A microfluidic chip-based assay outperforms common methods for quantification of cell-free DNA and RNA

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Abstract. Plasma and serum contain cell-free DNA and RNA and are common specimens used for pathology investigation. Circulating nucleic acids in plasma and serum (CNAPS) represent an important target for analyzing several conditions including prenatal diagnostics, tumors, infections, and inflammatory disorders. It is highly expected that plasma and serum contain potential biomarkers for the detection of cancers and other pathological conditions. The major challenges are that CNAPS are present in small quantities and are highly degraded, hampering their downstream analysis. For example, obtaining acceptable quality and quantity of CNAPS remains a bottleneck for advanced studies such as those using next-generation sequencing (NGS) platforms as the currently available platforms require nanogram to microgram levels of deoxyribonucleic acid or ribonucleic acid (DNA/RNA) input. This paper examines the use of various methods to quantify CNAPS, including quantitative real-time polymerase chain reaction (qRT-PCR), digital PCR (dPCR), fluorometric assay, gel electrophoresis, and Bioanalyzer. In particular, the results of CNAPS quantification using a fluorometer, a NanoDrop spectrophotometer, and a microfluidic chip-based assay were compared. It is asserted that spectrophotometry, fluorometric assay and gel electrophoresis lack sensitivity for detecting CNAPS; thus, highly sensitive methods such as qRT-PCR, dPCR, and Bioanalyzer are required.

1. Introduction

First discovered in 1869, nucleic acids were called "nuclein" consistent with their localization in the nucleus [1]. Plasma DNA was firstly reported 70 years ago by Mandel and Métais in 1948 [2] and in the last decade, researchers have been actively reporting small amounts of extracellular DNA and RNA fractions. Technical advancements in molecular biology have facilitated the characterization of circulating nucleic acids in plasma and serum (CNAPS) and the investigation of their roles in numerous pathological conditions. Previous studies reported the increased levels of circulating DNA in pregnancy, various types of malignancies, inflammatory disorders, trauma, and stroke [3,4]. It has also been shown that specific DNA/RNA fragments can be used as biomarkers for myocardial infarction, stroke, diabetes, and cancer; thus facilitating diagnosis, prognosis and monitoring of treatments [3].

The major difficulties when working with CNAPS are the low yield and poor quality, thus requiring highly efficient methods for isolation and quantification. The levels of circulating DNA in healthy subjects varied from undetectable to several hundreds of nanogram per milliliter of a sample

[5-8]. The poor quality of CNAPS is indicated by the high level of DNA and RNA degradation. Two studies [9,10] reported a prevalence of short DNA fragments (mostly <200 nt) in the plasma of healthy subjects whilst the circulating RNA detected is mostly small RNAs (up to 200 nt long) and miRNAs (~20 nt long) [11].

As the initial assessment in most of the laboratory experiments, quantification of CNAPS provides valuable information prior to downstream analysis. Therefore, the method for quantification should be simple, fast and accurate. Spectrophotometry based on ultraviolet absorption is the most common method used in the quantification of DNA and RNA yield after isolation procedures. Other methods of nucleic acids quantification include fluorometric assay, gel electrophoresis, quantitative real-time polymerase chain reaction (qRT-PCR), digital PCR (dPCR), and a microfluidic chip-based assay using Bioanalyzer. This study aimed to compare the results of CNAPS quantification using fluorometry, spectrophotometry, and microfluidic chip-based assays.

2. Experimental

There were two steps involved in this study in which the blood samples were donated by healthy volunteers and from patients recruited for a prospective study investigating infectious agents causing fever [12]. Plasma and serum were prepared in two different types of Vacuette® tubes (Greiner Bio-One); i.e. the purple-top tubes containing K2EDTA and the red-top tubes containing clot activator and were processed within three hours after collection by centrifugation for 15 minutes at 2,000 g.

In the first experiment, six healthy volunteers were recruited into the study. Samples were collected using various methods; i.e., by using a vacuum system or syringe and needle with or without the application of a tourniquet. The High Pure Viral Nucleic Acid kit (Roche Applied Science) was used to isolate the nucleic acids from the samples according to the manufacturer's instructions. The quantity of double-stranded DNA (dsDNA) was determined using SYBR Green I dye (Life Technologies) in a microplate fluorescence assay (MFA). The GeneRuler Express DNA ladder (Fermentas) was serially diluted with EB buffer (Qiagen) to make the following concentration of DNA standards: 0.25, 0.0125, 0.063, 0.031, 0.016 and 0,008 ng/µL. The buffer alone was served as a "blank" to give 0 ng/µL of DNA standard. Then, 25 µL of each DNA standards were prepared in the 384 well plates (Greiner) and mixed with an equal volume (25 µL) of SYBR Green I diluted in 1:1250 in EB buffer to give 50 µL per well. For the assay of test samples, 5 µL of each DNA tests was diluted in 20 µL EB buffer in the microplate well and mixed with 25 µL SYBR Green I solution as above. The plates were incubated for 10 min in the dark at room temperature. Following incubation, the fluorescence in each well was measured at room temperature using a spectrofluorometer (POLARStar Omega) fitted with 485/520 nm excitation/emission filter set. The experiment was conducted in duplicate and the average reading of fluorescence intensity was used for developing the standard curve and for calculating the level of DNA in test samples. The concentrations of dsDNA in the samples were compared by using Wilcoxon Signed-Rank test.

In the second experiment, plasma and serum were collected from patients with fever. The DNA Mini Kit (Qiagen) and TRIzol LS reagent (Life Technologies) were used to isolate plasma/serum DNA and RNA, respectively, with the addition of glycogen during RNA precipitation. Following this, the isolated DNA and RNA were randomly selected for quantification using methods other than fluorometry assay. A total of 41 DNA samples originated from plasma or serum of 21 participants and 63 RNA samples from 33 participants were quantified using a Nanodrop 2000 spectrophotometer (Thermo Scientific). All DNA and RNA samples were assessed using a microfluidic chip-based platform (the Agilent 2100 Bioanalyzer) with suitable kits; i.e., Agilent High Sensitivity DNA kit and Agilent RNA 6000 Pico kit (Agilent Technologies). Data were presented by using descriptive statistics and the representative results of the bioanalyzer were presented. The study protocol was approved by the Human Research Ethics Committee of James Cook University and Cairns Hospital (ID: H4456 and HREC/12/QCH/7, respectively).

3. Results and Discussions

The comparisons of the CNAPS quantity in healthy volunteers and in patients with fever measured by using various methods are presented as follows. It can be seen in Figure 1 that the concentrations of plasma/serum DNA were below 1500 ng/mL (or less than 1.5 ng/µL) whilst those in the whole blood are tripled. In particular, the levels of dsDNA in serum were higher than that in plasma ($p < 0.05$). The method of blood collection itself did not affect the DNA concentrations in the samples as it can be observed from the figure that there was no difference in DNA levels regardless the application of a tourniquet or vacuum system.
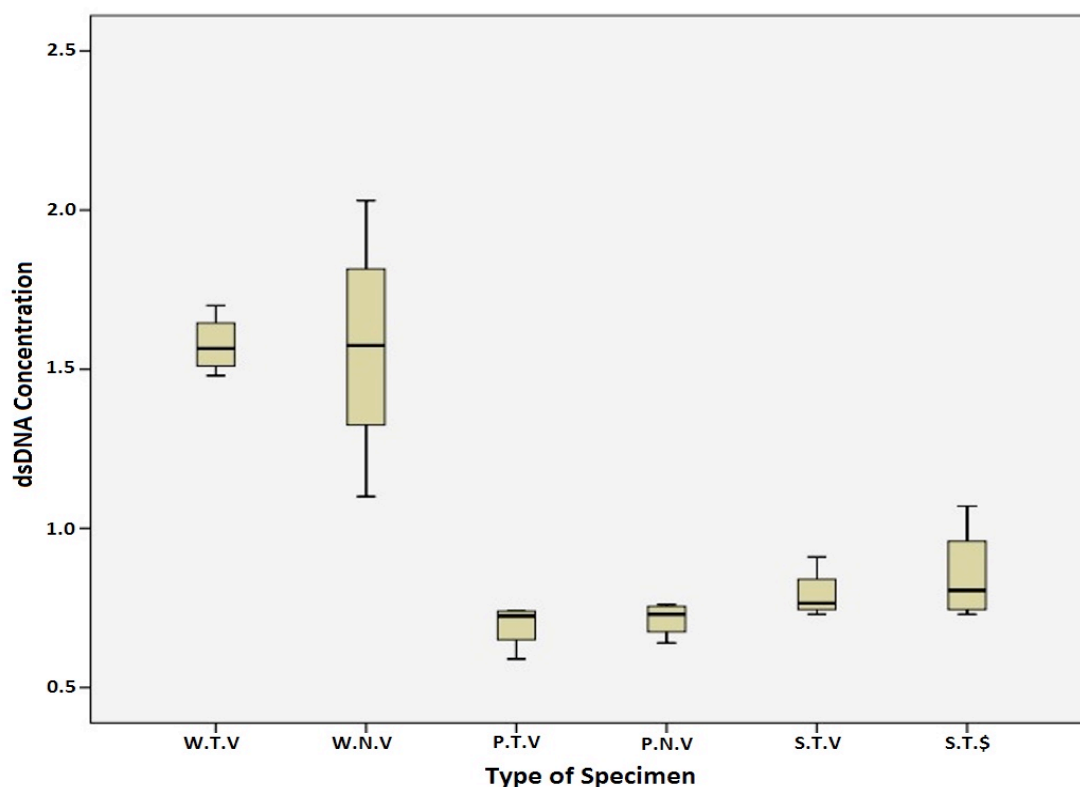

Figure 1. dsDNA concentration (ng/µl) in various blood specimens taken from healthy volunteers. W.T.V: whole blood, drawn by using a tourniquet and vacuum system; W.N.V: whole blood, drawn into a vacuum system without the use of tourniquet; P.T.V: plasma, collected with the use of tourniquet and vacuum system; P.N.V: plasma, collected into a vacuum system without the use of tourniquet; S.T.V: serum, collected with the use of tourniquet and vacuum system; S.T.$: serum, collected by using a syringe and needle with the use of tourniquet

The DNA levels in the plasma or serum of patients with fever measured by the Nanodrop 2000 spectrophotometry ranging from 1.4 until 12.4 ng/µL and the average concentration is 5.19 ± 3.57 ng/µL (data not shown). Among those, only 12 out of 41 (29%) samples are of acceptable quality, as indicated by the value of A 260/280, which is ≥ 1.8. As for the RNA levels, the concentrations are widely varied, ranging from 0.4 until 114.7 ng/µL and the average concentration is 12.79 ± 26.38 ng/µL (data not shown). Although the average of circulating RNA levels was generally detected higher than DNA, the quality was lower, only 8 out of 63 (12.7%) samples had the value of A 260/280 ≥ 1.8. In all DNA/RNA samples, the value of A 260/230 was less than 1, indicating contamination by phenolate ion, thiocyanates, and other organic compounds used during the nucleic acids isolation.

Figure 2 shows the results of CNAPS quantification using a microfluidic chip-based assay; i.e., using the Agilent 2100 Bioanalyzer. Data are presented in an easy-to-read format consisting of electropherograms, a gel-like image, and tabular results. In the bioanalyzer electropherograms, one cannot expect to see the 18S and 28S subunits as two distinct bands that show an acceptable quality of RNA. Instead, the whole degraded RNAs were grouped around the marker band and small peaks are usually present after the marker at 24–29 seconds. These peaks represented small RNA fragments about 100 bp that are especially noted when traditional RNA extraction methods using phenol are performed.
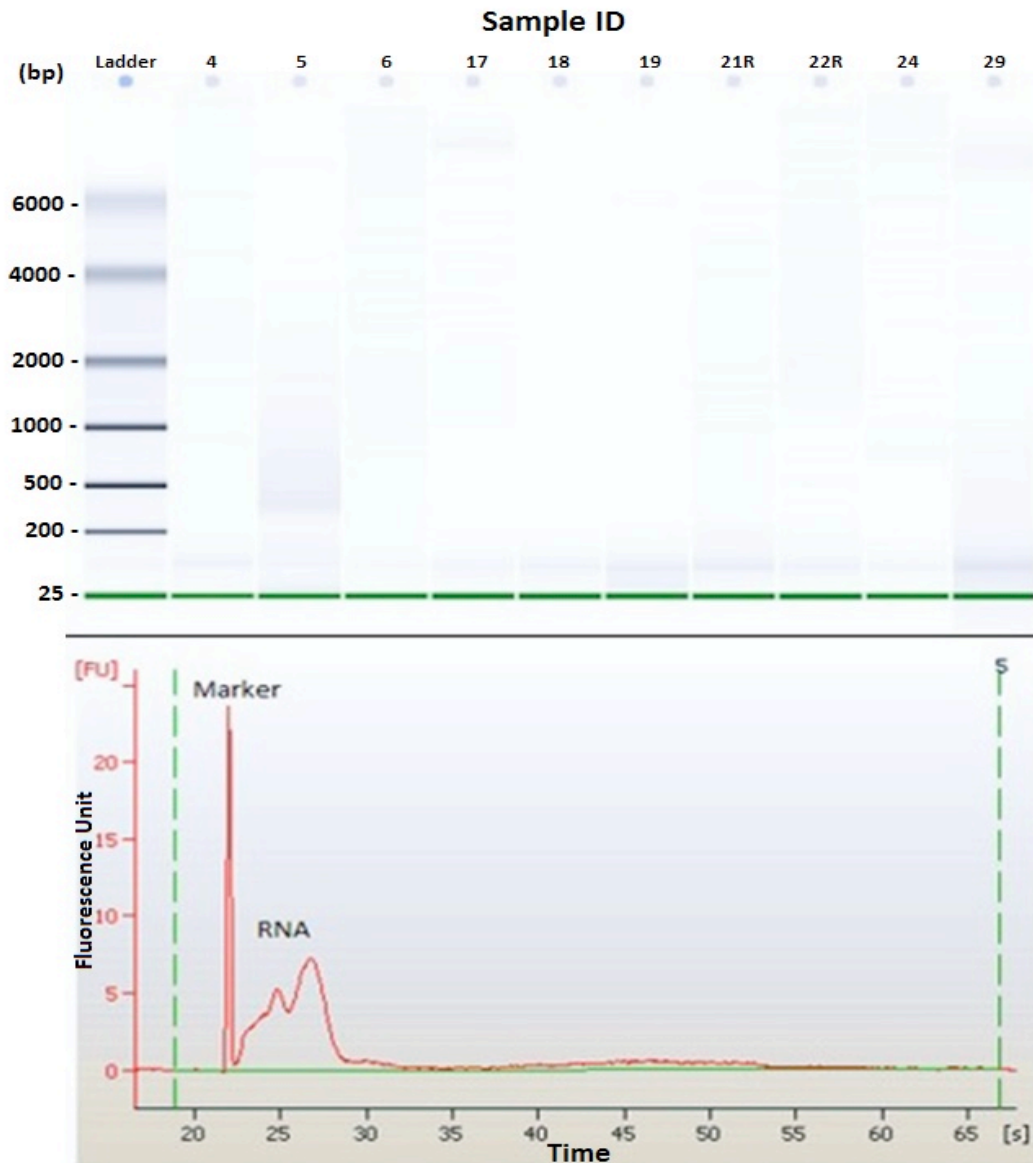

Figure 2. Typical quantity and quality of circulating nucleic acids, measured using Bioanalyzer with a detection limit of 200 pg per band. The low-molecular-weight smears in the gel-like image (above) indicate a low quantity (usually at picogram levels) of highly fragmented CNAPS. An electropherogram (below) from the plasma of a representative subject (ID# 005) indicates RNA of low quality in which the typical 18S and 28S subunits were not detectable, but all of the RNA was grouped around the marker band. In this sample, the RNA integrity number (RIN) is 2.4 (data not shown).

The origin of CNAPS is debatable but it is assumed that they probably derive from cells dead for apoptosis or necrosis as well as the results of the active release of newly synthesized DNA/RNA into circulation [13-16]. The low quantity and quality of CNAPS hinder their accurate quantification. Various methods of DNA and RNA quantification have been established and are discussed below.

Fluorometric assay utilizing dyes (fluorophores) that bind to a certain type of DNA or RNA has been used for quantification of nucleic acids. It is a simple and quick method, yet more sensitive than the spectrophotometric assay that measures nucleic acids' absorbance. A previous fluorometric study using PicoGreen reported that the total of circulating DNA was around 10 times higher than the levels of amplifiable DNA measured by qRT-PCR [7]. The study indicated that the fluorometry assay is sensitive enough to measure the levels of CNAPS, especially with the use of a highly sensitive dye such as PicoGreen. Indeed, PicoGreen and RiboGreen are recommended dyes for circulating cell-free DNA and RNA assay, respectively, due to their high sensitivity of detection [17,18]. SYBR Green I can be used as an alternative for quantification of circulating DNA since it has acceptable sensitivity but less expensive than PicoGreen. It is important to note that the yield of CNAPS is extremely variable from sample to sample (anywhere from less than 1 ng to more than 100 ng) so that some samples may fall below the limit of detection of fluorometry instruments, which is around 20 ng. This can produce false or non-consistent readings.

Spectrophotometry has poor sensitivity and the analysis is affected by the presence of contaminants (e.g., proteins, carbohydrates, salts, phenol, free nucleotides), contributing to the total absorbance of the sample and leading to overestimation of the concentration of CNAPS. Another problem is that purification of nucleic acid samples is required prior to spectrophotometry measurement in order to ensure accurate results. The purification of CNAPS can be problematic due to their short size. These small fragments of CNAPS pass through membrane filter used during column purification method, leading to significant loss of the isolated DNA/RNA. Thus, the spectrophotometry method is not applicable to analyze CNAPS as this method requires highly purified and adequate amount of nucleic acid samples.

The Agilent 2100 Bioanalyzer (Agilent Technologies) is a microfluidic chip-based assay that provides a more comprehensive analysis of circulating DNA and RNA including quantity, fragment size, and RNA integrity. Although the extent of CNAPS usually appears as faint smears, the results of bioanalyzer are far more accurate and more detail than fluorometry and spectrophotometry assays. In a situation when the CNAPS have been amplified, spectrophotometry or electrophoresis of DNA/RNA through agarose is a cheap and easy method to visualize the presence of DNA/RNA and often to estimate the quantity as well. Higher concentrations of agarose help to separate small DNA/RNA molecules; thus, visualization of CNAPS fragments can be conducted on a 1.5–2% (w/v) agarose gel.

Quantification of CNAPS using qRT-PCR usually involves the use of two fluorescent dyes (probes) and primers that are designed to amplify the particular gene of interest such as human telomerase reverse transcriptase (hTERT) [5], β-actin [19], or β-globin gene [20]. The presence of miRNA can be assessed using miR-21 gene [21], which is highly abundant and thus a good choice for control. Alternatively, RNU6 and miR-520d-5p [22] or random CNAPS such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes [23] could be used as an internal control for preventing false negative results when the study aims to look for pathogens' DNA/RNA in human plasma or serum using qRT-PCR. Although qRT-PCR is a fast method for gene quantification, its use is limited for targeting known genes so the total number of CNAPS within a sample is underestimated. In addition, the method requires standardization as the data generated by qRT-PCR are highly influenced by the instrument, user and experimental variability [24] so that comparison of the results cannot be made amongst different settings. It is also important to note that heparin can inhibit the detection of miRNAs in PCR-based assay so that other methods of miRNAs quantification should be considered in patients treated with heparin [25].

Digital PCR (dPCR) is an alternative method for quantification of a scarce amount of CNAPS. Unlike qRT-PCR which measures only amplifiable DNA, dPCR performs absolute measurement of CNAPS by single molecule counting. The method has been used for quantification of CNAPS in the

field of cancer research [26-29], prenatal testing [30-32], transplantation medicine [33] and virology [34-36]. The clinical utility of dPCR should be studied further, however, since the method can produce false positive results due to cross-reactivity [37] in addition to lower throughput and sensitivity compared with qRT-PCR [36,38].

4. Conclusion
The extent of low quantity and highly degraded CNAPS necessitates an optimal sample processing for the success of downstream analysis. Spectrophotometry, fluorometric assay and gel electrophoresis lack sensitivity for detecting CNAPS; thus, highly sensitive methods such as qRT-PCR, dPCR and bioanalyzer are required. The advantages and limitations of each method have been discussed.

Acknowledgements

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