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Are You a Hidden Heterozygote? Use of PCR to Genotype Brown vs. Blue-Eye-Color Alleles

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Alleles that differ by a single nucleotide polymorphism (SNP) can be detected and genotyped by PCR. The difference between blue vs. brown eyes is one such SNP and we have designed PCR primers to determine an individual student's eye color genotype. Using a simple procedure to isolate DNA from a cheek swab, followed by PCR and DNA gel electrophoresis, students are introduced to some common molecular biology procedures while being highly invested in learning the results of their experiment. This experiment can be conducted over two 1.5-2 hour lab periods and followed by primer design assignments to genotype other SNPs.

Keywords: PCR, forensics, eye-color

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Introduction

We begin with this exercise to familiarize students with DNA isolation, PCR and agarose gel electrophoresis before conducting more involved molecular biology experiments. Our course is a genetics lab course for upper level university students, but this particular exercise could be performed in first or second year lab courses as well. Student interest is high in this particular experiment because they get to test themselves. As we have very many brown-eyed students of Asian ancestry at our university, finding hidden blue alleles among these students is very intriguing to them. Because this is an introductory exercise, we do not assess the students on it. Also because some students may not want to test themselves, we allow students to test other volunteers.

This exercise can be conducted in two 1.5 to 2-hour lab periods: the first to isolate DNA and perform PCR, while the second lab period is when gel electrophoresis of the PCR products is performed. As we have two 3-hour lab periods each week, we conduct this experiment at the same time that students are working on some other experiments as well. For students who fail to get a result, we allow them to repeat the experiment if desired because the procedure is quick and easy and can be performed on the side while other experiments are ongoing.

Student Outline

Recently the mutation that causes blue eyes was discovered (Eiberg *et al*. 2008). Although more than one gene controls eye color there is a single gene which determines whether one has eyes in the brown/black range or blue/green/gray range. The polymorphism appears to be due to a SNP (single nucleotide polymorphism) in the upstream promoter/enhancer region of *OCA2*.

Brown: CATTAAATG
Blue: CATTAAGTG

A common PCR technique can be used to genotype a person's alleles. PCR primers can be designed which include the region of the SNP and preferentially bind to the brown vs. the blue allele. The site of the base pair change should be close to the 3' end of the primer. If the binding of the 3' end of a primer to the source DNA is not stable the PCR reaction will not proceed. In the primers below both of the forward primers will bind to the DNA around the site of the mutation. One primer is designed to bind preferentially to the brown sequence that contains an "A". The corresponding primer contains a "T" to bind to this "A". Another primer is designed which should bind preferentially to the blue sequence that contains a "G". The primer contains a "C" to bind to this "G". In addition to this single base pair difference a second deliberate mismatch is added into the primer sequence to make the binding less than perfect. With one single mismatch the DNA will still anneal depending on the temperature conditions. With two mismatches annealing will not occur (Fig. 1.).

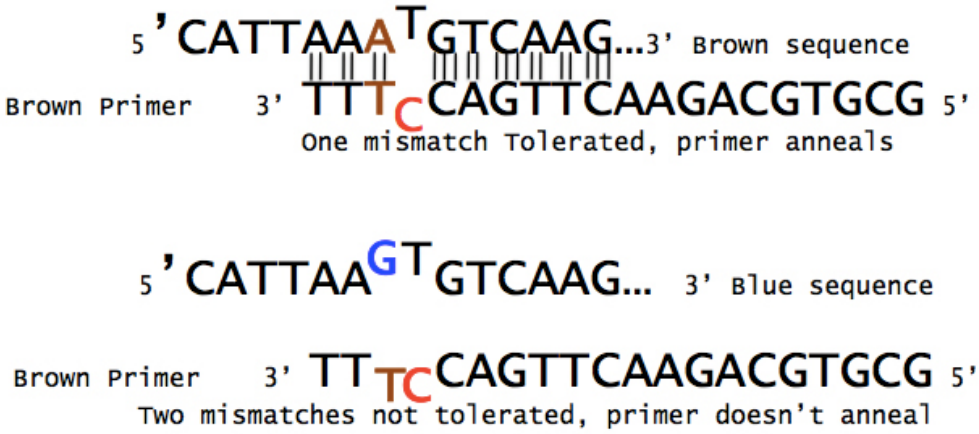

Figure 1. Diagram of the "brown" primer's ability to bind to DNA from brown-eyed individuals (top) vs. DNA from blue-eyed individuals (bottom).

By taking advantage of the differential specificity of the two primer sets to anneal to the DNA sequences should be homozygous for the blue mutation. If they only get a DNA fragment produced when using the brown primers, then the individual should be homozygous for the brown allele. If both PCR reactions produce products then the individuals should be heterozygous. As heterozygotes generally have brown eyes it could be of interest to learn that these individuals carry a blue allele and potentially could have blue-eyed children. When isolating DNA from a crime scene we routinely see that a series of PCR reactions are performed and the results are run through a database to see if the individual already exists in the DNA database commonly called "CODIS". As many individuals do not exist in that database one could still quickly learn the eye color of a suspect who left DNA at the crime scene.

Sequence of Primers:

Primer Set "brown"	-forward primer- 5'GCGTGCAGAA**C**tTGACCTTT3'
	-reverse primer- 5'AATTCAAAATGCCCCCAAGT3'
Primer Set "blue"	-forward primer- 5'GCGTGCAGAACTTGAC**C**cTT3'
	-reverse primer- 5'TGTGGTCACATGAGCTGTCC3'

*bold indicates mismatch position, while lowercase indicates SNP position

Note that the reverse primers for the two reactions have been chosen to be different from each other to cause fragments of distinct sizes to be amplified for each individual reaction. This is another control to differentiate between the two samples when loading and comparing them on a gel.

Experimental Procedure

DNA Extraction Steps

1) Swab the inside cheek of your mouth and air dry the swab at room temperature. Cut the tip of the swab (just some of the cotton off the end) into a 1.5 mL tube.
2) Add 20 µL of a 0.2 M NaOH solution into the tube, ensuring that the cotton is completely soaked.
3) Incubate the tube at 75°C for ten minutes. This can be done via water bath.
4) Remove the tube from incubation and add 180 µL of a 0.04 M Tris-HCl (pH 7.5) solution in order to stop the extraction.
5) Remove the fluid from the tube and transfer to another tube. Discard the remaining tube and cotton.
6) Store the tube with the extracted DNA in a fridge with temperature of 4°C or in a -20oC freezer.
*Above extraction method adapted from Rudbeck and Dissing (1998).

PCR Reactions

The blue and brown reaction mixes are made separately in different tubes:

"BLUE"	"BROWN"
2.5 µL of extracted DNA	2.5 µL of extracted DNA
2.0 µL 10X PCR buffer	2.0 µL 10X PCR buffer
1.0 µL forward blue primer	1.25 µL forward brown primer
1.0 µL reverse blue primer	1.25 µL reverse brown primer
4.0 µL 5mM MgCl2	4.0 µL 5mM MgCl2
0.5 µL dNTPs (10mM)	0.5 µL dNTPs (10mM)
8.6 µL dH2O	8.1µL dH2O
0.4 µL Taq polymerase	0.4 µL Taq polymerase
= 20 µL of solution per PCR tube	= 20 µL of solution per PCR tube

Note that not only are there separate primers for the blue versus brown reactions but we use different amounts of each (1 uL vs 1.25 uL) so that the final amount of water is also different. All primer concentrations are 25 pmol/uL.

PCR Conditions

1) 95°C for 3 minutes.
2) This next step will be repeated thirty times: Denature for 95°C for 30 seconds, Anneal at 56.1°C for 30 seconds, extend at 72°C for 45 seconds.
3) After thirty cycles, extension will be completed with 72°C for 6 minutes.
4) The PCR reaction is finished and the temperature will drop to 4°C indefinitely until it is retrieved from the machine.

Gel Electrophoresis

1) Make a 3% agarose gel using 10X SB buffer (Sodium Boric Acid from Brody and Kern (2004); Note that 1X SB buffer contains 5 mM disodium borate decahydrate) Add gel-red stain (from Biotium, a non-toxic replacement for ethidium bromide) into the gel to a concentration of 1/10 000 v/v (eg 40 ul of gel red for a 400 mL gel).
2) Add 2µL gel loading buffer e.g. Orange G to each PCR tube.
3) Pipet all of the PCR solution from each tube into the appropriate gel well. Each individual should have two wells representing themselves, one testing the products from the brown eye allele primers and one testing the products from the blue eye allele primers.
4) Observe the gel on the UV transilluminator with the cover in place.

Sample Student Results:

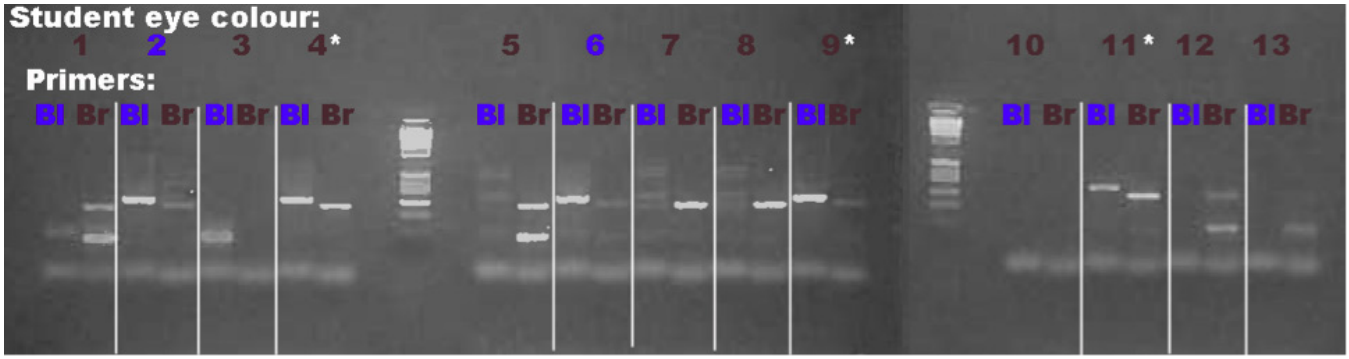

Figure 2. Student results for this PCR experiment. Student eye color is indicated by a blue or brown number at the top of the gel. Each individual had two sets of lanes where the blue (Bl) and brown (Br) primers were tested. Individuals 4, 9 and 11 were identified as heterozygotes.

Notes for the Instructor

1) Instruction on proper use of micropipets prior to this experiment is essential. Immediately following instruction, we suggest having groups compete for speed and accuracy in a series of skill-testing pipetting activities with a prize (e.g. winners have lowest pre-test scores dropped).

2) Have students complete a pre-test on both how PCR, primer annealing and primer design works, and ask about what controls are missing in this experiment (if a brown-eyed individual fails to have a blue PCR product, they still could be a heterozygote if their reaction failed).

3) As some students may not want to have their DNA tested, have "control" DNA from volunteers available e.g. yourself, TAs, or other interested individuals.

4) Follow up this exercise with a bioinformatics assignment where students find another SNP of interest (eg. in the dog genome database or in the genome of an organism used in the lab already) and have them design their own two sets of primers that could differentially amplify the two different alleles of the SNP in question.

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About the Authors

Jennifer Klenz is a Senior Instructor in the biology program at the University of British Columbia where she has been teaching for the past 15 years. She teaches an upper level genetics lab course that she created herself, as well as several large lecture classes in biology and genetics.

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