

**A)** If user didn’t enter Ref. sequence:

1) And didn’t enter any primer sequence, report “**please enter Ref. sequence**”

2) But did enter one or two primer sequences, evaluate the basic information of the user’s primers.

**B)** If user did enter Ref. sequence:

Ref. sequence format:

1. > seq name
2. fasta

1) a, t, g, c, and n will be changed to A, T, G, C, and N;

2) The sequence contains non-DNA characters, which will be omitted?

**C)** Evaluate Ref. sequence size (L/ref) and other basic information (below) before design primers:

L/ref: Ref. sequence size; L/min: minimum size of PCR product; L/max: maximum size of PCR product;

Region/F: region for F primer between Region/F/from and Region/F/to;

Region/R: region for R primer between Region/R/from and Region/R/to;

**Default:**

L/min = 1500; L/max = 8000;

Return primer pair number=10;

Tm/min=54; Tm/opt =56; Tm/max=58;

Region/F/from=1; Region/R/to= L/ref;

**Must:**

1. 20,000 ≥ L/max ≥ L/min ≥ 50 and 20,000 ≥ L/ref ≥ L/min ≥ 50;
2. 35.0 ≤ Tm/min ≤ Tm/opt ≤ Tm/max < 72.5; and confirm primer length based on the assigned Tm/min and Tm/max in the table

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Tm/min value | min primer length |  | Tm/max value | max primer length |
| 70.0 ≤ Tm/min < 72.5 | 24 |  | 70.0 ≤ Tm/max < 72.5 | 31 |
| 67.5 ≤ Tm/min < 70.0 | 23 |  | 67.5 ≤ Tm/max < 70.0 | 30 |
| 65.0 ≤ Tm/min < 67.5 | 22 |  | 65.0 ≤ Tm/max < 67.5 | 29 |
| 62.5 ≤ Tm/min < 65.0 | 21 |  | 62.5 ≤ Tm/max < 65.0 | 28 |
| 60.0 ≤ Tm/min < 62.5 | 20 |  | 60.0 ≤ Tm/max < 62.5 | 27 |
| 57.5 ≤ Tm/min < 60.0 | 19 |  | 57.5 ≤ Tm/max < 60.0 | 26 |
| 55.0 ≤ Tm/min < 57.5 | 18 |  | 55.0 ≤ Tm/max < 57.5 | 25 |
| 52.5 ≤ Tm/min < 55.0 | 17 |  | 52.5 ≤ Tm/max < 55.0 | 24 |
| 50.0 ≤ Tm/min < 52.5 | 16 |  | 50.0 ≤ Tm/max < 52.5 | 23 |
| 47.5 ≤ Tm/min < 50.0 | 15 |  | 47.5 ≤ Tm/max < 50.0 | 22 |
| 45.0 ≤ Tm/min < 47.5 | 14 |  | 45.0 ≤ Tm/max < 47.5 | 21 |
| 42.5 ≤ Tm/min < 45.0 | 13 |  | 42.5 ≤ Tm/max < 45.0 | 20 |
| 40.0 ≤ Tm/min < 42.5 | 12 |  | 40.0 ≤ Tm/max < 42.5 | 19 |
| 37.5 ≤ Tm/min < 40.0 | 11 |  | 37.5 ≤ Tm/max < 40.0 | 18 |
| 35.0 ≤ Tm/min < 37.5 | 10 |  | 35.0 ≤ Tm/max < 37.5 | 17 |

1. if Region/F/to > L/ref, then Region/F/to = L/ref; if Region/R/to > L/ref, then Region/R/to = L/ref;

1 ≤ Region/F/from ≤ Region/F/to - minimum primer length + 1;

1 ≤ Region/R/from ≤ Region/R/to - minimum primer length + 1;

Region/R/from + 2\*minimum primer length - Region/F/to -1 ≤ L/max;

Region/R/to - Region/F/from +1 ≥ L/min

Otherwise, report “the corresponding false”

**E1)** Evaluate GC distribution (≥ 100 bp and > 75%) and (forward and reverse) repeats (**the score value should be set at lower**).

Identify the fragments with special structure in the ref sequence:

1. Any ≥ 100-bp fragment with GC content > 75%;
2. Forward repeats (M/Mʹ---N/Nʹ);
3. Reverse repeats (≥ 100 bp and M/Nʹ---N/Mʹ);

**E2)** Find single-copy and multiple-copy regions:

**E3)** Identify the regions (≥ **10** bp) without E1-1, E1-2, and E1-3 in single-copy regions (E2):

