SITE-DIRECTED MUTAGENESIS

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BACKGROUND

- The site-directed mutagenesis is used to create mutant proteins through substitution, deletion or insertion of one or more amino acids. There exist multiple protocols for site-directed mutagenesis and alternative approach may be used. This protocol is designed for simple substitution/insertion/deletion and no additional kit is required.
- This protocol makes use of complementary primers, and PCR is used to amplify the whole plasmid (Note: this protocol involves whole-plasmid amplification, therefore, high-fidelity polymerase and smaller plasmid size is required).

NOTE

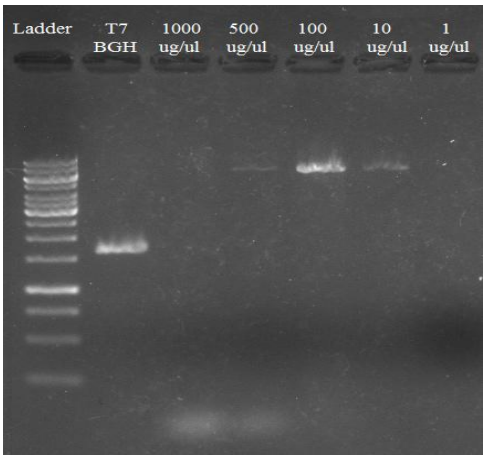
- Elongation time of KOD polymerase must be calculated for individual plasmids (refer to the figure below) (Novagen, 2003).

Enzyme	KOD HiFi DNA Polymerase	*Pfu* DNA Polymerase	*Taq* DNA Polymerase
Species	*Thermocccus kodakaraensis*	*Pyrococcus furiosus*	*Thermus aquaticus YT-1*
Fidelity* (mutation frequency)	0.0035	0.0039	0.013
Elongation rate (bases/second)	106–138	25	61
Processivity** (nucleotide bases)	> 300	< 20	not determined

* Fidelity was measured by the authors as mutation frequency in PCR products using a sensitive blue/white phenotypic assay with a 5.2 kbp lacZ plasmid as template (2).

** Processivity is defined as the number of nucleotides that can be extended in one catalytic reaction by one DNA polymerase molecule.

- Ensure that PCR thermocycler is set for your specific plasmid and primer Tm
- **DO NOT** increase the amount of polymerase. The polymerase stock contains glycerol that prevents efficient PCR



- Primer concentration is critical. Use 1 ul of 100ug/ul primer (1:10 – 1:100 dilutions depending on the primers)
- If you do not see a clear band (as shown above), use T7 and BGH as controls and run a diagnostic
- For diagnostics, please optimize for **primer concentration, thermocycler temperature conditions**.
- If you are getting smears in gel electrophoresis, decrease the elongation time.

EQUIPMENT

- Ice/ice bucket
- Thermocycler
- 50ul PCR tubes
- Plasmid DNA
- Forward and reverse primers
- 10X KOD Buffer
- 2mM dNTPs
- MgSO4
- KOD Polymerase
- Water
- DpnI and buffer

PROTOCOL

1. Prepare ice/bucket. All reagents should stay on ice (DO NOT take out KOD polymerase until the end).
2. Prepare plasmids so that there are 1ng/µl - 10ng/µl (and plasmid concentration should be 100ng/µl). NOTE: Optimize for primer concentrations (ranging from 1:10 to 1:100 dilutions).
3. When the regents are thawed, add the following
    a. 10 ng DNA – 1µl
    b. Primers (forward and reverse) – 1µl each
    c. 10X KOD Buffer - 5µl

   d. 2mM dNTPs - 5µl
   e. MgSO4 – 3µl
   f. Water - 33µl
4. Spin down the tubes for 2 seconds
5. Add 1µl of KOD polymerase
6. Immediately run a PCR using the following thermocycle conditions (Note: Please note that the temperatures suggested below should be changed depending on experiments).
   a. **INITIATION:** 2:00 (e.g. 95C)
   b. **DENATURATION:** (20 cycle) 0:20 (e.g. 95C)
   c. **ANNEALING:** (20 cycle) 0:10 (e.g. around 55C) (NOTE: This step is variable depending on the experiment. Refer to your primer Tm)
   d. **ELONGATION:** (20 cycle) 1:20 (e.g. 70C) (NOTE: The time depends on the length of your plasmid)
   e. **FINAL ELONGATION:** 5:00 (e.g. 70C)
   f. 4C
7. After obtaining the PCR products, prepare DpnI digestion (Note: DpnI is an enzyme that specifically cleaves methylated DNAs)
8. Add the following and incubate at 37C for 2 hours
   a. DpnI - 1µl
   b. DNA (1µg) -1µl
   c. 10X Buffer - 5µl
   d. Water - 43µl
9. Proceed to bacterial transformation stage (refer to Kyle Novakowski's protocol). Once colonies grow, extract their DNA and send it for sequencing

LINKS AND REFERENCES

- www.bowdish.ca/lab
- http://pef.aibn.uq.edu.au/wordpress/wp-content/blogs.dir/1/files/Support/Molecular_Cloning/Manuals/KOD_Hot_Start.pdf