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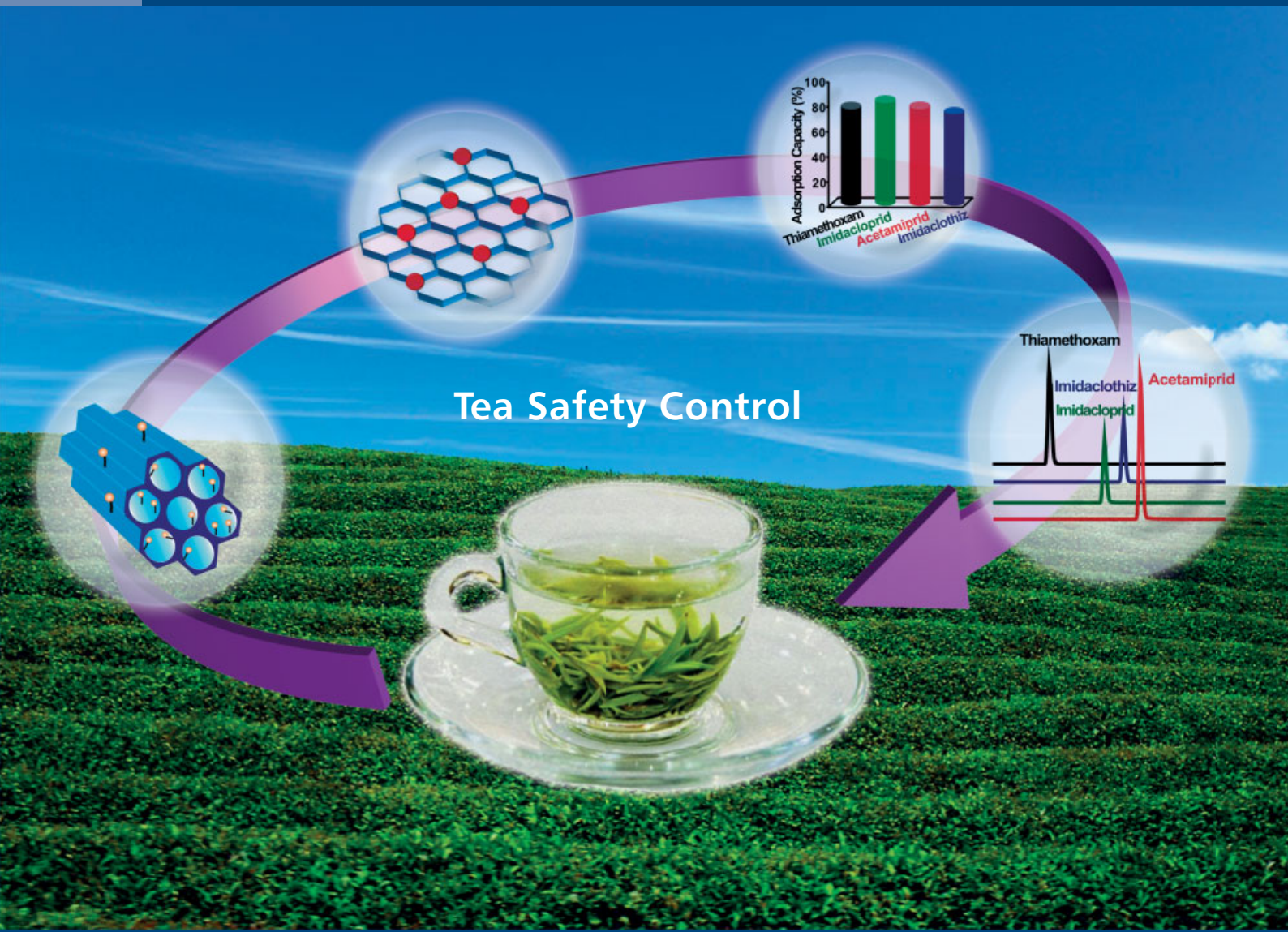

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Research Article

Gene analysis of multiple oral bacteria by the polymerase chain reaction coupled with capillary polymer electrophoresis

Capillary polymer electrophoresis is identified as a promising technology for the analysis of DNA from bacteria, virus and cell samples. In this paper, we propose an innovative capillary polymer electrophoresis protocol for the quantification of polymerase chain reaction products. The internal standard method was modified and applied to capillary polymer electrophoresis. The precision of our modified internal standard protocol was evaluated by measuring the relative standard deviation of intermediate capillary polymer electrophoresis experiments. Results showed that the relative standard deviation was reduced from 12.4–15.1 to 0.6–2.3%. Linear regression tests were also implemented to validate our protocol. The modified internal standard method showed good linearity and robust properties. Finally, the ease of our method was illustrated by analyzing a real clinical oral sample using a one-run capillary polymer electrophoresis experiment.

Keywords: Capillary polymer electrophoresis / Clinical diagnosis / Internal standard method / Oral bacteria / Quantitative analysis
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1 Introduction

The quantification of specific DNAs from oral bacteria is in urgent demand for medical research and dental health care. Clinically, the abnormal population of oral bacteria is of high risk to induce sickness in oral cavity [1], for example periodontal diseases and caries. It has been reported that oral bacteria are also risk factors for many diseases like adverse pregnancy outcome [2], diabetes mellitus [3], and cardiovascular and cerebrovascular diseases [4]. The analysis of specific genes originated from oral bacteria will provide incisive microbial information, which has attracted considerable interests among dental medical researchers [5, 8].

However, among the many practical instrumental analysis for quantifying DNAs there are still arguments about their quantitative efficiencies [9]. For example, UV spectrophotometry [10, 11] may count nonspecific biomolecules resulting in inaccurate quantitative analysis. MS [12, 13] determines the amounts and masses of DNAs by measuring the mass-to-charge ratio, which is highly sensitive and informative. But its sample preparing process is complicated because samples are supposed to be confined to volatile low-salt buffers. Quantitative polymerase chain reaction (qPCR), which is sometimes called real-time PCR [14, 15], quantifies target gene fragments by monitoring the fluorescence of PCR products, while it is realized to have high risk of unspecific PCR products and requires many steps of sample preparation.

During the past decades, capillary polymer electrophoresis (CPE) [16, 18] is recognized as a reliable and promising tool to identify and quantify specific genes. CPE is one of the various types of CE [19, 21]. Because its separation mechanism is based on simple physical interactions, which are colliding, hooking, and entangling between nucleic acid molecules and polymers, CPE possesses the

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Abbreviations: CPE, capillary polymer electrophoresis; **HEC**, Hydroxyethylcellulose; **RL**, resolution length; **TBE**, Tris-Boric acid-EDTA; **qPCR**, quantitative polymerase chain reaction

nucleic acid separation with high resolution. Since early 1990s, the resolution of CPE for ssDNA has reached the ultimate of one nucleotide [22]. Besides, CPE has an excellent compatibility with PCR because it requires no pretreatment for sample labeling or hybridization. It is able to distinguish target genes from unspecific PCR products and impurities in minutes. However, the superiority of CPE has not been widely realized in the quantitative analysis field. The most reported quantification protocols and applications are related to CZE [23] and CITP [24]. Till now, there is not sufficient research related to the CPE quantification of PCR products.

In this paper, we reported an innovative CPE protocol for the quantification of PCR products from three important periodontal pathogens, which were *Porphyromonas gingivalis* (*P.g*), *Treponema denticola* (*T.d*) and *Tannerela forsythia* (*T.f*) [25, 28]. Firstly, the analytical method of the quantitative CPE protocol was described in detail. Secondly, the separation reproducibility of CPE was examined by intermediate (run-to-run and day-to-day) tests. Linear regression tests were further implemented to evaluate the performance of the quantitative CPE protocol. Finally, a real clinical sample was analyzed using our analytical method to prove its facilitation and validation.

2 Materials and methods

2.1 protocol of CPE quantification

Our CPE system is based on fluorescence detection. Generally, the fluorescent intensity of analyte DNA is proportional to its quantity. To reduce errors between intermediate experiments and between instruments, internal standard was employed to provide self-calibration [29, 30]. From the relative fluorescent intensity between internal standard and analyte DNA, the quantity of analyte DNA can be calculated using simple mathematics. As our internal standard is also DNA, which is the same type of molecule with analytes, they have the same dye-conjunction ratio.

However, the internal standard method still needs modification. Because CPE system employs on-line detection system, the fluorescence signal is recorded as pin-point detection. The peak width of a DNA band depends on not only the width of itself, but also its migration velocity. The faster the DNA migrates, the narrower the peak width appears. The same DNA band migrating with different velocities will represent different peak areas. Therefore, the peak area is also related to the migration velocity of the DNA analyte. In the internal standard method, where the peak areas of two DNAs are compared, a velocity or time modification is supposed to be implemented, especially in short time analysis.

In the CPE process, DNAs with different sizes migrate at different velocities. The detector records the fluorescent signals from the DNA bands when they are migrating through the detection window (Fig. 1A). Controlled by a computer, the detector records the fluorescent signal in a series of short time intervals. The fluorescent intensities recorded at all these time intervals compose the profile of a band in electropherogram. To facilitate explanation, we represented a single electrophoretic band of a DNA analyte as successive sections from a_1 to a_m (Fig. 1B). During section a_1 passing by the detection window, several (n as assumed) time intervals have been proceeded. As a result, the fluorescent signal (I_1) of section a_1 is not only recorded in time interval 1, but also contributes to the signals in time intervals 2 to time interval n. Similarly, section a_i is recorded in time intervals i to time interval (i+n–1), totally n time intervals. The fluorescent signal I_j from a_i is recorded n times. The value of peak area of an electrophoretic band is the sum of the fluorescent signal at all the time intervals. Section a_1 contributes not one I_1, but nI_1 to the peak area, so do other sections (Fig. 1C). Therefore, the peak area observed in CPE is based on the multiplex measurement of fluorescence signal from the electrophoretic band (I) (Eq. (1)). The number n depends on two factors. One is the time duration (T_{dur}) for each section passing through the detection window, and another one is the sampling frequency (f) of the detector (Eq. (2)):

$$A = c\left(nI_1 + nI_2 + \ldots + nI_{j-1} + nI_j\right) = cnI \tag{1}$$

$$n = T_{dur} f \tag{2}$$

where A is the peak area of DNA analyte, c is a constant factor related to the collection efficiency of fluorescent intensity. Further on, T_{dur} relies on the length of detection window (L_d) and the migration velocity (v_m) of the analyte DNA:

$$T_{dur} = L_d / v_m = L_d t_m / L_e \tag{3}$$

where L_e is the effective length of capillary, t_m is the migration time of analyte DNA. From Eqs. (2) and (3), we discover that n is proportional to t_m of the DNA analyte.

According to reference [31], the quantity (Q) of analyte DNA is proportional to the fluorescent intensity (I) of its corresponding electrophoretic band (Eq. (4)):

$$Q = kI \tag{4}$$

where k is the factor related to the instrumental property, dye conjunction efficiency, injection parameter and other chemical properties. In practice, the detection and sample injection process introduces uncertainty errors to k among intermediate CPE runs, leading to problems for quantification precision and reducing reproducibility. The internal standard protocol [32] helps to provide a self-calibrated k as shown in Eq. (5). Thus the quantity of analyte can be yielded as Eq. (6):

$$k = Q_s / I_s \tag{5}$$

$$Q = Q_s\,(I / I_s) \tag{6}$$

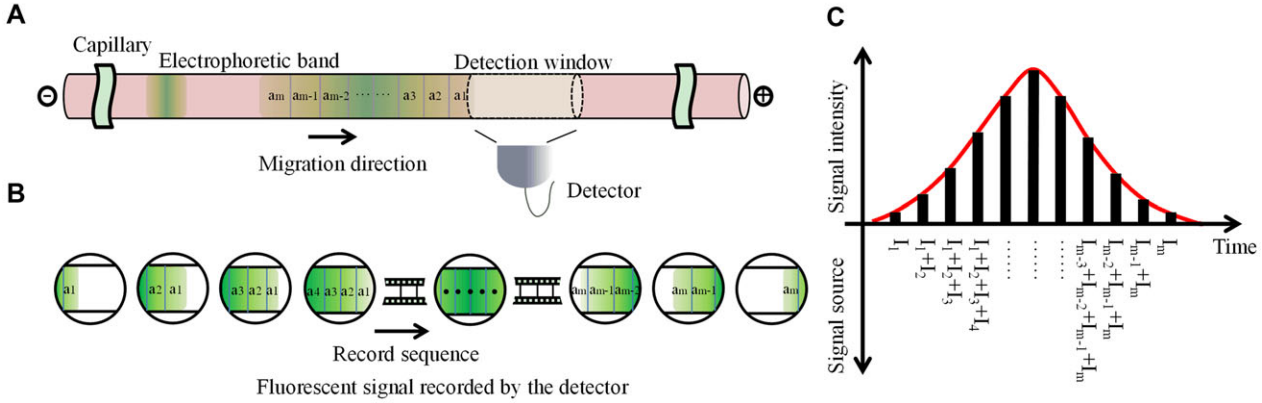

Figure 1. (A) On-column detection mode in CPE. The electrophoretic band was theoretically divided to adjoining sections. (B) Fluorescent signal recorded sequentially by the detector. (C) The electropherogram of analyte, the columns represented the fluorescent intensities recorded by the detector in sequence. This indicates the fluorescent signal of every section in Fig. 1A was multiplex recorded.

where Q_s is the known quantity of internal standard, I_s is its fluorescent intensity. Combining Eqs. (1)–(3) and (6), time modification is introduced when the peak areas in the electropherogram are compared:

$$Q = Q_s(At_{ms})/(A_s t_m) \quad (7)$$

where A and t_m are the peak area and migration time of analyte DNA, A_s and t_{ms} are the peak area and migration time of internal standard, respectively. According to Eq. (7), time modification is especially important in short time CPE analysis. The centers of two peaks may appear at a relative long interval (for example, t_{ms} is 1 min and t_m is 1.5 min), great error (49.9%) appears without time modification. For convenient elaboration, we define the time-modified peak area ratio as $At_{ms}/(A_s t_m)$.

2.2 Chemicals and CPE samples

A 100 bp DNA ladder marker, TBE and SpeedSTAR HS DNA Polymerase were purchased in Takara (Dalian, China). The 100 bp DNA ladder marker contained 11 double-strand DNA fragments with a size range from 100 to 1500 bp. Among the 11 DNA fragments, the mass of the 500 bp fragment was triple that of the other fragments. DNA samples were diluted with 0.5x TBE and kept in 4°C for short term storage. Hydroxyethylcellulose (HEC) polymer solution with 1% w/w concentration was prepared by mixing HEC powder (Polysciences, Warrington, PA, USA) with 0.5x TBE. This concentrated HEC solution was stirred for at least one week to make it homogenous. Its performance was stable for at least one year according to lab experience. Before use, the 1% HEC polymer was further diluted by 0.5x TBE to designed concentration. SYBR Green I (Invitrogen, Carlsbad, USA) was added to the HEC polymer solutions with a final concentration of 2x.

Positive-control bacteria, which were *P.g* (ATCC 33277), *T.d* (ATCC35405), and *T.f* (ATCC 43037), and clinical source sample (wild type) were prepared with Wizard Genomic DNA Purification (Promega, Madison WI USA) to extract the PCR templates. PCR reaction solutions were prepared to 20 μL using SpeedSTAR HS DNA Polymerase kit, containing 0.4 μL of each sample and 2 μM of each primer. The thermal circulation for target DNA amplification was 40 cycles of 95°C for 10 s (denaturation) and 64°C for 30 s (annealing and extension) with an initial cycle of 95°C for 2 min. Primers (Supporting Information 1) for specific DNA fragments were synthesized by Sangon Biotech Company (Shanghai, China). The PCR product of each bacterium contained target DNA fragments with size of 197 bp (*P.g*), 311 bp (*T.d*) and 641 bp (*T.f*). Before CPE experiments, three PCR products were mixed together and diluted in tubes to a series of concentration from 2 to 15%. Each tube contained 100 bp DNA ladder marker as the internal standard.

2.3 CPE instruments

In this work we demonstrated the quantification analysis with our home-made CE system [33]. The detection system of CE was constructed by a fluorescent excitation part and an emission collection part. The fluorescent excitation part was composed by a mercury lamp and an optical cube (U-MWIB-3, Olympus, Tokyo, Japan) on the microscope (DSY5000X, Aopu, Chongqing, China) to transmit light at 460–495 nm wavelength for the excitation of dye-nucleonic acid conjugate. The emission light was filtered by the optical filter and then collected by a PMT (H8249-101, Hamamatsu Photonics, Japan), which formed the emission collection part. LabVIEW software purchased from National Instrument (Austin, TX, USA) was employed to store raw electrophoretic data and control high voltage supplier for CPE. Fused-silica capillary (Polymicro, Phoenix, AZ, USA) with 75 μm ID was coated by polyacrylamide to eliminate EOF [34]. The total length and effective length of capillary was 7 and 12 cm, respectively. During the process of CE, the whole system was enclosed

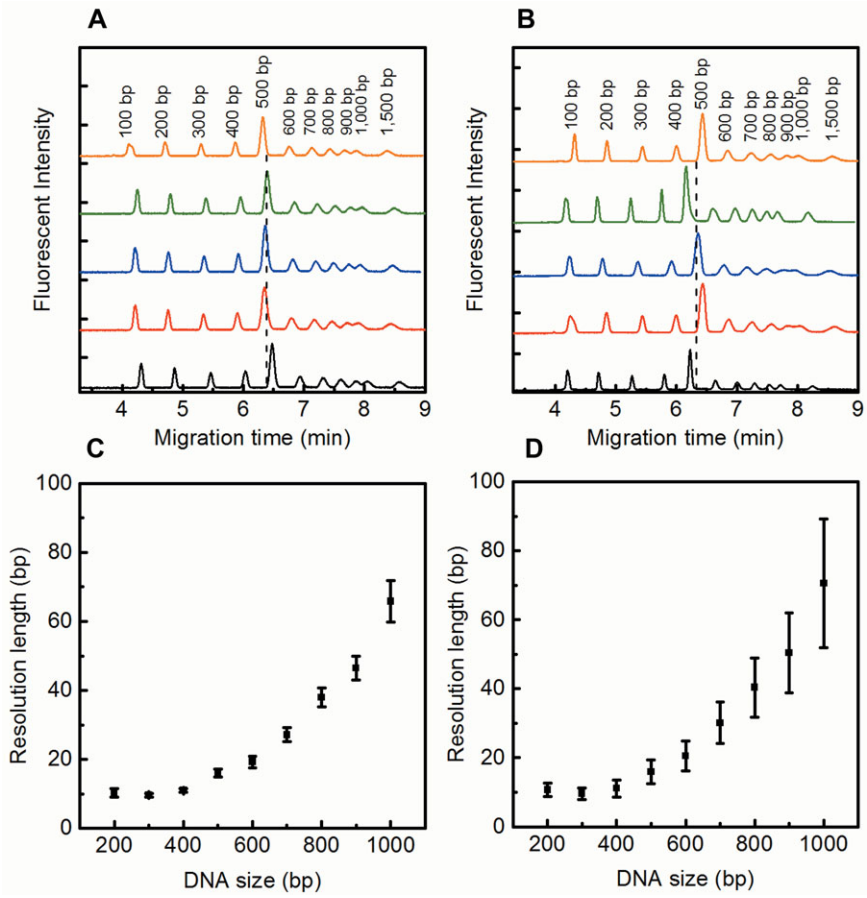

Figure 2. Electropherograms of 100 bp DNA ladder marker by CPE performed in (A) five sequential runs, (B) five different days. Resolution length with error bars which were calculated from (C) five sequential runs, (D) five runs performed on five different days. DNA sample was injected electrokinetically for 2.0 s at 100 V/cm. Polymer solution contained 0.3% w/w HEC and 0.5x TBE. CPE was performed under 100 V/cm electric field strength. The total length and the effective length of the capillary were 9.0 and 6.0 cm, respectively.

in a dark box at room temperature. After each run the capillary was flushed by ultrapure water to remove the remaining chemicals. The width, center and area of electrophoretic peaks were calculated by OriginPro software (OriginLab Corporation, Northampton, USA).

3 Results and discussion

3.1 Reproducibility of CPE quantification

A 100 bp DNA ladder marker with size range from 100 to 1500 bp was employed for run-to-run and day-to-day reproducibility testing. In these run-to-run and day-to-day CPE experiments, RSD [35] of peak area and time-modified peak area ratio were compared to demonstrate the modified internal standard protocol. The separation of DNA sample was performed in different days. On each day, five sequent runs were performed.

As shown in Fig. 2A and B, it was observed in the electropherogram that migration time shifted and peak widths differed among run-to-run and day-to-day experiments. This directly caused errors for the qualitative and quantitative determination. In Fig. 2C and D, the average resolution length (RL) [36] of each DNA fragment was demonstrated. We calculated the RL of each DNA fragment, and found out that the RL of 900 and 1000 bp DNA fragments were above 50 bp with standard error of 11%. To achieve accurate peak area integration, we selected 100 to 800 bp DNA fragments for reproducible CPE quantification.

The RSD of peak area and time-modified peak area ratio was employed to evaluate the precision of quantitative CPE. Raw electropherogram was approximated by Gaussian fitting (Supporting Information 2). Peak area of DNA fragment was computed by integrating the fitting curve. Then the time-modified peak area ratio of corresponding DNA fragment to internal standard was calculated. According to reference [35] the internal standard was supposed to have similar mobility as the analytes. At the same time, it should be well resolved from the analytes. Thus we selected the 500 bp DNA fragment as the internal standard, which was observed close to 200 to 800 bp DNA analytes in the electropherogram. Results were shown in Fig. 3A, for intermediate experiments processed in 5 days, the RSD of peak area was 12.4–15.1% (black bars). In contrast, the RSD of the time-modified peak area ratio was around 0.6–2.3% (shaded bars), which was preferable for quantitative analysis [37]. Regarding to run-to-run precision, the RSD was also greatly reduced by average 85.1%, which was from 11.6–13.0% to 1.0–3.3% (Fig. 3B).

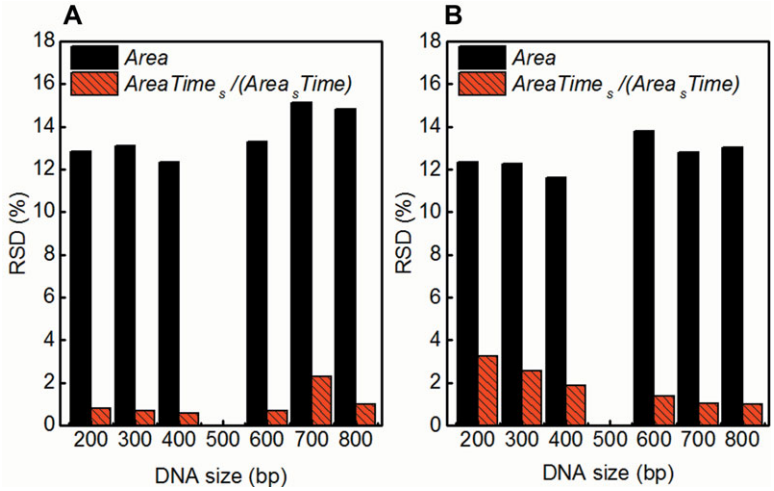

Figure 3. Comparison of RSD between peak area (Area) and time-modified peak area ratio (AreaTime$_s$/(Area$_s$Time)) after (A) 25 CPE experiments performed on five days, (B) five sequential CPE experiments, on each day five sequential runs were performed. Here, Area and Time represented the peak area and migration time of corresponding DNA analyte, respectively. Area$_s$ and Time$_s$ represented the peak area and migration time of internal standard, respectively.

3.2 Analyses of PCR products by CPE using modified internal standard

P.g, *T.d* and *T.f* bacteria are periodontal pathogens. The precise quantitative analysis of these three bacteria is indispensable in preventive medicine. To examine the validation of PCR-CPE quantification for oral bacteria, we employed the positive control PCR products of these three bacteria in liner regression test. PCR products were diluted to different concentrations ranging from 2–15% before CPE experiment. *Peak areas* of target DNA fragments were calculated from electropherograms (Supporting Information 3) and then plotted against the concentration of target DNA (Fig. 4).

From Fig. 4A we observed that the peak areas at 15% concentration were over half smaller than those at 10% concentration. We inferred that the rude PCR samples had impact on the fluorescent intensity of DNA analytes. Consequently, the peak area reduced when the concentration of PCR products increased. In this case, even if the liner relationship between peak area and the concentration of target DNA was observed, it was not convincible or reliable. In contrast, by using modified internal standard protocol, the liner relationship remained stable between time-modified peak area ratio and target DNA concentration (Fig. 4B). The regression plots passing through the origin had correlation coefficients (R^2) of 0.97, 0.99 and 0.96 for *P.g* *T.d* and *T.f*, respectively. This was because the internal standard compensated the impact factor from the rude PCR samples. Therefore, the impact from the implemental process of PCR products was avoided using modified internal standard protocol.

3.3 Qualitative and quantitative determination of target DNAs from clinical oral sample by CPE using modified internal standard protocol

A clinical saliva sample from the patient was analyzed by quantitative CPE using modified internal standard. Before CPE process, the PCR productions of saliva sample were

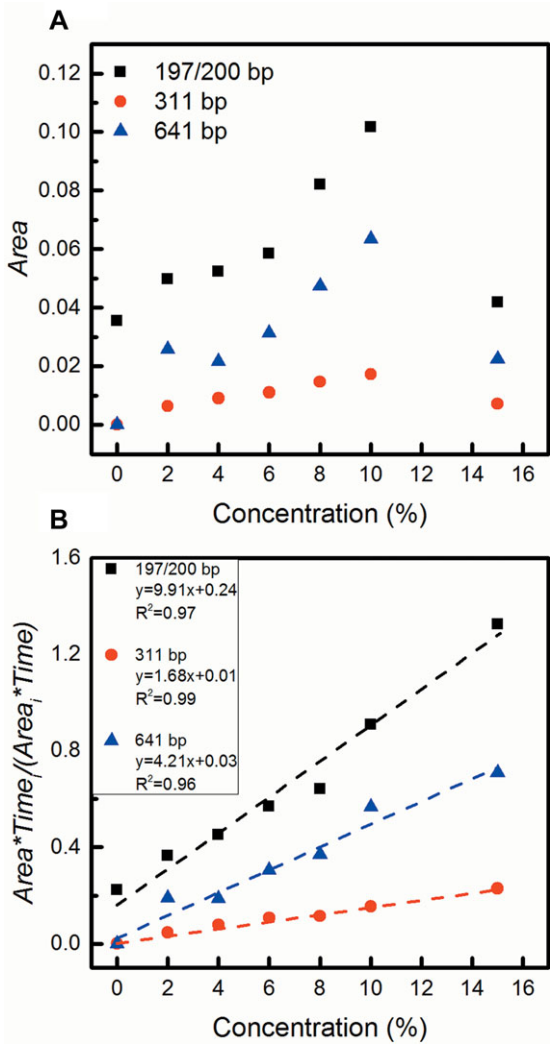

Figure 4. Liner regression test of CPE quantification by (A) measuring peak area and (B) measuring time-modified peak area ratio to internal standard. Electrophoretic peaks of 197 bp (*P.g*) and 200 bp overlapped, thus the corresponding peak area and peak area ratio referred to both DNA fragments.

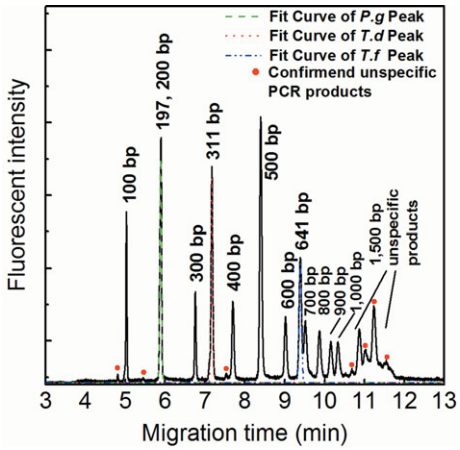

Figure 5. Electropherogram of the mixture of 100 bp DNA ladder marker and PCR products from clinical sample. Polymer solution contained 0.5% HEC with 0.5x TBE. Other conditions were the same with Fig. 2.

directly diluted and mixed with 100 bp DNA ladder marker. In the electropherogram (Fig. 5), the sizes of target DNAs were demonstrated as 197 bp (*P.g*), 311 bp (*T.d*) and 641 bp (*T.f*). And unspecific PCR products were observed in electropherogram. The concentrations of target DNAs were calculated by multiplying their time-modified peak area ratios by the concentration of internal standard as shown in Supporting Information Table S4. The peaks of 197 and 200 bp overlapped with each other. Thus the concentration of 197 bp was calculated by subtract the concentration of 197/200 bp with the concentration of 200 bp (0.5 ng/μL as designed).

4 Concluding remarks

This work demonstrated the quantification of PCR products using a modified internal standard protocol in CPE. This protocol quantified by the measuring time-modified peak area ratio to internal standard. We reported the RSD of time-modified peas area ratio and linear regression tests of the modified internal standard protocol. Results showed this modified internal standard protocol was precise and robust. The RSD was 0.6–2.3% for day-to-day experiments. In case of quantifying rude PCR product, the modified internal standard protocol was able to compensate the impact from concentrated PCR chemicals and showed a stable quantification performance. Generally, the PCR-CPE analysis is reliable and promising for the quantification of DNAs. Because no pretreatment is required for the CPE determination of PCR production, they have highly compatibility. CPE is able to separate target DNAs from unspecific products and allows multiple target gene determination by one-run experiment. This technology is highly recommended for clinical sample analysis and instrumental development.

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