

## Gene Expression Application Note No. 4

# Relative Quantification of miRNA Target mRNAs by Real-Time qPCR

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## 1 Introduction

MicroRNAs (miRNAs) are endogenous small non-protein coding RNAs which are primary regulators of gene expression in many basic cellular processes, including cell proliferation, cell differentiation and apoptosis. miRNA expression also plays a significant role in tumor growth. In general, miRNAs regulate target protein expression through translational control. However, the regulation of gene expression at the level of targeted mRNA degradation has also been reported. There is thus evidence that miRNAs can directly regulate specific intracellular mRNA concentrations by RNA degradation, as well as regulate mRNA levels by targeting the cascade of proteins that modulate the downstream transcriptional or post-transcriptional regulation of mRNAs.

In the present study, we focus on the miRNA hsa (Homo sapiens) miR-21 which is known to be upregulated in breast cancers and other tumors. Furthermore a functional role has been described for miR-21 in apoptosis and cell growth. To further investigate the role of human miR-21, we analyzed the influence of miR-21 levels on mRNA expression of three putative miR-21 target genes PDCD4, PTEN and TLR2 (see Figure 1).

## 2 Materials and Methods

### Cell culture

HeLa cells were maintained in DMEM supplemented with 1% L-glutamine, 1% penicillin/streptomycin, 10% FBS at +37°C in 5% CO<sub>2</sub>. Transfection was performed using miRNA 21 miRIDIAN mimic, miRNA mimic miRIDIAN control, miRNA 21 miRIDIAN inhibitor and miRNA miRIDIAN inhibitor control siRNAs (all at 50 nM, Dharmacon), and the X-tremeGENE siRNA Transfection Reagent (Roche), according to the manufacturer's protocol.

### RNA isolation and reverse transcription

Two days after transfection, total RNA was extracted from HeLa cells (three biological replicates per mimic miRNA) using the High Pure RNA Isolation Kit (Roche), according to the manufacturer's recommendations. In parallel, total RNA containing small size RNA was extracted (two biological replicates) using the High Pure miRNA Isolation Kit (Roche), with the one column protocol described in the package insert (see Figure 2). First strand cDNA synthesis was done on 500 to 1,000 ng total RNA per reaction using the Transcriptor First Strand cDNA Synthesis Kit (Roche) with a mixture of oligo(dT)<sub>18</sub> and random hexamer primers, according to the manufacturer's instructions. First strand cDNA synthesis of miRNA was performed using 4 ng total RNA containing small RNA in each reaction with the miRCURY LNA First-strand cDNA Kit (Exiqon).

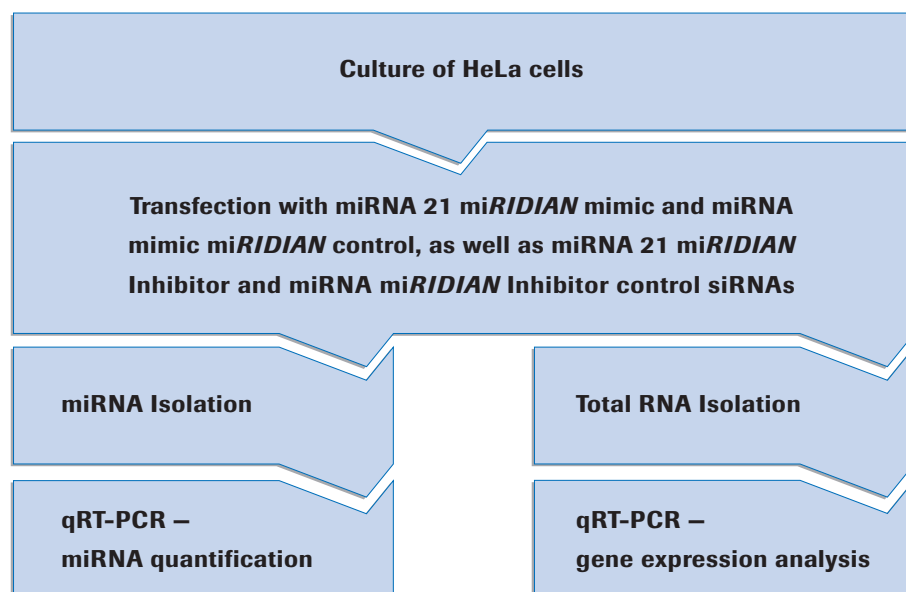
### qRT-PCR

#### Gene Expression Analysis

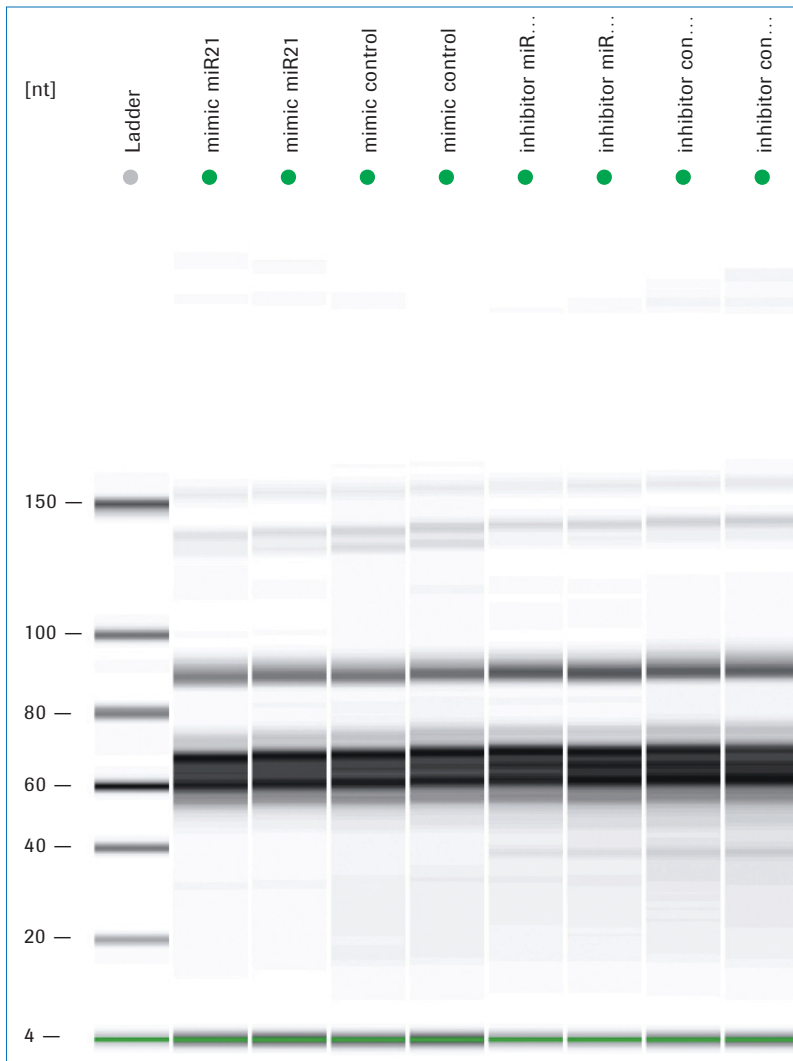
Quantitative RT-PCR was performed using fluorescent Universal ProbeLibrary (UPL) probes (Roche) and a ABI 7900HT Real-Time Instrument (Applied Biosystems), with 2x FastStart Universal Probe Master (Rox) PCR reagents (Roche) in a total reaction volume of 11 µl/well in 384-well format. The cDNA/RNA concentration was 10 ng per reaction. PCR conditions for the ABI 7900HT Real-Time Instrument (Applied Biosystems) were denaturation at +95°C for 15 minutes, 45 amplification cycles at +95°C for 15 sec, and a final elongation step at +60°C for 60 sec.

#### miRNA Quantification

miRNA qRT-PCR was performed with miRCURY LNA SYBR Green master mix, miRCURY LNA Endogenous control SNORD44 and LNA PCR primer for miR-21 (Exiqon), using the LightCycler® 480 Instrument (Roche), in a reaction volume of 20 µl in a 384-well plate with 1:10 diluted cDNA. PCR conditions for the LightCycler® 480 Instrument were denaturation at +95°C for 10 minutes, 40 cycles at +95°C for 10 sec, and elongation at +60°C for 20 sec. The fold change in putative target gene expression levels was determined using the  $\Delta\Delta C_t$  method (Livak and Schmittgen, 2001), with HPRT1 and GAPDH as reference (housekeeping control) genes. For hsa miR-21, non-targeted synthetic mimic- and inhibitor-miRIDIAN control miRNA was transfected into HeLa cells, serving as an experimental control.



**Figure 1:** Workflow for the analysis of high and low levels of hsa miR21 on the expression of putative miR-21 target genes, PDCD4, PTEN and TLR2.



**Figure 2:** Gel electrophoresis of small size RNA from different isolates of HeLa cells after transfection with the indicated reagents, miRIDIAN miRNA mimics or miRIDIAN miRNA Inhibitors, using an Agilent Bioanalyzer.

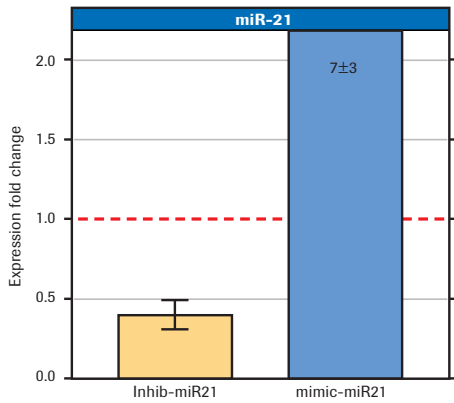
### 3 Results and Discussion

To analyze the effect of elevated levels of miR-21 on mRNA levels of several putative target genes (PDCD4, PTEN and TLR2), we quantified the abundance of some of the mRNAs reported to be regulated by miR-21 using Universal ProbeLibrary probes (Roche) with the ABI 7900HT Real-Time Instrument (Applied Biosystems). HeLa cells were transfected with mimics and inhibitors of miR-21, and respective controls (miRNA 21 *miRIDIAN* mimic, miRNA *miRIDIAN* mimic control, miRNA 21 inhibitor and miRNA Inhibitor control siRNAs), using the X-tremeGENE siRNA Transfection Reagent (Roche). *miRIDIAN* miRNA mimics are synthetic duplexes representing mature miRNAs that are able to regulate target mRNAs just like endogenous miRNAs. In contrast, the *miRIDIAN* miRNA inhibitors are nonhydrolyzable, single strand reverse complements of the mature miRNA that produce a decreased activity of endogenous miRNAs after transfection. This is most likely due to irreversible binding of the inhibitor to mature miRNA (Meister, Landthaler et al. 2004).

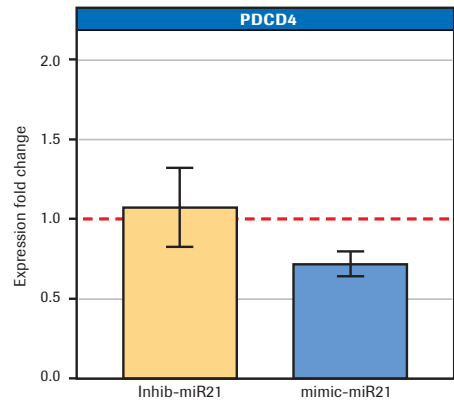
We quantified the effects the *miRIDIAN* mimics and inhibitors of miR-21 had on the levels of mature miR-21 using qRT-PCR on a LightCycler® 480 Instrument. miRNA was isolated using the High Pure miRNA Isolation Kit, and quantified using qRT-PCR with miRCURY LNA, and normalized for the small noncoding nucleolar RNA SNORD44. Transfection with 50 nM *miRIDIAN* mimic of miR-21 produced a 7.3 fold increase in miR-21 concentration in the HeLa cells compared to control cells treated with *miRIDIAN* mimic control (see Figure 3). In contrast, HeLa cells transfected with 50 nM *miRIDIAN* inhibitor of miR-21 showed a 77 % decrease in miR-21 levels (see Figure 3). This indicates that the X-tremeGENE siRNA Transfection Reagent (Roche) is an excellent tool for transporting both *miRIDIAN* mimics and *miRIDIAN* inhibitors into target cells. Moreover, we found that the High Pure miRNA Isolation Kit (Roche) is a valuable tool for the isolation of miRNAs for subsequent analysis of miRNA concentrations.

We investigated the expression of putative target mRNAs of miR-21, PDCD4, PTEN and TLR2, after using transfection with *miRIDIAN* mimics and inhibitors to upregulate and downregulate the intracellular miR-21 concentration. Total RNA was isolated using the High Pure RNA Isolation Kit (Roche), two days after transfection of HeLa cells with mimics or inhibitors of miR-21, and the respective controls. mRNA levels were quantified using qRT-PCR with UniversalProbeLibrary probes on a ABI 7900HT Real-Time Instrument (Applied Biosystems), with normalization of gene expression to the reference (housekeeping) genes HPRT1 and GAPDH. We found that overexpression of miR-21 leads to a significant reduction by 29 % in the mRNA levels of PDCD4 (encoding the human neoplastic transformation inhibitor) compared to controls (see Figure 4). This result confirms recent findings where miR-21 overexpression has been shown to affect PDCD4, both at the protein and the mRNA levels (Frankel, Christoffersen et al., JBC, 2008, 283).

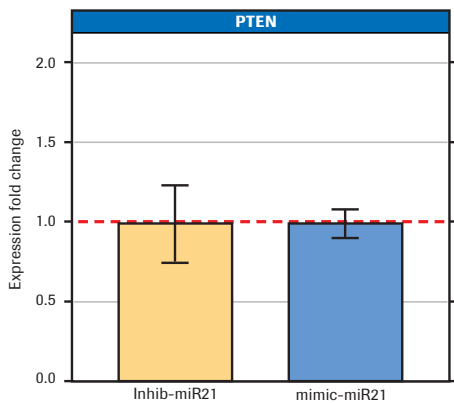
In contrast, no effect of miR-21 was observed on the levels of PTEN mRNA (encoding the human phosphatase and tensin homolog). This was true after both upregulating or downregulating miR-21 levels (see Figure 5). These findings correspond with recent observations, that miR-21 exerts an effect on PTEN at the protein level through translational effects, whereas the PTEN mRNA levels remain unchanged (Wickramasinghe et al. 2009, NAR). Interestingly, TLR2 (encoding toll-like receptor 2) mRNA levels were strongly downregulated by 57% after transfection with miR-21 inhibitor compared to inhibitor transfected control cells (see Figure 6). As both miR-21 and TLR2 are implicated to play a role in the regulation of apoptosis, TLR2 may be an important mediator of the anti-apoptotic activity of miR-21. It remains to be elucidated if TLR2 is a direct or indirect target of miR-21 activity.



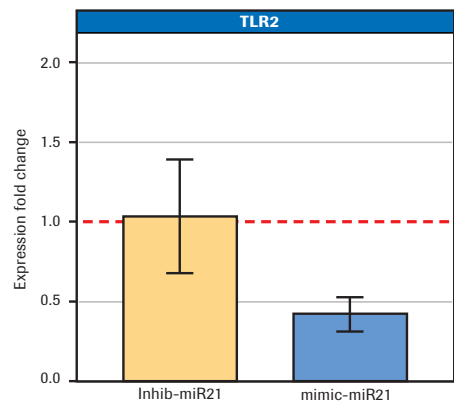
**Figure 3: High level change in the expression of miR-21.** Shown are expression levels of miR-21 after inhibition (yellow bar) or overexpression (blue bar) of miR-21, relative to the respective inhibitor and mimic control transfected cells (shown by the red dashed line = 1.0).



**Figure 4: Effect of miR21 modulation on PDCD4 gene expression.** Overexpression (blue bar) of mRNA-21 leads to reduced levels of PDCD4 mRNA, while downregulation (yellow bar) of miRNA-21 does not affect PDCD4 mRNA level, relative to the respective inhibitor and mimic control transfected cells (shown by the red dashed line = 1.0).



**Figure 5: Effect of miR21 modulation on PTEN gene expression.** The mRNA level of PTEN RNA does not change with different intracellular concentrations of miRNA-21. Shown are mRNA expression levels of PTEN after overexpression of miRNA-21 (yellow bar) and miRNA-21 inhibition (blue bar), respectively, relative to the respective inhibitor and mimic control transfected cells (shown by the red dashed line = 1.0).



**Figure 6: Effect of miR-21 modulation on TLR2 gene expression.** While inhibition of miRNA miR-21 (yellow bar) does not affect the level of TLR2 mRNA, overexpression of miR-21 (blue bar) leads to reduced levels of TLR2 mRNA, relative to the respective inhibitor and mimic control transfected cells (shown by the red dashed line = 1.0).

## 4 Conclusion

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In this study, we analyzed the influence of hsa miR-21 on the mRNA expression levels of three putative target genes, PDCD4, PTEN and TLR2. Human miR-21 response was modulated using exogenous miRNA miRIDIAN mimics and inhibitors (Dharmacon), which can easily be transfected into HeLa cells using the X-tremeGENE siRNA Transfection Reagent (Roche).

For analysis of miR21 levels the High Pure miRNA Isolation Kit produced high quality templates for subsequent qRT-PCR amplification using the miRCURY LNA First-strand cDNA Kit, miRCURY LNA SYBR Green Master Mix, miRCURY LNA Endogenous control SNORD44 and LNA miR-21 PCR primer (Exiqon), on the LightCycler® 480 Instrument (Roche).

For gene expression analysis of putative target genes, a workflow using the High Pure RNA Isolation Kit (Roche) for RNA isolation, Transcriptor First Strand cDNA Synthesis Kit (Roche) for reverse transcription, and Universal ProbeLibrary probes and FastStart Universal Probe Master (Rox) reagents (Roche) with the ABI 7900HT Real-Time Instrument (Applied Biosystems), was used to quantify mRNA levels after modulating intracellular miRNA.

In conclusion, this workflow of easy-to-establish procedures for gene expression analysis is sensitive enough to detect the subtle changes in mRNA levels typically induced by miRNAs.

## References

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## Ordering Information

Product	Cat. No.	Pack Size
<b>X-tremeGENE siRNA Transfection Reagent</b>	04 476 093 001	1 ml
<b>X-tremeGENE siRNA Transfection Reagent</b>	04 476 115 001	5 x 1 ml
<b>High Pure RNA Isolation Kit</b>	11 828 665 001	Up to 50 isolations
<b>High Pure miRNA Isolation kit</b>	05 080 576 001	Up to 50 isolations
<b>Transcriptor First Strand cDNA Synthesis Kit</b>	04 896 866 001	100 reactions
<b>Transcriptor First Strand cDNA Synthesis Kit</b>	04 897 030 001	200 reactions
<b>LightCycler® 480 II Instrument, 384 well</b>	05 015 243 001	1 instrument
<b>FastStart Universal Probe Master (Rox)</b>	04 913 957 001	10 x 1.25 ml (for 500 reactions of 50 µl final reaction volume)
<b>FastStart Universal Probe Master (Rox)</b>	04 914 058 001	10 x 5 ml (for 2000 reactions of 50 µl final reaction volume)
<b>Universal ProbeLibrary Set, Human</b>	04 683 633 001	1 Set
<b>Universal ProbeLibrary Set, Human Reference Gene Assays</b>	05 046 114 001	1 Set

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