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Enabling complete transcriptome sequencing

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RIBO COP™

Select and Deplete

rRNA Depletion Kit User Guide

Catalog Number:

037.24 (RiboCop rRNA Depletion Kit (Human/Mouse/Rat), 24 preps)

037.96 (RiboCop rRNA Depletion Kit (Human/Mouse/Rat), 96 preps)

042.08 (SENSE Total RNA-Seq Library Prep Kit for Illumina with RiboCop, 8 preps)

042.24 (SENSE Total RNA-Seq Library Prep Kit for Illumina with RiboCop, 24 preps)

042.96 (SENSE Total RNA-Seq Library Prep Kit for Illumina with RiboCop, 96 preps)

037UG073V0104

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When describing a procedure for publication using this product, please refer to it as the RiboCop rRNA Depletion Kit.

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1. Overview

Lexogen's RiboCop rRNA Depletion Kit enables removal of ribosomal RNA (rRNA) from human, mouse, and rat total RNA and is suited for Next Generation Sequencing (NGS) and similar applications. Samples are treated using a set of affinity probes for specific depletion of rRNA sequences. Number and positioning of probes are designed for applicability towards intact as well as fragmented input RNA. RiboCop probes efficiently remove ribosomal RNA and therefore afford a comprehensive view of transcriptome composition. Samples void of 28S, 18S, 5.8S, 45S, 5S, mt16S, mt12S ribosomal sequences are obtained within 2 hours of total processing time. No enzymatic reactions or mechanical shearing steps are involved, leaving full-length transcripts intact for downstream processing.

Affinity probes and 10 ng - 1 µg total RNA are mixed and denatured, facilitating access of probes to target sequences. Afterwards, hybridization is performed at elevated temperature. Depletion beads are conditioned and used to remove probes along with hybridized ribosomal RNA from solution. A final purification step using magnetic beads rounds off the procedure. Recovered RNA may be directly used for NGS library preparation, leaving enough material for optional quality control. The entire protocol is automation friendly by utilizing magnetic beads for depletion and purification purposes.

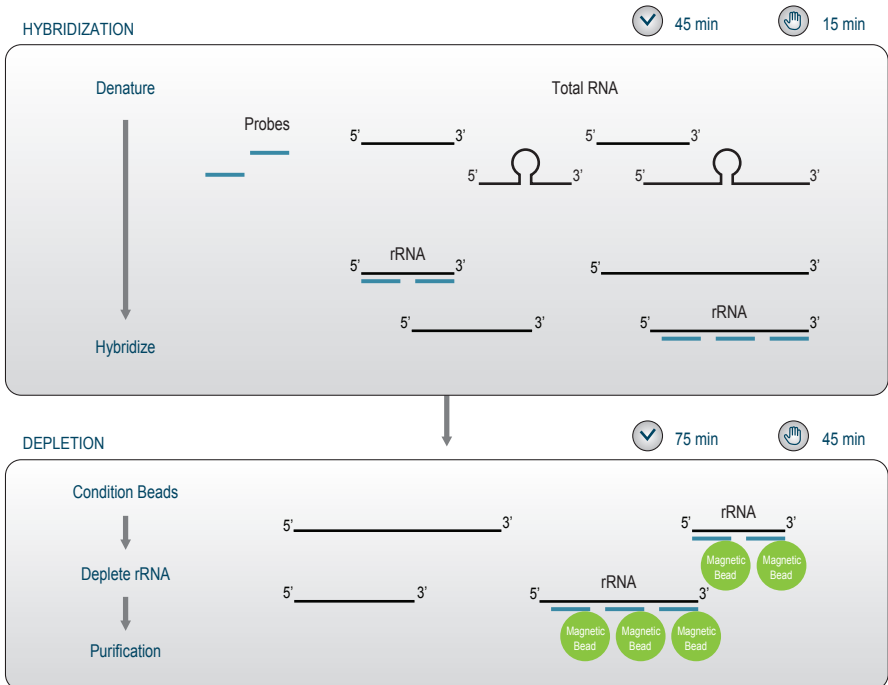


Figure 1. Schematic overview of the RiboCop workflow.

2. Kit Components and Storage Conditions

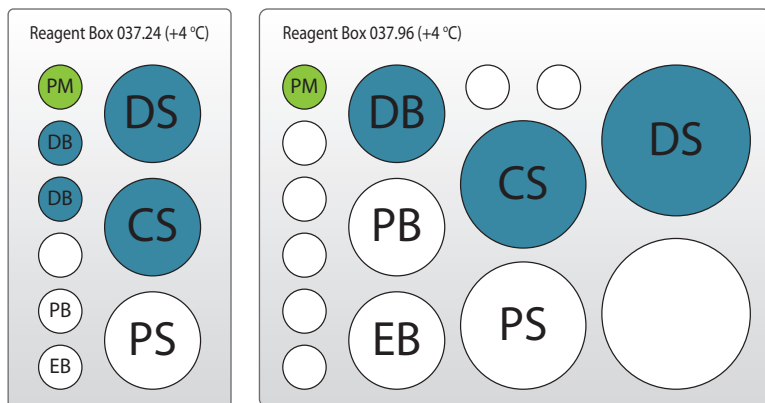


Figure 2. Location of kit components for 24 and 96 prep kits.

Kit Component	Tube Label	Volume* for		Storage
		24 preps	96 preps	
Depletion Solution	DS ●	8610 µl	34440 µl	+4 °C
Probe Mix	PM ●	150 µl	600 µl	-20 °C/+4 °C
Depletion Beads	DB ●	2070 µl	8280 µl	+4 °C
Conditioning Solution	CS ●	4110 µl	16440 µl	+4 °C
Purification Beads	PB ○	900 µl	3600 µl	+4 °C
Purification Solution	PS ○	6000 µl	24000 µl	+4 °C
Elution Buffer	EB ○	1320 µl	5280 µl	-20 °C/+4 °C

* including 10 % surplus

Upon receiving the RiboCop kit, store all components at +4 °C. **EB** ○ and **PM** ● can be stored either at +4 °C or -20 °C, avoid frequent freeze-thaw cycles. Before use, check the contents of **PS** ○ which may precipitate during shipping. If a precipitate is visible or the content appears milky, incubate at 37 °C until solution components dissolve completely.

3. User-supplied Consumables and Equipment

Ensure to have all necessary materials and equipment available before beginning RNA depletion. All reagents, equipment and labware must be free of nucleases and nucleic acid contamination.

Reagents

- 80 % fresh ethanol (washing of Purification Beads, **PB** ○).

Equipment

- Magnetic rack / plate.
- Benchtop centrifuge for spinning down liquids.
- Vortex mixer.
- Calibrated single-channel pipettes for handling 1 µl to 1000 µl volumes.
- Thermomixer.
- UV-spectrophotometer to quantify RNA.

Labware

- Suitable low-binding pipette tips (pipette tips with aerosol barriers recommended).
- 1.5 ml reaction tubes, low binding, certified ribonuclease-free.
- 200 µl PCR tubes or 96-well plates and caps or sealing foil.
- Benchtop cooler or ice pellets in ice box (for short-term storage of RNA).

Optional Equipment and Solutions

- Automated microfluidic electrophoresis station (Agilent Technologies 2100 Bioanalyzer).
- Benchtop fluorometer and appropriate assays (for RNA quality control).
- Agarose gels, dyes, and electrophoresis rig (for RNA quality control).
- RNaseZap.
- RNase inhibitor.

The complete set of materials, reagents, and labware for quality control is not listed. Consult Appendix A (p.13) for more information.

4. Guidelines

RNA Handling

- RNases are ubiquitous, and special care should be taken throughout the procedure to avoid RNase contamination.
- Use commercial ribonuclease inhibitors (i.e. RNasin, Promega Corp.) to maintain RNA integrity when storing samples.
- Use a sterile and RNase-free workstation or laminar flow hood if available. Please note that RNases may still be present on sterile surfaces, and that autoclaving does not completely eliminate RNase contamination. Before starting rRNA depletion, clean your work space, pipettes, and other equipment with RNase removal spray (such as RNaseZap, Ambion Inc.) according to manufacturer's instructions.
- Protect all reagents and your RNA samples from RNases on your skin by wearing a clean lab coat and fresh gloves. Change gloves after making contact with equipment or surfaces outside the RNase-free zone.
- Avoid speaking above opened tubes. Keep reagents closed when not in use to avoid airborne RNase contamination.

Bead Handling

- Beads are stored at +4 °C and must be resuspended before usage. Beads can be resuspended by pipetting up and down several times or by vortexing. When properly resuspended, the solution should have a uniform brown color with no visible clumping on the walls or bottom of the tube.
- Beads may stick to certain pipette tips, in which case removing the beads from the inside of the tip may be impossible. Avoid resuspending by repeated pipetting and instead resuspend by vortexing if this occurs with your tips.
- Beads are superparamagnetic and are collected by placing the plate / tube in a magnetic 96-well plate or a magnetic stand. The time required for complete separation will vary depending on the strength of your magnets, tube thickness, viscosity of the solution, and the proximity of the tube to the magnet. Separation time may need to be adjusted accordingly. When fully separated, the supernatant should be completely clear and the beads collected at one point or as a ring along the wall of the tube, depending on the magnet that was used.
- To remove the supernatant the plate / tube containing the beads has to stay in close contact with the magnet. Do not remove the plate / tube from the magnetic stand when removing the supernatant, as the absence of the magnet will cause the beads to go into suspension again.

- In general, beads should not be centrifuged during the protocol. However, should drops of fluid stay on the side of the reaction tube (e.g., after mixing by vortexing), centrifugation at 2,000 x g for 30 seconds should be carried out before placing the tube on the magnetic rack.
- Allowing the beads to dry out can damage them. Always keep the beads in suspension except for the short period after withdrawing the supernatant but before adding the next reagent. Beads can be resuspended by vortexing but make sure that beads are not deposited on the tube walls above the level of the liquid, where they can dry during incubation. If necessary, stuck beads can be collected by centrifuging the plate/tube briefly with an appropriate centrifuge.

General

- Unless explicitly mentioned, all steps should be carried out at room temperature (RT) between 20 °C and 25 °C. Results may be negatively impacted if the protocol is performed at temperatures outside this range. While reaction set-up is often performed at RT, incubation temperatures are explicitly defined and must be strictly adhered to.
- Ensure that adequate volumes of all reagents and the necessary equipment are available and set to the proper temperatures before beginning the protocol.
- Spin down solutions before opening tubes.
- Make sure to pre-heat thermomixers (dry bath incubators) well in advance.
- Perform all pipetting steps with calibrated pipettes and always use fresh tips. Pipette carefully to avoid foaming as some solutions contain detergents.
- Thaw all necessary buffers at room temperature or as indicated in the preparation tables at the beginning of each step of the detailed protocol. Mix reagents well by vortexing or pipetting repeatedly and centrifuge briefly with a benchtop centrifuge to collect contents before use.

5. Detailed Protocol

Preparation

Hybridization	Bead Conditioning and Depletion	Purification
DS – equilibrated at RT PM – equilibrated at RT	DB – stored at 4 °C CS – stored at 4 °C	PB – stored at 4 °C PS – stored at 4 °C 80 % EtOH – provided by user, prepare fresh! EB – thawed at RT or stored at 4 °C
Thermomixer set to 75 °C, 300 rpm	Thermomixer set to 60 °C, 400 rpm	Magnetic rack

REMARK: We recommend placing the Purification Beads (PB ○) and Purification Solution (PS ○) for step 17 at room temperature prior to starting the protocol to give them enough time to equilibrate.

Hybridization

Probes and total RNA are mixed, denatured, and hybridized.

- 1 Provide 10 - 1000 ng total RNA in a total volume of 5 µl. Dilute using RNase-free water, if required.
- 2 Add 60 µl Depletion Solution (DS ●).
- 3 Add 5 µl Probe Mix (PM ●) and vortex gently.
- 4 Put the tube to a thermomixer and denature at 75 °C for 5 minutes under gentle agitation at 300 rpm.
- 5 Decrease the temperature to 60 °C and incubate for 30 minutes under gentle agitation at 300 rpm. **REMARK:** Bead Conditioning (p.10) may be conducted during this incubation step. However, if more time is required for Bead Conditioning batched processing prior to Hybridization is suggested.

Bead Conditioning

Depletion Beads are conditioned for downstream application.

- 6 Resuspend and mix Depletion Beads (**DB** ●) properly. Transfer 75 μ l per reaction to a fresh tube.

- 7 Place the tube in a magnetic rack and let the beads collect for 2-5 minutes or until the supernatant is completely clear (depends on the magnet strength). Remove and discard the supernatant with a pipette while the tube remains in contact with the magnet.

- 8 Remove the tube from the magnetic rack and add 75 μ l Conditioning Solution (**CS** ●) per reaction. Resuspend the beads by vortexing briefly and transfer the tube to the magnetic rack. Incubate for 2 minutes. Remove and discard the supernatant.

- 9 Repeat this conditioning step once (for a total of two rounds of conditioning).

- 10 Remove the tube from the magnetic rack and resuspend the beads in 75 μ l of Depletion Solution (**DS** ●). Transfer the tube to the magnetic rack and let the beads collect for 2-5 minutes or until the supernatant is completely clear. Remove and discard the supernatant.

- 11 Repeat this washing step twice (for a total of three washes).

- 12 Resuspend the beads in 30 μ l of Depletion Solution (**DS** ●).

Depletion

Depletion Beads are used to remove probes along with hybridized ribosomal RNA from solution.

- 13 Spin down hybridized sample as soon as incubation (step 5) is finished. Add 30 μ l freshly conditioned beads. Mix by pipetting up and down 8 times. **REMARK:** Set pipette to large value for mixing but avoid formation of bubbles.

- 14 Put the tube back to the thermomixer and incubate at 60 °C for 15 minutes under gentle agitation at 400 rpm. Spin down.

- 15 Place the tube in a magnetic rack and let the beads collect for 5 minutes.

- 16 Recover and transfer 80 μ l of supernatant to a fresh tube without disturbing collected beads.

Purification

The depleted RNA is purified using magnetic beads. The Purification Beads (**PB** ○) and Purification Solution (**PS** ○) should equilibrate for 30 minutes at room temperature before use. **PB** ○ may have settled and must be properly resuspended before adding them to the reaction.

- 17 Add 32 μ l Purification Beads (**PB** ○) and 144 μ l Purification Solution (**PS** ○). Mix well by pipetting. Incubate for 20 minutes at room temperature.

- 18 Place the tube onto a magnetic rack and let the beads collect for 5-10 minutes or until the supernatant is completely clear. **REMARK:** In order to speed up collection of beads application of the strongest magnetic rack available is suggested.

- 19 Remove and discard the clear supernatant without removing the tube from the magnetic rack. Make sure that accumulated beads are not disturbed.

- 20 Add 30 μ l of Elution Buffer (**EB** ○), remove the tube from the magnet and resuspend the beads properly in EB. Incubate for 2 minutes at room temperature.

- 21 Add 66 μ l of Purification Solution (**PS** ○) to the beads / **EB** ○ mix to re-precipitate the sample. Mix thoroughly and incubate for 5 minutes at room temperature.

- 22 Place the tube onto a magnetic rack and let the beads collect for 2-5 minutes.

- 23 Remove and discard the clear supernatant without removing the tube from the magnetic rack. Make sure that accumulated beads are not disturbed.

- 24 Add 120 μ l of 80 % EtOH and wash the beads for 30 seconds. Leave the tube in contact with the magnet as beads should not be resuspended during this washing step. Remove and discard the supernatant.

- 25 Repeat this washing step once for a total of two washes. Make sure to remove the supernatant completely.

- 26 Leave the tube in contact with the magnet and let the beads dry for 5 -10 minutes or until all ethanol has evaporated. **ATTENTION:** Do not let the beads dry too long (visible cracks appear), as this will negatively influence the elution and hence the resulting yield.

- 27 Add 16 μ l of Elution Buffer (**EB** ○), remove the tube from the magnet and resuspend the beads properly in (**EB** ○). Incubate for 2 minutes at room temperature.

- 28 Place the tube onto a magnetic rack and let the beads collect for 2-5 minutes. Transfer 14 μ l of the supernatant into a fresh tube.

- 29 At this point, depleted RNA samples are ready for quality control and downstream application. (Appendix B, p.14)

6. Short Procedure

45 min

Hybridization and Bead Conditioning

<input type="checkbox"/>	Provide 10 ng - 1 µg total RNA in 5 µl.	
<input type="checkbox"/>	Add 60 µl DS ●.	
<input type="checkbox"/>	Add 5 µl PM ● and vortex gently.	Hybridization
<input type="checkbox"/>	Denature for 5 min at 75 °C / 300 rpm.	
<input type="checkbox"/>	Incubate for 30 min at 60 °C / 300 rpm.	
<input type="checkbox"/>	Resuspend DB ●, transfer 75 µl to a fresh tube.	
<input type="checkbox"/>	Place on magnet for 2 - 5 min, remove and discard supernatant.	
<input type="checkbox"/>	Resuspend in 75 µl CS ●, incubate 2 min on magnetic rack.	Bead Conditioning
<input type="checkbox"/>	Remove and discard supernatant. Repeat once.	
<input type="checkbox"/>	Resuspend in 75 µl DS ●, place on magnet for 2 - 5 min, remove and discard supernatant. Repeat twice.	
<input type="checkbox"/>	Resuspend in 30 µl DS ●.	

75 min

Depletion and Purification

<input type="checkbox"/>	Spin down hybridized sample. Add 30 µl conditioned beads. Mix by pipetting 8x up and down.	
<input type="checkbox"/>	Incubate for 15 min at 60 °C / 400 rpm. Spin down.	Depletion
<input type="checkbox"/>	Place on magnet for 2 - 5 min.	
<input type="checkbox"/>	Transfer 80 µl supernatant to a fresh tube.	
<input type="checkbox"/>	Add 32 µl PB ○ and 144 µl PS ○, mix well, incubate for 20 min at RT.	
<input type="checkbox"/>	Place on magnet for 5 - 10 min, remove and discard supernatant.	
<input type="checkbox"/>	Add 30 µl EB ○, mix well, incubate 2 min at RT.	
<input type="checkbox"/>	Add 66 µl PS ○, mix well, incubate 5 min at RT.	
<input type="checkbox"/>	Place on magnet for 2 - 5 min, remove and discard supernatant.	Purification
<input type="checkbox"/>	Wash the beads twice with 120 µl 80 % EtOH, 30 sec.	
<input type="checkbox"/>	Air dry beads for 5 - 10 min.	
<input type="checkbox"/>	Add 16 µl EB ○, mix well, incubate 2 min at RT	
<input type="checkbox"/>	Place on magnet for 2 - 5 min. Transfer 14 µl of the supernatant into a fresh tube.	

7. Appendix A: RNA Requirements and Results

Potential Contaminants

RNA samples should be free of salts, metal ions, and organic solvents, which can be carried over from RNA extraction. Several sources of contamination can be detected with a UV-Vis spectrophotometer. An acceptably pure RNA sample should have an A260/A280 ratio between 1.8 and 2.1. The A260/A230 ratio should also be greater than 1.8. Several common contaminants including proteins, chaotropic salts and phenol absorb strongly between 220 and 230 nm and can often be identified as peaks in this region. Contamination with any of these generates a lower A260/230 ratio. Phenol also has an absorption maximum between 250 and 280 nm, which overlaps that of nucleic acid, so high 230 nm absorbance combined with a biphasic or broad peak between 250 and 280 nm may indicate contamination with phenol rather than chaotropic salts.

RNA Integrity

High quality RNA-Seq data relies on high quality input RNA. Lexogen's SPLIT RNA Extraction Kit, Cat. No. 008.48 is recommended as it yields pure RNA that is free of gDNA. The integrity of an RNA sample may be assessed using a variety of methods. We recommend application of a microcapillary electrophoresis assay such as the RNA 6000 series for the 2100 Bioanalyzer (Agilent Technologies Inc.), although RNA quality can also be analyzed by denaturing agarose electrophoresis if such a device is not available. Most platforms will carry out an automated peak analysis and generate a quality score (RIN or RQN) in addition to the 28S/18S rRNA ratio.

Typical Results

Quality control of depleted RNA is recommended prior to conducting library preparation and sequencing runs. Successful rRNA depletion results in removal of (i.a.) 28S and 18S rRNA. These rRNA peaks usually dominate electropherograms of intact total RNA and should be absent after RiboCop depletion.

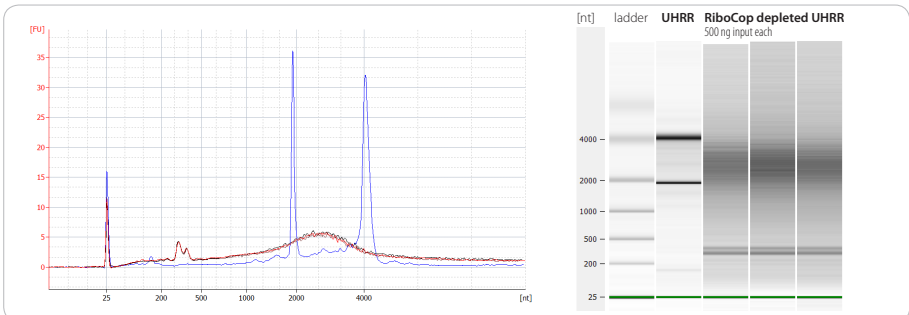


Figure 3. Overlay of RNA Bioanalyzer traces before and after RiboCop depletion. Blue trace: diluted total Universal Human Reference RNA, intact (RIN 9.1). Red, brown and black traces: RiboCop-depleted material. The three reactions shown here were performed using 500 ng UHRR as input each. 28S and 18S rRNA peaks are absent, indicating successful depletion. Depleted RNA is detected as a broad smear reaching beyond 4kb.

8. Appendix B: Downstream Processing

This protocol was tested extensively with Universal Human Reference RNA (UHRR) and Human Brain Reference RNA (HBRR) as well as mouse and rat samples. Depleted RNA samples are ideally suited for downstream NGS library preparation using Lexogen's SENSE Total RNA-Seq Library Prep Kit (Cat. No. 009, 042). 8 μ l of eluted RNA may be directly fed into library preparation, leaving enough material for optional quality control.

9. Appendix C: Revision History

Publication No.	Change	Page
037UG073V0100	Initial Release	
037UG073V0101	Consistency changes.	
037UG073V0102	Added section "Typical Results"	13
	Increased input to 1 μ g	4, 9
	Increased time on magnet in step 18	11
	Protocol available for human/mouse/rat	
037UG073V0103	Update of Figure 1	4
037UG073V0104	Lowered input amount to 10 ng	4, 9, 12

10. Notes

RiboCop rRNA Depletion Kit · User Guide

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