invitrogen™

RiboMinus™ Plant Kit for RNA-Seq

Catalog no.	Quantity	Store at 4ºC
A10838-02	2 prep	
A10838-08	8 prep	

Contents and Storage

The components included with the RiboMinus™ Plant Kit for RNA-Seq (Sequencing) are listed below. Sufficient reagents are included to perform 2 (Cat. no. A10838-02) or 8 (Cat. no. A10838-08) purifications. Upon receipt, **store all components at 4ºC**.

Components	A10838-02	A10838-08
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™ Plant 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™ Plant 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 (Lister *et al.*, 2008). 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 >80% 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™ Plant Kit for RNA-Seq allows for whole transcriptome isolation through selective depletion of plant abundant ribosomal RNA and includes probes designed to deplete nuclear-derived rRNAs (25/26S and 17/18S), chloroplast (23S and 16S), and mitochondrion (18S) 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™ Plant Kit to isolate RiboMinus™ RNA results in efficient (up to 99%) removal of plant nuclear (25/26S, 17/18S), chloroplast (23S, 16S), and mitochondrial (18S) rRNA molecules from up to 10 µg total RNA enabling whole transcriptome analysis without any interference from highly abundant rRNA.

System Overview

Total RNA is hybridized with plant rRNA sequence-specific 5′-biotin labeled oligonucleotide probes (RiboMinus™ Plant Probe, see next page) to selectively deplete plant 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 or RiboMinus™ Concentration Module.

Product Specifications

Starting Material:	1–10 µg total RNA (<10 µL)	Probe Contents:	6 probes each with specificity for plant nuclear (25/26S, 17/18S), chloroplast (23S, 16S), and mitochondrial (18S) rRNA
rRNA Removal:	Up to 99%	LNA™ Content:	Each probe contains 3 LNA™ monomers in the oligonucleotide
RiboMinus™ RNA Yield:	1–2 µg from 10 µg total RNA	Bead Binding Capacity:	~4,000 pmoles free biotin per mg RiboMinus™ Magnetic Beads
Probe Specificity:	Plant (next page)	Bead Size:	1 µm diameter
Probe Size:	22–25 oligonucleotides	Bead Concentration:	Approximately 10 mg/mL
Probe Label:	5′-biotin label	Magnet Particle:	Superparamagnetic polydisperse core-shell polystyrene particles

Intended Use

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

Part no: 100005390 MAN0000777 Rev. date: 8 Dec 2009

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.

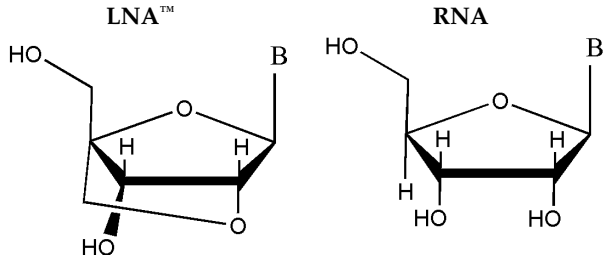
RiboMinus™ Plant Probe

The RiboMinus™ Plant probe is an oligonucleotide probe mixture containing 6 probes each specific for one or more of the conserved regions of the large cytosolic and chloroplast rRNA transcripts. The probes are designed to hybridize with highly conserved regions of the nuclear-derived rRNAs (25/26S and 17/18S), chloroplast (23S and 16S), and mitochondrion (18S) from a collection of model and important crop plants. 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 efficient depletion of rRNA using 1–10 µg total RNA as the starting material.

Materials Needed

- Total RNA (see below)
- RiboMinus™ Plant 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 Plant RNA Reagent (Cat. no. 12322-012), PureLink™ RNA Mini Kit (Cat. nos. 12183018A or 12183020), or TRIzol® Reagent (Cat. no. 15596-026) available from Invitrogen (for details, visit www.invitrogen.com).

You need 1–10 µg total RNA in no more than 10 µL for each reaction. Resuspend isolated total RNA in DEPC-treated water accordingly. 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 1–10 µg of your total RNA sample with the RiboMinus™ Plant 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:

Total RNA (1–10 µg):	<10 µL
RiboMinus™ Probe (15 pmol/µL):	10 µL
Hybridization Buffer:	100 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 the 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 and maintain the tube at 37ºC 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

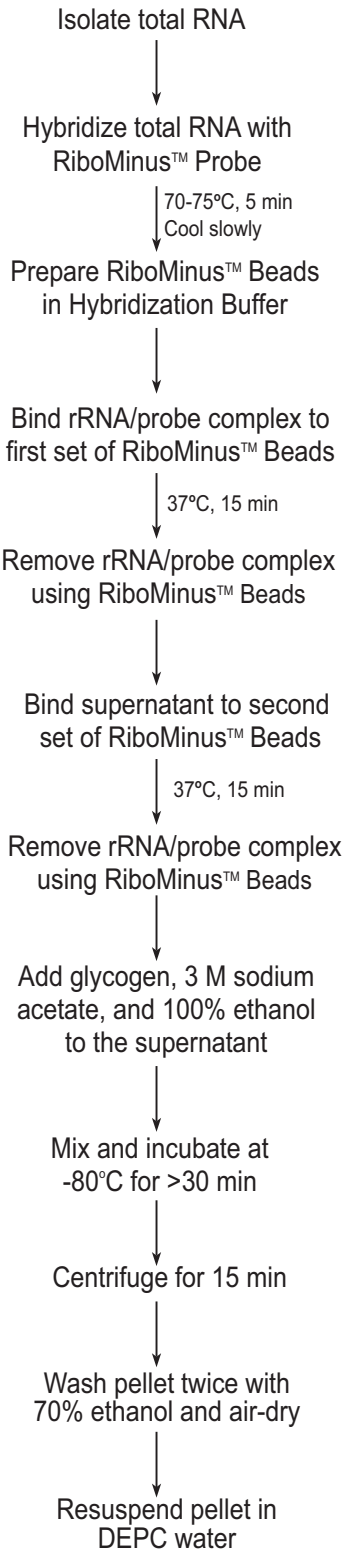
1. After the 37°C incubation step of the hybridized sample (above), briefly centrifuge the tube to collect the sample to the bottom of the tube.
2. Transfer the sample (~120 µL) to the prepared RiboMinus™ Magnetic beads from Step 9 (**Preparing Beads**, above). Mix well by pipetting up and down or low 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 ~320 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. 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.**
9. Transfer the supernatant (~ 320 µL) containing **RiboMinus™ RNA** to a new tube.

Concentrating RiboMinus™ RNA Using Ethanol Precipitation

To ensure recovery of smaller (<200 nt) RNA, concentrate the RiboMinus™ RNA using ethanol precipitation as described below or using a modified RiboMinus™ Concentration Module (Cat. no. K1550-05) protocol with 60% ethanol (protocol included with the kit).

1. Transfer the RiboMinus™ RNA sample into a clean, RNAse-free 1.5 mL or 2 mL microcentrifuge tube.
2. Add the following components to RiboMinus™ RNA:
   - 1 µL glycogen (20 µg/µL)
   - 1/10th sample (eluted RNA) volume (30 µL for this protocol) of 3 M sodium acetate
   - 2.5X sample volumes (750 µL for this protocol) of 100% ethanol

RiboMinus™ Workflow



Concentrating RiboMinus™ RNA Using Ethanol Precipitation, Continued

3. Mix well and incubate at –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 at ≥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 and proceed to desired downstream application or store RiboMinus™ RNA at –80°C.

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™ Plant Kit is of high-quality and is efficiently depleted in rRNA species.

To verify rRNA depletion, use a bioanalyzer or perform agarose gel electrophoresis of the sample. The efficiency of rRNA depletion in RiboMinus™ RNA, RNA degradation, and RNA concentration can be effectively analyzed using Agilent 2100 bioanalyzer with Agilent RNA 6000 Nano Kit. Agarose gel electrophoresis analysis can also show 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.

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 1–10 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™ Plant 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

Lister, R., O'Malley, R. C., Tonti-Filippini, J., Gregory, B. D., Berry, C. C., Millar, A. H., and Ecker, J. R. (2008) Highly Integrated Single-base Resolution Maps of the Epigenome in Arabidopsis. Cell *133*, 523-536

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*, 23-3

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.

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