Site-Directed Mutagenesis

Moriah Beck[1], Abby Jurgensmeier[1]
[1]Wichita State University

dx.doi.org/10.17504/protocols.io.pf4djqw

Mar 27, 2019

Working

Beck Lab

Abby Jurgensmeier

ABSTRACT

PROTOCOL STATUS

Working

We use this protocol in our group and it is working

MATERIALS

NAME	CATALOG #	VENDOR
PrimeSTAR GXL DNA Polymerase	R050A	
dNTP Mix 10 mM each	#R0191	Thermo Fisher Scientific
DMSO	D8418	Sigma

SAFETY WARNINGS

BEFORE STARTING

Design primers using the following set of guidelines (from Stratagene QuikChange protocol):

The mutagenic oligonucleotide primers for use in this protocol must be designed individually according to the desired mutation. The following considerations should be made for designing mutagenic primers:

- Both of the mutagenic primers must contain the desired mutation and anneal to the same sequence on opposite strands of the plasmid.
- Primers should be between 25 and 45 bases in length, with a melting temperature (Tm) of ≥78°C. Primers longer than 45 bases may be used, but using longer primers increases the likelihood of secondary structure formation, which may affect the efficiency of the mutagenesis reaction.

The following formula is commonly used for estimating the Tm of primers:

$$T_m = 81.5 + 0.41(\%GC) - 675/N - \%mismatch$$

For calculating T_m:

- N is the primer length in bases
- values for %GC and % mismatch are whole numbers

For calculating T_m for primers intended to introduce insertions or deletions, use this modified version of the above formula:

$$T_m = 81.5 + 0.41(\%GC) - 675/N,$$

where N does not include the bases which are being inserted or deleted.

- The desired mutation (deletion or insertion) should be in the middle of the primer with ~10–15 bases of correct sequence on both sides.
- The primers optimally should have a minimum GC content of 40% and should terminate in one or more C or G bases.

Additional Primer Considerations:

The mutagenesis protocol uses 125 ng of each oligonucleotide primer.

To convert nanograms to picomoles of oligo, use the following equation:

$$X \text{ pmoles of oligo} = \frac{\text{ng of oligo}}{330 \times \#\text{ bases in oligo}} \times 1000$$

For example, for 125 ng of a 25-mer: $\frac{125 \text{ ng of oligo}}{330 \times 25 \text{ bases}} \times 1000 = 15 \text{ pmole}$

- Primers need not be 5´ phosphorylated but must be purified either by fast polynucleotide liquid chromatography (FPLC) or by polyacrylamide gel electrophoresis (PAGE). Failure to purify the primers results in a significant decrease in mutation efficiency.
- It is important to keep primer concentration in excess. Stratagene suggests varying the amount of template while keeping the concentration of the primer constantly in excess.

Mix the following reagents in a small PCR tube:

1 5-50 ng template plasmid (typically this is 1 uL of miniprep DNA)
1-2.25 µL forward primer (0.5 µM or 125 ng)*
1-2.25 µL reverse primer (0.5 µM)*
4 µL dNTP mix (stock conc.)
10 µL 5x PrimeSTAR buffer
1 µL PrimeSTAR polymerase (*make sure this is not the 2X PrimeSTAR premix)
1.5 µL DMSO (optional)
Add autoclaved water to mixture to total at 50 µL

*Stock primers (with blue cap) need to be diluted 10 fold before use in PCR

Thermocycling

2 The tubes were put in the thermocycler with an initial denaturation temperature of 98°C for 30 seconds, followed by 16 cycles of denaturation at 98°C for 30 seconds, annealing at 55°C for 60 seconds, and extension at 72°C for 60-75 seconds/kb. A final extension at 72° for 10 minutes was followed by holding at 4°C.

Note: If SDM is unsuccessful, an annealing temperature of 68°C may need to be used.

DpnI Digest to destroy Wildtype plasmid

3 After PCR had been completed, 1 µL of DpnI should be added to the reaction tubes and allowed to incubate overnight at 37°C.

Perform Transformation of Smart cells:

4 Use 50-100 uL of cells per transformation (our lab stocks are stored in these volumes, but take note in your lab notebook of date of stock used and complete name of cells).

5 Remove Smart cells from the –80 °C freezer and place directly into your ice bucket. Thaw on ice for 15 minutes.

6 Add DNA from plasmid to be transformed. For ligations/PCR products use 10-50 uL of PCR reaction per 50-100uL of cells.

7 Incubate cells on ice with the DNA for 30 minutes. In the meantime make sure there is a 42°C water bath/block that has water in it.

Bring ice bucket with cells and a timer over to the water bath. Immerse tube of cells in 42°C bath for 45-60 seconds.

8

9 Remove tube from wate rbath and place directly on ice to recover for 2 minutes.

10 Add 900 uL of sterile LB (no antibiotics) and grow with shaking for 1 hour at 37 °C.

11 Spin cells in tabletop centrifuge for 1 minute at ~2,000 rpm, remove 700 uL of media and resuspend the cell pellet in the remaining media in the tube. Plate this onto the correct antibiotic LB plate.