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Applicability of Function Tested RealTime ready Assays for Gene Expression Analysis in Biomarker Research and Early Drug Development

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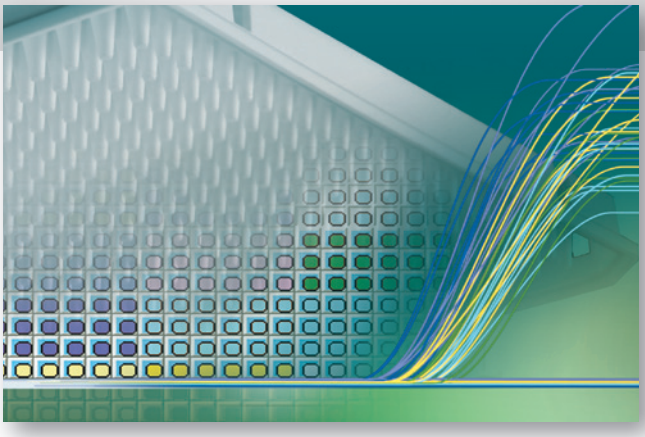


RealTime ready

Introduction

Personalized health care (PHC) uses gene-based information to understand requirements for health maintenance, disease prevention, and therapy, all tailored to an individual's genetic uniqueness. The identification and characterization of molecular biomarkers, used as indicators for a certain biological state, plays an important role in personalized healthcare (1). Biomarkers are therefore a central element in a drug's lifecycle, from target identification to drug application. Prostate-specific antigen (PSA), c-reactive protein (CRP), and HER2/neu are just a few examples of molecular biomarkers.

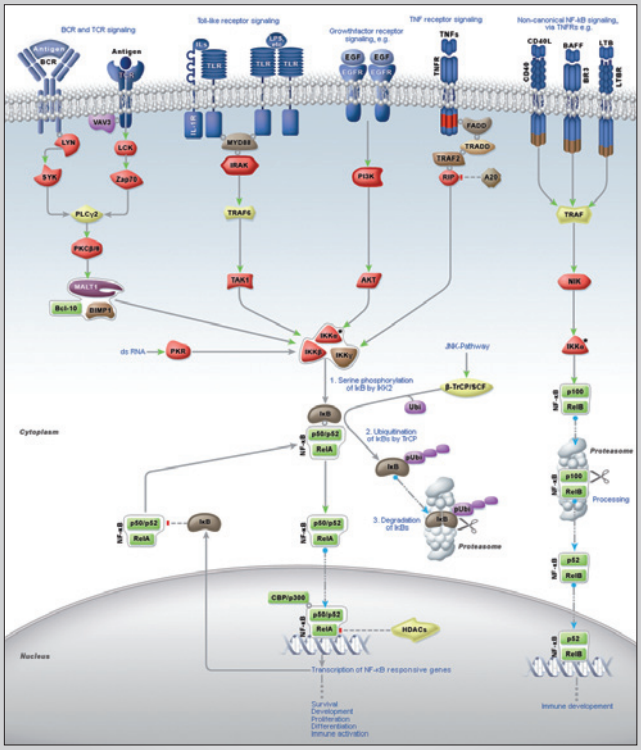
In biomarker research, the investigation of mRNA expression profiles in the context of complex biological pathways is of great interest. RealTime ready RT-qPCR assays and RealTime ready Configurator online portal facilitate the selection of relevant marker genes as well as gene expression profiling with function tested qPCR assays. RealTime ready Custom Panels on LightCycler® 480 Multiwell Plates contain pre-plated qPCR assays for human, mouse, or rat targets that have been selected by the user online within www.configurator.realtimeready.roche.com.

We have established workflows applicable for gene expression analysis in biomarker research with cell lines *(in vitro)* and xenograft mouse models *(in vivo)* as well as FFPET sections from various human research samples.

For life science research only.
Not for use in diagnostic procedures.

Therapeutic antibody compound development as an example of biomarker research and early clinical drug development

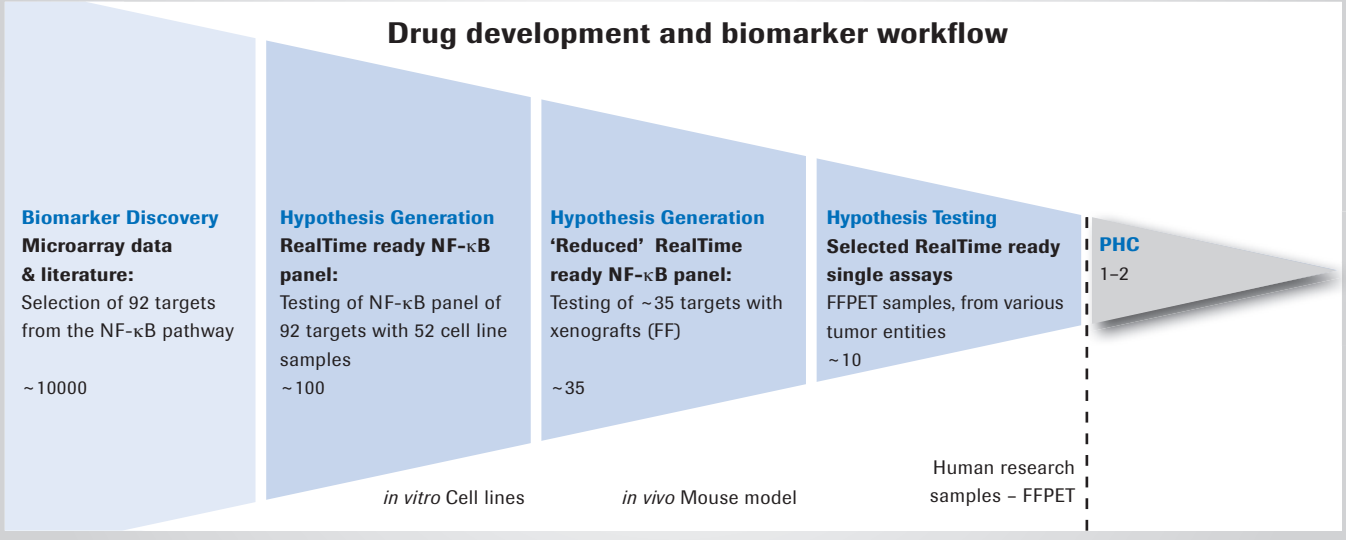
For a therapeutic antibody target, the biology and the downstream signal transduction pathways were analysed using literature and microarray data. Based on the results obtained, a RealTime ready NF-κB custom panel was assembled on the RealTime ready Configurator web portal using the "key word" search functions. For initial hypothesis generation, the RealTime ready NF-κB panel was used for *in vitro* gene expression analysis in 9 different tumor cell lines (treated versus untreated, at different time points; 54 samples in total). To confirm potential biomarkers, based on the cell line results, we applied a reduced NF-κB panel (35 parameters) to an *in vivo* mouse xenograft model. Based on the results obtained from the cell line and xenograft measurements, 13 potential candidate genes were identified. To test the hypothesis, 6 of these parameters were selected to analyze human FFPE samples. For these 6 parameters and selected reference genes, suitable for the tumor entities of interest (BC, CRC, NSCLC, sarcoma, RCC), RealTime ready qPCR assays were specifically developed and optimized to function with RNA isolated from FFPET.



Section of the interactive NF-κB pathway map from the RealTime ready Configurator, www.configurator.realtimeready.roche.com

RealTime ready qPCR Assays in biomarker research. Meeting the demands for different assay qualities.

Biomarkers are important throughout the entire development lifecycle of a therapeutic compound. The LightCycler® 480 real-time PCR platform in combination with RealTime ready qPCR custom panels is applied for biomarker research to generate a first hypothesis and for hypothesis testing (phase 0/I). The number of biomarker assays is reduced with progress in the assay development workflow.



Materials and Methods

Cell Culture

Tumor cell lines were cultured under standard conditions. The cells were treated with recombinant target for the therapeutic antibody or the therapeutic antibody, respectively. The tumor cell lines were harvested at different time points (0, 6, 24 hours) and stored in RNA later (Qiagen).

RNA Isolation from Cells

Total cellular RNA was isolated from the cell pellets using the MagNA Pure LC Instrument, the MagNA Pure LC RNA Kit – High Performance (Roche, Cat. No. 03 542 394 001), and the MagNA Pure LC protocol "RNA HP Cells". Cell lysates (1 x 10^6 cells in 600 µl RLT buffer [Qiagen, Cat. No. 79216]) were thawed and mixed, and 2 x 200 µl per lysate were transferred to MagNA Pure Sample Cartridge wells (200 µl per well). The isolated RNA from duplicate isolations of the same lysate, eluted in 50 µl each, was pooled.

RNA Isolation from FFPE Tissue

Total cellular RNA was isolated from a 10 µm section of FFPET using the High Pure FFPET RNA Isolation Kit (Roche, Cat. No. 06 650 775 001) an optimized version of the High Pure Parrafin Kit (Roche) with a fast and convenient standard protocol giving the same performance as a lengthy overnight Proteinase K digestion.

RNA Quality Control

The quality and quantity of each RNA preparation was analyzed using the NanoDrop Instrument.
For some samples, the integrity was analyzed using the Agilent Bioanalyzer and the RNA Nano Chip.

cDNA Synthesis

One microgram of total RNA was used for cDNA synthesis using the Transcriptor Universal cDNA Master (Roche, Cat. No. 05 893 151 001). Three separate 40 µl reactions per sample were set up.
Alternatively, serial RNA dilutions were reverse transcribed and the respective concentrations were used in the PCR.

Real-Time qPCR

Three cDNA synthesis reactions using one microgram total RNA each were pooled, diluted, and used as template for each RealTime ready Custom Panel Plate, 384 (Roche, Cat. No. 05 582 873 001).
A reaction mix containing the RNA was prepared using the LightCycler® 480 Probes Master (Roche, Cat. No. 04 707 494 001). The total PCR reaction volume per well was 10 µl, with a final RNA amount of 10 ng.
The LightCycler® 480 Software, Version 1.5 and the content .txt file provided by Roche for each panel enabled easy sample setup and analysis of the results.

Results and Discussion

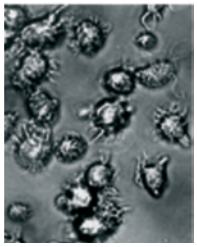
***In vitro* cell line studies with the NF-κB RealTime ready Custom Panel result in 35 differentially expressed genes.**

To generate a first hypothesis for predictive and pharmacodynamic markers, we used a multi-parameter panel (92 target and 4 housekeeping genes) covering the NF-κB pathway in a broad gene expression screening approach. The goal was also to establish a workflow with high convenience and throughput.

The first *in vitro* study was performed with tumor cell lines treated with the compounds of interest. A convenient and fast workflow was developed, starting with automated RNA isolation on the MagNA Pure LC Instrument, cDNA synthesis, and RT-qPCR on the LightCycler® 480 Instrument with the RealTime ready NF-κB custom panel in a 384-well format. The relative gene expression was analyzed.

Workflow description: *In vitro* studies with the NF-κB RealTime ready Custom Panel

Cell Culture



9 cell lines treated with recombinant target for the therapeutic antibody or the therapeutic antibody.

Sample Material

Cell pellets:
10^6 cells harvested in RLT buffer;
stored at -80°C

RNA Isolation

MagNA Pure LC instrument

MagNA Pure LC RNA Isolation Kit – High Performance

cDNA Synthesis

Transcriptor Universal cDNA Master

RT-qPCR

qPCR with RealTime ready NF-κB panel + LightCycler® 480 Probes Master

Selection of suitable reference genes for normalization of the target gene expression

For relative quantification analysis, the target gene expression is normalized to reference gene expression. This normalization should, ideally, compensate for variations in RNA/cDNA input amount and potential inhibitors in the cDNA synthesis or PCR amplification. The reference genes serve as endogenous controls of the sample material and are processed throughout the entire workflow along with the target genes. It is essential to select suitable reference genes for normalization to obtain reliable results. A suitable reference gene is characterized by stable, non-regulated gene expression in the sample material of interest (2, 3). 2 out of 4 reference genes were selected as described in Figure 1.

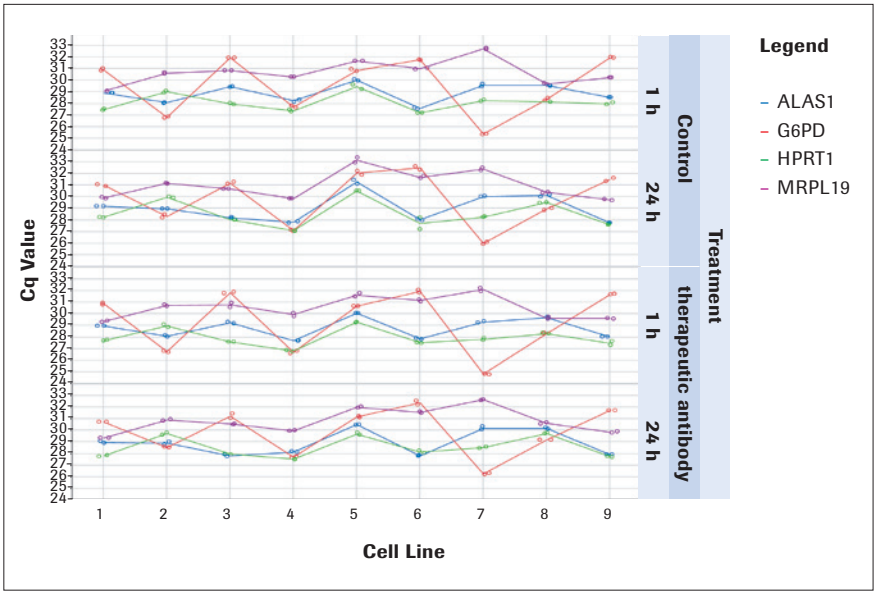


Figure 1: Gene expression analysis of 4 different reference genes in 9 different cell lines.
The absolute Cq values (duplicates) for 4 different reference genes are plotted for the 9 cell lines under investigation in this study. The PCR was performed starting with the identical cDNA pool. Two of the reference genes analyzed show a similar pattern: ALAS1 (aminolevulinate, delta-, synthase 1) and HPRT (hypoxanthine phosphoribosyltransferase 1). A third gene, MRPL19 (mitochondrial ribosomal protein L19), behaves quite comparably, while the fourth gene, G6PDH (glucose-6-phosphate dehydrogenase), exhibits a clearly distinct pattern. Therefore, we selected ALAS1 and HPRT as reference genes for relative gene expression analysis.

1: human renal cell adenocarcinoma
2: human kidney clear cell carcinoma cell line
3: human osteosarcoma; multipotential sarcoma
4: human pancreatic carcinoma, epithelial-like cell line
5: human breast cancer model; breast; mammary gland; pleural effusion
6: human pancreas adenocarcinoma
7: human lung carcinoma
8: bronchioloalveolar adenocarcinoma
9: cell line derived from a human prostate carcinoma xenograft

Differential gene expression results

In the gene expression analysis done with the NF-κB panel and RNA from 9 different cell lines derived from various tumor entities, 35 differentially expressed genes were identified when a normalization of Cq values to a combination of the 2 suitable reference genes (RG) was performed. The delta Cq was calculated as: Cq_RG – Cq_Target where Cq_RG is the mean value of Cq values of the reference genes ALAS and HPRT. The differential gene expression pattern of the receptor of the antibody target in the 9 cell lines is shown in Figure 2.

In vitro tumor cell line studies and *in vivo* xenograft studies with the RealTime ready NF-κB Custom Panel reveal a potential response prediction marker

Biomarkers for response prediction (clinical response) indicate whether or not an individual can optimally respond to a therapeutic compound (4). A well-known example is the couple HER2/neu and Herceptin.

To identify potential response prediction biomarkers, the relative gene expression results of the 9 selected cell lines (see Figure 2) were correlated with the tumor growth inhibition of the xenografted tumors in the mouse models. The cell lines harvested after 1 or 24 hours were untreated and the mice were treated with the therapeutic antibody. The gene expression results determined with the cell lines showed very good reproducibility and no effect from time or culture. Tumor growth inhibition (TGI) in the mouse models correlated well with relative gene expression for a potential biomarker in the cell lines, as shown in Figure 3.

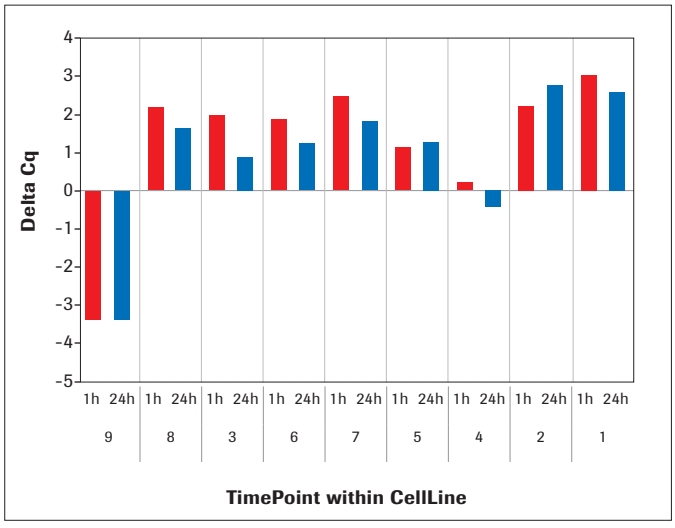


Figure 2: Gene expression of the receptor of the antibody target in 9 different cell lines.
The relative ratios (log 2 scale) were calculated as delta Cq = Cq_HK – Cq_ receptor of the antibody target, and are plotted for each cell line and time point. The normalization was performed against ALAS1 and HPRT1.
1: human renal cell adenocarcinoma
2: human kidney clear cell carcinoma cell line
3: human osteosarcoma; multipotential sarcoma
4: human pancreatic carcinoma, epithelial-like cell line
5: human breast cancer model; breast; mammary gland; pleural effusion
6: human pancreas adenocarcinoma
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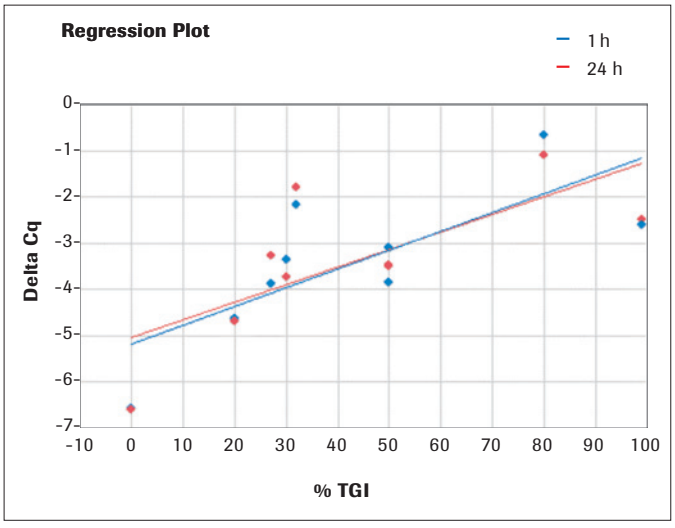


Figure 3: Gene expression of response prediction Biomarker 1 in cell lines correlates well to *in vivo* tumor growth inhibition (TGI) results.
Gene expression results from the RealTime ready NF-κB custom panel are plotted versus TGI *in vivo*. Increased gene expression of "biomarker 1" correlates with TGI.

In vivo xenograft studies confirm a potential pharmacodynamic marker identified *in vitro* using the RealTime ready NF-κB custom panel

Pharmacodynamic biomarkers (functional biomarkers) change after application of a therapeutic compound and show therapeutic response (4). They are important for defining the relevant dosage of the therapeutic compound. After selecting a set of parameters, the hypothetical biomarkers identified *in vitro* were verified using a mouse model. Xenograft-derived fresh frozen (FF) tissue samples were used to test for selected biomarkers. Figure 4 shows the relative gene expression ratios for a potential pharmacodynamic biomarker after dosing tumor bearing mice treated with therapeutic antibody or vehicle control. Reduced gene expression after drug application was observed.

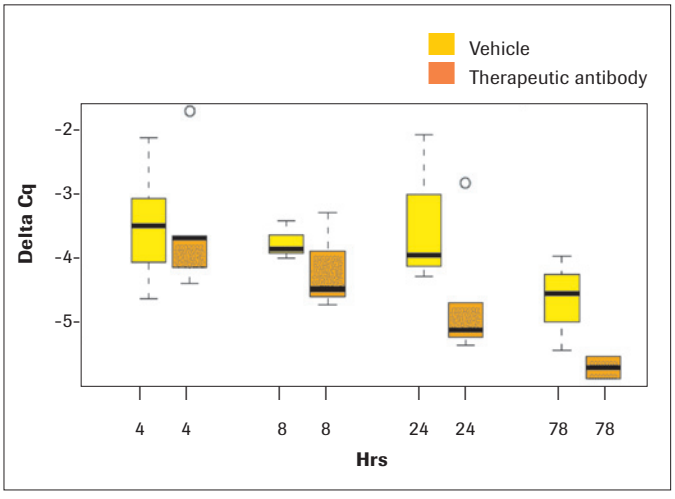


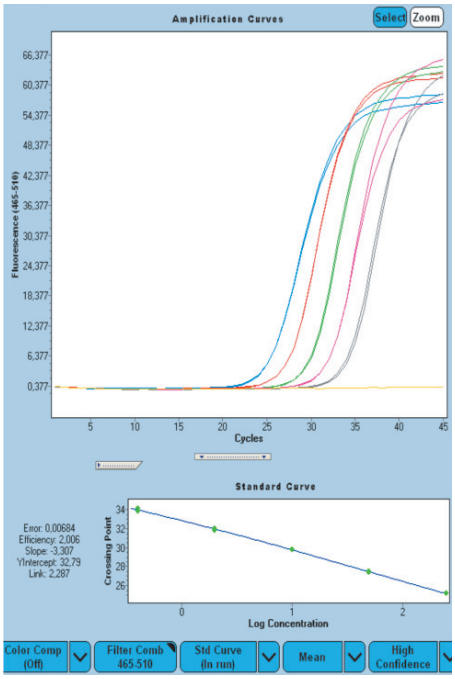
Figure 4: Relative gene expression ratios for potential Biomarker 2 plotted versus the response to treatment with the therapeutic antibody in a mouse xenograft model. The median gene expression levels on log 2 scale (n = 5 animals) are plotted.

Hypothesis testing of potential biomarkers using RealTime ready qPCR assays specifically developed for FFPET sample material

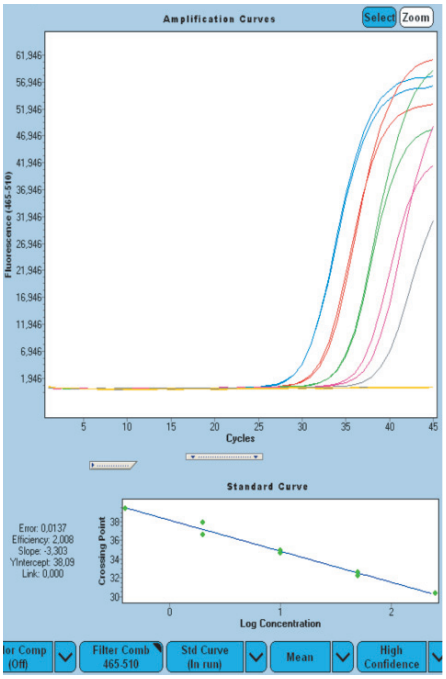
We used the first set of clinical research samples, formalin-fixed paraffin-embedded (FFPE) tissue derived from various human research samples. Tumor-derived FFPE tissue is the most relevant sample material for the development of therapeutic compounds in clinical trials or in biomarker research. RNA extraction from FFPE tissue (*e.g.*, 10 µm sections) using the High Pure FFPET RNA Isolation Kit is followed by RT-qPCR analysis, with specifically optimized and function tested RealTime ready assays.

RNA isolated from FFPET samples is fragmented due to formalin fixation and storage conditions (5). For FFPET-derived RNA, it is therefore important to use amplicons smaller than 100 bp to achieve good sensitivity and reproducibility. The RealTime ready assays are designed to amplify small amplicons. The amplification of all parameters should be RNA specific, without co-amplifying contaminating human genomic DNA. This can be achieved by primer-probe design that spans exon-intron boundaries. Also, any pseudogenes should not be co-amplified; this must be checked in control reactions with human genomic DNAs, as not all pseudogenes are known or published. RealTime ready assays were designed for and tested with RNA isolated from various FFPE-derived human research tumor samples (*e.g.*, BC, CRC). The optimal RealTime ready RT-qPCR assays were selected with respect to the following parameters: sensitivity, linearity, reproducibility, and specificity. Depending on the expression level of the parameters in the tissue of interest, the sensitivity could be further increased via specific priming applied for cDNA synthesis (see Figure 5). Reliable relative gene expression levels of a putative biomarker 4 had been measured after the selection of suitable reference genes for various tumor entities (6).

A) Biomarker 3/Kidney
Hexamer priming



B) Biomarker 3/FFPET BC
Hexamer priming



C) Biomarker 3/FFPET BC
Specific priming

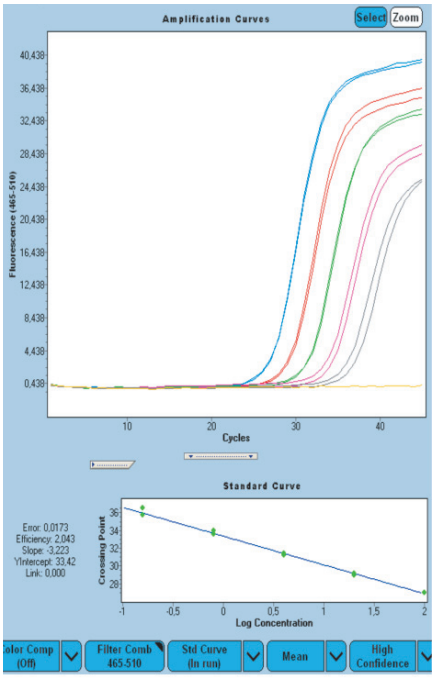


Figure 5: RealTime ready RT-qPCR amplification curves for potential "Biomarker-3". Serial dilutions of RNA templates were derived from kidney RNA **(A)** and FFPE breast cancer **(BC)** samples **(B and C).** The PCR started with 250 ng in 1:5 dilution steps with the lowest amount of 0.4 ng/PCR **(A and B),** or with 100 ng in 1:5 dilution steps with the lowest amount of 0.16 ng/PCR **(C).** In C, specific priming instead of hexamer priming for cDNA synthesis was chosen to increase sensitivity.

Cq values are shown in Table 1.

Kidney RNA/PCR	Cq	BC RNA/PCR	Cq	BC RNA/PCR	Cq
Hexamer Priming				Specific Priming	
250 ng	25.14	250 ng	30.33	**100 ng**	27.04
250 ng	25.15	250 ng	30.33	**100 ng**	26.99
50 ng	27.33	50 ng	32.15	20 ng	29.00
50 ng	27.43	50 ng	32.61	20 ng	29.11
10 ng	29.80	10 ng	34.65	4 ng	31.46
10 ng	29.73	10 ng	34.87	4 ng	31.18
2 ng	31.77	2 ng	36.58		33.95
				0.8 ng	33.57
2 ng	31.95	2 ng	37.86	0.8 ng	
0.4 ng	34.12	0.4 ng	39.37	0.16 ng	35.71
0.4 ng	33.82	0.4 ng	--	0.16 ng	36.52
Water	--	Water	--	Water	--

Table 1

Conclusions

RealTime ready RT-qPCR assays can be successfully applied in biomarker research for hypothesis generation with *in vitro* and *in vivo* models as well as for hypothesis testing with human FFPE research samples. Workflow protocols for the different sample materials have been set up and described. RNA from FFPE tissue samples can be isolated using the High Pure FFPET RNA Isolation Kit, and qRT-PCR analysis can be performed using RealTime ready dedicated assays.

Ordering Information

Product	Cat. No.	Pack Size
MagNA Pure LC 2.0	05 197 686 001	1 instrument
MagNA Pure LC RNA Kit – High Performance	03 542 394 001	192 reactions
High Pure FFPET RNA Isolation Kit	06 650 775 001	50 isolations
Transcriptor Universal cDNA Master	05 893 151 001	100 reactions
RealTime ready Custom Panel Plate 384–96	05 582 873 001	10 plates
LightCycler® 480 Probes Master	04 707 494 001	5 x 1 ml, up to 500 reactions

www.realtimeready.roche.com
www.lightcycler.com
www.magnapure.com

References

1. NIH Biomarker Definitions Working Group, Clin Pharmacol Ter 2001; **69:** 89–95.
2. Bustin, S.A., *et al.* (2009) Clin Chem 55 (4): 611.
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