Miltenyi Biotec

µMACS™ One-step cDNA Kit

For 20 reactions

Order no. 130-091-902

Miltenyi Biotec

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Contents

1. Description

Components

µMACS™ mRNA Isolation Kit containing:
1 mL Oligo (dT) MicroBeads
40 mL Lysis/Binding Buffer
20 mL Wash Buffer
2× 1.3 mL Elution Buffer
20 µ Columns
20 LysateClear Columns

µMACS™ cDNA Synthesis Module containing:
Lyophilized Enzyme Mix for 20 reactions containing Reverse Transcriptase and dNTPs
0.5 mL Resuspension Buffer for Enzyme Mix
15 mL Equilibration/Wash Buffer
0.5 mL cDNA Release Solution
5 mL cDNA Elution Buffer
100 µL Sealing Solution

Size

For 20 reactions.

Product format

Oligo (dT) MicroBeads: MicroBeads conjugated to oligo $(dT)_{25}$. Supension contains 0.1% SDS.

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

µ Columns (capacity: up to 10 µg mRNA).

Lyophilized Enzyme Mix: no reaction mix is present in the 4 corner wells.

Storage

Store mRNA Buffer Set Box containing buffers and MicroBeads protected from light at 2–8 °C. Do not freeze. The expiration date is indicated on the vial label.

Store µ Columns and LysateClear Columns at room temperature, dry, and protected from light. The expiration date is indicated on the vial label.

Store lyophilized Enzyme Mix and buffers for cDNA synthesis at 2–8 °C. Do not freeze. The expiration date is indicated on the vial label.

1.1 From cells and tissue to mRNA and cDNA in one step

Different cell types, or different developmental states of the same cell type exhibit distinct gene expression patterns. mRNA isolation is a standard procedure to obtain information regarding specific gene expression in cells or tissue. Gene expression analysis can serve as a research tool to ascertain the differential gene expression pattern that is relative to specific protein expression. It thus provides useful physiological information, i.e., signal transduction pathways within a certain cell or tissue type. To obtain reliable gene expression results, mRNA isolation has to be carried out rapidly as the mRNA molecules are susceptible to degradation. In addition, it is also critical to utilize a method that is very sensitive in order to detect those more rare transcripts to obtain a complete gene expression profile.

For analysis of mRNA expression, downstream applications such as RT-PCR, microarray analysis, Northern blotting, or cDNA synthesis are performed. However, for accurate gene expression analyses via these downstream applications, it is important that the mRNA isolation method does not affect the gene expression profile: DNA contaminations and degradation of the RNA during the isolation can lead to false results, contaminating rRNA lowers the efficiency of the reverse transcription, and mRNA is often lost during conventional precipitation and washing steps. Since mRNA represents only 1–5% of the total RNA fraction, it is recommended to use mRNA instead of total RNA for transcribing RNA into cDNA. With the µMACS™ mRNA Isolation Kit, mRNA can be isolated in 15 minutes directly from cells, while the Column Technology provides effective washing steps to minimize DNA or rRNA contamination. The µMACS cDNA Synthesis Module ensures a maximum yield of full-length cDNA due to a highly active Reverse Transcriptase combined with oligo (dT)-primed synthesis. In addition, downstream losses of cDNA product are eliminated as no further purification steps are required.

1.2 MACS® Technology for mRNA isolation and cDNA synthesis

µMACS mRNA Isolation Kits are developed for the direct isolation of mRNA without prior preparation of total RNA. With µMACS mRNA Isolation Kits full-length, intact mRNA from fresh, frozen, or cultured cells[1], animal[2,3] or plant tissue, whole blood or total RNA[4] can be obtained.

The isolation is achieved by using Oligo (dT) MicroBeads, which are added to a lysate prepared from cells or tissue. The magnetically labeled mRNA can then be isolated using µ or M Columns.

The mRNA can be used for any downstream application such as (quantitative) RT–PCR[5], cDNA synthesis[6,7], subtractive hybridization[8], Northern blotting, or microarray analysis.

After magnetic isolation, the mRNA can be eluted from the column in a small volume ready for downstream analysis.

Optionally, instead of eluting the mRNA, a subsequent cDNA synthesis can be performed on the column. The cDNA is then eluted from the column for downstream applications. With MACS® Technology, cDNA synthesis can be performed in one step without loss of material when compared to the difficult handling and extra purification steps of synthesis reactions carried out in tubes. This is especially important when working with small sample amounts.

For an overview of results for cDNA synthesis with µMACS One-step cDNA Kit or a standard method in a tube, please refer to figure 1 on page 8.

1.3 Kit capacities

- Isolation of up to 10 µg mRNA per isolation from a maximum of 1×10^7 cells, 30 mg human or animal tissue or 100 mg plant tissue, or 200 µg total RNA .
- Isolation of mRNA from up to 0.5 mL of human whole blood.
- Isolation of mRNA from up to 2×10^8 yeast protoplasts.
- cDNA synthesis with up to 10 µg mRNA.

1.4 Reagent and instrument requirements

- Mortar and pestle or homogenizer, if tissue is used as starting material.
- RNase-free tubes and pipette tips.
- thermoMACS™ Separator (# 130-091-136) for mRNA isolation with subsequent cDNA synthesis.
- Microcentrifuge suitable for 2 mL tubes.
- Sterile, RNase-free 21G needle and 1–5 mL syringes.
- (Optional) Antifoam A reagent (Sigma-Aldrich®) when a homogenizer is used.
- The Sealing Solution is also available as single reagent (µMACS Sealing Solution, # 130-091-160).

1. Description

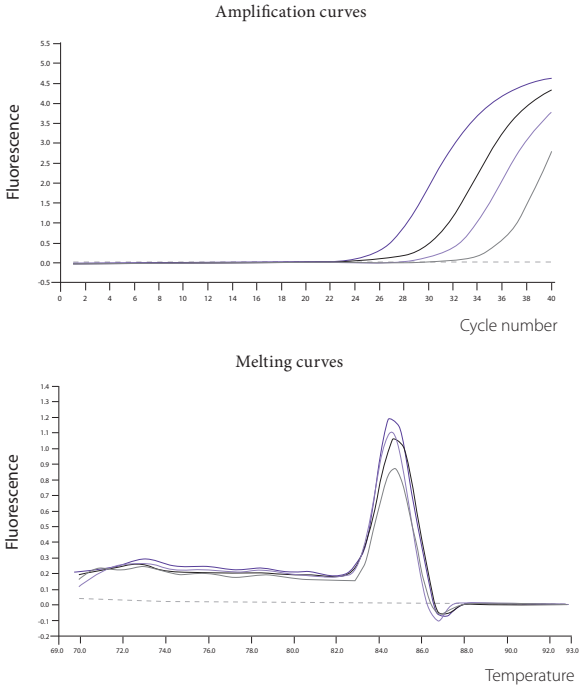

Figure 1: mRNA was isolated from 500 or 5 Jurkat cells, respectively, using µMACS mRNA Isolation Kit or a competitor magnetic mRNA isolation kit. cDNA was synthesized with the µMACS One-step cDNA Kit (Column) or by in-tube synthesis using a standard protocol (tube). For quantitative PCR, 10% of each cDNA was used for the amplification of a 87 bp GAPDH fragment using the LightCycler® with exon-spanning primers.

The negative control (NTC) was performed without template.

140-001-291.03

2. General protocol for mRNA isolation

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2.1 Before starting

▲ All buffers and MACS Columns included in the µMACS mRNA Isolation Kits are evaluated for the absence of RNase activity.

▲ All additionally required equipment must be RNase-free.

▲ It is extremely important to reduce the viscosity of the lysate. Insufficient reduction of viscosity may cause clogging of the column. For the most efficient lysis of cells, we recommend vigorous vortexing in a suitable tube for 3–5 minutes. Check if cells or tissue are fully lysed. If fuzzy material or clumps remain in the lysate, repeat mechanical shearing by passing the lysate several times through a 21G needle attached to a 1–5 mL syringe until all clumps are dissolved.

▲ To increase purity of mRNA, the number of washing steps can be increased.

▲ 1×10^7 cells or 30 mg human or animal tissue typically yield 1–10 µg mRNA. Some resting cells, e.g. lymphocytes, may contain significantly lower amounts of mRNA. For example, 1×10^7 peripheral blood mononuclear cells yield only 0.7 µg of mRNA.

▲ MACS µ Columns cannot be used for cell separations.

2.2 Supplied buffers

- **Lysis/Binding Buffer**: a high salt buffer containing 1% SDS.
- **Wash Buffer**: a low salt buffer containing NaCl, Tris-HCl, and EDTA.
- **Elution Buffer**

2.3 Sample preparation and magnetic labeling

Before starting

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

1. Harvest cells and centrifuge in a suitable tube. Remove the entire supernatant and resuspend cells completely by flicking the tube. Add **1 mL of Lysis/Binding Buffer per 10^7 cells** and lyse cells completely by vigorous vortexing for 3–5 minutes.
   ▲ **Note:** A complete lysis is extremely important for further steps.
2. (Optional) The foam which is caused during the lysis can be reduced by centrifuging the lysate for 3–5 minutes at ≥4,000×g.
3. Place a **LysateClear Column** in the centrifugation tube and apply sheared lysate sample on top of the LysateClear Column.
4. Centrifuge at ≥13,000×g for 3 minutes. The lysate is now contained in the centrifugation tube.

140-001-291.03

5. Add **50 µL Oligo (dT) MicroBeads per 1 mL tissue lysate** and mix. For the hybridization of mRNA to Oligo (dT) MicroBeads, further incubation is not necessary.
6. Proceed with magnetic separation (section 2.4).

2.3.2 Isolation of mRNA from human or animal tissue

Before starting

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

1. Grind tissue in a mortar on liquid nitrogen to a homogeneous powder. Prevent thawing of the powder. Alternatively, up to 250 mg of human or animal tissue can be homogenized by sonication in Lysis/Binding Buffer in a suitable tube.
   ▲ **Note:** To prevent foaming, 0.1% Antifoam A reagent can be included in the Lysis/Binding Buffer.

   Use a maximum of 30 mg tissue in 1 mL of **Lysis/Binding Buffer**.
2. Lyse sample completely by vigorous vortexing in a 15 mL tube for 3–5 minutes.
   ▲ **Note:** Do not lyse more than 250 mg human or animal tissue in one 15 mL tube to ensure **a complete lysis which is extremely important for further steps.**
3. To reduce viscosity of the lysate, **mechanical shearing** of DNA must be performed. Transfer lysate to a new tube by forcing it 2–5 times with maximum power through a 21G needle attached to a 1–5 mL syringe matching the lysate volume. Check that no fuzzy material or clumps remain in the lysate.
4. (Optional) The foam which is caused during the lysis can be reduced by centrifuging the lysate for 3–5 minutes at ≥4,000×g.
5. Place a **LysateClear Column** in the centrifugation tube and apply sheared lysate sample on top of the LysateClear Column.
6. Centrifuge at ≥13,000×g for 3 minutes. The lysate is now contained in the centrifugation tube.
7. Add **50 µL Oligo (dT) MicroBeads per 1 mL tissue lysate** and mix. For the hybridization of mRNA to Oligo (dT) MicroBeads, further incubation is not necessary.
8. Proceed with magnetic separation (section 2.4).

2.3.3 Isolation of mRNA from plant tissue

Before starting

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

1. Grind tissue in a mortar on liquid nitrogen to a homogeneous powder. Prevent thawing of the powder. Add a maximum of 500 mg homogeneous plant tissue to up to 5 mL of Lysis/Binding Buffer **(100 mg tissue/mL Lysis/Binding Buffer)**. Alternatively, up to 500 mg of plant tissue can be homogenized in up to 5 mL Lysis/Binding Buffer **(100 mg tissue/mL Lysis/Binding Buffer)** in a 15 mL tube.
   ▲ **Note:** To prevent foaming, 0.1% Antifoam A reagent can be included in the Lysis/Binding Buffer.
2. Lyse sample completely by vigorous vortexing in a 15 mL tube for 3–5 minutes.

   ▲ **Note:** Do not lyse more than 500 mg plant tissue in one 15 mL tube to ensure **a complete lysis which is extremely important for further steps.**
3. To reduce viscosity of the lysate, **mechanical shearing** of DNA must be performed. Transfer lysate to a new tube by forcing it 2–5 times with maximum power through a 21G needle attached to a 1–5 mL syringe matching the lysate volume. Check that no fuzzy material or clumps remain in the lysate.
4. (Optional) Remaining cell debris should be removed by centrifuging at ≥5,000×g for 5 minutes.
5. Place a **LysateClear Column** in the centrifugation tube and apply sheared lysate sample on top of the LysateClear Column.

   Small scale LysateClear Column: centrifuge at ≥3,000×g for 3 minutes. The lysate is now contained in the centrifugation tube.
6. Add **50 µL Oligo (dT) MicroBeads per 1 mL tissue lysate** and mix. For the hybridization of mRNA to Oligo (dT) MicroBeads, further incubation is not necessary.
7. Proceed with magnetic separation (section 2.4).

2.3.4 Isolation of mRNA from whole blood

Before starting

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

1. Transfer freshly drawn, anti-coagulated **peripheral blood** to a suitable tube.

   Use a maximum of 0.5 mL whole blood.
2. Dilute the blood with 1 mL **Lysis/Binding Buffer** per 0.5 mL whole blood.
3. Lyse cells completely by vigorous vortexing for 3–5 minutes.
   ▲ **Note: A complete lysis which is extremely important for further steps.**
4. To reduce viscosity of the lysate, **mechanical shearing** of DNA must be performed. Transfer lysate to a new tube by forcing it 2–5 times with maximum power through a 21G needle attached to a 5 mL syringe. Check that no fuzzy material or clumps remain in the lysate.
5. (Optional) The foam which is caused during the lysis can be reduced by centrifuging the lysate for 3–5 minutes at ≥4,000×g.
6. Place a **LysateClear Column** in the centrifugation tube and apply sheared lysate sample on top of the LysateClear Column.
7. Centrifuge at ≥13,000×g for 3 minutes. The lysate is now contained in the centrifugation tube.
8. Add 50 µL **Oligo (dT) MicroBeads** to the lysate and mix. For the hybridization of mRNA to Oligo (dT) MicroBeads, further incubation is not necessary.
9. Proceed with magnetic separation (section 2.4).

2.3.5 Isolation of mRNA from total RNA

Before starting

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

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

1. Heat freshly prepared total RNA for 3 minutes to 70 °C. Then, chill briefly on ice. For µ Columns up to 200 µg total RNA can be used.
2. Take the tube out of the ice and dilute total RNA with **at least 1 volume of Lysis/Binding Buffer**.
3. Add **25 µL Oligo (dT) MicroBeads per 100 µg total RNA** and mix. For less total RNA, also use 25 µL Oligo (dT) MicroBeads. For the hybridization of mRNA to Oligo (dT) MicroBeads, further incubation is not necessary.
4. Proceed with magnetic separation (section 2.4).

2.4 Magnetic separation of mRNA

Protocol for the magnetic isolation of mRNA

1. Place a MACS µ Column in the magnetic field of the 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. Apply lysate on top of the column matrix. Let the lysate pass through. Magnetically labeled mRNA is retained in the column.
4. Rinse column with 2×200 µL (total RNA sample: 1×200 µL) **Lysis/Binding Buffer** to remove proteins and DNA.
5. Rinse column with 4×100 µL **Wash Buffer** to remove rRNA and DNA.
6. Proceed with cDNA synthesis (section 3).

3. General protocol for cDNA synthesis

For mRNA isolation follow the protocols described in section 2. **Do not elute the mRNA!** After the washing step with the mRNA Wash Buffer proceed with the **cDNA Synthesis Kit** according to the protocol below.

1. Apply 2×100 µL **Equilibration/Wash Buffer** onto the column matrix.
2. Dissolve the **lyophilized Enzyme Mix** in 20 µL **Resuspension Buffer**.
   ▲ **Note:** It is not necessary to pipette the Enzyme Mix up and down more than twice.
3. Apply 20 µL resuspended **Enzyme Mix** on top of the column matrix.
4. To avoid evaporation apply 1 µL Sealing Solution directly on top of the column matrix.
5. Switch on the thermoMACS Separator and set to 42 °C.

6. Incubate for 1 hour.
7. Rinse column with 2×100 µL **Equilibration/Wash Buffer**.
8. Apply 20 µL **cDNA Release Solution** on top of the column matrix.
10. Incubate for 10 minutes at 42 °C.
11. Elute the synthesized cDNA with 50 µL **cDNA Elution Buffer**.
    ▲ **Note: The first drop already contains cDNA.**
    ▲ **Note:** To enhance cDNA recovery a second elution step with an additional 50 µL cDNA Elution Buffer can be performed.
12. Switch off thermoMACS Separator.

4. Tips & hints

For cDNA synthesis:

▲ The plastic film sealing the Enzyme Mix plate 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 such as RNaseZap (Ambion) to reduce the risk of contaminating the cDNA synthesis reaction with RNases. To pierce the plastic film, use a fresh pipette tip; to resuspend the Enzyme Mix, a new pipette tip should be used.

▲ Do not use any other solution than the Sealing Solution to seal the column. Mineral oils as used in PCR reactions do not work.

▲ For quantitative PCR reactions (e.g. LightCycler), we recommend using a maximum of 10–20% of the cDNA to obtain best results. The total amount of cDNA typically used should not exceed 1 ng; for more specific recommendations, please consult the manufacturer's manual of your quantitative PCR device.

▲ In rare cases, PCR reactions may fail due to residual mRNA bound to cDNA. This is a primer dependent process. In this case, we recommend using either RNase H to digest mRNA before the PCR reaction or choosing another pair of primers.

▲ For the RNAse H digestion, add 2 units RNAse H after washing the column (step 7), seal the column with 1 µL **Sealing Solution,** and incubate for 30 minutes at 37 °C in the thermoMACS Separator. Rinse column with 2×100 µL of **Equilibration/Wash Buffer** and proceed with the **cDNA Release Solution** (step 8).

▲ Depending on room conditions (e.g. temperature, humidity) the use of Sealing Solution is not necessary.

For mRNA isolation only:

▲ It is also possible with the µMACS One-step cDNA Kit to perform only the mRNA isolation and to elute the pure mRNA. An Elution Buffer is supplied with the kit.

▲ To achieve a high yield of mRNA, the Elution Buffer must be pre-heated to 70 °C. Place the heating block close to the µMACS Separator and work fast to avoid cooling of the Elution Buffer before applying it onto the column.

▲ The amount of mRNA yielded with one isolation can be determined by measuring the absorbance (A) at 260 nm. An absorbance of 1 corresponds to 40 µg RNA/mL when measuring with a 1 cm thick cuvette. Therefore, the yield of mRNA can be determined according to the formula:

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

▲ **Note:** The absorbance reading should have a value of ≥0.1 to ensure reliable analysis. For accurate results we recommend the usage of RNase-free disposable cuvettes with a small volume (50 µL), which allow the measurement of the undiluted mRNA eluate.

▲ The purity of the obtained mRNA can be determined by measuring the absorbance at 280 nm to determine possible protein content. The ratio A_{260}/A_{280} should be between 1.8 and 2.2 for pure mRNA.

▲ To obtain a higher mRNA concentration in the eluate, we recommend

the following protocol: Apply 20 µL pre-heated (70 °C) Elution Buffer. Discard the flow-through. Then apply 50 µL pre-heated Elution Buffer and collect the complete flow-through containing the mRNA.

Instead of the supplied Elution Buffer RNase-free water can be used.

▲ How to concentrate the eluted mRNA

1. Add 0.1 volume of RNase-free 3 M sodium acetate pH 5.2 and mix.
2. Add 3 volumes absolute ethanol, mix again.
3. Incubate for ≥1 hour at –70 °C or on dry ice.
4. Centrifuge at 14,000×g for ≥20 minutes at 4 °C.
5. Carefully remove the supernatant.
6. To remove residual salt add 1 ml RNase-free 75% ethanol.
7. Centrifuge at 14,000×g for 10 minutes at 4 °C.
8. Carefully remove the supernatant and dry the mRNA pellet.
9. Dissolve mRNA in an appropriate volume of buffer or RNase-free water.

▲ **Note:** If low amounts of mRNA are precipitated (<1 µg mRNA) use carriers, such as 10 µg of E. coli tRNA, or 20 µg of glycogen to precipitate the mRNA.

5. Troubleshooting

General hints to avoid contamination:

▲ 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.

▲ Change pipette tips between different washing buffers.

In case the column flow stops:

▲ We recommend removing the buffer from the column matrix and pipetting fresh buffer with force onto the column matrix.

▲ If solution at the column tip has evaporated after the cDNA synthesis, residual dried Enzyme Mix can be removed with a fresh pipette tip.

▲ (Optional) Omit Sealing Solution.

mRNA isolation:

▲ Work rapidly without interruptions to minimize mRNA degradation.

▲ Before eluting mRNA, the last drop should be taken away from the column tip with a RNase-free pipette tip.

▲ If the Wash or Elution Buffer does not run into the column: pipette the buffer up and down; avoid air bubbles.

▲ In case some lysate is left in the LysateClear Column after centrifugation (possibly to much cell material): centrifuge again.

▲ If the lysis of tissue is difficult to perform, reduce the amount of starting material in the next mRNA preparation.

cDNA synthesis:

▲ Always pipette the solutions directly onto the column matrix, especially when applying 1 µL Sealing Solution.

▲ Apply the sealing solution before switching on the thermoMACS Separator, to avoid evaporation and drying out of the column.

▲ Before eluting cDNA the last drop should be taken off from the column tip with a RNase-free pipette tip.

▲ To enhance recovery of cDNA the incubation time with the Release Reagent can be extended to a total of 30 minutes.

6. References

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