

Using Allele-specific Primer PCR to Determine Human ABO Blood Type Genotypes

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Genotyping individuals has moved from the clinical laboratory with implications on human health to commercial application in order to examine ancestry as well as non-health orientated traits. In this four-week laboratory exercise, students use allele-specific primer PCR in order to determine their genotype at the ABO blood group locus. The five most common alleles are examined in order to determine the genotype of each sample. Additionally, students were asked to troubleshoot any unexpected results as well as re-design the protocols in order to perform a multiplex PCR.

Keywords: Allele-specific primer PCR, ABO, genotyping, genetics

Introduction

PCR is one of the most pervasive techniques in molecular biology and other related disciplines. Students should be proficient in this technique prior to gaining an internship, research laboratory experience, or entering the workforce. Standard PCR whereby a target sequence is amplified is performed by most laboratories, but this exercise examines the role of primer annealing to differentiate between nearly identical primer binding sites. The principles of allele-specific primer PCR provide an opportunity for students to examine primer annealing and the ability/inability to generate a product to analyze their own ABO blood type genotypes. Additionally, sequenced genomes may be purchased to provide a substitute DNA

template if privacy or ethical concerns exist, and these genomes can be used as homozygous or heterozygous samples of the instructor's choosing. Introduction to codominance in a genetics prerequisite course should ensure that students have foundational knowledge of human ABO blood type prior to performing the laboratory exercise.

This laboratory exercise is described as a four-week project in which a three-hour laboratory period should be sufficient. Students in intermediate to upper level science courses are best suited to complete these experiments, especially following any previous experience with PCR and/or gel electrophoresis. There is minimal set-up required as aliquoting PCR reagents is the only significant task.

Student Outline

Objectives

- To perform allele-specific primer PCR
- To understand the effect of primer-template mismatches on annealing and extension
- To perform gel electrophoresis

Introduction

Genetic testing has been available for decades, but until recently it was typically performed at the request of a physician and for the diagnosis of disorders, determination of treatment, or carrier status (Katsanis and Katsanis 2013). The past few years have seen a rapid rise in direct-to-consumer genetic testing from companies, such as 23andMe and Ancestry. These companies test for genetic variation by determining the sequence at single nucleotide polymorphisms (SNPs) across the entire genome.

The human ABO phenotype is a classic genetic example of codominance. The four phenotypes of A, B, AB, and O are determined by a single gene that has three broad classes of gene variants (often referred to as I^A , I^B , and i in genetics textbooks). The O allele (i) is recessive to both of the A (I^A) and B (I^B) alleles, which exhibit codominance when inherited together. The ABO genetic system is often oversimplified in textbooks as there are multiple different alleles for A and O. In fact, there are five common alleles: A^1 , A^2 , B, O^1 , and O^2 (Yamamoto, *et al.* 2004).

The A^1 allele encodes a fully functioning glycosyltransferase that attaches N-acetylgalactosamine to the H antigen, whereas the A^2 allele produces an enzyme with weaker activity for N-acetylgalactosamine attachment. A single nucleotide deletion (C1061del) in the region that encodes the C-terminal portion of the enzyme results in this extended protein with reduced activity (Yamamoto, *et al.* 1992). The B allele differs from the A^1 allele by a series of substitutions that alters the transferase enzyme to prefer a galactose substrate instead of N-acetylgalactosamine, whereas a single base pair deletion (G261del) in the sixth exon produces the non-functional O^1 allele. The least common of these five alleles is O^2 which possesses a G-to-T transversion (G53T) early in the coding region and similarly produces a non-functioning enzyme. Figure 1 illustrates these SNPs and their position in the ABO gene.

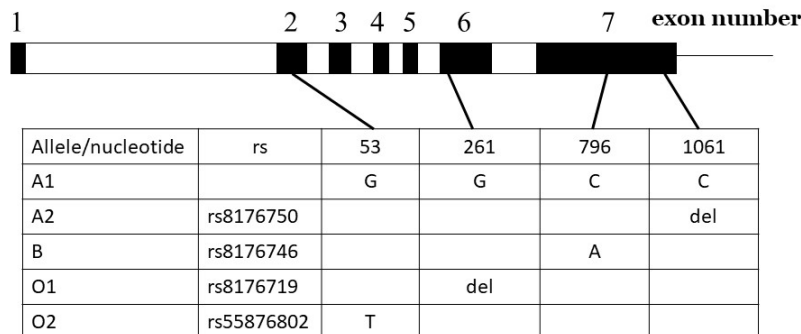


Figure 1. ABO gene SNP identity and location. A^1 SNP sequences are indicated, and an empty box represents no change when compared to the A^1 sequence. A deletion is shown as “del”. Reference SNP (rs) ID numbers are listed.

Restriction fragment length polymorphism (RFLP) testing uses restriction enzyme digestion to identify sequence differences (i.e., SNPs) that are present in sample DNA after PCR amplification. This technique is dependent on commercially available enzymes and is limited in its ability to test directly for the presence of the SNP. In many cases, scientists depend on linkage disequilibrium whereby they test for a particular SNP that is near the position about which they wish to gain knowledge. Since multiple SNPs tend to be co-inherited together in DNA, called a haplotype, the presence of a particular SNP allows for the inference of the presence of another SNP at a second locus. While this method is highly reliable, its utility/accuracy fails when recombination occurs in the DNA between the detected SNP and the second, inferred SNP.

In this multi-week laboratory exercise, you will be using allele-specific primer PCR in order to selectively amplify a single allele target; a companion reaction will amplify all other alleles in a separate tube. The O^1 allele will be used as an example of the experimental design. The 3' end of a forward primer will pair with either the allele of interest (O^1) or the other four alleles (Figure 2), and the same reverse primer will be present in each pair of reactions. An intentional mismatch in both

forward primers near the 3' end will act to destabilize the interaction at the 3' end (Hirotsu *et al.* 2010). Therefore, the O¹-specific primer will anneal exclusively to the O¹ allele template, whereas the non-O¹-specific primer will anneal with the A¹, A², B, and O² templates. An O¹O¹ homozygous sample will yield product in the O¹-specific reaction, but not the non-O¹-specific reaction. Any sample that does not contain an O¹ allele will only yield a product in the non-O¹-specific reactions. A sample that is heterozygous with a single O¹ allele (A¹O¹, A²O¹, BO¹, or O¹O²), will yield products in both the O¹-specific and non-O¹-specific reactions. Figure 3 shows example results for 4 samples with different genotypes. For an A¹A¹ sample, the genotype is determined by excluding the possibility of any of the other four alleles being present. As such, amplicons would appear in all four “non” reactions and no products in any of the allele-specific reactions.

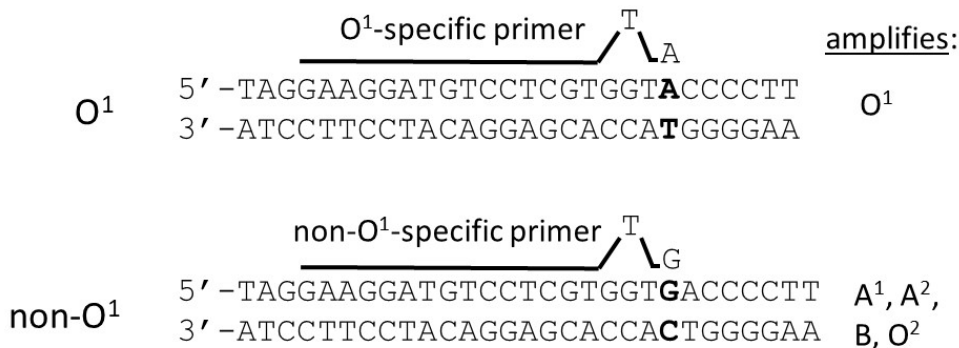


Figure 2. Annealing of O¹ and non-O¹ specific primers to their respective target sequences. The bolded G-C base pair is deleted in the O¹ allele.

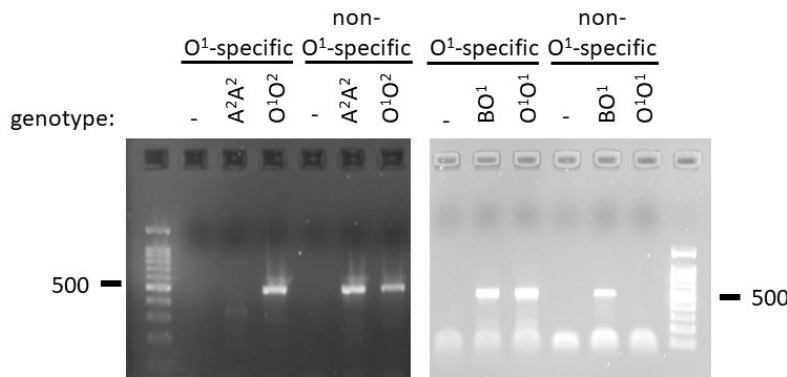


Figure 3. Sample data from O¹ PCR. A no-template control is indicated by a minus sign. Known genotypes are indicated above the gels.

Methods and Data Collection

Day 1: DNA Isolation

Genomic DNA will be isolated using the QiaAMP DNA Mini kit according to the manufacturer’s instructions. Students will collect buccal cells using a cotton swab. Subsequent DNA isolation will produce a genomic DNA sample that is sufficient to act as a template for polymerase chain reaction.

NOTE: students should not eat or drink for 30 minutes prior to swabbing their cheeks. Students will also need to briefly come to the laboratory at least 2 hours before the normal starting time in order to swab their cheeks. Bleach should not be used as a disinfectant for this lab.

1. Place open microcentrifuge tube into rack.

2. Remove cotton swab from packaging and scrape inside of each cheek six times.
3. Place the cotton swab in the microcentrifuge tube such that the cotton portion of the swab is facing the ceiling.
4. Allow the swab to air dry for two hours.
5. Place the cotton end of the swab in the microcentrifuge tube.
6. While keeping the cotton swab in the microcentrifuge tube, cut the plastic stem of the swab slightly above the cotton tip using a pair of scissors. Keeping the swab below the lip of the microcentrifuge tube will prevent the cotton tip from being thrown after the stem is cut.
7. Add 400 μL **phosphate-buffered saline (PBS)** by distributing the solution across the entire swab.
8. Add 20 μL **protease solution**.
9. Add 400 μL **Buffer AL** and close the lid of the microcentrifuge tube.
10. Mix by vortexing immediately for 15 seconds.
11. Incubate at 56°C for 10 minutes (optional: add cap locks to tubes before heating)
 - During the incubation, label a QIAamp Mini spin column with your initials.
 - Place the labeled column into a collection tube (note: collection tubes do not have lids).
12. Briefly centrifuge the sample to remove condensation from the lid.
13. Add 400 μL **ethanol (100%)** to the sample and mix briefly by vortexing.
14. Briefly centrifuge the sample to remove condensation from the lid.
15. Transfer 700 μL of the buccal swab solution into the spin column. Do not get the rim of the column wet.
16. Centrifuge at 8000 rpm for 1 minute.
17. Place the spin column into a new collection tube. Discard the filtrate.
18. Repeat steps 15-17. If the cotton swab solution does not contain 700 μL , transfer as much as you can reasonably pipet.
19. Add 500 μL **Buffer AW1** without wetting the rim of the spin column.
20. Centrifuge at 8000 rpm for 1 minute.
21. Place the spin column into a new collection tube. Discard the filtrate.
22. Add 500 μL **Buffer AW2** without wetting the rim of the spin column.
23. Centrifuge at top speed for 3 minutes.
24. Place the spin column into a new collection tube. Discard the filtrate.
25. Centrifuge at top speed for 1 minute. This will remove any residual solution prior to eluting your genomic DNA.
26. Place the spin column into a new microcentrifuge tube (with a lid) that has been labeled with your initials and the date on both the top and side of the tube.
27. Add 150 μL **Buffer AE** directly to the white filter disk on the bottom of spin column (note: don't allow the pipet tip to touch the white filter).
28. Incubate for 1 minute at room temperature.
29. Centrifuge at 8000 rpm for 1 minute.
30. Discard spin column; retain the eluate in the bottom of the microcentrifuge tube.
31. Cap the microcentrifuge tube and store at -20°C.

Day 2: PCR of B and A² Alleles

A. Prepare for PCR

1. While wearing gloves, consecutively number 20 PCR tubes on their sides immediately below the lip.
2. Place tubes on ice and add 1 μL of deionized water or genomic DNA to the following tubes making sure to change the micropipette tip between samples:

- 1: water
- 2: student DNA 1
- 3: student DNA 2
- 4: unknown 1-12 (record sample number)
- 5: unknown 1-12 (record sample number)
- 6: water
- 7: student DNA 1
- 8: student DNA 2
- 9: unknown 1-12 (record sample number)
- 10: unknown 1-12 (record sample number)

- 11: water
- 12: student DNA 1
- 13: student DNA 2
- 14: unknown 1-12 (record sample number)
- 15: unknown 1-12 (record sample number)
- 16: water
- 17: student DNA 1
- 18: student DNA 2
- 19: unknown 1-12 (record sample number)
- 20: unknown 1-12 (record sample number)

B. *B* Allele PCR

1. Each lab partner should prepare a single PCR master mix, either B or non-B specific.
2. Making sure to change tips before pipetting each reagent, assemble each PCR master mix in a separate 0.5 mL microcentrifuge tube by adding the reagents from the top to the bottom of the column in Table 1. Do not add the GoTaq DNA polymerase yet. Incubate the PCR master mix tubes on ice.
3. After all mixes have been assembled, the instructor will tell students to then add the polymerase to each master mix.

Table 1. Reaction components for *B* and non-*B* allele amplification.

	Stock conc.	Final conc.	Amount per reaction (μL)	B specific mix (μL), 5.5 reactions	Non-B specific mix (μL), 5.5 reactions
deionized water			12.8	70.4	70.4
GoTaq buffer	5X	1X	5.0	27.5	27.5
MgCl ₂	25 mM	3 mM	3.0	16.5	16.5
B specific forward primer	10 μM	0.5 μM	1.25*	6.9	N/A
B reverse primer	10 μM	0.5 μM	1.25	6.9	6.9
Non-B specific forward primer	10 μM	0.5 μM	1.25*	N/A	6.9
dNTPs	10 mM	0.2 mM	0.5	2.8	2.8
template DNA	10 ng/μL	0.4 ng/μL	1.0	N/A	N/A
GoTaq DNA polymerase	5 units/μL	0.025 units/μL	0.125	0.7	0.7

*Forward primers are present only in a single reaction.

4. Briefly vortex the PCR mix and centrifuge or tap tube on the bench to collect the liquid at the bottom of the tube.
5. Add 24 μL of the appropriate PCR master mix to the following PCR tubes changing tips between aliquots:

- 1-5: B specific master mix
- 6-10: non-B specific mix

6. Tightly cap the PCR tubes and place in the appropriate thermal cycler.

7. Amplify products using the following PCR conditions (with steps 2-4 repeated for 35 cycles):

1. 94°C for 3 minutes
2. 94°C for 30 seconds
3. 54°C for 30 seconds
4. 72°C for 30 seconds
5. 72°C for 5 minutes
6. 4°C hold

8. A^2 Allele PCR

1. Each lab partner should prepare a single PCR master mix, either A^2 or non- A^2 specific.
2. Making sure to change tips before pipetting each reagent, assemble each PCR master mix in a separate 0.5 mL microcentrifuge tube by adding the reagents from the top to the bottom of the column in Table 1. Do not add the GoTaq DNA polymerase yet. Incubate the PCR master mix tubes on ice.
3. After all mixes have been assembled, the instructor will tell students to then add the polymerase to each master mix.

Table 2. Reaction components for A^2 and non- A^2 allele amplification.

	Stock conc.	Final conc.	Amount per reaction (μ L)	A^2 specific mix (μ L), 5.5 reactions	Non- A^2 specific mix (μ L), 5.5 reactions
deionized water			12.8	70.4	70.4
GoTaq buffer	5X	1X	5.0	27.5	27.5
MgCl ₂	25 mM	3 mM	3.0	16.5	16.5
A^2 specific forward primer	10 μ M	0.5 μ M	1.25*	6.9	N/A
A^2 reverse primer	10 μ M	0.5 μ M	1.25	6.9	6.9
Non- A^2 specific forward primer	10 μ M	0.5 μ M	1.25*	N/A	6.9
dNTPs	10 mM	0.2 mM	0.5	2.8	2.8
template DNA	10 ng/ μ L	0.4 ng/ μ L	1.0	N/A	N/A
GoTaq DNA polymerase	5 units/ μ L	0.025 units/ μ L	0.125	0.7	0.7

*Forward primers are present only in a single reaction.

4. Briefly vortex the PCR mix and centrifuge or tap tube on the bench to collect the liquid at the bottom of the tube.
5. Add 24 μ L of the appropriate PCR master mix to the following PCR tubes changing tips between aliquots:

11-15: A^2 mix
16-20: non- A^2 mix

6. Tightly cap the PCR tubes and place in the appropriate thermal cycler.

7. Amplify products using the following PCR conditions (with steps 2-4 repeated for 35 cycles):

1. 94°C for 3 minutes
2. 94°C for 30 seconds
3. 62°C for 30 seconds
4. 72°C for 30 seconds
5. 72°C for 5 minutes
6. 4°C hold

Day 3: Gel Electrophoresis of B and A² PCR Reactions; PCR of O¹ and O² Alleles

A. Gel Electrophoresis

Caution: gloves should be worn whenever students may come in contact with ethidium bromide.

1. Prepare 600 mL of 1X TBE buffer (enough for pouring agarose gel and electrophoresis running buffer).
 - Add 120 mL 5X TBE to a graduated cylinder
 - Fill to 600 mL with deionized water
 - Cover the cylinder with parafilm and mix
2. Prepare a 1% agarose/1X TBE solution.
 - Pour 70 mL of 1X TBE into an Erlenmeyer flask
 - Add 0.7 g low-EEO agarose to the flask
 - Cover with plastic wrap and poke holes for ventilation
 - Dissolve agarose according to the instructor's microwave directions
 - Cool agarose solution-containing flask for 1 minute in a plastic beaker of cold water
 - After the solution has cooled, add 3.5 μ L of 0.5 μ g/mL ethidium bromide to the flask. Eject the tip into the ethidium bromide solid waste container for disposal.
 - Swirl solution gently in order to minimize bubbles
3. Pour 1% agarose/1X TBE gel.
 - Place gel tray in the casting tray
 - Place 2 combs in the gel tray (one at the top and one in the middle)
 - Pour the 1% agarose/1X TBE solution into the gel tray
 - Wait 20 minutes for the gel to solidify before continuing
4. Load the gel
 - Place the gel and gel tray into the gel rig, pour the remaining 1X TBE running buffer into the ends of the gel rig until the gel is covered by running buffer, and then carefully remove the combs. Don't pour the buffer directly on top of the gel.
 - Load 4 μ L of 100 bp DNA standard into the first well (on the left) in the top row.
 - Load 10 μ L of B and non-B PCR reactions into separate wells (following the 1-10 reaction order from Day 2) in the top portion of the gel
 - Repeat these last two steps for the A² and non-A² PCR reactions (following the 11-20 reaction order from Day 2) in the bottom portion of the gel (i.e., wells in the middle of the gel)
 - Record the order of loaded samples in your laboratory notebook
5. Running the agarose gel
 - Connect the electrodes to a power source (i.e., red to red; black to black) ensuring the gel within the rig is safely covered.
 - Run at a constant voltage of 10 volts/cm of gel length for 40 minutes
 - The expected sizes are 160 bp and 152 bp for the B and A² amplicons, respectively
 - Turn off the power supply and remove the gel for evaluation
 - Expose gel to UV excitation, photograph and save the resulting image in your group's folder

B. PCR of O^1 and O^2 Alleles

1. While wearing gloves, consecutively number 20 PCR tubes (21-40) on their sides immediately below the lip
2. Add 1 μL of water or genomic DNA to the following PCR tubes:

21: water	31: water
22: student DNA 1	32: student DNA 1
23: student DNA 2	33: student DNA 2
24: unknown 1-12 (record sample number)	34: unknown 1-12 (record sample number)
25: unknown 1-12 (record sample number)	35: unknown 1-12 (record sample number)
26: water	36: water
27: student DNA 1	37: student DNA 1
28: student DNA 2	38: student DNA 2
29: unknown 1-12 (record sample number)	39: unknown 1-12 (record sample number)
30: unknown 1-12 (record sample number)	40: unknown 1-12 (record sample number)

3. Assemble O^1 and non- O^1 reactions as described in Day 2 protocol (tubes 21-30 and Table 3)
4. Assemble O^2 and non- O^2 reactions as described in Day 2 protocol (tubes 31-40 and Table 4)
5. Both O^1 and O^2 reaction pairs have the same thermocycling conditions as the *B* allele PCR.

Table 3. Reaction components for O^1 and non- O^1 allele amplification.

	Stock conc.	Final conc.	Amount per reaction (μL)	O^1 specific mix (μL), 5.5 reactions	Non- O^1 specific mix (μL), 5.5 reactions
deionized water			12.8	70.4	70.4
GoTaq buffer	5X	1X	5.0	27.5	27.5
MgCl ₂	25 mM	3 mM	3.0	16.5	16.5
O^1 specific forward primer	10 μM	0.5 μM	1.25*	6.9	N/A
O^1 reverse primer	10 μM	0.5 μM	1.25	6.9	6.9
Non- O^1 specific forward primer	10 μM	0.5 μM	1.25*	N/A	6.9
dNTPs	10 mM	0.2 mM	0.5	2.8	2.8
template DNA	10 ng/ μL	0.4 ng/ μL	1.0	N/A	N/A
GoTaq DNA polymerase	5 units/ μL	0.025 units/ μL	0.125	0.7	0.7

*Forward primers are present only in a single reaction.

Table 4. Reaction components for O^2 and non- O^2 allele amplification.

	Stock conc.	Final conc.	Amount per reaction (μL)	O^2 specific mix (μL), 5.5 reactions	Non- O^2 specific mix (μL), 5.5 reactions
deionized water			12.8	70.4	70.4
GoTaq buffer	5X	1X	5.0	27.5	27.5
MgCl ₂	25 mM	3 mM	3.0	16.5	16.5
O^2 forward primer	10 μM	0.5 μM	1.25*	6.9	N/A
O^2 reverse primer	10 μM	0.5 μM	1.25	6.9	6.9
Non- O^2 forward primer	10 μM	0.5 μM	1.25*	N/A	6.9
dNTPs	10 mM	0.2 mM	0.5	2.8	2.8
template DNA	10 ng/ μL	0.4 ng/ μL	1.0	N/A	N/A
GoTaq DNA polymerase	5 units/ μL	0.025 units/ μL	0.125	0.7	0.7

*Forward primers are present only in a single reaction.

Day 4: Gel Electrophoresis of O^1 and O^2 PCR Reactions

1. Prepare another 1% agarose gel as indicated in Day 3 protocol.
2. Load 4 μL 100 bp DNA standard into the first well to the left in the top set of wells.
3. Load 10 μL of O^1 and non- O^1 specific PCR reactions into separate wells (following the 21-30 reaction order from Day 3) in the top portion of the gel.
4. Load 10 μL of O^2 and non- O^2 specific PCR reactions into separate wells (following the 31-40 reaction order from Day 3) in the bottom portion of the gel (i.e., wells in the middle of the gel).
5. Run the gel and record the results as described in Day 3 protocol.
6. The expected sizes are 514 bp and 371 bp for O^1 and O^2 amplicons, respectively.

Discussion

1. What effect does one versus two mismatches towards the 3' end of the primer have on amplification?
2. Would a similar effect occur if the mismatches were towards the 5' end of the primer? Design a simple experiment to test your hypothesis.
3. Multiplex PCR amplifies multiple targets in a single reaction. The targets should not overlap in multiplex PCR, and they should be easily distinguishable on a gel. Using a genome sequence of the *ABO* gene and mapping the location of the amplicons, you should determine which targets are appropriate for a multiplex reaction. Instead of the 8 reactions that were performed in this experiment, determine the lowest number of reactions that could give you the same results.
4. For the target(s) that are not appropriate for a multiplex reaction, how would you modify the procedure to convert them into multiplex targets.
5. If you got false positives (which would be found when 3 or more "specific" reactions yield a product), how would you modify the primers or procedure to prevent the false positive?

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Materials

PCR Reagents

GoTaq polymerase was purchased from Promega (Madison, WI) whereas primers were purchased from Eurofins Genomics (Louisville, KY). Human genomic DNA samples with specific genotypes were identified using the 1000 Genomes Browser (The 1000 Genomes Project Consortium 2015) and acquired as purified samples from the Coriell Institute for Medical Research (Camden, NJ). Samples were used directly in PCR reactions or mixed to mimic specific heterozygous genotypes. Human genomic DNA samples used in this study included: HG00133 (O^2O^2), HG00141 (O^1O^1), HG03814 (BB), HG02282 (A^2A^2), and HG00097 (A^1A^1).

Primers

A^2 specific forward:

5'-CCACCAGGCGGTCCGAGCCG-3'

Non- A^2 specific forward:

5'-CCACCAGGCGGTCCGAGCCC-3'

A^2 reverse:

5'-CGGGGCCTAGGCTTCTAGTTA-3'

B specific forward:

5'-ACGAGGGCGATTTCTACTGCA-3'

Non-B specific forward:

5'-ACGAGGGCGATTTCTACTGCC-3'

B reverse:

5'-CGCAGCAGGTACTTGTTTCAG-3'

O^1 specific forward:

5'-GAAGGATGTCCTCGTGTTA-3'

Non- O^1 specific forward:

5'-GAAGGATGTCCTCGTGTTG-3'

O^1 reverse:

5'-TCCAAGGACAGCAAACAGC-3'

O^2 specific forward:

5'-CCAAAATGCCACGCACTGCT-3'

Non- O^2 specific forward:

5'-CCAAAATGCCACGCACTGCG-3'

O^2 reverse: 5'-TTGGGACCCAGAATTGCCTTC-3'

Notes for the Instructor

1. Do not use bleach or acidic solutions for decontamination during the DNA isolation procedure. See QIAGEN handbook for proper disposal of solutions used during DNA isolation.
2. Set water bath to 56°C prior to DNA isolation on Day 1.
3. Genomic DNA concentrations should be checked by the instructor or student prior to performing PCR.
4. Instructors are encouraged to perform the exercise in advance in order to confirm annealing temperatures are optimized to produce the desired products. Otherwise, variability of thermocyclers may lead to aberrant results.

5. Multiplex PCR reactions targeting two different SNPs may be performed in a single reaction. This would be an appropriate experiment for more advanced students to develop.

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About the Authors

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Patrick Jungling is currently a senior Biology major at John Carroll University. He plans on furthering

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Kali Prillwitz is currently a junior Biology major at Alma College. After graduation, she plans on studying dental medicine and specializing in orthodontics.

Irfan Khan earned a BS in Biology at John Carroll University before attending Nova Southeastern University College of Pharmacy. He is currently a third-year pharmacy student.

Senan Susan earned a BS in Chemistry-Biochemistry Track at John Carroll University before attending Midwestern University College of Dental Medicine in Illinois. He is currently a first-year DMD student.

Jason Papciak earned a BA in Economics and French at the University of Pittsburgh before attending a post-baccalaureate program at John Carroll University. He is currently in an accelerated nursing program at Loyola University (Chicago).

Michael Martin is a Professor of Biology and Associate Dean of Sciences, Mathematics & Health at John Carroll University. He earned a BS in Biology from the University of Cincinnati and PhD in Cell and Molecular Biology from the University of Wisconsin-Madison. He teaches courses in the areas of Molecular Biology and Genetics. His research interests include laboratory education, yeast genetics, and cyanobacterial systematics.

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