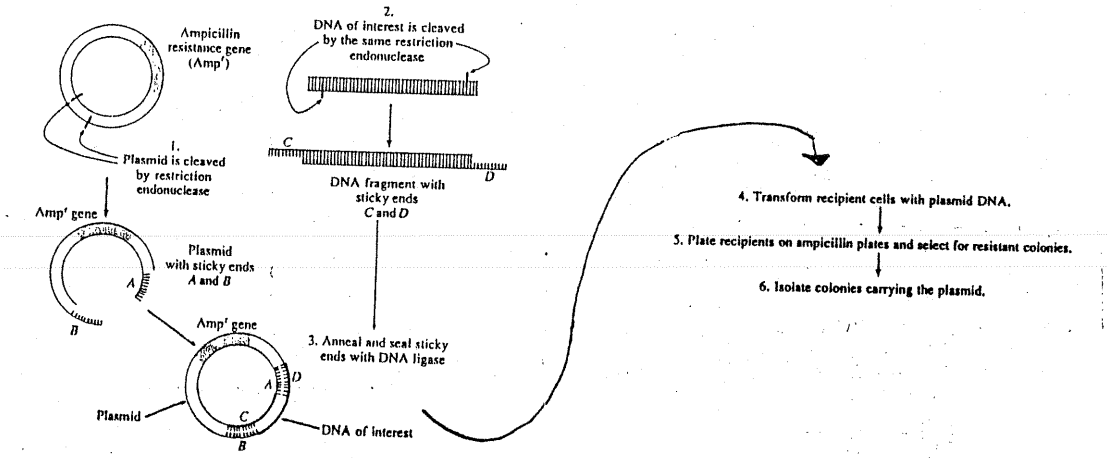
Laboratory 6: Molecular Biology

*discuss the rpinciples of bacterial transformation
*describe how to prepare competent *E. coli* cells
*discuss the mechanisms of gene transfer using plasmid vectors
*discuss the transfer of antibiotic resistance genes and tell how to select positively for transformed cells that are antibiotic resistant
*discuss the mechanisms of action for restriction endonucleases
*discuss how a plasmid can be engineered to include a piece of foreign DNA that alters the phenotype of the teransformed cells
*understand and be able to explain the principles of electrophoresis as they pertain to separating and identifying DNA fragments

E. coli is an ideal organism for use in molecular biology; grown easily, its circular chromosome contains 5 million base pairs (1/600 that of single human sperm). These bacteria also contain plasmids, small circular DNA molecules of 1000 to 200,000 base pairs; extrachromosomal, they replicate with the cell, and can be transferred during bacterial transformation. This phenomenon of transfer of DNA from one bacterium to another is normall rare, but it occurs best when cells are in a competence stage: humans have figured out a way to make cells competent by adding Ca^{+2} or Mg^{+2} . It also seems as if they take up DNA best after a short pulse of heat, so humans manipulate the temperature at which parts of this process are done.

A gene that would be easy to detect if transferred is antibiotic resistance. For example, *E. coli* is normally killed by ampicillin, but if a gene for ampicillin resistance was placed into it and worked, the bacteria should be able to thrive, even on plates with the antibiotic present. You can purchase plasmids which already have this gene inserted into them (see below the process).



To get the plasmid into the bacterial cells, you must treat them carefully with $CaCl_2$ to make competent, add plasmid while solutions are on ice; heat shock with "pulse of heat" in 42°C water bath for 90 seconds, then return to ice. Now streak bacteria on agar plates with nutrients plus ampicillin antibiotic and incubate for two days.

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If cells were "transformed", they should now contain the gene for resistance to ampicillin and should grow well on antibiotic-containing plates while non-transformed cells are killed. Visual inspection will show some colonies that are thriving; transformation efficiency is expressed as the number of resistant colonies per microgram of plasmid. That can be calculated if you observe 70 colonies after placing only 100 µL of broth there (originally there was 500 µL diluting 0.05 µgram of plasmid).

.05 µgram / 500 µliters = 0.0001 µgram/µliter

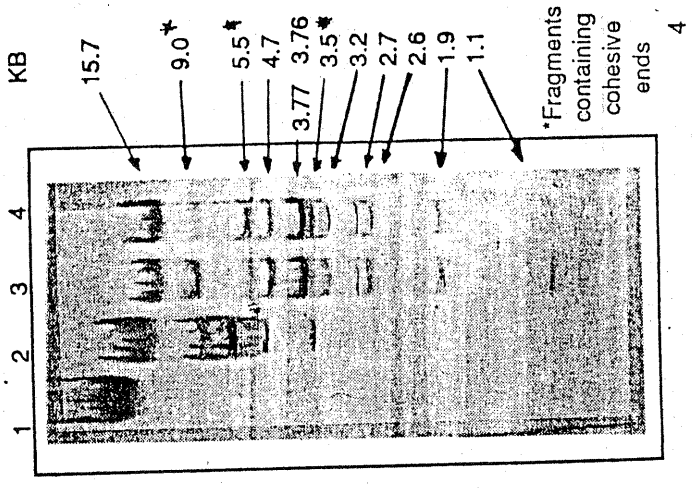
70 colonies from 100 µliters = .01 µgrams

∴ you could expect 7000 colonies per µgram

The restriction enzymes used to introduce fragments of "foreign" DNA into plasmids are more specifically restriction endonucleases. Several are very famous (EcoRI and HaeII...the first letter stands for the genus of the organisms from which it was isolated, the next two letters are the first of the species name, the fourth letter the strain -- may or may not have this -- and the Roman numerals indicate whether it was the fist enzyme isolated, the second, or so on....) These restriction endonucleases recognize specific 4-6 base pair DNA sequences, and many cleave the helix off-center to produce "sticky ends" or overhangs which allow recombination with other pieces with the same "sticky ends". If plasmids are cut with a particular enzyme and a wanted gene is spliced out with the same one, they may re-anneal and form a single circular strand of "recombinant DNA"!

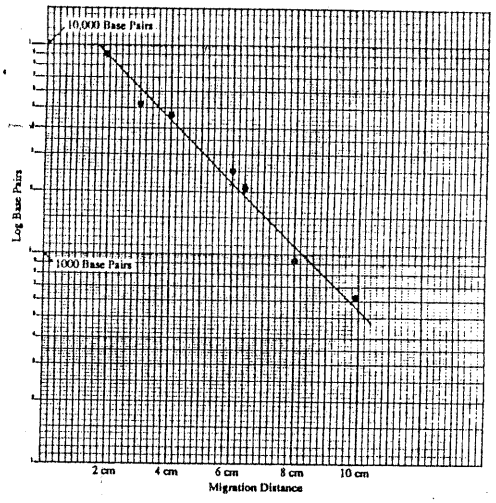
Restriction enzymes can be used to digest a sample of DNA and then the pieces electrophoresed; after migration of the DNA through an electrical field, the gel can be stained and a banding pattern corresponding to the number of sites recognized by the enzyme will be seen and an estimation of the base pair size can be made when compared to a known standard.

When any molecule enters an electrical field, the mobility or speed at which it will move is influenced by the charge of the molecule, the strength of the field, the size and shape of the molecule, and the density of the medium (gel in this case) through which it is migrating. All this makes it possible to separate heterogeneous populations of molecules. If all molecules start at the same place (parallel wells in the gel) and are run under identical conditions (in buffer, toward a positive electrode which attracts the negative phosphate groups of DNA), DNA fragments should separate into bands that migrate in inverse proportion to their size: the smallest run the farthest and fastest! Dyes help the molecule bands (groups of similar molecule fragments) show up more clearly.



By knowing how far DNA pieces of known base-pair size run, you can estimate the molecular size of all the bands on a gel! For example, if a 9,000 base-pair piece ran 2 cm from the start well, and a 620 base-pair piece ran 10 cm, you could estimate how large most other pieces were by plotting those points on semi-log paper/ a straight line there will predict the size by distance run with fair accuracy!

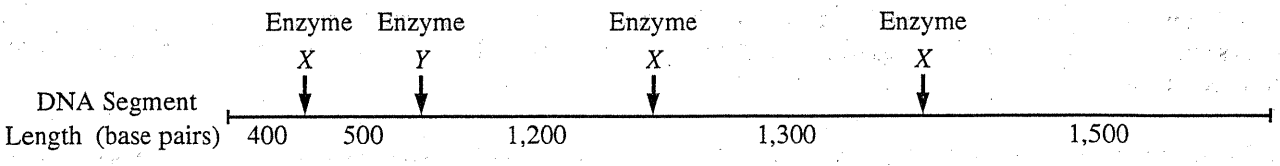
DNA Fragment Size vs. Migration Distance



Lab 6 R

3. Energy transfer occurs in all cellular activities. For 3 of the following 5 processes involving energy transfer, explain how each functions in the cell and give an example. Explain how ATP is involved in each example you choose.
   - Cellular movement
   - Active transport
   - Synthesis of molecules
   - Chemiosmosis
   - Fermentation

4. The diagram below shows a segment of DNA with a total length of 4,900 base pairs. The arrows indicate reaction sites for two restriction enzymes (enzyme *X* and enzyme *Y*).



(A) Explain how the principles of gel electrophoresis allow for the separation of DNA fragments.

(B) Describe the results you would expect from the electrophoretic separation of fragments from the following treatments of the DNA segment above. Assume that the digestions occurred under appropriate conditions and went to completion.

I. DNA digested with only enzyme *X*
II. DNA digested with only enzyme *Y*
III. DNA digested with enzyme *X* and enzyme *Y* combined
IV. Undigested DNA

(C) Explain both of the following.

(1) The mechanism of action of restriction enzymes

(2) The different results you would expect if a mutation occurred at the recognition site for enzyme *Y*.

Question 4 Standards

Overall Commentary for Question 4

This question expected students to focus on conceptual material in the area of molecular biology as well as on a laboratory experience in the AP Biology curriculum. The question was written with three distinguishable parts, the third of which is further subdivided into two. The first of these sections is on principles of gel electrophoresis; the second on expected results of an experiment; the third on the mechanisms of restriction enzymes and the effects of mutation at a restriction site.

In Part A, students could earn points for demonstrating understanding of the roles of electrical potential and charged particles in electrophoresis, for recognition of the rate/size relationship of the movement of fragments, for a description of calibration, and for the factors which affect resolution; an additional point could be awarded for an explanation of the use of apparatus. In Part B, one point was awarded for a correct description of each of the four expected sets of results. In Part C1, points were awarded for the mechanisms of recognition and cutting of the DNA, with a possible additional point for particular details. Since C2 asked for a change in results when mutation occurs, points were awarded for each of the two possible changes as well as for theoretical possibilities of how such a mutation might have altered the target sequence.

PART A. Explain how the principles of gel electrophoresis allow for the separation of DNA fragments. (4 point max.)

- Electricity ..... Electrical potential (charge, field) moves fragments
- Charge ..... Negatively charged fragments/ move (toward (+) anode) through gel/(-) charge due to phosphate groups
- Rate/size ..... Smaller fragments move faster (farther) relative to larger fragments/Describe logarithmic relationship
- Calibration..... DNA's of known molecular weights are used as markers/ standards
- Resolution ..... Depends on concentration of gel; is determined by pore size
- Apparatus ..... DNA is stained for visualization of bands/explains use of wells, gel material, tracking dye, buffers

PART B. Describe the results you would expect from electrophoretic separation of fragments from the following treatments of the DNA segment above. (4 point max.)

- Treatment I ..... Describe 400, 1300, 1500, 1700 bp fragments — or 4 bands — or correct diagram with explanation
- Treatment II ..... Describe 900, 4000 bp fragments — or 2 bands — or correct diagram with explanation
- Treatment III..... Describe 400, 500, 1200, 1300, 1500 bp fragments — or 5 bands — or correct diagram with explanation
- Treatment IV..... Describe 4900 bp fragment — or 1 band — or correct diagram with explanation

PART C1. The mechanism of action of the restriction enzymes (4 pt. max. for both C1 and C2; For a 10 must have at least one point from each section in part C)

- Recognition..... Binding of enzyme to target sequence/Specific short bp sequences of double stranded DNA are targeted/ Recognizes specific targets 4-8 bp long/Site may be palindromic
- Cutting ..... Enzyme cuts at *every* target location/may cut frequently or rarely
  Cuts but does not alter the sequence

Alternate: ..... One point may be given if instead of the above it is clear that the students says that the enzyme cuts at a specific point

- Detail Point..... Fragment lengths correspond to lengths between cutting sites/May generate blunt or sticky ends
  Methylation or modification
  Breaks the phosphodiester bond/Describe mechanism in living systems
  Restriction site may function as a genetic marker

PART C2. The different results ... if a mutation occurred at the recognition site for enzyme Y.

- Change in II..... Uncut/1 band (looks like IV)
- Change in III..... Like I: 4 bands

Alternate: ..... One point may be given instead of the above, if it is clear that the student says that Y sequence is no longer recognized *and* cut

- Detail Point..... Describes that RFLPs (markers) *might* correlate with phenotypic variation
  Y site might become an X site
  Deletion/Insertion at Y site — changes fragment length
  Silent alteration (pyrimidine > pyrimidine or purine > purine) in some target sequences

Question 4 Overview

How prepared were the students?

A large number (16-17%) of students did not respond or provided no biological information for Question 4. In addition, a significant number of students (22%) wrote, sometimes extensively, but were awarded 0 points because they provided no pertinent information, only incorrect information, or used genetic terminology but did not address the question. The remainder of the students' answers had scores distributed rather evenly across the total range. Evidently, according to students' comments, many students had no prior experience with a lab in molecular genetics or no significant conceptual material on gel electrophoresis or restriction enzymes. Some students with minimal preparation were still able to interpret the experimental design reflected in Part B of the question. The most prepared students could handle Part B with relative ease and also received, typically, three points on Part A and 2-3 points in Part C.

How were the standards applied to the sample student responses?

In order to assure that a student with a high total score had answered all parts of the question, each of the major sections had a maximum of 4 points and a score of 10 could only be given if both parts of C were addressed.

GOOD ESSAY (8 points): This is a reasonably good answer. The student describes the use of electrical current to move fragments and describes the use of the apparatus. Note that the relationship of fragment size to distance is given incorrectly. In Part B, three of the appropriate results are predicted, but the fourth is omitted. The student notes that restriction enzymes recognize and cut a sequence but does not elaborate on either of these. A detail point is given for the description of sticky ends produced by the cleavage. The final point is given for the description of why recognition and cutting no longer occur after the mutation, even though there is incorrect information in this paragraph.

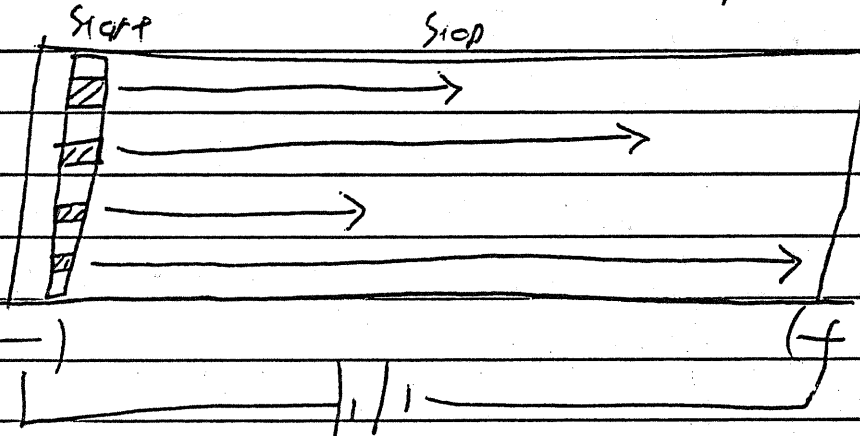
Our Sample →

EXCELLENT ESSAY 1 (10 points): This essay demonstrates proficiency. In Part A the points are for electricity moving fragments, for recognition of movement of negatively charged DNA toward the anode, and for the rate/size relationship. In Part B, all 4 points are awarded for correct predictions of results of all four treatments. In Part C, this student could receive four points although only three are needed to reach 10. These points are for the description of the mechanism's use in living systems, for the general description of the recognition/cutting functions of the enzymes, for a description of results when Y function is lost, and, finally, for the possibility that mutation could produce different fragment lengths.

EXCELLENT ESSAY 2 (10 points): The student describes three principles of gel electrophoresis: the use of electric potential to move fragments, the negative charge of DNA fragments, and the relationship of size to rate of movement. In Part B, all four results are correctly predicted, including the correct length of fragments. In the first section of Part C, the student receives a point for the general recognition/cutting function of the enzymes and another point for their role in living systems. In the second section of Part C, there is one point for the recognition that the sequence is no longer recognized and cut after mutation.

EXCELLENT ESSAY 1

Gel electrophoresis allows for the separation of DNA fragments by running DNA fragments through a gel, with a DC electric current moving the gel along at different speeds due to their molecular weight difference and charge difference. DNA is negatively charged, and a



constant voltage will cause the molecules with greater charge and lower molecular weight to migrate a longer distance per unit time. With the weight of certain stock dyes known, the weight and charge of the fragments could be then calculated.

The DNA digested with only enzyme X would separate into four strands, with the 400 base pair migrating the farthest, the 1300bp strand, the 1500 bp strand, and the 1700bp strand (enzyme Y does not cut between the 500 and 1200 strands) following in that order. If only enzyme Y is used, then there will be the 900bp strand migrating (400+500) the farthest, followed by the sluggish 4000bp strand. (1200+1300+1500)

Using both restriction enzymes X and Y produces 4 strands with 400, 500, 1200, 1300, and 1500 bp, and they will migrate fastest to slowest in that order, with the 400bp in front, followed by the 500bp, and so on. Undigested DNA will migrate little, as one giant band.

Restriction enzymes are found in bacteria as a defense against phage viruses. The enzymes cut DNA only at specific sequence locations, hence, each cut corresponds to a particular sequence. If a mutation occured at the recognition site of enzyme Y, then the enzyme Y will either not function at all, producing the results given if Y does no cutting, or cut the DNA based on a different sequence, providing strands of different lengths and hence different migration distances per unit time.

Lab 6 B
Transformation

QUESTION 2 1998

By using the techniques of genetic engineering, scientists are able to modify genetic material so that a particular gene of interest from one cell can be incorporated into a different cell.

- Describe a procedure by which this can be done.
- Explain the purpose of each step of your procedure.
- Describe how you could determine whether the gene was successfully incorporated.
- Describe an example of how gene transfer and incorporation have been used in a biomedical or commercial application.

A 1pt <u>obtaining gene</u>:

restriction enzymes
mRNA --> cDNA
aa seq--> DNA
isolating plasmid with choice gene

1 pt making <u>a packaging/ delivery system</u>
make vector (plasmid, virus, YAC ...);
gene gun; ligation to vector.

1 pt <u>incorporating</u>: transformation ($CaCl_2$, heat shock), viral infection, ligase, electroporation, PEG

1 pt <------\----------<u>elaboration</u> ---------->
(appropriate and detailed extra description: controlled experimental design, explanation of electrophoresis or use of radioactive/fluorescent probe)

B <u>Purpose</u> for 1-3 steps of procedure (Why)

1pt e.g. restriction enzymes to produce sticky ends; cDNA has no introns which bacteria can't splice.

1 pt so gene can be delivered to specific site; so plasmid can be taken up by host cell; so gene is placed with appropriate regulatory sequences.

1 pt change permeability (competence) of host; (covalently) bond piece into host DNA.

C 1 pt <u>how to determine gene incorporation / expression</u>
NOT phenotypic change alone

e.g. antibiotic resistance
protein assay
reporter genes
probe
color change
change in electrophoretic mobility
sequencing

1 pt <u>Elaboration</u> (detailed explanation of how, why it works, etc.; e.g. dideoxynucleotide method of gene sequencing.)

D 1 pt <u>Application</u> (one)

e.g. transgenic animal
herbicide resistance
gene therapy (specific)
FlavrSavr Tomato
monoclonal antibodies
insulin production
frost resistance
growth hormone
making clotting factor

1 pt <u>Elaboration</u> (not just second example; explanation of importance, how it is done. etc.)

Write in the box the number of the question you are answering on this page as it is designated in the examination.

2 A

2) Genetic engineering involves the transformation of foreign DNA into a type of host cell by means of recombinant DNA. Most often engineers work with plasmids; or circular pieces of DNA. In order to have a particular gene of one cell be incorporated into a different cell; one must splice that gene into a plasmid; and transform the plasmid into the different cell. The first step is to splice or cut the DNA containing the gene of interest using restriction enzymes (or endo nucleases). Each restriction enzymes is specific to a particular restriction site; meaning it cleaves (or cuts) at a particular nucleotide (or base pair) sequence. Then a vector (which is usually a plasmid) is spliced, cleaved, or cut with the same restriction enzyme. Vectors are a type of transport vehicle for a particular (foreign) gene to be incorporated into a cell. The vector, or plasmid must be cleaved by the same restriction enzyme in order to produce "stick ends" complementary to the ends on the gene. Sticky ends are vacant, or unpaired base of DNA; that want to hydrogen bond with complement (A→T & G→C) Now that the two pieces of DNA are cleaved with the restriction enzyme they must be mixed in order to have them pair up. Although this won't be a hundred percent; even a small amount of successful recombinant plasmid will suffice. Another thing that is necessary for the plasmid is a type of indicator to show successful transformation. A common indicator is an antibiotic resistance gene; such as the one on a plasmid (resistant to Ampicillan). Now the recombinant plasmid consists of a resistance gene, the gene of interest; and also a growth factor (gene) which allows the plasmid to replicate. After the plasmids are formed DNA ligase is introduced into the test tube. This enzymes

1pt A obtaining

1pt B

1pt A packaging

1pt elaboration

1pt B

2 A2

Write in the box the number of the question you are answering on this page as it is designated in the examination.

glues the sticky ends together by combining the phosphate and sugar molecules (phosphodiester bond). And also and ~~poteriase~~ to stop the function of the restriction enzyme. The newly recombinant plasmids now must be transfered into living cells (bacteria such as E coli) that are in lag phase; optimum time for bacteria to take up plasmids. The membrane of the bacteria must be weakened slightly. After the cells have been incubated and given time to take up plasmids, the cells are placed in a quick heat-shock. Now the bacteria cells are ready to be spreaded on agar. The LB broth (nutrient) agar should also include the antibiotic corresponding to the resistence gene; in this case ampicillin. This ensures that the plasmid transformed will carry the ampicillin restance gene; and that means it was a successful transformation. The petri dished should be incubated over night and observed

1pt B

1pt A incorporate

! C verification

1pt C elab

An example of a biomedical application is the production of insulin. Instead of retrevins it from cadaveres; scientists can put the gene for insulin production in a plasmid and incorporate into bacteria. As the bacteria reproduce so will the plasmid; and therfore the insulin gene which codes for gene production.

1pt D Applic.