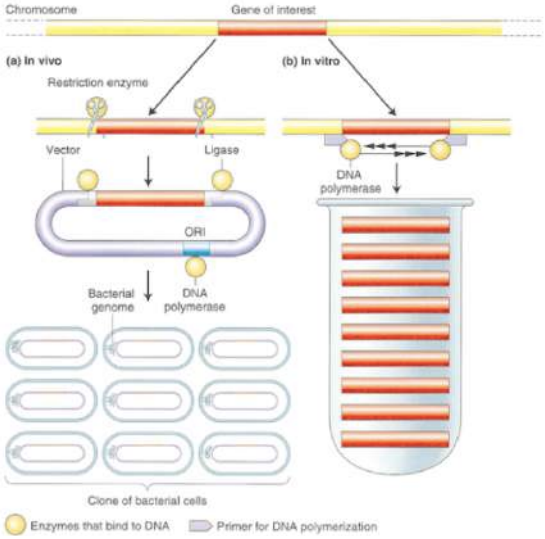
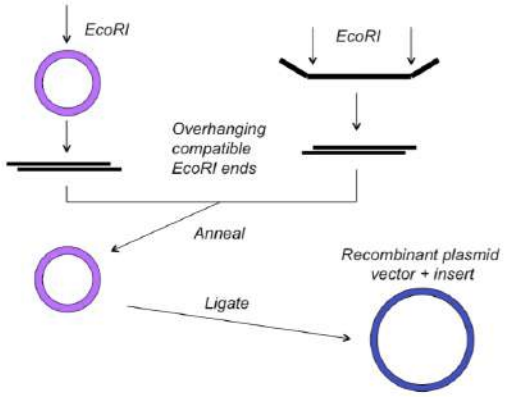
Week 5: Molecular Cloning Strategies

- 2 options for gene cloning:
    - Traditional recombinant plasmid cloning of the gene of interest
    - PCR amplification of the gene of interest within "bookended" primers
- Both yield many copies of the gene → same outcome
- In both, the gene cassette may be moved around



Recombinant Plasmid

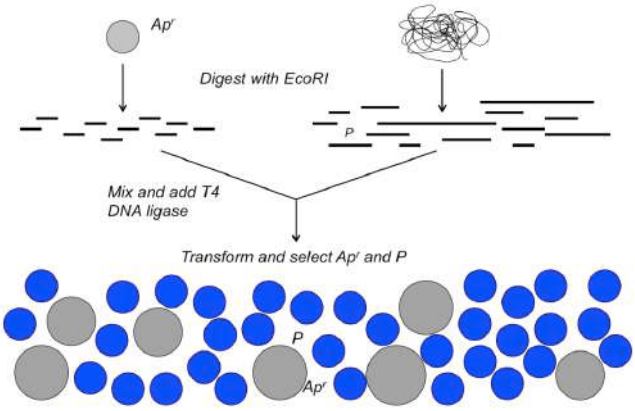
- A vector is linearised by digesting it at a unique restriction site, whilst preparing a source of linear insert DNA with the same or compatible restriction ends
    - E.g. vector + insert prepared with enzyme EcoRI
- Other ways of doing this:
    - Using enzymes that are isoschizomers of one another
    - Using any blunt-ended restriction enzyme
    - Using two different enzymes
- First 2 choices → the inserts will be aligned in either direction relative to the vector
- Last 2 choices → the new site in the recombinant plasmid might not be able to be cut with either original enzyme



Positive Selection

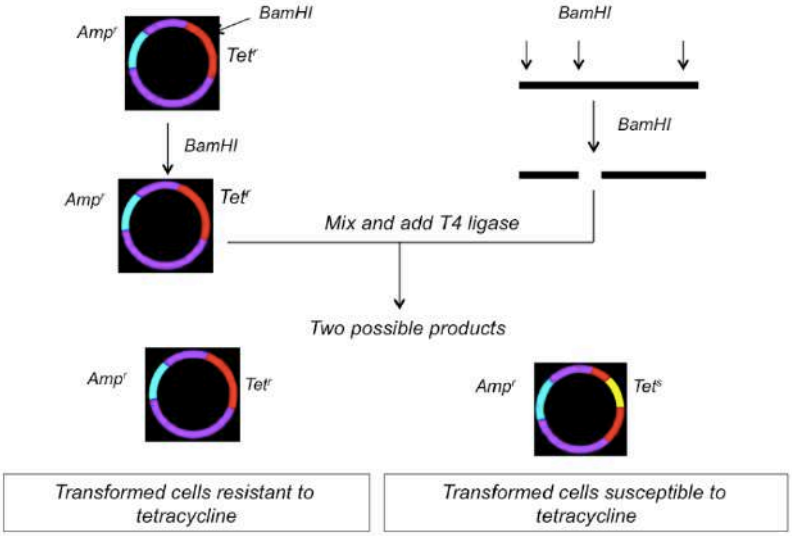
- Only 2 types of molecules are important + they are those that can be transformed:
    - Recircularised vectors (blue circles)
    - Recombinant circles (grey)

- The "background" of vector and various recombinants is unimportant bc we have a selectable phenotype 'P' for the clone we want
    - Phenotype is easily identified e.g. antibiotic resistance or colony colour
    - E.g. if phenotype is kanamycin resistance, only need to use agar plates with ampicillin and kanamycin to obtain the required recombinant clones
        - Not as easy in practice, may need to go through thousands of transformants to find the recombinant clone of interest
- <u>Want to reduce or eliminate the background so desired clones will be present</u>
- Successful molecular cloning = generating ONLY recombinant plasmids
- The linear concatamers are rendered redundant by using ideal ligation conditions
    - Problem = background of original vector plasmids that will represent the majority of transformants (~60-90%) depending on the sizes of the inserts in the recombinant plasmids
    - Bc small plasmids transform much more efficiently than larger ones
        - Rate about 1 log less per doubling in plasmid size



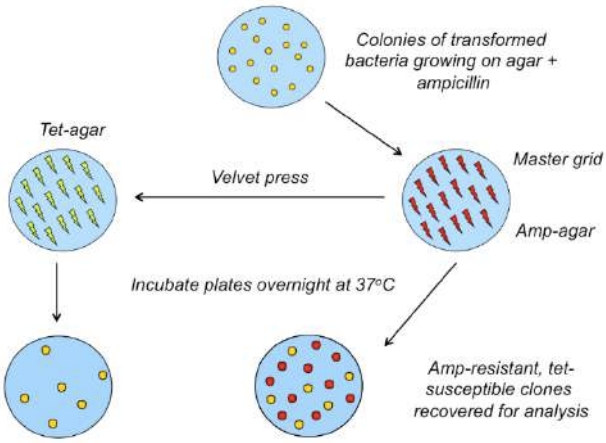
Insertional Inactivation

- II of antibiotic resistance gene = discriminate bet religated vectors and recombinants
- E.g. a DNA linear fragment with BamHI ends is ligated into the unique BamHI site in the tetracycline resistance gene. 2 results:
    - Recombinant plasmid result = susceptible to tetracycline bc the tet gene ORF sequence has been interrupted and the Tet protein cannot be made
        - ∴ cannot elicit tetracycline resistance
    - Original vectors that religate without an insert = still express Tet resistance



Velvet Press

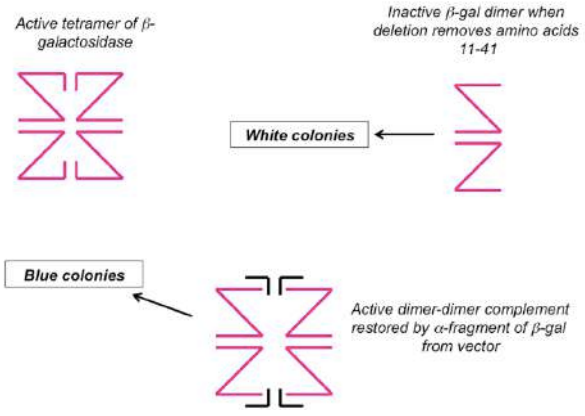
- Grid plating method to screen clones from insertional inactivation
- A master grid of transformants is patched onto an Amp-only agar plate
    - Plate is velvet-pressed onto a Tet-only agar plate
- Only vector transformants ("background") grow on the Tet plate
    - Actual recombinants are those that did not grow
    - These may be recovered from the master plate (red clones)
        - Determine by position on plate (look at last 2 plates in diagram)
- In practice, method is effective, but:
    - Requires a special vector (with 2 antibiotic markers)
    - Labour intensive + time consuming → needs fairly extensive screening to obtain a sizable number of recombinants (60-90% will be vectors)



Protein Complementation → 2 separate segments of the same original single polypeptide can associate in the same conformation and carry out the same function as the original

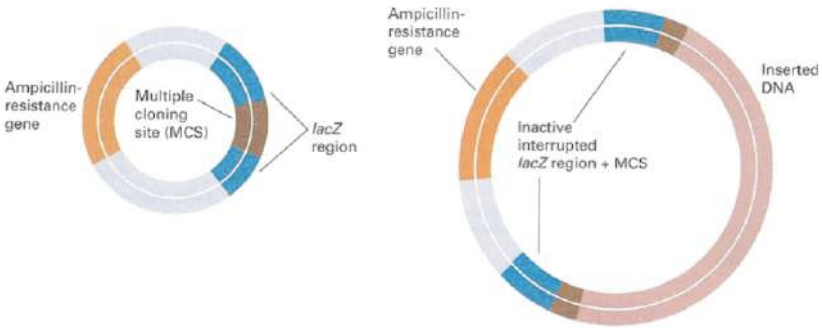
LacZa – B-Gal Complementation

- Most current vectors (plasmids + viruses) use this beta-galactosidase selection system for discriminating between religated vectors and recombinants
- Complementation B-galactosidase → breaks apart glycosidic bonds = blue colonies
- Complete enzyme only works as a <u>tetramer</u> or dimer of dimers
    - Alpha-peptide deletion = enzyme can only assemble as a dimer = inactive
        - Strain of E. coli with minor deletion (30aa's) affects folding ∴ cannot associate into tetramer, only dimers
        - ∴ cannot hydrolyse substrate = white colonies
- Alpha-peptide supplied by vector = combine with defective enzyme produced from host chromosome = generate an active tetramer by non-covalent complementation



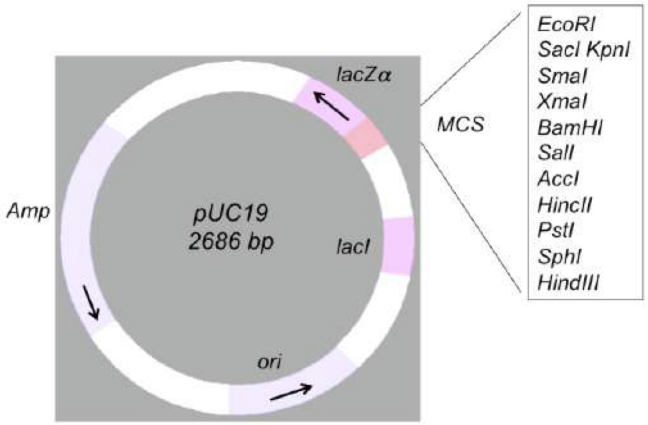
Plasmid Vector Optimised for Cloning

- Inactivated lacZ region by cloning into the MCS in the recombinant plasmid (right)
    - Basis of selection for recombinant clones from most modern vectors
- E.g. of 'insertional inactivation'
    - More suited to smaller vectors
    - Is direct (blue-white colony identification) vs. patched grids and extra antibiotic containing plates



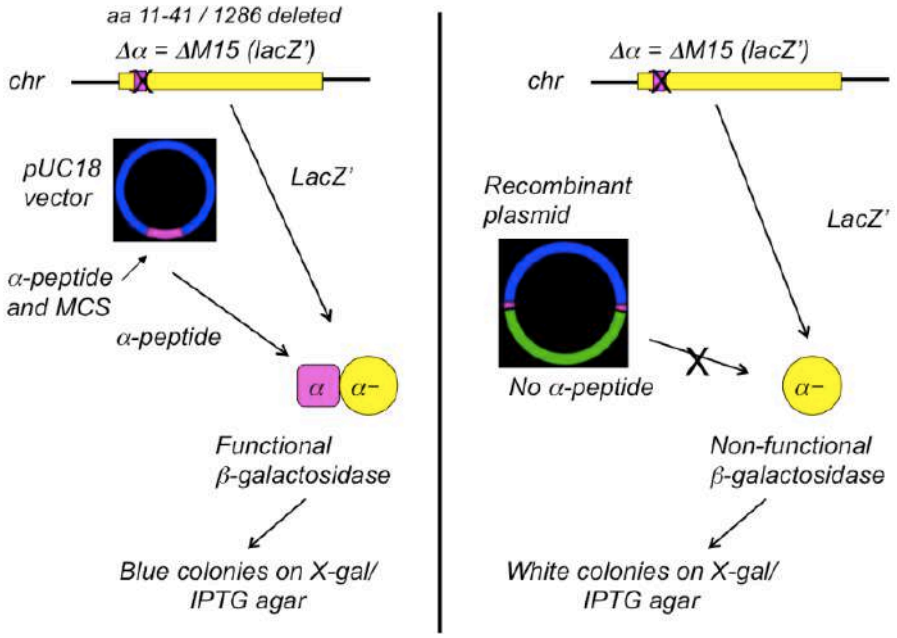
Plasmid Vector Features

- Elements of the blue-white LacZ selection system depicted in a typical cloning vector
- Inserts are ligated into the MCS to generate recombinant plasmids
    - MCS is within the short lacZ alpha-peptide sequence (codons for aa's 11-41) of the lacZ gene
        - Major portion being in the E. coli host chromosome
- Vector contains the promoter for lacZ → alpha-peptide sequence can be transcribed
    - Must be triggered by the inducer IPTG → present in agar selection plates
    - IPTG also induces transcription of the defective lacZ gene for beta-galactosidase in the host chromosome
    - Selection plates also contain:
        - Ampicillin → for plasmid-transformants selection
        - Beta-galactosidase chromogenic substrate X-gal → produces free indole when hydrolysed by a B-galactosidase enzyme = blue colonies



- Insertional inactivation also works
- Elements of the cloning vector and the host E. coli chromosome combine to generate the active enzyme by complementation
    - (Left) Religated vectors → supply an uninterrupted peptide that complements the defective enzyme = functional galactosidase = hydrolyse synthetic substrate X-gal = liberating indole = blue colonies containing vector plasmids

    - (Right) Insert ligated into the MCS of the vector → alpha-peptide is insertionally inactivated = defective galactosidase enzyme = white colonies containing recombinants
- Determined in the Amp-X-gal-IPTG agar plates the transformants are plated



- IPTG = inducer molecule
    - Removes the repressor from the operator-promoter on the vector pUC19
    - Enables read through uninterrupted transcription of the LacZ alpha-peptide
    - Complements defective beta-galactosidase from the chromosome
    - Enzyme is able to hydrolyse the synthetic substrate X-gal, releasing indole that gives the colonies a blue colour (vector background)
- In recombinants → no hydrolysis of X-gal and no indole or blue colour = white
- The blue/white system is often teamed with one of these vector reduction schemes
    - Reason → achieving complete vector elimination is never certain
        - There could be 1-2% of residual vectors that slip through the system
    - ∴ ensure that plated transformants are mostly recombinants by using the blue/ white selection agar plate system → most clones will be white and there could be a few blue colonies, and these are avoided

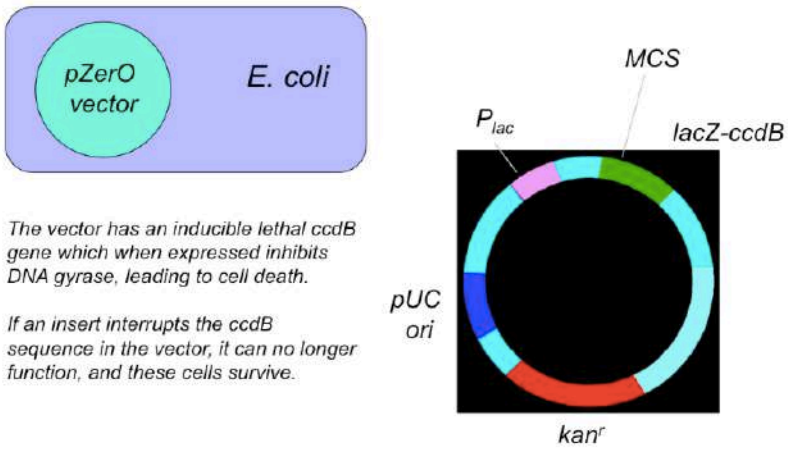
Central Ideas of Molecular Cloning

- Maximising the efficiency of the ligation reaction bet vector plasmid + DNA insert
- Reducing the religation of the vector + ∴ high vector background of transformants
- Modifying the restriction ends of the vector +/ DNA insert to enable broader and more flexible recombinant DNA possibilities
- Molecular cloning for multiple copies of genes PCR will do the same thing
    - Modifying DNA for cloning or research
    - Using a vector for expressing proteins

Zero Background

- Ways of controlling vector background:
    - Preventing the vector being regenerated
    - <u>Preventing vector's survival in transformants</u>

        - E. coli "Death Gene" → ccdB
        - Product of gene = ccdB protein → inhibits DNA gyrase = cell cannot repair or replicate its chromosome
- Vector "pZerO" has all of the usual regulatory elements of a cloning vector
- If the lacZ-ccdB MCS has a DNA fragment inserted, the ccdB sequence is interrupted by insertional inactivation
    - Recombinant plasmids will now survive in transformed cells
    - Religated vector will result in the death of the transformed cell
    - Vector background is absent and all colonies contain recombinant plasmids
- If ccdB is a lethal gene in the vector pZerO, how is it possible to introduce the plasmid into E. coli and make multiple copies of it for cloning purposes?
    - It will only cause death if it is transcribed and protein is made
        - ∴ must also induce promoter
    - Without inducer, promoter is dormant = no protein = cell survives

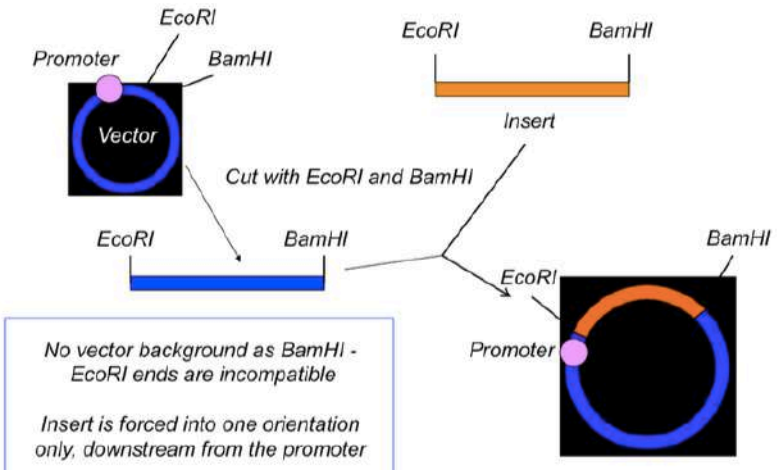


Ligation: Orientation of Ends

- For expression studies, it is essential that the insert (the orientation of the gene) is in the correct direction downstream from the vector's regulatory region and promoter

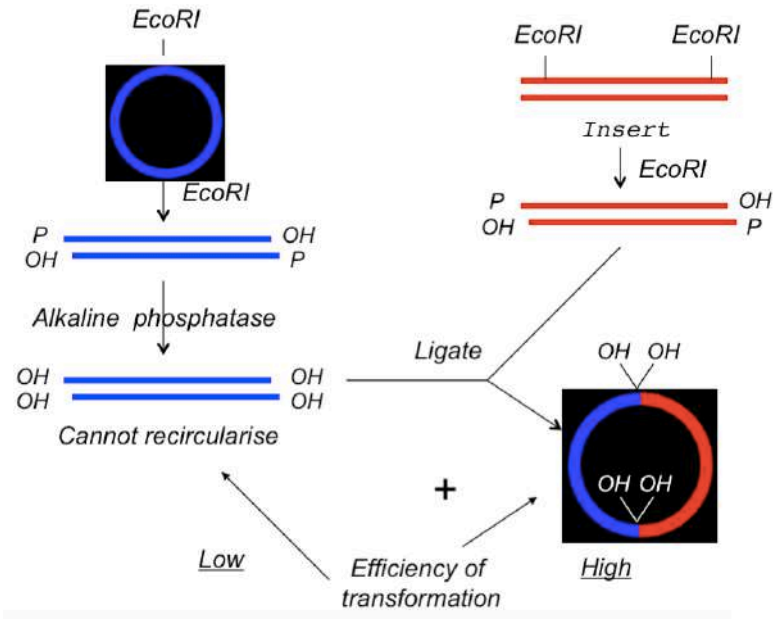
Directional/Forced Cloning

- Two important advantages:
    - Reducing vector background to zero so that most or all transformants will contain recombinant plasmids
        - Achieved by cutting the vector with two different + incompatible restriction enzymes
        - Insert is prepared with the same 2 incompatible sites on either end
        - Ligation reaction will generate the recombinant plasmid
        - No background bc the vector is incapable of religating → its ends contain incompatible restriction sites
        - ∴ all transformants will carry recombinant plasmids
    - Insert is ligated into the vector in ONE (forced) direction only
        - ∴ all recombinants (100%) are the same (100%)
        - Ensures that a cloned gene will have the correct orientation (5' to 3') with respect to the upstream vector promoter



Alkaline Phosphatase

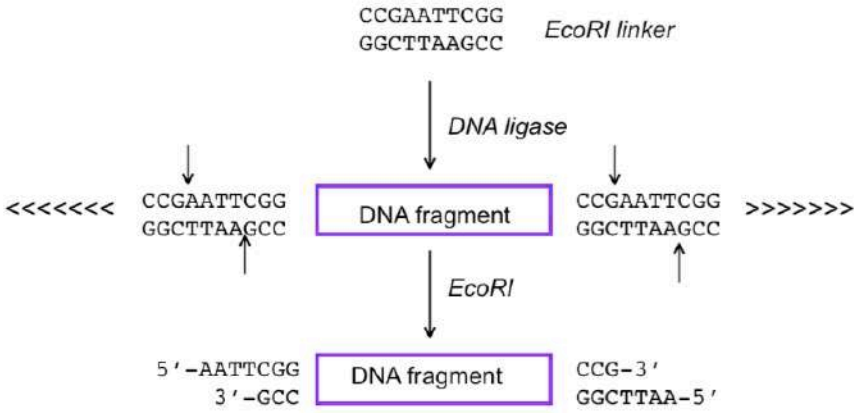
- Method for reducing vector background
- Alkaline phosphatase → removes 5' phosphates from the ends of DNA molecules
- E.g. vector and insert are both prepared with EcoRI ends
    - Linearised vector is then treated with alkaline phosphatase = removes the 2 diagonally opposite 5' phosphates
- Vector cannot be religated to itself = remains linear bc incapable of reforming the original phosphodiester bonds at the EcoRI cut site
    - ∴ vector background is reduced to zero
- Phosphate-free linearised vectors can be ligated to the insert bc the insert still has its two 5' phosphates
    - Circular recombinants may be formed with diagonally opposite phosphodiester bonds (one on each strand) and diagonally opposite non-ligated hydroxyl ends
    - Half-closed circular plasmids may be transformed into competent E. coli cells as efficiently as fully closed circular plasmids
    - Newly replicated recombinant plasmids in the cells will be fully closed circles



- Linkers and adaptors can increase the # of cloning options

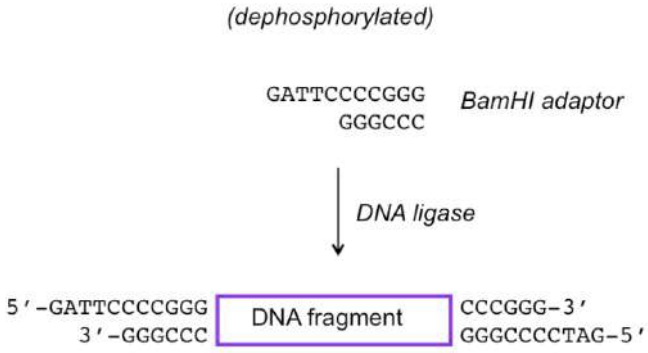
Modifying Restriction Sites: Linkers

- Converting restriction enzyme blunt ends to staggered ends
- Blunt-ended 'linkers' are ligated to the ends of the fragment
    - There is a range of linkers available that have internal restriction sites for most of the common enzymes that generate staggered ends
- E.g. an excess of linkers is used so that all of the available ends in the fragment sample will be 'linked'
    - Excess of linkers drives the reaction and also results in multiple end to end (tandem) linkers being attached
    - However, when the ligated linkers are subsequently cut with EcoRI, all of the added linkers are digested away
    - Only the last two that remain attached to the fragment remain
        - Have "designer" EocRI ends



Modifying Restriction Sites: Adaptors

- Converting restriction enzyme blunt ends to staggered ends
- Difference = adaptors have 'ready-made' restriction enzyme staggered sites that, once ligated to the blunt-ended fragment, require no further treatment other than now being used in a cloning scheme
    - ∴ adaptors cut out the need for the restriction reaction step to generate the enzyme site
- Adaptors must be dephosphorylated
    - 5' phosphates are removed by alkaline phosphatase digestion
    - Required to prevent the staggered ends of the adaptors from self-ligating, which would the require an additional restriction enzyme step

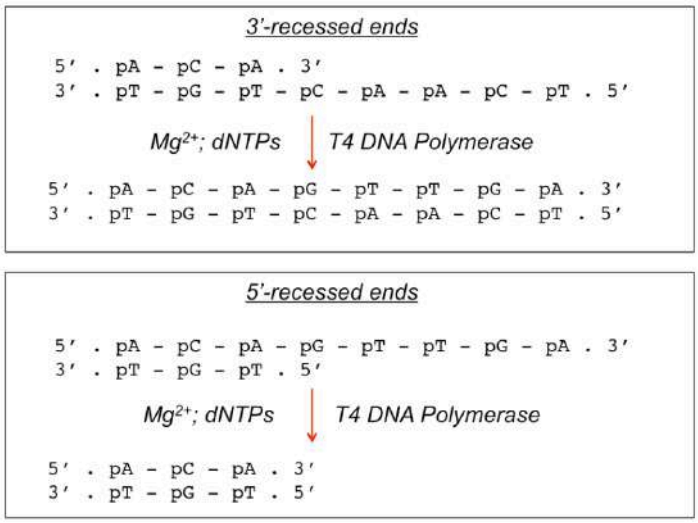


End-Filling

- Converting restriction enzyme staggered ends to blunt ends
- T4 DNA polymerase → end-fills either 3' or 5' recessed staggered ends

- Multifunctional enzyme
- Adds nucleotides to a single-stranded recessed 3’ end (top) or digest excess 3’ nucleotides (bottom) that overhang a staggered site
- Only adds in 5’ to 3’ direction → if it cannot fill in, snips off overhanging nucleotides

- Both types of staggered sites can be generated by different restriction enzymes



Methylation of Sites

- Mimics the methylase-restriction host bacterium system
- Methylase adds methyl groups to one of the nucleotide bases within the restriction site of it’s partner enzyme
  - ∴ prevents the host’s restriction enzyme from digesting its own DNA
- E.g. EcoRI methylase adds methyl groups to the opposite first adenine bases across the EcoRI site
  - Site is no longer a substrate for EcoRI as it is protected from enzyme binding and digestion
  - In vitro step used in MC scheme that requires a DNA molecule to be at a separate EcoRI site, but not at the now protected site, which could, for example, be within a gene sequence, whereas the non-protected EcoRI site would be external to the gene