

# **How to use PrimerExplorer V4**



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

## 1.1 Uploading the target sequence

The target sequence is uploaded in the PrimerExplorer V4 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 kbp. 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.

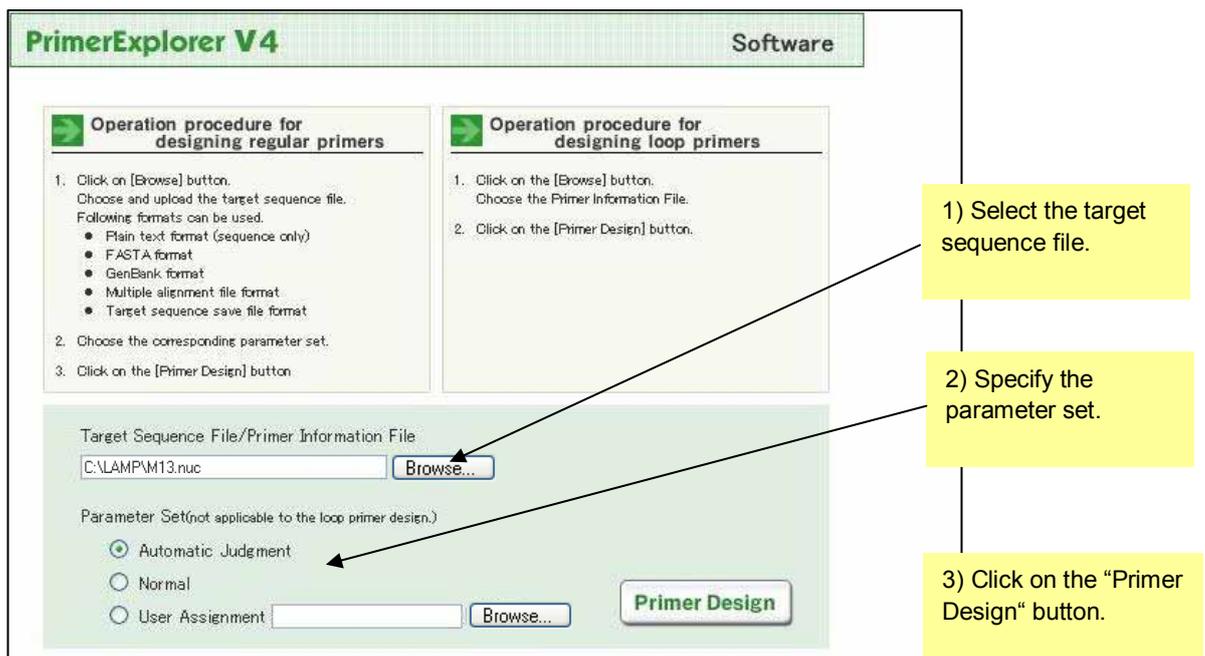


Figure1.1 PrimerExplorer V3 startup window

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 : C:\LAMP\M13.nuc

```

1 ACTAATCAAA GAGTATTGC TACAACGTT AATTGCGTG ATGGACAGC TCTTTTACT GGTGGCTCA CTGATTATAA 80
*****
81 AAACACTTCT CARGATTCTG GCGTACGTT CCTGTCTAAA ATCCCTTTAA TCGGCTCCT GTTTAGCTCC CGCTCTGATT 160
*****
161 CCAACGAGGA AAGCACGTTA TACGTGCTCG TCAAAGCAAC CATAGTACGC GCCCTGTAGC GCGGCATTAA GCGCGCGGG 240
*****
241 TGTGGTGGT ACGGCGAGC TGACCGCTAC ACTTGCCAGC GCCCTAGCG CCGCTCCTT CCGTTCTTC CCTTCCTTC 320
*****
321 TCGCCAGTT CGCCGCTTT CCCCGTCAAG CTCTAATCG GGGGCTCCCT TTAGGGTCC GATTTAGTGC TTTACGGCAC 400
*****
401 CTCGACCCA AAAAAGTTGA TTTGGGTGAT GGTTCACGTA GTGGCCATC GCCCTGATAG ACGGTTTTTC GCCCTTGAC 480
*****
481 GTTGGAGTCC ACGTTCTTTA ATAGTGGACT CTTGTTCCAA ACTGGAACAA CACTCAACCC TATCTCGGGC TATTCTTTTG 560
*****
561 ATTTATAGG GATTTTGCCG ATTTGGAAC CACCATCAA CAGGATTTTC GCTGCTGGG GCAACACAGC GTGGACCGCT 640
*****
641 TGCTCAACT CTCTCAGGC CAGGCGTGA AAGGCAATCA GCTGTTGCC GTCTGCTGG TGAARAAGAA ACCCACCCTG 720
*****

```

Number of Primer Candidates: F1=131, F2=187, F3=398, B1=215, B2=189, B3=393, F1P=209, B1P=243  
5 Primer set(s) were generated.

1. Generate  5 sets were generated.

2. Display

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

Set Mutation

Fixed Primer

Save Target

Design Option  
 Default  
 Common  
 Specific

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 is then shown in a separate window, and you can save that list in Excel format by clicking on the “Save List” button. Here, check the boxes for the primer sets you want to confirm in the left side of the window, and then click on the “Confirm” button. Another window then appears with detailed primer information of the checked sets (Figure 1.4). In this window, click on the “Primer Information” button for each primer set to save the information for that primer. The information should be used to design loop primer.



1. Push "Order" button in order to transfer to a Genome ORDER site.  
(Colored primers will be ordered.)
2. Push "Primer Information" button to download Primer Information format file.

Order

Design# 07011814002

**Primer Information**

1 ID:6 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 ACTACTGGCTGCTTCT  
 B3 784 802 19 60.31 -5.20 -4.90 0.58 GTCTGGCCAMATGAC  
 FIP 41 AGCTGACTGGTTGAAGCTCT-CCAGGAGTGCATAAGGA  
 BIP 40 CATGACTATGCTGCCCGACT-CACCTGTCTACGAGTTGC  
 F2 628 646 19 60.88 -6.10 -5.20 0.59 CCAGGAGTGCATAAGGA  
 F1c 668 689 22 66.68 -5.49 -5.93 0.55 AGCTGACTGGTTGAAGCTCT  
 B2 751 769 19 59.31 -5.50 -5.40 0.59 CACCTGTCTACGAGTTGC  
 B1c 709 729 21 64.31 -4.56 -6.57 0.57 CATGACTATGCTGCCCGACT

**Primer Information**

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 CGGACTGACACACACA  
 B3 1769 1706 19 59.00 -5.00 -5.75 0.56 AACTGGCTATGATGC  
 FIP 41 ACATAATGGTCCAGGGGCTG-TGAATGCTCTGGGTTTCG  
 BIP 40 CGGAGATGCTGCTGGCTAC-AATGACTCAGGGTAAATGAC  
 F2 1594 1613 20 59.76 -4.07 -5.30 0.50 TGAATGCTCTGGGTTTCG  
 F1c 1643 1663 21 65.39 -3.29 -7.42 0.57 ACATAATGGTCCAGGGGCTG  
 B2 1733 1752 20 59.64 -4.06 -5.40 0.50 AATGACTCAGGGTAAATGAC  
 B1c 1677 1696 20 65.39 -7.02 -5.42 0.65 CGGAGATGCTGCTGGCTAC

**Primer Information**

3 ID:6 dimer (minimum)dG=-2.23  
 label 5'pos 3'pos len Tm 5'dG 3'dG GCrate Sequence  
 F3 120 145 18 60.55 -5.94 -5.42 0.61 ACCCTGATCTCTTAGGC  
 B3 317 334 19 59.61 -6.54 -6.03 0.61 GCTCCAGTACGGAAC  
 FIP 40 GTGACTGGGATGCTGTGG-GCTTGGTATGCGCGTACTG  
 BIP 39 TATGGCTGCTCTAGGCTA-CAMAGCGTCCGACACTG  
 F2 150 169 20 60.49 -5.85 -4.23 0.55 GCTTGGTATGCGCGTACTG  
 F1c 198 217 20 65.19 -4.90 -6.19 0.65 GTGACTGGGATGCTGTGG  
 B2 270 296 18 60.06 -5.01 -5.05 0.61 CAMAGCGTCCGACACTG  
 B1c 210 238 21 65.98 -4.98 -6.50 0.57 TATGGCTGCTCTAGGCTA

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.

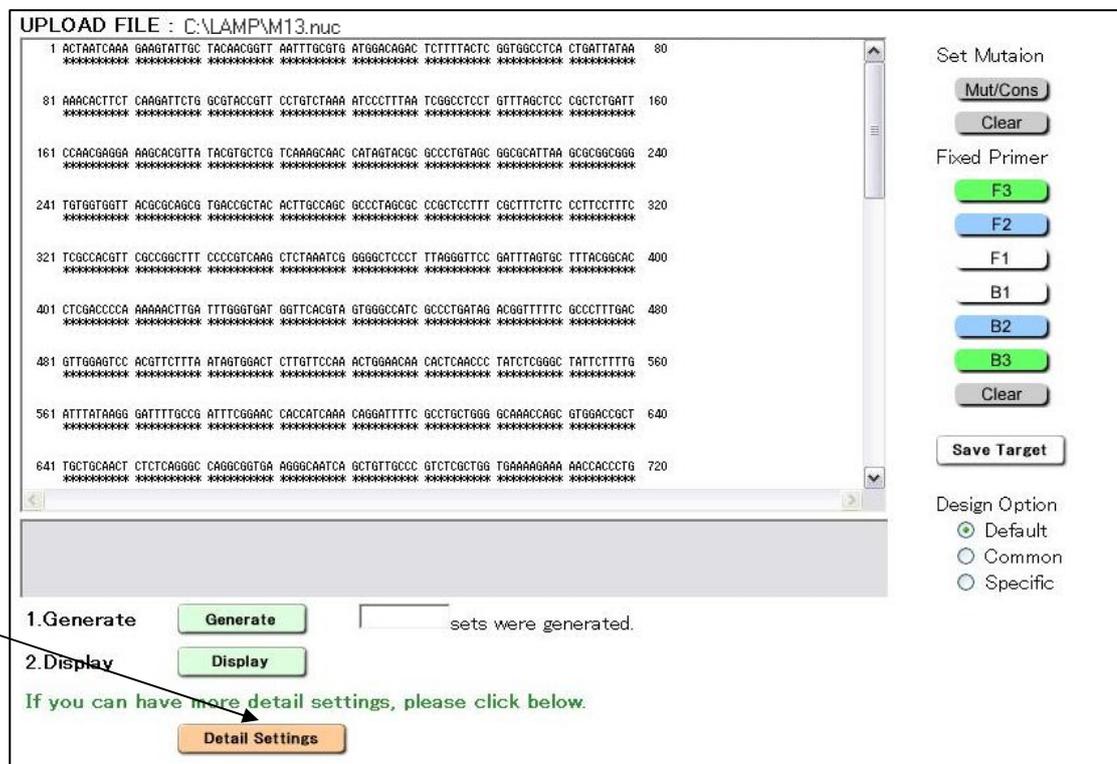


Figure 1.5 Primer design window

UPLOAD FILE :C:\LAMP\M13.nuc

```

1 ACTATCAGA GAGGATTCG TACAGCGTT AMTTTCGTA ATGAGCAGC TCTTTACTC GTFAGCTCA CTGATTATA 80
81 AAGACCTCT CAGGATTTG GGTACCGTT CTTCTTAAA ACCCTTTAA TGGGCTCTT GTTAGCTCC CACTCGATT 160
161 CCGACGAGA AAGCGCTTA TACTCTGTC TTAGAGCAG CATAGTCCG GCGCTTAGC GCGCATTAAG GCGGCGGAG 240
241 TGTGATGTT AGCGCAGG TACCGTAC ACTTGCAGC GCGCTAGAC CCGCTCTTT CACTTTCTTC CACTCTTTC 320
321 TGGCAGGTT CCGGCGCTT CCGCTCAG CTTAATATG GGGGCTCCT TTAGGTTCC GATTATAGC TTTGCGGAC 400
401 CTGACCGCA AAGACCTTA TTGGGTGAT GTTTCAGTA GTGGGATGC GCGCTATAG AGGTTTTC GCGCTTAGC 480
481 GTTGAATCC ACATCTTAA ATAGTGAAT CTTCTTCAA ACTGAGCAA CACTGACCC TATCTGAGC TATCTTTTG 560
561 ATTATGAGG GATTTTCGA GTTTCGAGC CCGCATGAA CAGATTTTC GCTCTTAGG GCGAGCGAC GTGAGCGCT 640
641 TCTGCACT CTTGAGG GCGGCTGA AAGCATCA GCTGTCCG GCTGTGCG TGAAGAAA AGCCACCTG 720

```

Number of Primer Candidates: F1=191, F2=167, F3=206, B1=215, B2=185, B3=293, F1P=205, B1P=243  
1000 Primer set(s) were generated.

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

2. Generate  
 1000 sets were generated.

3. Display  
 Page 1 Displayed. Sorting Rule None

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

Parameter Condition  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 [Kcal/mol]  
5'stability -3  
3'stability -4  
dimer check -2.5

Distances  
(F2-B2) 120 - 160  
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

Pecularity	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"/>
↓ low level	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"/>

Reset Parameter

Click on the "Generate" button.

Click on the "Display" button.

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

Figure 1.6 Expert mode

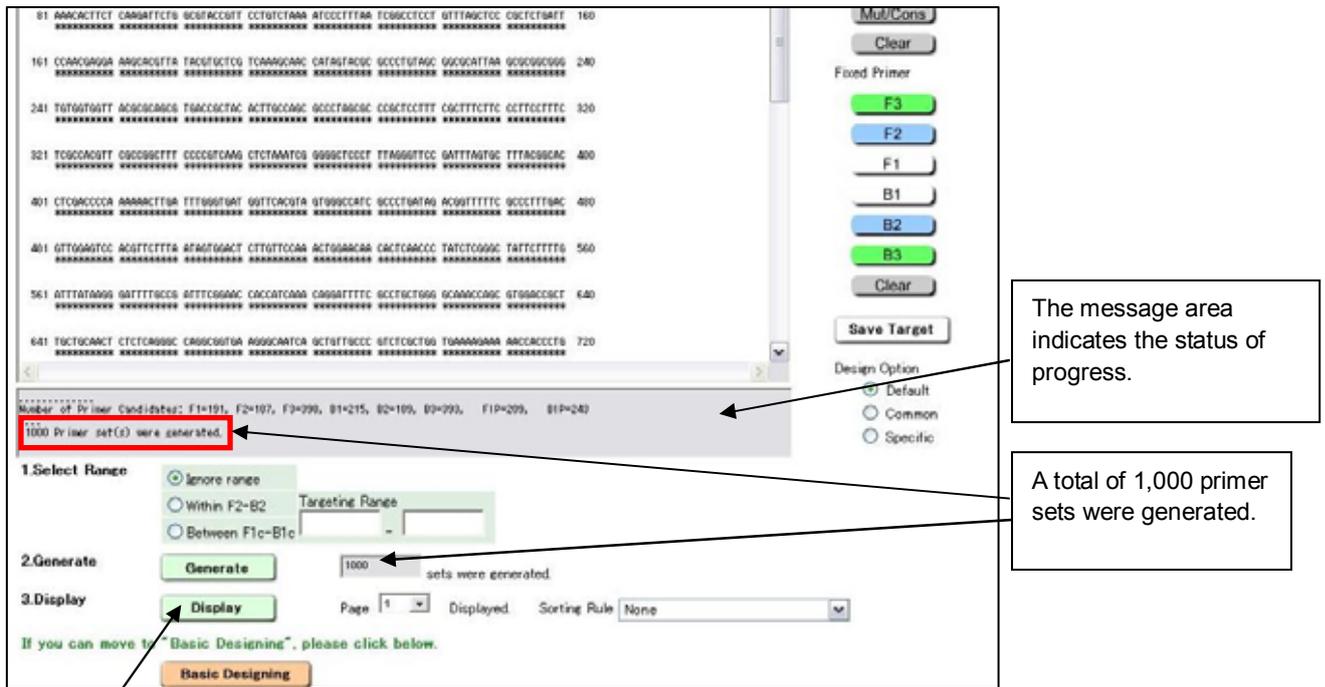


Figure 1.7 Primer design window

#### 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 1439 bp). (See Figure 1.5b) Several primers are then selected to compare the specific conditions.



Primer Set	Gene	Accession	Position	Orientation	Sequence	GC Content	Length
1	...	...	...	...	...	...	...
2	...	...	...	...	...	...	...
3	...	...	...	...	...	...	...
4	...	...	...	...	...	...	...
5	...	...	...	...	...	...	...
6	...	...	...	...	...	...	...
7	...	...	...	...	...	...	...
8	...	...	...	...	...	...	...
9	...	...	...	...	...	...	...
10	...	...	...	...	...	...	...
11	...	...	...	...	...	...	...
12	...	...	...	...	...	...	...
13	...	...	...	...	...	...	...
14	...	...	...	...	...	...	...
15	...	...	...	...	...	...	...
16	...	...	...	...	...	...	...
17	...	...	...	...	...	...	...
18	...	...	...	...	...	...	...
19	...	...	...	...	...	...	...
20	...	...	...	...	...	...	...
21	...	...	...	...	...	...	...
22	...	...	...	...	...	...	...
23	...	...	...	...	...	...	...
24	...	...	...	...	...	...	...
25	...	...	...	...	...	...	...
26	...	...	...	...	...	...	...
27	...	...	...	...	...	...	...
28	...	...	...	...	...	...	...
29	...	...	...	...	...	...	...
30	...	...	...	...	...	...	...
31	...	...	...	...	...	...	...
32	...	...	...	...	...	...	...
33	...	...	...	...	...	...	...
34	...	...	...	...	...	...	...
35	...	...	...	...	...	...	...
36	...	...	...	...	...	...	...
37	...	...	...	...	...	...	...
38	...	...	...	...	...	...	...
39	...	...	...	...	...	...	...
40	...	...	...	...	...	...	...
41	...	...	...	...	...	...	...
42	...	...	...	...	...	...	...
43	...	...	...	...	...	...	...
44	...	...	...	...	...	...	...
45	...	...	...	...	...	...	...
46	...	...	...	...	...	...	...
47	...	...	...	...	...	...	...
48	...	...	...	...	...	...	...
49	...	...	...	...	...	...	...
50	...	...	...	...	...	...	...
51	...	...	...	...	...	...	...
52	...	...	...	...	...	...	...
53	...	...	...	...	...	...	...
54	...	...	...	...	...	...	...
55	...	...	...	...	...	...	...
56	...	...	...	...	...	...	...
57	...	...	...	...	...	...	...
58	...	...	...	...	...	...	...
59	...	...	...	...	...	...	...
60	...	...	...	...	...	...	...
61	...	...	...	...	...	...	...
62	...	...	...	...	...	...	...
63	...	...	...	...	...	...	...
64	...	...	...	...	...	...	...
65	...	...	...	...	...	...	...
66	...	...	...	...	...	...	...
67	...	...	...	...	...	...	...
68	...	...	...	...	...	...	...
69	...	...	...	...	...	...	...
70	...	...	...	...	...	...	...
71	...	...	...	...	...	...	...
72	...	...	...	...	...	...	...
73	...	...	...	...	...	...	...
74	...	...	...	...	...	...	...
75	...	...	...	...	...	...	...
76	...	...	...	...	...	...	...
77	...	...	...	...	...	...	...
78	...	...	...	...	...	...	...
79	...	...	...	...	...	...	...
80	...	...	...	...	...	...	...
81	...	...	...	...	...	...	...
82	...	...	...	...	...	...	...
83	...	...	...	...	...	...	...
84	...	...	...	...	...	...	...
85	...	...	...	...	...	...	...
86	...	...	...	...	...	...	...
87	...	...	...	...	...	...	...
88	...	...	...	...	...	...	...
89	...	...	...	...	...	...	...
90	...	...	...	...	...	...	...
91	...	...	...	...	...	...	...
92	...	...	...	...	...	...	...
93	...	...	...	...	...	...	...
94	...	...	...	...	...	...	...
95	...	...	...	...	...	...	...
96	...	...	...	...	...	...	...
97	...	...	...	...	...	...	...
98	...	...	...	...	...	...	...
99	...	...	...	...	...	...	...
100	...	...	...	...	...	...	...

Figure 1.9 Primer Set List (full view)

## 1.5 Primer set selection

10-15 primer sets that amplify different regions in the target sequence are selected, and detail information on the sets is compared to select the appropriate primer sets. If the region to be amplified is pre-determined, then the primer sets that amplify that region are selected.

Here, we assume that “any region in the target sequence can be amplified.”

In the Primer Set List window (Figure 1.8a), primer sets are chosen that encompasses the entire length if possible. As an example, the ten sets, with the ID numbers 1, 5, 8, 12, 14, 15, 24, 30, 37 and 40, are selected. Check the boxes located to the left of each primer set and click on the “Details” button to open the Primer Set Details window.

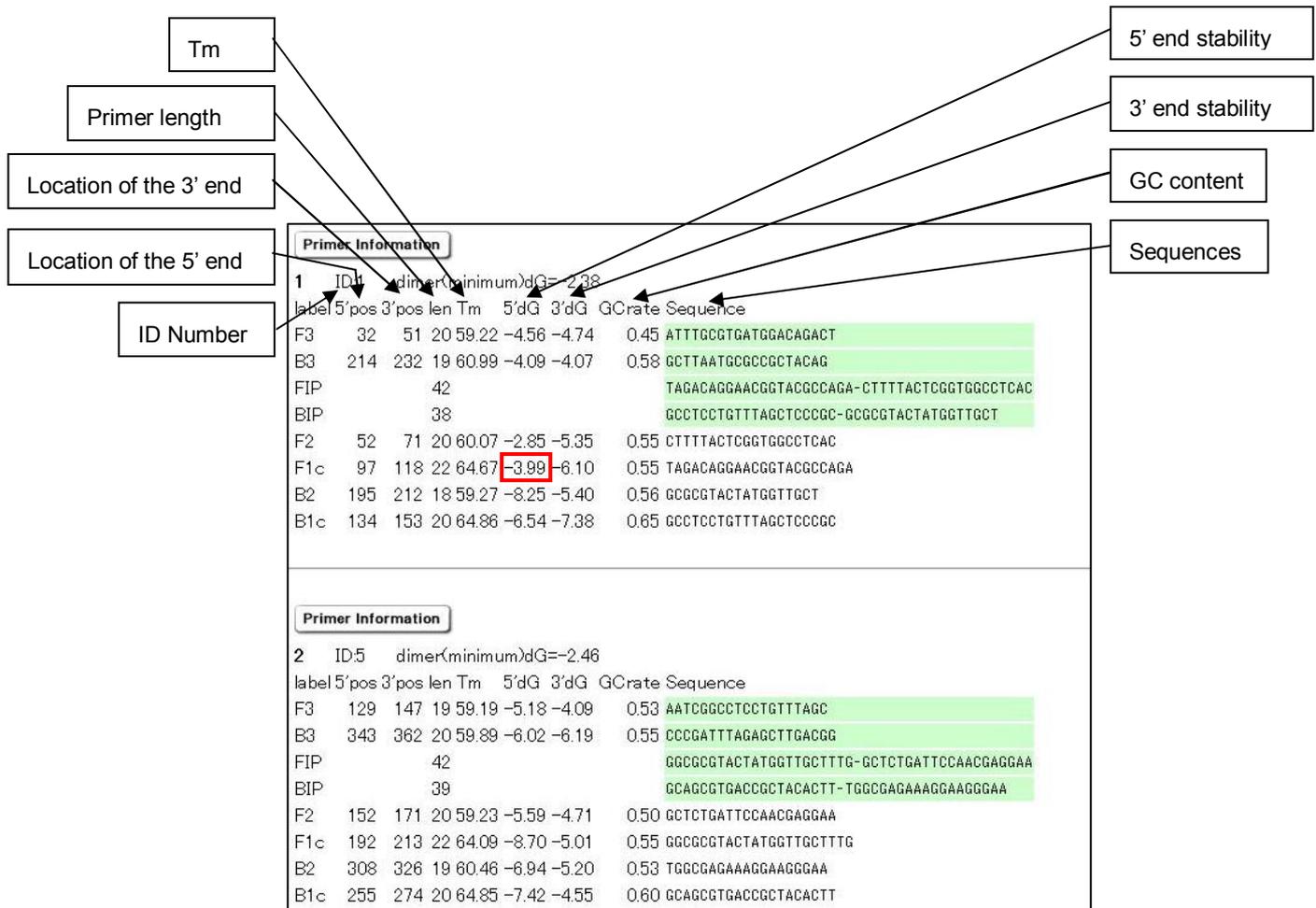


Figure 1.10 Primer Set Details window

In the window shown in Figure 1.10, check the stability of the 3' end at the region F2, 5' end at the region F1c, 3' end at the region B2, and the 5' end at the region B1c. As these are the starting positions of gene replication 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.

In the examples, in the set with ID number 1, the stability of the 5' end of F1c is  $-3.99$ , so this is eliminated because the end stability is inadequate. For the remaining sets, any set can be selected, but if possible, primer sets with the higher end stability should be selected. Here, the ID numbers 5, 8, 12, 30 and 40 were selected.

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. This will be explained further in the section on loop primer design; click on the “Primer Information” button to save the primer information. Follow the instructions in the window to specify the file name and location, and save the “primer information file.” (See Figure 1.12)

This is the end of regular primer design.

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

The screenshot shows two sections of primer information. The first section is for ID:1 with a dimer (minimum)dG of -2.38. It lists primers F3, B3, FIP, BIP, F2, F1c, B2, and B1c with their respective positions, lengths, Tm values, and GC rates. The second section is for ID:5 with a dimer (minimum)dG of -2.46, listing primers F3, B3, FIP, BIP, F2, and F1c with their respective parameters. In both sections, the primer sequences are highlighted in green.

Figure 1.11 Selected Primer Set window

The screenshot shows the same primer information window as Figure 1.11, but with a “File Download - Security Warning” dialog box overlaid. The dialog box asks, “Do you want to save this file?” and provides the following details: Name: PrimerInfo5143d8b2, Type: Unknown File Type, and From: primerexplorer.jp. There are “Save” and “Cancel” buttons. At the bottom of the dialog box, there is a warning icon and text: “While files from the Internet can be useful, this file type can potentially harm your computer. If you do not trust the source, do not save this software. What's the risk?”

Figure 1.12 Primer Information Save window

## 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 V3.

Enter the target sequence file, and after confirming that “Automatic Judgment” has been selected for the parameter set, click on the “Primer Design” button. (Figure not shown)

Click on the “Generate” button.

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

The setting includes a longer primer length and lower Tm.

Number of Primer Candidates: F1=694, F2=706, F3=1131, B1=675, B2=684, B3=1082, F1P=1423, B1P=1308  
1000 Primer set(s) were generated.

**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: **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

GC rate(%) 30 - 65

dG threshold [Kcal/mol]

5'stability	-3
3'stability	-4

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 (see Figure 2.1).

Next, click on the “Generate” button to design the primers. This will result in the generation of 1,000 primer candidates. (Figure not shown.)

Next, click on the “Display” button to display the results of the primer design. 170 sets of primers have been designed from the 5’ end to the 3’ end, and additional sets from the 171<sup>st</sup> set have been designed again from the 5’ end to the 3’ end. (See Figure 2.2)

The method described in Section 1 is then followed (See p.13 - 18) 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.

Confirm Save List DesignId 070606183331

Primer set: sorting rule [None]

Target DNA CTATTAGTAGAATTGATGCCACCTTTTCAGCTCGCGCCCCAAATGAAAATATAGCTAAACAGGTTATTGACCATTTGCGAAA  
 (Complement) gataatcatcttaactacggtgaaaagtcgagcggggtttacttttatatcgattgtccaataactggtaaacgcttt  
 CONSENSUS(\*) \*\*\*\*\*

Primer ID	dG(dimer)	11	21	31	41	51	61	71	81	91
[1]	-1.83	[1]	TGATGCC	ACCTTTTCAGC		GCCCCAAATGAAAAT	ATAGCT			
[2]	-2.32			[2]		GCCCCAAATGAAAAT	ATAGCTAAAC	AGGTTATTGACC	ATTTGCG	AAA
[3]	-2.32			[3]		GCCCCAAATGAAAAT	ATAGCTAAC	AGGTTATTGACC	ATTTGCG	AAA
[4]	-2.32			[4]		GCCCCAAATGAAAAT	ATAGCTAC	AGGTTATTGACC	ATTTGCG	AAA
[5]	-1.51			[5]		GCCCCAAATGAAAAT	ATAGCT	AGGTTATTGACC	ATTTGCGA	AAA
[6]	-1.51			[6]		GCCCCAAATGAAAAT	ATAGCT	GGTTATTGACC	ATTTGCGA	AAA
[7]	-1.69			[7]		CCAAATGAAAAT	ATAGCTAAAC	AGG		CGAAA
[8]	-2.46							[8]	GGTTATTGACC	ATTTGCGAAA
[9]	-2.46							[9]	GGTTATTGACC	ATTTGCGAAA
[10]	-1.11							[10]	AGGTTATTGACC	ATTTGCG
[11]	-1.11							[11]	AGGTTATTGACC	ATTTGCG
[12]	-2.46							[12]	GGTTATTGACC	ATTTGCGAAA
[13]	-2.46							[13]	GGTTATTGACC	ATTTGCGAAA
[14]	-2.46							[14]	GGTTATTGACC	ATTTGCGAAA
[15]	-2.46							[15]	GGTTATTGACC	ATTTGCGAAA
[16]								[16]		CGAAA
[17]										
[18]										
[19]	-2.32									

170 sets of primers have been designed from the 5' end to the 3' end and additional sets from the 171<sup>st</sup> 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)**

#### **1.1 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 V4, 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.

Option  
 Default  
 Common  
 Specific

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

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 AT rich Save Parameter Reset Parameter

Parameter	Value	Range
Length	F1c/B1c	19 - 25
	F2/B2	17 - 25
	F3/B3	17 - 25
T <sub>m</sub>	F1c/B1c	63 - 66
	F2/B2	58 - 61
	F3/B3	58 - 61
GC rate(%)	50	60
dG threshold		-3
5' stability		

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

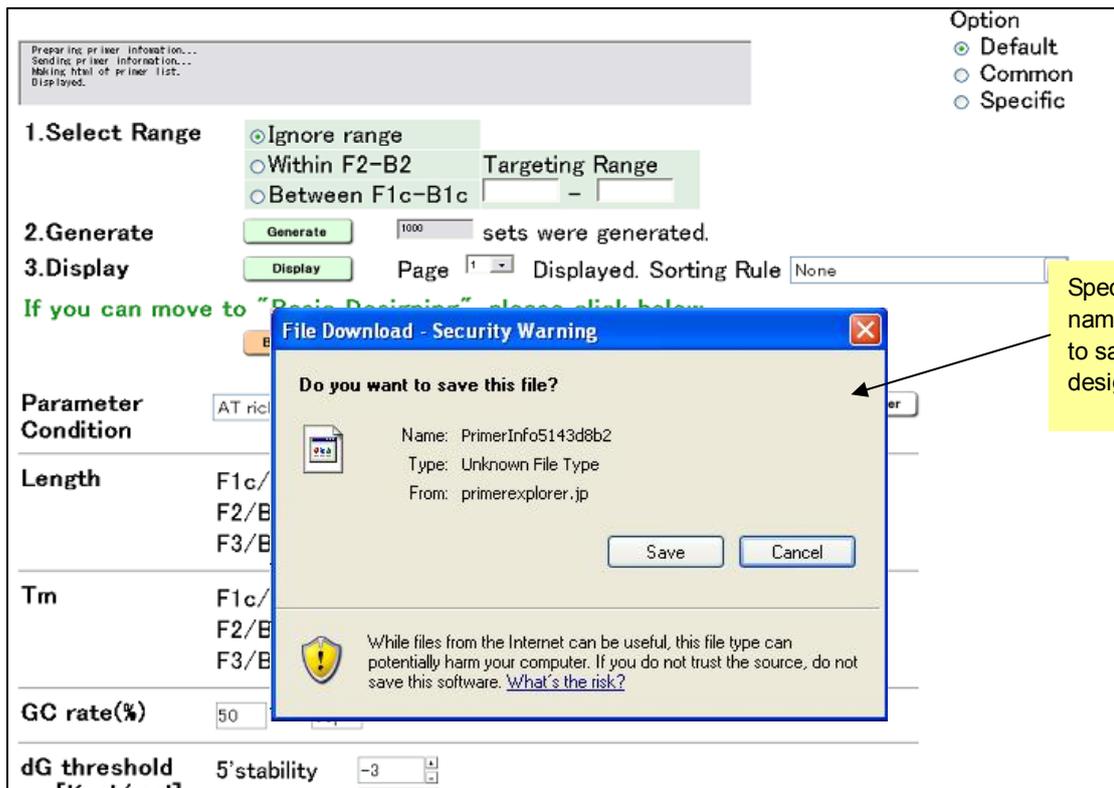


Figure 3.2 Saving the primer design conditions

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

Upload the target sequence in the startup window of the PrimerExplorer V4. 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.

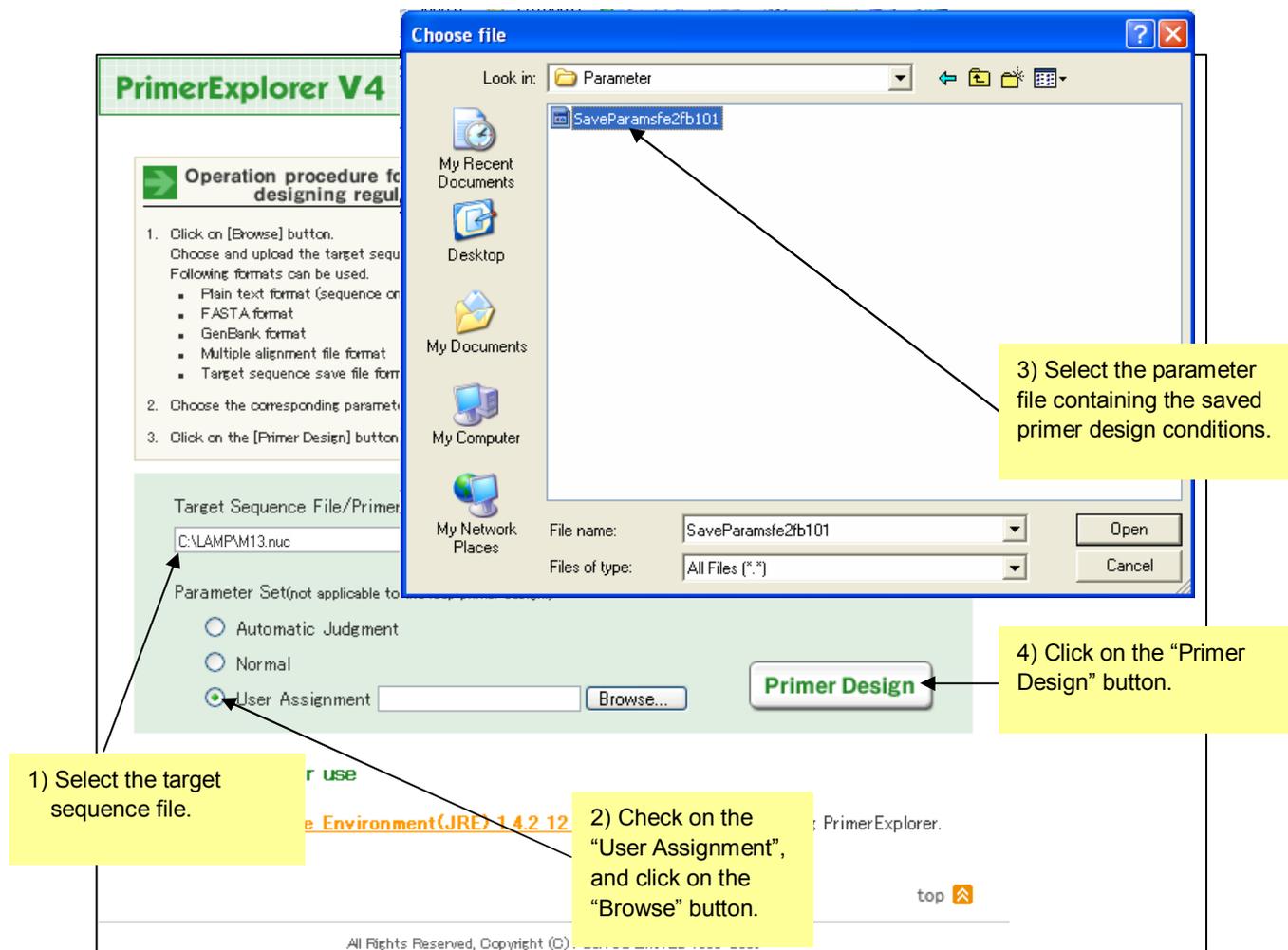


Figure 3.3 PrimerExplorer Ver. 4 startup window

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).

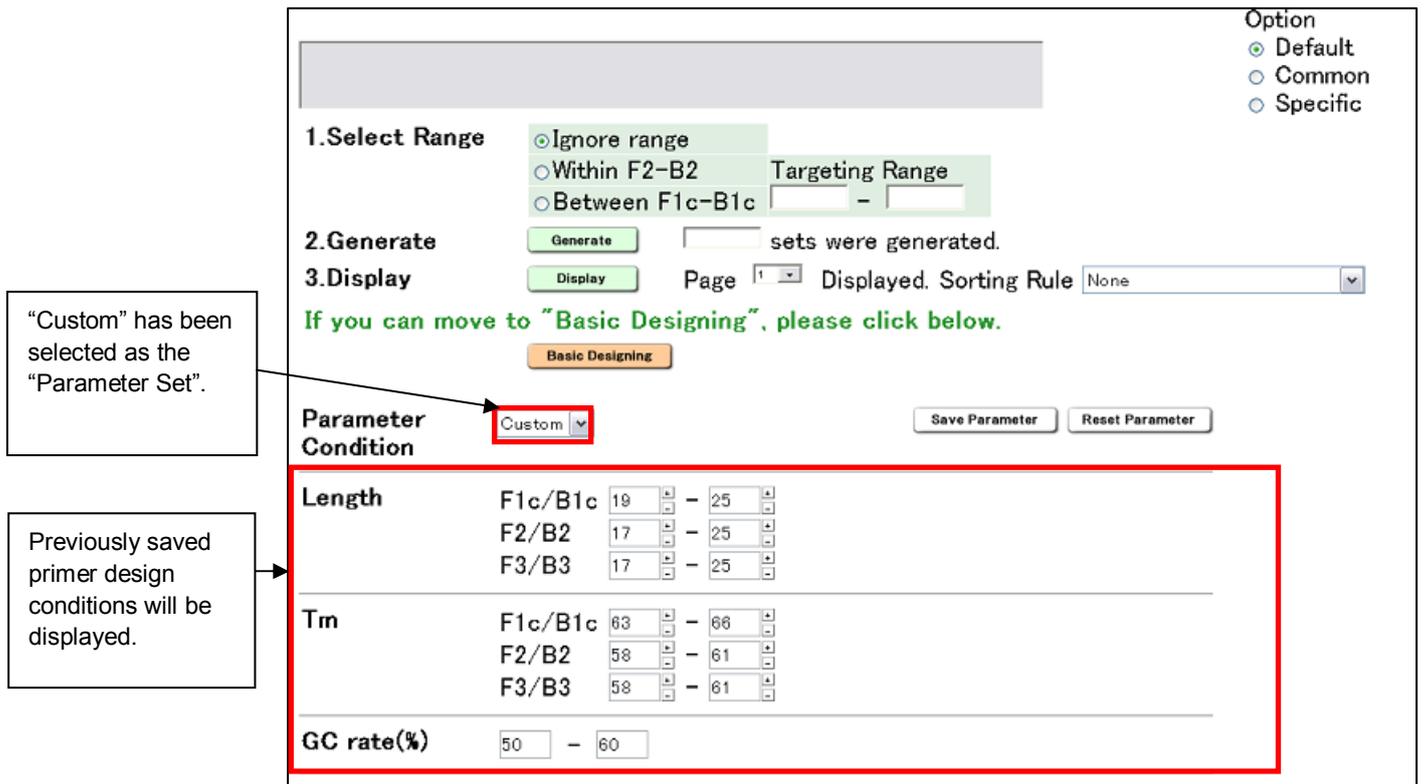


Figure 3.4 Primer design window

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)

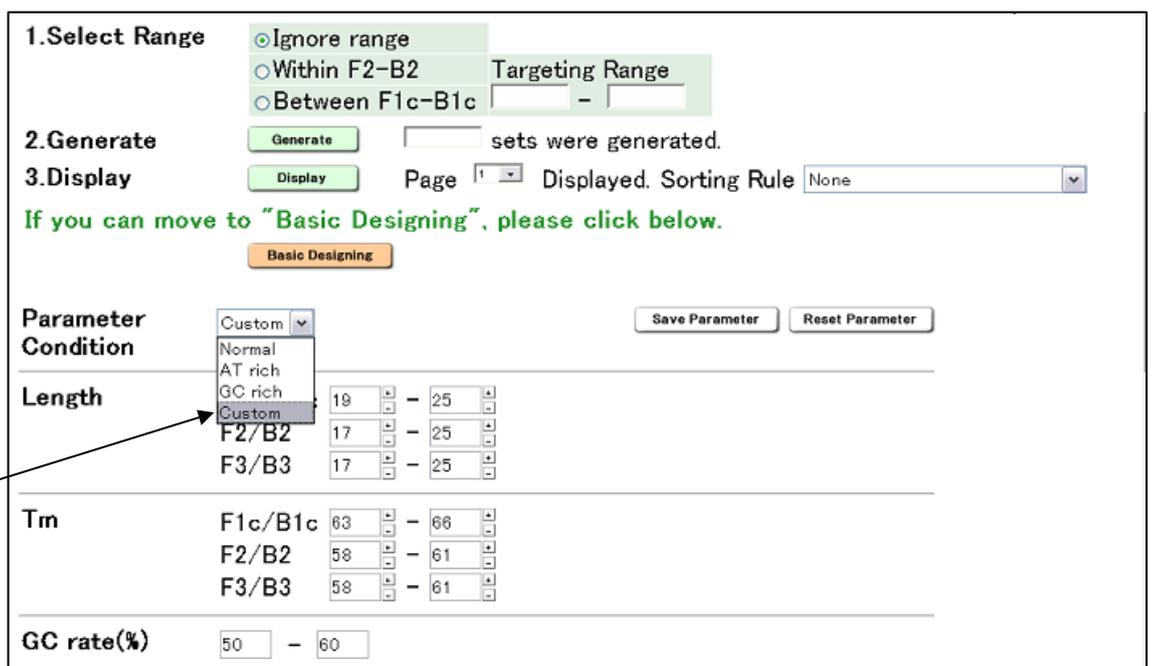


Figure 3.5 Changing the parameters

## 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.

UPLOAD FILE : C:\LAMP\M13.nuc

```
1  ACTAATCAA GAAGTATTGC TACACGGTT AATTTCGCTG ATGGACAGAC TCCTTTACTC GGTGGCCTCA CTGATTATAA 80
*****
81  AAACACTTCT CAAGATTCTG GCGTACCGTT CCTGTCTAAA ATCCCTTTAA TCGGCTCCTT GTTTAGCTCC CCGCTCGATT 160
*****
161  CCACGAGGA AAGCACGTTA TACGTGCTCG TCAAGCAAC CATAGTAGC GCCTGTAGC GCGGCATTAA GCGGCGCGG 240
*****
241  TGTGTGGTT ACGCGAGCG TGACCGCTAC ACTTGCCAGC GCCTAGCGC CCGCTCCTTT CGCTTCTTC CCTTCCTTC 320
*****
321  TCGCCAGTT CCGCGCTTT CCCCCTAAG CTCTAATCG GGGCTCCTT TTAGGGTCC GATTAGTGC TTTACGGAC 400
*****
401  CTCGACCCA AAAAATTGA TTTGGTGTG GATTACGTA GTGGCCATC GCCTGTAGC ACGTTTTC GCCTTTGAC 480
*****
481  GTTGGATCC AGTCTTTA ATAGTGAAT CTTGTCGA ACTGGAACA CACTGACCC TATCTGCGC TATCTTTTG 560
*****
561  ATTTATAGG GATTTTCCG ATTTGGAAC CACCATCAA CAGGATTTT GCCTGCTGG GCAACCGC GTGGCCGCT 640
*****
641  TGCTCAACT CTCACGGC CAGCGGTGA AGGCAATCA GCTGTTGCC GTCTCGCTG TGAAGAAGA ACCACCCCTG 720
*****
```

Set Mutation  
Mut/Cons  
Clear

Fixed Primer  
F3  
F2  
F1  
B1  
B2  
B3  
Clear

Save Target

Design Option  
Default  
Common

1) Specify the primer location.

2) Click on this button to specify the location F2

Figure 4.1 Primer design window

UPLOAD FILE : C:\LAMP\M13.nuc

```
1  ACTAATCAA GAAGTATTGC TACACGGTT AATTTCGCTG ATGGACAGAC TCCTTTACTC GGTGGCCTCA CTGATTATAA 80
*****
81  AAACACTTCT CAAGATTCTG GCGTACCGTT CCTGTCTAAA ATCCCTTTAA TCGGCTCCTT GTTTAGCTCC CCGCTCGATT 160
*****
161  CCACGAGGA AAGCACGTTA TACGTGCTCG TCAAGCAAC CATAGTAGC GCCTGTAGC GCGGCATTAA GCGGCGCGG 240
*****
241  TGTGTGGTT ACGCGAGCG TGACCGCTAC ACTTGCCAGC GCCTAGCGC CCGCTCCTTT CGCTTCTTC CCTTCCTTC 320
*****
321  TCGCCAGTT CCGCGCTTT CCCCCTAAG CTCTAATCG GGGCTCCTT TTAGGGTCC GATTAGTGC TTTACGGAC 400
*****
401  CTCGACCCA AAAAATTGA TTTGGTGTG GATTACGTA GTGGCCATC GCCTGTAGC ACGTTTTC GCCTTTGAC 480
*****
481  GTTGGATCC AGTCTTTA ATAGTGAAT CTTGTCGA ACTGGAACA CACTGACCC TATCTGCGC TATCTTTTG 560
*****
561  ATTTATAGG GATTTTCCG ATTTGGAAC CACCATCAA CAGGATTTT GCCTGCTGG GCAACCGC GTGGCCGCT 640
*****
641  TGCTCAACT CTCACGGC CAGCGGTGA AGGCAATCA GCTGTTGCC GTCTCGCTG TGAAGAAGA ACCACCCCTG 720
*****
```

Set Mutation  
Mut/Cons  
Clear

Fixed Primer  
F3  
F2  
F1  
B1  
B2  
B3  
Clear

Save Target

Design Option  
Default  
Common

Display of the specified primer location

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

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

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.

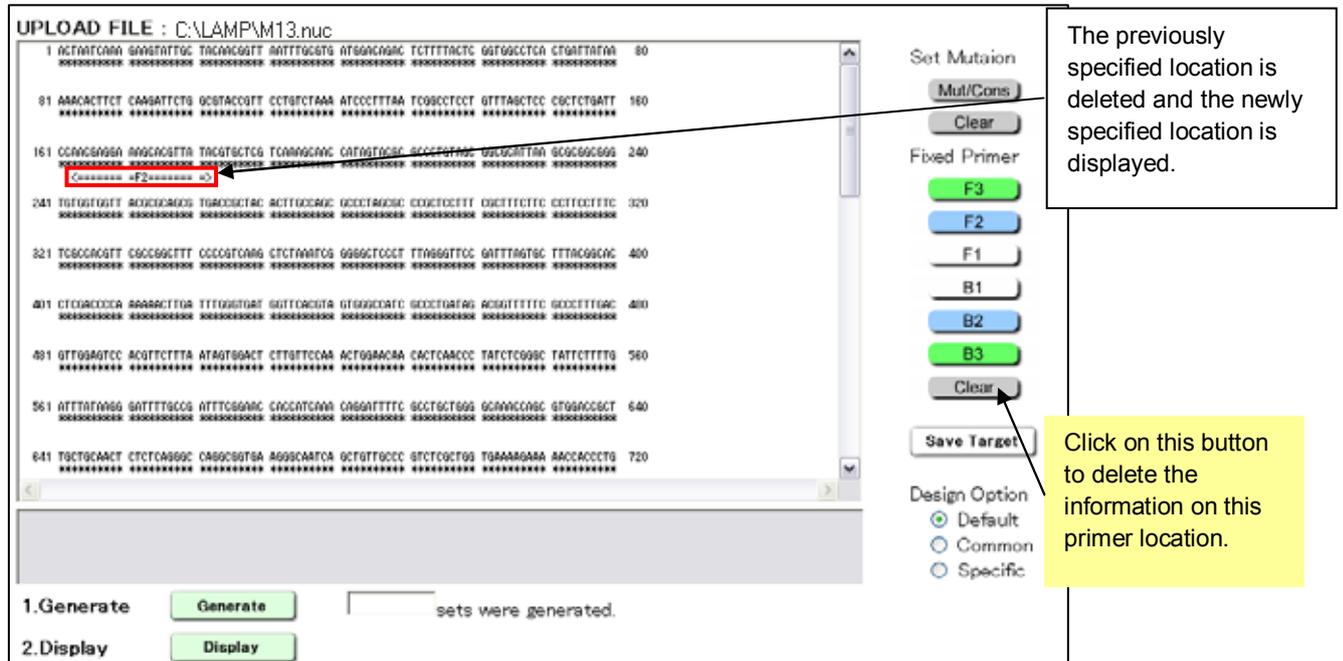


Figure 4.3 Primer design window

#### **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)

UPLOAD FILE : C:\LAMP\M13.nuc

```

1  ACTAATCAA GAGTATTGC TACACGGTT AATTTCGCG ATGGACAGAC TCTTTTACTC GGTGGCCTCA CTGATTATA 80
*****
81  AAACACTTCT CAAGATTCTG GCGTACCGTT CCTGTCTAAA ATCCCTTTAA TCGGCCTCCT GTTTAGCTCC GCGCTGATT 160
*****
161 CCACAGGGA AAGCACGTTA TACGTGCTCG TCAAGCAAC CATAGTACGC GCCCTAGAGC GCGCATTA A GCGCGCGGG 240
*****
241 TGTGGTGGTT ACGCGCAGCG TGACCGCTAC ACTTGCCAGC GCCCTAGAGC CCGCTCTTT GCTTTTCTTC CCTTCCTTC 320
*****
321 TGCCACGTT GCGCGCTTT CCCGTCAGG CTCTAATCG GGGGCTCCCT TTAGGGTCC GATTTAGTC TTTACGGAC 400
*****
401 CTCGACCCCA AAAAAGTTGA TTTGGGTGAT GGTTCACGTA GTGGGCCATC GCCCTAGTAG ACGGTTTTTC GCCCTTTGAC 480
*****
481 GTTGGAGTCC ACGTTCTTTA ATAGTGGACT GTTGTCCAA ACTGGAACAA CACTCAACCC TATCTCGGG TATTCITTTG 560
*****
561 ATTTATAAGG GATTTTCCG ATTTCCGAAC CACCATCAA CAGGATTTTC GCCTGCTGG GCAACCCAGC GTGACCCGCT 640
*****
641 TGCTGCACT CTCTAGGCG CAGCGGTGA AGGCAATCA GCTGTGCCG GTCTGCTGG TGAAGAGAA AACCCCTCG 720
*****

```

Number of Primer Candidates: F1=191, F2=187, F3=1, B1=215, B2=189, B3=393, FIP=209, BIP=243  
45 Primer set(s) were generated.

1. Select Range  
 Ignore range  
 Within F2-B2 Targeting Range 128 - 144  
 Between F1c-B1c

2. Generate  
Generate 45 sets were generated.

3. Display  
Display Page 1 Displayed. Sorting Rule None

Set Mutation: Mut/Cons, Clear  
Fixed Primer: F3, F2, F1, B1, B2, B3, Clear  
Design Option:  Default,  Common,  Specific

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.

Figure 4.4 Primer design window

Primer sets with the F3 specified have been designed.

on the check box to choose primer set.  
2. Push "Confirm" button to transfer to Primer Information page.  
3. Push "Save List" button to download Excel format file.

Confirm Save List

Primer set: sorting rule [None]

Primer ID	dG(dimer)	Primer	Primer	Primer	Primer	Primer	Primer	Primer	Primer	Primer
[1]	-2.13	[1]	TAATCGGCCTCCTGTTT	CGGCTCTGATTCCAACGAG						gtttccttggatcatccg
[2]	-2.13	[2]	TAATCGGCCTCCTGTTT	CGGCTGATTCCAACGAGG						gtttccttggatcatccg
[3]	-2.46	[3]	TAATCGGCCTCCTGTTT	GCTCTGATTCCAACGAGGAA						gtttccttggatcatccg
[4]	-2.13	[4]	TAATCGGCCTCCTGTTT	CGGCTCTGATTCCAACGAG						gtttccttggatcatccg
[5]	-2.13	[5]	TAATCGGCCTCCTGTTT	CGGCTCTGATTCCAACGAGG						gtttccttggatcatccg
[6]	-2.46	[6]	TAATCGGCCTCCTGTTT	GCTCTGATTCCAACGAGGAA						gtttccttggatcatccg
[7]	-2.13	[7]	TAATCGGCCTCCTGTTT	CGGCTCTGATTCCAACGAG						gtttccttggatcatccg
[8]	-2.13	[8]	TAATCGGCCTCCTGTTT	CGGCTGATTCCAACGAGG						gtttccttggatcatccg
[9]	-2.46	[9]	TAATCGGCCTCCTGTTT	GCTCTGATTCCAACGAGGAA						gtttccttggatcatccg
[10]	-2.13	[10]	TAATCGGCCTCCTGTTT	CGGCTCTGATTCCAACGAG						gtttccttggatcatccg
[11]	-2.36	[11]	TAATCGGCCTCCTGTTT	CGGCTGATTCCAACGAGG						gtttccttggatcatccg
[12]	-2.46	[12]	TAATCGGCCTCCTGTTT	GCTCTGATTCCAACGAGGAA						gtttccttggatcatccg
[13]	-2.13	[13]	TAATCGGCCTCCTGTTT	CGGCTCTGATTCCAACGAG						gtttccttggatcatccg
[14]	-2.36	[14]	TAATCGGCCTCCTGTTT	CGGCTGATTCCAACGAGG						gtttccttggatcatccg

Figure 4.5 Primer Set List window

## 5. Loop primer design

### 5.1 Uploading the primer information file

Return to the PrimerExplorer V3 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)

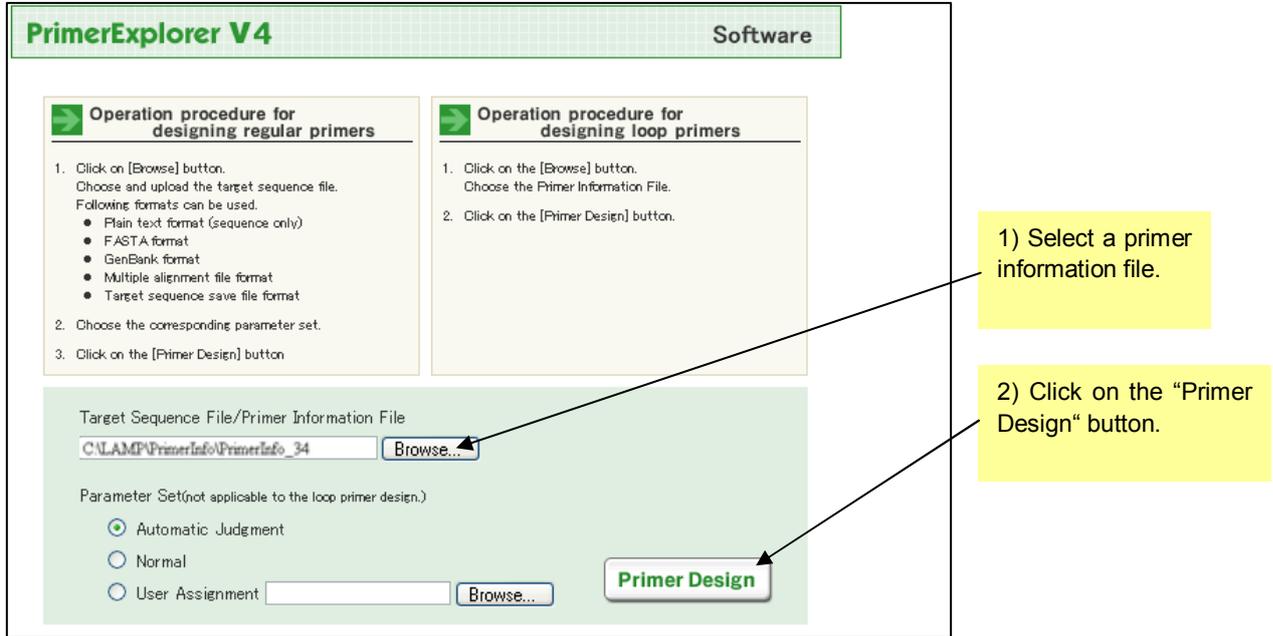


Figure 5.1 PrimerExplorer V3 startup window

### 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.

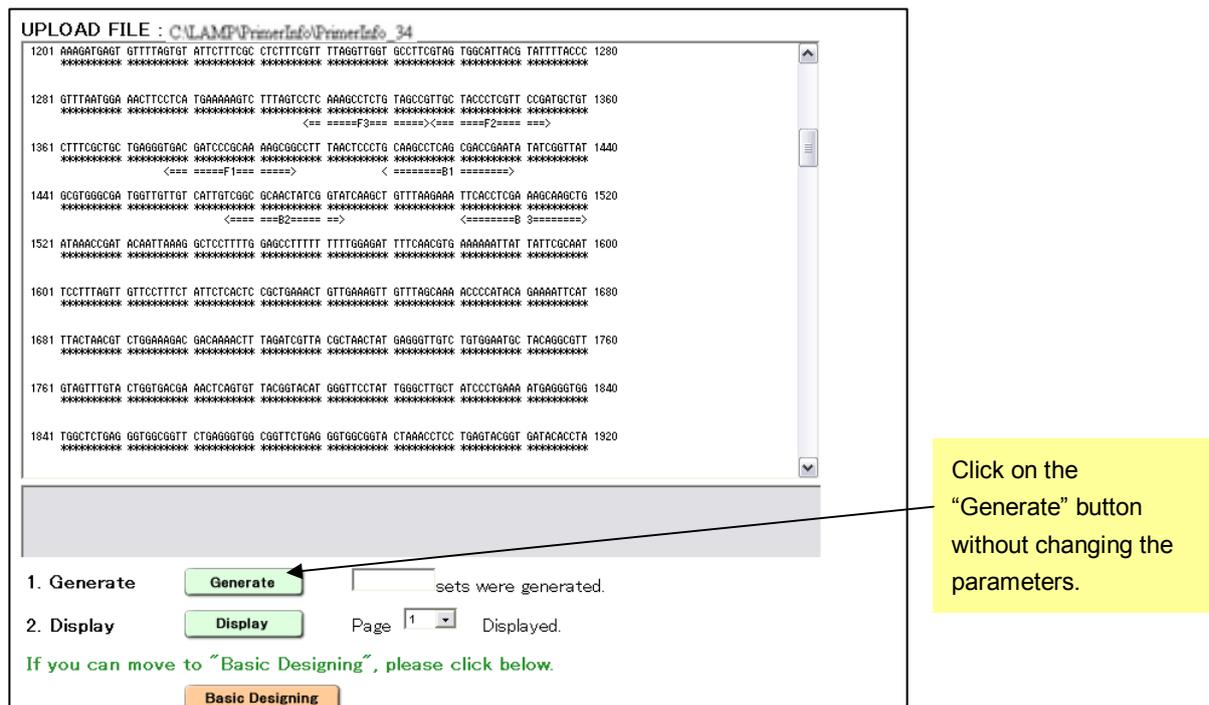


Figure 5.2 Loop primer design window

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

Forward-side loop primer

Backward-side loop primer

The screenshot displays a software interface for primer design. At the top, there are 'Confirm' and 'Save List' buttons. Below them, the 'Primer set' section shows a 'Target DNA' sequence with a highlighted region: CTC<sup>AAAGCCTCTGTAGCCGTTGC</sup>TACCCCTCGTTC<sup>CGA</sup>. Below the target DNA is a table of primer sets. The first six rows are checked, and a red box highlights the primer sequences for sets [1] through [6]. Below this table, it says '[Outputs: 6 sets] Displayed 1 - 6.' and 'DesignId 000312102710'. The bottom section shows the 'Backward-side loop primer' details, with a red box highlighting the primer sequences for sets [1] through [6]. Callout boxes with arrows point to various elements: '1) Check the boxes.' points to the checkboxes in the primer set table; '2) Click on the “Confirm” button.' points to the 'Confirm' button; 'Saved primer information locations' points to the primer sequences in both the primer set table and the loop primer details; 'Forward-side loop primer' points to the primer sequences in the primer set table; and 'Backward-side loop primer' points to the primer sequences in the loop primer details.

Primer ID	dimer	1301	1311	1321	1331	1341	1351	1361	1371	1381	1391	1401
[1]	-2.18							gaca	aaaa	gcagc	actccc	
[2]	-2.18							gaca	aaaa	gcagc	actccc	
[3]	-2.18							gaca	aaaa	gcagc	actccc	
[4]	-1.98							aca	aaaa	gcagc	actccc	
[5]	-1.98							aca	aaaa	gcagc	actccc	
[6]	-1.98							aca	aaaa	gcagc	actccc	

Primer ID	1411	1421	1431	1441	1451	1461	1471	1481	1491	1501	1511	1521	15
[1]				GGGCGA	TGGT	TGTG	CAT						
[2]				GGGCGA	TGGT	TGTG	CATT						
[3]				GGGCGA	TGGT	TGTG	CATTG						
[4]				GGGCGA	TGGT	TGTG	CAT						
[5]				GGGCGA	TGGT	TGTG	CATT						
[6]				GGGCGA	TGGT	TGTG	CATTG						

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.

Examine the detailed information to choose from these loop primer sets. Check the boxes to the left of all primer sets, and click on the “Confirm” button to display the Primer Set Details window.

## 5.2 Narrowing down the loop primer set candidates

Primer Set Details window (Figure 5.4) shows the detailed information on the six loop primer sets previously selected. This is the final screen for loop primer design.

ID	dimer(minimum)dG	Label	5'pos	3'pos	len	Tm	5'dG	3'dG	GCrate	Sequence
1	-2.18	LF	1357	1376	20	62.54	-5.70	-4.74	0.60	CCCTCAGCAGCGAAAGACAG
		LB	1445	1463	19	60.07	-7.38	-4.46	0.53	GGCCGATGGTTGTTGTCAT
2	-2.18	LF	1357	1376	20	62.54	-5.70	-4.74	0.60	CCCTCAGCAGCGAAAGACAG
		LB	1445	1464	20	60.75	-7.38	-4.06	0.50	GGCCGATGGTTGTTGTCATT
3	-2.18	LF	1357	1376	20	62.54	-5.70	-4.74	0.60	CCCTCAGCAGCGAAAGACAG
		LB	1445	1465	21	61.91	-7.38	-4.07	0.52	GGCCGATGGTTGTTGTCATTG
4	-1.98	LF	1358	1376	19	61.63	-5.70	-4.41	0.58	CCCTCAGCAGCGAAAGACA
		LB	1445	1463	19	60.07	-7.38	-4.46	0.53	GGCCGATGGTTGTTGTCAT
5	-1.98	LF	1358	1376	19	61.63	-5.70	-4.41	0.58	CCCTCAGCAGCGAAAGACA
		LB	1445	1464	20	60.75	-7.38	-4.06	0.50	GGCCGATGGTTGTTGTCATT
6	-1.98	LF	1358	1376	19	61.63	-5.70	-4.41	0.58	CCCTCAGCAGCGAAAGACA
		LB	1445	1465	21	61.91	-7.38	-4.07	0.52	GGCCGATGGTTGTTGTCATTG

Figure 5.4 Primer Set Details window

A close look at the forward-side loop primers will show that the ID numbers 1, 2, and 3 are the same and the ID numbers 4, 5 and 6 are the same. As an example from the former (1 to 3), the stability of the 3' end of the loop primer ID number 1 is -4.74. As an example from the latter (4 to 6), the stability of the 3' end of the loop primer ID number 4 is -4.41. Thus, the stability of the former three is better. Therefore, the choices will be further narrowed down from the former three.

Among the primers of the ID numbers 1, 2 and 3, we will now examine the stability of the 3' end of the backward side. Stability is -4.46 for ID number 1, -4.06 for ID number 2, and -4.07 for ID number 3, so that the loop primer set ID number 1 is the most stable. Among the six loop primer sets, ID number 1 is selected.