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ORIGINAL PAPER

Development of a mitochondrial DNA real-time polymerase chain reaction assay for quality control of pathogen reduction with riboflavin and ultraviolet light

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Background and Objectives Transfusion is associated with a risk of infection and alloimmunization. Pathogen reduction using riboflavin and UV light (Mirasol treatment) inactivates pathogens and leucocytes. With increasing adoption of the technology in clinical use, regulatory agencies have recommended the introduction of quality control measures to monitor pathogen reduction efficacy. We sought to develop a real-time PCR-based assay to document the impact of pathogen reduction on the mitochondrial genome in blood components.

Materials and Methods DNA was extracted from platelet and plasma components before and after treatment with riboflavin and UV light. Inhibition of PCR amplification of mitochondrial DNA (mtDNA) in short- and long-amplicon target regions, ranging from under 200 base pairs (bp) to over 1800 bp, was measured in treated relative to untreated components.

Results Pathogen reduction of platelets using riboflavin and UV light resulted in inhibition of PCR amplification of long-amplicon mtDNA targets, demonstrating approximately 1 log reduction of amplification relative to untreated products. Amplification of short-amplicon mtDNA targets was not affected by treatment. Evaluation of 110 blinded platelet samples from the PREPAReS clinical trial resulted in prediction of treatment status with 100% accuracy. Pathogen reduction of plasma components resulted in similar levels of PCR inhibition, while testing of 30 blinded plasma samples resulted in prediction of treatment status with 93% accuracy.

Conclusion A differential sized amplicon real-time PCR assay of mitochondrial DNA effectively documents nucleic acid damage induced by Mirasol treatment of platelets. The use of the assay for plasma product pathogen reduction requires further investigation.

Key words: mitochondrial DNA, pathogen reduction, plasma, platelets, quantitative PCR.

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Introduction

Emerging infections continue to pose a risk of transmission via transfusion, particularly for pathogens with an asymptomatic blood-borne phase and those for which there is no current intervention strategy [1]. Risk management depends primarily on donor questions and screening tests for specific pathogens. However, these strategies are reactive rather than proactive and are ultimately unsustainable as the number of questions and tests increases with the discovery of new pathogens that threaten blood safety. Pathogen reduction technologies

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have been developed as a generalized strategy to reduce the risk of transfusion-associated infections [2]. Several methods have been adopted, including leucoreduction, physicochemical methods for treatment of blood products and photochemical technologies that induce nucleic acid damage.

The Mirasol pathogen reduction technology (Terumo BCT, Lakewood, CO, USA) has been developed for platelets and plasma and relies on treatment with riboflavin and UV light to cause irreversible photo-oxidative damage to nucleic acids, primarily on guanine residues [3]. Riboflavin associates with nucleic acids, causing lesions upon exposure to UV light, and can sensitize the formation of hydroxyl radicals that induce base damage and strand breaks [4]. Evaluation of the pathogen reduction performance of the Mirasol technology using standard *in vitro* assays for infectivity or bioassays *in vivo* has shown a substantial reduction in the infectious load of a range of viruses, parasites and bacteria [5–9]. Moreover, the efficacy of Mirasol-treated blood products has been analysed in a series of *in vitro* and *in vivo* studies, indicating that both adequate platelet function and plasma protein quality are maintained after treatment [10–15]. Further, clinical trials evaluating Mirasol-treated platelets are currently ongoing [2].

While the use of infectivity assays to measure the extent of pathogen reduction is relevant for many pathogens that currently pose a risk to the blood supply, it can be time-consuming and burdensome and cannot assess efficacy for many relevant human pathogens where such assays are not available. With the goal of developing a generalizable, quantitative method to measure the impact of pathogen reduction, we developed a real-time PCR assay specific for mitochondrial DNA, which is a direct target of the Mirasol technology. Mitochondrial DNA, contained within platelet mitochondria and also detectable in plasma, can be damaged by treatment with riboflavin and UV light. Given that lesions occur within the nucleic acid molecule at a certain frequency, estimated at approximately one in every 350 base pairs [3], amplification of relatively long products should be impaired due to a block in polymerase-driven synthesis where damage is induced. Based on this principle, pathogen reduction has been shown to inhibit amplification of viral and parasitic DNA using a nested real-time PCR approach [16, 17]. Furthermore, a conventional PCR approach was used to document the effects of pathogen inactivation on mtDNA in platelet concentrates using the INTERCEPT Blood System (Cerus Corp., Concord, CA, USA), which relies on the use of amotosalen-HCl and UVA light to induce nucleic acid damage [18]. Since conventional PCR is not quantitative, we developed a real-time PCR assay that allows quantification of the relative amount of mtDNA in the absence or presence of treatment. Our results indicated that the extent of mtDNA damage induced by photochemical treatment can be quantified by real-time PCR, and this assay could be used to document the effects of pathogen reduction technologies both in research and development studies and as routinely employed quality assurance testing.

Materials and methods

Preparation of DNA from platelet-rich plasma

Platelet-rich plasma (PRP), derived from a single donor, was used as a source of mitochondrial DNA for validation experiments. Three EDTA tubes were drawn, and left to settle for two hours. The PRP supernatant was removed, pooled, aliquoted and stored at −80°C. DNA extraction from frozen PRP was performed using QIAamp DNA Blood Mini kits. Mitochondrial DNA copy number was calculated based on a standard curve of spectrophotometer-determined copy number vs. Cp using CoxF and CoxR primers, as previously described [19].

Preparation of platelets and plasma

Platelets were collected by apheresis on an automated blood collection system (Trima, Terumo BCT), suspended in plasma and allowed to rest for at least 2 h after collection prior to treatment and storage. Plasma products were derived from whole blood and frozen within 24 h of collection. The plasma was purchased from Bonfils Blood Center. Donors were selected and screened for collections as per local IRB approvals. Laboratory testing was completed on products as per blood bank standards.

Mirasol treatment

Purified mitochondrial DNA was spiked into Solution A (100 mM KCl, 10 mM Tris pH 8·3, 2·5 mM $MgCl_2$) at a concentration of $8{\cdot}6 \times 10^6$ copies/μl. 170 μl of mtDNA solution was combined with 20 μl of 500 μM riboflavin and 60 μl of saline to generate a final concentration of mtDNA roughly equivalent to that found in platelet units. Alternatively, 25 μl of 5× concentrated mtDNA was combined with 20 μl of 500 μM riboflavin and 205 μl of platelet-poor plasma (PPP). The untreated and UV controls were prepared by replacing riboflavin with an equal volume of saline. The UV and Mirasol-treated samples were plated in a 48-well plate at a volume of 250 μl per well. To mimic Mirasol treatment conditions of blood products in the illumination bag, the 48-well plate lid was replaced with bag material cut from a Mirasol illumination bag and a quartz cover. The plate was then treated

using a scaled UV energy dose equivalent to that delivered to a full-sized platelet or plasma unit [8]. Five independent experiments were conducted for saline spiking groups, and two independent sets of treatments were conducted for plasma spiking groups within this study.

For Mirasol treatment of blood products, illumination volumes (including riboflavin) were 230–332 ml for platelet units and 223–361 ml for plasma units. Samples were taken prior to riboflavin addition and/or illumination to serve as untreated controls. UV illumination (6·24 J/ml) was carried out according to the manufacturer's protocol (Terumo BCT). For energy dose kinetics experiments, units were illuminated to 10%, 25%, 50%, 75%, 90%, 100%, 110%, 125% and/or 150% of the standard energy dose. Samples were removed following illumination, aliquoted into cryogenic vials and stored at –80°C.

Real-time PCR amplification conditions

Frozen samples were thawed and duplicate DNA extractions were performed using QIAamp 96 DNA Blood kits (Qiagen Inc., Valencia, CA). DNA was extracted from 200 μl sample and eluted in 200 μl water. 10× PCR solution was added to a final concentration of 1× (100 mM KCl, 10 mM Tris pH 8·3, 2·5 mM $MgCl_2$, 1% Tween-20, 1% NP-40). Sequences of primers located in conserved mtDNA regions (Table 1) were taken from previously published reports [18, 20]. Real-time PCR reactions were performed in duplicate for each DNA extraction, with 15 μl of template in a 50-μl reaction containing 5 mM $MgCl_2$, 2 mM dNTPs (Bioline USA Inc., Taunton, MA, USA), 0·5 μM each primer, 0·25× SYBR Green I (Molecular Probes Inc., Eugene, OR, USA) and 2·5 U FastStart Taq (Roche Applied Science, Indianapolis, IN, USA). Real-time PCR was performed in a 96-well format on a sequence detection system (LightCycler 480 II, Roche) with the following cycle conditions: 1 min at 95°C followed by 45 cycles of 30 s at 95°C, 30 s at 60°C and 45 s at 72°C, with a melting curve analysis at the end of the reaction.

mtDNA PCR inhibition analysis

The DNA lysate from treated samples and 10-fold serial dilutions of the DNA lysate from untreated samples were amplified in parallel using the primers generating amplicons of different sizes. A standard curve for each target region was generated for each untreated sample and analysed by performing linear regression to calculate the relative amount of intact mtDNA in treated samples. Since PCR amplification of the 162-bp fragment was unaffected by treatment of blood products, this assay was used as an internal control reference for normalization of sample input. Log inhibition was calculated based on levels of intact mtDNA amplified using the primer pair generating a 1856-bp fragment, normalized using the 162-bp internal control assay.

Clinical samples

Samples from 55 platelet concentrates (each derived from five pooled buffy coats), suspended in plasma, were selected from the PREPAReS study (Sanquin Blood Supply, the Netherlands). One ml aliquots were taken before and after Mirasol treatment and thus generated 110 samples. The samples were recoded using a randomization list and thus randomized and blinded before shipment. All samples were stored at –30°C and shipped on dry ice to BSRI for mtDNA analysis. Samples were unblinded after mtDNA analysis results were reported to Sanquin.

Statistics

Samples with an undetectable mtDNA PCR signal were assigned a crossing point (Cp) value of 45. For analysis containing more than two groups, a repeated-measures one-way analysis of variance (ANOVA) was used in comparison to the untreated condition (for comparisons of Cp values) or to the 100% dose (for comparisons of log inhibition in energy dose kinetics experiments) with Dunnett's multiple comparisons post-test using a graphing and analysis software package (GRAPHPAD PRISM, GraphPad

Table 1 Real-time PCR primer sequences and characteristics of amplicons used to evaluate PRT effects

Primer pair	Forward primer DNA sequence (5′ → 3′)	Reverse primer DNA sequence (5′ → 3′)	Amplified mtDNA regions	Amplicon size (bp)
16SF1/16SR4[a]	GAATTAACTAGAAATAACTTTGCAAGG	CCAGCTATCACCAGGCTC	16S rRNA	162
16SF2/16SR12[a]	CAGACGAGCTACCTAAGAAC	GGCGCTTTGTGAAGTAGG	16S rRNA	1255
F361/R1769[b]	ACAAAGAACCCTAACACCAGC	GCCAGGTTTCAATTTCTATCG	Control region, 12S rRNA, 16S rRNA	1409
F361/R2216[b]	ACAAAGAACCCTAACACCAGC	TGTTGAGCTTGAACGCTTTC	Control region, 12S rRNA, 16S rRNA	1856

[a]The sequences of these primers were taken from Bruchmüller *et al.*, Transfusion 2005 [18].

[b]The sequences of these primers were taken from Levin *et al.*, Genomics 1999 [20].

Software, Inc., La Jolla, CA, USA). For analysis of plasma spiking studies, two-tailed unpaired *t*-tests were used to determine the significance of differences between two given groups.

Results

Development of real-time PCR inhibition assays for mtDNA

mtDNA modifications induced by the INTERCEPT Blood System pathogen inactivation technology were previously investigated using conventional PCR assays [18]. We evaluated the eight primer pairs used in that study for their performance in real-time PCR-based amplification of DNA extracted from PRP. We also included four additional assays derived from sequencing primers associated with a mtDNA standard reference material intended for quality control purposes [20]. All assays generated specific amplicons ranging in size from 162 to 1856 bp as observed by agarose gel electrophoresis (Fig. 1a) and by real-time PCR dissociation analysis (Fig. 1b). We chose four primer pairs spanning a range of amplicon lengths and targeting different regions in the mtDNA genome, including the control region, 12S and 16S rRNA genes, for further evaluation (Table 1).

To determine the sensitivity and dynamic range of the real-time PCR assays, DNA was extracted from PRP, diluted 10-fold from $10^{8.3}$ down to $10^{2.3}$ mtDNA copies per sample input, and amplified using real-time PCR with the four primer pairs. The assays resulted in a 4- to >6-log dynamic range. The lowest sample input was detected using the 162-bp and 1255-bp assays. Conversely, the 1409-bp and 1856-bp assays were less sensitive and required sample inputs of $10^{4.3}$ and $10^{5.3}$ mtDNA copies for detection, respectively (Fig. 1c).

As proof-of-concept that mtDNA damage induced by the Mirasol pathogen reduction technology (PRT) can be detected using our quantitative real-time PCR approach, we spiked DNA extracted from PRP into a saline solution

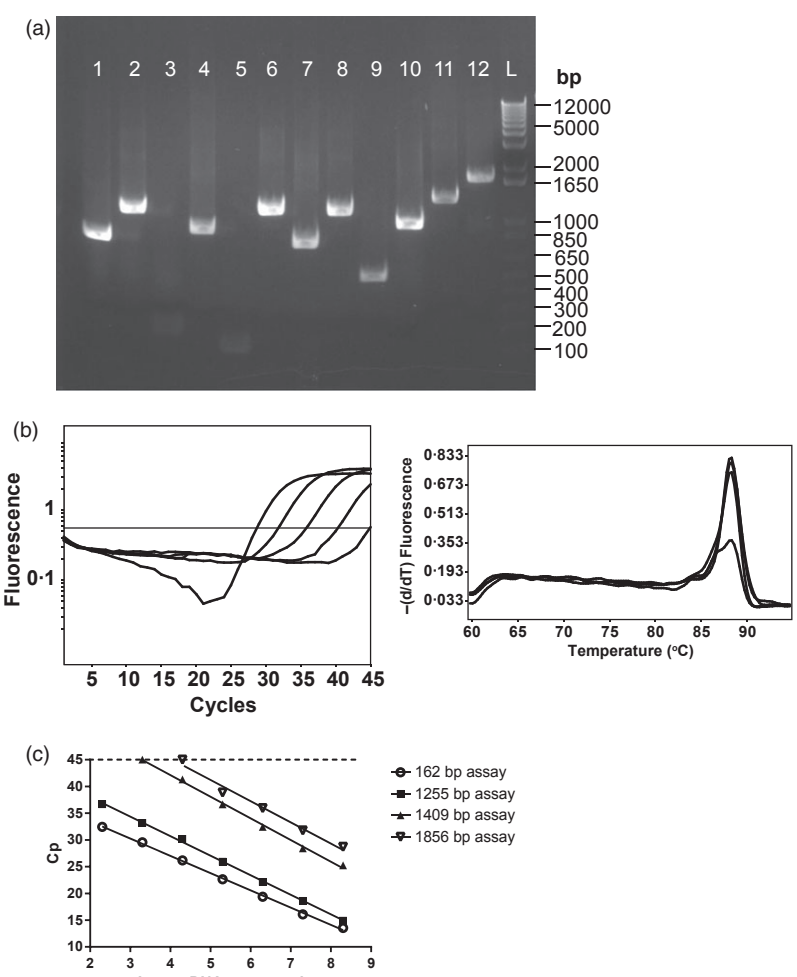

Fig. 1 Performance of mitochondrial DNA real-time PCR assays. (a) Agarose gel electrophoresis of PCR products from DNA extracted from platelet-rich plasma and amplified by real-time PCR using 12 primer pairs specific for human mtDNA. PCR products were run on a 1·5% agarose gel. Amplicon sizes in the indicated lanes are as follows: (1) 877 bp, (2) 1247 bp, (3) 236 bp, (4) 997 bp, (5) 162 bp, (6) 1255 bp, (7) 862 bp, (8) 1233 bp, (9) 561 bp, (10) 1065 bp, (11) 1409 bp, (12) 1856 bp. L = ladder (TrackIt 1 kb Plus DNA Ladder, Invitrogen). (b) Four 10-fold serial dilutions of platelet-rich plasma DNA were amplified using the 1856-bp mtDNA real-time PCR assay. Dissociation curves verify the specificity of the amplified products. (c) Standard curves are shown for four different mtDNA real-time PCR assays with variable amplicon lengths to determine the dynamic range and sensitivity of the assays. Cp, crossing point.

and exposed the DNA to Mirasol treatment. Controls included no treatment, riboflavin alone or UV alone. After DNA purification to remove the riboflavin, samples were amplified using real-time PCR with the four mtDNA primer pairs. The results of five independent experiments are shown in Fig. 2a. As predicted by the approximate frequency of lesion formation upon exposure to riboflavin and UV light, amplification of the shortest fragments was minimally inhibited by Mirasol treatment, whereas amplification of the long fragments was inhibited. The extent of PCR inhibition correlated with amplicon length, such that amplification of intermediate length sequences in the 560–880 bp range resulted in a 7–10 cycle increase in the Cp value (data not shown), whereas amplification of the 1409 bp and 1856 bp sequences was almost completely inhibited, corresponding to an increase of approximately 15 cycles in the Cp value after Mirasol treatment of purified DNA in saline solution. Treatment with riboflavin alone did not inhibit PCR amplification. In contrast, treatment with UV alone resulted in the inhibition of PCR amplification to a similar extent as Mirasol treatment.

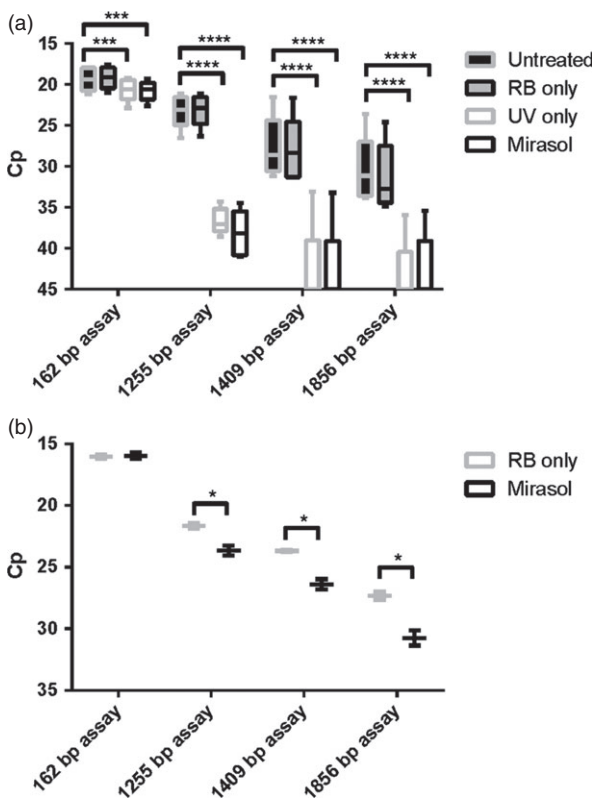

Fig. 2 Comparison of mtDNA PCR cycle number between untreated and Mirasol-treated DNA. (a) Platelet-rich plasma (PRP) DNA was spiked in saline solution and treated without or with riboflavin and/or ultraviolet light. PCR signals are compared between the four treatment conditions for each of four mtDNA real-time PCR assays generating amplicons of different lengths. Boxplots (median, 25% and 75% quartiles, and minimum to maximum values) show the cycle numbers for each condition ($N = 5$ separate experiments). Mean values were compared using a repeated measures one-way ANOVA with Dunnett's multiple comparison post-test, ***$P < 0{\cdot}001$, ****$P < 0{\cdot}0001$. (b) PRP DNA was spiked in platelet-poor plasma and treated with riboflavin with or without ultraviolet light. Boxplots (as above) show the cycle numbers for each condition (2 independent treatment wells per condition). Mean values were compared using a two-tailed unpaired *t*-test, *$P < 0{\cdot}05$.

To further investigate the feasibility of detecting mtDNA damage by the real-time PCR inhibition approach under treatment conditions that mimic the energy dose typically delivered to blood products, mtDNA was spiked into PPP and treated with riboflavin alone or Mirasol, followed by real-time PCR analysis (Fig. 2b). No PCR inhibition was observed using the short-amplicon assay, unlike the longer amplicon assays where the Cp value increased between two, three and four cycles, proportional to amplicon length, corresponding to approximately 1 log inhibition of amplification of the longest fragment. The Cp values observed with Mirasol treatment of PPP (Fig. 2b) were not as high as those with treatment of saline (Fig. 2a), probably due to exposure of DNA to increased energy, and thus increased damage, in the saline experiments.

Inhibition of mtDNA PCR following PRT treatment of blood products

Given that our assay reliably detected Mirasol-induced mtDNA damage, we then tested whether Mirasol treatment resulted in mtDNA damage when 11 platelet units (Fig. 3a) or 10 plasma units (Fig. 3b) were treated under conditions typically used for pathogen reduction of blood products. PCR amplification of the 162-bp sequence was not inhibited following treatment of platelets or plasma. Therefore, this assay was chosen as an internal control for sample input. Conversely, PCR amplification of the 1856-bp fragment resulted in a 3–5 cycle increase in the Cp value of the treated sample relative to the untreated sample, corresponding to a 1–1·5 log inhibition of amplification. To evaluate the influence of UV dose on the PCR inhibition assay, platelet (Fig. 3c) and plasma (Fig. 3d) samples were exposed to several different energy doses. Platelet and plasma samples exposed to 25%, 50% and 100% of the Mirasol energy dose had similar mean reductions of 0·2, 0·5 and 1·2 log, respectively. The extent of PCR inhibition reflected the UV illumination dose ranging from 10% to 150% of the standard clinical dose. For platelet samples exposed to the highest UV doses (Fig. 3c), amplification using the 1856-bp assay gave high Cp values that varied considerably between replicates, probably due to stochastic effects resulting from the low

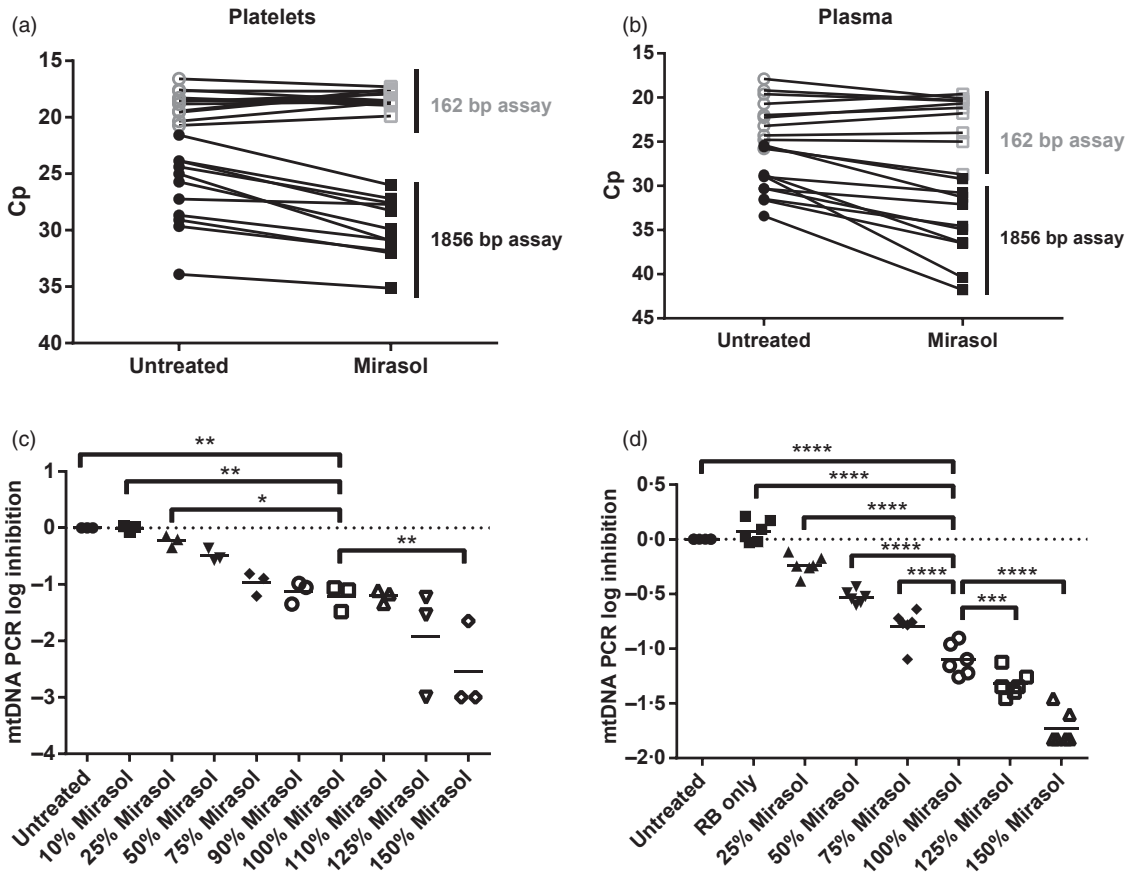

Fig. 3 mtDNA real-time PCR inhibition after Mirasol treatment of platelets and plasma. (a) Platelet units ($N = 11$) or (b) plasma units ($N = 10$) were untreated or treated with riboflavin and ultraviolet light (Mirasol). PCR signals are compared between the two treatment conditions for the 162-bp (grey open symbols) and 1856-bp (black solid symbols) mtDNA assays. mtDNA PCR inhibition was calculated after administration of different energy doses to (c) platelet units ($N = 3$) or (d) plasma units ($N = 6$). Mean values were compared using a repeated measures one-way ANOVA with Dunnett's multiple comparison post-test, $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$, $^{****}P < 0{\cdot}0001$.

level of intact target molecules. At 150% of the Mirasol energy dose, the assay showed inhibition of amplification to the limit of detection for most platelet and plasma samples.

Blinded evaluation of mtDNA PCR inhibition assay

Blinded analysis was performed using a coded panel of 110 samples, which included 55 pretreatment and 55 post-treatment samples, obtained from platelet products undergoing evaluation in the PREPAReS clinical trial (Table 2). For each sample, the difference in crossing point (dCp) between the 1856-bp fragment and the 162-bp fragment was used as a measure of mtDNA damage, such that the dCp should be higher for Mirasol-treated products relative to untreated products. Upon histogram analysis of the dCp values for the 110 blinded samples (Fig. 4a), we observed a population of 55 samples with dCp ranging from 5 to 8 and a second population of 55 samples with dCp ranging from 10 to 13. After breaking the code, we concluded that the mtDNA PCR inhibition assays predicted with 100% accuracy the treatment status of the samples. Based on this analysis, a dCp minimum threshold of 9 could be used for quality control testing to ensure nucleic acid damage induced by pathogen reduction treatment.

Fifteen paired plasma samples, each with pre- and post-treatment preparations, were similarly tested in a blinded fashion (Table 2). Histogram analysis of the dCp values did not reveal two clearly separate populations (Fig. 4b). Nonetheless, we predicted that the 15 samples with dCp ranging from 6 to 11 were untreated and the 15 samples with dCp ranging from 12 to 17 were treated. This prediction was 93% accurate, with the two samples that were incorrectly predicted having dCp values close to the midpoint (10·9 and 12·6 for treated and control samples, respectively, indicated by an asterisk in Fig. 4b). The

Table 2 Cp distribution of all blinded samples

	162-bp assay Cp		1856-bp assay Cp	
Product	**Range**	**Avg ± SD**	**Range**	**Avg ± SD**
Platelets	18·6–24·5	21·8 ± 1·1	25·4–36·7	30·8 ± 2·5
Plasma	20·3–29·6	26·1 ± 2·4	25·8–44·5	37·1 ± 4·3

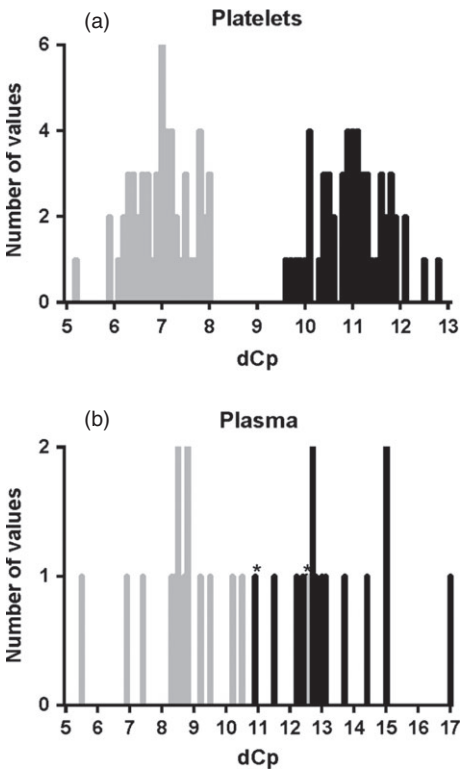

Fig. 4 Differential amplicon length mtDNA real-time PCR assay documenting the PRT process. Blinded samples from 55 platelet units (a) and 15 plasma units (b) before and after Mirasol treatment were amplified using the 162-bp and 1856-bp mtDNA real-time PCR assays. The difference in Cp (dCp) between the two assays was calculated for each sample and plotted on a histogram. Samples were identified as untreated (grey) or treated (black) after decoding. * indicates the two samples that were not predicted accurately (b).

quantity of mtDNA in the plasma samples was lower overall compared with the platelet samples (median Cp for the 1856-bp assay = 35·4 and 28·8 for untreated plasma and platelet samples, respectively). Stochastic effects during PCR amplification due to the low number of target mtDNA molecules in the plasma samples could account for the failure to differentiate between treated and control products. Thus, the assay may be suitable for quality control assessment of treatment-induced nucleic acid damage in plasma products upon further optimization of sample processing.

Discussion

In this study, we have demonstrated that a real-time PCR inhibition assay targeting two mitochondrial DNA fragments of different lengths can be used to document the impact of pathogen reduction after ultraviolet illumination and riboflavin treatment of platelet products and may be further optimized for plasma products. Due to the low probability of generating damage within small DNA fragments [3], the short product of 162 bp serves as an internal normalization control, whereas the long product of 1856 bp allows detection of PRT-induced damage that inhibits polymerase-driven DNA amplification. The assay is quantitative and sensitive to variations in UV energy dose, indicating that it can detect incomplete treatment as well as absence of treatment of blood components. While no difference in PCR inhibition was observed after UV treatment in the absence or presence of riboflavin, the addition of riboflavin is important for pathogen inactivation and prevention of DNA repair mechanisms [4, 21].

Given the high rate of polymorphism within mitochondrial DNA, the primer binding sites were located in highly conserved regions to minimize the potential for PCR failure [18, 20]. The modifications induced by ultraviolet light and riboflavin include oxidative lesions, mainly 8-oxodG and strand breaks. It is likely that the real-time PCR inhibition assay underestimates the amount of PRT-induced nucleic acid damage since 8-oxodG does not efficiently impede the advancement of *Taq* polymerase [22]. In fact, while real-time PCR amplification of mitochondrial DNA was inhibited by approximately 1 log after Mirasol treatment, infectivity assays for various pathogens exhibited on average 4 log reduction after treatment [3]. The higher stringency of infectivity assays, requiring relatively accurate fidelity during nucleic acid replication and translation of the entire genome for pathogen survival and spread, could account for the observed difference.

Compared to other methods used to detect nucleic acid damage, such as HPLC and end-point PCR, real-time PCR offers high sensitivity, wide dynamic range, high throughput and quantitative analysis. This rapid method is commonly used for infectious disease testing in blood banking settings. Previous studies employing pathogen-specific PCR amplification inhibition assays showed up to 4 log inhibition of HBV amplification after amotosalen-HCL and UVA light treatment of plasma [16], and 1–2 log inhibition of *P. falciparum* mitochondrial genome amplification after riboflavin and UV treatment of whole blood [17]. The molecular method used in those studies was based on a two-step amplification approach consisting of preamplification of a long-amplicon target for a prevalidated number of cycles using conventional PCR, followed

by quantification by real-time PCR using a nested primer pair. The sensitivity range using this approach depends on the number of preamplification cycles, and the use of nested primers for quantification increases the risk of PCR contamination. Furthermore, while this approach is useful for detecting nucleic acid damage for specific pathogens within infected blood units, it is not applicable for routine testing of transfusable units.

Based on our blinded analysis, a paired platelet sample taken before PRT is not needed for evaluation of treatment-induced nucleic acid damage. Rather, a small aliquot of the platelet product taken after PRT, in comparison with a single untreated control sample, would be sufficient for testing by real-time PCR using a mitochondrial DNA dual-amplicon system to establish whether or not the Mirasol treatment was effective. Recently, the European Directorate for the Quality of Medicines & Healthcare established a recommendation that blood centres introduce Quality Control measures to monitor the efficacy of pathogen reduction technologies [23].

In addition to the detection of mitochondrial DNA damage after Mirasol treatment, it may be useful to adapt the long-amplicon real-time PCR assays described here to investigate mitochondrial RNA damage induced by PRT as a measure of RNA inactivation. Recent work documented a decrease in micro-RNA and mRNA levels after INTERCEPT treatment of platelets without formation of cross-linked adducts in RNA species shorter than 300 nucleotides [24]. Decreases in platelet RNA function through cross-linking or other mechanisms might account for the loss of platelets observed in PRT clinical trials [2].

While the mtDNA real-time PCR inhibition assay was effective in documenting the impact of PRT for platelet products, further work is required for its application to plasma products. Although a less abundant source of mtDNA compared to platelets, plasma contains some residual platelets, intact mitochondria and free mtDNA [19]. The lower mtDNA signal and higher variability in plasma products impaired clean discrimination between untreated and treated samples during blinded analysis, such that one of fifteen samples from each of the two categories was incorrectly predicted. Nonetheless, we were able to predict treatment status with 93% accuracy, suggesting the assay could be further optimized by improving the DNA extraction process to concentrate the mtDNA, potentially by enriching for the residual platelet fraction using a 3000 g centrifugation step as previously described [19]. The concordance between levels of PCR inhibition seen for both platelets and plasma at similar energy doses suggests that additional validation work and testing of a larger number of plasma samples may indicate a comparable dCp cut-off value for plasma and platelets.

In summary, a mtDNA-based dual-amplicon real-time PCR assay was developed and is capable of quantifying the extent of mtDNA damage induced by photochemical treatment of blood products, with potential use by blood establishments as a quality control method to document the impact of pathogen reduction.

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References

1 Stramer SL, Dodd RY, AABB Transfusion-Transmitted Diseases Emerging Infectious Diseases Subgroup: Transfusion-transmitted emerging infectious diseases: 30 years of challenges and progress. *Transfusion* 2013; **53**:2375–2383

2 Prowse CV: Component pathogen inactivation: a critical review. *Vox Sang* 2013; **104**:183–199

3 Marschner S, Goodrich R: Pathogen reduction technology treatment of platelets, plasma and whole blood using riboflavin and UV light. *Transfus Med Hemother* 2011; **38**:8–18

4 Kumar V, Lockerbie O, Keil SD, *et al.*: Riboflavin and UV-light based pathogen reduction: extent and consequence of DNA damage at the molecular level. *Photochem Photobiol* 2004; **80**:15–21

5 Ruane PH, Edrich R, Gampp D, *et al.*: Photochemical inactivation of selected viruses and bacteria in platelet concentrates using riboflavin and light. *Transfusion* 2004; **44**:877–885

6 Cardo LJ, Salata J, Mendez J, *et al.*: Pathogen inactivation of *Trypanosoma cruzi* in plasma and platelet concentrates using riboflavin and ultraviolet light. *Transfus Apher Sci* 2007; **37**:131–137

7 Goodrich RP, Gilmour D, Hovenga N, *et al.*: A laboratory comparison of pathogen reduction technology treatment and culture of platelet products for addressing bacterial contamination concerns. *Transfusion* 2009; **49**:1205–1216

8 Keil SD, Kiser P, Sullivan JJ, *et al.*: Inactivation of *Plasmodium* spp. in plasma and platelet concentrates using riboflavin and ultraviolet light. *Transfusion* 2013; **53**:2278–2286
9 Vanlandingham DL, Keil SD, Horne KM, *et al.*: Photochemical inactivation of chikungunya virus in plasma and platelets using the Mirasol pathogen reduction technology system. *Transfusion* 2013; **53**:284–290
10 AuBuchon JP, Herschel L, Roger J, *et al.*: Efficacy of apheresis platelets treated with riboflavin and ultraviolet light for pathogen reduction. *Transfusion* 2005; **45**:1335–1341
11 Picker SM, Steisel A, Gathof BS: Effects of Mirasol PRT treatment on storage lesion development in plasma-stored apheresis-derived platelets compared to untreated and irradiated units. *Transfusion* 2008; **48**:1685–1692
12 Hornsey VS, Drummond O, Morrison A, *et al.*: Pathogen reduction of fresh plasma using riboflavin and ultraviolet light: effects on plasma coagulation proteins. *Transfusion* 2009; **49**:2167–2172
13 Cazenave JP, Folléa G, Bardiaux L, *et al.*: A randomized controlled clinical trial evaluating the performance and safety of platelets treated with MIRASOL pathogen reduction technology. *Transfusion* 2010; **50**:2362–2375
14 Smith J, Rock G: Protein quality in Mirasol pathogen reduction technology-treated, apheresis-derived fresh-frozen plasma. *Transfusion* 2010; **50**:926–931
15 Johansson PI, Simonsen AC, Brown PN, *et al.*: A pilot study to assess the hemostatic function of pathogen-reduced platelets in patients with thrombocytopenia. *Transfusion* 2013; **53**:2043–2052
16 Allain JP, Hsu J, Pranmeth M, *et al.*: Quantification of viral inactivation by photochemical treatment with amotosalen and UV A light, using a novel polymerase chain reaction inhibition method with preamplification. *J Infect Dis* 2006; **194**:1737–1744
17 El Chaar M, Atwal S, Freimanis GL, *et al.*: Inactivation of Plasmodium falciparum in whole blood by riboflavin plus irradiation. *Transfusion* 2013; **53**:3174–3183
18 Bruchmuller I, Janetzko K, Bugert P, *et al.*: Polymerase chain reaction inhibition assay documenting the amotosalen-based photochemical pathogen inactivation process of platelet concentrates. *Transfusion* 2005; **45**:1464–1472
19 Lauring AS, Lee TH, Martin JN, *et al.*: Lack of evidence for mtDNA as a biomarker of innate immune activation in HIV infection. *PLoS ONE* 2012; **7**: e50486
20 Levin BC, Cheng H, Reeder DJ: A human mitochondrial DNA standard reference material for quality control in forensic identification, medical diagnosis, and mutation detection. *Genomics* 1999; **55**:135–146
21 Martin CB, Wilfong E, Ruane P, *et al.*: An action spectrum of the riboflavin-photosensitized inactivation of Lambda phage. *Photochem Photobiol* 2005; **81**:474–480
22 Sikorsky JA, Primerano DA, Fenger TW, *et al.*: Effect of DNA damage on PCR amplification efficiency with the relative threshold cycle method. *Biochem Biophys Res Commun* 2004; **323**:823–830
23 Council of Europe, European Directorate for the Quality of Medicines & Healthcare, European Committee on Blood Transfusion (CD-P-TS): Guide to the preparation, use and quality assurance of blood components, 17th edition [Internet]. Strasbourg (France), 2013 [cited 2013 Sep 30]. p124. Available from: www.edqm.eu/en/blood-transfusion-guides-1608.html ISBN: 978-92-871-7637-0
24 Osman A, Hitzler WE, Meyer CU, *et al.*: Effects of pathogen reduction systems on platelet microRNAs, mRNAs, activation, and function. *Platelets* 2014; doi:10.3109/09537104.2014.898178 (Epub ahead of print)