Illumina® TotalPrep RNA Amplification Kit

(Cat #IL1791)

Instruction Manual

Manual Version 0606

Literature Citation When describing a procedure for publication using this product, we would appreciate that you refer to it as the Illumina® TotalPrep RNA Amplification Kit.

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I. Introduction

A. Background and Product Description

RNA amplification has become the standard method for preparing RNA samples for array analysis (Kacharmina et al. 1999, Pabon et al. 2001). The Illumina® TotalPrep RNA Amplification Kit (patent pending) 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 et al., 1990). The procedure consists of reverse transcription with an oligo(dT) primer bearing a T7 promoter using ArrayScript™, a reverse transcriptase (RT) engineered to produce higher yields of first strand cDNA than wild type enzymes. ArrayScript catalyzes the synthesis of virtually full-length cDNA, which is the best way to ensure production of reproducible microarray samples. The cDNA then undergoes second strand synthesis and clean-up to become a template for in vitro transcription with T7 RNA Polymerase. To maximize cRNA yield, Ambion's proprietary MEGAscript® in vitro transcription (IVT) technology along with biotin UTP (provided in the kit) is used to generate hundreds to thousands of biotinylated, antisense RNA copies of each mRNA in a sample. (In this Instruction Manual the antisense amplified RNA is referred to as cRNA, in scientific literature it is also commonly called aRNA.) The labeled cRNA produced with the kit was developed for hybridization with Illumina arrays.

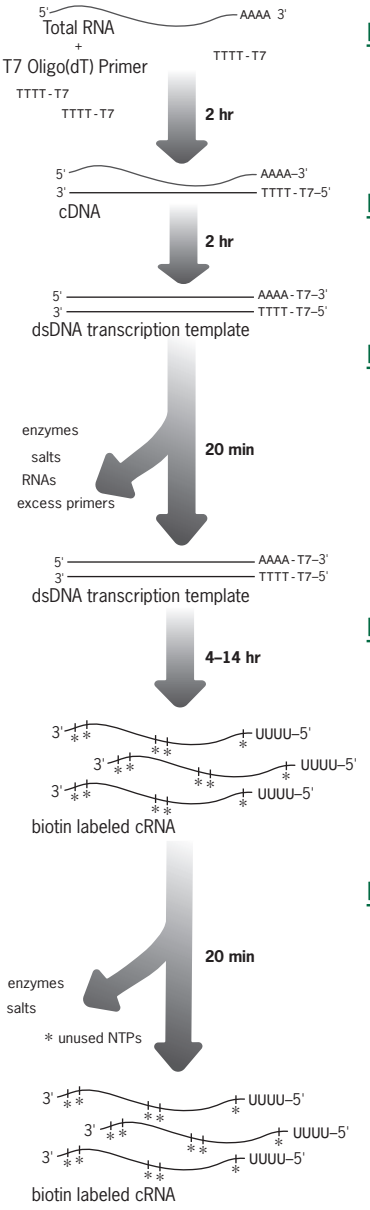
B. Procedure Overview

The Illumina TotalPrep RNA Amplification procedure is depicted in Figure 1.

- ***Reverse Transcription to Synthesize First Strand cDNA*** is primed with the T7 Oligo(dT) Primer to synthesize cDNA containing a T7 promoter sequence.
- ***Second Strand cDNA Synthesis*** converts the single-stranded cDNA into a double-stranded DNA (dsDNA) template for transcription. The reaction employs DNA Polymerase and RNase H to simultaneously degrade the RNA and synthesize second strand cDNA.
- ***cDNA Purification*** removes RNA, primers, enzymes, and salts that would inhibit in vitro transcription.
- ***In Vitro Transcription to Synthesize cRNA*** generates multiple copies of biotinylated cRNA from the double-stranded cDNA templates; this is the amplification and labeling step.

- ***cRNA Purification*** removes unincorporated NTPs, salts, enzymes, and inorganic phosphate. After purification, the cRNA is ready for use with Illumina's direct hybridization array kits.

Figure 1. Illumina TotalPrep RNA Amplification Procedure



II.C. Reverse Transcription to Synthesize First Strand cDNA

1. Bring RNA samples to 11 µl with Nuclease-free Water
2. Add 9 µl of Reverse Transcription Master Mix and place at 42°C
3. Incubate for 2 hr at 42°C

II.D. Second Strand cDNA Synthesis

1. Add 80 µl Second Strand Master Mix to each sample
2. Incubate for 2 hr at 16°C

Potential stopping point

II.E. cDNA Purification

Preheat Nuclease-free Water to 50–55°C

1. Add 250 µl cDNA Binding Buffer to each sample cDNA Binding Buffer to each sample
2. Pass the mixture through a cDNA Filter Cartridge
3. Wash with 500 µl Wash Buffer
4. Elute cDNA with a total of 19 µl 50–55°C Nuclease-free Water

Potential stopping point

II.F. In Vitro Transcription to Synthesize cRNA

1. Add 7.5 µl of IVT Master Mix to each cDNA sample, and mix
2. Incubate for 4–14 hr at 37°C
3. Add 75 µl Nuclease-free Water to each sample

Potential stopping point

II.G. cRNA Purification

Preheat Nuclease-free Water to 50–60°C

Assemble cRNA Filter Cartridges and tubes

1. Add 350 µl cRNA Binding Buffer to each sample
2. Add 250 µl 100% ethanol and pipet 3 times to mix
3. Pass samples through a cRNA Filter Cartridge(s)
4. Wash with 650 µl Wash Buffer
5. Elute cRNA with 100 µl preheated Nuclease-free Water

Potential stopping point

C. Materials Provided with the Kit and Storage Conditions

The kit contains reagents for 24 single-round amplification reactions. Properly stored kits are guaranteed for 6 months from receipt.

Store at –20°C in a non-frost-free freezer.

cDNA synthesis and in vitro transcription

Amount	Component
26 µl	T7 Oligo(dT) Primer*
51 µl	10X First Strand Buffer
205 µl	dNTP Mix
26 µl	RNase Inhibitor
27 µl	ArrayScript™
252 µl	10X Second Strand Buffer
51 µl	DNA Polymerase
26 µl	RNase H
64 µl	T7 10X Reaction Buffer
64 µl	T7 Enzyme Mix
64 µl	Biotin-NTP Mix
1.75 ml	Nuclease-free Water†
10 µl	Control RNA (1 mg/ml HeLa total RNA)

* The T7 Oligo(dT) Primer is available separately from Ambion; Cat #5710

† Store the Nuclease-free Water at –20°C, 4°C, or room temp.

cDNA and cRNA purification

Amount	Component	Storage
10 ml	Nuclease-free Water	any temp*
30 ml	Wash Buffer (Add 24 ml 100% ethanol before use)	4°C or room temp
7 ml	cDNA Binding Buffer	room temp†
9 ml	cRNA Binding Buffer	room temp
24	cRNA Filter Cartridges	room temp
48	cRNA Collection Tubes	room temp
24	cDNA Filter Cartridges + Tubes	room temp
24	cDNA Elution Tubes	room temp

* Store Nuclease-free Water at –20°C, 4°C, or room temp.

† The cDNA Binding Buffer may form a precipitate if stored below room temp. If a precipitate is visible, redissolve it by warming the solution to 37°C for up to 10 min and vortexing vigorously. Cool to room temp before use.

D. Materials Not Provided with the Kit

Lab equipment and supplies

- 100% Ethanol (to prepare the Wash Buffer), ACS reagent grade or equivalent proof
- Thermal cycler with adjustable temperature heated lid, hybridization oven, or heat blocks set at 42°C, 37°C, and 16°C. (See *Incubator recommendations* on page 9 for more information.)
- Vacuum centrifuge concentrator
- Vortex mixer
- Microcentrifuge
- Non-stick RNase-free 0.5 ml microcentrifuge tubes (e.g. Ambion Cat #12350)

Illumina arrays

Illumina arrays are available for purchase through Illumina, Inc., or one of its worldwide distributors. Arrays are available in a range of multi-sample formats (from as few as 6 to as many as 96), and can include custom and/or catalog content (such as the six-sample Whole Human Genome BeadChip). For further information please visit www.illumina.com or call Illumina Customer Solutions at 1.800.809.ILMN (toll-free) or 1.858.202.4566 (outside the USA).

Optional materials and equipment for RNA analysis

- Spectrophotometer, such as the NanoDrop® ND-1000A UV-Vis Spectrophotometer (www.nanoambion.com). With the NanoDrop Spectrophotometer, the user simply pipets 1.5–2 µl of sample onto the measurement pedestal; no dilutions or cuvettes are necessary. The NanoDrop Spectrophotometer performs all UV/Vis spectrophotometric analyses carried out by traditional spectrophotometers.
- (Optional) Reagents and apparatus for preparation and electrophoresis of agarose gels
- (Optional) RiboGreen® RNA Quantitation Assay and Kit (Molecular Probes Inc.)

E. Related Products Available from Ambion

Biotin-11-UTP and Biotin-16-UTP Cat #8450, 8451, 8452, 8453	Ambion's biotinylated UTPs are ideal for use as substrates in vitro transcription reactions, and can be utilized by a variety of RNA polymerases, including T7, T3, and SP6 RNA polymerases. Biotinylated RNA can be used in place of radioactively labeled RNA in many applications with detection via one of a variety of streptavidin-based methods.
FirstChoice® Total and Poly(A) RNA see our web or print catalog	Ambion 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. These RNAs are shown to be intact by denaturing agarose gel electrophoresis, Northern analysis, reverse transcription, and capillary electrophoresis using the Agilent 2100 bioanalyzer, and they are precisely quantitated. Please see our catalog or our website (www.ambion.com) for a complete listing.
RNA Isolation Kits see our web or print catalog	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 binding, and combination organic extraction/glass fiber filter binding kits.
MEGAclear™ Cat #1908	MEGAclear purifies RNA from transcription, and other enzymatic reactions yielding high quality RNA free of unincorporated nucleotides, enzymes and buffer components with close to 100% recovery of input RNA.
Millennium Markers™ and BrightStar® Biotinylated Millennium Markers™ Cat #7150 and 7170	Ambion's Millennium™ Markers are designed to provide very accurate size determination of single-stranded RNA transcripts from 0.5 to 9 kb and can be used in any Northern protocol. They are a mixture of 10 easy-to-remember sizes of in vitro transcripts: 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6 and 9 kb.
RNA 6000 Ladder Cat #7152	The RNA 6000 Ladder is a set of 6 RNA transcripts designed for use as reference standards with the RNA 6000 Lab Chip Kits and the Agilent 2100 bioanalyzer.

II. cRNA Amplification Protocol

A. Important Parameters for Successful Amplification

Input RNA quantity and IVT reaction incubation time

Consider both the amount of sample RNA you have and the amount of cRNA needed for your experiments when planning Illumina RNA Amplification experiments. These factors will influence how much input RNA is used, and how long to incubate the IVT reaction.

Accurate quantitation

For experiments where the cRNA yield from different samples will be compared, it is ***essential*** to accurately quantify the input RNA used in the Illumina RNA Amplification procedure. We recommend the NanoDrop 1000A Spectrophotometer for rapid, accurate quantitation of nucleic acids, however, any reliable RNA quantitation method such as traditional spectrophotometry or RiboGreen (Molecular Probes, Inc.) can be used.

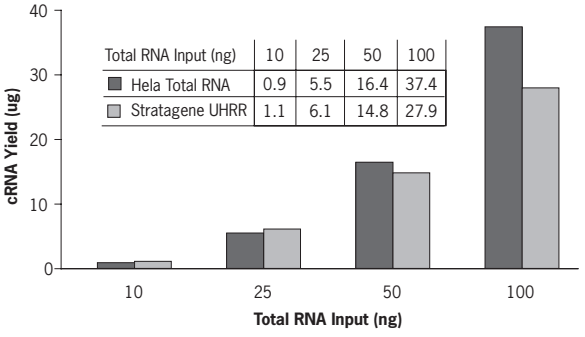
Recommended amount of input RNA

Recommended mass amount of total RNA	50–500 ng
Minimum mass amount of total RNA	25 ng
Maximum volume of RNA	11 µl

Determining input RNA amount and IVT reaction incubation time

The Illumina RNA Amplification procedure can accommodate a wide range of input RNA amounts, but for reproducible and comparable results, use a fixed amount of input RNA for all experiments. Tailor both the amount of input RNA and the IVT reaction time to produce the amount of cRNA needed for your microarray experiments. ***Illumina Sentrix arrays require 500 ng to 2 µg of cRNA for each hybridization.***

Figure 2 shows the yield of biotinylated cRNA from increasing amounts of both the Control RNA provided with the kit and Stratagene® Human Universal Reference RNA. In this experiment, input RNA amounts of 25 ng and more were sufficient to produce an excess of cRNA for use with Illumina Sentrix arrays. Note, however, that cRNA yield from Illumina TotalPrep RNA Amplification reactions may vary considerably depending on the integrity and purity of the RNA used in the reaction, and the other parameters discussed in these instructions.



Total RNA Input (ng)	10	25	50	100
Hela Total RNA	0.9	5.5	16.4	37.4
Stratagene UHRR	1.1	6.1	14.8	27.9

Figure 2. cRNA Yield with Different Amounts of Input RNA.

The indicated amounts of Control RNA (HeLa total RNA) or Stratagene® Human Universal Reference RNA were amplified in triplicate using a 14 hr IVT incubation times (average yield shown).

RNA purity

The quality of the RNA is the single most important factor affecting how efficiently an RNA sample will be amplified using the Illumina TotalPrep RNA Amplification Kit. RNA samples should be free of contaminating proteins, DNA, and other cellular material as well as phenol, ethanol, and salts associated with RNA isolation procedures. Impurities can lower the efficiency of reverse transcription and subsequently reduce the level of amplification. An effective measure of RNA purity is the ratio of absorbance readings at 260 and 280 nm. The ratio of A_{260} to A_{280} values should fall in the range of 1.7–2.1. RNA must be suspended in high quality water or TE (10 mM Tris-HCl, 1 mM EDTA).

RNA integrity

The integrity of the RNA sample, or the proportion that is full-length, is another important component of RNA quality. Reverse transcribing partially degraded mRNAs will typically generate relatively short cDNAs that can potentially lack portions of the coding region. RNA integrity can be evaluated by microfluidics analysis using the Agilent® 2100 bioanalyzer and an RNA LabChip® Kit. Primarily full-length RNA will exhibit a ratio of 28S to 18S rRNA bands that approaches 2:1. Using a bioanalyzer, the RNA Integrity Number (RIN) can be calculated to further evaluate RNA integrity. A new metric developed by Agilent, the RIN analyzes information from both the rRNA bands, as well as information contained outside the rRNA peaks (potential degradation products) to provide a more complete picture of RNA degradation states. Search for "RIN" at the following web address for more information:

www.chem.agilent.com

Denaturing agarose gel electrophoresis and nucleic acid staining can also be used to separate and visualize the major rRNA species. When the RNA resolves into discrete rRNA bands (i.e. no significant smearing

below each band), with the 28S rRNA band appearing approximately twice as intense as the 18S rRNA band, then the mRNA in the sample is likely to be mostly full-length. The primary drawback to gel electrophoresis is that microgram amounts of RNA must be sacrificed.

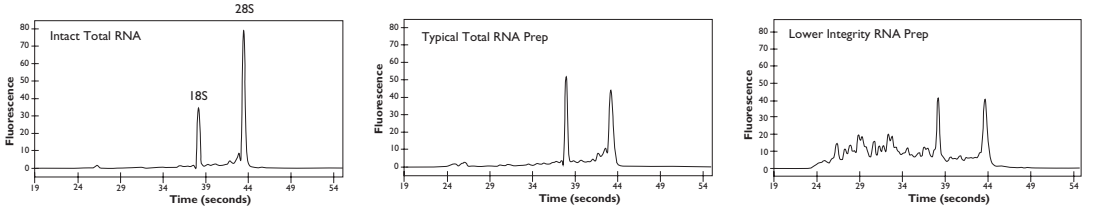


Figure 3. Bioanalyzer Images of Total RNA Preparations.

These electropherograms (from the Agilent 2100 bioanalyzer) show RNA samples with decreasing integrity that are all of sufficient quality to use as input for the Illumina TotalPrep RNA Amplification Kit. The trace labeled "Intact Total RNA" represents the ideal for a bioanalyzer trace of total RNA. Notice how the ribosomal RNA peaks are at a ratio of about 2 (28S:18S) in this sample; this represents ultrahigh quality RNA in terms of integrity. Most RNA samples will more closely resemble the center trace where there are nearly equal amounts of 28S and 18S rRNA. The trace on the right shows a fairly typical human RNA preparation with rRNA peaks that are lower than in the other two traces and where lower molecular weight degradation products become apparent. RNA samples with suboptimal integrity can yield meaningful array analysis results if the data are subjected to rigorous statistical analysis (Schoor et al. 2003).

Reaction incubation times should be precise and consistent

The incubation times for most of the enzymatic reactions in the protocol were optimized in conjunction with the kit reagents to ensure the maximum yield of nucleic acid product in each step—adhere to them closely. An exception is the IVT reaction, where a range of 4–14 hr incubation time is acceptable (step II.F.2 on page 15). Keep this IVT incubation time uniform if cRNA yield from different samples will be compared or if you want to have equal amplification of different samples. Although differences in IVT incubation time among samples has had very little, if any, effect on array results in our hands, we still recommend using a uniform IVT incubation time for the most reproducible amplification and array analysis.

Master mixes

We strongly recommend preparing master mixes for the Illumina RNA Amplification procedure. This approach reduces the effects of pipetting error, saves time, and improves reproducibility. Using master mixes is especially important when cRNA yield from different samples will be compared. Ambion provides a link for a web-based master mix calculator at the following address:

www.ambion.com/tools/illumina

Thorough mixing is very important for reproducibility

Below are specific instructions for mixing kit reagents, master mixes, and individual reactions. For maximum reproducibility and cRNA yield, follow these instructions closely.

- ***Mix each kit component after thawing***.
  Mix enzyme solutions by ***gently*** flicking the tube a few times before adding them to reactions. Thaw frozen reagents completely ***at room temperature*** (i.e. primers, nucleotides, and 10X buffers), then mix thoroughly by vortexing, and keep on ice before use.
- ***Mix master mixes by gentle vortexing***.
  After assembling master mixes, ***gently*** vortex to make a homogenous mixture without inactivating the enzyme(s).
- ***Mix individual reactions by pipetting and flicking the tube***.
  After adding master mixes or other reagents to individual reactions, pipet up and down 2–3 times to rinse reagents from the pipet tip. Then flick the tube with your finger 3–4 times to mix thoroughly, and finish by centrifuging briefly to collect the reaction at the bottom of the tube.

Incubator recommendations

- ***We recommend a calibrated hybridization oven or other constant temperature air incubator for most enzymatic reaction incubations.*** We do ***not*** recommend using heat blocks or water baths for any Illumina RNA Amplification reaction incubations.
  To avoid any potential influence on the reaction temperature from the tube holder, let tube holders equilibrate in the incubator for sufficient time or use a tube holder that doesn't touch the sides and bottoms of the tubes—for example a floating tube support.
- ***For the 16°C second strand synthesis reaction incubation (step II.D.2*** on page 12***), we recommend using a thermal cycler***.
  Ideally these reactions should be incubated in a calibrated thermal cycler with a lid temperature that matches the block temperature. Most machines do not have this feature, so if the lid temperature is static (~100°C), use it with the lid heat turned off or do not close the heated lid. Otherwise, heat from the lid will raise the temperature of the solution in the tube, compromising the reaction.

The Illumina RNA Amplification procedure is very sensitive to temperature; therefore use incubators that have been professionally calibrated according the manufacturer's recommended schedule. Variable or inaccurate incubation temperatures can limit cRNA synthesis. Preheat incubators if necessary so that the correct temperature has stabilized before reactions are placed in the incubator. It is also very important that condensation does not form in the reaction tubes during any of the incubations. Condensation changes the composition of reaction mixtures and can greatly reduce yield.

Maintaining consistency

Procedural consistency is very important for planning amplification experiments. Consider implementing a detailed procedural plan that will be used by everyone in the lab to maintain consistency. This type of plan will minimize variation due to subtle procedural differences that can influence RNA amplification and may complicate gene expression studies. The plan should include basic information such as the method of RNA isolation, the amount of RNA to use in the procedure, and how long to incubate the IVT reaction. It should also address specifics that are not often included in protocols such as which tubes, tube racks, and incubators to use for each step in the process. Finally, develop a consistent work flow. For example standardize stopping points in the method. The idea is to standardize all of the variables discussed in this section of the Instruction Manual and carefully follow all the protocol steps in order to maximize amplification consistency among samples.

Tubes: use RNase-free 0.5 ml nonstick tubes

It is most convenient to conduct the Illumina RNA Amplification procedure in 0.5 ml nonstick tubes (e.g. Ambion Cat #12350). These can be thin-wall (PCR) tubes or ordinary-weight nonstick tubes. 0.5 ml tubes are large enough to accommodate the cDNA Binding Buffer without having to transfer reactions to a larger tube. Their small size and nonstick properties also keep the reaction components at the bottom of the tube.

B. Prepare the Wash Buffer

Add 24 ml 100% ethanol (ACS reagent grade or equivalent proof) to the bottle labeled Wash Buffer. Mix well and mark the label to indicate that the ethanol was added.

C. Reverse Transcription to Synthesize First Strand cDNA

Incubator needed
42°C; hybridization oven or air incubator recommended

1. Bring RNA samples to 11 µl with Nuclease-free Water

a. Place a maximum volume of 11 µl of total RNA (50–500 ng is recommended) into a nonstick, sterile, RNase-free, 0.5 ml microcentrifuge tube.

b. Add Nuclease-free Water as necessary to bring all samples to 11 µl.

2. Add 9 µl of *Reverse Transcription Master Mix* and place at 42°C

a. At room temperature, prepare ***Reverse Transcription Master Mix*** in a nuclease-free tube. Assemble enough to synthesize first strand cDNA from all the RNA samples in the experiment, including ≤5% overage to cover pipetting error. We provide a master mix calculator on our website to calculate reagent amounts:

www.ambion.com/tools/illumina

At room temperature, assemble the ***Reverse Transcription Master Mix*** in the order shown:

*Reverse Transcription Master Mix (***for a single 20 µl reaction)**	
Amount	**Component**
1 µl	T7 Oligo(dT) Primer
2 µl	10X First Strand Buffer
4 µl	dNTP Mix
1 µl	RNase Inhibitor
1 µl	ArrayScript

b. Mix well by gently vortexing. Centrifuge briefly (~5 sec) to collect the ***Reverse Transcription Master Mix*** at the bottom of the tube and place on ice.

c. Transfer 9 µl of ***Reverse Transcription Master Mix*** to each RNA sample. Mix thoroughly by pipetting up and down 2–3 times, then flicking the tube 3–4 times, and centrifuge briefly to collect the reaction in the bottom of the tube.

d. Place the samples in a 42°C incubator.

3. Incubate for 2 hr at 42°C

Incubate reactions for 2 hr at 42°C (hybridization oven or air incubator is recommended). After the incubation, centrifuge briefly (~5 sec) to collect the reaction mixture at the bottom of the tube.

Place the tubes on ice and immediately proceed to *II.D. Second Strand cDNA Synthesis* starting on page 12.

D. Second Strand cDNA Synthesis

Incubator needed
16°C; thermal cycler recommended

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

a. On ice, prepare a ***Second Strand Master Mix*** in a nuclease-free tube in the order listed below. Assemble enough to synthesize second strand cDNA from all the samples in the experiment, including ≤5% overage to cover pipetting error. We provide a master mix calculator on our website to calculate reagent amounts:

www.ambion.com/tools/illumina

Assemble the ***Second Strand Master Mix*** on ice in the order shown:

*Second Strand Master Mix (***for a single 100 µl reaction)**	
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

b. Mix well by gently vortexing. Centrifuge briefly (~5 sec) to collect the mixture at the bottom of the tube and place on ice.

c. Transfer 80 µl of ***Second Strand Master Mix*** to each sample. Mix thoroughly by pipetting up and down 2–3 times, then flicking the tube 3–4 times, and centrifuge briefly to collect the reaction in the bottom of the tube.

d. Place the tubes in a 16°C thermal cycler. It is important to cool the thermal cycler block to 16°C before adding the reaction tubes because subjecting the reactions to temperatures >16°C will compromise cRNA yield.

2. Incubate for 2 hr at 16°C

Incubate 2 hr in a 16°C thermal cycler. If the lid temperature cannot be adjusted to match the 16°C block temperature, cover the reactions with the heated lid turned off, or if the lid cannot be turned off—do not cover the tubes with it. (Do not use a water bath or a heat block in a 4°C refrigerator for this incubation because the temperature will fluctuate too much.)

NOTE
You may want to preheat the Nuclease-free Water, for use in step II.E.4, during this incubation.

3. Place reactions on ice briefly or freeze immediately

After the 2 hr incubation at 16°C, place the reactions on ice and proceed to section *E. cDNA Purification* (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 (next section) before stopping.

E. cDNA Purification

IMPORTANT

All centrifugations in this purification procedure should be done at 10,000 x g (typically ~10,000 rpm) at room temperature.

cDNA Filter Cartridges should not be subjected to RCFs over 16,000 x g because the force could cause mechanical damage and/or may deposit glass filter fiber in the eluate.

Preheat Nuclease-free Water to 50–55°C

Before beginning the cDNA purification, preheat the 10 ml bottle of Nuclease-free Water to 50–55°C for at least 10 min.

IMPORTANT

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

1. Add 250 µl cDNA Binding Buffer to each sample

IMPORTANT

Check the cDNA Binding Buffer for precipitation before using it. If a precipitate is visible, redissolve it by warming the solution to 37°C for up to 10 min and vortexing vigorously. Cool to room temp before use.

Add 250 µl of cDNA Binding Buffer to each sample, and mix thoroughly by pipetting up and down 2–3 times, then flicking the tube 3–4 times. Follow up with a quick spin to collect the reaction mixture in the bottom of the tube. Proceed quickly to the next step.

2. Pass the mixture through a cDNA Filter Cartridge

Check that the cDNA Filter Cartridge is firmly seated in its wash tube (supplied).

a. Pipet the cDNA sample/cDNA Binding Buffer (from step 1) onto the center of the cDNA Filter Cartridge.

b. Centrifuge for ~1 min at 10,000 x g, or until the mixture is through the filter.

c. Discard the flow-through and replace the cDNA Filter Cartridge in the wash tube.

IMPORTANT

Make sure that the ethanol has been added to the bottle of Wash Buffer before using it in the next step.

3. Wash with 500 µl Wash Buffer

a. Apply 500 µl Wash Buffer to each cDNA Filter Cartridge.

b. Centrifuge for ~1 min at 10,000 X g, or until all the Wash Buffer is through the filter.

c. Discard the flow-through and spin the cDNA Filter Cartridge for an additional minute to remove trace amounts of Wash Buffer.

d. Transfer cDNA Filter Cartridge to a cDNA Elution Tube.

4. Elute cDNA with a total of 19 µl 50–55°C Nuclease-free Water

It is important to use Nuclease-free Water that is at 50–55°C for the cDNA elution. Colder water will be less efficient at eluting the cDNA, and using hotter water (≥58°C) may result in reduced cRNA yield.

a. Apply 10 µl of Nuclease-free Water (preheated to 50–55°C) to the center of the filter in the cDNA Filter Cartridge.

b. Leave at room temperature for 2 min and then centrifuge for ~1.5 min at 10,000 X g, or until all the Nuclease-free Water is through the filter.

c. Apply a second aliquot of 9 µl preheated Nuclease-free Water and centrifuge for 2 min. The double-stranded cDNA will now be in the eluate (~17.5 µl).

d. Proceed directly to section *F. In Vitro Transcription to Synthesize cRNA*, or place the cDNA at –20°C.

STOPPING POINT

The purified cDNA can be stored overnight at –20°C at this point if desired.

F. In Vitro Transcription to Synthesize cRNA

Incubator needed
37°C; hybridization oven or air incubator recommended

1. Add 7.5 µl of *IVT Master Mix* to each cDNA sample, and mix

a. At room temperature, prepare an ***IVT Master Mix*** by adding the following reagents to a nuclease-free microcentrifuge tube in the order listed below. Assemble enough to synthesize cRNA from all the samples in the experiment, including ≤5% overage to cover pipetting error. We provide a master mix calculator on our website to calculate reagent amounts:

www.ambion.com/tools/illumina

Assemble the ***IVT Master Mix*** at room temperature, in the order shown:

IVT Master Mix **for a single 25 µl reaction**	
Amount	**Component**
2.5 µl	T7 10X Reaction Buffer
2.5 µl	T7 Enzyme Mix
2.5 µl	Biotin-NTP Mix

b. Mix well by gently vortexing. Centrifuge briefly (~5 sec) to collect the ***IVT Master Mix*** at the bottom of the tube and place on ice.

c. Transfer ***7.5 µl of IVT Master Mix*** to each cDNA sample (volume ~17.5 µl). Mix thoroughly by pipetting up and down 2–3 times, then flicking the tube 3–4 times, and centrifuge briefly to collect the reaction mixture in the bottom of the tube.

Once assembled, place the tubes at 37°C.

2. Incubate for 4–14 hr at 37°C

The recommended IVT reaction incubation time is based on the amount of input RNA used in the amplification reaction:

Input RNA	Recommended IVT Incubation
100–500 ng	4–14 hr
<100 ng	14 hr

It is important to maintain a constant 37°C incubation temperature. We recommend incubating in a hybridization oven because it is extremely important that condensation does not form inside the tubes; this would change the reagent concentrations and reduce yield.

3. Add 75 µl Nuclease-free Water to each sample

Stop the reaction by adding 75 µl Nuclease-free Water to each cRNA sample to bring the final volume to 100 µl. Mix thoroughly by gentle vortexing.

Proceed to the cRNA purification step (below) or store at –20°C.

STOPPING POINT

The cRNA can be stored at –20°C at this point for several days.

G. cRNA Purification

This purification removes enzymes, salts and unincorporated nucleotides from the cRNA. At the end of the purification, the cRNA is eluted from the filter with Nuclease-free Water.

IMPORTANT

All centrifugations in this purification procedure should be done at 10,000 x g (typically ~10,000 rpm) at room temperature.

cRNA Filter Cartridges should not be subjected to RCFs over 16,000 x g because the force could cause mechanical damage and/or may deposit glass filter fiber in the eluate.

Preheat Nuclease-free Water to 50–60°C

Before beginning the cRNA purification preheat the 10 ml bottle of Nuclease-free Water to 50–60°C for at least 10 min.

Assemble cRNA Filter Cartridges and tubes

For each sample, place an cRNA Filter Cartridge into an cRNA Collection Tube, and set aside for use in step 3.

1. Add 350 µl cRNA Binding Buffer to each sample

Check to make sure that each IVT reaction was brought to 100 µl with Nuclease-free Water.

Add 350 µl of cRNA Binding Buffer to each cRNA sample. Proceed to the next step immediately.

2. Add 250 µl 100% ethanol and pipet 3 times to mix

IMPORTANT

It is crucial to follow these mixing instructions exactly, and to proceed quickly to the next step.

Add 250 µl of ACS reagent grade 100% ethanol to each cRNA sample, and mix by pipetting the mixture up and down 3 times. ***Do NOT vortex to mix and do NOT centrifuge.***

Proceed ***immediately*** to the next step as soon as you have mixed the ethanol into each sample. Any delay in proceeding could result in loss of cRNA because once the ethanol is added, the cRNA will be in a semi-precipitated state.

3. Pass samples through a cRNA Filter Cartridge(s)

a. Pipet each sample mixture from step 2 onto the center of the filter in the cRNA Filter Cartridge.

b. Centrifuge for ~1 min at 10,000 x g. Continue until the mixture has passed through the filter.

c. Discard the flow-through and replace the cRNA Filter Cartridge back into the cRNA Collection Tube.

4. Wash with 650 µl Wash Buffer

a. Apply 650 µl Wash Buffer to each cRNA Filter Cartridge.

b. Centrifuge for ~1 min at 10,000 x g, or until all the Wash Buffer is through the filter.

c. Discard the flow-through and spin the cRNA Filter Cartridge for an additional ~1 min to remove trace amounts of Wash Buffer.

d. Transfer Filter Cartridge(s) to a fresh cRNA Collection Tube.

5. Elute cRNA with 100 µl preheated Nuclease-free Water

a. To the center of the filter, add 100 µl Nuclease-free Water (preheated to 50–60°C).

b. Leave at room temperature for 2 min and then centrifuge for ~1.5 min at 10,000 x g, or until the Nuclease-free Water is through the filter.

c. The cRNA will now be in the cRNA Collection Tube in ~100 µl of Nuclease-free Water.

6. (Optional) Concentrate the purified cRNA

If necessary, concentrate the cRNA by vacuum centrifugation or by precipitation with ammonium acetate (NH_4OAc)/ethanol.

(Optional) Concentrate 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.

(Optional) Precipitate with 5 M NH_4OAc and ethanol

a. Add 1/10th volume of 5 M NH_4OAc to the purified cRNA. If the sample was eluted with 100 µl Nuclease-free Water as suggested, this will be 10 µl of 5 M NH_4OAc.

b. Add 2.5 volumes of 100% ethanol (275 µl if the cRNA was eluted in 100 µl). Mix well and incubate at –20°C for 30 min.

c. Microcentrifuge at top speed for 15 min at 4°C or room temperature. Carefully remove and discard the supernatant.

d. Wash the pellet with 500 µl 70% cold ethanol, centrifuge again, and remove the 70% ethanol.

e. To remove the last traces of ethanol, quickly respin the tube, and aspirate any residual fluid with a fine-tipped pipette or syringe needle.

f. Air dry the pellet.

g. Resuspend the cRNA pellet using the desired solution and volume.

III. Assessing cRNA Yield and Quality

A. cRNA Quantitation and Expected Yield

Assessing cRNA yield by UV absorbance

The concentration of a cRNA solution can be determined by measuring its absorbance at 260 nm. Ambion scientists recommend using the NanoDrop 1000A Spectrophotometer (www.nanoambion.com) because it is extremely quick and easy to use. No dilutions and no cuvettes are needed; just measure 1.5 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 by multiplying the A_{260} by the dilution factor and the extinction coefficient. (1 A_{260} = 40 µg RNA/ml)

A_{260} X dilution factor X 40 = µg RNA/ml

Assessing cRNA yield with RiboGreen®

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

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 influences 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. Empirical data obtained using the Control RNA in the Illumina TotalPrep RNA Amplification procedure is shown in Figure 2 on page 7.

B. Analysis of cRNA Size

The size distribution of cRNA can be evaluated using an Agilent 2100 bioanalyzer with Caliper's LabChip® technology, or by conventional denaturing agarose gel analysis. The bioanalyzer can provide a fast and accurate size distribution profile of cRNA samples, but cRNA yield should be determined by UV absorbance or RiboGreen analysis. To analyze cRNA size using a bioanalyzer, follow the manufacturer's instructions for running the assay using purified cRNA (from step II.G.5 on page 17). Instructions for denaturing agarose gel electrophoresis are provided on our website at the following address:

www.ambion.com/techlib/append/supp/rna_gel.html

Expected cRNA size

Agilent bioanalyzer analysis

The expected cRNA profile is a distribution of sizes from 250–5500 nt with most of the cRNA at 1000–1500 nt (see Figure 4). To compare bioanalyzer profiles of different cRNA samples, be sure to load equal mass amounts to get an accurate comparison.

Denaturing agarose gel analysis

Amplified cRNA should appear as a smear from 250 to 5000 nt. The average size of biotin labeled cRNA should be approximately 1200 nt.

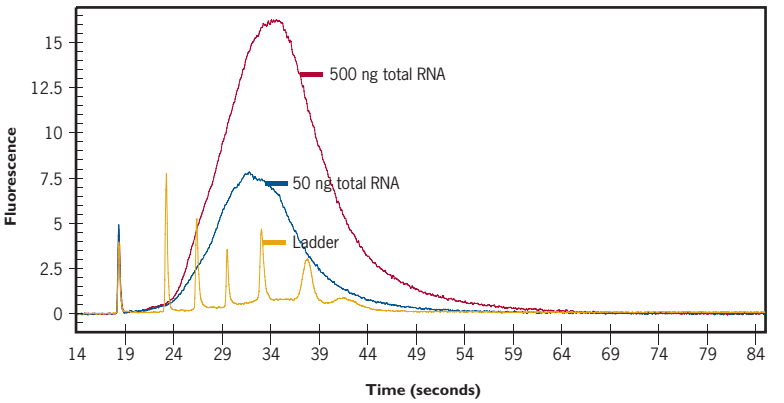


Figure 4. Bioanalyzer Analysis of cRNA made with the Illumina® TotalPrep RNA Amplification Kit.

This electropherogram displays the biotin labeled cRNA size distribution from amplification of 50 and 500 µg of the Control RNA using a 14 hr IVT reaction incubation.

IV. Troubleshooting

A. Positive Control Reaction

Control RNA amplification instructions

To establish if the kit is working properly, Control RNA consisting of 1 mg/ml HeLa cell total RNA is provided. ***Dilute the Control RNA 1:10*** by adding 1 µl of Control RNA to 9 µl Nuclease-free Water. Then ***use 1 µl of the diluted Control RNA (100 ng)*** in an Illumina RNA Amplification reaction; follow the protocol starting at step II.C.1 on page 10. At step II.F.2 on page 15, ***use a 14 hr incubation for the IVT reaction***. Continue with the procedure for making biotinylated cRNA through section II.G on page 16.

Analysis of the positive control amplification reaction

- After completing the cRNA purification, measure the A_{260} of the reaction product as described in section III.A. on page 19.
  The positive control reaction should produce ≥5 µg of cRNA.
  Be aware that often the positive control reaction cannot be compared to experimental reactions, because many experimental amplification experiments will use less than the 100 ng of input RNA used in the positive control reaction, and the cRNA yield will be proportionately lower. Also the Control RNA is of exceptional quality and purity, ensuring that it will amplify with extremely high efficiency.
- Also run a 2 µg aliquot of the reaction products on a denaturing agarose gel or analyze 100–200 ng on a bioanalyzer; ***the average size of the cRNA should be ≥1 kb***.

B. 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.

a. Incubation temperature(s) were incorrect

The incubation temperatures are critical for effective RNA amplification.

- Check the temperatures of all incubators used in the procedure with a calibrated thermometer.

- If a thermal cycler is used for incubation, check the accuracy of the adjustable temperature lid. If the lid temperature cannot be adjusted to match the desired reaction temperature, use the lid with the heat turned off, or do not use it to cover the reaction vessel(s).

b. 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 tube throws off the concentrations of the nucleotides and magnesium, which are crucial for good yield.

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

c. Nuclease-contaminated tubes, tips, or equipment

Using pipettes, tubes, or equipment that are contaminated with nucleases can cleave 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's RNase*Zap*® RNase Decontamination Solution (e.g., Cat #9780).

d. Absorbance readings were inaccurate

Confirm that your spectrophotometer is accurate by measuring the absorbance of an RNA or DNA sample of known concentration. Alternatively, assess the cRNA concentration by fractionating on an agarose gel adjacent to an RNA sample whose concentration is known. Comparing the ethidium bromide staining of the cRNA and control samples can approximate the concentration of the cRNA.

e. Incorrect dilution of the Control RNA

Confirm that the Control RNA was diluted as described in *Control RNA amplification instructions* on page 21.

C. Troubleshooting Low Yield and Small Average aRNA 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 in average aRNA size below ~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 Ambion's MEGAclear™ Kit (Cat #1908) to further purify it before reverse transcription.

Lower than suspected input RNA concentration

Measure A_{260} of your RNA sample again, 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 section *II.A. RNA integrity* on page 7 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 1–3% of total cellular RNA. 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 prep should always be considered to be a range from 10–30 ng per µg of total RNA (assuming good RNA quality).

V. Appendix

A. References

Kacharmina JE, Crino PB, Eberwine J (1999) Preparation of cDNA from single cells and subcellular regions. Methods Enzymol, 303: 3–18.

Pabon C, Modrusan Z, Ruvolo MV, Coleman IM, Daniel S, Yue H, Arnold LJ Jr. (2001) Optimized T7 amplification system for microarray analysis. Biotechniques 31(4): 874–879.

Schoor O, Weinschenk T, Hennenlotter J, Corvin S, Stenzl A, Rammensee H-G, and Stevanovic S (2003) Moderate degradation does not preclude microarray analysis of small amounts of RNA. Biotechniques 35:1192–1201.

Van Gelder RN, von Xastrow ME, Yool A, Dement DC, Barchas JD, Eberwine JH (1990) Amplified RNA synthesized from limited quantities of heterogeneous cDNA. Proc Natl Acad Sci USA, 87: 1663–1667.

B. Illumina TotalPrep RNA Amplification Kit Contents

Properly stored kits are guaranteed for 6 months from receipt. ***Store at –20°C*** in a non-frost-free freezer.

cDNA synthesis and in vitro transcription

Amount	Component
26 µl	T7 Oligo(dT) Primer*
51 µl	10X First Strand Buffer
205 µl	dNTP Mix
26 µl	RNase Inhibitor
27 µl	ArrayScript™
252 µl	10X Second Strand Buffer
51 µl	DNA Polymerase
26 µl	RNase H
64 µl	T7 10X Reaction Buffer
64 µl	T7 Enzyme Mix
64 µl	Biotin-NTP Mix
1.75 ml	Nuclease-free Water†
10 µl	Control RNA (1 mg/ml HeLa total RNA)

* The T7 Oligo(dT) Primer is available separately from Ambion; Cat #5710

† Store the Nuclease-free Water at –20°C, 4°C, or room temp.

cDNA and cRNA purification

Amount	Component	Storage
10 ml	Nuclease-free Water	any temp*
30 ml	Wash Buffer (Add 24 ml 100% ethanol before use)	4°C or room temp
7 ml	cDNA Binding Buffer	room temp†
9 ml	cRNA Binding Buffer	room temp
24	cRNA Filter Cartridges	room temp
48	cRNA Collection Tubes	room temp
24	cDNA Filter Cartridges + Tubes	room temp
24	cDNA Elution Tubes	room temp

* Store Nuclease-free Water at –20°C, 4°C, or room temp.

† The cDNA Binding Buffer may form a precipitate if stored below room temp. If a precipitate is visible, redissolve it by warming the solution to 37°C for up to 10 min and vortexing vigorously. Cool to room temp before use.

To obtain Material Safety Data Sheets

- Material Safety Data Sheets (MSDSs) can be printed or downloaded from our website by going to the following address and clicking on the link for the Illumina TotalPrep RNA Amplification Kit:
  www.ambion.com/techlib/msds
- Alternatively, e-mail us at MSDS@ambion.com to request MSDSs by e-mail, fax, or ground mail. Specify the Ambion catalog number of the kit(s) for which you want MSDSs and whether you want to receive the information by e-mail, fax, or ground mail. Be sure to include your fax number or mailing address as appropriate. If the mode of receipt is not specified, we will e-mail the MSDSs.
- Customers without internet access can contact our technical service department by telephone, fax, or mail to request MSDSs (contact information on the back of this booklet).

C. Quality Control

Functional Testing

The Control RNA is used in an Illumina RNA Amplification reaction following the instructions in section IV.A on page 21. The cRNA yield is assessed by measuring the A_{260} on the Nanodrop ND1000A spectrophotometer. The median size of the cRNA is assessed using the mRNA smear assay on the Agilent 2100 bioanalyzer.

Nuclease testing

Each component is tested in Ambion's rigorous nuclease assays:

RNase activity

A sample is incubated for 14–16 hr with ^{32}P-labeled RNA and analyzed by PAGE.

Non-specific endonuclease/nickase activity
A sample is incubated for 14–16 hr with supercoiled plasmid DNA and analyzed by agarose gel electrophoresis.

Exonuclease activity
A sample is incubated for 14–16 hr with supercoiled plasmid DNA and analyzed by agarose gel electrophoresis.

Protease testing

None detected in protein-containing components after a 14–16 hr incubation with 1 µg of protease substrate and analysis by fluorescence.