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 > 4 SNPs in 4 continuous bases > 3 SNPs in 4 continuous bases > 2 SNPs in 4 continuous bases > [G/C] > [A/T] > [G/T] > [C/A] > [C/T] > [G/A];

Manually select locus for primer design: if the two alleles have one SNP or one Indel (continuous symbol “-“, “--“,“---“,“----“, et al) 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;

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. Calculate 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:

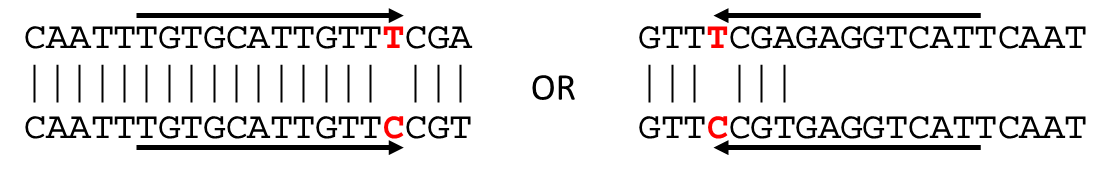
Calculate Tm value of each F primer and the average Tm value of each F primer pair;

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:

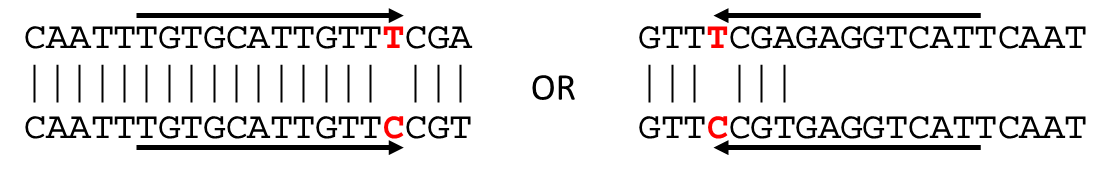
Calculate Tm value of each F primer and the average Tm value of each F primer pair;

Select the F primer pairs that both F primers have Tm value arranging from (≥) 54C to (≤) 60C;

If F primer pair number ≥ 1, preserve the F primer pair: Both F primers have Tm value arranging from (≥) 54C 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 (≥) 54C 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 (≥) 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:

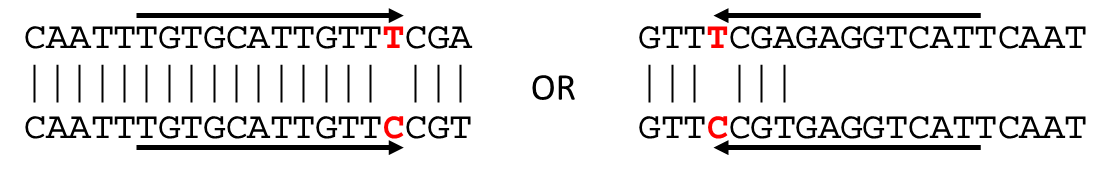
Calculate Tm value of each F primer and the average Tm value of each F primer pair;

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