PROTOCOL

ambion® | RNA
by life technologies™

Illumina® TotalPrep™ RNA Amplification Kit

life technologies™

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Part Number IL1791M Rev. E
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Illumina® TotalPrep™ RNA Amplification Kit

(Part Number AMIL1791)

Protocol

I. Introduction

IMPORTANT

Before using this product, read and understand the "Safety Information" in the appendix in this document.

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 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 cleanup to become a template for in vitro transcription with T7 RNA Polymerase. To maximize cRNA yield, Ambion® 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 protocol 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.

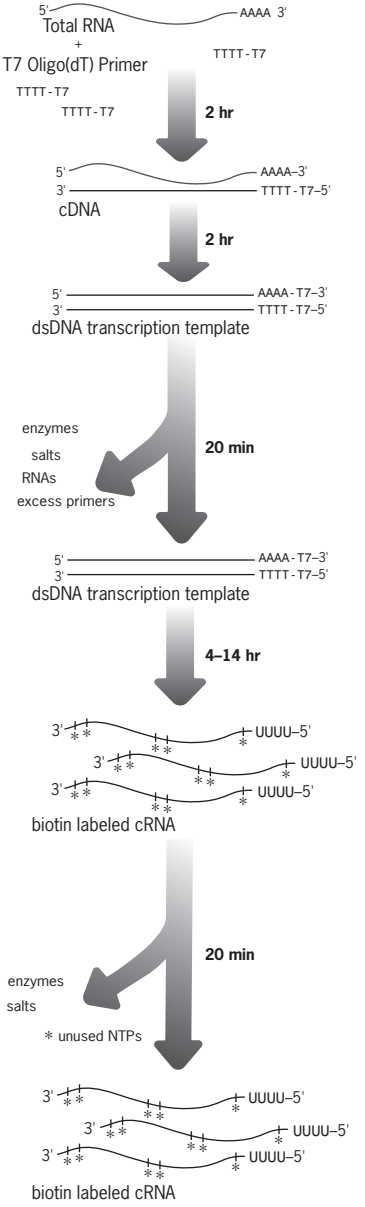
B. Procedure Overview

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 55°C
1. Add 250 µL 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 20 µL of 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

Before you begin: preheat Nuclease-free Water and assemble 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 200 µL 55°C 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.

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)

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

Some reagents may form a precipitate when stored at –20°C. If a precipitate is visible, redissolve it by warming the solution to room temperature with gentle mixing.

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
30	cDNA Filter Cartridges + Tubes	room temp
30	cDNA Elution Tubes	room temp

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

† The cDNA Binding Buffer may form a precipitate if stored below room temperature. 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 temperature 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 a temperature-adjustable heated lid (recommended), or hybridization ovens or incubators set at 70°C, 42°C, 37°C, and 16°C. (See "Thermal cycler recommended" on page 9 for more information.)
- Heat block set at 55°C, for preheating the water for cDNA and aRNA purification
- Vacuum centrifuge concentrator
- Vortex mixer
- Microcentrifuge
- Non-stick RNase-free 0.5 mL microcentrifuge tubes (e.g. P/N AM12350)
- RNase-free pipettors and tips, positive-displacement type recommended to increase the accuracy and precision of reaction inputs
- (Optional) Non-stick RNase-free tubes for storage of cDNA (e.g., AM 12450)

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-1000 or ND-8000 UV-Vis Spectrophotometer. Follow the manufacturer's instructions.
- (Optional) Agilent bioanalyzer and RNA LabChip Kits
- (Optional) Reagents and apparatus for preparation and electrophoresis of agarose gels
- (Optional) Quant-iT™ RiboGreen® RNA Assay Kit from Invitrogen (R11490) for use with a fluorescence microplate reader, standard spectrofluorometer, or filter fluorometer

E. Related Products

Biotin-11-UTP and Biotin-16-UTP P/N AM8450, AM8451, AM8452	Ambion® 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 RNA See web or print catalog for P/Ns	Life Technologies provides high quality total 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 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 RNAs are ready for use in any application that requires highly purified, intact RNA. See the catalog or website (www.invitrogen.com/ambion) for a complete listing of available FirstChoice RNAs.
RNA Isolation Kits See web or print catalog for P/Ns	Family of kits for isolation of total 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.
MEGAclear™ Kit P/N AM1908	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 P/N AM7150 and AM7170	Ambion 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 P/N AM7152	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 Procedure

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. The NanoDrop 1000A Spectrophotometer is recommended for rapid, accurate quantitation of nucleic acids, however, any reliable RNA quantitation method such as traditional spectrophotometry or RiboGreen (Invitrogen) 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.

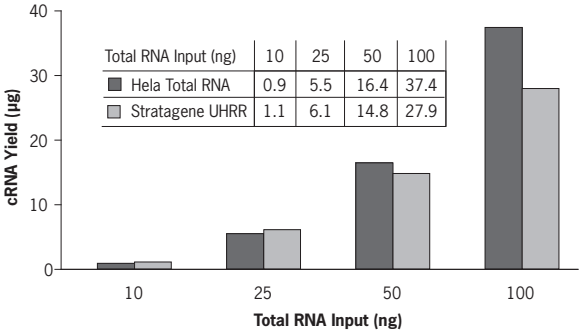


Figure 2. cRNA Yield with Different Amounts of Input RNA.

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 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 transcription of 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. 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.

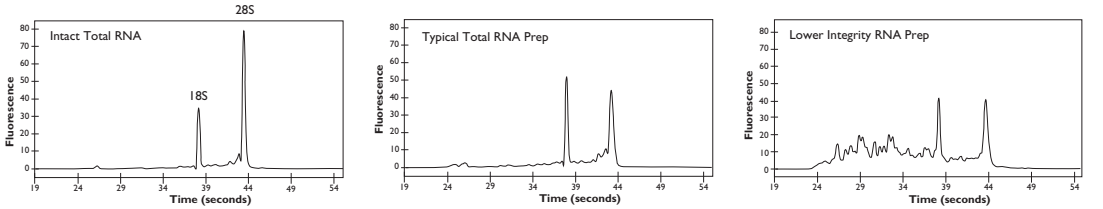


Figure 3. Bioanalyzer Images of Total RNA Preparations.

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 procedure 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 to 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.

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.

Thermal cycler recommended

The Illumina TotalPrep 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 reaction tubes 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 RNA Amplification reaction incubations. Allow the thermal cycler to equilibrate to the required temperature before placing the tubes 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.

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

NOTE

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

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 Protocol and carefully follow all the steps in order to maximize amplification consistency among samples.

Tubes: use 0.5 mL nonstick tubes

If a 60-well thermal cycler with temperature-adjustable lid is available, it is most convenient to conduct the Illumina TotalPrep RNA Amplification procedure in 0.5 mL nonstick tubes (for example, P/N AM12350). 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.

If your thermal cycler is equipped with a standard 96-well block, 0.2 mL non-stick tubes can be used.

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

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. 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. The required volumes for one reaction and the order of addition are shown in the table below. Multiply the number of reactions being performed by the volume of the component in the table, and by 1.05 to account for the necessary overage.

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 the thermal cycler, and start the run.

3. Incubate for 2 hr at 42°C

Incubate reactions for 2 hr at 42°C. 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 section *D. Second Strand cDNA Synthesis*.

D. 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

a. On ice, prepare a ***Second Strand Master Mix*** in a nuclease-free tube in the order listed below. Multiply the number of reactions being performed by the volume of the component in the table, and by 1.05 to account for the necessary overage.

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 and start the run.
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 55°C

Before beginning the cDNA purification, preheat a minimum of 20 µL per sample of Nuclease-free Water 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 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 temperature 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 20 µL of 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 20 µL of preheated Nuclease-free Water (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 min at 10,000 X g, or until all the Nuclease-free Water is through the filter. The double-stranded cDNA will now be in the eluate (~17.5 µL).

STOPPING POINT

The purified cDNA can be stored overnight at –20°C at this point if desired. Transfer the cDNA to a lidded, non-stick, RNase-free tube for storage.

F. 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 of *IVT Master Mix* to each cDNA sample, and mix

a. Transfer each cDNA sample from step 4.b on page 14 (volume ~17.5 µL) to a PCR tube compatible with your thermal cycler.

b. At room temperature, prepare an ***IVT Master Mix*** by adding the following reagents to a nuclease-free microcentrifuge tube in the order listed below. Multiply the number of reactions being performed by the volume of the component in the table, and by 1.05 to account for the necessary overage.

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

c. 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.

d. Transfer 7.5 µL of IVT Master Mix to each cDNA 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 mixture in the bottom of the tube.
Once assembled, place the tubes in the thermal cycler and start the run.

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

The reactions can be held post-IVT for up to 48 hr at 4°C, for convenience.

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

Incubator needed: heat block set at 55°C.

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.

Before you begin

- Before beginning the cRNA purification preheat a minimum of 200 µL of Nuclease-free Water to 55°C.
- 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 200 µL 55°C Nuclease-free Water

a. To the center of the filter, add 200 µL Nuclease-free Water (preheated to 55°C).

b. Incubate the samples in the 55°C heat block for 10 min (recommended).
Alternatively, incubate at room temperature for 2 min. This results in ~80% recovery of the aRNA.

c. Centrifuge for ~1.5 min at 10,000 x g, or until the Nuclease-free Water is through the filter.
The cRNA will now be in the cRNA Collection Tube in ~200 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 200 µL Nuclease-free Water as suggested, this will be 20 µL of 5 M NH_4OAc.

b. Add 2.5 volumes of 100% ethanol (550 µL if the cRNA was eluted in 200 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. The NanoDrop 1000A Spectrophotometer (www.nanodrop.com) is recommended because it is extremely quick and easy to use. Follow the manufacturer's instructions.

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® reagent

If a fluorometer or a fluorescence microplate reader is available, Invitrogen's 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 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 16). Instructions for denaturing agarose gel electrophoresis are provided at:

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

Expected cRNA size

Agilent bioanalyzer analysis

The expected cRNA profile is a distribution of sizes from 250 to 5500 nt with most of the cRNA at 1000 to 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.

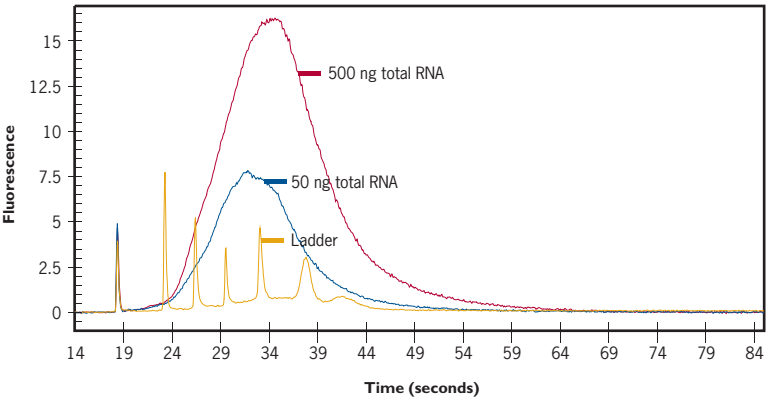


Figure 4. Bioanalyzer Analysis of cRNA made with the Illumina® TotalPrep™ RNA Amplification Kit.

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.

1. Dilute the Control RNA 1:10 by adding 1 µL of Control RNA to 9 µL Nuclease-free Water.
2. Use 2 µL of the diluted Control RNA (200 ng) in an Illumina RNA Amplification reaction; follow the procedure starting at step II.C.1 on page 11.
3. At step II.F.2 on page 15, use a ***14 hr*** incubation for the IVT reaction.
4. Continue with the procedure for making biotinylated cRNA through section II.G.

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 18.
  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 200 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.

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).

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.

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 RNase*Zap*® RNase Decontamination Solution (e.g., P/N AM9780).

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.

Incorrect dilution of the Control RNA

Confirm that the Control RNA was diluted as described in *Control RNA amplification instructions* on page 20.

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 the Ambion MEGAclear™ Kit (P/N AM1908) 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 *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. Quality Control

Functional Testing

The Control RNA is used in an Illumina RNA Amplification reaction following the instructions in section IV.A on page 20. 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

Relevant kit components are tested in the following nuclease assays:

RNase activity
Meets or exceeds specification when a sample is incubated with labeled RNA and analyzed by PAGE.

Nonspecific endonuclease activity
Meets or exceeds specification when a sample is incubated with supercoiled plasmid DNA and analyzed by agarose gel electrophoresis.

Exonuclease activity
Meets or exceeds specification when a sample is incubated with labeled double-stranded DNA, followed by PAGE analysis.

Protease testing

Meets or exceeds specification when a sample is incubated with protease substrate and analyzed by fluorescence.

C. Safety Information

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.*

1. 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.*

2. Biological hazard safety

WARNING

Potential Biohazard. *Depending on the samples used on the 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/

VI. Documentation and Support

A. Obtaining SDSs

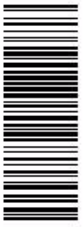
Safety Data Sheets (SDSs) are available from:
www.invitrogen.com/sds
or
www.appliedbiosystems.com/sds

B. Obtaining support

For the latest services and support information for all locations, go to:
www.invitrogen.com
or
www.appliedbiosystems.com

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
- 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



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