SAMPLE

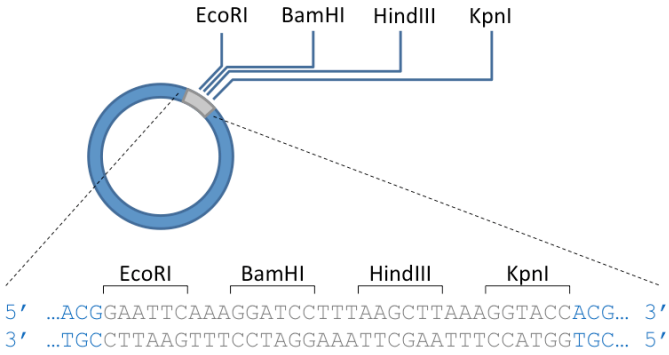
Lecture 18: Cloning – vectors and libraries

VECTORS

- **Definition** – a vector is a molecule used to transfer and replicate DNA put inside it.
- **Most commonly a plasmid, but doesn't have to be.** Can use viruses, cosmids and yaks.

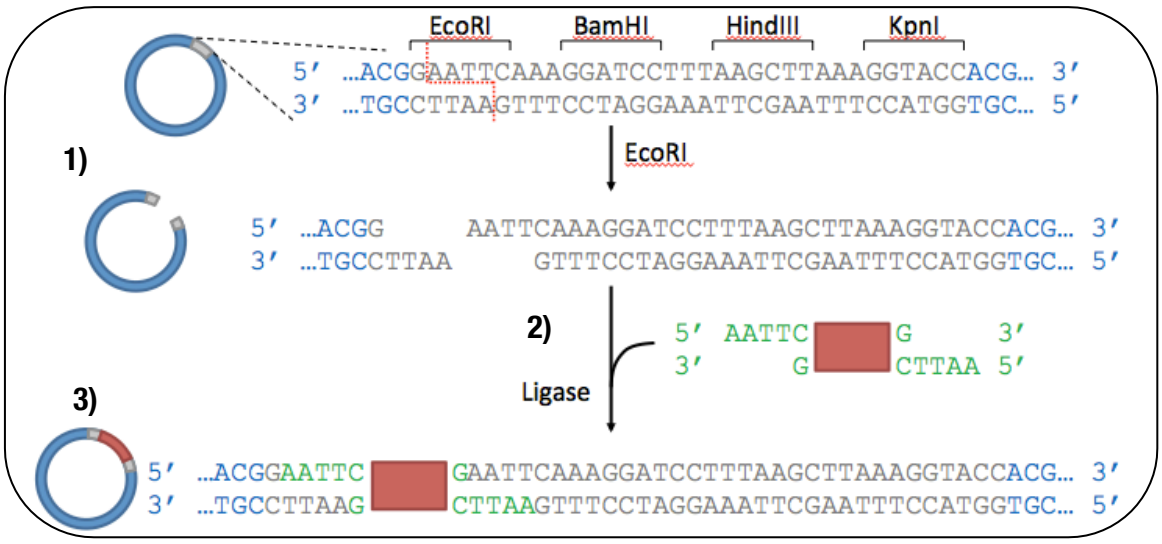
PLASMIDS

- Small, circular, ds DNA
- **Contains non-essential genes.** Essential genes (aka Genomic genes) are in the chromosome.
- Lab modifications
  - Make sure that the site the restriction enzyme recognises **only appears once** in the plasmid **(aka the sequence of the site is unique)**
  - This is so that the restriction enzyme doesn't cut the plasmid into pieces, making DNA insertion impossible.
  - **Multiple cloning site**: plasmids are designed to have a Multiple Cloning Site (MCS) or Polylinker, where recognised sequences of several different restriction enzymes sit close together.



STEP 1: DNA LIGATION INTO PLASMID

- General process
  1) Make cut in MCS with a restriction enzyme → sticky ends are formed.
  2) Insert DNA (**In Diagram**: the red rectangle) into plasmid (make sure it has **sticky ends** {the **AATTC** and **CTTAA** in the example below} so it can hybridise to the plasmid)
  3) Use **ligase** to stick ends together.



- Primer design
  - To **make a sticky end for ligation**, an extra sequence is added onto the **5' ends of primers** during PCR.
  - **This extra sequence is the restriction enzyme's recognition site**.
  - Extra sequence is not complementary and so hangs off during the process of PCR.

  - After PCR, you end up with **DNA of interest flanked by restriction sites**. (i.e. It has restriction sites attached to both ends)
  - By treating with the respective restriction enzyme (e.g. **EcoR1 recognises GAATTC**) it will **cut off extra bits** to create the sticky ends.

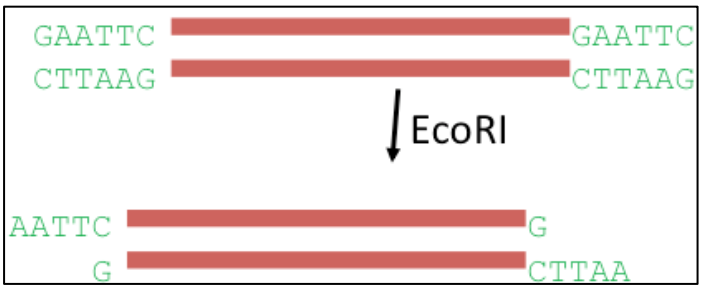


Diagram: EcoR1 recognising its restriction site (GAATTC). The actual cut is between the G and the A

  - **Cut plasmid with same enzyme**, bring DNA and plasmid together, bring ligase in to seal/attach backbone.
- DNA ligase
  - Once the DNA of interest forms H-bonds with the plasmid, it still isn't fully held in place.
  - This is because the backbones between the DNA strand and the Plasmid are not connected.
  - **Ligase forms the covalent phosphodiester bond that connects the backbones together**
  - Conditions for ligase to work:
    - **Sticky ends which are fully compatible** (every base pair including overhangs are complementary)
    - Or **blunt ends, which are always compatible** but harder to stick (no hydrogen bonds formed between bases).

STEP 2: AMPLIFICATION OF MODIFIED PLASMID

- We now want to make many copies of this modified plasmid by placing it into a bacterium.
- Steps:
  1) Bacteria made **competent*** (by treating with CaCl)→ plasmid taken up by **bacteria**

2) Grow bacteria on agar plate → bacteria grows into colonies, with each colony originating from a single bacterial cell

- **Plasmids have their own origin of replication , oriV** (V for "vegetative" , i.e. can make many copies of the plasmid).
- Therefore one bacterium can have multiple copies of the plasmid → shares the copies when the cell divides.

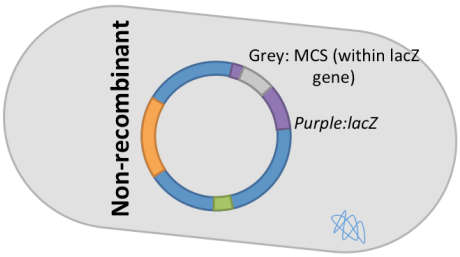
- ***competent**: "*Able to take in plasmids*"

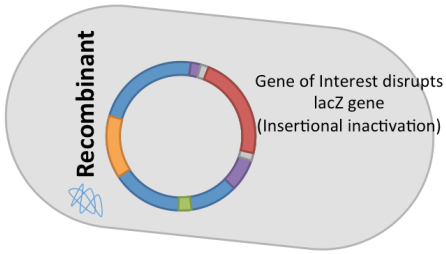
Step 3: Isolating the Transformants

- **Transformant:** "*A bacteria that has taken up the modified plasmid*"
- Looking at each colony, which one's have taken up the modified plasmid?
- **Our Plasmid has been engineered to have gene (ampR or bla) that grants resistance to the antibiotic ampicillin** (codes for beta-lactamase – enzyme that breaks down ampicillin).
- Therefore **transformants are resistant to ampicillin**
- Therefore place ampicillin on agar plate → **non-transformants will die**
- **Selectable Markers**: "*Genes that produce easily observed characteristics E.g. ampR gene"*
- But this doesn't tell us which bacteria have taken up plasmids with the desired gene/DNA.

Step 4: Selecting for Plasmids with DNA Insert

- Sometimes, inserting DNA into the plasmid (step 1) fails, and the plasmid simply sticks back together without taking up the gene. How do we test which bacteria is recombinant (i.e. has the insert)? Sev
- **Blue-white screening:**
  - Insert multiple cloning site within a gene (e.g. lacZ). This gene is known as a **screenable marker**.
  - If **no insert** placed → this gene is expressed → beta-galactosidase is made → breaks down X-gal to form a **blue compound**
  - If **insert successfully placed** → inserted DNA disrupts the reading frame of lacZ → no beta-galactosidase produced → no **blue colour**
  - Therefore: Place X-gal into agar jelly → colonies with recombinant DNA will remain white (I.e. will not turn blue)
- **Screenable Marker**: "*Genes that produce visibly different characteristics e.g. lacZ gene*"

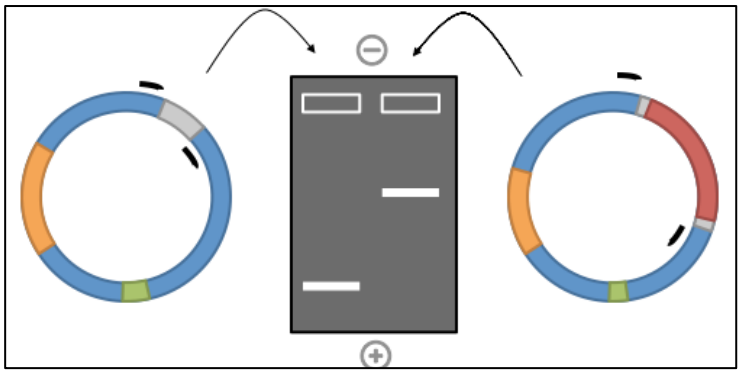




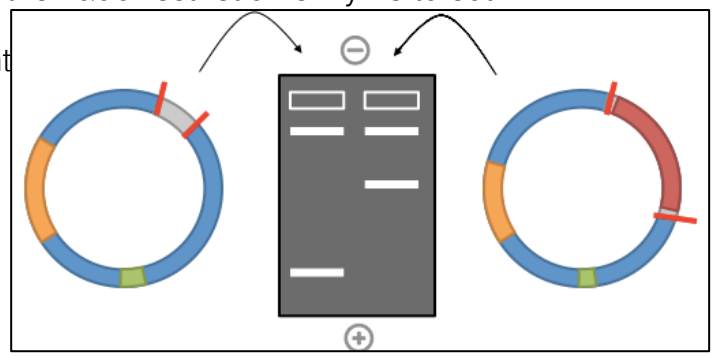
Step 4 cont.: Other Techniques

- **First method** – use gel electrophoresis.
  - Pick a colony, put on second plate (as a backup), and amplify in PCR tube, then put on gel.
  - If no gene inserted, plasmids are going to be small. If gene inserted, plasmids are going to be large.

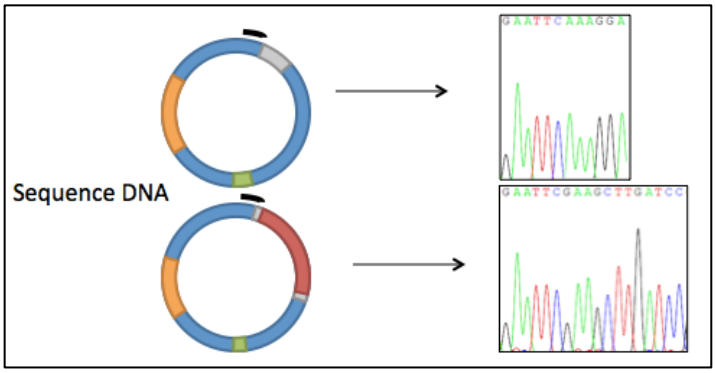
- This affects the distance travelled in gel.



– **Second method** – use **restriction enzymes.**
  - Grow culture, purify plasmid DNA, then add restriction enzyme to cut and use gel electrophoresis.
  - There will be one plasmid fragment and another separate fragment.
  - This fragment will have only the multiple cloning site, or the MCS + added gene, which will be larger.



– **Third method** – use **sequencing.**
  - This technology
  - Start from primer. Read the start of the MCS. The gene will show up if it has been inserted.



SUMMARY

– Plasmid Vectors used in laboratory are:
  - **Circular**
  - Have a **Multiple Cloning Site**: A site where the restriction site of many different Restriction Enzymes Exist
  - Are **small**: Modified Plasmid Vector must be small enough to get through cell's membrane and into the cell
  - Have their own origin of replication, **oriV**: V stands for "vegetative", as the plasmid can be replicated more than once, like a vegetable can be grown more than once
  - Has **selectable and screenable markers**