Site-Directed Mutagenesis Using Uracil-containing Double-Stranded DNA Templates and DpnI Digestion

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ABSTRACT

DpnI can cleave fully methylated parental DNA while leaving hemimethylated DNA intact. Based on this observation, we developed a rapid site-directed mutagenesis method using uracil-containing double-stranded DNA (dsDNA) templates and DpnI digestion. 38% mutation efficiency was achieved by DpnI treatment of the mutagenic strand extension reaction, and this was increased to 70-91% when uracil-containing dsDNA templates were used. This compares favorably to the most efficient current methods, but is simpler and does not require the use of single-stranded templates or phage vectors.

PROCEDURE

Uracil-containing plasmid preparation

1. Plasmids are transformed into *E. coli* CJ236 dut- ung- cells to produce uracil-containing dsDNA.

2. The bacteria were grown in LB media supplemented with 0.26µg/ml uridine and 100µg/ml ampicillin.

3. Plasmid DNA was purified using the preferred method.
Mutagenic strand extension

1. 1µg of the mutagenesis primer was phosphorylated using T₄ polynucleotide kinase and ATP.

2. Approximately 50 pM (0.2-0.8 µg) of phosphorylated mutagenesis primer is annealed to 100ng uracil-containing template in a solution containing 20mM Tris-HCl, pH7.5, 10mM MgCl₂ and 50mM NaCl in 20µl volume. Mixtures were heated to 100°C for 3 min and then quickly chilled in an ice water bath for 5 min., followed by incubation at room temperature for 30 min.

3. Alkali denature method was used to denature the large plasmids (>7-8 kb). 1-2 µg of plasmid in 20 µl volume was mixed with 4 µl of 2M NaOH. The mixture was incubated at room temperature for 10 min and neutralized with 4 µl of 3M NaOAc (pH 4.8). Denatured DNA was precipitated with ethanol, resuspended in H₂O and used immediately or stored at 4°C for later use. For annealing, the plasmid and primers was mixed and incubated at 37°C for 10min and then at room temperature for another 10 min.

4. Second-strand synthesis is performed by adding 3µl of a solution containing 100mM Tris-HCl, pH7.5, 5mM of each dNTP, 10mM ATP, and 20mM DTT, 1µl of T₄ DNA polymerase (3 units), 1µl of T₄ DNA ligase and 5µl ddH₂O (final volume 30 µl). Synthesis is performed for 1-2 hour at 37°C and then incubated at 70°C for ≥5 min to inactivate the enzyme.
**DpnI digestion of mutagenesis reaction and transformation**

1. After mutagenesis strand extension, DpnI is added to the reaction at a concentration of 5-10 units/µg template and incubated at 37°C for 1 hour. Higher concentration of DpnI may non-specifically destroy the newly-synthesized strands.

2. One third of the final reaction is used directly to transform 200µl of competent cells.

3. The final transformation reaction was spread onto LB plates containing 100µg/ml ampicillin and incubated at 37°C overnight.

4. Verify the mutants by sequencing.