# GenEdit™ Site-Directed DNA Mutagenesis Kit

For Medium and Large Plasmids

# User's Guide

**Catalog number 201321 (8 reactions), 201322 (16 reactions), and 201323 (24 reactions)**

**For Research Use Only**
**Not for Use in Clinical Diagnostic Procedures**

2013P03-103

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# Benefits of the GenEdit™ kit

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

Thank you for purchasing the GenEdit™ site-directed DNA mutagenesis kit. GenEdit is the most versatile DNA mutagenesis commercial kit available. The kit is designed for maximum efficiency to facilitate direct mutagenesis on DNA plasmids of up to 50 kilobase pairs (kbp) without sub-cloning. It supports insertions, deletions, substitutions and any combination thereof. This easy-to-use kit has many advantages, including significant savings in time, labor and cost over traditional DNA mutagenesis methods that require sub-cloning. With your GenEdit™ kit, you will enjoy several benefits, including:

- **Cloning vectors are not required.** Most mutagenesis projects involving large genes or plasmids require cloning a section of the target plasmid into smaller cloning vectors. GenEdit™ does not require cloning vectors.The mutagenesis is performed directly on your target plasmid.
- **Lengthy Sub-cloning procedure is not needed.** GenEdit™ replaces traditional sub-cloning and sequence-shuttling with a same-day series of PCR and circularization reactions followed by a cloning step, saving weeks of bench work.
- **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.
- **100% error-free plasmid backbone.** DNA polymerization in GenEdit™ is limited only to the mutagenesis target site, not the full gene or plasmid under investigation, avoiding the need for full plasmid sequence verification.
- **Requires minimal time to set up the reactions.** It takes only a few minutes to set up each reaction step.
- **Reduced costs.** By avoiding the steps involved in sub-cloning, such as colony selection, DNA isolation and purification, and a host of other lengthy steps, GenEdit™ saves weeks of laboratory personnel hours.
- **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.
- **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. GenEdit™ 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 GenEdit™ are premixed at optimal ratios and dispensed at 1x concentration in single reaction quantities.

### Storage temperature

The kit is shipped on dry ice. Upon arrival, please immediately store the FBT5α Super Competent cells in a -80 °C freezer. These cells are sensitive to 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.

## GenEdit™ kit

### Kit format

The GenEdit™ kit is available in 8 (Cat # 201301), 16 (Cat # 201301) or 24 (Cat # 201303) reaction formats. The PCR and circularization enzymes and 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 the user's convenience, all reagents that are needed to set up the PCR and circularization reactions come in their reaction tubes in a single-reaction format, eliminating pipetting errors. GenEdit™ 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 is dispensed in PCR tubes for convenient heat denaturation in standard thermocyclers, 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 are pre-aliquoted at 50µL/tube ready for heat shock transformation in the same tubes.

### 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) Three **Yellow** 8-tube strips of 0.2 mL reaction tubes (total 24 reactions) 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.
2) Two **Blue** 8-tube strips of 0.2 mL reaction tubes (total 16 reactions) each containing 15 µL of WideRange premixed circularization reagents, ready to receive up to 5 µL of linear DNA for circularization. For added convenience, this reagent's color is also **Blue**.

**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. Control mutagenesis of this plasmid produces convenient PCR products that can be analyzed using restriction enzymes for quick diagnostic purposes.
2) One 0.5 mL tube with a **Yellow** screw cap containing 20 µL of premixed Starter Primers.
3) One 0.5 mL tube with a **Red** screw cap containing 20 µL of Opener Primers.
4) One 0.5 mL tube with a **Gray** screw cap containing 10 µL of the *Nde*I restriction enzyme for optional $P_1$ and $P_5$ diagnostic tests.
5) One 0.5 mL tube with an **Orange** screw cap containing 10 µL of *Mlu*I restriction enzyme for optional $P_5$ diagnostic tests.
6) One 0.5 mL tube with a **White** screw cap containing 100 µL of 10x buffer for *Nde*I and *Mlu*I restriction enzymes.
7) One 0.5 mL tube with a **Purple** screw cap containing 20 µL of T4 DNA ligase at 1 unit per 1 µL for cloning.
8) One 0.5 mL tube with a **Brown** screw cap containing 50 µL of 10x T4 DNA ligase buffer.
9) Three 1.5 mL tubes with **Neutral** screw caps 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 $5 \times 10^9$ colony forming units per µg of pUC18 plasmid. They are included in the kit for the propagation of mutant plasmids. Each tube is individually labeled and contains 50 µL of transformation cells.

**Non-reagent components**

- Quick reference GenEdit™ protocol sheet.
- Quick reference cloning protocol sheet.

**Materials required but not provided**

- User's target plasmid.
- Plasmid-specific primers (see primer design section).
- Appropriate restriction enzymes for cloning the mutant and enriched PCR product into the user's target plasmid (see primer design section).
- DNA purification materials.
- 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 GenEdit™* method of DNA mutagenesis is based on the *Unrestricted Mutagenesis and Cloning* (URMAC) patented technology. The basic method involves two modules: module I is a series of three PCR and two circularization reactions followed by module II which is a cloning step. For convenience, the products of PCR and circularization reactions in module I are called Product 1 through Product 5 ($P_1$-$P_5$). The following illustration shows the steps involved:

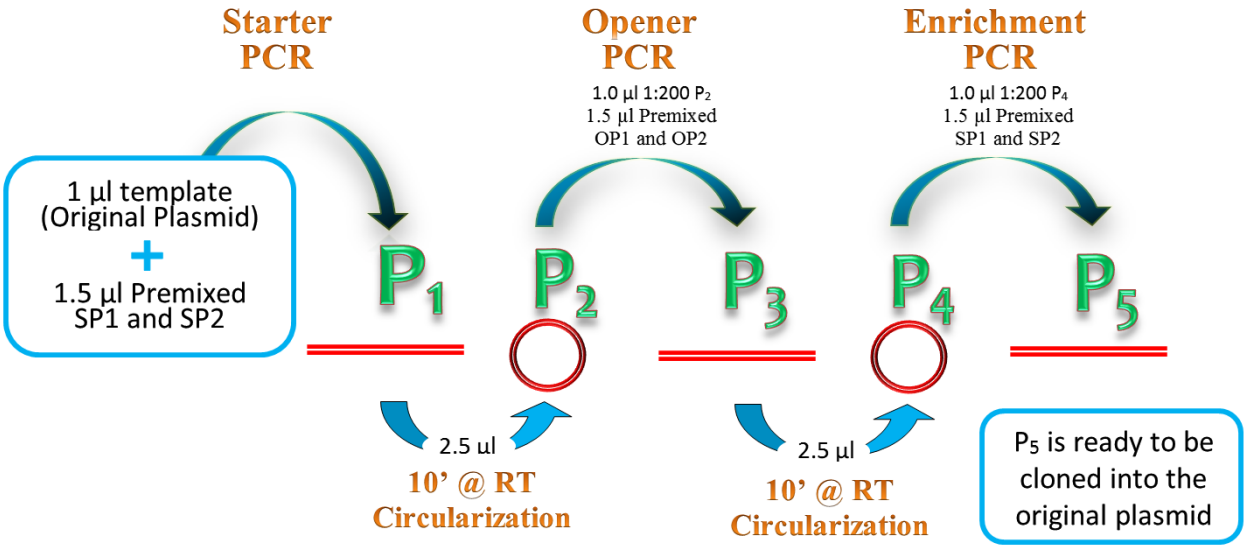



**Figure 1**.
Module I of GenEdit™ site-directed DNA mutagenesis: The target plasmid (Original Plasmid), DNA, and primers (Starter Primers, SP1 and SP2) are subjected to a standard PCR, called the "Starter PCR" resulting in product $P_1$. $P_1$ is circularized to form $P_2$ using the **Blue** premixed circularization reagent. Using a set of user-supplied mutagenic primers, $P_2$ is mutated to become $P_3$ in a mutagenic PCR. $P_3$ is circularized to form $P_4$ and amplified by a final enrichment PCR to give $P_5$.

Using conventional restriction digestion and T4 DNA ligation reactions, the final PCR product, $P_5$, is then cloned into the user's target plasmid. This is followed by colony screening to isolate the desired clones.

*US patent 8,206,909 and other pending patents.

## Expected results

Since only the mutagenesis target region is subjected to PCR amplification during the GenEdit™ procedures, the likelihood of a polymerization error in generating the mutation is small and most of the final clones will contain only the mutation of interest. The chances of obtaining error-free clones depend on the exact size of the DNA sequence being amplified; but for a typical 15,000 bp plasmid with an amplicon of 1,000 bp using our recommended protocol, over

97% of the final clones should be free from spontaneous polymerase errors. Over 99.99% of the backbone of the target plasmid is expected to be free from spontaneous errors since this sequence will not be subjected to *in vitro* DNA polymerization. Our DNA polymerase, FBT HotStart High Fidelity DNA polymerase, is one of the highest fidelity enzymes with an error rate around $4.5x10^{-7}$.

## Target plasmid minimum requirements

The target plasmid can be of any size as long as the following conditions are met:

1) The user's target plasmid sequence contains at least two unique restriction sites (referred to as "X" and "Y") flanking the mutagenesis target site. These sites will be used for cloning the mutant PCR product into the target plasmid.
2) The distance between the X and Y restriction sites should not be less than 150 bp and preferably, no more than 10,000 bp. An additional unique restriction site between X and Y is preferred for enhancing cloning efficiency, but it is not mandatory.
3) Prior knowledge of the full DNA sequence is not required; however, the sequences of the primer-binding regions and the identities of the two unique restriction sites flanking the mutagenesis target site must be known to allow designing the Starter and Opener Primers.

# GenEdit™ Protocol

Store the FBT5α Super Competent cells at -80 °C immediately upon arrival. Store the kit's PCR, circularization, 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 GenEdit™ protocol contains two modules. Module I for the PCR and circularization steps and module II the cloning steps. Under optimal conditions, module I can be completed in one day followed by 3-4 days to complete module II. A set of control reagents are included to verify success of the mutagenesis part of the procedure. Module I steps are a sequence of three PCR reactions punctuated by two circularization reactions. Module II steps for cloning and colony selection are standard laboratory procedures for which control reagents are not included in the GenEdit kit.

## Preparations and project design

Please visit First Biotech's online primer-design and enzyme-selection software utility http://www.FirstBiotech.com to plan your mutagenesis project. If you prefer to plan your project manually, please follow the procedure outlined in the steps below and in the primer design section of this user's guide.

1. Identify the position for the mutation in your plasmid ("Target" in Fig.2). This position will serve as the orientation point for the selection of restriction sites and primers. Now select two unique restriction sites (illustrated X and Y below) that flank your mutagenesis target. These restriction sites will be used at the end of the mutagenesis procedure to insert the final Enrichment PCR Product, $P_5$, into your plasmid. Make sure the distance between X and Y is more than 150 bp, but less than 10,000 bp. If available in your plasmid, select an additional restriction site(s) anywhere in-between the X and Y sites that do not occur elsewhere on the target plasmid. This site will be used at the end to disrupt the parental target region for better cloning results.

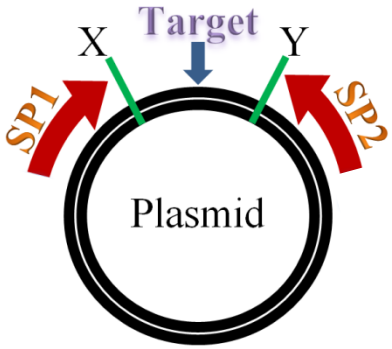



**Figure 2**:

**Selection of restriction sites and primers:** The X and Y restriction sites are selected to flank the desired mutagenesis target (Target). The Starter Primers SP1 and SP2 must

encompass both the Target and the restriction sites. The primer design section of this document provides additional details about primers selection.

   a. *In the control pUC18 plasmid, the two unique restriction enzyme sites chosen to flank the mutation Target are Pfo*I *(not provided) at position 47 and Eco*RI *(not provided) at position 451. The distance between these two sites is 404 bp.*

   b. *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. Using First Biotech's primer design software tool found at http://www.FirstBiotech.com (see primer design section below), design a primer pair that would amplify a stretch of DNA that includes the Target sequence and both unique flanking restrictions sites, X and Y. These primers are referred to as Starter Primers (SP1 and SP2).

   a. *The control SP1 and SP2 primers will produce a 531 bp product from pUC18.*

      *SP1 5'- ACACATGCAGCTCCCGGAGA, position 35-54 on pUC18*
      *SP2 5'- CTCACTCATTAGGCACCCCAGG, pos. 545-566 on pUC18*

3. Design a pair of Opener Primers (OP), also known as the Mutagenic Primers. The 3' ends of these two primers should face away from each other. The ends of the PCR product generated by the OP primers will be ligated, so these primers should include all the nucleotides that you want in the final construct.

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

   b. For an insertion, add the nucleotides you wish to insert at the 5' end of one or the other or both of the OPs. When inserting more than a few nucleotides, split the inserted sequence on the 5' ends of both OPs to balance the primers lengths (see primer design section below).

      *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, choose the location of the primers such that amplification would delete the sequence you wish to remove, then add your desired insertion sequence to the 5' end of one or the other OP, or split the insertion at the 5' ends of both primers.

4. Synthesize both primer pairs, SP and OP. The primers do not need to be phosphorylated.

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

   a. *The Control primers, SP1 and SP2, are diluted, premixed, and ready to use.*

6. 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 10 kbp plasmid should be diluted to 1 nanogram/µL.
    a. *The pUC18 control plasmid is provided at a concentration of 0.3 ng/µL, ready to use.*

## Module I: PCR and circularization reactions protocol

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

**1. $P_1$ Starter PCR (1st 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. Pre-mix 3 µL of SP1 with 3 µL SP2 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. Save the remaining 4.5 µL SP mixture on ice for the final enrichment PCR.
d. Mix for 2 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 to generate $P_1$.
    - 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 |
| 25 | 94 °C | 20 sec[a] |
| | (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 amplicon size (**not** the full plasmid size)

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

Table 2. Recommended thermocycling conditions for the control reactions

| Number of cycles | Temperature | Time |
|---|---|---|
| 1 | 94 °C | 2 min |
| 25 | 94 °C | 20 sec |
| | 60 °C | 30 sec |
| | 70 °C | 30 sec |
| 1 | 70 °C | 5 min |

f. Place $P_1$ on ice.

**2. $P_2$ 1st circularization**

a. Thaw a **Blue** tube at room temperature, then add 2.5 µL (the range is 1-5 µL) of **$P_1$** (the first PCR product) to it. The **Blue** tube contains the circularization premix. Vortex 2 seconds, then spin the contents down at 1500 rpm. There is no need to adjust the volume. Our circularization buffer has a wide dynamic range in which the enzyme remains functional.

b. Incubate 10 minutes at room temperature. If your expected PCR product size is above 1 kbp, increase this time by 5 min for each additional 1 kbp.

c. Dilute 1 µL of the resulting $P_2$ (the 1st ligation product) 1:200 by adding 1 µL **$P_2$** to 199 µL DNAse-free $dH_2O$ (provided). Mix by pipetting or shaking.

**3. $P_3$ Mutagenic PCR (2nd PCR)**

a. Add 1 µL of the 1:200 diluted $P_2$ to a new **Yellow** tube containing the PCR premix.

b. Pre-mix 1.5 µL of OP1 with 1.5 µL of OP2 in a separate tube, then take 1.5 µL of this mixture (75 pmol from each Opener Primer) and add it to the **Yellow** PCR tube. Mix 2 seconds by vortexing or pipetting, then spin the contents at 1500 rpm to collect them at the bottom of the tube. Use the appropriate PCR conditions for your specific primers (Table 1 may be used as a guide).
*As a control, the provided Opener Primers will result in an insertion of a new MluI site next to the native NdeI site in pUC18. The control PCR conditions for the mutagenic PCR reaction is the same as those for Starter PCR.*

**4. $P_4$ 2nd circularization**

a. Add 2.5 µL (1-5 µL) of $P_3$ ($2^{nd}$ PCR product) to one **Blue** tube, containing the circularization premix. Vortex 2 seconds, then spin the contents down at 1500 rpm.
b. Incubate 10 minutes at room temperature. If your PCR product size is above 1 kbp, increase this time by 5 min per each additional 1 kbp.
c. Dilute 1 µL of the resulting $P_2$ (the $2^{nd}$ ligation product) 1:200 by adding 1 µL $P_4$ to 199 µL DNAse-free $dH_2O$ (provided). Mix by pipetting or shaking.

5. **$P_5$ Final (Enrichment) PCR**
   a. Transfer 1 µL of 1:200 diluted $P_4$ into a new **Yellow** PCR tube containing PCR premix.
   b. Add 1.5 µL of the SP mixture that was saved from the $1^{st}$ PCR set up.
   c. Use the same PCR conditions used in the $1^{st}$ PCR reaction, unless the size of insertion or deletion affects the extension time.

$P_5$ can be stored at -20 °C until the user is ready to begin the cloning procedure as described below. Should you need more $P_5$, we recommend repeating step 5 above.

**Notes:**

- The enrichment PCR generally produces enough DNA for enzymatic digestion, purification, and cloning. If additional $P_5$ DNA is needed, we recommend repeating step 5 rather than further amplifying the $P_5$ product.
- A quick agarose gel analysis of your product following each PCR will tell you if modifications to the protocol are needed.
- Note that none of the above steps require DNA purification even when the $1^{st}$ or the $2^{nd}$ PCR products show bands in addition to the correct one. Generally, the last PCR results in a single band when resolved by agarose gel electrophoresis.
- Do not store $P_3$ (the mutagenic PCR) unless it is purified. This is to prevent degradation of the mutant PCR ends by the polymerase exonuclease activity. But you can safely store all other intermediate products at -20 °C.

## Module II: Standard procedure for cloning of $P_5$ into the target plasmid

This is a standard reaction to join the enriched PCR product ($P_5$) into the target plasmid. Before attempting to clone $P_5$, please make sure that your plasmid DNA is intact and free from interfering salts and solvents. Freshly-isolated plasmids produce more colonies with intact inserts than plasmids that have been in storage for an extended period of time.

A ligation reaction should have $1 \times 10^9$ (roughly 15 fmol) copies of cut and purified target plasmid plus $3 \times 10^9$ (~ 45 fmol) copies of cut and purified insert, in this case $P_5$.

The following formula can be used to convert double-stranded DNA from fmol to ng:

$$\text{Desired DNA amount in ng} = (\text{desired fmol})(\text{N})\left(\frac{660 \text{ fg}}{\text{fmol}}\right)\left(\frac{1 \text{ ng}}{10^6 \text{ fg}}\right)$$

Where N is the size of DNA in bp.

Alternatively, use Hallak's approximation:

**For 15 fmol of plasmid, the required ng of plasmid = plasmid size in kbp × 10**
**For 45 fmol of insert, the required ng of insert = insert size in kbp × 30**

The amount of cut and purified target plasmid DNA needed for ligation (i.e 15 fmol) expressed in nanograms equals the length of the plasmid in kbp multiplied by 10. Similarly, the amount of cut and purified $P_5$ DNA needed for ligation (i.e 45 fmol) expressed in nanograms equals the length of $P_5$ in kbp multiplied by 30.

For example, inserting a 300 bp DNA fragment into a 2.6 kbp plasmid backbone requires 2.6 × 10 = 26 ng of plasmid plus 0.3 x 30 = 9 ng of insert.

**Procedure**

1) In separate reactions, digest 1-2 µg of $P_5$ and the target plasmid using the X and Y restriction enzymes that were identified when you designed the primers.
2) If you selected an extra restriction site(s) that occurs only in between X and Y, use it to disrupt this target region in the target plasmid only. Do not digest $P_5$ with this extra enzyme.
3) Purify the cut DNA (by gel purification, ethanol precipitation or DNA resin-based column purification).
4) Quantify the amount of purified DNA using a sensitive DNA measuring method such as through the use of a NanoDrop™ instrument and adjust the DNA concentration to 50 µg/mL using $dH_2O$ (provided).

5) In a microfuge tube, combine the following: (Calculate the volumes used for a and b below so the correct amount of $H_2O$ can be added in step e.)

| Step | Quantity | Units | Description |
|---|---|---|---|
| a | 15 | fmol* | Purifed, digested plasmid backbone |
| b | 45 | fmol* | Purified, digested $P_5$ insert |
| c | 1 | µL | 10× buffer for T4 DNA ligase (provided) |
| d | 1 | µL | T4 DNA ligase, (provided) |
| e | To make 10 | µL | $dH_2O$ (provided) |

*See above for conversion to ng

6) Prepare a control reaction as in step 5 above but omitting the insert.
7) Incubate the reactions (control and test) at 16 °C for 16 hours.
8) Heat-inactivate the enzyme at 65 °C for 10 minutes.
9) Add 10 µl $H_2O$ to the reaction and mix.

**Notes:**

1) Avoid repeated freeze-thaw cycles of all reagents
2) When using a large plasmid over 18,000 bp, higher transformation efficiencies can be obtained by adding an ethanol precipitation step following the heat inactivation of the ligation reaction

**Transformation**

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 backbone-only control, use the same parameters that you use for your sample mutagenesis reaction
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, users 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 mutagenesis 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.

# Primer design

Please visit http://www.FirstBiotech.com to use First Biotech's Project Design Manager (PDM), a proprietary enzyme selection and primer design software tool. The PDM program will help you plan your mutagenesis project by selecting the appropriate restriction enzymes and optimal primers to use with your specific plasmid.

Site-directed DNA mutagenesis using the GenEdit™ protocol requires two sets of primers. The first set is the Starter Primers (SP1 and SP2, see figure 2 on page 7). The second set is the Opener Primers (OP1 and OP2, figures 3 and 4).

## Starter Primers: SP1 and SP2

SP1 and SP2 are used to generate a linear PCR product which includes the mutagenesis target site and two flanking restriction sites X and Y (Figure 2, page 7, Target). The length of SP1 and SP2 should be 18 to 25 nucleotides (nt) long depending on the original template complexity and size. The difference in melting temperature of SP1 and SP2 is recommended to be designed within 5 °C. The amplicon generated by SP1 and SP2 must be at least 150 bp but less than 10,000 bp. If the desired mutation is a deletion, or a substitution that reduces the size of the amplicon, the amplicon generated by SP1 and SP2 should be at least 150 plus the number of nucleotides by which the amplicon will be shortened through mutagenesis. For example, if the desired mutation is a deletion of 50 nucleotides, then the product of SP1 and SP2 amplification must be at least 200 nucleotides long.

## Opener Primers OP1 and OP2 (Mutagenic Primers)

The 3' ends of OP1 and OP2 are designed to face away from each other as shown in Figure 3 below. These primers will incorporate the intended mutation into the circularized PCR product of SP1 and SP2 amplification.

### 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.

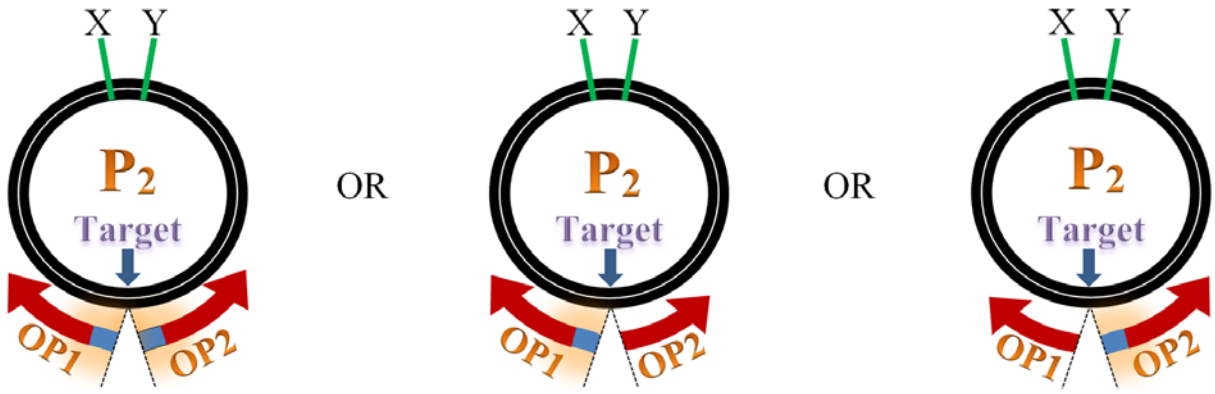



**Figure 3**
Strategies for designing Opener Primers: 3 possible ways of designing the insertion or substitution primers. The intended mutation(s) can be introduced through OP1, OP2 or both.

**Primers for deletions**
For deletions, the primers are designed to anneal to the region flanking the Target sequence. The maximum limit on the number of adjacent nucleotides that can be deleted all at once is estimated at 10,000 nucleotides.

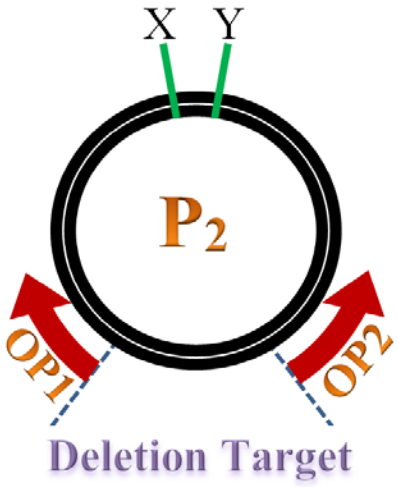
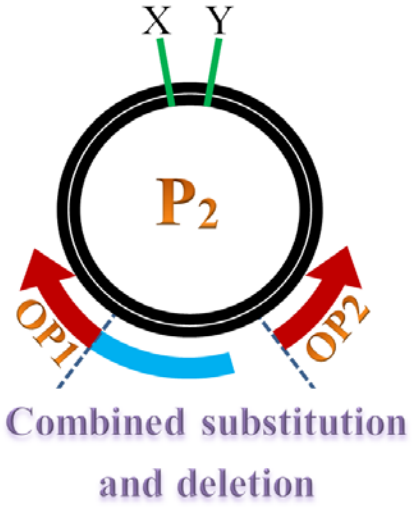



**Figure 4**
Placement of deletion primers on circularized Starter Product 1 ($P_2$). The OP1 and OP2 are designed to flank the sequence targeted for deletion (Deletion Target) with the primer's 3' ends facing away from each other.

**Primers for substitutions**

Substitution primers are designed as deletion primers with the addition of replacement nucleotides at their 5' ends. Figure 5 shows an illustration for placement of primers to achieve both substitution and deletion at the same time.




## Figure 5

Placement of OP primers to achieve both substitution and deletion at the same time. OP1 is placed at one side of the substitution target sequence and a replacement sequence is added to the 5' of the primer. The replacement sequence can be identical in size to the target sequence, or it can be shorter if a deletion is desired as depicted in this drawing. OP2 is placed on the other end of the target sequence. The replacement sequence can be placed on either OP1, OP2, or split on both. The OP1 and OP2 3' ends facing away from each other.

**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 | |
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| **Problem** | **Possible cause/solution** |
| **The Starter PCR product, $P_1$, is absent even for the control reagents.** | ▪ Make sure you added both the Starter 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 Starter 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. |
| **Control Starter PCR produced $P_1$ only for the control, but not for my plasmid.** | ▪ Make sure that you used template-specific SP primers. The provided control SP primers are designed only for the pUC18 plasmid.<br>▪ Check the concentration of your SP 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 amplicons that are over 10 kbp. The size of the target plasmid is irrelevant. |
| **The Starter product band is too faint.** | ▪ 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% |

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| | agarose gel. There should not be any streaking. |
| **Multiple bands appeared in addition to the expected Starter PCR band.** | ▪ As long as the band of interest is prominent, you can safely proceed to the circularization reaction without purifying the band of interest.<br>▪ 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. |
| **The Opener/mutagenic PCR product, $P_3$, is absent** | ▪ Make sure you used diluted $P_2$. Undiluted circularization premix is inhibitory for the PCR reaction.<br>▪ Check the melting temperatures of the Opener Primers. Your PCR annealing temperature should be the average melting temperatures of the two primers.<br>▪ If your amplicon size is larger than 1 kbp, increase the circularization time by 5 minutes for each additional 1 kbp. |
| **The enrichment product, $P_5$, is absent** | ▪ $P_5$ requires the same Starter Primers as $P_1$, therefore, the PCR conditions that worked for $P_1$ should be identical to that of $P_5$ unless you introduced a very large mutation that affected the sequence size by several hundred base pairs. If so, adjust the extension time accordingly. |
| **Cloning** | |
| **The concentration of $P_5$ after digestion and purification is too low** | ▪ DNA is lost during purification. If the flanking sequences after the X and Y cuts at the ends of the PCR product are under 100 bp, we recommend using PCR purification columns. The small DNA fragments are washed away during this purification leaving the internal sequence bound to the columns. This can be concentrated by eluting it in a small volume of tris EDTA buffer or $H_2O$.<br>▪ Larger flanking sequences require gel purification. This might require a greater starting quantity of $P_5$. We recommend obtaining more of $P_5$ by amplification of diluted $P_4$. |
| **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 minus 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.<br>▪ If your plasmid is over 15 kbp, colonies might take longer than 24 hrs to be clearly visible. Allow additional time for large plasmids, especially if your protocol calls for growing the plasmid at 30 °C rather than the standard 37°C. |

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| **I get only or mostly "empty" plasmids that do not contain the mutation of interest.** | 10) This is a common cloning problem. It occurs when the parental plasmid is not fully digested by the cloning restriction enzymes. To resolve it:<br>a. Use fresh restriction enzymes.<br>b. Make sure both enzymes are 100% active in the digestion buffer.<br>c. Make sure the appropriate digestion temperature was used; some enzymes require higher reaction temperature than others.<br>d. If possible, include a third restriction cut into the region being replaced by $P_5$ (between X and Y in the target plasmid). Do not use this enzyme to digest $P_5$.<br>11) If the digested parental plasmid produced bands that are too close to each other on the gel to separate with a scalpel, increase the electrophoresis time to allow better separation of bands before you cut and purify the plasmid backbone. |
| **There are too many colonies to select single clones** | 12) Make sure the appropriate selective antibiotic has been added to the nutrient agar to suppress irrelevant growth.<br>13) If the backbone-only, no-insert control plate contains a similar number of colonies compared to the test plate, then the parental plasmid was not digested appropriately. See the above solution for "empty" plasmids.<br>14) If test plate contains at least 2-fold or more colonies than the backbone-only control plate, then this is a good indication that your cloning was successful. You will only need to spread less transformed cells. 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