



Increase sensitivity of Illumina® BeadArray™ with

μMACS™ SuperAmp™ Technology

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Introduction

When working with rare cells, researchers are faced with several challenges, most notably isolating substantial RNA quantities from small cell populations.

The Miltenyi Biotec μMACS™ SuperAmp™ Protocol is a well-established method for RNA amplification of rare cells. The amplified cDNA can be labeled and hybridized to microarrays for gene expression profiling. This application note describes the labeling and hybridization protocol of samples amplified by SuperAmp™ Technology for processing on the Genome-Wide Expression Bead Chips from Illumina®.

Based on the well-established MACS® Technology, the μMACS SuperAmp Kit allows highly sensitive mRNA isolation, cDNA synthesis, and a million-fold amplification of mRNA-derived cDNA^{1,2} by global polymerase chain reaction (PCR).

The unique μMACS SuperAmp Protocol combines several features:

- Extremely small (50 nm) superparamagnetic MACS MicroBeads that instantly bind and label mRNA molecules from small samples
- MACS 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 owing to consistent annealing conditions and no primer competition.

Materials and methods

Cell sorting

Thymic epithelial cells of three different individuals were flow-sorted according to expression of a surface marker on a BD™ FACS Aria™ Cell Sorter. Six thousand cells of the positive as well as the negative fraction were collected into ice-cold PBS. For a technical replicate, another 6000 cells of each fraction were sorted from one of the samples subsequent to the first sort. After the sorting, cells were centrifuged at 1400 rpm for 8 min. The supernatant was aspirated and 5.4 μL of lysis buffer containing 0.18 μg/μL Proteinase K was added directly to the cell pellet. Lysis was performed at 45 °C for 10 min. For transportation, the cell lysate was snap frozen in liquid nitrogen, shipped on dry ice, and stored at –80 °C.

Alternatively, the cell lysate can be used directly for RNA isolation and amplification in case no storage or transportation is required.

RNA isolation and amplification

mRNA isolation and amplification were performed with the μMACS SuperAmp Kit according to the user manual. Shortly, the cells were incubated in 5.4 μL of lysis buffer containing 0.18 μg/μL Proteinase K for 10 min at 45 °C. The Proteinase K was inactivated for 1 min at 75 °C. Then, the lysate was incubated with magnetic μMACS SuperAmp MicroBeads and applied to a μ Column, placed in the magnetic field of a temperature-controlled permanent magnet, the thermoMACS™ Separator. The mRNA is retained in the column while effective washing steps remove all other cell components.

cDNA synthesis is performed in the same column for 45 min at 42 °C. The cDNA is washed and eluted for

subsequent tailing reactions. A tag is added by using a Terminal Deoxynucleotidyl Transferase (TdT) (MBI Fermentas).

For global PCR reaction (40 cycles), the Expand Long Template PCR System (Roche) was used. The amplification protocol utilizes only one primer which allows uniform annealing conditions for all transcripts. In addition, the cDNA is primed at multiple sites of comparable length both avoiding PCR bias due to different transcript length or unequal annealing conditions. PCR products were purified with High Pure PCR Product Purification Kit (Roche) and the purified DNA was quantified by photometrical measurement (NanoDrop™ ND1000).

The Klenow labeling protocol has to be adapted for the Illumina BeadArray Protocol. Kindly perform the Klenow labeling according to the following protocol instead of using the original user manual (differences are marked in bold):

Klenow labeling of amplified cDNA

Before starting

Heat two heating blocks to 99 °C and 37 °C, respectively.

1. Transfer 200 ng of the purified PCR product into a fresh 1.5 mL tube. Add Double Distilled Water (1.5-mL tube, blue cap) to adjust volume to **20 µL**.
2. Dissolve the Lyophilized Klenow Mix I of one well (microtiter plate labeled with one green line) with 20 µL Resuspension Buffer K (1.5-mL tube, green cap).
3. Add the 20 µL resuspended Klenow Mix I to the PCR product (total volume 40 µL). Vortex the tube and spin down.
4. Incubate reaction tube for 5 min 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 µL Resuspension Buffer K (1.5-mL tube, green cap).
6. Add 5 µL 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 **3 µL Biotin-11-dCTP** (3 nmol; e.g. from Perkin Elmer; product number: NEL538001EA).
8. Add 2 µL Klenow Fragment (10 units/µL, MBI Fermentas). Vortex and spin down.
9. Incubate tube for 2 hours at 37 °C.
10. Incubate for 5 min 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"). An 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 min 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

For Illumina BeadArray hybridization 150 ng Biotin-labeled DNA per microliter hybridization solution is used. The hybridization cocktail is prepared according to the BeadChip instructions. The arrays are hybridized, washed, and scanned according to the instructions with the exception of the hybridization temperature **reduced to 53 °C instead of 58 °C. In addition, the labeled DNA is boiled to 95 °C for 5 min prior to hybridization.** The hybridization temperature is reduced to adapt the hybridization conditions to the lower stability of the DNA-DNA pairs as compared to the cRNA-DNA hybrids for the standard Illumina protocol. For detailed instructions read the original BeadChip instructions. A short instruction is also given below:

Hybridization of Illumina BeadArray

Preheat the oven to **53 °C**.

Mix with HYB Reagents

Add RNase-free water to the Biotin-labeled DNA to a final volume as given in table 1, depending on the BeadChip type. Mix and leave at room temperature for 10 min to resuspend Biotin-labeled DNA.

Note: Place the HYB E1 buffer bottle at 55 °C for 10 min to dissolve any salts that may have precipitated in storage. Inspect the solution; if any salts remain undissolved, incubate at 55 °C for another 10 min. After cooling to room temperature, mix thoroughly before using.

Depending on the BeadChip Type, add the appropriate amount of HybE1 buffer to each Biotin-labeled DNA sample according to table 1.

BeadChip	Hyb E1 buffer	DNA amount	final volume
6-Sample BeadChip	20 µL	1.50 µg in 10 µL water	30 µL
8-Sample BeadChip	10 µL	0.75 µg in 5 µL water	15 µL

Table 1. Sample preparation overview for the different BeadChip formats. Hyb E1 contains all control oligo spikes needed.

Set up hybridization

Prepare Hyb Cartridge(s) according to the manufacturer's instructions. **Preheat the assay sample for 5 min at 95 °C to ensure complete denaturation of the samples.** Briefly, vortex and centrifuge to collect the liquid at the bottom of the tube. Allow sample to cool to room temperature. Pipet sample immediately after cooling to room temperature. Dispense final volume of your sample (table 1) onto the center of each array. Shake assembled Hyb Cartridge and check that bubbles move freely. Place Hyb Cartridges on the BeadChip Hyb shaker in pre-heated oven. Incubate for 16–20 hours at **53 °C**.

Prepare for high-temperature wash and overnight incubation

Pre-warm High-Temp Wash buffer at 55 °C overnight.

Prepare reagents

The next day, prepare Wash E1BC solution by adding 3 mL E1BC buffer to 1 L RNase-free water.

Pre-warm Block E1 buffer (4 mL/chip) to room temperature. Prepare Block E1 buffer (2 mL/chip) with streptavidin-Cy³ (2 µL of 1 mg/mL stock per chip). Use a single conical tube for all BeadChips. Store in dark until detection step.

Room-temperature Incubation

Using tweezers or powder-free gloved hands, place the BeadChip into the slide rack submerged in the staining dish containing 250 mL Wash E1BC solution. Using the slide rack handle, transfer the rack into the Hybex™ waterbath insert containing High-Temp Wash buffer.

High-temperature wash

Incubate statically for 10 min with the Hybex lid closed.

1st room-temperature wash

During the 10-min High-Temp Wash buffer incubation, add fresh 250 mL Wash E1BC solution to a clean staining dish. After the 10-min High-Temp Wash buffer incubation is complete, immediately transfer the slide rack into the above prepared staining dish. Briefly agitate using rack, then shake on orbital shaker for 5 min at the highest speed possible without allowing solution to splash out of dish.

Ethanol wash

Transfer rack to a clean staining dish containing 250 mL 100% ethanol. Briefly agitate using rack handle, then shake on orbital shaker for 10 min.

2nd room-temperature wash

Transfer rack to a clean staining dish containing fresh 250 mL Wash E1BC solution. Briefly agitate using rack handle, then shake on orbital shaker for 2 min.

Block

Pipette 4 mL Block E1 buffer into the Wash Tray(s). Transfer the BeadChip, face up into BeadChip Wash Tray(s) on rocker. Rock at medium speed for 10 min.

Detect

Pipette 2 mL Block E1 buffer + streptavidin-Cy3 into fresh Wash Tray(s). Transfer the BeadChip, face up into Wash Tray(s) on rocker. Place cover on tray and rock at medium speed for 10 min.

3rd room-temperature wash

Add 250 mL of Wash E1BC solution to a clean staining dish. Transfer the BeadChip to the slide rack submerged in the staining dish. Briefly agitate using rack, and then shake at room temperature on orbital shaker for 5 min.

Dry

Prepare centrifuge with plate holders, paper towels, and balance rack. Set speed to 275 rcf. Transfer rack of Bead-Chips from staining dish to centrifuge and spin at RT for 4 min. Store dry chips in slide box until scanned.

Results and discussion

Gene expression profiles were determined for two different cell fractions of thymic epithelial cells. For each fraction, 6000 flow-sorted cells were used for the SuperAmp amplification protocol. The cDNA yields at the end of the PCR reaction were typically in a range of 3 to 6 µg. Only 200 ng of the amplified cDNA is needed for the Klenow reaction, so there was sufficient material for further experiments from the same PCR source. At the end, the Klenow biotin labeling reaction yielded up to 6 µg labeled cDNA.

To assess the validity of the system, pairwise scatter plots (figure 1) and the corresponding Pearson correlation coefficient (R) (table 2) of gene expression profiles were calculated in pairs for the three samples and one technical replicate of one sample. The technical replicate started from the same cell population, but the cells were independently sorted, amplified, and hybridized.

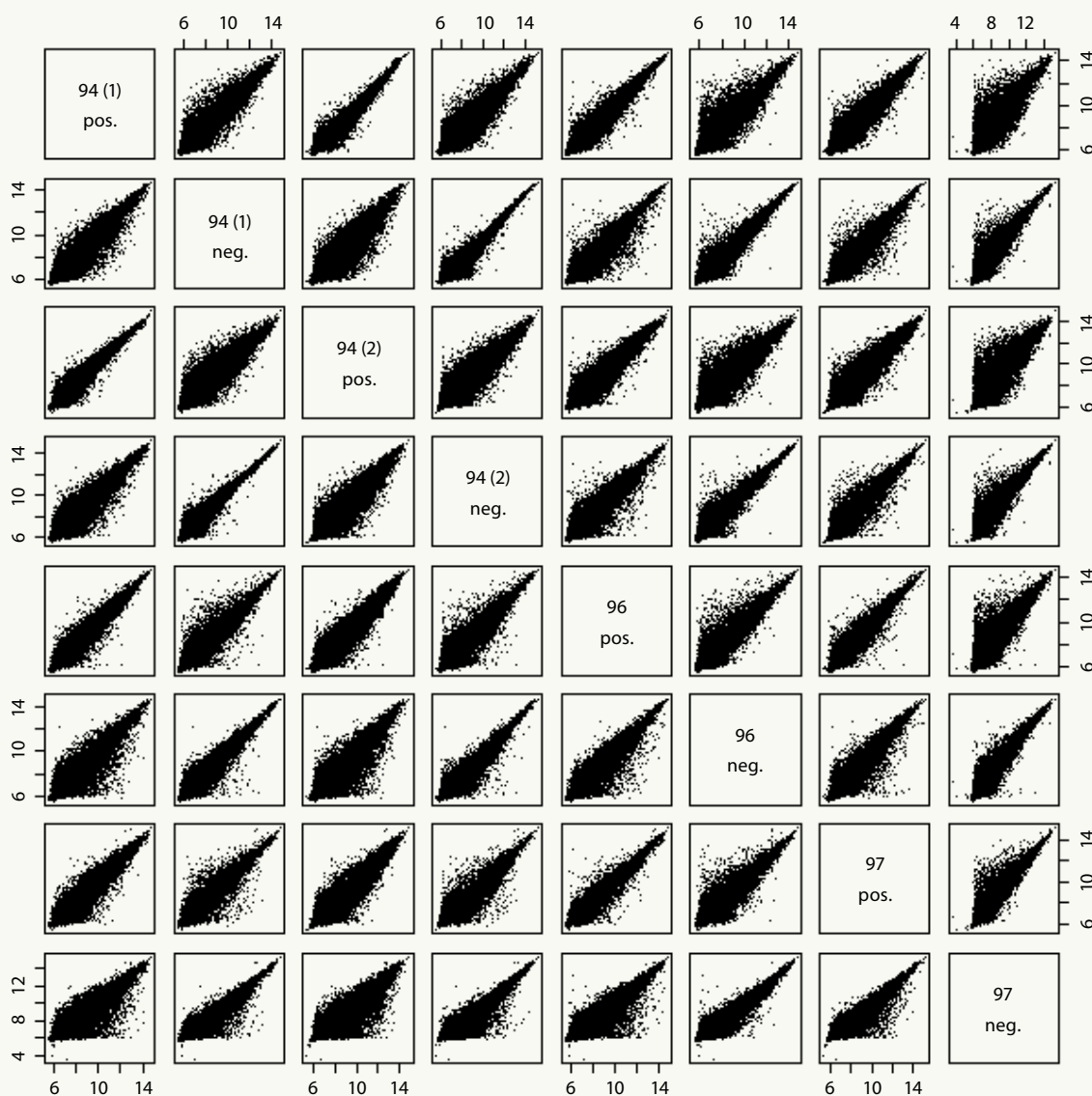


Figure 1. Pairwise scatter plots of the signal intensities of the positive (pos.) and negative (neg.) fractions of three samples (94, 96, 97) and one technical replicate of sample 94.

	94 pos.	94 neg.	94 (2) pos.	94 (2) neg.	96 pos.	96 neg.	97 pos.	97 neg.
94 pos.		0.96	0.98	0.96	0.98	0.95	0.97	0.93
94 neg.	0.96		0.95	0.98	0.97	0.98	0.97	0.97
94 (2) pos.	0.98	0.95		0.95	0.97	0.94	0.96	0.92
94 (2) neg.	0.96	0.98	0.95		0.96	0.97	0.97	0.96
96 pos.	0.98	0.97	0.97	0.96		0.96	0.98	0.94
96 neg.	0.95	0.98	0.94	0.97	0.96		0.96	0.97
97 pos.	0.97	0.97	0.96	0.97	0.98	0.96		0.96
97 neg.	0.93	0.97	0.92	0.96	0.94	0.97	0.96	

Table 2. Correlation coefficients of pairwise scatter plots of the signal intensities of the positive (pos.) and negative (neg.) fractions of three samples and one technical replicate of sample 94.

Gene	Sample 94 (-fold change with qPCR)	Sample 94 (-fold change with microarrays)	Samples 94 (2), 96, and 97 (-fold change with microarrays)	Adjusted p value
A	6.84	2.59	2.17	0.0054
B	14.17	3.78	4.02	0.0188
C	0.08	0.32	0.16	0.5000
D	66.72	2.41	3.00	0.0263
E	11.39	3.20	3.45	0.0041

Table 3. Comparison of n-fold changes between the two sorted subsets of five genes as tested by qPCR with the microarray results.

The correlations for the technical replicate (mean 0.98) as well as for the positive or negative fractions of the three samples (mean 0.97) indicate good reproducibility between replicate amplifications. The lower correlation of the positive fractions vs. the negative fractions (mean 0.95) indicates a higher number of differentially expressed genes between positive and negative fractions. This finding is supported by the fact that about 5.7% (mean) of the genes are detected as differentially expressed between positive and negative fraction (~ 2500 genes), whereas only 3.4% (mean) of the genes are detected as differentially expressed for the same fraction (positive or negative) of the biological replicates. The false positive rate can be estimated according to the differentially detected genes for the same fraction (positive or negative) of the technical replicates to be around 1.9% (mean).

Another indication for the reproducibility of the amplification are the set of housekeeping genes assigned according to the Illumina description. These genes have been found to be consistently amplified in all samples.

As shown in the Table 3, a set of 5 genes was tested using qPCR comparing the n-fold change between the positive and negative cell fractions of the sample 94. Gene A is encoding the epitope used for the cell sorting conforming with the found enrichment in the positive fraction.

Overall there is good correlation between both methods. The results indicate a general phenomenon when comparing expression changes of microarray experiments and qPCR experiments. Due to much higher dynamic range of the PCR method, the n-fold differences detected on the arrays are usually less pronounced compared to qPCR. However, significance and direction of n-fold change is comparable.

The results reported above highlight a single specific example of how the SuperAmp protocol can be used in conjunction with Illumina Bead Arrays for accurate gene expression analysis. We were able to amplify substantial amounts of cDNA from a sample of only 6000 flow-sorted cells.

This technique may prove especially useful for gene expression profiling of rare cell populations.

References

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