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# Introduction

## MarkMaker GUI

MarkMaker is a tool designed to facilitate the development of Amplification Refractory Mutation System (ARMS) or Cleaved Amplified Polymorphic Sequences (CAPS) markers at any given chromosomal regions based on resequencing data. The operation of MarkMaker is divided into two primary steps: selection of SNP positions and DNA marker development. In SNP selection, SNP positions displaying any genotype can be chosen. When it comes to DNA marker development, the user can choose between tri-ARMS, tetra-ARMS, and CAPS marker types. For reporting issues or making requests concerning this tool, kindly interact with us through “Issues” or “Pull requests” on GitHub (<https://github.com/SegawaTenta/DNAmarkMaker-GUI>).

# Preparation

- Install the following tool:  
Samtools (URL:<http://www.htslib.org>)
- Resequencing data:  
Reference fasta file  
Bam files
- Output folder

# How to use the GUI

## DNAmarkMaker

Output folder:

target\_SNP\_selection

A bam:   B bam:    
Reference fasta:   Chromosome:  Start:  End:   
Samtools path: samtools

A name: A B name: B  
Minimun depth: 10 Maximum depth: 99 Minimun MQ: 0 Minimun BQ: 13  
HeteroSelect ; Heterozygous simulation file:    
ProgenySNP ; Progeny bam:   Progeny SNP simulation file:

ARMS\_preparation

GC% min: 40 GC% opt: 50 GC% max: 60 Ln min: 17 Ln opt: 20 Ln max: 25 Tm min: 60 Tm opt: 62.5 Tm max: 65  
Max poly x: 4 Max self any: 8 Max self end: 3

tri\_ARMS

Make html:  Lower PCR max size: 700 Lower PCR min size: 100 SNPs distance max: 300 SNPs distance min: 100  
GC% min: 40 GC% opt: 50 GC% max: 60 Ln min: 17 Ln opt: 20 Ln max: 25 Tm min: 60 Tm opt: 62.5 Tm max: 65  
Max poly x: 4 Max self any: 8 Max self end: 3

tetra\_ARMS

Make html:  Fw&sRv bands max: 500 Fw&sRv bands min: 100 Rv&sFw max: 1000 Rv&sFw min: 600  
GC% min: 40 GC% opt: 50 GC% max: 60 Ln min: 17 Ln opt: 20 Ln max: 25 Tm min: 60 Tm opt: 62.5 Tm max: 65  
Max poly x: 4 Max self any: 8 Max self end: 3 Max compl any: 6 Max compl end: 3

CAPS

Restriction enzyme list:

Make html:  PCR max size: 1000 PCR min size: 500 Fragment min size: 200  
GC% min: 40 GC% opt: 50 GC% max: 60 Ln min: 17 Ln opt: 20 Ln max: 25 Tm min: 60 Tm opt: 62.5 Tm max: 65  
Max poly x: 4 Max self any: 8 Max self end: 3 Max compl any: 6 Max compl end: 3

## Here's how to specify files and folders in MarkerMaker using the GUI:

- Write the Full Path: You can directly input the full path of the file or folder in the input field.
- Use the Browse Button: You can click on the "Browse" button, which will open a file dialog.
- Drag and Drop: You can select the file or folder from your file explorer and drag it to the input field in the MarkerMaker GUI.

# How to use the GUI

**The configuration screen in MarkerMaker uses a color-coding system to distinguish different content types.**

Pale blue : Process commands

Pale red : Required options

Beige : Additional options

## Output Folder

The ‘Output Folder’ item, located at the top of the screen, must be specified for the execution of all process commands.

## Run

Once the necessary options have been entered, including the ‘Output Folder’ field and the desired process command, you can initiate the execution by clicking the ‘Run’ button, located at the bottom right of the screen.

# How to use the GUI

## MarkerMaker incorporates the following 5 process commands

Each process command is selected by a checkbox.

### **1. target\_SNP\_selection**

This command enables the selection of SNP positions of the required genotype from the BAM files of two cultivars.

### **2. ARMS\_preparation**

This command is used to design cultivar-specific primers.

### **3. tri-ARMS**

This command is employed to develop tri-ARMS markers.

### **4. tetra-ARMS**

This command is used to develop tetra-ARMS markers.

### **5. CAPS**

This command is utilized to develop CAPS markers.

# How to use the GUI

**Here's examples of how to process commands in MarkerMaker**

**Developing ARMS and CAPS markers**

1>>2>>3>>4>>5

**Developing tetra-ARMS markers**

1>>2>>4

**Developing CAPS markers**

1>>5

# Run

## 0. Specify the output folder:

Output folder:  [Browse](#)

### Output Folder:

Select an appropriate destination folder for storing the results generated by MarkerMaker.

 Please note that if you select a folder that has been used for output in the past, existing results may be overwritten. To avoid loss of previous data, ensure you select a different folder for each new run.

# Run

## 1. target\_SNP\_selection:

The screenshot shows a user interface for 'target\_SNP\_selection'. At the top, there's a header bar with the title. Below it is a form area with several input fields and buttons. The fields include 'A bam' and 'B bam' with 'Browse' buttons, 'Reference fasta' with a 'Browse' button, 'Samtools path' with a 'Browse' button, 'Chromosome' with 'Start' and 'End' inputs, and 'Samtools path' with a 'Browse' button. There are also dropdowns for 'A name' (set to 'A') and 'B name' (set to 'B'), and numerical inputs for 'Minimun depth' (10), 'Maximun depth' (99), 'Minimun MQ' (0), and 'Minimun BQ' (13). Buttons for 'HeteroSelect ; Heterozygous simulation file' and 'ProgenySNP ; Progeny bam' with their own 'Browse' buttons are also present.

### A bam

Select the bam file corresponding to one of the cultivars.

### B bam

Select the bam file for the alternative cultivar.

### Reference fasta

Choose the reference fasta file that was used for alignment.

### Chromosome

Input the number of the target chromosome.

### Start

Specify the starting position of the target chromosome.

### End

Specify the ending position of the target chromosome.

### Samtools path

Select the samtools file.

⚠ By default, the system presumes the path is correctly set. If the path isn't set or is incorrect, please ensure to input it correctly.

# Run

## 1. target\_SNP\_selection:

### A name

The name assigned to the A bam sample.

### B name

The name assigned to the B bam sample.

### Minimum depth (Default=10)

The minimum coverage-depth to be ensured during the processes of SNP selection and primer design.

### Maximum depth (Default=99)

The maximum coverage-depth to be ensured during the processes of SNP selection and primer design.

### Minimum MQ (Default=0)

The minimum value for mapping quality.

### Minimum BQ (Default=13)

The minimum value for base quality.

# Run

## 1. target\_SNP\_selection:

### HeteroSelect

#### Heterozygous simulation file

The file contains simulated confidence intervals for the expected allele dosage in the B bam. Users can specify simulations for diploid heterozygosity or tetraploid single SNP dosage. If not specified, select homozygous SNP positions.

### ProgenySNP

#### Progeny bam

The bam file for samples where heterozygosity is expected SNP positions.

#### Progeny genotype simulation file

The file contains simulated confidence intervals for the expected heterozygous SNPs in the 'Additional bam'. It is used to confirm whether the SNP-index value in the additional bam falls within these confidence intervals.

The simulation files for above options can be accessed from GitHub ([https://github.com/SegawaTenta/DNAmarkMaker\\_manual](https://github.com/SegawaTenta/DNAmarkMaker_manual)).

# Run

## 2. ARMS\_preparation

ARMS\_preparation

GC% min:	40	GC% opt:	50	GC% max:	60	Ln min:	17	Ln opt:	20	Ln max:	25	Tm min:	60	Tm opt:	62.5	Tm max:	65
Max poly x:	4	Max self any:	8	Max self end:	3												

### GC% min (Default=40)

The maximum GC content for the primer.

### GC% opt (Default=50)

The optimal GC content for the primer.

### GC% max (Default=60)

The minimum GC content for the primer.

### Ln min (Default=17)

Specify the minimum length of the primer.

### Ln opt (Default=20)

Specifies the optimal primer size.

### Ln max (Default=25)

Specify the maximum length of the primer.

### Tm min (Default=60)

The minimum Tm value for the primer.

### Tm opt (Default=62.5)

The optimal Tm value for the primer.

### Tm max (Default=65)

The maximum Tm value for the primer.

# Run

## 2. ARMS\_preparation

### **Max poly x (Default=4)**

The maximum length of a mononucleotide repeat, such as AAAAAA.

### **Max self any (Default=8)**

Controls the maximum allowable local alignment score when testing a primer for (local) self-complementarity, to avoid primers that can form secondary structures (like hairpins).

### **Max self end (Default=3)**

Controls the maximum allowable local alignment score for 3' end self-complementarity. This helps to avoid primers that can form secondary structures at the 3' end, which can affect the initiation of the PCR.

# Run

## 3. tri-ARMS

tri_ARMS									
Make html:	<input checked="" type="checkbox"/>	Lower PCR max size:	700	Lower PCR min size:	100	SNPs distance max:	300	SNPs distance min:	100
GC% min:	40	GC% opt:	50	GC% max:	60	Ln min:	17	Ln opt:	20
Max poly x:	4	Max self any:	8	Max self end:	3	Ln max:	25	Tm min:	60
						Tm opt:	62.5	Tm max:	65

### Make html

This setting determines if the program should output the results as an HTML file. If checked, the program will generate an HTML file that can be opened in a web browser, making it easier to view and interpret the results.

### Lower bands max size (Default=700)

The maximum expected size of the PCR product for bands observed under the another bands.

### Lower bands min size (Default=100)

The minimum expected size of the PCR product for bands observed under the product.

### SNPs distance max (Default=300)

The maximum distance allowed between SNPs.

### SNPs distance min (Default=100)

The minimum distance allowed between SNPs.

**Other options are similar to “ARMS\_preparation”**

# Run

## 4. tetra-ARMS

tetra\_ARMS

Make html: <input checked="" type="checkbox"/>	Fw&sRv bands max: 500	Fw&sRv bands min: 100	Rv&sFw max: 1000	Rv&sFw min: 600				
GC% min: 40	GC% opt: 50	GC% max: 60	Ln min: 17	Ln opt: 20	Ln max: 25	Tm min: 60	Tm opt: 62.5	Tm max: 65
Max poly x: 4	Max self any: 8	Max self end: 3	Max compl any: 6	Max compl end: 3				

### **Fw&sRv bands max (Default=500)**

The maximum size of the PCR product amplified common Fw and cultivar-specific Rv primers.

### **Fw&sRv bands min (Default=100)**

The minimum size of the PCR product amplified common Fw and cultivar-specific Rv primers.

### **Rv&sFw bands max (Default=1000)**

The maximum size of the PCR product amplified common Rv and cultivar-specific Fw primers.

### **Rv&sFw bands min (Default=600)**

The minimum size of the PCR product amplified common Rv and cultivar-specific Fw primers.

### **Max compl any (Default=6)**

Control the maximum allowable local alignment score when testing primer pairs for complementarity to each other.

### **Max compl end (Default=3)**

Controls the maximum allowable local alignment score for 3' end complementarity when testing primer pairs.

**Other options are similar to “ARMS\_preparation”**

# Run

## 5. CAPS

The screenshot shows the CAPS software interface. At the top, there is a blue header bar with the word "CAPS". Below it is a red toolbar containing a "Restriction enzyme list" input field, a "Browse" button, and several other input fields for PCR parameters: "Make html" (checked), "PCR max size: 1000", "PCR min size: 500", "Fragment min size: 200", "GC% min: 40", "GC% opt: 50", "GC% max: 60", "Ln min: 17", "Ln opt: 20", "Ln max: 25", "Tm min: 60", "Tm opt: 62.5", "Tm max: 65", "Max poly x: 4", "Max self any: 8", "Max self end: 3", "Max compl any: 6", and "Max compl end: 3".

### Restriction enzyme list

A file that provides a list of restriction enzymes along with their corresponding recognition sequences.

### PCR max size (Default=1000)

The maximum size of the PCR product before the process of restriction enzyme digestion takes place.

### PCR min size (Default=500)

The minimum size of the PCR product before the process of restriction enzyme digestion takes place.

### PCR fragment min size (Default=200)

The minimum size of the fragment following restriction enzyme digestion, assuming there is only one restriction enzyme site.

Other options are similar to “ARMS\_preparation” and “tetra\_ARMS”

# **Input file format**

## **Restriction enzyme list**

**EcoRI GAATTC**

**Alul AGCT**

**EcoT14I CCAAGG**

**EcoT14I CCATGG**

**EcoT14I CCTAGG**

**EcoT14I CCTTGG**

Input txt file contains the name of the restriction enzyme and its recognition sequence, separated by single spaces or tabs. In cases where there are multiple recognition sequences, such as with *EcoT14I*, please ensure to include all of them.

# Output

MarkMaker generates 6 types of folders according to process commands

```
<Output folder>
|---target_SNP_selection
|---ARMS_preparation
|---tri_ARMS
|---tetra_ARMS
|---CAPS
|---log
```

The log folder contains each process result and configuration values.

Explain the files output in each folder on the following pages

# Output

Example output file targeting between 1 and 2Mb of chromosome chr01

```
target_SNP_selection
|---SNP.txt
|---error_depth.txt
|---variant.txt
|---shared.chr01.1000000-2000000.seq
|---A.chr01.1000000-2000000.seq
|---B.chr01.1000000-2000000.seq
```

## **SNP.txt**

This file provides a list of positions chosen as target SNPs. Information regarding the chromosome name, position, the nucleotid detected in cultivar A, SNP-index value of cultivar A (which are all zeros), nucleotid specifically detected in cultivar B that differ from cultivar A, and the SNP-index value of cultivar B, are all tab-separated within this file.

## **error\_depth.txt**

This file lists positions where the coverage-depth is either 0, lower than the set value, or higher than the set value. Information about chromosome name, position, status of cultivar A, and status of cultivar B are tab-delimited within the file. The status is indicated by '0' if the coverage-depth equals 0, 'L' if it's lower than the set value, 'H' if it's higher than the set value, and '.' if it's within the range of the set value.

# Output

## **variant.txt**

This file provides variant information for each cultivar. Information about chromosome name, position, status of cultivar A, and status of cultivar B are tab-delimited within the file. The status is indicated by ‘v’ if two bases are detected, ‘M’ if three or more bases are detected, ‘+’ if an insertion is detected, ‘-’ if a deletion is detected, and ‘s’ indicates a homozygous SNP detected between cultivar A and cultivar B.

## **A.chr01.1000000-2000000.seq**

This file contains the sequence for chromosome region A01:1-2Mb for cultivar A. Locations where the coverage-depth of cultivar A is outside the set value or where cultivar A exhibits variants are denoted with ‘N’.

## **B.chr01.1000000-2000000.seq**

This file contains the sequence for chromosome region A01:1-2Mb for cultivar B. Locations where the coverage-depth of cultivar B is outside the set value or where cultivar B exhibits variants are denoted with ‘N’.

## **shared.chr01.1000000-2000000.seq**

This file provides a consensus sequence for the chromosome region chr01:1-2Mb. For positions that are SNP locations, and those where the coverage-depth is outside the set range or have a variant, the output is indicated as ‘N’.

# Output

ARMS\_preparation  
|---made\_primers.txt

## **made\_primers.txt**

This file describes the information about the designed cultivar-specific primers. It contains tab-delimited fields for chromosome name, position, cultivar A-specific Fw, Fw primer sequence for cultivar A, Tm value, GC% and length for cultivar A, cultivar B-specific Fw, Fw primer sequence for cultivar B, Tm value, GC% and length for cultivar B, cultivar A-specific Rv, Rv primer sequence for cultivar A, Tm value, GC% and length for cultivar A, cultivar B-specific Rv, Rv primer sequence for cultivar B, Tm value, GC% and length for cultivar B.

# Output

```
tri_ARMS
|---tri_site_sFw.txt
|---tri_site_sRv.txt
|---made_primers.txt
|---html
    |---chr01.1455448.1455575.sRv.html
    |--- . . .
```

## **tri\_site\_sFw.txt and tri\_site\_sRv.txt**

This file provides information about the primer combinations that can be used to design tri-ARMS markers. It contains tab-delimited fields for chromosome, one position, cultivar-specific primer sequence, Tm value, GC%, length, another position, another cultivar-specific primer sequence, Tm value, GC%, and length.

## **made\_primers.txt**

This file provides a summary of the designed primers. The file includes tab-delimited fields for chromosome, one position, cultivar-specific primer sequence, Tm value, GC%, length, another position, another cultivar-specific primer sequence, Tm value, GC%, length, common primer sequence, Tm value, GC%, length, and the start and end positions of the PCR product.

**'html' folder contains files that summarized each marker's information along with the resequencing results within the PCR amplified region. Please refer to 'HTML file' section for further details on the content of these html files.**

# Output

```
tetra_ARMS
|---tetra_site.txt
|---made_primers.txt
|---html
    |---chr01.1454535.html
    |--- . . .
```

## **tetra\_site.txt**

This file provides information about the primer combinations that can be used to design tri-ARMS markers. It contains tab-delimited fields for chromosome, one position, cultivar-specific primer sequence, Tm value, GC%, length, another position, another cultivar-specific primer sequence, Tm value, GC%, and length.

## **made\_primers.txt**

This file provides a summary of the designed primers. The file includes tab-delimited fields for chromosome, one position, cultivar-specific primer sequence, Tm value, GC%, length, another position, another cultivar-specific primer sequence, Tm value, GC%, length, common Fw sequence, [Position of sequence used to design Fw, length], Tm value, GC%, length, common Rv sequence, [Position of sequence used to design Rv, length], Tm value, GC%, length, and the start and end positions of the PCR product.

**'html' folder contains files that summarized each marker's information along with the resequencing results within the PCR amplified region. Please refer to 'HTML file' section for further details on the content of these html files.**

# Output

## CAPS

```
|---CAPS_site_cut_A.txt  
|---CAPS_site_cut_B.txt  
|---made_primers.txt  
|---html  
    |---chr01.1454535.html  
    |--- . . .
```

### **CAPS\_site\_cut\_A.txt** and **CAPS\_site\_cut\_B.txt**

These files contain a list of positions where the target SNP is located on the restriction enzyme site in either cultivar A or B. Each file includes tab-delimited fields for chromosomes, positions, bases of SNP positions, restriction enzymes, recognition sequences, the start and end positions of the restriction enzyme sites.

### **made\_primers.txt**

This file provides a summary of the designed primers. The file includes tab-delimited fields for chromosome, SNP position, restriction enzymes, recognition sequences, sample name containing restriction enzyme sites, starting and ending positions of restriction enzyme sites, Fw sequence, [Position of sequence used to design Fw, length], Tm value, GC%, length, Rv sequence, [Position of sequence used to design Rv, length], Tm value, GC%, length, and the start and end positions of the PCR product.

**'html' folder contains files that summarized each marker's information along with the resequencing results within the PCR amplified region. Please refer to 'HTML file' section for further details on the content of these html files.**

# HTML file

## ① tri ARMS marker

② Chromosome Position  
chr03 1455942-1456698

③ Target SNP  
1455966 (25) 1456264 (323)

④ Primers Sequence Length Tm GC%  
Rv TGGGCTCATAGCTACCAAGT 21 60.6 52.3  
I65\_Fw CGGATACTAATGAAGAGCTACGCC 25 60.6 48.0  
IS68\_Fw CGTGGGATGCGCGATACTTC 21 61.0 57.1

⑤ PCR product size  
757

## ⑥ I65

CGGATACTAA**T**GAAGAGCTACGGC**C**TGAACAAAACGCATAAT**C**CCAGTCTTATCCTATTCAACTCCTCATTGT**A**TTGCTGCTCCCTCGTTG  
>>>>>>>>>>>>>>>>  
**M**  
AAGTAAAATAA**A**TATAGCTATATATTATTCAAGATAAAAATAACTATATTAGAATATTAGA**A**ACAGAGAAAGTAGAAAGGAGCTCACGTT**A**GCC  
+LLLLLLL  
ATACCAC**T**AGTAACTATGTCAACCAATAGATCATACTCCTCGTATTAGCCATTAAACTGATGAGTATCGTAAGAAAGTTATAAAATCTCATTACAA  
L L  
TGGTGGGATGCGCGATATAGT**T**ATTACTATATTAA**C**AGTGTGGAAGATTAAGGGCGGTGCGCAATTGTATCTCGTACTGTCAAT  
LLLLLLLLLLLLLLLL  
>>>>>>>>>>>>  
CATAAGTTCAATT**C**ATCTATA**CC**CTTAAAAGTCTTAATATTGGTGCAAAGAATTGCCCCTTACCATGCTTTGAGTTTTAGAAATTG**C**AT  
LLLLLLLLLLLL  
CATTATGTTT**C**ATGACAATTGCTCTATCCATATTGAATATATGAGATTATT**CG**ATATATCTT**G**AAAATT**G**ATCGTCAATAGCTAAAAACAGT  
**M**  
TAGTTGAAACACATAACAATCATATGGAACATTTTATTATGAGTTATT**GG**ATATATCTT**G**AAAATT**G**ATCGTCAATAGCTAAAAACAGT  
CTATTACTCCAGGAGTACA**A**CTATAGATCAGGACAA**C**TTGGTAGCTATGAGCCCC  
<<<<<<<<<<<<

## IS68

CGGATACT**C**AGGAAGAGCT**G**CGG**T**TG**C**ACAAACCG**C**AT**G**ATTCCAGTCTTATCCTATTCAACTCCTCATTGT**A**TTGCTGCTCCCTCGTTG  
>>>>>>>>>>>>  
**M**  
AAGTAAAATAATT**G**CTATATATTATTCAAGATAAAA**C**TA**A**CTATATT**T****G**AA**A**TTTTAGAACAG**C**TA**A**TTAGAAAGGAGCTCACGTT**G**CC  
ATACCAC**T**AGTA**C**ATGTCAACCAATAGATCATA**C**GTCTCGTATTAGCCATTAAACTGATGAGTATCGTAAGAAAGTTATAAAATCTCATTACAA  
TGGTGGGATGCGCGATATAGT**C**ATTAC**C**ATATTAA**T**ACT**C**AGTGTGGAAGATTAAGGGCGGTGCGCAATTGTATCTCGTACTGTCAAT  
>>>>>>>>>>  
CATAAGTTCAATT**C**ATCTATA**CC**CTTAAAAGTCTTAATATTGGTGCAAAGAATTGCCCCTTACCATGCTTTGAGTTTTAGAAATTG**C**AT  
CATTATGTTT**C**ATGACAATTGCTCTATCCATATTGAATATATGAGATTATT**CG**TTATCTT**G**AAAATT**T**AA**C**CGTCAATAGCT**G**AAAACAGT  
**M**  
TAGTTGAAACACATAACAATCATATGGAACATTTTATTATGAGTTATT**GG**ATATATCTT**G**AAAATT**G**ATCGTCAATAGCT**C**AAAATATAGCTAT  
CTATTACT**T**AGGAGTACA**A**CTATAGATCAGGACAA**C**TTGGTAGCTATGAGCCCC  
<<<<<<<<<

① : DNA marker type.

② : PCR amplification chromosome positions.

③ : Target SNP Positions.

The numbers in ( ) represent the positions when the starting point of the PCR amplification region is set as 0.

# **HTML file**

## **④ : Primer Information**

This section lists the sequences, lengths, Tm values, and GC% of the generated primers. When a cultivar name is specified before 'Fw' and 'Rv' (like 'A\_Fw' or 'B\_Fw'), it indicates that the primers are cultivar-specific and are intended for ARMS markers.

## **⑤ : PCR Product Size**

This indicates the length of the longest DNA fragment generated from the PCR amplification resulting from all primer combinations.

# HTML file

## ⑥ : Resequencing Results

This section provides sequence information for each cultivar in the PCR-amplified region. The data is arranged in 100bp per line, split into top and bottom lines. The top lines represent the array and highlights variations: positions with two different detected base types are shown in green, while homozygous SNPs differing from the other cultivar are shown in red. The bottom lines indicate the positions of the primers and other related information. Fw primers are symbolized by '>', while Rv primers are symbolized by '<'. Cultivar-specific primers are color-coded in a manner similar to the Primer Information section. Positions detecting three or more types of bases are marked as 'M', positions with detected insertions are marked as '+', positions with deletions are marked as '-', positions with coverage-depth=0 are marked as '0', positions where coverage-depth is lower than the set value are displayed as 'L', and positions with a coverage-depth higher than the set value are displayed as 'H'. The asterisk (\*) is utilized exclusively in the design of tetra-ARMS markers. It signifies a position that simultaneously serves as the target position and the position of the 3' ends of the two cultivar-specific primers.

# HTML file

①

## CAPS marker

②

Chromosome Position  
chr03 1296317-1297234

③

Target SNP  
1296786 (470)

④

Restriction Sequence Sample

⑤

EcoRI GAATTC I65  
Primers Sequence Length Tm GC%  
Fw TGGTACGAAACTCATGCGTTCTTCT 23 62.4 47.8  
Rv ACCCTATGTACGCAATACCC 22 60.6 50.0

⑥

PCR product size  
917

⑦

### I65

TGGTACGAAACTCATGCGTTCTTCTTCTTGTGCTGAAAGCATGCATGCTGATATATTCTTTGGTACCAACTTTATCAGATGATATCATGTTATACCA  
>>>>>>>>>>>>  
ATGTTATGCAAAGCCCCAACATTATATATTATCTATAAGATTACTATTAAACGAACATATGTGTTATTCACCTTCTTAGTATGAAGGTTCATGCACAAAA  
CGAAAGTTATTACAATCCTTGTAAATTAAAGAATAATCGATGATAGAGAAGTCATTGGGTGAGAGAATGTTAGGGTACGCTGGATATCTAGAA  
AGGAAAATCTATCCTTGATGGAAAGGCAGGCCATTGGTAATGCTAGAAGATTCTCGCTCAGCTAGAAAAGAAAAGGCTAATAATGACATCAAAGAC  
CTTGCCTGGTAGAGAACCAAATTGGCCTACACCCACAAGAGCAAGAAAAGCTAGAAACATGACGAATTTCACACCGCATCTCGTCAATTAGCAGACT  
TTGCATCCAATAATTATTGCTCTTGTCTACGCATGATAAAAGAAATATAAGCTAGCGATCAAATGTTATGCTCTTCTATCATAGATTCTT  
GGTTTATTCTATTAGTTGGATAAGGAAGCTATAACATCCTTGATGTTATGCTAAACTAAATGTCTATCCAAAATAAAAGGTTGCAAACATGC  
CAAAAGATAACATACACCTATAGGTATGAAATTAAATACAGAATTCTCAAACACTAAATTGTTATTTGCTCTATGGAGTATG  
CGACAAAGAACTATTCTATGACAATTGTTATAAAGGGAAACTAACAGTGGCATTATGATAAGCAAGTTAAGGGTATATGTAACAAACGAAAGGGT  
<<<<

### IS68

TGGTACGAAACTCATGCGTTCTTCTTCTTCTTGTGCTGAAAGCATGCATGCTGATATATTCTTTGGTACCAACTTTATCAGATGATATCATGTTATACCA  
>>>>>>>>>>>  
ATGTTATGCAAAGCCCCAACATTATATATTATCTATAAAATTAAATTACCAAACATATGTGTTATTCACCTTCTTAGTATGAAGGTTCATGCACAAAA  
CGAAAGTTATTACAATCCTTGTAAATTGTTAGAGAATAATCAATGTTAGAGAAGTATCATTGGGTGAGATAATGTTAGGGTACGCTGGATATCTAGAA  
AGGAAAATCTATCTTGATGGAAAGGCAGGCCATTGGTAATGCTAGAAGATTCTCGCTCCCTAGAAAAGAACAGGCTCATATGACATCAAAGAC  
CTTGCCTGGTAGAGAACCAAATTGGCCTACACCCAAAGAGCAAGAAAAGCTAGAAACATGACGAACCTCACACCGCATATGTCATTGAGACT  
TTGCATCCAATAATTATTGCTCTTGTCTATGCTATGATAAAAGAAATATAAGCTAGCGATCAAATGTTATGCTCTTCTATCATAGATTCTT  
GGTTTATTCTATTAGTTGGATAAGGAAGCTATAACATCCTTCTATGTTATGCTAAACTAAATGTCTATCCAAAATAAAAGGTTGCAAACATGC  
CAAAAGATAACATACACCTATAGGTATGAAATTAAATACAGAATTCTCAAACACTAAATTGTTATTTGCTCTATGGAGTATG  
CGACAAAGAACTATTCTATGACAATTGTTATAAAGGGAAACTAAAAGTGGCATTATGATCAGTGAGCAAGTTAAGGGATATGTAACAAACGAAAGGGT  
<<<<

① : DNA marker type.

② : PCR amplification chromosome positions.

# **HTML file**

## **③ : Target SNP Positions.**

The numbers in ( ) represent the positions when the starting point of the PCR amplification region is set as 0.

## **④ : The name and sequence of the restriction enzyme to be used and the name of the sample to be cut**

## **⑤ : Primer Information**

This section lists the sequences, lengths, Tm values, and GC% of the generated primers.

## **⑥ : PCR Product Size:**

This refers to the length of the DNA fragment produced through PCR amplification prior to the process of restriction enzyme digestion.

# HTML file

## ⑦ : Resequencing Results

This section provides sequence information for each cultivar within the PCR-amplified region. The data is divided into 100bp segments per line, organized into upper and lower lines. The upper lines display the sequence, with positions where two distinct bases were detected highlighted in **green**, and homozygous SNPs differing from the alternate cultivar shown in **red**, while restriction enzyme sites are denoted in **purple**. The lower lines delineate the primer positions along with other pertinent information. Fw primers are indicated by '>', and Rv primers by '<'. Positions that detected three or more base types are designated as 'M', those with detected insertions are noted by '+', deletion positions are marked by '-', positions with a coverage-depth of zero are labeled '0', positions where coverage-depth is lower than the pre-set value are shown as 'L', and positions with a coverage-depth higher than the pre-set value are exhibited as 'H'.