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Optimizing the yield and utility of circulating cell-free DNA from plasma and serum

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ABSTRACT

Background: Cell-free DNA (CFDNA) in the plasma/serum of patients with cancer demonstrates tumour-associated genetic alterations, offering possibilities for diagnosis, prognostication and disease monitoring. There is wide variation in the reported levels of CFDNA, associated with different methods used to collect, process and analyze blood samples. We therefore evaluated different aspects of laboratory protocols for the processing and purification of CFDNA in clinical studies.
Methods: We evaluated and compared the QIAamp kit and a Triton/Heat/Phenol protocol (THP) for CFDNA purification. Total CFDNA was quantified by PicoGreen assay and SYBR-Green real-time PCR assay was used to amplify specific genes to estimate the efficiency of different protocols.
Results: The efficiency of DNA extraction was 18.6% using the standard QIAamp protocol and 38.7% using the THP method ($p<0.0001$, unpaired t-test). A modified QIAamp protocol that included a proteinase incubation stage and elution volumes up to 300 μl increased DNA yields, but was not as good as the THP method.
Conclusions: Blood samples should be kept at/or below room temperature (18 °C–22 °C) for no more than 2 h before plasma separation by double-spin. Because of its higher efficiency, low-cost and good-quality products, the THP protocol is preferred for extraction of CFDNA.

1. Introduction

Cell-free circulating DNA (CFDNA) has been studied in a wide range of physiological and pathological conditions, including pregnancy, trauma, inflammatory disorders and malignancy [1,2]. It is present in normal healthy individuals at low concentrations (ng/ml) [3]. Raised levels of CFDNA in cancer patients have been reported in many tumour types [2–5]. The finding that genetic and epigenetic changes typical of tumours can be detected in CFDNA from cancer patients, suggests that the excess CFDNA is of tumour origin. Although the precise mechanism of DNA release into the blood remains uncertain, it probably derives from a combination of apoptosis, necrosis and active release from tumour cells [6]. Such cell-free DNA has shown promise for improving early clinical diagnosis, prognostication and disease monitoring in inaccessible tumour types, such as lung cancer [7–11].

Although higher levels of CFDNA are consistently reported in cancer patients than healthy controls, there is considerable variation between studies. This may be attributable to differences in study design including selection of patient and control groups, and the methods used to extract and quantify CFDNA [10,12–14]. No agreed standards exist, and many publications fail to specify how samples were obtained, processed and analyzed. The problems of prolonged sample storage have been highlighted by a recent report that DNA levels in plasma stored at −80 °C declined by 30% per year [15]. Thus, this study was designed to evaluate the factors most likely to influence the yield of CFDNA from clinical samples.

Various methods have been used to purify CFDNA, including using modified salting-out [16], chromatography resins [16,17], magnetic beads [18], or guanidium thiocyanate [19]. The most popular is the QIAamp blood kit, which binds DNA to a silica-gel membrane, providing a fast and easy way to purify total DNA for polymerase chain reaction (PCR) analysis. We found that the recovery of CFDNA was less than 20% using the QIAamp DNA Midi Kit. Some authors have recommended using a predigestive buffer in plasma/serum samples to improve the results [16,18,20]. Therefore, we have evaluated standard and modified QIAamp protocols and compared them with a simple Triton/Heat/Phenol (THP) protocol. Because DNA is present in plasma/serum at such low concentrations (ng/ml), it is crucial to optimize laboratory protocols for the processing and extraction of CFDNA.

2. Materials and methods

2.1. Sample collection and preparation

To compare different CFDNA extraction protocols, commercial pooled human serum (SLI Ltd, UK) was spiked with reference genomic DNA (Sigma-Aldrich, UK) or a known amount of linearized bcl-2 plasmid reference DNA (final concentration in serum: 0, 50 and 100 ng/ml). For clinical samples, 20 ml peripheral blood was collected from healthy volunteers into EDTA tubes [21]. Plasma was separated by double centrifugation (800 *g* for 10 min, separation, and 1600 *g* for 10 min). Plasma aliquots were immediately frozen at −70 °C.

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2.2. CFDNA extraction

For the standard QIAamp method, DNA was purified using the QIAamp Blood DNA Midi Kit (Qiagen, UK) in accordance with the manufacturer's instructions. The protocol was also modified by incubating samples at 37 °C for 2 h with 400 mg/l proteinase K instead of the protease provided in the kit, and eluting the DNA with different volumes of elution buffer.

For the THP method, 500 µl of plasma/serum was mixed with 5 µl Triton X-100 (Sigma-Aldrich, UK) and heat denatured at 98 °C for 5 min. Samples were placed on ice for 5 min, then extracted with an equal volume of phenol–chloroform–isoamyl alcohol (25:24:1, v:v:v) (Sigma-Aldrich, UK) and centrifuged for 10 min at 14,000 *g*. The aqueous phase was precipitated overnight with 1/10 volume of 3 M NaOAc and 2.5 volume of 100% ethanol at −20 °C. The DNA pellet was washed with ethanol, air-dried and resuspended in 50 µl of ddH_2O.

The quality of the purified DNA was checked by conventional PCR amplification of the p53 gene. To determine if the different DNA extraction methods led to loss of small DNA fragments, the Quick-Load® 100-bp-DNA Ladder (New England Biolabs, UK) was spiked into pooled human serum. After re-purification, the ladder DNA was analyzed on 1.5% agarose gels with 0.5 µg/ml ethidium bromide.

2.3. Quantitative analysis of DNA

Quantification of total DNA was performed using the PicoGreen assay (Molecular Probes, Netherlands) following the manufacturer's instructions. Genomic (*h*GAPDH) [22] and plasmid (bcl-2) DNA were analyzed by SYBR-Green I fluorescence real-time PCR with an ABI7900 Sequence Detection Instrument (Applied Biosystems, USA). Melting curves of all tubes were observed for every reaction to ensure that only one product was present. All samples were performed in triplicate and the mean value was used for quantification. The qPCR efficiency was calculated from the slope of the standard curve (equation: efficiency $= 10^{(-1/\text{slope})} - 1$) and all correlation coefficients (r^2) were above 0.99.

2.4. Statistical analysis

Quantitative results were presented as mean ± standard deviation (SD). Differences were evaluated by two-tailed unpaired *t*-test (*p*-value) and correlations were evaluated using the Pearson correlation coefficient (*r*). The reproducibility of different protocols was evaluated by coefficient of variation (CV). The effects of time delay during blood sample processing on CFDNA concentration were analyzed by two-way ANOVA with interaction. GraphPad Prism 4.03 software was used for statistical analysis.

3. Results

3.1. Optimization of QIAamp protocol

Digesting or denaturing the plasma/serum proteins is one of the most important stages during the extraction of CFDNA. We compared the protease provided in the QIAamp Blood Kit with proteinase K for the purification of low quantities of CFDNA. Fig. 1 shows that pre-incubation of serum samples with proteinase K (400 µg/ml) at 37 °C for 1 h ($Q_{37\text{-}1}$, $P_{37\text{-}1}$) significantly increased the DNA recovery compared to no incubation (Q_N, P_N) (Q_N vs $Q_{37\text{-}1}$: $p = 0.003$; P_N vs $P_{37\text{-}1}$: $p = 0.002$) and there was significantly better DNA yield with proteinase K than the QIAgen protease (Q_N vs P_N: $p = 0.040$). However, longer incubation time (2 h) and incubation at 50 °C [23] did not improve the DNA yield further (data not shown).

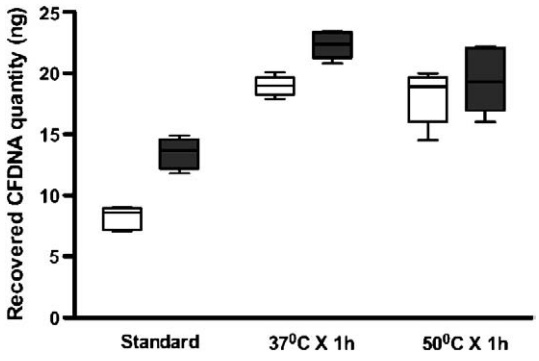

Fig. 1. Effects of different protease and incubation conditions on CFDNA yield. Pooled human serum samples spiked with reference DNA (final concentration: 100 ng/ml) were pre-incubated with the QIAgen protease = Q (□) and proteinase K = P (■) for 1 h at 37 °C or 50 °C prior to QIAamp DNA extraction. Results are shown as box plots for 5 replicates. The upper and lower limits of the boxes and the line across the boxes indicate the 90th and 10th percentiles and the mean, respectively. The upper and lower horizontal bars indicate the max and min. Standard: DNA extraction was processed according to the "blood and body fluid" protocol (no incubation time and 200 µl of elution buffer).

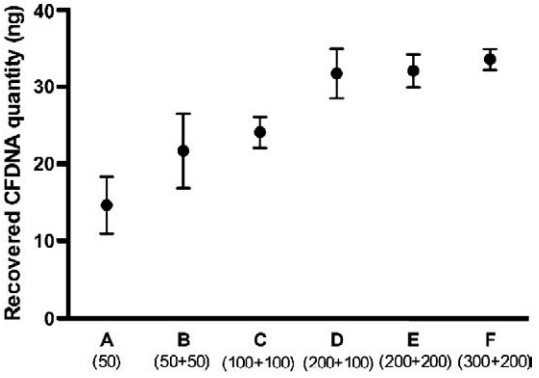

Fig. 2. Effects of different volume of elution buffer on CFDNA yield. Pooled human serum samples spiked with reference DNA (final concentration: 100 ng/ml) were pre-digested by proteinase K at 37 °C for 1 h, the DNA was isolated using the QIAamp DNA Midi Kit, then eluted in different volumes of buffer AE (provided in kit) from 50 µl (A) to 500 µl (F), in one or two aliquots, as indicated, columns were incubated 10 min at RT before centrifugation. Experiments were performed in 2 days and each day in 3 replicates. Data are presented as mean (dot) ± SD (error bar). Statistically differences were found between A and F ($p<0.001$), B and F ($p<0.001$), C and F ($p<0.001$), while means of groups D, E and F are not significantly different (D vs F, $p = 0.23$; E vs F, $p = 0.18$).

In the QIAamp midi Kit, use of 200 µl of elution buffer AE or ddH_2O is recommended. Some groups have routinely used as little as 50 µl of elution buffer [24]. We studied the effects of different volumes, composition and temperature of elution buffer on CFDNA recovery (Fig. 2). Increasing volumes of elution buffer between 50 (A) and 300 µl (D) led to significantly better DNA yield, but more than 300 µl did not further improve the recovery for 1 ml of initial serum sample. There was no significant difference in CFDNA yields with distilled water or AE buffer. Similarly, pre-warming the elution buffer at 37 °C or 50 °C did not significantly affect the CFDNA recovery ($p = 0.33$). Further, we investigated whether CFDNA recovery could be improved by reloading samples onto the QIAamp columns. CFDNA recovery from serum did not change significantly ($p = 0.27$) when samples were reloaded.

3.2. Evaluation of THP protocol for DNA extraction

In our initial studies, the recovery of CFDNA from plasma or serum with the QIAamp Midi Kit was less than 20%. We therefore evaluated the simple THP protocol. Pooled human serum was spiked with

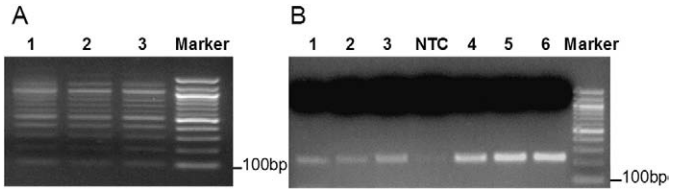

Fig. 3. Utility of extracted CFDNA for genetic analysis. A. Efficiency of extraction of small DNA fragments. A 100-bp-DNA ladder was spiked in pooled healthy human serum before DNA extraction by standard QIAamp (lane 1), modified QIAamp (lane 2) and THP (lane 3) protocols. The purified DNA was then analyzed on a 1.5% agarose gel. Marker = 100-bp-DNA ladder. Modified QIAamp protocol: proteinase K was applied instead of QIAgen protease with 1 h incubation at 37 °C and 300 µl of ddH_2O was used as elution buffer. B. Conventional PCR amplification of p53 gene in CFDNA purified by THP. Pooled human serum was spiked with 50 ng/ml (lane 1–3) and 200 ng/ml (lane 4–6) genomic DNA before THP extraction. The p53 gene primers were used to amplify a 225-bp fragment. Marker = 100-bp-DNA ladder; NTC = ddH_2O (negative control).

different amounts of genomic DNA and heated at 98 °C for 5 min, then direct PCR was performed on 5 μl samples using primers for the p53 gene. The lowest spiked concentration was 25 ng/ml and generated positive products consistently. No significant improvement was achieved by using a different protein digestion buffer (data not shown). Phenol extraction was used to obtain purified DNA solution for further analysis requiring good-quality DNA templates. Since CFDNA exists in fragments of varying size and QIAGEN columns might

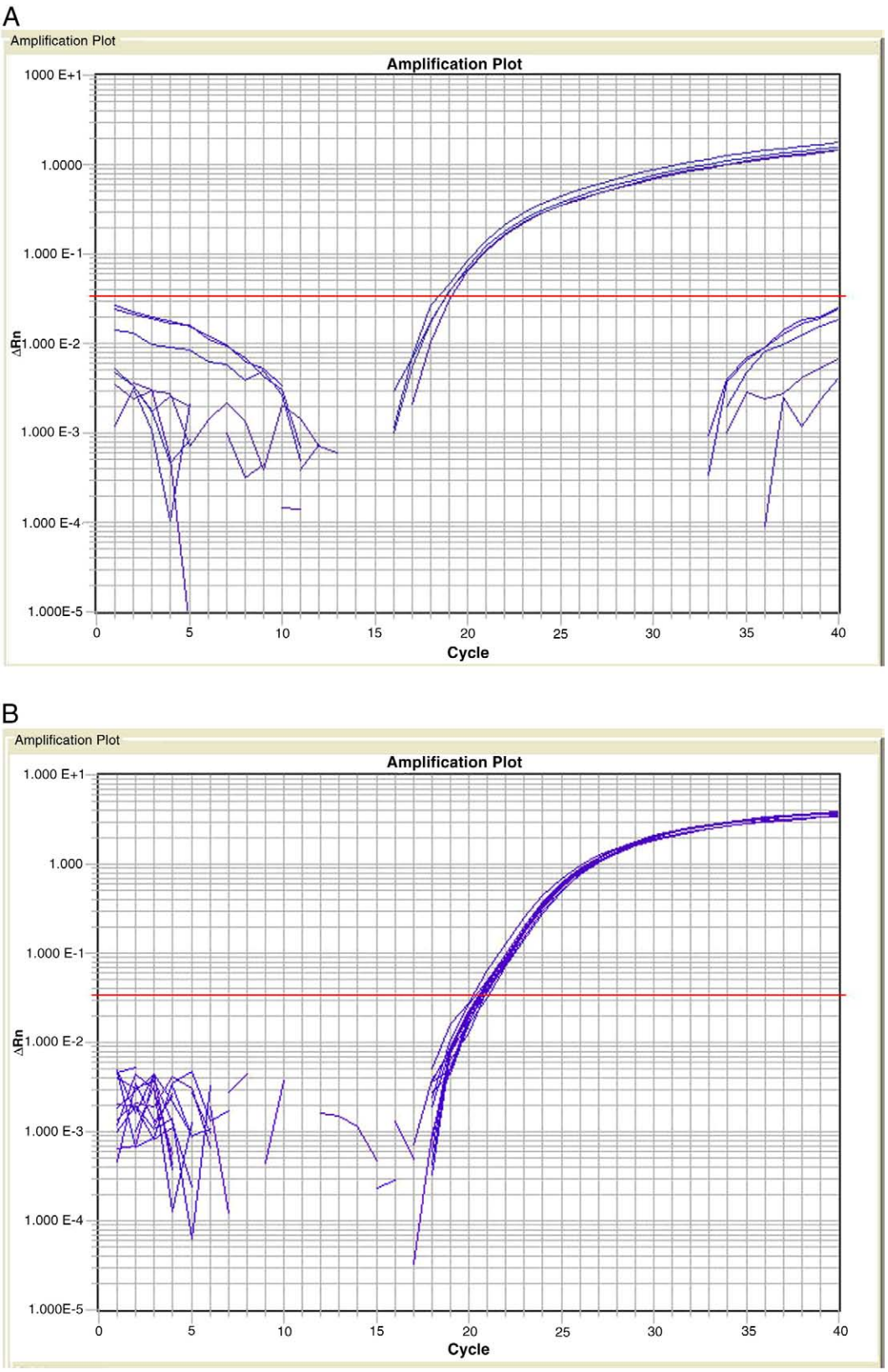

Fig. 4. SYBR-Green real-time PCR amplification curves for the hGAPDH gene in CFDNA. Pooled human serum CFDNA was extracted by (A) QIAamp or (B) THP. For both groups, CFDNA isolation was done in 5 replicates and each purified DNA sample was amplified in 3 replicates.

lose small DNA fragments (<150 bp) [20], we checked the ability of THP procedure to extract DNA fragments of different sizes. Fig. 3A shows that fragments as small as 100 bp were consistently obtained using the THP method. It is essential that DNA extraction methods used for CFDNA yield purified DNA of sufficient quality for PCR analysis. Fig. 3B shows that DNA extracted using the THP protocol from human serum spiked with 50 ng/ml and 200 ng/ml genomic DNA was suitable for PCR amplification of the p53 gene.

3.3. Comparison of the THP and standard QIAamp methods

We compared CFDNA extracted by the THP and QIAamp standard protocols from pooled healthy human serum using qPCR for the hGAPDH gene. Whereas 15/15 THP samples were successfully amplified, only 3/15 QIAamp samples were. The mean concentration of CFDNA obtained using the THP protocol was 4.73 ng/ml (range: 1.49–10.25 ng/ml), while that for the QIAamp standard protocol was 1.67 ng/ml (Fig. 4).

To determine the efficiency and reproducibility of these two different protocols, we added linearized bcl-2 plasmid DNA (reference DNA) into pooled human serum to achieve a final concentration of 50 ng/ml, close to the average concentration of most reported CFDNA in cancer patients. The reference DNA was re-purified by the two different extraction methods in 20 replicate samples (Fig. 5). Whereas 20/20 THP samples were successfully amplified by the SYBR-Green real-time PCR quantitative assay, only 14/20 QIAamp samples were successfully amplified. Fig. 5 shows that the efficiency of DNA extraction by the THP protocol ($38.74 \pm 7.15\%$; mean ± SD) was significantly better than that of the QIAamp protocol ($18.63 \pm 4.34\%$; mean ± SD; $p<0.0001$, unpaired t-test). The coefficients of variation were similar for the two protocols at 18.5% for THP and 23.3% for QIAamp. In repeated experiments, consistent results were obtained showing the efficiency of DNA extraction to be about 20% for the QIAamp protocol and about 40% for the THP protocol.

3.4. The effects of delays in blood processing and storage temperature

To optimize the yield of low-level CFDNA from serum and plasma, we considered the effects of delays in blood processing and storage temperature prior to DNA extraction. Each blood sample from 3 different healthy donors was divided into twelve aliquots and left at room temperature (RT, 18 °C–20 °C) or on ice (at 0 °C). Plasma separation and DNA extraction were processed at different time intervals after venepuncture using the THP protocol (Fig. 6). There was no significant difference in CFDNA yield for samples processed up to 2 h after venepuncture, but after this (4–25 h), the amount of recovered DNA increased. Two-way ANOVA showed the effect of time was considered extremely significant, but the temperature did not affect the CFDNA significantly. Time accounts for 70.4% of the total variance ($F = 15.80$; $p<0.0001$).

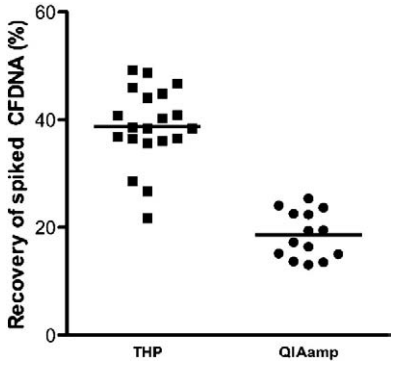

Fig. 5. Comparison of two methods for the isolation of CFDNA. CFDNA was extracted from pooled human serum (reference DNA: linearized bcl-2 plasmid DNA) using the THP procedure or the QIAamp standard protocol. DNA extraction was performed in 20 replicates and each sample was quantified by SYBR-Green real-time PCR in triplicate. Data are presented as scatter dot plot and the line indicates the mean value. The coefficients of variation (CV) of the THP and QIAamp groups were 18.5% and 23.3%, respectively.

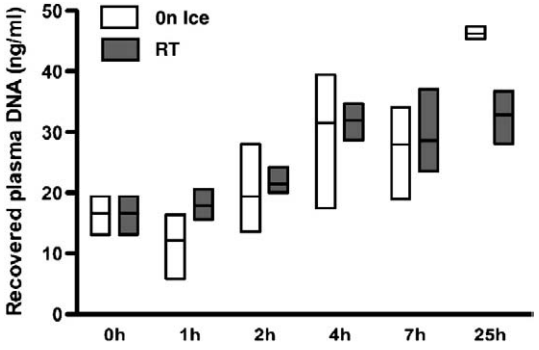

Fig. 6. Effects of delays and storage temperature before plasma separation on CFDNA yield. Blood samples were collected in EDTA-vacutainer tubes and held at RT (■) or on ice (□). Plasma separation and DNA extraction were processed using THP protocol at different time intervals after venepuncture, as indicated. Experiments were performed in triplicate. The upper and lower limits of the boxes and the line across the boxes indicate the max and min values and the mean, respectively.

4. Discussion

Published studies on CFDNA in cancer patients give remarkably little detail about the conditions under which clinical samples were obtained, transported, stored or processed, making comparisons of the reported results difficult. Very few studies have addressed the efficiency or reproducibility of their chosen DNA extraction methods. We were initially surprised to obtain DNA yields of less than 20%, but a detailed literature review showed that this was not unusual [16,17]. There is clearly a need to optimize DNA extraction from clinical samples where it is present at such low levels. Very low amounts of CFDNA have been found in healthy individuals, although some authors consider it undetectable in plasma [25]. We therefore spiked the pooled serum samples of healthy individuals with reference DNA for our studies, to achieve levels of CFDNA comparable to those reported in cancer patients [10].

A number of different methods have been used to quantify CFDNA, including DNA dipsticks, the PicoGreen double-stranded DNA assay, dot-hybridization or nick translation [10]. More recently, quantitative real-time PCR (QPCR) methods have been widely used including SYBR-Green, Taqman and allele specific real-time PCR. These methods have varying specificity and sensitivity [22]. Here we chose to use the PicoGreen and SYBR-Green assays to combine adequate sensitivity within the expected concentration range, with robust and easily optimized assays.

Although the QIAamp blood kit is the most popular method for extracting CFDNA, it does not appear very efficient at such low DNA concentrations. We sought to increase its efficiency by pre-incubating samples with a different protease to remove proteins. Proteinase K is a subtilisin-type protease particularly suitable for short digestion times. The predominant site of cleavage is the peptide bond adjacent to the carboxyl group of aliphatic and aromatic amino acids with blocked alpha amino groups. Raising the temperature of proteinase K from 37 °C to 50–60 °C can increase its activity several fold [23]. We found that proteinase K was more efficient than QIAgen protease, and that incubation at 37 °C gave better results than RT or 50 °C on CFDNA purification. One potential explanation is that higher temperature and longer incubation times accelerate DNA degradation or fragmentation and its impact cannot be made up by the increased activity of protease. We also tested another SDS-containing buffer recommended by Schmidt et al. [20], but discovered that molecular grade SDS from

several manufacturers is contaminated with variable amounts of DNA (results not shown). Hence this method cannot be recommended for the extraction of CFDNA.

We studied the effects of varying the type, temperature and volume of elution buffer in the QIAamp protocol. Some authors use small volumes of elution buffer (50 μl) to obtain a concentrated DNA solution for subsequent PCR analysis [24,26]. However, we found that using low volumes of elution buffer led to a significantly lower DNA yield. The yield could be significantly improved by eluting twice using two volumes of 200 μl and 100 μl, successively. However, the dilution in 300 μl might have rendered low CFDNA concentrations undetectable, leading to false-negative PCR results. We found no obvious difference when using distilled water or AE buffer, at RT or 40 °C. Because buffer AE has the potential to inhibit PCR reactions, sterile distilled water is preferred as elution buffer [27].

In the THP protocol, 98 °C incubation was used to inactivate PCR inhibitors and denature proteins in plasma/serum samples. Different times and temperatures were tested before choosing this. Since we had noticed substantial DNA contamination in SDS, Triton X-100 was used in the protein solubilization step. Direct conventional PCR was performed successfully on heat-denatured samples and able to detect 25 ng/ml DNA, indicating that this simple procedure offered sufficient protein digestion and inhibitor elimination for PCR analysis. The THP protocol gave a significantly higher yield of pure DNA solution for qPCR analysis than the QIAamp kit. The THP protocol showed high efficiency even with small DNA fragments as low as 100 bp. The abilities of these protocols to extract the CFDNA were evaluated by quantitative PCR. This showed that the THP products were more reproducible and consistent than the standard QIAamp protocol. In addition it is simple, relatively cheap and easy to perform and standardise. Phenol can present risks to the user, including burns on contact with the skin or mucous membranes, and carcinogenicity. It is therefore important to adhere to stringent protocols for its safe use in the laboratory. However, laboratory staff working with very low levels of DNA in human plasma and serum samples are familiar with careful procedures to avoid contamination of samples, and the risk of spillages in this setting is low.

The way clinical blood samples are handled before reaching the laboratory has significant impact on CFDNA yields. These findings will therefore be important in designing our future clinical studies. We found that recovered CFDNA from blood samples kept at RT was comparable to the samples on ice processed at the same time intervals (0 to 25 h), which is consistent with other observations [27,28]. However, delays in separating the plasma could increase the amount of recovered DNA. This contradicts the observations of Jung et al. [28], who reported that the DNA concentration in plasma did not change when blood samples were stored at RT for 8 h, or at 4 °C for 24 h. These contradictory results might be due to sampling at only two time points (8 and 24 h) at 4 °C in their study. It is likely that the increased DNA levels found at 25 h are attributable to contamination of plasma with genomic DNA from leukocyte lysis [3].

In summary, we recommend that blood samples for CFDNA analysis are collected into EDTA tubes and held at/or below RT (18 °C–22 °C) for no more than 2 h before separation of plasma. Plasma should be separated from whole blood samples by double centrifugation (800 *g* and 1600 *g* for 10 min, separately), avoiding leukocyte lysis. We prefer to isolate CFDNA immediately after plasma separation to minimize the effect of prolonged storage. Otherwise, samples should be aliquoted into small portions and stored at −70 °C before extraction because fragmentation of CFDNA might be caused by repeated freeze–thaw cycles. Because of its higher efficiency, low-cost, easy-of-handling and good-quality products for PCR analysis, we recommend the THP method for CFDNA extraction from limited clinical samples. The simplicity of the THP protocol should facilitate the genetic analysis of large populations. Of importance, the details of pre-analytic factors like sample collection and processing time should be specified in study protocols and taken into account during data analysis. We are now using this methodology in a study of CFDNA from lung cancer patients and controls.

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