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Determination of Interferon-Pathway–Related Gene Induction during RNAi

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Introduction

Experimental approaches using short interfering RNA (siRNA) molecules to specifically silence gene expression have become more widely used in recent years. As for all techniques in molecular and cellular biology, the importance of protocol optimization and the use of appropriate controls cannot be overestimated.

While the induction of interferon-pathway–related stimulatory processes during RNAi is usually attributed to long, double-stranded nucleic acids, it can also be a concern during transfection of short siRNA molecules or in other RNAi-related experiments. A number of parameters, including siRNA chemistry, transfection reagents, cell culture conditions, and siRNA concentration have the potential to cause interferon-pathway–related responses. These responses could interfere with the specific biological effect caused by the siRNA-mediated knockdown of the gene of interest, causing misleading results.

Here we describe assays for quantification of mRNAs which are derived from induced transcription of well characterized interferon-pathway–related genes, using quantitative, real-time RT-PCR. The assays are specific for STAT1, IL6, IFNα1, IFNβ1, IFIT1, IFITM1, OAS1, or OAS2. We performed the assays after cells were transfected with functional amounts of synthetic siRNAs or with polyinosinic-polycytidylic acid (poly(I)·poly(C)), which is a long, double-stranded RNA that induces the interferon pathway. Although poly(I)·poly(C) transfection caused dramatic induction of several interferon pathway genes in these cells, as indicated by the assays, such induction was not seen when siRNAs from QIAGEN were transfected.

We conclude that these assays are valuable tools for optimization of experimental conditions and for use as controls, ensuring successful RNAi experiments.

A set of QuantiTect® Primer Assays for analysis of the interferon response

- QuantiTect Primer Assays are bioinformatically validated gene-specific primer sets for real-time, quantitative RT-PCR using SYBR® Green based detection (Figure 1).
- QuantiTect Primer Assays were used for analysis of the interferon-pathway genes STAT1, IL6, IFNα1, IFNβ1, IFIT1, IFITM1, OAS1, and OAS2.

Principle of Real-Time PCR Using SYBR Green Detection

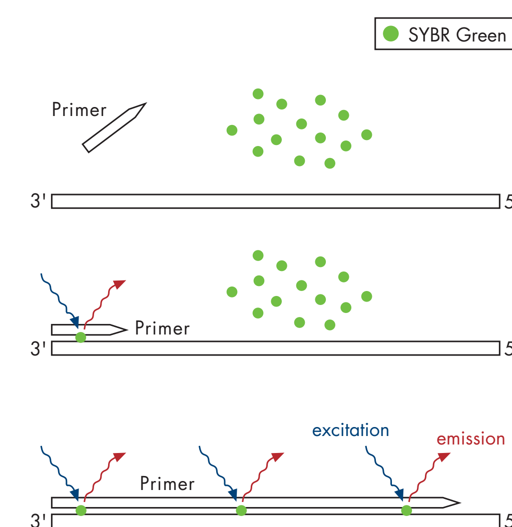


Figure 1 Primers bind to the nucleic acid template during the annealing step of PCR. Primers extend during the extension step and SYBR Green I dye binds to all double-stranded DNA molecules, emitting a fluorescent signal of a defined wavelength on binding.

Phosphorylation of eIF2α induced by poly(I)·poly(C) but not by siRNA

- Phosphorylation of eukaryotic translation initation factor 2A (eIF2α) is a key event that occurs during the interferon response, resulting in nonspecific downregulation of protein synthesis.
- The level of phosphorylated eIF2α (eIF2α~P) in MCF-7 cells was measured after transfection of interferon-response–inducing poly(I)·poly(C), CDC2 siRNA, and a nonsilencing siRNA that contains 3 mismatches to CDC2 mRNA (CDC2-mut siRNA).
- The level of phosphorylated eIF2α increased dramatically after transfection of poly(I)·poly(C). In contrast, very low levels of phosphorylated eIF2α were observed after siRNA transfection (Figure 2).

Western Blot Analysis of Phosphorylation of eIF2α

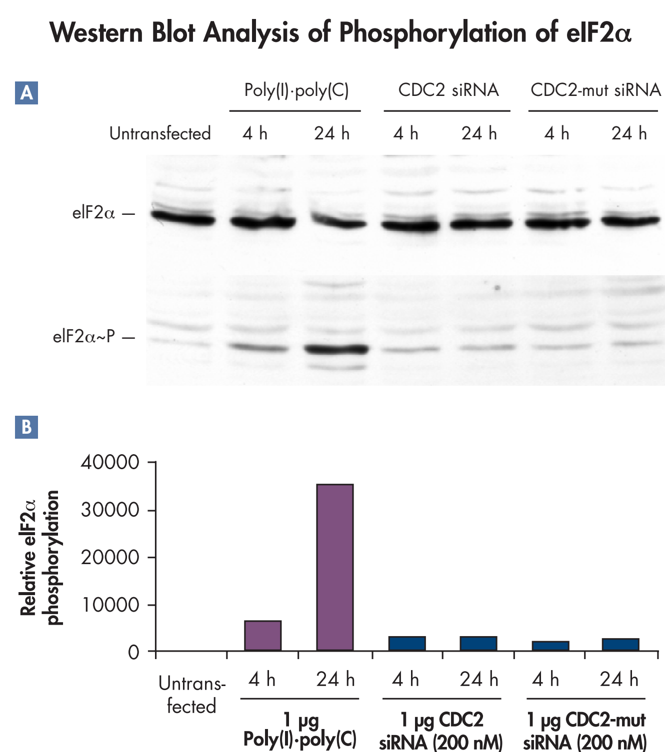


Figure 2 MCF-7 cells were transfected with 1 µg poly(I)·poly(C), 1 µg CDC2 siRNA, or 1 µg CDC2-mut siRNA (siRNA that contains 3 mismatches to CDC2 mRNA). After 4 and 24 hours, cells were analyzed by western blotting using antibody specific for eIF2α and eIF2α~P. **A** The resulting western blot is shown. **B** Quantification of western blot results.

Measurement of induction of interferon genes

- QuantiTect Primer Assays were developed to measure the expression of STAT1, IL6, IFNα1, IFNβ1, IFIT1, IFITM1, OAS1, and OAS2 after transfection of poly(I)·poly(C) or a nonsilencing control siRNA into HeLa or MCF-7 cells.
- Interferon-pathway–related genes were upregulated after transfection of poly(I)·poly(C). This demonstrates that these assays are a highly effective tool for control experiments investigating the interferon response (Figure 3A and B).
- None of the interferon-pathway–related genes were upregulated after transfection of highly pure siRNA from QIAGEN using HiPerFect Transfection Reagent. This demonstrates that QIAGEN siRNA and HiPerFect Reagent do not cause nonspecific RNAi effects via induction of the interferon response (Figure 3).
- Strong induction of the interferon response by poly(I)·poly(C) but not by siRNA was also shown at the protein level (Figure 3C). siRNA transfection did not induce STAT1 protein expression, even using high siRNA concentrations. In contrast, poly(I)·poly(C) strongly induced STAT1 expression, even using as little as 1 ng.

Interferon Gene Expression

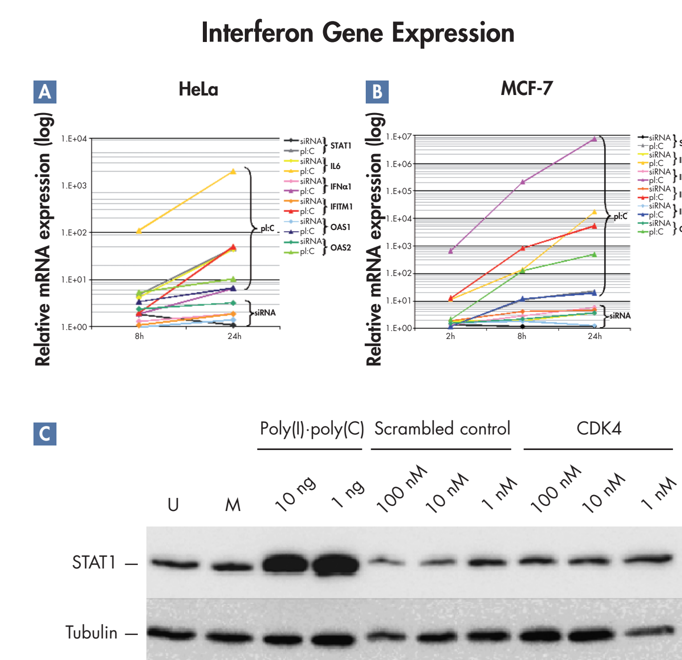
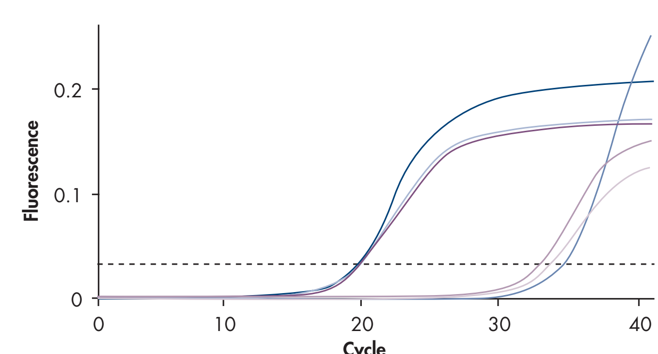


Figure 3 **A** HeLa or **B** MCF-7 cells were transfected with 1 µg poly(I)·poly(C) (abbreviated pI:C) or nonsilencing siRNA using HiPerFect Transfection Reagent. After 2 (MCF-7 only), 8, and 24 hours, gene expression was measured by quantitative, real-time RT-PCR using QuantiTect Primer Assays and the QuantiTect SYBR Green PCR Kit. **C** HeLa cells were transfected with a range of concentrations of poly(I)·poly(C), nonsilencing siRNA, or CDK4 siRNA using HiPerFect Transfection Reagent. After 48 hours, cells were analyzed by western blotting using STAT1-specific antibody and tubulin antibody (as an internal control). Untransfected (U) and mock transfected (M) cells were also analyzed.

Measurement of the interferon response in primary cells

- HUVEC were transfected with poly(I)·poly(C) or HP (HiPerformance) siRNA and the expression of IFNβ1 was determined after 72 hours, a typical timepoint for phenotypic knockdown evaluation. Knockdown was analyzed by quantitative, real-time RT-PCR.
- Poly(I)·poly(C) transfection induced IFNβ1 expression. siRNA transfection did not induce IFNβ1 expression, even after 72 hours in sensitive primary cells. Comparison with untreated cells showed that the viability of siRNA-transfected cells was not impaired.

Expression of Interferon Genes in Transfected Primary Cells



Transfection	C_T Value
HP siRNA	34.62
	33.78
	32.99
Poly(I)·poly(C)	19.78
	20.02
	20.05

Figure 4 HUVEC were transfected with 1 µg poly(I)·poly(C) or HP siRNA (nonsilencing) using HiPerFect Transfection Reagent. Three replicate experiments were performed. After 72 hours, the level of expression of IFNβ1 was measured by quantitative, real-time RT-PCR using the QuantiTect Primer Assay and the QuantiTect SYBR Green PCR Kit.

Conclusions

- Various reports have shown that interferon-related pathways can be induced after siRNA transfection, leading to nonspecific effects and misleading results.
- We have developed QuantiTect Primer Assays for quantitative, real-time RT-PCR of the interferon-pathway–related genes STAT1, IL6, IFNα1, IFNβ1, IFIT1, IFITM1, OAS1, and OAS2. These assays can be used as controls to determine the interferon response in RNAi experiments.
- Transfection with equimolar amounts of siRNA from QIAGEN or poly(I)·poly(C) using HiPerFect Transfection Reagent showed that the interferon response was not induced significantly by QIAGEN siRNA and HiPerFect Reagent.
- siRNA and HiPerFect Reagent are part of the range of RNAi solutions from QIAGEN that allow efficient knockdown without unwanted nonspecific effects.

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