invitrogen™

NCode™ miRNA Amplification System

For generating amplified senseRNA from small starting quantities of miRNA

Catalog no. MIRAS-20

Version B
12 January 2007
25-0918

User Manual

Table of Contents

Kit Contents and Storage

Shipping and Storage

The NCode™ miRNA Amplification System is shipped in two modules. The miRNA Amplification Module is shipped on dry ice, while the Purification Module is shipped at room temperature. Store the components of the miRNA Amplification Module at –20ºC and the components of the Purification Module at room temperature.

miRNA Amplification Module

Components should be stored at –20ºC. Reagents are provided for 20 amplification reactions.

Component	Amount
10X miRNA Reaction Buffer	110 µl
25 mM $MnCl_2$	100 µl
Poly A Polymerase	20 µl
Oligo(dT)24V	15 µl
10 mM ATP	20 µl
5X First-Strand Buffer (250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$)	200 µl
0.1 M DTT	100 µl
10 mM dNTP Mix	100 µl
RNaseOUT™ Recombinant Ribonuclease Inhibitor (40 U/µl)	25 µl
SuperScript™ III Reverse Transcriptase (200 U/µl)	40 µl
DEPC-treated Water	2 ml
10 mM dTTP	80 µl
Terminal Deoxynucleotidyl Transferase	40 µl
Klenow	20 µl
T7 Template Oligo	40 µl
100 mM ATP	30 µl
100 mM CTP	30 µl
100 mM GTP	30 µl
100 mM UTP	30 µl
10X T7 Reaction Buffer	80 µl
T7 Enzyme Mix (includes T7 RNA Polymerase in a proprietary formulation)	140 µl

cDNA Purification Module

Components should be stored at room temperature. Columns and tubes are provided for 20 purifications.

Item	Amount
cDNA Ultrafiltration Columns	20 columns
cDNA Ultrafiltration Tubes	2 × 20 tubes

Accessory Products

Additional Products

The NCode™ miRNA Amplification System is part of an integrated microRNA expression profiling system that includes miRNA isolation, amplification, purification, quantification, labeling, and array hybridization components. Additional products are available separately from Invitrogen. Ordering information is provided below.

For more information, visit our Web site at **www.invitrogen.com** or contact Technical Service (page 28).

Product	Quantity	Catalog no.
PureLink™ miRNA Isolation Kit	25 preps	K1570-01
PureLink™ Micro-to-Midi™ Total RNA Purification System	50 isolations	12183-018
Quant-iT™ Ribogreen® RNA Assay Kit	200–2,000 cuvette assays	R-11490
RediPlate™ 96 Ribogreen® RNA Quantitation Kit	96-well plate (8 × 12 strip wells)	R-32700
NCode™ miRNA Labeling System	20 labeling and hybridization reactions	MIRLS-20
NCode™ Multi-Species miRNA Microarray V2	5 slides	MIRA2-05
NCode™ Multi-Species miRNA Microarray Control V2	10 µl	MIRAC2-01
NCode ™ Multi-Species miRNA Microarray Probe Set V2	3 × 384-well plates / 500 pmol per well	MIRMPS2-01
Quant-iT™ Ribogreen® RNA Assay Kit	200–2,000 cuvette assays	R-11490
NCode™ SYBR® Green miRNA qRT-PCR Kit	10 polyadenylation/ 20 cDNA synthesis/ 100 qPCR reactions	MIRQ-100
NCode™ SYBR® GreenER™ miRNA qRT-PCR Kit	10 polyadenylation/ 20 cDNA synthesis/ 100 qPCR reactions	MIRQER-100
NCode™ miRNA First-Strand cDNA Synthesis Kit	10 polyadenylation/ 20 cDNA synthesis 50 polyadenylation/ 100 cDNA synthesis	MIRC-10 MIRC-50
RediPlate™ 96 Ribogreen® RNA Quantitation Kit	96-well plate (8 × 12 strip wells)	R-32700
UltraPure™ 20X SSC	1 liter 4 liters	15557-044 15557-036

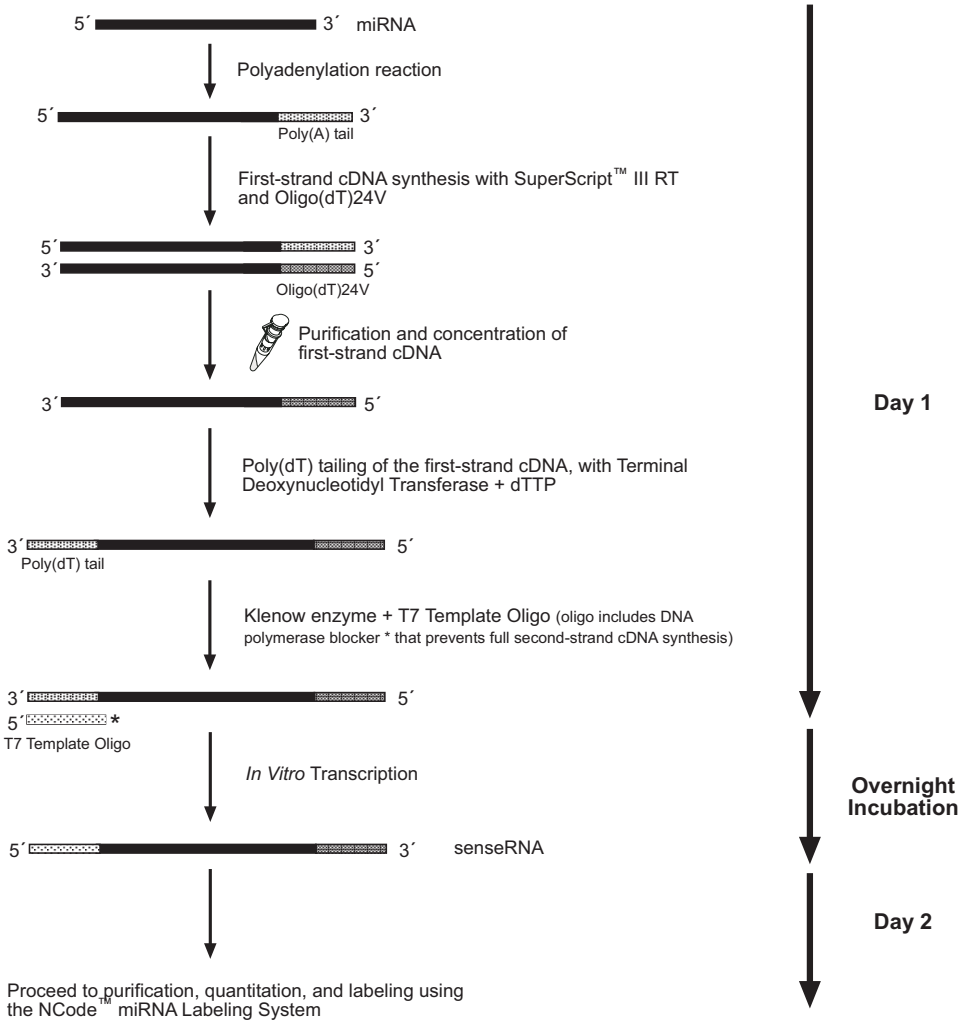
Introduction

Overview

The NCode™ miRNA Amplification System is a highly robust and efficient system for amplifying senseRNA molecules from minute quantities of purified microRNA (miRNA) to generate sufficient amounts of material for downstream research. The system provides consistent and accurate ≥1000-fold amplification of miRNA. The resulting amplified miRNA is in the sense orientation, for direct compatibility with miRNA probe sequences on microarrays. Amplified senseRNA is ideal for expression profiling from very small amounts of starting material because it preserves the relative abundance of the different miRNA sequences in the original sample, allowing you to compare relative quantities across experiments.

This system is designed for use with up to 30 ng of miRNA (isolated from 300–500 ng of total RNA) as starting material. The procedure allows for isolation and preparation of the sample in a single day, followed by *in vitro* transcription with an overnight incubation. The following day, you are ready to purify and label your amplified senseRNA population for downstream analysis.

Experimental Outline



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Introduction, continued

System Overview

After isolating small RNA from cells, tissue, or total RNA with the PureLink™ miRNA Isolation Kit, use the NCode™ miRNA Amplification System to amplify the enriched miRNAs as described below.

First you add a poly(A) tail to the miRNA using poly A polymerase and an optimized reaction buffer. Then you reverse-transcribe the tailed miRNA using SuperScript™ III RT, and purify and concentrate the resulting first-strand cDNA.

Next, you add a poly(dT) tail to the 3´ end of the first-strand product using terminal deoxynucleotidyl transferase, and synthesize and anneal a T7 promoter on the tailed cDNA using Klenow enzyme and a specially-designed T7 template oligo. Finally, you perform an *in vitro* transcription reaction with an overnight incubation to generate the amplified senseRNA.

Advantages of the System

- Optimized reagents and protocol ensure highly robust and reproducible reactions
- SuperScript™ III Reverse Transcriptase in the first-strand synthesis reaction ensures high specificity and yields of cDNA
- System generates amplified miRNA in the sense orientation, for direct compatibility with microarray probe sequences
- System includes all major reagents and materials for preparing amplified senseRNA for subsequent labeling and detection

MicroRNAs

MicroRNAs (miRNAs) are a recently discovered class of small, ~19–23-nucleotide non-coding RNA molecules. They are cleaved from hairpin precursors and are believed play an important role in translation regulation and degradation of target mRNAs by binding to partially complementary sites in the 3´ untranslated regions (UTRs) of the message (Lim, 2003). Recent experimental evidence suggests that the number of unique miRNAs in humans could exceed 800, though several groups have hypothesized that there may be up to 20,000 non-coding RNAs that contribute to eukaryotic complexity (Bentwich *et al.*, 2005; Imanishi *et al.*, 2004; Okazaki *et al.*, 2002).

Though hundreds of miRNAs have been discovered in a variety of organisms, little is known about their cellular function. They have been implicated in regulation of developmental timing and pattern formation (Lagos-Quintana *et al.*, 2001), restriction of differentiation potential (Nakahara & Carthew, 2004), regulation of insulin secretion (Stark *et al.*, 2003), and genomic rearrangements (John *et al.*, 2004).

Several unique physical attributes of miRNAs—including their small size, lack of poly-adenylated tails, and tendency to bind their mRNA targets with imperfect sequence homology—have made them elusive and challenging to study. In addition, strong conservation between miRNA family members means that any detection technology must be able to distinguish between ~22-base sequences that differ by only 1–2 nucleotides. Recent advances in spotted oligonucleotide microarray labeling and detection have enabled the use of this high-throughout technology for miRNA screening.

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Other Products in the NCode™ System

The NCode™ miRNA Amplification System was designed and developed in conjunction with the following products (for ordering information, see page vi):

- **The PureLink™ miRNA Isolation Kit** is designed to purify small (≤200 nt) cellular RNA molecules, including regulatory RNA molecules such as miRNA and short interfering RNA (siRNA). The kit uses a silica-based two-column system to enrich small RNA from various sample sources, including cells, tissues, and total RNA. The enriched miRNA from this kit can be used directly in the NCode™ miRNA Labeling System.
- **The NCode™ miRNA Labeling System** is a robust and efficient system for labeling and hybridizing miRNA to NCode™ microarrays for expression profiling analysis. Using this kit, you ligate a short, highly specific tag sequence to each miRNA, and then hybridize highly fluorescent Alexa Fluor® dye molecules to the tagged miRNA. The high specificity of the binding sequence and high fluorescence of the dye molecules ensure maximum signal and strong signal correlations.
- **The NCode™ Multi-Species miRNA Microarray V2** consists of 5 Corning® Epoxide-Coated Glass Slides, each printed with optimized probe sequences targeting all of the known mature miRNAs in miRBase, Release 9.0 (http://microrna.sanger.ac.uk), for human, mouse, rat, *D. melanogaster*, *C. elegans*, and Zebrafish. The probes were designed using an algorithm that generates miRNA sequences with enhanced hybridization properties (Goff *et al.*, 2005). Each slide comes blocked and ready to use.
- **The NCode™ Multi-Species miRNA Microarray Probe Set** includes the probe sequences provided on the microarray listed above, dried down in 384-well plates at 500 pmoles per well and ready for printing on standard DNA microarray surfaces.
- **The NCode™ Multi-Species miRNA Microarray Control** is a synthetic 22-nucleotide miRNA sequence that has been designed and screened as a positive control for use with NCode™ system. This sequence has been tested for cross-reactivity with endogenous miRNAs from model organisms, and is provided at a concentration compatible with endogenous miRNA expression levels.
- **The NCode™ SYBR® Green miRNA qRT-PCR Kit** provides qualified reagents for the detection and quantitation of miRNAs in real-time quantitative RT-PCR (qRT-PCR). This kit has been optimized for the detection and quantification of miRNA from 10 ng to 2.5 µg of total RNA using a SYBR® Green detection platform.
- **The NCode™ SYBR® GreenER™ miRNA qRT-PCR Kit** provides qualified reagents for the detection and quantitation of miRNAs in real-time quantitative RT-PCR (qRT-PCR). This kit has been optimized for the detection and quantification of miRNA from 10 ng to 2.5 µg of total RNA using a SYBR® GreenER™ detection platform.
- **The NCode™ miRNA First-Strand cDNA Synthesis Kit** provides qualified reagents for the polyadenylation of miRNAs from total RNA and synthesis of first-strand cDNA from the tailed miRNAs for use in real-time quantitative PCR (qPCR). This kit has been optimized for the detection and quantification of miRNA from 10 ng to 2.5 µg of total RNA using a SYBR® Green or SYBR® GreenER™ detection platform (sold separately).

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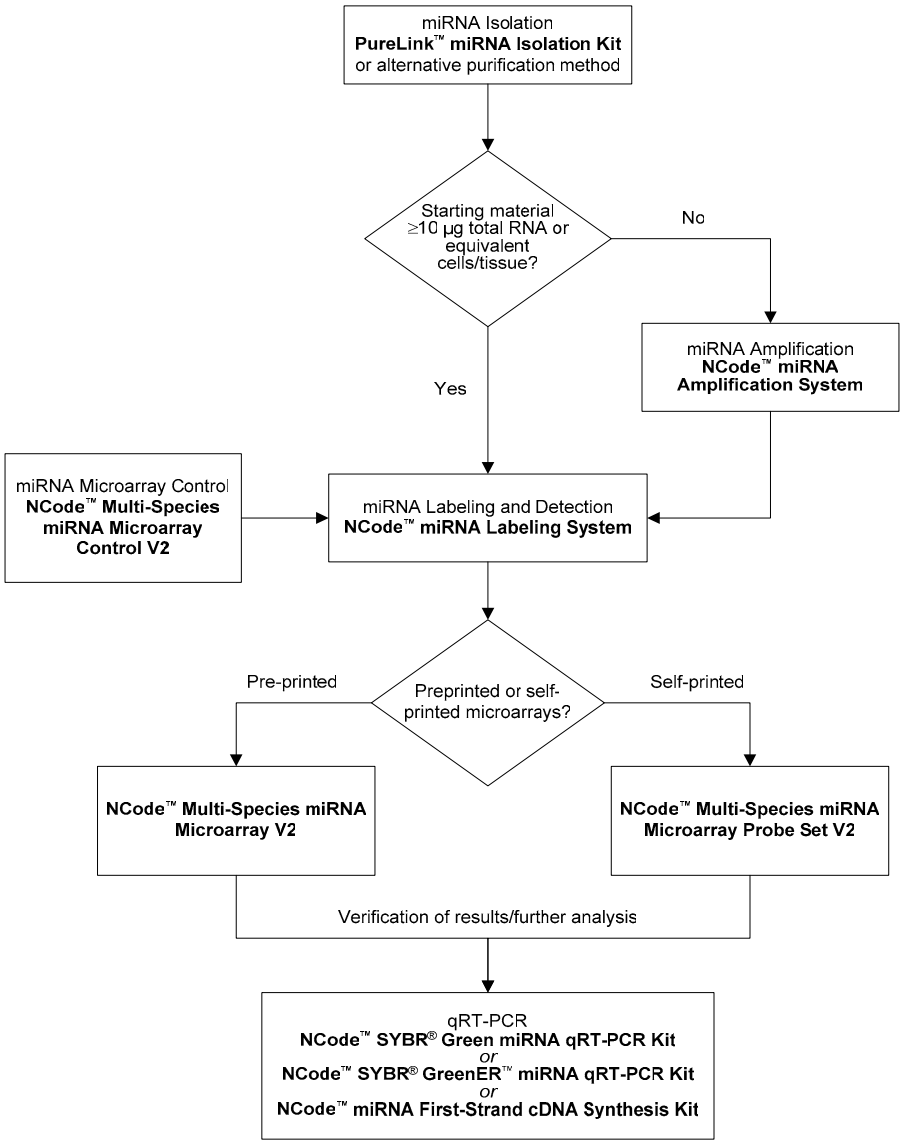
Introduction, continued

NCode™ System Workflow Diagram



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Materials Supplied by the User

In addition to the kit components, you should have the following items on hand before using this kit. Ordering information for Invitrogen products listed below is provided on page vi.

- 300–500 ng of total RNA or equivalent cells/tissue (for smaller amounts of starting material, see purification protocol starting on page 25)
- PureLink™ miRNA Isolation Kit (Invitrogen) *or* other miRNA isolation kit
- PureLink™ Micro-to-Midi™ Total RNA Purification System (Invitrogen) *or* other column-based total RNA purification system
- Quant-iT™ Ribogreen® RNA Assay Kit (Invitrogen) *or* RediPlate™ 96 Ribogreen® RNA Quantitation Kit (Invitrogen) *or* capillary or other small-volume spectrophotometer
- Optional: SpeedVac® or other concentrator may be required to concentrate sample prior to polyadenylation
- Thermal cycler with a heated lid *or* air incubator (heat block/water bath may be used for some but not all procedures)
- Microcentrifuge
- Vortex mixer
- 1.5-ml RNase-free microcentrifuge tubes
- RNase-free pipette tips
- 10 mM Tris, pH 8.0
- 0.1X TE buffer (1 mM Tris-HCl, 0.1 mM EDTA, pH 8.0)
- 1X TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0)
- 0.5 M NaOH/50 mM EDTA
- 1 M Tris, pH 8.0
- 100% ethanol
- 96–100% ethanol
- Ice

Product Qualification

This kit was verified using enriched miRNA in a standard amplification reaction as described in this manual. Equivalent quantities of amplified miRNA and nonamplified miRNA sample were assayed by qRT-PCR using primers for specific miRNA sequences. Cycle thresholds (CTs) and fold amplification were calculated and compared to determine sequence-specific amplification.

Methods

Isolating Small RNA

Introduction

In this step, you isolate small cellular RNA molecules from biological samples.

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.
- Use RNase-free microcentrifuge tubes. 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, to remove RNase contamination from surfaces. For further information on controlling RNase contamination, see (Ausubel *et al.*, 1994; Sambrook *et al.*, 1989).

Amount of Starting Material

The PureLink™ miRNA Isolation Kit can be used to isolate small RNA molecules from 300–500 ng of total RNA (or equivalent cells or tissue). For smaller amounts of starting material, you can use the cDNA Ultrafiltration Columns and Tubes provided in this kit or Microcon YM-100 columns and tubes from Millipore as described in the protocol starting on page 25.

Using the PureLink™ kit, 300–500 ng of total RNA or equivalent cells or tissue typically yields 15–30 ng of small RNA molecules, depending on the sample.

PureLink™ miRNA Isolation Kit

The NCode™ miRNA Amplification System was developed and optimized using enriched miRNA from the **PureLink™ miRNA Isolation Kit** (Invitrogen catalog no. K1570-01, see page vi). The PureLink™ kit provides columns, reagents, and protocols for isolating small RNA molecules from a variety of cell and tissue types, in small and large sample volumes. The PureLink™ kit may be used to isolate small RNA from 300–500 ng of total RNA, or equivalent cells or tissue.

Continued on next page

Isolating Small RNA, continued

Important

- When using the PureLink™ miRNA Isolation Kit to isolate small RNA, use the protocol on the following pages, which has been adapted from the standard PureLink™ protocol. Note that the following protocol uses **100% ethanol**, which removes more debris from the sample, enhancing the performance of this kit.
- We recommend starting with high-quality, isolated small RNA. We do not recommend using total RNA for amplification, labeling, and hybridization.
- The quality of the RNA is **critical** for amplification. In amplification, labeling, and array hybridization applications, the presence of contaminants in the RNA may reduce amplification yield and increase background fluorescence in microarrays. Carefully follow the recommendations below to prevent contamination.

Isolating Small RNA Using the PureLink™ miRNA Isolation Kit

The following protocol has been adapted from the PureLink™ miRNA Isolation Kit manual. See that manual for more details. The following protocol may be used to isolate up to 30 ng of miRNA from 300–500 ng of total RNA.

Materials needed:

- Components of the PureLink™ miRNA Isolation Kit (Invitrogen catalog no. K1570-01, see page vi)
- Total RNA sample
- 100% ethanol
- Microcentrifuge
- RNase-free pipette tips

Wash Buffer (W5): Prepare Wash Buffer (W5) for use by adding 40 ml of 96–100% ethanol to 10 ml of Wash Buffer (W5) included with the kit. Store the Wash Buffer (W5) with ethanol at room temperature.

Procedure:

1. Resuspend total RNA in 300 µl of Binding Buffer (L3) supplied with the PureLink™ kit. Mix well by vortexing or pipetting up and down.
2. Add 300 µl of 100% ethanol to the solution. Mix well by vortexing.
3. Add the complete solution (600 µl) to a Spin Cartridge (preinserted in a Collection Tube) from the PureLink™ kit.
4. Centrifuge the Spin Cartridge at 12,000 × *g* for 1 minute at room temperature to collect the flow-through. Remove and discard the Spin Cartridge. **Do not discard the flow-through.**
5. Add 400 µl of 100% ethanol to the flow-through and mix well by vortexing.
6. Transfer 500 µl of the sample from Step 5 to a **new** Spin Cartridge in a Collection Tube.

Procedure continued on next page

Continued on next page

Isolating Small RNA Using the PureLink™ miRNA Isolation Kit, continued

Procedure continued from previous page

7. Centrifuge the Spin Cartridge at 12,000 × *g* for 1 minute at room temperature.
8. Transfer the remaining sample (500 µl) from Step 5 to the Spin Cartridge from Step 6 and centrifuge at 12,000 × *g* for 1 minute at room temperature.
9. Discard the flow-through and re-insert the Spin Cartridge into the Collection Tube.
10. Add 500 µl of Wash Buffer (W5) prepared with ethanol (see above) to the Spin Cartridge.
11. Centrifuge 12,000 × *g* for 1 minute at room temperature.
12. Repeat Steps 10–11 one more time.
13. Discard the flow-through and place the Spin Cartridge into a Wash Tube supplied with the kit.
14. Centrifuge the Spin Cartridge at maximum speed for 2–3 minutes at room temperature to remove any residual Wash Buffer. Discard the Wash Tube.
15. Place the Spin Cartridge into a **clean** 1.7-ml Recovery Tube supplied with the kit.
16. Add 50–100 µl of sterile, RNase-free water (pH >7.0) supplied with the kit to the center of the Spin Cartridge (higher elution volumes may increase yield but will result in more dilute sample).
17. Incubate at room temperature for 1 minute.
18. Centrifuge the Spin Cartridge at maximum speed for 1 minute at room temperature.
19. The Recovery Tube contains purified small RNA molecules. Remove and discard the cartridge.

Note: The recovery of the elution volume will vary and is usually 90% of the elution buffer volume used.

Store the purified product at –80ºC, or proceed to quantification as described in the next section.

Quantifying Small RNA

Introduction

In this step, you determine the quantity of isolated small RNA prior to polyadenylation. This quantity is used to determine the amount of ATP to use in the polyadenylation procedure (see next page).

Quantifying the Amount of Small RNA

Isolated small RNA is typically too dilute to determine the quantity using A_{260} absorbance on a standard spectrophotometer. We recommend using the Quant-iT™ Ribogreen® RNA Assay Kit or the RediPlate™ 96 Ribogreen® RNA Quantitation Kit. Ordering information is provided on page vi.

Each kit provides highly accurate fluorescent quantification of minute quantities of RNA, in the range of 1–1,000 ng/ml. An undiluted sample of small RNA from the PureLink™ miRNA Isolation Kit should fall well within the linear range of the assay. The assay takes approximately 1 hour to complete.

Alternatively, a capillary or other small-volume spectrophotometer may be used to measure A_{260} absorbance.

After quantifying the small RNA, we recommend that you proceed directly to **Polyadenylation of miRNA** on page 10. The RNA may be stored at –80ºC if necessary.

Polyadenylation of miRNA

Introduction

After you have quantified the enriched miRNA, you are ready to add a poly(A) tail to the miRNA.

Before Starting

The following items are supplied in the miRNA Amplification Module:

- 10X miRNA Reaction Buffer
- 25 mM $MnCl_2$
- 10 mM ATP
- Poly A Polymerase
- DEPC-treated water

The following items are supplied by the user:

- Up to 30 ng of enriched miRNA per sample
- Optional: Depending on the amount of purified sample, a SpeedVac® Concentrator (Savant Instruments, Inc.) or similar instrument may be necessary to concentrate the sample
- 1 mM Tris, pH 8.0
- Microcentrifuge
- Heat block or water bath set at 37°C
- 1.5-ml RNase-free microcentrifuge tubes

Note

We do not recommend using the NCode™ Multi-Species miRNA Microarray Controls in the following amplification procedure. We recommend using these controls in the subsequent labeling procedure as described in the NCode™ miRNA Labeling System manual.

Important

The following reaction uses the **10 mM ATP** included in the kit, *not* the 100 mM ATP using in the *In Vitro* Transcription reaction (page 18). Be careful to select the vial of 10 mM ATP for use in the following reaction.

Continued on next page

Polyadenylation of miRNA, continued

Polyadenylation Procedure

Use the following procedure to add poly(A) tails to up to 30 ng of enriched miRNA:

1. Following quantification of the enriched miRNA as described on page 9, prepare an 18-µl volume of sample containing ≤30 ng of enriched miRNA using **one** of the following methods:

   - Aliquot up to 18 µl of eluate containing ≤30 ng of enriched miRNA into an RNase-free microcentrifuge tube. If necessary, add DEPC-treated water to increase the volume to 18 µl.

     OR

   - If the sample is extremely dilute, concentrate an amount of eluate containing ≤30 ng of enriched miRNA in a SpeedVac® Concentrator at low heat to a final volume of 18 µl. (**Note:** The elution volume from the PureLink™ miRNA Isolation Kit is 50–100 µl.) Transfer to an RNase-free microcentrifuge tube.

2. Dilute the **10 mM ATP** (see **Important** note on the previous page) as follows:

   - **For enriched miRNA samples between 1 and 30 ng**, dilute a volume of 10 mM ATP in 1 mM Tris (pH 8.0) according to the following formula:

     ATP dilution factor = 5000/____ ng of enriched miRNA

     Example: If you are starting with 5 ng of miRNA, the ATP dilution factor is 5000/5 ng = 1000. Dilute the 10 mM ATP 1:1000 by adding 1 µl of 10 mM ATP to 999 µl of 1 mM Tris, pH 8.0.

   - **For enriched miRNA samples less than 1 ng**, dilute the 10 mM ATP 1:5000 in 1 mM Tris, pH 8.0.

3. Add the following at room temperature to the tube of sample from Step 1:

Component	Volume
Tube from Step 1	18 µl
10X miRNA Reaction Buffer	2.5 µl
25 mM $MnCl_2$	2.5 µl
Diluted ATP (from Step 2)	1 µl
Poly A Polymerase	1 µl
Final Volume	25 µl

4. Mix gently and centrifuge the tube briefly to collect the contents.

5. Incubate the tube in a heat block or water bath at 37°C for 15 minutes.

After incubation, proceed immediately to **Reverse Transcription of Tailed miRNA**, next page.

Reverse Transcription of Tailed miRNA

Introduction

After you have polyadenylated the miRNA, you are ready to synthesize first-strand cDNA from the tailed miRNA.

Before Starting

The following items are supplied in the miRNA Amplification Module:

- Oligo(dT)24V primer
- 5X First-Strand Buffer
- 0.1 M DTT
- 10 mM dNTP Mix
- RNaseOUT™
- SuperScript™ III RT
- DEPC-treated water

The following items are supplied by the user:

- Polyadenylated miRNA from previous procedure
- 0.1X TE buffer (1 mM Tris-HCl, 0.1 mM EDTA, pH 8.0)
- 1X TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0)
- 0.5 M NaOH/50 mM EDTA
- 1 M Tris, pH 8.0
- Vortex mixer
- Incubator(s)/thermal cycler(s) set at 46ºC and 65ºC
- 1.5-ml RNase-free microcentrifuge tubes
- Ice

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.

Diluting Oligo(dT)24V Primer

The Oligo(dT)24V Primer provided in the kit must be diluted 1:10 in 0.1X TE buffer before use. We recommend diluting 10 µl of the Oligo(dT)24V in 90 µl of 0.1X TE buffer and preparing 20 × 5 µl single-use aliquots, to minimize free-thaw cycles. (Note that each kit contains 15 µl of undiluted oligo.)

You will need only 2 µl of the diluted Oligo(dT)24V per cDNA synthesis reaction.

Continued on next page

Reverse Transcription of Tailed miRNA, continued

First-Strand cDNA Synthesis

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

1. Briefly centrifuge the 25 µl of polyadenylated miRNA from Step 5, page 11, and place on ice.
2. If you haven't already done so, prepare a 1:10 dilution of Oligo(dT)24V primer as described on the previous page. Vortex and briefly centrifuge.
3. Add 2 µl of diluted Oligo(dT)24V primer to the tube of miRNA on ice. Mix and briefly centrifuge.
4. Incubate at 65ºC for 10 minutes, and then immediately transfer the tube to ice for 2 minutes.
5. Briefly vortex and centrifuge each of the following reagents, and then add them to the tube on ice, for a final reaction volume of 50 µl. For multiple reactions, prepare a master mix with a 5–10% overage to enable accurate pipetting:

Component	Volume
5X First-Strand buffer	10 µl
0.1 M DTT	5 µl
10 mM dNTP Mix	2.5 µl
RNaseOUT™ (40 U/µl)	1 µl
SuperScript™ III RT (200 U/µl)	2 µl
DEPC-treated water	2.5 µl

6. Mix the tube gently by hand (do not vortex) and incubate at 46°C for 1 hour.
7. Add 8.75 µl of 0.5 M NaOH/50mM EDTA to stop the reaction. Note that the reaction may turn to a brown color; this is normal.
8. Briefly vortex and centrifuge the tube to collect the contents.
9. Incubate the tube at 65ºC for 30 minutes to degrade the miRNA. Note that the reaction may turn from brown to clear; this is normal.
10. Neutralize the reaction by adding 12.5 µl of 1 M Tris, pH 8.0. Briefly vortex and centrifuge the tube.
11. Bring the reaction volume up to 100 µl by adding 28.75 µl of 1X TE buffer.

Proceed immediately to **Purification and Concentration of cDNA**, next page.

Purification and Concentration of First-Strand cDNA

Introduction

In this step, you purify and concentrate the first-strand cDNA using the cDNA Ultrafiltration Columns and Tubes provided in the kit.

Before Starting

The following items are supplied in the cDNA Purification Module:

- cDNA Ultrafiltration Columns
- cDNA Ultrafiltration Tubes

The following item is supplied in the miRNA Amplification Module:

- DEPC-treated water

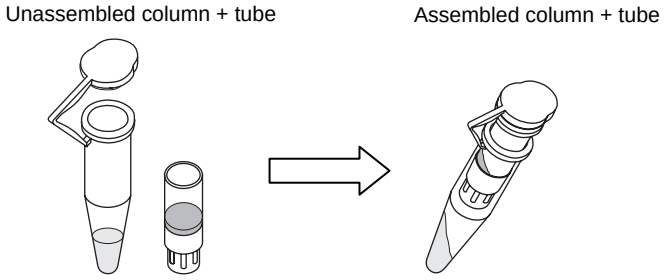
The following items are supplied by the user:

- 1X TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0)
- 10 mM Tris, pH 8.0
- Microcentrifuge

Purification Procedure

Use the following procedure to purify and concentrate the cDNA.

1. Insert a cDNA Ultrafiltration Column into a cDNA Ultrafiltration Tube. You will need a separate column and tube for each sample processed.



2. Pipette the 100 µl of cDNA from Step 11, page 13 onto the membrane in the center of the column. **Do not touch the membrane with the pipette tip.**
3. Secure the tube cap and insert the assembly in a centrifuge.

   Note: Align the cap strap toward the center of the rotor, and be sure to counterbalance the rotor with a similar device.
4. Centrifuge for 6 minutes at 13,000 × *g*.
5. Add 200 µl of 1X TE buffer to the column without touching the membrane. Pipette the buffer up and down ~5 times.
6. Secure the tube cap as before and centrifuge for 6 minutes at 13,000 × *g*.
7. Carefully separate the column from the tube and discard the flow-through. Insert the column in the same tube.

Procedure continued on next page

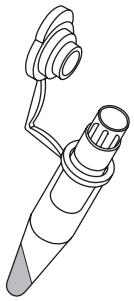
Continued on next page

Purification and Concentration of First-Strand cDNA, continued

Purification Procedure, continued

Procedure continued from previous page

8. Add 200 µl of 1X TE buffer to the column without touching the membrane. Gently pipette the buffer up and down 5 times.
9. Secure the tube cap as before and centrifuge for 6 minutes at 13,000 × *g*.
10. Carefully separate the column from the tube and discard the tube with the flow-through.
11. Add 5 µl of 10 mM Tris, pH 8.0, to the column membrane without touching the membrane. Gently tap the side of the column to mix.
12. Carefully place the column **upside-down** in a **new** cDNA Ultrafiltration Tube.



Column inserted upside-down in tube

13. Secure the tube cap and centrifuge for 3 minutes at 13,000 × *g*. **The eluate collected in the tube is your purified, concentrated cDNA.**
14. The volume of cDNA in the tube should be 5–10 µl. If necessary, bring the volume up to 10 µl with DEPC-treated water.

Proceed to **Tailing of First-Strand cDNA**, next page.

Tailing of First-Strand cDNA

Introduction

In this step, you add a poly(T) tail to the 3´ end of the purified cDNA using Terminal Deoxynucleotidyl Transferase and dTTP.

Before Starting

The following items are supplied in the miRNA Amplification Module:

- Terminal Deoxynucleotidyl Transferase
- 10 mM dTTP
- 10X miRNA Reaction Buffer
- DEPC-treated water

The following items are supplied by the user:

- 1.5-ml RNase-free microcentrifuge tubes
- Incubator or thermal cycler at 80ºC
- Heat block at 37ºC
- Ice
- Microcentrifuge

Tailing Procedure

Perform the following tailing reaction for each vial of purified cDNA:

1. Cap the tube containing the purified cDNA from Step 14, page 15, and heat-treat at 80ºC for 10 minutes. Chill on ice for 1–2 minutes, briefly centrifuge, and then return to ice.
2. In a separate RNase-free tube, add the following reagents and mix gently by hand. Amounts are provided per reaction. For multiple reactions, prepare a master mix with a 5–10% overage to enable accurate pipetting:

Component	Volume
10X miRNA Reaction buffer	2 µl
10 mM dTTP	4 µl
Terminal Deoxynucleotidyl Transferase	2 µl
DEPC-treated water	2 µl

3. Add the 10-µl reaction mix above to the cDNA from Step 1 for a final volume of 20 µl. Cap the tube, mix gently by hand, and briefly centrifuge.
4. Incubate in a 37°C heat block for 3 minutes.
5. Stop the reaction by heating at 80ºC for 10 minutes. Briefly centrifuge and cool to room temperature for 1–2 minutes.

Proceed immediately to **T7 Promoter Synthesis**, next page.

T7 Promoter Synthesis

Introduction

In this step, you synthesize a T7 promoter on the poly(dT) tail of the cDNA using Klenow enzyme.

Before Starting

The following items are supplied in the miRNA Amplification Module:

- T7 Template Oligo
- Klenow
- 10 mM dNTP Mix
- 10X miRNA Reaction Buffer

The following items are supplied by the user:

- Heat block or thermal cycler at 37ºC
- Ice
- Microcentrifuge

Note

The T7 Template Oligo includes a DNA polymerase blocker element that prevents complete second-strand synthesis of the cDNA.

T7 Promoter Synthesis Procedure

Perform the following synthesis reaction for each vial of tailed cDNA:

1. Add 2 µl of T7 Template Oligo to the tailed cDNA from Step 5, page 16, for a volume of 22 µl.
2. Incubate at 37°C for 10 minutes to anneal the strands.
3. To each reaction tube, add the following components for a final reaction volume of 25 µl. For multiple reactions, you can prepare a master mix of the following to enable accurate pipetting:

Component	Volume
10X miRNA Reaction buffer	1 µl
10 mM dNTP Mix	1 µl
Klenow	1 µl

4. Cap the tube, mix gently, and briefly centrifuge. Incubate at room temperature for 30 minutes.
5. Stop the reaction by heating at 65ºC for 10 minutes, and then place on ice.

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

In Vitro Transcription

Introduction

In this step, you generate senseRNA from the first-strand cDNA using T7 RNA Polymerase in a proprietary enzyme mix.

Before Starting

The following items are supplied in the miRNA Amplification Module:

- T7 Enzyme Mix
- 10X T7 Reaction Buffer
- 100 mM ATP
- 100 mM CTP
- 100 mM GTP
- 100 mM UTP

The following items are supplied by the user:

- Microcentrifuge
- Vortex mixer
- Air incubator set at 37ºC (see **Important** note below)

Note

Longer *in vitro* transcription incubation times will result in higher yields. For optimal results, we recommend performing an **overnight incubation** and proceeding with purification, quantitation, and labeling of the senseRNA on the following day.

Important

- The 4–16-hour incubation at 37ºC requires the use of an air incubator. **Do not use a heat block, water bath, or thermocycler for the incubation.** The reaction tube must be heated evenly throughout the incubation to avoid condensation on the tube lid. Heating methods that result in condensation may compromise the reaction.
- The following reaction uses the **100 mM ATP** included in the kit, *not* the 10 mM ATP used in the polyadenylation reaction (page 10). Be careful to select the vial of 100 mM ATP for use in this reaction.

Continued on next page

In Vitro Transcription, continued

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. Thaw the individual 100 mM NTPs (see **Important** note on the previous page) and T7 Enzyme Mix at room temperature, and hold at room temperature until use.
2. Thaw and warm the 10X T7 Reaction Buffer at 37ºC in an air incubator, then vortex briefly to dissolve any precipitates. Hold at room temperature.
3. Incubate the 25 µl of cDNA from Step 5, page 17 at 37ºC for 10 minutes to re-anneal the strands.
4. For each reaction, add the following components to the tube of cDNA at room temperature, for a final volume of 42 µl:

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

5. Cap the tube, mix gently by hand, and briefly centrifuge.
6. Incubate for 4–16 hours at 37ºC in an air incubator (do not use a heat block, water bath, or thermocycler; see **Important** note on the previous page).

Following incubation, the senseRNA may be stored at –80ºC. Otherwise, proceed to **Purification of the senseRNA**, page 20.

Purifying senseRNA

Introduction

Following preparation of the senseRNA, purify the sample according to the guidelines in this section.

PureLink™ Micro-to-Midi™ Total RNA Purification System

We recommend using the **PureLink™ Micro-to-Midi™ Total RNA Purification System** for cleanup of senseRNA samples (Invitrogen catalog no. 12183-018; see page vi). This kit has been extensively tested with the NCode™ miRNA Amplification System. The PureLink™ Micro-to-Midi™ System uses a silica-based membrane in a spin-column format, and can be used to purify high-quality RNA from very small quantities of sample.

Other small-sample RNA cleanup kits may also be appropriate for purification of senseRNA samples.

Purifying senseRNA using the PureLink™ Micro-to-Midi™ System

The following protocol has been adapted from the **Liquid Samples** cleanup protocol in the PureLink™ Micro-to-Midi™ Total RNA Purification System manual.

Materials needed:

- Components of the PureLink™ Micro-to-Midi™ Total RNA Purification System (Invitrogen catalog no. 12183-018; see page vi)
- 2-mercaptoethanol
- 96–100% ethanol
- Microcentrifuge
- 1.5-ml RNase-free microcentrifuge tubes
- RNase-free pipette tips

RNA Lysis Solution: Prepare the RNA Lysis Solution included with the system fresh for each use by adding 1% (v/v) 2-mercaptoethanol (*e.g.*, add 10 µl of 2-mercaptoethanol to every 1 ml of RNA Lysis Solution). Use 1 volume of freshly prepared RNA Lysis Solution for each volume of liquid sample.

Wash Buffer II: Before using the Wash Buffer II included with the system for the first time, add 60 ml of 96–100% ethanol directly to the bottle. Check the box on the Wash Buffer II label to indicate that ethanol was added.

Procedure:

1. To one volume of liquid sample (*e.g.*, 42 µl of senseRNA from Step 5, page 19), add one volume of RNA Lysis Solution prepared with 2-mercaptoethanol (see above) followed by the same volume of 96–100% ethanol (*e.g.*, to 42 µl of senseRNA, add 42 µl of RNA Lysis Solution followed by 42 µl of ethanol).
2. Mix by vortexing or pipetting up and down 5 times.
3. Pipette the sample onto the RNA Spin Cartridge, and centrifuge at 12,000 × *g* for 15 seconds at room temperature. Remove the cartridge from the tube, discard the flow-through, and re-insert the cartridge in the tube.

Procedure continued on the next page

Continued on next page

Purifying senseRNA using the PureLink™ Micro-to-Midi™ System, continued

Procedure continued from the previous page

4. Add 500 µl of Wash Buffer II prepared with ethanol (see previous page) to the spin cartridge. Centrifuge at 12,000 × *g* for 15 seconds at room temperature. Discard the flow-through, and re-insert the cartridge in the tube.
5. Repeat Step 4 once.
6. Centrifuge the spin cartridge at 12,000 × *g* for 1 minute at room temperature to dry the membrane with attached senseRNA.
7. Discard the collection tube, and insert the cartridge into an RNA Recovery Tube supplied with the kit.
8. To elute the senseRNA, add 30 µl of DEPC-treated water to the center of the spin cartridge, and incubate at room temperature for 1 minute.
9. Centrifuge the spin cartridge for 2 minutes at ≥12,000 × *g* at room temperature to collect the eluate. **The eluate contains your purified senseRNA.**

Prior to fluorescent labeling, calculate the yield of the purified senseRNA as described in **Quantifying senseRNA** on page 22. Alternatively, store the sample at –80ºC.

Quantifying senseRNA

Determining Yield Using an RNA Quantitation Kit

We recommend using the Quant-iT™ Ribogreen® RNA Assay Kit or the RediPlate™ 96 Ribogreen® RNA Quantitation Kit for highly sensitive quantitation of small amounts of RNA using a fluorescence microplate reader. Ordering information is provided on page vi.

Each kit provides highly accurate fluorescent quantification of minute quantities of RNA, in the range of 1–1,000 ng/ml. Use 1 µl of purified senseRNA in the quantitation reaction. See the product information sheet for each kit for detailed protocols.

Determining Yield Using A_{260} Absorbance

The following general protocol may be used to calculate the yield of the senseRNA by measuring A_{260} absorbance:

1. Aliquot 1 µl of the purified senseRNA into a clean cuvette (in most cases, the amount of senseRNA from the purification procedure is small enough that further dilution is not necessary).
2. Scan the sample at 260 nm using a UV/visible spectrophotometer. Be sure to blank the spectrophotometer using the sample elution buffer (*e.g.*, DEPC-treated water) before the reading.
3. **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, dilute the sample and re-scan. If the A_{260} reading is too low, use a lower dilution; if it's too high, use a higher dilution.
4. Transfer the sample back into the Recovery Tube for storage.
5. Calculate the yield of senseRNA using the formula below:

Total senseRNA 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 senseRNA at 1:50, and the A_{260} is 0.5, then 0.5 × 40 µg/ml RNA × 10 = 1000 µg/ml. In a 100 µl volume you would have 100 µg of senseRNA.

Determining Yield and Specificity Using qRT-PCR

Quantitative RT-PCR (qRT-PCR) may be performed on specific miRNA sequences to determine the level and specificity of the amplification reaction prior to array hybridization.

The NCode™ SYBR® Green miRNA qRT-PCR Kit provides qualified reagents for the sensitive detection and quantification of miRNA sequences.

Labeling and Hybridization

Introduction

After you have purified the senseRNA and determined the yield, you are ready to label the sample using the NCode™ miRNA Labeling System. For a description of this and other NCode™ products, see page 3.

Important

Note that you **do not need to add a poly(A) tail** to the amplified senseRNA prior to labeling with the NCode™ miRNA Labeling System. The senseRNA is already tailed. Proceed directly to the **Ligation of the Capture Sequence** protocol in the NCode™ miRNA Labeling System manual.

Amount of senseRNA

We recommend using 1.5 µg of senseRNA in each labeling reaction.

Appendix

Troubleshooting

Problem	Cause	Solution
Yield of enriched miRNA is low	Problems with the small RNA isolation procedure	Use the PureLink™ miRNA Isolation Kit for optimal results. See your miRNA isolation kit manual for additional troubleshooting information. (Note that we do not recommend using total RNA in place of small RNA with this kit.)
	Degraded starting material	Follow the guidelines on page 6 to prevent RNase contamination of the RNA preparation. Always use fresh samples or samples frozen at –80°C. If you are starting with total RNA, analyze it by agarose/ethidium bromide gel electrophoresis prior to isolation of small RNA.
Yield of senseRNA 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.
	Condensation formed in the *in vitro* transcription reaction tube	If condensation forms inside the tube during incubation, spin the tube briefly to remix the components, and perform the reaction in a different incubator. Note that the incubator must heat the tube evenly to avoid condensation on the tube lid. Do not use heat blocks or water baths.
	Poor quality RNA used or RNA is degraded	Check the quality of your RNA preparation (see page). 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 senseRNA amplification.
	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.
	Precipitates formed in 10X T7 Reaction Buffer	Vortex the buffer after warming to room temperature to avoid precipitation. If necessary, briefly heat to 37ºC to dissolve precipitates.

Isolating Small Amounts of Small RNA

Introduction

The standard range of starting material for this kit is 300–500 ng of total RNA or equivalent cells or tissue. If you are starting with smaller amounts of sample (down to 50 ng of total RNA), you can use the procedure in this section to isolate small RNA prior to amplification.

Note

For the following procedure, you can use *either* the cDNA Ultrafiltration Columns and Tubes provided in this kit, or order columns and tubes separately from Millipore Corporation.

Note that this kit includes only enough cDNA Ultrafiltration Columns and Tubes to perform 20 first-strand cDNA purifications described starting on page 14. If you use these columns and tubes in the following procedure, you will have fewer columns and tubes with which to perform the full amplification procedure.

Before Starting

Select one of the following:

- cDNA Ultrafiltration Columns and Tubes from the cDNA Purification Module

  or

- Microcon YM-100 Centrifugal Filter Unit, 100 columns and tubes, Millipore catalog no. 42413

The following additional items are supplied by the user:

- 50–500 ng of total RNA in a volume of ≤70 µl DEPC-treated water
- Microcentrifuge

Important

If you choose to use Millipore's Microcon YM-100 Centrifugal Filters, be careful to follow the procedure on the next page, *not* the manufacturer's protocol provided with the columns. The following procedure differs from the manufacturer's protocol, and has been optimized for use with this kit.

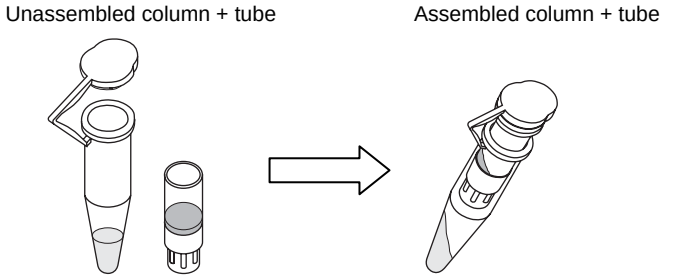
Continued on next page

Isolating Small Amounts of Small RNA, continued

Isolation Procedure

Use the following procedure to isolate small RNA from small amounts of total RNA.

1. Insert the filtration column into the specially designed snap-top tube. You will need a separate column and tube for each sample processed.



2. Pipette 50–500 µg of total RNA in a volume of ≤70 µl DEPC-treated water onto the membrane in the center of the column. **Do not touch the membrane with the pipette tip.**
3. Secure the tube cap and insert the assembly in a centrifuge.

   Note: Align the cap strap toward the center of the rotor, and be sure to counterbalance the rotor with a similar device.
4. Centrifuge for 6 minutes at 13,000 × *g*. **The eluate collected in the tube is your isolated small RNA.**

Proceed to **Quantifying Small RNA** on page 9.

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