Applied Biosystems

The Ambion® WT Expression Kit

For Affymetrix® GeneChip® Whole Transcript (WT) Expression Arrays

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Contents

Safety information

Note: For general safety information, see this Preface and Appendix A, "Safety" on page 29. When a hazard symbol and hazard type appear by a chemical name or instrument hazard, see the "Safety" Appendix for the complete alert on the chemical or instrument.

Safety alert words

Four safety alert words appear in Applied Biosystems user documentation at points in the document where you need to be aware of relevant hazards. Each alert word—**IMPORTANT, CAUTION, WARNING, DANGER**—implies a particular level of observation or action, as defined below:

IMPORTANT! – Indicates information that is necessary for proper instrument operation, accurate chemistry kit use, or safe use of a chemical.

CAUTION! – Indicates a potentially hazardous situation that, if not avoided, may result in minor or moderate injury. It may also be used to alert against unsafe practices.

WARNING! – Indicates a potentially hazardous situation that, if not avoided, could result in death or serious injury.

DANGER! – Indicates an imminently hazardous situation that, if not avoided, will result in death or serious injury. This signal word is to be limited to the most extreme situations.

MSDSs

The MSDSs for any chemicals supplied by Applied Biosystems or Ambion are available to you free 24 hours a day. For instructions on obtaining MSDSs, see "MSDSs" on page 30.

IMPORTANT! For the MSDSs of chemicals not distributed by Applied Biosystems or Ambion, contact the chemical manufacturer.

How to use this guide

Text conventions

This guide uses the following conventions:

- **Bold** text indicates user action. For example:

  Type **0**, then press **Enter** for each of the remaining fields.
- *Italic* text indicates new or important words and is also used for emphasis. For example:

  Before analyzing, *always* prepare fresh matrix.
- A right arrow symbol (▸) separates successive commands you select from a drop-down or shortcut menu. For example:

  Select **File ▸ Open ▸ Spot Set**.

  Right-click the sample row, then select **View Filter ▸ View All Runs**.

User attention words

Two user attention words appear in Applied Biosystems user documentation. Each word implies a particular level of observation or action as described below:

Note: – Provides information that may be of interest or help but is not critical to the use of the product.

IMPORTANT! – Provides information that is necessary for proper instrument operation, accurate chemistry kit use, or safe use of a chemical.

How to obtain support

For the latest services and support information for all locations, go to:

www.appliedbiosystems.com

At the Applied Biosystems web site, you can:

- Access worldwide telephone and fax numbers to contact Applied Biosystems Technical Support and Sales facilities.
- Search through frequently asked questions (FAQs).
- Submit a question directly to Technical Support.
- Order Applied Biosystems user documents, MSDSs, certificates of analysis, and other related documents.
- Download PDF documents.
- Obtain information about customer training.
- Download software updates and patches.

The Ambion® WT Expression Kit

Product information

Purpose of the product

The Ambion® WT Expression Kit enables you to prepare RNA samples for Affymetrix whole transcriptome microarray analysis. The kit generates sense-strand cDNA from total RNA for fragmentation and labeling using the Affymetrix GeneChip® WT Terminal Labeling Kit (PN 900671). The kit is optimized for use with human, mouse, and rat Affymetrix GeneChip® Sense Target (ST) Arrays.

The WT Expression Kit uses a reverse transcription priming method that specifically primes non-ribosomal RNA from your sample, including both poly(A) and non-poly(A) mRNA. Primers that avoid rRNA binding are designed by using a proprietary-oligonucleotide matching algorithm. These primer sequences provide complete and unbiased coverage of the transcriptome while significantly reducing the priming of rRNA.

Kit contents and storage

Component	For manual use only		For high-throughput automated use only		Storage
	10-Reaction kit (PN 4411973)	30-Reaction kit (PN 4411974)	24-Reaction kit (PN 4440537)	96-Reaction kit (PN 4440536)	
First-Strand Enzyme Mix	11 µL	33 µL	50 µL	150 µL	–20 °C
First-Strand Buffer Mix	44 µL	132 µL	160 µL	600 µL	–20 °C
Second-Strand Enzyme Mix	55 µL	165 µL	175 µL	650 µL	–20 °C
Second-Strand Buffer Mix	138 µL	413 µL	400 µL	1.45 mL	–20 °C
IVT Enzyme Mix	66 µL	198 µL	210 µL	850 µL	–20 °C
IVT Buffer Mix	264 µL	792 µL	750 µL	1.40 mL	–20 °C
Control RNA (1 mg/mL HeLa total RNA)	10 µL	10 µL	10 µL	10 µL	–20 °C
Nuclease-free Water	1.75 mL	1.75 mL	NA	NA	any temp‡
2nd-Cycle Buffer Mix	88 µL	264 µL	425 µL	1.45 mL	–20 °C
Random Primers	22 µL	66 µL	100 µL	300 µL	–20 °C
2nd-Cycle Enzyme Mix	88 µL	264 µL	275 µL	1.00 mL	–20 °C
RNase H	22 µL	66 µL	100 µL	300 µL	–20 °C
Nucleic Acid Purification Reagents					
Nucleic Acid Binding Buffer Concentrate	1.1 mL	3.3 mL	3.5 mL	12.5 mL	room temp
Nucleic Acid Binding Beads	220 µL	660 µL	700 µL	2.5 mL	4 °C§
Nucleic Acid Wash Solution Concentrate	10 mL	20 mL	15 mL	50 mL	room temp
Elution Solution	5 mL	5 mL	6 mL	22 mL	4 °C or room temp
Nuclease-free Water	10 mL	10 mL	NA	NA	room temp
Consumables					
8-Strip PCR Tubes & Caps (0.2-mL)	10	40	NA	NA	room temp
U-Bottom Plate	2	4	NA	NA	room temp
Reservoir	1	2	NA	NA	room temp

‡ Store the Nuclease-free Water at –20 °C, 4 °C, or room temp.
§ Do not freeze.

Required materials

Equipment

Item	Source
Agilent 2100 Bioanalyzer	Agilent Technologies, Inc.
Ambion Magnetic Stand-96	Ambion PN AM10027
Applied Biosystems Veriti® 96-Well Thermal Cycler	Applied Biosystems PN 4375786
Barnstead Titer Plate Shaker, Barnstead/Lab-Line	Major Laboratory Supplier (MLS)
Thermo Scientific NanoDrop® ND-8000 UV-Vis Spectrophotometer or Thermo Scientific NanoDrop® ND-1000 UV-Vis Single-Channel Spectrophotometer	NanoDrop Technologies
Microcentrifuge with adapter for PCR 8-strip tubes or for 96-well plates	MLS

Reagents and supplies

Item	Source
GeneChip® WT Terminal Labeling and Controls Kit. (For a list of kit components, go to: **www.affymetrix.com** and search for Ambion WT Expression Kit.)	Affymetrix PN 901524 (30 reaction) Affymetrix PN 901525 (10 reaction)
(Optional) Quant-iT™ RiboGreen® RNA Reagent	Invitrogen
(Optional) Quant-iT™ PicoGreen® RNA Reagent	Invitrogen
100% Ethanol (ACS grade or equivalent)	MLS‡
100% Isopropanol (ACS grade or equivalent)	MLS‡
RNA 6000 Nano Kit	Agilent Technologies, Inc. PN 5067-1511
(Optional) RNase*Zap*® RNase Decontamination Solution	Ambion PN AM9780 (250 mL) Ambion PN AM9784 (4.0 L)
(Optional) The RNA Storage Solution	Ambion PN AM7000 (10 × 1.0 mL) Ambion PN AM7001 (50 mL)

‡ For the MSDS of any chemical not distributed by Applied Biosystems, contact the chemical manufacturer. Before handling any chemicals, refer to the MSDS provided by the manufacturer, and observe all relevant precautions.

Day 1 workflow

Synthesize first-strand cDNA

Prepare the First-Strand Master Mix

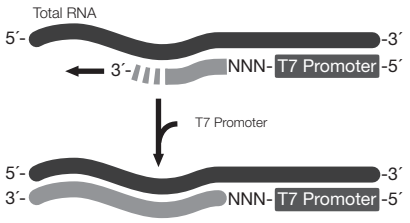
↓

Add the total RNA

↓

Incubate

↓



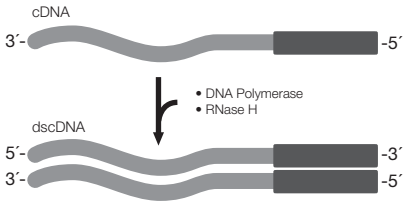
Synthesize second-strand cDNA

Prepare the Second-Strand Master Mix and add to sample

↓

Incubate

↓



Synthesize cRNA using in vitro transcription

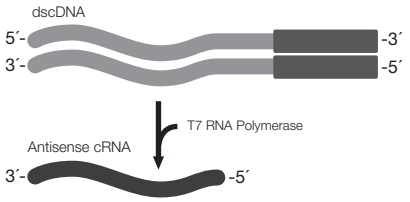
Prepare the IVT Master Mix and add to second-strand cDNA sample

↓

Incubate for 16 hours

↓

Stopping point



Day 2 workflow

Purify cRNA

Prepare the cRNA Binding Mix and add to each sample

↓

Add 100% isopropanol to each sample and shake gently

↓

Capture the Nucleic Acid Binding Beads and discard the supernatant

↓

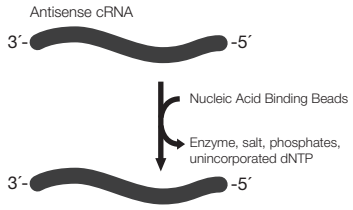
Wash twice with Nucleic Acid Wash Solution

↓

Elute cRNA with preheated Elution Solution

↓

Place the cRNA on ice briefly, or freeze immediately

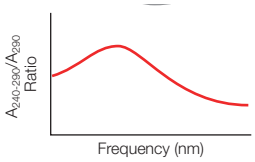


Assess cRNA yield and size distribution

Use 1.5 µL of cRNA to assess yield by UV absorbance

↓

Use 1 µL of cRNA to assess size distribution



Synthesize 2nd-cycle cDNA

Combine 10 µg of cRNA and the Random Primers

↓

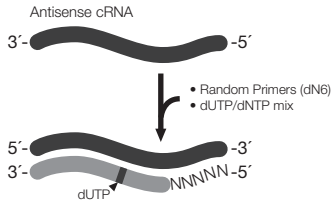
Incubate

↓

Prepare the *2nd-Cycle Master Mix* on ice and add to each sample

↓

Incubate



Day 2 workflow, continued

Hydrolyze using RNase H

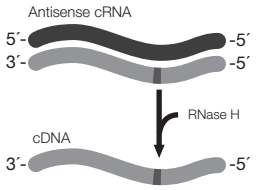
Add RNase H to the 2nd-Cycle cDNA

↓

Incubate

↓

Optional stopping point



Purify 2nd-cycle cDNA

Prepare the cDNA Binding Mix, add to each sample, and transfer to a U-bottom plate

↓

Add ethanol to each sample and shake gently

↓

Capture the Nucleic Acid Binding Beads and discard the supernatant

↓

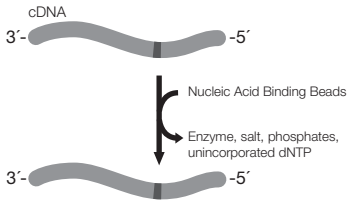
Wash twice with Nucleic Acid Wash Solution

↓

Elute the cDNA with preheated Elution Solution

↓

Place the cDNA on ice briefly, or freeze immediately

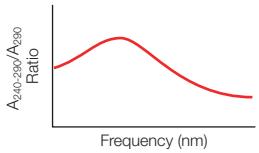


Assess cDNA yield and size distribution

Determine cDNA yield by UV absorbance

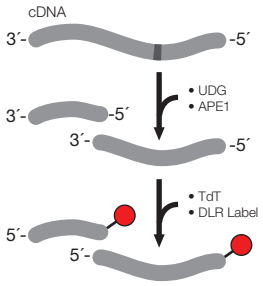
↓

(Optional) Use 1 µL of cDNA to assess size distribution



Fragment and label the single-stranded cDNA

For instructions on how to fragment the single-stranded cDNA, refer to the Affymetrix *GeneChip® WT Terminal Labeling and Hybridization User Manual* (PN 702880).



Procedure

Implement a plan to maintain procedural consistency

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

- Method of RNA isolation
- Amount of input RNA that is used for each tissue type
- Type of thermal cycler that is used
- Workflow stopping points

Equipment and reagent preparation

Recommended thermal cycler

Applied Biosystems recommends using the Applied Biosystems Veriti® 96-Well Thermal Cycler with 0.2-mL sample wells (PN 4375786) to prepare samples using the Ambion® WT Expression Kit. If you use a different instrument, 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

When using the Applied Biosystems Veriti® Thermal Cycler, set the idle temperature for the heated lid to 50 °C prior to starting Day 1, first-strand cDNA synthesis. Maintain this setting throughout Day 1. Prior to starting Day 2, 2nd-cycle cRNA denaturation, change the idle temperature for the lid to 75 °C. Maintain this setting throughout Day 2. Prior to starting cDNA fragmentation, change the idle temperature for the lid to 105 °C, which ensures that the temperature of the heated lid is at or near the required temperature for each step. For instructions on how to change the temperature settings of the Veriti thermal cycler, refer to the Applied Biosystems Veriti® Thermal Cycler User Guide (PN 4375799).

An alternate protocol may be used for thermal cyclers that lack a programmable heated lid. This is not the preferred method. Yields of cRNA may be greatly reduced if a lid heated to 105 °C is used during the Second-Strand cDNA Synthesis or during the In Vitro Transcription cRNA Synthesis steps. We recommend leaving the heated lid open during Second-Strand cDNA Synthesis. A small amount of condensation will form during the incubation. This is expected, and should not significantly decrease cRNA yields. For In Vitro Transcription cRNA Synthesis, we recommend incubating the reaction in a 40 °C hybridization oven if a programmable heated lid thermal cycler is unavailable.

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

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

Table 1 Thermal cycling methods

Method	Heated lid temp.	Alternate Protocol‡	Step 1	Step 2	Step 3	Step 4
First-Strand cDNA Synthesis	50 °C	105 °C	25 °C, 60 min	42 °C, 60 min	4 °C, 2 min	
Second-Strand cDNA Synthesis	RT or disable	Lid open	16 °C, 60 min	65 °C, 10 min	4 °C, 2 min	
In Vitro Transcription cRNA Synthesis	50 °C	40 °C oven	40 °C, 16 hrs	4 °C, Hold		
2nd-Cycle cRNA Denaturation	75 °C	105 °C	70 °C, 5 min	25 °C, 5 min	4 °C, 2 min	
2nd-Cycle cDNA Synthesis	75 °C	105 °C	25 °C, 10 min	42 °C, 90 min	70 °C, 10 min	4 °C, 2 min
RNase H Hydrolysis	75 °C	105 °C	37 °C, 45 min	95 °C, 5 min	4 °C, 2 min	

‡ For thermal cyclers that lack a programmable heated lid.

Prepare the Control RNA and total RNA

Prepare the Control RNA

For the following hazards, see the complete safety alert descriptions in "Chemical alerts" on page 32:

WARNING! BIOHAZARD. Control RNA.

To verify that the reagents are working as expected, a Control RNA sample (1 mg/mL total RNA from HeLa cells) is included with the kit.

To prepare the control:

1. Dispense 2 µL of Control RNA in 38 µL of nuclease-free water for a total volume of 40 µL.
2. Follow the "Synthesize first-strand cDNA" on page 13, but in Step 2, use 1 µL of diluted Control RNA (50 ng) in the control reaction.

Note: The positive control reaction should produce >20 µg of cRNA and >6 µg of 2nd-cycle cDNA.

Prepare your total RNA

Prepare your total RNA according to your laboratory's procedure.

Determine your input 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 the table below are based on total RNA from HeLa cells. Use these values as reference points for determining your optimal RNA input.

RNA input criterion	Total RNA (ng)
Recommended	100
Minimum	50
Maximum	500

Figure 1 on page 10 shows the cRNA yields with varying inputs of three RNAs: the HeLa Kit control, Stratagene® Universal Human Reference RNA (UHRR), and Ambion FirstChoice® Human Brain Reference RNA (HBRR). For each RNA, the plot displays the yields from eight titration points: 0 ng, 12.5 ng, 25 ng, 50 ng, 100 ng, 200 ng, 400 ng, and 800 ng.

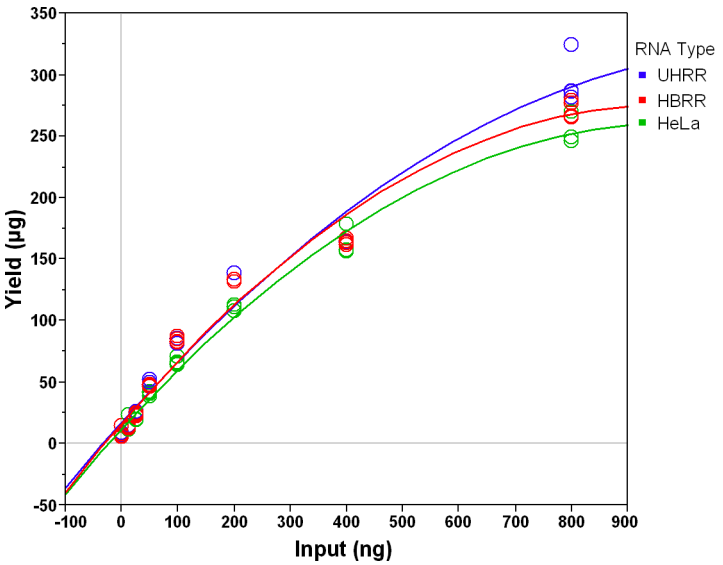


Figure 1 cRNA yields with varying inputs of three RNAs

Prepare the Poly-A RNA Controls

Note: To include premixed controls from the Affymetrix® GeneChip® Poly-A RNA Control Kit (PN 900433 or equivalent), add the reagents to the total RNA samples. Follow the instructions below. Ambion strongly recommends the use of poly-A controls for all reactions that will be hybridized to GeneChip® arrays.

Designed specifically to provide exogenous positive controls to monitor the entire target labeling process, a set of poly-A RNA controls supplied by Affymetrix can be added to the RNA prior to the First Strand Synthesis step.

Each eukaryotic GeneChip® probe array contains probe sets for several *B. subtilis* genes that are absent in eukaryotic samples (lys, phe, thr, and dap). These poly-A RNA controls are in vitro synthesized, and the polyadenylated transcripts for the *B. subtilis* genes are premixed at staggered concentrations. The concentrated Poly-A Control Stock can be diluted with the Poly-A Control Dil Buffer and spiked directly into RNA samples to achieve the final concentrations (referred to as a ratio of copy number) summarized in Table 2.

Table 2 Final concentrations of Poly-A RNA Controls when added to total RNA samples

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

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

The Poly-A RNA Control Stock and Poly-A Control Dil Buffer are provided in the Affymetrix® GeneChip® Poly-A RNA Control Kit (PN 900433 or equivalent) to prepare the appropriate serial dilutions based on Table 3. This is a guideline when 50, 100, 250, or 500 ng of total RNA is used as starting material. For starting sample amounts other than those listed here, calculations are needed in order to perform the appropriate dilutions to arrive at the same proportionate final concentration of the spike-in controls in the samples.

Table 3 Serial dilution of Poly-A RNA Control Stock

Total RNA input amount	Serial dilutions				Volume of 4th dilution to add to total RNA
	1st dilution	2nd dilution	3rd dilution	4th dilution	
50 ng	1:20	1:50	1:50	1:20	2 µL
100 ng	1:20	1:50	1:50	1:10	2 µL
250 ng	1:20	1:50	1:50	1:4	2 µL
500 ng	1:20	1:50	1:50	1:2	2 µL

Recommendation: Avoid pipetting solutions less than 2 µL in volume to maintain precision and consistency when preparing the dilutions.

IMPORTANT! Use non-stick RNase-free microfuge tubes to prepare all of the dilutions (not included).

For example, to prepare the Poly-A RNA dilutions for 100 ng of total RNA:

1. Add 2 µL of the Poly-A Control Stock to 38 µL of Poly-A Control Dil Buffer for the first dilution (1:20).
2. Mix thoroughly and spin down to collect the liquid at the bottom of the tube.
3. Add 2 µL of the first dilution to 98 µL of Poly-A Control Dil Buffer to prepare the second dilution (1:50).
4. Mix thoroughly and spin down to collect the liquid at the bottom of the tube.
5. Add 2 µL of the second dilution to 98 µL of Poly-A Control Dil Buffer to prepare the third dilution (1:50).
6. Mix thoroughly and spin down to collect the liquid at the bottom of the tube.
7. Add 2 µL of the third dilution to 18 µL of Poly-A Control Dil Buffer to prepare the fourth dilution (1:10).
8. Mix thoroughly and spin down to collect the liquid at the bottom of the tube.
9. Add 2 µL of this fourth dilution to 100 ng of total RNA.

Note: The first dilution of the Poly-A RNA controls can be stored up to 6 weeks in a non-frost-free freezer at – 20 ºC and frozen/thawed up to eight times.

Evaluate RNA quality

RNA quality affects how efficiently an RNA sample is amplified using this kit. High-quality RNA is free of contaminating proteins, DNA, phenol, ethanol, and salts. To evaluate RNA quality, determine its A_{260}/A_{280} ratio. RNA of acceptable quality is in the range of 1.7 to 2.1.

Evaluate RNA integrity

The integrity of the RNA sample, or the proportion that is full length, is an important component of RNA quality. Reverse transcribing partially-degraded mRNA may generate cDNA that lacks parts of the coding region.

Two methods to evaluate RNA integrity are:

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

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

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

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

Synthesize first-strand cDNA

In this reverse transcription procedure, total RNA is primed with engineered primers containing a T7 promoter sequence. The reaction synthesizes single-stranded cDNA containing a T7 promoter sequence.

Note: To include premixed controls from the Affymetrix® GeneChip® Poly-A RNA Control Kit (PN 900433 or equivalent), add the reagents to the total RNA samples. See "Prepare the Poly-A RNA Controls" on page 10 for more information.

1. Prepare a First-Strand Master Mix, then dispense 5 µL into a reaction tube/plate.

For the following hazards, see the complete safety alert descriptions in "Chemical alerts" on page 32:

WARNING! CHEMICAL HAZARD. First-Strand Buffer Mix and First-Strand Enzyme Mix.

IMPORTANT! Prepare master mixes for each step of the procedure to save time, improve reproducibility, and minimize pipetting error. Before preparing the master mixes, inspect the buffer mixes for precipitates. If necessary, warm the buffer mix(es) at <55 °C for 1 to 2 min, or until the precipitate is fully disolved. When preparing the master mixes, thoroughly vortex each buffer mix before use. Mix the enzymes by gently flicking the tubes a few times before use. Keep the buffer mixes at room temperature until needed to prevent re-precipitation. Keep the enzyme mixes on ice at all times.

a. **At room temperature**, prepare the First-Strand Master Mix in a nuclease-free tube. Combine the components in the sequence shown in the table below. Include 5% excess volume to correct for pipetting losses.

First-Strand Master Mix component	Volume for one reaction (µL)
First-Strand Buffer Mix	4
First-Strand Enzyme Mix	1
Total Volume	5

b. Mix thoroughly by gently vortexing. Centrifuge briefly (~5 sec) to collect the mix at the bottom of the tube. Proceed immediately to the next step.

c. Transfer 5 µL of the First-Strand Master Mix to the supplied PCR tubes.

2. Add 5 µL of total RNA, mix thoroughly by gently vortexing, centrifuge briefly, then proceed immediately to the next step.

a. Add 5 µL RNA to each tube or well containing the First-Strand Master Mix for a final reaction volume of 10 µL. If necessary, use nuclease-free water to bring the RNA to 5 µL.

Note: If you are adding Poly-A Spike Controls to your RNA, the volume of RNA must be less than 3 µL. If necessary, use a SpeedVac or ethanol precipitation to concentrate the RNA samples. For example, when performing the control RNA reaction, combine 1 µL of RNA (50 ng/µL), 2 µL of diluted Poly-A Spike Controls, and 2 µL of nuclease-free water.

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

3. Incubate for 1 hour at 25 °C, then for 1 hour at 42 °C, then for at least 2 min at 4 °C.

a. Incubate for 1 hr at 25 °C, then for 1 hr at 42 °C, then for at least 2 min at 4 °C in a thermal cycler using the First-Strand cDNA Synthesis Method that is shown in Table 1 on page 8.

b. Immediately after the incubation, centrifuge briefly (~5 sec) to collect the first-strand cDNA at the bottom of the tube/plate. Place the sample on ice for 2 min to cool the plastic, then proceed immediately to "Synthesize second-strand cDNA" on page 15.

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

Synthesize second-strand cDNA

In this procedure, single-stranded cDNA is converted to double-stranded cDNA, which acts as a template for transcription. The reaction uses DNA polymerase and RNase H to simultaneously degrade the RNA and synthesize second-strand cDNA.

1. Prepare a Second-Strand Master Mix, then add 50 µL to each sample.

For the following hazards, see the complete safety alert descriptions in "Chemical alerts" on page 32:

WARNING! CHEMICAL HAZARD. Second-Strand Buffer Mix, Second-Strand Enzyme Mix.

a. **On ice**, prepare the Second-Strand Master Mix in a nuclease-free tube. Combine the components in the sequence shown in the table below. Prepare the master mix for all the first-strand synthesis cDNA samples in the experiment. Include 5% excess volume to correct for pipetting losses.

Second-Strand Master Mix component	Volume for one reaction (µL)
Nuclease-free Water	32.5
Second-Strand Buffer Mix	12.5
Second-Strand Enzyme Mix	5
Total Volume	50

b. Mix thoroughly by gently vortexing. Centrifuge briefly (~5 sec) to collect the mix at the bottom of the tube and proceed immediately to the next step.

c. Transfer 50 µL of the Second-Strand Master Mix to each (10 µL) first-strand synthesis cDNA sample. Mix thoroughly by gently vortexing or flicking the tube 3 or 4 times. Centrifuge briefly to collect the reaction at the bottom of the tube/plate and proceed immediately to the next step.

2. Incubate for 1 hr at 16 °C, then for 10 min at 65 °C, then for at least 2 min at 4 °C.

a. Incubate for 1 hr at 16 °C, then for 10 min at 65 °C, then for at least 2 min at 4 °C in a thermal cycler using the Second-Strand cDNA Synthesis method for thermal cycling that is shown in Table 1 on page 8.

IMPORTANT! Disable the heated lid of the thermal cycler or keep the lid off during the second-strand cDNA synthesis.

b. Immediately after the incubation, centrifuge briefly (~5 sec) to collect the double-stranded cDNA at the bottom of the tube/plate. Place the sample on ice to cool the plastic, then immediately proceed to "Synthesize cRNA by In Vitro Transcription" on page 16.

Synthesize cRNA by In Vitro Transcription

In this procedure, antisense cRNA is synthesized and amplified by in vitro transcription (IVT) of the second-strand 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).

1. Prepare an IVT Master Mix, then dispense 30 µL to each sample.

For the following hazards, see the complete safety alert descriptions in "Chemical alerts" on page 32:

WARNING! CHEMICAL HAZARD. IVT Buffer Mix, IVT Enzyme Mix.

a. **At room temperature**, prepare an IVT Master Mix in a nuclease-free tube. Combine the components in the sequence shown in the table below. Prepare master mix for all the second-strand cDNA (60-µL) samples. Include 5% excess volume to correct for pipetting losses.

IVT Master Mix component	Volume for one reaction (µL)
IVT Buffer Mix	24
IVT Enzyme Mix	6
Total Volume	30

b. Mix thoroughly by gently vortexing. Centrifuge briefly (~5 sec) to collect the mix at the bottom of the tube, then proceed immediately to the next step.

c. Transfer 30 µL of the IVT Master Mix to each 60-µL Second-Strand cDNA sample. Mix thoroughly by gently vortexing, then centrifuge briefly to collect the reaction at the bottom of the tube/plate.

2. Incubate for 16 hr at 40 °C, then overnight at 4 °C.

a. Incubate the IVT reaction for 16 hr at 40 °C, then overnight at 4 °C in a thermal cycler using the In Vitro Transcription cRNA Synthesis method for thermal cycling shown in Table 1 on page 8.

b. After the incubation, centrifuge briefly (~5 sec) to collect the reaction at the bottom of the tube/plate.

3. Place the cRNA on ice briefly, or freeze immediately.

Place the reaction on ice, then proceed to "Purify cRNA" on page 17, or immediately freeze the samples at –20 °C for overnight storage.

STOPPING POINT. Samples can be stored overnight at –20 °C.

Purify cRNA

In this procedure, enzymes, salts, inorganic phosphates, and unincorporated nucleotides are removed to improve the stability of the cRNA.

Before beginning the cRNA purification:

- Preheat the bottle of Elution Solution to 50 to 58 °C for at least 10 min.
- Add 100% ethanol (ACS reagent grade or equivalent) to the bottle of Nucleic Acid Wash Solution Concentrate before use. This solution is referred to as Nucleic Acid Wash Solution.
- Make sure that the Nucleic Acid Binding Buffer Concentrate is completely dissolved. If not, warm the solution to <50 °C until the concentrate is solubilized.
- Vortex the Nucleic Acid Binding Beads vigorously before use to ensure that they are fully dispersed.

1. Prepare the cRNA Binding Mix.

For the following hazards, see the complete safety alert descriptions in "Chemical alerts" on page 32:

WARNING! CHEMICAL HAZARD. Nucleic Acid Binding Buffer Beads, Nucleic Acid Binding Buffer Concentrate.

At room temperature, immediately before use, prepare the cRNA Binding Mix in a nuclease-free tube for all the samples in the experiment. Follow the instructions in the table below.

IMPORTANT! Prepare only the amount that is needed for all samples in the experiment plus ~10% excess volume to compensate for pipetting losses.

cRNA Binding Mix component	Volume for one reaction (µL)
Nucleic Acid Binding Beads	10
Nucleic Acid Binding Buffer Concentrate	50

2. Add 60 µL of cRNA Binding Mix to each sample.

a. Add 60 µL of cRNA Binding Mix to each sample. Pipette up/down 3 times to mix.

b. Transfer each sample to a well of a U-Bottom Plate.

3. Add 60 µL of isopropanol to each sample, then shake gently for 2 min.

a. Add 60 µL of 100% isopropanol to each sample. Pipette up/down 3 times to mix.

IMPORTANT! Add isopropanol at this step. Do not use ethanol.

b. Gently shake for ≥2 min to thoroughly mix (setting 4 on the Lab-Line Titer Plate Shaker). The cRNA in the sample binds to the Nucleic Acid Binding Beads during this incubation.

4. Capture the Nucleic Acid Binding Beads and discard the supernatant.

a. Move the plate to a magnetic stand to capture the magnetic beads. When capture is complete (after ~5 min), the mixture is transparent, and the Nucleic Acid Binding 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.

b. Carefully aspirate and discard the supernatant without disturbing the magnetic beads, then remove the plate from the magnetic stand.

5. Wash twice with 100 µL of Nucleic Acid Wash Solution.

IMPORTANT! Make sure that 100% ethanol has been added to the bottle of Nucleic Acid Wash Solution Concentrate before using it.

a. Add 100 µL of Nucleic Acid Wash Solution to each sample, then shake at moderate speed for 1 min (setting 7 on the Lab-Line Titer Plate Shaker).

Note: Although the Nucleic Acid Binding Beads may not fully disperse during this step, this does not affect RNA purity or yield.

b. Move the plate to a magnetic stand and capture the Nucleic Acid Binding Beads as in the previous step.

c. Carefully aspirate and discard the supernatant without disturbing the Nucleic Acid Binding Beads, then remove the plate from the magnetic stand.

d. Repeat step 5a to step 5c to wash each sample again with 100 µL of Nucleic Acid Wash Solution.

e. Move the plate to a shaker and shake the plate vigorously for 1 min to evaporate residual ethanol from the beads (setting 10 on the Lab-Line Titer Plate Shaker). Dry the solution until no liquid is visible, yet the pellet appears shiny. Additional time may be required. Do not over-dry the beads.

6. Elute cRNA with 40 µL of preheated Elution Solution.

a. Add to each sample 40 µL of preheated (55 to 58 °C) Elution Solution to elute the purified cRNA from the Nucleic Acid Binding Beads. Incubate without shaking for 2 min.

b. Vigorously shake the plate for 3 min (setting 10 on the Lab-Line Titer Plate Shaker), then check to make sure that the Nucleic Acid Binding Beads are fully dispersed. If they are not, continue shaking until the beads are dispersed and/or pipette up/down 3 times. Pellets can be disrupted by manual pipetting using a single-channel P-200 or equivalent.

c. Move the plate to a magnetic stand to capture the Nucleic Acid Binding Beads.

d. Transfer the supernatant, which contains the eluted cRNA, to a nuclease-free multiwell plate.

7. Place the cRNA on ice briefly, or freeze immediately.

Place the reaction on ice, then proceed immediately to the "Synthesize 2nd-cycle cDNA" on page 21, or freeze the samples at –20 °C for overnight storage.

STOPPING POINT. Samples can be stored overnight at –20 °C.

Assess cRNA yield and size distribution

Expected cRNA yield

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

During development of this kit, using a wide variety of tissue types, 50 ng of input total RNA yielded 15 to 50 µg of cRNA. For most tissue types, the recommended 100 ng of input total RNA should provide >20 µg of cRNA.

1. Determine cRNA yield by UV absorbance.

Determine the concentration of a cRNA solution by measuring its absorbance at 260 nm. Applied Biosystems recommends evaluating the absorbance of 1.5 µL of cRNA sample using a NanoDrop® Spectrophotometer.

Alternatively, determine the cRNA concentration by diluting an aliquot of the preparation in TE (10 mM Tris-HCl pH 8, 1 mM EDTA) 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

2. (Optional) Determine cRNA yield using Quant-iT™ RiboGreen® RNA Reagent.

If a fluorometer or a fluorescence microplate reader is available, use the Quant-iT™ RiboGreen® RNA Reagent (Invitrogen) to measure RNA concentration. Follow the manufacturer's instructions.

(Optional) Expected cRNA size distribution

The expected cRNA profile is a distribution of sizes from 50 to 4500 nt with most of the cRNA sizes in the 200 to 2000 nt range. The distribution is quite jagged and does not resemble the profile observed when using a traditional dT-based amplification kit such as a MessageAmp™ kit. This step is optional.

1. (Optional) Determine cRNA size distribution using a bioanalyzer.

Applied Biosystems recommends analyzing cRNA size distribution using an Agilent 2100 Bioanalyzer, a RNA 6000 Nano Kit (PN 5067-1511), and mRNA Nano Series II assay. If there is sufficient yield, then load approximately 300 ng of cRNA per well on the bioanalyzer. If there is *not* sufficient yield, then use as little as 200 ng of cRNA per well. To analyze cRNA size using a bioanalyzer, follow the manufacturer's instructions.

Synthesize 2nd-cycle cDNA

In this procedure, sense-strand cDNA is synthesized by the reverse transcription of cRNA using random primers. The sense-strand cDNA contains dUTP at a fixed ratio relative to dTTP. 10 µg of cRNA is required for 2nd-cycle cDNA synthesis.

1. Prepare 10 µg of cRNA in a volume of 22 µL.

For the following hazards, see the complete safety alert descriptions in "Chemical alerts" on page 32:

WARNING! CHEMICAL HAZARD. 2nd-Cycle Buffer Mix, 2nd-Cycle Enzyme Mix.

On ice, prepare 455 ng/µL cRNA. This is equal to 10 µg cRNA in a volume of 22 µL. If necessary, use nuclease-free water to bring the cRNA sample to 22 µL.

If the cRNA is too dilute, then concentrate the cRNA by vacuum centrifugation. If vacuum centrifugation is required for one sample, then Applied Biosystems recommends drying all of the samples to reduce sample-to-sample variability. To concentrate the samples, place 10 µg of the sample in a tube, bring to a set volume of 30 µL using Nuclease-free water, dry the samples to ~15 µL, then bring the samples to a final volume of 22 µL using Nuclease-free water.

IMPORTANT! During vacuum centrifugation, check the progress of drying every 5 to 10 min. Remove the sample from the concentrator when it reaches the desired volume. Avoid drying cRNA samples to completion.

2. Combine 10 µg of cRNA and the Random Primers.

IMPORTANT! Prepare master mixes for each step of the procedure to save time, improve reproducibility, and minimize pipetting error. Before preparing the master mixes, inspect the buffer mixes for precipitates. If necessary, warm the buffer mix(es) at <55 °C for 1 to 2 min, or until the precipitate is fully disolved. When preparing the master mixes, thoroughly vortex each buffer mix before use. Mix the enzymes by gently flicking the tubes a few times before use. Keep the buffer mixes at room temperature until needed to prevent re-precipitation. Keep the enzyme mixes on ice at all times.

a. On ice, using supplied PCR tubes or plate, combine:
- 22 µL of cRNA (10 µg)
- 2 µL of Random Primers

b. Mix thoroughly by gently vortexing. Centrifuge briefly (~5 sec) to collect the reaction at the bottom of the tube/plate. Place on ice.

3. Incubate for
5 min at 70 °C, then
5 min at 25 °C, then
2 min at 4 °C.

a. Incubate in a thermal cycler using the 2nd-Cycle cRNA Denaturation method for thermal cycling that is shown in Table 1 on page 8.

b. After the incubation, centrifuge briefly (~5 sec) to collect the 2nd-Cycle cDNA at the bottom of the tube/plate.

4. Prepare the 2nd-Cycle Master Mix on ice, then add 16 µL to each sample.

a. **At room temperature**, prepare the 2nd-Cycle Master Mix in a nuclease-free tube. Combine the components in the sequence shown in the table below. Include 5% excess volume to correct for pipetting losses.

2nd-Cycle Master Mix component	Volume for one reaction (µL)
2nd-Cycle Buffer Mix	8
2nd-Cycle Enzyme Mix	8
Total Volume	16

b. Mix thoroughly by gently vortexing. Centrifuge briefly (~5 sec) to collect the mix at the bottom of the tube/plate. Proceed immediately to the next step.

c. Transfer 16 µL of 2nd-Cycle Master Mix to each (24-µL) cRNA/Random Primer sample. Mix thoroughly by gently vortexing. Centrifuge briefly to collect the reaction at the bottom of the plate/tube. Proceed immediately to the next step.

5. Incubate for 10 min at 25 °C, then 90 min at 42 °C, then 10 min at 70 °C, then for at least 2 min at 4 °C.

a. Incubate in a thermal cycler using the 2nd-Cycle cDNA Synthesis method for thermal cycling that is shown in Table 1 on page 8.

IMPORTANT! Cover the reactions with the heated lid at 75 °C.

b. Immediately after the incubation, centrifuge briefly (~5 sec) to collect the cDNA at the bottom of the tube/plate. Place the sample on ice and immediately proceed to "Hydrolyze using RNase H" on page 23.

Hydrolyze using RNase H

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

1. Add 2 µL of RNase H to the 2nd-Cycle cDNA.

For the following hazards, see the complete safety alert descriptions in "Chemical alerts" on page 32:

WARNING! CHEMICAL HAZARD. RNase H.

a. On ice, add 2 µL of RNase H to the 2nd-Cycle cDNA from above. Mix by pipetting up/down 3 times to ensure that all of the enzyme is dispensed from the pipet tip.

b. Mix thoroughly by gently vortexing. Centrifuge briefly (~5 sec) to collect the reaction at the bottom of the tube/plate. Proceed immediately to the next step.

2. Incubate for 45 min at 37 °C, then 5 min at 95 °C, then for at least 2 min at 4 °C.

a. Incubate in a thermal cycler using the RNase H Hydrolysis method for thermal cycling that is shown in Table 1 on page 8.

IMPORTANT! Cover the reactions with the heated lid at 75 °C.

b. After the incubation, centrifuge briefly (~5 sec) to collect the RNase H Hydrolyzed 2nd Cycle cDNA at the bottom of the tube/plate. Place the samples on ice, then proceed immediately to "Purify 2nd-cycle cDNA" on page 24.

STOPPING POINT. Samples can be stored overnight at –20 °C.

Purify 2nd-cycle cDNA

After synthesis, the second-strand cDNA is purified to remove enzymes, salts, and unincorporated dNTPs. This step prepares the cDNA for fragmentation and labeling.

Before beginning the cDNA purification:

- Preheat the bottle of Elution Solution to 50 to 58 °C for at least 10 min.
- Make sure to add ethanol to the bottle of Nucleic Acid Wash Solution Concentrate before use. This solution is referred to as Nucleic Acid Wash Solution.
- Make sure that the Nucleic Acid Binding Buffer Concentrate is completely dissolved. If not, warm the solution to <50 °C until the concentrate is solubilized.
- Vortex the Nucleic Acid Binding Beads vigorously before use to ensure they are fully dispersed.

1. Prepare the cDNA Binding Mix for the experiment.

At room temperature, prepare the cDNA Binding Mix in a nuclease-free tube for all the samples in the experiment following the instructions in the table below.

Note: Prepare only the amount needed for all samples in the experiment plus ~10% excess volume to compensate for pipetting losses.

cDNA Binding Mix component	Volume for one reaction (µL)
Nucleic Acid Binding Beads	10
Nucleic Acid Binding Buffer Concentrate	50

2. Add 18 µL of nuclease-free water and 60 µL of cDNA Binding Mix to each sample.

a. Add 18 µL of nuclease-free water to each sample for a final volume of 60 µL.

b. Add 60 µL of cDNA Binding Mix to each sample. Pipette up/down 3 times to mix.

c. Transfer each sample to a well of a U-bottom plate.

3. Add 120 µL of ethanol to each sample, then shake gently for 2 min.

a. Add 120 µL of 100% ethanol to each sample. Pipette up/down 3 times to mix.

IMPORTANT! Add ethanol in this step. Do not use isopropanol.

b. Gently shake for ≥2 min to thoroughly mix (setting 4 on the Lab-Line Titer Plate Shaker). The cDNA in the sample binds to the Nucleic Acid Binding Beads during this incubation.

4. Capture the Nucleic Acid Binding Beads, then discard the supernatant.

a. Move the plate to a magnetic stand to capture the magnetic beads. When the capture is complete (after ~5 min), the mixture is transparent, and the Nucleic Acid Binding Beads form pellets against the magnets in the magnetic stand. The exact capture time depends on the magnetic stand that you use.

b. Carefully aspirate and discard the supernatant without disturbing the magnetic beads, then remove the plate from the magnetic stand.

5. Wash twice with 100 µL of Nucleic Acid Wash Solution.

IMPORTANT! Make sure that ethanol is added to the bottle of Nucleic Acid Wash Solution Concentrate before using it.

a. Add 100 µL of Nucleic Acid Wash Solution to each sample, then shake the samples at moderate speed for 1 min (setting 7 on the Lab-Line Titer Plate Shaker).

Note: Although the Nucleic Acid Binding Beads may not fully disperse during this step; this does not affect DNA purity or yield.

b. Move the plate to a magnetic stand to capture the Nucleic Acid Binding Beads as in step 4 on page 25.

c. Carefully aspirate and discard the supernatant without disturbing the Nucleic Acid Binding Beads, then remove the plate from the magnetic stand.

d. Repeat step 5a to step 5c to wash a second time with 100 µL of Nucleic Acid Wash Solution.

e. Move the plate to a shaker, then shake the plate vigorously for 1 min to evaporate residual ethanol from the beads (setting 10 on the Lab-Line Titer Plate Shaker). Dry the solution until no liquid is visible, but the pellet appears shiny. Additional time may be required. Do not over-dry the beads.

6. Elute cDNA with 30 µL of preheated Elution Solution.

a. Elute the purified cDNA from the Nucleic Acid Binding Beads by adding 30 µL of preheated (55 to 58°C) Elution Solution to each sample. Incubate for 2 min at room temp. without shaking.

b. Vigorously shake the plate for 3 min (setting 10 on the Lab-Line Titer Plate Shaker). Make sure that the Nucleic Acid Binding Beads are fully dispersed. If they are not, continue shaking until the beads are dispersed and/or pipette up-and-down 3 times.

c. Move the plate to a magnetic stand to capture the Nucleic Acid Binding Beads.

d. Transfer the supernatant, which contains the eluted cDNA, to a nuclease-free multiwell plate.

7. Place the cDNA on ice briefly, or freeze immediately.

Place the reaction on ice, then proceed immediately to "Fragment and label the single-stranded cDNA" on page 27, or freeze the samples at –20 °C for overnight storage.

STOPPING POINT. Samples can be stored overnight at –20 °C.

Assess cDNA yield and size distribution

Expected cDNA Yield

During development of this kit, using a wide variety of tissue types, 10 µg of input cRNA yielded 6 to 9 µg of cDNA. For most tissue types, the recommended 10 µg of input cRNA should yield >6 µg of cDNA.

1. Determine cDNA yield by UV absorbance.

Determine the concentration of a cDNA solution by measuring its absorbance at 260 nm. We recommend evaluating the absorbance of 1.5 µL of cDNA sample using a NanoDrop® Spectrophotometer.

Alternatively, determine the cDNA concentration by diluting an aliquot of the preparation in TE (10 mM Tris-HCl pH 8, 1 mM EDTA) and reading the absorbance in a traditional spectrophotometer at 260 nm. Calculate the concentration in µg/mL using the equation below (1 A_{260} = 33 µg DNA/mL).

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

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

2. (Optional) Use Quant-iT™ PicoGreen® RNA Reagent to Assess cRNA yield.

If a fluorometer or a fluorescence microplate reader is available, use the Quant-iT™ PicoGreen® RNA Reagent (Invitrogen) to measure RNA concentration. Follow the manufacturer's instructions.

(Optional) Expected cDNA size distribution

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

1. (Optional) Determine cDNA size distribution using a bioanalyzer.

Applied Biosystems recommends analyzing cDNA size distribution using an Agilent 2100 Bioanalyzer, a RNA 6000 Nano Kit (PN 5067-1511), and mRNA Nano Series II assay. If there is sufficient yield, load approximately 250 ng of cDNA per well. If there is *not* sufficient yield, then use as little as 200 ng of cDNA per well. To analyze cDNA size using a bioanalyzer, follow the manufacturer's instructions.

Fragment and label the single-stranded cDNA

The Affymetrix GeneChip® WT Terminal Labeling Kit (PN 900671 or equivalent) is used for the fragmentation and labeling of the cDNA. Because the 2nd-cycle (sense-strand) cDNA contains dUTP, the kit uses uracil-DNA glycosylase (UDG) and apurinic/apyrimidinic endonuclease 1 (APE1) to recognize and fragment the cDNA at the unnatural dUTP residues. DNA is then labeled by terminal deoxynucleotidyl transferase (TdT) using the Affymetrix proprietary DNA Labeling Reagent.

For instructions on how to fragment and label the single-stranded cDNA, refer to the Affymetrix *GeneChip® WT Terminal Labeling and Hybridization User Manual* (PN 702880).

Troubleshooting

Observation	Possible cause	Solution
The positive control sample *and* your total RNA sample yield low levels of amplified cRNA product or low levels of appropriately sized cRNA product.	Incubation temperatures are incorrect or inaccurate.	Calibrate 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 not performed properly.	Perform the purification as described in this document and make sure that you use the correct alcohol.
	Pipettes, tubes, and/or equipment are contaminated with nuclease.	Remove RNases and DNases from surfaces using Ambion RNase*Zap*® RNase Decontamination Solution.
The positive control sample produces expected results, *but* your total RNA sample results in low levels of amplified cRNA/cDNA product.	The input total RNA concentration is lower than expected.	Repeat the A_{260} reading of your RNA sample. Use 100 to 200 ng of total RNA in the first-strand cDNA synthesis procedure.
	Your input RNA contains contaminating DNA, protein, phenol, ethanol, or salts, causing inefficient reverse transcription.	Phenol extract and ethanol precipitate your total RNA, or use the Ambion MEGAclear™ Kit (PN AM1908).
The positive control sample produces expected results *but* your total RNA sample results in low levels of appropriately sized 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. Refer to "Evaluate RNA integrity" on page 12.
	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).

Appendix A

Safety

This appendix covers:

General chemical safety

Chemical hazard warning

WARNING! CHEMICAL HAZARD. Before handling any chemicals, refer to the Material Safety Data Sheet (MSDS) provided by the manufacturer, and observe all relevant precautions.

Chemical safety guidelines

To minimize the hazards of chemicals:

- Read and understand the Material Safety Data Sheets (MSDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. (See "About MSDSs" on page 30.)
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood). For additional safety guidelines, consult the MSDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the MSDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

MSDSs

About MSDSs

Chemical manufacturers supply current Material Safety Data Sheets (MSDSs) with shipments of hazardous chemicals to new customers. They also provide MSDSs with the first shipment of a hazardous chemical to a customer after an MSDS has been updated. MSDSs provide the safety information you need to store, handle, transport, and dispose of the chemicals safely.

Each time you receive a new MSDS packaged with a hazardous chemical, be sure to replace the appropriate MSDS in your files.

Obtaining MSDSs

The MSDS for any chemical supplied by Applied Biosystems is available to you free 24 hours a day. To obtain MSDSs:

1. Go to **www.appliedbiosystems.com**, click **Support**, then select **MSDS**.
2. In the Keyword Search field, enter the chemical name, product name, MSDS part number, or other information that appears in the MSDS of interest. Select the language of your choice, then click **Search**.
3. Find the document of interest, right-click the document title, then select any of the following:
   - **Open** – To view the document
   - **Print Target** – To print the document
   - **Save Target As** – To download a PDF version of the document to a destination that you choose

Note: For the MSDSs of chemicals not distributed by Applied Biosystems, contact the chemical manufacturer.

Biological hazard safety

General biohazard

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. Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective equipment, which includes but is not limited to: protective eyewear, face shield, clothing/lab coat, and gloves. All work should be conducted in properly equipped facilities using the appropriate safety equipment (for example, physical containment devices). Individuals should be trained according to applicable regulatory and company/institution requirements before working with potentially infectious materials. Read and follow the applicable guidelines and/or regulatory requirements in the following:

- U.S. Department of Health and Human Services guidelines published in *Biosafety in Microbiological and Biomedical Laboratories* (stock no. 017-040-00547-4; **bmbl.od.nih.gov**)
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030; **www.access.gpo.gov/nara/cfr/waisidx_01/29cfr1910a_01.html**)
- Your company's/institution's Biosafety Program protocols for working with/handling potentially infectious materials

Additional information about biohazard guidelines is available at:

www.cdc.gov

Chemical alerts

For the definitions of the alert words **IMPORTANT**, **CAUTION**, **WARNING**, and **DANGER**, see "Safety alert words" on page v.

General alerts for all chemicals

Read the MSDS and follow the handling instructions. Wear appropriate eyewear, clothing, and gloves.

First Aid: If inhaled, remove to fresh air. In case of contact, flush thoroughly with water. If symptoms develop, get medical attention.

Specific chemical alerts

WARNING! First-Strand Buffer Mix causes eye, skin, and respiratory tract irritation. May be harmful if swallowed, inhaled, or absorbed through the skin. Avoid breathing vapor. Use with adequate ventilation. Avoid contact with eyes and skin.

WARNING! First-Strand Enzyme Mix may cause eye, skin, and respiratory tract irritation. May be harmful if swallowed. Avoid breathing vapor. Use with adequate ventilation. Avoid contact with eyes and skin.

WARNING! Second-Strand Buffer Mix causes eye, skin, and respiratory tract irritation. May be harmful if swallowed, inhaled, or absorbed through the skin. Avoid breathing vapor. Use with adequate ventilation. Avoid contact with eyes and skin.

WARNING! Second-Strand Enzyme Mix may cause eye, skin, and respiratory tract irritation. May be harmful if swallowed. Avoid breathing vapor. Use with adequate ventilation. Avoid contact with eyes and skin.

WARNING! IVT Buffer Mix causes eye, skin, and respiratory tract irritation. May be harmful if swallowed, inhaled, or absorbed through the skin. Avoid breathing vapor. Use with adequate ventilation. Avoid contact with eyes and skin.

WARNING! IVT Enzyme Mix may cause eye, skin, and respiratory tract irritation. May be harmful if swallowed. Avoid breathing vapor. Use with adequate ventilation. Avoid contact with eyes and skin.

WARNING! 2nd-Cycle Buffer Mix causes eye, skin, and respiratory tract irritation. May be harmful if swallowed, inhaled, or absorbed through the skin. Avoid breathing vapor. Use with adequate ventilation. Avoid contact with eyes and skin.

WARNING! 2nd-Cycle Enzyme Mix may cause eye, skin, and respiratory tract irritation. May be harmful if swallowed. Avoid breathing vapor. Use with adequate ventilation. Avoid contact with eyes and skin.

WARNING! BIOHAZARD. Control RNA. This product is a potential biohazard. Handle as if capable of transmitting infectious agents.

To clean spill site; wash spill site with 10% bleach and ventilate the area after material pick-up is complete. Soak up with inert absorbent material (e.g. sand, silica gel, acid binder, universal binder, or saw dust.

WARNING! RNase H may cause eye, skin, and respiratory tract irritation. May be harmful if swallowed. Avoid breathing vapor. Use with adequate ventilation. Avoid contact with eyes and skin.

WARNING! Nucleic Acid Binding Buffer Concentrate is harmful if swallowed, inhaled, or absorbed through the skin. Causes eye, skin, and respiratory tract irritation. Do not taste or swallow. Avoid breathing vapor. Use with adequate ventilation. Avoid contact with eyes and skin.

Contact with acids or bleach liberates toxic gases. **DO NOT ADD** acids or bleach to any liquid wastes containing Nucleic Acid Binding Buffer Concentrate.

WARNING! Nucleic Acid Binding Beads. This product contains sodium azide at a concentration that is considered non-hazardous according to OSHA regulations. Sodium azide may react with copper pluming to form highly explosive metal azides.

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.

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