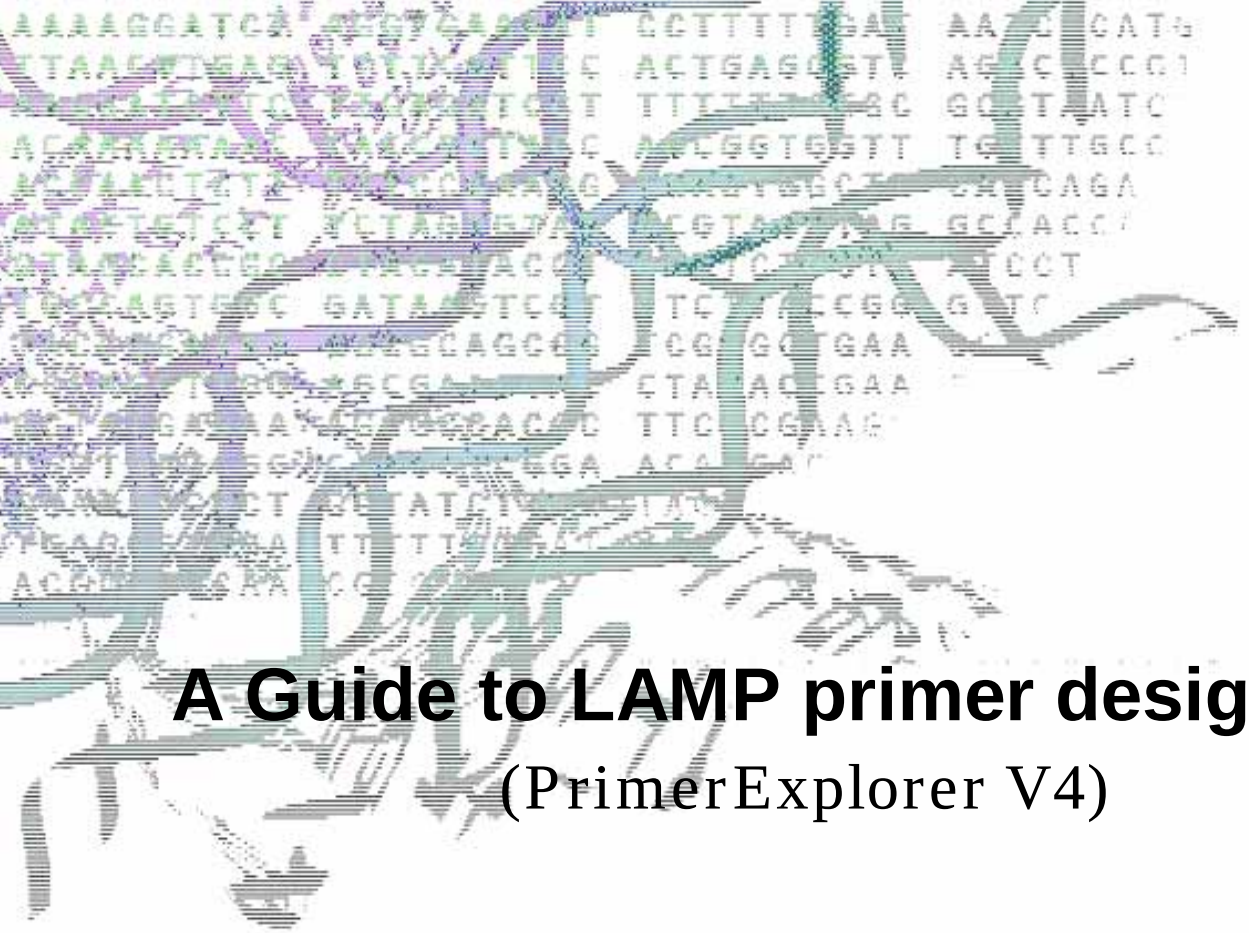


A Guide to LAMP primer designing

(PrimerExplorer V4)

Eiken Chemical Co., Ltd.

Contents

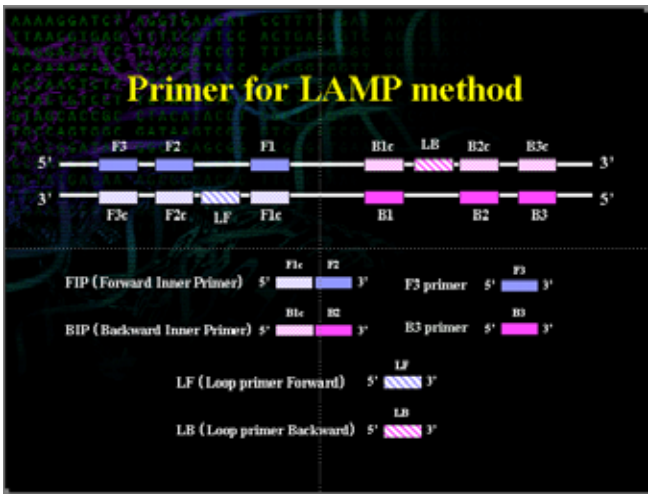
Key factors in designing LAMP primer

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.

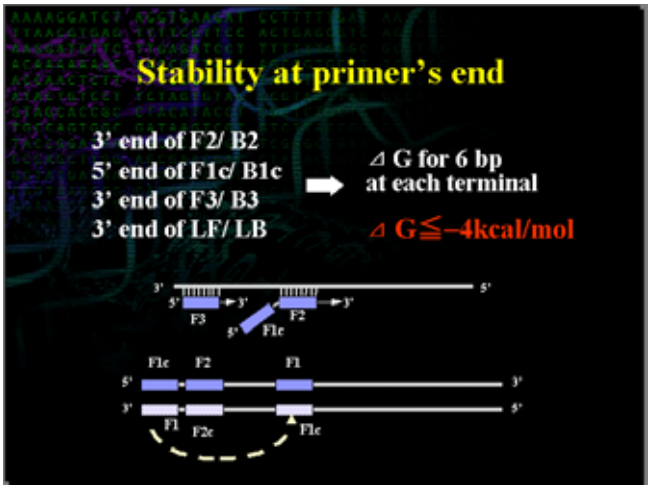
The calculated Tm is affected by experimental conditions such as the salt concentration and oligo concentration, so it is preferred that Tm be calculated under fixed experimental conditions (oligo concentration at 0.1 µM, sodium ion concentration at 50 mM, magnesium ion concentration at 4 mM).

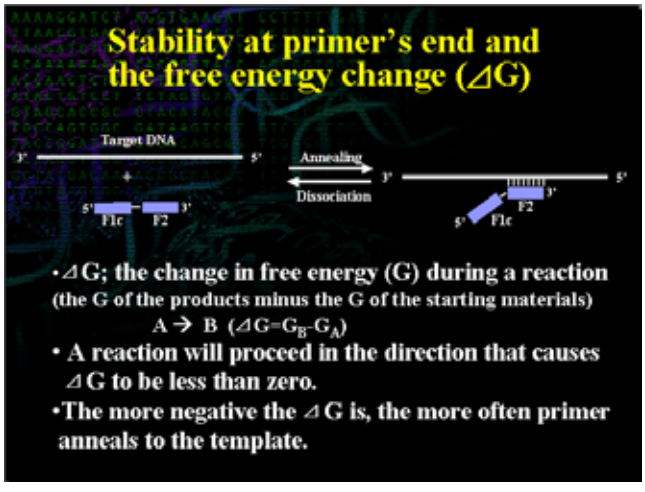
The Tm for each region is designed to be about 65°C (64 - 66°C) for F1c and B1c, about 60°C (59 - 61°C) for F2, B2, F3, and B3, and about 65°C (64 - 66°C) for the loop primers.

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

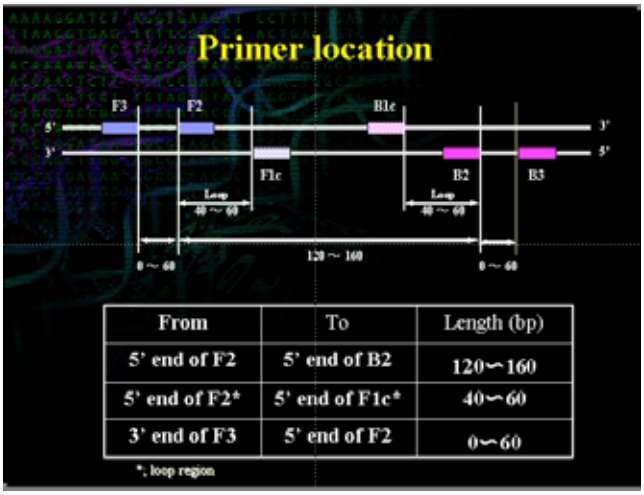
It is important, particularly for the Inner primer, that primers are designed so that they do not form secondary structures.

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.

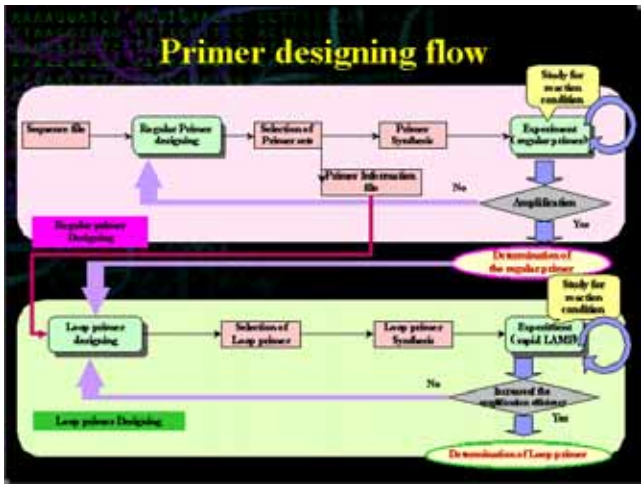


From	To	Length (bp)
5' end of F2	5' end of B2	120~160
5' end of F2*	5' end of F1c*	40~60
3' end of F3	5' end of F2	0~60

*; 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, 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 Ver.3	Primer Explorer Ver.4
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	○	○

* To specify primer regions, including for mutations and the locations of the mutations at those regions (5' end, internal, 3' end).

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.

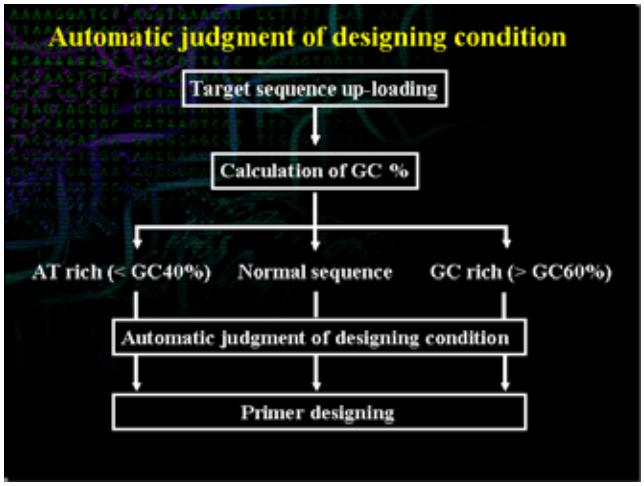
4.2 Standard method

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

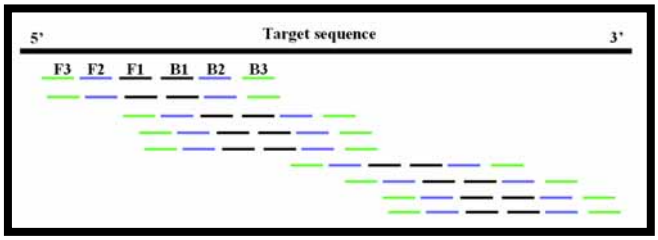
4.3 Automatic judgment



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

When the target sequence is loaded, 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.

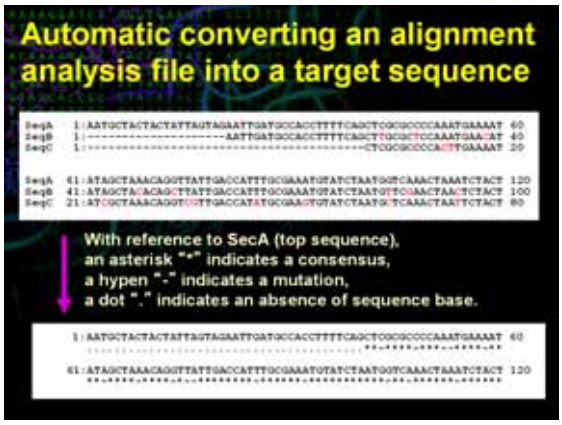
4.7 Primer design that takes the location of mutation into account

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 Application of multiple alignment results (ready in Ver.4)

PrimerExplorer Ver. 4 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 alignmnts 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.

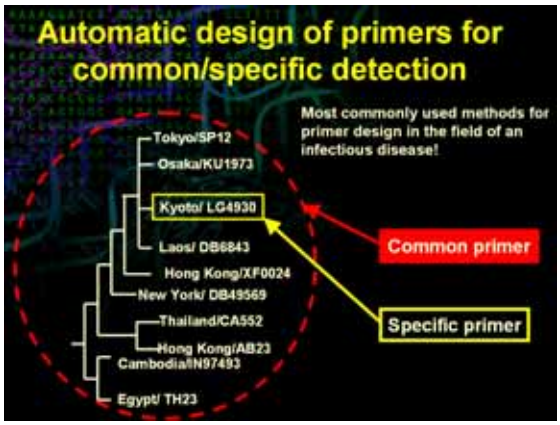


4.9 Automatic design of common primer (ready in Ver. 4)

By introducing mutations into the target sequence or uploading multiple alignment results, Ver. 4 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 (ready in Ver. 4)

By introducing mutations into the target sequence or uploading multiple alignment results, Ver. 4 enables automatic design of primers that recognize mutation sites at the end of their sequences (specific primers).



4.11 Saving the primer set list (ready in Ver. 4)

Ver. 4 displays the locations of the primers designed with reference to the target sequence in a view window, and allows for downloading the primer design results into an Excel file.

4.12 Saving the target sequence information (ready in Ver. 4)

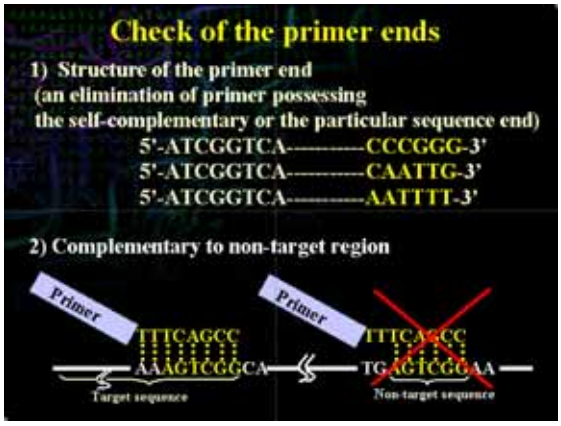
Ver. 4 can save not only gene sequence information but also the information of introduced mutations and specified fixed primers. It can also re-upload saved sequences to resume designing the primers.

4.13 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.

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

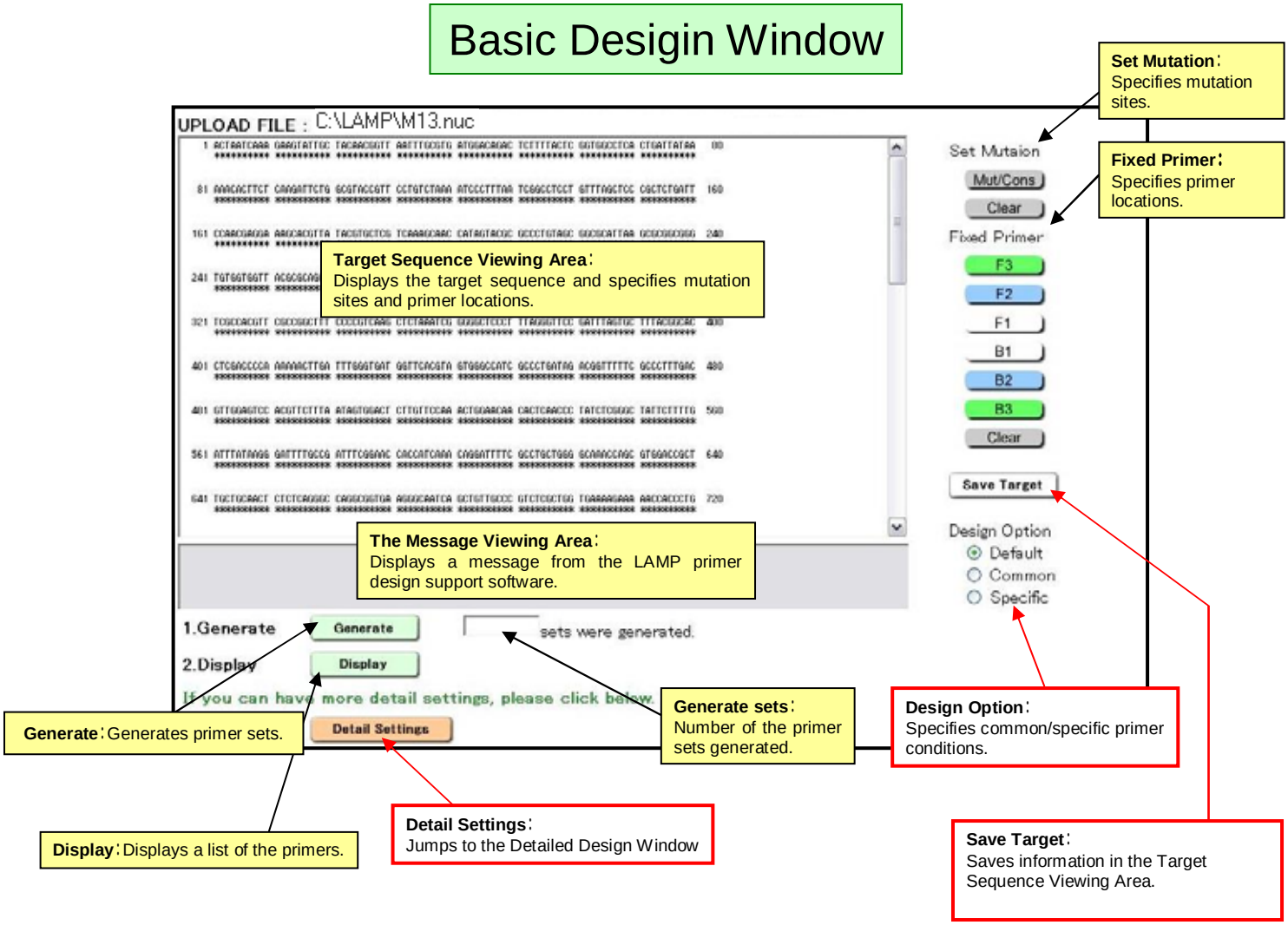


eliminated at the primer design step.

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.

Explanation of the PrimerExplorer V4 window

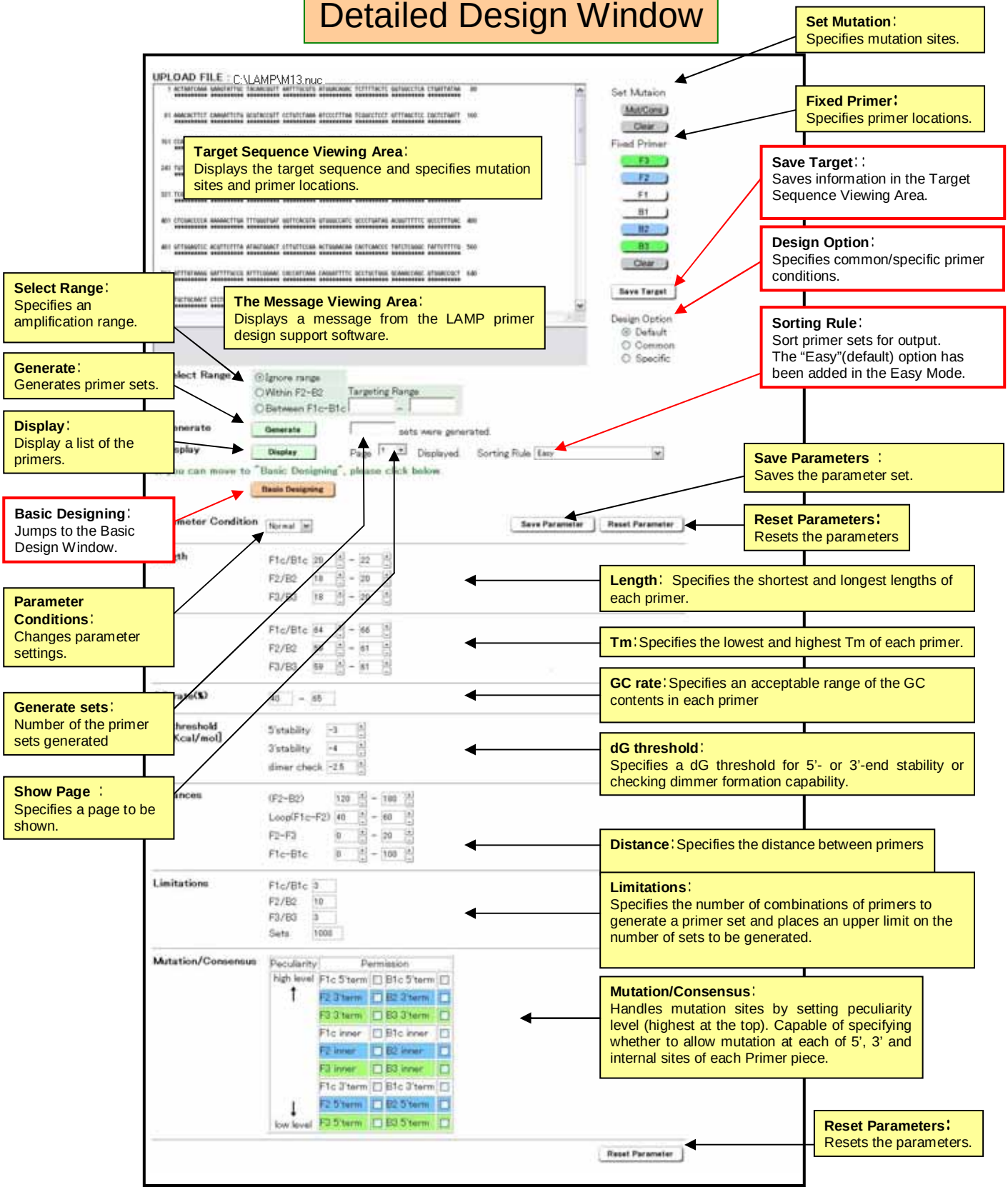
Explanation of standard primer design window



: Existing functions

: New functions

Detailed Design Window



Set Mutation:
Specifies mutation sites.

Fixed Primer:
Specifies primer locations.

Target Sequence Viewing Area:
Displays the target sequence and specifies mutation sites and 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.

The Message Viewing Area:
Displays a message from the LAMP primer design support software.

Sorting Rule:
Sort primer sets for output.
The "Easy"(default) option has been added in the Easy Mode.

Generate:
Generates primer sets.

Display:
Display a list of the primers.

Save Parameters :
Saves the parameter set.

Reset Parameters:
Resets the parameters

Basic Designing:
Jumps to the Basic Design Window.

Length: Specifies the shortest and longest lengths of each primer.

Parameter Conditions:
Changes parameter settings.

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

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

Generate sets:
Number of the primer sets generated

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

Show Page :
Specifies a page to be shown.

Distance:Specifies the distance between primers

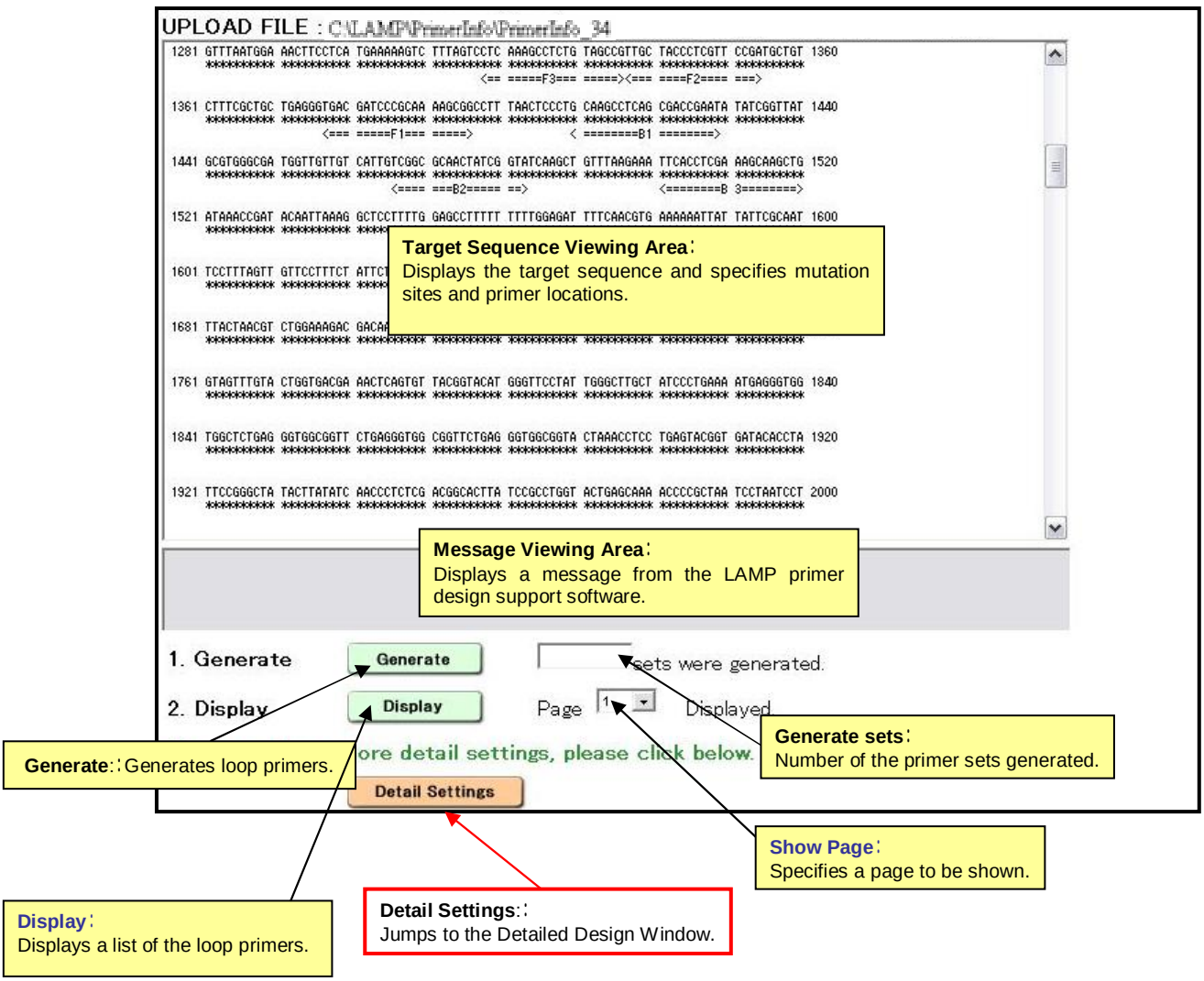
Limitations:
Specifies the number of combinations of primers to generate a primer set and places an upper limit on the number of sets to be generated.

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



: Existing functions

: New functions

Detailed Design Window