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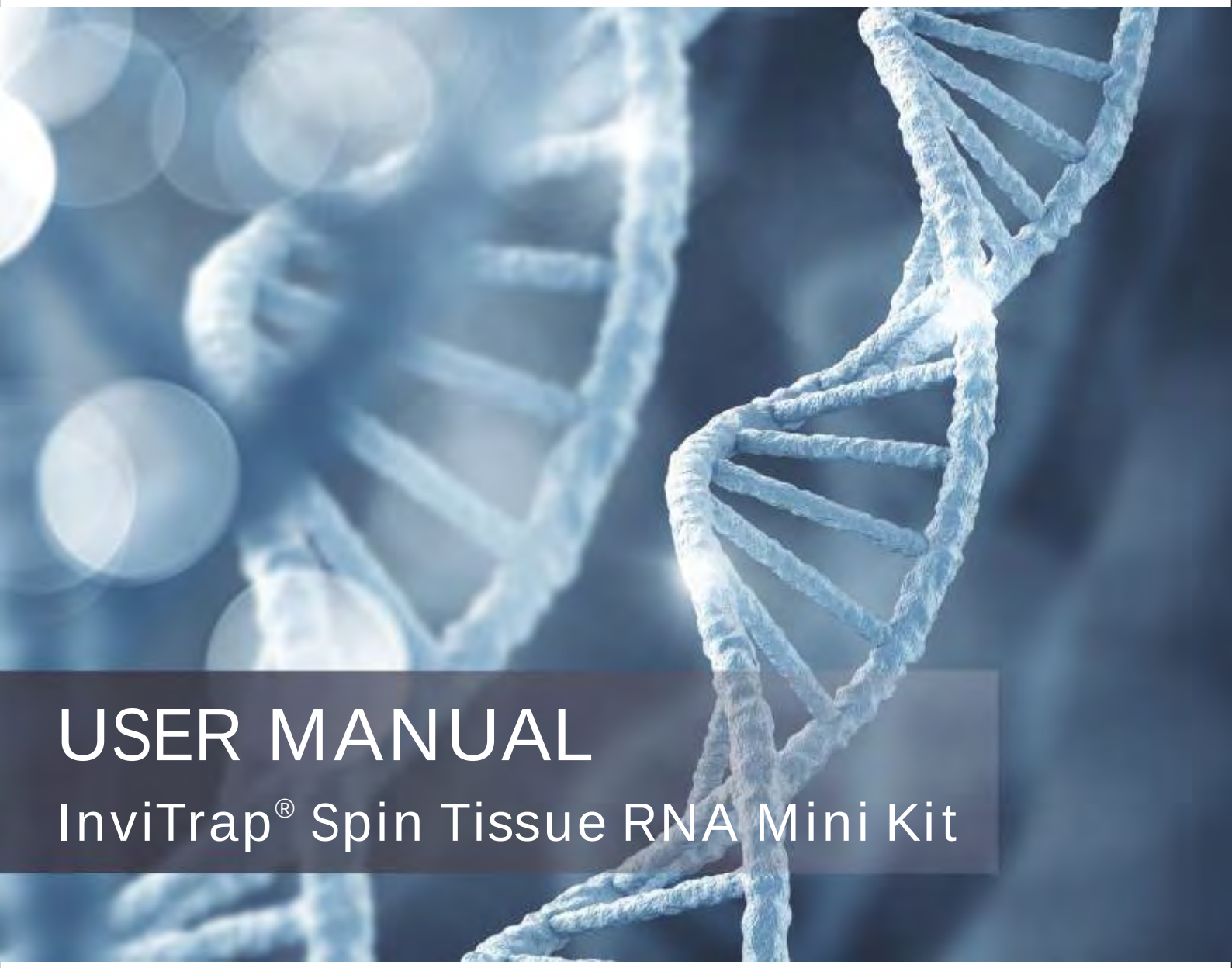


# USER MANUAL

## InviTrap® Spin Tissue RNA Mini Kit

for purification of total RNA from up to 20 mg fresh or frozen tissue sample, paraffin embedded tissue, RNA clean up and simultaneous protein isolation

CE IVD

REF 1062100300

Invitek Molecular GmbH · D-13125 Berlin

# Instruction for the InviTrap® Spin Tissue RNA Mini Kit

The **InviTrap® Spin Tissue RNA Mini Kit** provides a fast easy method for the preparation of high quality total cellular RNA from up to 20 mg of fresh or frozen tissue sample in a spin-filter format. Sources of tissue samples handled by the protocols in this manual are small amounts of various human and animal tissues (e.g. muscle, liver, heart, and brain), tissue sections from lung, spleen, or kidney, and paraffin embedded tissue samples. Multiple samples can be processed simultaneously in less than 1 hour. The kit can be used further for simultaneous isolation of total RNA and proteins as well as for RNA cleanup in Trizol® phases and for RNA isolation from paraffin- embedded tissue.

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.

A DNase-digestion step is not necessary.

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

The kit is neither validated for the isolation of total RNA from serum, plasma, 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 Tissue RNA Mini Kit 3
Symbols 4
Storage 4
Quality control 4
Intended use 5
Product use limitation 5
Safety information 6
Product characteristics of the InviTrap® Spin Tissue RNA Mini Kit 7
Principle and procedure 8
Sampling and storage of starting material 8
Influence of different methods of sample homogenization on the yield 10
Procedure 11
Lysis 11
Binding and removal of DNA 11
Binding total RNA 11
Removing residual contaminants 11
Elution 11
Important notes 12
Important points before starting a protocol 12
Preparing reagents and buffers 12
Equipment and reagents to be supplied by user 13
Scheme 14
Instructions 15
*Protocol 1:* Total RNA extraction from up to 20 mg of tissue 15
*Protocol 2:* Total RNA extraction from up to 20 mg of spleen, kidney, or lung tissue 17
*Protocol 3:* RNA “clean up” from Trizol aqueous phase 19
Supplemental protocol to advice RNA isolation from paraffin embedded tissue treated with formalin (paraffin slices) 20
Supplemental protocol for simultaneous isolation of RNA and proteins 21
Supplemental protocol for DNA digestion on the RTA Spin Filter 21
Troubleshooting 22
Appendix 24
General notes on handling RNA 24
Storage of RNA 25
Quantification of RNA 25
Purity of RNA 25
Ordering information 28

# Kit contents of the InviTrap® Spin Tissue RNA Mini Kit

| | **250 total RNA extractions** |
|---|---|
| **Catalogue No.** | 1062100300 |
| **Lysis Solution TR** | 250 ml |
| **Zirconia Beads I** | 5 |
| **Zirconia Beads II** | 5 |
| **Wash Buffer R1** | 80 ml<br>(final volume 160 ml) |
| **Wash Buffer R2** | 2 x 40 ml<br>(final volume 2 x 200 ml) |
| **Elution Buffer R** | 30 ml |
| **RTA Spin Filter Set** | 5 x 50 |
| **2.0 ml Receiver Tubes** | 10 x 50 |
| **RTA Receiver Tubes** | 5 x 50 |
| **Elution Tubes** | 5 x 50 |
| **Manual** | 1 |
| **Initial steps** | Add 80 ml 96 - 100% ethanol to the bottle **Wash Buffer R1**.<br>Add 160 ml 96 - 100% ethanol to each bottle **Wash Buffer R2.** |

## 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 Tissue RNA Mini Kit,** except the 1 M **DTT** solution (not provided)**,** should be stored at room temperature and are stable for at least 12 months.

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

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

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.

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

## Quality control and product warranty

Invitek Molecular warrants the correct function of the **InviTrap® Spin Tissue 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 Tissue 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 Tissue 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 www.invitek-molecular.com.

**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 Tissue RNA Mini Kit** is the ideal tool for reliable and fast manual isolation and purification of high quality total RNA from samples of up to 20 mg of fresh or frozen human and animal tissue sample. For reproducible and high yields an appropriate sample storage and quick operation under the rules for RNA operation is essential. The purified RNA is ready to use for *in vitro* diagnostic analysis only.

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, bacteria or yeast cells, nor for viruses. The performance of the kit in isolating and purifying total RNA from fecal samples has not been evaluated.

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

The included chemicals are only useable once.

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

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

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

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

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

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

The Product with its contents is unfit for consumption.

## Safety information

When and while working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles!

Avoid skin contact! Adhere to the legal requirements for working with biological material!

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

Invitek Molecular has not tested the liquid waste generated by the **InviTrap® Spin Tissue 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 Tissue 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

H302: Harmful if swallowed.
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 Tissue RNA Mini Kit

| Starting material | Yield | Time | Ratio |
|---|---|---|---|
| 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 |

The **InviTrap® Spin Tissue RNA Mini Kit** provides a fast and efficient way for reliable isolation of high quality total RNA from fresh or frozen human or animal tissue samples.

The **InviTrap® RNA isolation technology** simplifies total RNA isolation by combining efficient lysis of the starting material and inactivation of RNases with the efficient removal of genomic DNA with the speed and convenience of a spin technology. A specialized buffering system allows RNA species of sizes down to 200 base to bind to the Spin Filter membrane.

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 that immediately inactivate exogenous and endogenous RNases. The carrier material is also shearing high molecular components and reducing the viscosity during the lysis under permanent shaking. The out coming genomic DNA is fixed at the surface of mineral carrier particles provided within the **Lysis Solution TR** simultaneously to the cell lysis. Selective binding of the DNA is assured by optimized buffer conditions that reduce the undesired binding of the RNA to a minimum. The carrier particles loaded with the genomic DNA are removed by centrifugation. The RNA remains in the supernatant and is bound after adjustment of RNA binding conditions, subsequently onto the membrane of the RTA Spin Filter during a brief centrifugation step. Contaminants are removed by repeated wash steps and the purified total RNA can be eluted in a small volume of RNase free water. The isolated total RNA is ready to use and should be stored at - 80°C.

The particle size distribution of purified RNA is similar to those, gained using a CsCl - gradient. Due to the high purity, the isolated total RNA is ready to use for a broad panel of downstream applications like:

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

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

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

## Principle and procedure

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

1. disruption and homogenization of the starting material using beads of different size or other methods
2. cell lysis
3. selective binding of the genomic DNA to a specific carrier and separation of the DNA bound on the surface of the DNA binding mineral carrier particles by centrifugation
4. transfer of the supernatant into the RNA binding RTA Spin Filter, followed by the adjustment of the binding conditions
5. binding of the total RNA to the membrane, while proteins remain in the filtrate
6. washing of the membrane and elimination of contaminants and ethanol
7. elution of highly pure total RNA

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

This manual contains 3 protocols (+ 3 supplemental protocols) (see page 20 ff).

### Sampling and storage of starting material

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 25 $mm^2$ each 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 Tissue RNA Mini Kit** we recommend starting with one or 2 sections.

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 homogenization

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

### Disrupting and homogenizing of the tissue material are 2 distinct steps

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.

Some disruption methods simultaneously homogenize the sample, while others require an additional homogenization step.

Rotor–stator homogenizers and bead mills simultaneously disrupt and homogenize the tissue sample, whereas tissue are only disrupted using a mortar and pestle, and a separate homogenization step using a shredder spin column or a 20 gauge needle can be performed.

**Disruption and homogenization using rotor-stator homogenizer**

In the presence of **Lysis Solution TR**, rotor stator homogenizer thoroughly disrupt and simultaneously homogenize in 5 - 90 s, depending on the toughness of the sample. Often the use transparent disposables.

**Disruption and homogenization using a bead mill**

In bead-milling*, tissue can be disrupted by rapid agitation in the presence of beads and Lysis Solution. Disruption and simultaneous homogenization occur by the shearing and crushing action of the beads as they collide with the cells. Disruption efficiency is influenced by

- size and composition of beads
- ratio of buffer to beads
- amount of starting material
- speed of the mill
- disintegration time

A mixture of Zirconia beads with a diameter of 0.7 mm (Zirconia Beads I) and 2.4 mm (Zirconia Beads II) in a ratio in number of 2:1 Beads I : Beads II are optimal to use for tissue in combination with the **InviTrap® Spin Tissue 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 TR**, and it is recommended to continue as quickly as possible with the homogenization using a 20 gauge needle.

**This homogenization step will be supported in all InviTrap RNA Spin kits via the mineral carrier particles provided in the Lysis Solutions during the lysis step.**

*) Please refer to suppliers' guideline for further details

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

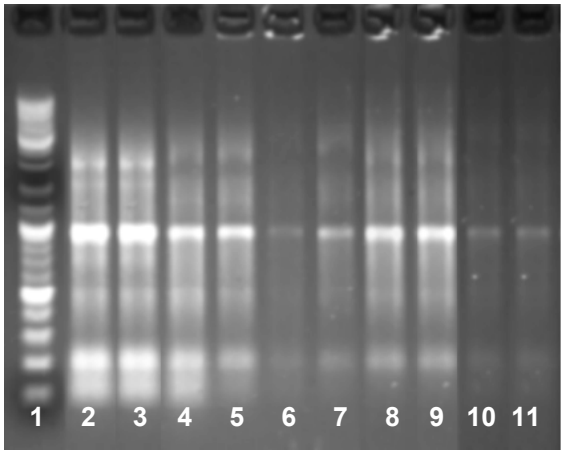
Total RNA was isolated from around 20 mg rat brain using the **InviTrap® Spin Tissue RNA Mini Kit** in combination with different method for the sample homogenization, see below the comparison of the yield.

| Table 1: Method of Homogenization | RNA yield from rat brain in % in comparison to the homogenization using Gyrator+ Zirconium Beads (100%) |
|---|---|
| Vortex + Zirconium Beads | 97.0% |
| Gyrator without Zirconium 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 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. In addition, 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 TR 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 Tissue 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 zirconium beads on a Gyrator* (similar to a mixer mill) (B)
Lane 6,7 homogenization using a Gyrator* (C)
Lane 8,9 homogenization using zirconium 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 and liquid $N_2$ |
|---|---|
| Gyrator/Bead Mill + Zirconium Beads | 90.5 % |
| Gyrator/Bead Mill | 45.7 % |
| Vortex + Zirconium Beads | 64.5 % |
| Vortex | 39.1 % |

## Starting amounts of samples

It is essential to use the correct amount of starting material in order to obtain optimal result.

For samples containing very high amounts of RNA, smaller amounts of starting material than shown in the kit protocol should be used in order to avoid exceeding the RNA binding capacity (It will be only possible by using more than 20 mg of these tissue samples).
For samples containing very low amounts of RNA, bigger amounts of starting material than shown in the kit protocol should be not used in order to avoid an inefficient lysis of the material.

**RNA binding RTA Spin Filter Specification**

| | |
|---|---|
| Maximum binding capacity | 100 µg nucleic acid, T 25°C |
| Maximum loading volume | 800 µl |
| Minimum elution volume | 30 µl |

***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.*

## Procedure

**Lysis**
After complete disruption and homogenization of the starting material, the cells 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**
By use of the Invitek Molecular principle, a 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 wash steps of the RNA binding RTA Spin Filter.

**Binding total RNA**
To adjust the RNA binding conditions 96 - 100% ethanol is added to the RNA containing supernatant. Following carefully mixing the solution is completely transferred onto the 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 30 - 60 µl **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-contaminations, we recommend the use of aerosol-barrier pipet tips.
- All centrifugation steps are carried out at room temperature.
- When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles.
- Discard gloves if they become contaminated.
- Do not combine components of different kits unless the lot numbers are identical.
- Avoid microbial contamination of the kit reagents.
- To minimize the risk of infections from potentially infectious material, we recommend working under laminar air-flow until the samples are lysed.
- This kit should only be used by trained personnel.

# Preparing reagents and buffers

## 1. Preparing buffers

| **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** |

***** 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 Solution TR

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

## Equipment and reagents to be supplied by user

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

- Microcentrifuge (≥ 13.500 x g (12.000 rpm))
- Ethanol (96-100%)
- Pipettes and filter tips
- DTT

Possible suppliers:

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

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

### Suppliers of equipment for disruption and homogenization

**Rotor–stator homogenizers can be purchased from:**

- BioSpec Products, Inc. (www.biospec.com): Tissue-Tearor™ homogenizer
- Charles Ross & Son Company (www.mixers.com)
- IKA (www.ika.de): ULTRA-TURRAX® dispersers
- KINEMATICA AG (www.kinematica.ch)
- Brinkmann Instruments, Inc.(www.brinkmann.com)
- POLYTRON® laboratory dispersing devices
- Omni International, Inc. (www.omni-inc.com)
- Silverson (www.silverson.com)
- VirTis (www.virtis.com)

**Bead-mill homogenizers and stainless steel and tungsten carbide beads can be purchased from:**

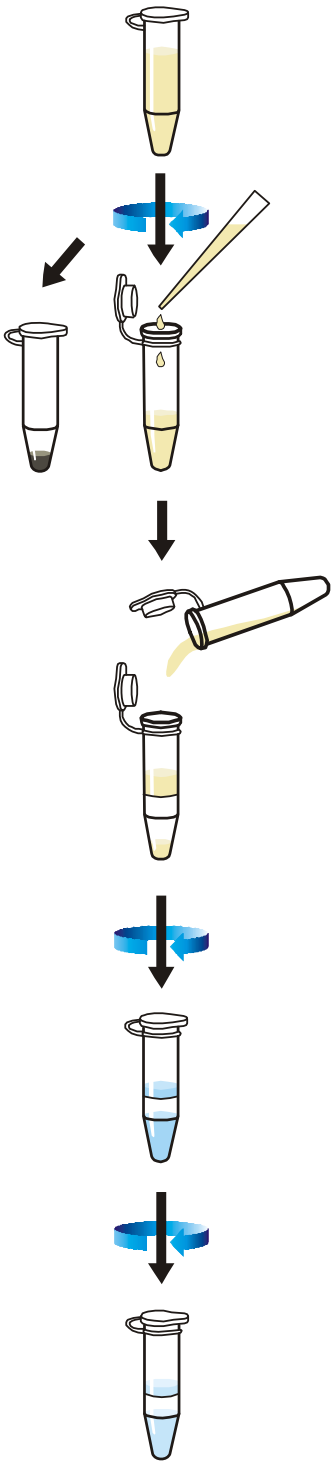
- QIAGEN (www.qiagen.com) TissueLyser system
- UniEquip (www.uniequip.com)

**Glass, stainless steel, and tungsten carbide beads can be purchased from:**

- Retsch (www.retsch.de)

## Scheme

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 500 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 once!**
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 1 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!**

# Instructions

## 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 up to 20 mg of tissue

***Important:*** *For extraction of total RNA from spleen, kidney, or lung tissue please use protocol 2.*

### 1. Disruption and lysis of the starting material (see page 10ff)

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 500 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 2*: Total RNA extraction from up to 20 mg of spleen, kidney, or lung tissue

### 1. Disruption and lysis of the starting material (see page 8 ff)

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 500 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 3:* 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 (11.000 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 RTA Spin Filter.

**6. First washing of the RTA Spin Filter**

Add 500 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 to advice RNA isolation 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 still remains 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, then dry the sample.
3. Centrifuge shortly and remove 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 ap 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. A 10 minute incubation under continuously agitation of the mixture at 80°C, 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 1.</u>**

Depending on the fixation in several cases no useable RNA is isolated we just can give an 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. In addition, 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.

## 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 Tissue 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 3 min at 11.000 x g (11.000 rpm) at 4°C. Discard the supernatant, be careful not to remove the pellet.

**4. Dissolving of proteins**

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

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

## Supplemental protocol for DNA digestion on the RTA Spin Filter

Totally DNA free sample for RT-qPCR applications or other protocols, where smallest amounts of DNA might disturb, can be realized using a DNase digestion directly on the RTA Spin Filter**.**

**Therefore, you have to change Step 5 in protocol 1 or 2 according to the following procedure:**

**Second washing of the RTA Spin Filter**

Add 600 µl **Wash Buffer R2** onto the RTA Spin Filter and centrifuge for 30 sec at 11.000 x g (11.000 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 RTA 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 30 sec at 11.000 x g (11.000 rpm). Reuse the Receiver Tube.

**Repeat this washing step once again and follow the standard procedure step 6, protocol 1.**

## Troubleshooting

| Problem/ Probable cause | Comments and suggestions |
|---|---|
| **Clogged Spin Filter** | |
| insufficient disruption or homogenization of the starting material | after lysis spin lysate to pellet unlysed tissue and continue with the protocol using the supernatant<br>increase g-force and/ or centrifugation time<br>all centrifugation steps should be conducted at room temperature |
| too much starting material | in subsequent preparations, reduce the amount of starting material and/ or increase volume of **Lysis Solution TR** |
| very viscous lysate, like a gel after addition of **Lysis Solution TR** to plant material | if too many tissue material including DNA has been used, the carrier with the bound DNA will be too viscous to pipet after homogenization<br>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 step 1 |
| **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<br>the use of a neutral buffer (10 mM Tris/HCl, pH 7.0) is recommended |
| protein contamination of RNA | use less sample<br>repeat washing step one |
| RNA sample is to strong diluted | sample concentration is out of the range of the photometer |
| **Little or no total RNA eluted** | |
| 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<br>elute a second time with 100 µl **Elution Buffer R**<br>incubate RTA Spin Filter at room temperature (15 - 25°C) for 5 min with RNase free water or **Elution Buffer R** prior centrifugation |
| no alcohol added to the **Wash Buffer R1** and **R2** | check that **Wash Buffer R1** and **Wash Buffer R2** concentrates were diluted with correct volume of 96 - 100% ethanol. Do not use denatured alcohol, which contains other substances such as methanol or methylethylketone,<br>repeat the purification procedure with a new sample |
| **DNA-contamination** | |
| too much starting material | reduce amount of starting material.<br>DNase digestion of the eluate containing the total RNA |
| no optimal homogenization of DNA binding carrier. | shake **Lysis Solution TR** carefully before use. |

| | |
|---|---|
| **RNA degraded**<br>inappropriate handling of the starting material | <br>the RNA purification protocol should be performed quickly (see also “General notes on handling RNA”, page 24)<br>plant material stored at - 80°C for later processing should be immediately frozen after cell harvest by liquid nitrogen treatment |
| **Lysis Solution TR** does not contain **DTT** | ensure that **DTT** has been added to the **Lysis Solution TR** |
| RNase contamination | check for RNase contamination of buffers<br>be certain not to introduce any RNase throughout the procedure or during further handling for analysis,<br>see appendix , ”General Notes on handling RNA” (page 24) |
| **Total RNA does not perform well in downstream-applications (e.g. RT-PCR)**<br>ethanol carryover during elution.<br>salt carryover during elution. | <br>increase g-force or centrifugation time when drying the Spin Filter.<br>ensure that **Wash Buffer R1** and **R2** are at room temperature.<br>check up **Wash Buffer R1** and **R2** for salt precipitates. If there are any precipitates solve these precipitates by careful warming. |

# Appendix

## General notes on handling RNA

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

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

- Electrophoresis tanks should be cleaned with detergent solution (e.g. 0.5% SDS), thoroughly rinsed with RNase free water, and then rinsed with ethanol and allowed to dry.
- Non-disposable 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 dd$H_2O$.
- 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 personnel trained in *in vitro* diagnostic laboratory practice.

## 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 determined by measuring the absorbance at 260 nm ($A_{260}$) in a spectrophotometer. Readings should be greater than 0.10 to ensure significance. An absorbance OD = 1.0 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 mM NaOH, 1 mM EDTA followed by washing with RNase free water. Use the buffer in which the RNA is diluted for calibration of the spectrophotometer.

An example of the calculation involved in RNA quantification:

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

Example: $A_{260}$ = 0.2

| | |
|---|---|
| Concentration of the RNA sample | = 40 µg/ml * $A_{260}$ * dilution factor<br>= 40 µg/ ml * 0.2 * 10<br>= 80 µg/ ml |
| Total amount | = concentration * volume of sample in ml<br>= 80 µg/ ml * 0.06 ml<br>= 4.8 µg of RNA |

## Purity of RNA

The ratio of the readings at 260 nm and 280 nm ($A_{260}/A_{280}$) provides an estimate of the purity of RNA with respect to the contaminants that absorb in the UV, such as protein. However, the $A_{260}/A_{280}$ ratio is influenced considerably by pH. Since water is not buffered, the pH and the resulting $A_{260}/A_{280}$ ratio can vary greatly. Lower pH results in lower $A_{260}/A_{280}$ ratio and reduced sensitivity to protein contaminations.* For accurate values, it is recommend to measure absorbance in 10 mM TrisHCl, pH 7.5. Pure RNA has an $A_{260}/A_{280}$ ratio of 1.9-2.1** in $ddH_2O$. 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 spectrophotometers.

## Denaturing agarose gel electrophoresis of RNA

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

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

### 1. Prepare the gel.

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

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

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

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

### 2. Prepare the RNA sample.

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

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

To 1-3 µg RNA, add 0.5-3 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).

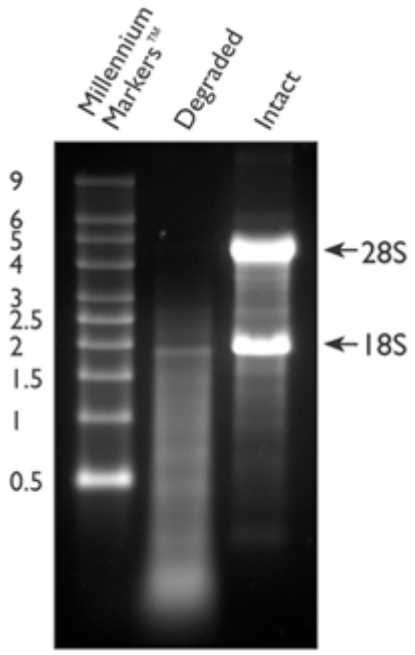



**Figure 1. Intact vs. Degraded RNA.** Two µg of degraded total RNA and intact total RNA were run beside Ambion's RNA Millennium Markers™ on a 1.5% denaturing agarose gel. The 18S and 28S ribosomal RNA bands are clearly visible in the intact RNA sample. The degraded RNA appears as a lower molecular weight 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 Tissue RNA Mini Kit | 250 preparations | 1062100300 |
| **Related Products** | | |
| InviTrap® Spin Universal RNA Mini Kit | 50 preps | 1060100200 |
| InviTrap® Spin Universal RNA Mini Kit | 250 preps | 1060100300 |
| InviTrap® Spin Cell RNA Mini Kit | 250 preparations | 1061100300 |

INVITEK
Molecular

Invitek Molecular GmbH
Röbert-Rössle-Str. 10
13125 Berlin

Phone: +49 30 94 89 29 01
Fax: +49 30 94 89 29 09
info@invitek-molecular.com

www.invitek-molecular.com

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