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Gene expression analysis in biomarker research and early drug development using function tested reverse transcription quantitative real-time PCR assays

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ABSTRACT

The identification of new biomarkers is essential in the implementation of personalized health care strategies that offer new therapeutic approaches with optimized and individualized treatment. In support of hypothesis generation and testing in the course of our biomarker research an online portal and respective function-tested reverse transcription quantitative real-time PCR assays (RT–qPCR) facilitated the selection of relevant biomarker genes. We have established workflows applicable for convenient high throughput gene expression analysis in biomarker research with cell lines *(in vitro* studies*)* and xenograft mouse models *(in vivo* studies*)* as well as formalin-fixed paraffin-embedded tissue (FFPET) sections from various human research and clinical tumor samples. Out of 92 putative biomarker candidate genes selected *in silico*, 35 were shown to exhibit differential expression in various tumor cell lines. These were further analysed by *in vivo* xenograft mouse models, which identified 13 candidate genes including potential response prediction biomarkers and a potential pharmacodynamic biomarker. Six of these candidate genes were selected for further evaluation in FFPET samples, where optimized RNA isolation, reverse transcription and qPCR assays provided reliable determination of relative expression levels as precondition for differential gene expression analysis of FFPET samples derived from projected clinical studies. Thus, we successfully applied function tested RT–qPCR assays in our biomarker research for hypothesis generation with *in vitro* and *in vivo* models as well as for hypothesis testing with human FFPET samples. Hence, appropriate function-tested RT–qPCR assays are available in biomarker research accompanying the different stages of drug development, starting from target identification up to early clinical development. The workflow presented here supports the identification and validation of new biomarkers and may lead to advances in efforts to achieve the goal of personalized health care.

1. Introduction biomarker research

Personalized health care (PHC) uses genetic information to understand requirements for health maintenance, disease prevention, and therapy. The aim of PHC is to select the right medicine at the right dose for the right patient at the right time [1–3]. The identification and characterization of molecular biomarkers, used as indicators for a certain biological state, plays an important role in PHC [4,5]. Biomarkers may serve for disease prognosis, the prediction of therapeutic response and monitoring duration and quality of response. Thus biomarkers have the potential to support clinical decisions, from diagnosis to treatment planning, to improve tailored treatment strategies to avoid over- or under treatment and adverse side effects and finally to enhance prognosis and cost effectiveness. For several years, the integration of biomarkers in drug development and their use as companion diagnostic in clinical practice is also strongly encouraged by regulatory authorities [6,7]. Therefore, biomarkers have become a central element in a drug's lifecycle, from target identification throughout pre-clinical and clinical development up to the application of the launched drug (Fig. 1).

Abbreviations: ALAS1, Aminolevulinate, delta- synthase-1; Cq, quantification cycle; FFPET, formalin-fixed paraffin-embedded tissue; HER2, human epidermal growth factor receptor 2; HPRT1, hypoxanthine phosphoribosyltransferase-1; PHC, personalized health care; qPCR, quantitative real-time PCR; RT–qPCR, reverse transcription qPCR; TGI, tumor growth inhibition.

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For example in breast cancer which displays extensive molecular heterogeneity the human epidermal growth factor receptor 2 (HER2/neu) represents a well established predictive molecular biomarker to differentiate HER2-overexpressing tumor subtypes in order to allow HER2 targeted therapy [4,5,8,9]. Today advancements in high throughput technologies significantly facilitate biomarker identification and constitute the basis to take full advantage of the potential biomarkers offer for PHC. Currently efforts are undertaken to standardize biomarker discovery, development and validation in order to rise the percentage of biomarkers passing the barriers for clinical application [1,10].

As fresh-frozen clinical tissue samples are largely limited as source for biomarker research the application of formalin-fixed paraffin embedded tissue (FFPET) specimens becomes important for gene expression analysis from pathology samples [11]. Formalin-fixation and paraffin embedding (FFPE) is the clinical standard for tissue fixation and processing for the purpose of diagnostic histology and long-time storage. For instance, in the clinical diagnosis of cancer patients FFPET samples have been routinely obtained for decades. Therefore, the existence of huge archives of FFPET specimens worldwide constitutes a valuable source of retrospective long term biological material for follow up biomarker discovery and validation and may provide material to sustained progress in the development of PHC.

In the present study we describe the use of function-tested RT–qPCR assays in order to discover appropriate biomarkers during pre-clinical and early clinical studies. In biomarker research, the investigation of mRNA expression profiles in the context of complex biological pathways is of great interest and represents a common initial step in the detection and identification of suitable biomarkers. The RT–qPCR technology is known to be highly specific and sensitive and in these aspects superior to the microarray technology [12–14] but the number of evaluated genes at one time is rather limited. Therefore, the goal of this study was also to establish a workflow offering high throughput and application convenience for efficient expression profiling.

In order to facilitate the selection of relevant biomarker genes as well as gene expression profiling we used a specialized online portal (RealTime ready Configurator, Roche Diagnostics) and the respective function tested RT-qPCR assays (function tested RealTime ready RT-qPCR assays). The gene panel selected using this tool (provided as a RealTime ready custom panel) were analysed by RT-qPCR on the LightCycler® 480 instrument for which the respective pre-plated assays were available. This study describes established workflows applicable for analysis of differential gene expression in biomarker research with cell lines (*in vitro*) and xenograft mouse models (*in vivo*) as well as with FFPET sections from various human research samples.

2. Overview of biomarker development workflow

The methodology applied to identify suitable biomarkers for the development of a therapeutic antibody compound is summarized in Fig. 2. For the target-specific therapeutic antibody the biology and the down stream signal transduction pathways were analysed using literature search and microarray data (mRNA expression data, unpublished in-house data). These *in silico* analyses revealed the involvement of the NF-κB pathway. Based on these results, we assembled a special panel of 92 NF-κB related genes of putative interest using the web portal (https://configurator.realtime-ready.roche.com) with the "keyword" search function.

To generate a first hypothesis, the NF-κB panel was used for *in vitro* gene expression analysis in nine different human tumor cell lines using function-tested RT-qPCR assays. To confirm potential biomarkers, based on the cell line results, we applied a reduced NF-κB panel (35 parameters) to *in vivo* mouse xenograft models. Based on the results obtained from the cell line and xenograft measurements, 13 potential candidate genes were identified. To test the hypothesis, six of these parameters were selected to analyze human FFPET samples. For these six parameters and selected reference genes, suitable for expression analysis in the tumor entities of interest, respective RT-qPCR assays were specifically developed and optimized to function with RNA isolated from FFPET. The aim of the preclinical and early clinical data generation was to select the most appropriate biomarkers for prospective hypothesis testing and ultimately for potential companion diagnostics (Dx) development.

3. Description of methods

3.1. Biomarker hypothesis generation in vitro: Workflow description for cell line studies

The workflow for *in vitro* cell culture studies with the selected NF-κB panel is described in Fig. 3 comprising the steps cell culture and sample preparation, RNA isolation, cDNA synthesis and qPCR. This workflow is designed for high throughput analysis required at the initial biomarker development step of hypothesis generation.

3.1.1. Cell culture

Commercially available tumor cell lines selected based on target receptor expression (1: human renal cell carcinoma; 2: human renal cell carcinoma; 3: human osteosarcoma; 4: human pancreatic carcinoma; 5: human breast cancer; 6: human pancreatic carcinoma; 7: human lung carcinoma; 8: human lung carcinoma; 9: human prostate carcinoma) 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 h) and immediately resuspended in RLT buffer (Qiagen) and stored at – 80 °C up to several months.

3.1.2. RNA Isolation from cells

Automated isolation of total cellular RNA was performed from the cell pellets using the MagNA Pure LC Instrument with the

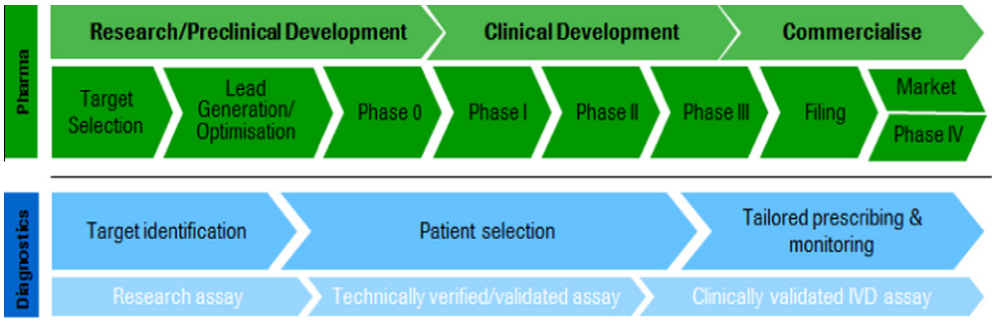


Fig. 1. Biomarkers are relevant for the entire development cycle of a therapeutic compound from target identification to drug application.

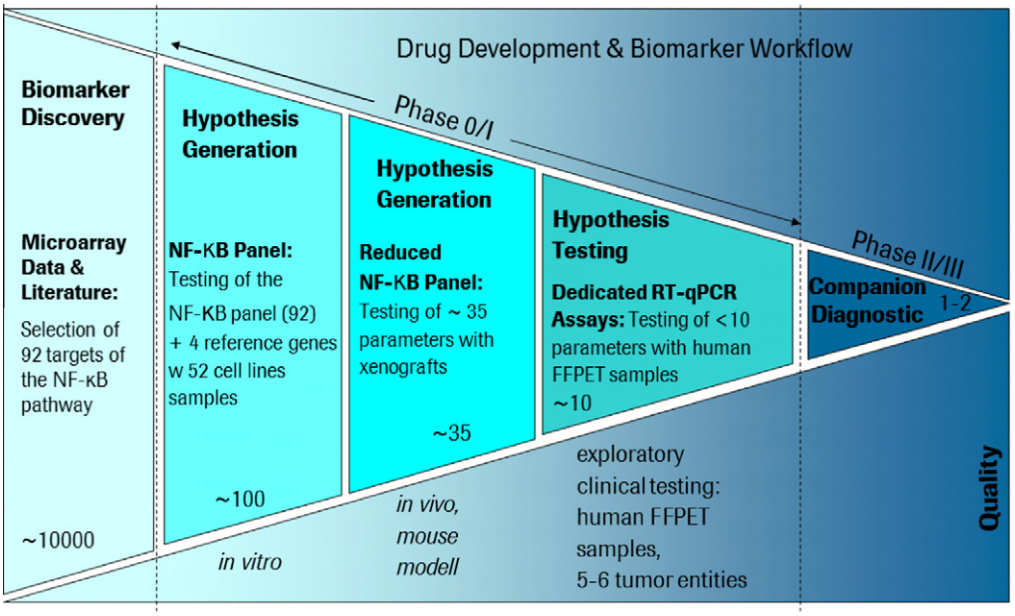


Fig. 2. Description of biomarker workflow. Biomarkers are important throughout the entire development lifecycle of a therapeutic compound. The real-time PCR platform in combination with pre-plated RT-qPCR custom panels was applied for biomarker research to generate a first hypothesis and for hypothesis testing. The number of biomarker assays is reduced with progress in the assay development workflow.

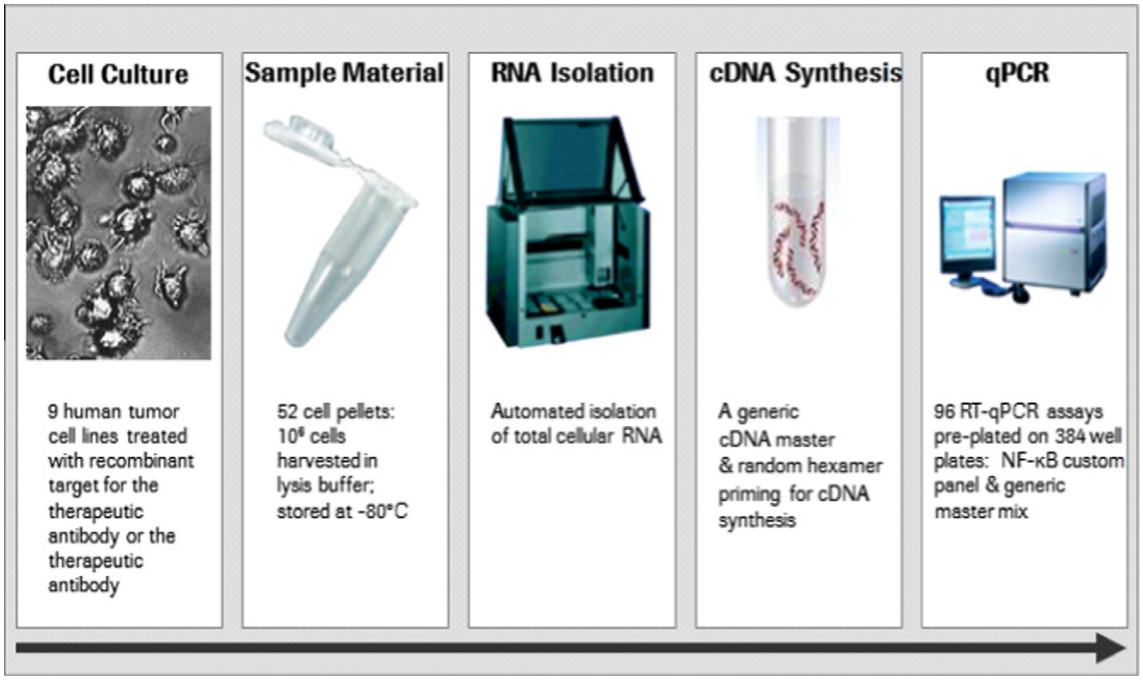


Fig. 3. Workflow description of *in vitro* studies with the NF-κB panel.

MagNA Pure LC RNA Kit – High Performance and the MagNA Pure LC protocol "RNA HP Cells" according to instruction manuals (Roche Diagnostics). In brief, samples of 1×10^6 cells lysed in 600 µl RLT buffer (Qiagen) were thawed and mixed. For each cell lysate isolations were performed in duplicate, thus 200 µl were transferred to each of two MagNA Pure sample cartridge wells. The procedure was based on proteinase K digestion to destroy remaining proteins and nucleases as well as the addition of magnetic glass particles which bind nucleic acids due to chaotropic salt conditions. Incubation with DNase I degraded genomic DNA. After several washing steps total RNA was eluted from magnetic glass particles using 50 µl of elution buffer per well at 70 °C and duplicate preparations were pooled. RNA quantity and quality were determined using the NanoDrop Instrument based on UV–Vis spectrophotometric measurement of absorbance (Thermo Fisher Scientific). The ratio 260/280 served to monitor protein contamination. Optionally, the quality and integrity of the RNA was evaluated by analysing RNA profiles

according to fragment length using the Agilent 2100 Bioanalyzer in conjunction with the RNA 6000 Nano Kit (see 3.3.1).

3.1.3. cDNA synthesis

1 μg of total RNA was used for cDNA synthesis using the Transcriptor Universal cDNA Master (Roche Diagnostics). For each RNA preparation reverse transcription was performed in triplicate in 40 μl reactions using random hexamer priming at 55 °C for 10 min according the manufacterer's instruction. Alternatively, serial RNA dilutions (1:5) were reverse transcribed and the resulting concentration levels were used in the PCR. As positive control, a commercial reference RNA (Human reference RNA from cancer cell lines, Stratagene or Human universal reference total RNA from human tissues, Clontech) was applied to monitor the performance of the complete procedure. cDNA was immediately used for PCR set up or stored at −20 °C.

3.1.4. qPCR using function tested RT-qPCR assays

Applied RT-qPCR assays were obtained as RealTime ready custom panel plate for 384 reactions (Roche Diagnostics) in a dried-down format in 384 well plates [15]. The pre-plated RT-qPCR assays contained target specific primers and a matching probe from the Universal ProbeLibary (UPL). The UPL is based on only 165 short hydrolysis probes, labeled at the 5′ end with fluorescein and at the 3′ end with a dark quencher dye. Each probe can cover up to several thousand binding sites and the whole set of 165 probes thereby covers ~99.9% of the human transcriptome. Incorporation of locked nucleic acids into the probe sequence increased the thermal stability and discriminative power [16].

The custom-designed assays were function tested by the manufacturer on the LightCycler® 480 Instrument (Roche Diagnostics) with a universal sample cDNA under standard PCR conditions and had to meet stringent quality control criteria. The amplification efficiencies accounted for 2.0 ± 0.2 and the linear dynamic range spanned at least three $\log_{10}$ steps with correlation coefficients (R^2) of the standard curve of 0.99–1.0. The specificity of amplification was controlled by gel electrophoresis. For RealTime ready assays the sequence accession number and the location of the amplicon is provided, due to confidentiality reasons of the target genes for the biomarkers assays in context with the compound developed, we could not publish these details, but in general the RealTime ready assays are in alignment with the MIQE guidelines. We performed the RealTime ready assay according to the standard procedures as given by the manufacturer.

Three cDNA synthesis reactions starting from 1 μg of total RNA each were pooled, diluted, and used as template for one PCR plate (384 reactions). The custom-designed plates contained the specific PCR reactions for the 92 identified target sequences of the NF-κB related gene panel which was initially selected with the aid of the online portal together with four reference genes, that had been identified in previous studies to be suitable after analysis with the GeNorm SW. The reference genes were selected to facilitate a later analysis of the relative gene expression ratios by normalizing target quantification cycle (Cq) values to reference gene Cq values (see also Fig. 6). The qPCR reaction mix was prepared from cDNA preparations using the LightCycler® 480 Probes Master (Roche Diagnostics) and 10 μl were added to each well. Each PCR reaction contained the cDNA input amount derived from 10 ng of total RNA. A cDNA preparation derived from the reference RNA (qPCR RNA, Stratagene) served as positive control, as no template control H_2O was used and was giving no cp call. To check for co-amplification of potential residual genomic DNA, 50 and 5 ng of genomic DNA from human whole blood (Roche Diagnostics) were applied as template. Sample setup, qPCR analysis and calculation of Cq values for relative quantification were performed by the LightCycler® 480 Software, Version 1.5 and the Sample Editor content ∗.txt file (Roche Diagnostics).

3.2. Biomarker hypothesis generation: Workflow description for in vivo xenograft studies

3.2.1. Xenograft studies and mouse model

Tumors were subcutaneously implanted into athymic immune-deficient nude mice (see also Section 3.1.1). After the tumors had developed the mice were treated with the therapeutic compound or vehicle control.

3.2.2. Analysis of tumor growth inhibition

The tumor growth inhibition (TGI) was assessed by measuring the tumor size after treatment with the therapeutic compound or vehicle. Tumor volumes of treated groups were presented as percentages of tumor volumes of the control groups (%T/C), using the formula: $100 \times ((T-T0)/(C-C0))$, where T represented mean tumor volume of a treated group on a specific day during the experiment, $T0$ represented mean tumor volume of the same treated group on the first day of treatment; C represented mean tumor volume of a control group on the specific day during the experiment, and $C0$ represented mean tumor volume of the same treated group on the first day of treatment. Tumor volume (in cubic millimeters) was calculated using the ellipsoid formula: $(D \times (d2))/2$ where 'D' represents the large diameter of the tumor, and 'd' represents the small diameter. Statistical analysis was determined by the rank sum test and One Way Anova and a post hoc Bonferroni t-test (SigmaStat, version 2.0, Jandel Scientific, San Francisco, CA). Differences between groups were considered to be significant when the probability value (p) was <0.05.

3.3. Biomarker hypothesis testing: Workflow description for FFPE tissue samples

The workflow for expression analysis of the pre-selected genes derived from the NF-κB panel in FFPET is described in Fig. 4 comprising the steps sample preparation, RNA isolation, cDNA synthesis and qPCR.

3.3.1. RNA extraction from FFPET

For assay establishment isolation of total cellular RNA started from FFPET samples. The FFPET samples were obtained from commercial vendors (Indivumed, Asterand). The FFPET tissue blocks were stored in the refrigerator (2–8 °C). The FFPET tissue blocks were quite different in age from a couple of months up to several years. The slides were typically stored at RT, curls are stored at −20 °C. The RNA was extracted from the FFPET samples applying the High Pure FFPET RNA Isolation Kit (Roche Diagnostics). The High Pure FFPET RNA Isolation Kit is based on nucleic acid binding to the surface of glass fleece in the presence of chaotropic salts. The de-paraffinization of samples was performed with xylene and ethanol during the initial steps of the procedure. 1x proteinase K digestion step at 85 °C for 30 min was applied, with a second addition of proteinase K at 55 °C for 30 min. This treatment reversed cross linking with proteins which had resulted from previous formalin fixation. DNase I treatment was performed on the High Pure column for 15 min at room temperature. A fraction of the isolated RNA was used for cDNA synthesis and the reminder of the RNA was pooled from 5 to 10 individual samples of the same tumor entity for purpose of assay development to minimize sample to sample variability. When we were generating controls to optimize the RT-qPCR assays we used 2 × 10 μm sections as curls from FFPET samples applying the High Pure FFPET RNA Isolation Kit (Roche Diagnostics). Briefly, each of the 10 μm curls were processed separately as described in the instruction of the manufacturer until the lysate is applied on the filter columns. At this step the two lysates were pooled and loaded on the identical filter column.

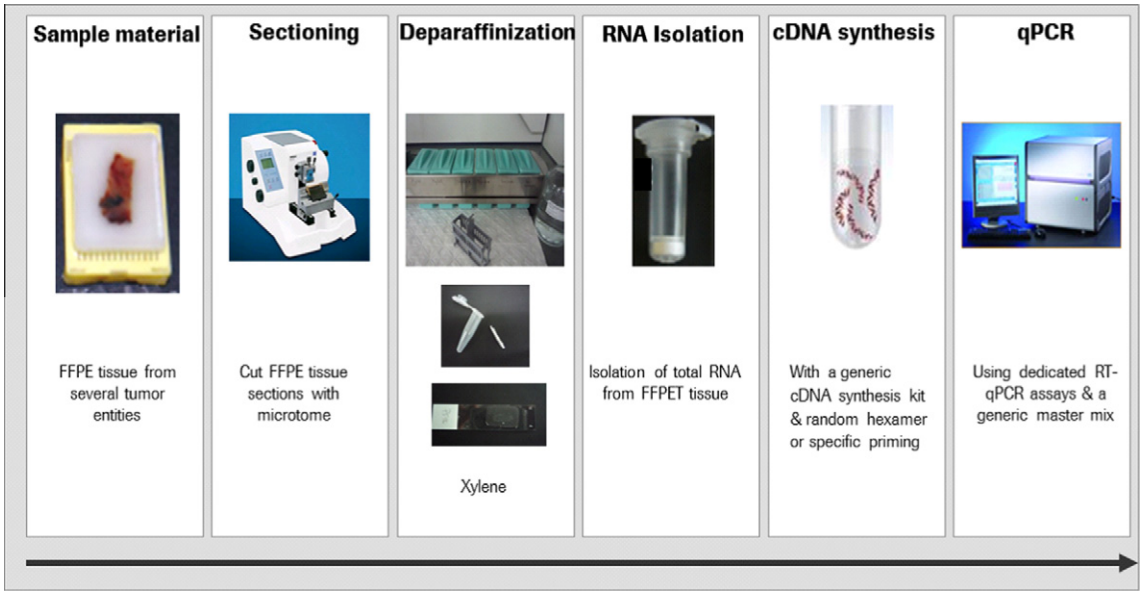


Fig. 4. Workflow description: Dedicated RT-qPCR assays verified with FFPET sample material.

RNA isolation from clinical study samples usually started from 3 to 4 μM FFPET sections fixed on microscope slides. The slides were stored at RT. In some cases pathological evaluation also recommended macrodissection (tumor content below 60–80%), i.e. areas with highest tumor content of the tissue sections were macrodissected and the macrodissected material was used for RNA extraction. In all studies a reference RNA was used as a positive control with predefined acceptance criteria (cq values) for each RT-qPCR assay.

For all FFPET samples RNA quantification was performed using the NanoDrop Instrument (see Section 3.1.2). Additionally, the integrity of isolated RNA was determined by the Agilent 2100 Bioanalyzer (Agilent Technologies) a system that uses a combination of microfluidics, capillary electrophoresis, and a fluorescence dye that binds to nucleic acids. Using this system, the quality control of isolated RNA was expressed according to the RNA Integrity Number (RIN) ranging from 1 to 10, with one being the most degraded profile and 10 being the most intact. One microliter of RNA preparations was analysed with the RNA 6000 Nano Chip (Agilent Technologies, for obtained RNA concentrations of 25–500 ng/μl) or the RNA 6000 Pico Chip (Agilent Technologies, for obtained RNA concentrations of 50–5000 pg/μl). This step was routinely performed during assay establishment, but was optional for analysis of FFPET derived from clinical studies in order to save material. Usually, the PCR result of positive control samples and of endogenous reference genes served for readout (see Section 4.1 and 4.5.3).

3.3.2. cDNA synthesis and priming

One microgram of isolated RNA was reverse transcribed into cDNA using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics) with standard random hexamer priming in 40 μl final reaction volume (see Section 3.1.3). For genes expressed at low levels the sensitivity of PCR detection could be increased by gene specific priming using the 0.2 μM sequence-specific reverse primer HPLC-purified (Metabion) instead of random hexamer priming for cDNA synthesis. Again the Transcriptor First Strand cDNA Synthesis Kit was applied according to manufacturer's instructions at 55 °C for 30 min. The cDNA was immediately used for PCR set up or stored at −20 °C.

3.3.3. qPCR using dedicated function-tested RT-qPCR assays

Due to enhanced fragmentation of RNA isolated from FFPET the primer pairs for target amplification were selected to generate amplicons smaller than 100 bps of length. Unspecific amplification from residual genomic DNA was avoided when the amplicons spanned introns (> 1000 bps). Primer design and a check for cross complementarity were performed with the LightCycler® Probe Design Software 2.0 Tool (Roche Diagnostics) and the primer specificity was checked *in silico* versus the public data base nr/Homo sapiens (ORGN). Target amplification was performed in duplicate with dedicated function tested RT-qPCR assays (Roche Diagnostics). 1:5 dilution series of cDNA generating input amounts of 50, 10, 2, 0.4, and 0.08 ng of cDNA were applied in 20 μl of PCR reaction volume in a 96 well plate format. cDNA preparations derived from the reference RNA (qPCR RNA, Stratagene) served as positive control and H_2O as no template control. As acceptance criteria for valid PCR results derived from dilution series of cDNA prepared from reference RNA four out of five dilution levels had to show linear amplification with efficiencies between 1.8 and 2.1. Data evaluation was automatically performed by the LightCycler® LC480 instrument and the LightCycler® 480 Software, Version 1.5. As acceptance criteria for valid PCR results derived from dilution series of cDNA prepared from FFPET sample material a minimum of three out of five dilution levels had to show linear amplification with efficiencies between 1.8 and 2.1.

For data evaluation, *C*q values of duplicate measurements were included which did not deviate for more than half a *C*q value. No template controls were included in each run and a valid result was given as no cp value called. Fluorescence growth curves had to follow typical steep amplification curve shapes as automatically evaluated by the LightCycler® software. Inter-run reproducibility of RT-qPCR was assessed by two operators and two LightCycler® 480 instruments. The acceptance criteria were determined using the serial dilutions series as described above, specifically for each target. The results were very much depending on the expression level of each target and the concentration. During the course of assay development agarose gel (4%) analysis was performed to assess absence of unspecific amplification products. Co-amplification of potential residual genomic DNA was checked with 50 and 5 ng of genomic DNA from human whole blood (Roche Diagnostics). As for most assays several primer probe sets were evaluated, those resulting in no cp call or a cp > 35 were selected, to exclude a positive signal by residual genomic DNA.

For evaluation of individual tumor entities the most stable reference genes for normalization were identified using the geNorm software [17].

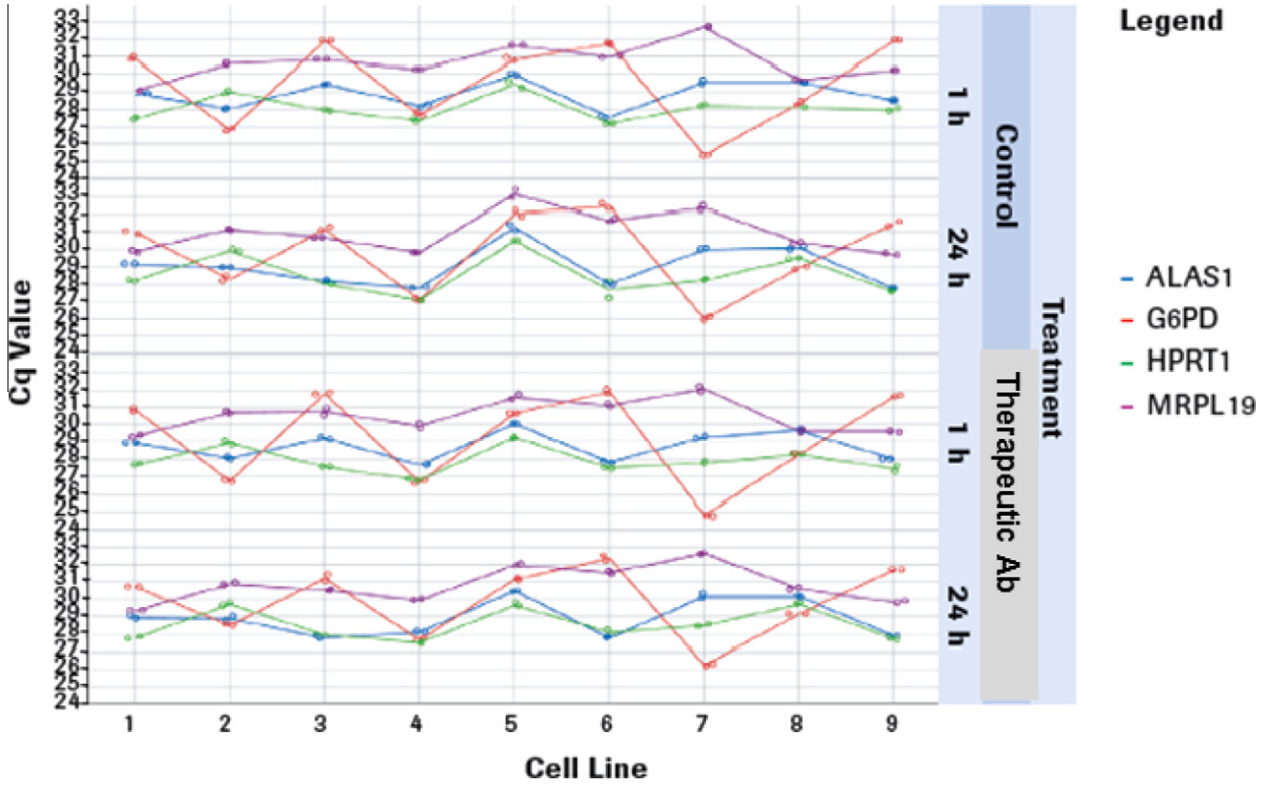


Fig. 5. Gene expression analysis of four different reference genes in nine cell lines. The Cq values evaluated from duplicate measurements for four different reference genes were plotted for nine cell lines (see numbers). ALAS1 (aminolevulinate, delta-, synthase 1) and HPRT1 (hypoxanthine phosphoribosyltransferase 1) were selected as best suitable reference genes for relative gene expression analysis. MRPL19 (mitochondrial ribosomal protein L19) and G6PDH (glucose-6-phosphate dehydrogenase) revealed a distinct pattern. Cell lines: 1: human renal cell carcinoma; 2: human renal cell carcinoma; 3: human osteosarcoma; 4: human pancreatic carcinoma; 5: human breast cancer; 6: human pancreatic carcinoma; 7: human lung carcinoma; 8: human lung carcinoma; 9: human prostate carcinoma.

For the dedicated RealTime ready assays the sequence accession number, the location of the amplicon is provided and the primer design is always checked not to co-amplify known pseudogenes, retropseudogenes or other homologs. Due to confidentiality reasons of the target genes for the biomarkers assays in context with the compound developed, we could not publish these details, but in general the RealTime ready assays are in alignment with the MIQE guidelines. We performed the RealTime ready assay according to the standard procedures as given by the manufacturer.

4. Results and discussion

4.1. Selection of reference genes for cell culture studies for normalization of target gene expression (Relative quantification)

Valid gene expression analysis by RT-qPCR should follow a set of guidelines for standardization [18–20]. For relative quantification analysis, the use of multiple endogenous reference genes to normalize target gene expression levels is commonly recommended. This normalization should compensate for variations in RNA/cDNA input amount and potential inhibitors of cDNA synthesis or PCR amplification. We applied endogenous reference genes as internal control of the sample material and processed them throughout the entire workflow in parallel to the target genes. However, the selection of suitable reference genes is essential for reliable and consistent quantification of the target gene expression. Suitable reference genes are characterized by stable, non-regulated gene expression in the sample material of interest and the use of the average of multiple internal control genes is recommended to minimize errors [17,18]. As the expression levels of commonly applied reference genes may vary particularly in tumor cell lines and tissue [21–23], the reference genes were experimentally selected according to their respective expression profiles in the cell lines and culture conditions under study. To this end, expression levels of four well-known reference genes, aminolevulinate, delta-, synthase-1 (ALAS1), hypoxanthine phosphoribosyltransferase-1 (HPRT1), mitochondrial ribosomal protein L19 (MRPL19) and glucose-6-phosphate dehydrogenase (G6PDH), were analysed according to Cq values during untreated control conditions and in parallel

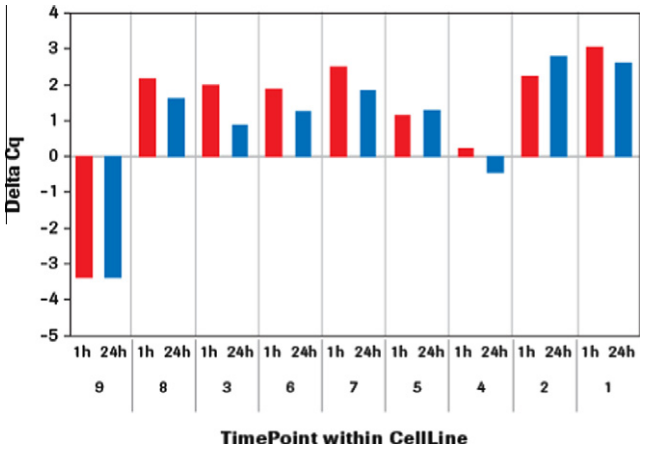


Fig. 6. Differential gene expression of the receptor of the antibody target in nine untreated different cell lines. The relative ratios (log 2 scale) were calculated as delta Cq = Cq (mean reference genes ALAS1 and HPRT1) – Cq (target receptor) and are plotted for each cell line and time point. Cell lines: 1: human renal cell carcinoma; 2: human renal cell carcinoma; 3: human osteosarcoma; 4: human pancreatic carcinoma; 5: human breast cancer; 6: human pancreatic carcinoma; 7: human lung carcinoma; 8: human lung carcinoma; 9: human prostate carcinoma.

during treatment with the therapeutic antibody at 1 and 24 h of culture time starting from identical cDNA pools (Fig. 5). ALAS1 and HPRT1 showed a similar expression pattern at quite comparable levels across the nine different cell lines, whereas MRPL19, revealed a more or less related pattern but at a lower level. In contrast, G6PDH exhibited a clearly distinct pattern and larger differences between the expression levels of the individual cell lines. Due to their similar and nearly constant expression patterns ALAS1 and HPRT1 were selected as reference genes for cell culture analysis. Relative quantification of target gene expression levels was obtained by normalizing the individual target gene Cq value against the mean Cq value of the ALAS1 and HPRT1 (delta Cq = mean Cq (referene genes) – Cq (target)).

4.2. Detection of differential gene expression of potential biomarkers in tumor cell lines (Hypothesis generation)

To generate a first hypothesis for predictive and pharmacodynamic biomarkers, we used a multi-parameter panel of 92 target genes and two reference genes covering the NF-κB pathway in a broad gene expression screening approach. *In vitro* gene expression analysis was performed in nine different tumor cell lines with cell cultures treated with the therapeutic antibody compound of interest and untreated controls at three different time points which resulted in 54 samples for each target gene. A convenient and fast workflow was developed, starting with automated RNA isolation, cDNA synthesis and function-tested RT-qPCR assays for the NF-κB panel in a 384-well format. In total 29 384 well plates were required for the high throughput analysis. The relative gene expression levels of the target receptor normalized against reference genes at baseline are shown exemplarily in Fig. 6. Seven cell lines displayed elevated expression levels, whereas one cell line showed a significantly lower expression level. In this way, the *in vitro* cell culture studies with the NF-κB gene panel performed on nine different cell lines resulted in the identification of a total of 35 differentially expressed genes.

4.3. Identification of a potential response prediction biomarker using in vitro tumor cell line and in vivo xenograft studies (Hypothesis generation)

Biomarkers for clinical response prediction indicate whether or not an individual person can optimally respond to a therapeutic compound [24]. A well-known example is the couple HER2/neu and Herceptin [8,9]. To verify the hypothesized response prediction biomarkers, the relative gene expression results of the 35 differentially expressed genes identified in the nine selected cell lines (see Fig. 6) were compared with the tumor growth inhibition of the xenografted tumors in the respective mouse models. The cell lines harvested after 1 or 24 h of culture were untreated and the mice were treated with the therapeutic antibody in order to analyse respective *in vivo* effects on tumor growth. The gene expression results of the cell lines showed very high reproducibility and were nearly stabile over culture time. The cell culture results were related to observed tumor growth inhibition (TGI) in the mouse model (Fig. 7). *In vitro* low expression levels of the target gene correlated with low rates of TGI and high gene expression levels in cell culture occurred together with high rates of TGI. Thus, the potential to inhibit tumor growth *in vivo* correlated well with the relative gene expression levels of the hypothesized predictive biomarker in the tumor cell line culture.

4.4. Identification of a potential pharmacodynamic biomarker using in vitro tumor cell line and in vivo xenograft studies (Hypothesis generation)

Pharmacodynamic biomarkers (functional biomarkers) change in response to application of a therapeutic compound, thus indicating therapeutic response [24]. They are important for defining the relevant dosage of the therapeutic compound. The hypothetical biomarkers identified *in vitro* were verified using a mouse model. Xenograft-derived tissue samples were used to test for selected biomarkers. Fig. 8 shows the relative gene expression ratios for a potential pharmacodynamic biomarker, initially derived from the NF-κB panel, in tumor bearing mice treated with different doses of the therapeutic antibody or vehicle control. After drug application the gene expression levels of the biomarker was observed to decrease with exposure time in treated mice.

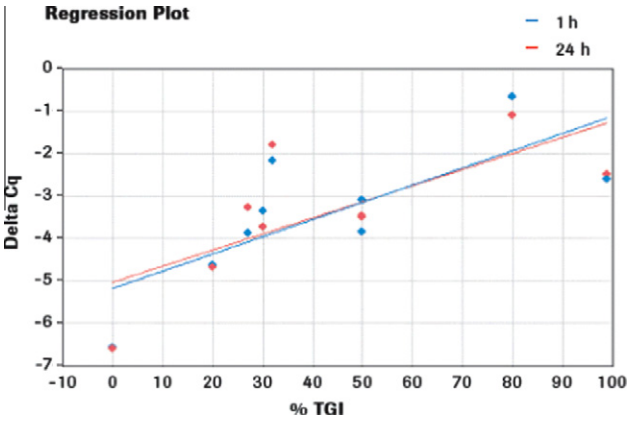


Fig. 7. Gene expression of a response prediction biomarker 1 in cell lines correlated with *in vivo* tumor growth inhibition (TGI) results. Gene expression results of biomarker 1 in a tumor cell line were plotted versus TGI in the respective *in vivo* mouse model.

4.5. Biomarker verification in human clinical FFPET samples (Hypothesis testing)

We used the first set of clinical research FFPET samples derived from various human tumor entities. Tumor-derived FFPET is commonly used as the most relevant sample material in patient monitoring during development of therapeutic compounds in clinical trials or in biomarker research. RNA extracted from FFPET was followed by cDNA synthesis and expression analysis with specifically optimized and dedicated function-tested RT-qPCR assays (see Fig. 4).

4.5.1. RNA quality and yield

FFPET is commonly known to represent poor material for many applications in molecular biology due to nucleic acid degradation,

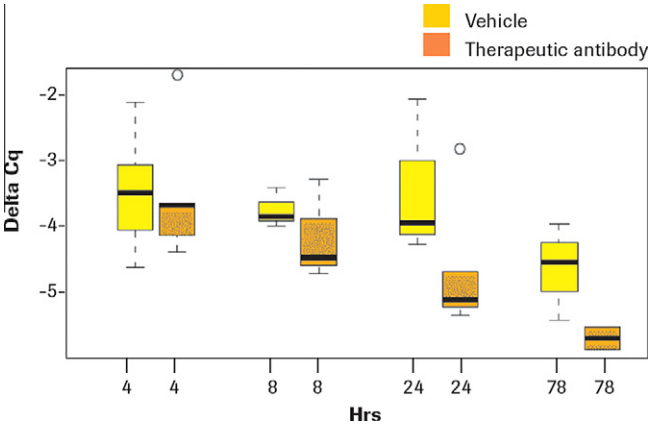


Fig. 8. Relative gene expression ratios for a potential pharmacodynamic biomarker 2 plotted versus time of exposure to the therapeutic antibody or the vehicle control in a mouse xenograft model. The median gene expression levels on log 2 scale ($n = 5$ animals) are displayed.

Table 1
RNA yields obtained from FFPET (2 × 10 μm sections) determined by NanoDrop.

Tumor entity	Concentration (ng/μl)	Yield (ng per 45 μl eluate)	Yield (μg)
BC RNA pool	174	7830	7.830
NSCLC RNA pool	143	6435	6.435
CRC RNA pool	320	14400	14.400
RCC RNA pool	139	6255	6.255
PAC RNA pool	121	5445	5.445
BC RNA Pool	69	3105	3.105

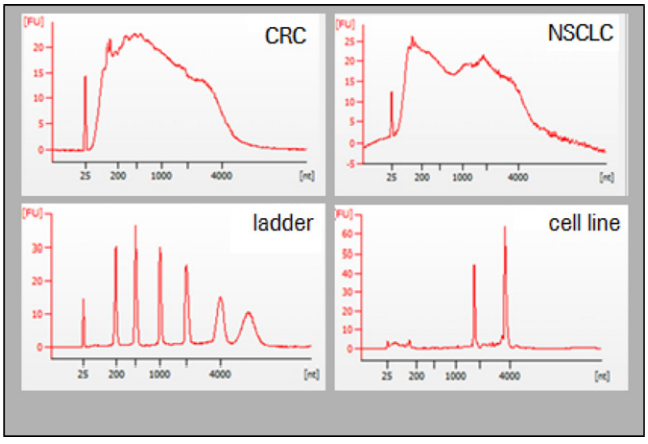
BC: breast cancer; NSCLC: non-small cell lung cancer; CRC: colorectal cancer; RCC: renal cell carcinoma; PAC: pancreatic cancer.

fragmentation and chemical modification [25]. Paraffin-embedding preserves tissue morphology and proteins very well but it does not totally prevent degradation of nucleic acids. Formalin-fixation causes chemical modifications by monomethylol additions to the RNA bases and cross-linkage with proteins [25,26]. Moreover, degradation of RNA can occur prior to formalin-fixation. Hence, time and storage of surgical material until fixation as well as conditions and time of fixation and storage afterwards are crucial factors for RNA integrity but difficult to standardize in clinical routine [14,27]. Therefore, RNA isolated from FFPET samples usually shows fragmentation and the degree of degradation may significantly vary of from sample to sample.

We performed RNA isolation from FFPET using the High Pure FFPET RNA Isolation Kit (Roche Diagnostics). This new kit uses a shorter protocol including optimized proteinase K digestion steps. These modifications proved to be faster and required less hands-on time, while results and performance were comparable. Proteinase K digestion is known to be a critical step affecting RNA extracted from FFPET [27,28]. The High Pure FFPET RNA Isolation Kit is a product update of the High Pure RNA Paraffin Kit that was recently reported to achieve high yields and quality of RNA isolations similar to the RNeasy FFPE kit (Qiagen) in a multicenter evaluation of different RNA extraction procedures from FFPET [29]. Moreover, the High Pure RNA Paraffin Kit was recommended especially for isolation from low input amounts of 10-year old FFPET samples [30]. The silica column-based purification method is also advantageous because it omits the time-consuming isopropanol precipitation step of phenol extraction-based methods. Typical examples of quantity and quality of our RNA preparations obtained from 2 × 10 μm FFPE tissue sections of different tumor entities are given in Table 1 and Fig. 9 showing RNA yields and electropherograms, respectively. Our RNA extraction procedure proved to be very efficient providing yields of high quality total RNA higher or comparable to reported values of similar applications [14,26,27,29]. For instance, a recovery of 2.55 μg per 20 μm colon control section [28] and up to 3 μg per 5 μm breast tumor FFPE section [31] have been described. However, comparability of results of different studies may be limited as RNA yields from FFPET samples may vary due to several factors such as specimen size, tumor fraction, the number of well-preserved cells per sample, fixation time and storage time and conditions [27]. With regard to RNA integrity, steep signals for ribosomal 18S and 28S rRNA fractions were typically obtained for profiles of high quality RNA resulting in RIN values higher than nine as displayed for RNA from cell culture material (Fig. 9, right lower panel). In contrast, RNA obtained from FFPET samples showed enhanced fragmentation resulting in RIN values in the range of 2.0–2.5 (Fig. 9, upper panels). However, our RNA preparations from FFPET samples were well suitable for further processing and provided reproducible RT-qPCR results as reaction conditions could be optimized for the amplification of short amplicons (see 4.4.3). Successful PCR analysis were also reported from FFPET samples with similar RNA profiles and low RIN values down to 1.4 using the same or alternative RNA isolation protocols [14,27,30].

4.5.2. Optimization of cDNA synthesis and RT-qPCR assays (FFPET)

As illustrated in Fig. 9 RNA isolated from FFPET samples was partly fragmented due to formalin fixation and storage conditions. qPCR reaction conditions for FFPET derived RNA and cDNA analysis, therefore, required specific optimization of dedicated function



Tumor entity	RIN	260/280	ng/μl
CRC	2.0	2.08	549
NSCLC	2.5	2.07	673
Cell line	9.6	2.13	314

RIN: RNA Integrity Number; NSCLC: non-small cell lung cancer; CRC: colorectal cancer

Fig. 9. Electropherograms obtained from FFPET-derived and cell line-derived RNA, respectively, generated by the Agilent 2100 Bioanalyzer according to RNA fragment length. The table above shows the corresponding RIN numbers, OD260/280 ratios and the final concentrations for each of the samples.

Table 2
Cq values determined by dedicated function-tested RT-qPCR assays for detection of biomarker 3 expression levels using random hexamer and specific priming.

Hexamer priming				Specific priming	
Kidney RNA (ng)/PCR (fresh tissue)*	Cq	BC RNA (ng)/PCR (FFPET)	Cq	BC RNA (ng)/PCR (FFPET)	Cq
250	25.1	250	30.3	100	27.0
250	25.2	250	30.3	100	27.0
50	27.3	50	32.2	20	29.0
50	27.4	50	32.6	20	29.1
10	29.8	10	34.7	4	31.5
10	29.7	10	34.9	4	31.2
2	31.8	2	36.6	0.8	34.0
2	32.0	2	37.9	0.8	33.6
0.4	34.1	0.4	39.4	0.16	35.7
0.4	33.8	0.4	-	0.16	36.5
Water	-	Water	-	Water	-

BC: breast cancer.
* RNA from kidney displayed high expression levels of biomarker 3 mRNA and served as positive control in these experiments, whereas biomarker 3 mRNA could not be detected at sufficient expression levels in the usually applied reference RNA.

Table 3
Relative gene expression levels of biomarker 4 in various tumor entities.

Tumor entity	Cq biomarker	Cq reference gene[a]	Delta Cq = Cq biomarker − Cq reference gene
NSCLC	32.4	31.5	0.9
BC	32.8	31.6	1.2
CRC	32.7	31.6	1.1
PAC	31.3	31.2	0.1
BC RNA pool	33.1	32.7	0.4

BC: breast cancer; NSCLC: non-small cell lung cancer; CRC: colorectal cancer; RCC: renal cell carcinoma; PAC: pancreatic cancer.
[a] MRPL19 served as reference gene except for CRC where HPRT1 was determined to be most suitable.

tested RT-qPCR assays with regard to sensitivity, linearity, reproducibility, and specificity of the amplification reactions. In terms of primer design, it was important to use target and reference gene amplicons smaller than 100 bps to achieve good sensitivity and reproducibility. In order to avoid amplification from potentially contaminating human genomic DNA the primer-probe design was chosen to spans introns (>1000 bp). To our experience the intron length spanned should exceed 1000 bps to largely eliminate unspecific PCR products which could otherwise affect relative quantification results of target sequences. Also, the amplification of any pseudogene sequences had to be considered and was routinely checked in control reactions with human genomic DNA. These controls are strongly recommended, because the existence and number of pseudogenes are often not known or published.

In cases of very low expression levels of the target gene in tissues of interest, the sensitivity of RT-qPCR assays could be further increased using specific instead of random hexamer priming for reverse transcription of RNA into cDNA. This phenomenon was recently also described in a multicenter validation of RNA extraction from FFPE tissue [29], although random hexamer priming is commonly regarded as favorable for fragmented and degraded RNA samples [27]. Generally, Cq values higher than 35 were more susceptible to imprecision in data analysis of amplification profiles and duplicate measurements showed higher variability. In our hands, specific cDNA priming using the reverse amplification primer was able to significantly improve sensitivity and reliability of relative quantification by RT-qPCR assays. In the example given in Table 2 the sensitivity for expression level analysis of a biomarker 3 in FFPET tumor samples from breast cancer (BC) was improved for more than three Cq values.

4.5.3. Differential gene expression in FFPET

For the early clinical studies FFPET samples derived from various tumor entities are the typical sample material. Different tumor entities may display significant variability in mRNA expression profiles also for reference genes [22,23]. Therefore, for the tumor entities under study, different candidate reference genes were analysed. Using the geNorm software [17] the most stable reference gene for the respective tumor and tissue type was evaluated. Accordingly, we established tumor-specific reference genes in order to allow most reliable determination of relative gene expression levels within each tumor entity as displayed in Table 3, exemplarily for putative biomarker 4. Thus, we demonstrated the feasibility to quantify relative gene expression levels of biomarkers in FFPET samples, which are currently applied in ongoing clinical studies. The material availability is usually very limited, when working with FFPET samples. In the studies only a few tissue sections on slides are available for RNA extraction and to perform all RT-qPCR experiments. It would be desirable to work with a set of reference genes comparable to the data we had been generating with the cell lines, but this procedure is, due to limited availability of the material, not always applicable. To achieve reliable results under this premise, the most stable reference, identified as described above by applying the geNorm SW [17], was measured. If enough RNA is available, we recommend, to measure 3–4 reference genes in parallel.

5. Conclusions

Dedicated function tested 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 FFPET research samples. Workflow protocols for the different sample material types have been established offering high convenience and high throughput. FFPET samples of various tumor entities subjected to optimized RNA isolation and evaluated by optimized reverse transcription and RT-qPCR assays yielded reliable results for relative expression analysis. Thus, function-tested RT-qPCR assays can be applied in biomarker research accompanying drug development up to early clinical development. Of course the clinical utility of the biomarker candidates identified pre-clinically need to be ex-

plored in the clinical setting. The presented workflow is able to improve the identification of new biomarkers and will aid to enhance PHC which promises redesigned therapeutic approaches that may optimize treatment according to a patients need.

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