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μMACS™ SuperAmp™ Kit

Order no. 130-093-242



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1. Description

1.1 Components and size

μMACS™ SuperAmp™ Starting Kit	Order no. 130-093-251
Components	1 μMACS SuperAmp Kit 1 thermoMACS™ Separator 1 MACS® MultiStand
Size	10 reactions
μMACS SuperAmp Kit	Order no. 130-093-242
Components	Reagent Box 1.25 mL of Lysis/Binding Buffer (1.5 mL tube, white cap) 20 μL of tRNA Solution (1.5 mL tube, violet cap) 50 μL of μMACS SuperAmp MicroBeads (brown glass bottle, black cap with white dot) 4 mL of Wash Buffer (15 mL bottle, orange-labeled cap) 4 mL of Equilibration/Wash Buffer (15 mL bottle, dark green-labeled cap) 0.2 mL of Resuspension Buffer C (1.5 mL tube, colorless cap) 10 μL of μMACS Sealing Solution (clear glass bottle, black cap) 2×1 mL of Tailing Wash Buffer (1.5 mL tube, yellow cap) 1 mL of Resuspension Buffer T (1.5 mL tube, brown cap) 0.6 mL of Resuspension Buffer P (1.5 mL tube, red cap) 0.22 mL of Double-distilled Water (1.5 mL tube, blue cap) 0.3 mL of Resuspension Buffer K (1.5 mL tube, green cap) 10× Lyophilized First-strand cDNA Mix (gray-labeled bag, plain microtiter plate)

20× Lyophilized Tailing Mix (brown-labeled microtiter plate)
 10× Lyophilized PCR Mix (red-labeled microtiter plate)
 10× Lyophilized Klenow Mix I (microtiter plate labeled with one green line)
 10× Lyophilized Klenow Mix II (microtiter plate labeled with two green lines)



▲ Resuspension Buffer P contains formamide.

R-phrase:

61 May cause harm to the unborn child.

S-phrases:

53 Avoid exposure - obtain special instructions before use.

45 In case of accident or if you feel unwell, seek medical advice immediately (show the label where possible).

Column Box

10 μ Columns, 20× 200 μL Tubes

Size 10 reactions

Product format μMACS SuperAmp MicroBeads: non-sedimenting MicroBeads conjugated to oligonucleotides.

Suspension contains 0.1% SDS.

Lyophilized First-strand cDNA Mix¹

Lyophilized Tailing Mix²

Lyophilized PCR Mix¹

Lyophilized Klenow I Mix¹

Lyophilized Klenow II Mix¹

¹ Ten single reaction mixes, reaction mix is omitted in two of the four corner wells.

² Twenty single reaction mixes, reaction mix is omitted in the four corner wells.

All buffers and MACS Columns included in the μMACS SuperAmp Kit needed for mRNA isolation and cDNA synthesis are evaluated for the absence of RNase activity.

Storage

Aliquot **tRNA Solution** in appropriate volumes to avoid multiple freezing and thawing and store aliquots at -20 °C. Do not thaw the tRNA Solution more than twice.

The **Reagent Box**—except for the tRNA Solution—with buffers, lyophilized mixes, and μMACS SuperAmp MicroBeads should be stored protected from light at 2–8 °C. **Do not freeze!**

Store **Column Box** with μ Columns and 0.2 mL tubes at room temperature, dry and protected from light.

The expiration dates are indicated on the labels.

1.2 Introduction

1.2.1 Background

Microarrays have become a standard tool to investigate gene expression for many genes in parallel. The gene expression profile can be used, for example, for the comparison of different cells or tissue. Different cell types can be characterized by their differences in the gene expression profile giving a detailed knowledge about changes occurring in, for example, diseased cells or due to drug treatment.

However, there are experimental settings where it is not possible to obtain more than a few cells, for instance when small surgical biopsies, laser capture microdissected (LCM) cells, or rare cell subsets are analyzed. Under these circumstances an efficient isolation and millionfold amplification of picogram amounts of RNA is required for microarray analysis.

Based on the well-established MACS Technology the μMACS SuperAmp Kit allows highly sensitive mRNA isolation, cDNA synthesis and millionfold amplification of cDNA^{1,2} by global polymerase chain reaction. The unique μMACS SuperAmp Protocol combines several features:

- Extremely small (50 nm) superparamagnetic MACS MicroBeads instantly bind and label mRNA molecules from small samples
- MACS Column Technology simplifies the required washing steps resulting in highly pure mRNA
- In-column cDNA synthesis and purification reduces loss of individual transcripts
- Small volumes facilitate optimal reaction kinetics
- Generated cDNA fragments have a uniform size to enable homogenous PCR amplification.
- Global PCR is driven by a single primer to ensure uniform amplification due to consistent annealing conditions

1.2.2 Principle of the μMACS™ SuperAmp™ Kit

The μMACS™ SuperAmp™ Kit is designed for efficient isolation and millionfold amplification of picogram amounts of RNA and thus allows gene expression analysis of 1 to 10,000 cells or from comparable amounts of tissue.

The μMACS SuperAmp Process is composed of the following five steps (see also figure 1 on pages 8 and 9).

Cell lysis, mRNA labeling, and in-column magnetic isolation of mRNA

With MACS Technology, pure mRNA can effectively be isolated from very small amounts of starting material. Added to the cell lysate, the superparamagnetic MACS MicroBeads instantly bind to the poly(A) residues of the mRNA. Then, the labeled cell lysate is applied to a

μ Column that is placed in the magnetic field of a thermoMACS Separator—a heatable permanent magnet. The magnetically labeled mRNA is retained in the strong magnetic field while effective washing steps remove all other cell components.

In-column cDNA synthesis and purification

cDNA synthesis and purification is performed in the same column used for mRNA isolation to avoid loss of material. This is crucial for reliable amplification of very small amounts of mRNA. The special and well balanced MicroBead mixture generates first-strand cDNA fragments of uniform size enabling uniform amplification during PCR.

Tailing of purified cDNA

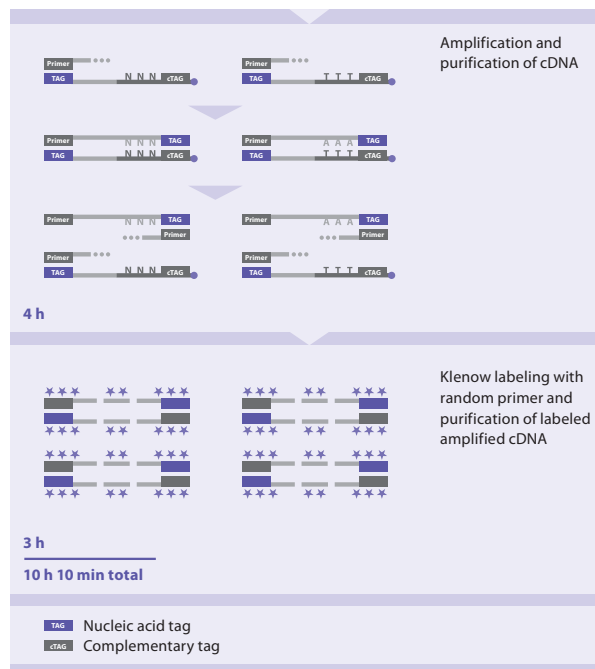
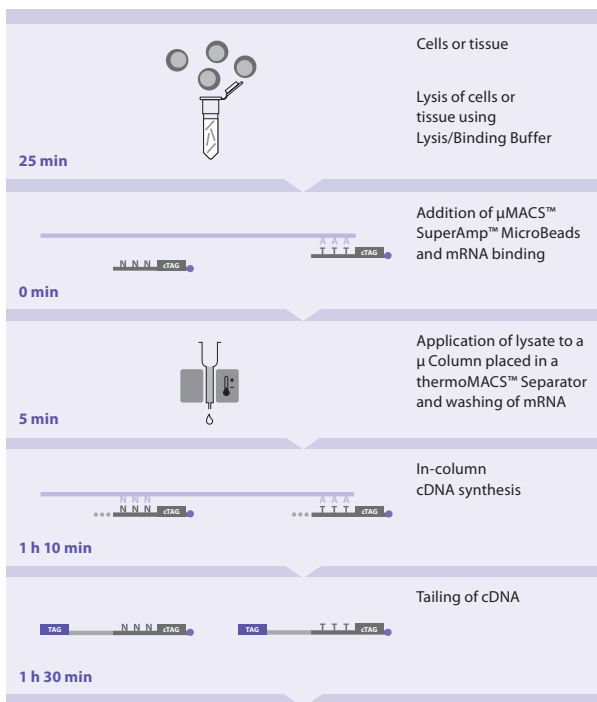
After the cDNA fragments are eluted from the column a tag is added to the 3' end of each cDNA fragment by utilizing a terminal deoxynucleotidyl transferase.

Amplification of cDNA by global PCR

A global PCR millionfold amplifies the uniform-sized cDNA fragments, resulting in sufficient target material for microarray hybridization. The PCR performed with a single primer guarantees unbiased amplification due to the uniform annealing temperature. The primer binding site at the 3' end has been added during cDNA tailing. The complementary sequence of the tag has been inserted at the 5' end of the cDNA fragments during cDNA synthesis.

Klenow labeling of amplified cDNA

After purification of the PCR products, a Klenow fragment labeling procedure with random primers in the presence of labeled nucleotides, for example, Cy3-dCTP or Cy5-dCTP, yields labeled DNA fragments. As sense and antisense strands are labeled, hybridization on either sense or antisense orientated microarrays is possible.

Figure 1: Principle of the μ MACS SuperAmplification Technology

1.2.3 Time schedule

It is recommended to schedule the amplification procedure for two days as proposed in the following scheme:

Day	Duration*	Procedure
Day 1	30 min	Cell lysis and magnetic isolation of mRNA
	1 h 10 min	In-column cDNA synthesis and purification
	1 h 30 min	Tailing of cDNA
	10 min	Preparation of global PCR
Overnight	3 h 20 min**	Amplification of cDNA by global PCR
Day 2	30 min	Purification of PCR products
	2 h 30 min	Klenow labeling of amplified cDNA
	30 min	Purification of labeled amplified cDNA
Total time	10 h 10 min	

* Includes typical handling times

** Depending on thermocycler

1.3 Kit capacities

The μ MACS SuperAmplification Kit is designed for efficient isolation and millionfold amplification of picogram amounts of mRNA and allows gene expression analysis of 1 to 10,000 cells or of comparable amount of tissue. When applying less than 10 cells it is recommended performing sufficient number of biological repeats to outbalance biological differences of individual cells like cell cycle status etc.

1.4 Reagent and instrument requirements

▲ All additionally required equipment and reagents must be RNase-free and free of DNA contamination!

- 1.5 mL RNase-free tubes and pipette tips
- RNase-removing solution, e.g. RNaseZAP®, Ambion Inc. # 9780 and 70% Ethanol for surface cleaning
- 15 mL conic tubes
- Phosphate-buffered saline (PBS)
- (Optional) Protector RNase Inhibitor (Roche # 3335399)
- Proteinase K, PCR-grade lyophilized, 25 mg (Roche # 3115836; working dilution: 5 μ g/ μ L) and RNase free water for reconstitution
- Terminal Deoxynucleotidyl Transferase (MBI Fermentas # EP0161; Takara # 2230A; MoBiTec # 2230A-TK)
- Expand Long Template PCR System (Roche # 1681834)
- High Pure PCR Product Purification Kit (Roche # 1732668)

- Klenow Fragment (10 units/ μ L; MBI Fermentas # EP0051)
- For labeling:
CyDye™ nucleotides (GE Healthcare: Cy3-dCTP, # PA53021; Cy5-dCTP, # PA55021, both 25 nmol, 1 nmol/ μ L)
Also, alternative markers like other fluorochrome or biotin-coupled dCTP as well as radioactively labeled α -dCTP can be used (volume should be 1 μ L per labeling reaction; amount of nucleotide might have to be optimized in respect to dCTP concentration and labeling efficiency/brightness)
- illustra™ CyScribe™ GFX™ Purification Kit (GE Healthcare # 27-9606-01)
- thermoMACS™ Separator (# 130-091-136) and MACS Multistand (# 130-042-303)
- Thermocycler for 0.2 mL tubes, with heatable lid
- Microcentrifuge suitable for 15 mL and 300 \times g centrifugation
- Microcentrifuge suitable for 1.5 mL and 0.2 mL tubes
- Spectrophotometer, e.g., Nanodrop™ ND1000 (Nanodrop Technologies)
- 2 \times heating blocks for 1.5 mL tubes suitable for 37 °C and 99 °C, respectively

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)
Total RNA, 8 reactions (# 130-075-102)

- μ MACS One-step cDNA Kit (# 130-091-902)
- μ MACS One-step cDNA Labeling Kit (# 130-092-443)
- μ MACS One-step T7 Template Kit (# 130-092-866)
- PIQOR™ Microarray Kits (topic-defined and custom):
www.miltenyibiotec.com
- a-Hyb™ Hybridization Station: www.miltenyibiotec.com
- MACS Products for cell separation: www.miltenyibiotec.com

2. Protocol for mRNA isolation and amplification

2.1 Sample preparation and cell lysis

Before starting

▲ The μ MACS SuperAmp Kit allows the amplification of mRNA from very small samples. The procedure is extremely sensitive to RNA or DNA contaminations. Make sure that all instruments and plasticware are free from RNase and contaminating RNA or DNA. Do not contaminate instruments or plasticware during the isolation or dissection of tissue or cells.

▲ Always keep the cell or tissue sample on ice before starting. In case the sample needs to be stored after preparation, quick-freeze sample in liquid nitrogen and store at -70 °C.

▲ Do not use RNA-stabilizing reagents.

▲ For general handling recommendations refer to chapter 3, Tips & hints.

▲ Read instruction manual of the thermoMACS Separator.

▲ Prepare a thermocycler for temperature incubations. Do not use a water bath because of the risk of RNase and cell contamination. It

is recommended to program the thermocycler in advance with the required temperatures to save time while performing the μ MACS SuperAmp Process. Program the following protocols on your PCR cyclor:

Program	Section	Temperature*	Duration
"lysis"	2.1.2 (step 4)	45 °C 75 °C	10 min 1 min
"denat."	2.4 (step 7)	94 °C 4 °C	4 min hold
"tailing"	2.4 (step 9)	37 °C 70 °C	1 hour 5 min
"SuperAmp" **	2.5 (step 5)	78 °C 94 °C 65 °C 68 °C 94 °C 65 °C 68 °C 68 °C 4 °C	30 sec 15 sec 30 sec 2 min } 20 cycles 15 sec 30 sec 2 min 30 sec + 10 sec/cycle } 21 cycles 7 min hold

* lid temperature should be 5 °C above block temperature.

** if possible, use different thermocycler or thermocycler block for lysis, denaturation and tailing than for global PCR to minimize risk of cross contamination (see chapter 3, Tips & hints).

2.1.1 Sample preparation

▲ The volume of the cells before lysis must not exceed 1 μ L. The cell suspensions should not contain more than 10,000 cells/ μ L. Cell suspensions containing more than 10,000 cells/ μ L may lead to inefficient lysis or can clog the μ Column.

2.1.1.1 Preparation of liquid samples with less than 10,000 cells in a maximum volume of 1 μ L

Directly start with step 1 in chapter 2.1.2, Cell lysis.

2.1.1.2 Preparation of liquid samples with less than 10,000 cells in a volume of 1 μ L to 200 μ L

▲ Pre-cool centrifuge to 4 °C.

1. Collect the cells (e.g. from FACS™ or MACS Cell Separation), ideally in ice-cold PBS, in the provided 0.2 mL tube.
2. Pellet the cells by centrifuging at 300 \times g for 10 minutes at 4 °C. Carefully aspirate the supernatant leaving the cells in approximately 1 μ L.
3. Continue with step 1 in chapter 2.1.2, Cell lysis.

2.1.1.3 Preparation of liquid samples with a volume larger than 200 μ L and/or a cell concentration higher than 10,000 cells/ μ L

▲ Pre-cool centrifuge to 4 °C.

1. Collect the cells, ideally in ice-cold PBS, in an appropriate tube for centrifugation.
2. Measure cell concentration.
3. Pellet the cells by centrifuging at 300 \times g for 10 minutes at 4 °C. Carefully aspirate the supernatant.

- Resuspend the cells in an appropriate volume of PBS to a concentration of max. 10,000 cells/ μ L.
- Continue with step 1 in chapter 2.1.2, Cell lysis.

2.1.1.4 Preparation of dry samples from microdissected tissue

- Collect the cells in a 0.2 mL tube as suggested by the supplier of the laser microdissection device.
 - ▲ **Note:** Do not microdissect more than 10,000 cells per tube.
- Add 6.4 μ L freshly prepared Incubation Buffer (refer to chapter 2.1.2) to the microdissected cells. Resuspend the cells by pipetting up and down 10–20 times.
- Incubate at 45 °C for 10 minutes in a thermocycler. If cells have been trapped in the tube cap, make sure that incubation temperature in the cap is 45 °C by heating the lid of the thermocycler to 45 °C. Spin down liquid at 2500 \times g.
- Incubate tube in a thermocycler for 1 minute at 75 °C and briefly spin down liquid at 1,000 \times g.
- Transfer cell lysate to the provided 0.2 mL tube.
- Continue with step 1 in chapter 2.2, In-column magnetic isolation of RNA.

2.1.2 Cell lysis

Before starting

- ▲ Assure that cell suspension from 2.1.1 is always on ice before adding the Incubation Buffer to prevent RNA degradation.
- ▲ Thaw tRNA Solution (violet cap) and the Proteinase K Solution on ice.

- ▲ Allow Lysis/Binding Buffer (1.5 mL tube, white cap), Wash Buffer (15 mL bottle, orange-labeled cap), and Equilibration/Wash Buffer (15 mL bottle, dark green-labeled cap) to adjust to room temperature.
- ▲ It is recommended to perform a no-cell control as described in chapter 3.5.

- Prepare the Incubation Buffer for 1–4 reactions in a 1.5 mL RNase-free tube by adding the following reagents in the indicated order. For more than four reactions, scale-up accordingly:
 - 25 μ L Lysis/Binding Buffer (1.5 mL tube, white cap)
 - 2 μ L tRNA Solution (1.5 mL tube, violet cap)
 - 1 μ L of the Proteinase K Solution (5 μ g/ μ L)
 Mix well.
 - ▲ **Note:** Do not pipette smaller volumes.
- Transfer 5.4 μ L of freshly prepared Incubation Buffer to the provided 0.2 mL tube. Add 1 μ L of cell suspension (with no more than 10,000 cells/ μ L). In case cells have been collected in the provided 0.2 mL tube (chapter 2.1.1.4), add 5.4 μ L of freshly prepared Incubation Buffer to the tube containing 1 μ L cell suspension. Pipette up and down twice. Vortex cell suspension thoroughly and briefly spin down liquid at 1,000 \times g.
 - ▲ **Note:** For subsequent hybridization of Affymetrix microarrays, please add 2 μ L of Poly-A RNA Control (Affymetrix # 900433; GeneChip® Poly-A RNA Control Kit) in a dilution of 1:5,000,000 for less than 100 cells and a dilution of 1:50,000 for more than 100 cells to the cell suspension. To prepare the dilutions use non-stick RNase-free microfuge tubes and avoid pipetting solutions less than 2 μ L in volume to maintain precision and consistency.
- Start “lysis” program on the thermocycler and wait until 45 °C have been reached.

- Place tube in the thermocycler and incubate 10 minutes at 45 °C followed by 1 minute at 75 °C.
- Directly proceed to chapter 2.2, In-column magnetic isolation of mRNA.

2.2 In-column magnetic isolation of mRNA

▲ Do not mix solutions containing mRNA or cDNA by pipetting up and down. Instead, vortex solution as indicated in the protocol. Pipetting may reduce the amount of nucleic acids due to adhesion of nucleic acids to plastic material.

- Add 5 μ L of μ MACS SuperAmp MicroBeads (brown glass bottle, black cap with white dot) to lysed cells from step 4 in chapter 2.1.2. and vortex. Briefly spin down liquid in a microcentrifuge at 1,000 \times g.
- Place a μ Column in the magnetic field of the thermoMACS Separator.
 - ▲ **Note:** 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.
- Prepare column by rinsing with 100 μ L of Lysis/Binding Buffer (1.5 mL tube, white cap).
 - ▲ **Note:** Columns are “flow stop” and do not run dry.
- Apply cell lysate with μ MACS SuperAmp MicroBeads from step 4 on top of the column matrix. Let the lysate pass through. Magnetically labeled mRNA is retained in the column.
- Rinse column with 4 \times 100 μ L of Wash Buffer (15 mL bottle, orange-labeled cap) to remove proteins, DNA, and rRNA.
- Directly proceed to chapter 2.3, In-column cDNA synthesis and purification.

2.3 In-column cDNA synthesis and purification

▲ For handling and resuspension of lyophilized mixes please refer to chapter 3, Tips & hints.

- Rinse column with 2 \times 200 μ L of Equilibration/Wash Buffer (15 mL bottle, dark green-labeled cap).
- Dissolve the Lyophilized First-strand cDNA Mix of one well (gray-labeled bag, plain microtiter plate) in 20 μ L Resuspension Buffer C (1.5 mL tube, colorless cap).
- (Optional) Add 1 μ L of RNase Inhibitor (e.g. Protector RNase Inhibitor from Roche) to the resuspended First-strand cDNA Mix.
- Apply the resuspended First-strand cDNA Mix on top of the column matrix.
- To avoid evaporation, apply 1 μ L of μ MACS Sealing Solution (clear glass bottle, black cap) directly on top of the column matrix.
 - ▲ **Note:** If small air bubbles are visible on the top of the column matrix, pierce these bubbles with a pipette tip and then apply the μ MACS Sealing Solution.
 - ▲ **Note:** Apply the sealing solution on top of the column matrix by slightly touching the matrix.
- 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.
- Switch on the thermoMACS Separator and set to 42 °C.
- Incubate for 45 minutes after temperature has been reached. The correct temperature is indicated by permanent red light.
- Switch off the thermoMACS Separator.
- Rinse column with 2 \times 100 μ L of Tailing Wash Buffer (1.5 mL tube, yellow cap).

▲ **Note:** If liquid at the column tip (column outlet) has evaporated during cDNA synthesis, residual reaction mix can dry up and block column flow. Remove dried reaction mix with a fresh pipette tip.

11. Directly proceed with chapter 2.4., Tailing of cDNA.

2.4 Tailing of cDNA

1. Dissolve the Lyophilized Tailing Mix of two wells (brown-labeled microtiter plate) each in 50 μ L Resuspension Buffer T (1.5 mL tube, brown cap)
2. Rinse column with 2 \times 50 μ L of freshly resuspended Tailing Mix.
3. Take off the last drop from the column tip with an RNase-free pipette tip. This will ensure a correct elution volume for subsequent reactions.
4. Remove column from the thermoMACS Separator and place it into a 15 mL conic tube (e.g. Falcon™ tube).

▲ **Warning!** Do not use 1.5 mL tubes as placing columns in 1.5 mL tubes in respective centrifuges can interfere with closing of the inner lid of the centrifuge or the columns can scratch inside of the lid during centrifugation.
5. Centrifuge tube with the column at 300 \times g for 10 seconds.
6. Transfer the eluate into a fresh 0.2 mL tube.
7. Place tube into a thermocycler and start “denat.” program for incubating for 4 minutes at 94 °C. Immediately set tube on ice for at least 1 minute.

▲ **Note:** This step denatures mRNA-cDNA hybrids to enhance the subsequent tailing.
8. Add 20 units of Terminal Deoxynucleotidyl Transferase (MBI

Fermentas, Takara, MoBiTec) to the sample, vortex, and briefly spin down liquid in a microcentrifuge at 1,000 \times g.

▲ **Note:** The final volume of the sample should reach approximately 30 μ L.

9. Start “tailing” program on the thermocycler and wait until 37 °C have been reached. Place tube in the thermocycler and incubate for 60 minutes at 37 °C followed by 5 minutes at 70 °C to inactivate the enzyme. Immediately place tube on ice.
10. Proceed to chapter 2.5, Amplification of cDNA by global PCR.

2.5 Amplification of cDNA by global PCR

1. Dissolve the Lyophilized PCR Mix (red-labeled microtiter plate) of one well in 60 μ L Resuspension Buffer P (1.5 mL tube, red cap).

▲ **Caution!** Resuspension Buffer P contains 5% formamide.
2. Add 7 μ L of Expand Long Template Buffer 1 (Roche) to the dissolved PCR Mix.
3. Add 3 μ L of Expand Long Template PCR System DNA Pol Mix (Roche).
4. Transfer 70 μ L of the above prepared PCR Mix to the tailed cDNA from chapter 2.4, step 10. Vortex tube and briefly spin down liquid in a microcentrifuge at 1,000 \times g.
5. Place the tube in a thermocycler and run “SuperAmp” PCR according to table on page 14.
6. After amplification, purify PCR products using High Pure PCR Product Purification Kit (Roche) following the instructions of the manufacturer with the modification of incubating the Elution

Buffer on the column for 4 minutes at room temperature before the last centrifugation step.

7. Measure DNA concentration of the purified sample using a spectrophotometer at 260 nm wavelength. The typical yield should be in a range of 3–6 μ g.
8. Proceed with chapter 2.6, Klenow labeling of amplified cDNA, or store amplified cDNA at –20 °C.

If samples are intended to be processed on Affymetrix microarrays please proceed with 2.7, Labeling procedure for Affymetrix microarrays.

2.6 Klenow labeling of amplified cDNA

Before starting

- ▲ Heat two heating blocks to 99 °C and 37 °C, respectively.
- ▲ Fluorescent dyes and labeled samples are light-sensitive. Whenever possible, keep the reactions including fluorescent dyes in the dark.
- ▲ If samples are intended to be processed on Affymetrix microarrays please proceed with 2.7, Labeling procedure for Affymetrix microarrays.

1. Transfer 200 ng of the purified PCR product from chapter 2.5 into a fresh 1.5 mL tube. Add Double Distilled Water (1.5 mL tube, blue cap) to adjust volume to 22 μ L.
2. Dissolve the Lyophilized Klenow Mix I of one well (microtiter plate labeled with one green line) with 20 μ L Resuspension Buffer K (1.5 mL tube, green cap).

3. Add the 20 μ L resuspended Klenow Mix I to the PCR product (total volume 42 μ L). Vortex the tube and spin down.
4. Incubate reaction tube for 5 minutes at 99 °C in a heating block. Immediately place tube on ice. Cool down heating block to 70 °C.
5. Dissolve the Lyophilized Klenow Mix II of one well (microtiter plate labeled with two green lines) with 10 μ L Resuspension Buffer K (1.5 mL tube, green cap).
6. Add 5 μ L resuspended Klenow Mix II to the reaction tube. Vortex, spin down, and place tube on ice. The residual resuspended Klenow Mix II can be discarded.
7. Add 1 μ L Cy3-dCTP or Cy5-dCTP (1 nmol; e.g. from GE Healthcare) or alternatively labeled dCTP to the tube.
8. Add 2 μ L Klenow Fragment (10 units/ μ L, MBI Fermentas). Vortex and spin down.
9. Incubate tube for 2 hours at 37 °C in the dark.
10. Incubate for 5 minutes at 70 °C to inactivate the enzyme. Briefly spin down liquid in a microcentrifuge.
11. Purify labeled DNA using illustra™ CyScribe™ GFX™ Purification Kit (GE Healthcare) following the instructions of the manufacturer's protocol (Product booklet Rev D 08/2007: chapter 5.2 “Protocol for purification of CyDye-labeled cDNA synthesized with the Amersham CyScribe First-Strand cDNA Labeling Kit”).

If two colour hybridizations (e.g. Cy3/Cy5) are intended respective samples should be pooled before purification. RNA degradation step is not necessary.

Briefly, capture labeled DNA using the appropriate capture buffer

without prior RNA degradation. Wash three times using the wash buffer given in the illustra™ CyScribe™ GFX™ Purification Kit and elute with 60 µL 65 °C Elution buffer. For increasing DNA yield, incubate the Elution buffer on the column for 5 minutes at room temperature before the last centrifugation step. It is not necessary to repeat the elution to further increase the yield.

12. Measure DNA concentration of the purified sample using a spectrophotometer at 260 nm wavelength.

▲ **Note:** The typical yield of the Klenow labeling reaction is in a range of 3–6 µg due to an up to 30-fold amplification of the template DNA.³

13. Proceed with hybridization of 3 µg labeled DNA on a DNA microarray following the instructions of the microarray manufacturer.

▲ **Note:** Be aware of denaturing the labeled double-stranded DNA samples before hybridization. In case of saturated signals, adjust the scanner settings or reduce the amount of labeled DNA used.

2.7 Labeling procedure for Affymetrix microarrays

The following procedure describes the generation of double-stranded, fragmented and labeled DNA for expression analysis using Affymetrix microarrays.

The sample preparation requires 3 different steps:

- The PCR product from chapter 2.5 is first used as template in Klenow fragment reactions, which incorporate deoxyuridine (dU) at predefined ratios into each DNA strand (chapter 2.7.1).
- The dU-incorporated DNA is fragmented using uracil DNA glycosylase (UDG) and apurinic/aprimidinic endonuclease (APE 1) enzymes (chapter 2.7.2).

- Fragmented DNA is then labeled using terminal deoxynucleotidyl transferase (TdT) and an Affymetrix DNA Labeling Reagent (chapter 2.7.3).

Then the fragmented, labeled, double-stranded DNA is ready to add to the hybridization cocktail and hybridize onto Affymetrix microarrays (chapter 2.7.4)⁴.

▲ **Note:** To obtain a minimum yield of 15 µg DNA for hybridization, two Klenow, fragmentation and labeling reactions have to be performed for each sample.

Additional reagents

- dNTP set, 4×25 µmol (MBI Fermentas # R0181; Peqlab # 20-2010)
- dUTP (MBI Fermentas # R0133)
- Tris (Sigma-Aldrich # 93362-250G)
- EDTA (Merck # 1.08418.0100)
- GeneChip® WT Double-Stranded DNA Terminal Labeling Kit (Affymetrix # 900812)

2.7.1 Klenow reaction of amplified cDNA

▲ For the hybridization of Affymetrix microarrays a typical yield of 15 µg DNA is required. Perform two Klenow reactions per sample as described below in order to receive enough material for hybridization.

▲ The Klenow Mix II used in this reaction differs from the standard Lyophilized Klenow Mix II, which is delivered with the µMACS SuperAmp Kit.

▲ Please do not use the Klenow Mix II delivered with the kit! Prepare the following Klenow Mix II, from now on called Klenow Mix II-affy:

Klenow Mix II-affy (for 2 reactions; total volume 10 µL):

- 1 µL 100 mM Tris, pH 8
- 1 µL 10 mM EDTA, pH 8
- 6 µL dNTP Mix (10 mM dATP, 10 mM dCTP, 10 mM dGTP, 8 mM dTTP, 2 mM dUTP)
- 2 µL Double Distilled Water

- a. Transfer 200 ng of the purified PCR product from chapter 2.5 into a fresh 0.2 mL tube. Add Double Distilled Water (1.5 mL tube, blue cap) to adjust volume to 23 µL.
- b. Dissolve the Lyophilized Klenow Mix I of one well (microtiter plate labeled with one green line; delivered with the kit) with 20 µL Resuspension Buffer K (1.5 mL tube, green cap; delivered with the kit).
- c. Add the 20 µL resuspended Klenow Mix I to the PCR product (total volume 43 µL). Vortex the tube and spin down.
- d. Incubate reaction tube for 5 minutes at 99 °C in a thermocycler. Immediately place tube on ice.
- e. Add 5 µL Klenow Mix II-affy to the reaction tube. Vortex, spin down, and place tube on ice
- f. Add 2 µL Klenow Fragment (10 units/µL, MBI Fermentas). Vortex and spin down.
- g. Incubate the reactions in a thermocycler (with heated lid) using the following protocol:
 - 37 °C for 2 hours
 - 70 °C for 5 minutes
 - 4 °C for 10 minutes
- h. Briefly spin down liquid in a microcentrifuge.

- i. Pool the duplicates and purify DNA products using High Pure PCR Product Purification Kit (Roche) following the instructions of the manufacturer. Use 60 µL Elution Buffer and incubate on the column for 4 minutes at room temperature before the last centrifugation step.
- j. Measure DNA concentration of the purified sample using a spectrophotometer at 260 nm wavelength.

▲ **Note:** The typical yield of two pooled Klenow reactions is in a range of 18–24 µg due to an up to 60-fold amplification of the template DNA.
- k. Proceed with chapter 2.7.2, Fragmentation of dU-incorporated DNA, or store DNA at –20 °C.

2.7.2 Fragmentation of dU-incorporated DNA

▲ This procedure requires the use of the GeneChip WT Double-Stranded DNA Terminal Labeling Kit (Affymetrix # 900812)⁴.

▲ Perform 2 fragmentation reactions for each sample (each with 7.5 µg DNA).

1. Transfer 7.5 µg DNA from chapter 2.7.1 into a fresh 0.2 mL tube and place tube on ice.
2. Add the following reagents to the DNA:
 - 4.8 µL 10× Fragmentation Buffer.
 - 1.5 µL UDG (10 units/µL).
 - 2.25 µL APE 1 (100 units/µL).
 - RNase-free Water to adjust volume to 48 µL.
 For multiple reactions prepare a sufficient mix (master mix).
3. Mix by flicking the tube and spin down.

- Incubate the reactions in a thermocycler (with heated lid) using the following protocol:
 - 37 °C for 1 hour
 - 93 °C for 2 minutes
 - 4 °C for 10 minutes
- Vortex, spin down the tubes, and transfer 45 µL of the sample to a new 0.2 mL tube. The remainder of the sample can be used for fragmentation analysis using, for example, a Bioanalyzer. For details about DNA size analysis refer to the instructions given in the Reagent Kit Guide that accompanies the RNA 6000 Nano LabChip® Kit.

▲ **Note:** Results of the Bioanalyzer analysis of fragmented DNA should show that the majority of fragmented DNA is between 25 to 200 bases, with the peak of the distribution between 25 to 100 bases⁴.
- Proceed with chapter 2.7.3, Labeling of fragmented dsDNA, or store DNA at -20 °C.

2.7.3 Labeling of fragmented dsDNA

▲ This Procedure requires the use of the GeneChip® WT Double-Stranded DNA Terminal Labeling Kit (Affymetrix # 900812)⁴.

▲ Perform 2 labeling reactions for each sample (each with 7.5 µg fragmented DNA).

- Add the following reagents to the 7.5 µg fragmented DNA from chapter 2.7.2:
 - 12 µL 5× TdT Buffer
 - 2 µL TdT (30 units/µL)
 - 1 µL 5 mM DNA Labeling Reagent
 - total volume 60 µL

- For multiple reactions prepare a sufficient mix (master mix).
- Mix by flicking the tube and spin down the tubes.
 - Incubate the reactions in a thermocycler (with heated lid) using the following protocol:
 - 37 °C for 1 hour
 - 70 °C for 10 minutes
 - 4 °C for 10 minutes
 - Proceed with chapter 2.7.4 for hybridization setup, or store labeled DNA at -20 °C.

2.7.4 Hybridization of labeled DNA on Affymetrix Microarrays

▲ This procedure requires the use of the GeneChip Hybridization, Wash, and Stain Kit (Affymetrix # 900720)⁴ and describes the composition of the hybridization cocktail for 49 Format Arrays.

- Pool duplicates from chapter 2.7.3 in a 1.5 mL RNase-free tube (total volume 120 µL) and use a speedvac to concentrate the sample to a volume below 109 µL.
- Determine the sample volume and add the following reagents to the labeled DNA:
 - 5 µL 3 nM Control Oligonucleotide B2
 - 15 µL 20× Eukaryotic Hybridization Controls (bioB, bioC, bioD, cre)
 - 21 µL DMSO
 - 150 µL 2× Hybridization Mix
 - RNase-free water up to 300 µL
 - Total volume 300 µL
- Mix by flicking the tube and spin down.

- Incubate the hybridization cocktail at 99 °C for 5 minutes. Cool to 45 °C for 5 minutes, and centrifuge at maximum speed for 1 minute.
- Inject the sample into the array through one of the septa.
- Place microarray in 45 °C hybridization oven, at 60 rpm, and incubate for 16 hours.
- Depending on the Fluidics Station the following protocols are recommended for washing and staining:
 - Fluidics Station 400: EukGE-WS2v5 / Midi_euk2v3
 - Fluidics Station 450: FS450_0001 / FS450_0002

3. Tips & hints

3.1 µ Columns

▲ Always insert µ Columns into the thermoMACS Separator from the front without touching the column tip (column outlet).

▲ Apply solutions, especially small volumes, directly onto the matrix in the column by touching the matrix with the pipette tip. Avoid buffer drops in the column reservoir: Aspirate residual drops with a pipette. Residual liquid might impair the reaction efficiency of cDNA reaction or tailing reaction.

▲ Always wait until liquid has completely run through the column before adding the next solution. µ Columns are “flow-stop”, and do not run dry.

3.2 Lyophilized mixes

The plastic foils sealing the lyophilized mix plates can either be peeled off by hand or pierced with a pipette tip.

▲ For peeling off, it is recommended to first cut the cover of an individual well with a scalpel. Then, peel off the film by hand wearing RNase-free gloves.

▲ To pierce the cover, it is recommended wiping the foil of the microtiter plate with an RNase-removing solution first to reduce the risk of contamination. Then, use a fresh pipette tip to pierce the plastic film; take another fresh pipette tip to resuspend the mix.

▲ For resuspension of lyophilized mixes, add the resuspension buffer into the well and gently pipette up and down until a clear solution is obtained. Avoid foam formation.

3.3 RNase-free work

▲ 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.

▲ Use an RNase-free pipette set and RNase-free pipette tips. Pipettes should also be free of contaminating DNA or cells. Reseal bags of plastic disposables immediately after removing the disposables needed for the respective step. Use a fresh tip for every pipetting step. Always use pipette tips that prevent aerosol formation.

▲ Change pipette tips always between different wash buffers.

▲ Always clean the bench with 70% ethanol and use RNase-removing solutions for decontamination before starting the protocol.

▲ It is recommended using RNase-removing solutions to eliminate RNases from glassware, plastic surfaces, countertops, and pipettors.

The solutions can be poured onto or wiped over surfaces. After rinsing twice with distilled water, materials are ready for use.

3.4 No DNase treatment after mRNA extraction

▲ It is not recommended to perform a DNase treatment to reduce residual genomic DNA after mRNA extraction. Potential RNase contamination of the DNase could reduce the RNA yield and decrease the reliability of the results.

▲ **Note:** The μ MACS SuperAmp Technology was developed to obtain highly pure mRNA devoid of genomic DNA and other contaminations and additional DNase treatment is not necessary.

3.5 No-cell control

▲ It is recommended to include a no-cell control during the μ MACS SuperAmp Procedure: In addition to the biological samples, set up a tube without any cell. This tube is from now on called “no-cell control”.

Treat this no-cell control tube as the tubes containing cells. Usually, even in the no-cell control amplified DNA is produced during PCR. The source of amplified DNA in this sample is contaminating bacterial DNA (see also chapter 3.6, Preventing PCR contamination). Label DNA from this no-cell control either with Cy3 or Cy5 as mentioned in the protocol. Hybridize the labeled no-cell control to a separate microarray. Hardly any signals should be found on this microarray. So even though the no-cell control SuperAmp PCR gives raise to substantial DNA, the amplified bacterial DNA does not impair the microarray results.

3.6 Preventing PCR contamination

▲ The laboratory area for sample preparation should be physically separated from the amplification and all following steps.

▲ **Note:** Ideally, the preparation steps for the PCR should be performed in a separate room than the PCR and the following steps.

▲ A dedicated set of pipettes, centrifuge for 0.2 mL and 1.5 mL tubes, centrifuge for 15 mL conical tubes, and disposables should be exclusively used for the pre-PCR steps.

▲ If possible, use a different thermocycler or thermocycler block for the sample preparation steps (all steps from cell lysis to tailing of the cDNA) than for the SuperAmp PCR to minimize the risk of carryover.

▲ Store all reagents used before PCR amplification separated from post PCR reagents and equipment. Use different laboratory coats and gloves before and after the PCR amplification.

▲ Always use pipette tips that prevent aerosol formation.

▲ Use a fresh tip for every pipetting step.

▲ Before starting, wipe pipette with an ethanol-soaked tissue.

▲ Regularly clean laboratory surfaces and instruments using reagents suitable for DNA decontamination, such as DNAZap, DNA-ExitusPlus, DNA Remover, License-to-kill, or bleach. Make sure that surfaces are resistant to bleach (10% sodium hypochloride) before use. Use single-use tissues for cleaning.

▲ Always use ultrapure water for reconstitution of the Proteinase K. Do not use autoclaved water as it might be contaminated with DNA if the autoclave is also used for sterilization of cultures.

▲ Most molecular biology enzymes contain minute amounts of bacterial DNA as they have usually been derived from bacteria. Therefore, also a negative control sample will yield substantial amounts of DNA (see 3.5, No-cell control).

4. Troubleshooting

▲ Refer to chapter 3, Tips & hints, for detailed information how to avoid contamination and degradation of mRNA.

Solution has dried out at column tip

In rare cases, a blockade of the column flow due to evaporation at the column tip (column outlet) can occur after heated incubation; remove residual dried reaction mix with a fresh pipette tip.

To minimize drying effects, apply the μ MACS Sealing Solution before switching on the thermoMACS Separator. Do not use any other solution than the μ MACS Sealing Solution to seal the column. Mineral oils, as used in PCR reactions, do not work.

Low global PCR yield

The SuperAmp PCR protocol has been optimized to reproducibly yield micrograms of DNA. In case of low yields, trivial causes, such as using a wrong PCR protocol, thermocycler being defect, or the impaired activity of the Expand Polymerase, might have disrupted the PCR reaction. Control your PCR protocol and thermocycler and avoid exposure of Expand Polymerase to room temperature for extended times or multiple freeze thaw cycles.

The PCR product might also get lost during purification. Use recommended DNA purification products to reduce the risk of DNA

loss. Add ethanol to the wash buffer as indicated in the instruction manual of the supplier.

Low yield or low incorporation rate after Klenow labeling

A low DNA yield (less than 3 μ g DNA) indicates a weak Klenow polymerase activity, as labeling with Klenow fragment generally leads to an up to 30-fold amplification of the template DNA.

The most likely cause for low yields during the Klenow labeling reaction is the Klenow enzyme itself. Use the indicated amount of Klenow enzyme from the recommended supplier. Avoid exposure of enzymes to room temperature for extended times or multiple freeze thaw cycles.

Labeled DNA might have been lost during sample clean up. Use recommended products for DNA purification to reduce the risk of DNA loss. Add ethanol to the wash buffer as indicated in the instruction manual of the supplier.

Some spectrophotometers, for example, Nanodrop ND1000, can determine the incorporation rate of labeled fluorescent dyes into DNA. The amount of dye incorporation should be larger than 0.8 pmol/ μ L.

Lower incorporation rates might occur if the amount of labeled dCTP used has been insufficient or the batch of labeled dCTP had been hydrolyzed, for example, by several freeze thaw cycles. Use 1 nmol labeled dCTP per reaction and split stocks to avoid repeated freeze thawing.

The incorporation rate can also appear to be low if fluorochromes had been bleached. Avoid exposure of the labeled dCTP as well as labeled DNA to direct light.

For labels other than Cy-dyes, the amount of labeled dCTP might have to be adjusted.

Number of detected genes on the microarray is low

The number of detected genes is usually comparable for SuperAmp amplified samples and samples without amplification using an appropriate number of cells. In case of very weak signals on the microarray or only a few detected genes, the lysis of the cells might have been insufficient. Lysis can be difficult if larger pieces of tissue have to be lysed or for some critical tissues like skin. It is recommended to visually control for complete lysis before applying μ MACS SuperAmp MicroBeads. In case of residual tissue pieces, vortex lysate thoroughly and repeat incubation for 10 minutes at 45 °C following 1 minute at 75 °C.

RNases can destroy RNA rapidly leading to loss of transcripts and less detected genes on the microarray. See chapter 3, Tips & hints for RNase-free work.

MACS MicroBeads are stable in solution but might decompose if frozen. mRNA isolation will not work if beads had been decomposed, leading to insufficient microarray signals. Do not freeze MACS MicroBeads.

Lyophilized mixes might have been dissolved incompletely impairing the amplification or labeling and giving weak signals on the microarray. Take care to gently pipette up and down resuspension buffers until lyophilized mixes are completely dissolved and clear solutions are obtained.

The reverse transcription might have been insufficient giving too short or too little cDNAs and therefore imperfect microarray results. It is important to keep the reverse transcription reaction at 42 °C for 45 minutes. Wait until thermoMACS Separator has reached the correct temperature before starting your timer.

The Terminal Deoxynucleotidyl Transferase enzyme might have been denatured. If the temperature of the tailing mix is too high when the

Terminal Deoxynucleotidyl Transferase is added, the high temperature can inactivate the enzyme.

cDNA without tails or with too short tails will be imperfectly amplified and can give raise to a small number of detected genes on the microarray.

Keep the tailing mix at least 1 minute on ice after denaturing the cDNA before adding the Terminal Deoxynucleotidyl Transferase.

The hybridization protocol for the microarray might have been suboptimal. Low numbers of detected genes might result from too stringent hybridization or washing conditions. Lowering the temperature during hybridization or washing as well as using appropriate buffers could improve the microarray performance. Usually, standard hybridization conditions used for labeled cDNA can also be used for SuperAmp samples.

Low correlation coefficients

The SuperAmp Amplification protocol has been developed to reproducibly amplify mRNA from tiny amounts of cells. For replicate experiments, correlation coefficients of above 0.8 are typically achieved. In case of varying replicate experiments, the number of cells used might have been too low. The fewer cells have been used, the more biological differences of individual cells, such as the cell cycle status or differentiation stage, will influence the expression profile. Avoid using less than 100 cells in order to level out effects of individual cells. Prevent losing cells due to, for example, insufficient centrifugation or repeated pipetting.

In addition, differences due to, for example, different patients, mouse strains, cancer types, inflammatory status, or impurities, like contaminating immune cells, will affect the expression profile of the

5. References

samples and can lead to low correlation coefficients. If possible, exclude most of the unwanted diversity to monitor gene expression differences of the distinctive cell types under examination. Another option is to increase the number of biological replicates to discriminate individual differences from characteristic gene expression changes of the cell types of interest.

DNA contamination from previous experiments might impair results by using the SuperAmp Kit. Especially amplicons achieved by using the SuperAmp Kit will be amplified very efficiently. So tiny amounts of carryover will bias the following expression profile. See chapter 3, Tips & hints to prevent PCR contamination.

Varying quality of the used microarrays as well as labile hybridization protocols might also decrease the reproducibility of the resulting expression profiles. To investigate the influence of the microarray platform, it is recommended to split labeled samples on at least two microarrays and comparing the results. The gained correlation coefficient will give an estimate of the maximum possible similarity of two identical samples.

5. References

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4. GeneChip® Whole Transcript (WT) Double-Stranded Target Assay Manual (Affymetrix, P/N 702179 Rev. 3)

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