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Application Note

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Life Science

A Quick and Efficient Method for Detecting Single-Nucleotide Mutations:

"PRIMA" Detection of Genome-Edited Mutants Using MultiNA™

Hiroyuki KAKUI [*1] [*2] [*3], Misako YAMAZAKI [*1], Kentaro K. SHIMIZU [*1] [*2], Yuji SOGABE [*4]

Life Science

■ Abstract

We developed a method that enables the detection of 1-base pair (bp) insertions/deletions (indels). This method is advantageous compared with existing methods such as the heteroduplex mobility assay (HMA). Although the HMA is an efficient method for genotyping mutants with small indels, it is difficult to distinguish sequences with 1-bp indels using the HMA. The probe-induced heteroduplex mobility assay (PRIMA) has made it possible to identify 1-bp differences with the use of 40-bp single-stranded DNA sequences with five deleted bases as a co-migrating probe. To genotype F2 individuals, the HMA requires two analysis runs to distinguish three genotypes (i.e., wild-type homozygous, heterozygous, and mutant homozygous); however, the PRIMA can distinguish three genotypes in a single run. It can also be used to detect single-nucleotide polymorphisms (SNPs). We succeeded in distinguishing SNPs with the PRIMA using a Shimadzu MultiNA automated microchip electrophoresis system.

1. Introduction

CRISPR/Cas9 is a genome-editing tool that is relatively easy to use in comparison with conventional gene transformation techniques, and it offers excellent efficiency and cost-performance. For these reasons, it has been used broadly in pharmaceutical, agricultural, and medical fields.

The standard CRISPR/Cas9 method often produces 1-bp insertions/deletions (indels); several methods are used to detect these mutations. DNA sequencing can reliably detect mutations in the target gene, but sequencing large numbers of samples is costly. On the other hand, the heteroduplex mobility assay (HMA) is a useful method to detect small indels. Because the HMA involves simple and cost-effective steps, polymerase chain reaction (PCR) and electrophoresis (e.g., polyacrylamide gel electrophoresis [PAGE] or microchip electrophoresis), it is easy to conduct in standard molecular laboratories. MultiNA is an automated electrophoresis system using microchips that can detect heteroduplex signals caused by the HMA method [(1)]. Therefore, the HMA using MultiNA is suitable for genotyping mutants with small indels from many samples, but distinguishing 1-bp indels is difficult. Here, we introduce the probe-induced heteroduplex mobility assay (PRIMA). With this assay, it is possible to identify 1-bp differences using short single-stranded DNA with five deleted base pairs as a co-migration probe. This Application Note describes the details of the PRIMA method using a MultiNA microchip electrophoresis instrument.

[*1] Department of Evolutionary Biology and Environmental Studies, University of Zurich
[*2] Kihara Institute for Biological Research, Yokohama City University
[*3] Laboratory of Plant Breeding, Graduate School of Agriculture, Kyoto University
[*4] Analytical & Measuring Instruments Division, Shimadzu Corporation

2. Detection of Heteroduplex Signals by the HMA

Heteroduplex signal patterns of the HMA were confirmed by using 1–7-bp-deleted sequences (Fig. 1). Primers 5300 and 5301 were designed to amplify a product of approximately 200 bp including the mutated region by PCR and mixed with the wild-type sequence. Heteroduplex signals were not detected from the 1-bp-deleted sequence and the wild type, highlighting the difficulty in distinguishing 1-bp differences using the conventional HMA method (Fig. 1).

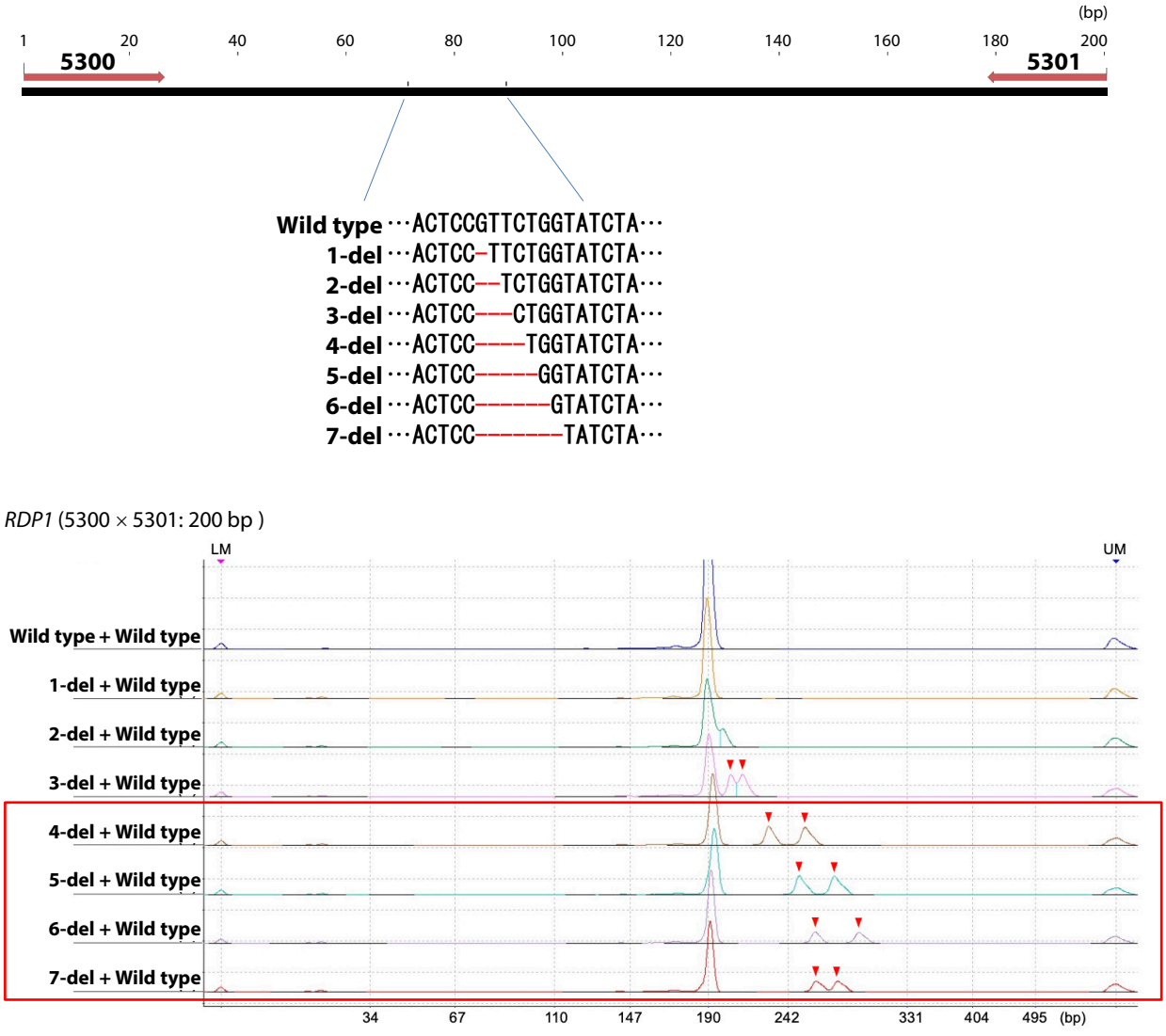


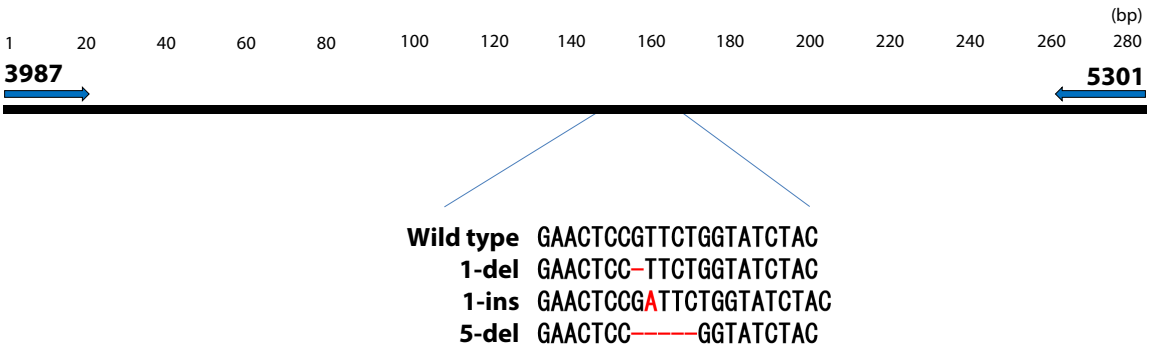
Fig. 1 Detection of Heteroduplex Signals with 1- to 7-bp Deletions by the HMA Method

In the formation of a heteroduplex, "Sample (1- to 7-bp deletion)" + "Wild type" were used.
A heteroduplex signal could not be acquired from the sequence with one deleted base (1-del). However, clear heteroduplex signals (▼) were obtained from the sequences with 4-, 5-, and 6-bp deletions (red box).

3. Detection of 1-bp Differences with the Precursive PRIMA (prePRIMA) Method

Although the HMA method could not detect heteroduplex signals from 1-bp deletions, clear heteroduplex peaks were produced from 4-, 5-, and 6-bp-deleted sequences (Fig. 1). Thus, we hypothesized that it would be possible to produce clear, distinguishable heteroduplex peaks by mixing a sequence with a 5-bp deletion as a probe. We prepared a probe containing a 5-bp deletion and conducted HMA analysis using a 1 bp-inserted (1-ins) or 1 bp-deleted (1-del) sequence. As a result, heteroduplex signals were detected from all sequences, and could be distinguished by the size of the heteroduplex signals (Fig. 2). This experiment confirmed that 1-ins/1-del sequences can be distinguished efficiently using a 5-bp-deleted sequence as a probe. We named this technique the prePRIMA method [2].

a



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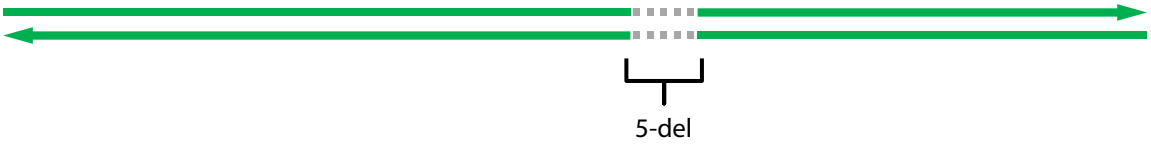

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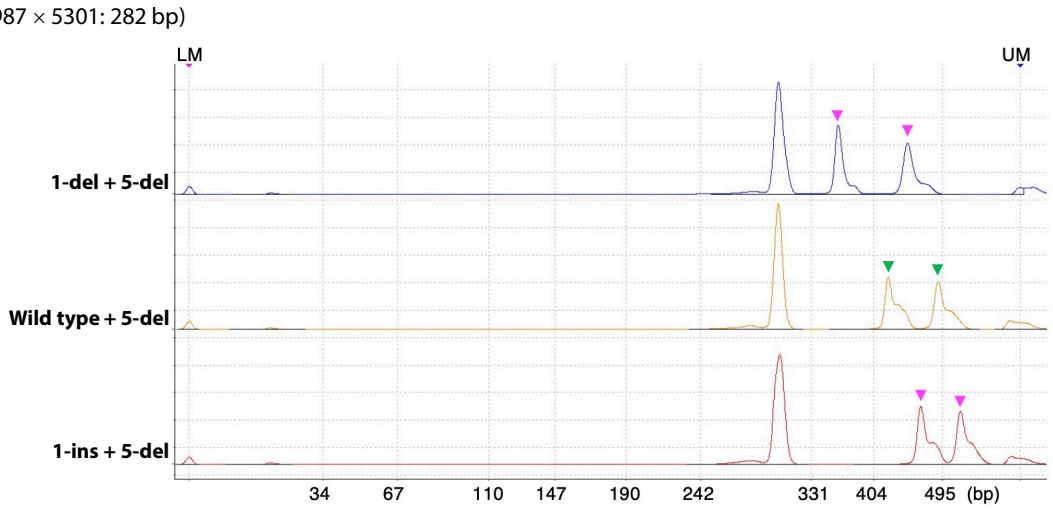


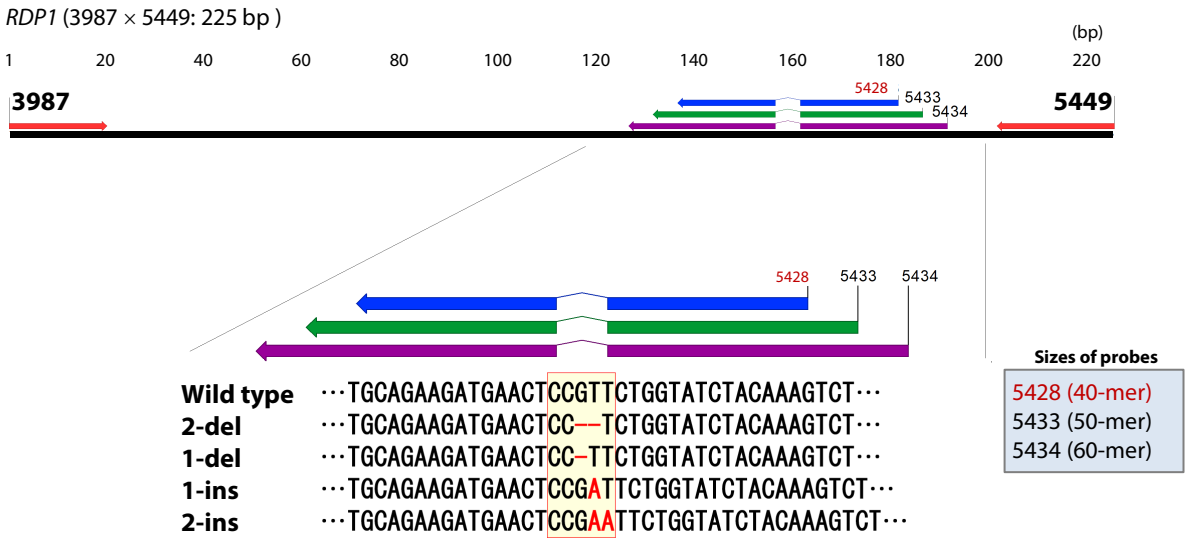
Fig. 2 Heteroduplex Signals from 1-bp Indels by the PrePRIMA Method

a: Sequences used in the experiment.
b: Full-length double-stranded probe with 5 deleted bases used in the prePRIMA.
c: Results of detection by MultiNA.
In the formation of heteroduplexes, "Sample (= 1-ins, 1-del, or wild type)" + "5-bp deleted sequence as probe" were used. Characteristic heteroduplexes signals (▼ or ▼) were detected from the wild-type, 1-del, and 1-ins sequences and the sequences could be distinguished.

4. Development of the PRIMA Method Using a 40-mer Single-Stranded DNA Probe

Although the prePRIMA method can clearly detect 1-bp differences, preparation of a 200-bp probe with a 5-bp deletion (Fig. 2b) is relatively complicated as it requires long primers or two-step PCR. To simplify this step, we tested single-stranded DNA with a shorter base sequence (40-, 50-, 60-mer) as short single-stranded DNA (sssDNA) probes. We found a 40-mer probe could produce heteroduplex signals from 1-bp indels (Fig. 3) [2]. We confirmed that this method was successful with six sequences from humans, bacteria, and plants [2]. We also confirmed that heteroduplex signals could be detected by MultiNA and PAGE; 40-mer sssDNA probes are easily obtained via custom oligo synthesis. Based on these findings, we named this method the PRIMA [2].

a



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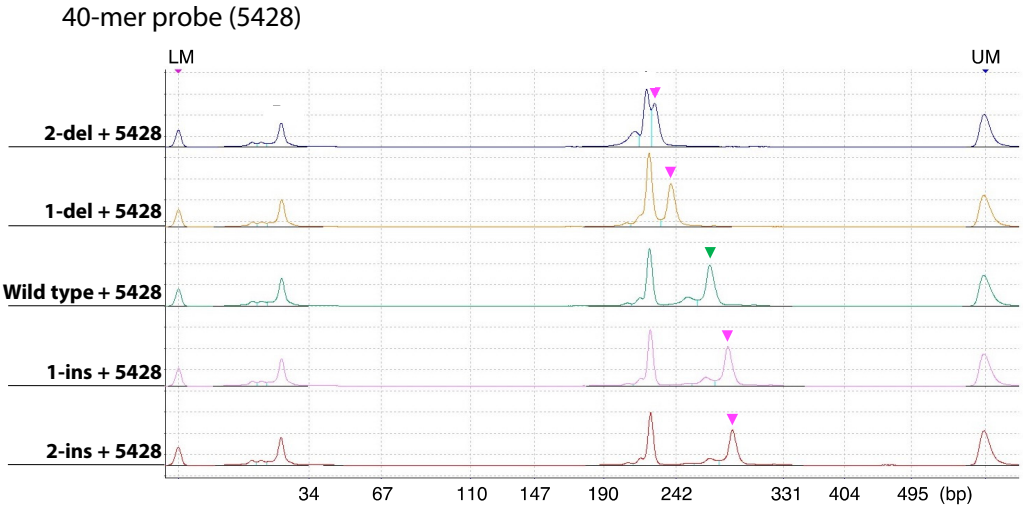


Fig. 3 Detection of Heteroduplex Signals Using Short Single-Stranded DNA (40-mer)

a: Sequence used in the experiment.
b: Results of detection by MultiNA.
Distinguishable heteroduplex signals (▼ or ▼) were detected using the 40-mer probe.

5. Protocol for the PRIMA Method (Fig. 4)

① Design of the primers and probe

Primers are designed to have a total length of approximately 200-bp with a mutation region in the central portion of the PCR product. The probe is designed as 40-mer single-stranded DNA. Since mutations are often produced in the 5-bp upstream of the protospacer adjacent motif (PAM) sequence in the CRISPR/Cas9 system [3], mutant sequences can be detected over a wider range by aligning the 5-bp deletions within this region. We recommend testing both forward- and reverse-strand probes for the first analysis because we currently cannot predict heteroduplex patterns based on primer and probe sequences.

② Amplification of the target sequence by PCR

The target sequence is amplified by standard PCR.

③ Construction of heteroduplexes

In this step, 9 µL of the PCR product and 1 µL of the 10 µM probe are mixed. After denaturation at 95˚C for 5 min, heteroduplex formation is induced by slowly cooling down to 25˚C (0.2˚C/s).

④ Detection of the signal by MultiNA

Then, 10 µL of the reannealed product is subject to electrophoresis by MultiNA. The data are checked using the MultiNA Viewer™.

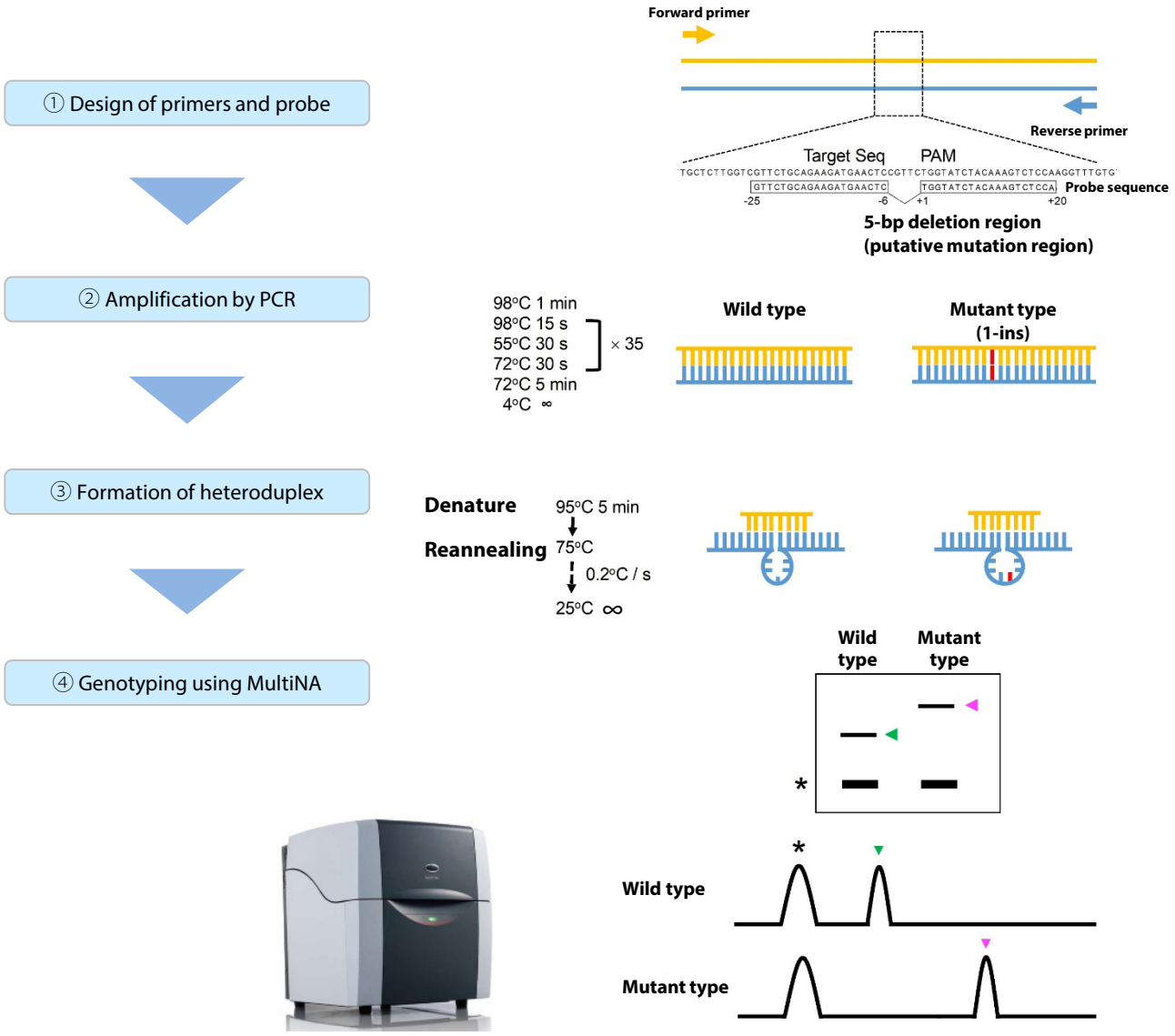
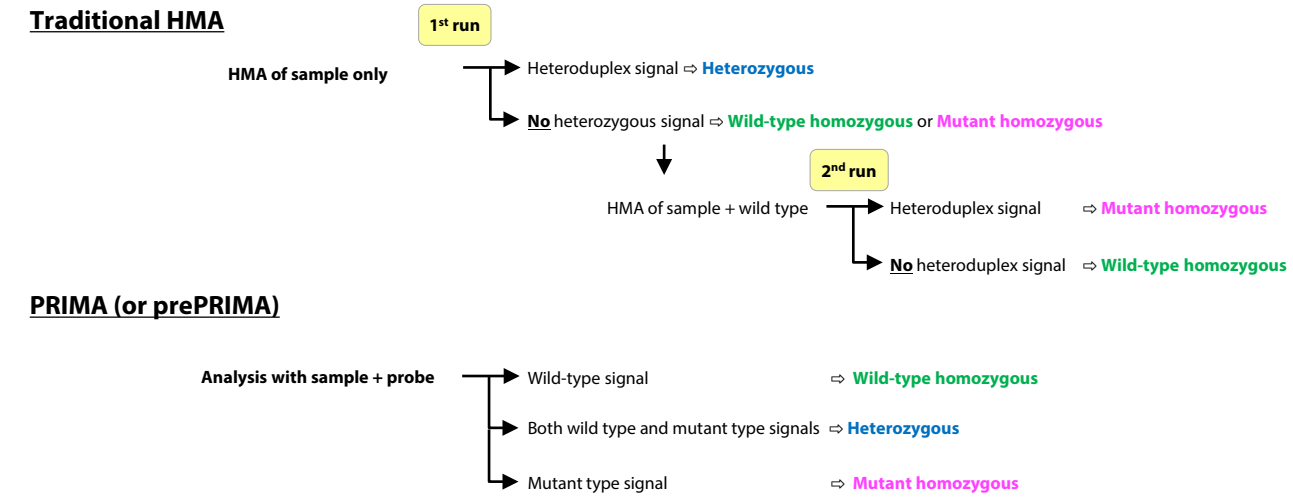


Fig. 4 The PRIMA Protocol

6. Example of the Application of the PRIMA Method ① One-Step Genotyping of Homozygous and Heterozygous Individuals

Analyses in which F1 individuals are maintained as heterozygous and homozygous mutants are selected by genotyping from F2 individuals have been conducted with both plants and animals. In traditional HMAs, the sample is first distinguished as either homozygous or heterozygous. For samples that were judged to be homozygous, it is also necessary to distinguish whether the sample is wild type or mutant homozygous, which means that two runs are necessary in the genotyping of F2 individuals [4] (Fig. 5a, top). By contrast, faster and simpler genotyping is possible by PRIMA analysis, as characteristic peaks can be detected as the homozygous wild type or mutant (Fig. 5a, bottom), and peaks for both the wild type and mutant can be detected from heterozygous individuals in a one-step analysis (Fig. 5b, c).

a



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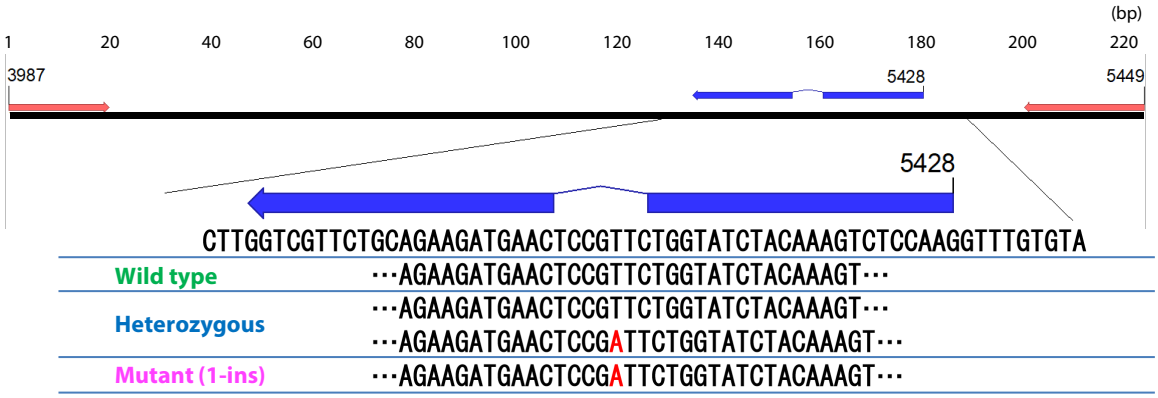

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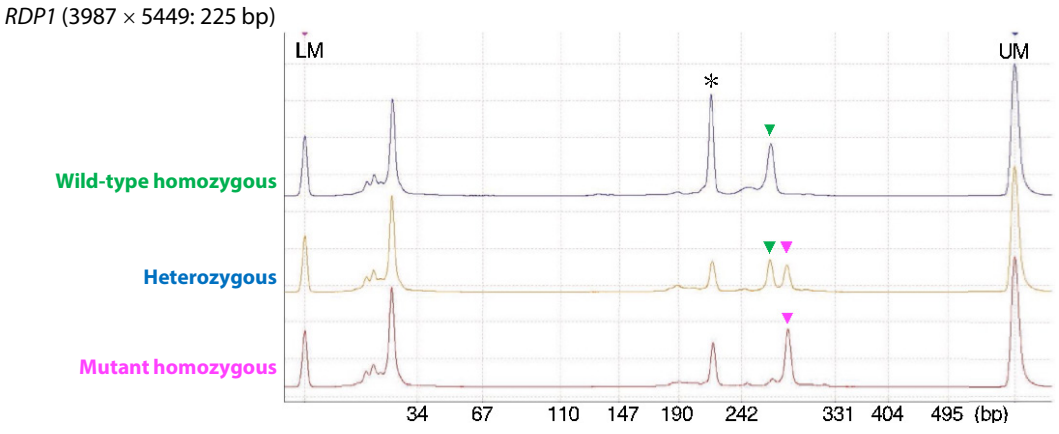


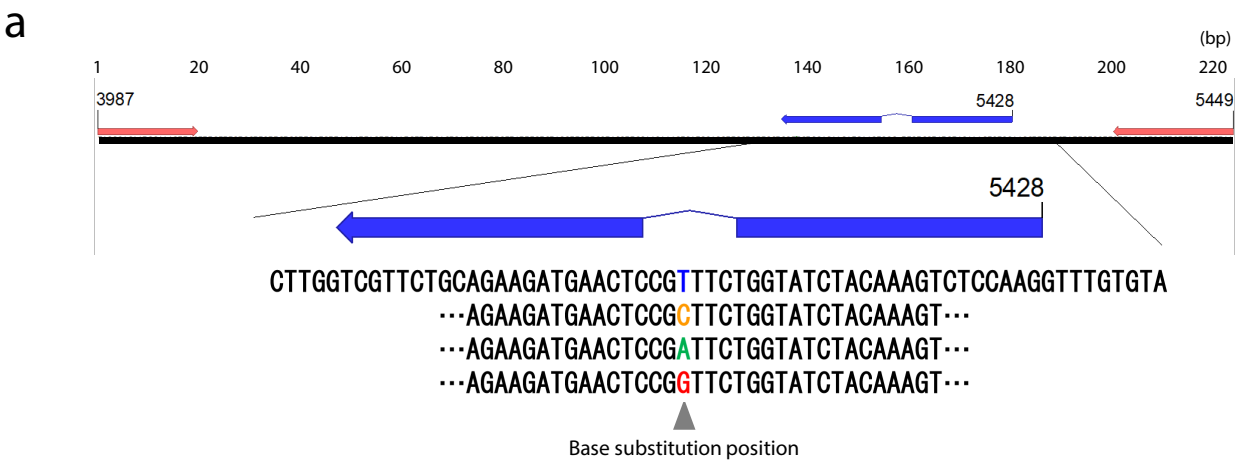
Fig. 5 One-Step Genotyping of F2 Individuals Using the PRIMA Method

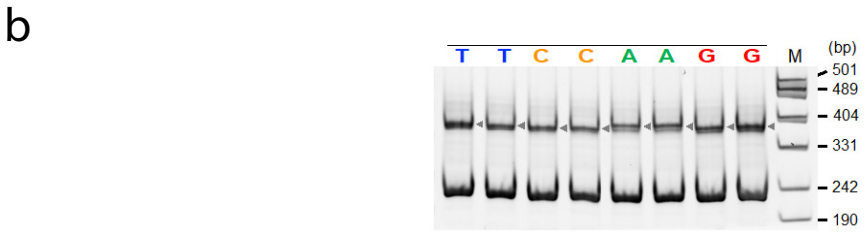
a: Screening strategies for F2 individuals by the HMA (top) and PRIMA (bottom).
b: Sequences and probes used in the experiment.
c: Detection of heteroduplex signals by MultiNA.
Distinguishable signals were detected in a single run [wild-type homozygous (▼); mutant homozygous (▼)].

7. Example of the Application of the PRIMA Method ② Detection of a Single-Nucleotide Polymorphism (SNP)

As described, 1-bp indels can be detected efficiently by PRIMA analysis. However, since SNPs can induce changes in heteroduplex structure [5], PRIMA may also be useful in the detection of SNPs.

To examine this possibility, we prepared a sequence containing a SNP (four-nucleotide variations: A/T/G/C) at the mutation position (Fig. 6a) and conducted a detection experiment using PRIMA. While it was not possible to distinguish the SNP by PAGE (Fig. 6b), characteristic peaks were detected from each of the bases by MultiNA analysis, demonstrating that it is possible to distinguish SNPs using the PRIMA method (Fig. 6c).





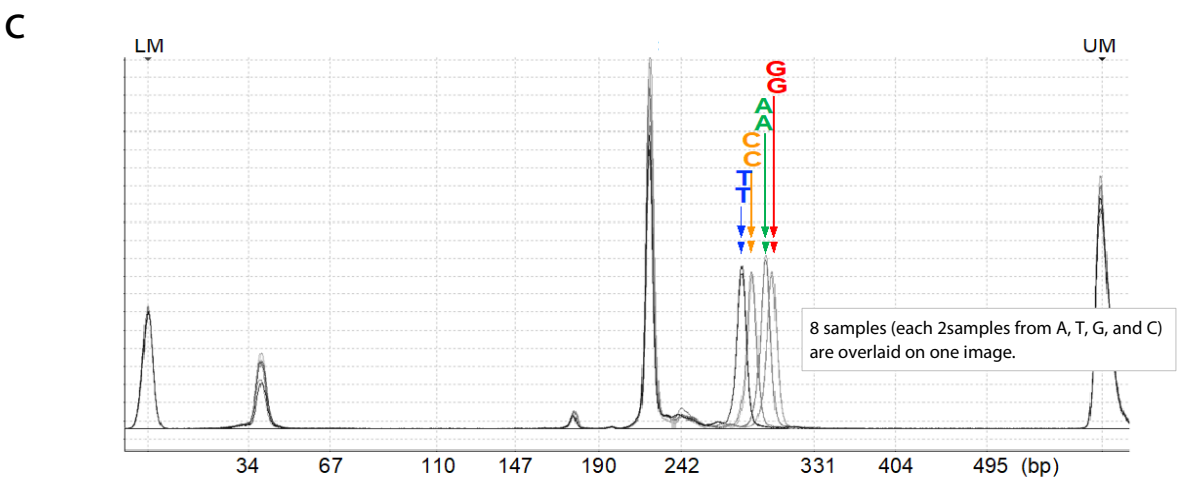


Fig. 6 Detection of SNPs by the PRIMA

a: SNP sequence and probe.
b: Detection by PAGE.
c: Detection by MultiNA.

MultiNA showed distinguishable signals, while PAGE did not.

8. Conclusion

Here, we introduced a new method, the PRIMA, to detect 1-bp differences. The PRIMA is a simple, fast, and cost-effective method because it is based on the HMA. We achieved one-step genotyping for F2 individuals and SNP typing with the PRIMA. While PRIMA analyses can also be carried out by PAGE, simpler and clearer results are possible using MultiNA. In particular, although SNPs could not be detected by PAGE, the individual bases could be clearly distinguished by MultiNA. We recommend testing several primers and/or probes for genotyping the first time because we sometimes found weak or no heteroduplex signals with some primer/probe combinations [(2)]. Accumulation of data on heteroduplex patterns may make it possible to predict appropriate primer/probe combinations in the future.

Single base pair indels and SNPs exist not only in genome-edited individuals but also as natural variations (natural polymorphisms) in genomes, indicating the broader applicability of the PRIMA (e.g., DNA fingerprinting).

<References>

(1) Shimadzu Application Note No. B65, Heteroduplex Mobility Assay (HMA) Using MultiNA.
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