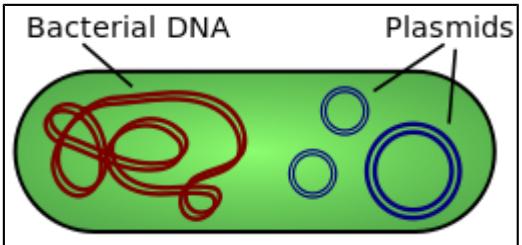
Bacterial Genetics and Complementation

George Beadle and Edward Tatum first described the concept that each gene corresponded to an enzyme in a metabolic pathway by exposing the yeast *Neurospoara crassa* to mutagenic conditions (**Beadle & Tatum, 1941**). Following these procedures Josua lederberg continued these studies with Tatum where they generated two mutants strains in *Escherichia coli* . These bacteria were **auxotrophs**, unable to generate some basic nutrients necessary to sustain their growth. The two strains were described as met^{-} bio^{-} Thr^{+} Leu^{+} Thi^{+} (Strain A) and Met^{+} Bio^{+} thr^{-} leu^{-} thi^{-} (Strain B). Strain A can sufficiently synthesize the amino acids threonine, leucine and the cofactor thiamine while deficient in producing the cofactor biotin and the amino acid methionine while the converse was true of Strain B. When either of these two strains were plated onto minimal media, no growth occurred. Supplementing minimal media with methionine and biotin permitted Strain A to grow as normal. When the two strains were mixed together and plated on minimal media, there was growth of bacteria. The two strains were capable of complementing each other in some way as if a sexual exchange of genetic material had occurred (**Lederberg & Tatum, 1946**).

Bacteria are equipped with all the necessary capacities to replicate DNA. Common bacterial species have bee adapted for use in the lab to carry DNA and propagate it for uses in biotechnology. In addition to chromosomal DNA of the bacterial genome, bacteria also have extrachromosomal DNA called **plasmids**. These plasmids replicate independently of the bacterial chromosome and can occur in high copy. These circular pieces of DNA are modified in labs to carry specific pieces of DNA so they can be studied or used for expression into proteins. Plasmids can naturally carry important traits, including antibiotic resistance. Plasmids are relatively small, ranging in size from 1000 bases to 1,000,000 bases long (1kb-1000kb).



Bacterial DNA usually exists as a large circular chromosome (red). Plasmids are extrachromosomal and autonomously replicating pieces of DNA (blue).
Credit: Spaully (CC BY-SA 2.5)

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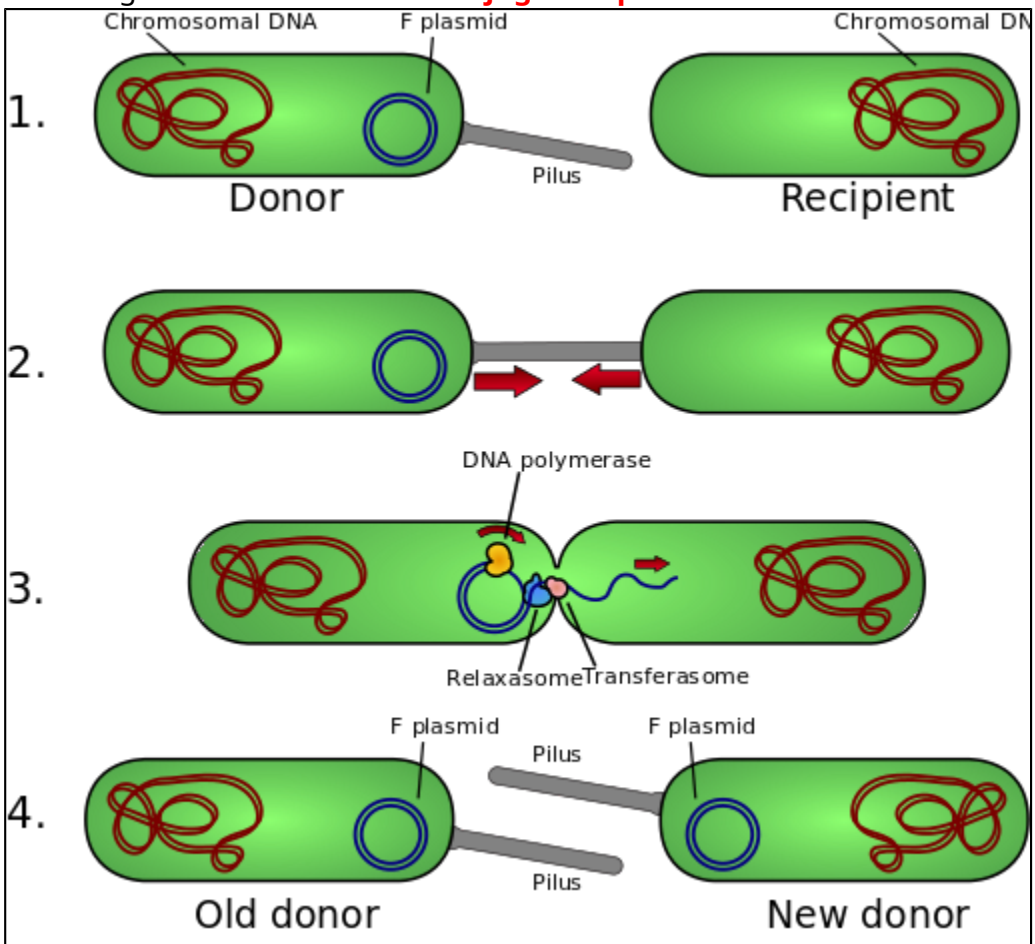
Through a process called **conjugation**, bacteria can “sexually” transfer genetic material to another by passing plasmids through a structure called a **conjugation pilus**.



Conjugation process between a plasmid bearing donor and a plasmid-less recipient. The donor creates a conjugation pilus to create a cytosolic bridge with the donor where the plasmid is replicated into the recipient through the rolling circle method of replication. The recipient then becomes competent to act as a donor.

Credit: Adenosine (CC-BY-SA 3.0)

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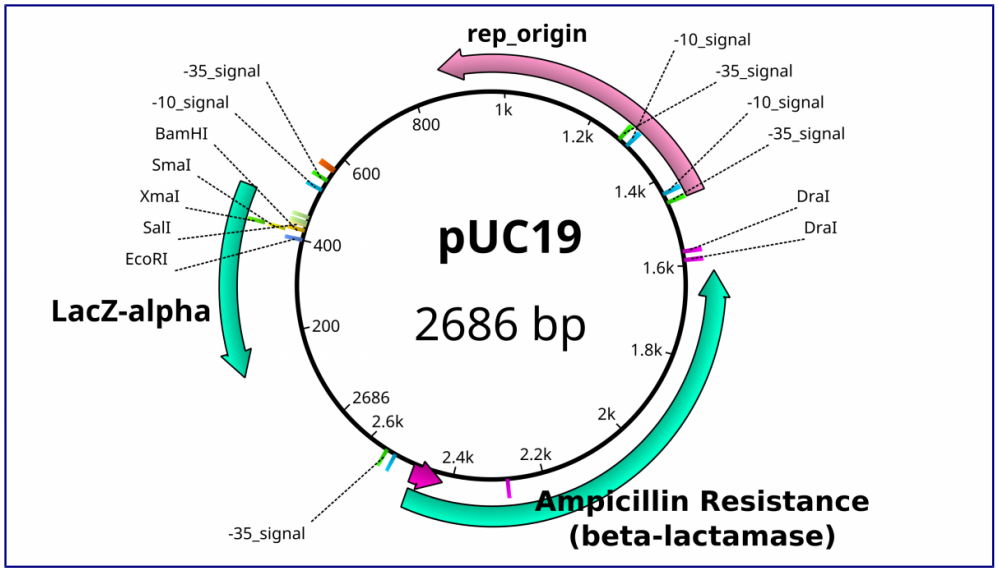
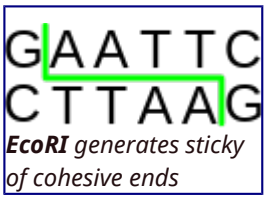
Features of Plasmids

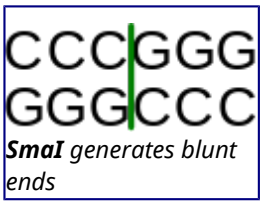
Plasmids that are designed by Biologists to shuttle pieces of DNA for study are referred to as **vectors**, because they *move* a piece of DNA.



These plasmid vectors have the same hallmarks as traditional plasmids with the capacity to replicate independently of the bacterial genome. The feature that allows these DNA's to replicate is called an **origin of replication** (ori) that is usually rich in A's and T's. However, these plasmid vectors have the additional properties that make them easy to work with and distinguishable from bacterial plasmids; a selection marker and a multiple cloning site. A **selection marker** usually comes in the form of a gene that encodes resistance to a specific antibiotic. In the pictured plasmid, Ampicillin resistance granted by the β-lactamase gene. The **multiple cloning site** (**MCS**), also known as the polylinker, is the location in which the DNA of interest is incorporated into the vector. MCSs are defined by a set of unique sites where the DNA can be cut by **restriction endonucleases** (**RE**). As the name implies, restriction enzymes are “restricted” in their ability to cut or digest DNA. The restriction that is useful to biologists is usually **palindromic** DNA sequences. Palindromic sequences are the same sequence forwards and backwards. Some examples of palindromes: RACE CAR, CIVIC, A MAN A PLAN A CANAL PANAMA. With respect to DNA, there are 2 strands that run antiparallelel to each other. Therefore, the reverse complement of one strand is identical to the other.



EcoRI *generates sticky of cohesive ends*



SmaI *generates blunt ends*

Restriction enzymes hydrolyze covalent phosphodiester bonds of the DNA to leave either “sticky/cohesive” ends or “blunt” ends. This distinction in cutting is important because an *EcoRI* sticky end can be used to match up a piece of DNA cut with the same enzyme in order to glue or ligate them back together. While endonucleases cut DNA, **ligases** join them back together. DNA digested with *EcoRI* can be ligated back together with another piece of DNA digested with *EcoRI*, but not to a piece digested with *SmaI*. Another blunt cutter is *EcoRV* with a recognition sequence of GAT | ATC.
By “cutting and pasting” DNA into vectors, we can introduce foreign or exogenous DNA into bacteria. This type of DNA is now called **Recombinant DNA** and is the heart of biotechnology.

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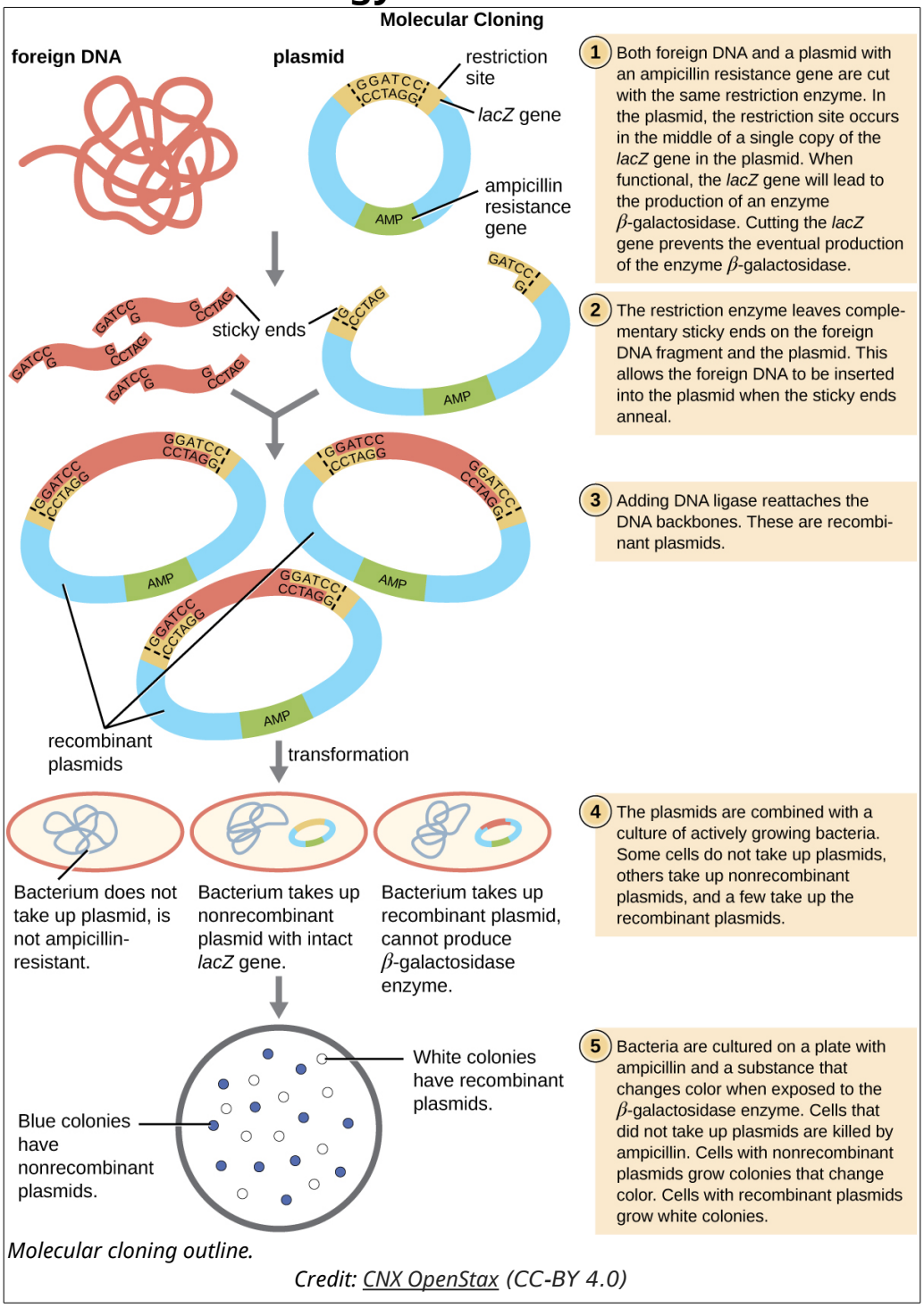
Recombinant DNA Technology



Molecular cloning outline.

Credit: CNX OpenStax (CC-BY 4.0)

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Additional Resources:

1. http://www.dnalc.org/resources/3d/20-mechanism-of-recombination.html
2. http://www.dnalc.org/view/16705-Animation-34-Genes-can-be-moved-between-species-.html

Questions for thought

1. Why do you think that origins of replication are made of A's and T's?
2. What is different about the types of bonds holding the double strands together versus phosphodiester bonds of the DNA backbone?
3. Can DNA digested with *SmaI* be ligated to DNA digested with *EcoRV*?
4. If so, which enzyme will be able to digest this new DNA?

References:

- *Beadle, G. W.; Tatum, E. L. (1941)*. "Genetic Control of Biochemical Reactions in Neurospora". *Proceedings of the National Academy of Sciences*. **27** (11): 499–506. doi:10.1073/pnas.27.11.499. PMC 1078370. PMID 16588492
- *Lederberg J, Tatum EL (1946). "Gene recombination in E. coli". Nature. **158** (4016): 558. doi:10.1038/158558a0*

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Alkaline Lysis

Once DNA is introduced and carried in bacteria, we would like to isolate the DNA again for further manipulation. In order to do so, bacteria containing the plasmid of interest is grown in a liquid culture of nutrient rich broth made of yeast extract called Luria-Bertani Broth (**LB**). These cultured bacteria are grown until they are of a high concentration over night. They are harvested through centrifugation and the broth is removed. The resulting pellet of bacteria is resuspended in a physiological buffer containing the chelator EDTA. A **chelator** is a chemical that removes divalent cations like Ca^{2+} or Mg^{2+} from solution. This is significant because divalent cations are necessary for DNA digesting enzymes to be active. By chelating the ions, the DNA we ultimately wish to purify will be safe from degradation.

After resuspension of the bacteria, an alkaline solution of 0.1N NaOH is mixed into the bacterial mix. This solution also contains an ionic detergent called sodium dodecyl sulfate (**SDS**) that aids in denaturing proteins and disrupting their interactions with the DNA. The mixture becomes viscous as the bacteria burst open and their contents leak into the solution. This basic solution is then neutralize with a potassium acetate buffer at pH5.5. As the solutions mix together, the pH approaches 7 and the potassium interacts with the SDS to cause a precipitation of the genomic chromosomal DNA and proteins. In order to separate the precipitate from the solution, the mixture is centrifuged at high speed to pellet the genomic DNA and protein. The **supernantant**, or solution, is transferred to a column containing a **silica membrane**. Under high salt conditions, DNA adheres to glass or silica. By passing the solution through this column, the plasmid DNA in the supernatant is trapped onto the silica membrane and removed from solution. Additional washes are used to removed stray contaminants and remove the excessive salt. Plasmid DNA is finally removed from the column through **elution** by a low salt buffer. This low salt buffer is Tris pH 8 with EDTA (**TE**). Plasmid DNA can be stored stably in TE buffer in the freezer for extended periods.

Exercise 1: Plasmid DNA Mini-Prep by Alkaline Lysis

1. **Inoculate** 2 ml of rich medium (LB, YT, or Terrific Broth) containing the appropriate antibiotic with a single colony of transformed bacteria. Incubate the culture overnight at 37°C with vigorous shaking. (This is what you were provided)
    1. Each group should take 2 cultures
2. **Centrifuge** culture tubes directly at maximum for 5 minutes.
    1. If incapable of spinning in these tubes, transfer 1.5 ml of the culture into a microfuge tube (Eppendorf Tube).
    2. Centrifuge at maximum speed for 30 sec.
3. When centrifugation is complete, pour the broth solution into a container of bleach
4. **Resuspend** the bacterial pellet in 250 µl of ice-cold P1 solution by vigorous shaking and transfer back into a microcentrifuge tube.
    - P1 is a physiological solution of 50mM Tris at pH 8
    - P1 contains a Chelator called EDTA
        - chelators bind up excess divalent cations that are required for DNAse activity
5. **Lyse**: Add 250µl of P2 solution to each bacterial suspension. Close the tube tightly, and mix the contents by inverting the tube gently five times. Do not vortex! Store the tube on ice.
    - This is the lysis buffer containing the detergent Sodium Dodecyl Sulfate and NaOH

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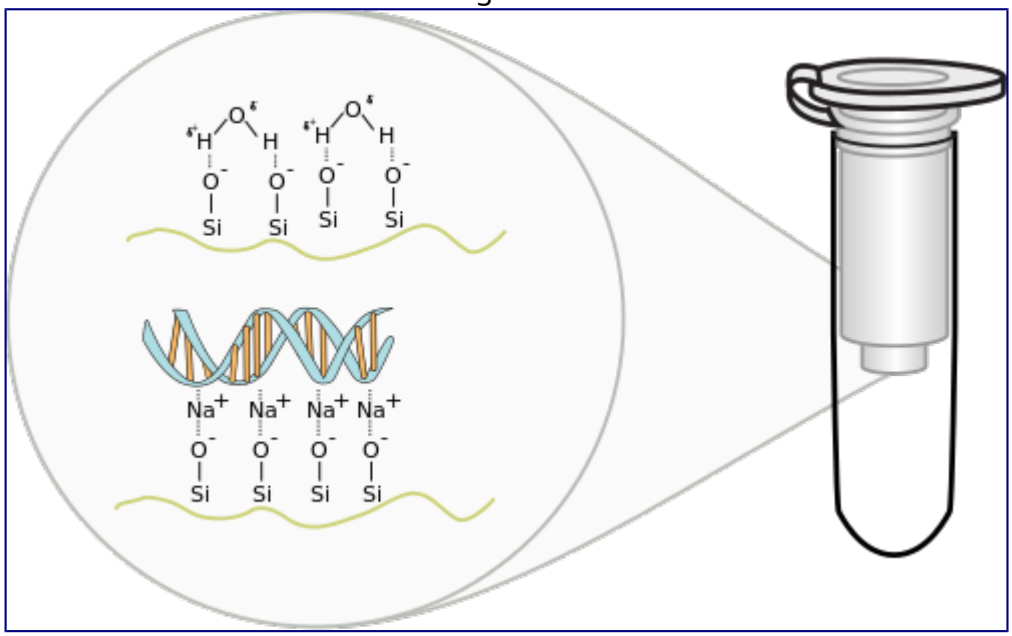
6. **Neutralize:** Add 350 µl of ice-cold P3 solution. Close the tube and disperse lysis solution by inverting the tube several times. Store the tube on ice for 3-5 minutes.
    - This is the neutralization buffer containing Potassium Acetate
    - Neutralization restores pH to near 7 and also causes the precipitation of genomic DNA and proteins into a gloopy mess (snot-like)
7. Centrifuge the bacterial lysate at maximum speed for 5 minutes in a microfuge.
    - Snot-like substances should be tightly packed into a pellet at the bottom of the tube after this step
    - the solution or supernatant contains the plasmid DNA
8. **Column Purification of DNA**: Transfer the supernatant to a fresh tube with silica-membrane column
    - DNA likes to bind to glass under high salt conditions
    - the white membrane is made of a glass fiber
    - 

9. Centrifuge the supernatant through column for 1 minutes at maximum speed in a microfuge.
    - The DNA will be bound to the membrane on the column (silica)
10. **Wash**: Discard flow-through and wash column with 500 µl PE. Centrifuge the supernatant through column for 1 minutes at maximum in a microfuge.
    - PE is a solution that helps to wash away the non-specifically bound substances
11. Discard flow-through. Wash Column with 700 µl PE. Centrifuge the supernatant through column for 1 minutes at maximum in a microfuge. Discard flow-through and repeat spin to dry column.
12. **Elute** the nucleic acids in 50 µl of TE (pH 8.0) by binding for 1 minute and spinning at maximum speed for 1 minute.

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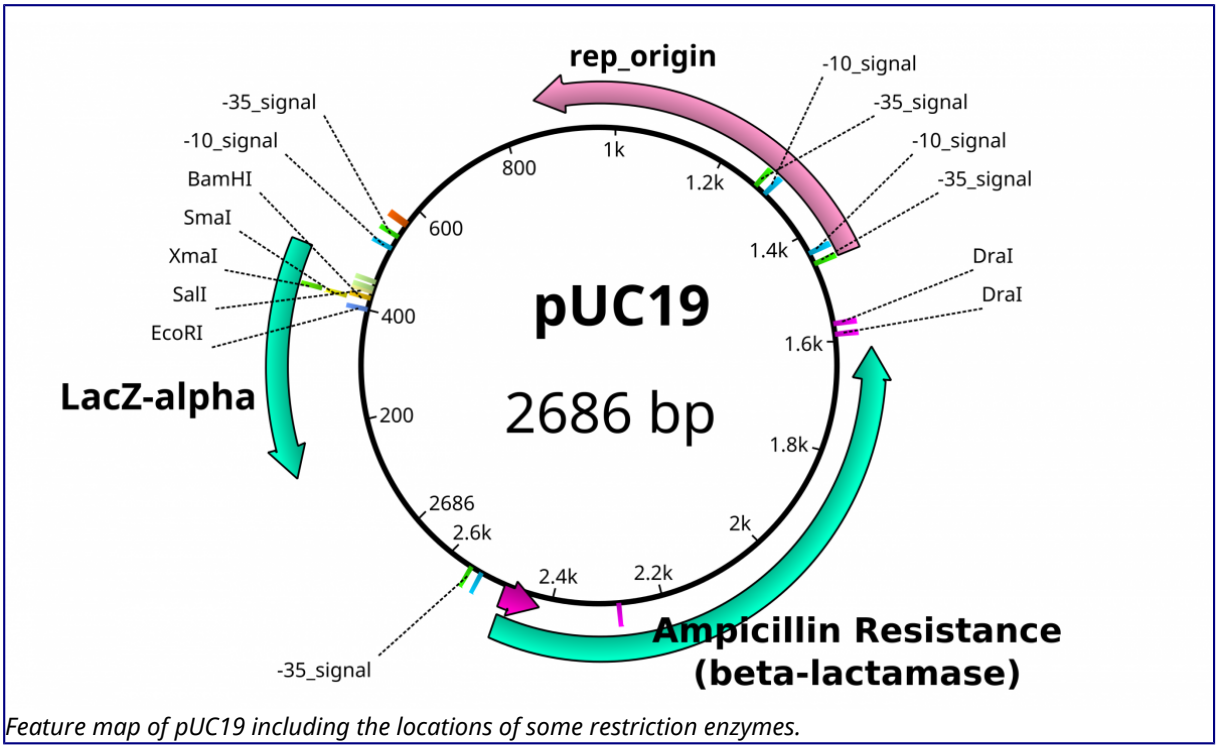
Identification of Plasmid DNA

Once plasmids are isolated, they require identification. Plasmid vectors have known sequences and are mapped of their major features. Knowing the sequence of these pieces of DNA means knowing the locations of RE digestion sites. By using Res, digesting plasmids into known sizes aids in verification of plasmid identity without the need to have the entire plasmid re-sequenced. A common plasmid is called pUC18 or pUC19. The “p” stands for plasmid, the “UC” stand for University of California (where it was designed) and 18 or 19 refer to the difference in the MCS. This plasmid is 2,686 base pairs or ~2.7kb (kilobase) long with a single *EcoRI* site in the MCS. Another plasmid of interest in learning Molecular Biology is called pGlo. This plasmid has a jellyfish gene in the MCS that codes for a protein that will fluoresce green when expressed under UV light. pGlo is 5.4kb long and contains a single *EcoRI* site. Once digested, by an enzyme, these plasmids can be identified based on size separation on an agarose gel. Usually, it is best to identify by using 2 different REs. Digestion is important before size comparisons since circular DNA migrates through agarose differently than linear DNA. Additionally, circular DNA can sometimes be “super-coiled” and lead to very rapid migration despite the size.



Feature map of pUC19 including the locations of some restriction enzymes.

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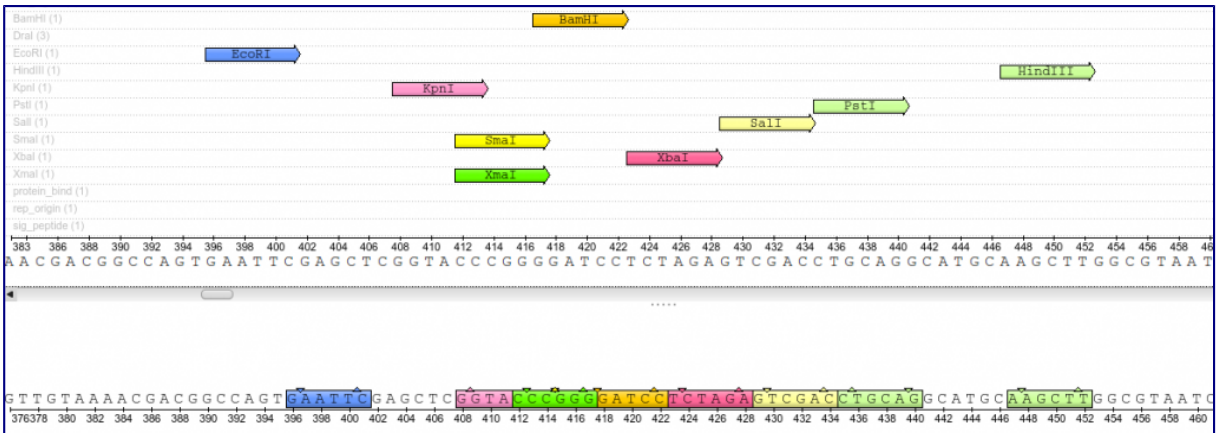
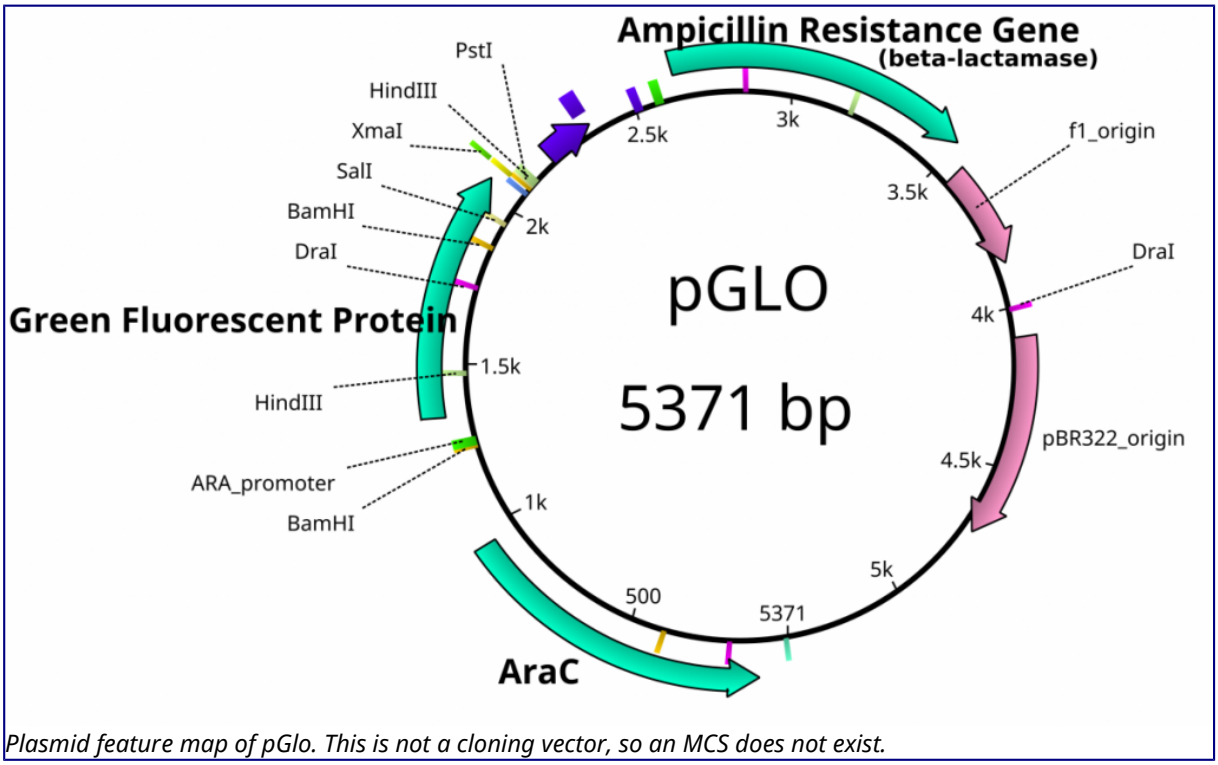


Close up view of the pUC19 Multiple Cloning Site (MCS). These restriction sites appear only once throughout the entirety of the plasmid sequence.



Plasmid feature map of pGlo. This is not a cloning vector, so an MCS does not exist.

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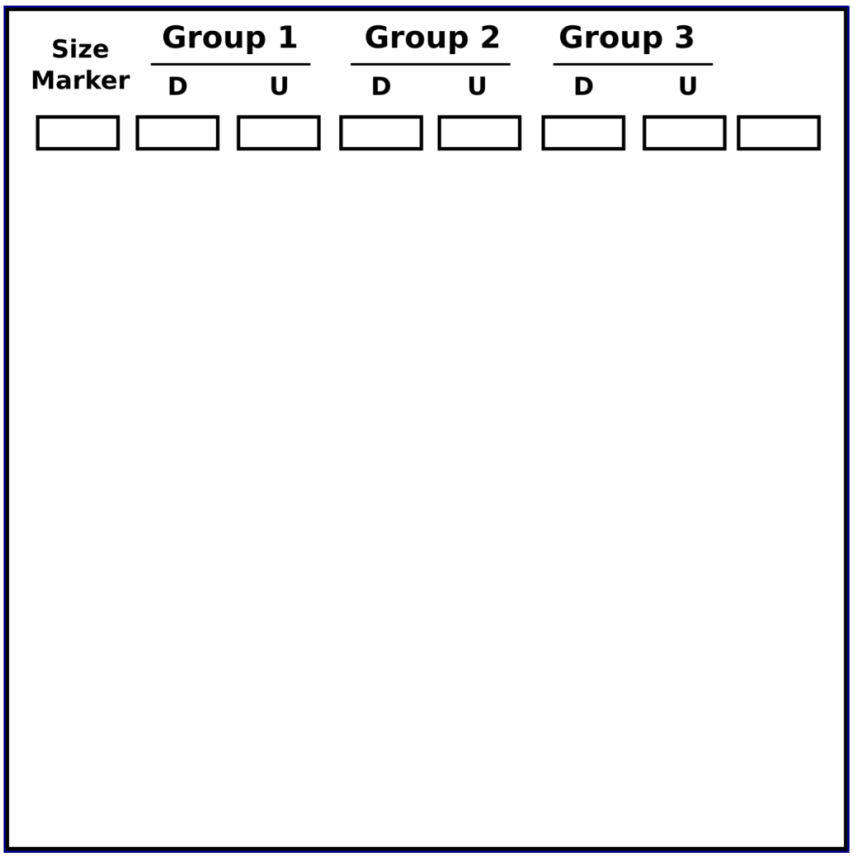
Exercise 2: Restriction Digestion Identification of Plasmids



1. The class should prepare 2X 0.8% agarose gels by preparing 0.4g agarose in 50ml TBE buffer
    1. Melt agarose solution by microwaving for 1 minute
    2. Add 5µl Sybr Safe solution into 100ml gel solution
    3. Pour this solution into a casting tray inside the refrigerator
    4. insert a comb
2. To a new tube add 2µl of plasmid DNA to 8µl of *Eco*RI fast digest mixture
    1. 1µl Fast Digest Buffer
    2. 1µl Fast Digest *Eco*RI enzyme
    3. 6µl H_2O
3. Incubate at 37°C for 10 minutes
4. add 2µl of loading buffer to digestion mixture
5. in a separate tube combine 3µl plasmid DNA with 2µl loading buffer and 7µl of H_2O
6. Load gel with an appropriate size ladder in the first lane, in the next lanes load the Digested plasmid, then the undigested plasmid.
    - 3 groups can load onto one gel
    - U=undigested plasmid
    - D=digested plasmid
7. Run gel at 110V for 30 minutes and visualize on a UV transilluminator
8. Document with your camera

Additional Resources

- For help on this problem, please try out the *In silico* digestion activity.

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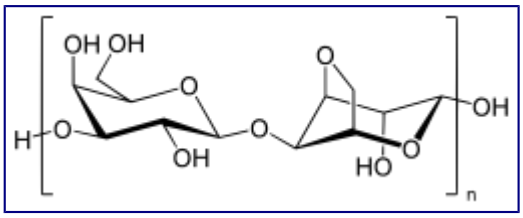
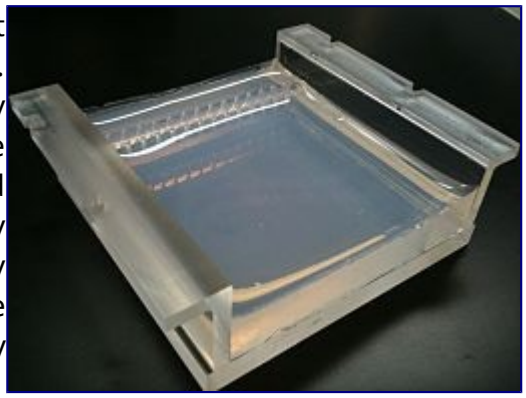
Agarose Gel Electrophoresis

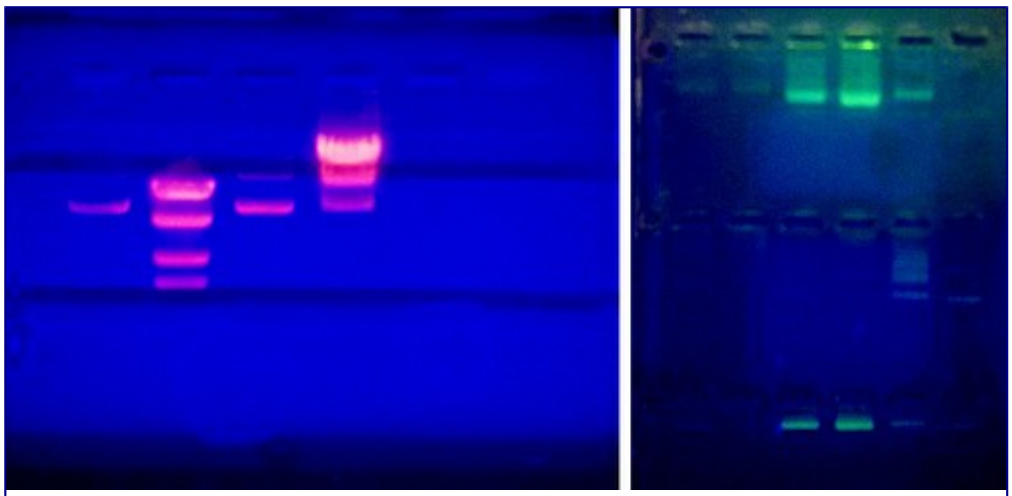


Agarose is a linear carbohydrate polymer purified from the cell walls of certain species of algae. Agar is a combination of the crude extract that contains agarose and the smaller polysaccharide agaropectin. When dissolved and melted in liquid, agarose strands become tangled together to form a netting that holds the fluid in a gel. Reduction of the fluid creates a higher percentage gel that is firmer and contains smaller pores within the netting.



Placing a **comb** within the melted agarose creates spaces that allow for the insertion of samples when the gel is solidified. Molecules can traverse through the pores as they are drawn by electrical currents. Charged compounds will migrate towards the electrode of opposite charge but migration rate will be influenced by the size of the molecules. Smaller compounds can easily traverse through the webbing while larger items are retarded by the pore size. Follow this simulation to get a better idea of how we use **Agarose Gel Electrophoresis** in molecular biology to study DNA fragments.

DNA molecules are not readily visible when **resolved** (separated) on an agarose gel. In order to visualize the molecules, a DNA dye must be administered to the gel. In research labs, a **DNA intercalating agent** called Ethidium Bromide is added to the molten gel and will bind to the DNA of the samples when run. Ethidium Bromide can then be visualized on a UV box that will fluoresce the compound and reveal bands where DNA is accumulated. Since Ethidium Bromide is known as a carcinogen, teaching labs will use a safer DNA intercalating agent known as Sybr Green. This can be visualized in a similar fashion, but will fluoresce a green color instead.



Agarose gels visualized on a UV transilluminator. Left shows a gel with Ethidium bromide. Right shows a gel with Sybr Green.

Agarose gels are made of and bathed in a buffered solution, usually of Tris-Borate-EDTA (**TBE**) or Tris-Acetate-EDTA (**TAE**). Regardless of buffer solution, the buffer provides necessary electrolytes for the current to pass through and maintain the pH of the solution.

DNA samples are prepared in a buffer similar to the solution that it will be run in to ensure that the phosphate backbone of the DNA remains deprotonated and moves to the positive electrode. Additionally, **glycerol** or another compound is added to this buffer in

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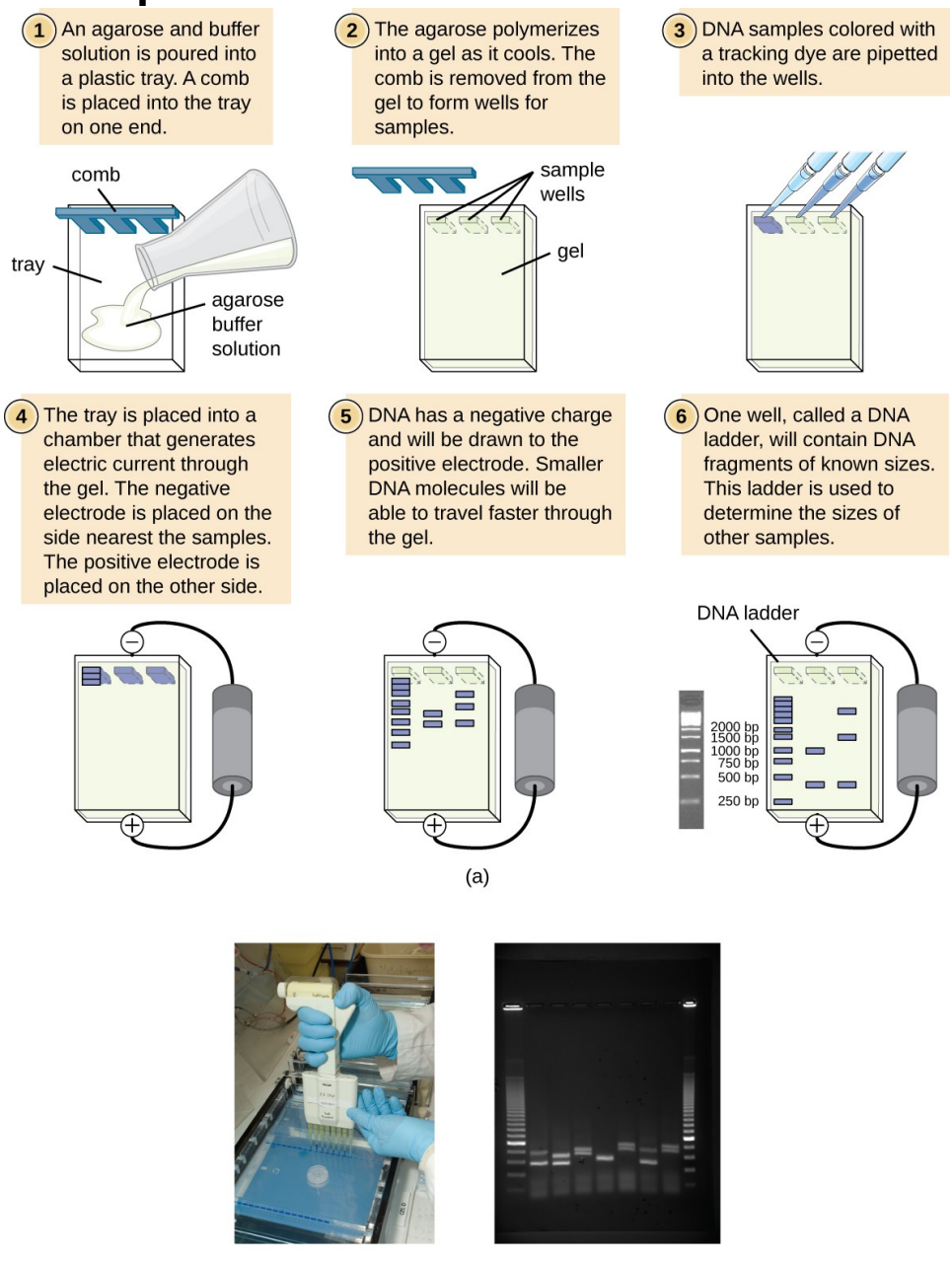
order for the solution to sink into the wells without spreading out. A dye is often included in this loading buffer in order to visualize the loading in the wells and to track the relative progression of gel.

Agarose Gel Set-up



External Resources

- Gel Electrophoresis Simulation
- The Structure of DNA http://www.nature.com/scitable/topicpage/discovery-of-dna-structure-and-function-watson-397
- http://learn.genetics.utah.edu/content/science/forensics/

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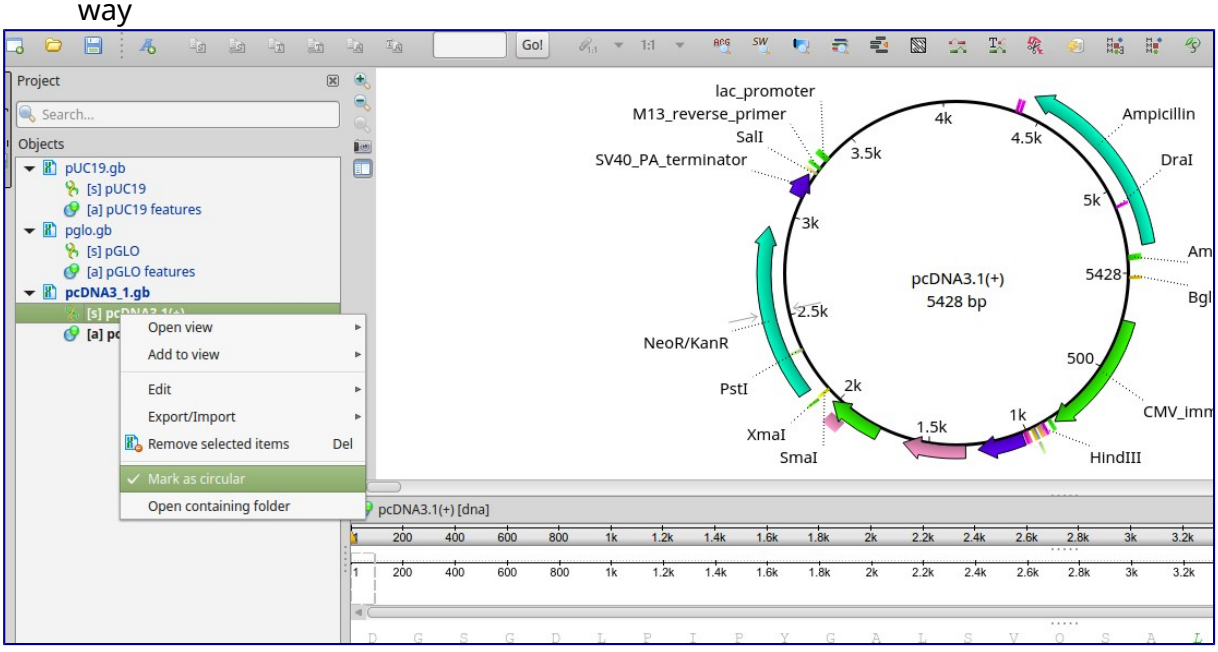
Concept

Restriction enzymes act as molecular scissors. The ones we use in Molecular Biology are those that cut within known sequences that occur often enough, yet rare enough to cut our DNA into analyzable fragments. Molecular Biologists often use 6-cutters. This means that the site of digestion is "restricted" to a recognition sequence of 6 nucleotides. These nucleotides are usually palindromic as discussed before.

Imagine a linear piece of DNA as a piece of string. When cutting the string once, you result in 2 pieces. Now consider a plasmid. This was already discussed to be a circular piece of DNA. With a circle, there are no ends. cutting the plasmid once results in 1 piece of DNA as opposed to 2. Keep this in mind when digesting circular plasmids. In this case, nucleotide 1 is adjacent to and contiguous with the last nucleotide of the sequence.

In silico digestion

1. This activity is meant to supplement Identification of DNA (activity)
2. Launch UGENE and open the following files:
    - **pGlo.gb**
    - **pUC19.gb**
    - **pcDNA3.1.gb**
    - **pUC with Insert**
3. In the **Objects** menu, right-click on the sequences and select "**Mark as circular**"
    - the sequences will now be treated as circular DNA
    - the first nucleotide and the last nucleotide become adjacent as a continuous sequence this way



4. From top menu, select **Actions** → **Analyze** → **Find restriction sites**

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- this will load a set of default restriction enzymes
- if none are loaded, select them individually (found in alphabetical order)
    - choose: BamHI, BglII, ClaI, DraI, EcoRI, EcoRV, HindIII, KpnI, PstI, SalI, SmaI, XbaI, XmaI, NotI

5. **Actions → Cloning → Digest into fragments**
    - Choose your enzyme
    - Try *Hind*III for each plasmid
    - Fragments will be added to the circular view and annotations for these fragments will be added
    - You can use this information to calculate how many fragments come from enzymes based on how many times they cut and by the nucleotide coordinates found in the annotation of the fragment

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