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Enabling complete transcriptome sequencing

RiBO COP™

Select and Deplete

rRNA Depletion Kit V1.2

User Guide

Catalog Number:
037 (RiboCop rRNA Depletion Kit V1.2 (Human/Mouse/Rat))
042 (SENSE Total RNA-Seq Library Prep Kit for Illumina with RiboCop)

037UG073V0202

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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. 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 28S, 18S, 5.8S, 45S, 5S, mt16S, and 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 1 - 1,000 ng of 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 that are hybridized to ribosomal RNA 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.

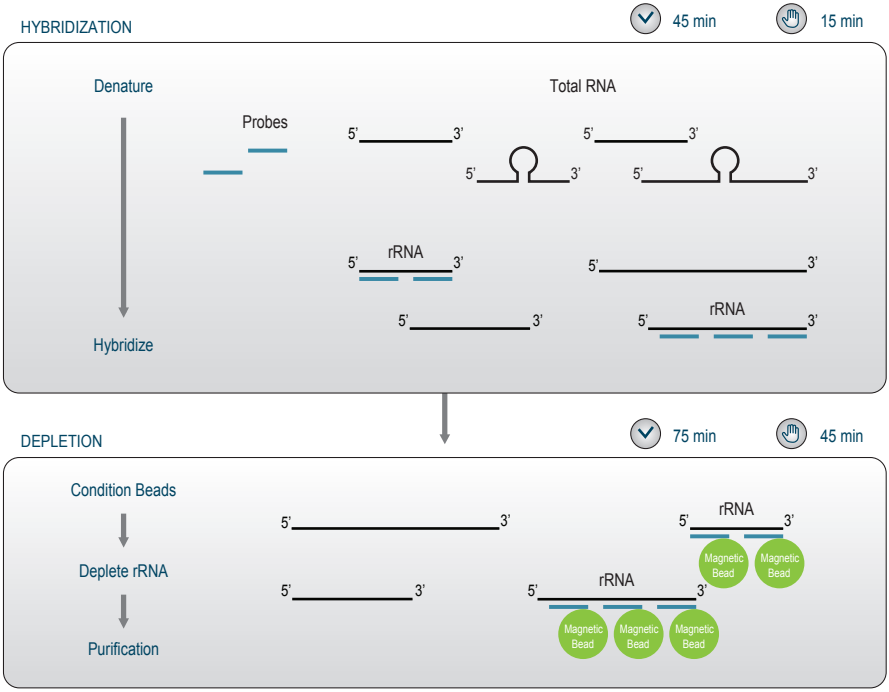

Figure 1. Schematic overview of the RiboCop workflow.

2. Kit Components and Storage Conditions

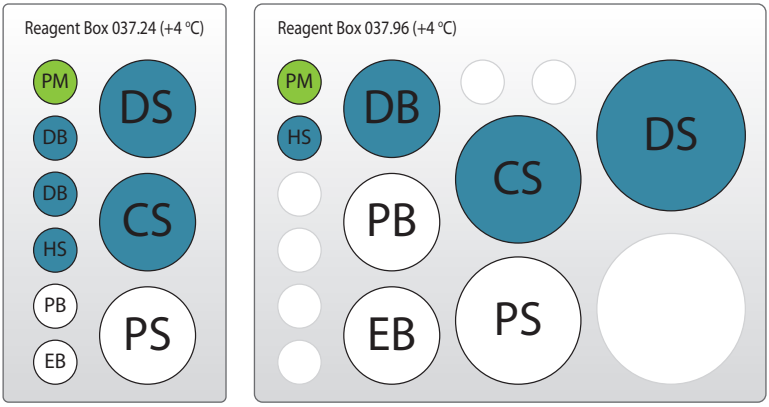

Figure 2. Location of kit components for 24 and 96 prep kits.

Kit Component	Tube Label	Volume* for		Storage
		24 preps	96 preps	
Hybridization Solution	**HS** ●	106 µl	423 µl	+4 °C
Probe Mix	**PM** ●	132 µl	528 µl	-20 °C/+4 °C
Depletion Beads	**DB** ●	1,980 µl	7,920 µl	+4 °C
Conditioning Solution	**CS** ●	3,960 µl	15,840 µl	+4 °C
Depletion Solution	**DS** ●	6,732 µl	26,928 µl	+4 °C
Purification Beads	**PB** ○	634 µl	2,535 µl	+4 °C
Purification Solution	**PS** ○	4,594 µl	18,375 µl	+4 °C
Elution Buffer	**EB** ○	1,109 µl	4,436 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

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 (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.
- 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.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. **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 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 on the 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.
- Equilibrate 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.
- **PB** O and **PS** O are viscous solutions which require care to pipette accurately. Quickly spin down the tubes to collect all liquid at the bottom of the tube. Be sure to pipette slowly and check the graduation marks on your pipette tips when removing an aliquot.

5. Detailed Protocol

Preparation

Hybridization	Bead Conditioning and Depletion	Purification
HS ● **PM** ● } equilibrated at RT	**DB** ● **CS** ● **DS** ● } equilibrated 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	Thermomixer set to 60 °C	Equilibrate all reagents to room temperature for 30 minutes prior to use

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

Probe Mix (**PM** ●) and total RNA are mixed, denatured, and hybridized.

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 (**PM** ●) and vortex gently.
4	Denature samples using a thermomixer at 75 °C for 5 minutes with gentle agitation at 400 rpm.
5	Decrease the temperature to 60 °C and incubate for 30 minutes under gentle agitation at 400 rpm. **NOTE:** If possible use a second thermomixer set to 60 °C and transfer the samples from step 4 directly to 60 °C. **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 (**DB** ●) must be conditioned before use.

6	Mix Depletion Beads (**DB** ●) properly. Transfer 75 µl 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	Add 75 µl Conditioning Solution (**CS** ●) per reaction. Remove the tube from the magnetic rack and resuspend the beads, then transfer the tube back 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	Add 75 µl Depletion Solution (**DS** ●). Remove the tube from the magnetic rack and resuspend the beads. 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 µl Depletion Solution (**DS** ●).

Depletion

Depletion Beads (**DB** ●) 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 µl freshly conditioned beads from step 12. Mix by pipetting up and down at least 8 times. **REMARK:** Set pipette to a larger value for mixing but avoid formation of air bubbles.
14	Put the tube back to the thermomixer and incubate at 60 °C for 15 minutes with 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 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 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 24 µl Purification Beads (**PB** ○) and 108 µl Purification Solution (**PS** ○) to the supernatant. 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:** The speed of bead collection depends on the strength of the magnet.
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 µl 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 µl 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 µl of 80 % EtOH and incubate 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. **NOTE:** When using 1.5 ml tubes use 150 µl of 80 % EtOH to ensure beads are fully covered in EtOH.
25	Repeat this washing step once for a total of two washes. Make sure the supernatant is removed 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:** 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 library yield.
27	Add 12 µl 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 or until the supernatant is completely clear. Transfer 10 µl of the supernatant into a fresh tube.
29	At this point, depleted RNA samples are ready for quality control (Appendix A, p.13) and downstream applications including NGS library prep (Appendix B, p.14).

6. Short Procedure

ATTENTION: Spin down solutions before opening tubes or plates!

45 min		Hybridization and Bead Conditioning
☐	Prepare 1 - 1,000 ng total RNA in 26 µl.	Hybridization
☐	Add 4 µl **HS** ●.	
☐	Add 5 µl **PM** ● and vortex gently.	
☐	Denature for 5 min at 75 °C / 400 rpm.	
☐	Incubate for 30 min at 60 °C / 400 rpm.	
☐	Resuspend **DB** ●, transfer 75 µl to a fresh tube.	Bead Conditioning
☐	Place on magnet for 2 - 5 min, remove, and discard supernatant.	
☐☐	Resuspend in 75 µl **CS** ●, incubate 2 min on magnetic rack. Remove and discard supernatant. Repeat once.	
☐☐☐	Resuspend in 75 µl **DS** ●, place on magnet for 2 - 5 min, remove and discard supernatant. Repeat twice.	
☐	Resuspend in 30 µl **DS** ●.	

75 min		Depletion and Purification
☐	Spin down hybridized sample. Add 30 µl conditioned beads. Mix by pipetting 8x up and down.	Depletion
☐	Incubate for 15 min at 60 °C / 400 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.	
☐	Add 24 µl **PB** ○ and 108 µl **PS** ○, mix well, incubate for 20 min at room temperature.	Purification
☐	Place on magnet for 5 - 10 min, remove, and discard supernatant.	
☐	Add 30 µl **EB** ○, mix well, incubate 2 min at room temperature.	
☐	Add 66 µl **PS** ○, mix well, incubate 5 min at room temperature.	
☐	Place on magnet for 2 - 5 min, remove, and discard supernatant.	
☐☐	Wash the beads twice with 120 µl 80 % EtOH, 30 sec.	
☐	Air dry beads for 5 - 10 min.	
☐	Add 12 µl **EB** ○, mix well, incubate 2 min at room temperature.	
☐	Place on magnet for 2 - 5 min. Transfer 10 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. These contaminants may have a negative impact on the efficiency of the protocol.

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 peaks. These rRNA peaks usually dominate electropherograms of intact total RNA and should be absent after RiboCop depletion as shown in Figure 3.

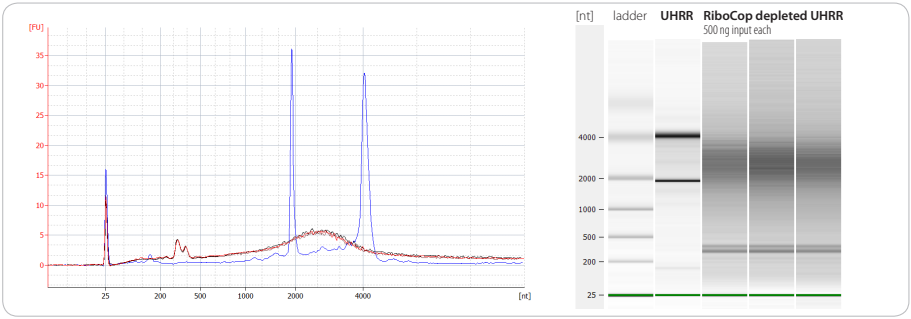

Figure 3. Overlay of RNA Bioanalyzer traces before and after RiboCop depletion. Blue trace: diluted total Universal Human Reference RNA (UHRR), intact (RIN 9.1). Red, brown, and black traces: RiboCop-depleted material. The three reactions shown here were performed using 500 ng UHRR as input each. 28S 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 µl of the eluted rRNA-depleted RNA may be directly used as input for library preparation, leaving enough material for optional quality control.

9. Appendix C: Revision History

Publication No. / Revision Date	Change	Page
037UG073V0202 Aug. 29, 2018	Use 150 µl of 80 % EtOH in step 24 when using 1.5 ml tubes.	11
	Reduced Revision History table to show updates from 2016 onwards.	14
037UG073V0201 Jul. 26, 2017	Consistency changes.	
	Added Attention note to step 16.	10
037UG073V0200 Aug. 19, 2016	Released RiboCop V1.2.	
	Lowered input amount to 1 ng.	5
	Introduction of Hybridization Solution (HS) in step 2.	5
	Adjusted rpm-values.	9
	Adjusted reaction volumes in step 16, 17, 27.	10, 11
037UG073V0104 Mar. 29, 2016	Lowered input amount to 10 ng.	4, 9, 12
037UG073V0100 Nov. 10, 2015	Initial Release.	

10. Notes

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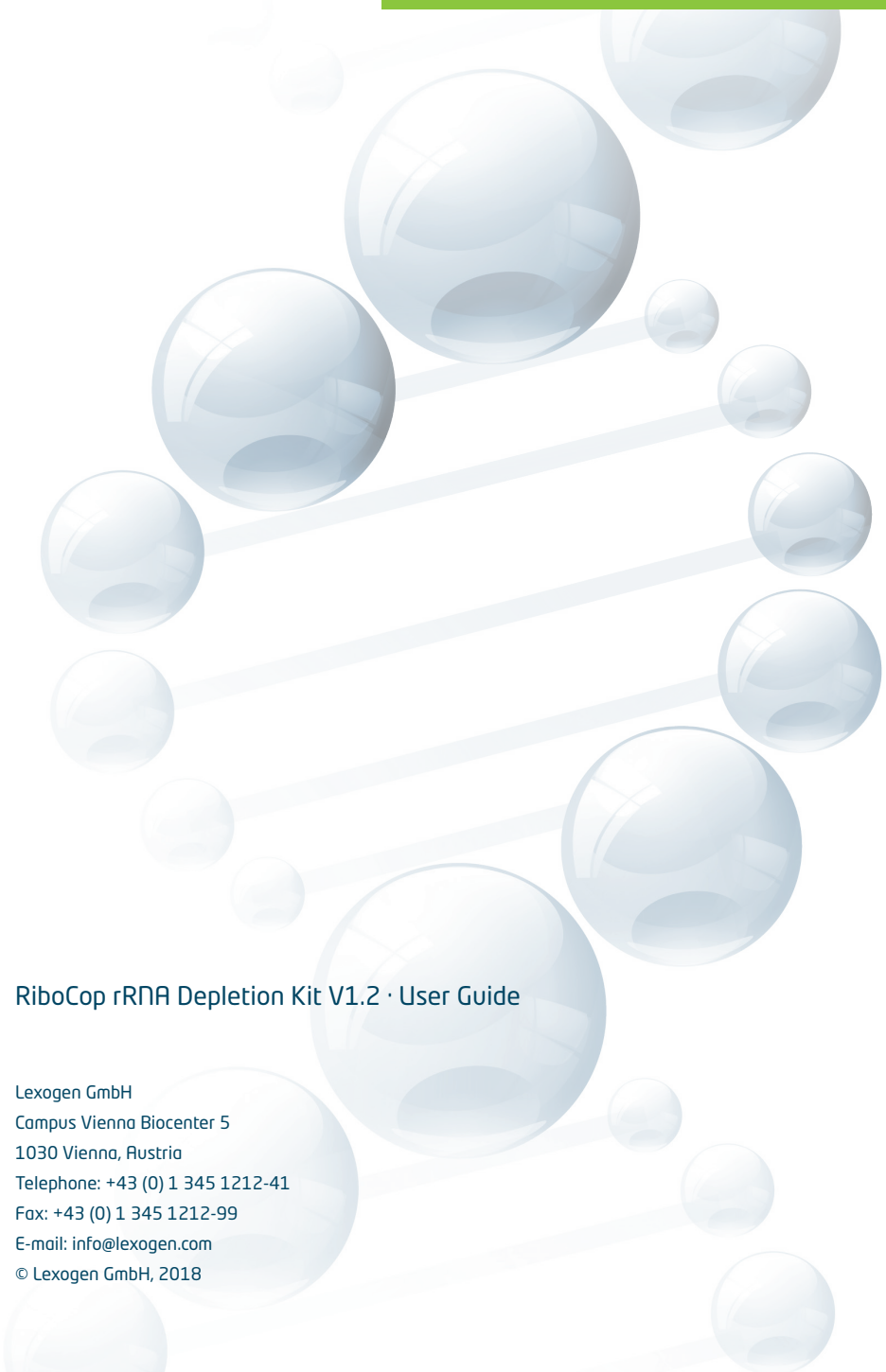

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