

Bi 1x, Spring 2009

Week 3

- **Session 1**
 - Restriction enzyme digestion
- **Session 2**
 - Agarose gel electrophoresis

Restriction enzyme digestion

Restriction enzymes are enzymes that bind to specific DNA sequences and cleave (“digest”) the DNA at or next to the binding site. You will become familiar with restriction enzymes during this week’s lab sessions on both bulk and single molecule scales.

Note: This document describes bulk restriction digests only.

Materials:

- Sterile, ultrapure water
 - pZE21-lacZ plasmid DNA
 - 10X NEB2 Buffer
 - 10X EcoRI Buffer
 - 10X BSA Solution
 - HindIII digested lambda phage DNA
- *EcoRI, HindIII, and KpnI restriction enzymes will be supplied by your TAs when you are ready to use them.*

Background:

Most useful restriction enzymes recognize 4-8 base pair restriction sites. These sites are symmetric, inverted repeats called palindromes. Shown below are the restriction sites of the three enzymes you will be using today: EcoRI, HindIII, and KpnI. Notice how the 5’ to 3’ sequence is identical on the top and bottom strands.



Some enzymes, like KpnI, produce sequences with 3’ overhangs upon cleavage. Others, like EcoRI and HindIII, produce 5’ overhanging ends. Additional enzymes can produce blunt sequences. It is also important to remember that restriction sequences are not necessarily unique to an enzyme—multiple enzymes often have the same recognition sequence. To look up the recognition sequences of different enzymes, you can consult the New England Biolabs (NEB) REBASE database (<http://rebase.neb.com/rebase/rebase.html>).

Restriction enzymes are generally supplied as a given number of units. These units correspond to a metric of enzymatic activity, as specified by the manufacturer. Today, you will be using enzymes from NEB, which uses the following definition for a “unit”:

One unit is defined as the amount of enzyme required to digest 1 μg of λ DNA in 1 hour at 37°C in a total reaction volume of 50 μl .

Lastly, restriction enzymes, like all enzymes, have certain optimal conditions for that must be met for full activity. Different restriction enzymes have different condition requirements—salt, metal, detergent, and additive concentrations can all have drastic effects on activity (though many enzymes are perfectly happy in generic buffers). One common additive (which you will be using today) is acetylated bovine serum albumin (BSA). BSA levels and other reaction conditions are usually optimized by the manufacturers, who supply specific buffers with each enzyme.

Today, you will familiarize yourself with restriction enzymes by digesting genomic DNA from the bacteriophage lambda (discussed in your prelab) as well as plasmid DNA. A restriction map of this plasmid has been supplied at the end of this document. You will run your samples on an agarose gel tomorrow to analyze the results of your digestions.

Protocol:

Today you will perform the following three restriction digests (per pair):

1. HindIII single digest of the plasmid pZE21-LacZ
2. KpnI/HindIII double digest of the plasmid pZE21-lacZ
3. EcoRI digest of HindIII digested (predigested) lambda phage DNA

Set the reactions up as follows, pipetting in the order the reagents are listed.

HindIII Single Digest of pZE21-lacZ plasmid

Reagent:	Amount:
Sterile water	19.5 μl
Plasmid (pZE21-LacZ) DNA (86 ng/ μl)	3.5 μl
NEB Buffer 2 (10X)	3 μl
BSA (10X)	3 μl
HindIII (10 units/ μl) *	1 μl

Total Volume: 30 μl

HindIII/KpnI Double Digest of pZE21-lacZ plasmid

Reagent:	Amount:
Sterile water	18.5 μl
Plasmid (pZE21-LacZ) DNA (86 ng/ μl)	3.5 μl
NEB Buffer 2 (10X)	3 μl
BSA (10X)	3 μl
KpnI (10 units/ μl) *	1 μl
HindIII (10 units/ μl) *	1 μl

Total Volume: 30 μl

EcoRI Digest of HindIII digested lambda DNA

Reagent:	Amount:
Sterile water	18.5 μl
HindIII digested lambda DNA (0.5 μg/μl)	3 μl
NEB EcoRI buffer (10X)	3 μl
BSA (10X)	3 μl
EcoRI (10 units/μl) *	2 μl

Total Volume: 30 μ l

* Restriction enzymes are supplied in a viscous, glycerol containing solution. Pipette with care!

After you have assembled your reactions, you should mix them, spin them down briefly, and place them at 37°C for the remainder of the lab session (~2 hours). Your TAs will freeze them afterwards for use during the next lab session.

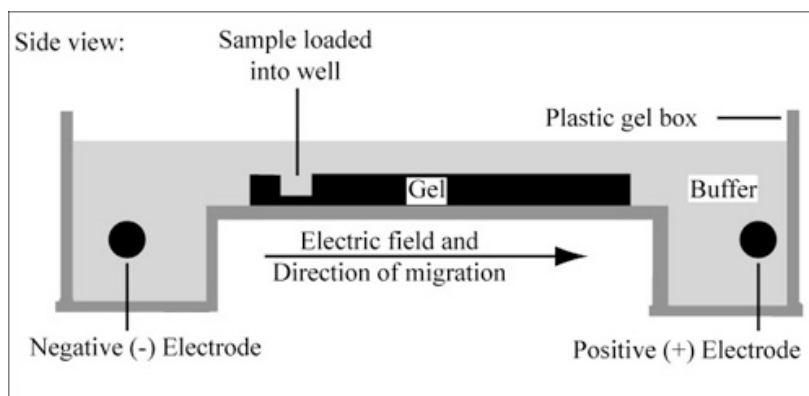
Session 2 – Agarose gel electrophoresis

Materials

- Your digestion reactions from session 1
- 6X DNA loading dye
- 1 kb and 100 bp ladders (pre-stained)
- HindIII digested lambda DNA (pre-stained)
- pZE21-lacZ DNA (pre-stained)

Background

Agarose gel electrophoresis is a powerful separation method frequently used to analyze DNA fragments generated by restriction enzymes. The agarose gel consists of microscopic pores that act as a molecular sieve. Samples of DNA are loaded into wells made in the gel during casting, as shown in the figure below.



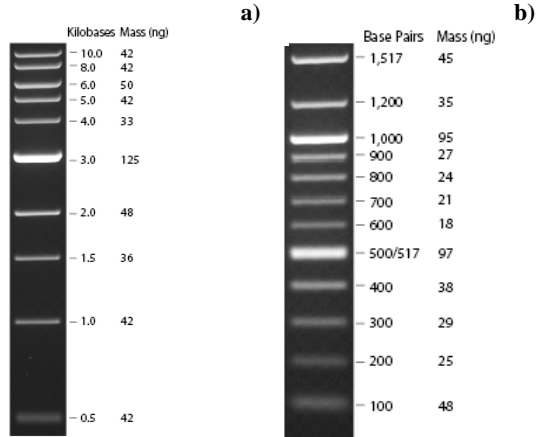
http://ocw.mit.edu/OcwWeb/Biological-Engineering/20-109Fall-2007/Labs/detail/mod1_2.htm

Since DNA has a strong negative charge at neutral pH, it migrates through the gel towards the positive electrode during electrophoresis. The DNA molecules are separated in the gel according to their size and shape. Linear DNA molecules are separated according to their size. The smaller the linear fragment, the faster it migrates. If the size of two fragments are similar or identical, they will migrate together in the gel. If DNA is cleaved many times, the wide range of fragments produced will appear as a smear after electrophoresis.

Circular DNAs such as plasmids are supercoiled. Supercoiled DNA has a more compact and entangled shape (like a twisted rubber band) than its corresponding non-supercoiled forms (linear, nicked and relaxed circles). When supercoiled DNA is cleaved by a restriction enzyme just once it unravels to its linear form. If supercoiled DNA is nicked (a phosphate bond is broken anywhere in the molecule, in either strand) it completely unravels to form a circle. Under the electrophoresis conditions being used in this experiment, supercoiled DNA migrates faster than its linear form and linear DNA migrates faster than its nicked circular form.¹

¹ EDVOTEK EDVO-kit #102 Manual

In order to determine the size of your DNA from the migration distance, you will use “DNA ladders,” which are molecular weight standards that consist of DNA fragments of known sizes. The ladders you will use today are shown below:



DNA Ladders

a) Example of 1 kb DNA ladder on a 0.8% agarose gel, stained with EtBr.

b) Example of the 100bp DNA ladder on a 1.3% agarose gel, stained with EtBr.

Protocol

You will run the following samples on a 1% TAE-Agarose gel (prepared by your TAs):

1. 100 bp DNA ladder
2. **EcoRI/HindIII lambda DNA double digest (from session 1)**
3. HindIII digest of lambda DNA (as a reference)
4. **pZE21-LacZ – HindIII single digest (from session 1)**
5. **pZE21-LacZ – KpnI/HindIII double digest (from session 1)**
6. Undigested pZE21-LacZ (as a reference)
7. 1 kb DNA ladder

1. Mix 20 µl of each of your reactions from session 1 with 4 µl of 6X DNA loading dye.
DNA loading dye consists of glycerol—which will help your solutions sink into the wells of the agarose gel—and reference dyes that help you determine how far your samples have migrated.

2. Load your samples on the gel in the order listed above.

- a. For samples that are supplied to you pre-stained (ladders, references), load 5 µl.
- b. For your own restriction digests from session 1, load 20 µl of sample/dye mixture.
- c. To load the gel, place your pipette beneath the buffer surface layer, part-way into your target well. Very slowly depress the pipette plunger to load your sample. As you depress the plunger, you will notice the sample fall into the well. Do not rush! This may cause the sample to flow out of the well.

Loading samples onto agarose gels is tricky! Ask your TA to demonstrate how to load a gel before attempting to do it yourself.