

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

When designing the primers, the user can change primer design conditions. The primer design conditions can be saved and revised.

#### 3.1 Changing and storing the primer design conditions

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.

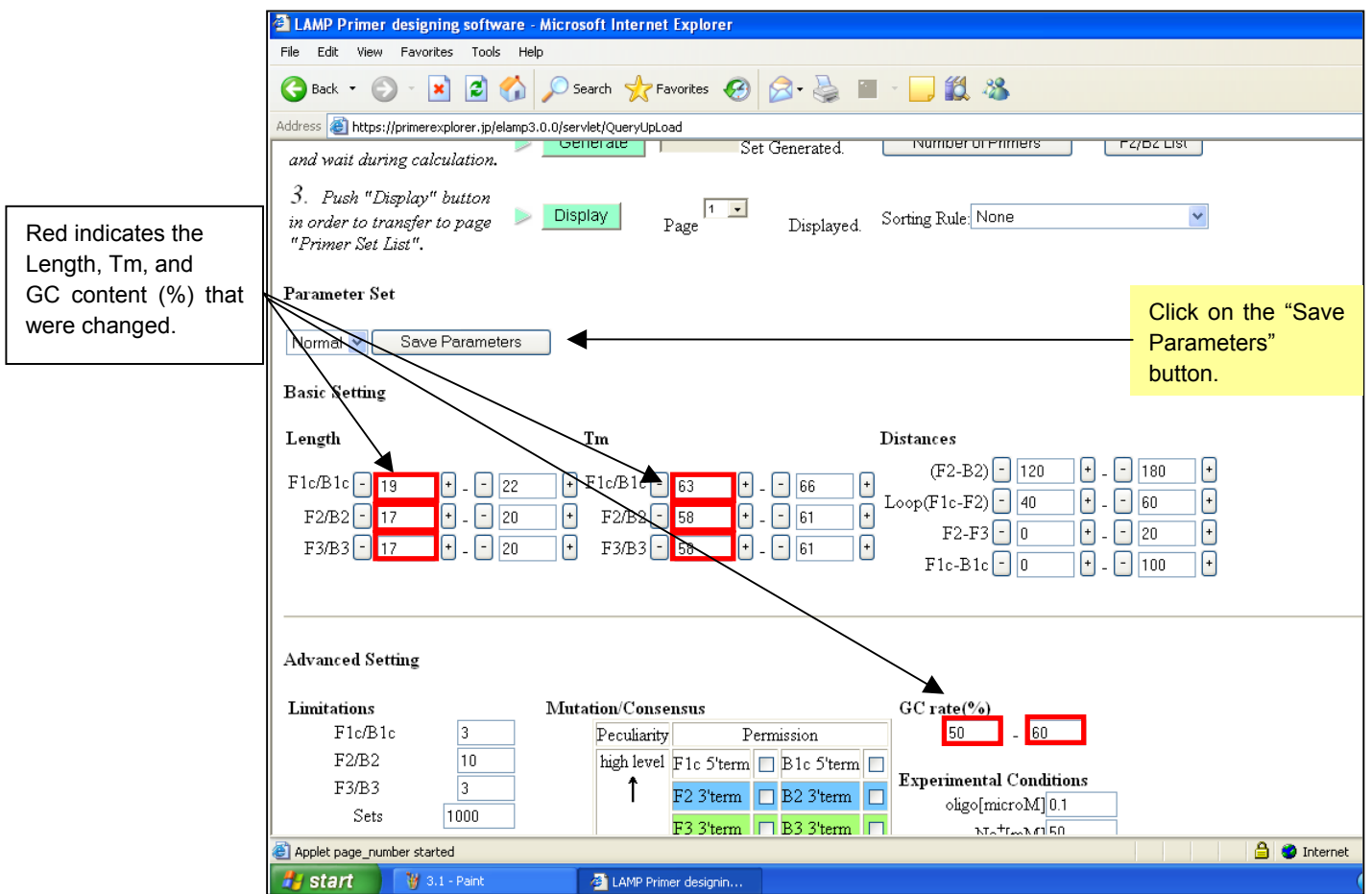


Figure 3.1 Changing the primer design conditions (Primer Design window)

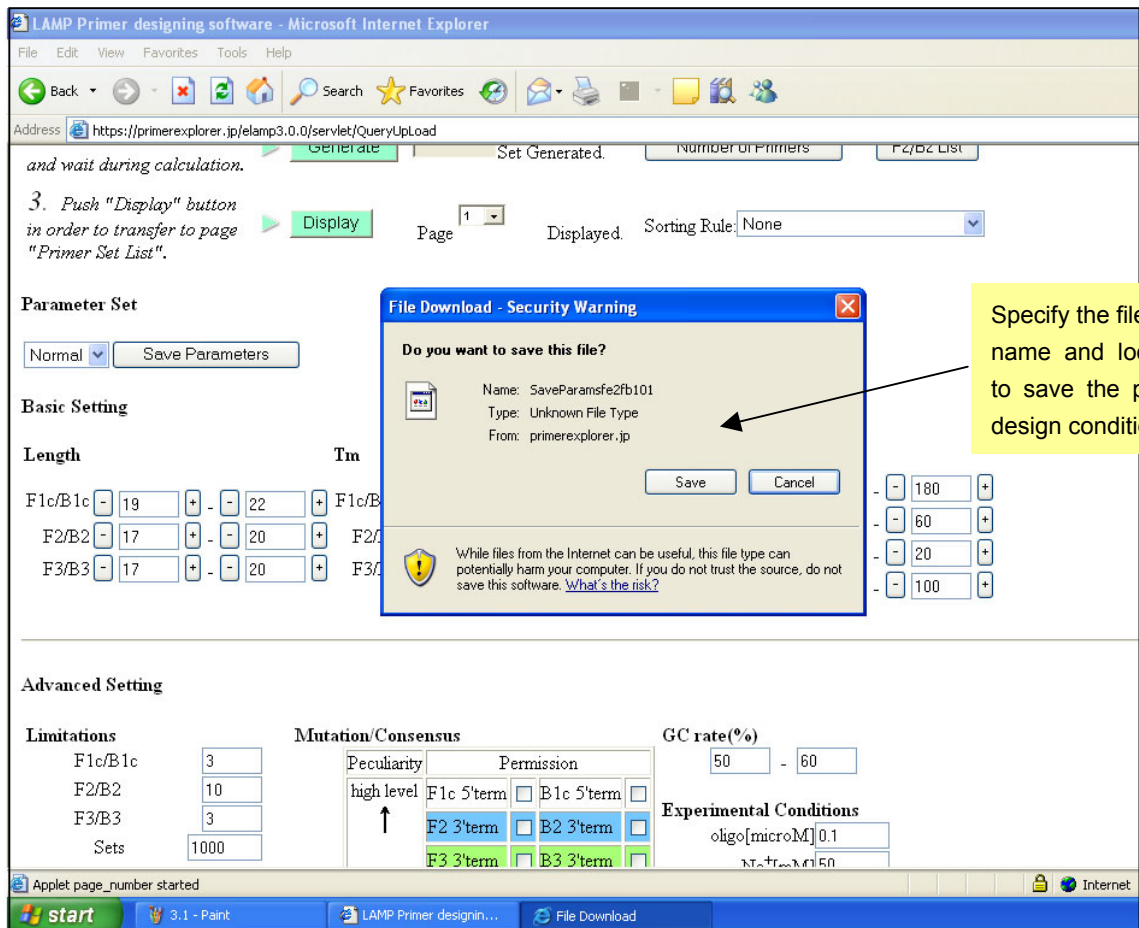


Figure 3.2 Saving the primer design conditions

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

Upload the target sequence in the startup window of the PrimerExplorer V3. 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.

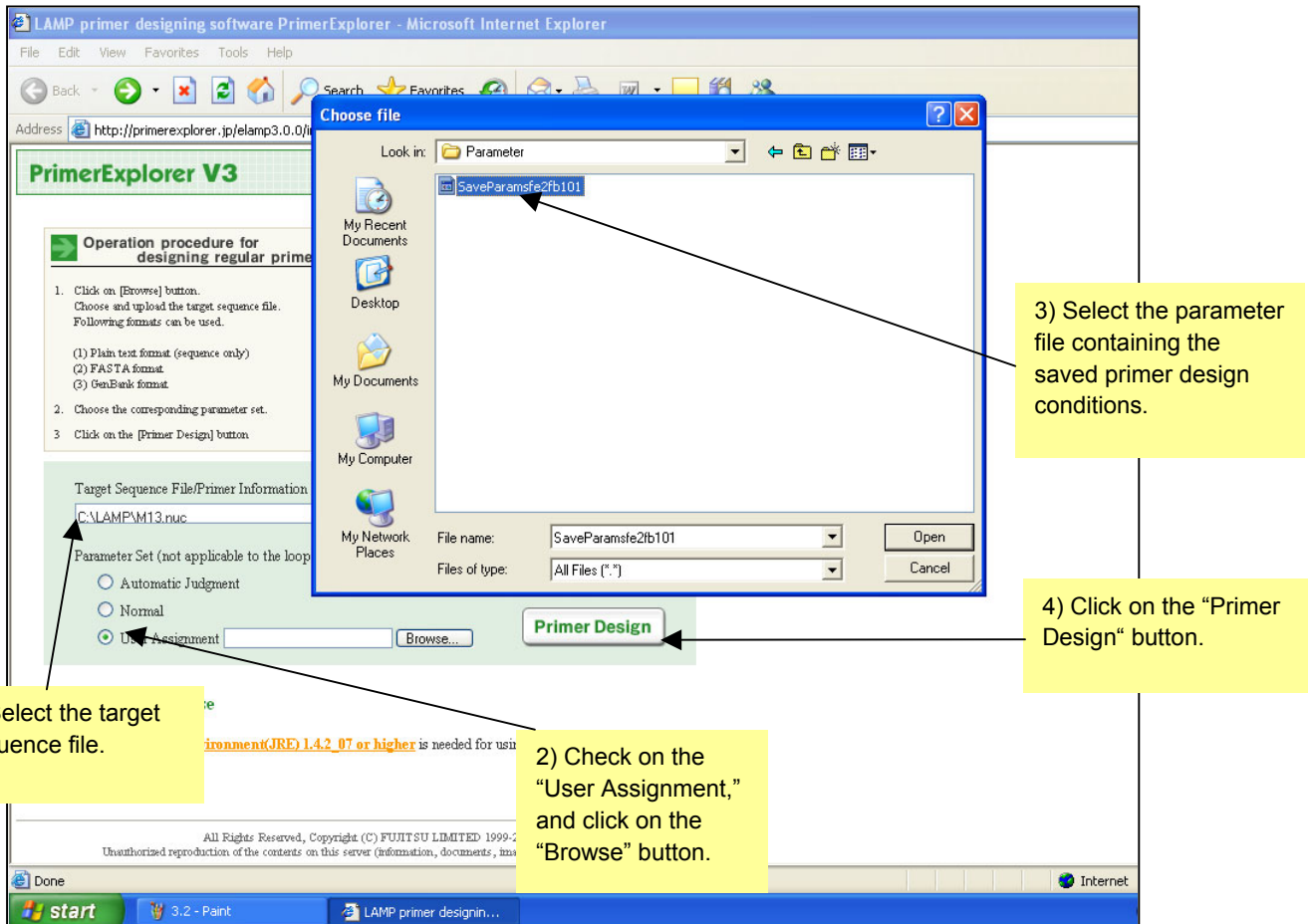


Figure 3.3 PrimerExplorer V3 startup window

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.12 - 14).

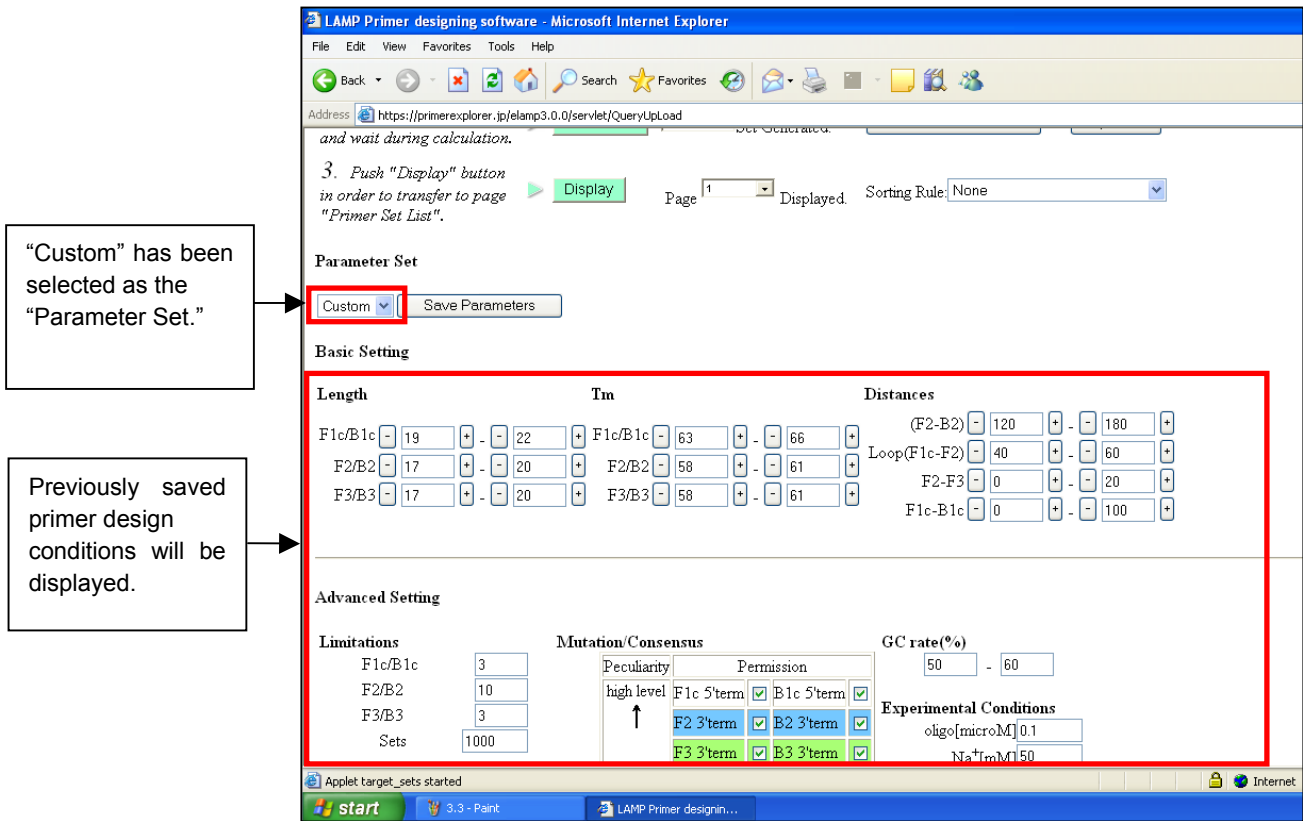


Figure 3.4 Primer design window

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)

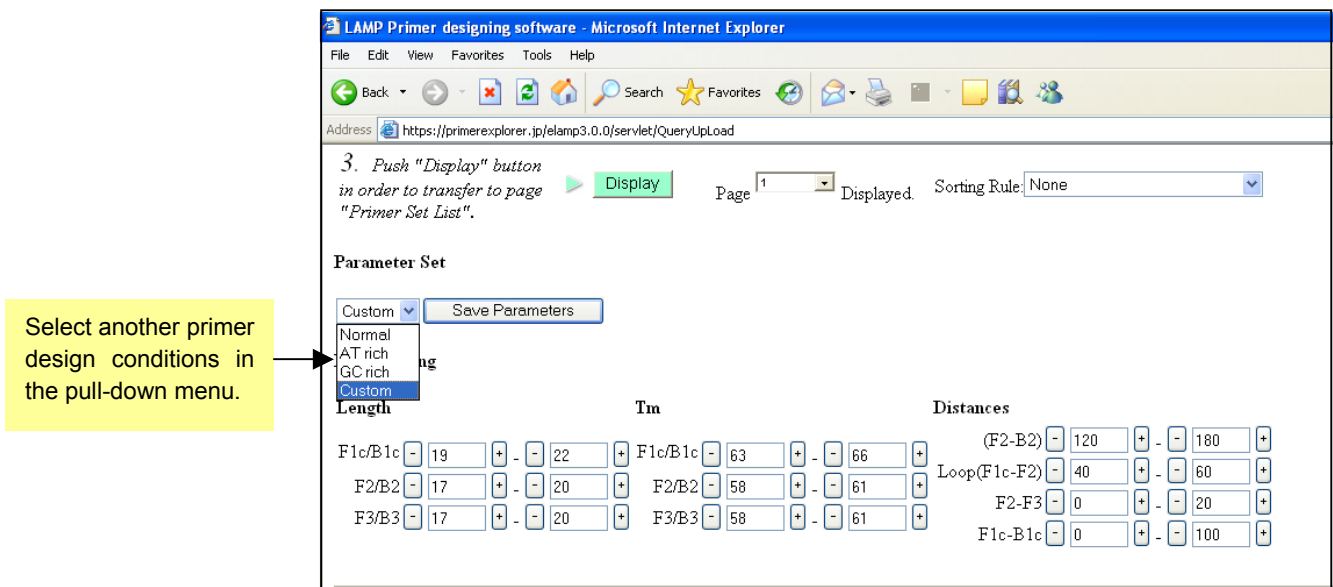


Figure 3.5 Changing the parameters

## 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 “F3” button is clicked, and as in Figure 4.2, the region specified as the location F3 will be displayed.

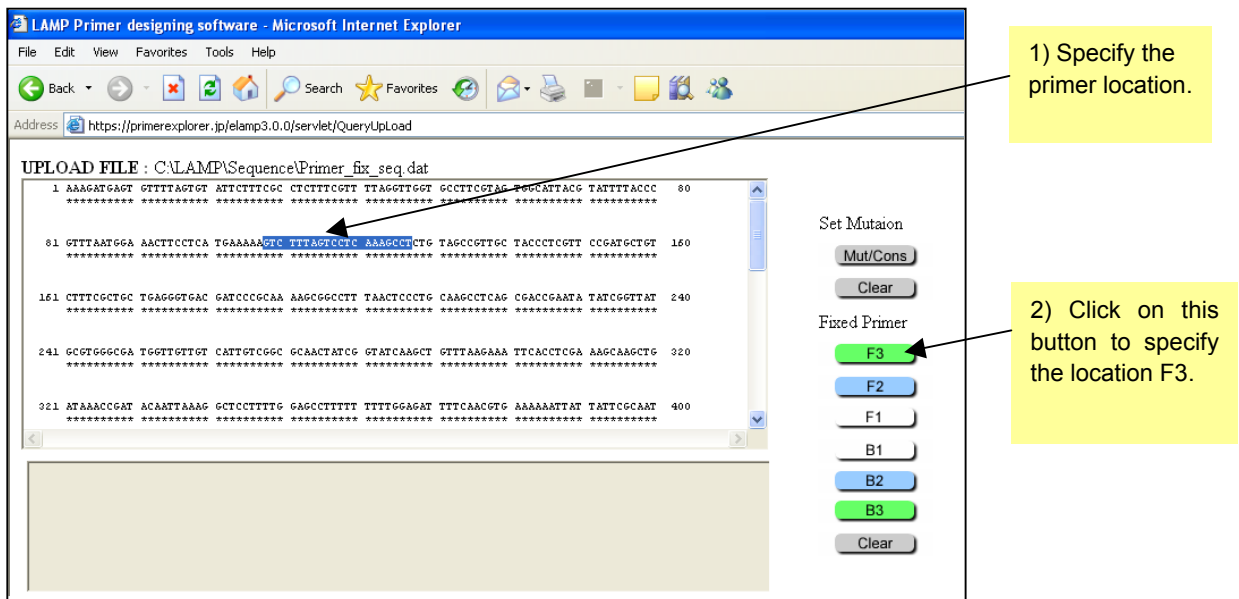


Figure 4.1 Primer design window

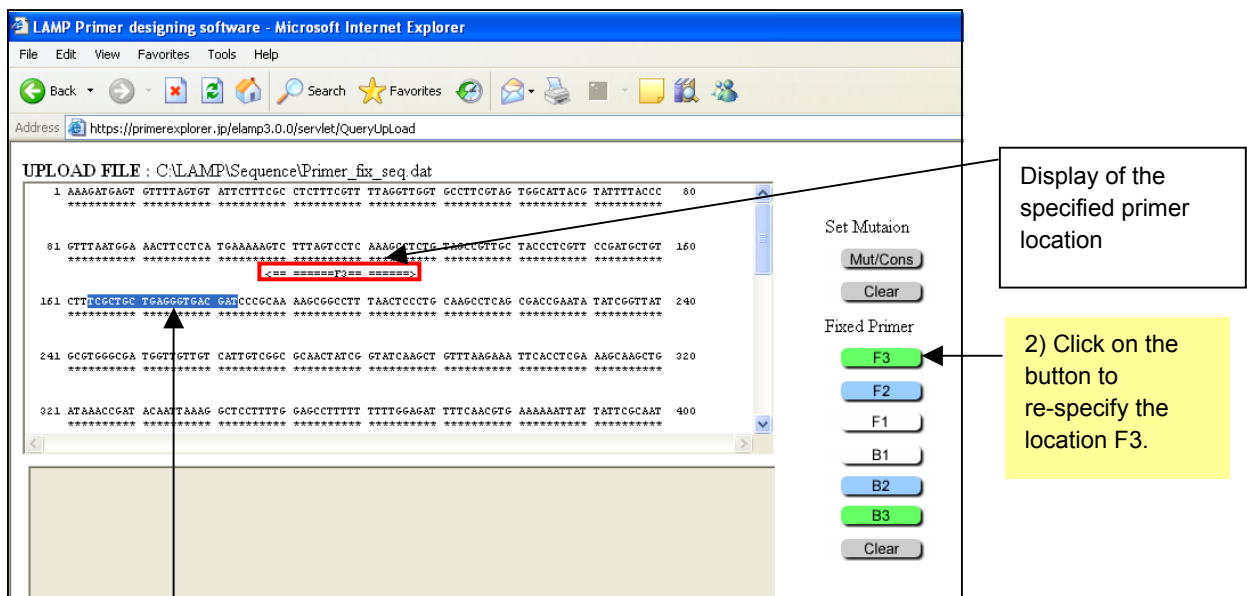


Figure 4.2 Window after specifying the primer location

1) To change the specified region, specify a new location F3.

To change the location F3 to some other location, specify another location as shown in Figure 4.2, and click on the “F3” button again. As shown in Figure 4.3, the new location is now specified as the location F3.

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

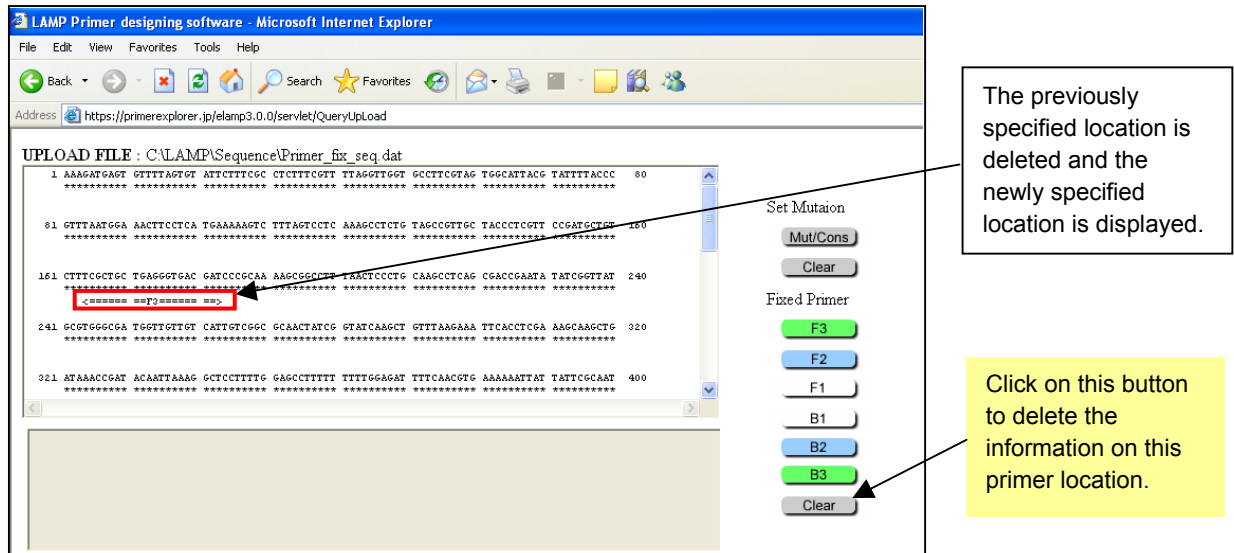


Figure 4.3 Window after re-specifying the primer location

#### 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)

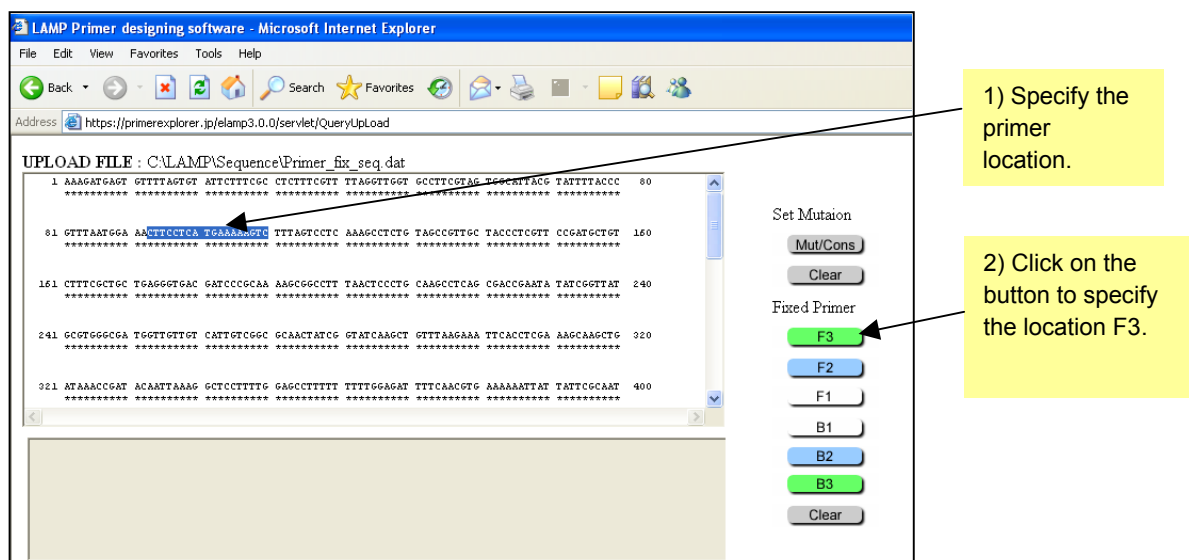


Figure 4.4 Primer design window

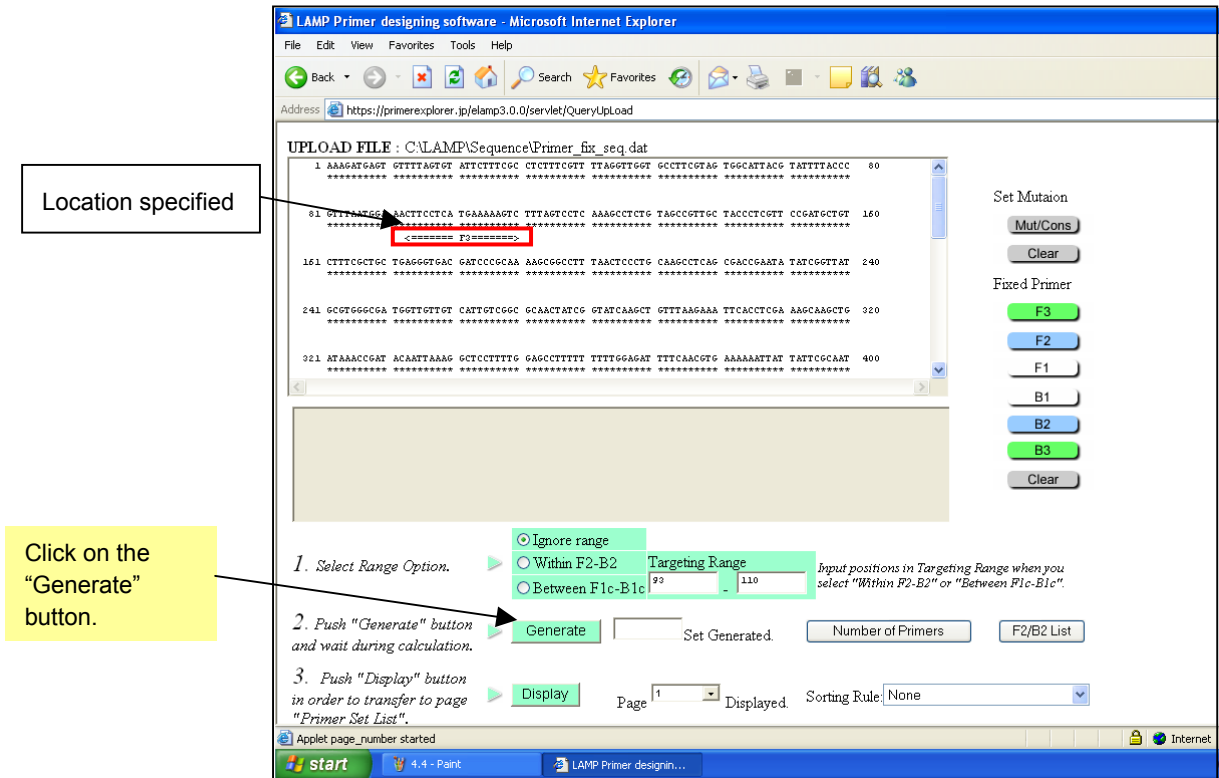


Figure 4.5 Primer design window (primer design after specifying the location)

After the primers have been designed, click on the "Display" button to display the Primer Set List window. (See Figure 4.6)

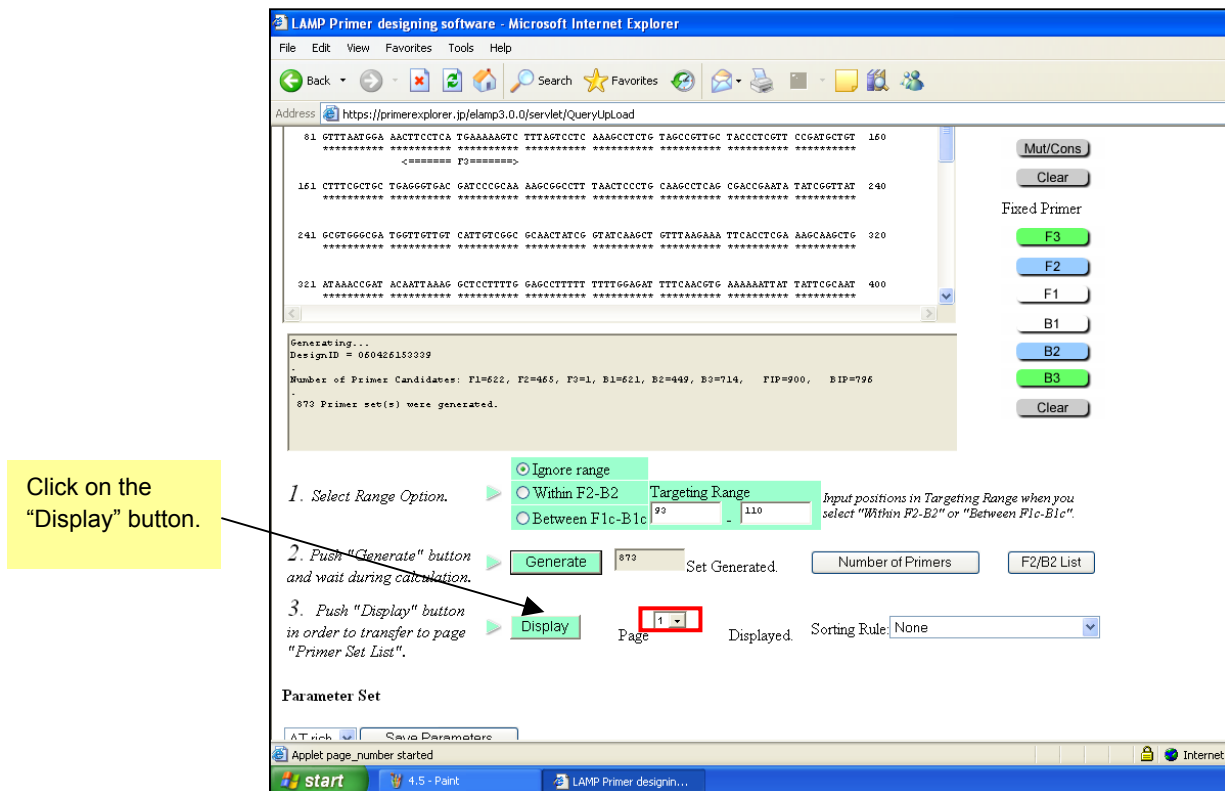


Figure 4.6 Primer design window (after primer design)

In Figure 4.7 Primer Set List window, the portion indicated in green capital letters corresponds to F3, which is in agreement with the previously specified location. Primer sets with the F3 specified have been designed.

Primer Set List

DesignId 060426153339

1. Turn on the check box of a primer set to see its detailed information.  
2. Push "Details" button in order to transfer to page "Primer Sets Details".

Details Sorting rule [None] / Displayed 1 - 100 / [outputs: 873 sets]

Primer set: sorting rule [None]

Target DNA CTTTAAATGGA AACTTCTCTCA TGA AAAAGTC TTTACTCTCTCA AAGCCTCTCTAGCCCTCTCTCCGATGCTCTCTTTTCCTCTCTGAGGGTGAACGATCCCGCAA AAGCGG  
(Complement) caaattaacctt gaaggagtc actttttcagaaatcagagagcttcggyagacatcggcaacgatgggagcaaggctacgacagaagagcgactcccactgctagggcgttttcggc  
CONSENSUS(\*) .....

Primer ID	dG(dimer)	Primer			
<input type="checkbox"/> [1]	-2.34	[11]	CTTCTCTCA TGA AAAAGTC TTTACTCTCTCA AAGCCTCTCTG	gctacgacagaagcgacgga	CGCAA AAGCGG
<input type="checkbox"/> [2]	-2.34	[12]	CTTCTCTCA TGA AAAAGTC TTTACTCTCTCA AAGCCTCTCTG	gctacgacagaagcgacgga	CGCAA AAGCGG
<input type="checkbox"/> [3]	-2.34	[13]	CTTCTCTCA TGA AAAAGTC TTTACTCTCTCA AAGCCTCTCTG	gctacgacagaagcgacgga	CGCAA AAGCGG
<input type="checkbox"/> [4]	-2.16	[14]	CTTCTCTCA TGA AAAAGTC TTTACTCTCTCA AAGCCTCTCTG	ctacgacagaagcgacgac	CGCAA AAGCGG
<input type="checkbox"/> [5]	-2.16	[15]	CTTCTCTCA TGA AAAAGTC TTTACTCTCTCA AAGCCTCTCTG	tacgacagaagcgacgact	CGCAA AAGCGG
<input type="checkbox"/> [6]	-1.97	[16]	CTTCTCTCA TGA AAAAGTC TTTACTCTCTCA AAGCCTCTCTG	gctacgacagaagcgacgga	AAGCGG
<input type="checkbox"/> [7]	-1.97	[17]	CTTCTCTCA TGA AAAAGTC TTTACTCTCTCA AAGCCTCTCTG	gctacgacagaagcgacgga	AAGCGG
<input type="checkbox"/> [8]	-2.16	[8]	CTTCTCTCA TGA AAAAGTC TTTACTCTCTCA AAGCCTCTCTG	gctacgacagaagcgacgga	AAGCGG
<input type="checkbox"/> [9]	-2.16	[9]	CTTCTCTCA TGA AAAAGTC TTTACTCTCTCA AAGCCTCTCTG	ctacgacagaagcgacgac	AAGCGG
<input type="checkbox"/> [10]	-2.16	[10]	CTTCTCTCA TGA AAAAGTC TTTACTCTCTCA AAGCCTCTCTG	tacgacagaagcgacgact	AAGCGG
<input type="checkbox"/> [11]	-2.34	[11]	CTTCTCTCA TGA AAAAGTC TTTACTCTCTCA AAGCCTCTCTG	gctacgacagaagcgacgga	CGG
<input type="checkbox"/> [12]	-2.34	[12]	CTTCTCTCA TGA AAAAGTC TTTACTCTCTCA AAGCCTCTCTG	gctacgacagaagcgacgga	CGG
<input type="checkbox"/> [13]	-2.34	[13]	CTTCTCTCA TGA AAAAGTC TTTACTCTCTCA AAGCCTCTCTG	gctacgacagaagcgacgga	CGG
<input type="checkbox"/> [14]	-2.16	[14]	CTTCTCTCA TGA AAAAGTC TTTACTCTCTCA AAGCCTCTCTG	ctacgacagaagcgacgac	CGG
<input type="checkbox"/> [15]	-2.16	[15]	CTTCTCTCA TGA AAAAGTC TTTACTCTCTCA AAGCCTCTCTG	tacgacagaagcgacgact	CGG
<input type="checkbox"/> [16]	-1.97	[16]	CTTCTCTCA TGA AAAAGTC TTTACTCTCTCA AAGCCTCTCTG	gctacgacagaagcgacgga	AAGCGG
<input type="checkbox"/> [17]	-1.97	[17]	CTTCTCTCA TGA AAAAGTC TTTACTCTCTCA AAGCCTCTCTG	gctacgacagaagcgacgga	AAGCGG

Primer sets with the F3 specified have been designed.

Figure 4.7 Primer Set List window (page 1)

From the primers designed, the primer sets are selected using the procedure described in Section 1 (see p.12 - 14).



## 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)

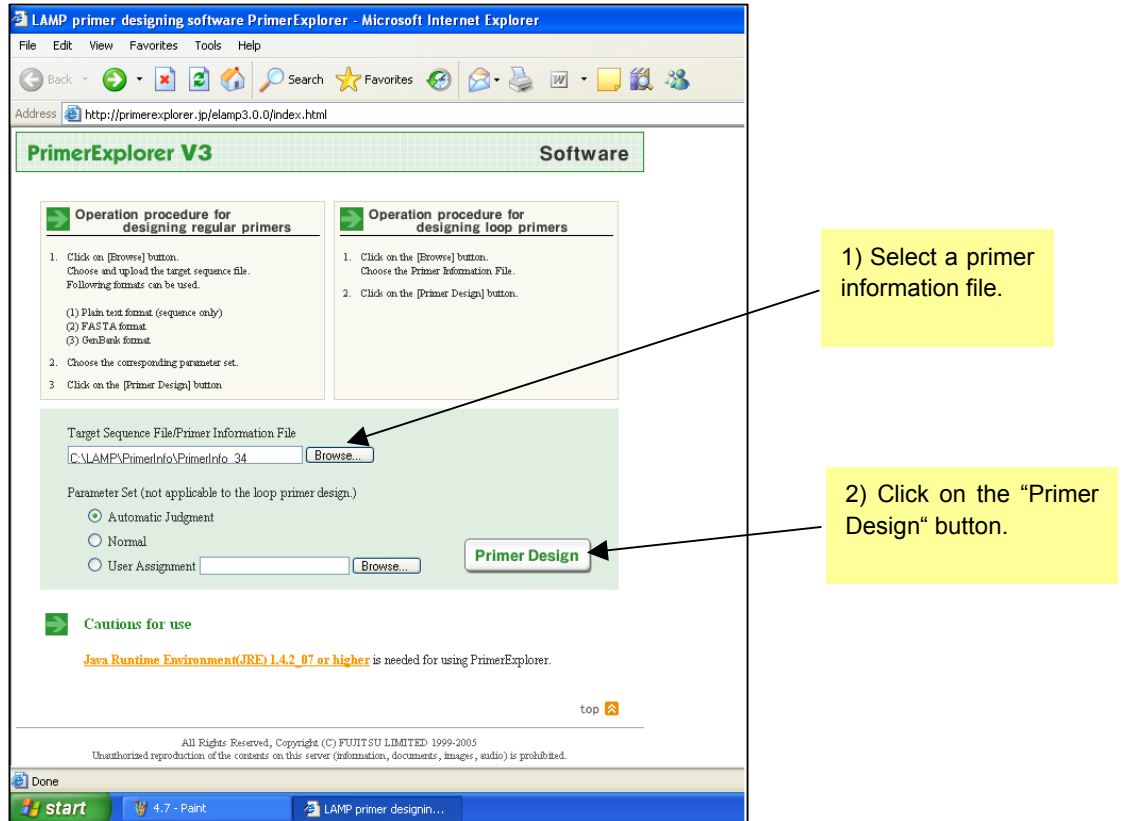


Figure 5.1 PrimerExplorer V3 startup window

### 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.

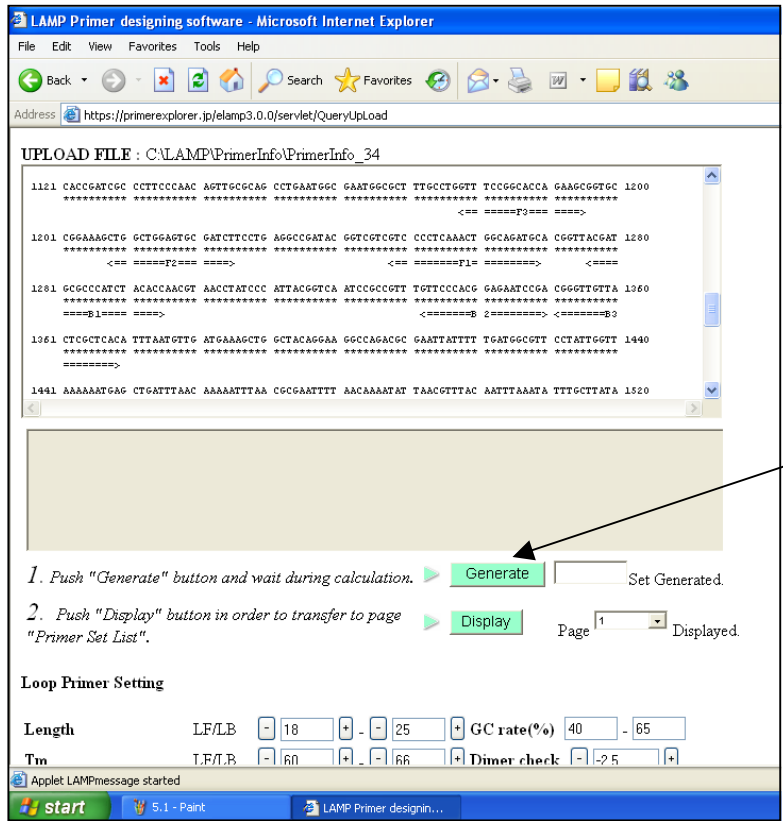


Figure 5.2 Loop primer design window

A total of 20 sets of primer will be generated. Click on the “Display” button to display the Primer Set List (See Figure 5.3)

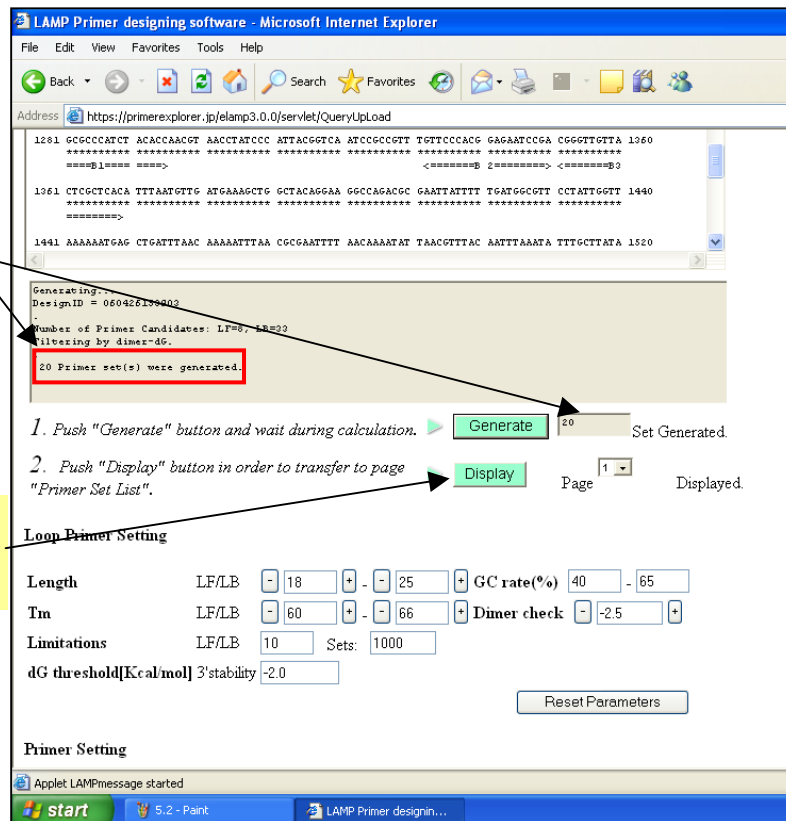


Figure 5.3 Loop primer design window (after primer design)

Figure 5.4 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.

**Primer Set List** *DesignId 021205164445*

1. Turn on the check box of a primer set to see its detailed information.  
2. Push "Detail" button in order to transfer to page "Primer Set Detail".

**Details** Displayed 1 - 6 / [outputs: 6 sets]

Primer ID(dimer)	1301	1311	1321	1331	1341	1351	1361	1371	1381	1391	140
<input checked="" type="checkbox"/> [1]	-2.18						gacagaaagcagcactccc				
<input checked="" type="checkbox"/> [2]	-2.18						gacagaaagcagcactccc				
<input checked="" type="checkbox"/> [3]	-2.18						gacagaaagcagcactccc				
<input checked="" type="checkbox"/> [4]	-1.98						acagaaagcagcactccc				
<input checked="" type="checkbox"/> [5]	-1.98						acagaaagcagcactccc				
<input checked="" type="checkbox"/> [6]	-1.98						acagaaagcagcactccc				

[outputs: ...]

**Forward-side loop primer**

**Backward-side loop primer**

Figure 5.4 Primer Set List window

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 "Details" button to display the Primer Set Details window.

### 5.3 Narrowing down the loop primer set candidates

Primer Set Details window (Figure 5.5) shows the detailed information on the six loop primer sets previously selected.

Primer Sets Details

1. Turn on the check box to make an order.  
2. Push "Confirm" button in order to transfer to page "Selected Pri

ID	dimer(minimum)dG	label	5'pos	3'pos	len	Tm	5'dG	3'dG	GCrate	Sequence
<input checked="" type="checkbox"/> ID:1	-2.18		1357	1376	20	62.54	-5.70	-4.74	0.60	CCCTCAGCAGCGAAAGACA
		LF								
		LB	1445	1463	19	60.07	-7.38	-4.46	0.53	GGCCGATGTTGTTGTCAT
<input type="checkbox"/> ID:2	-2.18		1357	1376	20	62.54	-5.70	-4.74	0.60	CCCTCAGCAGCGAAAGACA
		LF								
		LB	1445	1464	20	60.75	-7.38	-4.06	0.50	GGCCGATGTTGTTGTCATT
<input type="checkbox"/> ID:3	-2.18		1357	1376	20	62.54	-5.70	-4.74	0.60	CCCTCAGCAGCGAAAGACA
		LF								
		LB	1445	1465	21	61.91	-7.38	-4.07	0.52	GGCCGATGTTGTTGTCATTG
<input type="checkbox"/> ID:4	-1.98		1358	1376	19	61.63	-5.70	-4.41	0.58	CCCTCAGCAGCGAAAGACA
		LF								
		LB	1445	1463	19	60.07	-7.38	-4.46	0.53	GGCCGATGTTGTTGTCAT
<input type="checkbox"/> ID:5	-1.98		1358	1376	19	61.63	-5.70	-4.41	0.58	CCCTCAGCAGCGAAAGACA
		LF								
		LB	1445	1464	20	60.75	-7.38	-4.06	0.50	GGCCGATGTTGTTGTCATT
<input type="checkbox"/> ID:6	-1.98		1358	1376	19	61.63	-5.70	-4.41	0.58	CCCTCAGCAGCGAAAGACA
		LF								
		LB	1445	1465	21	61.91	-7.38	-4.07	0.52	GGCCGATGTTGTTGTCATTG

Figure 5.5 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. Check the primer ID number 1 and click on the "Confirm" button. The confirmed loop primer information is displayed and this is the final screen for loop primer design.

## 6. Primer design that takes the location of mutation into account

### 6.1 Uploading the target sequence

This section explains primer design for amplifying the wild type and mutant type strains together using common primers, or for selectively amplifying the mutant type only.

Open the PrimerExplorer V3 startup window, use the steps outlined in Section 1 to select the target sequence file (see p.8) and then click on the “primer design button.” (Figure not shown)

### 6.2 Designing primers that do not include the mutation by entering the location of the mutation over the target sequence

We now explain the design of primers that do not include the location of the mutation. Click on the “Mutation” button after specifying the location of the mutation in the target sequence in the primer design window (Figure 6.1). As indicated in Figure 6.2, the indicator of the location of the mutation changes from a star (\*) to a hyphen (-). This status indicates that the entry has been accepted. To delete this mutation information, click on the “Clear” button.

1) Specify the location of the mutation in the sequence.

2) Click on the “Mutation” button.

Figure 6.1 Primer design window

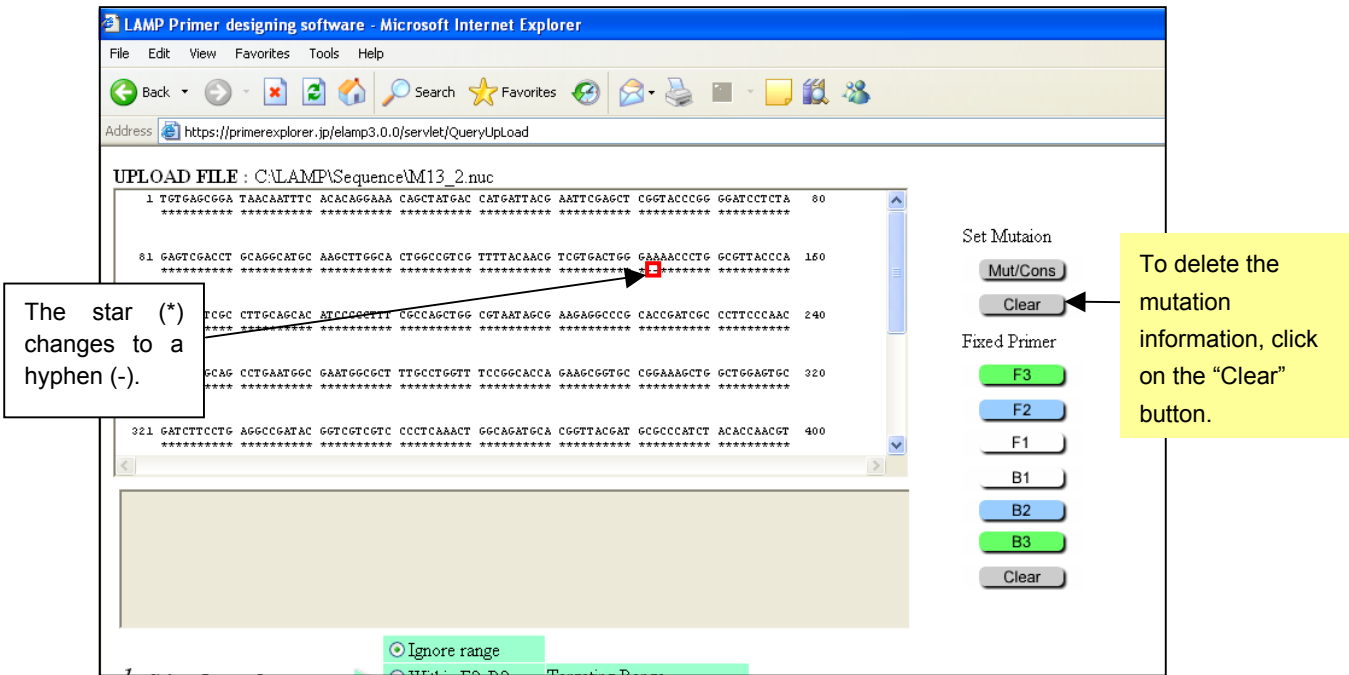


Figure 6.2 Window after entering the location of mutation

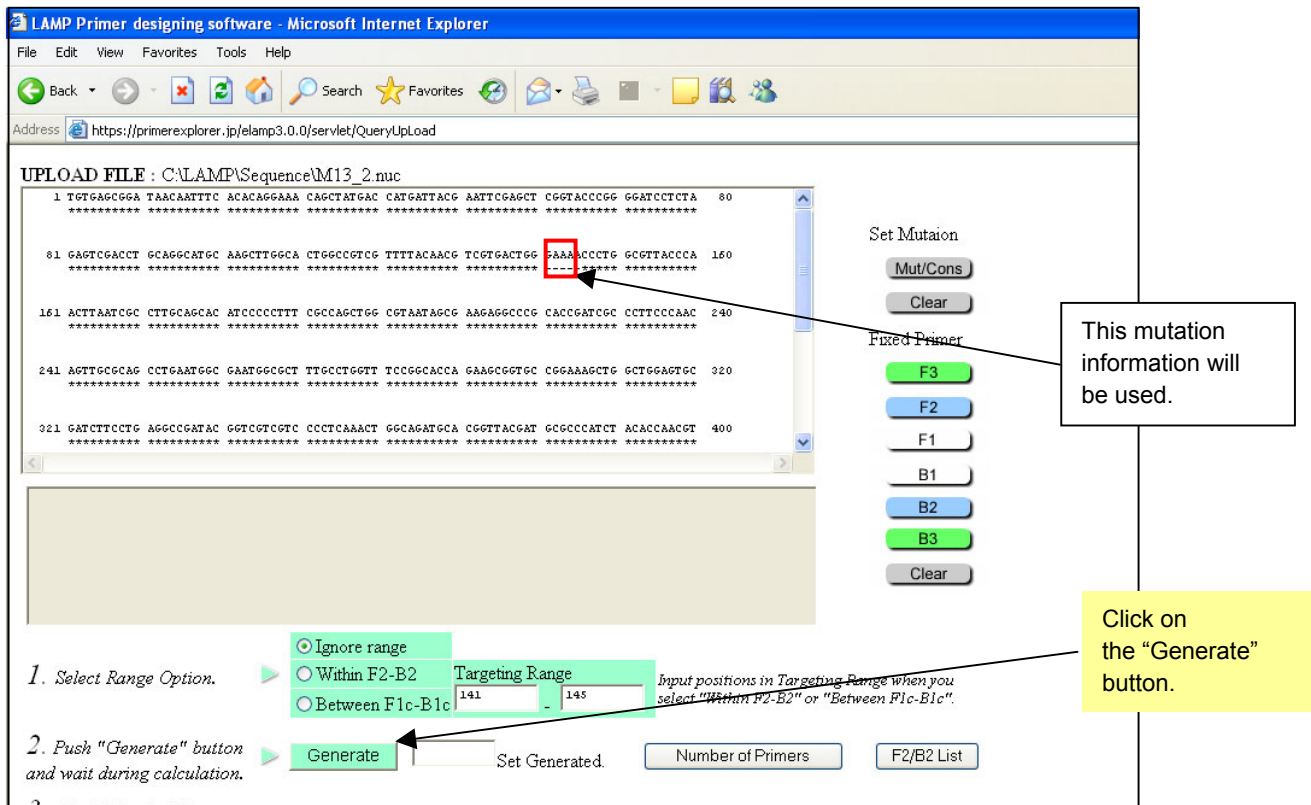


Figure 6.3 Window after entering site of mutation

Now, we will enter a mutation at another location (a different mutation). Here, the primers will be designed based on the re-entered mutation information (See Figure 6.3). The primers are designed to avoid including the mutation. 1000 primer sets have been designed. (See Figure 6.4)

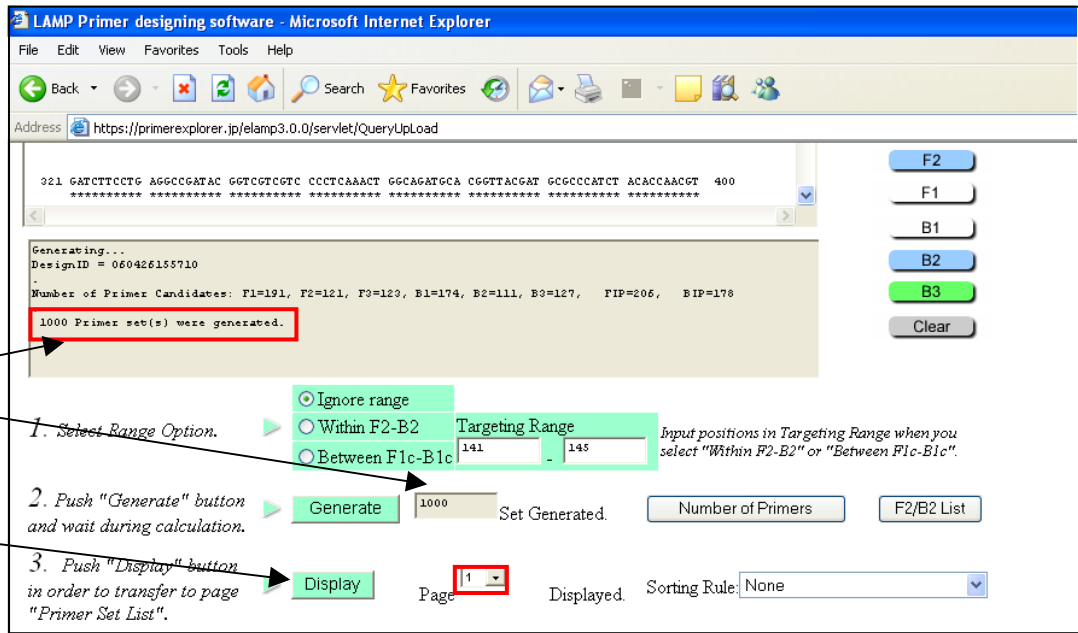


Figure 6.4 Primer design window

Next, click on the “Display” button to show the results. As indicated in Figure 6.5, the primers are designed to exclude mutation from the primer regions.

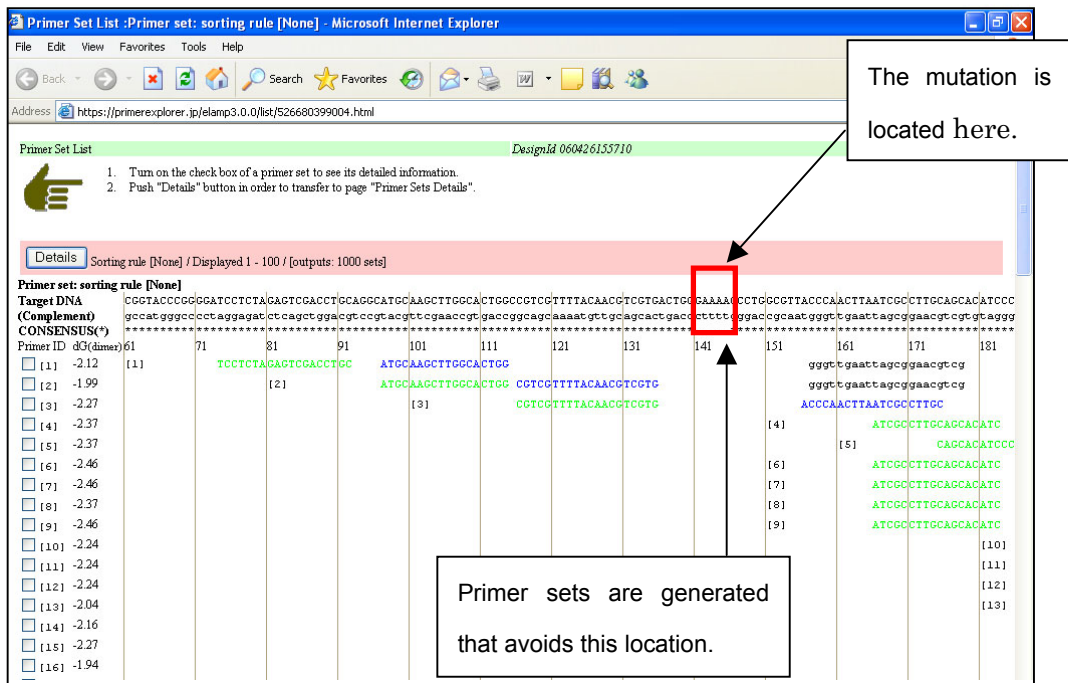


Figure 6.5 Primer Set List window (page 1)

For reference, Figure 6.6 shows the results obtained when the primer design was conducted without any mutation entry.

<Note>

The process of primer design when a mutation has been entered is to determine first the primer location for each of F1, F2, F3, B1, B2, and B3, then delete those candidates that include to the location of the mutation, and then use the remaining primer regions to compose the primer sets.

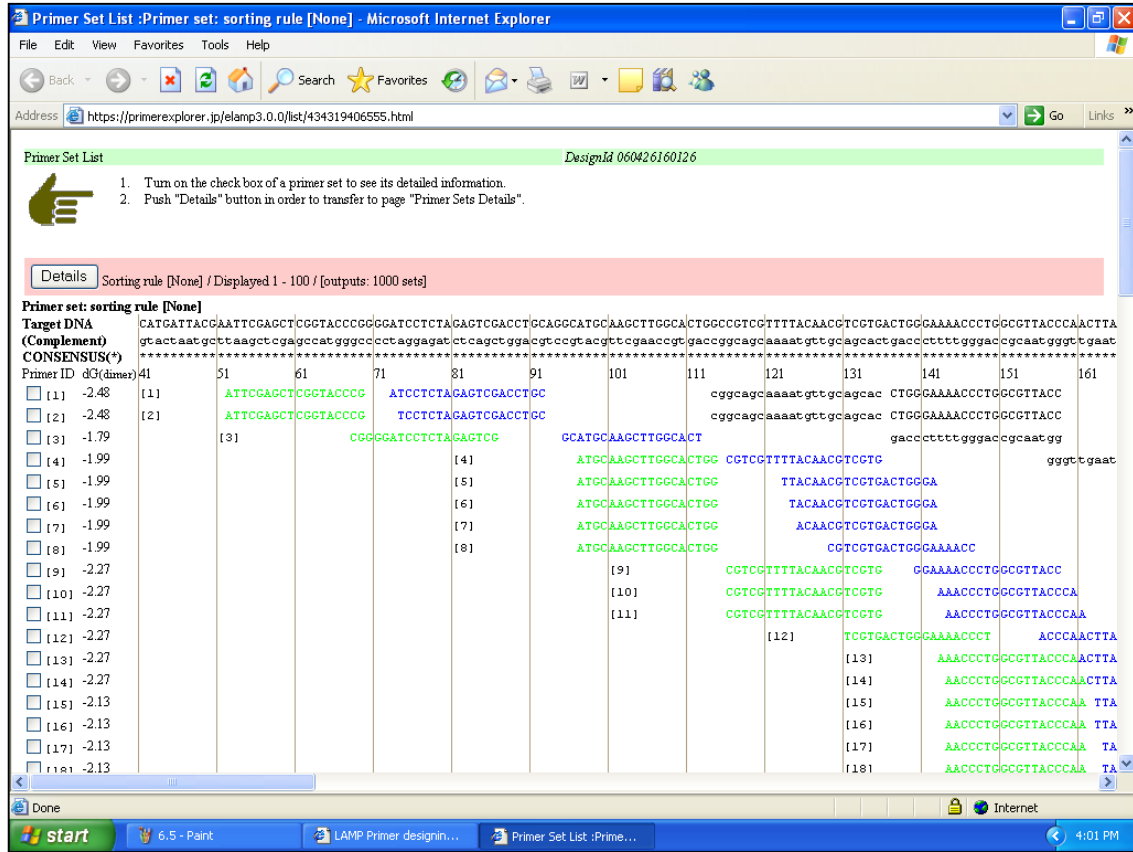


Figure 6.6 Primer set designed with no mutation entry

### 6.3 Designing primers with mutations at the 5' end or 3' end of the primer region

Here we discuss designing primers with mutations located at the 5' end and 3' end of the primer region. For example, in the example discussed above, if a mutation is located near 141 bp in the target sequence, then the number of primer set candidates for amplifying this region decreases dramatically. When amplifying the region that contains the mutation, it is preferred that the mutation not be located within the primer, but there are circumstances where such strict conditions greatly decreases the number of potential primers or prohibits primers from being designed at all. If the mutation is not present in the primer, then as indicated in Figure 6.6, many primer set candidates are generated such that they incorporate the location of the mutation. Thus, by using less stringent conditions to design the primers to allow the mutation to be present within the primer regions, many varieties of primer candidates can be generated. Then, primers are chosen such that the mutation has the least influence on the amplification.

In PrimerExplorer V3 it is possible to select the primer regions that contain the mutation. Regions that can be selected are 5' end, 3' end and the internal region for the F3, B3, F2, B2, F1c, and B1c. Since the 5' end at regions



F3, B3, F2, and B2 and the 3' end of regions F1c and B1c and the internal regions between 5' end and 3' end do not serve as the starting points of the amplification and are less susceptible to the effect of the mutation. When primer design can hardly be achieved, allow mutations at these regions to proceed to the primer design.

First, the primers are designed so that the mutation is located at the 5' end of the primer region. As in Figure 6.7, check the box for the 5' end at region F3 in the primer design window, and then click on the "Generate" button to design primers that include the mutation at the 5' end.

UPLOAD FILE : C:\LAMP\Sequence\M13\_2.nuc

1. Select Range Option.

2. Push "Generate" button and wait during calculation.

3. Push "Display" button in order to transfer to page "Primer Set List".

1) Check the "F3 5' term" box.

Use the same mutation information as in Figure 6.3

2) Click on the "Generate" button.

3) After the primers have been designed, click on the "Display" button.

Figure 6.7 Primer design window

<Note>

In case mutation regions have been specified using “Mutation/ Consensus” setting, the process of primer design will not eliminate primer regions that contain the specified mutation regions (for example F3 5' end) among the generated candidate primer by its filter. These primers incorporate with other remaining primer candidates to compose primer sets.

After the primers have been designed, click on the “Display” button to display the data. As indicated in the Figure 6.8 Primer Set List window, the mutation in the primer is indicated in red. Since we have specified that the mutation be present at the 5' end of the primer at region F3, we will design primers that include the mutation at the 5' end of region F3.

Primer Set List :Primer set: sorting rule [None] - Microsoft Internet Explorer

Address: https://primerexplorer.jp/elamp3.0.0/list/234557848539.html

Primer Set List DesignId 060426160726

1. Turn on the check box of a primer set to see its detailed information.  
2. Push "Details" button in order to transfer to page "Primer Sets Details".

Details Sorting rule [None] / Displayed 1 - 100 / [outputs: 1000 sets]

Primer set: sorting rule [None]

Target DNA CGGTACCCGGGATCCTCTAGACTCGACCTGCAGGCATGCAAGCTTGGCACTGGCCGTCGTTTTACAACCTCGTGACTGGAAAACCTGGCCGTACCCAACTTAATCGCCTTCAGCACATCCC  
(Complement) gccatgggccccttagyagatctcagctgyacgtccgtacgttcgaaccgtgacggcagc aaaatgtgcagcactgaccttttggacgc caatgggtgaattagcggaaactcgtgtaggg  
CONSENSUS(\*) \*\*\*\*\*

Primer ID	dG(dimer)	Primer
[1]	-2.12	TCCTCTAGACTCGACCTGC ATGCAAGCTTGGCACTGG
[2]	-1.99	ATGCAAGCTTGGCACTGG CGTGGTTTTACAACCTCGTG
[3]	-2.27	CGTGGTTTTACAACCTCGTG
[4]	-2.27	CCCTGGCGTTACCCAACTTAATCGCCTTCAGCAC
[5]	-2.27	CCCTGGCGTTACCCAACTTAATCGCCTTCAGCAC
[6]	-2.13	CCCTGGCGTTACCCAA TTAATCGCCTTCAGCAC
[7]	-2.13	CCCTGGCGTTACCCAA TTAATCGCCTTCAGCAC
[8]	-2.13	CCCTGGCGTTACCCAA TTAATCGCCTTCAGCAC
[9]	-2.13	CCCTGGCGTTACCCAA TTAATCGCCTTCAGCAC
[10]	-2.37	CCCTGGCGTTACCCAA AATCGCCTTCAGCAC
[11]	-2.37	CCCTGGCGTTACCCAA AATCGCCTTCAGCAC
[12]	-2.37	CCCTGGCGTTACCCAA ATCGCCTTCAGCAC
[13]	-2.37	CCCTGGCGTTACCCAA GCAGCACATCCC
[14]	-2.37	CCCTGGCGTTACCCAA CAGCACATCCC
[15]	-2.37	ATCGCCTTCAGCACATC
[16]	-2.37	CAGCACATCCC
[17]	-2.46	ATCGCCTTCAGCACATC
[18]	-2.46	ATCGCCTTCAGCACATC

Figure 6.8 Primer Set List window (page 1)

It is possible to select multiple regions that contain the mutation. Here, mutations are allowed at the 5' ends of locations F3 and F2.

As indicated in Figure 6.9, check the box for F3 5' end and the F2 5' end in the primer design window and then click on the “Generate” button. After the primers have been designed, click on the “Display” button to display the data. Primers with the mutations at the F3 5' end or the F2 5' end are designed. (See Figure 6.10)

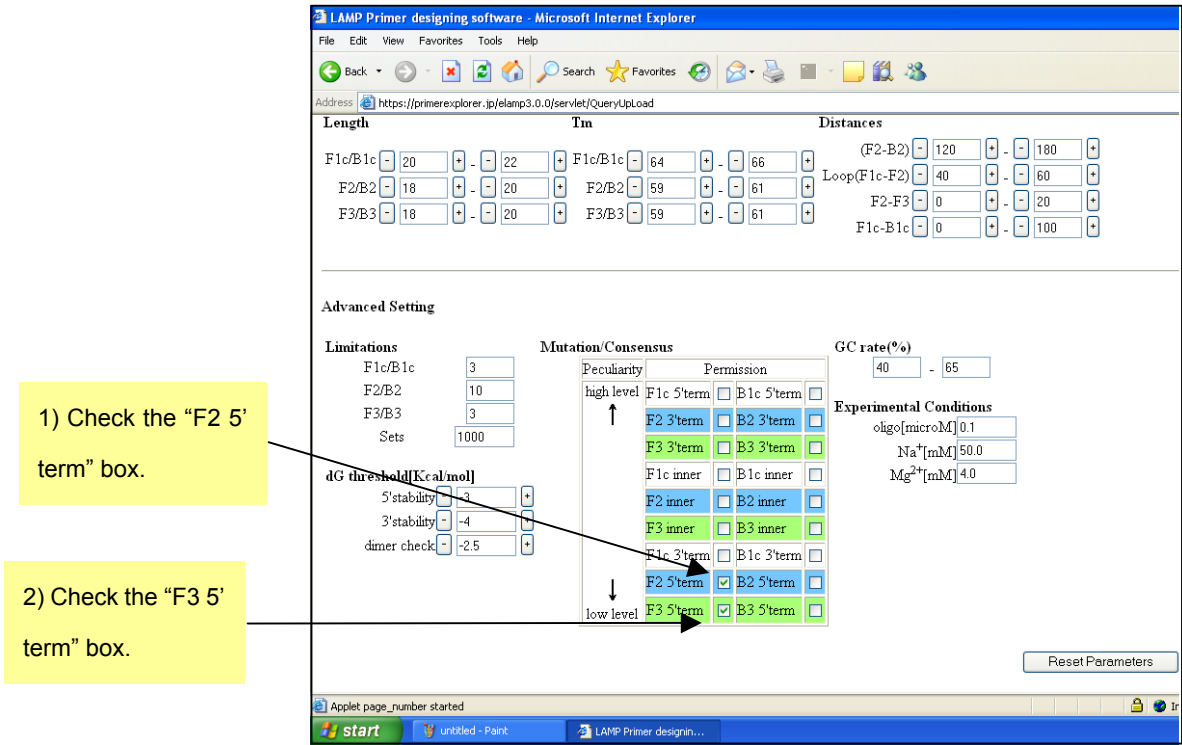


Figure 6.9 Primer design window

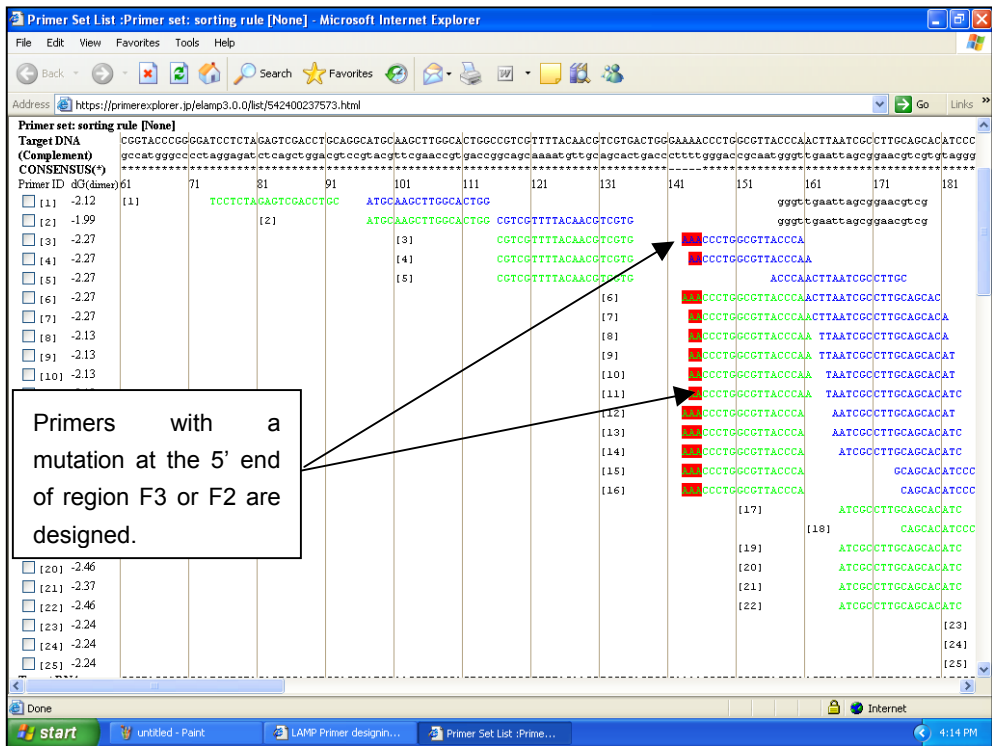


Figure 6.10 Primer Set List window (page 1)

Next, we design primers that contain a mutation at the 3' end of a primer region. As in Figure 6.11, in the primer design window, check the box at the 3' end of F2 region and click on the "Generate" button. After designing the primers, click on the "Display" button to display the results.

When a mutation is present at the 3' end of regions F2, F3, B2, or B3 or at the 5' end of regions F1c and B1c, the specificity of the primer to the wild type strain becomes low, and the specificity toward the mutant type strain becomes high. These locations serve as the replication starting points after annealing, so that the gene with the mutation is specifically amplified. Using this property, one can choose highly specific primers.

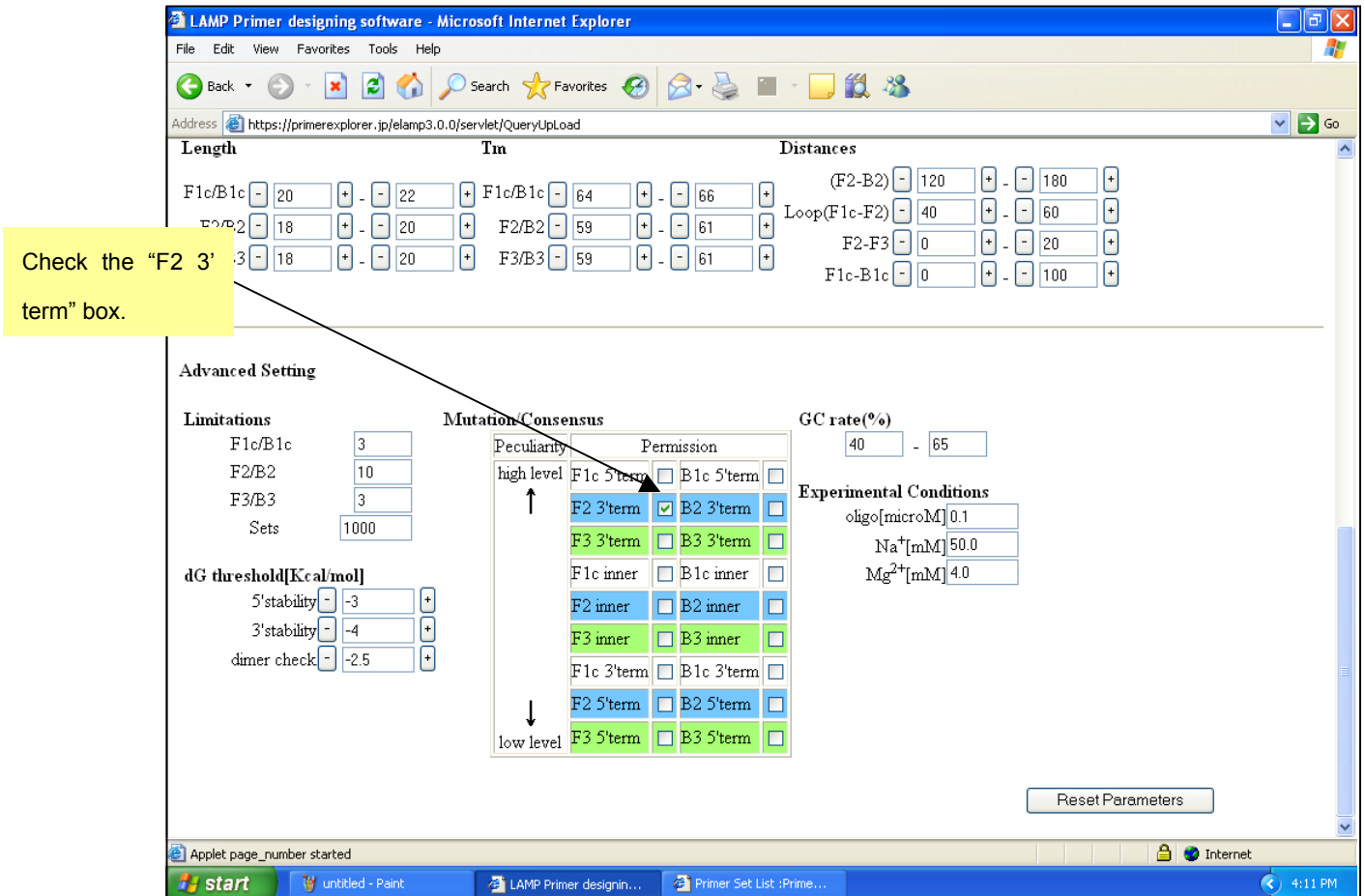


Figure 6.11 Primer design window

Figure 6.12 shows the result of primer designing that includes the mutation at the 3' end of F2 region.

The screenshot shows a web browser window titled "Primer Set List :Primer set: sorting rule [None] - Microsoft Internet Explorer". The address bar shows the URL: <https://primerexplorer.jp/elamp3.0.0/list/334688899519.html>. The main content area displays a list of primers with columns for Primer ID, dG (ΔG), and Target DNA. The Target DNA sequence is shown in a grid format with columns corresponding to primer positions (61, 71, 81, 91, 101, 111, 121, 131, 141, 151, 161, 171, 181). The primer sequences are listed in a table below the Target DNA. A callout box with an arrow points to the primer sequence "CTCCGTTTACAAGCTCGTG" at position 121, with the text "Mutation is located at the 3' end of F2".

Primer ID	dG (ΔG)	Target DNA
[1]	-2.12	TCCTCTAGAGTCGACCTGC
[2]	-1.99	ATGCAAGCTTGCCA CTGG
[3]	-1.99	ATGCAAGCTTGCCA CTGG CTCCGTTTACAAGCTCGTG
[4]	-1.99	ATGCAAGCTTGCCA CTGG TTACAAGCTCGTACTGG
[5]	-1.99	ATGCAAGCTTGCCA CTGG TACAAGCTCGTACTGG
[6]	-2.27	ATGCAAGCTTGCCA CTGG ACAAGCTCGTACTGG
[6]	-2.27	CTCCGTTTACAAGCTCGTG
[7]	-2.37	ACCCAACTTAATCGCCTTGC
[7]	-2.37	ATCGCCTTGCCAGCACATC
[8]	-2.37	CAGCACATCCC
[9]	-2.46	ATCGCCTTGCCAGCACATC
[10]	-2.46	ATCGCCTTGCCAGCACATC
[11]	-2.37	ATCGCCTTGCCAGCACATC
[12]	-2.46	ATCGCCTTGCCAGCACATC
[13]	-2.24	
[14]	-2.24	
[15]	-2.24	
[16]	-2.04	
[17]	-2.16	
[18]	-2.27	
[19]	-1.94	
[20]	-2.13	
[21]	-2.46	
[22]	-2.27	
[23]	-2.34	
[24]	-1.03	
[25]	-1.62	

Figure 6.12 Primer Set List window (page 1)

The method described in Section 1 is then followed (See p.12 - 14) to compare the primer information and select the primer sets.