Regular Article

Rapid and Cost-Effective Genotyping Protocol for Angiotensin-Converting Enzyme Insertion/Deletion (Ins/Del) Polymorphism from Saliva

Madoka Kisoi,[a] Miwako Moritsugu,[a] Miho Imai,[a] Kae Fukumoto,[a] Yui Sakaguchi,[a] Shigenori Murata,[a,b] Sayuri Kawai,[b] Atsushi Ichikawa,[b] and Kenji Kinoshita*[,a,b]

[a] *School of Pharmaceutical Sciences, Mukogawa Women's University; Kyuban-cho, Koshien, Nishinomiya, Hyogo 663–8179, Japan: and* [b] *Institute of Biosciences, Mukogawa Women's University; Kyuban-cho, Koshien, Nishinomiya, Hyogo 663–8179, Japan.*
Received February 8, 2019; accepted May 3, 2019

DNA extraction and purification have been generally considered to be required for PCR assay. We demonstrated a new protocol using biological specimens directly as templates for real-time PCR with melting curve analysis. We confirmed the melting curve analysis was particularly suitable for the identification of the insertion/deletion (Ins/Del) polymorphism of the angiotensin-converting enzyme (*ACE*) gene. The new protocol we developed can be set up using simple and complete PCR analysis including data interpretation in under four hours with additional advantages of application for large-scale clinical research, diagnostics, and epidemiological studies at low cost.

Key words angiotensin-converting enzyme (*ACE*) gene; melting curve analysis; real-time PCR; insertion/deletion (Ins/Del) polymorphism; water-soluble paper; oral mucosal cell

INTRODUCTION

Single nucleotide polymorphism (SNP), microsatellite polymorphism, copy number polymorphism (CNP), and insertion/deletion (Ins/Del) polymorphism have been associated with a predisposition to common diseases and individual variations in drug responses.[1–5] Various assays have been developed to analyze these polymorphisms, such as direct DNA sequencing after PCR amplification,[6] allele-specific primer-PCR,[7] PCR-restriction fragment length polymorphisms (PCR-RFLP),[8] TaqMan® PCR,[9,10] and a real-time PCR system with the melting curve analysis.[11,12] Generally, it is necessary for all of these genetic amplification methods to have sample preparation processes including extraction and purification of DNA from blood or saliva. The preparation processes for biological specimens are usually labor-intensive, time-consuming, and also simplified kits are costly to use for large-scale studies. Recently, we developed a simple SNP genotyping method in which all sample preparation processes were eliminated. We succeeded in using a dried blood sample on general filter paper for a PCR-RFLP method,[13] and a dried saliva template on water-soluble paper for a TaqMan® assay.[10]

The angiotensin-converting enzyme (ACE) is a central component of the renin-angiotensin system that controls blood pressure by regulating the volume of fluid in the body. Several studies have shown that approximately 50% of the variability of plasma ACE between individuals is the result of an Ins/Del polymorphism in intron 16 of the *ACE* gene on chromosome 17.[14–16] The Ins and Del alleles are due to the presence or absence of a 287 bp *Alu* sequence DNA, respectively. We developed here a rapid and cost-effective high-throughput Ins/Del polymorphism genotyping protocol of the *ACE* gene, to be suitable for practical use in clinical sites as well as in academic laboratories.

MATERIALS AND METHODS

Materials Water-soluble paper (120MDP) was purchased from Nippon Paper Papylia Co., Ltd. (Tokyo, Japan), and homemade sampling kits "DnaCapture" were prepared from Taiko Co., Ltd. (Nishinomiya, Japan). An oral care sponge swab was purchased from Osaki Medical Co. (Nagoya, Japan). Lancet (BD Microtainer® contact-activated lancet) was purchased from Becton Dickinson & Company (Franklin Lakes, NJ, U.S.A.). Biopsy punch (BPP-20F, BPP-40F) was purchased from Kai Industries Co., Ltd. (Tokyo, Japan). THUNDERBIRD® SYBR® qPCR Mix (QPK-201) was purchased from TOYOBO Co., Ltd. (Osaka, Japan). PowerUp® SYBR® Green Master Mix was purchased from Thermo Fisher Scientific Inc. (Waltham, MA, U.S.A.).

Subjects The development of the *ACE* Ins/Del genotyping protocol was based on real-time PCR with the melting curve analysis using seventeen healthy females' dried saliva and blood samples. For the large-scale study of the Japanese population in sufficient subjects, two hundred sixteen healthy female students at Mukogawa Women's University participated as volunteers. The Medical Ethics Committee of Mukogawa Women's University approved the study protocol. Informed consent was obtained from all subjects.

Sample Preparation The dried saliva samples were prepared as follows. The subjects were asked to rub their inner cheek surfaces with an oral care sponge swab to collect oral mucosal cells for their genotyping. The swabs including saliva specimens were applied to the DnaCapture on a part of water-soluble paper (120MDP) and were dried thoroughly for about an hour at room temperature. A small piece (4 mmϕ) of dried saliva sample for genotyping was cut with a biopsy punch (BPP-40F), directly suspended in 200 μL of distilled water, and heated at 95°C for 5 min. The supernatant of genome

* To whom correspondence should be addressed. e-mail: kenji_k@mukogawa-u.ac.jp

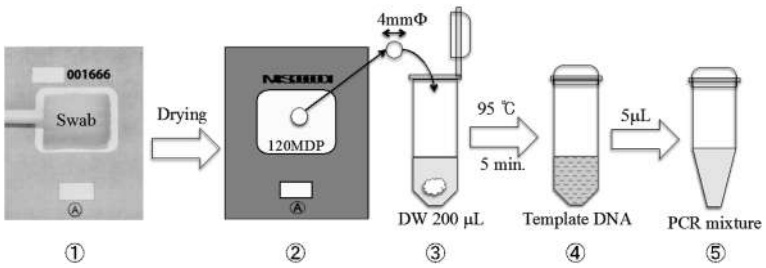

Fig. 1. Dried Saliva Sampling Process and Genetic Test Flow

① The oral mucosal cells in the oral care sponge swab were transferred to 120MDP on DnaCapture. ② The dried saliva on water-soluble paper was 4 mmϕ punch out by the biopsy trepan. ③ The punched specimen was poured into 200 μL of distilled water. ④ The suspension was treated on 95°C for 5 min. ⑤ 7.6 μL of supernatant was used for real-time PCR using SYBR green I.

DNA template was added to a PCR tube as shown in Fig. 1.

As control specimens, the blood samples were prepared as follows. Whole blood obtained by a finger prick using a lancet was dried onto DnaCapture for an hour at ambient room temperature. The dried blood sample was kept dried at room temperature until genotyping analysis. These samples were used as the DNA template, attached to a piece of water-soluble paper cut into a 2 mmΦ disk with a biopsy punch (BPP-20F), directly suspended in 200 μL of distilled water, and then heated at 95°C for 5 min.

Genotyping of *ACE* Gene Ins/Del Allele by Melting Curve Analysis To genotype Ins/Del polymorphism of the *ACE* gene, the real-time PCR SYBR GreenI was performed with the following primer sequences: 5′-CTG GAG ACC ACT CCC ATC CTT TCT-3′ and 5′-ATG TGG CCA TCA CAT TCG TCG TCA GAT-3′. The homozygous individual Del allele (Del/Del genotype) was identified by the presence of a single 190 bp PCR product. The homozygous Ins allele (Ins/Ins genotype) was identified by the presence of a single 490 bp PCR product. The heterozygous individual (Ins/Del genotype) was identified by the presence of both 190 and 490 bp PCR products. The resulting fragments were analyzed by automated microchip electrophoresis detection on MultiNA (MCE-202).

The amplification mixtures at a final volume of 20 μL, included 10 μL of THUNDERBIRD® SYBR® qPCR Mix (QPK-201), 0.4 μL of a 50 × ROX reference dye, each primer for the *ACE* gene (ACE-F and ACE-R) at a final concentration of 0.5 μM (1.0 μL of 10 μM Primers), 7.6 μL of the supernatant of genome DNA as template. The reactions were performed with the QuantStudio™ 12K Flex real-time PCR system (Thermo Fisher Scientific Inc.). The thermal cycling process was as follows: at 95°C for 1 min; 50 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 15 s, and extension at 72°C for 1 min. After the amplification was completed, melting curve analysis was performed by cooling the reaction to 60°C and then heating it slowly to 95°C, following the instructions from the manufacturer.

Endpoint Melting Curve Analysis for Large-Scale Population Study The amplification mixtures at a final volume of 10 μL included 5 μL of a PowerUp® SYBR® Green Master Mix, each primer for *ACE* gene (ACE-F and ACE-R) at a final concentration of 0.5 μM (0.5 μL of 10 μM Primers), and 4 μL of the supernatant of genome DNA as template. The reactions were performed with the Applied Biosystems GeneAmp® PCR System 9700. The thermal cycling process was same as mentioned above for the real-time PCR system. After the amplification was completed, melting curve analysis was performed with the QuantStudio™ 12K Flex real-time PCR system.

Processing Times and Cost for Endpoint Melting Curve Analysis When the researcher manually performed the genotyping of ACE Ins/Del polymorphism from ② (sample punching for 1 h) to ⑤ (liquid handling for 1 h), as shown in Fig. 1, the preparation for 216 dried saliva specimens could be carried out within 2 h until the PCR amplification processing. Following the PCR amplification reaction by simultaneously using three GeneAmp® PCR System 9700 at the same time for 1 h, the melting curve analyses were analyzed on the QuantStudio™ 12K Flex real-time PCR system. The total cost of *ACE* Ins/Del allele genotyping for each specimen was less than two U.S. dollars using the homemade sampling kits DnaCapture, the commercially available oral care sponge swab, and the real time PCR master mix. However, it does not include facilities costs, depreciation expenses and *etc.*

RESULTS

After real-time PCR using the dried saliva samples on water-soluble papers, the melting curve analyses were successfully performed to assay the genotyping of *ACE* Ins/Del polymorphisms. Melting curves from a part of endpoint analysis for the deletion Del/Del, insertion Ins/Ins, and Ins/Del alleles are shown in Fig. 2. It was identified easily for three types of genotype since each group was clearly separated. In Ins/Del genotyping of *ACE*, the homozygous PCR of the deletion type showed a single peak at 82.0 ± 0.1°C ($n = 11$), whereas the homozygous PCR of the insertion type showed a single peak at 89.8 ± 0.1°C ($n = 15$). Samples containing both alleles (heterozygotes) displayed two melting peaks as the respective homozygous samples at exactly the same temperatures (81.9 ± 0.1°C and 89.6 ± 0.1°C, $n = 20$).

It was also revealed that the saliva attached to the water-soluble papers could hold enough of the PCR product to be genotyped as shown from the amplification curves in Fig. 3. Successful amplifications were evident from the appearance of specific fluorescence and the display of derived melting curves. The mean *Ct* value of all specimens for the seventeen subjects was 25.1 ± 1.4 cycles for the dried saliva and 29.9 ± 1.4 cycles for the dried blood, respectively.

In addition, the amplified real-time PCR products analyzed by automated microchip electrophoresis detection on MultiNA (MCE-202, Shimadzu, Kyoto, Japan) of the PCR products verified the presence of only two PCR products (490 and/or 190 bp) as shown in Fig. 4.

With the endpoint melting curve analysis by the real time

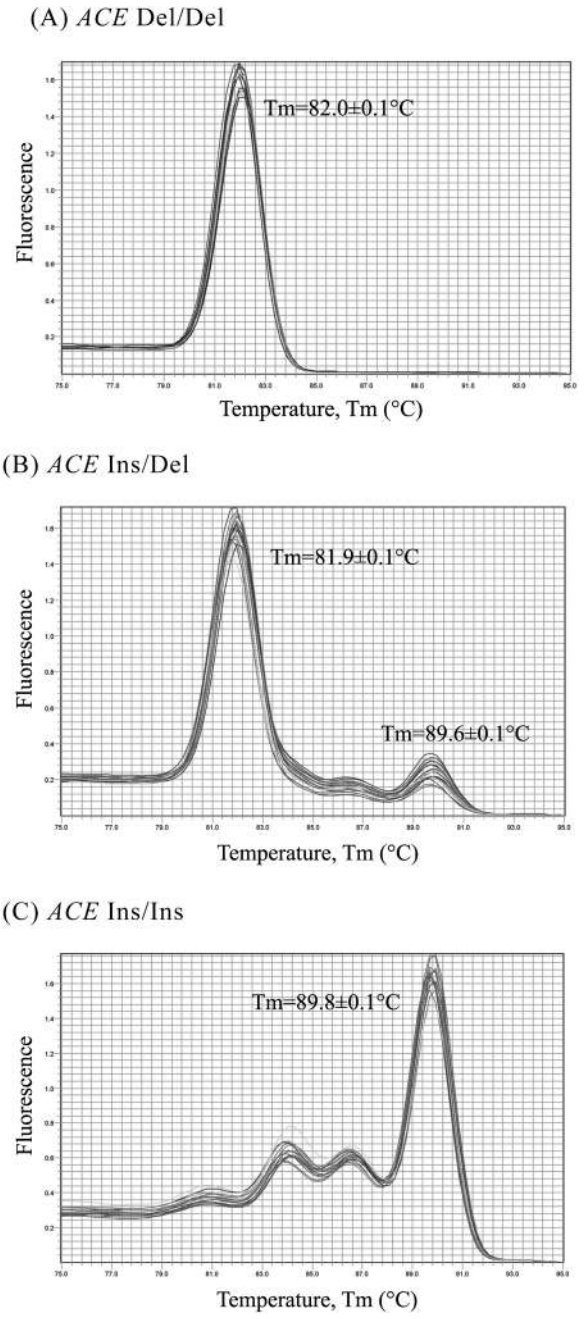

Fig. 2. Genotyping of Insertion/Deletion Polymorphism of ACE Gene by Endpoint Melting Curve Analysis

Melting curves for the (A) deletion homozygote Del/Del ($n = 11$), (B) heterozygote Ins/Del ($n = 20$), and (C) insertion homozygote Ins/Ins ($n = 15$) alleles. The melting curves were obtained after 50 cycles of PCR amplification with ACE primers in the presence of genomic DNA that has been genotyped. The first derivative of the fluorescence ($-dF/dT$) *versus* temperature shows different melting peaks.

PCR system after conventional PCR amplification, we were able to rapidly and accurately complete the genotyping of the 216 dried saliva specimens by the lists of the *Tm* (melting temperature) values. In general young Japanese women, the frequencies of the Ins and Del alleles are 64.8 and 35.2%; in the meantime, frequencies for the Ins/Ins, Ins/Del, and Del/Del genotypes are 45.8, 38.0, and 16.2% respectively. These genotype frequencies are similar to those found in previous studies of Japanese populations.[17)]

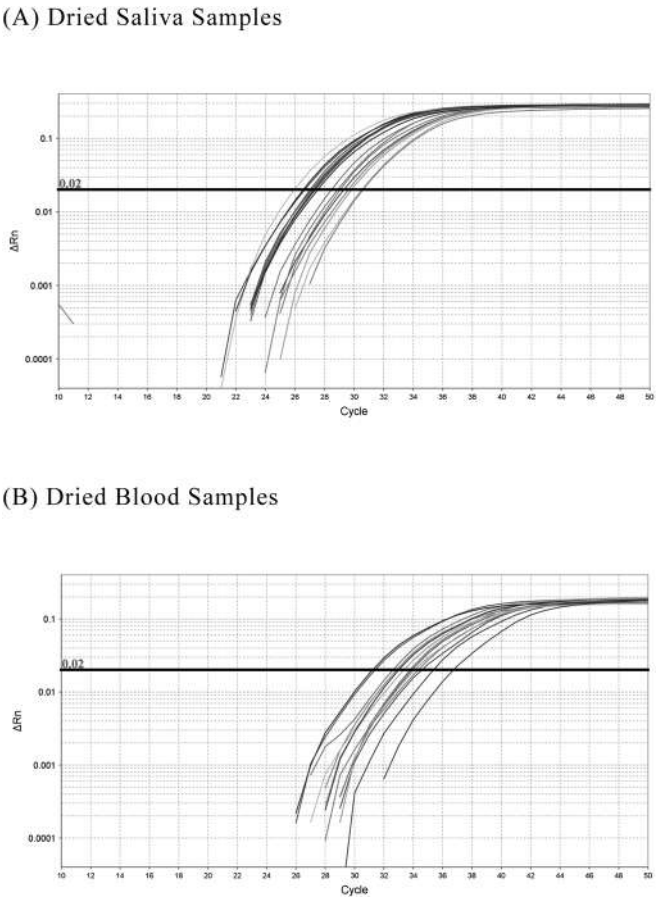

Fig. 3. Amplification Curves of *ACE* Gene by Real-Time PCR by SYBR® Green Fluorescence

The mean *Ct* values of dried saliva (A) and blood (B) samples for all seventeen specimens was shown at 24.9 ± 1.3 and 29.9 ± 1.4 cycles, respectively.

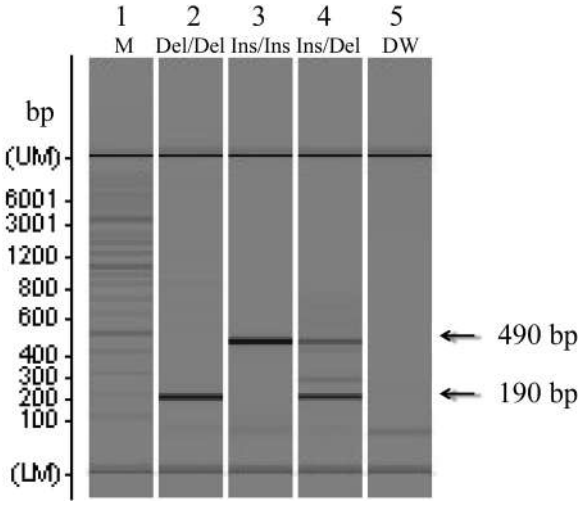

Fig. 4. Automated Microchip Electrophoresis Detection of *ACE* Ins/Del Genotypes

Lane 1: Marker, Lane 2: *ACE* Del/Del (190 bp), Lane 3: *ACE* Ins/Ins (490 bp), Lane 4: *ACE* Ins/Del (490 and 190 bp), Lane 5: DW, LM: lower marker, UM: upper marker.

DISCUSSION

In this study, we developed a cost-effective genotyping protocol for ACE Ins/Del polymorphism by real-time PCR method with the melting curve analysis using dried saliva

samples as templates. Because we have yet not to develop a cost-effective genotyping protocol for Ins/Del polymorphism, which has been frequently observed in human genome. Moreover, ACE Ins/Del polymorphism is known as useful information for the personalized treatment of cardiovascular and/or hypertension diseases. This is the first report on melting curve assay for Ins/Del genotyping protocol by adding reagents and unprocessed saliva samples simultaneously with no additional steps required before or after PCR. Therefore, this protocol can reduce the time and effort, as well as the risk of contamination. This high-throughput Ins/Del genotyping protocol is suitable for practical use in clinical sites as well as in academic laboratories. The real-time PCR-based protocol described in this study provides a rapid and sensitive way for detection of *ACE* Ins/Del polymorphism in clinical specimens.

The melting curve analysis results for the seventeen subjects were tested with a SYBR Green for *ACE* Ins/Del polymorphism. All samples performed very well with all assays. The average *Ct* value of all saliva were observed at 24.9 ± 1.3 cycle, on the other hand that of bloods were 29.9 ± 1.4 cycle. It was about five cycles difference. Therefore, the genomic DNA templates in saliva samples were good enough amounts to determine *ACE* Ins/Del polymorphism, and also the concentration of saliva samples were more than ten times higher from those of blood samples. It was suggested that most blood samples included PCR amplification with much higher levels of inhibitors than saliva.

However, both samples gave us the same results from the melting curve assay of *ACE* Ins/Del polymorphisms. Also, since the standard deviation of *Ct* values was within a narrow range, it was proved that the advantage for our sampling procedure using the DnaCapture.

Samples of either 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. Smaller sample size can also threaten the validity of statistical robustness. Inevitably, extracting oral mucosal cells in the saliva using a swab is more non-invasive than blood. Therefore, dried saliva samples are often more preferred as a source of genomic analysis, since sampling process is usually painless and non-invasive.

Moreover, its simplicity to sample will extend the capacity by allowing to self-sample, and to send the specimen to the laboratory for analysis. Considering the difficulties in collecting blood samples, we developed a new sampling method to collect dried saliva samples using water-soluble paper.[10,18,19] This sampling kit allows efficient sample collection and preservation, which sufficiently prepares for DNA amplification. It also simplified the sampling process; thus, an individual can easily learn to self-sample using this kit with instruction (Fig. 1). The saliva samples were able to be stored at room temperature more than three year later, and also, their genotyping still could be verified.

FTA (GE Healthcare UK Ltd.) technology,[20] which is most well-known as products for facilitates collection, transport, purification, and long-term, room-temperature storage of nucleic acids, all in a single device similar to that of DnaCapture. Processing time course for DNA preparation were compared between this study and FTA in Table 1. On the process for DNA purification for FTA technology, the punched piece has to be washed with plenty of water. This process also includes a risk of cross contamination. On the other hand, our protocol has great merit because of no need to be washing by distilled water.

Table 1. Comparison between Processing Time Course for DNA Preparation

Process for DNA preparation	FTA technology	This study
Sample punch 4 mmϕ	1 min	1 min
Washing with dedicated buffer	30 min	Not required
Heat treatment with DW	5 min	5 min

FTA technology, which is most well-known as products for facilitates collection, transport, purification, and long-term, room-temperature storage of nucleic acids, all in a single device, which is consists of two distinct chemistries, both of which have the ability to lyse cells on contact, denature proteins, and protect DNA from degradation.

In this endpoint melting curve analysis protocol using the real time PCR system by SYBR® Green assay method, the gene amplification was carried out with a general thermal cycler as a research facility related to molecular biology. We need to mention that this new protocol using the homemade sampling kit DnaCapture has a lower-cost and less reduction of processing time compared to the general genotyping process with DNA extraction and purification. All procedure related to the measurement of ACE Ins/Del of 216 saliva samples was completed within 4 h, and its consumable material cost was about 400 U.S. dollars. These were easier to handle than frozen liquid biopsy such as blood or saliva. Moreover, sample storage did not need facilities or any refrigeration equipment, and dried specimen could be easily transported through regular mail at room temperature. The risk of contamination had considerably decreased since the samples were solids. It is also of great value to use saliva as a template in some studies having healthy subjects since it is a non-invasive sampling method. By introducing automation such as the liquid handling automation system for genotyping reagents, it is also possible that further cost performance can be realized.

In summary, we constructed a new protocol to determine the genetic polymorphisms using real-time PCR and melting curve analysis. The genotyping of the *ACE* Ins/Del polymorphisms through simple sample preparation is a rapid and reliable protocol that is suitable for typing both small and large numbers of specimens. This new protocol may be used routinely in laboratories and allow clinicians an early assessment of individual drug efficacy. The pharmacogenomics (PGx) approach can be useful in drug development for targeting subgroups of patients genetically predisposed to high drug efficacy.

Acknowledgments Research reported in this publication was supported by the JSPS KAKENHI, Grant number JP16K08895. We thank the anonymous volunteers who participated in the blood and saliva sample collection.

Conflict of Interest The authors declare no conflict of interest.

REFERENCES

1) Risch N, Merikangas K. The future of genetic studies of complex

human diseases. *Science*, **273**, 1516–1517 (1996).
2) Sachidanandam R, Weissman D, Schmidt SC, Kakol JM, Stein LD, Marth G, Sherry S, Mullikin JC, Mortimore BJ, Willey DL, Hunt SE, Cole CG, Coggill PC, Rice CM, Ning Z, Rogers J, Bentley DR, Kwok PY, Mardis ER, Yeh RT, Schultz B, Cook L, Davenport R, Dante M, Fulton L, Hillier L, Waterston RH, McPherson JD, Gilman B, Schaffner S, Van Etten WJ, Reich D, Higgins J, Daly MJ, Blumenstiel B, Baldwin J, Stange-Thomann N, Zody MC, Linton L, Lander ES, Altshuler D. A map of human genome sequence variation containing 1.42 million single nucleotide polymorphisms. *Nature*, **409**, 928–933 (2001).
3) Kimpara T, Takeda A, Watanabe K, Itoyama Y, Ikawa S, Watanabe M, Takase S. Microsatellite polymorphism in the human heme oxygenase-1 gene promoter and its application in association studies with Alzheimer and Parkinson disease. *Hum. Genet.*, **100**, 145–147 (1997).
4) Sebat J, Lakshmi B, Troge J, Alexander J, Young J, Lundin P, Månér S, Massa H, Walker M, Chi M, Navin N, Lucito R, Healy J, Hicks J, Ye K, Reiner A, Gilliam TC, Trask B, Patterson N, Zetterberg A, Wigler M. Large-scale copy number polymorphism in the human genome. *Science*, **305**, 525–528 (2004).
5) Rigat B, Hubert C, Alhenc-Gelas F, Cambien F, Corvol P, Soubrier F. An insertion/deletion polymorphism in the angiotensin I-converting enzyme gene accounting for half the variance of serum enzyme levels. *J. Clin. Invest.*, **86**, 1343–1346 (1990).
6) Kim TH, Kim TJ, Lee HS, Uhm WS, Shin ES, Na YI, Jun JB. Single nucleotide polymorphism of COL6A1 in patients with ankylosing spondylitis. *J. Rheumatol.*, **35**, 1849–1852 (2008).
7) Ishiguro A, Kubota T, Soya Y, Sasaki H, Yagyu O, Takarada Y, Iga T. High-throughput detection of multiple genetic polymorphisms influencing drug metabolism with mismatch primers in allele-specific polymerase chain reaction. *Anal. Biochem.*, **337**, 256–261 (2005).
8) Obayashi K, Nakamura K, Kawana J, Ogata H, Hanada K, Kurabayashi M, Hasegawa A, Yamamoto K, Horiuchi R. *VKORC1* gene variations are the major contributors of variation in warfarin dose in Japanese patients. *Clin. Pharmacol. Ther.*, **80**, 169–178 (2006).
9) Borgiani P, Ciccacci C, Forte V, Sirianni E, Novelli L, Bramanti P, Novelli G. *CYP4F2* genetic variant (rs2108622) significantly contributes to warfarin dosing variability in the Italian population. *Pharmacogenomics*, **10**, 261–266 (2009).
10) Hayashida M, Ota T, Ishii M, Iwao-Koizumi K, Murata S, Kinoshita K. Direct detection of single nucleotide polymorphism (SNP) by the TaqMan PCR assay using dried saliva on water-soluble paper and hair-roots, without DNA extraction. *Anal. Sci.*, **30**, 427–429 (2014).
11) Lin MH, Tseng CH, Tseng CC, Huang CH, Chong CK, Tseng CP. Real-time PCR for rapid genotyping of angiotensin-converting enzyme insertion/deletion polymorphism. *Clin. Biochem.*, **34**, 661–666 (2001).
12) Minucci A, Canu G, Concolino P, Guarino D, Boccia S, Ficarra S, Capoluongo E. DNA from buccal swab is suitable for rapid genotyping of angiotensin-converting enzyme insertion/deletion (I/D) polymorphism. *Clin. Chim. Acta*, **431**, 125–130 (2014).
13) Hayashida M, Iwao-Koizumi K, Murata S, Yokoyama A, Kinoshita K. Genotyping of polymorphisms in alcohol and aldehyde dehydrogenase genes by direct application of PCR-RFLP on dried blood without DNA extraction. *Anal. Sci.*, **26**, 503–505 (2010).
14) Rigat B, Hubert C, Alhenc-Gelas F, Cambien F, Corvol P, Soubrier F. An insertion/deletion polymorphism in the angiotensin I-converting enzyme gene accounting for half the variance of serum enzyme levels. *J. Clin. Invest.*, **86**, 1343–1346 (1990).
15) Tiret L, Rigat B, Visvikis S, Breda C, Corvol P, Cambien F, Soubrier F. Evidence, from combined segregation and linkage analysis, that a variant of the angiotensin I-converting enzyme (*ACE*) gene controls plasma ACE levels. *Am. J. Hum. Genet.*, **51**, 197–205 (1992).
16) Wollinger LM, Dal Bosco SM, Rempel C, Almeida SEM, Berlese DB, Castoldi RP, Arndt ME, Contini V, Genro JP. Role of *ACE* and *AGT* gene polymorphisms in genetic susceptibility to diabetes mellitus type 2 in a Brazilian sample. *Genet. Mol. Res.*, **14**, 19110–19116 (2015).
17) Kondo H, Ninomiya T, Hata J, Hirakawa Y, Yonemoto K, Arima H, Nagata M, Tsuruya K, Kitazono T, Kiyohara Y. Angiotensin I-converting enzyme gene polymorphism enhances the effect of hypercholesterolemia on the risk of coronary heart disease in a general Japanese population: the hisayama study. *J. Atheroscler. Thromb.*, **22**, 390–403 (2015).
18) Iwao-Koizumi K., Ota T., Hayashida M., Yonetani Y., Nakata K., Kinoshita K., Koyanagi Y., Murata S. The *ACTN3* gene is a potential biomarker for the risk of non-contact sports injury in female athletes. *J. Mol. Biomark. Diagn.*, **S6** (2015). doi: 10.4172/2155-9929.S6-002.
19) Ota T, Kamada Y, Hayashida M, Iwao-Koizumi K, Murata S, Kinoshita K. Combination analysis in genetic polymorphisms of drug-metabolizing enzymes CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A5 in the Japanese population. *Int. J. Med. Sci.*, **12**, 78–82 (2015).
20) https://www.gelifesciences.com/en/us/shop/molecular-biology/sample-collection/cards-kits-and-collectors