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

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Background: **Accurate and precise quantification of mRNA in whole blood is made difficult by gene expression changes during blood processing, and by variations and biases introduced by sample preparations. We sought to develop a quantitative whole-blood mRNA assay that eliminates blood purification, RNA isolation, reverse transcription, and target amplification while providing high-quality data in an easy assay format.**
Methods: **We performed single- and multiplex gene analysis with multiple hybridization probes to capture mRNA directly from blood lysate and used branched DNA to amplify the signal. The 96-well plate singleplex assay uses chemiluminescence detection, and the multiplex assay combines Luminex-encoded beads with fluorescent detection.**
Results: **The single- and multiplex assays could quantitatively measure as low as 6000 and 24 000 mRNA target molecules (0.01 and 0.04 amoles), respectively, in up to 25 μL of whole blood. Both formats had CVs <10% and dynamic ranges of 3–4 logs. Assay sensitivities allowed quantitative measurement of gene expression in the minority of cells in whole blood. The signals from whole-blood lysate correlated well with signals from purified RNA of the same sample, and absolute mRNA quantification results from the assay were similar to those obtained by quantitative reverse transcription-PCR. Both single- and multiplex assay formats were compatible with common anticoagulants and PAXgene-treated samples; however, PAXgene preparations induced expression of known antiapoptotic genes in whole blood.**
Conclusions: **Both the singleplex and the multiplex assays can quantitatively measure mRNA expression directly from small volumes of whole blood. The assay offers an alternative to current technologies that depend on RNA isolation and is amenable to high-throughput gene expression analysis of whole blood.**
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The validity and reproducibility of mRNA quantification in peripheral blood affect the potential clinical application of biomarker research *(1)*. Limitations of technologies commonly used in peripheral blood gene expression analysis have hindered the wider application of genomics advances in the clinic *(2)*, *(3)*, and gene expression analysis in peripheral blood remains a challenge *(4)*.

Changes in gene expression during preanalytical handling are a major difficulty unique to peripheral blood mRNA analysis *(5–9)*. The blood-stabilizing reagent PAXgene was developed to prevent RNA degradation and time-dependent ex vivo induction of cytokines and immediate early-response genes *(5)*. An overall gene expression pattern distinct from regular blood leukocytes is found in PAXgene-stabilized blood *(10)*, and it has yet to be determined whether PAXgene reagent induces expression changes in blood samples *(5)*.

Accurate and precise quantification of mRNA in whole blood is made even more difficult by dependence on RNA purification and subsequent enzymatic manipulation. Standard RNA extractions from fresh or stabilized whole blood give RNA of variable yield and quality, which may lead to inconsistent gene expression patterns *(10–12)*. Indeed, although technologies such as microarrays and quantitative real-time PCR (RT-PCR)[1] are highly reproducible, their overall effectiveness has been hampered by inherent variations in sample preparation steps *(13–16)*. Of particular concern is the potential bias caused by

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[1] Nonstandard abbreviations: RT-PCR, quantitative real-time PCR; bDNA, branched DNA; CE, capture extender probe; LE, label extender probe; SA-PE, streptavidin-conjugated R-phycoerythrin; LPS, lipopolysaccharide; and IVT, in vitro transcript.

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reverse transcription *(17)*, a step common to both technologies.

An assay technology that involves minimal blood processing and does not require blood RNA isolation and enzymatic manipulation might overcome these challenges in blood gene expression analysis. Direct detection of target RNA in cell culture lysates has been described *(18, 19)*, but these methods have not been successfully applied to whole-blood analysis,

Sandwich hybridization coupled with signal instead of target amplification can bypass RNA isolation and labeling while achieving sufficient sensitivity. We report the development of a direct whole-blood RNA quantification method using branched DNA (bDNA) signal amplification technology (Bayer). bDNA is a hybridization-based method that uses multiple oligonucleotide probes to capture target RNA and multiple branched DNA multimers to amplify the signal *(20)*. The technology has been cleared by the US Food and Drug Administration for use in detecting HIV and hepatitis C virus mRNAs from viral particles purified from plasma *(20, 21)*, and it has also been used to directly detect RNA in cell culture lysates *(22)*. Initial attempts to apply bDNA to whole blood were unsuccessful, however, and partially purified RNA from the mononuclear fraction had to be used *(23)*. Our bDNA method directly measures the RNA in whole-blood lysates.

Materials and Methods

singleplex assay for whole blood

Fresh, anticoagulated (EDTA, heparin, or citrate as anticoagulant) blood from healthy donors obtained under Institutional Review Board–approved guidelines from Stanford Blood Center (Stanford, CA) was refrigerated and assayed within 1 h after blood draw. We added 1 to 25 μL of whole blood to lysis solution to a final volume of 150 μL containing 50% blood lysis buffer [200 mmol/L LiCl, 50 g/L lithium lauryl sulfate, 9 mmol/L EDTA, 50 mmol/L HEPES (pH 7.5), 2 g/L casein] and 1 g/L proteinase K. The mixture was shaken at 1200 rpm and 60 °C for 0.5 h in a heated shaker to lyse the cells. The blood lysate was assayed immediately or stored at −80 °C for months for later use. Target gene probe sets containing 150, 300, and 600 fmol of capture extender probe (CE), blocking probe, and label extender probe (LE), respectively, were added to the blood lysate, and the lysate was transferred to an assay well in a 96-well plate covalently coated with Capture Probe oligo (5′-CACTTCACTTTCTTTCCAAGAG-3′) and incubated for 16 h at 58 °C. Wells were washed 3 times with 200 μL of wash buffer (0.1× standard saline citrate containing 3 g/L lithium lauryl sulfate), followed by sequential hybridizations at 53 °C for 1 h with 100 μL of a 1:1000 dilution of bDNA amplifier (Bayer Diagnostics) and 46 °C for 1 h with 100 μL (50 fmol) of 3′-alkaline phosphatase–conjugated Label Probe oligo (5′-AAGTACGACAACCACATC-3′), with 3 washes after each incubation. After a final wash, the alkaline phosphatase substrate dioxetane (Bayer Diagnostics) was added to the wells and incubated at 46 °C for 30 min to develop the luminescent signal, which was detected with an Lmax microtiter plate luminometer (Molecular Device).

singleplex assay for PAXgene-stabilized blood

PAXgene-stabilized blood was prepared according to the manufacturer's protocol (PreAnalytiX); 9.5 mL of stabilized blood is equivalent to 2.5 mL of whole blood. After storage for 16 h at room temperature, the stabilized blood was centrifuged for 5 min at 3000*g*. The supernatant was removed by decanting or pipetting. The pellet was sequentially washed with H_2O (1000 μL/mL of stabilized blood) and 2 mol/L LiCl (400 μL/mL of stabilized blood) before being lysed at 60 °C with shaking for 30-min in T&C Lysis Solution (Epicentre; 265 μL/mL of stabilized blood) with 0.25 g/L proteinase K. Lysate was assayed immediately or stored at −80 °C for later use. We mixed 1 to 30 μL of lysate, corresponding to same volume of the original whole blood, with 75 μL of blood lysis buffer and H_2O to a final volume of 150 μL. Target gene probe sets containing 150, 300, and 600 fmol of CE, blocking probe, and LE, respectively, were added to the lysate. The mixture was transferred to an assay well in a 96-well plate coated with capture probes and incubated for 16 h at 58 °C. Subsequent steps were the same as after the 16-h hybridization step described for the singleplex assay for whole blood.

multiplex assay

A panel of oligonucleotide capture probes, each with a unique sequence of 15 bases, were synthesized with 5′-amino linker (BioSearch), and each was covalently linked to carboxylated fluorescently encoded beads (Luminex) according to the recommended conjugation procedure. Beads conjugated with different capture probes were pooled in equal proportions before use. We mixed 100 μL of whole-blood lysates or PAXgene blood lysates prepared as above with the multiplex panel probe sets and the pooled capture beads (2000 beads of each type) in a round-bottomed assay well and hybridized for 16 h at 58 °C (final volume in each well, 110 μL). The assay mixture was transferred to a MultiScreen filter plate (Millipore), and unbound material was filter-washed from the wells by washing 3 times with wash buffer. The plate was then hybridized at 53 °C for 1 h with 100 μL/well of a 3:1000 dilution of bDNA amplifier in amplifier diluent [3 mol/L tetramethylammonium chloride, 1 mL/L Sarkosyl, 50 mmol/L Tris-HCl, 4 mmol/L EDTA, 40 g/L dextran sulfate, 10 g/L bovine serum albumin, and 5 mL/L Micr-O-protect (Roche Molecular System)]. After the plate was filter-washed twice with wash buffer, it was incubated at 46 °C for 1 h with 100 μL/well of 150 fmol 5′-dT(Biotin)-conjugated label probe (Biosearch) diluted in Amplifier diluent without the dextran sulfate. After 2 washes, streptavidin-conjugated R-phycoerythrin (SA-PE;

Prozyme) at 6 mg/L diluted in SA-PE diluent (20 mmol/L Tris-HCl, 400 mmol/L lithium chloride, 1 mL/L Tween 20, 1 mL/L bovine serum albumin, and 5 mL/L Micr-O-protect) was added, and the plate was incubated at room temperature for 30 min. We washed the beads to remove unbound SA-PE and then analyzed them with the Luminex 100IS (Luminex) or Bio-Plex (Bio-Rad) system. The SA-PE fluorescence measured from each bead was proportional to the number of mRNA transcripts captured by the beads.

LIPOPOLYSACCHARIDE STIMULATION OF WHOLE BLOOD AND ABSOLUTE mRNA QUANTIFICATION BY MULTIPLEXED ASSAY OR QUANTITATIVE REAL-TIME PCR

We incubated fresh heparinized whole blood (Stanford Blood Center), with or without added 10 mg/L *Escherichia coli* lipopolysaccharide (LPS; Sigma), at 37 °C with mild shaking in a cell culture incubator for 30–125 min. Aliquots were removed and either lysed and assayed in multiplex according to the method described above or processed for total RNA extraction with a QIAamp RNA Blood Mini Kit (Qiagen) with on-column DNase digestion.

We prepared in vitro transcripts from commercial cDNA clones with sequences covering regions of the designed probes and the TaqMan amplicon. Purified transcripts were quantified with a Quan-iT RiboGreen RNA Assay Kit (Molecular Probes). We prepared a stock solution of 10 human cytokine and housekeeping gene transcripts mixed to a final concentration of 32 amol/μL each in 0.1 g/L yeast tRNA. Before gene quantification experiments, the stock solution was serially diluted 4-fold in 0.1 g/L yeast tRNA to generate a series of RNA calibrators.

For RNA quantification with multiplex assays, RNA calibrators from in concentrations from 40 to 0.01 amoles were assayed under conditions identical to the assays with whole-blood (20 μL) lysates. We obtained the RNA quantities (in attomoles) from the fluorescence intensities after fitting the calibration curve with the 5-parameter logistics algorithm provided by the Luminex instrument software package.

To determine RNA copy numbers by quantitative PCR, we reverse-transcribed 1 μL each of the RNA calibrators containing 32 to 0.008 amoles, as well as equivalent RNA (by volume) from 20 μL of whole blood, in 10-μL reactions with TaqMan reverse transcription reagents with random primers (Applied Biosystems). cDNA was amplified and detected in a Mx4000 PCR System (Stratagene) with TaqMan Universal PCR master mixture and TaqMan probes [Hs00174128_m1 for tumor necrosis factor (*TNF*),[2] Hs99999903_m1 for interleukin-1β (*IL1B*), and Hs00173626_m1 for vascular endothelial growth factor (*VEGF*); Applied Biosystems]. Each sample was reverse-transcribed in duplicate and each cDNA PCR-amplified in duplicate. The calibrations curves [threshold cycle vs log(copy number)] generated all have R^2 values >0.999. RNA copy numbers were calculated from threshold cycle values by use of the corresponding calibration curve.

ERYTHROCYTE LYSIS AND TOTAL RNA EXTRACTION

Erythrocytes in whole blood were lysed in erythrocyte lysis solution (Epicentre) according to the recommended protocol. After brief centrifugation, the supernatant was removed and the pellet resuspended in phosphate-buffered saline to the original volume of whole blood. Total RNA from whole blood or blood containing lysed erythrocytes was extracted with TriReagent BD (Molecular Research Center) according to the recommended protocol.

PROBE DESIGN FOR SINGLE- AND MULTIPLEX ASSAYS

We developed modified probe design software *(24)* to design probe sets for target genes in both single- and multiplex assays. For each target sequence, the software algorithm identified regions that could serve as annealing templates for CEs (5–7 per gene), LEs (10–15 per gene), or blocking probes. CE-LE, CE-bDNA, CE-label probe, and LE-capture probe interactions having highly negative ΔG values were removed to minimize nonspecific hybridization. The probe sets were essentially the same for both the single- and multiplex assays except for the portion of the CE probes that hybridized with the capture probe. We developed three 10-plex panels for the study of signal correlations between lysate and RNA. Probe sequences for each gene tested are listed in Table 1 of the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol52/issue7/.

DATA ANALYSIS AND STATISTICS

Three replicate assays (n = 3) were performed for all described experimental samples unless noted otherwise. For all samples, background signals in the absence of target mRNAs were determined and subtracted from

[2] *TNF*, tumor necrosis factor (TNF superfamily, member 2); *IL1B*, interleukin-1β; *VEGF*, vascular endothelial growth factor; *IL2*, interleukin-2; *IL8*, interleukin-8; *IL6*, interleukin-6; *CFLAR*, CASP8 and FADD-like apoptosis regulator; *EIF1*, eukaryotic translation initiation factor 1 (also known as *SUI1*); *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *ACTB*, actin, beta; *IFNG*, interferon, gamma; *CSF2*, colony stimulating factor 2 (granulocyte-macrophage); *RELB*, v-rel reticuloendotheliosis viral oncogene homolog B, nuclear factor of kappa light polypeptide gene enhancer in B-cells 3 (avian); *CDKN1A*, cyclin-dependent kinase inhibitor 1A (p21, Cip1); *NFKB1*, nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105); *NFKB2*, nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100); *RELA*, v-rel reticuloendotheliosis viral oncogene homolog A, nuclear factor of kappa light polypeptide gene enhancer in B-cells 3, p65 (avian); *NFKBIA*, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; *BAK*, BCL2-antagonist/killer; *FASL*, Fas ligand; *FAS*, Fas (TNF receptor superfamily, member 6); *PTK2B*, protein tyrosine kinase 2 beta (also known as *RAFTK*), *BAD*, BCL2-antagonist of cell death; *BCL2*, B-cell CLL/lymphoma 2; *IL6R*, interleukin-6 receptor; *BCL2L1*, BCL2-like 1 (also known as *BCL-XL*).

signals obtained in the presence of target mRNAs to obtain the net signal. Statistical significance of biological studies was tested with a two-tailed Student *t*-test. The limit of detection was determined as the amount of target in vitro transcript added to the assay that gave a net signal 3 times higher than the SD of the blank control.

Results and Discussion

OVERVIEW OF THE ASSAY

The technology quantifies mRNA by hybridizing ~20 independent oligonucleotide probes to 500–600 contiguous bases of target sequence. The assay amplifies the detection signal with bDNA multimers (Fig. 1). The multiple-probe-per-target design takes advantage of the fact that the interactions between contiguous probes and a target lead to stronger, more stable helix formation because of a base-stacking effect *(25)*. In addition, the "multidentate" interaction between the many capture probes on the solid surface and the multiple CEs bound to 1 target leads to stronger capture than any single-capture probe–CE interaction alone.

ASSAY PERFORMANCE

Interference from blood constituents such as proteins, DNA, and reticulocytes and high ribonuclease activity make quantifying RNA directly from whole blood difficult *(26)*. The original bDNA reagents and protocols from Bayer had high background and did not completely inhibit ribonuclease activity (data not shown). Lysis buffers based on chemotropic agents such as guanidine salts, however, interfere with subsequent hybridization and target capture. We therefore developed a lysis buffer and protocol (see *Materials and Methods*) that ensure complete inhibition of RNase activity while promoting robust and specific hybridization. To reduce the nonspecific assay background, we took advantage of the enhanced interaction between the capture surface (plate or bead) and the many CEs bound to one target (Fig. 1). We increased the hybridization temperature to destabilize the hybridization between a single CE and a surface capture probe while not affecting the stability of the multidentate interaction between the surface and multiple CEs. This modification improved the assay specificity. In addition, we limited the blood volume to no more than 25% of the final lysate and optimized assay conditions to maximize the signal-to-noise ratio.

To determine the analytical sensitivity and specificity of the optimized assay, we added increasing amounts of exogenous in vitro transcripts (IVTs) from *E. coli dap*B (not present in whole blood) during the lysis of a fixed volume of whole blood. Compared with IVT-only controls, exogenous transcript recovery rates from the whole-blood lysate were nearly 100% of known amounts (see Fig. 1a in the online Data Supplement). The assay signal was proportional to the amount of IVT in blood lysates. The assay can quantitatively measure as few as 6000 copies (0.01 amole) of target mRNA with a dynamic range spanning more than 3 logs, in the presence of a complex mixture from 25 μL of blood consisting of $\sim 0.2 \times 10^6$ leukocytes and 0.1×10^9 erythrocytes. We obtained a similar result by adding transcripts of interleukin-2 (*IL2*) that were not detectable in unstimulated whole blood (data not shown). When a fixed amount of IVT was assayed in the presence of increasing amount of blood lysates, the signals were similar (see Fig. 1b in the online Data Supplement), suggesting that nonspecific RNA or protein in blood lysates did not interfere with the assay. For detection of endogenous blood mRNA, we obtained quantitative responses for a variety of blood cell markers in up to 25 μL of whole blood (Fig. 2). The mean CV (representing data from 56 assays run in triplicate) was 5% (range, 0.3%–18%). The mean interrun CV was 9% (range, 0.4%–35%). Because blood cells were lysed and RNase was inactivated, the lysate can be stored long-term without degradation at −80 °C for future use (data not shown). Whole blood drawn into EDTA, citrate, heparin, and PAXgene-

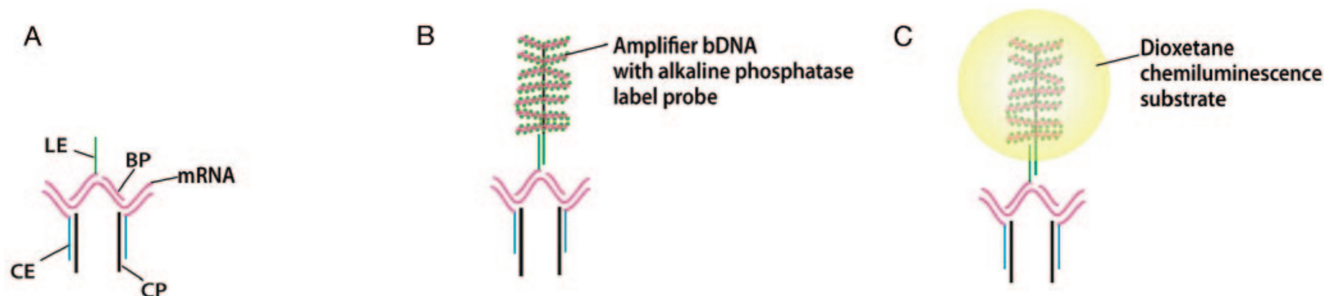

Fig. 1. Schematic of the assay.

(*A*), for each mRNA target, a set of oligonucleotide probes capable of hybridizing a contiguous regions of the target mRNA molecule is designed. Among these probes, some contain a common extension "tail" sequence that is independent of the target sequence but can interact with the solid support. These probes are called capture extenders (*CE*). Other probes contain a different common tail sequence that can interact with the detection system and are called label extenders (*LE*). The remaining probes containing only target specific sequences are called blockers (*BP* or *BL*). After cells are lysed and released mRNAs hybridize with their probes, multiple mRNA-bound capture extender tails hybridize to the complementary capture probes (*CP*) covalently attached to the solid support, leading to the capture of mRNA to the solid support. (*B*), a bDNA molecule *(20)* is then applied and hybridized to the tails of the label extenders. The brances of the molecule hybridize to oligonucleotide probes labeled with alkaline phosphatase (as shown, for singleplex) or biotin (for multiplex). (*C*), chemiluminescence (as shown, for singleplex) or fluorescence (multiplex) detection is used to measure signals generated from labeled probes after the luminescent substrate dioxetane (as shown, for singleplex) or fluorescent SA-PE (for multiplex) is added. The solid support is the surface of either a well (singleplex) or color-encoded bead (multiplex).

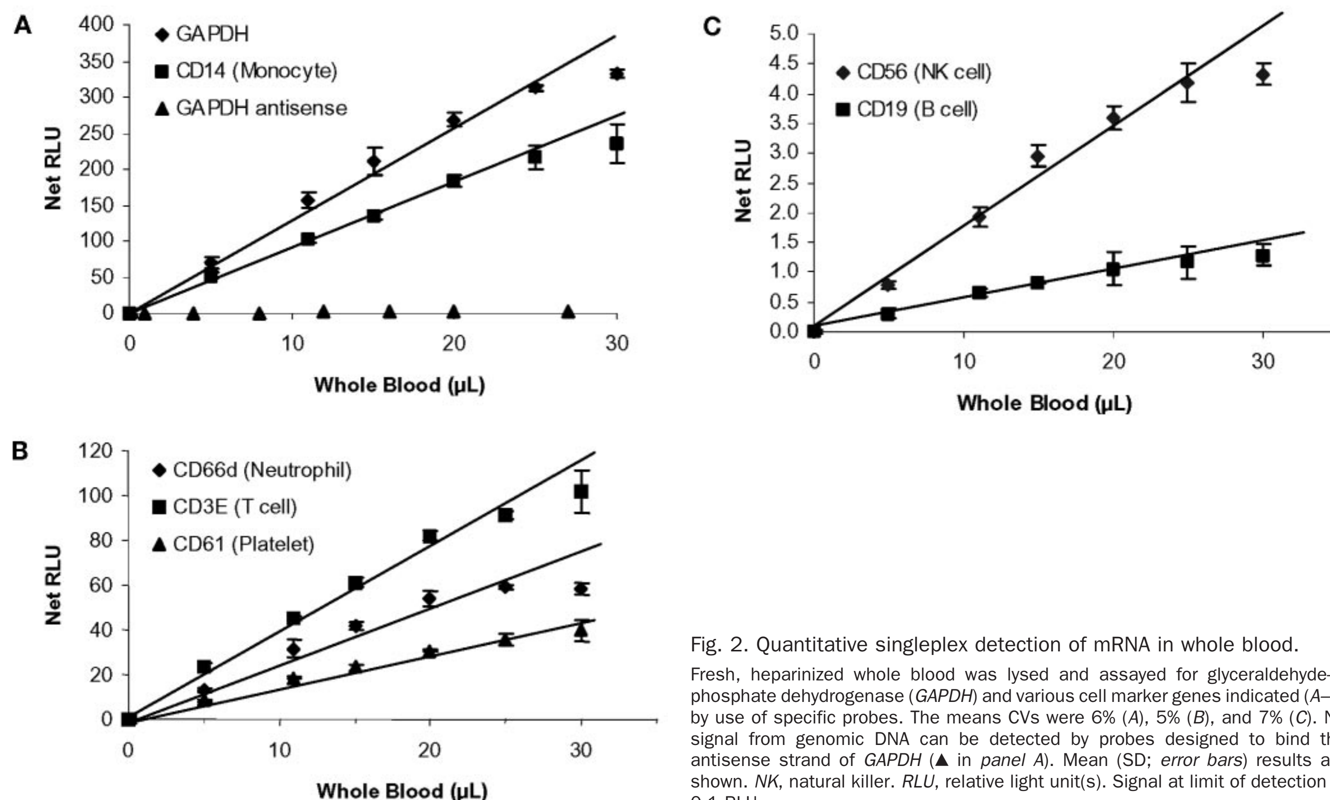

Fig. 2. Quantitative singleplex detection of mRNA in whole blood.

Fresh, heparinized whole blood was lysed and assayed for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and various cell marker genes indicated (*A–C*) by use of specific probes. The means CVs were 6% (*A*), 5% (*B*), and 7% (*C*). No signal from genomic DNA can be detected by probes designed to bind the antisense strand of *GAPDH* (▲ in *panel A*). Mean (SD; *error bars*) results are shown. *NK*, natural killer. *RLU*, relative light unit(s). Signal at limit of detection is 0.1 RLU.

containing tubes gave similar signals for the mRNAs tested (data not shown). Under the assay conditions, genomic DNA in the lysate remained double-stranded: probes for complementary strands did not produce a signal (Fig. 2A). These probes produced signals only when we used cDNA as the target (data not shown). These results indicate that the assay allows sensitive, specific, and direct quantification of RNA, without interference from blood constituents such as proteins, DNA, and the high concentrations of nonspecific RNAs.

In contrast to microarrays and RT-PCR, which rely on single-probe–target interaction for target capture or detection, our assay uses, on average, 20 probes (see Table 1 in the online Data Supplement) that hybridize to 500–600 contiguous bases of 1 target sequence. Under optimized assay conditions, both the capture and detection probes must hybridize to the same RNA molecule to produce a signal, and binding of multiple capture probes to the same RNA target is required for RNA capture on the solid surface. These conditions ensure high specificity of the assay in the complex mixture of whole-blood lysate. The assay also takes advantage of stronger, more stable helix formation from hybridization of contiguous probes to a target *(25)*, which leads to improved target capture and a lower assay CV. In the singleplex assay, >90% of the target molecules were captured on the solid surface, as indicated by the minimal signals from a second assay measuring the unbound supernatant (data not shown). The mean CV for replicate blood samples was routinely <10%. In comparison, the reported CV for replicate blood samples in microarray analysis is 37% *(10)*, and for TaqMan RT-PCR, the reported CV for copy numbers from replicate PAXgene-treated blood is ~16% *(5)*. Thus, our singleplex assay can efficiently detect gene expression in as little as 5 μL of whole blood, even for RNA expressed only in the minority blood cell types, such as natural killer cells and B cells (both ~300 cells/μL blood; Fig. 2C).

To simultaneously detect several distinct mRNAs in whole-blood lysate, we developed a multiplexed assay based on a Luminex bead–based multiplex platform *(27)* with modifications for higher stringency (see *Materials and Methods*). Target-specific hybridization occurs on the surface of different internally color-coded microbeads. When analyzed on the Luminex 100 IS flow system, the color code of each bead identifies the target gene being assayed, and the fluorescent intensities on the beads measure target concentration.

When we added increasing amounts of in vitro–transcribed *dap*B RNA to a constant volume of whole-blood lysate (25 μL), the signals were similar to those for IVT without lysate, and the specific responses in the presence and absence of whole blood showed good linearity, as in the singleplex plate assay (see Fig. 2 in the online Data Supplement). The multiplexed assay has a detection limit of 0.01–0.04 amoles (6000–24 000 copies) of target molecules in the complex mixture from up to 25 μL of whole blood, with a mean CV of 8.1%. The lower sensitivity relative to the singleplex assay is partly attributable to the

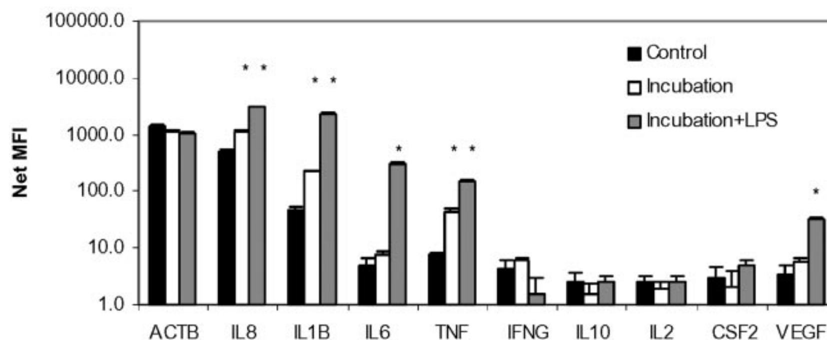

Fig. 3. Quantitative multiplex detection of mRNA in whole blood.

Fresh heparinized whole blood (100 μL) was incubated at 37 °C for 60 min with or without 10 mg/L LPS. To quantify cytokine gene expression with the multiplex assay, 13 μL was removed. Control (■) is blood sample at $t = 0$. * indicates significant difference from control ($P < 0.01$). Means (SD; *error bars*) are shown. *MFI*, mean fluorescence intensity. Signal at the limit of detection is 2.5 MFI.

use of fluorescence detection instead of the more sensitive chemiluminescence detection. We validated the multiplex assay by profiling multiple cytokine mRNAs in LPS-stimulated whole blood. Expression of *IL1B*, interleukin-8 (*IL8*), interleukin-6 (*IL6*), and *TNF* was significantly induced by LPS (Fig. 3), consistent with a previous report *(28)*. In addition, *VEGF* expression in whole blood was induced by LPS. Quantitative signals were detected in the lowest volume tested (4 μL; see Fig. 3 in the online Data Supplement). Interestingly, ex vivo incubation at 37 °C alone (no LPS) caused significant basal induction of *IL1B*, *IL8*, and *TNF* mRNA expression (Fig. 3), consistent with whole-blood responsiveness to environmental conditions.

To confirm that results from whole-blood lysate accurately represent RNA expression, we used phenol–chloroform to extract total RNA and assayed both the lysate and the purified RNA from the same sample for a group of cytokine and apoptosis genes whose copy numbers can be easily affected by sample handling *(29)*. Because no RNA isolation procedure can extract sufficient RNA from <25 μL of blood, we extracted 1 mL of whole blood and used an equivalent amount of RNA in the multiplex bDNA assay. We obtained good correlation between signals from whole-blood lysates and purified total RNA (Fig. 4A), which suggests that direct measurement is a reliable alternative to traditional methods of measuring isolated RNA. However, the signals from direct lysates were 3- to 10-fold stronger than those of purified RNA from equivalent amounts of blood (Fig. 4A and data not shown). The higher signals were not attributable to interference from erythrocytes because we obtained similar signals from lysates of blood containing lysed erythrocytes (Fig. 4B). Instead, the lower signals in RNA samples appeared to be attributable to RNA loss during the phenol–chloroform purification procedure, because the signals were similar after normalization against an exogenous transcript added to the lysate before RNA extraction (data not shown). Thus, the assay can reliably quantify multiple mRNAs directly from small volumes of whole blood.

Because the assay directly measures target RNA with the same hybridization efficiency for blood lysates and

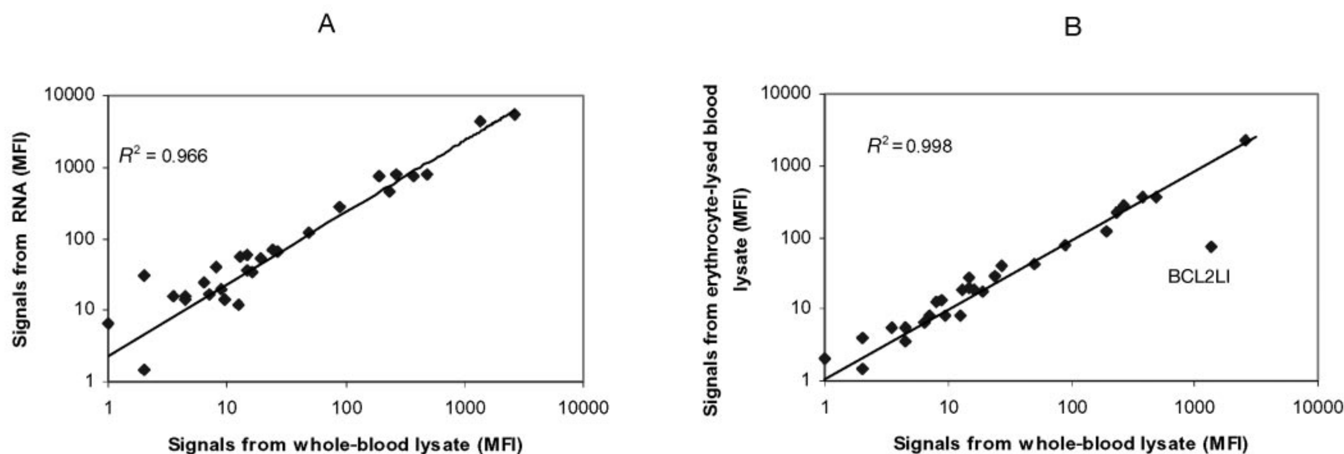

Fig. 4. Correlation of multiplex assay signals.

(*A*), correlation of signals from whole-blood (20 μL) lysate and from RNA of the same sample. Genes assayed in multiplex included *TNF*, *IL10*, *IL6*, *IL1B*, *IFNG*, *IL8*, *CSF2*, *GAPDH*, *RELB*, *A20*, *CDKN1A*, *NFKB1*, *NFKB2*, *RELA*, *NFKBIA*, *BAK*, *FASL*, *FAS*, *PTK2B*, *BAD*, *BCL2*, *IL6R*, *BCL2L1*, *ACTB*, and *CFLAR*. Equivalent RNA from 160 μL of whole blood was assayed to yield sufficient signals for most genes. Good correlation was evident except for signals approaching the limit of detection. The slope of the linear regression is 2.34, substantially lower than the expected slope of 8, suggesting RNA loss during phenol-chloroform extraction. (*B*), correlation of signals from lysates of whole blood and erythrocyte-lysed blood (both 20 μL). Erythrocyte lysis did not significantly change the expression of the genes listed for *panel A*, except for the substantial reduction *BCL2L1*, a gene expressed in erythrocytes that plays an important role in erythropoiesis *(35)*. The loss of *BCL2L1* signal thus verified successful erythrocyte lysis. The slope of the linear regression line, excluding *BCL2L1* (*outlier in panel B*), is 0.86, close to the expected slope of 1. *MFI*, median fluorescence intensity.

purified RNA (see Fig. 1a and Fig. 2 in the online Data Supplement), it enables absolute quantification of mRNA by use of calibration curves constructed with known amounts of RNA target transcripts. We demonstrated this capability by direct, multiplexed absolute quantification of 3 cytokine mRNAs in whole blood (see *Materials and Methods*) and compared those results with results obtained with the TaqMan RT-PCR method (Table 1). We used the same blood samples and RNA calibrators for both methods, and the results were in good agreement (R^2 = 0.999; see Fig. 4 in the online Data Supplement), with similar values and precisions (Table 1). However, because the multiplex assay did not involve RNA purification or 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 smaller amounts of sample and assay reagents than does RT-PCR.

Assessment of the effect of PAXgene stabilization on blood gene expression

Earlier reports suggested that blood stabilization by PAXgene is a slow process that may affect gene expression *(10–12)*. However, interference by excessive amounts of globin mRNA in reticulocytes made comprehensive microarray comparison of fresh and stabilized whole blood impossible *(10)*. Because our assay directly measures blood RNA expression and is not affected by excessive globin mRNA, we were able to assess the impact of PAXgene on blood gene expression. We purified total RNA with the PAXgene reagents and assayed both the lysate and the PAXgene-purified RNA from the same sample for the stress-sensitive genes mentioned above (see legend for Fig. 4A). We found that PAXgene treatment led to significant changes in gene expression compared with direct whole-blood measurement (Fig. 5). Antiapoptosis genes such as CASP8 and FADD-like apoptosis regulator (*CFLAR*) are induced by PAXgene reagent (see Fig. 5 in the online Data Supplement), whereas in direct lysates, lysates of erythrocyte-lysed blood, and phenol-purified RNA, normalized expression was comparable (Fig. 4). Other genes, such as eukaryotic translation initiation factor 1 (*EIF1*), a stress-inducible translation initiation factor *(30)*, were not induced in PAXgene blood (see Fig. 5 in the online Data Supplement), suggesting that the induction was relatively specific to apoptosis genes. An additional experiment assaying lysates of nucleic acid pellets formed in PAXgene blood (see *Materials and Methods*) also confirmed these results, indicating that increased amounts of these apoptosis genes are not artifacts of RNA extraction.

Table 1. Comparison of absolute mRNA quantification results.[a]

mRNA	Blood sample	Mean (SD) concentration, amoles: Multiplex	Quantitative PCR
TNF	Control	0.091 (0.006)	0.028 (0.003)
	LPS-treated	1.66 (0.12)	1.45 (0.18)
VEGF	Control	0.016 (0.007)	0.022 (0.001)
	LPS treated	0.15 (0.01)	0.41 (0.05)
IL1B	Control	0.30 (0.03)	0.13 (0.03)
	LPS treated	14.4 (0.56)	12.6 (1.5)

[a] Copies of *TNF*, *VEGF*, and *IL1B* mRNA in 20 μL of fresh whole blood (control) or whole blood incubated with 10 mg/L LPS at 37 °C for 60 min (LPS-treated) were determined either from whole blood lysates by the multiplex assay or from purified RNA by TaqMan quantitative PCR assay. See *Materials and Methods* for details. 1 amole = 6.02 × 10^5 copies.

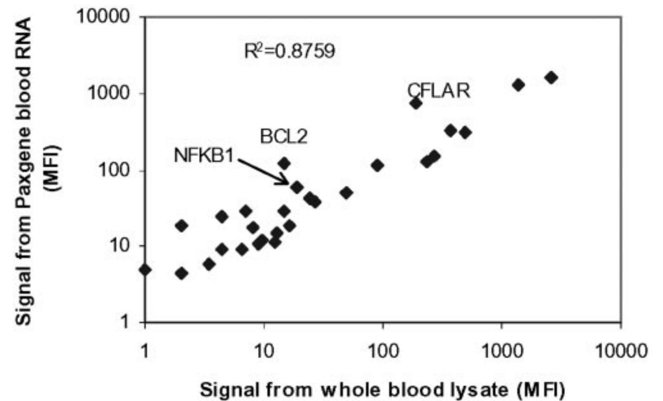

Fig. 5. Effect of PAXgene treatment on gene expression.

Correlation of multiplexed assay signals from whole blood (20 μL) lysate and from purified RNA of the same blood sample stabilized in PAXgene. mRNAs examined are listed in *Fig. 4A*. Equivalent RNA from 160 μL of whole blood stabilized in PAXgene was assayed to yield sufficient signals for most genes. The slope of the linear regression is 0.68, substantially lower than the expected slope of 8. *MFI*, median fluorescence intensity.

These observations agree with the finding that PAXgene treatment of human bone marrow aspirates induces gene expression *(31)*. Thus, for biomarker discovery, to be certain that the gene of interest is not affected by PAXgene, we recommend validation directly from blood without PAXgene treatment, particularly if the identified candidate biomarkers include *CFLAR (32)* or other apoptosis genes.

Blood biomarker studies often involve microarray profiling on a limited number of samples, followed by validation of dozens of genes in larger cohorts. Our singleplex and multiplex assays are both useful as follow-up assays for microarray validation. They have better sensitivity, precision, dynamic range, and sample throughput capability than microarrays, and the assay time from blood collection to mRNA detection is shorter. The Luminex bead platform enables simultaneous detection of up to 100 genes, a number that could be increased substantially by adapting the assay to higher multiplex formats. With minimal amounts of blood and processing required and no need to purify, label, or amplify the RNA targets, this assay overcomes many problems currently associated with gene expression profiling of whole blood *(2, 10, 33)*.

This assay also has several advantages over quantitative PCR, including no required RNA purification steps, the ability to multiplex, higher sample throughput, the ability to use much smaller volumes of blood, and overall better accuracy and precision because of direct measurement of target RNA. With the current design, the assay can directly detect as few as 6000 copies of target RNA. This detection limit is slightly higher than that of quantitative PCR. There are several ways the assay's detection limit can be lowered. One approach is to design additional LE probes per target to increase the number of bDNA amplifiers used to generate the signals. This approach is limited only by the assay cost. Another, more efficient approach is to improve signal amplification efficiency, e.g., by including "preamplifier" oligonucleotides that link each LE with multiple amplifier molecules *(34)*.

In summary, this method can simplify clinical gene expression analysis. RNA analysis can be performed with only a limited amount of blood, and the elimination of RNA purification is particularly useful for large-scale studies, for which sample throughput has been limited by laborious RNA purification or insufficient RNA yield. Taken together, the simple workflow, exceptional accuracy, and consistency of the results give this assay advantages over others for whole-blood gene expression analysis.

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