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GeneChip™ 3’ IVT Pico Kit Manual Workflow

USER GUIDE

for use with:
GeneChip™ Expression Arrays
GeneChip™ 3’ IVT Pico Kit

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

Products: GeneChip™ 3’ IVT Pico Kit

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

Products: GeneChip™ expression arrays

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Revision history

Table A Revision history of Pub. No. 703308

Revision	Date	Description
7	22 December 2020	Corrected an error in the 3’ Adaptor Synthesis, Step 3, thermal cycler protocols (Table 6).
6	28 September 2020	Corrected the volumes in Table 21 for the 3’ 169-format and Clariom™ 400-format arrays.
5	09 October 2019	Updated to the current document template, with associated updates to trademarks, logos, licensing, and warranty.

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GeneChip™ 3’ IVT Pico Kit

Product information

Purpose

The GeneChip 3’ IVT Pico Kit assay prepares hybridization ready targets from picogram to nanogram quantities of total RNA samples for gene expression profiling analysis with gene-level expression arrays including 3' IVT and Clariom S (Figure 1). Reverse transcription is initiated at the poly-A tail as well as throughout the entire length of RNA to capture both coding and multiple forms of non-coding RNA, making the GeneChip 3’ IVT Pico Kit ideal for amplification of intact, partially degraded, and compromised RNA samples. Complementary RNA (cRNA) 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 double-stranded cDNA (ds-cDNA) 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 3’ IVT Pico Kit is comprised of reagents and an assay 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 GeneChip 3’ IVT Pico 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.

To ensure high reproducibility in whole transcriptome amplification, we recommend using a starting amount of no less than 500 pg RNA template of purified total RNA from cells or tissues and 2 ng of purified total RNA from FFPE tissues. Depending on the copy number of the transcripts, it may be possible to use smaller amounts of RNA template (Table 1). 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.

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

Performance specifications

The GeneChip™ 3’ IVT Pico Kit and assay protocol has been verified to generate greater than 20 µg cRNA and greater than 6.6 µg of ds-cDNA from 500 pg of HeLa total RNA and 2 ng of 1—9 years old FFPE tissues.

Assay workflow

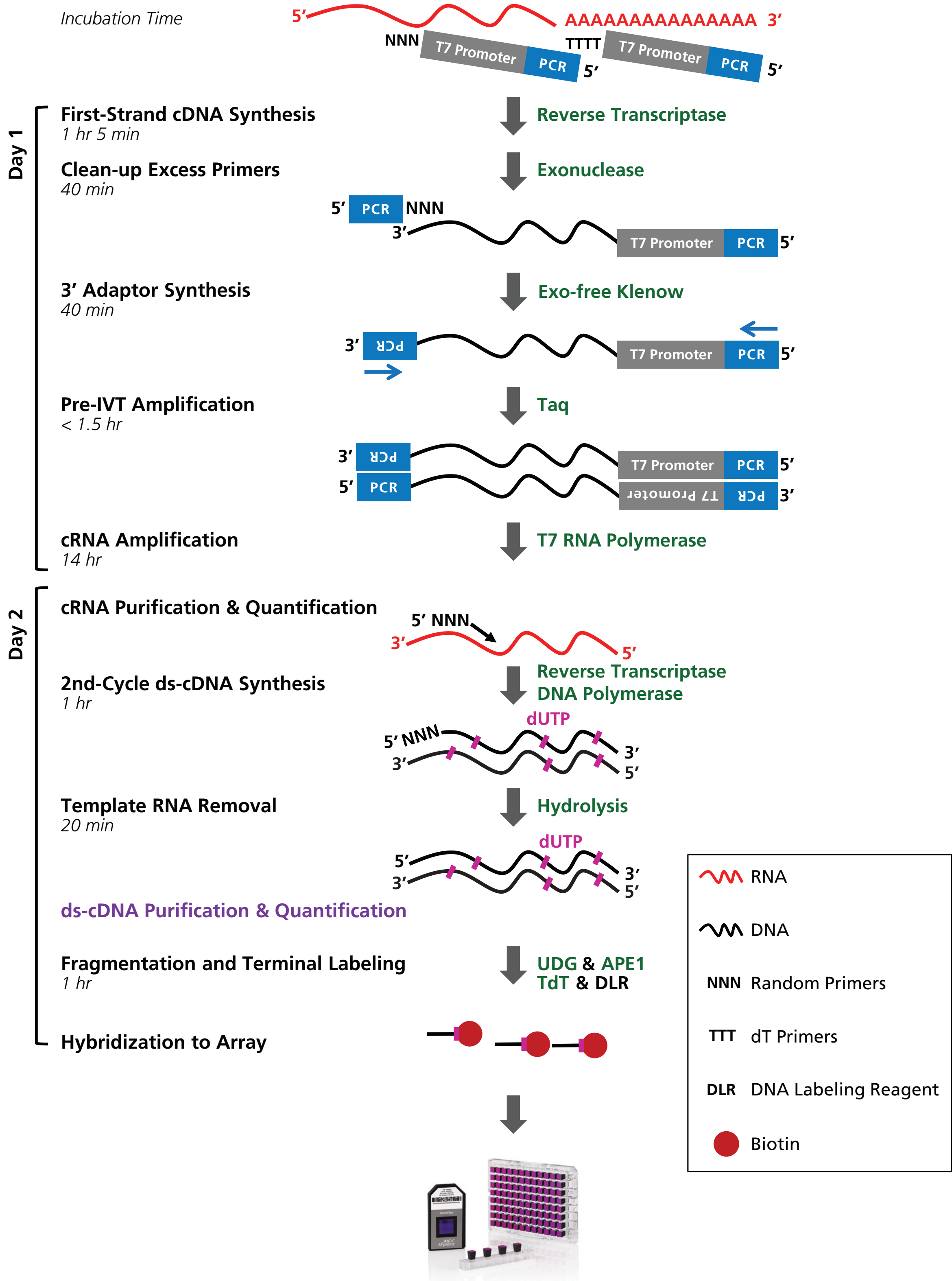
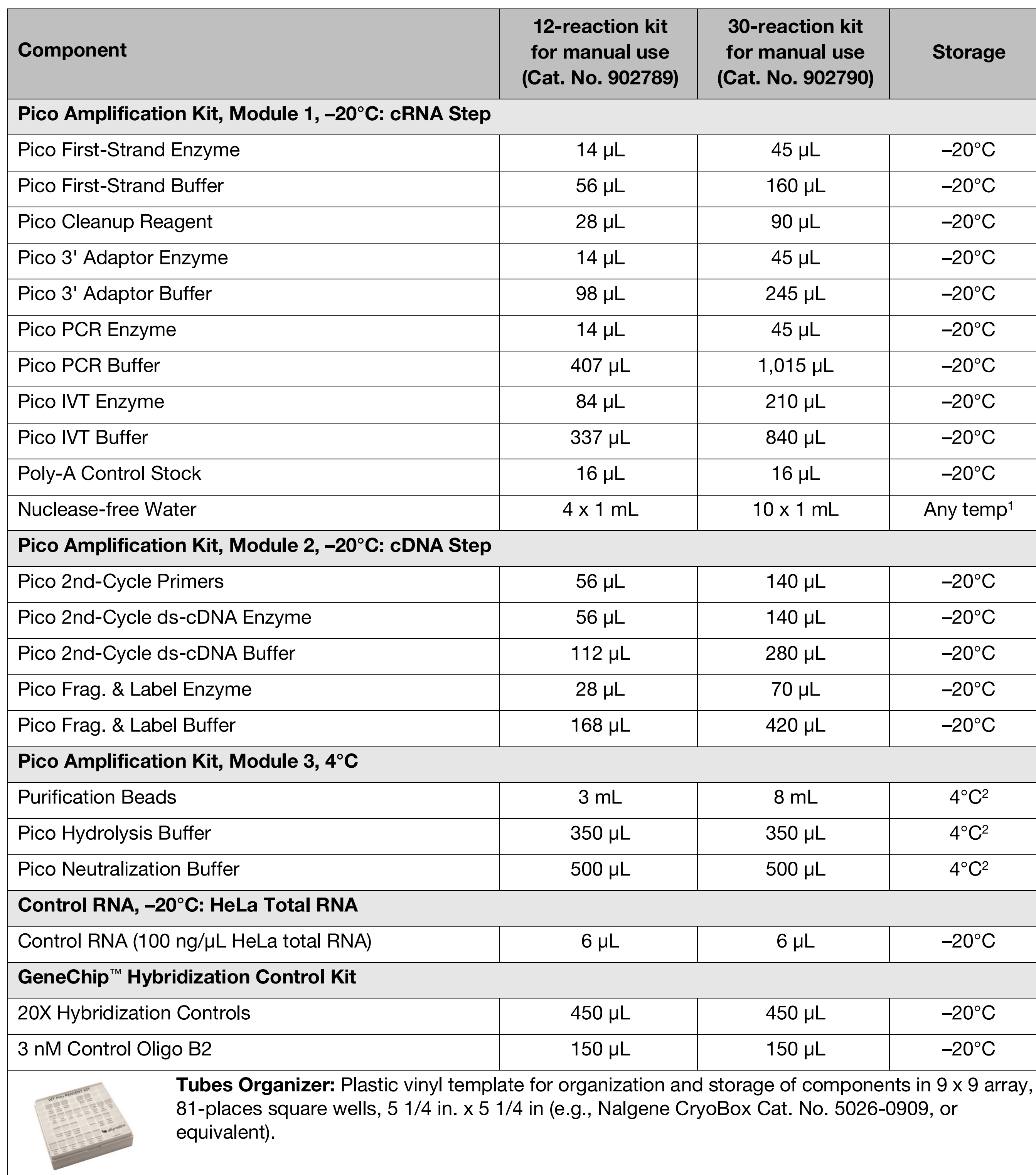


Figure 1 Pico amplification and labeling process.

Kit contents and storage

Table 2 GeneChip™ 3’ IVT Pico Kit contents and storage.

Component	12-reaction kit for manual use (Cat. No. 902789)	30-reaction kit for manual use (Cat. No. 902790)	Storage
Pico Amplification Kit, Module 1, –20°C: cRNA Step			
Pico First-Strand Enzyme	14 µL	45 µL	–20°C
Pico First-Strand Buffer	56 µL	160 µL	–20°C
Pico Cleanup Reagent	28 µL	90 µL	–20°C
Pico 3' Adaptor Enzyme	14 µL	45 µL	–20°C
Pico 3' Adaptor Buffer	98 µL	245 µL	–20°C
Pico PCR Enzyme	14 µL	45 µL	–20°C
Pico PCR Buffer	407 µL	1,015 µL	–20°C
Pico IVT Enzyme	84 µL	210 µL	–20°C
Pico IVT Buffer	337 µL	840 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 temp[1]
Pico Amplification Kit, Module 2, –20°C: cDNA Step			
Pico 2nd-Cycle Primers	56 µL	140 µL	–20°C
Pico 2nd-Cycle ds-cDNA Enzyme	56 µL	140 µL	–20°C
Pico 2nd-Cycle ds-cDNA Buffer	112 µL	280 µL	–20°C
Pico Frag. & Label Enzyme	28 µL	70 µL	–20°C
Pico Frag. & Label Buffer	168 µL	420 µL	–20°C
Pico Amplification Kit, Module 3, 4°C			
Purification Beads	3 mL	8 mL	4°C[2]
Pico Hydrolysis Buffer	350 µL	350 µL	4°C[2]
Pico Neutralization Buffer	500 µL	500 µL	4°C[2]
Control RNA, –20°C: HeLa Total RNA			
Control RNA (100 ng/µL HeLa total RNA)	6 µL	6 µL	–20°C
GeneChip™ Hybridization Control Kit			
20X Hybridization Controls	450 µL	450 µL	–20°C
3 nM Control Oligo B2	150 µL	150 µL	–20°C
 **Tubes Organizer:** Plastic vinyl template for organization and storage of components in 9 x 9 array, 81-places square wells, 5 1/4 in. x 5 1/4 in (e.g., Nalgene CryoBox Cat. No. 5026-0909, or equivalent).			

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

Required materials

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.

Instruments

Table 3 Instruments required for target preparation.

Item	Supplier
Magnetic Stand-96	Agencourt SPRI™Plate Super Magnet Plate (Beckman Coulter Genomics, Cat. No. A32782); Magnetic Stand-96 (Cat. No. AM10027); 96-well Magnetic-Ring Stand (Cat. No. AM10050); or equivalent magnetic stand
Microcentrifuge	MLS
Spectrophotometer and fluorometer • NanoDrop™ UV-Vis Spectrophotometer • Optional: NanoDrop™ Fluorospectrometer • Optional: Qubit™ 2.0 Fluorometer	 • or equivalent quantitation instrument • or equivalent quantitation instrument • or equivalent quantitation instrument
Optional: 2100 Bioanalyzer	Agilent Technologies, Inc., or equivalent DNA and RNA sizing instrument
Pipette	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™ instrument systems required for array processing.

Instruments	Source
GeneChip™ Hybridization Oven 645	Cat. No. 00-0331 (110/220V)
GeneChip™ Fluidics Station 450	Cat. No. 00-0079
GeneChip™ Scanner 3000 7G	Cat. No. 00-0212 (North America) Cat. No. 00-0213 (International)
GeneChip™ AutoLoader with External Barcode Reader	Cat. No. 00-0090 (GCS 3000 7G S/N 501) Cat. No. 00-0129 (GCS 3000 7G S/N 502)

Table 4 GeneChip™ instrument systems required for array processing. (Continued)

Instruments	Source
GeneAtlas™ System for array strips	
GeneAtlas™ Workstation	Cat. No. 90-0894
GeneAtlas™ Hybridization Station	Cat. No. 00-0380 (115VAC), Cat. No. 00-0381 (230VAC)
GeneAtlas™ Fluidics Station	Cat. No. 00-0377
GeneAtlas™ Imaging Station	Cat. No. 00-0376
GeneAtlas™ Barcode Scanner	Cat. No. 74-0015
GeneTitan™ System for array plates	
GeneTitan™ MC Instrument, NA/Japan includes 110v UPS	Cat. No. 00-0372
GeneTitan™ MC Instrument, Int'l includes 220v UPS	Cat. No. 00-0373
GeneTitan™ Instrument, NA/Japan includes 110v UPS	Cat. No. 00-0360
GeneTitan™ Instrument, Int'l Includes 220v UPS	Cat. No. 00-0363
GeneTitan™ ZeroStat AntiStatic Gun (for array plate processing)	Cat. No. 74-0014

Reagents and supplies

Table 5 Additional reagents and supplies required.

Item	Source
96-well round bottom microtiter plate	Costar, Cat. No. 3795 or equivalent
GeneChip™ Hybridization, Wash, and Stain Kit	Cat. No. 900720, 30 reactions
GeneAtlas™ Hybridization, Wash, and Stain Kit for 3' IVT Array Strips	Cat. No. 901531, 60 reactions
GeneTitan™ Hybridization, Wash, and Stain Kit for 3' IVT Array Plates	Cat. No. 901530, 96 reactions
GeneTitan™ Hybridization, Wash, and Stain Kit for WT Array Plates	Cat. No. 901622, 96 reactions
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
Nuclease-free Water (for preparing 80%ethanol wash solution)	Cat. No. 71786 or MLS
Optional: • RNA Quantification Kit For SYBR Green I and ROX™ Passive Reference Dye • RNA Quantification Kit For SYBR Green I and Fluorescein Passive Reference Dye	 • Cat. No. 902905; or equivalent reagents • Cat. No. 902906; or equivalent reagents
Optional: • Quant-iT™ RiboGreen™ RNA Reagent Kit • RNA HS Assay Kit	 • Cat. No. R11490; or equivalent reagents • Cat. No. Q32852; or equivalent reagents
Optional: • RNA 6000 Nano Kit • RNA 6000 Pico Kit	Agilent Technologies, Inc. • Cat. No. 5067-1511; or equivalent DNA and RNA sizing reagents • Cat. No. 5067-1513; or equivalent DNA and RNA sizing reagents
Optional: 96-well plate sealing film	MLS
Tough-Spots™	MLS
100% Ethanol (molecular biology grade or equivalent)	MLS[1]

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

2 Assay protocol

Procedural notes

Implement a plan to maintain procedural consistency

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

- Method of RNA purification ("Purify total RNA")
- RNA quality and integrity ("Evaluate RNA quality" and "Evaluate RNA integrity")
- Method of RNA quantitation ("Evaluate RNA quantity" on page 13)
- Equipment preparation ("Equipment preparation" on page 14)
- Reagent preparation ("Reagent preparation" on page 16)
- RNase contamination prevention ("RNase contamination prevention" on page 16)
- DNA contamination prevention ("DNA contamination prevention" on page 17)
- Workflow stopping points

Sample preparation

Purify total RNA

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

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.

Evaluate RNA quality

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

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

Evaluate RNA integrity

The integrity of the RNA sample, or the proportion that is full length, is an important component of RNA quality. Reverse transcribing degraded input RNA may generate cDNA that lacks exonic regions. While it is impossible to guarantee satisfactory results with all degraded samples, the GeneChip 3’ IVT Pico Kit can work with samples that are moderately to severely degraded.

Two methods to evaluate RNA integrity are:

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

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

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

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

Evaluate RNA quantity

Consider both the type and amount of sample RNA that are available when planning your experiment.

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

Determine RNA quantity by UV absorbance

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

Determine RNA quantity by fluorescence-based quantitation

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

Determine RNA quantity by Bioanalyzer

We do not recommend RNA concentration determination using a Bioanalyzer as it is not accurate, especially at RNA concentrations less than 25 ng/µL. The RNA 6000 Nano Kit may be used for total RNA quantity determination of high concentration samples (25 to 500 ng/µL) but the RNA 6000 Pico Kit should not be used for total RNA quantitation.

Determine RNA quantity by quantitative, real-time RT-PCR

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

Equipment preparation

Recommended thermal cycler

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

Program the thermal cycler

Set the temperature for the heated lid to or near the required temperature for each step. An alternate protocol may be used for thermal cyclers that lack a programmable heated lid, although this is not the preferred method. Yields of cRNA may be reduced if a heated lid is used during the 3' Adaptor cDNA Synthesis or during the *In Vitro* Transcription cRNA 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. This is expected and should not significantly decrease cRNA yields.

Incubation temperatures and times are critical for effective RNA amplification. Use properly calibrated thermal cyclers and adhere closely to the incubation times.

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

Table 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 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 ds-cDNA Synthesis	70°C or 105°C	25°C for 10 minutes	42°C for 50 minutes	70°C for 10 minutes	4°C hold	40 µL
RNA Alkaline Hydrolysis	70°C or 105°C	65°C for 20 minutes	4°C hold			47 µL
Fragmentation and Labeling	93°C or 105°C	37°C for 60 minutes	93°C for 2 minutes	4°C hold		30 µL or 60 µL
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

Tip: 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.

Kit component handling

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

IMPORTANT! Master mixes and samples should be mixed thoroughly by gently vortexing followed by a centrifuge briefly 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 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 Pre-Amplification Clean Area for performing all pre-amplification reaction setup:
    - Prepare total RNA sample
    - Prepare Poly-A RNA Controls
    - Prepare total RNA/Poly-A RNA Control mixture
    - Synthesize First-Strand cDNA
    - Synthesize 3' Adaptor cDNA
    - Synthesize Double-Stranded cDNA (reaction setup)
  - A Post-Amplification Area for performing all post-amplification reaction setup and concentration measurement:
    - Synthesize Double-Stranded cDNA (reaction incubation)
    - Synthesize cRNA by *In Vitro* Transcription
    - Purify cRNA
    - Synthesize 2nd-Cycle Double-Stranded cDNA
    - Hydrolyze RNA using Hydrolysis Buffer
    - Purify 2nd-Cycle Double-Stranded cDNA
    - Fragment and Label Double-Stranded cDNA
- Maintain a single direction workflow. Do not bring amplified products into the Pre-Amplification Clean Area.
- Keep dedicated equipment in each of the areas used for this assay protocol, including pipettes, ice buckets, coolers, etc. Do not move equipment back and forth between the areas.
- Concentration measurements can be performed in Post-Amplification Area.
- Clean and decontaminate work areas and instruments, including pipettes, with commercially available decontamination reagents.
- Always use filter tips for pipetting to reduce sample contamination.

Prepare control RNA

HeLa control RNA preparation

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

To prepare the Control RNA for positive control reaction:

1. On ice, dispense 2 µL of the Control RNA in 38 µL of Nuclease-free Water for a total volume of 40 µL (5 ng/µL).
2. Add 2 µL of the 1st Dilution (5 ng/µL) to 38 µL of Nuclease-free Water for a total volume of 40 µL (250 pg/µL).
3. Follow the "Prepare total RNA/Poly-A RNA control mixture" on page 20, but use 2 µL of the 2nd Dilution (500 pg) in the control reaction.

Note:

- Measure concentration of HeLa 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 >6.6 µg of 2nd-cycle ds-cDNA from 500 pg Control RNA.

Poly-A RNA control preparation

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 RNA Control Stock, prepare appropriate dilution of the Poly-A RNA Controls and add to the total RNA samples. Follow the "Prepare total RNA/Poly-A RNA control mixture" on page 20.
- Do not use the Poly-A Control Dil Buffer to prepare serial dilution of Poly-A RNA Controls because it may cause non-target amplification.
- Prepare serial dilution of Poly-A RNA Control Stock with Nuclease-free Water.

A set of poly-A RNA controls supplied by Thermo Fisher Scientific is designed specifically to provide exogenous positive controls to monitor the entire target preparation. It should be added to the RNA prior to the First-Strand cDNA Synthesis step.

Each eukaryotic GeneChip™ 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 RNA Control Stock and nuclease-free water are provided in the Pico Amplification Kit, Module 1 to prepare the appropriate serial dilutions based on Table 9. This is a guideline when ≤1, 2, 5, 10, 20, or 50 ng of total RNA is used as starting material. For starting sample amounts other than those listed here, calculations are needed in order to perform the appropriate dilutions to arrive at the same proportionate final concentration of the spike-in controls in the samples.

Table 9 Serial dilution of Poly-A RNA 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

Table 9 provides a guideline when the indicated amount of total RNA is used as starting material. For starting sample amounts other than those listed here and ≥1 ng, 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.

IMPORTANT!

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

For example, to prepare the Poly-A RNA dilutions for 500 pg of total RNA:

1. Add 2 µL of the Poly-A Control Stock to 98 µL of Nuclease-free Water for the First Dilution (1:50).
2. Add 2 µL of the 1st Dilution to 198 µL of Nuclease-free Water to prepare the Second Dilution (1:100).
3. Add 2 µL of the 2nd Dilution to 198 µL of Nuclease-free Water to prepare the Third Dilution (1:100).
4. Add 2 µL of the 3rd 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 Controls from Poly-A Controls provided in the GeneChip 3' IVT Pico Kit.

Prepare total RNA/Poly-A RNA control mixture

Prepare total RNA according to your laboratory's procedure. A maximum of 5 µL total RNA can be added to first-strand synthesis reaction. If you are adding Poly-A Spike Controls to your RNA, the volume of RNA must be 3 µL or less (Table 10). See "Poly-A RNA control preparation" on page 18 for more information. For example, when performing the Control RNA reaction, combine 2 µL of RNA (250 pg/µL), 2 µL of diluted Poly-A Spike Controls, and 1 µL of Nuclease-free Water.

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

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

Component	Volume for 1 reaction
Total RNA Sample (100 pg–50 ng)	variable
Diluted Poly-A RNA Controls (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 (ss-cDNA) with T7 promoter sequence at the 5' end.

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

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

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

Table 11 First-Strand Master Mix.

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

   b. Mix thoroughly by gently vortexing the tube. Centrifuge briefly to collect the mix at the bottom of the tube. Proceed immediately to the next step.
   c. On ice, transfer 5 µL of the First-Strand Master Mix to each tube or well.
2. Add total RNA to each First-Strand Master Mix aliquot.
   a. On ice, add 5 µL of the total RNA (Table 10) to each (5 µL) tube or well containing the First-Strand Master Mix for a final reaction volume of 10 µL.

      See "Prepare total RNA/Poly-A RNA control mixture" on page 20 for more information.
   b. Mix thoroughly by gently vortexing the tube. Centrifuge briefly to remove air bubbles and collect the reaction at the bottom of the tube or well, then proceed immediately to the next step.
3. Incubate for 5 minutes at 25°C, for 60 minutes at 42°C, and then for at least 2 minutes at 4°C.
   a. Incubate the first-strand synthesis reaction in a thermal cycler using the **First-Strand cDNA Synthesis** protocol that is shown in Table 6 on page 15.
   b. Immediately after the incubation, centrifuge briefly to collect the first-strand cDNA at the bottom of the tube or well.
   c. Place the sample on ice for 2 minutes to cool the plastic, then proceed immediately to the next step.
4. Add Cleanup Reagent to each cDNA sample.
   a. On ice, transfer 2 µL of Pico Cleanup Reagent to each (10 µL) cDNA sample for a final reaction volume of 12 µL. Pipet up and down twice and carefully eject all liquid from pipette tip to ensure complete transfer of the Cleanup Reagent.
   b. Mix thoroughly by gently vortexing the tube. Centrifuge briefly to remove air bubbles and collect the reaction at the bottom of the tube or well, then proceed immediately to the next step.

      Note: Centrifuge briefly to remove any air bubbles that may form during mixing.
5. Incubate for 30 minutes at 37°C, for 10 minutes at 80°C, and then for at least 2 minutes at 4°C.
   a. Incubate the first-strand cleanup reaction in a thermal cycler using the **Cleanup** protocol shown in Table 6 on page 15.
   b. Immediately after the incubation, centrifuge briefly to collect the first-strand cDNA at the bottom of the tube or well.
   c. Place the sample on ice for 2 minutes to cool the plastic, then proceed immediately to "Synthesize 3' adaptor cDNA" on page 22.

      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 there is approximately 15 minutes left on the thermal cycler you may start reagent preparation for 3' Adaptor cDNA Synthesis.

Synthesize 3' adaptor cDNA

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

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

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

   Table 12 3' Adaptor Master Mix.

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

   b. Mix thoroughly by gently vortexing the tube. Centrifuge briefly to collect the mix at the bottom of the tube and proceed immediately to the next step.
   c. On ice, transfer 8 µL of the 3' Adaptor Master Mix to each (12 µL) first-strand cDNA sample for a final reaction volume of 20 µL.
   d. Mix thoroughly by gently vortexing the tube. Centrifuge briefly to collect the reaction at the bottom of the tube or well, then proceed immediately to the next step.
2. Incubate for 15 minutes at 15°C, for 15 minutes at 35°C, for 10 minutes at 70°C, and then for at least 2 minutes at 4°C.
   a. Incubate the 3' Adaptor cDNA synthesis reaction in a thermal cycler using the **3' Adaptor cDNA Synthesis** protocol that is shown in Table 6 on page 15.

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

   b. Immediately after the incubation, centrifuge briefly to collect the 3' Adaptor cDNA at the bottom of the tube or well.
   c. Place the sample on ice, then proceed immediately to "Synthesize double-stranded cDNA".

   Tip: When there is approximately 15 minutes left on the thermal cycler you may start reagent preparation for Pre-IVT Amplification.

Synthesize double-stranded cDNA

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

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

   Table 13 Pre-IVT Amplification Master Mix.

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

   b. Mix thoroughly by gently vortexing the tube. Centrifuge briefly to collect the mix at the bottom of the tube and proceed immediately to the next step.
   c. On ice, transfer 30 µL of the Pre-IVT Amplification Master Mix to each (20 µL) 3' Adaptor cDNA sample for a final reaction volume of 50 µL.
   d. Mix thoroughly by gently vortexing the tube. Centrifuge briefly to collect the reaction at the bottom of the tube or well, then proceed immediately to the next step.

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

2. Incubate for 2 minutes at 95°C, for 6, 9 or 12 cycles of 30 seconds at 94°C and 5 minutes at 70°C, and then for at least 2 minutes at 4°C.
   a. Incubate the pre-IVT amplification reaction in a thermal cycler using the Pre-**IVT Amplification** protocol that is shown in Table 6 on page 15. See Table 7 on page 15 for Pre-IVT Amplification cycling guidelines based on sample type and the amount of starting total RNA input.
   b. Immediately after the incubation, centrifuge briefly to collect the ds-cDNA at the bottom of the tube or well.
   c. Place the sample on ice, then proceed immediately to "Synthesize cRNA by in vitro transcription" on page 24.

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

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

Synthesize cRNA by *in vitro* transcription

In this procedure, antisense RNA (complimentary RNA or cRNA) is synthesized and amplified by *in vitro* transcription (IVT) of the ds-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 ds-cDNA samples to room temperature for ≥5 minutes while preparing 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 IVT Master Mix.

   Note: This step is performed at room temperature.

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

   Table 14 IVT Master Mix.

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

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

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

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

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

   a. Incubate the IVT reaction in a thermal cycler using the ***In Vitro* Transcription cRNA Synthesis** protocol that is shown in Table 6 on page 15.

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

   c. Place the reaction on ice, then proceed to "Purify cRNA" on page 25, 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

In this procedure, enzymes, salts, inorganic phosphates, and unincorporated nucleotides are removed to prepare the cRNA for 2nd-cycle ds-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 Beads to a nuclease-free tube or container and allow the Purification Beads to equilibrate at room temperature. For each reaction, 140 µL plus ~10% overage will be needed.
- Prepare fresh dilutions of 80% ethanol wash solution each time from 100% ethanol (molecular biology grade or equivalent) and Nuclease-free Water in a nuclease-free tube or container. For each reaction, 600 µL plus ~10% overage will be needed.
- Transfer the cRNA sample to room temperature while preparing the Purification Beads.

Note:

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

1. Bind cRNA to Purification Beads.
   a. Mix the Purification Beads container by vortexing to resuspend the magnetic particles that may have settled. Transfer 140 µL of the Purification Beads to a clean well of a round bottom plate.
   b. Add 80 µL of cRNA sample to each (140 µL) Purification Beads, and mix by pipetting up and down 10 times.

      Tip:

      - Any unused wells should be covered with a plate sealer so that the plate can safely be reused.
      - Use multichannel pipette when processing multiple samples.
      - Set the pipette to 150 µL and pipet slowly when mixing to avoid bubble formation.

   c. Incubate for 10 minutes. The cRNA in the sample binds to the Purification Beads during this incubation.
   d. Move the plate to a magnetic stand to capture the Purification Beads. When capture is complete (after ~5 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 that you use and the amount of cRNA generated by *in vitro* transcription.
   e. Carefully aspirate and discard the supernatant without disturbing the Purification Beads. Keep the plate on the magnetic stand.

2. Wash the Purification Beads.
   a. While on the magnetic stand, add 200 µL of 80% ethanol wash solution to each well and incubate for 30 seconds.
   b. Slowly aspirate and discard the 80% ethanol wash solution without disturbing the Purification Beads.
   c. Repeat Step a and Step b twice for a total of 3 washes with 200 µL of 80% ethanol wash solution. Completely remove the final wash solution.
   d. Air-dry on the magnetic stand for 5 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.
3. Elute cRNA.
   a. Remove the plate from the magnetic stand. Add to each sample 27 µL of the preheated (65°C) Nuclease-free Water and incubate for 1 minute.
   b. Mix well by pipetting up and down 10 times.
   c. Move the plate to the magnetic stand for ~5 minutes to capture the Purification Beads.
   d. Transfer the supernatant, which contains the eluted cRNA, to a nuclease-free tube.
   e. Place the purified cRNA samples on ice, then proceed to "Assess cRNA yield", or immediately freeze the samples at –20°C for storage.

Note:

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

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

Assess cRNA yield

Expected cRNA yield

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

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

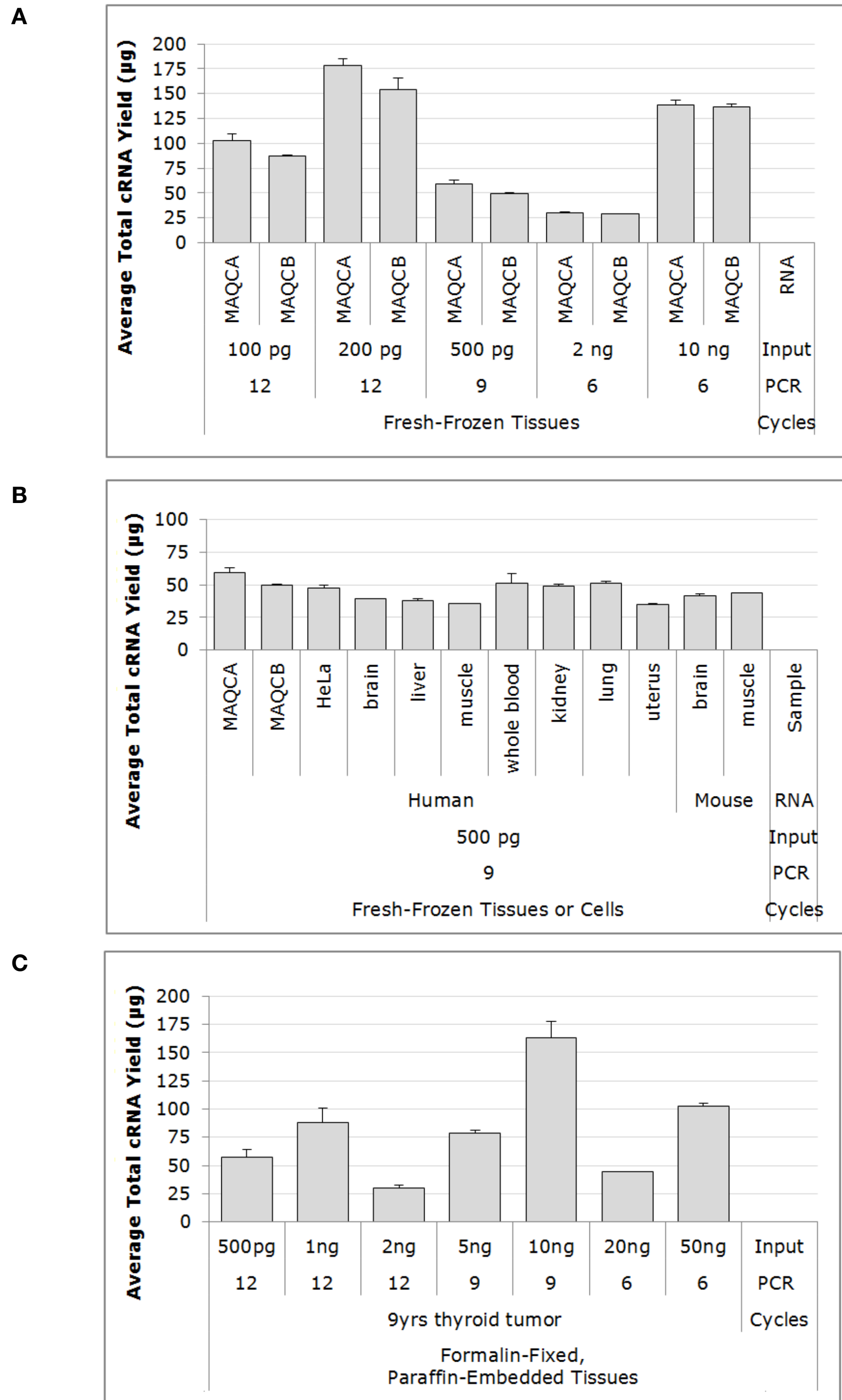


Figure 2 Average cRNA yield from MicroArray Quality Control (MAQC) (A), a variety of fresh-frozen tissues (B), and a 9 years old FFPE tissue (C) total RNA samples.

Determine cRNA yield by UV absorbance

Determine the concentration of a cRNA solution by measuring its absorbance at 260 nm. Use nuclease-free water as blank. We recommends using NanoDrop Spectrophotometers for convenience. No dilutions or cuvettes are needed; just use 1 to 1.5 µL of the cRNA sample directly. Samples with cRNA concentrations greater than 3,000 ng/µL should be diluted with Nuclease-free Water before measurement and reaction setup. Use the diluted cRNA as the input to prepare 20 µg cRNA in 2nd-cycle ds-cDNA synthesis reaction.

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

(Optional) Expected cRNA size distribution

The expected cRNA profile is a distribution of sizes from 200 nt to 1,000 nt. This step is optional.

Determine cRNA size distribution using a Bioanalyzer.

cRNA size distribution can be analyzed using an Agilent 2100 Bioanalyzer, a RNA 6000 Nano Kit (Cat. No. 5067-1511), and mRNA Nano Series II assay. If there is sufficient yield, then load approximately 500 ng of cRNA per well on the Bioanalyzer. To analyze cRNA size using a Bioanalyzer, follow the manufacturer's instructions.

Stopping Point. The purified cRNA samples can be stored overnight at –20°C.

Synthesize 2nd-cycle double-stranded cDNA

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

1. Prepare 20 µg of cRNA.

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

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

2. Prepare 2nd-Cycle ds-cDNA Master Mix.

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

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

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

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

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

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

3. Incubate for 10 minutes at 25°C, 50 minutes at 42°C, for 10 minutes at 70°C, and then for at least 2 minutes at 4°C.

    a. Incubate the 2nd-cycle synthesis reaction in a thermal cycler using the **2nd-Cycle ds-cDNA Synthesis** protocol that is shown in Table 6 on page 15.

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

    c. Place the sample on ice and proceed immediately to "Hydrolyze RNA using hydrolysis buffer" on page 29.

Hydrolyze RNA using hydrolysis buffer

In this procedure, Hydrolysis Buffer and heat hydrolyze the cRNA template leaving ds-cDNA.

1. Add Hydrolysis Buffer to each 2nd-cycle ds-cDNA sample.

    a. On ice, add 7 µL of the Pico Hydrolysis Buffer to each (40 µL) 2nd-cycle ds-cDNA sample for a final reaction volume of 47 µL.

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

2. Incubate for 20 minutes at 65°C, and then for at least 2 minutes at 4°C.

    a. Incubate the RNA hydrolysis reaction in a thermal cycler using the **RNA Alkaline Hydrolysis** protocol that is shown in Table 6 on page 15.

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

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

3. Add Neutralization Buffer to each hydrolyzed 2nd-cycle ds-cDNA sample.

    a. On ice, add 10 µL of the Pico Neutralization Buffer to each (47 µL) hydrolyzed 2nd-cycle ds-cDNA sample for a final reaction volume of 57 µL.

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

c. Place the sample on ice, then proceed to "Purify 2nd-Cycle double-stranded cDNA" on page 30, or immediately freeze the samples at –20°C for storage.

Stopping Point. The hydrolyzed ds-cDNA samples can be stored overnight at –20°C.

Purify 2nd-Cycle double-stranded cDNA

After hydrolysis, the 2nd-cycle ds-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 pellets.
- This entire procedure is performed at room temperature.

1. Bind ds-cDNA to purification beads.

   a. Mix the purification beads container by vortexing to resuspend the magnetic particles that may have settled. Transfer 100 µL of the purification beads to a clean well of a round bottom plate.

   b. Add 57 µL of 2nd-cycle ds-cDNA sample to each (100 µL) purification beads, and mix by pipetting up and down 10 times.

   Tip:

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

   Note: Do not add ethanol to ds-cDNA samples.

   c. Incubate for 10 minutes. The ds-cDNA in the sample binds to the purification beads during this incubation.

d. Move the plate to a magnetic stand to capture the purification beads. When capture is complete (after ~5 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 that you use and the amount of ds-cDNA generated by 2nd-Cycle ds-cDNA synthesis.

e. Carefully aspirate and discard the supernatant without disturbing the purification beads. Keep the plate on the magnetic stand.

2. Wash the purification beads.

a. While on the magnetic stand, add 200 µL of 80% ethanol wash solution to each well and incubate for 30 seconds.

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

c. Repeat Step a and Step b twice for a total of 3 washes with 200 µL of 80% ethanol wash solution. Completely remove the final wash solution.

d. Air-dry on the magnetic stand for 5 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-dry.

3. Elute ds-cDNA.

a. Remove the plate from the magnetic stand. Add to each sample 30 µL of the preheated (65°C) nuclease-free water and incubate for 1 minute.

Note: Elute with 25 µL of the preheated (65°C) nuclease-free water for samples for array plate hybridization.

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

c. Move the plate to the magnetic stand for ~5 minutes to capture the purification beads.

d. Transfer the supernatant, which contains the eluted ds-cDNA, to a nuclease-free tube.

e. Place the purified ds-cDNA samples on ice, then proceed to "Assess double-stranded cDNA yield", or immediately freeze the samples at –20°C for storage.

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

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

Assess double-stranded cDNA yield

Expected double-stranded cDNA yield

During development of this kit, using a wide variety of tissue types, 20 µg of input cRNA yielded 7 µg to 20 µg of ds-cDNA. For most tissue types, the recommended 20 µg of input cRNA should yield >6.6 µg of ds-cDNA. Figure 3 shows yield data for ds-cDNA produced with the kit from several different types of input RNA.

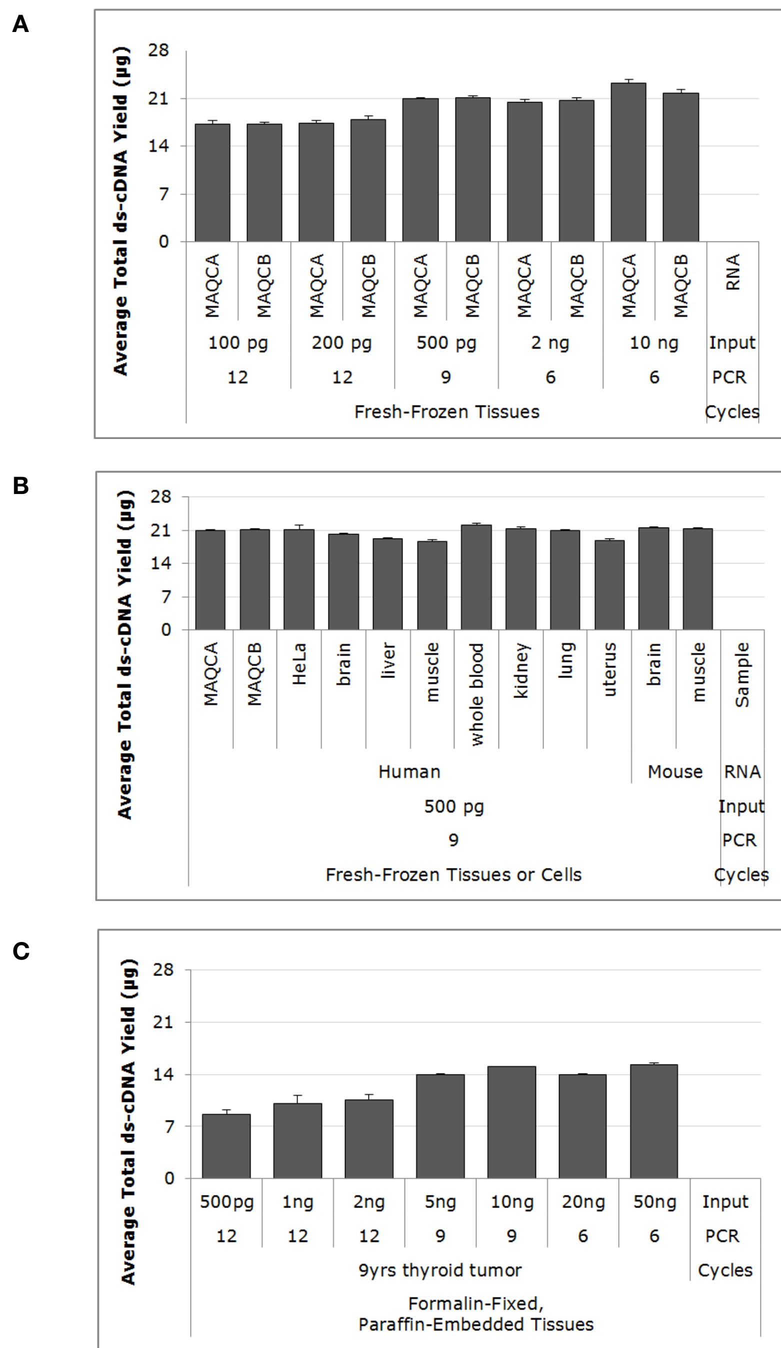


Figure 3 Average ds-cDNA yield from MicroArray Quality Control (MAQC) (A), a variety of fresh-frozen tissues (B), and a 9 years old FFPE tissue (C) total RNA samples.

Determine double-stranded DNA yield by UV absorbance

Determine the concentration of a ds-cDNA solution by measuring its absorbance at 260 nm. Use nuclease-free water as blank. We recommend using NanoDrop spectrophotometers for convenience. No dilutions or cuvettes are needed; just use 1 µL to 1.5 µL of the cDNA sample directly.

Alternatively, determine the ds-cDNA concentration by diluting an aliquot of the preparation in Nuclease-free Water and reading the absorbance in a traditional spectrophotometer at 260 nm. Calculate the concentration in µg/mL using the equation below (1 A_{260} = 50 µg DNA/mL).

A_{260} × dilution factor × 50 = µg DNA/mL

Note: The equation above applies only to ds-cDNA.

(Optional) Expected double-stranded cDNA size distribution

The expected cDNA profile does not resemble the cRNA profile. The expected cDNA profile is a distribution of sizes from 25—1,000 nt with most of the cDNA sizes in the 25—500 nt range. This step is optional.

Determine double-stranded cDNA size distribution using a Bioanalyzer

cDNA size distribution may be analyzed using an Agilent 2100 Bioanalyzer, an RNA 6000 Nano Kit (Cat. No. 5067-1511), and mRNA Nano Series II assay. If there is sufficient yield, load approximately 400 ng of cDNA per well. To analyze cDNA size using a bioanalyzer, follow the manufacturer’s instructions.

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

Note:

- Although 100 or 81/4-Format and 169-Format cartridge arrays use less of fragmented and labeled ds-cDNA in hybridization, the fragmentation and labeling reaction should be performed as described on page 34.
- The fragmentation and labeling reaction set-up is different for 3' cartridge arrays, Clariom S cartridge arrays and plate arrays as described on page 34.

Fragment and label double-stranded cDNA

In this procedure, the purified, ds-cDNA is fragmented by uracil-DNA glycosylase (UDG) and apurinic/apyrimidinic endonuclease 1 (APE 1) at the unnatural dUTP residues and breaks the DNA strand. The fragmented cDNA is labeled by terminal deoxynucleotidyl transferase (TdT) using the proprietary DNA Labeling Reagent that is covalently linked to biotin. 6.6 µg of ds-cDNA is required for fragmentation and labeling for 3′ cartridge array hybridization, and 5.5 µg of ds-cDNA is required for fragmentation and labeling for Clariom S array and plate array hybridization.

Fragment and label ds-cDNA

1. Prepare normalized ds-cDNA.

   On ice, prepare normalized ds-cDNA in appropriate volume of Nuclease-free Water depending on array type.

   Table 16 ds-cDNA normalization.

Array / Component	For 1 reaction		
	3' Cartridge all formats	Clariom™ S cartridge 400-format	All plates
ds-cDNA	6.6 µg	5.5 µg	5.5 µg
Nuclease-free water to	46 µL	22 µL	22 µL
ds-cDNA concentration	143.5 ng/µL	250 ng/µL	250 ng/µL

2. Prepare Fragmentation and Labeling Master Mix.

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

Table 17 Fragmentation and Labeling Master Mix.

Array / Component	For 1 reaction		
	3' cartridge all formats	**Clariom™ S cartridge 400-format**	**All plates**
Pico Frag. & Label Buffer	12 µL	6 µL	6 µL
Pico Frag. & Label Enzyme	2 µL	2 µL	2 µL
Master mix total volume	**14 µL**	**8 µL**	**8 µL**
Reaction total volume	**60 µL**	**30 µL**	**30 µL**

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

c. On ice, transfer the Fragmentation and Labeling Master Mix to each normalized ds-cDNA sample for the indicated final reaction volume.

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

3. Incubate for 1 hour at 37°C, for 2 minutes at 93°C, and then for at least 2 minutes at 4°C.

   a. Incubate the fragmentation reaction in a thermal cycler using the **Fragmentation and Labeling** protocol shown in Table 6 on page 15.

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

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

4. (Optional) The fragmented and labeled ds-cDNA sample can be used for size analysis using an Agilent 2100 Bioanalyzer, a RNA 6000 Nano Kit (Cat. No. 5067-1511), and mRNA Nano Series II assay. See the reagent kit guide that comes with the RNA 6000 Nano LabChip Kit for detailed instructions. The range in peak size of the fragmented samples should be approximately 40 to 70 nt.

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

3 Array hybridization

Cartridge array hybridization

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

See the *GeneChip™ Fluidics Station 450 User Guide GCC* (Pub. No. 08-0295), the *GeneChip™ Expression Wash, Stain, and Scan User Guide for Cartridge Arrays* (Pub. No. MAN0018114), and the *GeneChip™ Command Console™ User Guide* (Pub. No. 702569) for further detail.

Prepare ovens, arrays, and Sample Registration Files

1. Turn GeneChip Hybridization Oven on, set the temperature to 45°C and set to 60 rpm. Turn the rotation on, then allow the oven to preheat.
2. Equilibrate the arrays to room temperature immediately before use. Label the array with the name of the sample that will be hybridized.
3. Register the sample and array information into GCC.

Target hybridization setup for cartridge arrays

Reagents and materials required

- GeneChip™ Hybridization, Wash and Stain Kit. (Not supplied) For ordering information see Table 5 on page 11 or our website.
  - Pre-Hybridization Mix (Optional)
  - 2X Hybridization Mix
  - DMSO
  - Nuclease-free Water
  - Stain Cocktail 1
  - Stain Cocktail 2
  - Array Holding Buffer
  - Wash Buffer A
  - Wash Buffer B
- GeneChip™ Hybridization Control Kit
  - 20X Eukaryotic Hybridization Controls (*bioB, bioC, bioD, cre*)
  - Control Oligonucleotide B2 (3 nM)
- GeneChip expression cartridge array(s). (Not supplied.)

Procedure

1. (Optional) Warm the pre-hybridization buffer to room temperature.

   Aliquot ~220 µL of pre-hybridization buffer per array to be hybridized into a 1.5 mL tube to accelerate the equilibration to room temperature.
2. Prepare Hybridization Master Mix.
   a. At room temperature, thaw the components listed in Table 18.

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

b. Heat the 20X Hybridization Controls for 5 minutes at 65°C in a thermal cycler using the **Hybridization Control** protocol that is shown in Table 6 on page 15.

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

Table 18 Hybridization Master Mix for a single reaction.

Array Component	3’ 49 or 64-format	3’ 100 or 81/4-format	3’ 169-format	Final concentration	Clariom™ S 400-format	Final concentration
Control Oligo B2 (3 nM)	3.7 µL	2.5 µL	1.7 µL	50 pM	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	5 µL	1.5, 5, 25, and 100 pM respectively
2X Hybridization Mix	110 µL	75 µL	50 µL	1X	50 µL	1X
DMSO	22 µL	15 µL	10 µL	10%	7 µL	7%
Nuclease-free Water	13.3 µL	9 µL	6.3 µL		9.3 µL	
Total volume	**160 µL**	**109 µL**	**73 µL**		**73 µL**	

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

3. (Optional) Pre-hybridize array.

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

b. Wet the array with an appropriate volume of Pre-Hybridization Mix (Table 19) by filling it through the bottom left septa.

Table 19 Array cartridge volumes for pre-hybridization mix.

	3’ 49 or 64-format	3’ 100 or 81/4-format	3’ 169-format	Clariom™ S 400-format
Volume to load on array	200 µL	130 µL	80 µL	80 µL

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

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

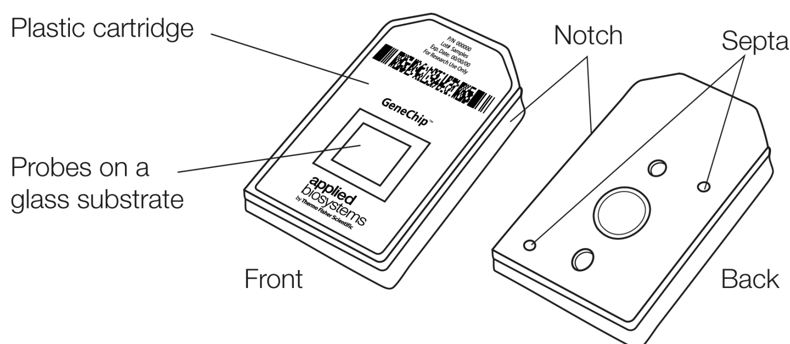


Figure 4 GeneChip cartridge array.

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

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

Table 20 Hybridization cocktail for a single array.

Array Component	3’ 49 or 64-format	3’ 100 or 81/4-format	3’ 169-format	Clariom™ S 400-format
Hybridization Master Mix	160 µL	109 µL	73 µL	73 µL
Fragmented and Labeled ds-cDNA	60 µL (6.6 µg)	41 µL (4.5 µg)	27 µL (3 µg)	27 µL (5 µg)
Total volume	**220 µL**	**150 µL**	**100 µL**	**100 µL**
ds-cDNA final concentration	**30 ng/µL**	**30 ng/µL**	**30 ng/µL**	**50 ng/µL**

   b. Mix thoroughly by gently vortexing. Centrifuge briefly to collect contents of the tube and proceed immediately to the next step.
   c. Incubate the hybridization cocktail reaction for 5 minutes at 99°C (tubes) or 95°C (plates), then for 5 min at 45°C in a thermal cycler using the **Hybridization Cocktail** protocol that is shown in Table 6 on page 15.
   d. After the incubation, centrifuge briefly to collect contents of the tube and proceed to Hybridization (Step 5).

5. Hybridize array.

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

a. (Optional) Remove the array from the hybridization oven. Vent the array with a clean pipette tip and extract the Pre-Hybridization Mix from the array with a micropipettor.

b. Refill the array with the appropriate volume (Table 21) of the hybridization cocktail (Step 4d), avoiding any insoluble matter at the bottom of the tube.

Table 21 Array cartridge volumes for Hybridization Cocktail.

Array	3’ 49 or 64-format	3’ 100 or 81/4-format	3’ 169-format	Clariom™ S 400-format
Volume to load on array	200 µL	130 µL	90 µL	90 µL

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

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

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

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

Note: During the latter part of the 16 hours hybridization prepare reagents for the washing and staining steps required immediately after completion of hybridization.

Wash and stain

For additional information about washing, staining, and scanning, see the *GeneChip™ Fluidics Station 450 User Guide GCC (Pub. No. 08-0295)*, the *GeneChip™ Expression Wash, Stain, and Scan User Guide for Cartridge Arrays* (Pub. No. MAN0018114), and the *GeneChip™ Command Console™ User Guide* (Pub. No. 702569).

1. Remove the arrays from the oven. Remove the Tough-Spots from the arrays.
2. Extract the hybridization cocktail mix from each array. (Optional) Transfer it to a new tube or well of a 96-well plate in order to save the hybridization cocktail mix. Store on ice during the procedure, or at –20°C for long-term storage.
3. Fill each array completely with Wash Buffer A.
4. Allow the arrays to equilibrate to room temperature before washing and staining.

   Note: Arrays can be stored in the Wash Buffer A at 4°C for up to 3 hr before proceeding with washing and staining. Equilibrate arrays to room temperature before washing and staining.

5. Place vials into sample holders on the fluidics station:

   a. Place 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.

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

Table 22 Fluidics protocol.

Array	3’ 49 or 64-format	3’ 100 or 81/4-format	3’ 169-format	Clariom™ S 400-format
Fluidics protocol	FS450_0001	FS450_0002	FS450_0003	FS450_0007

7. Check for air bubbles. If there are air bubbles, manually fill the array with Array Holding Buffer. If there are no air bubbles, cover both septa with 3/8” Tough-Spots. Inspect the array glass surface for dust and/or other particulates and, if necessary, carefully wipe the surface with a clean lab wipe before scanning.

Scan

The instructions for using the scanner and scanning arrays can be found in the *GeneChip™ Command Console User Guide* (Pub. No. 702569).

Array strip hybridization on the GeneAtlas™ Instrument

This section outlines the basic steps involved in hybridizing array strip(s) on the GeneAtlas™ System. The 2 major steps involved in array strip hybridization are:

- "Target hybridization setup for array strips" on page 40
- "GeneAtlas™ Software setup" on page 47

Note: If you are using a hybridization-ready sample, or re-hybridizing previously made hybridization cocktail continue the protocol from Step 4 on page 42.

IMPORTANT! Before preparing hybridization ready samples, register samples as described in "GeneAtlas™ Software setup" on page 47.

See the *GeneAtlas™ System User Guide* (Pub. No. 08-0306) for further detail.

Target hybridization setup for array strips

Reagents and materials required

- GeneAtlas Hybridization, Wash and Stain Kit for 3' IVT Array Strips. (Not supplied) For ordering information see Table 5 on page 11 or our website.
  - 1X Pre-Hybridization Mix
  - 1.3X Hybridization Mix Solution A
  - 1.3X Hybridization Mix Solution B
  - Nuclease-free Water
  - Stain Cocktail 1
  - Stain Cocktail 2
  - Array Holding Buffer
  - Wash Buffer A
  - Wash Buffer B
- GeneChip™ Hybridization Control Kit
  - 20X Eukaryotic Hybridization Controls (*bioB, bioC, bioD, cre*)
  - Control Oligonucleotide B2 (3 nM)
- Array strip and consumables (Not supplied)
  - 3' Array Strip(s)
  - 1 hybridization tray per array strip

Procedure

1. In preparation of the hybridization step, prepare the following:
    a. Pull the array strip from storage at 4°C so that it can begin to equilibrate to room temperature.
    b. Gather 2 hybridization trays per array strip.
    c. Set the temperature of the GeneAtlas Hybridization Station to 45°C. Push the start button to begin heating.
    d. Warm the pre-hybridization buffer to room temperature.

    Note: Aliquot ~500 µL of pre-hybridization buffer per array strip to be hybridized into an Eppendorf tube to accelerate the equilibration to room temperature.
2. In preparation of the hybridization master mix, prepare the following:
    a. Warm the following vials to room temperature on the bench:
        - 1.3X Hybridization Solution A
        - 1.3X Hybridization Solution B
    b. Vortex and centrifuge briefly (~5 seconds) to collect contents of the tube.
    c. Remove the following tubes from the GeneChip Hybridization Control Kit and thaw at room temperature:
        - Control Oligonucleotide B2 (3 nM)
        - 20X Eukaryotic Hybridization Controls
    d. Vortex and centrifuge briefly (~5 seconds) to collect contents of the tube.
    e. Keep the tubes of Control Oligonucleotide B2 (3 nM) and 20X Eukaryotic Hybridization Controls on ice.
3. Prepare Hybridization Master Mix.
    a. Heat the 20X Hybridization Controls for 5 minutes at 65°C in a thermal cycler using the **Hybridization Control** protocol that is shown in Table 6 on page 15.
    b. At room temperature, prepare the Hybridization Master Mix in a nuclease-free tube. Combine the appropriate amount of components in the sequence shown in the table below. Prepare the master mix for all the fragmented and biotin-labeled ds-cDNA samples in the experiment.

Table 23 Hybridization Master Mix.

Component	Volume for 1 array	Volume for 4 arrays (includes 10% overage)	Final concentration
Control Oligonucleotide B2 (3 nM)	2.5 µL	11 µL	50 pM
20X Hybridization Controls (*bioB, bioC, bioD, cre*)	7.5 µL	33 µL	1.5, 5, 25 and 100 pM, respectively
1.3X Hybridization Solution A	40 µL	176 µL	
1.3X Hybridization Solution B	75 µL	330 µL	
Total volume	**125 µL**	**550 µL**	

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

4. Pre-hybridize Array Strip.

   a. Gently pipette **120 µL** of Pre-Hybridization Buffer into the appropriate wells of the hybridization tray (Figure 5). Avoid generating air bubbles.

CAUTION! The center of the hybridization tray is not a sample well. It is important that you do not add anything to this area (Figure 5).

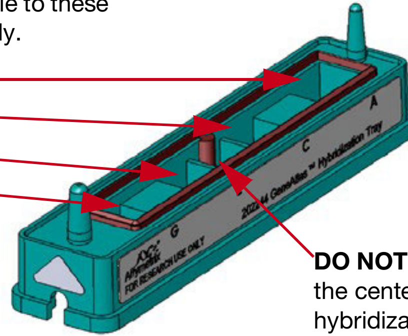
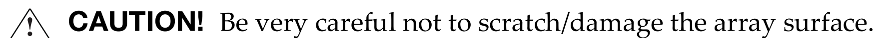


Figure 5 Location of sample wells on the hybridization tray.

   b. Remove the array strip from its foil pouch and carefully place it into the hybridization tray (Figure 6) making sure that there are no bubbles beneath the array strip.



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

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

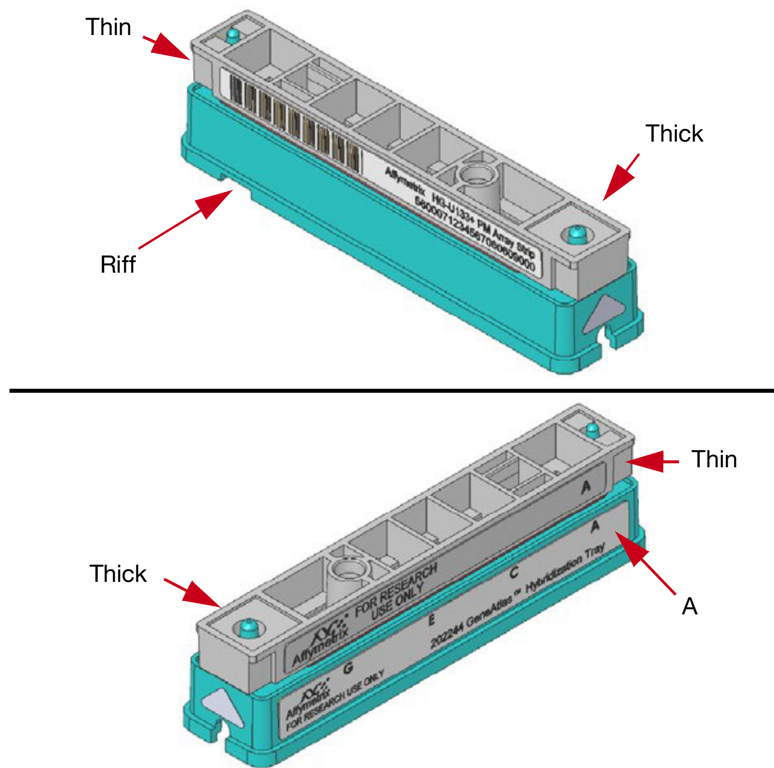


Figure 6 Proper orientation of the array strip in the hybridization tray.

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

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

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

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

> **WARNING!** Do not force the GeneAtlas Hybridization clamps up. To open, press down on the top of the clamp and simultaneously slightly lift the protruding lever to unlock. The clamp should open effortlessly. See Figure 7.

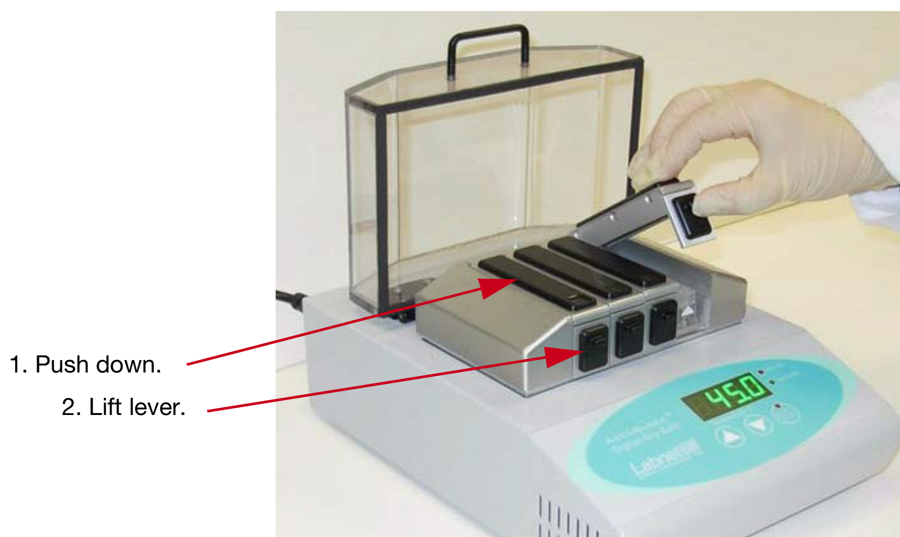


Figure 7 Opening the clamps on the GeneAtlas™ Hybridization Station.

5. Prepare Hybridization Cocktail.

   a. At room temperature, prepare the Hybridization Cocktail in the order as shown in Table 23 for all samples.

   Table 24 Hybridization Cocktail for a single array.

Component	Volume for 1 array	Final concentration
Hybridization Master Mix	125 µL	
Fragmented and Labeled ds-cDNA[1]	25 µL (4.6 µg)	30 ng/µL
Total volume	**150 µL**	

[1] Prepared according to the protocol on page 34.

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

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

   d. After the incubation, centrifuge briefly to collect contents of the tube or well and proceed to Hybridization (Step 6).

   e. Optional: the remainder of the hybridization cocktail can be stored at –20°C to supplement Hybridization Cocktail volume should a rehybridization be necessary. (See "Rehybridizing used cocktails" on page 51 for additional information.)

6. Hybridize the array strip.

   a. After 10-15 minutes of pre-hybridization, remove the array strip from the Hybridization Station and place on bench top keeping the arrays immersed in the pre-hybridization solution.

   b. Apply the **120 µL** of pre-heated hybridization cocktail (Step 5d) to the middle of the appropriate wells of a new clean hybridization tray (Figure 8).

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

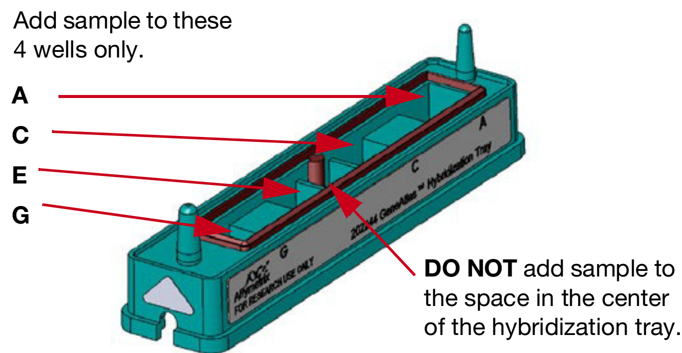


Figure 8 Location of the sample wells on the hybridization tray.

   c. Carefully remove the array strip from the hybridization tray containing the pre-hybridization buffer and place it into the hybridization tray containing the hybridization cocktail samples.

   d. Check for and remove any bubbles that were introduced. See Figure 9 for proper orientation of the array strip in the hybridization tray.

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

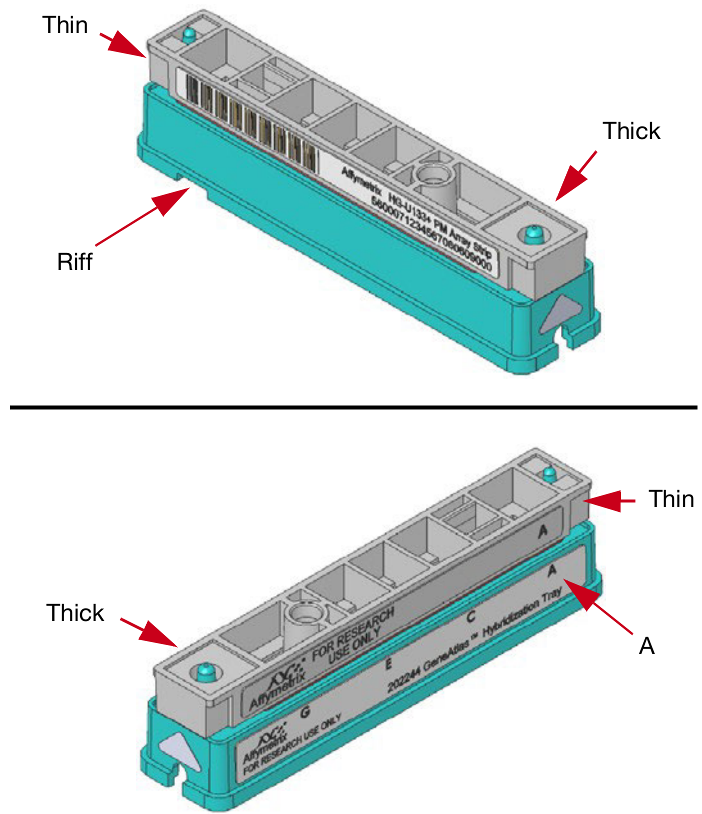
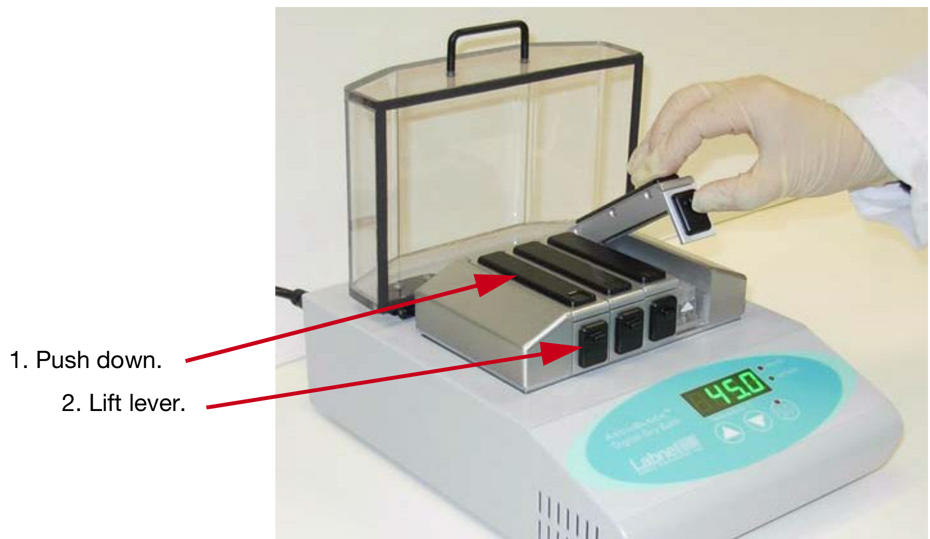


Figure 9 Proper orientation of the array strip in the hybridization tray.

e. Place the hybridization tray with the array strip into a slot in the Hybridization Station and close the clamp as shown in Figure 10.



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

Figure 10 Opening the clamps on the GeneAtlas™ Hybridization Station.

7. Proceed to "Hybridization software setup" on page 49.

GeneAtlas™ Software setup

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

- **Sample Registration**: Sample registration enters array strip data into the GeneAtlas Software and saves and stores the Sample File on your computer. The array strip barcode is scanned, or entered, and a Sample Name is input for each of the 4 samples on the array strip. Additional information includes Probe Array Type and Probe Array position.
- **Hybridization Software Setup**: During the Hybridization Software Setup the array strip to be processed is scanned, and the GeneAtlas Hybridization Station is identified with hybridization time and temperature settings determined from installed library files.

For additional information, see the *GeneAtlas™ System User Guide* (Pub. No. 08-0306).

Sample registration

The following information provides general instructions for registering GeneChip array strips in the GeneAtlas Software. For detailed information on Sample Registration, importing data from Excel and information on the wash, stain and scan steps, see the *GeneAtlas™ System User's Guide* (Pub. No. 08-0306).

1. Click **Start** → **Programs** → **Affymetrix** → **GeneAtlas** to launch the GeneAtlas Software.
2. Click the **Registration** tab.
   Figure 11 appears.

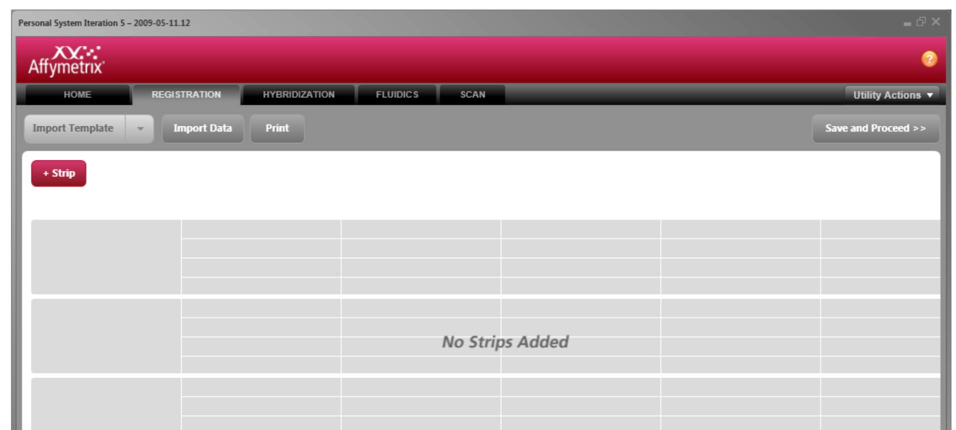


Figure 11 Registration tab of GeneAtlas™ Software.

3. Click the **+ Strip** button: .
   The Add Strip Window appears (Figure 12).

Figure 12 Add Strip window.

4. Enter or scan the array strip **Bar Code** and enter a **Strip Name**, then click **Add**.
   The array strip is added and appears in the Registration window (Figure 13).

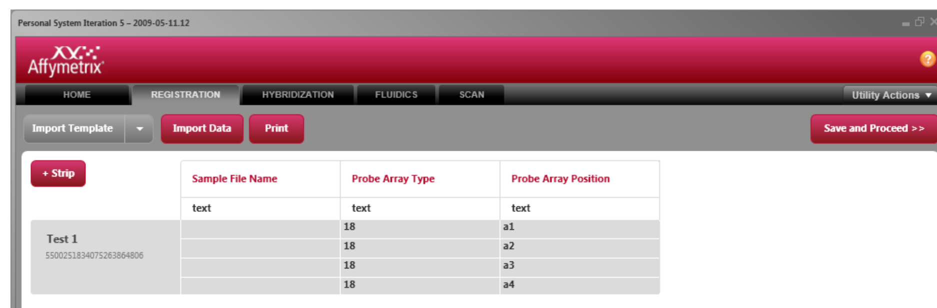
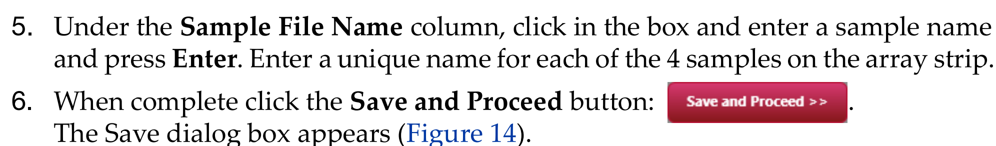


Figure 13 Array strip added to Registration window.

5. Under the **Sample File Name** column, click in the box and enter a sample name and press **Enter**. Enter a unique name for each of the 4 samples on the array strip.
6. When complete click the **Save and Proceed** button: .
   The Save dialog box appears (Figure 14).

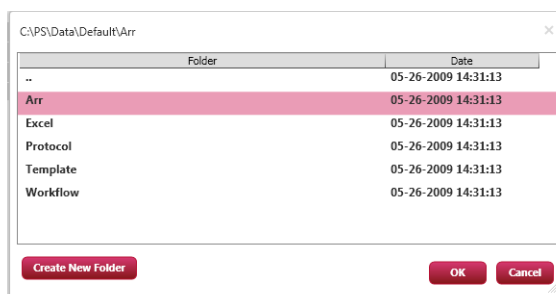
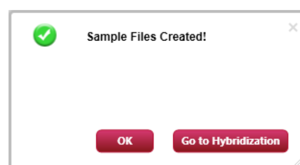


Figure 14 Save dialog.

7. In the Save dialog box, click to select a folder in which to save your data. Click **OK**.

   Your files are saved to the selected folder and a confirmation message appears.



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

   Note: You may enter a total of 4 array strips during the registration process. To add additional strips repeat Step 3 through Step 8.
9. Proceed to "Hybridization software setup" on page 49.

Hybridization software setup

All GeneChip array strips to be processed must first be registered prior to setting up the hybridizations in the GeneAtlas Software. See "Sample registration" on page 47 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. Setup hybridizations for 1 array strip at a time, staggered by 1.5 hours so that washing and staining can occur immediately after completion of hybridization for each array strip the next day.

---

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

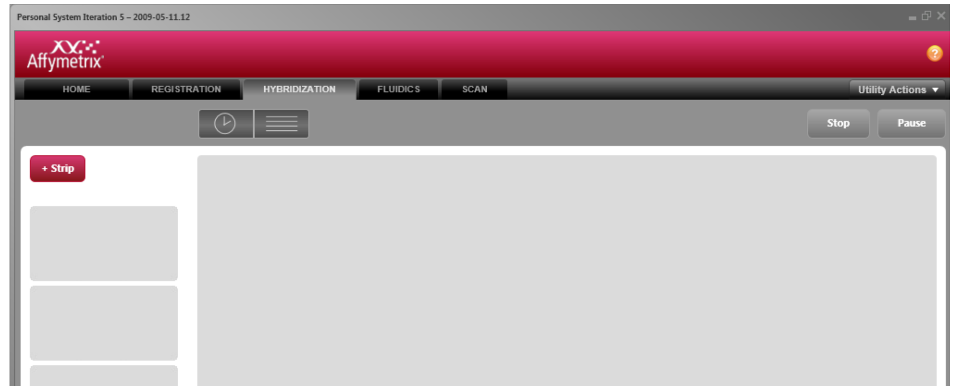


Figure 15 Hybridization window.

2. Click the **+ Strip** button:

.
   The Add Strip Window appears.

3. Scan or enter the **Bar Code** (required) of the array strip you registered.
   The **Strip Name** field is automatically populated.
4. From the **Instrument** drop-down box, select the correct hybridization station.
5. The **Time** and **Temperature** settings are automatically populated and are read from the installed library files.
6. *Do not* click **Start**. Proceed to "Target hybridization setup for array strips" on page 40.

7. With the hybridization tray and array strip already in the GeneAtlas Hybridization Station, click **Start**.

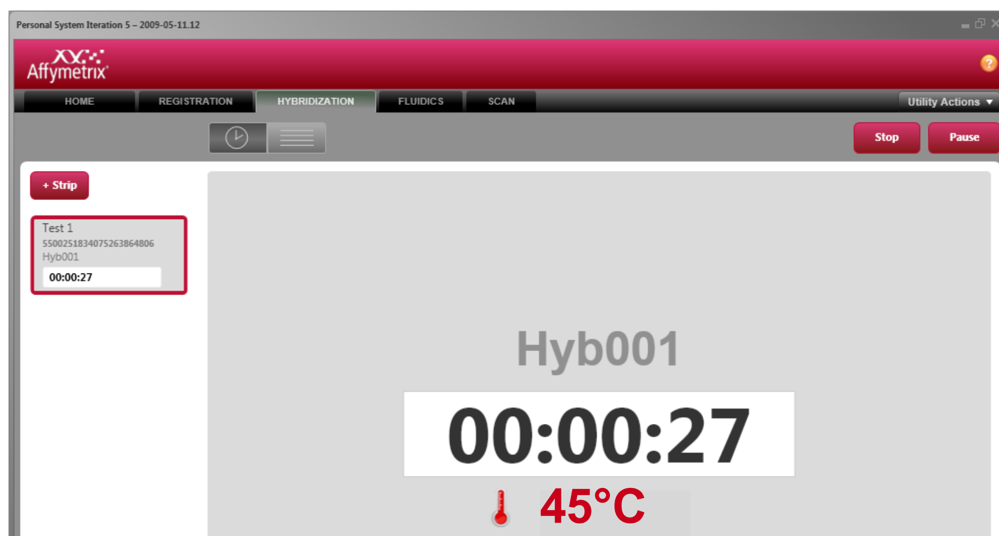


Figure 16 Hybridization countdown.

Note: The software displays the hybridization time countdown. This time is displayed with a white background (Figure 16). When the countdown has completed the display turns yellow and the time begins to count up (Figure 17).

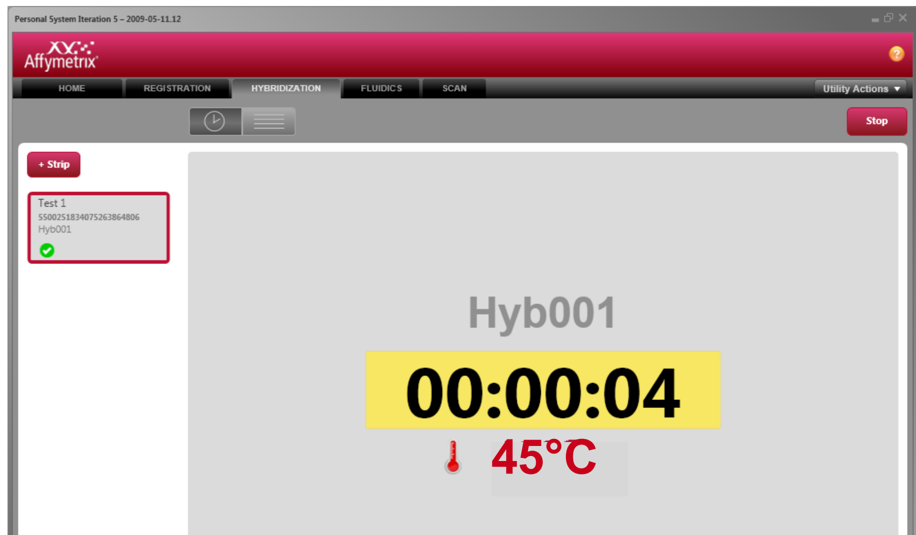


Figure 17 Hybridization count up.

8. When hybridization has completed, click the **Stop** button in the upper right corner.
   A confirmation message box appears (Figure 18).

Figure 18 Confirmation message.

9. Click **Yes** to complete hybridization.
10. Be sure to remove the hybridization tray from the Hybridization Station after the timer has completed the countdown, as the Hybridization Station does not shut down when the hybridization is complete.
11. Save the remaining hybridization cocktail in –20°C for future use (see "Rehybridizing used cocktails" on page 51 for additional information).
12. Immediately proceed to the GeneAtlas Wash, Stain, and Scan protocol. See the *GeneAtlas™ System User Guide* (Pub. No. 08-0306) for further detail.

Rehybridizing used cocktails

A used hybridization cocktail can be rehybridized to a new array if necessary. Collect the used hybridization cocktail immediately after the Fluidics run is completed, add to the remainder of the hybridization cocktail master mix from Step e on page 44 and store at –20°C.

For rehybridization, continue the protocol from Step 4 on page 42. The hybridization cocktail needs to be denatured again prior to reapplication to a new array strip.

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

Array plate hybridization in the GeneTitan™ Instrument

This chapter outlines the basic steps involved in hybridizing array plate(s) on the GeneTitan™ Instrument. The 2 major steps involved in array plate hybridization are:

- "Target hybridization setup for 3' array plates" on page 52
- "Processing array plates on the GeneTitan™ Instrument" on page 58

See the *GeneTitan™ Instrument User Guide for Expression Arrays Plates* (Pub. No. 702933) and *GeneChip™ Command Console™ User Guide* (Pub. No. 702569) for further detail.

Target hybridization setup for 3' array plates

Reagents and materials required

- GeneTitan™ Hybridization, Wash and Stain Kit for 3' Array Plates. (Not supplied) For ordering information see Table 5 on page 11 or our website.
  - 1.3X Hybridization Mix Solution A
  - 1.3X Hybridization Mix Solution B
  - Stain Cocktail 1 & 3
  - Stain Cocktail 2
  - Array Holding Buffer
  - Wash Buffer A
  - Wash Buffer B
- GeneChip™ Hybridization Control Kit
  - 20X Eukaryotic Hybridization Controls (*bioB, bioC, bioD, cre*)
  - Control Oligonucleotide B2 (3 nM)
- Array plate and consumables (not supplied)
  - 3' array plate(s)

Procedure

1. In preparation of the hybridization step, prepare the following:
   a. Warm the following vials to room temperature on the bench:
      - 1.3X Hybridization Mix Solution A
      - 1.3X Hybridization Mix Solution B
   b. Vortex and centrifuge briefly (~5 seconds) to collect contents of the tube.
   c. Remove the following tubes from the GeneChip Hybridization Control Kit and thaw at room temperature:
      - Control Oligonucleotide B2 (3 nM)
      - 20X Eukaryotic Hybridization Controls
   d. Vortex and centrifuge briefly (~5 seconds) to collect liquid at the bottom of the tube.
   e. Keep the tubes of Control Oligonucleotide B2 (3 nM) and the tube of 20X Eukaryotic Hybridization Controls on ice.
2. Prepare the Hybridization Master Mix & Cocktail.
   a. Heat the 20X Hybridization Controls for 5 minutes at 65°C in a thermal cycler using the **Hybridization Control** protocol that is shown in Table 6 on page 15.

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

Table 25 Hybridization Master Mix.

Component	Volume for 1 array	16-format[1]	24-format[1]	96-format[1]	Final concentration
Control Oligo B2 (3 nM)	2 µL	35.2 µL	52.8 µL	211.2 µL	50 pM
20X Eukaryotic Hybridization Controls (*bioB, bioC, bioD, cre*)	6 µL	105.6 µL	158.4 µL	633.6 µL	1.5, 5, 25 and 100 pM, respectively
1.3X Hybridization Solution A	32.3 µL	569.3 µL	853.9 µL	3,415.6 µL	
1.3X Hybridization Solution B	60 µL	1,056 µL	1,584 µL	6,336 µL	
Total volume	**100 µL**	**1,766 µL**	**2,649 µL**	**10,596 µL**	

[1] Includes ~10% overage to cover pipetting error.

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

3. Prepare Hybridization Cocktail.

a. At room temperature, prepare the Hybridization Cocktail in the order as shown in Table 25 for all samples.

Table 26 Hybridization Cocktail for a single array.

Component	Volume for 1 array	Final concentration
Hybridization Master Mix	100 µL	
Fragmented and Labeled ds-cDNA[1]	20 µL (3.7 µg)	30 ng/µL
Total volume	**120 µL**	

[1] Prepared according to the protocol on page 34.

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

c. Incubate the hybridization cocktail reaction for 5 minutes at 99°C (tubes) or 95°C (plates), then for 5 minutes at 45°C in a thermal cycler using the **Hybridization Cocktail** protocol that is shown in Table 6 on page 15.

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

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

f. Save the remaining hybridization cocktail in –20°C for future use.

g. Proceed to "Hybridization setup" on page 54.

Hybridization setup

This section describes the GeneTitan Setup protocol for 3' Array Plates. The reagent consumption per process on the GeneTitan™ Instrument for processing 3' array plates is shown in Table 28.

Table 27 The minimum volumes of buffer and rinse required to process on the GeneTitan 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 A	~920 mL	1,040 mL +	2,000 mL
Wash B	300 mL	450 mL	600 mL

Table 28 Volumes required to process 3' 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 3' Array Plates (Cat. No. 901530)		
		16-format	24-format	96-format
Wash A[1]	~920 mL	1	1	1
Wash B[1]	300 mL	1	1	1
Stain 1 and 3	105 µL/well	6	4	1
Stain 2	105 µL/well	6	4	1
Array Holding Buffer	150 µL/well	6	4	1

[1] Use GeneTitan™ Wash Buffers A and B Module (Part No. 901583) for ordering Wash A and B Buffers for additional plates.

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

Target hybridization setup for Clariom™ S Array Plates

Reagents and materials required

- GeneTitan™ Hybridization, Wash, and Stain Kit for WT Array Plates (not supplied). For ordering information see Table 5 on page 11 or our website.
  - 5X WT Hyb Add 1
  - 15X WT Hyb Add 4
  - 2.5X WT Hyb Add 6
  - Stain Cocktail 1 & 3
  - Stain Cocktail 2
  - Array Holding Buffer
  - Wash Buffer A
  - Wash Buffer B
- GeneChip™ Hybridization Control Kit
  - 20X Eukaryotic Hybridization Controls (*bioB, bioC, bioD, cre*)
  - Control Oligonucleotide B2 (3 nM)
- Array plate and consumables (not supplied)
  - Clariom S array plate(s) and trays

Procedure

Note: The "WT Hyb Add" reagent names were created to match the order in which reagents are added. For example, WT Hyb Add 4 is the fourth component added during preparation of the Hybridization Mix. WT Hyb Add 2, 3, and 5 are not used and are not part of the Hybridization Module.

1. In preparation of the hybridization step, prepare the following:
   a. Warm the following vials to room temperature on the bench:
      - 5X WT Hyb Add 1
      - 15X WT Hyb Add 4
      - 2.5X WT Hyb Add 6.
   b. Vortex and centrifuge briefly (~5 seconds) to collect contents of the tube.
   c. Remove the following tubes from the GeneChip Hybridization Control Kit and thaw at room temperature:
      - Control Oligonucleotide B2 (3 nM)
      - 20X Eukaryotic Hybridization Controls
   d. Vortex and centrifuge briefly (~5 seconds) to collect liquid at the bottom of the tube.
   e. Keep the tubes of Control Oligonucleotide B2 (3 nM) and the tube of 20X Eukaryotic Hybridization Controls on ice.
2. Prepare the WT Hybridization Master Mix & Cocktail.
   a. Heat the 20X Hybridization Controls for 5 minutes at 65°C in a thermal cycler using the **Hybridization Control** protocol that is shown in Table 6 on page 15.
   b. At room temperature, prepare the Hybridization Master Mix in a nuclease-free tube. Combine the appropriate amount of components in the sequence shown in the table below. Prepare the master mix for all the fragmented and biotin-labeled ds-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 29 Hybridization Master Mix.

Component	Volume for 1 array	24-format[1]	96-format[1]	Final concentration
5X WT Hyb Add 1	22 µL	580.8 µL	2,323.2 µL	1X
Control Oligo B2 (3 nM)	1.1 µL	29.04 µL	116.16 µL	30 pM
20X Eukaryotic Hybridization Controls (*bioB, bioC, bioD, cre*)	5.5 µL	145.2 µL	580.8 µL	1.5, 5, 25, and 100 pM, respectively
15X WT Hyb Add 4	7.3 µL	192.72 µL	770.88 µL	1X
Nuclease-free Water	0.1 µL	2.64 µL	10.56 µL	
Total volume	**36 µL**	**950.4 µL**	**3,801.6 µL**	

[1] Includes ~10% overage to cover pipetting error.

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

3. Prepare Hybridization Cocktail.

a. At room temperature, prepare the Hybridization Cocktail in the order as shown in Table 29 for all samples.

Table 30 Hybridization Cocktail for a single array.

Component	Volume for 1 array	Final concentration
Hybridization Master Mix	36 µL	
Fragmented and labeled ds-cDNA	30 µL	50 ng/µL
2.5X WT Hyb Add 6	44 µL	1X
Total volume	**110 µL**	

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

c. Incubate the hybridization cocktail reaction for 5 minutes at 99°C (tubes) or 95°C (plates), and then for 5 min at 45°C in a thermal cycler using the **Hybridization Cocktail** protocol that is shown in Table 6 on page 15.

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

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

f. Proceed to "Hybridization setup" on page 57.

Hybridization setup

This section describes the GeneTitan Setup steps for Clariom S array plates. The reagent consumption per process on the GeneTitan™ Instrument for processing Clariom S array plates is shown in Table 28.

Table 31 The minimum volumes of Buffer and Rinse required to process on the GeneTitan 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 A	~920 mL	1,040 mL +	2,000 mL
Wash B	300 mL	450 mL	600 mL

Table 32 Volumes required to process Clariom S 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 Clariom S Array Plates (Cat. No. 901622)	
		24-format	96-format
Wash A	~920 mL	1	1
Wash B	300 mL	1	1
Stain 1 and 3	105 µL/well	4	1
Stain 2	105 µL/well	4	1
Array Holding Buffer	150 µL/well	4	1

IMPORTANT! The instrument must have a minimum of 450 mL of Wash B in the Wash B reservoir of the instrument for each Clariom S array plate prior to starting hybridization, wash, stain, and scan process. The waste bottle should be empty.

Processing array plates on the GeneTitan™ Instrument

Follow the instructions provided in the *GeneTitan™ Instrument User Guide for Expression Array Plates* (Pub. No. 702933) to process HT array plates on the GeneTitan Instrument.

1. Use the anti-static gun on the wells of the stain tray labeled GeneTitan Stain Tray Part No. 501025.
   a. Place a stain tray on the table top.
   b. Hold the Zerostat 3 anti-static gun within 12" (30.5 cm) of the surface or object to be treated. Squeeze the trigger slowly for about 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.
   c. Repeat this procedure at several points across the surface of the stain tray.
2. Aliquot 105 µL per well of the Stain 1 into the GeneTitan Stain Tray.
3. Use the anti-static gun on the stain tray cover.
   a. Place a stain tray cover on the table top with the flat surface facing upward.
   b. Hold the Zerostat 3 anti-static gun within 12" (30.5 cm) of the surface or object to be treated. Squeeze the trigger slowly for about 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.
   c. Repeat this procedure at several points across the surface, covering the entire stain tray cover.
4. After removing the static electricity, place the cover on top Stain Tray 1.
5. After repeating Step 1, aliquot 105 µL per well of the Stain 2 into the GeneTitan Stain Tray.
6. After repeating Step 3, place cover on top of Stain Tray 2.
7. After repeating Step 1, aliquot 105 µL per well of the Stain 3 into the GeneTitan Stain Tray.
8. After repeating Step 3, place cover on top of Stain Tray 3.
9. Aliquot 150 µL per well of the Array Holding Buffer into all wells of the GeneTitan scan tray identified with the label **HT Scan Tray Part No. 500860** on the tray.
10. Use the fourth scan tray cover provided with the GeneTitan Consumable Upgrade kit to cover the scan tray.
11. Load all the consumables including the HT array plate into the GeneTitan Instrument following the instructions provided in the *GeneTitan™ Instrument User Guide for Expression Arrays Plates* (Pub. No. 702933).

IMPORTANT! It is important not to bump the trays while loading them into the GeneTitan Instrument. Droplets of the stain going onto the lid may result in a wicking action and the instrument gripper may be unable to remove the lids properly.

The remaining hybridization ready sample can be stored at –20°C in the hybridization tray using aluminum foil.

Troubleshooting and references

Troubleshooting

Table 33 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 procedure. Run an extra 1 or 2 amplification cycles during Pre-IVT Amplification.
	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 amount of total RNA in the First-Strand cDNA Synthesis procedure. Run and extra 1 or 2 amplification cycles during Pre-IVT Amplification.

Table 33 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 amount of total RNA in the First-Strand cDNA Synthesis procedure. Reduce amplification cycles by 1 or 2 cycles during Pre-IVT Amplification.
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 "Evaluate 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.

References

Aranda IV, R., Dineen, S.M., Craig, R.L., Guerrieri, R.A., and Robertson, M.J. 2009. Anal Biochem 387: 122-127.

Johnson, L.F., Abelson, H.T., Green, H., and S. Penman. 1974. Cell 1: 95–100.

Sambrook, J. and D.W. Russel. 2001. Extraction, purification, and analysis of mRNA from eukaryotic cells. In: Molecular cloning, a laboratory manual, third edition, Vol 1. Cold Spring Harbor, New York: Cold Spring Harbor Press.

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

cRNA purification photos

Magnetic Stand-96		96-Well Magnetic-Ring Stand
	Magnetic Stand	
	cRNA Binding to Beads	
	Bead Capture	
	Bead Washing	
	Ethanol Wash Solution Removal	

Figure 19 Photos of cRNA purification step (1 of 2).

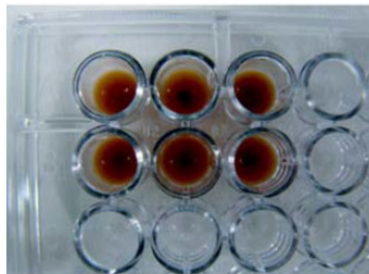
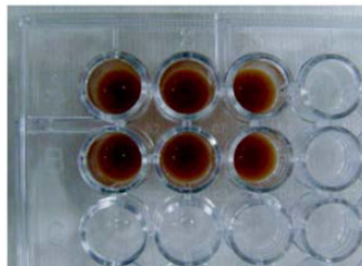
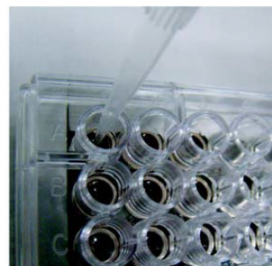
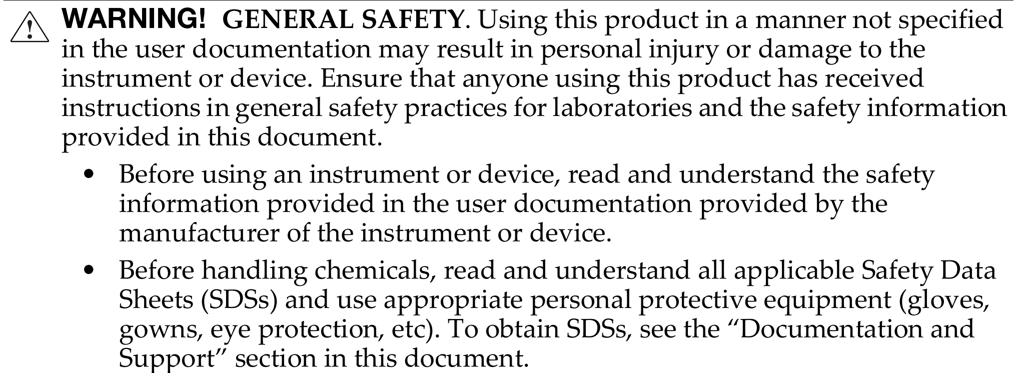
Magnetic Stand-96		96-Well Magnetic-Ring Stand
	cRNA Elution	
	Elution Step	

Figure 20 Photos of cRNA purification step (2 of 2).

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, etc). 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, and 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 adequate ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's 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 necessary) 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.

Biological hazard safety

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. All work should be conducted in properly equipped facilities using the appropriate safety equipment (for example, physical containment devices). Safety equipment also may 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: **www.cdc.gov/biosafety/publications/bmbl5/BMBL.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

- *GeneChip™ Pico Reagent Kit Quick Reference Card*, Pub. No. 703309
- *GeneAtlas™ System User Guide,* Pub. No. 08-0306
- *GeneTitan™ Instrument User Guide for Expression Array Plates User Guide*, Pub. No. 702933
- *GeneTitan™ Multi-Channel Instrument User Guide*, Pub. No. 08-0308
- *GeneChip™ Fluidics Station 450 User Guide GCC*, Pub. No. 08-0295
- *GeneChip™ Expression Wash, Stain, and Scan User Guide for Cartridge Arrays*, Pub. No. MAN0018114
- *GeneChip™ Command Console™ Software User Guide*, Pub. No. 702569
- *GeneChip™ Command Console™ 4.0 for GeneTitan™ and Gene Titan MC Instruments Quick Reference*, Pub. No. 702740
- *GeneChip™ Command Console™ 4.0 for Cartridge Arrays Quick Reference*, Pub. No. 702552
- *Expression Console™ Software User Guide*, Pub. No. 702387
- *Transcriptome Analysis Console™ (TAC) Software User Guide*, Pub. No. 703150

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