PALM® Laser Microdissection and Pressure Catapulting (LMPC) Technology at the Department of Pathology at PMRL-G
An Introduction to the Technology and its Application to Tissue Collection for Gene Expression Analyses of Different Mouse Tissues Using SuperAmp Amplification

H. Weiler[1], S. Gebel[1], K. Meurrens[2], B. Gerstmayer[3], A. Bosio[3]; [1]PHILIP MORRIS Research Laboratories GmbH, 51149 Cologne, Germany;
[2]PHILIP MORRIS Research Laboratories bvba, Leuven, Belgium; [3]Miltenyi Biotec GmbH, MACSmolecular Business Unit, 50829 Cologne, Germany
Horst.Weiler@pmintl.com

Introduction

Laser-based microdissection techniques have provided a major impetus to the sensitive and specific molecular analyses of diseased tissues. After being optimized over the last 10 years, they have now become a state-of-the-art tool for the collection of morphologically defined cell populations from a tissue section for downstream tissue analyses regarding genomics and proteomics.
One of the major advantages of using laser microdissection techniques to obtain specific cells for molecular analyses, especially from the viewpoint of the pathologist, is that the procedure is carried out under direct-light microscopic visualization of the cells.
The principle of the PALM® system is based on a pulsed ultra-violet (UV-A) laser of high-beam quality that is interfaced into the microscope and focused through an objective to a beam spot size of less than 1 µm in diameter for the cutting action. The principle of laser cutting is a locally restricted ablative process without heating of the adjacent material and results in a clear cut gap between the desired sample area and the surrounding tissue (**Figure 1**). The effective laser energy is concentrated on the minute focal spot only.
After microdissection, the isolated specimens are ejected out of the object plane (**Figure 2**) and catapulted directly into the cap of a common microfuge tube (**Figure 3**).
This is performed in an entirely non-contact manner with the help of a single defocused laser pulse.
Like a ball that is kicked into the goal, the sample is driven with high speed and can be "beamed" several millimeters away, even against gravity. Successful catapulting can be visualized within seconds by light microscopy.
In order to establish the above-mentioned technology at PMRL and to finally allow its application for downstream analyses of cigarette-smoke-induced tissue alterations, we are currently performing pilot studies of which preliminary results are presented.

Project Title: Gene expression analysis of different mouse tissues using SuperAmp amplification

Figure 1

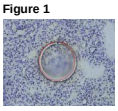


Figure 2

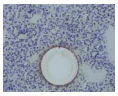
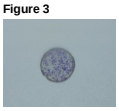


Figure 3



Materials, Methods, and Results

Animals and Treatment

A/J mice (Jackson Labs, Harbor, ME, USA) treated with urethane (1000 mg/kg body weight i.p.*) to induce lung tumors were killed 6 months after treatment. Lungs were removed and fixed in 4% buffered formaldehyde solution or snap frozen.

* treatment according to Stoner, G.:
Lung tumors in strain A mice as a bioassay for carcinogenicity of environmental chemicals. Exp. Lung Res. 17, 405-423 (1991)

1. SuperAmp Amplification of cDNA

Five different LMPC samples were directly collected in 0.2-ml polymerase chain reaction (PCR) tubes at PMRL, stored on dry ice and immediately transported to **Miltenyi Biotech**. The following samples were used for SuperAmp amplification.

Table 1 Summary of Samples

Sample ID	Type of Probe
1	left lung, mouse, tumor, 100000 µm²
2	left lung, mouse, tumor, 50000 µm²
3	left lung, mouse, normal tissue, 100000 µm²
4	right lung, mouse, tumor, 200000 µm²
5	right lung, mouse, normal tissue, 200000 µm²

2. Quality Control of SuperAmp-Amplified cDNA

SuperAmp amplification was performed according to **Miltenyi Biotech's** undisclosed protocol. Amplified cDNAs were quantified using ND-1000 spectrophotometer (NanoDrop Technologies, Inc).

Table 2 Summary of Obtained Amounts of cDNA

Sample ID	Concentration (ng/µl)	Ratio (260/280)	Amount cDNA (µg)
1	60.89	1.85	1.64
2	55.28	1.77	1.49
3	68.07	1.77	1.84
4	70.84	1.82	1.91
5	50.72	1.94	1.37

Integrity of all five library PCRs was checked via the *Bioanalyzer 2100* system (Agilent Technologies).

In **Figure 4**, the electropherogram and elution diagrams are shown.

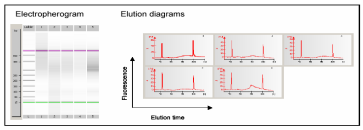


Figure 4 Electropherogram and elution diagrams of cDNAs. As a reference, the DNA ladder (in bp or nt) is shown on lane L (ladder). The lowest migrating band (green-colored) as well as the highest migrating band (purple-colored) represent internal standards (15 and 600 bp). Scaling of the y-axis is done automatically, relative to the strongest signal within a single run.

All library PCRs revealed Gaussian-type peaks. These peaks comprised DNA fragments with 200 to 500 bp in length as expected. All samples appeared to be successfully amplified. In the next step, quantitative PCR for two selected house-keeping genes was performed. This additional quality control step was done to ensure that library PCRs can be used as templates for real-time PCR experiments.

3. Validation of SuperAmp-Amplified Total RNA via Real-Time PCR

Five different library PCRs (**Table 1**) were used as templates for real-time PCR experiments.
Already established primer for the detection of two different house-keeping genes - GAPDH and beta microglobulin - were used. For each real-time PCR reaction, 10 ng cDNA template (library PCR) was used.

Table 3 Primer Sequences and Names Used for Real-Time PCR

Primer	Sequence
GAPDH forward	ACCTGCCAAGTATGATGACATCA
GAPDH reverse	TGCTGTTGAAGTCGCAGGAGACAA
beta microglobulin forward	GCCGAACATACTGAACTGCTACG
beta microglobulin reverse	GGATATAGAAAGACCAGTCCTTGCTG

3.1 Real-Time PCR

Real-time PCR is a PCR-based method which allows the relative and absolute quantification of gene expression in different samples. For this purpose, a fluorescent dye (SybrGreen) is added to the reaction. During the course of a PCR, the amount of amplified, double-stranded product increases exponentially (however, only if the gene was present in at least one copy per template) and finally reaches a plateau phase. SybrGreen intercalates into double-stranded PCR products. The increase in fluorescence that is being measured is directly proportional to the increase of the PCR product.

3.2 Determination of ct-Values

Expression levels of GAPDH and beta microglobulin were determined using the *SDS7000 Systems* (ABI, Applied Biosystems). To reach higher accuracy and to avoid fluctuations, all experiments were done in triplicates and repeated in an independent experiment once more (in triplicates).
A crucial point if performing real-time PCR experiments is the determination of the ct-value (cycle over threshold, see **Table 4**). ct-Values describe the PCR cycle in which the fluorescence intensity rises measurably above background for the first time. Based on these values, relative and absolute gene expression levels can be estimated. In the following table, the mean ct of triplicates from the house-keeping genes GAPDH and beta microglobulin are displayed. For each gene, melting curve analysis of amplified PCR products was performed to verify accuracy of the amplicon.

Table 4 ct-Values for GAPDH and Beta Microglobulin

Sample ID	GAPDH	Beta Microglobulin
1	15.02	10.75
1 (replicate)	15.63	10.28
2	16.37	33.64
2 (replicate)	17.37	37.07
3	16.93	10.51
3 (replicate)	16.97	10.93
4	13.13	11.44
4 (replicate)	11.01	10.42
5	27.68	9.61
5 (replicate)	25.56	9.86

Discussion

Tissues, especially diseased tissues, are complex 3-dimensional structures composed of heterogeneous mixtures of morphologically and phentotypically distinct cell types. The meaningful molecular analysis of morphologically and/or phenotypically distinct cell types from such tissues requires rapid, efficient, and accurate methods for obtaining specific populations of cells. LMPC provides an extremely valuable and sophisticated tool to fully utilize the power and sensitivity of modern molecular analytical technologies.

The data indicate that real-time PCR gene expression analysis can be performed if SuperAmp-amplified cDNA samples were used as template. Accuracy and reproducibility of corresponding replicate experiments are good. Interestingly, sample 2 appears to have almost "normal" GAPDH transcript levels (ct ~17) if compared to the other samples but hardly any expression of beta microglobulin (ct >33). In contrast, sample 5 shows only very low expression of GAPDH (ct ~26) but rather high expression of beta microglobulin (ct ~9.7). Whether the differential expression of these two genes reflects their different origins (sample 2: putative tumor sample; sample 5: putative normal sample) is currently speculative. Additional real-time PCR experiments analyzing the expression of other tumor-specific genes would be required in order to address this question.

It is noteworthy that for real-time PCR experiments - using sample 5 as template and analyzing beta microglobulin transcript levels - the reaction failed twice (in total, 4 experiments each as triplicates were performed). In **Table 4**, only the successfully applied PCRs and the corresponding ct-values for beta microglobulin are shown. We performed additional experiments using lower amounts of template (down to 1 or 0.1 ng). There, we have never encountered PCR-related problems indicating some kind of template-mediated inhibition of the reaction (data not shown).

It should also be mentioned that in the course of a small percentage of other "SuperAmp amplification projects" - semi-quantitative PCRs using other house-keeping genes like, e.g., Actin as a marker gene - failed from time to time. However, fluorescent labeling (using the same library PCR as template!) and subsequent hybridization on PIQOR arrays worked well and revealed good and concordant results to unamplified samples. This may indicate that the PCR reaction seems to be more error-prone in comparison to the fluorescent labeling reaction if library PCRs are used as template.

In summary, SuperAmp-amplified cDNAs (library PCRs) represent suitable templates for determining transcript expression levels of different genes via the real-time PCR platform. Ten ng template could be shown to work well in most of the analyzed cases. However, for some library PCRs, it seems to be advisable to reduce/titrate the amount of template to get optimal results.

Acknowledgement

Silke Töller and Thomas Grün for excellent technical assistance regarding LMPC.

presented at the Health Sciences Research Information Sharing Meeting, 2-4 November 2005, Lansdowne Resort, Lansdowne, VA, U.S.A.