Panomics

Accuracy, Precision, and Robustness of the QuantiGene Reagent System

Introduction

A variety of laboratory techniques are available to determine the expression levels of particular mRNAs present in biological samples. Depending on the laboratory resources available, the level of accuracy desired, and the number of targets to be measured, the researcher typically chooses between techniques such as quantitative PCR (Q-PCR), Northern blots, RNase protection analysis, or expression microarrays. Many of these technologies are cumbersome, time consuming, and require very specialized laboratory equipment and skills.

Because of the complexities of these technologies and their read-out formats, it is often challenging to obtain consistent results from laboratory to laboratory, and even from experiment to experiment in the same researcher's hands. This Application Note presents data on an alternative technology—the QuantiGene Reagent System, and the precision, accuracy and robustness of this method for quantitating RNA levels.

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 target-specific Probe Set. An overview of the assay is shown in Figure 1.

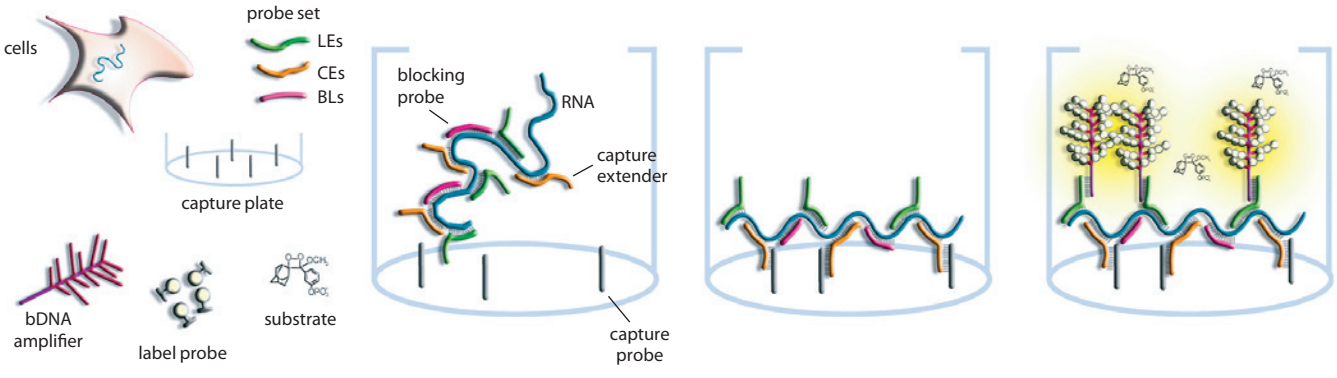
The Probe Set consists of three types of oligonucleotides, Capture Extenders (CEs), Label Extenders (LEs), and Blocking Probes (BLs), whose sequences are selected based on the sequence of the target mRNA.

The Capture Extender oligos (CEs) are approximately 40 nucleotides in length. Roughly half of the CEs' sequence is complementary to sections of the target mRNA, and the other half is complementary to the capture oligos 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 Amplifiers.

The final component of the Probe Set, the Blocking Probes, 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 300 to 500 nucleotides in length, creating a region of a completely double-stranded RNA/DNA hybrid molecule. 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 unusual 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

Figure 1. Overview of QuantiGene Reagent System Technology.

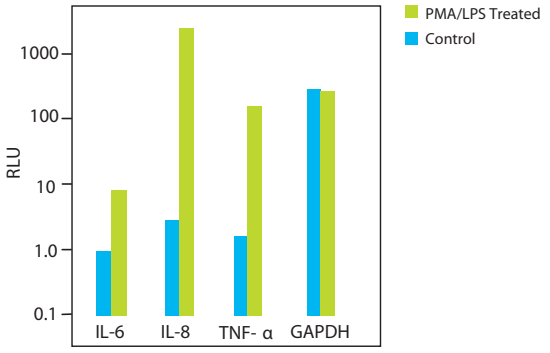


other CEs along the mRNA will also bind to the plate. The result is a very stable, specific hybridization event where the target mRNA is attached at multiple locations to the Capture Plate.

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.



	IL-6			IL-8			TNF-α			GAPDH		
RX	RLU	CV	Copy/cell	RLU	CV	Copy/cell	RLU	CV	Copy/cell	RLU	CV	Copy/cell
Control	0.955	7.0	12	2.89	6.5	24	1.66	3.3	8	303.3	2.0	12,000
PMA/LPS	8.244	6.0	103	2448.0	2.5	20,329	160.3	2.4	772	276.3	2.2	11,000

Figure 2. RNA quantitation of three inducible genes.

Materials and Methods

Sample Preparation

U937 cells were cultured in a growth media of RPMI 1640 with 10% FBS. For PMA/LPS induction experiment, U937 cells were first differentiated in the presence of 100 nM PMA in macrophage serum free media (SFM) (Gibco) for 48 hours and then stimulated with 1 µg/mL LPS for 4 hours in the growth media. Both control and PMA/LPS treated U937 cells were lysed in the QuantiGene Lysis Mixture and used for the QuantiGene assay.

Assay Procedure

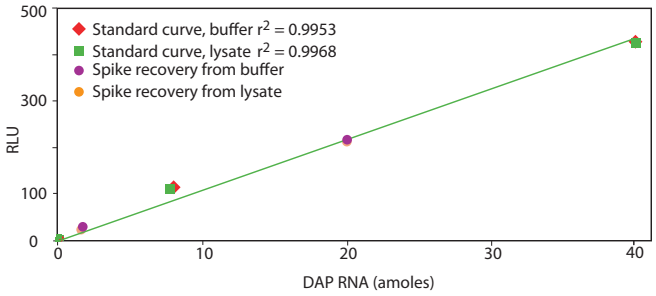
The standard QuantiGene kit protocol was followed. Briefly, the cell lysates were transferred to a capture well in the presence of a gene-specific probe set and then incubated at 53.0 °C overnight. Wells were washed. They were then incubated at 46.0 °C sequentially with an Amplifier and an alkaline phosphatase-linked Label Probe with a wash between the incubations. After a final wash, the luminescent alkaline phosphatase substrate dioxitane was added and was incubated at 46.0 °C for 30 min. The luminescence signal was detected using a Lmax luminometer.

Results

Precision

In order to characterize the performance capabilities of the QuantiGene Reagent System, the mRNAs from three cytokine genes and a housekeeping gene were measured both before and after stimulation of U937 cells with phorbol myristate acetate (PMA) for 48 hours, followed by lipopolysaccharide (LPS) for four hours. The cytokine genes were selected to provide a broad range of fold-induction. The literature indicates that when U937 cells are stimulated with PMA/LPS, the IL-6 gene is induced approximately 10-fold, the TNF-α gene is induced approximately 100-fold, and the IL-8 gene is induced approximately 1000-fold, while the expression of the GAPDH gene is unaffected. Following stimulation of the U937 with PMA/LPS, the mRNA levels of each gene were measured. The entire experiment was repeated on four different days, and on each day, the mRNA measurements were performed in triplicate. The results are shown in Figure 2. In this experiment, in vitro transcribed RNAs for these four genes were used in the QuantiGene Reagent System to construct a dose response standard curve, which serves as a reference to convert the luminescent signal to number of mRNA copy per cell.

The analysis of the data reveals coefficients of variation (CVs) that range from 2.0% to 7.0% over a 3-log range of relative luminescence (RLU) values. Coefficient of variation values below 10% are the hallmark of biological assays that are considered high precision, and give the researcher confidence in the reproducibility of their results.



Sample	RLU	Actual (amoles)	Calculated (amoles)	% Recovery
Buffer	22.86	1.6	1.5	93.8
	210.17	20.0	19.2	96.0
Lysate	21.92	1.6	1.5	93.8
	217.07	20.0	19.9	99.5

Figure 3. Recovery of DAP RNA spiked into lysates.

Accuracy

In order to measure the accuracy of the QuantiGene Reagent System, a dilution series of a bacterial control RNA (DAP) ranging from 0.01 to 40.00 attamoles, was added to samples of either buffer or HL60 human cell lysate. Measurements of the DAP RNA levels in these samples were determined, and standard curves in buffer (red) and in lysate (green) were plotted. Standard curve such as these can then be used to infer the attamoles of this same RNA in other samples by plotting the RLUs generated by the QuantiGene system.

Following the generation of these standard curves, two known concentrations of DAP RNA (1.6 and 20 amoles) were added into either buffer (purple) or HL60 lysate (orange), and the RLU measurements obtained using the QuantiGene Reagent System on these samples were plotted over these standard curves. The results are shown in Figure 3, where the table shows the percent recovery of the spiked RNA.

A technology that is 100% accurate would detect 100% of an mRNA species present in a sample. In this experiment, the QuantiGene Reagent System detected 93.8% of the 1.6 amole DAP RNA spike and 96% of the 20 amole DAP RNA spiked into a simple buffer solution.

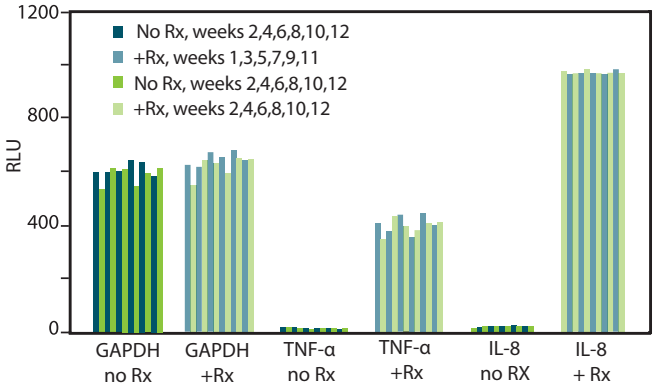
More notably, the QuantiGene Reagent System also detected 93.8% of the 1.6 amole of DAP RNA spike and 99.5% of the 20 amole DAP RNA spiked into an HL60 cell lysate, which presents a much higher complexity sample, and therefore a more challenging test of assay accuracy. The standard curves in this figure span a linear dynamic range of 3.5 logs and are identical in both buffer and lysate. This indicates that the molecular complexity of crude cellular lysates does not affect the assay accuracy, which in turn indicates that the QuantiGene Probe Set is recognizing a very specific target.

Robustness

To test the robustness of the QuantiGene Reagent System, U937 cells were treated with either vehicle (No Rx) or PMA/LPS (+Rx) to induce the TNF-α and IL-8 genes. The cells were then lysed, and the lysates frozen at –80°C in the absence of the Probe Set. At weekly intervals, the lysates were thawed, and the IL-8, TNF-α, and GAPDH mRNA levels were measured in triplicate, over a total of 12 weeks. The results of this experiment are shown in Figure 4, where each bar represents one week.

The data analysis for each group, shown in the table, indicates that over a 12-week period, the %CV ranged from 0.3% to 6.4% for assay replicates performed on a single day, and from 0.3% to 15.9% for assays performed over 12 weeks.

The ability of an assay to maintain such high intra-experimental and inter-experimental precision over a wide range of RLUs, despite working directly from crude cell lysates that have been previously frozen and thawed, demonstrates an unprecedented level of robustness for a technology that measures mRNA levels. It also demonstrates the excellent stability of mRNAs in the cell lysate mixture, even in the absence of the stabilization provided by the Probe Set.



Treatment Group	Intra % CV	Inter % CV
GAPDH control	2.31	6.05
GAPDH treated	5.79	7.69
TNF-α control	6.39	15.87
TNF-α treated	5.58	7.89
IL-8 control	3.28	15.34
IL-8 treated	0.31	0.35

Figure 4. RNA quantitation using cell lysates frozen over a 12-week period.

Discussion

In the experiments described above, the QuantiGene Reagent System demonstrated a high precision over a 3 log range of RLUs, with CVs of less than 10%. The spike recovery experiment demonstrated an accuracy of greater than 90%, despite assaying directly from crude cellular lysates.

The QuantiGene system was also shown to be highly robust and reproducible in experiments where repeated freeze-thaw cycles, and varying lysate complexities, had no effect on performance. The data presented indicate that the QuantiGene Reagent System is a powerful tool for quantitating RNA, with exceptional accuracy, precision, and robustness.

The QuantiGene Reagent System owes its power to three technical advantages. First, by eliminating the need to purify or amplify RNA, the process of going from cells to accurate RNA measurements is simplified. 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. Second, the assay is highly specific due to the cooperative hybridization, rather than simple hybridization, of a Probe Set uniquely designed for each mRNA.

Finally, the signal is amplified rather than the target, to achieve a final result that is proportional to the original target abundance. Other technologies that amplify the target can variably amplify different mRNAs and skew the final results.

The QuantiGene Reagent System is an alternative that offers many advantages compared to technologies commonly used to quantitate RNA. It is an alternative that gives the researcher confidence that the variation in mRNA levels measured in their experiments is the result of the biological variation under investigation, rather than variation in their experimental methods.

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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