

## ORIGINAL ARTICLE

# Standardized methods to generate mock (spiked) clinical specimens by spiking blood or plasma with cultured pathogens

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## Keywords

blood-borne, mock specimens, molecular quantification, pathogen spiking, standardized methods.

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This article is an informal communication and represents our own best judgment. These comments do not bind or obligate FDA.

2015/2507: received 3 December 2015, revised 11 January 2016 and accepted 20 January 2016

doi:10.1111/jam.13082

## Abstract

**Aims:** To demonstrate standardized methods for spiking pathogens into human matrices for evaluation and comparison among diagnostic platforms.

**Methods and Results:** This study presents detailed methods for spiking bacteria or protozoan parasites into whole blood and virus into plasma. Proper methods must start with a documented, reproducible pathogen source followed by steps that include standardized culture, preparation of cryopreserved aliquots, quantification of the aliquots by molecular methods, production of sufficient numbers of individual specimens and testing of the platform with multiple mock specimens. Results are presented following the described procedures that showed acceptable reproducibility comparing in-house real-time PCR assays to a commercially available multiplex molecular assay.

**Conclusions:** A step by step procedure has been described that can be followed by assay developers who are targeting low prevalence pathogens.

**Significance and Impact of the Study:** The development of diagnostic platforms for detection of low prevalence pathogens such as biothreat or emerging agents is challenged by the lack of clinical specimens for performance evaluation. This deficit can be overcome using mock clinical specimens made by spiking cultured pathogens into human matrices. To facilitate evaluation and comparison among platforms, standardized methods must be followed in the preparation and application of spiked specimens.

## Introduction

Diagnostics are a crucial component of the public health strategy to reduce the burden of infectious diseases. A series of diagnostic devices/platforms are being developed to detect National Institute of Allergy and Infectious Diseases (NIAID) Category A-C priority biodefense pathogens as well as other emerging agents, however,

development of diagnostics for these Medical Counter Measure-related pathogens is complicated by the low prevalence of many of these pathogens. As such, there may not be sufficient clinical samples available to conduct clinical sensitivity studies required for US Food and Drug Administration (FDA) clearance. To address this regulatory scientific challenge, the FDA could consider use of mock (spiked) clinical samples to support clinical

sensitivity studies for the evaluation of diagnostic devices that detect low prevalence pathogens. It is recommended that investigators consult with the FDA regarding this approach prior to undertaking this type of study. Please refer to the 'Highly Multiplexed Microbiological/Medical Countermeasure *In Vitro* Nucleic Acid Based Diagnostic Devices - Guidance for Industry and Food and Drug Administration Staff' document (<http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/ucm327293.htm>) for additional information regarding the potential use of spiked clinical samples. Development of general methods for generating spiked blood and plasma samples will assist diagnostics developers with performance evaluation studies when clinical specimens are not available. To facilitate the evaluation and comparison among platforms, standardized methods must be followed in the preparation and application of spiked specimens. These methods could also be used to generate mock (spiked) samples using other clinical matrices such as nasal wash or cerebrospinal fluid. This study was performed to present optimized methods and test them sufficiently to validate the methods for evaluation of molecular diagnostic devices.

## Materials and methods

In developing these methods, model blood-borne organisms representing two Gram-positive bacteria including a potential bioterror bacterium, a Gram-negative bacterium that is a surrogate for another potential bioterror bacterium, a protozoan parasite and an emerging virus, were used. This breadth of organism types allowed identification of common as well as unique procedures for each pathogen.

The common work flow began by performing repeated growth curves for each well-characterized source organism (bacterium, parasite and virus) under optimal conditions. Characterization of the source organism can include traditional tests routinely performed to identify a culture isolate and/or by obtaining the genomic sequence of the organism. Performing repeated growth curves was intended to identify the initial inoculum, time in culture and cell density at which the culture was in mid-exponential growth. Each of these parameters was specific to the species and strain in culture.

Bacteria and parasites were harvested at the mid-exponential growth point because the culture would be most uniform under these conditions. The harvested cells were aliquoted into vials and frozen under viable cryopreservation conditions that were appropriate for that species.

For viruses, the viral stock was a culture-supernatant collected from infected susceptible cells. The initial inocu-

lum (multiplicity of infection (MOI)) was allowed to grow for a predetermined number of days and supernatant was harvested at postinfection collection time and subjected to low-speed centrifugation to clear cell debris from the supernatant. This entire process must be optimized for each viral agent. The supernatant was aliquoted into a large number of single-use vials and frozen under cryopreservation conditions.

After the frozen stock aliquots were prepared, the next step was to determine the mean molecular concentration of pathogen copy number in the frozen aliquots by performing replicates of quantitative PCR on at least four aliquots. Note: if aliquots are stored for >6 months, an aliquot from this set should be tested to ensure the sample concentration has remained stable and unaffected by storage conditions.

To prepare spiked specimens, the quantity of the aliquot suspension that was necessary to achieve the final target concentration was added into the human matrix such as blood or plasma. Throughout this study, healthy human blood was obtained anonymously under the FDA IRB approved protocol 03-120B. Whole blood was collected at the NIH Department of Transfusion Medicine ([http://cc.nih.gov/dtm/our\\_services.html](http://cc.nih.gov/dtm/our_services.html)) aseptically by venipuncture, from deidentified, screened, healthy donors into 50-ml polypropylene screw cap tubes and is distributed within several hours to the FDA lab, heparinized and stored at 4°C for no more than 30 days before spiking and extraction. Other mock specimens could be similarly prepared with nasal wash, cerebro-spinal fluid or other relevant human matrix. The spiked specimens were mixed and a diagnostic sample was processed as a patient sample would be. Spiking was done with healthy donor matrix creating enough unique specimens for statistical analysis. A second set of spiked specimens was made using human matrix acquired from patients with fever or other signs and symptoms of disease as recommended by FDA/Center for Devices and Radiological Health ('[www.fda.gov/downloads/medicaldevices/deviceregulationandguidance/guidancedocuments/ucm327294.pdf](http://www.fda.gov/downloads/medicaldevices/deviceregulationandguidance/guidancedocuments/ucm327294.pdf),' accessed 1/7/2016). The patient's condition need not be identical to the symptoms of an infection with the pathogen under investigation; rather the general alteration of the blood or plasma seen in a symptomatic patient is the important quality. The primary criterion was body temperature >98.6°F. These specimens were obtained anonymously from BioReclamationIVT under approval by Schulman Associates IRB, which was further reviewed and approved by the FDA IRB under protocol 14-072B. The patient matrix can pose challenges to the diagnostic device not seen with the healthy donor matrix.

More specific methods will be described below for a collection of model organisms: *Bacillus anthracis*, *Staphy-*

*Staphylococcus aureus*, *Yersinia pseudotuberculosis*, *Leishmania donovani* and Dengue virus type 2.

### ***Bacillus anthracis* (Anthrax Spore Vaccine strain)**

#### *Determination of the growth curve*

All steps were performed using standard benchtop microbiology aseptic technique (Collins *et al.* 2004). Bacteria were streaked from a well-characterized stock onto a Luria–Bertani agar plate and incubated at 37°C overnight. To initiate the growth in culture, a single colony from the agar plate was inoculated into 10 ml of Superbroth (Quality Biological Inc., Gaithersburg, MD) in a sterile 50 ml conical tube and incubated at 37°C overnight with shaking at 220 rev min<sup>-1</sup>. The following morning, when the bacteria culture has reached saturated, stationary culture, it is necessary to dilute the culture at least 1/20 (v/v) to restart exponential growth. Approximately 0.5 ml of the overnight culture was inoculated into 10 ml of Superbroth to achieve an OD<sub>600</sub> of 0.1 in a 50 ml conical tube. This was incubated at 37°C with shaking and the OD<sub>600</sub> measured every hour until the optical density did not increase at the maximal rate (growth approaching stationary phase). Each of the samples was read immediately and was not placed on ice prior to measuring the OD<sub>600</sub>. Measurement of the growth curve was repeated twice more. We found the region of the growth curve where the slope of a line drawn through the plot of OD vs time in incubation was constant, indicating a uniform growth rate. We determined the time in culture and the optical density that was in the centre of the linear portion of the growth curve (exponential phase). Our results for *B. anthracis* (Anthrax Spore Vaccine was obtained from Colorado Serum Co., Denver, CO) were 2.5 h to an OD<sub>600</sub> of 1.0.

#### *Preparation of cryopreserved aliquots*

Liquid culture harvested at the chosen mid-exponential growth stage was mixed with sterile 100% glycerol to make the suspension 20% (v/v) glycerol. With frequent mixing to maintain a uniform suspension, 0.5 ml per tube was aliquoted into sterile labelled tubes that were tightly capped, making at least 50 aliquots. Tubes were placed immediately in crushed dry ice and stored at -80°C.

#### *Determination of molecular pathogen concentration of frozen aliquots*

The chosen method of quantification based on real-time PCR achieved high reproducibility and less variability among laboratories compared to culture-based methods. The pathogen concentration was calculated by PCR detection of a limiting dilution of the nucleic acid

extracted from the frozen aliquot and the concentration was expressed as PCR Detectable Units (PDU) per ml.

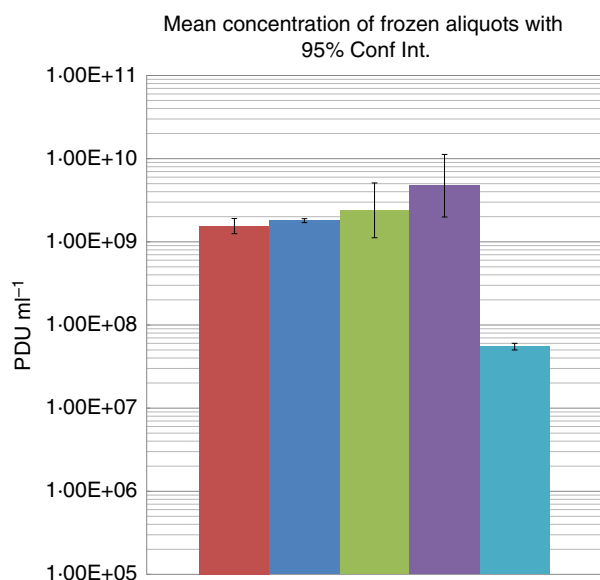
Steps included thawing a frozen aliquot, extracting total DNA from three separate 100 µl samples of the aliquot suspension by a chosen method (DNeasy kit, catalogue #69504; Qiagen, Valencia, CA, following the manufacturer's protocol was used for this step with all bacteria and eukaryotes in this study). The measurement was repeated with at least three other frozen aliquots of *B. anthracis* from the same batch. A series of 10-fold serial dilutions was made for each extracted DNA sample. Quantitative real-time PCR was performed on all the extracts of all the aliquots in the range of dilutions that span from 100% detectible down to below detectability. Our results for *B. anthracis* were between 1/10<sup>4</sup> and 1/10<sup>7</sup> dilutions of the DNA solution extracted from the frozen aliquots. Our assay was a Taqman style reaction targeting the Protective Antigen Gene on the pXO1 plasmid, which was specific for *B. anthracis* (Table 1).

The lowest dilution detectible for each DNA extraction was used to express the PDU ml<sup>-1</sup> in the frozen aliquot. For example, if 1/10<sup>6</sup> was the last dilution detectible, then there are 10<sup>6</sup> PDU in the undiluted volume of the nucleic acid sample corresponding to the amount of template added to the PCR reaction. If the volume of template was 1 µl, then the final concentration calculated for the extracted nucleic acid sample would be 1 × 10<sup>6</sup> PDU µl<sup>-1</sup>, better expressed as 1 × 10<sup>9</sup> PDU ml<sup>-1</sup>. The concentration in the frozen aliquot was calculated by the ratio of eluted volume to extracted volume times the PDU ml<sup>-1</sup> of the nucleic acid sample (eluted sample). For example in our procedure, the nucleic acid from 100 µl of aliquot suspension was eluted in 200 µl indicating a 2/1 ratio. Thus, there are 1 × 10<sup>9</sup> PDU ml<sup>-1</sup> × 2 = 2.0 × 10<sup>9</sup> PDU ml<sup>-1</sup> in the frozen aliquot. The values for each extraction of a frozen aliquot were used to evaluate the variability among the extractions for each aliquot. The mean PDU ml<sup>-1</sup> was calculated for each aliquot. Considering that the means arise from 10-fold dilutions, the mean and standard deviation among the aliquots are best calculated on the log transformed aliquot means. This standard deviation was used to calculate the 95% confidence interval (Sokal and Rohlf 1981) of the log transformed data (Mean ± 1.96 × SD/N<sup>0.5</sup>). The antilog of the mean of all the aliquot PDU ml<sup>-1</sup> log values and the antilog of the 95% confidence interval were the parameters of the frozen aliquots. The 95% confidence interval of the mean values for the aliquots should be less than twice the mean to insure sufficient reproducibility among aliquots. Once this condition was met, the mean of the PDU ml<sup>-1</sup> for the measured aliquots was used as the value for all remaining cryopre-

**Table 1** Real-time PCR assays and cycling conditions for detection of model blood-borne pathogens. All reactions were performed with a Bio-Rad CFX96 Real-Time PCR System. The four DNA genome pathogen reactions had primer concentrations of 200 nmol l<sup>-1</sup> in the final reaction volume and the reagent mix was Premix Ex Taq, catalogue #RR039A, Takara Bio, Kusatsu, Shiga, Japan

Organism	Forward primer	Probe	Reverse primer
<i>Bacillus anthracis</i>	CAGAATCAAGTCCCAGGGG Cycling profile	TCTCCTGAAAAATGGAGCACGGC (1) 95°, 1 min; (2) 95°, 15 s; (3) 60°, 1 min; step 2 > step 3, 40 cycles Probe concentration: 488 nmol l <sup>-1</sup> *	TCGGATAAGCTGCCACAAGG
<i>Staphylococcus aureus</i>	TAGGTGGCAAGCGTTATCCG Cycling profile	TTGAGCCGTGGGCTTTCACATCAGAC (1) 95°, 1 min; (2) 95°, 15 s; (3) 60°, 1 min; step 2 > step 3, 40 cycles Probe concentration: 400 nmol l <sup>-1</sup> *	TCCAGTTCCAATGACCCTCC
<i>Yersinia pseudotuberculosis</i>	GTAGTTTACTACTTTGCCGG Cycling profile	CCGCATGACCTCGCAAGAGC (1) 95°, 1 min; (2) 95°, 15 s; (3) 60°, 1 min; step 2 > step 3, 40 cycles Probe concentration: 269 nmol l <sup>-1</sup> *	GATTGAGCGTATTAATACTCA
<i>Leishmania donovani</i>	CCTATTTACACCAACCCCAAGT Cycling profile	RAAARKKVRTRCAGAAAYCCCGT (1) 95°, 2.5 min; (2) 95°, 10 s; (3) 56°, 30 s; step 2 > step 3, 40 cycles Probe concentration: 400 nmol l <sup>-1</sup> *	GGGTAGGGGCGTCTGCGAAA
Dengue virus type 2	CAGGYTATGGCACTGTCACGAT Cycling profile	CTCTCCGAGAACRGGCCTCGACTTCAA (1) 60°, 30 min; (2) 95°, 10 min; (3) 95°, 15 s; (4) 60°, 1 min; step 3 > step 4, 45 cycles Primer concentration: 500 nmol l <sup>-1</sup> , Probe concentration: 250 nmol l <sup>-1</sup> *, reaction mix: ABI RNA-to-Ct 1-Step Kit, catalogue #4393463 (Life Technologies Corporation, Grand Island)	CCATYTGACGACAACACCATCTC

\*The measurement of the concentration of a Taqman probe is complicated by the chemically attached fluorophore and quencher. Therefore, the optimal probe concentration in the final mix is chosen by titration.



**Figure 1** Bar graph showing the mean concentration of the set of frozen aliquots for each species was determined in PCR Detectible Units per millilitre. The 95% confidence intervals of the mean are indicated by error bars. (■) *Bacillus anthracis*, (■) *Staphylococcus aureus*, (■) *Yersinia pseudotuberculosis*, (■) *Leishmania donovani*, (■) Dengue 2.

served aliquots that were prepared as the same batch. In our study, the *B. anthracis* aliquot average (Fig. 1) was 1.54 × 10<sup>9</sup> PDU ml<sup>-1</sup> (1.25 × 10<sup>9</sup>–1.91 × 10<sup>9</sup>, 95% Confidence Interval).

#### Preparation of spiked specimens

An initial study was performed to determine the detectible range in the spiked specimens for a given extraction method and assay. An initial estimate was made from the range of concentrations detectible when the pure frozen aliquot was the starting material. Therefore, an appropriate quantity of a thawed aliquot was diluted in fresh human blood to a range of concentrations in PDU ml<sup>-1</sup> that, based on previous analytical studies, was detectible by the diagnostic test and included some dilutions in the series that should be below the limit of detection for the diagnostic test. The process of dilution to the desired concentration involved a number of dilution steps from the highly concentrated frozen stock. Therefore, a few steps were made in appropriate buffer such as Phosphate Buffered Saline. However, a dilution used as a specimen should have pathogen added such that the specimen volume was 99.9% blood. This was accomplished by making two 10-fold dilutions into blood before the final 10-fold dilution into the specimen. Three samples of each specimen in this initial dilution panel were then extracted using the Qiagen DNA Blood Mini Kit (catalogue #51104; Qiagen) or its equivalent and an undiluted sample assayed by PCR. These results indicated the detectible range near the limit of detection of the assay and platform. Further evaluation of the platform and assay was done with multiple specimens in the detectible range prepared from the additional frozen aliquots in the same manner.

To assess whether the diagnostic test had similar performance in ill patients, analysis of a series was performed by spiking blood samples obtained from individuals with symptoms similar to the very early stage of an infection (fever, malaise or flu-like symptoms). The study was performed as described above using the same frozen aliquots of pathogen spiked in the same concentrations tested for healthy donor specimens, the only difference being the source of donor matrix.

#### *Staphylococcus aureus* (ATCC # 29213)

##### *Determination of the growth curve*

A loop dipped in a vial of bacteria obtained from the American Type Culture collection (ATCC, Manassas, VA) was streaked on an LB plate then the procedure followed was identical to that described above for *B. anthracis*. In our culture of *Staph. aureus*, the mid-exponential growth point was OD<sub>600</sub> of 1.0, reached 2 h after the initial inoculation.

##### *Preparation of cryopreserved aliquots*

After harvesting the culture when it reached OD<sub>600</sub> of 1.0 in about 2 h, the procedure described for *B. anthracis* was followed.

##### *Determination of molecular pathogen concentration of frozen aliquots*

We tested the frozen aliquots and calculated the PDU ml<sup>-1</sup> proceeding as described above for *B. anthracis* using an appropriate PCR primer and probe set to detect *Staph. aureus*. Our assay was a Taqman style reaction targeting the 16S rRNA gene, which detected all Gram-positive bacteria (Table 1).

In our study, the *Staph. aureus* aliquot average (Fig. 1) was  $1.8 \times 10^9$  PDU ml<sup>-1</sup> for all four tested aliquots, thus no confidence interval was calculated.

##### *Preparation of spiked specimens*

Procedures for preparing spiked specimens were the same as described above for *B. anthracis*.

#### *Yersinia pseudotuberculosis* ATCC #29833, (Pfeiffer) Smith and Thal strain, surrogate for *Yersinia pestis*

##### *Determination of the growth curve*

A loop dipped in a vial of bacteria obtained from the ATCC was streaked on an LB plate, then the procedure followed was identical to that described above (37°C incubation) for *B. anthracis*. In our culture, *Y. pseudotuberculosis* reached an OD<sub>600</sub> of 1.0 in 4 h.

##### *Preparation of cryopreserved aliquots*

After harvesting the culture when it reached OD<sub>600</sub> of 1.0 in about 5 h, the procedure described for *B. anthracis* was followed.

##### *Determination of molecular pathogen concentration of frozen aliquots*

We tested the frozen aliquots and calculated the PDU ml<sup>-1</sup> proceeding as described above for *B. anthracis* using an appropriate PCR primer and probe set to detect *Y. pseudotuberculosis*. Our assay was a Taqman style reaction targeting the 16S rRNA gene (Table 1), which detected *Y. pseudotuberculosis* and *Y. pestis* (Drygin Iu *et al.* 1995) but does not react with all other Gram-negative bacteria according to Drygin Iu *et al.*

In our study, the *Y. pseudotuberculosis* aliquot average (Fig. 1) was  $2.39 \times 10^9$  PDU ml<sup>-1</sup> ( $1.12 \times 10^9$ – $5.11 \times 10^9$ , 95% Confidence Interval).

##### *Preparation of spiked specimens*

Procedures for preparing spiked specimens were the same as described above for *B. anthracis*.

#### *Leishmania donovani* (LD1S2D strain, CBER laboratory culture)

This strain of the protozoan parasite has been characterized by sequencing multiple gene segments and aligning to database genomic sequence (Duncan *et al.* 2001, 2009; Selvapandiyani *et al.* 2001; Debrabant *et al.* 2002; Goyal *et al.* 2006; Gannavaram *et al.* 2011, 2012, 2014; Lakhali-Naouar *et al.* 2012). Frozen aliquots of live organisms of this strain are available from the authors or any of the laboratories that are referenced above (Debrabant *et al.* 2004).

##### *Determination of the growth curve*

All steps were performed using sterile technique in a biosafety cabinet. A cryopreserved aliquot was thawed and placed in a plastic 25-cm<sup>2</sup> culture flask with 5 ml promastigote medium (Debrabant *et al.* 2004). Incubation occurred at 26°C for 24 h. The proportion of viable cells in the culture, indicated by their motility, was assessed by periodically observing the cells in the flask using a microscope. Culture growth continued for several passages until >90% of the parasites appeared motile. A dye exclusion test was performed to assess cell viability. One dye exclusion method (Chan *et al.* 2012) that worked well with the *Leishmania* cells required mixing acridine orange (AO; Sigma-Aldrich #A6014, St. Louis, MO) and propidium iodide (PI; Sigma-Aldrich #287075) with an aliquot of cells from the culture and observing them with a



fluorescent microscope. All cells will be fluorescent green by taking up the AO. Only the dead cells will take up the PI, appearing fluorescent red. When parasites were >90% viable, we proceeded with documenting the growth curve. Parasites were inoculated into 35 ml of culture medium in a 225-cm<sup>2</sup> flask at the concentration of  $1 \times 10^6$  cells ml<sup>-1</sup>. During incubation at 26°C, samples were taken every 6–12 h to measure the cell density. Measurement was made by automated microscopic image analysis (Vision Cellometer, Nexcelom Bioscience, Lawrence, MA). A haemocytometer and microscope or a Beckman/Coulter particle counter could also be used. We determined the time in culture and the cell concentration that were in the centre of the linear portion of the growth curve (exponential phase). Our results for *L. donovani* were 55 h and  $4 \times 10^7$  cells ml<sup>-1</sup>.

#### *Preparation of cryopreserved aliquots*

Liquid culture harvested at the chosen mid-exponential stage (30 ml) was centrifuged for 10 min at 2500 rev min<sup>-1</sup>, the liquid removed and the cells resuspended in 30 ml cryopreservation medium (standard culture medium + Foetal Bovine Serum (FBS) + Glycerol, mixed in a 6 : 3 : 1 ratio and sterile filtered). The suspension was aliquoted into sterile cryotubes, 0.5 ml each, mixing the suspension continually to maintain a uniform concentration. It was stored at room temperature for 30–60 min, then stored at –80°C overnight and transferred to a liquid nitrogen freezer for long-term storage.

#### *Determination of molecular pathogen concentration of frozen aliquots*

The *L. donovani* concentration was calculated by PCR detection of a limiting dilution of the nucleic acid extracted from the frozen aliquot and the concentration was expressed as PDU per ml. Steps included thawing a frozen aliquot and extracting total DNA from three separate 100 µl samples of the aliquot liquid by a chosen method (the Qiagen DNeasy kit, catalogue #69504; Qiagen, following the manufacturer's protocol). The process was repeated with at least three other frozen aliquots of *L. donovani* from the same batch. Each extracted DNA sample was diluted in 10-fold serial steps. All the extracts of all the aliquots in the range of dilutions that span from 100% detectable down to below detectability were tested by quantitative real-time PCR. Our results for *L. donovani* were between 1/10<sup>5</sup> and 1/10<sup>7</sup> dilutions of the DNA solution extracted from the frozen aliquots. Our assay was a Taqman style reaction targeting the minicircle DNA structure in the kinetoplast (Table 1). The primers bound in conserved regions and the probe was synthesized with degenerate bases to permit amplification of most species of *Leishmania* (Selvapandian et al. 2009).

In our study, the *L. donovani* aliquot average (Fig. 1) was  $4.73 \times 10^9$  PDU ml<sup>-1</sup> ( $2.74 \times 10^9$ – $6.51 \times 10^9$ , 95% Confidence Interval).

#### *Preparation of spiked specimens*

Procedures for preparing spiked specimens were similar to those described above for *B. anthracis*.

#### **Dengue virus type 2 (Strain New Guinea C, ATCC # VR-1584)**

##### *Growth conditions*

All steps were performed using sterile technique. Dengue virus type 2, strain New Guinea C (NGC), an RNA (+) sense genome virus was used as seed to infect susceptible *Aedes albopictus* C6/36 cells (# CRL-1660, ATCC). Cells were grown to about 80% confluence in T-150 culture flasks and incubated at 32°C in an atmosphere of 5% CO<sub>2</sub>, in minimum essential media (MEM; Life Technologies Corp., Grand Island, NY) plus 2% foetal bovine serum (FBS, Life Technologies Corp.) supplemented with L-glutamine (584 mg l<sup>-1</sup>), Fungizone (2.5 mg l<sup>-1</sup>) and Gentamicin (66 mg l<sup>-1</sup>). The number of C6/36 cells in a flask to be inoculated was estimated by counting the cells in a separate T-150 flask seeded and grown to 80% confluence in parallel. Cells were trypsinized and the cell suspension counted in a Countess Automated Cell Counter (Invitrogen, Grand Island, NY). DENV inoculum was prepared by diluting the viral seed to give a MOI = 0.01 in MEM, added to the C6/36 cell cultures and incubated for 1 h with gentle rocking every 15 min. After incubation, 20 ml of MEM + 2% FBS were added to each flask, the cells were returned to the incubator and observed daily for the appearance of a cytopathic effect. Supernatants were harvested at peak production of DENV NGC virus. The peak production was defined to be at day 5 postinfection based on multistep viral growth curves carried out in our laboratory. The cell culture supernatants were clarified to remove cell debris by centrifugation at 1000 g for 10 min at 4°C.

##### *Preparation of cryopreserved aliquots*

After collecting the supernatant from centrifugation, the virus suspension in culture medium was aliquoted 100 µl per tube into sterile labelled tubes that were tightly capped, making at least 50 aliquots. Tubes were placed immediately in crushed dry ice and transferred to a –80°C freezer for long-term storage.

##### *Determination of molecular pathogen concentration of frozen aliquots*

After a minimum of 24 h postfreezing, aliquots of the viral suspension were thawed and DENV RNA loads were

determined using an appropriate quantitative molecular assay.

In our study, the frozen aliquots were extracted with a QIAamp MinElute Virus Spin Kit (Qiagen) according to the manufacturer's instructions. The RNA sample was quantified with our TaqMan assay (Table 1, ABI Taqman RT-PCR Mix; Life Technologies, Carlsbad, CA) by limiting dilution.

The concentration in the aliquot was expressed as PDU per ml following the calculations as described for *B. anthracis*. Our result for the Dengue virus type 2 aliquots was  $5.5 \times 10^7$  PDU ml<sup>-1</sup> for each of the four tested aliquots (Fig. 1); thus no confidence interval was calculated.

#### Preparation of spiked specimens

Most diagnostic and blood donor screening devices for detection of viruses are currently optimized for using human plasma as the sample type. Therefore, these spiked specimens were prepared using plasma samples. Frozen aliquots of the virus stock were diluted into fresh human plasma to at least three different concentrations in PDU ml<sup>-1</sup> that included one near the limit of detection for the diagnostic test that resulted in 100% positive detection and two more 10-fold dilutions below that. Three samples of each specimen in this dilution panel were then extracted using the QIAamp MinElute Virus Spin Kit (Qiagen) or equivalent and tested for detectability with the platform and assay being evaluated. Further evaluation of the platform and assay were done with multiple copies of the specimens in this range prepared from the additional frozen aliquots in the same manner.

To assess whether the diagnostic test had similar performance in ill patients, analysis of a dilution series was performed using plasma samples obtained from individuals with early symptoms of infection (fever, malaise or flu-like symptoms).

## Results

### *Bacillus anthracis*

In our study analysing blood samples from healthy human donors spiked with *B. anthracis*, using the real-time PCR pXO1 assay (Table 1) bacteria were 100% detectible (10/10) at  $10^5$  PDU ml<sup>-1</sup> (Table 2), 55.6% detectible (5/9, one of the 10 specimens resulted in an invalid amplification curve) at  $10^4$  PDU ml<sup>-1</sup> and 10% (1/10) at  $10^3$ . Negative control samples extracted from unspiked blood were below cut-off in 80% of the replicates with the same PCR assay (8/10).

The same specimens were sent out with coded labels to a commercial diagnostic biotechnology laboratory, Diatherix Laboratories, Huntsville, AL, for independent test-

ing using target-enriched multiplex PCR (TEM-PCR), a proprietary method (US 7, 851 148 B2) developed by Diatherix Laboratories. This amplification technology in combination with detection on bar-coded magnetic beads (BMB; Applied Biocodes, Santa Fe Springs, CA) could simultaneously detect *B. anthracis*, *Staph. aureus*, *Y. pseudotuberculosis*, *L. donovani* and Dengue virus type 2 tested in this study. The TEM-PCR/BMB assay identified 10/10 (100%) spiked specimens correctly at  $10^5$  PDU ml<sup>-1</sup> (Table 2), at  $10^4$  PDU ml<sup>-1</sup>, 8/10 (80%) and at  $10^3$  1/10 (10%). One of the 20 Negative control samples extracted from unspiked blood had above cut-off reactivity for *B. anthracis* (95% specificity).

Blood samples were obtained commercially (BioreclamationIVT, Hicksville, NY) from patients with fever and signs and symptoms of flu-like illness. Spiked specimens were made following the same procedure used for healthy blood donors reproducing the same range of concentrations used with healthy donor blood. With *B. anthracis* spiked at  $10^5$  PDU ml<sup>-1</sup>, 6/7 (85.7%) were detected (Table 3), 2/7 (28.6%) at  $10^4$  PDU ml<sup>-1</sup> and 0/7 (0%) at  $10^3$  PDU ml<sup>-1</sup>. Negative control specimens made with the patient donor blood resulted in none reactive out of 10 controls (100% specificity).

The same specimens were sent out with coded labels to Diatherix Laboratories for independent testing. Using their multiplex nucleic acid test that is capable of detecting *B. anthracis*, spiked specimens at  $10^5$  PDU ml<sup>-1</sup> were all identified correctly 7/7 (100%), although one specimen spiked with *B. anthracis* showed above cut-off reactivity with both *B. anthracis* and *Y. pseudotuberculosis* (Table 3). This type of result that only occurs with multiplex assays is both true positive for *B. anthracis* and false positive for *Y. pseudotuberculosis*. At  $10^4$  PDU ml<sup>-1</sup>, 6/7 (85.7%) were correctly identified and at  $10^3$  PDU ml<sup>-1</sup>, 1/7 (14.3%). Negative control samples extracted from unspiked blood were all correctly identified 20/20 (100%).

### *Staphylococcus aureus*

In our study analysing blood samples from healthy human donors spiked with *Staph. aureus*, using our real-time PCR assay targeting the 16S rRNA gene (Table 1) the pathogen was 100% detectible (10/10) at  $10^6$  PDU ml<sup>-1</sup> (Table 2), 100% detectible (10/10) at  $10^5$  PDU ml<sup>-1</sup> and 10% detectible (1/10) at  $10^4$  PDU ml<sup>-1</sup>. Negative control samples extracted from unspiked blood were 10/10 (100%) below cut-off with the same PCR assay.

The same specimens were sent out with coded labels to Diatherix Laboratories, for independent testing. Using their multiplex nucleic acid test that is capable of detecting *Staph. aureus*, at  $10^6$  PDU ml<sup>-1</sup> 10/10 (100%) were

**Table 2** Healthy donor blood or plasma spiked specimen testing by the FDA laboratory and by Diatherix Laboratories. The numbers of replicates that met each condition based on Real-Time PCR (FDA) or target enriched multiplex PCR (Diatherix) are shown

Spiked Pathogen, concentration (PDU)	Pathogen identified	Diatherix result		FDA result	
		Pos	Neg	Pos	Neg
<i>Bacillus anthracis</i> , 10 <sup>3</sup> ml <sup>-1</sup>	<i>B. anthracis</i>	1	9	1	9
<i>B. anthracis</i> , 10 <sup>4</sup> ml <sup>-1</sup>	<i>B. anthracis</i>	8	2	5	4
<i>B. anthracis</i> , 10 <sup>5</sup> ml <sup>-1</sup>	<i>B. anthracis</i>	10	0	10	0
<i>Staphylococcus aureus</i> 10 <sup>4</sup> ml <sup>-1</sup>	<i>Staph. aureus</i>	3	7	1	9
<i>Staph. aureus</i> 10 <sup>5</sup> ml <sup>-1</sup>	<i>Staph. aureus</i>	10	0	10	0
<i>Staph. aureus</i> 10 <sup>6</sup> ml <sup>-1</sup>	<i>Staph. aureus</i>	10	0	10	0
<i>Yersinia pseudotuberculosis</i> , 10 <sup>2</sup> ml <sup>-1</sup>	<i>Y. pseudotuberculosis</i>	5	5	2	8
<i>Y. pseudotuberculosis</i> , 10 <sup>3</sup> ml <sup>-1</sup>	<i>Y. pseudotuberculosis</i>	8	2	9	1
<i>Y. pseudotuberculosis</i> , 10 <sup>4</sup> ml <sup>-1</sup>	<i>Y. pseudotuberculosis</i>	10	0	10	0
<i>Leishmania</i> , 10 <sup>3</sup> ml <sup>-1</sup>	<i>Leishmania</i>	4	6	6	4
<i>Leishmania</i> , 10 <sup>4</sup> ml <sup>-1</sup>	<i>Leishmania</i>	10	0	10	0
<i>Leishmania</i> , 10 <sup>5</sup> ml <sup>-1</sup>	<i>Leishmania</i>	10	0	10	0
Unspiked blood	Negative	2	18	4	36
Dengue virus type 2, 10 ml <sup>-1</sup>	Dengue virus	9	1	10	0
Dengue virus type 2, 10 <sup>2</sup> ml <sup>-1</sup>	Dengue virus	10	0	10	0
Dengue virus type 2, 10 <sup>3</sup> ml <sup>-1</sup>	Dengue virus	10	0	10	0
Unspiked plasma	Negative	0	10	0	10

**Table 3** Symptomatic donor-spiked specimen testing by the FDA laboratory and by Diatherix Laboratories. The numbers of replicates that met each condition based on Real-Time PCR (FDA) or target enriched multiplex PCR (Diatherix) are shown

Spiked Pathogen, concentration PDU ml <sup>-1</sup>	Pathogen identified	Diatherix result			FDA result	
		Pos	Neg	FP	Pos	Neg
<i>Bacillus anthracis</i> , 10 <sup>3</sup> ml <sup>-1</sup>	<i>B. anthracis</i>	1	6		0	7
<i>B. anthracis</i> , 10 <sup>4</sup> ml <sup>-1</sup>	<i>B. anthracis</i>	6	1		2	5
<i>B. anthracis</i> , 10 <sup>5</sup> ml <sup>-1</sup>	<i>B. anthracis</i>	7	0	1	6	1
<i>Staphylococcus aureus</i> 10 <sup>4</sup> ml <sup>-1</sup>	<i>Staph. aureus</i>	3	4		4	3
<i>Staph. aureus</i> 10 <sup>5</sup> ml <sup>-1</sup>	<i>Staph. aureus</i>	5	2		7	0
<i>Staph. aureus</i> 10 <sup>6</sup> ml <sup>-1</sup>	<i>Staph. aureus</i>	7	0	1	7	0
<i>Yersinia pseudotuberculosis</i> , 10 <sup>2</sup> ml <sup>-1</sup>	<i>Y. pseudotuberculosis</i>	1	6		0	7
<i>Y. pseudotuberculosis</i> , 10 <sup>3</sup> ml <sup>-1</sup>	<i>Y. pseudotuberculosis</i>	5	2		6	1
<i>Y. pseudotuberculosis</i> , 10 <sup>4</sup> ml <sup>-1</sup>	<i>Y. pseudotuberculosis</i>	7	0		7	0
<i>Leishmania</i> , 10 <sup>3</sup> ml <sup>-1</sup>	<i>Leishmania</i>	1	6		6	1
<i>Leishmania</i> , 10 <sup>4</sup> ml <sup>-1</sup>	<i>Leishmania</i>	4	3		6	1
<i>Leishmania</i> , 10 <sup>5</sup> ml <sup>-1</sup>	<i>Leishmania</i>	7	0		7	0
Unspiked blood	Negative	0	20		3	37
Dengue virus type 2, 10 ml <sup>-1</sup>	Dengue virus	5	2		0	7
Dengue virus type 2, 10 <sup>2</sup> ml <sup>-1</sup>	Dengue virus	7	0		6	1
Dengue virus type 2, 10 <sup>3</sup> ml <sup>-1</sup>	Dengue virus	7	0		7	0
Unspiked plasma	Negative	0	10		0	10

FP, false positive; PDU, PCR Detectible Units.

identified correctly (Table 2), at 10<sup>5</sup> PDU ml<sup>-1</sup>, 10/10 (100%) were identified correctly and at 10<sup>4</sup> PDU ml<sup>-1</sup>, 3/10 (30%), showing good correspondence with the in-house results. Negative control samples extracted from unspiked blood were correctly identified 20/20 (100%).

Blood samples were obtained commercially through BioreclamationIVT from patients with fever and signs

and symptoms of flu-like illness. Spiked specimens were made following the same procedure used for healthy blood donors reproducing the same range of concentrations. With *Staph. aureus* spiked at 10<sup>6</sup> PDU ml<sup>-1</sup>, 7/7 (100%) were detected (Table 3), 7/7 (100%) at 10<sup>5</sup> PDU ml<sup>-1</sup> and 4/7 (57.1%) at 10<sup>4</sup> PDU ml<sup>-1</sup>. Negative control specimens made with the patient donor



blood resulted in two reactive out of 10 controls (80% specificity).

The same specimens were sent out with coded labels to Diatherix Laboratories for independent testing. Using their multiplex nucleic acid test that is capable of detecting *Staph. aureus*, spiked specimens at  $10^6$  PDU ml<sup>-1</sup> were all identified correctly 7/7 (100%), although one specimen spiked with *Staph. aureus* showed above cut-off reactivity with both *Staph. aureus* and *Y. pseudotuberculosis* (Table 3). This type of result that only occurs with multiplex assays is both true positive for *Staph. aureus* and false positive for *Y. pseudotuberculosis*. At  $10^5$  PDU ml<sup>-1</sup>, 5/7 (71.4%) were correctly identified and at  $10^4$  PDU ml<sup>-1</sup>, 3/7 (42.9%). Negative control samples extracted from unspiked blood were correctly identified 20/20 (100%).

#### *Yersinia pseudotuberculosis*

In our study analysing blood samples from healthy human donors spiked with *Y. pseudotuberculosis*, using our real-time PCR assay targeting a 16S rRNA sequence unique to *Yersinia* species (Table 1), the pathogen was 100% detectible (10/10) at  $10^4$  PDU ml<sup>-1</sup>, 90% detectible (9/10) at  $10^3$  PDU ml<sup>-1</sup> and 20% detectible (2/10) at  $10^2$  PDU ml<sup>-1</sup> (Table 2). Among negative control samples extracted from unspiked blood, two were above cut-off with the same PCR assay (2/10, 20%).

The same specimens were sent out with coded labels to Diatherix Laboratories for independent testing. Using their multiplex nucleic acid test that is capable of detecting *Y. pseudotuberculosis*, at  $10^4$  PDU ml<sup>-1</sup>, 10/10 (100%) were identified correctly and at  $10^3$  PDU ml<sup>-1</sup>, 8/10 (80%) were identified correctly and at  $10^2$  PDU ml<sup>-1</sup>, 5/10 (50%), showing good correspondence with the in-house results (Table 2). Negative control samples extracted from unspiked blood were correctly identified 19/20 (95%).

Blood samples from patients with fever and signs and symptoms of flu-like illness were spiked following the same procedure used for healthy blood donors reproducing the same range of concentrations. With *Y. pseudotuberculosis* spiked at  $10^4$  PDU ml<sup>-1</sup>, 7/7 (100%) were detected (Table 3), 6/7 (85.7%) at  $10^3$  PDU ml<sup>-1</sup> and 0/7 (0%) at  $10^2$  PDU ml<sup>-1</sup>. Negative control specimens made with the patient donor blood resulted in none reactive out of 10 controls (100% specificity).

The same specimens made with blood from patients with fever and signs and symptoms of flu-like illness were sent out with coded labels to Diatherix Laboratories for independent testing. Using their multiplex nucleic acid test that is capable of detecting *Y. pseudotuberculosis*, spiked specimens at  $10^4$  PDU ml<sup>-1</sup> (Table 3) were all

identified correctly 7/7 (100%). At  $10^3$  PDU ml<sup>-1</sup>, 5/7 (71.4%) were correctly identified and at  $10^2$  PDU ml<sup>-1</sup>, 1/7 (14.3%). Negative control samples extracted from unspiked blood were correctly identified 20/20 (100%).

#### *Leishmania donovani*

In our study analysing blood samples from healthy human donors spiked with *L. donovani*, using our real-time PCR assay targeting the minicircle (Table 1) the pathogen was 100% detectible (10/10) at  $10^5$  PDU ml<sup>-1</sup> (Table 2), 100% detectible (10/10) at  $10^4$  PDU ml<sup>-1</sup> and 60% detectible (6/10) at  $10^3$  PDU ml<sup>-1</sup>. Negative control samples extracted from unspiked blood were all below cut-off with the same PCR assay (10/10).

The same specimens were sent out with coded labels to Diatherix Laboratories for independent testing. Using their multiplex nucleic acid test that is capable of detecting *L. donovani*, 10/10 (100%) were identified correctly at  $10^5$  PDU ml<sup>-1</sup>, at  $10^4$  PDU ml<sup>-1</sup>, 10/10 (100%) were identified correctly and at  $10^3$  PDU ml<sup>-1</sup>, 4/10 (40%), showing good correspondence with our results (Table 2). Negative control samples extracted from unspiked blood were identified as negative for *L. donovani*, 20/20 (100%).

Blood samples from patients with fever and signs and symptoms of flu-like illness were spiked following the same procedure used for healthy blood donors reproducing the same range of concentrations. With *L. donovani* spiked at  $10^5$  PDU ml<sup>-1</sup>, 7/7 (100%) were detected (Table 3), 6/7 (85.7%) at  $10^4$  PDU ml<sup>-1</sup> and 6/7 (85.7%) at  $10^3$  PDU ml<sup>-1</sup>. Negative control specimens made with the patient donor blood resulted in one reactive out of 10 controls (90% specificity).

The same specimens were sent out with coded labels to Diatherix Laboratories for independent testing. Using their multiplex nucleic acid test that is capable of detecting *L. donovani*, spiked specimens at  $10^5$  PDU ml<sup>-1</sup> (Table 3) were all identified correctly 7/7 (100%). At  $10^4$  PDU ml<sup>-1</sup>, 4/7 (57.1%) were correctly identified and at  $10^3$  PDU ml<sup>-1</sup>, 1/7 (14.3%). Negative control samples extracted from unspiked blood were correctly identified 20/20 (100%).

#### Dengue virus

In our study, the Dengue virus type 2 frozen stock diluted into fresh human plasma to  $10^3$  PDU ml<sup>-1</sup> was 100% detected (10/10) with our real-time PCR assay targeting the envelope region (Table 1) at an average cycle quantification (Cq) value of 28.6 (Table 2),  $10^2$  PDU ml<sup>-1</sup> was 100% detected (10/10) at an average Cq value of 32.1 and 10 PDU ml<sup>-1</sup> was 100% detected (10/10) at an average Cq value of 36.4. Although we

failed to prepare specimens with virus concentrations below the limit of detection in this assay, the log/linear increase in the Cq value shows the device's response to decreasing pathogen and the detection at another 10-fold dilution, 1 PDU ml<sup>-1</sup>, would not likely be 100%. Negative control samples extracted from unspiked plasma were all below cut-off (average Ct value 44.0) with the same PCR assay (19/19).

The same specimens were sent with coded labels to Diatherix Laboratories for independent testing. Using their multiplex nucleic acid test that is capable of detecting Dengue virus type 2, 100% (Table 2) were detected (10/10) at 10<sup>3</sup> PDU ml<sup>-1</sup> at an average mean fluorescence index (MFI) of 24 136. At 10<sup>2</sup> PDU ml<sup>-1</sup>, 10/10 (100%) were identified correctly with an average MFI of 22 864. At 10 PDU ml<sup>-1</sup>, 9/10 (80%) were detected with an average MFI of 14 147. As with the in-house RT-PCR, this range of virus concentration did not reach far enough below the limit of detection of this assay; however, a declining response showed that one more dilution probably would. Negative control samples extracted from unspiked blood were correctly identified 10/10 (100%).

Plasma samples from patients with fever and signs and symptoms of flu-like illness were spiked following the same procedure used for healthy donors reproducing the same range of concentrations. With Dengue virus type 2 spiked at 10<sup>3</sup> PDU ml<sup>-1</sup>, 7/7 (100%) were detected (Table 3), 6/7 (85.7%) at 10<sup>2</sup> PDU ml<sup>-1</sup> and 0/7 (0%) at 10 PDU ml<sup>-1</sup>. Negative control specimens made with the patient donor plasma resulted in none reactive out of 10 controls (100% specificity).

The same specimens were sent out with coded labels to Diatherix Laboratories for independent testing. Using their multiplex nucleic acid test that is capable of detecting Dengue virus type 2, spiked specimens at 10<sup>3</sup> PDU ml<sup>-1</sup> (Table 3) were all identified correctly 7/7 (100%). At 10<sup>2</sup> PDU ml<sup>-1</sup>, 7/7 (100%) were correctly identified and at 10 PDU ml<sup>-1</sup>, 5/7 (71.4%). Negative control samples extracted from unspiked blood were correctly identified 10/10 (100%).

## Discussion

A step by step procedure has been described that can be followed by assay developers who are targeting low prevalence pathogens. These types of assays and platforms are essential for detection of biothreat and emerging agents; yet clinical specimens to test their performance are difficult or impossible to obtain. Mock clinical specimens made by spiking cultured pathogens into the human matrix are a solution to that difficulty (FDA/CDRH 2014). If each developer is free to choose the most convenient procedure for making spiked specimens that may

differ substantially from other developers, evaluation of the relative performance will be difficult. Therefore, the intention of this study was to describe standardized methods and demonstrate how the spiked specimens could be used in evaluating an in-house real-time PCR assay and a commercial, multiplex assay.

The correspondence of our results with spiked specimens prepared by these methods and the results obtained using a commercially available multiplex assay to test the same specimens at another location demonstrates the reproducibility, practicality and utility of this approach to generate mock clinical specimens. Thus, following these methods, specimens can be prepared that could be used to assess the sensitivity, specificity and reproducibility of a diagnostic device to detect low prevalence viral, bacterial or protozoan pathogens in plasma, whole blood or other human matrices. The mock specimens were made in blood or plasma from healthy donors. Mock specimens were also made from these matrices obtained from patients with fevers and other flu-like symptoms. The importance of evaluating the performance with sick donor as well as healthy donor specimens is illustrated by the lower sensitivity and specificity of these two assay platforms that occurred with the sick donor specimens. Documenting the performance with the sick donor specimens comes closer to reproducing the true clinical specimens that the assay may be required to test.

Although the use of standardized methods to make and use mock specimens will substantially improve the evaluation and comparison of new devices intended to detect biothreat and emerging low prevalence pathogens, the performance measurements will not be absolute. Absolute comparison would require the establishment of internationally recognized reference standard material as the World Health Organization has done for common pathogens such as HIV (WHO *et al.* 2011) or *Trypanosoma cruzi* (Otani *et al.* 2011). As the cited documents will testify, establishment, storage and distribution of a reference standard is a time-consuming and costly venture only achieved when there is a broad consensus supporting the undertaking. At this time, the establishment of standardized spiking methods is an important first step.

In conclusion, device developers are encouraged to systematically follow the methods described here to evaluate the performance of new assays and platforms. Tabulated results from testing multiple, independently spiked specimens are intended for description of the characteristics of the new device.

## Acknowledgements

We wish to acknowledge that this work was supported by NIAID through an Interagency Agreement AAI13005-001

awarded to CBER/FDA. Members of the NIAID/Office of Biodefense, Research Resources, and Translational Research, Division of Microbiology and Infectious Diseases aided in the editing of the text. Yukiko Kozaki LEP/DETTD/CBER graciously assisted with statistical analysis. Healthy human blood and plasma was obtained thanks to the NIH Clinical Center, Department of Transfusion Medicine.

### Conflict of Interest

No conflict of interest is declared for any of the authors.

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