Concentrating RiboMinus™ RNA Using Ethanol Precipitation, Continued

3. Mix well and incubate at –20°C or –80°C for a minimum of 30 minutes.
4. Centrifuge the tube for 15 minutes at 12,000 × g at 4°C. Carefully discard the supernatant without disturbing the pellet.
5. Add 500 μl of 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 ~30–50 μl DEPC-treated water.

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 Quanti-Fl® 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 RNA 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 RNA 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.

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References


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 License No. 237: LNA™ Oligonucleotides

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

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Part no: 100004590 Rev. date: 8 Sep 2008
For technical support, email tech_support@invitrogen.com. For country-specific contact information, visit www.invitrogen.com.
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 to set low speed. A low speed centrifugation 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 reaising 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/rnaprobes

Each probe is single-stranded and contains 3 LNA™ (Locked Nucleic Acid) monomers at specific locations. The incorporation of LNA™ (see below for details on LNA™) into the oligonucleotide probe increases the RNA-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 Tm (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 complex with RNA 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 RNA 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. 18814-010) 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. 15966-02b) 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 purification 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 >30 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)
   - RiboMinus™ Probe (15 pmol/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. Incubate the tube at 37°C, above. Mix well by pipetting up and down or low speed vortexing.
4. 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.
5. Add 750 µl sterile, DEPC Water to the beads and resuspend beads by slow vortexing.
6. Place tube on a magnetic separator for 1 minute. Aspirate and discard the supernatant.
7. Repeat Steps 4–5 once.
8. Resuspend beads in 750 µl Hybridization Buffer and transfer 250 µl beads to a new tube for use at a later step.
9. Place the tube with 500 µl magnetic separator for 1 minute. Aspirate and discard the supernatant.
10. Resuspend beads in 200 µl Hybridization Buffer and keep the beads at 37°C until use.

Removing RNA

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 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 RNA-probe complex. Do not discard the supernatant. The supernatant contains RiboMinus™ RNA. RiboMinus™ RiboMinus™ Concentration Module (Cat. no. K1550-05), use a modified protocol with 50% ethanol available at www.invitrogen.com.
5. Place the tube with 250 µl beads from Step 7 (Preparing Beads, above) on a magnetic separator for 1 minute. Aspirate and discard the supernatant.
6. To this tube of beads, add ~50 µl supernatant containing RiboMinus™ RNA from Step 4, above. Mix well by pipetting up and down or slow speed vortexing.
7. Incubate the tube at 37°C for 15 minutes. During incubation, gently mix 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 µl) 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)
   - 1/10th 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
3. Mix and incubate at -20°C or -80°C for ~30 min
4. Centrifuge for 15 min
5. Wash pellet twice with 70% ethanol and air-dry
6. Resuspend pellet in DEPC water
**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

**RiboMinus™ Workflow**

- g of your total RNA sample with the RiboMinus™ Eukaryote Probe. To process >10 μg of total RNA, dilute your sample into two samples, each containing <10 μg total RNA.
- Always use proper microbiological aseptic techniques when working with RNA

**Preparing Beads**

- 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/rnaseq
- Each probe is single-stranded and contains 3 LNA™ (Locked Nucleic Acid) monomers at specific locations.

**LNA™ (Locked Nucleic Acid)**

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

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**RiboMinus™ Magnetic Beads**

- The RiboMinus™ Magnetic Beads are streptavidin-coated magnetic beads used for the removal of probe/rRNA complexes by binding to streptavidin-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 RNA or the probe alone.

**Materials Needed**

- Total RNA (see below)
- RiboMinus™ Eukaryote Kit for RNA-Seq
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- 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).
- 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 RNA 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 RNA using 2–10 μg total RNA as the starting material.

**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:
   - Total RNA (2–10 μg)
   - RiboMinus™ Probe (15 pmol/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.

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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 samples (~330 μl) to the prepared RiboMinus™ Magnetic beads from Step 9 (Preparing Beads, above). Mix well by pipetting up and down or slow speed vortexing.
3. Incubate the tube at 37°C for 15 minutes. During incubation, gently mix 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 discard the supernatant.
6. To this tube of beads, add ~500 μl supernatant containing RiboMinus™ RNA from Step 4, above. Mix well by pipetting up and down or slow speed vortexing.
7. Incubate the tube at 37°C for 15 minutes. During incubation, gently mix contents occasionally. Briefly centrifuge the tube to collect the sample to the bottom of the tube.
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- You can concentrate the RiboMinus™ RNA using ethanol precipitation that ensures recovery of smaller (~200 nM) RNA.
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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)
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3. Mix and incubate at −20°C or−80°C for >30 min
4. Centrifuge for 15 min
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6. Resuspend pellet in DEPC water
Concentrating RiboMinus™ RNA Using Ethanol Precipitation, Continued

3. Mix well and incubate at –20°C or –80°C for a minimum of 30 minutes.
4. Centrifuge the tube for 15 minutes at 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 ~30–50 μl DEPC-treated water.

Place RiboMinus™ RNA on ice to proceed to desired downstream application or store RiboMinus™ RNA at –80°C until use.

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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.

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