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Quantification of mRNA in Whole Blood by Assessing Recovery of RNA and Efficiency of cDNA Synthesis

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Background: **Current gene expression analysis relies on the assumption that the isolated RNA represents all species of mRNA in proportions equal to those in the original materials. No system is available for absolute quantification of mRNA.**

Methods: **We applied whole blood to 96-well filterplates to trap leukocytes. Lysis buffer containing cocktails of specific reverse primers and known concentrations of synthetic external control RNA (RNA34) was added to filterplates, and cell lysates were transferred to oligo(dT)-immobilized microplates for hybridization. We then synthesized the cDNA in the oligo(dT)-immobilized microplates from these primer sites and used the cDNA for real-time PCR. RNA34 acted as a universal control, and gene amplification results were converted to quantities of mRNA per microliter of whole blood after the recovery of RNA34 in each sample was determined.**

Results: **Under fully optimized conditions, both added RNA34 and native mRNA species exhibited ~10% recovery from whole blood to real-time PCR. When whole blood was stimulated ex vivo, changes in gene expression as low as 30%–40% were detected with statistical significance, and the experimental CVs were low (10%–20%).**

Conclusion: **This new system to estimate mRNA copies per microliter of whole blood may allow standardization of gene-expression–based molecular diagnostics.**

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Methods to quantify mRNA expression include Northern blot analyses *(1)*, RNase protection assay *(2)*, PCR *(3)*, nucleic acid sequence–based amplification *(4)*, and branched-DNA amplification *(5)*. With current real-time PCR methods, the amounts of various gene-specific cDNA sequences can be determined in each reaction vessel *(6)*. Because the efficiencies of RNA extraction and cDNA synthesis in each sample are unknown, however, the quantities of various mRNA molecules per milliliter blood *(7, 8)* or per cell *(9)* "determined" by these methods are presumed. Moreover, no information is available regarding whether the purified RNA or synthesized cDNA represents all species of mRNA as they exist in the starting materials. Although mRNA data are expressed as means and SD and statistical analysis is used, values are derived from multiple determinations of the final stage of gene amplification alone and therefore do not represent the whole procedure. Microarray chip technologies *(10)* can analyze many mRNA molecules simultaneously, but the results are dependent on the quality of purified RNA and amplified cDNA. The quantity of extracted RNA is measured by absorbance at 260 nm and the quality is assessed by gel electrophoresis to confirm the presence of rRNA bands *(1)*; however, whether the purified RNA represents all species of mRNA in the same proportions present in the original material is unknown.

The reporting of mRNA quantification results as quantities of target mRNA per microgram of total RNA may be inaccurate because mRNA represents only 1%–5% of total RNA. Furthermore, mRNA concentration can vary even when the total RNA concentration is constant. Yields of total RNA or mRNA also vary widely depending on the isolation method used *(7)*. In HIV viral load tests *(11)*, total RNA is extracted from patient plasma samples to which control RNA chosen to be similar in length and sequence to the target RNA is added. No control RNA that can act as a universal internal standard has been described.

Relative quantification by comparison of target gene data to data for housekeeping genes or rRNA is used

widely, although the quantities of control genes may change during experiments *(12)*. This method relies on the assumption that both target RNA and internal standard RNA are extracted with the same efficiency and have the same rate of cDNA synthesis. Because of a lack of standardization, however, it is difficult to compare results from one experiment with results obtained in another experiment or with published results *(13)*. As the number of gene expression studies continues to grow, the need for standardization becomes greater. We investigated a novel method to accurately quantify the amount of human leukocyte-specific poly(A)$^+$ mRNA per microliter of whole blood as a model for standard gene expression analysis.

Materials and Methods

Primers and TaqMan probes were designed by Primer Express (Applied Biosystems) and HYBsimulator (RNAture). DNA oligonucleotides were purchased from Applied Biosystems, IDT, Proligo, TriLink, or GeneScript. The sequences of TaqMan probes, primers, and templates are summarized in Table 1 of the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol52/issue4.

PREPARATION OF RNA

Template oligonucleotides and cDNA were amplified by PCR with T7 forward primers and oligo(dT) reverse primers (see Table 1 in the online Data Supplement) with 30–35 cycles of 95 °C for 30 s (denaturing), 55–65 °C for 30 s (annealing), with final extension at 72 °C for 1 min. The PCR products were analyzed by agarose gel electrophoresis to confirm a clear single band of the expected size. PCR products were then applied to spin columns (SigmaSpin; Sigma) to remove free nucleotides and primers, extracted twice with organic solvents (phenol, chloroform, and isoamyl alcohol; Invitrogen), and then subjected to ethanol precipitation. RNA was then synthesized with an in vitro transcription system (T7 RiboMax; Promega) at 37 °C for 30 min, followed by three 10-min DNase treatments. Each synthetic RNA was extracted with organic solvents and precipitated with ethanol as described above. The final RNA product was suspended in nuclease-free water, and the absorbance was measured at 260 nm [Ultroapec 3100 pro (Amersham) and DU7400 (Beckman-Coulter)]. The lengths of the RNAs [109 for RNA34, 775 for CD4, 473 for p21, and 982 for Fas ligand (FasL)[6]] were confirmed by microchip electrophoresis (iChip; Hitachi Chemical), and the peak areas were >70%. RNA34 was also synthesized by Dharmacon with 86% purity by HPLC analysis. The 40mer poly(A) tail was used because preliminary experiments showed similar recoveries for the 20-, 40-, and 60-mer (data not shown). The cDNA was synthesized from each RNA under different conditions [e.g., varying the concentration of Moloney murine leukemia virus (MMLV), the incubation time, and the primer/template ratio] to find the maximum yield, followed by TaqMan real-time PCR with known concentrations of HPLC-purified DNA oligonucleotides as calibrator. The molar quantities of cDNA were then determined as the amounts of RNA based on the assumption that cDNA synthesis efficiency was 100% under optimum conditions.

ASSAY PROCEDURE

The blood protocol used was approved by the Institutional Review Board. Blood samples were collected at the University of California, Irvine, and Hitachi General Hospital from adult volunteers after written informed consent was obtained. After collection samples were stored at 4 °C until use.

The assay procedure consists of 3 major steps, as shown in Fig. 1: (*a*) leukocyte isolation and lysis on filterplates; (*b*) mRNA isolation, reverse primer hybridization, and cDNA synthesis in oligo(dT)-immobilized microplates; and (*c*) real-time quantitative PCR. The custom 96-well filterplates were manufactured by Whatman or Pall by assembly with leukocyte reduction membranes (Leukosorb; Pall). These filterplates were placed over collection plates, and 150 μL of 5 mmol/L Tris (pH 7.4) was applied to wet the filter membranes. After centrifugation at 120*g* for 1 min at 4 °C to remove the Tris solution from the membranes, 50 μL of well-mixed whole blood sample was applied to each well and immediately centrifuged at 120*g* for 2 min at 4 °C. The wells were then washed once with 300 μL of phosphate-buffered saline. After centrifugation at 2000*g* for 5 min at 4 °C to remove the saline solution, 60 μL of stock lysis buffer [5 g/L *N*-lauroylsarcosine, 4× standard saline citrate, 10 mmol/L Tris-HCl (pH 7.4), 1 mmol/L EDTA, 1 mL/L IGEPAL CA-630 (substitute of NP-40), 1.79 mol/L guanidine thiocyanate (all from Sigma)], supplemented with 1 mL/L 2-mercaptoethanol (Bio-Rad), 0.5 g/L proteinase K (Pierce), 0.1 g/L salmon sperm DNA (5 Prime Eppendorf/Brinkman), 0.1 g/L *Escherichia coli* tRNA (Sigma), 5 nmol/L each of the specific reverse primers, and 10^9 molecules/L of synthetic RNA34 (as external control), was added to each well of the filterplates. The plates were then incubated at 37 °C for 10 min, placed over oligo(dT)-immobilized microplates (GenePlate; RNAture), and centrifuged at 2000*g* for 5 min at 4 °C. After overnight storage at 4 °C, the microplates were washed 3 times with 100 μL of plain lysis buffer and then 3 times with 150 μL of wash buffer [0.5 mol/L NaCl, 10 mmol/L Tris (pH 7.4) 1 mmol/L EDTA] at 4 °C.

cDNA was synthesized directly in each well by addition of 30 μL of buffer containing 1× reverse transcription buffer [50 mM KCl, 10 mM Tris-HCl (pH 8.3), 5.5 mM

[6] Nonstandard abbreviations: FasL, Fas ligand; MMLV, Moloney murine leukemia virus; PBMC, peripheral blood mononuclear cell; Ct, threshold cycle; ACD, acid-citrate-dextrose; PMA, phorbol 12-myristate 13-acetate; CaI, calcium ionophore; PHA, phytohemagglutinin-P; and IL-2, interleukin-2.

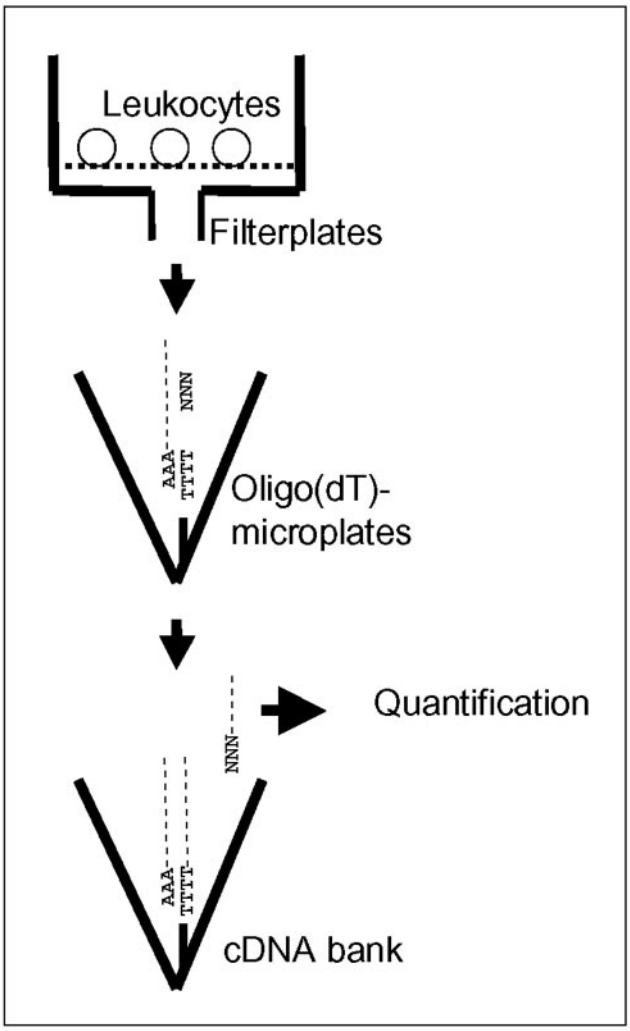

Fig. 1. Assay principle.

Whole blood is applied to 96-well filterplates to trap leukocytes. Lysis buffer containing both synthetic external control RNA and specific reverse primers is added to the filterplates, and cell lysates are transferred to oligo(dT)-immobilized microplates for hybridization. The cDNA is then synthesized in the oligo(dT)-immobilized microplates from these primer sites. The solution, which contains specific reverse primer–primed cDNA, is used for real-time PCR. The plate containing oligo(dT)-primed immobilized cDNA is stored as a cDNA bank.

$MgCl_2$, 1 mL/L Tween 20], 1.25 mM each deoxynucleoside triphosphate, 4 units of rRNasin, and 80 U of MMLV reverse transcriptase (Promega; without primers) and incubation at 37 °C for 2 h. From each 30-μL reaction, 4 μL of cDNA was transferred directly to 384-well PCR plates, and 5 μL of TaqMan universal master mixture (Applied Biosystems) and 1 μL of oligonucleotide cocktail (5 μM each of the forward and reverse primers, and 1–2 μM TaqMan probe) were added. PCR was carried out in a PRISM 7900HT (Applied Biosystems), with 1 cycle of 95 °C for 10 min followed by 45 cycles of 95 °C for 30 s, 55 °C for 30 s, and 60 °C for 1 min. Each gene was amplified in separate wells. The cycle threshold (Ct), i.e., the cycle at which certain amounts of PCR products (based on fluorescence) were generated, was determined with analytical software (SDS; Applied Biosystems).

CALCULATION OF RESULTS

To construct calibration curves for quantification, we synthesized long synthetic DNA oligonucleotides containing sequences of the forward and reverse primers and TaqMan probes for each target (see Table 1 in the online Data Supplement). Each oligonucleotide was purified by HPLC, and the purity was >95%. Each PCR contained 10–10^6 molecules/well of these template oligonucleotides. We used the calibration curve for control RNA34 generated by DNA oligonucleotides to convert the Ct values of the samples to molecules/PCR well (4 μL of cDNA), which were then multiplied by 7.5 (30 divided by 4) to obtain molecules/sample (30 μL of cDNA). We obtained the percentage recovery of RNA34 by dividing these values by the amounts of RNA34 in the original 60 μL of lysis buffer ($6 \times 10^5 = 10^7$/mL × 60 μL). For native mRNA (CD4, p21, and FasL), we determined the molecules/PCR well as described above with the respective calibration curves, and then converted these values to molecules per microliter of blood by multiplying by 7.5 (30 divided by 4), dividing by the percentage recovery of RNA34 in each sample, and dividing by the volume of blood added to each well of the filterplates (usually 50 μL). We calculated the mean (SD) from triplicate aliquots of whole blood and used the Student *t*-test for statistical analyses.

Results and Discussion

MATERIALS AND METHOD DEVELOPMENT

We used 3 criteria to make the system applicable to standardization: (*a*) application of whole blood without isolation of peripheral blood mononuclear cells (PBMCs), although eosinophils are a rich source of RNases *(14)*; (*b*) a high-throughput platform to facilitate statistical analysis from triplicate whole blood aliquots; and (*c*) a minimal number of manipulation steps to reduce between-sample variation *(8, 15–17)*. The third criterion is particularly important because a tiny variation in any step leads to substantial variation at the completion of exponential gene amplification procedures. Even if PCR variation is small in terms of Ct values, it becomes large when the log(Ct) is converted to number of molecules (linear). The assay procedure using filterplates to collect leukocytes was performed as described in the *Materials and Methods* and shown in Fig. 1. As shown in Fig. 1A of the online Data Supplement, filterplates captured more than 89% of leukocytes from whole blood and eliminated more than 87% of erythrocytes. This filterplate procedure is apparently simpler and has a higher throughput than conventional density gradient separation of PBMCs *(7, 9)*.

Leukocytes captured by the filterplate were ready for the subsequent lysis procedure. Because leukocytes were dispersed across the filterplate membrane, the application of lysis buffer was sufficient without any mechanical force. The resulting lysate was transferred to oligo(dT)-immobilized microplates (GenePlate) by centrifugation or vacuum (Fig. 1). This seamless process generated highly

reproducible results. In conventional assays, cells are precipitated by centrifugation and suspended in lysis buffer with vigorous vortex-mixing or pipetting to release mRNA from cell clumps. In this case, the strength of the mechanical mixing may introduce variations. The filterplate method is designed to eliminate this problem.

Because the lysis buffer contained a cocktail of specific reverse primers for the target genes, 2 independent hybridization reactions took place simultaneously: immobilized oligo(dT) hybridized with poly(A) tails of mRNA and specific reverse primers hybridized with the appropriate sites on mRNA (Fig. 1). After hybridization, nonhybridized RNA, DNA, primers, and other cellular materials were removed by aspiration, and cDNA was synthesized without additional primers. Unlike conventional cDNA synthesis in solution, this system did not carry free primers to PCR, and it eliminated the risk of inefficient asymmetric amplification. Interestingly, the specific primer–primed cDNA existed in solution without a heat denaturing step (see Fig. 1B in the online Data Supplement). By washing the microplates extensively with water after cDNA synthesis and using them directly for PCR, we successfully amplified cDNA from microplates with or without specific primers during the hybridization step (see Fig. 1C in the online Data Supplement). According to these data, specific primer–primed cDNA displacement occurred during synthesis of the oligo(dT)-primed cDNA (Fig. 1), a useful feature because the cDNA in solution is used for quantification of target mRNA and the microplate itself can be stored as a cDNA bank, as described previously *(18)*.

In preliminary studies, we isolated total RNA from filterplates with 2 common methods: organic extraction (phenol–guanidine) followed by ethanol precipitation *(19)*, and adsorption of RNA to silica particles followed by elution with nuclease-free water *(20)*. Because they involve multiple labor-intensive steps, these 2 methods showed wide variations when multiple blood aliquots were used as starting materials. The critical problem for quantification was variable recovery of specific mRNAs (data not shown), which we sought to eliminate with the present method.

ASSAY OPTIMIZATION

We identified the optimum reproducible conditions for quantification of 4 different target RNAs (control RNA34, CD4, p21, and FasL mRNA) from 50 μL of human whole blood. As shown in Fig. 2A of the online Data Supplement, heparin, acid-citrate-dextrose (ACD), and EDTA were acceptable as anticoagulants. Because ACD and EDTA chelate calcium, a critical component for many biological activities *(21)*, heparin was the choice of anticoagulant for subsequent studies when whole blood was to be used for stimulation ex vivo (Fig. 3). The stability of whole blood after blood drawing was a major concern. One commercial system (PAX gene; PreAnalytix) uses a special blood container in which the cells are lysed immediately and the released RNA is stabilized; however, this lysis buffer is not suitable for the isolation of poly(A)$^+$ RNA. Moreover, because a goal of this project was to quantify mRNA before and after gene induction processes ex vivo (Fig. 3), heparinized whole blood was stored at 4 °C and the changes in mRNA concentrations were examined. Although the concentrations of 3 native genes (CD4, p21, and FasL) fluctuated slightly after blood drawing, when blood was stored at 4 °C the concentrations stabilized and became constant after 2 h (see Fig. 2B in the online Data Supplement).

Poly(A)$^+$ mRNA capture by an oligo(dT) solid surface is usually performed at room temperature, but we performed the mRNA capture step at 4 °C because performance varied between 20 and 23 °C (see Fig. 2C in the online Data Supplement). Control RNA and FasL mRNA reached a plateau after 2 h of hybridization, whereas the other RNAs required at least 4–8 h (see Fig. 2D in the online Data Supplement). Thus, the mRNA capture step was performed at 4 °C overnight. The cDNA was synthesized without additional primers. Although short synthetic RNA and abundant RNA (CD4) required small quantities of reverse transcriptase, others required ~100 U of MMLV reverse transcriptase to reach a plateau (see Fig. 2E in the online Data Supplement). cDNA synthesis for 90 min at 37 °C was sufficient for all species of RNA tested. When MMLV was omitted from the reaction, no amplification was observed, suggesting that DNA contamination was negligible in this system. Assay sensitivity was proportional to the quantity of cDNA transferred to PCR. Commonly available buffers contain dithiothreitol to stabilize reverse transcriptase, which inhibits PCR. Omitting dithiothreitol from the reverse transcriptase buffer allowed cDNA volumes to be increased to 4 μL per 10-μL reaction (see Fig. 2F in the online Data Supplement). Because the assay procedure used 50 μL of whole blood/well, the 4 μL of cDNA in each PCR represented ~16 000 leukocytes, assuming 5000 leukocytes/μL of blood.

mRNA QUANTIFICATION

We sought to determine total assay efficiency for each sample by using known quantities of enriched control RNA34 and to use this efficiency to convert PCR results into quantities of target mRNAs in the original samples. This procedure is similar to those for HIV viral load tests *(11)* and assumes that the efficiency is identical between high- and low-abundance mRNA species and among different sequences.

To test for identical efficiencies for high- and low-abundance mRNA species, we added different quantities of control RNA34 to the system (Fig. 2A), confirmed that synthetic RNA34 was not amplified from human blood, and then quantified RNA34 by PCR. As shown in Fig. 3A in the online Data Supplement, dose-dependent recovery of RNA34 was observed for the tested range of 10^4 to 10^9 molecules/well. Because this range of RNA34 quantities was sufficiently small compared with the quantities of

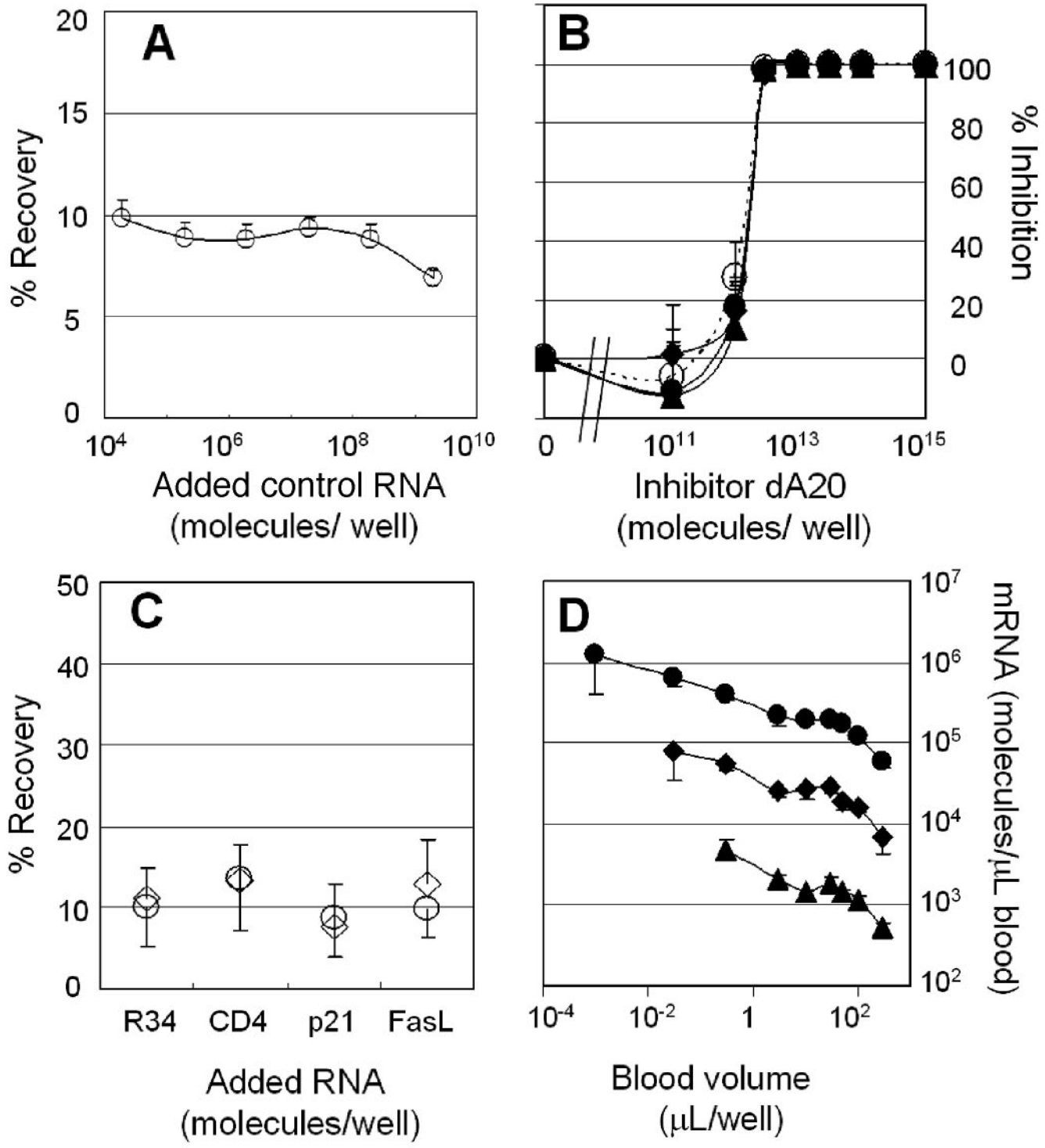

Fig. 2. Quantification of mRNA.

mRNA was quantified from triplicate aliquots (50 µL each) of whole blood, as stated in the *Materials and Methods*. Each symbol represents the mean (SD; *error bars*). (*A*), lysis buffer containing 10^4 to 10^9 molecules of synthetic RNA34 was used. The resulting Ct values (see Fig. 3A of the online Data Supplement) were converted to percentage recovery. (*B*), lysis buffer containing 0 to 10^{15} molecules of oligo$(dA)_{20}$ was used for competitive inhibition. The resulting Ct values (see Fig. 3B of the online Data Supplement) were converted to percentage inhibition. ○, RNA34; ●, CD4; ▲, p21; ♦, FasL. (*C*), lysis buffer containing 0 to 10^8 molecules of synthetic RNA34, CD4, p21, or FasL was used (see Fig. 3C of the online Data Supplement). The recovery of native mRNA was calculated as follows: mRNA recovery (%) = $(mRNA_1 - mRNA_0)/mRNA_A \times 100$, where $mRNA_1$ and $mRNA_0$ represent experimental mRNA values with or without synthetic RNA, respectively, and $mRNA_A$ represents the amount of synthetic RNA added to the lysis buffer. The mean (SD) from 4 persons was determined with (◇) or without (○) stimulation with 25 nmol/L phorbol ester and 1 µmol/L Cal at 37 °C for 2 h (see Fig. 5 in the online Data Supplement). (*D*), heparinized whole blood was diluted with phosphate-buffered saline, and 100 µL of each diluted sample was applied to a filterplate. Lysis buffer containing an equal quantity of control RNA34 was used. The resulting Ct values (see Fig. 3D in the online Data Supplement) were converted to molecules of mRNA/µL of blood. ●, CD4; ▲, p21; ♦, FasL.

total mRNA in 50 μL of blood, the concentrations of 3 other native mRNAs remained unchanged. When the Ct values were converted to number of RNA molecules and the percentage recovery was calculated, these values all became similar, ~10% (Fig. 2A), indicating that ~10% of the RNA34 added to the lysis buffer is recovered at the final step of TaqMan real-time PCR, a quantity that includes the yield of mRNA recovered on oligo(dT)-microplates (~30%), volume changes from 60 μL of lysis buffer per well to 30 μL of cDNA per well, and cDNA synthesis efficiency in the presence of crude blood mRNA. These data also suggest that the percentage recovery derived from one concentration of control RNA34 is applicable to any concentration of mRNA within the same samples.

To test for identical efficiency among different sequences, we carried out hybridization with or without oligo(dA) as a competitive inhibitor. All 4 RNAs exhibited almost identical inhibition curves, with an IC_{50} of ~3 × 10^{12} to 10^{13} molecules/well (Fig. 2B), although the expression these RNAs differed (see Fig. 3B in the online Data Supplement). This result indicates that the system is poly(A)-specific, a characteristic unique to poly(A)$^+$ RNA because of substantial variation between long- and short-RNA species in conventional total RNA purification methods (data not shown). The amounts of native CD4, FasL, and p21 mRNA recovered were all ~10% (Fig. 2C), although the lengths and sequences of these RNAs were different. The percentage recovery remained unchanged even when whole blood was stimulated with phorbol 12-myristate 13-acetate (PMA) and calcium ionophore A23187 (CaI; Sigma; see the section on ex vivo response). These data indicate that the system was sequence-independent even when certain mRNA concentrations changed widely after stimulation ex vivo. As shown Fig. 3D in the online Data Supplement, the Ct values for 3 native mRNAs approached a log-linear dependence on the volume of blood applied. Abundant mRNAs, such as CD4, were detectable even when as little as 0.001 μL of blood was used (1:10^5 dilution; 100 μL/well; Fig. 2D). Once the same data were converted to quantities per microliter blood, the values were consistent between volumes of 3 and 50 μL blood per well (Fig. 2D).

Quantification in this study was dependent on the DNA oligonucleotides used as calibrators for real-time PCR and the control RNA34 in the lysis buffer. Because single-stranded DNA oligonucleotides, double-stranded long DNA, and single-stranded cDNAs all exhibited identical amplification curves with this real-time PCR, DNA oligonucleotides were selected as calibrators because they are easily synthesized, easily purified by HPLC or gel electrophoresis, and are suitable for future standardization. Quantification of control RNA34 is difficult. The purity of in vitro–transcribed RNA34 was ~70% by capillary electrophoresis, although it showed a single band on agarose gel electrophoresis. Because of the instability of RNA, further purification steps may induce additional degradation. The cDNA was synthesized from each RNA under optimum conditions, and we determined the maximum amounts of cDNA by assuming that cDNA synthesis efficiency was 100%. Because RNA34 (109 bases), CD4 (775 bases), p21 (473 bases), and FasL (982 bases; not full length) all showed similar recoveries (Fig. 2C), we selected short RNA34 as a control.

baseline mRNAconcentrations in healthy adults

To determine the baseline concentrations of mRNA, we measured the concentrations of CD4, p21, and FasL in samples from 55 healthy individuals in 15 different experiments. The mean (SD) recovery of control RNA34 was 10.23 (0.64)%, giving an interassay CV of 6.3%, with an intraassay CV of 1%–22% for each determination from triplicate whole blood aliquots (see Fig. 4A in the online Data Supplement). According to the RNA34 recoveries for each sample, the mean (SD) baseline concentrations of CD4, p21, and FasL mRNA were 34 300 (3580), 399 (44), and 3440 (371) molecules/μL of blood, respectively; the corresponding CVs were 10%, 11%, and 11%. Although the number of leukocytes varied among individuals, leukocyte count is easily measured when mRNA copies per leukocyte are desired. Because of the low CVs, these data may serve as reference values for future molecular diagnostics. For example, CD4$^+$ leukocytes are HIV targets *(22)*, p21 is induced by exposure to radiation (Fig. 3, A and B) or anticancer drugs *(23)*, and FasL is a cytotoxic T-cell death ligand that induces apoptosis *(24)*.

According to BLAST (www.ncbi.nlm.nih.gov/BLAST/) or hybridization simulation analysis (HYBsimulator) *(25)*, the primer sequences used for CD4, p21, and FasL mRNA in this study did not amplify other mRNAs. Only intact full-length RNA or partially digested RNA that included the primer sites and poly(A)$^+$ tail were amplified. Because the results are expressed as mRNA molecules and not micrograms of mRNA, both full-length and digested RNA were counted equally. Because we do not know how each mRNA is digested in each cell, the reference mRNA values in Fig. 4 of the online Data Supplement are dependent on the primers and probes used.

ex vivo response

As a model system, we stimulated heparinized whole blood with PMA and CaI and quantified p21 and FasL mRNA to assess leukocyte responsiveness. As shown in Fig. 5A of the online Data Supplement, both p21 and FasL mRNA increased rapidly on stimulation with PMA and CaI, reaching a plateau with approximately a 10-fold increase after 90–120 min. The increases in p21 were more rapid than those in FasL (see Fig. 5A of the online Data Supplement). The baseline concentrations of p21 also increased during incubation at 37 °C ($P < 0.0001$), whereas FasL remained unchanged (see Fig. 5A of the online Data Supplement). Interestingly, even when heparinized whole blood was stored at 4 °C for 21 h, the responsiveness was

preserved (see Fig. 5B of the online Data Supplement), which provides flexibility in terms of diagnostic testing by allowing overnight shipment of blood samples at 4 °C.

CLINICAL APPLICABILITY

We use 2 different examples to demonstrate clinical applicability (Fig. 3). Two additional examples are shown in Fig. 6 of the online Data Supplement. We stimulated heparinized whole blood was with ionizing radiation and quantified the induction of p21 mRNA (Fig. 3, A and B). We evaluated p21 because it is induced by p53 activation during DNA damage and plays a primary role in cell-cycle arrest *(26)*. We observed >10-fold induction at 1 h after irradiation (10 Gy; Fig. 3A). Although the p21 mRNA concentration increased when blood was incubated at 37 °C without exposure to radiation, it exhibited significant induction with radiation and was a sensitive marker, increasing even on radiation exposure as low as 0.03 Gy (Fig. 3B). This test may be applicable in the identification of individuals at high risk for radiation sensitivity before

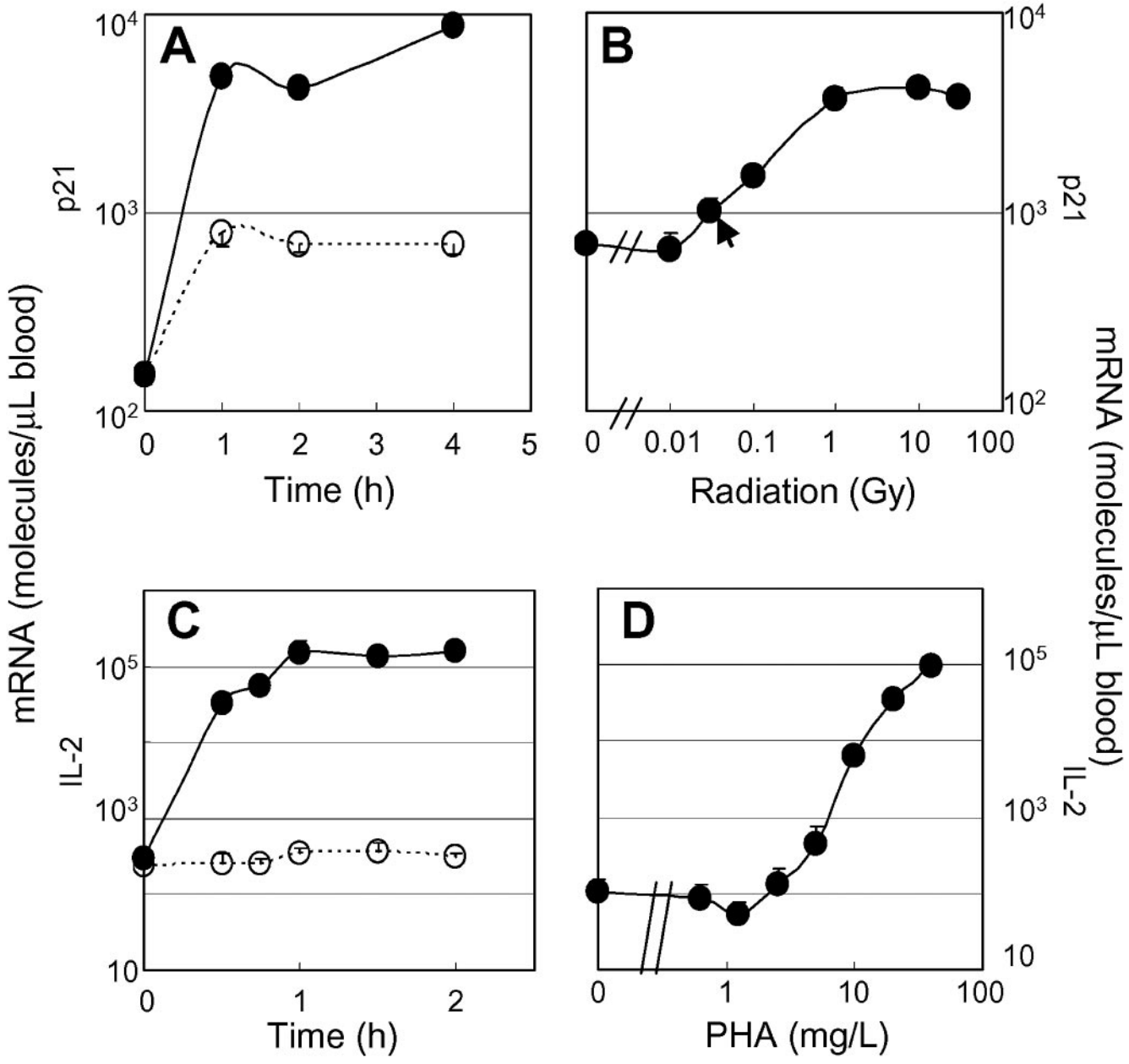

Fig. 3. Demonstration of clinical applicability.

Heparinized whole blood was stimulated with (●) or without (○) ionizing radiation (*A* and *B*) or PHA (*C* and *D*) and incubated at 37 °C. The concentrations of p21 (*A* and *B*) and IL-2 (*C* and *D*) mRNA were quantified as described in the *Materials and Methods*. (*A* and *C*), kinetics at 37 °C with 10 Gy of radiation or 40 mg/L PHA, respectively. (*B* and *D*), dose responses with 2 h of incubation at 37 °C. *Arrow* in *B* indicates $P < 0.05$. Each symbol is the mean (SD; *error bars*) from triplicate 50-μL aliquots of heparinized whole blood; in some cases, the error bars are not visible because they are smaller than the symbol.

radiation therapy or who are exposed to radiation in the work environment. This test may also be applicable as a screening tool to identify individuals at high risk for cancer, because radiation-induced p21 response is a model for quantifying DNA damage response in individuals.

When we stimulated heparinized whole blood with a mitogen [phytohemagglutinin-P (PHA)] to induce interleukin-2 (IL-2) mRNA, induction was increased ~1000-fold at 1 h (Fig. 3C), and the effect of PHA was dose dependent (Fig. 3D). IL-2 mRNA induction is one of the earliest events during blastogenesis of PBMCs *(27)*; thus, our findings correspond to results obtained in the PHA-induced [^{3}H]thymidine incorporation assay, a well-established test for immune deficiency diseases. Because the mRNA assay eliminates lengthy culture in artificial media, our results more closely reflect physiologic conditions and our assay might be applicable for quantifying individual responses to cyclosporin A, because the primary action of cyclosporin A is to inhibit IL-2 mRNA induction *(28, 29)*.

In conclusion, we present a unique approach to the quantification and standardization of gene expression analysis in whole blood. Our system is based on the use of oligo(dT)-immobilized microplates *(30, 31)* for high-throughput quantification and includes the use of a 96-well format for leukocyte isolation, cell lysis, reverse transcription, and real-time PCR quantification. The use of whole blood simplifies assay procedures and also allows physiologic ex vivo experiments, including complex cell-to-cell and cell-to-plasma interactions. Any gene transcript can be analyzed. Accurate, high-throughput mRNA quantification from whole blood may enable future drug discovery, toxicology testing, and tailored medicine based on assessment of individual responses.

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