Real-time quantitative PCR (rt-qPCR) is the method of choice for accurate, sensitive, and specific quantitation of nucleic acid sequences. Applications of this technology are numerous, both in molecular diagnostics and in virtually all fields of life sciences, including gene expression profiling, measurement of DNA copy number alterations, genotyping, mutation detection, pathogen detection, measurement of viral load, disease monitoring, and assessment of drug response. Several ingredients are essential to the successful and reliable completion of an rt-qPCR assay, such as careful primer design and evaluation, template preparation, the use of a robust normalization strategy, and accurate data analysis. This article describes how rt-qPCR can be implemented as a tool to monitor silencing efficiency and functional effects of RNA interference (RNAi)-mediated gene knockdown, using examples from our research on neuroblastoma. For detailed information on the experiments that contributed to this research, including instruments, reagents, and procedures, request bulletin 5692.
Neuroblastoma and the MYCN and TP53 Cancer Genes

Neuroblastoma is a childhood cancer derived from precursor cells of the adrenosympathetic system, arising in the adrenal medulla or in sympathetic ganglia. Although a relatively rare form of cancer, neuroblastoma is among the most fatal of childhood diseases. Indicators of mortality include age at diagnosis (the outcome for children with neuroblastoma is most favorable when diagnosed before the age of one year, even when the disease has metastasized), tumor stage, and level of MYCN protein activity (the most fatal clinicogenetic subtype of neuroblastoma is characterized by amplification of the MYCN oncogenic transcription factor) (Vandesompele et al. 2005). The mechanisms by which this transcription factor exerts its oncogenic activity and confers an unfavorable prognosis are poorly understood.

Another intriguing feature of neuroblastoma is the remarkably low frequency of TP53 mutations at diagnosis (Tweedle et al. 2003). Previous studies have shown that reactivation of the p53 pathway by the selective small-molecule MDM2 antagonist nutlin-3 constitutes a promising novel therapeutic approach for neuroblastoma (Van Maerken et al. 2006). To gain insight into the mechanism of action of these two pivotal genes in neuroblastoma pathogenesis and to create model systems for future exploration of targeted therapeutics in relationship to MYCN and TP53 status, RNAi was used as an experimental tool for suppressing the expression of these genes. Because neuroblastomas are notoriously difficult to transfect, we introduced an siRNA model with accurate detection of silencing efficiency and the resulting effects. In particular, for study of MYCN function, this model is believed to be more relevant, because traditional systems with forced overexpression of this gene in single-copy cells seem to lack the proper cellular context to mimic endogeneous amplification and hyperactivity. Our final goal is to disentangle MYCN’s transcriptional web, in order to interfere with its oncogenic signaling pathways, while leaving the beneficial pathways unaltered.

From Experimental Design to Analysis of an rt-qPCR Assay

Purity and integrity of the template are critical factors to the success of an rt-qPCR assay. Several commercial kits are available for producing clean RNA samples. Contaminants should be avoided or removed, as they can greatly influence the reverse-transcription step or the actual PCR. The presence of PCR inhibitors can be determined by a variety of methods, including the simple and fast PCR-based SPUD assay (Nolan et al. 2006). An oligonucleotide target sequence with no homology to human DNA is spiked into human RNA samples and a water control at a known concentration. rt-qPCR quantitation of the oligonucleotide template in both the RNA samples and the (negative) water control is indicative of possible enzymatic inhibitors present in the RNA extract. For assessment of RNA integrity, electrophoresis and PCR-based methods are available (Fleige and Pfaffl 2006, Nolan et al. 2006). Figure 1 shows an electropherogram of high-quality RNA assessed using the Experion™ automated electrophoresis system. Sharp peaks at 18S and 28S and no nonspecific peaks are desired results when determining whether or not RNA samples are intact.
To control for inevitable experimental variation due to factors such as the amount and quality of starting material, enzymatic efficiencies, and overall cellular transcriptional activity, use of a reliable normalization strategy in which these factors are taken into account is necessary. In principle, internal reference genes offer the best way to deal with the multiple sources of variables that might exist between different samples. A truly accurate normalization can only be achieved when multiple reference genes are utilized, as use of a single reference gene results in relatively large errors in a considerable proportion of the sample set (Vandesompele et al. 2002). Care should be taken when selecting the genes to be used for normalizing the expression levels since no universal set of always-applicable reference genes exists. Different sample origins and experimental manipulations might require another set of genes to be used as reference genes. The selection and validation of reference genes can be done using the geNorm algorithm (see sidebar), which determines the most stable genes from a set of tested candidate reference genes in a given sample panel and calculates a normalization factor (Vandesompele et al. 2002).

Bioinformatics-based quality assessment of newly designed rt-qPCR primers can considerably improve the likelihood of obtaining specific and efficient primers. A number of quality control parameters have been integrated in Ghent University’s RTPrimerDB in silico assay evaluation pipeline (Pattyn et al. 2006). This pipeline allows a streamlined evaluation of candidate primer pairs, with automated BLAST specificity search, prediction of putative secondary structures of the amplicon, indication of which transcript variants of the gene of interest will be amplified, and search for known SNPs in the primer annealing regions. This in silico evaluation, however, does not preclude the need for experimental validation after synthesis of the primers. Ideally, experimental evaluation addresses specificity, efficiency, and dynamic range of the assay using a broad dilution series of template (Figure 2).

Processing and analysis of the raw rt-qPCR data represent a multistep computational process of averaging replicate Ct values, normalization, and proper error propagation along the entire calculation track. This process might prove cumbersome and deserves equal attention as the previous steps in order to get accurate and reliable results. This final procedure has been automated and streamlined in Biogazelle’s qBasePlus software (www.biogazelle.com, see sidebar), a dedicated program for the management and analysis of rt-qPCR data (Hellemans et al. 2007).

**Fig. 2.** Experimental validation of newly designed rt-qPCR primers. A, PCR efficiency and dynamic range of the rt-qPCR assay was tested using a 4-fold serial dilution of six points of reverse transcribed human qPCR reference total RNA (64 ng down to 62.5 pg) and TPS3_P2 primers; B, specificity of the TPS3_P2 primers was assessed by generating a melting curve of the PCR product; C, standard curve and PCR efficiency estimation (including the error) according to the qBasePlus software. Cq, quantitation cycle value generated in RDML software (see sidebar).
rt-qPCR for Assessment of siRNA Silencing Efficiency: Anti-MYCNC siLentMer™ siRNA Duplexes

Human IMR-32 neuroblastoma cells were transfected with different anti-MYCNC siLentMer™ siRNA duplexes or a nonspecific control siRNA, and the MYCN transcript level was determined 48 hours posttransfection by rt-qPCR. Our results indicate the importance of primer location for evaluation of siRNA silencing efficiency, in agreement with a previous independent report (Shepard et al. 2005). The target mRNA sequence is cleaved by the RNA-induced silencing complex (RISC) near the center of the region complementary to the guiding siRNA (Elbashir et al. 2001). Complete nucleolytic degradation of the resulting fragments is not always guaranteed, which might result in underestimation of siRNA silencing efficiency if primers are used that do not span the siRNA target sequence, as observed for this gene (Figure 3).

In 2004, Jan Hellemans, a PhD student in the University’s Center for Medical Genetics laboratory, began automating the arduous mathematical computations associated with qPCR analysis by programming a few simple macros in Excel. These initial macros evolved into the qBase 1.0 qPCR data analysis software package (http://medgen.ugent.be/qbase/). Since then, several thousand copies have been downloaded and used worldwide. In 2007, the Excel version began being phased out by qBasePlus, a professional Java-based application that runs 20 times faster and is more intuitive than the original platform. All current versions of these programs are available at no charge, and even this latest tool developed by Biogazelle, a Ghent University spin-off company, will offer both free and reasonably priced licensing packages.

That these programs have revolutionized the synthesis of real-time PCR data is unquestionable. What is surprising, at least to Vandesompele, is that “what were once just tools to measure gene expression levels in scarce tumor biopsies from children with neuroblastoma in our laboratory, have now grown in scope to form an independent research line.” And while researchers in this lab continue to try to find new ways to combat neuroblastoma, so will they continue to discover tools to aid achievement of reliable and meaningful results through bioinformatics. Future plans include establishment of an international consortium to finalize a standard exchange format for real-time PCR data (coined RDML, previews of this effort can be seen at www.rdmli.org). In addition, they are developing a web-based primer design portal that will enable researchers to design high-quality assays in a high-throughput environment.
rt-qPCR for Monitoring of shRNA Silencing Efficiency and Functional Effects: Lentiviral-Mediated shRNA Knockdown of TP53

For generation of stable TP53 knockdown variants of neuroblastoma cell lines with wild-type p53, we infected IMR-32 and NGP cells with a lentiviral vector encoding an shRNA directed specifically against human TP53 (LV-h-p53) or against the murine Tp53 gene (LV-m-p53) as a control. Efficiency of TP53 gene silencing was evaluated by rt-qPCR using two different primer pairs (TP53_P1 and TP53_P2). Bars indicate mRNA expression levels of TP53 relative to the respective LV-m-p53 cells; error bars depict standard error of the mean (duplicated PCR reactions for TP53 and three reference genes).

Fig. 4. Assessment of shRNA-mediated TP53 knockdown efficiency by rt-qPCR. IMR-32 and NGP cells were infected with a lentivirus carrying an shRNA construct specific for either the human TP53 gene (LV-h-p53) or the murine Tp53 gene (LV-m-p53) as a control. Efficiency of TP53 gene silencing was evaluated by rt-qPCR using two different primer pairs (TP53_P1 and TP53_P2). Bars indicate mRNA expression levels of TP53 relative to the respective LV-m-p53 cells; error bars depict standard error of the mean (duplicated PCR reactions for TP53 and three reference genes).

Use of a high-performance real-time qPCR system is important to accurately measure the effectiveness of your siRNA knockdown. The CFX96™ real-time PCR detection system (used in the experiments discussed in this article) builds on the power and flexibility of the C1000™ thermal cycler, adding an easy-to-install interchangeable reaction module to create an exceptional real-time PCR system. The system’s thermal performance combined with an innovative optical design ensure accurate, reliable data. The powerful yet intuitive software accelerates every step of your real-time PCR research, shortening the time between getting started and getting great results.

The CFX96 system’s solid-state optical technology (six filtered LEDs and six filtered photodiodes) provides sensitive detection for precise quantitation and target discrimination. Scanning just above the sample plate, the optics shuttle individually illuminates and reads fluorescence from each well with high sensitivity and no crosstalk. The optical system always collects data from all wells during data acquisition, so you can enter or edit well information on your own schedule.

With the CFX96 system, you can:

- Be up and running fast — quick installation and factory-calibrated optics let you set up the system in seconds
- Perform more experiments — fast thermal cycling produces results in <30 minutes
- Save research time — thermal gradient feature lets you optimize reactions in a single experiment
- Minimize sample and reagent usage — reliable results are obtained with sample volumes as low as 10 µl
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Legend:

- TP53_P1 primers
- TP53_P2 primers

IMR-32 Cells

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Fig. 5. Functional validation of shRNA-mediated TP53 knockdown through rt-qPCR analysis of transcript levels of p53-regulated genes after nutlin-3 treatment. IMR-32 and NGP cells were infected with a lentiviral vector encoding an shRNA directed specifically against either the human TP53 gene (LV-h-p53) or the murine Trp53 gene (LV-m-p53). Cells were treated with 0, 8, or 16 µM nutlin-3 for 24 hr, and expression of BBC3 (PUMA) (A), and MDM2 (B), p53-regulated genes, and TP53 was determined by rt-qPCR. Two different primer pairs (TP53_P1 and TP53_P2) were used for quantitation of TP53 transcript levels (C, D). Bars indicate mRNA expression levels relative to the respective vehicle-treated (0 µM nutlin-3) LV-m-p53 infected cells, mean of two different rt-qPCR measurements; error bars show standard error of the mean.

Fig. 6. Functional validation of shRNA-mediated TP53 knockdown through cell viability analysis after treatment of IMR-32 and NGP cells with nutlin-3. Effect of nutlin-3 on viability of uninfected cells (A, D), LV-h-p53 infected cells (B, E), and LV-m-p53 infected cells (C, F). Exponentially growing cells were exposed to 0–32 µM of nutlin-3 for 24 (—), 48 (—), and 72 (—) hr, and the percentage cell viability with respect to vehicle-treated cells was determined. Error bars indicate standard deviation of mean cell viability values of three independent experiments.
**Conclusions**

rt-qPCR analysis provides a convenient and reliable method for evaluation of knockdown efficiency and functional consequences of RNAi-mediated gene silencing. Successful application of this monitoring tool requires careful attention to be given to all different steps in the rt-qPCR workflow, including primer design and evaluation, template preparation, normalization strategy, and data analysis, as discussed in this article.

Similar studies will be conducted in the future to evaluate results achieved using additional cell lines and varying combinations of multiple siLentMer duplexes, durations of effect, and concentrations of active siLentMer duplexes.

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