LEXOGEN

Enabling complete transcriptome sequencing

RiBOCOP™

Select and Deplete

rRNA Depletion Kits for Bacteria

User Guide

125 (RiboCop rRNA Depletion Kit for Mixed Bacterial Samples (META))
126 (RiboCop rRNA Depletion Kit for Gram Negative Bacteria (G-))
127 (RiboCop rRNA Depletion Kit for Gram Positive Bacteria (G+))

125UG246V0100

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1. Overview

Lexogen's RiboCop rRNA Depletion Kits for Bacteria enable removal of ribosomal RNA (rRNA) from bacterial total RNA and are suited for Next Generation Sequencing (NGS) and similar applications.

RiboCop uses a set of affinity probes for specific depletion of rRNA sequences. The number and positioning of probes are designed for compatibility with intact and fragmented input RNA. RiboCop probes efficiently remove ribosomal RNA and therefore afford a comprehensive view of transcriptome composition. Samples void of 23S, 16S, and 5S ribosomal sequences are obtained within 1.5 hours of total processing time. No enzymatic reactions or mechanical shearing steps are involved, leaving full-length transcripts intact for downstream processing.

Affinity probes are mixed with total RNA and denatured, facilitating access of probes to target sequences. Afterwards, hybridization is performed at elevated temperature. Depletion beads are washed and used to remove probes that are hybridized to rRNA from the solution. A final purification step using magnetic beads rounds off the procedure. Recovered RNA may be directly used for NGS library preparation, with sufficient volume for optional quality control. The entire protocol is automation-friendly, using magnetic beads for depletion and purification purposes.

The recommended input range for efficient depletion with RiboCop rRNA Depletion Kits for Bacteria is 1 - 1,000 ng of total RNA for monocultures of Gram Negative or Gram Positive bacteria using the respective G- and G+ Probe Mixes (Cat. No. 126 and 127, respectively). The META Probe Mix (Cat. No. 125) is primarily suited for depletion of 1 - 1,000 ng of total RNA from mixed bacterial samples (e.g., for meta-transcriptome analysis of complex communities or microbiome samples). In this case, depletion rates may vary depending on the complexity and species composition within the population subjected to depletion. The META Probe Mix may also be used at 1 - 100 ng when working with monocultures. RiboCop is suitable for intact or degraded RNA, including FFPE samples (Appendix A, p.15).

RiboCop is recommended for downstream NGS library preparation using the CORALL Total RNA-Seq Library Prep Kit (Cat. No. 095) or adapter ligation protocols such as Lexogen's Small RNA-Seq Library Prep Kit (Cat. No. 052).

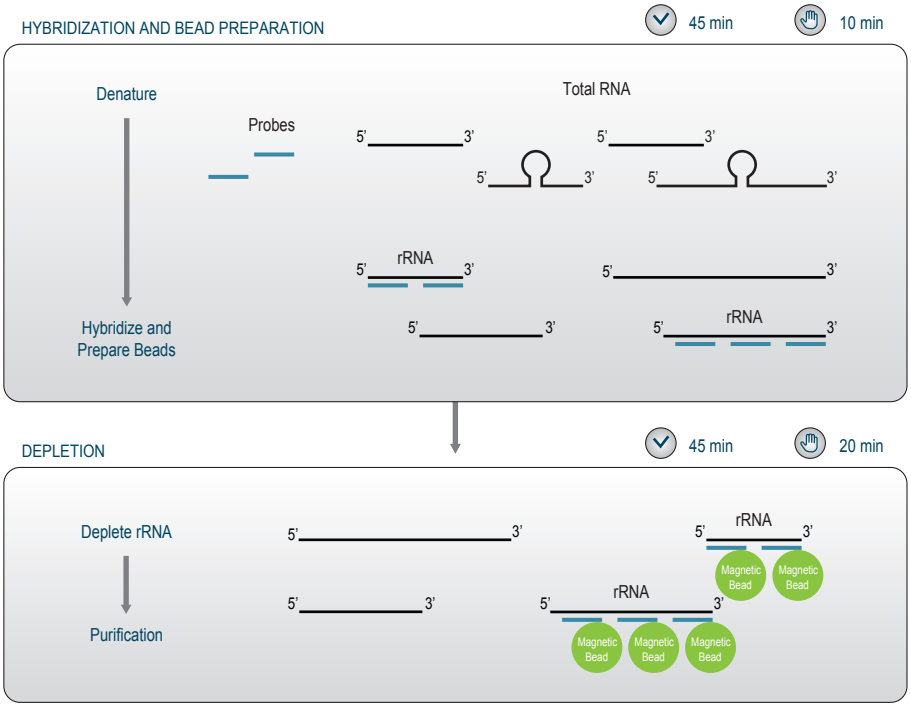

Figure 1. Schematic overview of the RiboCop workflow.

2. Kit Components and Storage Conditions

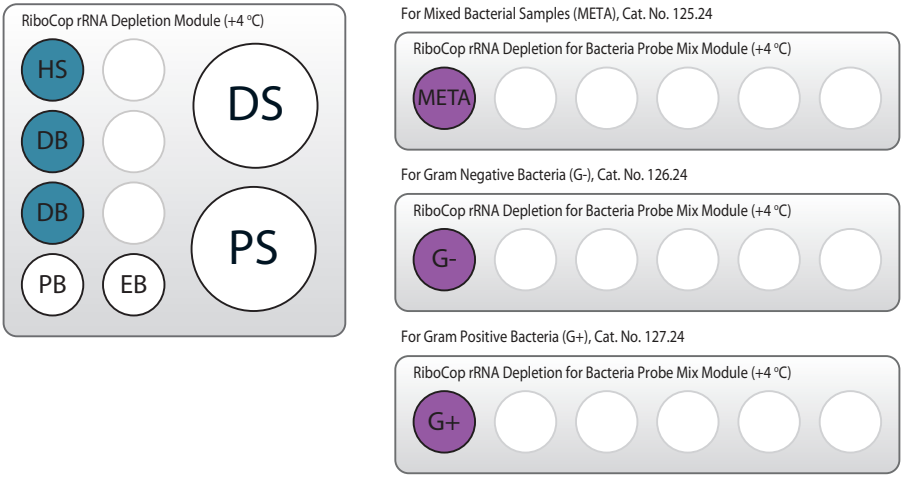

Figure 2. Location of kit components for 24 prep kits. RiboCop rRNA Depletion for Bacteria Kits are available for Mixed Bacterial Samples (META, Cat. No. 125.24) and for Gram Negative (G-, Cat. No. 126.24) or Gram Positive (G+, Cat. No. 127.24) Bacteria. Each RiboCop rRNA Depletion Kit for Bacteria consists of a Depletion Module and one of the three available Probe Mix Modules. 96 prep kits contain 4 Depletion Modules and 4 Probe Mix Modules. Trial kits contain reagents sufficient for 4 preps.

Kit Component	Tube Label	Volume*	Storage
		24 preps	
Hybridization Solution	**HS** ●	106 µl	+4 °C
Probe Mixes META, G-, or G+	**META / G- / G+** ●	132 µl	-20 °C/+4 °C
Depletion Beads	**DB** ●	1,980 µl	+4 °C
Depletion Solution	**DS** ○	6,732 µl	+4 °C
Purification Beads	**PB** ○	634 µl	+4 °C
Purification Solution	**PS** ○	4,594 µl	+4 °C
Elution Buffer	**EB** ○	1,109 µl	-20 °C/+4 °C

* including ≥10 % surplus

Upon receiving the RiboCop kit, store all components at +4 °C. **EB** ○ and Probe Mixes (**META**, **G-**, **G+**) ● can also be stored at -20 °C but frequent freeze-thaw cycles should be avoided. Before use, check the contents of **PS** ○ which may precipitate during shipping. If a precipitate is visible or the content appears turbid, incubate at 37 °C until solution components dissolve completely. Equilibrate to room temperature again before use.

3. User-Supplied Consumables and Equipment

Check to ensure that you have all of the necessary materials and equipment before beginning rRNA depletion. All reagents, equipment and labware must be free of nucleases and nucleic acid contamination.

Reagents

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

Equipment

- Magnetic rack or plate e.g., for 1.5 ml tubes: BcMag Magnetic separator-24, article# MS-03 from Bioclone; for 96-well plates: 96S Super Magnet Plate, article# A001322 from Alpaqua, or Dynamag™-96 Side/Side Skirted Magnets, article # 12331D/12027 from Thermo Fisher.
- Benchtop centrifuge for spinning down liquids.
- Vortex mixer.
- Calibrated single-channel pipettes for handling 1 µl to 1,000 µl volumes.
- Thermomixer.
- UV-spectrophotometer to quantify RNA.

Labware

- Suitable low-binding pipette tips (pipette tips with aerosol barriers recommended).
- 1.5 ml 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 and quantification).
- 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.15) 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. Well before starting a library preparation, clean your work space, pipettes, and other equipment with RNase removal spray (such as RNaseZap, Ambion Inc.) as per the manufacturer's instructions. **ATTENTION:** Do not forget to rinse off any RNaseZap residue with RNase-free water after usage. Residues of RNaseZap may damage the RNA.
- 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 of 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 or storage bottle.
- 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 plate or stand. The time required for complete separation will vary depending on the strength of your magnets, wall thickness of the wells / tubes, viscosity of the solution, and the proximity of the well / 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 well / 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 plate / stand when removing the supernatant, as the absence of the magnet will cause the beads to go into suspension again.

- When using a multichannel pipette to remove the supernatant, make sure not to disturb the beads. If beads were disturbed, ensure that no beads are stuck to the pipette tip opening and leave the multichannel pipette in the well for an extra 30 seconds before removing the supernatant. This way all beads can be recollected at the magnet and the clear supernatant can be removed.
- In general, beads should not be centrifuged during the protocol. However, should drops of fluid stay on the wall of the reaction tube / plate (e.g., after mixing by vortexing), centrifugation at 2,000 x g for 30 seconds should be carried out before placing the tube / plate on the magnetic stand / plate.
- Allowing the beads to dry out can damage them. Always keep the beads in suspension except for the short period after withdrawing the supernatant, and before adding the next reagent. Beads can be resuspended by vortexing, but make sure that beads are not deposited on the well / 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 a suitable benchtop centrifuge.

General

- The protocol can be performed in 1.5 ml tubes, PCR tubes, or PCR plates. When using PCR tubes or plates, shaking can be omitted and a thermocycler may be used. However, input RNA amounts may need to be reduced.
- Pre-heat thermomixers (or thermocyclers) in advance of starting. Thermomixers may take several minutes to cool from 75 °C to 60 °C. To increase the speed of the protocol, use two thermomixers: set to 75 °C for step 4 and 60 °C for step 5.
- Mixing may be performed by pipetting, or gentle brief vortexing. Mix until the sample is homogeneous.
- Perform all pipetting steps with calibrated pipettes and always use fresh tips. Pipette carefully to avoid foaming as some solutions contain detergents.
- Equilibrate all necessary buffers at room temperature or as indicated in the preparation tables at the beginning of the protocol. Mix reagents well by vortexing or pipetting repeatedly and spin down briefly before use.
- Ensure that adequate volumes of all reagents and the necessary equipment are available and set to the proper temperatures before beginning the protocol.
- 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.
- **PB** ○ and **PS** ○ are viscous solutions that require care to pipette accurately. Be sure to pipette slowly and check the graduation marks on your pipette tips when removing an aliquot.

5. Detailed Protocol RiboCop for Bacteria

Probe Mix Selection

The following Probe Mixes are available: META, G-, and G+. The G- and G+ Probe Mixes are specifically designed for efficient depletion of monocultures of Gram Positive and Gram Negative bacteria up to 1,000 ng total RNA input. The META Probe Mix is primarily suited for depletion of 1 - 1,000 ng of total RNA from mixed bacterial samples (e.g., for metatranscriptome analysis of complex communities or microbiome samples). In this case, depletion rates may vary depending on the complexity and species composition within the population subjected to depletion. The META Probe Mix may also be used at 1 - 100 ng when working with monocultures. It is recommended to use total RNA input amounts compatible to the library preparation kit used for library generation after rRNA depletion. Typically, 100 ng total RNA input is recommended for initial experiments.

Input type	Probe Mix	Input recommendations
Mixed Bacterial Samples	META	1 - 1,000 ng for mixed communities 1 - 100 ng for G- / G+ monocultures
Gram Negative Bacteria	G-	1 - 1,000 ng for Gram Negative monocultures
Gram Positive Bacteria	G+	1 - 1,000 ng for Gram Positive monocultures

Preparation

Hybridization	Bead Washing and Depletion	Purification
HS ● } equilibrated **PM** ● } at RT	**DB** ● } equilibrated **DS** ○ } at RT	**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 / 1,250 rpm	Thermomixer set to 60 °C / 1,250 rpm	Equilibrate all reagents to room temperature for 30 minutes prior to use

Hybridization

Probe Mix (**META**, **G-**, or **G+** ●) and total RNA are mixed, denatured, and hybridized.

ATTENTION: Important notes for RiboCop Hybridization Steps:

- If a thermomixer is not available, input amounts ≤250 ng of total RNA can be processed in PCR tubes or plates and incubated in a thermocycler, without shaking.
- Bead Washing can be performed during the incubation at step 5.
- Place the Purification Beads (**PB** ○) and Purification Solution (**PS** ○) for step 16 at room temperature prior to starting the protocol, to ensure these have at least 30 minutes to equilibrate.

1	Prepare 1 - 1,000 ng of total RNA in a total volume of 26 µl. Dilute using RNase-free water if required.
2	Add 4 µl Hybridization Solution (**HS** ●).
3	Add 5 µl Probe Mix (**META**, **G-**, or **G+** ●) and mix thoroughly until homogeneous.
4	Denature samples using a thermomixer at 75 °C for 5 minutes with agitation at 1,250 rpm.
5	Decrease the temperature of the thermomixer to 60 °C and incubate the samples for 30 minutes with agitation at 1,250 rpm. **OPTIONAL:** Transfer the samples from step 4 to a second thermomixer set to 60 °C.

Bead Washing

Depletion Beads (**DB** ●) must be washed before use. These steps may be performed as a batch for up to 6 samples.

ATTENTION: Mix Depletion Beads (**DB** ●) fully before use.

6	Transfer 75 µl Depletion Beads (**DB** ●) per reaction to a fresh tube.
7	Place the tube onto the magnet and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear. Remove and discard the supernatant with a pipette while the tube remains in contact with the magnet.
8	Add 75 µl Depletion Solution (**DS** ○) to the beads. Remove the tube from the magnet and resuspend the beads.
9	Place the tube back onto the magnet and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear. Remove and discard the supernatant.

10 Repeat this washing step once (for a total of two washes).

11 Resuspend the beads in 30 µl Depletion Solution (**DS** ○).

Depletion

Depletion Beads (**DB** ●) are used to remove the probe-hybridized ribosomal RNA.

ATTENTION: Avoid the formation of air bubbles while mixing.

12 Spin down the hybridized sample from step 5 and add 30 µl of freshly washed beads from step 11. Mix by pipetting up and down at least 8 times, or until homogeneous.

13 Put the sample back to the thermomixer and incubate at 60 °C for 15 minutes with agitation at 1,250 rpm.

14 Spin down briefly then place the sample on the magnet and let the beads collect for 5 minutes.

15 Recover and transfer 60 µl of the supernatant containing the rRNA-depleted RNA to a fresh tube. Avoid disturbing the collected beads to prevent rRNA contamination. **ATTENTION:** Do not throw away the supernatant.

Purification

The depleted RNA is purified using magnetic beads. The purification reagents (**PB** ○, **PS** ○, and **EB** ○) should equilibrate for 30 minutes at room temperature before use. The Purification Beads (**PB** ○) must be fully resuspended before use. Thorough mixing by pipetting or vortexing is recommended.

16 Add 24 µl Purification Beads (**PB** ○) and 108 µl Purification Solution (**PS** ○) to the supernatant. Mix well by pipetting. Incubate for 5 minutes at room temperature.

17 Place the sample onto a magnet and let the beads collect for 5 - 10 minutes or until the supernatant is completely clear. **NOTE:** The speed of bead collection depends on the strength of the magnet.

18 Remove and discard the clear supernatant without removing the sample from the magnet. Make sure that accumulated beads are not disturbed.

19	Add 120 µl of 80 % EtOH and incubate the beads for 30 seconds. Leave the sample on the magnet as beads should not be resuspended during this washing step. Remove and discard the supernatant. **ATTENTION:** When using 1.5 ml tubes use 150 µl of 80 % EtOH to ensure beads are fully covered by EtOH.
20	Repeat this washing step once for a total of two washes. Make sure the supernatant is removed completely.
21	Leave the sample in contact with the magnet and let the beads dry for 5 -10 minutes or until all ethanol has evaporated. **ATTENTION:** Dry the beads at room temperature only and do not let the beads dry too long (visible cracks appear), this will negatively influence the elution and the resulting RNA recovery.
22	Add 12 µl of Elution Buffer (**EB** ○), remove the sample from the magnet and resuspend the beads properly in **EB** ○. Incubate for 2 minutes at room temperature.
23	Place the sample onto the magnet and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.
24	Transfer 10 µl of the supernatant into a fresh tube. Depleted RNA is now ready for quality control and downstream use (e.g., for NGS library preparation). ☞ Safe stopping point. RNA can be stored at ≤-20 °C at this point.

6. Short Procedure RiboCop for Bacteria

ATTENTION: Spin down solutions before opening tubes or plates!

45 min Hybridization and Bead Washing

	Hybridization
☐	Prepare 1 - 1,000 ng total RNA in 26 µl.
☐	Add 4 µl **HS** ●.
☐	Add 5 µl Probe Mix (**META**, **G-**, or **G+**) ●, mix until homogeneous.
☐	Denature for 5 min at 75 °C / 1,250 rpm.
☐	Incubate for 30 min at 60 °C / 1,250 rpm.
	Bead Washing
☐	Resuspend **DB** ●, transfer 75 µl to a fresh tube.
☐	Place on magnet for 2 - 5 min, discard supernatant.
☐☐	Resuspend beads in 75 µl **DS** ○, incubate 2 min on magnet, discard supernatant. Repeat once.
☐	Resuspend beads in 30 µl **DS** ○.

45 min Depletion and Purification

	Depletion
☐	Spin down hybridized sample. Add 30 µl of washed beads. Mix by pipetting 8x, or until homogeneous.
☐	Incubate for 15 min at 60 °C / 1,250 rpm. Spin down.
☐	Place on magnet for 5 min.
☐	Transfer 60 µl supernatant to a fresh tube. **ATTENTION:** The supernatant contains the rRNA depleted RNA.
	Purification
☐	Add 24 µl **PB** ○ and 108 µl **PS** ○, mix well, incubate for 5 min at RT.
☐	Place on magnet for 5 - 10 min, discard supernatant.
☐☐	Wash the beads twice with 120 - 150 µl 80 % EtOH, 30 sec. **ATTENTION:** Use 150 µl for 1.5 ml tubes.
☐	Air dry beads for 5 - 10 min. **ATTENTION:** Do not over dry the beads!
☐	Add 12 µl **EB** ○, remove from magnet, mix well, incubate 2 min at RT.
☐	Place on magnet for 2 - 5 min, transfer 10 µl of the supernatant to a fresh tube.
	☛ Safe stopping point.

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 (e.g., proteins, chaotropic salts, and phenol) 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. Contaminants may have a negative impact on the efficiency of the protocol.

RNA Input Amount and Quality

The recommended input range for RiboCop is 1 - 1,000 ng of total RNA for monocultures using the G+ and G- Probe Mixes, and 1-100 ng for treating monocultures with the META Probe Mix. In addition, the META Probe Mix is suitable for depletion of complex, mixed community samples from 1 - 1,000 ng of total RNA. It is recommended to use 100 ng of total RNA for initial trials. RiboCop is suitable for intact or degraded RNA, including Formalin Fixed Paraffin-Embedded (FFPE) samples. Spike-in controls, including Lexogen's Spike-In RNA Variant Controls (SIRVs, Cat. No. 025, 050, 051), should be added to the RNA sample before RiboCop rRNA depletion (or prior to DNase treatment). The spike-in amount must be calculated relative to the target RNA fraction (e.g., ribo-depleted RNA). We recommend targeting 0.1 - 0.2 % of SIRV-mapped reads for FFPE samples, or 1 % for higher quality samples. For assistance with SIRV spike-in calculation, please contact support@lexogen.com.

8. Appendix B: Downstream Processing

This protocol was tested extensively with *E. coli* and *B. subtilis* total RNA and complex microbiome total RNA isolated from stool samples. Depleted RNA samples are ideally suited for downstream NGS library preparation using Lexogen's CORALL Total RNA-Seq Library Prep Kit (Cat. No. 095). Up to 10 µl of the eluted ribo-depleted RNA may be used as input for CORALL library preparation. RNA treated with RiboCop for Bacteria is compatible with all random-primed total RNA library prep kits and adapter ligation protocols.

9. Appendix C: Revision History

Publication No. / Revision Date	Change	Page
125UG246V0100 Feb. 17, 2020	Initial Release.	

10. Notes

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Associated Products:

022 (Purification Module with Magnetic Beads)
025, 050, 051 (SIRVs Spike-in RNA Variant Control Mixes)
037 (RiboCop rRNA Depletion Kit V1.3 (Human/Mouse/Rat))
052 (Small RNA-Seq Library Prep Kit for Illumina)
095 (CORALL Total RNA-Seq Library Prep Kit)

RiboCop rRNA Depletion Kits for Bacteria · User Guide

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