Promega

Technical Manual

GeneEditor™ in vitro Site-Directed Mutagenesis System

INSTRUCTIONS FOR USE OF PRODUCT Q9280.

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GeneEditor™ in vitro Site-Directed Mutagenesis System

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1. Description

The GeneEditor™ in vitro Site-Directed Mutagenesis System[a,b] is a high-efficiency system for the generation and selection of oligonucleotide-directed mutations. This system uses antibiotic selection to obtain a high frequency of mutants (Figure 1). Selection Oligonucleotides provided with the GeneEditor™ System encode mutations that alter the ampicillin resistance gene, creating constructs that confer new additional resistance to the GeneEditor™ Antibiotic

Selection Mix. In the GeneEditor™ System protocol, the Selection Oligonucleotide is annealed to a single- or double-stranded DNA template at the same time as a mutagenic oligonucleotide. Subsequent synthesis and ligation of the mutant strand link
the two oligonucleotides. The resistance to the GeneEditor™ Antibiotic Selection Mix encoded by this mutant DNA strand facilitates selection of the desired mutation. The system will work with any cloning vector that contains ampicillin resistance as a selectable marker. Mutants generated using this system retain ampicillin resistance and gain resistance to the GeneEditor™ Antibiotic Selection Mix.

This Technical Manual includes mutagenesis protocols for both double-stranded DNA (dsDNA) and single-stranded DNA (ssDNA). Double-stranded DNA is generally the template of choice, but ssDNA can be used for mutations where low efficiency is expected (Section 3.A). The efficiency of mutagenesis is enhanced by an initial transformation into the BMH 71-18 *mutS* Competent Cells provided with the system. This repair minus (*mutS*) strain of *E. coli* is used to avoid selection against the desired mutation. A second transformation into *E. coli* JM109 ensures proper segregation of mutant and wildtype plasmids and results in a high proportion of mutants. Use of the GeneEditor™ in vitro Site-Directed Mutagenesis System allows consistently high mutagenesis frequencies (often >90%) using either dsDNA or ssDNA.

Citations using the GeneEditor™ in vitro Site-Directed Mutagenesis System

For peer-reviewed articles that cite the use of the GeneEditor™ in vitro Site-Directed Mutagenesis System, visit: **www.promega.com/citations/**

2. Product Components and Storage Conditions

Product	Cat.#
GeneEditor™ in vitro Site-Directed Mutagenesis System	Q9280

Each system contains sufficient reagents to perform 30 mutagenesis reactions and sufficient Competent Cells to perform 20 transformations. Includes:

- 35µl Selection Oligonucleotide, Top Strand (labeled as "Top Select Oligo"; the top strand is considered to have the same sequence as the strand encoding the mRNA for β-lactamase.)
- 35µl Selection Oligonucleotide, Bottom Strand (labeled as "Bottom Select Oligo")
- 20µg pGEM®-11Zf(+) Vector
- 35µl *lacZ* Control Knockout Oligonucleotide, Bottom Strand (labeled as "*lacZ* Control Bottom Oligo")
- 75µl Annealing 10X Buffer
- 100µl Synthesis 10X Buffer
- 500u T4 DNA Polymerase
- 100u T4 DNA Ligase
- 2ml BMH 71-18 *mutS* Competent Cells (supplied in a separate bag)
- 2ml JM109 Competent Cells, High Efficiency (supplied in a separate bag)
- 20ml GeneEditor™ Antibiotic Selection Mix

We refer to the top strand as the strand of DNA that has the same sequence as the strand encoding the mRNA for β-lactamase for ampicillin resistance (Amp^r).

Storage Conditions: Store all Competent Cells at –70°C. See the expiration date on the tube label. For convenience, the Competent Cells are not packaged in the box with the other GeneEditor™ System components but are supplied in separate bags. Store all other system components at –20°C.

Store the GeneEditor™ Antibiotic Selection Mix in aliquots at –20°C, as it is sensitive to multiple freeze-thaw cycles. Thaw completely and mix thoroughly before preparing aliquots and before each use. Do not freeze-thaw the GeneEditor™ Antibiotic Selection Mix more than 5 times.

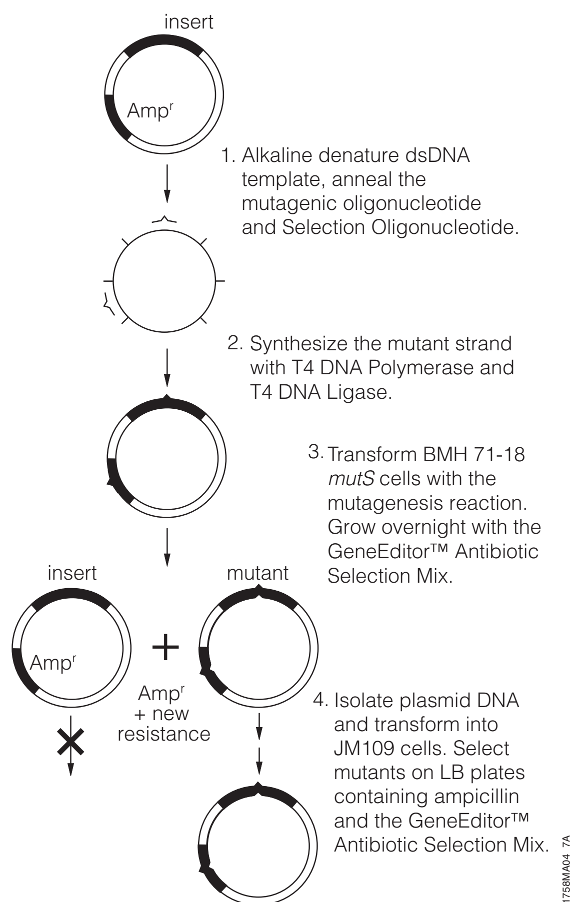

Figure 1. Schematic diagram of the GeneEditor™ in vitro mutagenesis procedure.

3. General Considerations

Site-directed mutagenesis is a valuable tool for the studies of DNA function, and protein structure and function. A number of different mutagenesis methods have been reported (1,2). Site-directed in vitro mutagenesis is accomplished by hybridizing a synthetic oligonucleotide that is complementary to the target template except for a region of mismatch near the center (3). This mismatched region contains the desired mutation. Following hybridization, the oligonucleotide is extended with DNA polymerase to create a double-stranded

structure. The nick is then sealed with DNA ligase, and the duplex structure is transformed into an *E. coli* host.

If no selection method is employed, the theoretical yield of mutants using this procedure is 50% (due to the semi-conservative mode of DNA replication). In practice, however, the mutant yield in the absence of selection may be much lower, often only a few percent or less. This is assumed to result from factors such as incomplete in vitro polymerization, primer displacement by the DNA polymerase used in the fill-in reaction and in vivo host-directed mismatch repair mechanisms that favor repair of the unmethylated newly synthesized DNA strand (4). The GeneEditor™ in vitro Site-Directed Mutagenesis System uses antibiotic resistance to select only for plasmid derived from the mutant strand, resulting in a greatly increased frequency of mutations.

3.A. Important Features of the GeneEditor™ in vitro Site-Directed Mutagenesis System

DNA Template Considerations

Mutagenesis can be performed using either double-stranded or single-stranded DNA templates. The double-stranded procedure is faster and is preferred for most situations. However, the single-stranded procedure may be more useful when trying to maximize the total number of transformants (e.g., when generating mutant libraries or when the mutagenic oligonucleotide is expected to have difficulty annealing to the template). Annealing problems can be caused by sequence mismatches at multiple locations, AT-rich regions that result in a low T_m or secondary structure in the oligonucleotide. A protocol for preparing ssDNA can be found in Chapter 3 of our *Protocols and Applications Guide*, Third Edition (5), as well as in the *Altered Sites® Mammalian Mutagenesis System Technical Manual* #TM041, available online at: **www.promega.com/tbs/**

The quality of the DNA template is important for all mutagenesis methods, as contaminants in the DNA preparation can inhibit synthesis of the second strand. The presence of contaminants can also lead to mispriming of the template and cause overestimation of DNA concentration. All of these effects can result in greatly reduced yields of the desired mutation. DNA prepared using the Wizard® *Plus* SV Minipreps DNA Purification System (Cat.# A1340) can be used with the GeneEditor™ System. Alternatively, refer to Section 7.A for a plasmid miniprep procedure.

It is also important that the DNA template be prepared from a strain that is compatible with the strains used in the mutagenesis procedure. Template DNA should be isolated from a modification (+) *E. coli* K12 strain, such as JM109, or it will be restricted by the modification (+) BMH 71-18 *mutS* strain used in this protocol (see Table 1). DNA isolated from HB101 or NM522 (modification minus strains) or BL21 (*E. coli* B strain) cells should not be used. If in doubt about the compatibility of your strain, transform the DNA into the JM109 Competent Cells provided with the GeneEditor™ System and prepare template DNA from these cells.

Table 1. Modification (+) *E. coli* Strains Compatible with BMH 71-18 *mutS*.

BJ5183	SK2287	MC1061	JM106
JM107	DH5α™	TG1	Q358
SK1590	JM109	JM105	Y1088
DH1	TB1	MM294	
JM108	JM103	XL1-Blue	

The GeneEditor™ in vitro Site-Directed Mutagenesis System is designed for use with vectors containing ampicillin resistance encoded by the TEM-1 β-lactamase (ampicillin resistance) gene. This β-lactamase gene is present on virtually all cloning vectors in common use. All of our pGEM® Vectors contain this gene. If you are in doubt about the compatibility of your particular vector, compare the sequence of the Selection Oligonucleotides to that of the β-lactamase gene in your vector to confirm hybridization. The Selection Oligonucleotides will match the region of the β-lactamase gene shown in Figure 2 except for a 7-nucleotide sequence that encodes resistance to the GeneEditor™ Antibiotic Selection Mix.

High Yield of Mutants

The use of antibiotic selection for the mutant strand yields a high percentage of mutants. This high frequency means that mutants can be identified by restriction analysis or direct sequencing of clones, eliminating the need to screen large numbers of colonies by hybridization.

The BMH 71-18 *mutS* strain (6) suppresses in vivo mismatch repair (7). In the GeneEditor™ System, this strain is used for the initial round of transformation to decrease the chance that the Selection Oligonucleotide mismatch or the mutagenic oligonucleotide mismatch will be repaired. Further information on the BMH 71-18 *mutS* and JM109 strains is provided in Section 7.B. Other *mutS*-containing strains such as ES1301 *mutS* can be used for the primary selection step.

Multiple Simultaneous Mutations

T4 DNA Polymerase is used in the synthesis reaction because it has high fidelity and does not displace the hybridized oligonucleotide (8–10). As a result, multiple site-directed mutations may be introduced simultaneously simply by annealing additional mutagenic oligonucleotides to the DNA insert (10). We have performed up to five simultaneous mutations with >30% efficiency using dsDNA and the GeneEditor™ System.

3.B. Ampicillin Resistance and Altered Substrate Specificity

Ampicillin and other β-lactam antibiotics prevent cell growth in *E. coli* by inhibiting cell wall synthesis. β-lactamases confer resistance to β-lactam antibiotics by degrading these compounds to an inactive form. The ability of the β-lactamase enzymes to degrade a variety of β-lactam antibiotics is

well-characterized (11). The wildtype TEM-1 β-lactamase found in most cloning vectors is proficient at degrading antibiotics such as ampicillin; consequently, cells expressing this β-lactamase exhibit substantial resistance to this antibiotic. The wildtype TEM-1 enzyme is much less proficient at degrading some other β-lactam antibiotics. Numerous mutations have been identified in the β-lactamase gene that alter the substrate specificity of the enzyme and give cells harboring these mutations increased resistance to alternate antibiotics (12). These mutations provide the basis of the mutant selection technique used in the GeneEditor™ System.

The GeneEditor™ System uses an optimized mixture of antibiotics to effectively select those cells expressing a mutant β-lactamase. Resistance to ampicillin is not lost, and cells can be grown using ampicillin selection in subsequent procedures. Resistant (mutant) cells can be killed if exposed to excessive amounts of the GeneEditor™ Antibiotic Selection Mix, so it is important to not exceed the amount specified in the protocol.

3.C. Design of Mutagenic Oligonucleotides

The mutagenic oligonucleotide and Selection Oligonucleotide used in the GeneEditor™ System must be complementary to the same strand of DNA in order to achieve coupling of the antibiotic selection to the desired mutation. Selection Oligonucleotides are provided for both strands of the ampicillin resistance gene. Refer to Section 7.B for sequences of the oligonucleotides provided in the system and to Figure 2 for guidelines on how to determine which oligonucleotide to use in your mutagenesis reaction.

The stability of the complex between an oligonucleotide and a DNA template is determined by the base composition of the oligonucleotide and the conditions under which it is annealed. In general, a 17–20-base oligonucleotide with a mismatch located in the center will be sufficient for single-base mutations. This gives 8–10 perfectly matched nucleotides on either side of the mismatch. For mutations involving two or more mismatches, oligonucleotides 25 bases or longer are needed to allow for 12–15 perfectly matched nucleotides on either side of the mismatch. Oligonucleotides 26–27 bases long have been used successfully to perform four-base insertions and deletions. Larger deletions require an oligonucleotide having 20–30 matched bases on either side of the mismatched region. To stabilize annealing between the oligonucleotide and template DNA, the 3´-end of the oligonucleotide should end with a G or a C nucleotide.

The annealing conditions required may vary with the base composition of the oligonucleotide. AT-rich complexes tend to be less stable than GC-rich complexes and may require a lower annealing temperature. Routinely, oligonucleotides can be annealed by heating to 75°C for 5 minutes followed by slow cooling to room temperature. Detailed discussions of oligonucleotide design and annealing conditions are provided in reference 13 and reference 14 (chapters 11 and 15).

3.D. Phosphorylation of Oligonucleotides

A significant increase in the number of mutants is observed when oligonucleotides are phosphorylated. The oligonucleotides provided in the GeneEditor™ System are 5´-phosphorylated. We also recommend phosphorylation of any mutagenic oligonucleotides used with this system (see Section 7.A for a protocol).

3.E. Template Denaturation

Alkaline denaturation, rather than heat denaturation, is recommended for DNA templates used with the GeneEditor™ System. The efficiency of mutagenesis decreases when using heat denaturation in conjunction with annealing of the mutagenic oligonucleotides. For example, the mutagenesis efficiency for the pGEM®-11Zf(+) Vector is 22.9–54.8% using heat denaturation compared to 73–75% using alkaline denaturation. This effect is especially pronounced when using low-copy-number vectors.

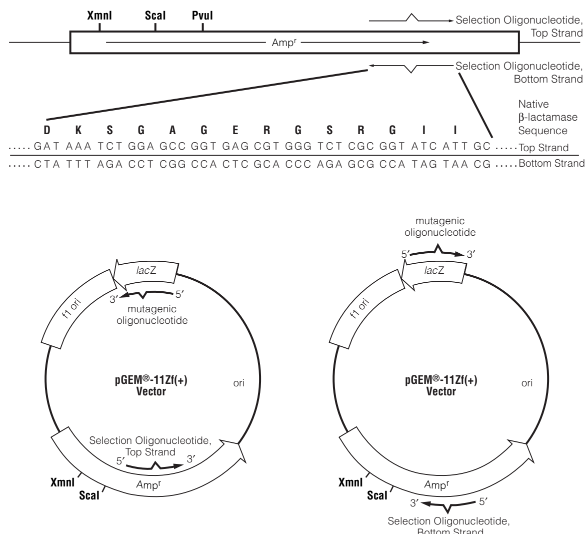

Figure 2. Hybridization location of Selection Oligonucleotides. The location and orientation of the Top and Bottom Strand Selection Oligonucleotides is shown relative to the ampicillin resistance gene in the pGEM®-11Zf(+) Vector. **The direction of the ampicillin resistance gene in any vector can be determined by the location of the unique restriction sites XmnI, ScaI and PvuI at the 5´-end.**

(!) We refer to the top strand as the strand of DNA that has the same sequence as the strand encoding the mRNA for β-lactamase for ampicillin resistance (Ampʳ).

The Top Strand Selection Oligonucleotide hybridizes to the bottom strand, covering the sequence indicated. To determine which Selection Oligonucleotide to use, determine which strand is encoded by your mutagenic oligonucleotide and the corresponding Selection Oligonucleotide. Both the selection and mutagenic oligonucleotides must hybridize to the same strand. The example shown indicates the proper oligonucleotide orientation for introducing the mutation in the gene encoding the *lac*Z α-peptide of the pGEM®-11Zf(+) Vector provided with the system.

4. Mutagenesis Protocol

The mutagenesis reaction involves annealing of the Selection Oligonucleotide (provided) and the mutagenic oligonucleotide(s) to the DNA template, followed by synthesis of the mutant strand with T4 DNA Polymerase and T4 DNA Ligase. The heteroduplex DNA is then transformed into the repair minus *E. coli* strain BMH 71-18 *mutS*, and the cells are grown in selective media to select for clones containing the mutant plasmid. Plasmids resistant to the novel GeneEditor™ Antibiotic Selection Mix are then isolated and transformed into the final host strain, JM109, using the same selection conditions.

4.A. Before You Begin

Before starting the procedure, prepare all of the reagents needed. Calculate the amount of mutagenic oligonucleotide needed (Table 2) and select the appropriate Selection Oligonucleotide (Figure 2) for your mutagenesis reaction.

Materials to Be Supplied by the User
(Solution compositions are provided in Section 7.C.)

- DNA template (see Section 3.A)
- mutagenic oligonucleotide, phosphorylated (see Section 7.A)
- sterile 17 × 100mm polypropylene tubes (Falcon Cat.# 2059 or equivalent)
- 0.8% agarose gel
- heating block or 75°C water bath
- 2M ammonium acetate (pH 4.6) (freshly prepared)
- 2M NaOH, 2mM EDTA (freshly prepared)
- ethanol (100% and 70%), 4°C
- TE buffer (pH 8.0)
- sterile, deionized water
- LB medium
- LB plates containing 125μg/ml ampicillin
- SOC medium

Table 2. Amount of Mutagenic Oligonucleotide Needed to Equal 1.25pmol.

Oligonucleotide Length	Oligonucleotide Equal to 1.25pmol
17mer	7.0ng
20mer	8.3ng
23mer	9.5ng
26mer	10.7ng
29mer	12.0ng

In general: ng of oligonucleotide = pmol of oligonucleotide × 0.33 × N, where N = length of oligonucleotide in bases.

Day 1

4.B. Hybridization to Template

Two options exist for hybridization of the selection and mutagenic oligonucleotides to the DNA template, depending on the nature of the template used. Double-stranded DNA must be denatured before it is used in the annealing reaction. This is accomplished by alkaline denaturation, not heat denaturation. In general, mutagenesis efficiencies for dsDNA templates are 60–90% when the *lacZ* Control Knockout Oligonucleotide is used as the mutagenic oligonucleotide. The pGEM®-11Zf(+) Vector supplied with the GeneEditor™ System must also be alkaline-denatured prior to performing control mutagenesis reactions.

Denaturation is not required when using a single-stranded DNA template for mutagenesis. The use of single-stranded DNA templates is recommended for the generation of large insertion and deletion mutations (>50 base pairs).

The amount of mutagenic oligonucleotide required for the annealing reactions may vary depending on the oligonucleotide length and base composition. The following guidelines provide a basis for individual optimization. Use the Selection Oligonucleotides at a 5:1 oligo:template molar ratio and the mutagenic oligonucleotides at a 25:1 oligo:template molar ratio. A typical reaction may contain approximately 200ng (0.05pmol) of dsDNA or 100ng of ssDNA.

Note: Selection and mutagenic oligonucleotides must hybridize to the same strand to achieve effective selection. Refer to Figure 2 for guidelines.

DNA Template Preparation

Alkaline Denaturation (dsDNA)

1. The following alkaline denaturation reaction generates enough DNA for 10 mutagenesis reactions using an experimental or control template.

dsDNA template or pGEM®-11Zf(+) Vector	0.5pmol (approx. 2µg)
2M NaOH, 2mM EDTA	2µl
sterile, deionized water to a final volume of	20µl

   In general: ng of dsDNA = pmol of dsDNA × 0.66 × N, where N = length of dsDNA in bases.

   Note: To ensure good DNA recovery, do not denature less than 0.5pmol of DNA.

2. Incubate at room temperature for 5 minutes.
3. Add 2µl of 2M ammonium acetate (pH 4.6) and 75µl of 100% ethanol (4°C).
4. Incubate at –70°C for 30 minutes.
5. Precipitate the DNA by centrifugation at top speed in a microcentrifuge for 15 minutes at 4°C.
6. Drain and wash the pellet with 200µl of 70% ethanol (4°C). Centrifuge again as in Step 5. Dry the pellet under vacuum.
7. Suspend the pellet in 100µl of TE buffer (pH 8.0). Analyze a 10µl sample of the denatured DNA on an agarose gel to verify that no significant loss has occurred before proceeding to the annealing reaction. Include nondenatured DNA of known concentration in a neighboring well to help quantify DNA losses and ensure that the DNA has been denatured. Denatured, single-stranded DNA will generally run faster than nondenatured, double-stranded DNA and appear more smeared.
8. Prepare the appropriate annealing reactions as described below.

ssDNA Templates

See Chapter 3 of our *Protocols and Applications Guide*, Third Edition (5), or the *Altered Sites® Mammalian Mutagenesis System Technical Manual* #TM041 for preparation of single-stranded template DNA.

Hybridization Reactions

Mutagenesis Reaction	
template DNA (dsDNA or ssDNA)	10µl (0.05pmol)
appropriate Selection Oligonucleotide (2.9ng/µl), phosphorylated	1µl (0.25pmol)
mutagenic oligonucleotide, phosphorylated (see Table 2)	xµl (1.25pmol)
Annealing 10X Buffer	2µl
sterile, deionized water to a final volume of	20µl

Control Reaction	
alkaline-denatured pGEM®-11Zf(+) Vector	10µl (0.05pmol)
Selection Oligonucleotide, Bottom Strand (2.9ng/µl)	1µl (0.25pmol)
lacZ Control Knockout Oligonucleotide, Bottom Strand (13.2ng/µl)	1µl (1.25pmol)
Annealing 10X Buffer	2µl
sterile, deionized water to a final volume of	20µl

Heat the annealing reactions to 75°C for 5 minutes and allow them to cool slowly to 37°C. Slow cooling minimizes nonspecific annealing of the oligonucleotides. We recommend cooling the reactions at about 1.5°C per minute to 37°C. For more information on annealing and oligonucleotide design, see Section 3.C and references 13 and 14.

Note: If you do not have access to a thermal cycler, the following annealing procedure may be used to slowly cool the annealing reactions.

1. Heat the reactions at 75°C for 5 minutes in a heating block or a beaker containing 200ml of water.
2. Place heating block or beaker at room temperature until it reaches 37°C (~30 minutes).

Optimal annealing temperatures and times may vary depending on the composition of the mutagenic oligonucleotide that is used. It may be necessary to try several annealing temperatures in order to optimize the mutagenesis efficiency for your oligonucleotide.

4.C. Mutant Strand Synthesis and Ligation

1. Once the annealing reactions have cooled to 37°C, spin briefly in a microcentrifuge to collect the contents at the bottom of the tube. Add the following components in the order listed:

Component	Volume
sterile, deionized water	5µl
Synthesis 10X Buffer	3µl
T4 DNA Polymerase, (5–10u)	1µl
T4 DNA Ligase, (1–3u)	1µl
final volume	30µl

2. Incubate the reaction at 37°C for 90 minutes to perform mutant strand synthesis and ligation.

   Note: Incubation times longer than 90 minutes are not recommended, as primer degradation can occur as dNTP levels are depleted.

4.D. Transformation of BMH 71-18 *mutS* Competent Cells

BMH 71-18 *mutS* Competent Cells should yield >10^7cfu/µg DNA for use in the following protocol. Follow this protocol as closely as possible to maximize the transformation efficiency of these cells. Additional BMH 71-18 *mutS* Competent Cells may be purchased from Promega (Section 7.D).

Note: Thaw the GeneEditor™ Antibiotic Selection Mix thoroughly and mix well before use. Aliquot the thawed material into 1–2ml amounts prior to refreezing to avoid multiple freeze-thaw cycles.

1. Prechill sterile 17 × 100mm polypropylene culture tubes on ice, one for each annealing reaction. **Note:** Use of a standard microcentrifuge tube reduces the transformation efficiency approximately twofold due to inefficient heat-shock treatment.
2. Place the frozen competent cells on ice for 5 minutes or until just thawed.
3. Gently mix the cells by flicking the tube and then transfer 100µl of the thawed BMH 71-18 *mutS* cells to each of the prechilled culture tubes.
4. Add 1.5µl of each mutagenesis reaction or control reaction (~10ng of template DNA) to 100µl of BMH 71-18 *mutS* Competent Cells. Move the pipette tip through the cells while dispensing. Quickly flick the tube several times.
5. Immediately place the tubes on ice for 10 minutes.
6. Heat-shock the cells for 45–50 seconds in a water bath at exactly **42°C. DO NOT SHAKE.**
7. Immediately place the tubes on ice for 2 minutes.
8. Add 900µl of room temperature LB broth without antibiotic to each transformation reaction, and incubate for 60 minutes at 37°C with shaking (approximately 225rpm).
9. Prepare overnight cultures by adding 4ml of LB containing 100µl of the GeneEditor™ Antibiotic Selection Mix to each transformation reaction (5ml total volume). It is important to thaw the Antibiotic Selection Mix completely and mix well before use. Incubate for 16–18 hours at 37°C with shaking (approximately 225rpm). The culture should be well aerated during growth to obtain sufficient cell densities. Tilt the tubes during growth or transfer the culture to a small Erlenmeyer flask. Use this culture for the plasmid miniprep (Section 4.E) and transformation (Section 4.F).

(!) Do not add volumes of GeneEditor™ Antibiotic Selection Mix greater than 100µl to the 5ml overnight culture. Unlike ampicillin, the antibiotics in this mix can inhibit growth of resistant cells when present in excess of the recommended levels, especially with low-copy-number plasmids. Some low-copy-number plasmids may require a decrease in antibiotic concentration. This must be determined empirically.

Notes:

1. Do not use BMH 71-18 *mutS* transformants as mutants. A second round of transformation (into JM109) should always be performed to avoid having a mixed population of mutant and wildtype DNA in the cell. BMH 71-18 *mutS* is not a stable host for long-term maintenance of plasmids.
2. Check overnight growth by measuring absorbance at 600nm. For the control reaction, 1ml of culture typically has an absorbance of ≥0.8. An absorbance of ≤0.2 indicates that the culture has not grown. Note that BMH 71-18 *mutS* cells grow very slowly and therefore require longer incubation times than most *E. coli* strains. BMH 71-18 *mutS* cells tend to aggregate when grown in the GeneEditor™ Antibiotic Selection Mix and often settle at the bottom of the culture tube.

Optional: Transformation Assay to Determine Efficiency of BMH 71-18 *mutS* Competent Cells

The following protocol is provided for those who wish to determine the efficiency of the BMH 71-18 *mutS* Competent Cells.

1. Thaw tubes of competent cells on ice, gently mix and then transfer 100µl to a prechilled, sterile 17 × 100mm polypropylene culture tube on ice.
2. Add 1µl of pGEM®-11Zf(+) Vector (diluted to 0.1ng/µl) to the tube. Mix gently by flicking the tube several times. Incubate on ice for 10 minutes.
3. Heat-shock the tube for 45–50 seconds in a water bath at exactly **42°C. DO NOT SHAKE.**
4. Immediately place the tube on ice for 2 minutes.
5. Add 900µl of SOC medium and incubate at 37°C with shaking (approximately 225rpm) for 60 minutes.
6. Plate 100µl on each of 2 LB plates containing 100µg/ml ampicillin. Incubate at 37°C overnight.
7. Count the number of colonies on each plate and calculate the mean number of colonies obtained. Calculate the transformation efficiency, taking the dilution factor into account, as follows:

   Example: After 100µl competent cells are transformed with 0.1ng uncut plasmid DNA, the transformation reaction is added to 900µl of SOC medium (0.1ng DNA/ml). From that volume, 100µl is plated on two plates (0.01ng DNA/100µl). If 200 colonies are obtained (average of two plates), the transformation efficiency is calculated as shown below:

$$\frac{200\text{cfu}}{0.01\text{ng}} = 2 \times 10^4\text{cfu/ng} = 2 \times 10^7\text{cfu/µg}$$

Day 2

4.E. Plasmid Miniprep

Purify plasmid DNA from the overnight culture above using the protocol provided in Section 7.A. Alternate purification methods, such as the Wizard® *Plus* SV Minipreps DNA Purification System (Cat.# A1340) also may be used for this step. Using the Wizard® *Plus* SV Minipreps DNA Purification System, high quality DNA is easily obtained from both EndA+ and EndA– strains. Please note that BMH 71-18 *mutS* is EndA+ and may not be compatible with all purification procedures.

4.F. Transformation into JM109 Competent Cells

1. Before beginning the transformation procedure, prepare plates by pouring molten LB agar containing 150µl of the GeneEditor™ Antibiotic Selection Mix and 2.5mg of ampicillin per 20ml of media. Prepare 2 plates for each transformation. Do not store plates containing the GeneEditor™ Antibiotic Selection Mix for more than one week at 4°C.

   Alternatively, prepare plates by evenly spreading 100µl of the GeneEditor™ Antibiotic Selection Mix onto 20–25ml LB agar plates containing 125µg/ml ampicillin. Allow the GeneEditor™ Antibiotic Selection Mix to soak into the plate for at least 20 minutes but for no longer than 2 hours before spreading the transformed cells.

   ! Do not add volumes of GeneEditor™ Antibiotic Selection Mix greater than those indicated. The antibiotics in this mixture can inhibit growth of resistant cells when provided in excess of the recommended levels.

   Prepare plates for the control reaction as above with the addition of IPTG (0.5mM final) and X-Gal (80µg/ml final) to screen for inactivation of the *lacZ* α-peptide. Mutant colonies will appear white.
2. Estimate the amount of DNA recovered from the plasmid miniprep. Run a portion of the DNA on an agarose gel and estimate the concentration by comparison with double-stranded DNA markers.
3. Prechill sterile 17 × 100mm polypropylene culture tubes on ice.
4. Place frozen JM109 Competent Cells on ice for 5 minutes or until just thawed.
5. Gently mix the cells by flicking the tube and transfer 100µl of the thawed JM109 cells to each of the prechilled culture tubes.

4.F. Transformation into JM109 Competent Cells (continued)

6. Add approximately 5–10ng of plasmid DNA to 100µl of JM109 Competent Cells. Move the pipette tip through the cells while dispensing. Quickly flick the tube several times.

   Note: It is important to limit the amount of DNA used in the transformation reaction to a maximum of 10ng to avoid co-transformation of cells with both wildtype and mutant plasmids. If necessary, quantitate the DNA concentration of the miniprep by measurement of absorbance at 260nm.

7. Immediately place the tube on ice for 30 minutes.
8. Heat-shock the cells for 45–50 seconds in a water bath at exactly **42°C.** **DO NOT SHAKE.**
9. Immediately place the tubes on ice for 2 minutes.
10. Add 900µl of room temperature SOC medium to each transformation reaction and incubate for 60 minutes at 37°C with shaking (approximately 225rpm).
11. For each tube, plate 100µl of cells on each of two LB plates prepared in Step 1, and incubate at 37°C for 12–14 hours. When using the high-efficiency competent cells provided with the GeneEditor™ System, it may be necessary to plate a lower volume of cells on each plate in order to obtain isolated colonies.

    Note: For best results, high-efficiency competent cells (>1 × 10^8cfu/µg) should be used.

4.G. Analysis of Transformants

The GeneEditor™ in vitro Site-Directed Mutagenesis System generally produces 60–90% mutants, so colonies may be screened by direct sequencing. Assuming that greater than 60% mutants are obtained, screening 5 colonies will give a greater than 95% chance of finding the mutation. Often restriction sites can be incorporated into the mutagenesis primers without altering the amino acid sequence. These sites can provide a quick screen to identify clones containing the desired mutation. Control mutagenesis reactions should give better than 60% white colonies when plated on IPTG and X-Gal indicator plates as described in Section 4.F.

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5. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: **www.promega.com**. E-mail: **techserv@promega.com**

Symptoms	Causes and Comments
No growth in overnight BMH 71-18 *mutS* culture as indicated by an absorbance at A_{600} of ≤0.2 for 1ml of culture	Excessive GeneEditor™ Antibiotic Selection Mix used: • Be sure to completely thaw and mix the GeneEditor™ Antibiotic Selection Mix before use. • Check GeneEditor™ Antibiotic concentration in selective media. • Reduce the amount of GeneEditor™Antibiotic Selection Mix used. Try using 50µl instead of 100µl in transformation selection.
	Low β-lactamase expression. Some vectors may have reduced β-lactamase expression and require reduced levels of the GeneEditor™ Antibiotic Selection Mix. Try using 50µl instead of 100µl in transformation selection.
	DNA derived from an *hsd* modification minus strain. BMH 71-18 *mutS* is restriction (+). DNA should be isolated from a modification (+) K12 strain (see Table 1) or it will be restricted by BMH 71-18 *mutS*. DNA isolated from modification minus strains (e.g., HB101 or NM522) or BL21 (*E. coli* B strain) cells should not be used.
	Incomplete denaturation of template DNA. Check that the NaOH solution used in the denaturation procedure was freshly prepared.
	Carryover of NaOH from the denaturation reaction. NaOH must be neutralized after the denaturation reaction. Use fresh ammonium acetate (pH 4.6) and check pH carefully.
	Poor recovery of DNA after alkaline denaturation. DNA not recovered after ethanol precipitation. Check denatured template on an agarose gel.
	Insufficient aeration of the culture. BMH 71-18 *mutS* cells tend to clump and grow poorly if insufficiently aerated. Slant the growth tubes or culture overnight in small Erlenmeyer flasks. Increase the shaker speed.

5. Troubleshooting (continued)

Symptoms	Causes and Comments
No growth in overnight BMH 71-18 *mutS* culture (continued)	Inaccurate DNA concentration. Confirm concentration by comparison with known standards on an agarose gel.
	DNA contaminants present. Further purify DNA by PEG precipitation if the A_{260}/A_{280} ratio is less than 1.8–2.0.
	Inadequate oligonucleotide hybridization. Incorrect oligonucleotide-to-template ratios. Check concentration of mutagenic oligonucleotide and DNA. Anneal more slowly.
	Inefficient synthesis or ligation of second-strand DNA. Low T4 DNA Polymerase or T4 DNA Ligase activity due to poor-quality DNA. Compare results to those of the control mutagenesis reaction.
	Contaminating DNA fragments in plasmid miniprep. Impure DNA may cause nonspecific priming. Make sure the DNA template is clean.
	Low competency of BMH 71-18 *mutS* cells ($<10^7$cfu/µg). Check competency with the pGEM®-11Zf(+) Vector provided. Use ampicillin only as the selection antibiotic.
No JM109 colonies	Excessive amount of GeneEditor™ Antibiotic Selection Mix used. Reduce the volume of GeneEditor™ Antibiotic Selection Mix used. Try using 50µl instead of 100µl on selective plates.
	Poor DNA recovery from BMH 71-18 *mutS* cells. BMH 71-18 *mutS* cells are *endA*+ and may not be compatible with all DNA purification procedures. Try an alternative method of plasmid preparation.
	Low-competence JM109 cells used. Use the high-efficiency competent cells provided with the system. Check competency with the pGEM®-11Zf(+) Vector provided. Use ampicillin only as the selection antibiotic.

5. Troubleshooting (continued)

Symptoms	Causes and Comments
JM109 antibiotic-resistant colonies, but low mutation frequency	Co-transformation of JM109 cells with both wildtype and mutant plasmids. Do not use more than 10ng DNA in the transformation of JM109 cells. Quantitate the amount of DNA in your miniprep by measurement of absorbance at 260nm.
JM109 antibiotic-resistant colonies, but no mutations	Mutagenic oligonucleotide not annealed to the same strand as the Selection Oligonucleotide. Recheck the orientation of the cloned insert. Repeat the mutagenesis reaction using the other Selection Oligonucleotide provided with the system.
	Inadequate annealing of mutagenic oligonucleotide to template DNA • Wrong oligonucleotide-to-template ratios used in the hybridization reaction. Check concentration and purity of mutagenic oligonucleotide by PAGE. • Increase annealing time. • Secondary structure in cloned insert or mutagenic oligonucleotide. Prepare template as single-stranded DNA. If necessary, redesign mutagenic oligonucleotide.
	Mutagenic oligonucleotide not incorporated into newly synthesized strand. Make sure that mutagenic oligonucleotide is phosphorylated.
	3´-end of oligonucleotide is not properly base-paired to template. Redesign oligonucleotide.
	Initial selection not performed with *mutS* strain. *mutS* phenotype is required for efficient coupling of mutations. Check for tetracycline resistance in BMH 71-18 strain.
	Problem with cloned insert. The BMH 71-18 strain is RecA+ and some constructs may undergo rearrangement. If possible, clone a small region for mutagenesis.

5. Troubleshooting (continued)

Symptoms	Causes and Comments
JM109 antibiotic-resistant colonies, but no mutations (continued)	The GeneEditor™ Antibiotic Selection Mix is no longer active. The GeneEditor™ Antibiotic Selection Mix should be stored in aliquots at –20°C, as it is sensitive to multiple freeze-thaw cycles. Check for the presence of mutant (white) colonies on the positive control plates (Section 4.F). The presence of white colonies on control plates indicates that the GeneEditor™ Antibiotic Selection Mix is functional.
JM109 antibiotic-resistant colonies are present around the edges at a higher density than in the center of the plate	GeneEditor™ Antibiotic Selection Mix and/or cells are unevenly spread on the plate. Make sure that the GeneEditor™ Antibiotic Selection Mix is spread to the edge of the plate.

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7. Appendix

7.A. Additional Protocols

5´-Phosphorylation of Oligonucleotides

Materials to Be Supplied by the User
(Solution compositions are provided in Section 7.C.)

- oligonucleotide to be phosphorylated
- T4 Polynucleotide Kinase (Cat.# M4101)
- Kinase 10X Buffer (supplied with the T4 Polynucleotide Kinase)
- ATP, 10mM

1. Add the following components to a microcentrifuge tube. Use the formula below to calculate the nanograms of nucleotide equivalent to 100pmol.

oligonucleotide	100pmol
Kinase 10X Buffer	2.5µl
T4 Polynucleotide Kinase	5u
ATP, 10mM	2.5µl
sterile, deionized water to a final volume of	25µl

   In general: ng of oligonucleotide = pmol of oligonucleotide × 0.33 × N, where N = length of oligonucleotide in bases.

2. Incubate the reaction at 37°C for 30 minutes.
3. Incubate the reaction at 70°C for 10 minutes to inactivate the T4 Polynucleotide Kinase.
4. The reaction products can be stored at –20°C or added directly to the annealing reaction (Section 4.B).

Plasmid Miniprep Procedure

Materials to Be Supplied by the User
(Solution compositions are provided in Section 7.C.)

- miniprep resuspension buffer
- 0.2M NaOH, 1% SDS (prepare fresh for each use)
- TE-saturated phenol:chloroform:isoamyl alcohol (25:24:1)
- chloroform:isoamyl alcohol (24:1)
- ethanol (100% and 70%)
- DNase-free RNase A
- 3M potassium acetate (pH 4.8)

1. Place 1.5ml of the overnight culture from Section 4.D, Step 9, into a microcentrifuge tube and centrifuge at 12,000 × *g* for 1 minute. The remainder of the overnight culture can be stored at 4°C.
2. Remove the medium by aspiration, leaving the bacterial pellet as dry as possible.
3. Suspend the pellet by vortexing in 100µl of ice-cold miniprep resuspension buffer.
4. Incubate for 5 minutes at room temperature.
5. Add 200µl of a freshly prepared solution containing 0.2M NaOH, 1% SDS to lyse the cells. Mix by inversion. **Do not vortex**. Incubate on ice for 5 minutes.
6. Add 150µl of ice-cold 3M potassium acetate solution (pH 4.8) to neutralize the lysate. Mix by inversion or gentle vortexing for 10 seconds. Incubate for 5 minutes on ice.
7. Centrifuge at 12,000 × *g* for 5 minutes.
8. Transfer the supernatant to a fresh tube, avoiding the white precipitate.
9. Add 1 volume of TE-saturated phenol:chloroform:isoamyl alcohol (25:24:1). Vortex for 1 minute and centrifuge at 12,000 × *g* for 5 minutes.
10. Transfer the upper, aqueous phase to a fresh tube and add 1 volume of chloroform:isoamyl alcohol (24:1). Vortex for 1 minute and centrifuge as in Step 9.
11. Transfer the upper, aqueous phase to a fresh tube and add 2.5 volumes of 100% ethanol. Mix and allow to precipitate for 5 minutes on dry ice.
12. Centrifuge at 12,000 × *g* for 5 minutes. Rinse the pellet with 70% ethanol (prechilled) and dry the pellet under vacuum.
13. Dissolve the pellet in 50µl of sterile deionized water. Add 0.5µl of 100µg/µl DNase-free RNase A and incubate for 5 minutes at room temperature.

14. The yield of plasmid DNA can be determined by electrophoresis on an agarose gel. A yield of 0.2–3µg of plasmid DNA may be expected, depending on the plasmid copy number.

7.B. Reference Information

Table 3. Sequence of Oligonucleotides Provided.

Description	Size	Sequence
Selection Oligonucleotide, Phosphorylated (Top Strand)	35mer	5´-d(pGATAAATCTGGAGCCTCCAAG GGTGGGTCTCGCGG)-3´
Selection Oligonucleotide, Phosphorylated (Bottom Strand)	35mer	5´-d(pCCGCGAGACCCACCCTTGGAG GCTCCAGATTTATC)-3´
lacZ Control Knockout Oligonucleotide (Bottom Strand)	32mer	5´-d(pGGGTAACGCCAGGGTTATTCC CAGTCACGACG)-3´

Description of Bacterial Strains

JM109

*endA*1, *recA*1, *gyrA*96, *thi*, *hsdR*17 (r_k–, m_k+), *relA*1, *supE*44, λ–, Δ(*lac-proAB*), [F´, *traD*36, *proAB*, *lacI*^{q}ZΔM15]

JM109 (15) is a useful host in which to clone pGEM® Vectors and for production of ssDNA from M13 or phagemid vectors. The strain grows well and is efficiently transformed by a variety of methods. Because JM109 is RecA– and lacks the *E. coli* K restriction system, undesirable restriction of cloned DNA and recombination with host chromosomal DNA are prevented. The *endA*1 mutation leads to an improved yield and quality of isolated plasmid DNA. JM109 High Efficiency Competent Cells are available from Promega (Section 7.D).

JM109 should always be maintained on minimal plates (M-9) supplemented with 1mM thiamine-HCl. This selects for the presence of the F´, which carries a nutritional requirement for growth (proline biosynthesis). Maintenance of the F´ is important for α-complementation and ssDNA yield.

Blue/White Color Screening

JM109 can be used for blue/white color screening of vectors encoding the *lacZ* α-peptide. This strain is deficient in β-galactosidase activity due to deletions in both genomic and episomal copies of the *lacZ* gene. The deletion in the episomal (F factor) copy of the *lacZ* gene (*lacI*^{q}ZΔM15) is located in the α-peptide region, and as a result, β-galactosidase activity can be complemented by addition of a functional α-peptide. The pGEM®-11Zf(+) Vector encodes the *lacZ* α-peptide, and cells carrying this plasmid are able to produce functional β-galactosidase.

When plated on indicator media containing X-Gal and IPTG, the host/plasmid combination will generate blue colonies. However, when the α-peptide is disrupted by incorporation of the control mutation, complementation does not occur and no β-galactosidase activity is produced. Therefore, bacterial colonies harboring the control mutants remain white.

BMH 71-18 *mutS*

thi, *supE*, Δ(*lac-proAB*), [*mutS*:Tn10] [F′, *proAB*, *lacI*^{q}ZΔM15]

BMH 71-18 *mutS* is a mismatch repair minus strain of *E. coli*. Use of a *mutS* strain prevents repair of the newly synthesized unmethylated strand (4,7) leading to high mutation efficiencies. BMH 71-18 *mutS* is RecA+, and as a result, inserts containing highly repetitive sequences may be unstable. BMH 71-18 *mutS* is tetracycline-resistant, due to the presence of Tn10. BMH 71-18 *mutS* is also restriction (+). Template DNA should be isolated from a modification (+) K12 strain or it will be restricted. For example, DNA isolated from HB101 or NM522 (modification minus strains) or BL21 (*E. coli* B strain) cells should not be used.

7.C. Composition of Buffers and Solutions

2M ammonium acetate (pH 4.6)

15.4g ammonium acetate

Dissolve the ammonium acetate in 50ml deionized water, bring to pH 4.6 with glacial acetic acid and bring final volume to 100ml with deionized water.

Annealing 10X Buffer

200mM Tris-HCl (pH 7.5)
100mM $MgCl_2$
500mM NaCl

ampicillin stock solution

50mg/ml in deionized water

Store at –20°C.

kinase 10X buffer

700mM Tris-HCl (pH 7.6)
100mM $MgCl_2$
50mM DTT

LB (Luria-Bertani) medium (1L)

10g Bacto®-tryptone
5g Bacto®-yeast extract
5g NaCl

Adjust pH to 7.5 with NaOH and autoclave.

LB plates plus ampicillin (1L)

Add 15g agar to 1 liter of LB medium. Adjust to pH 7.0 with NaOH. Autoclave. Allow the medium to cool to 55°C before adding ampicillin: 125μg/ml final concentration. Pour 20–25ml of medium into 85mm petri dishes. If necessary, flame the surface of the medium with a Bunsen burner to eliminate bubbles. Let the agar harden. Store at 4°C for up to one month.

Mg^{2+} stock solution, 2M (500ml)

101.5g $MgCl_2 \cdot 6H_2O$
123.3g $MgSO_4 \cdot 7H_2O$

7.C. Composition of Buffers and Solutions (continued)

miniprep resuspension buffer

25mM Tris-HCl (pH 8.0)
10mM EDTA
50mM glucose

0.2M NaOH 1% SDS
(prepare fresh for each use)

200μl 10M NaOH
1ml 10% SDS
8.8ml deionized water

2M NaOH 2mM EDTA
(prepare fresh for each use)

2ml 10M NaOH
40μl 500mM EDTA
7.96ml deionized water

3M potassium acetate (pH 4.8)

Dissolve 29.44g potassium acetate in 30ml of deionized water, bring to pH 4.8 with glacial acetic acid. Adjust volume to 100ml with deionized water.

SOC medium (100ml)

2.0g Bacto®-tryptone
0.5g Bacto®-yeast extract
1ml 1M NaCl
0.25ml 1M KCl
1ml 2M Mg^{2+} stock filter-sterilized
1ml 2M glucose, filter-sterilized

Add Bacto®-tryptone, Bacto®-yeast extract, NaCl and KCl to 97ml deionized water. Stir to dissolve. Autoclave and cool to room temperature. Add 2M Mg^{2+} stock and 2M glucose stock, each to a final concentration of 20mM. Filter the complete medium through a 0.2μm filter unit. The pH should be 7.0.

synthesis 10X buffer

100mM Tris-HCl (pH 7.5)
5mM dNTPs
10mM ATP
20mM DTT

TE buffer

10mM Tris-HCl (pH 8.0)
1mM EDTA

TE-saturated phenol:chloroform:isoamyl alcohol (25:24:1)

Mix equal parts of TE buffer and phenol and allow the phases to separate. Then mix 1 part of the lower, phenol phase with 1 part of chloroform:isoamyl alcohol (24:1).

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7.D. Related Products

GeneEditor™ System Components Available Separately

Product	Size	Cat.#
BMH 71-18 *mutS* Competent Cells (>10^7cfu/µg)	1ml (5 × 200µl)	L1201
T4 DNA Polymerase*	100u	M4211
	500u	M4215
T4 DNA Ligase*	100u (Weiss)	M1801
T4 Polynucleotide Kinase*	100u	M4101
Bacterial Strain BMH 71-18 *mutS*, Glycerol Stock	500µl	Q6321
Bacterial Strain JM109, Glycerol Stock	500µl	P9751
JM109 Competent Cells (>10^8cfu/µg)*	1ml (5 × 200µl)	L2001
JM109 Competent Cells (>10^7cfu/µg)	1ml (5 × 200µl)	L1001
GeneEditor™ Antibiotic Selection Mix	20ml	Q9291
Top Strand Selection Oligonucleotide	35µl	Q9321
Bottom Strand Selection Oligonucleotide	35µl	Q9301

*For Laboratory Use.

Related Products

Product	Size	Cat.#
R408 Helper Phage	5ml	P2291

Product	Size	Cat.#
Wizard® *Plus* SV Minipreps DNA Purification System	50 preps	A1340
	250 preps	A1470

For Laboratory Use.

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