Panomics

Sensitive and Quantitative Measurement of Gene Expression Directly from a Small Amount of Peripheral Whole Blood

Introduction

Peripheral blood is an easily accessible tissue from which surrogate biomarker discovery for a variety of diseases is being actively explored. However, accurate and precise quantification of mRNA in whole blood is made difficult by gene expression changes caused by sample handling,[1,2] as well as by the requirement of RNA isolation and subsequent enzymatic manipulation. Some components of blood, such as the heme compounds and globin mRNA, can cause variations in the quality of isolated RNA, the efficiencies of reverse transcription and PCR,[3] and the quality of microarray hybridization,[4] reducing the accuracy and reproducibility of mRNA quantitation.

In fact, when using the current technologies commonly available for expression profiling from blood, gene expression patterns can be strongly dependent on the choice of RNA preparation techniques themselves.[5] Blood gene expression profiling would therefore significantly benefit from any assay technology that requires minimal blood processing and is independent of RNA isolation and enzymatic manipulation requirements. Such technology has been unavailable to date.

A sandwich hybridization assay coupled with signal amplification, the QuantiGene® branched DNA (bDNA) technology offers an enzyme-free solution to measure RNA levels in cell culture lysates[6] and RNA viral particles purified from plasma.[7] However, since whole blood presents unique challenges not found in cultured cells, initial attempts to apply the bDNA technology to whole blood failed to generate specific signals. In these early attempts, the purification of RNA from the PBMC fractions was necessary.[8]

To overcome this problem, we have modified the reagents and protocols described in the original QuantiGene assay and adapted the assay specifically for direct RNA measurement in lysed whole blood (QuantiGene Blood), with excellent sensitivity and reproducibility. The workflow is simple and compatible with most blood collection methods (Figure 1). Importantly, the assay does not require blood processing (other than lysis) or RNA purification. Because signal detection is based on hybridization between target RNA and oligonucleotide probes, no enzymatic manipulation of the target is needed; the measured signal is directly proportional to target RNA, instead of its derivatives such as cDNA, cRNA or amplified product. The assay is offered in a single-plex format using multi-well plates and chemiluminescent detection (QuantiGene Blood), and in multiplex format (QuantiGene Plex Blood) by combining an encoded bead platform (Luminex xMAP®) with fluorescent detection.

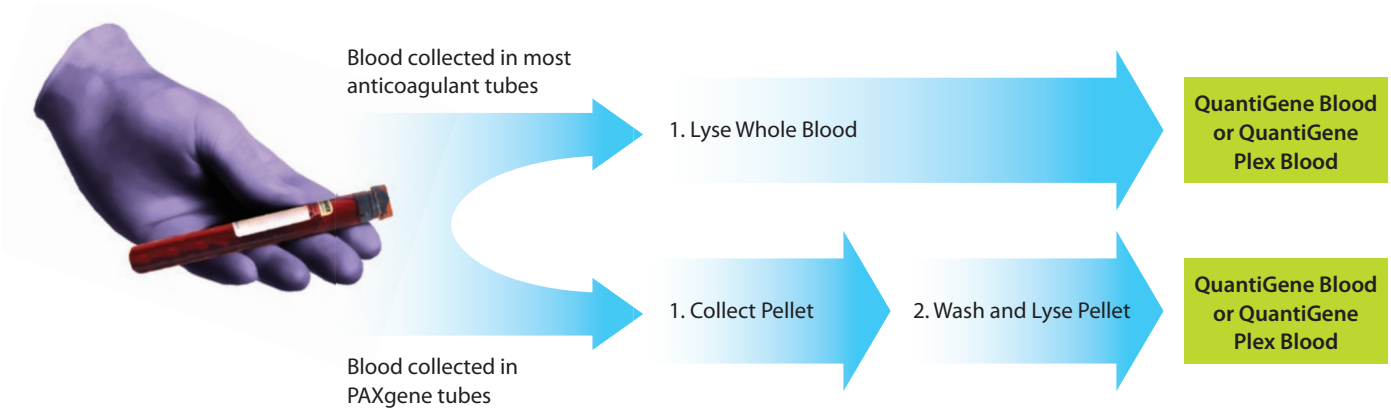

Figure 1. Workflow for Direct RNA Profiling from Whole Blood. Fresh whole blood collected in common anticoagulant tubes (e.g., EDTA, citrate or heparin) or stabilized in PAXgene tubes can be assayed for gene expression without RNA isolation.

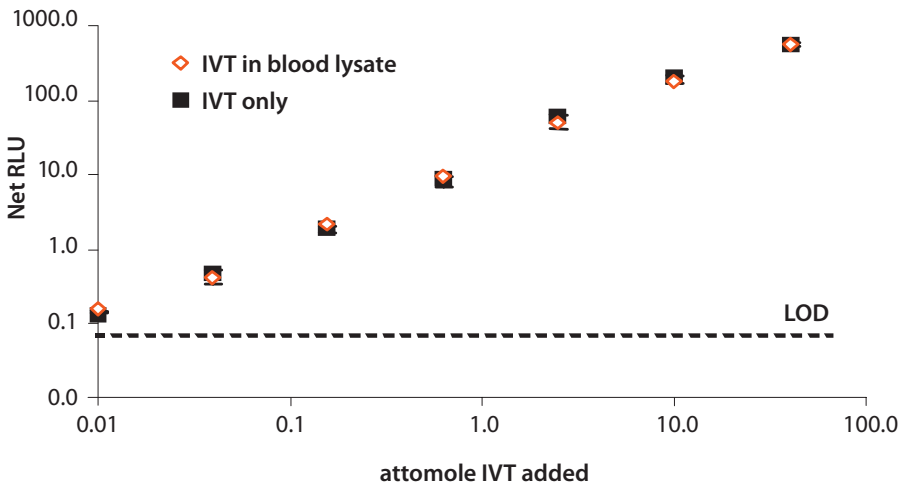

Figure 2. QuantiGene Blood is Insensitive to Interference from Blood Components. Detection of E.coli dapB gene in vitro transcripts (IVT) in the presence (diamond) and absence (square) of lysates from 25 µl whole blood, using QuantiGene Blood. Mean + SD values are graphed. R2 values for both curves are 0.99.

Results and Discussion

Performance of QuantiGene Blood on Whole Blood

QuantiGene Blood reagents and protocols completely inactivate RNase during lysis, while promoting robust and specific hybridization. This is demonstrated by the complete recovery of known amounts of an exogenous E.coli transcript when added to the whole blood lysate (Figure 2). The assay can specifically detect fewer than 6000 copies (0.01 attomole) of target mRNA, with a dynamic range spanning over 3 log, in the presence of a complex background from 25 µl whole blood. Nearly 100% of the target molecules were captured onto the solid capture surface, as indicated by the minimal signal obtained from a second assay measuring the unbound supernatant (data not shown).

For endogenous blood mRNA detection, we obtained quantitative responses for a variety of blood cell marker genes from up to 25 µl whole blood, beyond which signals started to deviate (Figure 3). The average Coefficient of Variation (CV) was only 5% (representing 56 assays in triplicates) with a range of 0.3%–18%.

Whole blood drawn in EDTA, citrate, heparin as well as PAXgene® tubes (Figure 4) can be used. Under the assay conditions, genomic DNA in the lysate remained annealed and did not contribute to the signal (data not shown). These results indicate that the assay provides sensitive and specific direct quantitation of RNA. Blood proteins, DNA and the high concentration of reticulocyte-specific RNAs such as globin do not interfere with the assay.

A distinctive feature of the QuantiGene assay is the use of, on average, 20 mRNA-specific oligos for each assay, that together hybridize to 500–600 bases of contiguous target sequence. Under the optimized assay conditions, both capture and detection probes must bind the same RNA molecule in order to achieve a signal, and multiple capture probes must bind the same RNA target in order to capture the RNA to the solid surface. This unique mechanism ensures high specificity of the assay in the complex background of a blood lysate.

The QuantiGene assay also takes advantage of the stronger, more stable helix formation resulting from the hybridization of a set of contiguous probes to a target.[9] This leads to improved probe capture and reduced assay CV. As a result, specific mRNA can be efficiently detected in as little as 5 µl whole blood, even for genes expressed only in the minority blood cell types such as NK cells and B cells (both ~300 cells/µl blood; data not shown). The average CV from replicate blood samples is routinely below 10%, indicating highly reproducible RNA measurements directly from whole blood.

Performance of QuantiGene Plex Blood

QuantiGene Plex experiments using in vitro transcripts added to whole blood lysates also gave excellent spike recovery and good linearity of response without interference from blood components (data not shown). The multiplexed assay has a limit of detection of 0.04 amol (25,000 copies) of target mRNA molecules per assay in a complex background of up to 25 µl whole blood, with an average CV of 8.1%. The multiplex assay was validated by profiling multiple inflammatory cytokine mRNAs in lipopolysaccharide (LPS) stimulated whole blood (Figure 5), an inflammation model that produces a characteristic gene expression induction.

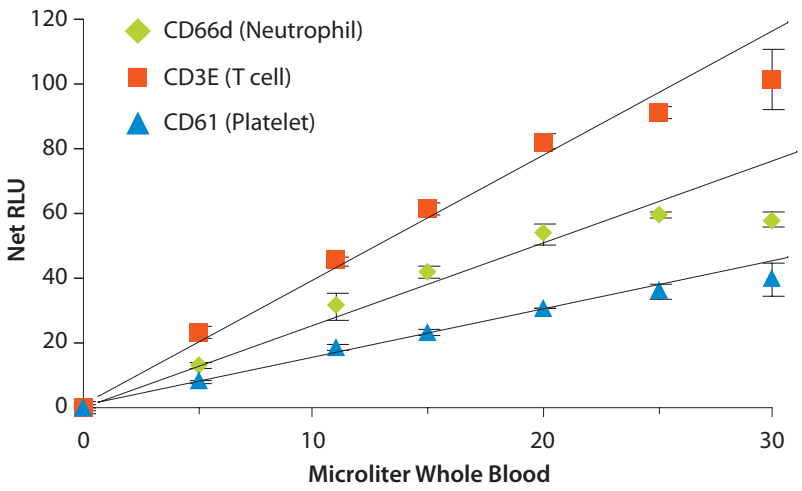

Figure 3. Quantitative Detection of Cell Marker Genes in Heparinized Whole Blood Using the QuantiGene Blood Kit.

Expression of IL-1ß, IL8, IL6, and TNF-α genes was significantly induced by LPS, as previously reported.[10] Interestingly, 37°C ex vivo incubation alone (no LPS) also caused significant basal induction of IL-1ß, IL8 and TNF-α mRNA expression, consistent with the sensitive nature of whole blood to environmental conditions.

To confirm that results from whole blood lysates accurately represent mRNA expression, we extracted total RNA using phenol-chloroform and assayed both the lysate and the RNA from the same sample, for a group of cytokine and apoptosis genes whose expression can be modulated during normal sample handling.[11] Since no known RNA isolation procedure can extract sufficient RNA from less than 25 µl blood, we used 1 ml whole blood for the extraction and took an equivalent amount of RNA. Good correlation was obtained between signals from whole blood lysates and purified total RNA (Figure 6), suggesting that this direct measurement is a reliable alternative to traditional methods of measuring isolated RNA. In addition, the signals from direct lysates are 3–10 times stronger than those of purified RNA from an equivalent amount of blood (Figure 6 and data not shown). The higher signals are not due to interference from red blood cells (RBC), as similar signals were obtained from RBC-lysed blood lysates (Figure 7). Instead, the reduced signal in purified RNA samples appears to be due to RNA loss during extraction, since signals are similar after normalization against an exogenous transcript added to the lysate before RNA extraction (data not shown). Thus, the assay can quantitate multiple mRNAs directly from a small volume of whole blood, including even for samples where blood volumes are insufficient for RNA extraction.

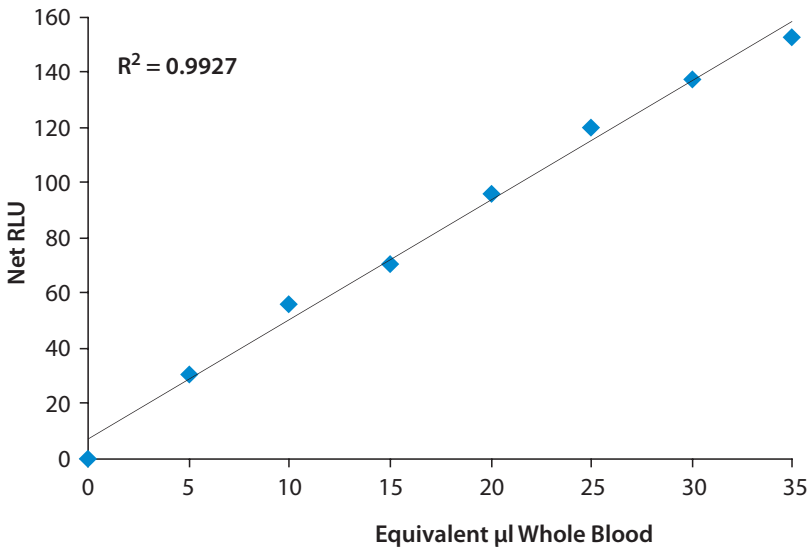

Figure 4. GAPDH Expression in PAXgene Stabilized Whole Blood. Nucleic acid pellet formed in PAXgene stabilized blood was lysed and assayed using the QuantiGene Blood Kit.

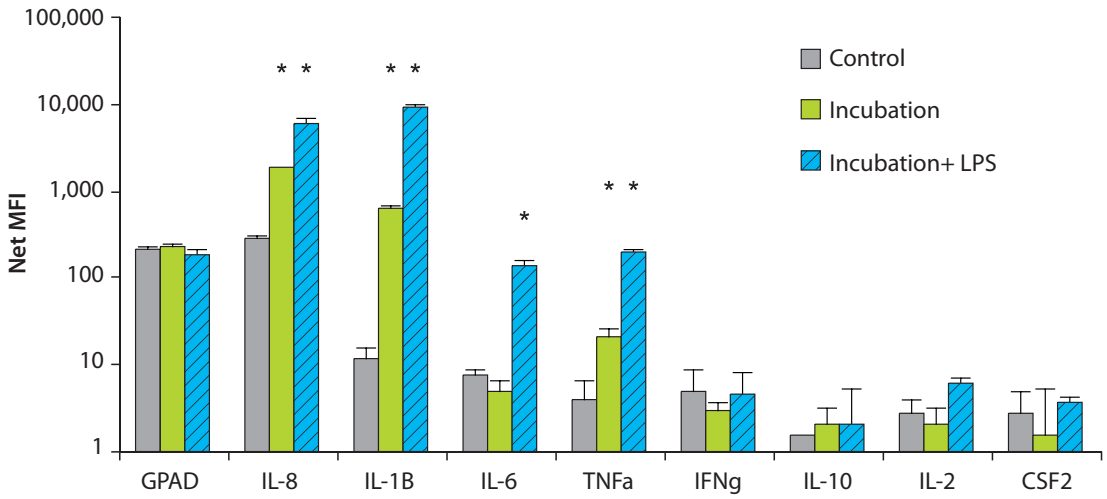

Figure 5. Multiplexed RNA Measurement in Whole Blood Using QuantiGene Plex Blood. Fresh heparinized whole blood was incubated at 37°C for 125 min with or without 10 µg/ml LPS. Sixteen microliters were removed and assayed in multiplex for cytokine gene expressions. Control is blood sample at t=0. * P<0.01 different from Control. Mean+S.D. are shown.

Absolute Quantitation Using Calibration Curves

Because the QuantiGene Blood assay directly measures target RNA with the same hybridization efficiency using either blood lysates or purified RNA (Figure 2), it enables absolute quantitation of mRNA by use of calibration curves with known amounts of RNA target transcripts. We demonstrate this capability with direct, multiplexed absolute quantitation of three cytokine mRNA in whole blood and compared with results from standard method of TaqMan® RT-PCR. Both methods used the same blood samples and RNA calibrators, and the results were in good agreement (Figure 8). However, since the multiplex assay did not involve RNA purification and enzymatic reactions, and has the ability to measure multiple genes and construct multiple calibration curves simultaneously, it has substantially higher sample throughput, is less labor-intensive, and requires much less sample and assay reagents than does RT-PCR.

Blood biomarker studies often involve microarray profiling on a limited number of samples, followed by the clinical validation of dozens of genes in a larger number of independent samples. QuantiGene Plex Blood, multiplexed bead assay, described here is well suited for such a validation workflow, given its capabilities for a middle level of multiplexing (up to 30 genes) and high sample throughput (100's to 1000's of samples per day).

The Luminex bead platform allows for simultaneous detection of up to 100 genes. However, the assay can in principle be adapted in higher multiplex format such as microarrays. With minimal amount of blood and processing required and no need to purify, label and amplify the RNA targets, the QuantiGene Plex Blood assay overcomes many of the problems currently associated with gene expression profiling of whole blood.[2,4,5]

In summary, QuantiGene Blood assays can significantly simplify blood gene expression analysis. Better accuracy and precision in quantitation is achieved due to direct measurement of target RNA. RNA analysis can be performed with only a limited amount of blood and the elimination of RNA purification is especially useful for large-scale studies, where sample analysis throughput had previously been limited by laborious RNA extractions or insufficient RNA yield. Taken together, the simple workflow, exceptional accuracy, and data consistency are significant advantages of this assay over other blood gene expression assays.

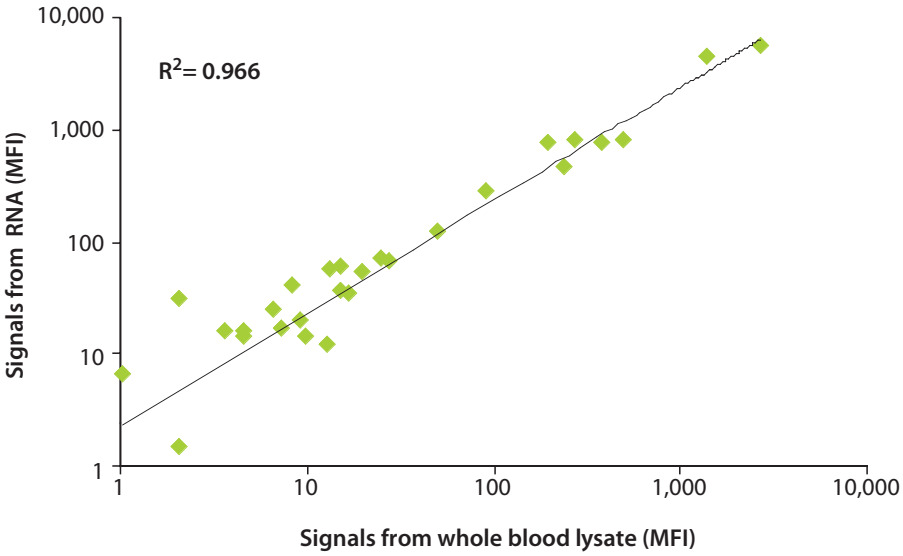

Figure 6. QuantiGene Plex Blood Signals from Whole Blood and Purified RNA. Correlation of QuantiGene Plex Blood signals from whole blood (20 µl) lysate and from RNA of the same sample. Genes assayed in multiplex included TNF-α, IL-10, IL-6, IL-1ß, IFNγ, IL-8, CSF2, GAPDH, RELB, A20, CDKN1, NFKB1, NFKB2, RELA, NFKBIA, BAK, FASL, FAS, RAFTK, BAD, BCL-2, IL6R, BCL-XL, ACTB and CFLAR. Equivalent RNA from 160 µl whole blood was assayed to yield sufficient signals for most genes. Good correlation was evident except for signals approaching LOD of 6 MFI. The slope of the linear regression is 2.34, substantially lower than the expected slope of 8, suggesting RNA is lost during extraction.

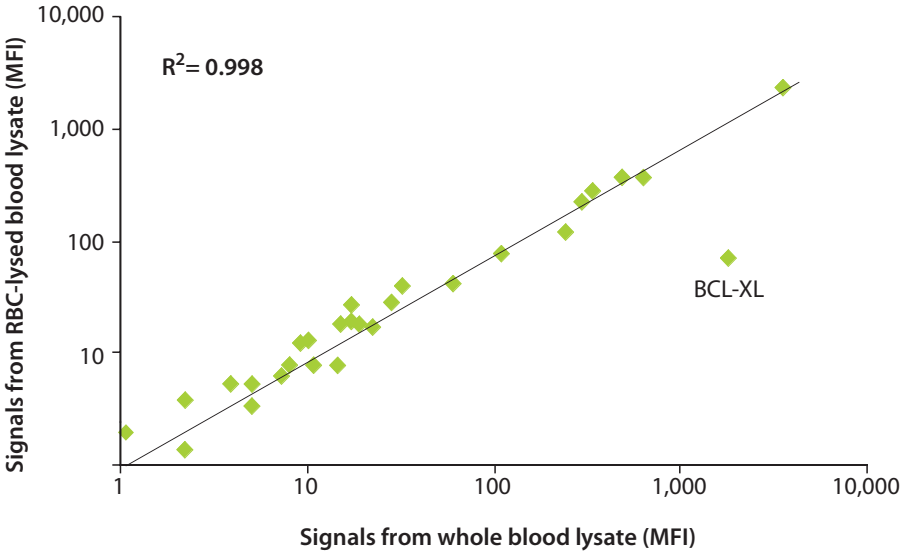

Figure 7. QuantiGene Plex Blood Signals from Whole Blood and RBC-Lysed Blood. Correlation of QuantiGene Plex Blood signals from whole blood and RBC-lysed blood (each 20 µl) lysates. The RBC lysis resulted in little change in expression of the genes listed in Figure 6, except for a significant reduction of BCL-XL, a gene expressed in erythrocytes with an important role for erythropoiesis. The loss of BCL-XL signal thus confirmed successful RBC lysis. Slope of the linear regression (excluding BCL-XL) is 0.86.

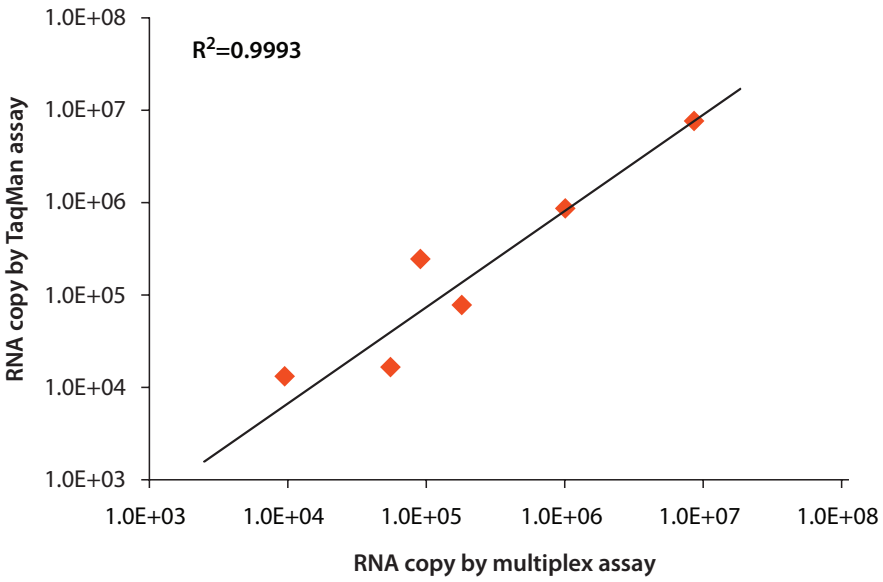

Figure 8. Correlation of Absolute mRNA Quantitation. A multiplex assay was performed on whole blood lysates to simultaneously determine copies of TNF, VEGF and IL-1ß mRNAs in 20 µl of control and LPS treated samples described in Figure 5, using a calibration curve of IVT mixtures. The results were correlated with TaqMan absolute quantitation assay results for the three genes in the same samples, using the same IVT mixtures as calibrators.

Materials and Methods

All reagents are provided in QuantiGene Blood or QuantiGene Plex Blood kits unless noted otherwise.

QuantiGene Assay for Whole Blood

Fresh, anticoagulated (EDTA, heparin or citrate as anticoagulant) blood from healthy donors (Stanford Blood Center, Stanford, CA) were refrigerated and assayed within 1 hour after blood drawn. 1–25 µl of whole blood was added to the lysis solution to a final volume of 125 µl containing 75 µl Blood Lysis Reagent A and 3 µl Blood Lysis Reagent B. The mixture was shaken vigorously at 60°C for 1 hour in a heated shaker to lyse the cells.

Target gene probe set containing CEs (capture extenders), BLs (blocking probes) and LEs (label extenders) was added to the blood lysate to a final volume of 150 µl and then transferred to a QuantiGene Plate for a 16 hour incubation at 58°C. Three washes with 300 µl Wash Buffer were followed by sequential hybridization at 46°C, 1 hour with 100 µl of 1:1000 dilution of Amplifier and 46°C, 1 hour with 100 µl of 1:1000 dilution of Label Probe, with two washes after each incubation.

After a final wash, substrate was added and incubated at 46°C for 30 min to develop luminescent signal, which was detected using a Lmax microtiter plate luminometer (Molecular Devices, Sunnyvale, CA).

QuantiGene Assay for PAXgene-Stabilized Blood

PAXgene stabilized blood was prepared according to manufacturer's protocol (PreAnalytix, Hobrechtikon, Switzerland). 9.0 ml of stabilized blood is equivalent to 2.5 ml of whole blood. After 16 hour storage at room temperature, the stabilized blood was centrifuged for 5 min at 3000 g. The supernatant was removed and the pellet sequentially washed with H_2O (1000 µl per ml of stabilized blood) and PAXgene Wash Buffer (400 µl per ml of stabilized blood), before being lysed at 60°C with shaking for 30 minutes in PAXgene Lysis Buffer (278 µl per ml of stabilized blood) with 0.4% Blood Lysis Reagent B.

One to thirty microliters of lysate, corresponding to the same volume of the original whole blood, was mixed with 75 µl of Blood Lysis Reagent A, probe set, containing of CEs, BLs, LEs, and Capture Buffer to a final volume of 150 µl. The mixture was transferred to an assay well in 96-well plate coated with Capture Probes and incubated for 16 hour at 58°C. Subsequent steps were the same as the post 16 hour hybridization steps described in QuantiGene Blood assay for whole blood.

QuantiGene Plex Assay

Luminex beads conjugated with different capture probes were pooled in equal proportion before use. One hundred microliters of whole blood lysates or PAXgene blood lysates containing 50 µl Blood Lysis Reagent A were mixed with the multiplex panel probe sets and the pooled capture beads (2000 beads each type) in a round bottom well and hybridized for 16 hours at 58°C in 110 µl final volume.

The assay mix was transferred to a filter plate and unbound material was filter-washed from wells three times with Wash Buffer. The plate was then hybridized at 54°C, 1 hour with 100 µl of 3:1000 dilution of Amplifier in Amplifier Diluent. After filter-washing twice with Wash Buffer, the plate was incubated at 54°C, 1 hour with 100 µl of biotinylated Label Probe diluted 3:1000 in Label Probe Diluent. After two washes, Streptavidin conjugated R-Phycoerythrin (SAPE) diluted 3:1000 in SAPE Diluent was added and the plate was incubated at room temperature for 30 min. The beads were washed to remove unbound SAPE, followed by analysis with Luminex 100IS system (Luminex) or Bio-Plex system (Bio-Rad, Hercules, CA). The level of SAPE fluorescence measured from each bead is proportional to the number of mRNA transcripts captured by the beads.

LPS Stimulation of Whole Blood and Absolute mRNA Quantitation

Fresh whole blood (Stanford Blood Center) with or without the addition of 10 µg/ml of E.coli LPS (Sigma) was incubated at 37°C with shaking in cell culture incubator for 30–125 minutes, before being lysed in the vessel and assayed in multiplex according to above described. For absolute mRNA quantitation, a series of IVT mixtures ranging from 0.01 to 40 amoles for each target was made. They were either assayed together with whole blood (20 µl) lysate using QuantiGene Plex Blood Kit, or assayed together with equivalent amount of total RNA from 20 µl blood using standard TaqMan RT-PCR procedures. For QuantiGene Plex Blood assay, quantities of RNA in blood were obtained after fitting the calibration curve with 5-parameter logistics algorithm. For TaqMan assay, quantities of RNA in blood were calculated from threshold cycle values by use of the linear calibration curves.

RBC Lysis and Total RNA Extraction

Red blood cells in whole blood were lysed in RBC Lysis Solution (Epicentre, Madison, WI) according to the manufacturer's recommended protocol. After a brief centrifugation, the supernatant was removed and the pellet resuspended in PBS up to the original volume of whole blood. Total RNA from whole or RBC lysed blood was extracted using TriReagent BD (Molecular Research Center, Cincinnati, OH) according to the manufacturer's recommended protocol.

References

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Ordering Information

QuantiGene Blood Reagent System

Product	Size	Catalog No.
QuantiGene Blood Assay Kit	2 – 96-well plate 10 – 96-well plate	QB0001 QB0002
QuantiGene Blood Sample Processing Kit Whole Blood	2 – 96-well plate 10 – 96-well plate	QB0100 QB0101
QuantiGene Blood Sample Processing Kit PAXgene Blood	192 samples*, 2 – 96-well plate 960 samples*, 10 – 96-well plate	QB0102 QB0103
QuantiGene Blood Probe Set	200 reactions 1,000 reactions	See website

*Each prepared PAXgene sample is sufficient for running 3 assay wells.

QuantiGene Plex Blood Reagent System

Product	Size	Catalog No.
QuantiGene Plex Blood Assay Kit	1 – 96-well plate 3 – 96-well plate 10 – 96-well plate	PB0001 PB0002 PB0003
QuantiGene Plex Blood Sample Processing Kit Whole Blood	1 – 96-well plate 3 – 96-well plate 10 – 96-well plate	PB0100 PB0101 PB0102
QuantiGene Plex Blood Sample Processing Kit PAXgene Blood	96 samples*, 1 – 96-well plate 288 samples*, 3 – 96-well plate 960 samples*, 10 – 96-well plate	PB0103 PB0104 PB0105
QuantiGene Plex Blood, Plex Set	1 – 96-well plate (3–30 plex) 3 – 96-well plate (3–30 plex) 10 – 96-well plate (3–30 plex)	See website

* Each prepared PAXgene sample is sufficient for running 3 assay wells.

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