Lab 3: First restriction digest and agarose gels (phage lambda).  

**Discuss:**

**Restriction endonucleases**
- Cut double stranded DNA
- Sequence specific
- Naturally occurring in bacteria; hundreds exist; protect from viral infection
- In vivo, sequence-specific methylases protect the bacterial DNA
- Recognition sequences are 4-8 bp, usually palindromic. Shorter site = more occurrences/cuts
- Sticky ends often created
- Handling: keep on ice; tiny volumes
- Activity measured in units: 1 unit cuts one microgram of DNA in one hour
  - Why use more units? Need less time; compensate for poor activity.
  - 10% volume rule: too much glycerol causes star activity
- Each has a specific buffer (primarily salt concentration) at which it is most active and accurate; cannot always combine multiple enzymes in one tube
- Most are most active at 37 but some work best at other temps

**Bacteriophage lambda:**
- Virus which infects E. coli
- Linear DNA genome of 48,502 bp
- See map for restriction sites of Bam, Eco, Hind.
- In a LINEAR molecule, 5 cut sites = 6 bands

**Gel electrophoresis:**
- DNA is a negatively charged molecule (phosphate groups in backbone)
- Will migrate toward positive pole under voltage
- RED = positive  BLACK = negative
- Performed in Tris-Borate-EDTA buffer (TBE) running buffer

**Agarose**
- A polysaccharide linear polymer
- Most types melt just before boiling and solidify when cooled into a gel like Jello or agar plates
- Formed into a “gel” with “wells” at one end for inserting DNA samples
- Placed in electrophoresis chamber, run current through it and DNA moves
- Acts like a sieve: higher concentration = smaller “holes”
- Large DNA molecules move more slowly (greater “drag”) than small DNAs & RNAs
- Choose a concentration (w/v %) appropriate to the size of DNA fragments you wish to resolve
- Generally can get resolutions in the 100 bp-10 kbp range depending on agarose concentration
  - To separate small DNAs: increase [agarose]; To separate large DNAs: decrease [agarose]
- DNA shape (conformation) affects mobility (more on this later)

**Loading/tracking dyes**
- Contains glycerol or 40% sucrose to weigh down the sample for loading
- Bromophenol blue: 300 bp  (fast) Purplish color
- Xylene cyanol: 4 kb (slow) blue

**“X” nomenclature**
- Restriction buffers are usually 10X
- Loading dye is 6X
- Means concentrated X-fold
- Can use C1V1 = C2V2 to solve where concentration is expressed in units X
- Working concentration is by definition 1X
Lab: Restriction analysis of plasmid DNA

Plasmids:
- Small, circular DNA molecules found in bacteria
- Physically independent of the host chromosome
- Must have an origin of replication (ori) to be maintained as the host divides
- Can be present in many copies per cell (each plasmid has a typical copy number)
- May carry genes for antibiotic resistance
- Commonly used as cloning vectors (carriers of foreign DNA in recombinant DNA work)
- Plasmids can be easily isolated from bacteria, cut, pasted, manipulated, studied, etc. and then reinserted back into new host bacteria by transformation
- Useful plasmids are fully sequenced so all restriction enzyme recognition sites are known
- Sites for enzymes which cut only once in the plasmid can be clustered in a small region called the multicloning site (MCS) or poly linker. Here, foreign DNA can be inserted without losing any of the plasmid DNA.

Methylation system:
- What protects bacterial chromosomes from their own restriction enzymes? Sequence-specific DNA methylases
- These DNA methylases recognize the same sequence as the cell’s restriction enzymes
- At these specific sites, a methyl group is added to a nucleotide. This protects from restriction enzyme digestion.
- Methylases are: sequence specific; and they do not cut DNA (they are not nucleases)

Lab 5: Rapid colony transformation

1. Preincubation: Cells are incubated at 0 degrees C, in presence of cations (Ca++)
2. Incubation: DNA is added to cells, more time at 0 degrees
3. Heat shock: Brief exposure to 42oC “thermal imbalance”

Mechanisms of action: ampicillin (inhibits peptidoglycan synthesis; bacteriostatic)
Kanamycin (inhibits protein synthesis; bactericidal)
(chapter 2 in text) How this relates to need for a recovery period.

Today’s lab uses bacteria taken from colonies on a plate. Efficiency of transformation for this method is very low.

Efficiency of transformation: # transformed colonies per microgram plasmid DNA
Competent cells: cells which have been treated to make them “transformable” (able to take up DNA)

For rapid colony method, expect $5 \times 10^3$ to $5 \times 10^4$

This is acceptable for purified, intact plasmid DNA.

However, ligated DNA (relaxed circular and linear plasmid DNAs) gives many fewer transformants so a transformation method with a better efficiency of transformation is required.

To prepare cells which are more competent (i.e., have a higher efficiency of transformation) must start with bacteria actively dividing in mid-log phase broth culture.
Satellite colonies: colonies that do NOT contain antibiotic-resistant bacteria but that grow in the “shadow” of resistant colonies because the antibiotic has been broken down locally in the agar

Plasmid conformations:
- Linear: plasmid with cuts in both strands
- Nicked circular: plasmid with one DNA strand cut or “nicked”; this releases the supercoiling and leaves a large, floppy circle with slow mobility in agarose.

When reading gels: consider
1. Conformation
2. Partial digests
3. Multimers / concatemers

Multimers are concatemers/fused products of several plasmids recombined together. Happens naturally if plasmids are cultivated in \textit{recA+} strains of bacteria. These plasmids are several times as large as the individual plasmid and therefore run very slowly in agarose (regardless of their conformation).

<table>
<thead>
<tr>
<th>Conformation</th>
<th>+ pAMP</th>
<th>no DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncut plasmid in well</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Multimer</td>
<td>LB</td>
<td>Lawn</td>
</tr>
<tr>
<td>Nicked circular</td>
<td>LB + amp</td>
<td>#</td>
</tr>
<tr>
<td>Linear</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Supercoiled</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All nonlinear conformations go away once plasmid is cut by a restriction endonuclease.

Lab 6: Purification of plasmid DNA: \textit{“Minipreps”}

Small scale, crude / dirty preps yield sufficient material for restriction analysis

When you need a lot of high quality (pure) plasmid, do cesium chloride prep. Under high centrifugation, CsCl2 forms a density gradient. Plasmid DNA will “float” at a particular level in the gradient and can be extracted in purified form.

E. coli cells transformed last time grew on ampicillin plates. Some resistant colonies were cultured overnight in nutrient broth with ampicillin (to maintain selective pressure). These small broth cultures will be spun down and plasmid isolated.

Note that cells must be fully resuspended in GTE before lysis with SDS/NaOH. Also, SDS is a detergent and will froth if vigorously mixed.

Roughly speaking, cells contain lipids, proteins, salts, chromosomal DNA, plasmid DNA and RNA.
Glucose/Tris/EDTA (GTE): Tris is a buffer. EDTA is a chelating agent. Often found in solutions for storing DNA. It chelates Mg++ which is a required cofactor for many nucleases; thus EDTA can minimize unwanted breakdown of DNA.

SDS/NaOH: Causes cell lysis. SDS is a detergent, dissolves lipids. NaOH denatures DNA, including plasmid DNA; however, closed circle plasmid strands stay intertwined.

KOAc (potassium acetate): Neutralizes the pH, allowing renaturation. Chromosome strands fail to align properly and form a partially hybridized tangle. KOAc also precipitates SDS and its associated lipids & proteins; chromosomal DNA is caught in this precipitate. Plasmid DNA & RNA remain in solution.

Isopropanol: Alcohol precipitates nucleic acids.

Wash to remove lingering SDS, salts.

Resuspend in Tris-EDTA.

RNase is frequently added to minipreps to eliminate the contaminating RNA. Interestingly, RNAses, unlike most enzymes, are highly heat resistant and are NOT inactivated by boiling (they can refold after denaturation and retain activity). This makes them a major contamination problem in labs which do work on RNA.