# Determining Human Blood Type by Non-Invasive Methods

***Michael P. Martin[1] and Stephen M. Detzel[2]***

[1]Biology Department
John Carroll University
20700 N. Park Blvd.
University Heights, OH 44118
216-297-4199
*mmartin@jcu.edu*

[2]Ohio University College of Osteopathic Medicine
Grosvenor Hall
Athens, OH 45701
*sd256706@ohio.edu*

**Abstract:** When teaching co-dominance in my genetics course, I discovered only 1 in 3 students knew their ABO blood type. This lab exercise allows students to determine their ABO blood type without drawing blood. Students isolate DNA from cells present in saliva and use this DNA as a template for PCR. Subsequently, the PCR products are digested to test for the identity of single nucleotide polymorphisms (SNPs) that indicate the presence of a particular allele. This method can differentiate among the most common alleles: $A^1$, $A^{1v}/A^2$, $B$, $O^1$, and $O^2$.

## Introduction

This laboratory exercise is intended for mid-level to upper-level courses that emphasize molecular biology techniques, such as Genetics, Biotechnology Lab, and Molecular Biology Lab. Students will learn various commonly-used molecular techniques during this multiple week lab exercise, including genomic DNA isolation, polymerase chain reaction, gel electrophoresis, and restriction digestion of DNA. Students will perform this exercise over the course of 5 lab periods; however, some steps may be done during the same class period to shorten the overall length. A single afternoon would be sufficient to prepare the few solutions that are needed, and reagents can be measured and stored during the autoclaving of solutions.

This exercise is also published in Biochemistry and Molecular Biology Education, but alternative protocols are presented here in the Appendix C. Assistance in analysis of data is also presented in Appendix D to aid instructors.

# Student Outline

Polymerase chain reaction (PCR) is an indispensable tool in molecular biology, genetics, forensics, and other fields. This technique is used to manipulate DNA for cloning and mutagenesis, and it can be used to detect genetic disorders, DNA fingerprinting, and identification of criminals from small samples of bodily fluids and hair (Sambrook, *et al.* 1989). PCR can amplify minute amounts of target DNA to produce millions of DNA copies through repeated cycles of DNA denaturation, oligonucleotide annealing, and DNA synthesis. Template DNA can come from any number of sources, including viral, bacterial, plasmid, and genomic DNA. In this laboratory exercise, students isolate their own genomic DNA from buccal epithelial (cheek) cells and white blood cells found in saliva. PCR of the gene that determines the ABO blood group phenotype and the subsequent digestion of these PCR products allow for determination of ABO blood type without drawing blood.

Haplotypes (haploid genotypes) are the set of genetic markers that exist on a chromosomal segment. Genetic markers can be genes, but they are more frequently variations in a single base pair that can be easily detected. These variations are called single nucleotide polymorphisms (SNPs), and these SNPs are useful in determining the genotype of an individual or tracking down the location of a gene that causes a particular disorder. It is estimated that 1% of the human genome consists of SNPs, but most of these SNPs do not cause a disorder. However, two SNPs may be in close proximity and certain variations may appear together at a high frequency (linkage disequilibrium). For example, SNP1 is present as an A or C, and SNP2 is present as a G or T. SNP1 doesn't cause any disorder, and there is no apparent evolutionary pressure to exist as either A or C. At some point in time, SNP2 is mutated from G-to-T in the chromosome that harbors an A at SNP1. When a T is present at SNP2, a non-functional protein is produced; this absence of protein function is only detrimental to the individual when both chromosomes cannot produce the protein, a classic example of an autosomal recessive disorder. If SNP2 cannot be easily assayed and SNP1 can be tested readily, then a researcher has another genetic marker to test in order to find the disorder-causing variation. If SNP1 is determined to be an A, then SNP2 is inferred to be a T. Therefore, the DNA from an unaffected individual could be tested to determine if they are a carrier of an autosomal recessive disorder. Students will perform this type of analysis, except they will determine the genotype for the ABO blood type gene.

The ABO blood group is the most medically important cell surface marker due to its antigenic properties that can lead to red blood cell agglutination, organ failure, and death (Yamamoto 2004). The surface of cells is decorated with proteins and lipids, and these macromolecules can be glycosylated which means they have sugars attached to them. Although the ABO blood groups were discovered in 1900 by Karl Landsteiner, another 70 years elapsed before scientists determined how the A and B antigen were formed (Ginsburg, 1972 and Tilley *et al*., 1978). The A and B antigens are terminal sugars that are added to proteins, lipids, and soluble oligosaccharides (Figure 1A); the enzyme that adds the terminal sugar is called a glycosyltransferase and is encoded by the *ABO* gene. The A transferase protein will attach the monosaccharide N-acetylgalactosamine, while the B transferase protein will add galactose to the H substrate. If an individual can join only one of these sugars to the H substrate, the A and B blood types will be observed, respectively. If an individual possesses both enzymatic activities, then AB is the blood

type. However, some individuals inherit two *ABO* alleles that produce non-functional glycosyltransferases; these individuals have an O phenotype.

In 1995, the *ABO* gene organization was determined (Yamamoto *et al.*, 1995 and Bennett *et al.*, 1995), and the gene is over 18 kilobases in size and contains 7 exons (Figure 1B). However, exons 6 and 7 harbor more than 75% of the protein-encoding sequence, and these exons possess 4 SNPs that are sufficient to differentiate among the most common *ABO* alleles: $A^1$, $A^{1V}/A^2$, $B$, $O^1$, and $O^2$ (the $A^{1V}$ and $A^2$ alleles cannot be differentiated by the protocols outlined in this exercise). A single base pair deletion in exon 6 of the $O^1$ allele converts the *Bst*E *II* restriction site to a *Kpn* I restriction site. This frameshift mutation results in a non-functional protein; therefore, this SNP is the only one that you will test that directly affects the glycosyltransferase function. All alleles will be digested by either *Kpn* I (for $O^1$ alleles) or *Bst*E *II* (non-$O^1$ alleles). Three SNPs in exon 7 allow for determination of $A^{1V}/A^2$, $B$, and $O^2$ alleles. Since the $O^1$ and $A^1$ alleles produce the same digestion pattern for exon 7, the exon 6 digestion is essential for $O^1/A^1$ identification. This exercise is based on findings from Olsson and Chester (1995).

In this laboratory exercise, students will isolate DNA from cells present in saliva. This DNA will be used as a template for PCR, and the PCR products will be digested to determine the genotype of each individual.

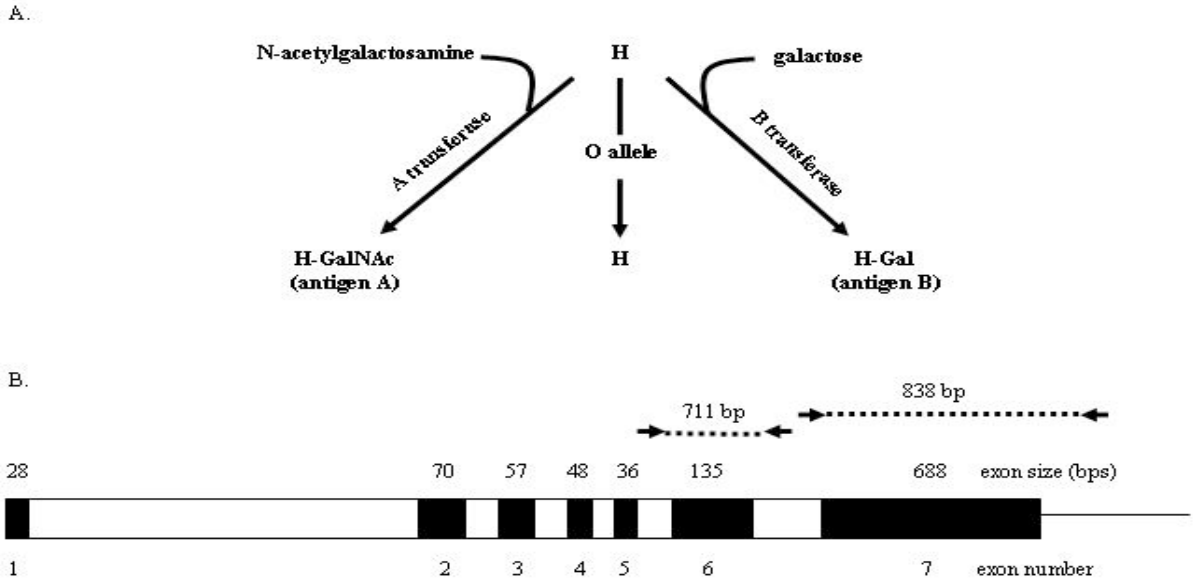


**Figure 1.** ABO blood type production. A. Biochemistry of antigen production. H = glycolipid or glycoprotein substrate, GalNAc = *N*-acetylgalactosamine, Gal = galactose. B. ABO gene organization. Primers are indicated with arrows. Product sizes are shown above the dashed line which represents the PCR product. Introns are represented by the open boxes. Exons are shown as filled boxes. The 3' untranslated region is illustrated as a solid line to the right of exon 7.

**Exercise schedule**

For this exercise, students will work in groups of two.

Day 1
- isolation of DNA from cheek cells

Day 2
- prepare agarose/EtBr gel
- run genomic DNA samples on gel to determine size and concentration

Day 3
- set up exon 6 and 7 PCR reactions
- put tubes into thermal cyclers and run reactions overnight

Day 4
- place PCR reaction tubes in the freezer

Day 5
- prepare agarose/EtBr gel
- run 10 µL of each reaction on gel and photograph
- set up exon 6 (*Kpn* I and *Bst*E II) and exon 7 (*Hpa* II) digests

Day 6
- run exon 6 and exon 7 digests on 4% agarose/EtBr gel and photograph

**Day 1**

*Isolation of DNA from cheek cells*

Each person will isolate DNA from their own cheek cells. It is extremely important to WEAR GLOVES during the entire process of DNA isolation as working with bodily fluids has the potential to transmit blood borne pathogens to others. You should be extremely cautious whenever you have an open cup or tube that contains cells or fluids. You should also wash your hands up to your elbows (if you are wearing short sleeves) whenever you remove your gloves. All tubes and cups that come into contact with bodily fluids should be disposed in biohazard bags for autoclaving, and all fluids should be put into a 10% bleach solution to decontaminate them.

Students will be given a tube containing 10 mL of 0.9% NaCl that has been sterilized. This salt solution will prevent cells from lysing prematurely. They will also get a small paper cup to transfer saliva back into the tube. They will also receive a 10% Chelex solution [10% Chelex, 50 mM Tris (pH 10.5-11.0)].

The instructor should beginning boiling water prior to student arrival to lab.

This protocol will result in an extract that contains genomic DNA. However, it is sufficiently pure to perform PCR.

1. Pour 10 mL of NaCl solution (0.9%) into the paper cup, and put the solution into your mouth. Vigorously swish around your mouth for 10 seconds. Spit the solution back into your cup and transfer it to the conical vial. Cap the vial.

2. Label your conical vial with your initials.
3. Spin the vial in at top speed in a clinical centrifuge for 10 minutes at room temperature.
4. Pour the supernatant back into the paper. Add 10% bleach and discard the solution in the sink.
5. Resuspend the Chelex by inverting the tube. It is critical that the beads are suspended in liquid prior to pipetting.
6. Transfer 500 µL of Chelex solution to the cell pellet and pipet up and down gently to resuspend the cell pellet fully.
7. Transfer 500 µL of Chelex/cell solution to a microcentrifuge tube that is labeled with your initials.
8. **Put on safety goggles.** Put your tube in a styrofoam float for 10 minutes in boiling water to lyse the cells. Open tubes after 30 seconds to relief pressure and place them back in the boiling water for remainder of the 10 minute incubation.
9. Put tubes on ice for 2 minutes.
10. Spin at top speed in a microcentrifuge for 30 seconds. This step will pellet the cellular debris, leaving DNA in solution.
11. Being extremely careful not to disturb the pellet, transfer 200 µL of supernatant to a new microcentrifuge tube. This tube should be labeled with your initials, "genomic DNA", and the date.
12. Place the tube at 4°C. Freeze/thawing genomic DNA can shear the DNA into smaller fragments.

**Day 2**

*Estimation of genomic DNA concentration*

1. Add 2 µL of your genomic DNA, 8 µL 1X TE, and 2 µL 6X loading dye to a microcentrifuge tube.
2. Thaw your tube of "uncut λ DNA". This tube contains 50 ng/µL uncut λ DNA.
3. Make up the following 3 tubes:

| Tube | uncut λ DNA | $dH_2O$ | 6X loading dye |
|---|---|---|---|
| 1 | 1 µL | 9 µL | 2 µL |
| 2 | 3 µL | 7 µL | 2 µL |
| 3 | 6 µL | 4 µL | 2 µL |

4. Load a 1% agarose/TBE gel in the following order:

| |
|---|
| a. λ DNA tube 1 |
| b. λ DNA tube 2 |
| c. λ DNA tube 3 |
| d. student DNA #1 |
| e. student DNA #2 |

5. Run the gel at a constant 150 volts for 45 minutes. Photograph as usual.
6. Estimate the concentration of genomic DNA by comparison to the known amounts in the lambda dilution lanes. Signal intensity is proportionate to the mass of DNA that is present.

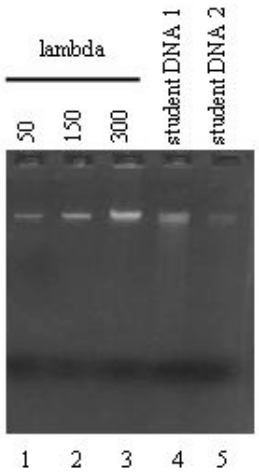


**Figure 2.** Genomic DNA visualization. Lanes 1-3 contain the indication amount (in ng) of uncut lambda DNA. Student DNA 1 and 2 were estimated to be appropriately 100 ng/µL and 50 ng/µL, respectively.

**Day 3**

*PCR of exons 6 and 7 from ABO blood type gene*

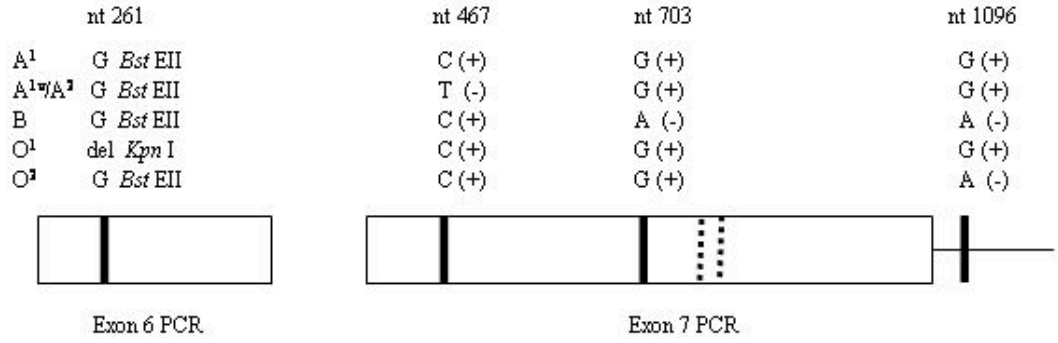


**Figure 3.** Single nucleotide polymorphisms in exons 6 and 7. The exon 6 digests will distinguish the $O^1$ allele from the $A^1$, $A^{1v}/A^2$, $B$, and $O^2$ alleles. Exon 7 SNPs will allow for the determination of the presence of $A^1$, $A^{1v}/A^2$, $B$, and $O^2$ alleles. del = deletion of nt 261. A "+" indicates digestion by *Hpa* II, whereas a "-" indicates no digestion by *Hpa* II. Dashed lines indicate the SNPs that result in a change in sugar attachment to the H substrate.

Groups will set up 2 PCR reactions for each sample: one for each of the student samples and one unknown. Along with a control reaction for each different PCR condition, this will result in 8 reactions per group.

1. Each group will make up the following cocktails. Set up a table in your lab notebook with the following information that will allow you set up the proper reactions:

| | exon 6 cocktail | exon 7 cocktail |
|---|---|---|
| $dH_2O$ | 153.75 µL | 153.75 µL |
| 10X buffer | 25 µL | 25 µL |
| 25 mM $MgCl_2$ | 15 µL | 15 µL |
| exon 6-1- *Eco* RI (10 pmol/µL) | 12.5 µL | ----- |
| exon 6-2 –*Hin*d III (10 pmol/µL) | 12.5 µL | ----- |
| Exon 7-3 (10 pmol/µL) | ----- | 12.5 µL |
| Exon 7-4 (10 pmol/µL) | ----- | 12.5 µL |
| 10 mM dNTPs | 5 µL | 5 µL |
| Taq polymerase | 1.25 µL | 1.25 µL |

Exon 6-1-*Eco* RI = 5'-GGAATTCGGGCTGGGAATGATTTG-3'
Exon 6-2 –*Hin*d III = 5'-GGAAGCTTGGTGTCCCCCTCCTGCTATC-3'
Exon 7-3 = 5'-CCCCGTCCGCCTGCCTTGCAG-3'
Exon 7-4 = 5'-GGGCCTAGGCTTCAGTTACTC-3'
- restriction sites are underlined

2. Add the reagents for each cocktail to a different microcentrifuge tube that is *ON ICE*. BE SURE TO CHANGE TIPS AFTER EACH REAGENT IS ADDED TO AVOID CONTAMINATION!!! You should start at the top and work towards to the bottom (water will be first, Taq polymerase will be last). To speed things up, add water to all tubes, then buffer to all tubes, etc. This will prevent you from changing the micropipettor setting after every reagent.
3. Add 5 µL template DNA or water (negative controls for contamination with DNA) to each labeled thin-walled PCR tube in the following fashion (put this table in your notebook):

| | tubes: |
|---|---|
| water | 1, 5 |
| student DNA 1 | 2, 6 |
| student DNA 2 | 3, 7 |
| unknown DNA | 4, 8 |

4. Mix the cocktail by briefly (~2 seconds) vortexing. Add 45 µL of cocktail to the following tubes:
    exon 6 cocktail: tubes 1-4
    exon 7 cocktail: tubes 5-8
5. (*Optional—necessary only if thermocycler does not have a heated lid*) Add 40 µL of light mineral oil to each tube. This oil will float on top of the reactions that you have made and prevent evaporation and condensation of water during the many cycles of heating.
6. Put your reactions into the appropriate thermal cycler.

Reaction conditions:

exon 6 PCR reactions
1. 94°C for 45 seconds
2. 53°C for 45 seconds

3. 72°C for 45 seconds
- 35 cycles of steps 1-3
72°C for 5 minutes (this step ensures complete synthesis of all strands)
4°C indefinitely

exon 7 PCR reactions
1. 94°C for 45 seconds
2. 60°C for 45 seconds
3. 72°C for 45 seconds
- 35 cycles of steps 1-3
72°C for 5 minutes (this step ensures complete synthesis of all strands)
4°C indefinitely

**Day 4**

Remove PCR reactions from the thermal cycler and place in your freezer box at -20°C.

**Day 5**

*A. Prepare and run agarose/ethidium bromide gel on PCR from last week*
1. Prepare 1% agarose/EtBr gel.
2. Remove 10 µL of each PCR reaction and place in labeled microcentrifuge tubes.
If mineral oil was added, do not push in the plunger of the micropipettor until the tip is below the light mineral oil layer. Once you gently expel the air from the tip, remove a portion of the PCR reaction. Be sure that you do not just draw the air back up into the tip as this will give you the incorrect amount to load on your gel.
3. Add 2 µL of 6X loading dye (-XC) to each microcentrifuge tube.
4. Prepare lambda *Bst*E II and pUC18 *Hae* III markers as usual (1 µL of marker, 9 µL of 1X TE, and 2 µL of 6X loading dye).
5. Load the gel and record the order of loading in your notebook.

| Lane | Sample |
|---|---|
| 1 | reaction 1 |
| 2 | reaction 2 |
| 3 | reaction 3 |
| 4 | reaction 4 |
| 5 | lambda *Bst*E II |
| 6 | pUC18 *Hae* III |
| 7 | reaction 5 |
| 8 | reaction 6 |
| 9 | reaction 7 |
| 10 | reaction 8 |

6. Run the gel at constant voltage of 150 volts for ~40 minutes and photograph as usual.
7. Estimate the concentration of DNA produced by each of the PCR reactions of exons 6 and 7.

This estimation is done by comparing the intensity of the PCR product to the pUC18 *Hae* III molecular weight marker. The 587 base pair band represents ~100 ng of DNA, while the 2323 base pair fragment in the lambda *Bst*E II digest is ~25 ng. If the PCR product and the 587 base pair fragment are approximately equal in intensity, then you have 100 ng of PCR product. In order to determine the concentration of DNA in your PCR reactions, you need to divide the mass (in ng) by the volume of DNA that was loaded (10 µL).

*B. Set up exon 6 and 7 digests*

Typically, students should digest as much DNA as possible in order to see the smallest fragments that are generated by exon digestion. You will make 3 premixes to digest exons 6 and 7 PCR products.

1. Digests of exons 6 and 7 will be assembled (15 µL total). Copy this table into your lab notebook prior to class.

| | 10X buffer | PCR product | 10X BSA | enzyme |
|---|---|---|---|---|
| Exon 6 (K) | 1.5 µL Promega J | 11.5 µL exon 6 | 1.5 µL | 0.5 µL *Kpn* I |
| Exon 6 (B) | 1.5 µL Promega D | 11.5 µL exon 6 | 1.5 µL | 0.5 µL *Bst*E II |
| Exon 7 (H) | 1.5 µL Promega A | 11.5 µL exon 7 | 1.5 µL | 0.5 µL *Hpa* II |

2. Make a premix for each reaction type (K, B, and H).

| | 10X buffer | 10X BSA | enzyme |
|---|---|---|---|
| Exon 6 (K) | 6 µL Promega J | 6 µL | 2 µL *Kpn* I |
| Exon 6 (B) | 6 µL Promega D | 6 µL | 2 µL *Bst*E II |
| Exon 7 (H) | 6 µL Promega A | 6 µL | 2 µL *Hpa* II |

3. Reagents should be added to the premix in the following order:
a) 10 X buffer
b) 10 X BSA
c) enzyme

4. Briefly mix each solution by pipetting up and down gently.
5. Add 3.5 µL of the appropriate premix to labeled microcentrifuge tubes.
6. Add 11.5 µL DNA to the microcentrifuge tubes and briefly centrifuge.
7. Place reactions exon 6 (K) and exon 7 (H) at 37°C for 2 hours
8. Place reaction exon 6 (B) at 60°C for 2 hours. This reaction should be briefly centrifuged every 30-45 minutes as evaporation and condensation under the cap will occur.
9. Once completed, place all reactions in your -20°C freezer box.

**Day 6**

*A. Run exon 7 digests on 4% agarose/EtBr gel and photograph*

1. A 4% agarose/TBE gel has been poured prior to class. Two groups will share each gel; one group will get the 4 lanes on the left and the other group will take the 4 lanes on the right.
2. Add 3 µL of 6X loading dye (-XC) to each of your digests from last week. Briefly centrifuge.
3. Prepare a sample of pUC18 *Hae* III as usual (1 µL of DNA, 9 µL of 1X TE, and 2 µL of loading dye –XC).
4. Once both groups are ready to load the gel, begin loading. Load all18 µL.

| Lane | Sample |
|---|---|
| 1 | pUC18 *Hae* III |
| 2 | student DNA 1 (group 1) |
| 3 | student DNA 2 (group 1) |
| 4 | unknown DNA (group 1) |
| 5 | pUC18 *Hae* III |
| 6 | student DNA 1 (group 2) |
| 7 | student DNA 2 (group 2) |
| 8 | unknown DNA (group 2) |

5. Run the gel at 95 volts for 100 minutes and photograph as usual. The bromophenol blue should travel 6-7 cm into the gel in order to resolve the 223 and 204 base pair bands.

```
375  atacgtggct ttcctgaagc tgttcctgga gacggcggag aagcacttca tggtgggcca
435  ccgtgtccac tactatgtct tcaccgacca gc***C***gg*ccgcg* gtgccccgcg tgacgctggg
495  ga*ccgg*tcgg cagctgtcag tgctggaggt gcgcgcctac aagcgctggc aggacgtgtc
555  catgcgccgc atggagatga tcagtgactt ctgcgagcgg cgcttcctca gcgaggtgga
615  ttacctggtg tgcgtggacg tggacatgga gttccgcgac cacgtgggcg tggagatcct
675  gactccgctg ttcggcaccc tgcaccc*cc****G****g* cttctacgga agcag*ccggg*g aggccttcac
735  ctacgagcg*c cggc*cccagt cccaggccta catccccaag gacgagggcg atttctacta
795  cCtgggggGg ttcttcgggg ggtcggtgca agaggtgcag cggctcacca gggcctgcca
855  ccaggccatg atggtcgacc aggccaacgg catcgaggcc gtgtggcacg acgagagcca
915  cctgaacaag tacctgctgc gccacaaacc caccaaggtg ctctcccccg agtacttgtg
975  ggaccagcag ctgctgggct ggcccgccgt cctgaggaag ctgaggttca ctgcggtgcc
1035 caagaaccac caggcggt*cc gg*aacc*C*gtg agcggctgcc aggggctctg ggagggctg*c*
1095 c***G****g*cagcccc gtccccctcc cgcccttggt tttagcagaa cgggtaaact ctgtttcctt
1155 tgtccgtcct gttgt
```

**Figure 3.** Exon 7 sequence for the $A^1$ allele. The primers that are used to amplify this sequence immediately flank this sequence. Numbering is according to the nucleotides present in exons, beginning with the A in the start codon. *Hpa* II restriction sites are italicized. SNPs in *Hpa* II sites are capitalized and are in bold lettering. The two SNPs (C796 and G803) that alter sugar-specificity in the *B* transferase are capitalized. These sites are not in *Hpa* II sites).

**Table 1.** Fragments produced by *Hpa* II digestion of exon 7 PCR products. A "+" indicates that the fragment will be present.

| Allele: | $A^1$ | $A^{1v}/A^2$ | $B$ | $O^1$ | $O^2$ |
|---|---|---|---|---|---|
| 309 bp | + | + | + | + | + |
| 223 bp | | | + | | |
| 204 bp | + | + | | + | + |
| 145 bp | | + | | | |
| 137 bp | | | + | | + |
| 119 bp | + | | + | + | + |
| 96 bp | + | + | | + | |

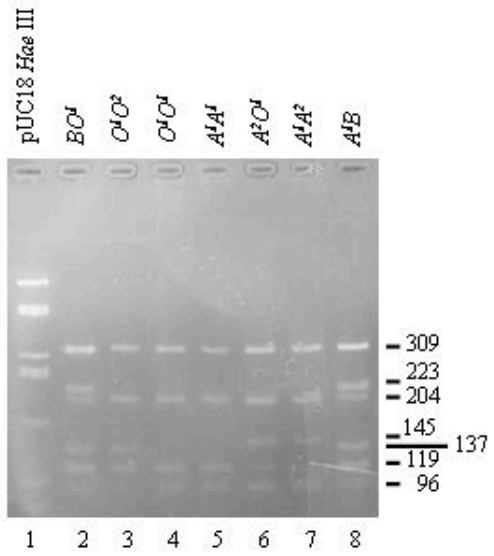


**Figure 4.** Results of exon 7 digestion by *Hpa* II. Exon 7 PCR products were digested by *Hpa* II for 2 hours followed by electrophoresis on a 4% high-resolution agarose/1X TBE gel. Sample genotypes are shown above each lane. The $A^2$ allele could also be $A^{1v}$.

*B. Run exon 6 digests on 1% agarose/EtBr gel and photograph*

1. Prepare a 1% agarose/EtBr gel.
2. Prepare an uncut sample of exon 6 PCR product. Remove 6 µL of DNA from the exon 6 PCR reactions and place it in a microcentrifuge tube. Add 9 µL of sterile water and add 3 µL of 6X loading dye (-XC) to each uncut sample.

3. Prepare a sample of pUC18 *Hae* III and lambda *Bst*E *II* as usual (1 µL of DNA, 9 µL of 1X TE, and 2 µL of loading dye –XC).

4. Load the gel in the following order and record this order in your notebook.:

| Lane | Sample |
|---|---|
| 1 | student DNA 1 exon 6 uncut |
| 2 | student DNA 1 exon 6 (K) |
| 3 | student DNA 1 exon 6 (B) |
| 4 | student DNA 2 exon 6 uncut |
| 5 | student DNA 2 exon 6 (K) |
| 6 | student DNA 2 exon 6 (B) |
| 7 | unknown DNA exon 6 uncut |
| 8 | unknown DNA exon 6 (K) |
| 9 | unknown DNA exon 6 (B) |
| 10 | lambda *Bst*E II |
| 11 | pUC18 *Hae* III |

5. Run the gel at a constant 150 volts for 40 minutes and photograph the gel.

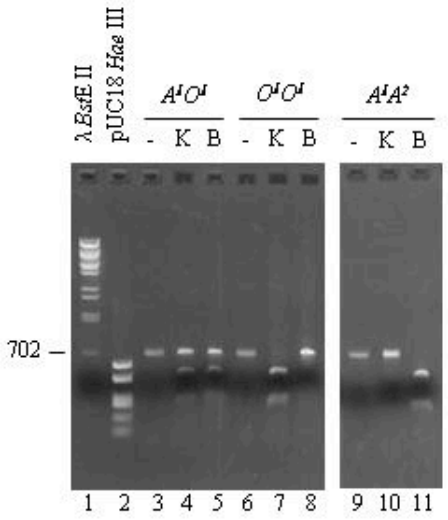


**Figure 5.** Results of exon 6 digest. Exon 6 PCR products were digested with *Kpn* I (K) or *Bst*E II (B) for 2 hours, and a "-" indicates uncut DNA (no enzyme). A 1% agarose/1X TBE gel was run for 40 minutes prior to photographing the gel. Genotypes are indicated above each sample. The $A^2$ allele could also be $A^{1v}$.

**Study Questions**

1. Rh D-negative individuals generate an immune response only when they are exposed to the Rh D antigen. However, incompatibility in the ABO blood group system occurs naturally in the absence of exposure to blood that has an A or B antigen. Why do individuals produce an immune response the first time they encounter the A or B antigen from human blood?

2. What chromosome possesses the ABO blood group gene? Is it on the long or short arm of this chromosome?
3. What major advantage does using a DNA polymerase from a thermophilic bacterium provide during a PCR reaction?
4. In rare cases an individual can possess the A and/or B transferase proteins (and their corresponding alleles), but they possess an O phenotype. What is this condition called and how does it occur?
5. What ABO phenotype illustrates the co-dominant nature of the ABO alleles?
6. What is the difference between the $A^1$ and $A^2$ alleles at the protein level? How does this difference affect the protein function?
7. In this exercise, the $A^{1v}$ and $A^2$ alleles are not differentiated. How would a research distinguish between the $A^{1v}$ and $A^2$ alleles?

## Materials

Laboratory chemicals were purchased from Research Organics (Cleveland, OH), Fisher (Pittsburgh, PA), and VWR (West Chester, PA). Taq DNA polymerase was acquired from USB (Cleveland, OH). Restriction enzymes, dNTPs, and λ *Bst*E II marker were purchased from Promega Corporation (Madison, WI). Primers used in PCR were ordered from Operon Biotechnologies (Huntsville, AL). Agarose, X-gal, IPTG, and pUC18 *Hae* III marker were acquired from Amresco (Solon, OH). Plasmid preparation kits were purchased from Qiagen (Valencia, CA). Chelex 100 resin was acquired from Bio-Rad (Hercules, CA). *E. coli* strain JM109 is free from New England Biolabs (Ipswich, MA) with a minimum purchase.

### Equipment and software

This exercise used the following equipment and software: microcentrifuge models 5415C and 5415D (Eppendorf), Bio-Rad DNA Engine PTC200 (Hercules, CA), BioMax QS710 and MP1015 gel electrophoresis systems, DC290 camera, and 1D version 3.6 gel analysis software (Kodak, Rochester, NY), model 300 power supplies (VWR), and EB-15 UV transilluminator (Ultra-Lum, Carlsbad, CA).

## Notes for the Instructor

### Expected background skills

Students will need to be highly proficient in micropipettor use; accurate measurement of small volumes will increase the likelihood of generating quality results. Alternatively, students may isolate DNA and add it to reactions that are prepared by the instructor. A background in restriction digests, agarose gel electrophoresis, and gel analysis would be useful, but it is not absolutely required.

## Safety issues

Collection of DNA from cells present in saliva is non-invasive. However, students should wear gloves whenever intact fluids are present. They should wash their hands and arms up to the elbow prior to leaving lab. Instructors should contact their committee that deals with human studies for any special regulations that their college possesses.

Ethidium bromide is a known carcinogen, and it should be handled with care. Your college may have special guidelines to dispose of ethidium bromide waste. Ethidium bromide decontamination protocols can be found in Sambrook *et al* (1989). We dispose of gels in a solid waste container, and contaminated buffer is stirred with a destaining bag from Amresco (Solon, Ohio). The destaining bag will bind ethidium bromide from solution; subsequently, decontaminated buffer can be washed down the sink.

Ethidium bromide-stained gels are visualized with UV light. UV light causes DNA lesions and is carcinogenic. UV safe goggles should be worn when UV light is being used.

## Common pitfalls

Instructors should use their PCR to optimize the cycling conditions as experience has shown that all machines do not generate a PCR product with these cycling conditions. If exon 7 amplification does not generate a large amount of product, it will be difficult to visualize *Hpa* II fragments that are smaller than 204 base pairs. In this case, students are instructed to use their exon 6 to determine whether an $O^1$ allele exists and to exclude the *B* allele if the 223 base pair fragment is not observed in the exon 7 digest. In addition, students are asked to determine the possibility of other alleles according to their allelic frequencies as shown in Yip (2000).

## Websites of interest

The genetic, biochemical, and immunological aspects of the ABO phenotypes are outlined in a website by Schroeder and Jensen: http://matcmadison.edu/is/hhps/mlt/mljensen/BloodBank/lectures/abo_blood_group_system.htm.

The National Center for Biotechnology Information (NCBI) gives information about the value and utility of SNPs. They also maintain a database of human SNPs that can be accessed through this website: http://www.ncbi.nlm.nih.gov/About/primer/snps.html

A good PCR animation can be found at: http://www.dnalc.org/ddnalc/resources/shockwave/pcranwhole.html

## Scheduling

*Isolation of genomic DNA*—2 hours total with 1 hour open during the initial incubation at 50°C.

*Gel electrophoresis of genomic DNA*—2 hours for gel preparation, electrophoresis, and photography with 20-30 minute open during electrophoresis. Alternatively, gels may be prepared by the instructor prior to class. Students can prepare samples and reduce the necessary time to 1 hour.

*PCR*—1 hour total followed by a 2.5-3 hours reaction that can be held overnight at 4°C in most PCR machines. The PCR reactions can be done on the same day as gel electrophoresis of the genomic DNA to save time.

*Gel electrophoresis of PCR products*—2 hours for gel preparation, electrophoresis, and photography with 20-30 minutes open during electrophoresis. Once again, instructors can prepare the agarose gels to reduce the time that students spend in lab.

*Restriction digests*—30 minutes to prepare the digests and 2 hours of incubation.

*Gel electrophoresis of restriction digests*—2 hours for gel preparation, electrophoresis, and photography with 20-30 minutes open during electrophoresis. The 4% agarose-TBE gel is prepared prior to the lab, and students load this gel immediately as it will take a longer time to run than the 1% agarose-TBE gel that has the exon 6 digests. Multiple groups can run their samples on the same gel to conserve materials.

## Acknowledgments

We would like to thank those individuals who provided samples to use in testing these protocols. We are grateful to James Lissemore for providing an inexpensive protocol to isolate genomic DNA from cells present in saliva. Finally, we thank John Carroll University's Graduate School for providing funding to purchase materials necessary for this exercise via a Faculty Instructional Grant to Dr. Martin.

## Literature Cited

Bennett, E. P., R. Steffensen, H. Clausen, D. O. Weghuis, A. Geurts van Kessel. 1995. Genomic cloning of the human histo-blood group ABO locus. Biochemical and Biophysical Research Communications, 206:318-325.

Ginsburg, V. 1972. Enzymatic basis for blood groups in man. Advances in Enzymology and Related Areas of Molecular Biology, 36:131-149.

Olsson, M. L. and M. A. Chester. 1995. A rapid and simple ABO genotype screening method using a novel $B/O_2$ versus $A/O_2$ discriminating nucleotide substitution at the ABO locus. Vox Sanguinis, 69:242-247.

Sambrook, J, E. F. Fritsch, T. Maniatis. 1989 Molecular Cloning: A Laboratory Manual. Second edition. Cold Spring Harbor Laboratory Press, New York.

Tilley, C. A., M. C. Crookston, J. H. Crookston, J. Shindman, H. Schachter. 1978. Human blood-group A- and H-specified glycosyltransferase levels in the sera of newborn infants and their mothers. Vox Sanguinis, 34:8-13.

Yamamoto, F., P. D. McNeil, S. Hakamori. 1995. Genomic organization of human histo-blood group *ABO* genes, Glycobiology, 5:51-58.

Yamamoto, F. 2004. Review: ABO blood group system-ABH oligosaccharide antigens, anti-A and anti-B, A and B glycosyltransferases, *ABO* genes. Immunohematology, 20:3-22.

Yip, S. P. 2000. Single-tube multiplex PCR-SSCP analysis distinguishes 7 common ABO alleles and readily identifies new alleles. Blood, 95:1487-1492.

## About the Authors

Mike Martin is an Assistant Professor in the Biology Department at John Carroll University, the Jesuit University in Cleveland. He earned his PhD in Cell and Molecular Biology from the University of Wisconsin-Madison following undergraduate work at the University of Cincinnati. His primary teaching load includes Principles of Biology I and II, Genetics, Introduction to Biotechnology Laboratory, and Molecular Cell Biology.

Steve Detzel is 2006 graduate of John Carroll University with a BS in Political Science. He is currently attending Ohio University's College of Osteopathic Medicine. He volunteered his summer to work on this project and made significant contributions to the optimization of this lab exercise.

# Appendix A: Recipes

*1X TE*, sterile
Mix the following and autoclave for 30 minutes:
10 mL 1 M Tris (pH 8)
2 mL 0.5 M EDTA, disodium salt (pH 8)
988 mL water

*6X loading dye (-XC)*, may be stored at 4°C
150 µL water
250 µL 1% bromophenol blue
600 µL 50% glycerol

*5X TBE*
54 g Tris base
27.5 g boric acid
20 mL 0.5 M EDTA, disodium salt (pH 8)
water to 1 liter
autoclave for 30 minutes

*1X TBE*, non-sterile, made immediately before using
120 mL 5X TBE
480 mL sterile water

*1% agarose/1X TBE gel*
In a flask (at least 250 mL), add 0.7 g agarose and 70 mL 1X TBE and cover with plastic wrap. Microwave at 50% power (test your microwave) for 2 minutes. This solution should be clear when agarose is in solution. Cool for 10 minutes at room temperature before adding 3.5 µL 10 mg/mL ethidium bromide. Swirl the flask and pour immediately into a gel casting rig that has a comb in it.

*4% agarose/1X TBE gel*
In a flask (at least 250 mL in size), add 2.0 g agarose (3:1 high-resolution blend from Ameresco) and 50 mL 1X TBE and cover with plastic wrap. Microwave at 50% power (test your microwave) for 2 minutes; rapid heating will cause bubble formation which will make visualization of the DNA band difficult. This solution should be clear when agarose is in solution. Cool for a couple of minutes at room temperature before adding 2.5 µL 10 mg/mL ethidium bromide. Swirl the flask and pour immediately into a gel casting rig that has a comb in it. This gel will solidify very quickly so you will need to work quickly once the agarose is in solution. If excessive bubbling occurs during microwaving and pouring the gel, you can use a razor blade to slice a thin layer from the top of the gel prior to taking a picture.

*0.9% NaCl* (Care should be taken to use glass wear that has not been used previously to eliminate the chance of introducing harmful chemicals that will be placed in the mouths of students)
4.5 g sodium chloride (table salt from the grocery store is preferable)
water to 500 mL
autoclave for 30 minutes

*10% Chelex, 50 mM Tris (pH 10.5-11.0)*

1 g Chelex
10 mL of 50 mM Tris base
swirl solution and check for pH of 10.5-11.0. Add NaOH or HCl to adjust the pH.

*50 mM Tris base (pH 10.5-11.0)*
0.6 g Tris base
100 mL sterile water
do NOT adjust the pH
autoclave for 30 minutes

*50 mg/mL ampicillin*
0.5 g ampicillin
water to 10 mL
filter sterilize through 0.22 μm filter into 1 mL aliquots
store at -20°C

*200 mg/mL IPTG* (Isopropylthio-β-D-galactoside)
2 g IPTG
water to 10 mL
filter sterilize through 0.22 μm filter into 1 mL aliquots
store at -20°C

*20 mg/ mL X-gal* (5-bromo-4 chloro-3-indolyl-β-D-galactoside)
Use a polypropylene or glass tube
100 mg X-gal
dimethylformamide to 5 mL
wrap tube in aluminum foil and store at -20°C

*LB liquid media*
5 g bacto-tryptone
2.5 g yeast extract
5 g NaCl
water to 500 mL
0.5 mL 1N NaOH
put 100 mL aliquots into glass bottles
autoclave for 30 minutes

*LB-amp plates*
5 g bacto-tryptone
2.5 g yeast extract
5 g NaCl
7.5 g agar
water to 500 mL in flask that is 1 L in size (add stir bar)
0.5 mL 1N NaOH
autoclave for 30 minutes
cool at room temperature until flask can be touched without gloves
add 1 mL 50 mg/mL ampicillin
stir briefly
pour 20 plates in a sterile fashion

*LB-amp/X-gal/IPTG plates*

Add 50 μL of 20 mg/mL X-gal and 12 μL of 200 mg/mL IPTG to an LB-amp plate
Spread the solution around the plate
Incubate at room temperature for 3 hours

*Competent cell preparation*

1. Inoculate *E. coli* strain JM109 into 5 mL LB media and shake overnight at 37°C.
2. Transfer the 5 mL overnight culture into 250 mL SOB media in a 2 L flask. Grow at 18°C overnight.
3. Harvest cells at $OD_{600} = 0.6$
4. Place on ice for 10 minutes.
5. Transfer cells to centrifuge bottles and spin 10 minutes at 3000 rpm in GSA rotor.
6. Pour off supernatant and resuspend pellet in 80 mL of ice-cold TB.
7. Incubate 10 minutes on ice.
8. Spin again at 3000 rpm in GSA rotor.
9. Pour off supernatant and resuspend pellet in 18.6 mL TB and 1.4 mL DMSO (7% final concentration of DMSO).
10. Incubate for 10 minutes on ice and leave tube on ice for the next step.
11. Aliquot 400 μL of cells into cold microcentrifuge tubes.
12. Immediately freeze aliquots in dry ice and place them at -80°C.

## Appendix B: Student worksheet

A.
*Kpn* I digests 37°C beginning time _____. ending time _____.
*Bst*E II digests 60°C beginning time _____. ending time _____.

B.
4% agarose gel
95 volts
initial current _____. final current _____.
beginning time _____. ending time _____.

Write the sample number and lane number at the top of each column. Place a check in the box if a fragment is observed in each sample.

| Fragments | | | | | | |
|---|---|---|---|---|---|---|
| 309 | | | | | | |
| 223 | | | | | | |
| 204 | | | | | | |
| 145 | | | | | | |
| 137 | | | | | | |
| 119 | | | | | | |
| 96 | | | | | | |
| Allele 1 | | | | | | |
| Allele 2 | | | | | | |

C. 1% agarose gel

95 volts
initial current _____. final current _____.
beginning time _____. ending time _____.

| Sample # | *Kpn* I digest | *Bst*E II digest | Allele 1 | Allele 2 |
|---|---|---|---|---|
| | | | | |
| | | | | |
| | | | | |
| | | | | |
| | | | | |
| | | | | |

D. Conclusions:

| Sample # | Allele 1 | Allele 2 |
|---|---|---|
| | | |
| | | |
| | | |
| | | |
| | | |
| | | |

## Appendix C: cloning and sequencing exon 6

Although cloning of the PCR products is not necessary to determine whether the $O^1$ allele exists; cloning and subsequently sequencing exon 6 incorporates another set of molecular techniques. Exon 7 could also be cloned, but the protocols have not been determined.

Restriction sites for common, inexpensive restriction sites have been engineered at the ends of primers exon6-1 and exon6-2 (see above for sequence). The *Eco*R I and *Hin*d III sites do not exist in the genome at these positions, but they allow for digestion of the exon 6 PCR product for cloning. This protocol would replace "day 5" from the normal schedule.

### Day 5

Students will be digesting the exon 6 PCR product with *Eco*R I and *Hin*d III in order to clone this fragment into the plasmid pUC19.

*Digestion of exon 6 PCR and pUC19*

1. Assemble the following reactions and record this table in your lab notebook:

| | Student DNA 1 | Student DNA 2 | Unknown DNA |
|---|---|---|---|
| 10X Promega buffer E | 1.5 µL | 1.5 µL | 1.5 µL |
| 10X BSA | 1.5 µL | 1.5 µL | 1.5 µL |
| Exon 6 PCR (student 1) | 11.0 µL | ---- | ---- |
| Exon 6 PCR (student 2) | ---- | 11.0 µL | ---- |
| Exon 6 PCR (unknown) | ---- | ---- | 11.0 µL |
| *Eco*R I (12 units/µL) | 0.5 µL | 0.5 µL | 0.5 µL |

| | | | |
|---|---|---|---|
| *Hin*d III (10 units/μL) | 0.5 μL | 0.5 μL | 0.5 μL |

2. Incubate the reactions at 37°C for 2 hours.
3. Thaw the ligase buffer completely just before it is needed. No white flakes should be visible. Store the buffer on ice until it is needed. Vortex briefly before using this buffer.
4. Assemble the following ligation reactions and record this table in your lab notebook:

| | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Sterile water | 2 μL | 2 μL | 2 μL | 2 μL | 2 μL |
| 10X ligase buffer | 17.5 μL | 17.0 μL | 7 μL | 7 μL | 7 μL |
| pUC19 (150 ng/μL) | 0.5 μL | 0.5 μL | 0.5 μL | 0.5 μL | 0.5 μL |
| Exon 6-E/H-student 1 | ---- | ---- | 10 μL | ---- | ---- |
| Exon 6-E/H-student 2 | ---- | ---- | ---- | 10 μL | ---- |
| Exon 6-E/H-unknown | ---- | ---- | ---- | ---- | 10 μL |
| T4 DNA ligase (3 units/μL) | ---- | 0.5 μL | 0.5 μL | 0.5 μL | 0.5 μL |

5. Incubate at 16°C overnight.

**Day 6**

Remove the ligations from the incubator and store at -20°C.

**Day 7**

*Transformation of bacterial cells with ligation reactions*

It is important that all manipulations during the bacterial transformation protocol occur under sterile conditions. The instructor will demonstrate how to handle tubes and plate cells while minimizing the opportunity for contamination to occur. Also, *E. coli* cells (JM109) that are competent to be transformed should be kept on ice as much as possible prior to step 5 in order to maximize their efficiency at picking up DNA from solution.

Students will perform a blue/white screen by plating the cells on LB-amp/X-gal/IPTG plates. Ligating an insert into the pUC19 plasmid disrupts the *lacZ* gene that produces the protein β-galactosidase. When an insert is present, no β-galactosidase is produced and colonies appear white. However, plasmids that contain no insert are able to produce β-galactosidase. This protein uses X-gal as a substrate and converts this colorless chemical to a chemical that is blue in color. The coloies that possess plasmids with no insert will appear blue. This screen allows for a visual inspection of the plate to guide students to choose colonies that are expected to have an insert. In this screen IPTG induces expression of β-galactosidase.

1. Thaw *on ice* one tube of JM109 cells for every 2 transformations. These tubes contain 400 mL of cells. Put 4 empty microcentrifuge tubes on ice.
2. When the JM109 cells are thawed, gently mix the cells by tapping the tube. Next, place 200 μL of cells into an empty microcentrifuge tube that has been pre-chilled.
3. Add DNA to the 7 tubes according to the list below:

| transformation | DNA |
|---|---|
| A | no DNA (negative control for transformation) |
| B | 1 μL pGEM-3Z (positive control for transformation) |
| C | 10 μLl ligation #1 |
| D | 10 μL ligation #2 |

| | |
|---|---|
| E | 10 µL ligation #3 |
| F | 10 µL ligation #4 |
| G | 10 µL ligation #5 |

4. Incubate 30 minutes on ice.
5. Incubate 2 minutes at 42°C.
6. Add cells to 0.6 mL of LB media at room temperature.
7. Incubate at 37°C for 45-60 minutes.
8. Plate 200 µL of cells on labeled LB-amp/X-gal/IPTG media.
8. After the solution has been absorbed by the media, place plates upside-down in the 37°C incubator overnight.
9. Remove plates in the morning (before 11 a.m.) to avoid satellite colony growth.

**Day 8**

1. Remove the plates from the incubator.
2. Count the colonies on each plate. Record the number of blue and white colonies in your lab notebook..
3. Wrap the plate sides with parafilm and place them in the refrigerator.

**Day 9**

Students will inoculate colonies into liquid LB-ampicillin (amp) media and grow them overnight. This step should be completed the afternoon before the normal lab meeting. LB-amp should be aliquoted for the students and stored in the refrigerator.

1. With an inoculating loop that has been flamed to sterilize it, pick a single white colony from plate E and put it into 3 mL of LB-amp. Shake the loop in the liquid.
2. Flame the loop again.
3. Repeat steps 1 and 2 until you have picked 4 white colonies from each of plates E, F, and G (12 total) and placed each colony in its own tube of LB-amp.
4. Incubate the cultures at 37°C in a shaking incubator overnight.

**Day 10**

Students will isolate their plasmids using a miniprep kit from Qiagen (Qiagen Spin Miniprep Kit). The kit reagents are proprietary, and this protocol taking from their kits. Cells are lysed in a basic solution that results in DNA denaturation; however, the plasmid DNA is entangled due to the small, circular structure of plasmids. Upon neutralization, the plasmid DNA strands will reanneal, while the vastly larger circular bacterial chromosome will not. Cellular debris that includes the chromosomal DNA will be precipitated, and the plasmid DNA will be bound to a resin. Following wasing, the plasmid DNA is eluted.

1. Remove 1.5 mL of media from a tube of your overnight culture and place into a labeled microcentrifuge tube.
2. Spin at top speed in a microcentrifuge for 2 minutes.
3. Remove the supernatant by pipeting and place in 10% bleach solution.
4. Fully resuspend the cell pellet in 250 µl of Buffer P1 by gently pipeting up and down. Make sure there are no clumps of cells present.
5. Add 250 µl of Buffer P2 and invert 4 times.
6. Add 350 µl of Buffer N3 and invert 4 times.

7. Spin at top speed for 10 minutes in a microcentrifuge. Wait for a few groups to be ready before starting the centrifuge.
8. Place spin columns in collection tubes (no lids on these tubes) and label the *spin column*.
9. Decant the supernatant into the appropriate spin column.
10. Spin for 1 minute at top speed.
11. Discard the flow through that is present in the collection tube.
12. Replace the spin column into the same collection tube.
13. Add 750 µl of Buffer PE to each spin column.
14. Spin for 1 minute at top speed.
15. Discard the flow through that is present in the collection tube.
16. Replace the spin column into the same collection tube.
17. Spin for 1 minute at top speed to remove any residual Buffer PE.
18. Discard the flow through that is present in the collection tube.
19. Place the spin column in a labeled new centrifuge tube. The lids will not close with the spin column in the microcentrifuge tube.
20. Add 50 µl of sterile, nuclease-free water by pipeting it onto the center of the resin, but you should not touch the resin with your pipet tip.
21. Wait 1 minute.
22. Spin at top speed for 1 minute.
23. Discard the spin column.
24. Close the microcentrifuge tubes and store them at -20°C.

**Sequencing**

The plasmid pUC19 contains M13 primer binding sites. Therefore, M13 forward and M13 reverse are standard primers that can be used in sequencing exon 6. Instructors should consult with their local sequencing facility for instructions regarding plasmid preparation (the places that I have used all prefer Qiagen-prepped plasmids) prior to initiating the lab exercise.

## Appendix D: Data analysis

*Exon 7 digests*

It is important to stress to students the value of data analysis in this exercise. If the individual is heterozygous, they need to remember that two columns from Table 1 would be merged to show a pattern of fragments. They should consider the results of the exon 6 digest before examining the exon 7 gel; it is helpful to have them give the possible combinations of fragments that could be observed on the exon 7 gel. For example, a student expects 4 specific fragments whenever the exon 6 PCR product is digested by *Kpn* I. Some fragments are diagnostic of a particular allele; the *B* allele produces a 223 base pair *Hpa* II fragment that appears in no other allele. It should be noted that the intensity of fragments can also be an indication of the allele combination that is present. If each fragment is present in a 1:1 ratio, then a decrease in fragment intensity would be expected moving down the gel from larger to smaller fragment sizes.

**Table 2.** Exon 7 fragment pattern for heterozygous samples. A single plus indicates that one allele will produce the fragment, while "++" indicates that both alleles will produce the fragment.

| | $A^1, A^2$ | $A^1, B$ | $A^1, O^1$ | $A^1, O^2$ | $A^2, B$ | $A^2, O^1$ | $A^2, O^2$ | $B, O^1$ | $B, O^2$ | $O^1, O^2$ |
|---|---|---|---|---|---|---|---|---|---|---|
| 309 bp | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| 223 bp | | + | | | + | | | + | + | |
| 204 bp | ++ | + | ++ | ++ | + | ++ | ++ | + | + | ++ |
| 145 bp | + | | | | + | + | + | | | |
| 137 bp | | + | | + | + | | + | + | ++ | + |
| 119 bp | + | ++ | ++ | ++ | + | + | + | ++ | ++ | ++ |
| 96 bp | ++ | + | ++ | + | + | ++ | + | + | | + |

*Ligation and transformation controls*

Ligation 1 = control for undigested pUC19
Ligation 2 = control for pUC19 that is digested by one enzyme
No DNA transformation = control for contamination/amp-resistance of JM109 cells
pGEM-3Z = control for ability of cells to be transformed and blue/white screen