A Guide to LAMP primer designing

（PrimerExplorer V3）

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Key factors in designing LAMP primer

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1. The LAMP primer

The design of LAMP primers is based on the six regions in the target sequence, designated in the Figure on the right from the 5'-end as F3, F2, F1, B1, B2, and B3.

Forward Inner Primer (FIP) consists of the F2 sequence (at its 3' end) that is complementary to the F2c region, and the same sequence as F1c region at its 5' end.

Furthermore, Forward loop primer is designed using the complementary strand corresponding to the region between F1 and F2, while Backward loop primer is designed using the complementary strand corresponding to the region between B1 and B2.
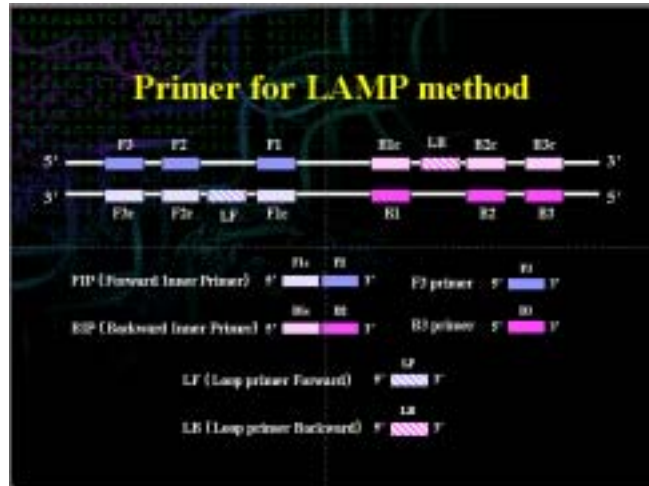
2. Key factors in the LAMP primer design

The four key factors in the LAMP primer design are the Tm, stability at the end of each primers, GC content, and secondary structure.

2.1 Tm

Tm is estimated using the Nearest-Neighbor method. This method is currently considered to be the approximation method that gives the value closest to the actual value.

The calculated Tm is affected by experimental conditions such as the salt concentration and oligo concentration, so it is preferred that Tm be calculated under fixed experimental conditions (oligo concentration at 0.1 µM, sodium ion concentration at 50 mM, magnesium ion concentration at 4 mM).

The Tm for each region is designed to be about 65°C (64 - 66°C) for F1c and B1c, about 60°C (59 - 61°C) for F2, B2, F3, and B3, and about 60°C for the loop primers.

2.2 Stability at the end of the primers

The end of the primers serves as the starting point of the DNA synthesis and thus must have certain degree of stability. The 3' ends of F2/B2, F3/B3, and LF/LB and the 5' end of F1c/B1c are designed so that the free energy is –4 kcal/ mol or less. The 5' end of F1c after amplification corresponds to the 3' end of F1, so that stability is important. (See lower left Figure).

The change in free energy (ΔG) is the difference between the product free energy and the reactant free energy.
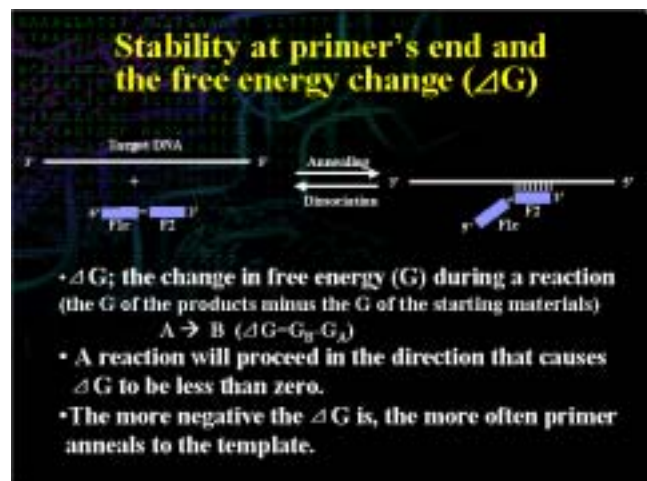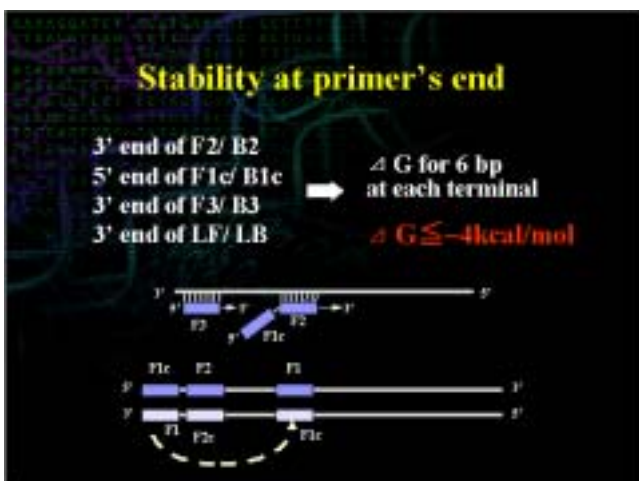
The reaction proceeds toward a negative change in free energy (∆G). The annealing between the primer and the target gene is an equilibrium reaction, and the annealing reaction proceeds with a smaller ∆G (see lower right Figure).

2.3 GC content

Primers are designed so that their GC content is between about 40% to 65%.

Primers with GC content between 50% and 60% tend to give relatively good primers.
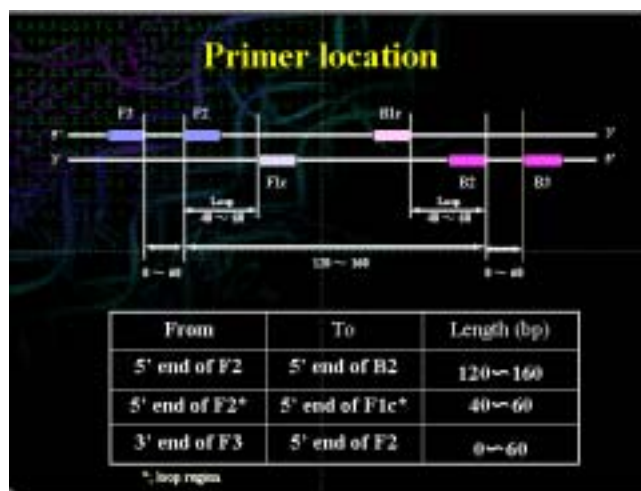
2.4 Secondary structure

It is important, particularly for the Inner primer, that primers are designed so that they do not form secondary structures.

To prevent the formation of primer dimers, it is also important to ensure that the 3' ends are not complementary.

2.5 Distance between primers

The primers are designed so that the distance from the end of F2 to the end of B2 (the region amplified by the LAMP method) is between 120 bases and 160 bases.

The primers are also designed so that the distance from the 5' end of F2 to the 5' end of F1 (the portion that forms the loop) is between 40 bases and 60 bases. The primers are also designed so that the distance between F2 and F3 is between 0 to 60 bases.

Primer location

From	To	Length (bp)
5' end of F2	5' end of B2	120~160
5' end of F2*	5' end of F1c*	40~60
3' end of F3	5' end of F2	0~60

*: loop region

3. The steps in LAMP primer design

As indicated by the figure on the right, the steps in primer design involve designing the regular LAMP primers (FIP, BIP, F3, B3) and using them in an actual amplification. They are then chosen as the LAMP primers if the amplification actually proceeds and the results are satisfactory. If the amplification does not occur or if the results are not satisfactory, the primers need to be re-designed.

When designing the loop primers, the loop primers are designed using the primer information file of the selected LAMP primers. If upon performing the actual reaction the rate of amplification increases, then they are chosen as the loop primers. If the results are not satisfactory,

Primer designing flow

the primers need to be re-designed. The loop primers are not the essential requirement for LAMP.

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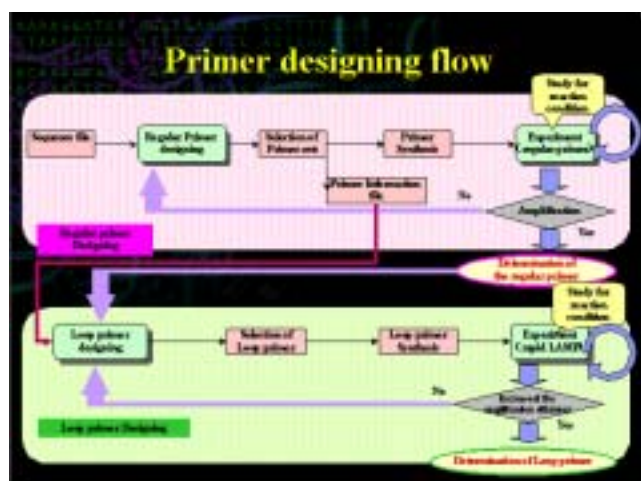
4. Designing primers using the PrimerExplorer

The individual functions are discussed below.

4.1 Standard method

The user enters the primer design conditions to design the primers.   The primer design conditions for a normal sequence (45%<GC<60%) has been entered as a default setting. If the target sequences are AT rich (GC content < 45%) or GC rich sequences (GC content > 60%), then the primers are designed with the Tm, Length, and GC content set as follows.

	Tm (ºC)	Length (mer)	GC content (%)
AT rich	>55	18-25	<45
GC rich	<68	15-22	>60

4.2 Automatic judgment

The steps in the automatic judgment are explained briefly in the Figure on the right.

When the target sequence is loaded, PrimerExplorer determines automatically the GC content of the target sequence. Based on the result, the sequence is classified as an AT rich sequence (GC% <45), normal sequence (45 < GC%< 60), or GC rich sequence (GC%> 60), and the primer design conditions are automatically selected. The design conditions are such that the Tm, Length, and GC content are set to fulfill conditions that have been optimized for a sequence, so that there is no need for the user to enter these values.
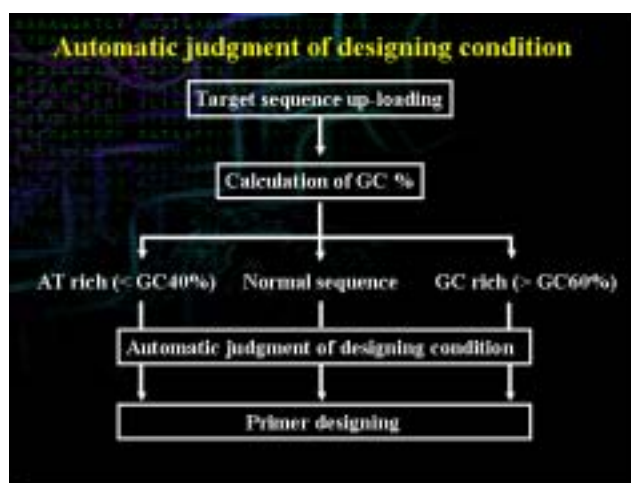
5. Primer design functions

5.1 Primer design that takes the location of mutation into account

When designing primers for mutations, the default option generates primers that are designed randomly, so that the primers designed may contain the mutation itself. In general, to amplify and detect the wild type and the mutation using common primers, select the primer sets whose sequence does not include the mutation point.

Under such circumstances, the primer design function that does not include mutation is used. If no appropriate primers are designed when this function is used, then the primers would be   designed under less stringent conditions that allow the mutation to be included in the 5' end or the 3' end.   It is possible to specify the primer regions allowing mutations and the position of the mutation at that region (5' end, internal, 3' end).

### 5.2 Primer design that specifies the primer location

This function permits specification of the region of each primer (F3, F2, F1, B1, B2, or B3) used in LAMP. This function is used if the region to be amplified or the regions of primers are known to be effective.

5.3 Loop primer design

After the regular LAMP primer set (FIP, BIP, F3, B3) has been determined, the loop primers, which reduce the amplification time and improve the specificity, can be designed. The loop primers are designed based on the primer information file of the regular primer set.

5.4 Primer design for the entire target region

It is now possible to design primers for the entire target region. When conducting the primer design, the primers are designed for FIP-BIP and F3 and B3 in the entire target region.   Next, for each FIP-BIP region, F3 and B3 are selected to form a primer set.   The generation of primer sets , which consist a combination of FIP-BIP with the F3 and B3, begins at the 5' end and proceeds until the 3' end is reached. Then, the primer design proceeds again from the 5' end to the 3' end, and each FIP- BIP can form primer set with a maximum of three combinations of F3-B3.
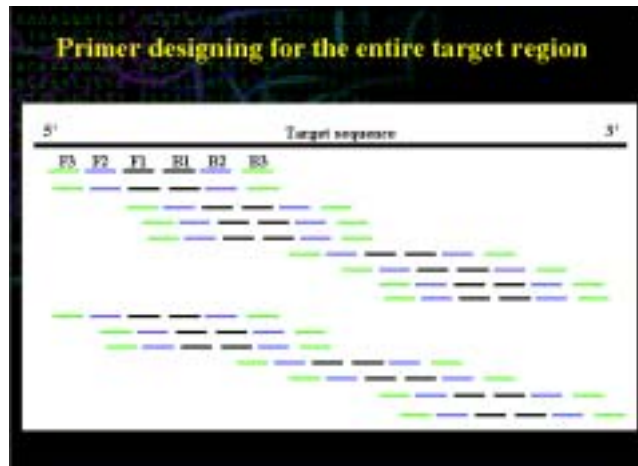
For each primer set with the same FIP-BIP region, various primer sets are designed for the entire target region.

5.5 Check of the primer ends

The primer's ends are checked automatically, and those primer sets possessing the complementary sequences or special sequences are automatically eliminated. A complementary sequence is defined as symmetric sequences (for example CCCGGG and GAATTC) and special sequences (for example, sequences containing the same nucleotide at the end such as CCGGGG and AATTTT). These can form primer dimers and thus are eliminated at the primer design step.

Complementarity against the target sequence is also checked. The ends of the primer candidates designed are compared to the target gene sequences, and if the end sequences of the primer candidates also exist in a location other than the amplification region of the target sequence, then that primer set is eliminated. This serves to eliminate primer sets that can cause nonspecific amplification.
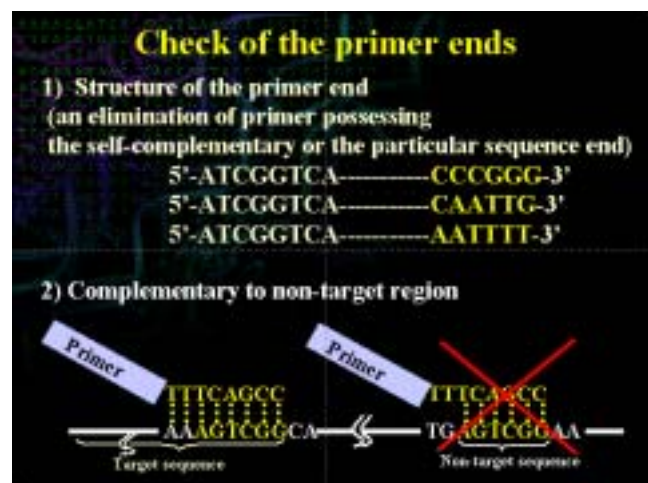
5.6 Saving the primer design conditions

The primer design conditions that the users manually have input can be saved as a file style and re-loaded.   When the sequence and the preserved primer design condition are input, the data previously obtained can be quickly displayed and the primer design working can be resumed easily.

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How to use PrimerExplorer V3

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

1.1 Uploading the target sequence

The target sequence is uploaded in the PrimerExplorer V3 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.
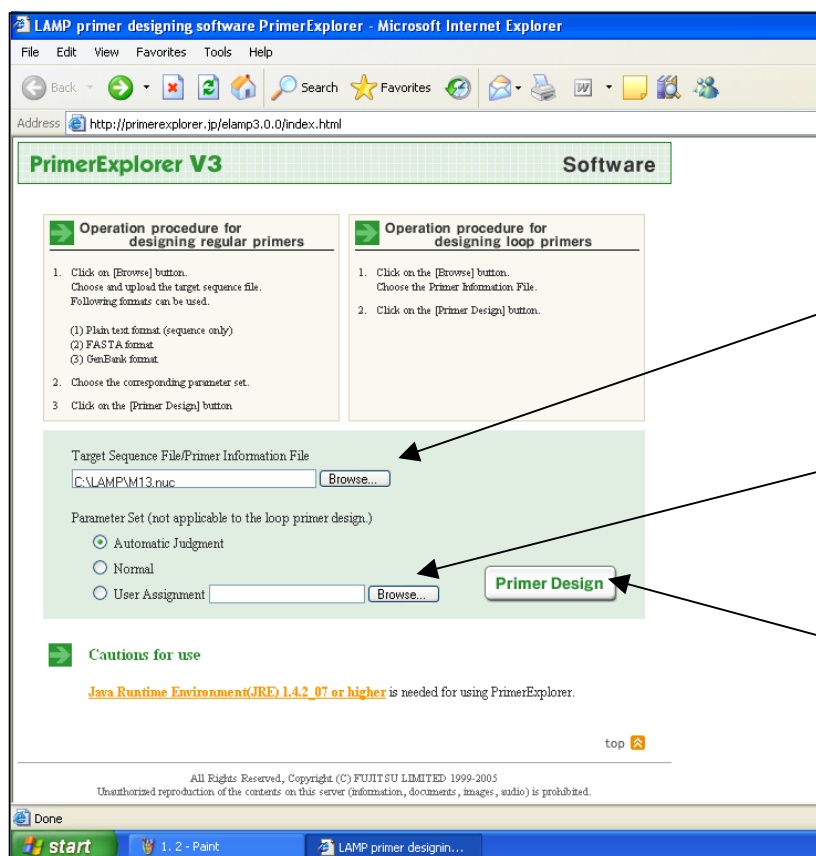
Figure1.1 PrimerExplorer V3 startup window

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

As an example, a portion of the M13 sequence (length 1969 bp, GC content = 48.2%) will be used to design the primers.

As indicated in the primer design window shown in Figure 1.2, "Parameter Set" of "Normal" has been selected. Normal parameter conditions are as indicated in Figure 1.3.

Next, click on the "Generate" button to start the primer design. (Figure 1.2)

Figure 1.2 Primer design window

Figure 1.3 Normal parameter conditions

When the primer design starts, the message area will indicate the status of progress in the primer design, as shown in Figure 1.4. 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. Clicking on the "Display" button will display the Primer Set List results.

Figure1.4 Window after completion of primer design under normal parameter conditions

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1.3 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, 59 primer sets have been designed. After the second round, primers are designed from set 60 to set 118. 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 1440 bp). (See Figure 1.5b) Several primers are then selected to compare the specific conditions.

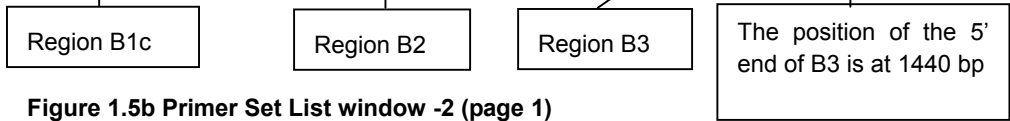**Figure 1.5a Primer Set List window -1 (page 1)**

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Figure 1.5b Primer Set List window -2 (page 1)

1.4 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 1.5a), primer sets are chosen that encompasses the entire length if possible. As an example, the ten sets, with the ID numbers 1, 5, 8, 9, 10, 14, 15, 17, 19 and 32, 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 1.6 Primer Set Details window

In the window shown in Figure 1.6, 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, 10, 14 and 17 were selected.

Next, we proceed to the next window to confirm the primers selected.   Check the boxes for the ID numbers 5, 8, 10, 14, and 17, and click on the "Confirm" button.

The primer information for the selected the ID numbers 5, 8, 10, 14, and 17 will again be displayed in the Selected Primer Set window (Figure 1.7a).   Confirm the conditions for the primers selected.

Under the ID number is the "Primer Information" button; this is used in designing the loop primers for the primer set chosen.   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 1.7b)

This is the end of regular primer design.

Figure 1.7a Selected Primer Set window

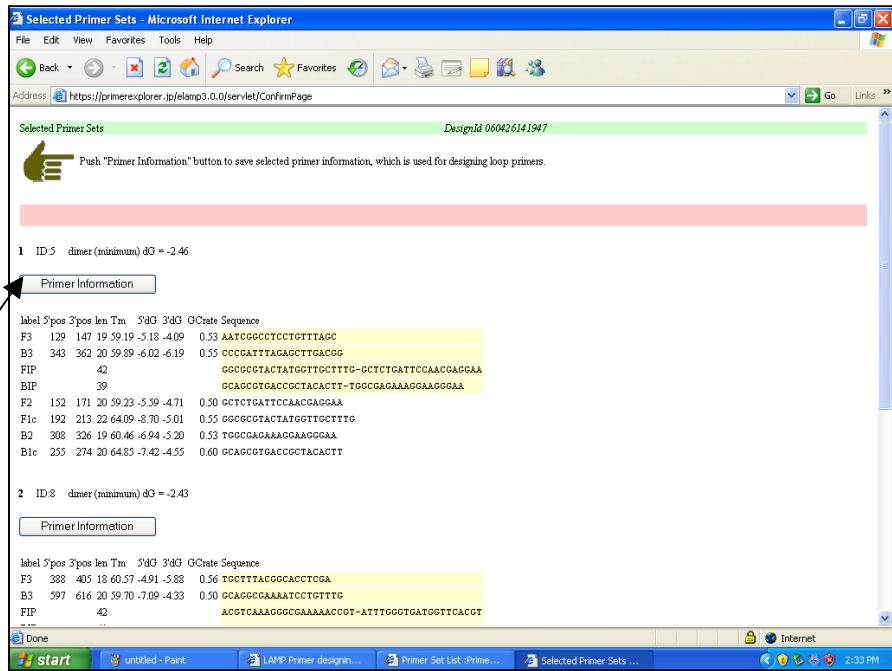To save the primer information for use in designing the loop primers, click on the "Primer Information" button.
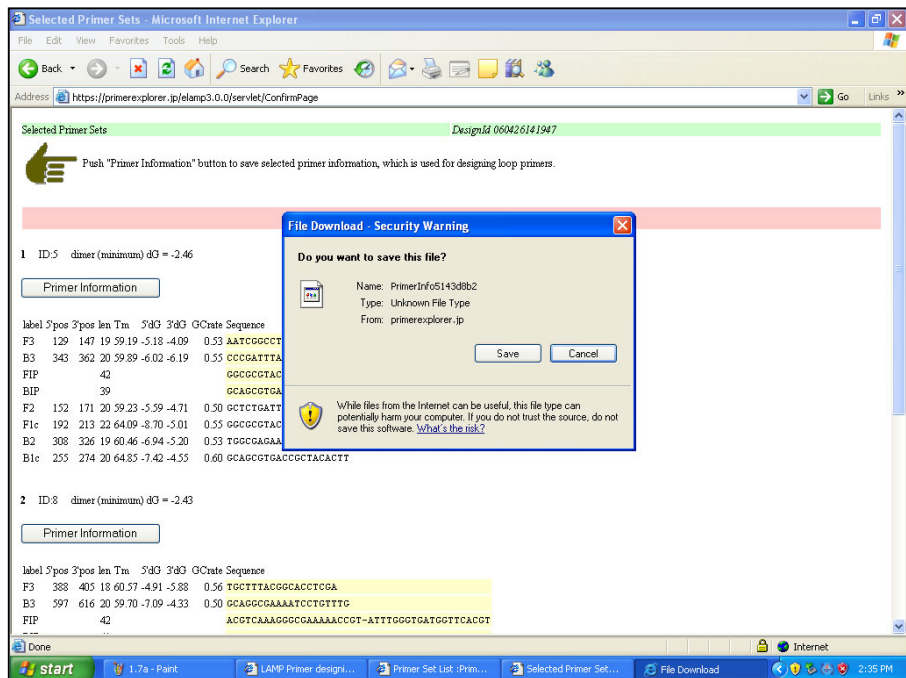

Figure 1.7b Primer Information Save window

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.   158 sets of primers have been designed from the 5' end to the 3' end, and additional sets from the 159th 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.12 - 14) 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 (page 2)

< 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.

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