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Enabling complete transcriptome sequencing

SPLIT

Fractions for pure RNA sequencing

RNA Extraction Kit
Instruction Manual

Catalog Number:
008 (SPLIT RNA Extraction Kit)

008IM062V0100

Instruction Manual

This instruction manual outlines the protocol for the SPLIT RNA Extraction Trial Kit (008.03). For more detailed information refer to the complete User Guide (008.48) which is available for download at our website (www.lexogen.com).

The SPLIT RNA Extraction Kit enables fast and extremely efficient extraction of RNA that is free of genomic DNA contamination without need for DNAse treatment thereby avoiding the high risk of concomitant RNA degradation. The RNA can be recovered as total RNA or split into a large (>150 nt) and a small RNA fraction (<150 nt), facilitating the analysis of e.g., mRNA and miRNA from the same sample. The RNA obtained is of highest purity and ideally suited to prepare libraries for RNA sequencing. The superior quality of SPLIT extracted RNA also renders it ideal for other high demanding applications such as full-length reverse transcription or sample preparation for microarray analysis.

SPLIT RNA Extraction Kit enables the extraction of total RNA or large RNA fraction only (single-fraction extractions) from 48 samples, or small and large RNA fractions can be isolated from 24 samples (dual-fraction extractions).

Please note that acidic phenol, chloroform, ethanol, and isopropanol have to be supplied by the user.

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1. Kit Components and Storage Conditions

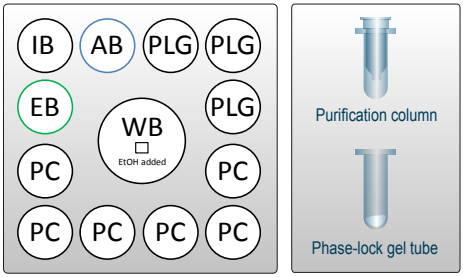


Figure 2. Location of kit contents. PLG, Phase-lock gel tubes; PC, purification column

Kit Component	Label	Requirement for		Storage
		3 single-fraction purifications	3 dual-fraction purifications	
Isolation Buffer	**IB**	1.2 ml	1.2 ml	+4 °C
Acidic Buffer	**AB**	0.45 ml	0.45 ml	+4 °C
Wash Buffer	**WB**	3.3 ml [1]	6.6 ml [1]	+4 °C
Elution Buffer	**EB**	0.15 ml [2]	0.3 ml [2]	+4 °C
Phase-lock gel tubes	**Phase-lock gel tubes**	3	3	+4 °C
Purification columns	**Purification columns**	3	6	+4 °C

[1] Including ethanol added by the user.
[2] For each RNA fraction, either EB or SB is required.
Buffers provided in the SPLIT kit include tolerance volumes.

Upon receiving the SPLIT kit, store it in a +2 - +8 °C refrigerator.

CAUTION: Phase-lock gel tubes must not be frozen.

IB is to be used at +4 °C. All other components (especially phase-lock gel tubes) should equilibrate to room temperature before use.

Check the contents of **IB**, **AB**, **WB,** and **SB** which may precipitate during shipping and storage. If a white precipitate is visible, incubate at 37 °C until buffer components dissolve completely.

Cat. No. 008.03 (3 extractions): Add 6 ml absolute ethanol to the bottle with Wash Buffer (**WB**) concentrate and shake to combine. This will yield 7.5 ml Wash Buffer (**WB**).

2. User-supplied Reagents

Check to ensure that you have all of the necessary material and equipment before beginning with the RNA extraction. All reagents, equipment, and labware must be free of nucleases and nucleic acid contamination.

Reagents

Reagent	Requirement for			Comment
	3 dual fraction purifications	3 large RNA only purifications	3 total RNA purifications	
Phenol solution pH 4.3	1.2 ml	1.2 ml	1.2 ml	e.g. Sigma-Aldrich P4682-100ML
Chloroform	0.6 ml	0.6 ml	0.6 ml	
Isopropanol	3.0 ml	0.6 ml	3.15 ml	2-Propanol
Ethanol abs.	6 ml	6 ml	6 ml	Added to **WB**

3. Detailed Protocol

3.1. Homogenization

Animal Tissue

Tissue	Weigh and Reduce Tissue	Homogenization
Animal tissue – freshly harvested, frozen, or thawed at +4 °C if stored in RNAlater	**Tweezers** – sterile **Scalpel** – sterile **Gauze pad** – sterile	**Isolation Buffer** (**IB**) - at +4 °C or on ice
Fume hood or laminar-flow cabinet	**Precision balance**	**Tissue grinder** – 0.1 ml, glass

Homogenization

Tissue is homogenized in a highly chaotropic solution.
This protocol is specific for hand-held tissue grinders (glass homogenizers with pestle) but can be easily adapted for other homogenization protocols. Optimally, the tissue should be stored at -20 °C in RNAlater (Ambion Inc.). Tissue frozen without preservation (RNAlater) must not be thawed before homogenization to maintain RNA integrity. To prevent cross-contamination, it is best to work in a fume hood or a laminar-flow cabinet that can be UV-irradiated.

1. Add 400 µl cold (+4 °C) Isolation Buffer (**IB**) into a glass tissue grinder.
2. Use sterile tweezers to transfer a tissue piece onto a fresh, sterile gauze pad. If RNAlater was used for conservation, dry the tissue by tapping onto the gauze pad.
3. Determine the weight of the tissue on a precision balance. The protocol is efficient for extraction of up to 100 µg of total RNA. See Appendix (p.13) for details on input and extraction efficiency.
4. Optional: Hard to homogenize tissue such as tendons or cartilage can be reduced using a scalpel to facilitate solubilization in the next steps.
5. Using tweezers, transfer the tissue pieces quantitatively into the Isolation Buffer (**IB**) in the tissue grinder.
6. Homogenize the tissue by carefully moving the pestle up and down. Simultaneous rotation helps to dissolve also larger pieces. Do not pull out the pestle completely to avoid foaming. The tissue is usually homogenized within 2 - 3 minutes; avoid extended homogenization and warming up of Isolation Buffer (**IB**).
7. Continue immediately with the phenol-chloroform extraction in 3.2.

After use, clean the tissue grinder thoroughly with a detergent such as DNA-ExitusPlus (AppliChem GmbH), then with ultra-filtered water and finally with 75 % ethanol.

Cell Culture

Cells	Solubilization
Cells – freshly harvested, frozen, or thawed at +4 °C if stored in RNAlater	**Isolation Buffer (IB)** – at +4 °C or on ice
Fume hood or laminar-flow cabinet	

Solubilization

Cells are solubilized in a highly chaotropic solution.
If cells are not harvested freshly, they can be pelleted, washed and stored in RNAlater (Ambion, Inc.) at -20 °C. To prevent cross-contamination, it is best to work in a fume hood or a laminar-flow cabinet that can be UV-irradiated.

1. Harvest, pellet and wash the cells. If they have been stored in RNA later, they should be pelleted at +4 °C and RNAlater removed with a pipette. The protocol is suitable for extraction of e.g., 10^6 cells of a human suspension cell culture.
2. Add 400 µl cold (+4 °C) Isolation Buffer (**IB**) to the cells.
3. Lyse the cells by carefully pipetting up and down. The cells are usually lysed within 1 - 2 minutes.
4. Continue with step 8, phenol-chloroform extraction in 5.2.

3.2. Phenol-Chloroform Extraction

	For each sample	Temperature
Phenol solution pH 4.3[1] **Acidic buffer (AB)** **Chloroform**[1]	400 µl 150 µl 200 µl	+4 °C RT RT
Phase lock gel tube **2 ml micro-tube**	1 1	RT RT
Centrifuge **Fume hood** **Vortex mixer**		+18 °C

[1] **Caution:** When working with phenol or chloroform always use a fume hood and discard waste according to applicable Health and Safety regulations.

Phenol-Chloroform Extraction

Utilizing a highly specific phenol-chloroform extraction, RNA is partitioned into the upper, aqueous phase whereas DNA and proteins are partitioned into the lower, organic phase.

8. For each sample, centrifuge 1 phase lock gel tube for 1 minute at 12,000 x g at 18 °C. This collects the gel on the bottom of the tube.
9. Transfer the homogenized tissue (cells) in Isolation Buffer (**IB**) into a phase lock gel tube.
10. Add 400 µl phenol solution pH 4.3 and mix by inverting the tube 5 times.
11. Add 150 µl Acidic Buffer (**AB**) and mix by pipetting.
12. Add 200 µl of chloroform.
13. Mix vigorously by 3 cycles of 5 seconds vortexing and 1 second pausing.
    ATTENTION: Vigorous vortexing is essential to disperse the chloroform efficiently and effectively separate all the phenol that will contain the gDNA and protein into the organic phase. Do not be afraid of shearing the gDNA. Even if this happens all DNA will separate into the lower organic phase irrespective of its size.
14. Incubate for 2 minutes at room temperature.
15. Centrifuge for 2 minutes at 12,000 x g at 18 °C.
16. Transfer the upper phase to a new 2 ml micro-tube by decanting.
17. For the purification of **total RNA**, proceed with **step 18 on p.8.** For the purification of the **large RNA fraction and – optionally – the small RNA fraction**, proceed with **step 18 on p.10.**

3.3. Column-based Purification

3.3.1. Purification of Total RNA

	For each sample	Temperature
Isopropanol **Wash Buffer (WB)**[1] **Elution Buffer (EB)**	~1050 µl 1100 µl 50 µl	RT RT RT
Purification column **Collection tube** **1.5 ml micro-tube**	1 1 1	RT RT RT
Centrifuge **Vortex mixer**		18 °C

[1] **Caution:** Discard waste containing guanidine isothiocyanate (**IB**), phenol and chloroform according to applicable Health and Safety regulations.

Column Loading

The total RNA is precipitated onto a silica column by addition of 1.75 x volume of isopropanol.

18. Determine the volume of the transferred upper phase in the 2 ml micro-tube and add isopropanol at 1.75 x of this volume (e.g., 1050 µl isopropanol to 600 µl sample). Mix by vortexing for 10 seconds.
19. Place a purification column in a collection tube.
20. Apply a maximum of 800 µl of the mixture from step 18 (aqueous phase with isopropanol) to the column.
21. Centrifuge for 20 seconds at 12,000 x g at 18 °C and discard the content of the collection tube.
22. Repeat steps 20 - 21 until the mixture is loaded completely.

Column Washing

The RNA is further purified by washing on the column.

23. Apply 500 µl of Wash Buffer (**WB**) to the column and centrifuge for 20 seconds at 12,000 x g at 18 °C. Empty the collection tube.
24. Apply 600 µl of Wash Buffer (**WB**) to the column and centrifuge for 20 seconds at 12,000 x g at 18 °C.
25. Empty the collection tube with a pipette and centrifuge for 1 minute at 12,000 x g at 18 °C.

26 Discard the collection tube and place the purification column in new 1.5 ml micro-tube. Make sure that no ethanol traces are carried to the new tube.

Elution of Total RNA

The RNA is eluted into an elution buffer.

27 Apply 10 - 50 µl Elution Buffer (**EB**) to the column. Incubate for 1 minute at room temperature.

28 Centrifuge for 1 minute at 12,000 x g at 18 °C.

29 Optional: For a second elution, place the purification column in a new 1.5 ml micro-tube and repeat steps 27 - 28.

30 At this point the total RNA is purified and ready for quality control (Appendix, p.13) and downstream applications.

31 Optional: Add RNase inhibitor (not included). Note that the RNase inhibitor might absorb at 230 nm, therefore use buffer with RNase inhibitor added as blank in OD measurements.

3.3.2. Purification of Large RNA / Small RNA Fractions

	Large RNA fraction	Large RNA and small RNA fraction	Temperature
Isopropanol **Wash Buffer (WB)**[1] **Elution Buffer (EB)**	~200 µl 1100 µl (2x) 50 µl	~800µl 2200 µl (2x) 100 µl	RT RT RT
Purification column(s) **Collection tube(s)** **2 ml micro-tube** **1.5 ml micro-tube(s)**	1 1 - 1	2 2 1 2	RT RT RT RT
Centrifuge **Vortex Mixer**			+18 °C

[1] **Caution:** Discard waste containing guanidine isothiocyanate, phenol and chloroform according to applicable Health and Safety regulations.

Column Loading

The large RNA fraction is precipitated onto a silica column by the addition of 0.33 x volume of isopropanol. The small RNA fraction will be in the flow-through.

18 Determine the volume of the aqueous phase, which may vary, depending on the tissue volume and volume transfer efficiency during homogenization and extraction. **ATTENTION:** For best reproducibility of the size cut-off it is essential to quantify the volume of the aqueous phase exactly.

19 Add isopropanol at 0.33 x of this volume (e.g. 200 µl isopropanol to 600 µl sample). Mix by vortexing for 10 seconds.

20 Place a purification column in a collection tube.

21 Apply a maximum of 800 µl of the mixture (aqueous phase / isopropanol) to the column.

22 Centrifuge for 20 seconds at 12,000 x g at 18 °C.

23 **OPTIONAL: If you want to also isolate the small RNA fraction pipette the flow-through into a 2 ml micro-tube. Otherwise discard the flow-through.**

24 Repeat steps 21 - 23 until the mixture is loaded completely.

Splitting into Fractions

The small RNA fraction is recovered by precipitation onto a new purification column with the addition of 1 x volume of isopropanol.

25 **LARGE RNA ONLY:** If you want to only isolate the large RNA fraction, you can discard the content of the collection tube. Place the purification column back into the collection tube and proceed with step 28.

26 **SMALL RNA PURIFICATION (optional)**. If you want to also isolate the small RNA fraction measure the volume of the flow-through in the 2 ml micro-tube and add the same volume isopropanol (e.g., 800 µl isopropanol to 800 µl flow-through). Mix by vortexing for 10 seconds.

27 **SMALL RNA PURIFICATION (optional).** For loading of the small RNA fraction, place a new purification column in a collection tube. Apply a maximum of 800 µl of the flow-through / isopropanol mixture to the column. Centrifuge for 20 seconds at 12,000 x g at +18 °C and discard the content of the collection tube. Repeat until loading of the mixture is complete. Washing and elution can now be performed for the large RNA fraction and the small RNA fraction in parallel.

Column Washing

The RNA fractions are further purified by washing on the column.
This protocol applies to both, the large RNA fraction on the original purification column and – optionally – the small RNA fraction on the second purification column.

28 Apply 500 µl of Wash Buffer (**WB**) to the purification column and centrifuge for 20 seconds at 12,000 x g at 18 °C. Empty the collection tube.

29 Apply 600 µl of Wash Buffer (**WB**) to the column and centrifuge for 20 seconds at 12,000 x g at 18 °C.

30 Empty the collection tube with a pipette and centrifuge for 1 minute at 12,000 x g at 18 °C.

31 Discard the collection tube and place the purification column in new 1.5 ml micro-tube. Make sure that no ethanol traces are carried to the new tube.

Elution of RNA Fractions

The RNA fractions are eluted into an elution or storage buffer.

32	Apply either 10 - 50 µl Elution Buffer (**EB**) or 10 - 50 µl Storage Buffer (**SB**) to each column. Incubate for 1 minute at room temperature.
33	Centrifuge for 1 minute at 12,000 x g at 18 °C.
34	Optional: For a second elution, place the purification column in a new 1.5 ml micro-tube and repeat steps 32 - 33.
35	At this point the large RNA fraction – and optionally, also the small RNA fraction – is purified and ready for quality control (Appendix B, p.20) and downstream applications.
36	Optional: Add RNase inhibitor (not included). See Appendix C (p.22) for RNA storage. Note that the RNase inhibitor might absorb at 230 nm, therefore use buffer with RNase inhibitor added as blank in OD measurements.

4. Appendix

Tissue Input and Extraction Efficiencies

If immediate RNA extraction is not possible, tissue samples can be either flash-frozen with liquid nitrogen and stored at -80 °C or preserved in RNAlater (Ambion, Inc.) and stored at -20 °C or -80 °C. Tissue / cells without RNAlater preservation must only be thawed during the homogenization step in cold Isolation Buffer (+4 °C) to keep RNases inactive.
RNA extraction efficiency for mouse liver is typically 4.0 - 4.5 µg total RNA / mg tissue (3.0 - 3.5 µg large RNA and 0.6 µg small RNA / mg tissue). A second elution can increase the overall yield by 5 - 20 %. The maximum binding capacity of the purification column is 100 µg RNA, which should not be exceeded for optimal results. For mouse liver tissue this translates into an upper limit of 20 - 25 mg input per extraction. Other tissues have different RNA content, and the input might have to be adjusted accordingly.

RNA Integrity

The integrity of an RNA sample can be assessed with a variety of methods (see Figure 3). We recommend the use of a microfluidics assay such as the RNA6000 series for the 2100 Bioanalyzer (Agilent Technologies Inc.), although RNA quality can also be assessed with denaturing agarose gel electrophoresis if such a device is not available. Most microfluidics platforms will carry out an automated peak analysis and generate a quality score (RIN or RQN) in addition to the 28S/18S rRNA ratio. The quality of RNA extracted with the SPLIT RNA Extraction Kit almost exclusively depends on the extraction source: a RIN of 10 and a 28S/18S rRNA ratio of 2.7 can be obtained from human cell culture. Extractions from tissue samples usually result in RNA with a RIN of 8.0 - 9.5.

Potential Contaminants

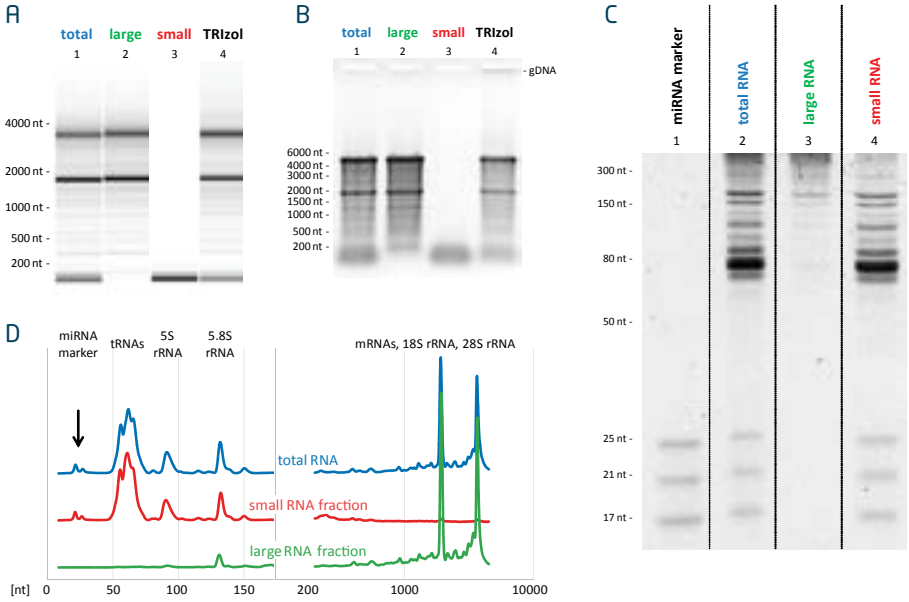
RNA samples should be free of salts, metal ions, and organic solvents, which can be carried over from the RNA extraction. Several sources of contamination can be detected with a UV-Vis spectrophotometer. An acceptably pure RNA sample should have an A260/A280 ratio between 1.8 and 2.1. The A260/A230 ratio 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.

Genomic DNA Contamination

The SPLIT RNA Extraction Kit was designed for minimizing the genomic DNA (gDNA) content in the RNA sample. gDNA is indistinguishable from RNA on a spectrophotometer, and many of the dyes used in RNA microfluidics assays stain single-stranded nucleic acids much more intensely

than double-stranded. Hence, low to moderate amounts of gDNA may not be readily visible with an RNA-specific microfluidics assay. We highly recommend examining all RNA samples on a denaturing agarose gel or using a fluorometric assay with DNA- and RNA-specific dyes to check samples for DNA contamination. On an agarose gel, gDNA can appear as either a dark mass which remains in the slot if relatively intact (see Figure 3B) or as a high molecular weight smear if it has been sheared during extraction.

Typical Results



E	Tissue	RNA yield	Efficiency*	RIN	A260/A280	A260/A230
Total RNA	19.8 mg	88.0 µg	4.43 µg/mg	8.2	1.98	2.22
Large RNA	19.8 mg	64.7 µg	3.26 µg/mg	8.3	2.00	2.28
Small RNA	19.8 mg	12.3 µg	0.62 µg/mg	N/A	2.00	2.06

Figure 3. Analysis of SPLIT kit extracted RNA. (A) Gel-like representation of Agilent Bioanalyzer traces. RNA from mouse liver stored in RNAlater was extracted either as total RNA (lane 1) or as large RNA and small RNA fractions (lanes 2 and 3). In the split sample RNAs shorter than 150 nt are confined to the small RNA fraction. A control sample was extracted following a TRIzol protocol (lane 4). This RNA sample contains a significant amount of genomic DNA (gDNA) that is not detected by the Bioanalyzer but becomes visible on a denaturing agarose gel as a slot-retained band. **(B)** RNA obtained with the SPLIT kit is free from detectable gDNA. **(C)** A miRNA marker was spiked into mouse liver homogenate, which was then extracted using the SPLIT kit. Analysis on a 15 % denaturing polyacrylamide gel demonstrates that small RNA down to at least 17 nt is efficiently recovered in the total RNA sample and in the small RNA fraction. The theoretical maximum spike-in RNA recovery amount was loaded in lane 1. **(D)** Bioanalyzer evaluation of miRNA-spiked samples on a small RNA chip (10 - 200 nt, linear scale) and on an RNA 6000 pico chip (200 - 500 nt, log scale). The traces from the two chips are shown alongside for illustrative purposes, the Y-axes do not correspond quantitatively. **(E)** The table shows key parameters of SPLIT RNA extractions from mouse liver that was stored in RNAlater. Efficiency is given in µg RNA per mg tissue.

5. Notes

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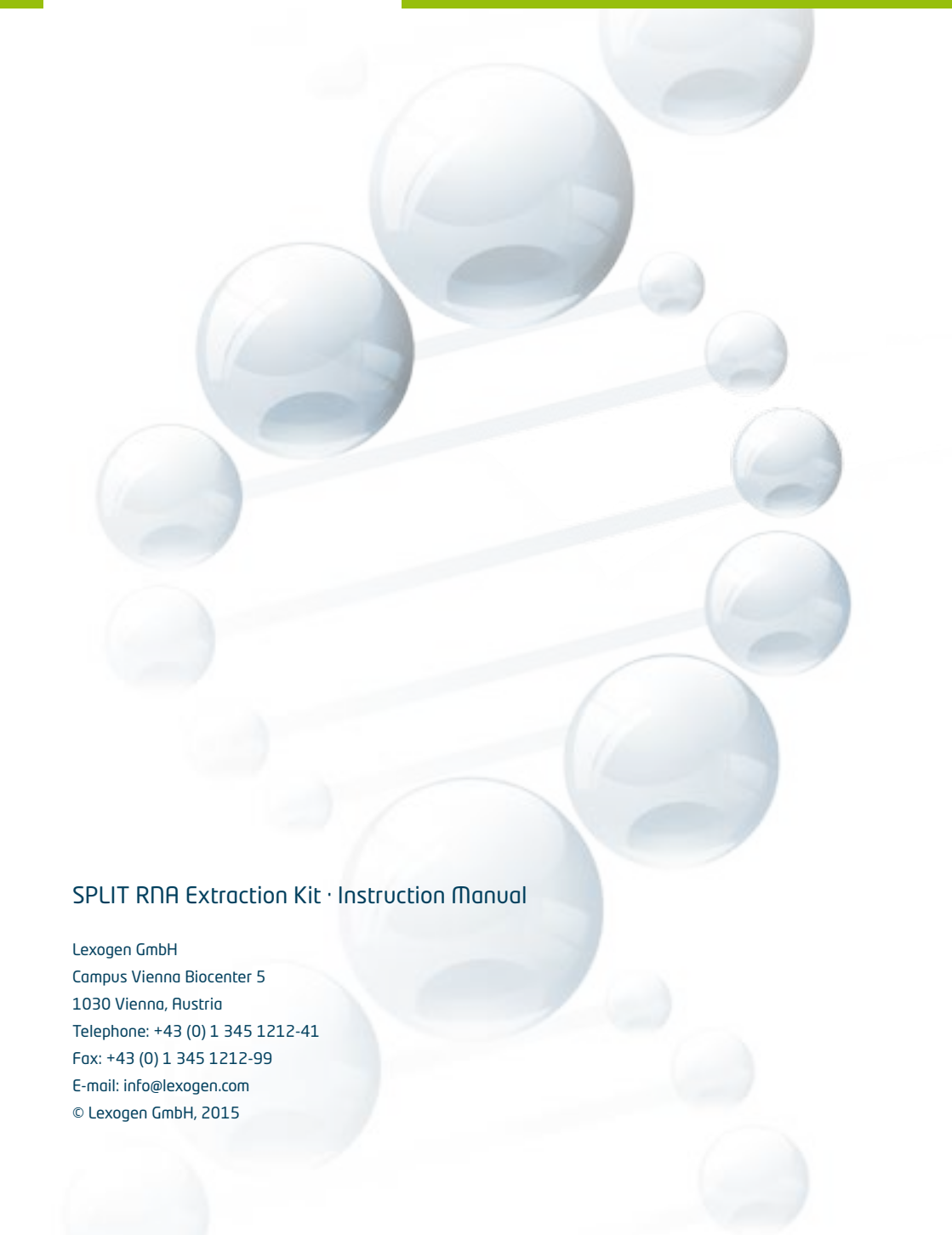

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