USER GUIDE

invitrogen™
by life technologies™

SuperScript® Indirect RNA Amplification System

For generating amplified amino-allyl antisense RNA from small starting quantities of RNA

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life technologies™

For Research Use Only. Not for diagnostic procedures.

Contents

Kit Contents and Storage

Shipping and Storage

The SuperScript® Indirect RNA Amplification System is shipped in three modules. The Core Module is shipped on dry ice, while the Purification Modules are shipped at room temperature. Store the components of the Core Module at –20°C and the components of the Purification Modules at room temperature.

Core Module

Components should be stored at -20°C. Reagents are provided for 20 labeling reactions.

Item	Components/Concentration	Amount
SuperScript® III Reverse Transcriptase	200 U/µL in 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.01% (v/v) NP-40, 50% (v/v) glycerol	40 µL
5X First-Strand Buffer	250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$	80 µL
DTT	0.1 M DTT in water	40 µL
10 mM dNTP Mix	dATP, dGTP, dCTP, and dTTP in DEPC-treated water	20 µL
T7-Oligo(dT) primer	In DEPC-treated water	20 µL
RNaseOUT™ Recombinant Ribonuclease Inhibitor	40 U/µL in 20 mM Tris-HCl (pH 8.0), 50 mM KCl, 0.5 mM EDTA, 8 mM DTT, and 50% (v/v) glycerol	20 µL
Control HeLa RNA	500 ng/µL	20 µL
E. coli DNA Polymerase I	10 U/µL in 50 mM potassium phosphate (pH 7.0), 100 mM KCl, 1 mM DTT, 50% (v/v) glycerol	80 µL
E. coli DNA Ligase	10 U/µL in 10 mM Tris-HCl (pH 7.4), 50 mM KCl, 0.1 mM EDTA, 1 mM DTT, 50% (v/v) Glycerol, 0.1% (w/v) Triton® X-100, and 200 µg/mL BSA	20 µL
E. coli RNase H	2 U/µL in 20 mM Tris-HCl (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT, 50 µg/mL BSA, 50% (v/v) glycerol	20 µL
5X Second-Strand Buffer	100 mM Tris-HCl (pH 6.9), 450 mM KCl, 23 mM $MgCl_2$, 0.75 mM β-NAD^+, 50 mM $(NH_4)_2SO_4$	600 µL
DEPC-treated Water	—	2 mL
T7 Enzyme Mix	Includes T7 RNA Polymerase (proprietary formulation)	140 µL
10X T7 Reaction Buffer	Proprietary formulation	80 µL
2X Coupling Buffer	Proprietary formulation	300 µL
DMSO	—	300 µL
DNase I	Amplification grade, 1 U/µL in 20 mM sodium acetate (pH 6.5), 5 mM $CaCl_2$, 0.1 mM PMSF, 50% (v/v) Glycerol	20 µL
100 mM ATP	In DEPC-treated water	30 µL
100 mM CTP	In DEPC-treated water	30 µL
100 mM GTP	In DEPC-treated water	30 µL
100 mM UTP	In DEPC-treated water	15 µL
50 mM aa-UTP	In DEPC-treated water	40 µL

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cDNA Purification Module

Components should be stored at room temperature.

Item	Amount
Low-Elution Volume Spin Cartridges (with collection tubes)	2 × 11 columns
cDNA Loading Buffer (must be combined with 100% isopropanol to create final buffer; see below)	9 mL
cDNA Wash Buffer (must be combined with 100% ethanol to create final buffer; see below)	4 mL
Recovery Tubes	20

aRNA Purification Module

Components should be stored at room temperature.

Item	Amount
PureLink® Spin Columns (with collection tubes)	42 columns
aRNA Binding Buffer (no additional preparation necessary)	2 × 4 mL
aRNA Wash Buffer (must be combined with 100% ethanol to create final buffer; see next page)	2 × 7 mL
DEPC-treated Water	20 mL
Amber Collection Tubes	4 × 11 tubes

Preparing cDNA Loading Buffer with Isopropanol

The cDNA Loading Buffer must be mixed with 100% isopropanol prior to use. The Loading Buffer plus isopropanol is stable for at least 6 months at room temperature.

Add the amount of isopropanol indicated below directly to the bottle of Loading Buffer. Be sure to mark the appropriate checkbox on the bottle to indicate that you have added the isopropanol.

	Amount
cDNA Loading Buffer	9 mL (entire bottle)
100% Isopropanol	3 mL
Total Volume	12 mL

Preparing cDNA Wash Buffer with Ethanol

The cDNA Wash Buffer must be mixed with 100% ethanol prior to use. The cDNA Wash Buffer plus ethanol is stable for at least 6 months at room temperature.

Add the amount of ethanol indicated below directly to the bottle of cDNA Wash Buffer. Be sure to mark the appropriate checkbox on the bottle to indicate that you have added the ethanol.

	Amount
cDNA Wash Buffer	4 mL (entire bottle)
100% Ethanol	12 mL
Total Volume	16 mL

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Preparing aRNA Wash Buffer with Ethanol

Each bottle of aRNA Wash Buffer must be mixed with 100% ethanol prior to use. The aRNA Wash Buffer plus ethanol is stable for at least 6 months at room temperature.

Add the amount of ethanol indicated below directly to each bottle of aRNA Wash Buffer. Be sure to mark the appropriate checkbox on the bottle to indicate that you have added the ethanol.

	Amount
aRNA Wash Buffer	7 mL (entire bottle)
100% Ethanol	21 mL
Total Volume	28 mL

Product Use

For research use only. Not intended for any human or animal diagnostic or therapeutic uses.

Introduction

Overview

Introduction

The SuperScript® Indirect RNA Amplification System is a highly robust and efficient system for amplifying mRNA from small starting quantities of total RNA or purified poly(A) RNA. This kit is based on the isothermal RNA amplification protocol developed in the laboratory of Dr. James Eberwine (Van Gelder et al, 1990). It uses SuperScript® III Reverse Transcriptase to synthesize first-strand cDNA primed with an anchored oligo(dT) primer containing a T7 promoter. Following second-strand synthesis and purification, the cDNA template is amplified via *in vitro* transcription using T7 RNA polymerase in an optimized enzyme and buffer formulation. This step generates antisense RNA (aRNA) molecules complementary to the original mRNA targets, incorporating amino-allyl UTP (aa-UTP) into the aRNA. The amplified aRNA is then ready for fluorescent-dye labeling and detection in gene expression profiling experiments.

Amplified aRNA is ideal for gene expression profiling from very small amounts of starting material because it preserves the relative abundance of the different mRNA sequences in the original sample, allowing you to compare relative quantities across experiments. This system is designed for use with 100–5000 ng of total RNA or 5–250 ng of poly(A) RNA as starting material. For optimal efficiency, we recommend using ≤1000 ng of total RNA.

Advantages of the System

- Optimized reagents and protocol ensure highly robust and reproducible reactions
- SuperScript® III Reverse Transcriptase in the first-strand synthesis reaction produces higher yields of cDNA and more full length products, resulting in more complete gene representation in the amplified probe population
- System generates aRNA with a greater average length than comparable kits.
- System includes all major reagents and materials for preparing amino-allyl-modified amplified RNA for subsequent labeling and detection

Advantages of SuperScript® III Reverse Transcriptase

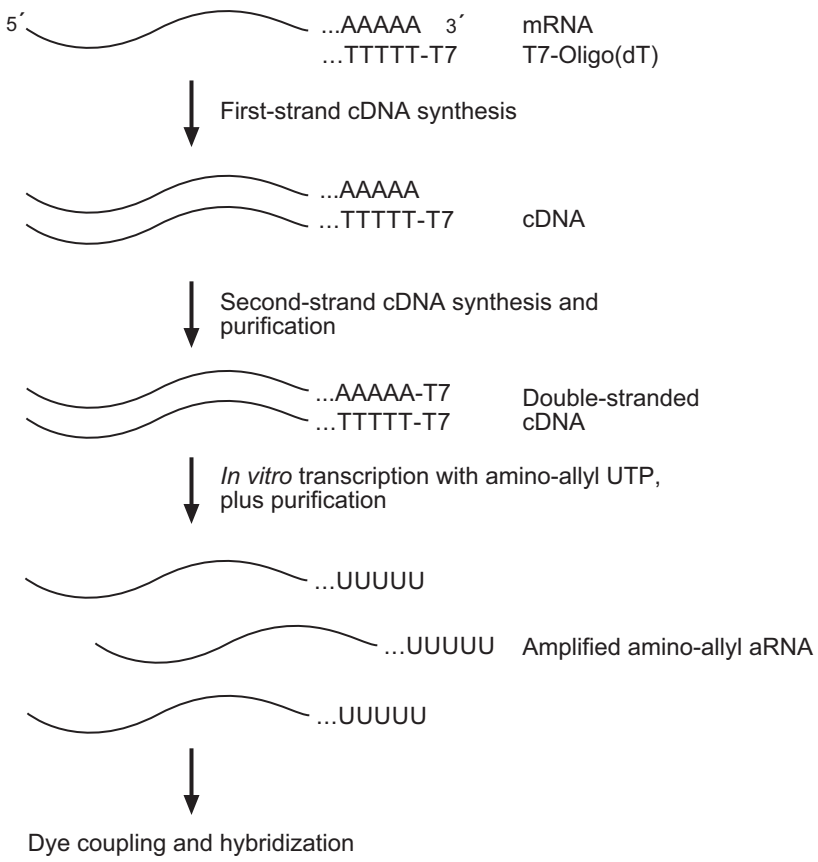
SuperScript® III Reverse Transcriptase is an engineered version of M-MLV RT with reduced RNase H activity and increased thermal stability. The enzyme can be used to synthesize first-strand cDNA from total RNA or mRNA at temperatures up to 55°C, providing increased specificity, higher yields of cDNA, and more full-length product than other reverse transcriptases.

The SuperScript® III RT in this kit is provided at an optimal concentration and used at an optimal temperature for first-strand cDNA synthesis.

Continued on next page

Overview, continued

Experimental Outline



T7-Oligo(dT)

The T7-Oligo(dT) primer consists of a bacteriophage T7 polymerase promoter sequence followed by a string of deoxythymidylic acid (dT) residues followed by a nucleotide "anchor" that allows each primer to anneal only at the 5′ end of the poly(A) tail of mRNA, providing more efficient cDNA synthesis. The sequence of the anchor varies among the primers in the mixture to allow binding to different template sequences.

Control RNA

Control HeLa RNA is included in the kit to help you determine the efficiency of the amplification procedure. We recommend that you perform the complete procedure using the control HeLa RNA if you are a first-time user of the system.

Methods for determining the aRNA yield and quality from the control HeLa RNA are provided on pages 13–18.

Continued on next page

Materials Supplied by the User

In addition to the kit components, you should have the following items on hand before using this kit.

- 100–5000 ng of total RNA or 5–250 ng of poly(A) RNA. (For optimal efficiency, use ≤1000 ng of total RNA.) Note that this kit has been optimized for use with total RNA, and purification of poly(A) RNA is not required in most cases.
- Fluorescent dyes (see section immediately following)
- Vortex mixer
- Microcentrifuge
- Speed-vac concentrator
- Spectrophotometer
- Recommended: Agilent 2100 bioanalyzer and RNA 6000 LabChip® Kit (for analyzing starting material and final aRNA product)
- *Optional:* Denaturing agarose gel (for analyzing starting material) and 1.2% agarose gel (for analyzing final aRNA product)
- Aerosol resistant pipette tips
- Air incubator or thermal cycler
- Refrigerated water bath
- 1.5-mL RNase-free microcentrifuge tubes
- Ice
- 100% Isopropanol
- 100% Ethanol

Fluorescent Dyes

This kit has been developed and tested with Alexa Fluor® dyes from Life Technologies (see page 21 for ordering information) and CyDyes™ from Amersham Biosciences. Alexa Fluor® 555 and Alexa Fluor® 647 dyes are compatible with commonly used microarray scanners, and provide greater signal correlation (R^2) values than the spectrally similar Cy™3 and Cy™5 dye pair, improving the resolution of two-color microarray gene expression assays. The exceptionally bright Alexa Fluor® dyes are also insensitive to pH and are highly water-soluble.

The table below shows the excitation and emission maxima and color of each Alexa Fluor® dye:

Dye	Excitation/Emission (nm)	Color
Alexa Fluor® 555	555/565	Orange Fluorescent
Alexa Fluor® 647	650/670	Far-Red Fluorescent

Methods

Isolating RNA

Introduction

High-quality, intact RNA is essential for full-length, high-quality cDNA synthesis. In this step, you isolate total RNA or poly(A) RNA using a method of choice.

Note

This kit has been optimized for use with total RNA, and purification of poly(A) RNA is not required in most cases.

Important

The quality of the RNA is **critical** for RNA amplification. In labeling and array hybridization applications, the presence of contaminants in the RNA may significantly increase background fluorescence in the microarrays. Carefully follow the recommendations below to prevent contamination.

General Handling of RNA

When working with RNA:

- Use disposable, individually wrapped, sterile plasticware.
- Use aerosol resistant pipette tips for all procedures.
- Use only sterile, new pipette tips and microcentrifuge tubes.
- Wear latex gloves while handling reagents and RNA samples to prevent RNase contamination from the surface of the skin.
- Use proper microbiological aseptic technique when working with RNA.
- Dedicate a separate set of pipettes, buffers, and enzymes for RNA work.
- Microcentrifuge tubes can be taken from an unopened box, autoclaved, and used for all RNA work. RNase-free microcentrifuge tubes are available from several suppliers. If it is necessary to decontaminate untreated tubes, soak the tubes overnight in a 0.01% (v/v) aqueous solution of diethylpyrocarbonate (DEPC), rinse the tubes with sterile distilled water, and autoclave the tubes.

You can use RNase *Away*™ Reagent, a non-toxic solution available from Life Technologies (see page 21), to remove RNase contamination from surfaces. For further information on controlling RNase contamination, see Ausubel, et al., 1994, and Sambrook, et al., 1989.

Amount of RNA

This system is designed for use with 100–5000 ng of total RNA or 5–250 ng of purified poly(A) RNA. For optimal efficiency, use ≤1000 ng of total RNA. Larger amounts of starting material may lead to a decrease in amplification efficiency, while smaller amounts may result in a decrease in amplification specificity.

Continued on next page

Isolating RNA

To isolate total RNA, we recommend TRIzol® Reagent (Chirgwin et al., 1979; Chomczynski and Sacchi, 1987), the Concert™ 96 RNA Purification System (for high-throughput purification), or the Micro-to-Midi Total RNA Purification System. To isolate mRNA, we recommend the Micro-FastTrack™ 2.0 or FastTrack® 2.0 mRNA Isolation Kits. Ordering information is provided on page 21.

After you have isolated the RNA, check the quality of your RNA preparation as described in **Checking the RNA Quality**.

Checking the RNA Quality

We recommend checking the quality of the RNA preparation using the Agilent 2100 bioanalyzer and an RNA 6000 LabChip® Kit, which is ideal for analyzing small quantities of RNA. You may also use agarose/ethidium bromide gel electrophoresis for larger quantities.

The Agilent 2100 bioanalyzer and RNA 6000 LabChip® Kit are suitable for analyzing very small quantities of RNA (as low as 200 pg). In the bioanalyzer graph for total human RNA, the 28S rRNA peak should be approximately twice the size of the 18S rRNA peak. If the peaks appear similar in size or if additional peaks appear on the graph, the RNA may be degraded.

Agarose Gel Electrophoresis is suitable for analyzing larger amounts of RNA (>500 ng total RNA). You can use a 1% agarose gel or a denaturing agarose gel (Ausubel et al., 1994). For total human RNA using a regular agarose gel, mRNA will appear as a smear from 0.5 to 9 kb, and 28S and 18S rRNA will appear as bands at 4.5 kb and 1.9 kb, respectively. The 28S band should be twice the intensity of the 18S band. If you are using a denaturing gel, the rRNA bands should be very clear and sharp.

If you do not load enough RNA, the 28S band may appear to be diffuse. A smear of RNA or a lower intensity 28S band with an accumulation of low molecular weight RNA on the gel are indications that the RNA may be degraded.

If you have problems with RNA quality, refer to **Troubleshooting** on page 19.

Storing RNA

After preparing the RNA, we recommend that you proceed directly to **First-Strand cDNA Synthesis** on page 6. Otherwise, store the RNA at –80°C.

cDNA Synthesis

Introduction

After you have isolated RNA and checked the quality of your RNA preparation, you are ready to synthesize double-stranded cDNA.

Before Starting

The following items are supplied by the user:

- 100–5000 ng of total RNA or 5–250 ng of poly(A) RNA (for optimal efficiency, use ≤1000 ng of total RNA)
- Vortex mixer
- Incubator or thermal cycler set at 46°C and 70°C
- Refrigerated water bath set at 16°C
- 1.5-mL RNase-free microcentrifuge tubes
- Ice

The following items are supplied in the Core Module of the kit:

- T7-Oligo(dT) primer
- 10 mM dNTP Mix
- 5X First-Strand Buffer
- 0.1 M DTT
- RNaseOUT™
- SuperScript® III RT
- *E. coli* DNA Polymerase I
- *E. coli* DNA Ligase
- 5X Second-Strand Buffer
- DEPC-treated water
- *E. coli* RNase H
- Control HeLa RNA; optional, see page 2

Note

For optimal results, the reagents used in second-strand cDNA synthesis should be ice-cold when they are added to the reaction tube.

RNaseOUT™ Recombinant RNase Inhibitor

RNaseOUT™ Recombinant RNase Inhibitor has been included in the system to safeguard against degradation of target RNA due to ribonuclease contamination of the RNA preparation.

Continued on next page

First-Strand cDNA Synthesis

The following procedure is for a single reaction. For multiple reactions, prepare a master mix with a 5–10% overage to enable accurate pipetting.

Note: If you are setting up a control reaction (recommended for first-time users), use 1 µL of the Control HeLa RNA supplied in the kit (500 ng/µL).

1. Mix and briefly centrifuge each component before use. In a 1.5-mL RNase-free tube, add the following:

Component	Sample
100–5000 ng of total RNA or 5–250 ng of mRNA*	≤9 µL
T7-Oligo(dT) Primer	1 µL
DEPC-treated water	to 10 µL

   *For optimal efficiency, use ≤1000 ng of total RNA. For the control reaction, use 1 µL of the supplied Control HeLa RNA (500 ng/µL).

2. Incubate the tube at 70°C for 10 minutes, and then place on ice for at least 1 minute.

3. Centrifuge the tube briefly to collect the contents, and add the following to the tube at room temperature:

Component	Volume
5X First-Strand buffer	4 µL
0.1 M DTT	2 µL
10 mM dNTP Mix	1 µL
RNaseOUT™ (40 U/µL)	1 µL
SuperScript® III RT (200 U/µL)	2 µL
Total Reaction Volume	20 µL

4. Mix gently and then centrifuge the tube briefly to collect the contents. Incubate the tube at 46°C for 2 hours.

5. Incubate the tube at 70°C for 10 minutes to inactivate the reverse transcriptase.

6. Centrifuge the tube briefly to collect the contents and place the tube on ice.

After incubation, proceed immediately to **Second-Strand cDNA Synthesis**, page 8.

Continued on next page

Second-Strand cDNA Synthesis

After first-strand synthesis, immediately perform the following second-strand synthesis reaction to generate double-stranded cDNA.

Note: The following procedure is for a single reaction. For multiple reactions, prepare a master mix with a 5–10% overage to enable accurate pipetting. For optimal results, reagents should be ice-cold when they are added to the reaction.

1. Add the following components to the reaction tube from step 6, previous page, on ice:

Component	Sample
DEPC-treated water	91 µL
5X Second-Strand Buffer	30 µL
10 mM dNTP Mix	3 µL
E. coli DNA Polymerase I (10 units/µL)	4 µL
E. coli DNA Ligase (10 units/µL)	1 µL
E. coli RNase H (2 units/µL)	1 µL
Total Volume	150 µL

2. Mix the contents gently by pipetting up and down.
3. Incubate the reaction mixture at 16°C for 2 hours. After incubation, place the tubes on ice.

The double-stranded cDNA can be stored at –20°C until you are ready to perform the rest of the procedure. Proceed to **cDNA Purification** on page 9.

cDNA Purification

Introduction

In this step, you purify the double-stranded cDNA using the spin columns provided in the kit.

Before Starting

The following items are supplied by the user:

- Microcentrifuge

The following items are supplied in the cDNA Purification Module:

- Low-Elution Volume Spin Cartridges, preinserted into collection tubes
- Recovery Tubes
- cDNA Loading Buffer (prepared with isopropanol as described on page v)
- cDNA Wash Buffer (prepared with ethanol as described on page v)
- DEPC-treated water

Purification Procedure

Use the following procedure to purify the cDNA.

1. Add 500 µL of cDNA Loading Buffer (prepared with isopropanol as described on page v) to the reaction tube from **Second-Strand cDNA Synthesis**, Step 3, page 8. The total volume in the tube should be 650 µL. Mix thoroughly by pipetting up and down.
2. Each Low-Elution Volume Spin Cartridge is preinserted into a collection tube. For multiple reactions, clearly label each collection tube, and then load the cDNA/buffer solution from Step 1 directly onto the Spin Column.
3. Centrifuge at 6,000 × g at room temperature in a microcentrifuge for 1 minute. Remove the collection tube and discard the flow-through.
4. Place the Spin Cartridge in the same collection tube and add 700 µL of cDNA Wash Buffer (prepared with ethanol as described on page v) to the column.
5. Centrifuge at 6,000 × g at room temperature for 1 minute. Remove the collection tube and discard the flow-through.
6. Place the Spin Cartridge in the same collection tube and centrifuge at 6,000 × *g* at room temperature for an additional 2 minutes to remove any residual Wash Buffer. Remove the collection tube and discard.
7. Place the Spin Cartridge into a new Recovery Tube (supplied in the kit).
8. Add 24 µL of DEPC-treated water to the center of the Spin Cartridge and incubate at room temperature for 2 minutes.
9. Centrifuge at 10,000 × *g* at room temperature for 1 minute to collect the purified cDNA. **The eluate contains the purified cDNA.**

Proceed to ***In Vitro*** **Transcription**, page 10.

In Vitro Transcription

Introduction

In this step, you generate aRNA from the double-stranded cDNA using T7 RNA Polymerase and amino-allyl UTP.

Before Starting

The following items are supplied by the user:

- Microcentrifuge
- Air incubator or thermal cycler set at 37°C (heat block is not recommended)

The following items are supplied in the Core Module of the kit:

- DEPC-treated water
- T7 Enzyme Mix
- 10X T7 Reaction Buffer
- DNase I (optional)
- 100 mM ATP
- 100 mM CTP
- 100 mM GTP
- 100 mM UTP
- 50 mM aa-UTP

Note

The yield of aRNA will increase with longer *in vitro* transcription incubation times, up to 14 hours. For maximum yield, an incubation time of at least 12 hours is recommended.

In Vitro Transcription Procedure

The following procedure is for a single reaction. For multiple reactions, prepare a master mix with a 5–10% overage to enable accurate pipetting.

1. The tube from Step 9, page 9, should contain ~22 µL of cDNA. Add the following, at room temperature:

Component	Volume
100 mM ATP	1.5 µL
100 mM CTP	1.5 µL
100 mM GTP	1.5 µL
100 mM UTP	0.75 µL
50 mM aa-UTP	2 µL
10X T7 Reaction Buffer	4 µL
T7 Enzyme Mix	7 µL
Total Reaction Volume	~40 µL

2. Gently mix and centrifuge briefly to collect the contents of the tube.
3. Incubate the tube at 37°C for 6–14 hours (incubate overnight for maximum yield).
4. **Optional:** Add 2 µL of DNase I to the tube. Gently mix and centrifuge briefly to collect the contents of the tube, and then incubate at 37°C for 30 minutes.

Proceed to **aRNA Purification**, page 11.

aRNA Purification

Introduction

In this step, you purify the aRNA or the dye-labeled aRNA.

Note

Sufficient components are provided for 20 purifications of aRNA ***and*** 20 purifications of dye-labeled aRNA.

Before Starting

The following items are supplied by the user:

- Microcentrifuge
- 100% ethanol

The following items are supplied in the aRNA Purification Module:

- PureLink® Spin Columns, preinserted into collection tubes
- Recovery Tubes
- aRNA Binding Buffer
- aRNA Wash Buffer (prepared with ethanol as described on page vi)
- DEPC-treated water

Purification Procedure

Use the following procedure to purify the aRNA from Step 4, page 10, or the dye-labeled aRNA from page 15.

1. Add aRNA Binding Buffer to the aRNA to bring the total volume to 200 µL. Add ~160 µL of buffer for aRNA (from page 10), or ~180 µL of buffer for dye-labeled aRNA (from page 15). Mix thoroughly by pipetting up and down.
2. Add 100 µL of 100% ethanol to the reaction tube. Mix thoroughly by pipetting up and down.
3. Each PureLink® Spin Column is preinserted into a collection tube. For multiple reactions, clearly label each collection tube, and then load the entire aRNA/buffer solution directly onto the Spin Column.
4. Centrifuge at 12,000 × g in a microcentrifuge for 15 seconds at room temperature. Remove the collection tube and discard the flow-through.
5. Place the Spin Column in the same collection tube and add 500 µL of aRNA Wash Buffer (prepared with ethanol as described on page vi) to the column.
6. Centrifuge at 12,000 × g for 15 seconds at room temperature. Remove the collection tube and discard the flow-through.
7. Repeat Steps 5–6.

Procedure continued on the next page

Continued on next page

Purification Procedure, continued

Procedure continued from the previous page

8. Place the Spin Column in the same collection tube and centrifuge at full speed for an additional 2 minutes to remove any residual Wash Buffer. Remove the collection tube and discard.
9. Place the Spin Column into a new collection tube (supplied in the kit).
10. Add 100 µL of DEPC-treated water to the center of the Spin Cartridge and incubate at room temperature for 1 minute.
11. Centrifuge at 12,000 × g for 2 minutes to collect the purified aRNA. **The eluate contains the purified aRNA.**

Prior to fluorescent labeling, calculate the yield of the aRNA as described in **Determining Yield** on page 13. Then proceed to **Labeling with Fluorescent Dye,** page 14. Alternatively, store the sample at –80° C.

Determining Yield

Determining Yield Using an RNA Quantitation Kit

You can use the Quant-iT™ RNA Assay Kit (Catalog no. Q-33140) or the RiboGreen® RNA Quantitation Kit (Catalog no. R-11490) for highly sensitive quantitation of small amounts of RNA using a fluorescence microplate reader.

See the product information sheet for each kit for detailed protocols. Use 1 µL of the purified aRNA from Step 11, page 12, in the quantitation reaction.

Determining Yield Using A_{260} Absorbance

The following general protocol may be used to calculate the yield of the aRNA using A_{260} absorbance:

1. Aliquot 1 µL of the purified aRNA from Step 11, page 12, into a clean cuvette and dilute it 1:10 to 1:100 using DEPC-treated water. As a general guideline, begin by diluting the aRNA sample 1:10 if you used ~100 ng of total RNA starting material and 1:100 if you used ~500 ng total RNA starting material.
2. Scan the sample at 260 nm using a UV/visible spectrophotometer. Be sure to blank the spectrophotometer using DEPC-treated water before the reading.

Note: The A_{260} reading should fall within the standard specification for the spectrophotometer (typically 0.1–1.0 OD). If it falls outside this range, adjust the dilution and re-scan. If the A_{260} reading is too low, use a lower dilution; if it's too high, use a higher dilution.

3. Transfer the sample back into the Recovery Tube for storage.
4. Calculate the yield of aRNA using the formula below:

   Total aRNA yield (µg/mL) = A_{260} × 40 µg/mL RNA × dilution factor × elution volume

   For example, if you diluted 1 µL of a 100 µL volume of aRNA at 1:50, and the A_{260} is 0.5, then 0.5 × 40 µg/mL RNA × 50 = 1000 µg/mL. In a 100 µL volume you would have 100 µg of aRNA.

Expected Yield for Control HeLa RNA

The expected yield of aRNA using the Control HeLa RNA (500 ng) and a 14-hour *in vitro* transcription reaction is >20 µg. If you do not achieve this yield for the control reaction, see **Troubleshooting** on page 19.

Labeling with Fluorescent Dye

Introduction

After you have purified the aRNA and determined the yield, you are ready to label the amino-allyl aRNA with a fluorescent dye of your choice. Labeling protocols are provided in this section for Alexa Fluor® dyes and CyDyes™.

Before Starting

The following items are supplied by the user:

- Microcentrifuge
- Speed-vac
- Vortex mixer
- Alexa Fluor® 555 Reactive Dye Decapack (Catalog no. A32756) /
  Alexa Fluor® 647 Reactive Dye Decapack (Catalog no. A32757) /
  Alexa Fluor® 555 and Alexa Fluor® 647 Reactive Dye Decapacks (Catalog no. A32755)

  or

  CyDye™ Post-Labeling Reactive Dye Pack (12 vials each Cy™3 and Cy™5) (Amersham Biosciences, #RPN 5661) /
  Cy™3 Mono-Reactive Dye Pack (Amersham Biosciences, #PA 23001) /
  Cy™5 Mono-Reactive Dye Pack (Amersham Biosciences, #PA 25001)

The following items are provided in the Core Module of the kit:

- 2X Coupling Buffer

Important

- Fluorescent dyes are sensitive to photobleaching. When preparing the reaction, be careful to minimize exposure of the dye solution to light. The dye coupling reaction must be incubated in the dark.
- When drying the sample down to ≤3 µL, be careful not to completely evaporate the sample. Overdrying may result in sample loss.

DMSO is hygroscopic and will absorb moisture from the air. Water absorbed from the air will react with the NHS ester of the dye and significantly reduce the coupling reaction efficiency. Keep the DMSO supplied in the kit in an amber screw-capped vial at -20°C, and let the vial warm to room temperature before opening to prevent condensation.

Note

Before proceeding with dye labeling, first calculate the yield of aRNA as described on page 13. Use that yield calculation to determine the volume that contains 5 µg of aRNA.

Continued on next page

Labeling Reaction with Alexa Fluor® Dyes

After you have calculated the yield of aRNA as described on page 13, follow the steps below to couple Alexa Fluor® dye to 5 µg of purified amino-allyl aRNA.

1. Aliquot a volume from Step 11, page 12 containing 5 µg of aRNA into a 1.5-mL RNase-free microcentrifuge tube and place in a speed-vac. Evaporate at low heat until the sample volume is reduced to ≤3 µL. **Be careful not to completely evaporate the sample. Overdrying may result in sample loss.**
2. Add 11 µL of DMSO directly to a vial of Alexa Fluor® Reactive Dye to resuspend the dye. Vortex thoroughly and then spin briefly to collect the contents.
3. Add 8 µL of 2X Coupling Buffer to the aRNA tube from Step 1 above.
4. Add the resuspended dye solution to the tube and vortex to mix thoroughly.
5. Incubate the tube at room temperature in the dark for 30 minutes.
6. Purify the dye-coupled aRNA using the aRNA purification protocol on page 11. Be careful to minimize exposure of the reaction to light. Collect the purified dye-labeled sample in an amber collection tube provided in the kit.

To calculate the amount of coupled dye, see page 17.

Labeling Reaction with CyDyes™

Follow the steps below to couple CyDye™ to the purified aRNA.

1. Aliquot a volume from Step 11, page 12, containing 5 µg of aRNA into a 1.5-mL RNase-free microcentrifuge tube and place in a speed-vac. Evaporate at low heat until the sample volume is reduced to ≤1 µL. Do not dry the sample completely, as it may be difficult to resuspend.
2. Prepare the Cy™3 or Cy™5 dye as follows:
   Individual reaction size (RPN 5661): Add 5 µL DMSO directly to each dye vial.
   Large size (PA 23001 and PA 25001): Add 45 µL DMSO directly to each dye vial.
3. Add 5 µL of 2X Coupling Buffer to the aRNA tube from Step 1 above.
4. Add 4 µL of the dye solution to the tube, vortex thoroughly to mix, and centrifuge briefly to collect the contents of the tube.
5. Incubate the tube at room temperature in the dark for 30 minutes. Store any unused dye solution according to manufacturer's directions.
6. Purify the dye-coupled aRNA as described in **aRNA Purification,** page 11. Be careful to minimize exposure of the reaction to light. Collect the purified dye-labeled sample in an amber collection tube provided in the kit.

After purification, you can proceed to array hybridization. To calculate the amount of coupled dye, see page 17.

Hybridization

Hybridization

After purification, you are ready to use the dye-coupled aRNA in any application of choice, including glass microarray hybridization. Follow the preparation and hybridization instructions for your specific application.

Calculating Amount of Coupled Dye

Introduction

After dye coupling (page 14) and purification to remove the uncoupled dye (page 11), use the formulas below to calculate the amount of coupled dye. (Formulas are provided for Alexa Fluor® dyes and CyDyes™).

Calculating the Amount of Coupled Dye

The following table shows the absorbance and baseline wavelengths for Alexa Fluor® dyes and CyDyes™:

Dye	Absorbance Wavelength	Baseline Wavelength
Alexa Fluor® 555	555 nm	650 nm
Alexa Fluor® 647	650 nm	750 nm
Cy™3	550 nm	650 nm
Cy™5	650 nm	750 nm

To calculate the amount of coupled dye:

1. Transfer a volume of purified, dye-coupled aRNA from Step 11, page 12, to a clean cuvette. Use an appropriate volume for your spectrophotometer. If you are using a 100-µL cuvette, transfer the entire sample; for smaller cuvettes, transfer an appropriate amount of sample.

   Note: The labeled aRNA must be purified as described on pages 11– 12 before scanning, as any unreacted dye will interfere with the detection of labeled aRNA.

2. Blank the spectrophotometer using DEPC-treated water, and then scan the sample at 240–800 nm. Wash each cuvette thoroughly between samples.

3. Calculate the amount of fluorescent dye using the following formulas:

   Alexa Fluor® 555 (pmole) = $(A_{555}–A_{650})/0.15 \times$ volume in µL

   Alexa Fluor® 647 (pmole) = $(A_{650}–A_{750})/0.24 \times$ volume in µL

   Cy™3 (pmole) = $(A_{550}–A_{650})/0.15 \times$ volume in µL

   Cy™5 (pmole) = $(A_{650}–A_{750})/0.25 \times$ volume in µL

4. Calculate the base-to-dye ratio using the following formulas:

   Base/dye ratio for Alexa Fluor® 555 =
   $\{(A_{260} – A_{320}) – [(A_{555} – A_{650}) \times 0.04]\} \times 150{,}000/(A_{555} – A_{650}) \times 6{,}600$

   Base/dye ratio for Alexa Fluor® 647 =
   $\{(A_{260} – A_{320}) – [(A_{650} – A_{750}) \times 0]\} \times 239{,}000/(A_{650} – A_{750}) \times 6{,}600$

   Base/dye ratio for Cy™3 =
   $\{(A_{260} – A_{320}) – [(A_{550} – A_{650}) \times 0.08]\} \times 150{,}000/(A_{550} – A_{650}) \times 6{,}600$

   Base/dye ratio for Cy™5 =
   $\{(A_{260} – A_{320}) – [(A_{650} – A_{750}) \times 0.05]\} \times 250{,}000/(A_{650} – A_{750}) \times 6{,}600$

Appendix

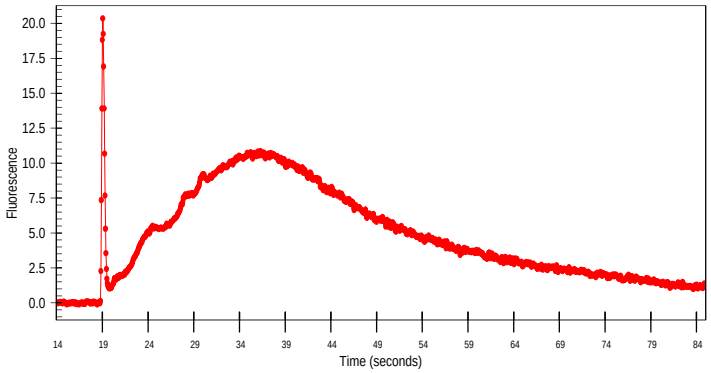
Determining aRNA Quality

Determining aRNA Quality Using the Agilent 2100 Bioanalyzer

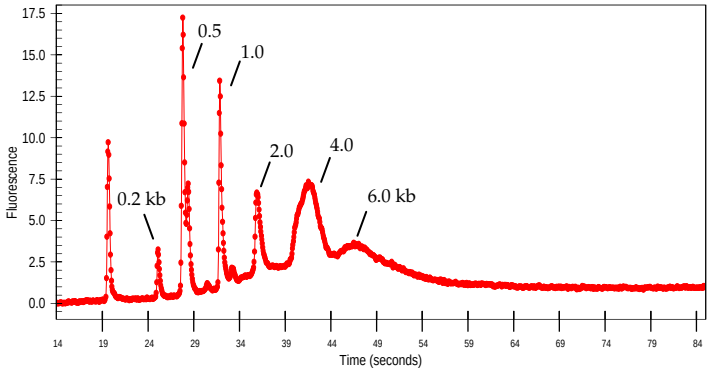
You can use the Agilent 2100 bioanalyzer with the RNA 6000 LabChip® Kit to analyze the aRNA sample. We do not recommend using the bioanalyzer to determine aRNA quantity (see page 13 for recommended quantitation methods).

To analyze samples using the bioanalyzer, use 1 µL of the purified aRNA from Step 11, page 12. The bioanalyzer graph for a typical amplification reaction will shown a population of aRNAs ranging from 0.2 kb to 4 kb, with a peak between 1 kb and 2 kb (average aRNA size >1 kb). An example is shown below, and an RNA ladder is provided for size comparison.

aRNA:



RNA 6000 Ladder (from Ambion, for use with the RNA 6000 LabChip® kits)**:**



Troubleshooting

Observation	Cause	Solution
28S and 18S bands are not observed after isolation of total RNA and agarose gel electrophoresis	Too little RNA loaded on the gel	To analyze total RNA by gel electrophoresis, you need at least 250 ng of RNA.
	RNA is degraded due to RNase activity	Follow the guidelines on page 4 to avoid RNase contamination. Use a fresh sample for RNA isolation.
28S band is diminished or low molecular weight RNA appears in the gel	RNA is degraded	Follow the guidelines on page 4 to avoid RNase contamination. Use a fresh sample for RNA isolation.
Yield of aRNA from the control reaction is low	Incubation temperatures were incorrect	Check the incubation temperatures of all the reactions.
	Incorrect reaction conditions used	Verify that all reaction components are included in the reaction and use reagents provided in the system. Verify the reaction conditions using the Control HeLa RNA provided in the kit.
	Condensation formed in the reaction tubes	If condensation forms inside the tubes during incubation, spin the tube briefly to remix the components, and perform the reaction in a different incubator (air incubation is recommended).
	Poor quality RNA used or RNA is degraded	Check the quality of your RNA preparation (see page 5). If RNA is degraded, use fresh RNA.
	RNase contamination	Use the RNaseOUT™ included in the kit to prevent RNA degradation.
	RT enzyme inhibitors are present in your RNA sample	Inhibitors of RT enzymes include SDS, EDTA, guanidinium chloride, formamide, sodium phosphate and spermidine (Gerard, 1994). Test for the presence of inhibitors by mixing 1 µg of Control HeLa RNA with 25 µg total RNA or 1 µg mRNA and compare the yields of aRNA amplification.
	Improper storage of SuperScript® III RT	Store the enzyme at -20°C.
	Reagents were not properly mixed before first-strand synthesis.	Repeat the procedure, being careful to briefly vortex and centrifuge each reagent before first-strand cDNA synthesis.

Continued on next page

Observation	Cause	Solution
Average aRNA size is <500 nucleotides for both sample RNA and control RNA reactions	Incubation temperatures were incorrect	Check the incubation temperatures of all the reactions.
	RNase contamination	Use the RNaseOUT™ included in the kit to prevent RNA degradation.
	Problem with gel analysis of aRNA	Improperly formulated agarose gels may provide inaccurate estimates of aRNA size. Analyze an RNA ladder in an adjacent lane to confirm the size of the aRNA products.
Average aRNA size is <500 nucleotides for sample RNA, but is >1 kb for control RNA reaction	Poor quality RNA used or sample RNA is degraded	Check the quality of your RNA preparation (see page 5). If RNA is degraded, use fresh RNA.
	Inefficient labeling due to improper purification	Follow all purification steps carefully and without modification.
	Starting amount of RNA is too low	Increase the amount of starting RNA.

Accessory Products

Additional Products

Many of the reagents in the SuperScript® Indirect RNA Amplification System, as well as additional reagents that may be used with this system, are available separately. Ordering information is provided below.

Product	Quantity	Catalog no.
Alexa Fluor® 555 reactive dye decapack	10 vials	A32756
Alexa Fluor® 647 reactive dye decapack	10 vials	A32757
Alexa Fluor® 555 and Alexa Fluor® 647 reactive dye decapacks	2 × 10 vials	A32755
RNase *Away*™ Reagent	250 mL	10328-011
TRIzol® Reagent	100 mL 200 mL	15596-026 15596-018
Concert™ 96 RNA Purification System	384 reactions	12173-011
Micro-to-Midi Total RNA Purification System	50 reactions	12183-018
FastTrack® 2.0 mRNA Isolation Kit	6 reactions 18 reactions	K1593-02 K1593-03
Quant-iT™ RNA Assay Kit	1000 reactions	Q-33140
RiboGreen® RNA Quantitation Kit	200–2000 reactions	R-11490
RNaseOUT™ Recombinant Ribonuclease Inhibitor	5000 units	10777-019
1.2% E-Gel® Starter Pack	6 gels + base	G6000-01
UltraPure™ DEPC-treated water	4 × 1.25 mL	10813-012

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