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Using Allele-specific PCR to Genotype Single Nucleotide Polymorphisms Associated with Eye Color Prediction

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Abstract

Human variation, including differences in skin, hair and eye pigmentation, have been a source of public interest and scientific investigation for hundreds of years. Recently, Walsh et al. (2012) extended our understanding of human pigmentation by showing a small number of genetic variants could be used to predict eye color in humans. This IrisPlex prediction system utilizes allele frequencies of six genetic markers to calculate the probability of blue, intermediate or brown eyes in an individual. Unfortunately, this method requires sequencing to determine the alleles present at each locus. For multiple samples, the cost and time expended may prohibit implementation in a classroom environment. Using allele-specific PCR, mismatch primers can determine the alleles present at these sites without the need for sequencing. We feel that these methods allow for the incorporation of eye color prediction into the curriculum of college laboratory courses and promote discussions concerning forensic science and human genetic diversity.

Keywords: Allele-specific PCR, forensics, human eye color

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INTRODUCTION

Teaching introductory genetic principles to students often starts with a discussion of Gregor Mendel's pioneering crosses with the garden pea, *Pisum sativum*. His efforts exploring several monogenic traits (with only two observed phenotypes) set the foundation for our understanding of gene inheritance and the effect of hybrid genotypes on observed phenotypes. Additional examples of simple Mendelian traits can be demonstrated in a variety of model organisms, providing opportunities for students to conduct experiments in real-time as well as seeing first-hand the offspring of true breeding and hybrid crosses. Translating these learning moments into discussions of human genetics, however, is more difficult due to a limited number of simple human traits that can be tested within a classroom laboratory environment. Our recent work (Calhoun et al. 2020) with DNA-based blood-type determination has provided educators with a relevant and familiar model for introducing extensions to Mendelian inheritance patterns such as codominance and multiple allelism, but other examples are needed.

In recent years, the genetics of human pigmentation has been a heavily investigated area of research with

published studies confirming the polygenic nature of human skin, hair, and eye color phenotypes (Liu et al. 2013, Pavan et al. 2019). Moreover, genetic variation within these loci has been investigated to evaluate which, if any, might help to predict an individual's pigmentation phenotypes from only DNA sources (Kayser et al., 2023).

One of the current prediction algorithms, called the IrisPlex system, incorporates the use of just six different single nucleotide polymorphism (SNP) genotypes to broadly predict an individual's eye color (Walsh et. al., 2012). While the multiplex nature of the reactions and the use of single base extension sequencing makes the published analysis efficient, the need to use capillary electrophoresis to process amplicons for genotype determination makes this method difficult to incorporate into an educational setting with limited equipment and resources available for investigations.

Methods such as allele-specific PCR that facilitate genotype determination without the need for sequencing would be advantageous in these situations. This method utilizes differences at the 3' end of a primer sequence that would either enhance or limit amplification depending upon the presence of complementary nucleotides (i.e., a specific allele) at each SNP locus. Successful amplification would thus correlate with the presence of a specific nucleotide allele in the DNA sequence being tested.

The experiments presented here will incorporate the use of allele-specific PCR to help individual students determine their own allele status at loci relevant to eye color phenotypes. These genotypes will then be used to "predict" the eye color as if the student sample was instead obtained from an unknown person (e.g., a suspect). As the data obtained is not impersonal or anonymous, students remain engaged with the project and are vested in determining their own genotypes at these loci. Moreover, the direct comparison students get to make between their own DNA and the color of their iris helps to make clear the impact of genotypes on phenotypes while simultaneously providing the context to discuss "prediction" as a probability and not a certainty, a highly relevant topic for all STEM students, not just those with forensic interests.

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STUDENT OUTLINE

Objectives

To perform allele-specific PCR
To understand the effect of primer-template mismatches on PCR efficiency
To gain an appreciation for forensic phenotype prediction of eye color

Introduction

Human variation, specifically externally visible characteristics (EVCs) such as skin, hair, and eye color, have been a source of public interest and scientific investigation for hundreds of years. The use of EVCs have been of particular interest to those in forensics where suspect identification relied initially on gray scale sketches and eyewitness accounts. The field of criminal investigation has progressed significantly in recent years, mainly in the area of Forensic DNA Phenotyping (FDP) where trace DNA collected as evidence can be used to predict not only eye, hair and skin color but also other traits such as eyebrow color, freckles, hair structure, hair loss in men, tall stature and an estimation of age (Kayser et al. 2023).

A broad range of variation within a phenotype of interest often indicates a polygenic inheritance pattern with genes exhibiting multiple alleles. In the specific case of EVCs including human pigmentation, this certainly holds true. But through intensive research into the genes and biochemical pathways that influence these phenotypes, many of the reasons why the human population is filled with a wonderfully diverse range of colors and shades in skin, hair and eye phenotypes are now known (Sturm 2009).

Recently, a small number of different genetic markers associated with 6 genes (HERC2, IRF4, SLC24A4, SLC45A2, TYR and OCA2) have been identified that are able to predict eye color with a high degree of probability (Walsh et al. 2012). These markers, called single nucleotide polymorphisms (SNPs), vary between individuals, and appear as a nucleotide substitution at a specific locus on one pair of your homologous chromosomes. For example, you may have two copies of the same nucleotide, say T/T, at one of the SNP sites. Someone else in class may be homozygous for the other allele on both of their chromosomes, say G/G. Still a third person could have one of each and be heterozygous, say G and T.

During this set of laboratory exercises, you will investigate your own DNA to characterize what alleles you have at each of these SNP loci. To accomplish this, you must first understand a general molecular biology technique called polymerase chain reaction (PCR), a method that uses the complimentary nature of DNA to replicate a small region of DNA billions of times. The first step in PCR is to denature double stranded DNA to yield single stranded templates. This is followed by the annealing (i.e., binding) of primers at both the start and end of the region to be copied and lastly, extending the primers by the DNA polymerase (i.e., Taq) to create new strands complimentary to the original templates. This three-step process is repeated numerous times in order to generate enough copies to visualize them.

Allele-specific (AS) PCR is different only in that one of the primers is designed specifically to anneal over a SNP nucleotide site (Figure 1). When the number of nucleotide mismatches increase on a primer, specifically at the 3' end, the efficiency of the PCR process decreases to a point that no specific DNA product would be generated. The primers for AS-PCR are designed to take advantage of this reduced efficiency and can be used to identify the presence or absence of a particular nucleotide at a SNP site. More simply, the appearance of a PCR product at the correct size indicates the presence of that specific SNP nucleotide whereas no PCR amplicon of the correct size indicates the absence of the allele.

Once you have determined your allele status at each of these SNP markers, we will then utilize an eye color prediction tool available on the web (https://hirisplex.erasmusmc.nl/) to "predict" your eye color. Clearly you can look at a mirror to determine this, but this exercise will help you to begin to understand one way that forensics is using trace DNA left behind at a crime scene to help authorities with suspect identification.

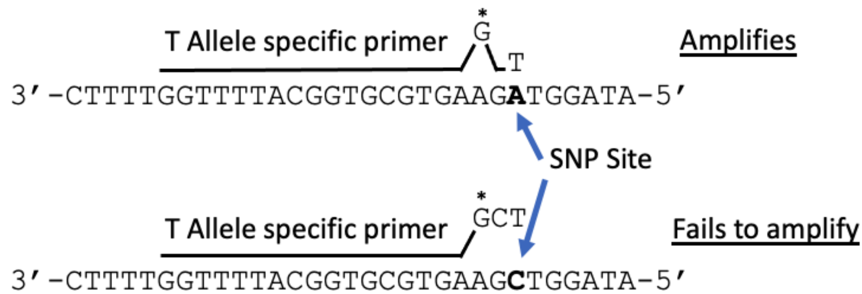

Figure 1. Allele-specific PCR Primer Design. The top and bottom primer are identical; only the template primer is annealing to is different (bolded nucleotides). When the terminal 1 or 2 nucleotides of the primer (-CT-3') can anneal to the template (top), amplification will occur indicating the presence of that specific allele. If the 3' nucleotides fail to anneal, amplification will also fail thus indicating the absence of an allele. The asterisk on the 3' end of the primer indicates an intentional mismatch that increases the specificity of the reaction.

Methods and Data Collection

Part A. DNA Isolation from buccal cells

You will be using a "swish and spit" method to collect buccal (cheek) cells before lysing the cells to collect the genomic DNA. Other methods, including professional forensic kits, can be used but we have found that this method is just as easy, costs less and produces similar qualities and quantities of DNA for PCR. While not critical for success, we have found that refraining from eating right before the lab (~30 mins) is preferable. Food that stains the mouth may also stain your DNA preparation while foods that stick to your teeth make for some unpleasant isolations. In particular, Crunchy Cheetos would be a striking example of what you would want to avoid…Yuck!! If needed, do a gentle pre-rinse with water to clean the mouth before starting. When you are ready, follow the procedure below to isolate your own DNA:

1. At all times, safety precautions should be followed. Make sure you are using gloves during the procedure as well as eye protection. All materials that came into contact with human tissues need to be disposed of properly in biohazard waste and/or sterilized. Watch out for broken glassware and the hot plate used for boiling our samples, burns can happen easily. Any accidents that happen need to be reported to the lab instructor immediately.

2. When ready, dump 30 mL of 0.9% saline into your mouth and vigorously swish for 30 seconds. Flush around the teeth and through all parts of the mouth. At the end, expel the saline swish back into a labeled 50mL conical centrifuge tube. Do not swallow the saline.

3. Transfer a 1 mL aliquot of the saline swish into a new 1.5 mL microcentrifuge tube. If the saline swish had time to settle, swirl the 50 mL centrifuge to redistribute the cells before taking this aliquot. Centrifuge at 6500 x g for 1 minute to pellet cells. Pipet to remove all but ~100 µL of the supernatant taking care to avoid disturbing the pellet.

4. Repeat the collection step (#3 above) two additional times but this time using the <u>same</u> 1.5 mL microcentrifuge tube as before. Pipet to remove all but ~100 µL of the supernatant after centrifuging for the last time. Remember to dispose the saline waste in a proper biohazard receptacle.

5. Re-suspend the cell pellet by pipetting the remaining saline up and down a few times. Try not to use the pipet tip itself to physically disrupt the cells, but the force of only the saline being pipetted. Once fully

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suspended again, transfer the cell suspension to a 2.0 mL microcentrifuge tube containing 200 µL of 10% Chelex. Mix by tapping the tube with your finger.

6. Put a boiling cap on microcentrifuge tube (to keep it from popping open) and boil the tube in a beaker of water for 10 minutes. Be careful to avoid burning yourself with splashing water or the hot plate.

7. After the boil, remove your tubes with tongs and centrifuge at 16,500 x g (i.e., max speed) for 3 minutes to completely pellet the cell debris and Chelex beads.

8. Transfer only the supernatant (your DNA!) to a new, clean 1.5 mL microcentrifuge tube. You can now use this sample for PCR or store this for later use at -20°C.

Part B. Perform PCR on isolated DNA

During the next set of exercises, you will be performing PCR at the six different SNP sites in order to determine what alleles you have inherited. To do this, you will be setting up two reactions for each site plus one negative control (i.e., a total of 13 reactions).

1. Each student should successively label 13 Polymerase Chain Reaction (PCR) tubes 1-13 on the exterior of the tube directly below the lid. Don't label the lids as these markings will be ruined due to the heated lid of the thermal cycler.

2. Assemble each PCR reaction into a separate 0.2 mL microcentrifuge tube by adding the reagents below starting with water and ending with DNA. For the Negative Control (Tube 13), you will be adding 1 µL of water instead of a primer mix. Make sure to write the following table into your lab notebook as well as detail any mistakes or observations you make during the assembly of these reactions. Changing tips between each step will be critical to ensure that contamination between reactions is minimized.

Table 1. Assembly of Reagents in PCR Reactions

Tube	Water	Platinum II Taq MasterMix (2X)	Primer Set (10uM each primer)	Student DNA
1	5.5 µL	7.5µL	1 µL of HERC2-T	1 µL
2	5.5 µL	7.5µL	1 µL of HERC2-C	1 µL
3	5.5 µL	7.5µL	1 µL of OCA2-A	1 µL
4	5.5 µL	7.5µL	1 µL of OCA2-G	1 µL
5	5.5 µL	7.5µL	1 µL of SLC24A4-T	1 µL
6	5.5 µL	7.5µL	1 µL of SLC24A4-C	1 µL
7	5.5 µL	7.5µL	1 µL of SLC45A2-C	1 µL
8	5.5 µL	7.5µL	1 µL of SLC45A2-G	1 µL
9	5.5 µL	7.5µL	1 µL of TYR-T	1 µL
10	5.5 µL	7.5µL	1 µL of TYR-C	1 µL
11	5.5 µL	7.5µL	1 µL of IRF4-T	1 µL
12	5.5 µL	7.5µL	1 µL of IRF4-C	1 µL
13	5.5 µL	7.5µL	1 µL of water	1 µL

3. After all the reactions have been assembled, cap the tubes carefully making sure that you don't damage the sides of the thin-walled PCR tubes. Finger-flick the tubes to make sure that the reactions are mixed and then centrifuge to ensure all liquid is returned to the bottom of the tubes.

4. Place the PCR tubes into the thermal cyclers following the directions of the instructor. Note: If you are using strip tubes, you will need to separate Tubes 9 and 10 from the rest of the tubes as they will be placed in a different thermal cycler, or run at a later time, using different annealing temperature conditions outlined below.

5. Start the thermal cycler program preprogrammed by the instructor after closing the heated lead on the reaction tubes.
    1. Denaturation at 94°C for 2 minutes.
    2. 3 Cycles of:
       Denaturation: 94°C for 10 seconds;
       Annealing: 64°C for 10 seconds;
       For TYR Only: **67°C for 10 seconds;**
       Extension: 72°C for 30 seconds.
    3. 3 Cycles of:
       Denaturation: 94°C for 10 seconds;
       Annealing: 61°C for 10 seconds;
       For TYR Only: **64°C for 10 seconds;**
       Extension: 72°C for 30 seconds.
    4. 32 Cycles of:
       Denaturation: 94°C for 10 seconds;
       Annealing: 58°C for 10 seconds;
       For TYR Only: **61°C for 10 seconds;**
       Extension: 72°C for 30 seconds.
    5. Incubation: 72°C for 2 minutes, then cool to 4°C.

Part C. Gel Electrophoresis of PCR Amplicons

Caution: gloves should be worn whenever you may come in contact with ethidium bromide (EtBr).

1. Prepare 1 L of 1X LB media (enough for pouring agarose gel and electrophoresis running media).
- Add 50 mL 20X LB to a 1 L graduated cylinder.
- Fill to 1 L with deionized water.
- Cover the cylinder with parafilm and mix.

2. Prepare a 1% agarose/1X LB solution.
- Pour 110 mL of 1X LB into a plastic Erlenmeyer flask with screw cap.
- Add 1 g low-EEO agarose to the flask.
- Cover flask with screw cap ensuring the cap is VERY loose. Do NOT tighten down.
- Dissolve agarose according to the instructor’s microwave directions.
- Cool agarose solution-containing flask under cold running water with continual but gentle stirring.
- After the solution has cooled enough to hold in gloved hand (~50°C), add 3 µL of 10 mg/mL EtBr to the flask. Eject the tip into the EtBr solid waste container for disposal.
- Swirl solution gently to mix EtBr, minimizing bubbles.

3. Pour 1% agarose/1X LB gel.
- Place gel tray in the casting tray.
- Pour the 1% agarose/1X LB solution into the gel tray.
- Place a 15 well comb in the gel tray using the top slots.
- Wait 20 minutes for the gel to solidify before continuing.

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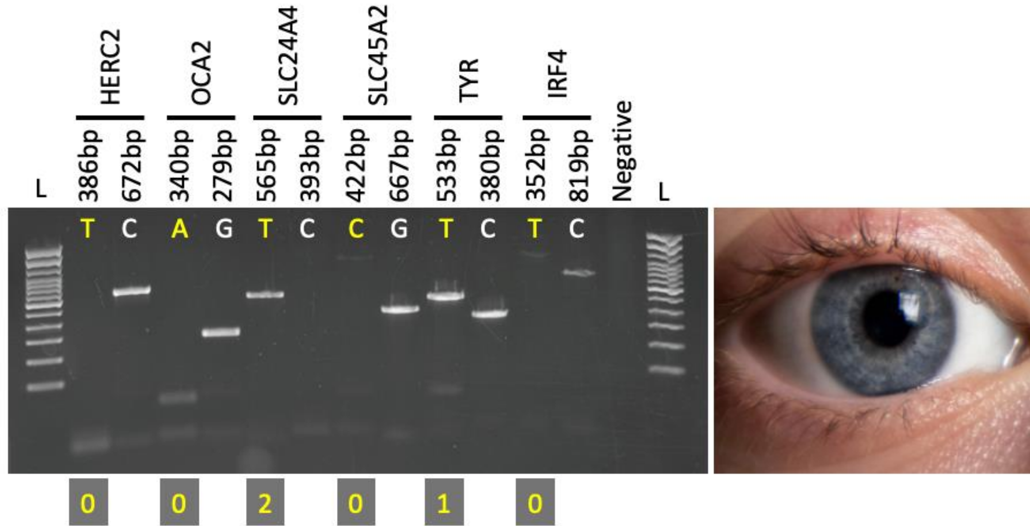
4. Load the gel with prepared samples.
   - Place the gel and gel tray with the wells oriented to the top (i.e., the black cathode side) of the gel rig. Pour the remaining 1X LB running buffer into the ends of the gel rig until the gel is covered by running buffer. Don't pour directly onto the gel. Now, carefully remove the comb so that the wells aren't damaged.
   - Add 3.7 µL of 5X loading media containing the orange G tracking dye to each PCR reaction you generated in Part B, mixing well.
   - Please look at Figure 2 in order to familiarize yourself with how the samples should be loaded into the gel. Using this guide, load your gel as follows: skip lane 1 of the gel (completely to the left) and then load 4 µL of each PCR reaction/1X loading media mix to the gel starting with lane 2 and continuing to lane 14. Be careful to not pierce the bottom of the well with your pipette tip; the bottoms are somewhat fragile. Then load 3 µL of the 100 bp DNA ladder into lanes 1 and 15.

A.



B.

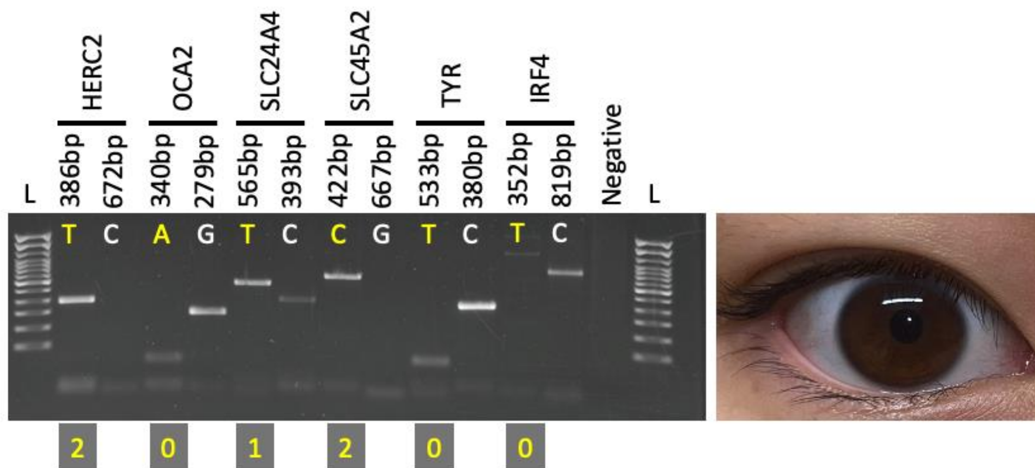


Figure 2. DNA loading order and example results. Each gene and the corresponding alleles should be amplified and loaded in the gel in the same order depicted here to aid in the analysis for eye color prediction later. The yellow nucleotides (top) and numbers (below) are the IrisPlex alleles of interest, and the number inherited by this individual, respectively. Representative A) blue-eyed and B) brown-eyed student results.

- Record the order you loaded your samples in your laboratory notebook. If you made a mistake in loading, or noticed anything unusual during this time, make sure to write these changes down in your laboratory notebook. Knowing what these “edits” were will be essential for your analysis later.

5. Running the agarose gel.
   - Connect the electrodes to a power source (i.e., red to the red; black to black) ensuring the gel within the rig is fully covered by the lid.
   - Run at a constant voltage of 200 volts (~20 volts/cm of gel length) for 30 minutes. You can follow the progress of the electrophoresis run by watching the orange G dye progress through the gel from the black cathode to the red anode. In terms of size, orange G runs through the gel at roughly where a 70bp piece of DNA would run.
   - Turn off the power supply, disconnect the leads and then remove the gel for evaluation. To do this, take the gel to the UV transilluminator (your instructor will show you where this is) and expose the gel to UV before taking a photograph to preserve the results. Save the image to your group’s folder before printing a copy that you should tape into your lab notebook.

Part D. Allele Determination

Using the photograph of your gel, determine for each gene/SNP which alleles you possess. To do this, start with Lane 2 and work your way to the right. Using the size marker on each side of your gel, determine if you have a product (band) in each lane at the CORRECT expected size. Please use Figure 2 for comparison. Mark your responses (either yes or no) in Table 2. If you answer “yes” for both alleles of a gene, you are heterozygous. If you answered “no” for one of the alleles, you are homozygous for the other allele (i.e., you inherited two of the same allele). If for any gene you answered “no” for both alleles, you will be unable to determine your allele status for that particular gene (NA). Complete the table to get “Your Answers” that you will use for the Eye Color Prediction in Part E.

Table 2. Summary of Student SNP Alleles for IrisPlex Prediction

Gene	Allele 1	Allele 2	Two Y’s	Two N’s?	Only allele 1 is Y	Only allele 2 is Y	Your Answers
HERC2	T	C					
Expected Size (bp)	386	672	Two Y’s	Two N’s?	Only “T” is Y	Only “C” is Y	
Band? Y or N			1	NA	2	0	
OCA2	A	G					
Expected Size (bp)	340	279	Two Y’s	Two N’s?	Only “A” is Y	Only “G” is Y	
Band? Y or N			1	NA	2	0	
SLC24A4	T	C					
Expected Size (bp)	565	393	Two Y’s	Two N’s?	Only “T” is Y	Only “C” is Y	
Band? Y or N			1	NA	2	0	
SLC45A2	C	G					
Expected Size (bp)	422	667	Two Y’s	Two N’s?	Only “C” is Y	Only “G” is Y	
Band? Y or N			1	NA	2	0	
TYR	T	C					
Expected Size (bp)	533	380	Two Y’s	Two N’s?	Only “T” is Y	Only “C” is Y	
Band? Y or N			1	NA	2	0	

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IRF4	T	C					
Expected Size (bp)	352	819	Two Y's	Two N's?	Only "T" is Y	Only "C" is Y	
Band? Y or N			1	NA	2	0	

Part E. Eye Color Prediction

Open up a browser on your computer and navigate to the following webpage: https://hirisplex.erasmusmc.nl/ This site has several prediction tools (Chaitanya et al., 2018, Walsh et al., 2014) for determining a variety of pigmentation phenotypes. We will only be using the eye color prediction system, IrisPlex, that is located approximately two thirds of the way to the bottom of the webpage. Using "Your Answers" from Table 2, click the corresponding allele number for each gene in the IrisPlex prediction tool (right below the brown eye), including NA for any gene that you weren't able to determine from your PCR results. Please note that the authors referred to SLC24A4 as LOC105370627 for this tool and at a minimum, you will need to have results for HERC2 for this prediction tool to work.
Now click, "Display Predicted Phenotype" to get the predicted probability of an eye color for a person with your exact set of alleles for these six SNPs. Figure 3 displays the outcomes for the two example students shown in Figure 2.

	Gene	SNP	Allele	No. of Alleles
1	*HERC2*	rs12913832	T	**0** 1 2 NA
2	*OCA2*	rs1800407	A	**0** 1 2 NA
3	*LOC105370627*	rs12896399	T	0 1 **2** NA
4	*SLC45A2*	rs16891982	C	**0** 1 2 NA
5	*TYR*	rs1393350	T	0 **1** 2 NA
6	*IRF4*	rs12203592	T	**0** 1 2 NA

Predicted phenotype	p-value	AUC Loss
blue eye	0.961	0
intermediate eye	0.029	0
brown eye	0.01	0

	Gene	SNP	Allele	No. of Alleles
1	*HERC2*	rs12913832	T	0 1 **2** NA
2	*OCA2*	rs1800407	A	**0** 1 2 NA
3	*LOC105370627*	rs12896399	T	0 **1** 2 NA
4	*SLC45A2*	rs16891982	C	0 1 **2** NA
5	*TYR*	rs1393350	T	**0** 1 2 NA
6	*IRF4*	rs12203592	T	**0** 1 2 NA

Predicted phenotype	p-value	AUC Loss
blue eye	0	0
intermediate eye	0.003	0
brown eye	0.997	0

Figure 3. Eye Color Prediction. The results of the IrisPlex prediction tool for eye color are displayed for the two example individuals shown in Figure 2. The eye color with the highest probability determines the eye color prediction, but to be reliable, this value must be above the 0.7 accuracy threshold as outlined by Walsh et al. (2012). The prediction here agrees with both individuals' eye colors as shown in Figure 2.

Discussion Questions

A. Prediction Tool and Individual Results:

1. Look at your eyes closely using a mirror or take a picture of your eyes and zoom into the photos to visualize the details of your irises.
    a. What colors do you see?
    b. Are the colors uniform across the iris?
    c. Do you have a "central ring" surrounding your pupil or any other unusual pigmentation features? When asked about the color or your eyes, do you include these features in what you say? Why/why not?

2. What did the IrisPlex prediction tool indicate was the most likely general color of your eyes?
    a. Does this agree with what you saw in your mirror/photo?
    b. Is this the same color you have listed on your driver's license/identification card?
    c. Does the distance away from you influence what an observer might say your eye color is?

3. Given the complexity of the details in your iris and the relatively small number of predicted colors from the Irisplex tool, what does this indicate about our overall understanding of how iris colors/patterns/details are generated?

B. Allele-specific PCR Methodology

4. What effect does one versus two mismatches towards the 3'end of the primer have on amplification?

5. Would a similar effect occur if the mismatches were towards the 5' end of the primer? Design a simple experiment to test your hypothesis.

6. Looking back at your gels. Did you get a product in the negative control (Lane 14)? If so, what might this mean about the results you obtained?

C. Molecular Biology

7. The central dogma, the flow of genetic information from DNA to RNA to Protein, is a fundamental principle in molecular biology.
    a. Where might the location of a SNP effect the first step (i.e., transcription) of this process?
    b. Consider ways in which a SNP could affect how a protein is translated.
        i. Does this SNP need to always occur in an exon sequence? Could it be in an intron?
        ii. Will all SNPs in an open reading frame (ORF) lead to an amino acid change in the protein? Why or why not?

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MATERIALS

Sherlock Tube Closures (USA scientific) – Cat # 1415-1508
Thermo Scientific Nunc 50mL Conical Centrifuge Tubes (Fisher Scientific) – Cat # 12-565-271
1.5mL microcentrifuge tube (Fisher Scientific) – Cat # 05-408-129
2.0mL graduated microtubes (GeneMate) – Cat # C-3261-1
0.2mL Snapstrip PCR Tubes with Dome Cap (GeneMate) – Cat # T-3035-2

Sterile Saline 0.9% NaCl (GeneMate) – Cat # MSDW-1000
DNA grade Water (Fisher Scientific) – Cat # BP2470-1
Chelex 100 Sodium form (Sigma) – Cat # C7901-50G
Platinum II Taq Hot-Start PCR Master Mix (2X) (Invitrogen) – Cat # 14000012
DNA oligo primers (Integrated DNA Technologies) - 25 nmole scale synthesis
Lithium Hydroxide (Amresco) – Cat # 0523-500G
Boric Acid (Fisher Scientific) – Cat # BP168-500
Orange G tracking dye (Sigma) – Cat # 861286-25G
Agarose (Fisher Scientific) – Cat # BP1356-500
100bp DNA Ladder (Invitrogen) – Cat # 15628050

NOTES FOR THE INSTRUCTOR

We feel that these experiments can be conducted in intermediate or high-level undergraduate classes and could be completed in four weeks with 3-4 hours of experimentation each week. Courses incorporating PCR and gel-electrophoresis techniques already, may be able to accelerate this timeline, while introductory undergraduate or even high school courses may need to simplify the procedure by reducing the number of genes (HERC2 analysis is required at a minimum) analyzed or taking more time to ensure the students are performing each of the tasks correctly. We believe there is a considerable amount of flexibility in how these experiments are presented to students and can be modified as needed to suit your laboratory schedule. Below is a tentative weekly schedule:

Week 1
- Introduction to DNA, PCR, and gel electrophoresis.
- Swish & Spit DNA Isolation – 45 minutes.
- Setup positive control PCR for student DNA isolation – 45 minutes.
- Run PCR overnight

Week 2
- Load positive control PCR on gels – 15 to 30 minutes.
- Run electrophoresis – 30 minutes to 1 hour.
- Introduction to Polygenic traits, specifically Eye Color.
- Take picture of gel and choose 1-2 student DNAs to continue for eye color analysis – 15 minutes.
- PCR set up for eye color determination – 1 hour.
- Run PCR overnight.

Week 3
- Load eye color PCR reactions on gels – 30 minutes.
- Run electrophoresis – 1 hour.
- Take picture of gel – 15 minutes.
- Determination of alleles for each gene – 30 minutes.

Week 4
- Introduction to IrisPlex eye color prediction.
- Utilize web prediction tool.
- Conclusions and discussion questions.

Setup time for instructors will depend upon the number of students/groups conducting the experiments. As a general guideline, we suggest that groups of three to four students would be optimal for the experiments outlined

here with two student DNAs analyzed (with different eye colors, if possible), but group size can be expanded or reduced depending upon desired learning outcomes and the available equipment in the laboratory. The most significant pre-laboratory task for instructors will be in aliquoting the PCR reagents, including the numerous allele-specific PCR primer set mixes (i.e., two for each of the six SNP loci, Appendix Table 1) for each group. While time can be saved by having primer set mixes shared between groups, we would highly advise against this to minimize contamination concerns.

If there are institutional restrictions in place that limit using student DNA directly, sequenced genomes can be purchased as homozygous or heterozygous DNA control samples. We have purchased DNA samples from the Coriell Institute for Medical Research for this purpose and have seen good results. Alternatively, donor DNA may be obtained from the instructors, or others volunteers, to create a panel of "suspects" with known eye color phenotypes.

The bioinformatics computer program, Geneious Prime (www.geneious.com) was utilized to evaluate the genomic context surrounding each of the six SNPs including the associated genes after downloading sequences from the Ensembl database (Cunningham et al. 2022). The Primer3 program (www.primer3.ut.ee) was used to design primers with the 3' terminal nucleotide of one primer residing exactly on the SNP site. A consistent mismatch was also introduced in this same primer at the 3rd base (2nd base for the SLC45A2 primers) from the 3' terminus, as demonstrated by Liu et al. (2012) to help to increase the specificity of the reaction. The companion primer for each reaction was determined by Primer3 with product size, primer length, and primer complexity taken into consideration. All of the primers used for this study and their sequences can be found in Appendix Table 2.

For gel electrophoresis, we use low molarity lithium borate (LB) solutions to prepare our agarose gels, along with the corresponding running and loading solutions, instead of traditional TAE/TBE buffers. LB media allow for higher voltages to be used during electrophoretic runs which results in faster separations of our PCR amplicons (Brody et al. 2004). This is particularly useful in classroom/laboratory settings where time is often at a premium.

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About the Authors

Athelia Gray graduated from Alma College with a B.S. in Biology and a B.A. in Dance in May, 2023. The bulk of the work on this project formed the basis for her thesis project, for which she was awarded honors in the Department of Biology. This Fall, Thea will be attending Thomas Jefferson University for her Master's degree in Human Genetics and Genetic Counseling.

Sophie Dech is a sophomore at Alma College with plans on completing a B.S. in Biology in May, 2026. While initially working on the eye color project, she has recently branched out on her own project evaluating metabolically relevant genetic loci with similar allele-specific PCR methodologies.

Tatym Plath graduated from Alma College with a B.S. in Biology in April, 2022. Despite working on several projects within the lab, she was vital in getting this project off the ground and training others as they started within the laboratory. Tatym is currently at Trine University working to obtain her Physician Assistant Master's degree.

Eric Calhoun has been instructing courses at Alma College since 2008 with a primary teaching focus on introductory and upper-level courses in cell biology, genetics, immunology, and cancer biology. Recently, Eric has expanded into interdisciplinary courses that discuss human biodiversity as well as medical and experimental abuses of disadvantaged populations.

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APPENDIX A

Table 1. Composition of Allele-specific Primer Set Mixes

Tube	Primer Set Mix Name	Allele-specific, Mismatch Primer	2ND PCR Primer	Product Size
1	HERC2-T	HERC2 (T) REV-MM	HERC2 FWD-S	386
2	HERC2-C	HERC2 (C) FWD-MM	HERC2 REV-L	672
3	OCA2-A	OCA2 (A) FWD-MM	OCA2 REV-L	340
4	OCA2-G	OCA2 (G) FWD-MM	OCA2 REV-S	279
5	SLC24A4-T	SLC24A4 (T) REV-MM	SLC24A4 FWD-L	565
6	SLC24A4-G	SLC24A4 (G) FWD-MM	SLC24A4 REV-S	393
7	SLC45A2-G	SLC45A2 (G) FWD-MM	SLC45A2 REV-L	422
8	SLC45A2-C	SLC45A2 (C) REV-MM	SLC45A2 FWD-S	667
9	TYR-A	TYR (A) FWD-MM	TYR REV-L	533
10	TYR-G	TYR (G) FWD-MM	TYR REV-S	380
11	IRF4-T	IRF4 (T) REV-MM	IRF4 FWD-S	352
12	IRF4-C	IRF4 (C) FWD-MM	IRF4 REV-L	819

Table 2. PCR Primer Sequences

	Primer Name	Primer Sequence
1	HERC2 (T) REV-MM	CCAGTTTCATTTGAGCATTGAA
2	HERC2 (C) FWD-MM	TAGCGTGCAGAACTTGATAC
3	OCA2 (A) FWD-MM	GGCATACCGGCTCTCTCA
4	OCA2 (G) FWD-MM	GGCATACCGGCTCTCTCG
5	SLC24A4 (T) REV-MM	ATCTGCTGTGACAAAGAAAA
6	SLC24A4 (G) FWD-MM	TTTAGGTCAGTATATTTTGAGG
7	SLC45A2 (G) FWD-MM	AAAACACGGAGTTGATGCGG
8	SLC45A2 (C) REV-MM	GTTGGATGTTGGGGCTCG
9	TYR (A) FWD-MM	CCTCAGTCCCTTCTCTGCAGCA
10	TYR (G) FWD-MM	CCTCAGTCCCTTCTCTGCAGCG
11	IRF4 (T) REV-MM	AAAAGTACCACAGGGGAATCTA
12	IRF4 (C) FWD-MM	ACTTTGGTGGGTAAAAGAAAGC
13	HERC2 FWD-S	GCCCACTTTTCTGTGGTACA
14	HERC2 REV-L	CAAGCTGGGGATAGCAAGAT
15	OCA2 REV-L	TTCTGCCACCAGCTTCTGAG
16	OCA2 REV-S	ACACGAGCTGGACTGGAATG
17	SLC24A4 FWD-L	CTAGGTGGAAGACAGGATGT
18	SLC24A4 REV-S	CCTCTGGATGTTCACAGTCTAA
19	SLC45A2 REV-L	CGATTGGCTGTAGCAGAAGA
20	SLC45A2 FWD-S	CACAGCCACCTGGACTTACA
21	TYR REV-L	CAGCATTGGTGAAGTTTTCTGC
22	TYR REV-S	GGTCTGTGGCCTGAGGAAAAGT
23	IRF4 FWD-S	ACATGGCCAGAGAACCACAG
24	IRF4 REV-L	TTGTGAACCTGCTAAAGGAGTG

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