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Journal of Chromatography B, 838 (2006) 122–128
JOURNAL OF CHROMATOGRAPHY B
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Comparisons between capillary zone electrophoresis and real-time PCR for quantification of circulating DNA levels in human sera

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Received 8 January 2006; accepted 16 April 2006
Available online 24 May 2006

Abstract

Background: Recently, some research results showed that the circulating DNA in serum or plasma had potential for the molecular diagnosis and prognosis of certain cancers. Several methods have been employed for the quantification of circulating DNA. However, the circulating DNA levels obtained by various methods exhibited considerable differences. Additionally, these methods were labor-extensive and time-consuming, and not suitable for the quantification of circulating DNA in numerous samples due to the use of commercial DNA extraction kits for the purification of circulating DNA. We presented a new method for the quantification of circulating DNA in sera by capillary zone electrophoresis (CZE) with laser-induced fluorescence detection (LIF).

Methods: In the present work, we want to make comparison between CZE-LIF assay and real time PCR for the quantification of circulating DNA levels. Linearity, intra and inter variability of two methods were evaluated.

Results: The intra and inter variability of circulating DNA quantification by real-time PCR were 7.3% and 14.92%, respectively. In CZE assay the intra and inter variability were 4.19% and 6.91%, respectively. The R.S.D. values of the same coated capillary and different coated capillaries were 5.14% and 9.02%, respectively. Our data showed that the circulating DNA levels obtained by two methods had a good correlation. Moreover, we further confirmed that blood samples collection, serum preparation and other treatment procedures had a significant impact on the DNA levels in sera.

Conclusion: Our data further illustrated that CZE-LIF is a simple, rapid and sensitive method for the quantification of circulating DNA in human sera, and well suitable for the analysis of a large number of samples in clinical diagnosis.

Keywords: Human sera; Capillary zone electrophoresis; Circulating DNA; Laser induced fluorescence; Quantification; Real-time PCR

1. Introduction

Cell-free DNA in the human bloodstream, also known as circulating DNA, was first observed by Mandal and Métais [1]. Little attention was paid to this innovative discovery until Tan et al. discovered the high levels of circulating DNA in patients with systemic lupus erythematosus [2]. Leon et al. first quantified circulating DNA amount using a sensitive radioimmunoassay [3], and found that circulating DNA levels in human sera were higher in patients with metastatic disease than those in healthy controls. Later, the elevated circulating DNA levels were found in some patients with various malignancies, such as lung cancer, head and neck cancer, breast cancer and gastrointestinal carcinomas [4–11]. More recently, an assay for Epstein Barr Virus (EBV) DNA is perhaps the closest to achieving clinical significance, which is closely associated with Nasopharyngeal carcinoma (NPC) in southern Asia [12]. In the last decades, more efforts have been devoted to the detection of mutation, loss of heterozygosity, microsatellites and methylation status of circulating DNA extracted from tumor tissues and plasmas or sera of patients with various tumors [13–16]. Therefore, circulating DNA is currently considered to be important for noninvasive diagnostic, prognostic and follow-up tests for cancer, especially the cell-free DNA level.

Several quantitative methods for circulating DNA have been developed, such as radio-immunoassays [3,4], spectrophotometry [17–19], fluorometric quantitative kit [20–23], competitive PCR [24] and real-time quantitative PCR [10,25,26]. All of these

Abbreviations: CE, capillary electrophoresis; CZE, capillary zone electrophoresis; LIF, laser induced fluorescence; R.S.D., relative standard deviation; ISTD, internal standard; Ct, threshold cycle

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doi:10.1016/j.jchromb.2006.04.026

methods demonstrated that average circulating DNA levels were significantly elevated in cancer patients compared to the healthy controls. However, the circulating DNA levels measured by different methods displayed considerable differences [2,20,24]. For example, using the same fluorometric quantitative method, several fold differences of circulating DNA levels measured by two groups were observed [21,26]. These were likely related to blood-processing protocols and quantitative methods. Therefore, Ziegler et al. and other researchers have already called on the standardization of methodology [27,28].

Recently, real-time PCR has become an important quantitative method in clinical analysis, which can detect the accumulation of amplified product in real time and provide accurate quantification in a widely dynamic range [29,30]. So far, this method is widely used for the quantification of circulating DNA in human serum and plasma. Unfortunately, template/primer combinations may be very sensitive to subtle temperature inhomogeneities that may exist in real-time PCR instruments [31]; and that the amplification efficiency can vary due to small variations in the reaction conditions, which maybe lead to unpredictable variation of the results.

We developed a new method for the quantification of circulating DNA by capillary zone electrophoresis with laser-induced fluorescence detection [32], and this method was characterized by automation, short analysis time, small sample and reagent requirement and extremely high sensitivity. However, it is not completely clear whether correlation of circulating DNA levels obtained by capillary zone electrophoresis (CZE)-laser-induced fluorescence detection (LIF) method with those measured by other methods (such as real-time PCR). In the present work, we want to further improve and evaluate CZE-LIF method for the quantification of circulating DNA, and make comparison between CZE-LIF method and real-time PCR assay for the quantification of circulating DNA levels from 50 normal serum samples.

2. Materials and methods

2.1. Instruments and chemical reagents

The quantification of circulating DNA was performed on a Beckman P/ACE MDQ system (Fullerton, CA, USA) with a LIF system. Fused-silica capillaries with 75 μm internal diameter (ID) were purchased from Yongnian Optical Fiber Factory (Yongnian, Hebei, P.R. China). The digests of λ-*Hin*dIII DNA were provided by Takara Co. (Japan). λ-DNA concentration was measured on a Hoefer DyNA Quant 200 fluorometer (Hoefer Pharmacia Biotech, San Francisco, CA, USA). Proteinase K was purchased from Merck (Darmstadt, Germany). SYBR Gold was from Molecular Probes (Eugene, OR, USA; concentration not given). QIAamp DNA Blood Mini Kit (QIAamp, Hilden, Germany) was used to extract circulating DNA from human serum. *N*,*N*,*N*′,*N*′-Tetramethylethylenediamine (TEMED) and ammonium peroxydisulfate (APS) were purchased from BIO-Rad Laboratories (Hercules, CA, USA). *N*-Trismethyl-3- aminopropanesulfonic acid (TAPS) and acrylamide were purchased from Sigma (St. Louis, MO, USA). Real-time PCR was performed on a MJ Opticon-2 (BIO-Rad Laboratories, Hercules, CA, USA). Primers, TaqMan Probe and heat-start Taq polymerase were purchased from Sangon (Shanghai, P.R. China).

2.2. Capillary coating

For the suppression of electroosmotic flow (EOF) and adsorption of DNA on the capillary inner surface, the inner wall of the capillary was coated with linear polyacrylamide using a modified procedure as described by Hjertén [33]. In brief, a new capillary was rinsed with methanol for 10 min firstly, etched with 1 M sodium hydroxide for 15 min, leached with 1 M hydrochloric acid for 15 min, and washed with water for 15 min, respectively. The capillary was first filled and left standing for 2 h with 50% acetic acid solution containing 10% γ-methacryloxypropy-trimethoxysilane, then for 2 h with degassed 3% acrylamide solution (5 mL 3% acrylamide solution containing 4 μL TEMED and 40 μL 10% APS).

2.3. Capillary zone electrophoresis procedure

A coated capillary (30 cm total length) was used for the quantification of circulating DNA. Before electrophoresis, TAPS buffer (80 mM, pH 7.5) containing SYBR Gold was pumped into the capillary by pressure. SYBR Gold has a good sensitivity and linearity in the low DNA concentration range, and its excitation wavelength matched with argon ion laser, therefore it was employed as the intercalating dye in our experiment. A LIF system (excitation wavelength, 488 nm; emission wavelength, 520 nm) was utilized for DNA detection. The DNA samples prepared by digesting the serum or extracted by the kits were injected by pressure. Between each run, the capillary was rinsed with distilled water and TAPS buffer for 1 min successively. Reversed polarity (12 kV) was used in electrophoresis.

2.4. Calibration curve of CZE

The digests of λ-*Hin*dIII DNA were used as standard DNA for calibration. λ-DNA was purified by ethanol precipitation, and then the total DNA concentration was measured on the Hoefer DyNA Quant 200 fluorometer. Serial dilutions of λ-DNA containing fluorescein (10^{-9} M, as an internal standard) were prepared as standard solutions. The peak areas of λ-DNA and fluorescein were measured by CZE-LIF. The calibration curve was generated by plotting various DNA concentrations versus peak area ratios of DNA to fluorescein.

2.5. Serum sample collection and isolation

The serum samples used in this study were from two collection ways. In the first, 12 blood samples were collected from students and staffs (healthy volunteer donors without malignant disease) in our laboratory, and the blood samples were stored for 2 h at room temperature before centrifugation. In the second, other 38 blood samples were offered by Shanghai Jiaotong University hospital (healthy students without malignant disease). They were collected sporadically, and the storage time of which

before centrifugation was not strictly controlled (within about 8 h at room temperature). In the collection of sera, all blood samples were centrifuged for 10 min at 3000 × *g*. Serum samples were stored at −20 °C until further processing.

DNA isolation from 200 μL serum was performed using a QIAamp DNA Blood Mini Kit (QIAamp, Hilden, Germany) according to the protocol recommended by the manufacturer. The extracted DNA was eluted with 100 μL of sterile water, and stored at −20 °C until further processing.

2.6. Pretreatment of serum samples

Four microliters of proteinase K (10 mg/mL) was added into the mixture of 50 μL serum and 98 μL 2 × Tris–borate–EDTA (TBE) buffer, and 50 μL distilled water, and then the mixture was incubated at 55 °C for 2 h. After digestion, the treated serum was generally diluted 10-fold, and a defined amount of fluorescein (10^{-9} M) was added as internal standard. The digested serum samples were used for the analysis of CZE.

2.7. CZE method

The DNA samples extracted by QIAamp blood kit (CZE^1) or digested serum samples (CZE^2) were diluted properly, and a defined amount of fluorescein (10^{-9} M) was added as internal standard. The concentrations of circulating DNA were estimated by the calibration curve.

2.8. Real-time quantitative PCR

We quantified the circulating DNA in serum using real-time quantitative PCR assay for the β-globin gene according to the procedure described in the reference [25]. In brief, the β-globin TaqMan system included two primers, β-globin-354F, 5′-GTG CAC CTG ACT CCT GAG A-3′; β-globin-445R, 5′-CCT TGA TAC CAA CCT GCC CAG-3′, and a dual-labelled fluorescent TaqMan probe, β-globin-402T, 5′-(FAM)AAG GTG AAC GTG GAT GAA GTT GGT GG(TAMARA)-3′, sequence data were taken from the Genebank sequence database No. U01317.

TaqMan amplification reactions were set up in a final reaction volume of 50 μL. Each reaction contained 5 μL of 10 × buffer; 100 nM of each of amplification primers; 66.67 nM of the fluorescent probe; 4 mM of the $MgCl_2$; 200 μM each of dATP, dTTP, dCTP, dGTP; 1 unit of heat-start Taq DNA polymerase; 10 μL of extracted DNA. The thermal cycling conditions were: 5 min at 95 °C to activate the enzyme, followed by 37 two-step cycles of 95 °C for 30 s, and 60 °C for 1 min. DNA amplifications were carried out in a 96-well reaction plate format in a MJ Opticon-2. Data were collected using a MJ Opticon detector and analyzed using the Opticon monitor™ analysis software (version 2.02). Each sample was analyzed in duplicate. Multiple water blanks were included in every analysis as a negative control.

The calibration curve was run in parallel and in duplicate with each analysis. For making a calibration curve, we extracted DNA from the whole blood of a normal person, measured the DNA concentration on a Hoefer DyNA Quant 200 fluorometer and serially diluted it. A conversion factor of 6.6 pg of DNA/diploid cell was used to calculate the copy number [59].

3. Results

3.1. Linearity and reproducibility of real-time PCR assay

We determined the dynamic range of real-time quantitative PCR using serial dilutions of the whole blood DNA. Fig. 1A shows amplification plots of fluorescence intensity against the PCR cycle. The amplification curves shift to the right as the whole blood DNA quantities reduce. Fig. 1B shows a plot of the threshold cycle (Ct) against the whole blood DNA quantity (the black dots •). A good linear relationship ($y = -3.32x + 36.92$) between the Ct (threshold cycle) values and the log copy numbers was obtained ($R^2 = 0.996$). All serum sample concentrations fell within the standard curve (the grey dots •), which were interpolated from the given Ct of any sample by this calibration standard curve. The measurement of the DNA sample isolated by the kits was preformed six times in the same TaqMan assay to determine the intra-assay variability. The inter-assay variability was assessed by six different TaqMan assays of the DNA sample isolated by the kit. The relative standard deviations of the intra-assay and inter-assay were 7.3% and 14.92%, respectively.

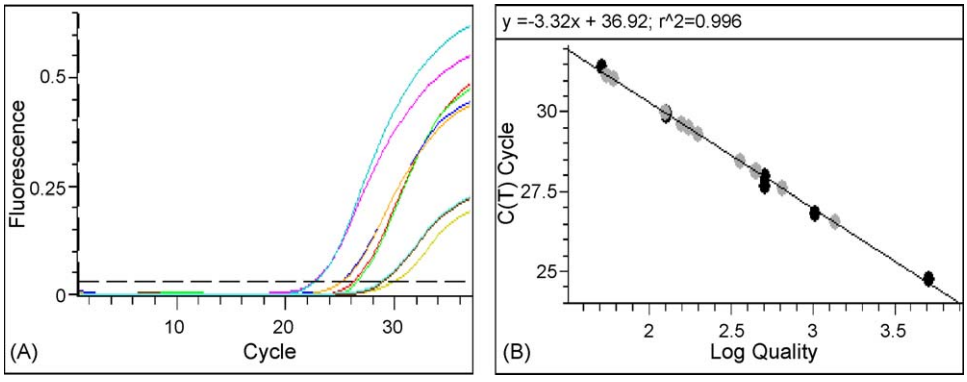

Fig. 1. Detection of DNA by real-time quantitative PCR. (A) Amplification plots of the real-time quantitative PCR for β-globin gene with serially diluted DNA; (B) standard curve for β-globin gene (log scale) plotting the threshold cycle (Ct) against known concentrations of serially diluted DNA (from 5151 to 51 copies).

3.2. Reproducibility and reliability of CZE method

The serum samples were first digested by proteinase K, and then were directly measured without purification for five replicate runs on the same capillary (same day) to assess the intra-assay variability. The inter-assay variability was evaluated by measuring the serum sample digested by proteinase K on the same capillary in five consecutive days. The R.S.D. values were 0.6% for migration time and 4.19% for peak area within in the same day, 1.38% for the migration time and 6.91% for the peak area for five days.

The reproducibility of the coated capillary was determined by the quantification of the DNA sample isolated using the kit for five replicate runs on the same coated capillary. The R.S.D. was also assessed by using six different coated capillary (using the DNA sample isolated using the kit). The R.S.D. values of the same coated capillary and different coated capillaries were 5.14% and 9.02%, respectively. Furthermore, a satisfactory recovery (89%) of the coated capillary was achieved [32]. The good reproducibility and recovery were attributed to a good covalent-coated capillary. Under this condition, the electroosmotic flow and the adsorptions of DNA were efficiently suppressed**.** Our data illustrated that CZE-LIF was practical and reliable for the quantification of circulating DNA in serum.

3.3. Evaluation of QIAamp DNA blood mini kit

The DNA sample was isolated using 10 spin columns from QIAamp DNA Blood Mini Kits. The concentrations of the 10 purified DNA samples were measured using CZE-LIF technique on the same coated capillary to evaluate the reproducibility of the kit. The R.S.D. of the kit was 14.37%. Moreover, this kit had a recovery from 82 to 91% and a sensitivity of 2 ng/mL [21].

3.4. CZE of circulating DNA

Due to the similar charge densities, different DNA fragments have the same electrophoretic mobility in CZE when the DNA molecules reach about 170 bp in size [34]. In principle, the total cell-free DNA amount can be detected by capillary zone electrophoresis (CZE). Fig. 2 shows the electropherograms of digests of λ-DNA and the digested serum sample in CZE. λ-DNA containing eight fragments from 125 bp to 23130 bp showed only one peak in CZE. For the digested serum sample, the same result was obtained. And that the migration time of the DNA was only about 3 min.

3.5. Calibration curve of DNA

The calibration curve of the peak area ratios (DNA/ISTD) versus DNA concentrations was obtained by CZE-LIF system (as shown in Fig. 3). A good linearity ($R = 0.9995$) was achieved from 0 to 40 ng/mL. Moreover, a detection limit of 0.5 ng/mL for DNA (S/N = 3) was obtained by CZE-LIF system, and the mass detection limit was about 1 fg of DNA. These data illustrated that the CZE-LIF was sensitive enough to directly quantify the circulating DNA levels in human sera.

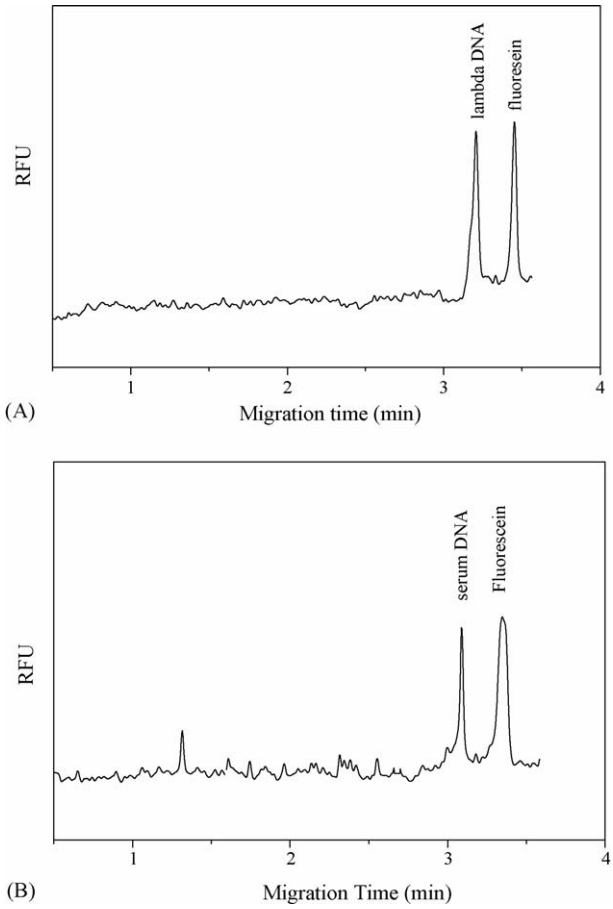

Fig. 2. The electropherogram of DNA and fluorescein (10^{-9} M) in CZE. (A) The digests of λ-DNA (9.8 ng/mL). (B) Serum DNA. Samples were introduced by pressure (6 s at 0.5 psi). The coated capillary (20 cm effective length, 75 μm i.d.) was used; filled with 80 mM TAPS containing SYBR Gold (1/10,000); temperature, 25 °C; applied voltage, −400 V/cm.

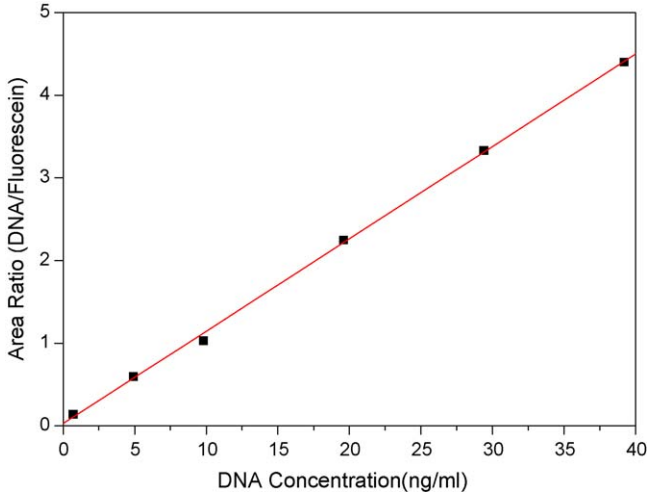

Fig. 3. Calibration curve for peak area ratios of the DNA and fluorescein to DNA concentrations. Fluorescein concentration was 1×10^{-9} mol/L, and DNA concentration varied from 0.5 to 40 ng/mL. Samples were introduced by pressure (6 s at 0.5 psi). The coated capillary (20 cm effective length, 75 μm i.d.) was used, filled with 80 mM TAPS containing SYBR Gold (1/10,000). Electrophoresis buffer was 80 mM TAPS (pH7.5), the temperature was 25 °C, and the applied voltage was −400 V/cm.

Table 1
Comparisons of the quantitative results of 12 serum samples with real-time PCR, CZE[1] and CZE[2]

Sample no.	Real-time PCR	CZE[1]	CZE[2]	Sample no.	Real-time PCR	CZE[1]	CZE[2]
1	49.48	40.12	45.92	7	94.79	101.60	92.64
2	120.43	119.98	125.60	8	43.68	40.30	37.01
3	71.95	79.10	83.43	9	34.87	55.02	62.60
4	29.90	32.52	45.90	10	121.68	149.13	112.70
5	59.40	36.98	33.68	11	122.59	134.21	140.50
6	28.43	27.84	33.30	12	35.52	33.13	40.13

	Real-time PCR	CZE[1]	CZE[2]
Average	67.73 ± 37.56 ng/mL	70.83 ± 44.30 ng/mL	71.12 ± 38.64 ng/mL

Fluorescein concentration as ISTD was 1×10^{-9} mol/L. Samples were introduced by pressure (6 s at 0.5 psi). The coated capillary (20 cm effective length, 75 μm i.d.) was used, filled with 80 mM TAPS containing SYBR Gold (1/10,000). Electrophoresis buffer, 80 mM TAPS (pH 7.5); temperature, 25 °C; applied voltage, −400 V/cm.

Table 2
Comparisons of the quantitative results of 38 serum samples with real-time PCR, CZE[1] and CZE[2]

Sample no.	Real-time PCR	CZE[1]	CZE[2]	Sample no.	Real-time PCR	CZE[1]	CZE[2]
1	277.03	225.20	251.83	20	131.11	140.58	117.15
2	180.17	204.48	174.58	21	111.16	156.11	157.20
3	209.14	220.70	228.42	22	329.47	317.79	335.32
4	266.98	254.28	265.96	23	164.72	119.98	103.18
5	216.56	246.87	201.01	24	181.96	189.16	220.20
6	295.56	376.79	315.65	25	78.26	80.14	90.80
7	104.63	127.80	179.50	26	230.41	226.53	228.46
8	183.91	200.57	246.30	27	170.55	170.88	182.23
9	362.40	303.54	313.06	28	146.58	163.62	147.83
10	251.34	214.34	266.21	29	204.55	194.26	229.33
11	162.96	177.40	134.78	30	127.09	144.80	135.17
12	122.59	134.21	140.50	31	71.22	52.64	77.43
13	263.85	273.25	285.20	32	24.31	48.28	50.23
14	234.50	212.66	232.12	33	200.79	183.82	189.37
15	206.86	230.12	210.20	34	130.38	149.16	128.54
16	219.25	212.03	198.01	35	275.21	240.50	296.52
17	312.70	304.44	326.40	36	119.29	133.98	116.45
18	169.40	208.25	200.32	37	216.43	231.40	232.08
19	100.11	122.41	113.10	38	112.71	124.10	116.45

	Real-time PCR	CZE[1]	CZE[2]
Average	188.58 ± 76.85 ng/mL	192.55 ± 70.99 ng/mL	195.71 ± 74.48 ng/mL

Conditions as in Table 1.

3.6. Comparisons of three methods for quantification of the circulating DNA levels

In order to assess whether three methods (real-time PCR, CZE[1] and CZE[2]) correlate with each other, we quantified the circulating DNA levels of 50 serum samples (healthy control). The results are shown in Tables 1 and 2. From Table 1, we knew that the mean levels obtained by three methods were 67.73 ± 37.56 ng/mL, 70.83 ± 44.30 ng/mL and 71.12 ± 38.64 ng/mL respectively. The data in Table 2 illustrated that the circulating DNA levels of most blood samples were much higher than those in Table 1. The mean results obtained by three methods were 188.58 ± 76.85 ng/mL, 192.55 ± 70.99 ng/mL and 195.71 ± 74.48 ng/mL, respectively. These data in Tables 1 and 2 demonstrated that three methods were consistent very well, although the results of DNA levels assayed by CZE[2] were slightly higher than those obtained by other two methods.

4. Discussion

Circulating DNA levels in human sera may be used as a diagnostic or prognostic marker for some cancers, as well as for other conditions such as pregnancy complications and trauma [35–41]. Several techniques have been employed for the quantification of circulating DNA, and they are mainly divided into two kinds of methods, including fluorometric methods and PCR-based quantitative assays. PCR-based quantitative assays hold much more promise as key tools for the quantification of circulating DNA, because they are more sensitive than fluorometric methods.

Competitive PCR entails the coamplification of each target sequence with a competitive synthetic DNA internal standard

to compensate the fluctuations of amplification efficiency. It is known to be a reliable and precise method [42]. However, this method needs a large amount of template DNA for analysis of a sample and also needs post-PCR analytical steps that maybe result in a risk of cross-contamination of sample with PCR products [43]. Real-time PCR has several advantages over the conventional PCR, such as increased test efficiency, large dynamic range and low risk of amplicon contamination [44,45]. So far, real-time PCR is widely applied for the quantification of circulating DNA in serum or plasma [10,46–53,60], and it has been generally considered as the gold standard. However, all these methods are generally based on the use of commercial DNA extraction kits for purification of the circulating DNA, which are labor-extensive and time-consuming, and thus they are not suitable for the large-scale quantification of circulating DNA samples in clinical diagnosis. Furthermore, many anticontamination measures have to be taken to prevent PCR contamination.

Compared to other quantitative methods, CZE-LIF method has many advantages, mainly including simplicity, automation, small sample and reagent requirement, low-cost and short analysis time and highly sensitivity. More importantly, the labor-extensive and time-consuming step for the extraction of circulating DNA from serum using commercial kits is not necessitated in CZE^2 method. In CZE, all DNA fragments have the nearly same electrophoretic mobility in free solution [54] and they co-migrate in the electric field, so that CZE can be used to determine the total DNA in serum [32]. Fig. 2 displays the electropherogram of the digests of λ-DNA and the digested serum sample. The result shows that the analysis time is about 5 min for one digested serum sample.

To further evaluate the performances of CZE-LIF method, the linearity, intra and inter variability were measured. A detection limit of 0.5 ng/mL for DNA (S/N = 3) was obtained, and this amount corresponded to about 1 fg of DNA, which indicated that CZE-LIF method was sensitive enough to quantify the circulating DNA in serum. Meanwhile, the good reproducibility and reliability of CZE were obtained. It should be pointed out that a good-quality coated capillary was very crucial for exact quantification. When the electroosmotic flow and the adsorptions of DNA were efficiently suppressed, the satisfactory results could be obtained.

Furthermore, we assayed the circulating DNA levels of 50 individual serum samples (healthy control) using three different methods. Comparisons of our data are shown in Tables 1 and 2. The mean DNA levels assayed by three methods in Table 1 were well agreement with the known data obtained by the commercial kit [20]. These results proved that CZE-LIF was very reliable for the quantification of circulating DNA. While the mean DNA concentrations in Table 2 were about 2.8-fold higher than those in Table 1. This cause was attributed to the fact that the blood storage time of 38 serum samples was not severely controlled before centrifugation. Lee et al. and other researchers also found that the concentrations of circulating DNA increased significantly in serum samples during blood sample storage before centrifugation, and that about three-fold increase in serum DNA level was observed in one day [55,56]. Our results also further confirmed that blood samples collection, serum preparation, and other treatment procedures had a significant impact on circulating DNA levels in sera [55,57,58]. The results suggest that optimization and equivalence of procedures are very necessary if any future application of plasma or serum DNA analysis attains diagnostic purposes.

In conclusion, our results illustrate that CZE-LIF assay is at least as sensitive as real-time PCR method and two methods are highly consistent with each other. The speed, simplicity, reliability and low cost of CZE-LIF method make it well suitable for the quantification of circulating DNA in human sera from clinical diagnosis.

Acknowledgements

This study was financially supported by the National Natural Science Foundation of China (No. 20271033, 90408014), the key project of National Natural Science Foundation of China (No. 20335020), and Mega-projects of Science Research for the 10th Five-Year Plan of MSTPRC (2004BA720A04).

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