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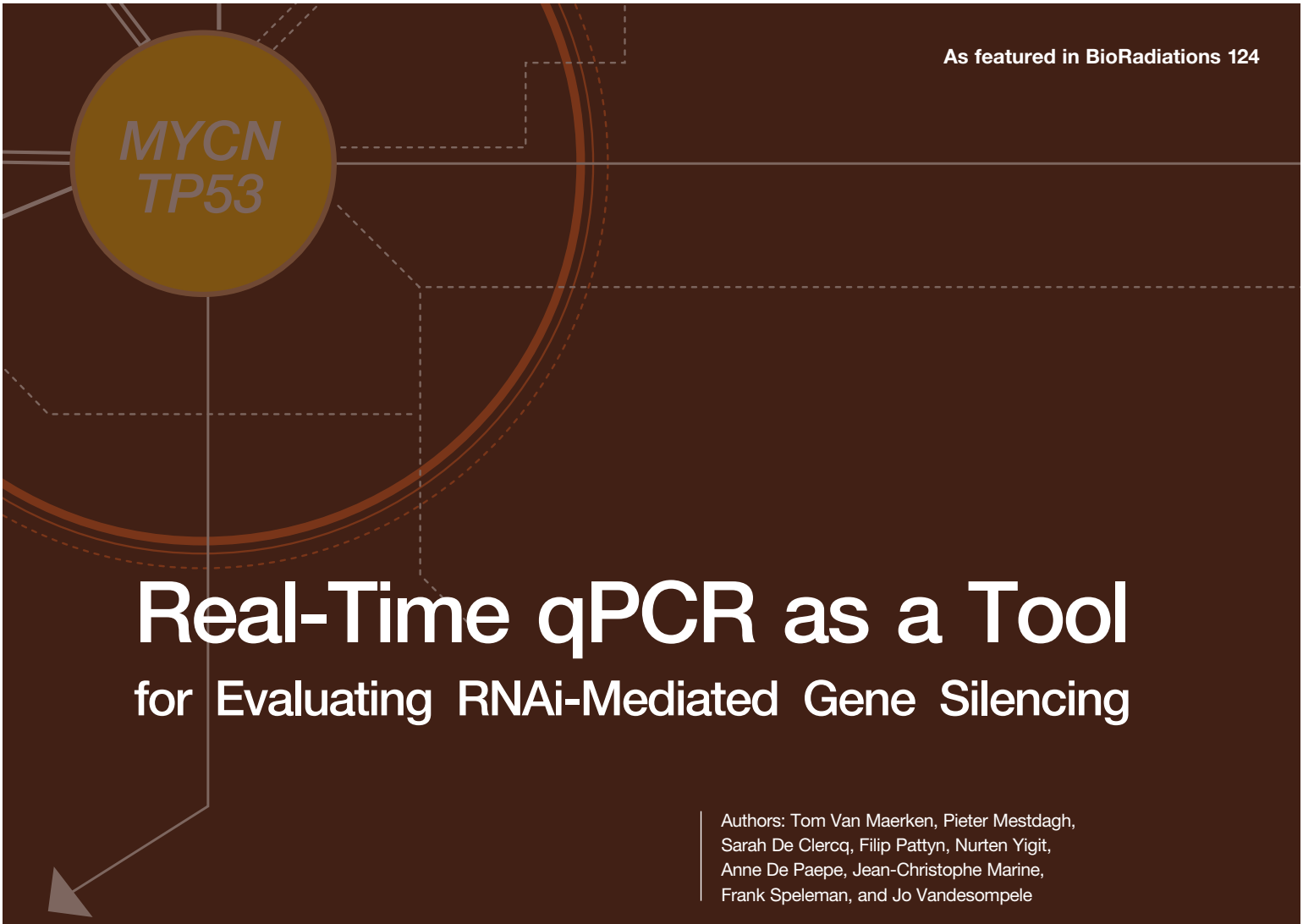


Real-Time qPCR as a Tool

for Evaluating RNAi-Mediated Gene Silencing

Authors: Tom Van Maerken, Pieter Mestdagh, Sarah De Clercq, Filip Pattyn, Nurten Yigit, Anne De Paepe, Jean-Christophe Marine, Frank Speleman, and Jo Vandesompele

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.

BIO-RAD

Neuroblastoma and the *MYCN* and *TP53* Cancer Genes

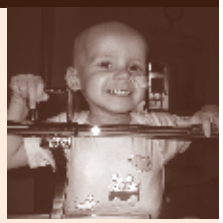
Neuroblastoma is a childhood cancer derived from precursor cells of the adrenosympathetic system, arising in the adrenal medulla or in sympathetic ganglia. Although a relatively rare form of cancer, neuroblastoma is among the most fatal of childhood diseases. Indicators of mortality include age at diagnosis (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 (Tweddle 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.

The Many Faces of Disease



With the focus of his research in neuroblastoma, a very deadly form of childhood cancer, Professor Jo Vandesompele often gets asked if he meets the children behind the research. His answer: "No, we see a tube." In fact, most researchers spend countless hours with analytical tools, but little time, if any, interacting with people affected by disease. That's why the scientists that comprise Vandesompele's lab at Ghent University in Belgium are introducing a pilot program, where parents of children who have died or are suffering from neuroblastoma will be invited to speak to researchers about their experiences.

"Most of us don't maintain a sense of what we're doing research for," says Vandesompele. "A sample is brought from a hospital lab. We begin extracting molecules and conducting procedures that have nothing to do with the child the sample came from, a child who might be dying. There's a disconnect there that shouldn't be." The parent program is meant to bridge this disconnect.

The idea sprang from travels to international conferences, where parents involved in disease-related support groups occasionally give talks. Vandesompele's colleagues realized that in terms of motivating progress toward a cure, even the world's best scientists can't match the words of a parent whose child has died. And it's not just that parents have heartbreaking tales to tell. It's also that they have a passion for raising money to support research, and that they're truly interested in what's happening in the field.

"Yes, we're doing science," says Vandesompele, "but being connected to the human aspects of research can motivate scientists to be much more precise, closer to perfect in what we are doing. Passion brings us to a level unattainable based on intellectual skills alone."

Soon, at least in Belgium, researchers will begin to be able to match a name and a face to a test tube.

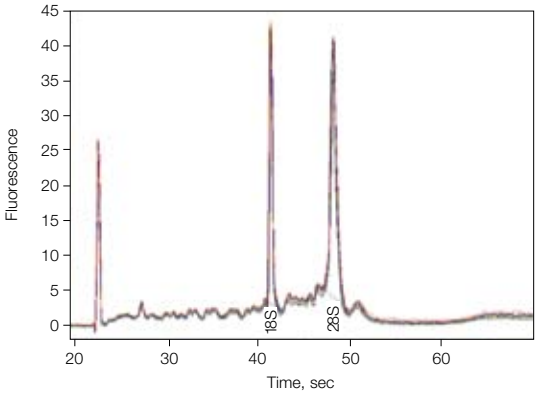


Fig. 1. Electropherogram generated using the Experion system depicting high-quality RNA sample.

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 C_T 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 qBase*Plus* software (www.biogazelle.com, see sidebar), a dedicated program for the management and analysis of rt-qPCR data (Hellemans et al. 2007).

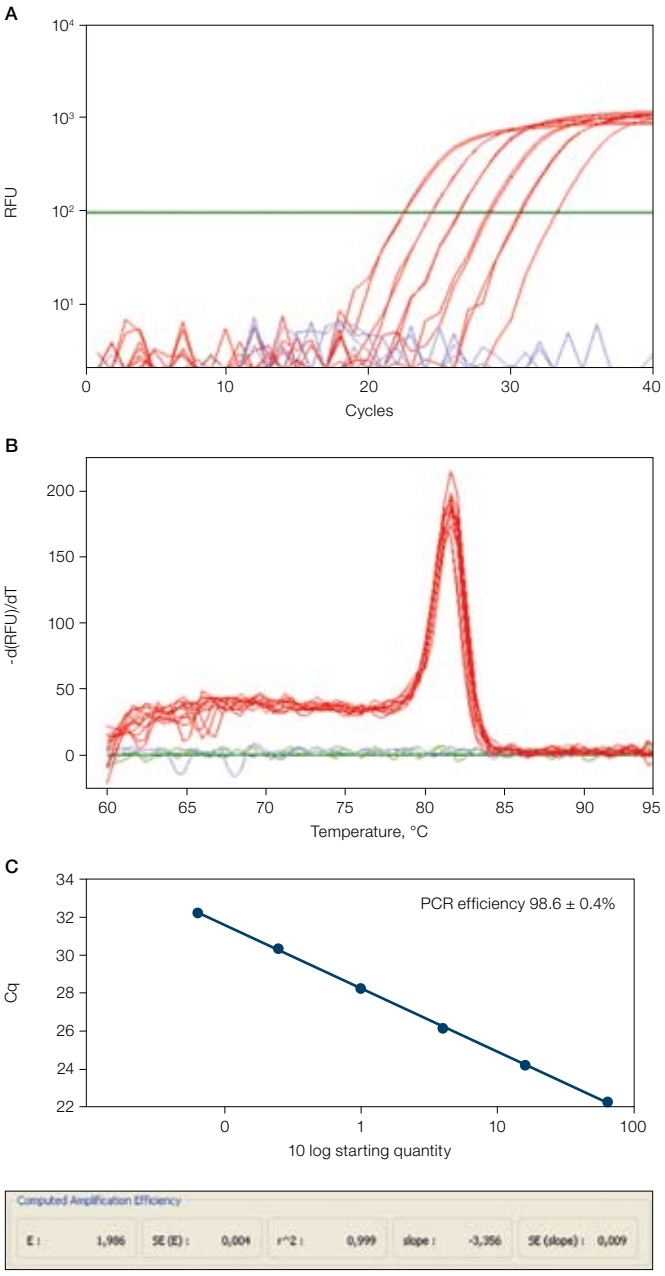


Fig. 2. Experimental validation of newly designed rt-qPCR primers. A, PCR efficiency and dynamic range of the rt-qPCR assay was tested using a 4-fold serial dilution of six points of reverse transcribed human qPCR reference total RNA (64 ng down to 62.5 pg) and TP53_P2 primers; **B**, specificity of the TP53_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 qBase*Plus* software. Cq, quantitation cycle value generated in RDML software (see sidebar).

Programming Progress

The year 2000 is a milestone that symbolizes movement toward the height of progress, particularly in science and technology. But back in 2000, Professor Jo Vandesompele (then a doctorate student beginning what would become a career devoted to the study of the genetics of neuroblastoma at Ghent University in Belgium) was attempting to conduct sophisticated analysis of genetic research results with rudimentary tools. "In 2000," says Vandesompele, "evaluating candidate reference genes with respect to their expression stability was impossible." Moreover, the concept of accurate normalization using multiple reference genes did not exist. "The problem of housekeeping gene variability was significantly underestimated at that time," he explains. In addition, he remembers calculating qPCR analyses by hand with Excel software, "a slow and error-prone process that required insight into mathematics and various quantitation models."

With no other solutions available, Vandesompele and colleagues set out to develop the first of many software, web, and database tools that continue to help drive progress in genetic research — not just in their lab, but in labs across the world. Launched in 2002, geNorm software is a tool used for the identification of stably expressed reference genes (**http://medgen.ugent.be/genorm/**). This launch was quickly followed by development of RTPrimerDB in 2003, a real-time PCR primer and probe database containing published and validated assays, as well as an integrated in silico PCR assay evaluation pipeline (**http://medgen.ugent.be/rtprimerdb/**).

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 qBase*Plus*, 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.rdml.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 Assessment of siRNA Silencing Efficiency: Anti-*MYCN* siLentMer™ siRNA Duplexes

Human IMR-32 neuroblastoma cells were transfected with different anti-*MYCN* 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).

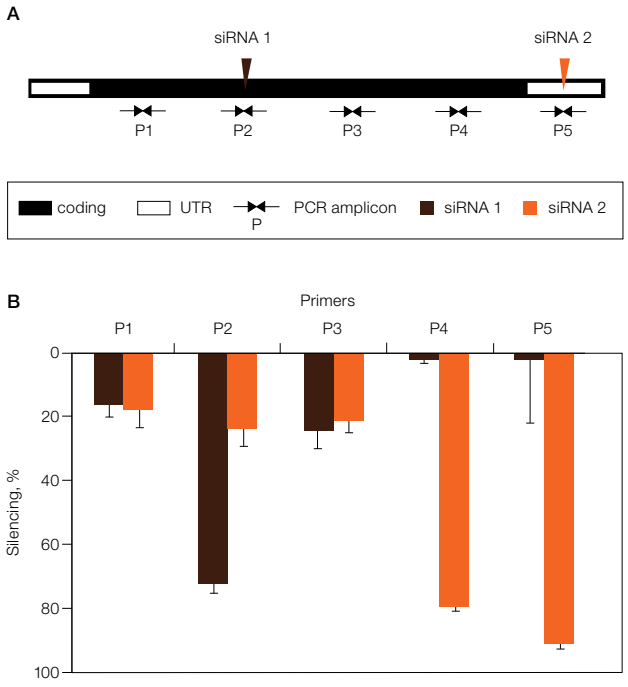


Fig. 3. Importance of primer location for rt-qPCR assessment of siRNA silencing efficiency. A, schematic representation of the *MYCN* mRNA structure with location of siRNA targeted sequences and primer binding sites; **B**, percentage silencing of *MYCN* gene expression measured by five different primer pairs (P1–P5) in IMR-32 cells 48 hr posttransfection with anti-*MYCN* siLentMer siRNA duplexes (siRNA 1 or siRNA 2), compared to cells transfected with a nonspecific control siRNA.

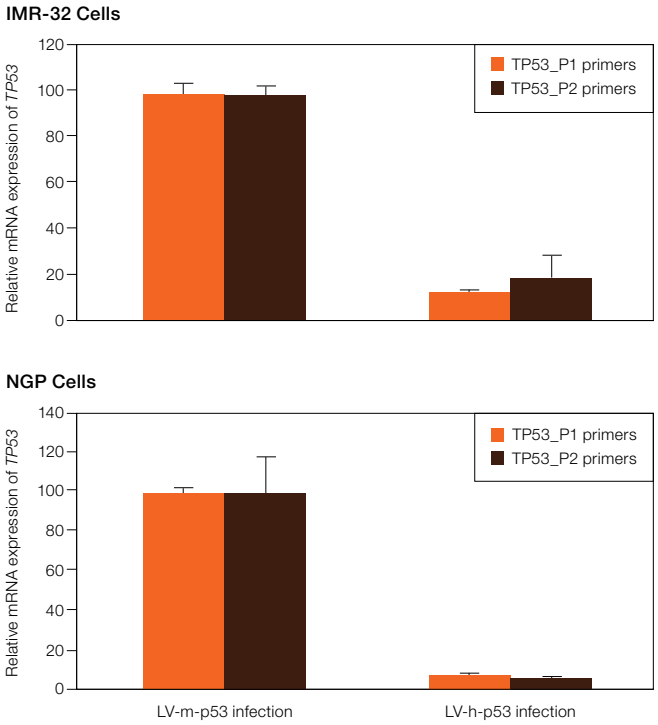


Fig. 4. Assessment of shRNA-mediated *TP53* knockdown efficiency by rt-qPCR. IMR-32 and NGP cells were infected with a lentivirus carrying an shRNA construct specific for either the human *TP53* gene (LV-h-p53) or the murine *Trp53* gene (LV-m-p53) as a control. Efficiency of *TP53* gene silencing was 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).

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 *Trp53* gene (LV-m-p53, negative control). rt-qPCR analysis with two different primer pairs demonstrated that expression of *TP53* was reduced by 81–87% in IMR-32-LV-h-p53 cells and by 92–94% in NGP-LV-h-p53 cells compared to the respective LV-m-p53 controls (Figure 4). Functionality of the *TP53* knockdown variants was validated by rt-qPCR and cell viability analysis after treatment of the cells with nutlin-3, a small-molecule compound that selectively disrupts the interaction between p53 and its negative regulator *MDM2*, resulting in stabilization and accumulation of the p53 protein and activation of the p53 pathway (Vassilev et al. 2004). Transactivation of p53 target genes such as *BBC3* (*PUMA*) and *MDM2* by nutlin-3 and nutlin-3 induced downregulation of *TP53* mRNA level, a consequence of the ability of the p53 protein to negatively regulate its own transcriptional expression after accumulation (Hudson et al. 1995), was largely prevented by lentiviral-mediated expression of shRNA against human *TP53* (Figure 5). At the cellular level, silencing of human *TP53* severely attenuated the nutlin-3 induced reduction in cell viability observed in nontransduced parental cells, in contrast to control infection with LV-m-p53 (Figure 6). These results firmly demonstrate potent and selective impairment of p53 function in IMR-32-LV-h-p53 and NGP-LV-h-p53 cells.

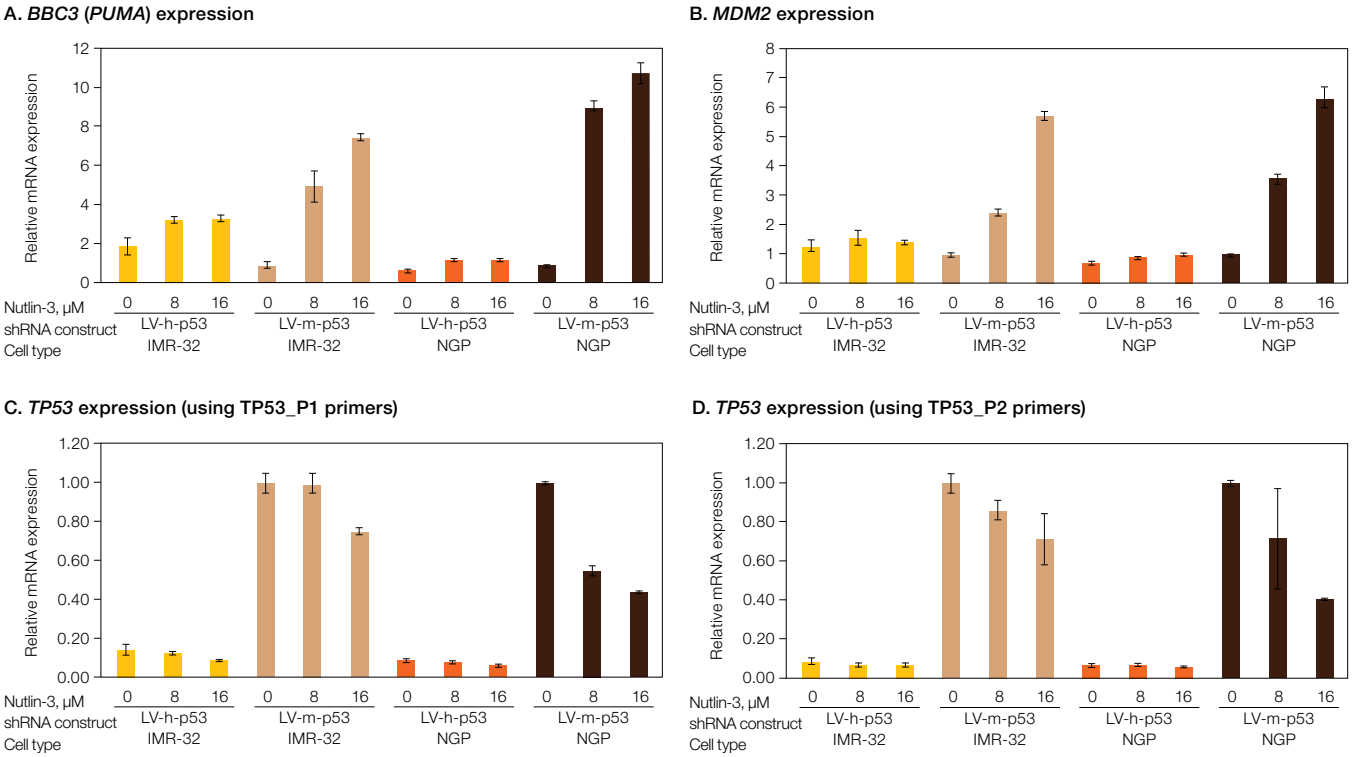


Fig. 5. Functional validation of shRNA-mediated *TP53* knockdown through rt-qPCR analysis of transcript levels of p53-regulated genes after nutlin-3 treatment. IMR-32 and NGP cells were infected with a lentiviral vector encoding an shRNA directed specifically against either the human *TP53* gene (LV-h-p53) 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.

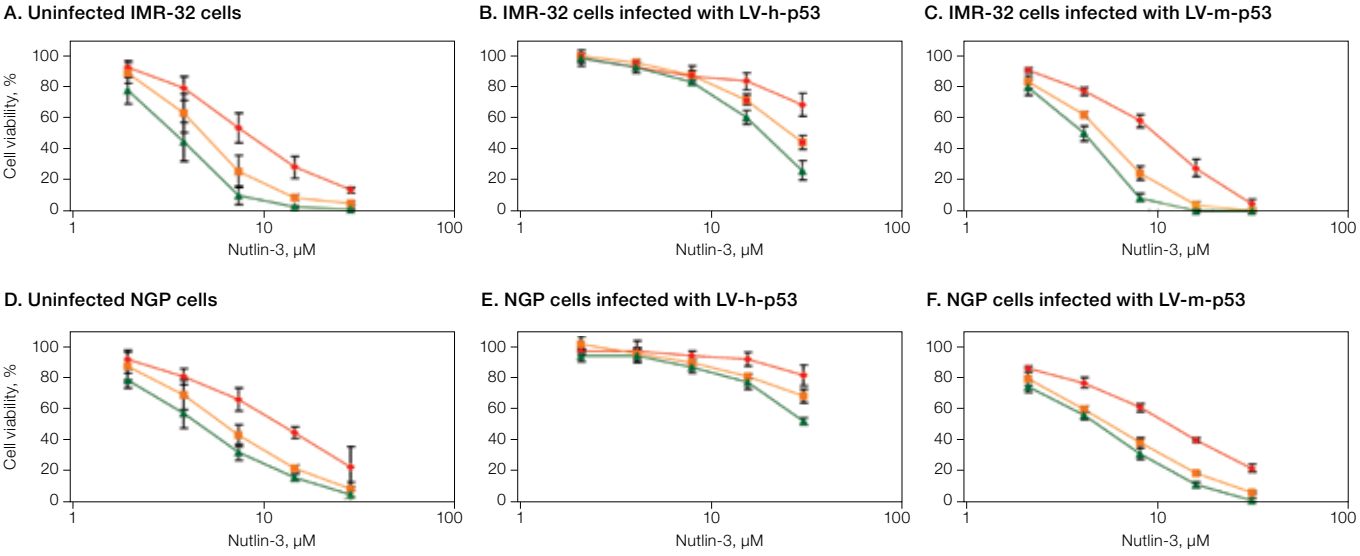


Fig. 6. Functional validation of shRNA-mediated *TP53* knockdown through cell viability analysis after treatment of IMR-32 and NGP cells with nutlin-3. Effect of nutlin-3 on viability of uninfected cells (**A**, **D**), LV-h-p53 infected cells (**B**, **E**) and LV-m-p53 infected cells (**C**, **F**). 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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Grant support: the Fund for Scientific Research – Flanders (FWO) grants 011F4004 (T. Van Maerken, Research Assistant), G.1.5.243.05 (J. Vandesompele) and G.0185.04; GOA grant 12051203; grants from the Belgian Foundation against Cancer (S. De Clercq), the Ghent Childhood Cancer Fund, and the Ghent University Research grant (B.O.F.) 01D31406 (P. Mestdagh).

Contact information: Jo Vandesompele, Center for Medical Genetics, Ghent University Hospital, MRB, De Pintelaan 185, 9000 Ghent, Belgium. Phone: 32-9-332-5187; Fax: 32-9-332-6549; E-mail: Joke.Vandesompele@UGent.be

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