

# Assessment of the IrisPlex Markers in Order for Eye Color Prediction in Iran (Tehran) Population

<sup>1,2</sup>Alireza Rafati, <sup>3</sup>Hassan Zamanifar, <sup>4</sup>Sayed Mostafa Hosseini

<sup>1</sup>PhD in Medical Biotechnology, Research Assistant, Department of Medical Biotechnology and Molecular Medicine, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran, <sup>2</sup>PhD in Medical Biotechnology, Research Assistant, Department of Medical Genetics, Sirjan School of Medical Sciences, Sirjan, Iran, <sup>3</sup>PhD in Molecular Genetics, Research Assistant, Department of Genetics, Rey Payam Noor University, Tehran, Iran, <sup>4</sup>PhD in Molecular Genetics, Associate Professor Chemical Injuries Research Center, Systems Biology and Poisonings Institute, Baqiyatallah University of Medical Sciences, Tehran, Iran

**How to cite this article:** Alireza Rafati, Hassan Zamanifar, Sayed Mostafa, \*Hosseini. Assessment of the IrisPlex Markers in Order for Eye Color Prediction in Iran (Tehran) Population. Indian Journal of Forensic Medicine & Toxicology / Vol 20 No. 1, January - March 2026

## Abstract

**Aim:** Today usually the crime scene is evaluated by using externally visible characteristics (EVCs) in forensic centers. EVCs such as eye color is a physical trait determined by feature the genetic of the iris. Evaluation of the accuracy of human, iris color prediction based on informative single nucleotide polymorphism (SNPs) can provide a useful intelligence tool to identify the suspect. In our current research, we analyzed a six informative SNP marker (IrisPlex) (rs12896399 rs16891982, rs1393350, rs12203592, rs12913832, rs1800407) prediction of human eye color in 106 individuals of Iranian population with different ethnicity. We hypothesized that IrisPlex marker evaluations could be a considerable tool in eye color prediction. Hence, we performed this assessment for the first time in Iran.

**Methods:** Peripheral blood samples were collected from 106 unrelated donors with informed consent in ophthalmology clinic of Baqiyatallah hospital. We analyzed a six informative SNP (rs12896399 rs16891982, rs1393350, rs12203592, rs12913832, rs1800407) with single base extension (SBE) assay (SNaPshot). The multinomial regression prediction model was used for these informative SNPs, for this model we calculated sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and the area under the receiver characteristic operating curves (AUC) to increase accuracy for eye color prediction.

**Results:** By Statistical analysis of logistic regression, the AUC value of IrisPlex markers was evaluated to determine eye color. AUC value for Dark eyes and blue eyes was  $0.99 \pm 0.0004$  and  $0.949 \pm 0.0100$  respectively and for intermediate eye colors was  $0.9505 \pm 0.017$ .

---

**Corresponding Author:** Sayed Mostafa Hosseini, Chemical Injuries Research Center, Systems Biology and Poisonings Institute, Baqiyatallah University of Medical Sciences, Tehran, Iran

**E-mail:** ar.rafati@sirums.ac.ir

**Submission:** Sep 3, 2025

**Revision:** October 13, 2025

**Published date:** January 29, 2026

---

**Conclusion:** We conclude that accuracy of IrisPlex predictive SNPs was acceptable in our study. However, the mere IrisPlex markers are not enough and some additional markers should be evaluably to increase the accuracy of eye color prediction.

**Keywords:** Eye color, SNP, EVCs, IrisPlex, forensic

## Introduction

After the introduction and rapid development of FDP (Forensic DNA Phenotyping) in forensic sciences, the ability accurately to predict the human's characteristics with DNA markers significantly increased<sup>1</sup>. Predictions of human EVCs (Externally visible characteristics) by analyzing informative SNP (single nucleotide polymorphism) in samples suggest a new perspective in the FDPs. Informative genes, including HERC2, OCA2, MC1R, SLC24A5, SLC45A2, TYR, TYRP1, ASIP, SLC24A4, TPCN2, KITLG, and IRF4 have been associated with eye, hair, and skin color in European populations and have been used in the forensic studies to predict eye color in target population<sup>2,3</sup>. The formation of eye color is dependent on eumelanine density in the iris, a with highest concentration of melanin in the dark eye color and the least concentrations in blue eyes<sup>4</sup>. Recent investigations have shown that a significant part of the iris color variation can be explained by SNPs in the human DNA, Containing the conserved region of HERC2 gene and the Proximity OCA2 gene on the chromosome 15. The other genes (SLC24A4, SLC45A2, TYR, and IRF4) involving other variations of eye colors<sup>5</sup>. Since to calculate potential differences may influence the eye color prediction value in various populations, we decided to evaluate the accuracy of the IrisPlex system in an Iranian population sample. Iran is a country in the Middle East. The Iranian population includes heterogeneous ethnic groups including: Persians (51%), Azeris (24%), Gilakis and Mazandarani (8%), Kurds (7%), Arabs (3%), Lurs (2%), Balochis (2%) and Turkmen (2%)(6). However, the distribution of ethnicity is homologous In Tehran. Today in forensic practice more EVCs is used to identify suspects in crime. This study consists of a multiplex genotyping system for the six informative SNP markers to predict eye color according to previous studies using a statistical prediction model<sup>6,7</sup>.

## Material and Methods

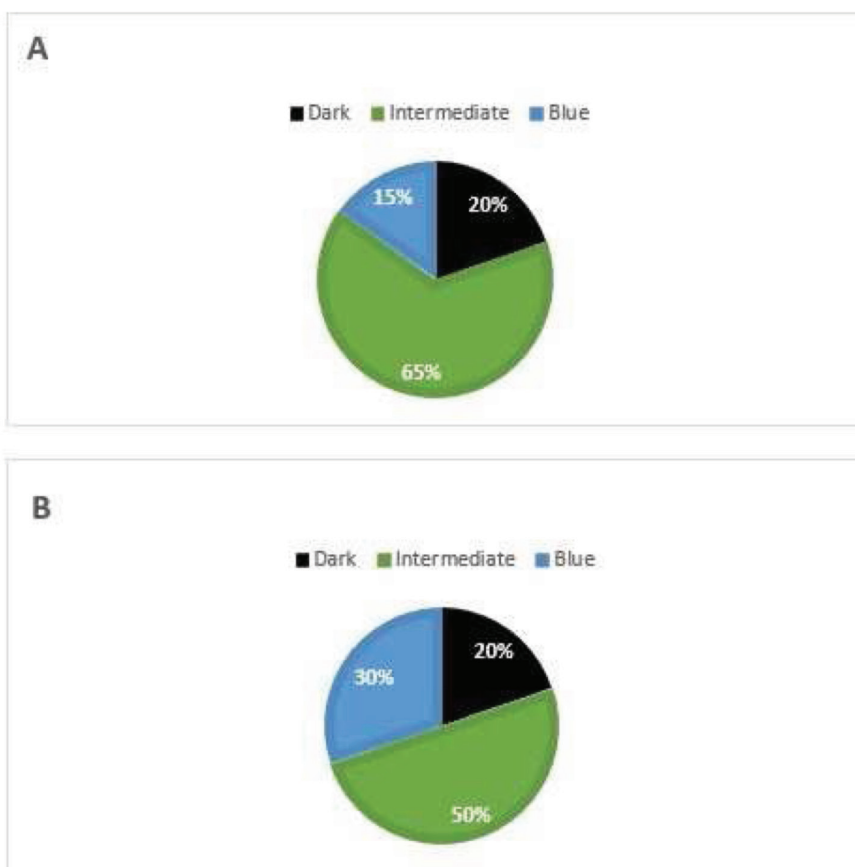
### Sample Collection and DNA Extraction

2 milliliters of venous blood on K2-EDTA anticoagulant were collected from each of 106 (61 males, 45 females) subjects randomly and after medical examination by an ophthalmologist, photographs were taken in macro-mood condition from each iris with a digital camera (Canon EOS.60D, Japan) in the Baqiyatallah DC Clinic. First, we classified pictures into three categories "Dark, Intermediate and Blue" In addition the consent was obtained from all participants. In this study, we performed DNA extraction by RGDE standard methods<sup>8</sup> To determine the concentration of extracted DNA, Nanodrop and the optical density (OD) was used and the ratio of light absorption (260/280) was calculated. The light absorption ratio (260/280) for all 106 samples were on average 1.6 to 1.8 and all samples were checked for contamination with protein.

### Multiplex Reaction and SNP Genotyping

In this investigation the Six SNP Primers (HERC2 - rs12913832, OCA2 - rs1800407, SLC24A4 - rs12896399, SLC45A2- rs16891982, IRF4 - rs12203592, and TYR - rs1393350) described in 2011 by Walsh et al were used<sup>2</sup> due to the impending ability to predict externally visible characteristics (EVCs). SNP fragments were amplified in standard PCR reaction with 1 µl (0.5-1 ng) extracted DNA from each person in 12 µl total reaction with 5 µl AmpliTaq Gold Master Mix (Applied Biosystems Corp) and 0.5 µl of each primer. PCR programs for this reaction were, according to Walsh et al protocols<sup>9</sup>. The PCR product was purified by USB ExoSAP (Affymetrix, Corp), then SNaPshot step using the SNaPshot™ Multiplex Kit (Applied Biosystems) with six SBE (Single Base Extension) primers, Primer sequences and thermocycling conditions was shown in table 1.

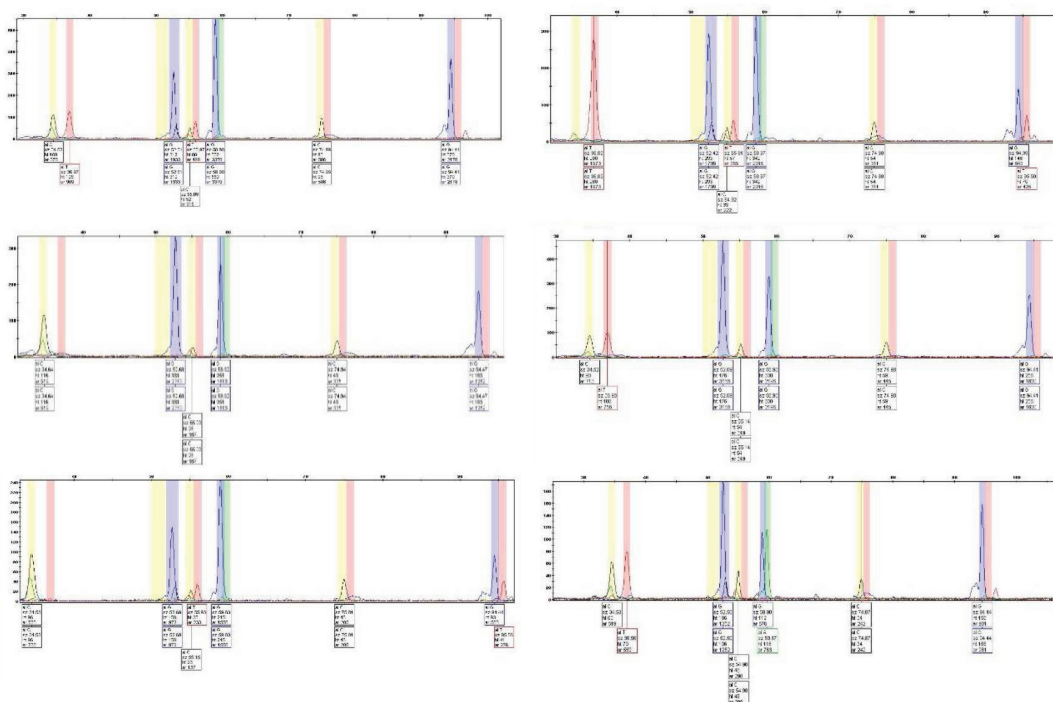




**Figure 1: Eye color Images of 16 sample contribute to studies, IMI index was calculated and classify (Blue, Dark, and Intermediate) by NoorGraphy native software.**

The SNaPshot reaction for all samples was performed according to the standard procedures with (15 ng) DNA for each SNaPshot reaction<sup>2</sup>due to the impending ability to predict externally visible

characteristics (EVCs. The results,after setting and optimizing were acceptable with high quality. Several different results are shown in Figure 2.



**Figure 2: Several SNaPshot results with different eye color and ethnic group**

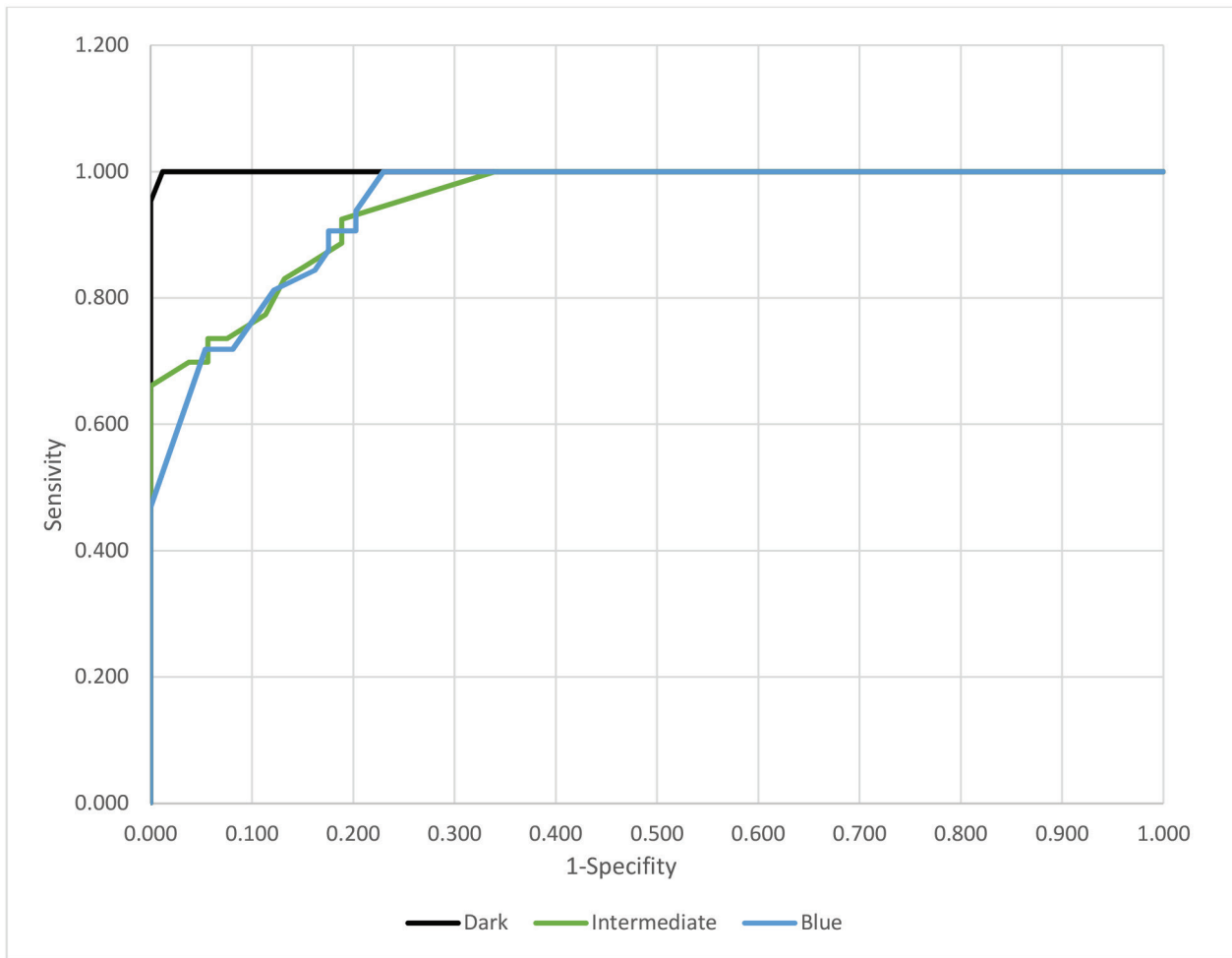
The SNaPshot tests of the multiplex reactions were performed by multinomial logistic regression standard analysis<sup>9</sup>. The output of this study, according to cross-tab test verified that the highest association between dark eye color and Fars ethnic, and high correlation between blue eye color and other ethnicities. In order to have accurate results of eye color we used AUC for each SNPs. As previous studies show rs12913832 SNP marker had the most

predictive allele in determining eye color with 98.2% correct results. In this study, we used eye color predictive models (The IrisPlex model). In addition, we also used two other variables (sex and ethnicity) along with the main variables (IrisPlex markers) to predict eye color. The AUC, sensitivity, specificity, PPV, and NPV in both tables are reported. The receiver operating characteristic curve for the IrisPlex model is shown in chart 2.

**Table 2. The statistical properties of the prediction model with IrisPlex variables for eye color (dark, intermediate and blue).**

	Dark	Intermediate	Blue
AUC	0.9997 ± 0.0004	0.9505 ± 0.017	0.949 ± 0.0100
Sensitivity <sup>1</sup>	100	73.06	87.5
Specificity <sup>1</sup>	98.82	92.45	82.43
PPV <sup>1</sup>	95.45	90.69	68.29
NPV <sup>1</sup>	100	77.77	98.84

Chart 2. Receiver operating characteristic curve analysis of all samples with a different ethnic group in Tehran, based on the IrisPlex prediction model.



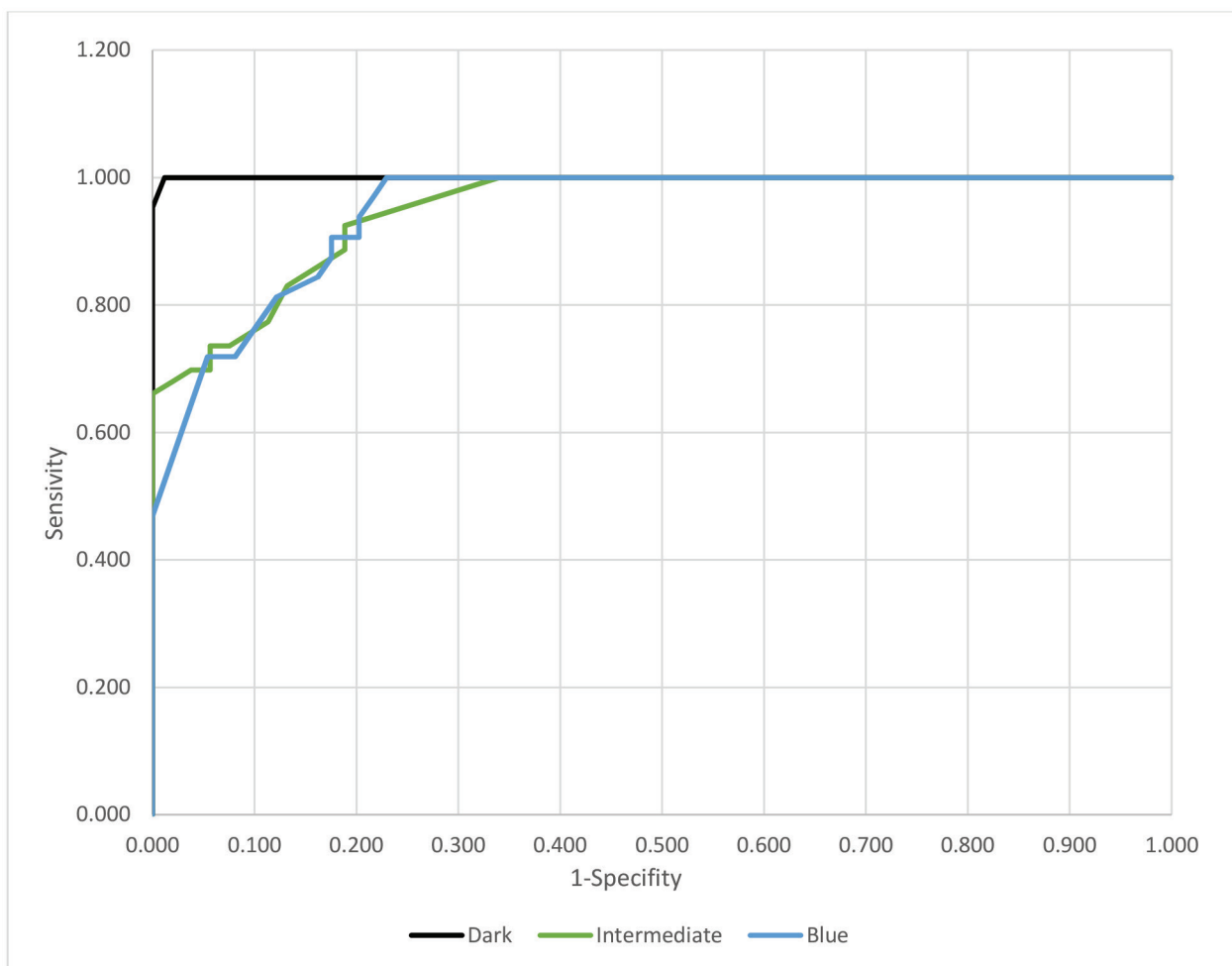
In contrast to in other models, we also used gender and ethnicity in the eye color prediction model. The percent of the AUC, sensitivity, specificity, particularly in dark eye color group was increased. Other characteristics of the IrisPlex model were

reported. The receiver operating characteristic curve for the first model is shown in chart 3. In this study, the addition of other factors (gender and ethnicity) improved the specificity and sensitivity of eye color prediction.

**Table 3. The statistical properties of the prediction IrisPlex model with gender and ethnicity for eye color (dark, intermediate and blue).**

	<b>Dark</b>	<b>Intermediate</b>	<b>Blue</b>
AUC	1 ± 0.00001	0.9505 ± 0.0154	0.9521 ± 0.0179
Sensitivity <sup>1</sup>	100	72.90	87.5
Specificity <sup>1</sup>	100	92.45	85.13
PPV <sup>1</sup>	100	91.30	71.79
NPV <sup>1</sup>	100	81.66	94.03

Chart 2. Receiver operating characteristic curve analysis of all samples with different ethnic group in Tehran, based on Irisplex predictive model with gender and ethnicity.



## Discussion

Human eye color is exceptionally polymorphic due to it being governed by multiple genes. This trait has been studied on a genetic level extensively, and some of the studies employed Europeans for genome wide association studies. Conversely, the IrisPlex system is also a commendable set of markers that due to its reliability and across multiple types of geographical locations is being validated to accurately target EVCs<sup>9,10</sup>. When it comes to genetic determinants of EVCs, the IrisPlex system successfully acts as an deterministic model which when combined with the precision of genetic data acts as a highly powerful with high accuracy of EVCs in forensic usage capabilities<sup>9,10</sup>. The said markers aforementioned are inclusive of 6 SNPs that are vital genes that greatly affect the control of pigmentation of the eye.

Some of the genes registered as major including *HERC2* rs12913832, *OCA2* rs1800407, *SLC24A4* rs12896399, *SLC45A2* rs16891982, *TYR* rs1393350 and *IRF4* rs12203592 eye color determining genes<sup>7</sup>. In the current study, SNaPshot method is deployed to undergo SNP genotyping process. SNaPshot is an ideal technique for this comparison owing to the fact that it has higher sensitivity and specificity during SNP detection. The rs12913832 SNP on the *HERC2* gene was previously associated with having the highest predictive power for eye color, and AUC values registering 98.2%, 85.2%, and 87.8% for dark, intermediate, and blue-colored eyes respectively. Such findings were replicated in our population because the data analyses showed that the TT genotype of rs12913832 was strongly associated with dark eyes, similar to other findings made in regions

including Egypt, Turkey, and Iraq<sup>11-13</sup>. In this study, we evaluated the genotype frequency of rs12913832 as TT (47%), CT (30%) and CC (23%). In previous studies, Persians (Fars ethnicities) accounted for about 61% of the total population of Iran<sup>13,14</sup>. The sample that we evaluated had a Fars ethnic distribution of 52.8%. The presence of such a large proportion of Fars people, coupled with their dominance in the population of Iran, indicates that the trend within the GWAS cohort is toward darker eye colors. This finding is consistent with the AUC results of our data analysis, where the most predictive accuracies were found for dark eye color. Results from the studies of other populations assist in validating these. For example, the application of AUC analysis in a similar study in the Netherlands revealed AUC values of 93% for brown eyes, 91% for blue and 73% for mixed eyes<sup>13</sup>. Similarly, work done in Iraq demonstrated 100% accuracy in recognizing dark eye color and 52.2% accuracy for blue eye color<sup>12</sup>. Although the dark and blue markers of the iris tackle their respective target in the eye color model very cohesively, the system fails to perform with equal finesse in regard to the intermediate eye colors. Hence the western population and diverse populations highlight a marked deficiency in the use of the systems in conjunction. This may be focused on modifying the marker set and further including the genetic and demographic components that may smoothen the predictions, especially for the intermediate eye color phenotypes<sup>9</sup>. In this investigation, a large number of participants are reported to be with intermediate eye color. However, there is a consistently high lack of sensitivity in providing a prediction for this category, similarly, guidance is required for development of new prediction systems for the determination of the intermediate type eye color as it is already present in literature. The prediction accuracy of the IrisPlex system can be further enhanced however the addition of new markers is required to improve upon this system. Recently three SNP were discovered that directly relate to the intermediate eye color and have been shown to be potentially effective for use in forensic studies. The IrisPlex system has a

number of drawbacks, including the fact this could potentially be a solution to some of those concerns. These markers could also assist not only improving the predictive accuracy to a certain extent, for providing intermediate eye color, but also extend the IrisPlex system functionality<sup>6,9</sup>. In 2020 for example, the HIrisPlex system of hair color prediction was presented, which is an enhancement of the previous methods of predicting EVC for forensic experts, as it employs an amalgamated set of SNP markers for the prediction of hair and eye color. That technique is relatively novel, but is potentially a great leap in terms of forensic genetics. We recommend that in the future scans of Iranian populations, the HIrisPlex markers be used for EVC prediction optimization purposes. An issue that has troubled researchers conducting FDP is the relative categorization of EVCs. This issue was addressed by use of a native software that was developed with the aim of classifying the eye color. This software lowered human errors significantly and thus increased the accuracy of the predictions made, especially in the case of blue eye color (Chart 1). Such technological advancements are vital in improving the dependability and reproducibility of forensic tools<sup>10,11,15</sup>. Because this research has been the first one ever conducted on EVCs in Iran it is important to explore a range of traits such as hair or skin color in the Iranian populations. Once more, as Iran has a fenestrated and diverse gene pool, our population has significant potential for these types of genetic studies. It is without doubt that interdisciplinary investigations in this discipline will not only broaden scientists' horizon concerning the genetic basis of a variety of phenotypic characters but also provide a basis for the application of these findings in forensic and anthropological investigations.

### Acknowledgements

We are grateful to all volunteers who helped us to conduct this study and to Dr. Amir Hashem Mohammadi for the eye photography. In addition, we thank all Noor Genetics Center employees. And finally, we thank Dr. Amir Saleh Khaje Hosseini for designing NoorGraphy software.

This manuscript has been prepared and conducted in accordance with the ethical and publication guidelines of ICMJE and COPE.

### Ethics Approval

Ethics this study was approved by the Ethics Committee of Baqiyatallah University of Medical Sciences, this study also had been recorded with IR. Bmsu.REC013950373 id number in the university ethics committee.

### Conflicts of Interest

The authors declare that there are no financial, personal, or professional conflicts of interest that could have influenced the results or interpretation of this study.

**Source of funding:** None

### Reference

- Kayser M, Branicki W, Parson W, Phillips C. Recent advances in Forensic DNA Phenotyping of appearance, ancestry and age. *Forensic Sci Int Genet.* 2023;65:102870. doi:10.1016/j.fsigen.2023.102870
- Walsh S, Liu F, Ballantyne KN, van Oven M, Lao O, Kayser M. IrisPlex: A sensitive DNA tool for accurate prediction of blue and brown eye colour in the absence of ancestry information. *Forensic Sci Int.* Published online 2011.
- Seo HJ, Cho S, Lee JH, Lyoo SH, Kim MY, Lee SD. Forensic DNA Phenotyping: A Review in Korean Perspective. *Korean J Leg Med.* 2017;41(2):23. doi:10.7580/kjlm.2017.41.2.23
- Passeron T, Mantoux F, Ortonne JP. Genetic disorders of pigmentation. *Clin Dermatol.* 2005;23(1):56-67. doi:10.1016/j.clindermatol.2004.09.013
- Sturm RA, Teasdale RD, Box NF. Human pigmentation genes: identification, structure and consequences of polymorphic variation. *Gene.* 2001;277(1-2):49-62. doi:10.1016/S0378-1119(01)00694-1
- Liu F, Van Duijn K, Vingerling JR, et al. Eye color and the prediction of complex phenotypes from genotypes. *Curr Biol.* 2009;19(5):R192-R193. doi:10.1016/j.cub.2009.01.027
- Sharafi Farzad M, Tomas C, Børsting C, et al. Analysis of 49 autosomal SNPs in three ethnic groups from Iran: Persians, Lurs and Kurds. *Forensic Sci Int Genet.* 2013;7(4):471-473. doi:10.1016/j.fsigen.2013.04.001
- Ali SM, Mahnaz S, Mahmood T. Rapid genomic DNA extraction (RGDE). *Forensic Sci Int Genet Suppl Ser.* 2008;1(1):63-65. doi:10.1016/j.fsigs.2007.12.001
- Walsh S, Wollstein A, Liu F, et al. DNA-based eye colour prediction across Europe with the IrisPlex system. *Forensic Sci Int Genet.* 2012;6(3):330-340. doi:10.1016/j.fsigen.2011.07.009
- Walsh S, Liu F, Wollstein A, et al. The HIrisPlex system for simultaneous prediction of hair and eye colour from DNA. *Forensic Sci Int Genet.* 2013;7(1):98-115. doi:10.1016/j.fsigen.2012.07.005
- Sari O I, Simsek SZ, Filoglu G, Bulbul O. Predicting Eye and Hair Color in a Turkish Population Using the HIrisPlex System. *Genes.* 2022;13(11):2094. doi:10.3390/genes13112094
- Al-Rashedi NAM, Mandal AM, ALObaidi LA. Eye color prediction using the IrisPlex system: a limited pilot study in the Iraqi population. *Egypt J Forensic Sci.* 2020;10(1):27. doi:10.1186/s41935-020-00200-8
- Rafati A, Hosseini M, Tavallaei M, Naderi M, Mohammadi AH, Bahmani H. Association of rs12913832 in the HERC2 Gene Affecting Human Iris Color Variation. 2015;12(1).
- Rafati A, Hosseini SM, Far HZ. Using the IrisPlex System for Eye Color Prediction on Skeletal Remains from the Past 30 Years. *J Forensic Sci Med.* 2023;9(4):367-370. doi:10.4103/jfsm.jfsm\_122\_22
- Navarro-López B, Baeta M, Suárez-Ulloa V, et al. Exploring Eye, Hair, and Skin Pigmentation in a Spanish Population: Insights from HIrisPlex-S Predictions. *Genes.* 2024;15(10):1330. doi:10.3390/genes15101330