Site-Directed Mutagenesis Using Altered \( \beta \)-Lactamase Specificity

Christine A. Andrews and Scott A. Lesley

1. Introduction

Site-directed mutagenesis (SDM) is a powerful tool for the study of gene expression/regulation and protein structure and function. Hutchinson et al. (1) developed a general method for the introduction of specific changes in DNA sequence, which involves hybridization of a synthetic oligonucleotide (ON) containing the desired mutation to a single-stranded DNA (ssDNA) target template. Following hybridization, the oligonucleotide is extended with a DNA polymerase to create a double-stranded structure. The heteroduplex DNA is then transformed into an \textit{Escherichia coli}, in which where both wild type and mutant strands are replicated. In the absence of any selection this method is very inefficient, often resulting in only a few percent of mutants obtained. Various strategies of selection have since been developed, which can increase mutagenesis efficiencies well above the theoretical yield of 50%. The methods of Kunkel (2), Eckstein (3), and Deng (4,5) employ negative selection against the wild-type DNA strand, in which the parental DNA is selectively degraded, either by growth in an alternate host strain, or by digestion with a nuclease or restriction enzyme. The methods of Lewis and Thompson (6) and Bonsack (7) utilize antibiotic resistance to positively select for the mutant DNA strand. This chapter describes a method for the positive selection of mutant strand DNA, which relies on the altered activity of the enzyme \( \beta \)-lactamase against extended spectrum cephalosporins (8).

Various amino acid substitutions in the active site of TEM-1 \( \beta \)-lactamase, the enzyme responsible for resistance to ampicillin (AMP), have been reported (9–17). These mutations alter the substrate specificity of the enzyme and result in increased hydrolytic activity of the enzyme against extended spectrum \( \beta \)-lactam
antibiotics and cephalosporins (Fig. 1). This increased activity results in increased resistance specific to cells expressing the mutant enzyme. The triple mutant, G238S:E240K:R241G, displays increased resistance to cefotaxime (9,10), and ceftriaxone (unpublished result), and is the basis for the selection strategy used in the GeneEditor™ Mutagenesis System. Residues 238, 240, and 241 are adjacent in the β-lactamase sequence, but are numbered according to the system of Ambler (18). The numbering system for amino acid residues starts at the N-terminus of the longest form of the TEM-1 gene from Bacillus licheniformis, and takes into account the postulated gaps necessary for optimal sequence alignment of the various forms of the TEM-1 gene.

Figure 2 is a schematic outline of the GeneEditor procedure. Double-stranded (ds) plasmid DNA is first alkaline denatured. Subsequently, two synthetic ONs are simultaneously annealed to the template. The first ON is the selection ON, which encodes the residue changes in the V-lactamase gene that result in increased resistance to the extended spectrum antibiotics. Table 1 shows the sequence of the selection ON, compared to the wild-type sequence in the V-lactamase gene. The second ON is the mutagenic ON, and codes for the desired sequence changes in the target DNA. This mutagenic ON hybridizes to the same DNA strand as the selection ON. Synthesis and ligation of the mutant strand by T4 DNA polymerase and T4 DNA ligase creates a heteroduplex, effectively linking the conferred antibiotic resistance with the desired
Fig. 2. Schematic diagram of the GeneEditor in vitro SDM procedure.

1. Alkaline denature dsDNA template, anneal mutagenic oligonucleotide and Selection Oligonucleotide.

2. Synthesize mutant strand with T4 DNA Polymerase and T4 DNA Ligase.

3. Transform BMH71-18 mutS cells with mutagenesis reaction. Grow overnight with the GeneEditor™ Antibiotic Selection Mix.

4. Isolate plasmid DNA and transform JM109. Select mutants on ampicillin plates containing the GeneEditor™ Antibiotic Selection Mix.
mutation in the target gene. The DNA is then transformed into a repair-deficient \textit{E. coli} \textit{mutS} host, such as BMH71-18 \textsuperscript{(19)}, followed by clonal segregation in a second host. Linkage of the antibiotic resistance to the desired mutation results in a high efficiency of mutagenesis. More than one mutagenic \textit{ON} may be annealed along with the selection \textit{ON}, to create several linked mutations on the same plasmid. The authors have effectively coupled seven separate mutations with the altered substrate specificity, in a single mutagenesis reaction, with 30\% efficiency.

The GeneEditor protocol can be used with any plasmid vector containing the \textit{TEM-1} \textit{β}-lactamase sequence (commonly designated the “amp” gene), without the need for subcloning into a specialized vector. A Basic Local Alignment Search Tool (BLAST) search of the vector database at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST/) indicates that the \textit{TEM-1} sequence is present in over 90\% of the commonly used cloning vectors. Guidelines for the design of mutagenic \textit{ON}s are discussed in the Notes section.

2. Materials

1. 2 \textit{M} \text{NaOH}, 2 \textit{mM} ethylenediamine tetraacetic acid (EDTA).
2. 2 \textit{M} Ammonium Acetate pH 4.6.
3. 70 and 100\% ethanol.
4. TE Buffer: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA.
5. Oligonucleotides, 5’ phosphorylated (see Table 1 and Note 1).
6. 10X Annealing buffer: 200 mM Tris-HCl, pH 7.5, 100 mM MgCl\textsubscript{2}, 500 mM NaCl.
7. 10X Synthesis buffer: 100 mM Tris-HCl, pH 7.5, 5 mM deoxyribonucleoside triphosphate (dNTPs), 10 mM adenosine triphosphate, 20 mm dithiothreitol.
8. T4 DNA Polymerase (10 U/\muL).
9. T4 DNA Ligase (5 U/µL).
11. Luria-Bertani (LB) media: 10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl.
12. SOC media (100 mL): 2 g tryptone, 0.5 g yeast extract, 1 mL of 1 M NaCl, 0.25 mL of 1 M KCl, 1 mL of 2 M Mg²⁺ stock (1 M MgCl₂·6H₂O/1 M MgSO₄·7H₂O), 1 mL of 2 M glucose.
13. Antibiotic selection mix: 5 mg/mL ampicillin/25 µg/mL cefotaxime/25 µg/mL ceftriaxone (Sigma, St. Louis, MO)/100 mM potassium phosphate, pH 6.0 (see Note 2).
16. Miniprep resuspension buffer: 25 mM Tris-HCl, pH 8.0, 10 mM EDTA, pH 8.0, 50 mM glucose.
17. Miniprep lysis buffer: 0.2 M NaOH, 1% sodium dodecyl sulfate. Prepare fresh.
18. Neutralization solution: 3.5 M potassium acetate, pH 4.8.
19. Ampicillin solution, 100 mg/mL in deionized H₂O, filter-sterilized.
20. LB agar (1.5%).

3. Methods

3.1. Step 1: Denaturation of dsDNA Templates

1. Set up the following alkaline denaturation reaction: 1.0 pmol (approx 2 µg for a 3-kb plasmid) dsDNA template; 2 µL of 2 M NaOH, 2 mM EDTA; sterile, deionized H₂O to a final volume of 20 µL. This generates enough DNA for 10 mutagenesis reactions. To ensure good recovery, do not denature less than 1.0 pmol dsDNA. In general, ng dsDNA = pmol dsDNA × 0.66 × N, where N = length of dsDNA in bases.
2. Incubate at room temperature for 5 min.
3. Add 2 µL of 2 M ammonium acetate, pH 4.6, and 75 µL of 100% ethanol.
4. Incubate at –70°C for 30 min.
5. Precipitate the DNA by centrifugation at top speed in a microcentrifuge for 15 min at 4°C.
6. Decant supernatant, and wash the pellet with 200 µL of 70% ethanol.
7. Centrifuge again as in step 5. Dry the pellet under vacuum.
8. Resuspend the pellet in 100 µL TE buffer pH 8.0. Analyze a 10-µL sample of the denatured DNA on an agarose gel, to verify that no significant loss occurred, before proceeding to the annealing reaction. Include non-denatured DNA of known concentration in a neighboring well, to help quantify DNA losses, and to ensure that the DNA has been denatured. Denatured, ssDNA will generally run faster than non-denatured dsDNA, and will appear more smeared. The denatured DNA may be stored at –20°C for up to several months, with no loss in mutagenesis efficiency.

3.2. Step 2: Annealing Reaction and Synthesis of Mutant Strand

1. Prepare the annealing reaction as outlined here: 0.10 pmol (200 ng) 10 µL denatured template DNA; 0.25 pmol 1 µL selection ON, phosphorylated (see Note 1);
1.25 pmol mutagenic ON, phosphorylated (see Note 1); 2 µL 10x annealing buffer; and sterile, deionized water to 20 µL.
2. Heat the annealing reaction to 75°C for 5 min, then allow to cool slowly to room temperature. Slow cooling minimizes nonspecific annealing of the ONs. Cooling the reactions at a rate of approx 1.5°C/min is recommended (see Note 3).
3. Once the annealing reactions have cooled to room temperature, spin briefly in a microcentrifuge to collect the contents at the bottom of the tube. Add the following components in the order listed (see Note 4): 5 µL sterile, deionized H2O; 3 µL of 10x synthesis buffer; 5–10 U T4 DNA polymerase; and 1–3 U T4 DNA ligase.
4. Incubate the reaction at 37°C for 90 min. Incubation times longer than 90 min are not recommended, because template degradation can occur as dNTP levels are depleted.

### 3.3. Step 3: Transformation into BMH71-18mutS Competent Cells

1. Prechill sterile 17 × 100 mm polypropylene culture tubes on ice (the use of microcentrifuge tubes reduces the transformation efficiency twofold because of inefficient heat-shock treatment).
2. Thaw competent BMH71-18mutS cells on ice. Add 1.5 µL mutagenesis reaction to 100 µL competent cells, and mix gently.
3. Incubate cells on ice for 10 min.
4. Heat-shock the cells for 45–50 s in a water bath exactly at 42°C.
5. Immediately place the tubes on ice for 2 min.
6. Add 900 µL room-temperature LB media, without antibiotic, to each transformation reaction, and incubate for 60 min at 37°C, with shaking.
7. Prepare overnight cultures by adding 4 mL LB media to each reaction (5 mL total), then add 100 µL antibiotic selection mix to each 5 mL culture (see Note 5).
8. Incubate overnight (16–20 h) at 37°C, with vigorous shaking (see Note 6).

### 3.4. Step 4: Plasmid DNA Miniprep Procedure

1. Place 3 mL overnight culture into an appropriate tube, and centrifuge at 12,000g for 5 min.
2. Remove the media by aspiration or decantation.
3. Resuspend the pellet by vortexing in 100 µL resuspension buffer.
5. Add 200 µL neutralization solution. Mix by inversion, and incubate on ice for 5 min.
6. Centrifuge at 12,000g for 5 min.
7. Transfer the supernatant to a fresh tube, avoiding the white precipitate.
8. Add 1 vol of TE-saturated phenol:chloroform:isoamyl alcohol (25:24:1). Vortex for 1 min then centrifuge at 12,000g for 2 min.
9. Transfer the upper aqueous phase to a fresh tube, and add 1 vol of chloroform:isoamyl alcohol (24:1). Vortex for 1 min, and centrifuge as in step 8.
10. Transfer the upper aqueous phase to a fresh tube, and add 2.5 vol of ice-cold 100% ethanol. Mix, and incubate on dry ice for 30 min.
11. Centrifuge at 12,000g for 15 min. Wash the pellet with cold 70% ethanol, and dry the pellet under vacuum.
12. Dissolve the pellet in 50 µL sterile, deionized H₂O.
13. Quantitate the DNA by taking a $A_{260}/A_{280}$ absorbance reading (see Note 7).

3.5. **Step 5: Transformation into JM109 and Clonal Segregation**

1. Before beginning the transformation procedure, prepare plates by pouring molten LB agar, containing 7.5 mL/L antibiotic selection mix and 1 mL/L 100 mg/mL ampicillin solution. Alternatively, plates may be prepared by evenly spreading 100 µL the antibiotic selection mix onto 20–25 mL LB agar plates containing 100 µg/mL AMP (see Note 8).
2. Prechill sterile 17 × 100 mm polypropylene culture tubes on ice (the use of microcentrifuge tubes reduces the transformation efficiency twofold because of inefficient heat-shock treatment).
3. Thaw competent JM109 cells on ice.
4. Add 5–10 ng BMH miniprep DNA (or a dilution of the miniprep DNA) to 100 µL competent cells, and mix gently.
5. Incubate cells on ice for 30 min.
6. Heat-shock the cells for 45–50 s in a water bath exactly at 42°C.
7. Immediately place the tubes on ice for 2 min.
8. Add 900 µL of room temperature SOC media, without antibiotic, to each transformation reaction, and incubate for 60 min at 37°C, with shaking.
9. Plate 100 µL transformation reaction onto each of two plates prepared in step 1 above. Incubate the plates at 37°C for 12–14 h. If cells are highly competent, it may be necessary to plate less than 100 µL, in order to obtain isolated colonies.
10. The GeneEditor mutagenesis system generally produces 60–90% mutants; therefore, colonies may be screened by direct sequencing. Engineering a restriction site into the mutagenic ON, if possible, may be useful to aid in screening. Assuming that greater than 60% mutants are obtained, screening five colonies will give greater than 95% chance of finding the desired mutation. Continued growth of the mutants is not necessary in the presence of the cephalosporin antibiotic selection mix, because the triple mutation in β-lactamase is stable. The authors do, however, recommend further outgrowth of the mutants in AMP, for maintenance of the plasmid DNA.
11. Notes 9–12 contain suggestions for troubleshooting the system, some of which have been observed during the development of the GeneEditor system.

4. **Notes**

1. The mutagenic ON and the selection ON must be complementary to the same strand of DNA, in order to achieve coupling of the antibiotic resistance to the desired mutation. Table 1 shows the sequence of the selection ON for the coding strand of the β-lactamase gene. This ON, or its complement, may be used, depending on the orientation of the cloned insert to be mutagenized. If the orientation of the cloned insert is not known, two separate mutagenesis reactions may be prepared, using each selection ON. Only one reaction will generate the target mutation.
The length and base composition of the mutagenic ON will depend on the nature of the desired mutation and the sequence of the template DNA. For single-base-pair substitutions, insertions, or deletions, a mutagenic oligonucleotide of about 20 bases is sufficient if the region of mismatch is located near the center. Larger mutations, particularly, large insertions or deletions, may require an ON having larger regions of complementarity on either side of the mismatched region. We have successfully used an ON of 90 bp to create a 50-bp insertion. This would allow for 20 perfectly matched bases on either side of the region of mismatch. However, the use of particularly large mutagenic ONs may decrease overall mutagenesis efficiency, because of formation of secondary structures within the ON.

To stabilize annealing between the ON and template DNA, and promote extension by T4 polymerase, the 3’ end of the ON should end with a G or a C nucleotide. A significant increase in the number of mutants is observed when ONs are phosphorylated. The authors recommend 5’ phosphorylation of both the selection ON, as well as any mutagenic ON used with this system.

2. Preparation and storage of the antibiotic selection mix:
   a. All three antibiotics are light-sensitive, and should be stored in the dark (i.e., foil-wrapped), both as a solution and in powder form.
   b. Powders should be stored at 4°C, and solutions at –20°C.
   c. All three antibiotics are members of the penicillin family of antibiotics, and as such have the potential to cause an allergic reaction in individuals who are sensitive to penicillin. The powders should be handled in a hood.
   d. The antibiotic selection mix should be filter-sterilized with a 0.22-µ filter, prior to use.
   e. The antibiotic selection mix is sensitive to freeze–thaw cycling. The authors recommend that the solution be aliquotted into single-use volumes of 1–2 mL and stored at –20°C.

3. Annealing conditions will vary for each mutagenic ON, and may need to be determined empirically. In general, longer ONs and G–C-rich ONs may require higher annealing temperatures; shorter ONs or A–T-rich ONs may require lower annealing temperatures. A slow cooling of the annealing reaction has been observed to give a higher overall mutagenic efficiency. The amount of ON used in the annealing reaction has been optimized for the selection ON, as well as for a number of mutagenic ONs. A 12.5:1 ON:template molar ratio for the mutagenic ON, and a 2.5:1 ON:template molar ratio for the selection ON are recommended for a typical reaction.

4. Add the components exactly in the order listed. Addition of the polymerase in the absence of dNTPs (in the synthesis buffer) can induce exonuclease activity associated with the polymerase, and result in degradation of the template.

5. Thaw the antibiotic selection mix thoroughly, and mix well, before use. Aliquot the thawed material into 1–2-mL amounts prior to refreezing, to avoid freeze–thaw cycles. Do not add greater than 100 µL antibiotic selection mix to the 5 mL overnight culture. Unlike AMP, the antibiotics in this mix can inhibit growth of resistant cells, when provided 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.

6. BMH71-18mutS cells grow very slowly, and therefore require longer incubation times than most E. coli strains. The overnight culture may take longer than 16–20 h to reach density. Optimal growth rate can be attained by maximizing aeration with vigorous shaking and slanting the tubes to increase surface area. BMH71-18mutS cells tend to aggregate when grown in the presence of the antibiotic selection mix and often settle at the bottom of the culture tube.

7. Limit the amount of DNA used in the second transformation reaction to a maximum of 10 ng, to avoid cotransformation of cells with both wild-type and mutant plasmids. The authors therefore recommend that the DNA miniprep be quantitated by measurement of $A_{260}$.

8. Pouring plates, containing the antibiotic selection mix, reduce edging effects that are often seen when plates are spread with the antibiotic mix. Uneven spreading may result in the growth of wild-type cells in areas with low antibiotic concentration, and, alternatively, in inhibition of growth of the mutant cells in areas of high antibiotic concentration. Poured plates should be prepared ahead of time and used within 1 wk when stored at 4°C.

9. **Problem:** No growth in the BMHmutS overnight culture. **Possible causes and suggestions:** β-Lactamase expression may be too low to overcome the effects of the antibiotics. This has been seen when using low-copy-number plasmids. Decrease the amount of antibiotic in the overnight culture to 50 μL instead of 100 μL. DNA used in the transformation is derived from an hsd modification minus strain, which is restricted by BMHmutS. Use DNA grown in a modification (+) K12 strain. Do not use strains such as HB101, MN522, or BL21 (E. coli B strain). Low competency of BMHmutS cells. Use only high-efficiency competent cells. Check competency by plating only on AMP.

10. **Problem:** No JM109 colonies. **Possible causes and suggestions:** Excessive amount of cephalosporin antibiotic selection mix used: Try using 50 μL instead of 100 μL on selective plates. Low competency of JM109 cells: Use only high-competency cells. Check competency by plating only on AMP.

11. **Problem:** JM109 antibiotic-resistant colonies, but low mutation frequency. **Possible causes and suggestions:** Co-transformation of JM109 cells with both wild-type and mutant plasmids. Do not use more than 10 ng DNA in the transformation reaction. Quantitate DNA in the miniprep by measuring the $A_{260}$ and dilute the DNA, if necessary.

12. **Problem:** JM109 antibiotic resistant colonies, but no mutations. **Possible causes and suggestions:** Mutagenic ON is not annealed to the same strand as the selection ON. Recheck the orientation of the cloned insert. Repeat the mutagenesis using the complementary selection ON. Inadequate annealing of mutagenic ON to template DNA. Secondary structure in cloned insert or mutagenic ON. Prepare template as ssDNA, and/or redesign mutagenic ON. Antibiotic selection mix is no longer active: Check for activity by plating JM109 cells transformed with an Amp$^\gamma$ plasmid on plates spread with 100 μL selection mix. If the selection
mix is active, no colonies should be obtained. Remake fresh antibiotic selection mix, if necessary.

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References


