

**Development of a Robust Genetic Test for Hyperkalemic
Periodic Paralysis (HYPP) in Quarter Horses**

Samantha L. Traver

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Submitted in partial fulfillment of the requirements
for Honors in the Program of Biochemistry

UNION COLLEGE

June, 2011

ABSTRACT

TRAVER, SAMANTHA L. Development of a robust genetic test for hyperkalemic periodic paralysis (HYPP) in quarter horses. Department of Biological Sciences, June 2011.

A single nucleotide substitution in a region of the skeletal muscle sodium channel gene (*SCN4A*) is known to cause an equine genetic disorder known as Hyperkalemic Periodic Paralysis (HYPP). The clinical effects of this disorder range from little or no symptoms to frequent episodes of muscle tremors, weakness, and/or complete collapse. Oligonucleotide primer pairs were designed for both the wild type and mutant alleles of the *SCN4A* gene for use in Amplification Refractory Mutation System-PCR (ARMS-PCR). These primers were tested with genomic DNA isolated from whole blood, saliva swabs, and hair of individual horses. It was determined that horse hair represented the most easily obtainable and reliable source for genomic DNA isolation. DNA was isolated from the hair of unaffected (wild-type) horses (N/N), a carrier for HYPP (N/H) and a homozygous mutant for the disease (H/H). As expected for the unaffected individual, a PCR product using the wild type-specific primer pair was generated, while the mutant-specific primer pair did not produce a product. DNA isolated from HYPP animals produced a PCR product with only the mutant-specific primer pair, and the carriers for HYPP produced a product in both wild type and mutant-specific PCR reactions. This robust DNA-based test was shown to generate an unambiguous assignment of the genetic status of the horses tested with respect to the HYPP trait.

ACKNOWLEDGEMENTS

I would like to thank:

My advisor, Stephen Horton for his assistance, guidance, and dedication to this research project as well as the sharing of his knowledge and expertise in this field of study. I have learned a great deal and would not have been able to successfully complete this project without his help and encouragement.

The Fox Research Lab and Matt Hanley (Horton lab) for their time, advice, and encouragement during weekly meetings throughout the process of this research project.

Bob Doyle, Jim Groff Quarter Horses, and Valley Quarter Horses for directing me to and providing me with carrier and mutant horses, whose DNA and HYPP statuses I was able to use to confirm the accuracy of this test.

Sharon Spier D.V.M, UC Davis for her knowledge, development of the current test, previous research and suggestions provided throughout the experiment.

The Internal Education Fund at Union College for funding this project.

The Union College Department of Biology for allowing me to utilize their resources, instrumentation, and supplies.

My parents, Ray and Cherrice Traver for their loving support and motivation to accomplish my goals in every aspect of life.

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INTRODUCTION

In March of 2007 the DNA sequence of the horse (*Equus ferus caballus*) genome became available to public databases, which provided ample opportunities for scientific and genetic researchers (Chowdhary and Raudsepp, 2004). Specifically, genetic diseases could now be more closely investigated and specific mutations could be identified and connected to such diseases. The breeding of horses is highly regulated among various breeds, and bloodlines are often diligently recorded and traced by breeders with hopes to produce the most optimal and successful offspring. Along with desirable characteristics being passed down through the lineages, genetic mutations manifest themselves the same way. With the sequencing of the horse genome and identification of certain mutations, this pattern of proliferation of genetic diseases can now be characterized and diagnostic tests can be provided to detect mutations in individual horses. If these mutations could be detected, the tendency to breed affected individuals could be reduced as a result, thus lowering the percentage of affected offspring and eventually decreasing the prevalence of certain harmful genetic diseases in the equine population.

HYPP Genotype

Hyperkalemic Periodic Paralysis (HYPP) is an autosomal dominant genetic disease that affects approximately 4% of all Quarter Horses (Spier, 2006). The disease is caused by a single nucleotide polymorphism (SNP), or substitution of cytosine to guanine in the skeletal muscle sodium channel gene (*SCN4A*) (Rudolph *et al.*, 1992). This mutation is found at position 14,273,176 on chromosome 11 (Rudolph *et al.*,

1992) and results in an amino acid substitution of phenylalanine to leucine in the SCN4A protein product. This substitution causes a conformational change in the sodium ion channel, thus leading to symptoms of varying severity regarding ion regulation, including that of potassium. The genetic mutation has been traced back to one individual, a Quarter Horse stallion called Impressive, who was a carrier of HYPP and was born in 1968. This particular stallion was highly bred due to his stunning conformation and muscle configuration (Finno *et al.*, 2009). HYPP statuses are denoted by abbreviations, as shown in Table 1 below.

HYPP Status	Genotype	Abbreviation
Affected	Homozygous for the mutant allele	HH
Carrier	Heterozygous for the mutant allele	NH
Wild-Type	Homozygous for the wild-type allele	NN

Table 1: Abbreviations for HYPP statuses and genotypes that will be used throughout this paper.

HYPP Phenotype

Clinical signs of HYPP can range from little or no symptoms to frequent episodes of muscle tremors, weakness, and/or complete collapse, often following a period of exercise (Spier, 2006). These episodes are caused by fluctuation of potassium levels in the blood that causes reactions of varying severity within the muscle (Spier, 2006). Many descendants of Impressive who are carriers of this genetic disease seem to have also inherited Impressive's physical characteristics such as defined muscle mass and attractive conformation, both of which are desirable traits in the halter-horse show industry. However, when two carrier individuals are bred, approximately one quarter of their progeny will have HYPP. A picture of

Impressive's well defined muscle mass and desirable conformation is shown in Figure 1 below.



Figure 1: HYPP carrier Impressive, born 1968, who was highly bred due to his desirable physical characteristics for halter show horse breeding and showing, such as defined muscle mass and correct conformation. (<http://crazyladyappaloosas.com/images/impresiveside.jpg>)

In the past, it was felt that the positive attributes of the carrier phenotype outweighed the risk of progeny having HYPP, and halter-horse show breeders sought out Impressive descendents. The negative attributes of HYPP seemed less significant to this particular industry since exercise is not necessary in halter-horse classes, so the episodes of HYPP would not affect the score and placement of the horse when being judged. In everyday life, however, the clinical signs and symptoms of HYPP are still very prevalent and very dangerous. In 1998 the American Quarter Horse Association (AQHA) recognized the negative affects of perpetuating the mutant allele, and made genetic testing mandatory for registration if the horses were descendents of Impressive (Spier, 2006). An increase in the use of genetic testing for HYPP carriers and selective breeding of only “clear” individuals could eventually eradicate the disease from the breed.

Amplification Refractory Mutation System - Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) is a widely used experimental technique, in which one can amplify regions of genomic DNA, or replicate a specific sequence of the original DNA template to create a large number of copies of that sequence or gene of interest. The process begins by the denaturing of a starting DNA template. Primers, or short strands of DNA sequences then anneal to the single stranded DNA and a DNA polymerase builds a new strand, using the original DNA as its template. This cycle occurs in fluctuating temperatures and repeats several times until the original DNA has been turned into hundreds of thousands of copies (Mullis *et al.*, 1989).

Amplification Refractory Mutation System- PCR (ARMS-PCR) can be used to identify single nucleotide polymorphisms, or different allelic genotypes by creating nucleotide-specific primer pairs for both the wild type and mutant alleles for a specific gene (Newton *et al.*, 1989). ARMS-PCR differs from general PCR in the generation of primers as well as the analysis of gel electrophoresis. With this technique, two PCR reactions are run with one genomic DNA sample; one with the wild-type specific primer pair and one with the mutant specific primer pair. The two primer pairs have a common downstream primer, and the upstream primers differ by the penultimate, or second to last nucleotide in the primer and the final nucleotide, or the nucleotide that varies between the wild-type and mutant allele of the gene (Ye *et al.*, 2001). This concept is shown below in Figure 2. The wild-type primer corresponds to the wild-type nucleotide at the site of the mutation in the genome, while the mutant primer corresponds to the mutated nucleotide at that position. Along

with this allelic specificity of the final nucleotide, changing the penultimate position is also important to ARMS-PCR to increase the instability of each primer pair to the DNA template (Ye *et al.*, 2001). This helps to ensure that the wild-type primer pair only anneals to and produces a band with a wild-type allele and the mutant primer pair only anneals to and produces a band with a mutant allele. The use of both primer pairs in two separate PCR reactions allows for the identification of differing alleles of certain genes containing a single nucleotide polymorphism.

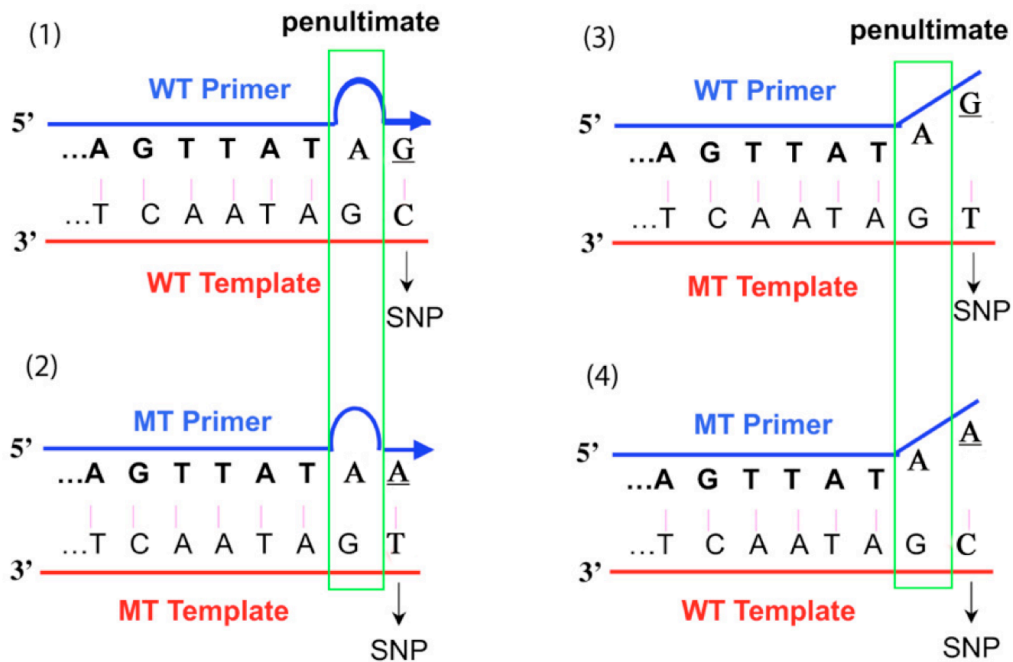


Figure 2: Variation of the penultimate, or second to last nucleotide increases the specificity of the two reactions. For example, wild type DNA templates still anneal accurately to wild type specific primer pairs because of the accurate pairing of the final nucleotide, while the mismatch of the penultimate nucleotide of the mutant primer would cause destabilization and yield a negative product, as anticipated. Thus, only wild type specific primer pairs anneal to wild type templates and only mutant specific primer pairs anneal to mutant templates.

Experimental Goals

Although there is currently a working diagnostic test for HYPP, the goal of this project was to create a different efficient, robust, and unambiguous DNA-based test for this disease. With multiple efficient and cost effective tests in conjunction with the new AQHA registration guidelines, breeders will be more likely to test their animals and selectively breed them, allowing for the eventual eradication of the disease. The current test involves PCR reactions that amplify a section of DNA that includes the cytosine to guanine substitution (Rudolph *et al.*, 1992). These PCR reactions are then followed by allele-specific oligonucleotide hybridizations. Analysis of these allele-specific hybridization reactions can determine which alleles the genomic DNA sample contained, depending on which oligonucleotide probe the PCR products bound to, thus providing the HYPP status of the individual (Rudolph *et al.*, 1992).

By using ARMS-PCR for my diagnostic test, the allele specificity lies within the PCR primers themselves, thus avoiding the need for oligonucleotide hybridization. The goal was to develop a test that provides unambiguous results after just two PCR reactions and gel electrophoresis analysis. Hair samples were obtained from previously tested NN, NH, and HH horses and their DNA was isolated for PCR. Both wild type and mutant specific primer pairs were designed and generated. These primer pairs were combined with each of the DNA samples in two different PCR reactions, which were analyzed with gel electrophoresis.

MATERIALS AND METHODS

Design of Oligonucleotide Primers

The National Center for Biotechnology Information, or NCBI, website (www.ncbi.nlm.nih.gov/) was used to access the horse genome, as well as to locate the sodium ion gene, *SCN4A*. The single nucleotide substitution was identified within the genome at nucleotide position 14273176. The region of the gene surrounding this mutation was used to design the allele specific oligonucleotide primer pairs, and is shown in Figure 3 below.

```
GAAGGAAGGGTCCCAGATTGAGGAAAGATGTCCACGGACCCCATAGAATGGCCTGGTCTGATA
GCTCTCCGCTCCCTGGCCTCCACCCTGCAACTTCACCACAAACCCTGATACCTCCTCTCCCTCT
CTCTCTCCCTCTCCTCTTTGTTATGCTGTCGTGTCATCCTGTGTCTCCACCTGTTCATGTCTGT
GTGGGCCCTGACCACTCCTCCACCCATATCCACCCATGTACATCCCTGCACTTCCATTCACTCTG
CAGAACAAGATCCAGGGCATGGTGTATGACTTTGTGACGAAGCAGGTGTTGACATCACGATC
ATGATCCTCATTTGCCTCAATATGGTCACCATGATGGTGGAGACGGACGACCAGAGCCAGCTCA
AGGTGGACATCCTGTACAACATCAATATGGTCTTCATCATCGTCTTCACGGGGGAGTGTGTGCT
CAAGATGTTCCGCCCTGCGCCAATACTACTTCACCGTTGGCTGGAACATCTCGACTTCGTGGTT
GTCATCCTGTCCATTGTGGGTGAGCAGGACCAGCCTGGGCTGAGTCATTCAGGAAGGCCTGGT
GCTCCCATTCTCGTGTATGCACACCTGCTCATGCACCTGGCATGATCACGCACACTCACACA
TACACATGTGTGTGTGCACCTGGCATTCACTTAATGCACAAAGGCACATGAGGATGGGTTC
ACAGGCAGCCTGTTGCCTGCACACACGTGAATACACCTCCAGAAACATTTACCTGGCTACTAG
ACACACACTCAGGTGCTCATGTGCTTACTGAAAAACGTAAGAAGACAGTTCAGTACCCACAC
ACACTGGAGAAGTGCGTGGACATGTGTAATTATGATAGTAATTAGCTACCATTATTGAGCATT
ACTGTTTGCCTGACGCTGTGCTATGCACTTTACAGGCATTA
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Figure 3: Relevant portion of the *Equus caballus* gene, *SCN4A*, which was used to design the allele-specific oligonucleotide primer pairs to be used in ARMS-PCR. The nucleotide shown in bolded red font is the variable nucleotide at position 14273176, which when substituted for guanine, results in HYPP.

From this genomic sequence, multiple primer pairs were created using the online program, Primer 3 (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). Since the goal of this experiment was to optimize diagnostic ARMS-PCR reactions to create a reliable test, multiple allele-specific primer pairs were necessary to ensure that the region of interest was amplified with allele-specificity dependent on the

primer pair used It was important that the primer pair that was selected for optimization was reliable, so having several pairs to choose from allowed for a greater pool and a higher likelihood of finding an effective pair. Primer pairs were created with allele-specific upstream primers with common downstream primers as well as allele-specific downstream primers with common upstream primers to again ensure that the most effective primer pair would be utilized for further optimization. The primers created are shown below in Table 2.

Specificity	Primer	Sequence (5' → 3')	Expected Product Size
WT Specific Upstream	HYP_WTU1	CACCGTTGGCTGGAACATCTAC	---
MUT Specific Upstream	HYP_MTU2	CACCGTTGGCTGGAACATCTAG	---
Common Downstream	HYP_D3	TCCAGTGTGTGTGGGTA CTGGAA	351 bp
Common Downstream	HYP_D4	ACATGTCCACGCACTTCTCCAGT	428 bp
Common Downstream	HYP_D5	AAAGTGCATAGCACAGCGTCAGG	451 bp
WT Specific Downstream	HYP_WTD6	ACAGGATGACAACCACGAAGTGG	---
MUT Specific Downstream	HYP_MTD7	ACAGGATGACAACCACGAAGTGC	---
Common Upstream	HYP_U8	TCCCACCTGTTTCATGTCCTGTGT	414 bp
Common Upstream	HYP_U9	TCCCTCTCTCTCTCCCTCTCCTC	482 bp
Common Upstream	HYP_U10	CACCCTGCAACTTCACCACAAAC	397 bp

Table 2: Sequences of wild type (WT) and mutant (MUT) primers designed and manufactured along with expected product size for the different combinations of upstream and downstream primers. The primer pairs that were later used for further optimization are shown in bold text.

Genomic DNA Isolation

Three sources of cells were obtained from various horses for isolation and purification of DNA: blood, saliva, and hair. This process of isolation was performed on each source using different protocols within the Qiagen QIAmp DNA Micro Kit. All isolated DNA samples were subjected to Nanodrop quantization to determine their nucleic acid concentrations and relative purity (260/280 ratios). This process

was done in order to ensure that the DNA was isolated properly and that the sample had a high enough concentration of DNA to be an effective template in ARMS-PCR.

The nucleic acid concentrations of all samples are shown below in Table 3.

Source	Designation	HYPP Status	ng/ μ L	260/280
Swab	S1	NN	32.1	1.93
Swab	S2	NN	15.0	2.16
Swab	S3	NN	6.3	1.71
Swab	S4	NN	9.6	2.18
Blood	B1	NN	6.4	1.42
Blood	B2	NN	7.2	1.11
Blood	B3	NN	22.5	1.62
Blood	B4	NN	27.3	1.65
Swab	S5	NN	10.9	2.16
Swab	S6	NN	20.9	2.04
Swab	S7	NN	25.3	2.02
Swab	S8	NN	29.1	1.92
Hair	H1	NN	54.0	1.52
Swab	S8	NH	28.4	1.63
Swab	S9	NH	20.4	1.86
Hair	H2	NN	169.9	2.04
Hair	H3	NH	77.3	1.99
Hair	H4	HH	257.4	2.00

Table 3: All DNA samples used throughout this experiment, the source that they came from, their previously tested HYPP allelic statuses, and their nucleic acid concentrations after isolation. The 260/280 ratios are also shown, which depicts the protein contamination of the nucleic acid sample, depending on its absorbance at 260nm vs. 280nm. NN indicates a wild-type individual, NH indicates a carrier for HYPP, and HH indicates an individual with the full-blown disease. The three samples that were used to confirm the reliability of the final test are shown in bolded text.

Optimization of Polymerase Chain Reaction

In order to use PCR as a diagnostic tool, the procedure must be reliable and reproducible with the chance of false negatives and false positives minimized. To accomplish this, several conditions of the PCR were varied and those that produced optimal results were used for the final test. The conditions that were varied throughout the optimization process were the primer pairs, the source of DNA

samples, the annealing temperature, and the magnesium concentrations within the reactions.

The first condition varied was the primer pairs. Each primer pair was tested with the SNPase PCR kit with 3 μ L of wild-type DNA, isolated from whole blood. The annealing temperature for all initial PCR reactions was set at 55°C and 0.45 μ L of 25 mM magnesium chloride was added to enhance the reactions. The primer pair that gave the strongest positive result with the wild-type specific primer and a negative result with the mutant specific primer was selected for further optimization.

Of the three possible DNA sources, hair from horses' manes was chosen for further experimentation. These isolated samples had significantly higher nucleic acid concentrations than samples from both blood and saliva, shown in Table 3. Along with this quantitative data, this source of genomic DNA was also chosen for practical reasons. Horsehair is very easy to obtain by the horse's owner, dismissing the need for a vet to come out to draw blood, as well as the need to purchase specific supplies, such as saliva collection swabs. Horsehair can be pulled by the owner and can be stored stably in a plastic bag, which can then be directly sent to a lab for testing. This method is the most convenient for horse owners wanting to test their horses, eliminates unnecessary intermediate steps, and reduces the cost of obtaining DNA for testing.

Magnesium concentrations within the PCR reactions were varied simultaneously with the annealing temperature throughout a series of PCR runs, again in order to optimize the clarity and visibility of positive products and to ensure that positive and negative reactions are clearly distinguishable from one another. The

annealing temperatures were varied between 50°C and 64°C and 0, 1, or 2µL of 25 mM MgCl₂ were added to various 15µL reactions.

Gel Electrophoresis

All completed 15µL PCR reactions were combined with 1.5µL of 10X Loading Dye. Each of these 16.5µL mixtures were then loaded into a 1% agarose gel in 10X TAE buffer and subjected to approximately 80 Volts of electrical current for at least 60-70 minutes.

When the PCR bands had migrated through the matrix, the entire gel was stained with Ethidium Bromide for 20 minutes and washed with Nanopure distilled water. This process allowed for the visualization of the bands formed within the gel using a UV Filter in the G:BOX gel documentation system for analysis.

Gene Sequencing

Despite the fact that the PCR products were all around the same size that we were expecting, to ensure that the products were in fact corresponding to the region of the genome we were intending to amplify, the products were sent out to be sequenced (Center for Functional Genomics, SUNY Albany). Two PCR products, one wild type and one mutant (from the same heterozygous carrier individual) were cut out of the gel, and a Gene Clean Turbo kit (Qbiogene) was used to purify the DNA within them and remove any excess nucleotides that may have been present in the reaction after PCR. The DNA sequences were then aligned with the horse genome using BLAT

(<http://genome.ucsc.edu>) to compare the sequence of the PCR products to the intended sequence of amplification.

RESULTS AND DISCUSSION

Optimization Analysis

The first step of PCR optimization was to determine which primer pair would produce reliable results and would be used in further steps for optimization. The primer pairs designed with allele-specific upstream primers and common downstream primers were used in 12 separate PCR reactions. Each common downstream primer was used with both the wild type and mutant specific upstream primers with DNA obtained from both blood and swabs. The DNA samples used in the PCR reactions were coded B4 and S1 from Table 3. These 12 PCR reactions were subjected to gel electrophoresis, and the ethidium bromide stained gel is shown in Figure 4. This analysis showed that of these 12 reactions, the upstream primer HYP_WTU1 produced strong bands with the common downstream primers HYP_D3 and HYP_D4, using wild type DNA from blood, as expected, and both downstream primers did not produce bands with the upstream primer HYP_MT2, as intended. HYP_WTU1 and HYP_MTU2, each paired with the common downstream primer HYP_D3, were chosen for further optimization because these primer pairs not only yielded the expected products with DNA obtained from blood, but were also the only primer pairs that yielded a visible product with the DNA obtained from swabs, seen

in Lane 7 of Figure 4. This suggests that these primer pairs are the most robust with various sources of DNA.

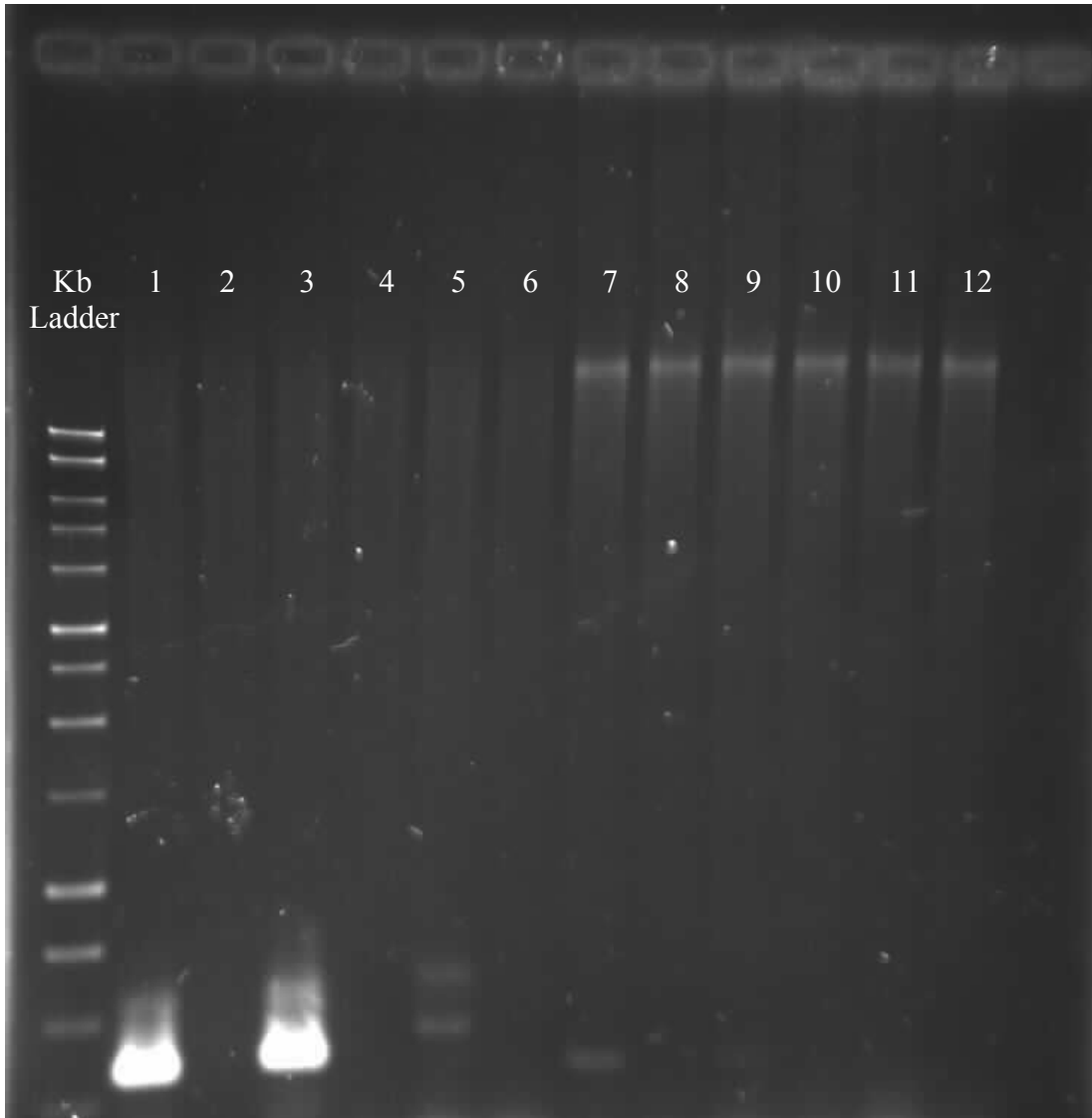


Figure 4: Gel electrophoretic analysis of 12 PCR reactions using allele-specific upstream primers (HYP_WTU1 and HYP_MTU2) with common downstream primers (HYP_D3, HYP_D4, and HYP_D5). All PCR products in odd numbered lanes were run with wild type specific primer pairs and all products in even numbered lanes were run with mutant specific primer pairs. Lanes 1-6 were run with B4 DNA and lanes 7-12 were run with S1 DNA. Lanes 1, 2, 7, and 8 were run using the HYP_D4 common downstream primer, lanes 3, 4, 9, and 10 were run using the HYP_D5 common downstream primer, and lanes 5, 6, 11, and 12 were run using the HYP_D6 common downstream primer.

A similar gel was produced from 12 additional PCR reactions, this time using the two allele-specific downstream primers with the three different common upstream primers, again with both B4 and S1 DNA. This gel is shown in Figure 5 below. This analysis showed that of these 12 PCR reactions and three different common upstream primers, only HYP_U8 was able to clearly differentiate between the wild type and mutant alleles since it produced a band with HYP_WTD6 and did not produce a band with HYP_MTD7, as expected. However, unlike the previous primer pair chosen from Table 2, however, these two primer pairs did not differentiate between the wild type allele and mutant allele with the S1 DNA. Therefore, due to this unreliability in preliminary testing, the previous primer pairs shown in Table 2, HYP_WTU1 with HYP_D3 and HYP_MTU2 with HYP_D3, were used for further optimization.

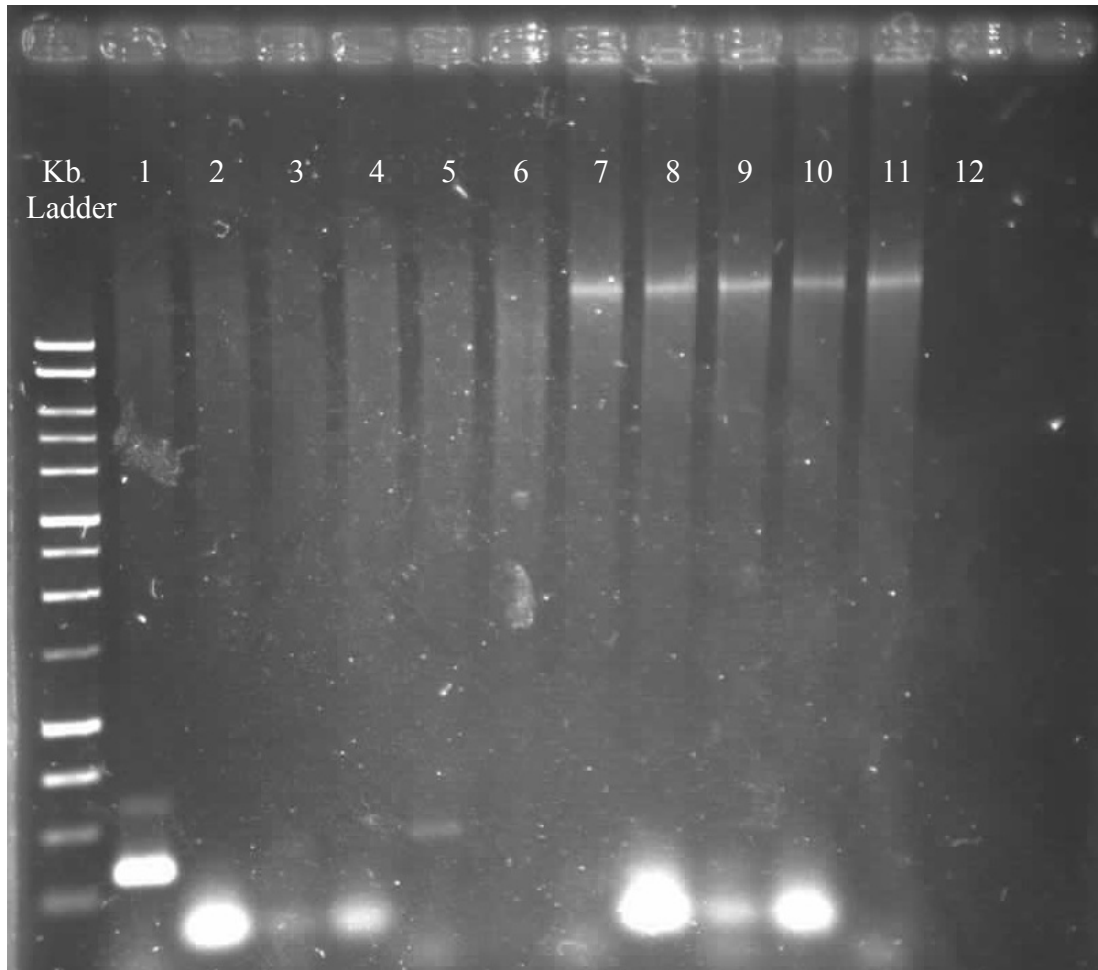


Figure 5: The analysis of 12 PCR reactions using allele-specific downstream primers (HYP_WTD6 and HYP_MTD7) with common upstream primers (HYP_U8, HYP_U9, and HYP_U10). All PCR products in odd numbered lanes were run with wild type specific primer pairs and all products in even numbered lanes were run with mutant specific primer pairs. Lanes 1-6 were run with B4 DNA and lanes 7-12 were run with S1 DNA. Lanes 1, 2, 7, and 8 were run using the HYP_U8 common upstream primer, lanes 3, 4, 9, and 10 were run using the HYP_U9 common upstream primer, and lanes 5, 6, 11, and 12 were run using the HYP_U10 common upstream primer. The PCR reaction designated to be loaded in Lane 12, (HYP_MTD7 and HYP_U10 with S1 DNA) was not loaded into the gel because there was not enough DNA left to have a complete PCR reaction.

Once the allele-specific primer pairs to be used for further optimization were chosen, the next experimental condition varied was the annealing temperature of the PCR reaction itself. Since the annealing temperature of the preliminary PCR test runs for all primer pairs was set at 55°C, a range of 50°C to 60°C was chosen to determine

if a higher or lower temperature would produce clearer or a higher yield of products. Both the wild type specific primer pair and mutant specific primer pair were run at 50°C, 55°C, and 60°C with both B4 and S1 DNA. The gel analysis of these PCR reactions is shown in Figure 6. Strong bands were produced with the wild type specific primer pair at all three temperatures using the DNA isolated from blood, however, only one temperature produced a faint band with the wild type primer pair and the DNA isolated from swabs. This very faint band can be seen in Lane 11 of Figure 6 and was produced by the PCR reaction run at 60°C. This analysis suggested that perhaps temperatures 60°C or higher would be most effective for the diagnostic test.

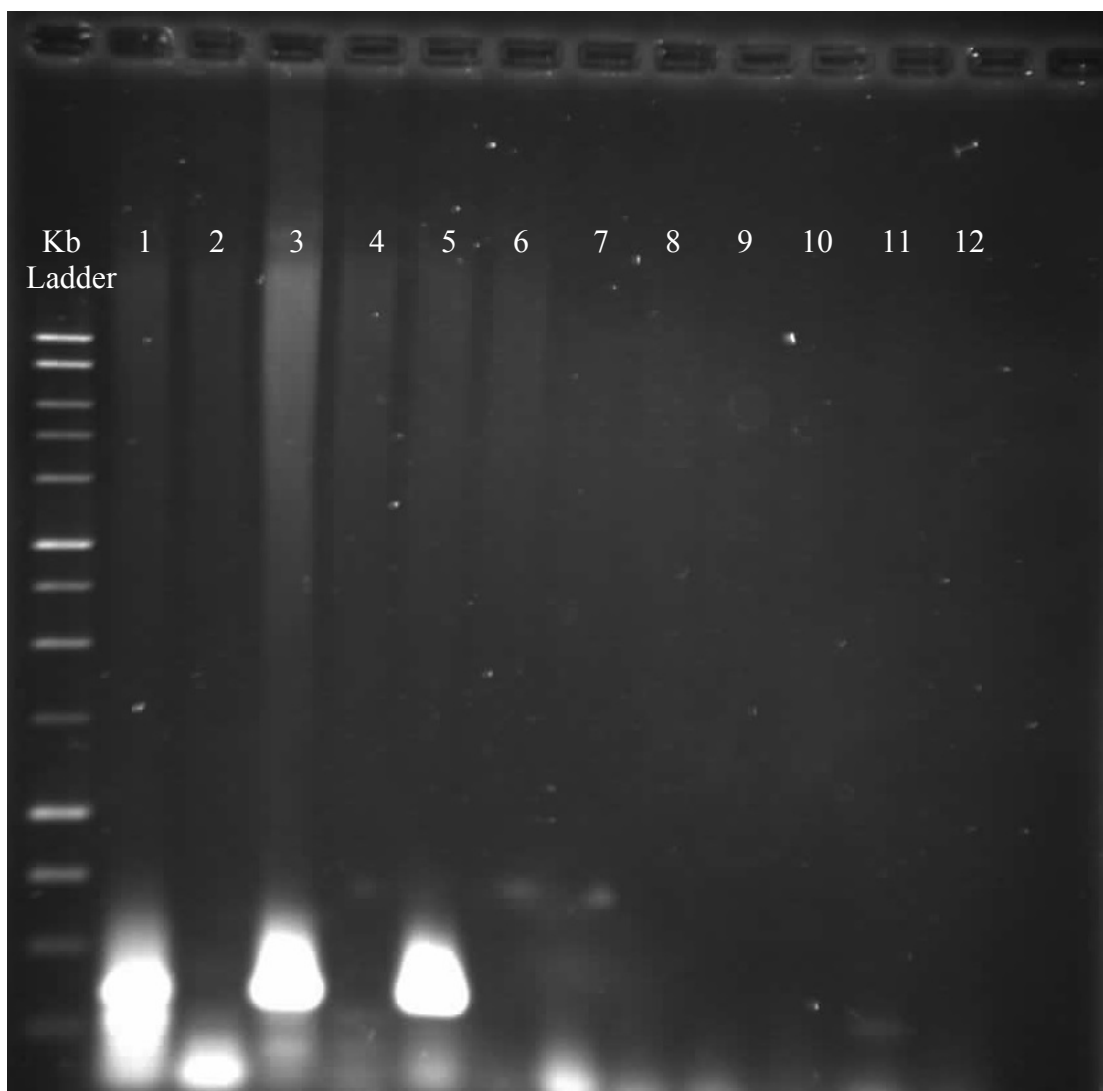


Figure 6: Gel analysis of various PCR reactions with a temperature gradient of 50°C, 55°C, and 60°C. All PCR products in odd lanes were run with the wild type specific primer pair and all PCR products in even lanes were run with the mutant specific primer pair. PCR products in Lanes 1-6 were run with DNA B4 and Lanes 7-12 were run with DNA S1. Lanes 1, 2, 7, and 8 were run at 50°C, Lanes 3, 4, 9 and 10 were run at 55°C, and Lanes 5, 6, 11, and 12 were run at 60°C.

Since the PCR reaction run with the DNA isolated from saliva at 60°C produced a faint band with the wild type primer pair, further temperature variation was done to determine if even higher temperatures would be more optimal. It was also decided that a specific Magnesium concentration might also produce a clearer product, so this too was varied within the next step of optimization. The gel analysis

of the PCR products from these two variations of higher annealing temperatures (60°C, 62°C, and 64°C) and amounts of 25 mM magnesium (0 µL, 1 µL, and 2 µL) per 15µL reaction are shown below in Figure 7. Since the PCR seemed more robust with DNA obtained from blood, only the extremes of each condition were tested as a control. The blood DNA was run twice at 60°C with 0µL or 2µL of 25 mM magnesium as well as twice at 64°C with 0µL and 2µL, shown in Lanes 1-4. Since at this point, the intention was to optimize PCR for the swab preparations, only the wild type primer pairs were used, and all three temperatures were run three times, once with each amount of magnesium, 0µL, 1µL, or 2µL. These nine reactions are shown in lanes 5-13. PCR reactions with 2µL of magnesium and DNA from blood produced bands at 60°C and 64°C. The only PCR reactions run with swab DNA that produced products were obtained with 1µL of magnesium at either 60°C or 64°C. The former band, seen in lane 6 of Figure 7 was the stronger band produced, so these conditions of 60°C and 1µL of magnesium were deemed optimal for swab preparations.

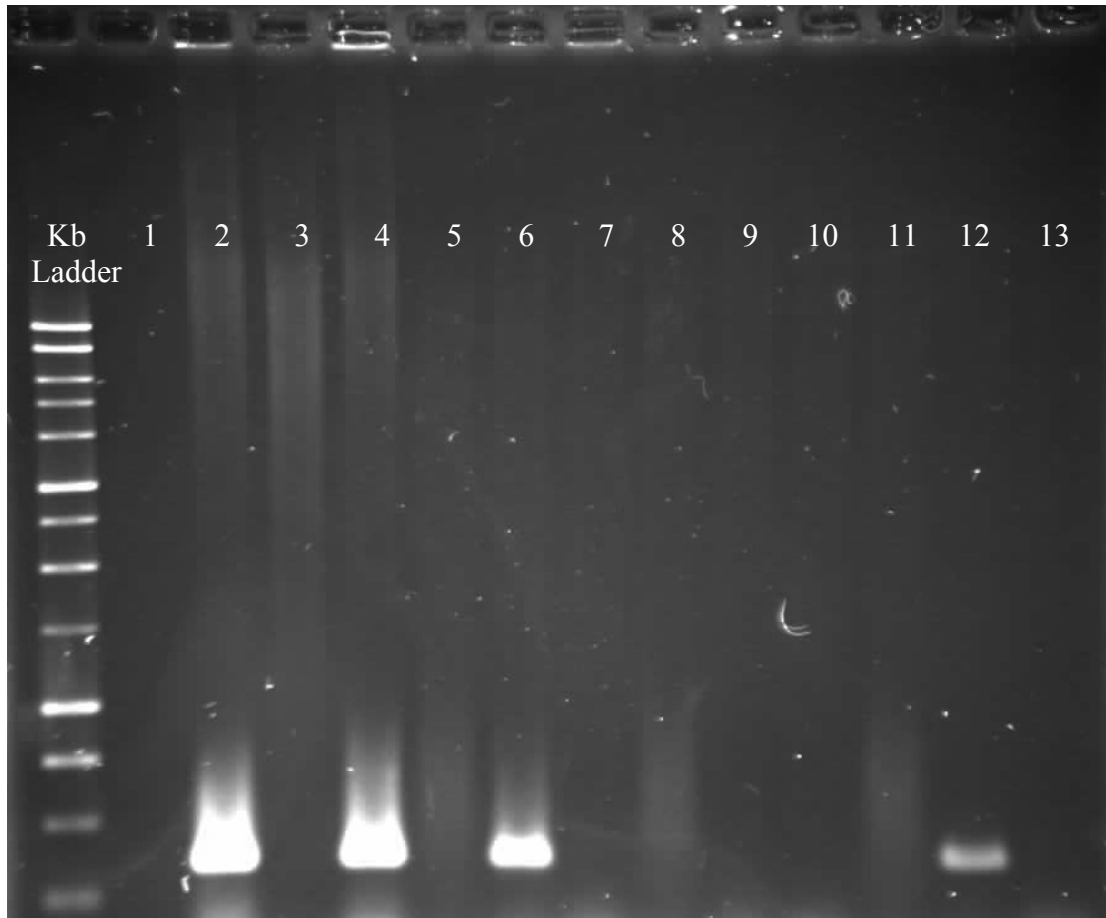


Figure 7: Gel analysis of various PCR reactions with a temperature gradient of 60°C, 62°C, and 64°C as well as the addition of 25 mM magnesium gradient in volumes of 0µL, 1µL, and 2µL. All lanes were run with the wild type primer pair, HYP_WTU1 and HYP_D3. Lanes 1-4 corresponded to DNA isolated from whole blood as controls and lanes 5-12 were run with DNA isolated from swabs. Lanes 1, 3, 5, 8, and 11 were run with 0µL of magnesium, lanes 2, 4, 7, 10 and 13 were run with 2µL of magnesium, and lanes 6, 9, and 12 were run with 1µL of magnesium. For the annealing temperature gradient, lanes 1, 2, 5, 6, and 7 were run at 60°C, lanes 3, 4, 11, 12 and 13 were run at 64°C, and lanes 8, 9 and 10 were run at 62°C.

Thus far, the most optimal conditions for wild type DNA obtained from swabs with the wild type allele-specific primer pair were 60°C and 1µL of MgCl₂ per 15µL PCR reaction. Three temperatures at five-degree intervals were tested, however, using the optimal 1µL of magnesium, the annealing temperature was again varied in a smaller interval of two degrees (58°C, 60°C, 62°C, and 64°C). During this optimization step, the mutant primer pair was also used with each set of conditions to

ensure that the PCR was still stringent enough to produce differentiated results among alleles. The analyses of these experimental reactions are shown in Figure 8. PCR reactions run at all four temperatures produced bands with the wild type primer pair, and did not yield products with the mutant primer pair, as expected. Since the 60°C reaction was consistent from the previous optimization experiment and was proven to differentiate the wild type allele from the mutant allele effectively, this temperature was used for future experimental tests.

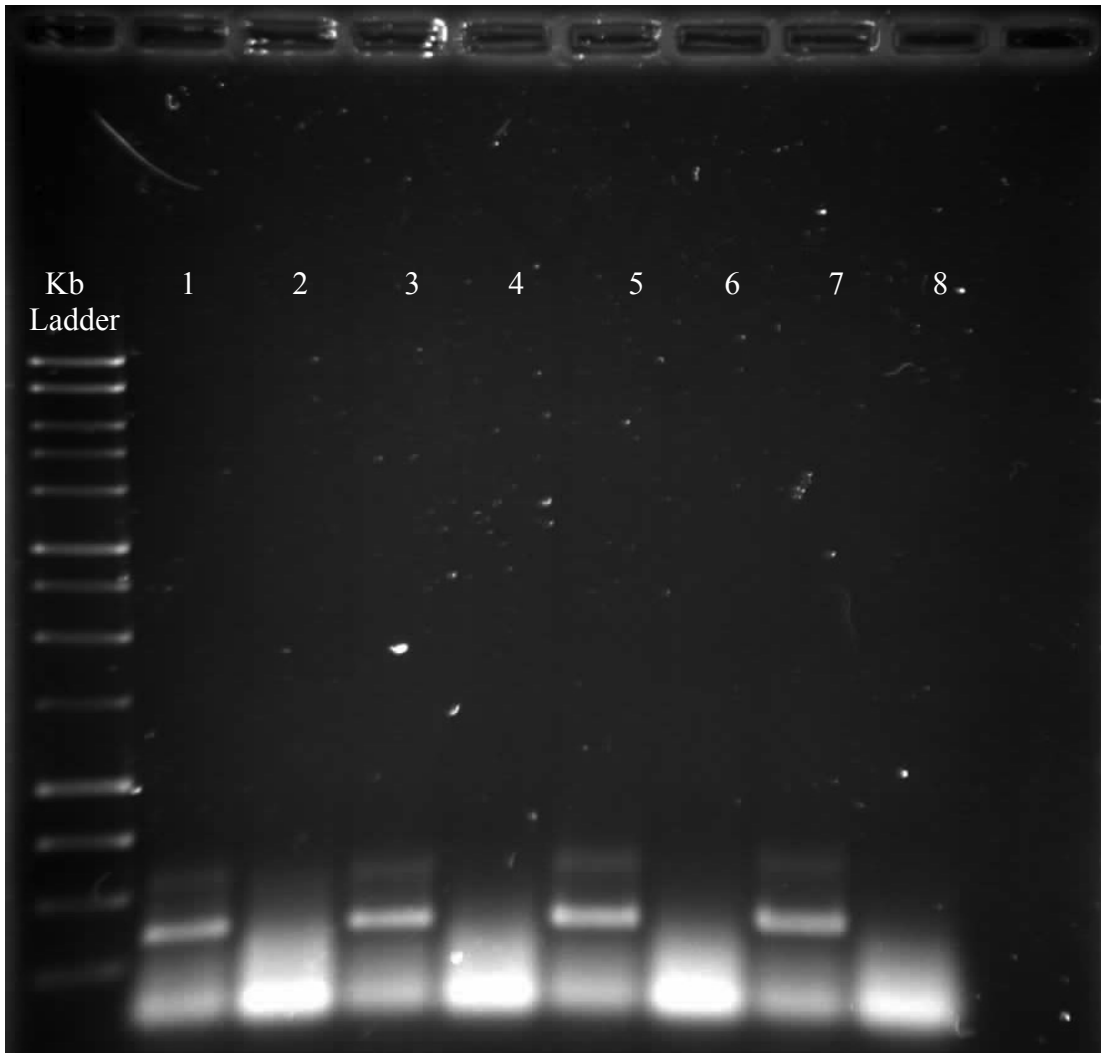


Figure 8: Gel analysis of eight PCR reactions with an annealing temperature gradient of 58°C, 60°C, 62°C, and 64°C. All PCR reactions were run with DNA isolated from wild-type swabs and with 1 μ L of 25 mM magnesium. All odd lanes were run with the wild type primer pair and all even lanes were run with the mutant primer pair. Lanes 1 and 2 were run at 58°C, lanes 3 and 4 were run at 60°C, lanes 5 and 6 were run at 62°C, and lanes 7 and 8 were run at 64°C.

At this point of the experimental process, horsehair was available for DNA isolation and optimization, as this was chosen to be the preferred method of obtaining DNA for testing due to its convenience to owners and high nucleic acid concentration after isolation. Using the optimal conditions for swab preparations as a starting point, the annealing temperature was again varied by a two degree interval of 60°C, 62°C

and 64°C with both primer pairs and wild type DNA obtained from horsehair. As what was found to be optimal for the PCR reactions with DNA obtained from swabs, 1µL of 25 mM magnesium was added to each reaction. The gel analysis of these six reactions can be seen below in Figure 9. PCR reactions run with the wild type primer pair at all three temperatures produced clear bands and PCR reactions run with the mutant primer pair at all three temperatures did not yield products, as intended. Since the reaction run at 60°C distinguished allelic genotype with all three genomic DNA isolations, produced unambiguous differentiation between the wild type and mutant products, and therefore seemed to be the most robust, it was decided that this temperature would be used for the final protocol.

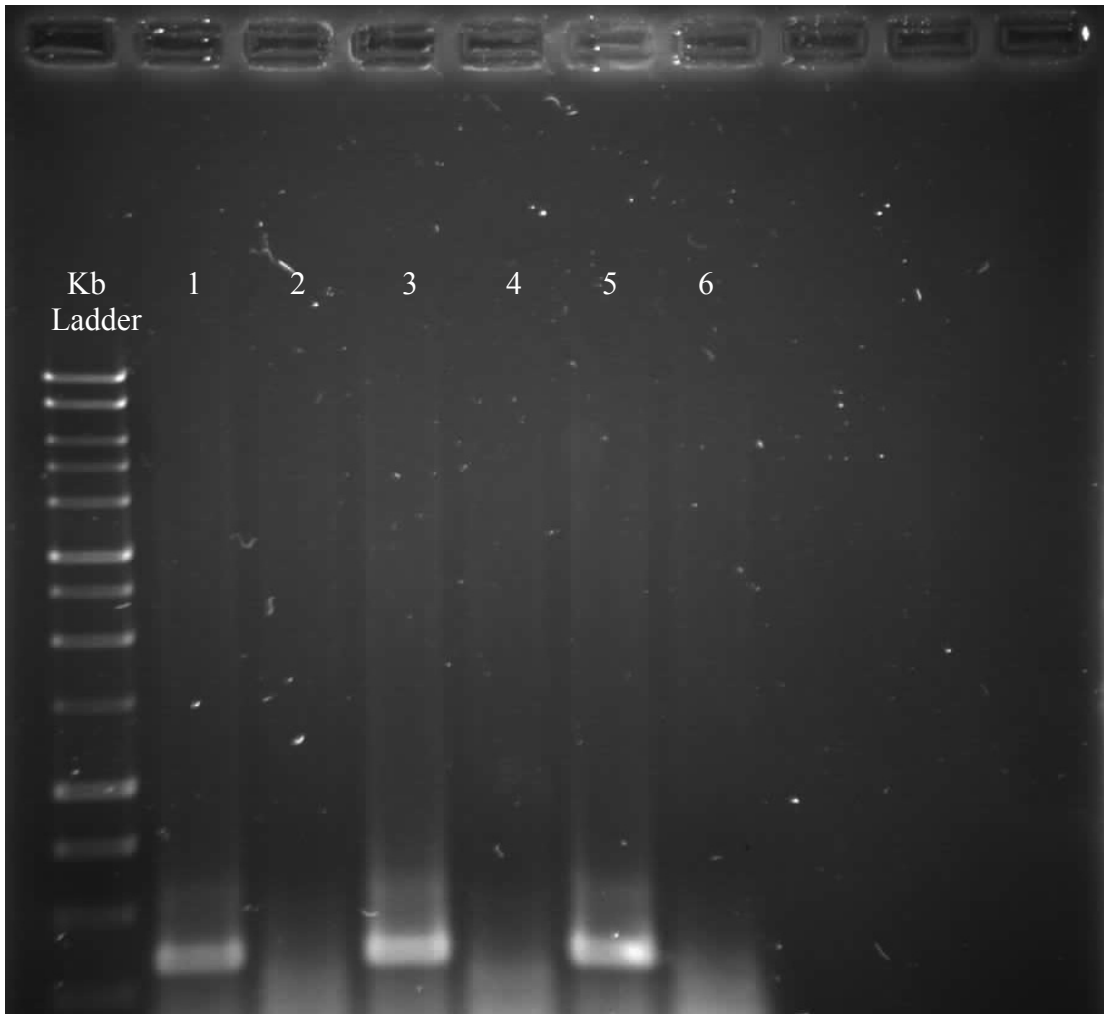


Figure 9: Gel analysis of a six PCR reactions with an annealing temperature gradient of 60°C, 62°C, and 64°C with DNA isolated from wild-type horsehair. All odd lanes were run with the wild type primer pair and all even lanes were run with the mutant primer pair. Reactions in lanes 1 and 2 were run at 60°C, lanes 3 and 4 were run at 62°C, and lanes 5 and 6 were run at 64C.

Confirmation of Final Protocol

From the optimization experiments, it was determined that with DNA isolated from wild type horsehair, an annealing temperature of 60°C and the addition of 1µL of 25 mM magnesium would produce the most optimal product yield when the PCR was run with the wild type primer pair and would not produce any significant product when the PCR was run with the mutant primer pair. This type of unambiguous result

was what was desired at the start of my experiments in coming up with a reliable and robust diagnostic test. The next step, however, was to be sure that these conditions would be just as effective with mutant or carrier DNA. The mutant primer pair had not yet been tested with a mutant allele. For this reason, horsehair from mutant and carrier individuals were obtained and DNA was isolated so that it could be demonstrated that the mutant-specific primer pair also worked effectively and under appropriate conditions.

Eight PCR reactions were run with four different DNA samples. B4 was used as a wild type control, since the DNA samples obtained from blood had seemed to yield the strongest product under various conditions. Wild type, carrier, and mutant DNA, H2, H3, and H4 from Table 3, respectively, were used to confirm that the conditions of the designed test were reliable for all HYPP allelic conditions. Each DNA sample was subjected to two PCR reactions: one with the wild type primer pair, and one with the mutant primer pair. These PCR reactions were run at 60°C with 1µL of 25 mM magnesium, and their analysis can be seen below in Figure 10. As seen in the gel, both wild type DNA samples only yielded PCR products with the wild type specific primer pair, the carrier DNA sample yielded PCR products with both primer pairs, suggesting that that DNA sample contained both alleles of the gene, and the mutant DNA sample yielded a PCR product only with the mutant specific primer pair. This experimental step was essential in showing that the protocol for this diagnostic test was in fact robust, reliable, and unambiguous.

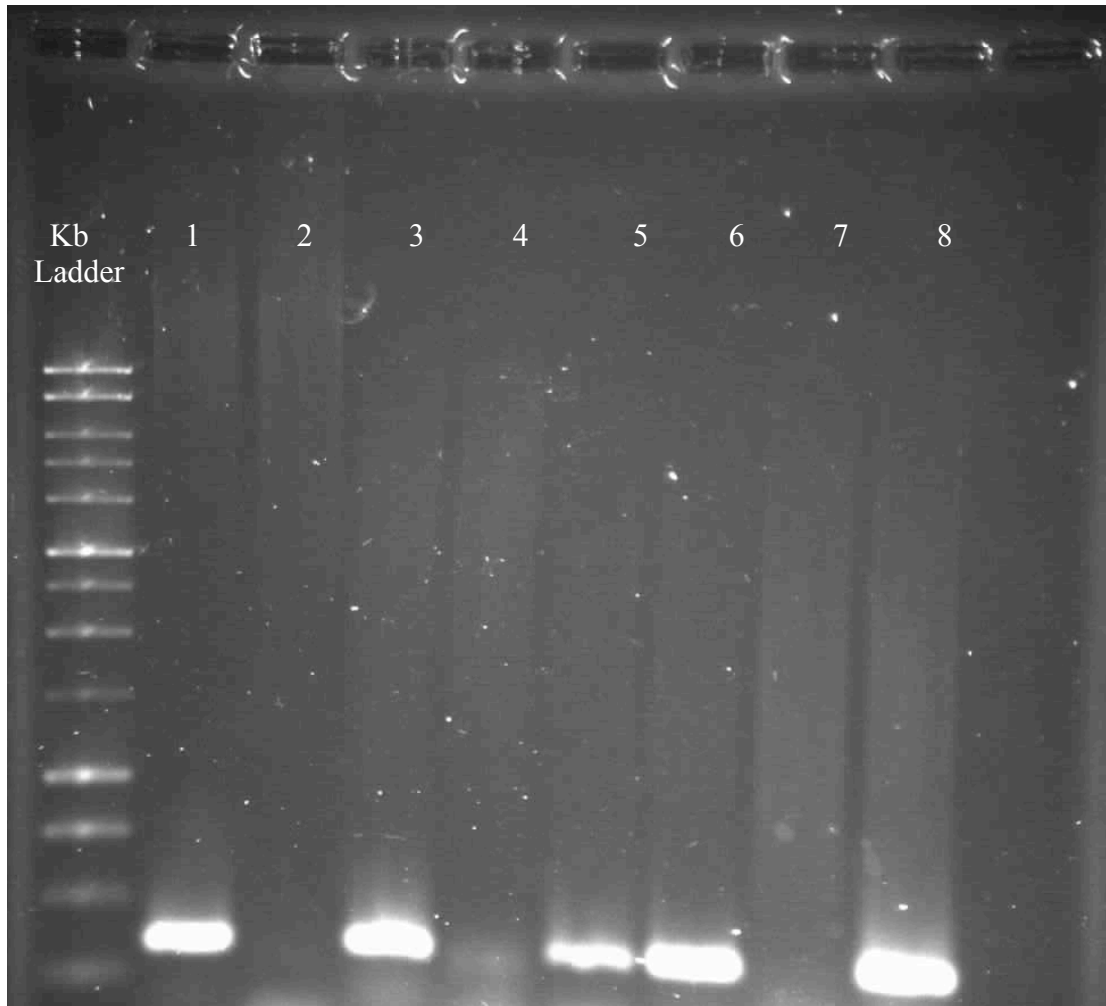


Figure 10: Gel analysis of eight PCR reactions using the final protocol conditions. All odd numbered lanes were run with the wild type specific primer pair and all even numbered lanes were run with the mutant specific primer pair. Lanes 1 and 2 were run with B4 DNA as a control, lanes 3 and 4 were run with H2 DNA (N/N), lanes 5 and 6 were run with H3 DNA (N/H) and lanes 7 and 8 were run with H4 DNA (H/H). All reactions were run with 1 μ L of 25 mM magnesium and at an annealing temperature of 60°C.

Sequencing Analysis

Although my final diagnostic test was shown to reliably differentiate between HYPP allelic statuses of the several DNA samples used for experimentation, wild type DNA from one of the PCR products was isolated and sent out for sequencing to ensure that the PCR reactions were amplifying the intended region of the genome.

The results of this sequencing were then aligned with the horse genome using BLAT, as shown in Figure 11 below. This alignment located the sequence of the PCR product to that portion of Chromosome 11 corresponding to the *SCN4A* gene locus, as intended. The sequenced product was 99.7% homologous to the desired region of the genome. This 0.3% difference was likely due to sequencing errors. These results conform that the PCR reactions were amplifying the intended region of the genome.



Figure 11: This figure shows the BLAT sequence alignment with the *SCN4A* gene from the horse genome to the sequence of the HYPP PCR product amplified from carrier horsehair DNA

Comparison to Current Test

In 1996, the AQHA ruled that all descendents of Impressive foaled after January 1, 1998 must be tested and must have his or her HYPP status disclosed on the registration certificate (www.aqha.com). Currently, the American Quarter Horse Association will accept HYPP test results from eight licensed laboratories. Dr. Sharon Spier was one of the main researchers at UC Davis to come up with the test currently in use. This current test involves a process of DNA isolation, PCR, gel electrophoresis, allele-specific oligonucleotide hybridization, duplicate filtering processes, autoradiography, and analysis (Rojas *et al.*, 1991; Rudolph *et al.*, 1992). In comparison to the test designed from this research, this current test is much less efficient. While the current test could take a matter of three to four days, this new test can be done in less than one day. While the allele-specificity of the current test lies within the oligonucleotide hybridization, or the fourth step of the process, the allelic specificity of this new test is present in the second step of the ARMS-PCR procedure I developed. This allows for more accurate results by minimizing unnecessary steps that may go wrong, producing false positive or false negative results. By minimizing these steps, the new test also minimizes materials and time needed, thus increasing the efficiency of time and money. Finally, this new test has been shown to be reliable and accurate with several different DNA samples, confirming its robust properties.

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