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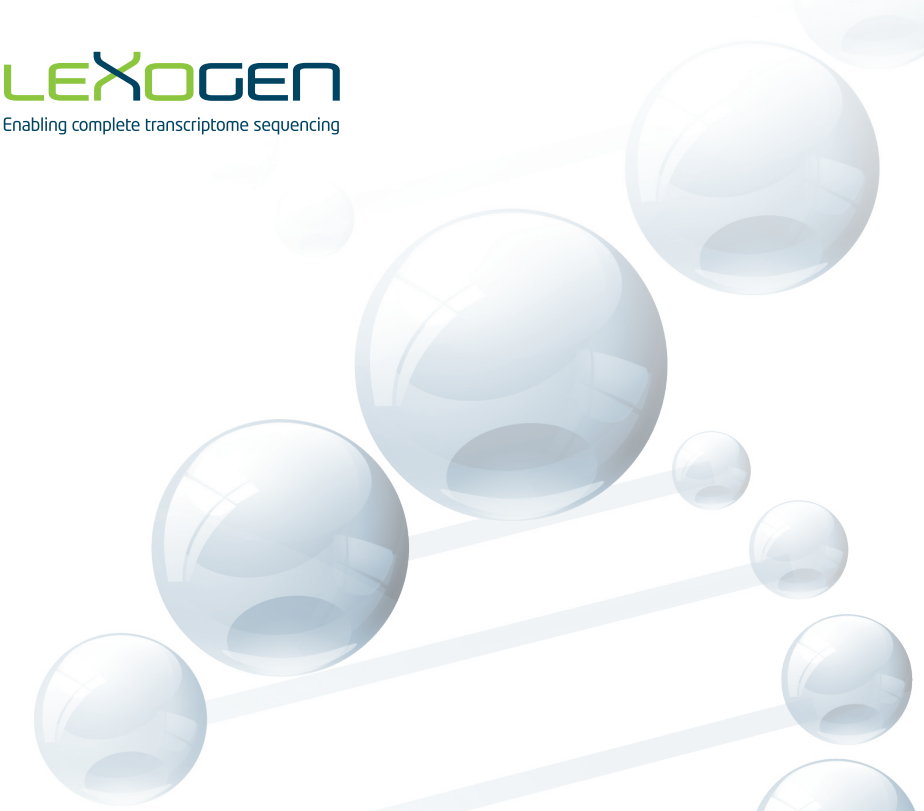
Enabling complete transcriptome sequencing



SPLIT

Fractions for pure RNA sequencing

RNA Extraction Kit

User Guide

Catalog Numbers:
008 (SPLIT RNA Extraction Kit)
099 (SPLIT RNA Extraction Kit for Blood)

008UG005V0310

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Important updates included in the SPLIT RNA Extraction Kit User Guide!

New changes to SPLIT RNA Extraction Kit and User Guide:

- A new SPLIT RNA Extraction Kit for Blood (Cat. No. 099) is now available. Kit components and protocols are included in this User Guide (see section 5.1.5.).
- Updated recommendations for RNA extraction from FACS/MACS sorted and cultured cells.
- Step 10: mix by inverting, step 11: mix by pipetting. Do not vortex.
- Step 13, and short protocol steps in Phenol Chloroform extraction: Mixing instruction changed to: mix by repeatedly inverting the tube for 15 seconds.
- Three washing steps with 500 µl **WB** buffer, at step 23 in 5.3.2. are now recommended.
- Attention note added to resuspend cell pellets in Isolation Buffer (**IB**) before storage.
- Step 27: **EB** or **SB** should be pre-warmed for 5 minutes at 70 °C to increase elution efficiency.

For further details please check the revision history at the end of the User Guide, or contact Lexogen technical support: support@lexogen.com, or +43 1345 1212-41.

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When describing a procedure for publication using this product, please refer to it as the SPLIT RNA Extraction Kit or SPLIT RNA extraction Kit for Blood.

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1. Overview

The SPLIT RNA Extraction Kits enable fast and highly efficient extraction of high quality, high purity RNA. 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 seamless library preparation 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 1.75x volume of isopropanol to the aqueous phase the entire total RNA will precipitate onto the silica carrier. When using only 0.33x volume isopropanol, large RNA with a lower limit of about 150 nucleotides will bind whereas the small RNA will be in the flow-through. By adding 1x 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 (Appendix A, p.21). For RNA extraction from human blood samples SPLIT RNA Extraction Kit for Blood (Cat. No. 099) is recommended. 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).

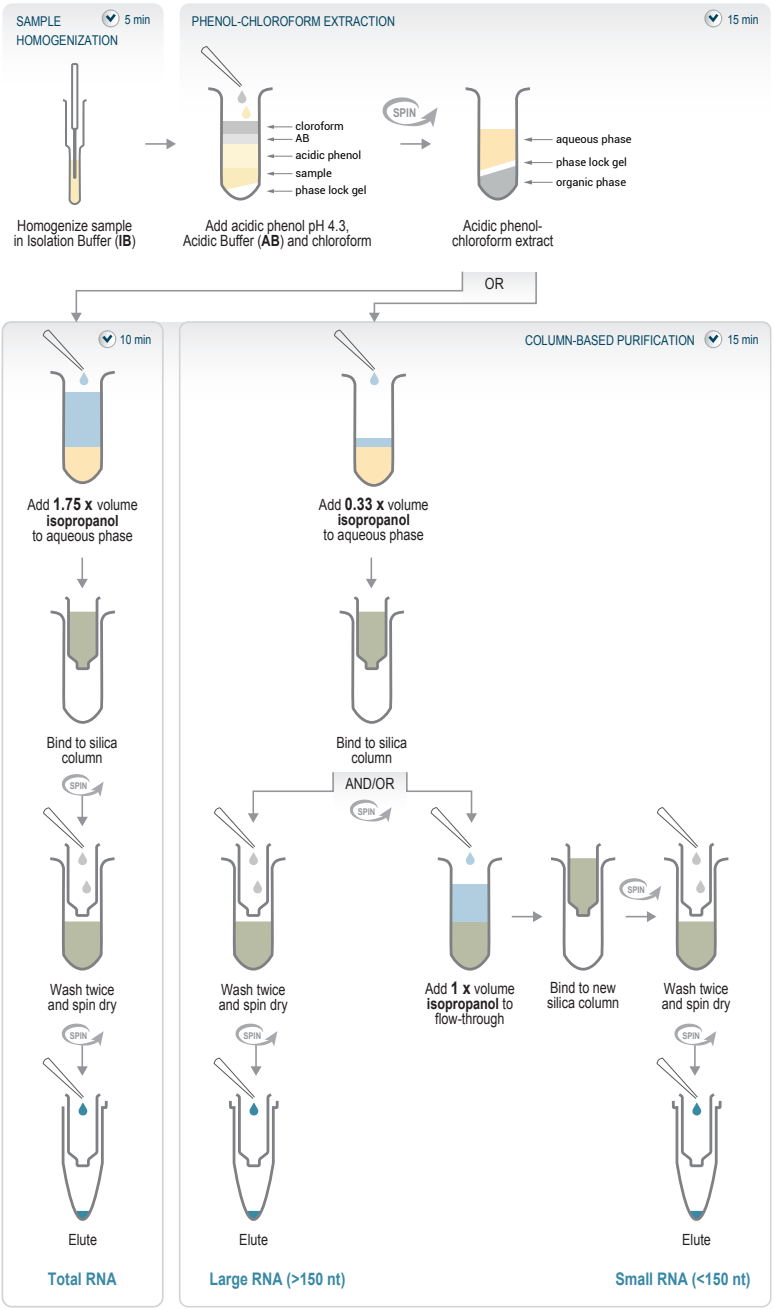


Figure 1. Schematic overview of the SPLIT workflow. The cut-off between large and small RNA fractions is at ~150 nucleotides.

2. Kit Components and Storage Conditions

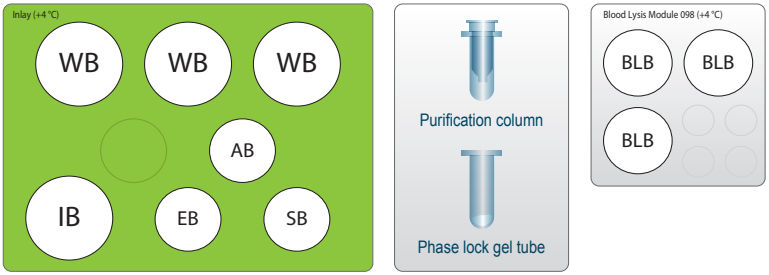


Figure 2. Location of kit components. The additional Blood Lysis Module is included in the SPLIT RNA Extraction Kit for Blood (Cat. No. 099) only.

Kit Component	Label	Volume*	Storage
Isolation Buffer	**IB**	21.1 ml	+4 °C
Acidic Buffer	**AB**	7.9 ml	+4 °C
Wash Buffer	**WB**	3 x 5 ml [1]	+4 °C
Elution Buffer	**EB**	2.6 ml [2]	+4 °C
Storage Buffer	**SB**	2.6 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
Blood Lysis Module included in the SPLIT RNA Extraction Kit for Blood (Cat. No. 099)			
Blood Lysis Buffer	**BLB**	3 x 13.2 ml	+4 °C

[1] Excluding ethanol (to be added by the user - see bottle for volume to add).
[2] For each RNA fraction, either EB or SB is required.

* Including ≥10 % surplus.

Upon receiving the SPLIT (Cat. No. 008) or SPLIT for Blood (Cat. No. 099) 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 be equilibrated to room temperature before use.
Check the contents of **IB**, **AB**, **WB**, **SB** which may precipitate during shipping and storage. If a white precipitate is visible, incubate at 37 °C until buffer components dissolve completely.

Add 25 ml absolute ethanol to each of the three bottles with Wash Buffer (**WB**) concentrate and shake to combine.

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) or VWR (Cat. No. 0981-100ML)
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 tubes).
- Calibrated single-channel pipettes for handling 10 µl to 1,000 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 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.

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

- Ensure that adequate volumes of all reagents and the necessary equipment are available before beginning the protocol.
- 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.
- 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 room temperature (20 - 25 °C). Results may be negatively impacted if the protocol is performed at temperatures outside of 18 - 25 °C.

- 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.

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 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 10 µg of total RNA. See Appendix A, p.21 for details on input and extraction efficiency.
4. **OPTIONAL:** Hard to homogenize tissues 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 at step 8 in 5.2. (p.15).

After use, clean the tissue grinder thoroughly with a detergent such as DNA-ExitusPlus™ (AppliChem GmbH), then rinse thoroughly 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 whilst frozen (i.e., in liquid nitrogen, or over dry ice) and homogenized in a highly chaotropic solution. Optimally, the plant material should be extracted immediately after harvesting. If storage of plant material is required, flash-freeze the sample in liquid nitrogen and store at -80 °C, or at -20 °C in RNAlater. Already ground 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 and homogenization methods may need to be optimized for different types of input plant material (e.g. waxy, low water content tissues). For more information please contact support@lexogen.com.

1 Determine the weight of the tissue on a precision balance, working under sterile conditions (e.g., use sterile tweezers for transfer). A range of 10 - 100 mg of plant tissue is a good starting point (see Appendix A, p.21).

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

3 Grind the tissue using pestle and mortar. This can be done in liquid nitrogen, or by grinding the frozen tissue in a 1.5 ml or 2 ml tube over dry ice.

4 Allow any 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 at step 8 in 5.2. (p.15).
OPTIONAL: ☞ Safe stopping point. Ground plant tissue in Isolation Buffer (**IB**) can be stored at -20 °C at this point.

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

5.1.3. Cultured Cells

Preparation

Cells	Solubilization
Cells – freshly harvested, FACS/MACS sorted or frozen	**Isolation Buffer (IB)** – at +4 °C or on ice
Fume hood or laminar-flow cabinet	

Solubilization

Cells are solubilized in a highly chaotropic solution. Lyse the cells fully in the Isolation Buffer (**IB**) and proceed directly to RNA extraction. Cell lysates in **IB** can also be stored at -80 °C prior to extraction.

NOTE: Recommendations for freshly harvested and FACS/MACS sorted cells: spin down to pellet the cells and wash twice with Phosphate Buffered Saline (1x PBS). Remove PBS completely before adding **IB**. To prevent cross-contamination, it is best to work in a fume hood or a laminar-flow cabinet that can be UV-irradiated.

ATTENTION: Do not store cells as cell pellets in **IB** without resuspending and lysing the cells beforehand. Cell lysis is required in order to maintain RNA integrity throughout storage in **IB**. Alternatively, snap freeze and store cell pellets after completely removing PBS and add **IB** when thawing to lyse cells before starting RNA extraction.

1 Harvest, pellet, and wash the cells. 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 1,000 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 at step 8 in 5.2. (p.15).

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 (aspirates, viral supernatants, plasma, urine etc.). Depending on the sample a homogenization step might be necessary or you can proceed directly to the phenol-chloroform extraction in 5.2. (p.15, 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.

NOTE: For sample types other than plasma, prior centrifugation may or may not be required. Use up to 200 µl of the liquid sample as input, then add 200 µl of Isolation Buffer (**IB**). For extraction of RNA from blood please see 5.1.5 Blood Samples.

ATTENTION: If the liquid sample volume is < 200 µl, add extra **IB** to bring the total volume of the sample / **IB** mix to 400 µl,

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 at step 8 in 5.2. (p.15).

5.1.5. Blood Samples

Preparation

Blood samples	Red Blood Cell Lysis	Solubilization
Blood sample - freshly collected in EDTA containing blood collection tube	**Blood Lysis Buffer (BLB)** - equilibrated to RT - protect from light!	**Isolation Buffer (IB)** - at +4 °C or on ice
Fume hood or laminar-flow cabinet	**1.5 ml or 2 ml tubes** **Centrifuge** – at RT	

The SPLIT RNA Extraction Kit for Blood (Cat. No. 099) includes the Blood Lysis Buffer (**BLB**) for the lysis of red blood cells from blood samples. **BLB** must be used prior to RNA extraction. Red blood cell lysis results in depletion of the majority of globin mRNA which account for 50 - 80 % of total RNA in blood samples. This protocol is recommended for RNA that will be used for downstream CORALL Total RNA-Seq (Cat. No. 095, 096, 117, 118, and 119), or SENSE mRNA-Seq (Cat. No. 001) library preparation. It can also be used in combination with QuantSeq 3′ mRNA-Seq Library Prep Kits (Cat. No.'s 015, 016, 113, 114, and 115) with or without the RS-Globin Block, *Homo sapiens* Module (Cat. No. 070).

To prevent cross-contamination, it is best to work in a fume hood or a laminar-flow cabinet that can be UV-irradiated.

NOTE: This protocol is verified for use with 50 - 250 µl of fresh human blood collected in EDTA containg blood collection tubes. Using 50 µl of whole blood is the maximum volume we could recommend when BLB is not used for SPLIT RNA Extraction (i.e. extracting RNA from whole blood).

ATTENTION: RNA quality can be severely affected by prolonged storage. Please contact support@lexogen.com if you wish to use this protocol with blood from other species, variable input blood volumes, or with blood exposed to other collection methods or storage conditions.

1. Equilibrate the Blood Lysis Buffer (**BLB**) to room temperature for 30 minutes before use. **ATTENTION:** protect from light!
2. Mix whole blood with Blood Lysis Buffer (**BLB**) at a 1:3 ratio in a 1.5 ml or 2 ml tube. Mix by inverting 5 - 6 times. **EXAMPLE:** For 50 µl human blood add 150 µl of **BLB**.
3. Incubate for 10 minutes at room temperature. Re-mix the solution 2 - 3 times during this incubation, by inverting the tube 5 - 6 times.
4. Centrifuge at 2,000 x g for 10 minutes at room temperature.
5. Remove and discard the supernatant. Do not disturb the pellet – some residual liquid can remain in the tube.
6. Add 400 µl of Isolation Buffer (**IB**) and mix well by pipetting to lyse the cells. **OPTIONAL:** ☞ Safe stopping point. Lysed cells can be stored in **IB** at -80 °C.
7. Continue immediately with the phenol-chloroform extraction at step 8 in 5.2. (p.15).

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 tube**	1 1	RT RT
Centrifuge – at 18°C **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 thoroughly by repeatedly inverting the tubes for 15 seconds (do not vortex!). **ATTENTION:** Thorough mixing is essential to disperse the chloroform efficiently and effectively separate all the phenol that will contain gDNA and protein into the organic and interphase.
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 18 - 25 °C if phase separation is incomplete.
16. Transfer the upper phase to a new 2 ml 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 isolating the small RNA fraction, the large RNA is retained on the column first and the small RNA fraction will be in the flow-through and is loaded onto a second column for purification.

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]**	~1,050 µl 1,100 µl 50 µl	~200 µl 1,100 µl 50 µl	~1,000 µl 1,100 µl 50 µl	~1,000 µl 2x 1,100 µl 2 x 50 µl	RT RT RT
Purification column **Collection tube** **2.0 ml tube** **1.5 ml 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 1.75x 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 1.75x of this volume. Mix by vortexing for 10 seconds. **EXAMPLE:** Add 1,050 µl isopropanol to 600 µl sample.

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 contents of the collection tube.

22 Repeat steps 20 - 21 until the mixture is loaded completely then proceed to column washing and elution at step 23 in 5.3.2. (p.18).

5.3.1.2. Column Loading of Large RNA

The large RNA fraction is precipitated onto a silica column by the addition of 0.33x 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 0.33x 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:** To isolate the small RNA fraction, pipette the flow-through into a 2 ml tube.

22 Repeat steps 20 - 21 until the mixture is loaded completely and then proceed to column washing and elution at step 23 in 5.3.2. (p.18). **REMARK:** If only the small RNA fraction is of interest, discard the spin-column containing the large RNA and collect the flow-through into the 2 ml tube. Continue to Column Loading of Small RNA at step 18 in 5.3.1.3 (below). Else discard the flow-through.

5.3.1.3. Column Loading of Small RNA

The flow-through obtained in steps 21 - 22 of 5.3.1.2 (Column Loading of Large RNA) contains the small RNA fraction and is recovered by precipitation onto a new purification column with the addition of 1x volume of isopropanol.

18 Determine the total volume of the flow-through in the 2 ml tube (from step 22, 5.3.1.2.) and add the same volume of isopropanol. Mix by vortexing for 10 seconds. **EXAMPLE:** 800 µl isopropanol to 800 µl flow-through.

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 then proceed to column washing and elution at step 23 in 5.3.2. (p.18).

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. Repeat this step twice for a total of three washes.

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

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

26 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.

27 Pre-warm the Elution Buffer (**EB**) or Storage Buffer (**SB**) for 5 minutes at 70 °C.

28 Add 10 - 50 µl of the pre-warmed Elution Buffer (**EB**) or Storage Buffer (**SB**) to the column and incubate for 1 minute at room temperature.

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

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

31 **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 for 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

	Homogenization
☐	Homogenize sample in 400 µl **IB**.
	Phenol-Chloroform Extraction
☐	Centrifuge 1 Phase Lock Gel tube for 1 min.
☐	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 by repeatedly inverting the tube for 15 sec. **ATTENTION:** Do not vortex!
☐	Incubate for 2 min at RT.
☐	Centrifuge for 2 min.
☐	Decant the upper phase into a 2 ml tube. **ATTENTION:** Do not transfer the upper phase by pipetting!

10 min Purification of Total RNA

	Column Loading Total RNA
☐	Measure volume of transferred upper phase.
☐	Add 1.75x 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.
	Column Washing
☐☐☐	Apply 500 µl **WB** and centrifuge for 20 sec. Empty collection tube. Repeat this step twice for a total of three washes.
☐	Centrifuge for 1 min to spin dry column.
	Elution
☐	Place purification column in a 1.5 ml tube.
☐	Pre-warm **EB** or **SB** for 5 min at 70 °C.
☐	Apply 10 - 50 µl of pre-warmed **EB** or **SB**, incubate for 1 min at RT.
☐	Centrifuge for 1 min.
☐	**OPTIONAL:** Add RNase inhibitor (not included).

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

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

	Homogenization
☐	Homogenize sample in 400 µl **IB**.
	Phenol-Chloroform Extraction
☐	Centrifuge 1 Phase Lock Gel tube for 1 min.
☐	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 by repeatedly inverting the tube for 15 sec. **ATTENTION:** Do not vortex!
☐	Incubate for 2 min at RT.
☐	Centrifuge for 2 min.
☐	Decant the upper phase into a 2 ml tube. **ATTENTION:** Do not transfer the upper phase by pipetting!

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

	Column Loading Total RNA
☐	Measure volume of transferred upper phase.
☐	Add 0.33x 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 tube if small RNA extraction is desired. If only small RNA extraction is desired, discard the spin column.
	Column Loading Smalll RNA
☐	Measure flow-through volume in 2 ml tube and add 1x vol. isopropanol. Mix by vortexing for 10 sec.
☐☐	Load max. 800 µl onto new purification column in a collection tube. Centrifuge for 20 sec. Discard flow-through and repeat until mixture is loaded completely.
	Column Washing
☐☐☐	Apply 500 µl **WB** to each column and centrifuge for 20 sec. Empty collection tube(s). Repeat this step twice for a total of three washes.
☐	Centrifuge for 1 min to spin dry column(s).
	Elution
☐	Place purification column(s) in a 1.5 ml tube.
☐	Pre-warm **EB** or **SB** for 5 min at 70 °C.
☐	Apply 10 - 50 µl of pre-warmed **EB** or **SB**, incubate for 1 min at RT.
☐	Centrifuge for 1 min.
☐	**OPTIONAL:** Add RNase inhibitor (not included).

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

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. Tissues / cells frozen 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). The maximum binding capacity of the purification column is 10 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 support@lexogen.com for information on protocol adaptations for other sample types.

The SPLIT RNA Extraction Kit for Blood (Cat. No. 099) includes the Blood Lysis Buffer (**BLB**), which is used for lysis of red blood cells in whole blood, prior to RNA extraction. Red blood cell lysis results in depletion of the majority of globin mRNA which accounts for 50 - 80 % of total RNA in blood. Thus, the SPLIT RNA Extraction Kit for Blood is the ideal solution for blood RNA-Seq applications, including downstream library preparation using the CORALL Total RNA-Seq Library Prep Kit (Cat. No.s 095, 096, 117, 118, and 119).

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.). However, 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.

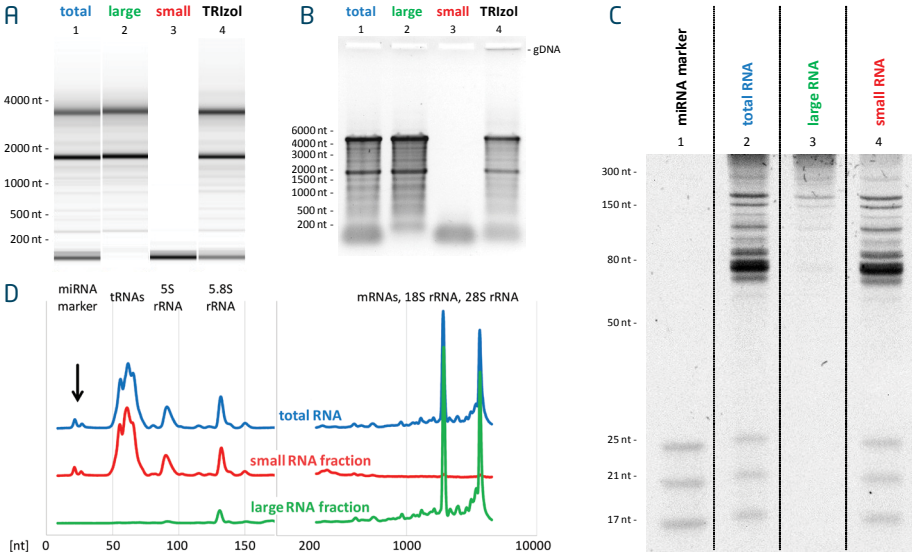
Potential Contaminants

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 these kits 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. / Revision Date	Change	Page
008UG005V0310 Jan. 10, 2020	Legal terms and conditions statements updated.	2
	Step numbers were corrected.	17-18
	Step 10: mix by inverting, step 11: mix by pipetting. Do not vortex.	15
	Step 13, and short protocol steps in Phenol Chloroform extraction: Mixing instruction changed to: mix by repeatedly inverting the tube for 15 seconds. ATTENTION note also updated.	15, 19 - 20
008UG005V0300 Jul. 29, 2019	Kit component and reagent volumes updated.	6
	Recommendations for cultured cells updated (do not store in RNAlater, lyse cell pellets in IB before storage).	12
	Protocol for SPLIT RNA Extraction for Blood (see 5.1.5) added.	14
	Step 23: Wash with 3x 500 µl **WB** (see 5.3.2.).	18, 19, 20
	Step 27: Pre-warm **EB** or **SB** for 5 minutes at 70 °C before Elution.	18, 19, 20
	Short Procedure steps updated, and reformatted.	19, 20
008UG005V0220 Mar. 22, 2016	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
008UG005V0211 May. 11, 2015	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 Aug. 27, 2014	Figure 3 updated to include miRNA spike-in experiment.	21
008UG005V0206 Jul. 8, 2014	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 1.75x 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 tube.	14, 17
	Short Procedures were adapted accordingly.	19 - 20
008UG005V0100 Aug. 19, 2013	Initial Release	

11. Notes

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016 (QuantSeq 3′ mRNA-Seq Library Prep Kit for Illumina (REV) with Custom Sequencing Primer)
025, 050, 051 (SIRVs Spike-in RNA Variant Control Mixes)
037 (RiboCop rRNA Depletion Kit)
039 (Poly(A) RNA Selection Kit)
070 (RS-Globin Block, *homo sapiens*)
095 (CORALL Total RNA-Seq Library Prep Kit)
096 (CORALL Total RNA-Seq Library Prep Kit with RiboCop)
113 (QuantSeq 3′ mRNA-Seq Library Prep Kit FWD with UDI 12 nt Set A1, (UDI12A_0001-0096), 1 rxn/UDI)
114 (QuantSeq 3′ mRNA-Seq Library Prep Kit FWD with UDI 12 nt Set B1, (UDI12B_0001-0096), 1 rxn/UDI)
115 (QuantSeq 3′ mRNA-Seq Library Prep Kit FWD with UDI 12 nt Set A1, (UDI12A_0001-0384), 1 rxn/UDI)
117 (CORALL Total RNA-Seq Library Prep Kit with UDI 12 nt Set A1, (UDI12A_0001-0096), 1 rxn/UDI
118 (CORALL Total RNA-Seq Library Prep Kit with UDI 12 nt Set B1, (UDI12B_0001-0096), 1 rxn/UDI)
119 (CORALL Total RNA-Seq Library Prep Kit with UDI 12 nt Sets A1-A4, (UDI12A_0001-0384), 1 rxn/UDI)

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