# EZ Mutation™ Site-Directed DNA Mutagenesis Kit

For Small Plasmids

## User's Guide

**Catalog number 201304 (8 reactions), 201305 (16 reactions), and 201306 (24 reactions)**

**For Research Use Only**

**Not for Use in Clinical Diagnostic Procedures**

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# EZ Mutation™ Site-Directed DNA Mutagenesis Kit

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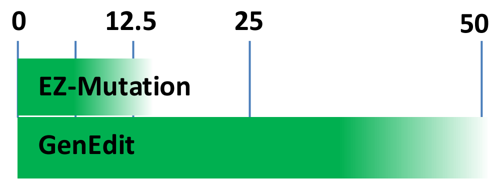
## Benefits of the EZ Mutation™ kit

Please read this entire document before starting your site-directed DNA mutagenesis project

Thank you for purchasing the EZ Mutation™ site-directed DNA mutagenesis kit. This kit is designed and recommended as a rapid method to introduce mutations into plasmids up to 8 kilobase pairs* (kbp) in size. Under certain conditions, such as the lack of sequence information, the EZ-Mutation™ kit can be used for larger plasmids up to 12 kbp.

* For plasmids over 6-8 kbp, we recommend using our First Mutation™ kit (cat# 201301, 201302 or 201303), which employs a different technology that prevents introduction of unwanted mutations in large plasmids. The solid green color of the bars below represents each kit's capability. The numbers represent target plasmid size in kilobase pairs.




**Salient features of the EZ Mutation™ site-directed mutagenesis kit:**

- **Speed.** Mutated plasmids with the EZ Mutation™ kit can be obtained the next day. Each step of the protocol requires minimal preparation.
- **Versatility.** The EZ Mutation™ kit can be used to make a wide variety of nucleotide sequence modifications to plasmids.
- **Convenience.** All key reagents come pre-mixed and distributed in the reaction tubes.
- **Restriction enzyme sites are not required.**
- **No special strain of *E. coli* is required for transformation.** The kit includes FBT5α Super Competent cells, but you may use other strains if your plasmid requires special handling.
- **Simple-to-follow instructions for both the novice and seasoned researcher.**
- **Requires minimal time to set up the reactions.** It takes only a few minutes to set up each reaction step.
- **No reagent freeze-thaw cycles.** Most enzymes, cells, and chemicals used in molecular biology are susceptible to deterioration and reduced functionality upon multiple freeze-thaw cycles. EZ Mutation™ single-reaction reagents remain frozen until use. The user will thaw only the tubes required for a given experiment.
- **Minimized pipetting errors.** Many PCR and other reactions fail due to imbalanced salt concentrations or skewed pH caused by inaccurate pipetting. The key reagents of EZ Mutation™ are premixed at optimal ratios and dispensed at a 1x concentration in single reaction quantities.
- **No cross-contamination of unused reagents.** The main reaction components of the kit are provided in single-reaction format. This format prevents cross-contamination of unused reagents.

## Storage temperature

The kit is shipped on dry ice. Upon arrival, please immediately store the FBT5α Super Competent bacterial cells in a -80 °C freezer. The efficiency of these cells deteriorates when they are stored at temperatures above -80 °C. For the best results, we recommend storing the cells on the lower rack of a chest freezer or away from the freezer's door in an upright style freezer. Store all other kit components in a non-frost-free freezer at -20 °C. The enzymes and chemicals in this kit are sensitive to repeated freeze-thaw cycles; therefore please remove only the reagents that are needed at the appropriate experimental step.

# EZ Mutation™ kit

## Kit format

The EZ Mutation™ kit is available in 8 (Cat # 201304), 16 (Cat # 201305) or 24 (Cat # 201306) reaction formats. The PCR and circularization buffers are premixed and dispensed into color-coded tubes in a separate box from the control reagents. The FBT5α Super Competent Cells are packaged in another container due to the requirement to store them at a different temperature.

For your convenience and to eliminate pipetting errors, all reagents needed to set up the PCR and circularization reactions come in their reaction tubes in a single-reaction format. The EZ Mutation™ components are color coded as follows:

a. The PCR premix comes in **Yellow** strips of eight 0.2 mL PCR tubes.
b. The DNA circularization premix comes in **Blue** strips of eight 0.2 mL tubes. The premix itself is also **Blue**. It comes in PCR tubes for convenient heat denaturation in any standard 96-well platform thermocycler, if needed.
c. The control reagents are supplied in 0.5 mL tubes with color-coded caps in a separate box as described under the kit components section below.
d. The FBT5α Super Competent cells come in 50 µl aliquots in separate tubes that are suitable for heat shock transformation, i.e., transferring the cells to another tube is not necessary.

## Kit components

The following components come with an 8-reaction kit (16 and 24 reaction kits are scaled up accordingly):

### Box 1, PCR and circularization reagents

1) One **Yellow** 8-tube strip of 0.2 mL reaction tubes containing premixed PCR reagents. Each tube contains 22.5 µL of FBT HotStart High Fidelity DNA polymerase in 1x buffer, ready to receive a total of 2.5 µL of template and primers.

**Box 2, Control reagents**

1) One 0.5 mL tube with a **Green** screw cap containing 20 µL of pUC18 control plasmid at 300 pg/µL.
2) One 0.5 mL tube with a **Red** screw cap containing 20 µL of control Opener Primers.
3) One 1.5 mL tube with a **Neutral** screw cap containing RNAse and DNAse-free water.

**Box 3, *E. coli* cells**

Eight tubes of First Biotech's FBT5α Super Competent cells. These cells are chemically competent with an efficiency range of $5 \times 10^8$ to $2 \times 10^9$ colony forming units per µg of pUC18 plasmid. They are included in the kit for propagating the mutated plasmids. Each tube is individually labeled and contains 50 µL of cells for transformation.

**Non-reagent components**

- Laminated quick reference EZ Mutation™ protocol card.
- Laminated quick reference transformation protocol card.

**Materials required but not provided**

- The target plasmid.
- Plasmid-specific Opener Primers (see primer design section).
- DNA electrophoresis system and materials.
- Bacterial growth media (liquid culture and agar plates), SOC, and appropriate resistance drugs.
- General laboratory equipment including a thermocycler, incubator, etc.

# Description of the technology

The EZ Mutation™ performs mutagenesis in three-steps. 1) A mutant copy of methylated target plasmid is generated using two synthetic oligonucleotide primers carrying the mutation(s) of interest on their 5' ends. The primers anneal to the mutagenesis target site in an inverse orientation, such that their 5' ends are proximal to each other to create a full synthetic copy of the target plasmid. 2) To select the newly synthesized copies, the parental plasmids are digested with the methylation-dependent restriction enzyme, *Dpn*I, which destroys the parental plasmid and leaves the mutant plasmid unaffected. In our triple-reaction formulation, degradation of the parental plasmid occurs concurrently with the phosphorylation and circularization reactions in the provided Blue tubes. The T4 polynucleotide kinase in the reaction buffer adds 5' phosphate groups to the PCR-generated copies, and then the T4 DNA ligase covalently closes the linear product to generate mutant plasmids. 3) The mutant plasmids are transformed into FBT5α Super Competent cells for propagation. Clones of transformed cells are then isolated on selective agar media plates. See Figure 1 for an illustration of the technique.

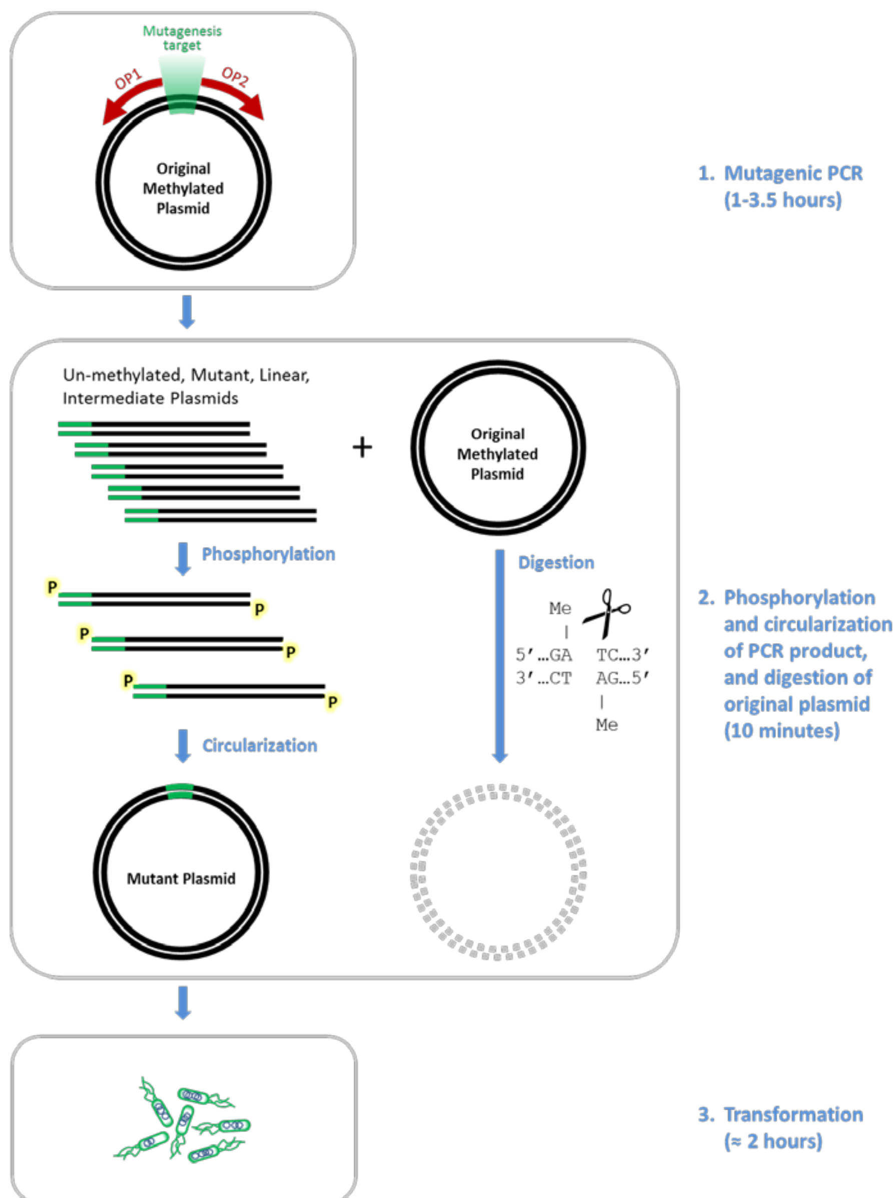



**Figure 1**.
Steps involved in EZ Mutation™ site-directed DNA mutagenesis. 1) The plasmid of interest is amplified by PCR with a set of Opener Primers (OP1 and 2) to produce un-methylated mutant linear intermediate plasmids. 2) This PCR product is transferred to a premixed buffer where the original methylated plasmid is digested with *Dpn*I. Concurrently, in the same reaction vessel, the mutant linear intermediate plasmids are phosphorylated, and then circularized. 3) The circular mutant plasmids are introduced into *E.coli* cells (or a host cell line of your choosing) by transformation for selection and propagation.

## Expected results

The chances of obtaining error-free clones depend on the size of the plasmid being amplified. For amplification of a 5 kbp plasmid using our recommended protocol, over 95% of the final

clones are expected to be free from spontaneous polymerase errors. This decreases to about 90% for 12 kbp plasmids. Our DNA polymerase, FBT HotStart High Fidelity DNA polymerase, is one of the highest fidelity enzymes with an error rate of $4.4 \times 10^{-7}$.

## Target plasmid minimum requirements

The EZ-Mutation kit works for plasmids up to 12 kbp in size, however, we recommend it for plasmids under 8 kbp. For larger plasmids, we recommend the use of First Biotech's First Mutation™ kit (catalog numbers 201301, 201302, and 201303) which uses a different technology to avoid introducing unwanted mutations in large plasmids. To use the EZ Mutation™ kit, the target plasmid must be isolated from $Dam^+$ cells such as FBT5α Super Competent cells. Prior knowledge of the full plasmid sequence is not necessary as long as the sequence of the region targeted for mutagenesis, to which the primers will anneal, is known. Aside from *Dpn*I restriction sites, no other restriction sites or special cell types are required.

# EZ Mutation™ Protocol

Store the FBT5α Super Competent cells at -80 °C immediately upon arrival. Store the kit's PCR and control reagents in a non-frost-free freezer at -20 °C. Quickly remove and thaw the tubes required to do **only one** mutagenesis step at a time. *A control test using the provided pUC18 plasmid and primers may be performed in parallel.*

The EZ Mutation™ kit provides a rapid method for mutagenesis of plasmids up to 8 kbp in length. A set of control reagents are included to verify success of the mutagenesis part of the procedure. The three biochemical steps of EZ Mutation™ are (1) a mutagenic PCR amplification, (2) a 10-minute reaction that degrades the original plasmid, phosphorylates the newly synthesized mutant linear intermediate plasmids, and circularizes the phosphorylated copies into functional plasmids, and (3) transformation into a host cell line for propagation.

## Preparations and project design

1. Identify the position of the nucleotide(s) to be mutated in your plasmid ("Mutagenesis target" in Fig.2). This position will serve as the orientation point for the selection of the Opener (mutagenic) Primers.

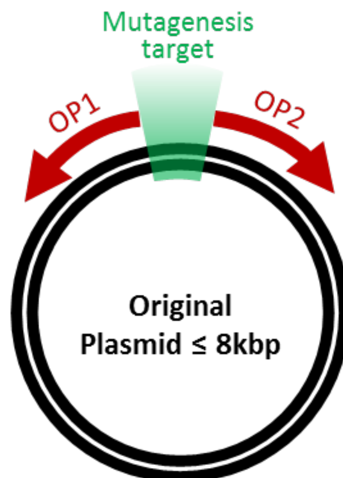



**Figure 2**:
Opener Primer positions: The Opener Primers, OP1 and OP2, flank the mutagenesis target with their 5' ends positioned at the ends of the mutagenesis target. For insertions and substitutions, the mutagenic nucleotide(s) will be appended to the 5' end(s) of the primer(s). The primer design section of this document provides additional details about primers selection.

   a. *In the pUC18 control plasmid, the mutation of interest is an insertion of a Mlu*I *(provided) restriction site next to the native Nde*I *(provided) restriction site at position 185.*

2. Use First Biotech's exclusive primer design software tool located at www.firstbiotech.com to design the primer pair that would incorporate your mutation of interest

   The 3' ends of the two Opener primers will face away from each other. The ends of the PCR product generated by the OP primers will be ligated, so these primers will include all the nucleotides that you want in the final construct.

   a. For a deletion, the primers will be designed to flank the target nucleotide or sequence that you wish to delete (see primer design section for an illustration).

   b. For an insertion, the nucleotides you wish to insert will be added to the 5' end of one or the other or both of the OPs. When inserting more than a few nucleotides, split the inserted sequence between the 5' ends of both OPs to balance the primers lengths (see primer design section below).
      i. *The pUC18 control insertion primers are:*
         *Forward OP1 5'- CATATGCGGTGTGAAATACCGCAC*
         *Reverse OP2 5'-* ***ACGCGT****GTGCACTCTCAGTACAAT*
         *The OP2 primer contains the Mlu*I *6 nucleotide insertion sequence (bold).*

   c. For a substitution, the location of the primer will be chosen such that amplification would delete the sequence you wish to remove, then your desired insertion sequence will be added to the 5' end of one or the other OP, or split the insertion at the 5' ends of both primers.

3. Once the primers are selected, you may order them from your preferred company.

4. Dissolve the primers in DNAse-free $dH_2O$ (provided) at 100 pmol/µL. Store the diluted primers at -20 °C.

   a. *The control primers, OP1 and OP2, are diluted, premixed, and ready to use.*

5. Dilute the target plasmid DNA, keeping the amount of starting DNA to a minimum. As a general guideline, use 100 picograms of DNA for every 1 kbp of plasmid. For example, a 5.5 kbp plasmid should be diluted to 550 pg/µL.

   a. *The pUC18 control plasmid is provided at a concentration of 0.3 ng/µL, ready to use.*

## Mutagenesis procedures

| The PCR cycling conditions given below have been tested for the included pUC18 plasmid and premixed Opener Primers. Please adjust the PCR conditions of your particular primers according to their complexity, size, and melting temperature (Tm). |
|---|

1. **Mutagenic PCR**
   a. For each PCR reaction, snap off one **Yellow** tube containing premixed PCR reagents from one of the 8-tube strips. A quick ½ turn with your fingers while the strip is still frozen will easily break the desired tube from the strip. Immediately

return the rest of the strip to -20 °C to avoid thawing at room temperature. Place the tube on ice. Its contents will thaw as you add other reagents.

b. Add 1 µL of your diluted plasmid (≤100 pg of DNA/kbp of plasmid).

c. Mix 1.5 µL of OP1 with 1.5 µL OP2 in a separate tube, then take 1.5 µL of this mixture (75 pmol from each primer) and add it to the **Yellow** PCR tube.

d. Mix for 5 seconds by vortexing or pipetting up and down, and then spin the contents down at 1,500 rpm.

e. Place the tube(s) in a thermocyler and run the program outlined in Table 1 below.

- Note that the annealing temperature and the extension time are critical parameters for a successful PCR and must be adjusted for each new set of primers for a given template. We recommend using the average melting temperatures ($T_m$) of your primers as your PCR annealing temperature.

Table 1. Recommended thermocycling conditions for sample reactions

| Number of cycles | Temperature | Time |
|---|---|---|
| 1 | 94 °C | 2 min |
| | 94 °C | 20 sec[a] |
| 20 | (Variable)[b] | 30 sec |
| | 70 °C | 1 min/kbp[c] |
| 1 | 70 °C | 5 min |

[a]Use 30 seconds for amplicons more than 1 kbp or GC rich

[b]We recommend using the average melting temp of the two primers

[c]Calculated based on the size of the full plasmid

- ***If running the pUC18 mutagenesis control:*** Place the Yellow tube with the PCR buffer, pUC18 plasmid, and control Opener Primers in a thermocyler and run the following program:

Table 2. Recommended thermocycling conditions for the control reaction

| Number of cycles | Temperature | Time |
|---|---|---|
| 1 | 94 °C | 2 min |
| | 94 °C | 20 sec |
| 20 | 60 °C | 30 sec |
| | 70 °C | 3 min |
| 1 | 70 °C | 5 min |

f. Place the product on ice.

## 2. Removal of original plasmid and generation of mutant plasmids

a. Thaw one **Blue** 0.2 mL tube per mutagenesis reaction at room temperature.
b. Add 5 µl PCR product to the **Blue** tube and mix gently (either by gentle pipetting or by flicking the tube and briefly spinning the contents back down).
c. Incubate the circularization mixture for 10 minutes at room temperature, then place on ice. The circularization product is now ready for transformation and it can be stored at -20°C for future use.

**Notes:**

- A quick agarose gel analysis of your product following the PCR step will tell you if modifications to the thermocycling protocol are needed. Be sure to store the PCR product at -20 °C while the gel is running.

### Standard transformation procedure

1. For each sample to transform, thaw one tube containing 50 µL of FBT5α Super Competent cells on ice for 8 minutes.

2. Add 1-5 µL of diluted ligation mix to the competent cells and mix by gently swirling the tube by holding the cap and swiveling at the wrist. Place the cells back on ice.
   - To prevent warming the cells, (1) minimize the length of time that the tubes are off ice, and (2) do not grasp the tubes from the bottom.
   - Do not mix the cells by vortexing or pipetting as they are very fragile.
   - For the mutated pUC18 control, use 1 µl of circularized product.
3. Incubate the cells with the ligated product on ice for 30 minutes.
4. Heat-shock the cells in a 42 °C water bath or heating block for 30 seconds then immediately transfer the tubes back on ice for 2 min.
   - If using a heating block, fill the well to be used with water while the cells are still incubating on ice to allow time for it to heat to 42 °C. The water will ensure optimal heat transfer from the block to the competent cells.
5. Add 250 µL of room-temperature SOC medium (not provided) to the cells and incubate at 37 °C for 1 hour, shaking horizontally at 225 rpm.
6. Spread the transformed cells on media agar plates with the appropriate selective antibiotic. As a suggestion, you may want to try spreading the cells on at least two plates, one with 100 µL of transformed cells, and another with 10 µL of cells diluted in 90 µL of SOC medium.
   - For the mutated pUC18 control, add 10 µL of transformed cells to 90 µL of SOC medium and spread all 100 µL of the mixture on an LB agar plate with 100 µg/mL ampicillin.
7. Incubate the plates overnight at a temperature suited for propagation of the plasmid of interest in *E. coli* (typically 37 °C) or your own host cell line.

# Primer design

Please visit WWW.FirstBiotech.com to use First Biotech's Project Design Manager (PDM) software tool. This online program will help you plan your mutagenesis project by selecting the optimal primers to use with your specific plasmid to incorporate your desired mutation with the highest rate of success.

In case you prefer to plan your mutagenesis project without using the online software tool, the following directions may be followed instead. Please contact us with any questions at Support@FirstBiotech.com.

Site-directed mutagenesis using the EZ Mutation™ kit requires one set of Opener Primers (also known as the mutagenic primers). Regardless of the type of mutation desired, the 5' ends of Opener Primers 1 and 2 (OP1 and OP2) are positioned proximal to each other as illustrated in Figures 3, 4, and 5, below.

## Opener Primers OP1 and OP2 (Mutagenic Primers)

**Primers for insertions**
The mutation of interest for insertions is built into OP1, OP2, or both at their 5' end(s). For insertions larger than 6 nucleotides, it is advisable to insert half of the insertion sequence into OP1 and the other half into OP2 to balance the annealing and melting temperature of the primers.
The limitation on the number of nucleotide insertions depends on the complexity of the original target and that of the intended mutations.

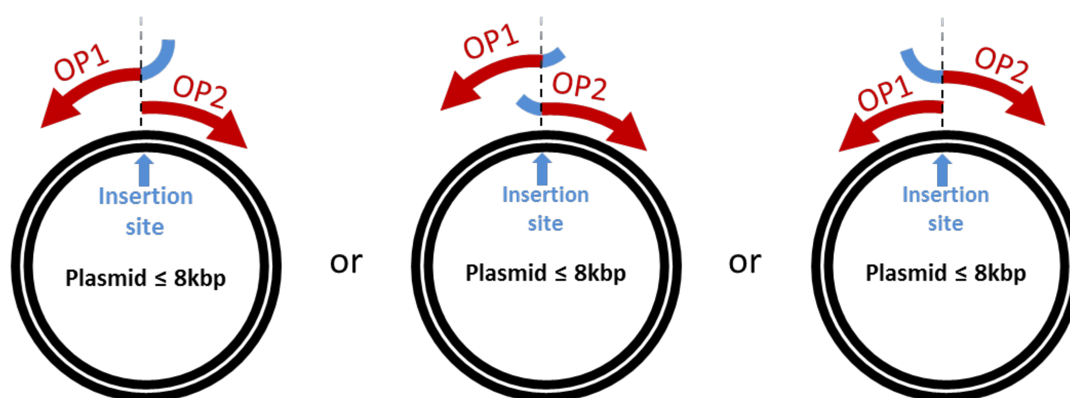



**Figure 3.**
Primer design for nucleotide insertions. The nucleotide(s) to be inserted (depicted as a blue section of the primer) may comprise the 5' end of OP1 (left), the 5' end of OP2 (right), or split in half and incorporated on the ends of both primers (center). The portion of the primers complementary to the user's plasmid is depicted in red. Dotted lines emphasize that there is no space, nor overlap between the complementary regions of the two primers.

**Primers for deletions**
For deletions, the primers are designed to anneal to the region flanking the Target sequence.

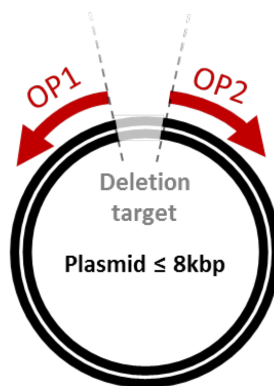



**Figure 4.**
Primer design for nucleotide deletions. OP1 and OP2 are designed to flank the sequence targeted for deletion (gray section of plasmid) with the 3' ends facing away from each other.

**Primers for substitutions**
Substitution primers are designed as deletion primers with the addition of replacement nucleotides at their 5' ends.

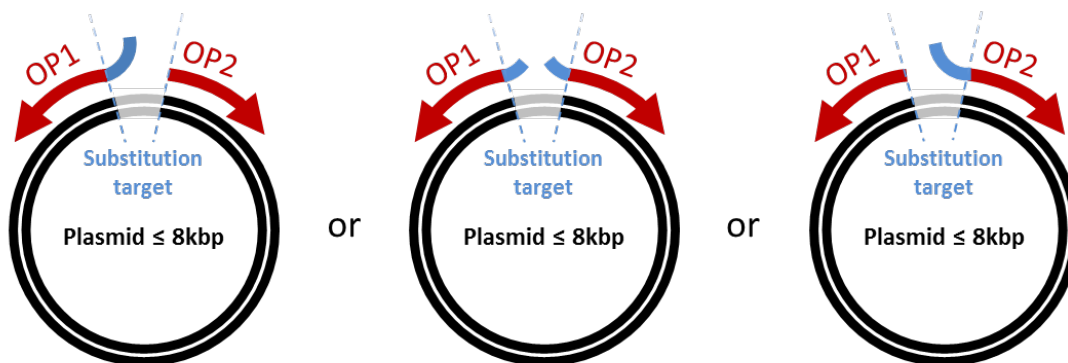



**Figure 5.**
Primer design for nucleotide substitutions. The nucleotide(s) (blue section of the primer) to replace the original sequence (gray section of plasmid) may be added to the 5' end of OP1 (left panel), the 5' end of OP2 (right panel), or split in half and incorporated on the ends of both primers (center panel).

**Considerations for OP1 and OP2**

1) When calculating the melting temperature (Tm) of OP1 and OP2, do not include the 5' additions to the primer size. The Tm should be calculated only for the 3' section that has perfect complementary sequence to the target plasmid.
2) For inserts of more than 6 nt, the complementary region should be 22-25 nt. Inserts longer than 50 nucleotides may require empirical optimization due to increased possibility of secondary structure formation and other complexities.

# Troubleshooting

| PCR | |
|---|---|
| **Problem** | **Possible cause/solution** |
| **The PCR product is absent even for the control reagents.** | ▪ Make sure you added both the Opener Primer mix and your target plasmid to a freshly thawed Yellow tube.<br>▪ Make sure your instruments are correctly calibrated. This is particularly important for the pipettors. Transferring a large amount of primers and template to the reaction tube could inhibit the PCR reaction.<br>▪ After programming your PCR machine and inserting the PCR tubes, make sure to start the program.<br>▪ Check the total volume of your PCR reactions. They should be 25 µl before and after thermocycling. Reduced volume before cycling indicates that the reagents were not spun down or were not stored in a non-frost-free freezer for an extended period of time. Reduced volume after thermocycling indicates that your PCR machine does not have an operational heated lid. Check the lid and try again. |
| **The PCR product is larger than anticipated, or occurs concurrently with a larger band.** | ▪ This could be due to non-specific priming. To resolve, try increasing the annealing temperature. Also, increasing the size of primers even by a single nucleotide might resolve the problem.<br>▪ Make sure there is no exogenous DNA contamination such as genomic DNA. This problem could be resolved by digesting template DNA with restriction enzyme(s) that do not cut in the amplicon region prior to PCR. |
| **The control PCR produced a product, but there was no product for my plasmid.** | ▪ Make sure that you used template-specific Opener Primers. The provided control Opener Primers are designed only for the pUC18 plasmid.<br>▪ Check the concentration of your primers and plasmid to ensure the correct dilutions were used.<br>▪ Adjust the annealing temperature to match the average melting temperatures of both primers.<br>▪ Make sure the correct extension time was used. Please allow 1 minute per kbp of amplicon size.<br>▪ Some amplicons may require experimental adjustments of selected primers, especially for larger amplicons. |
| **The PCR product band is too faint.** | ▪ Generally, this should not be a problem and may only result in fewer colonies of transformants on agar plates. Increasing the amount of transformants spread on the plate may be necessary.<br>▪ One or the other primer is not annealing efficiently to its target. Adjust the PCR parameters (annealing temperature and extension time) to match your primers and amplicon. Allow 1 minute extension time for each 1 kbp of your amplicon template.<br>▪ If the template concentration is extremely low, try increasing the template concentration.<br>▪ The plasmid could be contaminated with organic solvents such as ethanol or isopropanol. Make sure the template is |

| | |
|---|---|
| | pure of contaminants.<br>▪ The template may be degraded. Check the integrity of your plasmid DNA by running 100 -200 ng of your plasmid on a 1% agarose gel. There should not be any streaking. |
| **Multiple bands appeared in addition to the expected PCR band.** | ▪ If the band of interest is only minor or faint compared to other bands on the gel, repeat the PCR with increased annealing temperature by 5 °C at a time. |
| **Transformation and plating** | |
| **Problem** | **Possible cause/solution** |
| **There are no colonies, even for the control plasmid.** | ▪ Use ampicillin as the selection drug for the control pUC18, but use the appropriate drug for your plasmid. Check your plasmid specifications to determine the type of antibiotic that is required.<br>▪ Make sure the FBT5α Super Competent cells were stored at -80 °C.<br>▪ Check your plates to make sure the nutrient agar was not dry. This could occur if plates were incubated in a ventilated incubator with low humidity. |
| **Only the control plasmid produced colonies.** | ▪ Be sure the introduced mutation does not interfere with expression, stability, or function of the antibiotic resistance cassette on the plasmid. |
| **The mutation is not present after colony selection and screening.** | ▪ Make sure that the PCR produced the correct sized band on an agarose gel.<br>▪ Make sure the *Dpn*I has been added to the PCR product and the mixture has been incubated for 1 hour at 37 °C.<br>▪ Make sure that no more than 100 pg/kbp of plasmid has been used in the PCR. |
| **There are too many colonies to select single clones** | ▪ Make sure the appropriate selective antibiotic has been added to the nutrient agar to suppress irrelevant growth.<br>▪ Make sure the *Dpn*I has been added to the PCR product and the mixture has been incubated for 1 hour at 37 °C.<br>▪ Make sure that no more than 100 pg/kbp of plasmid has been used in the PCR.<br>▪ If the above has been followed, try spreading a smaller quantity (10- to 100-fold less) of transformants on another plate. |

For additional troubleshooting, please contact us at Support@FirstBiotech.com