MACS molecular

cDNA synthesis from whole blood using the µMACS cDNA Kit and subsequent KIR typing

Contents

1. Reagent requirements

- µMACS™ One-Step cDNA Kit (# 130-091-902).
- thermoMACS™ Separation Unit (# 130-091-136).
- 2 PAXgene® Blood RNA Tubes (PreAnalytiX®).
- RNase-free water.
- KIR Typing Kit (# 130-092-551 or # 130-092-584).
- DNase I (10 U/µL) (e.g. DNase I recombinant, RNase-free, Roche Applied Science, # 04716728001).
- DNase I 1× buffer.
- 96-well thermal cycler with heated lid.

2. Protocol

2.1 mRNA isolation and cDNA synthesis

▲ Warm all buffers of the µMACS cDNA Kit to room temperature.

▲ For mRNA isolation from whole blood, we recommend to use PAXgene® Blood RNA Tubes (PreAnalytiX®) for RNA stabilization in combination with the µMACS One-step cDNA Kit (# 130-091-902). Five milliliters of whole blood are sufficient per KIR typing test, two PAXgene RNA tubes are necessary.

1. Fill 2.5 mL of whole blood in each of two PAXgene Blood RNA Tubes.
2. Invert tubes 4–5 times and incubate tubes overnight at room temperature.
3. Pool the whole blood from the PAXgene Blood RNA Tubes in one 50 mL conical tube.
4. Centrifuge the tube at 5000×g for 10 minutes at room temperature.
5. Dissolve the pellet in 10 mL of RNase-free water. Centrifuge tube at 5000×g for 10 minutes at room temperature.
6. Dissolve the pellet in a final volume of 2 mL of Lysis/Binding Buffer provided in the µMACS One-step cDNA Kit.
7. Vortex 3 minutes.
8. To reduce viscosity of the lysate, mechanical shearing of DNA must be performed. Force the lysate 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.

   ▲ **Note:** A complete lysis is extremely important for further steps.
9. (Optional) The foam that is caused during the lysis can be reduced by centrifuging the lysate for 3–5 minutes at ≥4,000×g.
10. Place 2 LysateClear Columns in the centrifugation tube and apply half of the sheared lysate sample on top of each LysateClear Column (1 mL per LysateClear Column).
11. Centrifuge at ≥13,000×g for 3 minutes. The lysate is now contained in the centrifugation tube.
12. Add 50 µL of Oligo(dT) MicroBeads per 1 mL of lysate and mix. For the hybridization of mRNA to Oligo(dT) MicroBeads, further incubation is not necessary.
13. Place 2 µ Columns in a thermoMACS™ Separator.
14. Equilibrate µ Columns with 100 µL of Lysis/Binding Buffer.
15. Apply the cleared lysate onto the µ Columns.
16. Wash 2× with 200 µL of Lysis/Binding Buffer.
17. Set the temperature of the thermoMACS Separation Unit to 37 °C.
18. Wash 4× with 100 µL of Wash Buffer.
19. Wash with 100 µL of DNase 1× Buffer.
20. Apply 51 µL of DNase digest reaction mix on the µ Column (50 µL DNaseI 1× Buffer + 10 U (1 µL) DNase I.
21. Incubate 10 minutes at 37 °C.
22. Wash 2× with 200 µL of Lysis/binding Buffer.
23. Wash 4× with 100 µL of Wash Buffer.
24. Wash 2× with 100 µL of Equlibration/Wash Buffer.
25. Dissolve the lyophilized Enzyme Mix in 20 µL of Resuspension Buffer (one per µ Column).

    ▲ **Note:** It is not necessary to pipette the Enzyme Mix up and down more than twice.
26. Apply 20 µL of resuspended Enzyme Mix on top of the column matrix.
27. To avoid evaporation, apply 1 µL of Sealing Solution directly on top of the column matrix.
28. Discard a possibly remaining drop at the column tip with a sterile pipette tip.

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29. Set the temperature on the thermoMACS Separator to 42 °C.
30. Incubate for 1 hour.
31. Rinse column with 2×100 µL of Equilibration/Wash Buffer.
32. Apply 20 µL of cDNA Release Solution on top of the column matrix.
33. Incubate for 10 minutes at 42 °C.
34. Perform a pre-elution with 10 µL of Elution Buffer (discard the eluate).
35. Perform the elution with 30 µL of Elution Buffer and collect the eluate.
36. Combine the eluate of both µ Columns in one tube (around 50 µL eluate).

2.2 KIR typing—Set up PCR experiment

1. Take one KIR Typing PCR Plate from the refrigerator. Ensure that the lyophilized Enzyme Mix is at the bottom of each well. To place the lyophilizate at the tip of the well, gently slap the plate onto the bench.

   ▲ **Note:** Only plates at room temperature will be closed optimally by sealing foil.

   ▲ **Note:** If you perform KIR typing of only one sample, use a pair of scissors to cut the PCR plate between the columns (see fig. 1 in the KIR Typing Kit data sheet).

3. Place the plate in a plate holder and carefully remove cover foil.
4. Use one aliquot of Resuspension Buffer (600 µL) per experiment.
5. First, set up negative control (without cDNA): add 25 µL of Resuspension Buffer to well no. 22 (plate positions F3, F6, F9, or F12).
6. Add the pooled eluates from the cDNA synthesis to the remaining 575 µL of Resuspension Buffer
7. Vortex briefly.
8. For each test, dispense 25 µL of Resuspension Buffer-cDNA mix per well (21 wells per test). Pipette the buffer-cDNA mix closely to the top of the well onto the sidewalls. Please pay attention not to touch the lyophilized Enzyme Mix with the pipette tip!

   ▲ **Note:** The lyophilized Enzyme Mix will turn from yellow to purple.

9. Remove back side from an adhesive Sealing Foil, place foil over the top of the PCR plate, and press it onto the plate. Please make sure that the plate is completely sealed.

   ▲ **Note:** It is very important that the wells are tightly closed, especially at cut-off edges. High liquid loss may cause failure of the PCR reaction.

10. Check that the PCR mix is at the bottom of each well. Gently shake down the liquid to place it at the bottom, or centrifuge shortly.
11. Perform PCR reaction.

    Thermal cycler profile

    ▲ The thermal cycler profile is optimized for Eppendorf and MJ Research 96-well thermal cyclers with heated lid and with a temperature ramping rate of 3 °C/seconds. If you use other instruments, optimization of the thermal cycler profile may be necessary.

Step 1	1 cycle	95 °C	60 s
Step 2	35 cycles for cDNA	94 °C	20 s
		63 °C	20 s
		72 °C	90 s
HOLD		4 °C	

    Total reaction volume per well: 25 µL

11. After thermal cycling, remove plate and proceed directly to gel electrophoresis. For details see the KIR Typing Kit data sheet.

All protocols and data sheets are available at **www.miltenyibiotec.com**.

Warnings

Reagents contain sodium azide. Under acidic conditions sodium azide yields hydrazoic acid, which is extremely toxic. Azide compounds should be diluted with running water before discarding. These precautions are recommended to avoid deposits in plumbing where explosive conditions may develop.

Warranty

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