**Combination of the program-designed F and R primers:**

I=1 and J=1; total F primer number (FN) and total R primer number (RN);

1. Combine 20 F primers from (**I to I+19**) with all R primers (**1 to J+19**), and all F primers from (**1 to I-1**) with 20 R primers from (**J to J+19**);
2. Remove all primer pairs out of the size arranging from L/min to L/max (L/min ≤ “R primer end position - F primer first position + 1” ≤ L/max);
3. Calculate the **identity** of each primer pair (**don’t switch reverse and/or complement any primer**), remove the primer pairs having ≥ 10 contiguous identical bases or (short primer length - max identity) ≤ 4;

If primer pair number =0 and (**I+20** > FN and **J+20** > RN), then go to step **8**;

If primer pair number =0 and (**I+20** ≤ FN or **J+20** ≤ FN), **I = I+20 and J = J+20 and** go to step 1;

Otherwise, continue:

1. Evaluate the specificity of each F primer from (**I to I+19**) in Ref. sequence at the region from “0” to “Ref. end”, record the position and mismatch number of each F primer at each unexpected binding site (unexpected binding site: mismatch number ≤ 4), (SN4=, SN3=, SN2=, SN1=, and SN0=), then switch the F primer to its reverse complement, and evaluate the specificity of (reverse complement of the F primer) in whole Ref. sequence, record the position and mismatch number of (reverse complement of the F primer), (SN4=, SN3=, SN2=, SN1=, and SN0=);
2. Evaluate the specificity of each R primer from (**J to J+19**) in Ref. sequence at the region from “0” to “Ref. end”, record the position and mismatch number of each R primer, (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 Ref. sequence, record the position and mismatch number of (reverse complement of the R primer), (SN4=, SN3=, SN2=, SN1=, and SN0=);
3. Discard the primer pairs that contains unexpected binding site (SN2, SN1, and SN0) for F or R primer in target amplification region;

Discard the primer pairs that contains unexpected binding site for F (SN3 except “one mismatch at the 3’ end and two mismatches at 2nd, 3rd, or 4th from 3’ end) or R (SN3 except “one mismatch at the 5’ end and two mismatches at 2nd, 3rd, or 4th from 5’ end) primer in target amplification region;

Discard the primer pairs that contains unexpected binding site for F (SN4 except “one mismatch at the 3’ end and two or three mismatches at 2nd, 3rd, or 4th from 3’ end) or R (SN4 except “one mismatch at the 5’ end and two or three mismatches at 2nd, 3rd, or 4th from 5’ end) primer in target amplification region;

1. Select primer pairs:
2. Primer pairs having ≤ 6 contiguous identical bases and (short primer length-max identity) ≥ 10;
3. F primers having ≤ 6 contiguous complementarity and (primer length-max complementarity) ≥ 10;
4. R primers having ≤ 6 contiguous complementarity and (primer length-max complementarity) ≥ 10;

If primer pair number < 5, **I = I+20 and J = J+20 and** go to step 1;

Otherwise, continue:

1. Order primer pairs:
2. Primer pairs having ≤ 4 contiguous identical bases and (short primer length-max identity) ≥ 14;

F primers having ≤ 4 contiguous complementarity and (primer length-max complementarity) ≥ 14;

R primers having ≤ 4 contiguous complementarity and (primer length-max complementarity) ≥ 14;

1. Primer pairs having ≤ 5 contiguous identical bases and (short primer length-max identity) ≥ 12;

F primers having ≤ 5 contiguous complementarity and (primer length-max complementarity) ≥ 12;

R primers having ≤ 5 contiguous complementarity and (primer length-max complementarity) ≥ 12;

1. Primer pairs having ≤ 6 contiguous identical bases and (short primer length-max identity) ≥ 10;

F primers having ≤ 6 contiguous complementarity and (primer length-max complementarity) ≥ 10;

R primers having ≤ 6 contiguous complementarity and (primer length-max complementarity) ≥ 10;

1. Primer pairs having ≤ 7 contiguous identical bases and (short primer length-max identity) ≥ 8;

F primers having ≤ 7 contiguous complementarity and (primer length-max complementarity) ≥ 8;

R primers having ≤ 7 contiguous complementarity and (primer length-max complementarity) ≥ 8;

1. Primer pairs having ≤ 8 contiguous identical bases and (short primer length-max identity) ≥ 6;

F primers having ≤ 8 contiguous complementarity and (primer length-max complementarity) ≥ 6;

R primers having ≤ 8 contiguous complementarity and (primer length-max complementarity) ≥ 6;

1. Primer pairs having ≤ 9 contiguous identical bases and (short primer length-max identity) ≥ 5;

F primers having ≤ 9 contiguous complementarity and (primer length-max complementarity) ≥ 5;

R primers having ≤ 9 contiguous complementarity and (primer length-max complementarity) ≥ 5;

1. Others
2. Orderly select 5 primer pairs:

Select the 1st primer pair;

Select the 2nd primer pair that both F and R primers are not involved in the primer pairs above until to find 5 primer pairs;

If less than 5 primer pairs were found, select the other primer pairs from the 1st one in the remaining primer pairs

1. Stop the program and report results: