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REVIEW

Forensically relevant SNaPshot® assays for human DNA SNP analysis: a review

Bhavik Mehta[1] · **Runa Daniel**[2] · **Chris Phillips**[3] · **Dennis McNevin**[1]

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Abstract Short tandem repeats are the gold standard for human identification but are not informative for forensic DNA phenotyping (FDP). Single-nucleotide polymorphisms (SNPs) as genetic markers can be applied to both identification and FDP. The concept of DNA intelligence emerged with the potential for SNPs to infer biogeographical ancestry (BGA) and externally visible characteristics (EVCs), which together enable the FDP process. For more than a decade, the SNaPshot® technique has been utilised to analyse identity and FDP-associated SNPs in forensic DNA analysis. SNaPshot is a single-base extension (SBE) assay with capillary electrophoresis as its detection system. This multiplexing technique offers the advantage of easy integration into operational forensic laboratories without the requirement for any additional equipment. Further, the SNP panels from SNaPshot® assays can be incorporated into customised panels for massively parallel sequencing (MPS). Many SNaPshot® assays are available for identity, BGA and EVC profiling with examples including the well-known SNP*for*ID 52-plex identity assay, the SNP*for*ID 34-plex BGA assay and the HIrisPlex EVC assay. This review lists the major forensically relevant SNaPshot® assays for human DNA SNP analysis and can be used as a guide for selecting the appropriate assay for specific identity and FDP applications.

Keywords Single-nucleotide polymorphism (SNP) · SNaPshot · Forensic genotyping · Capillary electrophoresis (CE) · Forensic DNA phenotyping (FDP) · DNA intelligence

Introduction

Short tandem repeats (STRs) are the markers of choice for forensic human identification due to their highly polymorphic nature and, therefore, their ability to differentiate between individuals [1]. In the past decade, genome-wide association studies (GWASs) have flooded databases with novel single-nucleotide polymorphisms (SNPs) [2]. In addition, numerous studies have contributed population data associated with SNPs to these databases, creating a valuable scientific resource [3]. The forensic community has been utilising these resources to apply SNPs to forensic DNA analysis, for both human identification and intelligence. SNPs can offer some key advantages over STRs including lower mutation rates (ideal for ancestry affiliation), higher abundance in the human genome, short PCR amplicon length suitable for high multiplexing capability and the analysis of degraded DNA, amenity to high-throughput genotyping and application to many forensic applications outside of human identification [4–6]. SNPs can be classified according to their forensic application such as identity-informative SNPs (IISNPs) for human identification, lineage-informative SNPs (LISNPs) for inferring genealogies (especially useful in kinship analysis and paternity testing), ancestry-informative SNPs (AISNPs) for inferring biogeographical ancestry (BGA) and phenotypic-informative SNPs (PISNPs) for inferring externally visible characteristics (EVCs) (such as eye, hair and skin

✉ Bhavik Mehta
bhavik.mehta@hotmail.com

[1] National Centre for Forensic Studies, Faculty of Education, Science, Technology and Mathematics (ESTeM), University of Canberra, Bruce, ACT 2617, Australia

[2] Office of the Chief Forensic Scientist, Victoria Police Forensic Services Department, Macleod, VIC 3079, Australia

[3] Forensic Genetics Unit, Institute of Forensic Sciences, University of Santiago de Compostela, 15782 Santiago de Compostela, Spain

colours). IISNPs are less informative than the equivalent number of STRs because many of them are bi-allelic, and hence, 50–60 IISNPs are required to provide approximately the same discriminating power as 13 STRs [7]. In addition, bi-allelic markers have limited mixture resolution capability. Thus, while STRs are considered the gold standard for identification purposes, IISNPs can be used to supplement STR profiling and other LISNPs, AISNPs and PISNPs can provide valuable forensic intelligence by inferring lineage, BGA and EVCs, respectively, in cases when no STR inclusions are obtained and/or STR profiles are partial and non-informative [8].

The first PCR-based genotyping system interrogated SNPs at the HLA-DQA1 locus [9, 10]. The AmpliType® PM and DQA1 PCR amplification-reverse blot DNA typing system (Applied Biosystems, formerly PerkinElmer) was very popular in forensic laboratories nearly two decades ago [11, 12]. The system consisted of six loci and was developed as an alternative to the use of restriction fragment length polymorphisms (RFLPs) employing variable number tandem repeats (VNTRs). It was useful in casework applications, particularly when the evidentiary samples yielded low amounts of DNA or degraded DNA which could not be utilised for RFLP profiling [11, 13, 14]. However, it had a lower discrimination power than the RFLP method (~1:2000) due to the limited number of alleles available, was limited in its application to mixtures and was discontinued [12, 14]. VNTRs were later replaced by STRs [15] where DNA was amplified by PCR using commercial amplification kits and detected by capillary electrophoresis (CE) or 'genetic analysers'.

A variety of SNP genotyping techniques are available such as high resolution melting (HRM) analysis [16–18], TaqMan™ hybridisation probes (Applied Biosystems), invader technology [19], hybridisation microarrays [20], massively parallel sequencing (MPS) [21] and the SNaPshot® (Applied Biosystems) minisequencing method. Sobrino et al. [19] provided a comprehensive review on SNP genotyping methodologies [19]. Of these, SNaPshot®, based on minisequencing, has been most commonly applied to forensic DNA analysis due to its sensitivity and high multiplexing capability with the added advantage of not requiring additional equipment to that already utilised in forensic laboratories [22].

Background to the minisequencing method

Minisequencing is a genotyping method that falls under the broad category of primer extension techniques [19] which also includes other methods such as arrayed primer extension [23], primer oligo base extension [24] and pin-point assay [25]. In minisequencing, a detection primer is designed to anneal to the target DNA adjacent to the SNP of interest and is extended by a DNA polymerase using fluorescently labelled single nucleotides [19, 26]. The primer extension technique for detecting single nucleotides was developed in 1990 and was used mainly for diagnosis of genetic disorders and genotyping proteins [27, 28]. Earlier, singleplex minisequencing assays were performed using detection methods including gel and ELISA formats [26]. Later, multiplex assays that could simultaneously detect many sequence polymorphisms were developed [29]. The availability of enhanced detection methods including electrophoresis and fluorescence detection, matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry (MS) and microarrays [19] enabled multiplex SNP genotyping.

The development of a multiplex solid-phase fluorescent minisequencing assay for the detection of 12 mitochondrial DNA sequence polymorphisms showed the potential of this method for forensic applications [29]. This assay was subsequently validated for forensic casework [30]. The minisequencing assay was then applied to genotype markers associated with phenotypes. Grimes et al. [31] developed a multiplex minisequencing assay detecting 12 mutations in the human melanocortin 1 receptor (*MC1R*) gene of which eight had an association with the red hair colour phenotype. The availability of the robust and accurate SNaPshot® (Applied Biosystems) kit [32], which involves electrophoresis and a fluorescence detection method, has led to the development of a series of forensically relevant multiplex assays.

Principle and workflow of SNaPshot® assays

The SNaPshot® assay is the most common commercial minisequencing method that works on the principle of single-base extension (SBE). Firstly, the DNA template is subjected to multiplex PCR to generate the target amplicons containing the SNPs of interest. Purification of the PCR product is then performed by adding exonuclease I and shrimp alkaline phosphatase (SAP) to degrade unbound primers and unincorporated dNTPs, which would interfere with the subsequent SBE reaction. The 3′ end of the oligonucleotide SNaPshot® (detection) primer binds immediately adjacent to the SNP of interest and is extended by *Taq* DNA polymerase which incorporates a fluorescently labelled dideoxyribo nucleotide triphosphate (ddNTP) complementary to the base on the opposite strand at the SNP position. Each detection primer can be augmented with (non-binding) oligonucleotide tails at the 5′ end (for example, poly C or CT) that assist in the spatial separation of the SBE products when detected by CE [22, 33]. The manufacturer's protocol recommends a maximum 10-plex; however, highly multiplexed custom assays often incorporating in excess of 20 SNP targets have been developed [32, 34–37]. The SBE products are further purified by adding SAP to eliminate unincorporated ddNTPs that potentially interfere with fluorescence detection. The purified products are prepared for CE and spatially separated on genetic analysers [32, 33]. Software such as GeneMapper™

ID-X (Applied Biosystems) is used to analyse the data and genotype the samples. Figure 1 illustrates the principle and workflow of the SNaPshot® method.

Primer design for multiplex assays

Primer design is critical for the successful development of multiplex SNaPshot® assays. The PCR and SBE primers can be designed using freely available primer designing tools such as Primer 3 [38] for primer sequences, AutoDimer [39] and IDT OligoAnalyzer [40] for secondary structure analysis (including the formation of primer dimers) and NCBI primer blast [41] for specificity. It is important that all primers in the same multiplex have similar melting temperatures (±3 °C) as they will all be subject to the same PCR cycling conditions [42]. In the same way, GC content of all the primers in the multiplex should be in the range 40–70% [35]. The tails added to the 5′ end of the SBE primers should not bind to any region of the genome in order to avoid non-specific binding in the SBE assay [33].

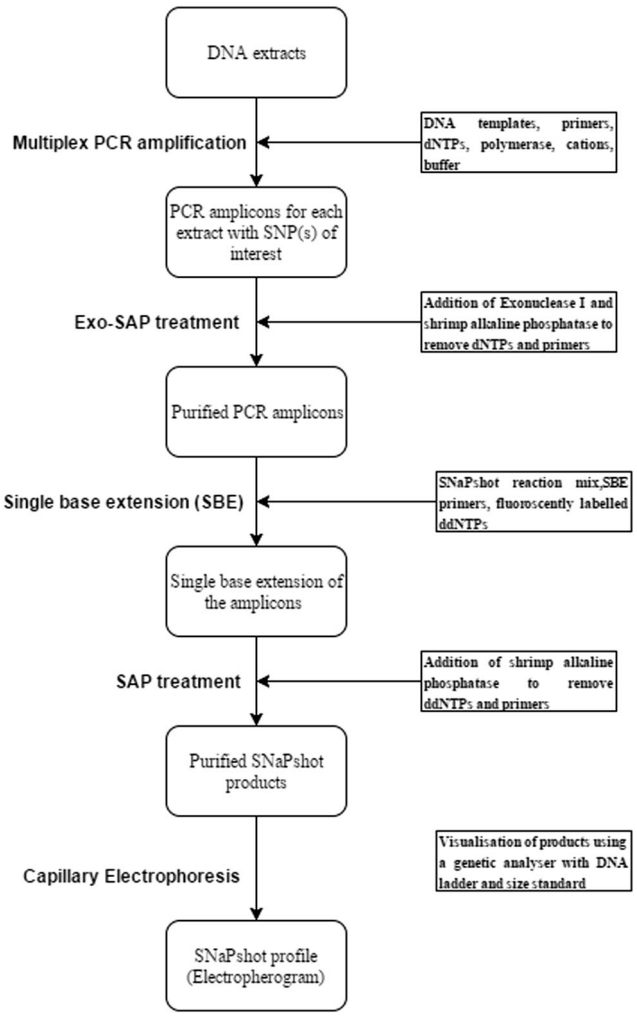

Fig. 1 SNaPshot® genotyping workflow including target amplification, enzymatic clean-up, single-base extension (*SBE*), second clean-up and electrophoresis

SNaPshot® multiplex optimisation

Each SNaPshot® multiplex assay requires optimisation in this order: multiplex PCR, multiplex SBE reaction and SBE product mobility. Generally, HPLC grade purified PCR and SBE primers are recommended for SNaPshot® assays to remove artefacts [43]. Prior to multiplex PCR optimisation, singleplex PCR is carried out to assess the performance of PCR primers and optimise assay conditions such as concentrations of primers, $MgCl_2$, dNTPs, *Taq* polymerase and thermal cycling conditions. The singleplex products are run on an agarose gel or microfluidic capillary electrophoresis (e.g. 2100 Bioanalyzer; Agilent Technologies) to verify the amplicon size distribution. The primers that yield low or no PCR product are redesigned at this stage. The multiplex PCR is then optimised, ensuring that all PCR products are more or less equally amplified [43, 44]. Exonuclease–shrimp alkaline phosphatase (ExoSAP–IT®; Affymetrix) purification of the optimised multiplex PCR products involves the removal of unbound primers and nucleotides that may hinder the subsequent analysis steps. After clean-up, the enzyme is inactivated by heating the product mixture to 80 °C for 15 min [35, 42, 45].

Singleplex SBE primer reactions without PCR template are performed to check for SBE primer self-extension. SBE primers should be redesigned if self-extension occurs. Multiplex PCR product is then added to singleplex SBE reactions to assess their specificity. The absence of one or more peaks requires redesign of the SBE primer. In order to proceed to multiplex SBE optimisation, the singleplex SBE products should show peaks higher than 500 relative florescence units (RFU) using the purified multiplex PCR amplicons [43]. During multiplex SBE optimisation, the concentration of SBE primers is adjusted depending on the signal intensities of the peaks. The sensitivity of the assay can be optimised by adjusting the volume of the SNaPshot® reaction mix (relative to DNA template amount), adjusting the number of PCR cycles and/or adjusting the number of SBE amplification cycles. Artefacts in the electropherogram may be due to factors such as poor quality of SBE primers, SBE primer interactions with other SBE primers, a non-specific PCR primer or very short PCR products. These artefacts can be neglected if they do not hinder accurate allele calling; otherwise, each SBE primer is removed serially from the multiplex to diagnose the cause of the artefact. A multiplex SBE reaction without PCR template can be carried out to identify any issues regarding the SBE primer interactions [33, 42].

It is also necessary to optimise the mobility of SBE primers to prevent SBE product overlap during CE. This optimisation

generally involves the redesign of the SBE primers with different length non-binding tails. It may also require redesign of both PCR and SBE primers and repetition of the entire process if the multiplex optimisation is unsuccessful [33].

CE and analysis

Purified SBE products are electrophoresed to obtain an electropherogram which is used to genotype samples. CE parameters including the dye set, type of polymer, capillary array length and run protocol are dependent on the type of platform used [33, 42]. For example, the 3500xl (Applied Biosystems) genetic analyser with POP-4 polymer and 36-cm array uses SNaPshot_pop4 default run protocol, GeneScan™ 120 LIZ™ Size Standard (Applied Biosystems) and Matrix Standard Set DS-02 (Applied Biosystems) for dye set E5. The electropherograms are analysed using fragment analysis software such as GeneMapper™ ID-X (Applied Biosystems) or GeneMarker™ (SoftGenetics®) [33]. The horizontal axis of the electropherogram represents the product length in the number of base pairs (bp), and the vertical axis represents the fluorescence signal intensity in RFUs. The product lengths derived from the horizontal axis will not correlate exactly with the SBE product lengths because the incorporated fluorophores will affect their electrophoretic mobilities. Each peak in the electropherogram represents the allele of a SNP with each of the four possible bases (A, C, G and T) labelled with a different fluorophore (ddATP-dR6G, ddCTP-dTAMRA™, ddGTP-dR110 and ddUTP-dROX™, respectively). Within the analysis software, bins and panels are used for genotype calling, and for each assay, different bins and panels are designed. Panels are determined based on the SNPs included in each assay while bins mark the bp range within which each peak falls. When a peak falls in a particular bin, the software makes a corresponding allele call. A single peak is obtained for homozygote genotypes, and two peaks (of different colours) are seen for heterozygous genotypes [22].

Forensically relevant SNaPshot® assays

Forensic SNaPshot® assays are here broadly classified into four categories based on their application such as IISNP assays, LISNP assays, AISNP assays and PISNP assays.

IISNP SNaPshot® assays

Forensic identity markers require the following characteristics: (i) minimal deviation from Hardy–Weinberg equilibrium (HWE), ensuring within-locus independence of alleles; (ii) minimal deviation from linkage equilibrium (LE), ensuring between locus independence; (iii) high heterozygosities to maximise the polymorphic nature of the loci, resulting in higher discrimination between genotypes; and (iv) these properties should apply both within and between multiple sub-populations [46]. One indication of this is that there is little genetic distance between these sub-populations, as measured by Wright's F_{ST}, the inbreeding coefficient within sub-populations relative to the total population [47]. IISNPs with these properties can be useful for providing identity information from degraded DNA or low DNA template amounts when STR profiling is not informative. Table 1 shows some of the potential IISNP assays.

Blood grouping assays

One of the earliest SNaPshot® assays differentiated between ABO blood groups. The multiplex assay was designed using six SNPs from the ABO gene. The assay correctly identified ABO genotypes when tested on casework samples involving bones, teeth and nails. ABO genotypes from semen-contaminated vaginal fluid casework samples were completely concordant with ABO phenotypes. This assay was species specific for human and higher-order primates. The assay was sensitive enough to generate ABO profiles from 0.1 ng of DNA [48].

Subsequently, a 17-plex SBE assay was developed to type all 10 of the known blood group systems. These SNPs represent the antigen and amino acid changes associated with each blood group. The method was tested on 29 samples with blood groups previously determined from hemagglutination, and it accurately predicted all the blood groups for all the samples. The assay was designed in three multiplexes (Multiplex I–Duffy and Dombrock blood group systems; Multiplex II–Landsteiner-Wiener, Colton, Scianna, Diego, Kidd, Lutheran and MN blood group systems; and Multiplex III–Kell and SS grouping systems) using genomic DNA extracted from 200 μl blood, and the turnaround time was 12 h for 32 samples [49].

Other (non-blood group) human identification SNP assays

One of the earliest human identification assays was a 24-plex SNaPshot® assay developed for the Korean population. The probability of identity for this assay was 2×10^{-10}, but the power of exclusion was 98.9% which is lower than that for Profiler Plus® (99.9%) [50]. The SNP*for*ID 52-plex assay, incorporating a set of two multiplexes (a 23-plex and a 29-plex) [35], provided a combined power of exclusion greater than 99.999% and a mean random match probability value in the order of 5×10^{-19}. The assay was forensically validated as a modified 49-plex assay [51] to be used in ISO 17025-accredited laboratories [52]. The 52-plex assay has also shown its versatility when applied in paternity testing [53]. It could be adapted by forensic casework laboratories as a supplementary method along with STR profiling and is useful for trace DNA amounts [54]. The 52-plex assay was tested in an Italian

Table 1 Identity-informative single-nucleotide polymorphism (IISNP) SNaPshot® assays

Assay	Target	Number of SNPs	Sensitivity (ng DNA)	Application	References
Blood grouping assays					
6-plex	Autosomal SNPs	6	0.1	ABO blood group genotyping	Doi et al. [48]
17-plex	Autosomal SNPs	17	–	Genotyping of 10 blood group systems	Palacajornsuk et al. [49]
Non-blood grouping assays					
SNP*for*ID 52-plex	Autosomal SNPs	52	0.5–70	Human identification	Sanchez et al. [35]
24-plex	Autosomal SNPs	24	1–2	Korean population identification	Lee et al. [50]
16-plex	X-SNPs	16	0.05–10	Identification of degraded samples	Oki et al. [60]
18-plex	Histone SNPs	18	0.078	Identification of highly degraded samples	Freire-Aradas et al. [61]
55-plex	Autosomal SNPs	55	0.125	Human identification	Wang et al. [22]

population, and the power of discrimination obtained was >99.99% with a 99.98% power of exclusion [55]. A sensitised version of SNP*for*ID 52-plex with increased SBE cycles (100 from 30) and use of the AmpFlSTR® SEfiler Plus™ Master Mix enhanced typing success from degraded and challenging DNA samples [56]. More recently, a 55-plex IISNP assay has been developed based on the Kidd 92 IISNP panel, enhanced from their earlier 44-plex assay [22, 57]. This assay was effective at 125 pg DNA with a power of exclusion >99.99% for the Hebei Han population [22].

IISNP SNaPshot® assays have also been developed for tri-allelic SNPs, which improve the resolution of degraded and mixed DNA samples. Westen et al. [58] developed a 16-plex SNP assay using Dutch sample sets. The assay was designed in three multiplexes of seven, four and five markers, and 15 SNPs were confirmed to be tri-allelic. When SGM Plus® and SNaPshot® profiles were compared, 14% of the alleles were lost with 5 min of UV degradation for the SGM plus® STR kit while 120 min of UV exposure was required for the same loss in the tri-allelic SNaPshot® assay. The first allelic loss for the SNaPshot® assay was observed after 60 min of UV irradiation. Resolution of two-person mixtures was possible for up to 1:8 ratios [58]. Another 20-plex SNaPshot® assay with tri-allelic SNPs generated reproducible identity profiles at 1 ng DNA input amount. The assay did not produce any profiles when tested on animal species including pig, chicken, rabbit, rat and loach fish [59]. A 16-X SNP SNaPshot™ assay (in combination with mini X-STRs) enhanced the identification of degraded DNA in Japanese samples. The sensitivity of the assay ranged from 50 pg to 10 ng DNA input amounts. The combined power of discrimination and the power of exclusion were greater than 99.99 and 99%, respectively [60].

The histone–DNA complexes of nucleosomes are known to be the sites preventing DNA degradation including apoptosis due to bacterial and environmental degradation [61]. An 18-plex SNaPshot® assay including the SNPs from these nucleosome regions had a sensitivity of 78 pg and was more effective than the SNP*for*ID 52-plex as well as the AmpFlSTR® Minifiler™ (Applied Biosystems) and AmpFlSTR® Identifiler™ (Applied Biosystems) STR assays for degraded DNA [61].

LISNP SNaPshot® assays

Lineage markers mostly include Y chromosome and mitochondrial (mt) DNA markers. Many Y-SNP and mtSNP SNaPshot® assays are available as shown in Tables 2 and 3. Before these, a solid-phase fluorescent minisequencing multiplex assay for 12 mtDNA polymorphism markers was developed and validated to identify British Caucasians and British Afro-Caribbeans [29, 30].

Y-SNP SNaPshot™ assays

In 2003, a 35-Y-SNP preliminary SNaPshot® minisequencing assay was developed with the intention of setting forensic parameters for SNaPshot® typing [62]. The sensitivity of the assay was in the range 100 pg to 10 ng DNA with an optimum of 1–2 ng. Reproducibility was demonstrated by concordant results for 194 male Danish samples typed in duplicate. The assay also illustrated the importance of primer design for SBE multiplex assays [62]. This work laid the platform for using SNaPshot® chemistry for a variety of other forensically relevant assays. The European SNP*for*ID consortium identified many potential Y-SNP and mtSNP for predicting lineages which assisted in the development of a series of SNaPshot® assays for haplogroup typing.

Vallone and Butler [63] examined 50 Y-SNPs able to differentiate US, African-American and Caucasian samples. Forty-two SNPs were typed using allele-specific hybridization

Table 2 Y chromosome lineage-informative single-nucleotide polymorphism (LISNP) SNaPshot® assays

Assay	Number of SNPs	Sensitivity (ng DNA)	Application	References
Global population assays				
Major Y chromosome haplogroup typing kit	29	0.25	Differentiates 12 world genealogies	Brión et al. [65]
28-plex	28	1–2	Differentiates major continental paternal lineages worldwide	van Oven et al. [66]
European population assays				
37-plex	37	0.05–1	Major clades of European sub-population	Onofri et al. [67]
Asian population assays				
16-plex assay	16	0.062–1	Differentiation of Asian haplogroup O	Park et al. [69]
Ancient DNA assay				
13 SNP aDNA assay	13	0.05–1	Ancient DNA samples	Bouakaze et al. [45]

(ASH) with flow cytometry detection, and 18 SNPs were typed using SBE with fluorescence detection. The SBE assay was designed in three multiplexes of six SNPs each. Ten SNPs were typed with both ASH and SBE methods. The results for both the genotyping methods were concordant, but the SBE method offered advantages of less time requirement and greater cost-effectiveness in re-analysing a sample in comparison to ASH. The study identified the need for additional Y-LISNPs. A comparative study between MALDI-TOF MS and SNaPshot® on eight Y-SNPs differentiating four European haplogroups also demonstrated the speed and accuracy of the SNaPshot® technique [64].

Global Y-SNP assays A major Y chromosome haplogroup typing kit consisting of 29 Y-LISNPs was subsequently developed which differentiated 1126 unrelated males from 12 worldwide populations into Y haplogroup lineages [65]. Only 12 SNPs were selected to divide the samples into 12 major clades, and the remaining SNPs subdivided some of these clades. This multiplex assay was subject to inter-laboratory validation using 10 human samples, and the assay overall defined 31 haplogroups. The Asian population samples were classified into six haplogroups out of 31, and 93% accuracy was reported for the detection of Southeast Asian population samples. Three haplogroups defined African samples with an additional haplogroup defining sub-Saharan African. Most of the African samples used in the study were of Somali origin. European samples were most abundant in the sample set and were classified into seven haplogroups. The assay had limitations in assigning admixed population

Table 3 Mitochondrial DNA (mtDNA) lineage-informative single-nucleotide polymorphism (LISNP) SNaPshot® assays

Assay	Number of SNPs	Sensitivity (ng DNA)	Application	References
Global population assays				
12-plex	12	0.007	Differentiates world genealogies	Nelson et al. [73]
71-plex	71	0.025	Differentiates R0 macro-haplogroup	Mosquera-Miguel et al. [74]
11-plex	11	1–2	Differentiation of genealogies in the Australian population	McNevin et al. [44]
36-plex	37	0.004	Differentiates 43 global haplotypes	van Oven et al. [75]
42-plex	42	0.01	Resolves Latin American admixture efficiently	Paneto et al. [76]
European population assays				
16-plex	16	0.0005–0.1	Differentiates West European haplogroups	Brandstätter et al. [77]
11-plex	11	0.0002–2	Resolution of European Caucasians	Vallone et al. [71]
17-plex	17	10	Differentiation of West Eurasian haplotypes	Quintáns et al. [78]
22-plex	22	–	Differentiates nine major European haplogroups	Köhnemann et al. [80]
Asian population assays				
20-plex	20		Differentiates the haplogroups in Andamanese populations	Endicott et al. [81]
32-coding mtSNP assay	32	5	Differentiates East Asian phylogeny	Álvarez-Iglesias et al. [82]

samples from Greenland and South America. This panel could differentiate the major population groups of the world but was more limited in differentiating closely related population groups [65].

More recently, a 28-Y-SNP SBE multiplex assay enabling the discrimination of major Y chromosome haplogroups worldwide has been developed. The assay was divided into two multiplexes to allow hierarchical typing. The recommended DNA amount was 1–2 ng, and further sensitivity tests were not conducted, but the PCR amplicon lengths were kept short in the range of 46–178 bp to make it suitable for degraded DNA samples. This assay can provide an assessment of the continental biogeographical male lineage only and requires additional SNPs for detailed phylogenetic classification [66].

European population Y-SNP assays A 37-Y-LISNP assay has been developed in six multiplexes for European lineage [67]. Two multiplexes with a total of 15 SNPs differentiate the major clades of the Y haplogroup tree (A-R) belonging to specific continents. The other four multiplexes differentiate European haplogroups. The sensitivity of the assays was in the range 50 pg–1 ng. The assay was shown to work on degraded DNA with quantities as low as 50 pg. The reproducibility of the assay was assessed by genotyping all the samples in duplicate which produced concordant results. The hierarchical multiplexes were designed in a way that at most, two amplification steps were required for determining the haplogroup for each sample. The first amplification step indicates the major continental clade, and the second amplification would depict the corresponding sub-clade of that sample. This assay was developed to differentiate closely related European population haplogroups but was not able to differentiate closely related population groups from other continents. This type of hierarchically designed multiplex assay could determine the specific genealogy of a sample in a forensic context [67].

Asian population Y-SNP assays A 16-Y-SNP SBE multiplex assay discriminating the males of haplogroup O mostly found in East and Southeast Asia has also been developed [68]. The assay uses smaller amplicon sizes in the range of 45–123 bp, making it applicable to degraded DNA. The assay could be used when a sample is found with a haplogroup O status from a global Y-SNP assay and further sub-lineage information is required [68]. Y-SNP miniplex assays are also available which can help dissect the high-occurrence haplogroups O and C in East Asian populations [69]. These Y-SNP miniplexes are a combination of four individual multiplexes with a total of 22 Y-SNPs. The first multiplex of six SNPs differentiates worldwide haplogroups. The other three multiplexes are designed to identify sub-haplogroups O, O3 and C. The sensitivity of the multiplexes was in the range of 62 pg–1 ng. When applied to an artificially degraded DNA, the assay produced concordant results with non-degraded controls, showing its reproducibility and reliability. The assay effectively typed 10 DNA samples from 55-year-old skeletal remains, and allele drop-in was not observed even when the amplification cycle number was increased from 33 to 35 or 37 cycles. This demonstrated the versatility of SNPs in comparison to STRs, which are prone to induce amplification errors when cycle numbers are increased. When used for typing 300 Korean samples, the assay correctly reported that the majority belonged to haplogroup O followed by haplogroup C. Haplogroup O3 signifies the migration patterns of modern East Asian populations, and this sort of information could be of forensic relevance [69].

Ancient DNA Y-SNP assays A 13-Y-SNP SNaPshot® assay designed specifically for typing of ancient DNA (aDNA) was developed on 11 bone samples from south Siberia [45]. As a single multiplex could not type any aDNA samples, it was redesigned in two PCR multiplexes of six and seven SNPs. This showed that a single multiplex assay with more markers might decrease amplification efficiency in the case of aDNA samples. Nine samples were successfully typed, and two very ancient samples failed to yield a result. The sensitivity of the assays was in the range of 50 pg–1 ng [45].

mtSNP SNaPshot® assays

Traditionally, when STR profiling fails, the typing of hypervariable parts of the mtDNA control regions (HV1 and HV2) often provides some identification information due to the high copy number of mtDNA. The inheritance of mtDNA is maternal, and due to the lack of recombination, it provides lower discrimination and identical HV1 and HV2 haplotypes are frequently encountered [70, 71]. The typing of mtSNPs in coding regions was used as an alternative to improve the discrimination power of mtDNA [72]. As a result, mtSNP typing can be used as a screening tool for eliminating multiple suspects or rapidly differentiating between many samples in high-volume cases.

Global mtSNP assays A 12-SNP multiplex assay defining the broad mtDNA haplogroups for different population samples requiring only 7 pg DNA to generate full profiles has been used to assign two World War II-era samples to their corresponding haplogroups [73]. A SNaPshot® assay defining different branches of macro-haplogroup R0 was designed using 71 mtSNPs in three multiplexes and was demonstrated to be accurate at 25 pg of DNA input amount. The assay was robust, and no contamination or amplification of NUMTs (nuclear mitochondrial DNA pseudo sequences) was found [74].

An Australian mtDNA SNP assay was used to assign 145 samples to 12 haplogroups using an 11-SNP multiplex assay [44]. The study demonstrated the difficulty in accounting for admixture with mtDNA markers. More recently, a 36-mtSNP

multiplex system has been employed to efficiently infer maternal ancestry at the continental level. The assay differentiated 43 different haplotypes with sensitivity down to 4 pg DNA. It was designed in three multiplexes of 12 SNPs each. The haplogroup assignment was consistent and concordant with full-sequence profiles [75].

Another 42-plex SNaPshot® assay was used to classify Latin American samples in an admixed population. The majority of the population (46.6%) was found to have African maternal lineage, 27.3% had European origin and 26.1% had an Asian origin. The complete profiles were obtained with only 10 pg DNA input amounts [76].

European population mtSNP assays A 16-SNP SNaPshot® mtSNP assay exists that is capable of discriminating between West European Caucasian haplogroups. The assay was developed in two equal multiplexes as a rapid screening method for elimination of multiple suspects. The power of discrimination and preliminary sensitivity were 88.6% and 25 pg, respectively [77]. Another 11-plex mtSNP assay was developed around the same time in an attempt to differentiate individuals with identical HV1/HV2 mitotypes in Caucasians [71]. The sensitivity of the assay was in the range of 0.2–2000 pg with an optimum of 1–2 pg for robust reproducibility. This assay displayed a limitation in detecting heteroplasmy with one highly ambiguous SNP [71]. A 17-plex SNaPshot® mtSNP assay was developed to allow differentiation of West Eurasian haplotypes in two PCR multiplexes. The first multiplex allocated samples to the most common European haplogroups, and the second multiplex differentiated the sub-haplogroups of the high-frequency European haplogroup H. Both the PCR multiplex products were combined in a single-SBE assay [78]. This assay separated haplogroup H into its sub-categories, thus complementing the 16-plex assay developed by Brandstätter et al. [77] and the 11-plex assay developed by Vallone et al. [71]. Grignani et al. [79] developed an assay using 25 mtSNPs to sub-type haplogroup H into sub-clades H1–H15. The assay was designed in two PCR multiplexes. Multiplex A was adapted from the 17-plex assay of Quintáns et al. [78] which separated H1–H7 sub-clades whereas multiplex B of eight SNPs differentiated H8–H15 sub-clades [79]. Another 22-mtSNP multiplex assay was developed to detect nine major European haplogroups and some of the sub-haplogroups [80].

Asian population mtSNP assays A 20-plex SNaPshot® assay was designed to target the M31 and M32 haplogroups found in Andaman Islanders using 20 ancient Andaman samples. The assay defined the fine structure of haplogroup M31, and two new sub-classes M31a1a and M31a1b were identified, supporting a division between greater Andamanese and Onge–Jarawa-speaking people. A sub-clade M32a1 was also identified to be specific to the Onge–Jarawa population [81]. A 32-coding mtSNP assay was developed to haplotype East Asian phylogeny with its Native American-derived branches [82]. A 15-plex mtSNP SNaPshot® assay was developed with a haplotype diversity of 0.9136, differentiating 28 haplotypes of the Chinese Yi population group. The assay showed the close relationship between the Chinese Yi and Bai populations [83].

Indigenous American population mtSNP assays Recently, a 26-plex SNaPshot® assay, AmericaPlex26, has been designed to genotype the human mitochondrial founder lineages of America [84]. The assay targeted sites within haplogroups A2, C, C1c, D, D4e, D2a and X, which commonly occur in American lineages. The assay was shown to work on degraded DNA and could be used as a screening tool to assess the sample preservation strategy and the presence of lineages other than above [84].

AISNP SNaPshot® assays

AISNPs are designed to distinguish between populations; therefore, their ideal characteristics are opposite to those of IISNPs. AISNPs have low heterozygosity and high F_{ST} between populations [7]. AISNP assays provide BGA information about the donor of a DNA sample (Table 4). As autosomal markers are co-inherited maternally and paternally, their advantage over lineage markers is that they are able to indicate recent admixture in individuals. In addition, mtDNA and Y chromosome markers will not reveal genetic inheritance from maternal grandfathers and paternal grandmothers which may bias ancestry estimates [42].

SNPforID 34-plex assay

The SNP*for*ID 34-plex SNaPshot® assay is a well-established ancestry-informative assay, differentiating between Europeans, Asians and Africans [42]. A naïve Bayesian classifier implemented via the *Snipper* web portal [85] can be used to estimate likelihood ratios of population membership. The assay has been demonstrated to be effective at 200 pg DNA amounts, but an optimum of 1–2 ng DNA was recommended [42].

The 11-M Madrid Bombings in 2004 represented a successful casework application of the SNP*for*ID 34-plex autosomal ancestry SNP assay. In this case, seven STR profiles from evidential samples were unmatched. Ancestry was assigned according to the 34-plex predictor model. Three samples were found to be of North African origin and one of European origin. The remaining three samples were not assigned, as their probabilities were lower than the predictor threshold. In one case, the 34-plex system revealed North African origin where Y and mtDNA loci did not. Later, familial searching of a Spanish DNA database indicated that this sample had an Algerian origin. This intelligence information led investigators to the suspect [86].

Table 4 Ancestry-informative single-nucleotide polymorphism (AISNP) SNaPshot® assays

Assay	Target	Number of SNPs	Sensitivity (ng DNA)	Application	References
SNP*for*ID 34-plex	Autosomal	34	0.2–2	Differentiation of continental populations	Phillips et al. [42]
Eurasiaplex	Autosomal	23	1	Differentiation of European and South Asian populations	Phillips et al. [36]
Pacifiplex	Autosomal	29	≥0.125	Differentiation of Oceania populations	Santos et al. [37]
EurEas_Gplex	Autosomal	14	0.5–2	Differentiation of European and East Asian populations and gender identification	Daca-Roszak et al. [88]
Global AIMs Nano	Autosomal	31	0.064	Differentiation of African, European, East Asian, Oceanian and Native American populations	de la Puente et al. [89]

Recently, the revised 34-plex assay was published, where the SNP rs727811 in the original panel was replaced by rs3827760 to improve resolution and performance of the assay [34]. The SNaPshot® assay design was re-optimised with new PCR and SBE primers. The amplification cycles for the PCR step were reduced from 35 to 30 and, for the SBE step, from 30 to 28. The assay includes two tri-allelic SNPs, which are useful for identifying contributors to a mixture. Samples with three or more admixed ancestries were difficult to resolve [34]. When applied to US population samples, the 34-plex assay showed that non-admixed samples and samples with two dominant co-ancestries were classified accurately. It was more difficult to identify ancestral populations in the highly admixed Hispanic samples [87].

Eurasiaplex assay

Eurasiaplex is a 23-plex SNaPshot® multiplex assay designed to complement the SNP*for*ID 34-plex assay [36]. Eurasiaplex, in combination with the 34-plex assay, differentiates Europeans and South Asians (especially west of Europe, India and Pakistan towards Afghanistan). However, misclassification errors were observed towards Eastern Europe (Turkey, South Caucasus) and were worst for Middle Eastern populations. This indicated that small-scale forensic multiplex assays are limited in discriminating continuous genetic variation among geographically close populations. The authors suggested that more than 100 SNPs would be needed for proper separation of Middle Eastern populations [36].

Pacifiplex

Pacifiplex was designed to complement the SNP*for*ID 34-plex assay in differentiating East Asians and Oceanian populations, in a 29-plex SNaPshot® multiplex assay [37]. The sensitivity of the assay was 125 pg, and the assay could be complementary to Y/mtDNA analyses if highly degraded and admixed samples are encountered. The assay potentially genotyped 50-year-old serum samples providing evidence of its versatility on challenging DNA samples. The combined Pacifiplex and SNP*for*ID 34-plex assay was able to differentiate Aboriginal Australians and Papua New Guineans [37].

EurEAs_Gplex

More recently, a 14-SNP sub-classification SNaPshot® assay known as EurEAs_Gplex has been published to be capable of discriminating European and East Asian ancestries along with gender identification [88]. The sensitivity of the assay was 500 pg, and the recommended optimum DNA input amount was 2 ng. This reduced SNaPshot assay was shown to differentiate continental populations when applied to artificially sonicated DNA samples [88].

Global AIMs Nano assay

The 31-plex nano SNaPshot assay is a compact version of the EUROFORGEN Global AIMs panel [89]. It consists of 28 bi-allelic and 3 tri-allelic SNPs and has been designed to differentiate between African, European, East Asian, Oceanian and Native American populations. The sensitivity of the assay enables the analysis of 64 pg that makes it suitable for degraded samples. The inclusion of tri-allelic SNPs may assist with mixture detection [89].

16-plex assay

A 16-plex SNaPshot® assay successfully inferred the BGA of six major ethnic population groups in Australia. Assignment accuracies of 93.5, 91.9, 100 and 94.1% were reported for classifying samples as Asian, Caucasian, sub-Saharan African and North African, respectively. The prediction accuracies for Middle Eastern (71.4%) and Continental Asian (82.8%) assignments were slightly lower. The sensitivity of the assay ranged from 140 pg to 2 ng DNA amounts in the evidentiary-type samples used for its development [90].

Native American admixed assays

A 128-SNP TaqMan AIM assay has been demonstrated to differentiate between American admixed populations [91]. A subset of 14 of these SNPs were combined in a SNaPshot® assay, developed as two multiplexes, to differentiate between African, European and Latin American populations, but it had limited ability to differentiate admixed Latin American populations from Southeast Brazil [92]. This smaller assay was designed as a cost-effective option for low-throughput labs [91, 92]. Another 28-plex SNaPshot assay was able to differentiate admixed Brazilian Native Amerindians (five regions in Brazil) and STRUCTURE multi-locus genotype clustering, which indicated that more than 90% of the admixed samples used in the study came from the European ancestry [93]. Similarly, a 24-plex SNaPshot assay developed by Corach et al. [94] was able to identify admixture in an Argentinean population with all samples used in the study displaying European and Native American admixture [94].

PISNP SNaPshot® assays

Prediction of EVCs can provide forensic intelligence about the physical characteristics of a DNA donor (such as eye, hair and skin colours) (Table 5). Currently, two well-established SNaPshot®-based phenotypic assays have been validated for the European population: IrisPlex [95] and HIrisPlex [96].

IrisPlex assay

IrisPlex is a blue and brown eye colour classification system comprised of six highly predictive eye colour SNPs [95, 97]. The sensitivity of the assay was 15–500 pg with reproducible profiles obtained at 31 pg DNA input amounts. The accuracy of blue and brown eye colour prediction was greater than 90% in a European population dataset when using a multiple logistic regression (MLR) prediction algorithm [95]. SBE primers for two SNPs (rs1800407 and rs12203592) were subsequently redesigned to increase the resolution at low template amounts and avoid sporadic effects encountered in the original IrisPlex assay [97]. Blind trials were performed on artificially created single-source and mixed (two contributors) casework-type samples from the blood, semen, saliva and touched surfaces. These revealed 100% genotyping consistency for single-source samples, but mixtures were difficult to detect due to the limited polymorphic nature of bi-allelic SNPs [97]. Non-blue and non-brown eye colours were classified as an intermediate category. The individual prediction of intermediate eye colour (such as green, grey and hazel) has lower prediction accuracy with the currently available SNPs and prediction tools [97].

The IrisPlex assay was also assessed in a Slovenian population, and this revealed prediction accuracies of 96.6, 91.3 and 79.6% for blue, brown and intermediate eye colours, respectively. The sensitivity (proportion of correct eye colour predictions) was highest for blue eye colour (93.6%) while brown and intermediate eye colours were less sensitive (58.1 and 0%, respectively). The zero sensitivity of the assay in predicting intermediate eye colour confirmed that more predictive markers are required. SNP rs1800407 that is claimed to be the next best predictive eye colour marker after rs121913832 had a weak effect on this population [98].

The IrisPlex system has been evaluated in a North American US population. The assay was performed in two PCR multiplexes of four and two SNPs (compared to the original one PCR multiplex) [95, 97], and then PCR products were pooled for a single-SBE multiplex reaction. Iris colour was determined using MLR as well as a Bayesian network model. This study had a greater number of intermediate eye colour phenotypes than in the original IrisPlex study, and hence, more inconclusive results were encountered. The Bayesian model offered better predictions than multinomial logistic regression (MLR) as well as offering the flexibility of calculating likelihood ratios which could be more convenient for reporting [99].

HIrisPlex assay

HIrisPlex is a 24-plex assay (23 SNPs and 1 INDEL) capable of predicting eye and hair colour collectively and includes the six IrisPlex SNPs. The sensitivity of the assay was in the range 31–500 pg with allelic dropout observed at 31 pg input amount. The profiles were reproducible at 63 pg template input [96]; hence, HIrisPlex demonstrated greater sensitivity

Table 5 Phenotype-informative single-nucleotide polymorphism (PISNP) SNaPshot® assays

Assay	Target	Number of SNPs	Sensitivity (ng DNA)	Application	References
IrisPlex	Autosomal	6	0.015–0.5	Eye colour prediction	Walsh et al. [97]
HIrisPlex	Autosomal	24	0.031–0.5	Hair and eye colour prediction	Walsh et al. [96]
8-Plex	Autosomal	8	≥0.1	Skin and eye colour prediction	Wurmbach [101]

than IrisPlex. Hair colour prediction was classified into four categories: blond, brown, red and black. The prediction accuracies were 69.5% for blond, 78.5% for brown, 80% for red and 87.5% for black hair colours in the European test dataset. The inaccurate predictions of age-related hair colour change, grey hair and intermediate eye colours were highlighted as major limitations of this tool. These limitations could be improved with the future discovery of DNA markers capable of resolving these highly variable traits [96].

The HIrisPlex assay has been tested on degraded, ancient DNA samples. Twenty-one tooth samples with ages ranging from 1 to 800 years and five contemporary bone samples were used. Of the 26 samples, 24 delivered full profiles with prediction accuracies consistent with those above for both eye and hair colours. The HIrisPlex profile from the DNA of a World War I Polish General revealed the same phenotype (blue eyes, blond hair) as mentioned in historical documents. The research provided evidence that accurate EVC prediction from degraded and ancient DNA depends on sample storage and environmental effects. The sensitivity of the assay remained 62 pg, as originally reported [96], except that one of the skeletal remains generated a full profile at 31 pg [100]. The study showed the applicability of HIrisPlex for skeletal and degraded remains.

8-plex assay

An 8-plex SNaPshot® assay was developed to predict eye and skin colour that had three SNPs in common with the IrisPlex assay. The sensitivity of the assay was shown to be 100 pg. Five of the eight SNPs were used in eye colour prediction and six in skin colour prediction. Skin colour is predicted in light, medium and dark categories using the predictor tool developed by the group using a training set of 803 independent samples. Eye colour is predicted into three categories: blue, brown and green. An error rate of 5% was estimated for eye colour prediction, and skin colour prediction was 62% accurate in European population samples [101, 102].

Other pigmentation assays

A SNaPshot® assay of 37 pigment-associated SNPs was developed to further understand the intermediate eye colour prediction and contained all six IrisPlex SNPs. The eye colour categories were divided into light and dark blue and brown colours, and the intermediate eye colour had a sub-category of green–hazel colour. The rs12913832–rs1129038 combination was able to identify light, blue, inter-light and green–hazel categories with sensitivities of 96.5, 98.5, 88.9 and 75.3%, respectively. There were four additional *HERC2* gene SNPs to improve the distinction of eye colour. The 13 SNPs used in the assay for intermediate eye colour were unable to provide a clear resolution, and it was noted that more informative prediction markers were required. This study used the Bayesian Snipper classifier for predictions of eye colours. The study also emphasised the need for a uniform procedure for eye colour phenotype documentation to reduce errors associated with the human perception of eye colour [103].

A 12-plex SNaPshot® assay was developed for eye and hair colour prediction in the Slovenian population and was published earlier than HIrisPlex. The results revealed a significant association of five SNPs out of 12 with eye and hair colour, and all five SNPs are included in the HIrisPlex assay. The optimal sensitivity of the assay was 1 ng, but if polymerase concentration was increased by five times, then the assay produced full profiles at 62 pg. Two prediction models (MLR and Bayesian network models) were developed based on the five most strongly associated SNPs for eye and hair colour prediction. The comparison between the two models showed that MLR was somewhat better than the Bayesian network model in making accurate predictions [104].

Combined ancestry and phenotypic SNaPshot® assays

SNaPshot® assays containing a combination of ancestry and phenotypic SNPs have been developed. These assays help to infer BGA and EVCs together depending on the SNPs included in the multiplex. One example is a 10-plex SBE assay that was developed on 27 modern human samples and then tested on 25 skeletal remains. The panel was selected from six candidate genes and comprised of four ancestry and eight phenotypic SNPs (a few SNPs overlapped for both ancestry and phenotype predictions). The probability estimates for modern human samples determined using STRUCTURE were mostly greater than 80% for inferring BGA except two Asian samples which indicated probabilities of approximately 70%. When tested on 25 degraded ancient DNA samples, the assay revealed that most were derived from European origins, one had equal contributions from European and Asian origins and two samples had Asian origins [105].

A 32-plex assay designed to complement the SNP*for*ID 34-plex was developed for more distinct Eurasian ancestry inference, and it comprised of 22 ancestry SNPs and 10 phenotypic SNPS for eye, hair and skin colour prediction. STRUCTURE analysis revealed this assay alone did not optimally differentiate South Asians from Europeans but, when combined with the 34-plex [34] and Eurasiaplex [36] assays, yielded better ancestry inference. The IrisPlex MLR prediction model could not adequately predict intermediate eye colour when tested on Turkish population samples, but Snipper's likelihood values were more indicative. However, the need for more informative intermediate eye colour predictive markers remained unchanged [106].

More recently, a SNaPshot® assay with 50 SNPs for inferring BGA and phenotypic traits in the US population was developed. The assay was designed in three multiplexes comprised of 32 AISNPs and 18 PISNPs. The ancestry inference

made using the Snipper model revealed 77% accuracy with 21.6% of samples inconclusive and 1.4% misclassified. Prediction using the published IrisPlex MLR model was made in two sets: Europeans and non-Europeans. At 0.7 thresholds, Europeans were predicted with 81% accuracy. The inaccurate predictions were mostly for subjects having intermediate eye colour that was misclassified either as blue or brown. The non-European set was mostly comprised of brown-eyed people, and hence, 99% accuracy was achieved with two intermediate eye colour samples not predicted correctly. The Bayesian Snipper model offered more flexibility than the regression model in cases with missing data [107].

Non-human SNaPshot® assays

SNaPshot® has also been applied to non-human forensic DNA analysis, and a few examples are listed here.

Forensic entomology

SNaPshot assays relevant to the forensic entomology field have been developed. For example, a 6-plex blowfly species identification assay differentiating seven common Calliphoridae blowflies found in the UK has been developed [108]. These blowflies are generally the first to populate cadavers, and species identification could assist in determining time of death. The SNPs are from the cytochrome oxidase I gene, and distinctive haplotypes were identified for each species.

Microbial forensics

A SNaPshot® multiplex assay of five SNP species-specific primers has been designed for the species identification of *Lactobacillus casei* group based on the conserved regions of the *dnaK* gene [109]. The assay consisted of group-specific and species-specific primers and was shown to successfully assign all 63 strains to *L. casei* group and explicitly differentiated all *L. casei* strains from *Lactobacillus paracasei* and *Lactobacillus rhamnosus* simultaneously [109].

Wildlife forensics

The prevention of wildlife trafficking and protecting endangered species is a major focus of wildlife forensics research. A mtSNP SNaPshot® assay designed to identify 11 tiger species and sub-species is one such example. Five SNPs were species specific and another six were sub-species specific with three primer pairs designed to amplify all 11 SNPs. The SBE reaction was performed using 11 SNP-specific primers. The method was 100% accurate when used to identify 15 tigers with a sensitivity of 0.26 pg. A specificity test shows this assay's potential for the identification of other big cat species (closely related to *Panthera*) in addition to tiger species [110].

Discussion and conclusions

STRs are considered to be the gold standard for human identification but are less suited to trace and damaged DNA than SNPs due to their long repeat sequences, resulting in larger PCR amplicons. Furthermore, SNPs can provide identity, lineage, ancestry and phenotype information. The SNaPshot® minisequencing assay is a versatile forensic SNP genotyping tool which can be easily integrated into operational forensic laboratories without any investments in additional equipment. The custom multiplex assays described here can be widely applied for forensic human DNA SNP analysis. The SNP*for*ID 52-plex IISNP assay could be used as a supplementary identity assay alongside conventional proprietary STR assays, especially where degraded and low amounts of DNA are involved [54]. SNP*for*ID 52-plex profiles have been obtained from highly degraded and complex samples including bones, teeth, crime scene samples and a decomposed and charred femur where current STR profiling systems failed or only produced partial profiles [111, 112]. The 52-plex assay can also be a useful complementary tool to STR profiling for resolving paternity cases [113]. Validation studies exist which demonstrate its applicability to difficult forensic casework samples [114].

Forensic DNA phenotyping (FDP), also known as molecular photofitting, refers to the process of predicting the BGA and EVCs of a donor of an evidentiary DNA sample. It can lead investigators to narrow a pool of suspect(s) in cases when STR profiling is uninformative. In these cases, investigators may alternatively use eyewitness statements which are known to be unreliable [115]. FDP has gained attention in the forensic community with the increasing discovery of potential markers and genes associated with many physical traits [116]. BGA and EVC information can be used as a molecular 'silent witness' [117]. With the development of more DNA-based intelligence assays providing information for more physical traits (including facial morphology), we are moving towards more accurate 'molecular photofits'. SNaPshot® lineage, ancestry and phenotypic assays are potential FDP tools capable of providing DNA intelligence information that would certainly help investigators to focus their resources more effectively and efficiently.

The number and types of SNaPshot® assays available enable users to adopt a hierarchical approach to the analysis of samples. The lineage-informative assays such as the 28-Y-LISNP [66] and 36-mt LISNP [75] assays can indicate major continental origins. If the sample is of European origin, the 37-Y-LISNP [67] assay could then be used to infer the specific European haplogroup. There is a range of mitochondrial paternal assays that can be selected to further differentiate European lineages such as the 22-plex assay differentiating nine major European clades, the 16-plex assay separating West European Caucasian clades [77], the 11-plex assay differentiating identical European mitotypes [71] and the 25-

mtSNP assay extricating clades H1 to H15 [79]. The ability to separate specific clades or sub-clades could be of significant importance in a mass disaster victim identification (DVI) case. Alternatively, a screening tool based on a subset of informative control region substitution sites can assist in eliminating a large proportion of samples from an investigation ahead of a more detailed sequence-based analysis. Such a tool has been established by a group at the Netherlands Forensic Institute (NFI) and has been evaluated for operational value by the European DNA Profiling (EDNAP) group in 2016 ([118], submitted manuscript).

The autosomal ancestry-informative SNP assays could also be applied to provide investigators with BGA information. The SNP*for*ID 34-plex [34, 42] SNaPshot® assay is a validated tool that can differentiate between three major world populations: Asian, African and European. The Global AIMs Nano 31-plex assay can be used to differentiate between African, East Asian, European, Oceanian and Native American populations [89]. There are also tools available to deconvolute admixed samples, which can complement the 34-plex assay. Eurasiaplex [36] and EurEas_Gplex [88] can urther assist in offering higher-resolution differentiation of Europeans and Asians (East Asians). Pacifiplex could play a critical role in differentiating Oceanian populations (such as Australian Aboriginals and Papua New Guinea) from global populations [37] which is useful for the analysis of samples in the Asia-Pacific region. These ancestry tools were applied in providing investigator leads in solving some high-profile cases. One such example is the 11-M bombings where the SNP*for*ID 34-plex assay confirmed the North African origin of an evidentiary sample that led investigators to a perpetrator [86]. The 34-plex assay was also employed in Operation Minstead, Britain's largest investigation, and provided investigators with evidence that the suspect was most likely to have admixed African origins from the Caribbean or mainland America [119]. Similarly, it was employed in the investigation of a murder in Madrid to confirm that the suspect was Moroccan which enabled police to narrow a pool of suspects to a few from many thousands [120]. Such assays could potentially be applied to cases of illegal trafficking of organ transplants [121]. Acceptance of the SNP*for*ID 34-plex assay by the forensic community was demonstrated in a global trial of binary AIMs assays [122].

In addition to ancestry prediction, intelligence can be generated using PISNP SNaPshot® assays to infer the EVC of the donor of an evidentiary DNA sample. The HIrisPlex system could be utilised to obtain eye and hair colour information, not only from pristine human DNA samples but also from ancient DNA, provided that prediction accuracy in non-human European populations is characterised [96, 123]. Elucidation of skin tone is possible using the 8-plex skin colour prediction tool [101, 102] with a 'HIrisPlex-S' system, capable of predicting eye, hair and skin colours, in development (Manfred Kayser and Susan Walsh, personal communication). There are numerous other EVCs of potential forensic value with associated SNPs such as male pattern baldness [124], hair texture [125], facial characteristics [126], fingerprint patterns [127] and age estimation [128]. Future SNaPshot® assays may incorporate some or all of these.

The detection of mixtures using bi-allelic SNPs remains challenging due to their low polymorphic nature consisting of only two alleles [96, 97]. For example, the mixture of two single-source samples with homozygote and heterozygote genotypes for a bi-allelic SNP would combine to generate a heterozygote genotype, indistinguishable from the original heterozygote contributor. Even if a mixture is suspected, deconvoluting the mixture may not be possible. This challenge could be overcome by including tri- or tetra-allelic SNPs in the assays, as for the SNP*for*ID 34-plex assay which contains two tri-allelic SNPs [34]. A Global ancestry-informative marker set developed by EUROFORGEN was purpose built with six tri-allelic SNPs to help identify mixed-source samples [129]. Further utilisation of tri-allelic SNPs associated with FDP will only improve the utility of SNaPshot® assays [58].

In recent times, MPS has been gaining popularity in the forensic community due to its ability to type large batteries of markers in multiple samples simultaneously [21, 130]. This technology has demonstrated potential to type identity, BGA and EVC markers together and hence can provide identity and FDP information in a single run [131]. However, SNaPshot® is a low-cost and time-efficient alternative to MPS for smaller-scale genotyping requirements and is ideal for laboratories that do not have the resources to consider MPS. A customised approach to MPS analysis of BGA and EVCs using the PCR products from existing SNaPshot® multiplexes has been demonstrated [21, 130]. Thus, even for labs that may adopt MPS, SNaPshot® assays remain useful and provide a flexible, modular approach to FDP (or identity) where population reference databases already exist for published SNaPshot® assays. This approach offers a reduction in costs associated with commercial panels.

In conclusion, SNaPshot® is an easily integrable and cost-effective SNP typing option for forensic laboratories with readily available forensic human and non-human DNA assays.

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Compliance with ethical standards

Conflict of interest The authors declared that they have no conflict of interest.

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