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SPEEDTOOLS TOTAL RNA EXTRACTION KIT

Designed for the rapid isolation of highly pure RNA from cells and tissue

Instructions for Use

(Ref. 21.210M/1/2)

PLEASE READ THE INSTRUCTIONS FOR USE THOROUGHLY BEFORE USING THE KIT, ESPECIALLY IF YOU ARE NOT FAMILIAR WITH THE PROTOCOL.

1. BASIC PRINCIPLE

The Kit is designed for the rapid isolation and purification of total RNA from cultured cells and tissue. The technology behind the Speedtools extraction kits is based on adsorption and desorption of nucleic acids to specially treated silica membrane in the presence of chaotropic salts.

The starting material i.e. cultured cells are lysed by incubation in a **lytic solution** containing large amounts of chaotropic ions. This lysis buffer immediately inactivates RNases, present in virtually all biological materials, and creates the appropriate conditions for **binding of nucleic acids to the silica membrane** of the RNA binding column. The obtained lysate is clarified by filtration through a filtering column. This step reduces the viscosity of the lysate which favour the process of binding to the RNA column. Contaminating DNA, which is also bound to the silica membrane, is removed by a **digestion step** in which a recombinant **DNase** is directly applied onto the column membrane. Simple **washing steps** with two different buffers remove salts, metabolites and macromolecular cellular components. **Pure RNA is finally eluted** under low ionic strength conditions with RNase-free H_2O.

The buffers formulated in the kit have been optimised to prevent degradation of the RNA during the isolation procedure. Thus, all the steps can be performed **at room temperature**.

The eluate with the **purified RNA** should be treated with maximum care because RNA is very sensitive to trace contaminations of RNases, often found on general lab ware, fingerprints and dust. To ensure RNA stability keep RNA **frozen** at –20°C for short-term or at –70°C for long-term storage.

2. KIT CONTENTS

SPEEDTOOLS TOTAL RNA EXTRACTION KIT			
Reagents	**10 Preps Ref. 21.210M**	**50 Preps Ref. 21.211**	**250 Preps Ref. 21.212**
LYSIS BUFFER LR	10 mL	25 mL	5 x 25 mL
WASH BUFFER WR1	15 mL	15 mL	5 x 15 mL
WASH BUFFER WR2 (concentrated)	6 mL	12 mL	5 x 12 mL
DESALTING BUFFER DBR	10 mL	25 mL	5 x 25 mL
BUFFER FOR rDNase	7 mL	7 mL	5 x 7 mL
rDNase, free of RNases (lyophilised)	1 vial (80U)	1 vial (200U)	5 x 1 vial (200U)
RNase-free H_2O	13 mL	13 mL	5 x 13 mL
FILTERING COLUMNS (violet ring)	10	50	5 x 50
RNA BINDING COLUMNS (blue ring) with Collection Tubes	10	50	5 x 50
COLLECTION TUBES (2 ml)	30	150	5 x 150
COLLECTION TUBES (1.5 ml)	10	50	5 x 50
PROTOCOL	1	1	5

3. KIT SPECIFICATIONS

Speedtools Total RNA Extraction Kit is recommended for the isolation of total RNA from **cultured cells** and **tissue**. Support protocols for the isolation of total RNA from **cell-free biological fluids**, **bacteria** and **yeasts** are included. The kit is also suitable for **clean-up of RNA** from reaction mixtures. Even biological samples which are difficult to process will yield high quality RNA with this kit (e.g. mouse liver or brain; tumor cell lines).

The Kit allows purification of pure RNA with an **$A_{260/280}$ ratio** generally **≥ 1.9** (measured in TE buffer, pH 7.5). Additionally, the isolated RNA from fresh high quality sample material usually has a **RIN** (RNA integrity number) **> 9.0**. However, RNA integrity strongly depends on the sample quality.

The amount of DNA contamination is minimal due to on-column digestion with rDNase. However, in very sensitive applications it might be possible to detect traces of DNA; in these cases a DNase treatment in solution is recommended. The probability of DNA detection with PCR increases with: the number of DNA copies per preparation and decreasing PCR amplicon size.

The standard protocol allows the purification of up **to 70 µg of total RNA** per RNA binding column from up to **5 x 10^6 cultured cells** or **30 mg of tissue**.

The isolated RNA is ready to use for applications like RT-PCR, Nothern, primer extension, arrays or RNase protection assays. The isolated RNA can be used as template in a reverse transcription PCR (RT-PCR) reactions. We recommend using lower quantities of sample, 1-10% of the eluate of total RNA prepared from 1 x 10^6 cultured cells or 10 mg of tissue is sufficient as starting material.

Table 1: General characteristics of the Kit	
Sample material	< 5 x 10^6 cultured cells < 10^8 yeast cells < 10^9 bacterial cells < 30 mg tissue
Average Yield	14 µg, from HeLa cells 70 µg, from bacterial cells
Elution Volume	40 - 120 µL
Binding Capacity	200 µg
A_{260}/A_{280}	1.9-2.1
RIN (RNA integrity number)	> 9
Time / Prep	30 min/6 preps
Spin Column	mini

The Kit can be used for preparing RNA from different amounts of sample material. For optimal results the volume of Lysis Buffer LR[1] (Step 1 of the Standard Protocol) and molecular grade ethanol (Step 4 of the Standard Protocol) should be adapted according to the **Table 2**. If 600 µL of Lysis Buffer LR and molecular grade ethanol is used an additional loading step will be required, load the sample onto the column in two successive centrifugation steps.

[1] The volume of Lysis Buffer LR included in the Kit is not sufficient to perform all preparations with 600 µl. If required, additional lysis buffer LR can be ordered separately.

Table 2: Recommended volumes of Buffer LR and Molecular Grade Ethanol			
Sample	**Amount**	**Buffer LR**	**Molecular Grade Ethanol**
Cultured animal or human cells	< 5 x 10^6 cells	350 µL	350 µL
Human or animal tissue	< 20 mg 20 - 30 mg*	350 µL 600 µL	350 µL 600 µL
Tissue stored in RNA*later*®	< 20 mg 20 - 30 mg*	350 µL 600 µL	350 µL 600 µL
Samples hard to lyse	< 5 x 10^7 cells*	600 µL	600 µL

Depending on sample type, the average yield is between 5 µg -70 µg total RNA (see Table 3); and the $A_{260/280}$ ratio, generally exceeds 1.9.

Table 3: Overview on average yields of the Kit	
Sample	**Average yield**
8 x 10^4 HeLa cells	1.5 µg
4 x 10^5 HeLa cells	4 µg
1 x 10^6 HeLa cells	14 µg
2 x 10^6 HeLa cells	21 µg
2.5 x 10^6 HeLa cells	25 µg
5 x 10^6 HeLa cells	50 µg

4. HANDLING, PREPARATION, AND STORAGE OF STARTING MATERIALS

RNA is not protected against degradation until the sample material is flash frozen or disrupted in the presence of RNase inhibiting or denaturing agents. Therefore it is important that samples are flash frozen in liquid N_2 immediately and stored at –70°C, or processed as soon as possible.

Samples can be stored in Lysis Buffer LR after disruption at –70°C for up to one year, at 4°C for up to 24 hours or up to several hours at room temperature. Frozen samples (without Lysis Buffer) are stable up to 6 months. Frozen samples in Buffer LR should be thawed slowly before starting with the isolation of RNA.

NOTE: Wear gloves at all times during the preparation and change them frequently.

CELL CULTURES

Cell lysis of suspension cell culture

Collect cells by centrifugation, discard the supernatant and resuspend the pellet in Lysis Buffer LR according to step 2 of the Standard Protocol.

Cell lysis of adherent growing cells in a culture dish

To allow full activity of the lysis buffer the culture medium has to be removed completely. Aspirate the culture medium, and continue immediately with the addition of Lysis Buffer LR to the cell-culture dish.

To trypsinize adherent growing cells

Aspirate the cell-culture medium, and wash the cells adding an equal amount of PBS. Aspirate PBS and add 0.1 – 0.3% trypsin in PBS. Incubate for an appropriate time to detach the cells from the dish surface. After cell detachment, add medium, transfer cells to an appropriate tube and pellet the cells by centrifugation for 5 min at 300 x *g*. Remove supernatant and add Lysis Buffer LR to the cell pellet.

ANIMAL TISSUES

If the animal tissue is solid it must be broken up mechanically. Depending on the disruption method, the viscosity of the lysed sample has to be reduced further for obtaining optimal results. For an efficient RNA

preparation all the RNA contained in the sample must be released from the cells by disruption, and that the viscosity of the sample must be reduced by homogenisation.
The most commonly used technique for disruption of animal tissues is grinding with a pestle and mortar. Grind the sample to a fine powder in the presence of liquid N_2. Take care that the sample does not thaw during the grinding process or after grinding during weighing. Add the frozen powder to an appropriate aliquot of Buffer LR containing β-mercaptoethanol and mix immediately. The broken-up tissue must then be homogenised with a Filtering Column or by passing ≥ 5 times through a 0.9 mm syringe needle.

Thawing of undisrupted animal tissue should be exclusively done in the presence of Buffer LR during simultaneous mechanical disruption. This ensures that the RNA is not degraded by RNases before the preparation has started. When using any kind of commercial homogeniser keep the rotor tip well submerged in the mixture in order to avoid excess foaming formation.

BACTERIA AND YEASTS

Bacteria and yeast have to be incubated in lysozyme or lyticase/zymolase solutions, respectively (see the support protocol in sections 8C and 8D). By this treatment, the robust cell walls of these organisms are digested or at least weakened, which is essential for effective cell lysis by Buffer LR. For microorganisms with extremely resistant cell walls – like some Gram-positive bacterial strains – it may be necessary to optimize the conditions of the treatment with the lytic enzymes or the cultivation conditions. After lysis, homogenisation is achieved by the use of the Filtering Columns or the syringe-needle method.

5. ELUTION PROTOCOLS

It is possible to adapt the elution method and volume of water used for the subsequent application of interest. In addition to the elution method described in each individual protocol (recovery rate about **70-90%**) several modifications are possible.

- ✓ **High yield:** Perform **two elution steps** with the volume indicated in the individual protocol. About **90-100%** of bound nucleic acid will be eluted.
- ✓ **High yield and high concentration:** Elute with the standard elution volume and apply the eluate once more onto the column for **re-elution**.

Eluted RNA should immediately be kept on ice for optimal stability to avoid degradation for the omnipresent RNases present in lab ware, fingerprints, dust, etc. For short-term storage freeze the purified RNA at –20°C, for long-term storage freeze at –70°C.

6. PREPARATION OF WORKING SOLUTIONS AND STORAGE CONDITIONS

NOTE: ***Buffers LR, WR1 and DBR*** **contain guanidine thiocyanate, wear gloves and goggles**.

- On arrival store lyophilized **rDNase** (free of RNases) at **4°C**.
- All other Kit components should be stored at room temperature (18-25°C)
- All kits components are stable up to the expiration date printed on the packaging label.

Before starting any protocol with this kit prepare the following reagents:

I. rDNase, free of RNases:

- ✓ **10 Preps Format:** Add **230 µL of RNase-free H_2O** to the rDNase vial and incubate for 1 min at room temperature.
- ✓ **5 Preps Format:** Add **540 µL of RNase-free H_2O** to the rDNase vial and incubate for 1 min at room temperature.

Gently swirl the vials to completely dissolve the DNase. Be careful not to mix ***rDNase*** *vigorously as it is* ***sensitive to mechanical agitation****.*

Dispense into aliquots and store at –20°C. The frozen working solution is stable for at least 6 months. Do not freeze/thaw more than three times.

II. Wash Buffer WR2:

- ✓ **10 Preps Format:** Add **24 mL of 96–100% molecular grade ethanol** to Buffer WR2 Concentrate. Mark the label of the bottle to indicate that ethanol was added.
- ✓ **50 Preps Format:** Add **48 mL of 96–100% molecular grade ethanol** to Buffer WR2 Concentrate. Mark the label of the bottle to indicate that ethanol was added.

Store the obtained Wash Buffer WR2 at room temperature (18-25°C) for at least one year.

7. REAGENTS AND EQUIPMENT REQUIRED BUT NOT PROVIDED

- Molecular Grade Ethanol (70% and 96-100%)
- Reducing agent (β-mercaptoethanol; DTT or TCEP)
- Centrifuge for microtubes
- Pipettes and sterile RNase-free tips
- 1.5 ml microtubes
- Equipment for sample disruption and homogenisation

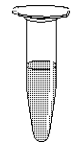
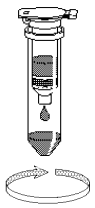
8. INSTRUCTION FOR USE

A. STANDARD PROTOCOL: Total RNA purification from cultured cells and tissue

Before starting the preparation:

- Check if Wash Buffer WR2 and rDNase were prepared according to Section 6.

STEP	DESCRIPTION		
1	**SAMPLE PREPARATION** ***Tissue*** Disrupt up to 30 mg of tissue ***Cultured cells*** Collect up to 5 x 10^6 eukaryotic cultured cells by centrifugation and lysed by addition of Lysis Buffer LR directly.		DISRUPT SAMPLE
2	**CELL LYSIS** Add **350 µL Buffer LR** and **3.5 µL β-mercaptoethanol** to the pellet of cells or to tissue and vortex vigorously. *For appropriate sample and lysis buffer amounts see Table 2.* ***Note:*** *As alternative to β-mercaptoethanol the reducing agent DTT or TCEP may be used. Use a final concentration of 10-20 mM within the Lysis Buffer LR.*		350 µL BUFFER LR + 3.5 µL β -mercapto ethanol
3	**FILTRATION OF THE LYSATE** Place a **Filtering Column** (violet ring) in a collection tube, **apply the lysate** and centrifuge for **1 min at 11,000 x *g***. In this way the lysate will be clarify and its viscosity will be reduced. *The lysate may be passed alternatively ≥ 5 times through a 0.9 mm needle (20 gauche) fitted to a syringe.* *In case of visible pellet formation transfer supernatant without any formed pellet to a new 1.5 ml microtube* ***Note:*** *To process higher amounts of cells (> 1 x 10^6) or tissue (>10 mg), the lysate should be homogenised using the 0.9 mm needle (20 gauche), followed by filtration through Filtering Column.*		Load lysate into a Filtering Column (violet ring) *1 min, 11,000 × g*

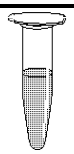
4	**ADJUST RNA BINDING CONDITIONS** Discard the filtering column and add **350 µL molecular grade ethanol 70%** to the clarified lysate and **mix** by pipetting up and down (5 times). *Alternative transfer flow-through into a new 1.5 ml microtube; add 350 µlL molecular grade ethanol (70%); and mix by vortexing (2 x 5 sec).* *After addition of molecular grade ethanol a stringy precipitate may become visible which will not affect the RNA isolation. Be sure to disaggregate any precipitate by mixing. Do not centrifuge the ethanolic lysate to avoid pelleting the precipitate.*		+ 350 µL 70% Molecular Grade Ethanol *Mix*
5	**BIND RNA** For each preparation, take one **RNA Binding Column** (blue ring) placed into Collection Tube. Pipet the **lysate mixture** up and down 2-3 times and **load** it into the column. Centrifuge for **30 sec at 11,000 × *g***. Discard Collection Tube with flow-through and place the column back into a new Collection Tube. *Maximal loading capacity of RNA Binding Column is 750 µL; repeat the procedure if larger volumes are to be processed*		Load mixture into a RNA binding column (blue ring) *30 sec, 11,000 × g*
6	**DESALT SILICA MEMBRANE** Add **350 µL Desalting Buffer DBR** and centrifuge at **11,000 x *g*** for **1 min** to dry the membrane. *Salt removal will make the following rDNase digest more efficient. If the column outlet has come into contact with the flow-through, discard the flow-through and centrifuge again for 30 sec at 11,000 x g.*		+ 350 µL BUFFER DBR 1 min, 11,000 × g
7	**ON-COLUMN DNA DIGESTION** Prepare **rDNase Reaction Mixture**: in a sterile 1.5 ml microcentrifuge tube. For each isolation add **10 µL reconstituted rDNase** to **90 µL Buffer for rDNase**. Mix by flicking the tube. Apply **95 µL rDNase Reaction Mixture** directly onto the center of the silica membrane of the column. Incubate at **room temperature** for **15 min**.		+ 95 µL rDNase Reaction Mixture RT 15 min
8	**WASH AND DRY SILICA MEMBRANE** **1st Wash** Add **200 µL Wash Buffer WR1** to the column. Centrifuge **30 secs** at **11,000 x g**. Place the column into a new Collection Tube (2 ml). *Buffer WR1 will inactivate the rDNase.* **2nd Wash** Add **600 µL Wash Buffer WR2** to the column. Centrifuge **30 secs** at **11,000 x g**. Discard flow through and place the column back into the Collection Tube. ***Note:** For efficient washing, make sure that residual buffer from previous steps is washed away with Buffer WR2* **3rd Wash** Add **250 µL Wash Buffer WR2** to the column. Centrifuge **2 min** at **11,000 x *g*** to dry the membrane completely. Discard flow through and place the column back into a 1.5 ml nuclease free tube).		+ 200 µL BUFFER WR1 *30 sec,11,000 xg* + 600 µL BUFFER WR2 *30 sec,11,000 × g* + 250 µL BUFFER WR2 *2 min,11,000 × g*

9	**ELUTE HIGHLY PURE RNA** Dispense **60 µL RNase-free H_2O** directly onto the silica membrane. Centrifuge **1 min** at **11,000 x g**. The eluate contains your pure RNA sample. *If higher RNA concentrations are desired, elution can be done with 40 µL RNase-free H2O. Overall yield, however, will decrease when using smaller volumes.* ***For further alternative elution procedures see Section 5.***		+ 60 µL RNase-free H_2O *1 min, 11,000 × g*

B. Total RNA preparation from biological fluids (e.g. serum, culture medium)

Before starting the preparation:

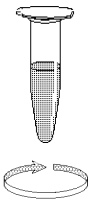
- Check if Wash Buffer WR2 and rDNase were prepared according to Section 6

STEP	DESCRIPTION		
1	**SAMPLE LYSIS** Sample homogenisation is not necessary. Add **350 µL Lysis Buffer LR** and **3.5 µL β-mercaptoethanol** to 100 µL of sample and vortex vigorously. ***Note:*** *As alternative to β-mercaptoethanol the reducing agent DTT or TCEP may be used. Use a final concentration of 10-20 mM within the Lysis Buffer LR.*		100 µL Starting material + 350 µL BUFFER LR+ 3.5 µl β-mercaptoethanol vortex
2	**ADJUST RNA BINDING CONDITIONS** Filtrate lysate is not necessary. Add **350 µL molecular grade ethanol (70%)** to the lysate and mix by vortexing.		+ 350 µL MOLECULAR GRADE ETHANOL (70%) vortex
	Proceed with Step 5 of the Standard Protocol*.*		

C. Total RNA preparation from paraffin embedded tissue

Before starting the preparation:

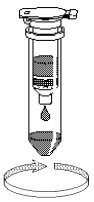
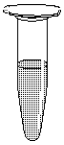
- Check if Wash Buffer WR2 and rDNase were prepared according to Section 6.
- Check if xylene is available.

STEP	DESCRIPTION		
1	**REMOVAL OF PARAFFIN** **Take 10 mg of disrupted tissue into a 1.5 mL microtube.** **Add** 300 µL xylene **and incubate** 5 min **with** constant mixing **at room temperature.** Centrifuge at max speed **(13,000 x *g*)** for **3 min** to pellet the **tissue.** Discard the xylene**.** **Repeat twice**, for a total of three xylene washes. Add **300 µL** of **molecular grade ethanol (96%)** to the tube and incubate **5 min** with constant **mixing** at room temperature. Centrifuge at max speed (**13,000 x *g*)** for **3 min** to pellet the tissue. **Discard the ethanol**. **Repeat once**, for a total of two ethanol washes		10 mg disrupted tissue + 300 µL Xylene 3 washes *3 min, 13,000x g* 96% molecular grade ethanol (300 µL) 2 washes *3 min, 13,000x g*
	Proceed with Step 1 of the Standard Protocol.		

D. Total RNA preparation from up to 10^9 bacterial cells

Before starting the preparation:

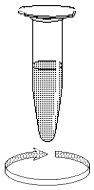
- Check if Wash Buffer WR2 and rDNase were prepared according to Section 6
- Prepare Lysozyme solution (not provided)
- Set an incubator or water bath to 37ºC

STEP	DESCRIPTION		
1	**SAMPLE PREPARATION** • For **Gram negative** strains resuspend the bacterial cell pellet in **100 µL TE buffer** (10 mM Tris-HCl, 1 mM EDTA; pH 8) containing **1 mg/ml lysozyme** by vigorous vortexing. Incubate at **37ºC** for **10 min.** • For preparation of RNA from **Gram-positive** bacteria, resuspend cells in **100 µL TE buffer** containing **2 mg/ml lysozyme**. It may be necessary to **optimize incubation** time and lysozyme concentration, depending on the bacterial strain. ***Note:*** *Because of the higher concentration of genome equivalents in a nucleic acid preparation of bacteria compared with eukaryotic material, it may be necessary to use a lower quantity of cells for the preparation.*		CELL PELLET + 100 µL BUFFER TE (with Lysozyme) 37ºC, 10 min
2	**CELL LYSIS** Add **350 µL Lysis Buffer LR** and **3.5 µL β-mercaptoethanol** to the cell suspension and vortex vigorously. ***Note:*** *As alternative to β-mercaptoethanol the reducing agent DTT or TCEP may be used. Use a final concentration of 10-20 mM within the Lysis Buffer LR.*		350 µL BUFFER LR + 3.5 µL β -mercapto ethanol
3	**FILTRATION OF THE LYSATE** Place a **Filtering Column** (violet ring) in a Collection Tube, **apply the lysate** and centrifuge for **1 min at 11,000 x *g***. In this way the lysate will be clarify and its viscosity will be reduced. *Alternatively, the lysate may be passed ≥ 5 times through a 0.9 mm needle (20 gauge) fitted to a syringe.* *In case of visible pellet formation transfer the flow-through without any formed pellet to a new 1.5 ml microtube.*		Load lysate into a filtering column (violet ring) *1 min, 11,000 × g*
4	**ADJUST RNA BINDING CONDITIONS** Discard the filtering column and add **350 µL molecular grade ethanol (70%)** to the clarified lysate and **mix** by vortexing.		+ 350 µL Molecular Grade Ethanol (70%) *Mix*
	Proceed with Step 5 of the Standard Protocol.		

E. Total RNA preparation from up to 5 x 10^7 yeast cells

Before starting the preparation:

- Check if Wash Buffer WR2 and rDNase were prepared according to Section 6.
- Prepare sorbitol and lyticase (or zymolase) solutions for homogenisation by enzymatic digestion or a swing-mill and glass beads for homogenisation by mechanical disruption

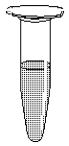
STEP	DESCRIPTION		
1	**SAMPLE PREPARATION** Two alternative protocols are given for homogenisation of yeast cells. Users may choose between an **enzymatic digestion** or a **mechanical homogenisation**. *Homogenisation by enzymatic digest is only recommended for fresh harvested cells, homogenisation by mechanical disruption may also be performed with yeast cell pellets, stored at - 70°C for several months.* ***Note**: Due to the much higher concentration of genome equivalents in a nucleic acid preparation of yeasts compared with cultured cells or tissue material, it may be necessary to use a lower quantity of cells for the preparation.* **A. <u>Homogenisation by enzymatic digest</u>** **Harvest 2-5 mL yeast culture** (5,000 x *g*; 10 min). Resuspend pellet in a fresh prepared **sorbitol/lyticase buffer** (50-100 U lyticase or zymolase in 1 M sorbitol/100 mM EDTA) and incubate at **30ºC** for **30 min**. Pellet the resulting spheroplast by centrifugation (**1,000 x *g***; **10 min**). Carefully discard supernatant *It may be necessary to optimize incubation time and lyticase/zymolase concentration, depending on the yeast strain.* ***Continue with Step 2 (Lysis step).*** **B. <u>Homogenisation by mechanical disruption</u>** **Harvest 2-5 mL yeast culture** (5,000 x *g*; 10 min). Wash with ice-cold water. Resuspend the cell pellet in a mixture of **350 µL Lysis Buffer LR** and **3.5 µL β-mercaptoethanol** Add **glass beads** Shake samples in a swing-mill at **30 Hz for 15 min.** ***Continue with Step 3 (Filtrate lysate).***		CELL PELLET + Sorbitol/ Lyticase Buffer 30ºC, 30 min 10 min,1,000 ×g CELL PELLET + 350 µL BUFFER LR + 3.5 µL β-mercapto ethanol + Glass beads shake
2	**CELL LYSIS** Add **350 µL Lysis Buffer LR** and **3.5 µl β-mercaptoethanol** to the cell suspension and **vortex** vigorously to lyse spheroplasts. ***Note:** As alternative to β-mercaptoethanol the reducing agent DTT or TCEP may be used. Use a final concentration of 10-20 mM within the Lysis Buffer LR.*		350 µL BUFFER LR + 3.5 µL β -mercapto ethanol vortex

3	**FILTRATION OF THE LYSATE** Place a **Filtering Column** (violet ring) in a collection tube **apply the lysate** and centrifuge for **1 min at 11,000 x *g***. In this way the lysate will be clarify and its viscosity will be reduced. *Alternatively, the lysate may be passed ≥ 5 times through a 0.9 mm needle (20 gauge) fitted to a syringe.* *In case of visible pellet formation recover and use flow-through of the filtering column without disturbing the pellet of undissolved cell debris.*		Load lysate into a filtering column (violet ring) *1 min, 11,000 x g*
	Proceed with Step 4 of the Standard Protocol.		

F. Clean-up of RNA from reaction mixtures

Before starting the preparation:

- Check if Wash Buffer WR2 was prepared according to Section 6.

STEP	DESCRIPTION		
1	**SAMPLE PREPARATION** **A)** Fill up **RNA samples** smaller than **100 µL with RNase-free H_2O** to 100 µL. **B)** If different samples with varying volumes between 100-200 µL are purified, RNA samples should be filled up with RNase-free H_2O to a **uniform volume**.		Prepare Sample
2	**PREPARATION OF LYSIS-BINDING PREMIX BUFFER** Prepare a Lysis-Binding Premix Buffer: **1 VOL Lysis Buffer LR + 1 VOL molecular grade ethanol** (96-100%). For each **100 µL RNA sample mix 300 µL of Lysis Buffer LR** and **300 µL molecular grade ethanol** (96-100%). *If multiple samples are processed, the preparation of a master with the premix buffer is recommended.*		Prepare Lysis-Binding Premix Buffer
3	**ADJUST RNA BINDING CONDITIONS** **Filtrate lysate is not necessary** For a **100 µl of RNA sample** add **600 µl** (6 volumes) **of Lysis-Binding Premix Buffer**. *Maximum loading capacity of RNA Binding Columns is 750 µl. Repeat the loading step if larger volumes are to be processed.* *After addition of molecular grade ethanol or Premix Buffer a stringy precipitate may become visible which will not affect the RNA isolation. Be sure to mix thoroughly and apply sample as homogenous solution onto the column.*		Reaction Mixture + Lysis-Binding Premix Buffer
	Proceed with Steps 5, 8, and 9 of the Standard Protocol. ***Steps 6 and 7 may be omitted in this protocol.***		

G. Total RNA preparation from RNAlater® treated samples

Before starting the preparation:

- Check if Wash Buffer WR2 was prepared according to Section 6.

STEP	DESCRIPTION		
1	**SAMPLE PREPARATION** **Remove** RNA*later*® solution and **cut** an appropriate amount of tissue.		Prepare Sample
2	**CELL LYSIS** Add **350 µL Lysis Buffer LR** and **3.5 µL β-mercaptoethanol** to the sample. Disrupt the sample material by using a homogenizer. ***Note:*** *As alternative to β-mercaptoethanol the reducing agent DTT or TCEP may be used. Use a final concentration of 10-20 mM within the Lysis Buffer LR.*		350 µL BUFFER LR + 3.5 µL β -mercapto ethanol
	Proceed with Step 3 of the Standard Protocol.		

H. rDNase digestion in solution

The **on-column rDNase digestion** in the standard protocol is already very efficient resulting in a minimal residual DNA content of the purified RNA. This DNA will not be detectable in most downstream applications. Despite this, there are still certain applications which require even lower contents of residual DNA. When downstream applications require low residual content of DNA, a rDNase digestion in solution is recommended. For example, for RT-PCR reaction in which:

- ✓ Primers do not differentiate between cDNA and contaminating genomic DNA.
- ✓ The target gene has a low expression level.
- ✓ Amplicons <200 bp.

DNA digestion in solution can efficiently destroy contaminating DNA. However, stringent RNase control and subsequent re-purification of the RNA (in order to remove buffer, salts, DNase and digested DNA) are usually required.

Before starting the preparation:

- Check if Wash Buffer WR2 was prepared according to Section 6.
- Set an incubator or water bath to 37ºC.

STEP	DESCRIPTION
1	**DIGEST DNA (Reaction Set-up)** Add **6 µL Buffer for rDNase** and **0.6 µL reconstituted rDNase** to **60 µL eluted** RNA. **Gently swirl the tube** in order to mix the solution and **spin down** (1 sec at 1,000 x g) to collect droplets of the solution at the bottom of the tube. *Alternatively premix 100 µL Reaction Buffer for rDNase and 10 µL rDNase and add 1/10 VOL to 1 VOL of RNA eluate.*
2	**INCUBATION** Incubate at **37ºC** for **10 min.**
3	**REPURIFICATION OF RNA** **Repurify RNA** with a suitable RNA clean-up *(Protocol 8.F)* or by ethanol precipitation. ***Ethanol precipitation**: Add 0.1 VOL of 3 M sodium acetate, pH 5.2 and 2.5 VOL of 96-100% molecular grade ethanol to 1 VOL of sample. Mix thoroughly and incubate several minutes (for high RNA concentration) to several hours (for low RNA concentration) at -20ºC or at 4ºC.Centrifuge for 10 min at max speed. **Wash RNA pellet with 70% molecular grade ethanol**. Dry RNA pellet and resuspend RNA in RNase-free H_2O.*

9. SIMULTANEOUS ISOLATION OF RNA AND DNA (RNA/DNA BUFFER TOOL SET)

The RNA/DNA Buffer Tool Set is a support set of buffers (Ref. 21.213 not included in the kit) for the isolation of RNA and DNA in conjunction with the Speedtools Total RNA Extraction Kit.

The technology behind this buffer set enables successive elution of DNA and RNA from one RNA Binding Column with low salt buffer and water, respectively.

After wash steps DNA and RNA eluted sequentially. DNA is eluted with a low salt buffer, which selectively elutes DNA and keeps RNA on the column. Eluted DNA is immediately ready for downstream applications without further purification. After DNA elution, residual DNA is digested on the RNA Binding Column as described in the standard protocol. The column is washed and pure RNA is eluted in RNase-free H_20.

10. TROUBLESHOOTING

Problem	Possible cause and suggestions
RNA is degraded/no RNA obtained	***RNase contamination*** • Create an RNase free working environment. Wear gloves during all steps of the procedure and change them frequently. Use nuclease free plastic materials. Keep tubes closed whenever possible during the preparation. Glassware should be oven-baked for at least 2 hours at 250ºC before use.
Poor RNA quality or yield	***Reagents not applied or restored properly*** • Reagents not properly restored. Add the indicated volume of RNase-free H_2O to rDNase vial, and 96% molecular grade ethanol to Wash Buffer WR2 (concentrated). Mix reagents following the instructions in Section 5. Reconstitute and store lyophilized rDNase according to the instructions given in Section 6. • Sample and reagents have not been mixed completely. Always vortex vigorously after each reagent has been added. • No ethanol has been added after lysis. Binding of RNA to the silica membrane is only effective in the presence of ethanol. ***Kit storage*** • Reconstitute and store lyophilized rDNase according to instructions given in Section 6. • Store other kit components at room temperature. Storage at low temperatures may cause salt precipitation in the buffers. • Keep bottles tightly closed in order to prevent evaporation or contamination. ***Ionic strength and pH influence A_{260} absorption as well as ratio A_{260}/A_{280}*** • For adsorption measurement, use 5 mM Tris, pH 8.5 as diluent. ***Sample material*** • Sample material not stored properly. Whenever possible, use fresh material. If this is not possible, flash freeze the samples in liquid N_2. Samples should always be kept at -70ºC. Never allow tissues to thaw before addition of Lysis Buffer LR. Perform disruption of samples in liquid N_2. • Insufficient disruption and/or homogenisation of starting material. Ensure thoroughly sample disruption and use the filtering columns for easy homogenisation of disrupted starting material.
Low A260 / A230 ratio	***Carry-over of guanidinium thiocyanate*** • Carefully load the lysate to the RNA Binding Column and try to avoid contamination of the upper part of the column and the column lid. • Make sure that residual Wash Buffer WR1 is washed away with Buffer WR2. This may be done by applying Buffer WR2 to the inner rim of the column.

Problem	Possible cause and suggestions
Clogged column/ Poor RNA quality or yield	***Sample material*** • Too much starting material. Overloading may lead to decreased overall yield. Reduce amount of sample material or use larger volume of Lysis Buffer LR. • Insufficient disruption and/or homogenisation of starting material. Ensure thoroughly sample disruption and use the filtering columns for easy homogenisation of disrupted starting material.
Contamination of RNA with genomic DNA	***rDNase not active*** • Reconstitute and store lyophilized rDNase according to instructions given in Section 6. ***DNase solution not properly applied*** • Pipette rDNase solution directly onto the center of the silica membrane. ***Too much cell material used*** • Reduce quantity of cells or tissue used. ***DNA detection system too sensitive*** • The amount of DNA contamination is significantly reduced during the on-column digestion with rDNase. Anyhow it cannot be guaranteed that the purified RNA is 100% free of DNA, therefore in very sensitive applications it might be possible to detect DNA. In this case a subsequent rDNase digestion in solution is recommended (Protocol H). • Use larger PCR targets (e.g. >500 bp) or intron spanning primers if possible.
Suboptimal performance of RNA in downstream experiments	***Carryover of ethanol or salt*** • Do not let the flow-through touch the column outlet after the second Wash Buffer WR2. Be sure to centrifuge at the corresponding speed for the respective time in order to remove ethanolic Buffer WR2 completely. • Check if Wash Buffer WR2 has been equilibrated to room temperature before use. Washing at lower temperatures reduces efficiency of salt removal by Buffer WR2. ***Store isolated RNA properly*** • Eluted RNA should always be kept on ice for optimal stability since trace contaminations of omnipresent RNases will degrade the isolated RNA. For short term storage freeze at -20ºC, for long term storage freeze at -70ºC.

11. ORDERING INFORMATION

SPEEDTOOLS KIT	10 PREPS	50 PREPS	250 PREPS
SPEEDTOOLS **DNA EXTRACTION** KIT	Ref. 21.130M	Ref. 21.131	Ref. 21.132
SPEEDTOOLS **TISSUE DNA EXTRACTION** KIT	Ref. 21.135M	Ref. 21.136	Ref. 21.137
SPEEDTOOLS **RNA VIRUS EXTRACTION** KIT	Ref. 21.140M	Ref. 21.141	Ref. 21.142
SPEEDTOOLS **FOOD DNA EXTRACTION** KIT	Ref. 21.175M	Ref. 21.176	Ref. 21.177
SPEEDTOOLS **PLANT DNA EXTRACTION** KIT	Ref. 21.170M	Ref. 21.171	Ref. 21.172
SPEEDTOOLS **TOTAL RNA EXTRACTION** KIT	Ref. 21.210M	Ref. 21.211	Ref. 21.212
SPEEDTOOLS **PCR CLEAN-UP** KIT	Ref. 21.200M	Ref. 21.201	Ref. 21.202
SPEEDTOOLS **PLASMID DNA PURIFICATION** KIT	Ref. 21.220M	Ref. 21.221	Ref. 21.222

12. PRODUCT USE RESTRICTION AND WARRANTY

1. Product for research purposes and *in vitro uses* only.
2. No claim or representation is intended for its use to identify any specific microorganism or for clinical use (diagnostic, prognosis, therapeutic, or blood bank).
3. It is rather the responsibility of the user to verify the use of the kit for a specific application range as the performance characteristic of this kit has not been verified for a specific microorganism.
4. The kit includes documentation stating specifications and other technical information. Follow the instructions in order to obtain highly pure RNA.
5. BIOTOOLS warrants to meet the stated specifications of the kit. Any product not fulfilling the specifications included in the product sheet will be replaced.
6. BIOTOOLS obligation and purchaser's rights under this warranty are limited to the replacement by BIOTOOLS of any product that is shown defective in fabrication, and that must be returned to BIOTOOLS, freight prepaid, or at BIOTOOLS' option, replacement of the purchasing price.
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