1. Calculate the positions of regions and gaps

1. Calculate the position of each region for **F** primer design:

Region I start position (region-FX0) = “region I first base position -10” (OR = 0, if “region I first base position - 10 < 0);

Region I end position (region-FY0) = “region I last base position -10”;

**Note: position means position in index**

1. Calculate the position of each region for **R** primer design:

Region I start position (region-RX0) = “region I first base position +10”;

Region I end position (region-RY0) = “region I last base position +10” (OR = “Ref. end position”, if “region I end base position + 10” > “Ref. end position”);

**Note: position means position in index**

1. Calculate the position of each gap for **F** primer design:

Gap I start position (gap-FX0) = “gap I first base position -10” (OR = 0, if “gap I first base position - 10 < 0);

Gap I end position (gap-FY0) = “gap I last base position -10”;

**Note: position means position in index**

1. Calculate the position of each gap for **R** primer design:

Gap I start position (gap-RX0) = “gap I first base position +10”;

Gap I end position (gap-RY0) = “gap I last base position +10” (OR = “Ref. end position”, if “gap I end base position + 10” > “Ref. end position”);

**Note: position means position in index**

2. Assign appropriate region to design primers.

2.1. If user didn’t assign any region to design primers;

2.2. If user assigned **one region** (position: M to N) for F primers and need to design **F and R** primers;

2.3. If user assigned **one region** (position: M to N) for R primers and need to design **F and R** primers;

2.4. If user assigned **the region** (M to N) for F primers and **the region** (X to Y) for R primers;

2.5. If user assigned **F primer** to design **R** primers;

2.6. If user assigned **R primer** to design **F** primers;

2.7. If user assigned **F and R primers**;

2.1 If user didn’t assign any region to design primers;

FA: select all the regions in the Ref. sequence arranging from “0” to the position “Ref. end position - L/min +1”, if the last selected region I cover the position “Ref. end position - L/min +1”, this region end position (region-FY0) = “Ref. end position - L/min +1”;

FB: select all the gaps in the Ref. sequence arranging from 0 to the position (Ref. end position - L/min +1), if the last selected gap cover the position (Ref. end position - L/min +1), this gap end position (gap-FY0) = “Ref. end position - L/min +1”;

RA: select all the regions in the Ref. sequence arranging from the position (L/min -1) to Ref. end position, if the first selected region cover the position (L/min -1), this region start position (region-RX0) = “L/min -1”;

RB: select all the gaps in the Ref. sequence arranging from the position (L/min -1) to Ref. end position, if the first selected gap cover the position (L/min -1), this gap first position (gap-RX0) = “L/min -1”;

If FA successful, then try RA:

If RA successful, combine F and R primers

If RA fail, try RB:

If RB successful, combine F and R primers

If RB fail, stop and report fail;

If FA fail, then try FB:

If FB fail, stop and report fail

If FB successful, then try RA

If RA successful, combine F and R primers

If RA fail, try RB:

If RB successful, combine F and R primers

If RB fail, stop and report fail;

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2.2. If user assigned **one region** (position: M to N) for F primers and need to design **F and R** primers;

FA: select all the regions in the user-assigned region (position: M to N), if the first selected region cover the position (M-1), this region first position (region-FX0) = “M-1”; if the last selected region cover the position (N-11), this region end position (region-FY0) = “N-11”;

FB: select all the gaps in the user-assigned region (position: M to N), if the first selected gap cover the position (M-1), this gap first position (gap-FX0) = “M-1”; if the last selected gap cover the position (N-11), this gap end position (gap-FY0) = “N-11”;

RA: select all the regions in the Ref. sequence arranging from the position (M+L/min -2) to the position Y (Y=“N+L/max -12” OR “Ref. end position”, if “N+L/max -12” > “Ref. end position”), if the first selected region cover the position (M+L/min -2), this region start position (region-RX0) = “(M+L/min -2)”; if the last selected region cover the position Y, this region end position (region-RY0) = “Y”;

RB: select all the gaps in the Ref. sequence arranging from the position (M+L/min -2) to the position Y (Y=“N+L/max -12” OR “Ref. end position”, if “N+L/max -12” > “Ref. end position”), if the first selected gap cover the position (M+L/min -2), this gap first position (gap-RX0) = “M+L/min -2”; if the last selected gap cover the position Y, this gap last position (gap-RY0) = “Y”;

If FA successful, then try RA:

If RA successful, combine F and R primers

If RA fail, try RB:

If RB successful, combine F and R primers

If RB fail, stop and report fail;

If FA fail, then try FB:

If FB fail, stop and report fail

If FB successful, then try RA

If RA successful, combine F and R primers

If RA fail, try RB:

If RB successful, combine F and R primers

If RB fail, stop and report fail;

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2.3. If user assigned **one region** (position: M to N) for R primers and need to design **F and R** primers;

FA: select all the regions in the Ref. sequence arranging from the position X (X=“M-L/max+10” OR “0”, if “M-L/max+10” < 0) to the position (N-L/min), if the first selected region cover the position X, this region first position (region-FX0) = “X”; if the last selected region cover the position (N-L/min), this region end position (region-FY0) = “N-L/min”;

FB: select all the gaps in the Ref. sequence arranging from the position X (X=“M-L/max+10” OR “0”, if “M-L/max+10” < 0) to the position (N-L/min), if the first selected gap cover the position X, this gap first position (gap-FX0) = “X”; if the last selected gap cover the position (N-L/min), this gap end position (gap-FY0) = “N-L/min”;

RA: select all the regions in the user-assigned region (position: M to N), if the first selected region cover the position (M+9), this region start position (region-RX0) = “(M+9)”; if the last selected region cover the position (N-1), this region end position (region-RY0) = “(N-1)”;

RB: select all the gaps in the user-assigned region (position: M to N), if the first selected gap cover the position (M+9), this gap first position (gap-RX0) = “M+9”; if the last selected gap cover the position (N-1), this gap last position (gap-RY0) = “(N-1)”;

If FA successful, then try RA:

If RA successful, combine F and R primers

If RA fail, try RB:

If RB successful, combine F and R primers

If RB fail, stop and report fail;

If FA fail, then try FB:

If FB fail, stop and report fail

If FB successful, then try RA

If RA successful, combine F and R primers

If RA fail, try RB:

If RB successful, combine F and R primers

If RB fail, stop and report fail;

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2.4. If user assigned **the region** (M to N) for F primers and **the region** (I to J) for R primers;

FA: select all the regions in the user-assigned region (position: M to N), if the first selected region cover the position (M-1), this region first position (region-FX0) = “M-1”; if the last selected region cover the position (N-1), this region end position (region-FY0) = “N-1”;

FB: select all the gaps in the user-assigned region (position: M to N), if the first selected gap cover the position (M-1), this gap first position (gap-FX0) = “M-1”; if the last selected gap cover the position (N-1), this gap end position (gap-FY0) = “N-1”;

RA: select all the regions in the user-assigned region (position: I to J), if the first selected region cover the position (I+9), this region start position (region-RX0) = “(I+9)”; if the last selected region cover the position (J-1), this region end position (region-RY0) = “(J-1)”;

RB: select all the gaps in the user-assigned region (position: I to J), if the first selected gap cover the position (I+9), this gap first position (gap-RX0) = “I+91”; if the last selected gap cover the position (J-1), this gap last position (gap-RY0) = “(J-1)”;

If FA successful, then try RA:

If RA successful, combine F and R primers

If RA fail, try RB:

If RB successful, combine F and R primers

If RB fail, stop and report fail;

If FA fail, then try FB:

If FB fail, stop and report fail

If FB successful, then try RA

If RA successful, combine F and R primers

If RA fail, try RB:

If RB successful, combine F and R primers

If RB fail, stop and report fail;

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2.5. If user assigned **F primer** to design **R** primers;

Evaluate the specificity of the F primer in whole Ref. sequence, record the position and mismatch number of each binding site (containing 4, 3, 2, 1, or 0 mismatches, and the corresponding site number SN4=, SN3=, SN2=, SN1=, and SN0=, respectively );

If SN0 =0, report “cannot find F primer binding site” and stop;

If SN0 =1 and Σ(SN4+SN3+SN2+SN1)=0, continue;

If SN0 =1 and Σ(SN4+SN3+SN2+SN1) ≥ 1, ask user “containing non-specific binding sites of the F primer, continue?

If SN0 ≥ 2, report “containing more than one binding sites of the F primer” and stop;

If user didn’t assign a region for R primers, retrieve the F primer position (from FM to FN) in index:

X=“FM+L/min -1”, and Y=“FM+L/max -1” OR “Ref. end position”, if “FM+L/max -1” > “Ref. end position”; if X>Y, report “amplicon size < L/min” and stop; Otherwise, continue:

RA: select all the regions in the Ref. sequence arranging from the position “X” to the position “Y”, if the first selected region cover the position “X”, this region start position (region-RX0) = X”; if the last selected region cover the position “Y”, this region end position (region-RY0) = “Y”;

RB: select all the gaps in the Ref. sequence arranging from the position “X” to the position “Y”, if the first selected gap cover the position “X”, this gap first position (gap-RX0) = “X”; if the last selected gap cover the position “Y”, this gap last position (gap-RY0) = “Y”;

If user assigned the region (position M to N) for R primers:

X= the bigger value between “FM+L/min -1” and “M+9”;

Y= the smallest value among “FM+L/max -1”, “Ref. end position”, and “N-1”;

if X>Y, report “amplicon size < L/min” and stop; Otherwise, continue:

RA: select all the regions in the Ref. sequence arranging from the position “X” to the position “Y”, if the first selected region cover the position X, this region start position (region-RX0) = “X”; if the last selected region cover the position Y, this region end position (region-RY0) = “Y”;

RB: select all the gaps in the Ref. sequence arranging from the position “X” to the position “Y”, if the first selected gap cover the position X, this gap first position (gap-RX0) = “X”; if the last selected gap cover the position Y, this gap last position (gap-RY0) = “Y”;

If RA successful, combine the assigned F primer and R primers

If RA fail, try RB:

If RB successful, combine the assigned F primer and R primers

If RB fail, stop and report fail;

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2.6. If user assigned **R primer** to design **F** primers;

Evaluate the specificity of the reverse complement of the R primer in whole Ref. sequence, record the position and mismatch number of each binding site (containing 4, 3, 2, 1, or 0 mismatches, and the corresponding site number SN4=, SN3=, SN2=, SN1=, and SN0=, respectively);

If SN0 =0, report “cannot find F primer binding site”;

If SN0 =1 and Σ(SN4+SN3+SN2+SN1)=0, continue;

If SN0 =1 and Σ(SN4+SN3+SN2+SN1) ≥ 1, ask user “containing non-specific binding sites of the F primer, continue?

If SN0 ≥ 2, report “containing more than one binding sites of the F primer” and stop;

If user didn’t assign a region for F primers, retrieve the position of R primer reverse complement (from RM to RN):

X=“RN-L/max +1” OR “0” if “RN-L/max +1” <0 and Y=“RN-L/min +1”; if X>Y, report “amplicon size < L/min” and stop; Otherwise, continue:

FA: select all the regions in the Ref. sequence arranging from the position “X” to the position “Y”, if the first selected region cover the position “X”, this region first position (region-FX0) = “X”; if the last selected region cover the position “Y”, this region end position (region-FY0) = “Y”;

FB: select all the gaps in the Ref. sequence arranging from the position “X” to the position “Y”, if the first selected gap cover the position “X”, this gap first position (gap-FX0) = “X”; if the last selected gap cover the position “Y”, this gap end position (gap-FY0) = “Y”;

If user assigned the region (position M to N) for F primers:

X= the bigger value between “RN-L/max +1” and “M-1”;

Y= the smaller value between “RN-L/min +1” and “N-11”;

FA: select all the regions in the Ref. sequence arranging from the position “X” to the position “Y”, if the first selected region cover the position “X”, this region first position (region-FX0) = “X”; if the last selected region cover the position “Y”, this region end position (region-FY0) = “Y”;

FB: select all the gaps in the Ref. sequence arranging from the position “X” to the position “Y”, if the first selected gap cover the position “X”, this gap first position (gap-FX0) = “X”; if the last selected gap cover the position “Y”, this gap end position (gap-FY0) = “Y”;

If FA successful, combine F primers and the assigned R primer

If FA fail, try FB:

If RB successful, combine F primers and the assigned R primer

If RB fail, stop and report fail;

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2.7. If user assigned **F and R primers**.

Evaluate the basic information of the user’s primers and calculate the PCR conditions