Panomics

Performance of the QuantiGene Reagent System for Predictive Toxicology

Introduction

Biomarkers have always been an important way to predict the toxic effects of compounds. Gene expression changes are a popular type of biomarker for the field of predictive toxicology. However, the quantitative requirements of predictive toxicology studies can strain the performance limits of many methods of RNA quantitation. Commonly used methods of RNA quantitation may be limited in accuracy and precision over the dynamic range required for predictive toxicology studies. Aside from such shortcomings in accuracy and precision, other characteristics of the commonly used methods for RNA quantitation are also problematic, such as complexity, low amenability to automation, poor reproducibility, and assay development difficulties.

Because of these issues, alternative methods are of general interest to the research community. Data are presented on a new alternative technology, the QuantiGene® Reagent System, and its performance for measuring changes in gene expression in predictive toxicology applications.

The QuantiGene Reagent System Technology

The QuantiGene system can measure RNA levels directly from crude cell lysates or tissue homogenates. The assay does not require RNA purification or target amplification, and is performed in standard 96-well plates directly from cellular lysates or tissue homogenates. In addition, the assay requires only standard laboratory equipment and skills. Detailed descriptions of the QuantiGene system technology, and the Probe Sets used in the experiments described in this Application Note, have been previously published, and are available at www.panomics.com.

Materials and Methods

Animals and Tissues

Male Fisher rats (150–200 grams) were treated for 4 hours, 1 day, 7 days, or 14 days with either vehicle alone or with a compound being evaluated for its potential toxic effects. Following sacrifice, liver and heart tissues were dissected and placed in 5 volumes of RNAlater buffer (Ambion). Two uL of 50 ug/uL Proteinase K (Panomics) was added to 600 uL Homogenizing Solution (Panomics) with up to 10 mg tissue. The tissue samples were homogenized, transferred to a microcentrifuge tube, and incubated at 65°C for 15 minutes, vortexing every 5 minutes. The tubes were centrifuged to remove debris, and the supernatant transferred to a new tube. For samples from which total RNA was purified, the protocol for the RNA Purification Kit (Epicentre) was performed according to the manufacturer's specifications.

RNA Quantitation

To measure gene expression levels, either tissue lysate or an RNA quantity equivalent to 1000 cells was transferred to a separate Capture Plate well for each gene to be tested. Probe Sets were designed for the biomarker genes and Gapd (Catalog No. PC-10036), which was used as a control to normalize the expression of the biomarkers. The Probe Sets were prepared according to the product instructions, and added to appropriate wells of the Capture Plates. The Capture Plates were sealed and incubated at 53°C for 18 hours. QuantiGene Amplifier and Label Probe were sequentially added, and the remainder of the protocol followed according to the product instructions (Panomics).
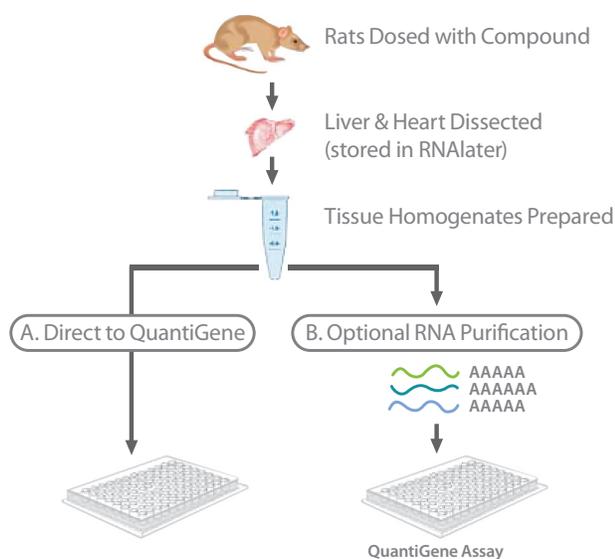
Figure 1. Experimental Workflow Using Tissue Homogenates Directly (A) or Using Purified RNA (B).
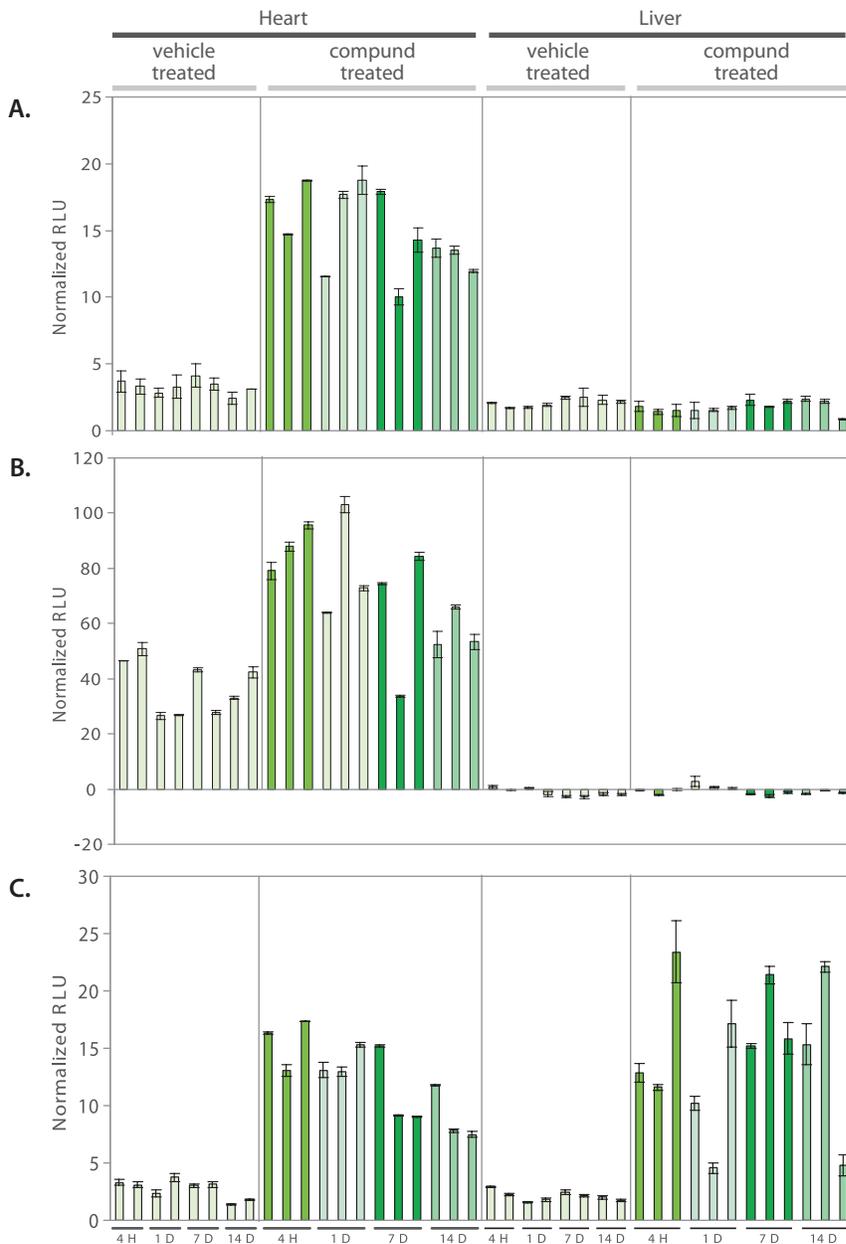
Results

Precision and Reproducibility

To evaluate the performance of the QuantiGene system, 8 rats were treated with vehicle and 12 rats were treated with a single bolus dose of the compound to be evaluated. Following treatment, heart and liver tissues were dissected from 2 vehicle treated and three compound-treated animals each at 4 hours (4 H), 1 day (1 D), 7 days (7 D) , and 14 days (14 D). Total RNA from those tissue samples was prepared. The expression of 18 biomarker genes and the control Gapd gene were measured using the QuantiGene system, with 4 replicates performed for each sample. The results for three of the bio-marker genes are shown in Figure 2, panels A, B, and C, where each bar represents the normalized average expression level of the biomarker gene from one tissue from one animal, and the error bars indicate one standard deviation.

A number of conclusions can be made from this data. First, Gene A is expressed at lower levels in heart and liver of untreated animals, and is induced as early as 4 hours after treatment in heart, but not liver, by the test compound. The average coef-ficient of variation (CV) for this experiment was 3.7%, demonstrating the high preci-sion of the QuantiGene system. Second, Gene B is expressed in heart of untreated animals, and is induced as early as 4 hours after treatment in heart, by the test compound. Expression of Gene B was extremely low in liver, and was not induced by the test compound. The average CV for this experiment was 2.7%, again demonstrating the high precision of the QuantiGene system. Finally, Gene C is expressed at lower levels in heart and liver of untreated animals, and is induced as early as 4 hours after treat-ment in heart and liver by the test compound. The average CV for this experiment was 4.2%.

In total, 18 biomarker genes were evaluated to assess the potential toxicity of the compound under investigation. Each biomarker gene was measured in two tissue samples from each of 20 animals, with four replicates performed per sample. The average Gapd level for a sample was used to normalize the average biomarker gene measurements, and the average CVs obtained for the entire set of

Figure 2. Effect of a Test Compound on the Expression of Genes A, B, and C. Liver- and Heart-Specific Gene Expression and Induction are Shown.

measurements performed for each of the biomarker genes was calculated. The results are shown in Figure 3.

The average CV obtained across the entire experimental program was only 5.6%, demonstrating the low variability of the QuantiGene system. The data shown in Figure 3 is a summary of the entire data set collected, and allows the evaluation of the routine performance of the QuantiGene system. Average CV values below 10% are the hallmark of biological assays that are considered to have high precision, and that provide the researcher confidence in the reproducibility of their results.
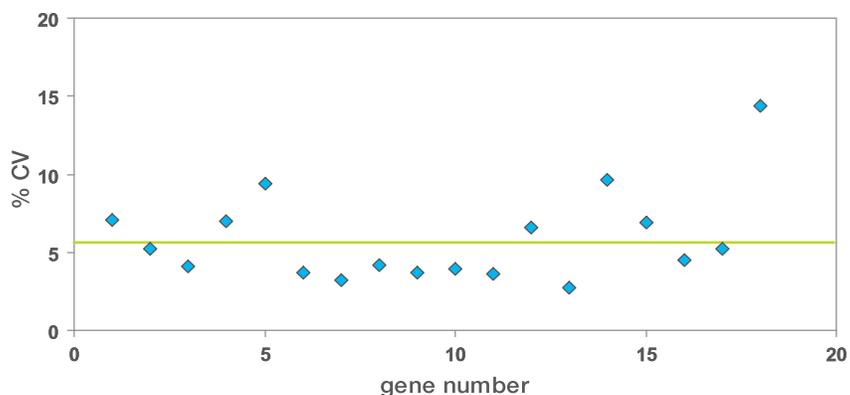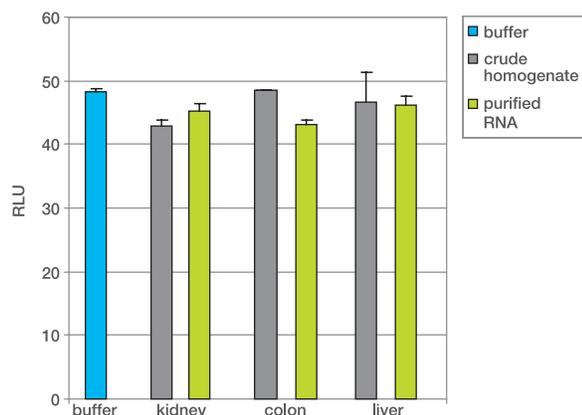
Figure 3. The Average CV Was 5.6% for the Entire Experimental Program.

Accuracy of the QuantiGene System Working Directly from Crude Tissue Homogenates

In order to compare the accuracy of the QuantiGene system when working from purified total RNA versus crude tissue homogenates, kidney, colon, and liver tissues were dissected from rat. Crude homogenates were prepared from half the tissue, total purified RNA was prepared from the other half, and aliquots equivalent to that obtained from 1000 cells were made. A known quantity (1 attomole) of the bacterial RNA DAP was spiked into a sample of buffer, each of the crude tissue homogenate aliquots, and each of the purified total RNA aliquots. The QuantiGene system was then used to measure the level of DAP RNA in each sample. Four replicate measurements were performed per sample, and the results are shown in Figure 4, where each bar shows the average DAP RNA measurement from one sample, and the error bars indicate one standard deviation.

This experiment was designed to determine the ability of the QuantiGene system to detect a known quantity of an RNA in solutions that contain an increasing level of molecular complexity. A technology that is 100% accurate would detect 100% of the mRNA species present in a sample. In this experiment, the QuantiGene system detected 94.4% of the DAP RNA spiked into purified total

RNA from kidney, 90.3% of the DAP RNA spiked into purified total RNA from colon, and 96.0% of the DAP RNA spiked into purified total RNA from liver. More notably, the QuantiGene system also detected 89.6% of the DAP RNA spiked into crude kidney homogenate, 100.3% of the DAP RNA spiked into crude colon homogenate, and 96.8% of the DAP RNA spiked into crude liver homogenate. These measurements made from crude tissue homogenates were not statistically different from the measurements made from purified total RNA. Crude tissue homogenates have a much h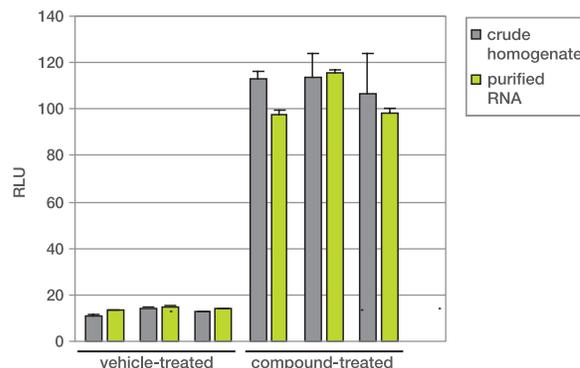igher molecular complexity, and therefore are a more challenging test of assay accuracy than purified total RNA. The high accuracy observed in this experiment indicates that the molecular complexity of crude tissue homogenates does not affect the accuracy of the QuantiGene system, indicating it is unnecessary to purify total RNA.

Robustness of the QuantiGene System Using Crude Tissue Homogenates in Predictive Toxicology Applications

In order to evaluate the robustness of the QuantiGene system when working from crude tissue homogenates, Pgm1 expression levels were measured in both crude liver homogenates and purified total liver RNA from rats treated with vehicle (3 animals) or a test compound (3 animals).

Four replicate measurements were performed for each sample. The results are shown in Figure 5, where each bar represents the average gene expression level of the Pgm1 gene from the liver of one animal, and the error bars indicate one standard deviation.

The Pgm1 expression measurements made from crude tissue homogenates were not statistically different from those made from purified total RNA. The variation that was observed is thought to arise from the variable loss of RNA that occurs during purification. The similarity of the data indicates that the molecular complexity of crude tissue homogenates does not affect the assay's performance, which in turn indicates that the purification of total RNA is unnecessary.

Figure 4. Comparison of the QuantiGene System Accuracy Using Crude Tissue Homogenates Versus Purified Total RNA as the Starting Material.

Figure 5. Comparison of the QuantiGene System Performance Using Crude Tissue Homogenates Versus Purified Total RNA as the Starting Material.

Discussion

Fifty percent of the compound attrition during drug development is attributed to issues related to the toxicity or ADME of the compound. In order to help alleviate this tremendous attrition burden on the industry, it is critical to develop methods to accurately predict toxicity and ADME issues earlier in the drug discovery process. The development of animal- and cell-based assays that predict the toxic effects of compounds in humans will require two scientific achievements. First, appropriate biomarkers must be identified, and second, highly quantitative and robust assays for these biomarkers that can be routinely used at some reasonable throughput must be developed.

In the experiments described above, the QuantiGene system demonstrated very high levels of accuracy, precision, and robustness in the types of studies that are commonly performed by researchers in the field of predictive toxicology. CVs for the QuantiGene measurements averaged just 5.6% for measurements made for each of 18 different genes in two tissues from 20 different animals. Other commonly used methods of RNA quantitation have such high inter-experimental and intra-experimental variation that CVs are not routinely reported.

The QuantiGene system demonstrated an average accuracy of 94.6%, even when assaying directly from crude tissue homogenates. In addition, the QuantiGene system was shown to be highly robust in experiments that compared the performance obtained using crude tissue homogenates to that of purified total RNA. These experiments clearly demonstrate that purification of RNA is not necessary to obtain accurate and precise gene expression measurements.

The data presented indicate that the QuantiGene Reagent System has exceptional accuracy, precision, and robustness for a technology used to measure gene expression, and is a powerful tool for predictive toxicology programs.

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
QuanitGene 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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