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TaqMan Custom SNP Genotyping Assays for Forensic DNA Phenotyping of Eye Color from Saliva Stains

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ABSTRACT

Single nucleotide polymorphisms (SNPs) can provide useful DNA evidence by evaluating genetic variation for phenotype prediction for criminal case investigation. The nature of SNPs allows for results to be obtained in severely degraded DNA that may not produce viable results via standard STR DNA profiling. SNP analysis by private forensic laboratories is commonly used but little published validation exists to aid one in determining the type of sample to submit or the chance of success using a SNP assay. Two key SNPs have been identified in blue/brown eye color determination: SNP rs12913832 located in the HERC2 gene and SNP rs1800407 located in the OCA2 gene. The IrisPlex software system allows for the prediction of eye color by inputting the genotypes at these SNPs obtained from custom TaqMan assays. This study focuses on eye-color determination as a model SNP system using saliva stain samples exposed to different laundering variables. Saliva samples from human donors of brown, blue, and hazel eyes were collected and used to stain fabric treated with one of the following conditions: unwashed, cold water only, cold water and bleach, hot water, and hot water and bleach. Custom Taqman assays were successful (100%) for unwashed saliva on fabric samples and partially successful for samples washed (16%) in cold water but not enough DNA was recovered for SNP testing from the other conditions. For blue and brown eyes, the IrisPlex system predicted accurately 100% of the time.

Keywords: Single nucleotide polymorphism; Taqman assay; Forensic DNA; Phenotyping; Eye color; Forensic biology; Saliva; Laundering; Human identification; Genetic variation; Pigmentation; Predictive software.

1. Introduction

Advancements in deoxyribonucleic acid (DNA) profiling have been vital to the growth of the forensic biology field. Traditionally, forensic science utilizes DNA profiles based on short tandem repeats (STRs), or short known sequences of nucleotides, typically 4 base pairs long, that are highly variable depending on an individual's genotype due to their high rate of mutation [1]. When analyzing numerous loci, (for example the 13 core loci required by the Combined DNA Index System or CODIS database), STR profiles provide an exceptionally high power of discrimination with a single profile unlikely to be duplicated in over a billion people [1].

However, there are two issues when using STR profiles in forensic casework that are avoided when considering DNA analysis of single nucleotide polymorphisms (SNPs) or single base variation. Primarily, the current system of STR DNA profiling is founded based on profile comparison. Traditional methods of DNA profiling will compare a recovered profile obtained from the scene to a known sample from a person of interest. This comparison will either confirm the perpetrator or provide exculpatory evidence excluding the person of interest as a source. Alternatively, the profile can be uploaded to the CODIS database and can yield an investigative lead only if the owner of that profile is already in the database. In cases where there are no known suspects and there are no matches in the CODIS database, the STR profile can provide no leads in an investigation. Alternatively, SNP profiles can provide phenotypic information for physical traits such as skin pigmentation, eye color, or hair color [2]. This information may provide investigators with a lead by forensic DNA phenotyping (FDP) and help them identify a potential suspect if the STR profile does not yield meaningful results.

The second concern surrounding the use of STR profiles in forensic science is the difficulty of working with poor-quality, degraded DNA. When evidence is exposed to environmental conditions or intentional destructive techniques, DNA is prone to degradation proportional to the length of the fragment of interest. For STR loci, particularly the longer STRs of 300-500 bases, degradation can lead to incomplete or entirely unusable STR profiles [3]. The benefit of SNP analysis is the ability to analyze highly degraded samples since SNPs look at a single nucleotide and can be detected in degraded fragments as small as 100 base pairs (bp) [2]. Additionally, SNP analysis simplifies the challenge associated with the interpretation of mixed samples as SNP analysis is not impacted by stutter artifacts that are common in STR analysis [2]. The ability of SNP analysis to provide investigative leads without comparison and achieve results from highly degraded DNA provides forensic scientists with the additional use of forensic DNA phenotyping for DNA samples from stored evidence in unsolved cases that may be used for facial reconstruction or forensic genetic genealogy (FGG) investigation.

In the field of forensic science, blood and semen are commonly encountered and isolated for DNA analysis. However, saliva as a source of biological evidence cannot be overlooked. Along with blood, saliva is the most common biological evidence found at a crime scene [4]. Detection of saliva presents challenges due to the clear nature of the body fluid. The invisible nature of saliva may allow this evidence to go largely unnoticed when a criminal disposes of more visible evidence such as weapons and blood stains [4]. Important forms of saliva evidence include clothing, gags, masks, cigarette butts, and drinking containers that can be useful as alternate evidence when the first round of DNA analysis on more visible evidence fails.

Saliva exhibits fluorescent properties due to the amino acid tryptophan (and other fluorophores), a key component of α-amylase, an enzyme in saliva [5]. This property allows investigators to use alternate light sources (ALS) in the 280 to 350 nm range to locate saliva that may not be visible to the naked eye [5]. After locating and collecting saliva, DNA can be extracted and analyzed. The key component of saliva that allows DNA analysis are the oral epithelial cells that are shed from the mouth and exit the body in the saliva [4]. Studies have shown that saliva-stained fabric remains a reliable source of DNA for up to 6 months before degradation begins and partial profiles can be obtained for older samples using as little as 50 µl of saliva [6]. The prevalence of saliva at crime scenes, the ability to locate saliva stains with ALS, and the success of saliva stains to produce a DNA profile from epithelial cells make saliva evidence a prime target for further investigation by SNP analysis.

This study specifically targets the phenotype of eye color, and the two main SNPs associated with eye color. Eye color does not follow traditional Mendelian genetic inheritance and is instead a result of a combination of sixteen different genes that work collaboratively to produce a single eye color during human development [7]. Studies have identified two major genes responsible for eye color: oculocutaneous albinism type 2 (OCA2) and HECT and RLD domain containing E3 ubiquitin protein ligase 2 (HERC2) [7]. The OCA2 gene codes for the P protein which is responsible for melanosome maturation that impacts the amount and quality of melanin present in the iris [7]. Albinism is associated with nonfunctional P protein and suggests that mutations in the OCA2 gene that codes for this protein can impact pigmentation. The HERC2 gene regulates OCA2 expression [7]. A mutation in either the gene OCA2 or the regulator protein produced by the HERC2 gene impact iris pigmentation. This study examines

the ability of SNP analysis to accurately predict eye color using DNA collected from saliva-stained fabric subjected to varying laundry treatment conditions that may cause DNA degradation.

1.1. Study Objectives

The following are the objectives of this study:

1) To determine the effect of laundry treatments (temperature, water, bleach) on the DNA recovery rates from saliva stains;

2) To determine the quality and quantity of DNA recovered from untreated and treated saliva stains;

3) To determine the impact of the laundry treatments on the ability to generate downstream SNP data;

4) To determine if DNA degradation impacts the accuracy of the IrisPlex software prediction for eye color from treated saliva stains;

5) To develop a predictive model for SNP testing success for saliva stains for use in criminal casework investigation.

2. Materials and Methods

2.1. Sample Collection

Three donors with varying eye colors were selected to determine how the SNPs rs12913832 and rs1800407 located at the HERC2 gene and OCA2 gene locations respectively withstand exposure to water and bleach applied to saliva stains. Prior to sample collection, all donors were given a consent form and a survey asking about perceived eye color and ancestry. The donor collection was approved by the University Institutional Review Board (IRB) for human subject testing. Saliva was used for this study. All participants were given 10 mL of ACT® Fluoride Mouthwash and were asked to swish for one minute before spitting it out. The donors waited 10 minutes before collecting saliva by spitting into a 50 mL sterile tube until ~20 mL of saliva was in the tube. At this time, an iPhone 14 Pro Max was used to photograph the eyes of the donors. All tubes were coded for anonymity and labeled and stored in a freezer until testing (-80 C).

2.2. Sample Treatment

A cotton t-shirt was cut into 8 x 8 cm squares using sterile scissors. A micropipette was used to add 20 µL of saliva to the center of 10 squares for each donor. A marker was used to trace the stain and label the samples with the donor number and letter associated with the treatment condition. All samples were left in a fume hood overnight until samples were completely dried before being individually wrapped in paper and stored at room temperature. A Mini Washing Machine (11L, Kwiwis) was used for sample treatment. Unwashed samples were individually transferred to a labeled paper bag and sealed. Samples were individually treated by adding one sample to the washing machine and filling it with tap water (~3,000 mL). The temperature was recorded, and samples were set to one 10-minute wash cycle. The machine was drained, and a 3-minute spin cycle was applied. The sample was removed from the machine and hung to dry overnight. The machine was cleaned with 10% bleach between every sample to prevent cross-contamination. The same process was repeated for cold water and bleach samples with 60 mL of bleach

added to the wash cycle. The average temperature for cold washing was 16.6 ºC. The process was repeated for hot water and hot water with bleach samples with an average temperature of 53.4 ºC.

2.3. DNA Extractions

Sterile scissors were used to remove stains from the fabric and sterile tweezers were used to add each sample to a labeled microcentrifuge tube. DNA extraction was conducted following the Invitrogen™ PureLink™ Genomic DNA Mini Kit blood spots and purification protocols per manufacturer's instructions. Modifications were made in step 2 as cotton was used instead of paper. In addition, the cotton sample was not transferred to a new microcentrifuge tube in step 5. No modifications were made to the purification protocol. DNA samples were stored at 4ºC after extraction.

2.4. DNA Quantification

The ultraviolet (UV) spectrophotometer (NanoDrop One C, Thermo Fisher Scientific) was used to determine the overall DNA concentration in each sample. The thirty microcentrifuge tubes containing the DNA samples were removed from the freezer and brought to room temperature. All samples were briefly vortexed and centrifuged to ensure a homogenous mixture. The instrument was cleaned with deionized water and 70% ethanol between each reading. The concentration of human specific DNA was determined using the Quantifiler™ Trio DNA Quantification Kit (Thermo Fisher Scientific). The QuantStudio™ 5 Real-Time PCR instrument (96-Well 0.2 mL Block) from Thermo Fisher Scientific was used to determine quantity. After quantity determination, the microcentrifuge tubes were closed and placed in the freezer (-20°C) to be used for further testing. Only samples with a human-specific DNA concentration above 0.2 ng/µL were further analyzed to ensure 2 ng of DNA template could be added to the polymerase chain reaction (PCR) reaction.

2.5. TaqMan SNP Assay for HERC2 and OCA2

The SNP genotyping assays for HERC2 and OCA2 were conducted following the TaqMan® SNP Genotyping Assays User Guide for TaqMan® Custom SNP Genotyping Assay. Before the well plate was loaded, the 40x Assay Working Stock for HERC2 (rs12913832) and OCA2 (rs1800407) PCR primers were diluted to 20x solutions by adding 9 µL of the stock and 9 µL of molecular biology grade water to a microcentrifuge tube for each stock. The tubes were labeled and vortexed. The reaction mix for each assay was prepared following the Wet DNA method for a 96-well plate. A sterile pipette was used to add the TaqMan® Master Mix and 20x Assay Working Stock to each well. The volume of DNA was calculated for each sample to include 2 ng of DNA in each well. Nuclease-free water was added for the total volume to be 25 µL in each well. For the negative controls, no DNA was added to the wells. After all the components of the reaction were added to the wells, the plate was sealed with adhesive film. The plate was centrifuged briefly to eliminate air bubbles in the reaction. The reaction plate was loaded into the QuantStudio™ 5 Real-Time PCR instrument. The genotyping experiment type was selected, and the PCR cycle was set per manufacturer recommendation. The alleles for the OCA2 and HERC2 were determined following designations in Table 1. The alleles were input into the IrisPlex system, a free software package that will predict eye color based on SNP detection, and the predicted phenotype was compared to the actual phenotype collected from camera images (Tables 2 and 5).

Table 1. SNP allele assignments for HERC2 and OCA2

SNP Assay	**HERC2 (rs12913832)**		**OCA2 (rs1800407)**	
Allele Call	Allele 1	Allele 2	Allele 1	Allele 2
Genotype	A	G	G	A

Table 2. Donor's perceived ancestry and perceived eye color

Participant	**Perceived Ancestry**	**Perceived Eye Color**
1	White/European	Blue
2	White/European	Hazel
3	White/ European	Brown

3. Results

3.1. DNA Recovery from Untreated and Laundered Saliva Stains

The temperature treatments for this study are listed in Table 3. The treatments consisted of unwashed control, cold water only, cold water with bleach, hot water only and hot water with bleach. No detergents were tested in this study. The variables are cold and hot water exposure and bleach exposure to DNA in saliva stains during machine washing.

Table 3. Temperature of treatment conditions

Treatment Condition	**Average Temperature (°C)**
Unwashed	N/A
Cold Water Only	16.8 + 0.3
Cold Water with Bleach	16.6 + 0.4
Hot Water Only	54.3 + 2.1
Hot Water with Bleach	53.3 + 1.4

As temperature increases or bleach is added to the wash cycle, DNA becomes less likely to give results in a SNP assay limiting the ability to predict eye color from saliva stains to just two environmental conditions (Figure 1). These conditions, however, are common for biological evidence that has been unwashed or exposed to rain or water environments. Donor 1 results showed the unwashed samples provided more DNA than the cold-water treatment. Donor 2 results showed the unwashed samples provided more DNA than the cold water, the hot water and hot water with bleach samples. Donor 3 results also showed the unwashed samples provided more DNA than the hot-water and cold-water treatments. There was no DNA recovered for cold water with bleach, hot water and hot water with bleach treated samples for Donor 1. There was less DNA recovered in hot water and hot water with bleach treated samples for Donor 2 compared to the cold water treated samples. There was less DNA recovered in hot water treated samples for Donor 2 compared to the cold water treated samples and no results for the bleach treatments.

For Donor 3, the unwashed samples provided statistically significantly more DNA than all tested conditions with a p-value of 0.013 for cold water and 0.009 for hot water treatments using a student t-test.

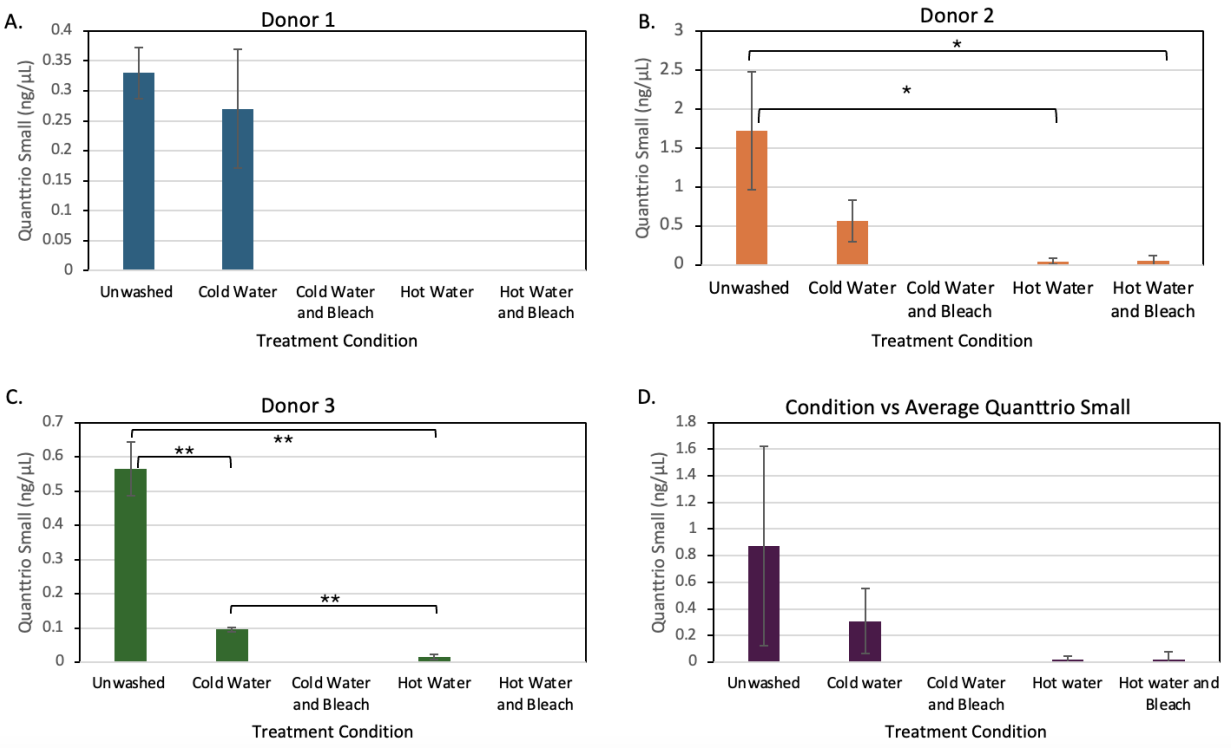


Figure 1. (A) Human specific DNA recovered for each condition for Donor 1, **(B)** Human specific DNA recovered for each condition for Donor 2, **(C)** Human specific DNA recovered for each condition for Donor 3, **(D)** Average human specific DNA recovered for each condition among all three donors. *Represents a 90% confidence interval, ** represents a 95% confidence interval.

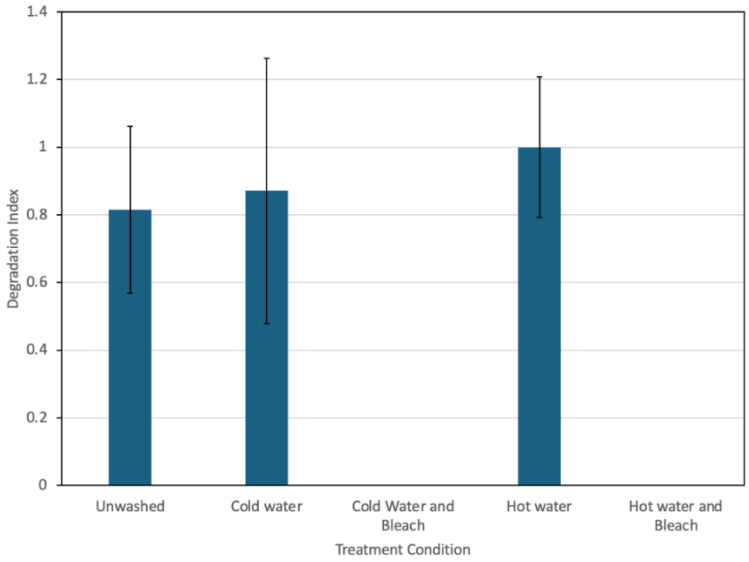


Figure 2. Median degradation index for each condition

The samples were treated using a countertop washing machine and tap water set to cold or hot to match the treatment condition. The average temperature of cold water was approximately 16.7 ºC and the hot water was approximately 54 ºC. Both temperatures fall below 70 ºC, the temperature at which DNA begins to denature. If not

due to denaturation or degradation, the decrease in DNA is likely a result of the DNA being dissolved and physically removed from the fabric during the washing process. This is confirmed by comparing the DNA recovered in unwashed samples and all treatment conditions. For all donors, the DNA recovered was the highest in unwashed samples with a 100% DNA recovered at a high enough concentration for further SNP testing. The recovery rate for DNA above 0.2 ng/µL of cold wash only and hot wash only was 67% and 0% respectively. Based on this analysis, unwashed samples provide the highest DNA quantity with the lowest degradation index (Figure 2). A degradation index with a value of 1 will typically produce a DNA result. Values greater than 1 may provide a partial or no DNA result.

3.2. Genotypic Probability Based on IrisPlex Software Analysis

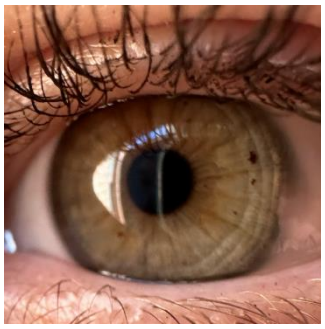
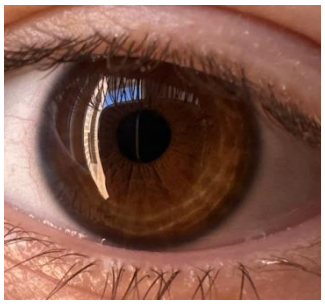
The SNP association to eye color pigmentation is determined by predictive software called IrisPlex. This software can accurately predict blue and brown eye colors in the absence of any other information such as race, ancestry or geographic association. For forensic evidence, this is valuable to generate trait information that is helpful for generating a composite sketch of a person of interest, for facial reconstruction of skeletal remains or for narrowing down a potential list of suspects to just those individuals that possess the same eye color trait. The genotype allele calls for the three donors are listed in Table 4 for both HERC2 (rs12913832) and OCA2 (rs1800407). For the donor 3A sample, UND stands for undetermined, but the second replicate assay determined the genotype call to be GG in the 3B replicate sample. For the Irisplex predictions for eye color from the saliva samples, the p-values were determined (Table 5). Donor 1 has a high scoring value of 0.91 for blue eye color prediction which is consistent with the camera image for that donor. Donor 2 values were spread across the three-color categories (blue, brown, intermediate) and therefore is undetermined but has a higher p-value score for brown pigmentation at 0.481. This is consistent with the hazel pigmentation for eye color from donor 2 which exhibits some brown color in the iris. Donor 3 has a p-value of 0.686 and a replicate assay value of 0.731 indicating a prediction of brown eye pigmentation which is consistent with the camera image.

Table 4. Genotype calls for HERC2 (rs12913832) and OCA2 (rs1800407)

Sample Name	**HERC2 Genotype***	**OCA2 Genotype**
Donor 1A	GG (blue)	GG (blue/grey)
Donor 1B	GG (blue)	GG (blue/grey)
Donor 2A	AG (brown)	AG (mixed)
Donor 2B	AG (brown)	AG (mixed)
Donor 3A	AG (brown)	UND.
Donor 3B	AG (brown)	GG (blue/grey)

*Genotypes were scored based on convention used in SNPedia.com. HERC2 genotypes control and supersede OCA2 genotypes. Other SNPs influence eye color during development as well.

Table 5. IrisPlex results and camera image of eye reference data for comparison

Sample Name	Blue Eye p-value	AUC Loss	Inter. Eye p-value	AUC Loss	Brown eye p-value	AUC Loss	Image of Eye
1A	**0.91**	0.024	0.058	0.06	0.032	0.02	
1B	**0.91**	0.024	0.058	0.06	0.032	0.02	
2A	0.278	0.024	0.241	0.06	0.481	0.02	
2B	0.278	0.024	0.241	0.06	0.481	0.02	
3A	0.153	0.03	0.162	0.071	**0.686**	0.029	
3B	0.125	0.024	0.144	0.06	**0.731**	0.02	

4. Discussions

The primary aim of this research was to explore the ability to perform SNP testing and use the IrisPlex predictive software on saliva stains that had been exposed to water treatments and/or bleach. The UV spectrophotometer was used as a preliminary screening of DNA quantity in the samples extracted from the treated fabric. The overall trend and condition of DNA was consistent for all donors with the most DNA being recovered in unwashed saliva stains followed by cold water treated samples. There was no DNA recovered for any sample treated with cold water and bleach. No DNA was recovered in Donor 1 hot water treated samples, but DNA was recovered for Donors 2 and 3 although in insufficient quantities to perform SNP testing. Aside from 1 sample from Donor 2, no DNA was recovered from the hot water and bleach treatment. When looking at the DNA recovered for each condition versus the donors, Donor 3 had more DNA for every treatment condition. One possible explanation of this trend lies in epithelial shedding rates. As epithelial cells are the primary source of DNA from saliva stains, a genetic difference in epithelial shedding can impact the DNA recovered from saliva stains in the same way it can impact the touch DNA recovery from handled samples.

Saliva is largely made up of water and is, therefore, water-soluble. In addition, the polarity of DNA allows DNA to dissolve in polar water. The spin of the washing machine aids in the mechanical removal of the DNA which dissolves in the water and is then emptied after the wash cycle. The higher temperature of hot water increases the solubility of DNA resulting in less DNA being recovered after treatment. These results agree with the expectation that a hot water wash will remove more DNA than a cold-water wash.

The Quantifiler Trio data showed that no samples treated with cold-water wash and bleach, or hot water and bleach had recoverable human specific DNA aside from one sample. Previous studies have determined that bleach adversely affects the recovery of DNA. When DNA is treated with bleach, DNA strands are cleaved and the bleach causes oxidative damage to the DNA, destroying the DNA. In combination with water and mechanical movement, it was expected that no DNA would be obtained from these samples.

The human-specific small locus DNA quantification data was used to establish the samples that were viable for SNP testing. A concentration of 0.2 ng/µL was used as the cutoff point as this was the lowest concentration that allowed for the addition of 2 ng of DNA while maintaining the 25 µL volume of the PCR reaction. This cutoff eliminated all the samples that were treated with bleach, hot water, and 33% of the samples that were treated with cold water. Despite some DNA being recovered in samples washed with hot water, the data shows that hot water washes do not leave enough DNA behind for further SNP analysis.

In previous research, it has been established that SNPs can be detected in degraded DNA. It has also been established that the SNPs rs1800407 and rs12913832 influence blue, brown and intermediate eye color determination. The SNP rs1800407 at the OCA2 gene is associated with green/hazel eyes with an A allele indicating green/hazel eyes. The OCA2 rs1800407 G allele is associated with blue/grey eye color. Mutations in HERC2 SNP rs12913832 are associated with blue eyes with allele A indicating brown eyes and allele G indicating blue eyes. The amplification of these alleles in the TaqMan® Custom SNP Genotyping Assay allowed for the accurate prediction of blue and brown eye color in our model system.

In Table 2, donors described their perceived eye color and ancestry. All donors are of European ancestry, eliminating ancestry as a potential variable. Table 5 contains images of the iris of each donor. The photograph of the iris of donor 1 shows a light blue/grey color throughout most of the iris. There appears to be slight light brown coloring near the pupil.

The overall perceived eye color is determined to be blue by the donor 1 survey. For donor 2, the outer iris appears pale green with dark brown spots on the upper right. Around the pupils, the iris is light brown with a darker brown above the pupils. The combination of green and brown classifies the eye color as hazel which agrees with the perceived eye color noted by the donor 2 survey. The iris of donor 3 is a solid medium brown color and can be classified as perceived brown as was indicated by donor 3 survey.

The IrisPlex system was able to accurately predict blue and brown eye color when HERC2 and OCA2 SNPs were determined. In this study, the Irisplex system was not able to accurately define the intermediate color type of hazel for donor 2, however, it did show that DNA recovered from untreated and cold water washed saliva stains is of sufficient quality and quantity to accurately predict the blue and brown eye color categories.

5. Conclusions

Previous research has established saliva as a valid source of DNA for human DNA identification methods. The DNA recovered from unwashed saliva stains provided the highest concentration and the lowest degradation index as expected. The corresponding SNP assays were the most successful using DNA from unwashed saliva-stained samples. The DNA recovered from cold water wash samples provided enough DNA to properly set up a PCR reaction, but the overall quality was not always high enough for SNP results to be obtained. No samples treated with bleach or washed in hot water provided DNA with a high enough concentration to conduct the SNP assay.

When examining the IrisPlex predictive software results, the IrisPlex system was able to accurately predict blue eye color for Donor 1 and brown eye color for Donor 3 using HERC2 alone or the HERC2 and OCA2 SNP data in combination. Although additional research is needed to fully understand the applications and limitations of SNP testing for forensic DNA phenotyping using saliva stains on fabric, this study illustrates the ability to accurately predict blue-brown phenotypic information for eye color to generate investigative information. This has important implications for cold case investigations since private forensic DNA phenotyping and SNP testing laboratories [Parabon NanoLabs, Inc., Othram Corp. (USA)] use this technology routinely in attempting to generate investigative leads for current and cold cases as well as for missing persons cases. Currently, there is no predictive strategy other than a quantity assessment to determine if a forensic sample is suitable for SNP analysis. This study defines the types of saliva stains commonly encountered in forensic casework that are most likely to succeed for forensic DNA phenotyping assays.

5.1. Recommendations

Some future directions for continued research include:

- Testing different types of body fluid stains such as blood, urine and semen for the impact of temperature, water and bleach on the ability to generate SNP data for comparison to saliva stains.
- Expanding the donor sample size to 50-100 individuals per eye color to increase the power of statistical analyses.
- Examining different forms of laundry machines and washing and rinsing cycles to determine the impact on SNP profiling results.
- Examining different forms of laundry detergent (powder, liquid, unit dose/pod, anionic, cationic, nonionic, amphoteric and phosphate-free) and bleach (chlorine-based and non-chlorine) for the impact on SNP testing results

5.2. Limitations

This study was limited by a small sample size and should be reconducted with multiple donors for each eye color to provide stronger support for the use of IrisPlex predictive software with saliva stains. It is also important to note that the simulated washing conditions did not accurately predict all laundry washing behavior. Typically, detergent is added when laundering clothing so this study cannot anticipate the impact of detergent on the ability to perform SNP testing. Furthermore, traditional large machine-washing cycles may last longer than 10 minutes and can result in less DNA than what this study recovered in the cold and hot water washes.

Declarations

Source of Funding

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Competing Interests Statement

The authors declare that they have no conflict of interest.

Consent for publication

The authors declare that they consented to the publication of this study.

Authors' contributions

Both the authors took part in literature review, analysis, and manuscript writing equally.

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