Mutagenesis

Why mutagenesis?

- Define the role of a gene
- Determine functionally important regions of a gene
- Improve or change the function of a gene product

Mutagenesis

- **Recombinant DNA technology** enables **isolate** and **modify** any desired gene.

Directed mutagenesis:

Point mutations

Insertion

Deletion

Standard Genetic Code

Standard Genetic Code

	T	C	A	G	
T	TTT Phe F	TCT Ser S	TAT Tyr Y	TGT Cys C	T
	TTC Phe F	TCC Ser S	TAC Tyr Y	TGC Cys C	C
	TTA Leu L	TCA Ser S	TAA Och *	TGA Opa *	A
	TTG Leu L	TCG Ser S	TAG Amb *	TGG Trp W	G
C	CTT Leu L	CCT Pro P	CAT His H	CGT Arg R	T
	CTC Leu L	CCC Pro P	CAC His H	CGC Arg R	C
	CTA Leu L	CCA Pro P	CAA Gln Q	CGA Arg R	A
	CTG Leu L	CCG Pro P	CAG Gln Q	CGG Arg R	G
A	ATT Ile I	ACT Thr T	AAT Asn N	AGT Ser S	T
	ATC Ile I	ACC Thr T	AAC Asn N	AGC Ser S	C
	ATA Ile I	ACA Thr T	AAA Lys K	AGA Arg R	A
	ATG Met M	ACG Thr T	AAG Lys K	AGG Arg R	G
G	GTT Val V	GCT Ala A	GAT Asp D	GGT Gly G	T
	GTC Val V	GCC Ala A	GAC Asp D	GGC Gly G	C
	GTA Val V	GCA Ala A	GAA Glu E	GGA Gly G	A
	GTG Val V	GCG Ala A	GAG Glu E	GGG Gly G	G

Leu = 6 codons
Pro = 4 codons
Gln = 2 codons
Trp, Met = 1 codon

ATG = initiation codon
TAG, TAA, TGA = non sense (stop) codons in DNA
UAA, UAG, UGA = stop codons in RNA

Single base change

- Single base change may lead to various alteration to the amino acid sequence
- The mutations may be:

Silent mutations: the triplet code is changed but the amino acid sequence in the protein is the same

5'-TCG-3' and 5'-TCC-3' both encode Ser

Mis-sense mutations: a codon change alters the amino acid encoded:

5'-TCG-3' – Ser

5'-ACG-3' – Thr

Non-sense mutations: An amino acid codon is changed to produce a stop codon

5'-TCG-3' – Ser

5'-TAG-3' – stop

Frameshift mutation: Additions or deletions

Insertions,Deletions

- Insertion or deletion of a base pair(s) into the coding sequence of a gene can have drastic implication on the encoded protein.
- Since the DNA code is read in triplets, the insertion or deletion of bases in multiples other than three will result in a frame-shift mutation
- Deletion or insertion of one or two bases into the coding sequence of a gene will alter the remainder of the sequence beyond the mutation in the gene
- Only deletion or insertion of multiples of three bases will leave the remainder of the protein sequence unaltered but will remove or insert amino acid

Site-Directed Mutagenesis

- **Simple mutation**: amplification of ds DNA plasmid using complementary oligos, carrying mutation of interest
- **Multiple mutations**: incorporation of desired mutations simultaneously in the same reaction

- A large amount of experimental procedures have been developed for directed mutagenesis of cloned genes.
- The procedures utilize:
- A **synthetic oligonucleotide** complementary to the area of the gene of interest but has the desired nucleotide change.

  (oligonucleotide is usually 10-30 nt long).

Bacteriophage M13

Use of oligonucleotides in creating site-directed mutations was introduced in laboratory of Michael Smith (Nobel prize for chemistry in 1993)

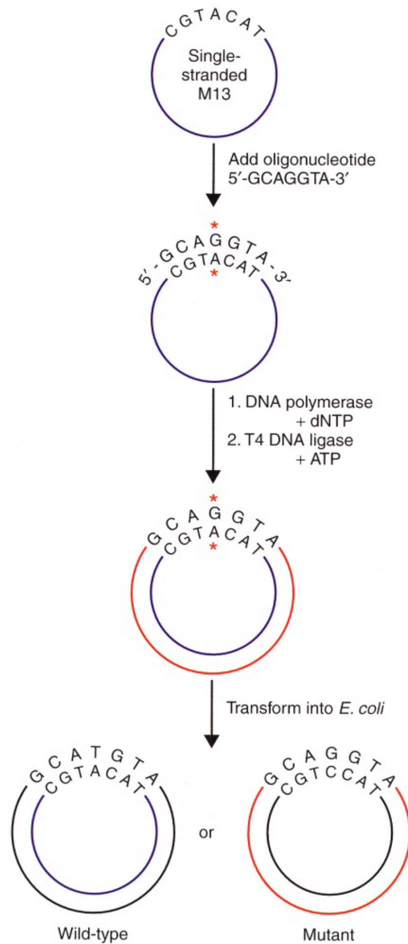
They used ss stranded M13 genomic DNA as a hybridization template for a synthetic oligo (1983)

- M13 undergoes a switch during its life cycle :

Single-stranded genome is converted to a double-stranded form

This is advantage for creating mutations within the newly synthetized DNA strand

Site Directed Mutagenesis using a Single-stranded DNA template: Primer extension mutagenesis



ss DNA from recombinant M13 bearing the target gene is used as a template for binding of oligonucleotide primer.

Primer hybridizes to the complementary sequence and introduces mutation.

The hybrid is then treated with DNA polymerase in the presence of deoxynucleoside triphosphates (dNTP) to synthetize a new M13 DNA complementary strand containing mutation.

The sugar-phosphate backbone of the new DNA circle is then completed using DNA ligase.

Double-stranded DNA is transformed into *E. coli.*

M13 plaques are produced

Identification of Mutant Plaques

- Because **DNA replicates semi-conservatively,** half the cells will have the mutant gene.
- Mutant plaques are identified by **DNA hybridization** using the oligo as probe.
- The procedure has been modified to enrich the number of mutant plaques

In general, the method is not efficient, screening of plaques is laborious

PCR-Amplified Oligonucleotide-Directed Mutagenesis

- PCR can be exploited both to introduce the desired mutation and to enrich for the mutated gene.
- This procedure uses forward and reverse PCR primers.
- Each pair of primers has one that is completely complementary to a portion of our gene and one that has a single nucleotide change for point mutation.
- Following the PCR, a high percentage of the plasmids produced will have the desired mutation.
- No specific plasmids required, all we need to know is the sequence of the cloned gene.

Mutagenesis using *Pfu* DNA polymerase

- The whole plasmid mutagenesis
- *Pfu* polymerases synthesize copies of the whole vector.

- The original template DNA without mutation is deleted by cleavage with endonuclease *DpnI*, which is specific for methylated and hemimethylated DNA izolated from *E. coli.* The mutated copies are not methylated.
- All DNA produced from most *E. coli* strains would be methylated; the template plasmid that is biosynthesized in *E. coli* will be digested, while the mutated plasmid, which is generated *in vitro* and is therefore unmethylated, would be left undigested.

- The procedure comprises: Thermal cycling to:

1) denature DNA template
2) anneal mutagenic primers containing desired mutation
3) extend primers with *Pfu* DNA polymerase

Mutagenesis using DNA polymerases

- High fidelity polymerases with low error rate, proofreading activity
- Pfu Turbo: is a mixture of cloned Pfu DNA polymerase and the exclusive thermostable ArchaeMaxx polymerase-enhancing factor that enhances PCR product yields and increases target length capability without altering DNA replication fidelity.
- PfuUltra high fidelity: genetically engineered mutant of Pfu polymerase: robust amplification of long and complex targets
- KOD: High fidelity DNA polymerase designed for accurate PCR amplification of DNA templates for general cloning and cDNA amplification applications.
- Phusion^{TM} : fusion of ds DNA binding domain to *Pyroccus*-like proofreading polymerase, generates blunt-ended products and amplifies GC-rich templates

Q^{5} High Fidelity polymerase – robust amplification of GC rich regions

Cloning of a region of interest into a plasmid

- Choosing the right vector (+/- tag, C- or N-terminal tag).

- Check that the restriction enzymes for insertion of sequences in the MCS do not cleave the sequence of interest

Primers

- Primers should be 15-30 nucleotides long and have a GC content of 40-60 %.
- The forward and reverse primer should have similar melting temperatures (Tm's).
- Try to avoid primer dimer and hairpin formation.
- For sticky end ligation remember to add additional nucleotides to the 5' side of the restriction site. The restriction site sequences should be followed by ≥ 15 bases that are homologous to the template DNA.
- The 3'-end of the primer has to end on an C or a G.
- For sticky end ligation (ratio vector : fragment; 1pmol : 3pmol)

Sticky ends

Blunt ends

Random mutagenesis

A technique for developing enzymes with novel properties, including altered substrate specificity, enantioselectivity, stability and reaction specificity.

There are many ways to create random mutant libraries, each has some advantages and disantanges:

Types:

Error-prone PCR

Error-prone Rolling circle amplification

Bacterial Mutator strains

Insertion mutagenesis

Chemical mutagenesis: Ethyl methanesulfonate, Nitrous acid

Error Prone PCR

- PCR is performed using polymerase with high error rate (up to 2%), to amplify the wild-type sequence. e.g.: *Taq* polymerase, which lacks proof reading activity, the error rate is higher.

- These heat stable DNA polymerases can occasionally **insert the wrong nucleotide** generating **mutations** during the PCR reaction, yielding randomly mutated products (Error Prone PCR).

- The error rate may be increased by modifying PCR conditions e.g
  DNA template concentration
  Adding unequal concentration of each Ntp
  Adding Mn^{2+}
  Increasing concentration of Mg^{2+}

Error-Prone PCR

- After amplification, the library of mutant coding DNA sequences must be cloned into a suitable plasmid and screen for altered function.

- The DNA from clones is isolated and sequenced.
- The size of the library is limited by the efficiency of the cloning step.

- Point mutations are the most common types of mutation in error prone PCR, deletions and frameshift mutations are also possible

Error-prone rolling circle amplification (RCA)

Rolling circle amplification (RCA):
an isothermal method that amplifies circular DNA by a rolling circle mechanism,

Random hexamers are used instead of specific primers:
they prime DNA synthesis nonspecifically

It uses the TempliPhi™ 100 Amplification Kit and Phi29 DNA polymerase.

TempliPhi™ uses an isothermal method for the exponential amplification of circular DNA.
Phi29 DNA polymerase is active at 30° C, enabling amplification to be performed at this temperature without the need for thermal cycling.

The amplified product can be used directly to transform a host strain.

Bacterial Mutator strains

The wild-type sequence is cloned into a plasmid and transformed into a mutator strain, such as *E.coli* XL1-Red , which is deficient in three of the primary DNA repair pathways (mutS, mutD and mutT).

During replication, this strain makes errors

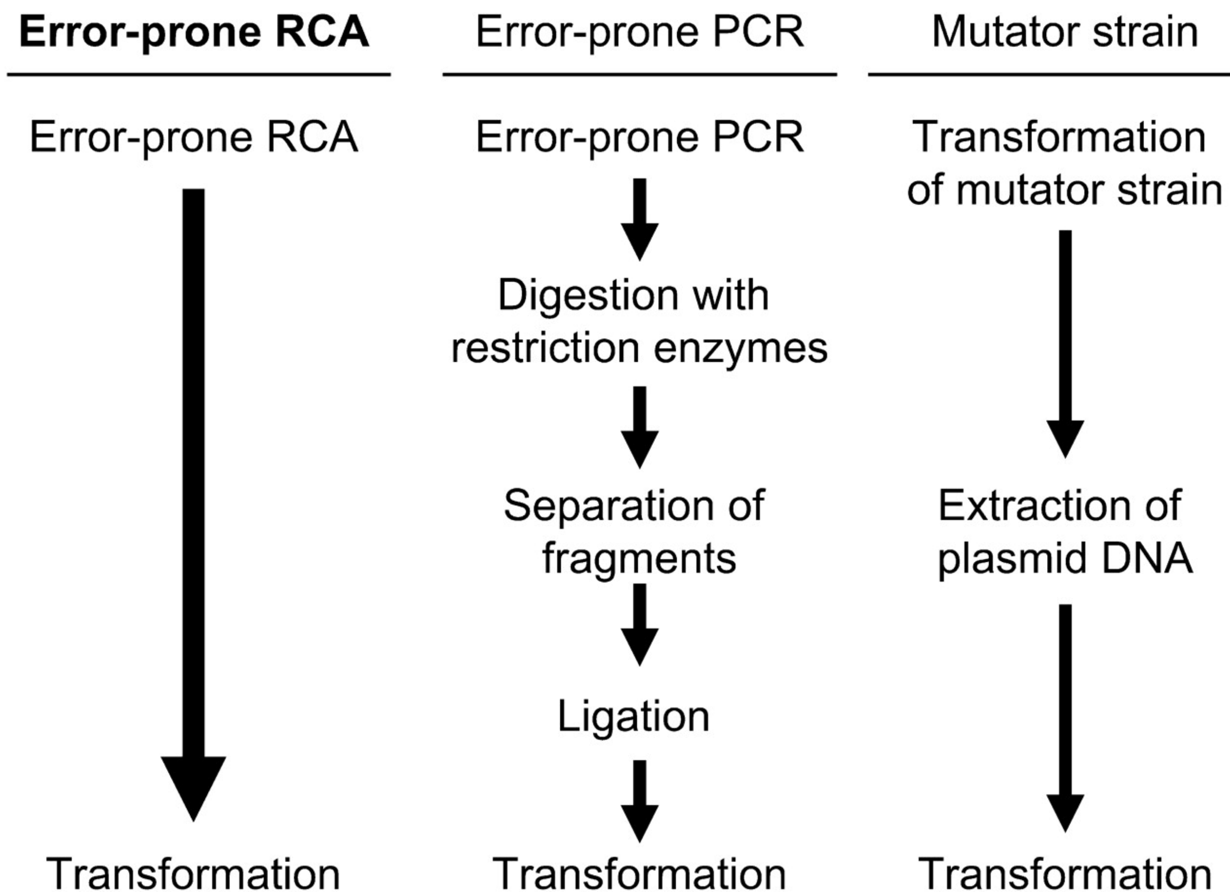
Each copy of the plasmid replicated in this strain has the potential to be different from the wild-type.

A wide variety of mutations can be incorporated including substitutions, deletions and frame-shifts.

Random mutation rate is 5000 times higher

The protocol for using the mutator strain is composed of two steps:
transformation of the mutator strain and recovery of the mutant from the transformant.
This protocol is much simpler than error-prone PCR, and a ligation step is unnecessary.

Comparison of conventional random mutagenesis methods.



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Insertion mutagenesis

The transposon-based system randomly inserts a short nt sequence (e.g. 5 codons) throughout a sequence of interest.

The insertion is random and a library of insertions is created

The system utilizes a hyperactive Tn5 transposase enzyme variant and transposon DNA

Magnesium is required for insertion into target DNA

Chemical Random Mutagenesis

Ethyl methanesulfonate (EMS): aklylates guanidine residues

In vivo mutagenesis in the cells.

In vitro: a PCR-amplified gene is treated with EMS before ligation into a plasmid.

Nitrous acid: de-aminates adenine and cytosine residues,
It causes transversion point mutations (A/T to G/C and vice versa).