invitrogen™

# RiboMinus™ Eukaryote Kit for RNA-Seq

| Catalog no. | Quantity | Store at 4ºC |
|---|---|---|
| A10837-02 | 2 prep | |
| A10837-08 | 8 prep | |

## Contents and Storage

The components included with the RiboMinus™ Eukaryote Kit for RNA-Sequencing (Seq) are listed below. Sufficient reagents are included to perform 2 (Cat. no. A10837-02) or 8 (Cat. no. A10837-08) purifications. Upon receipt, **store all components at 4ºC**.

| Components | A10837-02 | A10837-08 |
|---|---|---|
| RiboMinus™ Magnetic Beads (10 mg/ml) in phosphate buffered saline (PBS), pH 7.4 containing 0.01% Tween® 20 and 0.09% sodium azide | 2 ml | 6 ml |
| RiboMinus™ Eukaryote Probe in ultrapure water (15 pmol/µl) | 20 µl | 80 µl |
| Hybridization Buffer | 2.5 ml | 10 ml |
| DEPC-treated (RNase-Free) Water | 3 ml | 12 ml |

## Description

The RiboMinus™ Eukaryote Kit for RNA-Seq provides a novel and efficient method to isolate RNA molecules of the transcriptome devoid of large ribosomal RNA (rRNA) from total RNA for transcriptome analysis. The RiboMinus™ purification method is not dependent on the polyadenylation status or presence of a 5′-cap structure on the RNA which offer only a partial isolation of the transcriptome.

RNA-Seq is the digital interrogation of the transcriptome by next generation sequencing technology and provides detailed, high-throughput view of the transcriptome which is gaining increased attention in gene expression analysis. The first step in RNA-Seq is the isolation of whole transcriptome from total RNA. 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. Since large rRNA constitutes 90–95% RNA species in total RNA, whole transcriptome analysis without any contamination from rRNA is very difficult using existing RNA isolation methods and suggests the need for developing procedures that remove unwanted, abundant rRNA transcripts. The RiboMinus™ Eukaryote Kit for RNA-Seq allows for whole transcriptome isolation through selective depletion of eukaryote abundant ribosomal RNA molecules (18S, 28S) as well as small rRNA molecules (5S, 5.8S) from total RNA. The ribosomal RNA depleted RNA fraction is termed as RiboMinus™ RNA fraction and is enriched in polyadenylated (polyA) mRNA, non-polyadenylated RNA, pre-processed RNA, tRNA, 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.

Using RiboMinus™ Eukaryote Kit to isolate RiboMinus™ RNA results in efficient (up to 99.9%) removal of large 18S and 28S rRNA molecules from 10 µg total RNA enabling whole transcriptome analysis without any interference from highly abundant rRNA.

## System Overview

Total RNA is hybridized with eukaryote rRNA sequence-specific 5′-biotin labeled oligonucleotide probes (RiboMinus™ Eukaryote Probe for 18S, 28S, 5.8S, and 5S) to selectively deplete eukaryote abundant large ribosomal RNA molecules from total RNA. 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 ethanol precipitation.

## Product Specifications

| | | | |
|---|---|---|---|
| Starting Material: | 2–10 µg total RNA (<20 µl) | Probe Contents: | 2 probes each for 5S, 5.8S, 18S, 28S rRNA |
| rRNA Removal: | Up to 99.9% | LNA™ Content: | Each probe contains 3 LNA™ monomers in the oligonucleotide |
| RiboMinus™ RNA Yield: | ~1 µg from 10 µg human total RNA | Bead Binding Capacity: | >2,500 pmoles free biotin per mg RiboMinus™ Magnetic Beads |
| Probe Specificity: | Eukaryote (next page) | Bead Size: | 1 µm diameter |
| Probe Size: | 22–25 oligonucleotides | Bead Concentration: | 10 mg/ml |
| Probe Label: | 5′-biotin label | Magnet Particle: | Superparamagnetic polydisperse core-shell polystyrene particles |

Part no: 100004590 Rev. date: 8 Sep 2008

## General Guidelines

- Use disposable, individually wrapped, sterile plasticware and use sterile, new pipette tips and microcentrifuge tubes
- Wear latex gloves while handling reagents and RNA samples to prevent RNase contamination from the skin surface
- Always use proper microbiological aseptic techniques when working with RNA
- Use RNase *AWAY*® Reagent (Cat. no. 10328-011) to remove RNase contamination from surfaces
- During the mixing and washing steps with magnetic beads, mix beads by pipetting up and down or using a vortex set to low speed. A low speed centrifuge pulse may be required to remove beads stuck in the tube cap.
- 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.
- **Caution:** Sodium azide in the beads is toxic if ingested. Avoid pipetting by mouth. Sodium azide may react with lead and copper plumbing to form highly explosive metal azides. When disposing through plumbing drains, flush with large volumes of water to prevent azide buildup.

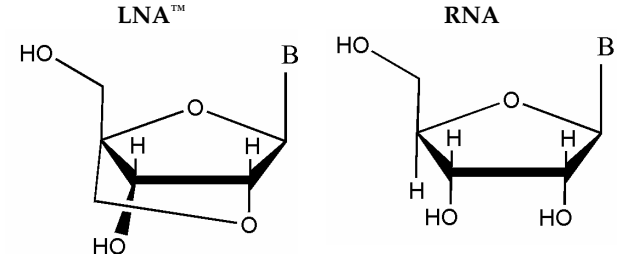
## RiboMinus™ Eukaryote Probe

The RiboMinus™ Eukaryote probe is an oligonucleotide probe mixture containing 2 probes each specific for 5S, 5.8S, 18S, and 28S rRNA. The probe is designed to hybridize with highly conserved regions of 5S, 5.8S, 18S, and 28S rRNA from several eukaryotic species such as human, mouse, rat, drosophila, yeast, and others with zero mismatches. For a detailed list of species specificity for these probes, visit www.invitrogen.com/rnapreps

Each probe is single-stranded and contains 3 LNA™ (Locked Nucleic Acid) monomers incorporated at specific locations. The incorporation of LNA™ (see below for details on LNA™) into the oligonucleotide probe increases the rRNA-probe stability. The 5′-end of each probe is conjugated to biotin to allow removal of rRNA/probe complexes by binding to streptavidin RiboMinus™ Magnetic Beads.

## 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 & Corey, 2001).




This configuration locks the sugar backbone resulting in an increase in $T_m$ (melting temperature). Incorporation of 3 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. The beads and hybridization conditions do not exhibit 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 >99.9% depletion of rRNA using 2–10 µg total RNA as the starting material.

## Materials Needed

- Total RNA (see below)
- RiboMinus™ Eukaryote Kit for RNA-Seq
- Magna-Sep™ Magnetic Particle Separator (Cat. no. K1585-01) or equivalent
- Sterile, RNase-free microcentrifuge tubes and water baths or heat blocks set to 70–75°C and 37°C
- Glycogen, 20 µg/µl (Cat. no. 10814-010) and 3 M sodium acetate in RNAse-free water
- 96–100% cold ethanol and 70% cold ethanol

## Preparing Total RNA

You need to isolate high-quality total RNA from samples using a method of choice prior to using this kit. We recommend isolating total RNA using the PureLink™ RNA Mini Kit (Cat. no. 12183018A or 12183020) or TRIzol® Reagent (Cat. no. 15596-026) available from Invitrogen (for details, visit www.invitrogen.com).

You need 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). If your downstream application requires DNA-free RNA, perform DNase-treatment of the total RNA before purifying RiboMinus™ RNA. Check the quality of your total RNA, including DNA contamination.

## Hybridization Step

Instructions are provided below to perform hybridization for 2–10 µg of your total RNA sample with the RiboMinus™ Eukaryote Probe. 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:

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|---|---|
| Total RNA (2–10 µg): | <20 µl |
| RiboMinus™ Probe (15 pmol/µl): | 10 µl |
| Hybridization Buffer: | 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. **Do not** cool samples quickly by placing tubes in cold water.
5. While the sample is cooling down, proceed to **Preparing Beads**.

## Preparing Beads

1. Resuspend RiboMinus™ Magnetic Beads in its bottle by thorough vortexing.
2. Pipet 750 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 separator for 1 minute. The beads settle to the tube side that faces the magnet. Gently aspirate and discard the supernatant.
4. Add 750 µl sterile, DEPC Water to the beads and resuspend beads by slow vortexing.
5. Place tube on a magnetic separator for 1 minute. Aspirate and discard the supernatant.
6. Repeat Steps 4–5 once.
7. Resuspend beads in 750 µl Hybridization Buffer and transfer 250 µl beads to a new tube for use at a later step.
8. Place the tube with 500 µl beads on a magnetic separator for 1 minute. Aspirate and discard the supernatant.
9. Resuspend beads in 200 µl Hybridization Buffer and keep the beads at 37°C until use.

## Removing rRNA

1. After the hybridized sample (above) has cooled to 37°C, briefly centrifuge the tube to collect the sample to the bottom of the tube.
2. Transfer the sample (~330 µl) to the prepared RiboMinus™ Magnetic beads from Step 9 (**Preparing Beads**, above). Mix well by pipetting up and down or low speed vortexing.
3. 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.
4. 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.**
5. Place the tube with 250 µl beads from Step 7 (**Preparing Beads**, above) on a magnetic separator for 1 minute. Aspirate and decant the supernatant.
6. To this tube of beads, add ~530 µl supernatant containing RiboMinus™ RNA from Step 4, above. Mix well by pipetting up and down or low speed vortexing.
7. 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.
8. Transfer the supernatant (~ 530 µl) containing **RiboMinus™ RNA** to a new tube.

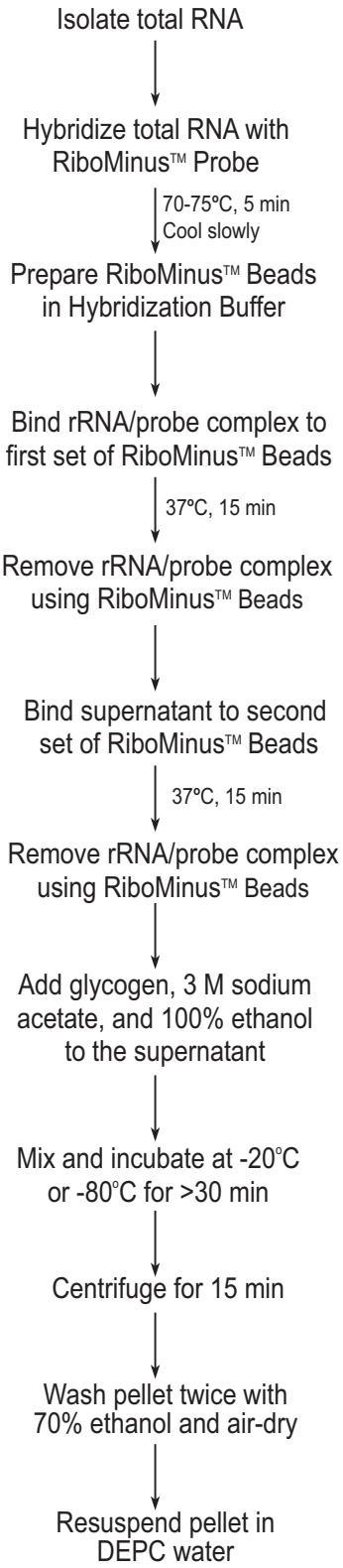
## Concentrating RiboMinus™ RNA Using Ethanol Precipitation

You can concentrate the RiboMinus™ RNA using ethanol precipitation that ensures recovery of smaller (<200 nt) RNA.

To concentrate RiboMinus™ RNA using PureLink™ RNA Mini Kit silica spin columns (Cat. no. 12183018A or 12183020) or RiboMinus™ Concentration Module (Cat. no. K1550-05), use a modified protocol with 50% ethanol available at www.invitrogen.com.

1. Transfer the RiboMinus™ RNA sample into a clean, RNAse-free 2 ml microcentrifuge tube.
2. Add the following components to RiboMinus™ RNA:
   - 1 µl glycogen (20 µg/µl)
   - 1/10$^{th}$ sample (eluted RNA) volume (53 µl for this protocol) of 3 M sodium acetate
   - 2.5X sample volumes (1,325 µl for this protocol) of 100% ethanol

**RiboMinus™ Workflow**



## Concentrating RiboMinus™ RNA Using Ethanol Precipitation, Continued

3. Mix well and incubate at –20ºC or –80°C for a minimum of 30 minutes.
4. Centrifuge the tube for 15 minutes ≥12,000 × g at 4°C. Carefully discard the supernatant without disturbing the pellet.
5. Add 500 µl 70% cold ethanol.
6. Centrifuge the tube for 5 minutes ≥12,000 × g at 4°C. Discard the supernatant without disturbing the pellet.
7. Repeat Steps 5–6 once.
8. Air-dry the pellet for ~5 minutes. Resuspend the RiboMinus™ RNA pellet in ~10–30 µl DEPC-treated water.
9. Place RiboMinus™ RNA on ice to proceed to desired downstream application or store RiboMinus™ RNA at –80°C until use.

## Analyzing RiboMinus™ RNA

The purified RiboMinus™ RNA is easily quantitated using UV absorbance at 260 nm or Quant-iT™ RNA Assay Kit. The RNA isolated using the RiboMinus™ Eukaryote Kit is of high-quality and is up to 99.9% depleted in rRNA species.

To verify rRNA depletion, perform agarose gel electrophoresis of the sample or use a bioanalyzer. Agarose gel electrophoresis analysis shows depletion of 18S and 28S rRNA bands as compared to a control sample. Absence of contaminating DNA and RNA degradation may also be confirmed by agarose gel electrophoresis. The efficiency of rRNA depletion in RiboMinus™ RNA, RNA degradation, and RNA concentration can also be analyzed using a bioanalyzer such as the Agilent 2100 bioanalyzer with an RNA LabChip®.

## Troubleshooting

| Problem | Cause | Solution |
|---|---|---|
| Low RNA yield | Low RNA content | Various tissues have different RNA content and the yield is dependent on the sample. |
| | Loss of pellet during ethanol precipitation | Remove supernatant from RNA pellet carefully. |
| 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™ Eukaryote Probe and RiboMinus™ Magnetic Beads for efficient removal of rRNA. |
| | Improper handling or drying of beads | To obtain best results with RiboMinus™ Magnetic Beads, follow the recommended guidelines 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 2 to prevent RNase contamination. |
| | Poor quality starting materials | Always use fresh samples or samples frozen at –80°C for total RNA isolation. 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** isolating RiboMinus™ RNA. |
| Inhibition of downstream enzymatic reactions | Presence of ethanol in purified RNA sample | For ethanol precipitation, make sure that ethanol is evaporated before resuspending the RiboMinus™ RNA pellet in DEPC-treated water. |

## References

Braasch, D. A., and Corey, D. R. (2001) Locked Nucleic Acid (LNA): Fine-tuning the Recognition of DNA and RNA. Chem Biol. *1*, 1-7

McTigue, P. M., Peterson, R. J., and Kahn, J. D. (2004) Sequence-dependent Thermodynamic Parameters for Locked Nucleic Acid (LNA)-DNA Duplex Formation. Biochemistry. *43*, 5388-5405

Ruan, Y., Le Ber, P., Ng, H., and Liu, E. (2004) Interrogating the Transcriptome. Trends Biotechnol. *22*, 23-30

## Quality Control

The Certificate of Analysis provides quality control information for this product, and is available by product lot number at www.invitrogen.com/support. Note that the lot number is printed on the kit box.

## Limited Use Label License No. 237: LNA™ Oligonucleotides

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