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

μMACS™ One-step cDNA Labeling Kit

Order no. 130-092-443

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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.

1. Description

1.1 Components and size

- ▲ μMACS™ One-step cDNA Labeling Kit (# 130-092-443)
- ▲ μMACS™ One-step cDNA Labeling Starting Kit (# 130-092-521)

Kit for 20 reactions includes

μMACS mRNA Isolation Kit

- 1 mL Oligo(dT) 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
- 2 × 1.3 mL Elution Buffer
- 20 μ Columns
- 20 LysateClear Columns

μMACS cDNA Labeling Module

- Lyophilized cDNA Labeling Mix Plate, black-rimmed frame, for 20 reactions containing Reverse Transcriptase and dNTPs
- Lyophilized RNase H Plate, white-rimmed frame, for 20 reactions
- 2 × 0.5 mL Resuspension Buffer for lyophilized enzymes
- 2 × 15 mL Equilibration/Wash Buffer
- 0.5 mL cDNA Release Solution
- 5 mL cDNA Elution Buffer
- 100 μL Sealing Solution

Product format **Oligo(dT) MicroBeads:** MicroBeads conjugated to oligo(dT)₂₅. Suspension contains 0.1% SDS.

LysateClear Columns (maximum reservoir volume: 1 mL; capacity: lysate from a maximum of 1×10^7 cells, 30 mg animal tissue or 100 mg plant tissue) and centrifugation tubes.

μ Columns (capacity: up to 10 μ g of mRNA).

Lyophilized cDNA Labeling Mix Plate: 20 single reaction mixes, no reaction mix is present in the four corner wells.

Lyophilized RNase H Plate: 20 single reaction mixes, no reaction mix is present in the four corner wells.

All buffers and MACS® Columns included in the μ MACS™ One-step cDNA Labeling Kits are evaluated for the absence of RNase activity.

Storage

Store mRNA Isolation Kit Box 1 containing buffers and MicroBeads protected from light at 4–8 °C. Do not freeze. The reagents are stable until the date indicated on the label.

Store cDNA Labeling Module Box containing lyophilized enzymes and buffers for cDNA synthesis and mRNA isolation at 4–8 °C. Do not freeze. The reagents are stable until the date indicated on the label.

Store Column Box 2 containing μ Columns and LysateClear Columns at room temperature, dry and protected from light.

1.2 mRNA isolation and cDNA labeling with MACS® Technology

Different cell types, or different developmental states of the same cell type exhibit distinct gene expression patterns. To analyze such patterns, mRNA isolated from cells or tissues can be transcribed into labeled cDNA and then hybridized to microarrays.

To obtain reliable gene expression profiles the mRNA isolation has to be carried out rapidly as the mRNA molecules are susceptible to degradation. Furthermore, the mRNA isolation method should not affect the gene expression profile: DNA contamination 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.

The second crucial step for accurate gene expression analysis is the generation of labeled cDNA. Direct cDNA labeling techniques incorporate fluorescent or radioactive nucleotides during cDNA synthesis. Direct labeling does not require extra steps such as covalent coupling, for example, with Aminoallyl-nucleotides. Finally, removal of unincorporated label by sample purification is required to reduce background signals.

The μ MACS One-step cDNA Labeling Kit enables the isolation of mRNA, immediate reverse transcription into labeled cDNA, and purification of the labeled cDNA. The whole procedure may be performed within 95 minutes. 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) MicroBeads, which are added to the cell lysate. The μ Column is placed

in the thermoMACS™ Separator, a heatable permanent magnet, then the cell lysate is applied. The MicroBead-mRNA complexes are retained in the strong magnetic field while effective washing steps minimize DNA or rRNA contamination.

Next, the dissolved cDNA Labeling Mix with Reverse Transcriptase is added onto the column together with user-supplied fluorescently or radioactively labeled dCTPs. The thermoMACS Separator is set to 42 °C to permit the labeling of cDNA during the in-column reverse transcription reaction. At the end of the reaction unincorporated nucleotides are removed with the supplied Wash Buffer, then purified, labeled cDNA is eluted.

Using MACS® Technology, cDNA can be synthesized in one step while loss of material, associated with tube-to-tube transfer and extra purification procedures, is avoided. This is especially important when working with small amounts of sample material.

To see a schematic view of in-column cDNA synthesis with μ MACS One-step cDNA Kit, refer to figure 1.

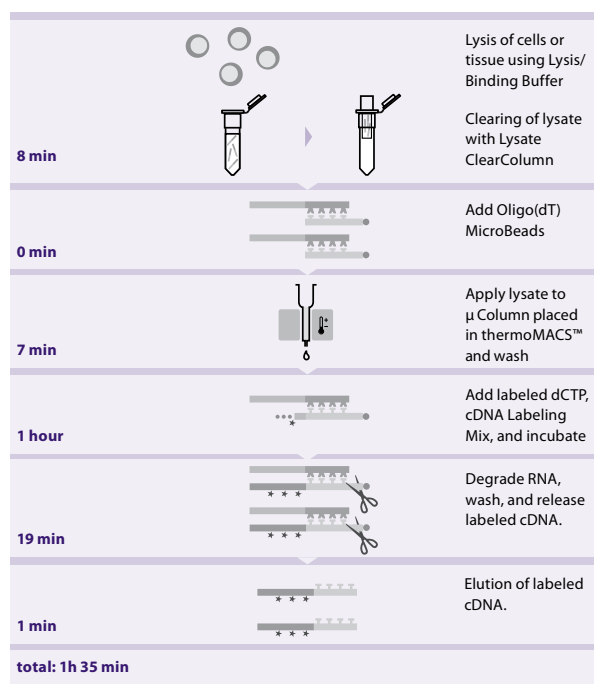


Figure 1: Principle of μ MACS™ One-step cDNA Labeling.

Reproducible μ MACS One-step cDNA Labeling

mRNA was isolated and cDNA was generated from the identical sample (10 mg mouse liver), labeled with either Cy3- or Cy5-dCTP, and both samples were hybridized to one microarray (fig. 2). The high regression-coefficient (r) of 0.994 demonstrates that both labeled cDNAs give nearly identical signals on the microarray. This scatter plot provides a visualization of the reproducibility and reliability of the μ MACS One-step cDNA Labeling Kit.

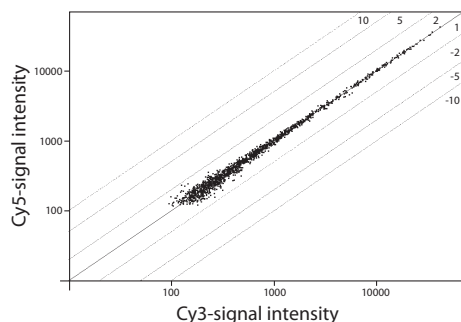


Figure 2: Self-self hybridization of probes generated from 10 mg mouse liver and labeled either with Cy3-dCTP or Cy5-dCTP.

1.3 Kit capacities

- This kit is for isolation of mRNA and labeling of cDNA from a maximum of 1×10^7 cells, 30 mg human and animal tissue, 100 mg plant tissue, or 200 μ g total RNA.
Cell numbers of 1×10^7 cells, or 30 mg human or animal tissue, typically yield 1–10 μ g mRNA, depending on transcriptional activity. Some resting cells, e.g. lymphocytes, may contain significantly lower amounts of mRNA. mRNA yields from 1×10^7 primary cells isolated with MACS[®] Technology such as CD19⁺, CD133⁺ and BDCA-4⁺ cells were 0.5–1 μ g. Using 30 mg mouse tissue, mRNA yields were 1–2 μ g for brain/heart/small intestine, approximately 3 μ g for spleen/kidney, and about 6 μ g for liver.
- In case of limited starting material below 5 μ g total RNA or the corresponding amount of cells or tissue, an RNA amplification and labeling procedure may be necessary.

1.4 Reagent and instrument requirements

- All additionally required equipment and reagents must be RNase-free
- CyDye[™] fluorescent nucleotides: Cy3-dCTP, # PA53021; Cy5-dCTP, # PA55021, both 25 nmol, GE Healthcare, or radioactive nucleotides: α [³²P]-dCTP, 10 μ Ci/ μ L, e.g. 250 μ Ci; 9.25 MBq; 3,000 Ci/mmol; 25 μ L, # AA0005, GE Healthcare. Radioactively-labeled probes should have a high specific activity. Use only fresh (less than one week old) α [³²P]-dCTP

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- For homogenization and lysis of tissue: mortar, pestle, and/or rotor-stator homogenizer
- For mechanical shearing of DNA to homogenize cells: sterile, RNase-free 21G needles and 1–5 mL syringes
- RNase-free tubes and pipette tips
- thermoMACS[™] Separator (# 130-091-136)
- Microcentrifuge suitable for 2 mL tubes
- (Optional) Antifoam A (1%, Sigma-Aldrich) can be added to prevent extensive foam formation during sample homogenization
- (Optional) RNase removing solution, e.g. RNaseZap[®], Ambion
- (Optional) Phosphate-buffered saline (PBS)

1.5 Related products

- μ MACS mRNA Isolation Kits:
Small Scale (10 reactions, # 130-090-276; 20 reactions, #130-075-201)
Large Scale (4 reactions, # 130-090-277; 8 reactions, # 130-075-101)
For Total RNA (8 reactions, # 130-075-102)
- μ MACS One-step cDNA Kit (# 130-091-902)
- a-Hyb[™] Hybridization Station for microarray processing (# 130-092-181)
- PIQOR[™] Microarray kits, sense (topic-defined and custom):
www.miltenyibiotec.com
- MACS[®] products for cell separation: www.miltenyibiotec.com
- PIQOR[™] HybChamb (# 130-091-726)

2. Protocol for cDNA labeling

All additionally required equipment must be RNase-free.

2.1 Sample preparation

μ MACS[™] One-step cDNA Labeling is compatible with the following sample types: adherent or suspension cell samples (section 2.1.1); human, animal or plant tissue (2.1.2). Also, it is used for isolation of mRNA from total RNA (2.1.3).

▲ Thorough sample homogenization and cell lysis, as well as the reduction of lysate viscosity, are very important (please see “Tips & 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 and/or EDTA before lysis (section 2.1.1.3). Use up to 1×10^7 cells.

Before starting

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

2.1.1.1 Lysis of suspension cells

- Harvest and centrifuge cells at low speed (200–300 \times g). Remove supernatant and (optional) wash cells once with cold (4 °C) PBS, centrifuge and remove 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.

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2. Add 1 mL 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 Lysate clearance, section 2.1.1.4.

2.1.1.2 Direct lysis of adherent cells

1. Remove cell culture medium, and rinse cells with cold (4 °C) PBS, remove supernatant.
2. Add 1 mL 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 Lysate clearance, section 2.1.1.4.

2.1.1.3 Detachment and subsequent lysis of adherent cells

1. Remove cell culture medium, rinse cells with PBS, and treat them with trypsin and/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 remove supernatant. 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 1 mL 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 Lysate clearance, section 2.1.1.4.

2.1.1.4 Lysate clearance

1. For more than 5×10^6 cells, 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 maximum pressure through a 21G needle attached to a 1–5 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 the 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 in-column magnetic isolation of mRNA, section 2.2.

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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.

Use a maximum of 30 mg human or animal tissue (spleen: 10 mg, heart: 15 mg, thymus: 5 mg). For many tissues, except e.g. lung, fat or skin, 30 mg corresponds to a 3 mm × 3 mm × 3 mm piece. Use up to 100 mg 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 skin: 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 1 mL Lysis/Binding Buffer.
 3. Immediately homogenize tissue using an appropriate method such as rotor-stator homogenizer.
- ▲ Note: Up to 30 mg 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 sheared lysate sample on top of the LysateClear Column placed in a centrifugation tube.

6. Centrifuge at $\geq 13,000 \times g$ for 3 minutes. The cleared lysate is collected in the centrifugation tube.
7. Proceed with in-column magnetic isolation of mRNA, section 2.2.

2.1.3 Sample preparation from total RNA

Use up to 200 µg of total RNA (max. volume 500 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 5 minutes to 70 °C. Then, chill briefly on ice.
2. Take the tube out of the ice and dilute total RNA with at least one volume of Lysis/Binding Buffer. If necessary, add Lysis/Binding Buffer to final minimum volume of 250 µL. The maximum volume of the column reservoir is 1 mL.

Volume of total RNA (µL)	20	100	150	250	500
Lysis/Binding Buffer (µL)	230	150	150	250	500

3. Proceed with in-column magnetic isolation of mRNA, section 2.2.

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2.2 In-column magnetic isolation of mRNA

- Place a μ Column in the magnetic field of thermoMACS Separator.
 - Prepare column by rinsing with 100 μ L Lysis/Binding Buffer and let buffer run through. Columns are "flow stop" and do not run dry.
 - Add Oligo(dT) MicroBeads to the lysate.
 - For cells and tissues: Add 50 μ L to the lysate as prepared in section 2.1.
 - For total RNA: Add 25 μ L Oligo(dT) MicroBeads per diluted 100 μ g total RNA as prepared in section 2.1. For less total RNA, use also 25 μ L Oligo(dT) MicroBeads.

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.
 - Apply lysate on top of the column matrix. Let the lysate pass through. Magnetically labeled mRNA is retained in the column.
 - Rinse column with 2 \times 200 μ L Lysis/Binding Buffer to remove proteins and DNA. Only for total RNA samples is one single rinse sufficient.
 - Rinse column with 4 \times 100 μ L Wash Buffer to remove rRNA and DNA.
- ▲ Do not elute the mRNA!**
- Proceed with cDNA labeling and purification (section 2.3).
 - ▲ Note:** Alternatively, mRNA isolation or DNase treatment can be performed. Please refer to the Appendix or to μ MACS mRNA Isolation Kit user manual for protocols.

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2.3 cDNA labeling and purification**2.3.1 Protocol for fluorescent cDNA labeling**

▲ Fluorescent dyes and labeled samples are light-sensitive material. Whenever possible, keep the reactions, including fluorescent dyes, in the dark.

- Apply 2 \times 100 μ L Equilibration/Wash Buffer onto the column matrix.
- Dissolve the Lyophilized cDNA Labeling Mix in 19 μ L Resuspension Buffer.
 - ▲ Note:** It is not necessary to pipette the Enzyme Mix up and down more than twice.
 - ▲ Note:** Please refer to "Tips & hints" for opening of the wells containing lyophilisates.
- Add 1 μ L Cy3-dCTP or Cy5-dCTP to cDNA Labeling Mix.
- Apply resuspended cDNA Labeling Mix directly 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.
- To avoid evaporation, apply 1 μ L Sealing Solution directly on top of the column matrix.
- Switch on the thermoMACS Separator and set to 42 $^{\circ}$ C. Incubate for one hour.
- Rinse column with 4 \times 200 μ L Equilibration/Wash Buffer.
 - ▲ Note:** If solution at the column tip (column outlet) has evaporated during cDNA labeling, residual dried reaction mix can block column flow. Remove dried reaction mix with a fresh pipette tip.
- Dissolve the Lyophilized RNase H with 20 μ L Resuspension Buffer.



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Apply resuspended RNase H on top of the column matrix. Incubate for 5 min at 42 $^{\circ}$ C.

▲ 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.

- Rinse column with 2 \times 100 μ L Equilibration/Wash Buffer.
- Apply 20 μ L cDNA Release Solution on top of the column matrix. Incubate for 10 minutes at 42 $^{\circ}$ C.
 - ▲ 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.
- Elute the synthesized cDNA by pipetting 50 μ L cDNA Elution Buffer onto column.
 - ▲ Note: The first drop already contains cDNA.**
 - ▲ Note:** To enhance recovery of labeled cDNA, a second elution step with an additional 50 μ L cDNA Elution Buffer can be performed. To reduce the volume of the eluate, please refer to "Appendix", section 5.2.
- Switch off thermoMACS Separator. (Optional) Store cDNA at -20° C.

2.3.2 Protocol for radioactive cDNA labeling**Before starting**

▲ Take appropriate safety measures when working with radioactive isotopes.

▲ Before used in downstream applications, radioactively labeled cDNA is usually denatured at high temperature, e.g. 95 $^{\circ}$ C. This step also degrades residual RNA template. Therefore, a further RNA digestion is omitted from this protocol.

- Apply 2 \times 100 μ L Equilibration/Wash Buffer onto the column matrix.
- Dissolve the Lyophilized cDNA Labeling Mix in 19 μ L Resuspension Buffer.
 - ▲ Note:** It is not necessary to pipette the Enzyme Mix up and down more than twice.
 - ▲ Note:** Please refer to "Tips & hints" for opening of the wells containing lyophilisates.
- Add 1 μ L α [32 P]-dCTP (10 μ Ci/ μ L; 3,000 Ci/mmol) to cDNA Labeling Mix.
- Apply resuspended cDNA Labeling Mix directly 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.
- To avoid evaporation, apply 1 μ L Sealing Solution directly on top of the column matrix.
- Switch on the thermoMACS Separator and set to 42 $^{\circ}$ C. Incubate for one hour.

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7. Rinse column with 8×100 µL Equilibration/Wash Buffer.
 - ▲ **Note:** If solution at the column tip (column outlet) has evaporated during the cDNA labeling, residual dried reaction mix can block column flow. Remove dried reaction mix with a fresh pipette tip.
8. Apply 20 µL cDNA Release Solution on top of the column matrix. Incubate for 10 minutes at 42 °C.
 - ▲ **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. Elute the synthesized cDNA by pipetting 50 µL cDNA Elution Buffer onto column.
 - ▲ **Note:** The first drop already contains cDNA.
 - ▲ **Note:** To enhance recovery of labeled cDNA, a second elution step with an additional 50 µL cDNA Elution Buffer can be performed. To reduce the volume of the eluate, please refer to "Tips & hints".
10. Switch off thermoMACS Separator. (Optional) Store cDNA at -20 °C.

In general, the average radioactively labeled cDNA from 1 µg mRNA yields 240,000 cpm from 10 µCi α^[32P]-dCTP and 470,000 cpm from 20 µCi α^[32P]-dCTP.

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 1–5 mL syringe until all clumps are dissolved and viscosity is reduced.

Solubilization of lyophilized enzyme

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 pipette tip to pierce the plastic film; to resuspend the enzyme mix a new pipette tip should be used.

Alternative RNA digestion

Instead of using RNase H, apply 20 µL of 100 mM NaOH solution on top of the column matrix. Incubate for 5 min at 42 °C.

Quantification of labeled cDNA

According to this one-step method mRNA is not eluted in between to measure its concentration. Therefore, we recommend closely monitoring the cell number or the weight of the tissues subjected to cDNA labeling. Note that the amount of mRNA depends on the transcriptional activity of cells or tissues and may vary. A protocol in the Appendix describes slide agarose gel electrophoresis for analyzing the quality of the labeled cDNA.

4. Troubleshooting

General hints to avoid contamination and degradation of mRNA

- ▲ Work rapidly 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
- ▲ Change pipette tips in case of contact with outer column housing and between different wash buffers

For troubleshooting of mRNA isolation, please see corresponding user manual on our website www.miltenyibiotec.com.

Some lysate is left in the LysateClear Column after centrifugation

Centrifuge again. In general, 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

Remove buffer. Use fresh buffer and pipette with force directly on top of column matrix. Alternatively, 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

Apply the Sealing Solution before switching on the thermoMACS Separator. In case solution has evaporated at the column tip (column outlet), remove residual dried reaction mix with a fresh pipette tip to avoid blockade of the column flow. Alternatively, the Sealing Solution can be omitted although this will lead to evaporation losses and may influence cDNA synthesis.

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. When applying small volumes, e.g. the Sealing Solution, pipette solution directly onto the column matrix.

Low fluorescence

Whenever possible, keep fluorescence-conjugated material in the dark to avoid bleaching of the dyes. Please pay attention to the stability of the dyes and aliquot the solutions to avoid frequent freezing and thawing. In case of using the labeled cDNA for microarray hybridization, scanner-derived bleaching may occur, mainly in the Cy5 channel, when the same slide is repeatedly scanned. This depends also on additional environmental factors¹.

Low efficiency of mRNA isolation/cDNA labeling

Monitor efficiency by adding specified amounts of control RNA to the lysate, e.g. a range of different concentration of various specific templates, e.g. bacterial RNA, from 2 fmol, 5 fmol, 10 fmol, and 20 fmol.

Reverse transcription should be performed at 42 °C. Take care that the thermoMACS™ Separator and its temperature sensor are not used in the direct proximity to any heat source or strong illuminants.

5. Appendix

5.1 Quality control of labeled cDNA

5.1.1 Agarose gel electrophoresis for cDNA analysis

This technique is recommended to determine incorporation of fluorescence-conjugated dCTP and removal of free Cy3- or Cy5-labeled nucleotides. The cDNA aliquots are separated by electrophoresis on a thin agarose gel, cut to the size of a glass slide, dried by placing blotting paper atop, and then visualized with a microarray scanner. Alternatively, a Typhoon™ Imager (GE Healthcare) can be used without prior drying or cutting of the gel.

1. Prepare thin 1% agarose gel in 1×TBE, or alternatively, in 1×TAE buffer.

▲ **Note:** The thinner the agarose gel the faster it will dry.

▲ **Note:** Do not use ethidium bromide in the gel or running buffer, it will affect the detection of fluorescence during scanning.

2. Adjust 1–5 µL (starting with approx. 1/50 of the eluate) of each labeled cDNA to a volume of 5 µL with cDNA Elution Buffer. Add 1 µL 50% glycerol and mix. cDNAs labeled with different fluorochromes can be pooled, since individual analysis is possible by scanning.

▲ **Note:** Do not use loading buffer containing dyes like bromophenol blue or xylene cyanol, as these will interfere with the detection of fluorescence during scanning.

▲ **Note:** To distinguish labeled cDNA from free Cy3- or Cy5-dCTP, load an aliquot of fluorescent dCTP onto the gel as well. Dilute aliquot 1:2,000 in a Tris-buffered solution and prepare for electrophoresis as described for cDNA samples.

3. Prepare RNA molecular weight marker using standard loading buffer. Apply the marker in a separate, distant lane.

▲ **Note:** After electrophoresis, this lane has to be cut off, stained separately with ethidium bromide (teratogenic substance, handle only with appropriate personal safety measures), and visualized with a transilluminator.

4. When not using a molecular weight marker, load an aliquot of buffer containing 10% glycerol and bromophenol blue into a well, several lanes separated from the cDNA samples to monitor electrophoresis.

5. Perform gel electrophoresis in 1×TBE or 1×TAE buffer, respectively, at 7.5 V/cm and protect the samples from light. Stop electrophoresis when the bromophenol blue dye has run 4–5 cm.

6. Place gel on a glass slide and cut off overlapping edges of the gel as well as the lane containing the bromophenol blue. To dry the agarose gel, place a strip of blotting paper, e.g. Whatman® 3MM Chr, on top of it for about 20–30 min.

7. Remove blotting paper. Scan the gel with a microarray scanner.

▲ **Note:** Pay attention to the limited space above the glass slide when inserting the gel.

▲ **Note:** For Cy3 detection, measure excitation with 532 nm laser. To detect Cy5, measure excitation with 633 nm laser.

8. Stain the gel containing RNA molecular marker with ethidium bromide and visualize it with a UV transilluminator.

▲ **Note:** The aliquot of synthesized cDNA is generally not visible by staining with ethidium bromide and UV visualization.

The lengths of generated cDNA molecules generally range between 400 and 1,500 bases. Residual free dye will appear as a bright band at the lower end of the gel.

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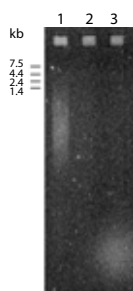


Figure 3: In lane 1, a pooled aliquot of 1/30 of each total cDNA labeling reaction volume from HepG2 and Jurkat cells (50 µg total RNA), labeled with Cy3 and Cy5, respectively, was run on an agarose gel and visualized as described in the above protocol. Lane 2 is empty. In lane 3, an aliquot of 1 µL Cy5-dCTP (dilution 1:2,000) was loaded. The bands of an RNA molecular weight marker are indicated.

5.1.2 cDNA quantification by UV measurement

UV measurement is frequently used for cDNA quantification; however, the cDNA concentration may be below the detection limit. A low A260/280 ratio (approximately 1.5) is due to the composition of the Release Solution and will have no adverse effect on downstream analysis.

5.1.3 Incorporation of fluorescent dyes

The incorporation rate of fluorescent dyes can be determined by simultaneous OD measurement at 260 nm, 550 nm and 650 nm using a spectrophotometer, e.g. ND 1000 NanoDrop Technologies, Inc. Please note that some labeled cDNA samples may be below threshold value for detection; however, these samples often give utilizable results in microarray hybridization experiments.

5.1.4 Quantification of radioactivity

Quantitate ³²P-labeled cDNA in a beta scintillation counter.

5.2 cDNA concentration

5.2.1 Speedvac Vacuum Concentrator

In case the volume of cDNA is too large, it is recommended to concentrate the cDNA solution by using a Speedvac Vacuum Concentrator at 45 °C. The time length depends on the volume of the cDNA solution.

▲ **Note:** Do not prolong Speedvac incubation when cDNA is dried.

▲ **Note:** No effect of the Elution Buffer's low salt content could be observed in microarray hybridization experiments.

5.2.2 Pre-elution to reduce cDNA volume

Pre-elution will reduce the cDNA volume from 50 µL to 30 µL.

▲ Please be aware that the reduced volume of the eluate can lower the cDNA yield.

1. As described in the cDNA labeling and purification protocol (section 2.3.1, step 10; 2.3.2, step 8), apply 20 µL cDNA Release Solution on top of the column matrix. Incubate for 10 minutes at 42 °C.

2. For pre-elution, apply 10 µL cDNA Elution Buffer directly onto the column matrix.

▲ **Note:** Before eluting cDNA, the last drop should be removed from the column tip with an RNase-free pipette tip.

3. To elute cDNA, apply 30 µL of Elution Buffer. Collect all drops into a fresh tube.

▲ **Note:** The first drop already contains cDNA.

4. Switch off thermoMACS Separator. (Optional) Store cDNA at –20 °C.

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5.2.3 cDNA precipitation

1. Add 0.1 volume of 3 M sodium acetate, pH 5.5 and mix.
2. (Optional) Add 1 μL glycogen (20 mg/mL).
 - ▲ **Note:** If low amounts of cDNA are precipitated, like $< 1 \mu\text{g}$ cDNA, use carriers, such as 20 μg of glycogen or 10 μg of *E. coli* tRNA, to precipitate the cDNA.
3. Add 2 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 . Carefully remove and discard the supernatant
 - ▲ **Note:** The cDNA pellet may not adhere tightly to tube.
6. To remove residual salt, add 1 mL 75% ethanol and vortex.
7. Centrifuge at $\geq 13,000\times g$ for 10 minutes at 4°C . Carefully remove the supernatant and dry the cDNA pellet.
9. Dissolve cDNA in an appropriate volume of (Tris-) buffer.

5.3 In-column removal of DNA traces

Traces of genomic DNA in RNA preparations, particularly in total RNA, can interfere with downstream applications. Although the μMACS mRNA isolation technology was developed to obtain highly pure mRNA devoid of genomic DNA, minimal amounts of residual genomic DNA cannot be entirely excluded. A DNase I treatment directly in the column following the μMACS mRNA isolation completely eliminates genomic DNA contamination.

- RNase-free DNase I (Ambion, # 2222, 2 U/ μL), 1 μL DNase I / reaction
- DNase buffer, RNase-free, supplied with DNase I; or prepare standard DNase I buffer, e.g. 10 \times DNase I Buffer:

100 mM Tris HCl (pH 7.5)
25 mM MgCl_2
5 mM CaCl_2

- Necessary volume of 1 \times DNase I Buffer/reaction: 150 μL

After rinsing column in step 6, section 2.2, In-column magnetic isolation of mRNA, proceed as described below.

1. Apply 100 μL 1 \times DNase I Buffer onto the column.
2. Add 1 μL DNase I to 50 μL 1 \times DNase I Buffer per reaction.
3. Apply DNase I reaction solution 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. Wash column with 2 \times 200 μL Lysis/Binding Buffer.
6. Wash column with 4 \times 100 μL Wash Buffer.
7. Proceed with cDNA labeling and purification, section 2.3.

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5.4 Elution of mRNA

Instead of proceeding with cDNA labeling, the mRNA can be eluted. This can be done after rinsing the column to remove rRNA and DNA (step 6, section 2.2, In-column magnetic isolation of mRNA).

Before starting

▲ Pre-heat Elution Buffer to 70°C . To achieve a high yield of mRNA, place the heating block close to the thermoMACS[®] Separator and work fast to avoid cooling of the Elution Buffer before applying it to the column.

1. Pre-elution: Apply 27 μL pre-heated (70°C) Elution Buffer with a fresh pipette tip for each pipetting step. Discard flow-through.
 - ▲ **Note:** Discard pipet tip after each dispense. Re-use of one pipette tip for multiple pipetting steps with hot buffer can change the pre-elution volume and thereby reduce the amount of eluted mRNA.
 - ▲ **Note:** For a consistent elution volume, remove any residual drop at the column tip by touching the column tip with the rim of the RNase-free tube or with an RNase-free pipette tip.

2. Elution: Place a fresh RNase-free tube under the column. Apply 50 μL of pre-heated Elution Buffer.

▲ **Note:** Collect residual drop at the column tip by touching the column tip with the rim of the RNase-free tube containing the eluate.

Alternative elution: To increase the mRNA yield by approximately 10%, apply a larger volume (75 μL) of pre-heated Elution Buffer.

▲ **Note:** The alternative elution will increase of volume of the eluate and decrease the mRNA concentration. Collect residual drop at the column tip by touching the column tip with the rim of the RNase-free tube containing the eluate.

6. Reference

1. Fare *et al.* (2003) Effects of atmospheric ozone on Microarray Data Quality. *Anal Chem.*, 2003 Sep 1; 75 (17): 4672-5

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