MACHEREY-NAGEL

User manual

Bioanalysis

RNA and protein purification

- NucleoSpin® RNA/Protein

November 2023 / Rev. 12

MACHEREY-NAGEL

www.mn-net.com

Total RNA and protein isolation

Protocol at a glance (Rev. 12)

NucleoSpin® RNA/Protein

1	**Homogenization of sample**	30 mg
2	**Cell lysis**	350 µL RP1 3.5 µL ß-mercaptoethanol
3	**Filtration of lysate**	11,000 x *g*, 1 min
4	**Adjust binding conditions**	350 µL ethanol (70 %)
5	**Bind RNA**	11,000 x *g*, 30 s

RNA Purification
(RNA bound to the silica membrane)

6	**Desalt silica membrane**	350 µL MDB 11,000 x *g*, 1 min
7	**Digest DNA**	95 µL DNase reaction mixture RT, 15 min
8	**Wash and dry silica membrane**	1st wash 200 µL RA2 2nd wash 600 µL RA3 11,000 x *g*, 30 s 3rd wash 250 µL RA3 11,000 x *g*, 2 min
9	**Elute highly pure RNA**	60 µL H_2O (RNase-free) 11,000 x *g*, 1 min

Protein Purification
(protein in the column flowthrough)

10	**Precipitate protein**	10 – 700 µL flowthrough 1 vol PP RT, 10 min 11,000 x *g*, 5 min
11	**Wash protein**	500 µL ethanol (50 %) 11,000 x *g*, 1 min
12	**Dry protein pellet**	RT, 5 – 10 min
13	**Prepare protein sample**	20 – 100 µL PSB-TCEP 95-98 °C, 3 min 11,000 x *g*, 1 min

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1 Components

1.1 Kit contents

	NucleoSpin® RNA/Protein		
REF	**10 preps 740933.10**	**50 preps 740933.50**	**250 preps 740933.250**
Protein Precipitator PP	9 mL	45 mL	225 mL
Protein Solving Buffer PSB (without reducing agent)	2 × 1 mL	7.5 mL	40 mL
Reducing Agent TCEP	2 × 14 mg	107 mg	5 × 107 mg
Lysis Buffer RP1	10 mL	25 mL	125 mL
Wash Buffer RA2	15 mL	15 mL	80 mL
Wash Buffer RA3 (Concentrate)*	6 mL	12 mL	3 × 25 mL
Membrane Desalting Buffer MDB	10 mL	25 mL	125 mL
Reaction Buffer for rDNase	7 mL	7 mL	30 mL
rDNase, RNase-free (lyophilized)	1 vial (size C)	1 vial (size D)	5 vials (size D)
RNase-free H_2O	13 mL	13 mL	60 mL
NucleoSpin® Filters (violet rings)	10	50	250
NucleoSpin® RNA/Protein Columns (light blue rings, plus Collection Tubes)	10	50	250
Collection Tubes (2 mL)	30	150	750
Collection Tubes (1.5 mL)	20	100	500
User manual	1	1	1

* For preparation of working solutions and storage conditions see section 3.

1.2 Reagents, consumables, and equipment to be supplied by user

Reagents

- 96 – 100 % ethanol (to prepare Wash Buffer RA3)
- 70 % ethanol (to adjust RNA binding conditions)
- 50 % ethanol (to wash protein pellet)
- Reducing agent (ß-mercaptoethanol, or DTT (dithiothreithol), or TCEP (Tris(2-carboxyethyl) phosphine hydrochloride) to supplement lysis buffer

Consumables

- 1.5 mL microcentrifuge tubes for sample lysis
- Disposable RNase-free tips

Equipment

- Manual pipettors
- Centrifuge for microcentrifuge tubes
- Vortex mixer
- Thermal heating block
- Equipment for sample disruption and homogenization
- Personal protection equipment (lab coat, gloves, goggles)

Additional material is furthermore needed for protein quantification, see section 6.1.

1.3 About this user manual

It is strongly recommended reading the detailed protocol sections of this user manual if the **NucleoSpin® RNA/Protein** kit is used for the first time. Experienced users, however, may refer to the Protocol at a glance instead. The Protocol at a glance is designed to be used only as a supplemental tool for quick referencing while performing the purification procedure.

All technical literature is available on the internet at ***www.mn-net.com***.

Please contact Technical Service regarding information about changes of the current user manual compared to previous revisions.

2 Product description

2.1 The basic principle

Introduction

Studies of gene expression at the level of transcription and translation by quantification of RNA and protein are often hampered by the small sample size and the necessity of different – often incompatible – techniques for RNA and protein isolation. Samples may comprise biopsies, tumors, tissues, transgene organisms and others. The **NucleoSpin® RNA/Protein** kit however enables isolation of RNA and protein from diverse sample types. Protein and RNA are isolated without splitting the sample prior to protein/RNA extraction. Thus, protein and RNA are obtained from one and the same sample and not from two similar portions of one sample. This is especially valuable for unique, small and precious samples. Isolated RNA is suitable for all common downstream applications. RNA isolated with the **NucleoSpin® RNA/Protein** kit is of identical quality as RNA isolated with the well proven NucleoSpin® RNA kit. Isolated protein is immediately suitable for SDS-PAGE, Western Blot analysis, and quantification with recommended methods.

RNA and protein isolation

One of the most important aspects in the isolation of RNA and protein is to prevent their degradation during the isolation procedure. With the **NucleoSpin® RNA/Protein** method, cells are lysed by incubation in a solution containing large amounts of chaotropic ions. This lysis buffer immediately inactivates virtually all enzymes (e.g., RNases and proteases) which are present in almost all biological materials. The buffer dissolves even hardly soluble protein, creates appropriate binding conditions which favor adsorption of RNA to the silica membrane and enables protein to pass the specially treated NucleoSpin® RNA/Protein Column virtually quantitatively. Expensive and harmful proteinase inhibitors or inhibitor cocktails are not necessary due to the denaturing properties of the lysis buffer. Contaminating DNA, which is also bound to the silica membrane, is removed by an rDNase solution which is directly applied onto the silica membrane during the preparation (RNase-free rDNase is supplied with the kit). Simple washing steps with two different buffers remove salts, metabolites and macromolecular cellular components. Pure RNA is finally eluted under low ionic strength conditions with RNase-free water (supplied).

Protein is isolated from the column flowthrough. Protein is precipitated in denatured form with a special buffer (Protein Precipitator PP) which effectively precipitates protein. After a washing step the protein pellet is dissolved in Protein Solving Buffer (PSB) containing the odourless reducing agent TCEP. The protein can thus readily be applied to SDS-PAGE analysis. **The kit is not recommended for isolation of native proteins.**

The RNA and protein preparation using **NucleoSpin® RNA/Protein** kits can be performed at room temperature. The RNA eluate, however, should be treated with care because RNA is very sensitive to trace contaminations of RNases, often found on general lab ware, fingerprints and dust. To ensure RNA stability keep RNA frozen at -20 °C for short term or -70 °C for long term storage. Recovered Protein dissolved in Protein Solving Buffer is unproblematic concerning stability.

Simultaneous isolation of RNA, protein, and DNA (NucleoSpin® RNA/DNA Buffer Set)

The NucleoSpin® RNA/DNA Buffer Set (see ordering information) is a support set for RNA and DNA isolation in conjunction with NucleoSpin® RNA, NucleoSpin® RNA XS, NucleoSpin® RNA Plant, or NucleoSpin® RNA/Protein.

This patented technology enables successive elution of DNA and RNA from one NucleoSpin® Column with low salt buffer and water respectively. DNA and RNA are immediately ready for downstream applications.

The combination of the NucleoSpin® RNA/DNA Buffer Set with NucleoSpin® RNA/Protein allows parallel isolation of RNA, DNA, and protein from one undivided sample.

2.2 Kit specifications

- **NucleoSpin® RNA/Protein** kits are recommended for the isolation of total RNA and protein from cultured cells and tissue. The NucleoSpin® RNA/Protein kits allow purification of pure RNA with an A_{260}/A_{280} ratio generally exceeding 1.9 (measured in TE buffer (pH 7.5)).
- The isolated RNA is ready to use for applications like reverse transcriptase-PCR* (RT-PCR), primer extension, or RNase protection assays.
- The isolated protein is ready to use for SDS-PAGE, Western Blot analysis and protein quantification with the Protein Quantification Assay (see ordering information).

* PCR is patented by Roche Diagnostics.

Table 1: Kit specifications at a glance		
Parameter	**NucleoSpin® RNA/Protein**	
Technology	Silica membrane technology	
Format	Mini spin column	
Sample material	$< 5 \times 10^6$ cells < 30 mg human / animal tissue < 100 mg plant tissue	
Use	For research use only	
	Total RNA	**Total protein**
Fragment size	> 200 nt	15 – 300 kDa
Typical yield	< 70 µg	< 1200 µg
A_{260}/A_{280}	1.9 – 2.1	–
Typical RIN (RNA integrity number)	> 9	–
Elution volume / Resolubilization volume protein	40 – 100 µL	10 – 100 µL
Preparation time (approx.)	30 min/6 preps	35 min/6 preps
Binding capacity	200 µg	–

- The standard protocol (section 5.1) allows purification of up to 70 µg of total RNA per **NucleoSpin® RNA / Protein Column** from up to 5×10^6 cultured cells, 30 mg of human/animal tissue, or 100 mg of plant tissue (see Table 1). The isolated RNA can be used as template in a RT-PCR-reaction. Generally, 1 – 10 % of the eluate of total RNA prepared from 1×10^6 cells or 10 mg of tissue is sufficient as template for RT-PCR. Intron-spanning primers for RT-PCR are preferable if possible.
- RNA prepared with **NucleoSpin® RNA / Protein** is generally free of residual DNA. However, minute traces of DNA may remain, if large amounts of material rich in nucleic acids are used. If the isolated RNA will be used as template in a RT-PCR-reaction, we recommend using lower quantities of sample material, depending on cell or tissue type (in the range of 1×10^6 cells or 10 mg of tissue resulting in about 20 µg of RNA).

- The kit can be used for preparing RNA from different amounts of sample material according to Table 2:

Table 2: Use of different amounts of sample material

Sample	Amount
Cultured animal cells (e.g., HeLa cells)	Up to 5×10^6
Animal tissue	Up to 30 mg
Bacteria	Up to 1×10^9
Yeast	Up to 5×10^7

- Depending on sample type, the average yield is around 5 – 70 µg total RNA (see Table 3). The A_{260}/A_{280} ratio, indicating purity of the RNA, generally exceeds 1.9.

Table 3: Overview on average yields of total RNA isolation using NucleoSpin® RNA/Protein

Sample	Average yield
8×10^4 HeLa cells	1.5 µg
4×10^5 HeLa cells	4 µg
1×10^6 HeLa cells	14 µg
2×10^6 HeLa cells	21 µg
2.5×10^6 HeLa cells	25 µg
5×10^6 HeLa cells	50 µg

Protein yield

Protein yield depends on sample type, amount and quality as well as on homogenization efficiency. Further, the utilized quantification method influences determined protein yield. The following values were determined with the MACHEREY-NAGEL Protein Quantification Assay and shall serve as a guideline for expected protein yield. It is assumed that the complete sample amount is processed, i.e. the complete lysed sample – after ethanol addition – is loaded onto the column and the complete 700 µL flow through is subjected to protein precipitation. Note that in many cases precipitation of only a portion of the column flow through (e.g.,100 µL) is recommended and will yield enough protein in terms of absolute amount and concentration for SDS-PAGE and Western Blot analysis.

As a guideline for appropriate precipitation volumes see section 2.4.

Table 4: Typical protein yield

Sample type and amount	Protein yield
Cultured human cells (e.g., HeLa, approx. 10^6 cells)	~ 50 – 150 µg
Plants (e.g., garden cress, approx. 100 mg)	~ 150 – 350 µg
Animal tissue (e.g., pig liver, approx. 30 mg)	~ 500 – 1200 µg

2.3 Handling, preparation, and storage of starting materials

RNA is not protected against digestion until the sample material is flash frozen or disrupted in the presence of RNase inhibiting or denaturing agents. Therefore it is important that samples are flash frozen in liquid N2 immediately and stored at -70 °C, or processed as soon as possible. Samples can be stored in Lysis Buffer RP1 after disruption at -70 °C for up to one year, at +4 °C for up to 24 hours or up to several hours at room temperature. Frozen samples are stable up to 6 months. Frozen samples in Buffer RP1 should be thawed slowly before starting with the isolation of total RNA.

Wear gloves at all times during the preparation. Change gloves frequently.

Cultured animal cells

are collected by centrifugation and directly lysed by adding Buffer RP1 according to step 2 of the standard protocol (see section 5.1).

Cell lysis of adherent growing cells in a culture dish:

Completely aspirate cell-culture medium, and continue immediately with the addition of Lysis Buffer RP1 to the cell-culture dish. Avoid incomplete removal of the cell-culture medium in order to allow full lysis activity of the lysis buffer.

To trypsinize adherent growing cells:

Aspirate cell-culture medium and add an equal amount of PBS in order to wash the cells. Aspirate PBS. Add 0.1 – 0.3 % trypsin in PBS and incubate for an appropriate time to detach the cells from the dish surface. After cell detachment, add medium, transfer cells to an appropriate tube (not supplied), and pellet by centrifugation for 5 min at 300 x *g*. Remove supernatant and continue with the addition of lysis buffer to the cell pellet.

Human / animal and plant tissues

are often solid and must therefore be broken up mechanically as well as lysed. Depending on the disruption method, the viscosity of the lysed sample has to be reduced further for optimal results. It is essential for efficient RNA preparation that all the RNA contained in the sample is released from the cells by disruption and that the viscosity of the sample is reduced by homogenization.

The most commonly used technique for disruption of animal tissues is grinding with a **pestle and mortar**. Grind the sample to a fine powder in presence of liquid N2. Take care that the sample does not thaw during or after grinding or weighing and add the frozen powder to an appropriate aliquot of Buffer RP1 containing ß-mercaptoethanol and mix immediately. The broken-up tissue must then be homogenized with a **NucleoSpin® Filter / Filter L** or by passing ≥ 5 through a 0.9 mm syringe needle.

Thawing of undisrupted animal tissue should exclusively be done in presence of Buffer RP1 during simultaneous mechanical disruption, for example, with a **rotor-stator homogenizer**. This ensures that the RNA is not degraded by RNases before the preparation has started. The spinning rotor disrupts and simultaneously homogenizes the sample by mechanical shearing within seconds up to minutes (homogenization time depends on sample). Take care to keep the rotor tip submerged in order to avoid excess foaming. To degenerate evolved foam, centrifuge 1 min at 400 x *g*. Select a suitably sized homogenizer (5 – 7 mm diameter rotors can be used for homogenization in microcentrifuge tubes).

Bacteria and yeasts

have to be incubated in lysozyme or lyticase/zymolase solutions, respectively (see support protocols in section 5). By this treatment the robust cell walls of these organisms are digested or at least weakened, which is essential for effective cell lysis by Buffer RP1. For microorganisms with extremely resistant cell walls – like some Gram positive bacterial strains – it may be necessary to optimize the conditions of the treatment with lytic enzymes or the cultivation conditions. After lysis, homogenization is achieved by the use of a **NucleoSpin® Filter** or the syringe-needle method.

2.4 Guideline for appropriate sample amount, precipitation, and resolubilization volume for protein isolation

The following Table 5 shall serve as a first guide for choosing appropriate amounts of sample material, precipitation volume, and resolubilization volume. Depending on sample type, and downstream application (e.g., Coomassie or silver stain, sensitivity of antibody, detection system) appropriate volumes might deviate from the table below and have to be determined experimentally.

Table 5: Guideline for appropriate sample amount

Amount of	Cultivated cells (e.g., HeLa)			Animal tissue (e.g., liver)			Plant tissue (e.g., garden cress leaf)		
Sample	10^6	10^5	10^4	30 mg	3 mg	0.3 mg	100 mg	10 mg	1 mg
Lysis Buffer RP1 incl. reducing agent	350 µL								
Ethanol	350 µL								
Column flowthrough to be precipitated*	35 µL	350 µL	700 µL	35 µL	350 µL	700 µL	35 µL	350 µL	700 µL
Volume of Protein Precipitator PP	35 µL	350 µL	700 µL	35 µL	350 µL	700 µL	35 µL	350 µL	700 µL
Buffer PSB used for protein pellet solubilization	100 µL	100 µL	20 µL	100 µL	100 µL	20 µL	100 µL	100 µL	20 µL
Protein sample to be analysed on SDS-PAGE with Coomassie stain	10 µL								
Protein sample to be analyzed on SDS-PAGE with silver stain	1 µL								
Protein sample analyzed on Western Blot	1 – 10 µL								

* Protein pellets with a diameter of up to approximately 1 – 2 mm in size are ideally suited for subsequent solubilization. Protein pellets exceeding volumes of approximately 10 µL should be avoided as large protein pellets are harder to dissolve than small pellets. To obtain small protein pellets, adapt the volume of column flow through in respect to the amount of sample material. Commonly small and even invisible protein pellets yield sufficient protein for SDS PAGE and Western Blot analysis.

Solubilization of protein pellets and reduction of protein disulfide bonds

The NucleoSpin® RNA/Protein kit provides a protein sample buffer (Protein Solving Buffer PSB) and the Reducing Agent TCEP.

The Protein Solving Buffer PSB is similar in composition and function to the buffer commonly known as "Laemmli" buffer. For most applications, PSB may be substituted by "Laemmli" buffer. However, for applications with large protein pellets (> approx. 1 mm, diameter) PSB is recommended.

TCEP is a powerful, multi-purpose and odourless reducing agent. It is non-volatile and unlike commonly used reducing agents like DTT and ß-mercaptoehanol resistant to air oxidation. TCEP reduces disulfide bonds as effectively as dithiothreitol (DTT). TCEP reduces even most stable water-soluble alkyl disulfides selectively and completely over a wide pH range.

Solubilization of TCEP in PSB according to the instruction, results in a PSB-TCEP solution with a concentration of 50 mM TCEP (see section 6.1 for composition). This provides sufficient molar excess to reduce peptide and protein disulfide bonds effectively within a few minutes (in a range up to a protein concentration of approximately 1 μg/μL).

2.5 Elution procedures

It is possible to adapt elution method and elution volume of water to the subsequent application of interest. In addition to the standard method described in the individual protocols (recovery rate about 70 – 90 %) there are several modifications possible.

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

Eluted RNA should immediately be put on ice and always kept on ice for optimal stability because almost omnipresent RNases (general lab ware, fingerprints, dust) will degrade RNA. For short term storage freeze at -20 °C, for long term storage freeze at -70 °C.

3 Storage conditions and preparation of working solutions

Attention:

Buffers RP1, RA2, and MDB contain chaotropic salt! Wear gloves and goggles!

CAUTION: Buffers RP1, RA2, and MDB contain guanidinium thiocyanate which can form highly reactive compounds when combined with bleach (sodium hypochlorite). DO NOT add bleach or acidic solutions directly to the sample-preparation waste.

- Store lyophilized **rDNase (RNase-free)** at +4 °C on arrival (stable up to 1 year).
- Store **Reducing Agent TCEP** at +4 °C on arrival.
- All other kit components should be stored at 15 – 25 °C and are stable up to one year. Storage at lower temperatures may cause precipitation of salts.
- Check that 70 % ethanol is available as additional solution to adjust binding conditions in the RP1-lysate.
- Check that 50 % ethanol is available as additional solution to wash the protein pellet.

Before starting any **NucleoSpin® RNA/Protein** protocol prepare the following:

- **rDNase (RNase-free):** Add indicated volume of RNase-free H_2O (see table below) to the rDNase vial and incubate for 1 min at room temperature. Gently swirl the vials to completely dissolve the rDNase. Be careful not to mix rDNase vigorously as rDNase is sensitive to mechanical agitation. Dispense into aliquots and store at -20 °C. The frozen working solution is stable for 6 months. Do not freeze/thaw the aliquots more than three times.
- **Wash Buffer RA3:** Add the indicated volume of 96 – 100 % ethanol (see table below) to Buffer RA3 Concentrate. Mark the label of the bottle to indicate that the ethanol is added. Store Wash Buffer RA3 at 15 – 25 °C for up to one year.
- **Protein Solving Buffer PSB** and **Reducing Agent TCEP:** For SDS-PAGE under reducing conditions (most common type of SDS-PAGE) transfer the indicated volume of PSB into each vial of TCEP (see table below). Mix gently to avoid foaming until TCEP is dissolved completely (this process will require several minutes). For better handling TCEP-PSB solution may be transferred back to the original PSB screw cap vial or a clean vial of your choice. TCEP reconstituted in PSB is stable for several days at 15 – 25 °C and several months at 4 °C. For long term storage keep at -20 °C.

- If SDS-PAGE under non-reducing conditions is intended consider the following:
  A: Omit addition the Reducing Agent TCEP to Buffer PSB.
  B: Omit addition of ß-mercaptoethanol to Lysis Buffer RP1.
- If other reducing agents than TCEP are preferred (e.g., DTT, ß-mercaptoethanol), appropriate amounts should be added to PSB. Please consider limited stability of DTT compared to TCEP.
- If PSB-TCEP is turbid, warm up PSB-TCEP to > 25 °C before use until solution is completely clear (i.e., all precipitate is dissolved completely). PSB-TCEP has a half-life of approximately 5 months if stored at 4 °C and approximately 7 months if stored at -20 °C.

	NucleoSpin® RNA/Protein		
	10 preps	**50 preps**	**250 preps**
REF	**740933.10**	**740933.50**	**740933.250**
Wash Buffer RA3 (Concentrate)	6 mL Add 24 mL ethanol	12 mL Add 48 mL ethanol	3 × 25 mL Add 100 mL ethanol to each vial
rDNase, RNase-free (lyphilized)	1 vial (size C) Add 230 μL RNase-free H_2O	1 vial (size D) Add 540 μL RNase-free H_2O	5 vials (size D) Add 540 μL RNase-free H_2O to each vial
Reducing Agent TCEP	2 × 14 mg Add 1 mL PSB each	107 mg Add 7.5 mL PSB	5 × 107 mg Add 7.5 mL PSB each

4 Safety instructions

When working with the **NucleoSpin® RNA/Protein** kit wear suitable protective clothing (e.g., lab coat, disposable gloves, and protective goggles). For more information consult the appropriate Material Safety Data Sheets (MSDS available online at ***www.mn-net.com/msds***).

Caution: Guanidin thiocyanate in Buffer RP1 and buffer RA2 can form highly reactive compounds when combined with bleach! Thus, do not add bleach or acidic solutions directly to the sample preparation waste.

The waste generated with the **NucleoSpin® RNA/Protein** kit has not been tested for residual infectious material. A contamination of the liquid waste with residual infectious material is highly unlikely due to strong denaturing lysis buffer but it cannot be excluded completely. Therefore, liquid waste must be considered infectious and should be handled and discarded according local safety regulations.

4.1 Disposal

Dispose hazardous, infectious or biologically contaminated materials in a safe and acceptable manner and in accordance with all local and regulatory requirements.

5 Protocols

5.1 Total RNA and protein purification from cultured cells and tissue

Joint protocol steps for RNA and protein purification.

Before starting the preparation:

- Check if Wash Buffer RA3, rDNase, and Reducing Agent TCEP were prepared according to section 3.

1 Homogenization of sample

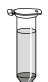
Disrupt up to **30 mg** of **human / animal tissue** or up to 100 mg of plant tissue (for homogenization methods see section 2.3).

Disrupt sample

Up to **5 × 10^6** eukaryotic **cultured cells** are collected by centrifugation and lysed by addition of Buffer RP1 directly.

2 Cell lysis

Add **350 µL Buffer RP1** and **3.5 µL ß-mercaptoethanol** (ß-ME) to the cell pellet or to ground tissue and vortex vigorously.



+ 350 µL RP1

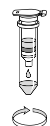
+ 3.5 µL ß-ME

Note: As alternative to ß-ME the reducing agent DTT or TCEP may be use instead of ß-ME. Use a final concentration of 10–20 mM DTT or TCEP within the Lysis Buffer RP1.

3 Filtration of the lysate

Reduce viscosity and clear the lysate by filtration through NucleoSpin® Filter: Place **NucleoSpin® Filter (violet ring)** in a Collection Tube, apply the mixture, and centrifuge for **1 min** at **11,000 x *g***.

The lysate may be passed alternatively ≥ 5 times through a 0.9 mm needle (20 gauge) fitted to a syringe.



11,000 x *g*, 1 min

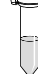
In case of visible pellet formation (depending on sample amount and nature), transfer supernatant without any formed pellet to a new 2 mL centrifuge tube (not included).

Important: To process higher amounts of cells (> 1 × 106) or tissue (> 10 mg), the lysate should first be homogenized using the 0.9 mm needle (20 gauge), followed by filtration through NucleoSpin® Filter.

4 Adjust RNA binding conditions

Discard the NucleoSpin® Filter and add **350 μL ethanol (70 %)** to the homogenized lysate and mix by pipetting up and down (approx. 5 times).

Alternatively, transfer flowthrough into a new 1.5 mL microcentrifuge tube (not provided), add 350 μL ethanol (70 %), and mix by vortexing (2 × 5 s).



+ 350 μL 70 % EtOH

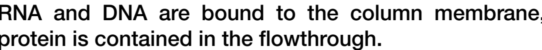
Mix

After addition of ethanol a stringy precipitate may become visible which will not affect the RNA isolation. Be sure to disaggregate any precipitate by mixing and to load all of the disaggregated precipitate on the column as described in step 5. Do not centrifuge at this stage in order to avoid sedimentation of any precipitate.

5 Bind RNA

For each preparation, take one **NucleoSpin® RNA/Protein Column (light blue ring)** placed in a Collection Tube and load the lysate. Centrifuge for **30 s** at **11,000 x *g***. Place the NucleoSpin® RNA/Protein Column in a new Collection Tube (2 mL).

Load sample



RNA and DNA are bound to the column membrane, protein is contained in the flowthrough.

11,000 x *g*, 30 s

Maximal loading capacity of NucleoSpin® RNA/Protein Columns is 750 μL. Repeat the procedure if larger volumes are to be processed.

For **RNA** isolation continue with step 6.

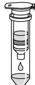
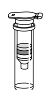
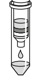
It is recommended to continue the RNA isolation protocol first and to perform the protein purification subsequently.

For **protein** isolation **recover flowthrough** and continue with step 10.

Recover flowthrough for protein isolation!

The protein containing flowthrough is stable for several hours at 4 – 8 °C.

Further steps for RNA purification (steps 6 – 9)

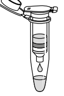
6	**Desalt silica membrane** Add **350 µL MDB** (Membrane Desalting Buffer) and centrifuge at **11,000 x *g*** for **1 min** to dry the membrane. *Salt removal will enhance the effectivity of the following rDNase digest much more effective. If the column outlet has come into contact with the flowthrough for any reason, discard the flowthrough and centrifuge again for 30 s at 11,000 x g.*		**+ 350 µL MDB**
7	**Digest DNA** **Prepare rDNase reaction mixture** in a sterile microcentrifuge tube (not provided): For each isolation, add **10 µL** reconstituted **rDNase** (also see section 3) to **90 µL Reaction Buffer for rDNase**. Mix by flicking the tube. Apply **95 µL rDNase reaction mixture** directly onto the center of the silica membrane of the column. Incubate at **room temperature** for **15 min**.		**+ 95 µL rDNase reaction mixture** **RT, 15 min**
8	**Wash and dry silica membrane** **1st wash** Add 200 µL Buffer RA2 to the NucleoSpin® RNA/Protein Column. Centrifuge for **30 s** at **11,000 x *g***. Place the NucleoSpin® RNA/Protein Column into a new Collection Tube (2 mL). *Buffer RA2 will inactivate the rDNase.*	 	**+ 200 µL RA2** **11,000 x *g*, 30 s**
	2nd wash Add **600 µL Buffer RA3** to the NucleoSpin® RNA/Protein Column. Centrifuge for **30 s** at **11,000 x *g***. Discard flowthrough and place the NucleoSpin® RNA/Protein Column back into the Collection Tube.		**+ 600 µL RA3** **11,000 x *g*, 30 s**
	3rd wash Add **250 µL Buffer RA3** to the NucleoSpin® RNA/Protein Column. Centrifuge for **2 min** at **11,000 x *g*** to dry the membrane completely. Place the NucleoSpin® RNA/Protein Column into an RNase-free Collection Tube (1.5 mL, supplied). *If for any reason, the liquid level in the Collection Tube has reached the NucleoSpin® RNA/Protein Column after centrifugation, discard flowthrough, and centrifuge again.*		**+ 250 µL RA3** **11,000 x *g*, 2 min**

9 Elute highly pure RNA

Elute the RNA in **60 µL RNase-free H_2O** (supplied) and centrifuge at **11,000 x *g*** for **1 min**.

If higher RNA concentrations are desired, elution can be done with 40 µL for the NucleoSpin® RNA/Protein kit. Overall yield, however, will decrease when using smaller volumes.

For further alternative elution procedures see section 2.5.



+ 60 µL RNase free H_2O

11,000 x *g*, 1 min

Further steps for protein purification (steps 10 – 13)

Perform steps 1 – 5 of the NucleoSpin® RNA/Protein kit standard protocol (homogenization, cell lysis, lysate filtration, adjusting of nucleic acid binding condition, and binding of nucleic acids to the NucleoSpin® RNA/Protein Column).

Use the NucleoSpin® RNA/Protein Column flowthrough of step 5 (i.e., the ethanolic lysate which has been passed throught the RNA binding column and is as such deprived of nucleic acids) as starting point for protein precipitation (step 10).

10 Precipitate protein

Transfer an appropriate amount (10 – 700 µL) of flow-though into a fresh Collection Tube (1.5 mL, supplied).

See section 2.4 as guideline for choosing an appropriate amount.

Add **one volume PP** (Protein Precipitator). Mix vigorously.

Incubate mixture at room temperature for approximately 10 min.

Note: *For samples of moderate to high protein content (e.g., 100 mg young plant leaf, 30 mg liver) this incubation step may be omitted. For samples of low to medium protein content (e.g., 15 mg young plant leaf) the 10 min incubation increases protein yield relative to no incubation significantly. An incubation of longer than one hour does not further increase protein yield.*

Centrifuge for **5 min** at **11,000 x *g***.

11 Wash protein

Remove supernatant by pipetting or decanting as complete as possible.

Add **500 µL** of **50 % ethanol** to the pellet (mixing or incubation at this step is not necessary).

Centrifuge **1 min** at **11,000 x *g***.

Remove supernatant by pipetting or decanting as completely as possible.

Note: Protein precipitate at this stage is quite different in appearance depending on kind and amount of starting material. The precipitate might appear as no visible pellet (e.g., for 10.000 cells, 0.3 mg liver and 1 mg leaf samples); a greenish tube wall coating on one side of the tube (e.g., for leaf material); green or white pellet at the bottom of the tube (e.g., for leaf and liver samples, respectively); green or white crumbs at one side of the inner wall of the centrifuge tube (e.g., for leaf and liver samples, respectively). If no precipitate is visible, mark the side of the tube where a precipitate is expected in order to avoid touching this side of the inner tube wall with the pipet tip during the washing step. See also section 2.3 how to avoid very large protein pellets.

12 Dry protein pellet

Dry precipitate for **5–10 min** at **room temperature**; keep lid open.

Note: Large pellets (e.g., complete precipitation of 700 µL column flowthrough form a 30 mg liver sample) need longer drying duration. Samples which are dried incomplete may cause problems when loading the sample onto the gel due to residual ethanol content. No problems with over-drying have been observed with small-sized pellets. See also section 2.4 how to avoid very large protein pellets.

13 Prepare protein sample

Add **20 – 100 μL PSB-TCEP** (Protein Solving Buffer containing reducing agent).

Assure that PSB-TCEP is clear (not turbid). If necessary, warm PSB-TCEP to > 25 °C to dissolve turbidity.

See section 2.4 as guideline for choosing an appropriate amount of PSB-TCEP for dissolving of protein pellets.

Disaggregate large and visible pellets with a pipet tip to facilitate subsequent protein dissolution; this is not necessary for small and invisible pellets.

Incubate for **3 min** at **95 – 98 °C** for complete protein dissolving and denaturation.

Let sample cool down to room temperature.

Centrifuge for **1 min** at **11,000 x *g*** to pellet residual insolvable material.

Note: Depending on sample amount and nature there might be no visible pellet of insolvable material up to large pellets of different size and structure. Do not disturb residual precipitates at this stage. Protein will be in the supernatant. Do not centrifuge samples at temperatures < 18 °C. SDS may precipitate at this temperature.

Recover supernatant for further analysis. See section 6.1 for suitable protein quantification methods.

Note: At this stage samples can be stored at -20 °C for several months or at +4 °C for several days. After storage, equilibrate sample to room temperature, mix, and then centrifuge briefly before withdrawal of sample aliquots. Repeated sample denaturing for 3 min at 95 – 98 °C is not necessary. Repetitive withdrawal, freezing, and thawing for at least three times has shown constant sample quality.

Due to the strong denaturing purification method protein is precipitated in denatured form with reduced solubility in water. Therefore resolubilization of the protein pellet in PSB-TCEP or in traditional Laemmli buffer is recommended. The use of Protein Solving Buffer PSB is not mandatory for dissolving protein. Alternatively to PSB, PSB-TCEP, or Laemmli buffer, precipitated protein can be dissolved in 1 % SDS or 8 M urea. Further, the protein pellet can be dissolved in urea/thiourea/CHAPS buffers as used for 2-D electrophoresis. However, depending on the target protein, the overall yield of solubilized protein may be reduced compared to PSB or PSB-TCEP used as dissolving agent.

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

Before starting the preparation:

- Check if Wash Buffer RA3, rDNase, and Reducing Agent TCEP were prepared according to section 3.

1	**Homogenization of sample** Not necessary!
2	**Cell lysis** Add **350 μL Buffer RP1** to **100 μL of sample** and vortex vigorously.
3	**Filtration of the lysate** Not necessary!
4	**Adjust RNA binding conditions** Add **350 μL ethanol (70 %)** to the lysate and mix by pipetting up and down (approx. 5 times).
	Proceed with step 5 of the NucleoSpin® RNA/Protein standard protocol (section 5.1).

5.3 Total RNA preparation from up to 10^9 bacterial cells

Before starting the preparation:

- Check if Wash Buffer RA3, rDNase, and Reducing Agent TCEP were prepared according to section 3.

1 Homogenization of sample

Resuspend the **bacterial cell pellet** (Gram negative strains) in **100 µL TE buffer** (10 mM Tris-HCl, 1 mM EDTA; pH 8) containing 0.2 mg/mL lysozyme by vigorous vortexing. Incubate at 37 °C for 10 min.

For preparation of RNA from Gram positive bacteria, resuspend cells in 100 µL TE containing 2 mg/mL lysozyme. Depending on the bacterial strain, it may be necessary to optimize incubation time and lysozyme concentration.

2 Cell lysis

Add **350 µL Buffer RP1** and 3**.5 µL ß-mercaptoethanol** (ß-ME) to the suspension and vortex vigorously.

As alternative to ß-ME the reducing agent DTT or TCEP may be used. Use a final concentration of 10 – 20 mM DTT or TCEP within the Lysis Buffer RP1.

3 Filtration of lysate

Reduce viscosity and turbidity of the solution by filtration through **NucleoSpin® Filters (violet rings)**. Place NucleoSpin® Filters in Collection Tubes, apply mixture, and centrifuge for **1 min** at **11,000 x *g***.

In case of visible pellet formation (depending on sample amount and nature) transfer supernatant without any formed pellet to a new 2 mL centrifuge tube (not included).

Alternatively, the lysate may be passed ≥ 5 times through a 0.9 mm needle (20 gauge) fitted to a syringe.

4 Adjust RNA binding conditions

Add **350 µL ethanol (70 %)** and mix by pipetting up and down (approx. 5 times).

Because of the much greater concentration of genome equivalents in a nucleic acid preparation of bacteria compared with eukaryotic material, it may be necessary to use a lower quantity of cells for the preparation.

Proceed with step 5 of the NucleoSpin® RNA/Protein standard protocol (section 5.1).

5.4 Total RNA preparation from up to 5 × 10^7 yeast cells

Before starting the preparation:

- Check if Wash Buffer RA3, rDNase, and Reducing Agent TCEP were prepared according to section 3.

1 Homogenization of sample

Harvest **2 – 5 mL YPD culture (5,000 x *g*; 10 min)**. Resuspend pellet in **sorbitol/lyticase buffer** (50 – 100 U lyticase or zymolase in 1 M sorbitol/100 mM EDTA) and incubate at **30 °C** for **30 min**. Pellet the resulting spheroplasts by centrifugation **(1,000 x *g*; 10 min)**.

It may be necessary to optimize incubation time and lyticase/zymolase concentration, depending on the yeast strain.

2 Cell lysis

Add **350 µL Buffer RP1** and **3.5 µL ß-mercaptoethanol** (ß-ME) to the suspension and vortex vigorously.

As alternative to ß-ME the reducing agent DTT or TCEP may be used. Use a final concentration of 10 – 20 mM DTT or TCEP within the Lysis Buffer RP1.

3 Filtration of lysate

Reduce viscosity and turbidity of the solution by filtration through **NucleoSpin® Filters**. Place NucleoSpin® Filters in Collection Tubes (2 mL), apply mixture, and centrifuge for **1 min** at **11,000 x *g***.

In case of visible pellet formation (depending on sample amount and nature), transfer supernatant without any formed pellet to a new microcentrifuge tube (not included).

Alternatively, the lysate may be passed ≥ 5 times through a 0.9 mm needle (20 gauge) fitted to a syringe.

Due to the much higher concentration of genome equivalents in a nucleic acid preparation of yeasts compared with cultured cells or tissue material, it may be necessary to use a lower quantity of cells for the preparation.

Proceed with step 4 of the NucleoSpin® RNA/Protein standard protocol (section 5.1).

5.5 Total RNA preparation from RNA*later*® treated samples

Before starting the preparation:

- Check if Wash Buffer RA3, rDNase, and Reducing Agent TCEP were prepared according to section 3.

1 Sample preparation

Remove RNA*later*® solution. Cut an appropriate amount of tissue.

2 Cell lysis

Add **350 µL Buffer RP1** and **3.5 µL ß-mercaptoethanol** (ß-ME) to the sample.

Disrupt the sample material by using, for example, rotor-stated homogenizers (for homogenization methods see section 2.3).

As alternative to ß-ME the reducing agent DTT or TCEP may be used. Use a final concentration of 10 – 20 mM DTT or TCEP within the Lysis Buffer RP1.

Proceed with step 3 of the NucleoSpin® RNA/Protein standard protocol (section 5.1).

5.6 rDNase digestion in solution

The on column rDNase digestion in the standard protocol is very efficient and resulting in minimal residual DNA content of the purified RNA. This DNA will not be detectable in most downstream applications. Despite this, there are still certain applications which require even lower contents of residual DNA. However, removal of DNA to a completely undetectable level is challenging and the efficiency of an on column DNA digestion is sometimes not sufficient for downstream applications requiring lowest residual content of DNA.

A typical example for such a demanding application is an RT-PCR reaction in which the primer molecules do not differentiate between cDNA (derived from RNA) and contaminating genomic DNA. Especially, if

- high copy number targets are analyzed (e.g., multi gene family, mitochondrial, plastidal or plasmid targets (from transfections))
- the target gene is of a very low expression level
- the amplicon is relatively small (< 200 bp).

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

The high quality, recombinant RNase-free DNase (rDNAse) in the NucleoSpin® RNA kits facilitates such a digestion in solution in order to remove even traces of contaminating DNA.

A Digest DNA (reaction setup)

Add **6 μL Reaction Buffer for rDNase** and **0.6 μL rDNase** to **60 μL eluted RNA**.

Alternatively, premix 100 μL Reaction Buffer for rDNase and 10 μL rDNase and add 1/10 volume to one volume of RNA eluate.

B Incubation

Incubate for **10 min** at **37 °C**.

C Repurification of RNA

Repurify RNA with a suitable RNA clean up procedure, for example, by use of the NucleoSpin® RNA Clean-up or NucleoSpin® RNA Clean-up XS kit (see ordering information) or by ethanol precipitation.

Ethanol precipitation, exemplary

Add 0.1 volume of 3 M sodium acetate, pH 5.2 and 2.5 volumes of 96 – 100 % ethanol to one volume of sample. Mix thoroughly.

Incubate several minutes to several hours at -20 °C or +4 °C.

Note: Choose long incubation times if the sample contains low RNA concentration. Short incubation times are sufficient if the sample contains high RNA concentration.

Centrifuge for 10 min at max. speed.

Wash RNA pellet with 70 % ethanol.

Dry RNA pellet and resuspend RNA in RNase-free H_2O.

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6 Appendix

6.1 Protein quantification

Quantification of protein dissolved in sample buffer such as PSB, PSB-TCEP, or traditional Laemmli buffer is occasionally required prior to SDS-PAGE and Western Blot analysis. However, major protein quantification assays are influenced by or are incompatible with SDS and/or reducing agents commonly present in protein sample buffers used for SDS-PAGE. A protein quantification procedure has to be chosen carefully to ensure appropriate compatibility of the method with the protein dissolution solution.

The NucleoSpin® RNA/Protein procedure allows several protein quantification methods at different steps of the procedure:

- Quantification of protein dissolved in PSB, PSB-TCEP or Laemmli buffer (recommended)
- Quantification of protein dissolved in alternative protein dissolution buffers (1 % SDS or 8 M urea)
- Quantification of protein within the column flowthrough (i.e., prior to protein precipitation, at step 5 of the standard protocol)

For most reliable results and convenience we recommend the MACHEREY-NAGEL Protein Quantification Assay (for ordering information see section 6.4) to quantify protein dissolved in PSB, PSB-TCEP, or Laemmli buffer.

Quantification of protein dissolved in PSB or PSB-TCEP

The concentation of protein dissolved in PSB, PSB-TCEP, or Laemmli buffer can be determined with several methods.

Below you find a choice of quantification methods, which are compatible with PSB, PSB-TCEP, and Laemmli buffer, but not all of the methods show the same sensitivity. The following list compares the different sensitivities and gives assistance to find out the most suitable protein quantification assay.

1: Protein Quantification Assay (highly recommended method!)

This is the most sensitive and convenient method for protein quantification in PSB or PSB-TCEB. Highly recommended due to sensitivity and high compatibility. For ordering information see section 6.4.

2: Method adapted from the publication Karlsson et al. 1994

For a detailed protocol of the Karlsson method, see page 35.

3: Pierce BCA Protein Assay Kit (reducing agent compatible)

Dilute the protein sample 1 :5 with water to enable compatibility.

4: Bio-Rad DC Protein Assay

Dilute the protein sample 1 :10 with water to enable compatibility.

* Method tested in MN laboratory for compatibility with PSB, PSB-TCEP and Laemmli buffer protein samples.

5: Bio-Rad RC DC Protein Assay

Dilute the protein sample 1 :5 with water to enable compatibility.

6: Serva ProtaQuant Assay Kit

According to manufacturer's instructions, this assay should be compatible with PSB, PSB-TCEP, and Laemmli buffer samples.

7: G-Biosciences SPNTM-Protein Assay

According to manufacturer's instructions, this assay should be compatible with PSB, PSB-TCEP, and Laemmli buffer samples.

8: Bio-Rad Protein Assay (Bradford)

This method has a very low tolerance towards SDS (0.1 % SDS for the Standard Assay Procedure). Therefore, PSB, PSB-TCEP Laemmli buffer samples have to be diluted considerably with water to reduce interference. After dilution of the sample 1 :20 with water protein can be quantified with the standard assay procedure. The microassay procedure however, is not compatible with such samples, even after 1 :50 dilution of the sample with water.

Compatibility of protein quantification methods with PSB and PSB-TCEP samples

#	Protein quantification assay	Input sample volume (e.g., PSB-TCEP sample)	Acceptable protein amount per assay	Protein concentration quantification range (undiluted PSB-TCEP sample)
1	Protein Quantification Assay* (highly recommended, REF 740967)	20 µL standard (1 – 60 µL optional)	0.6 – 20 µg	0.03 – 1 µg/µL (standard) 0.01 – 20 µg/µL (optional)
2	Karlsson protocol (recommended, see page 35 for details)*	20 µL standard (1 – 60 µL optional)	0.6 – 20 µg	0.03 – 1 µg/µL (standard) 0.01 – 20 µg/µL (optional)
3	Pierce BCA Protein Assay Kit (reducing Agent compatible)*	25 µL of a 1 :5 prediluted sample, corresponding to 5 µL original sample	3.125 – 50 µg	0.625 – 10 µg/µL

* Method tested in MN laboratory for compatibility with PSB, PSB-TCEP and Laemmli buffer protein samples.

** Method compatible with PLB-TCEP protein samples refering to manufacturer's product information. Not tested in MN laboratories

#	Protein quantification assay	Input sample volume (e.g., PSB-TCEP sample)	Acceptable protein amount per assay	Protein concentration quantification range (undiluted PSB-TCEP sample)
4	Bio-Rad DC Protein Assay*	100 µL of a 1:10 prediluted sample, corresponding to 10 µL original sample (standard assay) or	20 – 150 µg (standard)	2 – 15 µg/µL
		5 µL of a 1:10 prediluted sample, corresponding to 0.5 µL original sample (micro testtube assay)	1.0 – 7.5 µg (micro)	
5	Bio-Rad RC DC Protein Assay*	100 µL of a 1:5 prediluted sample, correspoding to 20 µL original sample or (standard assay)	20 – 150 µg (standard)	**1 – 7.5 µg/µL**
		25 µL of a 1:5 prediluted sample, corresponding to 5 µL original sample (micro testtube assay)	5.0 – 37.5 µg (micro)	
6	Serva ProtaQuant Assay Kit**	20 µL	5 – 35 µg	**0.25 – 1.75 µg/µL**
7	G-Bioscience SPNTM-Protein Assay**	1 – 10 µL	0.5 – 10 µg	**0.05 – 10 µg/µL**
8 a	Bio-Rad Protein Assay – Standard Assay Procedure* (Bradford)	100 µL of 1:20 prediluted sample, corresponding to 5 µL original sample	20 – 140 µg	**4 – 28 µg/µL**
8 b	Bio-Rad Protein Assay–Microassay Procedure* (Bradford)	Not recommended	–	–

Quantification of protein within the column flowthrough

Alternative to quantification of protein dissolved in PSB-TCEP, protein can be quantified within the ethanolic lysate column flowthrough. Knowledge of protein concentration in the column flowthrough helps to choose an appropriate volume for subsequent precipitation with Protein Precipatator PP. The following methods are suitable to quantify protein in the column flowthrough:

1: Bio-Rad Protein Assay (Bradford)

The standard assay procedure is compatibel with the column flowthrough, however, protein standards have to be prepared in an ethanolic lysate buffer (mix Buffer RP1 and ethanol (70 %) in a ratio of 1 :1). The microassay procedure is not compatible with the column-flowthrough!

2: Pierce BCA Protein Assay Kit (reducing agent compatible)

This method is compatible with the column flowthrough, however, protein standards have to be prepared in an ethanolic lysate buffer (mix Buffer RP1 and ethanol (70 %) in a ratio of 1 :1).

3: Bio-Rad DC Protein Assay

This method is compatible with the column flowthrough, however, protein standards have to be prepared in an ethanolic lysate buffer (mix Buffer RP1 and ethanol (70 %) in a ratio of 1:1).

4: Bio-Rad RC DC Protein Assay

This method is compatible with the column flowthrough, however, protein standards have to be prepared in an ethanolic lysate buffer (mix Buffer RP1 and ethanol (70 %) in a ratio of 1 :1).

5: Roti-Nanoquant Assay

This method is compatible with the column flowthrough, however, protein standards have to be prepared in an ethanolic lysate buffer (mix Buffer RP1 and ethanol (70 %) in a ratio of 1 :1).

Protein quantification in alternative protein pellet dissolution buffers

The use of the PSB or PSB-TCEP buffer is not mandatory for solving proteins.

Precipitated protein (protein pellets) may be dissolved in alternative solutions, such as **1 % SDS, 8 M urea**, or in **urea / thiourea / CHAPS buffers** as used for 2-D electrophoresis. However, depending on the target protein, the yield of solubilized protein may be reduced compared to PSB or PSB-TCEP. Check manufacturers product information to ensure compatibility of your protein quantification assay with your alternative protein dissolving solution.

Quantification of protein dissolved in sample buffer

Protein Quantification Assay (MACHEREY-NAGEL)

For most reliable results and convenience we recommend the MACHEREY-NAGEL Protein Quantification Assay to quantify protein dissolved in PSB, PSB-TCEP, or Laemmli buffer (for ordering information see section 6.4). Alternatively, the Karlsson protein quantification can be performed.

Protein quantification by Karlsson

The procedure presented below (based on the publication of Karlsson et al. 1994), is also suitable for quantification of protein dissolved in Protein Solving Buffer PSB-TCEP and may be followed alternatively.

Nucleic acids disturb protein quantification as described by Karlsson et al. 1994. Protein samples obtained with the NucleoSpin® RNA/Protein kit are virtually free of nucleic acids, thus, protein quantification is not affected.

Upon addition of TCA (Trichloracetic acid) to the sample, protein precipitates and causes turbidity. The degree of turbidity is used for quantification relative to a sample with known protein concentration. This test enables determination of protein concentration in the range 5 ng/μL–20 μg/μL by using variable sample volumes of 1 – 60 μL.

Recommended sample volume (protein dissolved in PSB-TCEP)	For protein concentration in the range of	Protein amount per well
60 μL	0.01 – 0.33 μg/μL	0.6 – 20 μg
20 μL	0.03 – 1.00 μg/μL	0.6 – 20 μg
1 μL	0.6 – 20 μg/μL	0.6 – 20 μg

For the primary determination of protein concentration of the sample measure different amounts, for example, 2 μL, 10 μL, and 50 μL. This assures that at least one value of the three amounts tested falls within the range of the calibration curve. Further, for a first rough estimation of expected protein yield also consider Table 4 in section 2.2 and Table 5 in section 2.4.

Materials

- TCA 60 % (Trichloracetic acid, not supplied)
- Protein Solving Buffer with reducing agent (PSB-TCEP, see note below)
- BSA (Bovine Serum Albumin, not supplied)
- Multititer plate (not supplied)

<u>Note:</u> The volume of PSB-TCEP, provided with the kit, might not be sufficient to quantify all isolated protein samples. Additional PSB-TCEP can either be ordered separately (see ordering information) or easily be prepared (see composition of PSB-TCEP below).

Composition of PSB-TCEP

- 125 mM BisTris (Bis-(2-hydroxyethyl)-imino-tris(hydroxymethyl)-methane)
- 2 % SDS (sodium dodecyl sulphate)
- 50 mM TCEP (Tris(2-carboxyethyl)phosphine Hydrochloride)
- 20 % glycerol
- 0.01 % bromophenol blue
- pH 6.8

Note: The composition of the previously used Protein Loading Buffer (PLB) has been improved and is now called Protein Solving Buffer PSB (reduced concentration of SDS and bromophenol blue).

The change in composition has increased the compatibility with protein quantification methods (see above). For details on the composition of previous Protein Loading Buffer (PLB) contact our technical service.

Method

Prepare a BSA stock solution with 40 mg/mL BSA in H_2O.

Prepare a BSA dilution series:

Tube	Add PSB to tube	Add BSA solution to tube	Resulting BSA concentration	Resulting BSA in 20 µL
#1	97.5 µL	2.5 µL BSA stock solution (40 mg/mL)	1 µg/µL	20 µg
#2	50 µL	50 µL from tube #1	0.5 µg/µL	10 µg
#3	50 µL	50 µL from tube #2	0.25 µg/µL	5 µg
#4	50 µL	50 µL from tube #3	0.125 µg/µL	2.5 µg
#5	50 µL	50 µL from tube #4	0.063 µg/µL	1.25 µg
#6	50 µL	50 µL from tube #5	0.031 µg/µL	0.625 µg
#7	50 µL	–	0 µg/µL	0 µg

Make sure that the protein concentration of your sample lies within the range of the largest (#1) and smallest (#6) value of the calibration curve in order to obtain valid measurements. Do not extrapolate beyond this range!

The prepared BSA dilution series is sufficient for subsequent determination of two calibration curves.

1	Add 20 µL of each dilution series sample #1 – 7 in microtiter plate wells.
2	Add 20 µL of samples (protein dissolved in PSB-TCEP) with unknown protein concentration to further wells (alternatively 1 – 60 µL).
3	Add 40 µL PSB-TCEP to each well. Final volume: 60 µL (alternatively add 0 – 59 µL if other volumes than 20 µL of sample are used in step 2).
4	Add 40 µL TCA (60 %) to each well.
5	Mix until complete colour change from blue to yellow.
6	Incubate for 30 min (± 3 min) at room temperature.
7	Measure extinction at 570 nm* .
8	Determine protein concentration of samples in relation to dilution series.

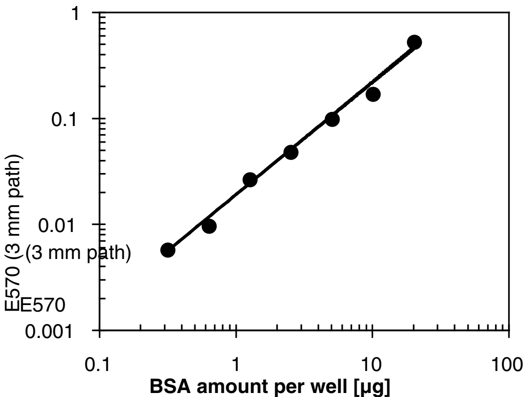

Figure 1 BSA standard curve for determination of protein in Protein Solving Buffer PSB.

* Measurement of extinction in the range of 530 – 700 nm is suitable and will typically result in correlation coefficients of 0.99 (concentration of BSA dilution series vs. obtained absorption values).

6.2 Troubleshooting

Problem	Possible cause and suggestions
RNA is degraded / no RNA obtained	*RNase contamination* • Create an RNase-free working environment. Wear gloves during all steps of the procedure. Change gloves frequently. Use of sterile, disposable polypropylene tubes is recommended. Keep tubes closed whenever possible during the preparation. Glassware should be oven-baked for at least 2 hours at 250 °C before use.
Poor RNA quality or yield	*Reagents not applied or restored properly* • Reagents not properly restored. Add the indicated volume of RNase-free water to rDNase vial and 96 % ethanol to Buffer RA3 Concentrate and mix. Reconstitute and store lyophilized rDNase according to instructions given in section 3. • Sample and reagents have not been mixed completely. Always vortex vigorously after each reagent has been added. • No ethanol has been added after lysis. Binding of RNA to the silica membrane is only effective in the presence of ethanol. *Kit storage* • Reconstitute and store lyophilized rDNase according to instructions given in section 3. • Store other kit components at room temperature. Storage at low temperatures may cause salt precipitation. • Keep bottles tightly closed in order to prevent evaporation or contamination. *Ionic strength and pH influence A_{260} absorption as well as ratio A_{260}/A_{280}* • For adsorption measurement, use 5 mM Tris pH 8.5 as diluent. Please see also: – Manchester, K L. 1995. Value of A_{260}/A_{280} ratios for measurement of purity of nucleic acids. Biotechniques 19, 208 – 209. – Wilfinger, W W, Mackey, K and Chomczyski, P. 1997. Effect of pH and ionic strength on the spectrophotometric assessment of nucleic acid purity. Biotechniques 22, 474 – 481.

Problem	Possible cause and suggestions
Poor RNA quality or yield (continued)	*Sample material* • Sample material was not stored properly. Whenever possible, use fresh material. If this is not possible, flash freeze the samples in liquid N2. Samples should always be kept at -70 °C. Never allow tissues to thaw before addition of Buffer RP1. Perform disruption of samples in liquid N2. • Insufficient disruption and/or homogenization of starting material: Ensure thorough sample disruption and use NucleoSpin® Filters for easy homogenization of disrupted starting material.
Clogged NucleoSpin® Column/Poor RNA quality or yield	*Sample material* • Too much starting material used: Overloading may lead to decreased overall yield. Reduce amount of sample material or use larger volume of Buffer RP1. • Insufficient disruption and/or homogenization of starting material: Ensure thorough sample disruption and use NucleoSpin® Filters/Filters L for easy homogenization of disrupted starting material.
Contamination of RNA with genomic DNA	*rDNase not active* • Reconstitute and store lyophilized rDNase according to instructions given in section 3. *rDNase solution not properly applied* • Pipette rDNase solution directly onto the center of the silica membrane. *Too much cell material used* • Reduce quantity of cells or tissue used.

Problem	Possible cause and suggestions
Contamination of RNA with genomic DNA (continued)	*DNA detection system too sensitive* • The amount of DNA contamination is effectively reduced during the on column digestion with rDNase. Anyhow, it can not be guaranteed that the purified RNA is 100 % free of DNA, therefore in very sensitive applications it might still be possible to detect DNA. The NucleoSpin® RNA / Plant system is checked by the following procedure: One million HeLa cells are subjected to RNA isolation according to the protocol. RNA eluate is used as template for PCR detection of a 1 kb fragment in a 30 cycle reaction. Generally, no PCR product is obtained while skipping the DNase digest usually leads to positive PCR results. The probability of DNA detection with PCR increases with: – the number of DNA copies per preparation: single copy target < plastidial / mitochondrial target < plasmid transfected into cells – decreasing of PCR amplicon size. • Use larger PCR targets (e.g., > 500 bp) or intron spanning primers if possible. • **Use support protocol 5.6 for subsequent rDNase digestion in solution.**
Suboptimal performance of RNA in downstream experiments	*Carry-over of ethanol or salt* • Do not let the flowthrough touch the column outlet after the second RA3 wash. Be sure to centrifuge at the corresponding speed for the respective time in order to remove ethanolic Buffer RA3 completely. • Check if Buffer RA3 has been equilibrated to room temperature before use. Washing at lower temperatures lowers efficiency of salt removal by Buffer RA3. *Store isolated RNA properly* • Eluted RNA should always be kept on ice for optimal stability since trace contaminations of omnipresent RNases (general lab ware, fingerprints, dust) will degrade the isolated RNA. For short term storage freeze at -20 °C, for long term storage freeze at -70 °C.
Trouble with resolubilization of precipitated protein in PSB-TCEP	*Protein pellets exceeding several millimeters in size are hard to dissolve.* • Use smaller volumes of column flowthrough for protein precipitation in order to obtain small sized pellets. Even invisible protein pellets commonly yield enough protein for SDS-PAGE and Western Blot analysis.

Problem	Possible cause and suggestions
Protein dissolved in PSB-TCEP escapes SDS-PAGE gel slot immediately after loading	*Protein pellet has not been dried sufficiently and contains residual ethanol.* • Increase drying time or decrease pellet size by precipitating a smaller volume of column flowthrough.
Unclear results with commonly used protein quantification systems	*Most commonly used protein quantification systems are incompatible with concentrations of SDS and/or reducing agents present in Protein Loading Buffer.* • Use a suitable quantification method as described in section 6.1. • If an other protein dissolution buffer than PSB or PSB-TCEP has used for dissolving the protein pellet, ensure compatibility of your buffer and quantification method of choice.
No protein precipitate (pellet) visible	*A small sample amount was used and/or a small volume of column flowthrough was used for precipitation.* • Formation of a visible protein pellet is not required for sufficient protein recovery. Even invisible protein pellets commonly yield enough protein for SDS-PAGE and Western Blot analysis.
PSB-TCEP turbid or partially solidified	*PSB-TCEP may form a precipitate at temperatures below 18 °C.* • Warm up ≥ 25 °C to dissolve turbidity completely.
No/low protein yield	*Protein was resolubilized in water* • Due to the strongly denatured form of the protein, the solubility in water is significantly reduced. Use PSB-TCEP for protein solubilization.

6.3 References

The following publications cite the use of the NucleoSpin® RNA/Protein kit:

Rodríguez-Jiménez FJ, Moreno-Manzano V, Lucas-Dominguez R, and Sánchez-Puelles JM (2008): Hypoxia Causes Down-Regulation of Mismatch Repair System and Genomic Instability in Stem Cells. Stem Cells, May 2008; 10.1634/stemcells. 2007 – 1016.

Bahn A, Hagos Y, Reuter S, Balen D, Brzica H, Krick W, Burckhardt BC, Sabolic I, and Burckhardt G (2008): Identification of a new urate and high affinity nicotinate transporter – human organic anion transporter 10 (hOAT10, SLC22A13). J. Biol. Chem. published 14 April 2008, 10.1074/jbc.M800737200

Weiske J, Albring KF, and Huber O (2007): The tumor suppressor Fhit acts as a repressor of ß-catenin transcriptional activity. PNAS, Dec 2007; 104: 20344 – 20349.

The following publications show the general usefulness of the parallel extraction of DNA, RNA, and protein from small and precious samples:

Coombs LM, Pigott D, Proctor A, Eydmann M, Denner J, and Knowles MA (1990): Simultaneous isolation of DNA, RNA, and antigenic protein exhibiting kinase activity from small tumor samples using guanidine isothiodyanate. Analytical Biochemistry 188, 338 – 343.

Banerjee S, Smallwood A, Chambers AE, and Nicolaides K (2003): Quantitative recovery of immunoreactive proteins from clinical samples following RNA and DNA isolation. BioTechniques 35 (3), 450 – 456.

Hoemann CD, Sun J, Chrzanowski V, and Buschmann MD (2002): A multivalent assay to detect glycosaminoglycan, protein, collagen, RNA, and DNA content in milligam samples of cartilage or hydrogel-based repair cartilage. Analytical Biochemistry 300, 1 – 10.

The following publication decribes the Reducing Agent TCEP:

Getz EB, Xiao M, Chakrabarty T, Cooke R and Selvin PR (1999): A comparison between the sulfhydryl reductants Tris(2-carboxyethyl)phosphine and Dithiothreitol for use in protein biochemistry. Analytical Biochemistry 273, 73 – 80.

The following publication describes a method for quantification of protein dissolved in sampel buffer such as PSB:

Karlsson JO, Ostwald K, Kabjörn C, and Andersson M (1994): A method for protein assay in Laemmli buffer. Analytical Biochemistry 219, 144 – 146.

6.4 Ordering information

Product	REF	Pack of
NucleoSpin® RNA/Protein	740933.10/.50/.250	10/50/250 preps
Protein Quantification Assay	740967.50/.250	50/250 preps
NucleoSpin® RNA	740955.10/.50/.250	10/50/250 preps
NucleoSpin® RNA XS	740902.10/.50/.250	10/50/250 preps
NucleoSpin® RNA Midi	740962.20	20 preps
NucleoSpin® RNA Clean-up	740948.10/.50/.250	10/50/250 preps
NucleoSpin® RNA Clean-up XS	740903.10/.50/.250	10/50/250 preps
NucleoSpin® RNA/DNA Buffer Set	740944	100 preps
NucleoSpin® RNA Blood	740200.10/.50	10/50 preps
Buffer RP1	740934.50	50 mL
Buffer RP1	740934.500	500 mL
Protein Solving Buffer Set PSB/TCEP	740941	1
TCEP	740395.107	107 mg
rDNase Set	740963	1
NucleoSpin® Filters	740606	50
Collection Tubes (2 mL)	740600	1000
Porablot		See price list
Blotting Paper		See price list

6.5 Product use restriction / warranty

All MACHEREY-NAGEL products are designed for their intended use only. They are not intended to be used for any other purpose. The description of the intended use of the products can be found in the original MACHEREY-NAGEL product leaflets. Before using our products, please observe the instructions for use and the safety instructions from the respective Material Safety Data Sheet of the product.

This MACHEREY-NAGEL product is carrying documentation stating specifications and other technical information. MACHEREY-NAGEL warrants to meet the stated specifications. The provided warranty is limited to the data specifications and descriptions as given in the original MACHEREY-NAGEL literature. No other statements or representations, written or oral, by MACHEREY-NAGEL's employees, agents or representatives, except written statements signed by a duly authorized officer of MACHEREY-NAGEL are authorized. They should not be relied upon by the costumer and are not a part of a contract of sale or of this warranty.

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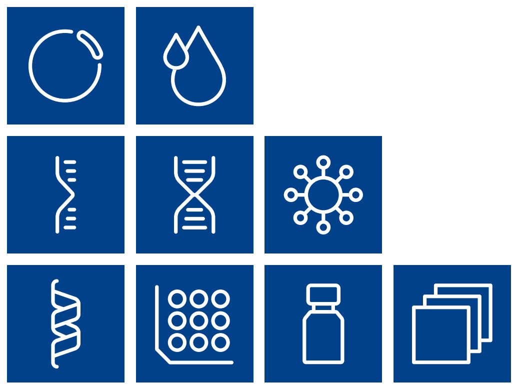
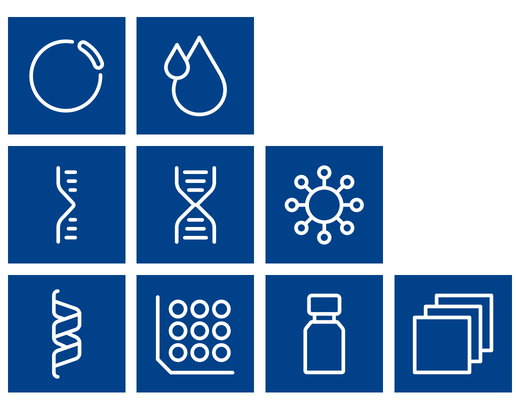


Plasmid DNA
Clean up
RNA
DNA
Viral RNA and DNA
Protein
High throughput
Accessories
Auxiliary tools



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