Measuring siRNA-mediated knockdown of the IL-8 gene using the QuantiGene Singleplex Assay

Introduction

Verifying the efficient inhibition of gene expression is a critical component of RNA interference (RNAi) studies. RNAi experiments have many sources of variation that make accurate quantitation of target mRNA difficult.

Variation in the potency and stability of small interfering RNA (siRNA), coupled with differences in transfection efficiency and protein turnover, results in inconsistent gene knockdown capabilities. Additionally, variation within the analytical method used to quantify gene knockdown may compound these factors.

Standardization of RNAi experiments would enable scientists to design the most effective conditions for gene knockdown, allowing results to be compared between laboratories. Invitrogen™ QuantiGene™ Singleplex Assays provide a reliable and reproducible RNA quantitation method. The QuantiGene Singleplex Assays are used to precisely measure siRNA-mediated knockdown in this application note, using the induction of IL-8 expression and its subsequent knockdown as a model system.

QuantiGene technology

QuantiGene assays use branched DNA (bDNA) technology for signal amplification, allowing for the direct measurement of mRNA levels from crude cell lysates or tissue homogenates. An overnight, cooperative hybridization between target mRNA and target-specific probe sets on a capture plate provides the foundation for subsequent bDNA signal amplification. The addition of a chemiluminescent substrate to the bDNA signal “tree” allows for signal detection by a luminometer.

The target-specific probe set consist of three types of oligonucleotides: capture extenders (CEs), label extenders (LEs), and blocking probes (BLs), whose collective sequences contiguously hybridize a 400–800 nucleotide region of target mRNA. This hybridization creates a region of double-stranded nucleic acid that is more stable than single-stranded RNA and will not form secondary structures.

Roughly, half of a CE’s sequence is complementary to a section of the target mRNA, while the other half is complementary to the capture probes that coat the bottom of the capture plate. There are typically six CEs designed per target mRNA. The CEs drive the assay’s specificity through cooperative hybridization. A CE binds both to the target mRNA and to a capture probe, increasing the likelihood that other CEs along the target mRNA will also hybridize to the capture probes. As a result, the target mRNA is captured at multiple locations in a very stable, specific hybridization event (Figure 1).

The LEs drive signal amplification through multiple hybridization events between the target mRNA and the preamplifier oligonucleotide (the “trunk” of the tree). One end of a LE is complementary to a section of the target mRNA, while the other end is complementary to the pre-amplifier. The LEs are designed to hybridize in pairs, such that each LE pair spans a continuous sequence both of the target mRNA as well as of the preamplifier. Both LEs in each pair need to properly hybridize to the target mRNA in order for the preamplifier to hybridize, creating an additional layer of specificity (Figure 1). There are typically six pairs of LEs designed per target.

The final component of the probe set, the BLs, hybridize any regions of the target mRNA that are not covered by CEs or LEs. Then the preamplifier, amplifier (the "branches"), and label probe (alkaline phosphatase) molecules are sequentially hybridized to build the signal amplification tree. Adding an alkaline phosphatase substrate produces a luminescence signal, which is detected by a luminometer (Figure 1).

In this fashion, the QuantiGene Singleplex Assays amplify the readout signal rather than the target. As a result, the signal is directly proportional to the quantity of target mRNA without the need for RNA purification or cDNA synthesis. The QuantiGene Singleplex Assays have a simple workflow (Figure 1) and are performed in standard 96- or 384-well plates.

Materials and methods

Induction of IL-8 gene expression by PMA in HeLa cells

Approximately 5,000 HeLa cells/well were plated in a 96-well plate in 200 µL of Gibco™ DMEM medium. At 70% confluence (16–24 hours post-plating), the cells were induced with 100 nM phorbol-12-myrstyl-13-acetate (PMA, CalBiochem™ product) in Gibco™ Serum-Free Medium (SFM) 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, to a working concentration of 5 µM. The sequences of the siRNAs are shown in Table 1. An siRNA transfection reagent was employed to generate an siRNA–transfection reagent complex at 37°C. The medium was removed from the HeLa cells, and the cells were washed once with phosphate buffered saline (PBS). Then 30 µL of transfection complex was added to each experimental well and incubated for 5 minutes at room temperature before adding SFM and incubating the cells for 2 hours at 37°C. Without removing the medium containing the transfection complex, 100 µL of fresh complete DMEM was added to each well. The cells were then incubated at 37°C for an additional 2 hours or 6 hours before RNA quantitation.

Table 1. Sequence of siRNAs used to target IL-8.

siRNA	Sequence
IL-8–sense	5´–ACCACCGGAAGGAACCAUCdTdT–3´
IL-8–antisense	5´–GAUGGUUCCUUCCGGUGGUdTdT–3´

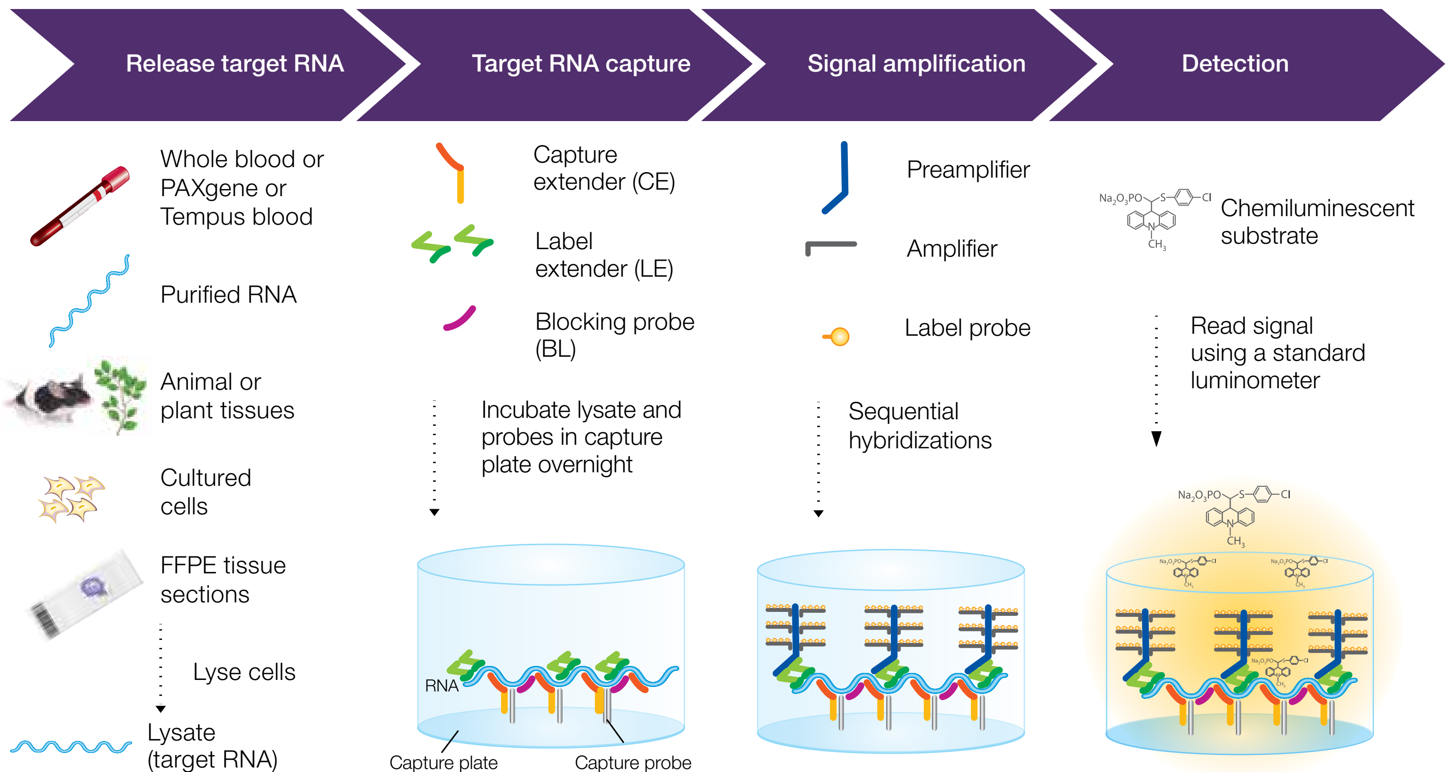

Figure 1. Overview of QuantiGene Singleplex Assay technology.

RNA quantitation

Briefly, the cells were lysed using the lysis mixture in a 2:1 ratio (2 parts medium and cells:1 part lysis mixture). A lysate volume equivalent to 1,000 cells was transferred to a separate capture plate well for each gene tested. Diluted lysis mixture (2 parts distilled water:1 part lysis mixture) was added to bring the capture well volumes to 90 µL/well. Then 10 µL of the appropriate working probe set was added, and the plate was sealed and incubated at 53°C overnight (18 hours). The probe set for the IL-8 mRNA hybridizes to 800 bp of the 1.2 kb mRNA, and entirely covers the regions targeted by the IL-8–specific siRNAs. Following the overnight probe hybridization, the capture wells were washed and sequentially hybridized with bDNA amplifiers and label probes at 46°C for 1 hour. After a final wash, dioxitane, a luminescent alkaline phosphatase substrate, was added and the cells were 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 assay 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*) mRNA, 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 expressed IL-8 mRNA ~1,000-fold higher than uninduced cells, while the expression of *PPIB* was unaffected (Figure 2).

Analysis of the data revealed coefficients of variation (CVs) over different experiments, ranging from 5% to 15% over a 3-logrithmic unit range of relative luminescence units (RLU) values (Table 2). 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) versus those seen at 8 hours (94% knockdown), even though they differ by only 5% (Figure 2, Table 2). Other common methods of RNA quantitation often produce two to three times the variability, which can dramatically obscure the experimental observations.

For example, quantitative PCR methods with high CVs may not allow the statistically significant distinction of a difference in knockdown 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 RNAi experiments.

Generating normalized gene expression measurements

The limited precision and reproducibility of commonly used methods of RNA quantitation lead to a degree of experimental error. The error generated by measuring a control gene further compounds the measurement error of the target gene level when normalized.

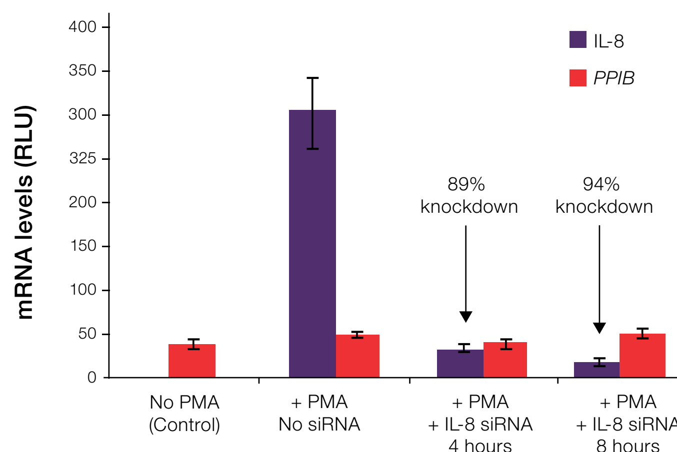

Figure 2. Measurement of IL-8 mRNA induction and siRNA-mediated knockdown. Following stimulation and transfection, the mRNA levels of each gene were measured. Four replicates were performed for each set of experimental conditions. Error bars indicate one standard deviation.

Table 2. Standard deviation and CV of siRNA-mediated knockdown of IL-8.

Treatment group	IL-8			*PPIB*		
	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 (89% 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%

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The QuantiGene Singleplex Assays are able to produce the high-precision measurements necessary to effectively normalize the level of one gene relative to another (Figure 3). The IL-8 and *PPIB* measurements made from the same replicate (i.e., the same batch of lysed cells) were converted to a ratio (IL-8:*PPIB* RLU) (Figure 3, Table 3). Analysis of the data generated by normalizing the IL-8 mRNA levels against the *PPIB* mRNA levels reveals CVs of 3–15% (Table 3). This high precision demonstrates that the exceptional reproducibility of the QuantiGene system allows for the clear distinction of siRNA effects that differ by as little as 5%. Furthermore, it indicates 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. Additionally, 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 for the effective comparison of data from experiment to experiment and from laboratory to laboratory.

Table 3. Standard deviation and CV of IL-8:*PPIB* RLU.

Treatment group	Average RLU ratio	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%

Discussion and conclusions

The QuantiGene Singleplex Assays demonstrate very high levels of precision and reproducibility in experiments commonly performed by researchers who utilize RNAi. CVs range from 5–15% for siRNA-mediated knockdown of IL-8 and from 3–15% for normalized gene expression data. These small CVs allow for 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 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 assay system is a powerful companion technology for experimental programs that employ siRNA technology. Indeed, such high precision, accuracy, and robustness are required of any technology that is a component of standardized methods for RNAi experiments.

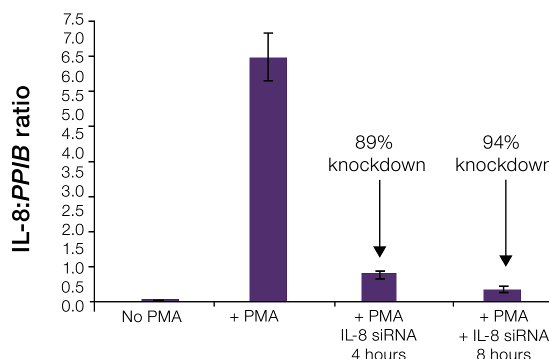

Figure 3. Normalized IL-8 gene expression levels. The IL-8 and *PPIB* measurements made from the same replicate were converted to an RLU ratio of IL-8:*PPIB*. This ratio was calculated for each of the four replicates for each of the four experimental conditions. Error bars indicate one standard deviation.

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