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TECHNICAL NOTE

The Ion AmpliSeq™ VISAGE (Visible Attributes through Genomics) Basic Tool Research Panel

Introduction

The purpose of this technical note is to introduce HID laboratories to the new Ion AmpliSeq™ VISAGE (Visible Attributes through Genomics) Basic Tool Research Panel (referred to herein as *VISAGE Basic Research Panel*), an MPS-based investigative panel developed in collaboration with the European VISAGE consortium to provide the forensic community with a single nucleotide polymorphism (SNP)-based multiplex for appearance (eye, hair and skin color) and biogeographic ancestry predictions.

This technical note informs the community of the following application of the VISAGE Basic Research Panel:

- Performance data for the VISAGE Basic Research Panel to highlight the following:
  - Sequencing run metric (ISP loading, total useable reads, etc.)
  - Marker read coverage
  - Concordance, sensitivity and specificity
- Workflow and system compatibility using the Ion Chef™ System and Ion GeneStudio™ S5 Systems, and Torrent Suite™ Software v5.10 and v5.12
- Genotyping and predictions using the following analysis tools:
  - Genotyping (Converge software v2.2)
  - Ancestry predictions (Converge v2.2 bootstrapping analysis and SNIPPER)
  - Visible trait predictions (HIrisPlex-S)
- Ordering information for the VISAGE Basic Research Panel and other HID community panels available through Ion AmpliSeq Designer

SNP Analysis Using Targeted MPS Applications

With the introduction of SNP analysis on MPS platforms, HID laboratories can now routinely ascertain biogeographic ancestry, paternal and maternal lineages and phenotypic traits[1-6] using informative genetic marker sets to assist investigators with new types of forensic investigative leads. By combining genetic marker sets in a single multiplex assay, researchers and practitioners can maximize investigative leads information as a supplement to conventional DNA profiling for human identification applications. The European VISAGE consortium[7], comprised of leading forensic genetics researchers, sought to expand the use of predictive markers for externally

visible traits and ancestry estimations to establish application use cases for forensic practitioners. The *Xavier et al. 2020* publication[5] provides a summary of panel performance and validation studies conducted to lay the groundwork for forensic casework interpretation.

Using MPS and Ion AmpliSeq chemistry, a single assay – capable of simultaneously interrogating hundreds genetic markers – can generate investigative information in a simple workflow with a range of forensic samples due to the scalable multiplexing capacity and small amplicon panel designs. The VISAGE Basic Research Panel consists of 153 SNPs that provide predictive information for externally visible characteristics (EVC) (41 SNPs for eye, hair and skin color from HIrisPlex-S) and continental biogeographical ancestry (115 SNPs; three overlap with the EVCs SNP set).

Data presented herein are derived from in-house verification testing using an Ion Chef/S5 workflow with the final formulation of the manufactured panel lot to ensure robust performance prior to making the panel available for purchase. Refer to the *Xavier et al.* 2020 publication for more detailed information on the panel development and design, assay performance, empirical test results, and validation studies conducted. Table 1 provides a current list of commercially available Thermo Fisher Scientific NGS community panels.

Table 1. Summary of commercially available Thermo Fisher Scientific NGS community panels

Panel	Application	Marker Type	Genetic Markers
Ion AmpliSeq HID Y-SNP Research v1	Identity & Investigative leads	Male lineage	781 Y-SNP's
Ion AmpliSeq™ PhenoTrivium Panel	Identity & Investigative leads	Ancestry, phenotype and male lineage	320 SNP's (200 autosomal + 120 Y-SNP's)
Ion AmpliSeq™ MH-74 Plex Research Panel	Identity	Autosomal microhaplotypes	230 SNP's (74 microhaplotyes)
Ion AmpliSeq™ VISAGE Basic Research Panel	Investigative leads	Autosomal	153 SNP's
Ion AmpliSeq DNA Phenotyping	Investigative leads	Autosomal	23 autosomal SNP's

Note: Panels were developed in collaboration with forensic genomics researchers and available on Ampliseq Designer. Refer to citations below for details on panel performance and validation studies performed.

Methods

For these studies, 12 male and female samples from the International Genome Sample Resource (IGSR) repository (Table 2) and 4 commonly used control DNA samples were used to examine panel performance. Population samples from the IGSR repository were chosen to cover a small range of populations (Finnish in Finland, Esan from Nigeria, Han Chinese, Indian Telugu in the UK, Peruvian in Peru, Colombian from Medellin, Colombia). M007 control DNA (European) was used as a positive control across each library pool and library replicates of 9947A (West Asia), HC-1017 (East Asian) and 2800M (ancestry could not be located for this control) were used to assess inter-library precision. To assess sensitivity of the assay, genomic DNA (gDNA) inputs of 50 pg – 1 ng were tested using 2800M control DNA.

Table 2. Samples Selected from International Genome Sample Resource (IGSR)

Sample	1000 Genomes Population Group	Reported Ancestry
HG00371	Finnish in Finland	European
HG00382	Finnish in Finland	European
HG03367	Esan from Nigeria	African
HG03369	Esan from Nigeria	African
HG00421	Han Chinese	East Asian
HG00418	Han Chinese	East Asian
HG04022	Indian Telugu in the UK	Southeast Asian
HG04017	Indian Telugu in the UK	Southeast Asian
HG02299	Peruvian in Peru	Admixture
HG02291	Peruvian in Peru	Admixture
HG01550	Colombian from Medellin, Colombia	Admixture
HG01497	Colombian from Medellin, Colombia	Admixture

Library preparation was performed with the Precision ID DL8 Kit using the VISAGE Basic Research Panel and the Ion Chef System following the custom SNP panel protocol outlined in the *Precision ID SNP Panels with the HID Ion S5/HID Ion GeneStudio S5 System Application Guide (MAN0017767)*. For library preparation, 150 uL of the VISAGE Basic Research Panel was added to Position A and B tubes of the DL8 reagents cartridge as described in the user guide. A DNA input amount of 1 ng (determined using the small autosomal target from the Quantifiler™ Trio DNA Quantification Kit) was used for library preparation sample set-up with the exception of the sensitivity study where 50 pg – 1 ng of gDNA was used. Cycling parameters for

library preparation on the Ion Chef were modified to accommodate the number of amplicons in the primer pool (Table 3). For the sensitivity study, the number of cycles was increased to 27 cycles for DNA input amounts ≤ 500 pg following the recommendations outlined in the '*Start the Ion Chef™ run*' section of the *Precision ID SNP Panels with the HID Ion S5/HID Ion GeneStudio S5 System Application Guide* (MAN0017767). A marker coverage comparison for 22 versus 27 amplification cycles is discussed in *Xavier et al*.

Table 3. Library Preparation Parameters

Ion Chef Library Preparation	
Library Preparation Cycle Number	22 cycles
Anneal & Extension Time	4 minutes
Manual Library Preparation	
Library Preparation Cycle Number	21 cycles
Anneal & Extension Time	4 minutes

Library pools were quantified using the 7500 Real-Time PCR System, HID Real-Time PCR Analysis Software v1.2, and Ion Library TaqMan Quantitation Kit, diluted and pooled to 30 pM for templating on an Ion 530 Chip using the Ion S5 Precision ID Chef & Sequencing Kit. Template plans were created following the protocol outlined in the '*Create a Planned Run for the Custom Ion AmpliSeq™ SNP Panel*' section of the *Precision ID SNP Panels with the HID Ion S5/HID Ion GeneStudio S5 System Application Guide (MAN0017767)*. Along with hg19 reference genome, the corresponding target and hotspot region BED files were used to setup templating runs (Table 4). Note that experiments performed using manual library preparation also followed the instructions provided in the Precision ID SNP Panels application guide mentioned above.

Table 4. Target & Hotspot Region BED Files

Type	Name
Target Region File	VISAGE_basic_targets_v1.0.bed
Hotspot File	VISAGE_basic_hotspot_v1.0.bed

Sequencing was performed on an Ion S5 XL System with Torrent Suite Software v5.10 and v5.12. Converge Software v2.2 with NGS Data Analysis module v1.2 was used for SNP genotyping with the target and hotspot files outlined above. Admixture analysis of the sequencing data was performed using two different analysis tools: 1) a custom implementation of the bootstrapping admixture analysis feature in Converge v2.2 and 2) SNIPPER v2.5. SNIPPER is a publicly available analysis tool for ancestry classification of individuals (http://mathgene.usc.es/snipper/). Figure 1 shows the associated analysis

workflows for this panel. DNA phenotype analysis was done through Erasmus University's HIrisPlex-S (HPS) webtool. The HPS tool is publicly available online at https://hirisplex.erasmusmc.nl/ and provides eye, hair and skin color predictions from SNP genotype data. SNP genotypes from Converge were converted to the corresponding input format for the HPS webtool using a custom excel worksheet.

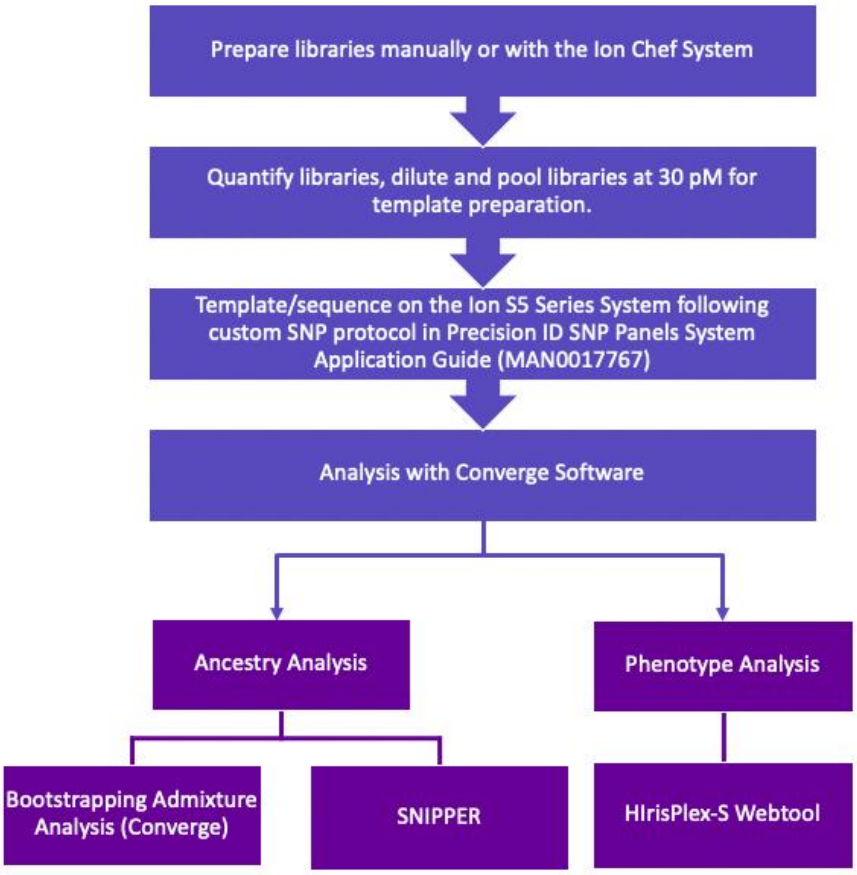


Figure 1. Analysis workflow for the Ion AmpliSeq VISAGE-Basic Research Panel

Results

Assay Performance. The baseline performance of the Ion AmpliSeq VISAGE-Basic Research Panel was examined using IGSR and control DNA samples with assay performance similar to the results provided in *Xavier et al*. Table 5 shows the average coverage of the panel for 8, 16 and 24 samples multiplexed on the Ion 530 chip. As expected, increasing the number of samples multiplexed per chip results in lower average coverage; however, the lowest coverage observed across all samples sequenced is still above the 100X threshold used by most laboratories. It is important to note that marker coverage is affected by the number of samples multiplexed per

sequencing run however marker performance should remain the same. Figure 2 shows average normalized marker coverage across 8, 16 and 24 samples and similar marker performance was obtained across the different configurations. Average coverage of the panel is still fairly high for 24 samples/530 chip at 6057X ± 1921X and >96% of markers falling within two standard deviations of the mean.

Table 5. Ion AmpliSeq VISAGE-Basic Research Panel Performance

Metric	8 Samples	16 Samples	24 Samples
Average coverage	17471 ± 5439	9237 ± 2894	6057 ± 1921
% of markers within 1SD of the mean	62.09%	66.01%	62.75%
% of markers within 2SD of the mean	99.35%	98.69%	96.73%
Lowest Coverage Observed Across Samples	4145	201 (1744)*	139 (1143)*

**Numbers provided in parentheses exclude HG01550 which was underrepresented, relative to total reads per sample, during sequencing.*

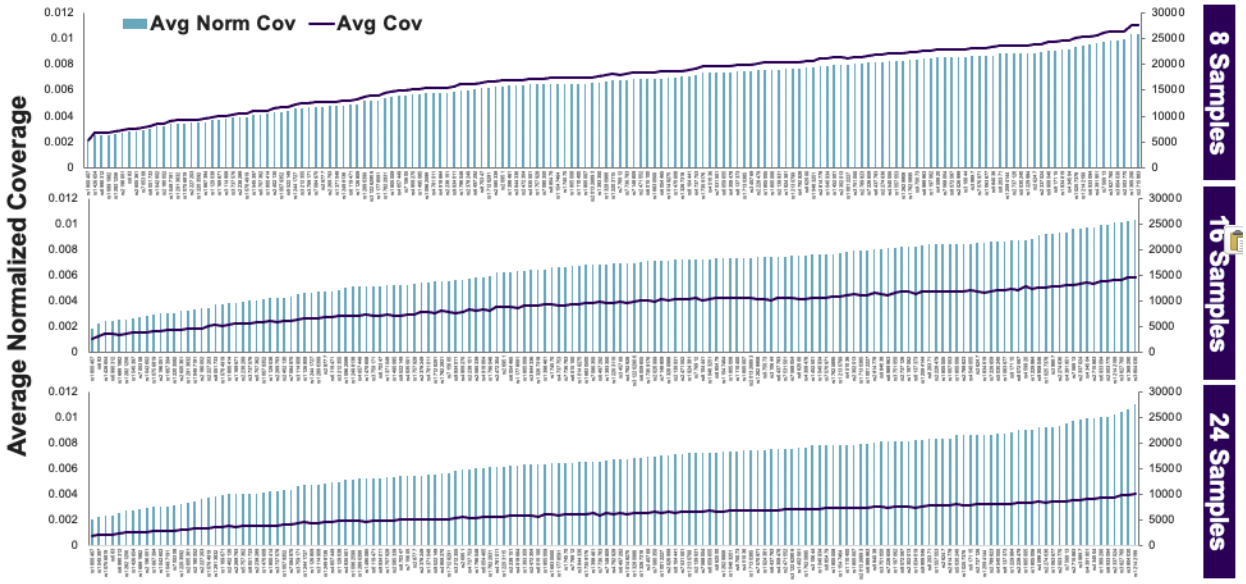
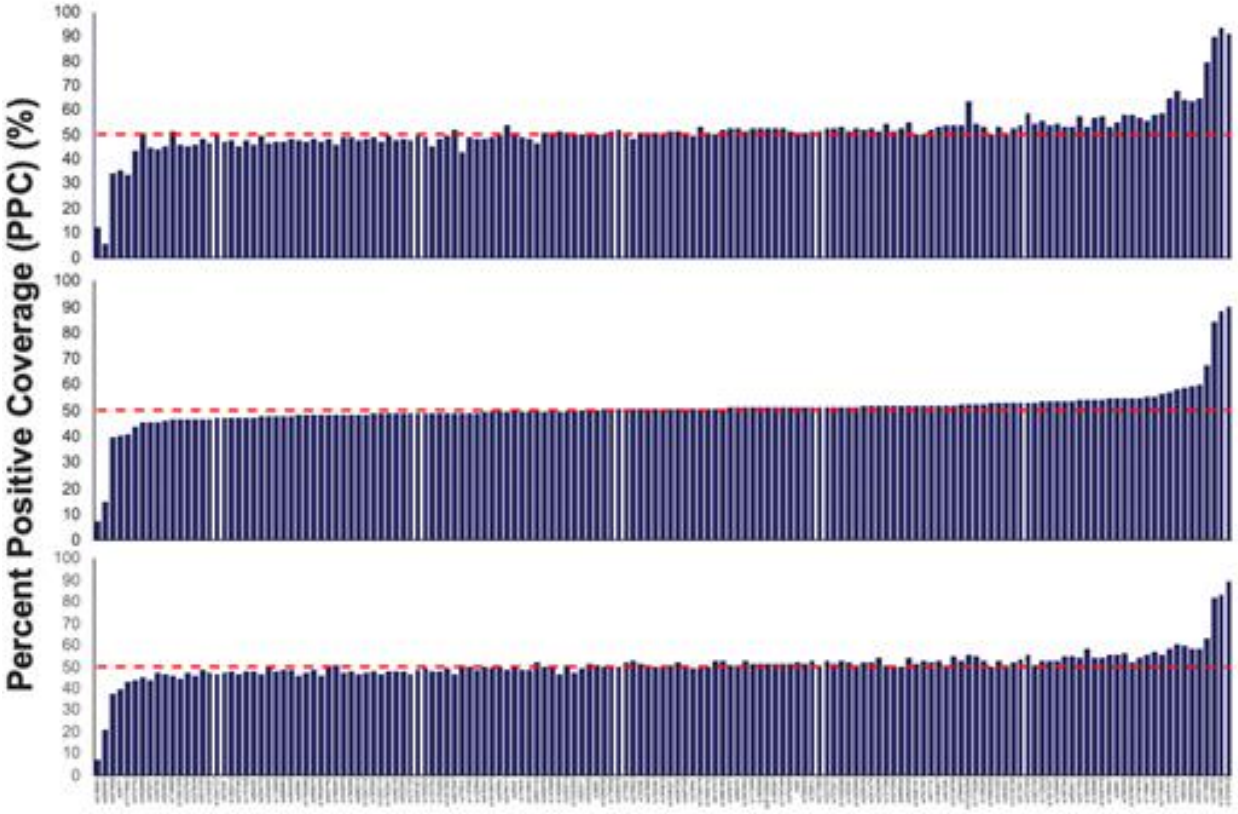


Figure 2. Average normalized coverage of the Ion AmpliSeq VISAGE-Basic Research Panel across 8, 16 & 24 Samples/530 chip. SNP coverage was normalized relative to total sample coverage. Average marker coverage is shown on the right y-axis and average normalized marker coverage is shown on the left y-axis.

Marker strand bias and major allele frequency (MAF) performance is shown in Figures 3 and 4. Strand bias, measured in percent positive coverage (PPC), refers to the ratio of forward reads to reverse reads for an amplicon/SNP with an optimal PPC value of 50%. Strand bias of the Ion AmpliSeq VISAGE Basic Research Panel is similar to the performance shown in *Xavier et al* with 88.2% of markers exhibiting PPC between 45% and 55% (for 16 samples/530 chip). MAF values help to identify imbalanced markers and are expected to be within 35-65% for heterozygotes genotypes and 95%-100% for homozygotes. For 8, 16 and 24 samples/chip, we observed 5, 20 and 31 instances, respectively, of MAF values outside of the expected 35%-65% range for heterozygotes. Markers with strand bias and/or allele imbalance (Tables 6 and 7) should be interpreted with caution. *Xavier et al.* provide additional guidelines and recommendations in interpreting flagged problematic SNPs.



	45% < PPC < 55%	30% < PPC < 70%
8 Samples	78.4%	96.1%
16 Samples	88.2%	96.7%
24 Samples	82.4%	96.7%

Figure 3. Marker strand bias of the Ion AmpliSeq VISAGE-Basic Research Panel for 8 (top), 16 (middle), & 24 (bottom) Samples/530 Chip. Results for 16 samples/530 chip are similar to those observed in Xavier et al with <12% of markers exhibiting strand bias outside of the optimal 45%-55% range. The red dotted line represents an optimal PPC of 50%. Panel performance remains the same irrespective of the number of samples multiplexed per 530 chip.

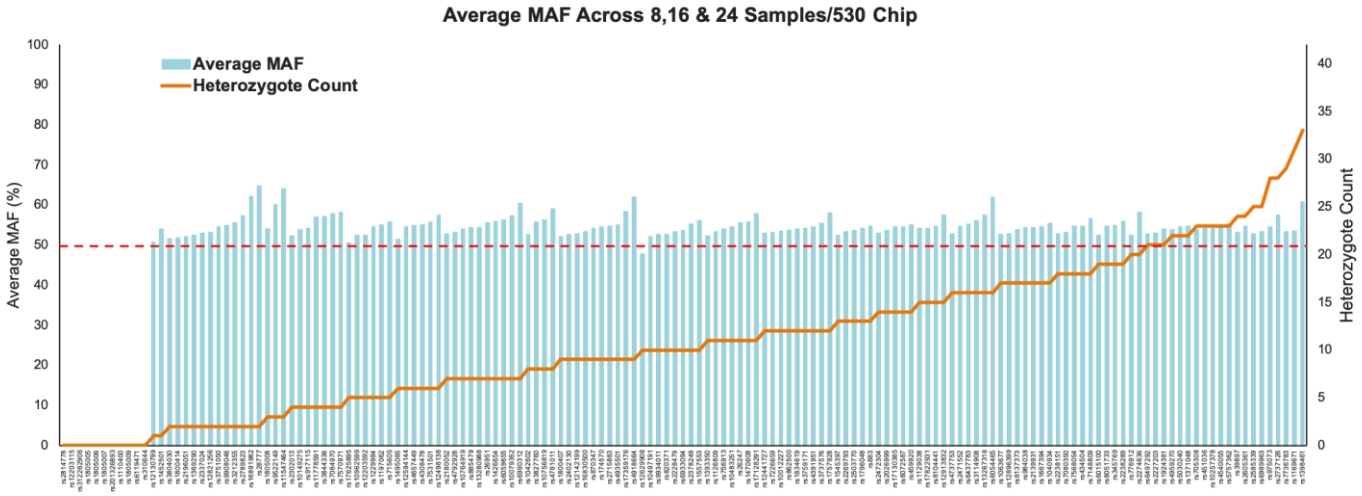


Figure 4. Average heterozygote MAF of markers in the VISAGE Basic Research Panel. MAF values of heterozygous genotypes (n=853 heterozygous genotypes; 42 samples total) were averaged across 8, 16 and 24 samples multiplexed per chip to obtain the average MAF for each marker. Markers where no heterozygous genotypes were observed across all samples are shown with a 0% MAF. The red dotted line represents an optimal MAF of 50%.

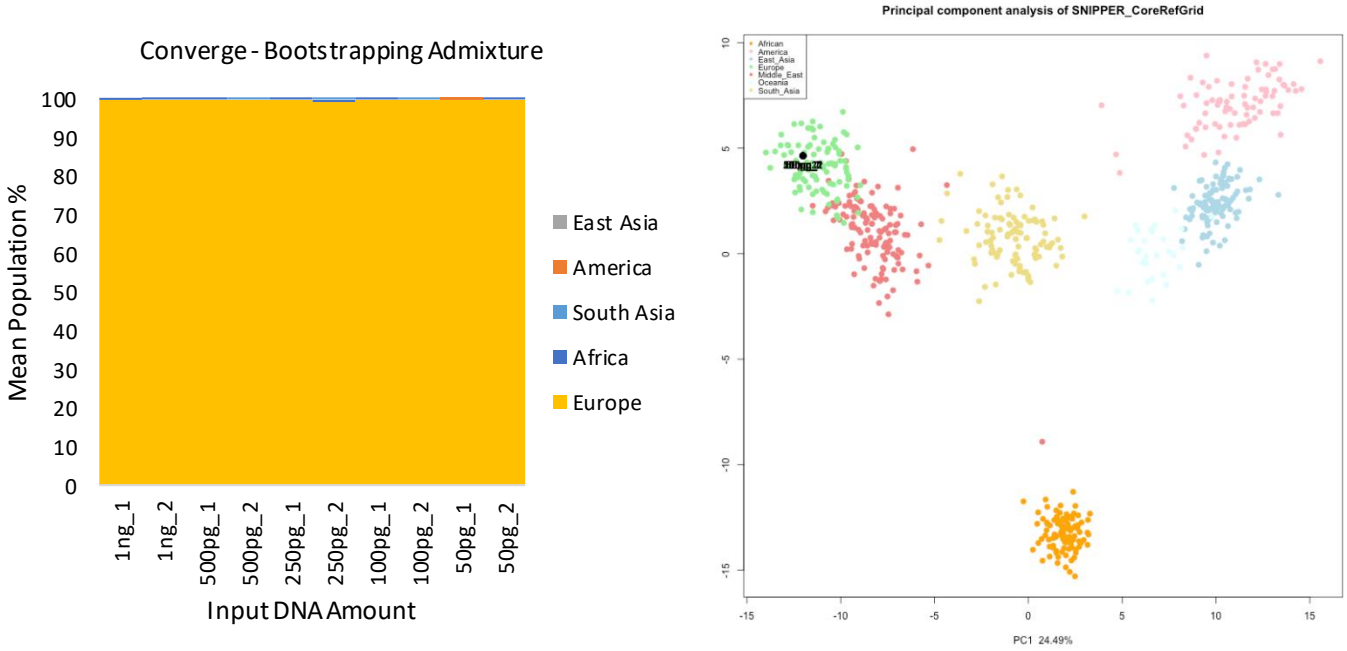
Table 6. Allele imbalance (outside 35-65% MAF) observed for 8, 16 & 24 samples per chip

Samples/530 Chip	Total # of Observed Heterozygotes	# of Instances of Imbalanced Heterozygotes (% of Total Observed Heterozygotes)
8	268	5 (1.87%)
16	572	20 (3.50%)
24	853	31 (3.63%)

Table 7. SNPs exhibiting strand bias (outside of 45% - 55% PPC)

SNPs <45% PPC	SNPs >55% PPC
rs2196051	rs1169671
rs2605361	rs1393350
rs2274636	rs17128291
rs28777	rs4781011
rs11778591	rs3737576
rs4767753	rs862500
	rs6054465
	rs9522149
	rs3114908
	rs2180052
	rs12498138
	rs10764919

Sensitivity. The VISAGE Basic Research Panel was sensitive down to 50 pg of DNA, with 100% SNP concordance observed across input DNA amounts from 1 ng – 50 pg. It is important to note that marker coverage is affected by the number of amplification cycles, samples multiplexed per chip, DNA quality and DNA quantity. As part of this, the increased number of cycles used for input DNA amounts ≤500 pg and small number of samples multiplexed per chip may have contributed to the overall genotyping success of samples ≤100 pg. Laboratories should internally validate chemistry workflow, including the number of amplification cycles, and analysis thresholds to accommodate for lower DNA input samples. Ancestry analysis was performed for the sensitivity series using a custom implementation of the bootstrapping admixture analysis on Converge v2.2 and SNIPPER PCA analysis using the VISAGE-Basic Tool SNIPPER Reference-Test Grids. Ancestry prediction results between Converge and SNIPPER were in agreement and predictions were consistent for all input DNA amounts tested (Figures 5A and 5B). Phenotype predictions were made using the HIrisPlex-S webtool (https://hirisplex.erasmusmc.nl/). SNP genotypes obtained from Converge were converted to the corresponding HirisPlex-S webtool input format using a custom excel worksheet.



Figures 5A and 5B. Ancestry and phenotype predictions for the 2800M sensitivity series. Ancestry results from bootstrapping admixture analysis (left) using 50% resampling size and 100 bootstrapping replications. SNIPPER PCA analysis of 2800M sensitivity series (right) with all sensitivity samples clustering with European reference samples.

Table 8: Phenotype predictions made using the HIrisPlex-S webtool using the 2800M reference genotype

Predicted phenotype		
	p-value	AUC Loss
blue eye	0.05	0
intermediate eye	0.114	0
brown eye	0.836	0
blond hair	0.458	0
brown hair	0.484	0
red hair	0.001	0
black hair	0.056	0
light hair	0.885	0
dark hair	0.115	0
very pale skin	0.006	0
pale skin	0.252	0
intermediate skin	0.717	0
dark skin	0.025	0
dark to black skin	0	0

Table 9: Phenotype predictions for all sensitivity samples with 2800M reference predictions boxed in red

				1 ng		500 pg		250 pg		100 pg		50 pg	
		Reference	Full AUC	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2
Eye Color	Blue	0.05	0.94										
	Intermediate	0.11	0.74										
	Brown	0.84	0.95										
Hair Color	Blond	0.46	0.81										
	Brown	0.48	0.74										
	Red	0.00	0.93										
	Black	0.06	0.86										
Hair Shade	Light	0.88	0.91										
	Dark	0.12											
Skin Color	Very Pale	0.01	0.83										
	Pale	0.25	0.76										
	Intermediate	0.72	0.78										
	Dark	0.02	0.98										
	Dark to Black	0.00	0.99										

= Similar P-Value with no AUC loss; Prediction in agreement with reference

Concordance. The VISAGE-Basic Research Panel contains 153 markers including an indel marker, rs312262906. As insertion/deletion calling capabilities are not available by Converge v2.2, the indel marker was excluded from concordance comparisons. Users are recommended to review the IGV read pile-up to call the insertion marker rs312262906. A total of 42 IGSR samples across 8, 16 and 24 Samples per 530 chip were used to establish genotype concordance (n=4104 genotypes). Genotype data from IGSR samples were compared to reference genotype data from the 1000 Genomes database and 99.9% of all genotypes were concordant. Four instances (0.10%) of discordance (in 2 unique samples) were observed with rs2737126 where genotyping was affected by unexpected low coverage observed in a small number of samples tested (n=3). Closer inspection of rs2737126 and the surrounding sequence content of the SNP in 3 samples shows a primer binding SNP upstream of rs2737126. The 3 IGSR samples contain a mutation in rs2567880 (refer to table below) which may have affected amplification of rs2737126. Library replicates of 9947A, HC-1017, M007 and 2800M control DNA were 100% concordant between replicates.

Table 10. Results for rs2737126 with 16 and 24 samples per 530 chip.

Sample	rs2567880 Genotype	Samples/530 Chip	rs2737126 Coverage	rs2737126 Ref Genotype	rs2737126 Called Genotype	MAF (%)	Flag
HG01550	CT	16	1845	GT	TT	97.72	
		24	1205	GT	TT	97.68	
HG03367	TT	16	256	GG	GG	89.45	MAF
		24	166	GG	GT	87.95	MAF
HG03369	TT	16	189	GG	GG	100	
		24	98	GG	GG	97.96	

Tertiary Analysis – Admixture Prediction. Genotype data from 16 samples per 530 chip were used for tertiary ancestry analysis using a custom implementation of the bootstrapping admixture analysis feature on Converge v2.2 with a reference data set created for the VISAGE Basic Research Panel markers with 1000 Genomes Phase III population data. Analysis parameters for bootstrapping analysis were varied at two levels: 1) the percentage of SNPs resampled (50% & 75%), 2) the number of bootstrapping replications (100, 200 & 500 replications). Varying the analysis parameters did not largely affect ancestry prediction with respect to the population attributions; however, laboratories are recommended to perform their own internal testing to identify analysis parameters and the corresponding interpretation guidelines.

Non-admixed samples were predicted to have single population attributions corresponding to the population origin of IGSR samples (Figure 6).

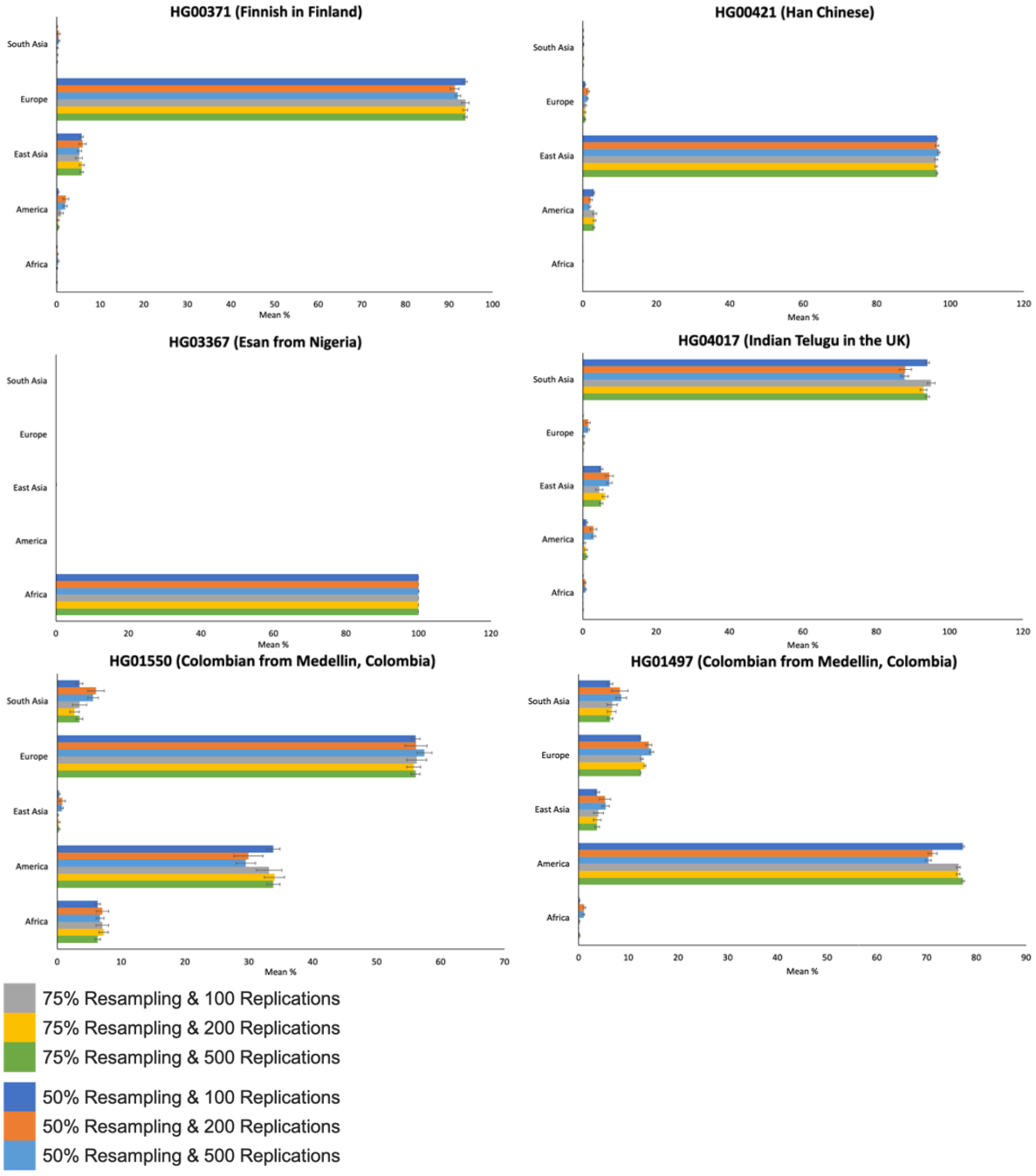


Figure 6. Ancestry predictions for IGSR samples used in the study. A custom reference data set was created for VISAGE Basic Research Panel using population data from 1000 Genomes Phase III categorized into 5 superpopulations (Africa, America, East Asia, Europe & South Asia). The resampling percentage and number of bootstrapping replications are listed. Bars represent the mean population % and error bars show 95% confidence interval.

Ancestry prediction was also performed using SNIPPER v2.5, the recommended analysis tool by Xavier et al, using the VISAGE-BT SNIPPER Reference-Test Grids. Ancestry classifications are shown in Figure 7 with similar predictions obtained for non-admixed samples as Converge. For samples with expected admixed American admixture, HG01497 and HG01550, SNIPPER PCA analysis assigned South Asia and Middle East ancestry attributions respectively with no admixture detected using the core reference grid of the reference data set.

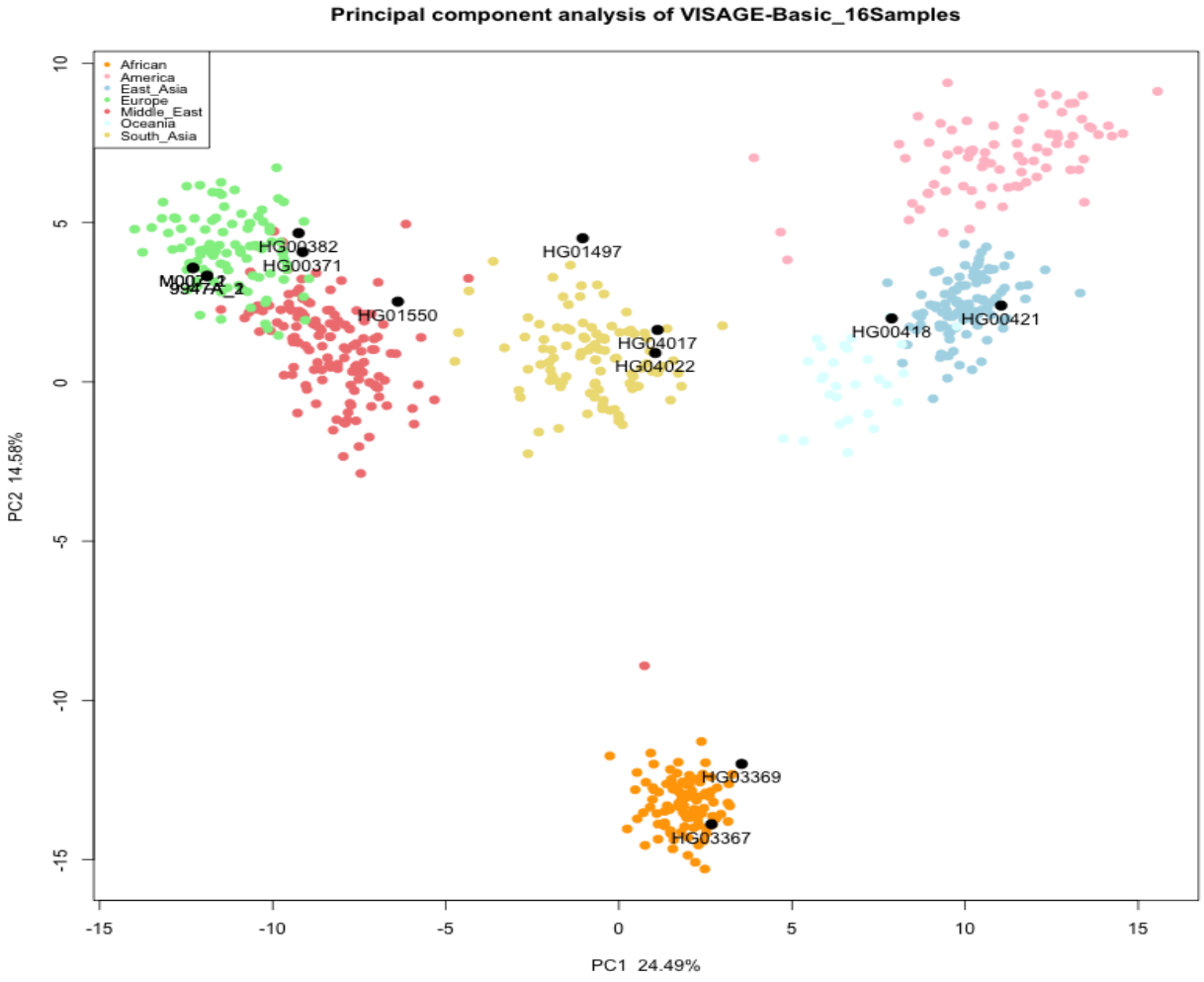


Figure 7. SNIPPER PCA analysis results of genotype data from 16 samples/530 chip analyzed using the Core Reference Grid of the VISAGE-BT SNIPPER Reference-Test Grids. Ancestry predictions were aligned with the reported population origins for non-admixed samples. For samples with expected American admixture (HG01497 & HG01550), samples were assigned South Asia and Middle East ancestry attributions respectively.

Admixed population data was added to the core reference grid and the samples with expected admixture were reanalyzed (Figure 8). After incorporating the 1KG Admixed population data, both samples were classified as admixed American.

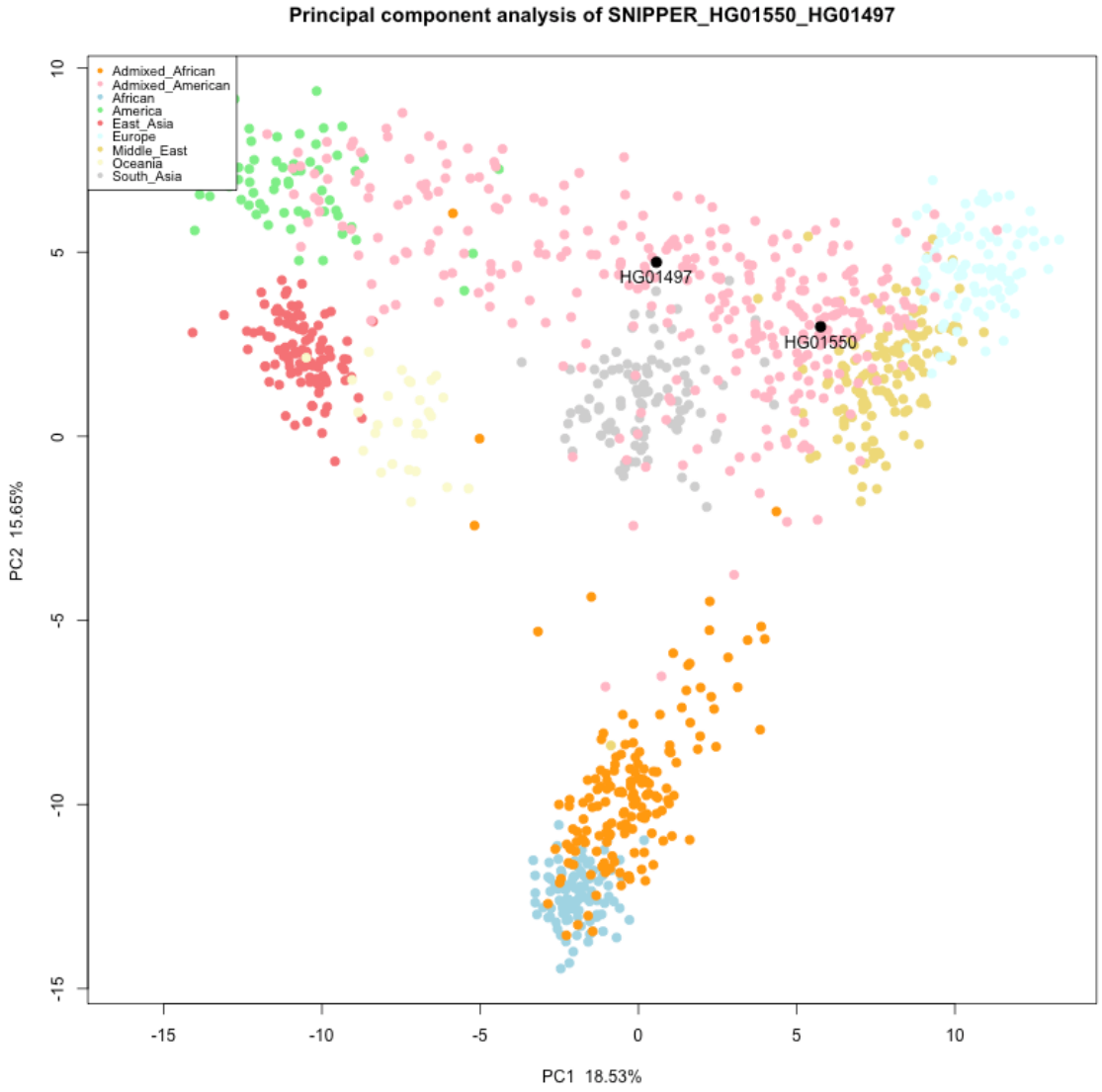


Figure 8. SNIPPER PCA analysis results of genotype data for HG01497 and HD01550 using 1KG Admixed Population data

Ordering information. To obtain the Ion AmpliSeq VISAGE Basic Research Panel, customers should visit Ion AmpliSeq Designer on thermofisher.com (https://www.ampliseq.com/login/login.action) to create a user account, login and purchase the panel through the website. The panel is catalogued under the Community Panel section of Ion AmpliSeq Designer.

Conclusions

Iterative panel development efforts between Thermo Fisher Scientific and the European VISAGE consortium have created an informative investigative tool that contains 153 SNP's to simultaneously predict appearance and continental biogeographic ancestry predictions in routine forensic DNA casework applications. *Xavier et al.* provide comprehensive test results for the Ion AmpliSeq VISAGE Basic Research Panel – including sequencing data benchmarks, sensitivity, concordance, mixtures, challenging and degraded samples and inhibitor tolerance – to demonstrate the robustness of this multiplex assay. The SNPs in this assay show practical utility in forensic investigations for practitioners who seek to assist law enforcement investigations by providing continental biogeographic ancestry as well as eye, hair, and skin color of an unknown crime scene sample.

The verification results provided in this technical note demonstrate performance of the final build of the panel in our hands and are intended as a guideline for those who perform testing with this panel. Multiplexing up to 24 samples per Ion 530 chip produced above 100X depth of coverage in all studies performed. Increasing the number of samples multiplexed per chip will result in lower average coverage, and as such, laboratories are recommended to perform their own internal studies with this panel to establish appropriate assay conditions and analysis thresholds.

As noted in *Xavier et al*., the VISAGE consortium intends to further refine the analytical tools for this panel to provide statistical predictions by converting resulting genotypes into probability estimates of eye, hair, skin color and continental biogeographic ancestry. In the interim, the publicly available HIrisPlex (https://hirisplex.erasmusmc.nl/) and SNIPPER (http://mathgene.usc.es/snipper/) tools support the tertiary analysis of this panel. Further developments to transition this research tool to routine forensic casework applications are underway.

References

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7. http://www.visage-h2020.eu/

Revision History

Revision	Date	Description
A	20 October 2021	Initial publication