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.

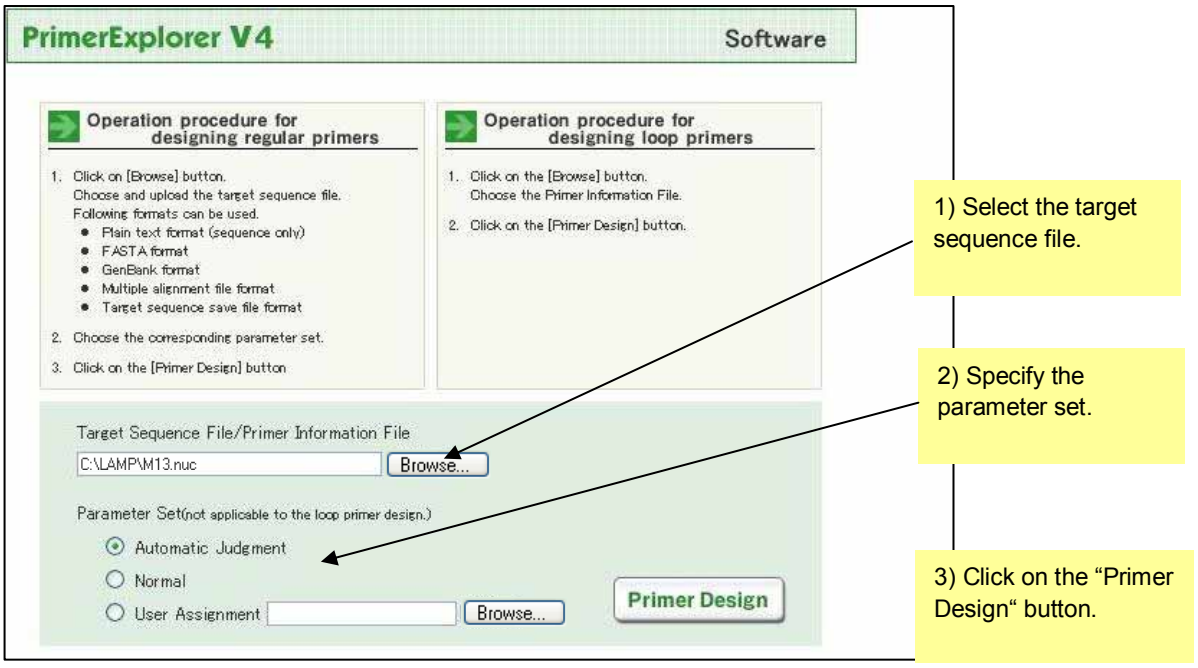

Figure1.1 PrimerExplorer V3 startup window

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).

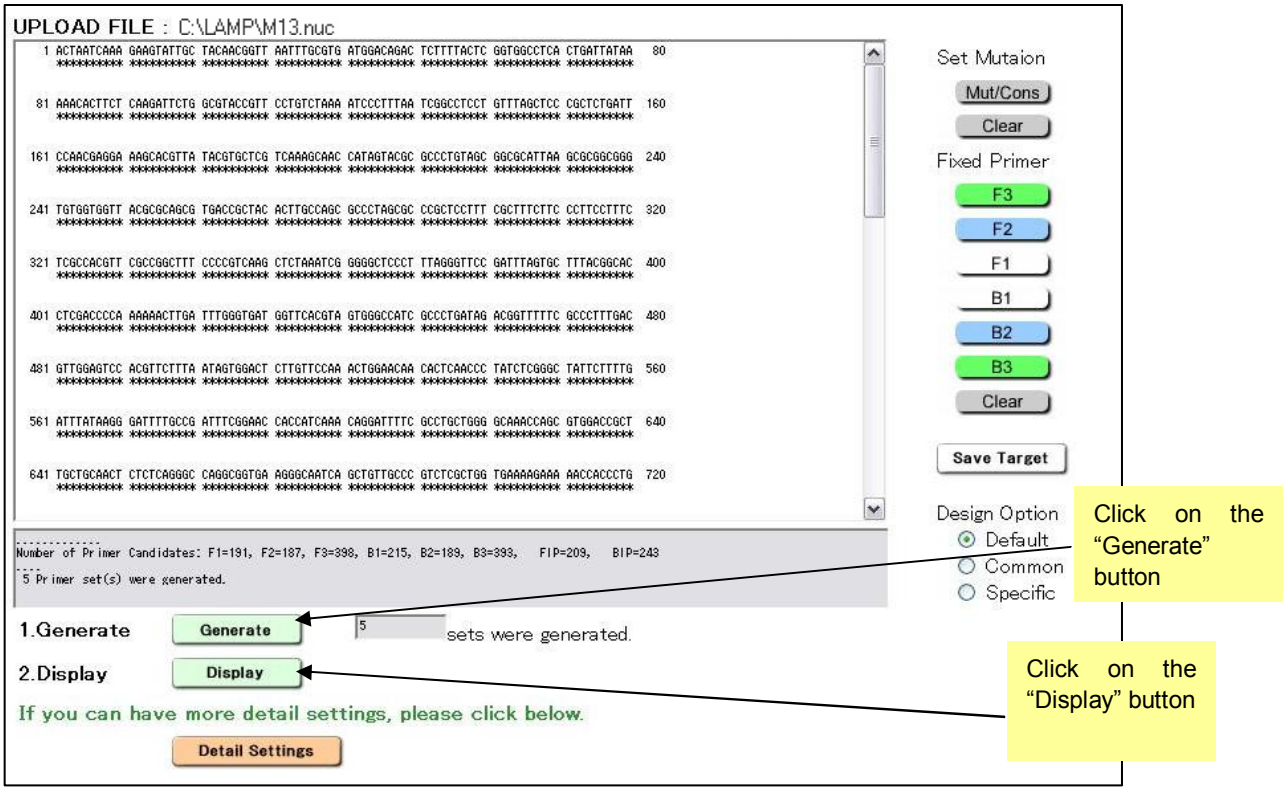

Figure1.2 Primer design window in Easy Mode

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.

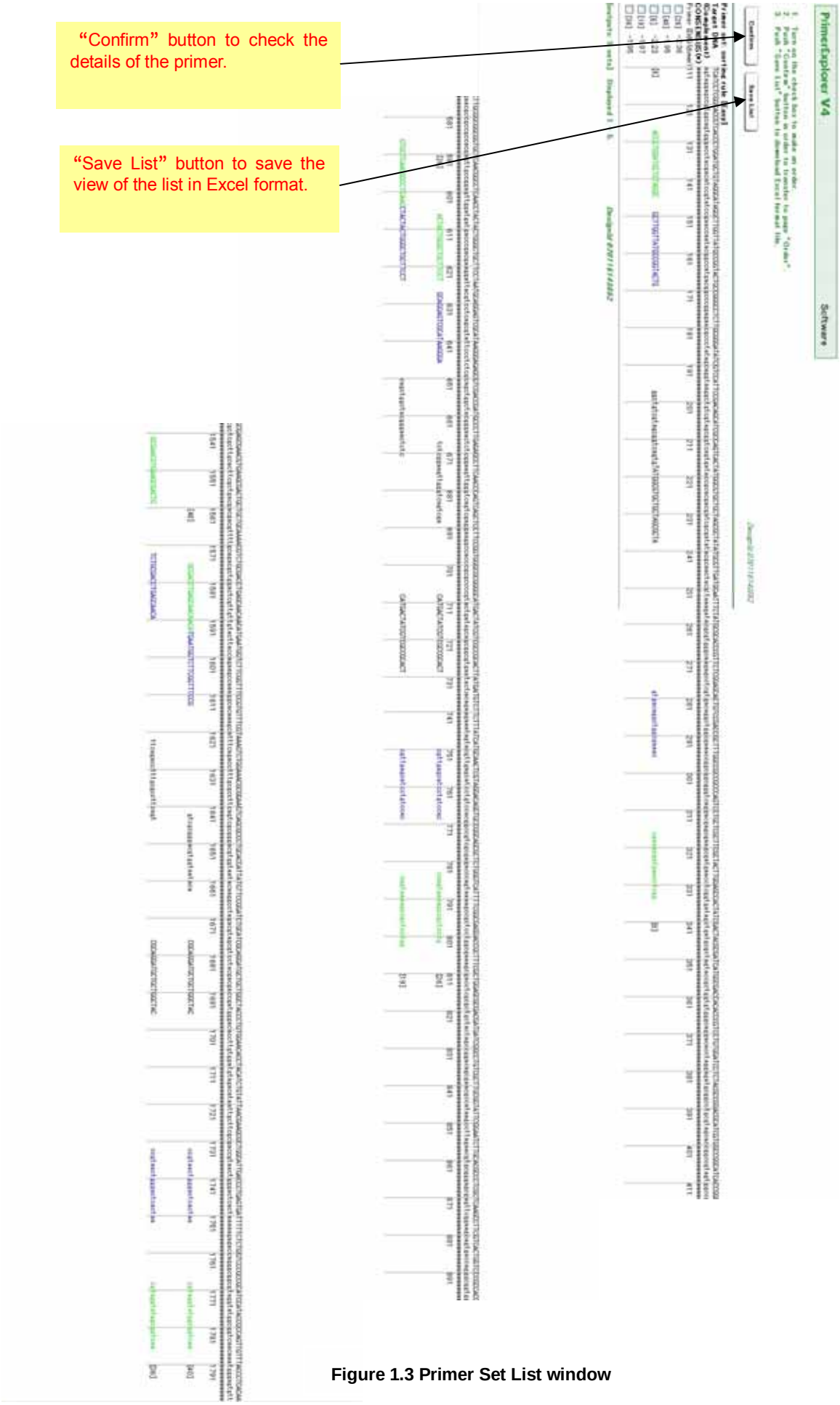

Figure 1.3 Primer Set List window

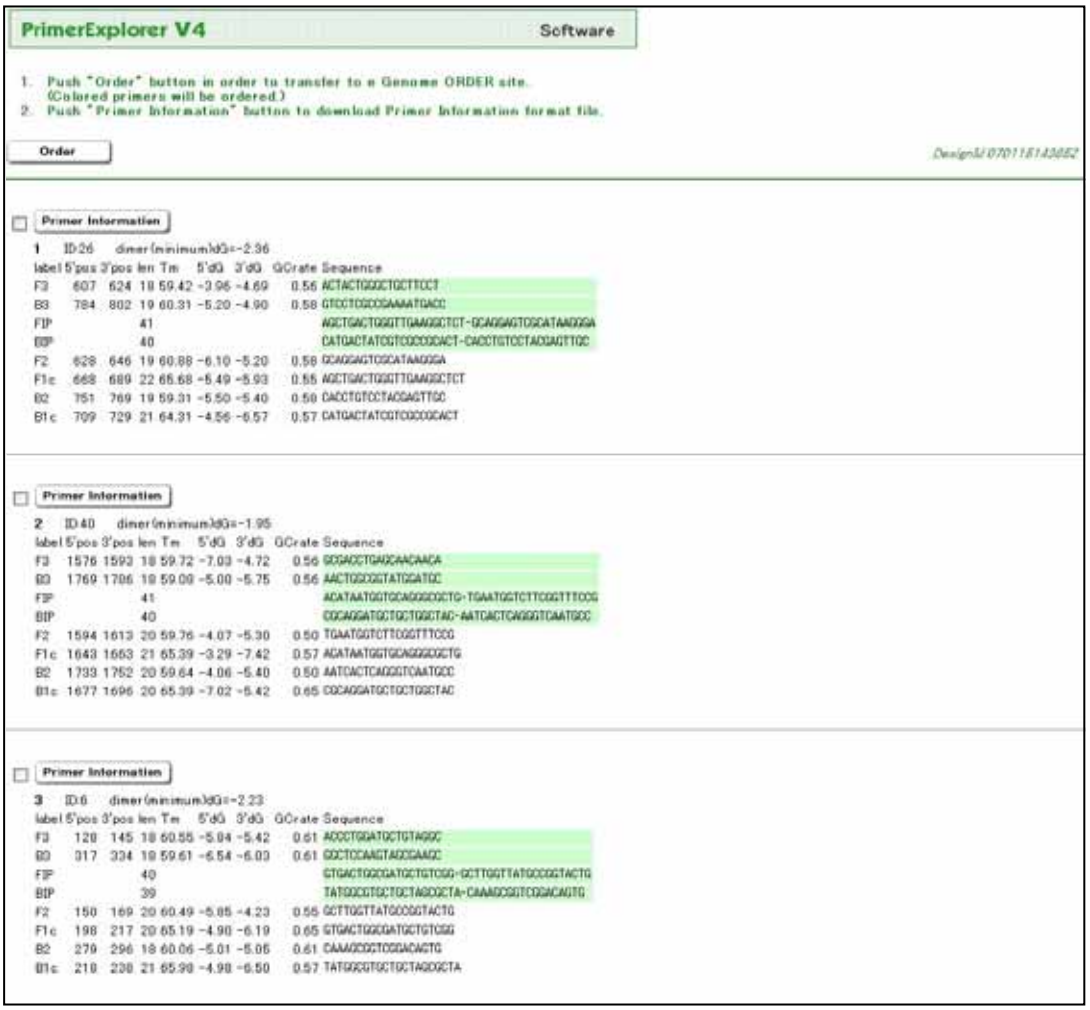

Figure 1.4 Primer Set Details window

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 ware designed (Figure 1.7). Clicking on the “Display” button will display the Primer Set List result.

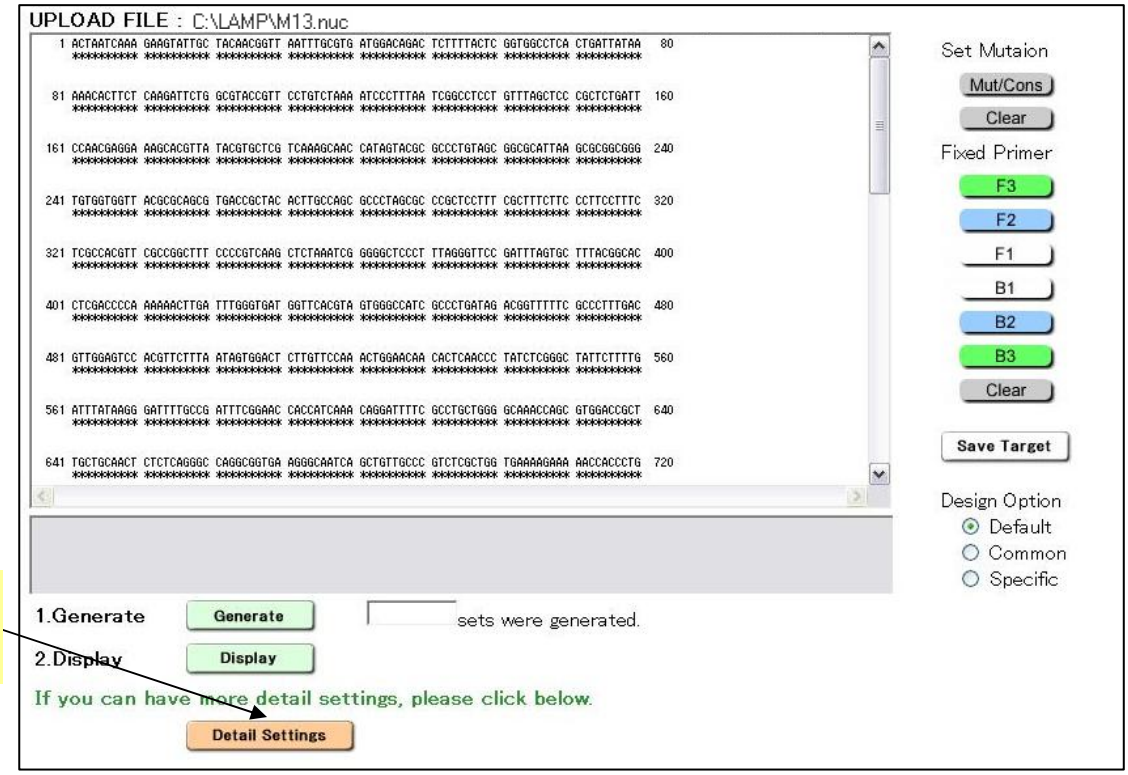

Figure 1.5 Primer design window

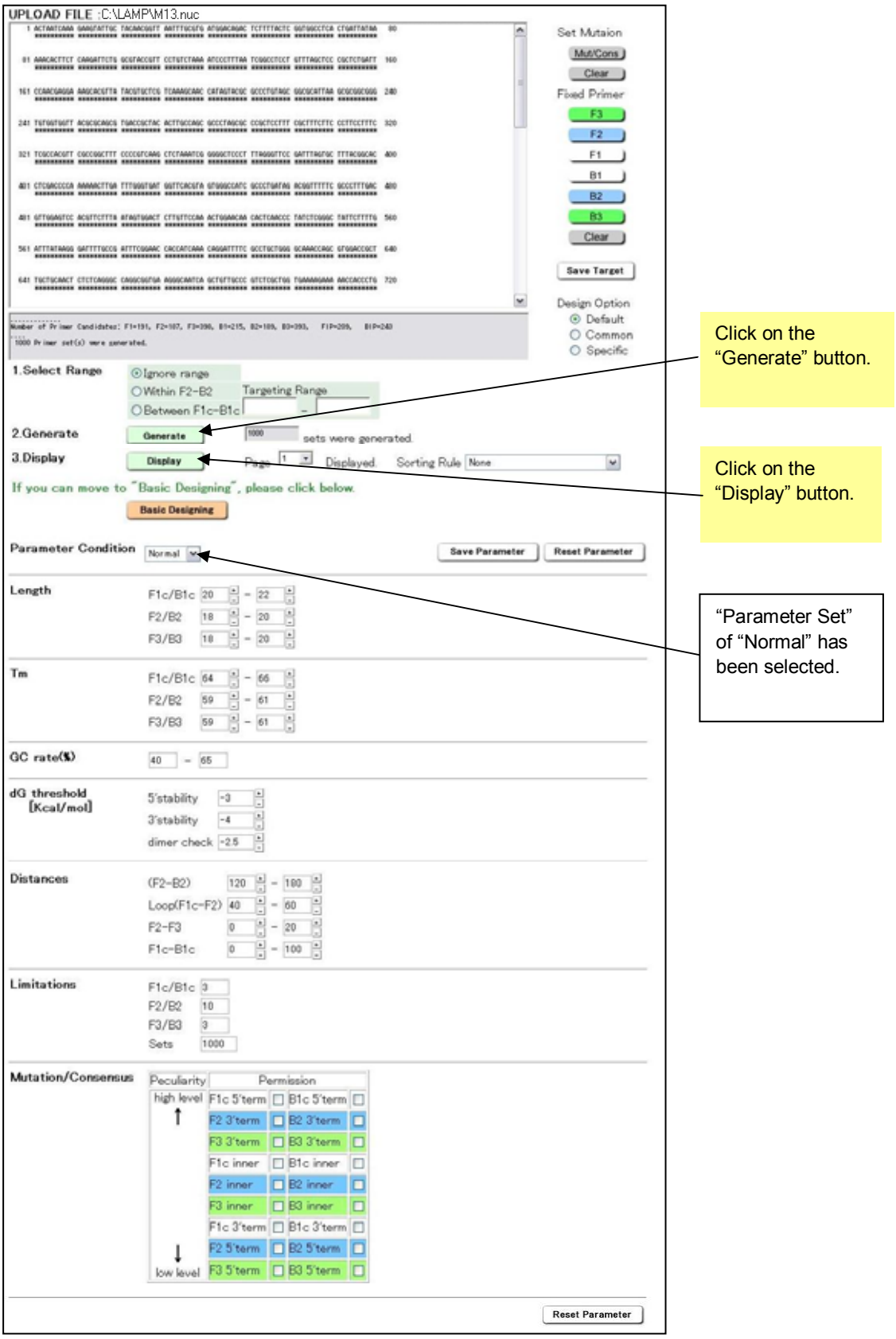

Figure 1.6 Expert mode

The message area indicates the status of progress.

A total of 1,000 primer sets were generated.

Click on the “Display” button to display the results.

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.

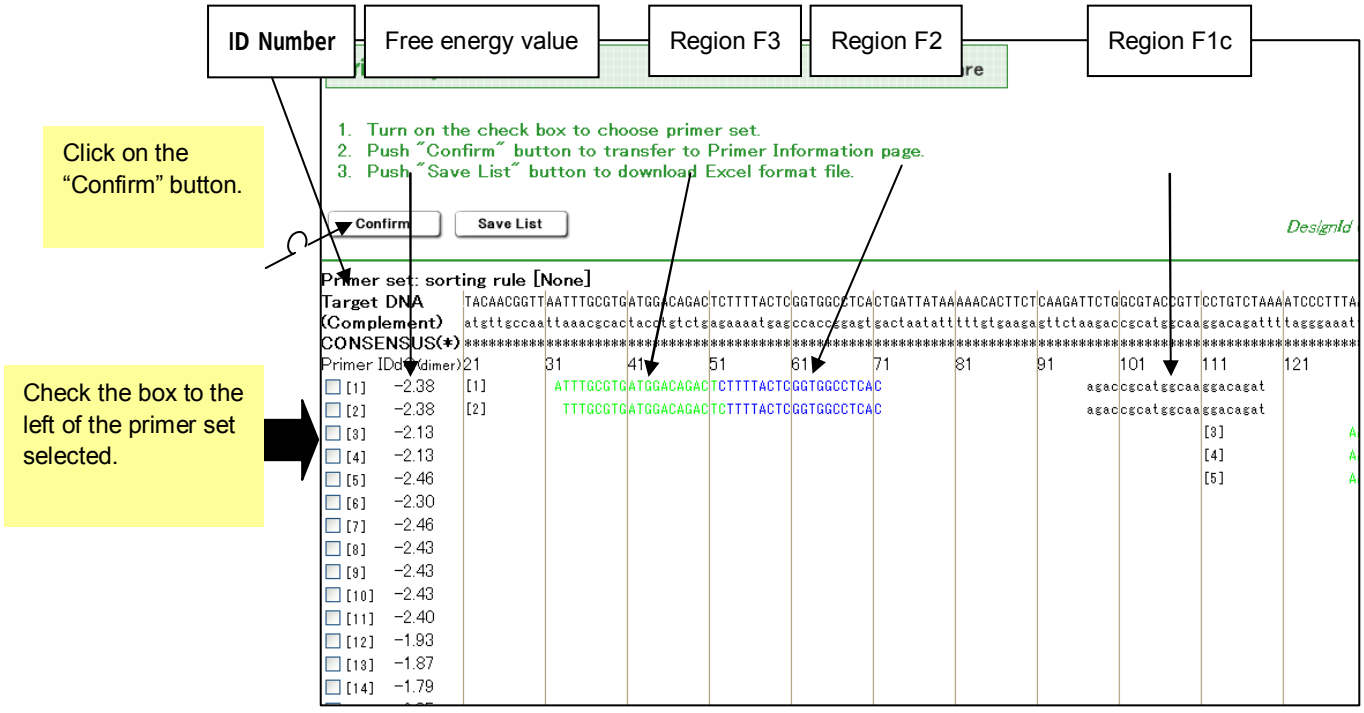

Figure 1.8a Primer Set List window-1 (page 1)

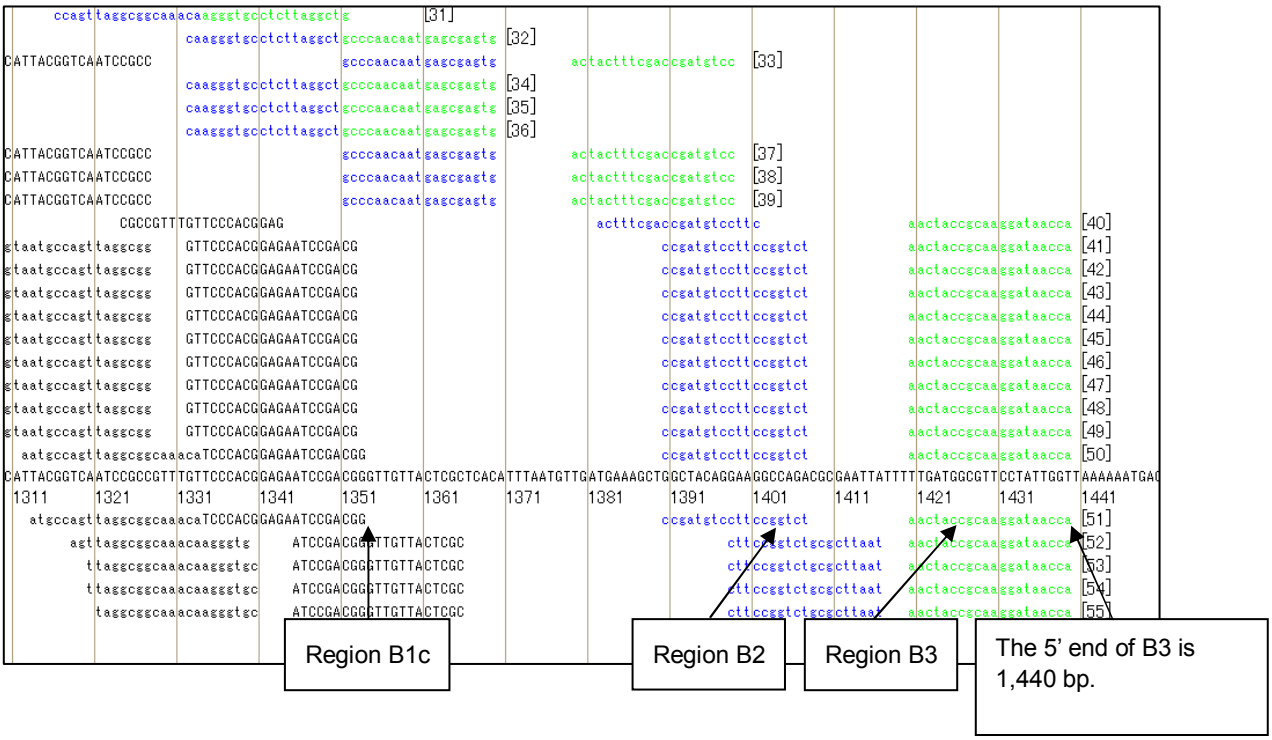

Figure 1.8b Primer Set List window-2 (page 2)

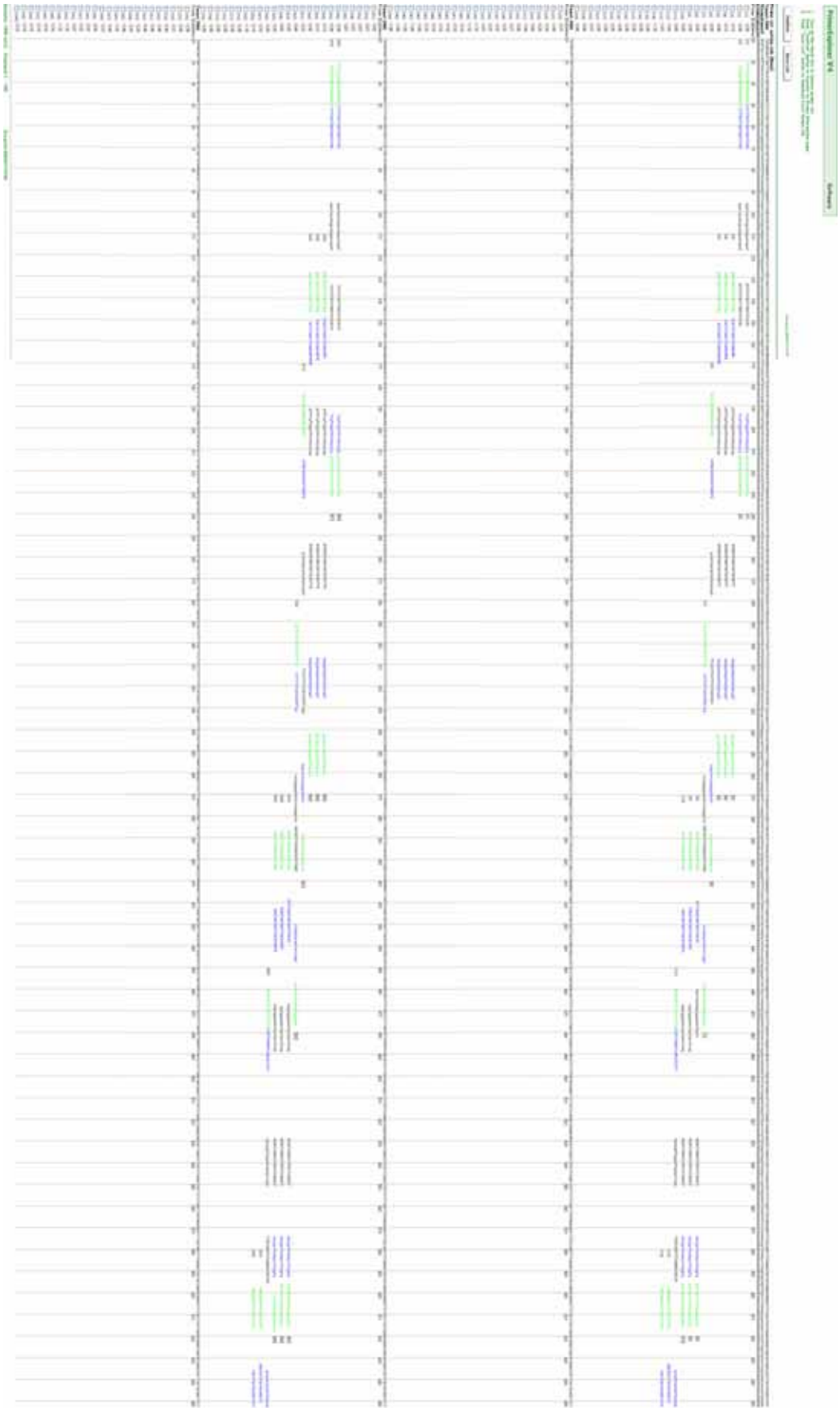

Figure 1.9 Primer Set List (full view)

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.

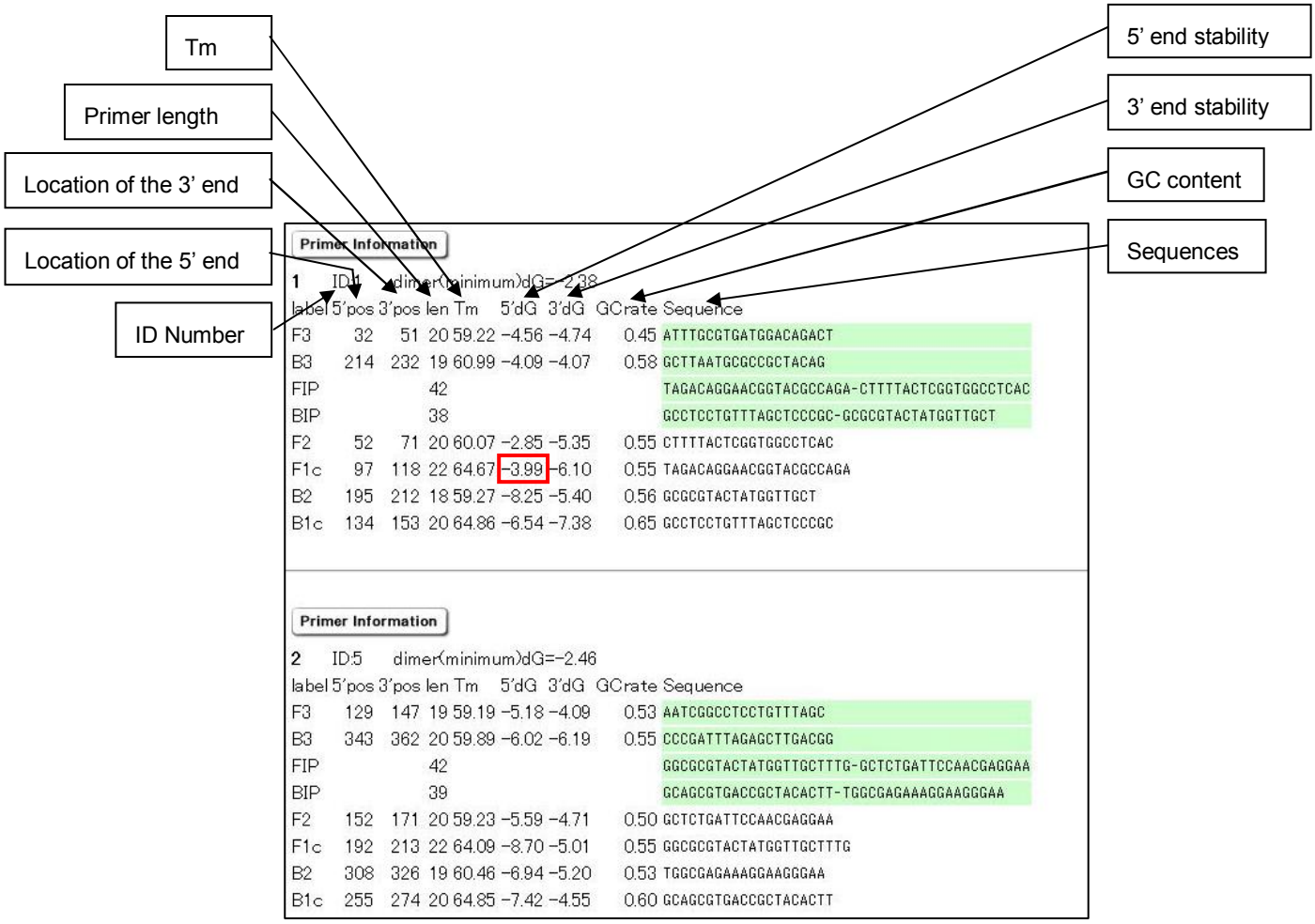

Figure 1.10 Primer Set Details window

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 ΔG (stability) is -4.0 kcal/mol or lower. For example the end with ΔG = -6.5 kcal/mol is more stable than the end with ΔG = -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.

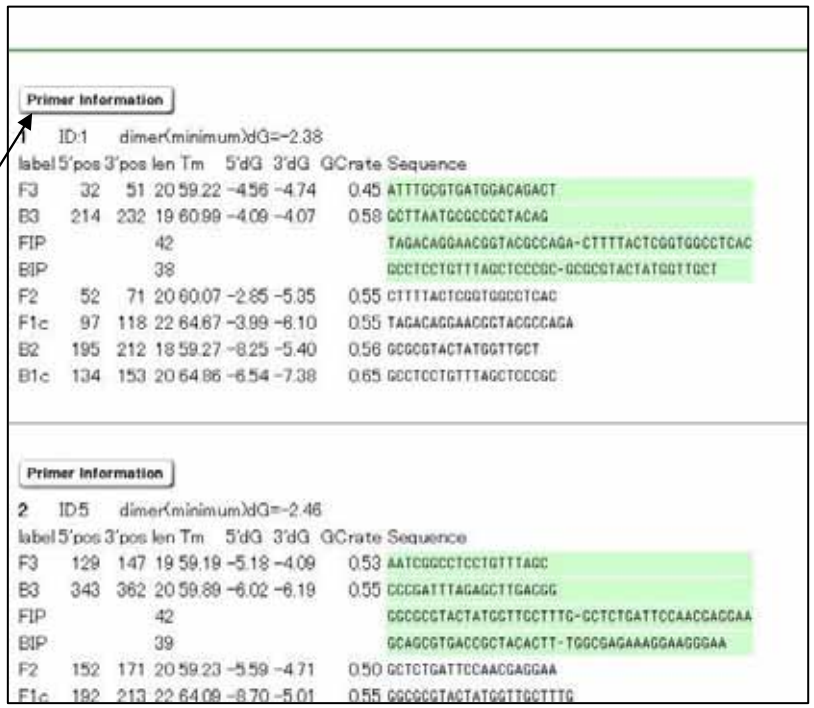

Figure 1.11 Selected Primer Set window

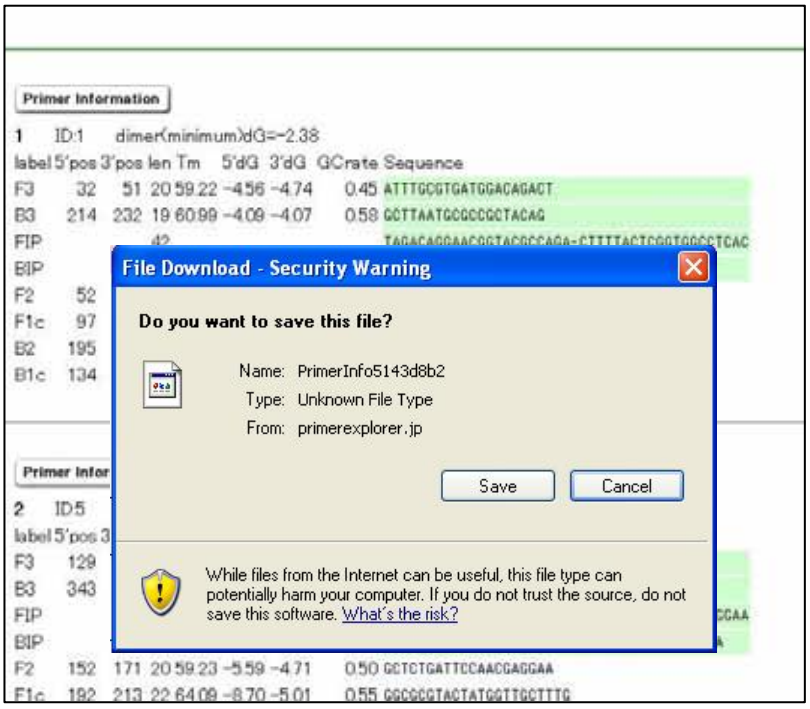

Figure 1.12 Primer Information Save window

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)

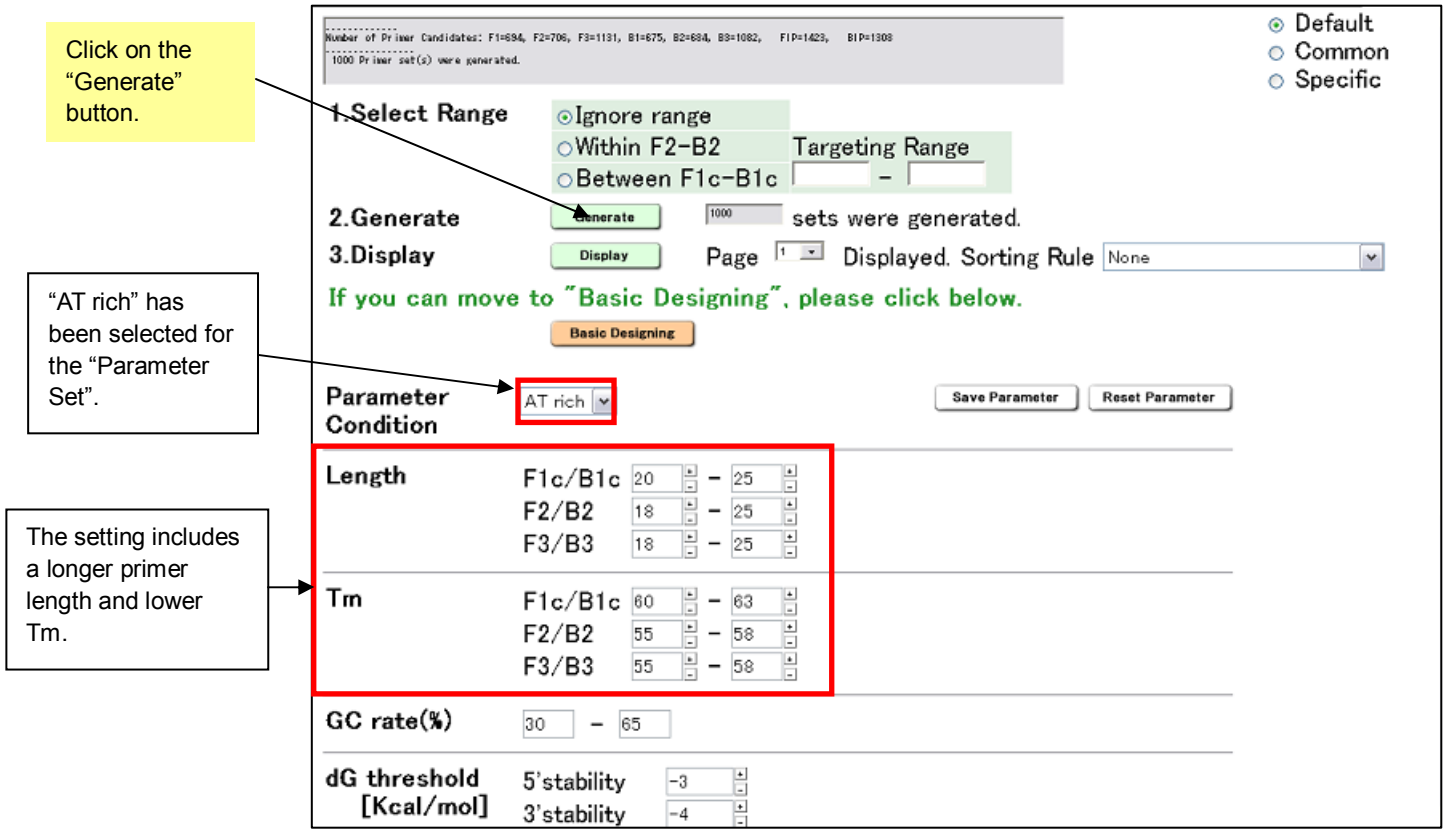

Figure 2.1 Primer Design window

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 171st 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.

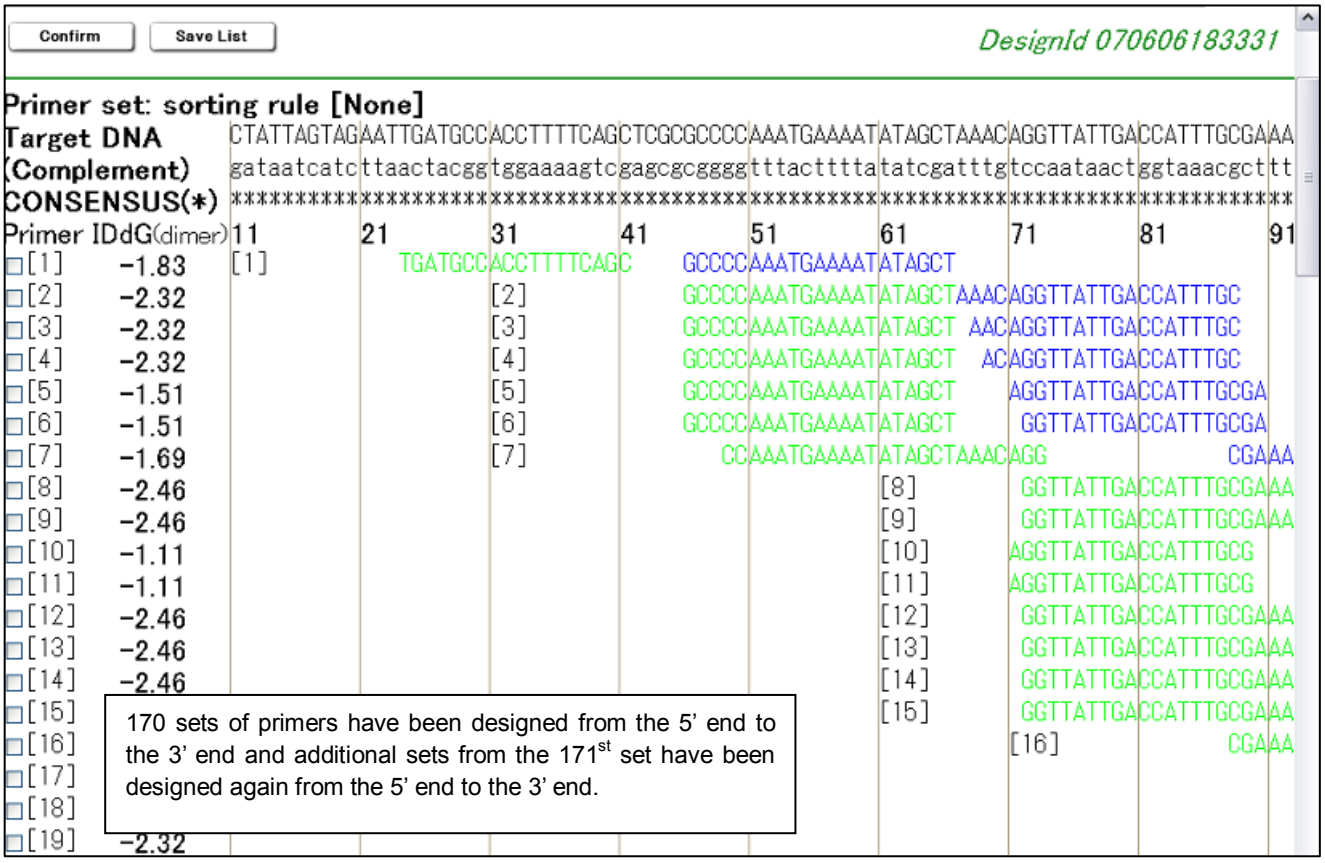

Figure 2.2 Primer Set List window

< 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 Tm 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 Tm value around 5°C higher than those of the other primers. When less stringent conditions (wider range of Tm's at each primer location) are used to design the primers, primer sets are generated, which consists of the primers with various Tm value. For this reason, the difference in the Tm in each primer region may be 3°C or less. Also, best results are obtained if the Tm'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 Tm should be adjusted.

a) For AT rich sequences

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

b) For GC rich sequences

In contrast, for GC rich sequences, the Tm is calculated to be higher than non-GC rich sequences of the same length. For this reason the Tm calculated from the default primer length may be higher than the default Tm upper limit, and prevent primers from being designed. Thus, the primer length should be decreased and/or the Tm should be increased. Because how the Tm 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 Tm 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, Tm, 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.

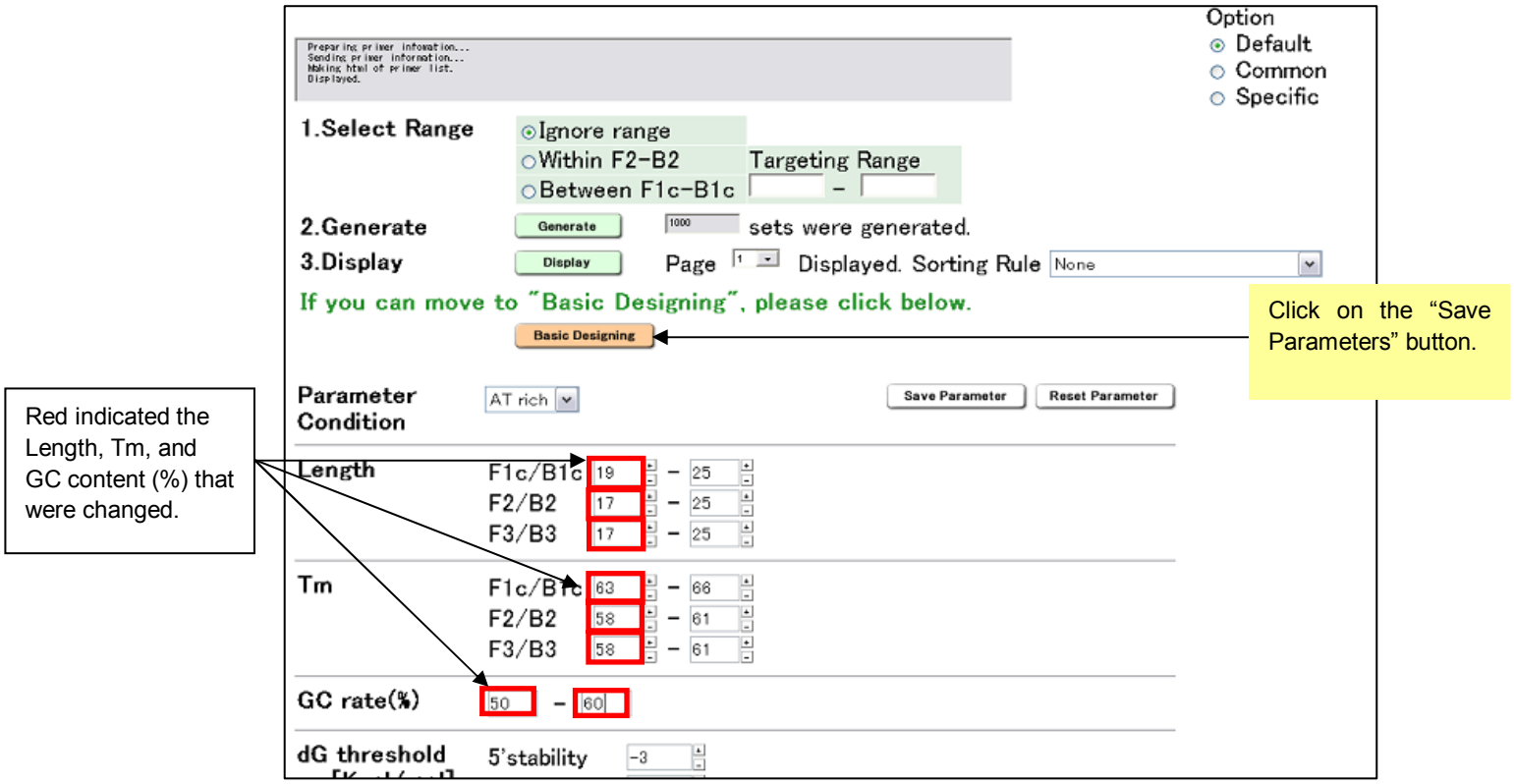

Figure 3.1 Changing the primer design conditions (Primer Design window)

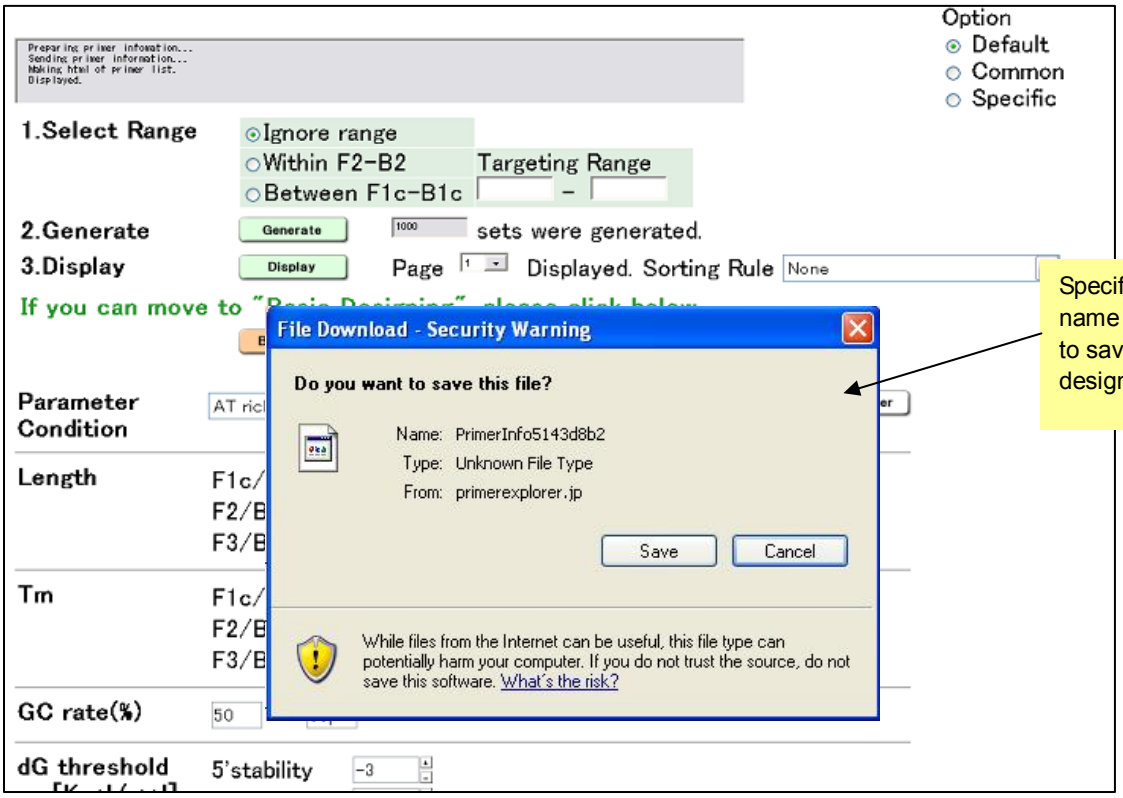

Figure 3.2 Saving the primer design conditions

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.

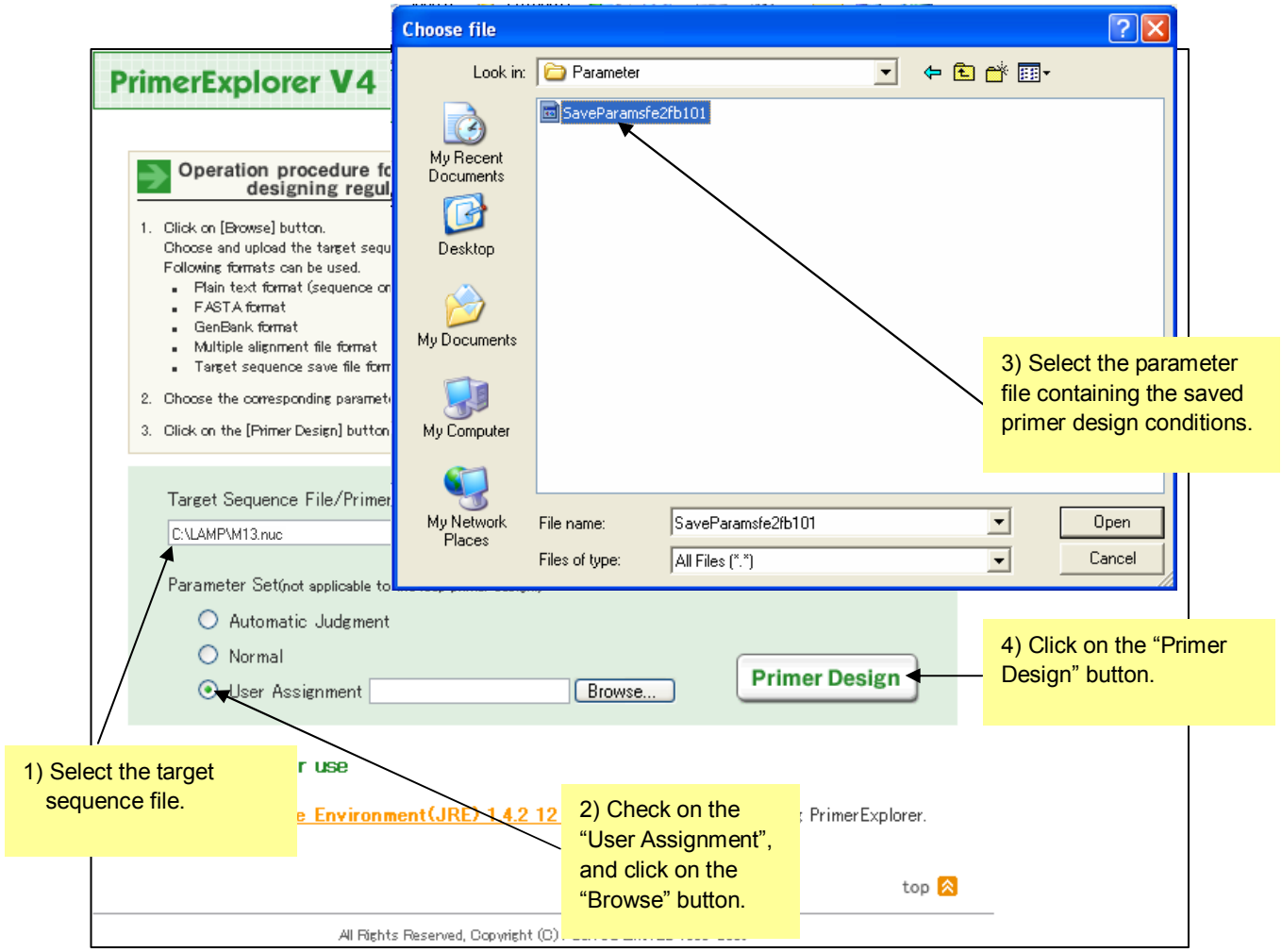

Figure 3.3 PrimerExplorer Ver. 4 startup window

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).

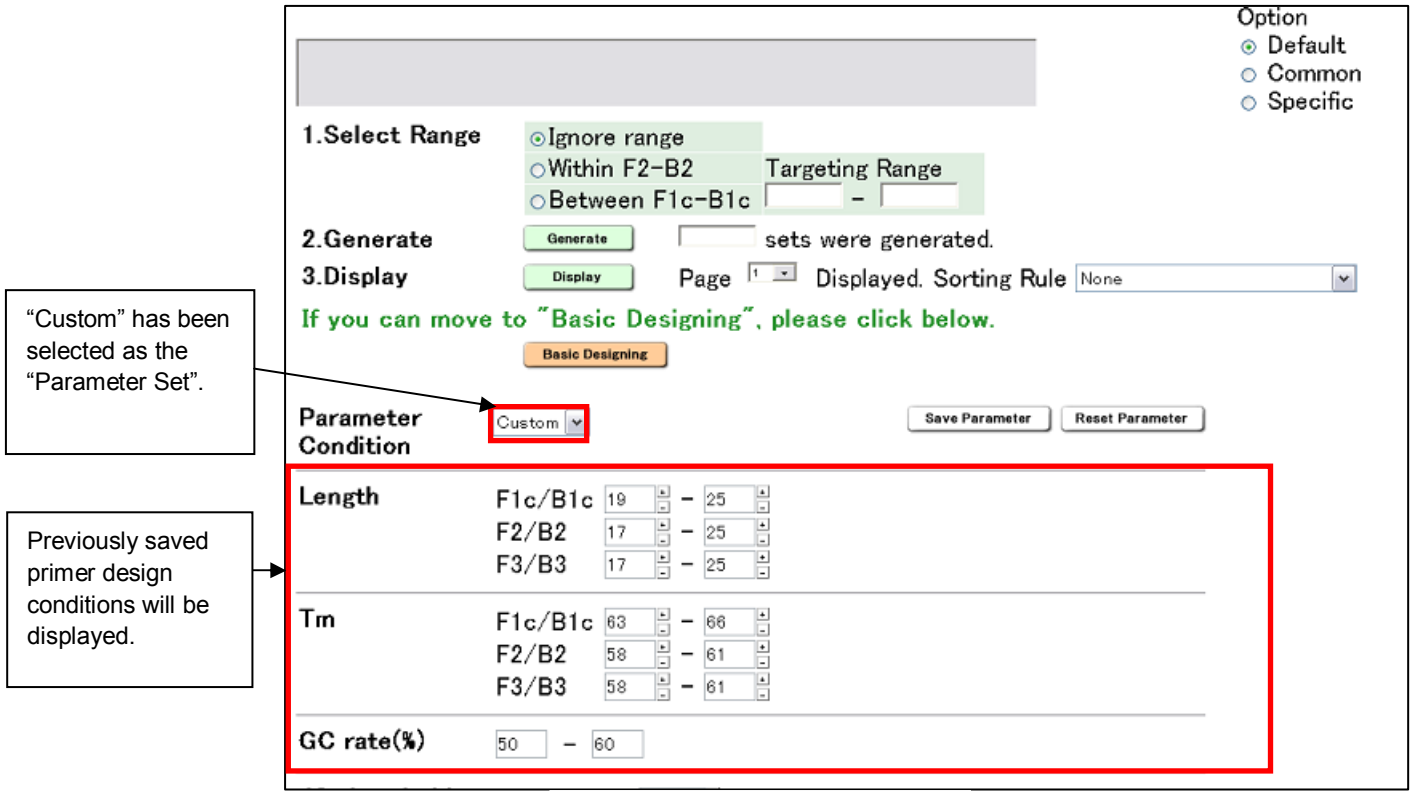

Figure 3.4 Primer design window

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)

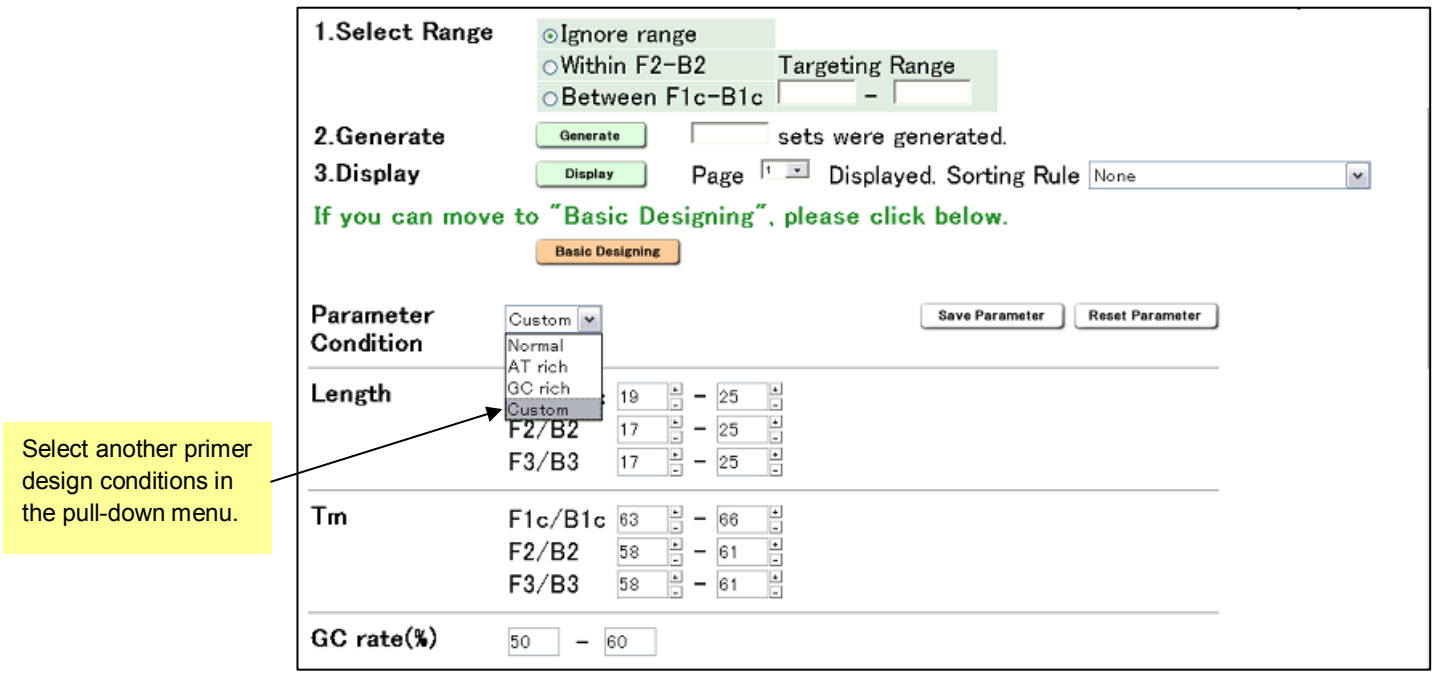

Figure 3.5 Changing the parameters

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.

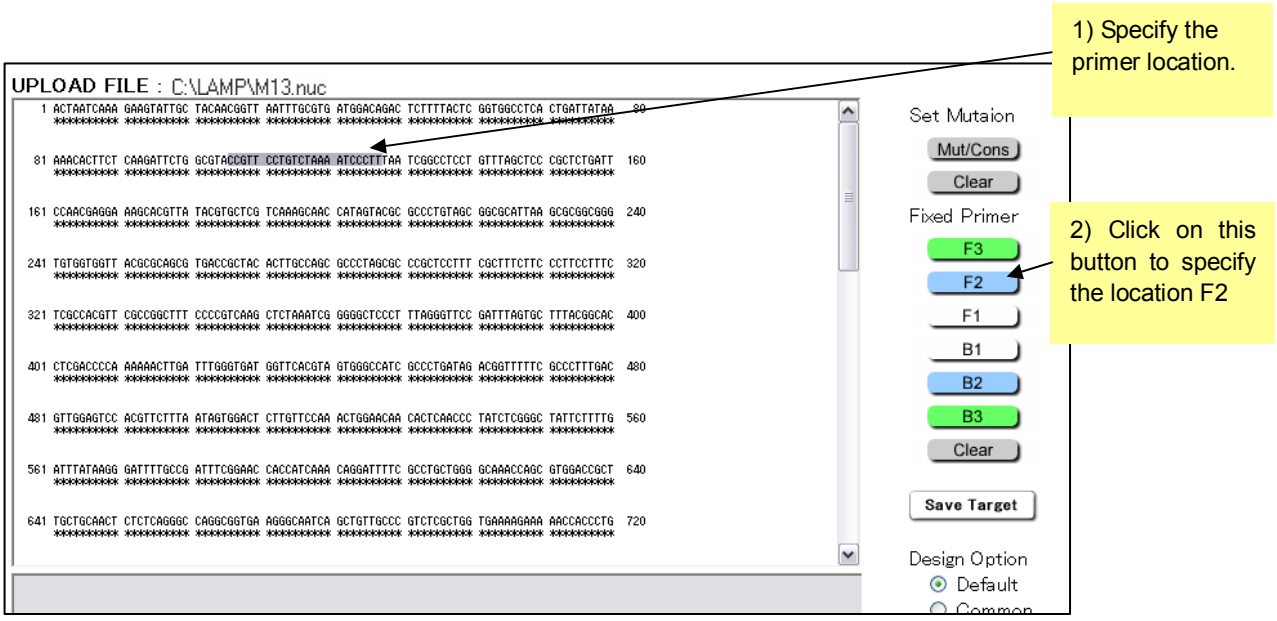

Figure 4.1 Primer design window

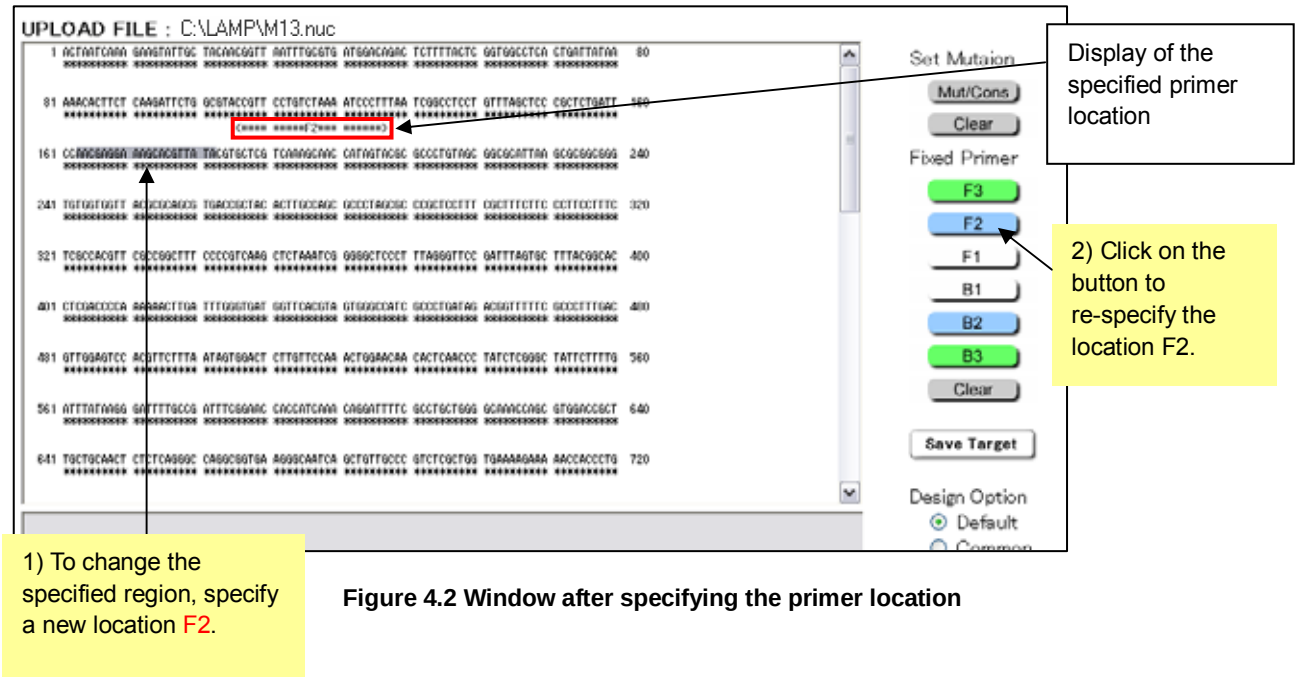

Figure 4.2 Window after specifying the primer location

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.

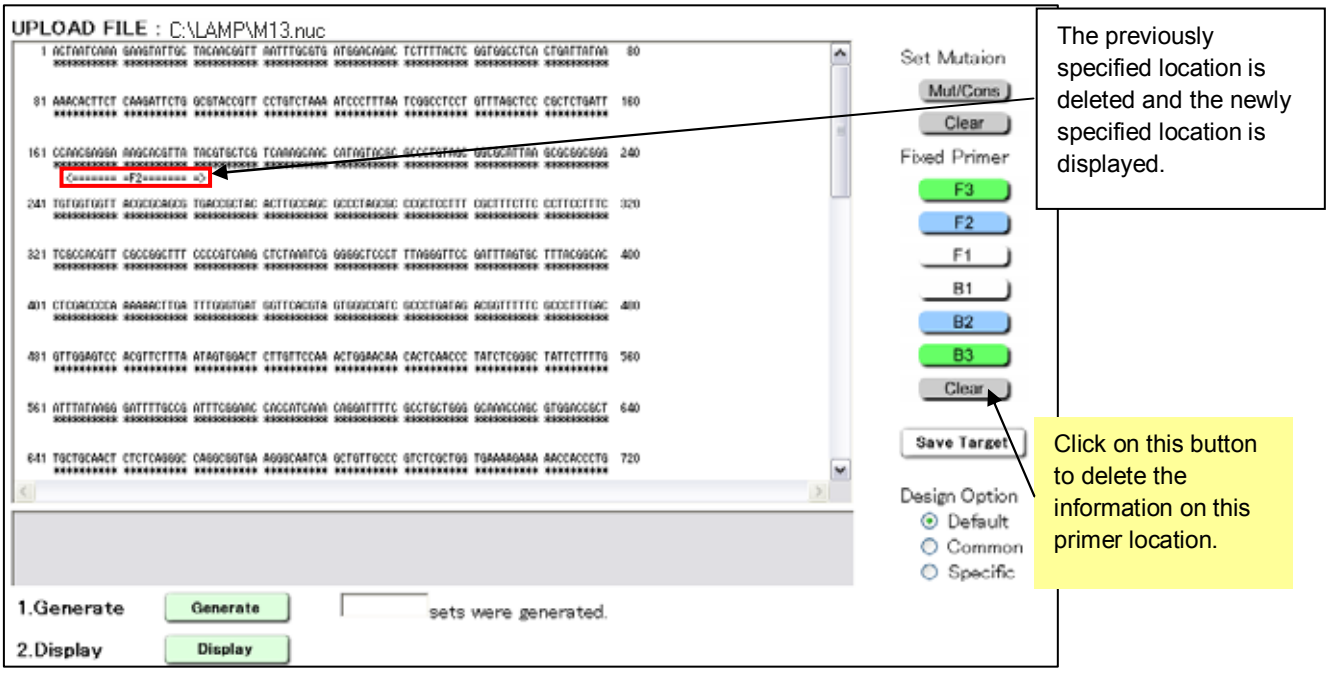

Figure 4.3 Primer design window

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)

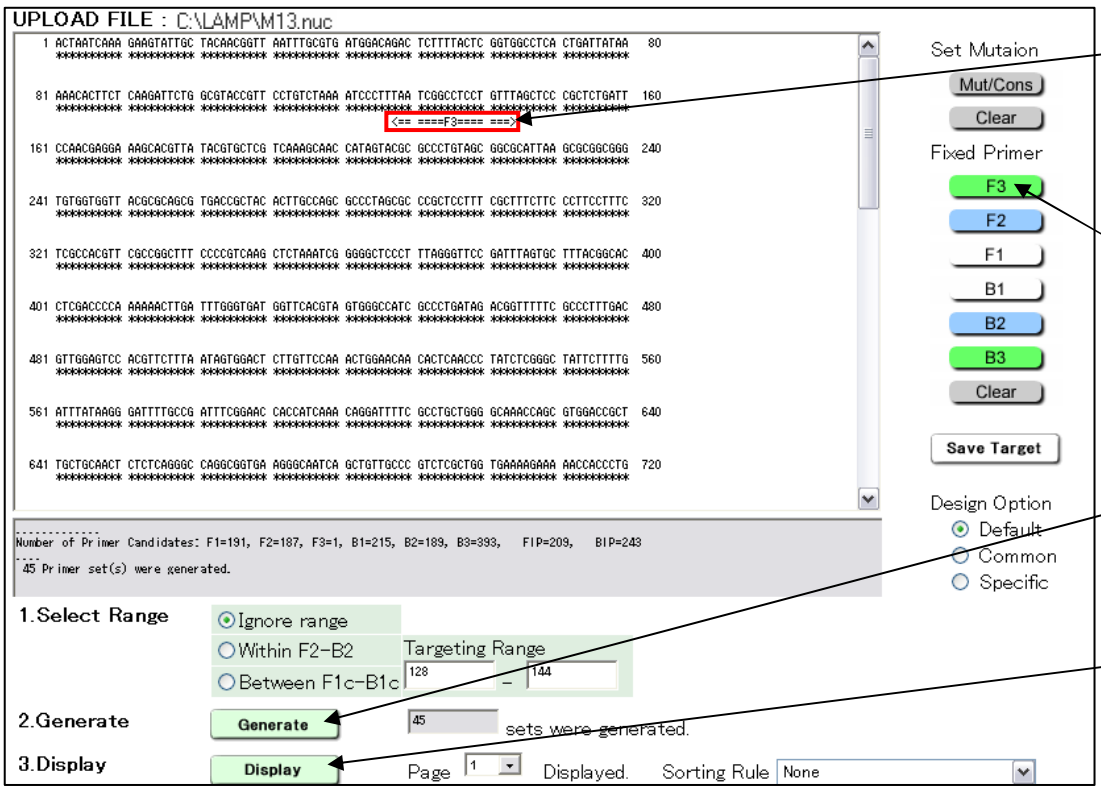

Figure 4.4 Primer design window

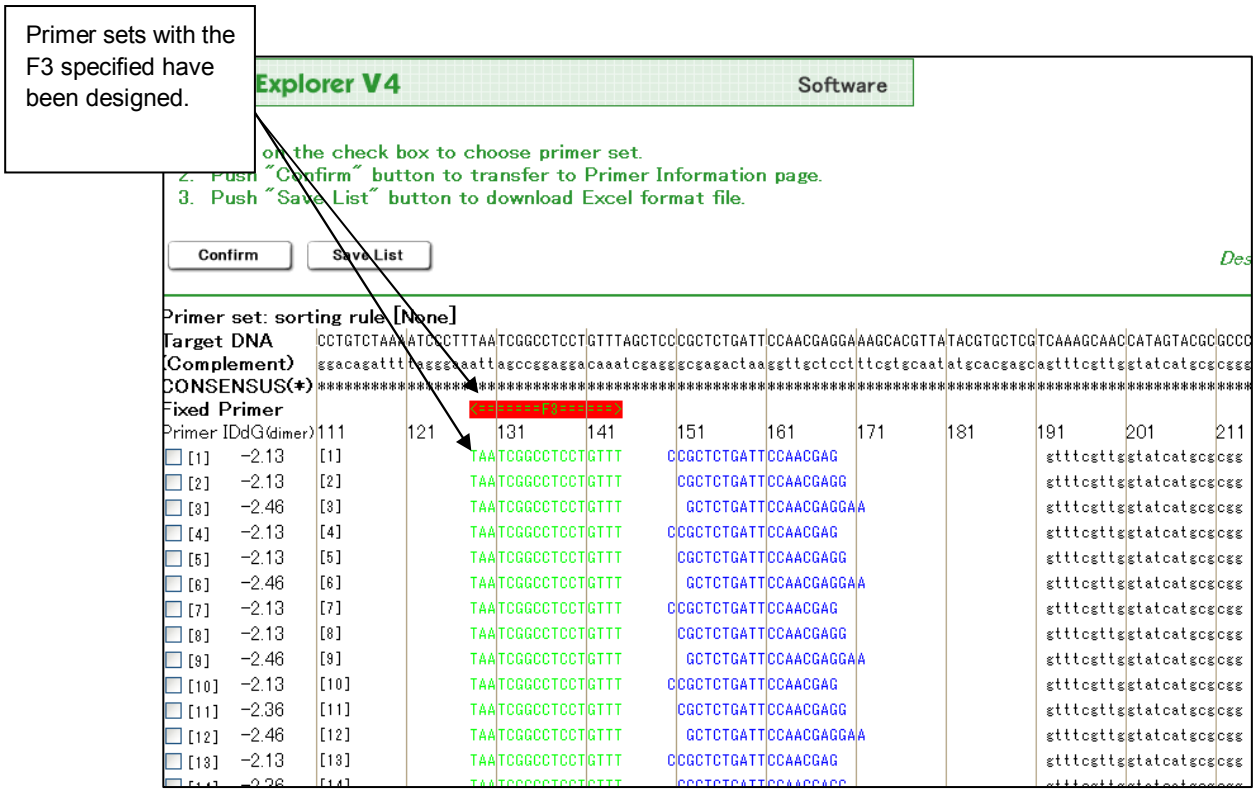

Figure 4.5 Primer Set List window

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)

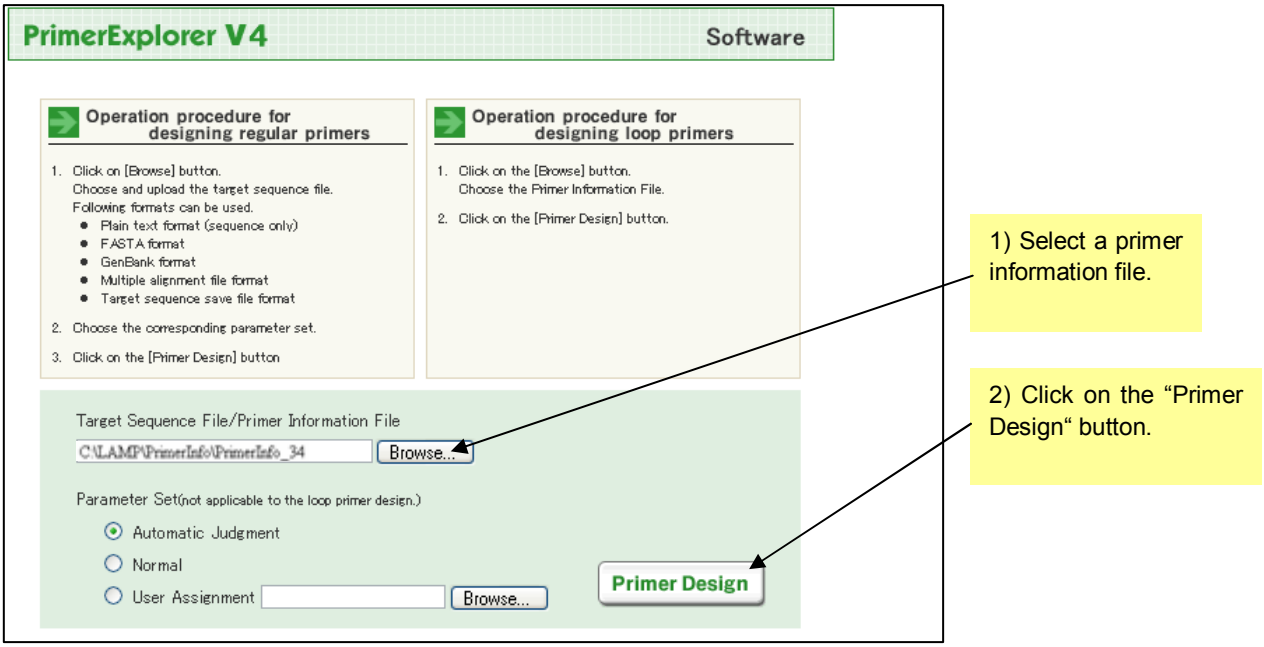

Figure 5.1 PrimerExplorer V3 startup window

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.

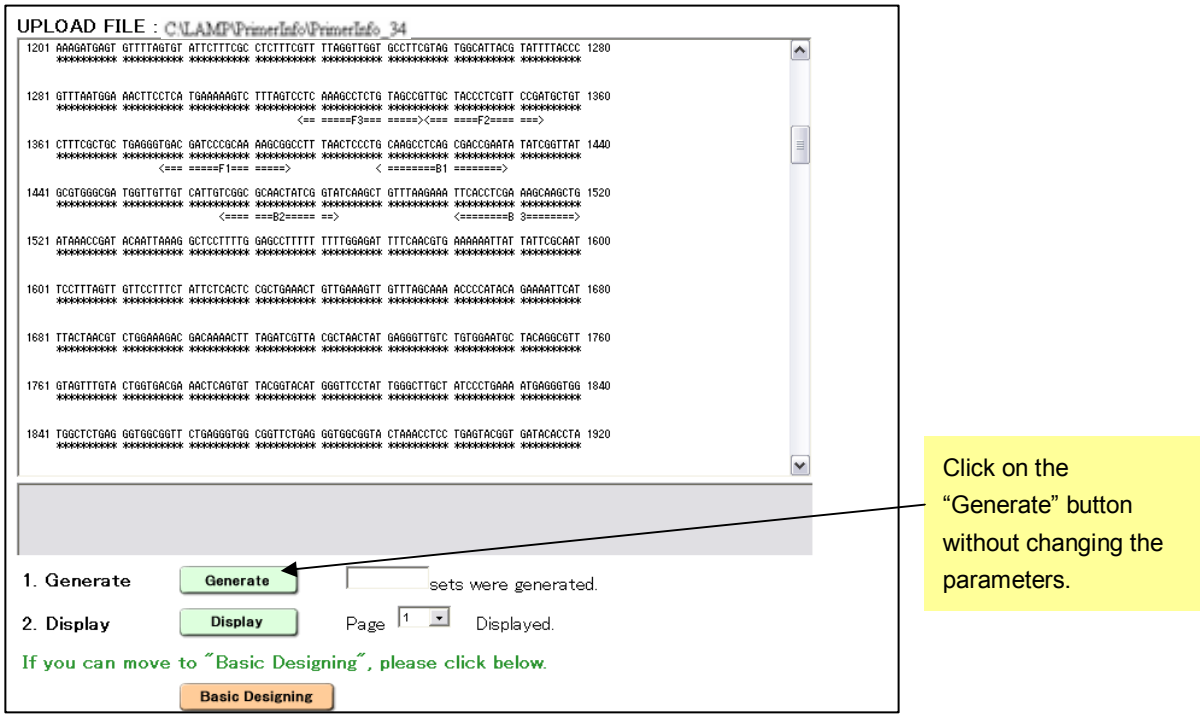

Figure 5.2 Loop primer design window

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)

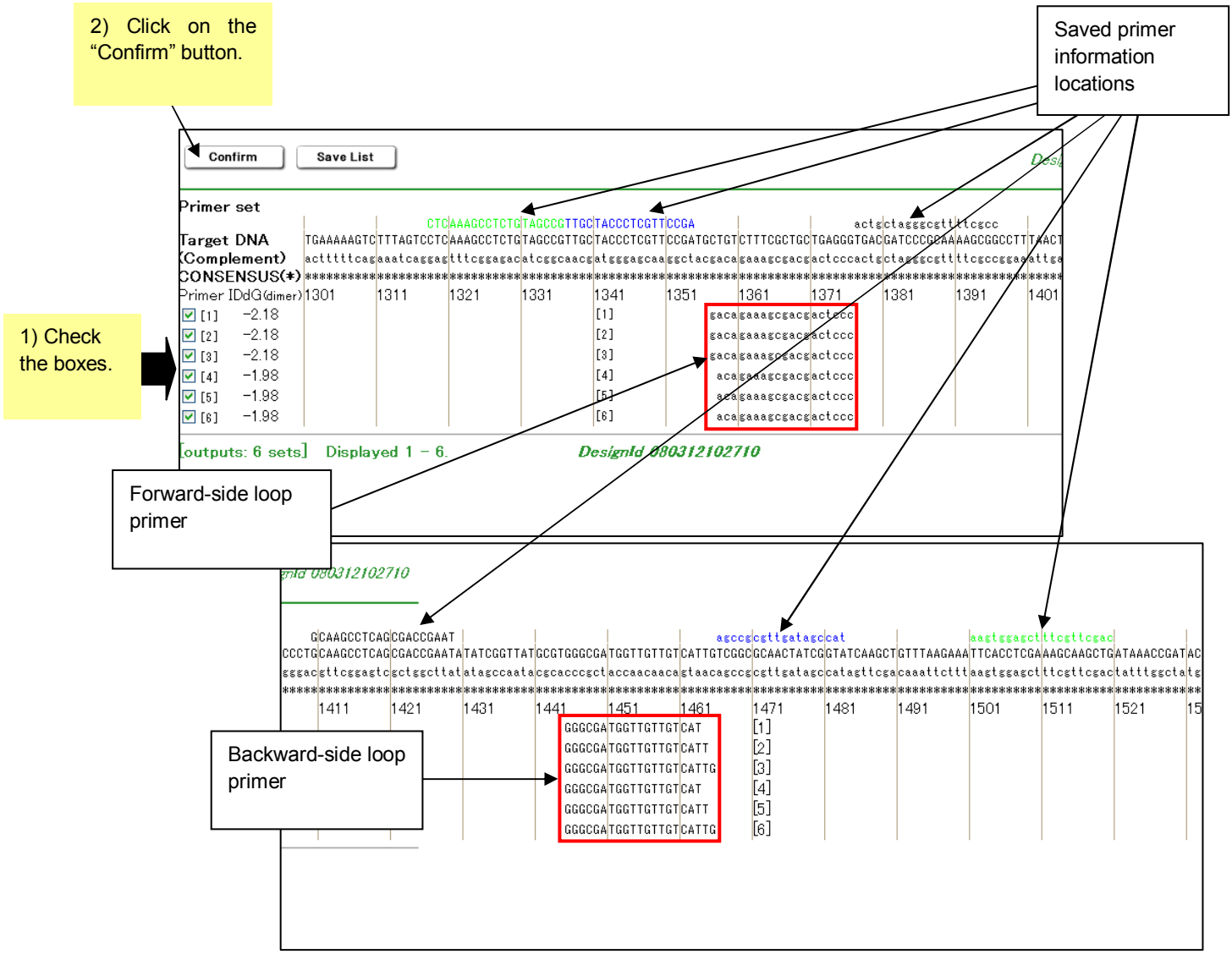

Figure 5.3 Loop primer design window (after primer design)

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.

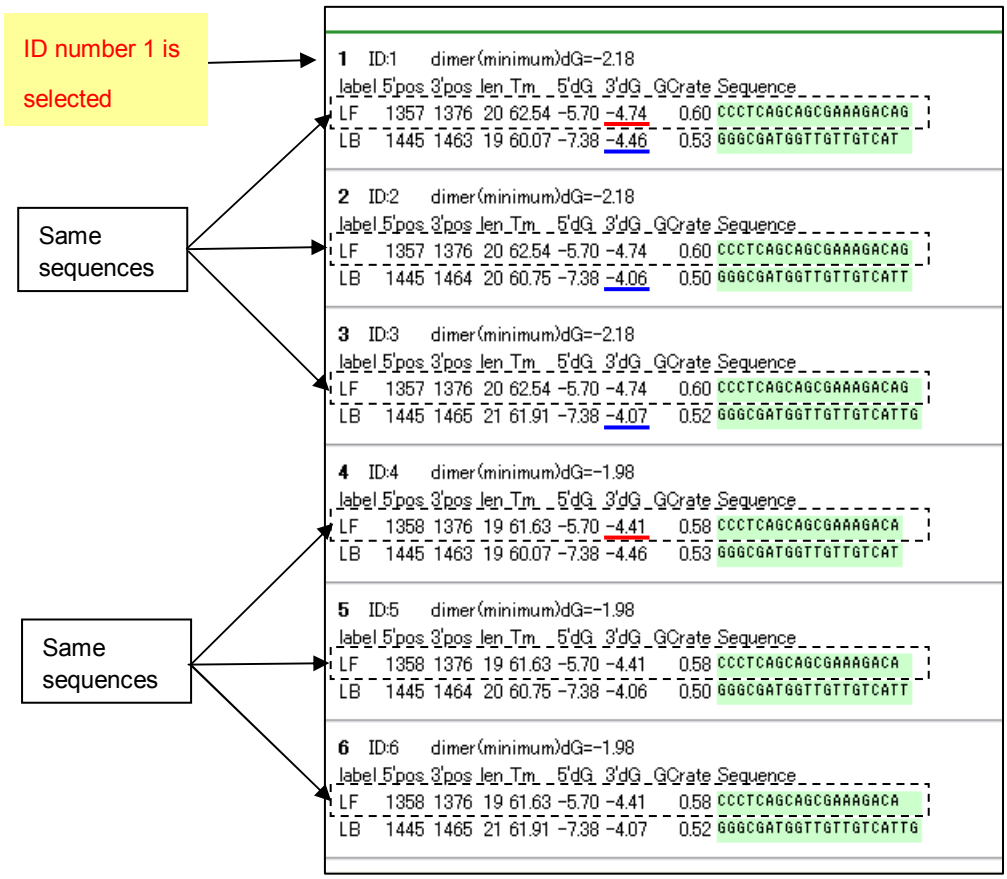

Figure 5.4 Primer Set Details window

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.