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Project Number	**Type of Document**
900_AX_IMI	Final Report
Title	
Site-Directed Mutagenesis	
Date	**Project Sponsor**
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Project Manager	**Lab/Data Technologist**

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Objective

The objective of this project was to create three different plasmid DNA constructs with three to four amino acid changes in each of the target gene TX-T with the use of site-directed mutagenesis.

Materials

Samples (provided by Client)
Plasmid DNA
Reference sequence of gene
Vector information

Reagents
QuickChange Multi Site-Directed Mutagenesis Kit (Stratagene)
BigDye Terminator Sequencing Kit, V 3.1 (Applied Biosystems Inc.)
CleanSeq Dye Terminator Removal Kit (Agencourt Bioscience Corp.)
QIAprep Spin Miniprep Kit (Qiagen)

Instruments
NanoDrop ND 1000 Spectrophotometer
BioRad C1000 Thermal Cycler
PRISM 3730XL DNA Analyzer (Applied Biosystems Inc.)
Biomek Automation Station (Beckman Coulter, Inc.)

Protocol

1. The amount and purity of the plasmid DNA provided by the client was determined on the NanoDrop ND1000 Spectrophotometer by measuring the absorbance of the DNA sample at 230 nm, 260 nm and 280 nm.

2. The identity of the plasmid DNA was verified by sequencing the insert containing the target gene. The sequences of the primers used to sequence the insert DNA were:

```
    T7:   5'- TAATACGACTCACTATAGGG -3'
   BGH:   5′- TAGAAGGCACAGTCGAGG   -3′
 TX-F1:   5′- GCTGGAGAATGGAGGTGGT  -3′
 TX-F2:   5′- TGGCCCTGGCCATTCACAT  -3′
 TX-F3:   5′- TGGCACTGAGACAATGAGAA -3′
 TX-F4:   5′- TGCCCTGGAATGCTGCTG   -3′
 TX-R1:   5′- ACAGCCAGCACCTCTCCC   -3′
 TX-R2:   5′- CCAGAGCTAGGATCACCA   -3′
 TX-R3:   5′- CTCACCCGCAGTTCTTGAT  -3′
```

3. Three different mutagenesis primers were designed to mutate three or four amino acids.

```
TX-T 1:  5′- CAGGTGAGGGTGCGCATCGAGGGGCGCGTGGCTGGCATTGGG -3′
TX-T 2:  5′- CAGGTGAGGGTGCGCTTGGTGCCGCGCGTGGCTGGCATTGGG -3′
TX-T 3:  5′- CAGGTGAGGGTGCGCGACGAGGTGGACGTGGCTGGCATTGGG -3′
```

4. Point mutations in these constructs were induced simultaneously into the parent plasmid with the QuickChange Multi Site-Directed Mutagenesis Kit.

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5. After PCR amplification, the PCR reaction was treated with the methylation sensitive restriction enzyme DpnI for 1 hr. at 37°C.

6. The reaction was then transformed into XL10-Gold ultracompetent cells, plated LB. ampicillin agar plates, and then Incubated at 37°C for 16 hours

7. Several colonies were then selected and grown overnight at 37_C with shaking in 3 ml SB (Super Broth) media.

8. Plasmid DNA was extracted from the cultured cells using a QIAprep Spin Miniprep Kit as per the manufacturer¢s instructions (Qiagen).

9. The DNA amount and purity was determined on the NanoDrop 1000 Spectrophotometer by measuring the absorbance of the purified DNA sample at 230 nm, 260 nm and 280 nm.

10. The clones were screened for the mutations by sequencing from both strands across the site with the putative mutations.

11. Once a clone was identified to contain the desired mutations, the remainder of the insert was sequenced using insert-specific sequencing primers described in Step 2.

Summary and Interpretation

Three constructs of the TX-T plasmid DNA were created that contained three to four amino acid changes each. Four different amino acids in the SEKS (Ser, Glu, Lys, Ser, in blue) sequence of the wild-type TX-T gene were mutated (in red). In the TX-T 1 construct, three amino acids were changed from serine (S) to Isoleucine (I), lysine (K) to glycine (G), and serine (S) to arginine (R), respectively. In the TX-T 2 construct, four amino acids were changed from serine (S) to leucine (L), glutamic acid (E) to valine (V), lysine (K) to praline (P), and serine (S) to arginine (R), respectively. In the TX-T 3 construct, three amino acids were changed from serine (S) to aspartic acid (D), lysine (K) to valine (V), and serine (S) to aspartic acid (D). Site-directed mutagenesis was used to change specific nucleotides as shown below (Fig.1).

Figure 1: Comparison of the putative amino acid sequence of the wild-type Tx-T gene and the three new constructs:

```
TX-T(wt)   CAGGTGAGGGTGCGCTCGGAGAAGTCCGTGGCTGGCATTGGG
            Q  V  R  V  R  S  E  K  S  V  A  G  I  G

 TX-T 1    CAGGTGAGGGTGCGCATCGAGGGGCGCGTGGCTGGCATTGGG
            R  R  V  V  Q  I  E  G  R  V  A  G  I  G

 TX-T 2    CAGGTGAGGGTGCGCTTGGTGCCGCGCGTGGCTGGCATTGGG
            R  R  V  V  Q  L  V  P  R  V  A  G  I  G

 TX-T 3    CGCAGGGTGGTGCAGGACGAGGTGGACGTGGCTGGCATTGGG
            R  R  V  V  Q  D  E  V  D  V  A  G  I  G
```

The point mutations were induced at once. The parent plasmid was initially transformed into dam^{+} cells to create a methylated plasmid DNA template. Plasmid DNA isolated from the dam^{+} cells was PCR amplified with a primer containing the point mutations. The parent DNA was eliminated from the reaction with the methylation-sensitive restriction enzyme DpnI, and the mutagenized plasmid DNA

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was used to transform cells. Clones were screened by DNA sequencing across the region with the putative mutations. One clone with each set of the appropriate mutations was identified and the remainder of the insert was sequenced to verify its integrity. The DNA sequencing data including electropherograms, alignments and consensus sequences is being sent under separate cover through over secure Web site.

In summary, three plasmid DNA constructs of TX-T were successfully created. The quantity and quality for the plasmid DNA for each construct is given below:

DNA Quantity and Quality

	Plasmid	A_{260}	A_{280}	A_{260}/A_{280}	A_{260}/A_{230}	DNA (ng/µl)
1	TX-T 1	2.90	1.55	1.87	2.18	145
2	TX-T 2	3.96	2.13	1.86	2.27	198
3	TX-T 3	4.50	2.43	1.85	2.29	225