A General Method for Scanning Photo-reactive Amino Acids

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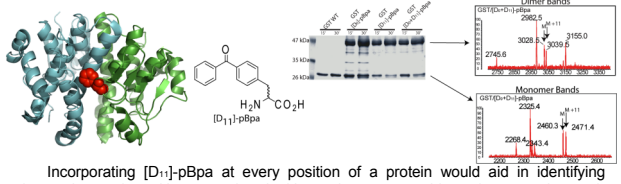
Abstract

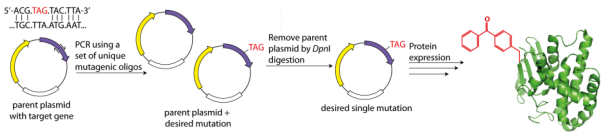
We have developed a method that facilitates the independent insertion of unnatural amino acids into every position within a protein sequence. These DNA libraries are created *in vitro* by first creating a double strand break by insertion of a transposon followed by removal and ligation of a reading frame selectable linker containing the amber codon, TAG. The described method results in the random replacement of a single amino acid codon with the amber codon, TAG. This library then allows us to control proteins with light by incorporating unique functional groups at the site of mutation. Scanning photo-reactive amino acids may allow for structural information to be obtained as well as developing new protein function efficiently. This method can also be adapted to scan any of the 20 natural amino acids.

Introduction

Rationally changing protein sequence can aid in the study of protein structure and function. Techniques used to create changes in a protein sequence include, Quickchange mutagenesis (Stratagene), error-prone PCR (1), DNA shuffling (2), and most recently introducing unique function through the incorporation of unnatural amino acids *in vivo* (3). For example, incorporation of the unnatural amino acid, *p*-benzoylphenylalanine (pBpa) allows for intrinsic protein-protein interactions to be covalently trapped (4). This allows for protein-protein interacting surfaces to be mapped using mass spectrometry. The properties of the *p*-benzoylphenylalanine are further enhanced by using an isotopically labeled analog, allowing for a unique fingerprint, M and M+11 to be obtained (5).

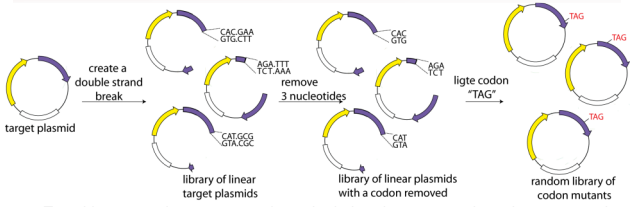


Incorporating [D_{11}]-pBpa at every position of a protein would aid in identifying unknown interactions. However when looking at how one would go about creating such mutations, every codon position of the protein would need to be mutated to a TAG. **Using traditional approaches, such as quickchange mutagenesis would not be practical. A 200 amino acid protein would require 400 unique oligonucleotides!**



For a 200 amino acid protein, repeat 199 times!

Mutagenesis without oligonucleotides



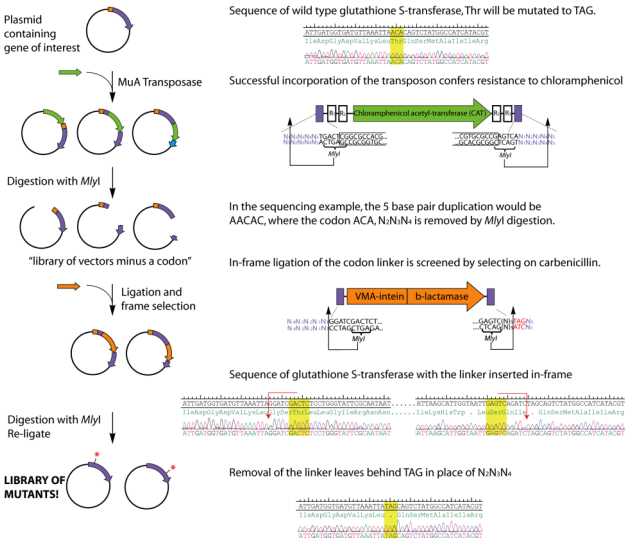
To achieve a random mutagenesis method, that does not require unique mutagenic oligonucleotides, the enzymatic properties of the MuA transposase were very appealing to us. It was thought that the transposase could be used to randomly insert throughout a gene allowing for insertion of a reading frame selectable linker to be inserted. The properties of the transposon have been used previously to create random codon deletions (6), insertions (7) and random 15 base pair insertions (8) into a gene. It has also been shown that the transposon inserts once per target plasmid and without sequence specificity (9). **However there is no control of creating only in-frame mutations.**

Codon Scanning Mutagenesis

We chose to test our method on glutathione S-transferase (GST), since it is a known homodimer and known to cross-link when pBpa is incorporated (10). We wanted to create a system that when the scanned codon was inserted inframe there would be translation of a selectable phenotype. Our design was based off of a non-biased reading frame selection plasmid reported by Lutz *et al* (11). The target gene, gst, is fused to the N-terminus of the VMA intein and the linker containing the scanning codon, contains the C-terminus of the VMA intein and β-lactamase.

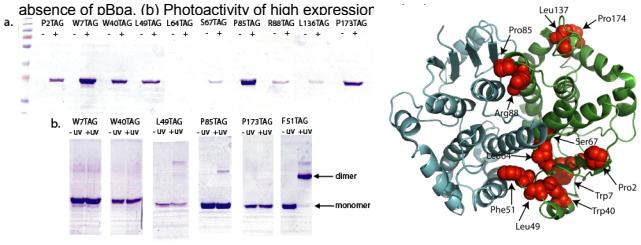
Once the transposon reaction has been completed and the transposon and 3 nucleotides are removed from the library by digesting with unique restriction endonucleases, *Mly*I. A linker, which replaces the random codon with the scanning codon and selects for the correct reading frame is then inserted. Removal of the linker with *Mly*I followed by intramolecular ligation leaves a codon scar with no additional nucleotides.

Codon Scanning Mutagenesis and Following a single clone through the process

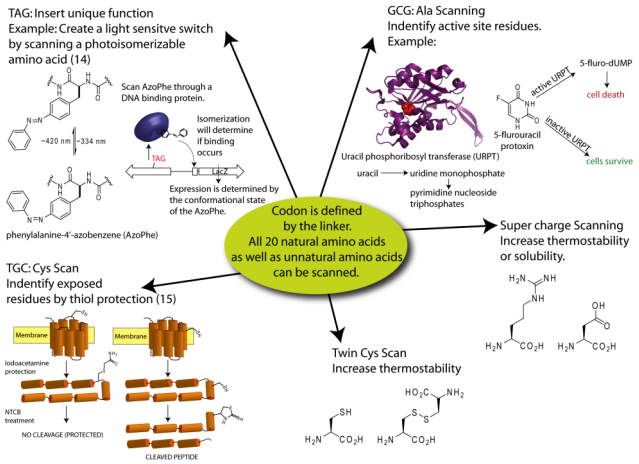


Expression with *p*-benzoylphenylalanine

From the library of TAG mutants, 10 were chosen to represent the scanned library. All amber mutants of GST were cloned into pBADmycHisA and transformed into E. coli with pSUP-*p*Bpa (12). All expressions were done on a 50 mL scale in LB media supplemented with 20 mM pBpa (racemic) (13). (a) 10 TAG mutants in the presence and absence of pBpa. (b) Photoactivity of high expressior



Future Applications



Conclusions

We have created a non-PCR based mutagenesis method, Codon Scanning Mutagenesis. This method allows for every possible position in a protein sequence to be mutated. There are no limitations in creating mutations, unlike traditional random mutagenesis methods, where amino acid mutations are limited by the redundancy of the genetic code.

Traditional Random Mutageneis:

GGG =Gly AGG=Arg

DNA Sequence: 5'GGA.TCC.ATC.CCC.ACC-3'

Protein Sequence: N-Gly.Ser.Ile.Pro.Thr-C

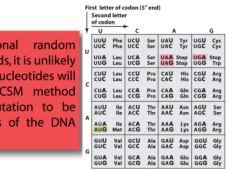
Codon Scanning Mutagenesis

GCG=Ala

DNA Sequence: 5'GGA.TCC.ATC.CCC.ACC-3'

Protein Sequence: N-Gly.Ser.Ile.Pro.Thr-C

With the traditional random mutagenesis methods, it is unlikely that 2 consecutive nucleotides will be changed. The CSM method allows for any mutation to be achieved, regardless of the DNA sequence.



This method is also user defined, not only is the codon to be scanned specifically selected but the number of times it is scanned is also determined by the user. By using codon scanning mutagenesis to incorporate [D_{11}]-*p*Bpa, identifying protein-protein interactions will be greatly enhanced. This method will also allow for the creating of proteins with improved or novel function that would otherwise not be feasible with traditional methods.

References

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