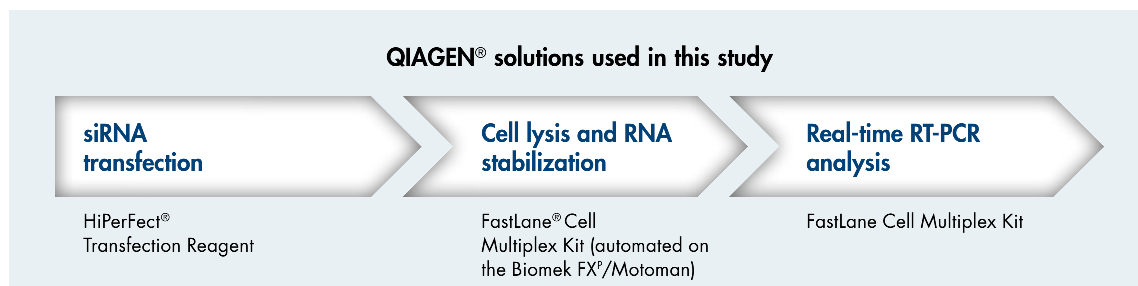
Newsletter
Gene Expression and Function Studies
15
March 2010

Automated process for siRNA screening

Short interfering RNAs (siRNAs) hold great promise as therapeutic drugs, since they can be designed to knock down the expression of specific genes. To ensure successful in vivo gene silencing, siRNAs need to be chemically modified or to be conjugated to a small molecule. Such modifications can, for example, improve potency, minimize off-target effects, and prevent degradation by endonucleases. Following the design of multiple chemically modified siRNAs for a particular target, in vitro studies need to be carried out to evaluate their potency. Such studies require the use of various cell lines and the establishment of optimal conditions for siRNA transfection. Since a huge range of parameters need to be tested, Pieczyk and coworkers from the siRNA lead finding laboratory of Hüsken at the Novartis RNAi Therapeutic Unit developed an automated process for optimizing transfection conditions. The same automated process can also be used for subsequent routine screening of siRNAs.



Transfection optimization experiments were carried out by the Novartis group using HeLa cells expressing enhanced yellow fluorescent protein (eYFP). With these cells, transfection of an eYFP-specific siRNA leads to gene knockdown that can be easily determined by measuring the reduction in fluorescence.

In initial experiments by the Novartis group, chemically modified siRNAs were transfected using HiPerFect Transfection Reagent under different conditions. Among the parameters that were varied, these included cell density; siRNA concentration; and ratio of transfection reagent to siRNA. Transfection was automated on the Biomek® FX^P/Motoman workstation (Beckman Coulter), and gene knockdown was measured using the Victor 2 plate reader (Perkin Elmer).

After determining the most promising conditions for transfection, experiments were repeated, replacing measurement of eYFP fluorescence with real-time RT-PCR analysis, which provides accurate quantification of endogenous gene knockdown. The entire procedure from cell lysis to real-time RT-PCR setup was automated using the combination of the FastLane Cell Multiplex Kit and the Biomek FX^P/Motoman workstation. The FastLane protocol is comprised of 4 automatable steps: cell wash; cell lysis with RNA stabilization and DNA removal;

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DNase inactivation; and real-time RT-PCR setup using cell lysate as RNA template. Duplex, real-time RT-PCR analysis of the target gene and a control housekeeping gene was carried out on the Applied Biosystems® 7900 Real-Time PCR System using TaqMan® Gene Expression Assays (Applied Biosystems).

Table 1. Impact of cell number and incubation time on knockdown efficiency

siRNA	Dose	HiPerfect/siRNA ratio	6000 cells/well 24 h	6000 cells/well 48 h	9000 cells/well 24 h	9000 cells/well 48 h
CM-2	**High**	H	+++	+++	++	+++
		M	+++	+++	++	+++
		L	++	++	+	++
CM-2	**2-fold lower**	H	++	+++	–	+++
		M	++	++	–	++
		L	–	–	–	–
CM-1	**High**	H	++	+++	+	+++
		M	++	+++	+	+++
		L	++	+++	–	+++
CM-1	**2-fold lower**	H	+	+++	+	+++
		M	+	++	–	++
		L	+	+	–	+

The knockdown achieved with the chemically modified siRNAs CM-1 and CM-2 at different siRNA concentrations, at different cell densities, and at different HiPerFect/siRNA ratios (**H**: high; **M**: medium; **L**: low) was determined by duplex, real-time RT-PCR (housekeeping control was hHPRT-1). Both siRNAs led to higher knockdown levels when applied to the lower cell number. +++: knockdown >94%; ++: knockdown >92%; +: knockdown >90%; –: knockdown <90%.

The results from the transfection optimization experiments revealed that cell density is an important parameter when transfecting HeLa cells with siRNA using HiPerFect Transfection Reagent (Table 1). For other cell lines and transfection reagents, the main parameter affecting transfection efficiency can be different (data not shown). Furthermore, this study by the Novartis group demonstrates the feasibility of high-quality, reproducible results in automated siRNA screening.

Visit www.qiagen.com/goto/siRNAFastLane to read the full-length article.

Ordering Information

Product	Contents	Cat. no.
HiPerFect Transfection Reagent (0.1 ml)*	Reagent for up to 33 transfections in 24-well plates or up to 133 transfections in 96-well plates	301702
FastLane Cell Multiplex Kit (200)	Buffers and reagents for real-time one-step RT-PCR direct from cell lysates (200 x 50 µl reactions)	216513

* Larger sizes available; please inquire. Genomewide siRNAs can be ordered at www.qiagen.com/GeneGlobe.

Comparing SYBR® Green master mixes

Real-time PCR and RT-PCR with SYBR Green detection is made convenient by master mixes from life science suppliers. A master mix typically contains all the necessary components for PCR, such as reaction buffer, DNA polymerase, dNTPs, and SYBR Green I dye. The user simply adds template and primers before starting PCR.

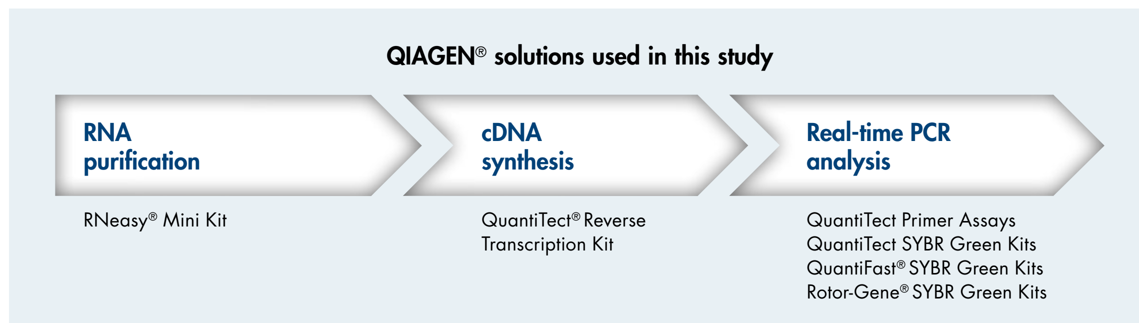
When evaluating master mixes from different suppliers, it is not sufficient to only compare C_T values. While lower C_T values appear to indicate more sensitive real-time PCR quantification, other parameters must also be considered. Firstly, it is necessary to check whether the master mixes provide specific amplification of the PCR product. Since SYBR Green dye binds all double-stranded DNA, it is critical to avoid primer–dimers and non-specific PCR products that can contribute to SYBR Green fluorescence. Secondly, it is important to compare the PCR efficiencies achieved with the master mixes. For quantification methods relying on high and comparable amplification efficiencies among several targets (e.g., the $\Delta\Delta C_T$ method), low PCR efficiencies will result in unreliable real-time PCR quantification.

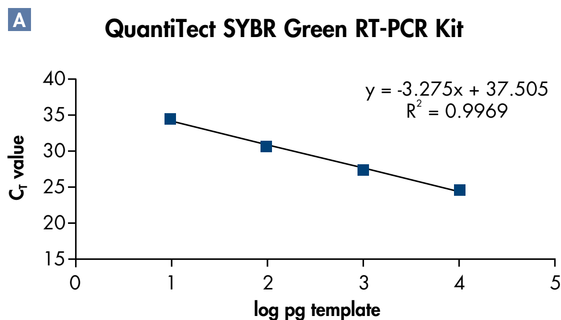
Comparing PCR specificity using a no template control

When performing real-time PCR, it is important to always include a no template control (NTC). This is a control reaction that contains all necessary components for PCR except the template. No SYBR Green fluorescence should be observed with the NTC, since amplification of the intended PCR product does not take place. Any SYBR Green fluorescence with the NTC indicates contamination of the reaction or the formation of primer–dimers (Figure 2).

Comparing PCR specificity using melting curve analysis

At the end of real-time PCR, melting curve analysis can be carried out on the real-time cycler to check the specificity of the reaction. In melting curve analysis, the amplified PCR product is gradually heated through a range of temperatures (e.g., from 65°C to 95°C) and SYBR Green fluorescence is continuously measured. Only a single peak, which represents the specific PCR product, should be observed. The presence of other peaks indicates the presence of primer–dimers and/or nonspecific PCR products.





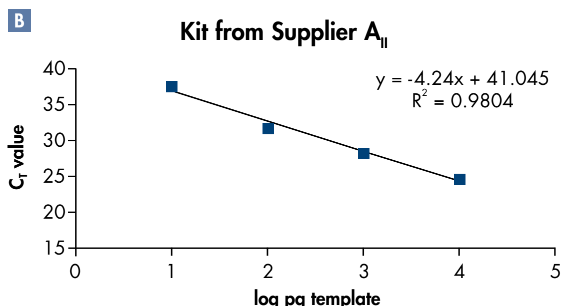


Figure 1. Standard curves. Real-time one-step RT-PCR was performed using the indicated kits in combination with the QuantiTect Primer Assay for human BAX (BCL2-associated X protein). Triplicate reactions were run on the ABI PRISM® 7000 using 4 tenfold dilutions of leukocyte RNA (10 ng to 10 pg). From the slopes of the standard curves, PCR efficiency was found to be higher for the QuantiTect Kit (102%) than for the kit from Supplier A_{II} (72.1%).

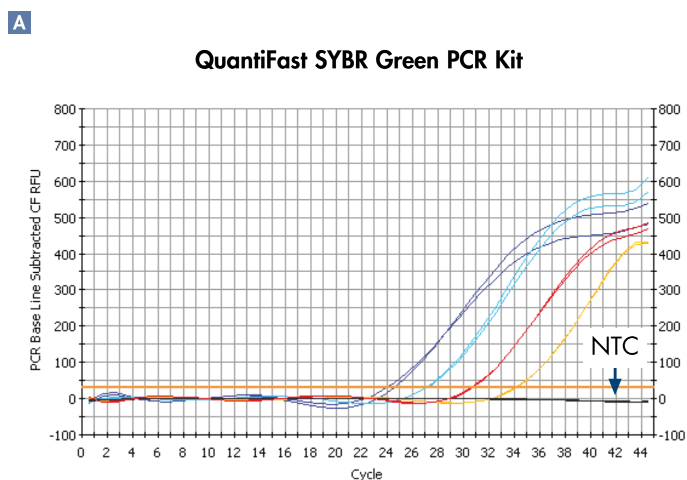
Comparing PCR efficiency

A PCR efficiency of 1 (or 100%) means that the amount of PCR product doubles with each amplification cycle. High PCR efficiency is essential for reliable real-time PCR quantification. PCR efficiency is determined by constructing a standard curve, where log template amount is plotted against the corresponding C_T value, and then determining the slope (Figure 1). From the value of the slope, PCR efficiency can be calculated according to the following formula:

$$E = 10^{(-1/S)} - 1$$

where E is PCR efficiency, and S is the slope of the standard curve. For reliable real-time PCR quantification, the value of the slope should be in the range of –3.3 to –3.8. This range corresponds to PCR efficiencies of 100% to 83.3%.

Visit www.qiagen.com/goto/SYBRGreenMM to read the full-length article.



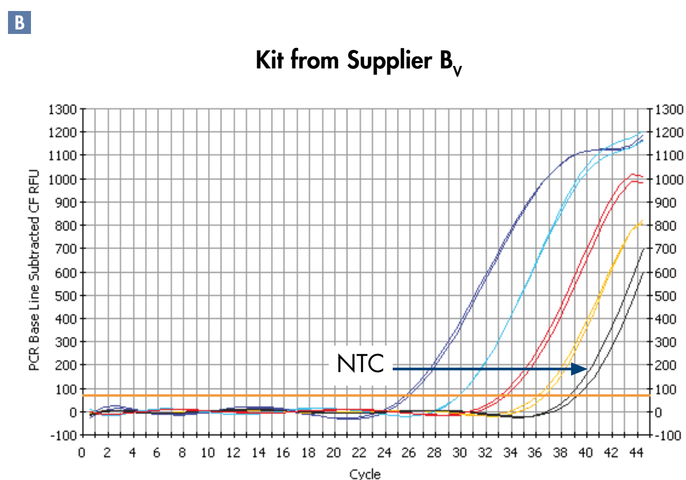


Figure 2. No template control. Real-time two-step RT-PCR was performed using the indicated kits in combination with the QuantiTect Primer Assay for human IL8 (interleukin 8). Duplicate reactions were run on the iCycler iQ® using 4 tenfold dilutions of leukocyte cDNA (100 ng to 100 pg) as well as an NTC. A fluorescent signal for the NTC was not observed with the QuantiFast Kit but with the kit from Supplier B_V, indicating contamination or primer–dimer formation.

Ordering Information

Product	Contents	Cat. no.
RNeasy Mini Kit (50)	Kit for 50 RNA minipreps	74104
QuantiTect Reverse Transcription Kit (10)	Trial kit for 10 x 20 µl reactions	205310
QuantiTect Primer Assays (200)	Primer set for 200 x 50 µl reactions	Varies*
QuantiFast SYBR Green PCR Kit (80)	Trial kit for 80 x 25 µl reactions	204052

* Visit www.qiagen.com/GeneGlobe to search for and order QuantiTect Primer Assays. Assays are available in tubes, 96-well plates, or 384-well plates..

Reliable detection of mature miRNAs and their precursors

microRNAs (miRNAs) have been shown to play an important role in regulation of gene expression in a variety of diverse biological processes. Often when studying miRNA expression, it is desirable to discriminate between levels of mature miRNAs, which are biologically active, and precursor miRNAs, which have not completed processing to become active molecules. The miScript PCR System enables detection of mature miRNA using a miScript Primer Assay or detection of precursor miRNA using a miScript Precursor Assay.

To verify that only mature miRNAs were being detected using a miScript Primer Assay, components of the miRNA processing machinery were knocked down prior to miRNA detection and the results were then compared to control samples. In addition, miScript Precursor Assays were used to detect precursor miRNA and these results were compared to mature miRNA levels, demonstrating differences in the relative levels of mature and precursor miRNA for different miRNAs.

miScript Primer Assays enable detection of mature miRNA only

Knockdown of critical components in the miRNA processing pathway was performed to test whether use of the miScript PCR System and a miScript Primer Assay detected mature miRNAs only. Dicer, Drosha, and DGCR8 are central to the miRNA processing mechanism. Their knockdown prevents accumulation of mature miRNAs in mammalian cells. Using the miScript PCR System and miScript Primer Assays, expression analysis of 4 miRNAs was performed following knockdown of each component and was compared to control cells. miRNA expression was detected in control cells. In contrast, significantly decreased miRNA expression was detected in cells in which miRNA processing components were knocked down (Figure 3). These results strongly indicate that the miScript PCR System can be used with a miScript Primer Assay to detect mature miRNAs only.

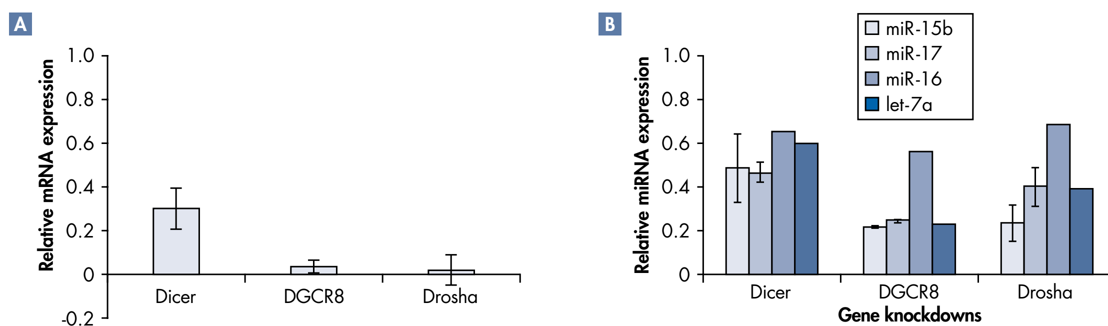


Figure 3. Knockdown of miRNA processing machinery reduces mature miRNA levels. Dicer, DGCR8, or Drosha, key miRNA maturation genes, were knocked down in primary mouse embryo fibroblasts (for experimental details, visit www.qiagen.com/literature/qiagennews/weeklyarticle/09_06/e13/). **A** Knockdown was confirmed by real-time RT-PCR. The mRNA level for each gene is shown relative to the mRNA level in control cells, which was set at 1. **B** miRNA expression analysis was carried out using the miScript PCR System and 4 miScript Primer Assays. miRNA expression levels are shown relative to expression in cells transfected with a control lentivirus, which was set at 1. These results show a decrease in detected miRNA expression after knockdown of miRNA processing genes. (Data kindly provided by Marzi, M.J. and Nicassio, F., IFOM-IEO Campus, via Adamello 16, Milan, Italy.)

QIAGEN solutions for microRNA research

miRNA purification	cDNA synthesis	Real-time PCR analysis
miRNeasy Mini Kit	miScript Reverse Transcription Kit	miScript SYBR Green PCR Kit miScript Primer Assays miScript Precursor Assays Rotor-Gene Q

miScript Precursor Assays expand the scope of miRNA research

The miScript PCR System can also be used for the detection of precursor miRNAs by using miScript Precursor Assays. Detection of precursor miRNA stem loops enables studies of miRNA biogenesis, regulation, and function. Variations in the relative levels of mature miRNAs and their precursors could reveal regulation at the level of miRNA biogenesis (Figure 4). In addition, mature miRNAs may have several possible precursor miRNAs and may be expressed from different precursor miRNAs under different conditions (Figure 5).

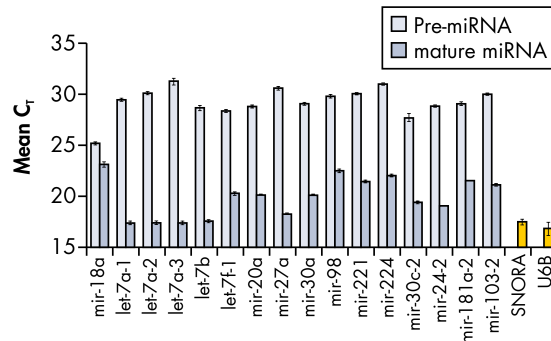
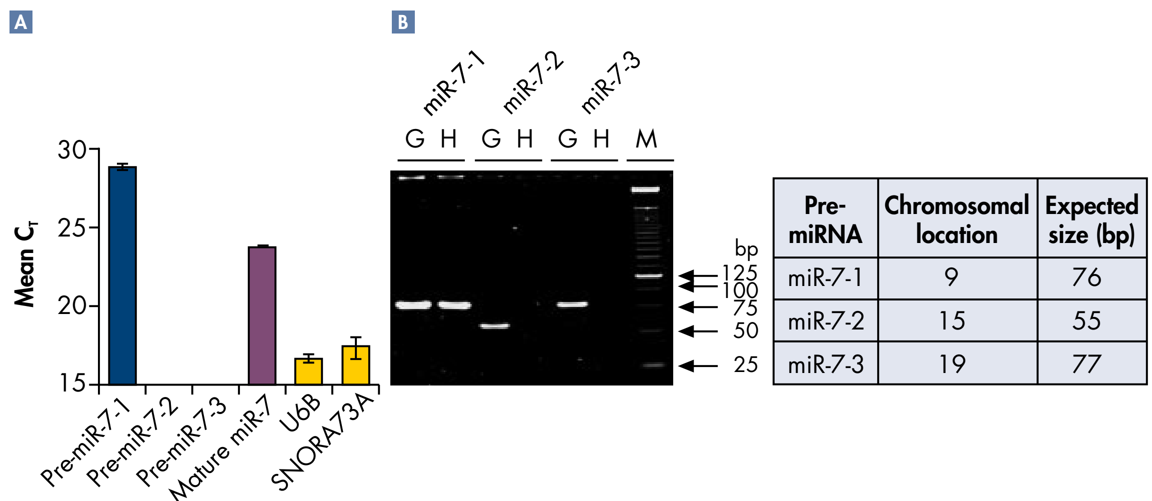


Figure 4. Detection of a panel of mature miRNAs and their precursors in HeLa S3 cells. RNA purified from HeLa S3 cells using the miRNeasy Mini Kit was used to synthesize cDNA for real-time PCR quantification of a range of mature and precursor miRNAs using the miScript PCR System. miScript Primer Assays were used for mature miRNA quantification. miScript Precursor Assays were used for precursor miRNA quantification. miScript Primer Assays for RNU6B and SNORA73A were used for normalization. Real-time PCR was carried out on the Rotor-Gene Q cycler.



Pre-miRNA	Chromosomal location	Expected size (bp)
miR-7-1	9	76
miR-7-2	15	55
miR-7-3	19	77

Figure 5. Expression analysis of mature miR-7 and its precursor stem loops (miR-7-1, miR-7-2, and miR-7-3) in HeLa cells. Three precursor miRNAs derived from 3 distinct chromosomal locations can form mature mir-7. **A** HeLa cDNA was used with the miScript PCR System for real-time PCR quantification of the 3 precursor miRNAs (using appropriate miScript Precursor Assays) or mature miR-7 (using a miScript Primer Assay). miScript Primer Assays for RNU6B and SNORA73A were used for normalization. Only one of the 3 miRNA precursors (miR-7-1) was expressed under these conditions. **B** Gel analysis of the PCR products confirmed that when HeLa cDNA was used as template (**H**), a PCR product was only detected for miR-7-1. In addition, as a positive control, the miScript Precursor Assays amplified PCR products of the expected sizes for each precursor from genomic DNA (**G**).

Conclusions

- Knockdown of miRNA processing genes significantly decreased mature miRNA levels as detected using the miScript PCR System with a miScript Primer Assay. This indicates that miScript Primer Assays detect mature miRNAs only.
- Use of miScript Precursor Assays for precursor miRNA detection demonstrated the potential for insights into miRNA regulation.

Find out more about the miScript PCR System at www.qiagen.com/miRNA!

Effects of low A_{260}/A_{230} ratios in RNA preparations on downstream applications

Silke von Ahlfen and Martin Schlumpberger
QIAGEN GmbH, Hilden, Germany

The efficiency of applications such as real-time RT-PCR and microarray analysis depends strongly on the purity of the RNA sample used. To assess RNA purity, a spectrophotometer is used to measure the absorbance of RNA at 260 nm and the absorbance of potential contaminants at 280 nm or 230 nm. An A_{260}/A_{280} ratio of 1.8–2.1 at pH 7.5 is widely accepted as indicative of highly pure RNA. Pure RNA should also yield an A_{260}/A_{230} ratio of around 2 or slightly above; however, there is no consensus on the acceptable lower limit of this ratio. Also, it has not been fully established which contaminants contribute to a low A_{260}/A_{230} ratio. Possible candidates include "salt", carbohydrates, peptides, and phenol (or aromatic compounds in general) (1).

In our experience, increased absorbance at 230 nm in RNA samples is almost always due to contamination with guanidine thiocyanate, a salt which absorbs very strongly at 220–230 nm and is present at very high concentrations in the lysis buffer or extraction reagent (e.g., TRIzol®) used in most RNA purification procedures. Our experiments show that the A_{260}/A_{230} ratio of an RNA sample is strongly reduced when guanidine thiocyanate is present even at submillimolar concentrations (Figure 6A). However, we also found that concentrations of guanidine thiocyanate of up to 100 mM in an RNA sample do not compromise the reliability of real-time RT-PCR, even when using PCR chemistries that are sensitive to inhibitors (Figure 6B). Similar observations have been reported by other researchers (2).

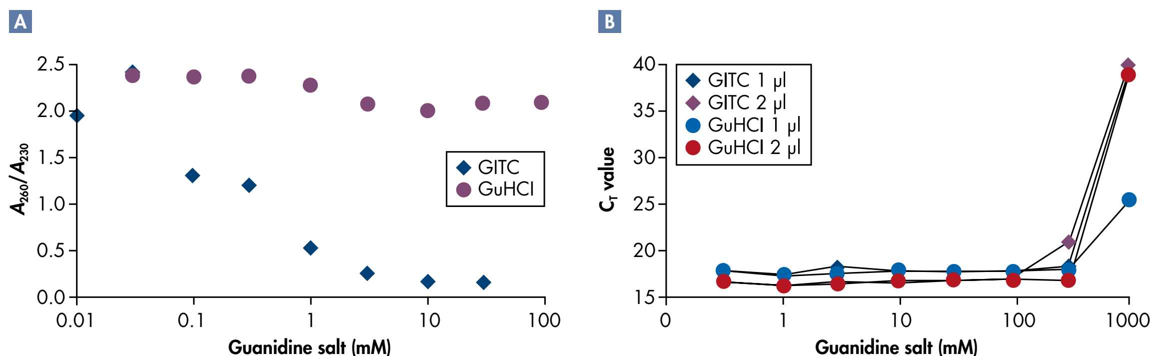


Figure 6. Effect of guanidine salt concentration on the A_{260}/A_{230} ratio and real-time RT-PCR. A Ratio of A_{260} to A_{230} for RNA samples (50 ng/µl) containing 0.03–100 mM guanidine hydrochloride (GuHCl) or 0.01–30 mM guanidine thiocyanate (GITC). B C_T values obtained from real-time one-step RT-PCR using a TaqMan Gene Expression Assay for beta-actin and a master mix from Supplier A_{II}. The reaction volume was 25 µl, and the template was either 1 µl or 2 µl of a 50 ng/µl RNA sample containing 0.3–1000 mM guanidine salt.

When establishing a suitable lower limit for the A_{260}/A_{230} ratio, it is important to remember that this ratio (and other absorbance ratios in general) also depends on RNA concentration. Trace amounts of contaminants will have virtually no effect on the ratio if the RNA is at a high concentration, but will have a major impact on the ratio if RNA is at a low or very low concentration. However, the most important factor is the amount of contaminant that is transferred to the downstream reaction (e.g., cDNA synthesis), rather than the absorbance ratio.

While our analysis has been performed using real-time RT-PCR, similar conclusions apply to microarray analysis and other applications that rely on cDNA synthesis as the first step — the step that is the most influenced by contaminants.

Note: Some sources have reported that the absorbance maximum for guanidine thiocyanate is around 260 nm, which is similar to that for RNA. This observation appears to be due to a saturation effect on the Nanodrop® spectrophotometer at high concentrations of the salt (Figure 7). At moderate or low salt concentrations, the absorbance maximum is around 220–230 nm, depending on the spectrophotometer.

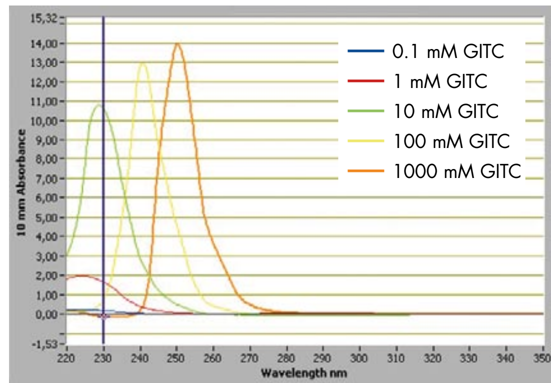


Figure 7. Effect of high guanidine thiocyanate concentrations on absorbance measurements made on the Nanodrop spectrophotometer. Absorbance measurements of 0.1, 1, 10, 100, or 1000 mM guanidine thiocyanate (GITC). The wavelength for peak absorbance appears to increase at high salt concentrations.

References

1. Luebbehusen, H. (2004) The Significance of the 260/230 Ratio in Determining Nucleic Acid Purity (www.bcm.edu/mcfweb/?PMID=3100)
2. Cicinnati, V.R., Shen, Q., Sotiropoulos, G.C., Radtke, A., Gerken, G., and Beckebaum, S. (2008) Validation of putative reference genes for gene expression studies in human hepatocellular carcinoma using real-time quantitative RT-PCR. BMC Cancer **8**, 350.

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