INVITEK
Molecular

USER MANUAL

InviTrap® Spin Universal RNA Mini Kit

for purification of total RNA from human and animal cells, bacteria and yeast cells, tissue samples, paraffin embedded tissue, whole blood samples (EDTA, citrate), RNA cleanup & simultaneous protein isolation

CE IVD

REF 1060100X00

Invitek Molecular GmbH · D-13125 Berlin

Instruction for the InviTrap® Spin Universal RNA Mini Kit

The **InviTrap® Spin Universal RNA Mini Kit** is a universal system for reliable and fast isolation of high quality total RNA from fresh or frozen samples of human or animal cell cultures (max. 1 x 10^7 cells), from up to 20 mg fresh or frozen human and animal tissue sample, and from up to 1.5 ml whole blood samples with anticoagulants, like citrate and EDTA (except heparin stabilized blood) in a 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 simultaneous isolation of total RNA and proteins as well as of total RNA and for RNA cleanup in Trizol® phases and paraffin embedded tissue.

DNase-digestion to remove contaminating genomic DNA is not necessary.

The **InviTrap® Spin Universal RNA Mini Kit supplemental protocols** are intended for life science research use only. Prior to using it for other purposes, the user must validate the system in compliance with the applicable law, directives, and regulations.

The kit is neither validated for the isolation of total RNA from serum, plasma, nor for viruses. The performance of the kit 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 Universal RNA Mini Kit

	50 total RNA extractions	250 total RNA extractions
Catalogue No.	1060100200	1060100300
Buffer EL concentrate	30 ml	4 x 30 ml
Lysis Solution TR	50 ml	250 ml
Zirconia Beads I	1	5
Zirconia Beads II	1	5
Wash Buffer R1	20 ml (final volume 40 ml)	80 ml (final volume 160 ml)
Wash Buffer R2	2 x 12 ml (final volume 2 x 60 ml)	2 x 40 ml (final volume 2 x 200 ml)
Elution Buffer R	15 ml	30 ml
DNA-Binding Spin Filter	50	5 x 50
RNA-RTA Spin Filter Set	50	5 x 50
2.0 ml Receiver Tubes	2 x 50	10 x 50
RTA Receiver Tubes	50	5 x 50
Elution Tubes	50	5 x 50
Manual	1	1
Initial Steps	Add 20 ml 96-100% ethanol to the bottle **Wash Buffer R1.** Add 48 ml 96-100% ethanol to each bottle **Wash Buffer R2.** Add 30 ml **Buffer EL concentrate** to 970 ml H_20. Label or sign the bottle with **Buffer EL**.	Add 80 ml 96-100% ethanol to the bottle **Wash Buffer R1**. Add 160 ml 96-100% ethanol to each bottle **Wash Buffer R2.** Add 30 ml **Buffer EL concentrate** to 970 ml H_20. Label or sign the bottle with **Buffer EL**.
	For protocol 1 (RNA from cells): Preparation of 10 ml/ 50ml/ 200 ml 70% ethanol. **Adjust Lysis Solution TR with 1/100 volume of 1M DTT**. Due to the instability of dissolved DTT under oxidative conditions, do not mix the whole **Lysis Solution TR** with DTT in case of the kits with 50 and 250 preparations. We recommend the preparation of a volume DTT-containing **Lysis Solution TR** shortly before carrying out the purifications adapted to the number of samples that will be processed. Store the remaining **Lysis Solution TR** and DTT separately in accordance to the storage instructions (see “Storage”, page 4). It is possible to replace DTT by β-Mercaptoethanol. In this case, adjust **Lysis Solution TR** with 1/100 volume of β-Mercaptoethanol as described above.	

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 Universal RNA Mini Kit**,** except **1 M DTT solution** (not provided) and **dissolved Buffer EL** 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.

Buffer EL: Dissolved Buffer EL must be stored at 2 - 8 °C.

Store **1 M DTT solution** (not provided) 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 Universal 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 Universal 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 Universal 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 Universal RNA Mini Kit** is the universal tool for reliable and fast isolation and purification of high quality total RNA from fresh or frozen samples of human or animal cell cultures (max. 1 x 10^7 cells)**,** for up to 20 mg of fresh or frozen human and animal tissue samples, and for up to 1.5 ml of whole blood sample with anticoagulants, like citrate and EDTA (*except heparin stabilized blood*) in a spin filter format. For reproducible and high yields an appropriate sample storage and quick operation under the rules for RNA operation is essential.

For some studies a simultaneous investigation of the RNA and of the sample specific proteins, 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, nor for viral RNA isolation. 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 DNase digestion mixtures, 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 Universal 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 Universal RNA Mini Kit** to which they apply are listed below as follows:

Lysis Solution TR

Danger

H302-H318-H332-H412-P280-305+P351+P338-EUH032

Wash Buffer R1

Warning

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

Buffer EL concentrate

Warning

H315-P280-P305+P351+P338

H302: Harmful if swallowed.
H315: Causes skin irritation
H318: Causes serious eye damage.
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 Universal RNA Mini Kit

Starting material	Yield	Time	Ratio
up to 1 x 10^7 human or animal cells (cultured cells)	up to 100 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} 1.6 - 2.0
up to 20 mg fresh or frozen tissue	up to 80 µg total RNA (depends on type and amount of starting material)	about 10 min after tissue lysis	A_{260} : A_{280} 1.6 - 2.0
up to 1.5 ml of whole blood sample containing common anticoagulants	up to 1 - 5 µg total RNA / ml of whole blood (depending on the amount of lymphocytes)	about 45 minutes	A_{260} : A_{280} 1.6 - 2.0

The **InviTrap® Spin Universal RNA Mini Kit** provides a fast and efficient way for reliable isolation of high quality DNA free total RNA from fresh or frozen samples of human or animal cell cultures, tissue samples and from whole blood. The content of leukocytes that can be processed in one preparation must not exceed 1 x 10^7. (1.5 ml whole blood of a healthy adult contains approximately 4.000-7.000 leukocytes / ml sample volume)

The kit allows a simultaneous isolation of proteins from the same sample.

During the **InviTrap®** procedure for purification of RNA from cell samples, a cell content higher than 1 x 10^7 should not be used-DNA contaminations and reduced RNA purity would be the consequence.

During the **InviTrap®** procedure for purification of RNA from tissue samples, the tissue material will be efficiently lysed using Zirconia beads to homogenize the tissue material under highly denaturating conditions.

In the procedure of the **InviTrap® Spin Universal RNA Mini Kit**, if a whole blood sample is processed, **Buffer EL** is provided that selectively lyses the red blood cells and leukocytes are recovered by centrifugation. This pellet later on is processed like other cell pellets.

Since the procedure relies on intact leukocytes, frozen blood cannot be used!

The **InviTrap® RNA isolation technology** simplifies total RNA isolation by combining efficient lysis of the starting material and the inactivation of exogenous and endogenous RNases. The genomic DNA is removed efficiently with the speed and convenience of a spin technology. The genomic DNA is fixed at the surface of the nucleic acid binding mineral carrier particles provided within the **Lysis Solution TR** simultaneously to the cell lysis of the sample. Selective binding of the DNA is assured by optimized buffer conditions that reduce the undesired binding of the RNA to a minimum. The mineral carrier particles loaded with the genomic DNA are removed by centrifugation or by centrifugation on spin filter surface. The RNA remains in the supernatant or filtrate and is after the adjustment of special binding conditions bound onto the membrane of the RNA-RTA 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.

A specialized buffering system allows RNA species of sizes down to 200 base to bind to the spin filter membrane. The particle size distribution of purified RNA is similar to those, gained using a CsCl - gradient.

The extracted RNA contains enriched mRNA.

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.

The purification procedure is rapid and does not require a phenol/ chloroform extraction. Only minimal interaction by the user is necessary. The procedure is designed to avoid sample-to-sample cross-contaminations. Traditional time-killing procedures can be replaced using the **InviTrap® Spin Universal RNA Mini Kit**.

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

Principle and procedure

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

- erythrocyte lysis (only in case of processing a whole blood sample) or
- disruption and homogenization of tissue samples using beads of different size or other methods or
- cell lysis in case of processing cells
- lysis of leukocytes (only in case of processing a whole blood sample)
- selective binding of the genomic DNA to a specific carrier and separation of the carrier bound contaminating DNA
- adjusting of RNA binding conditions
- transfer of the sample into the RNA binding RTA-Spin Filter Set
- binding of the total RNA to the membrane (optional: purification of proteins)
- washing of the membrane and elimination of contaminants and ethanol
- elution of high 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 water.

This manual contains 6 protocols and 5 supplemented protocols.

Sampling and storage of starting material

Cell samples
Best results are obtained using freshly extracted starting material, e.g. 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. The starting material can be stored also in **Lysis Solution TR** at - 80°C after cell lysis.

Tissue samples
Best results are obtained using fresh tissue samples. As long as samples are not shock frosted or are incubated with RNase inhibitors or denaturing reagents, the RNA is not secured. Therefore, it is essential, that cells are immediately flash frozen after cell harvest and are stored at - 80°C. RNA from deep frozen samples is stable for months. Frozen tissue should not be allowed to thaw while handling. RNA purification should be processed as soon as possible. Cells can be stored also in **Lysis Solution TR** at - 80°C after cell lysis.
Formalin-fixation and paraffin-embedding procedures according to standard protocols always cause a degradation of nucleic acids. The use of freshly cut sections with a thickness of up to 10 µm is recommended. Do not apply more than 8 sections with a surface area of 250 mm^2 in one purification run. Tissues with a high DNA content (e.g. thymus) request the use of fewer sections per preparation to avoid overloading the kit. To adjust the number of sections used in one preparation to the amount of nucleic acid bound by the **InviTrap® Spin Universal RNA Mini Kit** we recommend starting with one or 2 sections.

Whole blood samples
Best results are obtained using fresh blood samples that were collected in the presence of an anticoagulant like EDTA or citrate (*except heparin stabilized blood*) to get a real picture of the variety of RNA in the cell.
For optimal results, blood samples should be processed within a few hours after collection.
mRNAs from blood cells have different stabilities, e.g. shows the mRNA of some regulatory genes shorter half-live the mRNAs from most housekeeping genes. To ensure that the isolated RNA contains a representative distribution of mRNAs the blood sample should not be stored for long periods before isolating RNA.

***Note**: The **InviTrap® Spin Universal RNA Mini Kit** must not be used for frozen blood samples.*

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.

Sample preparation

Disrupting and homogenizing of the tissue material are 2 distinct steps (only in case of processing tissue samples)

The efficient and complete disruption and homogenization of the tissue samples is absolute essential for isolation of a high yield of total RNA.

The **disruption procedure**, the breakage of intercellular matrix, like cells walls, organelles and plasma membranes, is necessary to release the nucleic acids contained in the cell, thus inefficient disruption decreases the RNA yield. Different samples require different methods to achieve complete disruption

The **homogenization** means the reduction of the viscosity of the lysate after disruption. Contaminating genomic DNA and other cellular components of high molecular weight are sheared to form a homogenous lysate. Incomplete homogenization results in inefficient binding of RNA to the RTA Spin Filter membrane and therefore significantly reduced RNA yields.

It is possible to use a commercially available bead mills in combination with or without beads for the disruption and homogenization of the starting material. Alternatively, the starting material can be reduced to a fine powder in liquid nitrogen using a mortar and pestle.

A mixture of Zirconia beads with a diameter of 0.7 mm (Zirconia Beads I) and 1.2 mm (Zirconia Beads II) and a mixture of them in a ratio in number of 2:1 Beads I : Beads II are optimal to use for tissue in combination with the **InviTrap® Spin Universal RNA Mini Kit.** All other disruption parameters should be determined empirically for each application.

Disruption and homogenization using a mortar and pestle
To disrupt tissue using a mortar and pestle, freeze the sample immediately in liquid nitrogen, and grind to a fine powder under liquid nitrogen. Transfer the suspension (tissue powder and liquid nitrogen) into an RNase free tube, useful for this temperature range. Allow the liquid nitrogen to evaporate, but do not allow the sample to thaw. Add Lysis Solution, and it is recommended to continue as quickly as possible with the homogenization using a shredder column or a 20 gauge needle.

Erythrocyte lysis (only in case of processing whole blood samples)
The removal of the erythrocytes allows the RNA purification from the leukocytes. Human blood collected from healthy people contains approximately 1000 times more erythrocytes than leukocytes while only the latter one contain RNA. Due to the increased susceptibility of the erythrocytes to osmotic shock lysis by addition of a hypotonic buffer system the whole blood sample is directly charged with **Buffer EL** and thoroughly mixed. According to the special buffer conditions, the erythrocytes are selectively lysed while the leukocytes remain.

Procedure

Lysis
After complete removal of any supernatant, erythrocytes or culture medium or after complete disruption, the samples are directly charged with the **DTT-containing Lysis Solution TR** and thoroughly mixed. Shake the **Lysis Solution TR** to resuspend 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 intramolecular disulfide bridges.

Binding and removal of DNA
In general, DNase digestion is not necessary to remove the genomic DNA. Under lysis conditions the DNA is efficiently bound by the mineral carrier particles contained in **Lysis Solution TR**. The particle-bound DNA is removed from the solution by subsequent centrifugation. However, RNA applications that show an increased sensitivity to contaminations of DNA (e.g. caused by overloading the kit) require an optional DNase digestion. If the DNase digestion is carried out while running RNA purification protocol the DNase will be removed completely in the washing steps of the RNA-binding **RTA Spin Filter** (see supplemental protocols)

Binding total RNA
After removal of the genomic DNA from the lysate by removal of the DNA-binding mineral carrier particles the total RNA is found in the supernatant. To adjust the RNA-binding conditions 96 - 100% ethanol is added. Following carefully mixing the solution is completely transferred onto the RNA-binding **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.

Elution
Total RNA is eluted from the membrane using **Elution Buffer R** (or RNase free water). The eluted RNA is ready for 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 6). Do not use damaged kit components, since their use may lead to poor kit performance.

- Always change pipet tips between liquid transfers. To avoid cross-contamination, 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

1. Preparing buffers

50 total RNA-extractions:
Adjust **Lysis Solution TR** with 1/100 Volume of 1 M DTT*. Add 20 ml 96-100% ethanol to the bottle **Wash Buffer R1**. Add 48 ml 96-100% ethanol to each bottle **Wash Buffer R2**. Add 30 ml **Buffer EL Concentrate** to 970 ml H_20. Label the bottle with **Buffer EL.** Store **Buffer EL** at 4°C.
250 total RNA-extractions:
Adjust **Lysis Solution TR** with 1/100 Volume of 1 M DTT*. Add 80 ml 96-100% ethanol to the bottle **Wash Buffer R1**. Add 160 ml 96-100% ethanol to each bottle **Wash Buffer R2**. Add 30 ml **Buffer EL Concentrate** to 970 ml H_20. Label the bottle with **Buffer EL**. Store **Buffer EL** at 4°C.

* It is possible to replace DTT by β-Mercaptoethanol. Adjust Lysis Solution TR with 1/100 volume of β-Mercaptoethanol.

2. DTT

Adjust **Lysis Solution TR** 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 TR** with **DTT** in case of the kits with 50 and 250 preparations. We recommend the preparation of a volume **DTT-containing Lysis Solution TR** shortly before carrying out the purifications adapted to the number of samples that will be processed. Store the remaining **Lysis Solution TR** and **DTT** separately in accordance to the storage instructions (see “Storage”, Page 4).

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

3. Lysis Buffer TR

Shake **Lysis Solution TR** 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 TR** are stable at - 80°C for several months.

Important tips

Blood and body fluids of all human subjects are considered potentially infectious. All necessary precautions recommended by the Food & Drug Administration (in the USA), the Bundesseuchen-gesetz (in Germany), or the appropriate regulatory authorities in the country of use, should be taken when working with whole blood.

The maximum amount of human whole blood that can be processed (1.5 ml) has been determined for blood from healthy adults (approximately 4.000–7.000 leukocytes per microliter). Reduce amount appropriately if using blood with elevated numbers of leukocytes. A maximum of 1 x 10^7 leukocytes can be processed on a RTA Spin Filter. After erythrocyte lysis, all steps of this protocol should be performed at room temperature (RT), as quickly as possible.

Homogenized cell lysates from step 2 can be stored lysed under Lysis **Solution TR** at –70°C for several months. Frozen lysates should be incubated at 37°C in a water bath until completely thawed and salts are dissolved shortly prior isolation. Avoid prolonged incubation, which may compromise RNA integrity. Continue with step 3. The use of frozen leukocyte pellets (without addition of **Lysis Solution TR** is not recommended.

Frozen whole blood must not be used.

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 (≥ 12.000 rpm)
- Ethanol (96-100%)
- Tubes for erythrocytes lysis (e.g. 15 ml Falcon Tubes)
- Sterile; RNase-free pipet tips
- Disposable gloves
- DTT

Possible microcentrifuge suppliers:

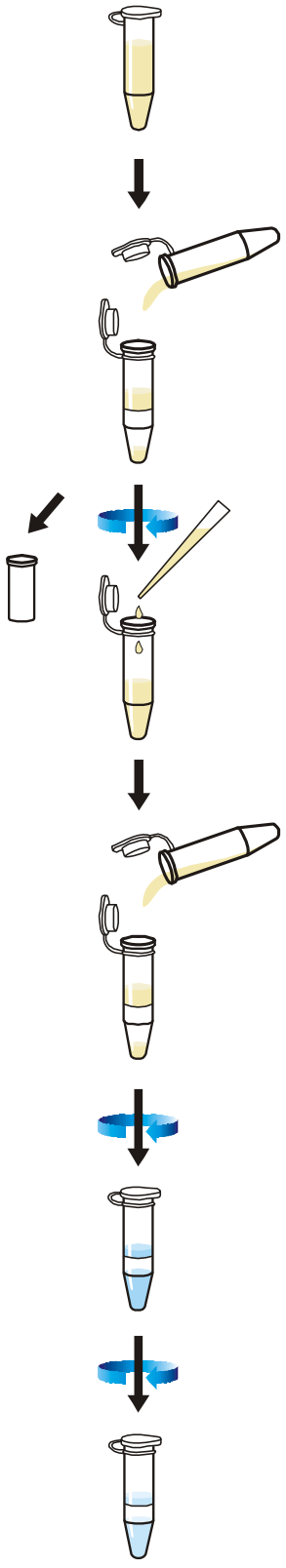
Eppendorf AG
22331 Hamburg, Germany
Phone: +49 (0) 40 53801 0
Fax: +49 (0) 40 53801 556
E-Mail: eppendorf@eppendorf.com
Internet: www.eppendorf.com

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

Instructions

Scheme: Total RNA extraction from human and animal cell culture

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 TR** by shaking and mix it with **DTT**.

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

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

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 1 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)

transfer the sample on the RNA-RTA Spin Filter, incubate for 1 min and centrifuge for 2 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 30 - 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!

Sample quantity

The maximal amount of pure total RNA that will be isolated depends on the applied starting material, its age, 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 this 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 Universal RNA Mini Kit** and avoid overloading the RNA-binding membrane an RNA-purification experiment with variation of the amount of starting material is recommended.

The RNA-binding membranes have a maximum binding capacity of approx. 100 µg.

The **InviTrap® Spin Universal RNA Mini Kit** purification procedure is optimized for the use of max. 1 x 10^7 human or animal cells.

Average yield of total RNA in dependence on the type of cell line using the InviTrap® Spin Universal 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
NIH3T3	human	6.5

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.

Important indications

The following notes are valid for all protocols:

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

Protocol 1: Total RNA extraction from cell culture

1. Harvesting cells (see also "Sample quantity", page 14)

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.

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 TR** (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 TR	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 TR** 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 TR	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 TR 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 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. (or use the DNA-Binding Spin Filter for simultaneous DNA isolation (see page 37).

4. Adjust RNA binding conditions

Add 250 µl (< 5 x 10^6 cells) or 500 µl (5 x 10^6 - 1 x 10^7 cells) 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 1 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

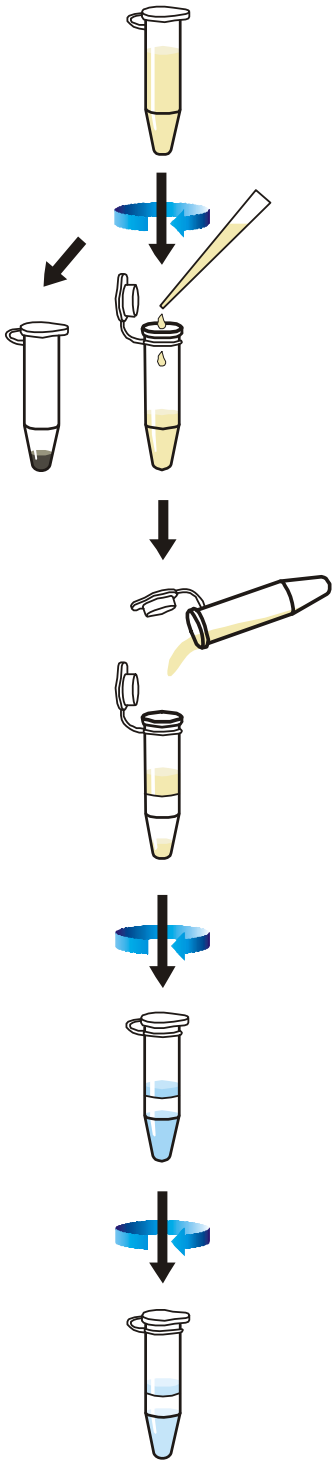
Discard the flow-through and put the RNA-RTA Spin Filter back to the RTA Receiver Tube. To eliminate any traces of ethanol, centrifuge for 4 min at maximum speed. Discard the RTA Receiver Tube.

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.

Scheme: Total RNA extraction from up to 20 mg tissue

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 TR** by shaking and mix it with **DTT**.

transfer the sample into an appropriate vial

add 600 µl **DTT-containing Lysis Solution TR** (in case of spleen, kidney, or lung tissue add 900 µl **Lysis Solution TR**)

homogenize completely the tissue sample using Zirconia beads

transfer the lysate in a 2 ml Receiver Tube
incubate the sample for an appropriate time under continuously shaking

centrifuge for 2 min at maximum speed in a microcentrifuge

transfer 500 µl of the supernatant carefully into a new 2 ml Receiver Tube and discard the DNA binding mineral particles to adjust the RNA binding conditions add 330 µl of 96 - 100 % **ethanol** to the supernatant and mix thoroughly by pipetting up and down

transfer the lysate completely to the RTA Spin Filter Set, incubate for 1 min and centrifuge for 2 min at 11.000 x g (11.000 rpm)

discard the flow-through and place the RTA Spin Filter back into the RTA Receiver Tube

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

pipet 700 µl **Wash Buffer R2** onto the RTA Spin Filter
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!
discard the flow-through and reuse the RTA Receiver Tube

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

transfer the RTA Spin Filter into the RNase-free Elution Tube
pipet 30 - 60 µl of **Elution Buffer R** onto the membrane of the RTA Spin Filter, incubate for 2 min and centrifuge for 1 min at 11.000 x g (11.000 rpm)

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

Influence of the different methods of sample homogenization on the yield using the InviTrap® Spin Universal RNA Mini Kit with rat brain

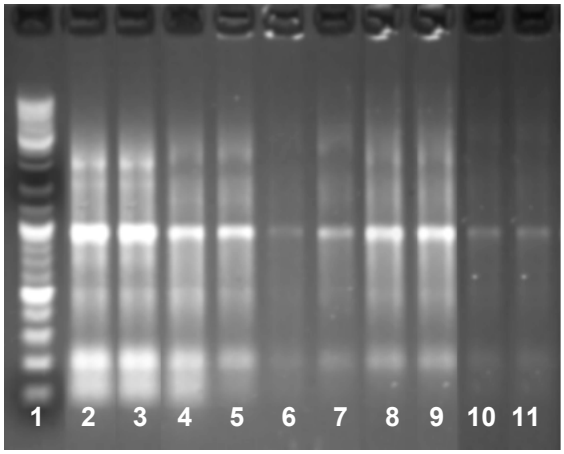
Total RNA was isolated from around 20 mg rat brain using the **InviTrap® Spin Universal RNA Mini Kit** in combination with different method for the sample homogenization, see below the comparison of the yield.

Table 1: Method of homogenization	RNA yield from rat brain in % in comparison to the homogenization using Gyrator+ Zirconia Beads (100%)
Vortex + Zirconia Beads	97.0%
Gyrator without Zirconia Beads	94.7%
Vortex	95.1 %
Mortar/ Pestle and liquid N_2	71.0%

Table 1 shows an example of one kind of tissue material following the theoretical predictions. In other tissues it may be different. The best method for disruption and homogenization depends on the kind of tissue used. The tissues differ in cell size, cell shape, DNA/RNA and protein contents as well as cell components. Also several tissue materials are very rigid like aorta, cartilage and bones etc. These materials obviously differ in behavior during homogenization from tissues like for example liver etc.

Brain tissue in several regions contains a lot axonal material, which has no nucleus respectively no DNA. A homogenization always is influenced by the DNA contents of the sample.

Therefore, here another example of the influence of different homogenization methods with liver tissue is shown below (fig. 1, table 2). Liver tissue contains very high contents of DNA and RNA and is simple to disrupt, so that here a treatment with mortar and pestle disrupt this material completely. During the further lysis step under shaking, the InviTrap RNA kit specific carrier material in the Lysis Solution shears efficient the high-molecular weight cellular components and create a homogeneous lysate, so that the RNA yield is also high when mortar and pestle will be used.



Total RNA was isolated from around 20 mg rat liver each using the **InviTrap® Spin Universal RNA Mini Kit** in combination with different method for the sample homogenization.

Lane 1 marker
Lane 2,3 homogenization with mortar and pestle under liquid nitrogen (A)
Lane 4,5 homogenization using zirconia beads on a Gyrator* (similar to a mixer mill) (B)
Lane 6,7 homogenization using a Gyrator* (C)
Lane 8,9 homogenization using zirconia beads on a vortex (D)
Lane 10,11 homogenization using a vortex (E)

(fig. 1)

The direct comparison shows that best result will be realized by using the mortar and pestle to homogenize the sample under liquid nitrogen for this tested sample.

Table 2: Method of homogenization	RNA yield from rat liver in % in comparison to the homogenization using mortar/ pestle (100%) and liquid N_2
Gyrator/Bead Mill + Zirconia Beads	90.5 %
Gyrator/Bead Mill	45.7 %
Vortex + Zirconia Beads	64.5 %
Vortex	39.1 %

Protocol 2: Total RNA extraction from up to 20 mg tissue

Important: *For extraction of total RNA from spleen, kidney, or lung tissue please use protocol 3.*

1. Disruption and lysis of the starting material (see page 9)

It is possible to use a commercially available rotor-stator homogenizer or bead mills for the disruption of the starting material or to disrupt the starting material using mortar and pestle in liquid nitrogen and grind the tissue sample to a fine powder.

Note: *Incomplete disruption and homogenization will lead to significantly reduced yields, and can cause clogging of the RTA spin column. Homogenization with a bead mill etc. generally results in higher RNA yields than with other methods.*

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

Disruption using a rotor-stator homogenizer/ vortexer / bead mill /gyrator

1. Transfer the weighed amount of fresh or frozen starting material in a suitable reaction vesicle for the homogenizer (not provided) and add about 10 Zirconia beads to the sample (Zirconia Beads I (small) : Zirconia Beads II (big) ratio = 2 :1)
2. Add 600 µl **DTT- containing Lysis Solution TR** (mixed before added).
3. Homogenize the sample.
4. Transfer the sample into the 2 ml Receiver Tube and place the sample under **Lysis Solution TR** for longer storage at –20°C or use the sample immediately for isolation of total RNA following the protocol step 2.

Disruption of the starting material using a mortar and pestle and liquid nitrogen

1. Transfer the weighed amount of fresh or frozen starting material under liquid nitrogen and grind the material to a fine tissue powder.
2. Transfer the powder into the 2 ml Receiver Tube
   Do not allow the sample to thaw!
3. Add 600 µl DTT- containing **Lysis Solution TR** (mixed before added) and incubate the sample for an appropriate time under continuously shaking until a homogenic lysate is visible
4. Finally place the sample under **Lysis Solution TR** for longer storage at –20°C or use the sample immediately for isolation of total RNA following protocol step 2.

Note: *To maximize the final yield of total RNA a complete disruption of tissue sample is important! Traces of DNA in the sample may be sheared by passing it through a 20 gauge needle. This may increase the lysis efficiency and the yield.*

2. Removal of genomic DNA and of beads

Centrifuge the 2 ml Receiver Tube containing the tissue lysates for 2 min at maximum speed in a microcentrifuge and transfer 500 µl of the supernatant carefully into a new 2.0 ml Receiver Tube. Add 330 µl 96 -100 % **ethanol** and mix thoroughly by pipetting up and down.
It is important to mix the lysate completely with ethanol.

3. Binding of the total RNA to the RTA Spin Filter

Transfer the lysate completely to the RTA Spin Filter Set. Incubate for 1 min and centrifuge for 2 min at 11.000 x g (11.000 rpm). Discard the flow-through and place the RTA Spin Filter back into the RTA Receiver Tube.

4. First washing of the RTA Spin Filter

Add 600 µl Wash Buffer R1 onto the 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. Transfer the RTA Spin Filter in a new RTA Receiver Tube.

5. Second washing of the RTA Spin Filter

Add 700 µl **Wash Buffer R2** onto the 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! Discard the flow-through and reuse the RTA Receiver Tube.

6. Drying of the RTA Spin Filter

To eliminate any traces of ethanol, centrifuge for 4 min at maximum speed. Discard the RTA Receiver Tube.

7. Elution of total RNA

Transfer the RTA Spin Filter into a RNase free Elution Tube and add 30 - 60 µl of **Elution Buffer R** directly onto the membrane of the RTA Spin Filter. Incubate for 2 min and centrifuge for 1 min at 11.000 x g (11.000 rpm). Discard the RTA Spin Filter and place the eluted total RNA immediately on ice.

Note: *Depending on the yield or the needed concentration of total RNA with different volumes of **Elution Buffer R** can be used. A lower volume of **Elution Buffer R** will increase the concentration of RNA and a higher volume of **Elution Buffer R** will lead to an increased yield but a lower concentration of total RNA. Please note, that the minimum of **Elution Buffer R** should be 30 µl.*

Protocol 3: Total RNA extraction from up to 20 mg spleen, kidney or lung tissue

1. Disruption and lysis of the starting material (see page 9)

It is possible to use a commercially available rotor-stator homogenizer or bead mills for the disruption of the starting material or to disrupt the starting material using mortar and pestle in liquid nitrogen and grind the tissue sample to a fine powder.

Note: *Incomplete disruption and homogenization will lead to significantly reduced yields, and can cause clogging of the RTA spin column. Homogenization with a bead mill etc. generally results in higher RNA yields than with other methods.*

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

Disruption using a rotor-stator homogenizer /vortexer/ bead mill /gyrator

1. Transfer the weighed amount of fresh or frozen starting material in a suitable reaction vesicle for the homogenizer (not provided) and add about 10 Zirconia beads to the sample (Zirconia Beads I (small) : Zirconia Beads II (big) ratio = 2 :1)
2. Add 900 µl **DTT- containing Lysis Solution TR** (mixed before added).
3. Homogenize the sample.
4. Transfer the sample into the 2 ml Receiver Tube and place the sample under **Lysis Solution TR** for longer storage at –20°C or use the sample immediately for isolation of total RNA following the protocol step 2.

Disruption of the starting material using a mortar and pestle and liquid nitrogen

1. Transfer the weighed amount of fresh or frozen starting material under liquid nitrogen and grind the material to a fine tissue powder.
2. Transfer the powder into the 2 ml Receiver Tube
   Do not allow the sample to thaw!
3. Add 900 µl **DTT- containing Lysis Solution TR** (mixed before added) and incubate the sample for an appropriate time under continuously shaking until a homogenic lysate is visible
4. Finally place the sample under **Lysis Solution TR** for longer storage at –20°C or use the sample immediately for isolation of total RNA following protocol step 2.

Note: *To maximize the final yield of total RNA a complete disruption of tissue sample is important! DNA in the sample may be sheared by passing it through a 20 gauge needle. This may increase the lysis efficiency and the yield.*

2. Removal of genomic DNA

Centrifuge the reaction tubes containing the tissue lysates for 2 min at maximum speed in a microcentrifuge and transfer the supernatant (app. 800 µl) carefully into a 2.0 ml Receiver Tube (not provided). Add 500 µl 96 -100 % ethanol and mix thoroughly by pipetting up and down. It is important to mix the lysate completely with ethanol.

3. Binding of the total RNA to the RTA Spin Filter

Transfer app. 750 µl of the lysate to a RTA Spin Filter. Incubate for 1 min and centrifuge for 2 min at 11.000 x g (11.000 rpm). Discard the flow-through and place the RTA Spin Filter back into the Receiver Tube. Reload the RTA Spin Filter with the residual volume of lysate and centrifuge again for 1 min. Discard the flow-through and place the RTA Spin Filter back into the Receiver Tube.

4. First washing of the RTA Spin Filter

Add 600 µl **Wash Buffer R1** onto the 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. Transfer the RTA Spin Filter in a new RTA Receiver Tube.

5. Second washing of the RTA Spin Filter

Add 700 µl **Wash Buffer R2** onto the 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. Discard the flow-through and reuse the RTA Receiver Tube.

6. Drying of the RTA Spin Filter

To eliminate any traces of ethanol, centrifuge for 4 min at maximum speed. Discard the RTA Receiver Tube.

7. Elution of total RNA

Transfer the RTA Spin Filter into a RNase free Elution Tube and add 30 - 60 µl of **Elution Buffer R** directly onto the membrane of the RTA Spin Filter. Incubate for 2 min and centrifuge for 1 min at 11.000 x g (11.000 rpm). Discard the RTA Spin Filter and place the eluted total RNA immediately on ice.

Note: *Depending on the extracted yield or the needed concentration of total RNA different volumes of **Elution Buffer R** can be used. A lower volume of **Elution Buffer R** will increase the concentration of RNA and a higher Volume of **Elution Buffer R** will lead to an increased yield but a lower concentration of total RNA. Please note, that the minimum of **Elution Buffer R** should be 30 µl.*

Protocol 4: Total RNA extraction from paraffin embedded tissue treated with formalin (paraffin slices)

Starting material: Paraffin-embedded tissue

Deparaffination:

1. Transfer the starting material into a 1.5 ml reaction tube (not provided). Add 0.5 ml **Octane** or **Xylene** and vortex carefully to dissolve the paraffin.
2. Centrifuge for 2 min at maximum speed to pellet down the tissue sample. Discard the supernatant very careful. This step should be repeated if any paraffin is still remained in the sample. Follow the dissolution until the tissue sample looks transparent (while paraffin is still white). A final washing step with 96 –100% **ethanol** is recommended, and then dry the sample.
3. Centrifuge shortly and remove of the ethanol by aspiration with pipette. Then incubate the open reaction tube at 52°C to evaporate the residual ethanol.
4. Do a lysis step with **Proteinase K**. Add 10 µl **Proteinase K** (40 mg / ml) and 90 µl of RNase free **TE-Buffer** under the presence of 10 mM **DTT** to the sample, mix completely by pipetting up and down and incubate at 48°C for 10 min. Mechanical grinding or a cutting of the material is recommended before or during lysis.

   The amount of proteinase depends on the proteinase used in the protocol, this protocol is giving an example, also consider the manufacturers recommendations.

5. 10 minutes incubation under continuously agitation at 80°C of the mixture partially reverses formalin crosslinking of the released nucleic acids. This improves RNA yield and quality as well as RNA performance in downstream enzymatic assays.

6. **The sample then has to be used complete (the liquid and the solid phase) <u>as sample in step 1, protocol 2.</u>**

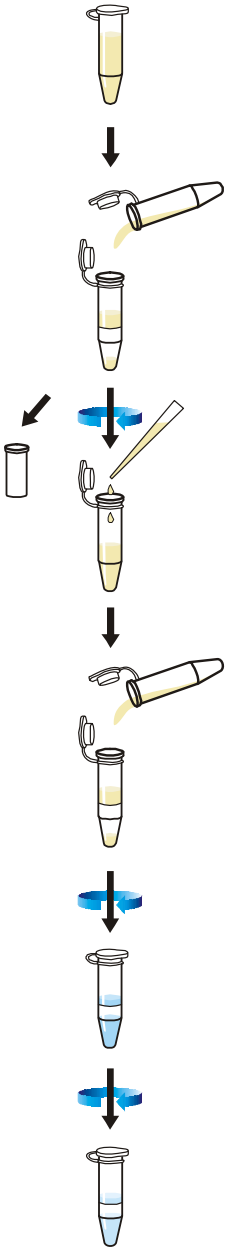
Depending of the fixation in several cases gives no useable RNA we just can give several advice:

Following advices can be given:

Non-pH adjusted formalin will lead to no RNA, the same problem occurs, if the sample was not fresh or was not stored correctly before the fixation procedure. Also incorrect fixation of the sample, for example residual water in the sample, leads to no RNA. Incorrect storage of the sample, for example high temperatures, humid surrounding lead to the same result.

Scheme: Total RNA extraction from up to 1.5 ml whole blood

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!

invert the blood sample 15 times, don't vortex !!
add 10 ml pre cooled (4°C) **Buffer EL** to 0.5 – 1.5 ml of whole blood in a 15 ml Tube (not provided), invert
incubate on ice for 15 min, invert 2 times during incubation
centrifuge 960 x g (3.000 rpm), 5 min, 4°C and remove the supernatant
add 5 ml **Buffer EL** to the pellet of leukocytes, mix by snipping with the finger and centrifuge again 5 min. at 960 x g (3.000 rpm) at 4°C, remove the supernatant that the white pellet is left

Note: Homogenize the carrier in the **Lysis Solution TR** by shaking and mix it with **DTT**.

resuspend the cell pellet in 900 µl **Lysis Solution TR**
pipet several times, transfer the sample into a 2 ml Receiver Tube, incubate for 5 min, vortex several times during incubation
centrifuge for 1 min. at 11.000 x g (11.000 rpm)

transfer the supernatant into a new 2 ml Receiver Tube
add 750 µl 96-100 % **ethanol** to the supernatant and mix by pipetting up and down

transfer 800 µl onto the 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 place the RTA Spin Filter back into the RTA Receiver Tube

transfer the residual sample into the same RTA Spin Filter and centrifuge again for 1 min at 11.000 x g (11.000 rpm)
discard the flow-through and place the RTA Spin Filter back into the RTA Receiver Tube

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

pipet 700 µl **Wash Buffer R2** onto the 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 RTA Spin Filter into a RNase free Elution Tube
pipet 30 - 60 µl of **Elution Buffer R** onto the membrane of the RTA Spin Filter, incubate for 2 min and centrifuge for 1 min at 11.000 x g (11.000 rpm)

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

Starting amounts of samples

It is essential to use the correct amount of starting material in order to obtain optimal result.
A maximum amount of 1.5 ml whole blood from healthy adults (typically 4.000-7.000 leukocytes per µl) can be processed on a single RTA spin column. For blood with elevated numbers of leukocytes, less than 1.5 ml must be used.
The maximum number of leukocytes that can be processed is 1 x 10^7 per spin column. If more leukocytes are processed, they will not be fully lysed and contaminants will not be completely removed, even if the volume of **the Lysis Solution TR** is increased.

Maximum RNA yields using the InviTrap® Spin RNA Kits are generally determined by two criteria: lysis volume and binding capacity of the spin column, Using the maximum amount of leukocytes that can be processed in the procedure (1 x 10^7), however, the binding capacity of the RTA Spin Filter is not usually attained due to the low RNA content of leukocytes

Note: *If the RNA binding capacity of the RTA Spin Filter is exceeded, yields of total RNA will not be consistent and less than the maximum possible total RNA may be recovered. If the starting material is incompletely lysed, the yield of total RNA will be lower than expected even if the binding capacity of the RTA Spin Filter is not exceeded. Yields can also vary due to the developmental stage, growth conditions etc of the sample source.*

Lysis of whole blood

Blood cells are lysed in two separate procedures, erythrocyte lysis and leukocyte lysis. Erythrocytes (red blood cells) of human blood do not contain nuclei and are therefore not important for RNA isolation since they do not synthesize RNA. The target of RNA isolation from whole blood is leukocytes (white blood cells), which are nucleated and therefore synthesize RNA. Leukocytes consist of 3 main cell types: lymphocytes, monocytes, and granulocytes.

Erythrocyte lysis

The removal of the erythrocytes allows the RNA purification from pure leukocytes. Blood of a healthy human contains approximately 1000 times more erythrocytes than leukocytes while only the latter one contain RNA of interest. Due to the increased susceptibility of the erythrocytes to osmotic shock lysis by addition of a hypo tonic buffer system the whole blood sample is directly charged with **Buffer EL** (provided in the kit) and thoroughly mixed. According to the special buffer conditions the erythrocytes are selectively lysed while the leukocytes still remain. Intact leukocytes are then recovered by centrifugation.
The conditions for selective lysis of erythrocytes in the InviTrap® procedure have been optimized to allow fast removal of erythrocytes without affecting the stability of the leukocytes. The erythrocyte lysis procedure can be scaled up for volumes of whole blood >50 µl.

A common alternative to erythrocyte lysis is Ficoll® density-gradient centrifugation. In contrast to erythrocyte lysis procedures, Ficoll density-gradient centrifugation only recovers mononuclear cells (lymphocytes and monocytes) and removes granulocytes. Mononuclear cells isolated by Ficoll density-gradient centrifugation can be processed with the **InviTrap® Spin Universal RNA Mini Kit**.

The erythrocyte lysis and Ficoll density-centrifugation procedures both rely upon intact blood cells, so fresh blood must be used.

Leukocyte lysis

After complete removal of the erythrocytes by centrifugation, the leukocytes are resuspended with the **DTT-containing Lysis Solution TR** and thoroughly mixed. Shake the **Lysis Solution TR** to resuspend the mineral carrier particles prior to use. Due to the highly denaturing lysis conditions leukocytes are quickly broken and RNases are inactivated simultaneously, allowing isolation of intact RNA the RNA is secured. **DTT** is added to inactivate the RNases by cleaving intramolecular disulfide bridges.

Isolation of total cellular RNA from leukocytes requires efficient disruption of cells and homogenization of lysate for optimal yield and purity. Traditional homogenization methods usually require a rotor–stator homogenizer or a syringe and needle. However, the **InviTrap® Spin Universal RNA Mini Kit** includes the carrier material in the **Lysis Solution TR**. The sample will be incubated with **Lysis Solution TR** 10 min shaking continuously. This treatment allows fast and simple homogenization of cell lysates without risk of cross-contamination.

Protocol 5: Total RNA isolation from 0.5 ml up to 1.5 ml whole blood

1. Erythrocyte lysis

Invert carefully the fresh sample minimum 15 times until all is one homogenous fraction. Do not vortex! Add 10 ml of cold (4°C) prepared **Buffer EL** to 0.5 ml – 1.5 ml of whole blood (max. 1x 10^7 leukocytes) in a 15 ml tube (e.g. 15 ml Falcon Tubes, not provided). Mix shortly but completely by inverting. Do not vortex!!!

Incubate on ice for 15 min. Mix briefly by inverting 2 times during incubation.

Note: *The cloudy suspension becomes translucent during incubation, indicating lysis of erythrocytes. If necessary, incubation time can be extended to 20 min.*

Please note, if you work with a fresh blood sample (up to 3 hours after taking of the sample ***extend the lysis time of erythrocytes to 45 min****. Please note that up to 1.5 ml of whole blood can be processed. If the expected amount of leukocytes is more than 1 x 10^7, reduce starting volume of the blood ample.*

Centrifuge at 960 x g (3.000 rpm) for 5 min at 4°C, remove the supernatant completely, but very carefully (leukocytes will form a visible cell pellet).

Note*: Leukocytes will form a pellet after centrifugation. Ensure that supernatant is completely removed e.g. by aspiration. Trace amounts of erythrocytes, which give the pellet the red tint, will be eliminated in the following wash steps.*

Add 5 ml of the cooled **Buffer EL** to the cell pellet, mix by snipping with the finger, and centrifuge again for 5 min at 960 x g (3.000 rpm) at 4°C. Remove the supernatant as complete as possible, including the red interface that only the small white pellet is left.

Note: *Incomplete removal of the supernatant will interfere with lysis and subsequent binding of RNA to the RTA spin column, resulting in lower yield and purity.*

All following steps were perform quickly and at RT

2. Leukocyte disruption

Important: *Before starting step 2 vortex* ***Lysis Solution TR*** *to resuspend the mineral carrier for DNA removal. Do not forget to add DTT to the* ***Lysis Solution TR****. Be aware that DTT in buffer is stable for at least 4 weeks (see page 12).*

Add 900 µl **Lysis Solution TR** (containing DTT) to the cell pellet. Pipet several times to complete remove any clumps of cells.

Note: *Lysis Solution TR contains carrier to bind the genomic DNA. The carrier with the bound DNA looks gel like. Don't confound this with clumps (See in the section "Troubleshooting")*

3. Binding of genomic DNA to the Carrier

Transfer the cell lysis suspension into a 2 ml Receiver Tube. Vortex for 10 sec and incubate for 5 min, vortex 3 – 5 times during incubation. Centrifuge the sample for 1 min at 11.000 x g (11.000 rpm) (a jelly pellet will be visible). Transfer the supernatant into a new 2 ml Receiver Tube; avoid carry over of the mineral particles with bound DNA.

Note: *During the continuously shaking, the kit specific carrier material shears more efficient the high-molecular weight cellular components and create a homogeneous lysate during vortexing several times.*

4. Adjust RNA binding conditions

Add 750 µl 96 - 100 % **ethanol** to the supernatant. Mix the suspension by pipetting several times.

Note: *A precipitate may form after the addition of ethanol. This will not affect the InviTrap® procedure.*

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

Transfer 800 µl of the lysate into a RTA Spin Filter without moistening the rim. Incubate for 1 min at RT. Centrifuge for 1 min at 11.000 x g (11.000 rpm). Discard the flow-through and place the RTA Spin Filter back into the RTA Receiver Tube. Transfer the residual sample into the same RTA Spin Filter, incubate for 1 min and centrifuge again at 11.000 x g (11.000 rpm) for 1 min. Discard the flow-through and place the RTA Spin Filter back into the RTA Receiver Tube.

6. First washing of the RTA Spin Filter

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

7. Second washing of the RTA Spin Filter

Add 700 µl **Wash Buffer R2** onto the 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.

8. Drying of the RTA Spin Filter membrane

To eliminate any traces of ethanol from the RNA, centrifuge for 4 min at maximum speed. Discard the RTA Receiver Tube.

9. Elution of total RNA

Transfer the RTA Spin Filter into a RNase free Elution Tube and add 30-60 µl of **Elution Buffer R** directly onto the membrane of the RTA Spin Filter. Incubate for 2 min and centrifuge for 1 min at 11.000 x g (11.000 rpm). Discard the RTA Spin Filter and place the eluted total RNA immediately on ice.

Note: *Depending on the yield or the needed concentration of total RNA, you can also elute with different volumes of **Elution Buffer R**. A lower volume of **Elution Buffer R** increases the concentration of RNA and a higher volume of **Elution Buffer R** leads to an increased yield but a lower concentration of total RNA. Please note, that the minimum of **Elution Buffer R** should be 30 µl.*

Protocol 6: RNA extraction from buffy coat from up to 2 ml whole blood (max. 0.3 ml) or from < 1 x 10^7 leukocytes

1. Treatment of buffy coat

Add fresh prepared buffy coat with max. 1 x 10^7 leukocytes in a 1.5 ml RNase free Receiver Tube (not provided) and spin the leukocytes down at 3000 x g for 3 min. Remove carefully the supernatant and follow than the protocol 1 from step 2: Leukocyte disruption

2. Leukocyte pellet

A common alternative to erythrocyte lysis is Ficoll® density-gradient centrifugation. In contrast to erythrocyte lysis procedures, Ficoll density-gradient centrifugation only recovers mononuclear cells (lymphocytes and monocytes) and removes granulocytes. Mononuclear cells isolated by Ficoll density-gradient centrifugation can be processed with the **InviTrap® Spin Blood RNA Mini Kit** starting with step 2 from protocol 1. The content of leukocytes should not be higher than 1 x 10^7.

Note: *Lysis Solution TR contains carrier to bind the genomic DNA. The carrier with the bound DNA looks gel like. Do not confound this with clumps .If the lysate is difficult to pipet, too many DNA is bound. See in the section "Troubleshooting" to solve the problem.*

3. Frozen leukocyte pellet

Lysed leukocytes, stored under **Lysis Solution TR** at - 80°C can be used for RNA isolation. Follow protocol from step 3 in protocol 1.
Also shock frozen leukocyte pellets (liquid nitrogen) stored at –80°C may be used. Follow protocol from step 2 in protocol 1.

Important: ***Material frozen at –20°C cannot be used!***

Troubleshooting

Problem/ Probable cause	Comments and suggestions
clogged RTA Spin Filter	
too much starting material	inefficient lysis and homogenization of the starting material Increase g-force and centrifugation time in subsequent preparations, reduce the amount of starting material/ and/or increase volume of **Lysis Solution TR**
	after lysis spin lysate to pellet debris and continue with the protocol using the supernatant
	all centrifugation steps should be conducted at room temperature
incomplete erythrocyte lysis,	extend incubation on ice to 20 min.
	the leukocyte pellet should be white and may contain residual traces of erythrocytes. However, if erythrocyte lysis is incomplete, the white pellet may not be visible and large amounts of erythrocytes will form a red pellet. If this happens, incubate for an additional 5–10 min on ice after addition of **Buffer EL** a second time
very viscous lysate, like a gel after addition of Lysis Solution TR	
to much starting material	if too many leukocytes, cells or tissue sample including DNA have 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 900 µl with **Lysis Solution TR**. Continue with the procedure from DNA removal
little or no total RNA eluted	
incomplete removal of cell culture medium.	make sure that the cell culture medium is complete removed after the cell harvest
carryover of erythrocytes	see ”Incomplete erythrocyte lysis” above
insufficient disruption or homogenization	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 of **Elution Buffer R**.
	incubate RTA Spin Filter at room temperature (15 – 25°C) for 5 min with RNase free water prior to centrifugation.

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 suspension of DNA binding carrier.	Shake **Lysis Solution TR** 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 31) cell pellets stored at - 80°C for later processing should be immediately frozen after cell harvest by liquid nitrogen treatment.
age of blood or tissue sample	blood or tissue samples stored for too long prior to RNA isolation. See ”Sample storage” (page 9)
lysis buffer does not contain DTT	ensure that **DTT** has been added to the **Lysis Solution** TR
handling	ensure that the protocol, especially during the first few steps, has been performed quickly. See Appendix , ”General Notes on handling RNA” (page 31)
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 31)
total RNA does not perform well in downstream-applications (e.g. RT-PCR)	
Ethanol carryover during elution	perform the recommended additional centrifugation step in the protocol to remove all traces of ethanol before eluting. 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
low A_{260}/A_{280} value	
RNA sample is diluted in H_2O	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

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 plastic ware 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.
- This kit should only be used by trained personnel.

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 concentration of RNA should be determinate 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 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: 100 µl
- Dilution = 20 µl of RNA sample + 180 µl of 10 mM Tris/HCl pH 7.0 (1/10 dilution).
- Measure absorbance of diluted sample in a 0.2 ml cuvette (RNase free): 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 * 0.1 ml = 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.

* 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 photospectrometers.

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, and 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 0.5-3 x volumes Formaldehyde Load Dye**.**

To simply check the RNA on a denaturing gel, as little as 0.5 X Formaldehyde Load Dye can be used, but to completely denaturate the RNA, e.g. for Northern blots, use 3 X 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).

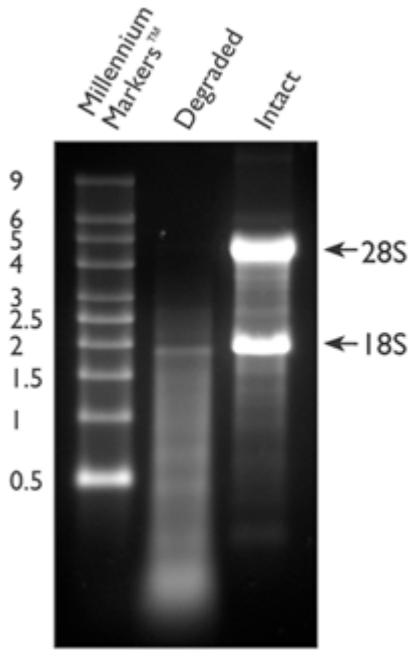


Figure 1. Intact vs. Degraded RNA. Two µg of degraded total RNA and intact total RNA were run beside Ambion's RNA Millennium Markers™ on a 1.5% denaturing agarose gel. The 18S and 28S ribosomal RNA bands are clearly visible in the intact RNA sample. The degraded RNA appears as a lower molecular weight 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 Universal RNA Mini Kit	50 preps	1060100200
InviTrap® Spin Universal RNA Mini Kit	250 preps	1060100300

Related products

InviTrap® Spin Cell RNA Mini Kit	250 preparations	1061100300
InviTrap® Spin Tissue RNA Mini Kit	250 preparations	1062100300

Supplemental not validated protocols, not for diagnostic use

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 Universal 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 RTA Spin Filter in the purification protocol (see *Protocol 1 +2*, step 3). 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 11.000 x g (11.000 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 4 min at maximum speed 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 Solutions, poisonous gas will be generated!!!***

Supplemental protocol for simultaneous isolation of RNA and DNA

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

Important Note: *The **Wash Buffer R1** and **R 2** as well 2.0 ml Receiver Tubes and 1.5 ml Elution Tubes for RNA purification provided with the kit are calculated for RNA isolation. The calculated amount does not include the needed buffers and plastic simultaneous DNA isolation. Please order additional Wash Buffers and tubes.*

Simultaneous DNA isolation from cells

See Protocol 1, Step 3.

Binding of 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. The eluate contains the total RNA.

Transfer the DNA Binding Spin Filter with the separated DNA bound to the carrier material into a new 2 ml Receiver Tube and start with the washing steps A, page 30.

Simultaneous DNA isolation from tissue samples

I) Protocol 2, step 2:

Removal of genomic DNA and of beads

Centrifuge the 2 ml Receiver Tube containing the tissue lysates at 11.000 x g (11.000 rpm) for 1 min in a microcentrifuge and transfer 500 µl of the supernatant carefully into a new 2.0 ml Receiver Tube for further RNA isolation. Remove the residual supernatant to waste. Add 600 µl **Wash Buffer R1** to the pellet and resuspend the pellet by pipetting up and down.

Put a DNA-Binding Spin Filter in a new 2 ml Receiver Tube. Transfer the resuspended DNA containing sample without the ziconia beads to the DNA-Binding Spin Filter and centrifuge for 1 min at 11.000 x g (11.000 rpm). Discard the flow-through and put the DNA-Binding Spin Filter back to the 2 ml Receiver Tube. Start with the washing steps B (page 30)

For big amounts of unlysed material:

which could clog the membrane the following procedure is recommended

II) Before starting Protocol 2, step 1

Please transfer 600 µl **DTT- containing Lysis Solution TR** (vigorously mixed before added) in a 2 ml Receiver Tube (not provided) and spin down the included carrier for 30 sec at 6.000 x g (8000 rpm). Store the carrier in the closed tube.

Use the carrier free **Lysis Solution TR** and proceed step 1 and 2 of the protocol 2.
Add 500 µl of the cleared supernant to the tube with the carrier and resuspend the carrier with the lysate.

Centrifuge the 2 ml Receiver Tube containing the tissue lysates with carrier for 2 min at maximum speed in a microcentrifuge and transfer the complete supernatant (wihtout carrier) carefully into a new 2.0 ml Receiver Tube. For DNA isolation, resuspend the carrier in 600 µl Wash Buffer R1 and follow from the Washing step A in the protocol below, page 30.

For RNA isolation use the supernatant and follow the protocol 2 step

3. Binding of the total RNA to the RTA Spin Filter, page 19.

Simultaneous DNA isolation from blood samples

Protocol 3 step 3. Binding of genomic DNA to the Carrier

Vortex the mixture from step 2 for 10 sec and incubate for 5 min at RT, vortex 3 – 5 times during incubation. Put a DNA-Binding Spin Filter in a new 2 ml Receiver Tube. 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. The eluate contains the total RNA.

Transfer the DNA-Binding Spin Filter with the separated DNA bound to the carrier material into a new 2 ml Receiver Tube and start with the washing steps A.

Washing Steps

Washing step A: First washing of the DNA-Binding Spin Filter

Add 600 µl **Wash Buffer R1** onto the DNA-Binding Spin Filter and centrifuge for 1 min at 11.000 x g (11.000 rpm). Discard the flow-through and the 2 ml Receiver Tube. Put the DNA-Binding Spin Filter in a new 2 ml Receiver Tube.

Washing step B: Second washing of the DNA-Binding Spin Filter

Add 700 µl **Wash Buffer R2** onto the DNA-Binding Spin Filter and centrifuge for 1 min at 11.000 x g (11.000 rpm). Discard the flow-through and reuse the 2 ml Receiver Tube.

Repeat this washing step once.

Drying of the DNA-Binding Spin Filter membrane

Discard the flow-through and put the DNA-Binding Spin Filter back to the 2 ml Receiver Tube. To eliminate any traces of ethanol, centrifuge for 4 min at maximum speed. Discard the 2 ml Receiver Tube.

Elution of genomic DNA

Transfer the DNA-Binding Spin Filter into a 1.5 ml Elution Tube and pipet 40-100 µl of **Elution Buffer R** or water directly onto the membrane of the DNA-Binding Spin Filter. Incubate for 2 min and centrifuge for 1 min at 11.000 x g (11.000 rpm). Discard the DNA-Binding Spin Filter and place the eluted genomic DNA in a refrigerator.

Supplemental protocol for digestion on the RTA Spin Filter

If you want to get a totally DNA free sample for RT-qPCR Applications or other protocols, where smallest amounts of DNA might disturb you may follow this protocol and do a DNase digestion directly on the Spin Filter**.**

Therefore, you have to change Step 7 in protocol 1 (Second washing of the RNA-RTA Spin Filter) according to the following procedure:

Add 600 µl **Wash Buffer R2** onto the RTA Spin Filter and centrifuge for 30 sec at 10.000 x g (10.500 rpm). Discard the flow through and put the 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 RTA Spin Filter at room temperature for 10 min.

Add 600 µl **Wash Buffer R2** onto the RTA Spin Filter incubate for 1 minute and centrifuge for 1 min at 11.000 x g (11.000 rpm). Reuse the Receiver Tube.

Repeat the second washing step once again.

Supplemental protocol for RNA “clean up” from Trizol aqueous phase

1. Starting the cleanup procedure

Add an equal volume of **DTT-containing Lysis Solution TR** to up to 350 µl of the Trizol aqueous phase in a 2 ml Receiver Tube. Mix thoroughly by pipetting up and down.

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

2. Binding of genomic DNA

Incubate the sample for 1 min and centrifuge at 11.000 x g (11.000 rpm) for 2 min.

3. Harvesting of supernatant

Pipet the supernatant into a new 2 ml Receiver Tube. Discard the Receiver Tube with the pellet.

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 RTA Spin Filter

Pipet the RNA containing sample resulting from step 4 directly onto the RTA Spin Filter. Incubate for 1 min and centrifuge at 11.000 x g (11.500 rpm) for 1 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 RTA Spin Filter.

6. First washing of the RTA Spin Filter

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

7. Second washing of the RTA Spin Filter

Add 700 µl **Wash Buffer R2** onto the 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. Discard the flow-through and reuse the RTA Receiver Tube.

8. Drying of the RTA Spin Filter membrane

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

Supplemental protocol for RNA “clean up” from liquids

1. Starting the cleanup-procedure for samples containing no DNA

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 TR** 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 TR). **Then continue with step 4**.

B) Purification of 200 µl sample from contaminations

Pipet 700 µl of **DTT-containing Lysis Solution TR** 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 200 µl of the sample (reaction mixture) to the flow-through (Lysis Solution TR). **Then continue with step 4**.

2. Starting the cleanup-procedure for samples containing contaminating DNA

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 TR** 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 TR** to 200 µl RNA sample. Mix thoroughly by pipetting up and down.

Important: ***Shake Lysis Solution TR 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 . 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.

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