LEXOGEN

Enabling complete transcriptome sequencing

SPLIT

Fractions for pure RNA sequencing

RNA Extraction Kit

User Guide

Catalog Numbers:
008 (SPLIT RNA Extraction Kit)
025 (SIRVs Spike-in RNA Variant Control Mixes)
037 (RiboCop rRNA Depletion Kit)
039 (Poly(A) RNA Selection Kit)

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1. Overview

The SPLIT RNA Extraction Kit enables a fast and highly efficient extraction of RNA that is free of genomic DNA contamination. The RNA can be recovered as total RNA or split into a large and a small RNA fraction, facilitating the analysis of e.g., mRNA and miRNA from the same sample. Thus the RNA obtained is ideal for seamlessly preparing libraries for Next Generation Sequencing of total RNA or its large and small fractions. Importantly, the SPLIT protocol does not require DNase treatment for the removal of genomic DNA and thereby avoids the high risk of concomitant RNA degradation. Furthermore, SPLIT recovers the complete RNA size ranges without loss of long RNAs as observed with some gDNA removal columns.

First, the sample is homogenized in a highly chaotropic isolation buffer which facilitates effortless and complete solubilization, and guarantees complete RNase inhibition.

Acidic buffer and acidic phenol are added to create a monophasic solution, a step that is essential for the efficient separation of genomic DNA into the organic phase. Chloroform is added, and phases are cleanly separated using phase lock gel tubes. The use of these tubes mitigates the risk of contaminating the upper aqueous phase that contains RNA with the lower phenol phase that contains DNA and protein.

The RNA is further purified on a silica column to eliminate trace amounts of phenol and to optionally fractionate the RNA. By adding 1.75 x volume of isopropanol to the aqueous phase the entire total RNA will precipitate onto the silica carrier. When using only 0.33 x volume isopropanol large RNA with a lower limit of about 150 nt will bind whereas the small RNA will be in the flow-through. By adding 1 x volume of isopropanol to this flow-through, also the small RNA can be recovered on another silica column.

With the SPLIT RNA Extraction Kit, either 48 samples can be extracted for their total or large RNA, or 24 samples for their small RNA fraction (with the large RNA alongside).

Protocols are given for RNA extraction from human cell culture, animal and plant tissue, as well as fluid samples. The extraction protocol can be easily adapted to a variety of RNA sources (Appendix A, p.21). Please note that acidic phenol, chloroform, ethanol, and isopropanol have to be supplied by the user.

The RNA obtained is of highest purity and ideally suited to prepare libraries for RNA sequencing. It is also superior for other high demanding applications such as full-length reverse transcription or sample preparation for microarray analysis (see Appendix B, p.22 for details on RNA quality).

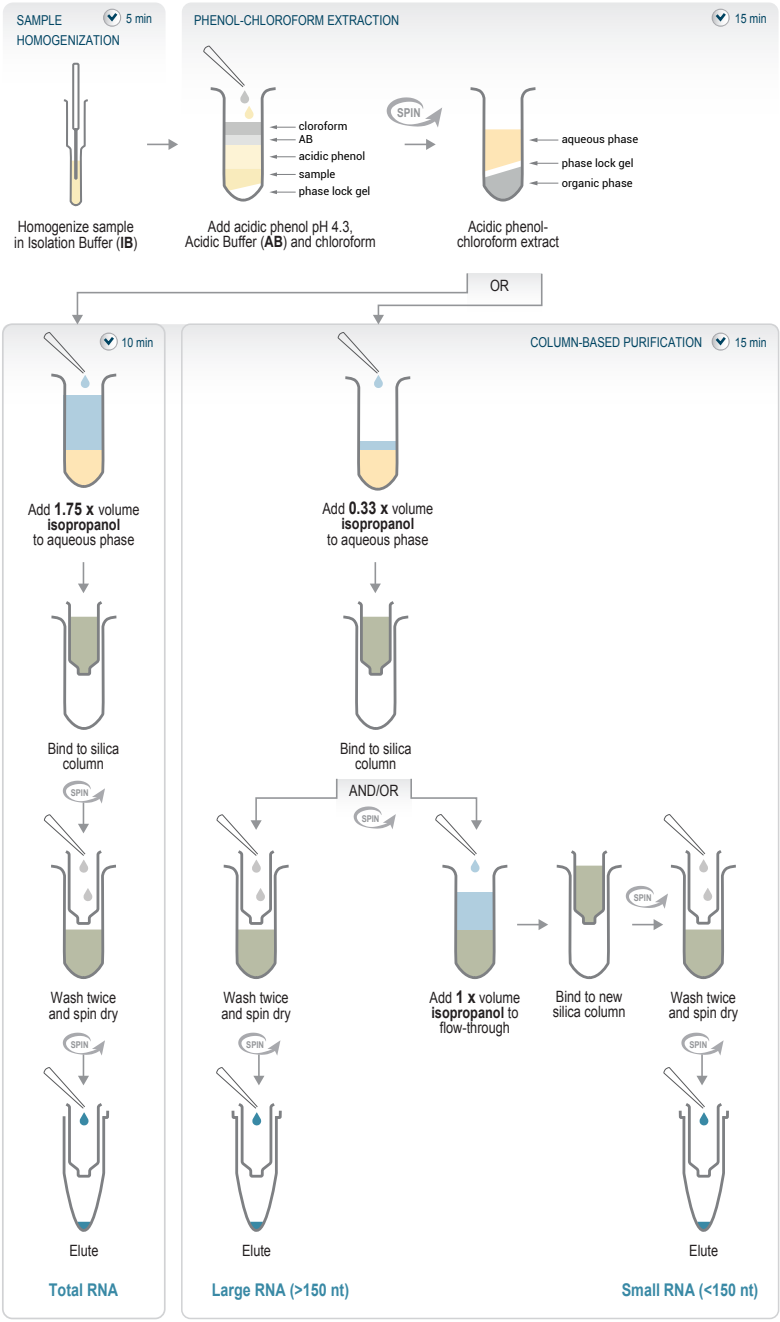


Figure 1. Schematic overview of the SPLIT workflow. The cut-off between large and small RNA fractions is at ~150 nt.

2. Kit Components and Storage Conditions

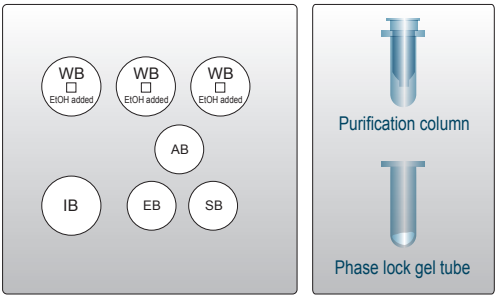


Figure 2. Location of kit contents.

Kit Component	Label	Volume / Amount Provided	Storage
Isolation Buffer	**IB**	19.2 ml	+4 °C
Acidic Buffer	**AB**	7.2 ml	+4 °C
Wash Buffer	**WB**	3 x 17.6 ml [1]	+4 °C
Elution Buffer	**EB**	2.4 ml [2]	+4 °C
Storage Buffer	**SB**	2.4 ml [2]	+4 °C
Phase lock gel tubes	**Phase lock gel tubes**	48	+4 °C or RT
Purification columns	**Purification columns**	48	+4 °C or RT

[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 a 10 % surplus.

Upon receiving the SPLIT kit, store it at +2 to +8 °C.
ATTENTION: Phase lock gel tubes must not be frozen. Optionally, they can be stored at room temperature together with the purification columns.

IB is to be used at +4 °C. All other components (especially phase lock gel tubes) should get 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. 008.48 (48 extractions): Add 20 ml absolute ethanol to each of the three bottles with Wash Buffer (**WB**) concentrate and shake to combine. This will yield 3 x 25 ml (75 ml) Wash Buffer (**WB**).

3. User-supplied Consumables and Equipment

Check to ensure that you have all of the necessary materials 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
	48 total RNA extractions	48 large RNA extractions	24 small RNA extractions (large RNA alongside)	
Phenol solution pH 4.3	19.2 ml	19.2 ml	9.6 ml	e.g. Sigma-Aldrich P4682-100ML
Chloroform	9.6 ml	9.6 ml	4.8 ml	
Isopropanol	~ 50.4 ml	~ 9.6 ml	~ 24.0 ml	2-Propanol
Ethanol abs.	3 x 20 ml	3 x 20 ml	3 x 20 ml	Added to **WB**

Equipment

- Fume hood for organic solvent handling.
- Benchtop centrifuge (12,000 x g, rotor compatible with 1.5 ml and 2.0 ml micro-tubes).
- Calibrated single-channel pipettes for handling 10 µl to 1000 µl volumes.
- Vortex mixer.
- UV-spectrophotometer to quantify RNA.

Labware

- Suitable pipette tips (pipette tips with aerosol barriers recommended).
- 1.5 ml and 2.0 ml micro-tubes with cap, low binding, certified ribonuclease-free.
- Benchtop cooler or ice pellets in ice box (for short-term storage of RNA).

Optional Equipment & Solutions

- 0.1 ml tissue grinder (hand-held homogenizer).
- Liquid nitrogen (for RNA extraction of plant tissue).
- Automated microfluidic electrophoresis station (Agilent Technologies 2100 Bioanalyzer).
- Agarose gels, dyes, and electrophoresis rig (for RNA quality control).
- DNA-ExitusPlus (AppliChem GmbH).
- RNaseZap.
- RNase inhibitor.

The complete set of materials, reagents, and labware for quality control is not listed. Consult 5.1. (p.10) for homogenization protocols and Appendix B (p.22) for more information on RNA quality.

4. Guidelines

Safety Information

- This kit is to be used with a phenol solution, which is toxic and corrosive and with chloroform, both of which may be health hazards if not handled properly. Phenol should not come in contact with skin, eyes, or the respiratory tract and may cause chemical burns to the exposed area. When working with the phenol solution and with chloroform, always work in a fume hood.
- The Isolation Buffer (**IB**) contains guanidine isothiocyanate, an irritant, which upon protocol completion is also present in flow-through and wash fractions. This chemical is harmful when in contact with the skin, inhaled, or ingested. Do not add bleach or acidic solutions directly to solutions or sample preparation waste that contains guanidine isothiocyanate, as reactive compounds and toxic gases are formed.
- Solutions containing isopropanol or ethanol are considered flammable. Use appropriate precautions when using these chemicals.
- For your protection, always wear a laboratory coat, gloves, and safety glasses when handling chemicals.
- Dispose buffers and chemicals in appropriate waste containers.
- Consult the appropriate Material Safety Data Sheets (MSDS) at www.lexogen.com, and contact your Environmental Health and Safety department for proper work and disposal guidelines.

General

- Unless explicitly mentioned, all steps should be carried out at a room temperature (RT) between 20 °C and 25 °C. Centrifugation should be performed at 18 °C to increase reproducibility. If a refrigerated centrifuge is not available, centrifugation can be carried out at RT. Results may be negatively impacted if the protocol is performed at temperatures outside of this range.
- Ensure that adequate volumes of all reagents and the necessary equipment are available before beginning the protocol.
- Perform all pipetting steps with calibrated pipettes and always use fresh tips. Pipette carefully to avoid foaming as the Isolation Buffer (**IB**) and the Storage Buffer (**SB**) contain detergents.
- Before you start, check all solutions for the formation of precipitate and if necessary, incubate at 37 °C until buffer components dissolve completely.
- The phenol solution pH 4.3 and the Isolation Buffer (**IB**) should be used at their storage temperature of +4 °C. All other components, especially the phase lock gel tubes, should be at room temperature.

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 an RNA extraction, clean your work space, pipettes, and other equipment with RNase removal spray (such as RNaseZap, Ambion Inc.) as per the 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 of the RNase-free zone.
- Avoid speaking above opened tubes. Keep reagents closed when not in use to avoid airborne RNase contamination.

5. Detailed Protocol

5.1. Sample Homogenization

5.1.1. Animal Tissue

Preparation

Tissue	Weigh and Reduce Tissue	Homogenization
Animal tissue – freshly harvested or 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 A (p.21) 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. Also, short incubation of tissue with Isolation Buffer (**IB**) prior to homogenization can help solubilization.
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 5.2. (p.14, step 8).

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

5.1.2. Plant Tissue

Preparation

Tissue	Weigh and Reduce Tissue	Homogenization
Plant tissue – freshly harvested or frozen at -80 °C or -20 °C in RNAlater or already grinded and frozen in **IB** at -20 °C	**Tweezers** – sterile **Scalpel** – sterile	**Liquid nitrogen** **Isolation Buffer** (**IB**) - at +4 °C or on ice
Fume hood or laminar-flow cabinet	**Precision balance**	**Pestle and mortar**

Homogenization

Plant material is disrupted in liquid nitrogen and homogenized in a highly chaotropic solution. Optimally, the plant material should be extracted immediately after harvesting. If storage of plant material is required, then shock-freeze the sample in liquid nitrogen and store at -80 °C or at -20 °C in RNAlater. Already grinded plant tissue can also be stored in Isolation Buffer (**IB**) 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.

REMARK: Disruption of plant material can also be done using other devices such as ball mills.

1 Determine the weight of the tissue on a precision balance. Make sure to work under sterile conditions (e.g., use sterile tweezers for transfer). 10 mg can be an initial plant input amount, with the upper limit determined by the binding capacity of the silica column (100 µg).

2 Quickly cut the plant tissue into small pieces using a scalpel and freeze in liquid nitrogen.

3 Still in liquid nitrogen, grind the tissue using pestle and mortar.

4 Allow the liquid nitrogen to evaporate.

5	Resuspend the tissue in 400 µl cold (+4 °C) Isolation Buffer (**IB**). Make sure to completely cover the tissue with **IB**.
6	**OPTIONAL:** Further homogenize the sample by carefully moving the pestle up and down. Do not pull out the pestle completely to avoid foaming. Avoid extended homogenization and warming up of Isolation Buffer (**IB**).
7	Continue immediately with the phenol-chloroform extraction in 5.2. (p.14, step 8).

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

5.1.3. Cell Culture

Preparation

Cells	Solubilization
Cells – freshly harvested or 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. SPLIT RNA extraction has also been successfully performed with 100 cells input.
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 immediately with the phenol-chloroform extraction in 5.2. (p.14, step 8).

5.1.4. Fluid Samples

Preparation

Fluid samples	Solubilization
e.g., plasma – freshly harvested	**Isolation Buffer (IB)** – at +4 °C or on ice
Centrifuge – at +4 °C	
Fume hood or laminar-flow cabinet	

Solubilization

The solubilization/homogenization step of the SPLIT protocol can be applied to a whole range of cells in fluids (blood, plasma, urine etc.) as long as the volume is reduced e.g. to 200 µl. Then, 200 µl Isolation Buffer is added. Depending on the sample a homogenization step might be necessary or you can proceed directly to the phenol-chloroform extraction in 5.2. (p.14, step 8). To prevent cross-contamination, it is best to work in a fume hood or a laminar-flow cabinet that can be UV-irradiated.

1. Centrifuge 300 - 400 µl of plasma at 12,000 x g for 5 minutes at 4°C to pellet the cell debris.
2. Transfer 200 µl of the supernatant to a new tube. Take care to avoid carry-over of cell debris.
3. Add 200 µl Isolation Buffer (**IB**) and mix properly.
4. Continue immediately with the phenol-chloroform extraction in 5.2. (p.14, step 8).

5.2. Phenol-Chloroform Extraction

Preparation

	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. The phase lock gel matrix will act as a barrier in between the two phases.

8. For each sample, centrifuge one 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. **ATTENTION:** Phase lock gel tubes should be equilibrated for 30 minutes at room temperature before use!
9. Transfer the homogenized sample 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. **ATTENTION:** Temperatures below 18 °C can negatively influence phase separation. Repeat centrifugation at correct temperature if phase separation is incomplete.

16 Transfer the upper phase to a new 2 ml micro-tube by decanting. **ATTENTION:** Do not transfer the upper phase by pipetting to avoid carry-over of the phase lock gel.

17 For the purification of **total RNA**, proceed with **step 18 in 5.3.1.1.** For the purification of the **large and small RNA fraction**, proceed with **step 18 in 5.3.1.2. ATTENTION:** For the small RNA fraction column loading of the large RNA must be performed first. The small RNA fraction will be in the flow-through then.

5.3. Column-based Purification

Preparation

	Total RNA	Large RNA	Small RNA	Small and Large RNA	Temperature
Isopropanol **Wash Buffer (WB)[1]** **Elution Buffer (EB) or Storage Buffer (SB)[2]**	~1050 µl 1100 µl 50 µl	~200 µl 1100 µl 50 µl	~1000 µl 1100 µl 50 µl	~1000 µl 2x 1100 µl 2 x 50 µl	RT RT RT
Purification column **Collection tube** **2.0 ml micro-tube** **1.5 ml micro-tube**	1 1 - 1	1 1 - 1	2 2 1 1	2 2 1 2	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.
[2] see Appendix C (p.24) whether **EB** or **SB** should be used for elution.

REMARK: Repeat centrifugation or increase centrifugation time if sample did not pass filter completely.

5.3.1. Column Loading

5.3.1.1. Column Loading of Total RNA

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

18 Determine the volume of the aqueous phase, which may vary, depending on the sample volume and volume transfer efficiency during homogenization and extraction. 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 and proceed with column washing and elution in 5.3.2. (p.17, step 23).

5.3.1.2. Column Loading of Large RNA

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 and can be further purified (see 5.3.1.3. Column Loading of Small RNA).

18	Determine the volume of the aqueous phase, which may vary, depending on the tissue volume and volume transfer efficiency during homogenization and extraction. Add isopropanol at 0.33 x of this volume (e.g., 200 µl isopropanol to 600 µl sample). Mix by vortexing for 10 seconds. **ATTENTION:** For best reproducibility of the size cut-off it is essential to quantify the volume of the aqueous phase exactly.
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. **ATTENTION: If you want to also isolate the small RNA fraction, pipette the flow-through into a 2 ml micro-tube. Else discard the flow-through**.
22	Repeat steps 20 - 21 until the mixture is loaded completely and proceed with column washing and elution in 5.3.2. (p.17, step 23). **REMARK:** If only the small RNA fraction is of interest to you, discard the spin-column now containing the large RNA and proceed with Column Loading of Small RNA of the flow-through (5.3.1.3.).

5.3.1.3. Column Loading of Small RNA

The small RNA will be in the flow-through of the large RNA fraction (see 5.3.1.2. Column Loading of Large RNA) and is recovered by precipitation onto a new purification column with the addition of 1 x volume of isopropanol.

18	Determine the volume of the flow-through in the 2 ml micro-tube (from step 22, 5.3.1.2. Column Loading of Large RNA) and add the same volume of isopropanol (e.g., 800 µl isopropanol to 800 µl flow-through). 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 and proceed with column washing and elution in 5.3.2. (p.17, step 23).

5.3.2. Column Washing and Elution of RNA

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. Empty the collection tube.

25 Centrifuge for 1 minute at 12,000 x g at 18 °C. **ATTENTION:** This step is essential to remove all traces of ethanol.

26 Discard the collection tube and place the purification column in new 1.5 ml microtube.

27 Make sure that no ethanol traces are carried to the new tube.

Elution of RNA

The RNA is eluted into an elution or storage buffer.

28 Apply either 10 - 50 µl Elution Buffer (**EB**) or Storage Buffer (**SB**) to the column.

29 Incubate for 1 minute at room temperature.

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

31 **OPTIONAL:** For a second elution, place the purification column in a new 1.5 ml microtube and repeat steps 28 - 30.

32 At this point the total RNA is purified and ready for quality control (Appendix B, p.22) and downstream applications.

33 **OPTIONAL:** Add RNase inhibitor (not included). See Appendix C (p.24) 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.

6. Short Procedure

6.1. Extraction of Total RNA

ATTENTION: All centrifugation steps are at 12,000 x g and 18 °C!

20 min	Homogenization and Phenol-Chloroform Extraction	
☐	Homogenize sample in 400 µl **IB**.	Homogenization
☐	Centrifuge 1 phase lock gel tube for 1 min.	Phenol-Chloroform Extraction
☐	Transfer homogenate into a phase lock gel tube.	
☐	Add 400 µl phenol solution pH 4.3, mix by inverting the tube 5 times.	
☐	Add 150 µl **AB**, mix by pipetting.	
☐	Add 200 µl chloroform and mix vigorously (3x 5 sec vortexing and 1 sec pausing).	
☐	Incubate for 2 min at RT.	
☐	Centrifuge for 2 min.	
☐	Decant the upper phase into a 2 ml micro-tube. **ATTENTION:** Do not transfer the upper phase by pipetting!	

10 min	Purification of Total RNA	
☐	Measure volume of transferred upper phase.	Column Loading Total RNA
☐	Add 1.75 x vol. isopropanol to the upper phase.	
☐	Mix by vortexing for 10 sec.	
☐☐	Load max. 800 µl onto purification column in collection tube. Centrifuge for 20 sec and discard flow-through. Repeat until mixture is loaded completely.	
☐	Apply 500 µl **WB** and centrifuge for 20 sec. Empty collection tube.	Column Washing
☐	Apply 600 µl **WB** and centrifuge for 20 sec. Empty collection tube.	
☐	Centrifuge for 1 min to spin dry column.	
☐	Place purification column in a 1.5 ml micro-tube.	Elution
☐	Apply 10 - 50 µl of either **EB** or **SB**, incubate for 1 min at RT.	
☐	Centrifuge for 1 min.	
☐	**OPTIONAL:** Second elution in new 1.5 ml micro-tube.	
☐	**OPTIONAL:** Add RNase inhibitor (not included).	

REMARK: Repeat centrifugation or increase centrifugation time if sample did not pass through the filter.

6.2. Extraction of Large and Small RNA Fractions

ATTENTION: All centrifugation steps are at 12,000 x g and 18 °C!

20 min Homogenization and Phenol-Chloroform Extraction

☐	Homogenize sample in 400 µl **IB**.	Homogenization
☐	Centrifuge 1 phase lock gel tube for 1 min.	Phenol-Chloroform Extraction
☐	Transfer homogenate into a phase lock gel tube.	
☐	Add 400 µl phenol solution pH 4.3, mix by inverting the tube 5 times.	
☐	Add 150 µl **AB**, mix by pipetting.	
☐	Add 200 µl chloroform and mix vigorously (3x 5 sec vortexing and 1 sec pausing).	
☐	Incubate for 2 min at RT.	
☐	Centrifuge for 2 min.	
☐	Decant the upper phase into a 2 ml micro-tube. **ATTENTION:** Do not transfer the upper phase by pipetting!	

15 min Purification of Large / Small RNA Fraction(s)

☐	Measure volume of transferred upper phase.	Column Loading Large RNA
☐	Add 0.33 x vol. isopropanol to the upper phase.	
☐	Mix by vortexing for 10 sec.	
☐☐	Load max. 800 µl onto purification column in collection tube. Centrifuge for 20 sec. Repeat until mixture is loaded completely. **ATTENTION:** Keep flow-through and transfer into a 2 ml micro-tube if small RNA extraction is desired. Else discard the flow-through. If only small RNA extraction is desired, the spin column now containing the large RNA fraction may be discarded.	
☐	Measure flow-through volume in 2 ml micro-tube and add 1 x vol. isopropanol. Mix by vortexing for 10 seconds	Column Loading Smalll RNA
☐☐	Load max. 800 µl onto new purification column in a collection tube. Centrifuge for 20 sec. Discard flow-through and repeat until loading is complete	
☐	Apply 500 µl **WB** to each column and centrifuge for 20 sec. Empty collection tube(s).	Column Washing
☐	Apply 600 µl **WB** and centrifuge for 20 sec. Empty collection tube(s).	
☐	Centrifuge for 1 min to spin dry column(s).	
☐	Place purification column(s) in a 1.5 ml micro-tube.	Elution
☐	Apply 10 - 50 µl of either **EB** or **SB**, incubate for 1 min at RT.	
☐	Centrifuge for 1 min.	
☐	**OPTIONAL:** Second elution in new 1.5 ml micro-tube.	
☐	**OPTIONAL:** Add RNase inhibitor (not included).	

REMARK: Repeat centrifugation or increase centrifugation time if sample did not pass through the filter.

7. Appendix A: Sample 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.

The SPLIT RNA Extraction Kit has been used for isolation of RNA from different organisms including animal (e.g., mouse, human) and plant tissues (e.g., A. thaliana, Picea abies), insects (e.g., drosophila), cell lines (e.g., human), fluid samples (e.g., plasma), and others (jellyfish, fungi, bacteria). Please contact us for more information (info@lexogen.com), we will gladly support you with protocol adaptations.

8. Appendix B: RNA Quality Control

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 homogenized according to 5.1.3. Extractions from tissue samples usually result in RNA with a RIN of 8.0 - 9.5.

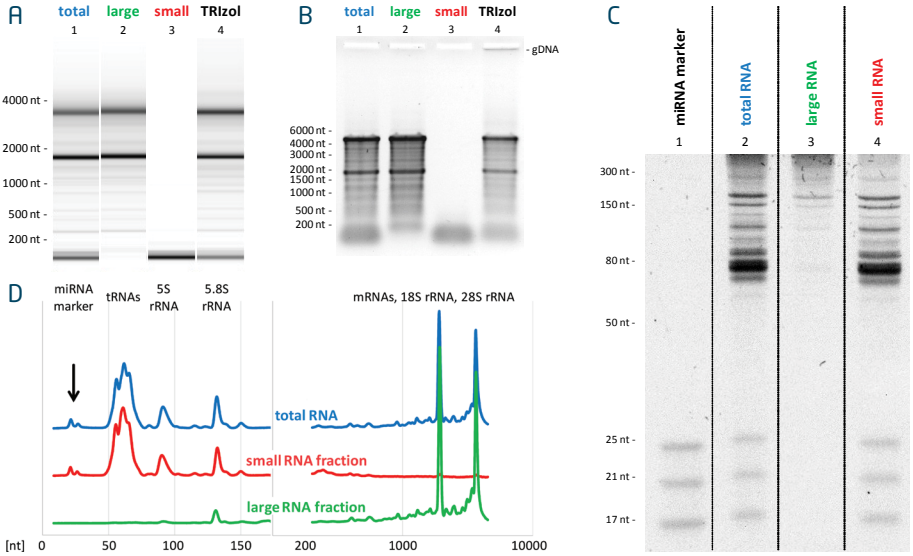
Potential Contaminants

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

* Efficiency is given in µg RNA per mg tissue.

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.

9. Appendix C: RNA Storage

After extraction, RNA can be stored in Elution Buffer (**EB**, 10 mM Tris-HCl pH 7.0) at -20 °C or -80 °C. This minimal buffer stabilizes the pH without any other components that might interfere with downstream applications. When eluting in **EB** we highly recommend the addition of RNase inhibitors to block any accidentally introduced RNases.

The Storage Buffer (**SB**, 10 mM Tris-HCl pH 7.0, 10 mM DTT and 0.1 mM EDTA) supplied with this kit can be used for intermediate storage of the RNA at -20 °C or -80 °C. DTT (antioxidant) and EDTA (chelating agent) both minimize the threat of RNA degradation, especially at non-freezing conditions. For long-term storage, we recommend keeping aliquots of the RNA as NaAc/ethanol precipitate at -80 °C to avoid accidental RNase contamination as well as RNA degradation due to freeze/thaw cycles.

We suggest checking the RNA quality after extended periods of storage for changes in integrity and quantity e.g., on a microfluidics system.

10. Appendix D: Revision History

Publication No.	Change	Page
008UG005V0220 2016-03-23	**CHANGES TO USER GUIDE -** The kit content was not changed.	
	New workflow overview graphic.	5
	Incorporation of homogenization protocols for plant tissue and fluid samples.	11, 13
	Column-based purification now one section for total and large/small RNA.	15 - 18
	Added tested RNA sources.	21
	Consistency changes.	
008UG005V0211 2015-05-08	**CHANGES TO USER GUIDE -** The kit content was not changed.	
	General text changes to account for consistency.	
	Note added on RNasin 230 nm absorption to be considered for OD blanking.	14, 17
	Optional RNasin addition added to Short Procedures.	18, 19
008UG005V0210 2014-08-27	**CHANGES TO USER GUIDE -** The kit content was not changed.	
	Figure 3 updated to include miRNA spike-in experiment.	21
008UG005V0206 2014-08-27	**CHANGES TO USER GUIDE -** The kit content was not changed.	
	The extra chloroform extraction was removed. Workflow, preparation table, number of PLG-tubes, volumes of user-supplied reagents and the phenol-chloroform extraction protocol were adapted accordingly.	5, 6, 7, 12
	Storage of all kit components can now be at +2 to +8 °C (+4 °C).	6
	Incubation and centrifugation times were shortened.	12 - 17
	Isopropanol volume increased to 1.75 x to maximize miRNA recovery.	13
	Max. loading volume of purification column increased from 600 µl to 800 µl.	13, 15, 16
	No re-elution but optional second elution into new micro-tube.	14, 17
	Short Procedures were adapted accordingly.	19 - 20
008UG005V0100 2013-08-19	Initial Release	

11. Notes

Please consult our Frequently Asked Questions page at https://www.lexogen.com/split-rna-extraction/#splitfaq for an updated list of relevant questions regarding the SPLIT kit. Furthermore, an up-to-date Troubleshooting Guide is available at www.lexogen.com.

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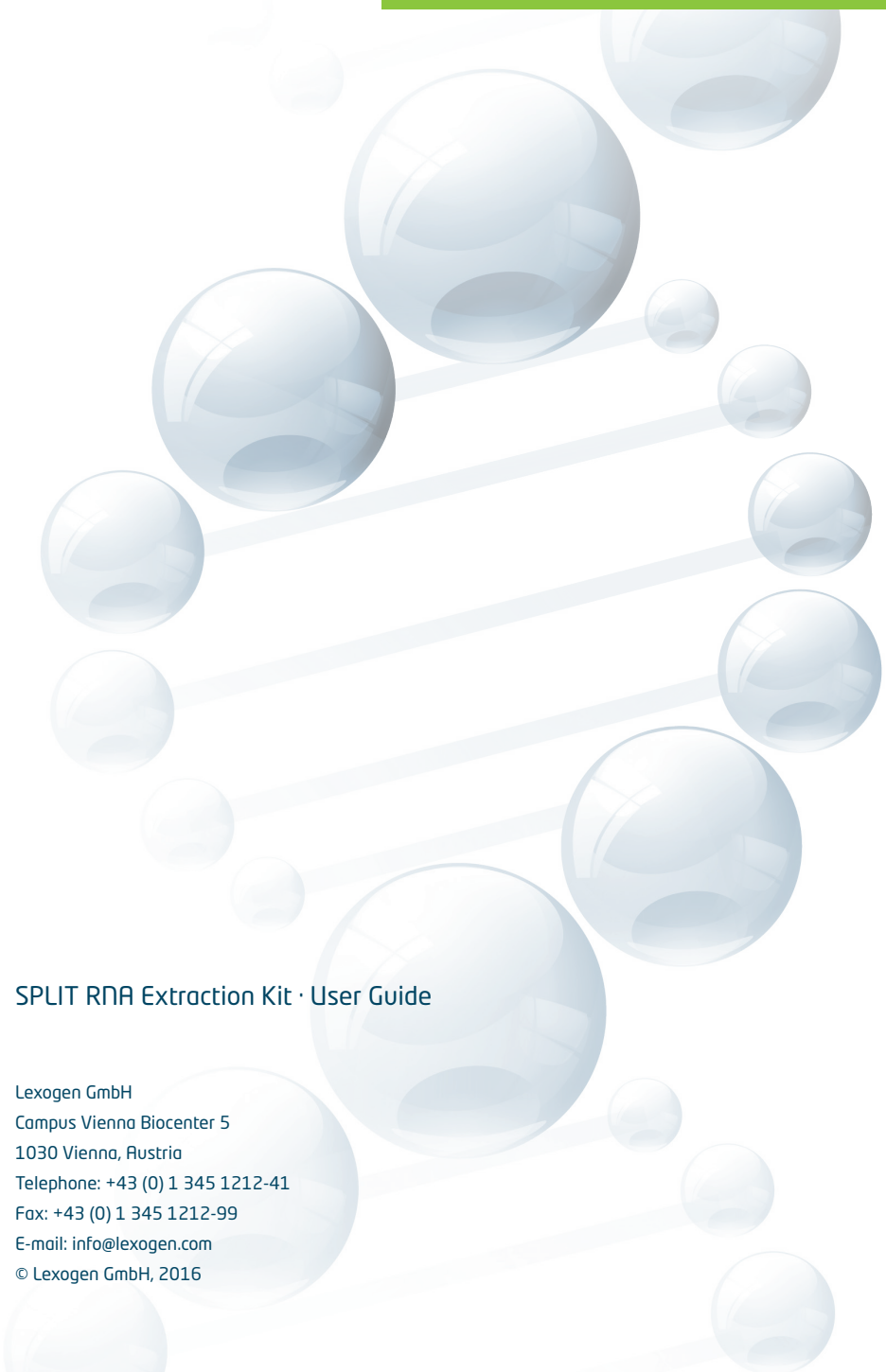
Enabling complete transcriptome sequencing



SPLIT RNA Extraction Kit · User Guide

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