invitrogen™

RiboMinus™ Transcriptome Isolation Kit (Human/Mouse)

For efficient transcriptome enrichment by depleting large ribosomal RNA from human and mouse total RNA

Catalog nos. K1550-02, K1550-05

Version C
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User Manual

Table of Contents

Experienced Users Procedure

Introduction

This quick reference protocol is included for experienced users of the RiboMinus™ Human/Mouse Transcriptome Isolation Kit. If you are a first time user, follow the detailed protocol in this manual.

Step	Action
Isolating Total RNA	Isolate high-quality total RNA from cells or tissues using a method of choice prior to using this kit. You will need 2-10 µg total RNA per reaction.
Selective Hybridization	Perform hybridization of your total RNA sample with the RiboMinus™ Human/Mouse Probe as below. 1. To a sterile, RNase-free 1.5 ml microcentrifuge tube, add: Total RNA (2-10 µg): <20 µl RiboMinus™ Probe (100 pmol/µl): 8 µl Hybridization Buffer (B5): 300 µl 2. Incubate the tube at 70-75°C for 5 minutes to denature RNA. Allow the sample to cool to 37°C slowly over a period of 30 minutes by placing the tube in a 37°C water bath. 3. Proceed to preparing beads, below.
Preparing RiboMinus™ Magnetic Beads	1. Resuspend the RiboMinus™ Magnetic Beads in its bottle by thorough vortexing. 2. Pipet 500 µl of the bead suspension into a sterile, RNase-free, 1.5-ml microcentrifuge tube. 3. Place the tube with the bead suspension on a magnetic stand for 1 minute. Gently aspirate and discard the supernatant. 4. Add 500 µl sterile, RNase-Free Water to the beads and resuspend beads. Place the tube on a magnetic stand for 1 minute. Gently aspirate and discard the supernatant. 5. Repeat Step 4 once. 6. Resuspend beads in 500 µl Hybridization Buffer (B5). Place the tube on a magnetic stand for 1 minute. Gently aspirate and discard the supernatant. 7. Resuspend beads in 200 µl Hybridization Buffer (B5) and keep the beads at 37°C until use.

Continued on next page

Experienced Users Procedure, Continued

Step	Action
Removing rRNA	1. Transfer ~328 µl of the cooled hybridized sample (from Step 3, previous page) to the prepared RiboMinus™ Magnetic beads from Step 7, previous page, and mix well. 2. Incubate the tube at 37°C for 15 minutes. During incubation, gently mix the contents occasionally. 3. Place the tube on a magnetic stand for 1 minute to pellet the rRNA-probe complex. The **supernatant** contains the RiboMinus™ RNA fraction. 4. Transfer the supernatant (~ 528 µl) to a tube capable of holding 3X volume of the supernatant.
Concentrating RiboMinus™ RNA using RiboMinus™ Concentration Module	See below for concentrating the RiboMinus™ RNA using the RiboMinus™ Concentration Module. Alternatively, you can concentrate the RiboMinus™ RNA using ethanol precipitation (page 17-18). 1. To the sample from Step 4, above, add 1X sample volume of the Binding Buffer (L3) and 1X sample volume of 96-100% ethanol. Mix well. 2. **Bind** ~700 µl of sample from Step 1 containing Binding Buffer (L3) and ethanol to the spin column. Centrifuge the column at ≥12,000 × g for 1 minute at room temperature. Discard the flow through. 3. Perform this binding step twice to bind the remaining sample from Step 1 onto the column and centrifuge the column at ≥12,000 x g for 1 minute at room temperature. Discard the flow through. 4. **Wash** the column with 200 µl Wash Buffer (W5) with ethanol (page 15). Centrifuge the column at ≥12,000 × g for 1 minute at room temperature. Discard the flow through. 5. **Repeat** the wash step once. 6. Discard the tube and place the column into a clean Wash Tube, supplied with the kit. 7. Centrifuge the column at maximum speed for 2–3 minutes at room temperature to remove any residual Wash Buffer (W5). Place the column in a 1.5-ml Recovery Tube. 8. **Elute** with 10-15 µl of RNase-Free Water. Incubate the column at room temperature for 1 minute. Centrifuge the column at maximum speed for 1 minute. *The Recovery Tube contains purified RiboMinus™ RNA.* 9. Store RiboMinus™ RNA at -80°C or place RiboMinus™ RNA on ice to proceed to desired downstream application.

Kit Contents and Storage

Types of Kits

This manual is supplied with the following products.

Product	Catalog no.
RiboMinus™ Transcriptome Isolation Kit (Human/Mouse)	K1550-02
RiboMinus™ Concentration Module	K1550-05

Shipping and Storage

All components of the RiboMinus™ Transcriptome Isolation Kit (Human/Mouse) and the RiboMinus™ Concentration Module are shipped at room temperature.

Upon receipt, store as follows:

- RiboMinus™ Transcriptome Isolation Kits at 4°C
- RiboMinus™ Concentration Module at room temperature

RiboMinus™ Transcriptome Isolation Kit

The components included in the RiboMinus™ Transcriptome Isolation Kit (Human/Mouse) are listed below.

Sufficient reagents are provided in the kit to perform 6 reactions.

Store the module at 4°C. For long-term storage, store the probe at -20°C.

Note: Some reagents are provided in excess.

Component	Amount
RiboMinus™ Magnetic Beads (12 mg/ml) in Phosphate Buffered Saline (PBS), pH 7.4 containing 0.01% Tween 20 and 0.09% sodium azide	3 ml
RiboMinus™ Human/Mouse Probe in ultrapure water (100 pmol/µl)	50 µl
Hybridization Buffer (B5)	6 ml
RNase-Free Water	6 ml

Continued on next page

Kit Contents and Storage

RiboMinus™ Concentration Module

The components included in the RiboMinus™ Concentration Module are listed below.

Sufficient reagents are provided in the kit to perform 6 reactions.

Store the Concentration Module at room temperature.

Note: Some reagents are provided in excess.

Component	Amount
Binding Buffer (L3)	3.3 ml
Wash Buffer (W5)	1.5 ml
RNase-Free Water	6.6 ml
Spin Columns with Collection Tubes	6
Wash Tubes (2.0 ml)	6
Recovery Tubes (1.5 ml)	6

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Product Qualification

The **RiboMinus™ Transcriptome Isolation Kit (Human/Mouse)** is functionally qualified as described below.

Purified total RNA (10 µg) from HeLa cells is subjected to ribosomal RNA depletion using the kit as described in this manual.

Agarose gel electrophoresis must show >95% depletion of 18S and 28S ribosomal bands from the purified sample as compared to control sample.

Bioanalyzer analysis of the purified sample must show <5% of peak area for 18S and 28S ribosomal bands as compared to peak area for the control sample.

RiboMinus™ Magnetic Beads

The binding capacity of the beads must be >2500 pmoles free biotin per mg of streptavidin-coated magnetic beads and must be free from bacterial contamination.

RiboMinus™ Human/Mouse Probe

The probes must contain the correct sequence and the locked nucleic acid (LNA™) at the specified position for each probe. Mass spectrometry analysis of probes must indicate the specified mass and HPLC analysis must indicate >85% purity. The probes must be RNase- and DNase-free.

Purchaser Notification

Limited Use Label License No: 237 LNA™ Oligonucleotides

LNA™ oligonucleotides are produced under a license from Exiqon A/S.

Accessory Products

Additional Products

The following products are also available from Invitrogen. For more details on these products, visit our Web site at www.invitrogen.com or contact Technical Service (page 24).

Product	Quantity	Catalog no.
RNase *AWAY*®	250 ml	10328-011
UltraPure™ DEPC-treated Water	1 L	750023
UltraPure™ DNase/RNase-Free Distilled Water	500 ml	10977-015
Quant-iT™ RNA Assay Kit	1000 assays	Q-33140
Micro-to-Midi™ Total RNA Purification System	50 reactions	12183-018
TRIzol® Reagent	100 ml	15596-026
DNase I	20,000 units	18047-019
DNase I, Amplification Grade	100 units	18068-015
Magna-Sep™ Magnetic Particle Separator	1	K1585-01
RiboMinus™ Yeast Transcriptome Isolation Kit (Yeast)	1	K1550-03
RiboMinus™ Transcriptome Isolation Kit (Bacteria)	1	K1550-04

Overview

Introduction

The RiboMinus™ Transcriptome Isolation Kit (Human/Mouse) provides a novel and efficient method to isolate RNA molecules of the transcriptome devoid of large ribososmal RNA (rRNA) from total RNA for transcriptome analysis. The purification method is not dependent on the polyadenylation status or presence of a 5′-cap structure on the RNA. See below for details on the purification protocol.

The isolation of RNA fraction depleted of ribososmal RNA is achieved by the selective removal of the large 18S and 28S human and mouse rRNA molecules from total RNA. The resulting rRNA depleted RNA fraction is termed as RiboMinus™ RNA (see next page for details).

Using the kit to isolate RiboMinus™ RNA results in efficient (>95%) removal of large 18S and 28S rRNA molecules from 10 µg total RNA enabling the analysis of the whole transcriptome without any interference from rRNA that account for ~90-95% RNA species in total RNA.

System Overview

The RiboMinus™ Transcriptome Isolation Kit (Human/Mouse) is based on the selective removal of human or mouse abundant large ribosomal RNA molecules (18S and 28S) from total RNA and concentrating the RiboMinus™ RNA enriched fraction.

Total RNA is hybridized with human and mouse rRNA sequence-specific 5′-biotin labeled oligonucleotide probes (RiboMinus™ Human/Mouse Probe). The rRNA/5′-biotin labeled probe complex is removed from the sample with streptavidin coated magnetic beads (RiboMinus™ Magnetic Beads).

The RiboMinus™ RNA sample is then concentrated using RiboMinus™ Concentration Module for a spin column-based centrifugation protocol (page 14) or using ethanol precipitation (page 17). The binding conditions of the spin column method are optimized for the RiboMinus™ RNA sample with ethanol and Binding Buffer (L3). The sample is loaded onto a spin column. The RiboMinus™ RNA binds to the silica-based membrane in the column and impurities are removed by thorough washing with Wash Buffer (W5). The RNA is then eluted in sterile RNase free water.

For details on RiboMinus™ Human/Mouse Probe and RiboMinus™ Magnetic Beads, see page 8.

Continued on next page

Overview, Continued

Advantages

Using the RiboMinus™ Transcriptome Isolation Kit (Human/Mouse) to isolate RiboMinus™ RNA (rRNA depleted RNA) provides the following advantages:

- Rapid and efficient isolation of high-quality RiboMinus™ RNA using probes specific to 18S and 28S human and mouse rRNA
- Specifically designed to isolate RiboMinus™ RNA enriched in polyadenylated (polyA) mRNA, non-polyadenylated RNA, pre-processed RNA, tRNA, and small rRNAs (5S rRNA, 5.8S rRNA)
- Minimal contamination from rRNA molecules
- Reliable performance of the RiboMinus™ RNA in downstream applications such as microarray analysis, cDNA library construction, and qRT-PCR

RiboMinus™ RNA

The large ribososmal RNA depleted RNA fraction is termed as RiboMinus™ RNA fraction.

The RiboMinus™ RNA fraction contains polyadenylated (polyA) mRNA, non-polyadenylated RNA, pre-processed RNA, tRNA, small rRNAs (5S rRNA, 5.8S rRNA), and may also contain regulatory RNA molecules such as microRNA (miRNA) and short interfering RNA (siRNA), snRNA, and other RNA transcripts of yet unknown function.

The RiboMinus™ RNA molecules are part of the transcriptome and are important in protein coding, signaling, structural support of subcellular elements, and transcriptional/post transcriptional regulation.

The transcriptome is defined as the complete collection of transcribed elements of the genome (Ruan *et al.*, 2004) and contains mRNA transcripts and non-mRNA transcripts including RiboMinus™ RNA. Transcriptome analysis is gaining increased attention in gene expression analysis. Since large rRNA constitutes 90-95% RNA species in total RNA, whole transcriptome analysis without any contamination from rRNA is very difficult and suggests the need for developing procedures for transcriptome isolation.

Continued on next page

Overview, Continued

Drawbacks of RNA Purification Methods

Current methods for RNA purification do not allow for efficient isolation of transcriptome. The total RNA purification methods result in enriching the large rRNA molecules while the mRNA purification methods use polyA-selection and/or cap-binding approaches that do not enrich for the complete transcriptome.

The RiboMinus™ Transcriptome Isolation Kit (Human/Mouse) is a novel method of isolating transcriptome and involves selective removal of large rRNA from total RNA. The isolated transcriptome is >95% depleted in rRNA and is enriched in all RNA transcripts of interest enabling whole transcriptome analysis.

Downstream Applications

The isolated RiboMinus™ RNA is suitable for use in downstream applications such as microarray analysis, qRT-PCR, and cDNA library construction

Product Specifications

System Specifications

Starting Material:	2-10 µg total RNA in <20 µl
rRNA Removal:	>95%
RiboMinus™ RNA Yield:	~1 µg from 10 µg total RNA

RiboMinus™ Probe Specifications

Probe Contents:	2 probes each for 18S and 28S rRNA
Probe Specificity:	Human and mouse
Probe Size:	18-19 oligonucleotides
Probe Label:	5′-biotin label
LNA™ Content:	Each probe contains 5-7 LNA™ monomers in the oligonucleotide
Probe Mixture Concentration:	100 pmol/µl

For details on the probe, see page 8.

RiboMinus™ Magnetic Bead Specifications

The RiboMinus™ Magnetic Beads are streptavidin-coated magnetic beads.

Bead Binding Capacity:	>2500 pmoles free biotin per mg RiboMinus™ Magnetic Beads
Bead Size:	1 µm diameter
Magnet Particle:	Superparamagnetic polydisperse core-shell polystyrene particles
Concentration:	12 mg/ml
Specific Gravity:	1.1-1.4 g/cm^3

For details on the beads, see page 9.

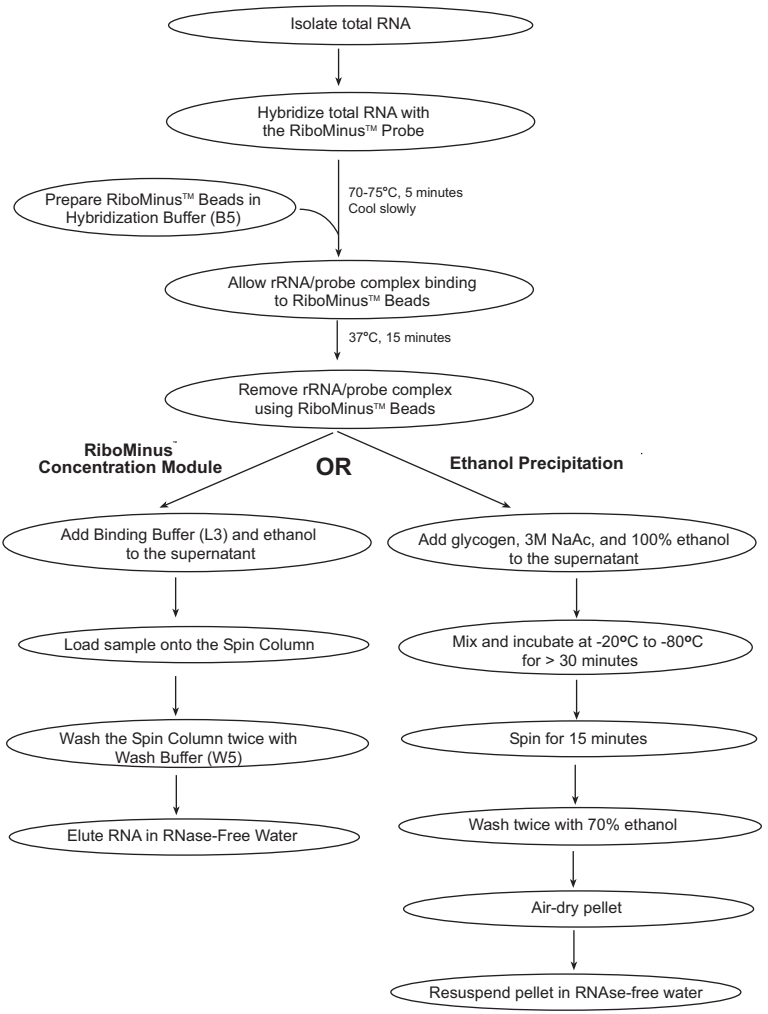
RiboMinus™ Concentration Module Specifications

Binding Capacity:	~5 µg nucleic acid
Column Reservoir Capacity:	700 µl
Wash Tube Capacity:	2.0 ml
Recovery Tube Capacity:	1.5 ml
Centrifuge Compatibility:	Capable of centrifuging at >10,000 x g
Elution Volume:	10-15 µl

Experimental Overview

Introduction

The flow chart for isolating transcriptome using the RiboMinus™ Transcriptome Isolation Kit (Human/Mouse) is shown below.



Preparing Total RNA

Introduction

You will need to isolate high-quality total RNA from cells or tissues using a method of choice prior to using this kit.

To obtain high-quality total RNA, follow the guidelines recommended below.

General Handling of RNA

Observe the following guidelines to prevent RNase contamination:

- Use disposable, individually wrapped, sterile plasticware
- 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
- Always use proper microbiological aseptic techniques when working with RNA
- Use RNase *AWAY*® Reagent (page x) to remove RNase contamination from surfaces

Total RNA Isolation

Total RNA can be isolated from tissue or cells using the method of choice. We recommend isolating total RNA using the Micro-to-Midi™ Total RNA Purification System or TRIzol® Reagent available from Invitrogen (see page x for ordering information).

You will use 2-10 µg total RNA in less than 20 µl for each reaction. Resuspend isolated total RNA in DEPC-treated water accordingly (≥0.5 µg/µl).

Check the quality of your total RNA, including DNA contamination (see below). Store your total RNA at -80°C and avoid repeated freezing and thawing of total RNA.

Important

If your downstream application requires DNA-free RNA, perform DNase-treatment of the total RNA **before** purifying RiboMinus™ RNA.

Continued on next page

Preparing Total RNA, Continued

Checking the Total RNA Quality

To check total RNA integrity, analyze ~0.5 µg of your RNA by agarose/ethidium bromide gel electrophoresis. You should see the following on an agarose gel:

- 28S rRNA band (5.0 kb for human; 4.7 kb for mouse) and 18S rRNA band (1.9 kb)
- 28S band should be approximately twice the intensity of the 18S band

Selective Hybridization and Removal of rRNA

Introduction

Instructions are provided in this section for selective hybridization of rRNA to the RiboMinus™ Probe and removal of rRNA using RiboMinus™ Magnetic Beads. See page 10 for an experimental outline.

RiboMinus™ Human/Mouse Probe

The RiboMinus™ Human/Mouse Probe is an oligonucleotide probe mixture containing 2 probes each specific for 18S rRNA and 28S rRNA (see page 4 for specifications). The probe is designed to hybridize with highly conserved regions of the human 18S and 28S rRNA. The probe also contains sufficient homology to hybridize efficiently against mouse rRNA.

Each probe is single-stranded and contains 5-7 LNA™ (Locked Nucleic Acid) monomers incorporated at specific locations. The incorporation of LNA™ (see next page for details on LNA™) into the oligonucleotide probe increases the depletion efficiency of the rRNA from the samples without increasing the amount of beads or probe concentration.

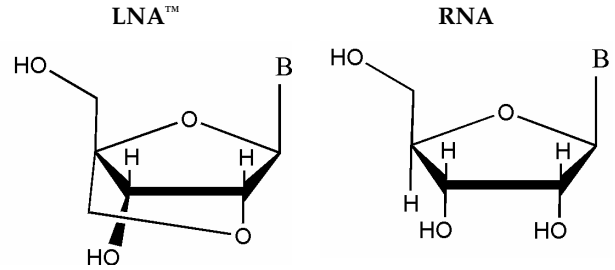
The 5′-end of each probe is conjugated to biotin to allow removal of rRNA/probe complexes by binding to stretptavidin RiboMinus™ Magnetic Beads (see next page).

Continued on next page

Selective Hybridization and Removal of rRNA, Continued

LNA™ (Locked Nucleic Acid)

The structure of the LNA™ (Locked Nucleic Acid) monomer (see figure below) consists of a ribonucleoside linked between the 2′ oxygen and 4′ carbon atom of the methylene ring (Braasch and Corey, 2001).



This configuration locks the sugar backbone resulting in an increase in T_m (melting temperature).

Incorporation of 5-7 LNA™ monomers into an oligonucleotide does not affect the ability of the oligonucleotide to bind DNA or RNA but increases the stability of the oligonucleotide/RNA complex (McTigue *et al.*, 2004). Oligonucleotides containing LNA™ are used in hybridization assays requiring high specificity and reproducibility.

RiboMinus™ Magnetic Beads

The RiboMinus™ Magnetic Beads are streptavidin-coated magnetic beads used for the removal of probe/rRNA complexes from the sample. The beads bind to the biotin-labeled probe complexed with rRNA or the probe alone.

The beads are 1 µm polystyrene beads with a magnetic core that is strong enough to separate the bound complex from the solvent in a short period of time (see page 4 for specifications). The beads do not promote non-specific binding of any other RNA molecules.

The size and the biotin binding capacity of the RiboMinus™ Magnetic Beads is optimized for use with this kit and results in >95% depletion of rRNA using 10 µg total RNA as the starting material. Avoid using any other streptavidin-coated magnetic beads with this kit.

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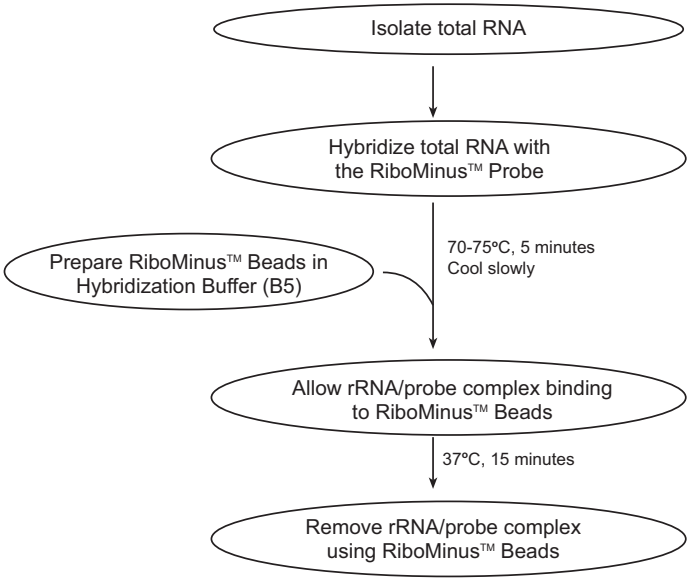
Selective Hybridization and Removal of rRNA, Continued

Experimental Outline

The figure below depicts the experimental outline for hybridization of rRNA to specific probes and removal of rRNA.



Selective Hybridization and Removal of rRNA, Continued

Materials Needed

You will need the following items:

- Total RNA (see pages 6-7)
- Magna-Sep™ Magnetic Particle Separator (page x) or equivalent
- RNase-free microcentrifuge tubes
- Water baths or heat blocks set to 70-75°C and 37°C
- Ice

Components supplied with the kit

- RiboMinus™ Magnetic Beads, keep on ice until use
- RiboMinus™ Human/Mouse Probe, keep on ice until use
- Hybridization Buffer (B5)
- RNase-Free Water

Follow the recommendations for handling the RiboMinus™ Magnetic Beads below for best results:

- During the mixing and washing steps of the magnetic beads, mix beads by using a vortex. A low speed centrifuge pulse may be required to remove beads stuck in the tube cap. **Avoid mixing by pipetting up and down as it results in bead loss.**
- During all washing steps with beads, add water or buffer to the tube containing beads while the tube is still on a magnetic stand to prevent drying of beads. Remove the tube from the magnet and resuspend the beads as described above. Do not allow the beads to dry as drying reduces the bead efficiency.
- To aspirate the supernatant after bead washing, place the pipette tip at the opposite side of the tube, away from the beads. Carefully remove the supernatant without disturbing or removing any beads.
- Do not submerge the magnetic stand in water. To clean the magnetic stand, spray the stand with ethanol and wipe it with a paper towel.

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Selective Hybridization and Removal of rRNA, Continued

Hybridization Step

Instructions are provided below to perform hybridization for 2-10 µg of your total RNA sample with the RiboMinus™ Human/Mouse Probe.

If you wish to process >10 µg total RNA sample, divide your sample into two samples, each containing <10 µg total RNA.

1. Set a water bath or heat block to 70-75°C.
2. To a sterile, RNase-free 1.5 ml microcentrifuge tube, add the following:

Total RNA (2-10 µg):	<20 µl
RiboMinus™ Probe (100 pmol/µl):	8 µl
Hybridization Buffer (B5):	300 µl

3. Incubate the tube at 70-75°C for 5 minutes to denature RNA.
4. Allow the sample to cool to 37°C slowly over a period of 30 minutes by placing the tube in a 37°C water bath. To promote sequence-specific hybridization, it is important to allow slow cooling.

   Avoid cooling the sample quickly by placing the tube in cold water.
5. While the sample is cooling down, prepare the magnetic beads as described below.

Preparing RiboMinus™ Magnetic Beads

Follow the recommendations on page 11 for handling beads and performing the washing steps.

1. Resuspend the RiboMinus™ Magnetic Beads in its bottle by thoroughly vortexing.
2. Pipet 500 µl of the bead suspension into a sterile, RNase-free, 1.5 ml microcentrifuge tube.

Procedure continued on the next page.

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Selective Hybridization and Removal of rRNA, Continued

Preparing RiboMinus™ Magnetic Beads, continued

Procedure continued from previous page.

3. Place the tube with the bead suspension on a magnetic separator for 1 minute. The beads will settle to the side of the tube that faces the magnet. Aspirate and discard the supernatant.
4. Add 500 µl sterile, RNase-Free Water supplied with the kit to the beads and resuspend beads by vortexing.
5. Place the tube on a magnetic separator for 1 minute. Gently aspirate and discard the supernatant.
6. Repeat Steps 4-5 once.
7. Resuspend beads in 500 µl Hybridization Buffer (B5). Place the tube on a magnetic separator for 1 minute. Gently aspirate and discard the supernatant.
8. Resuspend beads in 200 µl Hybridization Buffer (B5) and keep the beads at 37°C until use.

Removing rRNA

1. Set a water bath or heat block to 37°C.
2. After the hybridized sample (from Step 4, previous page) has cooled to 37°C, briefly centrifuge the tube to collect the sample to the bottom of the tube.
3. Transfer the sample (~328 µl) to the prepared RiboMinus™ Magnetic beads from Step 8, above. Mix well by vortexing the tube repeatedly.
4. Incubate the tube at 37°C for 15 minutes. During incubation, gently mix the contents occasionally. Briefly centrifuge the tube to collect the sample to the bottom of the tube.
5. Place the tube on a magnetic separator for 1 minute to pellet the rRNA-probe complex. **Do not discard the supernatant.** The supernatant contains RiboMinus™ RNA.
6. Transfer the supernatant (~ 528 µl) to a tube capable of holding 3X the volume of the supernatant.

Next Step

You can concentrate the RiboMinus™ RNA using RiboMinus™ Concentration Module with a rapid and optimized spin-based protocol (pages 14-16) or using ethanol precipitation (pages 17-18).

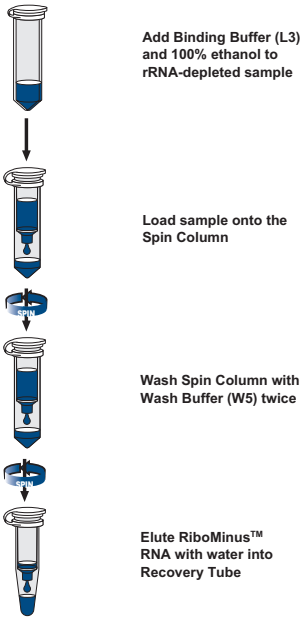
Using RiboMinus™ Concentration Module to Concentrate RiboMinus™ RNA

Introduction

The RiboMinus™ Concentration Module is designed for concentrating RiboMinus™ RNA purified with the RiboMinus™ Transcriptome Isolation Kit (Human/Mouse) using spin column-based centrifugation protocol in a total time of **10-15 minutes**. See next page for an experimental outline.

Experimental Outline

The figure below depicts the experimental outline for concentrating the RiboMinus™ RNA using a spin-column based centrifugation procedure.



Using RiboMinus™ Concentration Module to Concentrate RiboMinus™ RNA, Continued

Materials Needed

- RiboMinus™ RNA sample from Step 6, page 13
- 96-100% ethanol
- Microcentrifuge capable of centrifuging >12,000 x g

Components supplied with the RiboMinus™ Concentration Module

- Binding Buffer (L3)
- Wash Buffer (W5)
- RNase-Free Water
- Spin Column with Collection Tubes
- Wash Tubes
- Recovery Tubes

The RiboMinus™ Concentration Module Binding Buffer (L3) contains guanidine isothiocyanate. Always wear a laboratory coat, disposable gloves, and eye protection when handling buffers.

Do not add bleach or acidic solutions directly to solutions containing guanidine isothiocyanate or sample preparation waste as it forms reactive compounds and toxic gases when mixed with bleach or acids.

Before Starting

Add 6 ml 96-100% ethanol to 1.5 ml Wash Buffer (W5) included with the kit. Store the Wash Buffer (W5) with ethanol at room temperature.

Binding Step

1. To the sample from Step 6, page 13, add 1X sample volume of the Binding Buffer (L3) and 1X sample volume of 96-100% ethanol. Mix well by vortexing.
2. Load 700 µl sample containing Binding Buffer (L3) and ethanol to the column.
3. Centrifuge the column at ≥12,000 × g for 1 minute at room temperature. Discard the flow through.
4. Repeat the Steps 2 and 3 twice to load the remaining sample from Step 1 onto the column.
5. Proceed to **Washing Step**, next page.

Continued on next page

Using RiboMinus™ Concentration Module to Concentrate RiboMinus™ RNA, Continued

Washing Step

1. Add 200 µl Wash Buffer (W5) with ethanol (page 15) to the column.
2. Centrifuge the column at ≥12,000 × g for 1 minute at room temperature. Discard the flow through.
3. Repeat the wash step with 200 µl Wash Buffer (W5) with ethanol.
4. Discard the collection tube and place the column into a clean Wash Tube supplied with the kit.
5. Centrifuge the column at maximum speed for 2-3 minutes at room temperature to remove any residual Wash Buffer (W5). Discard the Wash Tube.
6. Proceed to **Elution Step**, below.

Elution Step

1. Place the Spin Column in a clean 1.5-ml Recovery Tube supplied with the kit.
2. Add 10-15 µl of RNase-Free Water to the center of the column. Incubate the column at room temperature for 1 minute.
3. Centrifuge the column at maximum speed for 1 minute at room temperature.

   The Recovery tube contains purified and concentrated RiboMinus™ RNA sample that is depleted of rRNA.
4. Place RiboMinus™ RNA on ice to proceed to desired downstream application or store RiboMinus™ RNA at -80°C until further use.

   See pages 19-21 to analyze yield and quality of the RiboMinus™ RNA.

Using Ethanol Precipitation to Concentrate RiboMinus™ RNA

Introduction

This section includes a protocol for **Ethanol Precipitation** to further concentrate the RiboMinus™ RNA purified with the RiboMinus™ Transcriptome Isolation Kit (Human/Mouse).

You will need at least 1 hour to perform the ethanol precipitation.

Materials Needed

- RiboMinus™ RNA sample (Step 7, page 13)
- Glycogen, 20 µg/µl (see page x)
- 3 M sodium acetate in RNAse-free water
- 96-100% cold ethanol
- 70% cold ethanol
- RNase-free water
- Sterile, RNase-free microcentrifuge tubes
- Microcentrifuge capable of centrifuging >12,000 x g

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Using Ethanol Precipitation to Concentrate RiboMinus™ RNA, Continued

Ethanol Precipitation

1. Transfer the sample into a clean RNAse-free 2 ml microcentrifuge tube.
2. Add the following components to the RiboMinus™ RNA:
   - 1 µl glycogen (20 µg/µl)
   - 1/10th sample volume (eluted RNA) of 3 M sodium acetate
   - 2.5X sample volumes of 100% ethanol
3. Mix well and incubate at -20 or -80°C for a minimum of 30 minutes.
4. Centrifuge the tube for 15 minutes ≥12,000 × g at 4°C.
5. Carefully discard the supernatant without disturbing the pellet.
6. Add 500 µl 70% cold ethanol.
7. Centrifuge the tube for 5 minutes ≥12,000 × g at 4°C.
8. Carefully discard the supernatant without disturbing the pellet.
9. Repeat Steps 6-8 once.
10. Air-dry the pellet for ~5 minutes (do not completely dry the pellet).
11. Resuspend the RNA pellet in ~10-30 µl RNase-free water.
12. Place RiboMinus™ RNA on ice to proceed to desired downstream application or store RiboMinus™ RNA at -80°C until further use.

See pages 19-21 to analyze yield and quality of the RiboMinus™ RNA.

Analyzing RiboMinus™ RNA

RNA Yield

The quantity of the purified RiboMinus™ RNA is easily quantitated using UV absorbance at 260 nm or Quant-iT™ RNA Assay Kit.

UV Absorbance

1. Dilute an aliquot of the small sample in 10 mM Tris-HCl, pH 7.0. Mix well. Transfer to a cuvette (1-cm path length).

   Note: The RNA must be in a neutral pH buffer to accurately measure the UV absorbance.

2. Determine the OD_{260} of the solution using a spectrophotometer blanked against 10 mM Tris-HCl, pH 7.0.

Calculate the amount of total RNA using the following formula:

Total RNA (µg) = OD_{260} x 40 µg/(1 OD_{260} x 1 ml) x dilution factor x total sample volume (ml)

The typical yield of RNA using the RiboMinus™ Human/Mouse Transcriptome Isolation Kit is ~1 µg RNA from 10 µg total RNA sample.

Quant-iT™ RNA Assay Kits

The Quant-iT™ RNA Assay Kit (see page x) provides a rapid, sensitive, and specific method for RNA quantitation with minimal interference from DNA, protein, or other common contaminants that affect UV absorbance readings.

The kit contains a state-of-the-art quantitation reagent and pre-diluted standards for standard curve. The assay is performed in a microtiter plate format and is designed for reading in standard fluorescent microplate readers.

Continued on next page

Analyzing RiboMinus™ RNA, Continued

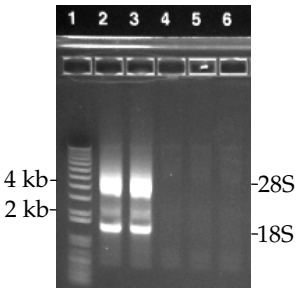
RNA Quality

The RNA isolated using the RiboMinus™ Transcriptome Isolation Kit (Human/Mouse) is of high-quality and is >95% depleted in rRNA species.

To verify rRNA depletion, perform agarose gel electrophoresis of the sample (see below) or use a bioanalyzer (next page).

Gel Electrophoresis to assess RNA Quality

Agarose gel electrophoresis analysis shows depletion of 18S and 28S rRNA bands as compared to a control sample (see below for an example). Absence of contaminating DNA and RNA degradation may also be confirmed by agarose gel electrophoresis.



RiboMinus™ RNA was purified using 10 µg total RNA from 293F cells as described in this manual. Samples (5% of total eluate) were analyzed on a 0.8% E-Gel® agarose gel and imaged to visualize RNA.

Lane 1: 1 µl 1 Kb Plus DNA Ladder

Lane 2: Control sample 1 (purification procedure performed in the absence of RiboMinus™ Beads and Probe)

Lane 3: Control sample 2 (purification procedure performed in the absence of RiboMinus™ Probe only)

Lanes 4-6: Purified RiboMinus™ RNA samples

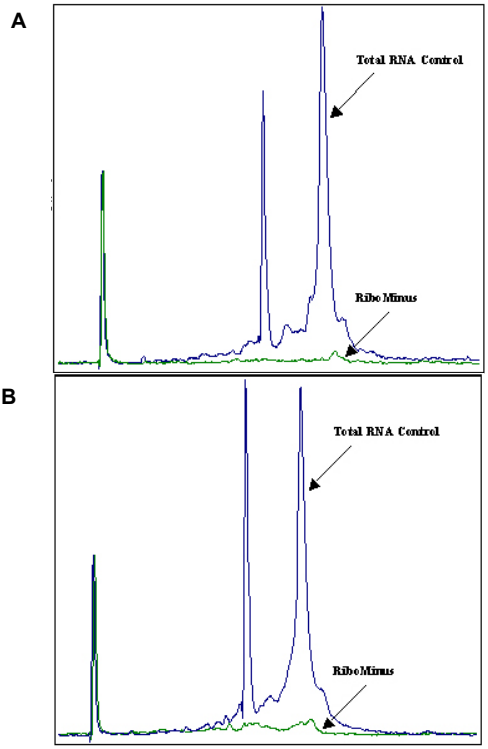
Lanes 4-6 show efficient removal of 18S and 28S rRNA bands from the purified RiboMinus™ RNA samples and Lane 3 shows absence of any non-specific rRNA removal.

Continued on next page

Analyzing RiboMinus™ RNA, Continued

Bioanalyzer to assess RiboMinus™ RNA Quality

The efficiency of rRNA depletion in RiboMinus™ RNA, RNA degradation, and RNA concentration can be analyzed using a bioanalyzer such as the Agilent 2100 bioanalyzer with an RNA LabChip®. In the example below, the bioanalyzer data was used to show efficient removal of 18S and 28S rRNA from total RNA.



RiboMinus™ RNA was purified from 10 µg total RNA from 293F cells (panel A) or from mouse liver (panel B) using RiboMinus™ Transcriptome Isolation Kit (Human/Mouse) as described in this manual. The total RNA control samples were obtained by omitting the RiboMinus™ Magnetic Beads in the reaction mixture. Aliquots of 2% of the final elution volume of RiboMinus™ RNA and control total RNA were subjected to the bioanalysis using Agilent 2100 bioanalyzer. The graph shows the removal of 18S and 28S rRNA from the purified RiboMinus™ RNA samples as compared to control samples.

Troubleshooting

Introduction

Review the table below to troubleshoot problems that you may encounter using the RiboMinus™ Human/Mouse Transcriptome Isolation Kit.

Problem	Cause	Solution
Low RNA yield	Low RNA content	Various tissues have different RNA content and the yield is dependent on the sample.
	Incorrect binding conditions when using the RiboMinus™ Concentration Module	For efficient binding of RiboMinus™ RNA to the spin column, always **add** 1X sample volume of the Binding Buffer (L3) and 1X sample volume of 100% ethanol to the sample prior to loading onto the Spin Column.
	Ethanol not added to Wash Buffer (W5)	Be sure to add 96–100% ethanol to Wash Buffer (W5) as described on page 15.
	Incorrect elution conditions	Add water to the center of the column and perform incubation for 1 minute with water before centrifugation.
	RNA quantitation performed with water	Be sure the RNA quantitation using UV absorbance is performed with 10 mM Tris-HCl, pH 7.0 (page 19) to accurately measure the UV absorbance.
	Loss of pellet during to ethanol precipitation	• Remove supernatant from RNA pellet carefully. • Use RiboMinus™ Concentration Module to concentrate the RiboMinus™ RNA.
Incomplete removal of rRNA	Too much total RNA used	The protocols in this manual are designed to purify RiboMinus™ RNA from 2-10 µg total RNA. If you are using more than 10 µg total RNA, divide the sample into two sample aliquots, each containing <10 µg total RNA for RiboMinus™ RNA purification.
	Low amount of magnetic beads or probe used	Be sure to use the recommended amounts of RiboMinus™ Probe and RiboMinus™ Magnetic Beads for efficient removal of rRNA.

Continued on next page

Troubleshooting, Continued

Problem	Cause	Solution
Incomplete removal of rRNA	Improper handling or drying of beads	To obtain the best results with RiboMinus™ Magnetic Beads, follow the guidelines on page 11 for washing and mixing the beads, and aspirating the supernatant. Do not allow the beads to dry as drying reduces the bead efficiency.
RNA degraded	RNA contaminated with RNase	Follow the guidelines on page 6 to prevent RNase contamination.
	Poor quality starting materials	Always use fresh samples or samples frozen at -80°C for isolation of total RNA. Be sure to check the quality of your total RNA prior to use.
Genomic DNA contamination	Total RNA contained genomic DNA	Perform DNase I digestion with the total RNA sample to remove any genomic DNA contamination **before** performing RiboMinus™ RNA purification.
Inhibition of downstream enzymatic reactions	Presence of ethanol in purified RNA sample	• Traces of ethanol from the RiboMinus™ Concentration Module Wash Buffer (W5) or from ethanol precipitation can inhibit downstream enzymatic reactions. • To remove Wash Buffer (W5), discard Wash Buffer flow through from the collection tube. Reinsert the spin column into the collection tube and centrifuge the spin column at maximum speed for 2-3 minutes to completely dry the column. • If performing ethanol precipitation, make sure that ethanol is evaporated before resuspending the RiboMinus™ RNA pellet in RNase-free water.

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Notes

Notes

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