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ORIGINAL ARTICLE

Homogeneous Assays for Single-Nucleotide Polymorphism Genotyping: Exo-proofreading Assay based on Loop-mediated Isothermal Amplification

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ABSTRACT

We have developed a new method Proofreading-LAMP (PR-LAMP) for genotyping single-nucleotide polymorphisms (SNPs) in combination with the use of nucleic acid amplification by loop-mediated isothermal amplification (LAMP) and the 3'-5' exonuclease proofreading (exo-proofreading) activity of DNA polymerase.

Using as a model a detection system for the SNP (G1951A) found in the human aldehyde dehydrogenase 2 (ALDH2) gene, typing primers with fluorescent-labeled 3' ends, and cloned DNA as a template, consisting of ALDH2*1, ALDH2*2, or a mixture of ALDH2*1 and ALDH2*2, and 3'-5' exo+ Pwo DNA polymerase were added to a LAMP reaction system, and amplification and the exo-proofreading reaction proceeded at 60℃ for 30 minutes, at the same time as which SNPs were detected by fluorescence polarization (FP). This procedure permitted evaluation by means of a single-step reaction at 60℃ for 30 minutes for ALDH2*1, ALDH2*1/*2, and ALDH2*2, and allowed a single nucleotide change to be distinguished with excellent reproducibility. Furthermore, the typing of human genomic samples, similar to the procedure for cloned DNA, yielded results that were in complete agreement with the results of PCR-RFLP.

PR-LAMP is accurate, simple, rapid, and robust, and is a flexible method that may be used in applications ranging from point-of-care testing (POCT) to high-throughput screening (HTS).

Key words: LAMP, SNP genotyping, exo-proofreading, fluorescence polarization

INTRODUCTION

Single nucleotide polymorphisms (SNPs) are variations that occur relatively evenly and very frequently across the entire genomic region, and have attracted attention as markers for investigating the predisposing factors of disease, drug resistance, and pharmacological efficacy. For that reason numerous SNP genotyping methods have been developed[1]. These include hybridization on microarrays[2),3)], exo-proofreading[4),5)] with primer extension, template-directed dye-terminator incorporation (TDI)[6)], pyrosequencing[7)], the Invader[8)] assay using nuclease, and oligonucleotide-ligation assays[9)] using ligation reactions.

Although these techniques were shown to be effective,

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their actual applications were hampered by their limited throughput capacity, operational complexity and high cost. After the completion of the Human Genome Project[10] and along with advances in the HapMap Project[11], enormous SNP information was obtained. To apply this information to pharmacogenomics research, a great number of genotyping is needed, which demands higher throughput capacity and lower operational cost. Furthermore, point-of-care testing (POCT) that expands these research results to each clinical site requires more convenient and rapid detection techniques.

Many SNP genotyping methods involve protocols that include Polymerase Chain Reaction (PCR) or other nucleic acid amplification techniques. Using 4 primers that recognize 6 regions, the loop-mediated isothermal amplification (LAMP)[12] method we have developed is a new amplification technique that proceeds via an isothermal reaction. The LAMP method has the following characteristics and may therefore be especially suited to POCT: i) it does not require a thermal cycler because the reaction proceeds isothermally; ii) it is highly specific because the primers contain numerous distinct regions; iii) it is rapid[13]; and iv) detection is simple due to the large quantities of amplification product[14]. In actual fact its simplicity has led to its application in various fields[15), 16), 17]. Therefore, we sought to develop a LAMP-based SNP genotyping method for application in POCT[18]. Using allele-specific primers, this method is simple and rapid, potentially yielding results in about 30 minutes after sample collection and utilizing the heat-treated blood sample as a direct template, pointing to its promise in POCT[18]. However, because it is necessary to investigate numerous allele-specific primers, it can take a considerable time to establish the assay system when typing large numbers of SNPs. Therefore, aiming at the development of a more flexible LAMP-based SNP genotyping method for many more SNP typing procedures, in which primer design was simple, and without losing the simplicity or rapidity of the LAMP method, we evaluated a new SNP genotyping method PR-LAMP that entails a combination of exo-proofreading and LAMP.

MATERIALS AND METHODS

Enzymes

TaKaRa ExTaq and *Pyrobest* DNA polymerase were obtained from TaKaRa. Pwo DNA polymerase was purchased from Roche Diagnostics. *Eco*RI and *Bst* DNA polymerase large fragment were purchased from New England Biolabs.

DNA samples

The cephalic hair follicle samples used in this study were collected from volunteers who gave informed consent. Human genomic DNA was extracted and purified using the ISOHAIR DNA extraction kit (NIPPON GENE) according to the manufacturer's protocol. The human ALDH2*1 gene was amplified from human genomic DNA (Roche Diagnostics) by polymerase chain reaction (PCR) and was cloned into pBluescriptII (Stratagene). The ALDH2*2 gene was prepared by site-directed mutagenesis of the ALDH2*1 gene.

Oligonucleotide primers

The oligonucleotide primers for LAMP were obtained from Operon Biotechnology.

Primers with 3'-amino-modified nucleotides were obtained from Japan Bio Services. The primers were synthesized using 3'-amino modifiers having a linker attached to the 5-position of terminal pyrimidine moiety (3'-Amino-Modifier C6-dC-CPG and 3'-Amino-Modifier-C6-dT-CPG; (Glen Research)). The resulting oligonucleotides were labeled using the following reaction conditions: 1 μg/μL custom synthesized oligonucleotide, 2 μg/μL succinimidyl ester dye (TAMRA). Labeled primers were purified by ethanol precipitation to remove a free dye.

PCR-RFLP analysis

ALDH2 genotyping was determined by a previously published method[19] (Chao et al. 1997). Human genomic DNA was amplified with *TaKaRa ExTaq* according to the manufacturer's instructions. The reactions were performed in an MJ Research PTC-200 Thermocycler using the following programs: 94℃ for 2 minutes followed by 30 cycles of 94℃ for 1 minute, 51℃ for 3 minutes, and 72℃ for 1 minute. At the end of the reaction, the reaction mixtures were held at 4℃ until further use.

PCR products were digested with *Eco*RI for 1 hour at 37℃ and were analyzed on 4% agarose gel, which was stained with SYBR-Green I (BioWhittaker Molecular Applications). DNA typing was based on the gel pattern of the restriction PCR fragments.

PR-LAMP

The Exo-proofreading assay based on LAMP was carried out in a reaction mixture of total volume 25 μl containing 1.6 μM each FIP (CCCCCAGCAGGCTGCAGGCATACACT) and BIP (CTGTTGGGGCTCAACAGACCCCA-

ATCC), 0.2 μM each F3 (GGAGTTGGGCGAGT) and B3 (TCCTGAACCTCTGGC), 0.8 μM LoopB (TCTGCTGGTGGCTC), 0.04 μM TAMRA-labeled typing primers for the *1 allele (CACAGTTTTCACTTC) and for the *2 allele (CACAGTTTTCACTTT), 400 μM each dNTP, 0.6 M Betaine (Sigma), 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 4 mM $MgSO_4$, 0.1% Tween 20, 8 U *Bst* DNA polymerase large fragment, 2 U Pwo DNA polymerase and 1 ng of human genomic DNA or 150 copies of cloned DNA as a template. The template DNA was heated at 95℃ for 3 minutes, and then chilled on ice before addition. Reactions were performed in a Loopamp real-time turbidimeter LA-200 (Teramecs)[20] followed by incubation at 60℃ for 30 minutes and heating at 80℃ for 5 minutes to terminate the reaction.

Fluorescence Polarization (FP) Measurement

After PR-LAMP, 8 μl of methanol was added to each tube[21] before it was transferred to a 384-well black assay plate (Corning) for FP measurement on a Polarion (Tecan) multi-mode plate reader in fluorescent polarization mode. The machine was set with a G-factor of 0.9 and the light filters 535 − 594 nm.

The fluorescence polarization value was calculated using the formula:

$$P = (Ivv - G. \cdot Ivh) / (Ivv + G. \cdot Ivh)$$

where *Ivv* is the emission intensity measured when the excitation and emission polarizers are parallel and *Ivh* is the emission intensity measured when the emission and excitation polarizers are oriented perpendicular to each other. The G-factor is a correction factor that allows for the different light transmission characteristics of the two polarizers in the vertical and horizontal orientations. The degree of polarization is expressed by the unit mP.

RESULTS

The principles of SNP typing by exo-proofreading are shown in Figure 1A. The detection primer (typing primer) for SNP genotyping has a fluorescent label at its 3' terminal nucleotide, and when primer extension is performed with this typing primer, the 3'-terminal nucleotide is removed by the exo-proofreading activity of DNA polymerase, and DNA that does not contain the fluorophore is synthesized when the 3'-terminal nucleotide and the template have a mismatched nucleotide (Figure 1A, right).

However, if the 3' terminus of the typing primer is a matched nucleotide, DNA that contains the fluorophore is synthesized, without the nucleotide being removed (Figure 1A, left). To make the most of the simplicity of the LAMP technique, fluorescence polarization (FP) detection[22], which permits homogeneous detection, was used. Because FP reflects the molecular weight of molecules that contain the fluorophore, it can be evaluated from the result of FP whether or not the fluorophore is included in the amplification product. In other words, when the template is mismatched, the fluorescence-labeled nucleotide of the typing primer is removed. Hence, because the post-reaction mixture contains fluorescence-labeled nucleoside and unreacted fluorescence-labeled typing primer, the FP value is comparable to or lower than that of the negative control (NC), and when there is a match with the template, the FP value of the mixture containing synthesized DNA that includes the fluorophore and unreacted fluorescence-labeled typing primer is greater than that of the NC.

Bst DNA-polymerase, a DNA synthetase with strand displacement activity, is required for amplification by LAMP. However, since *Bst* DNA-polymerase has no 3'-5' exonuclease activity, it is necessary to add a DNA synthetase with 3'-5' exonuclease activity to achieve exo-proofreading simultaneously (in 1 step) with the amplification reaction. Therefore, we first screened a number of DNA synthetases with 3'-5' exonuclease activity for possible combination with LAMP, finally selecting Pwo DNA polymerase (data not shown).

As a model to verify this SNP genotyping method by an exo-proofreading assay based on LAMP (PR-LAMP), we prepared a typing primer for the SNP (G1951A) found in the human ALDH2 gene (GenBank Accession No. NM_000690) (Figure 2). The LAMP amplification primer amplified both ALDH2*1 and *2, and the SNP site was designed to be located in the loop structure (Figure 1B, Figure 2). The typing primer was designed to recognize the same region as the loop primer used in ordinary LAMP, and the 3'-terminal nucleotide was labeled with TAMRA for detection.

The genotyping reaction by LAMP-based DNA amplification and exo-proofreading activity involves adding a typing primer and Pwo DNA polymerase to an ordinary LAMP reaction solution[23], and the cloned human genes ALDH2*1 (G), ALDH2*2 (A), or a mixture (G/A) of ALDH2*1 and ALDH2*2 in 1 step at 60℃ for 30 minutes, after which the fluorescence polarization in the reaction solution was measured. As shown in Figure 3A, the results of measurements for the NC, ALDH2*1 and ALDH2*2 as templates with ALDH2*1 typing primer (ALDH2_1951G) were 55 ± 11.4, 146 ± 10.2, and 21 ± 8.3 respectively, and the results of measurement for NC, ALDH2*1 and ALDH2*2 as templates

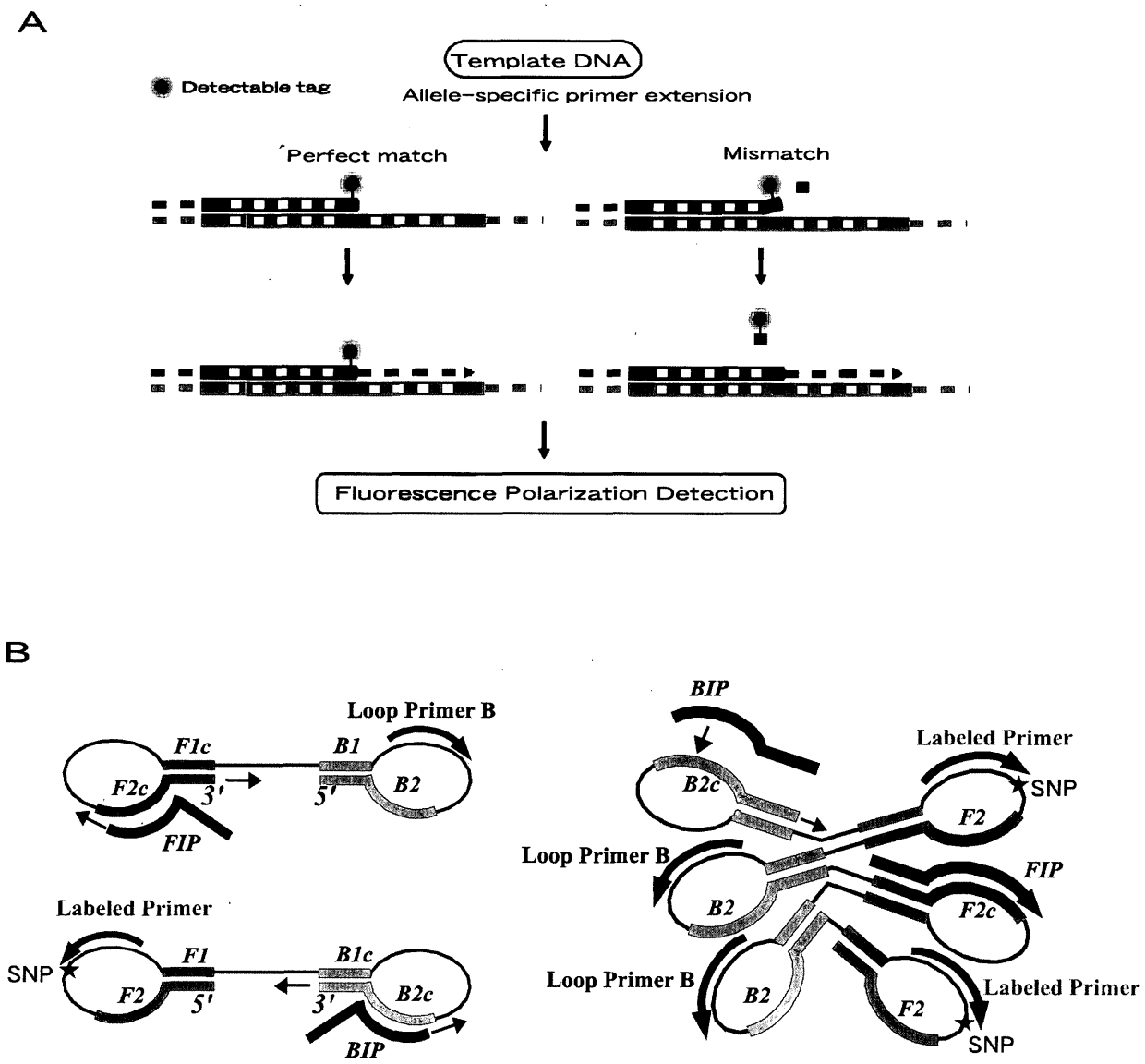

Figure 1

A Schematic representation of allele specific primer extension with exo-proofreading of a DNA polymerase. A primer is designed with its 3' terminus aligned at the polymorphic base of a DNA target and labeled with a detectable tag on the 3' nucleotide base. If the 3' end of the primer is matched with the target (left), the labeled nucleotide is retained and incorporated into the extension product. If the primer has a mismatch with the template (right), the labeled nucleotide is removed by the proofreading activity of the polymerase and no tag is incorporated into the extension product. The detection of genotypes can be accomplished by means of fluorescence polarization without any additional cleanup.

B Principle of the PR-LAMP method. The amplification product with a single strand part is produced in the process of DNA amplification by LAMP. LAMP primers are designed so that the polymorphic base of the DNA target is located in this single strand loop structure and allele specific primer extension is carried out with a labeled primer.

A Primer Design

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                                        *
GGAGTTGGGC GAGTACGGGC TGCAGGCATA CACTGAAGTG AAAActgtga gtgtgggacc
F3                    F2                typing primer

tgctgggggc tcagggcctg ttggggcttg agggtctgct ggtggctcgg agcctgctgg
F1c                   B1                loopB

gggattgggg tctgttgggg gctcggggcc tgccagaggt tcagga
  B2c                                 B3c
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B Primers for amplification and genotyping

Name	Sequence	Length	Label	Tm
Primers for LAMP				
FIP	5'-**CCCCCAGCAG**GCTGCAGGCATACACT-3'	26		
BIP	5'-**CTGTTGGGGCT**CAACAGACCCCAATCC-3'	27		
F3	5'-GGAGTTGGGCGAGT-3'	14		
B3	5'-TCCTGAACCTCTGGC-3'	15		
LoopB	5'-TCTGCTGGTGGCTC-3'	14		
Primers for genotyping				
ALDH2_1951G	5'-CACAGTTTTCACTT**C**-3'	15	TAMRA	41.6
ALDH2_1951A	5'-CACAGTTTTCACTT**T**-3'	15	TAMRA	41.5

Figure 2 Primer design for LAMP and genotyping.

A. The target regions for the LAMP primer and genotyping primer (typing primer) on the base sequence of ALDH2. The SNP bases indicated with an asterisk are G for ALDH2*1 and A for ALDH2*2. B. Shows the LAMP primer sequence and the typing primer sequence. The LAMP primers FIP and BIP are sequences in which F1c and F2 or B1 and B2c are complementary. The F1c and B1 portions of the primer are indicated in boldface. The typing primers are labeled with TAMRA on the 3'-terminal bases shown in boldface. ALDH2_1951G is the primer for *1 detection and ALDH2_1951A is the primer for *2 detection.

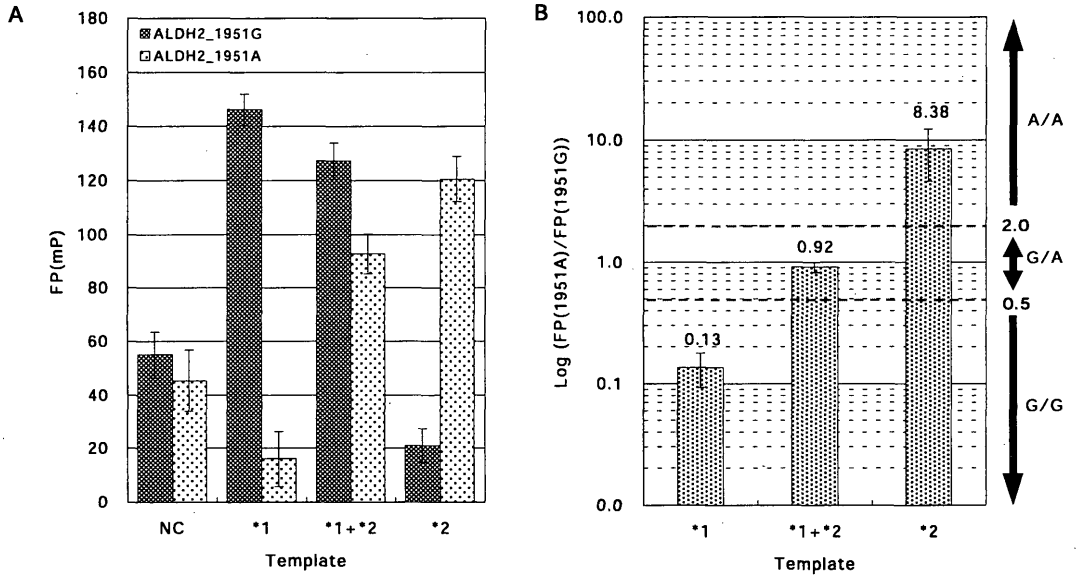


Figure 3 PR-LAMP performed on artificial samples.

Using the typing primer (ALDH2_1951G) matched to ALDH2*1 and the typing primer (ALDH2_1951G) matched to ALDH2*2, PR-LAMP was performed with the plasmid-cloned ALDH2 gene as the template (n = 8). A. This graph shows the mean values of the raw data. B. The ratio of the fluorescence polarization (FP) value measured for ALDH2_1951A and the value of FP measured for ALDH2_1951G is shown. The ratio of FP values was normalized with the FP values for the NC measured for each primer, and was calculated using the following formula.

FP ratio = FP (ALDH2_1951A) /FP (ALDH2_1951G). Mean values are displayed in the upper bars. NC: Negative Control.

with ALDH2*2 typing primer (ALDH2_1951A) were 45 ± 8.6, 16 ± 5.8, and 121 ± 6.3 respectively. Additionally, when the mixture of ALDH2*1 and ALDH2*2 was used as a template, the assay results for typing primers ALDH2_1951G and ALDH2_1951A were 127 ± 7.4 and 93 ± 6.7 respectively, demonstrating that highly-reproducible SNP detection was possible.

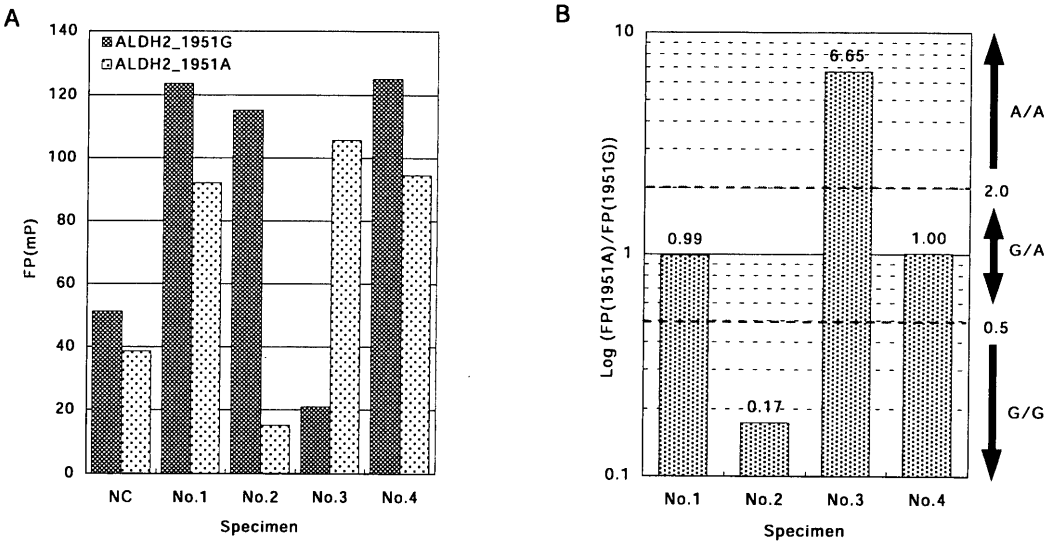
The FP values for each sample assayed with typing primers ALDH2_1951G and typing primer ALDH2_1951A shown in Figure 3A were normalized with the FP measured for NC. Comparison of those values shows that for ALDH2*1, the FP value measured for ALDH2_1951A was less than 1/2 of the FP value measured for ALDH2_1951G, and for ALDH2*2, the FP value was 2-fold or higher. Additionally, for the sample containing a mixture of ALDH2*1 and ALDH2*2, the result was approximately comparable. Hence, taking the ratio (ALDH2_1951A/ALDH2_1951G) of FP values measured for each primer, the cut-off value was determined to be < 0.5 for ALDH2*1/ ALDH2*1, $\geqq 0.5$-2.0 $\leqq$ for ALDH2*1/ ALDH2*2, and > 2.0 for ALDH2*2/ ALDH2*2 (Figure 3B). Based on these cut-off values, we genotyped samples of volunteer-derived human genomic DNA as a template. This revealed ALDH2*1/ ALDH2*1 in 1 sample, ALDH2*2/ ALDH2*2 in 1 sample, and ALDH2*1/ ALDH2*2 in 2 samples (Figure 4B). This was in complete agreement with the results of genotyping by PCR-RFLP (Figure 4C).

DISCUSSION

In this communication we have described a new method for SNP typing, known as PR-LAMP, that combines LAMP, exo-proofreading, and FP detection.

Two types of DNA polymerase were used in this method, *Bst* DNA polymerase in the LAMP reaction, and Pwo DNA polymerase, which has high exo-proofreading activity. However, *Bst* DNA polymerase is almost completely unable to participate in the extension reaction from the typing primer with the 3'-terminal labeled nucleotide (data not shown). Hence, because the extension reaction from the typing primer proceeds almost exclusively via Pwo DNA polymerase, the probability of miss-reading is low. In addition, the combination of PCR and exo-proofreading reaction means that the amplification primer and the typing primer are the same, and if there is a miss-reading from the typing primer, the reaction products thereof serve as the template for the typing primer, producing the possibility of misjudgment. In contrast, it is possible to separately select the amplification primer and the typing primer in LAMP. Hence, exo-proofreading chemistry may permit more accurate genotyping in combination with LAMP, as compared with PCR.

It was possible to use a number of different types of 3'-5' exo+DNA polymerase apart from Pwo DNA polymerase, such as *Pfu* DNA polymerase, Vent DNA polymerase, and *Pyrobest* DNA polymerase. The mixing ratio of 3'-5' exo-



C

Specimen	1	2	3	4
PR-LAMP	G/A	G/G	A/A	G/A
PCR-RFLP	G/A	G/G	A/A	G/A

Figure 4 PR-LAMP performed on genomic DNA.

An assay was performed for genomic DNA obtained from 4 human volunteers as a template, and using a typing primer (ALDH2_1951G) matched to ALDH2*1 and a typing primer (ALDH2_1951A) matched to ALDH2*2. A. The FP measured using ALDH2_1951G and the FP measured using ALDH2_1951A. B. The FP ratio calculated according to the same method shown in Figure 2. Cut-off values are shown on the right. C. Results of assay of samples by PR-LAMP and PCR-RFLP.

DNA polymerase and 3'-5' exo+DNA polymerase will vary according to the type and purpose of the enzymes[24), 25), 26)], and when we mixed Pwo DNA polymerase with *Bst* DNA polymerase in ratios ranging from 1/8 to 1/4 in the present method, there was a substantial difference in the signal produced between the reaction in which the 3' terminus was matched and the reaction in which a mismatch occurred (data not shown).

FP detection is reportedly also possible with an SNP genotyping method that entails a combination of PCR and exo-proofreading, but because of problems with reproducibility, it was found to be preferable to measure the fluorescence intensity in the amplification products purified after completion of the reaction[5)]. According to the protocol offered by the present LAMP method, it is possible to thoroughly distinguish differences of just one base by FP detection, and moreover, with excellent reproducibility (Figure 3 A). Because the quantity of amplification products of LAMP is approximately two orders of magnitude greater than that obtained with PCR[14)], most of the typing primers are involved in the reaction, and free typing primers are scarce after the completion of the reaction as compared with PCR, and furthermore, since the amplification product yielded by LAMP is a repeating structure of target sequences, the molecular weight is large, producing a substantial difference between the FP value of products that have incorporated fluorescence, and the FP value of free typing primer and fluorescent nucleosides eliminated by exo-proofreading, which may help explain the excellent reproducibility. Using FP for measurement means it will be possible to perform a homogeneous assay. This confers substantial advantages in that not only can the assay process be automated, but also that the contamination of amplification products can be avoided.

The present method, which combines LAMP for isothermal amplification at a temperature in the vicinity of 60℃ and exo-proofreading, is a rapid technique that permits detection in 30 minutes (Figures 3, 4) and can be carried out within a simple fluorescence-polarization detection equipment capable of incubation, therefore, expensive equipments are not required. Moreover, the present results show that SNPs were detected with the reaction proceeding in separate tubes with 2 typing primers labeled with TAMRA. However, it is possible in principle to label the 3' terminus with various fluorescent dyes (in fact, the same results have been obtained with Texas Red too), it may be possible to genotype a sample in 1 tube if the respective typing primers are labeled for example, with 2 colors of fluorescent dye with differing fluorescent wavelengths such as TAMRA and FAM. Finally, PR-LAMP is yet to be employed for typing genes other than ALDH2, but methods using exo-proofreading have already been described for typing various genes[5)]. There is another report that most miss-reading reactions in allele-specific PCR occur when the 3' terminus of the primer is a T[27)], but the *2 typing primer (ALDH2_1951A) used in this work had a T for the 3' terminal nucleotide. Accordingly, PR-LAMP may be used for the typing of various genes.

The results presented above demonstrate that PR-LAMP is accurate, rapid, simple, and robust, and promises to have application in a wide range of fields from the clinical setting to pharmacogenomics research.

In the present work, DNA samples were obtained from in-house volunteers who had given informed consent, and the research was approved by the in-house Ethical Review Board in accordance with the "Ethics Guidelines for Human Genome/Gene Analysis Research".

The handling of recombinant microorganisms in this research proceeded with institutional approval obtained in accordance with the "Law to Prevent the Proliferation of Recombinant Microorganisms".

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