Advanced primer design

6. Designing wild type and mutant type primers

In PrimerExplorer, it is possible to introduce mutations into the target sequence and then design primers. However, if there are too many mutations, the primer design conditions become too stringent and either the primers are not generated or the variety is insufficient. In such a case, one can design the primer with less stringent condition, for example reduce the number of the mutation point entered or completely eliminate the mutation sites from the target sequence. Appropriate primer sets could be selected, while identifying where the mutation points in the target sequence are located relative to the primer.

6.1 Detecting wild type and mutant type by amplification using common primers

In general, the primers are design to exclude mutation within the primer region, but if there are numerous mutations, it may not be possible to design primers that satisfy these conditions. For this reason, primers are designed that allow (contain) mutations and if possible, try to design primers that are not likely influenced by the mutation.

Under the principles of the LAMP reaction, F2 of FIP (or B2 of BIP) anneals to the target gene and initiates the gene synthesis. If the mutation is at the 3' end of F2 (B2), the DNA polymerase has difficulty in recognizing the double strand formed between the primer and the target gene, thus inhibit the gene amplification. Similar principles apply to the 5' end of F1c (B1c) and the 3' end of F3 (B3). Therefore, primers are selected so that mutations are not located in these regions.

On the other hand, if primers are selected so that the mutations are outside of the 3' end in F2 (B2), 5' end of F1c (B1c), or 3' end of F3 (B3), the primers are less susceptible to the effect of the mutation and the both wild type and mutant type are detectable by a common set of primers.

Thus, primers are selected by permitting the mutation to be located within the following locations (Table 6.1).

a) 3' end of F1c or B1c and in the internal region

b) 5' end of F2 or B2 and in the internal region

c) 5' end of F3 or B3 and in the internal region

Here, we will design common primers that detect M13 and its mutant. Figure 6.1 shows an alignment of the wild type and the mutant type. In the entire length of 510 bp there are seven mutations. The region containing these mutations is the target region for amplification.

Figure 6.2 shows an example of primer selection. Under default, primers have been designed for the wild type strain. Here, we focus on the 25 primer set candidates that contain the mutations and select the common primers. A star shows the locations of the mutations, and the locations of the mutations applicable to the primers designed are enclosed by a dotted line. This can be used to confirm the location of the primers for a given mutation. Table6-2 shows these results. The mark (plus, +) indicates the location of the primer region (5' end, internal region, 3' end) for each primer set (F3, F2, F1, B1, B2, B3) for a given mutation. It can be determined that No 1, No 6 - 11, and No 16 - 25 are the primers less susceptible to the effects of the mutations at amplification. These are selected from the primer list, and their detailed information is then assessed for a final selection of the primer sets.

6.2 Highly specific primers (specific primers that distinguish between wild-type and mutant type)

If the mutant type and wild type need to be distinguished, a method opposite to the aforementioned method is used. Thus, by selecting primers with the mutation in the locations mentioned below, one can select highly specific primers. If the primers include a mutation in this location, the mutant type is generally amplified while the wild type amplification is hindered, thus improving the specificity toward the mutant type (table 2.1).

a) 5’ end of F1c or B1c

b) 3’ end of F2 or B2

c) 3’ end of F3 or B3

As in (1), from the primer list designed under default, primer sets are selected that fulfill the above a), b), or c). In Table 2-2, No 2 – 5 and No 12 - 15 are mutant type-specific primer sets. Then, their detailed information is examined for final selection of the primer set.

Table 6.1 Select Common primers and specific

	F3			F2			F1c			B1c			B2			B3		
	5’ [1]	In[2]	3’ [3]	5’	In	3’	5’	In	3’	5’	In	3’	5’	In	3’	5’	In	3’
Common primer[4]	●	●		●	●			●	●		●	●	●	●		●	●	
Specific primer[5]			●			●	●			●					●			●

1) 5’ terminal region
2) internal region
3) 3’ terminal region
4) Common primer : Mutation sites that can be permitted for the amplification of wild type and mutant type using common primers.
5) Specific primer : Sites for mutation for distinguishing between wild type and mutant type.
6) ＋ : The mutation points are included.

```
M13_3.nuc      1:GCAGGCATGCAAGCTTGGCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTG 60
M13_3M1.nuc    1:GCAGGCATGCAAGCTTGGCACTGGCCGTCGTTTTGCAACGTCGTGACTGGGATAACCCTG 60

M13_3.nuc     61:GCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCG 120
M13_3M1.nuc   61:GCGTTACCCAACTTAATCGACTTGCAGCACATCCCGCTTTCGCCAGCTGGCGTAATAGCG 120

M13_3.nuc    121:AAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGCGCT 180
M13_3M1.nuc  121:AAGAGTCCCGCACCGATCGCCCTTCCCAACACTTGCGCAGCCTGAATGGCGAATGGCGCT 180

M13_3.nuc    181:TTGCCTGGTTTCCGGCACCAGAAGCGGTGCCGGAAAGCTGGCTGGAGTGCGATCTTCCTG 240
M13_3M1.nuc  181:TTGCCTGGTTTCCGGCACCAGAAGCGGTGCAGGAAAGCTGGCTGGAGTGCGATCTTCCTG 240

M13_3.nuc    241:AGGCCGATACGGTCGTCGTCCCCTCAAACTGGCAGATGCACGGTTACGATGCGCCCATCT 300
M13_3M1.nuc  241:AGGCCGATACGGTCGTCGTCCCCTCAAACTGGCAGATGCACGGTTACGATGCGCCCATCT 300

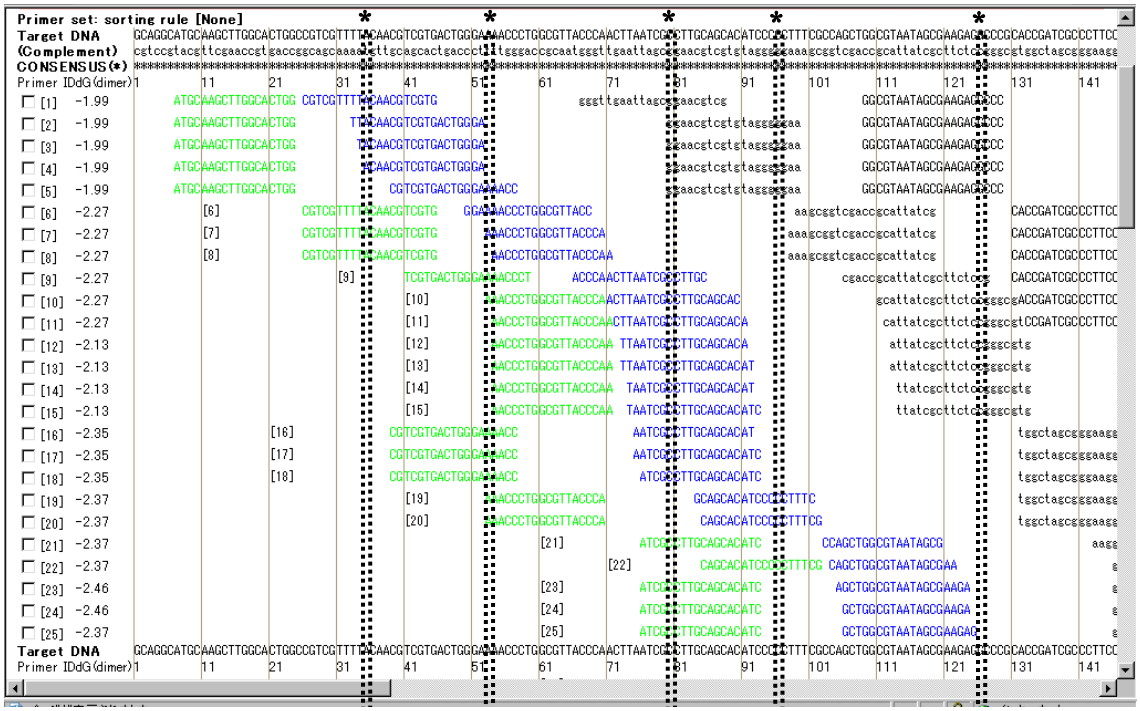
M13_3.nuc    301:ACACCAACGTAACCTATCCCATTACGGTCAATCCGCCGTTTGTTCCCACGGAGAATCCGA 360
M13_3M1.nuc  301:ACACCAACGTAACCTATCCCATTACGGTCAATCCGCCGTTTGTTCCCACGGAGAATCCGA 360

M13_3.nuc    361:CGGGTTGTTACTCGCTCACATTTAATGTTGATGAAAGCTGGCTACAGGAAGGCCAGACGC 420
M13_3M1.nuc  361:CGGGTTGTTACTCGCTCACATTTAATGTTGATGAAAGCTGGCTACAGGAAGGCCAGACGC 420

M13_3.nuc    421:GAATTATTTTTGATGGCGTTCCTATTGGTTAAAAAATGAGCTGATTTAACAAAAATTTAA 480
M13_3M1.nuc  421:GAATTATTTTTGATGGCGTTCCTATTGGTTAAAAAATGAGCTGATTTAACAAAAATTTAA 480

M13_3.nuc    481:CGCGAATTTTAACAAAATATTAACGTTTAC                              510
M13_3M1.nuc  481:CGCGAATTTTAACAAAATATTAACGTTTAC                              510
```

Figure 6.1 Alignment of wild type with mutant type



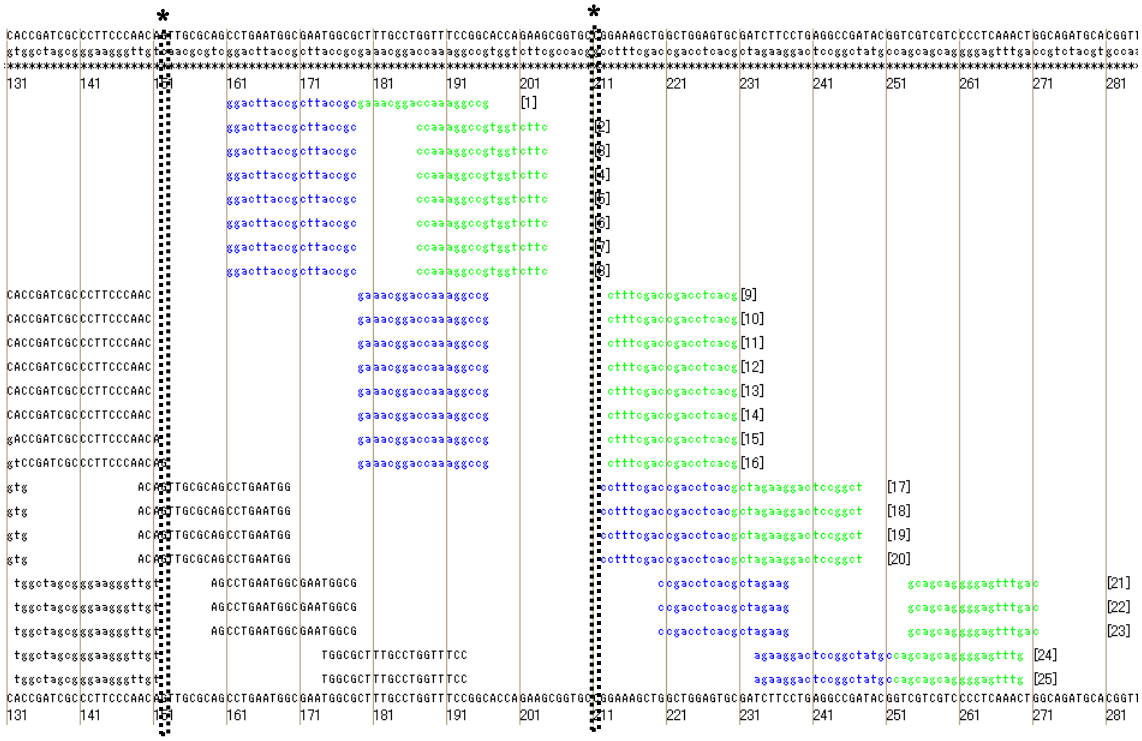


Figure 6.2 Primer sets and location of mutations

No.	F3			F2			F1c			B1c			B2			B3			Primer
	5'	In.	3'	5'	In	3'	5'	In.	3'	5'	In.	3'	5'	In.	3'	5'	In.	3'	
1					+			+				+							Common*
2				+			+		+			+							Specific**
3				+			+		+			+							Specific
4				+			+		+			+							Specific
5					+		+		+			+							Specific
6		+		+															Common
7		+																	Common
8		+																	Common
9		+			+				+										Common
10					+			+											Common
11					+			+				+							Common
12					+			+		+									Specific
13					+			+		+									Specific
14					+			+		+									Specific
15					+			+		+									Specific
16		+			+														Common
17		+			+														Common
18		+		+															Common
19					+														Common
20					+														Common
21	+							+											Common
22		+						+											Common
23	+							+											Common
24	+							+											Common
25	+							+											Common

Table 6.2 Primer sets depending on location of mutation

*Common : primer set candidates that can amplify both wild type and mutant type with a common primer set
**Specific : primer set that distinguishes mutant type from wild type

7. Primer design that takes the location of mutation into account

7.1 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 startup window, use the steps outlined in Section 1 to select the target sequence file (see p. 13) and then click on the “primer design button.” (Figure not shown)

7.2 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 7.1). As indicated in Figure 7.2, 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.

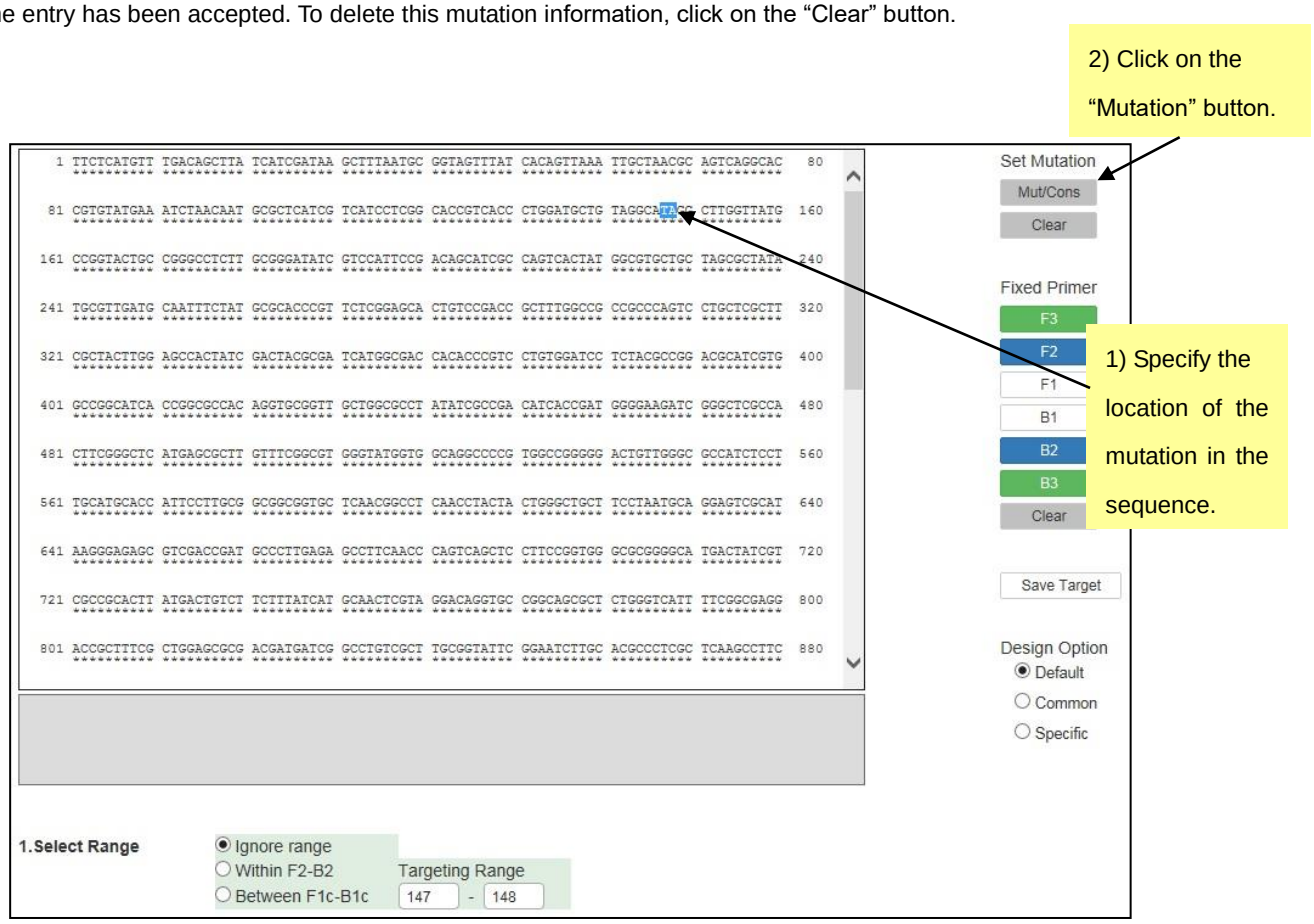


Figure7.1 Primer design window

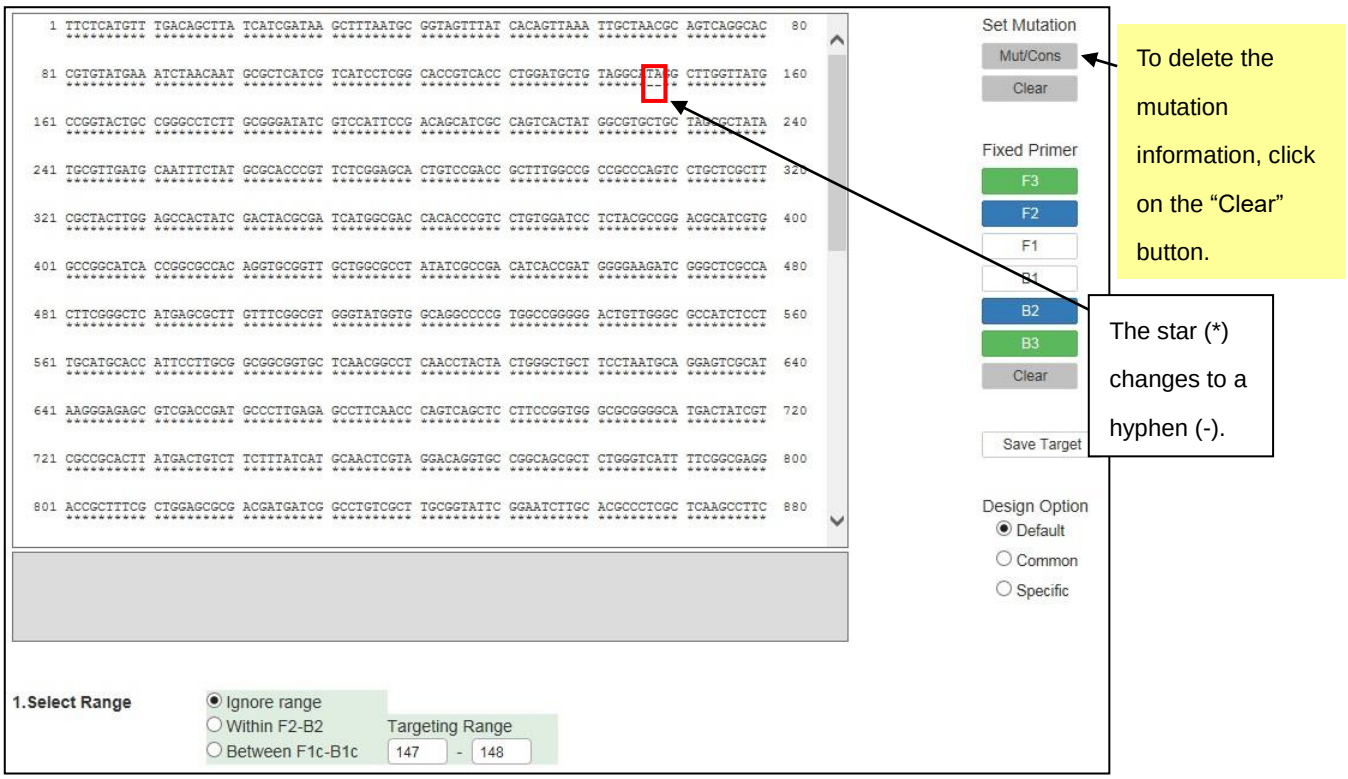


Figure7.2 Window after entering the location of mutation

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 7.3). The primers are designed to avoid including the mutation.

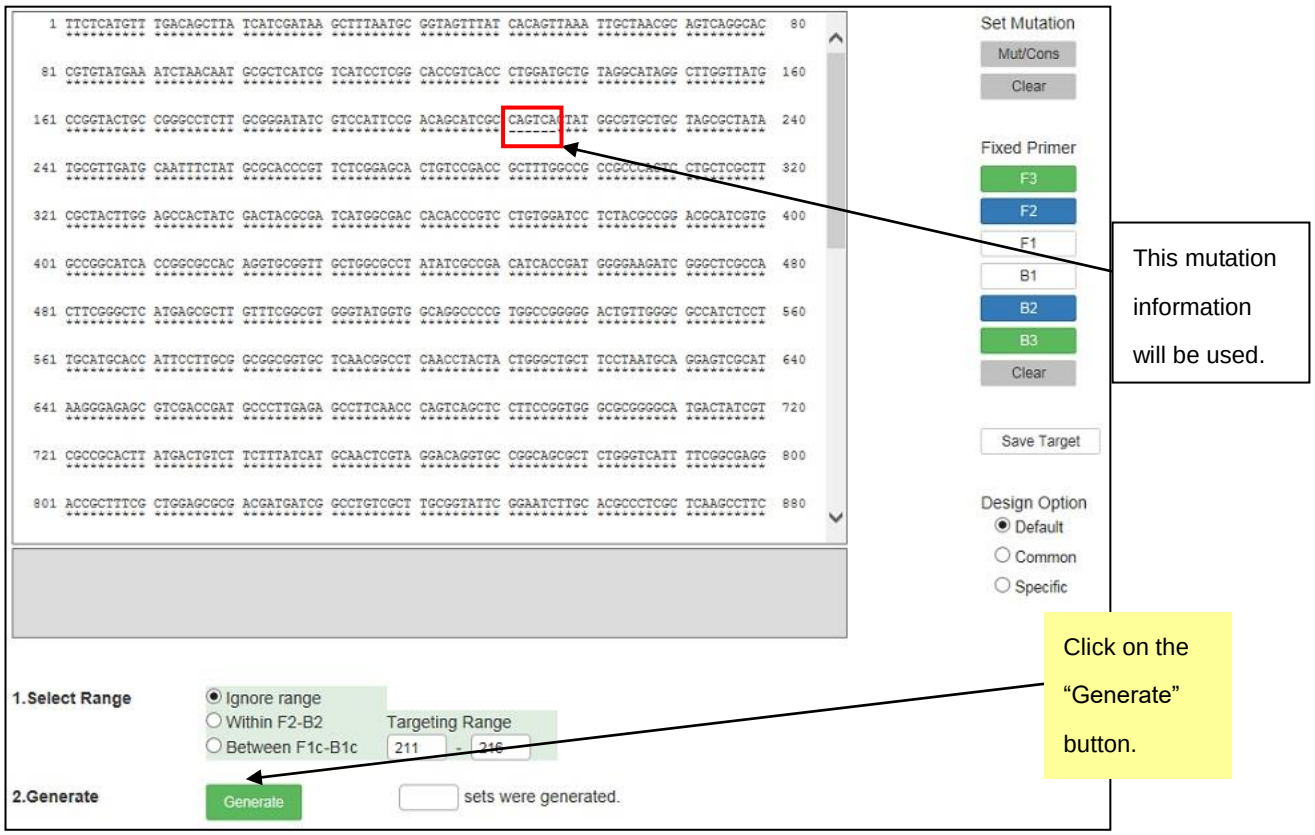


Figure7.3 Window after entering site of mutation

1,000 primer sets have been designed (See Figure 7.4).

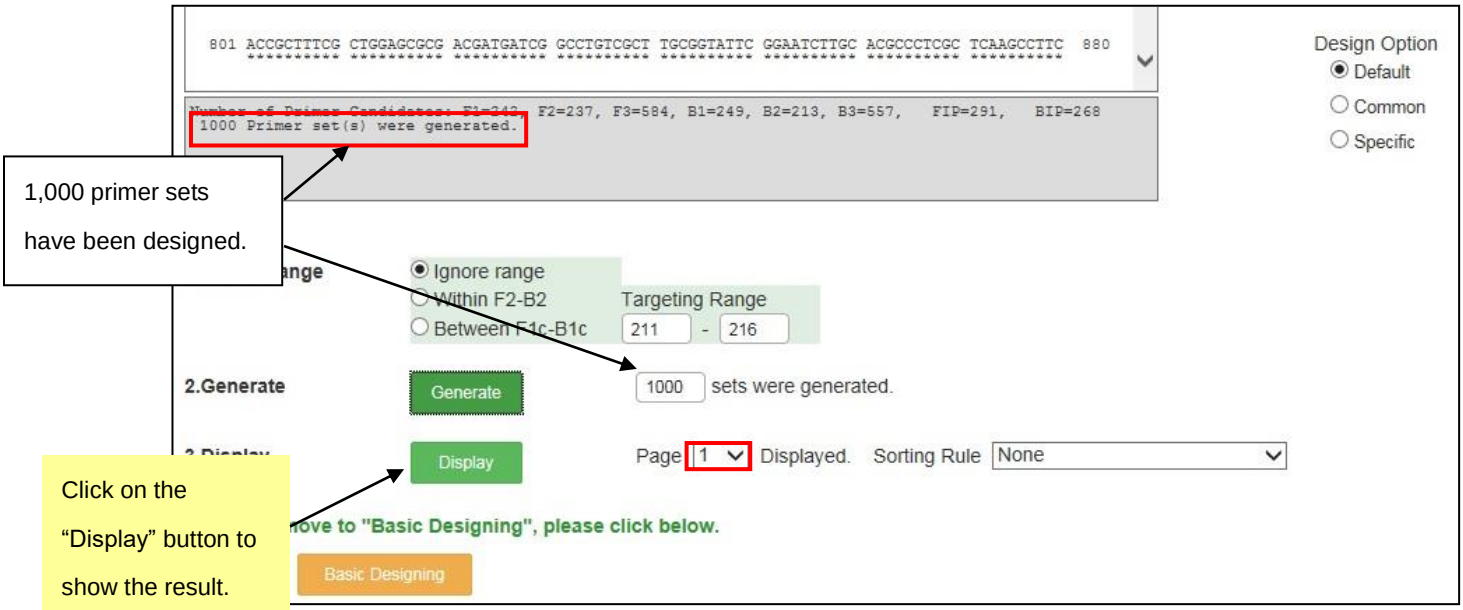


Figure7.4 Primer design window

Next, click on the "Display" button to show the results. As indicated in Figure 7.5, the primers are designed to exclude mutation from the primer regions.

<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.

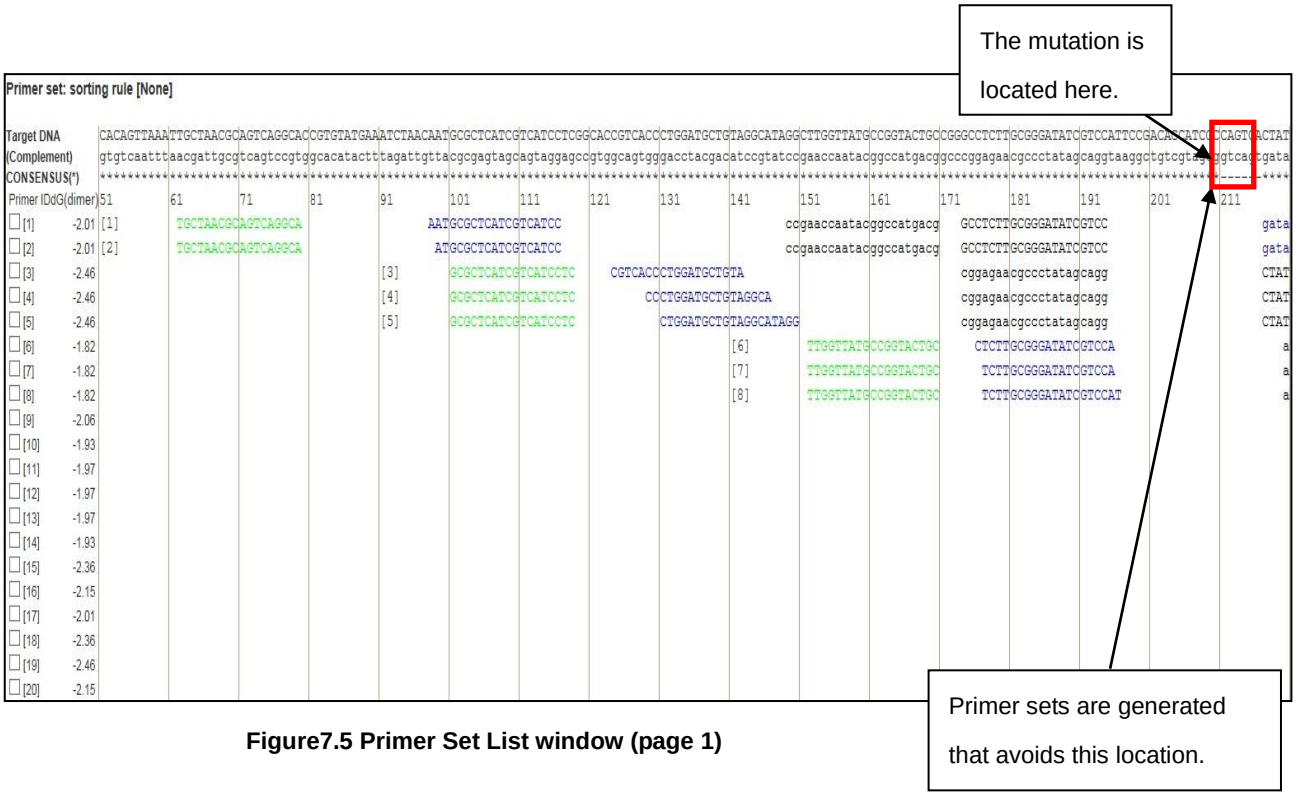


Figure7.5 Primer Set List window (page 1)

For reference, Figure 7.6 shows the results obtained when the primer design was conducted without any mutation entry.

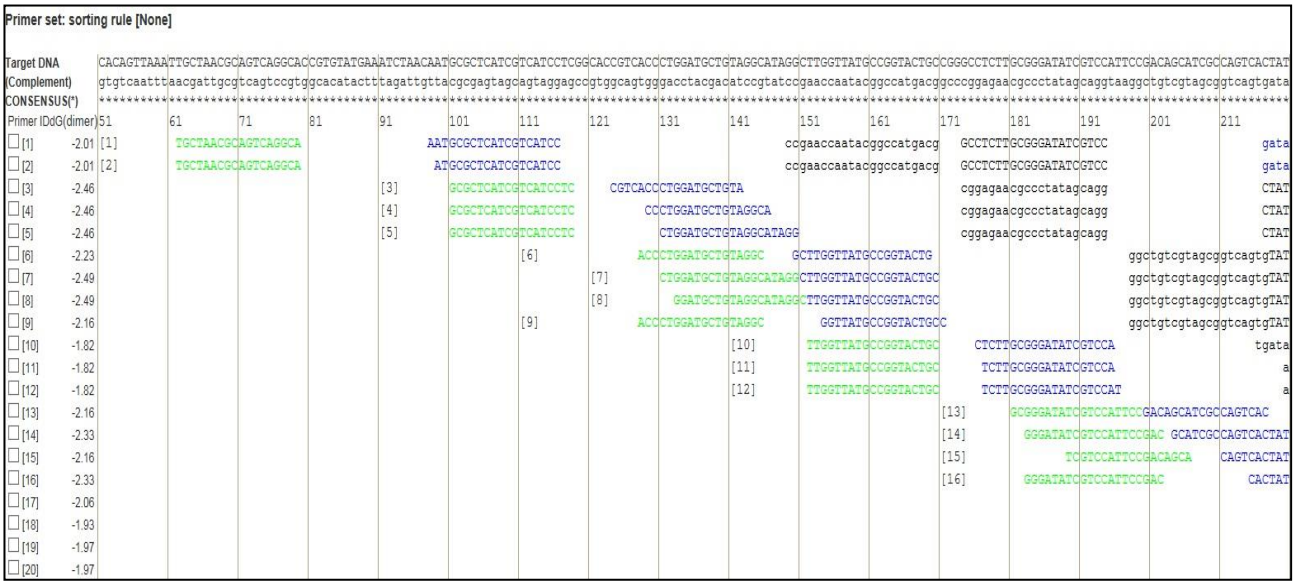


Figure7.6 Primer set designed with no mutation entry

7.3 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’ and 3’ ends of each primer region.

For example, if a mutation is introduced into a particular region of the target sequence as described in the preceding section, the number of primer set candidates for amplifying the gene containing this region decreases dramatically. When amplifying the region that contains the mutation, it is preferred that the mutation be not located within the primer; however, there are some cases in which such strict conditions dramatically reduce the number of potential primers, or even no primers are produced. If no mutations are present in the region, on the other hand, many primer set candidates incorporating the location of the mutation are designed, as shown in Figure 7.6. Hence, a wide variety of primer candidates are generated by using less stringent conditions to allow the mutation to be present within the primer region. The primers with the mutation having the minimal influence on the amplification are then chosen.

In PrimerExplorer, it is possible to select primer regions that contain the mutation. Regions that can be selected are the 5’ and 3’ ends, as well as the internal region for regions F3, B3, F2, B2, F1c, and B1c. Since the 5’ ends of regions F3, B3, F2, and B2, the 3’ ends of regions F1c and B1c, and the internal regions between the 5’ and 3’ ends do not serve as the starting points of the amplification, they are less susceptible to the influence of the mutation. If 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 shown in Figure 7.7, 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.

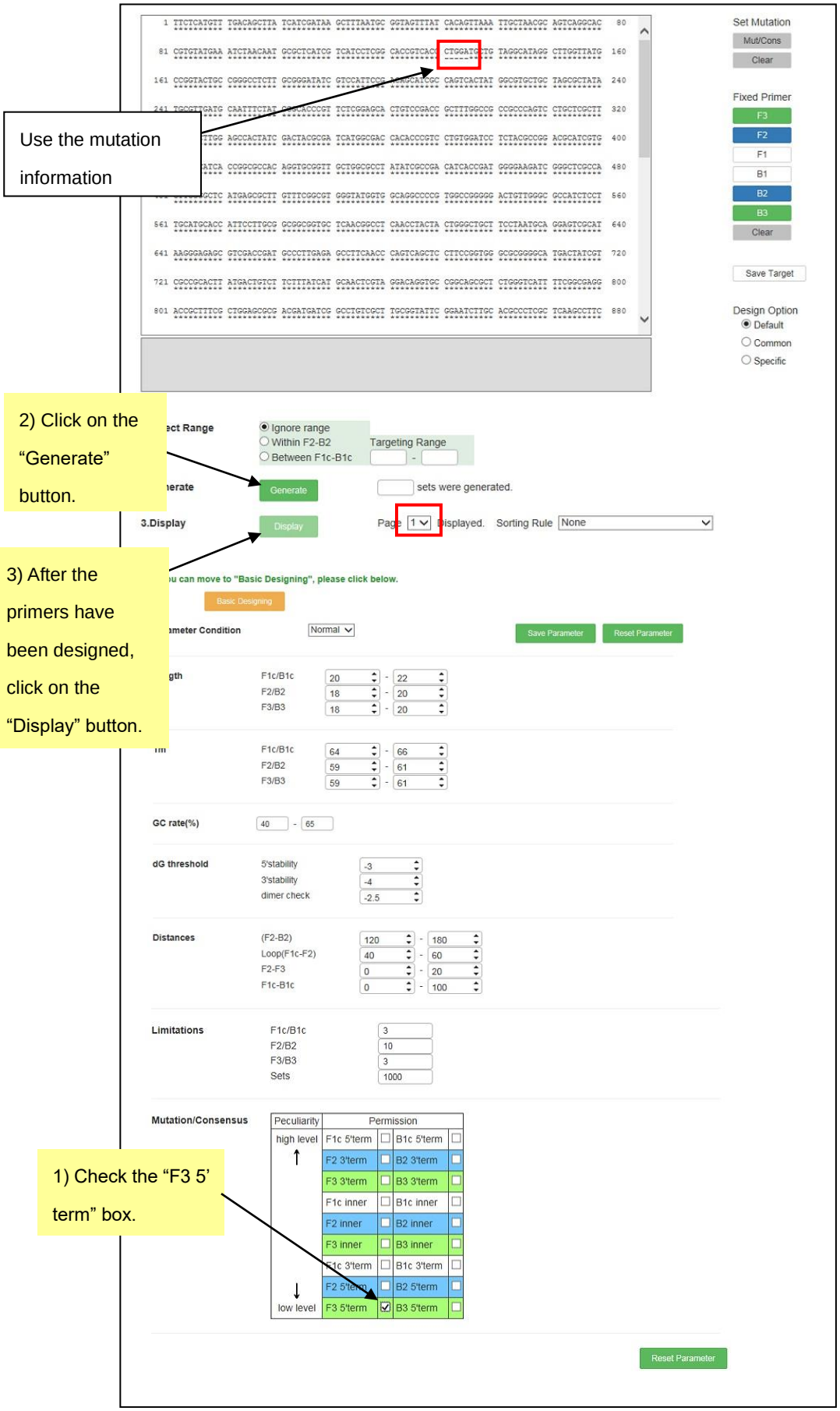


Figure7.7 Primer design window

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 regionF3, we will design primers that include the mutation at the 5’ end of region F3.

<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.

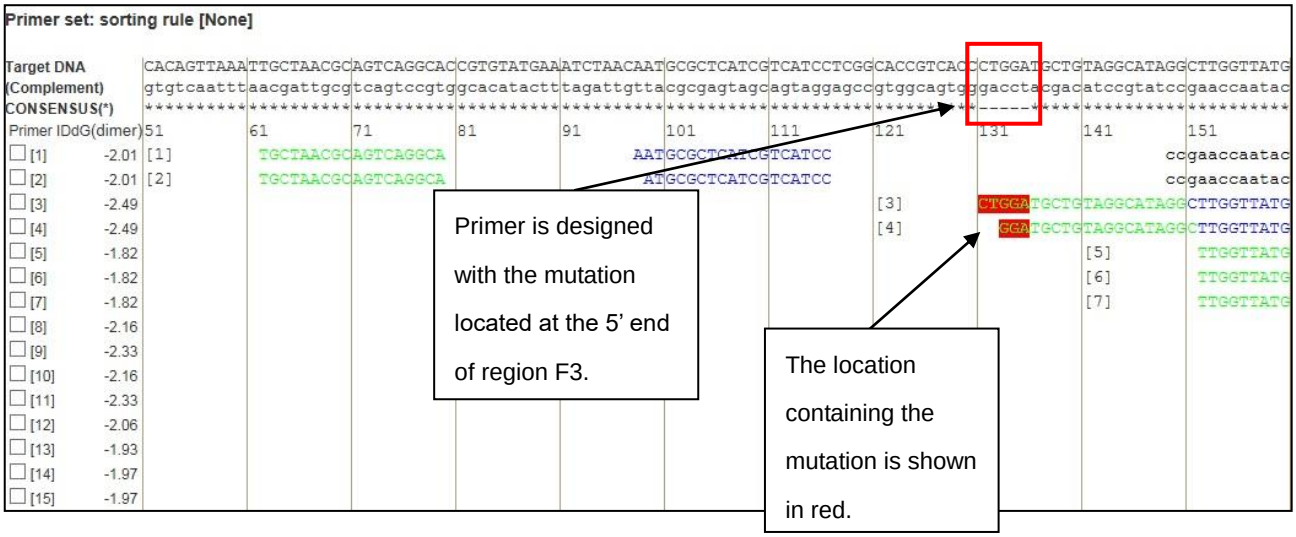


Figure7.8 Primer Set List (page 1)

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 7.9, 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 7.10)

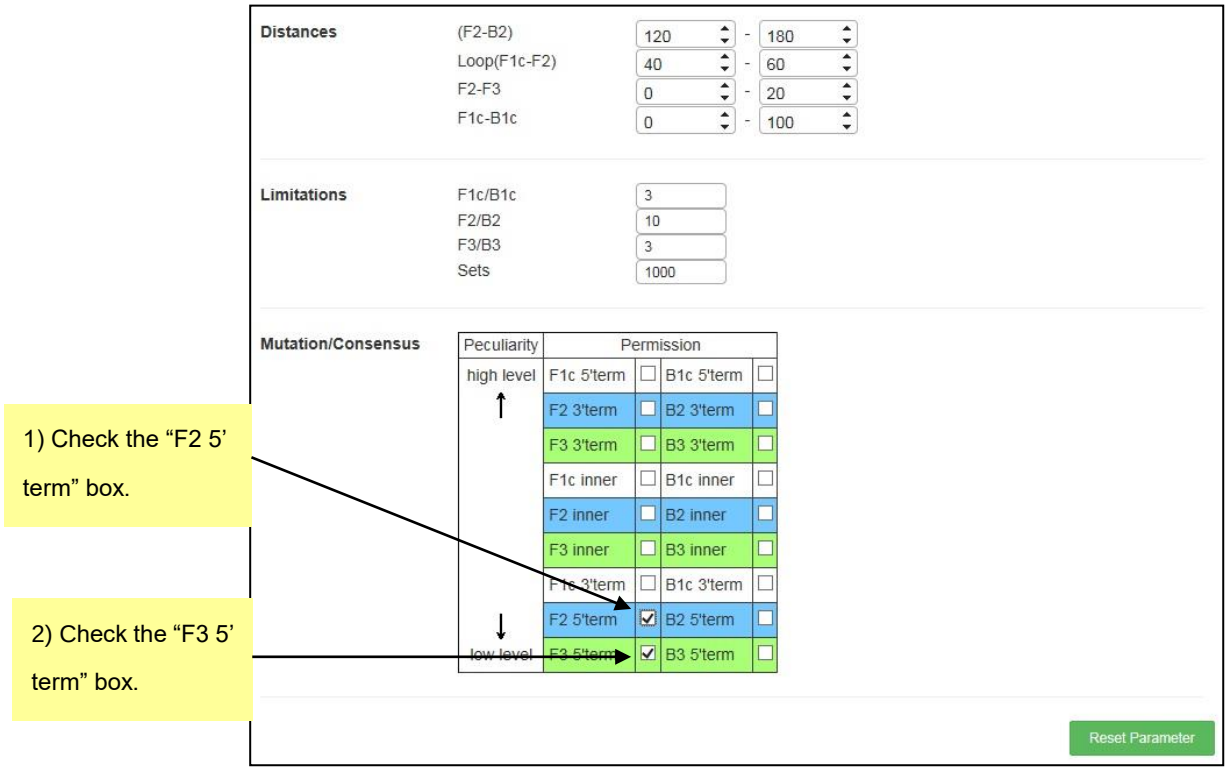


Figure7.9 Primer design window

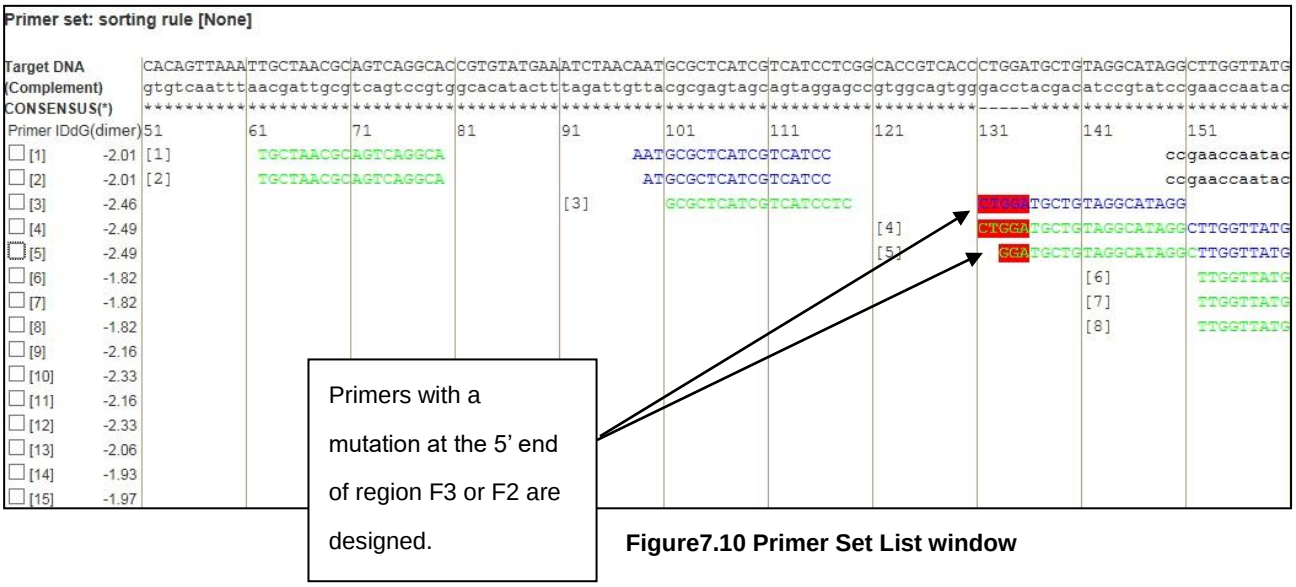


Figure7.10 Primer Set List window

The method described in Section 1 is then followed (See p.22 - 23) to compare the primer information and select the primer sets.

8. Designing common primer using multiple alignment results

8.1 Reading multiple alignment results

If alignment results are input in the same way for normal gene sequences, consensus sequences and mutation sites are displayed with reference to the genes at the top sequence. The alignment should be performed with the Genetyx or Clustal W program. Here, take an alignment of the three genes SeqA, SeqB, and SeqC for an example. Figure 8.1 shows an example alignment of SeqA, SeqB, and SeqC that was performed with Genetyx. Read the results in PrimerExplorer and click on the “Primer Design” button, and you will find that, as seen in the figure below, the window displays consensus sequences (*) and mutation sites (-) based on SeqA. Using three results, you can design primers (Figure 8.2).

```
SeqA            AATGCTACTACTATTAGTAGAATTGATGCCACCTTTTCAGCTCGCGCCCCAAATGAAAAT 60
SeqB            ----------------------AATTGATGCCACCTTTTCAGCTTGCGCTCCAAATGAACAT 40
SeqC            ------------------------------------------CTCGCGCCCCACTTGAAAAT 20
                                                           ** **** ***  **** **

SeqA            ATAGCTAAACAGGTTATTGACCATTTGCGAAATGTATCTAATGGTCAAACTAAATCTACT 120
SeqB            ATAGCTACACAGCTTATTGACCATTTGCGAAATGTATCTAATGTTCGAACTAACTCTACT 100
SeqC            ATCGCTAAACAGGTCGTTGACCATATGCGAAGTGTATCTAATGCTCAAACTAATTCTACT 80
                ** **** **** *  ******** ****** ********** ** ****** ******

SeqA            CGTTCGCAGAATTGGGAATCAACTGTTACATGGAATGAAACTTCCAGACACCGTACTTTA 180
SeqB            CGATCGCAGAATCGGAAATCAACTGTTACATGGAATGAACCTTCCAGACGCCGTACTTCA 160
SeqC            CGTTCGAAGAATTGGCAATCAACTGTAACATGAAATGAAACTTGCAGACACCGTACTTTA 140
                ** *** ***** ** ********** ***** ****** *** ***** ******** *

SeqA            GTTGCATATTTAAAACATGTTGAGCTACAGCACCAGATTCAGCAATTAAGCTCTAAGCCA 240
SeqB            GTTCCATATGTAACCCATGTTGAGCTACAGCACGAGTTTCAGCAATTAAGCTCTATGCGA 220
SeqC            GTTGCATATTTAAATCATGTTGAGCTACAGCAACAGATTCAGCACGTAAGCTCTAAGCCA 200
                *** ***** ***  *****************  ** *******  ********* ** * *

SeqA            TCCGCAAAAATGACCTCTTATCAAAAGGAGCAATTAAAGGTACTCTCTAATCCTGACCTG 300
SeqB            TCCGCAAAAATGACCTCTTATCATAAGGAGCAATTAACGGTACTTTCTAATCCTGACCTG 280
SeqC            TCCGCAACTGTGACCTCTTAACAAAAGGAGCACTTGAAGGTACTCTCTAATCGTGACATG 260
                *******   ********** ** ******** ** * * ****** ******* **** **

SeqA            TTGGAGTTTGCTTCCGGTCTGGTTCGCTTTGAAGCTCGAATTAAAACGCGATATTTGAAG 360
SeqB            TTGGAGTTTGCTTCTGGTCTGGTTCGCTTTGAAGCTCGACTTAAAACGCGATATTTGAAG 340
SeqC            TCGGAGTTAGCTTCCGGTCTGGTTCGCTTTGAAGCTCGAATCAACACGCGATAGTTGATG 320
                * ****** ***** ************************ * ** ******** **** *

SeqA            TCTTTCGGGCTTCCTCTTAATCTTTTTGATGCAATCCGCTTTGCTTCTGACTATAATAGT 420
SeqB            TCTTTCGGGGTTCCTCTTAATCTTCTTGATGCAATCCGTTTTGCTTCTGACTATAATAGT 400
SeqC            CCTTTCAGGCTTCCTCTGAATCTTTTTGATGCAATCGGCTTAGCTTCTGACTATATTAGT 380
                 ***** ** ******* ****** ********** * ** ************** ****

SeqA            CAGGGTAAAGACCTGATTTTTGATTTATGGTCATTCTCGTTTTCTGAACTGTTTAAAGCA 480
SeqB            CAGGGTAAAGACCTGATTTTTGATTAGTGGTCATTCTCGTTTGCTGAACTGTATAAAGCA 460
SeqC            CACGGTAAAGACCTGATTTATGATTTATGGTCATTCTCGTTTTCTGAACTGTTTAAGGCA 440
                ** ***************** *****  *************** ********* *** ***

SeqA            TTTGAGGGGGATTCA 495
SeqB            TTTGAGGC-------- 468
SeqC            TTAGAGGG-------- 448
                ** ****
```

Figure 8.1 Multiple alignment

Figure 8.2 Multiple alignment read window

8.2 Designing common primers

Check the “Common” button and press the “Generate” button, and you will find that five common primers are generally designed. As shown in Figure 8.3, sets of these primers are generated with mutations included at the 5’ end and internal region of F3, F2, B3, or B2, or at the 3’ end and internal region of F1c or B1c. Since the mutations are recognized at locations other than the starting points of amplification, these primers are relatively insusceptible to the mutations. Figure 8.4 shows the details of the primer sets.

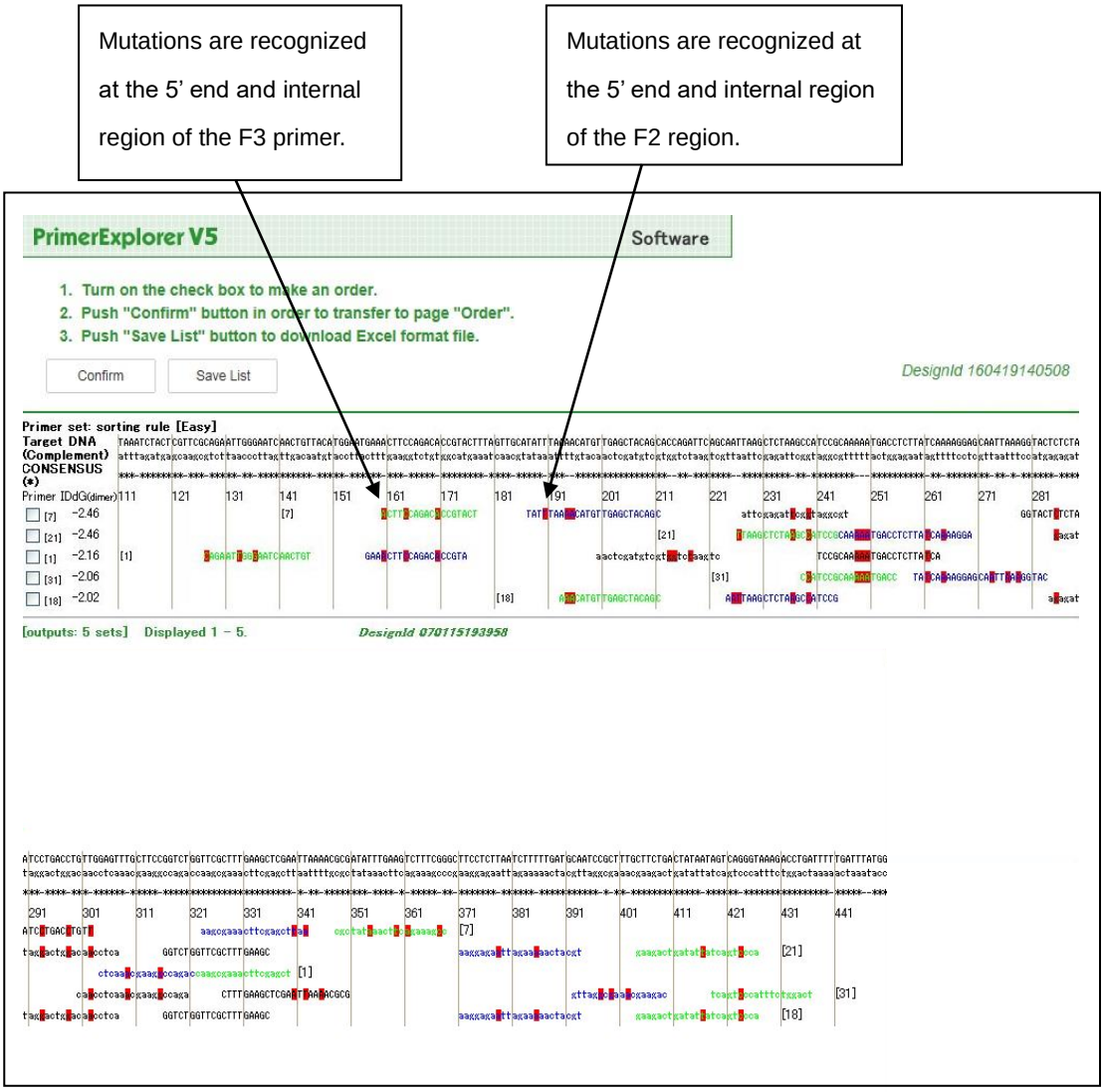


Figure 8.3 Primer Set List window

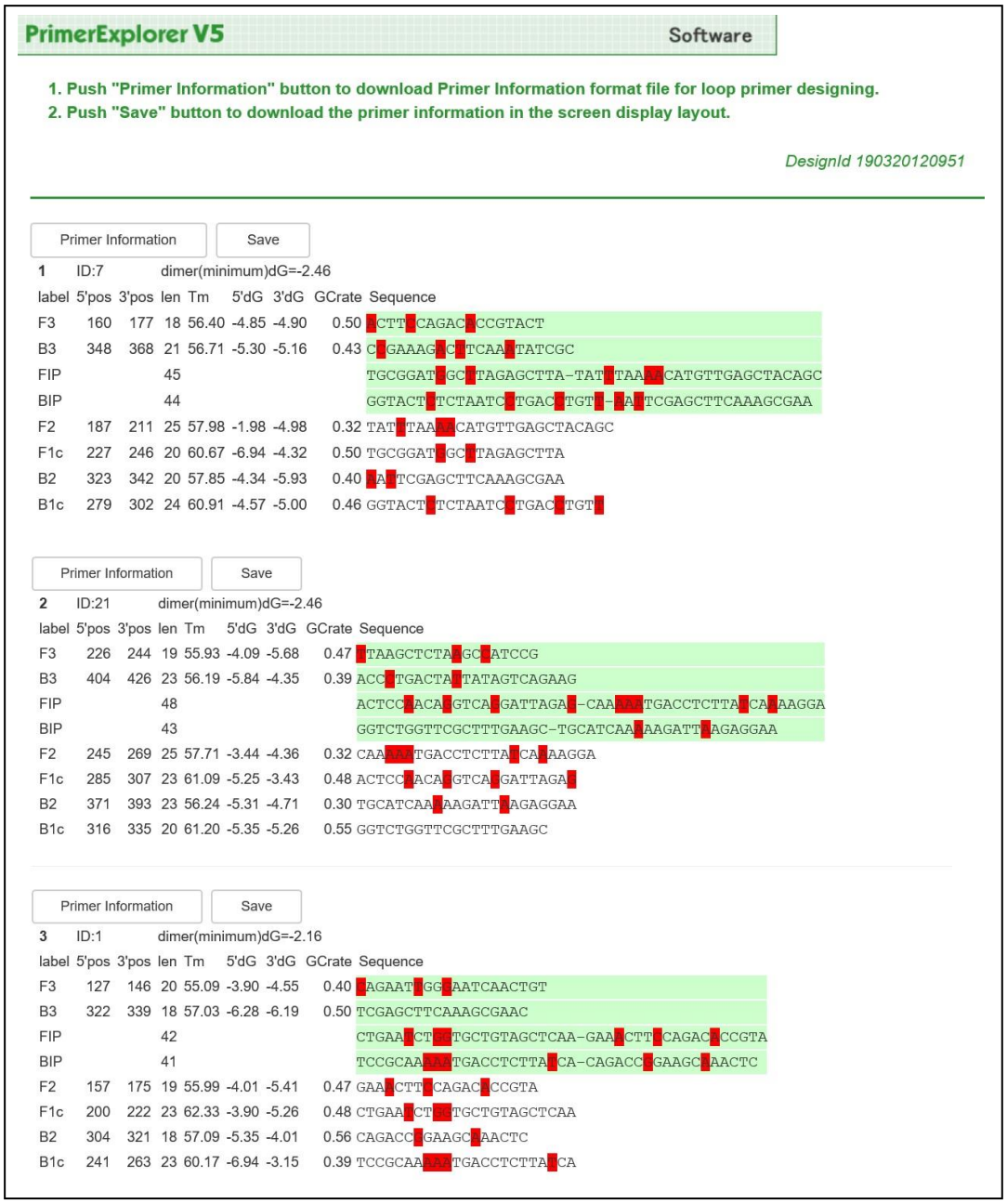


Figure 8.4 Primer Set Details window

9. Specific primer design

9.1 Design in Easy Mode

As shown in Figure 9.1, check the “Specific” button at the bottom right of the window and press the “Generate” button to automatically design specific primer sets.

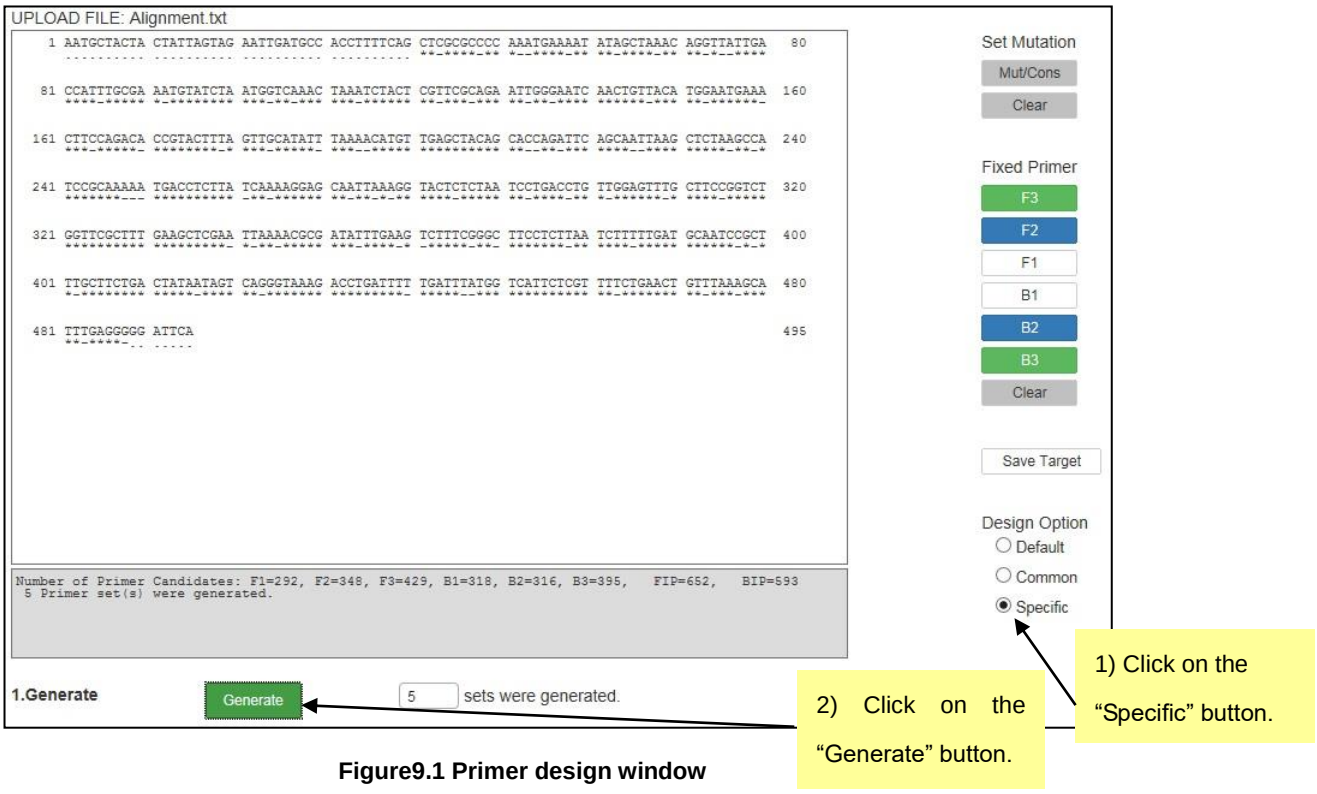


Figure9.1 Primer design window

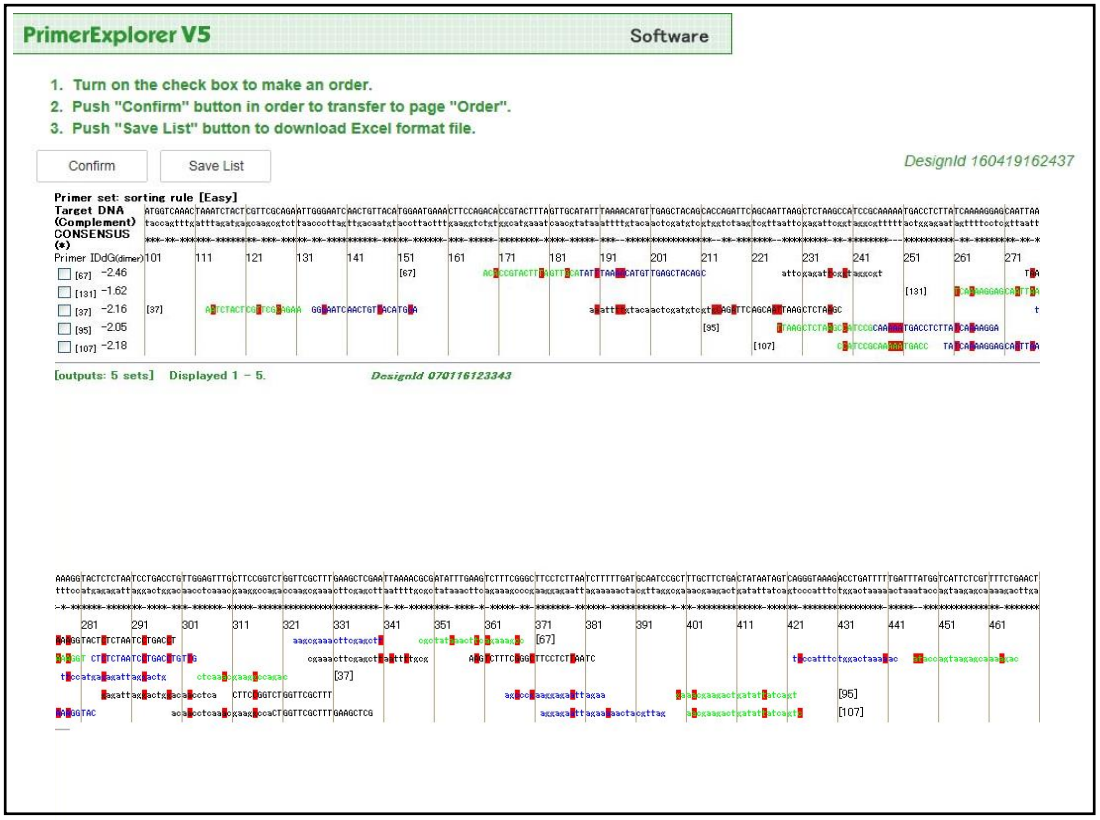


Figure9.2 Primer Set List window

As shown in the primer design result window in Figure 9.2, primer sets are generated that can recognize mutations sites at the 3' end of F3/B3 or F2/B2, or the 5' end of F1c/B1c. Figure 9.3 shows the details of the primer sets.

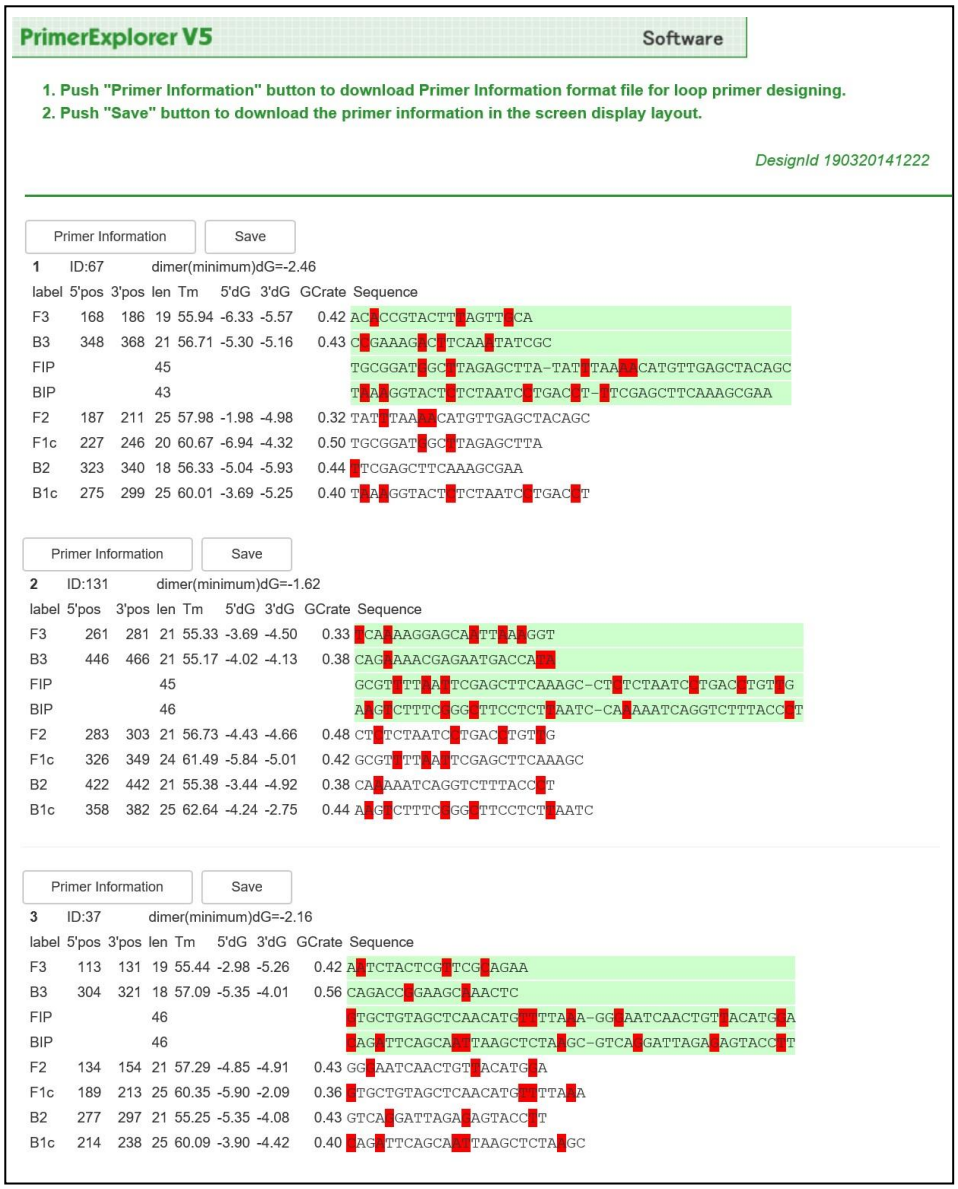


Figure 9.3 Primer Set Details window

9.2 Design in Expert Mode

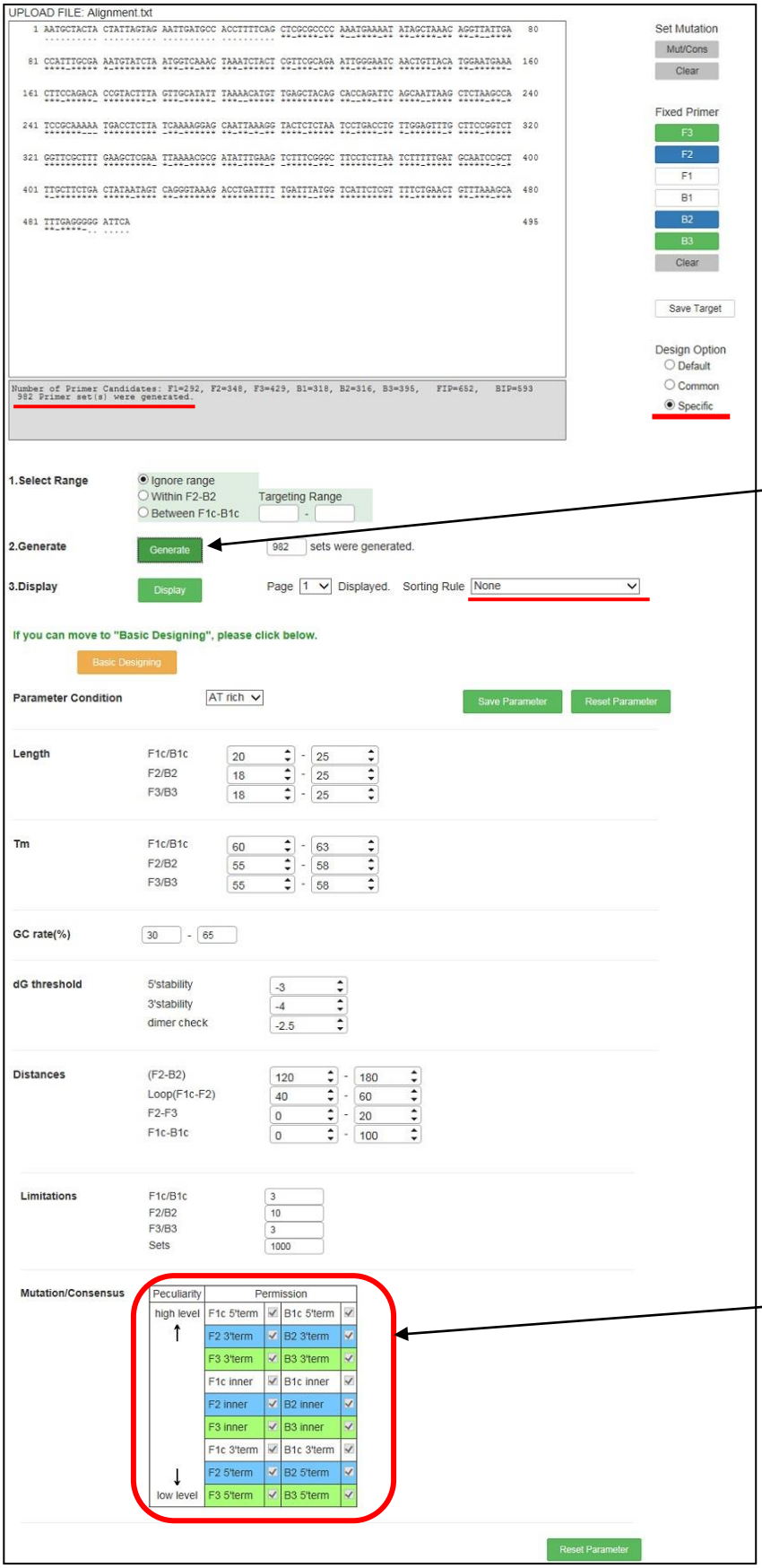


Figure9.4 Primer design window

As shown in Figure 9.4, Expert Mode enables designing with allowance for mutations to be included at the ends of each primer.

The results in Expert Mode are shown in Figure 9.5. Primer sets are generated that can recognize mutations sites at the 3’ end of F3/B3 or F2/B2, or at the 5’ end of F1c/B1c. Specific primers are generated from the 5’ end toward the 3’ end of the target genes. Primers are designed for the entire target region. If too many primer sets have been generated, you should set stricter design conditions and limit the number of primer sets to be generated. You can do this as instructed in p17-27, Section 1, to select the desired number of primer sets.

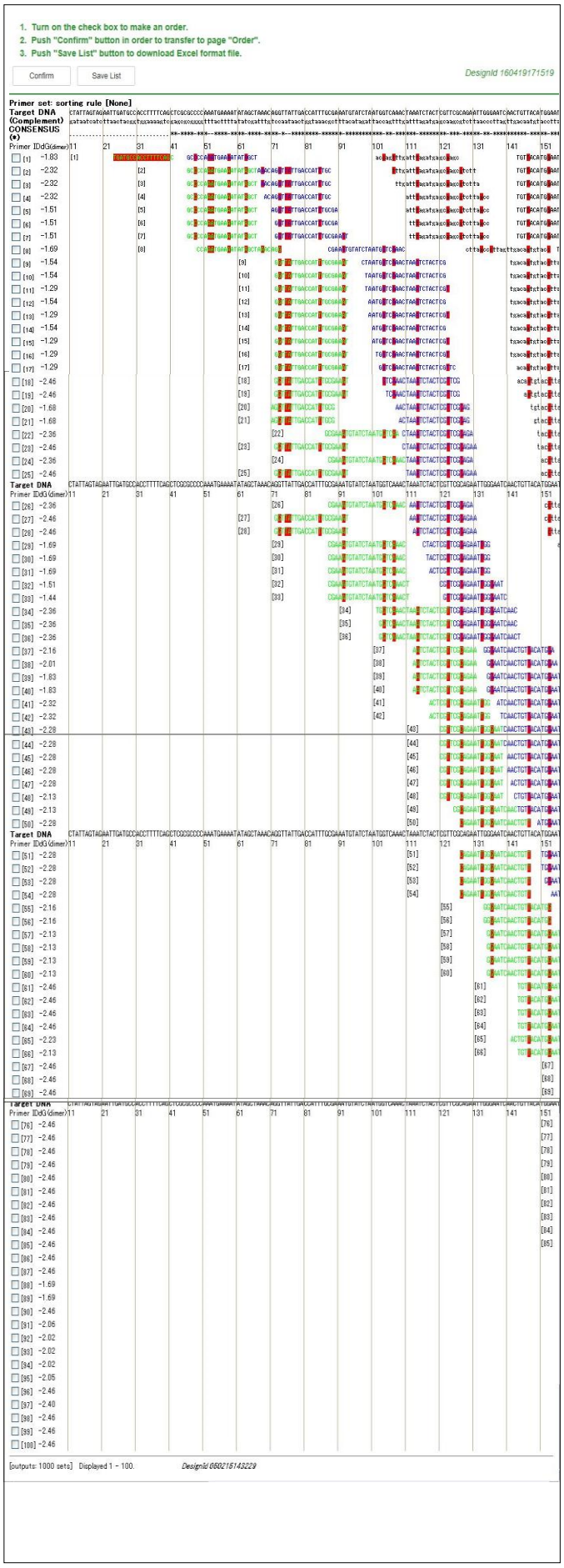
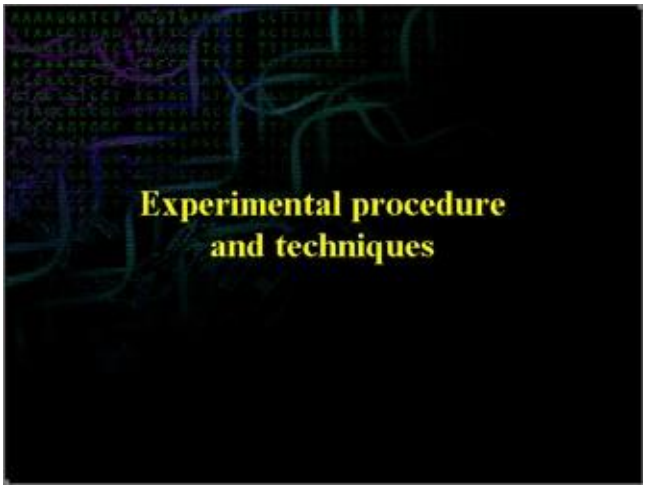
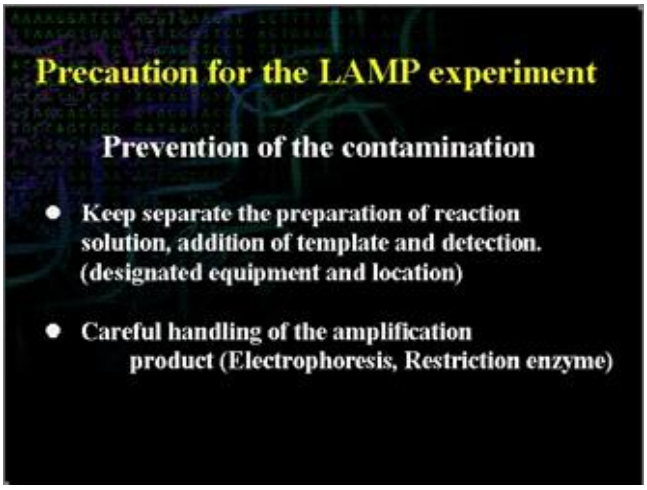


Figure 9.5 Primer Set List window

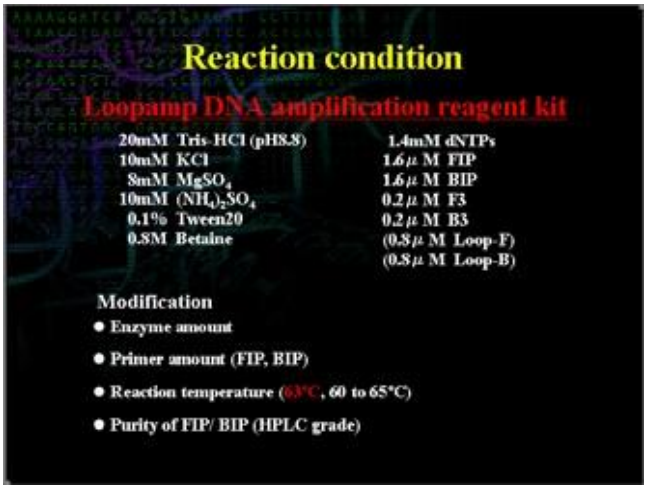
Experimental procedure and techniques of LAMP method

Experimental procedures and techniques

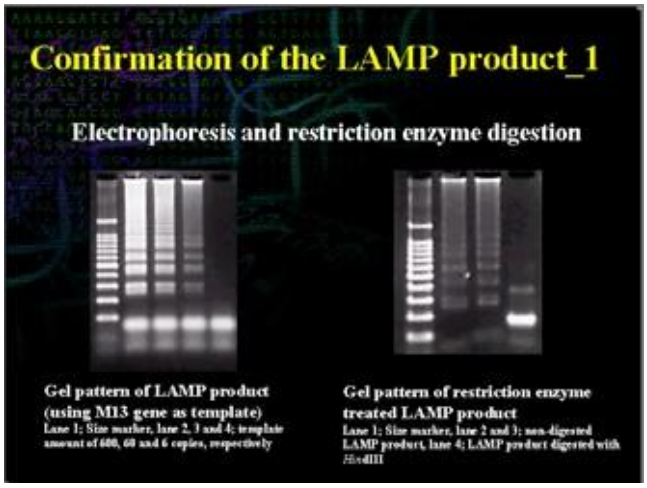




In conducting the LAMP method, the greatest care is needed to prevent contamination.
One preventive measure is to keep separate the preparation of reaction solutions, addition of template, and detection. The experimental equipment and experimental locations should be kept physically separate. If separately experimental locations are not available, at least conduct the preparation of reaction solutions and the addition of templates in separate clean benches. However, the detection should be done in a separate room.
Handling of the amplification product might cause contamination, thus sufficient care is needed when performing electrophoresis or restriction enzyme digests for the purpose of identifying the amplification products.

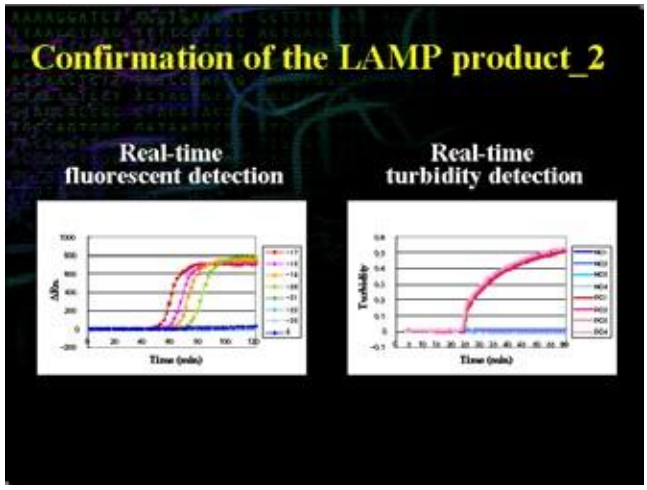


In the LAMP method, the amplification conditions of Loopamp DNA Amplification Kit are regarded as the fundamental experiment conditions.
To increase the amplification speed or sensitivity, consider the following factors.
- Amount of enzyme, amount of primer (particularly the Inner primer)
- Reaction temperature (In addition to the recommended temperature of 63°C, temperature in the 60 - 65°C range can also be considered)
- Purity of the Inner primer (for screening the primer sets, the de-salting grade purity is sufficient, but for further assessments, HPLC-grade purity should be used.)



A basic procedure for confirmation of LAMP product is restriction enzyme digest and electrophoresis.
The Figure on the left is the electrophoresis result of the LAMP products obtained using M13 as template. From the left are the size marker, 600 molecules, 60 molecules, 6 molecules of the template and negative control.

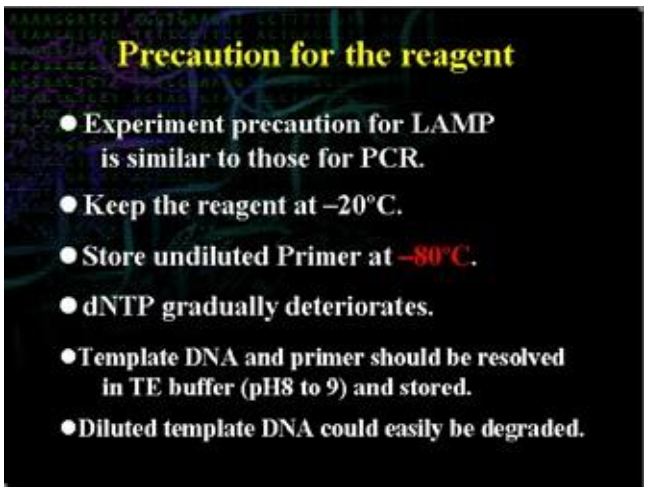
There exists a single *Hin*dIII site in the target M13 sequence. In the figure on the right, from the left are: size markers, two lanes of untreated sample, and a sample digested with *Hin*dIII. The apparent digestion result by *Hin*dIII treatment has confirmed the amplification of the target template.



A basic procedure for confirmation of LAMP is by electrophoresis, but this requires that the tube lid be opened after completion of the reaction, resulting in a high risk of contamination. Thus, we recommend that electrophoresis is only for the initial confirmation of amplification and afterwards detection can be conducted within the tube. Examples include a fluorescent real-time detection or real time turbidity detection.

The figure on the left shows the results obtained with real time fluorescent detection with M13 as the template. The amount of template is stated as 10^{-17} mol/tube to 10^{-23} mol/tube, and the speed of amplification relates to the quantity of the template.

The figure on the right is real time turbidity detection with λ DNA as the template. Samples NC1 to NC4 are negative controls, and PC1 to PC4 are positive controls, indicating a high degree of reproducibility.



There may be circumstances where the LAMP amplification proceeds quite effectively in the beginning, but as time passes it begins to perform poorly. In such circumstances, the deterioration of the reagents might be a possible cause, therefore, reagents should be handled with sufficient care. Generally the precautions required are similar to those for PCR, storing the reagents at -20°C, and storing the undiluted primer stock solution at -80°C. The substrate dNTP also deteriorates gradually and thus needs to be handled with care. If DNA such as the template or primer is reconstituted in water, the deterioration may be accelerated, thus they should be stored in a buffer such as TE. Specifically, the target template DNA at low concentration can easily be degraded and thus sufficient care is needed.

List of terms

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AT rich, GC rich:

The GC content of nucleic acids can vary for different creatures, or it can differ depending on whether the nucleic acid is derived from prokaryotic cell or non-prokaryotic cell. Those with a low GC content is said to be AT rich, and those with a high GC content is said to be GC rich.

bp:

Abbreviation for base pairs. Every nucleic base can specifically combine with its counterpart nucleic base through hydrogen bonds. This plays an important role in nucleic acid replication, transcription and interaction between mRNA and tRNA. In DNA the pairing is between adenine
(A) and thymine (T) or between guanine (G) and cytosine (C), while in RNA the pairing is between A and uracil (U), or between G and C. The length of double-stranded DNA is often expressed as the number of bases (bp).

dNTP:

A solution containing equal amounts of dATP, dTTP (or dUTP), dGTP and dCTP, and is used as substrate in nucleic acid synthesis. During nucleic acid synthesis, if the dNTP concentration is too high in the reaction solution, it is said that the mismatch of nucleotide will increase.

FASTA format:

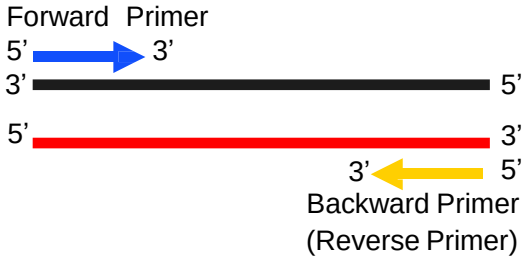
FASTA is a computer program that, through database searches, identifies similarities in gene or protein sequences. It is appropriate for searching through long sequences for homologies. The FASTA format is a format most often used in sequence analysis programs and is in the following format.

```
>AA987701(genbank-upd) ← The header is a comment beginning with > (sequence name or origin)
taaagaagtaagcctttatttccttgttttgca ← Second line and beyond is the data
tggcttcaaccttagctggggctgcagcagcac
>AA987701(genbank-upd) ← Multiple sequences are entered in this
format taaagaagtaagcctttatttccttgttttgca
tggcttcaaccttagctggggctgcagcagcac
```

Forward side, backward side:

At the start of DNA synthesis, primers are needed; PCR requires the minimum of two, while in the LAMP method the minimum of four primers are needed. With respect to the double-stranded DNA, with the coding region of the relevant gene as shown in the 5' end on the left and the 3' end on the right, the 5' → 3' direction is the forward side and the reverse is the backward side.

For PCR



For the LAMP method

⇨p.47 the LAMP method to legend (1)

GC content:

In expressing the base composition of nucleic acids, the proportion (percent) of G and C in the entire sequence. The GC content of primers is selected so that that it is not AT rich to ensure stability of binding to the target gene. For duplex nucleic acids, the base pairing is fixed, so this indicates the proportion of (G+C) in the entire sequence. The GC content is one index of a property of nucleic acids. The DNA GC content differs depending on the organism and in higher animals is in a narrow range centered at 42%, while in bacteria, it ranges from 75-25%.

GenBank format:

GenBank is an internationally known public DNA database maintained by the US NCBI (National Center for Biotechnology Information). GenBank uses the database entry format indicated below.

LOCUS	Locus name, sequence length, molecule type, GenBank division, and modification date
DEFINITION	Brief description of sequence
ACCESSION	Original accession number
KEYWORDS	Key words to describe the entry for searches
SOURCE	Organism from which DNA is derived
ORGANISM	Formal scientific name for the source organism
REFERENCE	Literature reference
COMMENT	Biological function or database information
FEATURES	Information about genes and gene products, as well as regions in the sequence
source	A region of sequence, source organism
misc_signal	A region of sequence, function or signal type
mRNA	A region of sequence, mRNA
CDS	A region of sequence, protein coding region
intron	A region of sequence, intron location
Mutation	Sequence change due to mutation within the sequence
BASE COUNT	The number of A, C, G, T, or other letter in a sequence
ORIGIN	Letter corresponding to sequence start
1 gaattcgata aatctctggt ttattgtgca gtttatggtt ccaaaatcgc	
51 atatactcac agcataactg tatatacacc caggggcgg aatgaaagcg	
//	Symbol indicating sequence end

***Hin*d III:**

Type of restriction enzyme often used in experimental procedures. This enzyme is produced from *Haemophilus influenzae* Rd and thus the enzyme is so named. The recognition sequence and location of the cuts are indicated below.

5'……A | AGCTT……3'
3'……TTCCA| A……5'

HPLC purification:

A purity grade for synthetic oligonucleotides.

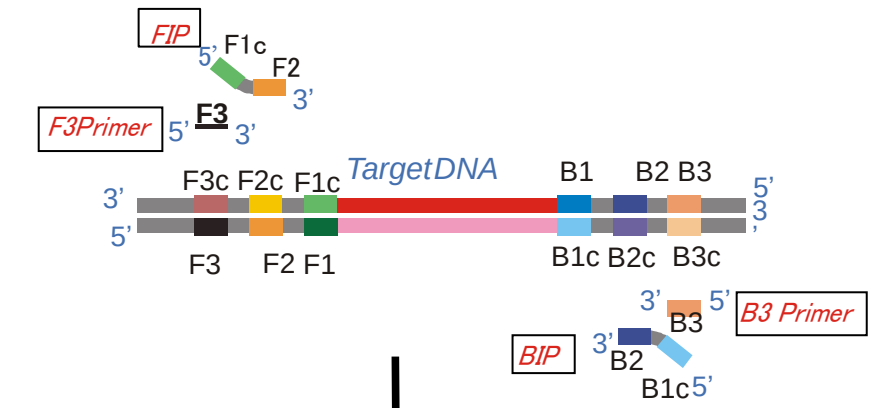
The LAMP method:

The LAMP method (Loop-mediated isothermal Amplification) is a simple, rapid, specific and cost-effective nucleic acid amplification method solely developed by Eiken Chemical Co., Ltd. As a gene amplification technology, compared to PCR, it has higher specificity and amplification efficiency, and has the advantage that it can perform the amplification at a constant temperature

around 65°C.

The two important features of isothermal amplification are: ① DNA synthesis occurs by DNA polymerase with strand displacement activity without the need for thermal cycles for double-strand denaturation ⇒ annealing - ⇒DNA synthesis ① Four primers (recognizing 6
distinct regions in the target gene) are used to amplify the gene, whereby the loops formed at the end of the synthesized strand proceed to self-primed DNA synthesis. The following Figure describes the steps in amplification.

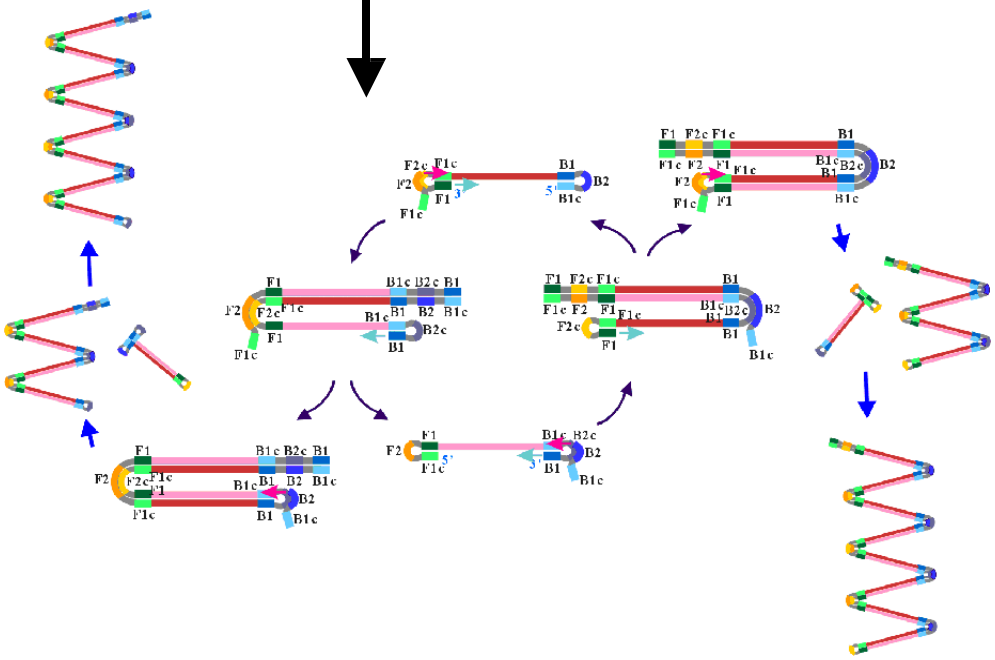
(1)



Through DNA polymerase with strand displacement activity, the following reactions proceed at a constant temperature of about 65°C.

(2)

(3)



By adding four primers (1), after a few steps of reaction, single stranded structure with a loop structure at each end is formed. (2) This serves as the starting point where primers will anneal to proceed to the amplification. As a result of this process, various sized structures consisting of alternately inverted repeats of the target sequence on the same strand are formed.

Loopamp DNA Amplification kit:

A reagent kit designed for LAMP amplification for research use. The contents are: the buffer, substrate and DNA polymerase with strand displacement activity. The user prepares the LAMP primers designed for the target gene of interest. The system can then be used in a variety of applications.

Loop primers:

The Loop Primers (either Loop Primer B or Loop Primer F), containing sequences complementary to the single stranded loop region (either between the B1 and B2 regions, or between the F1 and F2 regions) on the 5' end of the dumbbell-like structure, provide an increased number of starting points for DNA synthesis by the LAMP method. Loop primers provide an increased number of starting points for DNA synthesis resulting in shorter amplification time and higher specificity.

M13 phage:

Filamentous single-stranded DNA phage. Infects the host E coli through the F pili and is incorporated into the bacteria. In the host, the single-stranded DNA changes into a double-stranded replicative form, and this template is used to form single-stranded DNA. Once taken up by the newly formed progeny phage particles, the host bacteria do not lyse but rather releases phage particles. This phage is also useful as a cloning vector and is widely used to prepare single stranded DNA for use in DNA sequencing by the dideoxy method.

Nearest-Neighbor method:

A method for estimating the Tm of DNA and has now come to be the standard. The Tm is determined by taking into account the thermodynamic factors of the neighboring bases under the following formula.

$$Tm = \Delta H \times 1{,}000/(\Delta S + R \ln(C/4)) - 273.15 + 16.6 \log[Na^+]$$

R: gas constant = 1.987cal/^{o}C/mol

ΔH: enthalpy (kcal/mol)

ΔS: entropy (eu)

C: oligonucleotide concentration (M)

$[Na^+]$: sodium ion concentration

PCR:

PCR (polymerase chain reaction) involves repeated DNA synthesis reactions of a specified DNA sequence bracketed by 2 primers and can amplify the specified DNA region by several 10,000 folds. The amplification primers used are usually the synthetic oligonucleotides containing the bases at both ends of the region to be amplified, and the reaction involves repeated steps of 1) denaturation of double-stranded DNA, 2) annealing of oligonucleotide, and 3) DNA polymerase-mediated complementary strand synthesis (repeated usually 20-30 times). Developed by Cetus Co., in 1985.

TE buffer:

Nucleic acid buffer (10mM Tris- HCl (pH 8.0), 1 mM EDTA). Contains divalent metal ion (Mg^{2+} etc) chelator EDTA (2,2',2'',2'''-(Ethane-1,2-diyldinitrilo)tetraacetic acid, a chelator which can remove divalent metal ions in samples), which inhibits the divalent metal-ion dependent nucleases (nucleic acid degrading enzyme) and preserves the nucleic acids.

Tm:

Refers to the melting temperature of biological polymers. For nucleic acid in solution, elevation in temperature leads to destruction of the hydrogen bonds responsible for the base pairing. The temperature at which the DNA loses the double stranded structure, so that 50% is double stranded structure and 50% is single stranded, is the Tm. There are three hydrogen bonds in the GC pair and two in the AT pair, so that DNA with higher content of GC base pairs is resistant to thermal denaturation and thus has higher Tm. The efficiency of binding to primers is generally expressed as Tm.

Annealing:

After the double-stranded DNA has been denatured to the single-stranded form, annealing refers to the re-binding of the denatured single-stranded DNA back into a double-stranded DNA. The DNA-specific double stranded helix is formed, so that the annealing can be called renaturation. With double-stranded DNA, heat or alkaline treatment causes denaturation into single-stranded DNA. Denatured double stranded DNA under certain conditions forms the hydrogen bonds and forms a complete double helical structure.

Oligo concentration:

In the text, the oligo concentration in the slide on p43 refers to the oligonucleotide concentration, namely the primer concentration.

5' end, 3'end:

In nucleic acids all nucleotides are bound to the next nucleotide via phosphate diesterification between the 5-carbon on the 5-carbon sugar to the 3-carbon on the next sugar. At both ends these exist as -OH groups, called the 5' end and 3' end. For a given nucleic acid, the left hand side is generally the 5' end or upstream, while the right hand side is the 3' end or downstream.

Cloning:

Gene cloning refers to the isolation of DNA in which unfractionated DNA fragments are inserted into vectors to form recombinants, which are introduced and propagated in the hosts as colonies or plaques and then the desired DNA is identified, isolated and purified.

Free energy:

A type of thermodynamic coefficient. This refers to the thermodynamic equilibrium standard under standard experimental conditions. In systems where change of state is possible, the change tends to go in the direction of lowest free energy. As with chemical reactions, in the chemical equilibrium state, the free energy of the system is minimized. Currently used free energy terms include the Gibbs free energy and the Helmholtz free energy.

Restriction enzyme:

Name of enzyme that recognizes and cuts DNA at specific sequences. Classified as types I, II and III depending on the needed cofactors for enzyme activity and the type of cut made. Widely distributed among microorganisms, the enzyme type or recognition sequence differs depending on the microbial strain, so that numerous types are available.

Real-time turbidity detection:

Gene amplification is conducted by LAMP and its detection can be done by simultaneously monitoring the white turbidity caused by the existence of magnesium pyrophosphate, the amplification by-products. Thus, real time detection can be achieved. The detection of the magnesium pyrophosphate turbidity is facilitated by the efficiency and specificity of the LAMP amplification reaction.

Electrophoresis:

Method for separation and analysis of DNA. It takes advantage of the observation that when electric current applied to substances, it can result in the movement of substances to the positive or negative electrode. In applying the current, the buffer, filters, gels, carrier ampholytes, are used.

In nucleic acid electrophoresis, relatively large molecular weight DNA (60 - 100k bp) are separated by agarose gels, while smaller molecular weight DNA (1 k bp or less) are separated using acrylamide gels.

Secondary structure:

The primer secondary structure refers to the hairpin structure that can form if the primer is complementary to itself. Depending on the primer sequences, the likelihood for hairpin formation can differ greatly. If the primer itself forms hairpin structures, the primer becomes unable to bind to the target gene or can bind to unexpected genes, thus resulting in false positives.

Primer:

In general, the term primer refers to the oligonucleotide that forms a double strand with the target gene and supplies the 3'-OH needed to initiate the DNA polymerase-mediated elongation reaction. Through the activity of the DNA polymerase, the complementary nucleotides are added to the 3' –OH group of the primer on the template DNA sequence, so that the elongation proceeds from the 5' side to the 3' side.

Primer dimer:

This term refers to the structure that forms when a primer hybridizes to another primer. In the gene amplification method involving DNA synthesis in a test tube, it is necessary to have the primer concentration in the reaction mix at a concentration that is far greater than the Target gene concentration, so that if the structure of the primer permits the hybridization of a primer to itself, primer dimers can form and thus inhibit the hybridization to the Target gene.

Plain text format:

Containing only the sequences information in the following format.

```
ctcgaggact ggggaccctg caccgaacat ggagaacaca acatcaggat tcctaggacc
cctgctcgtg ttacaggcgg ggtttttctt gttgacaaga atcctcacaa taccacagag
tctagactcg tggtggactt ctctcaattt tctaggggga gcacccacgt gtcctggccc
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Mutation:

Refers to spontaneously occurring gene mutation. Changes occurring in the base sequence of a gene can result in a change in the genetic makeup. Gene mutation can be at the level of the genome, chromosome, a part of a chromosome, gene or nucleotide. Depending on how a gene has changed, the mutation can be classified also as a point mutation, deletion, duplication, inversion, insertion, translocation, etc. Mutations can occur in various forms, so its effect at the level of expression can vary greatly from noticeable significant changes to those only detectable after statistical analysis.

Terminal end stability:

The stability of the formation (ease of formation) of double stranded region formed between the template gene and each primer at the 3' end and the 5' end. The LAMP Primer designing software determines the stability as the ΔG (change in free energy) calculated by the Nearest-Neighbor method.

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