Biochemistry of the Gene
Laboratory Manual

Young Scholars Program
Summer, 2015

Young Scholars Program Biochemistry Schedule - Summer 2015
Lectures and Labs: 2:00-5:00 pm, 2061 King Life Sciences Building

Monday	Tues	Wednesday	Thurs	Friday
June 8	**9**	**10** **Chromosome Lab** Start blood cultures	**11**	**12** **Fruit fly Lab** Observe flies Start crosses 1 & 2
15 **Chromosome Lab** Process cells Group A: 2:00 Group B: 3:30	**16**	**17** **Chromosome Lab** Group A: Prepare slides Stain with Geimsa Observe slides **Fruit fly Lab** Group A: Remove parents	**18**	**19** **Chromosome Lab** Group B: Prepare slides Stain with Geimsa Observe slides **Fruit fly Lab** Group B: Remove parents
22 **Plasmid Lab** Isolate plasmid DNA	**23**	**24** **Plasmid Lab** Restriction enzyme digestion	**25**	**26** **Plasmid Lab** Gel of digests **Fruit fly Lab** Observe progeny cross 2 Start cross 3
29 **ABO Lab** Group A: Cheek DNA prep and PCR	**30**	**July 1** **ABO Lab** Group B: Cheek DNA prep and PCR **Fruit fly Lab** Remove parents	**2**	**3 Holiday**
6 **ABO Lab** Qiagen column **Plasmid Lab** Discussion of results	**7**	**8** **ABO Lab** Gel of PCR	**9**	**10** **ABO Lab** Prepare dilutions for sequencing
13 **Fruit fly Lab** Observe progeny of cross 3	**14**	**15** **ABO Lab** Results of sequencing Blood typing	**16**	**17 - Exam**

LAB 1: PREPARATION OF HUMAN MITOTIC CHROMOSOMES

I. Introduction

This protocol was adapted from the methods of the TC Chromosome Microtest Kit (Difco Laboratories) for use with Gibco's PB-MAX Karyotyping Media. The chromosomes to be examined are derived from cultured lymphocytes obtained from a few drops of blood. Proper procedures for the handling of human blood will be explained in detail prior to the lab and must be followed throughout the protocol.

II. Background Reading

A. The Basics (for week 2)

Read the following article. Download the PDF version, but be patient, it may take some time to fully download.
http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1921310/

B. Preparation of Karyotypes (for week 6)

http://www.molecularstation.com/molecular-biology-images/502-dna-pictures/95-human-karyotype.html

III. Materials

- Gibco's PB-MAX Karyotyping Media (2 ml aliquots in sterile culture tubes)
- Giemsa staining solution
- Trypsin solution
- Dulbecco's phosphate buffered saline (PBS)
- Colchicine solution (4 mg/ml in H_2O)
- Hank's solution
- Methanol : acetic acid fixative (3:1, chilled)
- Sterile blood lancets
- Sterile heparinized capillary tubes and suction device
- Rubbing alcohol and cotton swaps or prepackaged sterile swabs
- Band-aids
- Microscope slides
- Ice buckets and ice
- 37^{o}C Incubator
- ddH_20, prewarmed to 37^{o}C
- Pasteur pipettes with bulbs
- 10 ml disposable pipettes and fillers
- Swinging bucket clinical table top centrifuge
- 15 ml conical centrifuge tubes (clear polystyrene)

- slide staining and rinsing dishes
- slide warming oven

IV. Procedure

A. Day 1: Preparation and Incubation of Culture

1. Obtain a tube containing 2 ml of PB-MAX Karyotyping Media. Label the tube in two places with your initials and group letter (A or B).

2. Bring tube to the blood workstation. Clean finger with sterile swab soaked with rubbing alcohol and let dry completely.

3. Prick finger with sterile lancet.

4. Collect 2 capillary tubes of blood and transfer to medium in culture tube.

5. Close vial securely and mix tube by inverting 2-3 times.

6. Put culture tube into test tube rack. When all the cultures have been prepared, the instructor will put the tubes in the 37^{o}C incubator. Incubate for 3-5 days (5 days is best).

7. Every day or so, the instructor or TAs will check the color of the cultures. They should be pink before, during, and after the incubation period. If color becomes amber, the caps will be loosened to release CO_2 that accumulates during cell growth.

8. Two hours before processing the cells the instructor or TA will add 20 μl of a 4 mg/ml solution of colchicine to each culture (final concentration = 40 μg/ml).

9. Continue incubating the cultures at 37^{o}C until class.

B. Day 2: Harvesting and Fixing Cells

Note: When resuspending cells, disrupt the pellet by gently pipetting fluid onto the pellet. As clumps are dislodged, pipette them up and down gently but sufficiently to break up the aggregates. When pipetting with a Pasteur pipette, never suck or discharge an excess of air. The pressures generating by bubbling air can damage the cells.

10. Obtain a clean 15 ml conical tube (clear polystyrene). Label it with your initials and mark the tube at the level of 0.75 ml. Transfer your culture to the 15 ml tube with a clean Pasteur pipette. Save your pipette.

11. Collect cells by spinning for 10 min at setting 7 in the clinical centrifuge. (You should get a 0.2 to 0.3 ml reddish pellet.)

12. With your Pasteur pipette, carefully remove and discard all but 0.75 ml of supernatant above the pellet.

13. Add 4 ml of Hank's solution.

14. Resuspend cells by gently pipetting fluid up and down over pellet with the same Pasteur pipette.

15. Collect cells again by centrifugation for 5 min at setting 7. (The pellet should look the same as before.)

16. Remove all but 0.75 ml of the supernatant. (Do not leave more than 0.75 ml, less is even better.)

17. Add Hank's solution to a final volume of 1 ml.

18. Resuspend cells as before with the same Pasteur pipette.

19. Add 3 ml of 37^{o}C water, in 3 1-ml aliquots, with gentle agitation after each addition. The agitations are done by slowly and gently pipetting fluid and cells up and down three times. Begin timing a 10 min incubation period as soon as the first aliquot of water is added. When 3 ml of water has been added, continue incubating at 37^{o}C for a total of 10 min. Do not exceed 10 min.

20. Collect the cells by centrifugation for 10 min, setting 5. Carefully remove tubes from the centrifuge without disturbing the pellets and let tubes sit at room temperature for 5 min before proceeding to the next step. (The pellets should be white and much smaller then before. Because the red blood cells were lysed by the addition of the warm water, the supernatant should be reddish with hemoglobin.)

21. Carefully remove all but 0.75 ml of the supernatant without disturbing the pellet (again, do not leave more than 0.75 ml, and less is even better).

22. Add 4 ml of fresh, chilled, fixative carefully so as not to disturb the pellet. This is best done by pipetting the solution slowly down the side of the tube.

23. Incubate cells in fixative for 10 min at 4^{o}C (or on ice).

24. Resuspend the cells as before using the same Pasteur pipette.

25. Collect cells by centrifugation for 5 min at setting 5.

26. Being careful not to disturb this fragile pellet by shaking or jolting the tube, put tube on ice for 5 min. This allows more of the white blood cells to settle to the bottom of the tube.

27. Carefully remove all but 0.75 ml of the fixative. This pellet is extremely fragile and loose, so do not put your pipette tip below the 0.75 ml mark or anywhere near the pellet. You may see white material streaked up the side of the tube above the pellet. Try not to remove this material with the supernatant. These are white blood cells.

28. Gently add 4 ml of fresh fixative.

29. Gently resuspend cells in the fixative, and put tubes at 4^{o}C until next class period.

C. Day 3: Preparation of Slides

30. Spin cells for 5 min at setting 5. Let sit for 5 min.

31. Remove all but 0.75 ml of fixative, again being very careful not to disturb the pellet.

32. Resuspend the cells as before. Keep cells at 4^{o}C or on ice.

33. Obtain a clean microscope slide and label the frosted edge with your initials and the number 1. Place the slide in clean, chilled water on ice.

34. In rapid succession, shake excess water from the slide, wipe the underside of the slide with a Kimwipe, rest the slide at an angle by propping the end with your initials on a microtube rack, and add 2 drops of the cell suspension onto the top center of the slide by dropping from a distance of 5-10 inches above the slide.

35. Tip slide back and forth a few times to spread cells.

36. Ignite the fixative by bringing the slide into momentary contact with a flame. Your instructor or TA will demonstrate the proper technique. This is a critical step in determining the quality of your slides, so pay close attention to the instructions. The integrity of your chromosomes will be compromised by either too much flaming or not enough flaming.

37. As soon as the fixative is burned off, wave the slide vigorously a few times and then prop slide up at an angle to hasten drying.

Giemsa Staining of Slides

38. Place slide in staining dish with freshly prepared Giemsa staining solution for 12 min.

39. Rinse the slide through three dishes of distilled water and then air dry.

40. When slide is completely dry, put on microscope and examine with standard light optics. Scan slide with the 10x objective and take a closer look at chromosomes with

the 40x objective. Be very methodical in how you scan the slide. You may not have a lot of cells on the slide, but if you spend time looking, you should find some very nice chromosome spreads.

Additional directions for using the microscopes will be given to you before starting the lab. The parts of the microscope that you need to become familiar with for observing your slides are:

the on/off switch
the mechanisms to adjust the eyepiece focus and diameter
the coarse and fine focus knobs
the knob to raise and lower the condenser
the diaphragm on the condenser
the diaphragm on the base of the unit
the knobs to move the slide in two dimensions

41. If your slide is not satisfactory, try preparing another by repeating steps 33-40.

42. Once you have prepared a slide that you are satisfied with, search for 1 or two of the best spreads, and have your TA or instructor mark their location on the slide. When you are done, hand your slide into the instructor of TA.

The following steps are performed only if G-Banding with trypsin is desired.
(Do not use cells that are more than ~1 week old for this procedure. The cells need to be at a high density and as fresh as possible.)

43. Follow steps 33-37 to prepare two more slides (numbered 2 and 3) which will be used for G-banding. When those slides are dry, give them to your TA or instructor.

D. Day 4: G-Banding with Trypsin

44. The slides will be incubated overnight at 57^{o}C and then left at room temperature until you perform G-banding.

45. You should have 2 slides labeled with your initials and the numbers 2 and 3. Soak both slides in 0.2% trypsin solution warmed to 24^{o}C for 5-20 seconds (we are currently using 12 seconds).

46. Remove the slides from the trypsin solution and rapidly transfer them to cold Dulbecco's PBS. Dip them 3 times in the Dulbecco's solution to rinse off the trypsin.

47. Remove slides from Dulbecco's PBS, shake off any excess buffer, and transfer them to freshly made Giemsa stain. Incubate in the stain for 12 minutes.

48. After staining is complete, rinse slides through two trays of distilled water.

49. Shake off excess water and prop slides at an angle to facilitate drying.

50. When slides are completely dry, examine under the microscope. Look for chromosomes that are well spread AND have good banding patterns (distinct regions of light and dark staining along the length of the chromosomes). Have your instructor or TA mark one or two of your best spreads.

V. Solutions

- PB-MAX Karyotyping Medium is purchased from Gibco

- 100X Colchicine (MW = 399.4, 1X = 40 μg/ml)
  dissolve 40 mg of colchicine in 10 ml H_2O
  (Put 40 mg into 15 ml conical tube at -20^oC, and then add H_2O just before use)

- Fixative
  3 : 1 methanol : acetic acid (for 200 ml, 150:50)

- Giemsa Staining Solution
  First prepare Giemsa stain, 0.2 M Na_2HPO_4, and 0.1 M sodium citrate:

  Giemsa Stain
  0.7415 g dry stain in 100 ml methanol
  filter through Watman #1

  0.2 M Na_2HPO_4
  dissolve 2.84 g in 100 ml H_2O (anhydrous MW = 141.96)

  0.1 M sodium citrate
  dissolve 2.94 g in 100 ml H_2O (dihydrate MW = 294.1)

  Giemsa Staining Solution

Giemsa stain	10 ml
0.2 M Na_2HPO_4	6 ml
0.1 M sodium citrate	6 ml
methanol	6 ml
ddH_2O	200 ml

- Hank's Solution

  Start by preparing Hank's 20X Stock I and Hank's 20X Stock II

  Hank's 20X Stock I
  a) NaCl 40 g (MW = 58.44, 1X = 137mM)

KCl 2.0 g (MW = 74.56, 1X = 5.4 mM)
$MgSO_4$ 0.36 g (anhydrous MW = 120.37, 1X = 0.6 mM)
$MgCl_2 \cdot 6H_2O$ 0.5 g (MW = 203.31, 1X = 0.5 mM)
Dissolve in ~ 200 ml H_2O

b) $CaCl_2 \cdot 2H_2O$ 0.96 g (MW = 147, 1X = 1.3 mM)
Dissolve in ~30 ml H_2O

Mix solutions a and b and bring to final volume of 250 ml
Add 0.5 ml of chloroform

Hank's 20X Stock II

Na_2HPO_4 0.21 g (anhydrous MW = 141.96, 1X = 0.3 mM)
KH_2PO_4 0.3 g (MW = 136.09, 1X = 0.4 mM)
Dextrose 5.0 g (MW = 180.16, 1X = 5.6 mM)

Dissolve in ~200 ml H_2O
Bring volume to 250 ml
Add 0.5 ml of chloroform

Final dilution
1 : 1 : 18 Stock I : Stock II : H_2O
(For 200 ml, 10 : 10 : 180)
pH to 7.4 with 0.1N NaOH

- 10x Dulbecco's Phosphate Buffered Saline (500 ml)

KCl 1 g
KH_2PO_4 1 g
NaCl 40 g
Na_2HPO_4 5.75 g (or 21.7 g of $Na_2HPO_4 \cdot 7H_2O$)

Bring volume to 500 ml with H_2O. The pH of 10x should be ~6.8. When dilute with water to make 1x, the pH should be ~7.4.

- Trypsin Solution

Trypsin (1:250) 400 mg
Dulbecco's PBS 200 ml

LAB 2: FRUIT FLY GENETICS

I. Introduction

Most of our knowledge of the basic principles of genetics comes from studies of model genetic organisms. For over a hundred years, the fruit fly, *Drosophila melanogaster*, has been the premier model organism for studying transmission genetics and gene function. Its small size and short generation time enables researchers to grow large numbers of flies and study multiple generations in reasonably short time frames. It has an optimal balance between organismal complexity and genetic simplicity making it one of the best organisms for studying the genetics of complex biological phenomena. Finally, homologues for many, if not most, of its genes can be found in other organisms, including humans, making the genetic findings in *Drosophila* all the more relavent. In this lab you will learn how to use and manipulate fruit flies for genetic studies, and will perform experiments that will allow you to visualize some of the most fundamental and widespread principles of genetic inheritance.

II. Background Reading

A. Working with Fruit Flies (for week 1)

Read pages 1-11 of "Drosophila Guide: Introduction to the Genetics and Cytology of *Drosophila melanogaster*," by M. Demerec and B.P. Kaufmann, 10th edition, 1996. This publication is available free online on our course web site.

B. Basic Principles and Definitions of Inheritance (for weeks 2-3)

From this reading you should be able to understand and discuss the following terms and principles: chromosome, gene, allele, Law of Segregation, Law of Independent Assortment, diploid, haploid, homozygous, heterozygous, sex cells (the gametes; ie, the sperm and egg), F1, F2, genotype, phenotype, sex chromosomes, X chromosome, Y chromosome, hemizygous.

Start with "Mendel's Genetics" and "Probability of Inheritance" at:
http://anthro.palomar.edu/mendel/
Then read this article:
http://en.wikipedia.org/wiki/Mendelian_inheritance
Then this one:
http://www.nature.com/scitable/topicpage/Thomas-Hunt-Morgan-and-Sex-Linkage-452

C. Mitosis and Meiosis (for week 4)

The manipulation of chromosomes during asexual and sexual reproduction holds the key to understanding Mendelian Inheritance.

Go to the following site, click on "How Cell's Divide"and launch the interactive video.
http://www.pbs.org/wgbh/nova/baby/

D. Recombination and Gene Mapping (for week 5)

Study this article to learn what crossing over is and how to map genes by recombination:
http://www.nature.com/scitable/topicpage/Thomas-Hunt-Morgan-Genetic-Recombination-and-Gene-496

III. Materials

- Access to Fly Lab and tools for working with fruit flies
- 40 vials and 20 bottles of prepared media

IV. Procedure

A. Week 1

1. Examine wild type and mutant flies
2. Start cross 1: Bar female (virgin) x wsn male
   2-3 pairs of flies per vial, 2 vials per group
3. Start cross 2: wsn female (virgin) x Bar male
   2-3 pairs of flies per vial, 2 vials per group

B. Week 2

4. Discard parents of crosses 1 and 2 after larvae detected (5-8 days)

C. Week 3

5. Classify progeny from crosses 1 and 2 according to sex and phenotype.
   Choose the healthiest vial for each cross, and,
   score a minimum of 20 males and 20 females for each cross.
   Fill out Data Sheets 1 and 2 to be included with your reports.
6. Set up cross 3
   Each group should set up 2 bottles of this cross.
   Put 8-12 males and 8-12 females from cross 2 progeny into each bottle
   (the females do not have to be virgins).

D. Week 4

7. Discard parents of cross 3 after larvae detected (5-8 days), and add 2 cotton plugs to bottle.

E. Week 5

8. Classify progeny from cross 3 according to sex and phenotype.
   Score at **total of 100 male** and **10 female** progeny from the 2 bottles of this cross.
   Fill out Data Sheet 3 to be included with your report.

V. Data Sheets

Data Sheet 1: Cross 1 Results (Bar female x wsn male)
You may or may not need all of these rows.

Sex	Phenotype	Tally	Total
female			
female			
female			
female			
female			
female			
female			
female			
male			
male			
male			
male			
male			
male			
male			
male			

Data Sheet 2: Cross 2 Results (wsn female x Bar male)
You may or may not need all of these rows.

Sex	Phenotype	Tally	Total
female			
female			
female			
female			
female			
female			
female			
female			
male			
male			
male			
male			
male			
male			
male			
male			

Data Sheet 3: Cross 3 Results
You may or may not need all of these rows.

Sex	Phenotype	Tally	Total
female			
female			
female			
female			
female			
female			
female			
female			
male	+ + +		
male	B w sn		
male	+ w sn		
male	B + +		
male	+ + sn		
male	B w +		
male	B + sn		
male	+ w +		

VI. Fly Lab Report

Prepare an abbreviated report that describes the genetic crosses you performed, the results you obtained, and any and all conclusions you can make from your data concerning the nature of the genes and alleles that control the characteristics and traits under investigation. Justify these conclusions with clear and logical arguments and diagrams that also illustrate how the genes and alleles were inherited between parents and offspring in your crosses. Finally, describe the phenomena and principles that explain the complex results from Cross 3. What does that data tell you about the genes involved in these characteristics?

I do not want an Introduction or a Materials and Methods section. I do want thorough but CONCISE coverage of the genetics involved in your crosses.

LAB 3: ISOLATION AND CHARACTERIZATION OF PLASMID DNA

I. General Introduction

The current "genetic revolution" is actually the result of a long history of remarkable scientific achievements. In the late 1800's and early 1900's, Gregor Mendel and his successors determined that **heredity** was controlled by discrete factors (that we now call **genes**). Early cell biologists found a strong correlation between the behavior of genes and the behavior of cellular structures called **chromosomes**. Biochemists determined that chromosomes were made of both **DNA** and protein, and the work of Avery, McCarty, MacLeod, Hershey, Chase, and others demonstrated that it was the DNA of the chromosomes, and not the protein, that was the genetic material. In 1953, James Watson and Francis Crick proposed the double helix model for DNA, which explained the ability of DNA to store complex information for cell structure and function as well as the ability of DNA to replicate for the faithful transmission of hereditary traits from one generation to the next. Currently, sophisticated techniques are being exploited for isolating DNA, cloning genes, and determining how genes function. To comprehend fully these topics, you must understand some of the basic principles of DNA analysis. In this series of labs you will learn some simple methods for isolating and analyzing DNA. You will first extract plasmid DNA from bacteria. You will then digest (break apart) samples of your DNA with **restriction enzymes** in preparation for the analysis of the DNA by agarose gel **electrophoresis.**

II. Background Reading

For this lab, read to and through the section on "Gel Electrophoresis: Separating DNA Molecules of Different Lengths" of the following article.

http://www.ncbi.nlm.nih.gov/About/primer/genetics_molecular.html

III. Day 1: Isolation of Bacterial Plasmid DNA

A. Introduction

DNA is found in a variety of different sizes and configurations in different organisms. The chromosomes of higher organisms, such as man, contain long, linear molecules of DNA. In bacteria, the chromosomes are circular, as are the DNA molecules contained in these chromosomes. Although these circular DNA molecules are usually broken into linear fragments during purification, some bacteria also possess smaller extrachromosomal circular DNA molecules that are easier to isolate without breaking. These extrachromosomal DNA molecules are called **plasmids** and contain genes that are not essential to the bacteria but confer specialized functions such as resistance to antibiotics. You will be given two cultures of *Escherichia coli* from which you will isolate plasmid DNA. The plasmid in one of the cultures is an artificially modified plasmid that has been engineered for use as a **cloning vector**. The term **vector** refers to

its ability to take a piece of foreign DNA into a bacterial cell and allow that foreign DNA to be replicated -- the replication of foreign DNA in bacteria is, in essence, **gene cloning**. The plasmid in the other culture is a derivative of the same cloning vector that has actually been spliced (joined) to a piece of DNA originally isolated from rabbits. Plasmids that consist of DNA from two different biological sources (i.e., bacteria and rabbits) are called **recombinant plasmids.** Later you will learn how this recombinant plasmid was generated and will verify the presence of the rabbit DNA segment, but first you must isolate these two types of plasmid DNA. By centrifugation, you will concentrate the bacterial cells into a dense pellet at the bottom of a centrifuge tube. The cells will be suspended in a lysis buffer that contains lysozyme, an enzyme that degrades bacterial cell walls. Boiling the cells in this buffer for a short time breaks open the cells and releases the small circular plasmids. The larger bacterial chromosome is attached to the inside of the bacterial membrane in the living cell, and after cell lysis, remains attached to pieces of the membrane that form pellets when centrifuged. The plasmid DNA is then precipitated from the supernatants with isopropyl alcohol.

B. Materials

overnight culture of bacteria containing the cloning vector
overnight culture of bacteria containing the recombinant plasmid
STET buffer (Sucrose, Triton-X, EDTA, and Tris)
lysozyme solution (10 mg/ml in H_2O, made fresh)
TE (Tris, EDTA)
isopropyl alcohol
70% ethanol
microcentrifuge
vortex mixer
hot plate, dish, and microtube float (for boiling water bath)
4^oC refrigerator and -20^oC freezer
Pipetman and tips
1.5 ml microfuge tubes
waste beakers
ice buckets with ice
toothpicks
Bunsen burner and dissecting needle

C. Procedure (work in groups of two)

1. Label three 1.5 ml microfuge tubes *vector*, and three *r-plasmid*. Include your initials here and any time you are labeling tubes.

2. Transfer 1.4 ml of the bacterial culture containing the cloning vector into each of two of the *vector* tubes and 1.4 ml of the bacterial culture containing the recombinant plasmid into each of two of the *r-plasmid* tubes. This is most conveniently done by transferring 0.7 ml twice to each tube. **Make sure you swirl the cultures just**

before taking your samples to disperse the bacteria that have settled to the bottom of the flasks.

3. Place the microfuge tubes in the microcentrifuge such that the tube hinges point away from the center of the rotor. Do not forget to balance the rotor. Spin tubes for 5 minutes at the maximal speed. Pour the supernatants into waste beaker and then remove as much of remaining fluid as possible with a P200 Pipetman set to ~80 µl. DO NOT TOUCH THE PELLET WITH THE PIPET TIP!

4. Add 70 µl of STET buffer to each pellet. Vortex until the pellets are suspended. Add 5 µl lysozyme solution to each tube and vortex to mix. When adding the lysozyme, use the pipette tip to disrupt any bacterial pellet that did not suspend while vortexing.

5. Pierce the tops of the microfuge tubes with a flamed needle as will be demonstrated by your TAs, and then float the tubes in a boiling water bath for **exactly** 40 seconds. After boiling, spin the tubes for 1 minute in the microcentrifuge at maximum speed.

6. Add 70 µl TE to each tube. Spin tubes for another 10 minutes at maximum speed. At the bottom of each tube should now be a large, flocculent pellet under a small volume of fluid (the supernatant). Spear the flocculent pellet in each tube with a toothpick, slowly drag the pellets out of the tubes (allowing residual fluid to drain back to the bottom of the tube), and discard the pellets in an appropriate waste container.

7. Set the P-200 Pipetman to 25 µl and transfer the supernatants from the two *vector* tubes into a single, clean microfuge tube. Keep track of the total volume transferred (by counting how many 25 µl aliquots are transferred), and record that volume here: *vector* volume_____. In the same manner, combine the supernatants for the two *r-plasmid* preparations into a single tube: *r-plasmid* volume_____.

8. To each tube, add a volume of isopropyl alcohol that is equal to the supernatant volume recorded for that tube in the previous step. Vortex and incubate at -20^{o}C for 20 minutes or longer.

9. Spin tubes for 5 minutes in microcentrifuge (maximum speed setting), once again pointing the tube hinges away from the center of the rotor. Pour off supernatants into a waste beaker. Gently add 400 µl of 70% ethanol to each pellet. Spin for 5 minutes at maximum speed. Pour off supernatants, pop spin, and then remove as much of the remaining fluid as possible with a Pipetman and without disturbing the pellet. Invert tubes to dry on a Kimwipe for a minimum of 20 minutes.

10. Add 40 µl of TE to each pellet (your instructor or TAs may suggest a different volume after inspecting your pellets - check with them before adding the TE). Let tubes sit at room temperature for 5 minutes and then vortex briefly. Store tubes at 4^{o}C.

IV. Day 2: Restriction Enzyme Digestion of DNA Samples

A. Introduction

The manipulation and analysis of DNA has been greatly facilitated by a remarkable group of enzymes called **restriction enzymes**. These enzymes are found in bacteria and protect the bacteria from viral infection by cutting viral DNA into harmless pieces. The mechanism of cutting is such that the enzyme recognizes and cuts the DNA at a specific sequence of nucleotide base pairs. Each bacterial species that has been investigated has a different restriction enzyme that recognizes and cuts a different sequence of base pairs. Purified restriction enzymes are used by molecular biologists to cut large DNA molecules into smaller and more manageable pieces. An important property of restriction enzymes is that the ends of DNA fragments generated by restriction enzyme digestion have single-stranded extensions that are complementary to the extensions on any DNA fragments generated by the same restriction enzyme (see diagram below). Because of this property, fragments of DNA from different biological sources can be spliced (joined) together if they were generated by digestion with the same restriction enzyme. This is the basis of **gene cloning** which involves the splicing of a specific fragment of DNA into a restriction enzyme site in a cloning vector such as a bacterial plasmid. Upon reintroduction of the recombinant plasmid into a bacterial cell, the bacterium serves as a factory for the generation of large quantities of the cloned DNA for a variety of biochemical investigations.

This week you perform restriction enzyme digestions of your plasmid DNA preparations. Upon subsequent analysis of the DNA by gel electrophoresis, you will get a visual demonstration of the changes in the configuration of DNA that accompany restriction enzyme digestion and will see how it is possible to map restriction enzyme sites in cloned DNA. The digestions with restriction enzymes are simple to perform. Each reaction will consist of a small sample of one of your DNA preparations, a purified restriction enzyme purchased from a biological supply firm, a buffer mixture that provides the salts and pH to optimize the activity of the restriction enzyme, and ddH_2O to bring the volume of each reaction to 20 µl. The volume of the reaction is important because the buffer was designed to be diluted exactly 10 fold to provide the optimal enzyme conditions. The restriction enzymes we will use are PstI (isolated from the bacterium *Providencia stuartii*) and EcoRI (isolated from *Escherichia coli* strain RY 13). The base pair sequence that these enzymes recognize, and the reactions catalyzed by each enzyme are illustrated below.

```
5'----------CTGCAG----------3'   +   PstI  →   5'----------C          TGCAG----------3'
3'----------GACGTC----------5'                 3'----------GACGT          C----------5'

5'----------GAATTC----------3'   +   EcoRI  →  5'----------G          AATTC----------3'
3'----------CTTAAG----------5'                 3'----------CTTAA          G----------5'
```

B. Materials

BRL REact buffer 2
BRL REact buffer 3
PstI enzyme (10 units/µl)
EcoRI enzyme (10 units/µl)
10x gel juice
ddH_2O
Pipetman and tips
tabletop centrifuge
microfuge tubes
37°C water bath and microfuge tube floats
ice buckets and ice

C. Procedure

1. Label six 1.5 ml microfuge tubes *1-6*, and include your initials.

2. Set up restriction enzyme digests of your DNA samples by adding the ingredients in Table 1 to the tubes in the order shown (i.e., first add DNA to all tubes, then add water, then buffer, and then enzyme). Make sure that you spin your plasmid DNA tubes and the reaction buffer tubes for a few seconds in the microcentrifuge before taking samples from them. Also, put restriction enzyme stocks back in the ice bucket as soon as you are done with them.

3. Briefly vortex your reaction tubes, pop spin, and float them in the 37°C water bath for 60 min.

4. After the 60 min. incubation, add 2 µl of 10x gel juice to each tube, vortex and pop spin. Store tubes at 4°C in the refrigerator.

Table 1: Ingredients for restriction enzyme digestion of DNA samples

sample	1	2	3	4	5	6
DNA	r-plasmid DNA 4 µl	r-plasmid DNA 4 µl	r-plasmid DNA 4 µl	r-plasmid DNA 4 µl	vector DNA 4 µl	vector DNA 4 µl
ddH_2O	16 µl	13 µl	13 µl	12 µl	16 µl	13 µl
buffer	none	2 µl REact buffer 3	2 µl REact buffer 2	2 µl REact buffer 2	none	2 µl REact buffer 3
enzyme	none	1 µl EcoRI	1 µl PstI	1 µl PstI and 1 µl EcoRI	none	1 µl EcoRI

V. Day 3: Electrophoresis of Restriction Enzyme Digests and Uncut DNA Samples

A. Introduction

Today you will examine the restriction enzyme digestions you performed last session. Electrophoresis is one of the most common techniques used by cellular and molecular biologists. The basis of electrophoresis is that nucleic acids (DNA or RNA), or proteins coated with the negatively charged detergent sodium dodecyl sulfate, have uniform negative charges and migrate towards the positive pole in an electric field. If macromolecules are forced to migrate through a semisolid matrix (gel) formed by **polymers** such as **agarose** or **acrylamide**, they will worm their way through the microscopic pores within the polymer matrix, and small molecules will pass through the pores with greater ease and speed than larger molecules. This means that if a mixture of DNA molecules is electrophoresed for a fixed period of time, the molecules will separate according to size, with the smaller fragments migrating to a greater distance than the larger molecules. Because the distance of migration is inversely proportional to size, the actual sizes of the DNA fragments can be determined by comparing the distances they migrated to the migration of DNA fragments with known sizes (size standards).

You will see that the configuration of the DNA can also affect its ability to migrate through an electrophoretic gel. The plasmid DNAs you isolated earlier are primarily in the form of super-coiled circles in which the circular double helices are coiled up like an over wound yo-yo string (this super-coiling is actually in response to an under winding of the two strands of the double helix). The tightly compacted structure of the super-coiled circles allows them to migrate faster during electrophoresis than linear DNA with the same base pair composition. However, if one of the strands is broken, the other strand maintains the circular nature of the plasmid, but the coiling is reduced and the result is a relaxed circle. The bloated structure of the relaxed circle inhibits its migration through the agarose pores, and the relaxed circle migrates more slowly than a linear molecule of the same base pair composition. If both strands of the DNA molecule are broken the molecule is linearized (linear plasmid) and migrates at a speed that is intermediate between those of the super-coiled and relaxed circular forms. In addition to super-coiled, relaxed circles, and linear plasmids, even more slowly migrating forms are often found. These are called concatemers and occur when several circular plasmids are linked together like the links of a bracelet. With some help, you will be able to identify each of these forms in your undigested and digested plasmid preparations.

Visualization of the DNA is made possible by the addition of ethidium bromide to the agarose before it cools. Ethidium bromide inserts between the base pairs in double stranded DNA. When exposed to ultraviolet light, it fluoresces, revealing the location of the migrated DNA.

B. Materials

10x TAE
dd H_2O
agarose (standard electrophoresis grade)
ethidium bromide solution (10 mg/ml in H_2O)
DNA size markers in gel juice (6650, 4973, 3148, 2396, 1585, and 692 base pairs)
gel box (2), gel casting trays (2), gel combs (4), sealing tape
power supplies and cables
microwave oven
microcentrifuge
Pipetman and tips

C. Procedure

1. The gels will have been poured and solidified prior to the lab period . (While your gel is electrophoresing, we will demonstrate how they were prepared.) The TA will remove the combs and clean the gels by squirting buffer into the wells with a Pasteur pipette.

2. Each group of students should have six samples from the previous session ready for electrophoresis. The TA will show each group where to load their samples on the gel.

3. Spin the six tubes containing your DNA samples for 1 second (pop spin) in the microcentrifuge. Set the P-20 Pipetman to 18 µl. Slowly suck 18 µl of the first DNA sample into the pipette tip. If you don't have 18 µl of sample, reset the Pipetman to a smaller volume so as not to suck up any air. Carefully immerse the tip a few mm into the center of the first well assigned to your group. Make sure that the opening of the tip is in the buffer fluid and not touching the sides or the bottom of the well. **Slowly** expel the sample into the well, allowing the sample to gradually fall to the bottom of the well. Repeat for each of the remaining five samples.

4. Your instructor will fill one or two wells near your samples with a mixture of DNA fragments with known sizes to assist you in your subsequent analysis.

5. After all of the DNA samples are loaded into the wells, the TA will connect the power supply cables from the gel box to the power supply. He or she will then set the initial current to 12.5 milliamps and turn on the power supply.

6. After 20 minutes, the current will be raised to 40 milliamps. Electrophoresis will continue for 2-4 hours. Do not touch cables, gel, or electrophoresis buffer while the current is on.

7. The instructor or TAs will then photograph the gel, and copies of the photographs will be distributed to you during the next lab session.

VI. Lab Report - Isolation and Characterization of Plasmid DNA

1. Describe any problems you had with the protocol, or any mistakes or deviations you made in the protocol. How might these mistakes have affected your end results? Include a description of your samples just before you loaded them on the agarose gel. Did they have the correct volume? Were they uniform in volume? If the answer was no to either of these questions, what do you think you did wrong? How might this have affected your final results?

2. Briefly describe the purpose or rationale of each of the following protocol steps.

a. The addition of isopropyl alcohol during the isolation of plasmid DNA.

b. Boiling your bacteria for exactly 40 seconds during the isolation of plasmid DNA.

c. Incubating the restriction enzyme digests at 37^{o}C . (Why 37^{o}?)

d. Adding gel juice to your DNA samples before electrophoresis.

e. Loading your DNA samples at the negative pole of the gel during electrophoresis.

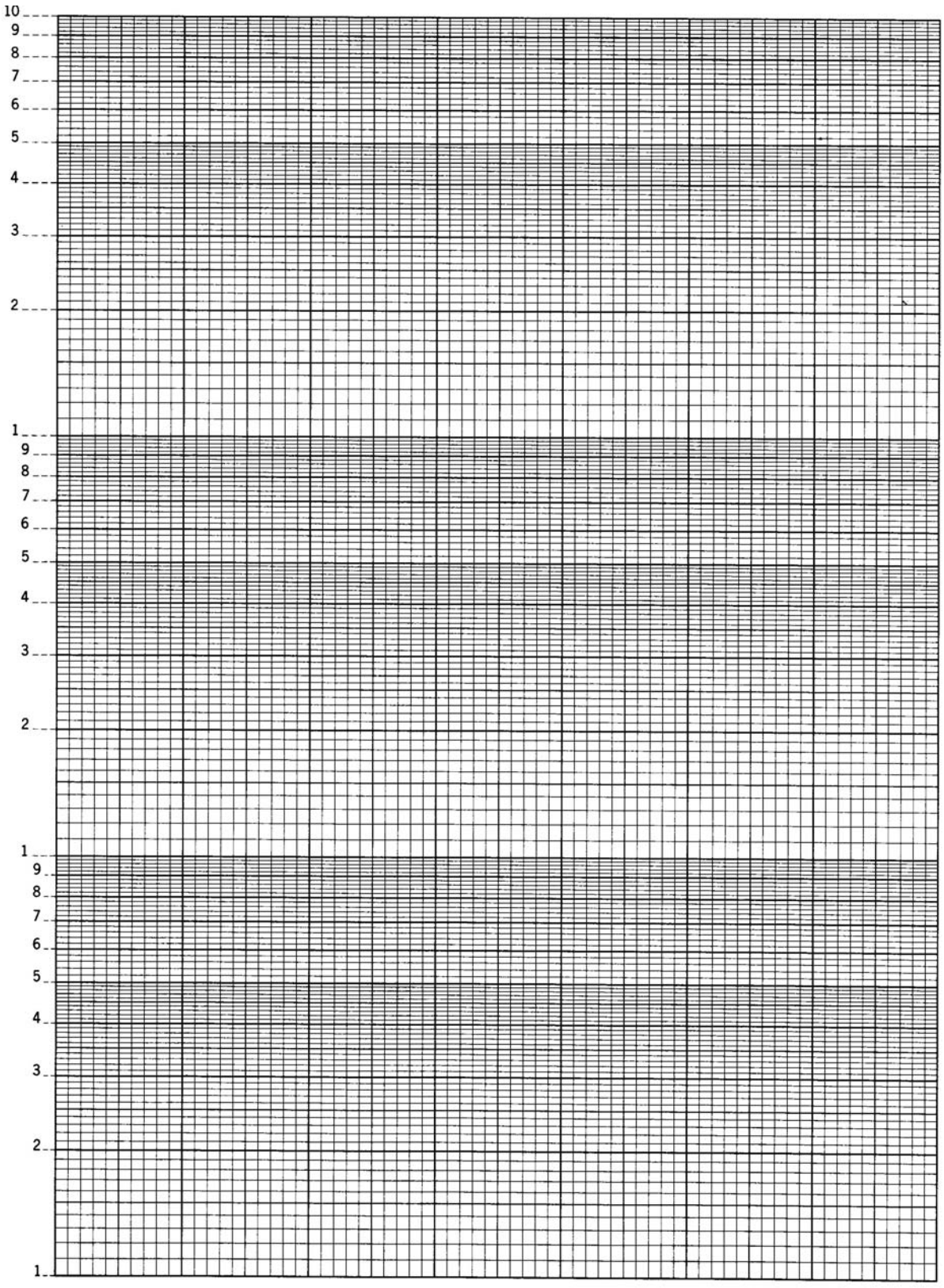
3. Data Analysis and Discussion

For questions 3a and 3b, you do not have to use your own digests for the analysis. Use the digests and nearby markers that you believe will give you the most accurate results.

a. On the photograph of the gel provided to you, label bands in one of the lanes with uncut vector that correspond to RNA and to the following forms of DNA: supercoiled circles, nicked circles, linearized plasmid, concatamers.

b. Use a metric ruler to measure the distances from the bottom of the wells to the bottom of each of the DNA fragments in the DNA size markers lane (labeled "M" on the gel). Use a piece of semi-log graph paper and plot the distance each fragment migrated (in mm on the standard scale) versus the size of the DNA fragment (in base pairs on the log scale). Use this standard curve to determine the sizes of each of the fragments in your plasmid lanes. Use this information to construct simple restriction enzyme maps of the vector plasmid and the recombinant plasmid (vector + insert).

c. Briefly describe an important concept or principle you learned from this lab. (This could be something you didn't know before, or that was previously vague to you but was clarified in this lab.)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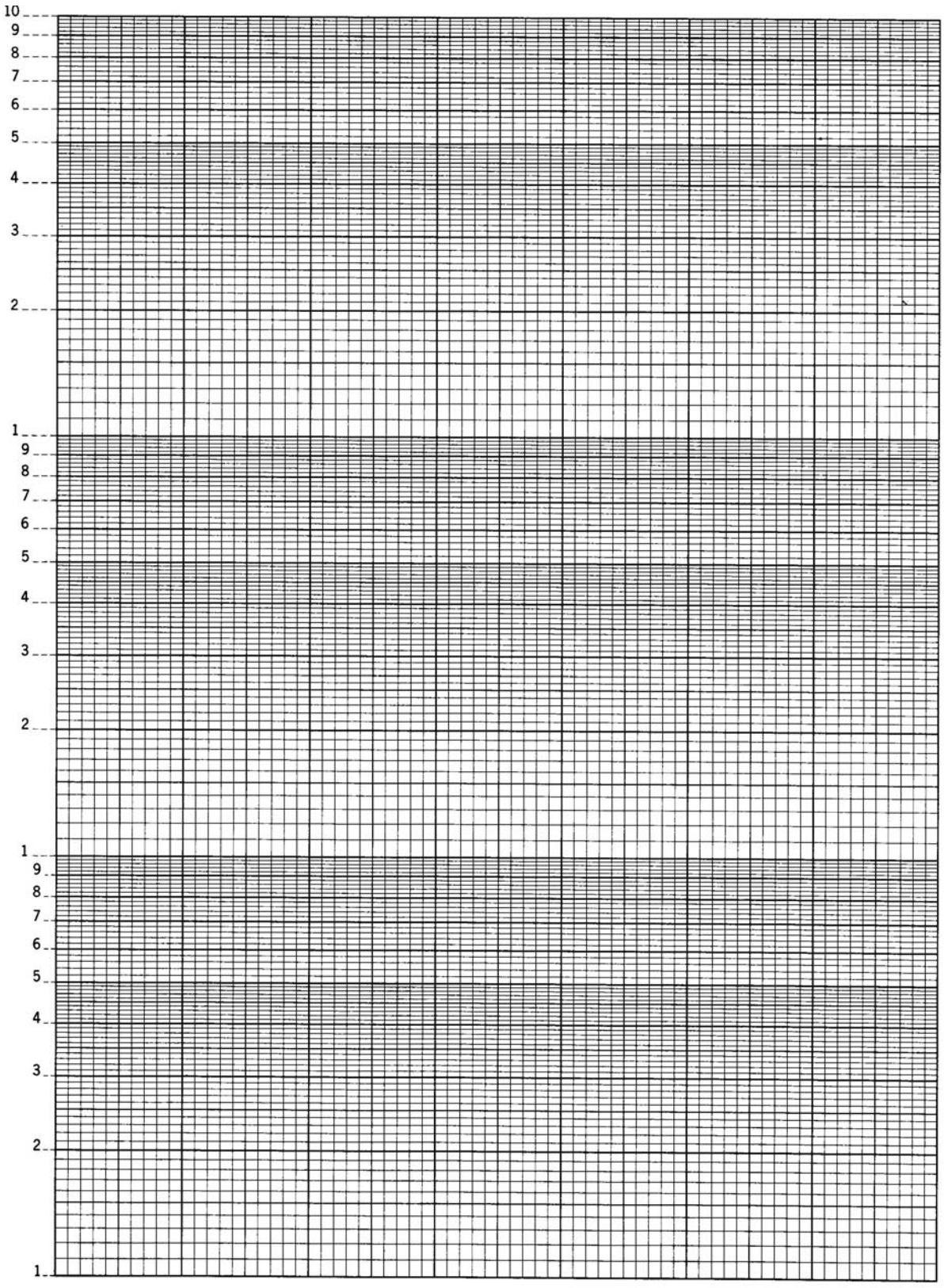
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LAB 4: ALLELIC TYPING OF THE ABO GENE BY PCR

I. General Introduction

On June 26, 2000 it was officially announced that the sequencing of the entire human genome had been completed. Deciphering the 3 billion base pairs of information provided by this project, and attempting to understand how this information is integrated and used during the growth and development of the human organism, will keep scientists busy for decades to come. But we do not have to wait for a full understanding of the workings of the human genome to take advantage of the wealth of sequence information that resulted from this project. This information and the powerful tools of molecular biology are already providing us with novel and sophisticated methods for augmenting medical practices. One example of this is the detection of disease causing alleles in adults or unborn fetuses to give parents the information they need to make difficult medical decisions and to help physicians better tailor medical procedures for the individual. To illustrate how allelic typing can be accomplished, we will each determine our allelic composition (genotype) for the gene that determines our ABO blood type. This procedure relies on PCR (the polymerase chain reaction), a technique that has revolutionized molecular biology and won Kary Mullis the 1993 Nobel Prize in chemistry. With this technique it is possible to specifically amplify one gene out of the entire genome, and produce enough copies of it to determine its nucleotide sequence by standard biochemical procedures. In our exercise we will amplify the ABO gene from total genomic DNA isolated from cheek epidermal cells. The amplification product will be purified and a sample of it will be examined by agarose gel electrophoresis. Upon verification that the amplification and purification were successful, it will be given to the FSU Sequencing Facility where it will be sequenced using automated technology. A comparison of this sequence with sequences published in the literature will enable you to determine the allele(s) you possess for the ABO gene.

II. Day 1: DNA Isolation and PCR

A. Introduction

In today's lab you will isolate DNA from a sample of your cheek cells and perform PCR to amplify the ABO gene. By vigorously rinsing your mouth with an isotonic saline solution, you should be able to collect several thousand cheek cells. Each of these cells contains 6.5 pg of DNA, but only a small fraction of this DNA corresponds to the segment of the ABO gene that we are investigating (less than 0.000007%). Even if you collect 10,000 cheek cells, you are starting your PCR reactions with less than 0.001 pg of DNA corresponding to the ABO gene. Nevertheless, after amplification you will have approximately 1 μg of pure ABO segment which is more than sufficient for electrophoresis and DNA sequencing. The polymerase chain reaction is described in Appendix 1, and the molecular biology of the ABO gene is described in Appendix 2. Both appendices should be studied before starting this lab.

B. Materials

gloves
sterile 1.5 and 0.5 ml microfuge tubes
extra fine point Sharpies
clean Pipetman and tips
clean tube racks and ice buckets
clean microcentrifuge
boiling water bath
thermocycler
sterile pipettes and pipette fillers
sterile disposable drinking cups
ice and ice buckets
0.9% NaCl (physiological saline)
10% Chelex in H_2O
sterile mineral oil
95% ethanol in squirt bottles
dilute Taq polymerase (2 U/10 μl: 0.4 μl Taq polymerase + 9.6 μl H_2O per reaction)

PCR reaction mix (for each reaction)

22.25 μl H_2O
5.0 μl 10X Taq Polymerase buffer
1.25 μl 50 mM $MgCl_2$ (2 mM $MgCl_2$ in final reaction)
0.5 μl 20 mM dNTPs (200 μM each in final reaction)
2.0 μl primer 3517 at 100 ng/μl (200 ng)
2.0 μl primer 3518 at 100 ng/μl (200 ng)

C. Procedure (wear gloves throughout this procedure)

1. Label two 1.5 ml microcentrifuge tubes with your initials, and a third with your initials and "*cheek DNA*." Also label a sterile drinking cup with your initials.

2. With a sterile pipette, transfer 10 ml of 0.9% NaCl into the drinking cup.

3. Pour the saline into your mouth and swish vigorously from cheek to cheek for 45 sec to 1 min.

4. Expel the saline back into the same drinking cup.

5. With P1000 Pipetman, transfer 1 ml of your cheek cell solution into each of the two 1.5 ml microcentrifuge tubes labeled just with your initials. ***Make sure you briefly swirl the solution in the drinking cup before taking your samples***.

6. Spin the tubes for 1 min in the microcentrifuge.
7. Carefully remove the supernatants with a P1000 Pipetman set to 1 ml. Do not disturb

the pellets, and leave just enough fluid (~25 μl) to completely cover the cell pellets. Discard the supernatants into appropriate waste containers.

8. Resuspend one of the pellets in the remaining fluid by pipetting the fluid and pellet up and down with a P200 Pipetman set to 25 μl.

9. Transfer the fluid and resuspended pellet to the tube with your other cell pellet, and resuspend the pellet in that tube. You should now have ~50 μl of fluid in one tube containing both of your resuspended pellets.

10. Vortex the 10% Chelex solution to get an even distribution of Chelex throughout the suspension, and then immediately transfer 200 μl of the Chelex suspension to your resuspended pellet using a P1000 Pipetman.

11. Vortex your tube for a few seconds, poke a hole in its top as instructed by your TA, and then incubate in a boiling water bath for 10 min. This breaks open the cheek cells and releases the DNA into solution.

12. Carefully remove the tube from the boiling water bath (*don't burn yourself*), vortex it for a few seconds, and then spin it for 1 min in the microcentrifuge.

13. There should be a pellet at the bottom of your tube that consists of cellular debris and the Chelex particles. *(The Chelex particles bind metal ions and other cellular factors that would inhibit the PCR reaction.)* Avoid this pellet and transfer 100 μl of the supernatant to the tube labeled with your initials and "*cheek DNA*."

14. Label a 0.5 ml microcentrifuge tube with your initials and the number *1* and another with your initials and the number *2*. Write your labels in two places on these tubes; once on the sides but near the top, and once on the actual tops of the tubes.

15. Using a P200 Pipetman, transfer 33 μl of the PCR reaction mix that has been prepared by your instructor to each of your 0.5 ml tubes. (Your TA might do this for you to avoid wasting this precious mixture.)

16. Using a P20 Pipetman, add 7 μl of your cheek cell DNA preparation to each of your tubes. Do not discard the tube with the remainder of your cheek cell DNA preparation. You will need this later. Instead, store it at 4^{o}C as directed by your TA.

17. Firmly hold the tubes at their top and flick the bottoms with your index finger to mix. Spin for a few seconds in the microcentrifuge. (Make sure you use the 0.5 ml tube adaptors that should already be in place in the microcentrifuge.) Overlay 40 μl of mineral oil to each tube by slowly pipetting the oil onto the inner wall of the microcentrifuge tube just above the level of the reaction mixture. Spin for a few seconds in the microcentrifuge. Keep the tubes at room temperature.

18. When all students have set up their reactions, the tubes will be put on the thermocycler. *The TA will include a control reaction tube with NO DNA.* After the initial heating at 94°C, the instructor will add 10 µl (2 units) of dilute Taq polymerase to each tube. The complete program for amplifying the ABO gene is as follows:

Hot Start
94°C 3 min (to denature the double-stranded DNA)
80°C 6 min (to keep the strands separated while adding the enzyme)
Thermocycle (x30)
94°C 30 sec (denature)
60°C 1 min (anneal primers)
72°C 1 min (extension)
Final extension
72°C 7 min
4°C hold

19. Upon completion of the program, the TA will store your tubes at 4°C.

III. Day 2: Product Purification

A. Introduction

The ultimate goal of this lab is to sequence the 213 bp fragment that was amplified from the ABO gene. The nucleotides and primers remaining in the PCR reaction would interfere with the sequencing reactions, so the amplified product needs to be purified from these unwanted substances. This will be accomplished using QIAquick columns made specifically for PCR purification. The column material is silica glass which binds DNA at high ionic strength. Diluting your sample in buffer PB raises the ionic strength to a level that causes the amplification product to bind to the column. While the DNA is bound to the column, it is washed with another buffer of high ionic strength (buffer PE) to remove all traces of unwanted substances from the reaction. Finally, the column is flushed with water which causes the DNA to become unbound and elute into a collection tube. The amplification product is now pure and ready to sequence.

Because DNA sequencing is a relatively expensive procedure, we want to make sure you have enough material to make an attempt at sequencing worthwhile. Next lab period, we will get a rough estimate of the success of your amplifications by analyzing aliquots of your reactions by gel electrophoresis. For this purpose you will prepare samples of your PCR reactions before the QIAquick column purifications, and another of the purified product, that will be used for electorphoresis next period.

B. Materials

Pipetman, tips, 1.5 ml microfuge tubes, racks
microcentrifuge and vortex
ice bucket and ice
gel juice
QIAquick columns and collection tubes
QIAquick buffers PB and PE
ddH_2O

C. Procedure (also see flow chart at the end of this procedure)

1. Label six 1.5 ml microfuge tubes with your initials and the following:
   PCR1 gel
   PCR2 gel
   PCR combined
   ABO elutant (Use a capless 1.5 ml tube for this)
   elutant gel
   Cheek DNA, no PCR

2. Retrieve your PCR reaction tubes from the 4°C refrigerator and give them a pop spin. Use the centrifuge labeled "after PCR" for this and all subsequent steps. Transfer 10 µl of one of your PCR reactions to the *PCR1 gel* tube, and 10 µl of your other PCR reaction to the *PCR2 gel* tube. Take your samples from the lower layer (aqueous layer) in the reaction tubes and try not to suck up any of the mineral oil (do not release the plunger on the Pipetman until the tip is completely past the oil and into the aqueous layer). Remove excess oil from the tip by scraping it against the inside of the tube opening as you pull out of the tube.

3. Add 1.5 µl of gel juice to the *PCR1 gel* and *PCR2 gel* tubes. Vortex briefly, spin for 5 seconds and store on ice. *These are "before column" samples of your PCR reactions to examine by gel electrophoresis.*

4. Transfer 35 µl from what remains in *each* of the PCR reaction tubes to the *PCR combined* tube. Again, try to avoid transferring any mineral oil with your samples. The 70 µl in this tube represents the bulk of your PCR reactions and will be purified by passage over a QIAquick column.

5. Add 5 volumes of Buffer PB to 1 volume of your combined PCR reaction. If you were able to collect 70 µl of combined reactions, you would add 350 µl of Buffer PB. Briefly vortex and spin for 5 sec.

6. Obtain a QIAquick column. Make sure that it comes attached to a 2-ml collection tube. Label the column and the collection tube with your initials. Apply your PCR

reaction + PB to the center of the top of the column matrix with a P1000 Pipetman. Centrifuge for 1 min.

7. Discard the fluid in the collection tube, and put the column back into the same tube.

8. Wash the DNA bound to the column by adding 0.75 ml Buffer PE to the column and centrifuging for 1 min.

9. Discard the flow through, replace column into same collection tube, and centrifuge for an additional 1 min. Discard the collection tube.

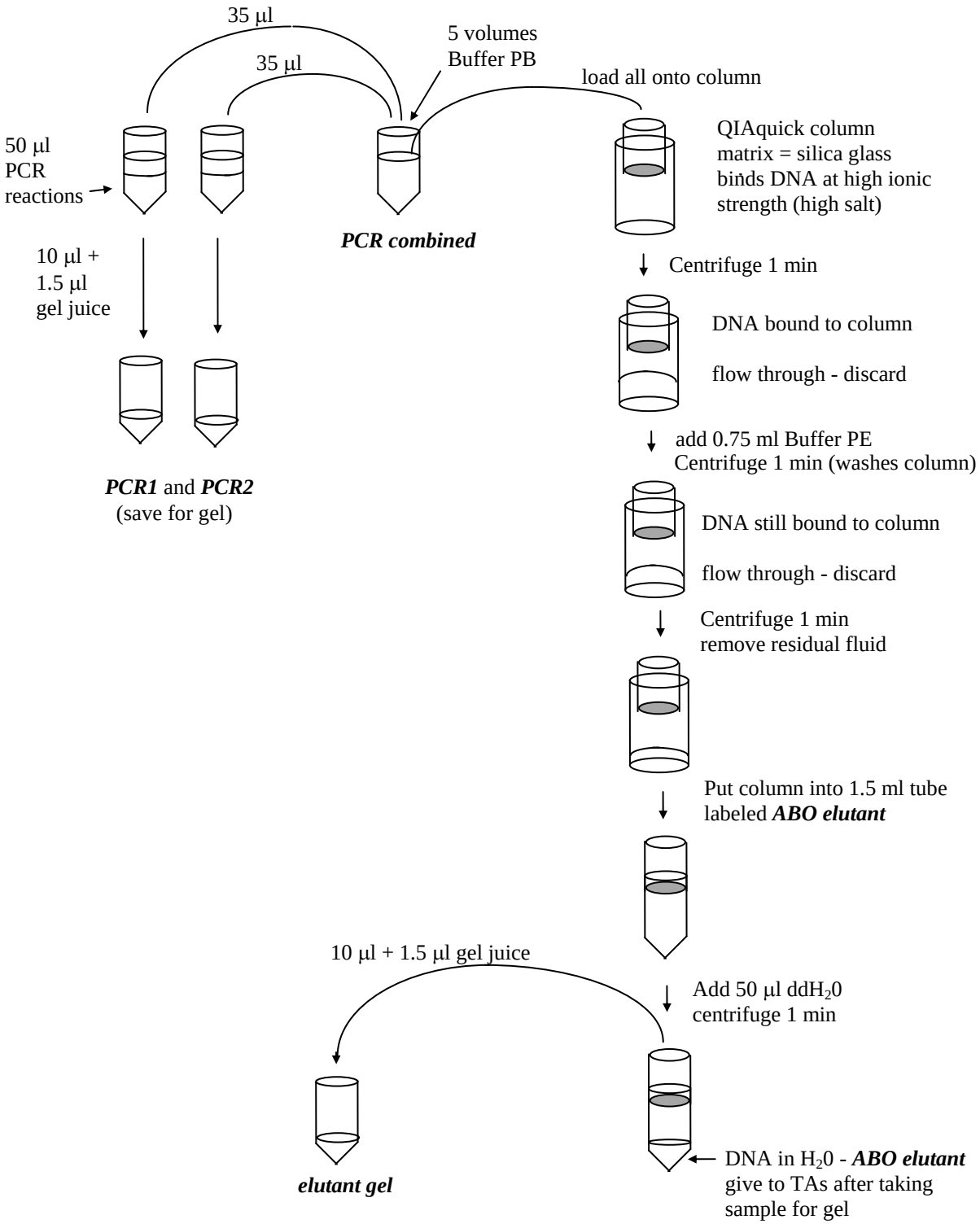
10. Place QIAquick column with bound DNA into the capless tube labeled *ABO elutant*. Add 50 µl ddH_2O to the center of the column matrix and centrifuge for 1 min. The flow through now contains your purified PCR product.

11. Transfer 10 µl from the *ABO elutant* tube to the tube labeled *elutant gel*. Add 1.5 µl of gel juice to this sample for the gel, vortex, and centrifuge for 5 sec. Store on ice. Insert a cap onto the *ABO elutant* tube, and store this tube as directed by the TAs.

12. Retrieve your original cheek DNA preparation from the refrigerator. Vortex and pop spin this tube and then transfer 10 µl of it to the tube labeled *cheek DNA, no PCR*. Add 1.5 µl of gel juice, vortex, and pop spin. Store on ice.

13. You should now have four tubes of samples on ice that will be examined by electrophoresis next period. Make sure these tubes are properly labeled, and store them as directed at 4^oC.

QIAquick Flow Chart



IV. Day 3: Electrophoresis

A. Introduction

Today you will examine the samples you prepared last lab period by electrophoresis on a 2.5% agarose gel. The results will help us determine how much of your purified product to use for the sequencing reactions.

B. Materials

Pipetman, tips, tubes, and racks
microcentrifuge and vortex
materials and equipment for 2.5% agarose gel electrophoresis (see Day 3 of Lab 1)

C. Procedure

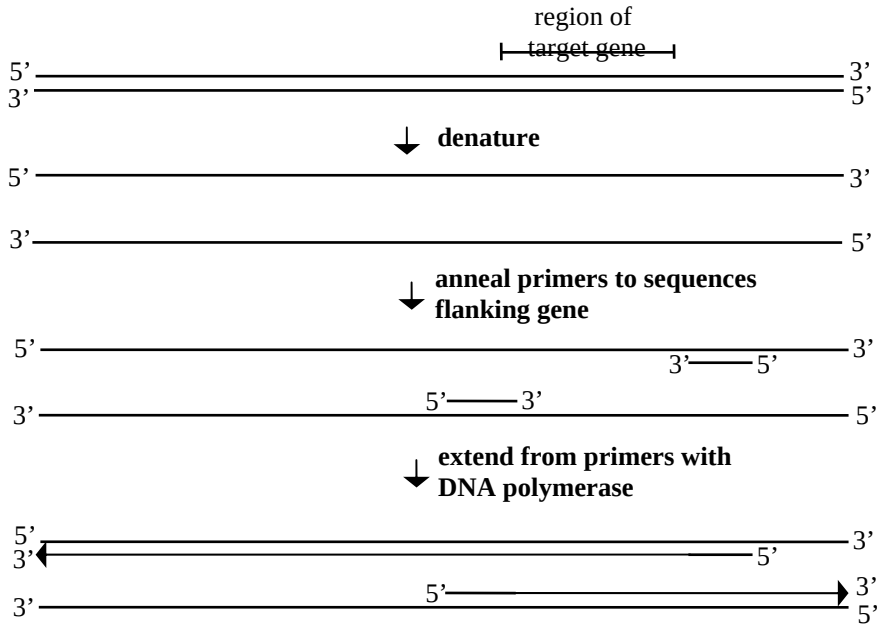
You should have four tubes of samples that you prepared last lab period to examine by electrophoresis. A 2.5% agarose gel in 1x TAE will be prepared for this purpose. Load 10 μl of your samples into the lanes assigned to you by the TAs. A photograph of the finished gel will be given to you during the next lab meeting. From these results we will determine how much of your *ABO elutant* is needed for DNA sequencing.

V. Sequencing

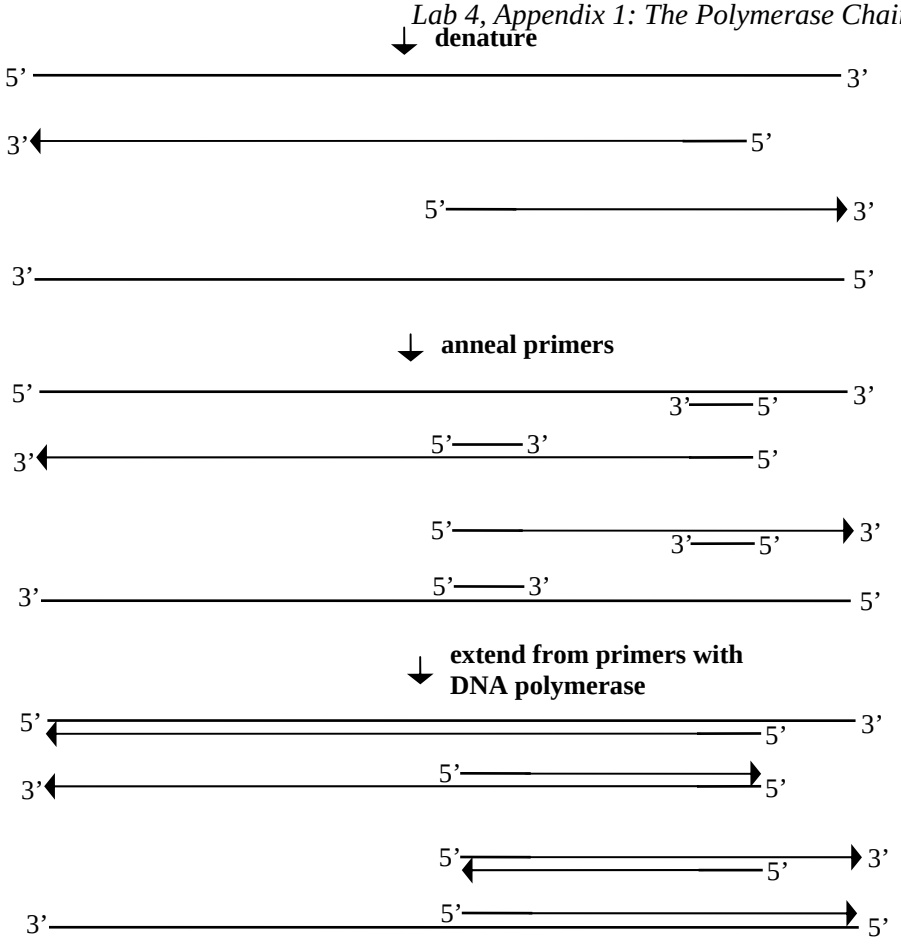
You will be instructed how much of your ABO elutant to put into a clean 1.5 ml microfuge tube to give to the DNA Sequencing Facility. The staff at this facility will perform automated sequencing of your amplification product using methods described in Appendix 3.

APPENDIX 1: THE POLYMERASE CHAIN REACTION

The polymerase chain reaction, or PCR, is a technique that allows for the amplification of a specific target DNA sequence within a larger population of DNA (such as the human genome). Using PCR, picogram quantities of target DNA can be amplified to yield microgram quantities for subsequent biochemical analysis. In order to perform PCR, the DNA sequence flanking both sides of the target must be known. (Note that PCR cannot be used as a substitute for cloning unknown genes!) This information is used to synthesize short oligonucleotides (single-stranded DNA, ~20 nucleotides long) that are complementary to these flanking sequences. These oligonucleotides will be used as "primers" to prime replication of the target DNA by DNA polymerase. (Note in the diagrams below that these primers are complementary to opposite strands of the double helix and thus their 3' ends point towards each other when annealed to their complementary sequence.). It should be easy to follow the first cycle of the replication process. The target DNA is **denatured** into single strands by incubation at 94C, the primers are allowed to **anneal** to their complementary sequences by reducing the temperature to 60C, and finally the temperature is elevated to 72C to allow DNA polymerase to replicate the target by **extending** from the 3' end of the primers. (The exact temperatures for each of these steps vary between different PCR protocols and are dependent on the nucleotide composition of the primers being used. The temperatures given here are those used in our ABO gene PCR protocol.)



What has just been described is one cycle of replication. The "chain reaction" part of PCR involves repeated cycles of replication, involving repeated denaturation, annealing, and extension steps, so that the replication products of one cycle become templates for replication in the next cycles. The result is an exponential increase in the amount of target DNA. The next cycle of PCR goes as follows:



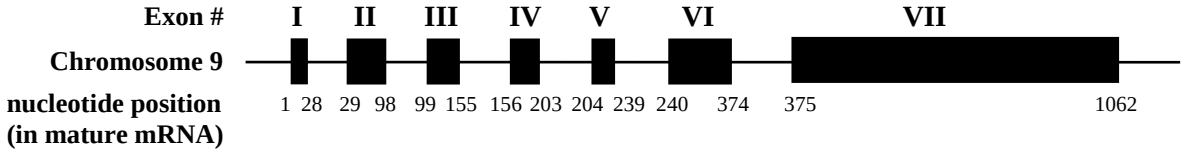
After two cycles of PCR, we have four double-stranded DNA molecules containing the gene we are amplifying. After another round, we would double that number again to eight. On your own, diagram the steps and products of another cycle of PCR.

This procedure could be performed using DNA polymerase from *E. coli* and by transferring the reaction tube to different water baths to achieve the various temperature shifts. However, this would be a very laborious procedure because you would have to shift tubes between water baths once every minute for 2-4 hours. It would also be very costly because every time you denatured the DNA at 94C, you would also irreversibly inactivate the polymerase, and would have to add fresh enzyme prior to the next extension step. Despite these inherent problems, PCR has gained widespread usage because of two developments that alleviate these problems. First, automated temperature cyclers (thermocyclers) alleviate the need of excessive manipulations by the investigator. Second, the use of a heat-stable DNA polymerase alleviates the need to add fresh enzyme prior to each extension step. This heat-stable polymerase was isolated from the thermophilic bacterium *Thermus aquaticus* and is called *Taq* DNA Polymerase. A single aliquot of *Taq* DNA Polymerase added at the beginning of the protocol will remain active through at least 30 cycles of PCR.

APPENDIX 2: MOLECULAR BIOLOGY OF THE ABO GENE

At the molecular level, the human ABO blood antigens are oligosaccharides that are attached to fatty acids protruding from the surface of red blood cells. These oligosaccharides are covalently attached to the fatty acids by enzymes (glycosyltransferases) encoded by the ABO gene. Three alleles exist for the ABO gene which encode very similar enzymes with nearly identical amino acid sequences. However, a few amino acid differences between the different allelic products result in significantly different activities. Specifically, the I^A allele encodes a glycosyltransferase that attaches N-acetylgalactosamine to an existing sugar on the fatty acid. The I^B allele encodes a glycosyltransferase that attaches galactose. The I^O allele produces a non-functional glycosyltransferase that cannot modify the existing sugar group. These different sugar group modifications are identified with antibodies; individuals who do not possess a specific type of modification produce antibodies against that modification. Thus, individuals who are I^AI^A produce the A type modification but not the B type modification and therefore have antibodies against the B antigen. I^AI^B individuals produce both types of modifications and do not have antibodies against either antigen. I^OI^O produces neither modification and has antibodies against both. Make sure you understand the genetics of the ABO blood type system by preparing a table of all the possible genotypes, the modifications that occur on their red blood cells, and the antibodies that each produce.

In 1990, researchers at the University of Washington cloned and sequenced representative I^A, I^B, and I^O alleles of the ABO gene to determine the differences between these alleles at the molecular level. This work has been extended by many research groups over the past twenty years resulting in a comprehensive understanding of the molecular biology of the ABO gene. The gene is located near the tip of the long arm of chromosome 9 (9q34) and has seven exons and six intervening sequences.



After transcription and RNA processing, the mature mRNA has a 1062 nt open reading frame that encodes a protein of 354 amino acids. There are only a handful of differences in the nucleotide sequences of the I^A, I^B, and I^O alleles. Most I^A and I^B alleles differ at 7 sites, each site having a single base-pair substitution, and the encoded proteins differ at four amino acid residues. The other nucleotide differences are neutral; due to the degeneracy of the genetic code they do not change the encoded amino acid. The four amino acid differences are responsible for the difference in sugar specificity of their glycosyltransferase activities. There are several variants of the I^O allele which have in common the deletion of a base pair found at position 261 of the I^A and I^B allele sequences. This single base pair deletion changes the reading frame in the mRNA and an entirely different amino acid sequence is generated after the point of the deletion. This

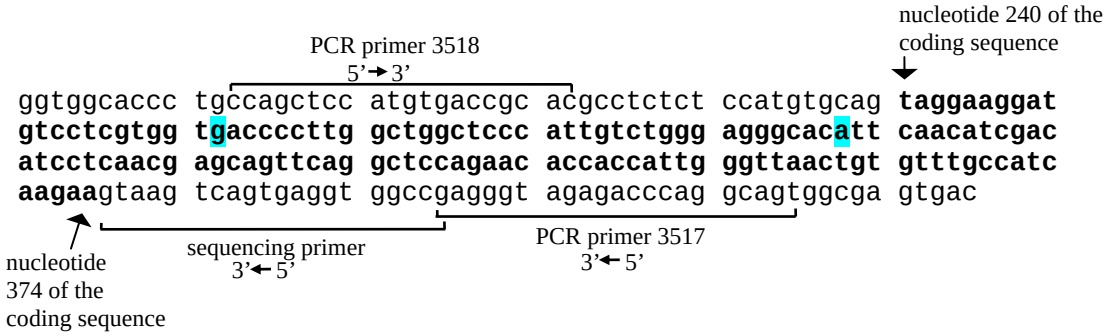
aberrant polypeptide lacks any glycosyltransferase activity. In addition to this critical deletion, the various I^O alleles have additional nucleotide differences from each other and from the sequences of the I^A and I^B alleles.

The nucleotide differences (at the mature mRNA level) between the I^A and I^B alleles, and two of the I^O variants, I^{O1} and I^{O2}, are summarized in the following table. Note that all of the differences between these alleles are confined to exons 6 and 7 which together encode the bulk of the polypeptide product.

	mRNA nucleotide position											
	exon 6		exon 7									
Allele	261	297	526	646	657	681	703	771	796	803	829	930
I^A	G	A	C	T	C	G	G	C	C	G	G	G
I^B	G	G	G[1]	T	T	G	A[1]	C	A[1]	C[1]	G	A
I^{O1}	--	A	nr[2]	T	C	G	G	C	C	G	G	nr[2]
I^{O2}	--	G	nr[2]	A	C	A	G	T	C	G	A	nr[2]

[1]These four positions are responsible for the four amino acid differences between the I^A and I^B allele products. Other nucleotide differences between these alleles have no effect on the amino acid sequence of the encoded protein.
[2]Information not reported in the literature.

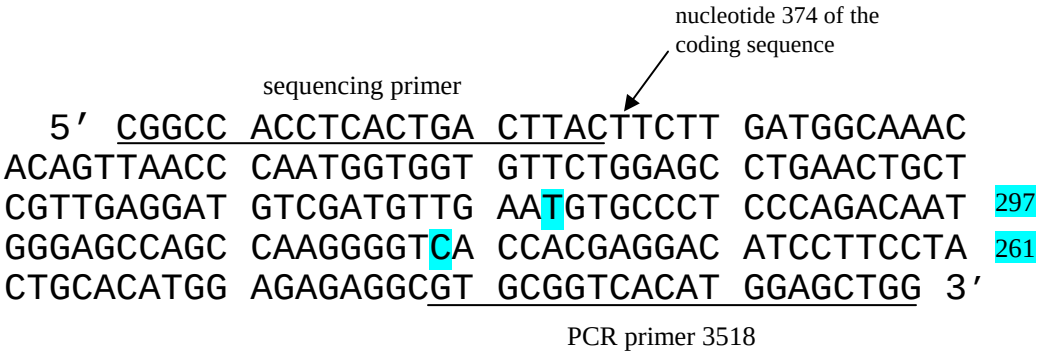
The strategy we will use to perform allelic typing takes advantage of the differences between alleles in exon 6. The I^A, I^B, and I^O alleles each have a characteristic pattern of nucleotides at positions 261 and 297, and the small size of the exon facilitates the process of PCR. Although much larger segments of DNA can be amplified, it is technically more difficult and more prone to ambiguities and artifacts. The complete sequence of exon 6 of the I^A allele and portions of the flanking introns (shown 5' to 3' at the RNA level) is as follows:



Additional notes: Exon sequence is boldfaced. Positions 261 and 297, which vary between alleles, are highlighted. The sequence shown is that of an I^A allele. The sequence of primer 3518 is identical to that of the coding strand shown. The sequences of primer 3517 and the sequencing primer are complementary to the coding strand.

Using primers 3518 and 3517, a 213 base-pair fragment is amplified which contains all of exon 6 and portions of both flanking introns. This fragment can then be sequenced as described in Appendix 3 and using the illustrated sequencing primer.

Interpreting your sequence. Because the sequencing primer is complementary to the coding strand shown in the diagram above, the sequence you obtain will also be the complement of the sequence shown. To facilitate your analysis, the actual sequence you will obtain (based on the sequence of the I^A allele) is shown below. Because the sequencing primer is located near the 3' end of the amplified segment, you are sequencing backwards through the coding region, and position 297 of the coding strand occurs earlier in the sequence than position 261. Also keep in mind that when determining your allelic composition by comparing your sequence to the sequence of the alleles listed in the table above, you must first take the complement of the sequence you read at positions 297 and 261. Thus, in the sequence shown below, the complement of the T and C at positions 297 and 261 are A and G, respectively, and based on the information in the allele table above, this corresponds to the I^A allele.



Finally, be aware of the fact that you are sequencing both of your alleles in a single reaction. If you are homozygous you will read a single nucleotide at each of the two diagnostic positions, and will have no trouble determining your genotype. However, if you are heterozygous, one or both positions will be "mixed." For example, if you are heterozygous I^AI^B, you will read a C at position 261, and both a T and a C at position 297. Before coming to class, try to complete the following table to make sure you have mastered the interpretation of your sequencing results.

nucleotides read at position 261	**nucleotides read at position 297**	**genotype / phenotype**
C/C	T/T	I^AI^A / blood type A
-/-	T/T	
C/-	T/T	
C/C	C/C	
-/-	C/C	
C/-	C/C	
C/C	T/C	I^AI^B / blood type AB
-/-	T/C	
C/-	T/C	

Keep in mind that the validity of characterizing ABO alleles by the sequence of exon 6 has not been firmly established. Although the vast majority of published alleles agree with these sequence assignments, some (< 5%) do not. Because of this you should not consider your results definitive. Nevertheless its accuracy of > 95% is on par with many other genetic tests actually being used in the medical profession. These tests are useful for screening patients, but the results must be confirmed by alternative means.

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Bennett, E. P., R. Steffensen, H. Clausen, D. O. Weghuis, and A. G. van Kessel, 1995. Genomic cloning of the human histo-blood group ABO locus. Biochem. Biophys. Res. Commun. **206**: 318-325.

Tsai, L.-C., L.-G. Kao, J.-G. Chang, H.-H. Lee, A. Linacre, and J. C.-I. Lee, 2000. Rapid identification of the ABO genotypes by their single-strand conformation polymorphism. Electrophoresis 2000 **21**: 537-540.

Yip, S.P., 2002. Sequence variation at the human ABO locus. Ann. Hum. Genet. **66**: 1-27.

Also see accession #s X84751 and AC000397 in the GenBank DNA sequence data base which can be accessed at Entrez Nucleotide on the internet at:
http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?db=Nucleotide
(select nucleotide in the search box and type the accession number in the larger dialog box near the top of the web page)

APPENDIX 3: DNA SEQUENCING

The DNA sequencing method currently in use (and used to sequence the entire human genome) was developed by Frederick Sanger in 1977. Sequencing by the "Sanger" method actually involves *replicating* the DNA you are attempting to sequence. The trick is to get replication to stop at specific nucleotides, and to then identify the positions of those nucleotides by determining the lengths of the truncated replication products. In the accompanying diagrams, the procedure is divided into four steps which are described in detail below.

1. Prepare replication template. One of the requirements for sequencing a fragment of DNA by the Sanger method is that the sequence of a portion of the DNA must already be known. The DNA to be sequenced is denatured, and a short oligonucleotide complementary to the known sequence is annealed to one of the strands of the denatured DNA. This forms a template complex suitable for replication by DNA polymerase.

2. Add components for in vitro replication. A mixture of these replication templates is divided into four tubes. The components for DNA replication, including dNTP precursors and the required salts and buffer are then added to each tube. One or more of the dNTPs are radioactively labeled to help visualize the replication products. Finally, each tube gets one of four special nucleotides called dideoxynucleotides (ddNTPs). These are identical to dNTPs with the exception that they have a 3'-H instead of a 3'-OH on their deoxyribose moiety. These nucleotides are recognized and used by DNA polymerase, and can be incorporated at the 3' end of a growing chain. However, once incorporated, DNA polymerase cannot further extend the chain because it needs a 3'-OH to attach to the next nucleotide. Without the 3'-OH, the chain stops growing, and dideoxynucleotides are also referred to as chain terminators. It is important to note that the ddNTPs are added to the reactions at much lower concentrations than the normal dNTPs, and because of that, DNA polymerase will incorporate the normal dNTP far more often than the ddNTP at any given position. But there are literally millions of active replication complexes in each reaction tube, and termination by incorporation of a ddNTP at each of the possible termination sites will occur in some fraction of these complexes.

3. Replication reactions. Replication is initiated by the addition of DNA polymerase. Each reaction tube has a different ddNTP and will thus truncate replication adjacent to different sites within the template. In the tube with ddCTP, incorporation of the chain terminator can occur whenever a complementary G is reached in the template. The incorporation of ddCTP occurs with low probability because of the low ratio of ddCTP to dCTP in the reaction mix, but there will be some extensions that stop at each and every G in the template. In this single reaction tube, extension fragments of a variety of different sizes will be produced. Because these fragments all start at the same position, defined by the location of the sequencing primer, their sizes are strictly dependent on the location of the G in the template where synthesis was truncated. Similar events occur in the other reaction tubes, but truncation is occurring at different template nucleotides (As, Ts, or

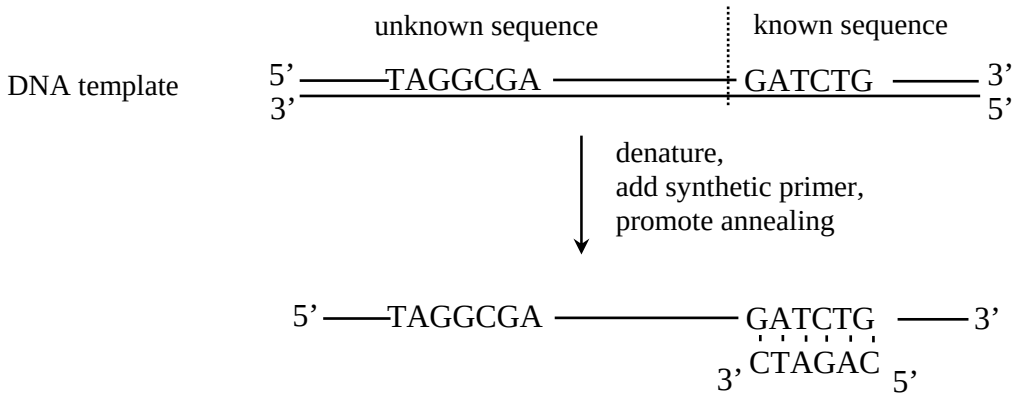
Cs). If the sizes of the truncated fragments could be determined in each tube, the position of every A, T, G, and C in the template would be identified.

4. Electrophoresis and visualization of replication products. The sizes of the truncated replication products in each reaction are determined by gel electrophoresis. A synthetic polymer, acrylamide, is used as the gel matrix for DNA sequencing because it has better resolving power than agarose. Following electrophoresis, the gel is exposed to a piece of X-ray film that is sensitive to the radiation emitted by the radioactive nucleotides. DNA fragments that have migrated to different distances appear as black bands on this "autoradiogram". While it is possible to use radioactively labeled DNA size markers to determine the sizes of the truncated fragments, it is easier to simply run the four reactions in adjacent lanes and read the sequence by reading up the gel. At any given position, the next higher band in the gel represents DNA that is one nucleotide longer, and the identity of that next nucleotide is determined by which lane it is found in.

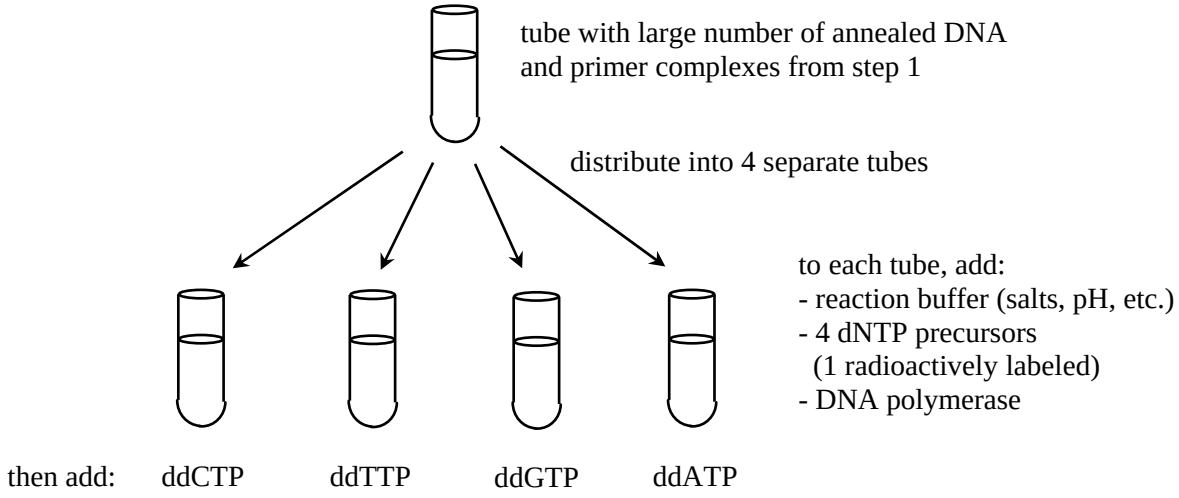
Automated sequencing. Automated DNA sequencing is very similar to the conventional chain termination method, but differs in one important respect. As described above, the signal source for conventional sequencing is a radioactive nucleotide. The signal source for automated sequencing is one of four fluorescent dyes attached to each of the four ddNTP chain terminators. Because each dye has a unique emission spectrum, a reaction containing all four chain terminators can be set up in a single reaction tube. This single reaction is fractionated on a single lane of an acrylamide gel which is monitored during electrophoresis by an automated fluorescence detector. This detector monitors each DNA fragment as it migrates through the gel and determines the wavelength of fluorescence it is emitting and thus the identity of its terminal dideoxynucleotide. This data is fed directly into a computer which assembles it into a sequence.

Sequencing by the Sanger Dideoxynucleotide Chain Termination Method

1. Prepare replication template

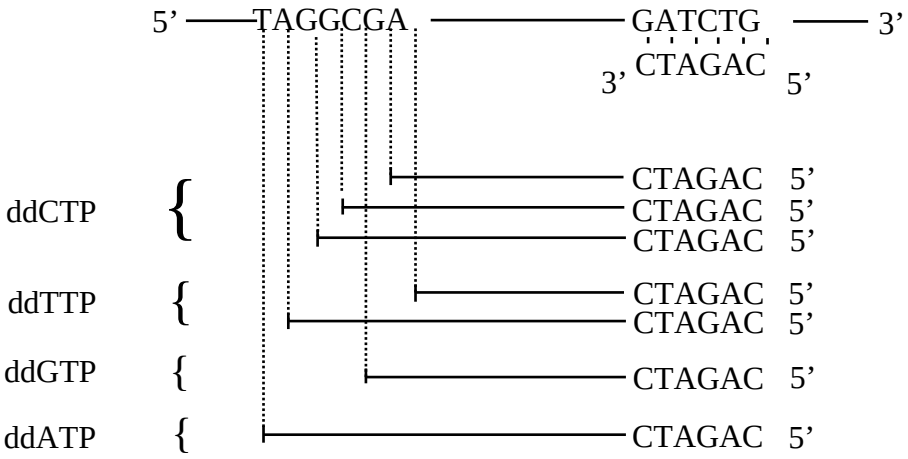


2. Add components for in vitro replication



Sequencing by the Sanger Dideoxynucleotide Chain Termination Method

3. Replication reactions



4. Electrophoresis and visualization of replication products