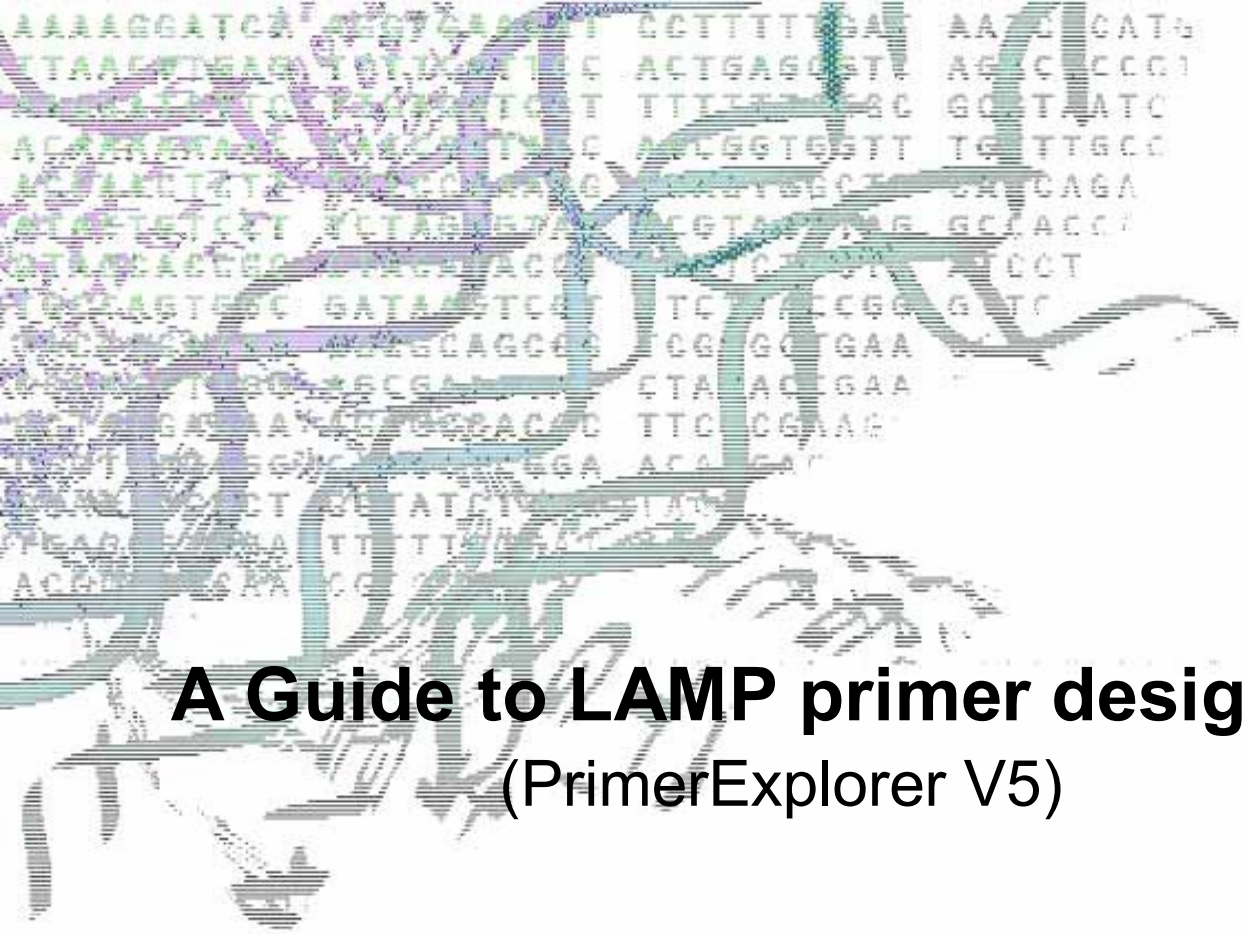

A Guide to LAMP primer designing

(PrimerExplorer V5)

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Experimental procedures and techniques

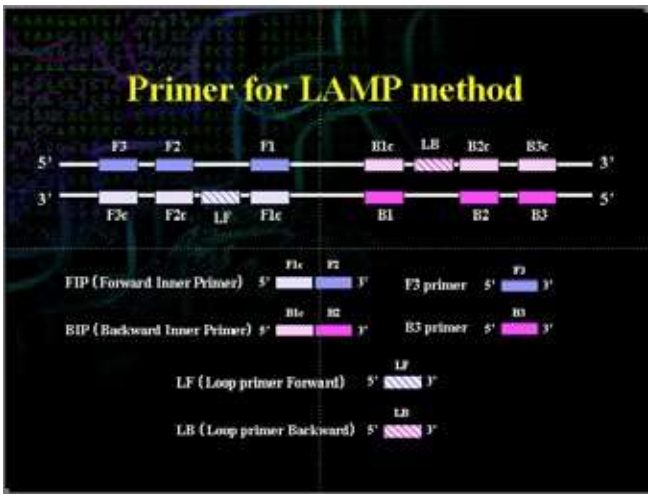
Key factors in designing LAMP primer

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.

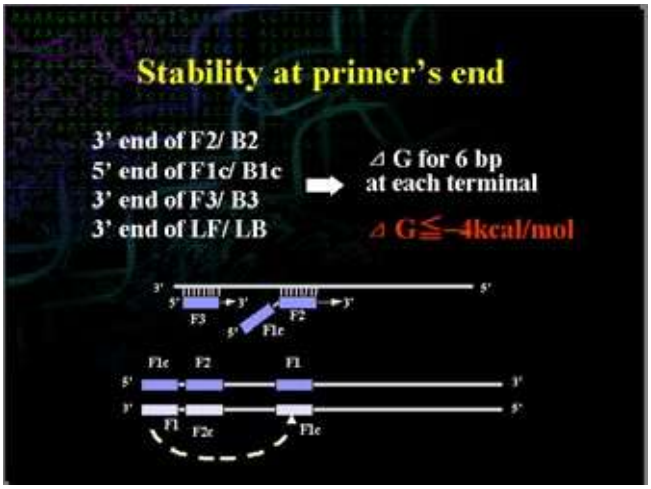
The calculated Tm is affected by experimental conditions such as the salt concentration and oligo concentration, so it is preferred that Tm be calculated under fixed experimental conditions (oligo concentration at 0.1 μM, sodium ion concentration at 50 mM, magnesium ion concentration at 4 mM).

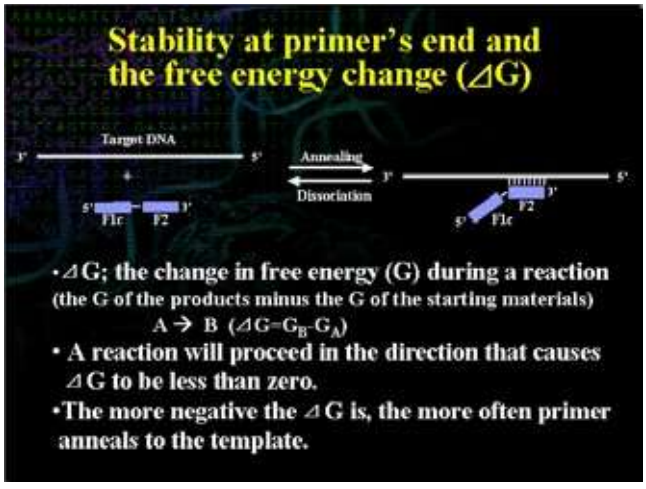
The Tm for each region is designed to be about 65°C (64 - 66°C) for F1c and B1c, about 60°C (59 - 61°C) for F2, B2, F3, and B3, and about 65°C (64 - 66°C) for the loop primers.

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 (ΔG). The annealing between the primer and the target gene is an equilibrium reaction, and the annealing reaction proceeds with a smaller ΔG (see lower right Figure on the previous page).

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**

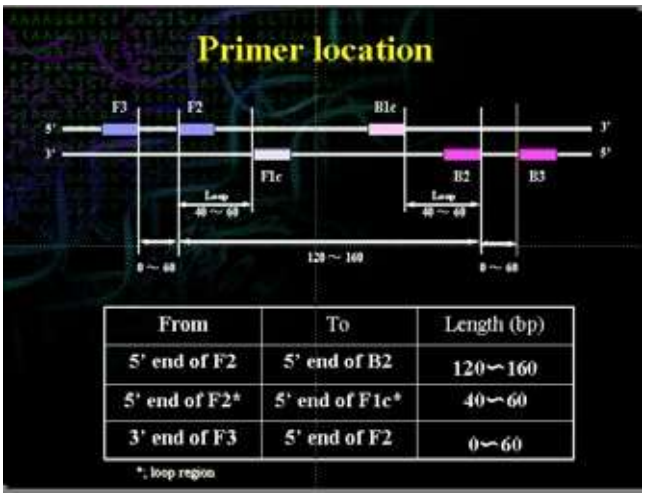
It is important, particularly for the Inner primer, that primers are designed so that they do not form secondary structures.

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**

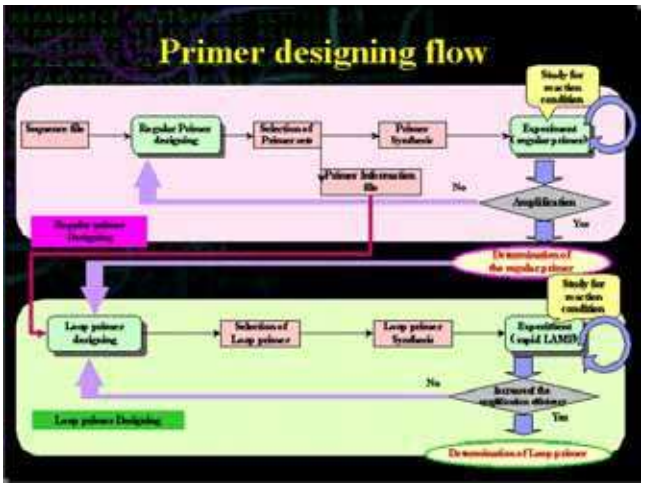
The primers are designed so that the distance from the end of F2 to the end of B2 (the region amplified by the LAMP method) is between 120 bases and 160 bases.

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.



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

4. PrimerExplorer functions

Currently, the two versions of Primer Explorer are available. The following table compares the functions of two versions.

Function \ Version	Primer Explorer V4	Primer Explorer V5
Switching between Easy and Expert Modes	○	○
Automatically narrowing down and prioritizing the primer set candidates	○	○
Standard design methods	○	○
Automatic determination of the primer design conditions	○	○
Design that takes the location of mutation into account	○	○
Designing primers with specified primer locations	○	○
Loop primer design	○	○
Primer design for the entire target region	○	○
Automatically designing common primers	○	○
Automatically designing specific primers	○	○
Inputting multiple alignment results	○	○
Saving primer set lists	○	○
Saving/uploading target sequence information	○	○
Check of the primer ends	○	○
Saving the primer set sequence information	×	○

The individual functions are discussed below.

4.1 **Easy Mode and Expert Mode**

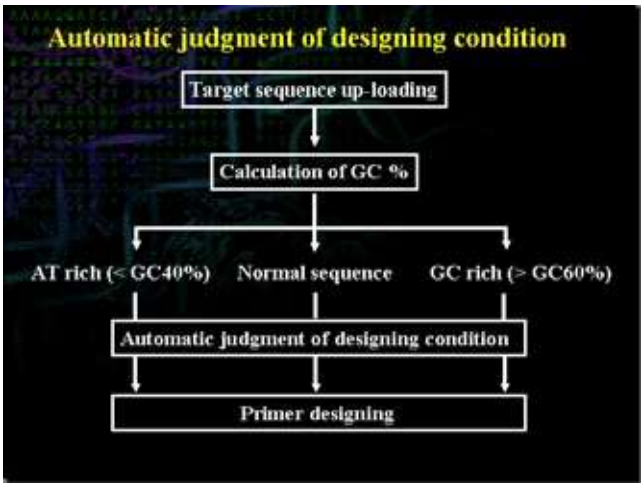
Easy Mode eliminates the need to change parameters, and displays five primer sets that are likely to have high amplification efficiency. It automatically narrows down and prioritizes the primer set candidates. Expert Mode is designed for primer set customization, allowing the user to change parameters and to specify the number of primer sets to be designed.

4.2 **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

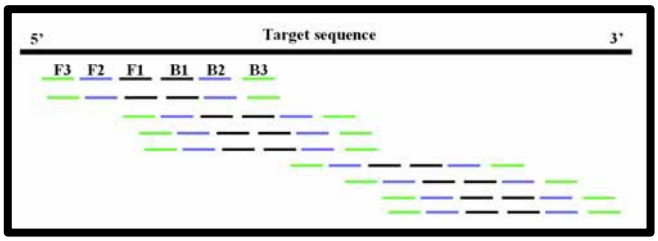
4.3 **Automatic judgment**



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

When the target sequence is loaded, The 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.

4.4 **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.

4.5 **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.

4.6 **Loop primer design**

After the regular LAMP primer set (FIP, BIP, F3, and 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.

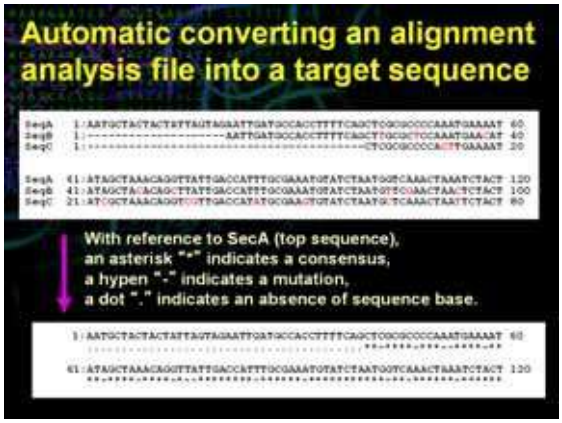
4.7 **Primer design that takes the location of mutation into account**

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).

4.8 **Inputting multiple alignments**

The PrimerExplorer can design two kinds of primers: one that can detect a set of multiple genes with various mutations (common primers) and another that can amplify only specific gene (specific primers). During the primer design phase, the program can input the results of multiple alignments of genes as they are. With reference to genes at the top sequence of the alignment, the program can identify mutation sites in each sequence and design primers as indicated at those sites.

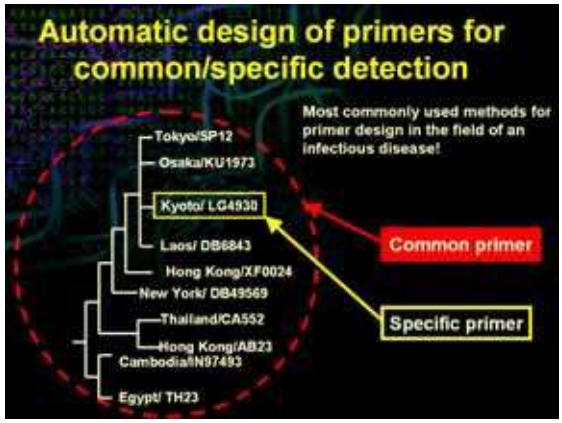


4.9 **Automatic design of common primer**

By introducing mutations into the target sequence or uploading multiple alignment results, The PrimerExplorer enables automatic design of primers in which the mutation sites will have little effect on amplification (common primers).

4.10 **Automatic design of specific primer**

By introducing mutations into the target sequence or uploading multiple alignment results, The PrimerExplorer enables automatic design of primers that recognize mutation sites at the end of their sequences (specific primers).



4.11 **Saving the Primer Set Design Result window**

A list of primer design results can be downloaded in an Excel file. The positions of the designed primers compared with the target sequence are displayed.

4.12 **Saving the target sequence information**

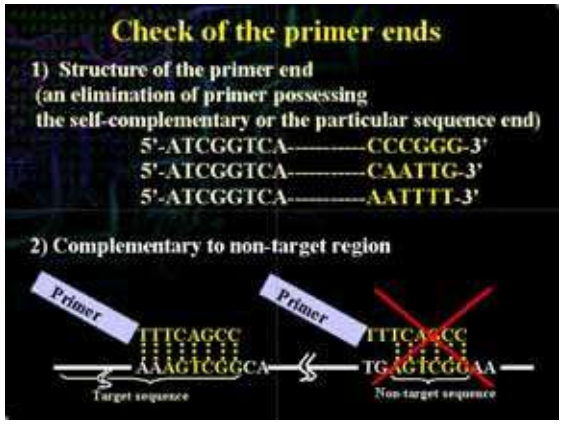
The PrimerExplorer can save information on the introduced mutations and information on the specified fixed primers, along with the gene sequence information. It is also possible to re-upload saved sequences to resume designing the primers.

4.13 **Saving the primer design conditions**

The primer design conditions can be saved and reloaded. Previously obtained data can be quickly displayed by inputting the old sequence information and reloading the design conditions used to obtain the information.

4.14 **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.

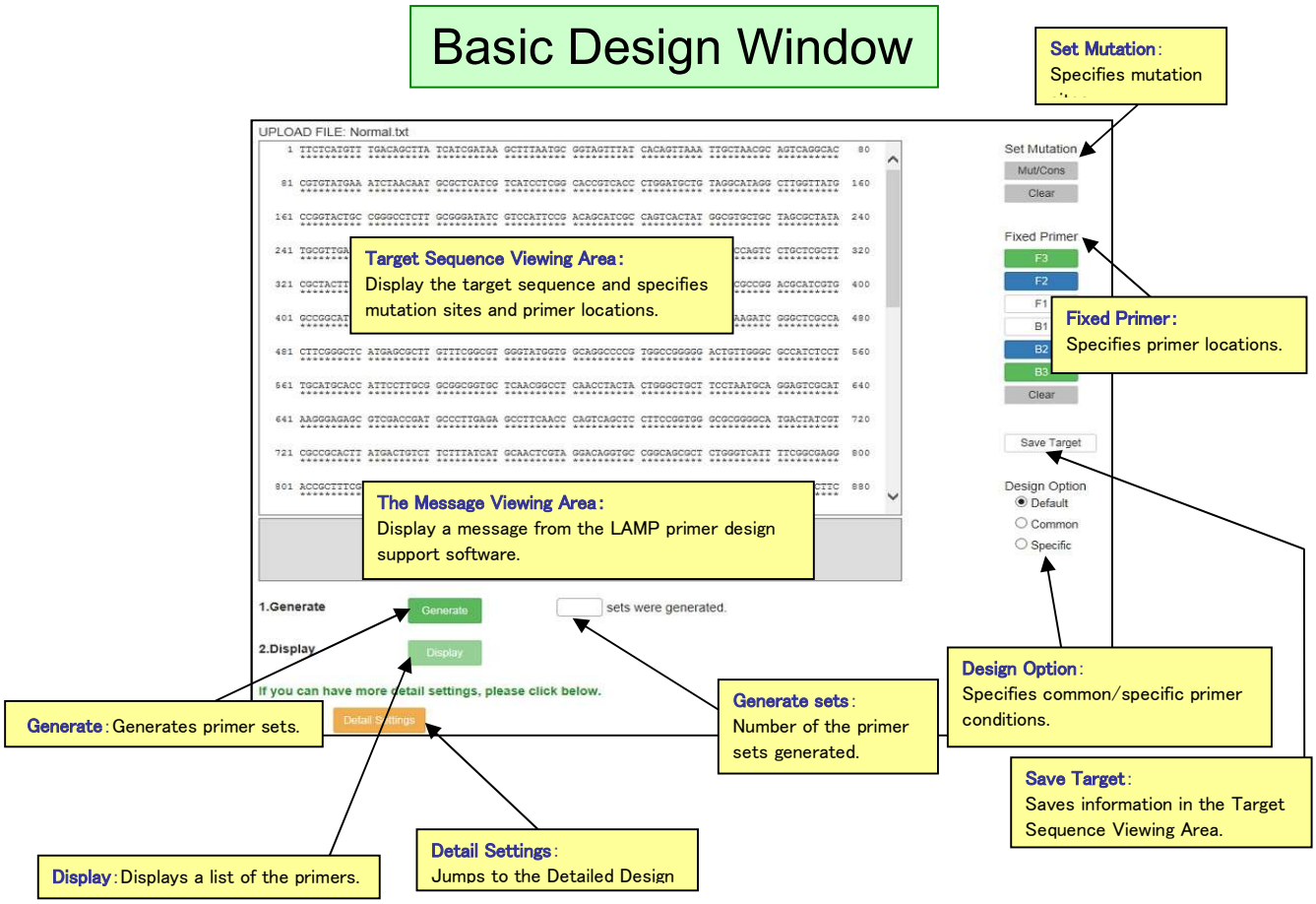
Complementarity against the target sequence is also checked. The ends of the primer candidates designed are compared to the target gene sequences, and if the end sequences of the primer candidates also exist in a location other than the amplification region of the target sequence, then that primer set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

4.15 **Saving the primer set sequence information (ready in V5)**

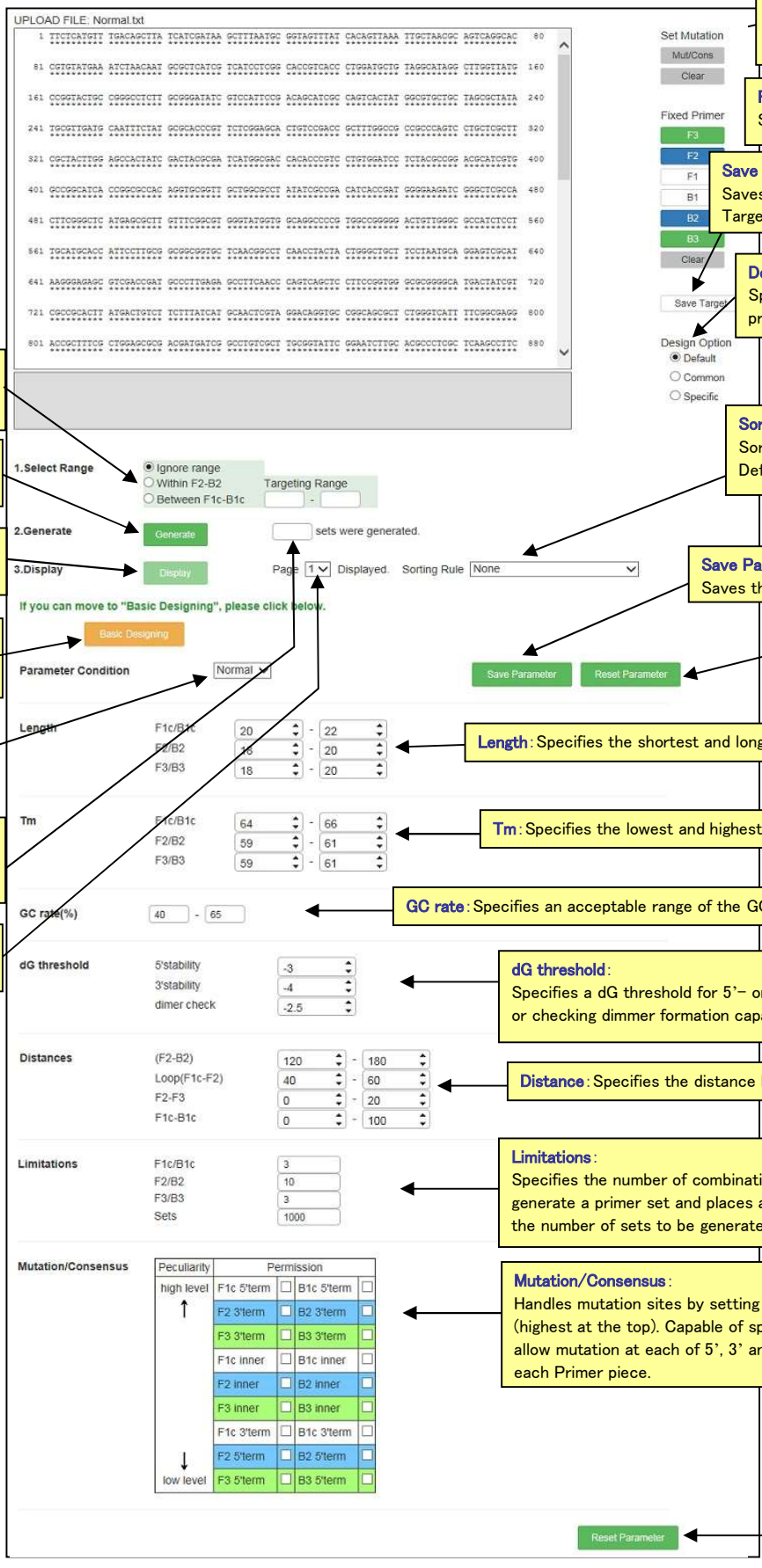
The primer sequence information can be downloaded in an Excel file. Basic information such as sequences and Tm values are displayed.

Explanation of the PrimerExplorer V5 window

Explanation of standard primer design window



Detailed Design Window



Set Mutation:
Specifies mutation sites.

Fixed Primer:
Specifies primer locations.

Save Target:
Saves information in the Target Sequence Viewing Area.

Design Option:
Specifies common/specific primer conditions.

Select Range :
Specifies an amplification range.

Generate:
Generates primer

Sorting Rule:
Sort primer sets for output. Default is "None"

Display:
Displays a list of the primers.

Save Parameters:
Saves the parameter

Basic Designing:
Jumps to the Basic Design Window.

Reset Parameters:
Resets the

Parameter Conditions:
Changes parameter settings.

Length: Specifies the shortest and longest lengths of each

Generate sets:
Number of the primer sets generated.

Tm: Specifies the lowest and highest Tm of each primer.

Show Page :
Specifies a page to be

GC rate: Specifies an acceptable range of the GC contents in each primer

dG threshold:
Specifies a dG threshold for 5'– or 3'–end stability or checking dimmer formation capability.

Distance: Specifies the distance between primers

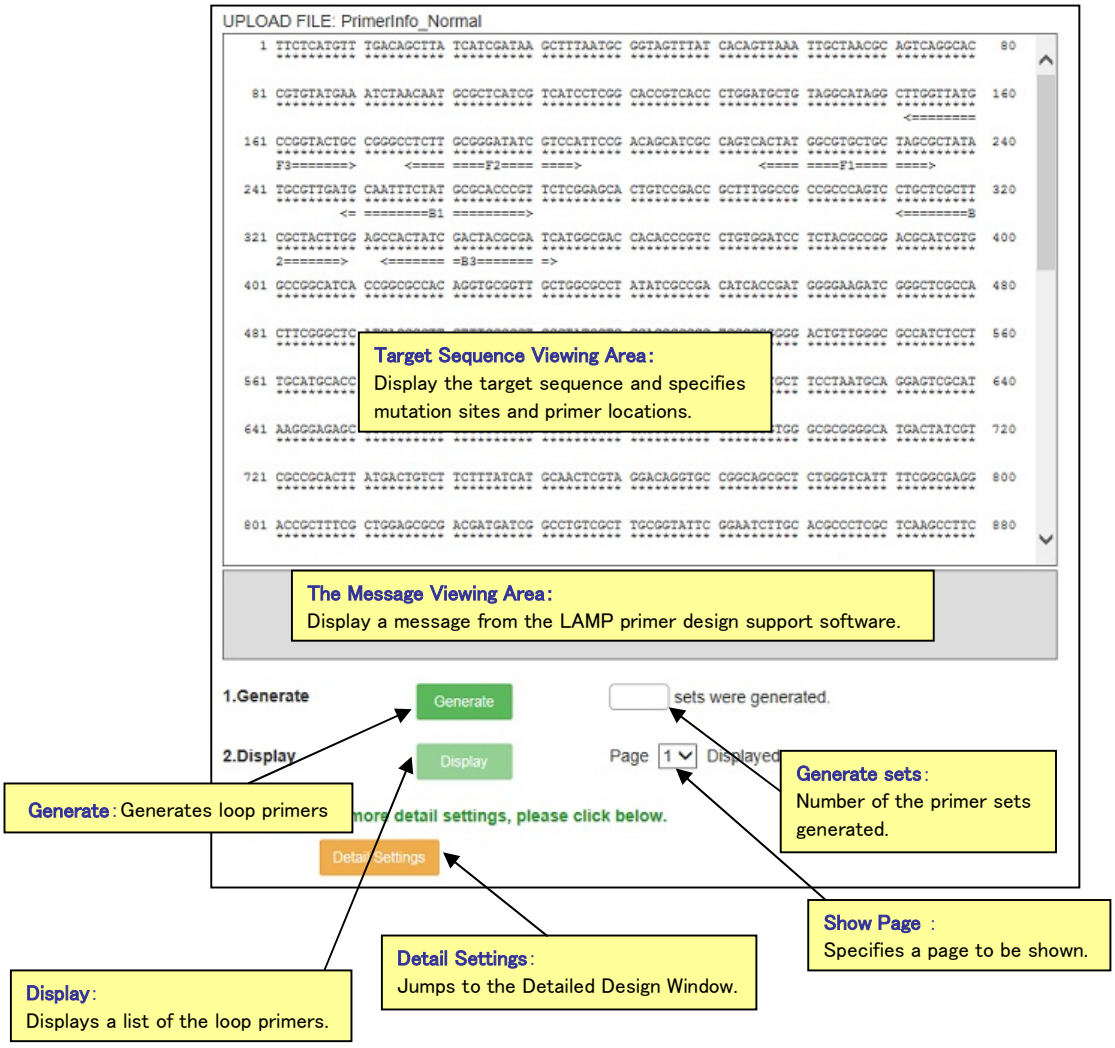
Limitations:
Specifies the number of combinations of primers to generate a primer set and places an upper limit on the number of sets to be generated.

Mutation/Consensus:
Handles mutation sites by setting peculiarity level (highest at the top). Capable of specifying whether to allow mutation at each of 5', 3' and internal sites of each Primer piece.

Reset Parameters:
Resets the parameters

Explanation of loop primer design window

Basic Design Window



Detailed Design Window