

Quantitation of residual WBCs in filtered blood components by high-throughput, real-time kinetic PCR

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BACKGROUND: The effort to eliminate transfusion complications associated with WBCs has led to the widespread use of filters able to reduce WBC concentrations to ≤ 0.1 WBC per μL blood. This has necessitated sensitive QC methods to quantitate residual WBCs in filtered units. One fast, effective method is DNA amplification using real-time kinetic PCR (kPCR). **STUDY DESIGN AND METHODS:** Two methods of preparation of standards were compared and used for the optimization of quantitative kPCR. The first involved spiking genomic DNA cell lysate into a diluent, followed by a series of 1 in 10 dilutions. The second involved spiking serial 1 in 10 dilutions of WBCs into twice-filtered fresh whole blood. Two hundred fifty filtered frozen whole-blood samples were amplified in duplicate to show the kPCR assay's reproducibility. Another 359 filtered frozen whole blood samples were used to compare data from kPCR with data from a standard PCR protocol using ^{32}P -labeled probe and autoradiography. All specimens were amplified for conserved *HLA DQ α* sequences.

RESULTS: Standards prepared by both methods gave reproducible and equivalent results. Quantitation of standards representing a dynamic range of 8×10^0 to 8×10^5 WBCs per mL, yielded standard deviations ranging from 0.59 cycle to 1.04 cycles (a one-cycle increase is equivalent to a twofold increase in WBC concentration). The scatter graph of the 250 samples tested in duplicate by kPCR generated a slope of 1.0122 and an R^2 value of 0.9265. The comparison of kPCR and ^{32}P -probe hybridization results on 359 clinical samples gave a scatter-graph slope of 0.9428 and an R^2 value of 0.8718, indicating excellent agreement of the methods over a 4-log dynamic range.

CONCLUSION: kPCR is a high-throughput, sensitive assay that could prove useful in routine quality assurance of the WBC reduction process.

Febrile transfusion reactions, alloimmunization to human WBC antigens, GVHD, and the development of CMV infections are only a few of the many complications that have been associated with transfused WBCs present in non-WBC-reduced, allogeneic blood.¹ In fact, regulatory agencies in several countries have already mandated transition to universal WBC-reduced (also called WBC-depleted) blood components. As methods for prestorage in-line filtration continue to evolve, more blood centers will move in the direction of large-scale whole-blood filtration.²

In order to prevent transfusion complications associated with WBCs, "4-log filters" have been developed and implemented that yield residual WBC counts at or below 10^5 to 10^6 WBCs per unit of RBCs (approx., 0.3-3.0 cells/ μL).^{1,3} In addition, experimental 6-log filters have been developed that yield 10^3 to 10^4 WBCs per unit of RBCs (0.003-0.030/ μL).⁴ The evolution of such filters has necessitated development of increasingly sensitive methods to quantitate residual WBCs for filter development, quality assurance, and QC purposes.³⁻¹³ Methods currently being used in the quantitation of residual WBCs

ABBREVIATIONS: C_T = cycle threshold; kPCR = kinetic PCR; VATS = Viral Activation Transfusion Study.

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include Nageotte hemocytometers,³⁻⁸ flow cytometry,^{3,8-11} RIA methods,³ microdroplet fluorochromatic assays,^{3,12} and automated volumetric capillary cytometry.^{3,13} These methods have reported sensitivities (i.e., limit of detection) ranging from 0.001 to 2.000 WBCs per μL .^{3,8} Although some of these methods can be sensitive enough to quantitate residual WBCs in 6-log filtered units, they tend to be expensive and labor intensive and are thus not ideal for screening large numbers of blood units.

We have reported adaptation of the PCR for QC of filtered units, applied to both fresh and frozen and thawed blood specimens.^{14,15} This adaptation uses the detection and quantitation of cell-associated genomic DNA (in genome equivalents/mL) as a variable for residual nucleated cells. Our original method required hybridization of PCR-amplified product with radioactive probes, autoradiography, and image analysis, with related safety concerns and expense.^{14,15} We report here the application of another PCR detection method, the kinetic (or real-time quantitative) PCR (kPCR),¹⁶⁻¹⁸ to achieve high throughput quantitation of as little as 0.008 WBCs per μL of filtered blood. Specifically, we have optimized the assay targeting a highly conserved region of *HLA DQ α* gene, which is present in two copies per WBC but not present in RBCs or platelets. Amplification of the *HLA DQ α* sequence (yielding a 242-bp product) in spiked standards was used to generate a calibration curve. Different concentrations of magnesium chloride, potassium chloride, primers, and polymerase (Amplitaq Gold Polymerase, Applied BioSystems, Foster City, CA) were evaluated to optimize the assay's sensitivity, dynamic range, and reproducibility. We then conducted a study to compare the new automated real-time detection system with the traditional ³²P-probe system to show that the new real-time detection system is a sensitive and accurate alternative for quantitation of low residual WBC concentrations in filtered blood.

MATERIALS AND METHODS

Preparation of DNA from blood

DNA lysate was obtained as described from frozen and thawed whole blood,¹⁵ provided by samples collected for the Viral Activation Transfusion Study (VATS).¹⁹⁻²¹ This lysate was centrifuged briefly to collect the sample at the bottom of the tube, heated at 100°C for 2 minutes to ensure the inactivation of the proteinase K (GIBCO BRL, Gaithersburg, MD), and centrifuged again at 7000 rpm for 15 seconds.

Preparation of DNA lysate-spiked standards

A set of standard samples for establishing a calibration curve was prepared with a crude lysate of A.301 cells

(NIH, Rockville, MD) (8×10^5 cells/mL). Five 1 in 10 serial dilutions of the A.301 lysate were made with an equal mixture of PCR Solutions A (100 mM KCl, 10 mM Tris base, 2.5 mM MgCl₂, pH 8.3) and B (10 mM Tris, pH 8.3, 2.5 mM MgCl₂, 1% Tween 20, 1% Nonidet P-40) as the diluent; 25 μL of each dilution was amplified to generate each cycle threshold (C_T)¹⁸ for calibration curves.

Preparation of blood-spiked samples

With use of an automated cell counter, a WBC count was performed in triplicate on an aliquot of unfiltered fresh whole blood. A calculated amount of this unfiltered blood, the source of WBCs, was then spiked into twice-filtered, freshly drawn (Sepacell RS2000, Baxter HealthCare, Deerfield, IL) whole blood giving a WBC concentration of 8×10^5 cells per mL filtered blood. Five 1 in 10 serial dilutions were then made with the twice-filtered, fresh whole blood as the diluent. The controls were stored at -80°C until processed as described elsewhere.¹⁵ In addition to the spiked filtered blood, kPCR was performed on twice-filtered and triple-filtered unspiked thawed whole blood to estimate the amount of background signal in the assay.

Amplification and quantitation of residual WBCs using kPCR

Twenty-five microliters of sample (equivalent to 125 μL of blood) was added to 50 μL of reaction mixture and overlaid with 40 μL of mineral oil (Perkin Elmer, Norwalk, CT). Microamp reaction tubes were kept in ice while the samples were added. The reaction mixture consisted of 15 mM KCl, 6 mM MgCl₂, 40 mM Tris HCl, pH 8.15; 0.2-percent Triton X100; 100 μg per mL nuclease-free BSA, 1 pmol per μL of each primer for *DQ α* amplifications (GH26 and GH27);²² 20 mM each dATP, dCTP, dGTP, and 40 mM dUTP, 2 μg per mL ethidium bromide (GIBCO BRL), and 0.15 U per μL (7.5 U/reaction) amplification reagent (Amplitaq Gold, Roche Molecular Systems, Alameda, CA). In addition to the samples, 25 μL of 40 mM EDTA was added to separate microamp tubes containing reaction mixture and mineral oil; the EDTA in these external fluorescence standards was used to prevent amplification. These EDTA "killed" reactions were imaged together with active PCRs to allow us to correct for fluctuations in illumination. After the samples were added, the microamp tubes were centrifuged at 3000 rpm for 1 minute. The samples were then amplified for 50 cycles consisting of 95°C for 30 seconds, then at 56°C for 30 seconds, and finally at 72°C for 30 seconds. Amplification was performed with a thermocycler (9600 Thermocycler, Perkin Elmer), with an attached module for monitoring fluorescence from the PCR products. During each annealing step, a UV light was used to excite the ethidium

bromide in the samples, and a CCD camera connected to the kPCR module was triggered to take an image of sample fluorescence.^{16,18} The fluorescence emitted from each sample was then digitized, saved, and quantitated.

kPCR analysis

After amplification was completed, data were imported into a data analysis spreadsheet. The EDTA "killed" reactions were used to normalize cycle-to-cycle variations in UV intensity. In addition, differences between samples were normalized by calculating the ratio between the fluorescence emitted by each sample and its initial background fluorescence. The analyzed data were then presented in graphic form, illustrating the relationship between the relative fluorescence and the cycle number as a fluorescence growth curve. In this form, an arbitrary fluorescence level was placed above the initial background. The point at which the arbitrary fluorescence level intersects the kinetic growth curve defines the C_T . The C_T , expressed as the fractional cycle value, was then used to estimate the DNA input relative to a calibration curve generated using samples with known amounts of starting material.^{16,18}

Quantitation of residual WBCs by PCR using a ³²P-labeled probe

Results obtained from kPCR were compared with those obtained from liquid oligomer hybridization using a ³²P-labeled probe.¹ In brief, a *DQ* α -specific probe (GH64)²² was end labeled at 37°C for 1 hour in a 50 μ L solution that consisted of 5 μ L of buffer (10 \times T4 Polynucleotide Kinase Buffer, New England Biologicals, Cambridge, MA), 25 units of enzyme (T4 Polynucleotide Kinase, New England Biologicals), 50 μ Ci of radio-labeled isotope (³²P-ATP, DuPont, Wilmington, DE), and 37.5 pMol of GH64. After incubation, the volume of the labeled probe mixture was adjusted to 2 mL by using a buffer solution containing 0.75 mM NaCl and 10 mM EDTA. For hybridization, 20 μ L of the diluted probe mixture was added to 75 μ L of postamplification sample, denatured at 95°C for 5 minutes, and incubated for an additional 5 minutes at 60°C. Electrophoresis was done on a 6-percent polyacrylamide gel for 30 minutes. The gel was exposed (X-OMAT, Kodak Scientific Imaging Film, Eastman Kodak Company, Rochester, NY). Autoradiographic signal intensities were measured and analyzed with software (BioImage Whole Band Analyzer Software, BioImage Millipore, Ann Arbor, MI); sample autoradiographic signals were analyzed relative to the signals given from the serially diluted standard curve.

RESULTS

Optimization of kPCR

Two kinds of standard samples were used to both optimize and calibrate kPCR for *HLA-DQ* α . The first involved

spiking a known amount of genomic DNA lysate into a diluent, followed by a series of 1 in 10 dilutions. The second involved spiking a known quantity of WBCs into twice-filtered fresh whole blood, again followed by a series of 1 in 10 dilutions. The WBC concentrations in the serial 1 in 10 dilutions covered the range of residual WBC concentrations found in filtered units.

We first examined variations of the following amplification conditions: magnesium, potassium chloride, primer, polymerase concentrations, annealing temperatures, and purified versus sterile, filtered water. The following magnesium chloride concentrations were used during optimization: 1, 2, 3, 4, 5, and 6 mM in reaction mixture. Overall, it was found that 4 or 5 mM MgCl₂ in the reaction mixture gave the most reproducible results and the best dynamic range. Potassium chloride, in the concentration range of 15 to 25 mM, gave the lowest C_T values, and therefore the highest amplification efficiency. Two types of water, nanopurified (nuclease-free water, Promega, Madison, WI) and sterile, filtered (0.1 μ M filter) water (Cell Culture Facility, University of California, San Francisco, CA) were compared. There was less nonspecific amplification with nanopurified water, thus enhancing the potential for detecting low copy numbers.

It was also found that 1 pMol per μ L of each *DQ* α primer (GH26 and GH27) yielded the best sensitivity and efficiency. It was found that some lots of primers were prone to generating nonspecific fluorescence increases during amplification, suggesting that the primer quality may be critical in determining the sensitivity of the assay; 7.5 U of polymerase per reaction yielded the best dynamic range and the most efficient amplification. Optimized reactions using the 1 in 10 dilution series of standards gave C_T s of three to four cycle differences between dilutions. This demonstrates good reaction efficiency because a doubling reaction requires 3.3 cycles to compensate for a 1 in 10 dilution.

Reproducibility of the standards

Both sets of standards, including spiking lysate into a diluent and spiking nonfiltered WBC-rich whole blood into filtered whole blood, gave reproducible and equivalent results with optimized amplification conditions. The calibration curves had standard deviations ranging from 0.42 cycle to 0.73 cycle (a one-cycle increase is equivalent to 100% increase), which indicates good reproducibility when amplifying high-input standards (8×10^4 to 8×10^1 copies/mL) prepared with solutions A and B as the diluent (Table 1A). Good reproducibility is also shown by the low standard deviation (0.55 cycle to 1.04 cycles) obtained from standards (8×10^5 to 8×10^0 copies/mL) prepared with twice-filtered blood as the diluent (Table 1B).

TABLE 1. Reproducibility of HLA-DQ α standard amplification by kPCR*

HLA-DQ-A (copies/mL)	DNA lysate-spiked standard†		Blood-spiked standard‡	
	C _T range	Mean \pm SD	CT range	Mean \pm SD
8 \times 10 ⁵	26.07-28.07	26.91 \pm 0.69	24.79-26.61	25.39 \pm 0.55
8 \times 10 ⁴	29.54-30.85	30.09 \pm 0.42	28.39-30.24	29.58 \pm 0.75
8 \times 10 ³	32.35-34.39	33.35 \pm 0.73	31.48-33.94	32.63 \pm 0.68
8 \times 10 ²	34.72-36.57	35.62 \pm 0.66	39.16-41.58	40.29 \pm 1.04
8 \times 10 ¹	27.40-38.71	35.74 \pm 3.48	44.57-41.70	43.09 \pm 0.94

† Standard prepared by diluting genomic DNA lysate with equal volumes of PCR Solutions A and B.

‡ Standards prepared by spiking fresh WBCs into twice-filtered whole blood, followed by a series of 10-fold dilutions.

* Reproducibility can be seen by the narrow C_T range per 10-fold dilution. C_T describes the point at which the kinetic curve crosses the arbitrary fluorescent level. One cycle increase is equivalent to a 2-fold or 100-percent increase.

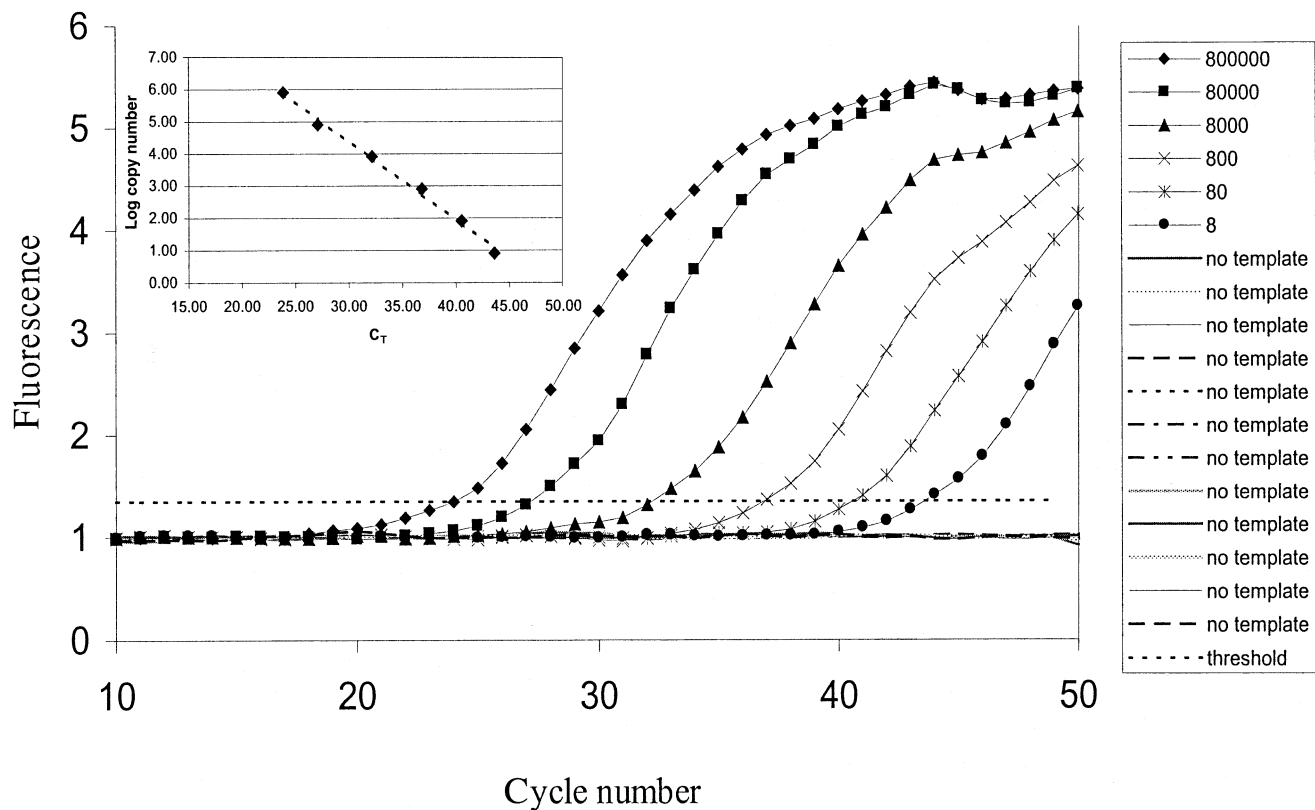


Fig. 1. Amplification of DQ α loci using dilutions of standards. Signals were not obtained after 50 cycles of amplification from reaction mixtures that did not contain DNA template. The insert plot shows the regression line of the standards, 10⁶ to 10¹, with the C_T plotted on the x axis and log copy number on the y axis.

Lower limit of detection and dynamic range of the assay

The lower limit of detection and dynamic range of the assay was determined with serial dilutions of genomic DNA. Figure 1 demonstrates sensitivity to 8 copies per mL. The 12 PCR mixtures that did not contain added template did not show amplification, demonstrating assay specificity. Furthermore, as shown in Figure 2, twice-filtered and triple-filtered unspiked whole blood yielded either no signal or a signal corresponding to less than 8 copies per mL.

Reproducibility of data obtained from VATS samples

Two hundred fifty filtered (\times 1) frozen whole blood samples obtained from the VATS repository were amplified with kPCR.²¹ In order to examine the reproducibility of the entire assay system, two aliquots of each frozen whole blood sample were processed (saponin lysis, proteinase K digestion) and amplified in separate runs. Figure 3 illustrates that there is a good correlation between both amplifications (slope = 1.0122 , y intercept = -0.0018 , R² = 0.9265).

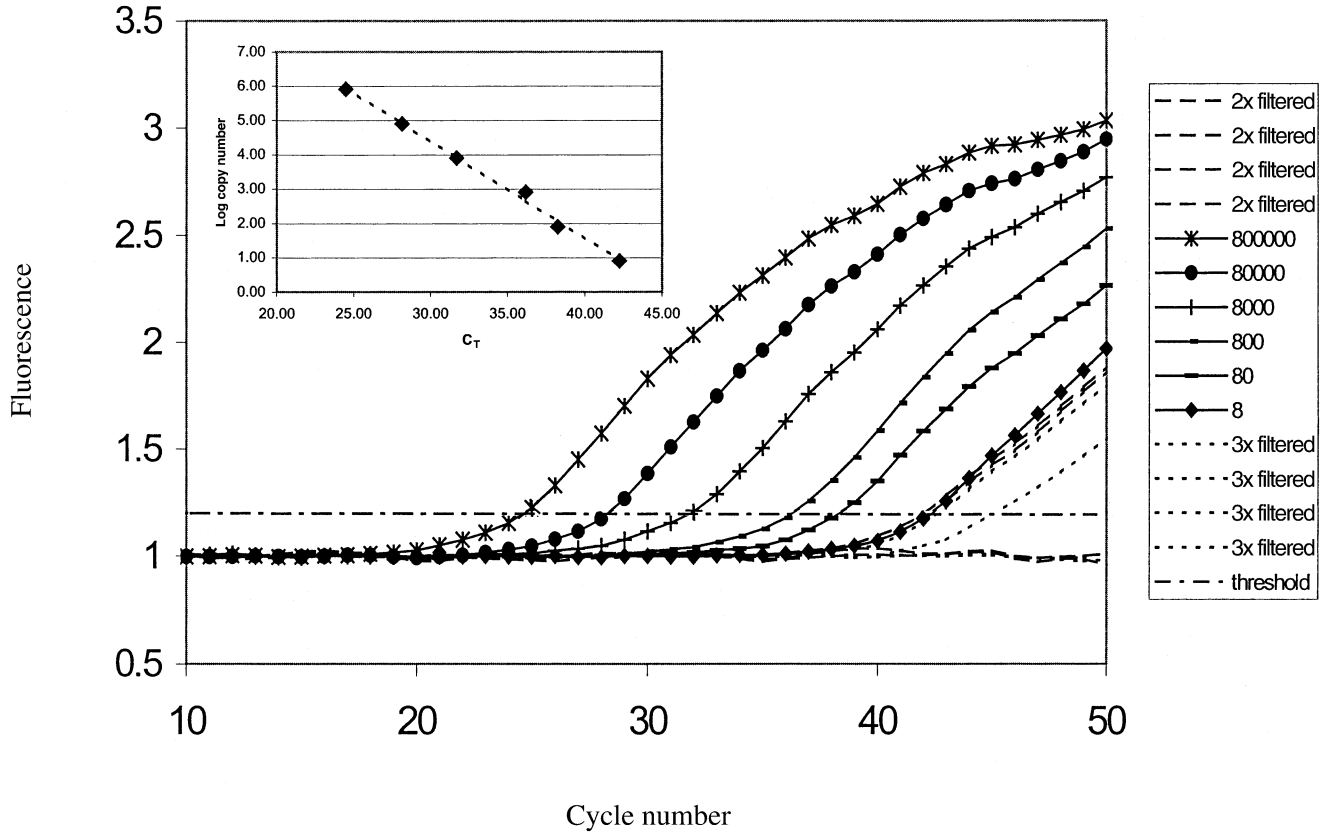


Fig. 2. Preparations of twice- and triple-filtered, unspiked whole blood were amplified for *DQα* and compared with amplified *DQα* standards. Twice-filtered and triple-filtered whole blood yielded either no signal or a signal of less than 1 copy per 25 μ L. The insert plot shows the regression line of the standards, 10^6 to 10^1 , with the C_T plotted on the x axis and log copy number on the y axis.

kPCR versus 32 P-probe-based detection

We had previously measured residual WBCs in a large number of filtered RBC preparations from VATS using the PCR hybridization to a 32 P-probe and gel electrophoresis method as detailed elsewhere.²¹ We used the kPCR assay to measure residual WBCs for 359 replicate VATS samples and compared the kPCR results with those from the standard protocol. A good correlation between the two sets of measurements was obtained, as seen in Figure 4 (slope = 0.9428 , Y intercept = 0.1152 , R^2 = 0.8718).

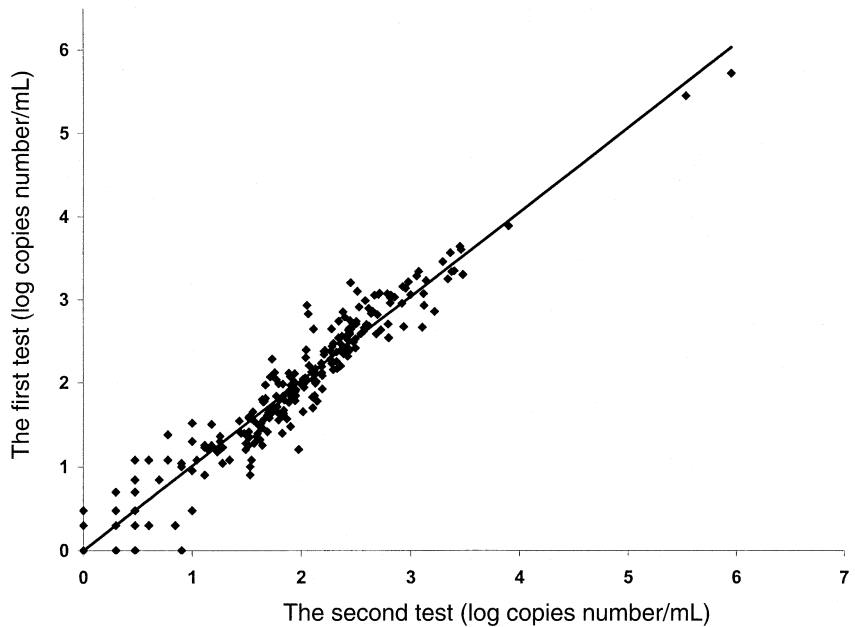


Fig. 3. *DQα* amplification of 250 filtered, frozen, whole-blood samples obtained from the VATS. The VATS samples were amplified twice to illustrate the reproducibility of the data obtained by using kPCR. A good correlation was obtained as can be seen by the y intercept (-0.0018), the slope (1.0122), and the R^2 value (0.9265).

DISCUSSION

WBC-reduction filtration of blood components is being mandated by many countries because of the many complications that can occur from transfused WBCs. Consequently, QC of filtration

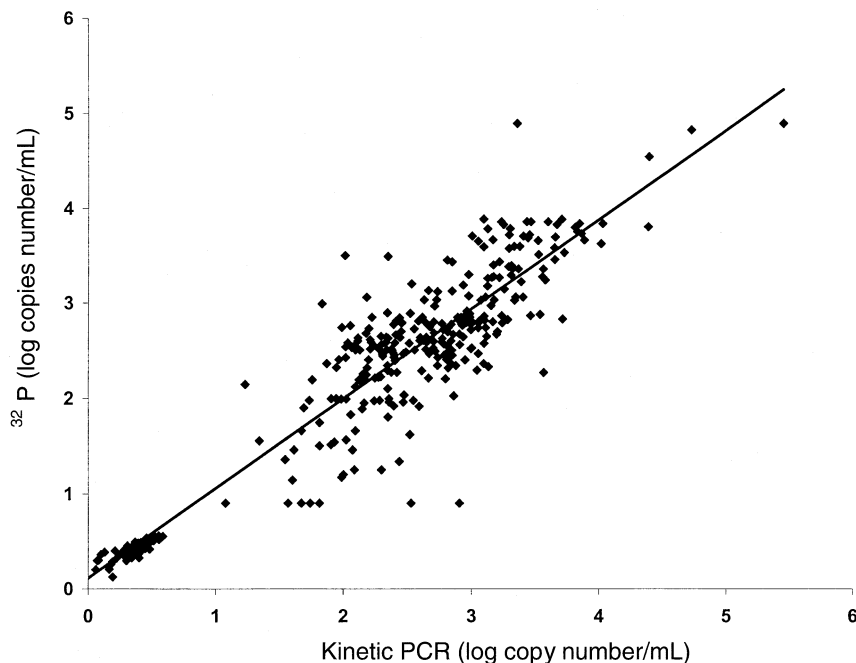


Fig. 4. Three hundred fifty-nine samples obtained from VATS were amplified for *DQ α* , using a protocol in which ^{32}P -labeled probe was used to detect and quantitate the PCR product, and the probe results were compared with those obtained from kPCR. A relatively good correlation was obtained between both sets of data, as shown by the y intercept (0.1152), slope (0.9428), and R^2 value (0.8718).

has become a significant issue for the blood banking community. Methods most frequently used for quantitating low concentrations of residual WBCs include Nageotte hemocytometers and flow cytometry methods. The lower detection limits reported for these two methods are 1.0 and 0.1 WBC per μL ,^{1,3,8} respectively. In contrast, the kPCR assay that we have optimized can detect as little as 0.008 WBC per μL . Using the protocol described here, we could detect as little as 8 to 80 cells per mL of whole blood, which translates into 2.4×10^3 to 2.4×10^4 WBCs per unit, far below the detection levels corresponding to the maximum accepted concentration of WBCs in WBC-reduced units (1×10^6 to 5×10^6 WBCs/unit). The assay also has a large dynamic range (≥ 5 logs), enabling quantitation of WBCs in "failed" RBC filtrates without a requirement for an alternate sample processing or counting method.

The automated detection system used in kPCR makes it a much less labor-intensive assay when compared with the use of Nageotte hemocytometers, flow cytometry methods, or other DNA amplification-based assays. In our laboratory, one technician is able to process at least 80 samples per day. Our previous procedure using amplification, oligomer hybridization with ^{32}P -labeled probes, electrophoresis, and quantitation using image analysis of autoradiographic signals took at least 2 days to complete a similar number of samples. In the

near future, it is expected that PCR sample preparation methods will be automated; once this occurs, one individual should be able to process, amplify, and analyze 400 to 500 samples per day. We estimate the cost of labor plus reagent would be approximately \$3.00 per unit at this scale. Thus, kPCR may be an ideal assay for obtaining rapid results when analyzing a large quantity of samples, such as for quality assurance of high-efficiency filters.

One major concern with kPCR is that the intercalator-based detection system does not differentiate between specific and nonspecific products, as does the more specific ^{32}P -labeled probe-based detection system. In our studies, the appearance of nonspecific products was delayed such that there was little or no impact on the sensitive detection of residual WBCs. This was accomplished in part by using lower concentrations of primers, as well as by keeping the reaction mixture cold (4°C) while adding samples.

In conclusion, our results demonstrate that kPCR is a high-throughput, sensitive assay that can be used in quantitating low concentrations of residual WBCs for QC of WBC-reduced blood components. With expanding applications of nucleic acid amplification assays in blood banks, and the development of increasingly efficient blood filters, this assay may prove applicable for routine quality assurance of the WBC-reduction process.

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