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This MACS® product is for *in vitro* research use only and not for diagnostic or therapeutic procedures.

µMACS™ One-step T7 Template Kit

User manual

Order no. 130-092-866

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The cover photo shows a replica of the DNA model built in 1953 by James D. Watson and Francis Crick at the Cavendish Laboratory in Cambridge. This model is located at Heureka, the Finnish Science Centre. Photography by Alexander Budde; © Miltenyi Biotec GmbH, Germany. Detailed information on the history of the Watson-Crick model can be found in: de Chadarevian, S. (2003) Relics, replicas and commemorations. Endeavour 27: 75–79.

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1. Description

1.1 Components and size

Kit for 20 reactions includes

Reagent Box

- 0.5 mL Oligo(dT)-T7 MicroBeads
- 40 mL Lysis/Binding Buffer: high-salt buffer containing 1% SDS
- 20 mL Wash Buffer: low-salt buffer containing NaCl, Tris-HCl, and EDTA
- 15 mL Equilibration/Wash Buffer
- 2×1 mL Elution Buffer: RNase-free H₂O
- 0.5 mL Resuspension Buffer I for lyophilized First-strand cDNA Mix
- 0.5 mL Resuspension Buffer II for lyophilized Second-strand cDNA Mix
- 20×Lyophilized First-strand cDNA Mix containing Reverse Transcriptase and dNTPs
- 20×Lyophilized Second-strand cDNA Mix containing E.coli DNA Polymerase I, E.coli DNA Ligase, RNase H, and dNTPs
- 100 µL IVT Enhancer
- 100 µL Sealing Solution

Column Box

- 20 µ Columns
- 20 LysateClear Columns

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Product format Oligo(dT)-T7 MicroBeads: non-sedimenting MicroBeads conjugated to oligo (dT)₂₃-T7. Suspension contains 0.1% SDS.

LysateClear Columns: maximal reservoir volume 1 mL.

First-strand cDNA Mix, lyophilized: 20 single reaction mixes, reaction mix omitted in the four corner wells.

Second-strand cDNA Mix, lyophilized: 20 single reaction mixes, reaction mix omitted in the four corner wells.

All buffers and MACS® Columns included in the μ MACS™ One-step T7 Template Kit are evaluated for the absence of RNase activity.

Storage Store Reagent Box containing lyophilized enzyme mixes, buffers, and MicroBeads protected from light at 4–8 °C. Do not freeze.

Store Column Box with μ Columns and LysateClear Columns at room temperature, dry and protected from light. The expiration dates are indicated on the labels.

1.2 MACS® Technology for antisense RNA (aRNA) synthesis

Different cell types, or different developmental states of the same cell type, exhibit distinct gene expression patterns. Methods like microarray analysis are commonly used to investigate such expression profiles. For these, microgram amounts of RNA are required. Thus, one of the most frequently emerging issues for researchers today is to generate sufficient RNA amounts from small biological samples, such as biopsies, microdissections or isolated, rare cells.

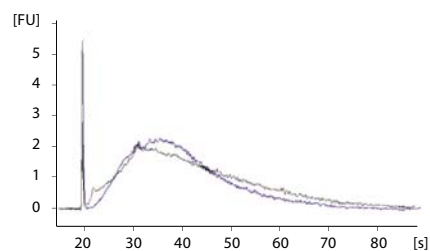


Figure 1A: Comparison of traditional versus μ MACS™ T7 RNA amplification T7 amplification products were generated from 1 μ g of mouse liver total RNA, reverse transcribed, and analyzed. Electropherograms of amplified RNA from the standard protocol in tubes is shown in purple and from the in-column procedure with μ MACS Technology in black (Bioanalyzer 2100, Agilent Technologies Inc).

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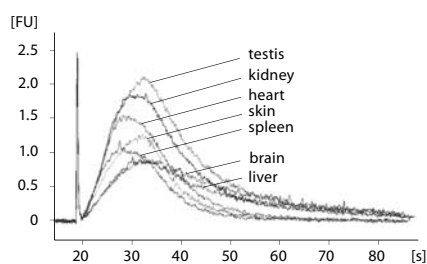


Figure 1 B: Electropherograms of amplified RNA from diverse tissues μ MACS™ One-step T7 template generation plus in-column *in vitro* transcription (MEGAscript® T7 Kit, Ambion Inc.) derived from mouse tissues.

Often, an amplification step is performed to overcome the limitation of small sample material. The most established method is the linear RNA-amplification based on T7 RNA polymerase.¹ T7-linear amplification generally results in antisense RNA accurately representing the initial mRNA profile. Traditional protocols, however, often describe a time-consuming process.

The μ MACS™ One-step T7 Template Kit enables the fast isolation of mRNA and the immediate synthesis of the T7-linked cDNA template for the in-column amplification with T7 polymerase. With μ MACS Technology, the whole procedure is performed in one column. Within approx. two and a half hours the T7 cDNA template is generated, and is ready for a subsequent in-column *in vitro* transcription (IVT) reaction within further three hours.

First, full-length intact mRNA is extracted directly from either cells or tissues without prior preparation of total RNA. The isolation of the mRNA is achieved by using superparamagnetic Oligo(dT)-T7 MicroBeads, which are added to the cell lysate. The labeled cell lysate is then applied to the μ Column that is placed in the thermoMACS™ Separator, a heatable permanent magnet. The MicroBead-mRNA complexes are retained in the strong magnetic field while effective washing steps minimize DNA or rRNA contamination. Next, a ready-to-use reverse transcriptase enzyme mix is added onto the column

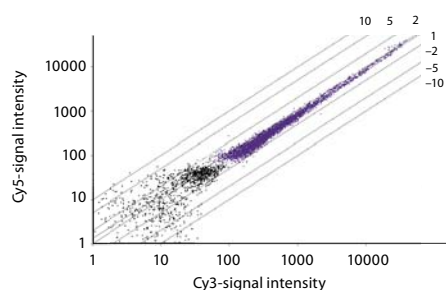


Figure 2: Amplification of two identical mouse liver samples

Two identical mouse liver tissues individually amplified with the μ MACS™ One-step T7 Template Kit in combination with the Microarray RNA Target Synthesis Kit (T7), Roche Diagnostic GmbH. Samples were labeled with Cy3 and Cy5 fluorescent dyes, respectively. 500 ng of each labeled aRNA were hybridized to PIQOR™ Immunology Microarray, mouse, antisense (1,076 genes). The resulting regression coefficient is 0.99.

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Amplification	A (liver) Traditional in tubes	B (spleen) Traditional in tubes	C (liver) μ MACS™ Kit in-column	D (spleen) μ MACS™ Kit in-column
	No. detected genes	Correlation		
A	976	1		
B	988	0.98	1	
C	952	0.93	0.91	1
D	956	0.93	0.92	0.99

Figure 3: Comparison of amplification in tubes versus in-column protocol
Samples from mouse liver and spleen, each 1 μ g total RNA, were amplified with a standard T7 amplification protocol in tubes (A, B) or with the μ MACS™ One-step T7 Template Kit (C, D). For *in vitro* transcription the MEGascript® T7 Kit, Ambion, was used. Labeling was performed with Cy3 (spleen) and Cy5 (liver). Labeled samples were hybridized to PIQOR Immunology Microarray, mouse, antisense (1,076 genes), and correlation values analyzed.

and the thermoMACS™ Separator is set to 42°C. During a 60-minute incubation first-strand cDNA is synthesized. Subsequently, a second-strand synthesis mixture containing *E. coli* DNA polymerase I, *E. coli* DNA ligase, and RNase H is added onto the column for the generation of double-stranded (ds) cDNA.

After purification of the ds cDNA, an *in vitro* transcription (IVT) reaction mixture containing T7 RNA polymerase (not included in the μ MACS One-step T7 Template Kit) is added onto the column, and

the thermoMACS™ Separator is set to 37 °C. Finally, amplified RNA is eluted, purified and ready for further application like labeling and microarray hybridization, e.g. with PIQOR™ Microarrays, antisense. For a scheme of in-column aRNA amplification, refer to figure 4.

The procedure of the μ MACS™ One-step T7 Template Kit addresses several aspects of reliable gene expression profiling: speed, purity, and reduced RNA loss. RNA isolation has to be carried out rapidly as RNA molecules are susceptible to degradation. DNA contaminations during the isolation can lead to false results, contaminating ribosomal RNA (rRNA) lowers the efficiency of the reverse transcription, and mRNA is often lost during precipitation and washing steps—especially when working with minor amounts of sample material.

The extremely small, paramagnetic Oligo(dT) MicroBeads enable instant labeling of mRNA while MACS® Column Technology facilitates efficient purification.

The next crucial step for accurate gene expression analysis from small sample material is to generate full-length, double-stranded cDNA bearing a T7 promoter sequence. The μ MACS One-step T7 Template Kit is designed to yield full-length ds cDNA using a highly active Reverse Transcriptase (fig. 1). The high reproducibility of the procedure is shown by self-versus-self hybridizations (fig. 2 and 3).

Using MACS Technology, mRNA can be isolated and aRNA synthesized in one column while minimizing loss of mRNA, cDNA, or aRNA associated with tube-to-tube transfer and with extra purification procedures. This is especially important when working with small amounts of sample material.

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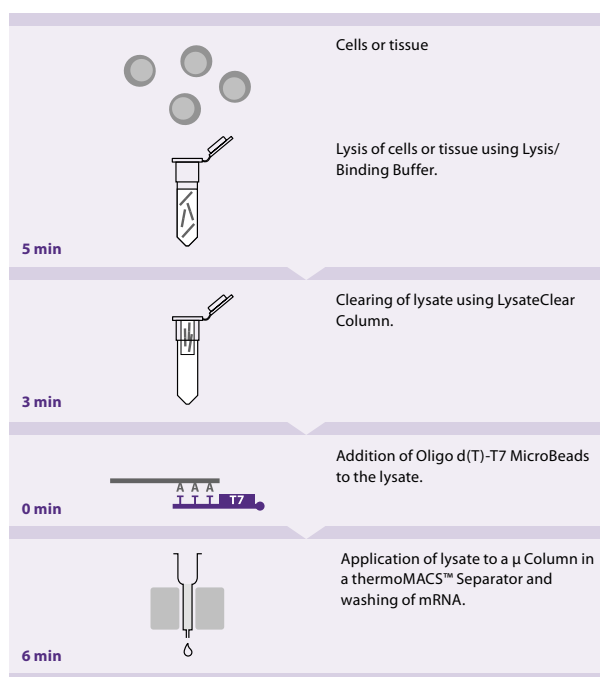
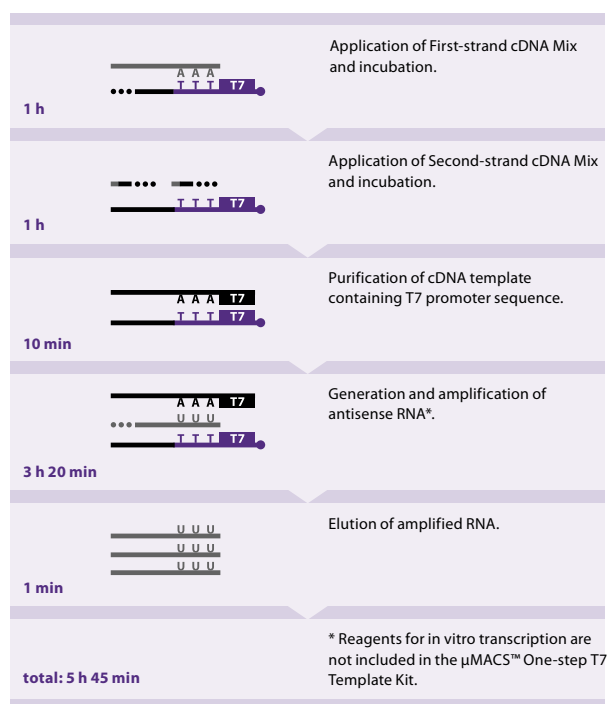


Figure 4: Principle of T7 template generation with MACS® Technology



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1.3 Kit capacities

This kit is for isolation of mRNA and ds cDNA synthesis from a maximum of 1×10^6 cells, 6 mg of human and animal tissue, or 40 μ g of total RNA.

Amplifications from 5×10^4 Jurkat cells or 250 ng of total RNA typically results in microgram amounts of amplified RNA which is sufficient for most applications.

1.4 Reagent and instrument requirements

All additionally required equipment and reagents must be RNase-free.

- For homogenization and lysis of tissue: mortar, pestle, and/or rotor-stator homogenizer
- For mechanical shearing of DNA to homogenize cells: sterile, 21G needles and syringes (1–5 mL)
- Tubes and pipette tips
- 70% Ethanol (EtOH)
- thermoMACS™ Separator (# 130-091-136)
- Microcentrifuge suitable for 2 mL tubes
- Suitable T7 *in vitro* transcription reagents, e.g. Megascript™ T7 Kit, Ambion Inc. or Microarray RNA Target Synthesis Kit (T7), Roche Diagnostics GmbH
- Suitable kit for the purification of amplified RNA, e.g. NucleoSpin® RNA II Kit, Macherey & Nagel GmbH

- (Optional) PrepProtect™ Stabilization Buffer (10 mL, #130-092-643; 100 mL, #130-092-642)
- (Optional) Antifoam A (1%), Sigma-Aldrich Inc. can be added to prevent extensive foam formation during sample homogenization
- (Optional) RNase removing solution, e.g. RNaseZap®, Ambion

1.5 Related Products

- μ MACS™ One-step cDNA Kit (# 130-091-902)
- IQOR™ Microarray Kits, antisense, topic-defined and custom: www.miltenyibiotec.com
- MACS® Products for cell separation: www.miltenyibiotec.com
- a-Hyb™ Hybridization Station: www.miltenyibiotec.com

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2. Protocol for mRNA isolation and in-column amplification

- ▲ All additionally required equipment must be RNase-free.

2.1 Sample preparation

μ MACS™ One-step T7 Template Kit is compatible with the following sample types: adherent or suspension cell samples (section 2.1.1); human, animal, or plant tissue (2.1.2); and eukaryotic total RNA (2.1.3).

- ▲ Thorough sample homogenization and cell lysis, as well as the reduction of viscosity of lysates, are very important. Please see chapter 3, Tips and hints.

2.1.1 Adherent or suspension cells

Suspension cells are lysed immediately after harvesting (section 2.1.1.1). Adherent cells can be lysed directly in the cell culture vessel (section 2.1.1.2), or detached with trypsin or EDTA before lysis (section 2.1.1.3). Use up to 1×10^6 cells.

Before starting

- ▲ Adjust Lysis/Binding Buffer and Wash Buffer to room temperature.

2.1.1.1 Lysis of suspension cells

1. Harvest and centrifuge cells at low speed. Aspirate entire supernatant and (optional) wash cells once with cold (4 °C) PBS,

centrifuge and aspirate supernatant. Resuspend cells in the residual liquid by flicking the tube.

- ▲ **Note:** After completely removing the supernatant, the cell pellet can be stored at -70 °C.

2. Add 200 μ L of Lysis/Binding Buffer. Lyse cells completely by vigorous vortexing for 3–5 minutes.

- ▲ **Note:** A complete lysis is extremely important for further steps.

3. Continue with section 2.1.1.4, Lysate clearance.

2.1.1.2 Direct lysis of adherent cells

1. Aspirate entire cell culture medium, rinse cells with cold (4 °C) PBS, and aspirate supernatant.

2. Add 200 μ L of Lysis/Binding Buffer. Collect the lysate with a rubber spatula and transfer it into a microfuge tube. Lyse cells completely by vigorous vortexing for 3–5 minutes.

- ▲ **Note:** A complete lysis is extremely important for further steps.

3. Continue with section 2.1.1.4, Lysate clearance.

2.1.1.3 Detachment and subsequent lysis of adherent cells

1. Aspirate entire cell culture medium, rinse cells with PBS, and treat them with trypsin or EDTA solution. When the cells have detached, add culture medium and transfer cells to a centrifuge tube.

2. Centrifuge at low speed and remove supernatant. (Optional) Wash cells with cold (4 °C) PBS, centrifuge and aspirate supernatant;

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resuspend cells in the residual PBS by flicking the tube.

▲ **Note:** After completely removing the supernatant, the cell pellet can be stored at -70°C .

3. Add 200 μL of Lysis/Binding Buffer. Lyse cells completely by vigorous vortexing for 3–5 minutes.

▲ **Note:** A complete lysis is extremely important for further steps.
4. Continue with section 2.1.1.4, Lysate clearance.

2.1.1.4 Lysate clearance

1. If fuzzy material and clumps remain in the lysate, or if the lysate is highly viscous (depending on cell type), **mechanical shearing of DNA** must be performed: Transfer lysate to a fresh tube by forcing it 2–5 times with maximal pressure through a 21G needle attached to a 1 mL syringe, matching the lysate volume. Check that no fuzzy material or clumps remain.
2. (Optional) The foam which is caused during the lysis can be reduced by centrifuging the lysate at $13,000\times g$ for 1–3 minutes.
3. Apply lysate on top of the LysateClear Column that is placed in a centrifugation tube. Centrifuge LysateClear Column at $\geq 13,000\times g$ for 3 minutes. The cleared lysate is collected in the centrifugation tube.
4. During centrifugation, proceed with column rinse and continue with section 2.2, In-column magnetic isolation of mRNA.

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2.1.2 Lysis of human, animal, or plant tissue

RNA from tissue tends to degrade quickly, especially when frozen samples thaw. Work fast until tissue is lysed completely. For storage of tissue samples, it is recommended to quick-freeze samples in liquid nitrogen and stabilize frozen samples in PrepProtect™ Stabilization Buffer, minimal incubation overnight at -20°C .

Use a maximum of 6 mg human or animal tissue (except e.g. spleen: 2 mg, heart: 3 mg, thymus: 1 mg). Use up to 20 mg of plant tissue.

Before starting

▲ Adjust Lysis/Binding Buffer and Wash Buffer to room temperature.

1. For hard tissue and tissue rich in connective tissue like muscle, heart, bone, and dermis: Grind tissue in a mortar on liquid nitrogen to a homogeneous powder. Prevent thawing of the powder.

For soft tissue: This can be lysed without grinding.

2. Add tissue to 200 μL of Lysis/Binding Buffer.
3. Immediately homogenize tissue using an appropriate method such as rotor-stator homogenizer.

▲ **Note:** Up to 6 mg of human or animal tissue can be handled in a 2 mL tube using a small rotor-stator (5 mm diameter).

▲ **Note:** A complete lysis is extremely important for further steps.
4. (Optional) The foam which is caused during lysis can be reduced by centrifuging the lysate at $13,000\times g$ for 1–3 minutes.



5. Apply lysate on top of the LysateClear Column that is placed in a centrifugation tube. Centrifuge at $\geq 13,000\times g$ for 3 minutes. The cleared lysate is collected in the centrifugation tube.
6. During centrifugation, proceed with column rinse and continue with section 2.2, In-column magnetic isolation of mRNA.

2.1.3 Sample preparation from total RNA

Use up to 40 μg of total RNA, maximal volume 100 μL .

Before starting

▲ For best mRNA preparations use freshly isolated, intact total RNA.

▲ Adjust Lysis/Binding Buffer and Wash Buffer to room temperature.

1. Heat total RNA for 3–5 minutes to 70°C . Then, chill briefly on ice.
2. Take the tube out of the ice and add 200 μL Lysis/Binding Buffer.
3. Proceed with section 2.2, In-column magnetic isolation of mRNA.

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2.2 In-column magnetic isolation of mRNA

1. Place a μ Column in the magnetic field of a thermoMACS™ Separator.
2. Prepare column by rinsing with 100 μL Lysis/Binding Buffer and let buffer run through. Columns are “flow stop” and do not run dry.
3. Add 20 μL of oligo(dT)-T7 MicroBeads to lysate prepared in section 2.1. Mix by pipetting up and down 2–3 times or vortex shortly. For the hybridization of mRNA to oligo(dT) MicroBeads, further incubation is not necessary.
4. Apply lysate on top of the column matrix. Let the lysate pass through. Magnetically labeled mRNA is retained in the column.
5. Rinse column with 2×200 μL of Lysis/Binding Buffer to remove proteins and DNA. Only for total RNA samples one single rinse is sufficient.
6. Rinse column with 4×100 μL of Wash Buffer to remove rRNA and DNA.
7. Proceed with section 2.3, In-column double-strand cDNA synthesis and purification.



2.3 In-column double-strand cDNA synthesis and purification

1. Rinse column with 2 × 100 µL of Equilibration/Wash Buffer.
2. Dissolve the lyophilized First-strand cDNA Mix in 20 µL of Resuspension Buffer I.
 - ▲ Note: It is not necessary to pipette the Enzyme Mix up and down more than twice.
 - ▲ Note: For opening of the wells containing lyophilisates, please refer to chapter 3, Tips & hints.
3. Apply 20 µL of resuspended First-strand cDNA Mix on top of the column matrix.
 - ▲ Note: Remove any residual drop at the column tip by touching the column tip with the rim of an RNase-free tube or with an RNase-free pipette tip.
4. To avoid evaporation, apply 1 µL of Sealing Solution directly on top of the column matrix.
5. Switch on the thermoMACS™ Separator and set it to 42 °C. Incubate for 45 minutes.
6. Switch off the thermoMACS Separator and let the columns cool down for further 15 minutes.
7. Dissolve the lyophilized Second-strand cDNA Mix in 25 µL of Resuspension Buffer II.
8. Apply 25 µL of resuspended Second-strand cDNA Mix on top of the column matrix.
 - ▲ Note: If solution at the column tip (column outlet) has evaporated during incubation, residual reaction mix can dry up and block column flow. Remove dried reaction mix with a fresh pipette tip.

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▲ Note: Remove any residual drop at the column tip by touching the column tip with the rim of an RNase-free tube or with an RNase-free pipette tip.

9. Incubate for 1 hour with thermoMACS™ Separator switched off (room temperature).
10. Rinse column with 2 × 200 µL of Lysis/Binding Buffer to remove enzymes and reaction components.
11. Rinse column with 4 × 100 µL of Wash Buffer.
12. Rinse column with 2 × 100 µL of Equilibration/Wash Buffer.
13. Proceed with section 2.4, In-column *in vitro* transcription.

2.4 In-column *in vitro* transcription**Before starting**

1. Prepare *in vitro* transcription mix: As the cDNA template is already immobilized in the column, substitute the regular cDNA template volume in the IVT mix with 5 µL of IVT Enhancer solution and prepare mix according to the manufacturer's instructions. Keep total volume at 20 µL.
 - ▲ Note: The volume of the immobilized cDNA in the column is virtually zero.
 - ▲ Note: The IVT Enhancer solution is supplied with this kit.
2. Apply 20 µL of *in vitro* transcription mix on top of the column matrix.
 - ▲ Note: Remove any residual drop at the column tip by touching the column tip with the rim of an RNase-free tube or with an RNase-free pipette tip.



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4. To avoid evaporation, apply 1 µL of Sealing Solution directly on top of the column matrix.
5. Switch on the thermoMACS™ Separator and set it to 37 °C. Incubate for 3 hours.
6. Elute the synthesized amplified RNA with 85 µL of Elution Buffer.
 - ▲ Note: If solution at the column tip (column outlet) has evaporated during incubation, residual reaction mix can dry up and block column flow. Remove dried reaction mix with a fresh pipette tip.
 - ▲ Note: Collect any residual drop at the column tip by touching the column tip with the rim of the collection tube.
7. Switch off thermoMACS™ Separator.
8. Proceed with section 2.5, Purification of *in vitro* transcription reaction.

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2.5 Purification of *in vitro* transcription reaction

The purification protocol for the *in vitro* transcription reaction below uses the NucleoSpin® RNA II Kit, for further information refer to NucleoSpin® RNA II Kit User Manual.

1. Add 350 µL of RA1 Buffer to the eluted amplified aRNA and mix.
2. Add 350 µL of 70% EtOH and mix by vortexing.
3. Transfer sample to NucleoSpin RNA column and centrifuge at 8,000 × g for 30 seconds. Discard flow-through.
4. Add 200 µL of Buffer RA2 to NucleoSpin column and centrifuge at 8,000×g for 30 seconds. Discard flow-through.
5. Add 600 µL of Buffer RA3 to NucleoSpin column and centrifuge at 8,000×g for 30 seconds. Discard flow-through and place column into a fresh collection tube.
6. Add 250 µL of Buffer RA3 to NucleoSpin column and centrifuge at 11,000×g for 2 minutes. Discard flow-through and place column into a fresh 1.5 mL microcentrifuge tube.
7. Add 60 µL of RNase-free H₂O to the membrane in the spin column.
8. Centrifuge for 1 minute at 11,000×g.
9. (Optional) Store the purified aRNA at -70 °C.



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3. Tips & hints

Sample lysis

Incomplete lysis and high viscosity will compromise mRNA yield, slow down column flow, or may cause clogging of the column. If fuzzy material or clumps remain in the lysate, or if the lysate is very viscous, shear sample mechanically: Pass the lysate several times through a 21G needle attached to a syringe (1–5 mL) until all clumps are dissolved and viscosity is reduced.

Solubilization of lyophilisates

The plastic film sealing the lyophilized enzyme mix plates can either be peeled off by hand or pierced with a pipette tip. In the case of piercing, we recommend wiping the foil with an RNase-removing solution to reduce the risk of contaminating the cDNA synthesis reaction with RNases. Use a fresh 1 mL pipette tip to pierce the plastic film; to resuspend the Enzyme Mix a new pipette tip (20 µL / 25 µL volume) should be used.

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4. Troubleshooting

General hints to avoid contamination and degradation of mRNA

- ▲ Work rapidly and without interruptions to minimize mRNA degradation.
- ▲ Always place the column in the magnet from the front to avoid contact of the column tip with the magnet.
- ▲ Do not touch the column tip with fingers or RNase-containing material.
- ▲ Change pipette tips in case of contact with outer column housing and between different wash buffers.

Incomplete lysis of sample material

If the lysis of tissue is difficult to perform, reduce the amount of starting material in the mRNA preparation or increase the Lysis/Binding Buffer volume to 400 µL.

Some lysate is left in the LysateClear Column after centrifugation

Centrifuge again. Please do not use more sample material than specified. Perform additional mechanical shearing steps for cell lysis.

Slow gravity flow of columns

The gravity flow of the columns depends on amount, tissue type (e.g. thymus and spleen can be problematic), and viscosity of sample material. Do not overload columns by using unspecified sample amounts which might lead to slowing of gravity flow. Inserting a DNA shearing step will improve gravity flow in the column.

Wash or Elution Buffer does not run into the column

Take off buffer. Use fresh buffer and pipette with force directly on top of column matrix; or, pipette buffer up and down, avoiding air bubbles.



The use of cold buffer on a pre-warmed column is not recommended due to the possibility of air bubble formation in the column matrix.

Evaporation and drying out of solution at column tip

Before switching on the thermoMACS™ Separator, apply the Sealing Solution. In rare cases, no liquid may be left in the column outlet due to evaporation during the heated incubation: Adjust the reaction volume by pipetting 3 µL of RNase-free H₂O on the column.

After an incubation, blockade of the column flow due to evaporation at the column tip (column outlet) can occur: Remove residual dried reaction mix with a fresh pipette tip. If necessary, additionally apply slight pressure on the top of the column.

Sealing Solution

Do not use any other solution than the Sealing Solution to seal the column. Mineral oils as used in PCR reactions do not work. Pipette small volumes, such as the Sealing Solution, directly on to the column matrix.

Low amount of aRNA

The use of an *in vitro* transcription kit containing high-quality T7 polymerase and reaction components such as nucleotides and RNase inhibitors is recommended, please see section 1.4, Reagent and instrument requirements. In case of low RNA content of sample material or RNA degradation, see chapter 4, Troubleshooting, General hints to avoid contamination and degradation of mRNA. Sealing the µ Columns with parafilm during incubation can decrease the risk of contamination with RNases.

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5. Appendix

5.1 In-column removal of DNA traces using DNase I

Traces of genomic DNA in RNA preparations, particularly in total RNA, can interfere with downstream applications such as microarray analysis or real-time PCR. The µMACS™ mRNA isolation technology was developed to obtain highly pure mRNA devoid of genomic DNA and other contaminations. However, even with this procedure minimal amounts of residual genomic DNA cannot be entirely excluded.

To completely eliminate genomic DNA contamination, a short DNase I treatment directly in the column following the µMACS mRNA isolation can be performed. No inactivation or precipitation step is required, as the DNase I is simply washed away from the column.

Reagent and instrument requirements

- RNase-free DNase I (Ambion Inc., # 2222, 2 U/µL); 1 µL DNase I / reaction
- DNase buffer, RNase-free, 150 µL 1× DNase I Buffer/reaction, supplied with DNase I; or prepare standard DNase I buffer as described below:

10× DNase I Buffer	100 mM Tris HCl (pH 7.5)
	25 mM MgCl ₂
	5 mM CaCl ₂



Protocol for in-column DNase I treatment

Perform the magnetic separation as described in section 2.2. After rinsing column with Wash Buffer (step 6), proceed with the following protocol.

1. Apply 100 μ L of 1 \times DNase I Buffer onto the column.
2. Prepare DNase I reaction solution.
Mix 1 μ L of DNase I with 50 μ L of 1 \times DNase I Buffer.
3. Apply DNase I reaction solution (51 μ L) onto the column.
4. Incubate DNase I in column for 1–2 minutes at room temperature.
▲ Note: Incubation time should not be extended as residual RNase activity in the DNase solution cannot be excluded.
5. Rinse column with 2 \times 200 μ L of Lysis/Binding Buffer.
6. Rinse column with 4 \times 50 μ L of Wash Buffer.
7. Proceed with section 2.3, In-column double-stranded cDNA synthesis and purification.

5.2 Quantification and quality control of aRNA

aRNA yield can be determined by measuring the UV absorbance (A) at 260 nm, if RNA concentrations > 5 ng/ μ L are expected. The measured A₂₆₀ should have a value of ≥ 0.1 to ensure reliable analysis. For accurate results with conventional spectrophotometers we recommend the usage of RNase-free disposable cuvettes with a small volume (50 μ L), which allow measurement of the undiluted aRNA eluate. An absorbance of 0.1 corresponds to 4 μ g RNA/mL (path length: 1 cm). Therefore, the yield of aRNA can be calculated as follows:

$$A_{260} \times 40 \times \text{dilution factor} = \mu\text{g aRNA/mL}$$

For UV measurements of very small samples, like aliquots of 1 μ L volume, instruments of NanoDrop Technologies Inc. can be used, e.g. NanoDrop ND-100.

To reduce the volume of the eluate and to concentrate the aRNA, please read section 5.3, How to concentrate eluted aRNA.

5.3 How to concentrate eluted aRNA

A standard procedure to reduce the volume of RNA samples is the usage of a Speedvac instrument. Since RNA is eluted with RNase-free H₂O, there is no danger of concentrating salt or EDTA in the sample.

Another approach to concentrate aRNA is precipitation, as described in the protocol below.

1. Add 0.1 volume of RNase-free 5 M ammonium acetate or 3 M sodium acetate pH 5.2 and mix.
2. (Optional) Add 1 μ L of glycogen (20 mg/mL).
3. Add 2.5 volumes of absolute ethanol and mix thoroughly by vortexing.
4. Incubate for 30 minutes at -70 °C or overnight at -20 °C.
5. Centrifuge at $\geq 13,000 \times g$ for 20–30 minutes at 4 °C.
6. Carefully remove and discard the supernatant. Attention, the RNA pellet may not adhere tightly to tube.
7. To remove residual salt, add 1 mL of RNase-free 75% ethanol and vortex.
8. Centrifuge at $\geq 13,000 \times g$ for 10 minutes at 4 °C.
9. Carefully remove the supernatant and dry the aRNA pellet.
10. Dissolve aRNA in an appropriate volume of buffer or RNase-free water.

6. Reference

1. Van Gelder R.N., von Zastrow M.E., Yool A., Dement D.C., Barchas J.D., Eberwine J.H. (1990) Amplified RNA synthesis from limited quantities of heterogeneous cDNA. Proc. Natl. Acad. Sci. (USA) 87: 1663–1667.

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