Recovery of Small DNA Fragments from Serum Using Compaction Precipitation

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Abstract

Background: While most nucleic acids are intracellular, trace amounts of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), including micro RNAs, can also be found in peripheral blood. Many studies have suggested the potential utility of these circulating nucleic acids in prenatal diagnosis, early cancer detection, and the diagnosis of infectious diseases. However, DNA circulating in blood is usually present at very low concentrations (ng/ml), and is in the form of relatively small fragments (<1,000 bp), making its isolation challenging.

Methods: Here we report an improved method for the isolation of small DNA fragments from serum using selective precipitation by quaternary ammonium compaction agents. A 151 bp fragment of double-stranded DNA from the *Escherichia coli* bacteriophage lambda served as the model DNA in our experiments. DNA was serially diluted in serum until undetectable by conventional polymerase chain reaction (PCR), before being enriched by compaction precipitation.

Results: Starting with concentrations two to three orders of magnitude lower than the PCR-detectable level (0.01 ng/ml), we were able to enrich the DNA to a detectable level using a novel compaction precipitation protocol. The isolated DNA product after compaction precipitation was largely free of serum contaminants and was suitable for downstream applications.

Conclusions: Using compaction precipitation, we were able to isolate and concentrate small DNA from serum, and increase the sensitivity of detection by more than four orders of magnitude. We were able to recover and detect very low levels (0.01 ng/ml) of a small DNA fragment in serum. In addition to being very sensitive, the method is fast, simple, inexpensive, and avoids the use of toxic chemicals.

Introduction

It is well known that trace amounts of cell-free, circulating deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and micro RNA are present in human blood [1]. The prospect of obtaining prognostic or diagnostic information from these nucleic acids is highly attractive, and previous studies have already established that, for instance, the circulating concentrations of specific nucleotide sequences are significantly higher in prostate, breast and lung cancer patients [2,3,4]. There also is extensive work on the value of circulating, trace fetal DNA in the mother's blood, both as a predictive and as a diagnostic marker [5,6]. Current methods of isolating cell-free circulating DNA and RNA suffer from low sensitivity and often use toxic chemicals such as phenol and chloroform [7,8,9,10,11]. Here we report a new, highly-sensitive, simple and inexpensive method to selectively isolate and concentrate small DNA from serum using compaction agents.

Compaction agents are small, cationic molecules which interact selectively with the phosphate backbone and groove of nucleic acid molecules. This interaction can change the conformation of nucleic acids into a compact form which can precipitate out of solution [12]. In the past, compaction agents have been used to selectively precipitate DNA from complex mixtures [13,14,15,16,17].

Materials and Methods

Trace DNA Analyte

To simulate small circulating nucleic acids in blood, we spiked a 151 bp lambda genomic polymerase chain reaction (PCR) amplicon at different concentrations into human serum (Gulf Coast Regional Blood Center, Houston, TX). The fragment was chosen so that the necessary primers would have no significant homology to any human sequences, and was prepared by PCR amplification using total lambda genomic DNA (Affymetrix, Cleveland, OH) as the template. The primers used were: F1, 5′-GGCTTCGGTCCCTTCTGT-3′, and R1, 5′-CTCTTCCA-GAAACGACTCCAG-3′ (IDT, Coralville, IA).

In addition, beta-globin-encoding DNA, normally found at low levels in human plasma [18], was used to test the effectiveness of
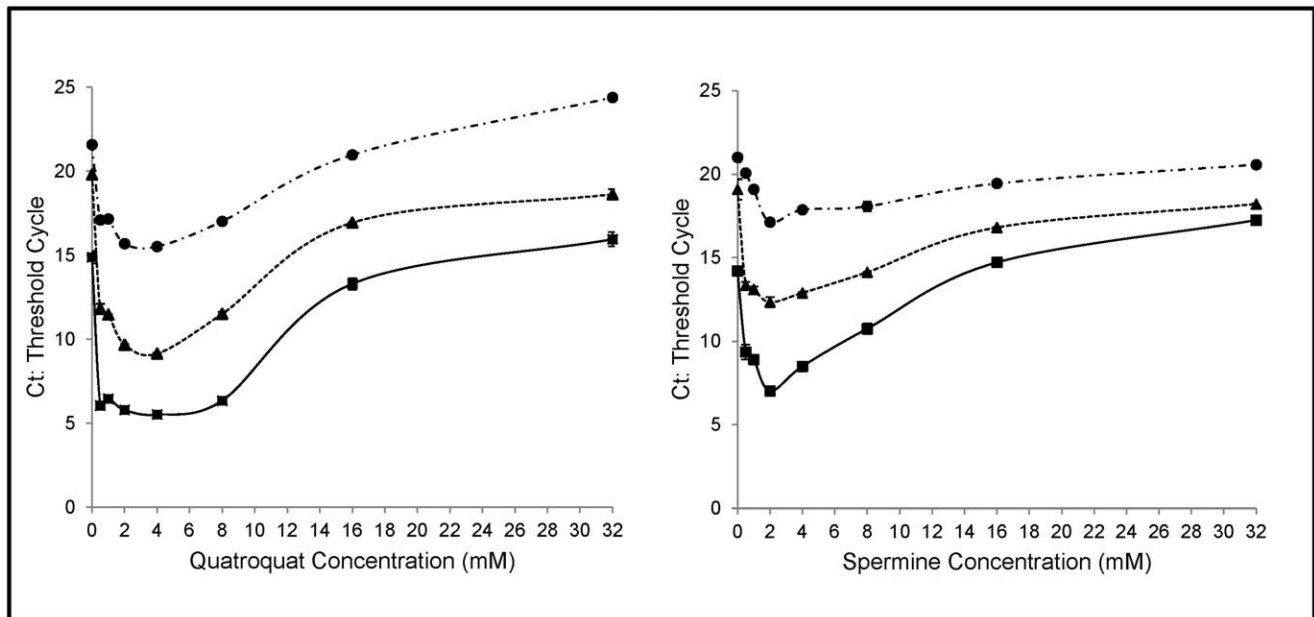
Figure 1. Effect of compaction agent concentration on DNA precipitation. Three sets of human serum samples containing 10, 1, and 0.1 ng/ml of lambda DNA were heat treated and compaction precipitated with various concentrations of spermine and quatroquat.
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various methods of isolating circulating DNA. For its amplification we used the forward primer 5′-ACACAACTGTGTTCAC-TAGC-3′ and the reverse primer 5′-CAACTTCATCCACGTT-CACC-3′ (IDT, Coralville, IA) [18].

Compaction Precipitation

Quatroquat (QQ SBST[M] 8915, Sachem, Austin, TX) and spermine-HCl (S1141, Sigma, St. Louis, MO) were used as compaction agents. Various protocols were tested to determine the most effective and efficient method as discussed below. In the final recommended protocol, human serum samples (1 ml) are diluted with nuclease-free water (1.5 ml, OmniPur, EMD Chemicals, Gibbstown, NJ) and heated at $95°C$ for 15 min to non-specifically precipitate serum proteins. The sample is then vortexed 5 sec, then centrifuged at $16,000 \times g$ for 15 min to remove the protein precipitate. The supernatant is recovered and compaction agent is added to a concentration of 2.5 mM. After 15 min incubation on an end-to-end rotator, the samples are again centrifuged at $16,000 \times g$ for 15 min. The supernatant is discarded and the DNA pellet is washed with 100 µl Wash Buffer (50% isopropanol, 300 mM NaCl, 10 mM $MgCl_2$) and 100 µl 75% ethanol, with centrifugation at $16,000 \times g$ for 10 min after each wash. The pellet is then dried in a SpeedVac, before finally being dissolved in 20 µl 10 mM Tris-HCl, pH 7.4.

Real-time PCR Detection

Real-time PCR for the detection and measurement of DNA was conducted using the Brilliant II SYBR Green® QPCR reagent (Agilent, Santa Clara, CA) in an Mx3000P QPCR system (Agilent). After compaction precipitation, the samples were subjected to real-time PCR without further purification. The original lambda DNA served as a positive control. Primers were the same primers used in preparing the template. All PCRs were set up with 1 µl of sample in 25 µl total volume. PCR conditions were: $95°C$ for 10 min followed by 35 cycles of 45 s denaturation

at $95°C$, 1 min annealing at $55°C$, followed by 30 s extension at $72°C$.

Results and Discussion

We first investigated the effect of varying the concentration of the compaction agent concentration. Ten ng/ml of a 151 bp lambda model DNA amplicon was serially diluted in serum. The samples were diluted with 1.5 volumes of water and heated to $95°C$ for 15 min. Denatured aggregated proteins were then removed by centrifugation. The supernatants after centrifugation were precipitated with compaction agents at concentrations between 0 and 32 mM. After analysis of the spiked samples in the various reactions by real-time PCR, we identified an optimal concentration range for each of our compaction agents of 1–5 mM. As shown in Figure 1.A, higher concentrations of spermine noticeably reduced the sensitivity of the PCR. The results for quatroquat showed the same effect (Figure 1.B). It is known that at low and optimal concentration compaction agents interact with the negatively charged DNA backbone to form intermolecular aggregates [14].

Variation of the incubation time of the sample with the compaction agent showed that it had no significant effect on the sensitivity of the subsequent PCR. Serum samples were spiked with 10 ng/ml target DNA, diluted with 1.5 volumes of water, heat-treated as described above, and then divided into two sets, one for each compaction agent. Each set was then subdivided into seven reactions which were treated with 2 mM compaction agent for incubation times ranging from 1 min to 15 hr. Analysis by real-time PCR showed no benefit of prolonged incubation times. In fact, an incubation time of 1 min gave virtually the same level of sensitivity (Ct = 6.68 for spermine, and 6.13 for quatroquat) as a 15 hr incubation (Ct = 6.69 for spermine, and 6.35 for quatroquat) for both compaction agents. We believe that the brief incubation time is a significant advantage of the method; the protocol is simple and can be completed in less than 1.5 hours.

To examine the need for the heat treatment in the protocol, serum samples were aliquoted into two treatment sets: heat treated and not heat treated. Without heat treatment samples subjected to one round of compaction precipitation were detected 3 to 5 cycles later than samples with heat treatment (Table 1). The results showed that heat treatment improved the sensitivity of the real-time PCR. We attribute these results to the removal of inhibitory serum components such as immunoglobulin G and lactoferrin [19,20] from the PCR by heat. Our real-time PCR results showed that the heat treatment effectively removed these inhibitors, improving the limit of detection by at least an order of magnitude.

In optimizing the procedure, we tested a number of variations of the main protocol. As outlined, the original protocol involved heat treatment followed by one round of compaction precipitation. The results were significantly better than heat treatment alone (Table 1). With heat treatment, two rounds of compaction precipitation with either spermine or quatroquat did not improve the outcome (Table 1). Without heat treatment, two rounds of compaction precipitation gave virtually the same results as heat treatment with one round of compaction precipitation. Thus, when heating is not desirable, a second round of compaction precipitation can be substituted.

The possible effect of inter-specimen variation on the compaction precipitation method was examined using 20 distinct serum samples. The samples were divided into four sets, one for each spiked DNA concentration, ranging from 0.01–10 ng/ml. The samples were subjected to purification by compaction precipitation with spermine as described above, followed by real-time PCR. The real-time PCR analysis after compaction precipitation showed no significant variation in DNA recovery among the donors at any of the four DNA concentrations. The average ($\pm$SD) Ct values were 6.6 ± 0.2, 10.3 ± 0.7, 15.8 ± 0.9, and 17.6 ± 0.4 for spiked DNA concentrations of 10, 1, 0.1, and 0.01 ng/ml, respectively. Similar results were obtained with quatroquat, the average ($\pm$SD) Ct values were 6.6 ± 0.3 for 10 ng/ml, 10.3 ± 0.9 for 1 ng/ml, 14 ± 1 for 0.1 ng/ml, and 17.1 ± 0.5 for 0.01 ng/ml. These results demonstrated the high reproducibility (average coefficient of variation = 5.4%) of the compaction precipitation method across donors.

Compaction precipitation also was compared with the well-developed commercial QIAamp Circulating Nucleic Acid Kit. Pooled serum was spiked with DNA in various concentrations and subjected to DNA purification with compaction precipitation and QIAamp Circulating Nucleic Acid Kit. The results showed that the QIAamp kit was less than 1 cycle more sensitive than our method at 10 ng/ml spiked DNA, and was 2–3 cycles more sensitive at lower concentrations. In an effort to demonstrate the general applicability of the method, the naturally present beta-globin gene was also amplified from blood samples purified by various means. Using the QIAamp Kit, the beta-globin gene was detected at Ct values of 27.2 ± 0.2, with spermine it was detected at Ct values of 28.1 ± 0.4, and with quatroquat at Ct values of 28.0 ± 0.2. Thus, the QIAamp kit was only about one cycle more effective than our compaction precipitation method.

These results were encouraging, since our method is new and not fully optimized. The QIAamp kit employs a large amount of carrier RNA (greater than 1 μg/ml) to enhance recovery, though exogenous RNA would be undesirable in some downstream applications. The addition of carrier RNA to our method did not improve the recovery yield. The cost per sample for the QIAamp kit is hundreds of times higher than compaction precipitation, making our method an attractive choice for high throughput application.

In summary, compaction precipitation proved to be a highly effective method for the isolation and concentration of small DNA fragments from serum. In our final protocol, the combination of heat treatment with one round of compaction precipitation using either spermine or quatroquat improved the sensitivity, especially in samples with initial DNA concentrations between 1–10 ng/ml, the typical concentration range for small DNAs in human blood [21]. Compaction precipitation improved detectability for that concentration range by at least 23 cycles, which is 2^{23} or 6 to 7 orders of magnitude better than heat treatment alone (Table 1). The overall sensitivity of the PCR was also increased by more than five orders of magnitude; specifically, we were able to recover and detect 10 pg/ml of a small DNA fragment in serum. The yield of this method is not subject to significant inter-specimen variation and its initial performance is comparable to that of a widely-used commercial product, at a fraction of the cost. Furthermore, the method is fast, simple, applicable to larger or multiple samples, and avoids the use of hazardous chemicals.

Author Contributions

Conceived and designed the experiments: BV KA US RW. Performed the experiments: BV KA. Analyzed the data: BV KA US RW. Wrote the paper: BV US RW.

Table 1. Detection of small DNA in serum after compaction precipitation.

Treatments*		DNA concentration			
		0.01 ng/ml	0.1 ng/ml	1 ng/ml	10 ng/ml
No Heat	No additives	ND	ND	ND	ND
	Sp	21.5 ± 0.4	18 ± 1	16.5 ± 0.7	14 ± 1
	QQ	22.14 ± 0.01	18 ± 1	16.9 ± 0.3	13.5 ± 0.1
	SP (twice)	18.9 ± 0.1	16.0 ± 0.2	12.8 ± 0.5	7.73 ± 0.06
	QQ (twice)	18.61 ± 0.08	16.0 ± 0.1	11.8 ± 0.1	8.56 ± 0.01
Heat	Heat only	ND	ND	33.3 ± 0.2	29.9 ± 0.1
	SP	18.7 ± 0.1	15.7 ± 0.6	11.6 ± 0.2	7.8 ± 0.6
	QQ	18.6 ± 0.1	14.6 ± 0.2	10.37 ± 0.07	6.5 ± 0.2
	SP (twice)	19.5 ± 0.3	16.1 ± 0.1	12.24 ± 0.08	8.4 ± 0.2
	QQ (twice)	17.92 ± 0.05	14.0 ± 0.3	10.1 ± 0.2	6.5 ± 0.1

*Unless otherwise noted ('twice') all samples went through one round of the compaction precipitation protocol. Heat: 10 min 95°C. SP: spermine (2 mM), QQ: quatroquat (2 mM), ND: Not detectable.
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