Note

Development of an Inexpensive and Rapid Operation Device for High-Throughput Real-Time Quantitative PCR-Based *CYP2D6* CNV Genotyping

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The *CYP2D6* gene is the most well characterized gene involved in drug metabolism and is known to have both gene duplication and deletion variants. We report an optimized method for the determination of copy number variation (CNV) in the *CYP2D6* gene by a novel purification process for a real-time quantitative PCR. This high-throughput low-cost method accurately determines CNV in the *CYP2D6* gene enabling reliable estimates of disease prediction in large epidemiological samples.

Key words copy number variation (CNV); *CYP2D6*; pharmacogenomics (PGx); real-time quantitative PCR; water-soluble paper; saliva

INTRODUCTION

The developments of genotyping methods to analyze DNA variations have contributed enormously to medicine, especially for understanding genes responsible or susceptible to various diseases. Genetic variations can be classified into five major classes: variable number of tandem repeat (VNTR), short tandem repeat or microsatellite (STR), insertion/deletion (I/D) polymorphism, single nucleotide polymorphism (SNP), and copy number variation (CNV). CNV is known to be the primary source of variation in the human genome, since it involves deletion or multiplication of DNA segments, and is the important factor for understanding susceptibility against diseases. CNV is similar to a type of genomic structural variation, which refers to DNA segments with sizes ranging from one-kilo base to several mega bases in which deletion, insertion, inversion, duplication, and complex recombination occur.[1–4] Knowing the combination of variants within a given haplotype, and the diploid content in an individual, plays a key and important role when studying drug metabolism, response, and adverse reactions.

Among CYP enzymes, CYP1, 2, and 3 families are primary drug metabolizing enzymes and responsible for the metabolism of more than 70% of the drugs in clinical use.[5–7] Most of them are highly polymorphic, allowing clinically significant modifications of drug metabolizing capacities. Understanding the combinations of genetic variations is crucial for studying drug metabolism, response, and adverse reactions. Metabolism by the *CYP2D6* is known to be one of the major genetic polymorphisms influencing drug-metabolizing activity. Various drugs playing an important role in current medicine, such as antidepressants, anticancer drugs, beta-blockers, and antipsychotics, are metabolized by CYP2D6.[8,9]

The CNV of the *CYP2D6* gene is the determinant to cause decreased or increased drug metabolism. Genetically, *CYP2D6* is highly polymorphic, and the variants are SNPs, as well as CNV resulting from *CYP2D6* gene deletion or multiplication. Understanding the characteristics of the *CYP2D6* gene locus, genotypes and phenotypes, has an enormous potential in pharmacogenomics (PGx).[10] However, the current CNV genotyping method requires DNA purification for the use of real-time quantitative PCR, which can put constraints on research, since it is time-consuming and expensive. Further improvement or simplification of the methods currently available will accelerate and broaden research to be conducted using CNV genotyping. Namely, genotyping methodologies that are fast, accurate, cost-effective, and that also allow broad marker coverage are the key factors to support and stimulate the growing number of PGx studies being conducted.

The objective of this study is to introduce a new PGx method that allows quick, accurate, and inexpensive testing. We introduce a more comprehensive and robust workflow for high-throughput PGx genotyping experiments, from sample preparation to data analysis, using a real-time quantitative PCR system, which is better than previous methods that require extraction and purification procedures. The new method described here is the first report to use unprocessed biological specimens directly as templates for TaqMan® CNV assay. We believe our new technology will reduce the difficulties to conduct large-scale association studies in various fields.

Samples of either whole blood or saliva are collected for epidemiological studies involving the analysis of DNA using high-throughput genotyping methods. Generally, blood collection is not always possible or feasible, particularly in case-control studies when requesting blood can potentially reduce control subjects to participate. A smaller sample size can also threaten the validity of statistical robustness. Inevitably, extracting oral mucosal cells from the saliva using a swab is less invasive than blood sampling. Therefore, dried saliva samples are often preferred as a source of genomic analysis, since the sampling process is usually painless and non-invasive.[11] Moreover, its simplicity to sample will extend the capacity by

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allowing self-sampling and sending the specimen to the laboratory for analysis.

MATERIALS AND METHODS

Materials An oral care sponge swab was purchased from Osaki Medical Co. (Nagoya, Japan). Biopsy punch (BPP-40F) was purchased from Kai Industries Co., Ltd. (Tokyo, Japan). TaqMan® Genotyping PCR Master Mix and TaqMan® Copy Number Assay (assay id: Hs00010001_cn [Ex9]) with an internal control–ribonuclease (RNase) TaqMan® Copy Number Reference Assay (assay id: 4403326) were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, U.S.A.).

Homemade Sampling Kit Water-soluble paper (60MDP) was purchased from Nippon Paper Papylia Co., Ltd. (Tokyo, Japan), and homemade sampling kits were prepared from SUN-OAK Co., Ltd. (Tokyo, Japan). Considering the difficulties in collecting blood samples, we developed a new sampling method to collect dried saliva samples using water-soluble paper.[11–13] This sampling kit allows efficient sample collection for DNA amplification and stable preservation for long term at room temperature. It also simplified the sampling process. Thus, an individual can easily learn how to self-sample using this homemade sampling kit with instruction (Fig. 1).

Subjects *CYP2D6* genotyping was based on a real-time quantitative PCR for the TaqMan® Copy Number Assays Protocol (Thermo Fisher Scientific). A total of 22 healthy female subjects participated as volunteers in this study. The Medical Ethics Committee of Mukogawa Women's University approved the study protocol (No. 13–30). Informed consent was obtained from all the subjects.

Sample Preparation For the validation of this method, we further quantified the performance of high-throughput real-time quantitative PCR-based CNV genotyping directly from oral mucosal cells, excluding the process of a general DNA purification. The results of this study will not only be informative for genotyping, but also will contribute to transition from low-throughput to high-throughput PCR-based experiments in other genotyping fields. With quantification of robustness of our new method, we introduce a new device, which has sufficiently overcome the shortcomings of previous methods, by employing direct collection of dried saliva

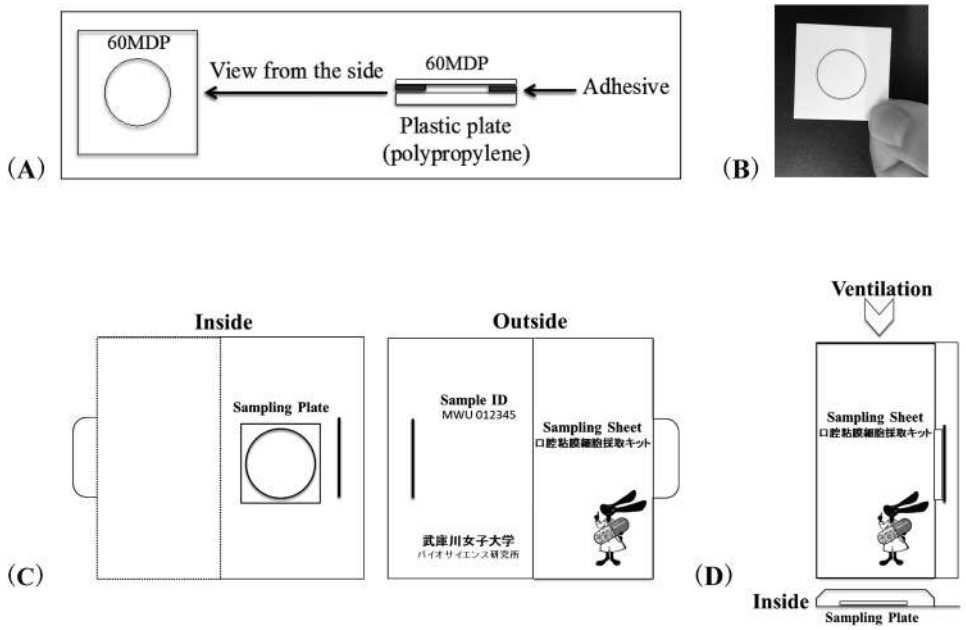

Fig. 1. Development of Homemade Sampling Kit Design

(A) A homemade sampling kit, in which water-soluble paper (60MDP) was fixed on a plastic plate (polypropylene: PP, 3 × 3 cm) with an adhesive except for the diameter of about 2 cm in the center part. (B) The picture of the sampling plate. (C) The homemade sampling kit design. (D) After sampling, the kit was folded in half to allow spontaneous drying of the saliva on the water-soluble paper. Therefore, the folded kit has a hollow structure.

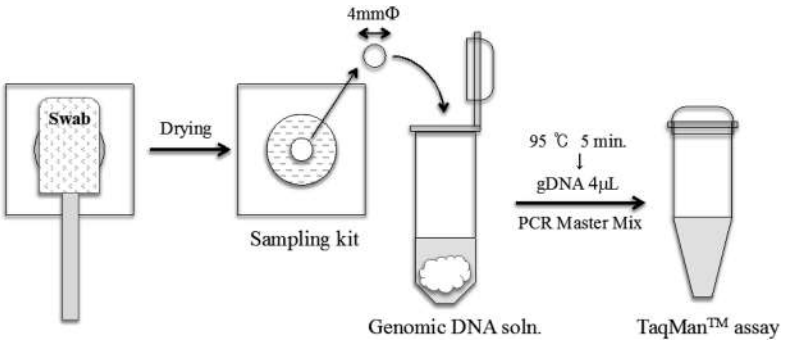

Fig. 2. Sampling Process and Genetic Test Flow

samples. The dried saliva samples were prepared as follows: (1) the subjects were asked to rub their inner cheek surfaces with a swab to collect oral mucosal cells for genotyping, then (2) the oral mucosal cells included in saliva specimens were applied to the water-soluble paper, and (3) dried thoroughly for around 1 h at room temperature. A small piece of a dried saliva sample for genotyping was used by cutting off and punching with a 4 mm diameter Biopsy Punch (BPP-40F), and was directly suspended in 100 μL of distilled water, and heated at 95°C for 5 min. This supernatant of genome DNA template was added directly to a PCR tube and real-time PCR based on TaqMan® assay was carried out excepting the usual extraction and purification procedure for genome DNA from the saliva (Fig. 2).

Genotyping of *CYP2D6* Gene Copy Number Variation To assess the *CYP2D6* gene copy number, we used a commercial quantitative TaqMan® Copy Number Assay. (assay id: Hs00010001_cn [Ex9]) with an internal control-RNase TaqMan® Copy Number Reference Assay (assay id: 4403326). All assays were performed in triplicate in a QuantStudio™ 12K Flex Real-Time PCR System. The assays were performed with TaqMan® Genotyping PCR Master Mix using 4 μL of genomic DNA in a 10 μL reaction. The cycling conditions were: 95°C for 10 min for initial denaturation and enzyme activation, followed by 40 cycles each of 95°C for 15 s and 60°C for 1 min. Relative quantification was performed using CopyCaller Software, following the comparative $\Delta\Delta Ct$ method (Fig. 3).

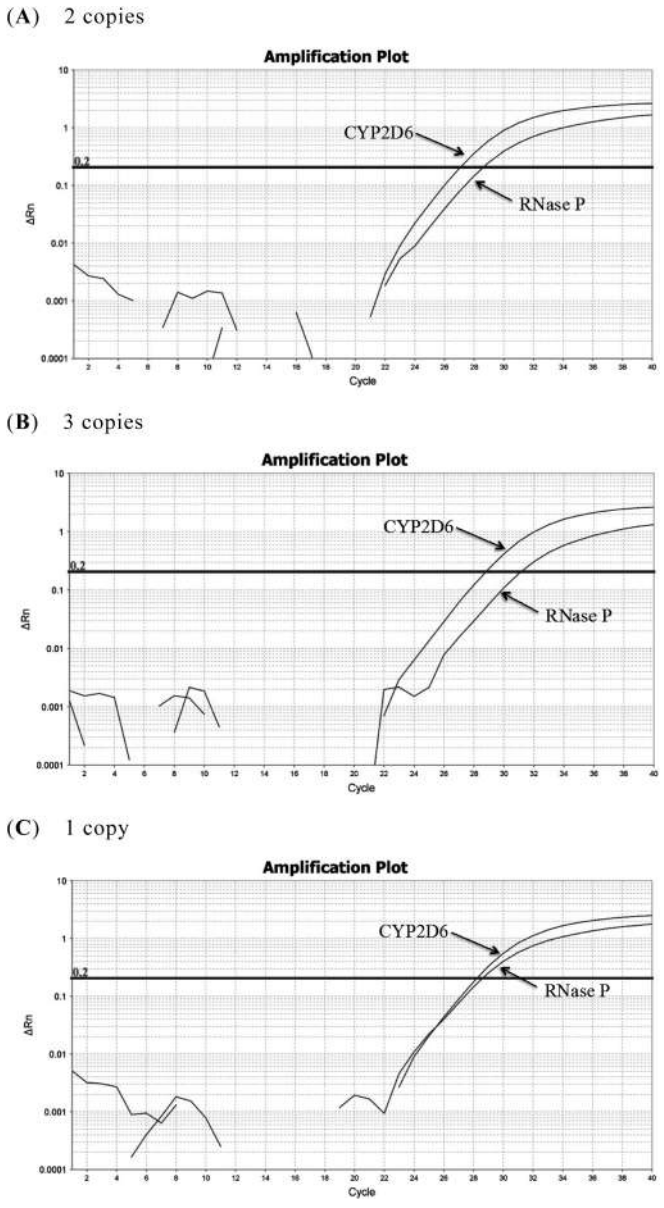

Fig. 3. Amplification Plots of *CYP2D6* and *RNase P* Genes

The ΔRn (reflecting the quantity of fluorescent probe degraded) is plotted *versus* cycle number. (A) Samples with two copies of *CYP2D6*. In (B) samples with duplication, the Ct value of *CYP2D6* decreased compared to *RNase P* whereas in (C) samples with deletion, the Ct value of *CYP2D6* increased compared to *RNase P*.

RESULTS AND DISCUSSION

To confirm the *CYP2D6*5* allele (whole gene deletion), we performed long-range PCR, which has been established previously by Ota *et al.*[14] (data not shown). The copy number analysis for the 22 subjects were tested by a TaqMan® Copy Number Assay for CYP2D6. All samples performed very well with all assays. The data is reported as the estimated number of *CYP2D6* copies, with the error bars representing the estimated copy numbers range. As a quality control measure, we repeated all three assays within 10% of the samples. All repeated assays yielded concordant results (Fig. 4). This result showed that the copy number variation noted with this sample set was diverse, showing CNV of at least one gene. This genomic DNA had sufficient purity and concentration for usage in TaqMan® Copy Number Assays. The average *Ct* value of *RNase P* of all dried saliva samples were observed at 29.9 cycle ±1.2 (Fig. 5). The genomic DNA copy number in the reaction was calculated from 10^4 to 10^5 copies by a calibration curve (data not shown).

It should be noted, this method of using a new device leads to a low-cost and a reduction of operation time compared to methods that are popular. Actually, it takes an average of 2.5 h and approximately 100 U.S. dollars to measure the CNV of the 22 saliva samples. However, experience shows that it took an average of 15 h and 500 U.S. dollars to measure the same 22 samples by conventional methods using a real-time quantitative PCR, because a saliva sample needs the high-quality DNA solution to be adjusted to similar concentrations of DNA in PCR for CNV analysis.

We have developed an optimized CNV genotyping protocol using the water-soluble paper for a real-time quantitative PCR system excluding a general genomic DNA purification process. The results of this study have demonstrated that using saliva (oral mucosal cells) directly to perform real-time quantitative PCR-based genotyping is possible.

The sample-to-CNV procedure with CNV genotyping on a real-time quantitative PCR-based high-throughput platform yielded an accurate process applicable to large-scale genotyping operations. Moreover, even after six months, our method gave us exactly the same copy number variation results, and samples of oral mucosal cells were only stored at room temperature.

This study has shown that we have developed a novel DNA preparation procedure for accurate real-time quantitative PCR-based genotyping, and the direct use of dried saliva samples can therefore be used to further expedite the entire genotyping process, especially when combined with a high-throughput liquid handling platform. We believe that using our proposed

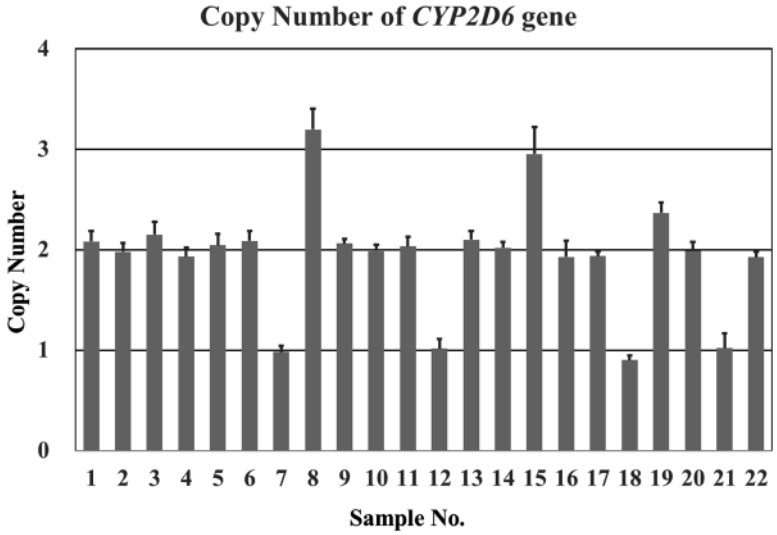

Fig. 4. Comparison of Estimated *CYP2D6* Gene Copy Numbers

Each sample was assayed in triplicate for the *CYP2D6* exon 9. The data presented are the mean *CYP2D6* copy numbers with the error bars representing the range of copy numbers estimated for each assay.

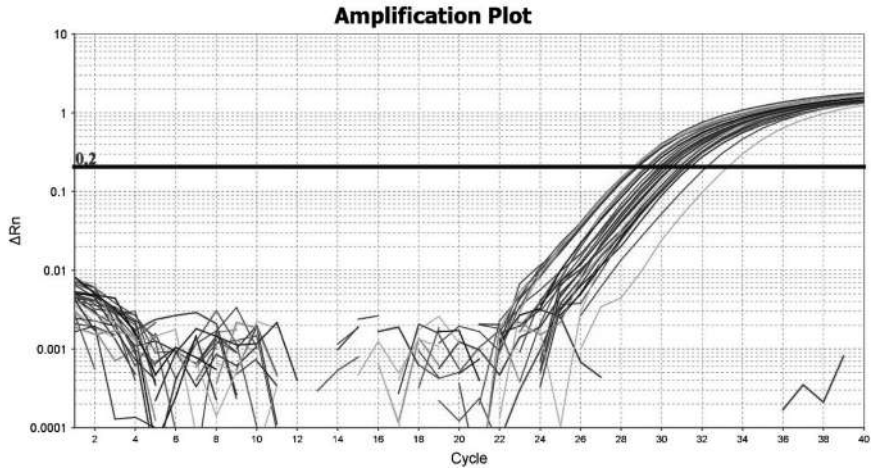

Fig. 5. Amplification Curves of *RNase P*

The ΔRn (reflecting the quantity of fluorescent probe degraded) is plotted *versus* cycle number.

method simplifies current genotyping methods, allowing further usage in diagnostic settings where a large number of samples are analyzed daily using real-time quantitative PCR by TaqMan® copy number assay. For further improvement, usage of samples directly for high-throughput genotyping with the exclusion of a general DNA purification will continue to be investigated by our lab. Nevertheless, our results demonstrate this technique is highly feasible as a low-cost alternative of CNV genotyping. We hope our new methodology will be applied to perform routine genotyping in a truly high-throughput system.

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Conflict of Interest The authors declare no conflict of interest.

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