

Recitation Notes for RDM Day 1

1. **Module Overview** –

- **RDM = Recombinant DNA Methods**

What does that mean? Recombinant DNA methods means “recombining” pieces of DNA in ways that don’t occur in nature. Most DNA we work with in this module is in the form of plasmid DNA (circular double stranded DNA that can grow in bacteria). We are going to manipulate this plasmid DNA in various ways:

- cut DNA (How do we do this? By using **restriction enzymes**)
- separate DNA fragments by size (What is this called? **Agarose gel electrophoresis**)
- purify DNA fragments
- join fragments to make a new plasmid (What enzyme catalyzes this reaction? **Ligase**)
- put plasmid back into bacteria to grow (What is this called? Bacterial transformation)
- purify lots of plasmid from bacteria (Called **miniprep**)

We are also going to learn how to amplify a specific piece of DNA by using **PCR (polymerase chain reaction)**.

These techniques are going to be incredibly important for you in just about any biology lab.

Two projects in this module

- **Subclone gfp gene into expression vector - express GFP in E. coli to give green bacteria**
- **PCR analysis of Ara⁻ mutants from Genetics Module**

RDM1 – Restriction mapping, restriction digests, and agarose gel electrophoresis

RDM2 – Purify digested DNA, ligate to form new plasmid; inoculate mutant (from GEN) for PCR

RDM3 - Bacterial Transformation with plasmid; PCR amplification of mutant (from GEN)

RDM3.5 – Identify bacteria that have taken up plasmid

RDM4 - Calculate transformation efficiency; miniprep of plasmid DNA; analyze PCR products

RDM5 – Restriction digests and agarose gel electrophoresis of new plasmid to confirm subcloning

RDM6 – Transform bacteria for expression with plasmid

RDM7 – Detect GFP expression in bacteria, calculate transformation efficiency

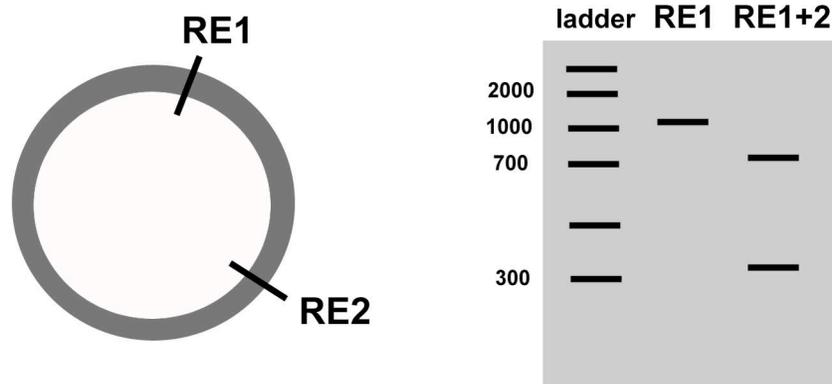
2. Restriction Mapping

This establishes a physical “map” of restriction sites (sites where a particular restriction enzyme cuts) on a fragment on DNA.

* Restriction “mapping” gives information about the relative position of DNA sequences on a small scale (0.5-5kb), whereas “mapping” the *ara* gene in between the *leu* and *thr* genes in GEN gave information about the relative position of DNA sequences on a larger scale (i.e. within 100kb).

To construct a restriction map, cut a plasmid using different combinations of **restriction enzymes**. Run cut DNA on an **agarose gel** to separate the DNA fragments, and compare sizes to known ladder to determine sizes of each fragment.

Sample restriction mapping problem to do while gels are running:



Cutting with RE1 gives one band, a little more than 1000 bases. Thus, this is the size of the entire plasmid. When digested with both RE1 and RE2, you get two bands, one about 700 bases and one a little more than 300 bases ($700 + 300 = 1000$). Thus, RE2 site is 300 bases away from RE1.

We are going to construct restriction maps of the plasmids **pUC19** and **pUGFP**.

pUGFP is the pUC19 vector with the *gfp* gene inserted (which we will be moving into another plasmid, **pET** - that has a promoter so that *gfp* can be expressed).

*pET and pUC19 are “vectors” that we use to move our gene of interest (*gfp*). We’ve used phage (lambda 1205, P1) as “vectors” before in GEN.

3. Restriction Digests

The purpose of using a restriction enzyme (RE), which is an endonuclease, is to cut DNA in particular locations to generate specific DNA fragments.

There are normally 4-5 components in a given reaction.

1- **DNA** which will be digested. Must have specific site for enzyme. Also needs to be free of contaminants such as phenol, alcohol, excessive salts, which interfere with enzyme activity.

2- **the enzyme- RE are fragile! Need to be treated carefully just like other proteins (i.e. β -galactosidase).** **ALWAYS on ice.** New pipette each time you go into tube; hold by top of tube. Enzyme is stored in 50% glycerol, so that it does not freeze at -20 degrees Celsius and the enzyme retains its function. However, more than 5% glycerol in a digest reaction will disrupt the proper reaction- either there will not be digestion, or the enzyme will exhibit star activity (which is term for cleavage at sites other than the defined recognition sequence of the enzyme). Some enzymes are more likely to exhibit star activity than others, for instance Eco RI; thus it is important to not add too much enzyme- it is better to avoid over-digestion and keep glycerol concentration lower. Also low ionic strength and high pH also contribute to star activity, so important to use proper buffer (see below). One important point about the enzyme- you should add it last. All reaction components should be mixed gently but thoroughly, and then the enzyme added and mixed again. Enzyme should never exceed 10% of total reaction volume.

3- **buffer** – the restriction enzyme requires a particular ionic strength and pH. Buffers contain various salts and other components which the enzyme needs in order to function optimally. Tris – buffer; MgCl₂ – Mg is cofactor for RE. NaCl to maintain ionic strength, mimics cellular conditions. 0.025% Triton X-100 (detergent)- keeps proteins and DNA from sticking to sides of tube.

4- **BSA**- some enzymes require bovine serum albumin for optimal activity, because it's good to have other proteins around to take up space. None of the enzymes we are using require it though (adding it never harms the reaction).

5- **water**- to achieve proper ratios of each component.

The recommended incubation temperature for most RE is 37 degrees Celsius.

We are working with two different digests today – what is the difference between an **analytical digest** versus a **preparative digest**? Analytical is on a small scale, for answering a question (for instance, where do restriction enzymes cut a plasmid?). Preparative is on a large scale, for preparing DNA fragments for further manipulation.

Today you are analyzing results from an analytical digest that the TAs performed already, and then you will be performing your own preparative digests that you will use on Day 2.

4. Agarose Gel Electrophoresis

This is a method used to separate nucleic acids on the basis of size (and shape, in some cases). DNA gels are made of **agarose**, a highly purified agar, heated and dissolved in a buffer solution. The agarose molecules, when cooled, form a matrix with pores between them. The more concentrated the agarose solution, the tighter the matrix and the smaller its pores.

An electric current applied across the **gel** drives the negatively charged DNA molecules through the gel toward the positive pole of the electric field. As DNA molecules move through the pores in the matrix, longer molecules encounter more resistance and move more slowly. Given the same amount of time to move through the gel, shorter fragments will migrate farther.

DNA shape can also have an effect – if all DNA is linear, then DNA will migrate on basis of size alone. But if plasmid DNA is **supercoiled** and very compact, then it will run **FASTER** than linear of the same size. If plasmid DNA is circular **nicked** (one strand cut, the other strand uncut), then it will run **SLOWER** than linear of same size, since the nicked strand causes it to drag through the pores more slowly.

In the chamber and power supply, red pole – positive charge; black pole – negative charge. A handy rhyme to remember which way to run the DNA on your gel – “back on black, run to red.”

*Running a gel for determining DNA fragment size is similar to running the SDS-PAGE gel (Coomassie stained) from PBC Day 4 (to find protein sizes).

If you are analyzing plasmid DNA that was only partially digested by the RE, how would you be able to tell? What do partial digests look like on gel? You would see bands that are also present in the uncut plasmid (always run them side-by-side), the bands that are uncut would be lighter than expected for size, and if you add up the sizes of the bands in each lane, they won't add up correctly to the right size of the plasmid.

Some extra notes:

Visualize with UV light/ethidium bromide – EtBr is mutagen! Wear gloves! Wear goggles/face shields when working with UV light boxes.

Graph 1 kb ladder bands and phi marker on semilog paper at home.

Save your leftover ladders, 6X buffer, TE, etc in freezer box for use later in module.

For calculating concentrations, always remember

Volume Initial x Concentration Initial = Volume Final x Concentration Final

How much 6X sample buffer to add to 20uL of sample so final concentration sample buffer is 1X?

$$(x \text{ uL})(6X) = (x + 20\text{uL})(1X)$$

$$6x = 20 + x$$

$$5x = 20; \quad x=4\text{uL}$$