Assessment of nucleic acid modification induced by amotosalen and ultraviolet A light treatment of platelets and plasma using real-time polymerase chain reaction amplification of variable length fragments of mitochondrial DNA

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BACKGROUND: Pathogen inactivation methods are increasingly used to reduce the risk of infections after transfusion of blood products. Photochemical treatment (PCT) of platelets (PLTs) and plasma with amotosalen and ultraviolet A (UVA) light inactivates pathogens and white blood cells through formation of adducts between amotosalen and nucleic acid that block replication, transcription, and translation. The same adducts block the amplification of nucleic acids using polymerase chain reaction (PCR) in a manner that correlates with the number of adducts formed, providing a direct quality control (QC). Current QC measures for PCT rely on indirect methods that measure the delivered UVA dose or percent residual amotosalen after illumination, rather than directly measuring nucleic acid modification.

STUDY DESIGN AND METHODS: Endogenous mitochondrial DNA (mtDNA), which is detectable in PLT and plasma units, was chosen as a target for the quantification of photochemically induced modifications. DNA was extracted from untreated or amotosalen and UVA–treated PLTs or plasma, and mtDNA fragments of variable lengths were quantified using a real-time PCR inhibition assay.

RESULTS: PCT induced increasing real-time PCR inhibition of mtDNA amplification for larger amplicon sizes. Amplification was unaffected by treatment with amotosalen or UVA alone, whereas up to 3 log inhibition was observed after PCT. Blinded PCR testing of a panel of 110 samples each, from PLT or plasma components prepared for routine use within a blood center, allowed 100% discrimination between untreated and treated units.

CONCLUSION: Our initial findings indicate that an adequately sensitive, quantitative real-time PCR inhibition assay targeting mtDNA could provide a valuable tool to confirm and monitor PCT.

The recent emergence and geographic expansion of viral threats to blood safety have underscored the value of wider implementation of pathogen inactivation (PI) technologies to contain such threats.[1,2] The INTERCEPT system for PI of platelets (PLTs) and plasma has been in use in European blood centers over the past decade and has recently gained Food and Drug Administration approval in the United States.[3-5]

ABBREVIATIONS: C_T = threshold cycle; EFS = Établissement Français du Sang; mtDNA = mitochondrial DNA; PCT = photochemical treatment; PI = pathogen inactivation.

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This work was supported by research funding from Cerus Corporation.

This article was published online on Oct 7 2015. An error was subsequently identified. This notice is included in the online and print versions to indicate that both have been corrected 12 Oct 2015.

Received for publication May 26, 2015; revision received July 22, 2015; and accepted August 31, 2015.

doi:10.1111/trf.13360

TRANSFUSION 2016;56;410–420

The importance of PI for addressing blood safety concerns has been recognized by regulatory authorities as evidenced by their approval for investigational device exemption studies using INTERCEPT to address chikungunya and dengue virus blood safety risks and for treatment of Ebola virus convalescent plasma.[6,7]

PI using the INTERCEPT system is based on photochemical treatment (PCT) of blood components with amotosalen and UVA light that induce adducts and cross-links in nucleic acids at an estimated frequency of 1/83 bp, thus preventing nucleic acid replication, transcription, and translation and leading to PI.[8-14] The degree of PI correlates with the concentration of amotosalen, as well as the dose of UVA light delivered.[15] Quality assurance for illuminated products relies on monitoring using photodiode sensors inside the instrument that measure the UVA dose administered during each treatment. UVA indicator labels are also available, providing a visual check of proper illumination based on a color change on the label.[16] While such process controls can accurately measure performance of the bulbs, they are unable to track the downstream effects of illumination, which may be attenuated by other factors such as the container material and product volume or composition. The PCT process can additionally be tracked by high-performance liquid chromatography (HPLC)-based quantification of percent residual amotosalen after illumination,[17] and this method is used in some European centers for quality control (QC) of the inactivation process. Indeed, it has been shown that residual amotosalen, measured by HPLC, correlates linearly with the extent of *Klebsiella pneumoniae* inactivation.[18] Nonetheless, such an approach remains indirect since it does not quantify modifications to the target molecule itself. In addition, the HPLC approach requires sampling at two time points: after addition of amotosalen, before UVA treatment; and after UVA treatment, before compound adsorption. These sampling time points fall outside the routine workflow for most blood centers, thus impeding ease of implementation.

An alternative approach to directly measure nucleic acid modification induced by PCT is long-amplicon real-time polymerase chain reaction (PCR) whereby enzyme progression is inhibited upon encountering adducts or cross-links. Mitochondrial DNA (mtDNA) is an appropriate target for such an approach, given that it is a collateral target of PI and, unlike nuclear DNA, is present in PLTs. Previous work showed that amplification of mtDNA is inhibited after treatment of PLT concentrates with amotosalen and UVA, using endpoint PCR.[19,20] This proof-of-principle study demonstrated that inhibition of PCR amplification of mtDNA could be used to monitor the success of the photochemical inactivation process. However, the semiquantitative and low-throughput aspects of the endpoint PCR method are poorly suited to routine testing in the blood banking setting. Conversely, real-time PCR has the advantages of being high-throughput and quantitative, and similar methodologies are widely used for nucleic acid testing (NAT) of blood products. Its potential application as a routine QC test for nucleic acid modification after illumination is significant given recent regulatory agency recommendations to implement such an assay.[21] Here we assess the use of long-amplicon real-time PCR for analysis of mtDNA modification after treatment of PLTs with amotosalen and UVA.

MATERIALS AND METHODS

PLT and plasma processing

PLTs, plasma, and whole blood units were obtained from an accredited blood bank and shipped to Cerus Corp. (Concord, CA) for processing and treatment. Apheresis PLTs collected in plasma were combined with PLT additive solution (AS; InterSol, Fenwal Inc., Lake Zurich, IL) at a ratio of 35% plasma/65% InterSol. PLTs were prepared from either individual or pooled apheresis units. An aliquot was sampled from each preparation for PLT count. All PLT units met the guard band requirements for INTERCEPT treatment ($0.60 \times 10^9 - 2.35 \times 10^9$ PLTs/mL). For production of nonleukoreduced whole blood–derived PLTs, eight PLT units prepared by the PLT-rich plasma method were pooled and split into two matching units, one of which was leukoreduced (SEPACELL PLS-5A, Fenwal Inc.), while the other unit was not leukoreduced. White blood cells (WBCs) and PLTs were counted using a hematology analyzer (ADVIA 120, Siemens Healthcare Diagnostics, Deerfield, IL).

Plasma units were prepared either individually from apheresis collections or ABO-identical whole blood collections were pooled and split. Samples from Établissement Français du Sang (EFS) Alsace were derived from leukoreduced buffy coat PLTs and apheresis plasma. Sample panels were blinded and coded at Cerus before shipment. All samples were stored frozen and shipped on dry ice to Blood Systems Research Institute for mtDNA analysis.

DNA isolation from WBCs

Whole blood–derived WBCs from a single donor were used as a source of mtDNA for proof-of-concept spiking studies. Approximately 150×10^6 WBCs were processed as previously described.[22] Briefly, pelleted cells were resuspended in 1 mL of PCR Solution A (100 mmol/L KCl, 10 mmol/L Tris, pH 8.3, 2.5 mmol/L $MgCl_2$) and 200 μL of saponin lysis solution (0.4% saponin in 0.5% NaCl, pH 7.4) and agitated for 5 minutes to lyse the residual red blood cells (RBCs). After two washes with PCR Solution A, DNA was extracted from the cell pellet by adding 100 μL of Solution A, 100 μL of Solution B (10 mmol/L Tris, pH 8.3, 2.5 mmol/L $MgCl_2$, 1% Tween 20, 1% NP-40), and 25 μg of proteinase K and incubating overnight at 60°C. The proteinase K was inactivated by heating at 95°C for 30 minutes. The DNA lysate volume was adjusted to approximately 1.5 mL using Solution A:Solution B 1:1 (vol/vol). Based on the assumption that each WBC contains about

400 copies of mtDNA,[23] the concentration of the DNA lysate stock solution used from spiking studies was approximately 4×10^{10} mtDNA copies/mL.

INTERCEPT treatment

WBC-derived DNA was spiked 1:80 (vol/vol) into 35% plasma/65% InterSol at an estimated concentration of 5×10^8 mtDNA copies/mL, similar to the concentration of mtDNA found in PLT units. Fifteen millimoles per liter amotosalen was added to achieve a 150 μmol/L concentration. After being mixed, the suspensions were dispensed into six-well plates at 3.6 mL per well in duplicate and treated with 3 J/cm^2 UVA in an illuminator (Model FX1019, Nova Biomedical, Waltham, MA). Samples were taken before amotosalen addition and/or UVA treatment to serve as controls. Two-hundred microliters of the amotosalen-containing samples were removed for HPLC analysis to verify illumination, whereas the remaining volume was used for real-time PCR analysis. Treatment of PLTs was performed in PL2410 bags on mini- (30 mL) or full-size (>300 mL) units. Samples were removed before and after illumination. In the latter case, aliquots were sampled either before or after passage through the compound adsorption device. Treatment of plasma was performed on full-size units and aliquots were sampled as described for PLTs. For apheresis plasma, single units were treated, whereas for whole blood–derived plasma, pools were split into 2 or 3 units and treated separately. After collection, all samples were stored at −80°C before testing.

Real-time PCR

DNA was extracted and amplified as previously described.[24] In brief, DNA extractions from 200-μL samples were performed using silica membrane-based purification in 96-well plate or individual spin column formats (QIAamp 96 DNA Blood kits and QIAamp DNA Blood Mini kit columns, respectively, Qiagen Inc., Valencia, CA). For plasma samples, 1.8 mL of plasma was centrifuged at $21{,}000 \times g$ for 15 minutes, followed by aspiration of all but 200 μL of sample before DNA extraction. Real-time PCR reactions using SYBR Green chemistry were performed in 96-well plates on a sequence detection system (LightCycler 480 II, Roche Applied Science, Indianapolis, IN). The sequences of the primers are listed in Supplementary Table 1 (available as supporting information in the online version of this paper). Forty-five cycles of amplification were performed, with a melting curve analysis at the end of the reaction to confirm specificity of the amplified products. Samples with an undetectable signal were assigned a threshold cycle (C_T) value of 40, given that no signal was observed between 40 and 45 cycles of amplification. Serial dilutions of DNA from untreated samples were amplified to assess efficiency of the PCR assays based on the slope of the standard curve ($E = 10^{-1/\text{slope}} - 1$).

RESULTS

PCT inhibits real-time PCR amplification of mtDNA

To evaluate the effect of amplicon size on inhibition of mtDNA PCR after treatment with amotosalen and UVA, we developed real-time PCR assays targeting regions between 73 and 1409 bp. The PCR primers are designed to target conserved sequence regions to allow amplification and sequencing of mtDNA standard reference materials for quality assurance purposes.[25] We combined the primers to generate short- and long-amplicon real-time PCR assays. To examine the linear dynamic range of the different size amplicon mtDNA assays, DNA was extracted from PLT units and serially diluted 10-fold before amplification. As expected, the efficiency of the real-time PCR assays decreased with increasing amplicon size. The 73- and 561-bp amplicon assays demonstrated linear dynamic ranges of at least 5.6 and 5.0 log, respectively, whereas the assays generating amplicons longer than 1 kb had a 4.6 log dynamic range (Fig. 1 and data not shown). While the 73- and 561-bp amplicon assays reached 100 and 95% efficiency, respectively, the longer amplicon assays only showed 80% efficiency, based on the slope of the standard curve. The expected sizes of the amplified products were confirmed by agarose gel electrophoresis during assay development (data not shown). Specificity of the amplified products was routinely confirmed in each experiment by real-time PCR dissociation analysis. Negative controls ensured the lack of contamination.

Next we wanted to see if mtDNA real-time PCR amplification using the different size amplicon assays was inhibited by treatment with amotosalen and UVA. We spiked purified human WBC DNA into 35% plasma/65% PLT AS (InterSol). After laboratory-scale treatment (3 mL volume) with 150 μmol/L amotosalen and 3 J/cm^2 UVA, the C_T increased by a mean of 7.4 and 17.1 cycles for the 73- and 561-bp amplicon assays, respectively, whereas amplification was completely inhibited for the 1065-bp amplicon assay (Fig. 2). Treatment with either amotosalen, or UVA alone, did not inhibit PCR amplification. These results demonstrate that inhibition of mtDNA PCR amplification after PCT of spiked WBC DNA increases with amplicon length. Preliminary work indicated that the short amplicon assay is sensitive to variations in plasma content and UVA dose (data not shown). Further amotosalen and UVA dose-response experiments are needed to evaluate the sensitivity of the mtDNA inhibition assay to suboptimal PCT conditions.

Different size amplicon mtDNA real-time PCR assays quantify DNA modifications induced by PCT of PLTs

We then investigated the ability of the PCR inhibition assays to detect modifications in endogenous mtDNA within PLT units treated with amotosalen and UVA.

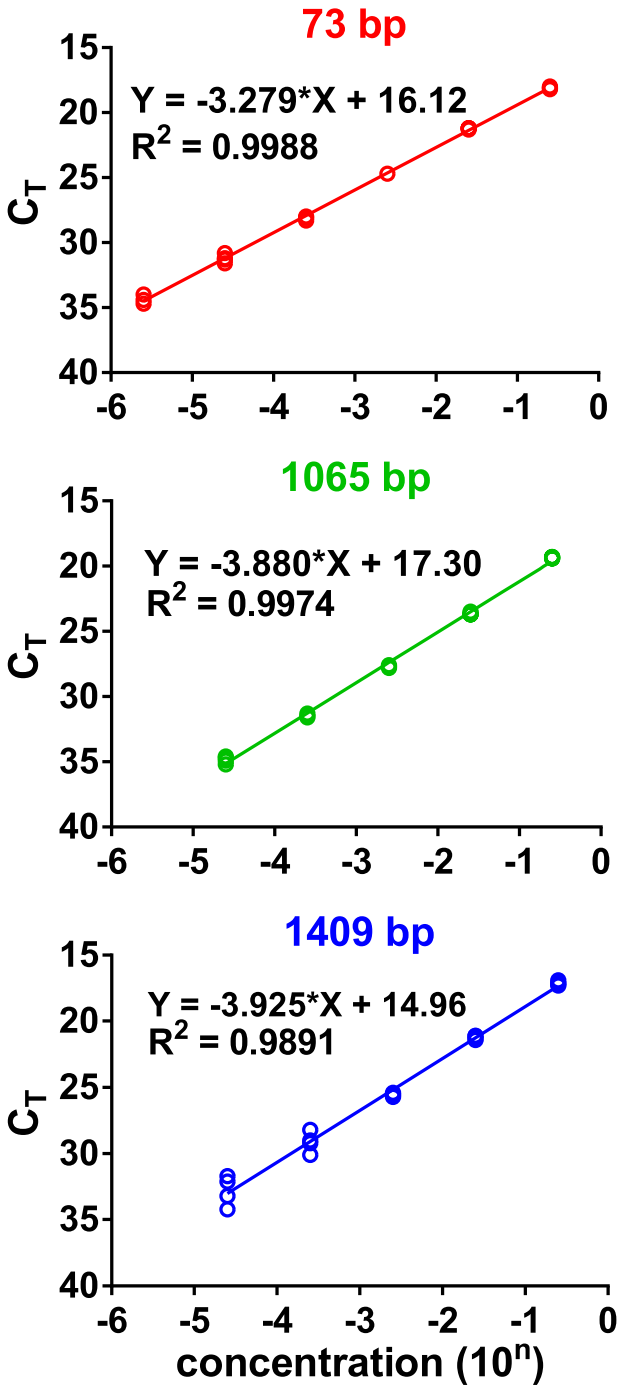

Fig. 1. Dynamic range and efficiency of mtDNA-specific real-time PCR assays. DNA was extracted from PLTs in duplicate, serially diluted 10-fold, and amplified in duplicate wells per sample using mtDNA assays targeting variable amplicon lengths. Note the decreasing amplification efficiency with increasing amplicon length based on the slope of the standard curve.

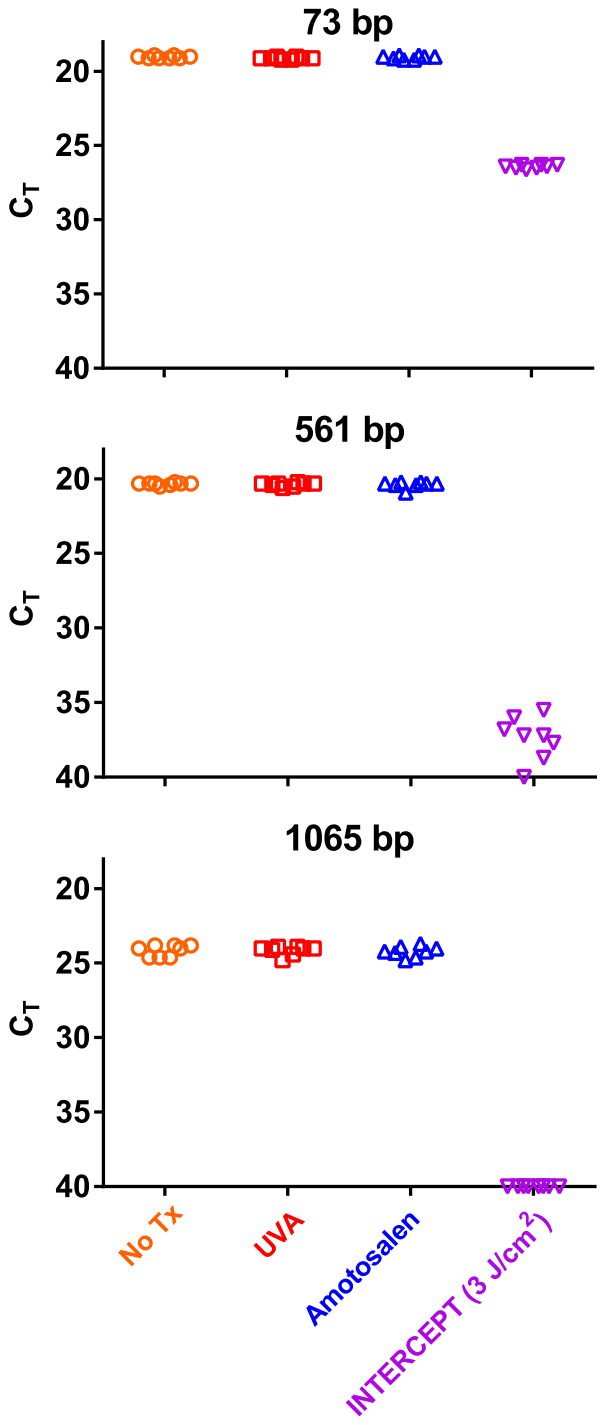

Fig. 2. INTERCEPT treatment of human WBC DNA spiked into 35% plasma/65% InterSol inhibits PCR amplification using mtDNA-specific real-time PCR assays, based on proposed mechanism of action. Control treatment conditions included exposure to UVA or amotosalen alone. Data represent two independent treatments, with duplicate DNA extractions from each treatment and duplicate amplification wells from each sample. Tx = treatment.

Feasibility studies were performed using PLT miniunits (30 mL) prepared in 35% plasma/65% InterSol. After addition of amotosalen and UVA illumination of PLTs, PCR

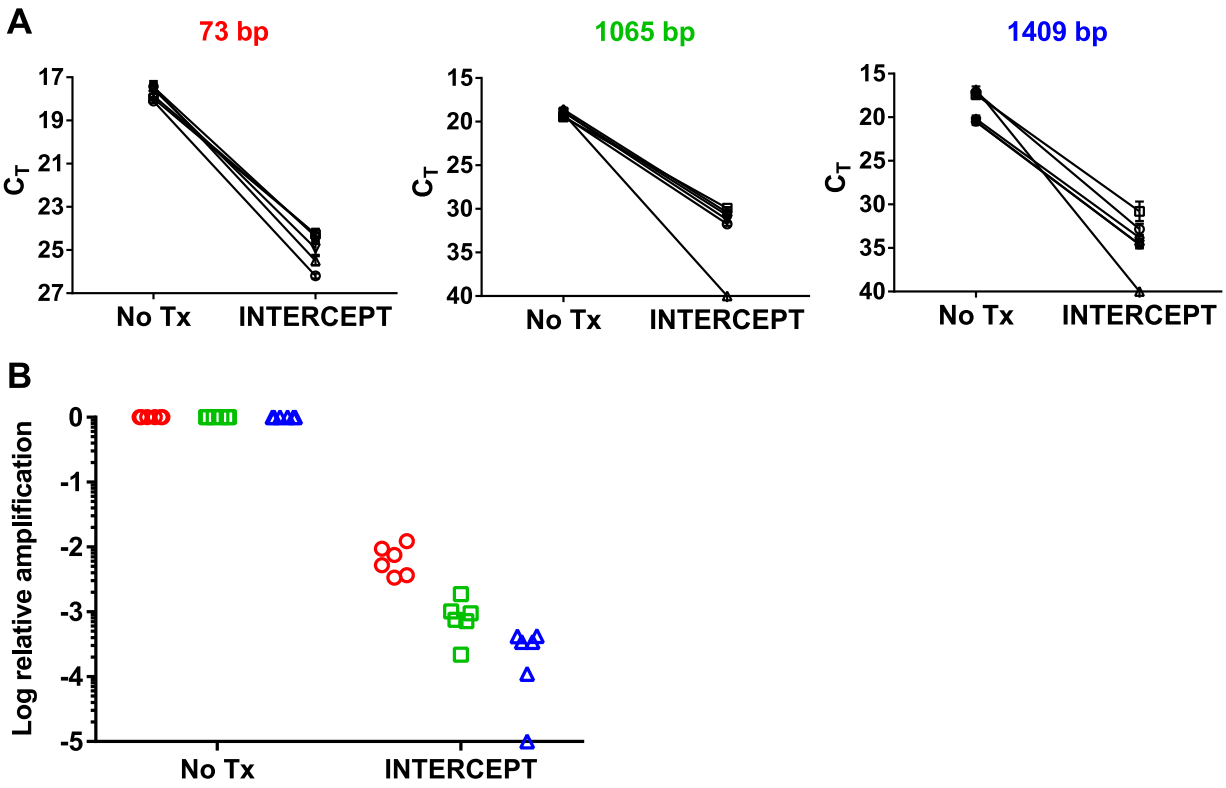

Fig. 3. mtDNA real-time PCR inhibition assay documents INTERCEPT treatment of PLT products. Mean C_T values from four replicates are shown for six individual PLT units before and after INTERCEPT treatment (A). Relative amplification was calculated from a standard curve generated using 10-fold serial dilutions of DNA from each untreated PLT unit (B). The PCR signal for the long-amplicon assays was no longer detectable at a 5 log dilution of samples from the untreated units, with the exception of 1 unit where amplification with the 1065-bp assay could not be detected at a 4 log dilution. The standard curve from a representative unit is shown in Fig. 1. Tx = treatment. (○) 73 bp; (□) 1065 bp; (△) 1409 bp.

amplification of mtDNA was increasingly inhibited according to amplicon size. The median C_T increase was 7.2, 12.1, and 14.1 cycles for 73-, 1065-, and 1409-bp amplicons, respectively (Fig. 3A), corresponding to an amplification reduction by 2.2, 3.1, and 3.4 log (Fig. 3B) calculated by relative quantification based on serial dilutions of matched untreated samples (as shown for a representative unit in Fig. 1).

To test whether the assays could reproducibly distinguish treated and untreated PLT samples, analysis was initially performed using a blind panel of 20 samples, consisting of 10 pretreatment and 10 posttreatment samples and including several duplicate sample aliquots. After PCR amplification using short- and long-amplicon mtDNA assays, two populations were clearly distinguishable based on their C_T values (Fig. 4A). Ten samples had C_T values ranging from 16 to 19 for the 73- and 1065-bp amplicons and from 20 to 22 for the 1409-bp amplicon. Another population of nine samples had C_T value ranges of 22 to 25 for the 73-bp amplicon, 28 to 36 for the 1065-bp amplicon, and 34 or higher for the 1409-bp amplicon. One sample (depicted in gray in Fig. 4) did not fall within either of the two populations observed after PCR amplification using the short-amplicon assay, likely due to low DNA recovery from that sample. We previously reported that the ΔC_T value between long- and short-amplicon assays can be used as a measure of mtDNA modification, such that the difference is larger for photochemically treated relative to untreated blood products.[24] Based on ΔC_T analysis comparing the 1065- and 73-bp amplicons, we predicted that the 10 samples with a ΔC_T value of 0 to 2 were untreated, and the nine samples with a ΔC_T value of 5 or higher were treated (Fig. 4B). After breaking the code, we concluded that the mtDNA PCR inhibition assays were 100% accurate in predicting the treatment status of the samples. In addition, the results of the ΔC_T analysis were reproducible, such that duplicate sample aliquots gave very similar values (shown boxed in Fig. 4B).

To further validate the ΔC_T approach using the 1065- and 73-bp amplicon assays, we analyzed a second blinded panel of 110 samples, consisting of 10 pretreatment and 100 posttreatment samples obtained from PLT products prepared for routine use by EFS Alsace, France. Blinded analysis indicated that one population of 10 samples had

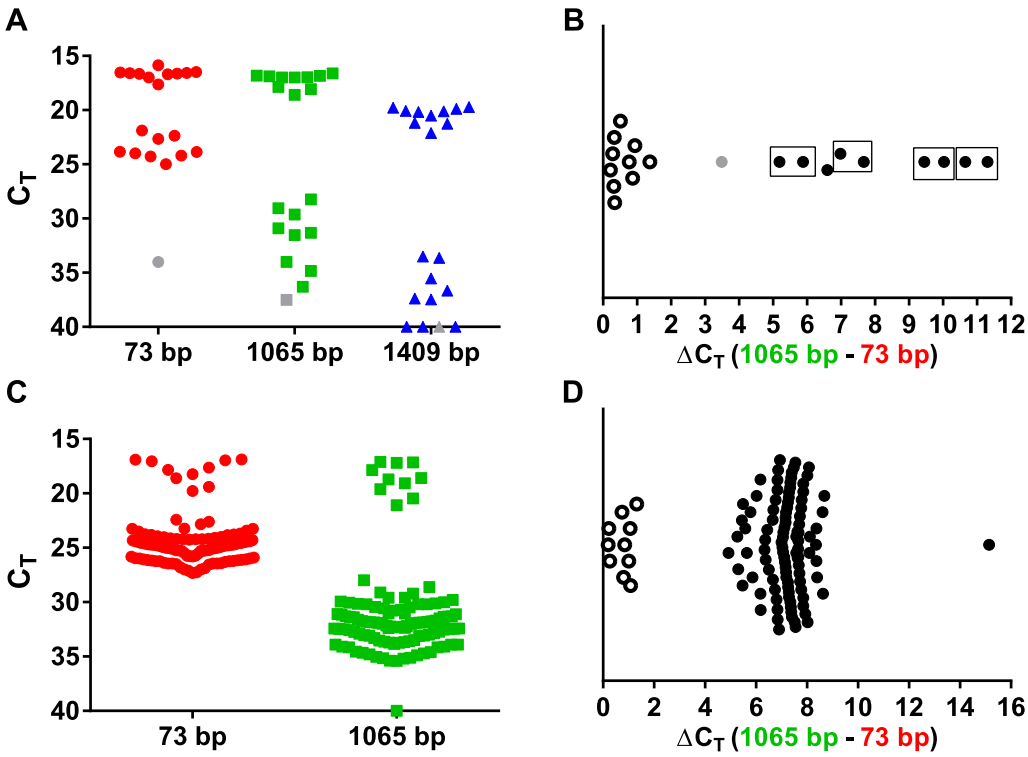

Fig. 4. Performance of mtDNA-specific real-time PCR inhibition assay on PLT samples blinded to treatment status (n = 20 for A and B; n = 110 for C and D). Mean C_T from four (A) or six (C) replicates and ΔC_T (B, D) values are shown. Boxes in (B) indicate duplicate blinded samples. Gray symbol indicates a sample with low DNA recovery. Based on the ΔC_T values, samples were predicted as either untreated (open symbols) or INTERCEPT treated (closed symbols).

C_T values ranging from 17 to 20 and 17 to 21 for the 73- and 1065-bp amplicons, respectively, whereas another population of 100 samples had C_T value ranges of 22 to 27 for the 73-bp amplicon and 28 or higher for the 1065-bp amplicon (Fig. 4C). Thus, 10 samples had ΔC_T values of less than 2 and 100 samples had ΔC_T values of at least 5 (Fig. 4D). We confirmed that these represented untreated and treated samples, respectively, after breaking the code.

Furthermore, the effect of WBC content on the mtDNA PCR inhibition assay was evaluated by comparing nonleukoreduced (4.1×10^7 WBCs/unit) and leukoreduced ($<10^6$ WBCs/unit) PLTs. Eight PLT-rich plasma units were pooled and split into a pair of 4-unit PLT pools with or without leukoreduction before treatment. Similar mtDNA PCR inhibition was observed during treatment of nonleukoreduced and leukoreduced PLTs (Table 1).

TABLE 1. Effect of WBC content on mtDNA PCR inhibition in treated PLTs

Characteristics	Leukoreduced PLTs	Nonleukoreduced PLTs
WBC count	$<10^6$/unit	4.1×10^7/unit
PLT count	2.62×10^{11}/unit	2.87×10^{11}/unit
73-bp amplicon C_T*		
Before treatment	15.03 ± 0.66	14.36 ± 0.05
After treatment	21.02 ± 0.43	20.54 ± 0.13
1065-bp amplicon C_T*		
Before treatment	16.52 ± 1.16	15.30 ± 0.22
After treatment	27.92 ± 0.35	27.84 ± 0.47
ΔC_T (1065 bp – 73 bp)†		
Before treatment	1.50 ± 0.61	0.94 ± 0.19
After treatment	6.90 ± 0.19	7.30 ± 0.53

* Mean ± SD of quadruplicate PCRs (duplicate DNA extractions each amplified in duplicate PCRs).
† Mean ± SD of duplicate DNA extractions (ΔC_T of each extraction calculated based on the difference between the mean C_T of the long- and short-amplicon assays).

Inhibition of mtDNA amplification with PCT of plasma

To determine whether the PCR inhibition assay can also be applied to detect mtDNA modification in PCT-treated plasma, which contains lower amounts of mtDNA compared to PLTs (likely derived from residual PLTs, free mitochondria, and mtDNA),[26] we measured the C_T value in plasma before and after PCT in the absence or presence of a concentration step via centrifugation before DNA extraction. Centrifugation allowed an enhanced detection of unmodified mtDNA compared to modified mtDNA, thus improving the differentiation between untreated and treated units (Fig. 5A). Using the enhanced detection method, analysis of a blind panel of 110 plasma samples

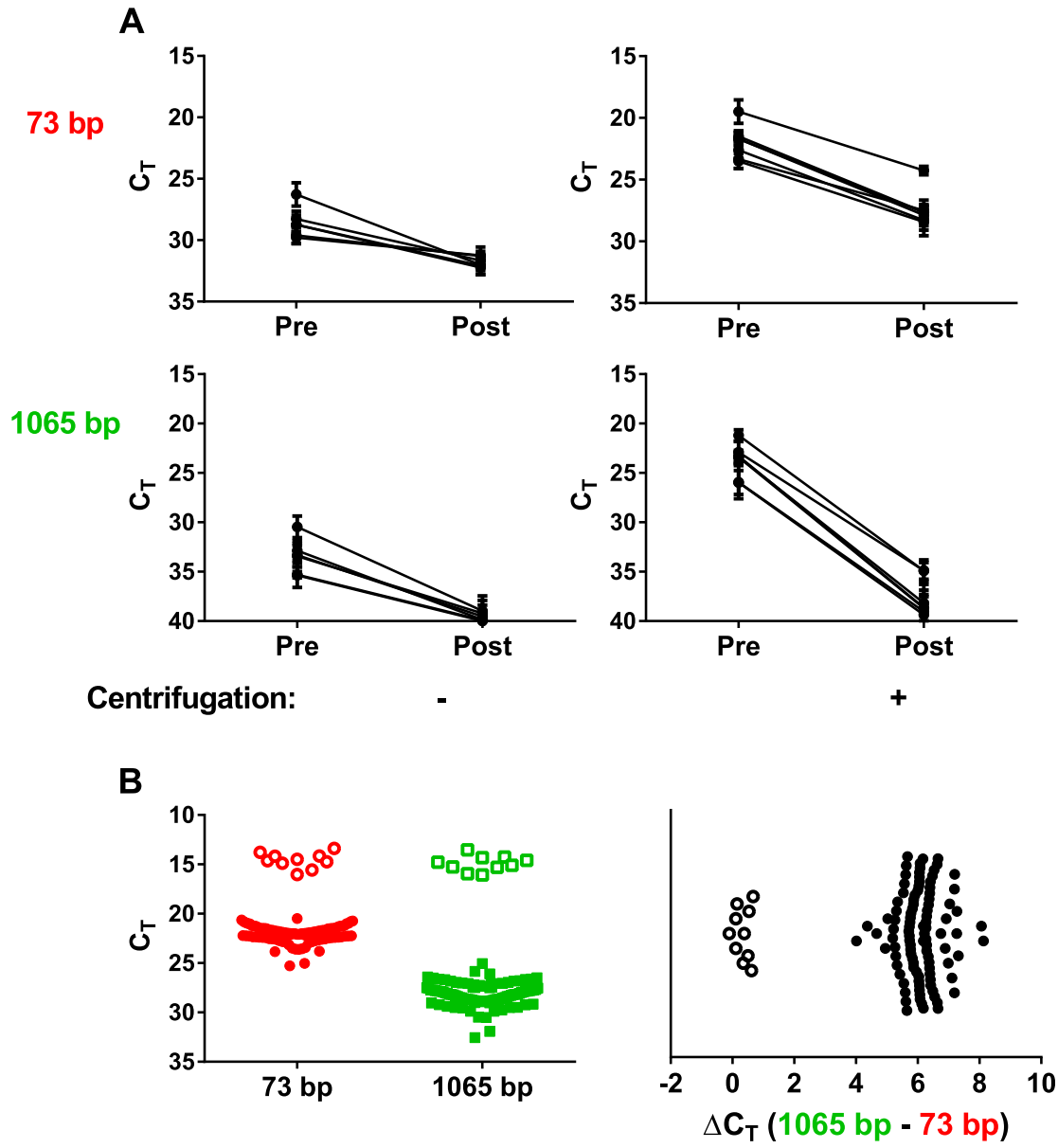

Fig. 5. Performance of mtDNA-specific real-time PCR inhibition assay on plasma samples. Two sample processing methods in the absence or presence of a concentration step via centrifugation were evaluated (A). Plasma samples blinded to treatment status (n = 110) were processed after centrifugation (B). Mean C_T from two replicates (left) and ΔC_T (right) values are shown. Based on the ΔC_T values, samples were predicted as either untreated (open symbols, n = 10) or INTERCEPT treated (closed symbols, n = 100).

obtained from EFS Alsace revealed two populations with ΔC_T values of less than 1 and 4 or more (Fig. 5B), corresponding to untreated and treated samples, respectively.

DISCUSSION

Pathogen reduction technologies have been available and in routine use in Europe over the past decade, and the INTERCEPT Blood System is now licensed in the United States. The goal of these technologies is to significantly reduce the infectious load of a broad spectrum of pathogens within blood components, thus minimizing the infectious risk associated with transfusion. Indeed, hemovigilance programs in France and Switzerland have noted the absence of septic reactions associated with transfusion of INTERCEPT-treated blood products compared to an ongoing risk of transfusion-transmitted bacterial infection mainly associated with conventional PLT products (2.6 to 6.1 per 100,000).[27-32] In rare instances, breakthrough viral

infections have been reported after PI for viruses, either not covered or covered by the respective technologies.[33,34] These cases underscore the need for developing and using QCs of pathogen reduction technologies to ensure the proper setup and continuous application of the technologies in blood centers. Such methods will reduce process failure that could provide an explanation for breakthrough infection and will provide an additional tool for investigations of infection imputability.

We show here that nucleic acid modification induced by amotosalen and UVA treatment of PLTs and plasma is clearly detectable using a real-time PCR inhibition assay targeting mtDNA. Inhibition of mtDNA amplification increases with amplicon size and is not greatly affected by variations in WBC content, similar to previous observations using endpoint PCR.[19] No inhibition was observed with amotosalen or UVA alone, consistent with the chemical mechanism of action of the PI system. This finding was dissimilar to the other photochemical technology evaluated by our approach, where significant modification of the nucleic acids was observed even for the UV alone arm.[24] It would be interesting to evaluate other technologies (Theraflex UVC and Theraflex MB, MacoPharma, Tourcoing, France) used for PI of PLTs and plasma, respectively, as well as technologies under development for pathogen reduction in whole blood (Mirasol, Terumo BCT, Lakewood, CO) and RBCs (S-303 FRALE, Cerus Corp.), to see whether additional mechanistic insights are offered by this method. It has been shown that treatment of PLT concentrates containing high levels of contaminating WBCs with amotosalen and UVA light results in genomic DNA modification at the level of one covalent adduct per 83 bp based on liquid scintillation.[13] In parallel, more than 1000-fold inhibition of PCR amplification of a 242-bp fragment in the HLA-DQα locus was observed. Additionally, PCR inhibition studies measuring viral targets showed approximately 1 log reduction in amplification of short fragments (244 bp for parvovirus B19; 495 bp for HBV) after amotosalen and UVA treatment.[35] For mtDNA, the adduct density after INTERCEPT treatment of leukoreduced PLT concentrates was calculated at one adduct per 268 bp based on liquid scintillation,[20] and PCR amplification of a 166-bp mtDNA fragment was unaffected, although quantitation was not performed.[19] In contrast, our results are consistent with a high frequency of mtDNA modification by amotosalen and UVA treatment such that amplification of a 73-bp fragment was inhibited approximately 100-fold.

A large difference in PCR amplification of a long fragment was observed when comparing untreated to treated samples. However, the use of a single long-amplicon target for QC requires the collection of paired samples before and after PCT and does not control for potential failures during DNA extraction. To internally control for variations during extraction and demonstrate proper DNA recovery from treated blood products, we show that a ΔC_T approach comparing amplification of a short and long fragment is informative for documenting successful INTERCEPT treatment. The short-amplicon assay would indicate any potential problems with DNA recovery, which could be addressed by performing replicate extractions. Although we observed a reduction in amplification of a 73-bp fragment located within the mitochondrial 16S rRNA gene, amplification of longer fragments was more severely inhibited. Ideally, an internal control region that is unaffected by INTERCEPT treatment would allow optimal differentiation between untreated and treated blood products using a ΔC_T approach. Further work is required to evaluate whether real-time PCR amplification of short fragments within other regions of the mitochondrial genome is less sensitive to inhibition after INTERCEPT treatment.

Bruchmüller and colleagues[19] designed a pass-or-fail PCR system based on 28 cycles of amplification of an approximately 1-kb mtDNA target to distinguish between untreated and INTERCEPT-treated samples, since a signal was observed after more than 28 PCR cycles. Consistent with their data, we detect a signal after 31 cycles of amplification of a 1065-bp mtDNA fragment in INTERCEPT-treated PLTs, compared to detection after only 19 cycles of amplification in untreated PLTs, corresponding to 3 log reduction in amplification. Similarly, a 2 log reduction in PCR amplification of approximately 1-kb targets was observed for parvovirus B19 treated with INTERCEPT under nonlimiting conditions, whereas up to 6 log reduction in amplification was observed for a 4.3-kb target using endpoint PCR, approximating the observed reduction of B19 infectivity.[35,36] It is unlikely that increasing the size of the mtDNA long fragment will significantly improve the level of PCR inhibition observed in our system due to the loss of efficiency and limited ability of real-time PCR to generate long amplicons. Indeed, we observed only a small incremental increase in PCR inhibition comparing a 1065- and 1409-bp amplicon (3.1 log vs. 3.4 log inhibition).

We have previously shown that treatment of PLTs and plasma with riboflavin and UV light does not affect amplification of a 162-bp target within mtDNA, whereas amplification of a 1856-bp target was inhibited about 10-fold.[24] Here we show a stronger effect of amotosalen and UVA treatment on PCR inhibition of mtDNA amplification, in terms of both the magnitude of reduction and the amplicon size. The different nucleic acid modifications induced by the two technologies may account for the observed differences in PCR inhibition. Photoactivated amotosalen forms adducts with nucleic acids, resulting in bulky photoadducts or interstrand cross-links that prevent helical unwinding, as well as monoadducts,[37] and thus inhibit replication by *Taq* polymerase. Conversely, riboflavin and UV light induce oxidation of guanine residues; this type of

lesion may not efficiently block polymerase advancement.[38] Additionally, the weak binding of riboflavin to nucleic acids makes the delivery of the damage less efficient, resulting in damage that is more consistent with diffusible species modification generated in the bulk of the solution, in contrast to technologies of the same nature, such as methylene blue and visible light, that are characterized by strong binding to nucleic acids and larger damage and pathogen reduction.[39] It is thus important to note that the criteria for PCR inhibition-based QC assays differ between the various pathogen reduction technologies, as do their mechanisms of action.

Although the frequency of nucleic acid modification induced by amotosalen and UVA treatment is relatively high, it does not affect viral RNA or DNA detection in blood products using NAT.[40] The size of the amplicons used for NAT is typically quite small, approximately 100 bp, to maximize the sensitivity of detection. Therefore, PI would have little to no impact on detection.[41] Furthermore, samples for NAT are typically removed before PCT, thus obviating concerns regarding the effect of PI on viral detection using routine NAT; however, NAT assays are often performed for confirmation or molecular characterization of viruses using plasma derived from frozen plasma or PLT components, which may have been subjected to PI.[42-45] Although preliminary work by our group indicated that PI has minimal impact on sensitivity of commercial NAT assays (data not shown), this issue warrants further study and caution when attempting to amplify pathogen nucleic acids from PI and particularly INTERCEPT-treated blood components.

In this study, we show that INTERCEPT treatment of nonleukoreduced PLTs was similarly effective, based on mtDNA inhibition, as treatment of matched leukoreduced PLTs. This finding demonstrates the efficacy of inactivation in the presence of large amounts of excess genomic material derived from high levels of residual WBCs. INTERCEPT treatment of WBCs in PLT concentrates abrogates clonal T-cell proliferation implicated in graft-versus-host disease (GVHD).[13] These findings served to support the approval by a number of European agencies for the use of INTERCEPT to reduce the risk of transfusion-associated GVHD.

While the results of this study indicate that the mtDNA PCR inhibition assay can clearly detect treatment errors resulting from the omission of amotosalen or UVA, further work is needed to characterize the robustness of the assay to detect suboptimal amotosalen or UVA doses that could potentially arise from operator error.

In conclusion, this study describes a valuable quantitative method for documenting successful nucleic acid modification induced by amotosalen and UVA treatment, applicable to both PLTs and plasma. A single small-volume sample removed after treatment is required for the assay using the ΔC_T approach, in comparison to the need for a control pretreatment or untreated sample to confirm PI efficacy. Further development of a duplex assay capable of detecting both amplicon targets within a single well may enhance its utility as a QC method for PI, although the added costs associated with fluorogenic probes may outweigh the benefit of a single-well system.

ACKNOWLEDGMENTS

We thank Mr Hervé Isola and Dr Christian Gachet of EFS Alsace for providing samples obtained from INTERCEPT-treated PLT and plasma products prepared under standard center SOPs.

CONFLICT OF INTEREST

KD, GC, JMG, and AS are employed by Cerus. SB received support for travel costs from Cerus. The other authors have disclosed no conflicts of interest.

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SUPPORTING INFORMATION

Additional Supporting Information can be found in the online version of this article at the publisher's website:
Supplementary Table 1. Sequences of mtDNA real-time PCR primers.