Research Overview

Quantitative Real Time Polymerase Chain Reaction in Drug Development

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ABSTRACT> Measurements of the number of copies of DNA or mRNA with the quantitative polymerase chain reaction (qPCR) have transformed the drug development process. This transformation is driven by the information these measurements have contributed for a better understanding of the molecular definition of disease and of the mechanisms of efficacy and toxicity for new drugs. As this information is translated into accurate genomic biomarkers of efficacy and toxicity, drug development processes supported by these measurements are becoming more efficient. This transformation is exemplified in the conversion of P450 enzyme activity measurements to gene expression in drug metabolism studies, the measurement of cytokine and chemokine genomic expression levels as clinical markers, and the identification and evaluation of genomic biomarkers of nephrotoxicity. A good understanding of factors affecting qPCR measurements can simplify their implementation, as will high-throughput platforms for these assays. Drug Dev. Res. 62:151–158, 2004. © 2004 Wiley-Liss, Inc.

Key words: qPCR; qRT-PCR; TaqMan; SYBR; drug development

INTRODUCTION

The polymerase chain reaction (PCR) has been in use for almost two decades [Mullis et al., 1986]. This discovery enabled detection of a few copies of DNA or of cDNA through a reverse-transcriptase reaction for the measurement of gene expression levels. Thermal cyclers, reagents, and primer design software were quickly developed [Carothers et al., 1989] supporting the spread of this technology in academic, industrial, and government laboratories. The initial application of PCR focused on a qualitative use linked to electrophoretic product identification.

Several approaches were developed before quantitative, real time PCR [Henco and Heibey, 1990; Landgraf et al., 1991] in order to convert the qualitative information in a PCR gel into quantitative results. While PCR products can be accurately quantified in gels, there is a fundamental limitation that prevents an accurate quantification of PCR products from reflecting accurately the original quantity of DNA or cDNA in a sample. Endpoint measurements of PCR products after 40 amplification cycles have a weak correlation with the original number of copies of template in the sample and this correlation is only attainable over a limited dynamic range [Powell and Kroon, 1992; Ferre, 1992]. Quantitative, real time PCR (qPCR) [Heid et al., 1996; Wittwer et al., 1997] was developed over the last decade as a tool to accurately measure template copy number. The theory behind this method is straightforward and only assumes that PCR efficiency is constant and close to 100% throughout the amplification cycles used [Livak and Schmittgen, 2001]. The number of copies of template in a sample is determined through the amplification cycle (Ct) at which the amplification product can be initially detected. Detection chemistries such as TaqMan [Livak et al., 1995] and SYBR Green

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and specificity for hybridization to individual sequences, while universal protocols for qPCR are developed around a level of PCR efficiency and the requisite binding of fluorescent probes such as TaqMan that minimize detection of non-specific amplification products. This is the fundamental difference between these two genomic technologies and a key determinant for the eventual adoption of qPCR in a regulatory context for diagnostic and pharmaceutical applications.

Differences in hybridization platforms can lead to significant differences in hybridization results. In a recent study [Goodsaid et al., 2004] reported as part of the collaborative work of the International Life Science Institute Health and Environmental Science Institute (ILSI/HESI) Genomics Committee, quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was used to investigate discrepant results between an NIEHS cDNA and Affymetrix oligo rat platforms used to evaluate hepatic gene expression changes in rats exposed to methapyrilene. Caldesmon cDNA platform hybridization results showed decreases in gene expression results compared with their controls. By contrast, the Affymetrix oligonucleotide platform showed increases in expression levels for these samples. In the case of caldesmon, there was a 74-base sequence in the cDNA clone showing a >10-fold suppression relative to the day 7 high-dose methapyrilene-pooled control. These data demonstrated the importance of qRT-PCR to confirm key hybridization results as well as to understand sources of discrepancies resulting from different hybridization platforms.

**TRANSFORMATION OF DRUG METABOLISM MEASUREMENTS BY QRT-PCR**

How good is the correlation between mRNA and expressed protein levels? Mechanistic studies for drug efficacy and safety ultimately require results that can be extrapolated to the function of proteins and enzymes in this mechanism. Cytochrome P450 (CYP) gene expression changes have been measured previously using cultured cells [Bowen et al., 2000; Burczynski et al., 2001]. Bowen et al. [2000] showed that quantitative gene expression for CYP 1A1 and 3A1 induction could be determined for human hepatocytes in culture, but no correlation with functional testing was included [Burczynski et al., 2001]. Burczynski et al. [2001] found a good correlation between quantitative gene expression for the measurement of CYP induction in rat hepatocytes with LC/MS/MS-based CYP enzyme assays. Results published previously [Pan et al., 2000] using CD1 mice have shown that quantitative gene expression measurements for CYP enzyme induction in liver tissues are possible for CYP3A11, CYP2B10,
CYP2D9, CYP2E1, and CYP1A2. This study, however, did not comprehensively characterize biochemical changes in these P450 enzymes.

A recent study [Goodsaid, 2003] compared temporal biochemical and gene transcription changes in rat liver cytochrome P450. A conventional approach to assess cytochrome P450 (CYP) induction in preclinical animal models involves daily dosing for at least a week followed by Western blot and/or enzyme activity analysis. This study simultaneously assessed gene expression by qRT-PCR, along with Western blots and enzyme activity assays as a time course in an in vivo model. Rats were dosed daily for 8 days with model inducers of CYP1A, CYP2B, CYP3A, or CYP4A. Liver P450 levels were measured after 0.5, 1, 2, 4, and 8 days of dosing by qRT-PCR, Western blot and enzyme activity. The results of the study showed that CYP1A, CYP3A, and CYP4A genes were maximally induced very rapidly (0.5–1 day), whereas the CYP2B gene was maximally induced after a lag time of 4 days. In all cases, fold changes in induction detected by qRT-PCR were greater than fold changes in protein levels and enzyme activities. Maximal persistent and larger fold changes observed by qRT-PCR either preceded or occurred simultaneously with maximal sustained fold changes in protein levels as measured by Western blots and enzyme activity assays. These data showed that qRT-PCR provides increased sensitivity and specificity over conventional assays and may be key information for reliable assessment of drug-related changes in CYP induction during the transition from discovery to toxicology studies.

Mapping expression levels of P450 enzyme and P-glycoprotein genes in different rat tissues has also been reported with qRT-PCR [Lindell et al., 2003]. Expression of CYP2B1, CYP2C6, CYP2C11, CYP2D1, and Pgp was found to be similar in the liver and in the small intestine. Higher expression levels were detected for CYP1A2, CYP2A3, CYP2E1, and CYP3A1 in the liver and for CYP1A1 in the small intestine. This study underscored the need to study further the contribution of the small intestine to the bioavailability of drugs metabolized by P450 enzymes expressed in this rat tissue.

**GENOMIC BIOMARKERS OF EFFICACY**

One of the most important drug development applications of qPCR has been in the measurement of biomarkers of drug efficacy. Measurements of gene expression levels for chemokines and chemokine receptors are good models both for the advantages of measuring mRNA in conjunction with protein product levels as well as for the conversion of genomic biomarkers identified in hybridization experiments into qRT-PCR assays. qRT-PCR assays for chemokines and chemokine receptors have redefined standards for sensitivity and specificity in the detection of these analytes. These assays have also broadened the biological matrices and context in which assays for these analytes may be performed.

An earlier applications of qRT-PCR in this area was an investigation of IL-18 receptors and their ligands [Debets et al., 2000]. qRT-PCR was used in this work to show that Th1 but not Th2 cells are unique in that they coexpress IL-1R-related protein and IL-1R accessory protein, which is required for high-affinity binding to IL-18. Anti-IL-1R accessory protein antibodies were also shown in this work to be strong antagonists for IL-18. qRT-PCR was also applied in an investigation of the expression of chemokines and chemokine receptors in two Th1-mediated murine models of inflammatory bowel disease [Scheerens et al., 2001]. Increases in gene expression levels were measured for a set of these genes in chronically inflamed colons of IL-10−/− mice when compared to wild type mice. Reversal of colitis in IL-10−/− mice by anti-IL-12 monoclonal antibody was followed by inhibition of some, but not all of these genes. These genes were identified as LIX, lymphotactin, MCP-1, MIG, MIP-3alpha, and MIP-beta.

In addition to tests for previously identified chemokine and chemokine receptor biomarkers of efficacy, these applications have also accelerated the characterization of new cytokines. Cytokine IL-27 is a member of the IL6-IL12 family of cytokines, and induces proliferation of naïve CD4(+)+ T cells and the generation of a Th1-type adaptive immune response. It binds to cytokine receptor WSX-1, and qRT-PCR demonstrated [Pflanz et al., 2004] the induction of a subset of inflammatory cytokines in primary human mast cells and monocytes in response to IL-27 stimulation.

qRT-PCR tests for cytokine gene expression have been used to study the differential regulation of IL-4 expression and degranulation by anti-allergic compounds in rat basophilic leukemia (RBL-2H3) cells in an in vivo mouse model [Matsubara et al., 2004]. Olopatadine is an anti-allergic drug that functions as a histamine H(1) antagonist and inhibits both mast cell degranulation and the release of arachidonic acid metabolites in various types of cells. This study measured the inhibition of IL-4 expression by olopatadine and other anti-allergic drugs. It concluded that suppression of degranulation and arachidonic acid release by a compound correlated well with the inhibition of Ca\(^{2+}\) influx through receptor-operated channels but not with their reduction of IL-4 expression, suggesting that additional
mechanisms determine the levels of cytokine expression in mast cells.

Another common application of qRT-PCR is the accurate measurement of the time dependence of gene expression for a subset of genes previously identified from hybridization experiments. An example of this application is in the determination of the kinetics for expression of genomic biomarkers of beta-adrenergic induced cardiomyopathy in mice [Gaussin et al., 2003]. Beta-adrenergic receptor blockade therapy is used in the treatment of heart failure. In mice overexpressing beta1A or beta2A receptors, or protein kinase A, increased expression levels were measured at the onset of cardiomyopathy for uncoupling protein 2 (UCP2, a protein involved in mitochondrial membrane potential) and four-and-a-half LIM domain protein-1 (FHL1, a member of the LIM protein family). Treatment blocking beta adrenergic receptors reversed the cardiomyopathy and suppressed the increased expression of these genes.

An example of the direct application of qRT-PCR for the validation of biomarkers of efficacy has been published for the validation of P-selectin as a biomarker of atherogenesis [Molemaar et al., 2003]. This validation as a candidate target for the development of anti-atherogenic therapies required expression measurements in the aortic arch and other tissues of apoE-deficient (apoE−/−) mice by qRT-PCR. Results of these measurements showed that p-selectin expression increased with age up to 14-fold higher in apoE−/− than in control mice, correlating with the development of lesions in apoE−/− mice.

Applications of qRT-PCR in the investigation of genomic biomarkers for the development of anti-cancer drugs have focused on several models for efficacy. Taxol antiangiogenic effects in a human epithelial ovarian carcinoma cell line were studied [Hata et al., 2004] by measuring the effect of Taxol on expression levels for angiopoietin-1, a major ligand for the endothelium-specific tyrosinase kinase receptor Tie-2 and an important regulator of endothelial cell survival. This study showed that expression levels for angiopoietin-1 were significantly decreased by exposure to 2 nM Taxol for 168 h. The conclusion of this study was that exposure of ovarian cancer cells to a low concentration of Taxol may inhibit vascular regression, which is considered the trigger for angiogenesis. Angiogenesis was also studied with qRT-PCR in human bone marrow [Kumar et al., 2004]. This was a study of the angiogenic potential of bone marrow plasma as a function of expression of VEGF, bFGF, and their receptors in plasma cells from patients with different clinical profiles for myeloma. The study found no significant difference in expression levels for these genes in plasma cells from different patients. It concluded that angiogenic potential correlated with tumor burden rather than increased expression of VEGF/bFGF.

**GENOMIC BIOMARKERS OF TOXICITY**

Broader benefits associated with novel biomarker testing are possible if these have been evaluated across multiple species of interest in pre-clinical drug safety evaluation. Davis et al. [2004] recently showed that correlations with rat kidney toxicity of gene expression changes reported by the ILSI Genomics Committee [Amin et al., 2004] are also observed by qRT-PCR in Cynomolgus monkeys. This work further showed that expression changes in some of these genes, particularly the Kidney Injury Molecule (KIM-1) [Han et al., 2002] and clusterin [Correa-Roter et al., 1998], are highly predictive of the development of tubular necrosis. Early detection of toxicity in kidney tissues from the measurement of changes in transcript levels of KIM-1 may also be possible in urine from the measurement of changes in protein product levels of KIM-1 [Han et al., 2002].

The study by Davis et al. [2004] emphasizes the need to identify safety biomarkers at doses and protocol lengths that match applications for biomarkers. It anchors changes reported in levels of genomic biomarkers on histological data and supports claims showing that novel biomarkers are either more sensitive or specific than currently accepted markers. It also provides validation across species for the proposed biomarkers. Gene expression measurements across species are possible with qRT-PCR because primers can be designed for sequences conserved across these species. In the report by Davis et al. [2004], the expression of several monkey genes was measured with primers designed for sequences conserved between rat and human genes. High-efficiency qRT-PCR requires a match between primer design for conserved regions from two known sequences and the actual sequence of a third, previously unknown, genomic sequence. The requirement for an approximation of 100% efficiency in qRT-PCR makes the sequence match an absolute requirement for this genomic platform and also facilitates its validation.

The slope of a graph showing the C_{T} value as a function of template dilution for the known and unknown sequences can be used to validate the choice of primer sequences. An ever-increasing knowledge of genomic sequences for species of interest in drug safety evaluation will reduce the need for this approach of primer design for qRT-PCR.

qRT-PCR has also been applied in the measurement of genomic biomarkers of gastrointestinal toxicity...
inhibitors can block cleavage of several transmembrane proteins including amyloid precursor protein and the cell fate regulator Notch-1. The inhibition of APP processing leads to blockage of the generation of amyloid beta peptides, which is why gamma secretase inhibitors are considered candidates for therapy of patients with Alzheimer’s disease. A mechanism-dependent effect of some gamma-secretase inhibitors is gastrointestinal toxicity through cell population changes in the ileum of rat models, and microarray analysis was used in this study to identify a number of genes whose expression levels correlate with the development of these endpoints. qRT-PCR was used for the accurate quantification of adipin and Hes-1, the most important biomarkers identified in this study. In vitro results from 3T3-L1 pre-adipocytes reported in this study demonstrated that gamma secretase inhibitors inhibit Hes-1 expression while up-regulating adipin expression and blocking pre-adipocyte differentiation.

Clinical Microbiology and Virology

Applications of qPCR

Accurate measurements of gene expression have found multiple clinical microbiology applications relevant to drug development. qPCR assays have been developed recently for the detection of Francisella tularensis [McAvin et al., 2004], specific genetic lineages of Anaplasma phagocytophilum in Ixodes ricinus ticks [Polin et al., 2004], periodontopathic bacteria Tannarella forsythensis and Fusobacterium spp. in periodontal pockets [Suzuki et al., 2004], Candida albicans in concentrated oral rinse cultures [White et al., 2004], Shigella [Thiem et al., 2004], human herpesvirus 8 and Epstein-Barr virus [Friedrichs et al., 2004], parvovirus mutant [Hokynar et al., 2004], and severe acute respiratory syndrome (SARS) coronavirus [Hourfar et al., 2004; Drosten et al., 2004; Hui et al., 2004].

Drug resistance gene expression has also been measured by qRT-PCR in Candida albicans [Frade et al., 2004]. The sensitivity and dynamic range of these assays was also compared in this work with that for the corresponding Northern hybridization assays. The correlation between qRT-PCR and Northern hybridization was excellent, except for detection of low levels of CDR2 expression by qRT-PCR but not by Northern hybridization and the linear response by qRT-PCR for a 200-fold induction of MDRI that was inaccurately quantified by Northern hybridization.

Tests for detection and Clarithromycin resistance of Helicobacter pylori were evaluated in gastric biopsy specimens by qPCR [Lascols et al., 2004]. The study found the sensitivity for detection of H. pylori by qPCR to be better (97.0% at a specificity of 94.6%) than those for detection by culture (90.9%) or histology (87.9%). qPCR was capable of quantification for as little as 300 (minimum detection of 30) and a dynamic range of $10^6$ H. pylori. qPCR also correctly sorted clarithromycin susceptible from resistant strains at a concordance of 98.2% with current methods.

High-Throughput Platforms for qRT-PCR

High-throughput screening applications in quantitative gene expression require both valid cell models as well as platforms with the sensitivity and dynamic range that will lead to accurate prioritization of candidate compounds. Most of the results reviewed here for in vitro studies have focused on biomarkers of efficacy. Efforts to assemble efficient high-throughput qRT-PCR measurement protocols have also focused on biomarkers of safety [Pinhasov et al., 2004] and drug metabolism [Perez et al., 2003]. At Johnson & Johnson Pharmaceutical Research [Pinhasov et al., 2004], robotic systems have been seamlessly merged for RNA isolation and purification, reverse-transcriptase reaction, and delivery of sample and reagents to a 384-well plate. This configuration is flexible, leading to either a maximum number of samples or a maximum number of genomic targets. Target identification/validation, structure-activity (SAR) studies, compound selection for efficacy studies, and biomarker identification have run in this high-throughput genomic platform.

Critical Issues in the Development of qRT-PCR Assays

The development of qRT-PCR assays is closely linked to a few straightforward rules that were validated early in the development of this technology [Livak and Schmittgen, 2001]. The “plug-and-play” character of qRT-PCR requires the following:

1. A Taq polymerase enzyme reaction at maximum efficiency. The chemical matrix in commercially available master mixes is optimized for maximum rate and stability of different Taq enzymes.
2. Primer and probe design with accurate $T_m$'s for the chemical matrices in commercially available master mixes. Primer and probe design software product such as Primer Express™ [Livak and Schmittgen, 2001] are matched with master mixes supplied by each vendor.
3. Maximum PCR efficiency throughout the range of $C_T$ over which expression results are needed. This is critical for relative quantification. Specific methods have been developed [Meijerink et al., 2001] to
compensate for the effect of sample matrix effects on these measurements.

4. **Choice of endogenous control gene for relative quantification.** The only reason why an endogenous control gene should be chosen is that its expression remains unchanged throughout a study and depends exclusively on the total amount of RNA in a sample. An excellent choice as an endogenous control gene is 18S RNA because its expression level is essentially constant. An endogenous control gene should not be chosen for the proximity of its expression to that of the target gene. This approach will prevent accurate multiplexing.

5. **Disengagement of the reverse transcriptase and Taq polymerase reactions.** The optimization of master mixes for these two reactions is only possible if they are considered independently of each other. Single-step qRT-PCR chemistries compromise the sensitivity and dynamic range of qRT-PCR. A valuable asset for any lab interested in quantitative gene expression measurements is the long-term storage of relatively stable reverse transcription cDNA products for individual test samples.

**THE LOGIC OF QPCR AND THE FUTURE OF GENE EXPRESSION MEASUREMENTS IN DRUG DEVELOPMENT**

Success for the high-throughput screening applications summarized here is closely linked to the unique logic of qPCR (Table 1). qPCR has succeeded in multiple applications because it was conceived from the very beginning as a "plug-and-play" method with a universal and generic biochemical matrix. Regardless of the commercial source of qPCR reagents, this basic physicochemical logic has been retained, so that any assay for any genomic target may be assembled with primer and probe design software for high-efficiency PCR reactions. Hybridization platforms, whether oligonucleotide- or cDNA-based, are often proprietary in menu, configuration, labeling, and hybridization chemistries, and in hybridization and analysis hardware and software. Hybridization platforms also focus on the density of information available from hundreds of simultaneous gene expression results but not necessarily on the sensitivity, specificity, and dynamic range of the method. A clear measure of these is essential for the long-term development of validated GLP protocols for gene expression measurements.

While format densities for qPCR do not yet exceed a range of hundreds of genes or samples, there is no limitation inherent in the platform that would prevent successful development of higher density plates or microfluidic devices. The ease with which validated GLP protocols may be developed with qPCR suggests that this platform will become a future standard for gene expression measurements in drug development.

**REFERENCES**

Amin RP, Vickers AE, Sistare F, Thompson KL, Roman RJ, Lawton M, Kramer J, Hamadeh HK, Collins J, Grisson S, Bennett L, Tucker CJ, Wild S, Kind C, Orefio V, Davis JW, Curtiss S, Naciff JM, Cunningham M, Tennant R, Stevens J, Car B, Bertram TA, Afshari CA. 2004. Identification of putative gene-based markers of renal toxicity. Environ Health Perspect 112:465–479.

Atkins SD, Clark IM. 2004. Fungal molecular diagnostics: a mini review. J Appl Genet 45:3–15.

Bell AS, Ranford-Cartwright LC. 2002. Real-time quantitative PCR in parasitology. Trends Parasitol 18:337–342.

Bowen WP, Carey JE, Miah A, McMurray HF, Munday PW, James RS, Coleman RA, Brown AM. 2000. Measurement of cytochrome CYP gene induction in human hepatocytes using quantitative real-time reverse-transcription-polymerase chain reaction. Drug Metab Disp 28:781–788.

Burczynski ME, McMillian M, Parker JB, Bryant S, Leone A, Grant E, Thorne J, Zivin Z, Zivin RA, Johnson MD. 2001. Cytochrome P450 induction in rat hepatocytes assessed by quantitative real-time reverse-transcription polymerase chain reaction and the RNA invasive cleavage assay. Drug Metab Disp 29:1243–1250.

Carothers AM, Urlaub, G, Mucha J, Grunberger D, Chasin LA. 1989. Point mutation analysis in a mammalian gene: rapid preparation of total RNA, PCR amplification of cDNA and Taq sequencing by a novel method. Biotechniques 7:494–499.
Correa-Roter R, Ibarra-Rubio ME, Schwochau G, Cruz C, Silkensen JB, Pedraza-Chaverri J, Chmielwski D, Rosenberg ME. 1998. Induction of clusterin in tubules of nephrotic rats. J Am Soc Nephrol 9:33–37.

Davis JW, Goodsaid FM, Brla C, Obert LA, Mandakas G, Garner CE, Collins ND, Smith RJ, Rosenblum IY. 2004. Real time PCR quantification of drug resistance gene expression in a nonhuman primate model of antibiotic-induced nephrotoxicity. Toxicol Appl Pharmacol epub June 2004. doi: 10.1016/j.taap.2004.02.001.

Debets R, Timans JC, Churakovska T, Zurasovs S, de Waal Malefyt R, Moore KW, Abrams JS, O’Garra A, Bazan JF, Kastelein RA. 2000. IL-18 receptors, their role in ligand binding and function: anti-IL-1RacPL antibody, a potent antagonist of IL-18. J Immunol 165:4950–4956.

De Mello AJ. 2001. DNA amplification: does “small” really mean “efficient”? Lab on a chip. Antiviral Research 1:24N–29N.

Drosten C, Chiu LL, Panning M, Leong HN, Preiser W, Tam JS, Davis JW, Goodsaid FM, Bral C, Obert LA, Mandakas G, Correa-Roter R, Ibarra-Rubio ME, Schwochau G, Cruz C, Heid CA, Stevens J, Livak KJ, Williams PM. 1996. Real time PCR: reality vs. myth. PCR Methods Appl 8:263–264.

Friedrichs C, Neyts J, Gaspar G, Clercq ED, Wutzler P. 2004. Evaluation of antiviral activity against human herpesvirus 8 (HHV-8) and Epstein-Barr virus (EBV) by a quantitative real-time PCR assay. Antiviral Res. 62:121–123.

Gaussian V, Tomlison JE, Depre C, Engellhardt S, Antos CL, Takagi G, Hein L, Topper JN, Liggett SB, Olson EN, Lohe M, Valter SF, Valter DE. 2003. Common genomic response in different murine models of beta-adrenergic-induced cardiomyopathy. Circulation 108:2926–2933.

Goodsaid FM. 2003. Genomic biomarkers of toxicity. Curr Opin Drug Discov Dev 6:41–49.

Goodsaid FM, Palamanda JR, Montgomery D, Mandakas G, Gu C, Li Z, You X, Norton L, Smith R, Chu I, Soares T, Alton K, Ishnani N, Rosenblum IY. 2003. Assessment of Temporal Biochemical and Gene Transcription Changes in Rat Liver Cytochrome P450. Utility of Real-Time Quantitative RT-PCR. Pharm Res 20:1373–1380.

Goodsaid FM, Smith R, Rosenblum IY. 2004. Quantitative PCR deconstruction of discrepancies between results reported by different hybridization platforms. Environ Health Perspect 112:456–459.

Hanco K, Heibey M. 1990. Quantitative PCR: the determination of template copy numbers by temperature gradient gel electrophoresis. Nucleic Acids Res. 18:6733–6734.

Hokynar K, Norja P, Laitinen H, Palomaki P, Garbarg-Chenou A, Hedman K, Soderlund-Venermo M. 2004. Detection and differentiation of human parvovirus variants by commercial quantitative real-time PCR tests. J Clin Microbiol 42:2013–2019.

Hourfar MK, Roth WK, Seifried E, Schmidt M. 2004. Comparison of two real-time quantitative assays for detection of severe acute respiratory syndrome coronavirus. J Clin Microbiol. 42:2094–2100.

Hui RK, Zeng F, Chan CM, Yuen KY, Peiris JS, Leung FC. 2004. Reverse transcriptase PCR diagnostic assay for the coronavirus associated with severe acute respiratory syndrome. J Clin Microbiol 42:1994–1999.

Jaeger U, Kainz B. 2003. Monitoring minimal residual disease in AML: the right time for real time. Ann Hematol 82:139–147.

Josefsson A, Livak K, Gyllensten U. 1999. Detection of human papillomavirus by using the fluorescent 5’ exonuclease assay. J Clin Microbiol 37:490–496.

Kumar S, Witzig TE, Timm M, Hang J, Wellik L, Kimlinger TK, Greipp PR, Rajkumar SV. 2004. Bone marrow angiogenic activity and expression of angiogenic cytokines in myeloma: evidence favoring loss of marrow angiogenesis inhibitory activity with disease progression. Blood 104:1159–1165.

Landelgraf A, Reckmann B, Pingoud A. 1991. Quantitative analysis of polymerase chain reaction (PCR) products using primers labeled with biotin and fluorescent dye. Anal Biochem 193:231–235.

Lascals C, Lamarque D, Costa JM, Copie-Bergman C, Le Glannec JM, Deforges L, Sousy CJ, Petit JC, Delchier JC, Tankovic J. 2003. Fast and accurate quantitative detection of Helicobacter pylori and identification of clarithromycin resistance mutations in H. pylori isolates from gastric biopsy specimens by real-time PCR. J Clin Microbiol 41:4573–4577.

Lee LG, Livak KJ, Mullah B, Graham BJ, Vinayak RS, Woudegeren TM. 1999. Seven-color, homogeneous detection of six PCR products. Biotechniques 27:342–349.

Lindell M, Lang M, Lemmerness H. 2003. Expression of genes encoding for drug metabolizing cytochrome P450 enzymes and P-glycoprotein in the rat small intestine; comparison to the liver. Eur J Drug Metab Pharmacokinet 28:41–48.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25:402–408.

Livak KJ, Flood SJ, Marmaro J, Giusti W, Deetz K. 1995. Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. PCR Methods Appl 4:357–362.

Maremucci G, Livak KJ, Bi W, Stroup MP, Bloomfield CD, Caligiuri MA. 1998. Detection of minimal residual disease in patients with AML/ETO-associated myeloid leukemia using a novel quantitative reverse transcription polymerase chain reaction assay. Leukemia 12:1482–1489.

Matsuhara M, Masaki S, Ohnori K, Karasawa A, Hasegawa K. 2004. Differential regulation of IL-4 expression and degranulation by anti allergic olopatadine in rat basophilic leukemia (RBL-2H3) cells. Biochem Pharmacol 67:1315–1326.
Matsuki T, Watanabe K, Tanaka R. 2003. Genus- and species-specific PCR primers for detection and identification of bifidobacteria. Curr Issues Intest Microbiol 4:61–69.

McAvin JC, Morton MM, Roudabush RM, Atchley DH, Hickman JR. 2004. Identification of Francisella tularensis using real-time fluorescence polymerase chain reaction. Mil Med 169:330–333.

Meierink J, Mandigers C, van de Locht L, Tonissen E, Goedsaid E, Raemaekers J. 2001. A novel method to compensate for different amplification efficiencies between patient DNA samples in quantitative real-time PCR. J Mol Diagn 3:55–61.

Molenaar TJ, Twisk J, de Haas SA, Petersen N, Vogelaar BJ, van Leenwen SH, Michon IN, van Berkel TJ, Kuiper J, Biessen EA. 2003. P-selectin as a candidate target in atherosclerosis. Biochem Pharmacol 66:859–866.

Morrison TB, Weis JJ, Wittwer CT. 1998. Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification. Biotechniques 24:954–962.

Mullis K, Faloona F, Scharf S, Saiki R, Erlich H. 1986. Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. Cold Spring Harb Symp Quant Biol 51:263–273.

Niesters HG. 2002. Clinical virology in real time. J Clin Virol 3:S3–12.

Pan J, Xiang Q, Ball S. 2000. Use of a novel real-time quantitative reverse transcription-polymerase chain reaction method to study the effects of cytokines on cytochrome CYP mRNA expression in mouse liver. Drug Metab Disp 28:709–713.

Peretz G, Tabares B, Jover R, Gomez-lechon MJ, Castell JV. 2003. Semi-automatic quantitative RT-PCR to measure CYP induction by drugs in human hepatocytes. Toxicol In Vitro 17:643–649.

Pflanz S, Hibbert L, Mattson J, Rosales R, Vaisberg E, Bazan JF, Phillips JH, McClanahan TK, de Waal Malefyt R, Kastelein RA. 2004. WSX-1 and glycoprotein 130 constitute a signal-transducing receptor for IL-27. J Immunol 172:2225–2231.

Polin H, Hufnagl P, Hanschmidt R, Gruber F, Ladurner G. 2004. Molecular evidence of anaplasma phagocytophilum in Ixodes ricinus ticks and wild animals in Austria. J Clin Microbiol 42:2285–2286.

Powell EE, Kroon PA. 1992. Measurement of mRNA by quantitative PCR with a nonradioactive label. J Lipid Res 33:609–614.

Rajeevan MS, Vernon SD, Taysavang N, Unger ER. 2001. Validation of array-based gene expression profiles by real-time (kinetic) RT-PCR. J Mol Diagn 3:26–31.

Scheerens H, Hessel E, de Waal Malefyt R, Leach MW, Rennick D. 2001. Characterization of chemokines and chemokine receptors in two murine models of inflammatory bowel disease: IL-10−/− mice and Rag-2−/− mice reconstituted with CD4+ CD45Rbhigh T cells. Eur J Immunol 31:1465–1474.

Searfoss GH, Jordan WH, Calligaro DO, Galbreath EJ, Schirtzinger LM, Berridge BR, Gao H, Higgins MA, May PR, Ryan TP. 2003. Adipsin, a biomarker of gastrointestinal toxicity mediated by a functional gamma-secretase inhibitor. J Biol Chem 278: 46107–46116.

Slack JL, Bi W, Livak KJ, Baubier N, Yu M, Clark M, Kin SH, Gallagher RE, Willman CL. 2001. Pre-clinical validation of a novel, highly sensitive assay to detect PML-RARalpha mRNA using real-time reverse-transcription polymerase chain reaction. J Mol Diagn 3:141–149.

Suzuki N, Yoshida A, Saito T, Kawada M, Nakano Y. 2004. Quantitative microbiological study of subgingival plaque by real-time PCR shows correlation between levels of Tannarella forsythensis and Fusobacterium spp. J Clin Microbiol 42:2255–2257.

Thiem VD, Sethabutr O, Von Seidlein L, Van Tung T, Canh DG, Chien BT, Tho LH, Lee H, Hoang HS, Hale TL, Clemens JD, Mason C, Trach DD. 2004. Detection of Shigella by a PCR assay targeting the ipaH gene suggests increased prevalence of Shigellosis in Nha Trang, Vietnam. J Clin Microbiol 42:2031–2035.

Walker NJ. 2001. Real-time and quantitative PCR: applications to mechanism-based toxicology. J Biochem Mol Toxicol 15:121–127.

White PL, Williams DW, Kuriyama T, Samad SA, Lewis MA, Barnes RA. 2004. Detection of Candida in concentrated oral rinse cultures by real-time PCR. J Clin Microbiol 42: 2101–2107.

Winer J, Jung CK, Shakel I, Williams PM. 1999. Development and validation of real-time quantitative reverse transcriptase-polymerase chain reaction for monitoring gene expression in cardiac myocytes in vitro. Anal Biochem 270:41–49.

Wittwer CT, Hermann MG, Moss AA, Rasmussen RP. 1997. Continuous fluorescence monitoring of rapid cycle DNA amplification. Biotechniques 22:130–138.