Population Screening for Missense Mutations in the HFE Gene Associated with Hereditary Hemochromatosis

by
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"When I look at your heavens, the work of your fingers, the moon and the stars, which you have set in place, what is man that you are mindful of him, the son of man that you care for him?" – Psalm 8:3-4 (ESV).

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Abstract

Hereditary hemochromatosis, one of the most common genetic disorders in individuals of Northern European descent, is associated with missense mutations C282Y, H63D, and S65C in the *HFE* gene found on chromosome 6. An autosomal recessive disease affecting the metabolism of iron, hemochromatosis can result in serious complications such as cirrhosis of the liver, liver cancer, diabetes, heart problems, persistent abdominal pain, and extreme fatigue. If detected early, symptoms of hemochromatosis are preventable and treatable by phlebotomy, making hemochromatosis an attractive candidate for population screening. Genetic testing can identify high-risk individuals, allowing them to make informed decisions about preventative measures.

A goal of this project was to develop a reliable genetic test for three mutations (C282Y, H63D, and S65C) associated with hereditary hemochromatosis in order to screen volunteers from Houghton College, a Christian liberal arts college in western New York. Following DNA isolation from epithelial cheek cells of 132 volunteers, these single nucleotide polymorphisms (SNPs) were tested for using a tetra-primer amplification refractory mutation system (ARMS PCR), followed by fragment analysis on an ABI Prism 310 Genetic Analyzer. Genotyping for each SNP revealed a carrier rate of 13.2% for C282Y, 26.6% for H63D, and 1.6% for S65C. Compound heterozygotes, C282Y/H63D and C282Y/S65C, were also represented in the population at 1.6% and 0.78%, respectively. These genotypic frequencies are comparable to published values for Caucasian populations, but since this is a small, non-random population, the observed genotypic frequencies cannot be extrapolated to represent any other population.

An additional aim of this project was to investigate the bioethical implications of population screening. Although initially considered a “poster-child” for genetic testing, recent

research on the disease has cast a negative light on suggestions regarding the implementation of wide-scale genetic screening. Reduced penetrance and variable expressivity, as well as diet, age, and gender, significantly impact the expression of the mutant phenotype. Thus, bioethicists question if the benefits of population screening for this disease outweigh the risks of discrimination and undue anxiety.

In this study bioethical perspectives on genetic testing were surveyed, motivated by a desire to educate the campus about genetic diseases and especially the bioethical issues at stake in genetic testing. Survey data of 105 participants indicate overall optimism regarding the promise of genetic testing, mixed with hesitance in the application of genetic testing technologies, as well as a need for further education on genetic testing issues.

Introduction

Modern genetics is transforming medical care. While today's medicine focuses on treating preexisting chronic conditions, preventative medicine envisions a future where physicians will be able to predict and prevent these diseases long before they inflict suffering.[1,2] Genomic medicine may be one avenue to reach these commendable goals. Following in the footsteps of the Human Genome Project, the search to identify the function of each gene is yielding results that allow scientists to correlate gene variants with medical conditions.[1] For the first time in history, genetic testing for a multitude of conditions is available, allowing individuals to glimpse a faint image of their genetic future.

Genetic testing, as defined by the National Institutes of Health's 1997 Genetic Testing Task Force report, is "the analysis of human DNA, RNA, chromosomes, proteins, and certain metabolites in order to detect heritable disease-related genotypes, mutations, phenotypes or karyotypes for clinical purposes."[3] By this definition, genetic testing includes not only DNA-based testing, but any testing that could indicate inherited abnormalities.

In current practice, this testing is used in carrier screening, prenatal testing, neonatal screening, and confirmation of medical diagnoses. For example, chorionic villus sampling (CVS) or amniocentesis can be used to obtain genetic material for testing while still in utero.[4] Moreover, newborn screening programs have also been implemented in every state with required tests for cystic fibrosis, phenylketonuria, sickle cell anemia, maple syrup urine disease, and galactosemia to name a few.[5] Often just a few drops of blood obtained from the heel of the infant and placed on a Guthrie card are sufficient for a wide array of tests. Newborn screening is considered a life-saving practice as many symptoms of these diseases can be prevented with early detection and proper treatment.

Phenylketonuria (PKU) is a prime example of the value of newborn screening. PKU is an autosomal recessive disease in which the amino acid phenylalanine cannot be broken down, resulting in a toxic build-up that causes damage to the central nervous system.[6] However, if detected at birth, these symptoms can be avoided merely by restricting phenylalanine in the diet.[6]

Testing for some adult-onset conditions is also possible, such as in the cases of Huntington's disease or familial breast cancer associated with *BRCA1* and *BRCA2* mutations. Huntington's disease is a progressive neurodegenerative autosomal dominant disease that usually strikes an individual during his or her third or fourth decade of life.[7] With a 50% chance of inheriting this disease from their parent, children of Huntington's patients often want to know if they carry the mutant allele for Huntington's. Yet since no cure is available for this disease, genetic testing provides no clear preventative advantage. In contrast, individuals found to carry mutations associated with familial breast cancer do have some preventative options. Frequent screening or removal of breast tissue can minimize the risk of cancer.[8] However, it must be noted that not all individuals with BRCA1 or BRCA2 mutations will develop breast cancer. The lifetime risk of breast cancer associated with these mutations has been estimated to range from 26 to 85% penetrance.[8]

Genetic testing has also been linked with the concept of personalized medicine.[9] The goal of personalized medicine is to treat each patient and his or her disease as unique, tailoring treatment to the individual's molecular profile.[9] In today's medical practice, medications are generally prescribed with a "one drugs fits all" mentality.[10] In reality, many genetic factors play a role in drug response, in addition to the effects of age, nutrition, and environment. As of 1998 adverse drug reactions (ADR) were about the fifth leading cause of death in the United States.[11] If scientists better understood the molecular basis of why some patients respond positively to

drug therapy when others are unaffected or worse yet, experience life-threatening reactions, it would be possible to adapt individual medical care appropriately. Genetic testing can play a role here, helping physicians determine how gene variants correlate with drug reactions. For example, the chemotherapy drugs 6-mercaptopurine, 6-thioguanine, and azathioprine can cause severe toxicity and death in cancer patients who encode nonfunctional variants of a protein called thiopurine methyltransferase (TPMT). Thus, many cancer treatment centers now attempt to identify through genotyping the 0.33% of cancer patients who are unable to process these drugs.[10]

Understanding the prevalence of gene variants that affect how drugs are metabolized can also play a vital role in drug development. Scientists need to consider if the drug target in question is known to significantly vary amongst individuals or ethnic groups. For example, the cytochrome P450 enzymes that are responsible for detoxification and elimination of drugs from the body provide an illustration of the important variance between patients. Small differences in the family of P450 genes can dramatically affect the ability of the human body to process drugs. In 6 to 10% of Caucasians, homozygosity for a nonfunctional CYP2D6 allele of a P450 gene impairs the metabolism of many drugs such as debrisoquine and metoprolol that are used to treat hypertension. Similarly, the CYP2C19 variant that affects 2 to 5% of Caucasians produces a truncated nonfunctional protein, generating sensitivity to omeprazole (for the treatment of ulcers) and diazepam (for anxiety).[10] These are just a few examples of the many genes that are known to impact how a patient responds to a medication. The search for gene variants that impact drug metabolism is not an easy task, but an essential one to protect patients from harmful side-effects.

It is evident that genomic medicine has much to offer, but numerous limitations – scientific, legal, social, and ethical objections – impede the implementation of genetic testing.

First of all, in some ways, genomics is still in its infancy. The central dogma of genetics seems so simple: DNA is transcribed to RNA, which is translated to protein. But the actual relationship of genotype to phenotype is far from simple! Correlating a certain genotype with clinical symptoms is not an easy task because genotype does not always "equal" phenotype. For example, epigenetic modifications can turn genes "on" or "off", thus affecting phenotype.[12] Alternative splicing, a process by which different RNAs are produced from the same DNA sequence, can also impact the expected gene expression. In humans, an intriguing example of this is in the *bcl-x* gene, in which alternative splicing can create two different mRNAs that actually have antagonistic functions; one form initiates cell death, while the alternative form inhibits it.[13]

Variable expressivity and incomplete penetrance likewise influence gene expression. Variable expressivity is the idea that a certain gene may manifest itself in slightly different ways in each affected individual. While one patient may have severe symptoms, another patient with the same mutation may have only mild symptoms. Incomplete (or reduced) penetrance is a related concept, where one individual with a mutation may experience no negative side-effects, while another individual with the same mutation may have symptoms. Ultimately, this variation is likely due to the interaction of environment and/or other genes modifying the phenotype.

Unfortunately, the majority of diseases, such as cardiovascular disease, type II diabetes, hypertension, and Alzheimer's, are not simple monogenic diseases; such diseases are termed complex or multifactorial, meaning they are caused by mutations in more than one gene and/or environmental factors.[14] The effects of diet, age, and environment cannot be considered negligible here. Genetic testing for such conditions is usually without merit because innumerable factors can influence the clinical manifestation of a complex disease. In such a

case, genetic testing can only offer a hint of whether an individual may have a predisposition toward developing the condition. The glimpse of the future offered by genetic testing is often only a blurry view.

Social and ethical objections also hinder the implementation of wide scale genetic testing. Many fear the dangers of both false positives and false negatives. For example, if an individual incorrectly tested positive for Huntington's disease, she might spend the rest of her life wondering when the disease would strike, causing unnecessary anxiety and psychological harm. Or consider genetic testing for cystic fibrosis: a couple may be tested before conceiving to determine if they are carriers of cystic fibrosis. The wife may know she carries a mutation, while the husband tests negative for any identifiable mutations. They are relieved until they discover their newborn has cystic fibrosis. How is this possible? It is essential to understand genetic testing usually does not identify *every* mutant allele. The husband in this case may have carried an extremely rare mutation that was not detected by the test, but was passed to their child.

Furthermore, although mutations are usually considered deleterious, this is not universally true. Mutations can confer a protective advantage against certain pathogens or other environmental factors. For example, it has been hypothesized that cystic fibrosis (CF) mutations are relatively common because in the heterozygous form, CF mutations provide a protective advantage against pathogens such as *Vibrio cholerae* or *Escherichia coli*, which produce toxins that can induce diarrhea and subsequent dehydration.[15] Likewise, it is thought that hemochromatosis mutations are so common because at one point in history, excessive iron absorption would have been favorable to prevent anemia. Genetic testing may be able to determine if a mutation is present, but it cannot definitively say that the mutation is disease-causing in nature.

Discrimination based on genetic test results is also feared. A 1999 study by the Secretary's Advisory Committee on Genetic Testing discovered the greatest public concerns regarding genetic testing involved discrimination in employment and insurance coverage.[2] Countless stories can be narrated about cases of genetic discrimination: couples that lost their insurance when they decided not to abort a diseased child, individuals denied the option to adopt because of a genetic condition, or loss of an employment opportunity when a genetic condition was revealed.[16] However, as of 2008 with President George W. Bush's signature on the Genetic Information Nondiscrimination Act (GINA), this will hopefully be a problem of the past. Under this law, it is illegal to discriminate for health insurance or employment based on genetic information.[17]

Concerns about eugenics also slow the endorsement of genetic testing. Historically, the eugenics movement is not foreign to the United States.[18] In Justice Oliver Wendell Holmes' notorious 1927 statement justifying forced sterilization, he declared, "three generations of imbeciles are enough."[19] Prior to this, Indiana was the first state in 1907 to allow for involuntary sterilization for the ''prevention of the procreation of 'confirmed criminals, idiots, imbeciles, and rapists."[20] Unfortunately, these were not isolated cases, but widespread policies reflecting a desire to improve society through eugenics as it was thought that these traits were genetically determined. In modern times, it has been alleged that preimplantation genetic diagnosis and prenatal testing are also forms of a "new eugenics."[21, 22] By not implanting embryos or aborting fetuses that carry known genetic mutations, parents have more control over the genetic makeup of their offspring than ever before.

In light of the above limitations and considerations, it is natural to question when population screening is appropriate. Ten criteria formulated by Gunner Jungner and James

Wilson in 1968 have often been considered the “gold standard” in such decisions.[23] These criteria are displayed in Table 1.

Table 1: 1968 Jungner and Wilson Criteria for Population Screening from WHO report *Principles and practice of screening for disease*[23]

1. **The condition sought should be an important health problem.**
2. **There should be an accepted treatment for patients with recognized disease.**
3. **Facilities for diagnosis and treatment should be available.**
4. **There should be a recognizable latent or early symptomatic stage.**
5. **There should be a suitable test or examination.**
6. **The test should be acceptable to the population.**
7. **The natural history of the condition, including development from latent to declared disease, should be adequately understood.**
8. **There should be an agreed policy on whom to treat as patients.**
9. **The cost of case-finding (including diagnosis and treatment of patients diagnosed) should be economically balanced in relation to possible expenditure on medical care as a whole.**
10. **Case-finding should be a continuing process and not a “once and for all” project.**

Hereditary hemochromatosis, one of the most common genetic diseases in individuals of Northern European descent, is a condition for which population screening has been controversial. At first glance, hemochromatosis is an immensely attractive candidate for genetic testing.[24] An autosomal recessive disease associated with mutations in the *HFE* gene found on chromosome 6, hemochromatosis is characterized by iron overload due to an inability of the HFE protein to

properly interact with the transferrin iron receptor to regulate the uptake of iron into the cell.[25] HFE also functions as an iron sensor in the body that acts to regulate the expression of protein hepcidin that is essential to proper iron metabolism. The missense mutations C282Y, H63D, and S65C each cause a change of one amino acid in the HFE protein, interfering with the protein's regulatory functions, eventually causing iron build-up in the body. With early detection, serious symptoms such as liver cirrhosis, liver cancer, diabetes, heart problems, persistent abdominal pain, and extreme fatigue are easily prevented by regular phlebotomy. Unfortunately, due to the mild nature of early symptoms, hemochromatosis is often not diagnosed until organ damage has occurred.[26] The primary mutation implicated in hereditary hemochromatosis is C282Y, for which about 83% of hemochromatosis patients are homozygous.[27] About 1 in 10 Caucasians carry the C282Y mutation.[27] Compound heterozygotes (C282Y/H63D or C282Y/S65C), H63D homozygotes, and S65C homozygotes are considered less clinically significant than C282Y and are usually associated with milder forms of hemochromatosis.[28, 29]

Genetic testing can identify individuals who carry these mutations associated with hemochromatosis, but can only assert that these individuals are *predisposed* to hereditary hemochromatosis. This disease shows a wide range of expressivity and is considered to have low penetrance, especially in premenopausal women.[24] It has been estimated that of C282Y homozygotes only about 28.4% of males and 1.2% of females will develop symptoms of iron overload.[30] Environmental factors and other modifier genes have also been implicated in the expression of the mutant phenotype, thus further complicating the simple genotype-equals-phenotype scenario. However, genetic testing can alert high-risk individuals to their status, thus facilitating early clinical detection, and prevention of symptoms.

Thus far, based on Jungner and Wilson's criteria for population screening (Table 1), widespread implementation of hemochromatosis screening has been rejected on the basis of its low penetrance, controversy over the natural history of the disease, and how to best treat asymptomatic individuals who test positive for mutations. Fears about the psychosocial impact of testing for mutations that have such a low penetrance are especially of concern.[2, 8]

The purpose of the following study was to develop a relatively simple and reliable genetic test for three missense mutations (C282Y, H63D, and S65C) associated with hereditary hemochromatosis using tetra-primer ARMS PCR followed by fragment analysis using capillary electrophoresis, while surveying attitudes on the Houghton College campus regarding genetic testing for this condition. As Houghton College is 94% Caucasian,[31] the rate of carriers for each mutation should be comparable to literature values for white populations; in studies of Caucasians, about 10% carry C282Y,[27] 24% carry H63D,[32] and 2-3% carry S65C.[33]

Literature Review

Historical View

The genetic disease hemochromatosis was first described in 1865 by the French doctor Armand Trousseau who noticed a link between liver cirrhosis, diabetes, and bronze pigmentation of the skin.[34] In 1889 the German pathologist Friedrich Daniel von Recklinghausen discovered that the cause of the bronze pigmentation was in fact iron. Believing that iron originated from the blood, he termed the condition "haemochromatose." The next breakthrough in the study of hemochromatosis came in 1935 when a scientist at the University of Birmingham hypothesized that hemochromatosis was an inherited disease, caused by an inborn error of iron metabolism.[34]

However, it was not until 1996, when John Feder and a team of scientists mapped the causative mutation to a gene found on the short arm of chromosome 6, that the genetic basis of hemochromatosis would be established.[27] This gene, initially called the *HLA-H* gene (now the *HFE* gene) is about 13 kilobases in size and contains seven exons that code for a protein 343 amino acids in length. Feder et al. also identified two missense mutations in the *HFE* gene that are associated with hereditary hemochromatosis.[27] A missense mutation occurs when a single nucleotide is substituted for a different nucleotide, resulting in a change in the amino acid sequence. The first and most clinically relevant mutation, found on 85% of patient chromosomes and abbreviated as C282Y, changes a cysteine amino acid (abbreviated "C") for a tyrosine (abbreviated "Y") at position 282 of the protein.[27] Feder et al. found that in 178 hemochromatosis patients, 83% of probands were homozygous for this mutation, thus supporting the hypothesized role of the *HFE* gene in hemochromatosis.[27] Among Caucasians, C282Y has a carrier frequency of about 1 in 10, but only 1 in 200 to 500 individuals is homozygous for this mutation.[32] A second mutation was also discovered that mutates a histidine amino acid to an

aspartate at position 63 of protein. As this mutation is carried by about 24% of the general Caucasian population, Feder et al. were not convinced of the clinical role of this mutation. It is currently believed that the H63D mutation's effect is minimal except when combined with other mutations.[35]

In the years following the discovery of the *HFE* gene, it has become apparent that the penetrance (the percentage of individual carrying the mutation who develop symptoms) of the C282Y mutation is lower than initially estimated. Among males the penetrance of the C282Y homozygous genotype has been estimated to be as low as 2%[36] to as high as 28.4%.[30] Studies indicate that compound heterozygotes for C282Y/H63D have a reduced risk of disease with only about 0.5-2.0% of individuals ever developing symptoms; H63D homozygotes are suspected to have an even lower penetrance.[37] A third mutation, S65C, which converts a serine to a cysteine, has been implicated in mild cases of hemochromatosis, but the penetrance of this mutation has been difficult to ascertain due to its rarity, being carried by only 2-3% of the population.[29] It should be acknowledged that the statistics surrounding these studies of penetrance are controversial. Difficulties defining what constitutes "iron-overload" have led to divergence in diagnostic criteria and inclusion criteria in the above population studies, resulting in confusion regarding the true penetrance of the disease.[24] Additionally, since hemochromatosis is considered an adult-onset disease, symptoms typically manifest after age 40; longitudinal studies are needed to assess if asymptomatic C282Y homozygotes will remain disease-free. Regarding the penetrance of hemochromatosis mutations, it is also essential to note that men are at greater risk than premenopausal women of developing symptoms, as menstruation helps manage iron levels.[30]

After the novel discovery of the *HFE* gene associated with hemochromatosis, the search for genes associated with hemochromatosis did not cease. With the low penetrance of observed mutations and the realization that not all hemochromatosis-like conditions could be linked to *HFE,* the status of hemochromatosis as a monogenic disease was brought into question.[38] Although *HFE*-related hemochromatosis is the most common form, other genes have been shown to cause hemochromatosis (see Table 2). In the first years of the 21st century, these rare forms were intensely studied in the hope of better understanding iron metabolism and the reduced penetrance of hemochromatosis. It has been suggested that variants of these genes or other related genes act as modifier genes, impacting phenotypic expression of the *HFE* genotype.[38] Current classification systems list at least five distinct types of inherited hemochromatosis, each associated with different genes that produce varying clinical presentations. Only the most common form, type I hereditary hemochromatosis, associated with mutations in the *HFE* gene will be addressed in this paper. For a summary of non-*HFE* hemochromatosis, see Table 2.

Secondary iron overload imitating hemochromatosis can also occur. Liver diseases such as alcohol-related liver disease and hepatitis may cause iron-overload, as can the ingestion of too much iron through diet, iron supplements, or medications.[37]

Classification	Gene	Protein	Mode of Inheritance	Clinical Presentation	OMIM Accession ID
Juvenile Hemochromatosis (Type 2A)	*HJV*	Hemojuvelin	Autosomal recessive	• Earlier age of onset (before age 30) • More severe form • Affects both genders	602390
Juvenile Hemochromatosis (Type 2B)	*HAMP*	Hepcidin	Autosomal recessive	• See Type 2A	613313
TFR2-related HH (Type 3)	*TFR2*	Transferrin receptor 2	Autosomal recessive	• Earlier age of onset than Type 1 • Slower progression than Type 2	604250
Ferroportin disease (Type 4)	*SLC40A1/ ferroportin1/ IREG1/MTP1*	Ferroportin	Autosomal dominant	• Late onset • Iron-overload of macrophages	606069
Type 5 Hemochromatosis	*H-ferritin*	H-subunit of ferritin	Autosomal dominant	• Extremely rare	134770

Table 2: Summary of inherited forms of hemochromatosis. The above table summarizes the types of non-*HFE* hemochromatosis, indicating the causative gene, the protein encoded by that gene, the mode of inheritance, and how the indicated form differs from type I hemochromatosis.[26,37,38] An Online Mendelian Inheritance in Man (OMIM) accession ID number for each condition is also listed to facilitate further research on these rare diseases and related genes (http://www.ncbi.nlm.nih.gov/omim).

Clinical Aspects and Molecular Basis of Iron Metabolism

In order to truly understand the clinical aspects of hemochromatosis, scientists must first understand the molecular basis of iron metabolism. Iron is an essential nutrient in the body, necessary for oxygen transport and storage by hemoglobin and myoglobin and required by cytochromes involved in oxidative phosphorylation. Iron is capable of accepting and donating

electrons readily, cycling between ferric (Fe^{3+}) and ferrous (Fe^{2+}) states. Although this property makes this element useful in a biological system, it can be damaging in excess. Ferrous iron can react with peroxides to generate radicals that are damaging to the cell. As radicals can cause harm to lipids, proteins, and nucleic acids, iron metabolism must be carefully regulated.[39]

The average adult male has around 3000-4000 mg of iron in his body.[40] However, only 20 mg are needed each day for the synthesis of new red blood cells, which accounts for about 80% of the body's iron demand (see Figure 1) .[39] Only 1-2 mg of dietary iron need to be absorbed each day because very little iron is lost from the body, with the main sources of loss occurring through blood loss and sloughing of epithelial cells. As the natural mechanisms for iron loss are negligible, regulation of the body's iron intake from food is essential. Normally only about 10% of dietary iron is absorbed,[41] but an individual suffering from hemochromatosis may absorb two to three times as much iron by the gastrointestinal mucosa.[42] Over time, this increased absorption results in a build-up of iron in the heart, liver, pancreas, skin, joints, and testes that can only be diminished through routine phlebotomy; removal of 400-500 mL of blood can eliminate as much as 200-250 mg of iron from the body. Following phlebotomy, iron is released from the liver, helping to prevent organ damage.[35]

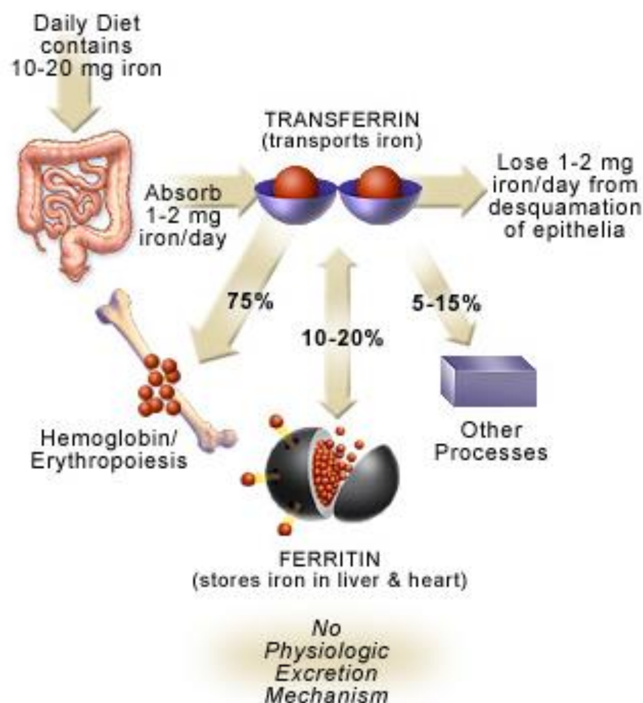

Figure 1: Normal Iron Absorption and Metabolism. Image retrieved from: http://www.cdc.gov/ncbddd/hemochromatosis/training/pathophysiology/ iron_cycle_popup.htm)

With this simple treatment, hemochromatosis patients have a normal life expectancy. But without this intervention, hemochromatosis can result in a multitude of symptoms. Early symptoms, such as abdominal pain, tiredness, weakness, and weight loss, are nonspecific and generally mild. However, as iron-overload increases, patients may suffer from cirrhosis of the liver, liver cancer, diabetes, congestive heart failure, heart arrhythmias, arthritis, and hypogonadism. In type I hemochromatosis, these symptoms usually do not develop until after age 40, although iron levels may be high long before this.[37]

When symptoms are indicative of iron-overload, hemochromatosis is primarily diagnosed biochemically by three methods: serum ferritin levels, percent transferrin saturation, and/or liver biopsy. When too much iron is stored in the organs, ferritin is released into the blood stream, resulting in an elevated serum ferritin concentration of greater than 300 µg/L in men and 200

μg/L in women. As transferrin is the iron transporter in the plasma, a high level of transferrin saturation is also evidence of iron-overload; transferrin saturation of greater than 45% is indicative of hemochromatosis. Liver biopsies are useful as well, not only to determine hepatic iron levels, but to also to detect the presence of liver cancer, fibrosis, and cirrhosis.[35] Genetic testing is often used to confirm the diagnosis of hemochromatosis.[37]

But how do these mutations in the *HFE* gene impact iron metabolism and why does iron accumulation occur in hemochromatosis? To answer these questions, the functions of several proteins involved in iron metabolism must be examined. Normally, Fe^{3+} is absorbed from the diet by cells called enterocytes that line the intestinal villi. The transport of iron across the apical membrane requires a protein called divalent metal transport 1 (DMT1) and the reduction of Fe^{3+} to Fe^{2+}. These enterocytes can either store iron as ferritin (a protein that sequesters the reactive iron) or transfer it across the basolateral membrane to reach the plasma, depending upon the body's needs. Ferritin is a multimeric protein that consists of L and H subunits that can surround an iron core of up to 4500 iron atoms. Ferritin's storage of iron protects the cell from damage due to iron's reactivity (see Figure 1). The protein ferroportin is located at the basolateral membrane where it acts to mediate iron export to the blood; the ferroxidase ceruloplasmin (Cp) is also required for iron export. This abundant plasma protein oxidizes Fe^{2+} back to Fe^{3+}, at which point the iron is picked up by a transferrin (Tf). While iron storage is associated with ferritin, iron transport is accomplished by transferrin. Tf has a high affinity for iron and keeps iron in a nonreactive state during its transport to body tissues.[39,42] Figure 2 provides an illustration of this pathway.

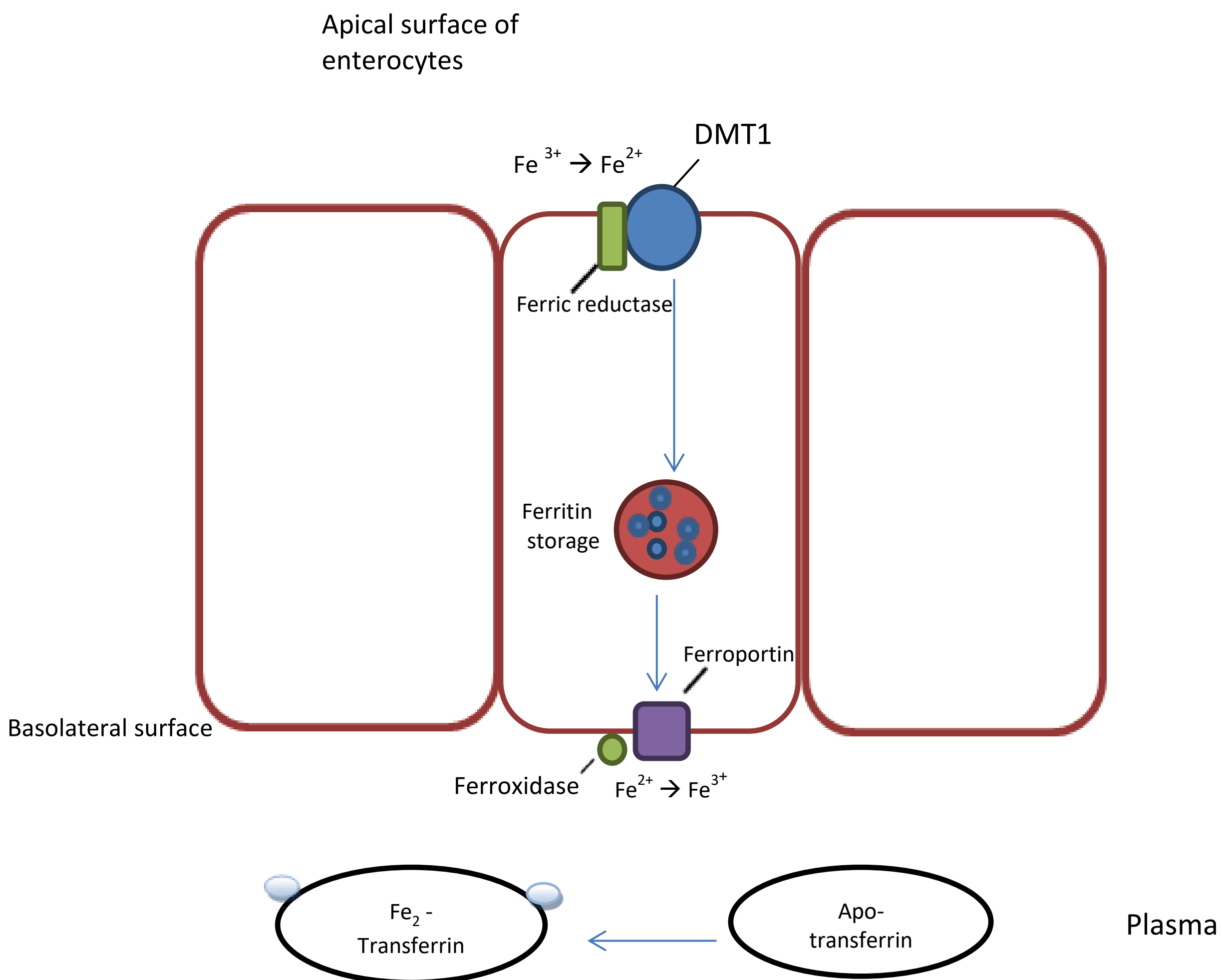

Figure 2: Iron Transport across the Intestinal Epithelium. Dietary iron is absorbed by intestinal cells called enterocytes that reside in the duodenal epithelium. The apical surface of the enterocytes faces the lumen of the intestine. After Fe^{3+} is reduced to Fe^{2+} by a ferric reductase, the iron transporter DMT1 moves iron across the membrane. In the enterocytes, iron can either be stored as ferritin or transported into the plasma by ferroportin. Here iron is oxidized and loaded onto a transferrin protein, which is part of the Transferrin Cycle discussed in Figure 3.[39, 42]

The Transferrin Cycle (Figure 3), the method by which iron is delivered to cells and transferrin is recycled, is a necessary part of iron metabolism. As iron is tightly bound to transferrin, receptor-mediated endocytosis is used to internalize the entire complex, extract the needed iron, and return the unbound transferrin to the plasma. In receptor-mediated endocytosis, the transferrin receptor binds the iron-transferrin complex and is brought into the cell via a

clathrin-coated pit that becomes an endosome. Protons are then pumped into the endosome by a proton pump. This results in a conformational change in the transferrin and its receptor, allowing the release of iron. The Fe^{3+} is once again reduced and then transported into the cytoplasm, where it can either be stored as ferritin or be directed to the mitochondria for heme production. The unbound transferrin and the transferrin receptor are recycled to the cell surface, where the entire cycle can be repeated.[39, 42]

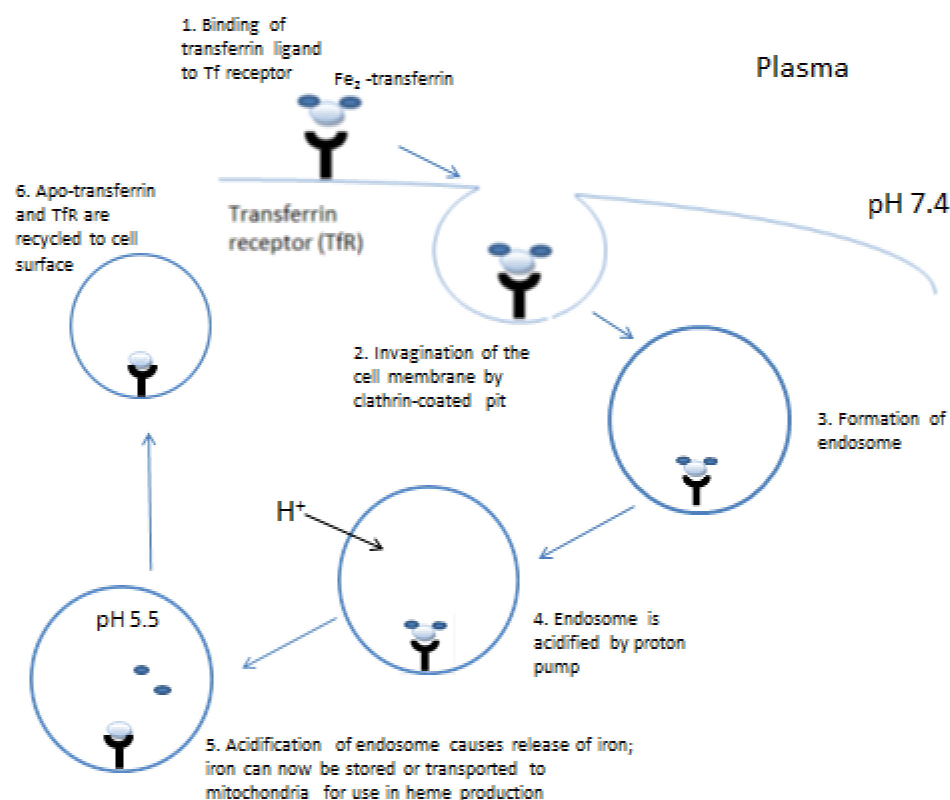

Figure 3: The Transferrin Cycle. The Transferrin Cycle plays an essential role in delivering iron to tissues and rejuvenating the supply of available transferrin. This process is an example of receptor-mediated endocytosis, in which iron-bound transferrin binds to the transferrin receptor and is brought into the cell by a clathrin-coated vesicle. Acidification of the endosome by proton pumps causes a change in the conformation of the transferrin-transferrin receptor complex, resulting in the release of iron. Iron is transported into the cytoplasm and then either used in the mitochondria or stored.

What role does the HFE protein play in this entire system? The HFE protein normally complexes with the transferrin receptor, lowering its affinity for iron-binding; this association

appears to be important in iron regulation, reducing the amount of iron brought into body tissues. However, in hemochromatosis, the C282Y mutation prevents the formation of a key disulfide bond (Figure 4). This disulfide bond is critical for HFE's ability to bind to a molecule called beta-2-microglobulin (β_2m) that allows the cell to transport HFE to the cell surface where it would normally complex with the transferrin receptor. Without the HFE bound to the transferrin receptor, receptor-mediated endocytosis of iron increases.[25, 41]

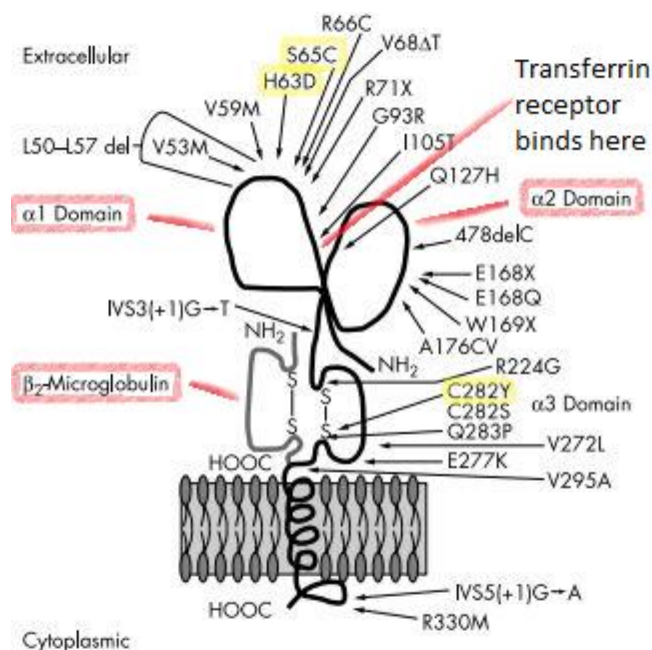

Figure 4. HFE/β_2-microglobulin complex. The above figure, adapted from Feder et al. and Robson et al. shows the position of known *HFE* mutations. C282Y interferes with a disulfide bridge needed for binding β_2-microglobulin, thus preventing expression at the cell surface. H63D and S65C affect the α1 domain that is associated with binding to the transferrin receptor. These mutations impact the ability of the HFE protein to act as an iron sensor and negatively modulate transferrin's affinity for iron.[43]

The H63D mutation also impacts this relationship between HFE and the transferrin receptor. The H63D mutant protein normally interacts with β_2-microglobulin and is found on the cell surface. However, studies have shown that the H63D mutation alters the ability of HFE to interact appropriately with the transferrin receptor. The H63D protein cannot as effectively

decrease the transferrin receptor's affinity for iron as the wild-type HFE can, but it is nonetheless partially functional. Thus, from a biochemical level, it is possible to see why the C282Y mutation would result in a more severe case of hemochromatosis compared to H63D.[25]

Yet this does not fully explain why hemochromatosis patients seem to take in 2-3 times more dietary iron. How can a protein that modulates the transferrin receptor affect iron uptake in the intestine – when the Transferrin Cycle is not even functional on the apical surface of enterocytes?[39] Another protein called hepcidin is integral to the understanding of how HFE can significantly impact iron homeostasis. Encoded by the *HAMP* gene, hepcidin is an antimicrobial peptide that is only 25 amino acids long, but plays essential roles both in disrupting bacterial cell membranes and regulating iron metabolism. Hepatic expression of hepcidin normally increases in response to iron-overload and inflammation. Hepcidin functions to regulate iron uptake from the intestines by blocking iron transport by the ferroportin protein found at the basolateral membrane of enterocytes. Thus, when more hepcidin is present, less iron can be absorbed into the blood. This regulation maintains homeostasis because when iron-overload occurs in healthy individuals, more hepcidin is produced to prevent further iron absorption, returning levels to normal. But counter-intuitively, hepcidin is actually found in lower than expected concentrations in hemochromatosis patients with iron-overload. Therefore, scientists have hypothesized that the HFE protein does more than modulate TfR. It is thought that HFE acts as a sensor of iron in the body, and when iron levels are high, the normal HFE protein can stimulate expression of more hepcidin. But when mutations in the HFE protein impact its ability to work properly as an iron sensor, it cannot promote the production of much-needed hepcidin. Thus, HFE is hypothesized to indirectly influence iron uptake through the regulation of hepcidin synthesis. It is believed

that all forms of hemochromatosis ultimately decrease hepcidin synthesis, resulting in iron-overload.[40, 41]

Molecular Methods for Genetic Testing

Countless molecular methods are used in genetic testing today. As these tests provide vital information to physicians and their patients, methods must be fast, sensitive, specific, relatively simple to run, and inexpensive. Two approaches to genetic testing can be taken: screening for known mutations versus scanning. The screening method searches only for common known mutations. In contrast, scanning a gene or genes is more comprehensive, attempting to identify any mutation.[44] Obviously, scanning for mutations is the more desirable method, but not always as practical in the laboratory.

Screening methods frequently are polymerase chain reaction (PCR)-based. PCR is a technique commonly used by molecular biologists to make millions of copies of a segment of DNA. In a standard PCR, template DNA is mixed with primers that bind to the region of interest and a thermostable DNA polymerase called *Taq* polymerase that adds free nucleotides to the 3' end of the primer. A multiplex PCR, a reaction in which more than one DNA segment can be amplified simultaneously, allows tests for several mutations to be run in one reaction. The method of screening used may vary depending on the type of mutation for which a laboratory is testing. For example, two-thirds of cases of Duchenne muscular dystrophy (DMD) are caused by deletions in the dystophin gene. As these deletions usually affect exons 3-8 and 44-60, a multiplex PCR can be used to amplify each of these exons. The resulting PCR products can then be separated by size using gel electrophoresis, and if any exons are missing, it can be assumed that a deletion has occurred.[45] Unfortunately, this method does not test for all DMD mutations.

Some cases of muscular dystrophy are known to be caused by frameshift mutations or nonsense mutations that result in a truncated protein.[4]

Smaller deletions such as the ΔF508 mutation that is a 3-base pair deletion in the *CFTR* gene associated with cystic fibrosis can also be identified by this method. If the region containing the deletion is amplified via PCR and then separated using electrophoresis, the mutant PCR product will migrate through the gel faster than the wild-type sequence, allowing clear genotyping.

Some genetic diseases, such as Huntington's disease, Fragile-X, and spinocerebellar ataxia type 2, are caused by trinucleotide repeat expansions (TREs) and detectable by similar means. TREs result when a specific three nucleotide sequence is repeated an abnormal number of times. For example, in the Huntington gene, the sequence C-A-G is normally repeated 10 to 30 times, but when this sequence is repeated more than 36 times, neurodegenerative disease is likely to result.[46] TREs can be tested for by designing primers that bind on either side of the expansion site. Amplification using PCR will yield products of varying sizes, allowing determination of the number of repeats.

Another technique called restriction enzyme fragment polymorphism analysis (RFLP) is used to test for mutations that create or destroy a restriction enzyme digestion site. Restriction enzymes function by cutting only at specific DNA sequences. Thus, if a DNA sequence of interest is amplified and then cut with restriction enzymes, only the sequence that contains the restriction enzyme recognition site will be cut, producing two fragments of shorter length that can be separated using electrophoresis. RFLP analysis has been used to test for the most common sickle cell mutation that substitutes an adenine nucleotide for thymine. This mutation destroys a Dde I restriction site, thus allowing differentiation between the wild-type and mutant

alleles after digestion.[47] Again, while this technique is useful for certain point mutations that create or destroy recognition sites, it only capable of identifying a known mutation.

The above techniques are just a few of the many molecular methods of screening genes for known mutations. Techniques that scan a whole gene or genes can be more challenging because it can be difficult to ascertain whether a gene variant is actually deleterious to protein function. SSCP (single-strand conformation polymorphism) testing is a method based on the migration of denatured PCR products in a gel with non-denaturing conditions that allows for the formation of secondary structures. Migration rates of the single strands vary based on sequence and their resulting secondary structure conformations, allowing separation of mutants from wild-type fragments.[44] However, this method will show differences between sequences that in reality could encode for the same protein.

In contrast, the protein truncation test (PTT) only detects differences that impact the protein. In this assay, PCR fragments are used as templates for *in vivo* transcription and translation to make polypeptides. When these proteins are separated using gel electrophoresis, truncated proteins can be identified. This is useful because a large percentage of genetic diseases are caused by proteins that are missing needed amino acids. PTT is limited though; small changes in amino acid sequence may not be detected by separation.[4, 44]

A third method of scanning for mutations uses denaturing gradient gel electrophoresis (DGGE). It has been found that partially denatured DNA fragments migrate much slower than fragments in their native state. Using DGGE, DNA is run in a gel of denaturing agents. As the DNA denatures, its migration will slow to a stop. The point at which the DNA can no longer migrate is highly dependent on its sequence. Thus, it is possible to identify mutations based on comparisons with the wild-type sequence's migration. Although this technique is sensitive,

rapid, and simple to perform once conditions are established, it can be difficult to find the optimal electrophoresis conditions.[4]

In this project, the mutations being tested for in the *HFE* gene were all characterized as missense mutations, meaning they result from a single nucleotide change in the DNA sequence that affects the HFE protein's amino acid structure. Since the goal of this project was to identify known mutations that may be of predictive clinical value, screening (instead of scanning the *HFE* gene) for mutations previously associated with hemochromatosis was the focus. To detect the C282Y, H63D, and S65C mutations, a technique called tetra-primer amplification refractory mutation system (ARMS) PCR was used, in conjunction with touchdown PCR. To confirm the validity of this method, DNA sequencing was also carried out on exon 2 and exon 4 of the *HFE* gene where these mutations are located.

Tetra-primer ARMS PCR is a method first described by Ye et al. 2001 that allows for genotyping of practically any single nucleotide polymorphism (SNP) such as the missense mutations described above.[48] (This technique is a slight modification of an earlier method of classical ARMS PCR reported on by Newton et al. 1989.)[49] The basic principle behind ARMS PCR is that primers with a mismatch to the DNA template at the 3' end of the primer are unable to amplify the intended sequence. The DNA polymerase used in PCR adds new nucleotides to the 3' end of a primer, but cannot do this in the case of a 3' mismatch. Therefore, allele-specific primers (ASO) that only amplify a particular polymorphism can be designed for use in ARMS, in addition to outer primers that are not allele-specific.

Perhaps the best way to understand this technique is to consider an example. In Figure 5 below, an illustration of primer binding sites and expected product sizes for the C282Y ARMS PCR is shown. Two primers pairs are designed for tetra-primer ARMS PCR. The two outer

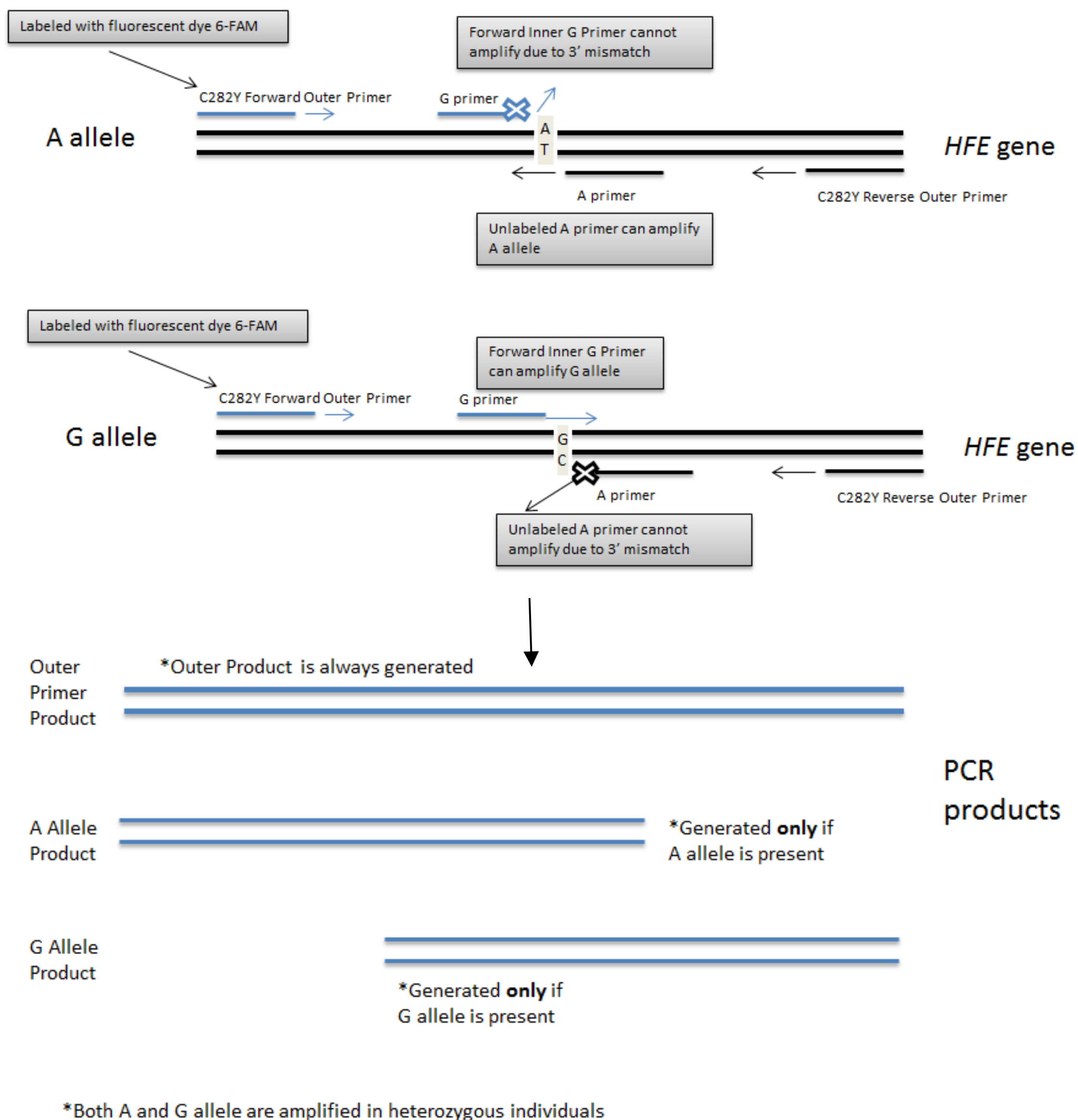

Figure 5: Tetra-Primer ARMS PCR Amplification of the C282Y Mutation on Exon 4. For the above method of testing for a single nucleotide polymorphism (SNP), both inner and outer sets of primers are designed. The inner primers are allele-specific, meaning the forward inner primer is capable of amplifying only the G allele, whereas the reverse inner primer can only amplify the A allele. The outer primers should be capable of amplification regardless of what SNPs are present, providing a control to determine if the PCR is working. If an individual is homozygous, two PCR products are amplified, one allele-specific inner product and one outer product, whereas if an individual is heterozygous, both allele-specific inner products will be amplified along with the outer product. Following PCR, capillary electrophoresis is used to separate mutant from wild-type alleles for genotyping. (Figure adapted from Ye et al. 2001)[48]

primers are not allele-specific and should always result in amplification of a large outer product. This is an important control that verifies the outer primers are binding correctly to the DNA and that the PCR is functioning properly. The inner primers are allele-specific; one of these primers is designed to amplify the mutant A allele, while the other only amplifies the wild-type G allele. A second mismatch at position -2 from the 3' end of these inner primers is also introduced in tetra-primer ARMS PCR in order to increase the specificity of annealing to only the desired allele. These inner primers run in opposite directions, allowing for the production of different sized products for mutant versus wild-type alleles. In both primer pairs, only the forward primers are fluorescently labeled for subsequent fragment analysis.

Therefore, if an individual was homozygous wild-type, only the G allele specific inner primer would be capable of annealing to the sequence. The fluorescently labeled G allele forward inner primer and reverse outer primer would amplify a segment 196 base pairs long, while the fluorescently labeled forward outer primer and the reverse outer primer would also produce a product of 317 base pairs in length in the same PCR. However, if an individual is homozygous for C282Y, the A allele reverse inner primer would bind instead of the G allele primer. This reverse inner primer and the fluorescently labeled forward outer primer would form a 176 bp product, in addition to the outer product of 317 base pairs. In the case of a heterozygous individual, both inner primers would be capable of binding, and thus products of 176, 196, and 317 would all be produced. In this project, capillary electrophoresis using an ABI Prism 310 Genetic Analyzer was used to separate the DNA fragments for genotyping. With this system, genotyping is relatively simple and rapid.

The main problems with ARMS PCR are associated with the initial design of the assay. It is essential for primers to be well-designed to avoid self-complementarity or secondary

structures that may interfere with amplification. The melting temperatures of the primers must all be similar in order to prevent preferential amplification of one allele. Additionally, in assays at least four primers are being used at all times; therefore, artificial products such as primer dimers may be problematic. For this reason, touchdown PCR is typically used in conjunction with tetra-primer ARMS PCR. Touchdown reduces artificial amplification by running the first PCR cycle with an annealing temperature 5-10°C higher than the average melting temperature of the inner primers, followed by a decrease of 1°C per cycle for the next 10 cycles. With these initial higher annealing temperatures, amplification can only occur when the primer very closely matches the template, reducing non-specific amplification.

Following genotyping by ARMS PCR, sequencing is necessary to confirm the accuracy of the method for the tested SNPs. Sequencing reactions use one sequencing primer per reaction, unlabeled deoxynucleotidetriphosphates (dNTPs) as well as fluorescently labeled dideoxynucleotidetriphosphates (ddNTPs). ddNTPs lack a free 3' hydroxyl group, which is required for the formation of the phosphodiester bond that links nucleotides. Thus, incorporation of ddNTPs into a growing strand terminates further synthesis, creating a collection of DNA fragments that all differ by one base pair (depending on where the ddNTP was incorporated). Separation of these fragments by capillary electrophoresis allows the researcher to determine the sequence of the desired gene as a laser identifies each nucleotide by its attached fluorescent dye. Sequencing is an effective method for genetic testing, but unfortunately, it is both time consuming and expensive. Additionally, it is difficult to determine if any mutations found are normal polymorphisms or disease-causing mutations.

Simple, rapid, inexpensive, yet sensitive and specific genetic tests must be developed if progress in the field of genomic medicine is to be achieved. Yet genetic testing methods often

must strike a balance between the practical screening for known mutations and the practice of scanning for any mutations.

Materials and Methods

Volunteer Recruitment and DNA Collection

Epithelial cheek cell samples from 132 volunteers on the Houghton College campus were collected between May 2010 and January 2011. Volunteers were asked to rub the inside of their cheeks for 30 seconds with a cytology brush (Fisher Scientific Cat#22-263357), which was then stored in 600 µl of 50 mM NaOH until DNA extraction. Recruitment was entirely voluntary, and all samples were labeled with a randomly generated 6-digit number to ensure the absolute anonymity of volunteers. Eligible participants were students, faculty, staff, or community members with a minimum age of 18. All volunteers were asked to sign the Informed Consent document (see Appendix B).

DNA Isolation and Quantification

Following sample collection, DNA was isolated from epithelial cheek cell samples. Samples were first heated at 95℃ for 5 minutes to initiate cell lysis and then vortexed for 30 seconds. The heating and vortex steps were repeated, followed by scraping the side of the tube with the brush. After the cytology brush was discarded, 100 µl of 1M Tris (VWR) at a pH of 7.0 were added to each sample to balance the pH. The pH of each sample was then tested to ensure that the samples were at an optimal pH (between 7.0 and 8.0) for PCR. Next, 300 µl of this crude DNA were added to a clean labeled tube; the remainder of the crude was stored at -20°C as a reserve supply. 30 µl of 3M Sodium Acetate Buffer pH 5.2 and 650 µl of cold 96%-100% ethanol were added to each sample and placed at -20°C for a minimum of 15 minutes. After centrifugation at full speed for 15 minutes, the supernatant was removed. 200 µl of cold 70% ethanol were added to each sample to dislodge the pellets, which contained genomic DNA.

Samples were centrifuged again for 5 minutes, and the 70% ethanol was removed and discarded appropriately. This 70% ethanol-centrifugation step was repeated to remove any salt from the precipitated DNA. The speed vac (LabConco Cat#7811020) was then used to evaporate any remaining ethanol from each sample. After the samples were dried, 100 µl of TE buffer (10 mM Tris, 1mM EDTA (VWR) at pH 8) were added to prevent DNA degradation. DNA was quantified in ng/µl using a Thermo-Fisher Nanodrop 2000. Samples were diluted to a final concentration of about 10-40 ng/µl using 0.1x TE buffer and stored at 4°C until further use.

In later DNA extractions, a more expeditious isolation method was utilized. After the heating and vortexing steps described above, 1 M Tris pH 7 was added to balance pH. pH was then tested using pH paper. Half of this crude DNA sample was placed at -20°C as a reserve supply, while the rest was centrifuged at 13000xg for 5 minutes. The supernatant was then transferred to a fresh 1.7 mL tube, quantified, and diluted as described above. Over the course of the project it was observed that optimal DNA amplification occurred at a DNA concentration of about 15ng/µl; thus, later DNA samples were carefully diluted to 15ng/µl (instead of the more broad concentration range initially used).

Tetra-primer ARMS Touchdown PCR

Tetra-primer ARMS PCR is described in Ye et al. 2001 as an effective method for genotyping single nucleotide polymorphisms (SNPs) such as the missense mutations tested for in this paper. SNPs occur when a single nucleotide varies at a particular location in the DNA of a species, such as in the case of the C282Y mutation, where a guanine nucleotide is mutated to adenine. ARMS PCR is based on the principle that a primer must exactly match the DNA template at the 3’ end in order to amplify the area of interest because DNA polymerases can only

add new nucleotides to the free hydroxyl group at the 3' end of the primer. This technique requires the use of two primer pairs consisting two inner allele-specific primers and two outer primers.[48]

Primers for tetra-ARMS PCR were designed using the following website: http://cedar.genetics.soton.ac.uk/public_html/primer1.html (referenced by Ye et al. 2001). The *HFE* sequences for exon 2 and exon 4 with the positions of the SNPs specified were inputted, allowing the computer-generated design of primer pairs for ARMS PCR. Ye et al. 2001 also advocates introducing a second mismatch at the second position from the 3' end of the inner primers to enhance specificity (which the online software automatically included in the inner primers).

Kibbe 2007 describes the design of an online oligonucleotide properties calculator[50] found at http://www.basic.northwestern.edu/biotools/oligocalc.html. Primer self-complementarity and melting temperature (T_m) were determined using this online tool; the nearest neighbor formula for melting temperature was used to estimate the melting temperature of the primers. As the interaction between bases on different strands depends somewhat on the neighboring bases, this formula factors in how neighboring bases can impact binding of the primer. The primer sequences and melting temperatures generated by the online software are displayed below in Table 3.

In order to multiplex C282Y and H63D mutations, different fluorescent dyes were attached to the 5' end of the primers for each mutation. C282Y forward primers were labeled with the 6-FAM dye (blue), while H63D and S65C forward primers were labeled with the HEX (green) and TAMRA dyes (yellow), respectively. Only the forward primers were labeled in each

primer pair for detection on the genetic analyzer. See Figure 5 for graphic representation of primer binding sites.

	Primer Name	Sequence (5'→3')	T_m
C282Y Primers	C282YForwardInnerG	**6-FAM** ACCCCCTGGGGAAGAGCAGAGATATACTTG	63°C
	C282YReverseInnerA	ATCCAGGCCTGGGTGCTCCACCTTGT	64°C
	C282YForwardOuter	**6-FAM** CCCAGAACATCACCATGAAGTGGCTGAA	62°C
	C282YReverseOuter	CTCAGCCCACCCCCTAACAAAGAGCAGA	64°C
H63D Primers	H63DForwardInnerC	**HEX** TGGATGACCAGCTGTTCGTGTTCTATGTTC	62°C
	H63DReverseInnerG	CGGGGCTCCACACGGCGACTCTCTTC	66°C
	ForwardOuter	**HEX** AGGCCTGTTGCTCTGTCTCCAGGTTCACAC	65°C
	ReverseOuter	AGGTGAGGCCCCCTCTCCACATACCCTT	64°C
S65C Primers	S65CForwardInnerA	**TAMRA** ACCAGCTGTTCGTGTTCTATGATCATGTGA	62°C
	S65C ReverseInnerT	GGAGTTCGGGGCTCCACACGGCGTCA	66°C
	S65CForwardOuterRed	**TAMRA** AGGCCTGTTGCTCTGTCTCCAGGTTCACAC	65°C
	ReverseOuter	AGGTGAGGCCCCCTCTCCACATACCCTT	64°C

Table 3: PCR Primers and T_M. Table 3 shows PCR primer sequences for each of the genetic tests and estimated melting temperatures calculated using the nearest neighbor formula. It was advantageous to find primers all with similar melting temperatures as cycling profiles were identical for each test.

Ye et al. also advocate the necessity of using touchdown PCR with tetra-primer ARMS PCR to reduce the amplification of non-specific products. Touchdown PCR reduces non-specific amplification by running the first PCR cycle with an annealing temperature 5-10°C higher than the average melting temperature of the inner primers, followed by a decrease of 1°C per cycle for the next 10 cycles. With these initial higher annealing temperatures, amplification can only occur when the primer very closely matches the template, reducing non-specific amplification.

Due to the fact that the same outer primers were chosen for both H63D and S65C, these mutations could not be multiplexed successfully. Thus, C282Y and H63D PCRs were

multiplexed, while S65C PCRs were run alone. Fortunately, the melting temperatures of all of the primers used were relatively close to each other; thus, the same cycling conditions could be used for all primers (see Table 4 for PCR cycling conditions). PCRs were also run using a 10:1 inner: outer primer concentration to enhance the amplification of the inner allele-specific products. Each 20 µl reaction included approximately 30 ng of DNA, 20 pmoles of each outer primer (Integrated DNA Technologies), 2 pmoles of each inner primer, 10 µl 2X BioMix (Bioline Cat#25011), and distilled water up to the 20 µl volume. A negative control reaction that did not contain any DNA template was always run to ensure no contamination would affect the results. Expected product sizes are displayed in Table 5.

PCR Conditions for C282Y, H63D, and S65C	94˚C for 5 minutes
	12 cycles of:
	    94˚C for 30 seconds
	    74˚C for 30 seconds (decreasing by 1˚C per cycle)
	    72˚C for 30 seconds
	18 cycles:
	    94˚C for 30 seconds
	    62˚C for 30 seconds
	    72˚C for 30 seconds
	72˚C for 10 minutes
	Hold 4˚C forever

Table 4: Tetra-Primer ARMS Touchdown PCR Cycling Conditions. The above conditions were generally used for both the C282Y/H63D multiplex and S65C PCRs. The actual number of cycles and extension times were increased for some DNA templates that did not amplify as readily. The touchdown cycling helped decrease the presence of artificial products by ensuring that only the most specific products could be amplified in the first few cycles.

	Wild-Type Allele	Mutant Allele	Outer Product
C282Y (6 FAM)	196	176	317
H63D (HEX)	207	158	310
S65C (TAMRA)	201	164	310

Table 5: Expected Product Sizes in base pairs. For each of the SNPs, the expected product sizes are listed above. The outer product should be amplified in all PCR reactions, whereas the allele-specific inner primers can only amplify if a particular SNP is present. For homozygous individuals, two products should be produced: one inner allele-specific product and the outer product. For heterozygous individuals, three products should be produced: one for each inner allele-specific product and the outer product. Forward primers for each mutation were labeled with different color dyes: C282Y primers were labeled with 6 FAM (a blue dye), while H63D and S65C primers were labeled with HEX (green), and TAMRA (yellow), respectively.

Fragment Analysis Using the ABI Prism 310 Genetic Analyzer

Following amplification of the *HFE* gene, fragment analysis was performed using the ABI PRISM 310 Genetic Analyzer (Applied Biosystems Cat#4330878). Capillary electrophoresis is used to separate the fragments based on size; smaller fragments migrate faster than larger fragments. To prepare samples for analysis on the genetic analyzer, a stock solution of 16 µl of HiDi Formamide (Applied Biosystems Cat#4311320) and 0.35 µl LIZ 500 GeneScan molecular weight marker (Applied Biosystems Cat#4311320) multiplied by number of samples for analysis, was mixed thoroughly. 15 µl of stock solution were added into each 0.5 ml genetic analyzer tube (Applied Biosystems Cat#401957) along with 2µl of PCR product and capped with a septum (Applied Biosystems Cat#401956). Samples were vortexed and centrifuged briefly, followed by a 3 minute incubation in a 95°C water bath to disrupt hydrogen bonding between DNA strands. Finally, samples were placed on ice for 1 minute, then briefly vortexed and centrifuged before loading in the genetic analyzer sample tray.

Applied Biosystems 310 Data Collection software was used to collect fragment analysis data from the genetic analyzer. Following capillary electrophoresis, GeneMapper v.4.0 software was used for data analysis to determine fragment sizes produced in each sample. Genotyping

was accomplished by comparing fragments to expected sizes indicated in Table 5. Allelic and genotype frequencies were also calculated and compared to those expected for Hardy-Weinberg equilibrium, as well as to published frequencies.

Sequencing

To validate the accuracy of genotyping by ARMS PCR, sequencing of a representative sample of each genotype was completed. Sequencing requires the amplification of the sequence of interest using unlabeled outer sequencing primers, followed by sequencing reactions using unlabeled inner sequencing primers (one primer per reaction). Outer sequencing primers were designed to bind a few base pairs before the forward and reverse outer primers used in ARMS PCR. These primers would be used in the initial amplification step, following by sequencing reactions using the ARMS outer primers. (The ARMS *outer* primers were used in sequencing as *inner* sequencing primers. For the forward outer primers in ARMS that were fluorescently labeled, *unlabeled* versions of the oligos were used in sequencing). The primers used in sequencing are displayed in Table 6 and 7. Note that C282Y is found on exon 4, whereas H63D and S65C are found on exon 2; the H63D and S65C mutations are only six nucleotides apart and can therefore be sequenced together.

Sequence of Interest	Outer Sequencing Primers		
	Primer Name	**Sequence 5'→3'**	**T_M**
Exon 2 (H63D and S65C)	HFE Exon 2 Forward	CCCTCCTACTACACATGGTTAA	51 ˚C
	HFE Exon 2 Reverse	AAGCTCTGACAACCTCAGGA	53 ˚C
Exon 4 (C282Y)	HFE Exon 4 Forward	TCGGGCCTTGAACTACTACC	53 ˚C
	HFE Exon 4 Reverse	ACAGAAAAAGCAAGTTAAAGCC	51 ˚C

Table 6: Outer Sequencing Primers. The above primers were used to amplify the exon of interest prior to setting up sequencing reactions. This extra step provides a cleaner template for sequencing reactions and also prevents mis-priming during sequencing that could interfere with the readability of derived sequence. Exon 2 primers were used to amplify the H63D and S65C SNPs, while Exon 4 primers were used for C282Y.

Sequence of Interest	Inner Sequencing Primers		
	Primer Name	**Sequence 5'→3'**	**T_M**
Exon 2 (H63D and S65C)	Seq. ForOuter Exon 2	AGGCCTGTTGCTCTGTCTCCAGGTTCACAC	65 ˚C
	Reverse Outer	AGGTGAGGCCCCCTCTCCACATACCCTT	64 ˚C
Exon 4 (C282Y)	Seq. C282Y ForOuter Exon 4	CCCAGAACATCACCATGAAGTGGCTGAA	62 ˚C
	C282Y ReverseOuter	CTCAGCCCACCCCCTAACAAAGAGCAGA	64 ˚C

Table 7: Inner Sequencing Primers. The above primers were used in sequencing reactions. One primer was added to each reaction along with dNTPs and fluorescently labeled ddNTPs. When ddNTPs are incorporated into a growing chain, synthesis is terminated, creating a mixture of fragments that all vary in size by one base. Capillary electrophoresis then separates these fragments, allowing determination of the nucleotide sequence.

Each PCR reaction using outer sequencing primers (for amplification of both exon 2 and exon 4 of the *HFE* gene) was carried out in a 20 µl volume, using 30 ng DNA, 20 pmoles of each forward and reverse primer (Integrated DNA Technologies), 10 µl of 2x BioMix (Bioline 25011), and distilled water up to the desired volume (see Table 8 for PCR conditions).

Amplification of Exon 2 and Exon 4 using Outer Sequencing Primers:	94˚C for 5 minutes 30 cycles: 94˚C for 30 seconds 50˚C for 30 seconds 72˚C for 30 seconds 72˚C for 10 minutes Hold 4˚C forever

Table 8: PCR Conditions. The above PCR conditions were used for amplification of both Exon 2 and Exon 4. PCR product was purified and then run on an agarose gel to determine if fragment of expected size (about 300 bps) was amplified before proceeding with sequencing reactions.

Following PCR, DNA was purified using a QiaQuick PCR Purification kit (Qiagen Cat#28104). DNA was then quantified using the Nanodrop as described previously and run on a 1% agarose gel to determine if amplification of desired product had occurred. The expected products were all about 300 bp long as expected.

Next, sequencing reactions were set-up using 10 ng PCR product, 2 µl BigDye Terminator Ready Reaction Mix v. 3.1 (Applied Biosystems), 1 µl 5X Big Dye sequencing buffer (Applied Biosystems), 3.2 µl of 1 µM sequencing primer (Integrated DNA Technologies), and distilled water up to 10 µl volume. Cycle sequencing was performed in a thermal cycler under the following conditions: 1 minute at 96°C followed by 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes, and then held at 4°C until next step. These samples were then purified and prepared for analysis by following the "Preparing Sequencing Reactions for Analysis by Genetic Analyzer" protocol found in Appendix A." After cycle sequencing,

although most sequencing reactions were performed using Houghton facilities, a few sequencing reactions were sent to Cornell University's Life Science Core Laboratories Center for sequencing on an Applied Biosystems Automated 3730 DNA Analyzer. After running analysis on genetic analyzer, sequences were analyzed using Finchtv (http://www.geospiza.com/Products/finchtv.shtml) and BiologyWorkbench (http://workbench.sdsc.edu/) and compared to the *HFE* wild-type sequence provided by GenBank (http://www.ncbi.nlm.nih.gov/gene/3077) to determine the presence or absence of the mutation for which the DNA was being tested. Sequencing results were then compared to ARMS PCR results to confirm the accuracy of the genetic testing methodology.

Upon completion of the project, participants were informed of their results. Individuals who wish to be retested were directed to a CLIA (Clinical Laboratory Improvement Amendment of 1988)-certified lab for follow-up testing. Individuals were advised of the penetrance of each of the genotypes based on current literature. If any individuals had an at-risk genotype, they would be advised to see a medical doctor or genetic counselor for further phenotypic testing to prevent disease.

Survey Design

In consultation with honors advisor Dr. Matthew Pelletier and the assistance of Dr. Paul Young of the Houghton College Psychology Department, a survey assessing the bioethical perspectives of participants was developed. This survey (see Appendix D) attempted to determine if participants were well-informed about the genetic disease being tested for, how they planned to use the information derived from genetic testing, and their overall perspective on the use of genetic testing in society. The survey was administered through the online survey

company, www.surveymonkey.com, by email invitation to participants who had previously donated their DNA. Participants were required to agree to the Informed Consent document in Appendix D, which guaranteed participants anonymity and confidentiality. Survey responses were in no way linked to participant name or email address.

Results

Fragment Analysis

Of the 132 DNA samples collected from the Houghton College campus, 129 of these samples were successfully genotyped for C282Y, H63D, and S65C SNPs using fluorescently labeled forward primers with tetra-primer ARMS PCR. Expected product sizes are listed in Table 5. Genotyping of each SNP was done by separating ARMS PCR products via capillary electrophoresis on an ABI Prism 310 Genetic Analyzer. The analyzer detects fluorescently labeled fragments and determines the size of each PCR product by comparing the migration rate to the LIZ500 fluorescently labeled orange molecular weight standard. (See Figures 6-12 for representative electropherograms of each generated genotype.)

The number of each genotype detected was recorded and the genotypic frequencies were calculated for comparison to expected literature values for Caucasian populations. As Houghton College is primarily of European descent, genotypic frequencies should be comparable. See Table 9 and 10 for calculations of genotypic frequencies and the frequency of compound heterozygotes. In the collected samples no homozygous individuals were found. However, a C282Y homozygous individual was identified by the 2010 Human Genetics and Disease class using RFLP analysis; this sample was tested as shown below using ARMS PCR. Genotyping was consistent between the two assays. In addition, a sample of S65C carrier DNA was obtained from Michigan State University; this sample tested positive for S65C as expected.

	C282Y	**H63D**	**S65C**
Homozygous wild-type	112 (86.8%)	94 (73.4%)	127 (98.4%)
Carriers (Heterozygous for mutation)	17 (13.2%)	34 (26.6%)	2 (1.6%)
Homozygous for mutation	0 (0%)	0 (0%)	0 (0%)
Totals:	129	128	129

Table 9: Genotypic Frequencies. The above genotypic frequencies were observed using the tetra-primer ARMS PCR assay developed. These frequencies are qualitatively similar to genotypic frequencies for Caucasian populations.

Compound Heterozygotes:	
C282Y/H63D	2 (1.6%)
C282Y/S65C	1 (0.78%)

Table 10: Frequency of Compound Heterozygotes. As compound heterozygotes have a greater risk of developing hereditary hemochromatosis than the general population, it is important to recognize the frequency of these genotypes as well.

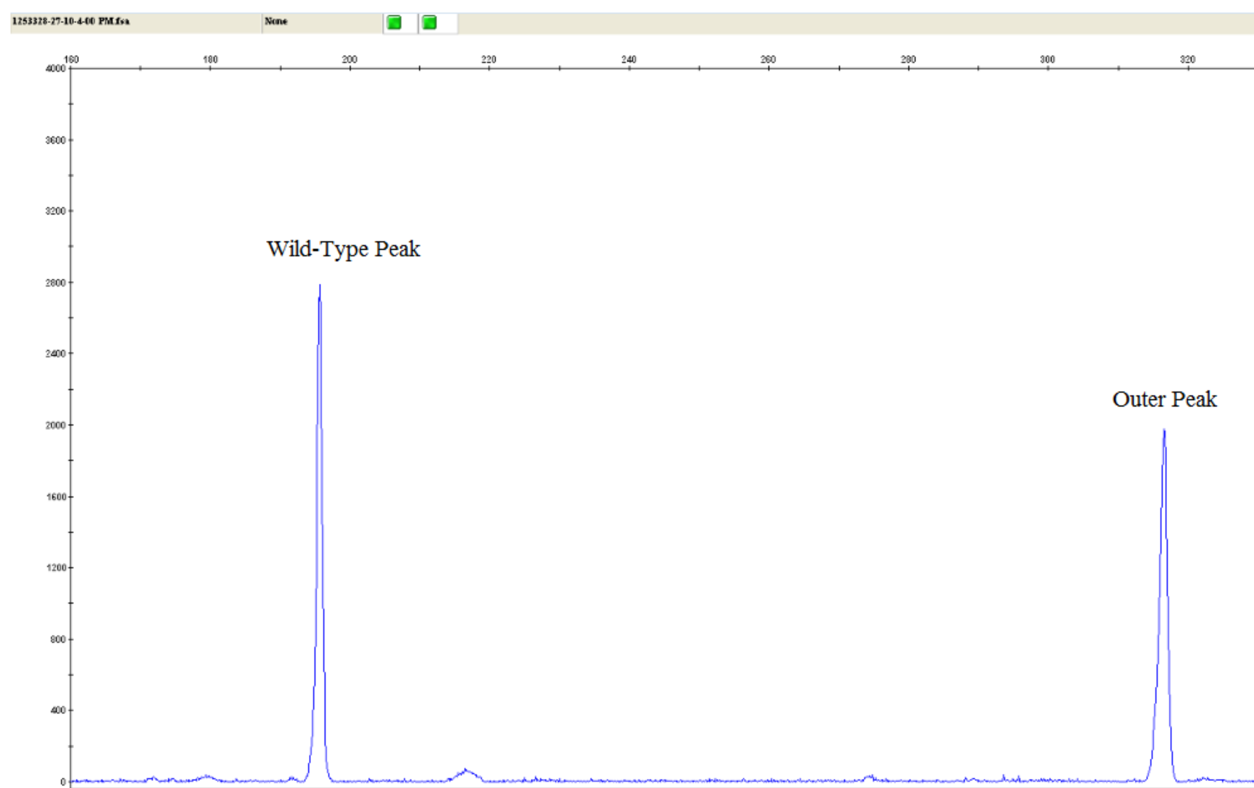

Figure 6: Wild-type for C282Y Test. The above electropherogram shows fluorescently labeled 6FAM (blue) DNA fragments appearing at about 196 and 317 base pairs in length. (The x-axis indicates the size of PCR product in base pairs.) These PCR product sizes indicate that the outer primers were functional, and only the allele-specific primer for the wild-type sequence was capable of annealing to the DNA template. Therefore, this individual is homozygous wild-type for the C282Y test. 86.8% of the Houghton population were wild-type for this SNP.

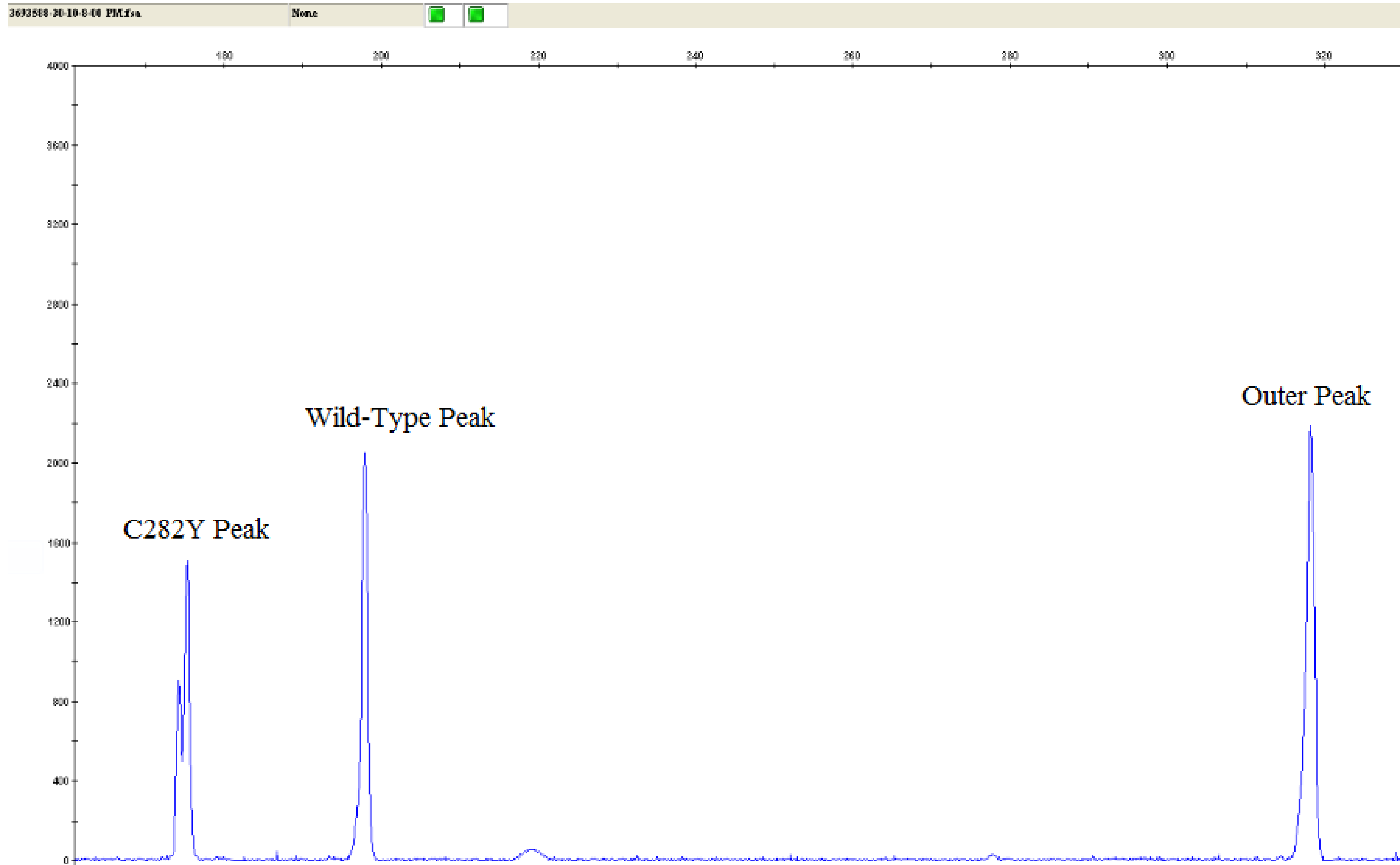

Figure 7: C282Y Heterozygote. The above electropherogram shows fluorescently labeled blue DNA fragments appearing at about 176, 196 and 317 base pairs in length. (The x-axis indicates the size of PCR product in base pairs.) These PCR product sizes indicate that the outer primers were functional and both allele-specific primers for the wild-type and mutant sequences were capable of annealing to the DNA template. Therefore, this individual is heterozygous for the C282Y HFE protein. 13.2% of tested samples were carriers for this mutation.

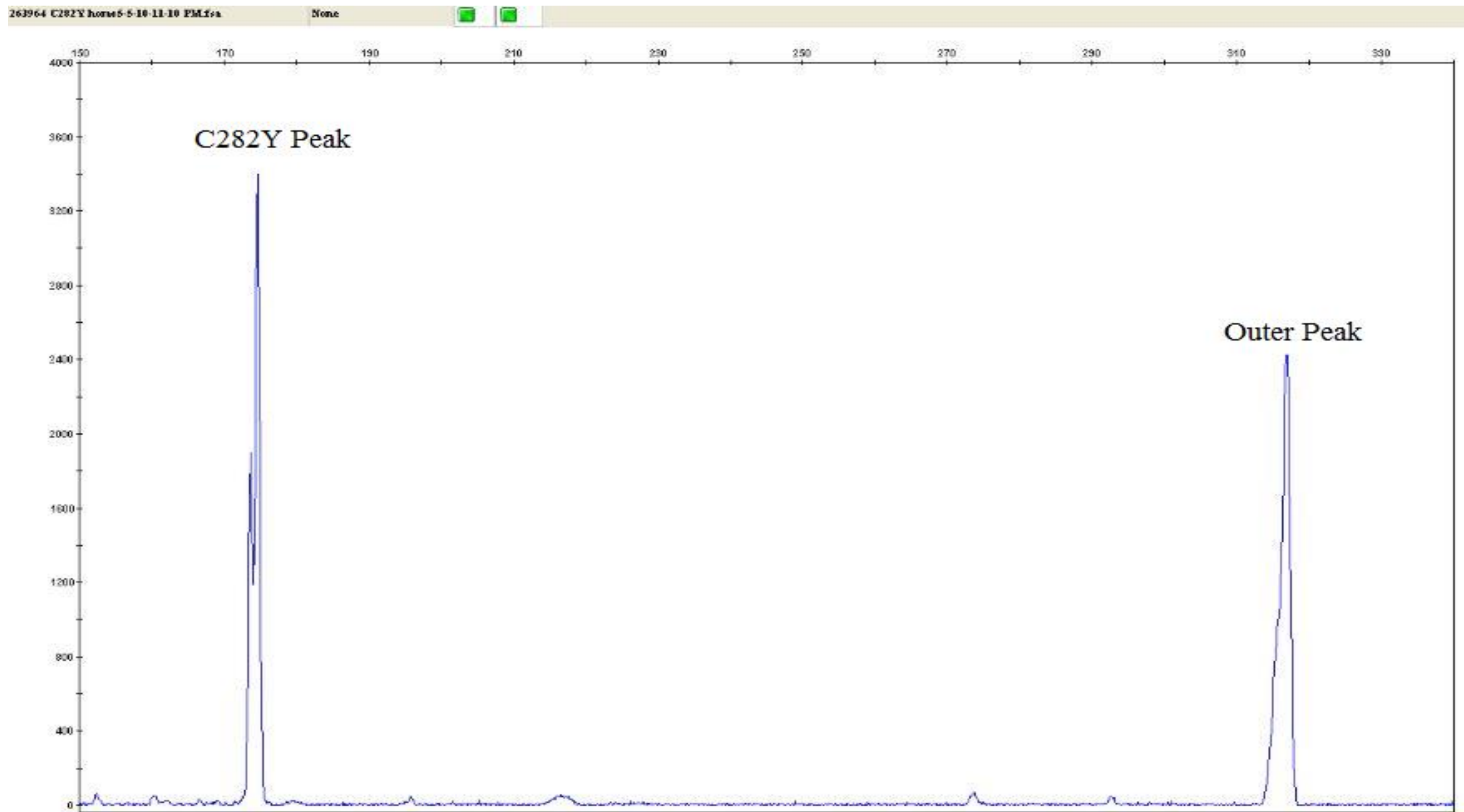

Figure 8: Homozygous for C282Y. The above electropherogram shows fluorescently labeled blue DNA fragments appearing at about 176 and 317 base pairs in length. No wild-type allele is shown at 196 bps. (The x-axis indicates the size of PCR product in base pairs.) These PCR product sizes indicate that the outer primers were functional, and only the allele-specific primer for the mutant C282Y sequence was capable of annealing to the DNA template. Therefore, this individual is homozygous for the mutation C282Y on exon 4 of the *HFE* gene. This genotype is consistent with prior testing of this sample by the 2010 Human Genetics and Disease class using RLFP analysis.

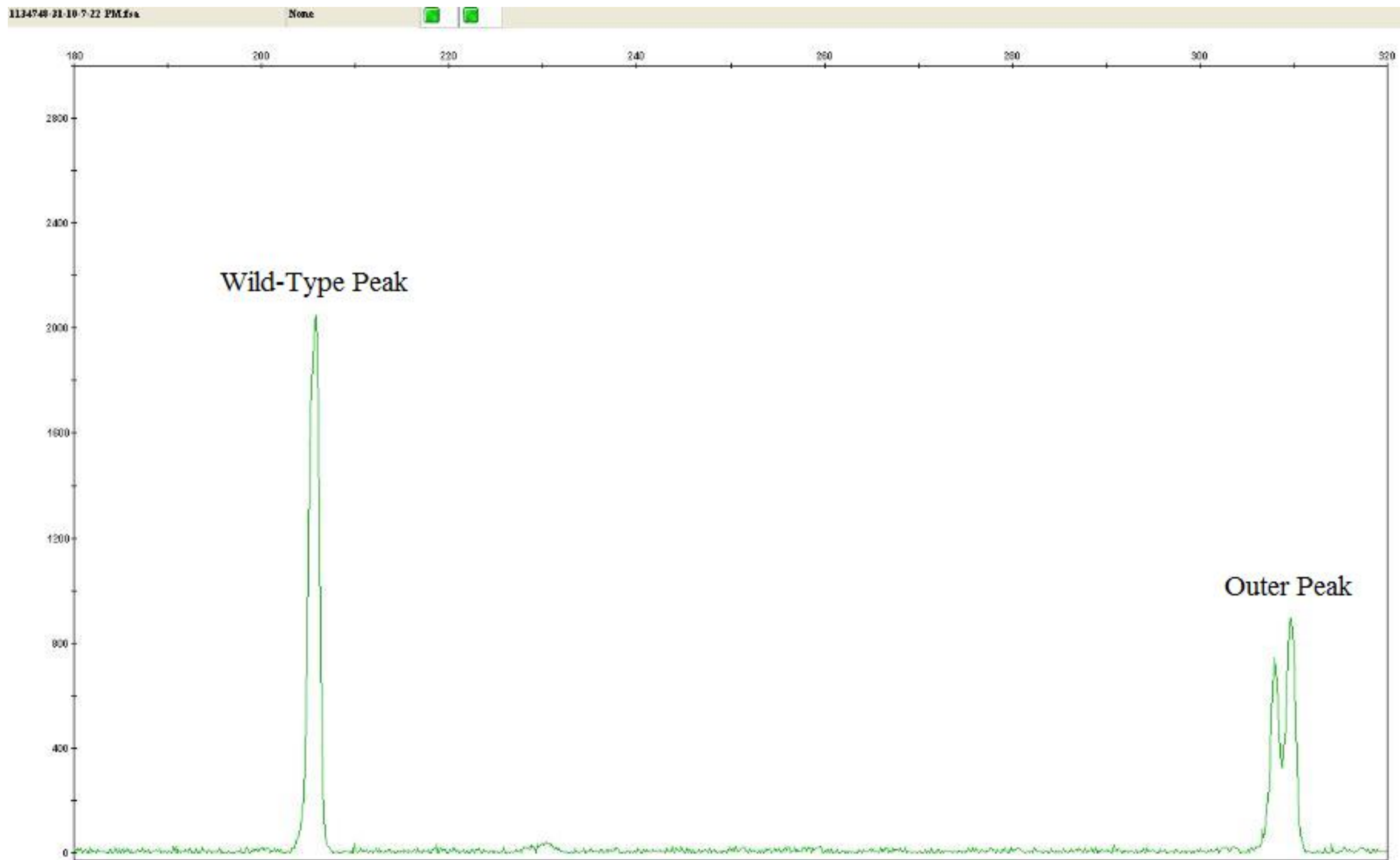

Figure 9: Wild-Type at Site of H63D Mutation. The above electropherogram shows green peaks at 207 and 310. Therefore, this individual is homozygous wild-type at the site of the H63D because a 158 bp mutant allele was not amplified. (The x-axis indicates the size of PCR product in base pairs.) Although these peaks are similar in size to PCR products produced for C282Y and S65C testing, primers for each mutation are labeled with different fluorescent dyes to aid differentiation. 73.4% of the Houghton population were wild-type for this SNP on exon 2 of the *HFE* gene.

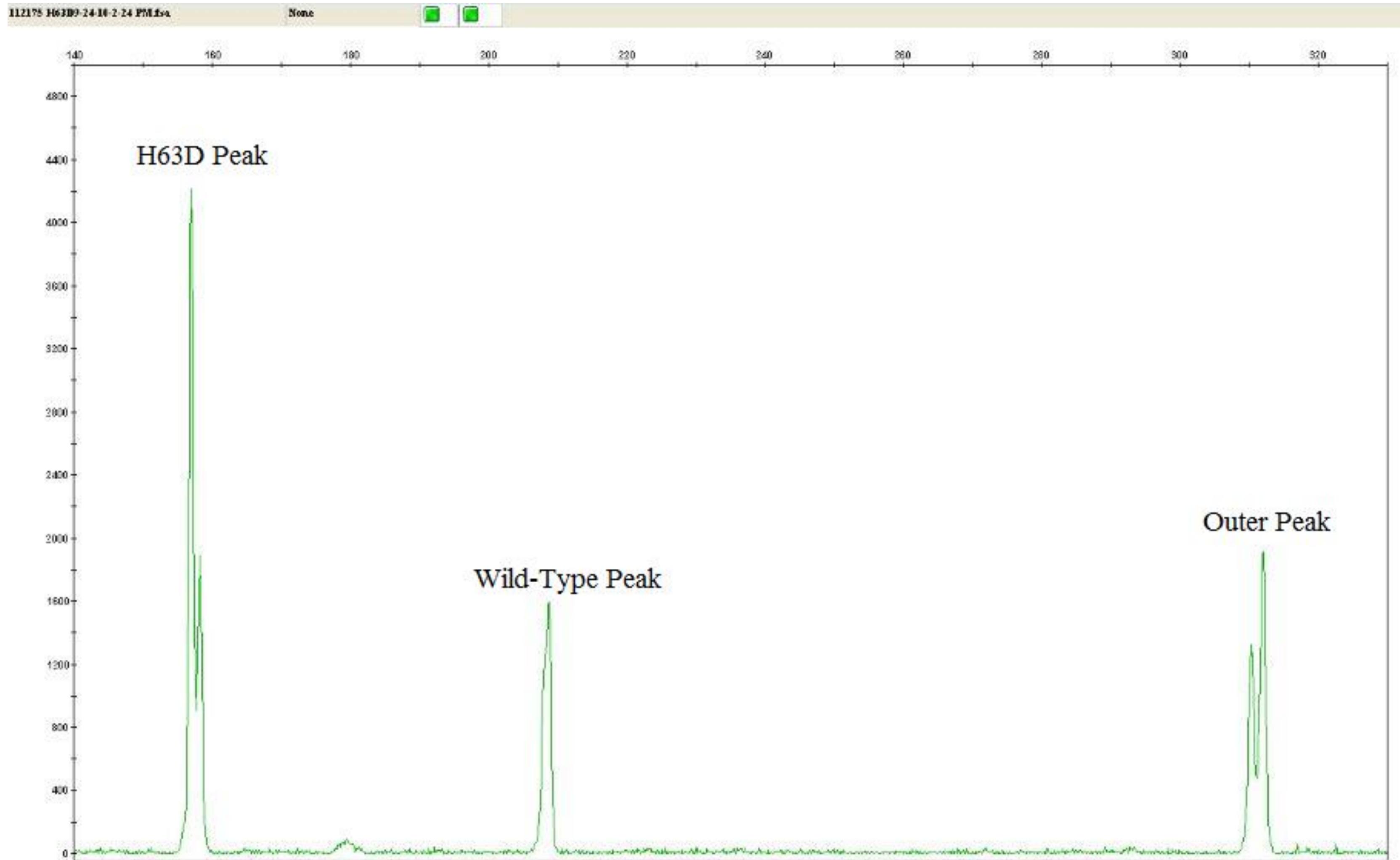

Figure 10: Heterozygous for H63D. The above individual is among the 26.6% of the population found to carry the H63D mutation, a mutation that is normally only clinical relevant is cases of compound heterozygosity. This PCR successfully amplified the H63D allele at 158, the wild-type allele at 207, and the outer non-allele specific product at 310 bp, indicative of an individual carrying both the wild-type and mutant form of the gene. The x-axis indicates the size of PCR product in base pairs.

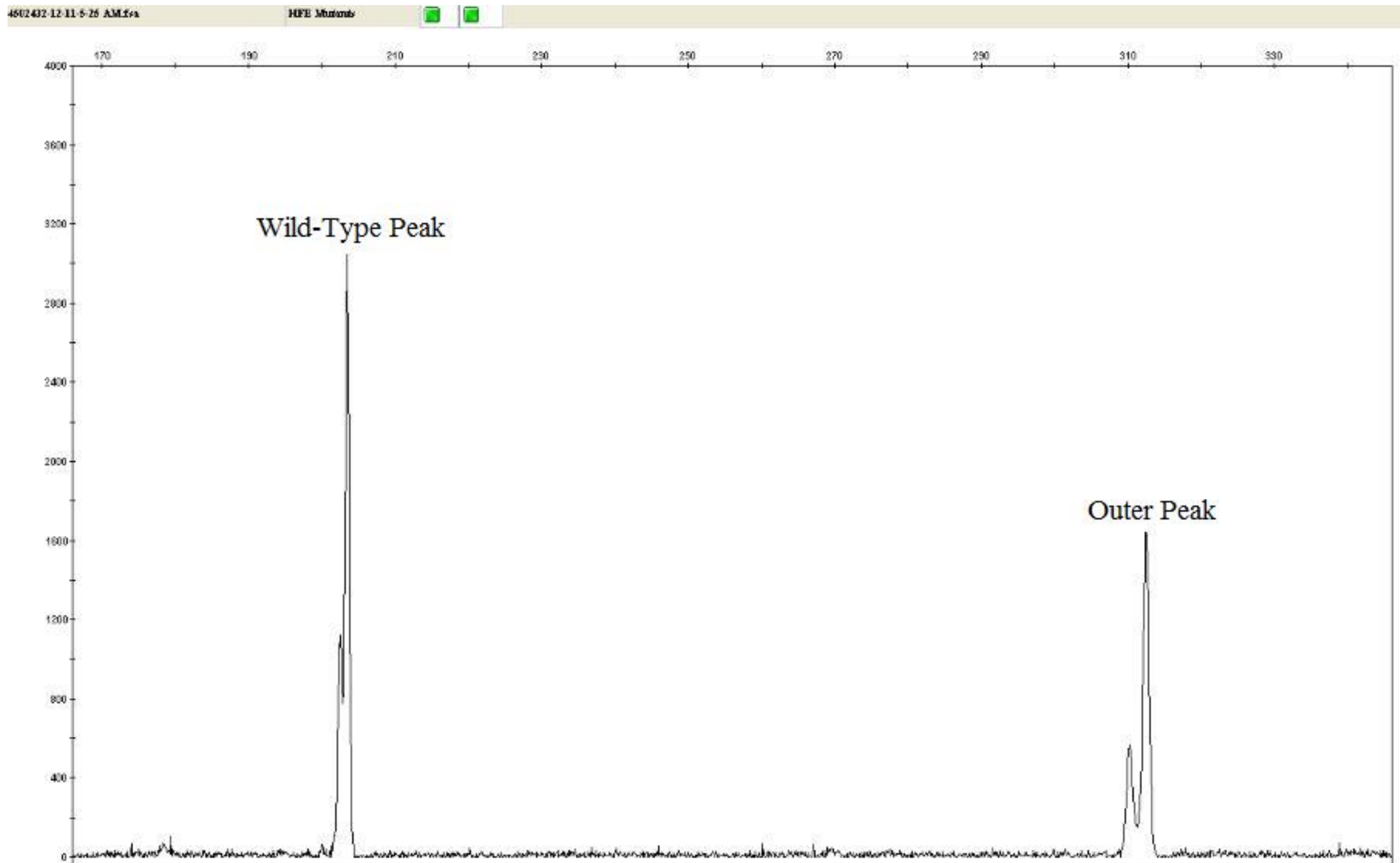

Figure 11: Wild-Type for S65C SNP. The above electropherogram shows peaks at 201 and 310 that are labeled with the TAMRA fluorescent dye that appears yellow on the genetic analyzer. Yellow peaks are colored black on Gene Mapper electropherograms to facilitate viewing. The size of the yellow fragments indicate that this individual is homozygous wild-type for the S65C SNP; no mutant allele is present at 164 bp. 98.4% of the Houghton population were wild-type for this SNP.

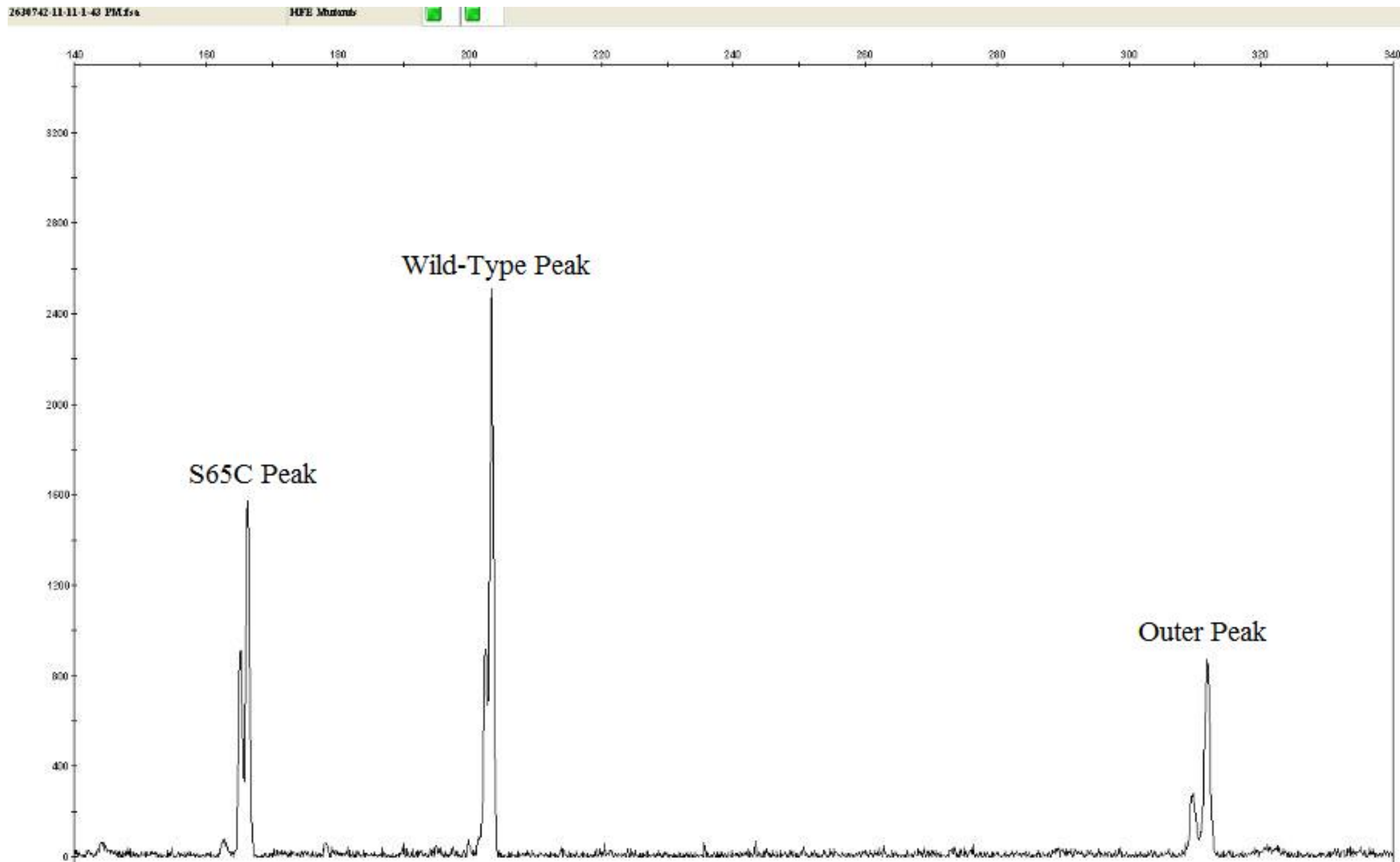

Figure 12: Heterozygous for S65C. This sample shows amplification of both the S65C and the wild-type allele, in addition to an outer non-allele specific product. Therefore, this individual is heterozygous for the S65C mutation. Only 1.6% of tested individuals, in addition to a sample from Michigan State University's DNA Diagnostic Lab (courtesy of Dr. Sainan Wei), tested positive for the presence of this allele.

Sequencing

Sequencing was an essential component of this project to confirm the genotyping derived from tetra-primer ARMS PCR. Due to the time intensive and expensive nature of sequencing, not all samples could be sequenced. Nor could the entirety of the 13,000 bp *HFE* gene be sequenced to scan for mutations. Instead, sequencing efforts were focused on 300-bp regions of exon 2 and 4 that contained the SNPs of interest. DNA samples from six individuals were sequenced to determine the accuracy of genotyping by ARMS. Amongst these six samples, both mutant and wild-type forms of each SNP were observed by ARMS. Results of sequencing confirmed the accuracy of genotyping by ARMS as both methods yielded the same results. Electropherograms below (Figures 13-15) give representative samples of sequencing data. Homozygous individuals provide the best DNA template as only one peak is evident per base. Heterozygous individuals are slightly more difficult to genotype by sequencing as they are indicated by the presence of a mixed base; two smaller peaks are present in heterozygous individuals instead of one large peak.

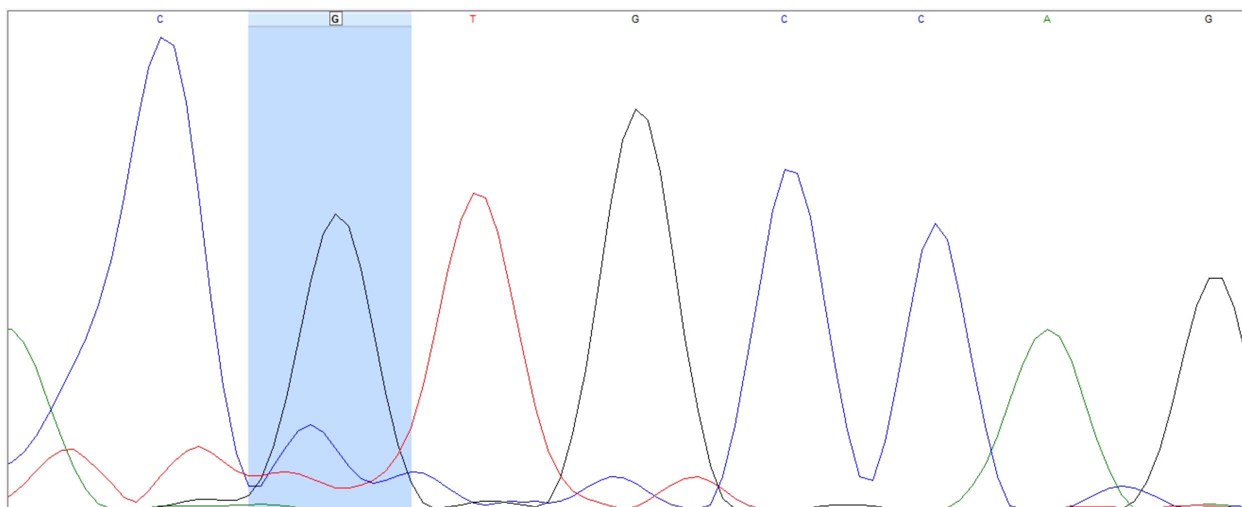

Figure 13: Sequence Analysis of the Wild-type Allele at the Site of the C282Y Mutation. This sample indicates the individual is homozygous wild-type at the site of the C282Y mutation. C282Y mutates a guanine to an adenine at this position. Highlighted above it is possible to see the presence of a single black guanine peak, indicating wild-type.

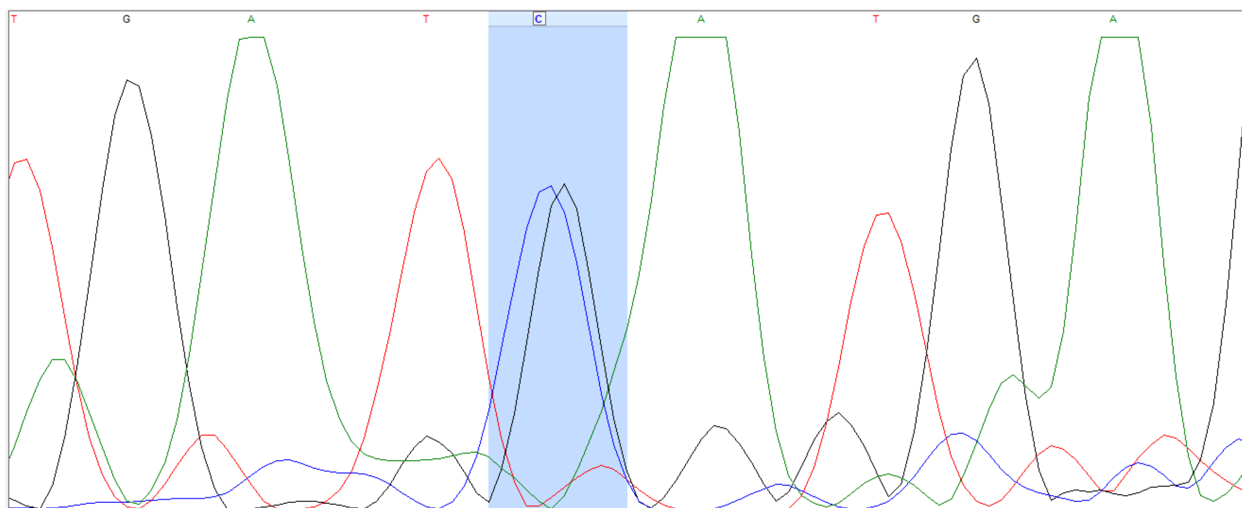

Figure 14: Mixed Base for H63D Indicates Heterozygosity. The above highlighted region contains two peaks, indicative of a mixed base for H63D found on exon 2. The wild-type peak is blue for cytosine, while the H63D is black for guanine. H63D mutates a cytosine to a guanine.

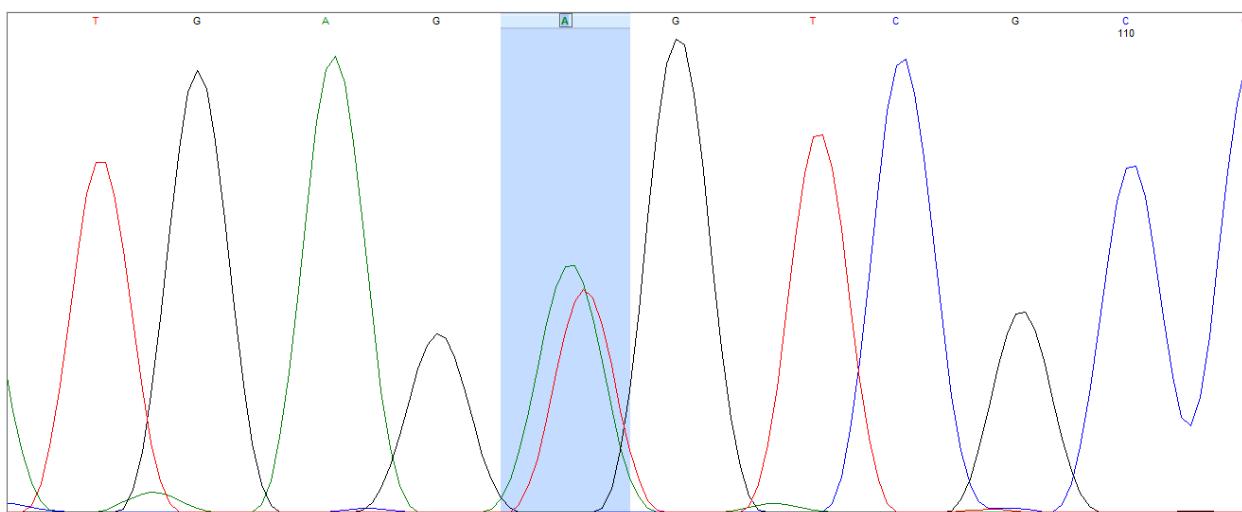

Figure 15: Mixed Base for S65C Indicates Heterozygosity. The above highlighted region contains two peaks, indicative of a mixed base for S65C found on exon 2, just six base pairs away from the site of the H63D SNP. The wild-type peak is green for adenine, while the S65C peak is red for thymine. S65C mutates an adenine to a thymine.

Allelic Frequencies

After confirming the validity of this genetic testing method by sequencing, the expected frequency of genotype was calculated based on the model given by Hardy-Weinberg equilibrium equation ($p^2 + 2pq + q^2 = 1$). This was done particularly to determine if the observed distribution of genotypes was reasonable. Values p and q represent the frequency of each allele in a population; the frequency of all alleles in a population should add to 1. The observed allelic frequency of p (the dominant allele) was calculated by adding the frequency of the homozygous dominant genotype to half of the frequency of heterozygotes, while the observed frequency of q was determined by adding the frequency of homozygous recessive individuals to half the frequency of heterozygotes. In the Hardy-Weinberg equation ($p^2 + 2pq + q^2 = 1$), homozygous dominant wild-type individuals are represented by p^2; the frequency of heterozygous individuals is 2pq, while homozygous recessive mutants are q^2. Using this equation, it is possible to calculate the expected distribution of genotype frequency under a Hardy-Weinberg model based on the observed allelic frequencies. See Table 11 for calculations.

	C282Y	**H63D**	**S65C**
Observed Frequency of Dominant Allele (p)	0.93	0.87	0.99
Observed Frequency of Recessive Allele (q)	0.066	0.13	0.0078
Expected Frequency of Homozygous Dominant Genotypes (p^2) Assuming Hardy-Weinberg Distribution	0.87	0.75	0.98
Expected Frequency of Heterozygous Genotype (2pq) Assuming Hardy-Weinberg Distribution	0.12	0.23	0.015
Expected Frequency of Homozygous Recessive Genotypes (q^2) Assuming Hardy-Weinberg Distribution	0.0043	0.018	0.000060

Table 11: Calculation of Allelic Frequencies. Using the genotypic frequencies in Table 9, observed allelic frequencies of the dominant (p) and recessive (q) alleles for each SNP were calculated. Based on these observed frequencies, the expected distribution of genotypic frequencies could be calculated for the population using the Hardy-Weinberg model. Hardy-Weinberg assumes homozygous dominant individuals are represented in the population by p^2, heterozygotes by 2pq, and homozygous recessive mutants by q^2.

Genetic Testing Survey Results

In February of 2011, an online survey was administered via www.surveymonkey.com to 105 individuals (an 80% response rate out of 132 total individuals) who had donated DNA to the genetic testing phase of the project. The goal of this survey was to assess attitudes about genetic testing, as well as to assess participants' understanding of the genetic abnormality for which they were being tested. Individuals were advised of the confidentiality and anonymity of the survey and allowed to skip any question they did not wish to answer. Tables 12-14 below list the demographics of the surveyed population, background knowledge regarding hemochromatosis, and summarize the most pertinent elements of survey responses.

	Responses:	**Comments:**
Major:	27 different majors represented 22% Unknown/Undeclared major 53% Science majors represented 25% Non-science majors	*Science major defined as: Biology, Biochemistry, Chemistry, General Science, Math, Physics, Psychobiology *Double majors where one degree was science were counted as science majors
Grade Level:	13% Freshman 19% Sophomore 29% Juniors 28% Seniors 11% Not Applicable	
Age:	Age range from 18-54 years 94% of those surveyed were between 18-23 years	
Gender:	37% Male 61% Female 2% No response	
Religion:	12% Baptist 28% Nondenominational 6% Presbyterian 6% Roman Catholic 15% Wesleyan 2% Atheist/No religion	*A minimum of 16 denominations were represented in the survey population *Other represented Protestant groups each made up less than 6% of those surveyed

Table 12: Demographic Data of 105 Survey Respondents. As a Christian liberal arts college, Houghton College's demographics are unique. 98% of respondents self-identify as Christian with only a small minority associating themselves with Atheism/No Religion. As a college campus, most individuals range from 18-23 years of age with a few outliers. The male:female ratio is also typical of the Houghton campus. Most interesting is the high percentage of science majors who participated in this study. 53% of participants declared a science major.

Question	Responses	Comments
My primary motivation for participating in this study was (choose one):	• 81% indicated that their primary motivation was "to help with a research project" • 12% wanted to know if they carried any disease-causing mutations • 2% were interested in the disease • 5% Other response	
Before participating in this study, had you heard of hereditary hemochromatosis?	• 65% had never heard of hemochromatosis prior to this study	• It was expected that a relatively high number of individuals on campus would have heard of HH due to prior research on the disease last spring by the Human Genetics and Disease class.
Does anyone in your close family (siblings, parents, grandparents, first cousins) have hereditary hemochromatosis?	• 0.95% (1 out of 105 participants) had a close relative with hemochromatosis	
Have you known anyone with hereditary hemochromatosis?	• 8% of participants knew someone with the disease	• 100% of participants who answered "yes" to this question were able to define hereditary hemochromatosis
If you can, please briefly describe what hereditary hemochromatosis is:	• 47% of all respondents were able to define HH correctly • 48% of all science majors responded correctly • 50% of biology majors responded correctly • 23% of non-science majors responded correctly	• "Correct" responses were generously graded with the expectation that respondents would mention iron accumulation or HH symptoms in a correct answer. • 3% of respondents admitted to using an Internet source in their definition. These answers were still counted as "correct."
The usual treatment for hereditary hemochromatosis is:	• 56% answered correctly by indicating "Giving blood" as the treatment out of a list of options • 37% incorrectly answered that "There is no treatment"	a. Other possible choices in this multiple choice question were: a. Antibiotics b. Antiviral medications c. Chemotherapy f. There is no treatment
Hereditary hemochromatosis is usually caused by:	• 90% were able to identify this as a multifactorial genetic disease	• Other possible choices in this multiple choice question were: a. Bacteria b. Virus c. Carcinogens e. The cause is unknown

Table 13: Background Information of Respondents. The above table summarizes responses to background questions. The purpose of these questions was to determine level of exposure to information about hemochromatosis.

Statement:	Agree	Neutral	Disagree	Comments
I plan to tell my family about the results of this test.	58%	6%	6%	• 0% of participants chose "Strongly disagree"
If I test positive for hereditary hemochromatosis, I will ask my doctor for further information about preventing potential symptoms of the disease.	87%	7%	4%	• 0% of participants chose "Strongly disagree"
I fear genetic testing will lead to discrimination (in health insurance or employment, etc.) if I test positive.	9%	31%	60%	• 0% of participants chose "Strongly agree"
I fear genetic testing will eventually lead people to abort "imperfect" offspring.	41%	24%	33%	
I fear genetic testing will lead people to select for certain traits when using reproductive technologies such as in vitro fertilization (IVF).	56%	23%	18%	
Parents should not be allowed to use genetic testing to select for the physical traits of their children, such as gender, eye color, or height.	67%	16%	15%	
Parents should be allowed to use genetic testing to select for traits in their offspring if it allows them to have children who are disease-free.	28%	34%	35%	
If I carried a disease-causing mutation for hereditary hemochromatosis, I would decide to not have children.	11%	26%	57%	
If I test positive for hereditary hemochromatosis, I will encourage my relatives to be tested.	62%	27%	6%	
If I knew I carried any disease-causing mutations that would affect my children, I would be sure to have my spouse tested before having children.	68%	23%	7%	
If both my spouse and I carried a disease-causing mutation for a severe untreatable genetic disease such that 25% of our children would have the disease, I would still plan to have children.	27%	43%	28%	
If both my spouse and I carried a disease-causing mutation for a treatable genetic disease, such that 25% of our children would have the disease, I would still plan to have children.	63%	28%	7%	
It is morally acceptable to abort a fetus if genetic testing shows the child has a severe genetic defect and will likely only live a painful 6 months after birth.	12%	17%	68%	

Table 14: Survey Responses for Genetic Testing Survey from February 2011. Above is a summary of pertinent elements regarding genetic testing survey responses. Participants were given a statement and asked to rate their agreement or disagreement using the following scale: strongly agree, agree, neutral, disagree, strongly disagree, or NA. The above summary is very much condensed; "strongly agree" and "agree" responses were consolidated into the "Agree" column, as were "strongly disagree" and "disagree" responses.

Discussion

Human molecular genetics is transforming medicine by providing early detection of genetic conditions, estimation of disease risks, and essential data about gene variants that affect drug metabolism. A multitude of uses are currently available for predictive genetic testing, but often these are limited by scientific, social, ethical, and legal objections. A primary goal of this project was to develop a relatively simple and reliable genetic test for three mutations (C282Y, H63D, and S65C) associated with hereditary hemochromatosis in order to screen 132 volunteers from the campus of Houghton College, a small Christian liberal arts college in western New York. These single nucleotide polymorphisms (SNPs) were tested for using a tetra-primer amplification refractory mutation system (ARMS) touchdown PCR, followed by capillary electrophoresis to separate PCR fragments and thus determine the genotype for each SNP.

However, this project also aimed to examine the socio-ethical implications of genetic testing for hereditary hemochromatosis. Although initially considered a "poster-child" for genetic testing, recent research in the field of hemochromatosis has cast a negative light on suggestions regarding the implementation of wide-scale genetic screening. The effects of modifier genes, diet, age, and gender significantly impact the expression of the disease phenotype in hereditary hemochromatosis. Thus, bioethicists are left to question if the benefits of population screening for this disease outweigh the risks of discrimination, undue anxiety, and fears of eugenics. In this study, 105 volunteers who had previously donated DNA to the project were surveyed to assess bioethical perspectives on genetic testing within the Houghton population. By offering genetic testing to the Houghton community, the project offered a vehicle to encourage campus discussion of bioethical issues and education about genetic diseases, while providing participants with information that could be used in preventative care.

This project successfully developed a tetra-primer ARMS PCR genetic test for three missense mutations in the *HFE* gene associated with type I hereditary hemochromatosis, a condition characterized by excessive absorption of dietary iron that leads to iron-overload and organ damage. Out of 132 DNA samples collected, 129 were genotyped by this method, which was calculated to cost about $1.75 per sample. The genetic testing methodology was then validated by sequencing relevant portions of exons 2 and 4 of the *HFE* gene. Six samples were sequenced, confirming each of the genotypes determined by ARMS PCR. Of the three samples that failed to be genotyped by ARMS PCR, the outer PCR products could not be amplified. The pH and concentration of these samples were checked to ensure that they were within desired parameters. Failed samples were initially re-diluted in TE buffer, and subsequently in Tris buffer (in case the EDTA was inhibiting the DNA polymerase in PCR), but amplification was still unsuccessful, even after the addition of PCR cycles and an increase in final extension time. It is hypothesized that these samples failed due to DNA degradation, making it unfeasible to amplify the larger outer fragments.

The first mutation tested for, C282Y, converts a cysteine to a tyrosine at position 282 of the resulting protein and abrogates a disulfide bond necessary for localization to the cell membrane. Carried by about 1 in 10 Caucasians, this mutation is usually considered the most clinically significant and is found on 85% of hemochromatosis patient chromosomes.[27] H63D, in contrast, is more prevalent (carried by 24% of Caucasians) but yields milder phenotypic effects. This mutation is thought to be clinically relevant when carried along with another mutation such as C282Y. The third mutation and least studied due to its rarity (carried by only 2-3% of the population) converts a serine to a cysteine, and like H63D is thought to cause only mild cases of iron-overload unless combined with another mutation.

Genotyping for each SNP revealed a carrier rate of 13.2% for C282Y, 26.6% for H63D, and 1.6% for S65C in the Houghton population. Compound heterozygotes, C282Y/H63D and C282Y/S65C, were also represented in the population at 1.6% and 0.78%, respectively. No homozygous individuals were found in the population of 129, which is not completely unexpected. Of the above participants found to carry mutations in the *HFE* gene, the predicted risk of disease is low. Although estimates vary by study, Allen et al. reported in a 2008 paper that among males the penetrance of the C282Y homozygous genotype is about 28.4%, while penetrance in females is a low as 1.2%.[30] Other studies indicate that compound heterozygotes for C282Y/H63D have an even more reduced risk of disease with only about 0.5-2.0% of individuals ever developing symptoms. H63D homozygotes are suspected to have an even lower likelihood of developing symptoms,[37] while the penetrance of S65C has been challenging to establish in light of its rarity. Therefore, of the above observed genotypes, the highest risk groups for developing iron-overload are the compound heterozygotes (C282Y/H63D and C282Y/S65C). But according to the above studies, it is likely that only about 0.5-2.0% of these individuals will ever develop symptoms.

Using a Hardy-Weinberg model, it was also possible to determine the expected frequency of the homozygous recessive genotype from the observed allelic frequencies, and thus assess the likelihood of finding individuals with the at-risk genotype. According to the distribution of allelic frequencies in the Hardy-Weinberg model, 0.43% of the population was expected to be homozygous for C282Y, which in a population of 129 individuals means that less than 1 person should have this genotype. For H63D, the expected frequency was 1.8% for homozygotes, or about 2 people out of 129. As for the rare S65C, the probability of finding a homozygous individual was negligible. With an observed allelic frequency of 0.0060%, it is predicted that

only six people out of a population of 100,000 are homozygous for S65C. From these results, it is not unusual for this small study of 129 individuals to detect no homozygotes. However, it must be noted that although these genotypic and allelic frequencies are qualitatively comparable to published values for Caucasian populations, this is a small, non-random population, and therefore, the observed genotypic frequencies cannot truly be extrapolated to represent any other population.

Invitations to the survey in Appendix D were sent out in February 2011 to participants who donated their DNA to the genetic testing project. Although it was realized that this pool of participants was likely more favorably disposed to genetic testing than the general population (as they had donated DNA to the project), useful data were still obtained about their knowledge and attitudes regarding genetic testing. Several observations were made based on the survey responses listed in Table 12-14.

First of all, the demographic data of the survey should be noted. Overall, the demographics of participants seem to be representative of the Houghton College campus. 94% of participants were between ages 18-23. 98% of respondents self-identified with a Christian denomination, while 2% described themselves as an atheist/no religion. An unusually high percentage of science majors participated in this study however: 53% of participants were science majors, 25% were non-science majors, and 22% were undeclared/not applicable. It was expected that respondents with a science background would be better educated about genetic testing than the average college student and perhaps more interested in promoting the application of genetic testing.

The administration of this survey attempted to answer several questions about the tested cohort: Did individuals understand the testing? Why did they choose to participate? How do

they feel about the applications of genetic testing? And lastly, how will these results, if at all, affect their lifestyle choices?

Scientists and bioethicists often question whether individuals that undergo genetic testing have enough understanding of the disease and associated genes to truly comprehend the meaning of their results. For this reason, genetic counselors are essential to the progress of genomic medicine. After all, genetic testing is somewhat pointless in a medical sense if the patient and/or their physician does not understand the evaluated risk, and may in fact be harmful, causing unnecessary anxiety or conversely, lack of appropriate lifestyle modification. In this study, prior to volunteering, 65% of the population had never heard of hemochromatosis. Only one individual was aware of a close relative with hemochromatosis, but 8% of volunteers did know someone with the disease. When asked to define hemochromatosis, 47% of respondents were able to define the condition appropriately, with some mention of iron-overload or other symptoms. 56% of individuals were also able to select the treatment for hemochromatosis from a list of five choices, and 90% were able to identify this disease as a multifactorial genetic disease. Considering that only 35% of the tested population initially had even heard of hemochromatosis, it could be said that this project did promote the education of participants. However, it is still of concern that 10% of the tested population were unable to identify hereditary hemochromatosis as a multifactorial *genetic* disease out of five options on a survey about *genetic* testing following the donation of DNA to test for a *genetic* disease. The importance of the internet as a source of medical information for 18-23 year olds is also apparent in examining survey answers. 3% of individuals outright admitted to researching the disease prior to answering the knowledge-based questions. These answers were counted as correct since the individual clearly invested effort to understand the disease for which they were being tested.

Additionally, it was predicted that science majors would be more interested in the disease and better equipped to understand the genetic testing process and hereditary hemochromatosis in general due to prior experience in the sciences. This hypothesis was supported by the data. Biology majors did have the highest percent of correct answers when asked to define hemochromatosis (50% correct), followed closely by 48% of all science majors answering correctly. In contrast, 77% of non-science majors answered this question incorrectly. This statistic indicates that scientists obviously need to do a better job of communicating scientific findings to the general public. However, this is difficult when even 50% of biology majors do not have the requisite knowledge. Overall, 47% of participants were capable of defining hemochromatosis appropriately, which is encouraging if it is considered that 65% of the test population had never heard of hemochromatosis prior to the study; at least 12% more of the participants can now explain what hemochromatosis is due to this project.

But perhaps the lack of knowledge regarding hemochromatosis is related to a general lack of interest in the disease being studied. When asked to identify their primary motivation for participation, volunteers overwhelmingly (81%) indicated that their primary motivation for donating their DNA was "to help with a research project." This indicates several points about volunteers. Overall, they obviously were motivated not by interest in the science or what mutations they carry. It appears then that altruism was the primary motivator.

The second idea this indicates is the general lack of concern about the negative effects of genetic testing that bioethicists are often apprehensive of, i.e. discrimination, false negatives/false positives, undue anxiety, fear of discovering a mutation, eugenics, etc. This hypothesis is supported by the perspectives of respondents in the agree/disagree section. 0% of participants strongly agreed with the statement, "I fear genetic testing will lead to discrimination

(in health insurance or employment, etc.) if I tested positive." A mere 9% agreed with this statement, and 60% disagreed. In addition, when asked if respondents would encourage relatives to be tested if they find they carry a mutation, 62% agreed with this statement. Only 6% disagreed. 68% also indicated that if they carried any disease-causing mutations, they would want to have their spouse tested, whereas only 7% disagreed. It seems reasonable to suggest that participants have an overall positive attitude about the promise of genetic testing or they would not encourage their relatives and/or spouse to also be tested.

Perhaps due to the overall youth of the population and the technological environment most of these individuals grew up, it is conceivable that these participants are not as fearful of biotechnology as an older population might be. It would be interesting to survey a wider age range to determine if this is an accurate hypothesis. However, due to the inherent bias of only surveying DNA donors, in reality this trend may not be significant at all. Further studies of those who did not volunteer would be required to state this with certainty.

Despite this optimism in the surveyed group, respondents still expressed concern about the applications of genetic testing technologies. While only 9% said they personally feared discrimination, a much higher 41% did fear the idea that genetic testing would lead to abortion of "imperfect" offspring. 68% of individuals indicated that it was not "morally acceptable to abort a fetus" – even if genetic testing shows that the fetus has a severe genetic defect and will die at 6 months of age. This statistic is most assuredly influenced by the strong condemnation of abortion by most Christian traditions. 56% also agreed that genetic testing would lead to the selection of certain traits using reproductive technologies such as in vitro fertilization. 67% thought that parents should **not** be allowed to use genetic testing to select for children with desirable physical traits such as gender, eye color, or height, but the use of this same technology

was much more acceptable to the population when used to prevent disease. In the case of disease, only 35% of the population rejected this use of technology as immoral. The impulse to prevent disease in our offspring is strong, but it is ironic that the technology that allows for it is based on the same technological principles as selecting for blond hair and blue eyes, which many would consider a new form of eugenics.

The final question answered by this survey was simply how will these results affect lifestyle choices of participants? If they test positive, will they engage in measures to prevent symptoms of iron-overload? 58% indicated that they would tell their family about the results of this test, and 87% agreed that if they tested positive for mutations, they would ask their doctors for further information about preventing potential symptoms. Some individuals went even further, agreeing that they would not have children if they tested positive. If they carried a mutation for a "severe untreatable genetic disease such that 25% of their children would have the disease," 28% of individuals would not have children. However, this is matched by an almost equal number of individuals (27%) who would still plan to have children; a relatively large number (43%) were undecided. In contrast, for a "treatable genetic disease," only 7% said they would not have children, while 63% indicated they would desire children. From these statistics, it is apparent that genetic testing is considered useful to this population; information regarding genetic predispositions would seem to impact their lifestyle choices. Nonetheless, as one respondent said, these questions are "very difficult to answer, and for many of them it is hard to know what I would do until I was actually faced with that situation. For some of these questions I don't believe there is a single 'right' answer, and it would also depend on God's leading in a particular situation." Although respondents may have one answer they strongly agree with now, their decisions are likely to change over time. It would be fascinating to determine how and if

perspectives would change after the respondents find out their genetic testing results, and in the long term, to assess how many individuals actually followed through on their expressed intentions.

As Houghton is a unique rural community influenced by its commitment to the Christian faith, it is predicted that survey results would vary tremendously if this study was instead conducted at an urban secular university. A comparison of the similarities and differences between universities would be informative. Determining the extent to which our Christian faith impacts our bioethical views is important in defining ourselves as a Christian community, as well as in outlining the burgeoning field of "Christian bioethics."

In conclusion, participants indicate overall optimism regarding the promise of genetic testing, mixed with hesitance in the application of genetic testing technologies, as well as a need for further education on genetic issues. It is essential before any wide-scale population screening for hemochromatosis commences that more is known about the scientific basis of hemochromatosis and why some individuals show symptoms and others do not, despite having the same genotype. The risks and benefits of genotypic testing should also be compared to the value of phenotypic testing for this condition.

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