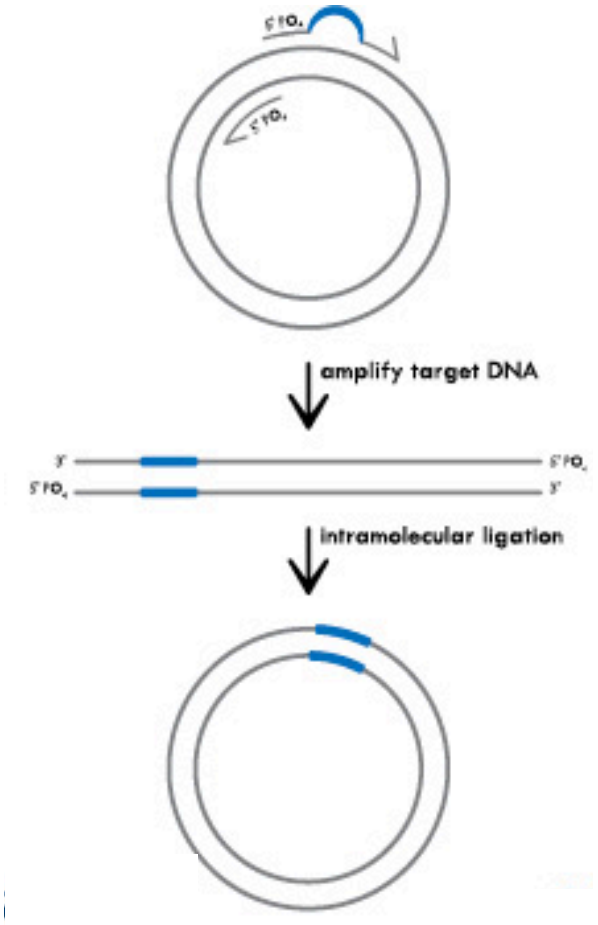
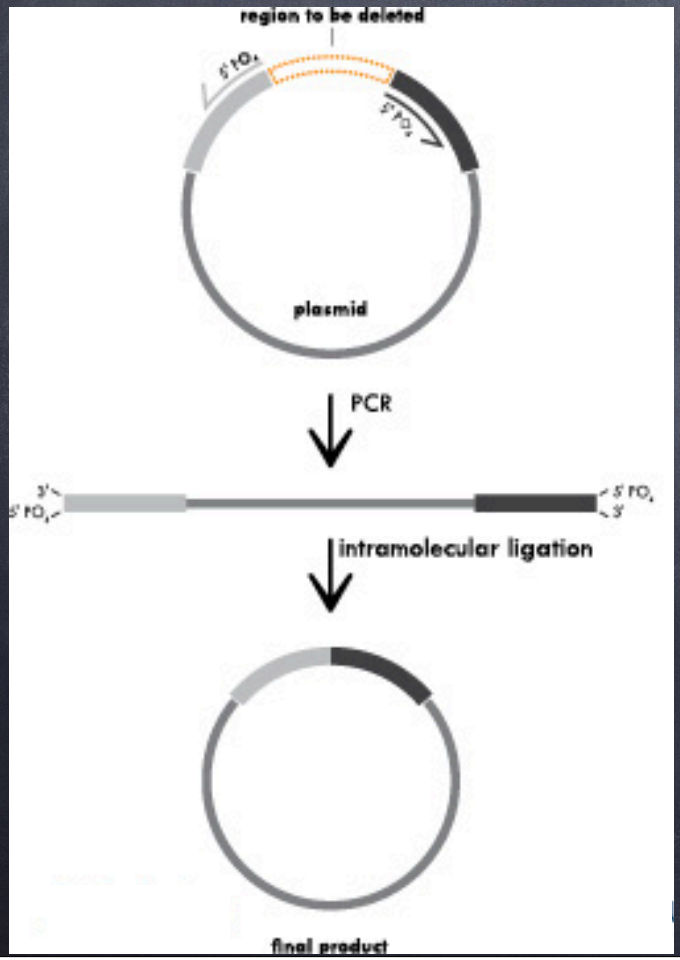
Technique 3:
Inverse PCR for site-directed mutagenesis

The primers used are 5'-phosphorylated to allow ligation of the amplicon ends after PCR. A high fidelity DNA polymerase that creates blunt-ended products is used for the PCR to produce a linearized fragment with the desired mutation, which is then recircularized by intramolecular ligation.

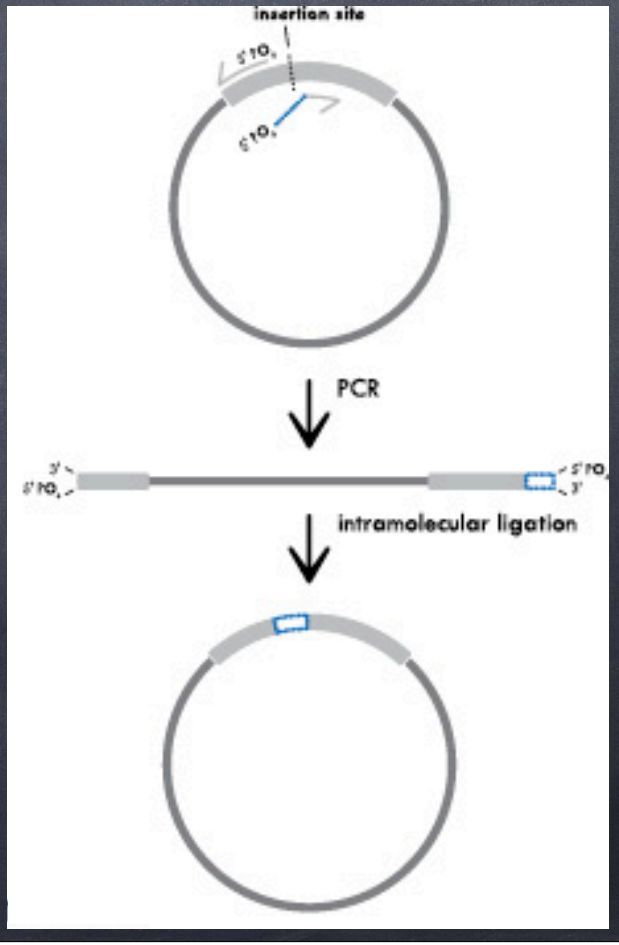


Technique 3:
Inverse PCR for site-directed mutagenesis

Primers that hybridise to regions on either side of the area to be deleted are used.



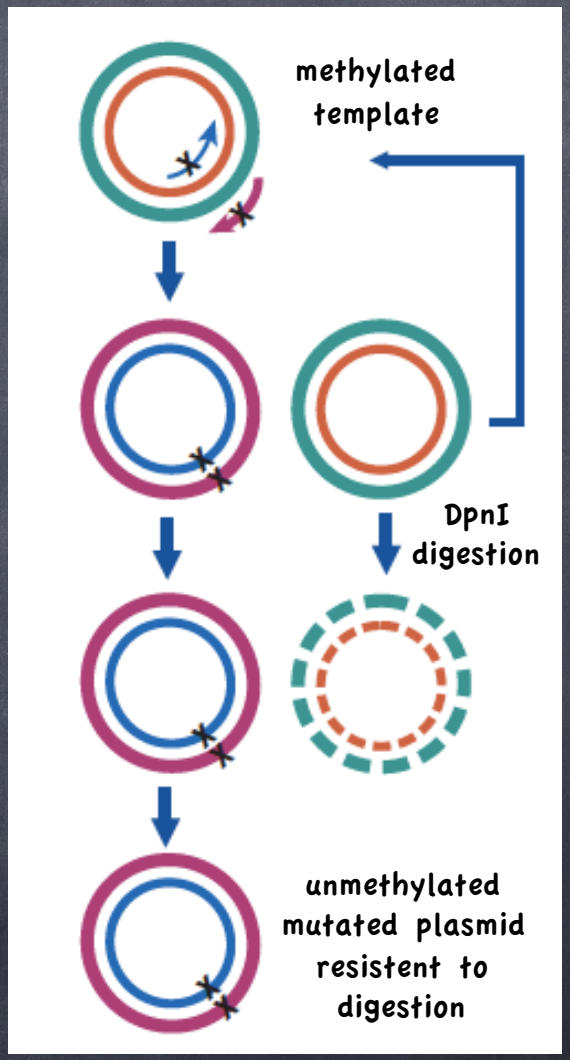
One primer contains the additional sequence that will be inserted (blue line).



Technique 3: Inverse PCR for site-directed mutagenesis

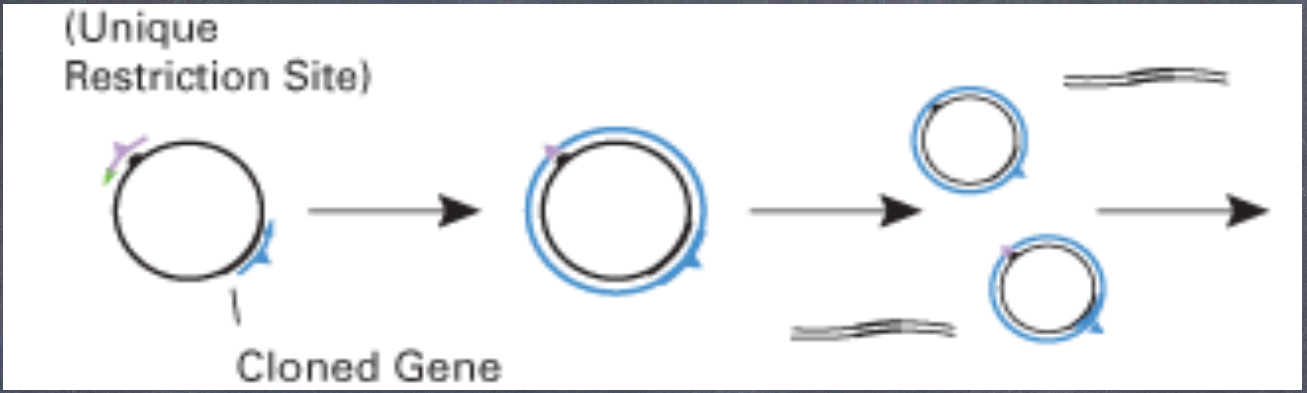
Mutated-plasmid selection

Digest parental methylated and hemimethylated DNA



From Stratagene

Use a selection primer to suppress a unique restriction site on the plasmid

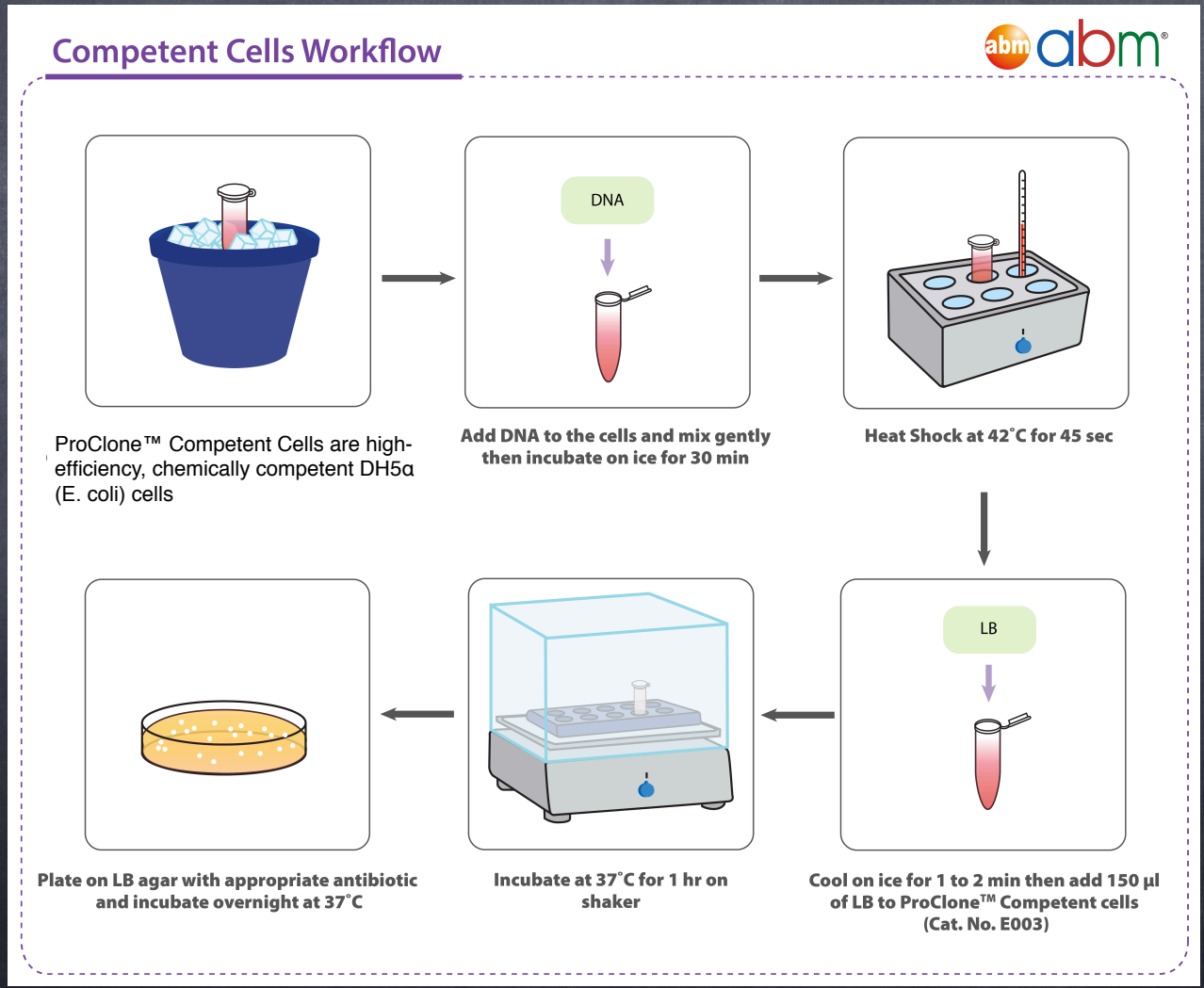


The Transformer Kit uses two oligonucleotide primers which are simultaneously annealed to one strand of a denatured double-stranded template. One primer introduces the desired mutation and the other mutates the unique restriction site in the plasmid, creating a new restriction site or eliminating the site completely. Elongation by T4 DNA polymerase, which lacks strand displacement activity, results in the incorporation of both mutations in the same newly synthesized strand. The DNA is then digested with a restriction enzyme that cuts at the original restriction site. The uncut, mutated DNA will transform E. coli more efficiently than the linear DNA with no mutations.

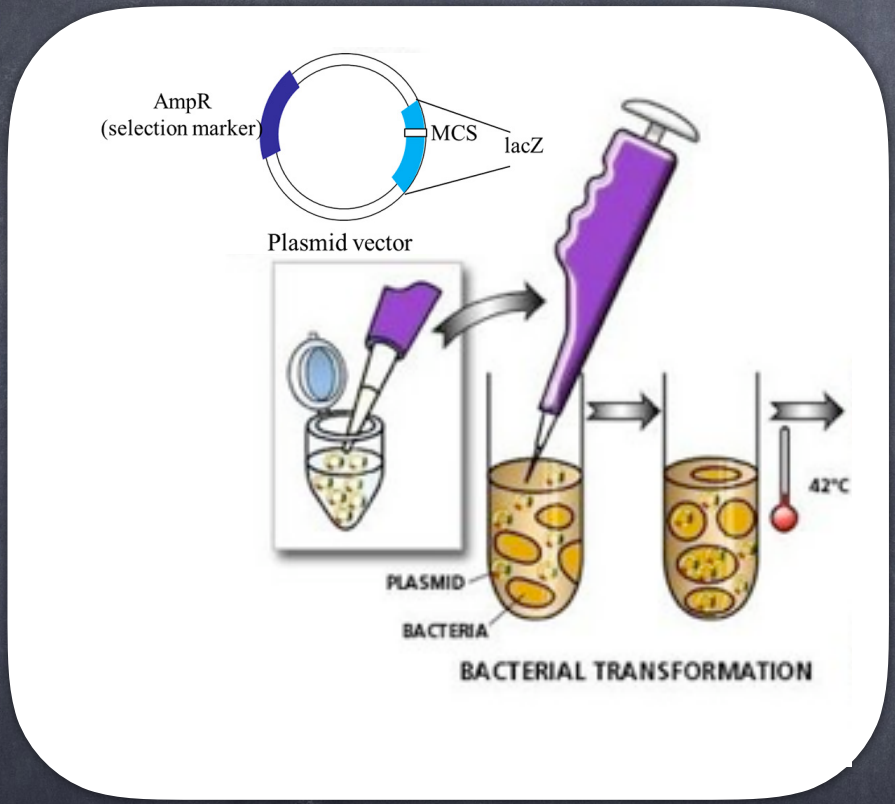
(from Clontech)

Next step following recombinant vector production?

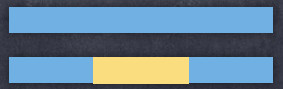
Bacterial transformation



Antibiotic selection



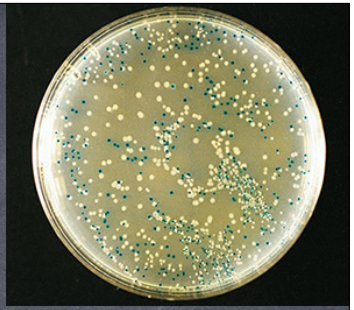
Ampicillin



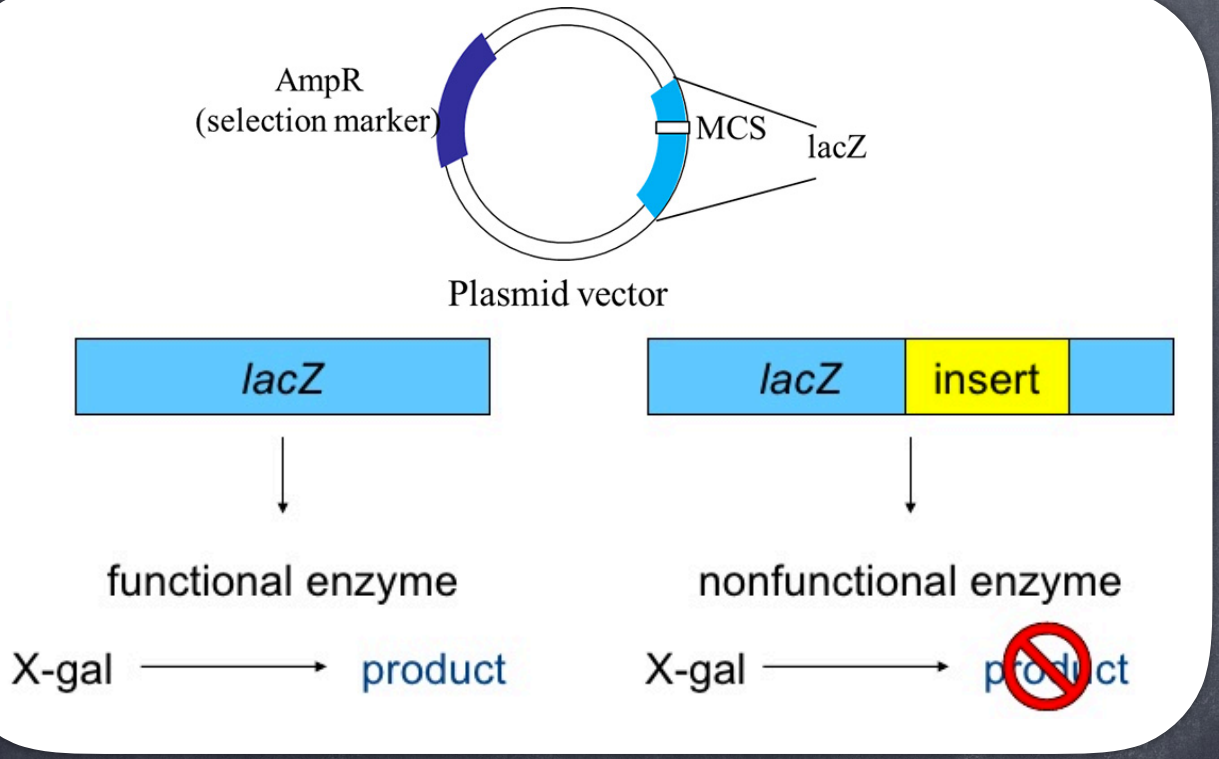
Growth of transformed colonies (with plasmid)

- plasmid w/o insert
- plasmid with insert

Blue-white color screening



select white colonies

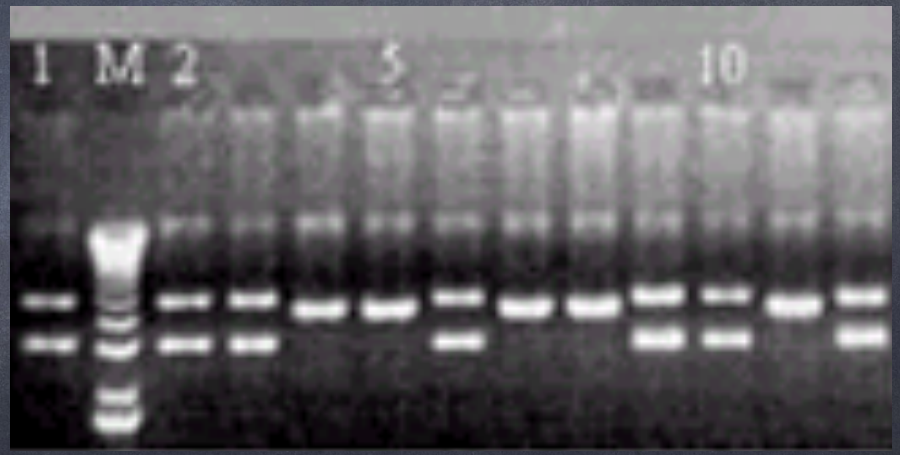


Transformed colonies w/o insert

Transformed colonies with insert

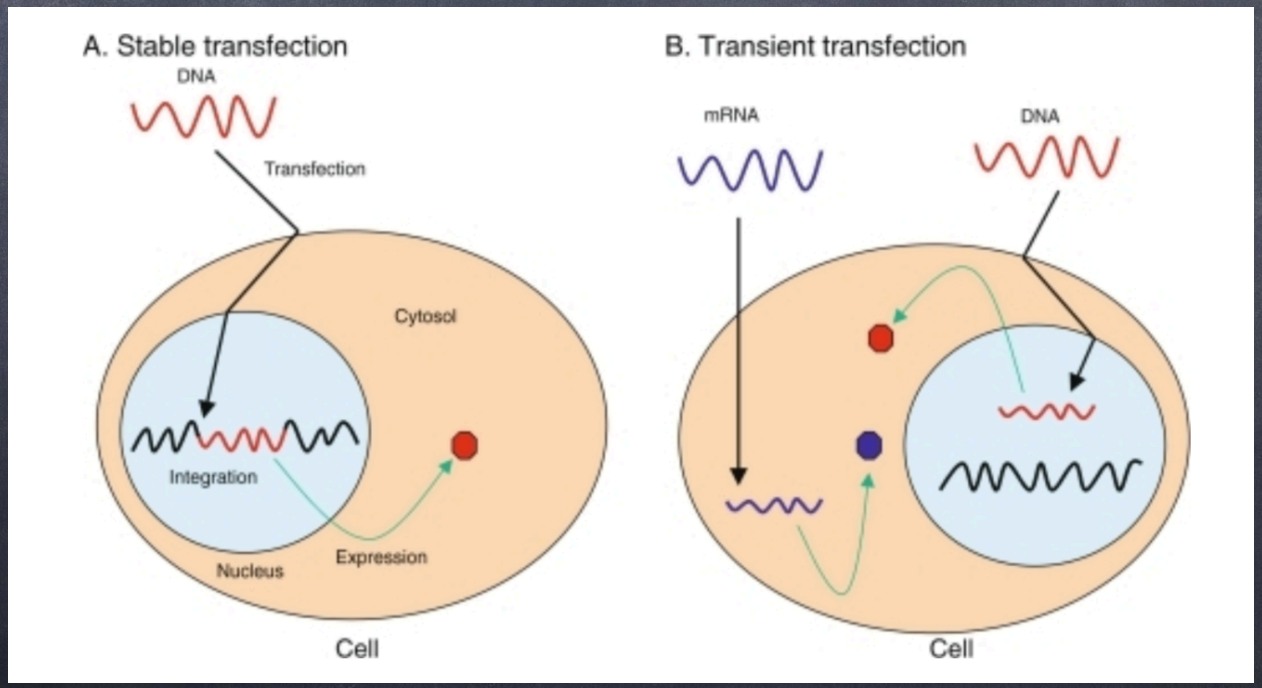
Analysis of Mutants

Miniprep and enzymatic digestion



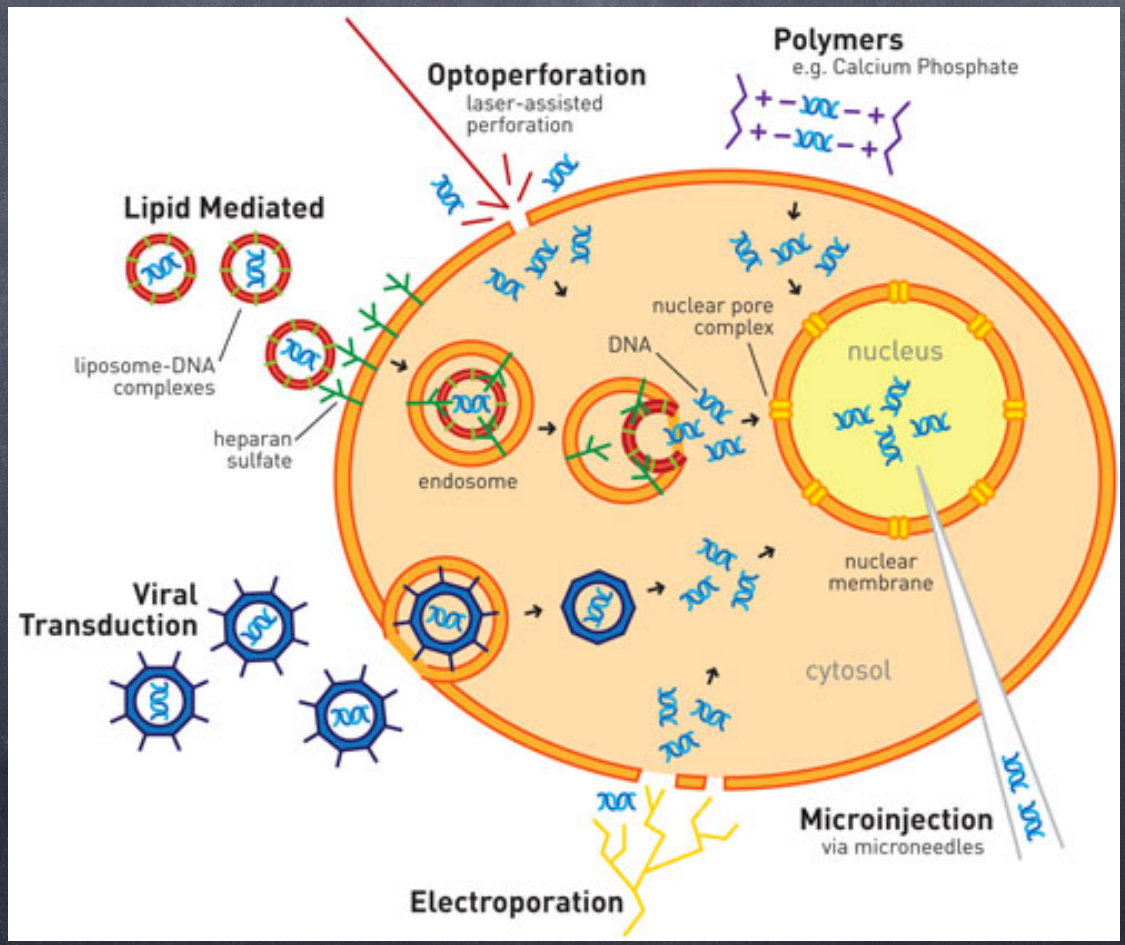
Eukaryote cell transfection

Stable and transient transfectants



Transfection methods

Protocols can vary greatly depending on the characteristics of the cell line you use and the desired effects.



Transient transfections

Transiently transfected cells express the foreign gene but do not integrate it into their genome. Thus the new gene will not be replicated. These cells express the transiently transfected gene for a finite period of time, usually several days, after which the foreign gene is lost through cell division or other factors.

Not interested in biological response	→	transient transfection of Cos cells
Interested in short term biological response	→	transient transfection of specific cells

Stable transfections

Generating stably transfected cells begins with a transient transfection, followed by an infrequent but important and serendipitous process. In a small proportion of transfected cells, the foreign gene is integrated into the cells' genome. The hallmark of stably transfected cells is that the foreign gene becomes part of the genome and is therefore replicated. Descendants of these transfected cells, therefore, will also express the new gene, resulting in a stably transfected cell line.

A common selection method is to cotransfect the new gene with another gene for antibiotic resistance (such as the neomycin resistance gene, neo) and then treat the transiently transfected cells with the appropriate antibiotic for selection (such as geneticin or G418 for neotransfected cells). Only the stably transfected cells with resistance to the antibiotic will survive in longterm cultures, allowing for the selection and expansion of the desired cells.

Interested in long term biological response	→	Stable transfection of specific cells

Not interested in biological response → Transient transfection of Cos cells

Molecular level (protein extract)

- To study protein-protein interaction → co-IP, FRET
- To study protein modification (phosphorylation...) → WB, FRET
- To study the effect of dominant mutations → WB, co-IP
- To study the effect of dominant-negative mutations WB, co-IP

Interested in short term biological response → Transient transfection of specific cells

Interested in long term biological response → Stable transfection of specific cells

Cellular level

- To study the effect of dominant mutations → phenotype
- To study the effect of dominant-negative mutations → phenotype

Dominant mutations

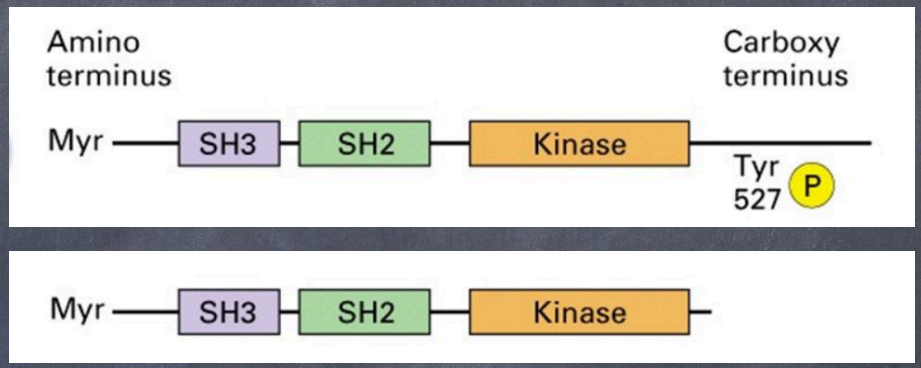
Definition:

A mutation whose gene product is constitutively active .

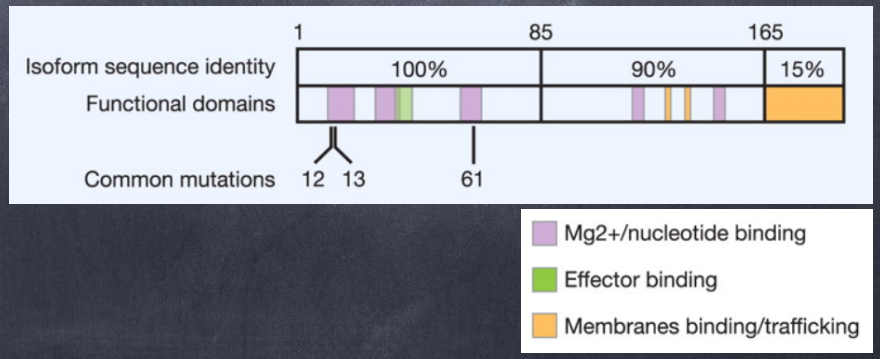
The product overcomes the wild-type product

Examples of dominant mutation:

Oncogenic mutations



Oncogenic RAS mutations at residues G12, G13 and Q61 render them constitutively active (GTP-RAS) as they are impaired in their intrinsic and GAP-mediated GTP hydrolysis



Dominant negative mutations

Definition:

A mutation whose gene product adversely affects the normal, wild-type gene product within the same cell.

This usually occurs if the product can still interact with the same elements as the wild-type product, but block some aspect of its function.

Examples of dominant negative mutation:

- A mutation in a transcription factor that removes the activation domain, but still contains the DNA binding domain. This product can then block the wild-type transcription factor from binding the DNA site leading to reduced levels of gene activation.

- A protein that is functional as a dimer.
  A mutation that removes the functional domain, but retains the dimerization domain would cause a dominate negative phenotype, because some fraction of protein dimers would be missing one of the functional domains.

Opened-questions

- Explain experimental results obtained using IP or co-IP techniques, with or W/O Tag-proteins, Y-2H and site-directed mutagenesis

- Choose among IP, co-IP, tagged-proteins, Y-2H and site-directed mutagenesis the appropriate approach to address a specific experimental question, defending your choice

Assignment for next Wednesday:

Regulators	Differences in ERK activity	Cellular responses
Temporal regulators PKC Rap1 Sprouty	Activity Sustained ERK activation Time Transient ERK activation	Differentiation PC12 cells Proliferation

Is the difference in the kinetics due to the rate of internalisation of the receptor?

How would you proceed to answer this question?