Bioanalysis

MACHEREY-NAGEL

User manual

Total DNA, RNA, and protein isolation

■ NucleoSpin® TriPrep

November 2023 / Rev. 07

Total DNA, RNA, and protein isolation

Protocol at a glance (Rev. 07)

NucleoSpin® TriPrep

1	Homogenize sample	Up to 30 mg
2	Lyse sample	350 µL RP1 3.5 µL ß-mercaptoethanol (or comparable reducing agent)
3	Filtrate lysate	1 min, 11,000 x *g*
4	Adjust DNA and RNA binding conditions	350 µL ethanol (70 %)
5	Bind DNA and RNA	Load sample 30 s, 11,000 x *g*

DNA and RNA Purification
(both bound to the silica membrane)

6	Wash silica membrane	1st and 2nd wash each: 500 µL ***DNA Wash*** 1 min, 11,000 x *g*
7	Dry membrane	RT, 3 min (with open lid)
8	Elute DNA	100 µL ***DNA Elute*** Incubate 1 min 1 min, 11,000 x *g*
9	Digest residual DNA	95 µL DNase reaction mixture RT, 15 min
10	Wash and dry silica membrane	1st wash 200 µL RA2 2nd wash 600 µL RA3 30 s, 11,000 x *g* 3rd wash 250 µL RA3 2 min, 11,000 x *g*
11	Elute highly pure RNA	60 µL H_2O (RNase-free) 1 min, 11,000 x *g*

Protein Purification
(protein in the column flow-through)

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

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Table of contents

1 Components

1.1 Kit contents

	NucleoSpin® TriPrep		
REF	**10 preps 740966.10**	**50 preps 740966.50**	**250 preps 740966.250**
Lysis Buffer RP1	10 mL	25 mL	125 mL
Buffer DNA Wash (Concentrate)*	12 mL	12 mL	5 × 12 mL
Buffer DNA Elute	1.2 mL	12 mL	3 × 12 mL
Wash Buffer RA2	15 mL	15 mL	80 mL
Wash Buffer RA3 (Concentrate)*	6 mL	12 mL	3 × 25 mL
RNase-free H_2O	13 mL	13 mL	60 mL
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
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)
NucleoSpin® Filters (violet rings)	10	50	250
NucleoSpin® TriPrep 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

- 50 % ethanol (to prepare Buffer DNA Wash)
- 70 % ethanol (to adjust RNA binding conditions)
- 96 – 100 % ethanol (to prepare Wash Buffer RA3)
- 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 and DNA elution)
- Disposable RNase-free pipette 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® TriPrep** 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 combined with verification of genomic sequence (e.g., transgene integration site) are often hampered by the small sample size and the necessity of different – often incompatible – techniques for DNA, RNA, and protein isolation. Samples may comprise biopsies, tumors, tissues, transgene organisms, and others. The **NucleoSpin® TriPrep** kit enables isolation of DNA, RNA, and protein from diverse sample types. DNA, RNA, and protein are isolated without splitting the sample prior to extraction. Thus, DNA, RNA, and protein are obtained from one and the same sample and not from three similar portions of one sample. This is especially valuable for unique, small, and precious samples. DNA and RNA are eluted separately from the NucleoSpin® TriPrep Column, with a low salt buffer and water, respectively. Isolated DNA and RNA are suitable for all common downstream applications. Isolated protein is suitable for SDS-PAGE, Western Blot analysis, and quantification.

DNA, RNA, and protein isolation

One of the most important aspects in the isolation of DNA, RNA, and protein is to prevent their degradation during the isolation procedure. With the **NucleoSpin® TriPrep** 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., DNases, RNases, proteases, and phosphatases) which are present in almost all biological materials. The buffer dissolves even hardly soluble protein, creates appropriate binding conditions which favor adsorption of DNA and RNA to the silica membrane, and enables protein to pass the specially treated NucleoSpin® TriPrep Column virtually quantitatively. Expensive and harmful proteinase inhibitors or inhibitor cocktails are not necessary due to the denaturing properties of the lysis buffer. After two special washing steps, DNA is eluted with a low salt buffer (DNA Elute) which selectively elutes DNA and keeps RNA quantitatively on the column. Eluted DNA is immediately ready for downstream applications without further purification. DNA elution prior to RNA elution does neither compromise RNA quantity nor quality. RNA isolated with the **NucleoSpin® TriPrep** kit is of identical quality as RNA isolated with the well proven NucleoSpin® RNA kit. Residual DNA still 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.**

DNA, RNA, and protein preparation using 1 kits can be performed at room temperature. The DNA and RNA eluates, 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. DNA can be stored at 4 °C for short term and at -20 °C for long term storage. 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.

2.2 Kit specifications

- **NucleoSpin® TriPrep** kits are recommended for the isolation of total DNA, RNA, and protein from cultured cells, tissue, and other biological samples.

Table 1: Kit specifications at a glance

Parameter	NucleoSpin® TriPrep		
Format	Mini spin column		
Sample material	< 5 × 10^6 cultured cells*, < 30 mg human / animal tissue*, < 100 mg plant tissue*		
	Total RNA	**Total DNA**	**Total protein**
Fragment size	> 200 b	< 30 kbp	15 – 300 kDa
Typical yield	< 70 µg	< 6 µg	< 1200 µg
A_{260}/A_{280}	1.9 – 2.1	1.7 – 1.9	–
Typical RIN (RNA integrity number)	> 9	–	–
Elution volume (RNA and DNA) Resolubilization volume (protein)	40 – 120 µL	100 µL	10 – 100 µL
Preparation time	30 min/6 preps	45 min/6 preps (RNA + DNA)	35 min/6 preps
Binding capacity	200 µg	10 µg**	–
Use	For research use only		

DNA characteristics

- Isolated DNA is of high molecular weight and typically exceeds 20 kb.
- DNA is commonly stable, even at 37 °C for 2 h with or without addition of a typical restriction enzyme buffer, showing the absence of DNases.
- DNA is digestable with restriction enzymes and suitable for PCR.

* For samples larger than approx. one million cells or 5 mg tissue, DNA yield does not increase lineary with the sample amount. DNA yield may decrease utilizing five million cells, 30 mg tissue, or more. Typically however, DNA yield is still sufficient for PCR analysis.

** Binding capacity of DNA ≤ 10 µg, strongly depending on RNA amount bound to the membrane

RNA characteristics

- The **NucleoSpin® TriPrep** kit allows 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.
- RNA of high integrity can be isolated with NucleoSpin® TriPrep kit. RIN (RNA Integrity Number) of RNA isolated from fresh high quality sample material (e.g., eukaryotic cells or fresh mouse liver) generally exceeds 9.0. However, RNA integrity strongly depends on the sample quality. RNA integrity was examined using the Agilent 2100 Bioanalyzer in conjunction with the RNA 6000 Nano or Pico assay.
- RNA prepared with **NucleoSpin® TriPrep** 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).

Protein characteristics

- Small (17 kDa) to large (250 kDa) proteins, as well as glycoproteins, membrane proteins, lipoproteins, phosphorylated proteins, and structural proteins have been analyzed successfully.
- The isolated protein is ready to use for SDS-PAGE, Western Blot analysis and protein quantification with the Protein Quantification Assay (see ordering information).

Typical yields of DNA, RNA, and protein

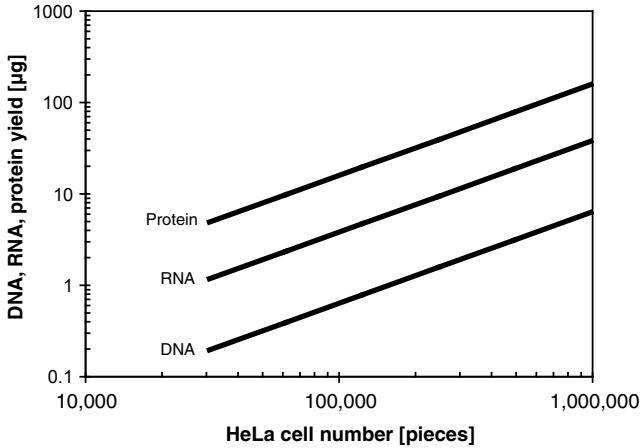


Figure 1 DNA, RNA, and protein yield from different amounts of HeLa cells
DNA, RNA, and protein were isolated from different amounts of HeLa cells. For cell numbers exceeding 250,000 only a fraction of the protein solution was used for precipitation and quantification; total protein yield was calculated.

DNA, RNA, and protein were isolated as described in Figure 1. Obtained correlation coefficients between HeLa sample amount and DNA, RNA, and protein yield are shown in Table 2.

Table 2: Correlation between sample amount, nucleic acid and protein yield

	$3 \times 10^4 - 5 \times 10^5$ cells	$3 \times 10^4 - 1 \times 10^6$ cells
DNA	> 0.99	> 0.95
RNA	> 0.98	> 0.98
Protein	> 0.99	> 0.99

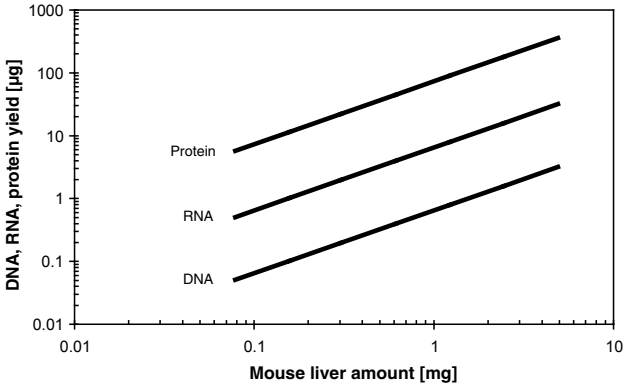


Figure 2 DNA, RNA, and protein yield from different amounts of mouse liver tissue
DNA, RNA, and protein were isolated from different amounts of mouse liver tissue. For tissue amounts exceeding 2.5 mg only a fraction of the protein solution was used for precipitation and quantification; total protein yield was calculated.

DNA, RNA, and protein were isolated as described in Figure 2. Obtained correlation coefficients between sample amount and DNA, RNA, and protein yield are shown in Table 3.

Table 3: Correlation between sample amount, nucleic acid and protein yield

	0.08 – 1.25 mg mouse liver	**0.08 – 2.5 mg mouse liver**	**0.08 – 5 mg mouse liver**
DNA	> 0.99	> 0.95	> 0.67
RNA	> 0.98	> 0.98	> 0.98
Protein	> 0.99	> 0.99	> 0.99

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 MACHEREY-NAGEL's Protein Quantification Assay (see ordering information) and shall serve as a guideline for expected protein yield. It is assumed that the complete sample amount is processed, that is 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 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** or by passing ≥ 5 times 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 RNA is not degraded by RNases before the preparation starts. 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 yeast have to be incubated in lysozyme or lyticase / zymolase solutions, respectively. 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 using a NucleoSpin® Filter or the syringe-needle method.

2.4 Guideline for appropriate sample amount, precipitation volume, 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	350 µL	350 µL	350 µL	350 µL	350 µL	350 µL	350 µL	350 µL
Ethanol (70 %)	350 µL	350 µL	350 µL	350 µL	350 µL	350 µL	350 µL	350 µL	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 analyzed by SDS-PAGE with Coomassie stain	10 µL	10 µL	10 µL	10 µL	10 µL	10 µL	10 µL	10 µL	10 µL
Protein sample to be analyzed by SDS-PAGE with silver stain	1 µL	1 µL	1 µL	1 µL	1 µL	1 µL	1 µL	1 µL	1 µL
Protein sample to be analyzed by Western Blot	1 – 10 µL	1 – 10 µL	1 – 10 µL	1 – 10 µL	1 – 10 µL	1 – 10 µL	1 – 10 µL	1 – 10 µL	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® TriPrep 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) 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 for DNA

Elution of DNA is carried out under selective conditions to make sure that only DNA is eluted while RNA is still bound to the membrane. The DNA washing solution DNA Wash and the DNA elution buffer DNA Elute are finely tuned to achieve this. Therefore, the DNA elution volume should only be altered moderately, in the range of 60 – 150 µL. Furthermore, the temperature of the DNA Elute solution shall not exceed 30 °C, otherwise RNA will partly elute with the DNA Elute solution. DNA Elute solution may stay for 1 min up to 15 min on the column, a 1 – 5 min incubation time is recommended. Eluted DNA is immediately ready for downstream applications without further purification.

2.6 Elution procedures for RNA

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 and to avoid RNA degradation by almost omnipresent RNases (general lab ware, fingerprints, dust). 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 and RA2 contain chaotropic salt. Wear gloves and goggles!

CAUTION: Buffers RP1 and RA2 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.

Storage conditions:

- 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 room temperature (18 – 25 °C) and are stable up to one year. Storage at lower temperatures may cause precipitation of salts.

Before starting any **NucleoSpin® TriPrep** protocol prepare and consider the following:

- 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 and to prepate Buffer DNA Wash.
- Check that 96 – 100 % ethanol is available to prepare Wash Buffer RA3.
- The **DNA Wash** solution is delivered as a concentrate. To prepare the final DNA Wash solution add four volumes of 50 % ethanol to the DNA Wash Concentrate as indicated in the table below. Mark the label of the bottle to indicate that the ethanol is added. Keep the bottle tightly close to avoid evaporation of ethanol.
- Due to its composition the **DNA Elute** solution (DNA elution buffer) does not inhibit DNases, that is DNA Elute does not contain substances (e.g., EDTA) to complex divalent cations. Therefore, be aware not to contaminate DNA Elute with DNases! Further, due to its composition, DNA Elute solution does not inhibit microbial growth. Therefore, make sure not to contaminate DNA Elute with any source of microbial contamination.
- **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 -18 °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 room temperature (18 – 25 °C) for up to one year. Keep the bottle tightly close to avoid evaporation of ethanol.

- **Protein Solving Buffer PSB and Reducing Agent TCEP:** For SDS-PAGE under reducing conditions (most common type of SDS-PAGE) transfer PSB from one vial to one vial of the Reducing Agent TCEP. Mix gently to avoid excessive foaming until the reducing agent is dissolved completely (this process will require several minutes)*. Protein Solving Buffer containing Reducing Agent TCEP (PSB-TCEP) is stable for several days at room temperature (18 – 25 °C) and several months at 4 °C. For long term storage of PSB-TCEP keep at -20 °C.
- If SDS-PAGE under non-reducing conditions is intended consider the following:
  A: Omit addition of the Reducing Agent TCEP to Buffer PSB.
  B: Omit addition of ß-mercaptoethanol (or other reducing agent) 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® TriPrep		
REF	**10 preps** **740966.10**	**50 preps** **740966.50**	**250 preps** **740966.250**
Buffer DNA Wash (Concentrate)	12 mL Add 48 mL 50 % ethanol	12 mL Add 48 mL 50 % ethanol	5 × 12 mL Add 48 mL 50 % ethanol to each vial
Wash Buffer RA3 (Concentrate)	6 mL Add 24 mL 96 – 100 % ethanol	12 mL Add 48 mL 96 – 100 % ethanol	3 × 25 mL Add 100 mL 96 – 100 % 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	2 × 107 mg Add 7.5 mL PSB each

* For 50 and 250 prep kits: For better handling, PSB-TCEP may be transferred into the original PSB vial (withscrew cap).

4 Safety instructions

When working with the **NucleoSpin® TriPrep** 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 http://www.mn-net.com/msds).

Caution: Guanidinium thiocyanate in buffer RA2 and RP1 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® TriPrep** 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 and Proteinase K treatment but it cannot be excluded completely. Therefore, liquid waste must be considered infectious and should be handled and discarded according to 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 DNA, RNA, and protein purification from cultured cells and tissue

Joint protocol steps for DNA, RNA, and protein purification.

DNA purification: steps 1 – 8

RNA purification: steps 1 – 11

Protein purification: steps 1 – 5 and 12 – 15

Before starting the preparation:

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

1 Homogenize 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).

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

Disrupt sample

2 Lyse sample

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

<u>Note:</u> 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 (e.g. ad 7 – 14 µL of a 500 mM DTT or TCEP solution).

+ 350 µL RP1

+ 3.5 µL ß-ME

3 Filtrate lysate

Reduce viscosity and clear the lysate by filtration through NucleoSpin® Filter: Place **NucleoSpin® Filter (violet ring)** in a Collection Tube (2 mL), 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.

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).

<u>Important:</u> To process higher amounts of cells (> 1 × 10^6) or tissue (> 10 mg), the lysate should first be homogenized using the 0.9 mm needle (20 gauge), followed by filtration through NucleoSpin® Filter.

1 min 11,000 x *g*

4 Adjust DNA and RNA binding conditions

+ 350 µL 70 % EtOH

Mix

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).

After addition of ethanol a stringy precipitate may become visible which will not affect the RNA isolation. Make sure to disaggregate any precipitate by mixing and 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 DNA and RNA

Load sample

30 s
11,000 x *g*

For each preparation, take one **NucleoSpin® TriPrep 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® TriPrep Column in a new Collection Tube (2 mL).

RNA and DNA are bound to the column membrane. Protein is in the flowthrough.

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

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

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

For **protein** isolation recover flowthrough and continue with step 12.

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

6 Wash silica membrane

1st wash

Add **500 µL DNA Wash** to the NucleoSpin® TriPrep Column. Centrifuge for **1 min** at **11,000 x *g***. Discard flowthrough and reuse the Collection Tube.

+ 500 µL DNA Wash

1 min 11,000 x *g*

2nd wash

Add again **500 µL DNA Wash** to the NucleoSpin® TriPrep Column. Centrifuge for **1 min** at **11,000 x *g***. Discard Collection Tube with flowthrough.

Chaotropic salt is removed by these washing steps. DNA and RNA are still bound to the membrane. The membrane is prepared for subsequent DNA elution.

+ 500 µL DNA Wash

1 min 11,000 x *g*

7 Dry membrane

Insert the NucleoSpin® TriPrep Column into a 1.5 mL microcentrifuge tube (not provided). Open the lid of the column and let it stand for 3 min.

This step ensures removal of residual ethanol.

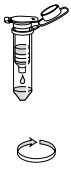
RT 3 min

(with open lid)

8 Elute DNA

Add **100 µL DNA Elute** directly onto the membrane and **incubate** for **1 min**. Elute DNA by centrifugation for **1 min** at **11,000 x *g***.

The temperature of the DNA Elute solution shall not exceed 30 °C, otherwise RNA will partly elute with the DNA Elute solution. DNA Elute solution may stay for 1 min up to 15 min on the column before DNA is eluted. A 1 – 5 min incubation time is recommended. Eluted DNA is immediately ready for downstream applications without further purification.



+ 100 µL DNA Elute

Incubate 1 min

1 min 11,000 x *g*

Further steps for RNA purification (steps 9 – 11)

9 Digest residual DNA on-column

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**.

Do not centrifuge, directly proceed with step 10. Depending on sample type and amount approximatively 50 – 90 % of the DNA is eluted in the DNA elution step. Residual DNA on the column is digested on-column with rDNase.

+ 95 µL rDNase reaction mixture

RT 15 min

10 Wash and dry silica membrane

+ 200 µL RA2

30 s
11,000 x *g*

1st wash

Add **200 µL Buffer RA2** to the NucleoSpin® TriPrep Column. Centrifuge for **30 s** at **11,000 x *g***. Place the NucleoSpin® TriPrep Column into a new Collection Tube (2 mL).

Buffer RA2 will inactivate the rDNase.

+ 600 µL RA3

30 s
11,000 x *g*

2nd wash

Add **600 µL Buffer RA3** to the NucleoSpin® TriPrep Column. Centrifuge for **30 s** at **11,000 x *g***. Discard flowthrough and place the NucleoSpin® TriPrep Column back into the Collection Tube.

+ 250 µL RA3

2 min
11,000 x *g*

3rd wash

Add **250 µL Buffer RA3** to the NucleoSpin® TriPrep Column. Centrifuge for **2 min** at **11,000 x *g*** to dry the membrane completely. Place the NucleoSpin® TriPrep 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® TriPrep Column after centrifugation, discard flowthrough and centrifuge again.

11 Elute highly pure RNA

+ 60 µL RNase-free H_2O

Incubate 1 min

1 min
11,000 x *g*

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. Overall yield, however, will decrease when using smaller volumes.

For further alternative elution procedures see section 2.6.

Further steps for protein purification (steps 12 – 15)

Use the NucleoSpin® TriPrep Column flowthrough from step 5 (i.e., the ethanolic lysate which has been passed throught the NucleoSpin® TriPrep Column) as starting point for protein precipitation.

12 Precipitate protein

Transfer an appropriate amount (10 – 700 µL) of flowthrough 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***.

13 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 consistence of 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 (for e.g. 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 pipette tip during the washing step. See also section 2.4 how to avoid very large protein pellets.

14 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 from a 30 mg liver sample) need longer time for drying. 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.

15 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 pipette 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 overal yield of solubilized protein may be reduced compared to PSB or PSB-TCEP used as dissolving agent.

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

Before starting the preparation:

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

1 Prepare sample

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

2 Lyse sample

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).

Note: 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® TriPrep standard protocol (section 5.1).

5.3 rDNase digestion in solution

The on-column rDNase digestion in the standard protocol is very efficient and results in minimal residual DNA content of the purified RNA. This DNA will not be detectable in most downstream applications, but 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® TriPrep 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 Incubate sample

Incubate for **10 min** at **37 °C**.

C Repurify 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, section 6.4) 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.

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 sensitivies 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 28

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.

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
4	Bio-Rad DC Protein Assay*	100 µL of a 1:10 prediluted sample, corresponding to 10 µL original sample (standard assay) or 5 µL of a 1:10 prediluted sample, corresponding to 0.5 µL original sample (micro testtube assay)	20 – 150 µg (standard) 1.0 – 7.5 µg (micro)	2 – 15 µg/µL

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

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

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)
5	Bio-Rad RC DC Protein Assay*	100 µL of a 1:5 prediluted sample, correspoding to 20 µL original sample or (standard assay) 25 µL of a 1:5 prediluted sample, corresponding to 5 µL original sample (micro testtube assay)	20 – 150 µg (standard) 5.0 – 37.5 µg (micro)	1 – 7.5 µg/µL
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
8a	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
8b	Bio-Rad Protein Assay – Microassay Procedure* (Bradford)	Not recommended	–	–

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

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

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. Knowlege 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 a ethanolic lysate buffer (mix Buffer RP1 and ethanol (70 %) in a ratio of 1:1). The microassay procedure is not compatible the column-flowthrough!

2: Pierce BCA Protein Assy Kit (reducing agent compatible)
This method is compatible with the column flowthrough, however, protein standards have to be prepared in a 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 a 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 a 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 a 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**, or **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 quantifiction 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)

Note: 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.

* 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).

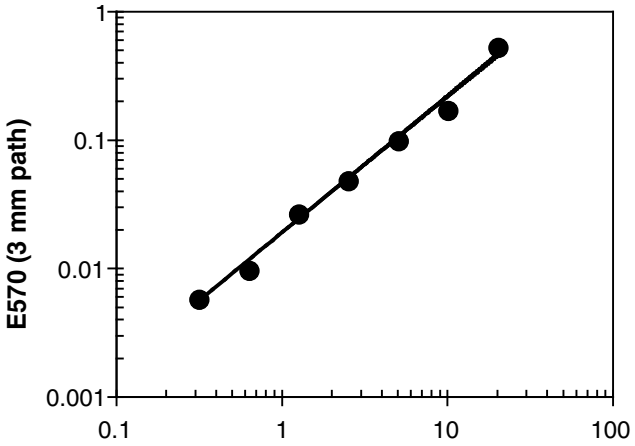


Figure 3 BSA standard curve for determination of protein in Protein Solving Buffer PSB.

6.2 Troubleshooting

Problem	Possible cause and suggestions
DNA is contaminated with RNA	*Buffer temperature* • DNA elution buffer DNA Elute exceeded 30 °C during application. Use DNA Elute with a temperature preferentially of 18 – 25 °C.
DNA yield lower than RNA yield	*Sample material* • DNA and RNA yield depend very much on sample material. Ratio of RNA yield to DNA yield may vary from approximately 1 – 20.
DNA degrades upon storage	*DNase contamination* • DNA elution buffer DNA Elute does not contain divalent cation complexing substances (e.g., EDTA). Therefore, DNA is not protected against DNases. Keep DNA Elute solution clean and avoid any contamination. As a precaution, keep DNA on ice for short term or at -20 °C for long term storage. • Some sample materials may contain DNase traces which are not sufficiently washed away by the standard procedure. Perform a wash step of the column with Buffer RA2 after loading the lysate onto the column and before starting the washing steps with DNA Wash solution: add 500 µL Buffer RA2 onto the column, centrifuge 1 min at 11,000 x *g* and continue with DNA Wash washing steps.
Suboptimal performance of DNA in downstream application	*Divalent cations* • Eluted DNA contains small amounts of divalent cations. If the downstream application comprises, for example 50 % DNA eluate of the final reaction volume the divalent cations introduced into the reaction by the DNA eluate may alter the performance. Decrease the divalent cation concentration of the reaction by 1 – 5 mM for compensation.
Low DNA yield for large sample amounts	*Sample amount to large* • Depending on the type of sample and its DNA content, DNA yield may not increase proportional to increased sample amount. Sample amounts larger than, for example 5 mg tissue or 106 cultured cells may yield less DNA than smaller sample amounts. Use smaller sample to ensure good correlation between sample amount and DNA yield.
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.

Problem	Possible cause and suggestions
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, KL, 1995. Value of A_{260}/A_{280} ratios for measurement of purity of nucleic acids. Biotechniques 19, 208 – 209. - Wilfinger, WW, Mackey, K and Chomczyski, P, 1997. Effect of pH and ionic strength on the spectrophotometric assessment of nucleic acid purity. Biotechniques 22, 474 – 481. *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 for easy homogenization of disrupted starting material.

Problem	Possible cause and suggestions
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. *DNA detection system too sensitive* • The amount of DNA contamination is significantly reduced during the on-column rDNase digestion. Anyhow we can not guarantee that the purified RNA is 100 % free of DNA. In very sensitive applications it might be possible to detect DNA. The NucleoSpin® TriPrep 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 fragment is obtained if the rDNase is applied, however, a strong PCR fragment is obtained if rDNase is omitted. 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 PCR amplicon size. • Use larger PCR targets (e.g., > 500 bp) or intron spanning primers if possible. • Use support protocol 5.3 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.

Problem	Possible cause and suggestions
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.
Protein dissolved in PSB-TCEP flows out of 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 Solving Buffer.* • Use a suitable quantification method as described in section 6.1 . • If an other protein dissolution buffer than PSB or PSB-TCEP was 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 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 publications cite the use of the NucleoSpin® kits for DNA, RNA, and protein isolation:

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 publication describes 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 sample buffer such as PSB:

1 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® TriPrep*	740966.10/.50/.250	10/50/250
NucleoSpin® RNA/Protein	740933.10/.50/.250	10/50/250
Protein Quantification Assay	740967.50/.250	50/250
NucleoSpin® RNA	740955.10/.50/.250	10/50/250
NucleoSpin® RNA XS	740902.10/.50/.250	10/50/250
NucleoSpin® RNA Midi	740962.20	20
NucleoSpin® RNA Clean-up	740948.10/.50/.250	10/50/250
NucleoSpin® RNA Clean-up XS	740903.10/.50/.250	10/50/250
NucleoSpin® RNA/DNA Buffer Set*	740944	100
Buffer RP1	740934.50	50 mL
Buffer RP1	740934.500	500 mL
Protein Solving Buffer Set PSB/TCEP	740941	1 set
rDNase Set	740963	1 set
NucleoSpin® Filters	740606	50
Collection Tubes (2 mL)	740600	1000
Porablot	See price list	
Blotting Paper	See price list	

Visit ***www.mn-net.com*** for more detailed product information.

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.

Liability for all possible damages that occur in any connection with our products is limited to the utmost minimum as stated in the general business terms and conditions of MACHEREY-NAGEL in their latest edition which can be taken from the company's website. MACHEREY-NAGEL does not assume any further warranty.

Products and their application are subject to change. Therefore, please contact our Technical Service Team for the latest information on MACHEREY-NAGEL products. You may also contact your local distributor for general scientific information. Descriptions in MACHEREY-NAGEL literature are provided for informational purposes only.

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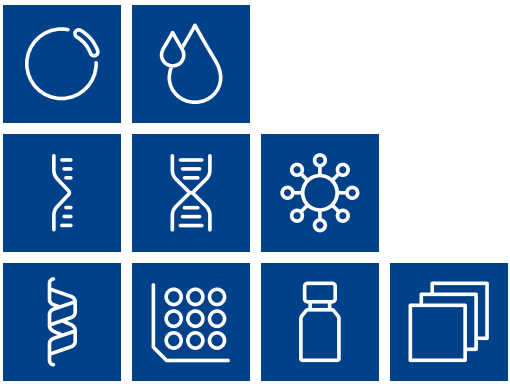
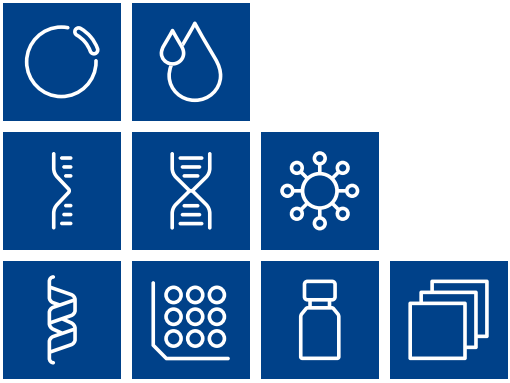


Plasmid DNA
Clean up
RNA
DNA
Viral RNA and DNA
Protein
High throughput
Accessories
Auxiliary tools



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