Original Article

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Quick Response Code:

Website: www.ajts.org

DOI: 10.4103/ajts.AJTS_90_19

Comparison of UV spectrometry and fluorometry-based methods for quantification of cell-free DNA in red cell components

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Abstract:

BACKGROUND: Stress and shear force applied on blood components during processing and storage may induce cellular damage leading to release of cell-free DNA (cfDNA). In this study, we have compared ultraviolet (UV) spectrophotometry with UV-induced fluorescence for the quantification of cfDNA in red cell supernatant.

MATERIALS AND METHODS: cfDNA was extracted from 200 μL sample of supernatants from 99 packed red blood cells using QIAamp DNA Blood Mini Kit (Qiagen, Germany). Quantification of cfDNA was done using two different methods: one based on spectrophotometry (NanoDrop 2000c, ThermoFisher Scientific, USA) and another based on fluorometry (Qubit 2.0, Life Technologies, ThermoFisher Scientific, USA). Interassay variability of both the methods was estimated using serial dilutions of standard with known DNA concentration.

RESULTS: DNA quantification by both the methods was close to actual amount of known standard in dilutions with higher concentration of DNA (21.68 to 2.71 ng/μl). While at higher dilutions, quantification by NanoDrop was neither precise nor accurate. Median cfDNA concentration in the study units was found to be 1.60 ng/μl (25^{th}–75^{th} percentile range: 1.10–2.10) by UV spectrophotometry (NanoDrop) compared to 0.080 ng/μl (25^{th}–75^{th} percentile range: 0.050–0.130) by fluorometry (Qubit).

CONCLUSION: Due to high interassay variability between the two methods and the better precision and accuracy of Qubit, it is recommended that fluorometry-based method be used for the quantification of cfDNA in blood components.

Keywords:

Cell-free DNA, fluorometry, quantification, red cell components, spectrophotometry

Introduction

Allogeneic blood transfusion has an immunomodulatory capacity on its recipients through accumulation of immunologically active substances with blood storage. This response is accentuated with storage duration and partially attenuated with leukoreduction.[1] Many immunologically active substances, including cytokines and inflammatory lipids, have been identified in packed red blood cells (PRBCs), and these mediators have been reported to accumulate with blood storage.[2,3] A number of *in vitro* studies have examined the potential role of stored PRBCs to accentuate the innate immune response of neutrophils[4,5] leading to adverse outcomes in transfusion recipients.

During refrigerated storage of red blood cell (RBC) units, the RBCs undergo numerous physicochemical changes, collectively referred to as the RBC storage lesion, which affects the quality, function, and *in vivo* survival of the transfused RBCs.[6-8] Recently,

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Submission: 23-08-2019
Accepted: 30-08-2019
Published: 03-12-2019

How to cite this article: Khetan D, Gupta N, Chaudhary R, Shukla JS. Comparison of UV spectrometry and fluorometry-based methods for quantification of cell-free DNA in red cell components. Asian J Transfus Sci 2019;13:95-9.

introduced concept of damage-associated molecular patterns (DAMPs)[9,10] involves release of endogenous immunogenic molecules due to damage to a cell and the loss of integrity of its cell membrane. DAMPs act through pattern recognition receptors.[11,12] Stress and shear force applied on blood components during processing and storage may induce cellular damage leading to release of DAMPs. Cell-free DNA (cfDNA) is one of the DAMPs identified to mediate various inflammatory conditions, and it is now clear that cfDNA exerts both protective and harmful effects in the host.[13,14] Unlike, routine practice where extraction of DNA is done after inducing cellular lysis, resulting in release of all the intracellular DNA in the extraction media, DNA released in red cell supenatnat due to shear stress of component preparation process will be limited in quantity. Sensitivity of analytical method used is therefore important to assess if certain amount of cfDNA is released during preparation and storage of blood components.

The quantification of DNA is a critical step in all molecular protocols of science laboratories including polymerase chain reaction amplification and next-generation sequencing. There are a range of methods available for the quantification of DNA including absorbance, agarose gel electrophoresis, and fluorescent DNA-binding dyes.

This study was performed to compare ultraviolet (UV) spectrophotometry with UV-induced fluorescence for the quantification of cfDNA in red cell supernatant.

Materials and Methods

This prospective study was conducted at the Department of Transfusion Medicine, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow, between the period from January 2017 to October 2018, after getting the approval from the Institute Research Committee and Institutional Ethics committee.

Red cell component prepared from a total of 99 whole blood donations were included in the study. Whole blood collected from nonremunerated voluntary blood donors selected as per the Drugs and Cosmetics Act, 1940, was collected in quadruple CPD/SAGM 450 ml blood bags (Terumo Penpol, Japan) and processed by buffy coat-depletion method as per the departmental standard operating procedure (SOP) using Cryofuge 6000i (Heraeus) and Terumo Automatic Component Extractor – II.

PRBCs were stored in the standard refrigerated storage conditions as per departmental SOP for the sampling period (21 days, standard shelf life is 42 days). After completion of sampling as detailed below, the red cell components were included in inventory for issue to patients for transfusion.

Sampling of supernatant from study units

Samples for the study were withdrawn from individual red cell components on the day of preparation. PRBCs were centrifuged (1050 rpm × 9 min, Cryofuge 6000i, Heraeus) at 4°C, and 1 ml of supernatant was withdrawn under closed sterile conditions in microcentrifuge tubes. The collected red cell supernatant was given hard spin (3000 rpm × 5 min) to remove any remaining cells from the collected red cell supernatant. The clear cell-free supernatant after the hard spin was collected and stored at or below −40°C till further analysis.

DNA extraction

DNA extraction from 200 µL of stored red cell supernatants was done using QIAamp DNA Blood Mini Kit (Qiagen, Germany), as per manufacturer's instruction for spin protocol, and the extracted DNA was diluted in 200 µL of elution buffer.

Quantification of cell-free DNA

Concentration of cfDNA was measured by two methods:

Cell-free DNA quantification using spectrophotometer

NanoDrop 2000c Spectrophotometer (ThermoFisher Scientific, USA) was used for measuring DNA concentration as per manufacturer's instructions using 1–2 µL of extracted DNA sample.

Cell-free DNA quantification using benchtop fluorometer

Estimation of cell-free DNA (cfDNA) was done using 10 µL of extracted DNA sample on Qubit 2.0 Fluorometer (Life Technologies, ThermoFisher Scientific, USA) using the Qubit dsDNA HS Assay Kit as per manufacturer's instruction.

Assessment of interassay variability

Sample of known concentration of DNA (21.68 ng/µl) from the Department of Molecular Biology, SGPGI was used to test the interassay variability of the two methods of DNA quantification used in the study. Serial dilutions of the known test sample were made in microcentrifuge tubes using the doubling dilution technique. Elution buffer (AE, DNA Blood Mini Kit, Qiagen) was used as the diluting medium. The different dilutions were tested 20 times each by the benchtop fluorometer (Qubit 2.0) and spectrophotometer (NanoDrop 2000c). Mean DNA concentration and variance by both the methods at different dilutions were then estimated to assess the interassay variability.

Statistical analysis

Data management was done using Microsoft Excel (Microsoft Technologies, USA). The data have been presented as median with percentile (ranges from 25th to 75th percentile). The data were analyzed using SPSS statistics software (Version 20, IBM Corp., New

York, USA) to find out significant change or association. Regression analysis was done to assess agreement between both the methods of DNA quantification.

Results

Interassay variability

Interassay variability was done using serial doubling dilution of a sample with known concentration of DNA. Mean ± standard deviation and variance at different dilutions by both the methods are shown in Table 1.

As shown in Figure 1, DNA quantification by both the methods was close to estimated concentration of DNA at lower dilutions (neat, two fold, four fold, and eight fold), i.e., in samples with higher concentration of DNA (21.68–2.71 ng/μl). At higher dilutions (sixteen fold and above, DNA concentration range: 1.35–0.16 ng/μl), quantification by NanoDrop was neither precise nor accurate, while quantification by Qubit was found to be precise and close to estimated concentration of DNA at the tested dilution.

As shown in Figure 1, DNA quantification by NanoDrop after 16-fold dilutions (with estimated concentration of DNA ≤ 1.35 ng/μl) was not found to be reliable.

Quantification of cell-free DNA in red cell supernatants

Of the total 99 study units, cfDNA could be quantified by both the methods in 92 units only; in rest of the seven units, the quantity of cfDNA was too low to be quantified by either of the two methods.

Median cfDNA concentration in the study units was found to be 1.60 ng/μl (25^{th}–75^{th} percentile range: 1.10–2.10) by UV spectrophotometry (NanoDrop) compared to 0.080 ng/μl (25^{th}–75^{th} percentile range: 0.050–0.130) by fluorometry (Qubit). Quantity of cfDNA was thus found to be significantly higher by NanoDrop compared to Qubit method.

There was no agreement between the two methods as is evidenced by the median and percentile values. Regression analysis revealed poor agreement between the two methods ($R^2 = 0.021$) over the concentration range of cfDNA observed across the study samples.

Discussion

In this study, the cfDNA quantification was done using two methods, that is, spectrophotometer (NanoDrop 2000c) and benchtop fluorometer (Qubit 2.0) [Figure 2]. During quantification, it was noticed that these two methods had no agreement for the

Table 1: Comparison of NanoDrop and Qubit for cell-free DNA quantification using known standard (ng/μl)

Dilution	Parameter	Qubit	NanoDrop
Neat (21.68 ng/μl)	Mean±SD	18.09±0.116	21.7±0.311
	Variance	0.014	0.097
Two fold (10.84 ng/μl)	Mean±SD	9.02±0.044	9.65±0.216
	Variance	0.002	0.047
Four fold (5.42 ng/μl)	Mean±SD	3.96±0.023	5.04±0.131
	Variance	0.001	0.017
Eight fold (2.71 ng/μl)	Mean±SD	2.076±0.10	2.97±0.211
	Variance	0.000	0.044
Sixteen fold (1.35 ng/μl)	Mean±SD	1.037±0.008	11.43±3.154
	Variance	0.000	9.947
Thirty-two fold (0.67 ng/μl)	Mean±SD	0.541±0.003	3.77±0.473
	Variance	0.000	0.223
Sixty-four fold (0.33 ng/μl)	Mean±SD	0.270±0.002	2.12±0.194
	Variance	0.000	0.037
One hundred and twenty-eight fold (0.16 ng/μl)	Mean±SD	0.124±0.002	1.67±0.168
	Variance	0.000	0.032

SD=Standard deviation

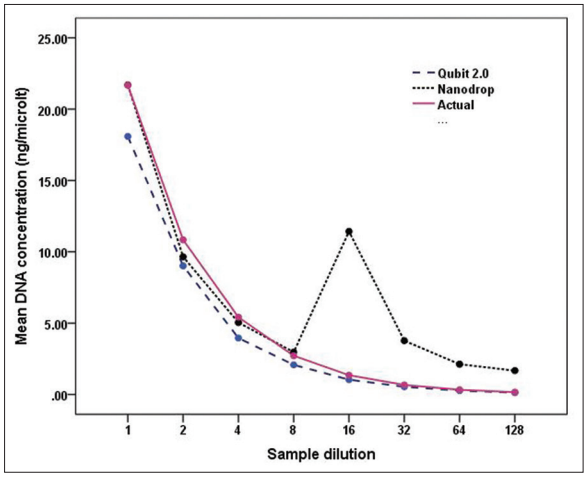

Figure 1: Mean values by NanoDrop and Qubit at different dilutions against the actual concentration in the sample

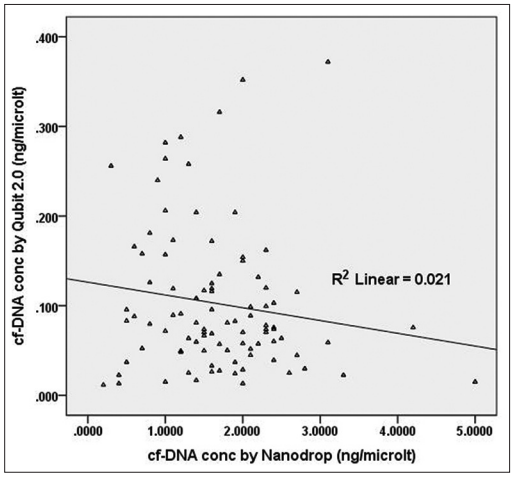

Figure 2: Scatter plots showing comparison of cell-free DNA estimation by NanoDrop and Qubit 2.0NanoDrop and Qubit 2.0

concentration of cfDNA in the study samples. NanoDrop is based on the principle of spectrophotometer, a method to measure how much a chemical substance absorbs light by measuring the intensity of light as a beam of light passes through sample solution. Each chemical compound absorbs or transmits light over a particular wavelength range. Measurement of light absorbed or transmitted thus can be used to measure the amount of a known chemical substance. Spectrophotometry is one of the most commonly used methods for quantitative estimation of chemicals in various fields including chemistry, physics, biochemistry and other clinical settings.

Unlike Nanodrop, Qubit 2.0 is based on the principle of fluorometry where quantitation of DNA, RNA and protein is done using highly sensitive and accurate fluorescence dyes. When compared to spectrophotometers, UVinduced fluorescence due to intercalating dyes is a more precise and sensitive method of quantifying DNA. The intercalating dye interacts specifically with double-stranded DNA and therefore estimation is not effected by contaminating proteins or ribo-nucleic acid molecules. The usable concentration range for NanoDrop as per the manufacturer is 0.4–15,000 ng/μL. It cannot selectively measure dsDNA/ssDNA/RNA when the others are present as it measures the total absorbance of the sample.[15] Nucleic acids have absorbance maxima at 260 nm. Historically, the ratio of this absorbance maximum to the absorbance at 280 nm has been used as a measure of purity in both DNA and RNA extractions. Inaccurate ratios may be encountered at very low concentrations (<10 ng/μl) of nucleic acids. All these factors might contribute to the inaccurate concentrations of cfDNA given by NanoDrop.[16]

Interassay variability was done using serial doubling dilution of a sample with known concentration of DNA. At lower concentrations or higher dilutions (sixteenfold and above), quantification by NanoDrop was neither precise nor accurate, while quantification by Qubit was found to be precise and close to estimated concentration of DNA at the tested dilution. As explained above, the higher detection range of NanoDrop and its inability to selectively measure dsDNA/ssDNA/RNA may be responsible for this high inter-assay variability at lower concentration of DNA in tested sample.

In case of Qubit, use of dyes selective for dsDNA, RNA, and protein minimizes the effects of contaminants in the sample that affects the quantitation. The illumination and detection technologies used in fluorometer allow using as little as 1 μL of sample and still achieve high levels of accuracy, even with very dilute samples.

Gong and Li[17] have reported the average yields of DNA from 200 μL of fresh whole blood, frozen blood, and dry blood to be 7.21 ± 0.84 μg, 6.30 ± 0.59 μg, and 2.15 ± 0.25 μg, respectively. The quantity of DNA extracted from 200 μL of our study samples was much lower, average 0.32 μg as quantified by NanoDrop and 0.016 μg as quantified by Qubit method. The difference in amount of extracted DNA is because of the conscious efforts in the present study to perform DNA extraction on study samples after removing all the cellular components.

Conclusion

Concentration of cfDNA in red cell supernatants in the present study were found to be in the range where quantification by Nanodrop (Spectrophotometry) was found to be inaccurate.. Due to high interassay variability between the two methods and the better precision and accuracy of Qubit, it is recommended that fluorometry-based method be used for quantification of cfDNA in blood components.

Acknowledgment

The authors would like to acknowledge all blood donors who consented to participate in this study. We also acknowledge the support form Research Cell and Ethics Committee of the Institute in supporting the study.

Financial support and sponsorship

This study was financially supported by Research Cell, SGPGI.

Conflicts of interest

There are no conflicts of interest.

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