6

Production of Randomly Mutated Plasmid Libraries Using Mutator Strains

Annalee W. Nguyen and Patrick S. Daugherty

1. Introduction

A variety of methods have been developed for random mutagenesis of genes and whole plasmids to generate genetic diversity for directed evolution experiments *(1)*. In particular, bacterial strains exhibiting unusually high rates of spontaneous DNA mutagenesis, or mutator strains, can be used to generate large, diverse plasmid libraries. Propagation of plasmids within relatively stable mutator strains has been shown to provide a rich spectrum of single base substitutions, insertions, and deletions throughout the entire plasmid *(2)*. A significant advantage of using mutator strains to create random libraries is the accessibility of the procedure. Specialized equipment or cloning techniques are not required. Researchers can potentially create a large random library in two or three days, with minimal effort or prior experience in recombinant DNA techniques.

Mutator strains are typically characterized by their genetic deficiencies in DNA proofreading and editing machinery. The most commonly occurring deficiencies involve mutations in the *mutD*, *mutS*, and *mutT* genes. Specifically, *mutD* mutations can hinder the 3'-5' exonuclease activity of DNA polymerase III, thereby preventing repair of incorrectly incorporated bases *(3)*. The resulting mutations are predominantly transitions (85%), but also include transversions (10%), and frameshifts (5%) *(2,4)*. In minimal media, *mutD* strains exhibit a mutation rate 10 to 100 times that of wild-type, while cultures in rich media have up to 10^3 to 10^5-fold increase in mutation rate over wild-type *(2)*. The *mutS* mutation disables DNA mismatch repair, resulting in transitions and transversions *(5)*. Finally, *mutT* mutations prevent the degradation of 8-oxodGTP in mismatches involving A:G *(6)*, resulting in mainly AT-CG transversions *(2)*. While many bacterial strains with *mutD* mutations have been

From: *Methods in Molecular Biology, vol. 231: Directed Evolution Library Creation: Methods and Protocols*
Edited by: F. H. Arnold and G. Georgiou © Humana Press Inc., Totowa, NJ

used for mutagenesis (e.g., GM4708 *[7]*, CC954 *[8]*, mutD5-FIT *[9]*), the widely used and commercially available mutator strain, XL1-RED (Stratagene; La Jolla, CA) includes *mutD*, *mutS*, and *mutT* mutations. The combination of these three alleles elevates mutagenesis rates to on average 0.5 mutations per kb after 30 generations of growth in XL1-RED ***(10)***.

While mutator strains provide significant advantages in some applications, they possess a few notable drawbacks. First, the mutator phenotype is intrinsically unstable and must be monitored. The mutations that cause higher frequency mutagenesis are also responsible for slow growth rates of the mutator strains (e.g., doubling time of about 90 min ***[10]***). Thus, clones with reduced mutator phenotypes will quickly overgrow a culture, thereby preventing further mutagenesis. Second, multiple days of growth are required for more than a couple of mutations per gene. High-frequency mutagenesis may be required for faster evolutionary improvement ***(11)***, in which case, PCR-based mutagenesis is less time consuming. Lastly, the inability to target mutations within a single gene may present problems in evolutionary selections where changes in promoter sequence and the plasmid origin may result in increases in protein production, rather than improvement of the activity of interest ***(10)***.

The following protocol outlines the basic procedure to create a library using a bacterial mutator strain.

2. Materials

1. *E. coli* mutator strain (frozen stock or heat shock competent).
2. M9 minimal medium: 1X M9 salts, pH 7.4, 2 m*M* $MgSO_4$, 100 μ*M* $CaCl_2$, 0.4% D-(+)-glucose, 5 μg/mL thiamine-HCl, 1% casamino acids.
3. M9 minimal medium plates: M9 minimal medium plus 15 g/L agar.
4. Plasmid DNA including the gene of interest (*see* **Note 4**).
5. Antibiotic.
6. Chemical inducer.
7. β-mercaptoethanol.
8. SOC media.
9. Luria Bertani (LB) Broth.
10. Sterile plastic loop or spatula.
11. Mini-prep kit (Qiagen; Valencia, CA), or equivalent.
12. 10 m*M* Tris-HCl, pH 8.5.
13. V Series nitrocellulose filter discs, 0.025 μm pore size (Millipore; Bedford, MA).
14. 0.1 cm gap electroporation cuvet and electroporation apparatus.
15. Electrocompetent cells (non-mutator).
16. Sequencing primer.

3. Methods

The following methods describe 1) the preparation of competent mutator cells, 2) the transformation of the mutator strain with a plasmid, 3) the growth

of the mutator strain resulting in mutagenesis, 4) the transfer of the mutated plasmid to a stable strain, and 5) the initial analysis of the library.

3.1. Preparing Competent Cells

1. Streak mutator strain to M9 minimal medium plates and grow at 37°C to obtain single colonies (*see* **Note 1**).
2. After 36 h, colonies will be rough-edged and various sizes. Choose approximately 20 colonies at random for inoculation of a single 400 mL M9 minimal medium culture.
3. Grow the 400 mL mutator strain culture to an OD_{600} of 0.35–0.38.
4. Make heat shock competent cells according to the *Current Protocols in Molecular Biology* procedure ***(12)***.

3.2. Transformation of Mutator Strains (see Note 2)

1. Thaw 50 μL heat shock cells per transformation on ice.
2. Pre-heat SOC to 42°C.
3. For each transformation, aliquot 50 μL of cells to a 1.5 mL eppendorf tube and place on ice (*see* **Note 3**).
4. Add β-mercaptoethanol to the cells to a final concentration of 25 m*M*.
5. Gently swirl the tube to mix and incubate cells on ice for 10 min.
6. Add 10–50 ng of plasmid DNA (*see* **Note 4**) in less than 5 μL to the cells and incubate the mixture on ice for approximately 30 min.
7. Transfer the tubes to a 42°C water bath for 45 s.
8. Incubate the tubes on ice for 2 min.
9. Add 1 mL of SOC, pre-warmed to 42°C, to each tube and transfer the entire contents to a culture tube. Shake culture (250 rpm) at 37°C for 1 h.
10. Plate 200 μL aliquots of each transformation on LB plates with antibiotic and repressor appropriate to the plasmid.
11. Incubate the plates at 37°C for 30 h.

3.3. Propagation of the Mutator Strains

1. More than 100 colonies should be present on the plate containing 200 μL of transformation culture. Add 1–2 mL of LB to the 200 μL plate and scrape the colonies from the agar with a sterile plastic loop or spatula. Resuspend the cells in LB on the plate by pipetting up and down. Transfer the cell suspension to 10 mL of LB.
2. Measure the OD_{600} of the cell mixture and dilute the cells in 200 mL LB, with appropriate antibiotics, to a calculated OD_{600} of 0.0005 (*see* **Note 5**).
3. Monitor the OD_{600} of the culture to determine the number of generations the cells have been grown (*see* **Note 6**). This is determined using the equation:

$$N = N_0 \times 2^n$$

where N is the final number of cells, N_0 is the initial number of cells and n is the number of generations.

4. The culture should be rediluted while it is still in log phase to minimize overgrowth of non-mutator cells. An appropriate point for dilution is at an OD_{600} between 2.0 and 2.5 (approx 12 generations). Redilute the culture to a calculated OD_{600} of 0.0005 in 200 mL of LB with antibiotic and repressor.
5. Repeat dilution and growth of the cells until the desired number of generations is reached (fewer than 35 generations is recommended). To obtain a high yield of plasmid DNA, adjust the culture volume and dilution factor to ensure that the culture will be near saturation when the cells are harvested.

3.4. Transfer of the Library to a Stable Strain

1. After growth to the desired number of generations, prepare a plasmid DNA miniprep using 3 mL of culture, and elute the plasmid into 50 μl of 10 m*M* Tris-HCl buffer, pH 8.5 (*see* **Note 7**).
2. Place a nitrocellulose filter "shiny side up" on sterile water in a petri dish. Place 5 μL of the isolated supercoiled plasmid on the filter and leave undisturbed for 1–2 h to desalt the DNA.
3. The volume of the droplet containing plasmid will increase over the desalting period. Remove 5 μL of DNA from the filter and place in a 1.5 mL eppendorf tube on ice.
4. Chill an electroporation cuvet (0.1 cm gap) on ice.
5. Pre-heat SOC to 37°C.
6. Thaw 35 μL of electrocompetent cells on ice and add to the tube containing the desalted DNA.
7. Transfer the cell/DNA mixture to the chilled electroporation cuvet and remove any bubbles by gently tapping the bottom of the cuvette on the counter about 5 times. Leave the cuvet on ice.
8. Set the electroporation apparatus to 100 Ω, 50 μF, and 1.8 kV. Wipe off any water on the sides of the cuvet and place it in the electroporation chamber. Pulse the cells. The time constant should be near 5.0 ms.
9. Immediately after electroporation, rinse the cuvet twice with 0.5 mL SOC, prewarmed to 37°C. After each rinse, transfer the contents of the electroporation cuvet to a culture tube.
10. Incubate the transformed cells at 37°C with 250 rpm shaking for 1 h.

3.5. Analysis of Library

3.5.1. Size

1. Once the transformation has recovered for 1 h, make a 1:1000 dilution in 1 mL of SOC. Plate 100 μL and 10 μL aliquots of the 1:1000 dilution on LB plates with antibiotic and repressor. Add 100 μL of SOC to the 10 μL aliquot before spreading to ensure even dispersal of the smaller culture volume.
2. Grow overnight at 37°C.
3. Count the number of colonies on each plate and calculate the total library size represented in the transformation.

3.5.2. Diversity (This step is optional, but recommended)

1. Depending on the expected mutational load, individually culture 10–30 clones (isolated from plates in **Subheading 3.5.1.**) in 5 mL of LB with antibiotic and repressor.
2. Mini-prep each clone and sequence with an appropriate sequencing primer.
3. Analyze sequencing data to obtain the mutation rate (mutations/gene, % mutated).

3.5.3. Screening/Selection

3.5.3.1. PLATES

1. After 1 h of recovery, the transformed library may be directly plated on appropriate growth or selection media (with inducer) and incubated as required.

3.5.3.2. LIQUID

1. Add the remaining transformation mixture to 10 mL of LB with antibiotic (non-inducing conditions) for overnight growth.
2. Once the culture has reached saturation, subculture 1:100 in appropriate media with antibiotic and grow for 2–3 h, then induce as appropriate.

4. Notes

1. The *mutD* mutation is suppressed when grown on minimal media (owing to the absence of thymidine *[2]*). Any cell culture should be carried out on minimal media prior to introduction of the target plasmid to minimize the probability of loss of the mutator genotype.
2. The transformation protocol is adapted from literature provided by Stratagene with the purchase of XL1-Red cells.
3. Heat shock protocols often recommend the use of 100 μL cells and Falcon 2059 polypropylene tubes, but the authors have obtained high-efficiency transformations using 50 μL cells and typical 1.5 mL polypropylene eppendorf tubes.
4. The *mutD* mutation affects only DNA polymerase III, and the rate of mutagenesis is significantly lower when colE1-type origin plasmids are used. ColE1 origins of replication rely on DNA polymerase I for replication and should not be used for efficient mutagenesis of the target gene *(8)*.
5. The volume and dilution factor may be adjusted for convenience, but maintenance of a high level of library diversity is important. Each dilution will reduce the diversity of the library by selecting only a small portion of the entire population for further propagation. The guidelines described here involve further growth of approximately 10^8 cells per dilution.
6. Appropriate dilutions must be made to keep the OD readings in the range that correlates linearly with concentration. Normally this range is Abs 0.01 to 1.
7. If a higher mutational frequency is desired, the mini-prepped DNA can be retransformed to the mutator strain by repeating **Subheading 3.2–3.4., step 1** as required.

References

1. Brakmann, S. (2001) Discovery of superior enzymes by directed molecular evolution. *Chembiochem* **2,** 865–871.
2. Cox, E. C. (1976) Bacterial mutator genes and the control of spontaneous mutation. *Annu. Rev. Genet.* **10,** 135–156.
3. Miller, J. H. (1998) Mutators in *Escherichia coli. Mutat. Res.* **409,** 99–106.
4. Schaaper, R. M. (1988) Mechanisms of mutagenesis in the *Escherichia coli* mutator *mutD5*: role of DNA mismatch repair. *Proc. Natl. Acad. Sci. USA* **85,** 8126–8130.
5. Hsieh, P. (2001) Molecular mechanisms of DNA mismatch repair. *Mutat. Res.* **486,** 71–87.
6. Fowler, R. G. and Schaaper R. M. (1997) The role of the *mutT* gene of *Escherichia coli* in maintaining replication fidelity. *FEMS Microbiol. Rev.* **21,** 43–54.
7. Palmer, B. R. and Marinus M. G. (1991) DNA methylation alters the pattern of spontaneous mutation in *Escherichia coli* cells (*mutD*) defective in DNA polymerase III proofreading. *Mutat. Res.* **264,** 15–23.
8. Stefan, A., et al. (2001) Directed evolution of beta-galactosidase from *Escherichia coli* by mutator strains defective in the 3'→5' exonuclease activity of DNA polymerase III. *FEBS Lett.* **493,** 139–143.
9. Coia, G., et al. (1997) Use of mutator cells as a means for increasing production levels of a recombinant antibody directed against Hepatitis B. *Gene* **201,** 203–209.
10. Greener, A., Callahan, M., and Jerpseth, B. (1997) An efficient random mutagenesis technique using an *E. coli* mutator strain. *Mol. Biotechnol.* **7,** 189–195.
11. Daugherty, P.S., et al. (2000) Quantitative analysis of the effect of the mutation frequency on the affinity maturation of single chain Fv antibodies. *Proc. Natl. Acad. Sci. USA* **97,** 2029–2034.
12. Seidman, C.E., Struhl, K., Sheen, J., and Jessen, T. (1997) Introduction of Plasmid DNA into Cells, in *Current Protocols in Molecular Biology* (Ausubel, F.M., Brent, R., Kingston, R. E., et al., eds.), John Wiley & Sons Inc. Hoboken, NJ, pp. 1.8.1–1.8.2.