invitrogen™

SuperScript™ RNA Amplification System

For generating amplified mRNA from small starting quantities of RNA

Catalog nos. L1016-01

Version E
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User Manual

Table of Contents

Kit Contents and Storage

Shipping and Storage

The SuperScript™ RNA Amplification System is shipped in two modules. The Core Module is shipped on dry ice, while the Purification Module is shipped at room temperature. Upon receipt, store the components of the Core Module at -20ºC and store the components of the Purification Module at room temperature.

Core Module

The Core Module includes enough reagents for 20 reactions. The components of the Core Module should be stored at -20ºC.

Component	Description	Amount
SuperScript™ III Reverse Transcriptase	200 U/µl	40 µl
5X First-Strand Buffer	250 mM Tris-HCl (pH 8.3, room temp), 375 mM KCl, 15 mM $MgCl_2$	90 µl
Dithiothreitol (DTT)	0.1 M DTT in water	50 µl
10 mM dNTP Mix	dATP, dGTP, dCTP, and dTTP in DEPC-treated water	80 µl
T7 Oligo(dT) primer	In DEPC-treated water	20 µl
RNaseOUT™ Recombinant Ribonuclease Inhibitor	40 U/µl	30 µl
Control HeLa RNA	500 ng/µl in HE buffer	20 µg
E. coli DNA Polymerase	10 U/µl	90 µl
E. coli DNA Ligase	10 U/µl	25 µl
E. coli RNase H	2 U/µl	25 µl
5X Second-Strand Reaction Buffer	100 mM Tris-HCl (pH 6.9), 450 mM KCl, 23 mM $MgCl_2$, 50 mM $(NH_4)_2SO_4$, stabilizers	700 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
DNase I	Amplification grade, 1 U/µl	40 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	30 µl

Continued on next page

Kit Contents and Storage, continued

Purification Module

The Purification Module includes enough reagents and columns for 20 reactions. The components of the Purification Module should be stored at room temperature

Item	Amount
cDNA Loading Buffer (you must add 100% isopropanol to create the final buffer; see below)	9 ml
cDNA Wash Buffer (you must add 100% ethanol to create the final buffer; see below)	4 ml
aRNA Binding Buffer (no additional preparation is necessary)	4.5 ml
aRNA Wash Buffer (you must add 100% ethanol to create the final buffer; see below)	7 ml
DEPC-treated Water	5 ml
Spin Cartridges	42
Recovery Tubes	42

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 six 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.

Component	Amount
cDNA Loading Buffer	9 ml (entire bottle)
100% Isopropanol	3 ml
Final 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 six 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
Final Volume	16 ml

Continued on next page

Preparing aRNA Wash Buffer with Ethanol

The aRNA Wash Buffer must be mixed with 100% ethanol prior to use. The aRNA Wash Buffer plus ethanol is stable for at least six 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
Final Volume	28 ml

Accessory Products

Additional Products

The following related products are available separately from Invitrogen. To order, visit www.invitrogen.com or contact Technical Support (see page 21).

Product	Quantity	Catalog no.
SuperScript™ Indirect RNA Amplification System	20 reactions	L1016-02
RNase *Away*™ Reagent	250 ml	10328-011
PureLink™ Micro-to-Midi Total RNA Purification System	50 reactions	12183-018
PureLink™ 96 RNA Purification System	384 reactions	12173-011
TRIzol® Reagent	100 ml 200 ml	15596-026 15596-018
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 and base	G6000-01
UltraPure™ DEPC-treated water	4 × 1.25 ml	10813-012

Overview

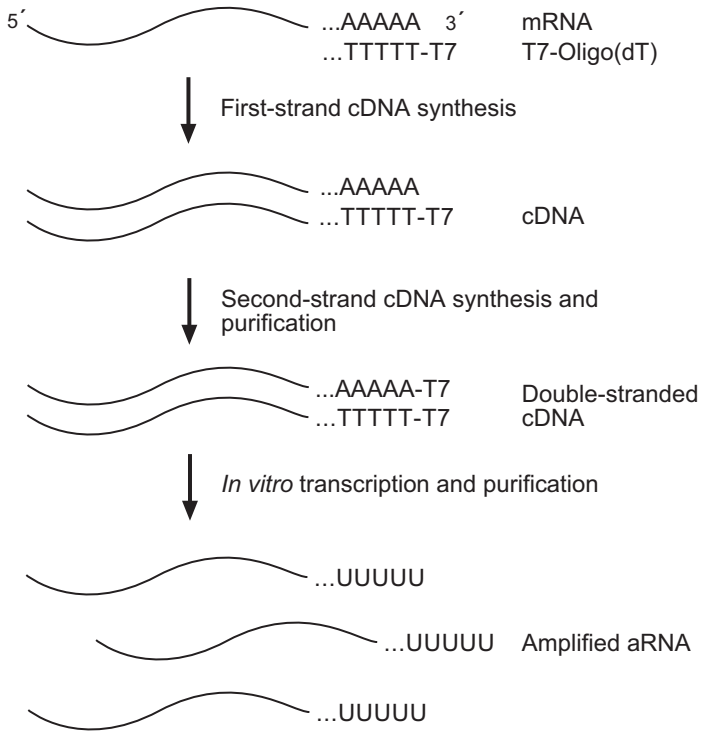
Introduction

The SuperScript™ 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 transcribes antisense RNA (aRNA) molecules complementary to the original mRNA targets. The amplified aRNA is then ready to use in applications such as gene expression profiling.

Amplified RNA 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 has been optimized for use with 100–5000 ng of total RNA or 5–250 ng of poly(A) RNA as starting material. The amplified unlabeled RNA can be subsequently labeled by reverse transcription. Alternatively, amino-allyl-modified nucleotides can be incorporated into the RNA in the *in vitro* transcription reaction for subsequent labeling and detection.

Experimental Outline



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Overview, continued

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 complete representation of the mRNA population from very small amounts of starting material
- System generates aRNA with a greater average length than comparable kits.
- System includes all major reagents and materials for preparing amplified RNA

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 55ºC, 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.

T7-Oligo(dT)

T7-Oligo(dT) primer is a mixture of primers, each consisting 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 14–15.

Continued on next page

Overview, continued

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. Note that this kit has been optimized for use with total RNA, and purification of poly(A) RNA is not required in most cases.
- Amino-allyl-modified nucleotides (**optional** for use in the *in vitro* transcription reaction)
- 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

Product Qualification

This kit was verified using 500 ng of total HeLa RNA in a standard amplification reaction as described in this manual. After purification, the amount of aRNA was calculated using A_{260} absorbance as described on page 14. The quality of the aRNA was verified using agarose gel electrophoresis. The representation of a group of housekeeping genes of various expression levels was confirmed using the aRNA in quantitative RT-PCR with Certified LUX™ Primer Sets.

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. 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 Invitrogen (see page 19), 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 optimized for use with 100–5000 ng of total RNA or 5–250 ng of purified poly(A) 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, continued

Isolating RNA

To isolate total RNA, we recommend the PureLink™ Micro-to-Midi Total RNA Purification System, TRIzol® Reagent, or (for high-throughput applications) the PureLink™ 96 RNA Purification System. To isolate mRNA, we recommend the FastTrack® 2.0 mRNA Isolation Kits or the FastTrack® MAG mRNA Isolation Kits. Ordering information is provided on page 19.

After you have isolated the RNA, check the quality of your RNA preparation as described on the following page.

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 16.

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 –80ºC.

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
- Vortex mixer
- Incubator or thermal cycler set at 46ºC and 70ºC
- Refrigerated water bath set at 16ºC
- 1.5-ml RNase-free microcentrifuge tubes
- Ice

The following items are supplied in the kit:

- T7-Oligo(dT) primer
- 10 mM dNTP Mix
- 5X First-Strand Buffer
- 0.1 M DTT
- RNaseOUT™
- SuperScript™ III RT
- DNA Polymerase I
- DNA Ligase
- 5X Second-Strand Buffer
- DEPC-treated water
- 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

cDNA Synthesis, continued

First-Strand cDNA Synthesis

The following procedure is designed to convert 100–5000 ng of total RNA or 5–250 ng of purified poly(A) RNA into first-strand 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.

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 the control reaction, use 1 µl of the supplied Control HeLa RNA (500 ng/µl).

2. Incubate the tube at 70ºC 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 70ºC 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**, next page.

Continued on the next page

cDNA Synthesis, continued

Second-Strand cDNA Synthesis

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

Note: For multiple reactions, prepare a master mix with a 5–10% overage to enable accurate pipetting.

Note: 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
DNA Polymerase I (10 units/µl)	4 µl
DNA Ligase (10 units/µl)	1 µl
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 16ºC for 2 hours. After incubation, place the tubes on ice.

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

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
- Speed-vac

The following items are supplied in the kit:

- Spin Cartridges pre-inserted into collection tubes
- Recovery Tubes
- cDNA Loading Buffer **plus isopropanol** (see page vi for preparation)
- cDNA Wash Buffer **plus ethanol** (see page vi for preparation)
- DEPC-treated water

Purification Procedure

Use the following procedure to purify the cDNA.

Note: Before starting the procedure, be sure to add isopropanol to the cDNA Loading Buffer supplied in the kit and ethanol to the cDNA Wash Buffer supplied in the kit as described on page vi.

1. Add 500 µl of cDNA Loading Buffer prepared as described on page vi 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 Spin Cartridge is pre-inserted into a collection tube. Load the cDNA/buffer solution directly onto the Spin Cartridge.
4. Centrifuge at 12,000 × *g* at room temperature in a microcentrifuge for 1 minute. Remove the collection tube and discard the flow-through.
5. Place the Spin Cartridge in the same collection tube and add 700 µl of cDNA Wash Buffer prepared as described on page vi to the column.
6. Centrifuge at 12,000 × *g* at room temperature for 2 minutes. Remove the collection tube and discard the flow-through.
7. Place the Spin Cartridge in the same collection tube and centrifuge at 12,000 × *g* at room temperature for an additional 4 minutes. Remove the collection tube and discard the flow-through.
8. Place the Spin Cartridge into a new Recovery Tube (supplied in the kit).
9. Add 100 µl of DEPC-treated water to the center of the Spin Cartridge and incubate at room temperature for 2 minutes.
10. Centrifuge at 12,000 × *g* at room temperature for 1 minutes to collect your purified cDNA. **The eluate contains your purified cDNA.**
11. Place the eluate in a speed-vac and evaporate at low to medium heat until the sample volume is reduced to ≤20 µl. **Be careful not to overdry the sample, as this may result in sample loss.**

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

In Vitro Transcription

Introduction

In this step, you generate aRNA from the double-stranded cDNA using T7 RNA Polymerase. An alternative protocol is provided for generating aRNA with amino-allyl UTP for subsequent indirect labeling with fluorescent dyes.

Before Starting

The following items are supplied by the user:

- Microcentrifuge
- Air incubator or thermal cycler set at 37ºC (heat block is not recommended)
- Optional: 50 mM amino-allyl UTP

The following items are supplied in 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

Note

The yield of aRNA will increase with longer *in vitro* transcription incubation times, up to 16 hours. For maximum yield, an incubation time of at least 12 hours is recommended.

In Vitro Transcription — Unlabeled aRNA

Use the following procedure to generate unlabeled aRNA. See the following page for a procedure to generate amino-allyl aRNA. For multiple reactions, prepare a master mix with a 5–10% overage to enable accurate pipetting.

1. Add DEPC-treated water to the tube containing purified cDNA from Step 11, page 9, to bring the total volume to 23 µl.
2. Add the following to the tube 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	1.5 µl
10X T7 Reaction Buffer	4 µl
T7 Enzyme Mix	7 µl
Total Reaction Volume	40 µl

Protocol continued on next page

Continued on next page

In Vitro Transcription, continued

In Vitro Transcription — Unlabeled aRNA, continued

Protocol continued from previous page

3. Gently mix by hand and centrifuge briefly to collect the contents of the tube.
4. Incubate at 37ºC for 6–16 hours.
5. **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 37ºC for 30 minutes.

Proceed to **aRNA Purification**, page 12.

In Vitro Transcription — Amino-Allyl aRNA

Use the following procedure to generate aRNA with amino-allyl UTP for subsequent labeling with fluorescent dye and array hybridization.

For multiple reactions, prepare a master mix with a 5–10% overage to enable accurate pipetting.

1. Add DEPC-treated water to the tube containing purified cDNA from Step 11, page 9, to bring the total volume to 22 µl.
2. Add the following to the tube 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
10X T7 Reaction Buffer	4 µl
T7 Enzyme Mix	7 µl
Amino-allyl UTP (50 mM)	1.75 µl
Total Reaction Volume	40 µl

3. Gently mix and centrifuge briefly to collect the contents of the tube.
4. Incubate the tube at 37ºC for 6–16 hours.
5. **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 37ºC for 30 minutes.

Proceed to **aRNA Purification**, page 12.

aRNA Purification

Introduction

In this step, you purify the aRNA using the spin columns provided in the kit.

Before Starting

The following items are supplied by the user:

- Microcentrifuge
- Speed-vac
- 100% ethanol

The following items are supplied in the kit:

- Spin Cartridges pre-inserted into collection tubes
- Recovery Tubes
- aRNA Binding Buffer
- aRNA Wash Buffer **plus ethanol** (see page vii for preparation)
- DEPC-treated water

Purification Procedure

Use the following procedure to purify the aRNA. **Note:** Before starting the procedure, be sure to add ethanol to the aRNA Wash Buffer supplied in the kit as described on page vii.

1. Add 160 µl of aRNA Binding Buffer to the reaction tube from Step 6 on page 11 or 11. The total volume should be 200 µl. 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 Spin Cartridge is pre-inserted into a collection tube. Load the entire aRNA/buffer solution directly onto the Spin Cartridge.
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 Cartridge in the same collection tube and add 500 µl of aRNA Wash Buffer prepared as described on page vii 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.
8. Place the Spin Cartridge in the same collection tube and centrifuge at full speed for an additional 2 minutes to dry the column. Remove the collection tube and discard the flow-through.

Procedure continued on next page

Continued on next page

aRNA Purification, continued

Purification Procedure, continued

Procedure continued from previous page

9. Place the Spin Cartridge into a new Recovery Tube (supplied in the kit). Note: For fluorescent-labeled aRNA, we recommend using an amber recovery tube (not supplied) to avoid photobleaching.
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 at room temperature to collect your purified aRNA. **The eluate contains your purified aRNA.**

To calculate the yield of aRNA, proceed to **Determining aRNA Yield** on page 14.

To label amino-allyl aRNA with fluorescent dye, proceed to **Dye Coupling to Amino-Allyl aRNA,** page 16.

Stored the sample at –80º C.

Appendix

Determining aRNA Yield

Determining aRNA Yield Using an RNA Quantitation Kit

You can use the Quant-iT™ RNA Assay Kit from Molecular Probes (Cat. no. Q-33140) or the RiboGreen® RNA Quantitation Kit from Molecular Probes (Cat. 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 aRNA 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

   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 using the Control HeLa RNA (500 ng) provided in the kit depends on whether the aRNA is labeled or unlabeled, as well as the length of the *in vitro* transcription reaction. If you do not achieve the minimum yields specified below for the control reaction, see **Troubleshooting** on page 16.

Note: The expected yields listed below assume a 14-hour *in vitro* transcription reaction.

Type of aRNA	Expected Yield
Unlabeled	> 35 µg
Amino-allyl labeled	> 20 µg

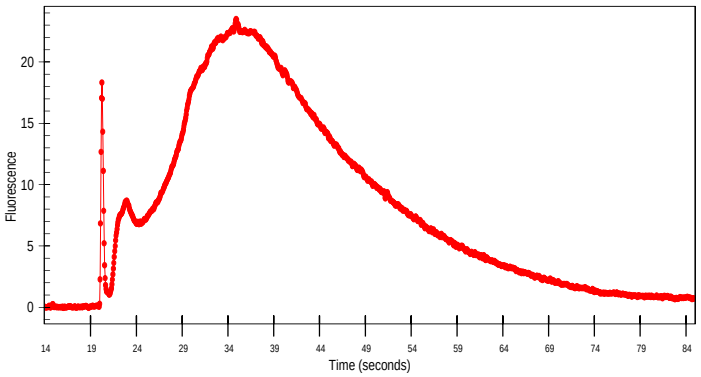
Determining aRNA Quality

Determining aRNA Quality Using the Agilent 2100 Bioanalyzer

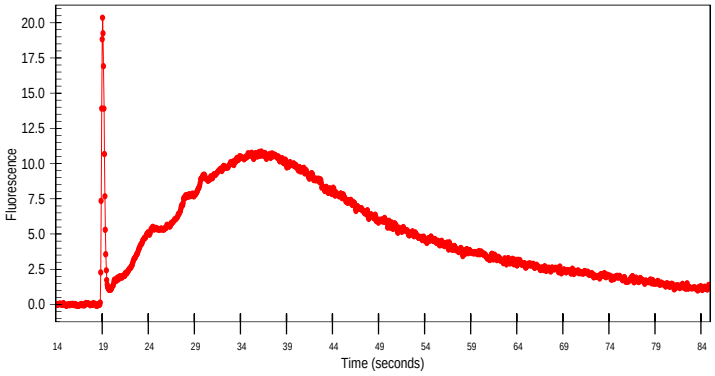
You can use the Agilent 2100 bioanalyzer with the RNA 6000 LabChip® Kit to analyze you aRNA sample. We do not recommend using the bioanalyzer to determine aRNA quantity (see the previous page for recommended quantitation methods).

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). Examples are shown below, and an RNA ladder is provided for size comparison.

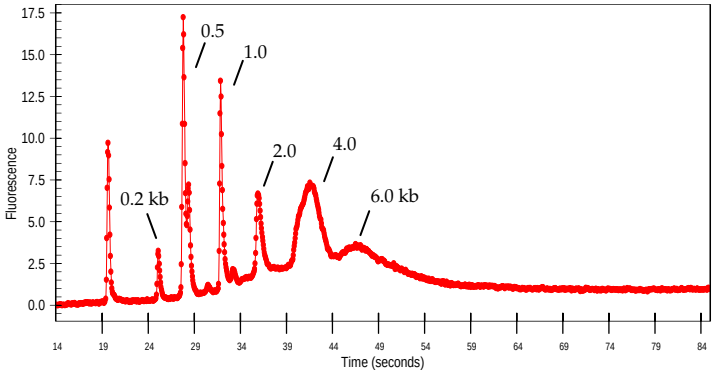
Unlabeled aRNA



Amino allyl-labeled aRNA:



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



Dye Coupling to Amino-Allyl aRNA

Introduction

This section provides a general protocol for labeling amino-allyl aRNA with Cy3™ or Cy5™ monofunctional, NHS-reactive fluorescent dyes.

Note

You must first calculate the yield of amino-allyl aRNA as described on page 14 before proceeding to dye coupling.

Before Starting

The following items will be used in the procedure:

- Speed-vac
- Sodium tetraborate decahydrate (500 g, Fisher, cat. no. S248-500)
- HCl
- 0.22-micron syringe filter or vacuum-flask filter
- DMSO
- DEPC-treated water
- Fluorescent dye (see above)
- Purification components listed on page 12

Coupling Buffer

To prepare the Coupling Buffer used in the labeling reaction:

1. Dissolve 3.8 g of sodium tetraborate decahydrate in 100 ml of DEPC-treated water.
2. Adjust the pH with HCl to 8.5.
3. Sterilize with a 0.22-micron syringe filter (for individual reaction volumes) or vacuum-flask filter.

Dye Information

This kit has been validated with the following dyes and dye packs:

CyDye Post-Labeling Reactive Dye Pack (12 vials each Cy3™ and Cy5™) (Amersham Biosciences, #RPN 5661)
Cy3™ Mono-Reactive Dye Pack (Amersham Biosciences, #PA 23001)
Cy5™ Mono-Reactive Dye Pack (Amersham Biosciences, #PA 25001)

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.

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 -20ºC, and let the vial warm to room temperature before opening to prevent condensation.

Continued on next page

Dye Coupling to Amino-Allyl aRNA, continued

Coupling Reaction

Follow the steps below to couple fluorescent dye to your amino-allyl aRNA.

1. Calculate the yield of amino-allyl aRNA as described on page 14. Determine the volume of purified sample that contains 5 µg of amino-allyl aRNA.
2. Aliquot the volume containing 5 µg of amino-allyl 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. Be careful not to overdry.
3. Add 5 µl of Coupling Buffer to the tube (see recipe on previous page).
4. Prepare the Cy3™ or Cy5™ 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. Use 5 µl of this DMSO/dye solution in the next step.
5. Add 5 µl of the DMSO/dye solution to the tube from Step 3 above.
6. Mix well and incubate the tube at room temperature in the dark for 30–45 minutes. Store any unused dye solution according to manufacturer's directions.
7. Purify the dye-coupled amino-allyl aRNA as described in **aRNA Purification,** page 12. Be careful to minimize exposure of the reaction to light. We recommend collecting the purified dye-labeled sample in an RNase-free amber collection tube.

To calculate the amount of coupled dye, see below.

Calculating the Amount of Coupled Dye

The following table shows the absorbance and baseline wavelengths for Cy3™ and Cy5™ dyes:

Dye	Absorbance Wavelength	Baseline Wavelength
Cy3™	550 nm	650 nm
Cy5™	650 nm	750 nm

To calculate the amount of coupled dye:

1. Transfer the **undiluted** purified dye-coupled amino-allyl aRNA from Step 11, page 12, into a clean cuvette, and scan at 240–800 nm using a UV/visible 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 page 12 before scanning, as any unreacted dye will interfere with the detection of labeled aRNA.
2. Calculate the amount of fluorescently labeled aRNA using a formula below:

   Cy3™ (pmole) = $(A_{550}–A_{650})/0.15 \times 100$ (elution volume)

   Cy5™ (pmole) = $(A_{650}–A_{750})/0.25 \times 100$ (elution volume)

Troubleshooting

Problem	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 inhibitors are present in your RNA sample	Inhibitors of RT 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 -20ºC.
	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

Troubleshooting, continued

Problem	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

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