

## Measuring siRNA-Mediated Knockdown of the IL-8 Gene Using the QuantiGene Reagent System

### Introduction

Different siRNA oligonucleotides and constructs have variable effectiveness in knocking down the expression of the cognate target gene. This variability, coupled with variation in transfection efficiencies, and differences in protein turnover and stability, result in the substantial variability observed in RNA interference (RNAi) experiments.

Compounding these factors are variability and inaccuracy in the commonly used methods for measuring gene expression, which are thought to arise from differences in sample loss during the RNA purification steps, as well as sequence-specific and random biases that occur during the amplification process. These sources of variation and error make the commonly used methods for RNA quantitation difficult to standardize.

One way to minimize assay variation is to establish a set of standards for RNA interference experiments that would enable scientists to understand and deconvolute

the sources of variation in order to design the most effective experimental conditions for gene expression knockdown. Such standards would permit RNAi experiments to be compared from day to day, and between different laboratories.

An important component of standardized methods for RNA interference experiments will be a highly quantitative and robust method for measuring the gene expression changes caused by RNAi. Because current methods for RNA quantitation have limitations to their accuracy, precision, and robustness, are cumbersome to perform, or are challenging to develop for particular mRNAs, alternative methods are of general interest to the research community.

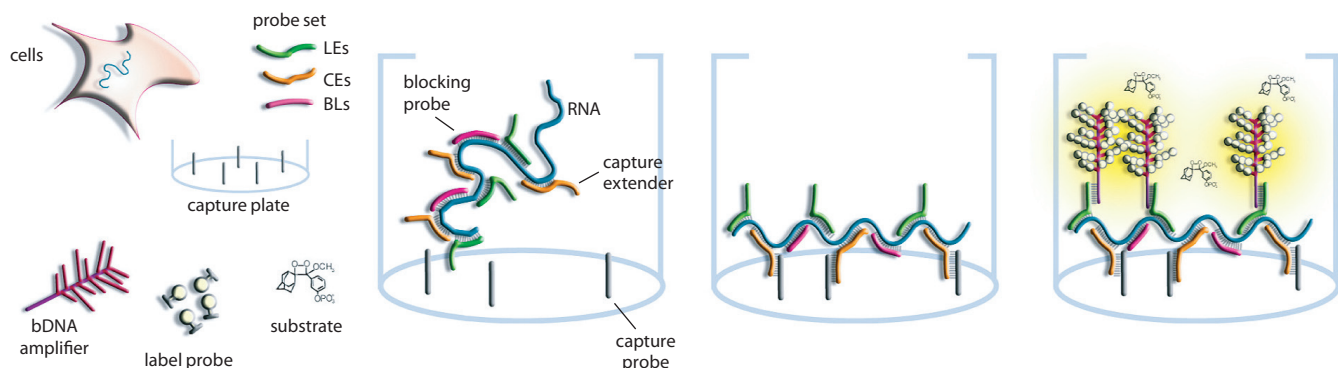
This Application Note presents such an alternative method. The QuantiGene® Reagent System and its performance in measuring both induction of gene expression as well as siRNA-mediated knockdown are presented. The induction of IL-8 gene expression, and its knockdown in HeLa cells, are employed as a model system.

### The QuantiGene Reagent System Technology

The QuantiGene Reagent System measures mRNA levels directly from crude cell lysates or tissue homogenates. It is built upon the branched DNA technology, which relies on cooperative hybridization between a target mRNA and a specific Probe Set. The Probe Sets consist of three types of oligonucleotides, Capture Extenders (CEs), Label Extenders (LEs), and Blocking Probes (BLs), whose sequences are selected based on the sequence of the target mRNA. The Capture Extender oligos (CEs) are approximately 40 nucleotides in length.

Roughly half of the CE's sequence is complementary to sections of the target mRNA, and the other half is complementary to the Capture Probes that are immobilized onto the bottom of the Capture Plates. The Label Extender oligos (LEs) are also approximately 40 nucleotides in length. Again, roughly half of the LEs' sequence is complementary to sections of the target mRNA, and the other half is complementary to the branched DNA

Figure 1. Overview of the QuantiGene Reagent System Technology.



Amplifiers. The final component of the Probe Set, the Blocking Probes (BLs), are used to hybridize across any regions of the target mRNA sequence that are not covered by CEs and LEs. Probe Sets are specifically designed so that together these three oligo types hybridize contiguously to a region typically 400 to 800 nucleotides in length, creating a region of completely double-stranded RNA/DNA hybrid. This double stranded molecule is much more stable than single stranded RNA, and does not form the secondary structures that single stranded mRNAs form. These two attributes contribute to the assay's extraordinary robustness. The CEs drive the assay's specificity through cooperative hybridization. Cooperative hybridization occurs when a CE probe binds both to the target mRNA and to the Capture Plate, making it much more likely that the second, third, and other CEs along the target mRNA will also bind to the plate.

The result is a very stable, specific hybridization event where the target mRNA is attached at multiple locations to the Capture Plate. The LEs drive signal amplification through multiple hybridization events between the target mRNA and the Amplifier. Each LE binds to a specific region of the target mRNA as well as to the "trunk" region of the Amplifier. In turn, the "branches" of the Amplifier have binding sites for the Label Probe (alkaline phosphatase). Upon addition of alkaline phosphatase substrate, a 45-fold amplification of luminescence signal is achieved for each Amplifier molecule associated with the target mRNA.

In this fashion, the QuantiGene Reagent System enables amplification of the readout signal rather than the target. As a result, a readout signal that is directly proportional to the quantity of target mRNA is achieved without the need for purification and amplification of the target mRNA.

The QuantiGene system is performed in standard 96-well plates and has a simple workflow that requires only standard laboratory equipment and skills. Detailed descriptions of the QuantiGene Reagent System, and the Probe Sets used in the experiments described in this Application Note, have been previously published, and are available at [www.panomics.com](http://www.panomics.com).

## Materials and Methods

### Induction of IL-8 Gene Expression by PMA/LPS in HeLa Cells

Approximately 5000 HeLa cells/well were plated in a 96-well plate in 200  $\mu$ L of DMEM medium (Invitrogen). At 70% confluence (16–24 hours), the cells were induced with a 100 nM final concentration of Phorbol-12-myristyl-13-acetate (PMA, CalBiochem) in SFM media (Gibco) for 2 hours.

### Transfection of IL-8 siRNA

siRNAs specific for the IL-8 mRNA (Trilink Biotechnologies) were diluted in Tris-EDTA buffer, pH 8.0 (Ambion) to a working concentration of 5  $\mu$ M. The sequences of the siRNAs are shown in the table below. A novel peptide based delivery reagent, DeliverX siRNA Transfection Reagent, was employed in this study. According to the vendor's specifications, a siRNA/transfection reagent complex was formed at 37°C. The media was removed from the HeLa cells and cells were washed once with PBS. Then 30  $\mu$ L of transfection complex was added to each experimental well and incubated for 5 min at room temperature before adding serum free media (30  $\mu$ L/well). The cells were incubated in the serum free media for 2 hours at 37°C. Without removing the media containing the transfection complex, 100  $\mu$ L of fresh complete DMEM was added to each well. The cells were then incubated at 37°C for additional 2 or 6 hours before RNA quantitation.

siRNA	Sequence
IL-8 – sense	5' – ACCACCGGAAGGAACCAUCdTdT – 3'
IL-8 – antisense	5' – GAUGGUUCCUCCGGUGGUdTdT – 3'

### RNA Quantitation

The standard QuantiGene User Manual was followed. Briefly, the cells were lysed using Lysis Mixture in a 2:1 ratio (2 parts media/cells to 1 part Lysis Mixture). A Lysate volume equivalent to 1000 cells was transferred to a separate Capture Plate well for each gene to be tested, Diluted Lysis Mixture (2 parts distilled water to 1 part Lysis Mixture) was added to bring Capture well volumes to 90  $\mu$ L/well, 10  $\mu$ L of the appropriate Working Probe Set was added, the plate was sealed and incubated at 53°C overnight (18 hours). The Probe Set for the IL-8 mRNA hybridizes to 800 nucleotides of the 1.2 kb mRNA, and entirely covers the regions targeted by the IL-8 specific siRNAs. Following the overnight hybridization, the Capture wells were washed and sequentially hybridized with bDNA Amplifier and Label Probe at 46°C for 1 hour. After a final wash, the luminescent alkaline phosphatase substrate dioxitane was added and incubated at 46°C for 30 minutes. The luminescence signal was detected using a Lmax luminometer (Molecular Devices).

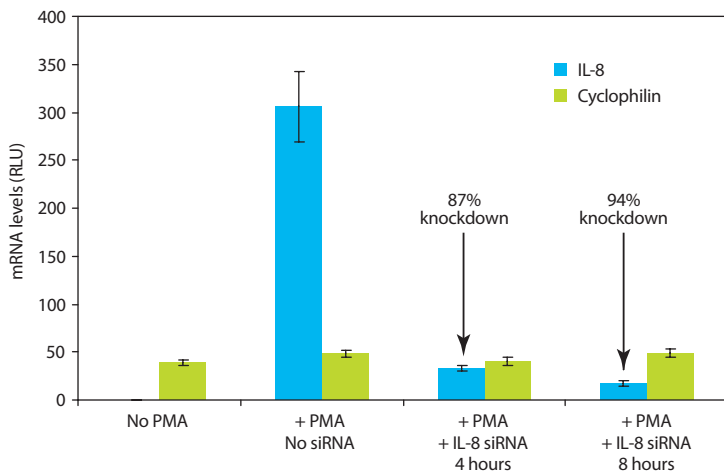
## Results

### Measurement of siRNA Effects

In order to demonstrate the precision of the QuantiGene Reagent System for measuring siRNA mediated knockdown of gene expression, HeLa cells were induced with PMA to express the IL-8 gene, and then transfected with the IL-8 specific siRNAs. Cyclophilin B (PPIB) gene mRNA, which is considered to be constitutively and stably expressed, was used as a control. Under the conditions used in these experiments, HeLa cells stimulated with PMA for 2 hours induce the expression of the IL-8 mRNA approximately 1000-fold, while the expression of the Cyclophilin B gene is unaffected. Following stimulation and transfection, the mRNA levels of each gene were measured, with 4 replicates performed for each set of experimental conditions. The results are shown in Figure 2, where the error bars indicate one standard deviation.

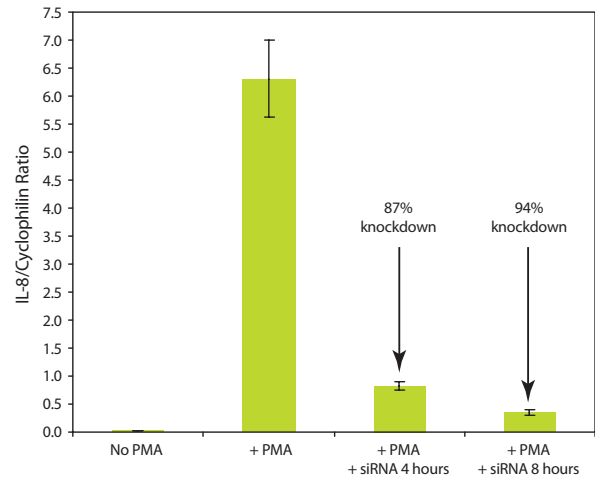
Analysis of the data reveals coefficients of variation (CVs) over different experiments that range from 5% to 15% over a 3-log range of relative luminescence (RLU) values. These small CVs are indicative of the high precision of the QuantiGene system, and allow the clear distinction of the effects of the siRNA seen at 4 hours (89% knockdown) from the effects seen at 8 hours (94% knockdown), even though they differ by only 5%. Other common methods of RNA quantitation often produce two to three times the variability, which can dramatically obscure the experimental observations.

**Figure 2.** Measurement of IL-8 mRNA Induction and siRNA-Mediated Knockdown.



Treatment Group	IL-8			Cyclophilin B		
	Average RLU	Standard Deviation	% CV	Average RLU	Standard Deviation	% CV
No PMA	0.07	0.03	5%	39.19	2.54	6%
+ PMA	305.90	36.37	12%	48.55	3.47	7%
+ PMA, IL-8 siRNA (4 hours)	32.95 (87% knockdown)	2.76	8%	40.21	4.30	11%
+ PMA, IL-8 siRNA (8 hours)	17.27 (94% knockdown)	2.62	15%	49.50	4.20	8%

**Figure 3.** Normalized IL-8 Gene Expression Levels.



Treatment Group	Average RLU	Standard Deviation	%CV
No PMA	0.02	0.0005	3%
+ PMA	6.31	0.6884	11%
+ PMA, IL-8 siRNA (4 hours)	0.82	0.0705	9%
+ PMA, IL-8 siRNA (8 hours)	0.35	0.0535	15%

For example, quantitative PCR methods with high CVs may not allow the statistically significant distinction of a difference in knockdown effect of only 5%. The data from this experiment demonstrate the excellent precision and robustness of the QuantiGene system for measuring the effects of siRNAs in RNA interference experiments.

### Generating Normalized Gene Expression Measurements

The limited precision and reproducibility of commonly used methods of RNA quantitation lead to a certain degree of experimental error in the measurements that are made. The error in the measurement of a control gene further compounds the error in the measurement of a target gene level when the control gene levels are used to normalize the target gene levels.

In order to demonstrate the ability of the QuantiGene system to produce the high-precision measurements necessary to effectively normalize the level of one gene relative to another, the data from the experiment described above was analyzed in the following way. The IL-8 and Cyclophilin B measurements made from the

same replicate (i.e., the same batch of lysed cells) were converted to a ratio (IL-8 RLU to Cyclophilin B RLU). This ratio was calculated for each of the four replicates for each of the four experimental conditions. The results of this analysis are shown in Figure 3, where the error bars indicate one standard deviation.

Analysis of the data generated by normalizing the IL-8 mRNA levels against the Cyclophilin B mRNA levels reveals CVs of 3% to 15%. This high precision demonstrates that the exceptional reproducibility of the QuantiGene system allows the clear distinction of siRNA effects that differ by as little as 5%, and that the QuantiGene system is an excellent technology for measuring normalized mRNA levels.

A technology that uses housekeeping gene expression to normalize other gene expression levels must be sufficiently accurate and precise, or the experimental variation will obscure the biological variation under investigation. Experimental variation in RNAi studies arises from a number of sources, including liquid handling error, variation in cell numbers, variation in cell viability, and transfection efficiency. RNA quantitation methods that involve RNA purification and amplification add a significant source of experimental variability that can obscure the measurement of the siRNA efficiency.

When using a highly precise and robust method for RNA quantitation, such as the QuantiGene system, the other sources of experimental variation can be effectively addressed by normalizing the target gene expression levels to a housekeeping gene level. The precision provided by the QuantiGene system therefore allows the effective comparison of data from experiment to experiment, and from laboratory to laboratory.

## Discussion

In the study described above, the QuantiGene Reagent System demonstrates very high levels of precision and reproducibility in experiments that are commonly performed by researchers who utilize RNAi. CVs range from 5% to 15%, and from 3% to 15% for normalized gene expression data. These small CVs allow the clear distinction of the effects of siRNAs that differ by as little as 5%.

Other common methods of RNA quantitation are often two to three times as variable, which can dramatically obscure the biological variation under investigation. Because their inter-experimental and intra-experimental variation is so high, CVs are not routinely reported for experiments that use the common methods of RNA quantitation.

In addition to providing high precision, the QuantiGene system simplifies the process of going from cells to RNA measurements by eliminating the need to purify or amplify RNA. The elimination of these steps results in reduced handling time, higher retention of sample, and the elimination of biases introduced by reverse transcription and PCR amplification.

The high level of reproducibility and precision observed in these experiments demonstrates that the QuantiGene Reagent System is a powerful companion technology for experimental programs that employ siRNA technology. Indeed, such high precision, accuracy, and robustness is required of any technology that would become a component of standardized methods for RNA interference experiments.

## Ordering Information

Product	Size	Catalog No.
QuantiGene Explore Kit	2 – 96-well plate (192 assays)	QG0001
QuantiGene Discover Kit	10 – 96-well plate (960 assays)	QG0002
QuantiGene Discover XL Kit*	50 – 96-well plate (4,800 assays)	QG0003
QuantiGene Screen Kit**	50 – 96-well plate (4,800 assays)	QG0004

\* Configured to allow processing of 1 or more plates/run

\*\* Configured for high throughput applications requiring the processing of 10 plates/run

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