The Fleischman Lab

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Title: Site-Directed Mutagenesis via QuikChange II XL

Introduction

Site-directed mutagenesis is a useful tool for introducing mutations into specific regions of plasmid DNA. We frequently use this technique to generate plasmids coding for mutant proteins (e.g. $Jak2^{V617F}$, $Calr^{Ins5}$, $Calr^{Del52}$), but it may also be used for other purposes, such as adding tags (e.g. HA or FLAG) or restriction enzymes.

The protocol described here has been optimized for our lab. The mutagenesis reaction volume is halved to reduce costs, and the reaction and cycling parameters have been altered to improve yield for large plasmids. To save time, we use Z-competent (a.k.a. Mix & Go) DH5α cells as opposed to the XL-10 Gold cells supplied with the kit. While we have described the Z-competent cell method in this protocol, other *E. coli* strains and transformation methods may be used (e.g. some of our plasmids require the use of ccdB Survival cells). As validation of the mutated plasmid is extremely important, we have included brief procedures for plasmid propagation and sequencing.

Please note that insertions and deletions are limited to roughly 20-25 bp. For larger indels, we recommend sequential mutagenesis reactions. Additionally, the primers designed with the QuikChange tool may not be specific enough for all sequences, and even high-fidelity polymerases such as Pfu Ultra have the potential to introduce unintended base pair changes. For these reasons, mutated plasmids must be screened for unintended mutations by fully sequencing the gene of interest.

For more information, especially if you are new to site-directed mutagenesis, please read the full protocol for the QuikChange II XL kit:
www.agilent.com/cs/library/usermanuals/public/200521.pdf

To design primers for site-directed mutagenesis, use the QuikChange primer design tool:
www.genomics.agilent.com/primerDesignProgram.jsp

Materials

1. Plasmid DNA
2. Site-directed mutagenesis primers (100 ng/µl), designed using the QuikChange primer design tool at http://www.genomics.agilent.com/primerDesignProgram.jsp
3. Molecular-grade water
4. Agilent QuikChange II XL site-directed mutagenesis kit
5. PCR tubes or tube strips
6. Thermal cycler
7. Competent bacterial cells (Z-competent or heat shock)
8. 1.5 ml microcentrifuge tubes
9. LB-agar plates with appropriate antibiotic(s)
10. LB broth with appropriate antibiotic(s)
11. Sterile 14 ml culture tubes
12. Miniprep kit (we use Zymo's ZR Plasmid Miniprep-Classic kit)
13. Sequencing primers (10 µM)
14. 37°C incubator
15. 37°C shaking incubator

<table>
<tr><th>Protocol</th><th>A.</th><th>Notes</th></tr>
<tr><td>1.</td><td>Prepare reactions site-directed mutagenesis:
<table>
<tr><th>Reagent</th><th>Volume</th></tr>
<tr><td>10x reaction buffer</td><td>2.5 µl</td></tr>
<tr><td>F primer (100 ng/µl)</td><td>0.625 µl</td></tr>
<tr><td>R primer (100 ng/µl)</td><td>0.625 µl</td></tr>
<tr><td>dNTP mix</td><td>0.5 µl</td></tr>
<tr><td>Quiksolution</td><td>1.5 µl</td></tr>
<tr><td>H_2O</td><td>16.25 µl</td></tr>
<tr><td>Plasmid DNA (10 ng/µl)</td><td>2.5 µl</td></tr>
<tr><td>Pfu Ultra</td><td>0.5 µl</td></tr>
</table></td><td>The Quiksolution (DMSO) thaws very slowly. Remove from the freezer well before preparing the reactions.

Do not allow the dNTP mix or Pfu Ultra to stay out of the freezer for longer than is necessary.</td></tr>
<tr><td>2.</td><td>Flick the tubes to mix and spin down briefly. Place in the thermal cycler and set the cycling parameters as follows:
<table>
<tr><td>1x</td><td>95°C</td><td>2.5 min</td></tr>
<tr><td>18x</td><td>95°C</td><td>1 min</td></tr>
<tr><td></td><td>60°C</td><td>1 min</td></tr>
<tr><td></td><td>68°C</td><td>2 min/kb</td></tr>
<tr><td>1x</td><td>68°C</td><td>7 min</td></tr>
<tr><td>1x</td><td>4°C</td><td>∞</td></tr>
</table></td><td>For small plasmids (<5 kb), extension time may be reduced to 1 min/kb.</td></tr>
<tr><td>3.</td><td>Add 0.5 µl DpnI to each tube. Flick the tubes to mix and spin down briefly. Incubate in the thermal cycler at 37°C for 1 hour.</td><td>Do not allow the DpnI to stay out of the freezer for longer than is necessary.</td></tr>
<tr><td></td><td>B. Transform bacteria (Z-competent method)</td><td></td></tr>
<tr><td>1.</td><td>Pre-heat two LB-agar plates containing the appropriate antibiotic(s) per transformation at 37°C for 1 hour or until agar is warm to the touch.</td><td>Plates should be agar-side up to prevent condensation from dripping onto the agar surface.</td></tr>
<tr><td>2.</td><td>Transfer 2 µl of plasmid to a 1.5 ml microcentrifuge tube and incubate on ice for 15 minutes.</td><td></td></tr>
<tr><td>3.</td><td>Thaw an aliquot of Z-competent DH5α cells on ice. Flick to mix and spin down briefly, then gently pipette up and down 2-3 times to resuspend cells.</td><td>Not all plasmids are compatible with DH5α cells. Be sure you are using the correct strain and transformation method for your plasmid.</td></tr>
<tr><td>4.</td><td>Transfer 25 µl of competent cells to the pre-chilled tube containing the plasmid and incubate on ice for 5 minutes.</td><td>Return unused competent cells to the -80°C freezer.</td></tr>
<tr><td>5.</td><td>Pipette 10 µl of the transformation mixture into the center of a pre-warmed LB-agar plate. Use a sterilized spreader to gently and evenly spread the cells over the entire surface of the plate.</td><td>Sterilize spreader by dipping in 100% EtOH and lighting on fire. Allow to cool for 20 seconds before using.</td></tr>
<tr><td>6.</td><td>Sterilize the spreader and repeat step 5 with the remaining transformation mixture (≈17 µl) on a second plate.</td><td></td></tr>
<tr><td>7.</td><td>Return plates to the incubator. Incubate overnight. Do not incubate more than ~16-18 hours to prevent formation of satellite colonies.</td><td>Plates should be agar-side up to prevent condensation from dripping onto the agar surface.</td></tr>
</table>

	C. Isolate and analyze plasmids	
1.	Prepare LB media containing the appropriate antibiotic(s). Prepare 14 ml culture tubes by adding 3 ml LB+antibiotic per tube.	
2.	Pick one colony per tube by using a sterile 200 µl pipette tip to gently touch the colony. Eject the pipette tip into the culture tube. Cap loosely and incubate in the shaker at 37°C 250 rpm for 8-16 hours or until media becomes cloudy.	*Only pick isolated colonies.* *Longer culture times generally produce more concentrated minipreps.*
3.	Miniprep 1-2 ml of bacterial culture using the Zymo ZR Plasmid Miniprep-Classic kit.	*The protocol is posted above the bacteria bench.*
4.	Measure DNA concentration on the NanoDrop. Prepare sequencing reactions for up to 6 colonies per mutagenesis reaction: Plasmid DNA | 600 ng Primer (10 µM) | 2 µl H_2O | to 12 µl	*Plasmid DNA concentration must be 60 ng/µl or greater for sequencing.* *Choose a sequencing primer that reads the region of your intended mutation.*
5.	Analyze sequencing results for a) successful mutagenesis, and b) absence of unintended base pair changes. If mutagenesis was successful, choose one plasmid for further validation. Prepare sequencing reactions as in step 4 for additional sequencing primers as needed to read the complete region of interest. Ensure no unintended base pair changes were made before using the altered plasmid in downstream applications.	*Tip: Align the sequencing results to the intended sequence using BLAST to search for unintended base pair changes.*