INVITEK
Molecular

USER MANUAL

InviTrap® Spin Cell RNA Mini Kit

for purification of total RNA from up to 1 x 10^7 human & animal cells, up to 1 x 10^9bacteria cells, up to 5 x 10^7 yeast cells; RNA clean up and simultaneous protein isolation

CE IVD

REF 1061100300

Invitek Molecular GmbH · D-13125 Berlin

Instruction for the InviTrap® Spin Cell RNA Mini Kit

The **InviTrap® Spin Cell RNA Mini Kit** is the ideal tool for the isolation of high quality total RNA from samples of human or animal cell cultures (max. 1 x 10^7 cells), bacteria suspensions (max. 1 x 10^9 cells) and yeast suspensions (max. 5 x 10^7 cells) in a convenient spin-filter format. The patented Invitek Molecular technology combines an efficient lysis of the starting material with the rapid inactivation of RNases, the stabilization of the total RNA and the almost complete separation of the DNA. The kit can be used further for RNA Cleanup and simultaneous isolation of total RNA and proteins.

A DNase-digestion step is not necessary.

The purified total RNA is ready to use for *in-vitro* diagnostic use.

The kit is neither validated for the isolation of total RNA from serum, plasma, blood, nor for viruses. The performance of the kits in isolating and purifying total RNA from fecal samples has not been evaluated.

CE **IVD** Compliance with EU Directive 98/79/EC on *in vitro* medical devices.

Not for *in-vitro* diagnostic use in countries where the EU Directive 98/79/EC on in vitro medical devices is not recognized.

Contents

Kit contents of the InviTrap® Spin Cell RNA Mini Kit

	250 total RNA extractions
Catalogue No.	1061100300
Lysis Solution R	190 ml
Wash Buffer R1	80 ml (final volume 160 ml)
Wash Buffer R2	2 x 40 ml (final volume 2 x 200 ml)
Elution Buffer R	60 ml
DNA-Binding Spin Filter	5 x 50
RNA-RTA Spin Filter Set	5 x 50
2.0 ml Receiver Tubes	5 x 50
RTA Receiver Tubes	5 x 50
Elution Tubes	5 x 50
Manual	1
Initial steps	Preparation of 200 ml 70% ethanol. Add 80 ml 96-100% ethanol to the bottle **Wash Buffer R1**. Add 160 ml 96-100% ethanol to each bottle **Wash Buffer R2.**
	Adjust Lysis Solution R with 1/100 volume of 1M DTT. Due to the instability of dissolved DTT under oxidative conditions do not mix the whole **Lysis Solution R** with DTT in case of the kits with 50 and 250 preparations. We recommend the preparation of a volume DTT-containing **Lysis Solution R** shortly before carrying out the purifications adapted to the number of samples that will be processed. Store the remaining **Lysis Solution R** and DTT separately in accordance to the storage instructions (see “Storage”, page 6). It is possible to replace DTT by β-Mercaptoethanol. In this case, adjust **Lysis Solution R** with 1/100 volume of β-Mercaptoethanol as described above.
	Preparation of TE-buffer (10 mM Tris/ HCl; 1 mM EDTA pH 8.0). (only for bacteria)
	Preparation of 1 M Sorbitol/0.1 M EDTA-solution pH 7.4. (only for yeast)

Symbols

 Manufacturer

 Lot number

Attention: *Do not combine components of different kits, unless the lot numbers are identical!*

 Catalogue number

 Expiry date

 Consult operating instructions

 Temperature limitation

 Do not reuse

 Humidity limitation

Storage

All buffers and kit contents of the **InviTrap® Spin Cell RNA Mini Kit**, except **1 M DTT solution** (not provided) should be stored at room temperature and are stable for at least 12 months.

Room temperature (RT) is defined as range from 15-30°C.

Before every use, make sure that all components have room temperature. If there are any precipitates within the provided solutions solve these precipitates by warming carefully (up to 30°C).

Wash Buffers charged with ethanol should be appropriately sealed and stored at room temperature.

Store the **1 M DTT solution** at - 20°C to prevent oxidative damage. Under this condition, the solution of **1 M DTT** is stable for 12 months. If the kit components are consumed in more than one run it is recommended to aliquot the **1 M DTT solution** and minimize the number of freezing and thawing cycles.

Quality control and product warranty

Invitek Molecular warrants the correct function of the **InviTrap® Spin Cell RNA Mini Kit** for applications as described in this manual. Purchaser must determine the suitability of the Product for its particular use. Should any Product fail to perform the applications as described in the manual, Invitek Molecular will check the lot and if Invitek Molecular investigates a problem in the lot, Invitek Molecular will replace the Product free of charge.

Invitek Molecular reserves the right to change, alter, or modify any Product to enhance its performance and design at any time.

In accordance with Invitek Molecular's EN ISO 13485 certified Quality Management System the performance of all components of the **InviTrap® Spin Cell RNA Mini Kit** have been tested separately against predetermined specifications routinely on lot-to-lot to ensure consistent product quality.

If you have any questions or problems regarding any aspects of **InviTrap® Spin Cell RNA Mini Kit** or other Invitek Molecular products, please do not hesitate to contact us. A copy of Invitek Molecular's terms and conditions can be obtained upon request or are presented at the Invitek Molecular webpage.

For technical support or further information please contact:

from Germany **+49-(0)30-9489-2901/ 2910**
from abroad **+49-(0)30-9489-2907**

or contact your local distributor.

Intended use

The **InviTrap® Spin Cell RNA Mini Kit** is the ideal tool for reliable and fast manual isolation and purification of high quality total RNA from samples of human or animal cells (cell culture)**,** bacteria or yeast suspensions. For reproducible high yields an appropriate sample storage and quick operation under the rules for RNA operation is essential. The purified RNA is ready to use for *in vitro* diagnostic analysis only.

For some studies a simultaneous investigation of the RNA and of the cell, bacteria or yeast strain specific proteins, synthesized during the cultivation period is necessary. The kit includes an isolation protocol of RNA and intracellular proteins from the same sample.

The isolation protocol and all buffers are optimized to assure a high yield as well as a high purity of purified total RNA. All manual work is reduced to a minimum.

THE PRODUCT IS INTENDED FOR USE BY PROFESSIONALS ONLY, SUCH AS TECHNICIANS, PHYSICIANS AND BIOLOGISTS TRAINED IN MOLECULAR BIOLOGICAL TECHNIQUES. It is designed to be used with any downstream application employing enzymatic amplification or other enzymatic modifications of RNA followed by signal detection or amplification. Any diagnostic results generated by using the sample preparation procedure in conjunction with any downstream diagnostic assay should be interpreted with regard to other clinical or laboratory findings.

To minimize irregularities in diagnostic results, adequate controls for downstream applications should be used.

The kit complies with EU Directive 98/79/EC on in vitro medical devices. However, it is not for in-vitro diagnostic use in countries where the EU Directive 98/79/EC on in vitro medical devices is not recognized.

Product use limitation

The kit is neither validated for the isolation of total RNA from serum, plasma, blood, tissue nor for viruses. The performance of the kit in isolating and purifying total RNA from fecal samples has not been evaluated.

The kit was not tested on its ability to desalinate RNA or for RNA purification from enzymatic reactions, like Proteinase digestion, RNA ligation or labeling reactions.

The included chemicals are only useable once.

Differing of starting material or flow trace may lead to inoperability; therefore, neither a warranty nor guarantee in this case will be given, neither implied nor express.

The user is responsible to validate the performance of the Invitek Molecular Product for any particular use. Invitek Molecular does not provide for validation of performance characteristics of the Product with respect to specific applications.

Invitek Molecular products may be used e.g. in clinical diagnostic laboratory systems under following conditions:

- If used in the US, based on the condition that the complete diagnostic system of the laboratory has been validated pursuant to CLIA' 88 regulations.
- For other countries based on the condition that the laboratory has been validated pursuant to equivalents according to the respective legal basis.

All Products sold by Invitek Molecular are subject to extensive quality control procedures (according to EN ISO 13485) and are warranted to perform as described herein. Any problems, incidents or defects shall be reported to Invitek Molecular immediately upon detection thereof.

The chemicals and the plastic parts are for laboratory use only; they must be stored in the laboratory and must not be used for purposes other than intended.

The Product with its contents is unfit for consumption.

Safety information

When and while working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles! Avoid skin contact! Adhere to the legal requirements for working with biological material!

For more information, please consult the appropriate material safety data sheets (MSDS). These are available online in convenient and compact PDF format at www.invitek-molecular.com for each Invitek Molecular Product and its components. If buffer bottles are damaged or leaking, **WEAR GLOVES, AND PROTECTIVE GOGGLES** when discarding the bottles in order to avoid any injuries.

Invitek Molecular has not tested the liquid waste generated by the **InviTrap® Spin Cell RNA Mini Kit** procedures for residual infectious materials. Contamination of the liquid waste with residual infectious materials is highly unlikely, but cannot be excluded completely. Therefore, liquid waste must be considered infectious and be handled and discarded according to local safety regulations.

European Community risk and safety phrases for the components of the **InviTrap® Spin Cell RNA Mini Kit** to which they apply are listed below as follows:

Lysis Solution R


Warning

H315-H319-P280-P305+P351+P338

Wash Buffer R1


Warning

H302-H332-H412-P280-P305+P351+P338-EUH032

H302: Harmful if swallowed.
H315: Causes skin irritation.
H319: Causes serious eye irritation.
H332: Harmful if inhaled.
H412: Harmful to aquatic life with long lasting effects.
P280: Wear protective gloves/protective clothing/eye protection/face protection.
P305+P351+P338: IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
EUH032: Contact with acids liberates very toxic gas.

Emergency medical information can be obtained 24 hours a day from infotrac:

outside of USA: 1 – 352 – 323 – 3500
in USA : 1 – 800 – 535 – 5053

Product characteristics of the InviTrap® Spin Cell RNA Mini Kit

Starting material	Yield	Time for preparation	Ratio
up to 1 x 10^7 human or animal cells (cultured cells) up to 1 x 10^9 cells of Gram-positive or Gram-negative bacteria up to 5 x 10^7 yeast cells	up to 150 µg total RNA (e.g. 10 - 15 µg total RNA from 5 x 10^5 NIH 3T3 fibroblasts)	15 - 20 minutes	A_{260} : A_{280} 1.7 - 2.1

The **InviTrap® Spin Cell RNA Mini Kit** provides a fast and efficient way for reliable isolation of high quality total RNA as well as the isolation of the proteins from samples of human or animal cells (cell culture)**,** bacteria, or yeast. The **InviTrap® Spin Cell RNA Mini Kit** can also be used for an efficient RNA cleanup and the simultaneous isolation of total RNA and proteins.

The **InviTrap® Spin Cell RNA Kit** simplifies total RNA isolation by combining efficient lysis of the starting material and the inactivation of exogenous and endogenous RNases with the efficient removal of the genomic DNA. The genomic DNA is fixed at the surface of the mineral carrier particles provided within the **Lysis Solution R** simultaneously to the cell lysis. Selective binding of the DNA is assured by optimized buffer conditions and the mineral carrier particles loaded with the genomic DNA are removed by centrifugation on the spin filter surface. The RNA remains in the filtrate and is after the adjustment of special binding conditions bound onto the membrane of the RNA-RTA Spin Filter / RNA-Binding Spin Filter. Contaminants are removed by repeated washing steps and the purified total RNA can be eluted in a small volume of RNase free water. The isolated total RNA is ready to use and should be stored at - 80°C.

The extracted RNA contains enriched mRNA. The particle size distribution of purified RNA is similar to those, gained using a CsCl - gradient.

Due to the high purity, the isolated total RNA is ready to use for a broad panel of downstream applications like:

- Northern Blotting,
- RNA dot blots,
- cDNA-library,
- *in vitro* translation,
- RT-PCR*,
- DDRT-PCR*,
- or TaqMan® analysis and array technologies.

For technical support or further information, please contact: +49-(0)30-9489-2901/ 2907/ 2910 or your local distributor.

* The PCR process is covered by US Patents 4,683,195, and 4,683,202 and foreign equivalents owned by Hoffmann-La Roche AG.

Important indications

Sample quantity

The maximal amount of pure total RNA that will be isolated depends on the applied starting material, storage conditions and intracellular RNA level.
To assure a high yield of pure total RNA the number of cells given in this manual should not be exceeded. Direct cell counting is recommended. If more than the allowed cell numbers will be processed, it is suggested to split the sample. In that case, the volumes of Wash- and Elution Buffer need not to be aligned.
To adjust the number of cells in a special application to the RNA binding capacity of the **InviTrap® Spin Cell RNA Mini Kit** and to avoid an overloading the RNA binding membrane, an RNA purification experiment with variation of the amount of starting material is recommended. The RNA Mini kit-binding membranes have a maximum binding capacity of approx. 100 µg.

The **InviTrap® Spin Cell RNA Mini Kit** purification procedure is optimized for the use of

Human and animal cells: max. from 1 x 10^7

Average yield of total RNA in dependence on the type of cell line using the InviTrap® Spin Cell RNA Mini Kit:

Cell Line	Source	Yield [µg] from 1 x 10^5 cells
HeLa	human cervical carcinoma	2.0
Jurkat	human T-cell leukemia	1.7
MRC 5	human	5.0

Growth area and number of HeLa cells in various cell-culture plates:

Cell culture plate	Growth area in cm^{2*}	Number of cell**
96 well	0.3 – 0.6	4 - 5 x 10^4
48 well	1.0	1.3 x 10^5
12 well	4.0	5.0 x 10^5
6 well	9.5	1.2 x 10^6

* Growth area varies slightly depending on the supplier.
** Confluent growth is assumed. Values are reported per well.

Depending on the used cell lines, lysates may become viscous if a large number of cells is lysed. High viscosity always causes clogged membranes and leads to a reduced yield and quality of the isolated RNA. Therefore, it is suggested to perform a preliminary viscosity analysis of the desired cell line depending on the cell number.

Bacteria cells: from up to 1 x 10^9

The achievable yield of purified total RNA depends strongly on the type of bacteria, the culture medium and the growth phase. Up to 1 x 10^9 cells taken from minimal media can be used. The use of higher numbers of cells results in inefficient lysis and a reduced yield of isolated RNA.

The determination of the cell number in a given bacteria culture is often done photometric by measurement of the optical density (OD). The measured OD should be below 0.3 to exclude a loss of significance and therefore incorrect values of cell density. Due to the varying effects of the bacterial species, the growth phase and the type of culture medium to the light scattering properties of the sample the measured OD values and calculated cell numbers should be ensured by parallel counting of viable cells on agar plates before starting the first RNA purification procedure.

Yeast cells: from up to 5 x 10^7
In strong connection to the type of strain and the growth conditions 30 - 100 µg RNA can be purified from 5 x 10^7 yeast cells.

To get optimal results in RNA-purification yeast cells should be harvested in the log-phase growth. Although the photometrical determination of the cell density of yeast cultures is common, an additional plating experiment is recommended to ensure the measured values of light scattering before starting the first RNA purification procedure. An enzymatic or mechanical disruption of yeast cell is possible. This manual contains a protocol for enzymatic breakage of the yeast cell wall using Lyticase.

Principle and procedure

The **InviTrap® Spin Cell RNA Mini Kit** procedure comprises the following steps:

- cell lysis
- selective binding of the genomic DNA to a specific carrier and additional separation of the bound DNA on the surface of the DNA-Binding Spin Filter
- adjustment of the RNA binding conditions and transfer of the sample into the RNA-RTA Spin Filter/ RNA-Binding Spin Filter
- binding of the total RNA to the membrane, while proteins remain in the filtrate
- washing of the membrane and elimination of contaminants and ethanol
- elution of highly pure total RNA

Repeated wash steps make sure that contaminations and enzyme inhibitors are efficiently removed and highly purified RNA is eluted in **Elution Buffer R** or RNase free water.

This manual contains 6 protocols (+ 2 supplemental protocols) (see page 16-30).

Sampling and storage of starting material
Best results are obtained using freshly extracted human or animal cells, bacteria or yeast cells. As long as the samples are not shock frosted with liquid nitrogen or are incubated with RNase inhibitors or denaturing reagents, the RNA is not secured and the gene expression profiles based on the RNA isolation and quantification will provide a false result. Therefore, it is essential, that cells are immediately flash frozen subsequent to the cell harvest using liquid nitrogen and are stored at - 80°C. RNA contained in such deep frozen samples is stable for months. RNA purification should be processed as soon as possible. Cells can be stored also in **Lysis Solution R** at - 80°C after cell lysis.

Invitek Molecular will be released of its responsibilities if other sample materials than described in the Intended Use are processed or if the sample preparation protocols are changed or modified.

Procedure

Lysis
After complete removal of the culture medium, the cells are directly charged with the **DTT-containing Lysis Solution R** and thoroughly mixed. Shake the **Lysis Solution R** to resuspended the mineral carrier particles prior to use. Due to the strong denaturing lysis conditions cells are quickly broken and RNases are inactivated simultaneously. The RNA is secured. **DTT** is added to inactivate the RNases by cleaving intermolecular disulfide bridges.

Binding and removal of genomic DNA
DNase digestion is not necessary to remove the genomic DNA. Under the provided lysis conditions, the DNA is efficiently bound by the mineral carrier particles contained in **Lysis Solution R**. The DNA containing carrier remains at the surface of the membrane of the DNA-Binding Spin Filter by subsequent centrifugation, while the RNA remains in the filtrate.

If a DNase digestion is required e.g. caused by overloading the kit, this step is carried out after the binding of the RNA to the spin filter. All DNA fragments and the DNase will be removed completely in the following wash steps (see page 26).

Binding total RNA
To adjust the RNA binding conditions 70% **ethanol** is added to the RNA containing filtrate. Following carefully mixing the solution is completely transferred onto the RNA-RTA Spin Filter and the RNA is bound on the membrane by centrifugation.

Removing residual contaminants
Contaminants are efficiently washed away using **Wash Buffer R1** and **R2**, while the RNA remains bound to the membrane of the RNA-RTA Spin Filter.

Elution
High quality total RNA is eluted from the membrane using 40 - 100 µl **Elution Buffer R** (or RNase free water). The eluted RNA is ready to use in different subsequent applications.

Important notes

Important points before starting a protocol

Immediately upon receipt of the Product, inspect the Product and its components as well as the package for any apparent damages, correct quantities and quality. If there are any unconformities you have to notify Invitek Molecular in writing with immediate effect upon inspection thereof. If buffer bottles are damaged, contact the Invitek Molecular Technical Services or your local distributor. In case of liquid spillage, refer to “Safety Information” (see page 7). Do not use damaged kit components, since their use may lead to poor kit performance.

- Always change pipet tips between liquid transfer, to avoid cross-contaminations, we recommend the use of aerosol-barrier pipet tips.
- All centrifugation steps are carried out at room temperature.
- When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles.
- Discard gloves if they become contaminated.
- Do not combine components of different kits unless the lot numbers are identical.
- Avoid microbial contamination of the kit reagents.
- To minimize the risk of infections from potentially infectious material, we recommend working under laminar air-flow until the samples are lysed.
- Trained personnel should only use this kit.

Preparing reagents and buffers

250 total RNA-extractions:
adjust **Lysis Solution R** with 1/100 volume of 1 M DTT preparation of 200 ml 70 % ethanol add 80 ml 96-100% ethanol to the bottle **Wash Buffer R1** add 160 ml 96-100% ethanol to each bottle **Wash Buffer R2** preparation of TE-Buffer (10 mM Tris/ HCl ; 1 mM EDTA pH 8,0) (only for bacteria) preparation of a 1 M Sorbitol/0.1 M EDTA-solution pH 7.4 (only for yeast)

DTT
Adjust **Lysis Solution R** with 1/100 volume of 1 M **DTT**. Due to the instability of dissolved **DTT** under oxidative conditions, do not mix the whole **Lysis Solution R** with **DTT** in case of the kits with 50 and 250 preparations. We recommend the preparation of a volume **DTT- containing Lysis Solution R** shortly before carrying out the purifications adapted to the number of samples that will be processed. Store the remaining **Lysis Solution R** and **DTT** separately in accordance to the storage instructions (see “Storage”, page 6).

It is possible to replace **DTT** by β-Mercaptoethanol. In that case, adjust **Lysis Solution R** with 1/100 volume of β-Mercaptoethanol as described above.

Lysis Solution R
Shake **Lysis Solution R** gently before use to homogenize the DNA-binding mineral carrier particles! Wait a short time because of foam formation! Cell lysates stored in **Lysis Solution R** are stable at - 80°C for several months.

Equipment and reagents to be supplied by user

When working with chemicals, always wear a suitable lab coat, disposable gloves and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDS). (See our webpage: www.invitek-molecular.com)

- Microcentrifuge (≥ 13.400 x g (12.000 rpm))
- Ethanol (96-100%)
- Tris (only for bacteria)
- EDTA (only for bacteria and yeast)
- Lysozyme (only for bacteria)
- Sorbitol (only for yeast)
- ß-Mercaptoethanol (only for yeast)
- Lyticase (only for yeast)
- Thermoshaker (only for yeast)
- Pipettes and filter tips
- DTT

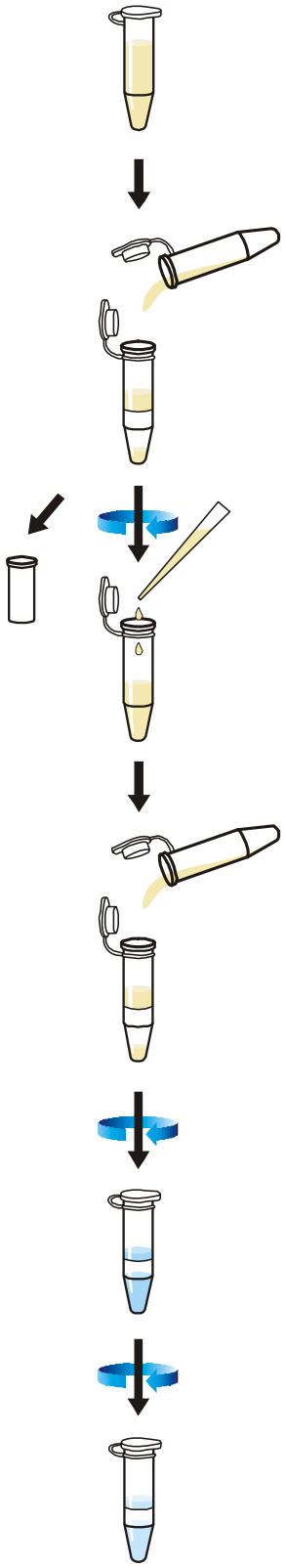
Possible suppliers:

Eppendorf AG
22331 Hamburg, Germany
Phone: +49 (0) 40 53801 0
Fax: +49 (0) 40 53801 556
E-Mail: eppendorf@eppendorf.com
Internet: www.eppendorf.com

SIGMA Laborzentrifugen GmbH
37507 Osterode am Harz, Germany
Phone: +49-5522-5007-0
Fax: +49-5522-5007-12
E-Mail: info@sigma-zentrifugen.de
Internet: www.sigma-zentrifugen.de

Scheme of the InviTrap® Spin Cell RNA Mini Kit

Please work quickly and perform all extraction steps at room temperature (RT)!



Please read the protocols carefully prior to the start of the preparation procedure!

Note: Homogenize the carrier in the **Lysis Solution R** by shaking. Mix it with DTT.

transfer the sample into a 1.5 ml reaction tube (not provided) and add 350 µl DTT-containing **Lysis Solution R**

Note: For 5 x 10^6 - 1 x 10^7 human or animal cells add 700 µl DTT-containing **Lysis Solution R.**

mix thoroughly by pipetting up and down

place a DNA-Binding Spin Filter into a 2.0 ml Receiver Tube

transfer the whole lysate onto the DNA-Binding Spin Filter, incubate for 1 min at RT and centrifuge for 2 min at 11.000 x g (11.000 rpm)

discard the DNA-Binding Spin Filter

add the recommended volume of **ethanol** [1 volume of 70% ethanol] (see Protocol 1), 250 µl 96 -100% ethanol (see Protocol 2 and 3)] to the filtrate and mix thoroughly by pipetting up and down

transfer the sample on the RNA-RTA Spin Filter, incubate for 1 min and centrifuge for 1 min at 11.000 x g (11.000 rpm)
discard the flow-through and reuse the RTA Receiver Tube

pipet 600 µl **Wash Buffer R1** onto the RNA-RTA Spin Filter and centrifuge for 1 min at 11.000 x g (11.000 rpm)
discard the flow-through and put the RNA-RTA Spin Filter in a new RTA Receiver Tube

pipet 700 µl **Wash Buffer R2** onto the RNA-RTA Spin Filter and centrifuge for 1 min at 11.000 x g (11.000 rpm)
discard the flow-through and reuse the RTA Receiver Tube
(Repeat this washing step!)

to eliminate any traces of ethanol, centrifuge for 4 min at maximum speed
discard the RTA Receiver Tube

transfer the RNA-RTA Spin Filter into a RNase free Elution Tube
pipet 40 - 100 µl of **Elution Buffer R** onto the membrane of the RTA Spin Filter, incubate for 2 min at RT and centrifuge for 1 min at 11.000 x g (11.000 rpm)

discard the RNA-RTA Spin Filter
place the eluted total RNA immediately on ice!

Note: *The centrifugation steps were made with the **Centrifuge 5415 D from Eppendorf.** The indicated **rpm amounts** are referring to this centrifuge.*

Instructions

Protocol 1: Total RNA extraction from human and animal cell culture

1. Harvesting cells (see also "Sample quantity", page 10)

Cells grown in suspension: Spin up to 1 x 10^7 cells for 5 min at 240 x g (1.500 rpm). Discard the supernatant and remove all media completely.

Cells grown in a monolayer: In large culture vessels (dishes > ∅ 35 mm, flasks > 12.5 cm^2) detach cells by trypsination. Transfer the cells to a centrifuge tube and sediment by centrifugation at 240 x g (1.500 rpm) for 5 min. Remove the supernatant completely.
In small culture vessels (96-, 24-, 12-, 6- well plates, ∅ 35 mm dishes, 12.5 cm^2 flasks) discard the media completely and continue with the lysis immediately.

Bacteria: Spin up to 1 x 10^9 bacteria cells for 5 min at 2.500 x g (5.000 rpm) in a centrifuge tube. Discard the supernatant and remove all media completely.

Yeast cells: Spin up to 5 x 10^7 yeast cells for 5 min at 2.500 x g (5.000 rpm) in a centrifuge tube. Discard supernatant and remove all media completely.

Important: *Incomplete removal of the cell culture media will inhibit the lysis and dilute the lysate, which will affect the binding of RNA to the RNA- RTA Spin Filter.*

2. Cell Disruption

Cell pellet:

To loosen the cell pellet flick the tube and add **DTT-containing Lysis Solution R** (volume as given in table 1). Mix thoroughly by pipetting up and down. No cell clumps should be visible before proceeding with the next step.

Table 1:

Lysis Solution R	Number of pelleted cells
350 µl	< 5 x 10^6 cells
700 µl	5 x 10^6 - 1 x 10^7 cells

Monolayer cells:

Add **DTT-containing Lysis Solution R** to the monolayer cells (volume as given in table 2). Collect the cell lysate with a rubber policeman. Mix thoroughly by pipetting up and down. No cell clumps should be visible before proceeding with the next step.

Table 2:

Lysis Solution R	Size of the culture vessels
350 µl	96-; 24-; 12-well-plates
700 µl	6-well plates; ∅ 35 mm dishes; 12.5 cm^2 flasks

Important: ***Shake Lysis Solution R gently before use!*** *Wait a short time because of foam formation!*

Note: *DNA in the sample may be sheared by passing it through a 20 gauge needle. This may increase the lysis efficiency and the yield.*

3. Binding of the genomic DNA to the DNA-Binding Spin Filter

Pipet the lysate resulting from step 2, including any precipitate which may have formed, directly onto the DNA-Binding Spin Filter placed in a 2 ml Receiver Tube (with lid). Incubate the sample for 1 min and centrifuge at 11.000 x g (11.000 rpm) for 2 min. Discard the DNA-Binding Spin Filter.

4. Adjust RNA binding conditions

Add 1 volume of 70 % **ethanol** to the RNA containing lysate and mix thoroughly by pipetting up and down.

5. Binding of the total RNA to the RNA-RTA Spin Filter

Pipet the RNA containing sample resulting from step 4 directly onto the RNA-RTA Spin Filter. Incubate for 1 min and centrifuge at 11.000 x g (11.000 rpm) for 2 min. Discard the flow-through and reuse the RTA Receiver Tube. If the volume of the RNA-containing sample exceeds 700 µl, divide the sample and load aliquots into the RNA-RTA Spin Filter.

6. First washing of the RNA-RTA Spin Filter

Add 600 µl **Wash Buffer R1** onto the RNA-RTA Spin Filter and centrifuge for 1 min at 11.000 x g (11.000 rpm). Discard the flow-through and the RTA Receiver Tube. Put the RNA-RTA Spin Filter in a new RTA Receiver Tube in step 7.

7. Second washing of the RNA-RTA Spin Filter

Add 700 µl **Wash Buffer R2** onto the RNA-RTA Spin Filter and centrifuge for 1 min at 11.000 x g (11.000 rpm). Discard the flow-through and reuse the RTA Receiver Tube.

Repeat this washing step once.

8. Drying of the RNA-RTA Spin Filter membrane

Discard the flow-through and put the RNA-RTA Spin Filter back to the RTA Receiver Tube. To eliminate any traces of ethanol, centrifuge for 4 min at maximum speed. Discard the RTA Receiver Tube.

9. Elution of total RNA

Transfer the RNA-RTA Spin Filter into a RNase-free Elution Tube and pipet 40-100 µl of **Elution Buffer R** directly onto the membrane of the RNA-RTA Spin Filter. Incubate for 2 min and centrifuge for 1 min at 11.000 x g (11.000 rpm). Discard the RNA-RTA Spin Filter and place the eluted total RNA immediately on ice.

Protocol 2: Total RNA extraction from Gram-positive or Gram-negative bacteria

1. Harvest bacteria (see also "Sample quantity", page 10)

Spin up to 1 x 10^9 bacteria cells for 5 min at 2.500 x g (5.000 rpm) in a centrifuge tube. Discard the supernatant and remove all media completely.

2. Lysis of the bacterial cell wall with Lysozyme

Important: *Immediately before use, dissolve **Lysozyme** (concentration according to table 3) in TE-buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0)!*

To loosen the bacteria cell pellet flick the tube and add 100 µl **Lysozyme-containing TE-buffer**. Mix gently by vortexing and incubate at RT.

Different **Lysozyme** concentrations and incubation times are required for Gram-positive and Gram-negative bacteria (see table 3). The amount of **Lysozyme** and the incubation time may also be modified depending on the bacterial strain.

A careful optimization of cell wall digestion is the basis for high total RNA yield!

table 3:

Lysozyme-concentration in TE-Buffer		**Incubation time at RT**
Gram-negative bacteria	400 µg/ml	5-10 min
Gram-positive bacteria	3.000 µg/ml	10-30 min

3. Disrupt bacteria cells by adding Lysis Solution R

Add 350 µl DTT-containing **Lysis Solution R** to the Lysozyme-treated sample. Mix thoroughly by pipetting up and down. No cell clumps should be visible before proceeding with the next step.

Important: ***Shake Lysis Solution R gently before use!*** *Wait a short time because of foam formation!*

Note: *DNA in the sample may be sheared by passing it through a 20 gauge needle. This may increase the lysis efficiency and the yield.*

4. Binding of genomic DNA to the DNA-Binding Spin Filter

Pipet the lysate (450 µl), including any precipitate which may have formed, directly onto the DNA-Binding Spin Filter placed in a 2 ml Receiver Tube (with lid). Incubate for 1 min and centrifuge at 11.100 x g (11.000 rpm) for 2 min. Discard the DNA-Binding Spin Filter.

5. Adjust RNA binding conditions

Add 250 µl 96-100% **ethanol** to the RNA containing lysate and mix thoroughly by pipetting and down.

6. Binding of the total RNA to the RNA-RTA Spin Filter

Transfer the RNA containing sample resulting from step 5 directly into the RNA-RTA Spin Filter. Incubate for 1 min and centrifuge at 11.000 x g (11.000 rpm) for 2 min. Discard the flow-through and reuse the RTA Receiver Tube. If the volume of the RNA containing sample exceeds 700 µl, divide the sample and load aliquots into the RNA-RTA Spin Filter.

7. First washing of the RNA-RTA Spin Filter

Add 600 µl **Wash Buffer R1** onto the RNA-RTA Spin Filter and centrifuge for 1 min at 11.000 x g (11.000 rpm). Discard the flow-through and put the RNA-RTA Spin Filter in a new RTA Receiver Tube in step 8.

8. Second washing of the RNA-RTA Spin Filter

Add 700 µl **Wash Buffer R2** onto the RNA-RTA Spin Filter followed by centrifugation for 1 min at 11.000 x g (11.000 rpm). Discard the flow-through and reuse the RTA Receiver Tube.

Repeat this washing step once.

9. Drying of the RNA-RTA Spin Filter membrane

Discard the flow-through and put the RNA-RTA Spin Filter back to the RTA Receiver Tube. To eliminate any traces of ethanol, centrifuge for 4 min at maximum speed. Discard the RTA Receiver Tube.

10. Elution of total RNA

Transfer the RNA-RTA Spin Filter into a RNase-free Elution Tube and pipet 40 - 100 µl of **Elution Buffer R** directly onto the membrane of the RNA-RTA Spin Filter. Incubate for 2 min and centrifuge for 1 min at 11.000 x g (11.000 rpm). Discard the RNA-RTA Spin Filter and place the eluted total RNA immediately on ice.

Protocol 3: Total RNA extraction from yeast

1. Harvest yeast cells (see also "Sample quantity", page 10)

Spin up to 5 x 10^7 yeast cells for 5 min at 2.500 x g (5.000 rpm) in a centrifuge tube. Discard the supernatant and remove all media completely.

Note: *For enzymatic lysis of yeast, the use of freshly harvested cells is recommended.*

2. Lysis of the yeast cell wall with Lyticase (preparation of spheroplasts)

Important: *Immediately before use, dissolve 50 U* ***Lyticase*** */ 1 x 10^7 yeast cells and add 0.1 % β-Mercaptoethanol in 1 M Sorbitol / 0.1 M EDTA-solution pH 7.4!*

To loosen the yeast pellet flick the tube and add 100 µl **Lyticase-containing 1 M Sorbitol / 0.1M EDTA-solution** pH 7.4. Mix gently by vortexing and incubate for 10 - 30 min at 30°C with gentle shaking (thermoshaker).

The enzymatic lysis of yeast cells is based on the digestion of the cell wall with **Lyticase** (optional: Zymolase) and the generation of spheroplasts in an isotonic buffer system. Due to considerable differences among the various yeast strains the concentration of **Lyticase**, the incubation time and the buffer composition given here can only be an example. The creation of spheroplasts protects the total RNA from degradation until the transfer of the sample in RNA-stabilizing conditions provided by **Lysis Solution R** (see step 3).

A careful optimization of spheroplast preparation is the basis for high total RNA yield!

3. Disrupt yeast cells by adding Lysis Solution R

Add 350 µl DTT-containing **Lysis Solution R** to the Lyticase-containing sample. Mix thoroughly by pipetting up and down. No cell clumps should be visible before proceeding with the next step.

Important: ***Shake Lysis Solution R gently before use!*** *Wait a short time because of foam formation!*

Note: *DNA in the sample may be sheared by passing it through a 20 gauge needle. This may increase the lysis efficiency and the yield.*

4. Binding of genomic DNA to the DNA-Binding Spin Filter

Transfer the lysate from step 3 (450 µl), including any precipitate which may have formed, directly onto the DNA-Binding Spin Filter placed in a 2 ml Receiver Tube (with lid). Incubate for 1 min and centrifuge at 11.400 x g (11.000 rpm) for 2 min. Discard the DNA-Binding Spin Filter.

5. Adjust RNA binding conditions

Add 250 µl 96-100% **ethanol** to the RNA containing lysate and mix thoroughly by pipetting up and down.

6. Binding of the total RNA to the RNA-RTA Spin Filter

Pipet the RNA containing sample resulting from step 5 directly onto the RNA-RTA Spin Filter. Incubate for 1 min and centrifuge at 11.400 x g (11.000 rpm) for 2 min. Discard the flow-through and reuse the RTA Receiver Tube.

If the volume of the RNA containing sample exceeds 700 µl, divide the sample and load aliquots into the RNA-RTA Spin Filter.

7. First washing of the RNA-RTA Spin Filter

Add 600 µl **Wash Buffer R1** onto the RNA-RTA Spin Filter and centrifuge for 1 min at 11.000 x g (11.000 rpm). Discard the flow-through and put the RNA-RTA Spin Filter in a new RTA Receiver Tube.

8. Second washing of the RNA-RTA Spin Filter

Add 700 µl **Wash Buffer R2** onto the RNA-RTA Spin Filter and centrifuge for 1 min at 11.000 x g (11.000 rpm). Discard the flow-through and reuse the RTA Receiver Tube.

Repeat this washing step once.

9. Drying of the RNA-RTA Spin Filter membrane

Discard the flow-through and put the RNA-RTA Spin Filter back to the RTA Receiver Tube. To eliminate any traces of ethanol, centrifuge for 4 min at maximum speed. Discard the RTA Receiver Tube.

10. Elution of total RNA

Transfer the RNA-RTA Spin Filter into a RNase-free Elution Tube and pipet 40 - 100 µl of **Elution Buffer R** directly onto the membrane of the RNA-RTA Spin Filter. Incubate for 2 min and centrifuge for 1 min at 11.100 x g (11.000 rpm). Discard the RNA-RTA Spin Filter and place the eluted total RNA immediately on ice.

Protocol 4: RNA Cleanup from liquids

1. Starting the cleanup-procedure **<u>for samples containing no DNA</u>**

This step is useful if your RNA is not contaminated by DNA.

A) Purification of 100 µl sample from contaminations

Pipet 350 µl of **DTT-containing Lysis Solution R** directly onto the DNA-Binding Spin Filter (without lid) placed in a 2 ml Receiver Tube (with lid). Centrifuge at 13.400 x g (12.000 rpm) for 2 min. Discard the DNA-Binding Spin Filter. Take the flow-through for the following cleanup-procedure. Just add 100 µl of the sample (reaction mixture) to the flow-through (Lysis Solution R). **Then continue with step 4**.

B) Purification of 200 µl sample from contaminations

Pipet 700 µl of **DTT-containing Lysis Solution R** directly onto the DNA-Binding Spin Filter (without lid) placed in a 2 ml Receiver Tube (with lid). Centrifuge at 13.400 x g (12.000 rpm) for 2 min. Discard the DNA-Binding Spin Filter. Take the flow-trough for the following cleanup-procedure. Just add 200 µl of the sample (reaction mixture) to the flow-through (Lysis Solution R). **Then continue with step 4**.

2. Starting the cleanup-procedure **<u>for samples containing contaminating DNA</u>**

This procedure useful if your RNA is contaminated by DNA

A) Purification of 100 µl sample from contaminations

Add 350 µl of **DTT-containing Lysis Solution R** to 100 µl RNA sample. Mix thoroughly by pipetting up and down.

B) Purification of 200 µl sample from contaminations

Add 700 µl of DTT-containing **Lysis Solution R** to 200 µl RNA sample. Mix thoroughly by pipetting up and down.

<u>Important:</u> ***Shake Lysis Solution R gently before use!*** *Wait a short time because of foam formation!*

3. Binding of genomic DNA to the DNA-Binding Spin Filter

Pipet the whole lysate (450 µl or 2 x 450 µl), including any precipitate which may have formed, directly onto the DNA-Binding Spin Filter placed in a 2 ml Receiver Tube (with lid). Incubate for 1 min and centrifuge at 11.000 x g (11.000 rpm) for 2 min. Discard the DNA-Binding Spin Filter.

Note: *On the surface of the membrane of DNA-Binding Spin Filter is a gelly material visible. This is the DNA bound to the Carrier.*

4. Adjust RNA binding conditions

Add 250 µl (100 µl sample volume) respectively 500 µl (200µl sample volume) 96 - 100% ethanol to the RNA containing lysate and mix thoroughly by pipetting and down.

5. Binding of the RNA to the RNA-RTA Spin Filter

Transfer the whole RNA containing sample resulting from step 4 directly into the RNA-RTA Spin Filter. Incubate for 1 min and centrifuge at 11.000 x g (11.000 rpm) for 2 min. Discard the flow-through and reuse the RTA Receiver Tube.

If the volume of the RNA-containing sample exceeds 700 µl, divide the sample and load aliquots into the RNA-RTA Spin Filter.

6. First washing of the RNA-RTA Spin Filter

Add 600 µl **Wash Buffer R1** onto the RNA-RTA Spin Filter and centrifuge at 11.000 x g (11.000 rpm) for 1 min. Discard the flow-through and put the RNA-RTA Spin Filter in a new RTA Receiver.

7. Second washing of the RNA-RTA Spin Filter

Add 700 µl **Wash Buffer R2** onto the RNA-RTA Spin Filter followed by centrifugation at 11.000 x g (11.000 rpm) for 1 min. Discard the flow-through and reuse the RTA Receiver Tube.

Repeat this washing step once.

8. Drying of the RNA-RTA Spin Filter membrane

Discard the flow-through and put the RNA-RTA Spin Filter back to the RTA Receiver Tube. To eliminate any traces of ethanol, centrifuge for 4 min at maximum speed. Discard the RTA Receiver Tube.

9. Elution of total RNA

Transfer the RNA-RTA Spin Filter into a RNase-free Elution Tube and pipet 40 - 100 µl of **Elution Buffer R** directly onto the membrane of the RNA-RTA Spin Filter. Incubate for 2 min and centrifuge for 1 min at 11.000 x g (11.000 rpm). Discard the RNA-RTA Spin Filter and place the eluted total RNA immediately on ice.

Protocol 5: RNA Cleanup from Trizol aqueous phase

1. Starting the cleanup procedure

Add 350 µl **DTT-containing Lysis Solution R** to up to 350 µl of the Trizol aqueous phase. Mix thoroughly by pipetting up and down.

Important: ***Shake Lysis Solution R gently before use!*** *Wait a short time because of foam formation!*

2. Binding of the genomic DNA to the DNA-Binding Spin Filter

Pipet the whole lysate resulting from step 1, including any precipitate which may have formed, directly onto the DNA-Binding Spin Filter placed in a 2 ml Receiver Tube (with lid). Incubate the sample for 1 min and centrifuge at 11.000 x g (11.000 rpm) for 2 min. Discard the DNA-Binding Spin Filter.

4. Adjust RNA binding conditions

Add 1 volume of 96-100 % **ethanol** to the RNA containing lysate and mix thoroughly by pipetting up and down.

5. Binding of the total RNA to the RNA-RTA Spin Filter

Pipet the RNA containing sample resulting from step 4 directly onto the RNA-RTA Spin Filter. Incubate for 1 min and centrifuge at 11.000 x g (11.000 rpm) for 2 min. Discard the flow-through and reuse the RTA Receiver Tube.
If the volume of the RNA containing sample exceeds 700 µl, divide the sample and load aliquots into the RNA-RTA Spin Filter.

6. First washing of the RNA-RTA Spin Filter

Add 600 µl **Wash Buffer R1** onto the RNA-RTA Spin Filter and centrifuge for 1 min at 11.000 x g (11.000 rpm). Discard the flow-through and put the RNA-RTA Spin Filter in a new RTA Receiver Tube.

7. Second washing of the RNA-RTA Spin Filter

Add 700 µl **Wash Buffer R2** onto the RNA-RTA Spin Filter and centrifuge for 1 min at 11.000 x g (11.000 rpm). Discard the flow-through and reuse the RTA Receiver Tube.

Repeat this washing step once.

8. Drying of the RNA-RTA Spin Filter membrane

Discard the flow-through and put the RNA-RTA Spin Filter back to the RTA Receiver Tube. To eliminate any traces of ethanol, centrifuge for 4 min at maximum speed. Discard the RTA Receiver Tube.

9. Elution of total RNA

Transfer the RNA-RTA Spin Filter into a RNase-free Elution Tube and pipet 40-100 µl of **Elution Buffer R** directly onto the membrane of the RNA-RTA Spin Filter. Incubate for 2 min and centrifuge for 1 min at 1.100 x g (11.000 rpm). Discard the RNA-RTA Spin Filter and place the eluted total RNA immediately on ice.

Supplemental Protocol for simultaneous isolation of RNA and proteins

The starting material for RNA purifications is often limited and in some cases the isolation of proteins from the same sample is necessary. Following the given protocol below the simultaneous preparation of RNA and proteins is possible using the **InviTrap® Spin Cell RNA Mini Kit.**

Important Note: *The buffer solutions for RNA purification provide very denaturing conditions due to the contained salts. Proteins isolated according to the protocol below are denatured and can be analyzed by SDS-PAGE or Western Blot. Experiments requiring the native state of the proteins (e.g. interaction assays) are excluded.*

1. Collection of protein containing material

The protein fraction is found in the flow-through of the RNA-RTA Spin Filter in the purification protocol (see *Protocol 1*, step 5; *Protocol 2 +3*, step 6). Measure the volume of the flow-through.

2. Precipitation of proteins

Add the three fold volume of ice cold **acetone** and mix well by vortexing.
Centrifuge for 10 min at 13.400 x g (12.000 rpm) at 4°C.
Discard the supernatant. Be careful not to remove the pellet!

3. Washing step

Add 500 µl of ice cold 96-100% **ethanol** and centrifuge for 3 min at 11.000 x g (11.000 rpm) at 4°C.
Discard the supernatant, be careful not to remove the pellet.

4. Dissolving of proteins

Dissolve the protein pellet by suspending in a buffer solution suitable for the subsequent application. For SDS-PAGE, directly dissolve proteins in 1-fold Laemmli Buffer and heat them at 99°C for 5 min.

ATTENTION: ***Never try a TCA-Precipitation from RNA-Lysis Buffers, poisonous gas will be generated!***

Supplemental Protocol for DNA digestion on the RNA-RTA Spin Filter

For special needs, applications or other protocols, where smallest amounts of DNA might disturb, please follow this protocol and do a DNase digestion directly on the RNA-RTA Spin Filter**.**

Therefore you have to change Step 7 in protocol 1 (Second Washing Step) or

you have to change Step 8 in Protocol 2 and 3 according to the following procedure:

Second washing of the Spin Filter

Add 550 µl **Wash Buffer R2** onto the RNA-RTA Spin Filter with the ound RNA and centrifuge for 30 sec at 11.000 x g (11.000 rpm). Discard the flow-through and put the RNA-RTA Spin Filter back into the RTA - Receiver Tube. Add 10 µl of a DNase reaction mixture directly in the center of the Spin Filter membrane.

A typical reaction mix is for example 1 µl of **DNase I** (50 u) enzyme in 9 µl 1 x **DNase reaction buffer**, but here follow the advice of the manufacturer. Do not exceed the reaction mix volume to more than 10 µl! Incubate the RNA-RTA Spin Filter at room temperature for 10 min.

Add again 550 µl **Wash Buffer R2** onto the RNA-RTA Spin Filter incubate for 1 minute and centrifuge for 1 min at 11.000 x g (11.000 rpm).

Reuse the RTA - Receiver Tube and repeat this washing step once again.

Troubleshooting

Problem/ Probable cause	Comments and suggestions
clogged Spin Filter	
too much starting material	reduce the amount of starting material in subsequent preparations
	increase g-force and / or centrifugation time
insufficient disruption or homogenisation of the starting material	after lysis spin lysate to pellet debris and continue with the protocol using the supernatant
	all centrifugation steps should be conducted at room temperature
very viscous lysate, like a gel after addition of **Lysis Solution R**	if too many cells including DNA has been used, the carrier with the bound DNA will be too viscous to pipet after homogenization in this case, divide the sample into two aliquots and adjust the volumes of each aliquot to 700 µl with **Lysis Solution R;** continue with the procedure from step 3
A_{260}/A_{280} value below 1.7	
protein contamination of RNA	use less sample
	repeat washing step one
RNA sample is diluted in H_2O with wrong pH	do not use RNase free water to dilute the sample for measuring the RNA purity
	the use of a neutral buffer (10 mM Tris/HCl, pH 7.0) is recommended. (see also “Quantification of RNA”, page 35)
RNA sample is to strong diluted	sample concentration is out of the range of the photometer
little or no total RNA eluted	
sample stored incorrectly	the sample should be stored at – 80°C
incomplete removal of cell culture medium	make sure that the cell culture medium is complete removed after the cell harvest
insufficient disruption or homogenisation	reduce the amount of starting material, overloading the kit reduces the yield
incomplete elution	prolong the incubation time with **Elution Buffer R** to 5-10 min or repeat elution step once again
	elute a second time with 100 µl **Elution Buffer R**
	incubate RTA Spin Filter at room temperature (15 - 25°C) for 5 min with RNase free water or **Elution Buffer R** prior centrifugation
no alcohol added to the **Wash Buffer R1** and / or **Wash Buffer R2**	check that **Wash Buffer R1** and / or **Wash Buffer R2** concentrates were diluted with correct volume of 96-100% ethanol. Do not use denatured alcohol, which contains other substances such as methanol or methylethylketone.
	repeat the purification procedure with a new sample

Problem/ Probable cause	Comments and suggestions
DNA-contamination	
too much starting material	reduce amount of starting material DNase digestion of the eluate containing the total RNA
no optimal homogenization of DNA binding carrier	shake **Lysis Solution R** carefully before use
RNA degraded	
inappropriate handling of the starting material	the RNA purification protocol should be performed quickly (see also “General notes on handling RNA”, page 34) cell pellets stored at - 80°C for later processing should be immediately frozen after cell harvest by liquid nitrogen treatment or under **Lysis Solution**
Lysis Solution does not contain **DTT**	ensure that **DTT** has been added to the **Lysis Solution R**
RNase contamination	check for RNase contamination of buffers, be certain not to introduce any RNase throughout the procedure or during further handling for analysis see appendix , ”General Notes on handling RNA” (page 34)
total RNA does not perform well in downstream-applications (RT-PCR)	
ethanol carryover during elution	increase g-force or centrifugation time when drying the **RTA Spin Filter**
salt carryover during elution	ensure that **Wash Buffer R1** and **R2** are at room temperature check up **Wash Buffer R1** and **R2** for salt precipitates, if there are any precipitates solve these precipitates by careful warming

Appendix

General notes on handling RNA

RNA is far less stable than DNA. It is very sensitive to degradation by endogenous RNases in the biological material and exogenous RNases, which are permanently present everywhere in the lab. To achieve satisfactory qualitative and quantitative results in RNA preparations, contaminations with exogenous RNases has to be reduced as much as possible. Avoid handling bacterial cultures, cell cultures or other biological sources of RNases in the same lab where the RNA purification is to be carried out.

All glassware should be treated before use to ensure that it is RNase-free. Glassware should be cleaned with detergent, thoroughly rinsed and oven baked at 240°C for four or more hours before use. Autoclaving alone will not completely inactivate many RNases. Oven baking will both inactivate RNases and ensure that no other nucleic acids (such as Plasmid DNA) are present on the surface of the glassware. You can also clean glassware with 0.1% DEPC (diethyl pyrocarbonate). The glassware has to stand 12 hours at 37°C and then autoclave or heat to 100°C for 15 min to remove residual DEPC.

- Electrophoresis tanks should be cleaned with detergent solution (e.g. 0.5% SDS), thoroughly rinsed with RNase-free water, and then rinsed with ethanol and allowed to dry.
- Non-disposable plasticware should be treated before use to ensure that it is RNase-free. Plastic ware should be thoroughly rinsed with 0.1 M NaOH, 1 mM EDTA followed by RNase-free water. You can also take chloroform-resistant plastic ware rinsed with chloroform to inactivate RNases.
- All buffers must be prepared with RNase-free ddH_2O.
- When working with chemicals, always wear a suitable lab coat, disposable gloves and protective goggles.
- Change gloves frequently and keep tubes closed.
- All centrifugation steps are carried out at room temperature.
- To avoid cross contamination cavity seams should not be moisten with fluid.
- Reduce the preparation time as much as possible.
- Use only sterile, disposable polypropylene tubes throughout the procedure (these tubes are generally RNase free).
- Keep isolated RNA on ice.
- Do not use kit components from other kits with the kit you are currently using, unless the lot numbers are identical.
- To minimize the risk of infections from potentially infectious material, we recommend working under laminar air-flow until the samples are lysed.
- Personnel trained in in vitro diagnostic laboratory practice should only use this kit.

Storage of RNA

Purified viral RNA can be stored at -80°C and is stable for several years at this condition.

Quantification of RNA

The concentraton of RNA should be determinated by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer. Readings should be greater than 0.10 to ensure significance. An absorbance of 1 unit at 260 nm corresponds to 40 µg of RNA per ml. This relation is valid only for measurements at neutral pH. The ratio between absorbance values at 260 nm and 280 nm gives an estimate of RNA purity (see below).

When measuring RNA samples, make sure that cuvettes are RNase-free, esp. if the RNA is to be recovered after spectrophotometry. This can be accomplished by washing cuvettes with 0.1 N NaOH, 1 mM EDTA followed by washing with RNase-free water. Use the buffer in which the RNA is diluted for calibration of the spectrophotometer.

An example of the calculation involved in RNA quantification:

- volume of RNA sample: 60 µl
- example for dilution = 5 µl of RNA + 45 µl of ddH_2O (1/10 dilution) for measurement in a 50 µl cuvette.

Example: A_{260} = 0.2

Concentration of the RNA sample	= 40 µg/ml * A_{260} * dilution factor = 40 µg/ml * 0.2 * 10 = 80 µg/ml
Total amount	= concentration * volume of sample in ml = 80 µg/ml * 0.06 ml = 4,8 µg of RNA

Purity of RNA

The ratio of the readings at 260 nm and 280 nm (A_{260}/A_{280}) provides an estimate of the purity of RNA with respect to the contaminants that absorb in the UV, such as protein. However, the A_{260}/A_{280} ratio is influenced considerably by pH. Since water is not buffered, the pH and the resulting A_{260}/A_{280} ratio can vary greatly. Lower pH results in lower A_{260}/A_{280} ratio and reduced sensitivity to protein contaminations.* For accurate values, it is recommend to measure absorbance in 10 mM Tris Cl, pH 7.5. Pure RNA has an A_{260}/A_{280} ratio of 1.9-2.1** in 10 mM Tris/HCl pH 7.5. Always be sure to calibrate the spectrophotometer with the same solution.

For determination of the RNA concentration, however, it is recommend diluting the sample in a buffer with neutral pH since the relationship between absorbance and concentration (A_{260} reading of 1 = 40 µg/ml of RNA) is based on an extinction coefficient calculated for RNA at neutral pH.

Using an Agilent Bioanalyzer: RNA of good quality shows RIN of 7-10.

* Wilfinger, W.W.,Mackey, M., and Chomczynski, P. (1997) Effect of pH and ionic strength on the spectrophotometric assessment of nucleic acid purity. Bio Techniques 22, 474

**Values up to 2.3 are routinely obtained for pure RNA (in 10 mM Tris/HCl, pH 7.5) with some spectrophotometers.

Denaturing agarose gel electrophoresis of RNA

The overall quality of an RNA preparation may be assessed by electrophoresis on a denaturing agarose gel; this will also give some information about RNA yield. A denaturing gel system is suggested because most RNA forms extensive secondary structure via intramolecular base pairing, and this prevents it from migrating strictly according to its size. Be sure to include a positive control RNA on the gel so that unusual results can be attributed to a problem with the gel or a problem with the RNA under analysis. RNA molecular weight markers, RNA sample known to be intact, or both, can be used for this purpose.

The given denaturing agarose gel method for RNA electrophoresis is modified from "Current Protocols in Molecular Biology", Section 4.9 (Ausubel et al., eds.).

1. Prepare the gel.

a. heat 1 g agarose in 72 ml water until dissolved, then cool to 60°C.
b. add 10 ml 10X MOPS running buffer, and 18 ml 37% formaldehyde (12.3 M).

WARNING: Formaldehyde is toxic through skin contact and inhalation of vapors. Manipulations involving formaldehyde should be done in a chemical fume hood.

10X MOPS running buffer: 0.4 M MOPS, pH 7.0
0.1 M sodium acetate
0.01 M EDTA

c. pour the gel using a comb that will form wells large enough to accommodate at least 25 µl.
d. assemble the gel in the tank, and add enough 1X MOPS running buffer to cover the gel by a few millimeters. Then remove the comb.

2. Prepare the RNA sample.

Heat denature samples at 65-70°C for 5-15 min.

Denaturation for 5 min is typically sufficient for simply assessing RNA on a gel, but a 15 min denaturation is recommended when running RNA for a Northern Blot. The longer incubation may be necessary to completely denature the RNA.

To 1-3 µg RNA, add 0.5-3x volumes Formaldehyde Load Dye**.**

To simply check the RNA on a denaturing gel, as little as 0.5x Formaldehyde Load Dye can be used, but to completely denaturate the RNA, e.g. for Northern blots, use 3X volumes of Formaldehyde Load Dye. Ethidium bromide can be added to the Formaldehyde Load Dye at a final concentration of 10 µg/ml. Some size markers may require significantly more than 10 µg/ml ethidium bromide for visualization. To accurately size your RNA, however, it is important to use the same amount of ethidium bromide in all the samples (including the size marker) because ethidium bromide concentration affects RNA migration in agarose gels.

3. Electrophoresis

Load the gel and electrophorese at 5-6 V/cm until the bromophenol blue (the faster-migrating dye) has migrated at least 2-3 cm into the gel, or as far as 2/3 the length of the gel.

4. Results

Visualize the gel on a UV transilluminator. (If ethidium bromide was not added to the Formaldehyde Load Dye, the gel will have to be post-stained and destained.)

Intact total RNA run on a denaturing gel will have sharp 28S and 18S rRNA bands (eukaryotic samples). The 28S rRNA band should be approximately twice as intense as the 18S rRNA band (Figure 1, lane 3). This 2:1 ratio (28S:18S) is a good indication that the RNA is intact. Partially degraded RNA will have a smeared appearance, will lack the sharp rRNA bands, or will not exhibit a 2:1 ratio. Completely degraded RNA will appear as a very low molecular weight smear (Figure 1, lane 2). Inclusion of RNA size markers on the gel will allow the size of any bands or smears to be determined and will also serve as a good control to ensure the gel was run properly (Figure 1, lane 1). Note: Poly(A) selected samples will not contain strong rRNA bands and will appear as a smear from approximately 6 kb to 0.5 kb (resulting from the population of mRNAs, and depending on exposure times and conditions), with the area between 1.5 and 2 kb being the most intense (this smear is sometimes apparent in total RNA samples as well).

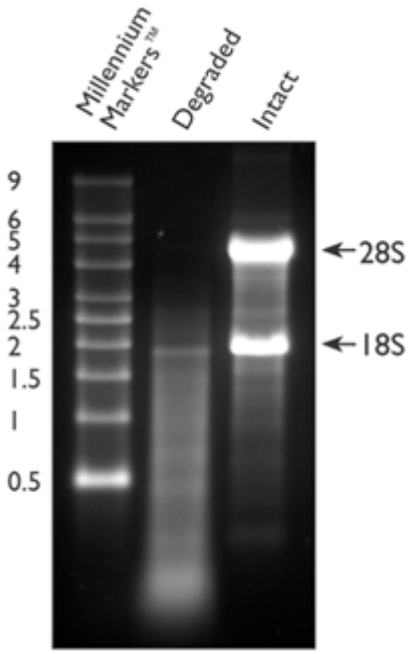


Figure 1. Intact vs. Degraded RNA. Two µg of degraded total RNA and intact total RNA were run beside Ambion's RNA Millennium Markers™ on a 1.5% denaturing agarose gel. The 18S and 28S ribosomal RNA bands are clearly visible in the intact RNA sample. The degraded RNA appears as a lower molecular weight smear.

Generally, at least 200 ng of RNA must be loaded onto a denaturing agarose gel in order to be visualized with ethidium bromide. Some RNA preparations, such as those from needle biopsies or from laser capture microdissected samples, result in very low yields. In these cases, it may be impossible to spare 200 ng of RNA to assess integrity. Alternative nucleic acid stains, such as SYBR® Gold and SYBR® Green II RNA gel stain from Molecular Probes, offer a significant increase in sensitivity over ethidium bromide. Using a 300 nm transilluminator (6 x 15-watt bulbs) and a special filter, as little as 1 ng and 2 ng of RNA can be detected with SYBR Gold and SYBR® Green II RNA gel stain, respectively.

Ordering information

Product	Package size	Catalogue No.
InviTrap® Spin Cell RNA Mini Kit	250 preparations	1061100300

Related products

InviTrap® RNA Cell HTS 96 Kit / C	24 x 96 preparations	7061300400
InviTrap® Spin Universal RNA Mini Kit	50 preparations	1060100200
InviTrap® Spin Universal RNA Mini Kit	250 preparations	1060100300

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