Gene Expression Application Note No. 3

Step-by-Step Workflow for Small Sample Quantitative Real-Time PCR Confirmation of Microarray Results

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1 Introduction

Ovarian cancer is one of the most common gynecological malignancies in women with a very high mortality rate. The high mortality rate is caused in large part by the late stage of diagnosis and patient chemical resistance to platinum-based chemotherapy, contributing to a relapse in 90 percent of the patients after a 'debulking' operation [1]. At this time, there are no clinical or histopathological parameters for predicting platinum resistance with sufficient accuracy. It would be a great leap forward for platinum-resistant cancer patients to find an efficient method for distinguishing between platinum-chemoresistant and platinum-chemosensitive tumours for postoperative platinum-based therapy. Alternative, and probably more effective, chemotherapy treatments could be applied in those cases where platinum resistance has been detected.

The starting point of our research project was an in-house expression microarray analysis to identify biological differences between platinum-resistant and platinum-sensitive ovarian cancers. For that purpose, we compared ten chemoresistant and ten chemosensitive cancer tissue samples to identify differentially expressed genes that could explain the mechanisms that lead to resistance to the most common adjuvant chemotherapy. Approximately 100 transcripts with differential expression were detected. Several genes were selected from this list to confirm microarray results using real-time qPCR.

To verify our findings, we chose the robust Roche workflow for small sample quantitative real-time PCR analysis, which includes tissue lysis, RNA isolation, cDNA synthesis and amplification, coupled with the LightCycler® 480 System and data analysis. The relative expression of eight selected target genes and five housekeeping genes was measured and compared with the findings of the microarray analysis.

2 Materials and Methods

Single 10 µm thick frozen sections were cut from fresh frozen ovarian cancer tissue samples from ten platinum-resistant and ten platinum-sensitive patients using the Kryotom CM3050 S (Leica). The **MagNA Lyser** using **MagNA Lyser Green Beads** was used for optimal homogenization and lysis of the tissue and sample preparation before for RNA isolation.

Total RNA was isolated from all 20 samples using the **High Pure RNA Tissue Kit** according to the instructions provided in the package insert. RNA quality and quantity were verified using the NanoDrop® 1000 spectrophotometer (NanoDrop Technologies) and Bioanalyzer 2100 (Agilent Technologies, Inc.). The Bioanalyzer 2100 allows estimation of RNA quality using capillary electrophoresis to calculate the RNA-Integrity-Number (RIN).

Thirty nanograms of high quality RNA were reversely transcribed and cDNA was synthesized using the **LightCycler® RNA Pre-Amplification Kit.** In addition to the 20 samples, both a non-template control (NTC), a sample containing water instead of RNA, and a RT-minus control, a sample containing master mix without the RT enzyme, were processed. The LightCycler® RNA Pre-Amplification Kit synthesizes cDNA from research samples with very low amounts of RNA, such as small tissue sections, using Ribo-SPIA technology. This approach features linear, isothermal amplification of total RNA while retaining the relative representation of each transcript in the original RNA sample.

Subsequent quantitative real-time PCR was performed for eight target (*APOC1, COL3A1, CSPG2, EGFR, F2R, HLA-DMB, HOXB7* and *PPAPDC1A*) and five reference genes (*ACTB, G6PD, GAPD, PGK1* and *TBP*) chosen from the **Universal ProbeLibrary Set, Human Reference Gene Assays.** Until now, there has been no standard reference gene set for ovarian cancer. To find the most suitable transcripts for normalization of our samples, we measured several housekeeping genes. The Universal ProbeLibrary Assay Design Center (https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp?id=UP030000) was used to create gene-specific, intron-spanning primer sets for the UPL assays (see **Table 1**); the provided ranking helped us to pick out the most promising designs. The efficacy and uniqueness of the primer design was verified using the BLAST application of the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Amplification analysis was performed using the LightCycler® 480 System in 384-well plate format, in combination with the **Universal ProbeLibrary Set, Human** and the **LightCycler® 480 Probes Master** in 10 µl PCR total reaction volumes (comprising 2 µl template, 1.9 µl H_2O, 0.1 µl UPL, 5 µl 2x master and 1 µl primer with final concentration: 0.5 µM). Triplicates for each cDNA sample were done for quantification purposes, as well as one corresponding RT-minus control and one NTC for each transcript. A positive control cDNA was diluted 1:2 five times in duplicates to evaluate PCR efficiency. The PCR program described in the LightCycler® 480 Probes Master manual was used for real-time qPCR to quantify all 13 transcripts.

Figure 1: Workflow for small sample quantitative LightCycler® 480 Real-Time PCR of fresh frozen ovarian cancer tissue samples.

Transcript	Primer Sequence forward	Primer Sequence reverse	Probe No.
APOC1	TTCTGTCGATCGTCTTGGAA	TCAGCTTATCCAAGGCACTG	23
COL3A1	TCAAGGCTGAAGGAAATAGCA	CATTCCCCAGTGTGTTTCG	65
CSPG2	GGACTGATGGCAGCACACT	TCTTCTCCAGCAGAAAAGAAGC	20
EGFR	CAGCCACCCATATGTACCATC	AACTTTGGGCGACTATCTGC	42
F2R	TGAGACTGGGGCCACTACAT	ACATGATCAGTTCACAGCCAAT	58
HLA-DMB	GCTCCTGAGCCCATCCTT	GAAACCTTCAGGGTCTGCAT	26
HOXB7	CTACCCCTGGATGCGAAG	CAGGTAGCGATTGTAGTGAAATTCT	1
PPAPDC1A	GCGACTACAAGCATCACTGG	GGGTTTATGGCAAGCTGTGT	82

Table 1: Probe numbers and sequences for gene-specific and intron-spanning primer sets for UPL assays.

LightCycler® 480 Software was used to estimate the overall quality of all PCR results with the help of the amplification and calculated regression curve profile of the samples in reference to the assay controls (RT-minus control and NTC). The dilution series was used to calculate the PCR efficiency which, in combination with the performance of the negative controls (RT-minus control, NTC), allowed the evaluation of the PCR amplification.

The LightCycler® 480 Software also carried out the absolute quantification of the 13 transcripts on three 384-well plates; these results were transferred to qBase Software [2] to define the best suited reference genes for normalization and to calculate the relative expression of the genes of interest.

3 Results and Discussion

We used the described Roche workflow (see **Figure 1**) for confirmation of our in-house microarray analysis to demonstrate the straightforwardness, ease of operation and reliability of each of the successive steps.

To ensure using the same amount and excellent quality of RNA for each reverse transcription, it was very important to verify both RNA quality and quantity after tissue lysis, homogenization and isolation. The measured RNA-Integrity-Numbers with a mean RIN of 8.66 ± 0.38, indicated very good quality RNA (see **Figure 2**) with a low standard deviation. We used the LightCycler® RNA Pre-Amplification Kit for high quality cDNA synthesis from RNA from just a single tissue section. This method is a huge advantage because small frozen tissue samples can be rare and very valuable. Thirty nanograms of high quality RNA were used for each cDNA synthesis and amplification of each sample; the resulting cDNA was diluted 1:10 for subsequent quantitative LightCycler® 480 Real-Time PCR.

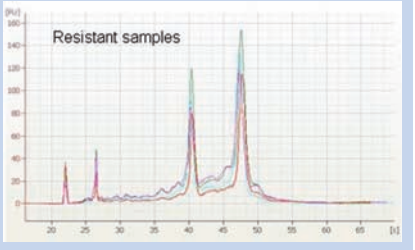
The establishment of each of the eight Universal ProbeLibrary Assays was performed using a dilution series to produce a standard curve. In nearly all cases, the best ranked primer design produced successful amplification, characterized by high PCR efficiencies. Several assays required a new primer design, which was uncomplicated with the help of the Probefinder Software Design Center, resulting in new designs using the same PCR conditions for all assays (see **Figure 3A** and **3B**).

The quantitation of the 13 transcripts in the RNA isolated from each of the 20 samples (see **Figure 3C** and **3D**), and data analysis with qBase Software was very straightforward. Using qBase Software, ACTB, GAPD and PGK1 were identified as the best fitting reference genes for normalization (Mean M (geNorm) of 1.02). Normalized data were used for the estimation of absolute expression and calculation of relative expression of eight selected genes in each of the 20 ovarian cancer tissue samples (see **Figure 4**).
Our findings clearly demonstrate the modified expression of those transcripts in platinum-resistant ovarian cancer tissues in comparison to platinum-sensitive tumour samples. Positive confirmation of each of the surveyed differential expressed transcripts was accomplished using quantitative LightCycler® 480 Real-Time PCR, thus easily verifying the results of gene profiling using microarrays.

A

Platinum-resistant ovarian cancer samples		Platinum-sensitive ovarian cancer samples	
Sample	RNA-Integrity-Number	Sample	RNA-Integrity-Number
1	8.6	11	8.7
2	8.0	12	8.5
3	8.2	13	8.4
4	8.7	14	8.5
5	8.5	15	9.0
6	8.2	16	9.2
7	8.8	17	8.5
8	8.1	18	8.6
9	9.1	19	9.1
10	9.0	20	9.4

B



C

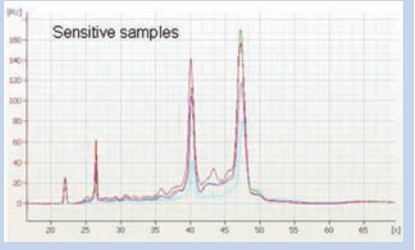


Figure 2: Measurement of RNA quality ranking using the Agilent Bioanalyzer results in a RNA Integrity Number (RIN) between 10 (highest) and 1 (lowest). Panel **A** shows the measured RNA-Integrity-Numbers in the 10 platinum-sensitive and 10 platinum-resistant ovarian cancer tissues.

Panels **B** and **C** show an electropherogram overlay of several platinum-resistant (**B**) and platinum-sensitive (**C**) ovarian tumour samples. The first big peak at 40 sec marks 18S rRNA, the second peak at 47 sec 28S rRNA; these signals reflect the good quality of total RNA obtained from the fresh frozen ovarian tissue samples.

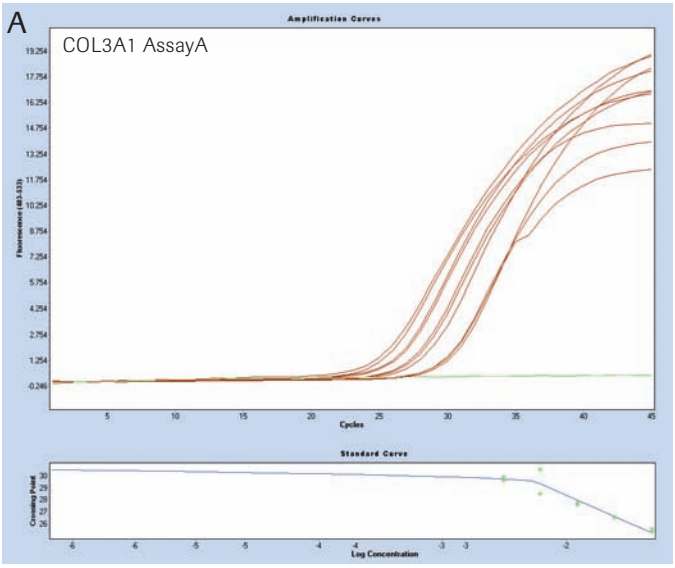
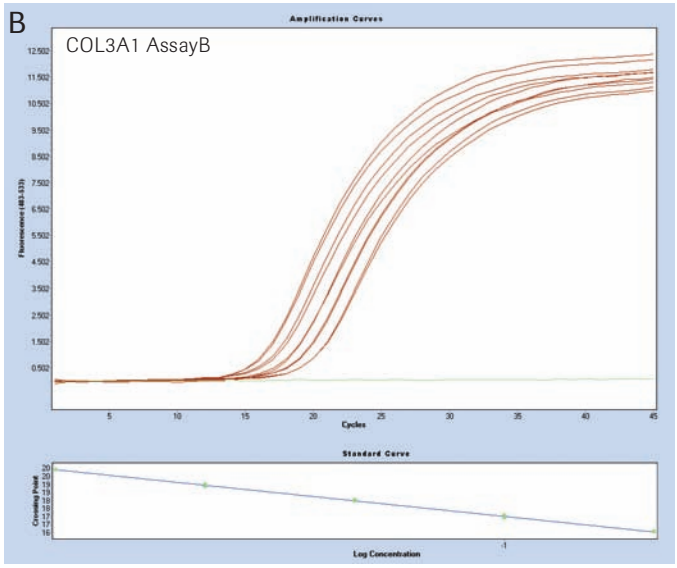
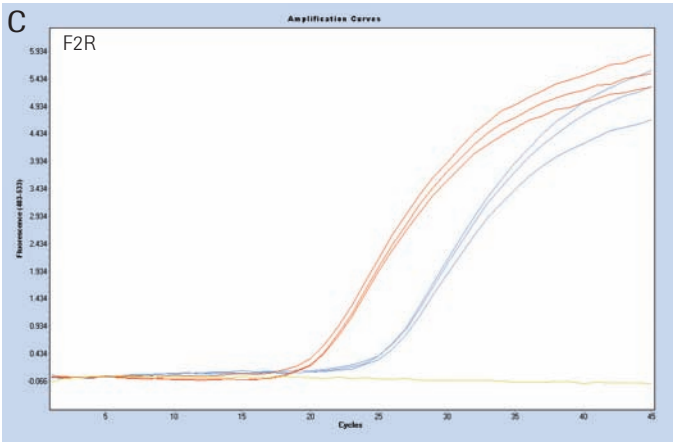


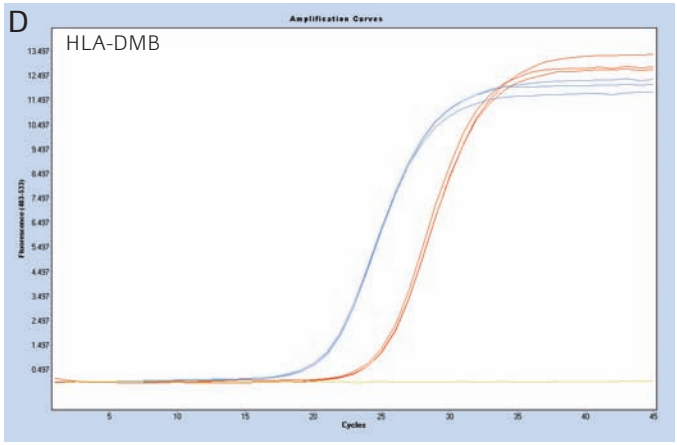
Figure 3: Establishment and quantification of Universal ProbeLibrary (UPL) assays for COL3A1, F2R and HLA-DMB.
(A) Amplification curves of serial dilutions of a positive control cDNA. The best ranked primer design showed dispersing replicates and an suboptimal regression curve and resulted in an efficiency of 1.663.



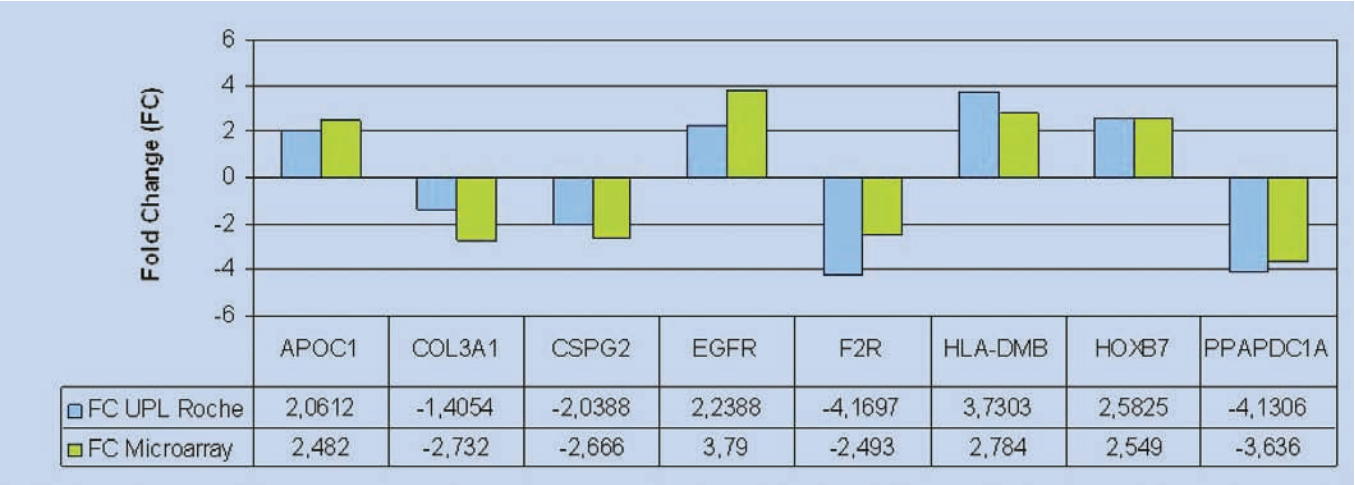
(B) A second primer design lead to satisfactory amplification curves and comparable low variation replicates. The generated regression curve showed the efficiency of 2.055.



(C) Results of sample measurement of F2R. **Red** graphs display triplicates of platinum-sensitive sample 16; **blue** curves describe related amplification of platinum-resistant sample 6.



(D) Amplification curves of sample measurement of HLA-DMB. **Blue** graphs show early amplification of platinum-resistant sample 2; **red** curves display triplicates of platinum-sensitive sample 16.



	APOC1	COL3A1	CSPG2	EGFR	F2R	HLA-DMB	HOXB7	PPAPDC1A
FC UPL Roche	2,0612	-1,4054	-2,0388	2,2388	-4,1697	3,7303	2,5825	-4,1306
FC Microarray	2,482	-2,732	-2,666	3,79	-2,493	2,784	2,549	-3,636

Figure 4: Relative expression of analyzed transcripts.
Example illustration of results from the gene expression analyses using either microarray (**green**) or quantitative real-time PCR (**blue**) .
The **y-axis** describes the fold change of the transcripts; the eight confirmed genes are listed on the **x-axis**. The fold change data are summarized in the table below.

4 Conclusions

We used an integrated workflow of Roche Applied Science featuring mechanical disruption of frozen tissue sections followed by total RNA isolation and pre-amplification. For the subsequent qRT-PCR analysis on a LightCycler® 480 Real-Time PCR Instrument, we performed Universal Probe Library assays and quantified gene expression in a set of 20 fresh frozen ovarian cancer samples. Starting from very small sample amounts, we easily used this workflow culminating in LightCycler® 480 Real-Time PCR with 384-well plates, to measure all the eight chosen transcripts, thus confirming the corresponding microarray data.

References

1. McGuire WP et al. (1996) N Engl J Med 334(1):1-6

2. Hellemans J et al. (2007) Genome Biol. 8(2):R19

Ordering Information

Product	Cat. No.	Pack Size
MagNA Lyser Instrument, 220V	03 358 976 001	1 instrument
MagNA Lyser Green Beads	03 358 941 001	100 tubes
High Pure RNA Tissue Kit	12 033 674 001	50 isolations
LightCycler® RNA Preamplification Kit	05 190 894 001	32 reactions
LightCycler® 480 II Instrument, 384 well	05 015 243 001	1 instrument
LightCycler® 480 Probes Master	04 707 494 001	500 reactions, 20 µl volume
LightCycler® 480 Probes Master	04 887 301 001	10 x 500 reactions, 20 µl volume
Universal ProbeLibrary Set, Human	04 683 633 001	1 Set
Universal ProbeLibrary Set, Human Reference Gene Assays	05 046 114 001	1 Set

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