1. Acceptable sequence format:

Format 1 (SNP sequences: this format can be set to generate STAR primers for multiple SNPs one time):

1.1 One SNP sequence one time

>SNP sequence 1

NNNNNNNNNNNN**[N1/N2]**NNNNNNNNN

OR

NNNNNNNNNNNN**[N1/N2]**NNNNNNNNN

Note: [**N1/N2**] is the default SNP format, N1 and N2 must be A, T, C, G, a, t, c, or g;

Standard sequence name: >sample (Any line starts with > and no space after >);

Preserve [**N1/N2**] and the sequence name if having and automatically run:

1 Small letters (a, t, g, c, n) will be changed to capital letters (A, T, G, C, N), respectively;

2 Other letters (such as R, K) will be changed to N;

3 Digit numbers (0,1,2,3,4,5,6,7,8,9) and other symbols will be omitted.

Then follow user’s selection (automatically or manually select).

1.2 Multiple SNP sequences one time

>SNP sequence 1

NNNNNNNNNNNN**[N1/N2]**NNNNNNNNN

>SNP sequence 2

NNNNNNNNNNNN**[N1/N2]**NNNNNNNNN

>SNP sequence 3

NNNNNNNNNNNN**[N1/N2]**NNNNNNNNN

Note: clarify the sequence based on format 1.1 and then automatically or manually select the sequence for designing primers, one sequence by one sequence:

Format 2 (2 allele sequences, blast against each other in our program and switch to format 3):

>Allele 1

NNNNNNNNNNNN**N1**NNNNNNNNN

>Allele 2

NNNNNNNNNNNN**N2**NNNNNNNNN

Note: Automatically generate 3 lines (sequence 1, variation check (symbol |), and sequence 2);

If broken, add symbol “–” and/or then the missing bases in the broken region.

Format 3 (alignment format):

Query 1 TGCTCTTTGAATGAAATTTGAGGTCATTCAATTTGTGCATTGTT**T**CGAAACCTTGATTTT

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

Sbjct 1 TGCTCTTTGAATGAAATTTGAGGTCATTCAATTTGTGCATTGTT**C**CGAAACCTTGATTTT

Note: Automatically generate 3 lines (sequence 1, variation check (symbol |), and sequence 2).

Then:

3.1 Find any variation locus based on variation check (symbol |) line (symbol | indicate same base and “space” indicate variation locus) and record the position in index;

3.2 Identify SNPs and Indels;

Sequence 1 and sequence 2 have bases (A, T, C, or G) at the same position in index indicate SNP;

Sequence 1 OR sequence 2 have symbol “-” at the same position in index indicate Indel.

Then follow user’s selection (automatically or manually select).

2. Automatically or manually select locus for designing primers:

2.1 “Automatically select”

Combine SNPs based on the results in format 3:

**≥5** apart from any two adjacent SNP positions will be identified as **two SNPs**;

**≤4** apart from any two adjacent SNP positions will be identified as **one SNP**;

E.g. SNP (50), SNP (58), SNP (60), SNP (62), SNP (64), SNP (70), SNP (100); The number in () indicate SNP position in index;

SNP (100)- SNP (70)=30 **≥5**, SNP (100) as an independent SNP-U (100) and SNP-D (100);

Note: SNP (100) can be used to design two pairs of F STAR-primers (**u**pstream and **d**ownstream), and thus designated as SNP-U (100) and SNP-D (100). Here, need reverse and complement between SNP-U and SNP-D (e.g. SNP-U (100) = [A/G], then SNP-D (100) =[T/C]).

SNP (70)- SNP (64)=6 **≥5**, SNP (70) as an independent SNP-U (70) and SNP-D (70);

SNP (64)- SNP (62)=2 **≤4**, then;

SNP (62)- SNP (60)=2 **≤4**, then;

SNP (60)- SNP (58)=2 **≤4**, then;

SNP (58)- SNP (50)=8 **≥5**, thus having SNPs: SNP-U (58), SNP-U (58-60), SNP-U (58-60-62), SNP-U (58-60-62-64); and SNP-D (58-60-62-64), SNP-D (60-62-64), SNP-D (62-64), SNP-D (64);

SNP (50)- 0=50 **≥5**, SNP (50) as an independent SNP-U (50) and SNP-D (50);

Thus, there have loci: SNP-U (100), SNP-D (100), SNP-U (70), SNP-D (70), SNP-U (58), SNP-U (58-60), SNP-U (58-60-62), SNP-U (58-60-62-64), SNP-D (58-60-62-64), SNP-D (60-62-64), SNP-D (62-64), SNP-D (64), SNP-U (50), and SNP-D (50);

Combine Indels based on the results in format 3 (contiguous symbol “-” as an indel).

Then order variation loci: **indel (≤20 bases)** > **≥ 4 SNPs** > 3 SNPs > 2 SNPs > **indel (≥21 bases)** > [G/C] > [A/T] > [G/T] > [C/A] > [C/T] > [G/A] and then generate STAR primers for ≤ 3 loci.

Order indel variation loci based on the position in index;

Order SNP variation loci based on the bigger SNP numbers, then smaller value (max SNP position – min SNP position), then [G/C] > [A/T] > [G/T] > [C/A] > [C/T] > [G/A], then the position in index;

If **≥2** loci having same SNP numbers, order loci based on the smaller value to bigger value (max SNP position – min SNP position);

If **≥2** loci having same value (max SNP position – min SNP position), order loci based on the SNP (the last one for U and the first one for D): [G/C] > [A/T] > [G/T] > [C/A] > [C/T] > [G/A];

If **≥2** loci having same SNP, order loci based on the smaller position in index;

Thus: SNP-D (**58**-60-62-64) or SNP-U (58-60-62-**64**) > SNP-D (**60**-62-64) or SNP-U (58-60-**62**) > SNP-U (58-**60**) or SNP-D (**62**-64) > SNP-U (50), SNP-D (50), SNP-U (58), SNP-D (64), SNP-U (70), SNP-D (70), SNP-U (100), or SNP-D (100);

2.2 “Manually select”

Combine Indels based on the results in format 3 (contiguous symbol “-” as an indel) and then show all variation loci for selection (each SNP as a locus):

Switch Indel locus into SNP locus for F primer design:

1. If the deleted or inserted base number ≤ 20:

1.1 Stretch the two allele sequences from the 1st matched base at the upstream **(based on the variation check line)** towards 3’ direction one base by one base (eight times, generate eight allele pairs);



1.2 Remove the allele pairs having same base at their 3’ end (Remove allele pairs 1, 2, 7, and 8);

Remove the allele pairs having only **one** different base at the stretched region (Remove allele pair 3);

Remove the allele pairs that any allele having ≥ 5 As (AAAAA), Ts, Gs, or Cs in the stretched region);

1.3 If the remaining allele pair number =0, try the other variation locus; otherwise, continue:

1.4 Order the allele pairs based on the different base number in the last four bases (4 different bases, then 3, and then 2); if having more than one allele pair with same different base number in the last four bases, order these allele pairs based on the stretched base number from min to max;

1.5 Preferentially select the 1st allele pair to design F primer pair following SNP principle (**F primer design for SNP; next page**);

1.6 If successful, design R primer; otherwise, try the next allele pair until to the last allele pair;

2. If the deleted or inserted base number ≥21:

2.1 Select (6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20) bases at the downstream of the indel locus from the 1st matched base towards 3’ direction and generate 15 allele pairs;

**6-bp allele 1: NNNNNN**

**6-bp allele 2: NNNNNN**

**7-bp allele 1: NNNNNNN**

**7-bp allele 2: NNNNNNN**

……

**19-bp allele 1: NNNNNNNNNNNNNNNNNNN**

**19-bp allele 2: NNNNNNNNNNNNNNNNNNN**

**20-bp allele 1: NNNNNNNNNNNNNNNNNNNN**

**20-bp allele 2: NNNNNNNNNNNNNNNNNNNN**

2.2 Stretch each allele pair towards 5’ direction one base by one base (make each allele containing 17 through 26 bases, e.g. 6-bp allele pair, Ref. **F primer design for SNP; next page**);

**F1 NNNNNNNNNNNNNNNNN**

**F2 NNNNNNNNNNNNNNNNN**

**F1 NNNNNNNNNNNNNNNNNN**

**F2 NNNNNNNNNNNNNNNNNN**

**F1 NNNNNNNNNNNNNNNNNNN**

**F2 NNNNNNNNNNNNNNNNNNN**

**F1 NNNNNNNNNNNNNNNNNNNN**

**F2 NNNNNNNNNNNNNNNNNNNN**

**F1 NNNNNNNNNNNNNNNNNNNNN**

**F2 NNNNNNNNNNNNNNNNNNNNN**

**F1 NNNNNNNNNNNNNNNNNNNNNN**

**F2 NNNNNNNNNNNNNNNNNNNNNN**

**F1 NNNNNNNNNNNNNNNNNNNNNNN**

**F2 NNNNNNNNNNNNNNNNNNNNNNN**

**F1 NNNNNNNNNNNNNNNNNNNNNNNN**

**F2 NNNNNNNNNNNNNNNNNNNNNNNN**

**F1 NNNNNNNNNNNNNNNNNNNNNNNNN**

**F2 NNNNNNNNNNNNNNNNNNNNNNNNN**

**F1 NNNNNNNNNNNNNNNNNNNNNNNNNN**

**F2 NNNNNNNNNNNNNNNNNNNNNNNNNN**

2.3 Remove the F primer pairs that any F primer:

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

having ≥ 8 contiguous A (AAAAAAAA), Ts, Gs, or Cs;

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

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

2.4 Remove all allele pairs having ≤ 4 different bases;

2.5 Calculate Tm value of part2.1 (the selected bases) and part2.2 (the stretched bases) **in each F primer**; Remove the F primer pairs that any F primer having (Tm of part2.1) – (Tm of part2.2) < 5;

2.6 Calculate Tm value of each F primer and Remove the F primer pairs that any F primer having Tm value < 53C or >60C;

2.7 Calculate the average Tm value of each F primer pair and cluster all F primer pairs into 3 groups: group 1 (53C ≤ average Tm value < 55C); group 2 (55C ≤ average Tm value <57C); group 3 (57C ≤ average Tm value <60C);

2.8 Order F primer pairs in group 1 then group 2 and then group 3 based on different base number from max to min;

2.9 Select the 1st F primer pair and then design R primer (No nucleotide substitution is required); if F primer pair number =0, try another variation locus;

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 contiguous A (AAAAAAAA), Ts, Gs, or Cs;

2.3) having ≥ 4 As or Ts or ≥ 5 Gs, or Cs in the last 6 bases at the 3’ end;

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

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

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

4. Determine the SNP module:

If the combined SNP number =1 (indicate SNP number in parenthesis, see 2.1), go to **one SNP module**;

If the combined SNP number =2 (indicate SNP number in parenthesis, see 2.1), go to **two SNP module**;

If the combined SNP number ≥3 (indicate SNP number in parenthesis, see 2.1), go to **multiple SNP module**;

One SNP module: the combined SNP number =1 at 3’ end:

Calculate Tm value of each F primer, the average Tm value of each F primer pair, and total SNP number of each F primer pair; Select F primer pairs having total SNP number ≥ 4;

1. If the selected F primer pair number =0, go to step 2; otherwise, select the F primer pairs that both F primers have Tm value arranging from (≥) 53C to (≤) 60C (53C ≤ Tm ≤ 60C);

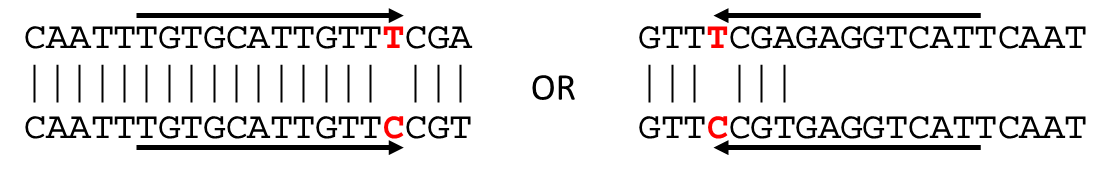
If total F primer pair number=0, go to step 2; Otherwise, preserve the F primer pair with average Tm close to **53C** as F primer pair (No nucleotide substitution is required) and then design R primer;

2. Select the F primer pairs that both F primers have Tm value arranging from (≥) 54C to (≤) 58C (54C ≤ Tm ≤ 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 substituted for G allele, and the G/C distal to 3ʹ end will be substituted for C allele

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 substituted for G allele, and the A/T distal to 3ʹ end will be substituted for C allele.

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;

4th 3rd 2nd 1st (SNP at 3ʹ end) principle of base substitution: A→C, T→C, G→A, and C→T;

IF A/T **A/T** **A/T** [C/G] Then **2nd** base for G allele and **3rd** base for C allele;

IF **A/T** **A/T** G/C [C/G] Then **3rd** base for G allele and **4th** base for C allele;

IF **A/T** G/C **A/T** [C/G] Then **2nd** base for G allele and **4th** base for C allele;

IF G/C **A/T** **A/T** [C/G] Then **2nd** base for G allele and **3rd** base for C allele;

IF A/T **G/C** **G/C** [C/G] Then **2nd** base for G allele and **3rd** base for C allele;

IF **G/C** A/T **G/C** [C/G] Then **2nd** base for G allele and **4th** base for C allele;

IF **G/C** **G/C** A/T [C/G] Then **3rd** base for G allele and **4th** base for C allele;

IF G/C **G/C** **G/C** [C/G] Then **2nd** base for G allele and **3rd** base for C allele;

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;

4th 3rd 2nd 1st (SNP at 3ʹ end) principle of base substitution: A→C, T→C, G→A, and C→T;

IF A/T **A/T** **A/T** [C/T] Then **2nd** base for C allele and **3rd** base for T allele;

IF A/T **A/T** **G/C** [C/T] Then **2nd** base for C allele and **3rd** base for T allele;

IF **A/T** G/C **A/T** [C/T] Then **2nd** base for C allele and **4th** base for T allele;

IF G/C **A/T** **A/T** [C/T] Then **2nd** base for C allele and **3rd** base for T allele;

IF A/T **G/C** **G/C** [C/T] Then **2nd** base for C allele and **3rd** base for T allele;

IF G/C **A/T** **G/C** [C/T] Then **2nd** base for C allele and **3rd** base for T allele;

IF **G/C** **G/C** A/T [C/T] Then **3rd** base for T allele and **4th** base for C allele;

IF G/C **G/C** **G/C** [C/T] Then **2nd** base for C allele and **3rd** base for T allele;

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;

4th 3rd 2nd 1st (SNP at 3ʹ end) principle of base substitution: A→C, T→C, G→A, and C→T;

IF A/T **A/T** **A/T** [C/A] Then **2nd** base for C allele and **3rd** base for A allele;

IF A/T **A/T** **G/C** [C/A] Then **2nd** base for C allele and **3rd** base for A allele;

IF **A/T** G/C **A/T** [C/A] Then **2nd** base for C allele and **4th** base for A allele;

IF G/C **A/T** **A/T** [C/A] Then **2nd** base for C allele and **3rd** base for A allele;

IF A/T **G/C** **G/C** [C/A] Then **2nd** base for C allele and **3rd** base for A allele;

IF G/C **A/T** **G/C** [C/A] Then **2nd** base for C allele and **3rd** base for A allele;

IF **G/C** **G/C** A/T [C/A] Then **3rd** base for C allele and **4th** base for A allele;

IF G/C **G/C** **G/C** [C/A] Then **2nd** base for C allele and **3rd** base for A allele;

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;

4th 3rd 2nd 1st (SNP at 3ʹ end) principle of base substitution: A→C, T→C, G→A, and C→T;

IF A/T **A/T** **A/T** [G/T] Then **2nd** base for G allele and **3rd** base for T allele;

IF A/T **A/T** **G/C** [G/T] Then **2nd** base for G allele and **3rd** base for T allele;

IF **A/T** G/C **A/T** [G/T] Then **2nd** base for G allele and **4th** base for T allele;

IF G/C **A/T** **A/T** [G/T] Then **2nd** base for G allele and **3rd** base for T allele;

IF A/T **G/C** **G/C** [G/T] Then **2nd** base for G allele and **3rd** base for T allele;

IF G/C **A/T** **G/C** [G/T] Then **2nd** base for G allele and **3rd** base for T allele;

IF **G/C** **G/C** A/T [G/T] Then **3rd** base for T allele and **4th** base for G allele;

IF G/C **G/C** **G/C** [G/T] Then **2nd** base for G allele and **3rd** base for T allele;

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;

4th 3rd 2nd 1st (SNP at 3ʹ end) principle of base substitution: A→C, T→C, G→A, and C→T;

IF A/T **A/T** **A/T** [G/A] Then **2nd** base for G allele and **3rd** base for A allele;

IF A/T **A/T** **G/C** [G/A] Then **2nd** base for G allele and **3rd** base for A allele;

IF **A/T** G/C **A/T** [G/A] Then **2nd** base for G allele and **4th** base for A allele;

IF G/C **A/T** **A/T** [G/A] Then **2nd** base for G allele and **3rd** base for A allele;

IF A/T **G/C** **G/C** [G/A] Then **2nd** base for G allele and **3rd** base for A allele;

IF G/C **A/T** **G/C** [G/A] Then **2nd** base for G allele and **3rd** base for A allele;

IF **G/C** **G/C** A/T [G/A] Then **3rd** base for G allele and **4th** base for A allele;

IF G/C **G/C** **G/C** [G/A] Then **2nd** base for G allele and **3rd** base for A allele;

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;

4th 3rd 2nd 1st (SNP at 3ʹ end) principle of base substitution: A→C, T→C, G→A, and C→T;

IF **A/T** **A/T** A/T [A/T] Then **3rd** base for T allele and **4th** base for A allele;

IF **A/T** **A/T** G/C [A/T] Then **3rd** base for T allele and **4th** base for A allele;

IF **A/T** G/C **A/T** [A/T] Then **2nd** base for T allele and **4th** base for A allele;

IF G/C **A/T** **A/T** [A/T] Then **2nd** base for T allele and **3rd** base for A allele;

IF A/T **G/C** **G/C** [A/T] Then **2nd** base for T allele and **3rd** base for A allele;

IF **G/C** A/T **G/C** [A/T] Then **2nd** base for T allele and **4th** base for A allele;

IF **G/C** **G/C** A/T [A/T] Then **3rd** base for T allele and **4th** base for A allele;

IF **G/C** **G/C** G/C [A/T] Then **3rd** base for T allele and **4th** base for A allele;

Two SNP module: the combined SNP number =2 at 3’ region:

Calculate Tm value of each F primer, the average Tm value of each F primer pair, and total SNP number of each F primer pair; Select F primer pairs having total SNP number ≥ 3;

1. If the selected F primer pair number =0, go to step 2; otherwise, select the F primer pairs that both F primers have Tm value arranging from (≥) 53C to (≤) 60C (53C ≤ Tm ≤ 60C);

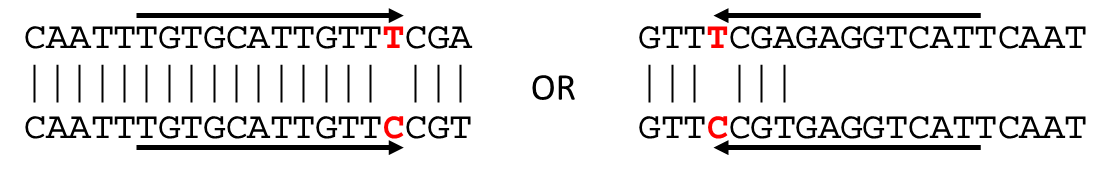
If total F primer pair number=0, go to step 2; Otherwise, preserve the F primer pair with average Tm close to **53C** as F primer pair (No nucleotide substitution is required) and then design R primer;

2. Select the F primer pairs that both F primers have Tm value arranging from (≥) 54C to (≤) 58C (54C ≤ Tm ≤ 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 (at 3ʹ end)

1.1 The additional SNP at 2nd from 3ʹ end, select the 3rd, 4th, or 5th base for substitution following this principle (see below): A→C, T→C, G→A, and C→T;

**A. The additional SNP is [C/G] or [A/T]:**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

A/T **A/T** **A/T** [SNP] [C/G] Then **3rd** base for G allele and **4th** base for C allele;

**A/T** **A/T** G/C [SNP] [C/G] Then **4th** base for G allele and **5th** base for C allele;

**A/T** G/C **A/T** [SNP] [C/G] Then **3rd** base for G allele and **5th** base for C allele;

G/C **A/T** **A/T** [SNP] [C/G] Then **3rd** base for G allele and **4th** base for C allele;

A/T **G/C** **G/C** [SNP] [C/G] Then **3rd** base for G allele and **4th** base for C allele;

**G/C** A/T **G/C** [SNP] [C/G] Then **3rd** base for G allele and **5th** base for C allele;

**G/C** **G/C** A/T [SNP] [C/G] Then **4th** base for G allele and **5th** base for C allele;

G/C **G/C** **G/C** [SNP] [C/G] Then **3rd** base for G allele and **4th** base for C allele;

**B. The additional SNP is [C/A], [C/T], [G/A], or [G/T]:**

**B1. G or C allele couples with C allele (indicates one allele (G2 or C2) C1 ∞ another allele (A2 or T2) G1)**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

A/T **A/T** **A/T** [SNP] [C/G] Then **3rd** base for G allele and **4th** base for C allele;

A/T **A/T** **G/C** [SNP] [C/G] Then **3rd** base for C allele and **4th** base for G allele;

A/T **G/C** **A/T** [SNP] [C/G] Then **3rd** base for G allele and **4th** base for C allele;

G/C **A/T** **A/T** [SNP] [C/G] Then **3rd** base for G allele and **4th** base for C allele;

A/T **G/C** **G/C** [SNP] [C/G] Then **3rd** base for G allele and **4th** base for C allele;

G/C **A/T** **G/C** [SNP] [C/G] Then **3rd** base for C allele and **4th** base for G allele;

G/C **G/C** **A/T** [SNP] [C/G] Then **3rd** base for G allele and **4th** base for C allele;

G/C **G/C** **G/C** [SNP] [C/G] Then **3rd** base for G allele and **4th** base for C allele;

**B. The additional SNP is [C/A], [C/T], [G/A], or [G/T]:**

**B2. A or T allele couples with C allele (indicates one allele (A2 or T2) C1 ∞ another allele (G2 or C2) G1)**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

A/T **A/T** **A/T** [SNP] [C/G] Then **3rd** base for G allele and **4th** base for C allele;

A/T **A/T** **G/C** [SNP] [C/G] Then **3rd** base for G allele and **4th** base for C allele;

A/T **G/C** **A/T** [SNP] [C/G] Then **3rd** base for C allele and **4th** base for G allele;

G/C **A/T** **A/T** [SNP] [C/G] Then **3rd** base for G allele and **4th** base for C allele;

A/T **G/C** **G/C** [SNP] [C/G] Then **3rd** base for G allele and **4th** base for C allele;

G/C **A/T** **G/C** [SNP] [C/G] Then **3rd** base for G allele and **4th** base for C allele;

G/C **G/C** **A/T** [SNP] [C/G] Then **3rd** base for C allele and **4th** base for G allele;

G/C **G/C** **G/C** [SNP] [C/G] Then **3rd** base for G allele and **4th** base for C allele;

1.2 The additional SNP at 3rd from 3ʹ end, select the 2nd, 4th, or 5th base for substitution following this principle (see below): A→C, T→C, G→A, and C→T;

**A. The additional SNP is [C/G] or [A/T]:**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

A/T **A/T** [SNP] **A/T** [C/G] Then **2nd** base for G allele and **4th** base for C allele;

**A/T** **A/T** [SNP] G/C [C/G] Then **4th** base for G allele and **5th** base for C allele;

**A/T** G/C [SNP] **A/T** [C/G] Then **2nd** base for G allele and **5th** base for C allele;

G/C **A/T** [SNP] **A/T** [C/G] Then **2nd** base for G allele and **4th** base for C allele;

A/T **G/C** [SNP] **G/C** [C/G] Then **2nd** base for G allele and **4th** base for C allele;

**G/C** A/T [SNP] **G/C** [C/G] Then **2nd** base for G allele and **5th** base for C allele;

**G/C** **G/C** [SNP] A/T [C/G] Then **4th** base for G allele and **5th** base for C allele;

G/C **G/C** [SNP] **G/C** [C/G] Then **2nd** base for G allele and **4th** base for C allele;

**B. The additional SNP is [C/A], [C/T], [G/A], or [G/T]:**

**B1. G or C allele couples with C allele (indicates one allele (G3 or C3) C1 ∞ another allele (A3 or T3) G1)**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

A/T **A/T** [SNP] **A/T** [C/G] Then **2nd** base for G allele and **4th** base for C allele;

A/T **A/T** [SNP] **G/C** [C/G] Then **2nd** base for C allele and **4th** base for G allele;

A/T **G/C** [SNP] **A/T** [C/G] Then **2nd** base for G allele and **4th** base for C allele;

G/C **A/T** [SNP] **A/T** [C/G] Then **2nd** base for G allele and **4th** base for C allele;

A/T **G/C** [SNP] **G/C** [C/G] Then **2nd** base for G allele and **4th** base for C allele;

G/C **A/T** [SNP] **G/C** [C/G] Then **2nd** base for C allele and **4th** base for G allele;

G/C **G/C** [SNP] **A/T** [C/G] Then **2nd** base for G allele and **4th** base for C allele;

G/C **G/C** [SNP] **G/C** [C/G] Then **2nd** base for G allele and **4th** base for C allele;

**B. The additional SNP is [C/A], [C/T], [G/A], or [G/T]:**

**B2. A or T allele couples with C allele (indicates one allele (A3 or T3) C1 ∞ another allele (G3 or C3) G1)**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

A/T **A/T** [SNP] **A/T** [C/G] Then **2nd** base for G allele and **4th** base for C allele;

A/T **A/T** [SNP] **G/C** [C/G] Then **2nd** base for G allele and **4th** base for C allele;

A/T **G/C** [SNP] **A/T** [C/G] Then **2nd** base for C allele and **4th** base for G allele;

G/C **A/T** [SNP] **A/T** [C/G] Then **2nd** base for G allele and **4th** base for C allele;

A/T **G/C** [SNP] **G/C** [C/G] Then **2nd** base for G allele and **4th** base for C allele;

G/C **A/T** [SNP] **G/C** [C/G] Then **2nd** base for G allele and **4th** base for C allele;

G/C **G/C** [SNP] **A/T** [C/G] Then **2nd** base for C allele and **4th** base for G allele;

G/C **G/C** [SNP] **G/C** [C/G] Then **2nd** base for G allele and **4th** base for C allele;

1.3 The additional SNP at 4th from 3ʹ end, select the 2nd, 3rd, or 5th base for substitution following this principle (see below): A→C, T→C, G→A, and C→T;

**A. The additional SNP is [C/G] or [A/T]:**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

A/T [SNP] **A/T** **A/T** [C/G] Then **2nd** base for G allele and **3rd** base for C allele;

**A/T** [SNP] **A/T** G/C [C/G] Then **3rd** base for G allele and **5th** base for C allele;

**A/T** [SNP] G/C **A/T** [C/G] Then **2nd** base for G allele and **5th** base for C allele;

G/C [SNP] **A/T** **A/T** [C/G] Then **2nd** base for G allele and **3rd** base for C allele;

A/T [SNP] **G/C** **G/C** [C/G] Then **2nd** base for G allele and **3rd** base for C allele;

**G/C** [SNP] A/T **G/C** [C/G] Then **2nd** base for G allele and **5th** base for C allele;

**G/C** [SNP] **G/C** A/T [C/G] Then **4th** base for G allele and **5th** base for C allele;

G/C [SNP] **G/C** **G/C** [C/G] Then **2nd** base for G allele and **3rd** base for C allele;

**B. The additional SNP is [C/A], [C/T], [G/A], or [G/T]:**

**B1. G or C allele couples with C allele (indicates one allele (G4 or C4) C1 ∞ another allele (A4 or T4) G1)**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

A/T [SNP] **A/T** **A/T** [C/G] Then **2nd** base for G allele and **3rd** base for C allele;

A/T [SNP] **A/T** **G/C** [C/G] Then **2nd** base for C allele and **3rd** base for G allele;

A/T [SNP] **G/C** **A/T** [C/G] Then **2nd** base for G allele and **3rd** base for C allele;

G/C [SNP] **A/T** **A/T** [C/G] Then **2nd** base for G allele and **3rd** base for C allele;

A/T [SNP] **G/C** **G/C** [C/G] Then **2nd** base for G allele and **3rd** base for C allele;

G/C [SNP] **A/T** **G/C** [C/G] Then **2nd** base for C allele and **3rd** base for G allele;

G/C [SNP] **G/C** **A/T** [C/G] Then **2nd** base for G allele and **3rd** base for C allele;

G/C [SNP] **G/C** **G/C** [C/G] Then **2nd** base for G allele and **3rd** base for C allele;

**B. The additional SNP is [C/A], [C/T], [G/A], or [G/T]:**

**B2. A or T allele couples with C allele (indicates one allele (A4 or T4) C1 ∞ another allele (G4 or C4) G1)**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

A/T [SNP] **A/T** **A/T** [C/G] Then **2nd** base for G allele and **3rd** base for C allele;

A/T [SNP] **A/T** **G/C** [C/G] Then **2nd** base for G allele and **3rd** base for C allele;

A/T [SNP] **G/C** **A/T** [C/G] Then **2nd** base for C allele and **3rd** base for G allele;

G/C [SNP] **A/T** **A/T** [C/G] Then **2nd** base for G allele and **3rd** base for C allele;

A/T [SNP] **G/C** **G/C** [C/G] Then **2nd** base for G allele and **3rd** base for C allele;

G/C [SNP] **A/T** **G/C** [C/G] Then **2nd** base for G allele and **3rd** base for C allele;

G/C [SNP] **G/C** **A/T** [C/G] Then **2nd** base for C allele and **3rd** base for G allele;

G/C [SNP] **G/C** **G/C** [C/G] Then **2nd** base for G allele and **3rd** base for C allele;

1.4 The additional SNP at 5th from 3ʹ end, select the 2nd, 3rd, or 4th base for substitution following this principle (see below or same to [C/G] SNP in one SNP module): A→C, T→C, G→A, and C→T;

**A. The additional SNP is [C/G] or [A/T]:**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

[SNP] A/T **A/T** **A/T** [C/G] Then **2nd** base for G allele and **3rd** base for C allele;

[SNP] **A/T** **A/T** G/C [C/G] Then **3rd** base for G allele and **4th** base for C allele;

[SNP] **A/T** G/C **A/T** [C/G] Then **2nd** base for G allele and **4th** base for C allele;

[SNP] G/C **A/T** **A/T** [C/G] Then **2nd** base for G allele and **3rd** base for C allele;

[SNP] A/T **G/C** **G/C** [C/G] Then **2nd** base for G allele and **3rd** base for C allele;

[SNP] **G/C** A/T **G/C** [C/G] Then **2nd** base for G allele and **4th** base for C allele;

[SNP] **G/C** **G/C** A/T [C/G] Then **3rd** base for G allele and **4th** base for C allele;

[SNP] G/C **G/C** **G/C** [C/G] Then **2nd** base for G allele and **3rd** base for C allele;

**B. The additional SNP is [C/A], [C/T], [G/A], or [G/T]:**

**B1. G or C allele couples with C allele (indicates one allele (G5 or C5) C1 ∞ another allele (A5 or T5) G1)**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

[SNP] A/T **A/T** **A/T** [C/G] Then **2nd** base for G allele and **3rd** base for C allele;

[SNP] A/T **A/T** **G/C** [C/G] Then **2nd** base for C allele and **3rd** base for G allele;

[SNP] A/T **G/C** **A/T** [C/G] Then **2nd** base for G allele and **3rd** base for C allele;

[SNP] G/C **A/T** **A/T** [C/G] Then **2nd** base for G allele and **3rd** base for C allele;

[SNP] A/T **G/C** **G/C** [C/G] Then **2nd** base for G allele and **3rd** base for C allele;

[SNP] G/C **A/T** **G/C** [C/G] Then **2nd** base for C allele and **3rd** base for G allele;

[SNP] G/C **G/C** **A/T** [C/G] Then **2nd** base for G allele and **3rd** base for C allele;

[SNP] G/C **G/C** **G/C** [C/G] Then **2nd** base for G allele and **3rd** base for C allele;

**B. The additional SNP is [C/A], [C/T], [G/A], or [G/T]:**

**B2. A or T allele couples with C allele (indicates one allele (A5 or T5) C1 ∞ another allele (G5 or C5) G1)**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

[SNP] A/T **A/T** **A/T** [C/G] Then **2nd** base for G allele and **3rd** base for C allele;

[SNP] A/T **A/T** **G/C** [C/G] Then **2nd** base for G allele and **3rd** base for C allele;

[SNP] A/T **G/C** **A/T** [C/G] Then **2nd** base for C allele and **3rd** base for G allele;

[SNP] G/C **A/T** **A/T** [C/G] Then **2nd** base for G allele and **3rd** base for C allele;

[SNP] A/T **G/C** **G/C** [C/G] Then **2nd** base for G allele and **3rd** base for C allele;

[SNP] G/C **A/T** **G/C** [C/G] Then **2nd** base for G allele and **3rd** base for C allele;

[SNP] G/C **G/C** **A/T** [C/G] Then **2nd** base for C allele and **3rd** base for G allele;

[SNP] G/C **G/C** **G/C** [C/G] Then **2nd** base for G allele and **3rd** base for C allele;

2 For [C/T] SNP

2.1 The additional SNP at 2nd from 3ʹ end, select the 3rd, 4th, or 5th base for substitution following this principle (see below): A→C, T→C, G→A, and C→T;

**A. The additional SNP is [C/G] or [A/T]:**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

A/T **A/T** **A/T** [SNP] [C/T] Then **3rd** base for T allele and **4th** base for C allele;

A/T **A/T** **G/C** [SNP] [C/T] Then **3rd** base for C allele and **4th** base for T allele;

A/T **G/C** **A/T** [SNP] [C/T] Then **3rd** base for T allele and **4th** base for C allele;

G/C **A/T** **A/T** [SNP] [C/T] Then **3rd** base for T allele and **4th** base for C allele;

A/T **G/C** **G/C** [SNP] [C/T] Then **3rd** base for T allele and **4th** base for C allele;

G/C **A/T** **G/C** [SNP] [C/T] Then **3rd** base for C allele and **4th** base for T allele;

G/C **G/C** **A/T** [SNP] [C/T] Then **3rd** base for T allele and **4th** base for C allele;

G/C **G/C** **G/C** [SNP] [C/T] Then **3rd** base for T allele and **4th** base for C allele;

**B. The additional SNP is [C/A], [C/T], [G/A], or [G/T]:**

**B1. G or C allele couples with C allele (indicates one allele (G2 or C2) C1 ∞ another allele (A2 or T2) T1)**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

A/T **A/T** **A/T** [SNP] [C/T] Then **3rd** base for T allele and **4th** base for C allele;

A/T **A/T** **G/C** [SNP] [C/T] Then **3rd** base for C allele and **4th** base for T allele;

A/T **G/C** **A/T** [SNP] [C/T] Then **3rd** base for T allele and **4th** base for C allele;

**G/C** A/T **A/T** [SNP] [C/T] Then **3rd** base for T allele and **5th** base for C allele;

**A/T** G/C **G/C** [SNP] [C/T] Then **3rd** base for C allele and **5th** base for T allele;

G/C **A/T** **G/C** [SNP] [C/T] Then **3rd** base for C allele and **4th** base for T allele;

G/C **G/C** **A/T** [SNP] [C/T] Then **3rd** base for T allele and **4th** base for C allele;

G/C **G/C** **G/C** [SNP] [C/T] Then **3rd** base for T allele and **4th** base for C allele;

**B. The additional SNP is [C/A], [C/T], [G/A], or [G/T]:**

**B2. A or T allele couples with C allele (indicates one allele (A2 or T2) C1 ∞ another allele (G2 or C2) T1)**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

A/T **A/T** **A/T** [SNP] [C/T] Then **3rd** base for T allele and **4th** base for C allele;

**A/T** G/C **A/T** [SNP] [C/T] Then **3rd** base for T allele and **5th** base for C allele;

G/C **A/T** **A/T** [SNP] [C/T] Then **3rd** base for T allele and **4th** base for C allele;

A/T **G/C** **G/C** [SNP] [C/T] Then **3rd** base for T allele and **4th** base for C allele;

**G/C** A/T **G/C** [SNP] [C/T] Then **3rd** base for T allele and **5th** base for C allele;

**G/C** **G/C** A/T [SNP] [C/T] Then **4th** base for T allele and **5th** base for C allele;

G/C **G/C** **G/C** [SNP] [C/T] Then **3rd** base for T allele and **4th** base for C allele;

**A/T** **A/T** G/C [SNP] [C/T] Then **4th** base for T allele and **5th** base for C allele;

2.2 Additional SNP at 3rd from 3ʹ end, select the 2nd, 4th, or 5th base for substitution following this principle (see below): A→C, T→C, G→A, and C→T;

**A. The additional SNP is [C/G] or [A/T]:**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

A/T **A/T** [SNP] **A/T** [C/T] Then **2nd** base for C allele and **4th** base for T allele;

A/T **A/T** [SNP] **G/C** [C/T] Then **2nd** base for C allele and **4th** base for T allele;

**A/T** G/C [SNP] **A/T** [C/T] Then **2nd** base for C allele and **5th** base for T allele;

G/C **A/T** [SNP] **A/T** [C/T] Then **2nd** base for C allele and **4th** base for T allele;

A/T **G/C** [SNP] **G/C** [C/T] Then **2nd** base for C allele and **4th** base for T allele;

G/C **A/T** [SNP] **G/C** [C/T] Then **2nd** base for C allele and **4th** base for T allele;

**G/C** **G/C** [SNP] A/T [C/T] Then **4th** base for T allele and **5th** base for C allele;

G/C **G/C** [SNP] **G/C** [C/T] Then **2nd** base for C allele and **4th** base for T allele;

**B. The additional SNP is [C/A], [C/T], [G/A], or [G/T]:**

**B1. G or C allele couples with C allele (indicates one allele (G3 or C3) C1 ∞ another allele (A3 or T3) T1)**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

A/T **A/T** [SNP] **A/T** [C/T] Then **2nd** base for C allele and **4th** base for T allele;

A/T **A/T** [SNP] **G/C** [C/T] Then **2nd** base for C allele and **4th** base for T allele;

**A/T** **G/C** [SNP] A/T [C/T] Then **4th** base for C allele and **5th** base for T allele;

**G/C** **A/T** [SNP] A/T [C/T] Then **4th** base for T allele and **5th** base for C allele;

**A/T** G/C [SNP] **G/C** [C/T] Then **2nd** base for C allele and **5th** base for T allele;

G/C **A/T** [SNP] **G/C** [C/T] Then **2nd** base for C allele and **4th** base for T allele;

**G/C** **G/C** [SNP] A/T [C/T] Then **4th** base for T allele and **5th** base for C allele;

G/C **G/C** [SNP] **G/C** [C/T] Then **2nd** base for C allele and **4th** base for T allele;

**B. The additional SNP is [C/A], [C/T], [G/A], or [G/T]:**

**B2. A or T allele couples with C allele (indicates one allele (A3 or T3) C1 ∞ another allele (G3 or C3) T1)**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

A/T **A/T** [SNP] **A/T** [C/T] Then **2nd** base for C allele and **4th** base for T allele;

**A/T** **A/T** [SNP] G/C [C/T] Then **4th** base for T allele and **5th** base for C allele;

**A/T** G/C [SNP] **A/T** [C/T] Then **2nd** base for C allele and **5th** base for T allele;

G/C **A/T** [SNP] **A/T** [C/T] Then **2nd** base for C allele and **4th** base for T allele;

A/T **G/C** [SNP] **G/C** [C/T] Then **2nd** base for C allele and **4th** base for T allele;

**G/C** A/T [SNP] **G/C** [C/T] Then **2nd** base for C allele and **5th** base for T allele;

**G/C** **G/C** [SNP] A/T [C/T] Then **4th** base for T allele and **5th** base for C allele;

G/C **G/C** [SNP] **G/C** [C/T] Then **2nd** base for C allele and **4th** base for T allele;

2.3 The additional SNP at 4th from 3ʹ end, select the 2nd, 3rd, or 5th base for substitution following this principle (see below): A→C, T→C, G→A, and C→T;

**A. The additional SNP is [C/G] or [A/T]:**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

A/T [SNP] **A/T** **A/T** [C/T] Then **2nd** base for C allele and **3rd** base for T allele;

A/T [SNP] **A/T** **G/C** [C/T] Then **2nd** base for C allele and **3rd** base for T allele;

**A/T** [SNP] G/C **A/T** [C/T] Then **2nd** base for C allele and **5th** base for T allele;

G/C [SNP] **A/T** **A/T** [C/T] Then **2nd** base for C allele and **3rd** base for T allele;

A/T [SNP] **G/C** **G/C** [C/T] Then **2nd** base for C allele and **3rd** base for T allele;

G/C [SNP] **A/T** **G/C** [C/T] Then **2nd** base for C allele and **3rd** base for T allele;

**G/C** [SNP] **G/C** A/T [C/T] Then **3rd** base for T allele and **5th** base for C allele;

G/C [SNP] **G/C** **G/C** [C/T] Then **2nd** base for C allele and **3rd** base for T allele;

**B. The additional SNP is [C/A], [C/T], [G/A], or [G/T]:**

**B1. G or C allele couples with C allele (indicates one allele (G4 or C4) C1 ∞ another allele (A4 or T4) T1)**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

A/T [SNP] **A/T** **A/T** [C/T] Then **2nd** base for C allele and **3rd** base for T allele;

A/T [SNP] **A/T** **G/C** [C/T] Then **2nd** base for C allele and **3rd** base for T allele;

**A/T** [SNP] **G/C** A/T [C/T] Then **3rd** base for C allele and **5th** base for T allele;

**G/C** [SNP] **A/T** A/T [C/T] Then **3rd** base for T allele and **5th** base for C allele;

**A/T** [SNP] G/C **G/C** [C/T] Then **2nd** base for C allele and **5th** base for T allele;

G/C [SNP] **A/T** **G/C** [C/T] Then **2nd** base for C allele and **3rd** base for T allele;

**G/C** [SNP] **G/C** A/T [C/T] Then **3rd** base for T allele and **5th** base for C allele;

G/C [SNP] **G/C** **G/C** [C/T] Then **2nd** base for C allele and **3rd** base for T allele;

**B. The additional SNP is [C/A], [C/T], [G/A], or [G/T]:**

**B2. A or T allele couples with C allele (indicates one allele (A4 or T4) C1 ∞ another allele (G4 or C4) T1)**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

A/T [SNP] **A/T** **A/T** [C/T] Then **2nd** base for C allele and **3rd** base for T allele;

**A/T** [SNP] **A/T** G/C [C/T] Then **3rd** base for T allele and **5th** base for C allele;

**A/T** [SNP] G/C **A/T** [C/T] Then **2nd** base for C allele and **5th** base for T allele;

G/C [SNP] **A/T** **A/T** [C/T] Then **2nd** base for C allele and **3rd** base for T allele;

A/T [SNP] **G/C** **G/C** [C/T] Then **2nd** base for C allele and **3rd** base for T allele;

**G/C** [SNP] A/T **G/C** [C/T] Then **2nd** base for C allele and **5th** base for T allele;

**G/C** [SNP] **G/C** A/T [C/T] Then **3rd** base for T allele and **5th** base for C allele;

G/C [SNP] **G/C** **G/C** [C/T] Then **2nd** base for C allele and **3rd** base for T allele;

2.4 Additional SNP at 4th from 3ʹ end, select the 2nd, 3rd, or 5th base for substitution following this principle (see below): A→C, T→C, G→A, and C→T;

**A. The additional SNP is [C/G] or [A/T]:**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

[SNP] A/T **A/T** **A/T** [C/T] Then **2nd** base for C allele and **3rd** base for T allele;

[SNP] A/T **A/T** **G/C** [C/T] Then **2nd** base for C allele and **3rd** base for T allele;

[SNP] **A/T** G/C **A/T** [C/T] Then **2nd** base for C allele and **4th** base for T allele;

[SNP] G/C **A/T** **A/T** [C/T] Then **2nd** base for C allele and **3rd** base for T allele;

[SNP] A/T **G/C** **G/C** [C/T] Then **2nd** base for C allele and **3rd** base for T allele;

[SNP] G/C **A/T** **G/C** [C/T] Then **2nd** base for C allele and **3rd** base for T allele;

[SNP] **G/C** **G/C** A/T [C/T] Then **3rd** base for T allele and **4th** base for C allele;

[SNP] G/C **G/C** **G/C** [C/T] Then **2nd** base for C allele and **3rd** base for T allele;

**B. The additional SNP is [C/A], [C/T], [G/A], or [G/T]:**

**B1. G or C allele couples with C allele (indicates one allele (G5 or C5) C1 ∞ another allele (A5 or T5) T1)**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

[SNP] A/T **A/T** **A/T** [C/T] Then **2nd** base for C allele and **3rd** base for T allele;

[SNP] A/T **A/T** **G/C** [C/T] Then **2nd** base for C allele and **3rd** base for T allele;

[SNP] **A/T** **G/C** A/T [C/T] Then **3rd** base for C allele and **4th** base for T allele;

[SNP] **G/C** **A/T** A/T [C/T] Then **3rd** base for T allele and **4th** base for C allele;

[SNP] **A/T** G/C **G/C** [C/T] Then **2nd** base for C allele and **4th** base for T allele;

[SNP] G/C **A/T** **G/C** [C/T] Then **2nd** base for C allele and **3rd** base for T allele;

[SNP] **G/C** **G/C** A/T [C/T] Then **3rd** base for T allele and **4th** base for C allele;

[SNP] G/C **G/C** **G/C** [C/T] Then **2nd** base for C allele and **3rd** base for T allele;

**B. The additional SNP is [C/A], [C/T], [G/A], or [G/T]:**

**B2. A or T allele couples with C allele (indicates one allele (A5 or T5) C1 ∞ another allele (G5 or C5) T1)**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

[SNP] A/T **A/T** **A/T** [C/T] Then **2nd** base for C allele and **3rd** base for T allele;

[SNP] **A/T** **A/T** G/C [C/T] Then **3rd** base for T allele and **4th** base for C allele;

[SNP] **A/T** G/C **A/T** [C/T] Then **2nd** base for C allele and **4th** base for T allele;

[SNP] G/C **A/T** **A/T** [C/T] Then **2nd** base for C allele and **3rd** base for T allele;

[SNP] A/T **G/C** **G/C** [C/T] Then **2nd** base for C allele and **3rd** base for T allele;

[SNP] **G/C** A/T **G/C** [C/T] Then **2nd** base for C allele and **4th** base for T allele;

[SNP] **G/C** **G/C** A/T [C/T] Then **3rd** base for T allele and **4th** base for C allele;

[SNP] G/C **G/C** **G/C** [C/T] Then **2nd** base for C allele and **3rd** base for T allele;

3 For [C/A] SNP

3.1 Additional SNP at 2nd from 3ʹ end, select the 3rd, 4th, or 5th base for substitution following this principle (see below): A→C, T→C, G→A, and C→T;

**A. The additional SNP is [C/G] or [A/T]:**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

A/T **A/T** **A/T** [SNP] [C/A] Then **3rd** base for C allele and **4th** base for A allele;

A/T **A/T** **G/C** [SNP] [C/A] Then **3rd** base for C allele and **4th** base for A allele;

A/T **G/C** **A/T** [SNP] [C/A] Then **3rd** base for A allele and **4th** base for C allele;

G/C **A/T** **A/T** [SNP] [C/A] Then **3rd** base for C allele and **4th** base for A allele;

A/T **G/C** **G/C** [SNP] [C/A] Then **3rd** base for C allele and **4th** base for A allele;

G/C **A/T** **G/C** [SNP] [C/A] Then **3rd** base for C allele and **4th** base for A allele;

G/C **G/C** **A/T** [SNP] [C/A] Then **3rd** base for A allele and **4th** base for C allele;

G/C **G/C** **G/C** [SNP] [C/A] Then **3rd** base for C allele and **4th** base for A allele;

**B. The additional SNP is [C/A], [C/T], [G/A], or [G/T]:**

**B1. G or C allele couples with C allele (indicates one allele (G2 or C2) C1 ∞ another allele (A2 or T2) A1)**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

A/T **A/T** **A/T** [SNP] [C/A] Then **3rd** base for C allele and **4th** base for A allele;

A/T **A/T** **G/C** [SNP] [C/A] Then **3rd** base for C allele and **4th** base for A allele;

A/T **G/C** **A/T** [SNP] [C/A] Then **3rd** base for A allele and **4th** base for C allele;

**G/C** A/T **A/T** [SNP] [C/A] Then **3rd** base for A allele and **5th** base for C allele;

**A/T** G/C **G/C** [SNP] [C/A] Then **3rd** base for C allele and **5th** base for A allele;

G/C **A/T** **G/C** [SNP] [C/A] Then **3rd** base for C allele and **4th** base for A allele;

G/C **G/C** **A/T** [SNP] [C/A] Then **3rd** base for A allele and **4th** base for C allele;

G/C **G/C** **G/C** [SNP] [C/A] Then **3rd** base for C allele and **4th** base for A allele;

**B. The additional SNP is [C/A], [C/T], [G/A], or [G/T]:**

**B2. A or T allele couples with C allele (indicates one allele (A2 or T2) C1 ∞ another allele (G2 or C2) A1)**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

A/T **A/T** **A/T** [SNP] [C/A] Then **3rd** base for C allele and **4th** base for A allele;

**A/T** **A/T** G/C [SNP] [C/A] Then **4th** base for C allele and **5th** base for A allele;

**A/T** G/C **A/T** [SNP] [C/A] Then **3rd** base for C allele and **5th** base for A allele;

G/C **A/T** **A/T** [SNP] [C/A] Then **3rd** base for C allele and **4th** base for A allele;

A/T **G/C** **G/C** [SNP] [C/A] Then **3rd** base for C allele and **4th** base for A allele;

**G/C** A/T **G/C** [SNP] [C/A] Then **3rd** base for C allele and **5th** base for A allele;

**G/C** **G/C** A/T [SNP] [C/A] Then **4th** base for C allele and **5th** base for A allele;

G/C **G/C** **G/C** [SNP] [C/A] Then **3rd** base for C allele and **4th** base for A allele;

3.2 Additional SNP at 3rd from 3ʹ end, select the 2nd, 4th, or 5th base for substitution following this principle (see below): A→C, T→C, G→A, and C→T;

**A. The additional SNP is [C/G] or [A/T]:**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

A/T **A/T** [SNP] **A/T** [C/A] Then **2nd** base for C allele and **4th** base for A allele;

A/T **A/T** [SNP] **G/C** [C/A] Then **2nd** base for C allele and **4th** base for A allele;

**A/T** G/C [SNP] **A/T** [C/A] Then **2nd** base for C allele and **5th** base for A allele;

G/C **A/T** [SNP] **A/T** [C/A] Then **2nd** base for C allele and **4th** base for A allele;

A/T **G/C** [SNP] **G/C** [C/A] Then **2nd** base for C allele and **4th** base for A allele;

G/C **A/T** [SNP] **G/C** [C/A] Then **2nd** base for C allele and **4th** base for A allele;

**G/C** **G/C** [SNP] A/T [C/A] Then **4th** base for C allele and **5th** base for A allele;

G/C **G/C** [SNP] **G/C** [C/A] Then **2nd** base for C allele and **4th** base for A allele;

**B. The additional SNP is [C/A], [C/T], [G/A], or [G/T]:**

**B1. G or C allele couples with C allele (indicates one allele (G3 or C3) C1 ∞ another allele (A3 or T3) A1)**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

A/T **A/T** [SNP] **A/T** [C/A] Then **2nd** base for C allele and **4th** base for A allele;

A/T **A/T** [SNP] **G/C** [C/A] Then **2nd** base for C allele and **4th** base for A allele;

**A/T** **G/C** [SNP] A/T [C/A] Then **4th** base for C allele and **5th** base for A allele;

**G/C** **A/T** [SNP] A/T [C/A] Then **4th** base for A allele and **5th** base for C allele;

**A/T** G/C [SNP] **G/C** [C/A] Then **2nd** base for C allele and **5th** base for A allele;

G/C **A/T** [SNP] **G/C** [C/A] Then **2nd** base for C allele and **4th** base for A allele;

**G/C** **G/C** [SNP] A/T [C/A] Then **4th** base for C allele and **5th** base for A allele;

G/C **G/C** [SNP] **G/C** [C/A] Then **2nd** base for C allele and **4th** base for A allele;

**B. The additional SNP is [C/A], [C/T], [G/A], or [G/T]:**

**B2. A or T allele couples with C allele (indicates one allele (A3 or T3) C1 ∞ another allele (G3 or C3) A1)**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

A/T **A/T** [SNP] **A/T** [C/A] Then **2nd** base for C allele and **4th** base for A allele;

**A/T** **A/T** [SNP] G/C [C/A] Then **4th** base for C allele and **5th** base for A allele;

**A/T** G/C [SNP] **A/T** [C/A] Then **2nd** base for C allele and **5th** base for A allele;

G/C **A/T** [SNP] **A/T** [C/A] Then **2nd** base for C allele and **4th** base for A allele;

A/T **G/C** [SNP] **G/C** [C/A] Then **2nd** base for C allele and **4th** base for A allele;

**G/C** A/T [SNP] **G/C** [C/A] Then **2nd** base for C allele and **5th** base for A allele;

**G/C** **G/C** [SNP] A/T [C/A] Then **4th** base for C allele and **5th** base for A allele;

G/C **G/C** [SNP] **G/C** [C/A] Then **2nd** base for C allele and **4th** base for A allele;

3.3 Additional SNP at 4th from 3ʹ end, select the 2nd, 3rd, or 5th base for substitution following this principle (see below): A→C, T→C, G→A, and C→T;

**A. The additional SNP is [C/G] or [A/T]:**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

A/T [SNP] **A/T** **A/T** [C/A] Then **2nd** base for C allele and **3rd** base for A allele;

A/T [SNP] **A/T** **G/C** [C/A] Then **2nd** base for C allele and **3rd** base for A allele;

**A/T** [SNP] G/C **A/T** [C/A] Then **2nd** base for C allele and **5th** base for A allele;

G/C [SNP] **A/T** **A/T** [C/A] Then **2nd** base for C allele and **3rd** base for A allele;

A/T [SNP] **G/C** **G/C** [C/A] Then **2nd** base for C allele and **3rd** base for A allele;

G/C [SNP] **A/T** **G/C** [C/A] Then **2nd** base for C allele and **3rd** base for A allele;

**G/C** [SNP] **G/C** A/T [C/A] Then **3rd** base for C allele and **5th** base for A allele;

G/C [SNP] **G/C** **G/C** [C/A] Then **2nd** base for C allele and **3rd** base for A allele;

**B. The additional SNP is [C/A], [C/T], [G/A], or [G/T]:**

**B1. G or C allele couples with C allele (indicates one allele (G4 or C4) C1 ∞ another allele (A4 or T4) A1)**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

A/T [SNP] **A/T** **A/T** [C/A] Then **2nd** base for C allele and **3rd** base for A allele;

A/T [SNP] **A/T** **G/C** [C/A] Then **2nd** base for C allele and **3rd** base for A allele;

**A/T** [SNP] **G/C** A/T [C/A] Then **3rd** base for C allele and **5th** base for A allele;

**G/C** [SNP] **A/T** A/T [C/A] Then **3rd** base for A allele and **5th** base for C allele;

**A/T** [SNP] G/C **G/C** [C/A] Then **2nd** base for C allele and **5th** base for A allele;

G/C [SNP] **A/T** **G/C** [C/A] Then **2nd** base for C allele and **3rd** base for A allele;

**G/C** [SNP] **G/C** A/T [C/A] Then **3rd** base for C allele and **5th** base for A allele;

G/C [SNP] **G/C** **G/C** [C/A] Then **2nd** base for C allele and **3rd** base for A allele;

**B. The additional SNP is [C/A], [C/T], [G/A], or [G/T]:**

**B2. A or T allele couples with C allele (indicates one allele (A4 or T4) C1 ∞ another allele (G4 or C4) A1)**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

A/T [SNP] **A/T** **A/T** [C/A] Then **2nd** base for C allele and **3rd** base for A allele;

**A/T** [SNP] **A/T** G/C [C/A] Then **3rd** base for C allele and **5th** base for A allele;

**A/T** [SNP] G/C **A/T** [C/A] Then **2nd** base for C allele and **5th** base for A allele;

G/C [SNP] **A/T** **A/T** [C/A] Then **2nd** base for C allele and **3rd** base for A allele;

A/T [SNP] **G/C** **G/C** [C/A] Then **2nd** base for C allele and **3rd** base for A allele;

**G/C** [SNP] A/T **G/C** [C/A] Then **2nd** base for C allele and **5th** base for A allele;

**G/C** [SNP] **G/C** A/T [C/A] Then **3rd** base for C allele and **5th** base for A allele;

G/C [SNP] **G/C** **G/C** [C/A] Then **2nd** base for C allele and **3rd** base for A allele;

3.4 Additional SNP at 5th from 3ʹ end, select the 2nd, 3rd, or 4th base for substitution following this principle (see below): A→C, T→C, G→A, and C→T;

**A. The additional SNP is [C/G] or [A/T]:**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

[SNP] A/T **A/T** **A/T** [C/A] Then **2nd** base for C allele and **3rd** base for A allele;

[SNP] A/T **A/T** **G/C** [C/A] Then **2nd** base for C allele and **3rd** base for A allele;

[SNP] **A/T** G/C **A/T** [C/A] Then **2nd** base for C allele and **4th** base for A allele;

[SNP] G/C **A/T** **A/T** [C/A] Then **2nd** base for C allele and **3rd** base for A allele;

[SNP] A/T **G/C** **G/C** [C/A] Then **2nd** base for C allele and **3rd** base for A allele;

[SNP] G/C **A/T** **G/C** [C/A] Then **2nd** base for C allele and **3rd** base for A allele;

[SNP] **G/C** **G/C** A/T [C/A] Then **3rd** base for C allele and **4th** base for A allele;

[SNP] G/C **G/C** **G/C** [C/A] Then **2nd** base for C allele and **3rd** base for A allele;

**B. The additional SNP is [C/A], [C/T], [G/A], or [G/T]:**

**B1. G or C allele couples with C allele (indicates one allele (G5 or C5) C1 ∞ another allele (A5 or T5) A1)**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

[SNP] A/T **A/T** **A/T** [C/A] Then **2nd** base for C allele and **3rd** base for A allele;

[SNP] A/T **A/T** **G/C** [C/A] Then **2nd** base for C allele and **3rd** base for A allele;

[SNP] **A/T** **G/C** A/T [C/A] Then **3rd** base for C allele and **4th** base for A allele;

[SNP] **G/C** **A/T** A/T [C/A] Then **3rd** base for A allele and **4th** base for C allele;

[SNP] **A/T** G/C **G/C** [C/A] Then **2nd** base for C allele and **4th** base for A allele;

[SNP] G/C **A/T** **G/C** [C/A] Then **2nd** base for C allele and **3rd** base for A allele;

[SNP] **G/C** **G/C** A/T [C/A] Then **3rd** base for C allele and **4th** base for A allele;

[SNP] G/C **G/C** **G/C** [C/A] Then **2nd** base for C allele and **3rd** base for A allele;

**B. The additional SNP is [C/A], [C/T], [G/A], or [G/T]:**

**B2. A or T allele couples with C allele (indicates one allele (A5 or T5) C1 ∞ another allele (G5 or C5) A1)**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

[SNP] A/T **A/T** **A/T** [C/A] Then **2nd** base for C allele and **3rd** base for A allele;

[SNP] **A/T** **A/T** G/C [C/A] Then **3rd** base for C allele and **4th** base for A allele;

[SNP] **A/T** G/C **A/T** [C/A] Then **2nd** base for C allele and **4th** base for A allele;

[SNP] G/C **A/T** **A/T** [C/A] Then **2nd** base for C allele and **3rd** base for A allele;

[SNP] A/T **G/C** **G/C** [C/A] Then **2nd** base for C allele and **3rd** base for A allele;

[SNP] **G/C** A/T **G/C** [C/A] Then **2nd** base for C allele and **4th** base for A allele;

[SNP] **G/C** **G/C** A/T [C/A] Then **3rd** base for C allele and **4th** base for A allele;

[SNP] G/C **G/C** **G/C** [C/A] Then **2nd** base for C allele and **3rd** base for A allele;

4 For [G/T] SNP

4.1 Additional SNP at 2nd from 3ʹ end, select the 3rd, 4th, or 5th base for substitution following this principle (see below): A→C, T→C, G→A, and C→T;

**A. The additional SNP is [C/G] or [A/T]:**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

A/T **A/T** **A/T** [SNP] [G/T] Then **3rd** base for T allele and **4th** base for G allele;

A/T **A/T** **G/C** [SNP] [G/T] Then **3rd** base for G allele and **4th** base for T allele;

A/T **G/C** **A/T** [SNP] [G/T] Then **3rd** base for T allele and **4th** base for G allele;

G/C **A/T** **A/T** [SNP] [G/T] Then **3rd** base for T allele and **4th** base for G allele;

A/T **G/C** **G/C** [SNP] [G/T] Then **3rd** base for T allele and **4th** base for G allele;

G/C **A/T** **G/C** [SNP] [G/T] Then **3rd** base for G allele and **4th** base for T allele;

G/C **G/C** **A/T** [SNP] [G/T] Then **4th** base for T allele and **4th** base for G allele;

G/C **G/C** **G/C** [SNP] [G/T] Then **3rd** base for T allele and **4th** base for G allele;

**B. The additional SNP is [C/A], [C/T], [G/A], or [G/T]:**

**B1. G or C allele couples with G allele (indicates one allele (G2 or C2) G1 ∞ another allele (A2 or T2) T1)**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

A/T **A/T** **A/T** [SNP] [G/T] Then **3rd** base for T allele and **4th** base for G allele;

A/T **A/T** **G/C** [SNP] [G/T] Then **3rd** base for G allele and **4th** base for T allele;

A/T **G/C** **A/T** [SNP] [G/T] Then **3rd** base for T allele and **4th** base for G allele;

**G/C** A/T **A/T** [SNP] [G/T] Then **3rd** base for T allele and **5th** base for G allele;

**A/T** G/C **G/C** [SNP] [G/T] Then **3rd** base for G allele and **5th** base for T allele;

G/C **A/T** **G/C** [SNP] [G/T] Then **3rd** base for G allele and **4th** base for T allele;

G/C **G/C** **A/T** [SNP] [G/T] Then **3rd** base for T allele and **4th** base for G allele;

G/C **G/C** **G/C** [SNP] [G/T] Then **3rd** base for T allele and **4th** base for G allele;

**B. The additional SNP is [C/A], [C/T], [G/A], or [G/T]:**

**B2. A or T allele couples with G allele (indicates one allele (A2 or T2) G1 ∞ another allele (G2 or C2) T1)**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

A/T **A/T** **A/T** [SNP] [G/T] Then **3rd** base for T allele and **4th** base for G allele;

**A/T** **A/T** G/C [SNP] [G/T] Then **4th** base for T allele and **5th** base for G allele;

**A/T** G/C **A/T** [SNP] [G/T] Then **3rd** base for T allele and **5th** base for G allele;

G/C **A/T** **A/T** [SNP] [G/T] Then **3rd** base for T allele and **4th** base for G allele;

A/T **G/C** **G/C** [SNP] [G/T] Then **3rd** base for T allele and **4th** base for G allele;

**G/C** A/T **G/C** [SNP] [G/T] Then **3rd** base for T allele and **5th** base for G allele;

**G/C** **G/C** A/T [SNP] [G/T] Then **4th** base for T allele and **5th** base for G allele;

G/C **G/C** **G/C** [SNP] [G/T] Then **3rd** base for T allele and **4th** base for G allele;

4.2 Additional SNP at 3rd from 3ʹ end, select the 2nd, 4th, or 5th base for substitution following this principle (see below): A→C, T→C, G→A, and C→T;

**A. The additional SNP is [C/G] or [A/T]:**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

A/T **A/T** [SNP] **A/T** [G/T] Then **2nd** base for G allele and **4th** base for T allele;

A/T **A/T** [SNP] **G/C** [G/T] Then **2nd** base for G allele and **4th** base for T allele;

**A/T** G/C [SNP] **A/T** [G/T] Then **2nd** base for G allele and **5th** base for T allele;

G/C **A/T** [SNP] **A/T** [G/T] Then **2nd** base for G allele and **4th** base for T allele;

A/T **G/C** [SNP] **G/C** [G/T] Then **2nd** base for G allele and **4th** base for T allele;

G/C **A/T** [SNP] **G/C** [G/T] Then **2nd** base for G allele and **4th** base for T allele;

**G/C** **G/C** [SNP] A/T [G/T] Then **4th** base for T allele and **5th** base for G allele;

G/C **G/C** [SNP] **G/C** [G/T] Then **2nd** base for G allele and **4th** base for T allele;

**B. The additional SNP is [C/A], [C/T], [G/A], or [G/T]:**

**B1. G or C allele couples with G allele (indicates one allele (G3 or C3) G1 ∞ another allele (A3 or T3) T1)**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

A/T **A/T** [SNP] **A/T** [G/T] Then **2nd** base for G allele and **4th** base for T allele;

A/T **A/T** [SNP] **G/C** [G/T] Then **2nd** base for G allele and **4th** base for T allele;

**A/T** **G/C** [SNP] A/T [G/T] Then **4th** base for G allele and **5th** base for T allele;

**G/C** **A/T** [SNP] A/T [G/T] Then **4th** base for T allele and **5th** base for G allele;

**A/T** G/C [SNP] **G/C** [G/T] Then **2nd** base for G allele and **5th** base for T allele;

G/C **A/T** [SNP] **G/C** [G/T] Then **2nd** base for G allele and **4th** base for T allele;

**G/C** **G/C** [SNP] A/T [G/T] Then **4th** base for T allele and **5th** base for G allele;

G/C **G/C** [SNP] **G/C** [G/T] Then **2nd** base for G allele and **4th** base for T allele;

**B. The additional SNP is [C/A], [C/T], [G/A], or [G/T]:**

**B2. A or T allele couples with G allele (indicates one allele (A3 or T3) G1 ∞ another allele (G3 or C3) T1)**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

A/T **A/T** [SNP] **A/T** [G/T] Then **2nd** base for G allele and **4th** base for T allele;

**A/T** **A/T** [SNP] G/C [G/T] Then **4th** base for T allele and **5th** base for G allele;

**A/T** G/C [SNP] **A/T** [G/T] Then **2nd** base for G allele and **5th** base for T allele;

G/C **A/T** [SNP] **A/T** [G/T] Then **2nd** base for G allele and **4th** base for T allele;

A/T **G/C** [SNP] **G/C** [G/T] Then **2nd** base for G allele and **4th** base for T allele;

**G/C** A/T [SNP] **G/C** [G/T] Then **2nd** base for G allele and **5th** base for T allele;

**G/C** **G/C** [SNP] A/T [G/T] Then **4th** base for T allele and **5th** base for G allele;

G/C **G/C** [SNP] **G/C** [G/T] Then **2nd** base for G allele and **4th** base for T allele;

4.3 Additional SNP at 4th from 3ʹ end, select the 2nd, 3rd, or 5th base for substitution following this principle (see below): A→C, T→C, G→A, and C→T;

**A. The additional SNP is [C/G] or [A/T]:**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

A/T [SNP] **A/T** **A/T** [G/T] Then **2nd** base for G allele and **3rd** base for T allele;

A/T [SNP] **A/T** **G/C** [G/T] Then **2nd** base for G allele and **3rd** base for T allele;

**A/T** [SNP] G/C **A/T** [G/T] Then **2nd** base for G allele and **5th** base for T allele;

G/C [SNP] **A/T** **A/T** [G/T] Then **2nd** base for G allele and **3rd** base for T allele;

A/T [SNP] **G/C** **G/C** [G/T] Then **2nd** base for G allele and **3rd** base for T allele;

G/C [SNP] **A/T** **G/C** [G/T] Then **2nd** base for G allele and **3rd** base for T allele;

**G/C** [SNP] **G/C** A/T [G/T] Then **3rd** base for T allele and **5th** base for G allele;

G/C [SNP] **G/C** **G/C** [G/T] Then **2nd** base for G allele and **3rd** base for T allele;

**B. The additional SNP is [C/A], [C/T], [G/A], or [G/T]:**

**B1. G or C allele couples with G allele (indicates one allele (G4 or C4) G1 ∞ another allele (A4 or T4) T1)**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

A/T [SNP] **A/T** **A/T** [G/T] Then **2nd** base for G allele and **3rd** base for T allele;

A/T [SNP] **A/T** **G/C** [G/T] Then **2nd** base for G allele and **3rd** base for T allele;

**A/T** [SNP] **G/C** A/T [G/T] Then **3rd** base for G allele and **5th** base for T allele;

**G/C** [SNP] **A/T** A/T [G/T] Then **3rd** base for T allele and **5th** base for G allele;

**A/T** [SNP] G/C **G/C** [G/T] Then **2nd** base for G allele and **5th** base for T allele;

G/C [SNP] **A/T** **G/C** [G/T] Then **2nd** base for G allele and **3rd** base for T allele;

**G/C** [SNP] **G/C** A/T [G/T] Then **3rd** base for T allele and **5th** base for G allele;

G/C [SNP] **G/C** **G/C** [G/T] Then **2nd** base for G allele and **3rd** base for T allele;

**B. The additional SNP is [C/A], [C/T], [G/A], or [G/T]:**

**B2. A or T allele couples with G allele (indicates one allele (A4 or T4) G1 ∞ another allele (G4 or C4) T1)**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

A/T [SNP] **A/T** **A/T** [G/T] Then **2nd** base for G allele and **3rd** base for T allele;

**A/T** [SNP] **A/T** G/C [G/T] Then **3rd** base for T allele and **5th** base for G allele;

**A/T** [SNP] G/C **A/T** [G/T] Then **2nd** base for G allele and **5th** base for T allele;

G/C [SNP] **A/T** **A/T** [G/T] Then **2nd** base for G allele and **3rd** base for T allele;

A/T [SNP] **G/C** **G/C** [G/T] Then **2nd** base for G allele and **3rd** base for T allele;

**G/C** [SNP] A/T **G/C** [G/T] Then **2nd** base for G allele and **5th** base for T allele;

**G/C** [SNP] **G/C** A/T [G/T] Then **3rd** base for T allele and **5th** base for G allele;

G/C [SNP] **G/C** **G/C** [G/T] Then **2nd** base for G allele and **3rd** base for T allele;

4.4 Additional SNP at 5th from 3ʹ end, select the 2nd, 3rd, or 4th base for substitution following this principle (see below): A→C, T→C, G→A, and C→T;

**A. The additional SNP is [C/G] or [A/T]:**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

[SNP] A/T **A/T** **A/T** [G/T] Then **2nd** base for G allele and **3rd** base for T allele;

[SNP] A/T **A/T** **G/C** [G/T] Then **2nd** base for G allele and **3rd** base for T allele;

[SNP] **A/T** G/C **A/T** [G/T] Then **2nd** base for G allele and **4th** base for T allele;

[SNP] G/C **A/T** **A/T** [G/T] Then **2nd** base for G allele and **3rd** base for T allele;

[SNP] A/T **G/C** **G/C** [G/T] Then **2nd** base for G allele and **3rd** base for T allele;

[SNP] G/C **A/T** **G/C** [G/T] Then **2nd** base for G allele and **3rd** base for T allele;

[SNP] **G/C** **G/C** A/T [G/T] Then **3rd** base for T allele and **4th** base for G allele;

[SNP] G/C **G/C** **G/C** [G/T] Then **2nd** base for G allele and **3rd** base for T allele;

**B. The additional SNP is [C/A], [C/T], [G/A], or [G/T]:**

**B1. G or C allele couples with G allele (indicates one allele (G5 or C5) G1 ∞ another allele (A5 or T5) T1)**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

[SNP] A/T **A/T** **A/T** [G/T] Then **2nd** base for G allele and **3rd** base for T allele;

[SNP] A/T **A/T** **G/C** [G/T] Then **2nd** base for G allele and **3rd** base for T allele;

[SNP] **A/T** **G/C** A/T [G/T] Then **3rd** base for G allele and **4th** base for T allele;

[SNP] **G/C** **A/T** A/T [G/T] Then **3rd** base for T allele and **4th** base for G allele;

[SNP] **A/T** G/C **G/C** [G/T] Then **2nd** base for G allele and **4th** base for T allele;

[SNP] G/C **A/T** **G/C** [G/T] Then **2nd** base for G allele and **3rd** base for T allele;

[SNP] **G/C** **G/C** A/T [G/T] Then **3rd** base for T allele and **4th** base for G allele;

[SNP] G/C **G/C** **G/C** [G/T] Then **2nd** base for G allele and **3rd** base for T allele;

**B. The additional SNP is [C/A], [C/T], [G/A], or [G/T]:**

**B2. A or T allele couples with G allele (indicates one allele (A5 or T5) G1 ∞ another allele (G5 or C5) T1)**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

[SNP] A/T **A/T** **A/T** [G/T] Then **2nd** base for G allele and **3rd** base for T allele;

[SNP] **A/T** **A/T** G/C [G/T] Then **3rd** base for T allele and **4th** base for G allele;

[SNP] **A/T** G/C **A/T** [G/T] Then **2nd** base for G allele and **4th** base for T allele;

[SNP] G/C **A/T** **A/T** [G/T] Then **2nd** base for G allele and **3rd** base for T allele;

[SNP] A/T **G/C** **G/C** [G/T] Then **2nd** base for G allele and **3rd** base for T allele;

[SNP] **G/C** A/T **G/C** [G/T] Then **2nd** base for G allele and **4th** base for T allele;

[SNP] **G/C** **G/C** A/T [G/T] Then **3rd** base for T allele and **4th** base for G allele;

[SNP] G/C **G/C** **G/C** [G/T] Then **2nd** base for G allele and **3rd** base for T allele;

5 For [G/A] SNP

5.1 Additional SNP at 2nd from 3ʹ end, select the 3rd, 4th, or 5th base for substitution following this principle (see below): A→C, T→C, G→A, and C→T;

**A. The additional SNP is [C/G] or [A/T]:**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

A/T **A/T** **A/T** [SNP] [G/A] Then **3rd** base for G allele and **4th** base for A allele;

A/T **A/T** **G/C** [SNP] [G/A] Then **3rd** base for G allele and **4th** base for A allele;

A/T **G/C** **A/T** [SNP] [G/A] Then **3rd** base for A allele and **4th** base for G allele;

G/C **A/T** **A/T** [SNP] [G/A] Then **3rd** base for G allele and **4th** base for A allele;

A/T **G/C** **G/C** [SNP] [G/A] Then **3rd** base for G allele and **4th** base for A allele;

G/C **A/T** **G/C** [SNP] [G/A] Then **3rd** base for G allele and **4th** base for A allele;

G/C **G/C** **A/T** [SNP] [G/A] Then **3rd** base for A allele and **4th** base for G allele;

G/C **G/C** **G/C** [SNP] [G/A] Then **3rd** base for G allele and **4th** base for A allele;

**B. The additional SNP is [C/A], [C/T], [G/A], or [G/T]:**

**B1. G or C allele couples with G allele (indicates one allele (G2 or C2) G1 ∞ another allele (A2 or T2) A1)**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

A/T **A/T** **A/T** [SNP] [G/A] Then **3rd** base for G allele and **4th** base for A allele;

A/T **A/T** **G/C** [SNP] [G/A] Then **3rd** base for G allele and **4th** base for A allele;

A/T **G/C** **A/T** [SNP] [G/A] Then **3rd** base for A allele and **4th** base for G allele;

**G/C** A/T **A/T** [SNP] [G/A] Then **3rd** base for A allele and **5th** base for G allele;

**A/T** G/C **G/C** [SNP] [G/A] Then **3rd** base for G allele and **5th** base for A allele;

G/C **A/T** **G/C** [SNP] [G/A] Then **3rd** base for G allele and **4th** base for A allele;

G/C **G/C** **A/T** [SNP] [G/A] Then **3rd** base for A allele and **4th** base for G allele;

G/C **G/C** **G/C** [SNP] [G/A] Then **3rd** base for G allele and **4th** base for A allele;

**B. The additional SNP is [C/A], [C/T], [G/A], or [G/T]:**

**B2. A or T allele couples with G allele (indicates one allele (A2 or T2) G1 ∞ another allele (G2 or C2) A1)**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

A/T **A/T** **A/T** [SNP] [G/A] Then **3rd** base for G allele and **4th** base for A allele;

**A/T** **A/T** G/C [SNP] [G/A] Then **4th** base for G allele and **5th** base for A allele;

**A/T** G/C **A/T** [SNP] [G/A] Then **3rd** base for G allele and **5th** base for A allele;

G/C **A/T** **A/T** [SNP] [G/A] Then **3rd** base for G allele and **4th** base for A allele;

A/T **G/C** **G/C** [SNP] [G/A] Then **3rd** base for G allele and **4th** base for A allele;

**G/C** A/T **G/C** [SNP] [G/A] Then **3rd** base for G allele and **5th** base for A allele;

**G/C** **G/C** A/T [SNP] [G/A] Then **4th** base for G allele and **5th** base for A allele;

G/C **G/C** **G/C** [SNP] [G/A] Then **3rd** base for G allele and **4th** base for A allele;

5.2 Additional SNP at 3rd from 3ʹ end select the 2nd, 4th, or 5th base for substitution following this principle (see below): A→C, T→C, G→A, and C→T;

**A. The additional SNP is [C/G] or [A/T]:**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

A/T **A/T** [SNP] **A/T** [G/A] Then **2nd** base for G allele and **4th** base for A allele;

A/T **A/T** [SNP] **G/C** [G/A] Then **2nd** base for G allele and **4th** base for A allele;

**A/T** G/C [SNP] **A/T** [G/A] Then **2nd** base for G allele and **5th** base for A allele;

G/C **A/T** [SNP] **A/T** [G/A] Then **2nd** base for G allele and **4th** base for A allele;

A/T **G/C** [SNP] **G/C** [G/A] Then **2nd** base for G allele and **4th** base for A allele;

G/C **A/T** [SNP] **G/C** [G/A] Then **2nd** base for G allele and **4th** base for A allele;

**G/C** **G/C** [SNP] A/T [G/A] Then **4th** base for G allele and **5th** base for A allele;

G/C **G/C** [SNP] **G/C** [G/A] Then **2nd** base for G allele and **4th** base for A allele;

**B. The additional SNP is [C/A], [C/T], [G/A], or [G/T]:**

**B1. G or C allele couples with G allele (indicates one allele (G3 or C3) G1 ∞ another allele (A3 or T3) A1)**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

A/T **A/T** [SNP] **A/T** [G/A] Then **2nd** base for G allele and **4th** base for A allele;

A/T **A/T** [SNP] **G/C** [G/A] Then **2nd** base for G allele and **4th** base for A allele;

**A/T** **G/C** [SNP] A/T [G/A] Then **4th** base for G allele and **5th** base for A allele;

**G/C** **A/T** [SNP] A/T [G/A] Then **4th** base for A allele and **5th** base for G allele;

**A/T** G/C [SNP] **G/C** [G/A] Then **2nd** base for G allele and **5th** base for A allele;

G/C **A/T** [SNP] **G/C** [G/A] Then **2nd** base for G allele and **4th** base for A allele;

**G/C** **G/C** [SNP] A/T [G/A] Then **4th** base for G allele and **5th** base for A allele;

G/C **G/C** [SNP] **G/C** [G/A] Then **2nd** base for G allele and **4th** base for A allele;

**B. The additional SNP is [C/A], [C/T], [G/A], or [G/T]:**

**B2. A or T allele couples with G allele (indicates one allele (A3 or T3) G1 ∞ another allele (G3 or C3) A1)**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

A/T **A/T** [SNP] **A/T** [G/A] Then **2nd** base for G allele and **4th** base for A allele;

**A/T** **A/T** [SNP] G/C [G/A] Then **4th** base for G allele and **5th** base for A allele;

**A/T** G/C [SNP] **A/T** [G/A] Then **2nd** base for G allele and **5th** base for A allele;

G/C **A/T** [SNP] **A/T** [G/A] Then **2nd** base for G allele and **4th** base for A allele;

A/T **G/C** [SNP] **G/C** [G/A] Then **2nd** base for G allele and **4th** base for A allele;

**G/C** A/T [SNP] **G/C** [G/A] Then **2nd** base for G allele and **5th** base for A allele;

**G/C** **G/C** [SNP] A/T [G/A] Then **4th** base for G allele and **5th** base for A allele;

G/C **G/C** [SNP] **G/C** [G/A] Then **2nd** base for G allele and **4th** base for A allele;

5.3 Additional SNP at 4th from 3ʹ end, select the 2nd, 3rd, or 5th base for substitution following this principle (see below): A→C, T→C, G→A, and C→T;

**A. The additional SNP is [C/G] or [A/T]:**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

A/T [SNP] **A/T** **A/T** [G/A] Then **2nd** base for G allele and **3rd** base for A allele;

A/T [SNP] **A/T** **G/C** [G/A] Then **2nd** base for G allele and **3rd** base for A allele;

**A/T** [SNP] G/C **A/T** [G/A] Then **2nd** base for G allele and **5th** base for A allele;

G/C [SNP] **A/T** **A/T** [G/A] Then **2nd** base for G allele and **3rd** base for A allele;

A/T [SNP] **G/C** **G/C** [G/A] Then **2nd** base for G allele and **3rd** base for A allele;

G/C [SNP] **A/T** **G/C** [G/A] Then **2nd** base for G allele and **3rd** base for A allele;

**G/C** [SNP] **G/C** A/T [G/A] Then **3rd** base for G allele and **5th** base for A allele;

G/C [SNP] **G/C** **G/C** [G/A] Then **2nd** base for G allele and **3rd** base for A allele;

**B. The additional SNP is [C/A], [C/T], [G/A], or [G/T]:**

**B1. G or C allele couples with G allele (indicates one allele (G4 or C4) G1 ∞ another allele (A4 or T4) A1)**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

A/T [SNP] **A/T** **A/T** [G/A] Then **2nd** base for G allele and **3rd** base for A allele;

A/T [SNP] **A/T** **G/C** [G/A] Then **2nd** base for G allele and **3rd** base for A allele;

**A/T** [SNP] **G/C** A/T [G/A] Then **3rd** base for G allele and **5th** base for A allele;

**G/C** [SNP] **A/T** A/T [G/A] Then **3rd** base for A allele and **5th** base for G allele;

**A/T** [SNP] G/C **G/C** [G/A] Then **2nd** base for G allele and **5th** base for A allele;

G/C [SNP] **A/T** **G/C** [G/A] Then **2nd** base for G allele and **3rd** base for A allele;

**G/C** [SNP] **G/C** A/T [G/A] Then **3rd** base for G allele and **5th** base for A allele;

G/C [SNP] **G/C** **G/C** [G/A] Then **2nd** base for G allele and **3rd** base for A allele;

**B. The additional SNP is [C/A], [C/T], [G/A], or [G/T]:**

**B2. A or T allele couples with G allele (indicates one allele (A4 or T4) G1 ∞ another allele (G4 or C4) A1)**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

A/T [SNP] **A/T** **A/T** [G/A] Then **2nd** base for G allele and **3rd** base for A allele;

**A/T** [SNP] **A/T** G/C [G/A] Then **3rd** base for G allele and **5th** base for A allele;

**A/T** [SNP] G/C **A/T** [G/A] Then **2nd** base for G allele and **5th** base for A allele;

G/C [SNP] **A/T** **A/T** [G/A] Then **2nd** base for G allele and **3rd** base for A allele;

A/T [SNP] **G/C** **G/C** [G/A] Then **2nd** base for G allele and **3rd** base for A allele;

**G/C** [SNP] A/T **G/C** [G/A] Then **2nd** base for G allele and **5th** base for A allele;

**G/C** [SNP] **G/C** A/T [G/A] Then **3rd** base for G allele and **5th** base for A allele;

G/C [SNP] **G/C** **G/C** [G/A] Then **2nd** base for G allele and **3rd** base for A allele;

5.4 Additional SNP at 5th from 3ʹ end, select the 2nd, 3rd, or 4th base for substitution following this principle (see below): A→C, T→C, G→A, and C→T;

**A. The additional SNP is [C/G] or [A/T]:**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

[SNP] A/T **A/T** **A/T** [G/A] Then **2nd** base for G allele and **3rd** base for A allele;

[SNP] A/T **A/T** **G/C** [G/A] Then **2nd** base for G allele and **3rd** base for A allele;

[SNP] **A/T** G/C **A/T** [G/A] Then **2nd** base for G allele and **4th** base for A allele;

[SNP] G/C **A/T** **A/T** [G/A] Then **2nd** base for G allele and **3rd** base for A allele;

[SNP] A/T **G/C** **G/C** [G/A] Then **2nd** base for G allele and **3rd** base for A allele;

[SNP] G/C **A/T** **G/C** [G/A] Then **2nd** base for G allele and **3rd** base for A allele;

[SNP] **G/C** **G/C** A/T [G/A] Then **3rd** base for G allele and **4th** base for A allele;

[SNP] G/C **G/C** **G/C** [G/A] Then **2nd** base for G allele and **3rd** base for A allele;

**B. The additional SNP is [C/A], [C/T], [G/A], or [G/T]:**

**B1. G or C allele couples with G allele (indicates one allele (G5 or C5) G1 ∞ another allele (A5 or T5) A1)**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

[SNP] A/T **A/T** **A/T** [G/A] Then **2nd** base for G allele and **3rd** base for A allele;

[SNP] A/T **A/T** **G/C** [G/A] Then **2nd** base for G allele and **3rd** base for A allele;

[SNP] **A/T** **G/C** A/T [G/A] Then **3rd** base for G allele and **4th** base for A allele;

[SNP] **G/C** **A/T** A/T [G/A] Then **3rd** base for A allele and **4th** base for G allele;

[SNP] **A/T** G/C **G/C** [G/A] Then **2nd** base for G allele and **4th** base for A allele;

[SNP] G/C **A/T** **G/C** [G/A] Then **2nd** base for G allele and **3rd** base for A allele;

[SNP] **G/C** **G/C** A/T [G/A] Then **3rd** base for G allele and **4th** base for A allele;

[SNP] G/C **G/C** **G/C** [G/A] Then **2nd** base for G allele and **3rd** base for A allele;

**B. The additional SNP is [C/A], [C/T], [G/A], or [G/T]:**

**B2. A or T allele couples with G allele (indicates one allele (A5 or T5) G1 ∞ another allele (G5 or C5) A1)**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

[SNP] A/T **A/T** **A/T** [G/A] Then **2nd** base for G allele and **3rd** base for A allele;

[SNP] **A/T** **A/T** G/C [G/A] Then **3rd** base for G allele and **4th** base for A allele;

[SNP] **A/T** G/C **A/T** [G/A] Then **2nd** base for G allele and **4th** base for A allele;

[SNP] G/C **A/T** **A/T** [G/A] Then **2nd** base for G allele and **3rd** base for A allele;

[SNP] A/T **G/C** **G/C** [G/A] Then **2nd** base for G allele and **3rd** base for A allele;

[SNP] **G/C** A/T **G/C** [G/A] Then **2nd** base for G allele and **4th** base for A allele;

[SNP] **G/C** **G/C** A/T [G/A] Then **3rd** base for G allele and **4th** base for A allele;

[SNP] G/C **G/C** **G/C** [G/A] Then **2nd** base for G allele and **3rd** base for A allele;

6 For [T/A] SNP

6.1 Additional SNP at 2nd from 3ʹ end, select the 3rd, 4th, or 5th base for substitution following this principle (see below): A→C, T→C, G→A, and C→T;

**A. The additional SNP is [C/G] or [A/T]:**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

A/T **A/T** **A/T** [SNP] [T/A] Then **3rd** base for T allele and **4th** base for A allele;

**A/T** **A/T** G/C [SNP] [T/A] Then **4th** base for T allele and **5th** base for A allele;

**A/T** G/C **A/T** [SNP] [T/A] Then **3rd** base for T allele and **5th** base for A allele;

G/C **A/T** **A/T** [SNP] [T/A] Then **3rd** base for T allele and **4th** base for A allele;

A/T **G/C** **G/C** [SNP] [T/A] Then **3rd** base for T allele and **4th** base for A allele;

**G/C** A/T **G/C** [SNP] [T/A] Then **3rd** base for T allele and **5th** base for A allele;

**G/C** **G/C** A/T [SNP] [T/A] Then **4th** base for T allele and **5th** base for A allele;

G/C **G/C** **G/C** [SNP] [T/A] Then **3rd** base for T allele and **4th** base for A allele;

**B. The additional SNP is [C/A], [C/T], [G/A], or [G/T]:**

**B1. G or C allele couples with T allele (indicates one allele (G2 or C2) T1 ∞ another allele (A2 or T2) A1)**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

A/T **A/T** **A/T** [SNP] [T/A] Then **3rd** base for T allele and **4th** base for A allele;

A/T **A/T** **G/C** [SNP] [T/A] Then **3rd** base for T allele and **4th** base for A allele;

A/T **G/C** **A/T** [SNP] [T/A] Then **3rd** base for A allele and **4th** base for T allele;

G/C **A/T** **A/T** [SNP] [T/A] Then **3rd** base for T allele and **4th** base for A allele;

A/T **G/C** **G/C** [SNP] [T/A] Then **3rd** base for T allele and **4th** base for A allele;

G/C **A/T** **G/C** [SNP] [T/A] Then **3rd** base for T allele and **4th** base for A allele;

G/C **G/C** **A/T** [SNP] [T/A] Then **3rd** base for A allele and **4th** base for T allele;

G/C **G/C** **G/C** [SNP] [T/A] Then **3rd** base for T allele and **4th** base for A allele;

**B. The additional SNP is [C/A], [C/T], [G/A], or [G/T]:**

**B2. A or T allele couples with T allele (indicates one allele (A2 or T2) T1 ∞ another allele (G2 or C2) A1)**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

A/T **A/T** **A/T** [SNP] [T/A] Then **3rd** base for T allele and **4th** base for A allele;

A/T **A/T** **G/C** [SNP] [T/A] Then **3rd** base for A allele and **4th** base for T allele;

A/T **G/C** **A/T** [SNP] [T/A] Then **3rd** base for T allele and **4th** base for A allele;

G/C **A/T** **A/T** [SNP] [T/A] Then **3rd** base for T allele and **4th** base for A allele;

A/T **G/C** **G/C** [SNP] [T/A] Then **3rd** base for T allele and **4th** base for A allele;

G/C **A/T** **G/C** [SNP] [T/A] Then **3rd** base for A allele and **4th** base for T allele;

G/C **G/C** **A/T** [SNP] [T/A] Then **3rd** base for T allele and **4th** base for A allele;

G/C **G/C** **G/C** [SNP] [T/A] Then **3rd** base for T allele and **4th** base for A allele;

6.2 Additional SNP at 3rd from 3ʹ end, select the 2nd, 4th, or 5th base for substitution following this principle (see below): A→C, T→C, G→A, and C→T;

**A. The additional SNP is [C/G] or [A/T]:**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

**A/T** **A/T** [SNP] A/T [T/A] Then **4th** base for T allele and **5th** base for A allele;

**A/T** **A/T** [SNP] G/C [T/A] Then **4th** base for T allele and **5th** base for A allele;

**A/T** G/C [SNP] **A/T** [T/A] Then **2nd** base for T allele and **5th** base for A allele;

G/C **A/T** [SNP] **A/T** [T/A] Then **2nd** base for T allele and **4th** base for A allele;

A/T **G/C** [SNP] **G/C** [T/A] Then **2nd** base for T allele and **4th** base for A allele;

**G/C** A/T [SNP] **G/C** [T/A] Then **2nd** base for T allele and **5th** base for A allele;

**G/C** **G/C** [SNP] A/T [T/A] Then **4th** base for T allele and **5th** base for A allele;

**G/C** **G/C** [SNP] G/C [T/A] Then **4th** base for T allele and **5th** base for A allele;

**B. The additional SNP is [C/A], [C/T], [G/A], or [G/T]:**

**B1. G or C allele couples with T allele (indicates one allele (G3 or C3) T1 ∞ another allele (A3 or T3) A1)**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

**A/T** **A/T** [SNP] A/T [T/A] Then **4th** base for T allele and **5th** base for A allele;

**A/T** **A/T** [SNP] G/C [T/A] Then **4th** base for T allele and **5th** base for A allele;

**A/T** **G/C** [SNP] A/T [T/A] Then **4th** base for T allele and **5th** base for A allele;

**G/C** **A/T** [SNP] A/T [T/A] Then **4th** base for A allele and **5th** base for T allele;

**A/T** **G/C** [SNP] G/C [T/A] Then **4th** base for T allele and **5th** base for A allele;

**G/C** **A/T** [SNP] G/C [T/A] Then **4th** base for A allele and **5th** base for T allele;

**G/C** **G/C** [SNP] A/T [T/A] Then **4th** base for T allele and **5th** base for A allele;

**G/C** **G/C** [SNP] G/C [T/A] Then **4th** base for T allele and **5th** base for A allele;

**B. The additional SNP is [C/A], [C/T], [G/A], or [G/T]:**

**B2. A or T allele couples with T allele (indicates one allele (A3 or T3) T1 ∞ another allele (G3 or C3) A1)**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

**A/T** **A/T** [SNP] A/T [T/A] Then **4th** base for T allele and **5th** base for A allele;

**A/T** **A/T** [SNP] G/C [T/A] Then **4th** base for T allele and **5th** base for A allele;

**A/T** **G/C** [SNP] A/T [T/A] Then **4th** base for A allele and **5th** base for T allele;

**G/C** **A/T** [SNP] A/T [T/A] Then **4th** base for T allele and **5th** base for A allele;

**A/T** **G/C** [SNP] G/C [T/A] Then **4th** base for A allele and **5th** base for T allele;

**G/C** **A/T** [SNP] G/C [T/A] Then **4th** base for T allele and **5th** base for A allele;

**G/C** **G/C** [SNP] A/T [T/A] Then **4th** base for T allele and **5th** base for A allele;

**G/C** **G/C** [SNP] G/C [T/A] Then **4th** base for T allele and **5th** base for A allele;

6.3 Additional SNP at 4th from 3ʹ end, select the 2nd, 3rd, or 5th base for substitution following this principle (see below): A→C, T→C, G→A, and C→T;

**A. The additional SNP is [C/G] or [A/T]:**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

**A/T** [SNP] **A/T** A/T [T/A] Then **3rd** base for T allele and **5th** base for A allele;

**A/T** [SNP] **A/T** G/C [T/A] Then **3rd** base for T allele and **5th** base for A allele;

**A/T** [SNP] G/C **A/T** [T/A] Then **2nd** base for T allele and **5th** base for A allele;

G/C [SNP] **A/T** **A/T** [T/A] Then **2nd** base for T allele and **3rd** base for A allele;

A/T [SNP] **G/C** **G/C** [T/A] Then **2nd** base for T allele and **3rd** base for A allele;

**G/C** [SNP] A/T **G/C** [T/A] Then **2nd** base for T allele and **5th** base for A allele;

**G/C** [SNP] **G/C** A/T [T/A] Then **3rd** base for T allele and **5th** base for A allele;

**G/C** [SNP] **G/C** G/C [T/A] Then **3rd** base for T allele and **5th** base for A allele;

**B. The additional SNP is [C/A], [C/T], [G/A], or [G/T]:**

**B1. G or C allele couples with T allele (indicates one allele (G4 or C4) T1 ∞ another allele (A4 or T4) A1)**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

**A/T** [SNP] **A/T** A/T [T/A] Then **3rd** base for T allele and **5th** base for A allele;

**A/T** [SNP] **A/T** G/C [T/A] Then **3rd** base for T allele and **5th** base for A allele;

**A/T** [SNP] **G/C** A/T [T/A] Then **3rd** base for T allele and **5th** base for A allele;

**G/C** [SNP] **A/T** A/T [T/A] Then **3rd** base for A allele and **5th** base for T allele;

**A/T** [SNP] **G/C** G/C [T/A] Then **3rd** base for T allele and **5th** base for A allele;

**G/C** [SNP] **A/T** G/C [T/A] Then **3rd** base for A allele and **5th** base for T allele;

**G/C** [SNP] **G/C** A/T [T/A] Then **3rd** base for T allele and **5th** base for A allele;

**G/C** [SNP] **G/C** G/C [T/A] Then **3rd** base for T allele and **5th** base for A allele;

**B. The additional SNP is [C/A], [C/T], [G/A], or [G/T]:**

**B2. A or T allele couples with T allele (indicates one allele (A4 or T4) T1 ∞ another allele (G4 or C4) A1)**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

**A/T** [SNP] **A/T** A/T [T/A] Then **3rd** base for T allele and **5th** base for A allele;

**A/T** [SNP] **A/T** G/C [T/A] Then **3rd** base for T allele and **5th** base for A allele;

**A/T** [SNP] **G/C** A/T [T/A] Then **3rd** base for A allele and **5th** base for T allele;

**G/C** [SNP] **A/T** A/T [T/A] Then **3rd** base for T allele and **5th** base for A allele;

**A/T** [SNP] **G/C** G/C [T/A] Then **3rd** base for A allele and **5th** base for T allele;

**G/C** [SNP] **A/T** G/C [T/A] Then **3rd** base for T allele and **5th** base for A allele;

**G/C** [SNP] **G/C** A/T [T/A] Then **3rd** base for T allele and **5th** base for A allele;

**G/C** [SNP] **G/C** G/C [T/A] Then **3rd** base for T allele and **5th** base for A allele;

6.4 Additional SNP at 5th from 3ʹ end, select the 2nd, 3rd, or 4th base for substitution following this principle (see below): A→C, T→C, G→A, and C→T;

**A. The additional SNP is [C/G] or [A/T]:**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

[SNP] **A/T** **A/T** A/T [T/A] Then **3rd** base for T allele and **4th** base for A allele;

[SNP] **A/T** **A/T** G/C [T/A] Then **3rd** base for T allele and **4th** base for A allele;

[SNP] **A/T** G/C **A/T** [T/A] Then **2nd** base for T allele and **4th** base for A allele;

[SNP] G/C **A/T** **A/T** [T/A] Then **2nd** base for T allele and **3rd** base for A allele;

[SNP] A/T **G/C** **G/C** [T/A] Then **2nd** base for T allele and **3rd** base for A allele;

[SNP] **G/C** A/T **G/C** [T/A] Then **2nd** base for T allele and **4th** base for A allele;

[SNP] **G/C** **G/C** A/T [T/A] Then **3rd** base for T allele and **4th** base for A allele;

[SNP] **G/C** **G/C** G/C [T/A] Then **3rd** base for T allele and **4th** base for A allele;

**B. The additional SNP is [C/A], [C/T], [G/A], or [G/T]:**

**B1. G or C allele couples with T allele (indicates one allele (G5 or C5) T1 ∞ another allele (A5 or T5) A1)**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

[SNP] **A/T** **A/T** A/T [T/A] Then **3rd** base for T allele and **4th** base for A allele;

[SNP] **A/T** **A/T** G/C [T/A] Then **3rd** base for T allele and **4th** base for A allele;

[SNP] **A/T** **G/C** A/T [T/A] Then **3rd** base for T allele and **4th** base for A allele;

[SNP] **G/C** **A/T** A/T [T/A] Then **3rd** base for A allele and **4th** base for T allele;

[SNP] **A/T** **G/C** G/C [T/A] Then **3rd** base for T allele and **4th** base for A allele;

[SNP] **G/C** **A/T** G/C [T/A] Then **3rd** base for A allele and **4th** base for T allele;

[SNP] **G/C** **G/C** A/T [T/A] Then **3rd** base for T allele and **4th** base for A allele;

[SNP] **G/C** **G/C** G/C [T/A] Then **3rd** base for T allele and **4th** base for A allele;

**B. The additional SNP is [C/A], [C/T], [G/A], or [G/T]:**

**B2. A or T allele couples with T allele (indicates one allele (A5 or T5) T1 ∞ another allele (G5 or C5) A1)**

5th 4th 3rd 2nd 1st Positions for base substitution **based on the SNP at 3ʹ end**

[SNP] **A/T** **A/T** A/T [T/A] Then **3rd** base for T allele and **4th** base for A allele;

[SNP] **A/T** **A/T** G/C [T/A] Then **3rd** base for T allele and **4th** base for A allele;

[SNP] **A/T** **G/C** A/T [T/A] Then **3rd** base for A allele and **4th** base for T allele;

[SNP] **G/C** **A/T** A/T [T/A] Then **3rd** base for T allele and **4th** base for A allele;

[SNP] **A/T** **G/C** G/C [T/A] Then **3rd** base for A allele and **4th** base for T allele;

[SNP] **G/C** **A/T** G/C [T/A] Then **3rd** base for T allele and **4th** base for A allele;

[SNP] **G/C** **G/C** A/T [T/A] Then **3rd** base for T allele and **4th** base for A allele;

[SNP] **G/C** **G/C** G/C [T/A] Then **3rd** base for T allele and **4th** base for A allele;

Multiple SNP Module: the combined SNP number ≥ 3 at 3’ end:

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 (≥) 53C to (≤) 60C (53C ≤ Tm ≤ 60C);

If F primer pair number ≥ 1, preserve the F primer pair having min average Tm value;

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 (≥) 53C 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