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Rapid screening of phenylketonuria using a CD microfluidic device

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ABSTRACT

We herein present a compact disc (CD) microfluidic chip based hybridization assay for phenylketonuria (PKU) screening. This CD chip is composed of a polydimethylsiloxane (PDMS) top layer containing 12 DNA hybridization microchannels, and a glass bottom layer with hydrogel pad conjugated DNA oligonucleotides. Reciprocating flow was generated on the CD chip through a simple rotation-pause operation to facilitate rapid DNA hybridization. When rotated the CD chip, the sample solution was driven into the hybridization channel by centrifugal force. When stopped the CD chip, the sample plug was pulled backward through the channel by capillary force. The hybridization assay was firstly validated with control samples and was then used to analyze 30 clinical samples from pregnant women with suspected PKU fetus. The on-chip DNA hybridization was completed in 15 min with a sample consumption as low as 1.5 μL, and the limit-of-detection (LOD) of DNA template was 0.7 ng/μL. Among the 30 samples tested, V245V mutation was identified in 4 cases while R243Q mutation was detected in one case. Results of the hybridization assay were confirmed by DNA sequencing. This CD-chip based hybridization assay features short analysis time, simple operation and low cost, thus has the potential to serve as the tool for PKU screening.

1. Introduction

Phenylketonuria (PKU), an inherent metabolic disorder caused by a deficiency in a specific enzyme (phenylalanine hydroxylase) which prevents the body from utilizing phenylalanine, almost always leads to irreversible damage to the central nervous system and causes mental retardation [1]. To date, almost 500 different disease-causing mutations in the PAH gene have been identified and reported to the PAH mutation consortium database (http://www.pahdb.mcgill.ca). The occurrence of PKU varies among ethnic groups and geographic regions worldwide. In China, one case of PKU occurs in every 10,000–15,000 newborns. Since women with poorly controlled PKU during a pregnancy put their baby at risk for delayed development, mental retardation, poor head growth (microcephaly), poor overall growth, heart defects, and other structural birth defects, the prenatal PKU screening becomes a very necessary and crucial means. Conventional PKU screening strategies include polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) [2,3], denaturing gradient gel electrophoresis (DGGE) [4,5], allele-specific oligonucleotide assay (ASO) [6], PCR restriction fragment length polymorphism (PCR RFLP) [7] and DNA sequencing. However, these methods can hardly meet the demand of clinic PKU screening in terms of analysis time, cost, etc. Therefore, there has been increasing interest in development of rapid, low cost, and efficient methods for PKU screening.

In recent years, DNA microarray with the advantages such as high-throughput and robust nature has become one of the most popular analytical techniques for detections of genetic mutation [8]. Whereas, the workflows for microarray based assay are rather labor-and-time intensive, which include partially separated amplification, labeling, hybridization, washing and detection. The recently developed microfluidic technologies promise the possibility of integrating all the analytical steps related to a sample analysis on a microchip [9–11]. Reports on merging DNA microarray into microfluidics have shown a particularly useful way to apply such microfluidic matrixes, or "dynamic arrays" which let one perform all possible combinatorial assays on a set of reagents while realizing significant economies of scale in both pipetting, labor and reagent consumption [12–17]. Although these microfluidic-array hybridization devices are superior in performance, to operate them still requires sophisticated fluid control schemes, such as pumps and valves. Besides, sample consump-

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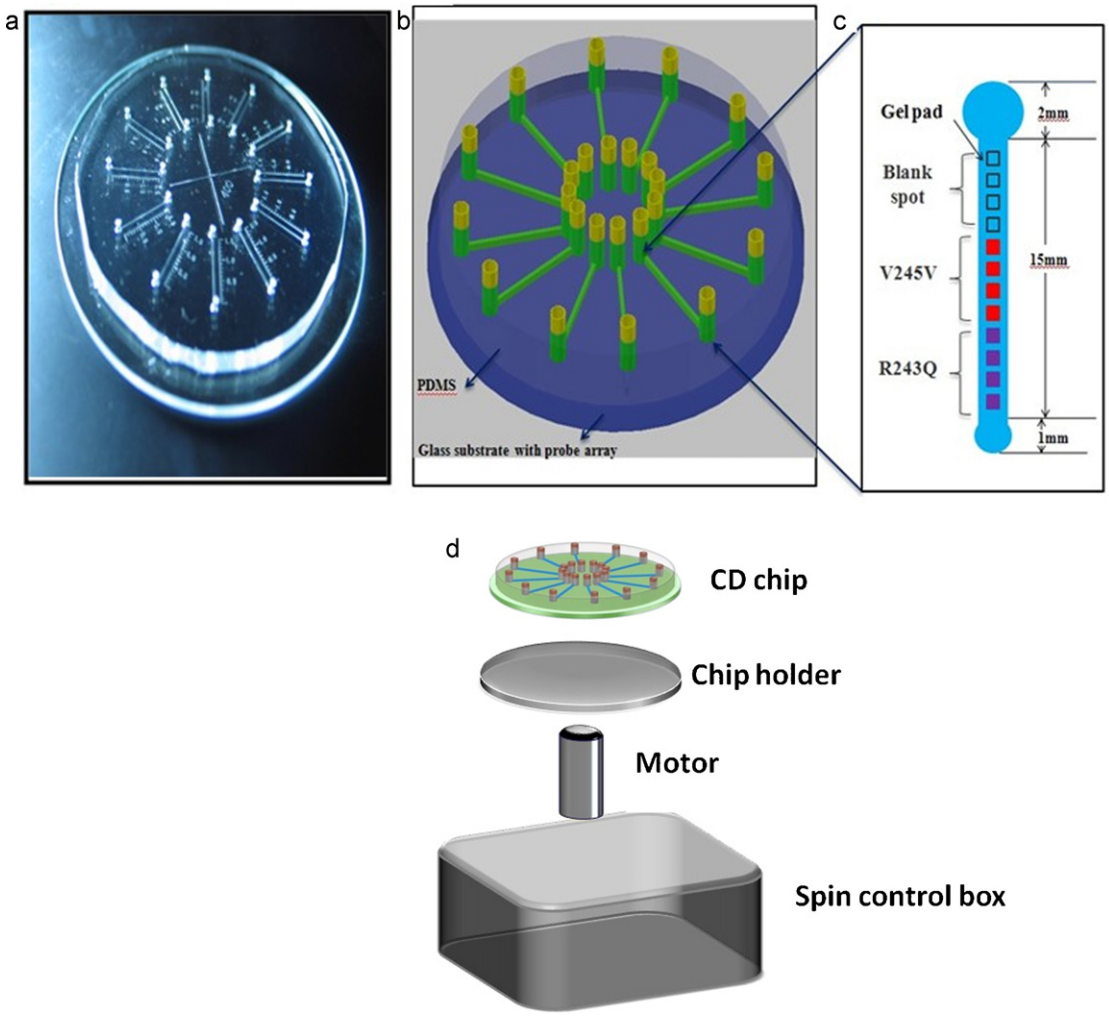

Fig. 1. Schematic representation of the CD microfluidics for DNA hybridization. (a) The photograph of the CD chip. (b) The two-layer structure of the CD microfluidic chip: a top PDMS layer containing 12 hybridization microchannels, and a bottom glass layer with immobilized DNA arrays. (c) The DNA arrays included R243Q, V245V and the negative control probes. (d) Exploded view of the spin system.

tions in these systems are usually considerable as a result of the large dead volumes. Recent works showed that the centrifugal microfluidics might become an alternative to these systems, since centrifugal force actuate the fluid without the requirement of any external pumps, cumbersome fluidic connectors, or sophisticated liquid handling instruments. Madou and coworkers reported a CD-based microfluidic-DNA microarray platform for infectious disease analysis, presenting an elegant solution to automate and speed up microarray hybridization [18]. Our previous studies also described automatic genetic analysis assays on a CD chip, in which reciprocating flow was generated in the micro-channel to perform a dynamic hybridization. It was demonstrated that the dynamic type hybridization increased hybridization efficacy and therefore improved hybridization signals comparing to the conventional flow-through type hybridization [19,20]. With advantages such as simple operation, short analysis time and parallel sample processing capacity, the reciprocating-flow based hybridization open up an effective solution apply to these microfluidic-DNA microarray platforms. In this work, an on-chip reciprocating-flow based hybridization method was developed for the analysis of clinic PKU samples. Our results showed that CD microfluidic based method provided more precise PKU information regarding clinically relevant symptoms with a new high-throughput screening format.

2. Experimental

2.1. Design and fabrication of CD microfluidic chip

The CD microfluidic chip consists of two layers: a top PDMS layer contains 12 DNA hybridization functional units and a bottom glass one with immobilized DNA probe array. The size of hybridization channel was 15 mm × 400 μm × 46 μm (length × width × depth) (Fig. 1). Dimensions of the hybridization channel were designed to result in a low flow resistance according to the principle of the capillary pump [21], while the lower height of the hybridization channel was designed to improve hybridization efficiency by reducing the diffusion distances.

The PDMS layer was fabricated using the standard soft lithography technology [22]. In brief, PDMS precursor and crosslinker (Sylgard 184, Dow Corning, USA) were mixed at a ratio of 10:1 by weight. The mixture was then poured onto a silicon wafer, degassed and cured at 80 °C for 2 h. The cured PDMS replica was gently peeled off from the wafer, and holes were then punched on the PDMS film as reservoirs. The PDMS slab was further covered by a stencil mask to leave the hybridization channels. After exposing to oxygen plasma for 2 min, the PDMS slab was ready for bonding to the glass plate.

Table 1
Oligonucleotide probes, primers and targets used in this study.

Acronym	Length	Sequence (5′–3′)
E7A	22-mer	CAAACCTCATTCTTGCAGCAGG
E7B	22-mer	ACTACCAAAGGTCTCCTAGTGC
E7C	22-mer	CAAGAAATCCCGAGAGGAAAGC
R243Q	27-mer	NH_2-AAAAAAAAAAACAGGTTGGAGGCGGAA
V245V	27-mer	NH_2-AAAAAAAAAAAGCTACAGGTCGGAGGC
T245	18-mer	FITC-GCCTCCGACCTGTAGCTT
T243	18-mer	FITC-TTCCGCCTCCAACCTGTT

The glass substrate with DNA probe arrays was prepared using a photo patterning method [23,24]. The glass plate (75 mm × 75 mm) was firstly treated with bind-silane (Sangon, Shanghai, China) and then aligned to a repel-silane (Sangon, Shanghai, China) treated glass cover of the same size. Between the two plates was a "U" shape interlayer that formed a space of 20 μm height. 100 μL of precursor solution (5% acrylamide–bisacylamide 19:1, w:w, 0.002% methylene blue, 1.2% N, N, N, N′-tetramethylethylenediamine and 40% glycerol in 0.1 mol/L sodium phosphate buffer, pH 7.5) was added to the space. The glass was then aligned with a mask and exposed to UV light. Therefore, polyacrylamide gel pads (100 μm × 100 μm × 20 μm) were formed through the polymerization reaction (Fig. 1c). After that, the cover was taken off and the glass plate with patterned gel pads was washed with deionized water for 5 min. The gel pads were further activated by treating with 12.5% glutaraldehyde (Sangon, Shanghai, China) in 0.1 M sodium phosphate buffer (pH 7.5) at 40 °C for 12 h. After washing with deionized water and drying by N_2 blowing, the glass plate was sealed with the oxygen plasma treated PDMS layer. By introducing NH_2-labeled oligonucleotides into the microchannels and incubating then there for 12 h at 37 °C and 100% humidity, DNA microarray was prepared on the gel pads. Subsequently, 0.1 M $NaBH_4$ was introduced into the microchannels and reserved for 20 min at 4 °C to reduce the residual aldehydes. Finally, the glass wafer was washed with deionized water and dried by N_2 blowing.

2.2. Subjects

Five unrelated Chinese Han patients from southern China with PKU were selected as the positive controls. Their phenotypes were classified based on the pretreatment plasma phenylalanine (phe) levels. Accordingly, the 5 patients were identified as classic PKU (phe > 1200 μmol/L). Blood samples from 30 suspected PKU infant carried pregnant women (with a reproductive history of PKU infant or a pre-treatment plasma phe level >600 μmol/L) with gestation times ranging from 7 to 16 weeks were analyzed. An explanation of the study was given to the participants, and a standard informed consent was reviewed and approved by the Guangzhou Ethical Committee of Human Genetic Resources.

2.3. Oligonucelotide synthesis and PAH gene amplification

R243Q and V245V mutation in exon 7 are the most common mutation sites of PAH gene in China (http://www.pahdb.mcgill.ca). Probe V245V and R243Q were designed to detect these two mutations. The proved oligonulceotide was modified with an amine group at 5′-end that spaced from the matching region. T243 and T245 were positive control oligonucleotides labeled with fluorescein at 5′-end, which were complementary to probe R243Q and V245V, respectively. The sequences of the primers (Table 1) were designed on the basis of known homo PAH sequences. Primer E7A and E7C were combined to yield a 134 bp fragment for hybridization, while primer E7B and E7C were used to amplify a 285 bp fragment for sequencing. The amplicons covered both R243Q and V245V regions.

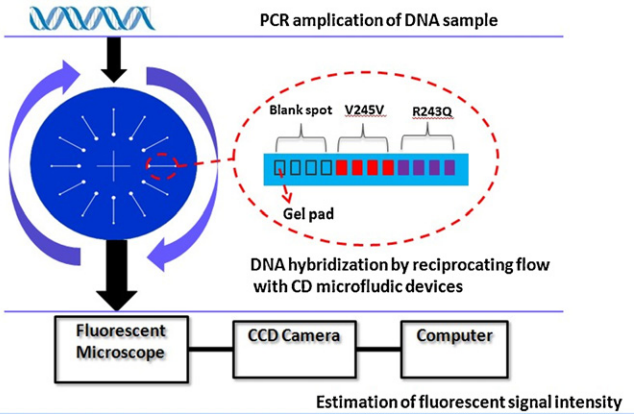

Fig. 2. Experimental flow of the CD microfluidics-based phenylketonuria (PKU) screening.

Genomic DNA was isolated from the peripheral blood with a genome DNA rapid extraction kit (Guangzhou DaHui Biotech Co, China, Ltd.). The extracted DNA (~500 ng) was added to a 25 μL PCR system, containing 2 U Taq-DNA polymerase, 2.5 μL 10 × PCR buffer, 200 μM PCR Fluorescein Labeling Mix (Roche, Ltd.), 1.5 mM $MgCl_2$ stock solution, 13.5 μL distilled water, and 600 nM total primer sets. During PCR amplification, FITC-labeled dUTP were incorporated into the amplification products. Thermal cycling conditions were as follows: 10 min at 95 °C for denaturation, followed by 35 cycles of 95 °C, 30 s for denaturation, 60 °C, 60 s for annealing and 72 °C, 60 s for extension.

2.4. Hybridization and fluorescence detection on the CD microfludic chip

After the PCR amplification, 5 μL reaction product was mixed with 5 μL hybridization buffer (5 × SSC + 0.2% SDS + 46% formamide) and was then heated at 95 °C for 10 min. Afterwards, sample solutions (1.5 μL) were added to the inlet reservoirs. The DNA hybridization was carried out at room temperature. In the hybridization, the reciprocating-flow was generated through the repetitive rotation and pause of the CD chip: the CD chip was firstly rotated and then stopped by a spin control box (Fig. 1d). After hybridization, the CD device was spun for another 10 s at 1500 rpm to drain out the sample solution, and finally 1.5 μL washing buffer (1 × SSC) was loaded into the hybridization channel and spun for 5 min to remove the non-specifically bound probes.

The fluorescence images of the hybridization region were obtained on a fluorescence microscope (Olympus, IX71) equipped with a CCD camera (Fig. 2). The average fluorescence of each hybridization region was corrected for device fluorescence as well as illumination fluctuations and nonuniformities by subtracting the average local background fluorescence [25].

2.5. DNA sequencing

The amplicons of PAH gene from 30 pregnant women were sequenced on an ABI Prism 3100 sequencer using the BigDye Terminator Cycle Sequencing Genetic analysis Kit, version 3.1 (Applied Biosystems). The sequencing data were aligned to the known homo PAH sequences to determine whether the amplicons contain mutation(s).

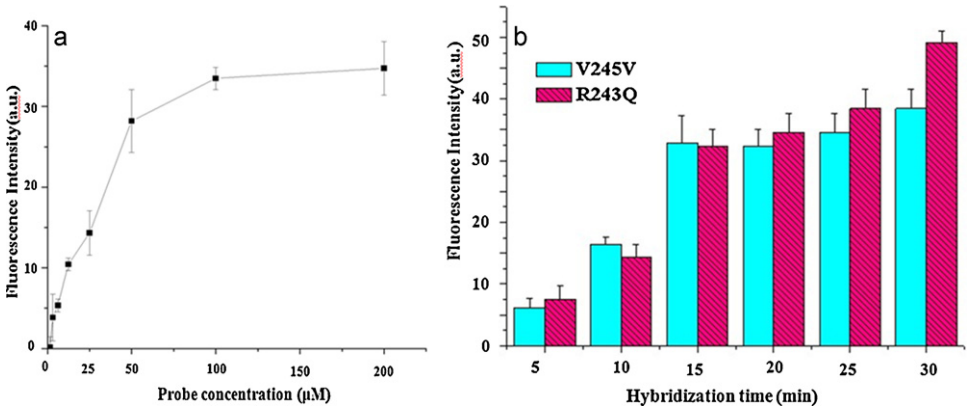

Fig. 3. Hybridization conditions of the CD microfluidic device. (a) Hybridization signal growth at various concentrations of probe solution. The PCR products from the PKU patients with V245V mutation were hybridized to V245V probe for 15 min. (b) Hybridization signal growth at various hybridization times. Two PCR products from the PKU patients with V245V or R243Q mutation were hybridized to V245V and R243Q probes for different times (5 min, 10 min, 15 min, 20 min, 25 min, and 30 min). The fluorescence intensities were calculated by the average of 4 probe spots. Error bar represented standard deviation.

3. Results and discussion

3.1. Reciprocating flow-based hybridization on the CD microfluidic chip

The on-chip reciprocating flow-based hybridization uses the principle of enhanced hybridization efficiency resulted from the increasing of contact between the sample molecules and the immobilized probes, since the movement of DNA molecules is enhanced as the effect of intensified convection.

The reciprocating flow was generated on the CD chip through the combination of centrifugal force and capillary action. The centrifugal force (F_c) acting on the fluid in the microchannel with cross-sectional area (S), and length (Δr) can be described as:

$$F_c = \rho S \, \Delta r \omega^2 \bar{r} \tag{1}$$

where ρ is the density of the liquid, ω is the angular velocity, $\bar{r}$ is the average distance of the liquid in the channels from the center of the disk [26]. The flow resistance (R) of the liquid on channel can be given as:

$$R = \frac{12\mu \ \Delta r}{wh^3} \tag{2}$$

where μ is the fluid viscosity, w is the width of the microchannel, and h is the depth of the microchannel. The volumetric flow rate (Q) of the liquid driven by the centrifugal force can be calculated as:

$$Q = \frac{F_c}{SR} = \frac{\rho\omega^2 \bar{r} w h^3}{12\mu} \tag{3}$$

In previous studies using the centrifugal microfluidics, the hydrophobic valves were usually used for the controlled release of sample/reagents. However, the hydrophobic valve was not available in the hybridization assay. The inactivation of hydrophobic valves was ascribed to the decrease of surface tension that caused by the presentation of SDS surfactant in the hybridization buffer. Therefore, the hydrophobic valve was not employed in our CD chip.

3.2. Optimizing experimental conditions of the on-chip hybridization assay

Experimental conditions including rotational frequency, probe concentration and hybridization time were optimized.

According to the formula (3), flow rate in the reciprocating flow-based hybridization is proportional to the square of angular velocity. Therefore, higher rotational frequency increases flow rate but shortens the binding time. In our previous work, it was found that the hybridization signals were improved by increasing the rotational frequency to a certain extent. However, rotational frequency higher than 1200 rpm became a disadvantage factor for hybridization efficiency, as the high rotational frequency resulted in an increased flow rate and consequently decreased binding time. Besides, liquid could be spun out of the microchannel at too high rotational frequency. The hybridization signals obtained with various rotational frequencies (600 rpm, 900 rpm, 1000 rpm, 1200 rpm, 1300 rpm and 1500 rpm) were compared, and 1200 rpm was defined as the optimal frequency. When rotated the CD device at 1200 rpm, the flow rate Q was calculated to be 3.879 μL/s and therefore the centrifugation time was estimated to be 3 s. By monitoring the movement of the sample plugs vs. time on a CCD camera, the liquids were found to be transported from the edge to the center of the CD chip in 4 s as the effect of capillary action during rotation intervals. In total, a back and forth movement of the sample fluids was completed within 7 s.

The on-chip hybridization process was then validated using the T235 and T245 positive controls. The reciprocating flow-based hybridization assay was proved by fluorescence detection. The effect of probe concentration on hybridization efficiency was investigated with the fluorescence labeled samples. Different concentrations (1.5, 3.1, 6.125, 12.5, 25, 50, 100, and 200 μM) of V245V probe were immobilized onto the gel pads and then hybridized with a control positive V245V mutation sample for 15 min. As shown in Fig. 3a, the intensity of hybridization signal increased with probe concentrations ranging from 1.5 μmol/L to 100 μmol/L. Further increased probe concentration to 200 μmol/L did not generate significantly higher hybridization signal intensity. Therefore, the probe concentration of 100 μmol/L was used in the subsequent work.

Furthermore, the hybridization signal with respect to hybridization times (5–30 min) was investigated. It was observed that longer hybridization time in the reciprocating-flow hybridization assay facilitated higher hybridization signal intensity (as shown in Fig. 3b). To balance analysis speed and detection sensitivity, 15 min hybridization time was used in this experiment for rapid hybridization of clinical DNA samples as the signal intensity at the time point approached to the maximum value. It was also found that hybridization time more than 30 min was not applicable, since the volume of liquid obviously reduced as the effect of evaporation loss.

3.3. Performance of the on-chip hybridization assay

The limit-of-detection (LOD) of the on-chip hybridization assay was determined by analyzing PCR products under the optimized

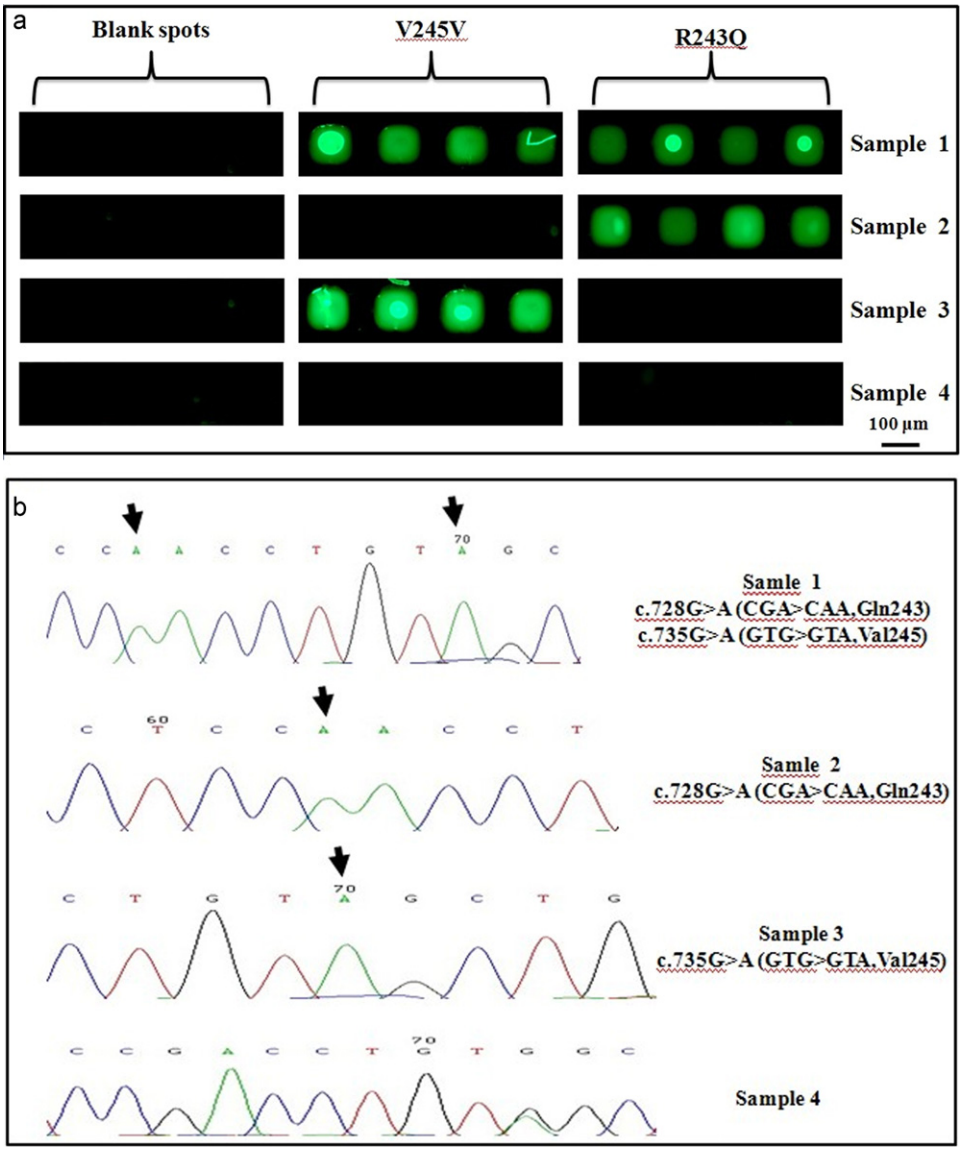

Fig. 4. Investigation of hybridization specificity with control samples. (a) Hybridization signals of the 4 clinical samples. Sample 1–3 were DNA templates from PKU patients. Sample 1 was defined as V245V (+)/R243Q (+), sample 2 was defined as R243Q (+) and sample 3 was R243Q (+). Sample 4 was a negative control with no fluorescent signal detected. (b) Sanger sequencing data of the above 4 samples.

experimental conditions. A series of PCR products were prepared by amplifying different concentrations of DNA templates (0.07 ng/μL, 0.35 ng/μL, 0.7 ng/μL, 3.5 ng/μL and 7 ng/μL). The equivalent of as little as 0.35 ng/μL of the starting material was not sufficient to produce an unambiguous hybridization signal using the criteria of S/N >3, therefore the LOD of DNA templates was determined to be 0.7 ng/μL. This detection limit was comparable to those obtained with microfluidic devices that were more complex to manufacture and required much longer hybridization times [16]. In comparison with the results obtained on conventional microarray hybridization platforms, the analysis time of the CD chip assay was obviously shortened without the loss of detection sensitivity [27].

In conventional DNA microarray analysis, a lengthy post-hybridization washing step is usually required to reduce non-specific binding [28]. In contrast, the reciprocating flow-based hybridization employs only a 5 min washing step. So we examined whether the current method ensured satisfied hybridization specificity. The hybridization specificity was investigated with 4 control samples, 3 positive controls and a negative one. For all the 4 cases, no fluorescence was detected on the blank spots. Among the 3 PKU samples tested, one case was defined as R243Q (+)/V245V (+), while the other two cases were identified as R243Q (+) and V245V (+), respectively. No fluorescence was detected in the parallel negative control assay, because the DNA sample did not contain the mutation. Results of the on-chip hybridization assay obtained from 3 PKU samples as well as the negative control were compared to the corresponding DNA sequencing data (Fig. 4). These results completely accorded with the DNA sequencing data, demonstrating the high specificity of the hybridization assay.

3.4. PKU screening using the CD microfluidic device

Blood samples from 30 suspected PKU infant carried pregnant women at high risk for birth defects were analyzed using the optimized hybridization protocol. Our device allowed the analysis of 12 samples one time, with each sample being processed in an individual channel. In each run, the whole hybridization assay was completed in 20 min. Therefore, the whole analysis took ~60 min. Among the 30 samples tested, V245V mutation was identified in 4 cases while R243Q mutation was detected in one case. When com-

pared to the corresponding DNA sequencing data, the 4 V245V (+) cases were confirmed to have the mutation, while the R243Q (+) one was found to be false-positive. The false-positive result could be explained by the fact that our probe design is inappropriate for the case, as the hybridization-based method cannot identify all the cases [29].

In comparison with conventional PKU screening methods, such as PCR-RFLP, PCR-SSCP and DGGE, the CD microfluidics based method is superior in terms of analysis time and operation convenience. Although DNA sequencing is currently the gold standard in PKU screening, the method has many disadvantages such as labor intensive operations, long analysis time (6–12 h), and high cost. In comparison, the CD chip based assay features simplified operation, fast analysis and low cost. The application of the centrifugal microfluidics dramatically reduced the time and costs required for a PKU diagnosis, and at the meantime improved specificity and accuracy of the detection. In addition, due to its rapid prototyping and disposability, the microfluidic chip may be very useful in large-scale clinical screening of PKU.

4. Conclusions

We developed a PKU screening method based on a CD microfluidic device. Using the principle of reciprocating flow, a simple DNA hybridization was performed through the rotation/pause operation of the CD chip. Under optimized experimental conditions, the on-chip hybridization process was completed in 15 min and achieved a LOD as low as 0.7 ng/μL. In view of the advantages of short analysis time, simple operation, high sensitivity and low cost, the microfluidics based method offers an alternative tool for clinical prenatal screening of PKU.

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