USER GUIDE

ambion®
by life technologies™

Illumina® TotalPrep™-96 RNA Amplification Kit

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Illumina® TotalPrep™-96 RNA Amplification Kit

IMPORTANT! Before using this product, read and understand the information the "Safety" appendix in this document.

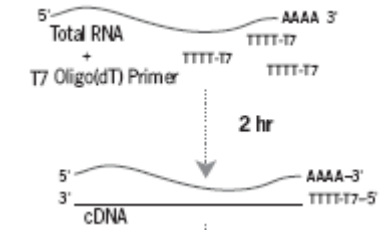
Introduction

Product description and background

RNA amplification has become the standard method for preparing RNA samples for array analysis. The Illumina® TotalPrep™-96 RNA Amplification Kit is a complete system for generating biotinylated, amplified RNA for hybridization with Illumina Sentrix arrays. It is based on the RNA amplification protocol developed in the laboratory of James Eberwine (Van Gelder, 1990). The procedure begins with reverse transcription with an oligo(dT) primer bearing a T7 promoter using ArrayScript™ reverse transcriptase. The cDNA then undergoes second strand synthesis and cleanup to become a template for in vitro transcription with T7 RNA Polymerase. Life Technologies proprietary MEGAscript® in vitro transcription (IVT) technology is used to generate hundreds to thousands of biotinylated, antisense RNA copies of each mRNA in a sample, known as "cRNA" or "aRNA". The labeled cRNA produced with the kit is ideal for hybridization with Illumina Sentrix arrays.

Figure 1 Illumina® TotalPrep™-96 RNA Amplification Procedure Overview

Reverse Transcription to Synthesize First Strand cDNA

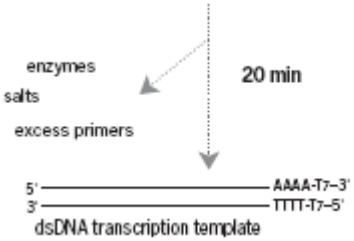


1. "Adjust the volume of ≤ 500 ng RNA to 11 µL" on page 11
2. "Add 9 µL Reverse Transcription Master Mix" on page 11
3. "Incubate 2 hr at 42°C" on page 11

Second Strand cDNA Synthesis

1. "Add 80 µL Second Strand Master Mix to each sample" on page 12
2. "Incubate 2 hr at 16°C" on page 12

cDNA Purification



1. "Add 180 µL cDNAPure, transfer to a U-Bottom Plate, and shake for 2 min" on page 13
2. "Capture the magnetic beads and discard the supernatant" on page 13
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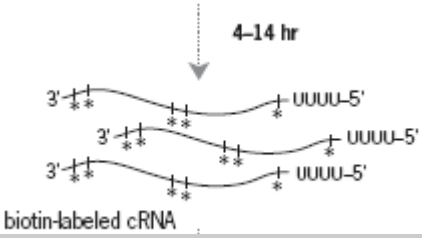
5. "Transfer 17.5 µL cDNA to wells of a new PCR Plate" on page 14

In Vitro Transcription to Synthesize Biotin-labeled cRNA



1. "Add 7.5 µL IVT Master Mix to each sample" on page 14
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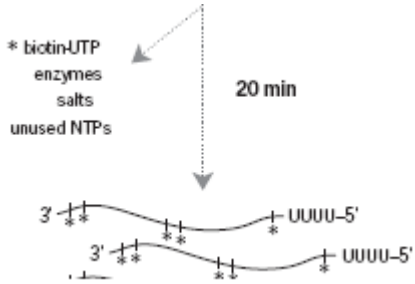
cRNA Purification



1. "Add 70 µL cRNA Binding Mix and 95 µL ethanol" on page 15
2. "Transfer to U-Bottom Plate and shake gently for 2 min" on page 15
3. "Capture the RNA Binding Beads and discard supernatant" on page 15
4. "Wash twice with 100 µL cRNA Wash Solution" on page 15
5. "Elute cRNA with 40–100 µL 55°C cRNA Elution Buffer" on page 16

Materials provided with the kit and storage conditions

Components for 96 single-round amplification/biotin labeling reactions are included with the Illumina® TotalPrep™-96 RNA Amplification Kit.

Do not store in a frost-free freezer.

Amount	Component	Storage
110 µL	T7 Oligo(dT) Primer	–20°C
110 µL	ArrayScript™ Reverse Transcriptase	–20°C
220 µL	10X First Strand Buffer	–20°C
880 µL	dNTP Mix	–20°C
110 µL	RNase Inhibitor	–20°C
110 µL	RNase H	–20°C
1.1 mL	10X Second Strand Buffer	–20°C
220 µL	DNA Polymerase	–20°C
275 µL	T7 Enzyme Mix	–20°C
275 µL	T7 10X Reaction Buffer	–20°C
275 µL	Biotin-NTP Mix	–20°C
10 µL	Control RNA	–20°C
20 mL	cDNAPure†	4°C
1.1 mL	RNA Binding Beads†	4°C
40 mL	cDNA Wash Buffer Concentrate Add 32 mL 100% Ethanol before use	room temp
5.5 mL	cRNA Binding Buffer Concentrate	room temp
450 µL	Bead Resuspension Solution	room temp
25 mL	cRNA Wash Solution Concentrate Add 20 mL 0f 100% Ethanol before use	room temp
10 mL	cRNA Elution Buffer	room temp
8 ea	U-Bottom Plate	room temp
8 ea	PCR Plate	room temp
20 mL	Nuclease-free Water	any temp‡

† Do not freeze.

‡ Store Nuclease-free Water at –20°C, 4°C, or room temperature.

Materials not provided with the kit

- General laboratory equipment, including microcentrifuge, and spectrophotometer
- RNase-free pipettors and tips, positive-displacement type recommended to increase the accuracy and precision of reaction inputs
- 100% Ethanol (ACS reagent grade or equivalent)

- Thermal cycler with temperature-adjustable heated lid (recommended), or hybridization ovens or incubators set at 70°C, 42°C, and 37°C. (See"Thermal cycler recommended" on page 8 for more information.)
- Heat block set at 55°C, for preheating the water for cDNA and cRNA purification
- Sealing aluminum foil or tape for incubating reactions in 96-well plates
- Magnetic stand for 96-well plates: We recommend the 96-well Magnetic-Ring Stand (Cat. no. AM10050) for its high-strength magnets and quality design.
- Orbital shaker for 96-well plates such as the Barnstead/Lab-Line Titer Plate Shaker (VWR #57019-600 or Fisher #14-271-9)

RNA amplification protocol

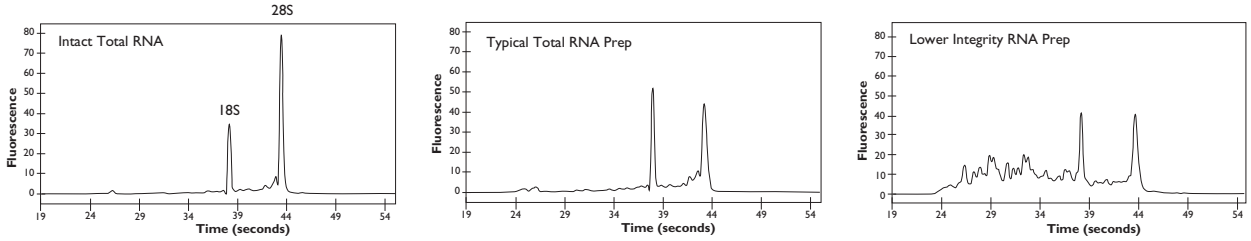
Important parameters for successful amplification

RNA integrity

The integrity of the RNA sample, or the proportion of full-length mRNA, can have a significant impact on the amplification reaction. Reverse transcribing partially degraded mRNAs will typically generate relatively short cDNAs that lack the sequence upstream of the break in the RNA molecule.

The most common method for evaluating the relative integrity of mRNA is to measure the ratio of the 28S and 18S ribosomal RNAs (rRNA) using an RNA LabChip Kit with an Agilent® 2100 Bioanalyzer® instrument. The RNA LabChip fractionates RNA molecules according to size, and the relative amounts of 18S and 28S rRNA can be determined. The ratio of the 28S to 18S rRNA peaks will approach 2:1 in RNA samples composed of primarily full-length RNA (Figure 2).

Figure 2 Bioanalyzer® Instrument Images of Increasingly Degraded Total RNA. These electropherograms from the Agilent® 2100 Bioanalyzer® instrument show progressive RNA degradation. Notice that the ribosomal RNA (rRNA) peaks are at a ratio of about 2:1 (28S:18S) in an intact total RNA sample (left). Total RNA that is somewhat degraded shows nearly equal amounts of 28S and 18S rRNA (middle). Further degradation decreases the rRNA peaks, and degradation products become apparent in the lower molecular weight range (right).



Thermal cycler recommended

The Illumina® TotalPrep™-96 RNA Amplification procedure is very sensitive to temperature; variable or inaccurate incubation temperatures can limit aRNA synthesis. It is also very important that condensation does not form in the plate wells during any of the incubations. Condensation changes the composition of reaction mixtures, which can greatly reduce yield.

- A thermal cycler with a temperature adjustable heated lid is recommended.

A calibrated thermal cycler, with a temperature-adjustable heated lid, is recommended, for the greatest temperature control and stability during Illumina® TotalPrep™-96 RNA Amplification reaction incubations. Allow the thermal cycler to equilibrate to the required temperature before placing the plates in the block for incubation. Follow the recommended settings for the lid temperatures. Too high a lid setting may inhibit the reaction; too low a setting may cause condensation.

Note: Even if you use a hybridization oven or incubator for most of the Illumina® TotalPrep™-96 RNA Amplification reactions, a thermal cycler is strongly recommended for the 16°C second-strand synthesis reaction incubation (step 2. on page 12). Turn off the heated lid if it cannot be adjusted to match the 16°C block temperature.

If your thermal cycler does not have a temperature-adjustable lid, or a thermal cycler is unavailable, calibrated hybridization ovens or incubators (at constant temperature) may also be used. Preheat incubators so that the correct temperature has stabilized before reactions are placed in the incubator.

- Heat blocks or water baths are not recommended for Illumina TotalPrep-96 RNA Amplification reaction incubations.

Orbital shaker settings

We recommend using an orbital shaker with a 2–3 mm orbit. Using a shaker with too large an orbit will cause the liquid to spill out of the wells, while too small of an orbit may not mix the sample effectively.

Determine the maximum speed that can be used in this procedure by filling the wells of a 96-well plate with 290 µL of water. Turn the shaker on and increase the speed slowly while observing the movement of the water in the wells. Note the maximum speed at which the plate can be shaken without causing the water to splash or spill out of the wells. When shaking experimental samples, always increase the orbital shaker speed slowly to avoid splashing. Always choose the lowest speed that is strong enough for complete mixing.

Mix each kit component before use

- Mix enzyme solutions by gently flicking the tube a few times before adding them to master mixes.
- Thaw frozen reagents completely at room temperature, then mix thoroughly by vortexing, and place on ice.

Master mixes

We strongly recommend preparing master mixes containing ~10% extra, instead of pipetting components individually. This reduces the effects of pipetting error, saves time, and improves reproducibility.

When using a multichannel pipettor to dispense small volumes of master mix (<2 mL), we recommend that you minimize the void volume by pipetting master mix into a PCR plate instead of using a reservoir.

Reagent preparation

1. Add 32 mL 100% ethanol to the cDNA Wash Buffer Concentrate

   Add 32 mL 100% ethanol (ACS reagent grade or equivalent) to the bottle labeled cDNA Wash Buffer Concentrate. Mix well and mark the label to indicate that the ethanol was added. This solution will be referred to as ***cDNA Wash Buffer*** in these instructions. Store at room temperature.

2. Add 20 mL 100% ethanol to the cRNA Wash Solution Concentrate

   Add 20 mL 100% ethanol (ACS reagent grade or equivalent) to the bottle labeled cRNA Wash Solution Concentrate. Mix well and mark the label to indicate that the ethanol was added. This solution will be referred to as ***cRNA Wash Solution*** in these instructions. Store at room temperature.

3. Prepare cRNA Binding Mix for the experiment

   IMPORTANT! Prepared cRNA Binding Mix can be stored at room temperature for one week; prepare only the amount needed for all samples in the experiment plus ~10% overage to cover pipetting error.

   a. First, mix RNA Binding Beads with Bead Resuspension Solution and ethanol

      Mixing beads with the Bead Resuspension Solution will facilitate bead dispersion in Binding Mix. At room temperature, combine Bead Resuspension Solution with RNA Binding Beads in a nuclease-free tube and mix briefly. Then add 100% ethanol and mix thoroughly by vortexing.

Combine the following (for a single reaction):	
RNA Binding Beads†	10 µL
Bead Resuspension Solution	4 µL
Mix briefly, then add:	
100% ethanol (ACS reagent grade)	6 µL
Mix well by vortexing.	

† Mix the RNA Binding Beads by vortexing before dispensing.

   b. Next, add cRNA Binding Buffer Concentrate

      At room temperature, add cRNA Binding Buffer Concentrate to the bead mixture from the previous step in a nuclease-free container. Mix by vortexing:

cRNA Binding Mix (for a single reaction)	
Component	**Amount**
cRNA Binding Buffer Concentrate	50 µL
RNA Binding Bead Mixture (from step 3.a.)	20 µL

      This mixture is referred to as cRNA Binding Mix in these instructions.

Reverse transcription to synthesize first strand cDNA

Incubator needed

Thermal cycler programmed as follows:

Temp	Time	Cycles
42°C (50°C lid)	2 hr	1
4°C	hold	—

It is important to follow the lid temperature recommendation; higher lid temperatures may inhibit reverse transcription, while lower temperatures may cause condensation, resulting in changes to the reaction composition.

1. Adjust the volume of ≤ 500 ng RNA to 11 µL
   a. Place up to 500 ng of total RNA (typically 50–500 ng) into the wells of a PCR Plate (provided with the kit).
   b. Add Nuclease-free Water to a final volume of 11 µL.
2. Add 9 µL ***Reverse Transcription Master Mix***

   At room temperature, assemble a Reverse Transcription Master Mix in a nuclease-free tube in the order shown in the table below. Prepare enough master mix for all samples in the experiment plus ~10% extra.

Amount	Component
2 µL	10X First Strand Buffer
4 µL	dNTP Mix
1 µL	T7 Oligo(dT) Primer
1 µL	RNase Inhibitor
1 µL	ArrayScript™ Reverse Transcriptase

   a. Mix well by gently vortexing. Centrifuge ~5 sec to collect the master mix at the bottom of the tube and place it on ice.
   b. Transfer 9 µL of the Reverse Transcription Master Mix to each RNA sample, and mix by gently pipetting up and down 3–4 times. Cover the reactions with aluminum sealing foil.

      To facilitate the transfer process, you can distribute the master mix into 8 wells of a PCR plate and use an 8-channel pipettor to transfer the master mix.
3. Incubate 2 hr at 42°C

   Place the plate in the thermal cycler, start the run, and incubate the reaction for 2 hr at 42°C. If condensation is apparent after the incubation, centrifuge the plate briefly (~5 sec) to collect the samples at the bottom of the wells.

   Place the plate on ice and immediately proceed to the second strand cDNA synthesis.

Second strand cDNA synthesis

Incubator needed

Thermal cycler programmed as follows:

Temp	Time	Cycles
16°C (heat-disabled lid, or no lid)	2 hr	1
4°C	hold	—

Disable the heated lid of the thermocycler if the lid temperature cannot be set in the range 16°C to room temperature. If the lid is too hot, inhibition of the reaction may occur.

1. Add 80 µL ***Second Strand Master Mix*** to each sample

   On ice, prepare a Second Strand Master Mix by adding reagents to a reservoir in the order listed in the table below. Assemble enough master mix for all samples in the experiment plus ~10% overage to allow for pipetting error.

Amount	Component
63 µL	Nuclease-free Water
10 µL	10X Second Strand Buffer
4 µL	dNTP Mix
2 µL	DNA Polymerase
1 µL	RNase H

   a. Mix gently by rocking the reservoir back and forth.

   b. Transfer 80 µL of Second Strand Master Mix to each sample in the PCR Plate. Mix by gently pipetting up and down 3–4 times. Cover the reactions with aluminum sealing foil.

2. Incubate 2 hr at 16°C

   a. Place the PCR Plate in the 16°C thermal cycler, start the run, and incubate for 2 hr.

   It is important to cool the thermal cycler to 16°C before placing the PCR Plate inside; subjecting the reactions to temperatures >16°C could compromise cRNA yield.

   b. After the 2 hr incubation at 16°C, place the reactions on ice and proceed to the next section (below), or immediately freeze reactions at –20°C. Do not leave the reactions on ice for more than 1 hr.

STOPPING POINT. This is a potential overnight stopping point (at –20°C), but it is better to complete the cDNA purification before stopping.

cDNA purification

Before beginning the cDNA purification:

- Remove the cDNAPure from the refrigerator and allow it to equilibrate to room temperature for 30 min before use.
- Shake the container of cDNAPure to fully resuspend the magnetic beads before use. cDNAPure is a slurry of magnetic beads in binding buffer. The magnetic beads will settle over time.

- Preheat a minimum of 20 µL per sample of Nuclease-free Water to 55°C.

IMPORTANT! Preheat the Nuclease-free Water to a maximum of 55°C; temperatures above 58°C can partially denature the cDNA, compromising final aRNA yield.

1. Add 180 µL cDNAPure, transfer to a U-Bottom Plate, and shake for 2 min
   a. Add 180 µL of resuspended cDNAPure to each second strand cDNA synthesis reaction.
   b. Transfer the samples from the PCR Plate to a U-Bottom Plate.

      Note: Adding the cDNAPure to the reactions and then transferring the samples (as opposed to simply transferring samples into wells that already contain cDNAPure), facilitates good mixing of the cDNAPure with the cDNA.

   c. ***Gently*** shake for at least 2 min to thoroughly mix the sample with the cDNAPure (setting 4 on the Lab-Line Titer Plate Shaker). The cDNA in the samples will bind to the magnetic beads in the cDNAPure during this incubation.
2. Capture the magnetic beads and discard the supernatant
   a. Move the U-Bottom Plate to a magnetic stand and capture the magnetic beads, for ~5 min. When capture is complete, the mixture becomes transparent and the beads will form pellets against the magnets in the magnetic stand. The exact capture time depends on the magnetic stand used.
   b. Carefully aspirate the supernatant without disturbing the magnetic beads. Discard the supernatant.
   c. Remove the plate from the magnetic stand.

      For effective washing, it is critical to remove the plate from the magnetic stand before adding cDNA Wash Buffer (next step).
3. Wash twice with 150 µL cDNA Wash Buffer

   Make sure that ethanol has been added to the bottle of cDNA Wash Buffer Concentrate before using it.
   a. Add 150 µL cDNA Wash Buffer to each sample and shake at ***moderate*** speed for 1 min (setting 7 on the Lab-Line Titer Plate Shaker).

      Note: The beads will not completely resuspend at this point because of the low surface tension of the cDNA Wash Buffer.

   b. Move the plate to a magnetic stand, and capture the magnetic beads as in the previous step.
   c. Carefully aspirate and discard the supernatant without disturbing the magnetic beads, and remove the plate from the magnetic stand.
   d. Repeat the above substeps to wash a second time with 150 µL of cDNA Wash Buffer.

e. Move the plate to a shaker and shake ***vigorously*** for 2 min to evaporate residual ethanol from the plate (setting 10 on the Lab-Line Titer Plate Shaker).

   Do not overdry the beads as this may make them difficult to resuspend in the next step.

4. Elute cDNA with 20 µL 55°C Nuclease-free Water

   a. Elute the cDNA from the magnetic beads by adding 20 µL preheated 55°C Nuclease-free Water to each sample.

   b. ***Vigorously*** shake the plate for 3 min, then check to make sure the magnetic beads are fully dispersed. If they are not, continue shaking until the beads are dispersed.

   c. Move the plate to the magnetic stand, and capture the magnetic beads until the mixture becomes transparent.

5. Transfer 17.5 µL cDNA to wells of a new PCR Plate

   Carefully transfer 17.5 µL of the supernatant, which contains the eluted cDNA, into wells of a new PCR Plate (supplied).

STOPPING POINT. The purified cDNA can be stored overnight at –20°C, if desired.

In vitro transcription to synthesize cRNA

Incubator needed

Thermal cycler programmed as follows:

Temp	Time	Cycles
37°C (default lid; 100–105°C)	4–14 hr; see step 2.	1
4°C	hold	—

It is important to use a thermal cycler with a heated lid (100–105°C), to maintain uniform temperature, and because it is extremely important that condensation does not form inside the tubes; this would change the reagent concentrations and reduce yield.

1. Add 7.5 µL ***IVT Master Mix*** to each sample

   a. Assemble the ***IVT Master Mix*** at room temperature in a nuclease-free tube in the order shown in the table below. Prepare enough master mix for all samples in the experiment plus ~10% extra to cover pipetting error.

Amount	Component
2.5 µL	Biotin-NTP Mix
2.5 µL	T7 10X Reaction Buffer
2.5 µL	T7 Enzyme Mix

   b. Mix well by gently vortexing. Centrifuge ~5 sec to collect the master mix at the bottom of the tube and place it on ice.

   c. Transfer 7.5 µL of IVT Master Mix to each sample; mix by pipetting up and down twice. Cover the reactions with aluminum sealing foil.

2. Incubate the reaction for 4–14 hr at 37°C

   The minimum recommended incubation time is 4 hr. The maximum incubation time is 14 hr.

cRNA purification

Before beginning the cRNA purification:

- Preheat the cRNA Elution Buffer to 55°C.
- Make sure the cRNA Binding Mix was assembled and resuspended as described in section "Reagent preparation" on page 10.

1. Add 70 µL cRNA Binding Mix and 95 µL ethanol
   a. Swirl the prepared cRNA Binding Mix to resuspend the magnetic beads and add 70 µL to each sample.
   b. Add 95 µL of 100% ethanol to each sample.
2. Transfer to U-Bottom Plate and shake gently for 2 min
   a. Transfer each sample to a well of a U-Bottom Plate (provided with the kit).

      Note: As in the previous purification, adding the magnetic bead mixture to the reactions and then transferring the samples facilitates good mixing of the RNA Binding Beads with the cRNA.

   b. ***Gently*** shake for ≥2 min to thoroughly mix (setting 4 on the Lab-Line Titer Plate Shaker). The cRNA in the samples will bind to the RNA Binding Beads during this incubation.
3. Capture the RNA Binding Beads and discard supernatant
   a. Move the plate to a magnetic stand and capture the magnetic beads, for ~5 min. When capture is complete, the mixture becomes transparent and the RNA Binding Beads will form pellets against the magnets in the magnetic stand. The exact capture time depends on the magnetic stand used.
   b. Carefully aspirate and discard the supernatant without disturbing the magnetic beads; then remove the plate from the magnetic stand.
4. Wash twice with 100 µL cRNA Wash Solution

   Make sure that ethanol has been added to the bottle of cRNA Wash Solution Concentrate before using it.
   a. Add 100 µL cRNA Wash Solution to each sample, and shake at ***moderate*** speed for 1 min (setting 7 on the Lab-Line Titer Plate Shaker).

      Note: The RNA Binding Beads may not fully disperse during this step; this is expected and will not affect RNA purity or yield.

   b. Move the plate to a magnetic stand and capture the RNA Binding Beads as in the previous step.
   c. Carefully aspirate and discard the supernatant without disturbing the RNA Binding Beads and remove the plate from the magnetic stand.
   d. Repeat the above substeps to wash a second time with 100 µL of cRNA 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).

   Do not overdry the beads as this may make them difficult to resuspend in the next step.

5. Elute cRNA with 40–100 µL 55°C cRNA Elution Buffer

   a. Elute the purified cRNA from the RNA Binding Beads by adding 40–100 µL preheated (55°C) cRNA Elution Buffer to each sample.

   b. ***Vigorously*** shake the plate for 3 min (setting 10 on the Lab-Line Titer Plate Shaker) then check to make sure the RNA Binding Beads are fully dispersed. If they are not, continue shaking until the beads are dispersed.

   c. Move the plate to a magnetic stand, and capture the RNA Binding Beads.

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

   e. Store purified cRNA at –20°C overnight or at –80°C for long-term storage.

      Note: For long-term storage, keep the cRNA at –80°C and minimize repeated freeze-thawing. Splitting samples into 5–20 µg aliquots is a good way to prevent multiple freeze-thaws.

Evaluation of reaction products

cRNA quantitation and optional sample concentration

Assessing yield by UV absorbance

The concentration of a cRNA solution can be determined by measuring its absorbance at 260 nm. We recommend using NanoDrop Spectrophotometers for convenience. No dilutions or cuvettes are needed; just measure 2 µL of the cRNA sample directly.

Alternatively, the cRNA concentration can be determined 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. Find the concentration in µg/mL using the equation shown below. (1 A_{260} = 40 µg RNA/mL)

$$A_{260} \text{ X dilution factor X } 40 = \text{µg RNA/mL}$$

Assessing yield with the RiboGreen® assay

If a fluorometer or a fluorescence microplate reader is available, the RiboGreen fluorescence-based assay for RNA quantitation (Invitrogen) is a convenient and sensitive way to measure RNA concentration. Follow the manufacturer's instructions for the RiboGreen assay.

Expected yield

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

(Optional) Concentrate the purified cRNA

If necessary, concentrate the cRNA by vacuum centrifugation. If the heater on the vacuum centrifuge has different settings, use medium or low. Check the progress of drying every 5–10 min, and remove the sample from the concentrator when it reaches the desired volume. Avoid drying cRNA samples to completion.

Analysis of cRNA size

The size distribution of cRNA can be evaluated using an Agilent® 2100 Bioanalyzer® instrument with the Agilent® RNA 6000 Nano Kit (part number 5067-1511), or by conventional denaturing agarose gel analysis. The Bioanalyzer® instrument can provide a fast and accurate size distribution profile of cRNA samples, but yield should be determined by UV absorbance or RiboGreen analysis. To analyze cRNA size using a Bioanalyzer® instrument, follow the manufacturer's instructions for running the assay using purified cRNA.

Expected cRNA size

We recommend analyzing cRNA size distribution using an Agilent® Bioanalyzer® instrument and RNA 6000 Nano Kit loaded with 300 ng of cRNA per well. The expected profile is a distribution of sizes from 250–5500 nt, with most of the reaction products between 850–1500 nt.

Troubleshooting

Positive control reaction

Control RNA amplification instructions

To verify that the process is working as expected, a Control RNA sample isolated from HeLa cells is provided with the kit.

1. Dilute 2 µL of the Control RNA into 18 µL of Nuclease-free Water.
2. Use 1 µL of the diluted Control RNA (100 ng) in an Illumina® TotalPrep™-96 RNA Amplification reaction; follow the protocol starting at step "Reverse transcription to synthesize first strand cDNA" on page 11.
3. At step 2. on page 15, use a 14 hr incubation for the IVT reaction.
4. Continue with the procedure through section "cRNA purification" on page 15.

Expected results

- The positive control reaction should produce ≥5 µg of cRNA.
- The average size of the cRNA should be ≥1000 nucleotides.

Factors that affect both the positive control and experimental samples

If the positive control reaction yield or amplification product size does not meet expectations, consider the following possible causes and troubleshooting suggestions. These suggestions also apply to problems with amplification of experimental RNA.

Incubation temperature(s) were incorrect

The incubation temperatures are critical for effective RNA amplification. Use only properly calibrated thermal cyclers for the procedure.

Condensation formed in the tube during the reaction incubation(s)

Condensation occurs when the cap of the reaction vessel is cooler (e.g., room temperature) than the bottom of the tube. As little as 1–2 µL of condensate in an IVT reaction changes the concentrations of the nucleotides and magnesium, which can reduce yields.

The best way to avoid condensation is to use a thermal cycler with the recommended lid temperature settings for all incubations.

If you see condensation, spin the plate briefly and mix the reaction gently. Move the plate to an incubator where condensation does not occur or is minimized.

Nuclease-contaminated tubes, tips, or equipment

Using pipettes, tubes, or equipment that are contaminated with nucleases can result in cleavage of the RNA or DNA being generated at each step in the procedure. This will reduce the size of the cRNA products and decrease cRNA yield. Both RNases and DNases can be removed from surfaces using Ambion® RNase*Zap*® RNase Decontamination Solution (Cat. no. AM9780).

Incomplete resuspension or dispersion of magnetic beads

In general, the cDNAPure and the RNA Binding Beads will disperse more easily when the temperature of the mixture is warmer than 20°C.

1. Be sure to resuspend the RNA Binding Beads just before each step in preparing the cRNA Binding Mix (step 3. on page 10).
2. Make sure that magnetic bead mixtures are fully resuspended before adding them to cDNA or cRNA samples for purification procedures (steps 1. on page 13, and 1. on page 15).
3. For efficient elution of purified reaction products, make sure that magnetic beads are fully resuspended in the elution solution (steps 4. on page 14 and 5. on page 16).
4. Avoid overdrying magnetic beads before eluting the cDNA or cRNA (in steps 3 e. on page 14 and 4.e. on page 16) because this may make the beads more difficult to resuspend. If the beads are inadvertently overdried, increase the mixing time (to 10 min) during the elution step to allow the beads to rehydrate.

Loss of the cDNAPure and/or the RNA Binding Beads

Since the reaction products are purified by immobilization on the cDNAPure and the RNA Binding Beads, any magnetic bead loss during the cDNA or cRNA purifications will result in lower final cRNA yield. Magnetic beads can be lost by incomplete magnetic bead capture and/or by inadvertently aspirating beads when removing supernatant from captured beads.

To determine whether cDNAPure or RNA Binding Beads have been aspirated along with supernatant, it may be helpful to collect all supernatants (except the final cDNA and cRNA-containing supernatant) in a single container. Observe the color of the collected supernatant; if magnetic beads are present, the solution will be light brown.

To prevent aspiration of magnetic beads in subsequent experiments, reduce the aspiration speed and make sure that the tip openings are not touching the bottom of the well when removing supernatant from captured cDNAPure and RNA Binding Beads.

To avoid incomplete magnetic bead capture, make sure that the solution becomes transparent before removing supernatant. This is especially important during the cDNA Purification in section "cDNA purification" on page 12, because the reaction mixture may be somewhat viscous.

Troubleshooting low yield and small average cRNA size

Consider the following troubleshooting suggestions if the positive control reaction produced the expected results, but amplification of your experimental samples results in less cRNA than expected or average cRNA size below approximately 500 nt.

Impure RNA samples

RNA samples with significant amounts of contaminating DNA, protein, phenol, ethanol, or salts are reverse transcribed poorly and subsequently generate less cRNA than pure RNA samples. Phenol extract and ethanol precipitate your RNA, or use the Ambion® MEGAclear™ Kit (Cat. no. AM1908) to further purify the samples before reverse transcription.

Lower than expected input RNA concentration

Take another A_{260} reading of your RNA sample or try using more RNA in the cRNA amplification procedure.

RNA sample integrity is poor

RNA that is partially degraded generates cDNA that is relatively short. This will reduce the average size of the cRNA population and subsequently reduce the yield of cRNA. You can assess the integrity of an RNA sample by determining the size of the 18S and 28S rRNA bands and the relative abundance of 28S to 18S rRNA (See *"RNA integrity" on page 8* for more information).

The mRNA content of your total RNA sample is lower than expected

Different RNA samples contain different amounts of mRNA. In healthy cells, mRNA constitutes 1–10% of total cellular RNA (Johnson 1974, Sambrook and Russell 2001). The actual amount of mRNA depends on the cell type and the physiological state of the sample. When calculating the amount of amplification, the starting mass of mRNA in a total RNA sample should always be considered within a range of 10–30 ng per µg of total RNA (assuming good RNA quality).

Bead carryover in eluted sample

If cDNAPure or RNA Binding Beads are carried over into the eluate containing the RNA, they will cause the solution to be light brown in color.

To avoid bead carryover, allow sufficient time for bead capture and aspirate slowly and carefully when transferring eluent from the beads. Also avoid touching the bottom of the well with the pipette tip when removing solutions from captured beads.

A Supplemental Information

Related products available from Life Technologies

96-well Magnetic-Ring Stand Cat. no. AM10050	The 96-well Magnetic-Ring Stand features 96 powerful ring-shaped magnets arranged to cradle each well of a 96-well plate for quick, thorough bead capture. Captured magnetic beads form evenly distributed donut-shaped pellets with a large hole in the center. This capture pattern facilitates both supernatant removal and subsequent bead resuspension. The stand is suitable for high throughput applications conducted with multichannel pipettors or with robotic liquid handlers. However, because the pellets will be evenly distributed around the edge of the wells, it may require practice for efficient manual removal of supernatants.
Magnetic Stand-96 Cat. no. AM10027	The Magnetic Stand-96 has powerful magnets positioned to capture beads to one side of the well. This capture pattern makes it very easy to remove supernatants manually without disturbing the beads, and therefore may be preferred by beginning users. In some applications, however, pellets formed with the Magnetic Stand-96 may be difficult to resuspend. If this occurs, we recommend the 96-well Magnetic-Ring Stand (Cat. no. AM10050).
FirstChoice® Total and Poly(A) RNA	Life Technologies provides high quality total and poly(A) RNA from a variety of human, mouse and rat tissues and from human cell lines. DNA is removed with a stringent DNase treatment, and the purity and integrity of these RNAs are verified by Agilent® Bioanalyzer® instrument evaluation, denaturing agarose gel electrophoresis, or Northern analysis. FirstChoice® Total RNA is prepared by methods that quantitatively recover small RNAs (miRNA, siRNA, and snRNA). FirstChoice® Total and Poly(A) RNAs are ready for use in any application that requires highly purified, intact RNA.
RNA Isolation Kits	Family of kits for isolation of total or poly(A) RNA. Included in the product line are kits using classical GITC and acidic phenol, one-step disruption/denaturation, phenol-free glass fiber filter or magnetic bead binding, and combination kits.
GLOBINclear™ Whole Blood Globin Reduction Kits Cat. nos. AM1980, AM1981	The GLOBINclear™ Whole Blood Globin Reduction Kits employ a novel, non-enzymatic technology to remove >95% of the globin mRNA from whole blood total RNA samples. The resulting mRNA is a superior template for RNA amplification and synthesis of labeled cDNA for array analysis. Kits are available for treatment of human or mouse/rat whole blood total RNA.

Quality control

Functional Testing

The Control RNA is used in an Illumina TotalPrep-96 RNA Amplification reaction following the instructions in section II starting on page 6. Reaction yield is assessed by measuring the A_{260} on a spectrophotometer. The median size of the cRNA is assessed using the mRNA smear assay on the Agilent® 2100 Bioanalyzer® instrument.

Nuclease testing

Relevant kit components are tested in the following nuclease assays:

RNase activity

A sample is incubated with labeled RNA and analyzed by PAGE.

Nonspecific endonuclease activity

A sample is incubated with supercoiled plasmid DNA and analyzed by agarose gel electrophoresis.

Exonuclease activity

A sample is incubated with labeled double-stranded DNA, followed by PAGE analysis.

Protease testing

A sample is incubated with protease substrate and analyzed by fluorescence.

B

Safety

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

WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. 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:

In the U.S.:
- U.S. Department of Health and Human Services guidelines published in Biosafety in Microbiological and Biomedical Laboratories found at: **www.cdc.gov/biosafety**
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030), found at: **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**

In the EU:
- Check local guidelines and legislation on biohazard and biosafety precaution and refer to the best practices published in the World Health Organization (WHO) Laboratory Biosafety Manual, third edition, found at: **www.who.int/csr/resources/publications/biosafety/WHO_CDS_CSR_LYO_2004_11/en/**

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Documentation and Support

Obtaining SDSs

Safety Data Sheets (SDSs) are available from **www.lifetechnologies.com/support**.

Note: For the SDSs of chemicals not distributed by Life Technologies, contact the chemical manufacturer.

Obtaining support

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

www.lifetechnologies.com/support

At the website, you can:

- Access worldwide telephone and fax numbers to contact Technical Support and Sales facilities
- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support (**techsupport@lifetech.com**)
- Search for user documents, SDSs, vector maps and sequences, application notes, formulations, handbooks, certificates of analysis, citations, and other product support documents
- Obtain information about customer training
- Download software updates and patches

Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale found on Life Technologies' website at **www.lifetechnologies.com/termsandconditions**. If you have any questions, please contact Life Technologies at **www.lifetechnologies.com/support**.

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