Gene expression profiling of a single cell using laser capture microdissection and OpenArray technology

- A workflow is presented here for gene expression profiling of a single cell or low number of cells using real-time PCR
- No RNA isolation is required during the process
- Simultaneous analysis of 648 genes is performed using the Applied Biosystems™ TaqMan™ OpenArray™ Human Cancer Panel, making it possible to extract a large amount of gene expression data quickly and efficiently

Figure 1. Workflow for LCM using a single cell or low number of cells on the QuantStudio™ 12K Flex system with Applied Biosystems™ TaqMan™ OpenArray™ plates.

Introduction

Current gene expression profiling studies use groups of purified cells collected from whole tissues, flow cytometry, micromanipulation, or laser capture microdissection (LCM) methods. When analyzing gene expression profiles of a group of cells, the average profile may not be a true representation of the many different profiles that could exist in a pure cell population (e.g., in different states of growth, differentiation, or activation). As a result, the transcriptional variability of individual cells and any insight into the relationship between specific genes in single cells or between individual cells may be lost. To fully understand tissue and cellular heterogeneity, it is necessary to conduct measurements at single-cell resolution.

Techniques for single-cell collection include flow cytometry and micromanipulation. With these harvesting techniques, *in situ* cell location information is lost because these methods involve disruption and dissociation of the cells from the tissue. However, this is not the case with samples collected by LCM. With LCM, the tissue section is visualized by staining, and the cell or cells of interest are removed from the surrounding tissue. The *in situ* location of the cell or cells can provide valuable context and insight that can facilitate the interpretation of gene expression results.

In this application note, we describe a simple and rapid end-to-end workflow for quantitative gene expression analysis from a single cell or small number of cells (Figure 1). LCM provides a rapid and reliable method to procure purified cell populations from a heterogeneous tissue sample, allowing a targeted approach to genomic and proteomic profiling. The Applied Biosystems™ ArcturusXT™ LCM System provides the power of two microdissection lasers in a single, microscope-based platform. The exclusive infrared (IR) capture laser enables a gentle capture technique that preserves biomolecule integrity and is ideal for single cells or a small number of cells. The optional ultraviolet (UV) laser permits unprecedented speed and precision, and is well suited for microdissection of larger numbers of cells and dense tissue structures.

When coupled with a high-throughput gene expression profiling platform such as the Applied Biosystems™ OpenArray™ system, the ArcturusXT LCM System facilitates confirmation and screening of a large number of genes from a single LCM sample. Applied Biosystems™ OpenArray™ technology provides an effective solution for gene expression analysis with a small reaction volume for each assay (33 nL) and allows either a large sample or assay set to be analyzed in a single qRT-PCR run.

Methods

Tissue preparation and LCM

The ArcturusXT LCM System workflow is simple and intuitive, and allows sample custody throughout the LCM process. After loading the slides onto the microscope stage, cells or areas of interest are located, marked, and then microdissected onto an Applied Biosystems™ CapSure™ HS LCM Cap using the tools in the software user interface (Figure 2). The microdissected areas can be confirmed by inspecting the LCM cap prior to downstream processing for genomic or proteomic analysis.

Frozen sections, 8–10 µm in thickness, were prepared from a human breast carcinoma tissue sample embedded in Tissue-Tek™ O.C.T.™ Compound (Sakura Finetek). The sections were mounted onto Applied Biosystems™ PEN (polyethylene naphthalate) Membrane Glass Slides and kept cold on dry ice or stored at –70°C. For microdissection, the slides were removed from –70°C and stained with the Applied Biosystems™ HistoGene™ LCM Frozen Section Staining Kit following the protocol indicated in the user manual. The stained slides and CapSure HS LCM Caps were then loaded onto the ArcturusXT LCM System. Using the features in the Applied Biosystems™ ArcturusXT™ Software, the tumor areas were located, and 1, 10, or 100 individual tumor cells were marked for IR laser capture microdissection. For comparison, whole tissue sections representing a mixed cell population sample were processed.

Cell lysis and RNA extraction

The Invitrogen™ Ambion™ Single Cell-to-C_T™ qRT-PCR Kit, optimized for use with small quantities of total RNA, was used to process the LCM samples for qRT-PCR analysis with some minor modifications (Figure 3). The LCM samples were lysed according to the protocol provided in the user manual. To accommodate the 10 µL volume of lysis solution, the Applied Biosystems™ ExtracSure™ Sample Extraction Device was coupled with the CapSure HS cap and then the lysis solution was added to the buffer well. To collect the lysate upon the completion of the incubation steps, a microcentrifuge tube was attached to the ExtracSure device, and then the assembly was centrifuged at 800 x *g* for 2 minutes. The ExtracSure device and LCM cap was detached from the microcentrifuge tube, and the lysate was either subjected to reverse transcription or stored at –20°C.

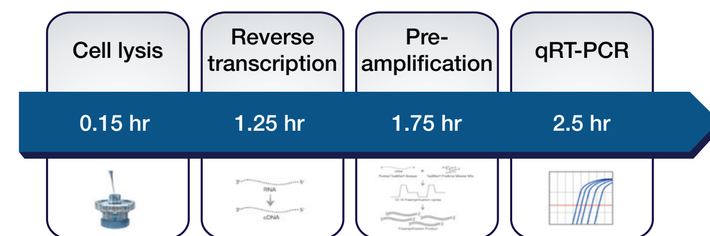

Figure 3. The Invitrogen™ Ambion™ Single Cell-to-C_T™ kit enables sample preparation to qRT-PCR results in about 6 hours.

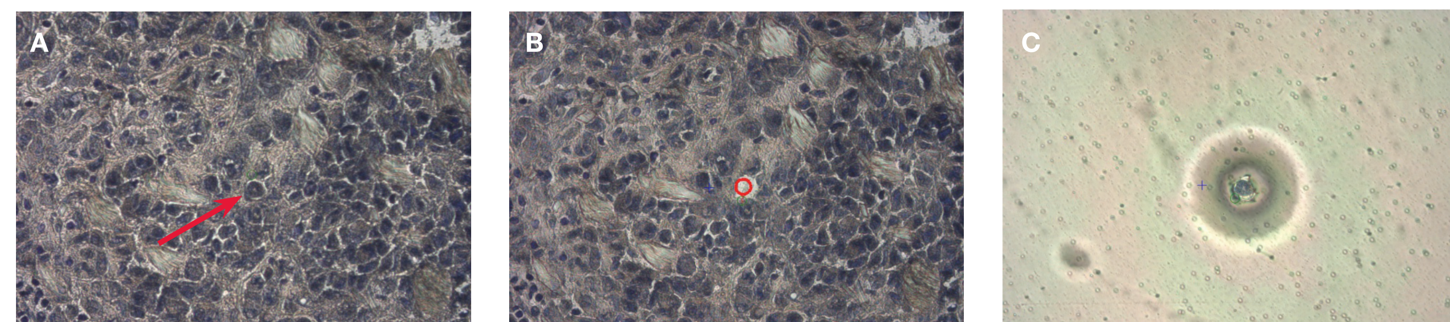

Figure 2. Laser capture microdissection of a single cell. (A) Frozen tissue section stained with Applied Biosystems™ Histogene™ Staining Solution before microdissection; red arrow indicates cell of interest. **(B)** The same tissue section is shown with the missing single cell after microdissection. **(C)** The single LCM cell on the CapSure HS LCM Cap.

For the tissue section samples, one section was processed using 100 µL of the Single Cell-to-C_T kit lysis buffer. RNA was isolated from a second section using the Applied Biosystems™ PicoPure™ RNA Isolation Kit.

Reverse transcription and preamplification

All LCM samples as well as the tissue section samples were reversed transcribed using the reagents and protocol provided with the Single Cell-to-C_T kit. For the tissue section sample processed with the Single Cell-to-C_T kit, an 11 µL aliquot was taken from the lysate for the reverse transcription. For the RNA isolated from the tissue sections, the lysates were diluted so as to provide 100 pg and 10 pg of RNA per reverse transcription reaction. The cDNA was then preamplified using the Single Cell-to-C_T kit PreAmp Mix and the TaqMan OpenArray Human Cancer Assay Pools (Pool A and B) in the volumes stated in the manual. Standard preamplification thermal cycling conditions were followed as specified in the Single Cell-to-C_T kit user manual, with the exception of the number of cycles, which was increased from 14 to 16 cycles.

qRT-PCR analysis

The preamplification product was diluted 10-fold with nuclease-free water and then mixed with an equal volume of the Applied Biosystems™ TaqMan™ OpenArray™ Real-Time PCR Master Mix. The samples were then loaded into the TaqMan OpenArray Human Cancer Panel using the Applied Biosystems™ OpenArray™ AccuFill™ System. Real-time PCR was performed using the OpenArray™ gene expression program on the Applied Biosystems™ QuantStudio™ 12K Flex Real-Time PCR System. Data analysis was completed using Applied Biosystems™ QuantStudio™ 12K Flex software.

Results

To determine the expression differences in a small number of cells, we analyzed the results obtained with 1, 10, and 100 LCM-captured cells with the 648-assay OpenArray Human Cancer Panel. Representative amplification curves for one replicate and eight genes are shown in Figure 4. For each of these genes, we were able to obtain good amplification of reference genes (18S rRNA, *B2M*, *GAPDH*, and *UBC*) as well as genes implicated in various types of breast cancer (*ERBB2*, *AR*, *RHOA*, and *TP53*). This indicates that the Single Cell-to-C_T kit is able to extract useful RNA from LCM-collected cells.

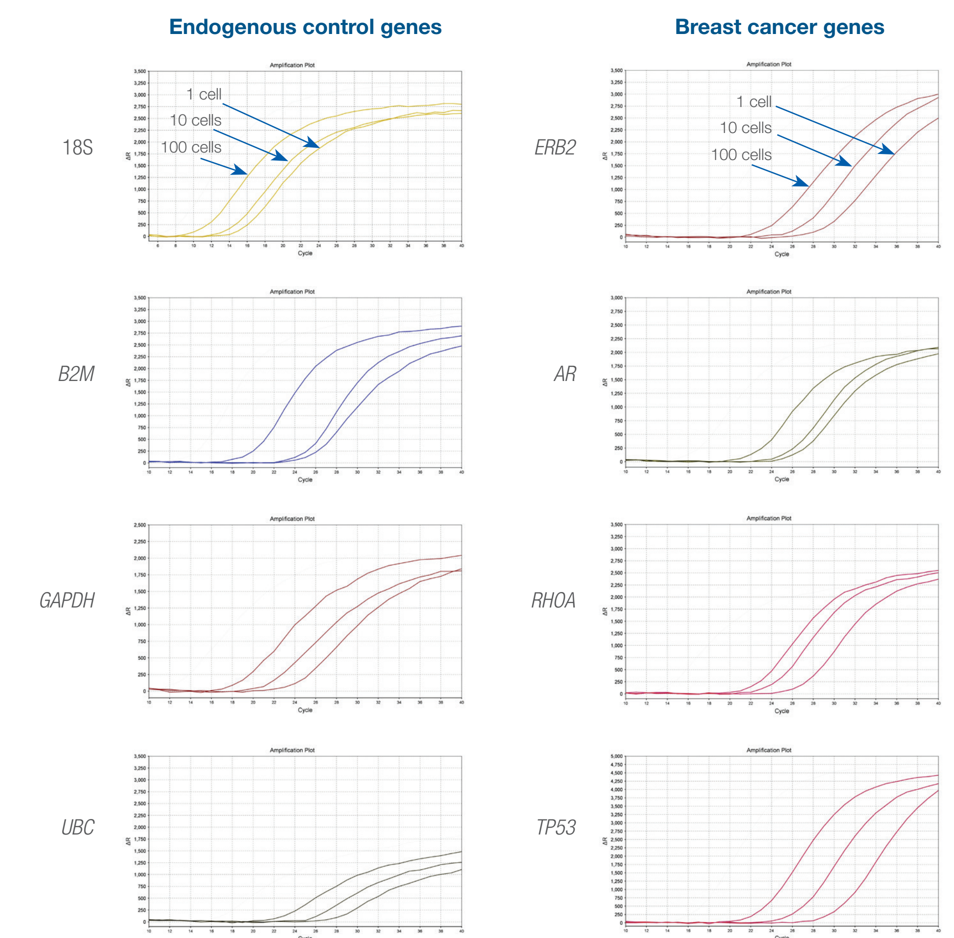

Figure 4. Gene expression profiles for a selected set of genes from the TaqMan OpenArray Human Cancer Panel. One sample each of 1, 10, and 100 LCM cells were run together on a single OpenArray plate. For each gene, the C_t value increases with decreasing cell numbers. In each profile, the left curve represents 100 LCM cells, the middle curve represents 10 LCM cells, and the right curve represents 1 LCM cell, as indicated in the graphs for the 18S rRNA and *ERB2* genes.

	Unfiltered	Filtered ($C_t \leq 30$)
1 LCM cell	144–208	123–173
10 LCM cells	246–288	222–246
100 LCM cells	449–566	420–548

Table 1. The number of genes detected (out of 648 total) with the TaqMan OpenArray Human Cancer Panel.

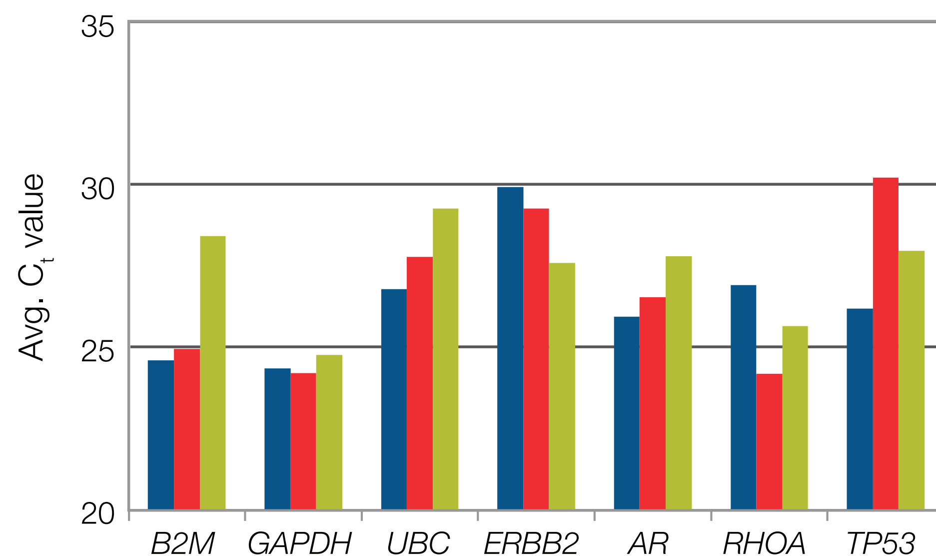

Figure 5. Comparison of gene expression from 3 single-cell samples. Cell-to-cell gene expression variation is observed in endogenous control genes (*B2M, GAPDH, UBC*) as well as breast cancer–related genes (*AR, RHOA, TP53*).

As the number of cells input into the reaction decreases, we expect to see two things—that the C_t value for detectable transcripts should increase, and that the total number of transcripts detectable should decrease. For each of the amplification curves there was a shift to the right when fewer cells were used. Furthermore, the number of detectable transcripts decreased, from an average of 507 transcripts detected in two replicates of 100 cells to an average of 176 transcripts detected in four replicates of single cells (Table 1).

The expression levels of seven genes in three single-cell collections were analyzed (Figure 5). Although differences in the pattern of expression of the tumor-associated genes might be expected, there are also significant differences in the expression pattern of the three housekeeping genes. This reflects the heterogeneity of expression in individual cells, and suggests that using individual housekeeping genes for normalization could produce aberrant results.

To illustrate the heterogeneity of expression in single cells, we performed a clustering and heat map analysis of the TaqMan OpenArray panel results. In addition to the LCM samples, we included clustering data from the average C_t value of (1) the LCM-collected samples, (2) small amounts of purified bulk RNA from the same sample, and (3) two whole-tissue scrapes from adjacent sections of the sample. A portion of the heat map is shown in Figure 6. In the single-cell profiles, there are large differences between cells, both in the presence and in the expression levels of genes. This confirms that individual cells can vary tremendously in their behaviors. As more cells are analyzed, the expression profile moves closer to the average of 100 cells and the whole tissue scrapes. Note that the bulk RNA and whole tissue scrapes were not obtained from purified tumor cells. These samples probably have a significant contribution from unrelated nontumor cells that were in the specimen. RNA from these unrelated cells may then have contributed to the differences in clustering of these samples.

Conclusions

We describe a workflow that can analyze gene expression patterns from a single cell isolated by LCM. The IR laser and the CapSure LCM caps that are unique to the ArcturusXT LCM System allow confirmation of the presence of the single microdissected cell prior to proceeding with downstream analysis. Unlike other platforms, this gentle microdissection method maintains the integrity of biological molecules. This facilitates analysis of truly single cells and obviates the need to pool several microdissected single cells to ensure enough intact material is collected for gene expression analysis.

The ability to analyze gene expression levels in single cells in preserved tissue offers tremendous potential for understanding the finer details of biology. Unlike flow cytometry or other disruptive cell sorting techniques, LCM is the only mechanism that can isolate single cells while preserving surrounding cells and tissue. This provides a mechanism to isolate both tumor cells and normal cells from the same tissue sample.

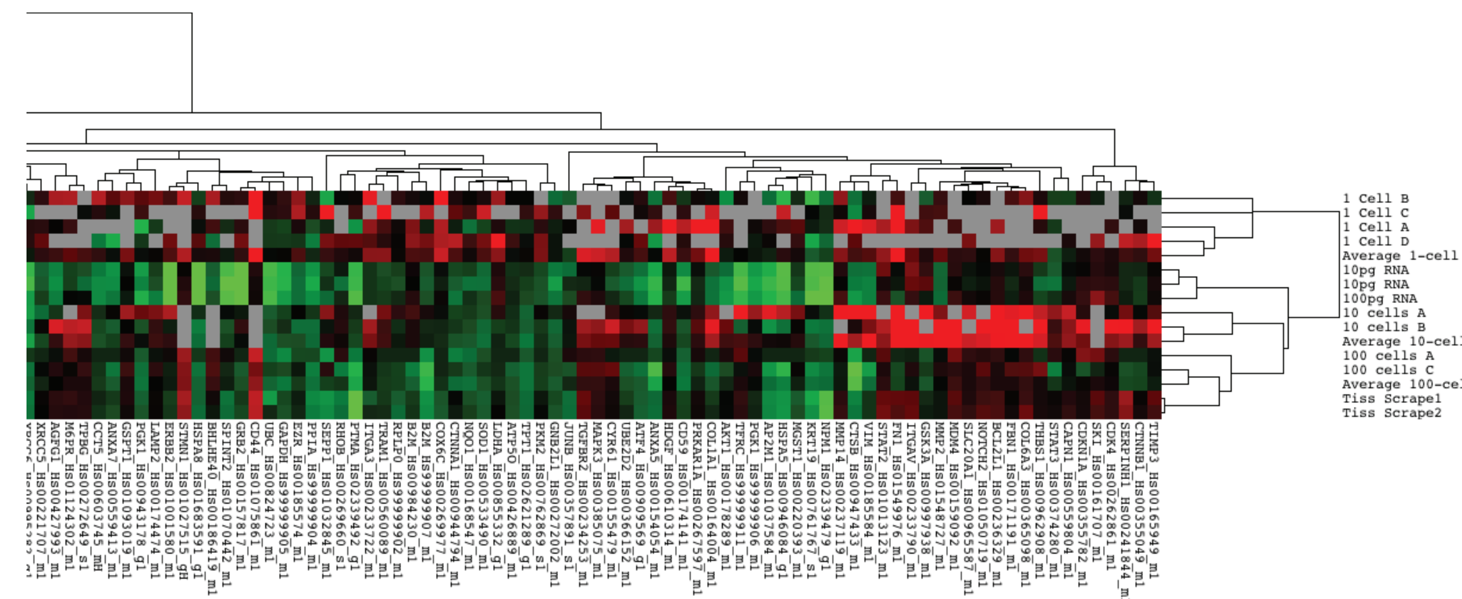

Figure 6. Gene expression clustering. Partial heat map of genes expressed from small numbers of LCM cells, tissue scrapes, and bulk purified RNA from the same tissue. Red indicates greater expression than median, green indicates less expression than median, and grey is not detectable. Note that there is a large amount of hetereogeneity among individual cells, but as more cells are analyzed, the expression pattern of both the level and number of genes expressed approaches the pattern seen in 100-cell samples.

A tissue's expression profile is ultimately the sum of the cells in that tissue, but there can be significant variation among individual cells in the abundance of transcripts. Although we show that individual cells do indeed have very different expression profiles, combining the profiles of many cells approaches the average profile.

In most cases, analysis of gene expression experiments requires a reference transcript that is used to normalize for RNA input amount. Given the heterogeneity of gene expression in single cells, however, normalization to a single reference gene will produce inconsistent or uninterpretable results. Thus, the best way to compare gene expression levels in single cells is to ensure that individual cells are collected and analyzed. Since single-cell LCM ensures that discrete cells are collected, normalization to reference genes is not required.

This application note has illustrated a simple and rapid workflow for analysis of single cells captured by LCM. The ArcturusXT LCM System facilitates the collection of individual cells with minimal damage to target molecules. The Single Cell-to-C_T kit provides a one-step method for converting transcripts in these collected cells into amplifiable cDNA. Finally, the TaqMan OpenArray panels allow investigators to query hundreds of transcripts from a single isolated cell. This powerful combination enables investigators to more efficiently work to better understand the complexities of normal biology and disease.

References

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3. Abruzzese RV, Fekete RA (2013) Single cell gene expression analysis of pluripotent stem cells. *Methods Mol Biol* 997:217–224.
4. Elowitz MB, Levine AJ, Siggia ED, Swain PS (2002) Stochastic gene expression in a single cell. *Science* 297:1183–1186.

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Ordering information

Product	Cat. No.
CapSure HS LCM Caps	LCM0214
PEN Membrane Glass Slides	LCM0522
HistoGene LCM Frozen Section Staining Kit	KIT0401
Single Cell-to-C_T qRT-PCR Kit	4458237
ExtracSure Sample Extraction Device	LCM0208
TaqMan OpenArray Human Cancer Panel	4475371
OpenArray AccuFill System	4457243
ArcturusXT LCM System	thermofisher.com/arcturus
QuantStudio 12K Flex Real-Time PCR System	thermofisher.com/quantstudio

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