**R primer design:**

1. Retrieve the data of min base (M) and max base (N) in the amplified base number (amplicon length);
2. Select allele1 Ref. sequence at the region from (the position of F1 primer 3ʹ end + 1+ M) to (the position of F1 primer 3ʹ end + 1+ N);
3. Confirm the small region(s) having ≥ 10 contiguous identical bases between the two alleles in the selected region (from step 2) based on the alignment data;
4. Generate R primer pair groups in each small region and 1st group start from the -8th position and the last group from the last base based on the alignment result (e.g.).



Each group contains 10 R primer pairs from -8th position and each primer having 18 to 27 bases;

Example:

Allele1-R: NNNNNNNNNNNNNNNNNN (-8)-(+10)

Allele2-R: NNNNNNNNNNNNNNNNNN (-8)-(+10)

Allele1-R: NNNNNNNNNNNNNNNNNNN (-8)-(+11)

Allele2-R: NNNNNNNNNNNNNNNNNNN (-8)-(+11)

Allele1-R: NNNNNNNNNNNNNNNNNNNN (-8)-(+12)

Allele2-R: NNNNNNNNNNNNNNNNNNNN (-8)-(+12)

Allele1-R: NNNNNNNNNNNNNNNNNNNNN (-8)-(+13)

Allele2-R: NNNNNNNNNNNNNNNNNNNNN (-8)-(+13)

Allele1-R: NNNNNNNNNNNNNNNNNNNNNN (-8)-(+14)

Allele2-R: NNNNNNNNNNNNNNNNNNNNNN (-8)-(+14)

Allele1-R: NNNNNNNNNNNNNNNNNNNNNNN (-8)-(+15)

Allele2-R: NNNNNNNNNNNNNNNNNNNNNNN (-8)-(+15)

Allele1-R: NNNNNNNNNNNNNNNNNNNNNNNN (-8)-(+16)

Allele2-R: NNNNNNNNNNNNNNNNNNNNNNNN (-8)-(+16)

Allele1-R: NNNNNNNNNNNNNNNNNNNNNNNNN (-8)-(+17)

Allele2-R: NNNNNNNNNNNNNNNNNNNNNNNNN (-8)-(+17)

Allele1-R: NNNNNNNNNNNNNNNNNNNNNNNNNN (-8)-(+18)

Allele2-R: NNNNNNNNNNNNNNNNNNNNNNNNNN (-8)-(+18)

Allele1-R: NNNNNNNNNNNNNNNNNNNNNNNNNNN (-8)-(+19)

Allele2-R: NNNNNNNNNNNNNNNNNNNNNNNNNNN (-8)-(+19)

1. Remove the R primer pairs **in the first 16 groups (skip this step for all other groups)**:

5.1) if Allele1-R having bases overlapped with F1 (based on the base position); or

5.2) if Allele2-R having bases overlapped with F2 (based on the base position);

1. Remove the R primer pairs (**for all groups**):

6.1) if any primer containing “N”; or

6.2) if “the position of Allele2-R start base”- “the position of F2 end base” > “N-1”; or

6.3) containing SNPs between each primer pair;

1. Preserve one primer in each primer pair;
2. Remove the primers:

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

8.2) having ≥ 8 As, Ts, Gs, or Cs;

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

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

1. Calculate Tm value of each primer and preserve all primers having Tm value arranging from 53C to 62C (53C ≤ Tm ≤ 62C); if primer number=0, stop and report results; Otherwise, continue;
2. Preserve the primers having Tm value arranging from **53**C to 58C (**53**C ≤ Tm < 58C) in cluster 1; and the others (58C ≤ Tm ≤ 62C) in cluster 2;
3. Calculate primers in cluster 2;
4. If primer number =0 in cluster 2, **go to step 25**; Otherwise, continue:
5. Calculate R primer self-complementarity (each R primer against its reverse sequence, **not the reverse complement sequence**), record the maximum contiguous complementarity and the maximum complementarity; e.g. and remove the primers having ≥ 10 contiguous complementarity or (primer length - max complementarity) ≤ 4; If primer number =0, **go to step 25**; Otherwise, continue:
6. Order R primers:

14.1) R primers having 9 contiguous (G and/or C) or 11 contiguous (A and/or T);

14.2) R primers having 7 As, Ts, Gs, or Cs;

14.3) R primers having 5 di-nucleotide **(AG, AC, TG, TC, GA, GT, CA, CT)** R**epeats;**

14.4) R primers having (GC% > 75% or GC% < 25%);

14.5) having ≥ 8 contiguous complementarity or (primer length - max complementarity) ≤ 6;

14.6) R primers having 8 contiguous (G and/or C) or ≥ 9 contiguous (A and/or T);

14.7) R primers having 6 As, Ts, Gs, or Cs;

14.8) R primers having 4 di-nucleotide **(AG, AC, TG, TC, GA, GT, CA, CT)** R**epeats;**

14.9) R primers having (GC% > 70% or GC% < 30%);

14.10) having 7 contiguous complementarity or (primer length - max complementarity) ≤ 8;

14.11) R primers having ≥ 6 contiguous (G and/or C) or ≥ 7 contiguous (A and/or T);

14.12) R primers having 5 As, Ts, Gs, or Cs;

14.13) R primers having 3 di-nucleotide **(AG, AC, TG, TC, GA, GT, CA, CT)** R**epeats;**

14.14) R primers having (GC% > 65% or GC% < 35%);

14.15) having 6 contiguous complementarity or (primer length - max complementarity) ≤ 10;

14.16) R primers having 6 A/T or 5 G/C in the first seven bases;

14.17) R primers having 4 A/T or 3 G/C in the first four bases;

14.18) having 5 contiguous complementarity or (primer length - max complementarity) ≤ 12;

14.19) R primers having ≥ 4 contiguous (G and/or C) or ≥ 5 contiguous (A and/or T);

14.20) R primers having 4 As, Ts, Gs, or Cs;

14.21) R primers having (GC% > 60% or GC% < 40%);

14.22) having 4 contiguous complementarity or (primer length - max complementarity) ≤ 14;

14.23) Remaining;

1. Order all R primers from the last sub-group (14.23) to the first sub-group (14.1);

If any sub-group having ≥ 2 primers, order primers based on primer base number: 22>21>23>20>24>19>25>18>26>27

1. Evaluate the specificity of 1st R primer in allele 1 Ref. sequence at the region from “0” to “Ref. end”, record the position and mismatch number of the R primer at each unexpected binding site (unexpected binding site: mismatch number ≤ 4), (SN4=, SN3=, SN2=, SN1=, and SN0=), then switch the R primer to its reverse complement, and evaluate the specificity of (reverse complement of the R primer) in whole allele1 Ref. sequence, record the position and mismatch number of (reverse complement of the F primer), (SN4=, SN3=, SN2=, SN1=, and SN0=);
2. Discard the R primer if containing any conditions in target amplification region:

17.1) containing unexpected binding site (SN2, SN1, and SN0); or

17.2) containing unexpected binding site (SN3 and SN4) for the R primer (**except** “one mismatch at the 5’ end and two mismatches at 2nd, 3rd, or 4th from 5’ end) and reverse complement of the R primer (**except** “one mismatch at the 3’ end and two mismatches at 2nd, 3rd, or 4th from 3’ end);

1. If the R primer was discarded, try the next R primer until to the last primer; Otherwise, continue:
2. Evaluate the specificity of 1st R primer in allele 2 Ref. sequence at the region from “0” to “Ref. end”, record the position and mismatch number of the R primer at each unexpected binding site (unexpected binding site: mismatch number ≤ 4), (SN4=, SN3=, SN2=, SN1=, and SN0=), then switch the R primer to its reverse complement, and evaluate the specificity of (reverse complement of the R primer) in whole allele2 Ref. sequence, record the position and mismatch number of (reverse complement of the F primer), (SN4=, SN3=, SN2=, SN1=, and SN0=);
3. Discard the R primer if containing any conditions in target amplification region:

17.1) containing unexpected binding site (SN2, SN1, and SN0); or

17.2) containing unexpected binding site (SN3 and SN4) for the R primer (**except** “one mismatch at the 5’ end and two mismatches at 2nd, 3rd, or 4th from 5’ end) and reverse complement of the R primer (**except** “one mismatch at the 3’ end and two mismatches at 2nd, 3rd, or 4th from 3’ end);

1. If the R primer was discarded, try the next R primer until to the last primer; Otherwise, continue:
2. Evaluate the specificity of the R primer in non-specific sequence:

23.1) find the R primer position in non-specific sequence based on the index of the non-specific alignments;

23.2) find the core region (max contiguous identical base number), if having ≥ 2 cores, based on the core closely next to the R primer 5ʹ end;

23.3) Stretch the sequence and calculate the mismatched base position and number;

If having ≥ 5 mismatched bases, evaluate the specificity of the R primer in the next non-specific sequence until to the last non-specific sequence;

If having 3 or 4 mismatched bases and (“one mismatch at the 5’ end and two mismatches at 2nd, 3rd, or 4th from 5’ end), evaluate the specificity of the R primer in the next non-specific sequence until to the last non-specific sequence;

Otherwise, check F1 and F2 primer specificity:

If both F1 and F2 primers having ≥ 5 mismatched bases or having 3 or 4 mismatched bases and (“one mismatch at the 3’ end and two mismatches at 2nd, 3rd, or 4th from 3’ end), evaluate the specificity of the R primer in the next non-specific sequence until to the last non-specific sequence;

Otherwise, discard the R primer and evaluate the next R primer;

1. If R primer number=3, report the result, otherwise, check the next R primer;
2. If all R primers were checked and 1≤ R primer number ≤2, report the result, otherwise, continue:
3. Calculate primers in cluster 1;
4. If primer number =0 in cluster 1, **stop and report fail**; Otherwise, continue:
5. Retrieve the 1st R primer in cluster 1, add C, G, CG, GC, CGC, GCG at 3ʹ end, respectively; generate 3 primers (e.g.);

NNNNNNNNNNNNNNNNNN

NNNNNNNNNNNNNNNNNNC

NNNNNNNNNNNNNNNNNNG

NNNNNNNNNNNNNNNNNNCG

NNNNNNNNNNNNNNNNNNGC

NNNNNNNNNNNNNNNNNNCGC

NNNNNNNNNNNNNNNNNNGCG

1. Remove the primers having ≥ 28 bases;
2. Remove the primers having Tm value >62C;
3. Retrieve the 2nd R primer in cluster 1 and repeat step 27 to 29, until to the last R primer;
4. Calculate R primer self-complementarity (each R primer against its reverse sequence, **not the reverse complement sequence**), record the maximum contiguous complementarity and the maximum complementarity; e.g. and remove the primers having ≥ 10 contiguous complementarity or (primer length - max complementarity) ≤ 4; If primer number =0, **stop and report fail**; Otherwise, continue:
5. Order R primers:

32.1) R primers having 9 contiguous (G and/or C) or 11 contiguous (A and/or T);

32.2) R primers having 7 As, Ts, Gs, or Cs;

32.3) R primers having 5 di-nucleotide **(AG, AC, TG, TC, GA, GT, CA, CT)** R**epeats;**

32.4) R primers having (GC% > 75% or GC% < 25%);

32.5) having ≥ 8 contiguous complementarity or (primer length - max complementarity) ≤ 6;

32.6) R primers having 8 contiguous (G and/or C) or ≥ 9 contiguous (A and/or T);

32.7) R primers having 6 As, Ts, Gs, or Cs;

32.8) R primers having 4 di-nucleotide **(AG, AC, TG, TC, GA, GT, CA, CT)** R**epeats;**

32.9) R primers having (GC% > 70% or GC% < 30%);

32.10) having 7 contiguous complementarity or (primer length - max complementarity) ≤ 8;

32.11) R primers having ≥ 6 contiguous (G and/or C) or ≥ 7 contiguous (A and/or T);

32.12) R primers having 5 As, Ts, Gs, or Cs;

32.13) R primers having 3 di-nucleotide **(AG, AC, TG, TC, GA, GT, CA, CT)** R**epeats;**

32.14) R primers having (GC% > 65% or GC% < 35%);

32.15) having 6 contiguous complementarity or (primer length - max complementarity) ≤ 10;

32.16) R primers having 6 A/T or 5 G/C in the first seven bases;

32.17) R primers having 4 A/T or 3 G/C in the first four bases;

32.18) having 5 contiguous complementarity or (primer length - max complementarity) ≤ 12;

32.19) R primers having ≥ 4 contiguous (G and/or C) or ≥ 5 contiguous (A and/or T);

32.20) R primers having 4 As, Ts, Gs, or Cs;

32.21) R primers having (GC% > 60% or GC% < 40%);

32.22) having 4 contiguous complementarity or (primer length - max complementarity) ≤ 14;

32.23) Remaining;

1. Order all R primers from the last sub-group (32.23) to the first sub-group (32.1);

If any sub-group having ≥ 2 primers, order primers based on primer base number: 22>21>23>20>24>19>25>26>27

1. Evaluate the specificity of 1st R primer in allele 1 Ref. sequence at the region from “0” to “Ref. end”, record the position and mismatch number of the R primer at each unexpected binding site (unexpected binding site: mismatch number ≤ 4), (SN4=, SN3=, SN2=, SN1=, and SN0=), then switch the R primer to its reverse complement, and evaluate the specificity of (reverse complement of the R primer) in whole allele1 Ref. sequence, record the position and mismatch number of (reverse complement of the F primer), (SN4=, SN3=, SN2=, SN1=, and SN0=);
2. Discard the R primer if containing any conditions in target amplification region:

17.1) containing unexpected binding site (SN2, SN1, and SN0); or

17.2) containing unexpected binding site (SN3 and SN4) for the R primer (**except** “one mismatch at the 5’ end and two mismatches at 2nd, 3rd, or 4th from 5’ end) and reverse complement of the R primer (**except** “one mismatch at the 3’ end and two mismatches at 2nd, 3rd, or 4th from 3’ end);

1. If the R primer was discarded, try the next R primer until to the last primer; Otherwise, continue:
2. Evaluate the specificity of 1st R primer in allele 2 Ref. sequence at the region from “0” to “Ref. end”, record the position and mismatch number of the R primer at each unexpected binding site (unexpected binding site: mismatch number ≤ 4), (SN4=, SN3=, SN2=, SN1=, and SN0=), then switch the R primer to its reverse complement, and evaluate the specificity of (reverse complement of the R primer) in whole allele2 Ref. sequence, record the position and mismatch number of (reverse complement of the F primer), (SN4=, SN3=, SN2=, SN1=, and SN0=);
3. Discard the R primer if containing any conditions in target amplification region:

17.1) containing unexpected binding site (SN2, SN1, and SN0); or

17.2) containing unexpected binding site (SN3 and SN4) for the R primer (**except** “one mismatch at the 5’ end and two mismatches at 2nd, 3rd, or 4th from 5’ end) and reverse complement of the R primer (**except** “one mismatch at the 3’ end and two mismatches at 2nd, 3rd, or 4th from 3’ end);

1. If the R primer was discarded, try the next R primer until to the last primer; Otherwise, continue:
2. Evaluate the specificity of the R primer in non-specific sequence:

23.1) find the R primer position in non-specific sequence based on the index of the non-specific alignments;

23.2) find the core region (max contiguous identical base number), if having ≥ 2 cores, based on the core closely next to the R primer 5ʹ end;

23.3) Stretch the sequence and calculate the mismatched base position and number;

If having ≥ 5 mismatched bases, evaluate the specificity of the R primer in the next non-specific sequence until to the last non-specific sequence;

If having 3 or 4 mismatched bases and (“one mismatch at the 5’ end and two mismatches at 2nd, 3rd, or 4th from 5’ end), evaluate the specificity of the R primer in the next non-specific sequence until to the last non-specific sequence;

Otherwise, check F1 and F2 primer specificity:

If both F1 and F2 primers having ≥ 5 mismatched bases or having 3 or 4 mismatched bases and (“one mismatch at the 3’ end and two mismatches at 2nd, 3rd, or 4th from 3’ end), evaluate the specificity of the R primer in the next non-specific sequence until to the last non-specific sequence;

Otherwise, discard the R primer and evaluate the next R primer;

1. If R primer number=3, report the result, otherwise, check the next R primer;

If all R primers were checked and 1≤ R primer number ≤2, report the result;

If all R primers were checked and R primer number =2, stop and report fail;

1. Add tails to 5ʹ end of F primers

Check “TATGAC” overlapped bases with 5ʹ end of F1 primer and 5ʹ end of F2 primer (check 6 times for each F primer, e.g.) and preserve the max overlapped base number;

F1: GACNNNNNNNNNNN1

F2: GACNNNNNNNNNNN2

Then having 3 overlapped bases (GAC) with F1 and F2 primers, respectively;

Amplicon 1 = the position of R primer 3ʹ end (in allele 1) - the position of F1 primer 5ʹ end +1-overlapped base number;

Amplicon 2 = the position of R primer 3ʹ end (in allele 2) - the position of F2 primer 5ʹ end +1- overlapped base number;

1. If Amplicon 1- Amplicon 2 ≥ 8,

Tail 1 will be removed the overlapped bases at 3ʹ end and then add to F1 primer;

Tail 2 will be removed the overlapped bases at 3ʹ end and then add to F2 primer;

1. If 1≤ Amplicon 1- Amplicon 2 ≤ 7,

Tail 2 will be removed the overlapped bases at 3ʹ end and then add to F1 primer;

Tail 1 will be removed the overlapped bases at 3ʹ end and then add to F2 primer;

1. If Amplicon 1- Amplicon 2 = 0,

Tail 1 or 2 will be removed the overlapped bases at 3ʹ end and then add to F1 or F2 primer following the principle in the paper;

1. If -7≤ Amplicon 1- Amplicon 2 ≤ -1,

Tail 1 will be removed the overlapped bases at 3ʹ end and then add to F1 primer;

Tail 2 will be removed the overlapped bases at 3ʹ end and then add to F2 primer;

1. If Amplicon 1- Amplicon 2 ≤ -7,

Tail 2 will be removed the overlapped bases at 3ʹ end and then add to F1 primer;

Tail 1 will be removed the overlapped bases at 3ʹ end and then add to F2 primer;

1. Report F primer pairs and the reverse-complement of R primer