**3. Primer design**

**FA module to design F primers in regions:**

1. Generate F primer groups in each selected region (from “region-FX0” to “region-FY0”). Each selected region contains “(region-FY0) - (region-FX0) +1” F primer groups and Each F primer group contains primers with length arranging from min primer length to max primer length, in which the min primer length and the max primer length are confirmed based on Tm/min and Tm/max (e.g. Tm/min=54 and Tm/max=58, then primer length arranging from min primer length (17) to max primer length (26)). (**May combine step 1 and step 2 and step 3**)

Example:

(21-37) NNNNNNNNNNNNNNNNN

(21-38) NNNNNNNNNNNNNNNNNN

(21-39) NNNNNNNNNNNNNNNNNNN

(21-40) NNNNNNNNNNNNNNNNNNNN

(21-41) NNNNNNNNNNNNNNNNNNNNN

(21-42) NNNNNNNNNNNNNNNNNNNNNN

(21-43) NNNNNNNNNNNNNNNNNNNNNNN

(21-44) NNNNNNNNNNNNNNNNNNNNNNNN

(21-45) NNNNNNNNNNNNNNNNNNNNNNNNN

(21-46) NNNNNNNNNNNNNNNNNNNNNNNNN**N**

1. Remove all primers containing “N”;
2. Remove all primers having ≥ 10 contiguous (G and/or C) or ≥ 12 contiguous (A and/or T);
3. Remove all primers having ≥ 8 As, Ts, Gs, or Cs;
4. Remove all primers having ≥ 6 di-nucleotide **(AG, AC, TG, TC, GA, GT, CA, CT)** R**epeats**;
5. Remove all primers having (GC% > 80% or GC% < 20%);
6. Calculate Tm value of each primer and preserve all primers with Tm arranging from Tm/min to Tm/max;
7. Calculate F primer self-complementarity (each F 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 F primer number = 0, “**stop and back to “assign appropriate region to design primers**”; Otherwise, continue:
8. If user didn’t assign region for F primers, go to step 10;

Otherwise, select the last “max primer length - 11” groups and delete all the primers don’t fully fall in the user assigned region (between “Region/F/from **-1**” and “Region/F/to **-1**” to match **index**):

If F primer number = 0, “**stop and back to “assign appropriate region to design primers**”; Otherwise, continue:

1. Order F primers:

10.1) F primers having 9 contiguous (G and/or C) or 11 contiguous (A and/or T);

10.2) F primers having 7 As, Ts, Gs, or Cs;

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

10.4) F primers having (GC% > 75% or GC% < 25%);

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

10.6) F primers having 6 As, Ts, Gs, or Cs;

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

10.8) F primers having (GC% > 70% or GC% < 30%);

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

10.10) F primers having 5 As, Ts, Gs, or Cs;

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

10.12) F primers having (GC% > 65% or GC% < 35%);

10.13) F primers having 6 A/T or 5 G/C in the last seven bases;

10.14) F primers having 4 A/T or 3 G/C in the last four bases;

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

10.16) F primers having 4 As, Ts, Gs, or Cs;

10.17) F primers having (GC% > 60% or GC% < 40%);

10.18) Remaining;

1. Order F primers in each sub-step (order F primers at step 10 from 10.1 to 10.18):

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

11.2) F primers having 7 contiguous complementarity or (primer length - max complementarity) ≤ 8;

11.3) F primers having 6 contiguous complementarity or (primer length - max complementarity) ≤ 10;

11.4) F primers having 5 contiguous complementarity or (primer length - max complementarity) ≤ 12;

11.5) F primers having 4 contiguous complementarity or (primer length - max complementarity) ≤ 14;

1. Order primers in each sub-sub-step (order F primers at step 11 from 11.1 to 11.5) based on 3ʹ end base “A”, then “T”, then “G”, and then “C”;
2. Order all F primers from the last one to the first one;

**RA module to design R primers in regions):**

1. Generate R primer groups in all the selected regions. Each selected region contains “(region-RY0) - (region-RX0) +1” and each primer group contains primers with length arranging from min primer length to max primer length, in which the min primer length and the max primer length are confirmed based on Tm/min and Tm/max (e.g. Tm/min=54 and Tm/max=58, then primer length arranging from min primer length (17) to max primer length (26)). (**May combine step 1 and step 2**)

Example:

NNNNNNNNNNNNNNNNN (21-37)

NNNNNNNNNNNNNNNNNN (20-37)

NNNNNNNNNNNNNNNNNNN (19-37)

NNNNNNNNNNNNNNNNNNNN (18-37)

NNNNNNNNNNNNNNNNNNNNN (17-37)

NNNNNNNNNNNNNNNNNNNNNN (16-37)

NNNNNNNNNNNNNNNNNNNNNNN (15-37)

NNNNNNNNNNNNNNNNNNNNNNNN (14-37)

NNNNNNNNNNNNNNNNNNNNNNNNN (13-37)

**N**NNNNNNNNNNNNNNNNNNNNNNNNN (12-37)

1. Remove all primers containing “N”;
2. Remove all primers having ≥ 10 contiguous (G and/or C) or ≥ 12 contiguous (A and/or T);
3. Remove all primers having ≥ 8 As, Ts, Gs, or Cs;
4. Remove all primers having ≥ 6 di-nucleotide **(AG, AC, TG, TC, GA, GT, CA, CT)** R**epeats**;
5. Remove all primers having (GC% > 80% or GC% < 20%);
6. Calculate Tm value of each primer and preserve all primers with Tm arranging from Tm/min to Tm/max;
7. 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 F primer number = 0, “**stop and back to “assign appropriate region to design primers**”; Otherwise, continue:
8. If user didn’t assign region for R primers, go to step 10;

Otherwise, select the first “max primer length - 11” groups and delete all the primers don’t fully fall in the user assigned region (between “Region/R/from **-1**” and “Region/R/to **-1**” to match **index**):

If R primer number = 0, “**stop and back to “assign appropriate region to design primers**”; Otherwise, continue:

1. Order R primers:

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

10.18) Remaining;

1. Order R primers in each sub-step (order R primers at step 10 from 10.1 to 10.18):

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

11.2) R primers having 7 contiguous complementarity or (primer length - max complementarity) ≤ 8;

11.3) R primers having 6 contiguous complementarity or (primer length - max complementarity) ≤ 10;

11.4) R primers having 5 contiguous complementarity or (primer length - max complementarity) ≤ 12;

11.5) R primers having 4 contiguous complementarity or (primer length - max complementarity) ≤ 14;

1. Order primers in each sub-sub-step (order R primers at step 11 from 11.1 to 11.5) based on 5ʹ end base “T”, then “A”, then “C”, and then “G”;
2. Order all F primers from the last one to the first one;

**FB module to design F primers in gaps:**

1. Generate F primer groups in each selected gap (from “gap-FX0” to “gap-FY0”). Each selected region contains “(gap-FY0) - (gap-FX0) +1” F primer groups and Each F primer group contains primers with length arranging from min primer length to max primer length, in which the min primer length and the max primer length are confirmed based on Tm/min and Tm/max (e.g. Tm/min=54 and Tm/max=58, then primer length arranging from min primer length (17) to max primer length (26)). (**May combine step 1 and step 2 and step 3**)

Example:

(21-37) NNNNNNNNNNNNNNNNN

(21-38) NNNNNNNNNNNNNNNNNN

(21-39) NNNNNNNNNNNNNNNNNNN

(21-40) NNNNNNNNNNNNNNNNNNNN

(21-41) NNNNNNNNNNNNNNNNNNNNN

(21-42) NNNNNNNNNNNNNNNNNNNNNN

(21-43) NNNNNNNNNNNNNNNNNNNNNNN

(21-44) NNNNNNNNNNNNNNNNNNNNNNNN

(21-45) NNNNNNNNNNNNNNNNNNNNNNNNN

(21-46) NNNNNNNNNNNNNNNNNNNNNNNNN**N**

1. Remove all primers containing “N”;
2. Remove all primers having ≥ 10 contiguous (G and/or C) or ≥ 12 contiguous (A and/or T);
3. Remove all primers having ≥ 8 As, Ts, Gs, or Cs;
4. Remove all primers having ≥ 6 di-nucleotide **(AG, AC, TG, TC, GA, GT, CA, CT)** R**epeats**;
5. Remove all primers having (GC% > 80% or GC% < 20%);
6. Calculate Tm value of each primer and preserve all primers with Tm arranging from Tm/min to Tm/max;
7. If user didn’t assign region for F primers, go to step 9;

Otherwise, select the last “max primer length - 11” groups and delete all the primers don’t fully fall in the user assigned region (between “Region/F/from **-1**” and “Region/F/to **-1**” to match **index**):

If F primer number = 0, “**stop and back to “assign appropriate region to design primers**”; Otherwise, continue:

1. Check the specificity of each F primer in not-target alignment:

Search the 1st F primer binding site in the 1st non-target alignment **based on the index** (if having gaps (indicate “-”) in the corresponding site, then based on the biggest core region, if having ≥ 2 cores, based on the core closely next to the F primer 3ʹ end), if the F primer (having 3ʹ end mismatch) or (having ≥ 2 mismatches); search the next non-target alignment until to the last non-target alignment, otherwise, remove this primer and try the next F primer;

1. Calculate F primer self-complementarity (each F 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 F primer number = 0, “**stop and back to “assign appropriate region to design primers**”; Otherwise, continue:
2. Order F primers:

10.1) F primers having 9 contiguous (G and/or C) or 11 contiguous (A and/or T);

10.2) F primers having 7 As, Ts, Gs, or Cs;

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

10.4) F primers having (GC% > 75% or GC% < 25%);

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

10.6) F primers having 6 As, Ts, Gs, or Cs;

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

10.8) F primers having (GC% > 70% or GC% < 30%);

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

10.10) F primers having 5 As, Ts, Gs, or Cs;

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

10.12) F primers having (GC% > 65% or GC% < 35%);

10.13) F primers having 6 A/T or 5 G/C in the last seven bases;

10.14) F primers having 4 A/T or 3 G/C in the last four bases;

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

10.16) F primers having 4 As, Ts, Gs, or Cs;

10.17) F primers having (GC% > 60% or GC% < 40%);

10.18) Remaining;

1. Order F primers in each sub-step (order F primers at step 10 from 10.1 to 10.18):

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

11.2) F primers having 7 contiguous complementarity or (primer length - max complementarity) ≤ 8;

11.3) F primers having 6 contiguous complementarity or (primer length - max complementarity) ≤ 10;

11.4) F primers having 5 contiguous complementarity or (primer length - max complementarity) ≤ 12;

11.5) F primers having 4 contiguous complementarity or (primer length - max complementarity) ≤ 14;

1. Order primers in each sub-sub-step (order F primers at step 11 from 11.1 to 11.5) based on 3ʹ end base “A”, then “T”, then “G”, and then “C”;
2. Order all F primers from the last one to the first one;

**RB module to design R primers in regions):**

1. Generate R primer groups in all the selected regions. Each selected region contains “(region-RY0) - (region-RX0) +1” and each primer group contains primers with length arranging from min primer length to max primer length, in which the min primer length and the max primer length are confirmed based on Tm/min and Tm/max (e.g. Tm/min=54 and Tm/max=58, then primer length arranging from min primer length (17) to max primer length (26)). (**May combine step 1 and step 2**)

Example:

NNNNNNNNNNNNNNNNN (21-37)

NNNNNNNNNNNNNNNNNN (20-37)

NNNNNNNNNNNNNNNNNNN (19-37)

NNNNNNNNNNNNNNNNNNNN (18-37)

NNNNNNNNNNNNNNNNNNNNN (17-37)

NNNNNNNNNNNNNNNNNNNNNN (16-37)

NNNNNNNNNNNNNNNNNNNNNNN (15-37)

NNNNNNNNNNNNNNNNNNNNNNNN (14-37)

NNNNNNNNNNNNNNNNNNNNNNNNN (13-37)

**N**NNNNNNNNNNNNNNNNNNNNNNNNN (12-37)

1. Remove all primers containing “N”;
2. Remove all primers having ≥ 10 contiguous (G and/or C) or ≥ 12 contiguous (A and/or T);
3. Remove all primers having ≥ 8 As, Ts, Gs, or Cs;
4. Remove all primers having ≥ 6 di-nucleotide **(AG, AC, TG, TC, GA, GT, CA, CT)** R**epeats**;
5. Remove all primers having (GC% > 80% or GC% < 20%);
6. Calculate Tm value of each primer and preserve all primers with Tm arranging from Tm/min to Tm/max;
7. If user didn’t assign region for R primers, go to step 9;

Otherwise, select the first “max primer length - 11” groups and delete all the primers don’t fully fall in the user assigned region (between “Region/R/from **-1**” and “Region/R/to **-1**” to match **index**):

If R primer number = 0, “**stop and back to “assign appropriate region to design primers**”; Otherwise, continue:

1. Check the specificity of each R primer in not-target alignment:

Search the 1st R primer binding site in the 1st non-target alignment **based on the index** (if having gaps (indicate “-”) in the corresponding site, then based on the biggest core region, if having ≥ 2 cores, based on the core closely next to the R primer 5ʹ end), if the R primer (having 5ʹ end mismatch) or (having ≥ 2 mismatches); search the next non-target alignment until to the last non-target alignment, otherwise, remove this primer and try the next R primer;

1. 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 F primer number = 0, “**stop and back to “assign appropriate region to design primers**”; Otherwise, continue:
2. Order R primers:

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

10.18) Remaining;

1. Order R primers in each sub-step (order R primers at step 10 from 10.1 to 10.18):

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

11.2) R primers having 7 contiguous complementarity or (primer length - max complementarity) ≤ 8;

11.3) R primers having 6 contiguous complementarity or (primer length - max complementarity) ≤ 10;

11.4) R primers having 5 contiguous complementarity or (primer length - max complementarity) ≤ 12;

11.5) R primers having 4 contiguous complementarity or (primer length - max complementarity) ≤ 14;

1. Order primers in each sub-sub-step (order R primers at step 11 from 11.1 to 11.5) based on 5ʹ end base “T”, then “A”, then “C”, and then “G”;
2. Order all F primers from the last one to the first one;