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Novel high-speed droplet-allele specific-polymerase chain reaction: Application in the rapid genotyping of single nucleotide polymorphisms

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ABSTRACT

Background: Single nucleotide alterations such as single nucleotide polymorphisms (SNP) and single nucleotide mutations are associated with responses to drugs and predisposition to several diseases, and they contribute to the pathogenesis of malignancies. We developed a rapid genotyping assay based on the allele-specific polymerase chain reaction (AS-PCR) with our droplet-PCR machine (droplet-AS-PCR).
Methods: Using 8 SNP loci, we evaluated the specificity and sensitivity of droplet-AS-PCR. Buccal cells were pretreated with proteinase K and subjected directly to the droplet-AS-PCR without DNA extraction. The genotypes determined using the droplet-AS-PCR were then compared with those obtained by direct sequencing.
Results: Specific PCR amplifications for the 8 SNP loci were detected, and the detection limit of the droplet-AS-PCR was found to be 0.1–5.0% by dilution experiments. Droplet-AS-PCR provided specific amplification when using buccal cells, and all the genotypes determined within 9 min were consistent with those obtained by direct sequencing.
Conclusions: Our novel droplet-AS-PCR assay enabled high-speed amplification retaining specificity and sensitivity and provided ultra-rapid genotyping. Crude samples such as buccal cells were available for the droplet-AS-PCR assay, resulting in the reduction of the total analysis time. Droplet-AS-PCR may therefore be useful for genotyping or the detection of single nucleotide alterations.

1. Introduction

Single nucleotide alternations such as single nucleotide polymorphisms (SNPs) or single nucleotide mutations are useful genetic markers for molecular diagnosis, prognosis, drug response, and predisposition to diseases. SNPs in *CYP2C9* and *VKORC1* greatly influence the effective warfarin maintenance dose [1,2]. Warfarin therapy is typically commenced soon after the diagnosis of a disease is made. Furthermore, *EGFR* or *KRAS* mutations in non-small cell lung cancer are critical to predict the response to tyrosine kinase inhibitors therapy [3–6]. Especially in terms of drug response and selection of molecular-targeted therapy, rapid identification of SNPs or mutations is clinically significant.

Several methods have been developed to detect single nucleotide alternations. Direct sequencing is generally used to determine SNP or mutation genotype, but analysis processes are labor-intensive and time-consuming. High resolution melting (HRM) analysis allows researchers to carry out scanning of known and unknown single nucleotide alternations and multiplex analysis [7]. However, accurate allele discrimination depends on strict temperature control. Allele-specific PCR (AS-PCR) analysis is only capable of detecting known SNPs or mutations, but has high specificity and sensitivity [8]. Unlike direct sequencing and HRM analysis, AS-PCR is applicable to quantitative assessment in addition to qualitative assessment [9–11].

We previously developed a novel droplet-PCR machine, which has better thermal conductivity than the conventional PCR machine and enables rapid PCR amplification [12,13]. We subsequently applied the novel high-speed droplet-PCR machine technology for the detection of influenza virus and *PML–RARA* fusion gene.

In the present study, we developed droplet-allele specific polymerase chain reaction (droplet-AS-PCR) for a rapid SNP genotyping with our droplet-PCR machine. Furthermore, to reduce the time of sample preparation before PCR, we used crude buccal cells without DNA extraction and examined the reactivity of the droplet-AS-PCR.

2. Materials and methods

2.1. Novel real-time droplet-PCR machine

The novel droplet-PCR machine (Seiko Epson, Nagano, Japan) has 2 connected heater blocks, which regulate the temperature of each

Abbreviations: SNP, single nucleotide polymorphism; AS-PCR, allele specific-polymerase chain reaction; droplet-AS-PCR, droplet-allele specific-polymerase chain reaction; HRM analysis, high resolution melting analysis; PB, peripheral blood.

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end of the reaction tube (Fig. 1). The reaction temperature of the 2 heater blocks are controlled consistently during denaturation and annealing/extension. To perform a rapid temperature transition, the reaction tube is filled with silicone oil, allowing the droplet of PCR mixture to move easily in the tube during the mechanical rotation of the machine with the 2 connected heater blocks. Once the droplet transfers from one end to another owing to gravitation, the machine with the 2 heater blocks inverts to return the droplet. Therefore, 1 μL of the PCR mixture in the droplet state is able to perform shuttle PCR in the reaction tube. In addition, the novel droplet-PCR machine integrates a fluorescence detector that allows monitoring the amount of PCR products after each extension step [12].

2.2. DNA extraction

Genomic DNA was extracted from peripheral blood (PB) obtained from 7 healthy volunteers using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA) according to manufacturer's instructions.

This study was approved by the institutional review board of the Shinshu University.

2.3. Selection of SNPs and design of primers and probes for droplet-AS-PCR assay

We selected 8 SNPs located on different regions of the genome [14] (Table 1). For each SNP allele, we designed allele-specific primers that included a SNP-matched nucleotide in the 3′-end and a template-mismatched nucleotide at the −2 position from the 3′-end, and TaqMan probe including fluorescein amidite at the 5′-end nucleotide and quencher (Black Hole Quenchers) at the 3′-end nucleotide. We previously reported the mutation-allele specific PCR using primers including mismatch nucleotides, and analyzed the specificity and sensitivity of the assays using the modified primers in detail [9–11]. These pilot studies indicated that the primers that only included SNP/mutation-matched nucleotide at the 3′ end provided the non-specific amplification, but primers that included mismatch nucleotide in −2 position or penultimate base, provided the specific amplification retaining high sensitivity. According to the pilot studies, we used primers that included mismatched nucleotide introduced at the −2 position in this study. The sequences of primer and probe sets are listed in Table 1.

2.4. Direct sequencing

The genotypes at 8 SNP loci were confirmed using genomic DNAs obtained from PB by direct sequencing from both directions on an automatic DNA sequencer (ABI3100 Genetic Analyzer, Applied Biosystems, Foster City, CA). The genomic DNAs obtained from PB were used for evaluating the reactivity and sensitivity of the droplet-AS-PCR assay using the SNP genotype-specific primers and probes.

2.5. Droplet-AS-PCR

The droplet-AS-PCR reaction mixture contained genomic DNA (50 ng/μL), Platinum Taq DNA polymerase (Life Technologies, Grand Island, NY), 800 nmol/L of primer designed as above, 300 nmol/L of TaqMan probe, and reaction buffer composed of Tris–HCl pH 9.0, KCl and $MgCl_2$, in a total of 10 μL. One microliter of the reaction mixtures was used for the droplet-AS-PCR assay [12,13]. The reaction conditions used in the present study were as follows: 95 °C for 10 s and 35 cycles at 95 °C for 4 s and 58–66 °C for 8 s. The droplet-AS-PCR assays for the 8 SNP loci were performed in triplicate measurements using three aliquots prepared from a single PB preparation. In this study, using the positive samples, the SNP types of which were pre-determined by direct

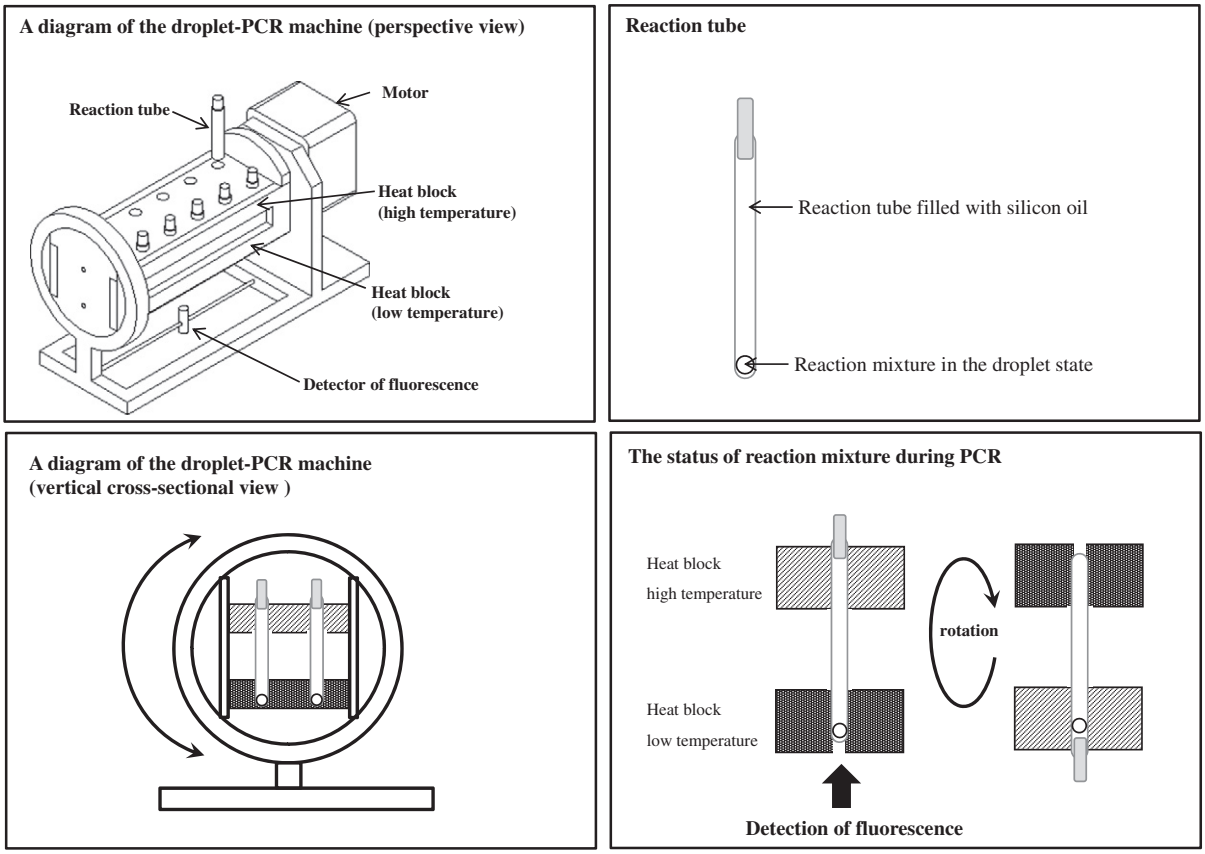

Fig. 1. A diagram of the droplet-PCR machine. The diagram of the droplet-PCR machine was cited from our previous report [12].