Acceptable character includes any digit (0,1,2,3,4,5,6,7,8,9) and special letters (A, T, G, C, N, a, t, g, c, n):

1. Acceptable sequence format:

Format 1 (SNP sequence):

NNNNNNNNNNNN[**N1/N2**]NNNNNNNNN

Format 2 (2 allele sequences, need blast in our program):

>Allele 1

NNNNNNNNNNNN**N1**NNNNNNNNN

>Allele 2

NNNNNNNNNNNN**N2**NNNNNNNNN

Format 3 (alignment):

Query 1 TGCTCTTTGAATGAAATTTGAGGTCATTCAATTTGTGCATTGTT**T**CGAAACCTTGATTTT

|||||||||||||||||||||||||||||||||||||||||||| |||||||||||||||

Sbjct 1 TGCTCTTTGAATGAAATTTGAGGTCATTCAATTTGTGCATTGTT**C**CGAAACCTTGATTTT

2. Ensure SNP or Indel:

SNP (no difference in length):

TGCTCTTTGAATGAAATTTGAGGTCATTCAATTTGTGCATTGTTTCGAAACCTTGATTTT

|||||||||||||||||||||||||||||||||||||||||||| |||||||||||||||

TGCTCTTTGAATGAAATTTGAGGTCATTCAATTTGTGCATTGTTCCGAAACCTTGATTTT

Indel (difference in length, symbol: “-“):

TGCTCTTTGAATGAAATTTGAGGTCATTCAATTTGTGCATTGTT**-**CGAAACCTTGATTTT

|||||||||||||||||||||||||||||||||||||||||||| |||||||||||||||

TGCTCTTTGAATGAAATTTGAGGTCATTCAATTTGTGCATTGTTCCGAAACCTTGATTTT

OR

TGCTCTTTGAATGAAATTTGAGGTCATTCAATTTGTGCATTGTTTCGAAACCTTGATTTT

|||||||||||||||||||||||||||||||||||||||||||| |||||||||||||||

TGCTCTTTGAATGAAATTTGAGGTCATTCAATTTGTGCATTGTT**-**CGAAACCTTGATTTT

3. Automatically and manually select locus for designing primers

Automatically select locus for designing primers: **indel** > **more than 3 (≥ 4) continuous SNPs** > 3 SNPs in 4 continuous bases > 2 SNPs in 4 continuous bases > [G/C] > [A/T] > [G/T] > [C/A] > [C/T] > [G/A];

**Indel:** continuous symbol “-“, “--“,“---“,“----“, et al or **broken e.g.**

Query 1 CGAGGACTATAGTCTCAGGAGAGGTGGGCATGGTGCCTCAGGATCGGAAGTGTGGACGCT **60**

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||

Sbjct 1 CGAGGACTATAGTCTCAGGAGAGGTGGGCATGGTGCCTCAGGATCGGAAGTGTGGACGCT **60**

Query **81** CGAGGACTATAGTCTCAGGAGAGGTGGGCATGGTGCCTCAGGATCGGAAGTGTGGACGCT 140

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||

Sbjct **61** CGAGGACTATAGTCTCAGGAGAGGTGGGCATGGTGCCTCAGGATCGGAAGTGTGGACGCT 120

**more than 3 (≥4) continuous SNPs:** Select the first 4 SNPs to design F primers

Manually select locus for primer design: if the two alleles have one SNP only (**note**) or one Indel only, directly switch to “automatically model” and then pop out the results; otherwise our program will pop-out the second interface for user selecting the target locus such as that in Dr. sun’s version;

Note: if having more than 1 (≥2) SNP at any conditions, will be treated as multiple SNPs

F primer design for SNP (**here I show the F primer design at the upstream of SNP only**)

1. Generate 10 F primer pairs in each SNP site: stretch base (16-25) to upstream from SNP site;

**F1 NNNNNNNNNNNNNNNNN1**

**F2 NNNNNNNNNNNNNNNNN2**

**F1 NNNNNNNNNNNNNNNNNN1**

**F2 NNNNNNNNNNNNNNNNNN2**

**F1 NNNNNNNNNNNNNNNNNNN1**

**F2 NNNNNNNNNNNNNNNNNNN2**

**F1 NNNNNNNNNNNNNNNNNNNN1**

**F2 NNNNNNNNNNNNNNNNNNNN2**

**F1 NNNNNNNNNNNNNNNNNNNNN1**

**F2 NNNNNNNNNNNNNNNNNNNNN2**

**F1 NNNNNNNNNNNNNNNNNNNNNN1**

**F2 NNNNNNNNNNNNNNNNNNNNNN2**

**F1 NNNNNNNNNNNNNNNNNNNNNNN1**

**F2 NNNNNNNNNNNNNNNNNNNNNNN2**

**F1 NNNNNNNNNNNNNNNNNNNNNNNN1**

**F2 NNNNNNNNNNNNNNNNNNNNNNNN2**

**F1 NNNNNNNNNNNNNNNNNNNNNNNNN1**

**F2 NNNNNNNNNNNNNNNNNNNNNNNNN2**

**F1 NNNNNNNNNNNNNNNNNNNNNNNNNN1**

**F2 NNNNNNNNNNNNNNNNNNNNNNNNNN2**

2. Remove the F primer pairs that any F primer:

2.1) having ≥ 10 contiguous (G and/or C) or ≥ 12 contiguous (A and/or T);

2.2) having ≥ 8 As, Ts, Gs, or Cs;

2.3) having ≥ 6 di-nucleotide **(AG, AC, TG, TC, GA, GT, CA, CT)** R**epeats**;

2.4) having (GC% > 80% or GC% < 20%);

3. If F primer pair number =0, try the downstream sequence; otherwise, continue:

4. Calculate the SNP numbers at the 2nd, 3rd, and 4th positions from 3’ end in the first F primer pair;

If SNP number =0 at the 2nd, 3rd, and 4th positions from 3’ end, go to **one SNP module**;

If SNP number =1 at the 2nd, 3rd, and 4th positions from 3’ end, go to **two SNP module**;

If SNP number >1 at the 2nd, 3rd, and 4th positions from 3’ end, go to **three SNP module**;

Module: one SNP between the two allele sequences:

Calculate Tm value of each F primer and the average Tm value of each F primer pair;

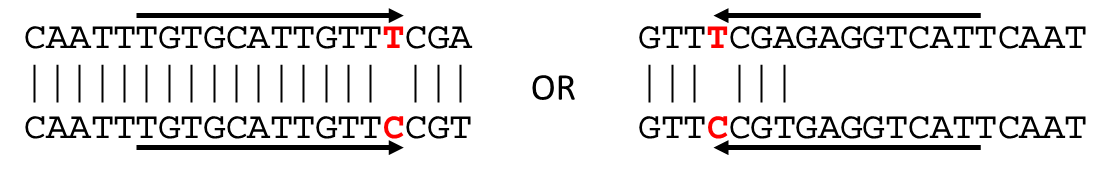
Calculate total SNP number of each F primer pair and select F primer pairs having total SNP number ≥ 4;

1. If the selected F primer pair number ≥ 1, select the F primer pairs that both F primers have Tm value arranging from (≥) 53C to (≤) 60C;
   1. If total F primer pair number ≥ 1, preserve the F primer pair with average Tm close to 58C as F primer pair and then design R primer;
   2. If total F primer pair number =0, go to step 2;
2. Select the F primer pairs that both F primers have Tm value arranging from (≥) 54C to (≤) 58C;

If F primer pair number ≥ 1, preserve the F primer pair with average Tm close to 58C and go to **substitute base**;

If F primer pair number = 0, select the F1 primer (in the 10 F1 candidates) and F2 primer (in the 10 F2 candidates) both with Tm value: 1) close to 56C **and** 2) arranging from (≥) 54C to (≤) 58C;

If F1 primer number = 0 or F2 primer number = 0, **stop** and then try the downstream sequence,



Otherwise, combine the two F primers as F primer pair and go to **substitute base**;

Substitute base

1 For [C/G] SNP

1.1 Three G/C bases at 2nd, 3rd, and 4th from 3ʹ end: substitute the 2nd base for G allele and 3rd base for C allele following this principle: A→C, T→C, G→A, and C→T;

1.2 Two G/C bases and one A/T base at 2nd, 3rd, and 4th from 3ʹ end: select G/C to be substituted following this principle: A→C, T→C, G→A, and C→T. The G/C proximal to 3ʹ end will be treated as 2nd, and the G/C distal to 3ʹ end will be treated as 3rd.

1.3 One G/C base and two A/T bases at 2nd, 3rd, and 4th from 3ʹ end: select A/T to be substituted following this principle: A→C, T→C, G→A, and C→T. The A/T proximal to 3ʹ end will be treated as 2nd, and the A/T distal to 3ʹ end will be treated as 3rd.

1.4 0 G/C base (three A/T bases) at 2nd, 3rd, and 4th from 3ʹ end: substitute the 2nd base for G allele and 3rd base for C allele following this principle: A→C, T→C, G→A, and C→T;

2 For [C/T] SNP

2.1 Three G/C bases at 2nd, 3rd, and 4th from 3ʹ end: substitute the 2nd base for C allele and 3rd base for T allele following this principle: A→C, T→C, G→A, and C→T;

2.2 Two G/C bases and one A/T base at 2nd, 3rd, and 4th from 3ʹ end: select G/C to be substituted following this principle: A→C, T→C, G→A, and C→T.

If A/T base at 2nd from 3ʹ end, substitute the 3rd base for T allele and 4th base for C allele;

If A/T base at 3rd from 3ʹ end, substitute the 2nd base for C allele and 4th base for T allele;

If A/T base at 4th from 3ʹ end, substitute the 2nd base for C allele and 3rd base for T allele;

2.3 One G/C base and two A/T bases at 2nd, 3rd, and 4th from 3ʹ end: select A/T to be substituted following this principle: A→C, T→C, G→A, and C→T.

If G/C base at 2nd from 3ʹ end, substitute the 3rd base for T allele and 4th base for C allele;

If G/C base at 3rd from 3ʹ end, substitute the 2nd base for C allele and 4th base for T allele;

If G/C base at 4th from 3ʹ end, substitute the 2nd base for C allele and 3rd base for T allele;

2.4 0 G/C base (three A/T bases) at 2nd, 3rd, and 4th from 3ʹ end: substitute the 2nd base for C allele and 3rd base for T allele following this principle: A→C, T→C, G→A, and C→T;

3 For [C/A] SNP

3.1 Three G/C bases at 2nd, 3rd, and 4th from 3ʹ end: substitute the 2nd base for C allele and 3rd base for A allele following this principle: A→C, T→C, G→A, and C→T;

3.2 Two G/C bases and one A/T base at 2nd, 3rd, and 4th from 3ʹ end: select G/C to be substituted following this principle: A→C, T→C, G→A, and C→T.

If A/T base at 2nd from 3ʹ end, substitute the 3rd base for C allele and 4th base for A allele;

If A/T base at 3rd from 3ʹ end, substitute the 2nd base for C allele and 4th base for A allele;

If A/T base at 4th from 3ʹ end, substitute the 2nd base for C allele and 3rd base for A allele;

3.3 One G/C base and two A/T bases at 2nd, 3rd, and 4th from 3ʹ end: select A/T to be substituted following this principle: A→C, T→C, G→A, and C→T.

If G/C base at 2nd from 3ʹ end, substitute the 3rd base for C allele and 4th base for A allele;

If G/C base at 3rd from 3ʹ end, substitute the 2nd base for C allele and 4th base for A allele;

If G/C base at 4th from 3ʹ end, substitute the 2nd base for C allele and 3rd base for A allele;

3.4 0 G/C base (three A/T bases) at 2nd, 3rd, and 4th from 3ʹ end: substitute the 2nd base for C allele and 3rd base for A allele following this principle: A→C, T→C, G→A, and C→T;

4 For [G/T] SNP

4.1 Three G/C bases at 2nd, 3rd, and 4th from 3ʹ end: substitute the 2nd base for G allele and 3rd base for T allele following this principle: A→C, T→C, G→A, and C→T;

4.2 Two G/C bases and one A/T base at 2nd, 3rd, and 4th from 3ʹ end: select G/C to be substituted following this principle: A→C, T→C, G→A, and C→T.

If A/T base at 2nd from 3ʹ end, substitute the 3rd base for T allele and 4th base for G allele;

If A/T base at 3rd from 3ʹ end, substitute the 2nd base for G allele and 4th base for T allele;

If A/T base at 4th from 3ʹ end, substitute the 2nd base for G allele and 3rd base for T allele;

4.3 One G/C base and two A/T bases at 2nd, 3rd, and 4th from 3ʹ end: select A/T to be substituted following this principle: A→C, T→C, G→A, and C→T.

If G/C base at 2nd from 3ʹ end, substitute the 3rd base for T allele and 4th base for G allele;

If G/C base at 3rd from 3ʹ end, substitute the 2nd base for G allele and 4th base for T allele;

If G/C base at 4th from 3ʹ end, substitute the 2nd base for G allele and 3rd base for T allele;

4.4 0 G/C base (three A/T bases) at 2nd, 3rd, and 4th from 3ʹ end: substitute the 2nd base for G allele and 3rd base for T allele following this principle: A→C, T→C, G→A, and C→T;

5 For [G/A] SNP

5.1 Three G/C bases at 2nd, 3rd, and 4th from 3ʹ end: substitute the 2nd base for G allele and 3rd base for A allele following this principle: A→C, T→C, G→A, and C→T;

5.2 Two G/C bases and one A/T base at 2nd, 3rd, and 4th from 3ʹ end: select G/C to be substituted following this principle: A→C, T→C, G→A, and C→T.

If A/T base at 2nd from 3ʹ end, substitute the 3rd base for G allele and 4th base for A allele;

If A/T base at 3rd from 3ʹ end, substitute the 2nd base for G allele and 4th base for A allele;

If A/T base at 4th from 3ʹ end, substitute the 2nd base for G allele and 3rd base for A allele;

5.3 One G/C base and two A/T bases at 2nd, 3rd, and 4th from 3ʹ end: select A/T to be substituted following this principle: A→C, T→C, G→A, and C→T.

If G/C base at 2nd from 3ʹ end, substitute the 3rd base for G allele and 4th base for A allele;

If G/C base at 3rd from 3ʹ end, substitute the 2nd base for G allele and 4th base for A allele;

If G/C base at 4th from 3ʹ end, substitute the 2nd base for G allele and 3rd base for A allele;

5.4 0 G/C base (three A/T bases) at 2nd, 3rd, and 4th from 3ʹ end: substitute the 2nd base for G allele and 3rd base for A allele following this principle: A→C, T→C, G→A, and C→T;

6 For [T/A] SNP

6.1 Three G/C bases at 2nd, 3rd, and 4th from 3ʹ end: substitute the 3rd base for T allele and 4th base for A allele following this principle: A→C, T→C, G→A, and C→T;

6.2 Two G/C bases and one A/T base at 2nd, 3rd, and 4th from 3ʹ end: select G/C to be substituted following this principle: A→C, T→C, G→A, and C→T.

If A/T base at 2nd from 3ʹ end, substitute the 3rd base for T allele and 4th base for A allele;

If A/T base at 3rd from 3ʹ end, substitute the 2nd base for T allele and 4th base for A allele;

If A/T base at 4th from 3ʹ end, substitute the 2nd base for T allele and 3rd base for A allele;

6.3 One G/C base and two A/T bases at 2nd, 3rd, and 4th from 3ʹ end: select A/T to be substituted following this principle: A→C, T→C, G→A, and C→T.

If G/C base at 2nd from 3ʹ end, substitute the 3rd base for T allele and 4th base for A allele;

If G/C base at 3rd from 3ʹ end, substitute the 2nd base for T allele and 4th base for A allele;

If G/C base at 4th from 3ʹ end, substitute the 2nd base for T allele and 3rd base for A allele;

6.4 0 G/C base (three A/T bases) at 2nd, 3rd, and 4th from 3ʹ end: substitute the 3rd base for T allele and 4th base for A allele following this principle: A→C, T→C, G→A, and C→T;

Module: two SNP between the two allele sequences:

Calculate Tm value of each F primer and the average Tm value of each F primer pair;

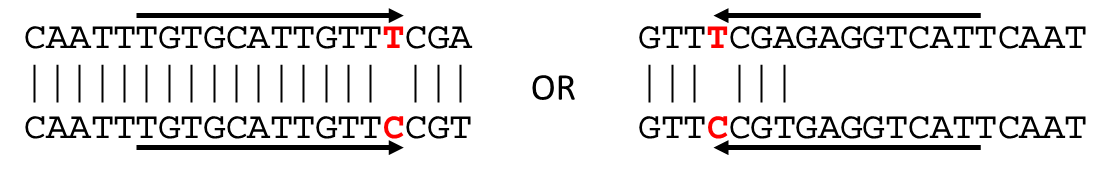
Calculate total SNP number of each F primer pair and select F primer pairs having total SNP number ≥ 3;

1. If the selected F primer pair number ≥ 1, select the F primer pairs that both F primers have Tm value arranging from (≥) 53C to (≤) 60C;
   1. If total F primer pair number ≥ 1, preserve the F primer pair with average Tm close to 58C as F primer pair and then design R primer;
   2. If total F primer pair number =0, go to step 2;
2. Select the F primer pairs that both F primers have Tm value arranging from (≥) 54C to (≤) 58C;

If F primer pair number ≥ 1, preserve the F primer pair with average Tm close to 58C and go to **substitute base**;

If F primer pair number = 0, select the F1 primer (in the 10 F1 candidates) and F2 primer (in the 10 F2 candidates) both with Tm value: 1) close to 56C **and** 2) arranging from (≥) 54C to (≤) 60C;

If F1 primer number = 0 or F2 primer number = 0, **stop** and then try the downstream sequence,



Otherwise, combine the two F primers as F primer pair and go to **substitute base**;

Substitute base

1 For [C/G] SNP

1.1 Additional SNP at 2nd from 3ʹ end:

If the additional SNP is [C/G] or [A/T], substitute the 3rd base for G allele and 4th base for C allele following this principle: A→C, T→C, G→A, and C→T;

If the additional SNP is [C/A], [C/T], [G/A], or [G/T]:

If having two [C or G] at 3rd and 4th positions **or** having two [A or T] at 3rd and 4th positions: substitute the 3rd base for G allele and 4th base for C allele following this principle: A→C, T→C, G→A, and C→T;

If having one [C or G] and one [A or T] at 3rd and 4th positions: **FOCUS ON THE ADDITIONAL SNP**, substitute the [C or G] base for [C or G] allele and the [A or T] base for [A or T] allele following this principle: A→C, T→C, G→A, and C→T;

1.2 Additional SNP at 3rd from 3ʹ end:

If the additional SNP is [C/G] or [A/T], substitute the 2nd base for G allele and 4th base for C allele following this principle: A→C, T→C, G→A, and C→T;

If the additional SNP is [C/A], [C/T], [G/A], or [G/T]:

If having two [C or G] at 2nd and 4th positions **or** having two [A or T] at 2nd and 4th positions: substitute the 2nd base for G allele and 4th base for C allele following this principle: A→C, T→C, G→A, and C→T;

If having one [C or G] and one [A or T] at 3rd and 4th positions: **FOCUS ON THE ADDITIONAL SNP**, substitute the [C or G] base for [C or G] allele and the [A or T] base for [A or T] allele following this principle: A→C, T→C, G→A, and C→T;

1.3 Additional SNP at 4th from 3ʹ end:

If the additional SNP is [C/G] or [A/T], substitute the 2nd base for G allele and 3rd base for C allele following this principle: A→C, T→C, G→A, and C→T;

If the additional SNP is [C/A], [C/T], [G/A], or [G/T]:

If having two [C or G] at 2nd and 3rd positions **or** having two [A or T] at 2nd and 3rd positions: substitute the 2nd base for G allele and 3rd base for C allele following this principle: A→C, T→C, G→A, and C→T;

If having one [C or G] and one [A or T] at 2nd and 3rd positions: **FOCUS ON THE ADDITIONAL SNP**, substitute the [C or G] base for [C or G] allele and the [A or T] base for [A or T] allele following this principle: A→C, T→C, G→A, and C→T;

2 For [C/T] SNP

2.1 Additional SNP at 2nd from 3ʹ end:

If the additional SNP is [C/G] or [A/T]:

If having two [C or G] at 3rd and 4th positions **or** having two [A or T] at 3rd and 4th positions: substitute the 3rd base for T allele and 4th base for C allele following this principle: A→C, T→C, G→A, and C→T;

If having one [C or G] and one [A or T] at 3rd and 4th positions: substitute the [C or G] base for C allele and the [A or T] base for T allele following this principle: A→C, T→C, G→A, and C→T;

If the additional SNP is [C/A], [C/T], [G/A], or [G/T]:

If having two [C or G] at 3rd and 4th positions **or** having two [A or T] at 3rd and 4th positions: substitute the 3rd base for T allele and 4th base for C allele following this principle: A→C, T→C, G→A, and C→T;

If having one [C or G] and one [A or T] at 3rd and 4th positions **(FOCUS ON THE TWO SNPs)**:

If one allele has two [C or G] and other has two [A or T], substitute the [C or G] base for the allele having two [C or G] bases and the [A or T] base for the allele having two [A or T] bases following this principle: A→C, T→C, G→A, and C→T;

If one allele has one [C or G] and one [A or T], other also has one [C or G] and one [A or T], substitute the 3rd base for T allele and 4th base for C allele following this principle: A→C, T→C, G→A, and C→T;

2.2 Additional SNP at 3rd from 3ʹ end:

If the additional SNP is [C/G] or [A/T]:

If having two [C or G] at 2nd and 4th positions **or** having two [A or T] at 2nd and 4th positions: substitute the 2nd base for C allele and 4th base for T allele following this principle: A→C, T→C, G→A, and C→T;

If having one [C or G] and one [A or T] at 2nd and 4th positions: substitute the [C or G] base for C allele and the [A or T] base for T allele following this principle: A→C, T→C, G→A, and C→T;

If the additional SNP is [C/A], [C/T], [G/A], or [G/T]:

If having two [C or G] at 2nd and 4th positions **or** having two [A or T] at 2nd and 4th positions: substitute the 2nd base for C allele and 4th base for T allele following this principle: A→C, T→C, G→A, and C→T;

If having one [C or G] and one [A or T] at 2nd and 4th positions **(FOCUS ON THE TWO SNPs)**:

If one allele has two [C or G] and other has two [A or T], substitute the [C or G] base for the allele having two [C or G] bases and the [A or T] base for the allele having two [A or T] bases following this principle: A→C, T→C, G→A, and C→T;

If one allele has one [C or G] and one [A or T], other also has one [C or G] and one [A or T], substitute the 2nd base for C allele and 4th base for T allele following this principle: A→C, T→C, G→A, and C→T;

2.3 Additional SNP at 4th from 3ʹ end:

If the additional SNP is [C/G] or [A/T]:

If having two [C or G] at 2nd and 3rd positions **or** having two [A or T] at 2nd and 3rd positions: substitute the 2nd base for C allele and 3rd base for T allele following this principle: A→C, T→C, G→A, and C→T;

If having one [C or G] and one [A or T] at 2nd and 3rd positions: substitute the [C or G] base for C allele and the [A or T] base for T allele following this principle: A→C, T→C, G→A, and C→T;

If the additional SNP is [C/A], [C/T], [G/A], or [G/T]:

If having two [C or G] at 2nd and 3rd positions **or** having two [A or T] at 2nd and 3rd positions: substitute the 2nd base for C allele and 3rd base for T allele following this principle: A→C, T→C, G→A, and C→T;

If having one [C or G] and one [A or T] at 2nd and 3rd positions **(FOCUS ON THE TWO SNPs)**:

If one allele has two [C or G] and other has two [A or T], substitute the [C or G] base for the allele having two [C or G] bases and the [A or T] base for the allele having two [A or T] bases following this principle: A→C, T→C, G→A, and C→T;

If one allele has one [C or G] and one [A or T], other also has one [C or G] and one [A or T], substitute the 2nd base for C allele and 3rd base for T allele following this principle: A→C, T→C, G→A, and C→T;

3 For [C/A] SNP

3.1 Additional SNP at 2nd from 3ʹ end:

If the additional SNP is [C/G] or [A/T]:

If having two [C or G] at 3rd and 4th positions **or** having two [A or T] at 3rd and 4th positions: substitute the 3rd base for C allele and 4th base for A allele following this principle: A→C, T→C, G→A, and C→T;

If having one [C or G] and one [A or T] at 3rd and 4th positions: substitute the [C or G] base for C allele and the [A or T] base for A allele following this principle: A→C, T→C, G→A, and C→T;

If the additional SNP is [C/A], [C/T], [G/A], or [G/T]:

If having two [C or G] at 3rd and 4th positions **or** having two [A or T] at 3rd and 4th positions: substitute the 3rd base for C allele and 4th base for A allele following this principle: A→C, T→C, G→A, and C→T;

If having one [C or G] and one [A or T] at 3rd and 4th positions **(FOCUS ON THE TWO SNPs)**:

If one allele has two [C or G] and other has two [A or T], substitute the [C or G] base for the allele having two [C or G] bases and the [A or T] base for the allele having two [A or T] bases following this principle: A→C, T→C, G→A, and C→T;

If one allele has one [C or G] and one [A or T], other also has one [C or G] and one [A or T], substitute the 3rd base for C allele and 4th base for A allele following this principle: A→C, T→C, G→A, and C→T;

3.2 Additional SNP at 3rd from 3ʹ end:

If the additional SNP is [C/G] or [A/T]:

If having two [C or G] at 2nd and 4th positions **or** having two [A or T] at 2nd and 4th positions: substitute the 2nd base for C allele and 4th base for A allele following this principle: A→C, T→C, G→A, and C→T;

If having one [C or G] and one [A or T] at 2nd and 4th positions: substitute the [C or G] base for C allele and the [A or T] base for A allele following this principle: A→C, T→C, G→A, and C→T;

If the additional SNP is [C/A], [C/T], [G/A], or [G/T]:

If having two [C or G] at 2nd and 4th positions **or** having two [A or T] at 2nd and 4th positions: substitute the 2nd base for C allele and 4th base for A allele following this principle: A→C, T→C, G→A, and C→T;

If having one [C or G] and one [A or T] at 2nd and 4th positions **(FOCUS ON THE TWO SNPs)**:

If one allele has two [C or G] and other has two [A or T], substitute the [C or G] base for the allele having two [C or G] bases and the [A or T] base for the allele having two [A or T] bases following this principle: A→C, T→C, G→A, and C→T;

If one allele has one [C or G] and one [A or T], other also has one [C or G] and one [A or T], substitute the 2nd base for C allele and 4th base for A allele following this principle: A→C, T→C, G→A, and C→T;

3.3 Additional SNP at 4th from 3ʹ end:

If the additional SNP is [C/G] or [A/T]:

If having two [C or G] at 2nd and 3rd positions **or** having two [A or T] at 2nd and 3rd positions: substitute the 2nd base for C allele and 3rd base for A allele following this principle: A→C, T→C, G→A, and C→T;

If having one [C or G] and one [A or T] at 2nd and 3rd positions: substitute the [C or G] base for C allele and the [A or T] base for A allele following this principle: A→C, T→C, G→A, and C→T;

If the additional SNP is [C/A], [C/T], [G/A], or [G/T]:

If having two [C or G] at 2nd and 3rd positions **or** having two [A or T] at 2nd and 3rd positions: substitute the 2nd base for C allele and 3rd base for A allele following this principle: A→C, T→C, G→A, and C→T;

If having one [C or G] and one [A or T] at 2nd and 3rd positions **(FOCUS ON THE TWO SNPs)**:

If one allele has two [C or G] and other has two [A or T], substitute the [C or G] base for the allele having two [C or G] bases and the [A or T] base for the allele having two [A or T] bases following this principle: A→C, T→C, G→A, and C→T;

If one allele has one [C or G] and one [A or T], other also has one [C or G] and one [A or T], substitute the 2nd base for C allele and 3rd base for A allele following this principle: A→C, T→C, G→A, and C→T;

4 For [G/T] SNP

4.1 Additional SNP at 2nd from 3ʹ end:

If the additional SNP is [C/G] or [A/T]:

If having two [C or G] at 3rd and 4th positions **or** having two [A or T] at 3rd and 4th positions: substitute the 3rd base for T allele and 4th base for G allele following this principle: A→C, T→C, G→A, and C→T;

If having one [C or G] and one [A or T] at 3rd and 4th positions: substitute the [C or G] base for G allele and the [A or T] base for T allele following this principle: A→C, T→C, G→A, and C→T;

If the additional SNP is [C/A], [C/T], [G/A], or [G/T]:

If having two [C or G] at 3rd and 4th positions **or** having two [A or T] at 3rd and 4th positions: substitute the 3rd base for T allele and 4th base for G allele following this principle: A→C, T→C, G→A, and C→T;

If having one [C or G] and one [A or T] at 3rd and 4th positions **(FOCUS ON THE TWO SNPs)**:

If one allele has two [C or G] and other has two [A or T], substitute the [C or G] base for the allele having two [C or G] bases and the [A or T] base for the allele having two [A or T] bases following this principle: A→C, T→C, G→A, and C→T;

If one allele has one [C or G] and one [A or T], other also has one [C or G] and one [A or T], substitute the 3rd base for T allele and 4th base for G allele following this principle: A→C, T→C, G→A, and C→T;

4.2 Additional SNP at 3rd from 3ʹ end:

If the additional SNP is [C/G] or [A/T]:

If having two [C or G] at 2nd and 4th positions **or** having two [A or T] at 2nd and 4th positions: substitute the 2nd base for G allele and 4th base for T allele following this principle: A→C, T→C, G→A, and C→T;

If having one [C or G] and one [A or T] at 2nd and 4th positions: substitute the [C or G] base for G allele and the [A or T] base for T allele following this principle: A→C, T→C, G→A, and C→T;

If the additional SNP is [C/A], [C/T], [G/A], or [G/T]:

If having two [C or G] at 2nd and 4th positions **or** having two [A or T] at 2nd and 4th positions: substitute the 2nd base for G allele and 4th base for T allele following this principle: A→C, T→C, G→A, and C→T;

If having one [C or G] and one [A or T] at 2nd and 4th positions **(FOCUS ON THE TWO SNPs)**:

If one allele has two [C or G] and other has two [A or T], substitute the [C or G] base for the allele having two [C or G] bases and the [A or T] base for the allele having two [A or T] bases following this principle: A→C, T→C, G→A, and C→T;

If one allele has one [C or G] and one [A or T], other also has one [C or G] and one [A or T], substitute the 2nd base for G allele and 4th base for T allele following this principle: A→C, T→C, G→A, and C→T;

4.3 Additional SNP at 4th from 3ʹ end:

If the additional SNP is [C/G] or [A/T]:

If having two [C or G] at 2nd and 3rd positions **or** having two [A or T] at 2nd and 3rd positions: substitute the 2nd base for G allele and 3rd base for T allele following this principle: A→C, T→C, G→A, and C→T;

If having one [C or G] and one [A or T] at 2nd and 3rd positions: substitute the [C or G] base for G allele and the [A or T] base for T allele following this principle: A→C, T→C, G→A, and C→T;

If the additional SNP is [C/A], [C/T], [G/A], or [G/T]:

If having two [C or G] at 2nd and 3rd positions **or** having two [A or T] at 2nd and 3rd positions: substitute the 2nd base for G allele and 3rd base for T allele following this principle: A→C, T→C, G→A, and C→T;

If having one [C or G] and one [A or T] at 2nd and 3rd positions **(FOCUS ON THE TWO SNPs)**:

If one allele has two [C or G] and other has two [A or T], substitute the [C or G] base for the allele having two [C or G] bases and the [A or T] base for the allele having two [A or T] bases following this principle: A→C, T→C, G→A, and C→T;

If one allele has one [C or G] and one [A or T], other also has one [C or G] and one [A or T], substitute the 2nd base for G allele and 3rd base for T allele following this principle: A→C, T→C, G→A, and C→T;

5 For [G/A] SNP

5.1 Additional SNP at 2nd from 3ʹ end:

If the additional SNP is [C/G] or [A/T]:

If having two [C or G] at 3rd and 4th positions **or** having two [A or T] at 3rd and 4th positions: substitute the 3rd base for G allele and 4th base for A allele following this principle: A→C, T→C, G→A, and C→T;

If having one [C or G] and one [A or T] at 3rd and 4th positions: substitute the [C or G] base for G allele and the [A or T] base for A allele following this principle: A→C, T→C, G→A, and C→T;

If the additional SNP is [C/A], [C/T], [G/A], or [G/T]:

If having two [C or G] at 3rd and 4th positions **or** having two [A or T] at 3rd and 4th positions: substitute the 3rd base for G allele and 4th base for A allele following this principle: A→C, T→C, G→A, and C→T;

If having one [C or G] and one [A or T] at 3rd and 4th positions **(FOCUS ON THE TWO SNPs)**:

If one allele has two [C or G] and other has two [A or T], substitute the [C or G] base for the allele having two [C or G] bases and the [A or T] base for the allele having two [A or T] bases following this principle: A→C, T→C, G→A, and C→T;

If one allele has one [C or G] and one [A or T], other also has one [C or G] and one [A or T], substitute the 3rd base for G allele and 4th base for A allele following this principle: A→C, T→C, G→A, and C→T;

5.2 Additional SNP at 3rd from 3ʹ end:

If the additional SNP is [C/G] or [A/T]:

If having two [C or G] at 2nd and 4th positions **or** having two [A or T] at 2nd and 4th positions: substitute the 2nd base for G allele and 4th base for A allele following this principle: A→C, T→C, G→A, and C→T;

If having one [C or G] and one [A or T] at 2nd and 4th positions: substitute the [C or G] base for G allele and the [A or T] base for A allele following this principle: A→C, T→C, G→A, and C→T;

If the additional SNP is [C/A], [C/T], [G/A], or [G/T]:

If having two [C or G] at 2nd and 4th positions **or** having two [A or T] at 2nd and 4th positions: substitute the 2nd base for G allele and 4th base for A allele following this principle: A→C, T→C, G→A, and C→T;

If having one [C or G] and one [A or T] at 2nd and 4th positions **(FOCUS ON THE TWO SNPs)**:

If one allele has two [C or G] and other has two [A or T], substitute the [C or G] base for the allele having two [C or G] bases and the [A or T] base for the allele having two [A or T] bases following this principle: A→C, T→C, G→A, and C→T;

If one allele has one [C or G] and one [A or T], other also has one [C or G] and one [A or T], substitute the 2nd base for G allele and 4th base for A allele following this principle: A→C, T→C, G→A, and C→T;

5.3 Additional SNP at 4th from 3ʹ end:

If the additional SNP is [C/G] or [A/T]:

If having two [C or G] at 2nd and 3rd positions **or** having two [A or T] at 2nd and 3rd positions: substitute the 2nd base for G allele and 3rd base for A allele following this principle: A→C, T→C, G→A, and C→T;

If having one [C or G] and one [A or T] at 2nd and 3rd positions: substitute the [C or G] base for G allele and the [A or T] base for A allele following this principle: A→C, T→C, G→A, and C→T;

If the additional SNP is [C/A], [C/T], [G/A], or [G/T]:

If having two [C or G] at 2nd and 3rd positions **or** having two [A or T] at 2nd and 3rd positions: substitute the 2nd base for G allele and 3rd base for A allele following this principle: A→C, T→C, G→A, and C→T;

If having one [C or G] and one [A or T] at 2nd and 3rd positions **(FOCUS ON THE TWO SNPs)**:

If one allele has two [C or G] and other has two [A or T], substitute the [C or G] base for the allele having two [C or G] bases and the [A or T] base for the allele having two [A or T] bases following this principle: A→C, T→C, G→A, and C→T;

If one allele has one [C or G] and one [A or T], other also has one [C or G] and one [A or T], substitute the 2nd base for G allele and 3rd base for A allele following this principle: A→C, T→C, G→A, and C→T;

6 For [A/T] SNP

6.1 Additional SNP at 2nd from 3ʹ end:

If the additional SNP is [C/G] or [A/T], substitute the 3rd base for T allele and 4th base for A allele following this principle: A→C, T→C, G→A, and C→T;

If the additional SNP is [C/A], [C/T], [G/A], or [G/T]:

If having two [C or G] at 3rd and 4th positions **or** having two [A or T] at 3rd and 4th positions: substitute the 3rd base for T allele and 4th base for A allele following this principle: A→C, T→C, G→A, and C→T;

If having one [C or G] and one [A or T] at 3rd and 4th positions: **FOCUS ON THE ADDITIONAL SNP**, substitute the [C or G] base for [C or G] allele and the [A or T] base for [A or T] allele following this principle: A→C, T→C, G→A, and C→T;

6.2 Additional SNP at 3rd from 3ʹ end:

If the additional SNP is [C/G] or [A/T], substitute the 2nd base for T allele and 4th base for A allele following this principle: A→C, T→C, G→A, and C→T;

If the additional SNP is [C/A], [C/T], [G/A], or [G/T]:

If having two [C or G] at 2nd and 4th positions **or** having two [A or T] at 2nd and 4th positions: substitute the 2nd base for T allele and 4th base for A allele following this principle: A→C, T→C, G→A, and C→T;

If having one [C or G] and one [A or T] at 3rd and 4th positions: **FOCUS ON THE ADDITIONAL SNP**, substitute the [C or G] base for [C or G] allele and the [A or T] base for [A or T] allele following this principle: A→C, T→C, G→A, and C→T;

6.3 Additional SNP at 4th from 3ʹ end:

If the additional SNP is [C/G] or [A/T], substitute the 2nd base for T allele and 3rd base for A allele following this principle: A→C, T→C, G→A, and C→T;

If the additional SNP is [C/A], [C/T], [G/A], or [G/T]:

If having two [C or G] at 2nd and 3rd positions **or** having two [A or T] at 2nd and 3rd positions: substitute the 2nd base for T allele and 3rd base for A allele following this principle: A→C, T→C, G→A, and C→T;

If having one [C or G] and one [A or T] at 2nd and 3rd positions: **FOCUS ON THE ADDITIONAL SNP**, substitute the [C or G] base for [C or G] allele and the [A or T] base for [A or T] allele following this principle: A→C, T→C, G→A, and C→T;

Module: three SNP between the two allele sequences:

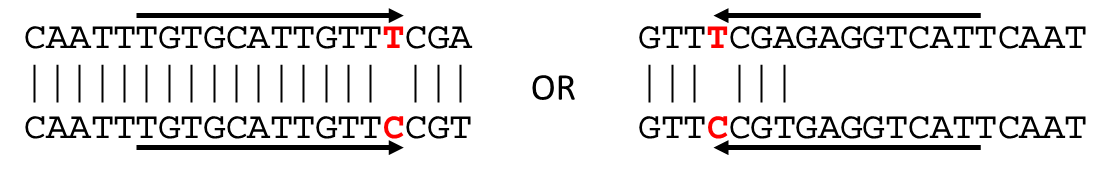
Calculate Tm value of each F primer and the average Tm value of each F primer pair;

Select the F primer pairs that both F primers have Tm value arranging from (≥) 52C to (≤) 60C;

If F primer pair number ≥ 1, preserve the F primer pair: Both F primers have Tm value arranging from (≥) 52C to (≤) 58C and average Tm close to 58C ~~and go to~~ **~~substitute base~~**; Otherwise, preserve the F primer pair: Both F primers have Tm value arranging from (≥) 52C to (≤) 60C and average Tm close to 58C ~~and go to~~ **~~substitute base~~**;

If F primer pair number = 0, select the F1 primer (in the 10 F1 candidates) and F2 primer (in the 10 F2 candidates) both with Tm value: 1) close to 56C **and** 2) arranging from (≥) 52C to (≤) 60C;

If F1 primer number = 0 or F2 primer number = 0, **stop** and then try the downstream sequence,



Otherwise, combine the two F primers as F primer pair (No nucleotide substitution is required);

F primer design for Indel (**here I show the F primer design at the upstream of Indel only**)

Please see PPT named how to design AMAS-primers for Indel\_20180719