PCR-RFLP for genotyping candidate *P. falciparum* artemisinin resistance SNPs MAL10-688956 and MAL13-1718319 v1.1

Procedure

WWARN Procedure

Procedure ID: MOL07
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Molecular Module, WWARN

**Version History**

<table>
<thead>
<tr>
<th>Version number</th>
<th>Revision(s) &amp; reason for amendment</th>
<th>Release date</th>
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<td>1.0</td>
<td>Creation of procedure</td>
<td></td>
</tr>
<tr>
<td>1.1</td>
<td>Fixed photo credits and added PCR run times</td>
<td>18/12/2012</td>
</tr>
</tbody>
</table>

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WorldWide Antimalarial Resistance Network (WWARN)

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WWARN Procedure: PCR-RFLP for genotyping SNPs on MAL10 and MAL13 v1.1
Page 2/14
1. Purpose .......................................................................................................................... 4
2. Scope ............................................................................................................................. 4
3. Abbreviations .................................................................................................................. 4
4. Duties and Responsibilities ........................................................................................... 4
5. Materials and Equipment ............................................................................................... 5
   Table 1. Primary (1⁰) and secondary (2⁰) forward (F) and reverse (R) PCR primers .5
5.2 Equipment .................................................................................................................... 6
6. Procedure ......................................................................................................................... 6
   6.1 PCR .............................................................................................................................. 6
   Table 2. Master Mix calculation for Primary and Secondary PCR ................................. 6
   for MAL10-688956 and MAL13-1718319 ................................................................. 6
   6.2 RFLP .......................................................................................................................... 8
   6.3 Agarose gel electrophoresis ....................................................................................... 9
7. References ......................................................................................................................... 10
Appendix A 3D7 sequences of MAL10-688956 and MAL13-1718319 ......................... 11
Appendix B Restriction sites and DNA controls for MAL10-688956 and MAL13-1718319 ................................................................. 13
Appendix C Standard curves for restriction digest of MAL10-688956 and MAL13-1718319 ........................................................................................................................................ 14
1. Purpose

This procedure is designed to genotype point mutations on chromosome 10 (MAL10-688956 in the 3’ untranslated region of the gene encoding DNA polymerase delta catalytic subunit PF10_0165) and 13 (MAL13-1718319 in the RAD5 homologue gene MAL13P1.216) of *Plasmodium falciparum*, identified in a genome-wide association study by Takala-Harrison et al, 2012\(^1\).

2. Scope

This procedure is intended for use in molecular studies of DNA extracted from dried blood spots or whole blood samples for genotyping of *P. falciparum* infections. It describes the genotyping procedure for two SNPs, one at position 688956 on chromosome 10 (MAL10) and one at position 1718319 on chromosome 13 (MAL13). Full gene sequences are given in Appendix A and restriction sites are given in Appendix B. This procedure is applicable for well-equipped laboratories with staff familiar with PCR, RFLP, and agarose gel electrophoresis.

3. Abbreviations

- bp: base pairs
- DNA: Deoxyribonucleic Acid
- PCR: Polymerase Chain Reaction
- µL: microlitre
- mL: millilitre
- ng: nanogram
- pg: picogram
- RFLP: Restriction Length Fragment Polymorphism
- SNP: Single Nucleotide Polymorphism

4. Duties and Responsibilities

N/A
5. Materials and Equipment

5.1 Materials

I. General
- Micropipets and tips (10 µL, 200 µL and 1000 µL)
- 1.5 mL centrifuge tubes
- PCR tubes with caps
- Disposable gloves
- Fine tip marker pens
- Nuclease-free water

II. PCR
- 10X PCR buffer (MgCl₂-free)
- MgCl₂ (concentration varies)
- dNTP (concentration varies)
- Taq DNA Polymerase (5U/µL)
- Primers (see Table 1)
- Parasite DNA standards: 3D7 and V1/S strains at 25 pg/µL

III. RFLP
- Restriction enzymes: NsiI (10U/µL), MsiI (5U/µL)
- New England Biolabs Buffer B7003S (NEBuffer 3) and B7004S (NEBuffer 4)

IV. Agarose gel electrophoresis
- 6X Xylene cyanol loading dye
- Ethidium bromide (10 mg/mL)
- Agarose
- 1X TBE (Tris/Borate/EDTA) buffer
- 50 bp or 100 bp size standard with xylene cyanol loading dye added
- Parafilm

Table 1. Primary (1⁰) and secondary (2⁰) forward (F) and reverse (R) PCR primers

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Chromosome</th>
<th>1⁰/2⁰</th>
<th>F/R</th>
<th>Sequence (5’ – 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAL10-EF</td>
<td>10</td>
<td>1⁰</td>
<td>F</td>
<td>TGTTATGAATAGGGATTGTCC</td>
</tr>
<tr>
<td>MAL10-ER</td>
<td>1⁰</td>
<td>R</td>
<td>GGAACATTATGCGATCAAC</td>
<td></td>
</tr>
<tr>
<td>MAL10-IF</td>
<td>2⁰</td>
<td>F</td>
<td>GACGAGCATAAATTTGGAAGATAGC</td>
<td></td>
</tr>
<tr>
<td>MAL10-IR</td>
<td>2⁰</td>
<td>R</td>
<td>TTATATGTAATGCGGATTGAGAATGG</td>
<td></td>
</tr>
<tr>
<td>MAL13-EF</td>
<td>13</td>
<td>1⁰</td>
<td>F</td>
<td>GAAATAGGATGATGAGAGATG</td>
</tr>
<tr>
<td>MAL13-ER</td>
<td>1⁰</td>
<td>R</td>
<td>CTAATTAGGGATGATGACCATC</td>
<td></td>
</tr>
<tr>
<td>MAL13-IF</td>
<td>2⁰</td>
<td>F</td>
<td>AGGAAGCAGGAGGGATGAGC</td>
<td></td>
</tr>
<tr>
<td>MAL13-IR</td>
<td>2⁰</td>
<td>R</td>
<td>TTCTAAAATAAACACATTGCATGACA</td>
<td></td>
</tr>
</tbody>
</table>
5.2 Equipment
- Thermocycler
- Gel electrophoresis apparatus including chamber and power pack
- Microwave to melt agarose

6. Procedure

6.1 PCR
I. Prepare Primary PCR Master Mixes in a 1.5 mL centrifuge tube according to the volumes calculated using Table 2. Include enough reactions for DNA controls (3D7 and V1/S) and negative (no template) controls.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Stock Conc. (may vary)</th>
<th>Final Conc.</th>
<th>Vol. X 1 sample</th>
<th>Vol. X N samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Nuclease-free water</td>
<td></td>
<td></td>
<td>19.50</td>
<td></td>
</tr>
<tr>
<td>2. 10X PCR Buffer</td>
<td>10X</td>
<td>1X</td>
<td>2.50</td>
<td></td>
</tr>
<tr>
<td>3. MgCl₂</td>
<td>25 mM</td>
<td>1 mM</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>4. dNTP</td>
<td>25 mM</td>
<td>1 mM</td>
<td>0.20</td>
<td></td>
</tr>
<tr>
<td>5. Primer - F</td>
<td>10 uM</td>
<td>0.1 µM</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>6. Primer - R</td>
<td>10 uM</td>
<td>0.1 µM</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>7. Taq Polymerase</td>
<td>5U/µL</td>
<td>0.06 U</td>
<td>0.30</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>24 µl</td>
<td></td>
</tr>
</tbody>
</table>

II. Label PCR tubes and add 24 µl Primary Master Mix to each tube.

III. Add 1 µl of template DNA to each tube. Seal and run PCR in thermocycler according to the conditions listed in Table 3.
Table 3. PCR thermocycling conditions for Primary PCR for MAL10-688956 and MAL13-1718319

<table>
<thead>
<tr>
<th>Step no.</th>
<th>Cycle</th>
<th>Temperature (°C)</th>
<th>Time (min)</th>
<th>No. of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Initial Denaturation</td>
<td>95</td>
<td>15:00</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Denaturation</td>
<td>95</td>
<td>0:30</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Annealing</td>
<td>50</td>
<td>0:45</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Extension</td>
<td>72</td>
<td>1:00</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Final extension</td>
<td>72</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>Hold</td>
<td></td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

IV. Prepare Secondary PCR Master Mixes in a 1.5 mL centrifuge tube according to the volumes calculated using Table 2.

V. Label PCR tubes and add 24 µl Secondary Master Mix to each tube.

VI. Add 1 µl of Primary PCR product to each tube. Seal and run PCR in thermocycler according to the conditions listed in Table 4.

Table 4. PCR thermocycling conditions for Secondary PCR for MAL10-688956 and MAL13-1718319

<table>
<thead>
<tr>
<th>Step no.</th>
<th>Cycle</th>
<th>Temperature (°C)</th>
<th>Time (min)</th>
<th>No. of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Initial Denaturation</td>
<td>95</td>
<td>15:00</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Denaturation</td>
<td>95</td>
<td>0:30</td>
<td>30</td>
</tr>
<tr>
<td>3</td>
<td>Annealing</td>
<td>55</td>
<td>0:45</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Extension</td>
<td>72</td>
<td>1:00</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Final extension</td>
<td>72</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>Hold</td>
<td></td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

VII. Run an agarose gel of undigested Secondary PCR product to ensure amplification has been successful (See Section 6.3).

NOTE: PCR product may be stored at 4 °C for up to 1 week or at −20 °C or -80 °C for long-term storage.
6.2 RFLP

I. Prepare the restriction digest Master Mix according to the volumes calculated using Table 5.
   - Enzyme NsiI with NEBuffer 3 is used to digest MAL10
   - Enzyme MsiI with NEBuffer 4 is used to digest MAL13

Table 5. Restriction digest Master Mix for MAL10-688956 and MAL13-1718319

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Nuclease-free water</td>
<td></td>
<td></td>
<td>14.50</td>
<td></td>
</tr>
<tr>
<td>2. 10X NEBuffer 3 / 4</td>
<td>10X</td>
<td>1X</td>
<td>2.00</td>
<td></td>
</tr>
<tr>
<td>3. NsiI / MsiI</td>
<td>10U/µL / 5U/µL</td>
<td>0.25 U / 0.125 U</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>17 µl</td>
<td></td>
</tr>
</tbody>
</table>

II. Label PCR tubes and add 17 µl Restriction digest Master Mix to each tube.

III. Add 3 µl of Secondary PCR product to each tube. Seal and run in thermocycler according to the conditions listed in 6.

Table 6. Restriction digest conditions for MAL10-688956 and MAL13-1718319

<table>
<thead>
<tr>
<th>Locus</th>
<th>Step no.</th>
<th>Temperature (°C)</th>
<th>Time (min)</th>
</tr>
</thead>
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<tr>
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<td>1</td>
<td>37</td>
<td>60:00</td>
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<td></td>
<td>2</td>
<td>80</td>
<td>20:00</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>4</td>
<td>hold</td>
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<tr>
<td>MAL13-1718319</td>
<td>1</td>
<td>37</td>
<td>60:00</td>
</tr>
<tr>
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<td>2</td>
<td>65</td>
<td>20:00</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>4</td>
<td>hold</td>
</tr>
</tbody>
</table>
6.3 Agarose gel electrophoresis

I. Make a 2% agarose gel:
   a. Dissolve 2 grams of agarose and 100 mL of 1X TBE in the microwave.
   b. Cool, then add 4 µL Ethidium bromide and gently swirl to mix.
   c. Pour into assembled gel tray with comb(s) and leave at room temperature for 30 minutes to set.

II. Load the gel:
   a. Place the gel in gel apparatus and fill to line with 1X TBE.
   b. Place 3-4 µL dots of loading dye on Parafilm; 1 dot per sample to be loaded.
   c. Carefully pipet 3-5 µL undigested secondary PCR product or 20 µL digested product to each dot of loading dye. If loading digested product next to undigested product for each sample (see Figure 1), load the same amount of undigested product as was added to the digestion reaction (in this case, 3 µL).
   d. Load each dye + product mixture into wells. Add 4-5 µL 50 bp or 100 bp size standard + loading dye to wells on either side of the products.

III. Run gel at 100 volts for 60 minutes and view using a UV transilluminator.

![Agarose gel electrophoresis image]

**Figure 1.** Undigested (a) and digested (b) PCR products for MAL10-688956 (top) and MAL13-1718319 (bottom). 1a and b: 3D7 @ 50 pg/µL, 2a and b: 3D7 @ 10 pg/µL, 3a and b: 3D7 @ 0.5 pg/µL, 4a and b: V1/S @ 50 pg/µL, 5a and b: V1/S @ 10 pg/µL, 6a and b: V1/S @ 0.5 pg/µL, 7a and b: 1º PCR negative control, 8a and b: 2º PCR negative control.

Image source: Sandra Mon, University of Maryland, Baltimore.

WWARN Procedure: PCR-RFLP for genotyping SNPs on MAL10 and MAL13 v1.1
Page 9/14
7. References

Appendix A 3D7 sequences of MAL10-688956 and MAL13-1718319

2000 bp sequences flanking candidate marker SNPs from 3D7 complete genome are given. Positions 688956 of MAL10 and 1718319 of MAL13 are shown in red.

>MAL10: 687956-689956

AAAGTATATTGCCCATTTTATTATAAATTTTTTTTTTTTATTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT

WWARN Procedure: PCR-RFLP for genotyping SNPs on MAL10 and MAL13 v1.1 Page 11/14
GAAACGTTCCTCTCGAATCCATTTTCCATACCTGTTTGATTAATAATATA

>MAL13: 1717319-1719319

TCTACTTTAGTTTCTGAATATAAAAAATACGTTAATAAATAAAAATCTC
AAAAGCAGAAAAATAATAATAAATG[7/G]AGTACATATAACAATAAAGAATCATTT
TTAAAGATGTATCAGTACTACGAAAAAAAAGATGAACAACATCTT
ATGAAAACTGTGTTAAATAGTGG[7/C]AATAAAAATAATGATAATAATTTTT
TTTGGATAAAAAAGACTAAATACAAATGCTATATAATATGAAAGAATAC
CTTTTATAAAATATACATGGAAGAAATAATAATGATGAGGCTCATGTT
ATTAAGAATAAAAATTCGATTTCAATCAGTTGCAGTGGGAAATTAGGAG
AGAAAGAAATTGGGTTTAACTGGAAACACCAACCAAAATTTTCTATATTTG
ATATATTTGCTTTTGGTTTTAGAAAAACCAATATGTGCTATCT
GAATGGTGAAATAAAGAAAATGTTAGATTATGTAATAAAGAAATAATTAA
TTTAGCTTTTAGATGTTTGGTCCAGAAGATTTTCTACCTATATTTTAAAGAAA
GAACCCAAAAATCCAAAAAAATGAATATCTTCTATAATATCTTGGC
AAAAAAATAATACATTTTAGAAAAATGAAATTTCATTAGAAGAAGAAGA
TTTTTATAAGGCATTTTTTATTAGAAGAAAAAATCCCAAATATGCTATC
TGACTATGGAAGATGCTTAAATCTCTATGTTTACAAACTTTTTA
TTAAGGGTAAGAACATTGTTGGTCTCTCTTGTGGTTATTCTTCCAGGC
AAAAAAAATGGAAGATAAGGAAATAAAGATGATGAGGCTTATTAAAAAAATA
AAACTGATAAGGAAAGCAACCGGTGACAGTCTACTAAAAAGTAATATAG
AATAAAGACAGCTGAAGAGGTAATTTAAAGTGAAACAATAAATGGA
TGAAGAATAATTGAATAAAATTTATGATGGAACGATCTTTTTAAATAATG
G[7/T]TGAGAATAATATAGGAAATAATATATTATTTTCTTTAGATC
ACCAAAATCCCCAAATATAGTGTAGTTAT[7/G]CAAAAGAATTTGGAAAACCTT
AAAAAGTTGAAATGTGCATGCAATTTGTTATTGGAATAGGCTCATCAT
ATCCCTTAAATAGGATTGCTTTTCTATATATGCAAATATTTATTTATCTT
GGATGAAATTATTAAAAAGAAAAAGATAATTTTCGTATATTCA
ACAAAGGAAAATATTTTGGGACAGGTTAAAGATGCTAGAATGAAAAAAGA
ATTACATATTGTTGCTTTTCCAAATGGAGAGTTTCTTAAAATTTAG
AAAAAGTATAAAACCTTATAATATAGCAGAATAAAATATATTAGGTGTTCA
TGGACCTTGGAAAGAAAAAGCAACATTAAATTGTTGCTAATAAACAAA
GGGAAAAATATATCAACGAGGCTAGGTTAAAACACCTTGTATTTG
TACTGTAGAAAATTTTGGAGAAAGTTTTGGTTATGTCTTAAAGAGCT
GGAGGGTGAGGTGAGTTAATTAAATCTGTATCATCAAAAAGATATATTATGAG
TTTGTGGGAAATCCACGCTATAAGAAGATCAGTACAGTGAATACATA
GAATAGGCAATAAAAGAAAGATAATTTTAAATTTTGGGTAGAAAA
ACAGTAGAAGAAAGACAAAAACATATACAAAGAATAATACACGC
TAACCAAAATTTTGACCAAGAGGGAAATAAATATGACTGAAATGAA
TTGATCCGAGAAATAGGAAATGAGTACCTTTTATATATGTTCCAAAAGAT
TGGATACAGATGAAATAAAAAAAAAACTTTAAAAACATTATC

WWARN Procedure: PCR-RFLP for genotyping SNPs on MAL10 and MAL13 v1.1
Page 12/14
Appendix B Restriction sites and DNA controls for MAL10-688956 and MAL13-1718319

SNPs shown in red.

**MAL10-688956**
- **SNP Location:** 688956
- **Restriction Enzyme:** NsiI
- **Buffer:** NEBuffer 3
- **Recognition Site:** ATGCAT
- **Complement:** TACGTA
- **Digests:** 3D7-type (ATGCAT)
  - Digested product sizes: 143 bp, 165 bp
- **Undigested:** V1/S-type (ATGCAA)
  - Undigested product size: 309 bp

**MAL13-1718319**
- **SNP Location:** 1718319
- **Restriction Enzyme:** MslI
- **Buffer:** NEBuffer 4
- **Recognition Site:** CAYNNNNRTG
- **Complement:** GTRNNNNYAC
- **Digests:** 3D7-type (CATTCCAATG)
  - Digested product sizes: 139 bp, 141 bp
- **Undigested:** V1/S-type (CATTCCATTG)
  - Undigested product size: 281 bp
Appendix C Standard curves for restriction digest of MAL10-688956 and MAL13-1718319

A) MAL10-688956

B) MAL13-1718319

Undigested (a) and digested (b) PCR products at 3D7:V1/S ratios: 1a and b: 100:0, 2a and b: 90:10, 3a and b: 80:20, 4a and b: 70:30, 5a and b: 60:40, 6a and b: 50:50, 7a and b: 40:60, 8a and b: 30:70, 9a and b: 20:80, 10a and b 10:90, 11a and b: 0:100.

Image source: Sandra Mon, University of Maryland, Baltimore.

WWARN Procedure: PCR-RFLP for genotyping SNPs on MAL10 and MAL13 v1.1 
Page 14/14