CHAPTER NINETEEN

Site-Directed Mutagenesis

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Contents

Abstract

Site-directed mutagenesis is a PCR-based method to mutate specified nucleotides of a sequence within a plasmid vector. This technique allows one to study the relative importance of a particular amino acid for protein structure and function. Typical mutations are designed to disrupt or map protein–protein interactions, mimic or block posttranslational modifications, or to silence enzymatic activity. Alternatively, noncoding changes are often used to generate rescue constructs that are resistant to knockdown via RNAi.

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1. THEORY

Forward and reverse primers are centered on the desired base change(s) and overlap completely in both directions. Extension of these primers around the entire plasmid creates a copy of the template with the inserted mutation. This mutated plasmid then serves as a template for further PCR amplification. Since plasmids grown in *E. coli* are *dam* methylated, the restriction endonuclease Dpn I, which is specific to methylated and hemimethylated DNA, is used to digest the original parent template. The remaining plasmid is then transformed into *E. coli* for selection and DNA isolation.

2. EQUIPMENT

PCR Thermocycler
Water bath (37 and 42 °C)
Shaking incubator
Incubator
Micropipettors
Micropipettor tips
0.2-ml thin-walled PCR tubes
1.5-ml polypropylene tubes
15-ml sterile snap-cap tubes
Petri plates

3. MATERIALS

PCR primers
Plasmid DNA (with desired sequence to be mutated)
dNTPs (100 mM each of dATP, dCTP, dGTP, and dTTP)
10× High-fidelity DNA polymerase buffer
High-fidelity *Pfu*-based DNA polymerase
Ultrapure water
DpnI
Chemically competent cells (e.g., XL 10-Gold ultracompentent cells, Stratagene)
Lysogeny Broth (LB media)
Petri plates

3.1. Solutions & buffers

Step 1 10 mM dNTP mix

Component	Final Concentration	Stock	Amount
dATP	10 mM	100 mM	100 µl
dCTP	10 mM	100 mM	100 µl
dGTP	10 mM	100 mM	100 µl
dTTP	10 mM	100 mM	100 µl

Add purified water to 1 ml

4. PROTOCOL

4.1. Preparation

PCR primers must be designed and synthesized prior to starting this protocol (see Explanatory chapter: PCR -Primer design).

Design forward and reverse primers containing the desired mutation(s). Primers should be 25–45 nucleotides long leaving at least 10–15 nucleotides of perfect matching sequence at both ends. In general, more point mutations being made will require longer primers. For example, a single base change probably requires only 25-mers. If three bases are to be mutated, the primers should be about 35–38 bases in length. Ideally, terminate primers with C or G and maintain a minimum GC content of 40%. Additional primers are needed for sequencing through the insert in order to verify mutations made and check for nonspecific mutations.

4.2. Duration

Preparation	Variable
Protocol	5–6 h + overnight incubation
	2 h + DNA sequencing

See Fig. 19.1 for the flowchart of the complete protocol.

5. STEP 1 SETTING UP AND RUN THE PCR

5.1. Overview

Set up and run the PCR using the primers containing the mutation(s) and a high-fidelity thermostable DNA polymerase.

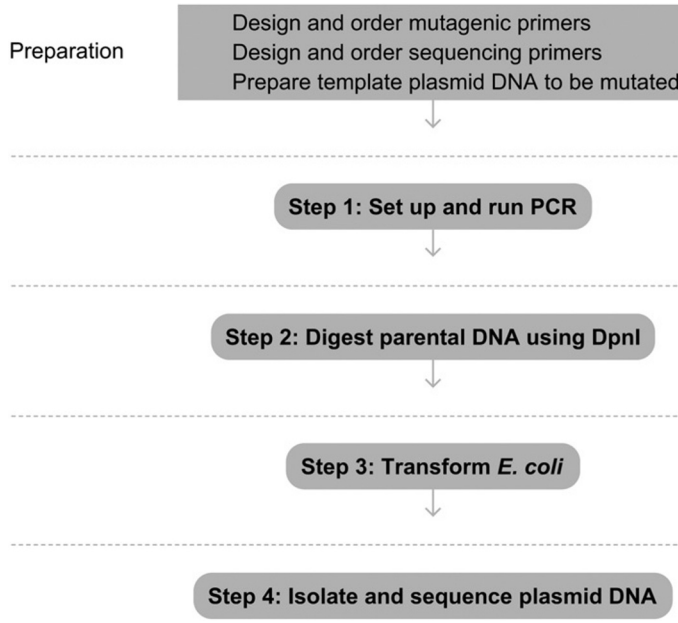

Figure 19.1 Flowchart of the complete protocol, including preparation.

5.2. Duration

about 2 h

1.1 Set up the reaction mixture on ice. Add to a 0.2-ml thin-walled PCR tube:

1 µl template (10 ng μl^{-1})
5 µl 10× Polymerase Buffer
2.5 µl Forward primer (50 ng μl^{-1})
2.5 µl Reverse primer (50 ng μl^{-1})
1 µl dNTP mix (10 mM)
37.5 µl purified water
0.5 µl DNA Polymerase

1.2 Run the PCR with the following cycling parameters:

At 98 °C for 30 s (Initial denaturation of the template)
Repeat for 18 cycles:
At 98 °C for 10 s (Denature)
At 60 °C for 30 s (Anneal)
At 68 °C for 1 min per kb length of entire plasmid (Extension)

5.3. Tip

Add the enzyme last, immediately before starting the thermocycler. If setting up more than one reaction, make a master mix and dispense into tubes already containing the specific primers and/or template.

5.4. Tip

The annealing temperature can vary from 55 to 60 °C according to the primer T_m.

5.5. Tip

The extension temperature for Taq DNA polymerase is 72 °C. However, the recommended extension temperature for many high-fidelity polymerases is 68 °C. Check the product information sheet of the high-fidelity thermostable DNA polymerase being used.

See Fig. 19.2 for the flowchart of Steps 1 and 2.

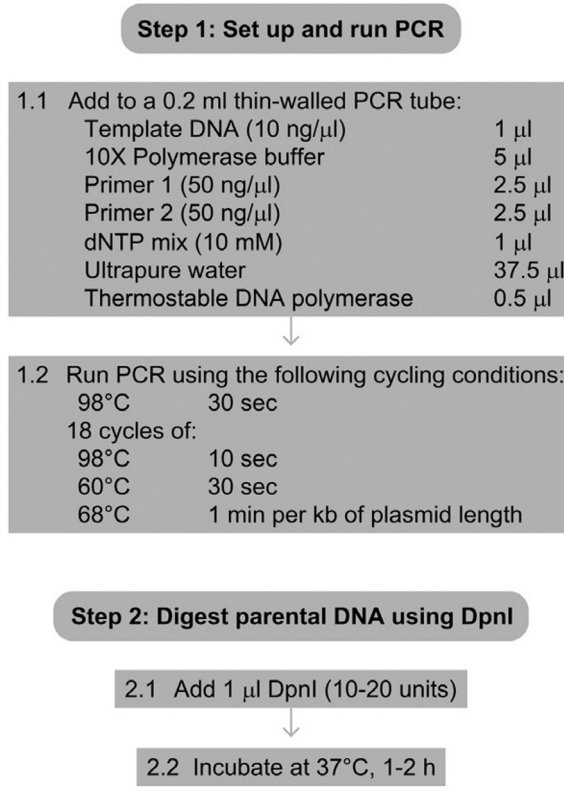

Figure 19.2 Flowchart of Steps 1 and 2.

6. STEP 2 DIGESTION OF TEMPLATE DNA

6.1. Overview

Parental strands of DNA will be digested using DpnI, which cuts methylated and hemimethylated DNA.

6.2. Duration

1–2 h

2.1 Add 1 μl of Dpn I restriction enzyme (10–20 units).
2.2 Incubate at 37 °C for 1–2 h.

7. STEP 3 TRANSFORMATION INTO CHEMICALLY COMPETENT *E. COLI*

7.1. Overview

The mutated plasmid is transferred into chemically competent *E. coli* via heat-shock (see Transformation of Chemically Competent *E. coli*, or alternatively see Transformation of *E. coli* via electroporation if you are using electrocompetent cells). The transformed bacteria are then grown on media containing the appropriate antibiotic for the plasmid.

7.2. Duration

2 h + overnight incubation

3.1 Let the competent cells thaw on ice.
3.2 Add 5 μl of the reaction to 50 μl of chemically competent cells. Mix gently to avoid damaging the cells (do not pipette up and down).
3.3 Incubate on ice for 30 min.
3.4 Heat shock at 42 °C for 1 min.
3.5 Incubate tube on ice for 2 min.
3.6 Add 1 ml of room temperature LB (or other medium, e.g., SOC).
3.7 Incubate at 37 °C for 1 h with shaking (100–150 rpm).
3.8 Centrifuge bacteria at low speed (~7500 rpm) for 2 min.
3.9 Aspirate all but 100 μl of the LB.
3.10 Carefully resuspend the pellet and spread all the 100 μl evenly onto a selective plate.
3.11 Incubate overnight at 37 °C with the plate turned upside down.

7.3. Tip

XL 10-Gold ultracompetent cells are recommended for their high transformation efficiency.

7.4. Tip

Check the supplier's instructions for the optimal length of time for the heat shock.

See Fig. 19.3 for the flowchart of Steps 3 and 4.

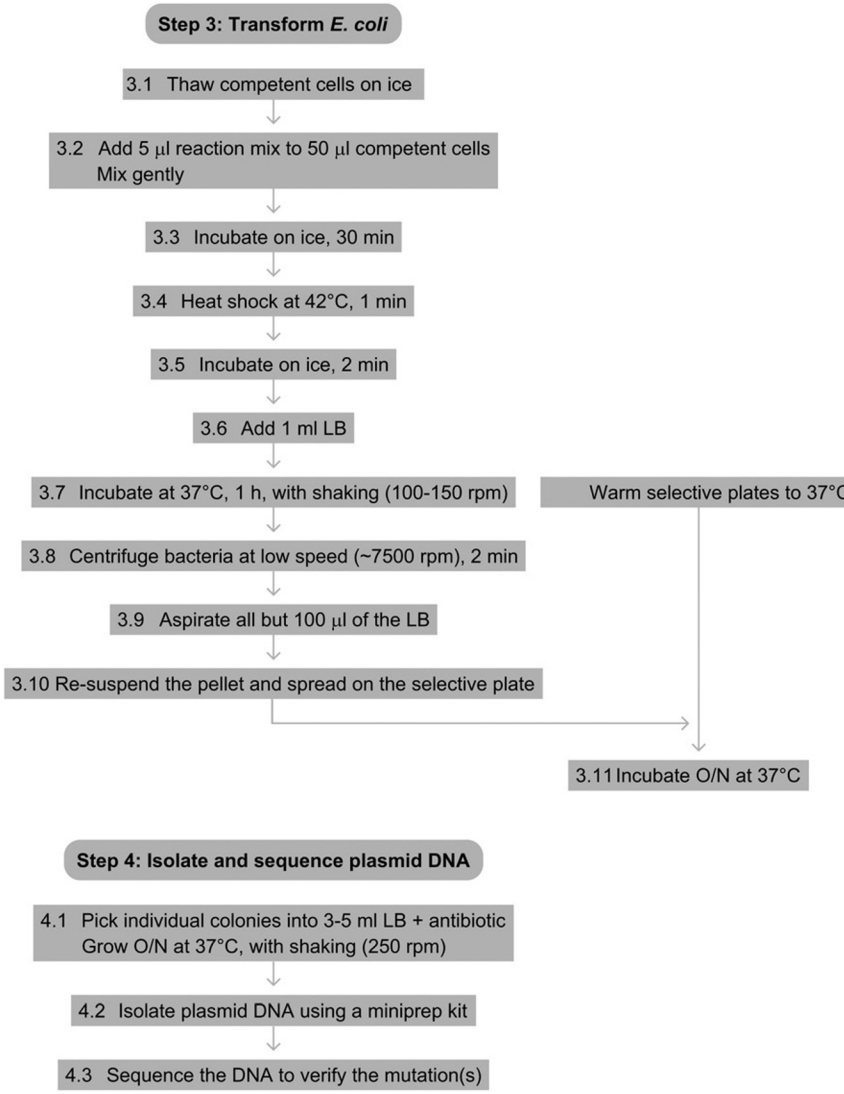

Figure 19.3 Flowchart of Steps 3 and 4.

8. STEP 4 COLONY SCREENING

8.1. Overview

Pick single colonies, grow overnight, and isolate plasmid DNA using a miniprep kit (alternatively see Isolation of plasmid DNA from bacteria). Sequence the DNA to verify that the mutations have been made.

8.2. Duration

15 min + overnight growth
1 h + DNA sequencing

4.1 Pick single colonies into 3–5 ml LB + antibiotic in a 14-ml snap-cap tube. Grow at 37 °C overnight with shaking (250 rpm).

4.2 Isolate plasmid DNA using a plasmid miniprep kit according to the manufacturer's instructions.

4.3 Sequence the DNA to verify that appropriate base changes have been made.

8.3. Tip

Since PCR is used to amplify the whole plasmid, it is important to sequence the entire insert in order to check for PCR-induced mutations. Using a high-fidelity polymerase will minimize this possibility. Alternatively, one could sequence the smaller region containing the desired mutations and subclone this region back into the original vector.

REFERENCES

Referenced Protocols in Methods Navigator
Explanatory chapter: PCR -Primer design
Transformation of Chemically Competent *E. coli*
Transformation of *E. coli* via electroporation
Isolation of plasmid DNA from bacteria