Mix.

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**	

9. Mix thoroughly by gently vortexing. Centrifuge briefly to collect the mix, then proceed immediately to the next step.

10. Prepare the Hybridization Cocktail in the order as shown in Table 22 for all samples, while at room temperature.

Table 22 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	25 ng/µL
2.5X WT Hyb Add 6	60 µL	1X
Total volume	**150 µL**	

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

12. Incubate the Hybridization Cocktail reaction for 5 minutes at 99°C (tubes) or 95°C (plates), and then for 5 minutes at 45°C in a thermal cycler using the Hybridization Cocktail protocol that is shown in Table 6.

13. Centrifuge briefly to collect contents of the tube or well and proceed immediately to the next step after the incubation.

Hybridize the array strip

1. Apply 120 µL of Hybridization Cocktail to the middle of the appropriate wells of a new clean hybridization tray (Figure 5).

IMPORTANT! Do not add more than 120 µL of Hybridization Cocktail to the wells. Adding more than 120 µL could result in cross-contamination of the samples.

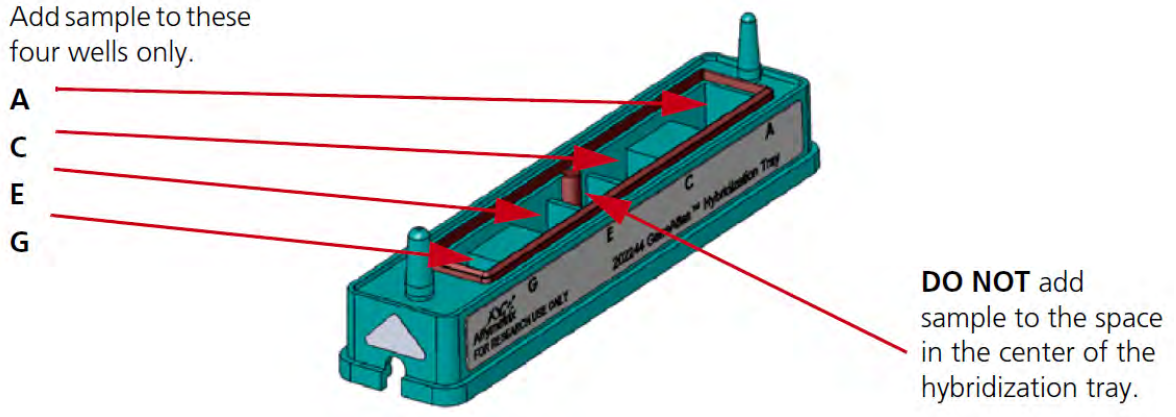


Figure 5 Location of the sample wells (A, C, E, and G) on the hybridization tray.

2. Carefully remove the array strip and protective cover from its foil pouch and place on bench (Figure 6).

IMPORTANT! Leave the array strip in the protective cover.

Figure 6 Array strip in protective tray.

3. Place the array strip into the hybridization tray containing the Hybridization Cocktail samples (Figure 7). See Figure 8 for proper orientation of the array strip in the hybridization tray.

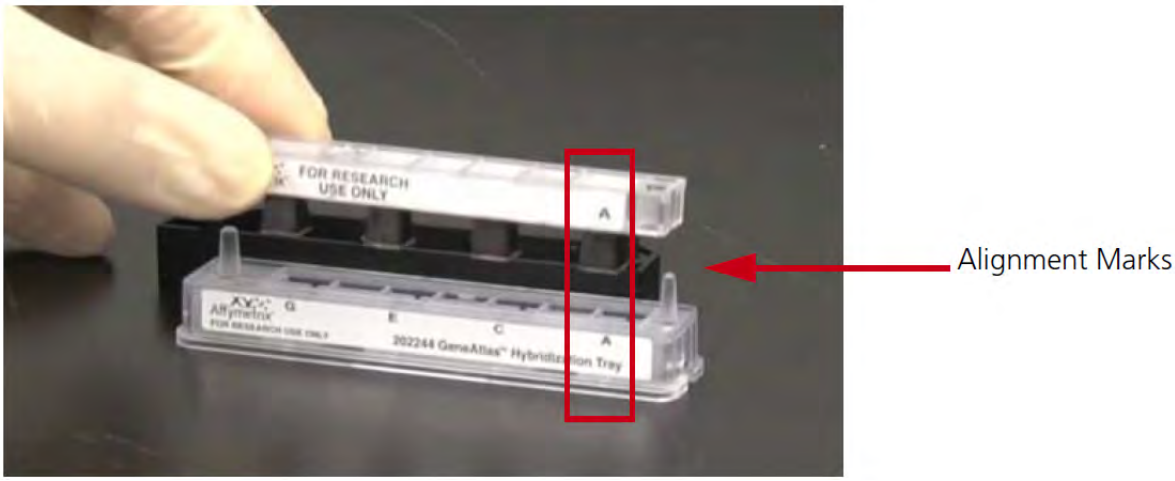


Figure 7 Placing the array strip into the hybridization tray.

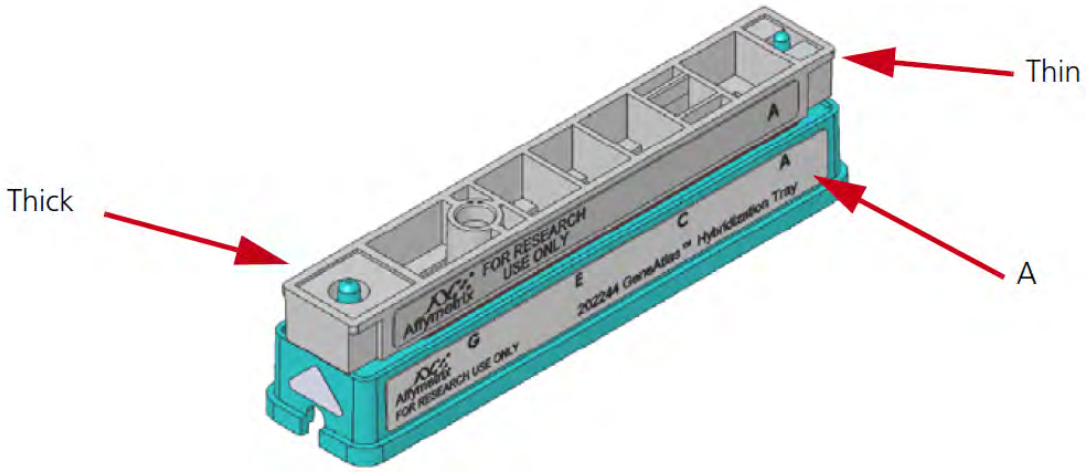


Figure 8 Proper orientation of the array strip in the hybridization tray.

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

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

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

5. 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.

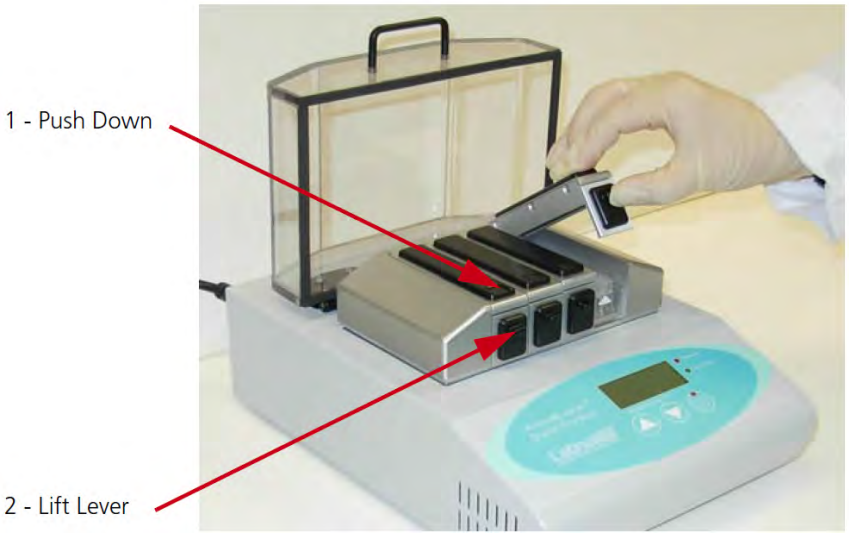
IMPORTANT! Insertion of the array strip and air bubble removal should be performed quickly to avoid drying of the array surface.

- 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. If no air bubbles are observed, proceed to the next step.

6. Open a GeneAtlas™ Hybridization Station clamp by applying pressure to the top of the clamp while gently squeezing inward. Lift the clamp to open, while squeezing (Figure 9).

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 9 .

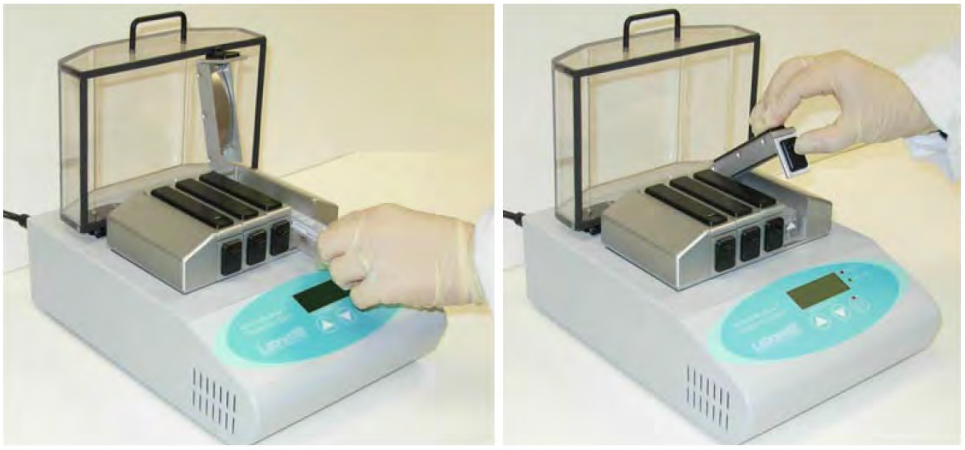
IMPORTANT! The hybridization temperature for GeneAtlas™ WT array strips is 48°C.



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

Figure 9 Opening the clamps on the GeneAtlas™ Hybridization Station.

7. Place the hybridization tray with the array strip into a clamp inside the GeneAtlas™ Hybridization Station and close the clamp as shown in Figure 10.



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

Figure 10 Laterally inserting an array strip and closing the clamp of the GeneAtlas™ Hybridization Station .

8. Proceed to Set up the Hybridization Software on page 51.

GeneAtlas™ Instrument Control 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 set up in the GeneAtlas™ Instrument Control 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*.

Register a sample

The following information provides general instructions for registering array strips in the GeneAtlas™ Instrument Control Software. For detailed information on sample registration, importing data from 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™ Instrument Control Software.

2. Click the **Registration** tab (Figure 11).

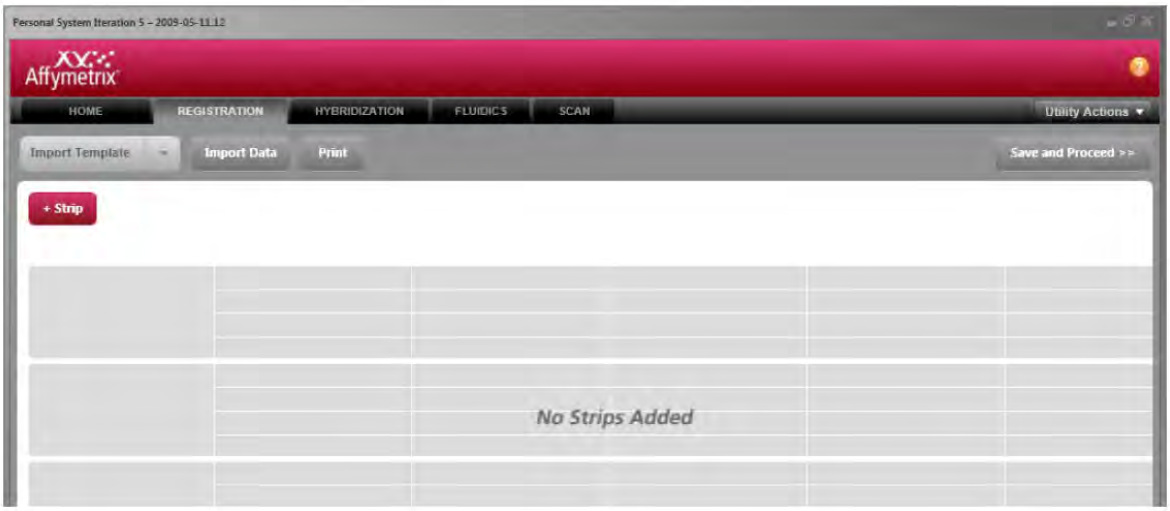


Figure 11 Registration tab of GeneAtlas™ Instrument Control Software.

3. Click **+ Strip**: + Strip .
   The **Add Strip** window 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** tab.

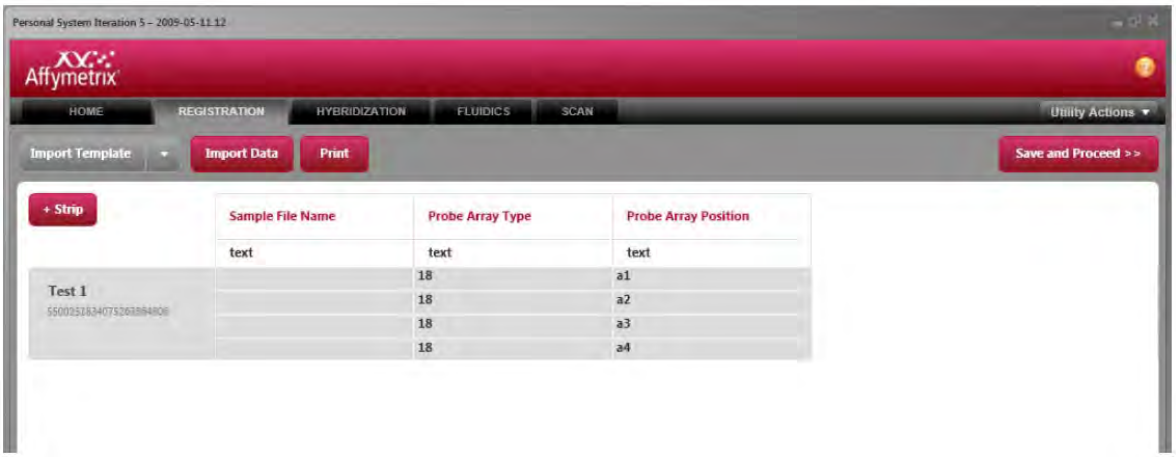
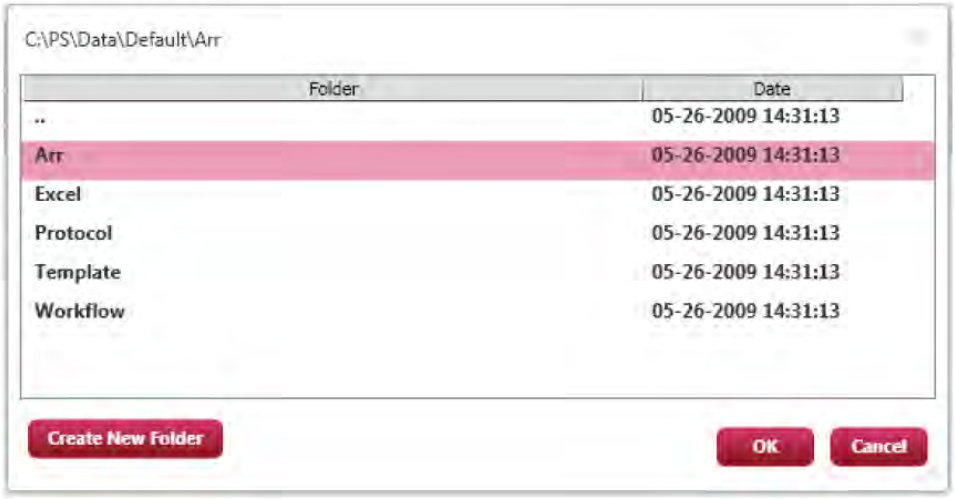


Figure 12 Array strip added to the Registration tab.

5. Click in a box under the **Sample File Name** column, enter a sample name, then press **Enter**.
   A unique name is required for each of the 4 samples on the array strip.

6. Click **Save and Proceed**  when complete.
   The **Save** dialog box appears.



7. In the **Save** dialog box, click to select a folder, 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. Repeat step 3 through step 8 to add additional array strips.

9. Proceed to "Set up the hybridization software" on page 51.

Set up the hybridization software

All array strips to be processed must first be registered prior to setting up the hybridizations in the GeneAtlas™ Instrument Control Software. See "Register a sample" on page 49 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. On the GeneAtlas™ Instrument Control Software interface, navigate to the **Hybridization** tab (Figure 13).

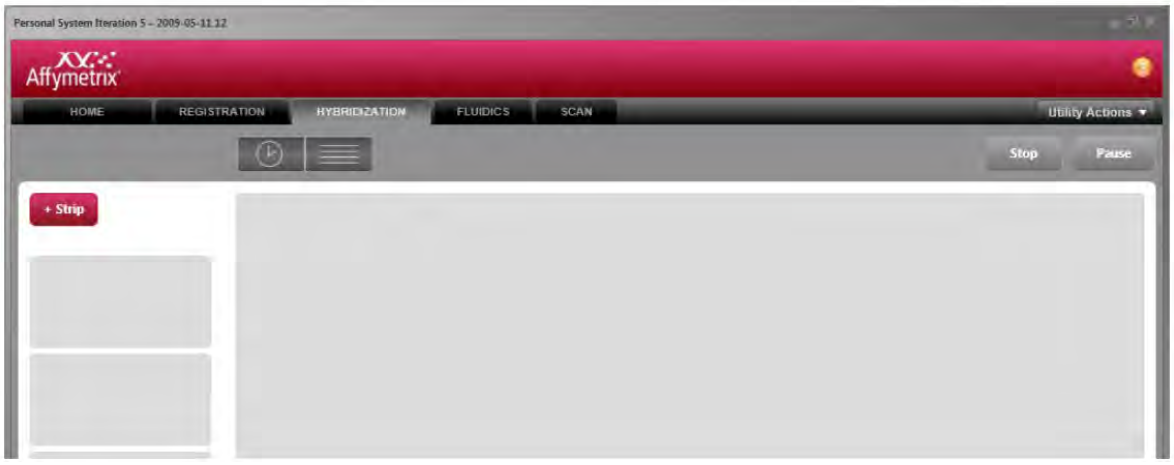


Figure 13 Hybridization tab.

2. Click **+ Strip** .

   The **Add Strip** dialog box appears.

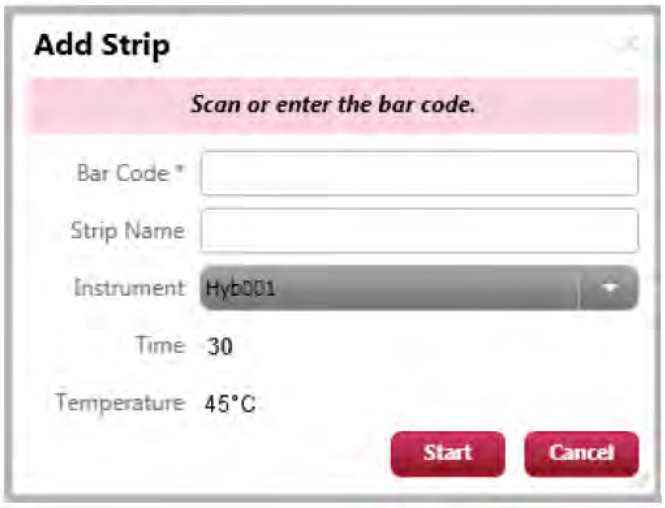


Figure 14 Add Strip dialog box.

3. Scan or enter the **Bar Code** (required) of the array strip you registered.

   The **Strip Name** field is automatically populated.

4. In the **Add Strip** dialog box, click **Start**.

   Ensure that the hybridization tray and array strip are already in the GeneAtlas™ Hybridization Station.

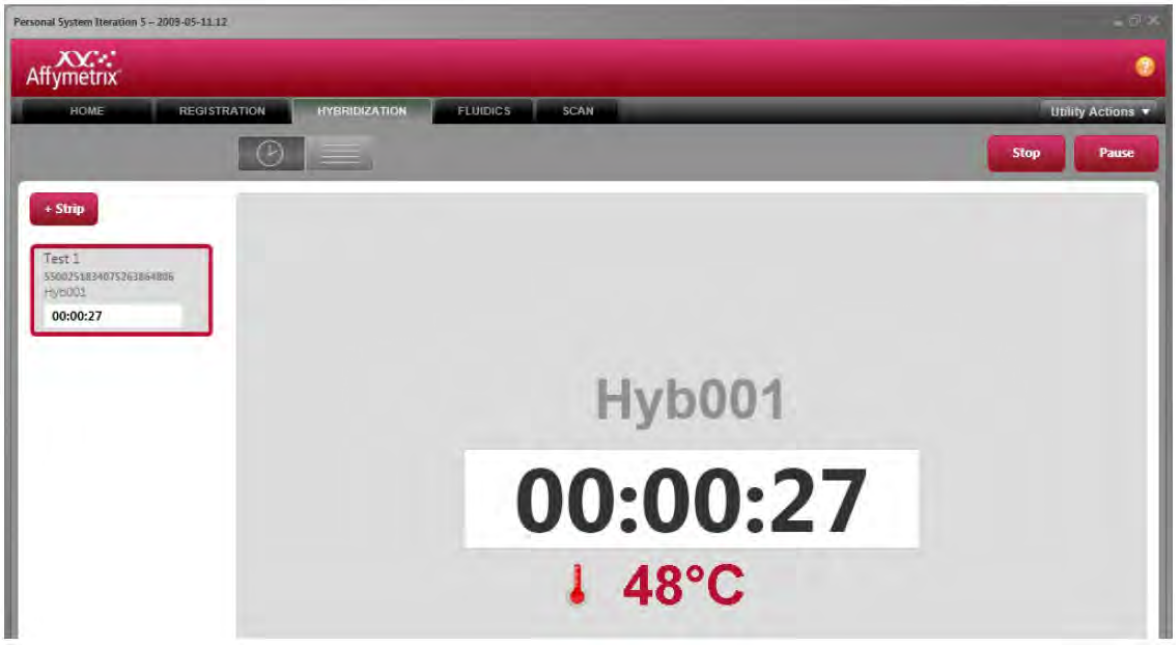


Figure 15 Hybridization countdown.

Note: The software displays the hybridization time countdown. This time is displayed with a white background (Figure 15). When the countdown has completed the display turns yellow and the time begins to count up.

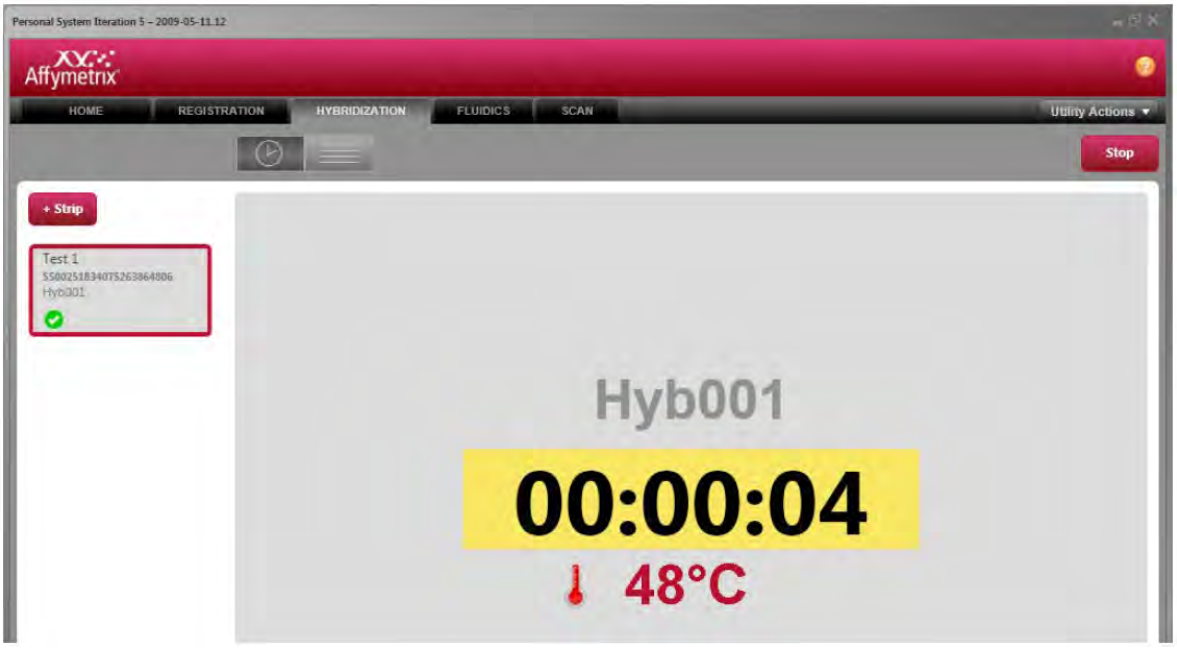


Figure 16 Hybridization count up

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

6. In the confirmation 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 and store the remaining Hybridization Cocktail at –20°C for future use.

8. Immediately proceed to the GeneAtlas™ Wash, Stain, and Scan protocol.
   See the *GeneAtlas™ System User Guide*.

Array plate hybridization on the GeneTitan™ MC Instrument

This chapter 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 54.
- "Hybridization setup for array plates" on page 56
- "Process WT array plates on the GeneTitan™ MC Instrument" on page 57.

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 4 is the fourth component added during preparation of the Hybridization Mix. WT Hyb Add 2, 3, and 5 are not used and are not part of the Hybridization Module.

- GeneTitan™ Hybridization, Wash, and Stain Kit for WT Array Plates. (Not supplied. See Table 5).
  - 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
- Array plate and consumables (Not supplied)
  - WT array plates and trays

Prepare Hybridization Cocktail and Hybridization Master Mix for array plates

1. 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
2. Vortex and centrifuge briefly (~5 seconds) to collect contents of the tube.

3. Remove the following tubes from the GeneChip™ Hybridization Control Kit and thaw at room temperature:
   - 3 nM Control Oligo™ B2
   - 20X Hybridization Controls
4. Vortex and centrifuge briefly (~5 seconds) to collect liquid at the bottom of the tube.
5. Keep the tubes of 3 nM Control Oligo™ B2 and the tube of 20X Hybridization Controls on ice.
6. Heat the 20X Hybridization Controls for 5 minutes at 65°C in a thermal cycler.
   The Hybridization Control protocol that is shown in Table 6 can serve as a reference.
7. Prepare the Hybridization Master Mix in a nuclease-free tube, while at room temperature. Combine the appropriate volume of components in the sequence shown in the following table. Prepare enough Hybridization 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 contents at the bottom of the tube.

Table 23 Hybridization Master Mix.

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 to cover pipetting error.

8. Mix thoroughly by gently vortexing and centrifuge briefly to collect the mix, then, proceed immediately to the next step.

9. Prepare the Hybridization Cocktail in the order shown in the following table for all samples, while at room temperature.

Table 24 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	25 ng/µL
2.5X WT Hyb Add 6	48 µL	1X
Total volume	**120 µL**	

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

IMPORTANT! Complete step 1 through step 10 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.

11. 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 6 can serve as a reference.

12. Centrifuge briefly to collect contents of the tube or well after the incubation, then proceed immediately to the next step.

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

14. Proceed to "Hybridization setup for array plates" on page 56.

Hybridization setup for array plates

This section describes the GeneTitan™ 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 26.

Table 25 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 26 Volumes required to process WT array plates per run.

Reagent	Amount required for 1 array plate	Number of plates that can be processed using the GeneTitan™ Hybridization, Wash, and Stain Kit for WT Array Plates (901622)		
		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 (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 sub-steps 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 can 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

Troubleshooting

Troubleshooting

Table 27 Troubleshooting possible problems.

Observation	Possible Cause	Solution
The positive control sample and your total RNA sample yield low levels of amplified cRNA product.	Incubation conditions are incorrect or inaccurate.	Calibrate your thermal cycler.
	Condensation formed in the tubes during the incubations.	Check 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 manual.
	Pipettes, tubes, and/or equipment are contaminated with nucleases.	Remove RNases and DNases from surfaces using RNase decontamination solution.
The positive control sample produces expected results, but your total RNA sample results in low levels of amplified cRNA product.	The input total RNA concentration is lower than expected.	Repeat the concentration measurement of your RNA sample.
		Increase amount of total RNA in the First-Strand cDNA Synthesis protocol.
		Run an extra 1 or 2 amplification cycles during the Pre-IVT Amplification protocol.
	Your input RNA contains contaminating DNA, protein, phenol, ethanol, or salts, causing inefficient reverse transcription.	Phenol extract and ethanol precipitate your total RNA.
	Your input FFPE RNA sample has poor quality due to modification and degradation.	Increase the amount of total RNA in the First-Strand cDNA Synthesis protocol.
		Run an extra 1 or 2 amplification cycles during the Pre-IVT Amplification protocol.

Table 27 Troubleshooting possible problems. *(continued)*

Observation	Possible Cause	Solution
The positive control sample produces expected results, but your total RNA sample results in high levels of amplified cRNA product.	The input total RNA concentration is higher than expected.	Repeat the concentration measurement of your RNA sample.
		Decrease the amount of total RNA in the First-Strand cDNA Synthesis protocol.
		Reduce amplification cycles by 1 or 2 cycles during Pre-IVT Amplification protocol.
The positive control sample produces expected results but your total RNA sample results in low levels of cRNA/cDNA product.	The total RNA integrity is partially degraded, thereby generating short cDNA fragments.	Assess the integrity of your total RNA sample by determining the size of the 18S and 28S rRNA bands and the relative abundance of 28S to 18S rRNA. See “Evaluation of RNA integrity” on page 13.
	The mRNA content of your total RNA sample is lower than expected.	Verify the mRNA content of your total RNA. **Note:** In healthy cells, mRNA constitutes 1 to 10% of total cellular RNA (Johnson, 1974; Sambrook and Russel, 2001).
The positive control sample produces expected results but your total RNA sample results in cDNA product higher than 20 µg.	The input cRNA amount is higher than expected.	Repeat the concentration measurement of your cRNA sample. cRNA concentrations greater than 3,000 ng/µL should be diluted with Nuclease-free Water before measurement and reaction setup.

B cRNA purification photos

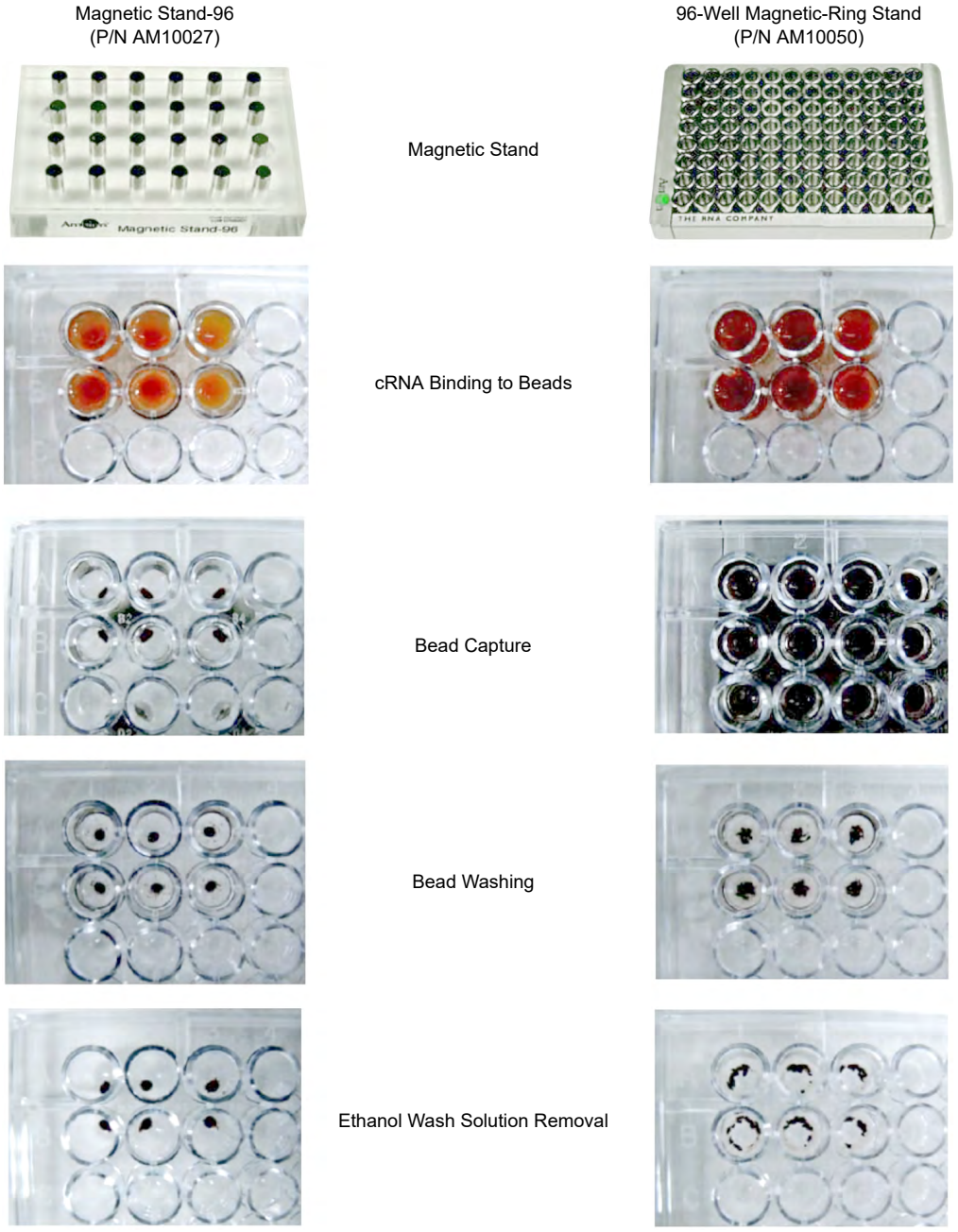


Figure 17 Photos of the cRNA purification step (1 of 2).

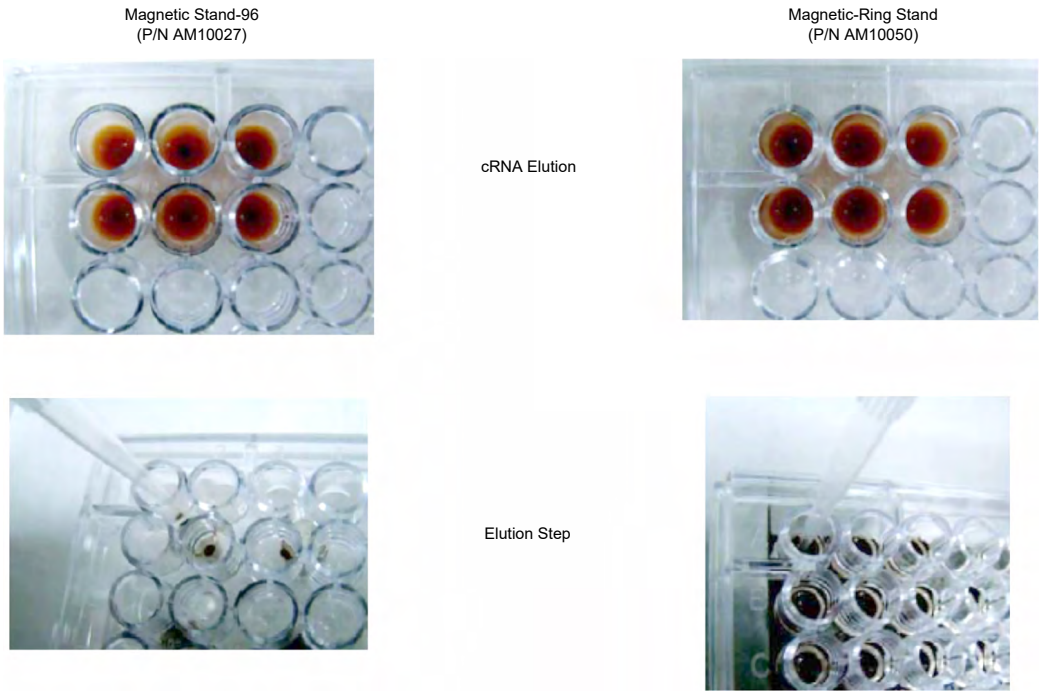


Figure 18 Photos of the cRNA purification step (2 of 2).

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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  - 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**.