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SNPs in forensic genetics: a review on SNP typing methodologies

Beatriz Sobrino[a,b,*], María Brión[a,b], Angel Carracedo[a,b]

[a]*Institute of Legal Medicine, University of Santiago de Compostela, San Francisco s/n, 15782 Santiago de Compostela, Spain*
[b]*National Genotyping Center (CeGen), University of Santiago de Compostela, Hospital Clínico Universitario, 15706 Santiago de Compostela, Spain*

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Abstract

There is an increasing interest in single nucleotide polymorphism (SNP) typing in the forensic field, not only for the usefulness of SNPs for defining Y chromosome or mtDNA haplogroups or for analyzing the geographical origin of samples, but also for the potential applications of autosomal SNPs. The interest of forensic researchers in autosomal SNPs has been attracted due to the potential advantages in paternity testing because of the low mutation rates and specially in the analysis of degraded samples by use of short amplicons.

New SNP genotyping methods, chemistries and platforms are continuously being developed and it is often difficult to be keeping up to date and to decide on the best technology options available. This review offers to the reader a state of the art of SNP genotyping technologies with the advantages and disadvantages of the different chemistries and platforms for different forensic requirements.

Keywords: Single nucleotide polymorphism (SNP); SNP genotyping; DNA typing; Forensic genetics

1. Introduction

Single nucleotide polymorphisms (SNPs) represent the most abundant class of human polymorphisms. It is still difficult to give an estimate of the number of SNPs in the human genome, but in the different public and private databases more than five million SNPs have been collected and around four million SNPs have been validated, this is to say they have been confirmed to be polymorphic in one or various major population groups. Their abundance, despite their simplicity and rather limited polymorphic content is the main reason for their current enormous interest in the medical field, since they can be used as markers to identify the genes that underlie complex diseases and to realize the full potential of pharmacogenomics in analyzing variable response to drugs.

The mapping of the human genome has made possible to develop a haplotype map in order to better define human SNP variability. The haplotype map or "HapMap" (www.hapmap.org) will be a tool that will allow researchers to find genes and genetic variations that affect health and disease. The HapMap project will be valuable because it will reduce the number of SNPs required to examine the entire genome for association with a phenotype from the 10 million SNPs that are expected to exist to roughly 500,000 tag SNPs [1].

In the forensic field the interest of SNP is continuously increasing. The reason is that SNPs have a number of characteristics that make them very appropriate for forensic studies: first, they have very low mutation rates and this is

* Corresponding author. Tel.: +34 981 582327; fax: +34 981 580336.
E-mail address: beaiml@usc.es (B. Sobrino).

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interesting for paternity testing, second they are very suitable for analysis using high throughput technologies and this interesting for data basing and for automation and specially they can be analyzed in short amplicons and, in general, short sizes are desirable since the size of the amplified product is critical for the successful analysis of degraded samples. Preliminary data on the WTC identification shows the potential of this type of markers [2,3].

SNPs have also some limitations, first the number of SNPs required is around four times the number of STRs (on average) (http://dna-view.com/SNPpost.htm#Zero%20database) [4,5], so around 60 well balanced SNPs are necessary to have a similar discrimination power than the new multiplexes in use in the forensic field. Second the cost advantages are still not clear although for high-throughput the actual cost of SNPs could be as low as €0.01, clearly better than the cost per STR using commercial kits. Anyway paternity testing is simple and cost efficient using STRs. In addition experience on STRs has accumulated during the last 10 years. For instance mutations or polymorphisms in flanking regions are increasingly more known for STRs and can be a problem for SNPs since an extensive validation in population groups is required for an increased number of markers [6].

Whether SNPs will replace STRs as the primary method of choice in the forensic field is a matter of conjecture at present but there is no doubt that they are useful for some specific applications specially for the definition of Y chromosome and mtDNA haplogroups, for the analysis of the population origin of a sample [7] or for the analysis of common place characteristics [8]. Standardization and interlaboratory validation assays will be key for the use of SNPs in the forensic field.

There is no one ideal method for SNP typing and the selection of an appropriate technique depends on the user requirements. For forensics such requirements would include a good multiplexing capacity and a high accuracy, while for large scale genotyping projects high throughput and low cost are essential.

New genotyping methods, chemistries and platforms are continuously being developed and it is often difficult to be keeping up to date and to decide on the best technology options available. And this is the aim of the article: to offer to the reader a state of the art of SNP genotyping technologies and chemistries showing the advantages and disadvantages for the different forensic applications.

2. DNA typing methodologies

Over the past years, a large number of different SNP typing technologies have been developed based on various methods of allelic discrimination and detection platforms. Due to the rapid and continue evolution of this field it is often difficult to keep up to date and to make informed decisions on the best options available.

To understand each of the technologies, it is important to distinguish between allelic discrimination reactions, assay formats and detection methods. Products of the allelic discrimination reactions can be detected with more than one method, and the same detection method can analyze products obtained with different reactions or assay formats (Table 1). The majority of SNP genotyping assays can be assigned to one of four groups based on molecular mechanism: allele specific hybridization, primer extension, oligonucleotide ligation and invasive cleavage. There are several detection methods for analyzing the products of each type of reaction (fluorescence, luminescence, mass measurement, etc...). There are two different categories related with the assay format, overall referred with the detection: homogenous reactions, when they occur in solution, and reactions on solid support such as glass slide, a chip, a bead, etc.... In general, homogenous reactions are more amenable to automation because there are not separation or purification steps after the allele discrimination reaction. However, the major drawback is the limited multiplex capability. In contrast, reactions on solid-support have greater multiplex capability but further manipulations are required.

2.1. Allele specific hybridization

Allele specific hybridization, also known as allele specific oligonucleotide hybridization (ASO), is based on distinguishing between two DNA targets differing at one nucleotide position by hybridization [9]. Two allele-specific probes are designed, usually with the polymorphic base in a central position in the probe sequence. Under optimized assay conditions, only the perfectly matched probe-target hybrids are stable, and hybrids with one-base mismatch are unstable (Fig. 1).

ASO probes with reverse dot-blot formats were used to detect the first polymorphisms analysed by PCR in the forensic field and they are still used in some laboratories although they have now largely been substituted by STR analysis. To take full advantage of new ASO probe formats for SNP typing it is necessary to use detection methods which provide high accuracy, high sensitivity and high throughput.

2.1.1. Homogeneous hybridization using fret

Fluorescence resonance energy transfer (FRET) occurs when two fluorescent dyes are in close proximity to one another and the emission spectrum of one fluorophore overlaps the excitation spectrum of the other fluorophore [10].

These genotyping methods combine allele discrimination using ASO probes with real-time PCR reactions and quantification. Therefore, in addition to the probes for genotyping, two PCR primers are required. The increase in fluorescence can be measured in real-time during the PCR or when the PCR has finished. There are several variations based on the same principle.

2.1.1.1. LightCycler® (Roche). Two specially designed sequence specific oligonucleotides labelled with fluorescent dyes are applied for this detection method. Probe 1 carries a fluorescein label at its 3′ end and probe 2 carries another label

Table 1
This table shows the detection method that can be used for each allelic discrimination reaction

Detection Method / Allelic Discrimination Reaction	Electrophoresis (fluorescence)	FRET	Fluorescence polarization	Arrays (fluorescence)	Mass spectrometry	Luminescence
Allelic-specific hybridization		✓	✓	✓		
Primer extension	✓	✓	✓	✓	✓	✓
Oligonucleotide Ligation	✓	✓		✓		
Invasive cleavage		✓	✓		✓	

FRET, fluorescence resonance energy transfer.

(LC Red) at the 5′ end. The sequences of the two oligonucleotides are selected such that they hybridize adjacent to one another on the DNA target. When the oligonucleotides are hybridized, the two fluorescence dyes are positioned in close proximity to each other. The first dye (fluorescein) is excited and emits green fluorescent light and with the two dyes in close proximity, the emitted energy excites the adjacent LC Red which subsequently emits fluorescent light (Fig. 2A). This energy transfer, referred to as FRET is highly dependent on the spacing between the two dye molecules. Only if both molecules are within a distance of 1–5 nucleotides is the energy transferred at a high enough efficiency. The intensity of the light emitted by the dye LC Red is filtered and measured by the optics in the LightCycler® instrument. The increasing amount of measured fluorescence is proportional to the increasing amount of DNA generated during the ongoing PCR process. Since LC Red only emits a signal when both oligonucleotides are hybridized, the fluorescence measurement is performed after the annealing step.

One of the probes is arranged with the polymorphic base in a central position and the other must be adjacent to allow for FRET. It is well known that a single mismatch can significantly reduce the melting temperature of the oligonucleotide, and this reduction primarily depends on the length of the oligonucleotide and the position of the mismatch. The reduced melting temperature is measured by performing a melting curve analysis. This method may, of course, also be applied by using hybridization probes matching the mutant and having a mismatch with the wild type.

More than one SNP can be simultaneously genotyped combining the use of the different fluorescent labels with the design of probes with different melting temperatures. The first version of the LightCycler® only detects two fluorescent labels, but the new version of the instrument allows the use of four different labels. A four-plex of Y-chromosome SNPs has been described [11]. In this paper, the utility of this technology for forensic purposes is discussed. The main advantage of this system is the high sensitivity [11].

2.1.1.2. 5′ exonuclease activity: TaqMan® assay (Applied Biosystems). The TaqMan® assay is based in the 5′ nuclease activity of Taq polymerase that displaces and cleaves the oligonucleotide probes hybridized to the target DNA, generating a fluorescent signal [12,13]. Two TaqMan® probes that differ at the polymorphic site are required; one probe is complementary to the wild-type allele and the other to the variant allele. These probes have different fluorescent dyes attached to the 5′ end and a quencher attached to the 3′ end [14]. When the probes are intact, the quencher interacts with the fluorophore by FRET, quenching their fluorescence. During the PCR annealing step, the TaqMan® probes hybridize to the target DNA. In the extension step, the 5′ fluorescent dye is cleaved by the 5′ nuclease activity of the Taq polymerase, leading to an increase in fluorescence of the reporter dye (Fig. 2B). Mismatch probes are displaced without fragmentation. The genotype of a sample is determined by measuring the signal intensity of the two different dyes.

A 3-plex has been described [15] using six different reporter dyes. However, the detection was performed in a luminescence spectrometer after PCR, because the real-time PCR instrument normally used in this assay is not able to measure more than four dyes.

Another detection method that can be used in 5′ nuclease assays is fluorescence polarization (FP) [16]. When a fluor-

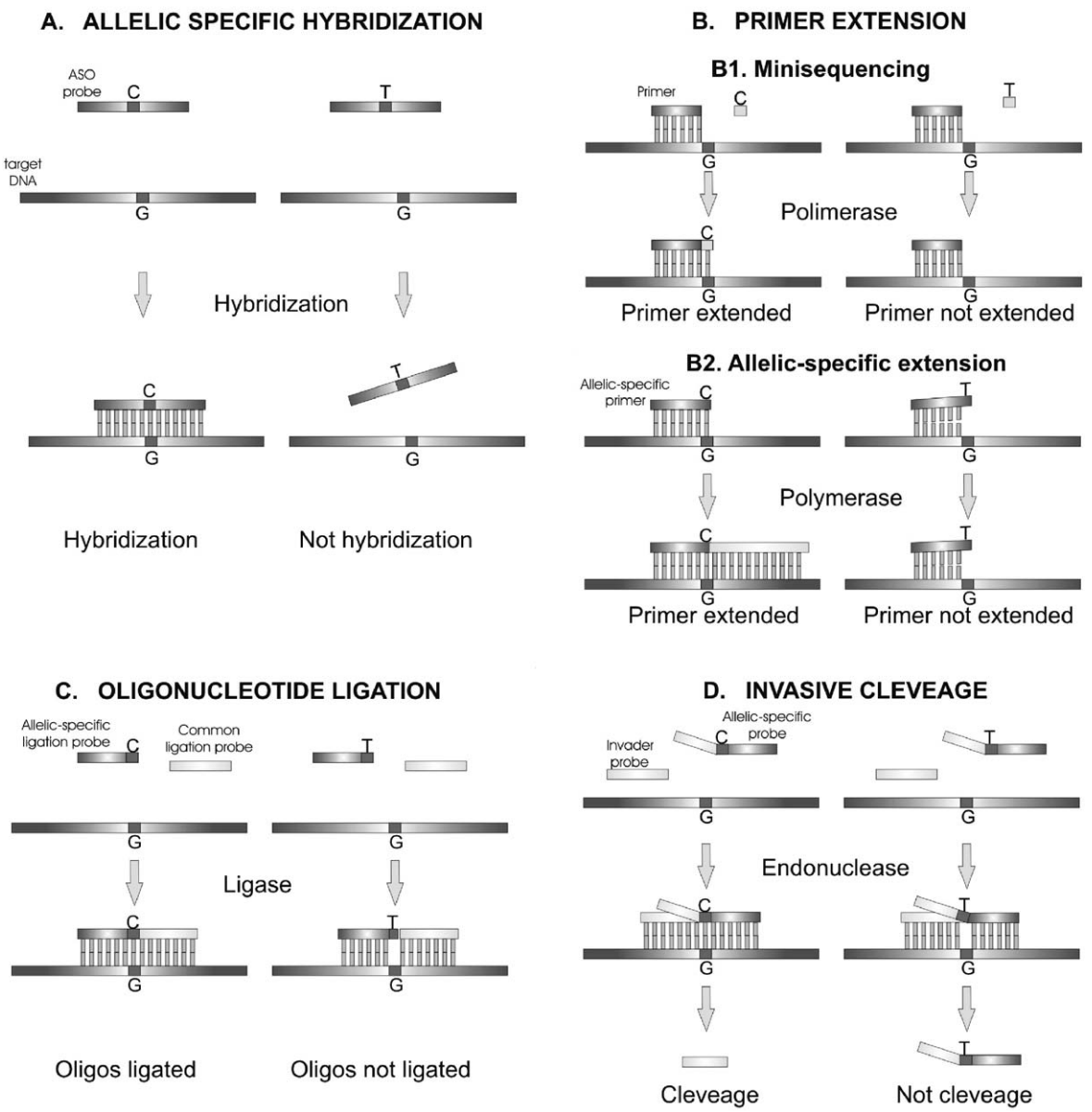

Fig. 1. Illustration of the allelic discrimination reactions. (A) Hybridization with allelic-specific oligonucleotides (ASO): two ASO probes are hybridized with the target DNA that contains the SNP. Under optimized conditions, only the perfectly matched probe-target hybrids are stable. (B) Primer extension reactions: minisequencing (B1) and allelic-specific extension (B2). (B1) minisequencing: a primer anneals to its target DNA immediately upstream to the SNP and is extended with a single nucleotide complementary to the polymorphic base. (B2) allelic-specific extension: the 3′ end of the primers is complementary to each allele of the SNP. When there is a perfect match the primer is extended. (C) oligonucleotide ligation assay (OLA): two allelic-specific probes and one common ligation probe are required per SNP. The common ligation probe hybridized adjacent to the allelic-specific probe. When there is a perfect match of the allelic-specific probe, the ligase joins both allelic-specific and common probes. (D) Invasive cleavage: the oligonucleotides required called invader probe and allelic-specific probes, anneal to the target DNA with an overlap of one nucleotide. When the allelic-specific probe is complementary to the polymorphic base, overlaps the 3′ end of the invader oligonucleotide, forming the structure that is recognized and cleaved by the Flap endonuclease, releasing the 5′ arm of the allelic-specific probe.

ophore is excited by plane-polarized light, its emissions remains polarized if the molecule is still and the angle between the exciting plane and the emitting plane is a function of the mass of the molecule when other parameters are kept constant. In principle, any genotyping method in which the product of the allelic discrimination reaction is substantially larger or smaller than the starting fluorescent molecule can use FP as a detection method. Because the

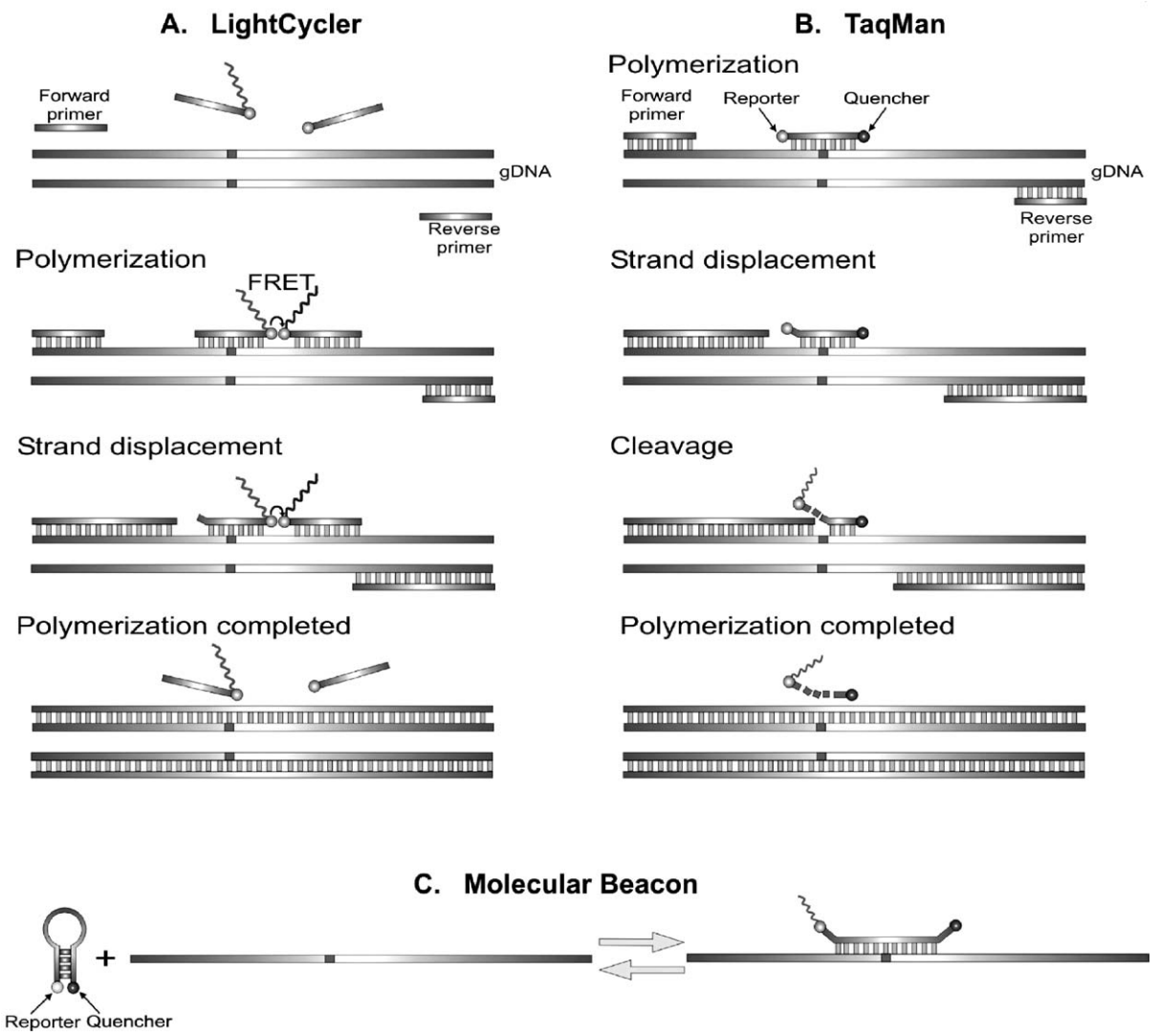

Fig. 2. Representation of different SNP genotyping methods by real-time PCR and FRET detection. (The diagrams show only one ASO probe per SNP, instead of the two required to simplify the picture.) (A) LightCytcler: two sequence specific oligonucleotides labelled with fluorescent dyes are designed, one of them carries a fluorescein label at its 3′ end and another one carries another label (LC Red) at the 5′ end. The sequences of the two oligonucleotides hybridize adjacent to one another on the DNA target. When the oligonucleotides are hybridized, the two fluorescence dyes are positioned in close proximity to each other. The first dye (fluorescein) is excited and emits green fluorescent light and with the two dyes in close proximity, the emitted energy excites the adjacent LC Red which subsequently emits fluorescent light. This energy transfer is referred to as FRET. The increasing amount of measured fluorescence is proportional to the increasing amount of DNA generated during the ongoing PCR process. The polymorphic base is genotyped performing a melting curve analysis, due to one of the probes involved in the process is an ASO probe. If there is a mismatch the melting temperature of the oligonucleotide is reduced significantly. (B) TaqMan; this assay is based in the 5′ nuclease activity of Taq polymerase. When the probes are intact, the quencher interacts with the fluorophore by FRET, quenching their fluorescence. In the extension step, the 5′ fluorescent dye is cleaved by the 5′ nuclease activity of the Taq polymerase, leading to an increase in fluorescence of the reporter dye. Mismatch probes are displaced without fragmentation. (C) Molecular beacon: the probe adopts a hairpin-loop conformation when is not hybridized to the target, and the fluorophore is quenched by the quencher. When the molecular beacon is hybridized to a perfectly complementary target, the fluorophore and the quencher are separated and fluorescence is emitted.

probe used for genotyping is labelled with a fluorescent dye, and the starting probe has a much higher molecular weight than the cleavage products, the FP changes drastically in a positive reaction.

2.1.1.3. Molecular beacons. Molecular beacons are oligonucleotides probes that have two complementary sequences flanking the complementary sequence to the target DNA, with a fluorophore in the 5′ end and a quencher in the 3′ end

(Fig. 2C). The probe adopts a hairpin-loop conformation when not hybridized to the target, and the fluorophore is quenched by the quencher, and therefore, no fluorescence is emitted. When the molecular beacon is bound to a perfectly complementary target, the fluorophore and the quencher are separated and fluorescence appears [17]. For SNP typing, two molecular beacons are used, one specific for the wild type allele and the other specific for the mutant allele. Each of them is labelled with different fluorophores allowing allelic discrimination in one PCR reaction [18].

Different targets can be detected in the same reaction. This is accomplished using different molecular beacons for each target and attaching a different colour fluorophore to each [19]. The number of different fluorophores that can be used in the same reaction is limited by the detection capability of available instruments. Instruments that can perform PCR while simultaneously monitoring fluorescence in real-time use a monochromatic light source such as a laser or light-emitting diodes. By using wavelength-shifting molecular beacons this problem is solved. These probes emit fluorescent light in a distinct range of colours although excited by the same monochromatic light source [20]. This approach increases the multiplex capability for SNP typing.

The principal advantage of homogenous hybridization methods is that no post-PCR process is necessary, PCR and detection being performed in the same reaction. This allows for high-throughput genotyping of a few SNPs in many samples but the method has the serious drawback of limited multiplexing capability.

2.1.2. Array hybridization and fluorescence detection

In this approach short oligonucleotides are attached to a solid support, to create a microarray, and are hybridized with fluorescent labelled PCR products containing the SNP sequence. This is more suitable to analyze many SNPs in parallel. However, the efficiency of the hybridisation and the stability of hybrids depend not only on the polymorphic site but also on the SNP flanking sequence [21]. Therefore, it is very difficult to design optimum conditions to simultaneously analyse a large number of SNPs. This difficulty is overcome in the GeneChip® system (Affymetrix) using tens of ASO probes for each SNP. The probes include all possible sequences at the polymorphic site and some nucleotides that flank the SNP: a technique referred to as tiling strategy [22,23]. The GeneChip® is produced with parallel light-directed chemistry to synthesize specific oligonucleotide probes covalently bound at defined locations on a chip [24,25]. This method is designed for genotyping a large number of SNPs, at a scale that far exceeds current forensic requirements.

2.2. Primer extension

Primer extension is based on the ability of DNA polymerase to incorporate specific deoxyribonucleotides complementary to the sequence of the template DNA. There are several variations of the primer extension reaction; however they can be divided into two main types of reaction. Firstly there is the minisequencing reaction or single nucleotide primer extension where the polymorphic base is determined by the addition of the ddNTP complementary to the base interrogated by a DNA polymerase. Secondly there is the allele-specific extension where the DNA polymerase amplifies only if the primers have a perfect match with the template (Fig. 1). One other alternative approach for DNA sequencing is pyrosequencing, which is also based on the DNA polymerase reaction [26,27].

2.2.1. Minisequencing

In the minisequencing reaction a primer that anneals to its target DNA immediately adjacent to the SNP is extended by a DNA polymerase with a single nucleotide that is complementary to the polymorphic site [28–30] (Fig. 1). This method is based on the high accuracy of nucleotide incorporation by DNA polymerases.

The detection of SNPs in the human genome, and also in other large genomes, requires the previous PCR amplification of the region that flanks the variable site. This is a requirement in most of the technologies used for genotyping SNPs. Before the minisequencing reaction, in order to obtain the specific product from the primer extension, it is necessary to remove the excess PCR reagents, such as PCR primers and dNTPs, from the previous PCR amplification.

There are different technologies for analyzing the primer extension products. The use of labelled nucleotide or unlabelled nucleotide, ddNTP combined with dNTP or only ddNTP in the minisequencing reaction depends on the method selected for detecting the products.

The capability of multiplexing depends also on the technology used. The most common technologies used for analyzing minisequencing products are electrophoresis and fluorescence detection, matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) and microarrays.

2.2.1.1. Electrophoresis and fluorescence detection: SNaPshot™ (Applied Biosystems). One of the most common commercial technologies based on minisequencing reaction followed by electrophoresis and fluorescence detection is the SNaPshot™ kit from Applied Biosystems. The SNaPshot™ multiplex single base extension reaction uses fluorescent ddNTPs. An unlabelled primer is positioned with the 3′ end at the base immediately upstream to the SNP site and is extended with a single ddNTP labelled with a fluorescent dye. Each ddNTP is assigned one fluorescent dye. Multiplex reactions can be accomplished by spatial separation of the minisequencing products using ''tails'' at the 5′ end of the SNaPshot™ primers with varying lengths of non-human sequence. The products are then separated electrophoretically in an automated capillary DNA sequencer. It is possible to perform a 10-plex according to the manufacturer's protocol, but larger multiplexes have been developed [31,32].

2.2.1.2. MALDI-TOF MS. Matrix assisted laser desorption/ionization time-of-flight mass spectrometry measures the molecular weight of the products formed in a minisequencing reaction. Therefore, it is the most direct method of detection compared to the other assay formats that infer the identity of the products by monitoring the fluorescence emitted by labelled molecules. The mass of the base added to the extended primer is determined by the incremental mass of ddNMP residues added [33]. The smallest mass difference is between ddA and ddT: 9 Da. The resolution of MALDI-TOF MS is very high; therefore it is possible to distinguish which ddNTP has been incorporated in the primer extension.

Minisequencing products are deposited onto a matrix on the surface of a plate or a chip. The matrix and the DNA product are hit with a pulse from a laser beam, in a process known as desorption. Energy from the laser beam is transferred to the matrix and it is vaporized resulting in a small amount of the DNA product being expelled into a flight tube. The DNA product is subsequently accelerated towards a detector as it becomes charged when an electrical field pulse is applied to the flight tube. The time between application of the electrical field pulse and collision of the DNA product with the detector is referred to as the time of flight. This is a very precise measure of the molecular weight of the DNA products, as molecular mass correlates directly with time of flight: lighter molecules flying faster and hitting the detector quicker than heavier molecules. Specific software converts this time of flight into an exact mass.

There are several approaches for typing SNPs with MALDI-TOF. The PROBE assay (MassEXTEND, Sequenom) combine the use of ddNTP with dNTPs in the extension reaction to increase mass differences between the alleles of a SNP [34]. In the PinPoint assay (Applied Biosystems) only ddNTPs are used [33]. Despite the fact that the smallest mass difference of 9 Da can be detected, it is difficult to distinguish between A/T, A/A and T/T genotypes [35].

One limitation of MALDI-TOF analysis is the purity of the sample required by the assay. This problem is solved in the GOOD assay [36], which increases the sensitivity using modification primers. Although this requires more reaction steps, all the reagents can be added to a single tube.

If the minisequencing products have non overlapping mass, the assay can be multiplexing [37]. This can be achieved adding a non human tail sequence at the 5′ end of the primer, as in the SNaPshot™ reaction. A 12-plex has been described by Ross et al. [38] and even a 20-plex by Kim et al. [39].

2.2.1.3. Microarrays and fluorescence detection. The microarray format is also suitable for genotyping SNPs with minisequencing, which can be performed on the chip surface or in solution. In the first case, minisequencing primers are attached to a chip (Fig. 3A). This reaction is also known as arrayed primer extension (APEX) [40,41]. The primers are extended by a DNA polymerase with labelled ddNTPs and the microarray is scanned to measure fluorescence. In the second case, the SNPs are genotyping by a single base extension using minisequencing primers with a unique sequence tag at the 5′ end [42,43]. Each SNP has a distinct identifying tag. The product of the multiplex minisequencing reaction performed in solution is hybridized to the reverse complementary sequences of the tags arrayed onto the chip (Fig. 3B).

In Pastinen et al. [44], an ‘‘array of arrays’’ format for slides was described. This allows the analysis of up to 80 different samples per slide by creating different hybridization chambers with silicon rubber grids.

2.2.1.4. Fluorescence polarization. As previously described, FP can be used for detecting any allelic discrimination product where the initial and the final molecules have different sizes. In the case of minisequencing, the primer is extended by the dye-terminator specific for the allele present on the template, increasing approximately 10-fold the molecular weight of the fluorophore [45]. This assay is performed in singleplex.

2.2.2. Pyrosequencing

Pyrosequencing is a sequencing-by-synthesis method. This technology uses an enzyme cascade system, consisting of four enzymes and specific substrates, to produce light whenever a nucleotide is complementary to the template DNA strand [26,27]. This light signal is detected, the base registered, and the next nucleotide added. If the added nucleotide is not complementary to the next base in the template no light will be generated. The detection is based on the pyrophosphate released during the DNA polymerase reaction. The reaction mixture consists of single stranded DNA with an annealed primer, DNA polymerase, ATP sulfurylase, luciferase and apyrase. The four nucleotides are added to the mixture in a defined order (i.e. CGAT). If the added nucleotide forms a base pair, the DNA polymerase incorporates the nucleotide and pyrophosphate will consequently be released. The released pyrophosphate will be converted to ATP by ATP sulfurylase. Luciferase then uses the ATP to generate detectable light. The light intensity is proportional to the number of incorporated nucleotides. The excess of each nucleotide will be degraded by apyrase. If the added nucleotide does not form an incorporated base pair with DNA template no light will be produced.

Pyrosequencing provides rapid real-time determination of 20–30 bp of target DNA. With this technology, the SNP alleles are determined, including adjacent base positions as a built in control.

The limiting factors of pyrosequencing are the template preparation required and the degree of multiplexing. Prior to analysis, PCR products need to be converted to single stranded template onto which a sequencing primer is annealed. This is achieved using biotinylated PCR products.

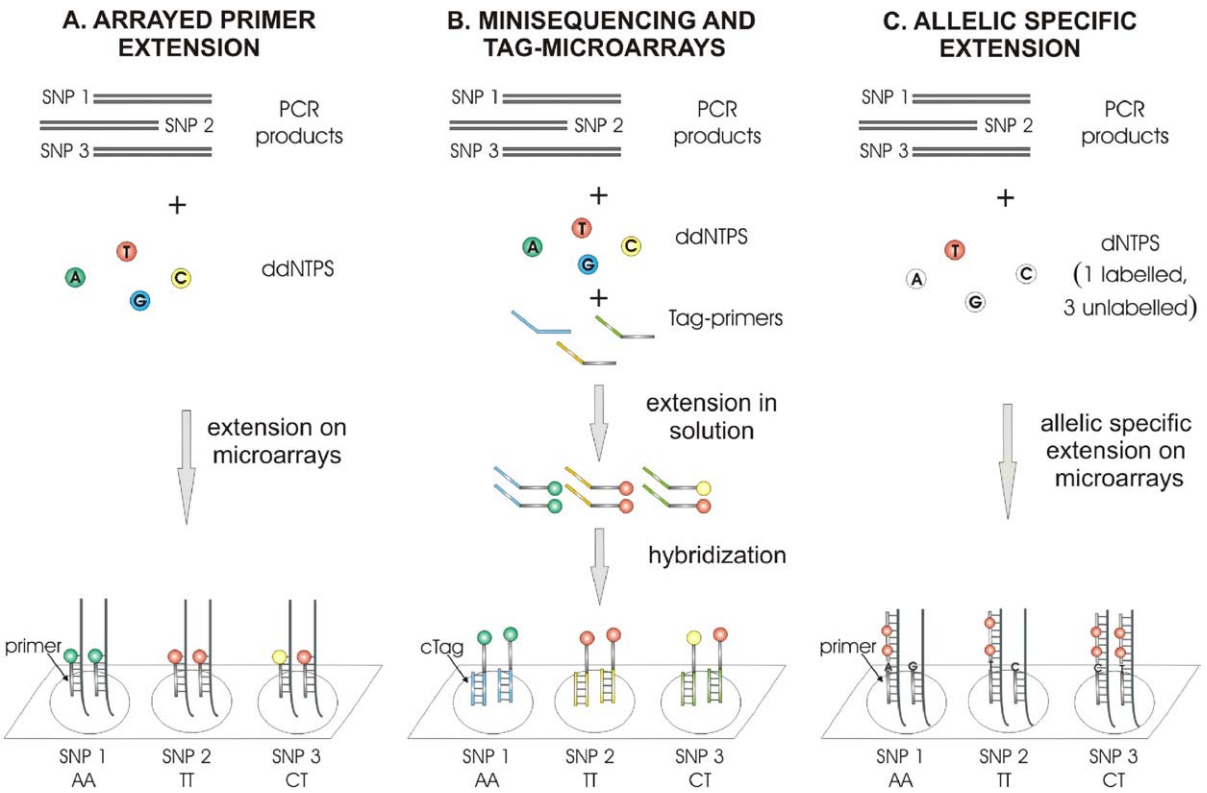

Fig. 3. SNP typing technologies with microarrays. (A) Arrayed primer extension: the minisequencing primers are attached on the chip surface, therefore, the minisequencing reaction is performed on the chip. (B) Minisequencing and tag-microarrays: the minisequencing reaction is performed in solution using primers with a tag at the 5′ end. The minisequencing products are hybridized to the microarrays created with the reverse and complementary sequences of the tags (cTags). (C) Allelic specific extension: allelic-specific primers are attached onto the microarray. When there is a perfect match with the target DNA the primers are extended.

In addition, as with real time PCR, the method has the problem of limited multiplex capability.

2.2.3. Allele-specific extension

Allele-specific extension relies on the difference in extension efficiency of DNA polymerase between primers with matched and mismatched 3′ ends. DNA polymerase extends a primer only when the 3′ end is perfectly complementary to the DNA template (Fig. 1). Two primers are required, one for each allele of a SNP. By detecting which primer forms the product, the genotype of a sample can be determined. The product of this reaction can be detected on a microarray using fluorescently labelled nucleotides [44] (Fig. 3C).

Another variant of this reaction is the use of a common reverse primer, in addition to the allelic-specific primers: where the matching primer allows the amplification of a specific allele in a sample. In this case, the reaction is called allele-specific PCR. The detection of the appropriate PCR products allows the genotyping of the sample [46,47]. The use of tag-primers allows the specific identification of each PCR product, in some cases based on melting curve analysis [48] and in other cases FRET is used as detection method [49].

2.3. Allele specific oligonucleotide ligation

DNA ligase is highly specific in repairing nicks (a missing phosphodiester bond) in the DNA. Landergren et al. [50] described the oligonucleotide ligation assay (OLA) as a method for SNP typing based on the ability of ligase to covalently join two oligonucleotides when they hybridize next to one another on a DNA template. The OLA assay requires that three probes were designed, one common and two allele specifics. The common probe anneals to the target DNA immediately downstream of the SNP. One allelic probe has at the 3′ end the nucleotide complementary to one allele, with the other allelic probe complementary to the alternative allele. These two allelic probes compete to anneal to the DNA target adjacent to the common probe. This generates a double stranded region containing a nick at the allele site. Only the allelic probe perfectly matched to the target will be ligated to the common probe by the DNA ligase (Fig. 1). The use of thermostable DNA ligase allows

repeated thermal cycles, resulting in a linear increase in ligation products. If both strands of genomic DNA are used as targets for oligonucleotide hybridization, the increasing of ligation products can be exponential. This reaction is called ligase chain reaction (LCR) [51]. Two sets of oligonucleotides, complementary to each target strand, are used. The ligation products from the first ligation reaction can then be the targets for the next round of ligation.

Different assay formats have been developed for detecting the ligated product. Use of biotin on the common probe and a reporter group on the allelic specific probe allows for product capture and detection [50]. Other assays have replaced the biotin with mobility modifiers and the allelic specific probes have been labelled with different fluorescent dyes, enabling the ligation products to be discriminated by size and colour [52]. The use of mobility modifiers and fluorescent dyes allows a multiplexed OLA to be performed where the products of this reaction are resolved electrophoretically under denatured conditions with fluorescent detection. The mobility modifiers allow the precise regulation of the mobility of each ligation product regardless of oligonucleotide length. This strategy has been termed sequence-coded separation by Grossman et al. [52]. In this assay a PCR is performed before the OLA reaction. However, the coupled amplification and oligonucleotide ligation (CAL) procedure [53] combines multiplex amplification and SNP typing in one reaction. This is achieved using PCR primers with high melting temperatures and oligonucleotide ligation probes with lower melting temperatures. All the reagents are added simultaneously to one tube, minimizing the manual work and allowing easy automation. During the first stage of the reaction the PCR primers anneal to the DNA target and amplification occurs at a temperature above the melting temperature of the oligonucleotide ligation probes. In the second stage the temperature is lowered, allowing the probes to hybridize and for ligation to occur. Ligation products are detected using a fluorescence DNA sequencer.

A variation of the CAL assay is the dye-labelled oligonucleotide ligation (DOL) [54]. In this case the ligation products are detected by monitoring FRET in real time. Three dye-labelled ligation oligonucleotides are needed for each biallelic marker. The common probe is labelled at 5′ end with a donor dye, and the allele-specific probes have an acceptor dye at the 3′ end. When ligation occurs, an increase in FRET is detected.

More recent technologies as SNPlex™ (Applied Biosystems) and Illumina genotyping system (Illumina Inc.) are based on the specificity of ligases. Both technologies perform the allelic discrimination reaction directly on genomic DNA and ligation products are amplified simultaneously by universal PCR. In the SNPlex™ system, fragmented gDNA is interrogated directly by a set of three unlabelled ligation probes per SNP in a multiplex reaction including up to 48 assays (Fig. 4). After genotype specific ligation, a PCR amplification is performed, using two universal PCR primers, one of them carries a biotin molecule. Biotinylated amplicons are bound to streptavidin-coated plates to be converted in single-stranded PCR products. The encoded genotype information is read utilizing a set of common ZipChute probes. These probes are fluorescently labelled, they have a unique sequence that is complementary to a specific portion of the single-stranded PCR product, and they contain mobility modifiers. ZipChute probes are hybridized to the complementary sequences of the amplicons, eluted and detected by electrophoretic separation on a capillary electrophoresis instrument. The system is currently configured at 48-plex per reaction; however, Applied Biosystems has announced that the future system enhancements include increased throughput through higher multiplexed reactions, such as 96- and 192-plex per reaction.

On another hand, Illumina genotyping reaction (www.illumina.com) is based on ligation but also on allelic-specific extension in a process called GoldenGate™ assay. In this case the ligation products contain address sequences for hybridization to probes on an array. The products are fluorescently labelled, with different colour being used for each allele of a SNP. Following hybridization to the array, the ratios of the two allele-specific signals are used to determine the genotype of each SNP. The detection relies on the BeadArray™ technology, which is based on fiber-optic substrates with randomly assembled arrays of beads [55]. Each bead represents a sensor element for a particular DNA-sequence. The system allows analysing up to 1152 SNPs per assay.

Another approach of the ligation assay is the use of padlock probes. They are linear oligonucleotides designed so that the two end segments, connected by a linker region, are both complementary to a target sequence [56]. Upon hybridization to a target DNA the two probe ends become juxtaposed and can be joined by a DNA ligase if there is a perfect match. As a consequence, the linear oligonucleotide is circularized by ligation. If there is a mismatch the ligation does not occur, allowing the identification of sequence variations. Padlock probes have been applied to analyse SNPs directly on genomic DNA without previous amplification being used as template in rolling circle amplification (RCA) [57–59]. Sets of padlock probes can be applied to analyze large number of SNPs in parallel being detected after hybridization on tag arrays [60,61].

2.4. Invasive cleveage

The Invader® assay (Third Wave™ Technology) is based on the specificity of recognition, and cleavage, by a Flap endonuclease, of the three-dimensional structure formed when two overlapping oligonucleotides hybridize perfectly to a target DNA [62,63].

The two oligonucleotides required, called ‘‘invader oligonucleotide’’ and ‘‘probe’’, anneal to the target DNA with an overlap of one nucleotide. The invader oligonucleotide is complementary to the sequence on the 3′ side of the SNP. The probe is designed with the allelic base at the overlapping

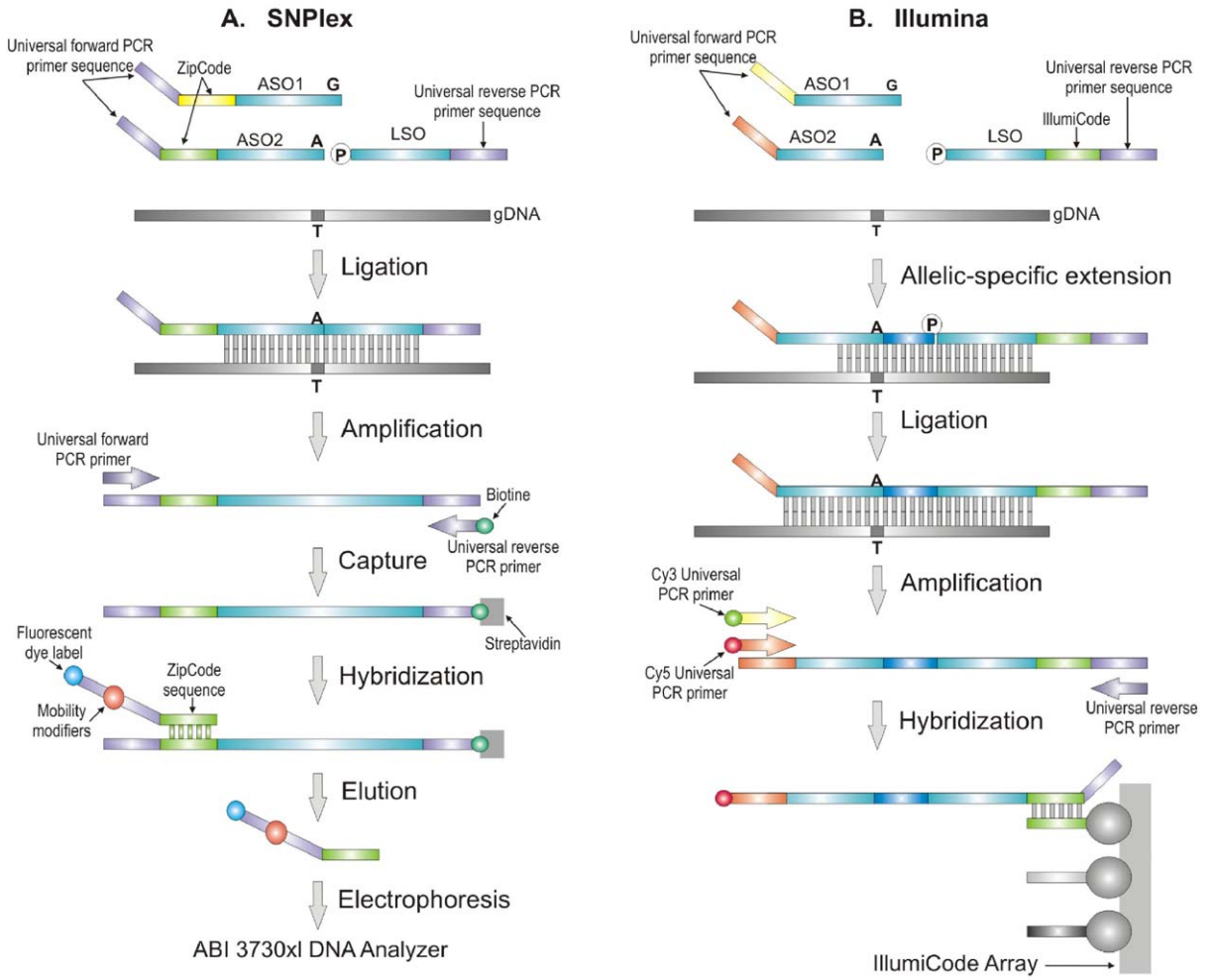

Fig. 4. SNPlex™ and Illumina™ assays for a single SNP. (A) SNPlex: gDNA is interrogated directly by a set of three probes per SNP, two allelic-specific probe (ASO) and one ligation probes (LSO). The ligase joins the ends of the ASO probe complementary to the gDNA and the LSO, forming a ligation product which is used to be amplified with universal PCR primers, one of them biotinilated. Biotinylated amplicons are bound to streptavidin-coated plates. Single-stranded PCR products are interrogated by a set of universal ZipChute probes. These probes are fluorescently labelled, they have a unique sequence that is complementary to a specific portion of the single-stranded PCR product, and they contain mobility modifiers. After elution ZipChute probes are electrophoretically separated on a capillary electrophoresis instrument. (B) Illumina: The process is very similar to the SNPlex but in this case there is an allelic-specific extension in addition to the ligation reaction. There are two universal forward primers, one labelled with Cy3 and another with Cy5. After amplification the PCR products are hybridized to an IllumiCode array, which is made by beads with oligonucleotides attached on their surface. These oligonucleotides are complementary to a portion of the LSO probes called IllumiCode. The array is scanned to detect fluorescence.

fragment, and contains two regions, one complementary to one of the alleles of the SNP and the sequence on the 5′ side of the polymorphic site and a non complementary 5′ arm region (Fig. 1). When the allelic base is complementary to the base in the probe, the probe overlaps the 3′ end of the invader oligonucleotide, forming the structure that is recognized and cleaved by the Flap endonuclease, releasing the 5′ arm probe. If there is a mismatch, the structure formed will not be recognized by the Flap endonuclease and cleavage will not occur. This specificity of substrate structure recognition enables detection of single nucleotide mutations. The 5′ arm probe serves as an invader oligonucleotide in a secondary cleavage reaction with a signal probe.

Performing the invasive reaction at elevated temperatures allows for rapid denaturation of the probe after cleavage, enabling the endonuclease to produce multiple cleaved probes per target DNA molecule. Because of this, the signal is amplified and, together with the secondary cleavage reaction, the amplification is exponential. Therefore, the Invader assay is a genotyping method without the requirement of previous PCR amplification [62,64,65], but a large amount of target DNA is needed. In order to increase the

sensitivity, this assay can also be performed with a PCR step before the invader reaction, known as PCR–Invader assay [66].

Different detection methods can be used, involving different designs of probe. When FRET is the detection method, a reporter dye is placed on the 5′ arm and the quencher is placed on the complementary region of the probe [64,66]. The cleavage event removes the fluorophore and enhances fluorescence. With fluorescence polarization detection, the probe is labelled with a fluorophore at the 5′ end. When the probe is cleaved, the molecular weight of the fluorophore decreases with the corresponding decrease in FP [67]. If mass spectrometry is the detection method, probes are designed with different numbers of nucleotides [68].

3. Discussion

SNP genotyping technologies have been developed rapidly in the last few years. As a result, a great variety of different SNP typing protocols have become available for researchers, however there is no single protocol that meets all research needs. Different aspects should be taken into account to determine which technology is the most suitable for forensic purposes, such as the sensitivity, the reproducibility, the accuracy, the capability of multiplexing and the level of throughput. It is also important to have in mind the flexibility of the technology, the time-consumption and the cost, considering both the equipments required and the cost per genotype.

The level of throughput required depends on each application. Some applications use few SNP markers but a large sample size, other applications require large number of SNPs in a few samples and finally, there are other applications that need large number of both SNPs and samples. For forensic purposes, a medium throughput is required for paternity testing and criminal casework, but a high throughput is necessary to implement criminal DNA databases.

An important limiting step for forensic genetics in all these technologies is the amount of DNA required per genotype. There are some techniques that interrogate the SNP directly on genomic DNA, without a previous PCR, such as the SNPlex, Illumina and the Invader assay. In these cases, the minimum amount of DNA required for the analysis should be determined, but it is always higher than when a previous PCR is performed. For the other technologies requiring a PCR before the allelic discrimination reaction, the development of multiplex PCR is essential, not only for the small amount of DNA available to be analysed in the majority of the criminal caseworks but also from the throughput point of view. In critical cases, the use of whole genome amplification (WGA) should be explored [69] prior to start with the analysis of the selected markers to avoid the problem of the lack of enough amount of DNA.

Another important issue in forensic genetics is the analysis of mixtures. Due to the biallelic nature of most the SNPs it will be more difficult to detect the presence of a mixture in a sample using these markers. Therefore, the possibility of the quantification of each allele in a sample can help in the determination of the contribution of each component in a mixed profile. Some technologies such as mass spectrometry and pyrosequencing allow the possibility of some quantification. This feature is routinely used for estimating allele frequencies in pooled samples, but could be an advantageous feature useful in forensic genetics.

In technologies based on homogenous hybridization with FRET detection such as the LightCycler, TaqMan and Molecular Beacon, PCR and allelic discrimination reaction are performed in the same reaction. This advantage avoids further manipulation steps, favouring the automation and throughput of the process, especially when the high-throughput equipments for TaqMan assays are used. The main drawback is the limited multiplexing capability. As a consequence, these technologies are a good option for validating candidate SNPs and for building the criminal DNA databases, but not for being used as routine technology in forensic casework.

The minisequencing technologies are at this moment the most popular methods in forensic laboratories, especially the SNaPshot, because the detection is performed on an automatic capillary electrophoresis instrument, that it is also used for STRs analysis. Several works have been done in different forensic laboratories related with the analysis of SNPs with this technology [31,32,70,71]. The multiplex capability is feasible for forensic requirements, but a lot of work is needed to optimize the design and concentration of the primers for PCR and minisequencing reaction of each set of SNPs. The other detection methods such as microarrays and MALDI-TOF seem to be suitable for forensic purposes, and there are also some works in those directions [72,73]. However, the main drawback of these technologies is that specific equipment is required, in contrast with SNaPshot. Furthermore, the multiplex capability of MALDI-TOF is lower than with the other minisequencing technologies. The main problem of microarrays detection is that the reproducibility and validation is more difficult.

Pyrosequencing has the limitation of the multiplex capability, which is very low, and the automation, because several steps need to be performed before the detection. The main advantage is the possibility of quantification the contribution of each allele, a very useful feature in the analysis of mixtures profiles.

The multiplex capability of several technologies based on OLA is very high, even above the forensic requirements. The main problem of these technologies is that the allelic discrimination reaction is performed directly on genomic DNA. This is a big advantage when there is enough amount of DNA for the analysis but not for criminal casework. These technologies have been recently described and more work is needed to know if they can be used for forensic purposes.

The technologies that reach the criteria required for forensic purposes need to be explored to determine their

limitations and the possibility of being used in forensic genetics. It is difficult that one technology fit all the requirements and therefore it is not an easy task to define which technology is the best for SNP typing in forensic genetics. Furthermore, there is a rapid technological progress, due to biotechnology companies are making a big effort in developing new strategies for SNP typing, making more difficult to choose the appropriate method for specific applications. Probably, different technologies will be used for routine casework, where the DNA amount and integrity are much more critical, than for paternity testing or for creating criminal DNA databases. Furthermore, it is possible to make differences between autosomal-SNPs and Y-chromosome and mitochondrial SNPs regarding the number of markers that it is necessary to analyze per sample and the strategy of the analysis. Therefore, the multiplex requirements are not the same for both applications and also the appropriate technology could be different for different types of SNPs. In general, all the technologies that have limited multiplexing capability should be excluded as candidates for routine forensic analysis; however they could be useful for estimating the allele frequencies of the SNPs selected and also for creating large criminal DNA databases.

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