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Review

Massively parallel sequencing techniques for forensics: A review

DNA sequencing, starting with Sanger's chain termination method in 1977 and evolving into the next generation sequencing (NGS) techniques of today that employ massively parallel sequencing (MPS), has become essential in application areas such as biotechnology, virology, and medical diagnostics. Reflected by the growing number of articles published over the last 2–3 years, these techniques have also gained attention in the forensic field. This review contains a brief description of first, second, and third generation sequencing techniques, and focuses on the recent developments in human DNA analysis applicable in the forensic field. Relevance to the forensic analysis is that besides generation of standard STR-profiles, DNA repeats can also be sequenced to look for polymorphisms. Furthermore, additional SNPs can be sequenced to acquire information on ancestry, paternity or phenotype. The current MPS systems are also very helpful in cases where only a limited amount of DNA or highly degraded DNA has been secured from a crime scene. If enough autosomal DNA is not present, mitochondrial DNA can be sequenced for maternal lineage analysis. These developments clearly demonstrate that the use of NGS will grow into an indispensable tool for forensic science.

Keywords:
DNA analysis / Forensics / Massively parallel sequencing / Short tandem repeat / Single nucleotide polymorphism
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1 Introduction

Massively parallel sequencing (MPS) has gained a lot of attention over the last decade. A MPS technique is defined by the National Cancer Institute dictionary of genetic terms as 'a high-throughput method used to determine a portion of the nucleotide sequence of an individual's genome. This technique utilizes DNA sequencing technologies that are capable of processing multiple DNA sequences in parallel.' (https://www.cancer.gov/publications/dictionaries/genetics-dictionary).

MPS is often named next generation sequencing (NGS), to distinguish the new developments from previous DNA sequencing methods. Multiple reviews have been reported on the principles, performance, advantages, and disadvantages of NGS techniques [1–9]; however, only a few papers discuss applications of NGS for forensic applications [10–12]. This topic is the focus of this review.

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Abbreviations: **ddNTPs**, di-deoxynucleotidetriphosphates; **dNTPs**, deoxynucleotidetriphosphates; **em-PCR**, emulsion PCR; **MPS**, massively parallel sequencing; **mtDNA**, mitochondrial DNA; **NGS**, next generation sequencing; **SMS**, single molecule sequencing; **ZMW**, zero-mode waveguide

A variety of important developments in first, second, and third generation sequencing are depicted in Fig. 1. It is clear that first generation sequencing, with especially Sanger sequencing developed in 1977, dominated the market for a long time. In the years 2005–2007 several second generation systems were launched onto the market, such as the Solexa Genome Analyzer and the SOLiD® system. The so-called third generation sequencers started with the launch of the Helicos system in 2007. Lin and co-workers gave an overview of some recent NGS techniques and patents in 2008 [13].

An overview of the characteristics, such as the sequencing principle, the read length, the throughput, and the run time, of the three generations of MPS techniques is given in Table 1. Note that the read length, throughput, and run time are subjective to fast changes because the field is advancing fast, therefore this table only gives an indicative comparison between the various generations of sequencing techniques and not an exact evaluation of the limits of each method.

1.1 Terminology

Important characteristics of the different sequencing techniques are the read length, the coverage, and the depth. A read is the sequence of bases of a single molecule of DNA, whereas the read length is the actual number of sequenced bases

Color Online: See the article online to view Figs. 1–3 in color.

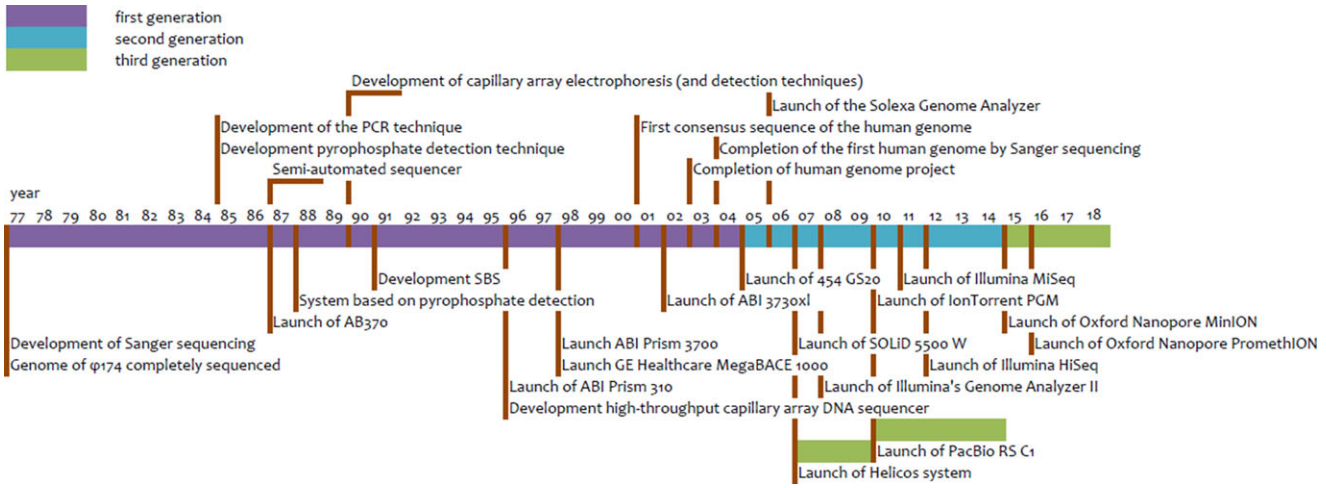

Figure 1. Timeline of the most imsportant developments regarding MPS [1–4, 6, 13–15].

Table 1. Characteristics of the different sequencing techniques (first, second, and third generation). The read length range covers the read length at the introduction of the technique till the read length which can be obtained nowadays. Throughput and run time are for the machine that has currently the highest capacity. Throughput, reads and sequence data; AB, Applied Biosystems™; em-PCR; LT, Life Technologies; SBS, sequencing by synthesis [1, 3, 5, 7–9, 15–17]

Generation	Method	Launch	Technique	Read length (nt)	Throughput and run time	Comments
I	Sanger	1977	Cloning/chain termination	25–1200	96, 84 Kb, 2 h	First commercialized by AB (now LT)
II	454	2005	em-PCR/SBS/pyrosequencing	100–1000	1 million, 0.7 Gb, 24 h	Purchased by Roche in 2007
	Solexa/HiSeq®/MiSeq®	2006	Bridge PCR/SBS/reverse termination	36–300	6 billion, 1.8 Tb, several days	Solexa purchased by Illumina® in 2007
	SOLiD®	2007	em-PCR/ligation/probes	35–75	6 billion, 320 Gb, 1–2 weeks	Purchased by AB in 2006 (now LT)
	Ion Torrent™	2010	em-PCR/ion-sensitive SBS/pH change	200–400	60–80 million, 50 Gb, 2 h	Purchased by LT in 2010
III	PacBio®	2010	SMRT®/ZMW wells	8000–20000	350000, 7Gb, 0.5–6 h	
	(Oxford) nanopore	2014	Ion current shift	9545–200000	100000, 2–4 Tb up to 48 h	

read in one run. The coverage is described as the number of short reads that overlap within a specific genomic region, while the depth is defined as the amount of reads of the same region.

Most of the second-generation sequencing techniques are based on sequencing by synthesis. This is the serial extension of a primed template by an enzyme, which is either a polymerase (e.g. 454) or a ligase (e.g. SOLiD®). Another term used often in sequencing is homopolymer, which is actually a polymer with a series of identical components, in the MPS terminology it means a repetitive DNA sequence.

2 First generation sequencing

Sanger sequencing was developed in 1977 by Frederick Sanger, who was awarded the Nobel Prize in Chemistry in 1980 [1]. This method, which is now known as the first generation sequencing, is rather similar to PCR, because a ssDNA template, a DNA primer, DNA polymerase, and deoxynucleotidetriphosphates (dNTPs) are also required to perform the reaction. Furthermore, di-deoxynucleotidetriphosphates (ddNTPs) are needed, which can be incorporated into the newly synthesized DNA strand, just as normal dNTPs, but with termination of the elongation process as a result. Therefore this method is also known as the chain termination method. The reaction is carried out in fourfold, whereby in each tube (besides the normal dNTPs) only one of the four labeled ddNTPs is added in a relatively low concentration [18]. Nowadays the sequence can be determined by fluorescently labeled ddNTPs and capillary (gel) electrophoresis (Fig. 2A) [14, 19–21]. Sanger sequencing can produce longer reads than most of the second generation techniques, which produce short reads.

3 Second generation sequencing

The development of PCR in 1985 has led to major improvements in instrumentation, because alternatives for the principle of Sanger sequencing became available [1, 2]. The new systems such as 454, SOLiD®, and Ion Torrent™ make use of a cell free system, whereas for Sanger sequencing bacterial cloning of DNA fragments was required [3, 8, 14]. Because this resulted in faster and cheaper methods which are based on parallel analysis, giving higher throughput, the term 'next generation sequencing' was introduced. Also new sequencing methods have been developed, such as pyrosequencing and virtual terminator chemistry [1–3, 5, 13]. Drawback of the higher throughput is the reduced accuracy of each short read [8, 22]; therefore, with these techniques it is not possible to read a complete DNA sequence of a genome, but only small DNA fragments [5].

In 1986 Ansorge and co-workers developed a method and an instrument for automated DNA sequencing without the need for radioactive labels [4, 23, 24]. They used a fluorescently labeled primer to generate a nested set of DNA fragments, which become fluorescent because of the incorporated labeled primer. By the use of gel electrophoresis in combination with a laser to excite the bands, it was possible to detect as low as 0.1 fmol per band and to read 250–300 bases within 6 h [23].

A wide variety of second generation sequencing machines have been launched on the market, such as the GE Healthcare (previously Amersham) MegaBACE™ system [25], the system of Intelligent Bio-Systems Inc. (subsidiary of Qiagen®) [26, 27], sequencing with nanoballs by Complete Genomics™ [28, 29], and the Polonator (developed by the research group of Church at Harvard) [8, 30]. In this review only the most used and advanced techniques will be discussed in detail, i.e. the 454 system (Roche) and the systems from Illumina® and Applied Biosystems™/Life Technologies.

3.1 454

The technology on which the 454 sequencing technique is based was patented in 1989 by Melamede [31]. The GS FLX from 454 Life Sciences (later on Roche), the first NGS technique on the market, is based on pyrosequencing. This implies that detection takes place by analysis of the signal emitted from the nucleotide that is incorporated in the new DNA strand (Fig. 2B). This is also called sequencing by synthesis. Several chemical reactions occur (Fig. 2B) that result in the emission of light detected by a camera, from which the sequence can be constructed. dNTPs that do not match are degraded by a pyrase. When the correct nucleotide is incorporated in the newly synthesized strand, the pyrophosphate is converted to adenosine triphosphate with the help of the enzyme sulfurylase. Next, luciferase converts the adenosine triphosphate and luciferin to oxyluciferin, accompanied by light emission [32–34]. The use of SBS ensures that detection and analysis via electrophoresis is no longer needed [2, 4, 16, 35].

A drawback is that library preparation is required before sequencing can take place. The DNA solution is fragmented and so-called adaptors, 44-base primer sequences, are added to both ends of each DNA fragment. At one end adapter 'A' is ligated and on the other end adapter 'B', which differs from A in nucleotide sequence. The exact sample preparation steps can be found in the supplementary material of the article of Margulies et al. [36]. The aim of these adapters is to capture the DNA fragments on a solid surface [16]. In the 454 sequencer the solid surface is provided by 26 μm beads used for emulsion PCR (em-PCR) (Fig. 2C), which means that the DNA is amplified on the bead inside a water droplet surrounded by an oil solution [6, 14]. An important feature of em-PCR is the bias-free amplification of single DNA molecules acquired by entrapping them in lipid microreactors [20]. The beads, after em-PCR covered with multiple copies of the template DNA, are loaded in a fiber-optic slide with recessed 75 pL wells, where each well houses one bead [2, 35, 36]. Roche stopped the production of the 454 system at the end of 2016 [1, 3].

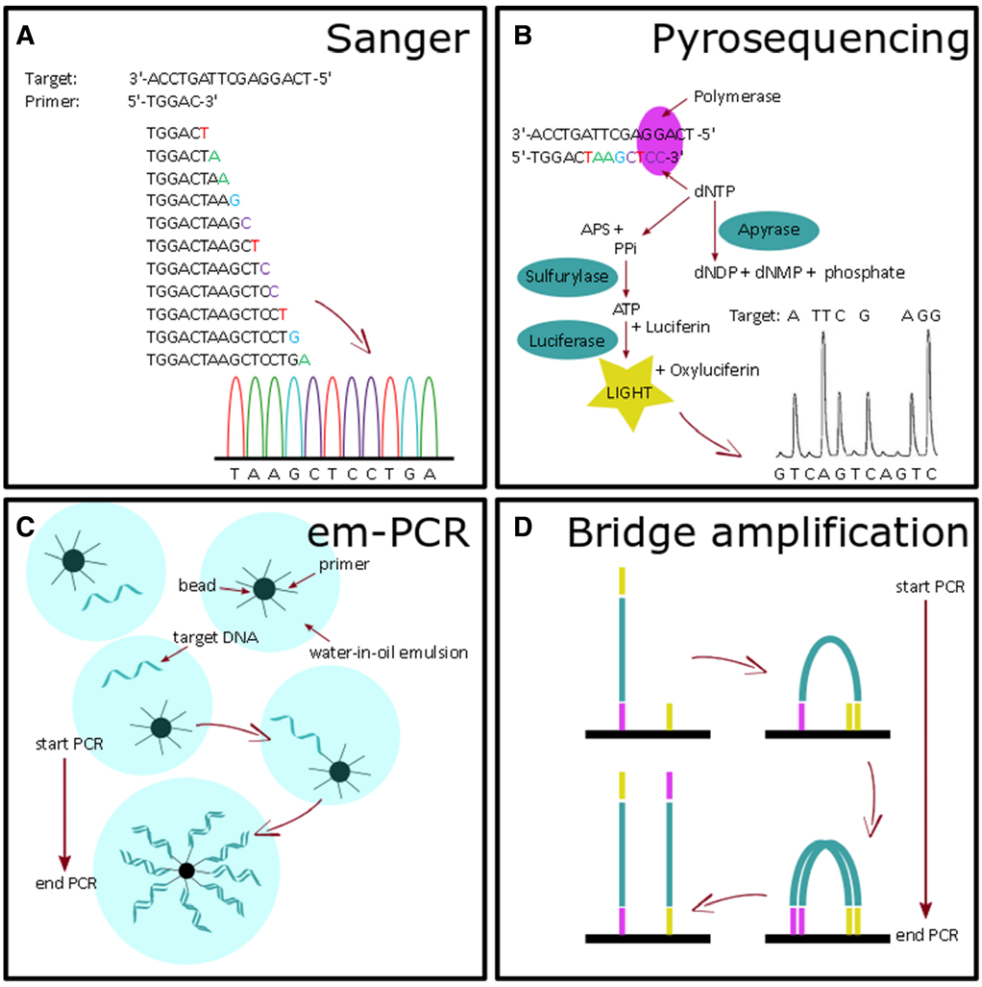

Figure 2. Overview of several DNA sequencing techniques with the principle of (A) Sanger sequencing, (B) pyrosequencing (e.g. 454), (C) em-PCR (e.g. 454, SOLiD® and Ion Torrent™) and D) bridge amplification/cluster PCR (e.g. Solexa).

3.2 Illumina

Also Solexa (acquired by Illumina® in 2007 (https://www.illumina.com/science/technology/next-generation-sequencing/illumina-sequencing-history.html) released a sequencing system based on sequencing-by-synthesis and the use of reversible dye terminators [3, 4]. Just as with the 454 system, adapters are placed at the ends of the DNA sequence. Whereas the 454 system used beads and em-PCR, Solexa makes use of a planar solid glass support [7, 9]. Sequences complementary to the adapters 'A' and 'B' are present on the full inside of flow cell lanes [37]. When the other end of the target DNA also hybridizes to the complementary sequence present on the support, a bridge structure is created (Fig. 2D) [4, 14]. Each bridge-amplified cluster contains a unique DNA template, which is primed and sequenced [9]. The ddNTPs have different fluorescent labels and a removable blocking group. By completing the template one base at a time and recording the fluorescent signal with a CCD camera, the DNA sequence can be determined [1]. Higher throughput was achieved by implementing a faster camera, faster polymerases, higher occupancy, and monoclonality within each well and patterned flow cells with fixed nanowells [3]. Drawback of the reversible dye termination SBS technique is the short read length, because 100% efficiency of base incorporation and cleavage in each cycle is hard to obtain [9]. The HiSeq® series, and later the MiSeq® series, succeeded the Solexa Genome Analyzer [1].

3.3 Applied Biosystems

Over the years Life Technologies, which joined Invitrogen™ to become Applied Biosystems™ in 2008, took over several companies that brought sequencing systems to the market (e.g. SOLiD® and Ion Torrent™). Nowadays Applied Biosystems™ is part of Thermo Fisher Scientific.

3.3.1 SOLiD

SOLiD® is an abbreviation of Sequencing by Oligo Ligation Detection, a system based on the Polonator technology, and brought to the market by Agencourt in 2006 and later by Applied Biosystems™ [8,14,16]. This technique was patented by McKernan et al. in 2006 [38]. The system is based, as the name suggests, on sequencing by ligation, which means that a probe sequence is bound to a fluorophore which hybridizes to

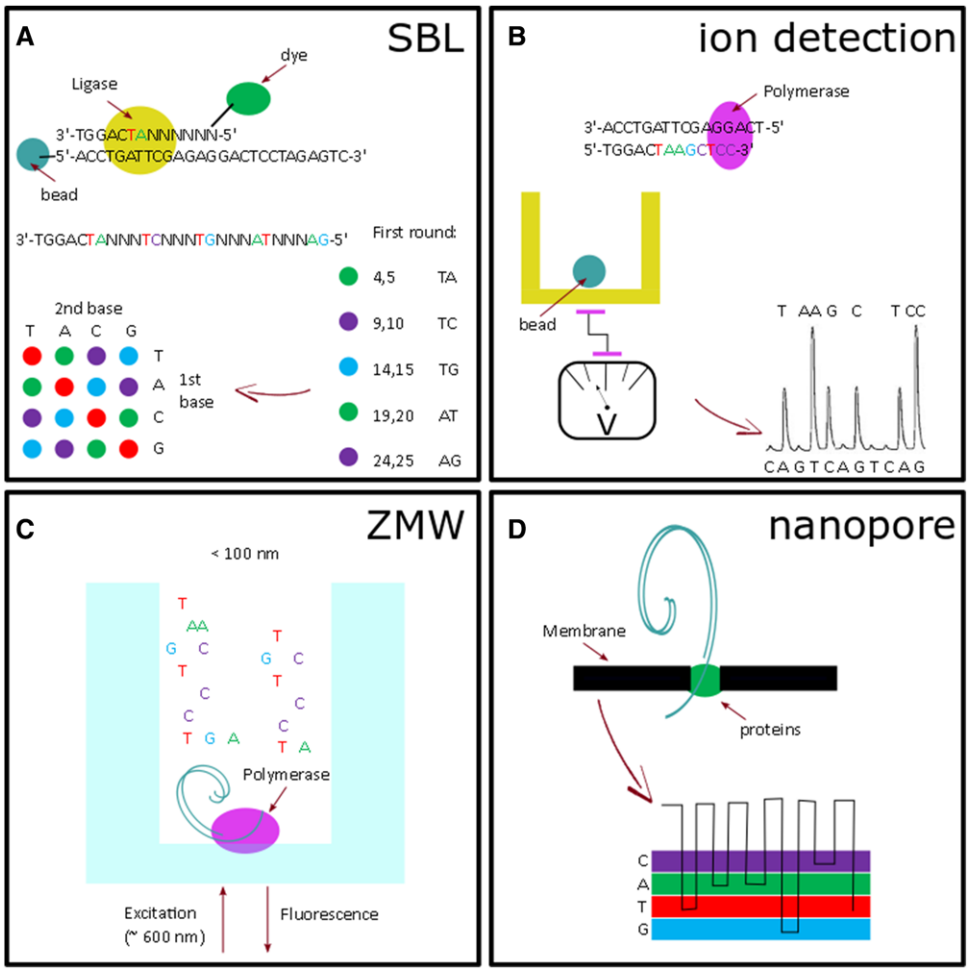

Figure 3. Overview of several DNA sequencing techniques with the principle of (A) sequencing by ligation (SBL, e.g. SOLiD®), (B) ion detection (e.g. Ion Torrent™), (C) zero-mode waveguides (ZMWs, e.g. PacBio®) and (D) nanopores (e.g. Oxford Nanopore).

a DNA fragment and is ligated to an adjacent oligonucleotide for imaging (Fig. 3A) [1, 15]. This method utilizes em-PCR with small magnetic beads of 1 μm [2, 6, 39]. The technique makes use of oligonucleotides of eight nucleotides present in all possible variations of the complementary DNA strand, with on the 4th and 5th bases a specific fluorescent label [6]. Next, the ligated octamer is cleaved after the fifth base, whereby the fluorescent label is removed and the next ligation cycle can start [4]. Therefore in the first round the bases are determined on positions 4–5, 9–10, 14–15, etc. In the next round a primer is used with one less base to sequence the positions 3–4, 8–9, 13–14, etc. [3, 16].

3.3.2 Ion Torrent

At the end of 2010 the company Ion Torrent™ introduced their Ion Personal Genome Machine™ (PGM™) (developed by Jonathan Rothburg after leaving 454) [1, 21]. This SBS method also starts with em-PCR on beads (Fig. 2C), which, in a later stage of the process are kept in position in the wells of a chip/picowell plate [9,21,40]. The detection method is not based on fluorescence, but uses the pH change upon addition of a nucleotide in a sequence. When the new nucleotide is bound, upon pyrophosphate cleavage, it releases a proton that is detected by monitoring the potential (Fig. 3B) [1]. The well plate ensures that proton release can be localized and retained. The signal is proportional to the amount of protons released, which makes it possible to sequence homopolymeric regions of the template DNA. Data collection is carried out by a complementary metal-oxide semiconductor (CMOS) sensor array chip with the sensor surface present at the bottom of the well plate. These chips can measure millions to billions of simultaneous sequencing reactions [40]. Measuring the potential is faster, cheaper and can be accomplished with smaller instruments than systems based on fluorescence read-out [1].

4 Third generation sequencing

Although second generation sequencing techniques are based on amplification, with the next-next-next (or third generation) methods single molecules are read in real time. Therefore these techniques are much faster and longer reads can be generated than with the previous generations of sequencing techniques [1, 3, 5, 20, 41]. Single molecule

sequencing (SMS) technologies can be grouped in three categories. The first category is SBS methods, whereby single molecules of DNA polymerase are observed at the moment they are synthesizing a single DNA molecule (e.g. PacBio®). Nanopore sequencing techniques are the second category (e.g. Oxford Nanopores) and the third category consists of methods that use direct imaging of individual DNA molecules by means of advanced microscopy (e.g. VisiGen) [41, 42]. The first SMS technique was developed by the laboratory of Quake and commercially launched by Helicos in 2007, which went bankrupt in 2012 [4, 22, 43]. The systems of PacBio® (fluorescent signal detection) and Oxford Nanopore (current measurements) are also based on SMS and are described in more detail below. Other third generation sequencing systems, such as Heliscope from Helicos [8,42,44], VisiGen (later Life Technologies/Invitrogen™ [4, 45], and Starlight (also known as the code name for VisiGen, a patent interference case involving Pacific Biosciences®, Life Technologies, and Helicos resulted in discontinuation of this technique) [7, 46] will not be discussed here, because these systems are either not available or not mentioned in literature anymore.

4.1 PacBio

The system of Pacific Biosciences® (PacBio® in short) makes use of Single Molecule Real-Time® (SMRT®) technology [47, 48]. The instrument, available since late 2010, is the first sequencer for individual DNA molecules and real-time detection [7]. In contrast to the previously described methods, this technique is suitable for long-read sequencing [15]. The method is based on fluorescent labeling and SBS and the sequence is read real time. Glass wells of less than 100 nanometers in diameter are coated with a metal film, forming zero-mode waveguides (ZMWs) because no propagation modes exist in these tiny wells [5, 49, 50]. The volume of these wells is in the order of zeptoliters and the polymerase (ϕ29) is immobilized beforehand on the bottom of the well. A high-resolution camera at the bottom of the ZMWs records the fluorescence of the nucleotide that is incorporated in real time [16, 49]. Because of the design of the ZMWs, the lowest 30 nm of the well is illuminated, which enables the detection of the incorporation of the fluorescent nucleotides by the polymerase [41].

4.2 Nanopore-based systems

With the use of nanopores a single DNA molecule can be read and this can be done, similar to other third generation techniques, without the need for amplification or expensive fluorescent labels [51]. The detection principle of nanopore-based systems relies on the ion current, which is generated when a charged molecule, such as a DNA molecule, passes through a nanoscale pore in a membrane. The membrane separates two chambers, which are filled with a conductive electrolyte. A voltage is used to drive the DNA strand through the pore, resulting in a changing ionic current (Fig. 3D). In theory the four different bases would produce four distinct current levels, which can be used for sequencing the DNA strand. Transport through the pore is usually realized by α-haemolysin. The transport of ssDNA through a pore can reach velocities of about 1 nucleotide/μs. To reduce the velocity of a DNA strand through a pore, α-haemolysin can be replaced by *Mycobacterium smegmatis* porin A [51, 52]. A motor protein is attached to the pore and a transport protein provides the DNA translocation (denaturation of the dsDNA to become ssDNA) through the pore [5, 15, 21].

The MinION™ is a nanopore system commercially released by Oxford Nanopore Technologies in 2014. This device weighs only 100 gram and can be connected to a computer via USB [5, 53].

5 MPS in forensics

In the annual report of the Netherlands Forensic Institute (NFI) of 2015 the need for forensic awareness of NGS was highlighted. The forensic laboratory for DNA analysis at the University of Leiden is the first institute in the world that was accredited to use NGS technology in forensic DNA analysis. As a consequence the probability of false positive matches in DNA profiling decreased and it has become easier to distinguish the different DNA profiles in a complex mixture. With the use of NGS technology the distinctive character of the profiles increases and therefore also the value of the evidence (i.e. the random match probability will be lower) (https://dnadatabank.forensischinstituut.nl/binaries/dna-jaarverslag-2015_tcm37-87649.pdf).

In 2015, Iozzi and co-workers reported that the use of NGS technologies in forensics has been limited to sporadic pilot studies [54]. This was confirmed by a literature review of the period December 2012–June 2015 by Alvarez-Cubero et al. [11]. At that time sufficient read lengths for a complete DNA profile could only be obtained with pyrosequencing [11, 55, 56].

A survey in 2017 among 33 European forensic laboratories showed that 52% of these laboratories already bought one or more NGS machines. At the moment the MiSeq®/NextSeq and the Ion Torrent™ PGM™/S5 are most often purchased. The technology of NGS is mainly used for SNP markers for identity or ancestry, but also for autosomal STR markers (i.e. DNA profiling) [57].

By introducing new sequencing techniques and systems, as well as forensic assays/kits, MPS gathered a lot of attention over the last 2–3 years. Kits, such as the HID-Ion AmpliSeq™ Identity (SNP) Panel (Thermo Fisher Scientific) and the ForenSeq™ DNA Signature Prep Kit (Illumina® for (X/Y-)STRs and SNPs for identity, biogeographical ancestry, and phenotyping applications are commercially available and comprehensively tested by forensic researchers [10, 56, 58]. Furthermore, mitochondrial DNA (mtDNA) can be sequenced to obtain more information about, for example, the maternal lineage [10, 56, 59]. In the review of Børsting and co-workers from 2015 NGS technology was called "the

future of forensic DNA analysis" [56]. Although there are many more applications of NGS in forensics, in this review only the use of NGS techniques and systems for human DNA analysis will be discussed.

5.1 STR

Because STRs are used to obtain a DNA profile that can be compared with profiles in a database, it must be possible with every NGS technology to sequence these repeats [10, 56]. To increase the random match probability more STR markers are needed, but with conventional analysis techniques (i.e. PCR in combination with capillary electrophoresis) the amount of markers is limited. With NGS methods these limitations do not exist [10]. Moreover, NGS of STR markers is also very suitable for samples with a low quantity of DNA or degraded samples [60]. With NGS not only the number of repeats (the classical STR profile) can be analyzed, but also polymorphisms can be determined [11]. In order to compare STR-MPS data with STR profiles obtained by CE, the nomenclature must be well established. The International Society for Forensic Genetics suggested minimal nomenclature requirements. The standard nomenclature for CE obtained profiles is not suitable for sequence differences (e.g. transversions, insertions, and deletions) between alleles [61]. When more information on the paternal inheritance is required, Y-STRs can be used [10].

Van Neste and co-workers performed STR-profiling on a Roche GS FLX sequencer, since 2012 it was the only technique that could generate full read lengths of 400–500 bp that are required for STR-profiling. The results of both single contributor samples and multiple-person mixtures were compared with the AmpFISTR® Profiler Plus® kit from Applied Biosystems™. A known disadvantage of the Roche GS FLX is the high error rate on homopolymers, which are usually widely present in most STR amplicons. Reducing all homopolymers to a single base with software correction did not influence the data analysis because of homopolymer sequencing errors; however, it must be noted that not all information present in these homopolymers becomes available with this approach. Van Neste et al. concluded that using Roche GS FLX technology is not ideal for sequencing multiplexed STR amplicons, because of this high homopolymer sequencing error rate [55]. Li and co-workers have used the Precision ID GlobalFiler™ NGS STR panel from Thermo Fisher Scientific with the Ion Torrent™ PGM™ for paternity testing with mismatched STR loci. The benefit of using NGS is that length polymorphisms, as well as detailed sequencing polymorphism variations can be analyzed, which is not possible with conventional CE analysis. With the obtained data, by including enough STR loci, the certainty of paternity can be supported [62]. When annealing and/or elongation of a primer cannot take place, because of rare variants in the DNA at the primer binding place, dropout of one or both alleles occurs. This situation is also known as null alleles or silent alleles and hinders STR profiling. Yao and co-workers determined these sequence variations at several STR loci by the use of Sanger sequencing [63]. An overview of articles published on forensic STR analysis by the use of MPS is given in Table 2.

5.2 SNP

SNPs are single-base variances in a DNA sequence, such as substitutions, insertions, and deletions [74]. STRs show a high heterozygosity, but a higher mutation rate compared to SNPs [56, 75]. SNPs are used in forensics for paternity/ancestry testing (e.g. with Y-SNPs) and to determine phenotypical characteristics of an individual [10,76,77]. May 30/31 2017 was the opening of a EU Horizon 2020-funded project called VISAGE, VISible Attributes through Genomics, which aims at the allocation of previous and establishment of new DNA predictors. These predictors can give information on appearance, age, and ancestry. In addition a forensically validated prototype tool based on MPS will be developed for simultaneous analysis of these DNA predictors, which is also suitable for trace DNA (www.visage-h2020.eu). An overview of articles published on forensic SNP analysis by the use of MPS is given in Table 3.

In 2015 Wang et al. screened 44 loci for new forensic marker microhaplotypes. Subsequently 25 loci were used to sequence with a MiSeq®. One microhaplotype locus showed three SNPs and is therefore suitable as forensic marker [75]. Elwick et al. compared two forensic kits, i.e. the HID-Ion AmpliSeq™ Library kit (Ion Torrent™ system) and the ForenSeq™ DNA Signature Prep kit (MiSeq® system), on the inhibitory effect of humic acid, melanin, hematin, collagen and calcium. With both kits an inhibitory effect was observed for high concentrations (30.3 ng/μL) of humic acid. Hematin and calcium showed the highest inhibition with the AmpliSeq™ kit (a kit for only SNPs), whereas melanin and collagen affected the ForenSeq™ kit the most (a kit for STRs and SNPs) [78]. Apaga et al. compared these two sequencing systems for their performance on 83 SNP markers. The MiSeq® was used in combination with the ForenSeq™ DNA Signature Prep kit and the HID-Ion PGM™ in combination with the HID-Ion AmpliSeq™ Identity Panel. The concordance between the two kits was 99.7% [79]. The HID-Ion AmpliSeq™ Library kit was used by Hollard et al. in a real forensic case. A carbonized body was found and no direct physical description or personal belongings could be used for identification of the body. The obtained DNA profile did not result in a match with the database. However, by using the HID-Ion AmpliSeq™ Ancestry Panel, in combination with the Ion PGM™, 165 SNPs were sequenced and a probable origin could be determined [80]. Ambers et al. used the combination of STR and SNP analysis to characterize 140-year-old skeletal remains. Almost all STRs and SNPs could be typed with the Ion Torrent™ PGM™ in combination with the HID-Ion AmpliSeq™ Identity Panel, proving that MPS is suitable for samples which are limited in both quantity and quality [81]. In Table 4 an overview is given of articles published on kits that combine forensic STR and SNP analysis by the use of MPS.

Table 2. Overview of forensic STR analysis by means of MPS techniques. The used machine, kit, and most important conclusion(s) are summarized

Machine	Kit and no. of markers	Most important conclusion(s)	Year	Ref.
Biotage® AB Pyrosequencing PSQ™96MA	Custom primers, 7 Y-STRs	Advantage to observe sequence variants	2009	[64]
Genome analyzer IIx	Phusion polymerase and custom primers, 13 STRs	Maximum read length of 150 bp is not suitable for long STRs	2012	[65]
454 GS FLX	AmpFISTR® Profiler Plus®, 10 STRs	Not ideal for multiplexed STRs	2012	[55]
454 GS Junior	miniSTR markers, 13 STRs	Potential of NGS, but data analysis is challenging	2014	[60]
MiSeq®	MiSeq® v2 (2 × 250 bp), 18 STRs	With 250 pg input DNA balanced heterozygote allele coverage ratio's	2015	[66]
Ion Torrent™ PGM™	Custom primers, 13 Y-STRs	Many sequence variants with the same sequence length detected	2015	[67]
Ion Torrent™ PGM™	HID STR 10-plex, 10 STRs	With 50 pg input DNA full profiles can be obtained	2015	[68]
MiSeq®	PowerSeq™ Auto System, 22 STRs	6 loci show more than double the number of alleles by sequence than by length	2016	[69]
Ion Torrent™ PGM™	Early Access STR Kit 1, 25 STRs	With 100 pg input DNA full profiles can be obtained	2016	[70]
Ion Torrent™ PGM™	HID STR 10-plex, 10 STRs	Better alternative to predict stutter ratio presented	2017	[71]
MiSeq® FGx™	ForenSeq™ DNA Signature Prep, 27 STRs + 24 Y-STRs	High recovery rates and concordance with CE data	2017	[72]
Ion Torrent™ PGM™	Precision ID GlobalFiler™ NGS STR panel, 29 STRs + 1 Y-STRs	The certainty of paternity can be supported	2017	[62]
MiSeq® FGx™	PowerSeq™ Systems Prototype Auto/Y, 23 STRs + 23 Y-STRs	Improved workflow for forensics obtained	2018	[73]

Table 3. Overview of forensic SNP analysis by means of MPS techniques. The used machine, kit and most important conclusion(s) are summarized

Machine	Kit and no. of markers	Most important conclusion(s)	Year	Ref.
Ion Torrent™ PGM™	HID-Ion AmpliSeq™ Identity Panel, 136 SNPs + 33 Y-SNPs	With 25–100 pg of input DNA 90–95% of the genotypes can be obtained	2015	[82]
MiSeq®	GeneRead™ DNAseq Targeted Panels V2, 140 SNPs	Accurate SNP call with high degree of coverage	2016	[76]
Ion Torrent™ PGM™	HID-Ion AmpliSeq™ Identity Panel v2.3, 90 SNPs + 29 Y-SNPs	With 31 pg of input DNA consistent profiles can be obtained	2016	[83]
Ion Torrent™ PGM™	HID-Ion AmpliSeq™ Identity Panel, 90 SNPs + 34 Y-SNPs	With 100 pg of input DNA full profiles can be obtained	2016	[84]
Ion Torrent™ PGM™	HID-Ion AmpliSeq™ Identity Panel, 90 SNPs + 34 Y-SNPs	MPS good tool for paternity testing and human identification	2017	[74]
Nanopore MinION™	SNPforID protocol, 52 SNPs	51/52 loci in correspondence to genotype with the TruSeq™ kit	2017	[85]
Ion Torrent™ PGM™	Custom primers, 233 SNPs + 9 Y-SNPs + 31 X-SNPs	With 1 ng input DNA reproducible results can be obtained	2017	[86]
Ion Torrent™ PGM™	HID-Ion AmpliSeq™ Library, 90 SNPs + 34 Y-SNPs	Reaction highly inhibited by hematin	2017	[78]
Ion Torrent™ PGM™	HID-Ion AmpliSeq™ Identity Panel, 83 SNPs	Lower sample-to-sample variation compared to the ForenSeq™ kit	2017	[79]
Ion Torrent™ PGM™	SNP-ID kit, 136 SNPs + 34 Y-SNPs	Full genotype concordance with a similar SNP panel	2017	[87]
Ion Torrent™ PGM™	Precision ID Identity Panel, 90 SNPs + 34 Y-SNPs	With ≥ 0.2 ng of pure DNA or forensic samples reliable and reproducible genotyping	2017	[88]
Ion Torrent™ PGM™	Precision ID Identity Panel, 165 SNPs	Japanese, Okinawa Japanese, and East Asians could not be differentiated	2018	[89]
Ion Torrent™ PGM™	Precision ID Identity Panel, 165 SNPs	3/165 loci consistently poor performance	2018	[90]

Table 4. Overview of forensic STR and SNP analysis by means of MPS techniques. The used machine, kit, and most important conclusion(s) are summarized

Machine	Kit and no. of markers	Most important conclusion(s)	Year	Ref.
MiSeq® FGx™ BETA	ForenSeq™ DNA Signature, 63 STRs + 95 SNPs	100% correct allele assignment with 1 ng of input DNA complete and correct profile	2015	[54]
MiSeq® FGx™	ForenSeq™ DNA Signature Prep, 59 STRs + 172 SNPs	Complete concordance with PCR-CE (STR) phenotypical/ancestry predictions are not always correct	2015	[77]
MiSeq® desktop sequencer	ForenSeq™ DNA Signature Prep (Beta Version) 63 STRs + 95 SNPs	With 1 ng of input DNA full profiles advantage to observe sequence variants	2016	[91]
MiSeq® FGx™	ForenSeq™ DNA Signature Prep, 58 STRs + 174 SNPs	Some loci are prone to more sequence errors	2017	[92]
MiSeq® FGx™	ForenSeq™ DNA Signature Prep, 59 STRs + 172 SNPs	Robust method for forensics	2017	[93]
MiSeq® FGx™	ForenSeq™ DNA Signature Prep, 58 STRs + 94 SNPs	99.7% concordance between this kit and the HID-Ion AmpliSeq™ Identity kit	2017	[79]
MiSeq® FGx™	ForenSeq™ DNA Signature Prep, 58 STRs + 94 SNPs	Higher inhibition to melanin and collagen compared to HID-Ion AmpliSeq™ Library kit	2017	[78]
MiSeq® FGx™	ForenSeq™ DNA Signature Prep, 60 STRs + 174 SNPs	With 50 pg of input DNA reproducible genotypes	2017	[94]
MiSeq® FGx™	ForenSeq™ DNA Signature Prep, 59 STRs + 172 SNPs	100% accuracy in STR allele calling >99.1% accuracy in SNP typing	2017	[95]
MiSeq® FGx™	ForenSeq™ DNA Signature Prep, 59 STRs + 172 SNPs	Loci with higher read numbers perform better	2017	[96]
MiSeq® FGx™	ForenSeq™ DNA Signature Prep, 58 STRs + 94 SNPs	99.98% concordance with commercial STR and CE kits	2017	[97]

5.3 mtDNA

When a limited amount of DNA is available, mtDNA can be used to obtain forensically relevant information, because the maternal lineage can be investigated with this type of DNA [10, 59]. The whole mitochondrial genome can be sequenced in one run [98]. Besides the multiple copies present per cell, other advantages of mtDNA are the absence of recombination across many generations and the accumulation of mutations over time [11]. On 16 October 2006, the EDNAP mtDNA Population Database (EMPOP) was launched for frequency estimation of mtDNA sequences. The goal of this database is to collect, perform quality control, and give a searchable presentation of mtDNA haplotypes around the globe [99], (https://empop.online/). However, in 2011 Bandelt and co-workers concluded that NGS, e.g. with the Roche 454 FLX, was not yet suitable for the forensic casework. Comparison of mtDNA sequences of different tissues from one person varied so much that the minimum quality standard could not be guaranteed [100]. Nevertheless, the 454 Roche instrument was used in 2013 by Bekaert and coworkers to sequence the control region of the mitochondrial genome. A 100% concordance with Sanger sequencing was observed [101]. In 2014 Mikkelsen and co-workers compared the Roche 454 GS Junior with Sanger sequencing for determination of the nucleotide sequence for hypervariable regions in the mtDNA, HV1, and HV2. They found almost full concordance between all 72942 compared sites, with homopolymers as most often miscalled. The accuracy of sequences of four, five, and six identical bases is 95, 95, and 85%, respectively. By visual inspection of the data, most of the artifacts can be identified and manually changed [102]. Kim et al. used the Roche 454 GS Junior for the analysis of mixtures of mtDNA. They were successful in obtaining sequences from about 1 pg genomic DNA or 100–500 mtDNA copies. The sequences analyzed showed concordance with the Sanger sequencing results and it was possible to distinguish three contributors in a mixture sample [103]. Samples were taken from 194 mother–child pairs from a Chinese population and sequenced with the Ion Torrent™ system by Ma et al. in 2018. They concluded that the inheritance of the mtDNA variants of each mother–offspring pair was as expected. They also noted that by the use of MPS methods more insight can be gained about mtDNA [59]. Gouveia and co-workers evaluated the Precision ID mtDNA Whole Genome Panel from Applied Biosystems™, which is specifically developed for mtDNA in forensic applications. The 162 amplicons were sequenced with the Ion S5™ system from Ion Torrent™. They found concordance of the haplotypes between this kit and system and previously obtained results with Sanger sequencing [104]. The MinION™ and the MiSeq® platforms have been evaluated by Lindberg and co-workers for the analysis of human mtDNA. As it is known from the specifications of both techniques, the MiSeq® shows a greater depth of coverage than the MinION™. For single source samples a great overall concordance (≥0.940) was seen between the two techniques, while for the analyzed mixture it was 0.875, which

makes the MinION™ less suitable for the analysis of mixtures compared to the MiSeq®. Gallimore and co-workers have sequenced head and pubic hears, common forensic traces, to look for the presence of heteroplasmy (mutations in the mtDNA) and compared the outcome with samples from blood cells. They concluded that the variant ratios of heteroplasmy from hairs can be somewhat different than observed in blood cells. The analysis was carried out with the MiSeq® system in combination with the PowerSeq™ Mito Nested System Prototype kit [105]. Wai and co-workers have tested the Early Access AmpliSeq™ Mitochondrial Panel with the Ion Torrent™ to analyze the performance of the kit with degraded DNA. To mimic degraded samples, DNA samples were heat-treated. Although the amplicon coverage decreased with longer heating times, haplogroup variants could still be reliably assessed, even with highly degraded samples [106]. Yao and co-workers published a study in 2018 in which they used the Ion Torrent™ PGM to sequence the complete mitochondrial genome of six samples from three forensic cases. By the use of the Precision ID mtDNA Whole Genome Panel >99% of the sequencing reads from the aged forensic samples could be mapped to the reference sequence [107]. With the use of the Ion Chef for template preparation and the Ion S5 for sequencing in combination with the Precision ID mtDNA Whole Genome Panel it was possible to sequence the complete mitochondrial genome [98]. By sequencing mtDNA with MPS techniques, it might be possible to analyze mixtures. Churchill and co-workers used the Ion Torrent™ PGM™ in combination with the Precision ID mtDNA whole Genome Panel to sequence two-person mixtures in various ratios. From 1:1 to 20:1 the major contributor's haplotype could be identified correctly. The SNPs from the minor contributor's haplotype were identified in the 1:1, 5:1, and 10:1 mixtures only [108].

5.4 Other markers

The type of tissue or body fluid, the chronological age of a person, and differentiation between identical twins is possible by analyzing DNA methylation [12, 109]. Vidaki and co-workers used the state of methylation of the DNA to estimate the age of people. They used the MiSeq® to sequence 1156 whole blood samples from people in the age group of 2–90 years. Although the prediction method can be improved, the obtained accuracy was lower than the original model - it is a promising technique for forensic applications [110]. With the current technique of STR-typing it is not possible to distinguish between DNA profiles from identical twins, on the other hand, with the use of NGS technology this has become possible (https://dnadatabank.forensischinstituut.nl/binaries/dna-jaarverslag-2015_tcm37-87649.pdf). Weber-Lehmann and co-workers have looked at extremely rare mutations, which can only be analyzed by MPS. To investigate this, they sequenced the DNA of monozygotic male twins and also from the wife and child of one of the twins with the HiSeq® 2000. Total 12 potential SNPs were found, which were present in the father and child, but not in the DNA of the uncle [111]. Drawback of the MinION™ is the low accuracy and the high error rate (~12%), being higher than the expected differences between two people [5, 53]. Nevertheless, Zaaijer and co-workers have developed a method for the reidentification (e.g. to identify samples of victims of mass disaster or human trafficking) of human samples within 3 min. This was illustrated by analyzing 91 SNPs of a human cell line, obtaining a confidence level of 99.9% [53].

6 Concluding remarks

The use of MPS for forensic (human) DNA analysis appears to be very promising. Not only a DNA profile with STR markers can be obtained, but also ancestry and phenotype can be determined by the use of SNPs. Besides autosomal DNA also mtDNA or the state of methylation of the DNA can be analyzed with MPS systems. Especially from samples low in quantity and quality significantly more information can be obtained with MPS than with the conventional techniques.

Nowadays a wide variety of sequencing systems is commercially available. Since Applied Biosystems™, Invitrogen™ and Life Technologies are part of Thermo Fisher Scientific at present, this company is market leader in MPS systems with machines as the Genetic Analyzer series and the Ion Torrent™ system. The latter system is widely applied for the forensic SNP analysis, as it is also reflected in Table 3. Moreover, the Illumina's® MiSeq® system is quite popular for especially a combination of STR and SNP analysis, as can be seen in Table 4.

A major limitation is the long measurement time that is currently still needed by MPS systems. One run, with steps like DNA isolation, library generation and data analysis, can easily take several days, as it is also reflected in Table 1. Especially data analysis, database management, and the lack of a clear nomenclature are important issues [57]. These challenges are also mentioned by Van Neste and co-workers, who plead for the use of one single system to process all the data acquired with sequencing [112]. Another important reason for forensic laboratories not to implement MSP techniques yet is the costs of machines and kits, as mentioned in a survey of 2017 [57]. Nevertheless, as a logical consequence of the enormous growth in publications on MPS for forensic (human) DNA analysis over the last years, the first real case reports have just recently appeared in literature.

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7 References

[1] Liu, L., Li, Y., Li, S., Hu, N., He, Y., Lin, D., Lu, L., Law, M., *BioMed Res. Int. 2012*, 2012, http://doi.org.10.1155/2012/251364.

[2] Kircher, M., Kelso, J., *Bioessays* 2010, *32*, 524–536.

[3] Van Dijk, E. L., Auger, H., Jaszczyszyn, Y., Thermes, C., *Trends Genet.* 2014, *30*, 418–426.

[4] Ansorge, W. J., *New Biotechnol.* 2009, *25*, 195–203.

[5] Kchouk, M., Gibrat, J.-F., Elloumi, M., *Biol. Med.* (Aligarh) 2017, *9*, 395.

[6] Zhang, J., Chiodini, R., Badr, A., Zhang, G., *J. Genet. Genomics* 2011, *38*, 95–109.

[7] Glenn, T. C., *Mol. Ecol. Resour.* 2011, *11*, 759–769.

[8] Shendure, J., Ji, H., *Nat. Biotechnol.* 2008, *26*, 1135–1145.

[9] Stranneheim, H., Lundeberg, J., *Biotechnol J.* 2012, *7*, 1063–1073.

[10] Yang, Y., Xie, B., Yan, J., *GPB* 2014, *12*, 190–197.

[11] Alvarez-Cubero, M. J., Saiz, M., Martínez-García, B., Sayalero, S. M., Entrala, C., Lorente, J. A., Martinez-Gonzalez, L. J., *Ann. Hum. Biol.* 2017, *44*, 581–592.

[12] Budowle, B., Schmedes, S. E., Wendt, F. R., *Forensic Sci. Med. Pat.* 2017, *13*, 342–349.

[13] Lin, B., Wang, J., Cheng, Y., *Recent Pat. Biomed. Eng.* 2008, *1*, 60–67.

[14] Kumar, B. R., in: A., Munshi (Eds.), *DNA sequencing – Methods and Applications*, InTech, Rijeka 2012, pp. 3–14.

[15] Goodwin, S., McPherson, J. D., McCombie, W. R., *Nat. Rev. Genet.* 2016, *17*, 333–351.

[16] Myllykangas, S., Buenrostro, J., Ji, H. P., in: N., Rodríguez-Ezpeleta, M., Hackenberg, A. M., Aransay (Eds.), *Bioinformatics for High Throughput Sequencing*, Springer Science+Business Media, LLC, 2012, pp. 11–25.

[17] Berglund, E. C., Kiialainen, A., Syvänen, A.-C., *Invest. Gen.* 2011, *2*, 23.

[18] Sanger, F., Nicklen, S., Coulson, A. R., *Proc. Nat. Acad. Sci.* 1977, *74*, 5463–5467.

[19] Rogers, Y.-H., Venter, J. C., *Nature* 2005, *437*, 326–327.

[20] Schuster, S. C., *Nat. Med.* 2008, *5*, 16.

[21] Heather, J. M., Chain, B., *Genomics* 2016, *107*, 1–8.

[22] Reis-Filho, J. S., *Breast Cancer Res.* 2009, *11*, S12.

[23] Ansorge, W., Sproat, B. S., Stegemann, J., Schwager, C., *J. Biochem. Biophys. Meth.* 1986, *13*, 315–323.

[24] Ansorge, W., Process for sequencing nucleic acids, 1992, US Patent 5,124,247.

[25] Koumi, P., Green, H. E., Hartley, S., Jordan, D., Lahec, S., Livett, R. J., Tsang, K. W., Ward, D. M., *Electrophoresis* 2004, *25*, 2227–2241.

[26] Ju, J., Li, Z., Edwards, J. R., Itagaki, Y., Massive parallel method for decoding DNA and RNA, 2015, US Patent 9,133,511.

[27] Ju, J., Kim, D. H., Bi, L., Meng, Q., Bai, X., Li, Z., Li, X., Marma, M. S., Shi, S., Wu, J., Edwards, J. R., Romu, A., Turro, N. J., *Proc. Nat. Acad. Sci.* 2006, *103*, 635–640.

[28] Drmanac, R., Sparks, A. B., Callow, M. J., Halpern, A. L., Burns, N. L., Kermani, B. G., Carnevali, P., Nazarenko, I., Nilsen, G. B., Yeung, G., Dahl, F., Fernandez, A., Staker, B., Pant, K. B., Baccash, J., Borcherding, A. P., Brownley, A., Cedeno, R., Chen, L., Chernikoff, D., Cheung, A., Chirita, R., Curson, B., Ebert, J. C., Hacker, C., Hartlage, R., Hauser, B., Huang, S., Jigan, Y., Karpinchyk, V., Koenig, M., Kong, C., Landers, T., Le, C., Liu, J., McBride, C. E., Morenzoni, M., Morey, R. E., Mutch, K., Perazich, H., Perry, K., Peter, B. A., Peterson, J., Pethiyagoda, C. L., Pothuraju, K., Richter, C., Rosenbaum, A. M., Roy, S., Shafto, J., Sharanhovich, U., Shannon, K. W., Sheppy, C. G., Sun, M., Thakuria, J. V., Tran, A., Vu, D., Wait Zaranek, A., Wu, X., Drmanac, S., Oliphant, A. R., Banyai, W. C., Martin, B., Ballinger, D. G., Church, G. M., Reid, C. A., *Science* 2010, *327*, 78–81.

[29] Porreca, G. J. *Nat. Biotechnol.* 2010, *28*, 43–44.

[30] Shendure, J., Porreca, G. J., Reppas, N. B., Lin, X., McCutcheon, J. P., Rosenbaum, A. M., Wang, M. D., Zhang, K., Mitra, R. D., Church, G. M., *Science* 2005, *309*, 1728–1732.

[31] Melamede, R. J., Automatable process for sequencing nucleotide, 1989, US Patent 4,863,849.

[32] Froehlich, T., Heindl, D., Roesler, A., Heckel, T., Miniaturized, high-throughput nucleic acid analysis, 2014, US Patent 8,822,158.

[33] Ronaghi, M., *Genome Res.* 2001, *11*, 3–11.

[34] Nyrén, P., Lundin, A., *Anal. Biochem.* 1985, *151*, 504–509.

[35] Rothberg, J. M., Leamon, J. H., *Nat. Biotechnol.* 2008, *26*, 1117–1124.

[36] Margulies, M., Egholm, M., Altman, W. E., Attiya, S., Bader, J. S., Bemben, L. A., Berka, J., Braverman, M. S., Chen, Y.-J., Chen, Z., Dewell, S. B., Du, L., Fierro, J. M., Gomes, X. V., Goodwin, B. C., He, W., Helgesen, S., He Ho, C., Irzyk, G. P., Jando, S. C., Alenquer, M. L. I., Jarvie, T. P., Jirage, K. B., Kim, J.-B., Knight, J., Lanza, J. R., Leamon, J. H., Lefkowith, S. M., Lei, M., Li, J., Lohman, K. L., Lu, H., Makhijani, V. B., McDade, K., McKenna, M., Myers, E., Nickerson, E., Nobile, J. R., Plant, R., Puc, B. P., Ronan, M. T., Roth, G. T., Sarkis, G. J., Simons, J. G., Simpson, J. W., Srinivasan, M., Tartaro, K. R., Tomasz, A., Vogt, K. A., Volker, G. A., Wang, S. H., Wangy, Y., Weiner, M. P., Yu, P., Begley, R. F., Rothber, J.M., *Nature* 2005, *437*, 376.

[37] Buermans, H. P. J., Den Dunnen, J. T., *Biochim. Biophys. Acta* 2014, *1842*, 1932–1941.

[38] McKernan, K., Blanchard, A., Kotler, L., Costa, G., Reagents, methods, and libraries for bead-based sequencing, 2016, US Patent 9,493,830.

[39] Hutchison, C. A. III, *Nucleic Acid Res.* 2007, *35*, 6227–6237.

[40] Merriman, B., Rothberg, J. M. *Electrophoresis* 2012, *33*, 3397–3417.

[41] Schadt, E. E., Turner, S., Kasarskis, A., *Hum. Mol. Genet.* 2010, *19*, R227–R240.

[42] Thompson, J. F., Milos, P. M., *Genome Biol.* 2011, *12*, 217.

[43] Braslavsky, I., Hebert, B., Kartalov, E., Quake, S. R., *P. Natl. A Sci.* 2003, *100*, 3960–3964.

[44] Thompson, J. F., Steinmann, K. E., *Curr. Protoc. Mol. Biol.* 2010, Supplement 92.

[45] Hardin, S., Gao, X., Briggs, J., Willson, R., Tu, S.-C., Methods for real-time single molecule sequence determination, 2008, US Patent 7,329,492.

[46] Perkel, J. M., *BioTechniques* 2010, *49*, 875–878.

[47] Roberts, R., Carneiro, M. O., Schatz, M. C., *Genome Biol.* 2013, *14*, 405.

[48] Chin, C.-S., Alexander, D. H., Marks, P., Klammer, A. A., Drake, J., Heiner, C., Clum, A., Copeland, A., Huddleston, J., Eichler, E. E., Turner, S. W., Korlach, J. *Nat. Methods* 2013, *10*, 563–569.

[49] Levene, M. J., Korlach, J., Turner, S. W., Foquet, M., Craighead, H. G., Webb, W. W., *Science* 2003, *299*, 682–686.

[50] Eid, J., Fehr, A., Gray, J., Luong, K., Lyle, J., Otto, G., Peluso, P., Rank, D., Baybayan, P., Bettman, B., Bibillo, A., Bjornson, K., Chaudhuri, B., Christians, F., Cicero, R., Clark, S., Dalal, R., deWinter, A., Dixon, J., Foquet, M., Gaertner, A., Hardenbol, P., Heiner, C., Hester, K., Holden, D., Kearns, G., Kong, X., Kuse, R., Lacroix, Y., Lin, S., Lundquist, P., Ma, C., Marks, P., Maxham, M., Murphy, D., Park, I., Pham, T., Philips, M., Roy, J., Sebra, R., Shen, G., Sorenson, J., Tomaney, A., Travers, K., Trulson, M., Vieceli, J., Wegener, J., Wu, D., Yang, A., Zaccarin, D., Zhao, P., Zhong, F., Korlach, J., Turner, S., *Science* 2009, *323*, 133–138.

[51] Schneider, G. F., Dekker, C., *Nat. Biotechnol.* 2012, *30*, 326–328.

[52] Venkatesan, B. M., Bashir, R., *Nat. Nanotechnol.* 2011, *6*, 615.

[53] Zaaijer, S., Gordon, A., Speyer, D., Piccone, R., Groen, S. C., Erlich, Y., *eLife* 2017, *6*, e27798.

[54] Iozzi, S., Carboni, I., Contini, E., Pescucci, C., Frusconi, S., Nutini, A. L., Torricelli, F., Ricci, U., *Forensic Sci. Int. Genet. Suppl. Ser.* 2015, *5*, e418–e419.

[55] Van Neste, C., Van Nieuwerburgh, F., Van Hoofstat, D., Deforce, D., *Forensic Sci. Int. Genet.* 2012, *6*, 810–818.

[56] Børsting, C., Morling, N., *Forensic Sci. Int. Genet.* 2015, *18*, 78–89.

[57] Alonso, A., Müller, P., Roewer, L., Willuweit, S., Budowle, B., Parson, W., *Forensic Sci. Int. Genet.* 2017, *29*, e23–e25.

[58] Mehta, B., Venables, S., Roffey, P., *Int. J. Legal Med.* 2018, *132*, 125–132.

[59] Ma, K., Zhao, X., Li, H., Cao, Y., Li, W., Ouyang, J., Xie, L., Liu, W., *Forensic Sci. Int. Genet.* 2018, *32*, 88–93.

[60] Scheible, M., Loreille, O., Just, R., Irwin, J., *Forensic Sci. Int. Genet.* 2014, *12*, 107–119.

[61] Parson, W., Ballard, D., Budowle, B., Butler, J. M., Gettings, K. B., Gill, P., Gusmão, L., Hares, D. R., Irwin, J. A., King, J. L., de Knijff, P., Morling, N., Prinz, M., Schneider, P. M., van Neste, C., Willuweit, S., Phillips, C. *Forensic Sci. Int. Genet.* 2016, *22*, 54–63.

[62] Li, H., Zhao, X., Ma, K., Cao, Y., Zhou, H., Ping, Y., Shao, C., Xie, J., Liu, W., *Forensic Sci. Int. Genet.* 2017, *31*, 155–159.

[63] Yao, Y., Yang, Q., Shao, C., Liu, B., Zhou, Y., Xu, H., Zhou, Y., Tang, Q., Xie, J., *Leg. Med.* 2018, *30*, 10–13.

[64] Edlund, H., Allen, M., *Forensic Sci. Int. Genet.* 2009, *3*, 119–124.

[65] Bornman, D. M., Hester, M. E., Schuetter, J. M., Kasoji, M. D., Minard-Smith, A., Barden, C. A., Nelson, S. C., Godbold, G. D., Baker, C. H., Yang, B., Walther, J. E., Toernes, I. E., Yan, P. S., Rodriguez, B., Bondschuh, R., Dickens, M. L., Young, B. A., Faith, S. A., *Biotech. Rapid Dispatch.* 2012, *2012*, 1–6.

[66] Zeng, X., King, J., Stoljarova, M., Warshauer, D. H., LaRue, B. L., Sajantila, A., Patel, J., Storts, D., Budowle, B., *Forensic Sci. Int. Genet.* 2015, *16*, 38–47.

[67] Zhao, X., Ma, K., Li, H., Cao, Y., Liu, W., Zhou, H., Ping, Y., *Forensic Sci. Int. Genet.* 2015, *19*, 192–196.

[68] Fordyce, S. L., Mogensen, H. S., Børsting, C., Lagacé, R. E., Chang, C.-W., Rajagopalan, N., Morling, N., *Forensic Sci. Int. Genet.* 2015, *14*, 132–140.

[69] Gettings, K. B., Kiesler, K. M., Faith, S. A., Montano, E., Baker, C. H., Young, B. A., Guerrieri, R. A., Vallone, P. M., *Forensic Sci. Int. Genet.* 2016, *21*, 15–21.

[70] Guo, F., Zhou, Y., Liu, F., Yu, J., Song, H., Shen, H., Zhao, B., Jia, F., Hou, G., Jiang, X., *Forensic Sci. Int. Genet.* 2016, *28*, 82–89.

[71] Vilsen, S. B., Tvedebrink, T., Mogensen, H. S., Morling, N., *Forensic Sci. Int. Genet.* 2017, *28*, 82–89.

[72] Just, R. S., Moreno, L. I., Smerick, J. B., Irwin, J. A., *Forensic Sci. Int. Genet.* 2017, *28*, 1–9.

[73] Montano, E. A., Bush, J. M., Garver, A. M., Larijani, M. M., Wiechman, S. M., Baker, C. H., Wilson, M. R., Guerrieri, R. A., Benzinger, E. A., Gehres, D. N., Dickens, M. L., *Forensic Sci. Int. Genet.* 2018, *32*, 26–32.

[74] García, O., Soto, A., Yurrebaso, I., *Forensic Sci. Int. Genet.* 2017, *28*, e8–e10.

[75] Wang, H., Zhu, J., Zhou, N., Jiang, Y., Wang, L., He, W., Peng, D., Su, Q., Mao, J., Chen, D., Liang, W., Zhang, L., *Forensic Sci. Int. Genet. Suppl. Ser.* 2015, *5*, e233–e234.

[76] Grandell, I., Samara, R., Tillmar, A. O., *Int. J. Legal Med.* 2016, *130*, 905–914.

[77] Hussing, C., Børsting, C., Mogensen, H. S., Morling, N., *Forensic Sci. Int. Genet. Suppl. Ser.* 2015, *5*, e449–e450.

[78] Elwick, K., Zeng, X., King, J., Budowle, B., Hughes-Stamm, S., *Int. J. Legal Med.* 2017, 1–13.

[79] Apaga, D. L. T., Dennis, S. E., Salvador, J. M., Calacal, G. C., De Ungria, M. C. A., *Sci. Rep.* 2017, *7*, 398.

[80] Hollard, C., Keyser, C., Delabarde, T., Gonzalez, A., Vilela Lamego, C., Zvénigorosky, V., Ludes, B., *Int. J. Legal Med.* 2017, *131*, 351–358.

[81] Ambers, A. D., Churchill, J. D., King, J. L., Stoljarova, M., Gill-King, H., Assidi, M., Abu0Elmagd, M., Buhmeide, A., Budowle, B., *BMC Genomics* 2016, *17*, 21.

[82] Eduardoff, M., Santos, C., de la Puente, M., Gross, T. E., Fondevila, M., Strobl, C., Sobrino, B., Ballard, D., Schneider, P. M., Carracedo, Á., Lareu, M. V., Parson, W., Philipos, C., *Forensic Sci. Int. Genet.* 2015, *17*, 110–121.

[83] Elena, S., Alessandro, A., Ignazio, C., Sharon, W., Luigi, R., Andrea, B., *Forensic Sci. Int. Genet.* 2016, *22*, 25–36.

[84] Guo, F., Zhou, Y., Song, H., Zhao, J., Shen, H., Zhao, B., Liu, F., Jiang, X., *Forensic Sci. Int. Genet.* 2016, *25*, 73–84.

[85] Cornelis, S., Gansemans, Y., Deleye, L., Deforce, D., Van Nieuwerburgh, F., *Sci. Rep.* 2017, *7*, 41759.

[86] Zhang, S., Bian, Y., Chen, A., Zheng, H., Gao, Y., Hou, Y., Li, C. *Forensic Sci. Int. Genet.* 2017, *27*, 50–57.

[87] de la Puente, M., Phillips, C., Santos, C., Fondevila, M., Carracedo, Á., Lareu, M. V., *Forensic Sci. Int. Genet.* 2017, *28*, 35–43.

[88] Meiklejohn, K. A., Robertson, J. M., *Forensic Sci. Int. Genet.* 2017, *31*, 48–56.

[89] Nakanishi, H., Pereira, V., Børsting, C., Yamamoto, T., Tvedebrink, T., Hara, M., Takada, A., Saito, K., Morling, N., *Forensic Sci. Int. Genet.* 2018, *33*, 106–109.

[90] Pereira, V., Mogensen, H. S., Børsting, C., Morling, N., *Forensic Sci. Int. Genet.* 2017, *28*, 138–145.

[91] Churchill, J. D., Schmedes, S. E., King, J. L., Budowle, B., *Forensic Sci. Int. Genet.* 2016, *20*, 20–29.

[92] Almalki, N., Chow, H. Y., Sharma, V., Hart, K., Siegel, D., Wurmbach, E., *Electrophoresis* 2017, *38*, 846–854.

[93] Xavier, C., Parson, W., *Forensic Sci. Int. Genet.* 2017, *28*, 188–194.

[94] Silvia, A. L., Shugarts, N., Smith, J., *Int. J. Legal Med.* 2017, *131*, 73–86.

[95] Jäger, A. C., Alvarez, M. L., Davis, C. P., Guzmán, E., Han, Y., Way, L., Walichiewicz, P., Silva, D., Pham, N., Caves, G., Bruand, J., Schlesinger, F., Pond, S. J. K., Varlaro, J., Stephens, K. M., Holt, C. L., *Forensic Sci. Int. Genet.* 2017, *28*, 52–70.

[96] Sharma, V., Chow, H. Y., Siegel, D., Wurmbach, E., *PLoS One* 2017, *12*, e0187932.

[97] Devesse, L., Ballard, D., Davenport, L., Riethorst, I., Mason-Buck, G., Syndercombe Court, D., *Forensic Sci. Int. Genet.* 2018, *34*, 57–61.

[98] Churchill, J. D., Peters, D., Capt, C., Strobl, C., Parson, W., Budowle, B., *Forensic Sci. Int. Genet.* 2017, *6*, e388-e389.

[99] Parson, W., Dür, A. *Forensic Sci. Int. Genet.* 2007, *1*, 88–92.

[100] Bandelt, H.-J., Salas, A. *Forensic Sci. Int. Genet.* 2012, *6*, 143–145.

[101] Bekaert, B., Massoli, C., Anandarajah, A., van de Voorde, W., Decorte, R., *Forensic Sci. Int. Genet. Suppl. Ser.* 2013, *4*, e111–e112.

[102] Mikkelsen, M., Frank-Hansen, R., Hansen, A. J., Morling, N., *Forensic Sci. Int. Genet.* 2014, *12*, 30–37.

[103] Kim, H., Erlich, H. A., Calloway, C., *Croat. Med. J.* 2015, *56*, 208–217.

[104] Gouveia, N., Brito, P., Bogas, V., Bento, A. M., Balsa, F., Serra, A., Lopes, V., Sampaio, L., São Bento, M., Cunha, P., Porto, M. J., *Forensic Sci. Int. Genet. Suppl. Ser.* 2017, *6*, e167–e168.

[105] Gallimore, J. M., McElhoe, J. A., Holland, M. M., *Forensic Sci. Int. Genet.* 2018, *32*, 7–17.

[106] Tak Wai, K., Barash, M., Gunn, P., *Electrophoresis* 2018, https://doi.org/10.1002/elps.201700371.

[107] Yao, L., Xu, Z., Zhao, H., Tu, Z., Liu, Z., Li, W., Hu, L., Wan, L., *Leg. Med.* 2018, *32*, 27–30.

[108] Churchill, J. D., Stoljarova, M., King, J. L., Budowle, B., *Int. J. Legal Med.* 2018, *132*, 1263–1272.

[109] Naue, J., Hoefsloot, H. C. J., Kloosterman, A. D., Verschure, P., *Forensic Sci. Int. Genet.* 2018, *33*, 17–23.

[110] Vidaki, A., Ballard, D., Aliferi, A., Miller, T. H., Barron, L. P., Syndercombe Court, D., *Forensic Sci. Int. Genet.* 2017, *28*, 225–236.

[111] Weber-Lehmann, J., Schilling, E., Gradl, G., Richter, D. C., Wiehler, J., Rolf, B., *Forensic Sci. Int. Genet.* 2014, *9*, 42–46.

[112] van Neste, C., Vandewoestyne, M., Van Criekinge, W., Deforce, D., Van Nieuwerburgh, F., *Forensic Sci. Int. Genet.* 2014, *9*, 1–8.

