

# **How to use PrimerExplorer V5**



# 1. Primer design using M13 as the template (Target)

## 1.1 Uploading the target sequence

The target sequence is uploaded in the PrimerExplorer V5 startup window (Figure 1.1).

First, click on the “Browse” button to select the target sequence file. The target sequence entered is set to less than 2 k bp. Three types of file formats are supported, plain text format (sequence only), FASTA format, and GenBank format.

Next, a parameter set (primer design conditions) is chosen from one of the three below.

1) Automatic Judgment: Based on the GC content of the target sequence, the initial parameter setting is specified. If the GC content is 45% or less, the “AT rich” parameters are used; if greater than 60%, the “GC rich” parameters are used. For all others, the “Normal” parameters are used.

2) Normal: The user enters the primer design conditions manually to design the primers. As the default conditions, the “Normal” parameters from 1) above are displayed.

3) User Assignment: Click on the [Browse...] button on the right and specify the parameter file of primer design conditions saved on the PC. The specified parameter file will be used as the initial setting to design the primers.

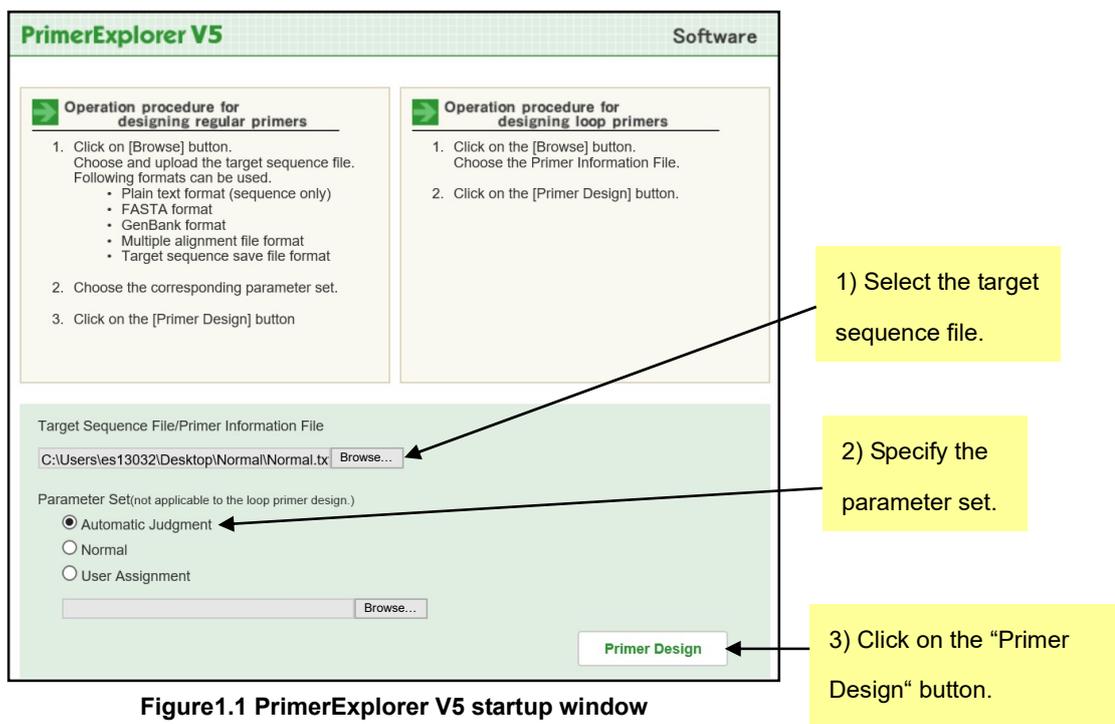


Figure 1.1 PrimerExplorer V5 startup window

The default parameter set is “automatic judgment.” In “automatic judgment,” the GC content of the target sequence is automatically calculated, and the primer design conditions are automatically selected in the following primer design conditions (“Normal sequences primer design conditions” “GC rich sequences primer design conditions,” “AT rich sequences primer design conditions”).

Next, click on the “Primer Design” button.

## 1.2 Designing the primer (Easy Mode)

As an example, a portion of the M13 sequence (length 1969 bp, GC content = 48.2%) will be used to design the primers. Click on the “Generate” button (Figure 1.2). This mode eliminates the need to change parameters and displays five primer sets that are likely to have high amplification efficiency. It automatically narrows down and prioritizes primer set candidates. The “Generate sets” box shows that five primer sets have been designed. Clicking on the “Display” button will display the Primer Set List results (Figure 1.3).

UPLOAD FILE: Normal.txt

1 TTCTCATGTT TGACAGCTTA TCATCGATAA GCCTTAAATGC GGTAGTTTAT CACAGTTAAA TTGCTAACGC AGTCAGGCAC 80  
81 CGGTATAGAA ATCTAACAAAT GCGCTCATCG TCATCCTCGG CACGGTCAGC CTGGAATGCTG TAGGCATAGG CTGGTATAG 160  
161 CCGTACTGTC CGGGCGCTCT GCGGGAATAC GTCCATTCCG ACAGCATCGC CAGTCACIAI GCGGTGCTGC TAGCGCIATA 240  
241 TCGGTATGATG CAATTTCTAT GCGCACCGGT TCTGGGAGCA CTGTCCGACC GCTTTGGCGG CCGCCAGATC CTGCTGCTT 320  
321 CGTACTTGG AGCACTAAT GACTAGCGGA TCATGGGAC CACACDDTTC CTGTGATCC TCTACGCGG ACCCATCGTG 400  
401 GCGGCAATCA CCGGCGCAC AGGTGGGGIT GCTGGGCGCT ATATGGCGGA CATCACCGAT GGGGARGATC GGGCTGGCCA 480  
481 CTTCGGGCTC ATGAGCGCCT GTTTGGGCGT GGGTATGGTG GCAGGCCCCG TGGCGGGGGG ACIGTIGGGC GCCATCTCCT 560  
561 TGCATGCACC ATTCTTTGGG GCGGCGTGC TCAACGGCTT CAACTACTA CTGGGCTGCT TCCTAATGCA GGAGTGGCAT 640  
641 AAGGAGAGAC GTCGACCGAT GCGCTTGAGA GCTTCAGCC CAGTCAGCTC CTTCGGTGG GCGGCGGGCA TGACTATGCT 720  
721 CCGCGCACIT ATGACTGCTT TCTTATCAT GCAACTCGTA GCACAGTGC GCGCAGCGCT CTGGGTCATT TTGGGAGAG 800  
801 ACCGGTTCG CTGGAGCGCG AGGATGATCG GCCTGTGCTT TCGGTATTC GGAATCTTGC ACGCCCTCGC TCAAGCCCTC 880

Set Mutation  
Mut/Cons  
Clear

Fixed Primer  
F3  
F2  
F1  
B1  
B2  
B3  
Clear

Save Target

Design Option  
 Default  
 Common  
 Specific

1.Generate  sets were generated.

2.Display

If you can have more detail settings, please click below.

Click on the “Generate” button

Click on the “Display” button

Figure 1.2 Primer design window in Easy Mode

A list of primer sets designed on the target sequence appears in a separate window, and you can save the list in an Excel file by clicking the “Save List” button. Here, check the button at the left end of the window, and click on the “Confirm.” The Primer Set Details window appears with detailed primer information for the checked primer sets (Figure 1.4). Check for problematic findings in each parameter. Click on the “Primer Information” for each primer set to save the information for that primer. This information should be used to design loop primers. In this window, clicking the “Save” button allows the sequence information of each primer to be set in an Excel file.



1. Push "Primer Information" button to download Primer Information format file for loop primer designing.
2. Push "Save" button to download the primer information in the screen display layout.

DesignId 190319111918

To save the primer sequence information, click on the "Save"

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**1** ID:26 dimer(minimum)dG=-2.36

| label | 5'pos | 3'pos | len | Tm    | 5'dG  | 3'dG  | GCrate | Sequence                                  |
|-------|-------|-------|-----|-------|-------|-------|--------|-------------------------------------------|
| F3    | 607   | 624   | 18  | 59.42 | -3.96 | -4.69 | 0.56   | ACTACTGGGCTGCTTCCT                        |
| B3    | 784   | 802   | 19  | 60.31 | -5.20 | -4.90 | 0.58   | GTCCTCGCCGAAAATGACC                       |
| FIP   |       |       | 41  |       |       |       |        | AGCTGACTGGGTGAAGGCTCT-GCAGGAGTCGCATAAGGGA |
| BIP   |       |       | 40  |       |       |       |        | CATGACTATCGTCGCCCACT-CACCTGTCCTACGAGTTGC  |
| F2    | 628   | 646   | 19  | 60.88 | -6.10 | -5.20 | 0.58   | GCAGGAGTCGCATAAGGGA                       |
| F1c   | 668   | 689   | 22  | 65.68 | -5.49 | -5.93 | 0.55   | AGCTGACTGGGTGAAGGCTCT                     |
| B2    | 751   | 769   | 19  | 59.31 | -5.50 | -5.40 | 0.58   | CACCTGTCCTACGAGTTGC                       |
| B1c   | 709   | 729   | 21  | 64.31 | -4.56 | -6.57 | 0.57   | CATGACTATCGTCGCCCACT                      |

To save the primer information for use in designing the loop primers, click on the "Primer Information" button.

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**2** ID:40 dimer(minimum)dG=-1.95

| label | 5'pos | 3'pos | len | Tm    | 5'dG  | 3'dG  | GCrate | Sequence                                   |
|-------|-------|-------|-----|-------|-------|-------|--------|--------------------------------------------|
| F3    | 1576  | 1593  | 18  | 59.72 | -7.03 | -4.72 | 0.56   | GCGACCTGAGCAACAACA                         |
| B3    | 1769  | 1786  | 18  | 59.08 | -5.00 | -5.75 | 0.56   | AACTGGCGGTATGGATGC                         |
| FIP   |       |       | 41  |       |       |       |        | ACATAATGGTGCAGGGCGCTG-TGAATGGTCTTCGGTTTCCG |
| BIP   |       |       | 40  |       |       |       |        | CGCAGGATGCTGCTGGCTAC-AATCACTCAGGGTCAATGCC  |
| F2    | 1594  | 1613  | 20  | 59.76 | -4.07 | -5.30 | 0.50   | TGAATGGTCTTCGGTTTCCG                       |
| F1c   | 1643  | 1663  | 21  | 65.39 | -3.29 | -7.42 | 0.57   | ACATAATGGTGCAGGGCGCTG                      |
| B2    | 1733  | 1752  | 20  | 59.64 | -4.06 | -5.40 | 0.50   | AATCACTCAGGGTCAATGCC                       |
| B1c   | 1677  | 1696  | 20  | 65.39 | -7.02 | -5.42 | 0.65   | CGCAGGATGCTGCTGGCTAC                       |

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**3** ID:6 dimer(minimum)dG=-2.23

| label | 5'pos | 3'pos | len | Tm    | 5'dG  | 3'dG  | GCrate | Sequence                                  |
|-------|-------|-------|-----|-------|-------|-------|--------|-------------------------------------------|
| F3    | 128   | 145   | 18  | 60.55 | -5.84 | -5.42 | 0.61   | ACCCTGGATGCTGTAGGC                        |
| B3    | 317   | 334   | 18  | 59.61 | -6.54 | -6.03 | 0.61   | GGCTCCAAGTAGCGAAGC                        |
| FIP   |       |       | 40  |       |       |       |        | GTGACTGGCGATGCTGTCCG-GCTTGGTTATGCCGGTACTG |
| BIP   |       |       | 39  |       |       |       |        | TATGGCGTGCTGCTAGCGCTA-CAAAGCGGTCGGACAGTG  |
| F2    | 150   | 169   | 20  | 60.49 | -5.85 | -4.23 | 0.55   | GCTTGGTTATGCCGGTACTG                      |
| F1c   | 198   | 217   | 20  | 65.19 | -4.90 | -6.19 | 0.65   | GTGACTGGCGATGCTGTCCG                      |
| B2    | 279   | 296   | 18  | 60.06 | -5.01 | -5.05 | 0.61   | CAAAGCGGTCGGACAGTG                        |
| B1c   | 218   | 238   | 21  | 65.98 | -4.98 | -6.50 | 0.57   | TATGGCGTGCTGCTAGCGCTA                     |

Figure 1.4 Primer Set Details window

### 1.3 Designing the primer (Expert Mode)

Although Easy Mode enables you to design primers with some capability, Expert Mode allows you to design with better capability or customized primers. To jump to the Expert Mode (Figure 1.6), click on the “Detail Setting” button in Easy Mode window (Figure 1.5). The default parameter set is “automatic judgment”. In “automatic judgment”, the GC content of the target sequence is automatically calculated, and the primer design conditions are automatically selected in the following primer design conditions (“Normal sequences primer design conditions”, “GC rich sequences primer design conditions” and “AT rich sequences primer design conditions”). As indicated in the primer design window, “Parameter Set” of “Normal” has been selected. Normal parameter conditions are as indicated in Figure 1.6.

Next, click on the “Generate” button to start the primer design. When the primer design starts, the message area will indicate the status of progress in the primer design. The number of primer candidates for each region that fulfills the parameter conditions is displayed, as well as the number of inner primers (FIP, BIP) for each region. Based on these data, the primer sets are created. In this example, a total of 1,000 primer sets were designed (Figure 1.7). Clicking on the “Display” button will display the Primer Set List result.

The screenshot displays the primer design interface. At the top, it shows the uploaded file name "Normal.txt" and a target DNA sequence with positions 1 to 880. On the right side, there are three sections of controls: "Set Mutation" with "Mut/Cons" and "Clear" buttons; "Fixed Primer" with buttons for F3, F2, F1, B1, B2, B3, and "Clear"; and "Design Option" with radio buttons for "Default" (selected), "Common", and "Specific". Below the sequence, there are two main buttons: "Generate" and "Display". A text box indicates "sets were generated." Below these buttons, there is a "Detail Settings" button. A yellow callout box on the left points to the "Detail Settings" button with the text: "Click on the 'Detail Settings' button."

Figure 1.5 Primer design window

UPLOAD FILE: Normal.txt

```

1 TTCCTATGTT TGACAGCTTA TCAICATATA GCTTTAATCC GGTAGTTTAT CACAGTTAAA TTGCTAACGC ATCCAGGCAC 80
81 CCGTATATGA ATCTAACAT GGGCCATGCG TGACCTCCGG CACCCCTCAC CTGGATCCCG TAGCCATAGC CTGGTTATAG 160
161 CCGGTACTGCG CCGGCTCTTT GCGGAAATAC TTGCATTCOD ACAGCATDCC CAGTCAATAT GCGCTCCTCC TAGCCCTATA 240
241 TGCTTGAATG CAATTTCTAT GGGCAACCGT TGTGGAGCCA CTGTCGACCC GGTITGGGCG CCGCCCAATC CTGCTGCTTT 320
321 GCGTACTGCG AGCATATAC GACTACCGGA TCGAGCGGAC CACAGCCGTC CTGGGATGCT TTATAGCGCG AGCCATCGCG 400
401 GCGGATGTC CCGGCGCAC AGTTCGGTGT GCTTGGCTCT ATATGGCGCA CATGACCGAT GGGGAAAGAT GGGCTGCGCA 480
481 CTTCGGCTC ATGAGCGCTT GTTTGGGCTT GGTATGGTGT GCGAGCCGCG TGGCGGGGCG ACTGTTGGGC GGCATCTCTT 560
561 TGATGCGAC ATTCCTTGG GCGGCGGTCG TGAAGCGGCT CAACCTACTA CTGGGCTGCT TCCATAAGCA GAGTGGCAT 640
641 AAGGAGAGG GTGACCGAT GCGCTTGAAG GCTTCAACC CAGTCAGCTC CTTCGGTGG CCGCGGGGCA TGACTATGCT 720
721 CCGCCACTT ATGACTGTT TCTTATCAT GGAATCTGTA GGCAGGTCG CCGGACCGCT CTGGGTCATT TTGCGGAGAG 800
801 ACCGCTTTC CTGGAGCGCG AGCATATGCG GCGTCTGCTT TGGGTTATC GGAATCTTCC ACCGCTTCC TCAAGCTTTC 880

```

Number of Primer Candidates: F1=251, F2=242, F3=593, B1=253, B2=219, B3=668, F1P=318, B1P=248  
1000 Primer set(s) were generated.

Set Mutation  
Mut/Cons  
Clear

Fixed Primer  
F3  
F2  
F1  
B1  
B2  
B3  
Clear

Save Target

Design Option  
Default  
Common  
Specific

1. Select Range  
 Ignore range  
 Within F2-B2  
 Between F1c-B1c Targeting Range

2. Generate  
Generate 1000 sets were generated.

3. Display  
Display Page 1 Displayed. Sorting Rule None

If you can move to "Basic Designing", please click below.  
Basic Designing

Parameter Condition Normal Save Parameter Reset Parameter

Length  
F1c/B1c 20 - 22  
F2/B2 18 - 20  
F3/B3 18 - 20

Tm  
F1c/B1c 64 - 66  
F2/B2 59 - 61  
F3/B3 59 - 61

GC rate(%) 40 - 65

dG threshold  
5'stability -3  
3'stability -4  
dimer check -2.5

Distances  
(F2-B2) 120 - 180  
Loop(F1c-F2) 40 - 60  
F2-F3 0 - 20  
F1c-B1c 0 - 100

Limitations  
F1c/B1c 3  
F2/B2 10  
F3/B3 3  
Sets 1000

Mutation/Consensus

| Peculiarity | Permission                                                              |
|-------------|-------------------------------------------------------------------------|
| high level  | F1c 5'term <input type="checkbox"/> B1c 5'term <input type="checkbox"/> |
| ↑           | F2 3'term <input type="checkbox"/> B2 3'term <input type="checkbox"/>   |
|             | F3 3'term <input type="checkbox"/> B3 3'term <input type="checkbox"/>   |
|             | F1c inner <input type="checkbox"/> B1c inner <input type="checkbox"/>   |
|             | F2 inner <input type="checkbox"/> B2 inner <input type="checkbox"/>     |
|             | F3 inner <input type="checkbox"/> B3 inner <input type="checkbox"/>     |
|             | F1c 3'term <input type="checkbox"/> B1c 3'term <input type="checkbox"/> |
|             | F2 5'term <input type="checkbox"/> B2 5'term <input type="checkbox"/>   |
| ↓           | F3 5'term <input type="checkbox"/> B3 5'term <input type="checkbox"/>   |
| low level   |                                                                         |

Reset Parameter

Click on the "Generate" button.

Click on the "Display" button.

"Parameter Set" of "Normal" has been selected.

Figure 1.6 Expert Mode

The screenshot displays the 'Primer design window' with the following components:

- Target Sequence:** A DNA sequence starting with '1 TTCTCA... 80' and ending with '801 ACCGCTTTC... 880'.
- Design Options:**
  - Set Mutation:** Buttons for 'Mut/Cons' and 'Clear'.
  - Fixed Primer:** Radio buttons for 'F3' (green), 'F2' (blue), 'F1' (white), 'B1' (white), 'B2' (blue), 'B3' (green), and 'Clear'.
  - Design Option:** Radio buttons for 'Default' (selected), 'Common', and 'Specific'.
  - Save Target:** A button to save the current target.
- Summary:** A box indicating '1000 Primer set(s) were generated.' and 'F2=242, F3=593, B1=253, B2=213, B3=568, F1P=318, B1P=268'.
- Configuration:**
  - 1. Select Range:** Radio buttons for 'Ignore range' (selected), 'Within F2-B2', and 'Between F1c-B1c'. A 'Targeting Range' input field is present.
  - 2. Generate:** A 'Generate' button and a text field showing '1000 sets were generated.'
  - 3. Display:** A 'Display' button, a 'Page' dropdown menu set to '1', and a 'Sorting Rule' dropdown set to 'None'.

Annotations in the image include:

- A yellow box pointing to the 'Display' button with the text: 'Click on the "Display" button to display the results.'
- A box pointing to the summary text: 'The message area indicates the status of progress.'
- A box pointing to the '1000 sets were generated' text: 'A total of 1,000 primer sets were generated.'

**Figure 1.7 Primer design window**

#### 1.4 Displaying the results

Primer Set List window (Figures 1.5a, 1.5b) shows the ID number of each primer set on the left, and to its right the change in free energy, which indicates the propensity for dimer formation. A low value of the change in free energy results in a higher likelihood of dimer formation and thus the primer set is unacceptable. Green capital letters indicate the region F3, blue capital letters indicate the region F2, black lower-case letters indicate the region F1c, black capital letters indicate the region B1c, blue lower-case letters the region B2, and green lower-case letters the B3 region.

The primer set is designed with the 5' end of F2 as the origin, and primer sets that fulfill the primer design conditions are displayed for the entire target sequence from the 5' end toward the 3' end. For each region F2, ones from other regions (regions F3, F1c, B1c, B2 and B3) are determined and displayed. After displaying the primers designed for the target sequence from the 5' end to the 3' end, the design is re-started from the 5' end to the 3' end. This operation is repeated until 1,000 primer design candidates are generated.

In this example, the length of the input target is 1,969 bp, and after the first round from the 5' end to the 3' end, 55 primer sets have been designed. After the second round, primers are designed from set 56 to set 110. The 5' end of the region F2 included in the final primer set after the first round is at 1,281 bp (the 5' end of F3 is 1,439 bp). (See Figure 1.5b) Several primers are then selected to compare the specific conditions.

**PrimerExplorer V5** Software

ID number

Free energy value

Region F3

Region F2

2) Click on the "Confirm" button.

Confirm    Save List

DesignId 160412104924

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**Primer set: sorting rule [None]**

**Target DNA** CACAGTTAAATTGCTAACGGAGTCAGGCACCGTGTATGAAATCTAACAATGCGCTCATCGTCATCCTCGGCACCGTCACCCT

**(Complement)** gtgtcaatttaacgattgcgtcagtcocgtgcacatactttagattgtttacgcgagtagcagtaggagccgtggcagtgagg

**CONSENSUS(\*)** xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx

| Primer ID                     | dG(dimer) | 51  | 61                 | 71 | 81 | 91  | 101                 | 111 | 121 | 131      |
|-------------------------------|-----------|-----|--------------------|----|----|-----|---------------------|-----|-----|----------|
| <input type="checkbox"/> [1]  | -2.01     | [1] | TGCTAACGCAGTCAGGCA |    |    |     | AATGCGCTCATCGTCATCC |     |     |          |
| <input type="checkbox"/> [2]  | -2.01     | [2] | TGCTAACGCAGTCAGGCA |    |    |     | ATGCGCTCATCGTCATCC  |     |     |          |
| <input type="checkbox"/> [3]  | -2.46     |     |                    |    |    | [3] | GGCTCATCGTCATCCTC   |     |     | CGTACCCT |
| <input type="checkbox"/> [4]  | -2.46     |     |                    |    |    | [4] | GGCTCATCGTCATCCTC   |     |     | CCCT     |
| <input type="checkbox"/> [5]  | -2.46     |     |                    |    |    | [5] | GGCTCATCGTCATCCTC   |     |     | CT       |
| <input type="checkbox"/> [6]  | -2.23     |     |                    |    |    |     |                     | [6] |     | ACCCT    |
| <input type="checkbox"/> [7]  | -2.49     |     |                    |    |    |     |                     |     | [7] | CT       |
| <input type="checkbox"/> [8]  | -2.49     |     |                    |    |    |     |                     |     | [8] |          |
| <input type="checkbox"/> [9]  | -2.16     |     |                    |    |    |     |                     | [9] |     | ACCCT    |
| <input type="checkbox"/> [10] | -1.82     |     |                    |    |    |     |                     |     |     |          |
| <input type="checkbox"/> [11] | -1.82     |     |                    |    |    |     |                     |     |     |          |
| <input type="checkbox"/> [12] | -1.82     |     |                    |    |    |     |                     |     |     |          |

1) Check the box to the left of the primer set selected.

Figure 1.8a Primer Set List window-1 (page 1)

戻る    進む    中止    更新    ホーム    検索    お気に入り    メイン

アドレス(D) <https://mercury.netlaboratory.com/lamp2.2.0/list/618951282593.html>

|                      |                        |                    |                    |            |            |                     |        |      |
|----------------------|------------------------|--------------------|--------------------|------------|------------|---------------------|--------|------|
|                      |                        | caaggstgctcttaggct | gccaacaatgagcgastg | [39]       |            |                     |        |      |
|                      |                        | caaggstgctcttaggct | gccaacaatgagcgastg | [40]       |            |                     |        |      |
| ATTACGGTCAATCCGCC    |                        |                    | gccaacaatgagcgastg |            |            | actacttcgaccgatgtcc |        | [41] |
| ATTACGGTCAATCCGCC    |                        |                    | gccaacaatgagcgastg |            |            | actacttcgaccgatgtcc |        | [42] |
| ATTACGGTCAATCCGCC    |                        |                    | gccaacaatgagcgastg |            |            | actacttcgaccgatgtcc |        | [43] |
| ATTACGGTCAATCCGCCG   |                        |                    | gccaacaatgagcgastg |            |            | actacttcgaccgatgtcc |        | [44] |
| taatgccagttaggcgg    | GTTCCCACGGAGAATCCGACG  |                    |                    |            |            | acttcgaccgatgtccttc |        |      |
| taatgccagttaggcgg    | GTTCCCACGGAGAATCCGACG  |                    |                    |            |            | acttcgaccgatgtccttc |        |      |
| taatgccagttaggcgg    | GTTCCCACGGAGAATCCGACG  |                    |                    |            |            | acttcgaccgatgtccttc |        |      |
| taatgccagttaggcgg    | GTTCCCACGGAGAATCCGACG  |                    |                    |            |            | acttcgaccgatgtccttc |        |      |
| taatgccagttaggcgg    | GTTCCCACGGAGAATCCGACG  |                    |                    |            |            | acttcgaccgatgtccttc |        |      |
| taatgccagttaggcgg    | GTTCCCACGGAGAATCCGACG  |                    |                    |            |            | acttcgaccgatgtccttc |        |      |
| taatgccagttaggcgg    | GTTCCCACGGAGAATCCGACG  |                    |                    |            |            | acttcgaccgatgtccttc |        |      |
| ATTACGGTCAATCCGCCGTT | TGTTCCCACGGAGAATCCGACG | CGGGTTGTTA         | CTCGCTCACA         | TTTAATGTTG | GATGAAAGCT | GGCTACAGGAA         | GGCCAG |      |
| 1311                 | 1321                   | 1331               | 1341               | 1351       | 1361       | 1371                | 1381   | 1391 |
| 1401                 |                        |                    |                    |            |            |                     |        |      |

Region B1c

Region B2

Region B3

The 5' end of B3 is 1440bp.

Figure 1.8b Primer Set List window-2 (page 2)



## 1.5 Primer set selection

Three to five or more primer sets that amplify different regions in the target sequence are designed, and their actual reactivities are compared to select the appropriate primer sets. If the region to amplify is pre-determined, the primer sets that amplify the region are selected.

If the appropriate primer is selected from among the multiple primer sets designed in the same region, detail information is compared. Here is an example of comparing two primer sets (ID number 1 and 2) in the Primer Set List window (Figure 1. 8a). First, check the boxes located to the left of each primer set, and click on the “Details” to open the Primer Set Details window (Figure 1.10).

Primer Information    Save

| 1     | ID:1  | dimer(minimum)dG=-2.01 |     |       |       |       |        |                                            |
|-------|-------|------------------------|-----|-------|-------|-------|--------|--------------------------------------------|
| label | 5'pos | 3'pos                  | len | Tm    | 5'dG  | 3'dG  | GCrate | Sequence                                   |
| F3    | 62    | 79                     | 18  | 60.91 | -4.49 | -6.25 | 0.56   | TGCTAACGCAGTCAGGCA                         |
| B3    | 250   | 268                    | 19  | 59.11 | -6.85 | -4.56 | 0.53   | GGGTGCGCATAGAAATTGC                        |
| FIP   |       |                        | 41  |       |       |       |        | GCAGTACCGGCATAACCAAGCC-AATGCGCTCATCGTCATCC |
| BIP   |       |                        | 39  |       |       |       |        | GCCTCTTGCGGGATATCGTCC-GCTAGCAGCAGCCATAG    |
| F2    | 98    | 116                    | 19  | 59.84 | -5.73 | -4.76 | 0.53   | AATGCGCTCATCGTCATCC                        |
| F1c   | 149   | 170                    | 22  | 65.71 | -4.98 | -5.85 | 0.59   | GCAGTACCGGCATAACCAAGCC                     |
| B2    | 217   | 234                    | 18  | 59.71 | -5.23 | -4.07 | 0.61   | GCTAGCAGCAGCCATAG                          |
| B1c   | 174   | 194                    | 21  | 64.55 | -5.93 | -6.04 | 0.62   | GCCTCTTGCGGGATATCGTCC                      |

Primer Information    Save

| 2     | ID:2  | dimer(minimum)dG=-2.01 |     |       |       |       |        |                                           |
|-------|-------|------------------------|-----|-------|-------|-------|--------|-------------------------------------------|
| label | 5'pos | 3'pos                  | len | Tm    | 5'dG  | 3'dG  | GCrate | Sequence                                  |
| F3    | 62    | 79                     | 18  | 60.91 | -4.49 | -6.25 | 0.56   | TGCTAACGCAGTCAGGCA                        |
| B3    | 250   | 268                    | 19  | 59.11 | -6.85 | -4.56 | 0.53   | GGGTGCGCATAGAAATTGC                       |
| FIP   |       |                        | 40  |       |       |       |        | GCAGTACCGGCATAACCAAGCC-ATGCGCTCATCGTCATCC |
| BIP   |       |                        | 39  |       |       |       |        | GCCTCTTGCGGGATATCGTCC-GCTAGCAGCAGCCATAG   |
| F2    | 99    | 116                    | 18  | 59.08 | -6.97 | -4.76 | 0.56   | ATGCGCTCATCGTCATCC                        |
| F1c   | 149   | 170                    | 22  | 65.71 | -4.98 | -5.85 | 0.59   | GCAGTACCGGCATAACCAAGCC                    |
| B2    | 217   | 234                    | 18  | 59.71 | -5.23 | -4.07 | 0.61   | GCTAGCAGCAGCCATAG                         |
| B1c   | 174   | 194                    | 21  | 64.55 | -5.93 | -6.04 | 0.62   | GCCTCTTGCGGGATATCGTCC                     |

Figure 1.10 Primer Set Details window

In the window displayed as in Figure 1.10, check for the stability of the 3' end at region F2, the 5' end at region F1c, the 3' end at region B2, and the 5' end at region B1c, in each primer set. Since these are the starting points for gene amplification by primers, their end stability is important. Specifically, check to see whether the  $\Delta G$  (stability) is  $-4.0$  kcal/mol or lower. For example, the end with  $\Delta G = -6.5$  kcal/mol is more stable than the end with  $\Delta G = -4.0$  kcal/mol.

There is a "Primer Information" button above each ID number (Figure 1.11). This button should be used to design loop primers for the primer set selected. To save the primer sequence information, click on the "Save" (Figure 1.11). Click on the "Primer Information" to save the primer information to be used to explain loop primer design.

The screenshot shows two panels of primer information. The top panel is for ID:1 and the bottom for ID:2. Both have a dimer(minimum)dG=-2.01. The 'Primer Information' button is highlighted with a yellow box and an arrow pointing to it. The 'Save' button is also highlighted with a yellow box and an arrow pointing to it. The primer sequences are listed in a table with columns for label, 5'pos, 3'pos, len, Tm, 5'dG, 3'dG, GCrate, and Sequence. The sequences are highlighted in green.

| ID | label | 5'pos | 3'pos | len | Tm    | 5'dG  | 3'dG  | GCrate | Sequence                                   |
|----|-------|-------|-------|-----|-------|-------|-------|--------|--------------------------------------------|
| 1  | F3    | 62    | 79    | 18  | 60.91 | -4.49 | -6.25 | 0.56   | TGCTAACGCAGTCAGGCA                         |
|    | B3    | 250   | 268   | 19  | 59.11 | -6.85 | -4.56 | 0.53   | GGGTGCGCATAGAAATTGC                        |
|    | FIP   |       |       | 41  |       |       |       |        | GCAGTACCGGCATAACCAAGCC-AATGCGCTCATCGTCATCC |
|    |       |       |       |     |       |       |       |        | GCCTCTTGCGGGATATCGTCC-GCTAGCAGCACGCCATAG   |
|    |       |       |       |     | 59.84 | -5.73 | -4.76 | 0.53   | AATGCGCTCATCGTCATCC                        |
|    |       |       |       |     | 65.71 | -4.98 | -5.85 | 0.59   | GCAGTACCGGCATAACCAAGCC                     |
| 2  | F3    | 62    | 79    | 18  | 60.91 | -4.49 | -6.25 | 0.56   | TGCTAACGCAGTCAGGCA                         |
|    | B3    | 250   | 268   | 19  | 59.11 | -6.85 | -4.56 | 0.53   | GGGTGCGCATAGAAATTGC                        |
|    | FIP   |       |       | 40  |       |       |       |        | GCAGTACCGGCATAACCAAGCC-ATGCGCTCATCGTCATCC  |
|    | BIP   |       |       | 39  |       |       |       |        | GCCTCTTGCGGGATATCGTCC-GCTAGCAGCACGCCATAG   |
|    | F2    | 99    | 116   | 18  | 59.08 | -6.97 | -4.76 | 0.56   | ATGCGCTCATCGTCATCC                         |
|    | F1c   | 149   | 170   | 22  | 65.71 | -4.98 | -5.85 | 0.59   | GCAGTACCGGCATAACCAAGCC                     |
|    | B2    | 217   | 234   | 18  | 59.71 | -5.23 | -4.07 | 0.61   | GCTAGCAGCACGCCATAG                         |
|    | B1c   | 174   | 194   | 21  | 64.55 | -5.93 | -6.04 | 0.62   | GCCTCTTGCGGGATATCGTCC                      |

Figure 1.11 Selected Primer Set

## 2. Primer design for AT-rich sequences

In this section, primers will be designed for an AT rich gene sequence. We will use a portion of a viral gene of 1,140 bp in length and GC content = 34.5%.

Upload the target sequence in the Startup window of the PrimerExplorer.

Enter the target sequence file, and after confirming that “Automatic Judgement” has been selected for the parameter set, click on the “Primer Design” button. Click on the “Detail Settings” button to change to the Expert Mode (Figure not shown).

Click on the “Generate”

“AT rich” has been selected for the “Parameter Set”.

The setting includes a longer primer length and lower Tm.

Design Option  
 Default  
 Common  
 Specific

Number of Primer Candidates: F1=292, F2=348, F3=429, B1=318, B2=316, B3=395, FIP=652, BIP=593  
 1000 Primer set(s) were generated.

1. Select Range  
 Ignore range  
 Within F2-B2 Targeting Range  
 Between F1c-B1c

2. Generate  
 Generate 1000 sets were generated.

3. Display  
 Display Page 2 Displayed. Sorting Rule None

If you can move to “Basic Designing”, please click below.

Basic Designing

Parameter Condition **AT rich** Save Parameter Reset Parameter

|        |         |    |   |    |
|--------|---------|----|---|----|
| Length | F1c/B1c | 20 | - | 25 |
|        | F2/B2   | 18 | - | 25 |
|        | F3/B3   | 18 | - | 25 |
| Tm     | F1c/B1c | 60 | - | 63 |
|        | F2/B2   | 55 | - | 58 |
|        | F3/B3   | 55 | - | 58 |

Figure 2.1 Primer Design window

The GC content of the sequence was automatically calculated, and the sequence was determined to be AT rich. Thus, “AT rich” was automatically selected as the “Parameter Set.” The setting calls for a longer primer and a lower Tm value (see Figure 2.1).

Next, click on the “Generate” to design the primers. This will result in the design of 1,000 primer candidates (not illustrated). Subsequently, click on the “Display” to display the results of the primer design.

At this point, 147 primer sets have been designed from the 5’ end to the 3’ end. Additional primer sets from the 148th set have been designed again from the 5’ end to the 3’ end (see Figure 2.2).

The method described in Chapter 1 (see pp. 18-23) is then followed to compare the primer information, and to select the primer sets. It should be confirmed that at each primer region, the differences in the Tm between F1 and F2 and between B1 and B2 are about 5°C.

DesignId 160412160037

Primer set: sorting rule [None]

|                               |                                                                                                     |     |                    |                       |    |      |                      |                        |    |              |
|-------------------------------|-----------------------------------------------------------------------------------------------------|-----|--------------------|-----------------------|----|------|----------------------|------------------------|----|--------------|
| Target DNA                    | CTATTAGTAGAATTGATGCCACCTTTTCAGCTCGCGCCCCAAATGAAAATATAGCTAAACAGGTTATTGACCATTGCGGAAATGTATCTAATGGTCAAA |     |                    |                       |    |      |                      |                        |    |              |
| Complement                    | gataatcatcttaactacggtggaaaaagtcgagcgcgggtttacttttatatcgattgtccaataactggtaaacgctttacatagattaccagtttg |     |                    |                       |    |      |                      |                        |    |              |
| CONSENSUS(*)                  | *****                                                                                               |     |                    |                       |    |      |                      |                        |    |              |
| Primer ID(dimer)              | 11                                                                                                  | 21  | 31                 | 41                    | 51 | 61   | 71                   | 81                     | 91 | 101          |
| <input type="checkbox"/> [1]  | -1.83                                                                                               | [1] | TGATGCCACCTTTTCAGC | GCCCCAAATGAAAATATAGCT |    |      |                      |                        |    | accagtttg    |
| <input type="checkbox"/> [2]  | -2.32                                                                                               |     |                    |                       |    |      |                      | ATTGACCATTTC           |    | tttg         |
| <input type="checkbox"/> [3]  | -2.32                                                                                               |     |                    |                       |    |      |                      | ATTGACCATTTC           |    | ttg          |
| <input type="checkbox"/> [4]  | -2.32                                                                                               |     |                    |                       |    |      |                      | ATTGACCATTTC           |    |              |
| <input type="checkbox"/> [5]  | -1.51                                                                                               |     |                    |                       |    |      |                      | ATTGACCATTTCGA         |    |              |
| <input type="checkbox"/> [6]  | -1.51                                                                                               |     |                    |                       |    |      |                      | ATTGACCATTTCGA         |    |              |
| <input type="checkbox"/> [7]  | -1.51                                                                                               |     |                    |                       |    |      |                      | ATTGACCATTTCGAAAT      |    |              |
| <input type="checkbox"/> [8]  | -1.69                                                                                               |     |                    |                       |    |      |                      | CGAAATGTATCTAATGGTCAAA |    |              |
| <input type="checkbox"/> [9]  | -1.54                                                                                               |     |                    |                       |    | [9]  | GTTATTGACCATTTCGAAAT |                        |    | CTAATGGTCAAA |
| <input type="checkbox"/> [10] | -1.54                                                                                               |     |                    |                       |    | [10] | GTTATTGACCATTTCGAAAT |                        |    | TAATGGTCAAA  |
| <input type="checkbox"/> [11] | -1.29                                                                                               |     |                    |                       |    | [11] | GTTATTGACCATTTCGAAAT |                        |    | TAATGGTCAAA  |
| <input type="checkbox"/> [12] | -1.54                                                                                               |     |                    |                       |    | [12] | GTTATTGACCATTTCGAAAT |                        |    | AATGGTCAAA   |
| <input type="checkbox"/> [13] | -1.29                                                                                               |     |                    |                       |    | [13] | GTTATTGACCATTTCGAAAT |                        |    | AATGGTCAAA   |
| <input type="checkbox"/> [14] | -1.54                                                                                               |     |                    |                       |    | [14] | GTTATTGACCATTTCGAAAT |                        |    | ATGGTCAAA    |
| <input type="checkbox"/> [15] | -1.29                                                                                               |     |                    |                       |    | [15] | GTTATTGACCATTTCGAAAT |                        |    | ATGGTCAAA    |
| <input type="checkbox"/> [16] | -1.29                                                                                               |     |                    |                       |    | [16] | GTTATTGACCATTTCGAAAT |                        |    | TGGTCAAA     |
| <input type="checkbox"/> [17] | -1.29                                                                                               |     |                    |                       |    | [17] | GTTATTGACCATTTCGAAAT |                        |    | GGTCAAA      |
| <input type="checkbox"/> [18] | -2.46                                                                                               |     |                    |                       |    | [18] | GTTATTGACCATTTCGAAAT |                        |    | GTCAA        |
| <input type="checkbox"/> [19] | -2.46                                                                                               |     |                    |                       |    | [19] | GTTATTGACCATTTCGAAAT |                        |    | TCAA         |

147 sets of primers have been designed from the 5' end to the 3' end and additional sets from the 148th set have been designed again from the 5' end to the 3' end.

Figure 2.2 Primer Set List window

< Note >  
 For GC rich sequences, the parameter set for GC rich sequences are automatically selected and the primers are designed to cover the entire target sequence.

### **3. Changing the primer design conditions (parameter)**

#### **(Precautions in primer design)**

##### **3.1 When too many primer sets are generated**

a) Adjust the primer GC content.

When the primer GC content is 50 - 60%, favorable amplification performance will be obtained experimentally. Thus, the conditions are adjusted so that the GC content is in this range. Narrowing the permitted range for the GC content will be able to reduce the number of candidates.

b) The differences in the  $T_m$  are set to about 5°C for the primers (regions F2 and F1c, regions B2 and B1c).

In the LAMP reaction process, F1 (B1) and F1c (B1c) each self-anneal to form a loop structure, which serves as the starting structure for amplification. To facilitate forming this loop, set F1c (B1c) at a  $T_m$  value around 5°C higher than those of the other primers. When less stringent conditions (wider range of  $T_m$ 's at each primer location) are used to design the primers, primer sets are generated, which consists of the primers with various  $T_m$  value. For this reason, the difference in the  $T_m$  in each primer region may be 3°C or less. Also, best results are obtained if the  $T_m$ 's match between regions F2 and B2, regions F1c and B1c, and regions F3 and B3.

##### **3.2 When too few primer sets are generated**

If only small number of primer sets is generated for GC rich or AT rich sequences, it is plausible that the primer design conditions for the given target sequence are too stringent. In PrimerExplorer V5, the primer design conditions are automatically selected for GC rich or AT rich sequences, but for some sequences, in spite of these conditions only a few primer sets are generated. In such cases, the range of primer length or the range of  $T_m$  should be adjusted.

a) For AT rich sequences

For AT rich sequences, the  $T_m$  is calculated to be lower than non-AT rich sequences of the same length. For this reason the  $T_m$  based on the default primer length may be lower than the lower limit of default  $T_m$  value, and prevent primers from being designed. Thus, the primer length should be increased and/ or the  $T_m$  should be decreased.

b) For GC rich sequences

In contrast, for GC rich sequences, the  $T_m$  is calculated to be higher than non-GC rich sequences of the same length. For this reason the  $T_m$  calculated from the default primer length may be higher than the default  $T_m$  upper limit, and prevent primers from being designed. Thus, the primer length should be decreased and/or the  $T_m$  should be increased. Because how the  $T_m$  or the length is adjusted would be determined on a case-by-case basis, the length of each primer should be changed by one base at a time and the  $T_m$  should be changed 1°C at a time. Once a large number of primers have been generated, then stop the adjustment and select the primers.

### 3.3 Changing and storing the primer design conditions

When designing the primers, the user can change primer design conditions. The primer design conditions can be saved and revised. In this example (Figure 3.1), the Length, T<sub>m</sub>, and GC content (%) have been changed. To save the primer design conditions, click on the “Save Parameters” button. As indicated in Figure 3.2, the program will ask how the conditions should be saved. Save the primer design conditions by specifying the file name and location.

The screenshot shows the 'Primer Design' window with the following sections:

- Design Option:** Radio buttons for Default (selected), Common, and Specific.
- 1. Select Range:** Radio buttons for Ignore range (selected), Within F2-B2, and Between F1c-B1c. A 'Targeting Range' field is present.
- 2. Generate:** A green 'Generate' button and a text field showing 'sets were generated'.
- 3. Display:** A green 'Display' button, a 'Page' dropdown (set to 1), 'Displayed' text, and a 'Sorting Rule' dropdown (set to None).
- Basic Designing:** An orange button with the text 'Basic Designing'.
- Parameter Condition:** A dropdown menu set to 'AT rich'.
- Length:** A table of input fields for F1c/B1c, F2/B2, and F3/B3. The values 19, 17, and 17 are highlighted in red boxes.
- T<sub>m</sub>:** A table of input fields for F1c/B1c, F2/B2, and F3/B3. The values 63, 58, and 58 are highlighted in red boxes.
- GC rate(%):** Input fields for 50 and 60, both highlighted in red boxes.
- Buttons:** Green 'Save Parameter' and 'Reset Parameter' buttons.

Annotations:

- A yellow box on the right points to the 'Save Parameter' button with the text: 'Click on the "Save Parameters" button.'
- A white box on the left points to the red-highlighted input fields with the text: 'Red indicated the Length, T<sub>m</sub>, and GC content (%) that were changed.'

Figure 3.1 Changing the primer design conditions (Primer Design window)

### 3.4 Using the saved primer design conditions for the primer designing

Upload the target sequence in the startup window of the PrimerExplorer. Next, check on “User Assignment” in the parameter set and click on the “Browse” button to select the parameter file containing the primer design conditions.

Click on the “Primer Design” button.

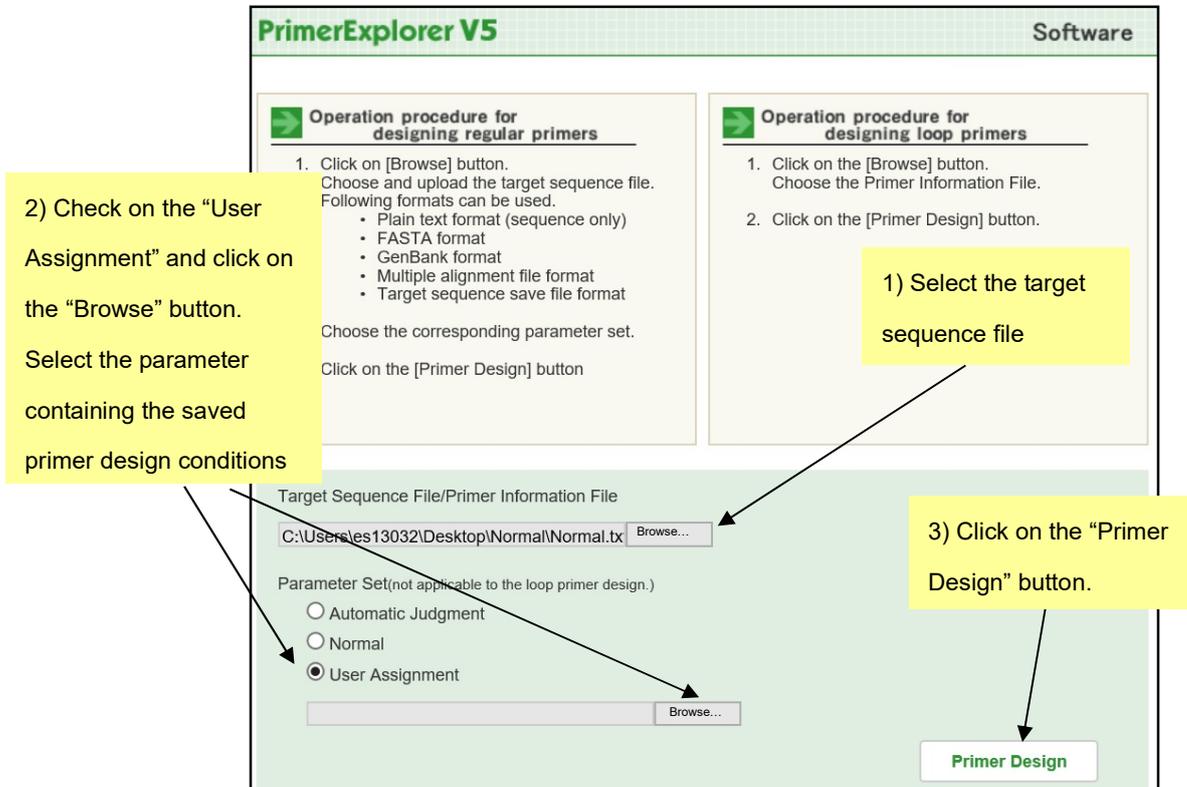
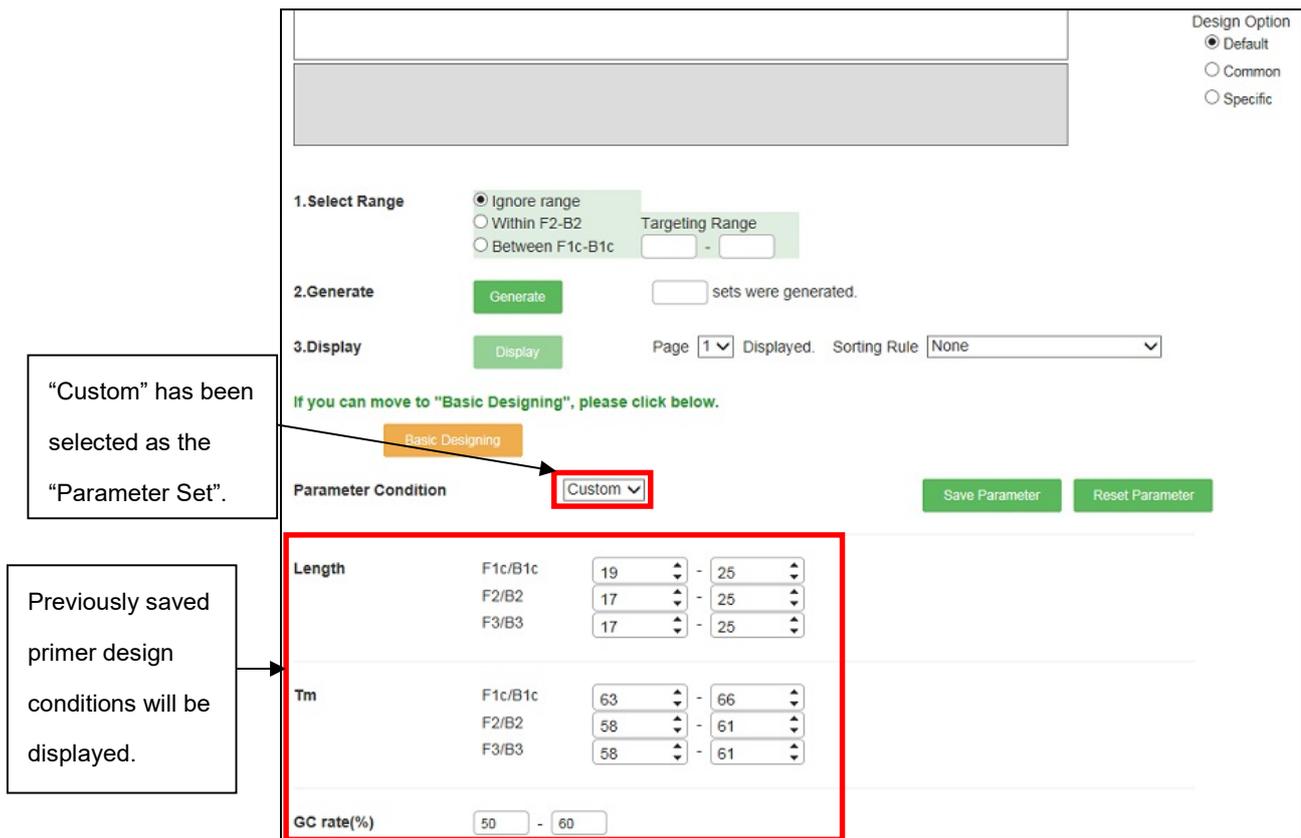


Figure 3.2 PrimerExplorer Ver. 5 startup window

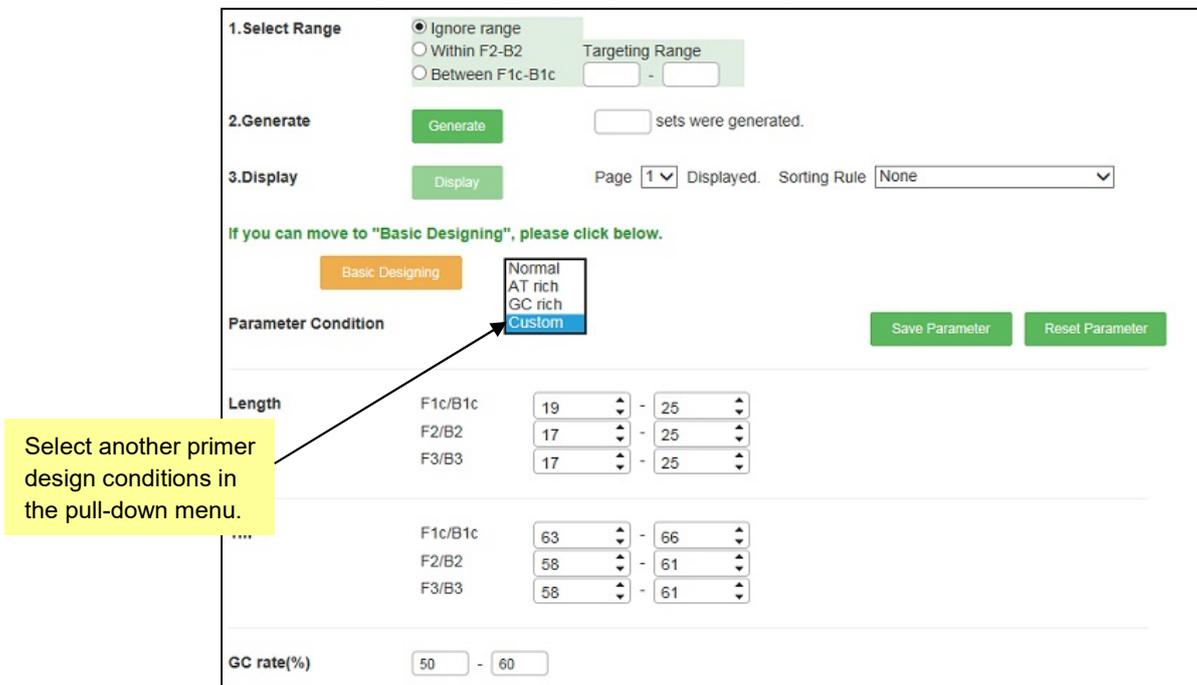
The primer design window (Figure 3.4) will display the previously saved (Figure 3.2) primer design conditions. Here, the “Parameter Set” is displayed as “Custom.”

Next, click on the “Generate” button to design the primers. The primers are selected using the procedures described in Section 1 (see p.13 – 18).



**Figure 3.4 Primer design window**

Even if a "User specified" parameter of "Custom" has been selected, it is possible to switch to other primer design conditions (Normal, AT rich, GC rich). To do this, select another desired primer design conditions in the pull-down menu in "Parameter Set" prior to designing the primers. (See Figure 3.5)



**Figure 3.5 Changing the parameters**

## 4. Designing primers with specified primer locations

### 4.1 Specifying the primer locations in the target sequence

Primer can be designed for a specified primer location if the region is known to be easily amplified by PCR, or if the region to be amplified is pre-determined, or if it is desired to use the primers or primer locations used in PCR.

As in Figure 4.1, specify the primer location by clicking on the “primer location” button. The Figure shows that the “F2” button is clicked, and as in Figure 4.2, the region specified as the location F2 will be displayed.

1) Specify the primer location.

2) Click on this button to specify the location F2

Figure 4.1 Primer design window

1) To change the specified region, specify a new location F2.

2) Click on the button to re-specify the location F2.

Display of the specified primer location

Figure 4.2 Window after specifying the primer location

To change the location F2 to some other location, specify another location as shown in Figure 4.2, and click on the “F2” button again. As shown in Figure 4.3, the new location is now specified as the location F2.

The locations can be changed as above. To delete the information at this primer location, click on the “Clear” button to delete.

The previously specified location is deleted  
and the newly specified location is displayed.

The screenshot shows a primer design window with a DNA sequence on the left and a control panel on the right. The DNA sequence is displayed in 80-base increments, with a red box highlighting a primer location at approximately position 200. The control panel on the right includes a 'Set Mutation' section with 'Mut/Cons' and 'Clear' buttons, a 'Fixed Primer' section with buttons for 'F3', 'F2', 'F1', 'B1', 'B2', 'B3', and 'Clear', and a 'Save Target' button at the bottom. A yellow callout box with an arrow points to the 'Clear' button in the 'Fixed Primer' section, containing the text: 'Click on this button to delete the information on this primer location.'

**Figure 4.3 Primer design window**

#### **4.2 Specify the primer location to be designated for primer design**

Now we design primers in which the primer location has been pre-specified. Here, as indicated in Figure 4.4, the location F3 has been pre-specified prior to the primer design. Specify the primer location by clicking on the “F3” button, and once the specified location has been displayed, then click on the “Generate” button to design the primers. (See Figures 4.4, 4.5)

1) Specify the primer location.

2) Click on this button to specify the location F3

Click on the "Generate" button.

Click on the "Display" button.

1. Select Range  
 Ignore range  
 Within F2-B2 Targeting Range 208 - 227  
 Between F1c-B1c

2. Generate  
 116 sets were generated.

3. Display  
 Page 1 of 1 Displayed. Sorting Rule: None

Figure 4.4 Primer design window

Primer sets with the F3 specified have been designed.

Confirm Save List

Primer set: sorting rule [None]

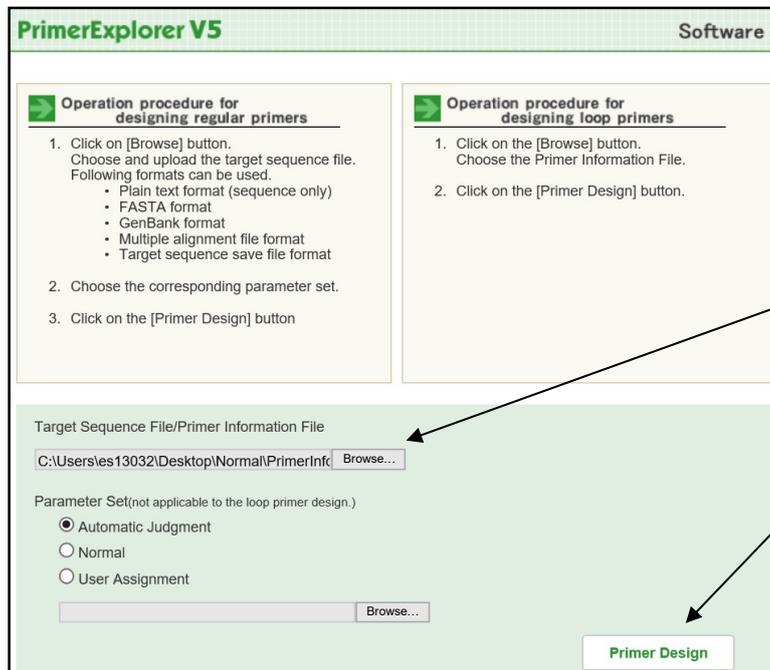
| Target DNA (Complement) CONSENSUS(*) | TCAACGGCCTCAACTACTACTGCGCTGCTTCCTAATGCAGGAGTCGCATAAGGGAGAGCGTCGACCGATGCCCTT |      |                      |                      |           |     |     |     |  |  |  |  |
|--------------------------------------|-----------------------------------------------------------------------------|------|----------------------|----------------------|-----------|-----|-----|-----|--|--|--|--|
| Fixed Primer                         | agttgccggagttgatgatgaccgcagcaaggattacgtcctcagcgtattcctctctgcagctggctacgggaa |      |                      |                      |           |     |     |     |  |  |  |  |
| Primer ID(dimer)                     | 191                                                                         | 201  | 211                  | 221                  | 231       | 241 | 251 | 261 |  |  |  |  |
| <input type="checkbox"/> [1]         | -1.63                                                                       | [1]  | CTACTGGGCTGCTTCCTAAT | GCAGGAGTCGCATA       | AAGGGA    |     |     |     |  |  |  |  |
| <input type="checkbox"/> [2]         | -2.46                                                                       | [2]  | CTACTGGGCTGCTTCCTAAT | CAGGAGTCGCATA        | AAGGGAGA  |     |     |     |  |  |  |  |
| <input type="checkbox"/> [3]         | -2.15                                                                       | [3]  | CTACTGGGCTGCTTCCTAAT | AGGAGTCGCATA         | AAGGGAGAG |     |     |     |  |  |  |  |
| <input type="checkbox"/> [4]         | -2.13                                                                       | [4]  | CTACTGGGCTGCTTCCTAAT | TCGCATAAAGGGAGAGCGT  |           |     |     |     |  |  |  |  |
| <input type="checkbox"/> [5]         | -2.45                                                                       | [5]  | CTACTGGGCTGCTTCCTAAT | TCGCATAAAGGGAGAGCGTC |           |     |     |     |  |  |  |  |
| <input type="checkbox"/> [6]         | -2.45                                                                       | [6]  | CTACTGGGCTGCTTCCTAAT | CGCATAAAGGGAGAGCGTC  |           |     |     |     |  |  |  |  |
| <input type="checkbox"/> [7]         | -1.06                                                                       | [7]  | CTACTGGGCTGCTTCCTAAT | GCAGGAGTCGCATA       | AAGGGA    |     |     |     |  |  |  |  |
| <input type="checkbox"/> [8]         | -2.46                                                                       | [8]  | CTACTGGGCTGCTTCCTAAT | CAGGAGTCGCATA        | AAGGGAGA  |     |     |     |  |  |  |  |
| <input type="checkbox"/> [9]         | -2.41                                                                       | [9]  | CTACTGGGCTGCTTCCTAAT | AGGAGTCGCATA         | AAGGGAGAG |     |     |     |  |  |  |  |
| <input type="checkbox"/> [10]        | -2.41                                                                       | [10] | CTACTGGGCTGCTTCCTAAT | TCGCATAAAGGGAGAGCGT  |           |     |     |     |  |  |  |  |
| <input type="checkbox"/> [11]        | -2.45                                                                       | [11] | CTACTGGGCTGCTTCCTAAT | TCGCATAAAGGGAGAGCGTC |           |     |     |     |  |  |  |  |
| <input type="checkbox"/> [12]        | -2.45                                                                       | [12] | CTACTGGGCTGCTTCCTAAT | CGCATAAAGGGAGAGCGTC  |           |     |     |     |  |  |  |  |

Figure 4.5 Primer Set List window

## 5. Loop primer design

### 5.1 Uploading the primer information file

Return to the PrimerExplorer startup window and re-load the previously saved “primer information file”. Click on the “Browse” button to select the file, and then click on the “Primer Design” button. (See Figure 5.1)



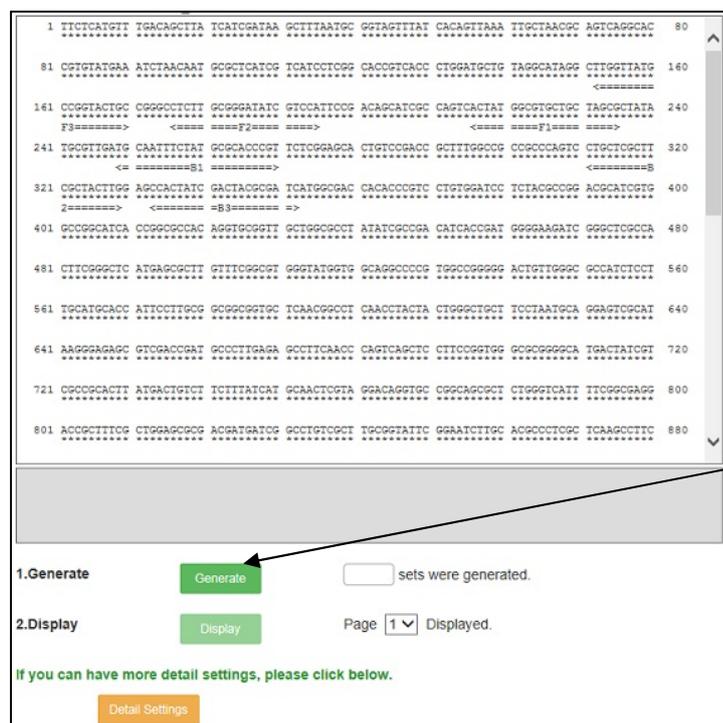
1) Select a primer information file.

2) Click on the “Primer Design” button.

Figure 5.1 PrimerExplorer V5 startup window

### 5.2 Designing loop primers

After uploading the primer data file, the loop primer design window will be displayed as shown in Figure 5.2 on the next page. Keep the parameters as default and click on the “Generate” button.



Click on the “Generate” button without changing the parameters.

Figure 5.2 Loop primer design window

A total of 24 sets of primer will be generated. Click on the “Display” button to display the Primer Set List (See Figure 5.3)

2) Click on the “Confirm” button.

1) Check the boxes.

Saved primer information locations

Confirm Save List DesignId 160413160245

Primer set

Target DNA  
 Complement  
 CONSENSUS(\*)

Primer IDdG(dimer) 141 151 161 171 181 191 201 211 221 231

[1] -2.95

Forward-side loop primer

ggctgtcgtagcgggtca

DesignId 160413160245

Backward-side loop primer

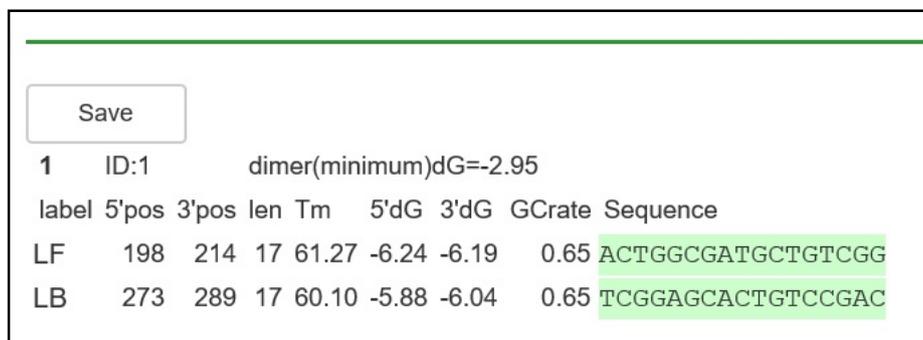
TGCAATTTCTATGCGCACCCGT  
 TCGGAGCACTGTCCGAC [1]

**Figure 5.3 Loop primer design window (after primer design)**

Figure 5.3 shows the results as a Primer Set List. At the top is the location of the saved primer information, underneath is the target sequence, and at the bottom are the loop primers. To examine the detailed information regarding these loop primer sets, check the boxes to the left of primer sets, then click on the “Confirm” to open the Primer Set Details window.

### 5.3 Detailed information on the loop primer sets

The Primer Set Details window (Figure 5.4) shows the detailed information regarding the loop primer sets previously selected.



The screenshot shows a window titled "Primer Set Details" with a "Save" button at the top left. Below the button, the following information is displayed:

| label | 5'pos | 3'pos | len | Tm    | 5'dG                   | 3'dG  | GCrate | Sequence           |
|-------|-------|-------|-----|-------|------------------------|-------|--------|--------------------|
| 1     | ID:1  |       |     |       | dimer(minimum)dG=-2.95 |       |        |                    |
| LF    | 198   | 214   | 17  | 61.27 | -6.24                  | -6.19 | 0.65   | ACTGGCGATGCTGTCCGG |
| LB    | 273   | 289   | 17  | 60.10 | -5.88                  | -6.04 | 0.65   | TCGGAGCACTGTCCGAC  |

Figure 5.4 Primer Set Details window

After more than one loop primer set has been designed, follow the method described in Chapter 1 (see pp. 17-27) to select a primer set.