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Genetic Variations That May Increase Your Resistance to Malaria

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Most biology students are informed of the classic relationship between sickle cell anemia and resistance to malaria, an infection by a parasitic protist. However, most students are unaware of other genes that can confer resistance. This exercise explores how the minor alleles of four genes, IL-3, Duffy, PKLR, and G6PD have been shown to contribute to malaria resistance. Students genotype the class (anonymously) using PCR coupled with differential restriction enzyme digest followed by statistical analysis that compares local versus worldwide frequencies of the alleles (based on Hardy-Weinberg Equilibrium) and a discussion on the biochemical and physiologic basis for genetic resistance.

Keywords: SNP Genotypes, *Plasmodium*, IL-3, Duffy, PKLR, G6PD, Hardy-Weinberg

Introduction

There are about 3 million Single Nucleotide Polymorphisms (SNPs) in the human genome. Therefore, it can be calculated that about one in a thousand bases varies across the human population. While most of these single base pair variations have little to no effect on human phenotypes, some contribute to human disease, including the propensity to be infected by pathogens.

Malaria, an infection caused by protists from the genus *Plasmodium*, is the fifth leading cause of death worldwide. However, North Americans rarely consider the implications of this disease because of its low-prevalence in the local population. Therefore, while most biology students are informed of the classic relationship between sickle cell anemia and resistance to malaria, most are unaware of the multitude of other genes that can confer a level of resistance.

This exercise explores how minor alleles of four genes, IL-3, Duffy, PKLR, and G6PD have been shown to contribute to malaria resistance. These resistance alleles are not rare in a typical North American population. Students anonymously genotype the class for the frequency of the unique alleles using PCR coupled with differential restriction enzyme digest. They statistically compare the results with the results on HapMap and investigate how these genes could biochemically and physiologically affect susceptibility to malaria. Additionally, students can discuss why these genes do not follow the distribution pattern of sickle cell anemia as well as the important issues of environmental versus genetic resistance to parasites.

The exercise presented here in full is intended to take three weeks for upper level biology students with some experience in molecular biology methods. However, this can be tailored to the level of detail required; from simply analyzing provided data, to actually genotyping the class and using appropriate statistics. In addition, though not included in this current manuscript, the development of the genotyping method can be used as an exercise in bioinformatics. Depending on the extent of the exercise used, the methods contained can take anywhere from an hour-long activity to a three-week long laboratory module.

Although the methods have been tested thoroughly and simplified from the original design, the exercise does require that the instructor have some basic molecular genetics knowledge including the basics of DNA extraction, PCR, gel electrophoresis, and restriction enzyme digests. With this background knowledge, laboratory preparation will require mostly the dilution and aliquotting of readily acquired reagents as would be typical of other standard PCR-based activities.

The depth of the discussion of the exercise can also vary as per use of the activity. This would also depend on the breadth of knowledge of the instructor in the topics. However, an instructor with an appropriate background would not need much more specific knowledge than is presented in the review article by Lopez et al. (2010). Additional helpful information is provided in PowerPoint form for instructional use.

Student Outline

Genetic Variations That May Increase Your Resistance to Malaria

The general perception is that humans acquire parasitic diseases simply by coming into contact with the parasite. However, within human populations that are in constant contact with parasitic organisms, there is significant variation in levels of infection. It has been well documented that host organisms can vary in their susceptibility to parasites because of their genotype (Shaw and Quinnell 2009, Peacock 2010). Malaria is a human parasitic disease that has been thoroughly studied in this realm (Prudencio et al. 2007, Tibayrenc 2007, Weatherall 2008, Carpenter et al. 2009, Verra et al. 2009)

Malaria is a disease caused in humans by various species *Plasmodium*, a type of protist. Its life cycle is relatively complex and requires a mosquito as an intermediate host (Figure 1). Infection in the human stage requires that the parasite enter red blood cells and overtake the cellular mechanisms. However, many human genes can prevent this from happening (Lopez et al. 2010).

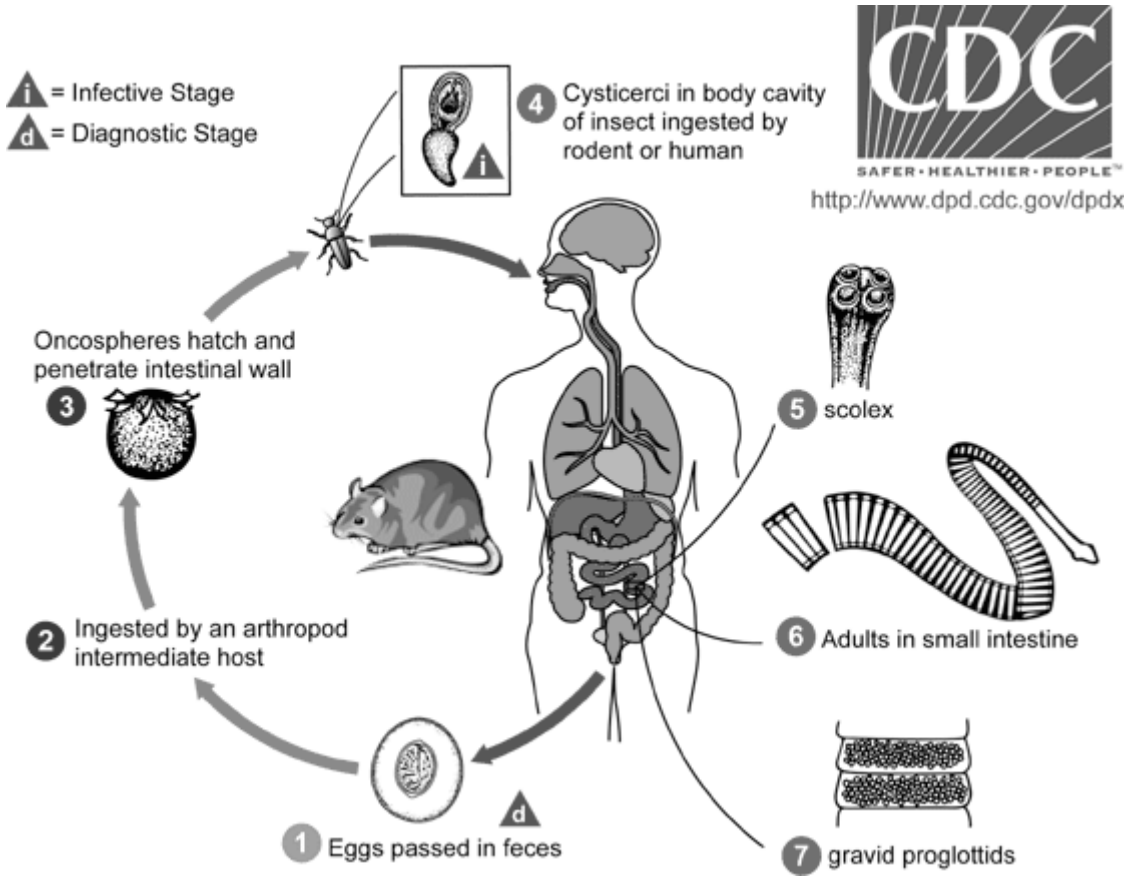

Figure 1. The life cycle of the malaria-causing *Plasmodium* genus. (From: **http://www.dpd.cdc.gov/DPDx/HTML/ImageLibrary/Malaria_il.htm**)

It only takes slight differences in the sequence of these genes to change the resulting proteins and make it difficult for the *Plasmodium* to manipulate our systems. In fact, many of the genetic differences that confer a degree of resistance to malaria are only a single base pair in the entire sequence, an occurrence known as a Single Nucleotide Polymorphism (SNP). In this lab exercise you will be responsible for genotyping the whole class for one of five SNPs (in four genes) as shown in Table 1 below.

Table 1. A summary of the SNPs examined in this exercise. Includes the gene name, the chromosome on which the gene is found (and the locus name if applicable), the size of the PCR product, the enzyme used to cut the PCR product and the band sizes expected after the restriction digest.

					Gel pattern	
Gene	**Chr.** (Locus)	**PCR product**	**Enzyme**	**Cuts**	**Wild type bp**	**Mutant bp**
IL3	**5**	477	HpyCH4IV	Mutant	477	249/228
Duffy	**1**	726	SfaNI	Wild	555/171	726
G6PD	**X**	701	EaeI	Wild	290/209/204	499/204
PKLR	**1** (1529A)	512	StyI	Mutant	512	275/237
	1 (1456T)	512	BsmAi	Wild	346/166	512

Generally the versions of the genes (alleles) responsible for resistance to malaria are most prevalent in areas where the disease is the most widespread. A classic example of this is the distribution of the sickle cell anemia gene which causes a terrible disease in the homozygous state, but confers malarial resistance in the heterozygous state. Since malaria is ubiquitous on the African continent, the sickle cell variation of the hemoglobin gene is selected in African populations. Therefore, the frequency of the mutant allele that causes sickle cell anemia is significantly more prevalent in African populations. However, not all malarial resistance genes predominate in Africans. Contrary to this expectation, the four SNPs chosen for this study are predicted to be quite common in Caucasian populations (Table 2).

Table 2. Allele frequencies for the SNPs in this exercise, if known.

			Frequency	
Gene	**Chr.** (Locus)	**PCR product**	**Major**	**Minor**
IL3	**5**	477	C 0.79	T 0.21
Duffy	**1**	726	A 0.517	G 0.483
G6PD	**X**	701	C unknown	G unknown
PKLR	**1** *1529A*	512	G unknown	A unknown
	1456T	512	C 0.992	T 0.008

Procedure

I. DNA Extraction (WEAR GLOVES!)

a. Obtain a collection swab for your instructor and label the casing with your name and date.

b. Rinse your mouth with water thoroughly.

c. Swab the inside of EACH cheek twenty times.

d. Immediately put the swab into the extraction solution in the screw-capped tube. Swish the swab in the solution at least five times and squeeze against the side before withdrawing the swab. DO NOT TOUCH the swab with your hands.

e. Tightly close the tube, vortex for 10 seconds.

f. Incubate for 10 minutes at 65°C.

g. Vortex again for 15 seconds.

h. Heat the tube in the 98°C for 2 minutes.

i. Vortex for 15 more seconds.

j. Quantify the DNA extraction using spectrophotometry with your instructor's help.

II. PCR(make sure you get the proper primer set)

a. In a 0.2 ml PCR tube, mix the following:

Sterile water	9.5 µl
GoTaq Master Mix	12.5 µl
Upstream primer (1:10)	0.5 µl
Downstream primer (1:10)	0.5 µl
DNA template (1:10)	2 .0 µl

Give your tube to your instructor to be put in the thermal cycler.

III. Flash Gel Check

a. In a fresh 0.2 ml tube mix 10 µl of your PCR sample with 2 µl of the loading dye.

b. Load 7 µl of this mix into the Lonza FlashGel cartridge.

c. Your instructor will start and photograph the gel.

IV.Restriction Enzyme Digest

a. In a 0.2 ml PCR tube, mix the following:

Sterile water	17 µl
10x FastDigest Buffer	2 µl
PCR product	10 µl
Enzyme	1 µl

b. Mix gently and spin down.

c. Incubate in the water block at the temperature appropriate for your enzyme.

V. Agarose Gel Electrophoresis

a. Create a 1.5% gel by mixing 1.5g of agarose for every 100 ml of 1x TBE

b. Microwave the mix to boiling, let cool until the flask can be handled.

c. Make sure the gel mold is set-up and the proper comb is added. Pour the agarose slowly into the mold and let set until solid.

d. Mix 10 ml of your restriction digest with 2 ml of the loading dye containing GRGReen.

e. Load 10 ml of this mix into the agarose gel.

f. Run the gel for 20-30 minutes @ 120V.

g. Document the gel with either UV or blue light.

Results and Discussion

1. Calculate the allele frequencies for your gene in our population using the principles of Hardy-Weinberg (and assuming our population is in equilibrium).
2. Using a Chi-squared test, determine if this frequency is different than what has been shown previously (see your instructor if this is not known).
3. Using Lopez et al. (2010) as a start, research your gene (hint: find and read OTHER sources). **Thoroughly** explain how your SNP could affect the likelihood that a human could contract malaria.
4. Discuss your personal level of resistance to malaria considering all of the class's data.
5. Discuss why the class should NOT rely completely on this data to determine if they will get malaria in the future (assuming they come into contact with a vector).

References

Carpenter, D., I. Rooth, A. Farnert, H. Abushama, R. J. Quinnell and M. A. Shaw. 2009. Genetics of susceptibility to malaria related phenotypes. *Infection, Genetics and Evolution*, 9: 97-103.

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Materials

DNA Extraction (for 25 students)

1. BuccalAmp extraction kit (Epicenter, #BQ0908SCR)
2. 4-8 Vortexers
3. Heat block at 65°C
4. Heat block at 98°C
5. 1-4 UV Spectrophotometers

PCR (for 25 students)

1. 25, 0.2 ml PCR tubes (USA Scientific, #1402-8120)
2. 8 sets of micropipettors with tips
3. 25 ml sterile water (Sigma, #95284)
4. 25 reactions of GoTaq Master Mix (Promega, M7133)
5. 10 nMoles of each primer set (Integrated DNA Technologies)

a. IL3
5'-CCG ACG CCT GCC CCA CAC CAC-3'
5'-GGG ATC CAC GGG CTG AGC TCT TAC CAT-3'

b. Duffy
5'-TCC CCT CCC ACC TGC CCC TCA AT-3'
5'-AAT GGC AAC AAG ACA AAG ATG GCA AGA CAG-3'

c. PKLR
5'-CTC GTT CAC CAC TTT CTT GC-3'
5'-GAG GCA AGG CCC TTT GAG TG-3'

d. G6PD
5'-GTC TTC TGG GTC AGG GAT-3'
5'-GGA GAA AGC TCT CTC TCC-3'

Restriction Digest (for 25 students)

1. Heat block at 37°C
2. 25, 0.2 ml tubes (USA Scientific, #1402-8120)
3. 25 ml sterile water (Sigma, #95284)
4. Restriction Enzymes (New England BioLabs)
   a. 10000U HpyCH4IV (#R0619S)
   b. 300U SfaNI (#R0172S)
   c. 3000U EaeI (#R0508S)
   d. 3000U StyI (#R0500S)
   e. 1000U BsmAI (#R0529S)

Gel Electrophoresis (for 25 students)

1. Agarose (Sigma, #A4718) (2 grams per 100 ml TBE Buffer)
2. 1 L 1X TBE Buffer
3. 25 ml GRGreen Loading Dye (LabGene Scientific, #IV-1034)
4. UV Light Source
5. 1 100bp Extended Ladder (Lonza #50327)

Flash Gel Electrophoresis (for 25 students)

1. FlashGel Starter Kit (Lonza # 57026)
2. 2-3 FlashGels (Lonza, #57067)
3. 1 FlashGel Ladder (Lonza, #57033)

Notes for the Instructor

1. Materials for this course including the PowerPoint file are found in my Drop Box. Simply email me at: **annyezerski@kings.edu** for access.
2. The student handouts are designed for a sophomore level course where it assumed that students have had previous experiences with the major techniques. Instructors may opt to include more introductory information.
   a. For basic background on molecular biology techniques, I recommend the DNA Learning Center: **http://www.dnalc.org**
   b. Here is a nice little video on SNPs: **http://biology-animations.blogspot.com/2008/02/snp-animation.html**
3. I do NOT include a background on the genes because in my course, the students are responsible for researching the function of their gene and how a SNP within that gene may affect resistance to malaria.
   a. I have included some background on each of the four genes in my PowerPoint.
   b. Lopez et al. (2010) is an excellent starting point for background information. I give the students this paper. A PDF of this article in included in my Drop Box.
   c. Briefly:
      i. **IL-3** (Interleukin 3) is involved in the differen tiation of white blood cells and therefore might affect how the immune system attacks the *Plasmodium* invader.

ii. **Duffy:** a common red blood cell receptor known to be used specifically by to gain entry into the cell.

iii. **G6PD** (Glucose-6 Phosphate Dehydrogenase) is a vital part of the glycolysis pathway and known to have a role in oxidative stress. One study suggested that people with G6PD deficiency tended to have a stronger response to *Plasmodium* infection.

iv. **PKLR** (Pyruvate Kinase; Liver, Red Blood Cell): minor allele provides less energy for liver and RBC's thus reducing the ability of the parasite to hijack this energy for DNA replication

4. Scheduling

   a. As a three-week module I recommend the following schedule:

      i. Week 1: Section I (DNA extraction and quantification) and Section II (PCR set-up. End with students giving samples to instructor for thermal cycling

      ii. Week 2: Section III (FlashGel Check) and Section IV (Restriction Digest). End with enzyme incubation.

      iii. Week 3: Section V (Agarose Gel Electrophoresis of RE digests). Data collection and analysis

      iv. *N.B. This can vary greatly when choosing FlashGels over standard gels or quicker restriction enzymes (i.e. FastDigest from Fermentas)*

5. Ethical issues

   a. Check with your university's Institutional Review Board (IRB) for their standards of DNA collection and use.

   b. Although IRB approval is technically not required in educational situations, the following recommendations are made:

   c. Have students label their sample immediately with a number that you provide. Make sure they record this, but never refer to the samples by anything other than sample number.

   d. Immediately destroy the DNA samples by autoclaving after lab unless you have the students complete an appropriate IRB form to keep their DNA.

   e. Although resistance to malaria has little meaning in the context of a North American population, it is vital to discuss that the results of this genotyping will not guarantee resistance to malaria in those countries that harbor an active population of *Plasmodium* species. Students should take the same precautions against malaria when visiting countries with malaria prevalence regardless of the outcome of their genotyping.

   f. This exercise could also serve as a basis for an activity on the ethics of DNA collection, genotyping, genetic disease resistance, etc.

6. Student micropipetting is notoriously inaccurate. Expect 50% success. I always collect the DNA and set-up the reactions on my own (which can be done using high-throughput methods that require little time), to serve as back-up data.

7. I usually sub-divide the class so that each sub-group genotypes one SNP using the DNA from the whole class

8. DNA extraction

   a. It is very important that the students understand the concepts and results of contamination, especially increase in apparent heterozygotes that result from cross-contamination.

   b. DNA can be frozen for future use. If frozen at -70°C it is useful almost indefinitely with this procedure (but refer to Ethics section).

   c. DNA can be quantified using UV spectrophotometry. Absorbance at 260 nm can be used for a rough quantification (Absorbance@260 x 50 x dilution factor = DNA concentration in µg/ml). Typically DNA should be diluted 1:100 before spectrophotometry if this extraction method is used.

   d. It is not necessary to dilute the DNA exactly for these procedures to work. However, a 1:10 dilution routinely works well.

9. PCR

   a. The PCR program takes about 2 hours on an MJ Research Thermocycler.

   b. Touchdown Program (please check with me since I am still optimizing the program).

      i. 94°C 3 minutes
      ii. 94°C 30 sec
      iii. 65°C 30 sec (-1°C each cycle)
      iv. 72°C 60 sec
      v. 12 cycles
      vi. 94°C 30 sec
      vii. 50°C 30 sec
      viii. 72°C 60 sec
      ix. 20 cycles
      x. Final 10 minute extension at 72°C

   c. You can use FlashGels at any time to check the progress of the PCRs, but keep in mind that they are much more expensive per gel than a standard agarose gel. (They are also "flashy" and impressive). **www.lonza.com**

10. Restriction Digest
    a. I get the enzymes from New England Biolabs, but Fermentas is producing more and more Fast-Digest versions of enzymes which take only five minutes. If time is an issue, it might be worth checking their website.
    b. All restriction enzymes have an incubation temperature of 37°C with an inactivation of 65°C with the exception of BsmAI which uses 55°C incubation and 80°C inactivation.
11. Gels
    a. FlashGels are fast but expensive. Althernatively, a standard agarose gel method can be used.
    b. A 2.0% agarose gel is best for scoring but will take over an hour to run.
    c. Gels can be stained with any standard stain including Ethidium bromide. I recommend GRGreen because of its sensitivity, cost, and low risk.
    d. A quicker gel using SB buffer instead of TBE can be used in the case of time constraints.
12. Scoring
    a. I prefer to give all students the complete data set. That number is around 100 for our classes.
13. Bioinformatics
    a. I personally designed all genotyping methods for this exercise using the LaserGene suite of programs from DNAStar.
        i. These are serious research-grade programs that are very expensive. An educational version was available but had to be renewed and reinstalled each year.
        ii. Alternatively, if you want the students to do their own genotyping design, programs from NCBI are available for free: **http://www.ncbi.nlm.nih.gov/guide/data-software/#howto_**
        iii. If you are interested, I can work with you on a Bioinformatics exercise.
14. Statistics
    a. As an exercise in allele frequencies, students can compare their results to known results from HAPMAP (The international SNP Database)
        i. Data can be found at HAPMAP itself: **http://hapmap.ncbi.nlm.nih.gov/** but NCBI's SNP database is easier and clearer to use: **http://www.ncbi.nlm.nih.gov/snp**
    b. The frequencies in this exercise are based on the standard European population (CEU) because it best represents my student population.

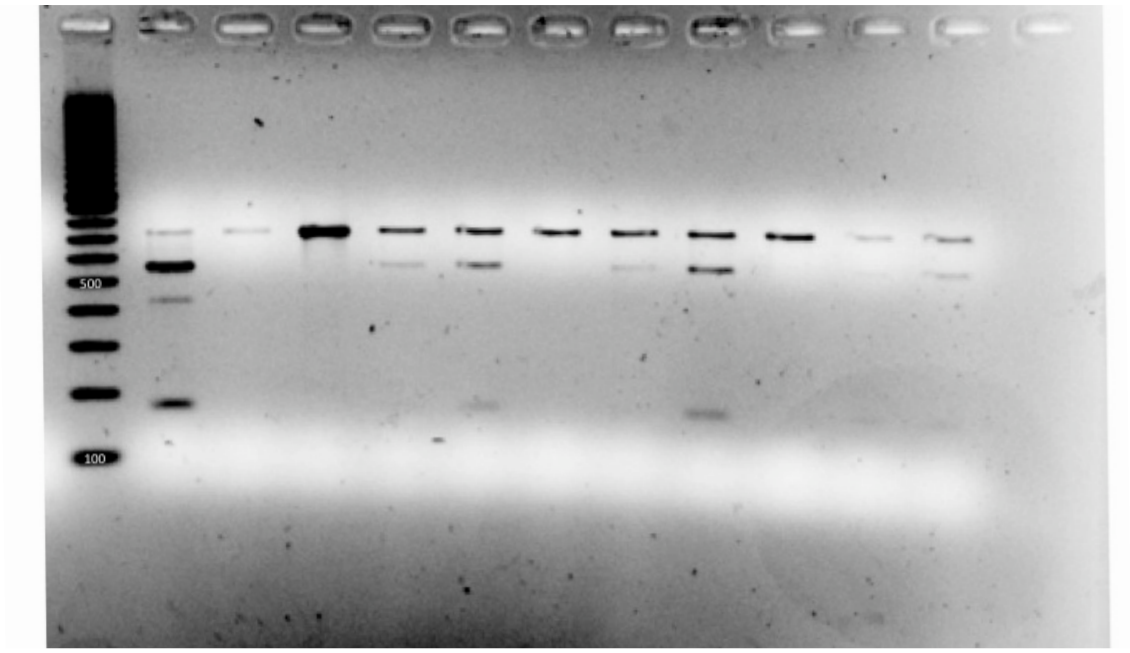

Figure 2. Amplified Duffy gene cut with SfaNI which cuts the wild type and does not cut the mutant. First lane is Extended 100bp Ladder (Lonza). Scored bands are at 726 (uncut), 555 and 171 (cut).

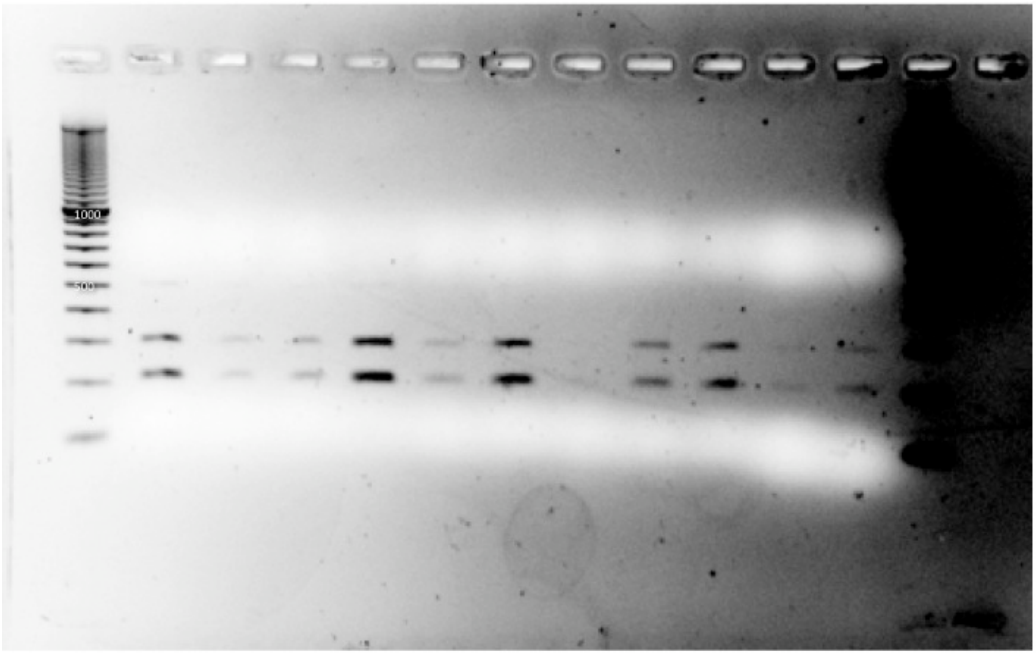

Figure 3. Results from the G6PD amplification and digest with EaeI. PCR product in 701 bp, but the enzyme cuts the wild type into three fragments at 290, 209, and 204 bp while the mutant allele is cut into only two fragments at 499 and 204. Lanes 2 and 5 show a faint band at 499 indicating one copy of the mutant allele and thus a heterozygous genotype. No homozygous mutants are seen, but the actual allele frequency for this SNP is not known.

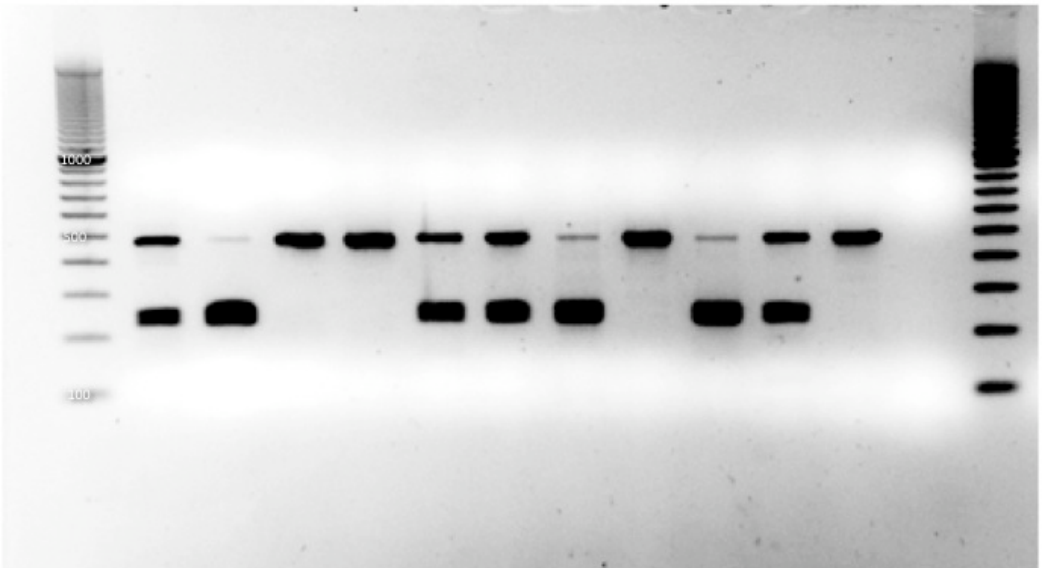

Figure 4. Results of the IL-3 PCR and digest with HpyCH4IV. Wild type is uncut and shows a band at 477 bp. The mutant is cut and shows two bands very close to each other at 249 bp and 228 bp. No homozygous mutants are seen, but the mutant allele has a frequency of about 21% in the population.

i. You may want to use frequencies from other populations if your student population is not primarily from European descent.

ii. Both databases also provide frequencies for other populations (i.e. Chinese, Japanese, African) if available.

iii. Some frequencies for these particular SNPs do not yet have adequate information. In these cases, you can assume equal allele frequencies in order to complete the following exercise.

c. Comparison is done via a Chi-Square statistic using COUNTS (not percentages) of the genotypes in the populations.

i. Observed counts are the actual genotypes determined by the above exercise

ii. Expected counts are found by multiplying HAPMAP frequencies by the class sample size

d. Do not be surprised if you class is significantly different from HAPMAP. Some SNPs do not have a lot of population data yet.

Sample Results

Results of PCR followed by restriction digest for the four genes of interest run on a 2% agarose gel with TBE buffer. Images are of the photonegative for better viewing and less toner use when printing.

Acknowledgements

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About the Author

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