



Electrochemical biosensors for analysis of DNA point mutations in cancer research

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Abstract

Cancer is a genetic disease induced by mutations in DNA, in particular point mutations in important driver genes that lead to protein malfunctioning and ultimately to tumorigenesis. Screening for the most common DNA point mutations, especially in such genes as *TP53*, *BRCA1* and *BRCA2*, *EGFR*, *KRAS*, or *BRAF*, is crucial to determine predisposition risk for cancer or to predict response to therapy. In this review, we briefly depict how these genes are involved in cancer, followed by a description of the most common techniques routinely applied for their analysis, including high-throughput next-generation sequencing technology and less expensive low-throughput options, such as real-time PCR, restriction fragment length polymorphism, or high resolution melting analysis. We then introduce benefits of electrochemical biosensors as interesting alternatives to the standard methods in terms of cost, speed, and simplicity. We describe most common strategies involved in electrochemical biosensing of point mutations, relying mostly on PCR or isothermal amplification techniques, and critically discuss major challenges and obstacles that, until now, prevented their more widespread application in clinical settings.

Keywords Cancer diagnostics · Biomarker · DNA point mutation · Single nucleotide variation · Electrochemical biosensor · Isothermal amplification

Introduction

DNA point mutation, sometimes referred to as single nucleotide variation (SNV), is a substitution of a single nucleotide for another, arising either from spontaneous DNA replication errors or from exogenous mutagen sources (radiation, chemicals, etc.). Depending on the position, point mutations can be “neutral” (if the mutation is in a non-coding DNA region), “silent” (mutation does not change the amino

acid sequence, usually located at the third position of the codon), “missense” (resulting in a codon coding for different amino acid), and “nonsense” (resulting in a premature stop codon and thus truncated protein). Even the single mutation event may cause the resulting protein to lose its function (loss-of-function) or to acquire a new function or ability (gain-of-function). Therefore, a number of point mutations are closely associated with the onset of various diseases, especially cancer, as well as with individual response to the therapy [1]. In the next section “DNA point mutations in cancer biology”, we will give an overview of the most frequent DNA point mutations and their association with the carcinogenesis process.

Although their effects can be deleterious, point mutations represent very subtle changes in the DNA structure, making their analysis quite challenging. Nowadays, the next-generation sequencing (NGS) is a widely used tool for complex genomic profiling of tumor samples, including high-throughput screening of a large panel of chosen point mutations as well as discovery of new SNVs in human genome [2]. However, NGS assays are still expensive and time-consuming, and their complexity and huge amount of provided data are

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not easily implemented into routine clinical practice. There are also numerous low-throughput methods targeting individual point mutations, such as amplification-refractory mutation system PCR (ARMS-PCR), denaturing gradient gel electrophoresis (DGGE), high-resolution melting (HRM) analysis, or restriction fragment length polymorphism (RFLP), which principles are explained in the section “[Standard methods of detection and commercial kits](#).” These techniques are faster and cheaper than NGS, but they still require relatively large instrumentation and skilled personnel to perform the analysis. Novel analytical tools that would meet demands of personalized medicine at the point-of-care are thus needed. In this context, electrochemical (EC) biosensors featuring inexpensive and simple miniaturized instrumentation, rapid measurement times, or low consumption can be attractive alternatives to current methods [3–6], as detailed in the last section “[Electrochemical biosensors: advantages and challenges](#).” In that section, we searched for recent publications on various platforms, such as Web of Science, PubMed, or Google Scholar, with multiple search terms, including “DNA point mutation,” “single nucleotide variation,” “biosensor,” “electrochem*,” and their combinations. The list, however, is not meant to be exhaustive, and certain papers could have been unintentionally omitted. Despite this, we believe that our selection is a reliable representation of current trends in the field of EC biosensors for DNA point mutation detection.

DNA point mutations in cancer biology

DNA point mutations are commonly divided into hereditary (germline) or somatic, based on a type of cell where the mutation occurs. If the mutation occurs in germinal haploid cells (gametes), it is called *hereditary* and can be passed to an offspring who then carries the point mutation in all cells. *Somatic* mutations, on the other hand, are found in diploid non-germline body cells (i.e., somatic cells) and are not passed to an offspring.

Hereditary mutations

Germline mutation in a specific gene may result in a hereditary cancer syndrome that poses an elevated risk of cancer that runs in the family. Only 5–10% of all cancers are hereditary; the rest are due to mutations in genes of somatic cells [7]. There are hundreds of susceptibility genes described so far, but mutations in most of them are very rare [8]. Perhaps the most frequently mutated gene in germinal cells that is associated with cancer (and perhaps most frequently targeted in biosensors) is *TP53* gene. Other frequently mutated genes include *BRCA1*, *BRCA2*, *PTEN*, *APC*, *CDKN2A*, and *KIT*.

The *TP53* gene encodes a phosphoprotein nicknamed as the “guardian of the genome” [9]. The p53 protein acts in cells as a transcription factor by binding to a p53 response element in promoter regions to regulate expression of its target genes. By this way, p53 protein influences expression of approximately 400 different genes [10] that participate mainly in cell cycle arrest, DNA repair, apoptosis, and inhibition of angiogenesis [11]. Germline mutations in *TP53* gene create molecular background of a cancer predisposition disorder called Li-Fraumeni syndrome [12] that negatively influences risk of developing certain tumors, such as breast carcinomas, soft tissues sarcomas, brain tumors, adrenal gland tumors, and bones sarcomas [13–15]. A lifetime cancer risk of individuals with germline mutations in *TP53* gene increases for breast cancer from 12 to 28%, soft tissues sarcoma from less than 1 to 14%, brain tumors from less than 1 to 13%, adrenal gland cancer from 3 to 11%, and bones sarcomas from less than 1 to 8% compared to a lifetime cancer risk of general population [16]. Based on data from the COSMIC v96 catalogue [17, 18], the mutations in *TP53* gene were detected in approx. 33% of almost thirty thousand cancer samples that were tested across many independent studies. The most frequent type of mutation is a missense substitution that leads to the change of corresponding amino acid [9]. The six most frequent and well-characterized mutations occur in amino acids R175, Y220, G245, R248, R273, and R282 [19].

BRCA1 and *BRCA2* genes are tumor suppressor genes that code multi-domain proteins of same names. Both participate in maintenance of DNA integrity [20]. Pathogenic germline mutations in *BRCA1* and *BRCA2* genes contribute to the development of familial/hereditary breast and ovarian cancer [21–23]. A lifetime cancer risk of individuals with germline mutations in *BRCA1* (as compared to general population) increases for breast cancer from 12% to 70–80%, while in case of *BRCA2* mutations up to 50–60%. Regarding ovarian cancer, this risk increases from 1.37 to 50% in case of *BRCA1* mutations and to 30% in case of *BRCA2* mutations [20]. Based on data from the COSMIC v96 catalogue [17, 18], mutations in *BRCA1* gene were detected in almost 7% of samples, predominantly comprising point mutations (approx. 75%), and *BRCA2* mutations in 1.75% of tested samples, again comprising mostly point mutations (approx. 80%).

Somatic mutations

The most common cause of a cancer is the presence of somatic mutation, giving rise to so-called sporadic cancer. Mutations appear quite frequently, and the most common well-known factors include smoking, exposition to ultraviolet radiation, oncoviral infections, and age. More than one hundred genes have been identified that may promote or control carcinogenicity when altered by intragenic mutations. Most tumors contain from two to eight of these “driver”

mutations. The first mutation that gives a normal epithelial cell a growth advantage is called “gatekeeping.” For instance, in colon tumors, this is usually a mutation in the *APC* gene [24], followed by mutations that allow cell proliferation, such as in the *KRAS* gene [25], and by a clonal expansion of cells and other mutations in genes such as *TP53*, *PIK3CA*, or *SMAD4*, which lead to malignant transformation of the tumor and its metastasis [26].

In addition to “driver” mutations, there are also passenger mutations which usually do not give cells any selective advantage, and their number often correlates with age [27]. It was reported that most somatic mutations are single base substitutions (95%), of which 90.7% lead to missense changes, 7.6% to nonsense changes, and 1.7% affect splice sites or untranslated regions around the start or stop codon [28]. In general, the genes most frequently involved in the development of cancer fall into

three broad areas: tumor suppressor genes (e.g., *TP53*, *BRCA2*, *APC*, *PTEN*), oncogenes (e.g., *Ras* family, *EGFR* family, *myc* family, *PIK3CA*) and DNA repair genes (e.g., *BRCA1*, *BRCA2*, *MMR* genes). According to Vogelstein et al., all known driver genes can be classified into signaling pathways that are involved in three main biological processes: cell survival, cell fate, and genome maintenance; their examples are shown in Fig. 1 [28].

APC is a tumor suppressor gene that can be deregulated in both germinal and somatic cells. The *APC* gene encodes a 310 kDa protein APC (adenomatous polyposis coli), a negative regulator of Wnt/ β -catenin signaling, that controls β -catenin concentrations and interacts with E-cadherin [29]. *APC* germline mutations are associated with familial adenomatous polyposis (FAP), which often leads to the development of colorectal cancer [30], and somatic mutations of *APC* are present in up to 80% of sporadic colorectal cancers

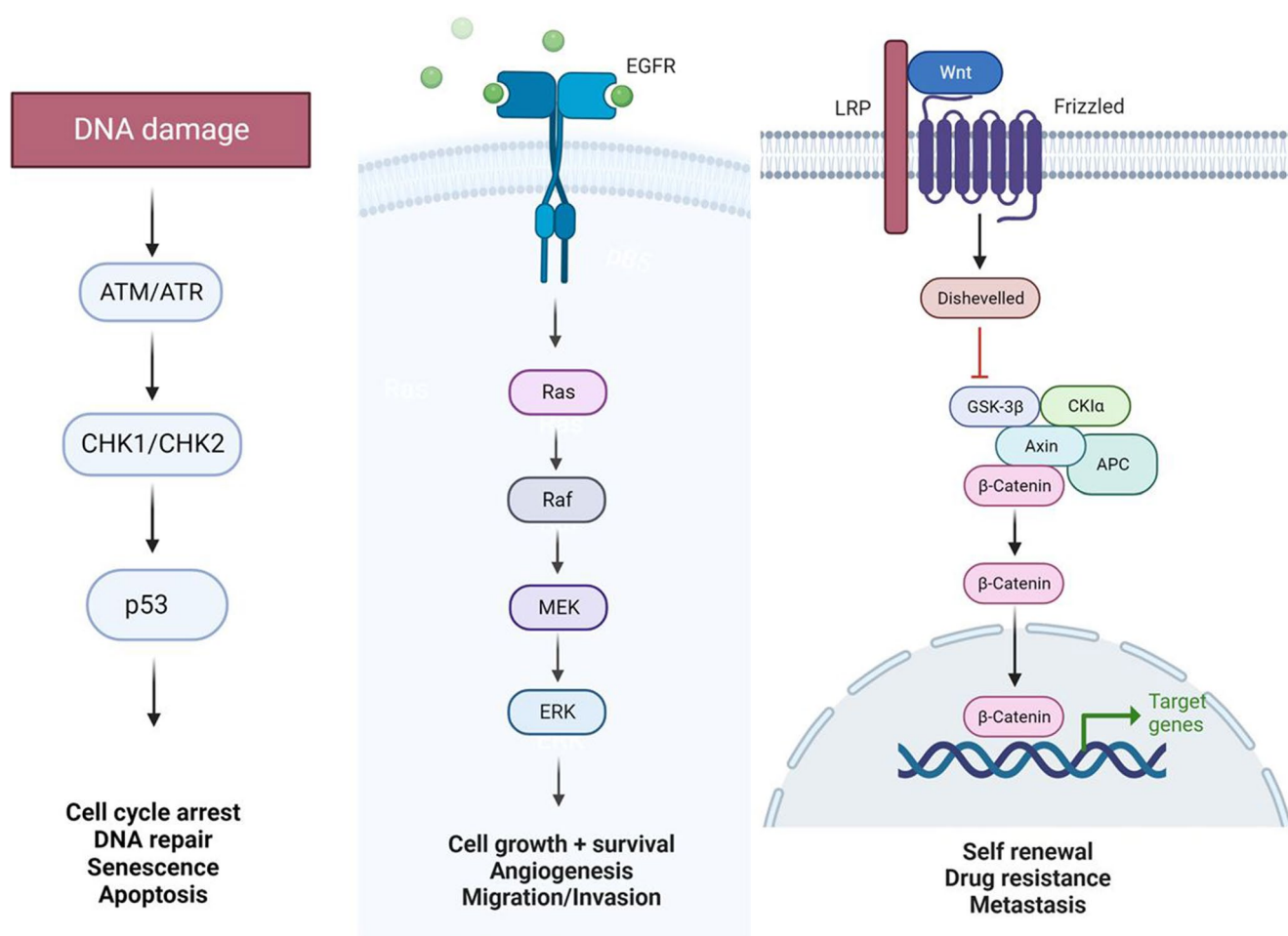


Fig. 1 Simplified signaling pathways where point mutations in associated driver genes are often connected to carcinogenesis. (Left) Point mutations in *TP53* lead to malfunctioning p53 protein, disrupting G2/M, and G1 cell cycle checkpoints or DNA repair processes. (Middle) Point mutations in *KRAS* or *BRAF* genes may lead to constant activation of Ras or Raf onco-

proteins, promoting cell growth or migration. (Right) Point mutations in *APC* gene block its tumor suppressor role in β -catenin destruction, allowing cell proliferation or metastasis. Created with [BioRender.com](https://www.biorender.com)

[31]. *APC* mutations, most of which are concentrated in exon 15, generally do not cause loss of the entire protein, but up to 90% of mutations generate premature stop codons and result in stable truncated gene products that can lose original functions and gain new ones [32].

KRAS gene is one of the most studied oncogenes. It encodes the *KRAS* protein that belongs to the Ras family of small GTPases and is one of the most frequently mutated oncogenes in human tumors (20–30%), occurring in up to 90% of pancreatic cancers [33] and 45% of colorectal cancers [31]. Oncogenic mutations lead to constant activation of the *KRAS* protein and thus affect cell proliferation and viability, autophagy, or tumor stroma (by activating fibroblasts and recruiting various inflammatory cells) and also initiate epithelial-mesenchymal transition, thus supporting migration and invasion of tumor cells [33]. Most activating mutations are found in codons 12 and 13 of exon 2 (80% are G12D, G12V, G12A, G12C, and G13D in amino acid notation or G35A, G35T, G35C, G34T, and G38A in nucleotide notation, respectively) [34]. Clinical data indicate that mutations in *KRAS* codon 12 or 13 are the main predictive biomarkers of resistance to anti-EGFR therapy in patients with metastatic colorectal cancer [35].

EGFR gene encodes a 170 kDa transmembrane glycoprotein that belongs to the ErbB family of transmembrane receptor tyrosine kinases. Ligand binding to the cell surface induces EGFR dimerization, which results in EGFR tyrosine kinase activation and trans/autophosphorylation of the receptor. Activated EGFR receptors (homodimers and heterodimers) form signaling complexes with many proteins and initiate the activation of various signaling pathways, including Ras/ERK, PI3K/Akt, PLC- γ 1, Src, and STAT [36]. Deregulation of the EGFR signaling cascade, either due to overexpression or constitutively activating mutations, occurs in many types of cancer (breast, lung, colorectal and esophageal cancer, head and neck cancer, glioma and glioblastoma) [37]. Alterations of the *EGFR* gene are very diverse and include amplifications, deletions, insertions, single nucleotide polymorphisms, methylations or copy number variations [38]. For example, EGFR plays an important role in the pathogenesis of lung cancer, and EGFR kinase domain mutations occur in 10–40% of samples. The most common *EGFR* activating mutations in non-small cell lung cancer include deletions in exon 19 (amino acids 747–750, comprising 45% of all EGFR mutations), exon 21 L858R point mutations (40%), and in-frame insertions in exon 20 (5–10%).

BRAF gene encodes a serine/tyrosine kinase belonging to the Raf protein family, where B-Raf (v-RAF murine sarcoma viral oncogene homolog B; B-type RAF kinase) is the most efficient activator of MEK and is also the most frequently mutated [39]. More than 90% of activating BRAF mutations constitute only a single V600E substitution mutation where

thymine is substituted by adenine at the position 1799. This alteration has been identified in up to 7% of human cancers, mainly in melanoma (50%), metastatic colorectal cancer (10%), thyroid papillary cancer (50%), and non-small cell lung cancer (3%) [40]. The V600E substitution leads to a constitutive activation of MAPK signaling pathway. *BRAF* mutations are associated with a poor prognosis in metastatic colorectal tumors, and mortality is almost threefold compared to patients without *BRAF* mutation [41]. Since both BRAF and KRAS act in the EGFR signaling pathway, dual mutations of both proteins are rarely seen in tumors [42].

Standard methods of detection and commercial kits

Due to an importance of SNVs both in physiological and pathological processes, many techniques were developed for their analysis. Below, we describe their basic principles and list main advantages and disadvantages.

Next-generation sequencing

As we mentioned in the “[Introduction](#)”, NGS as a method of choice is a highly precise, high-throughput screening tool for parallel analysis of hundreds of SNVs. Advantages include cheap design, high sensitivity, large amount of generated data, and possibility to discover novel variants. Its disadvantage over Sanger’s sequencing is a time-consuming and less effective analysis of low number of targets and requirement for large data storage. Commonly used NGS platform for genomic profiling that includes also point mutation analysis is *Illumina sequencing*, using sequence coverage of 30–40 $\times$ [43]. This platform uses sequencing-by-synthesis approach and bridge amplification. The DNA fragments are modified with adapters at their terminal ends. During the bridge PCR, DNA strand hybridizes to the adapters and forms a bridge at the bottom of the reaction chamber. Then, a complementary strand is synthesized. This process is repeated until several thousand copies of the DNA fragments are made [44]. The SNV analysis is performed after Illumina sequencing using different software programs, for example, MiSeq Reporter Software (Illumina) [45] or SeqNext Software (JSI Medical Systems). The disadvantage of this technology is the inability to distinguish repeating regions in the genome, such as trinucleotide repeats, which are found in many disorders. Other NGS platforms include Ion Torrent (Thermo Fisher), which generates thousands of reads detecting a release of hydrogen ion during sequencing, and 4th generation sequencing platforms, such as PacBio Sequencing and/or Oxford Nanopore Sequencing, which both provide high amount of long reads.

Protein-assisted methods

PCR products can be characterized by different banding patterns in a technique called *restriction fragment length polymorphism* (RFLP). This method involves specific enzymatic restriction and staining of the amplicons with dye. Then, the digested amplicons are separated by size with gel electrophoresis. RFLP is relatively simple but there is a possibility of some sequences to go undetected. The reason for this is a limited number of restriction sites that are available. There is also a limitation when several SNPs are targeted at the same time, which can be solved by using two different restriction enzymes in one reaction [46, 47]. When combined with PCR, it can be a valuable technique for genotyping analyses, species identification, and determination of intraspecies variation [47, 48].

MutS proteins are increasingly used for the detection of SNVs because of their ability to discriminate mismatch base pairs in DNA repair processes. These proteins recognize the damaged DNA and initiate repair process. They can be found both in prokaryotes and eukaryotes and recognize the base–base mismatch or small loops in base pairs. For example, MutS α was shown to recognize single-base mismatches and is mainly responsible for their reparation. Specific domains of this protein encircle the mismatched DNA while inducing conformational changes [49]. The binding occurs due to a higher affinity for mismatched base pairs. This binding can be detected by using fluorescent MutS (labeled with fluorescent dye) or biotinylated tag [50].

Methods based on melting differences

DNA separation can be achieved by many techniques. The golden standard for many laboratories is *denaturing gradient gel electrophoresis* (DGGE), separating DNA fragments of the same size and different melting points. The principle lies in the detection of mutations based on the separation of homoduplexes (fully complementary strands) and heteroduplexes (harboring a mismatch) in gradient gel. Heteroduplexes due to their incomplete pairing and sequence mismatches have reduced stability when exposed to denaturation. Denaturation reduces mobility, resulting in an effective separation of homo/heteroduplexes, which can be seen as different peaks in electrophoretograms [51]. The downside of this technology is poorer mutation detection in GC rich segments; therefore, usefulness depends on the DNA fragment sequence [52]. Connecting DGGE with capillaries or microchips would increase the sensitivity and specificity of the analysis, making it a low-cost and simple-executed detection method.

HRM (*high-resolution melting*) is a powerful technique suitable to differentiate SNVs by making use of the annealing and melting properties of DNA. DNA sequences are

analyzed by monitoring the change in fluorescence of the DNA binding dye. When increasing the temperature to the melting point, dsDNA separates into single strands, i.e., is melted. HRM can detect small sequence variations, such as single base pair differences, due to the change in melting profiles of the analyzed sequences [53]. When combined with PCR methods, the dye is added prior or post to the amplification reaction, and the products are then exposed to rapid melting analysis [50, 54].

The advantages of HRM in contrast to other melting techniques are a fast analysis of large amounts of PCR products, as well as simplicity. This undoubtedly proved its usefulness in clinical laboratory applications [55]. On the other hand, this method appears to have a low ability to discriminate between sequences that show only a subtle variation in their melting profiles. This could be a challenge in neutral base-pair differences (genomic alterations that do not change the final amount of genetic material, i.e., when the GC content remains the same) because of the slight changes in T_m (≤ 0.4 °C) [50].

Methods based on PCR

PCR is one of the most widely used techniques in molecular biology and as such has many variations suited for certain needs, including SNV detection. In that case, however, SNV detection requires modified protocols and the use of specific primers or probes that are fully complementary to the target DNA (either wild-type or mutated).

Real-time PCR provides accurate detection of PCR amplification and is commonly used in most laboratories. Real-time PCR monitors the point in the reaction cycle where the target DNA amplification is detected. This happens by using different fluorescent reporter molecules, such as fluorescent dyes (e.g., SYBR Green), a fluorescein-labeled oligonucleotides, or a fluorescent TaqMan probes. If the probe, for example, is bound to the desired DNA sequence and then degraded, fluorescence signal is emitted [56, 57]. Compared to the end-point PCR, quantification steps in real-time PCR are simplified into a continuous measurement of the fluorescence intensity of the PCR product after each cycle [57].

ARMS-PCR (*amplification refractory multiplication system PCR*) is an alteration of PCR with high accuracy, widely used in genetic diagnostic analyses. It has higher efficiency due to precisely designed allele-specific primers, which are complementary to a target DNA but with added mismatch base at the 3' end. This modification results in a match of one primer to a wild-type allele and the other to the mutant allele, hence allowing a preferential amplification and detection of SNV sequences [50, 58]. The main benefit of ARMS-PCR is a fast detection of mutations or SNVs. Furthermore, it can be used for analyzing mutations where the restriction enzyme is

not available and thus cannot be detected by RFLP. The shortcoming of this method is the efficiency of amplification of desired sequences because of various reaction conditions that may differ for each fragment. The risk of primer-dimer formation is also very high. All this combined, with the limitation in multiplexing capability, the cost of instruments (e.g., thermal cyclers), and the complicated design of suitable primers, makes its use in point-of-care applications rather limited. Nowadays, other techniques offer a solution for some of ARMS-PCR disadvantages [58, 59], one of them being isothermal amplification techniques (IATs) that are described in greater detail in a subsection “IAT-based EC biosensors”.

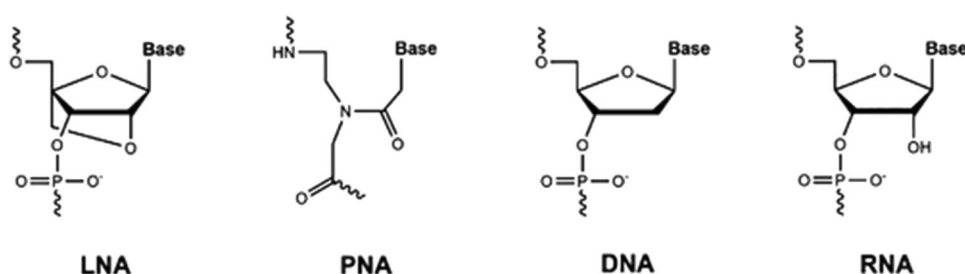
Digital PCR (dPCR) enables direct clonal amplification and subsequent quantification of nucleic acids and can be used, e.g., for SNVs detection, either as a chip-based (cdPCR) or droplet-based systems (ddPCR). cdPCR is based on microfluidics, and this proprietary technology enables consistent and automated compartmentalization of a sample into microchambers, where an absolute quantification takes place. On the other hand, ddPCR is based on water and oil emulsion droplet technology. The amplification takes place within each droplet, discriminating droplets containing at least one target copy (positive) that show increased fluorescence in contrast to the negative ones. The probes are labeled with fluorescent dye, one binding to the wild-type allele and the other to the mutant allele. If the DNA target sequence is present in the droplet, amplification occurs, and the reporter emits a fluorescent signal. As a result, absolute quantification of the target DNA is obtained. By improved separation of droplet populations due to the changes in amplicon length, Miotke et al. were able to detect SNVs in *BRAF* gene from cell lines and patients with colorectal cancer. Authors used single-color ddPCR that involves single fluorescent dye, giving rise to positive and negative populations based on the mutation status [60]. The limitations in ddPCR are the cost as well as limited range for detection. Higher concentrations lead to loss of linearity [61]. Despite this, the advantage of absolute quantification makes this method useful in detecting mutations of numerous genes.

LNA/PNA probes

To improve mismatch recognition, synthetic DNA analogs have been developed that possess altered backbone structure but same base pairing abilities. Most common are *locked nucleic acids* (LNA) and *peptide nucleic acids* (PNA), both having remarkably high binding affinities towards complementary DNA sequences and thus improved mismatch recognition abilities, making them ideal candidates when analyzing DNA point mutations, especially in techniques relying on melting temperature differences (such as HRM), as well as in PCR (Fig. 2).

LNA is a modified RNA nucleotide where 2'-O and 4'-C atoms of the ribose are connected by a methylene bridge. This is the reason for less flexible ring which results in enhanced hybridization stability. If some of the nucleotides in DNA oligomers are replaced by these locked forms, T_m may increase by 3–5 °C [50]. The short probes can distinguish mismatch target sequences because of the difference in match and mismatch complementary sequences when it comes to the thermal stability. This is crucial for SNV detection, where these highly specific probes are mostly used. However, these probes are quite expensive and often form secondary structures, making the design of LNA probes a challenging task [50]. PNA consists of a polyamide (poly-N-(2-aminoethyl)glycine) backbone, resembling negatively charged phosphate backbone of regular nucleic acids, with an exception of being uncharged. The neutral PNA increases thermal stability in PNA-DNA base pairs. They are more single-base specific, making them ideal for SNVs targeting. T_m can be lowered by 8–20 °C if a single-base mismatch is present near the PNA-DNA or PNA-RNA duplex [50, 62]. For example, Sun et al. showed that 16-nt long wild-type PNA probe had T_m around 70 °C in a complex with fully complementary wild-type RNA, while in a complex with mismatched RNA, the T_m was around 10–20 °C lower [63]. PNAs are often used in a process called PNA clamping where amplification of wild-type DNA sequences is greatly reduced during PCR. This leads to a lower noise and better sensitivity when detecting mutated DNA sequence. A drawback of PNA clamping is a possible amplification of PNA probes themselves, which can be solved by using high-fidelity DNA polymerases [50].

Fig. 2 Molecular structures of LNA and PNA. Structures of DNA and RNA are shown for comparison. Reprinted with permission from [50]. Copyright (2019) American Chemical Society



Commercial kits

There are many commercial kits available, mostly based on NGS or PCR using different strategies with different performance. Some studies provided kit comparisons, for instance, Morrison et al. who compared single-gene tests [64] or Zhao et al. who compared diagnostic tests for *BRAF* mutation [65]. They allow for a simple and effective way to analyze SNVs and to detect many mutations in parallel. Examples of such kits are given in Table 1.

Electrochemical biosensors: advantages and challenges

Despite a huge progress in above-mentioned methods in recent years, a gap remains in achieving rapid, inexpensive, and easy-to-use diagnostic tools fulfilling a needs of personalized medicine at the point-of-care. As we mentioned in the Introduction, EC methods are capable of achieving these goals, and many authors have already developed interesting EC-based schemes targeting most frequent DNA point mutations (listed in Table 2). These schemes usually involve either PCR or isothermal amplification techniques (IATs), which rapidly amplify DNA at constant temperature without a need for thermal cycler [103, 104]. Moreover, some assays introduced enzyme-free strategies whereby high sensitivity was achieved in a different way than by amplifying DNA using polymerases.

PCR-based EC biosensors

Usually, PCR is a first choice when analyzing point mutations in DNA. EC assays often involve PCR amplification of the DNA fragment where the mutation site is present, followed by a specific capture of the amplicons by a mutation-specific probe. This was shown, for instance, for point mutations in *TP53* [75], *KRAS* [74], *EGFR* [69, 70, 105], or *BRCA1* genes [68]. For example, Xu et al. reported EC biosensor that analyzes in-frame deletions in exon 19 of an *EGFR* gene [69]. Authors employed λ -exonuclease to digest PCR products in order to generate single-stranded targets for improved hybridization efficiency. They showed feasibility of the assay by testing four patient samples with non-small cell lung carcinoma, and the results were in concordance with direct sequencing method. For its potential application in clinical settings, larger cohort of patients along with healthy individuals would need to be tested.

Pingarron's group developed an assay using disposable screen-printed carbon electrodes to distinguish R175H *TP53* mutation from a *wild type* (wt) [79]. The electrodes were functionalized with reduced graphene oxide-carboxymethylcellulose hybrid nanomaterial and hairpin capture probes complementary to the wt sequence; hence, mutated sequence did not hybridize to the probe, yielding signals similar to blank. Authors analyzed endogenous *TP53* status in MCF-10A, MCF-7, and SK-BR-3 breast cancer cell lines by targeting cDNA obtained from total RNA after cDNA synthesis, albeit without further PCR amplification of the cDNA. This interesting approach gives a rapid YES/NO information whether the gene has a wt sequence or not, and it would be interesting to see whether other mutations are distinguishable as well.

Table 1 List of some commercially available kits and assays for SNV analysis

Commercial kit	Mutations detected	Instrumentation
THxIDTM- BRAF1 (bioMérieux, Inc.)	V600E, V600K	Real-time PCR, ABI7500 FastDx
therascreen BRAF Pyro Kit5 (QIAGEN)	Codon 600: V600A, V600E, V600G, V600M, Codons 464–469: G464E, G464V, G466E, G466V, G469A, G469E, G469V	Pyrosequencing, Pyromark Q24 System
INFINITI BRAF Assay (AutoGenomics, Inc.)	BRAF V600A, V600D, V600E, V600KRM	Multiplex PCR
ddPCR™ <i>KRAS G12/G13</i> Screening Kit (BioRad)	G12A, G12C, G12D, G12R, G12S, G12V, G13D	ddPCR
ddPCR <i>BRAF V600</i> Screening Kit (BioRad)	V600E, V600K, V600R	ddPCR
cobas® <i>EGFR</i> Mutation Test v2 (Roche)	42 mutations in exons 18, 19, 20 and 21 of the <i>EGFR</i> gene including the T790M mutation	PCR
BRAF 600/601 StripAssay® (BioVendor LM)	V600A, V600D, V600E, V600E, V600G, V600K, V600M, V600R, V601E	PCR, reverse-hybridization
Oncomine BRCA Research Assay (Thermo Fisher Scientific)	SNVs, InDels, large exon/gene deletions/ duplications	Ion Torrent next generation sequencing system
TruSight Oncology 500 and TruSight Oncology 500 High-Throughput (Illumina)	BRAF, BRCA1, BRCA2, PTEN, EGFR, PIK3CA, TP53 and many others	NextSeq 500, NextSeq 550, or NextSeq 550Dx (research mode) Systems

Table 2 A list of EC assays coupled to various amplification reactions for determining DNA point mutations associated with cancer

Gene	Mutation	Amplification reaction/strategy	Limit of detection	Sample	Reference
<i>PCR-based approaches</i>					
<i>BRAF</i>	V600E	ARMS-PCR	0.8% of mutated sequence in excess of a wild type	HT29 and SW480 colorectal cell lines	[66]
<i>BRAF</i>	V600E	ARMS-qPCR	2 copies of mutated ctDNA	HT29 and SW480 colorectal cell lines	[67]
<i>BRCA1</i>	3746insA 1942delA 3459G>T	PCR	N/A	Whole blood of control and breast cancer patients	[68]
<i>EGFR</i>	exon 19 del	PCR	N/A	Lung cancer patients	[69]
<i>EGFR</i>	L858R exon 19 del (delE746-A750)	ARMS-PCR	30 pg of DNA	Plasma ctDNA of NSCLC ^a patients	[70]
<i>EGFR</i>	L858R	PCR with CRISPR-based detection	0.1 pg/ μ L	A549 and H1975 cancer cell lines	[71]
<i>EGFR</i>	T790M	Asymmetric PCR coupled with CRISPR/Cas12a detection system	100 aM	Synthetic oligos spiked in human serum	[72]
<i>Hras</i>	G12V	PCR	100 fmol	T24 urinary bladder carcinoma cell line and ASTC-a-1 lung cancer cell line, and blood from healthy donor	[73]
<i>KRAS</i>	G12D G13D	PCR	0.58 ng/ μ L	SK-UT-1 uterine cancer cell line	[74]
<i>TP53</i>	P72R R175H R273H	PCR	0.1 fmol	Peripheral blood	[75]
<i>TP53</i>	R273H R273C G245S	PCR	N/A	Glioblastoma cell lines U251 and Onda 10	[76]
<i>TP53</i>	R175H	PCR and copper-free click chemistry mediated cyclic ligation	7.7 fM	Synthetic oligos spiked in human serum	[77]
<i>TP53</i>	wild type	PCR	5.9 pM	Peripheral blood	[78]
<i>TP53</i>	R175H	Detection of cDNA from total RNA	3.4 nM for scsp53 and 2.9 nM for lcp53	MCF-10A control cell line and MCF-7, SK-BR-3 breast cancer cell lines	[79]
<i>IAT-based approaches</i>					
<i>BRAF</i>	V600E	LAMP	20 copies / μ L	Spiked human serum	[80]
<i>EGFR</i>	exon 19 deletion	ESDR	0.13 pM	Spiked human serum	[81]
<i>EGFR</i>	L858R	RPA	3.3 aM	Blood serum of 25 NSCLC ^a patients	[82]
<i>KRAS</i>	A11P A11R	Exonuclease III-assisted target recycling and RCA	0.28 fM	Synthetic oligos spiked in human serum	[83]
<i>KRAS</i>	G12D G12A G12C G12S	RCA	3 aM	Synthetic oligos spiked in human serum	[84]
<i>PIK3CA</i>	H1047R	SDA	0.001% of mutated sequence in a wild type	Synthetic oligos spiked in human serum	[85]
<i>TP53</i>	R273H	RCA	2 amol	Synthetic oligos	[86]

Table 2 (continued)

Gene	Mutation	Amplification reaction/strategy	Limit of detection	Sample	Reference
<i>TP53</i>	Not specified	Cascade signal of nick-endonuclease assisted target recycling and hyper-branched-RCA	0.02 fM	Synthetic oligos spiked in human serum	[87]
<i>TP53</i>	Not specified	Cascade signal of nick-endonuclease assisted target recycling and RCA	0.23 fM	Synthetic oligos spiked in human serum	[88]
<i>TP53</i>	Not specified	Cascade signal of nick-endonuclease assisted target recycling and RCA	0.25 fM	Synthetic oligos spiked in human serum	[89]
<i>TP53</i>	R175H	Toehold-mediated strand displacement reaction	0.42 fM	Synthetic oligos spiked in human serum	[90]
<i>Non-enzymatic approaches</i>					
<i>BRCA1</i>	5382insC	Thiolated DNA capture probes on gold electrodes	4.6×10^{-20} M	Peripheral blood	[91]
<i>BRCA1</i>	185delAG	Graphene-doped Mn_2O_3 nanofibers platform	0.8 pM	Synthetic oligos	[92]
<i>BRCA1</i>	N/A	Self-assembled ferrocene-cored poly(amidoamine) dendrimers	0.38 nM	Synthetic oligos	[93]
<i>EGFR</i>	Point mutations (exon 18) deletions (exon 19) insertions (exon 20)	Combinatorial PNA probes and PNA clamps on nanostructured microelectrodes	N/A	Circulating nucleic acids from serum of NSCLC ^a patients	[94]
<i>EGFR</i>	T790M	Ligation chain reaction	0.75 aM	Synthetic oligos spiked in human serum	[95]
<i>KRAS</i>	G12D G12V G12A G12S G12C G12R G13D	PNA probes and PNA clamps on nanostructured microelectrodes	1 fg/ μL	Circulating nucleic acids from serum of lung cancer and melanoma patients	[96]
<i>KRAS</i>	G12D	Anchor-like biosensor with two probes	100 pM	Synthetic oligos spiked in human serum	[97]
<i>KRAS</i>	G12D	Mg^{2+} -dependent DNase coupled to hybridization chain reaction	0.5 fM	Synthetic oligos spiked in human serum	[98]
<i>PIK3CA</i>	E545K	Nest hybridization	3 pM	Blood from breast cancer patients and healthy control	[99]
<i>TP53</i>	N/A	3D-nanosheets with two hairpin probes	3 fM	Synthetic oligos spiked in a FBS and cellular homogenate solution	[100]
<i>TP53</i>	N/A	Enzyme-free Cd^{2+} -dependent cascade amplification	6.3 fM	Synthetic oligos spiked in human serum	[101]
<i>TP53</i>	N/A	PNA-DNA hybrids on gold electrode	6.82×10^{-10} M	Synthetic oligos	[102]

^aNSCLC, non-small cell lung cancer

Not all assays utilized mutation-specific probes. For instance, an interesting approach was developed by Horaikova et al. for analysis of *TP53* mutations [76]. Instead of DNA, they targeted *TP53* mRNAs in cell lines harboring R273H, R273C or G245S hot spot mutations. They reversely transcribed RNA into cDNA and amplified it with PCR; PCR amplicons then served as templates for primer extension reaction, whereby the polymerase incorporated biotinylated dCTPs only when the primer hybridized with a wt sequence (carrying G in a mutation site). If a mismatch was present, tagged nucleotides were not attached, yielding low voltammetric signals (Fig. 3). It would be interesting to see, however, how this approach works in clinical settings. Later, Situ et al. used ARMS-PCR, where one of the primers was complementary to mutated sequence, leading to preferential amplification of mutant alleles which carried biotin tags, detectable via streptavidin/alkaline phosphatase reporter system [66]. The assay was applied to colorectal cancer cells to analyze V600E *BRAF* mutation, detecting as low as 0.8% of mutant alleles in excess of the wt background.

IAT-based EC biosensors

IATs are highly sensitive and rapid enzymatic techniques that amplify both DNA and RNA at constant temperatures (usually from room temperature up to 65 °C). In contrast to PCR, they may be performed in a thermoblock (or even at the benchtop) and often tolerate various PCR inhibitors. On the other hand, similarly to PCR a nonspecific amplification from contaminated sample remains an issue, and great precautions must be taken when handling DNA or RNA samples. Most common IATs include rolling circle amplification (RCA), loop-mediated isothermal amplification (LAMP), recombinase polymerase amplification (RPA), or strand displacement amplification (SDA) [103, 104].

RCA is a common IAT choice when dealing with point mutations. During RCA, target DNA or RNA hybridizes to both ends of a specially designed padlock probe carrying a phosphate modification at its 5'-end. Upon hybridization, adjacent ends of the padlock probe are covalently joined in a process of ligation, yielding circular DNA that serves as a template for subsequent RCA. The first generation of RCA was a linear type utilizing special DNA polymerase (Phi29, Bst, or Vent exo-DNA) or RNA polymerase (T7 RNA polymerase) and a single primer [106, 107]. Later on, hyperbranched RCA was developed by introducing second primer to produce hyperbranched amplicons [106, 108]. Further modifications include nicked RCA that produces ultra-high forest like amplicons [108] or circle-to-circle RCA, all of which led to great improvement in sensitivity [109]. Since RCA is especially useful for short targets, it is commonly applied for ultrasensitive detection of micro-RNAs [110–112]. For point mutations, it is advisable to

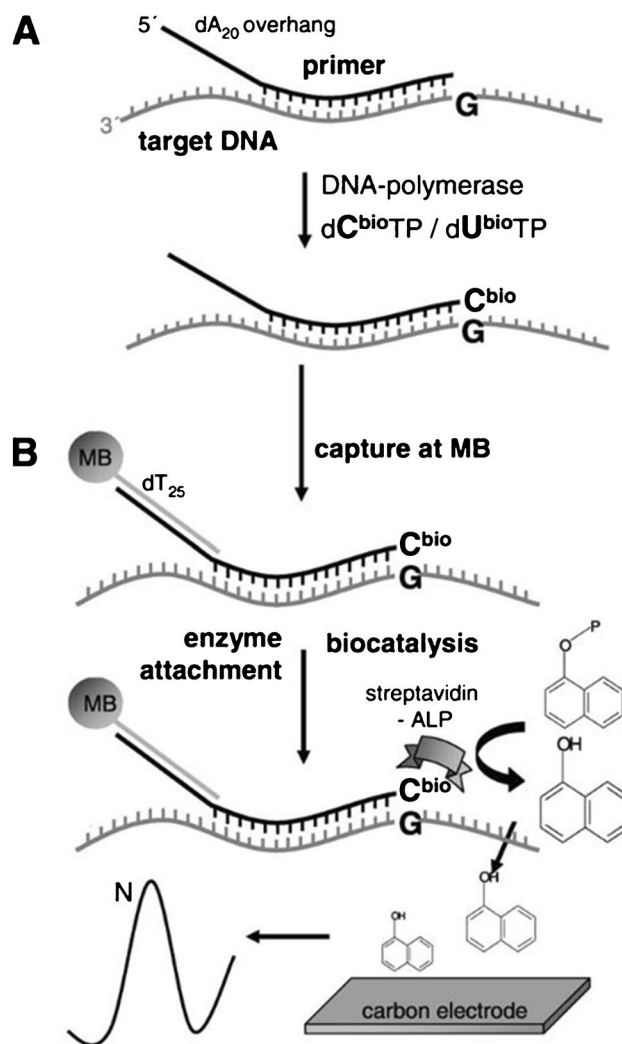


Fig. 3 **A** Incorporation of biotinylated nucleotides (dC^{bio}TP/dU^{bio}TP) into DNA using primer extension based on the first free nucleotide in the target template following the primer sequence. **B** Enzyme-linked electrochemical assay with magnetic beads (MB) coupled to a conjugate of streptavidin with alkaline phosphatase (ALP). The substrate, 1-naphthyl phosphate, was enzymatically converted into an electroactive indicator 1-naphthol that was detected by voltammetry at a carbon electrode. Reprinted from [76] with permission from Wiley-VCH

design the padlock probe in such a way that its 3'-terminal base faces the mismatched base in the target, preventing ligation to occur [86, 113]. Some RCA-based EC assays targeting point mutations are listed in Table 2. For instance, Wang et al. [89] developed a dual amplified electrochemical assay to detect mutant *TP53* gene. Authors employed a signal enhancement strategy using the nicking endonuclease-assisted target recycling and RCA to produce G-quadruplex/hemin complexes measurable at gold electrode. The assay was able to detect spiked mutant *TP53* gene sequence in 10x diluted human serum, which makes it potentially useful for clinical applications, but it first needs to be tested on

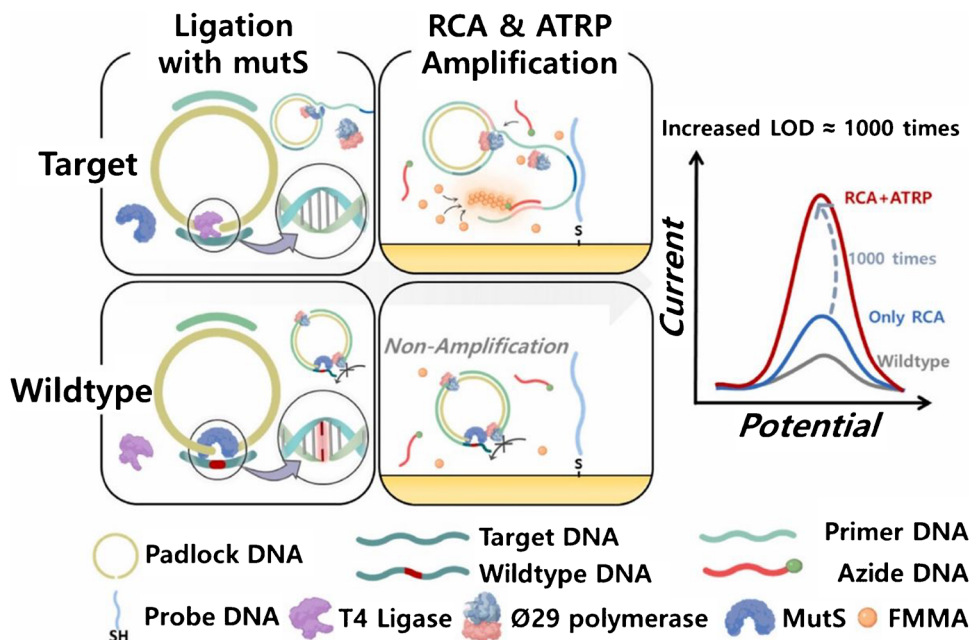
real tumor samples with mutated *TP53* to prove its usefulness. In another assay, authors combined RCA with MutS protein to improve mismatch recognition and with atomic transfer radical polymerization (ATRP) reaction to increase sensitivity of detecting *KRAS* point mutation [84]. MutS protein bound to the mismatch and interfered with the ligation and subsequent RCA; on the other hand, fully complementary DNA was ligated, followed by a combined RCA/ATRP amplification that led to around 1000-fold sensitivity improvement over conventional RCA (Fig. 4). Again, authors only spiked the synthetic oligo into a human serum, and thus, it is difficult to evaluate assay feasibility in clinical samples from patients. Actually, the behavior in clinical samples can be very different, especially taking into account that the long genomic DNA containing analyzed mutation would probably need to be digested into shorter fragments for more efficient RCA [114–116].

LAMP is an enzymatic IAT that is most effective in a temperature range between 55 and 70 °C. It requires 4–6 primers which makes the reaction highly sequence-specific and generates products of variable lengths, ranging from hundreds to few thousand bp. LAMP is widely used for pathogen analysis not only in a fluorescent or colorimetric format [117–120], but also in electrochemical assays to detect viruses, such as human papillomavirus (HPV) [121–124] and hepatitis virus B [125], and bacteria (*E. coli*, *Salmonella*, etc.) [126, 127]. Primer design is more complicated as compared to PCR, including SNP analysis (Fig. 5), and thus not many EC assays used LAMP for point mutation detection (Table 2). In fact, most assays were fluorimetric or colorimetric, targeting, e.g., mutations in *TP53*, *KRAS*, *BRAF*, or *EGFR* genes [129, 130]. An exception is a work

by Wang et al. that used a glucometer for analysis of V600E *BRAF* mutation, where the V600E mutation was localized within the F loop [80]. LAMP products were discriminated using mutation-specific probes immobilized on magnetic beads surface that were originally hybridized to invertase-labeled antisense strands. Since the mutation was designed to bind to the toehold domain of the probe, only upon the combination of V600E LAMP products and invertase/probe/magnetic beads conjugates, the toehold initiated strand exchange reaction, liberating invertase-tagged strand for glucose monitoring. Unfortunately, the assay was tested only by spiking into 10% human serum. This step seems to be quite common replacement of patient samples, but it is not sufficient enough. We highly encourage using either clinical samples, or if not available, at least cancer cell lines with defined point mutations.

RPA is an IAT quite similar to PCR, as it often employs identical forward and reverse primers, but instead of a Taq polymerase, it uses combination of three enzymes: recombinase, single-stranded DNA-binding protein, and strand-displacing polymerase. RPA is performed under constant temperature, usually between 37 and 42 °C, but it may function also at the room temperature. An interesting RPA-based approach for SNP detection in *Myosin Heavy Chain 7* gene [131] used solid-phase primer elongation, ferrocene-labeled nucleotides, and square wave voltammetry detection from fingerprick blood samples. Blood samples were diluted in EDTA solution, shortly heated and introduced directly into RPA reaction without a need for DNA isolation. This assay showed a tremendous potential of RPA coupled to electrochemistry for SNP diagnostics from a single drop of blood, although it was not focused on cancer. SDA is an IAT

Fig. 4 Working protocol of a modified RCA process using MutS proteins for mismatch recognition and atomic transfer radical polymerization (ATRP) for sensitivity enhancement. Reprinted from [84] with permission from Elsevier



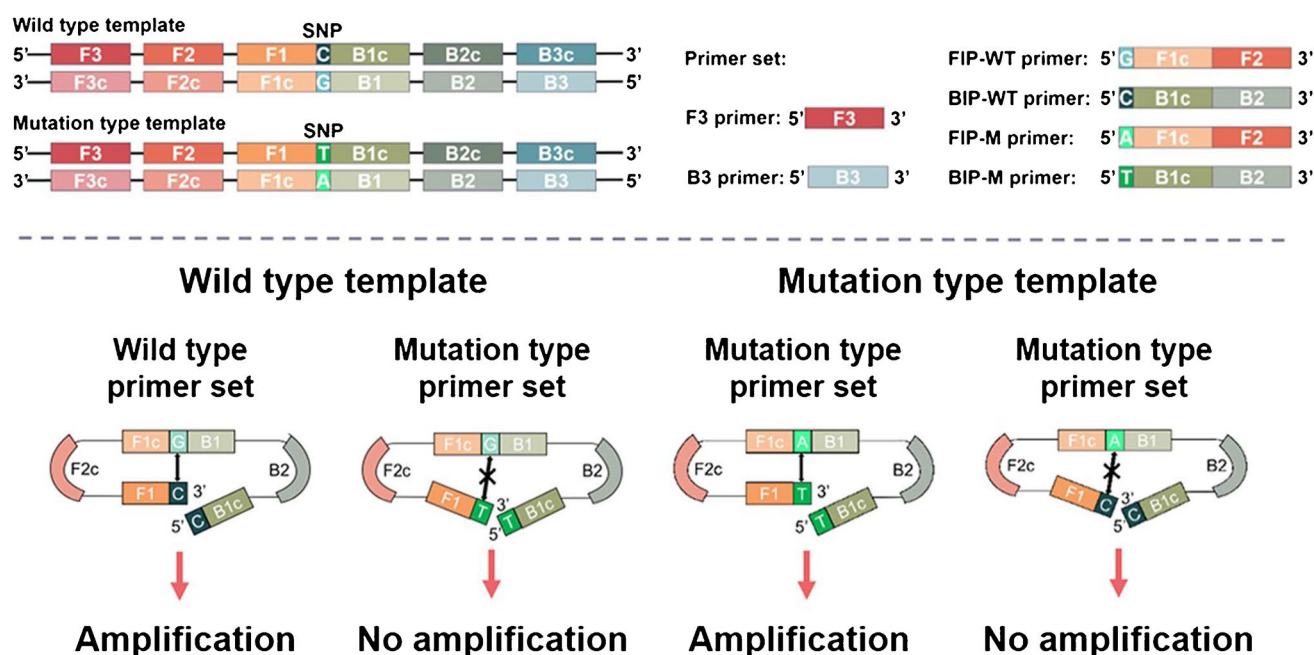


Fig. 5 Scheme of a LAMP-based assay for SNPs analysis. Reprinted from [128] with permission from Elsevier

combining principles of PCR and RPA. Similarly to RPA, SDA is performed under temperature close to 37 °C, but there is a need of initial denaturation step (same as in PCR) followed by polymerase addition. This technique has been used for identification of SNPs in combination with *NbsI* restrictase and coupled to EC measurement. Wang et al. developed target-triggered SDA for detection of *PIK3CA* gene mutation with immobilization on surface of gold electrode and sensitive EC measurement of methylene blue oxidation, but similarly to many other papers, only spiked human serum was used to demonstrate feasibility in clinical settings [85]. *Entropy-driven strand displacement reaction (ESDR)* is an enzyme-free reaction based on principles of DNazyme digestion and entropy-driven strand displacement [132]. Innovative strategy used by Chen et al. for recognition of *EGFR* mutation in circulating tumor DNA biomarker was presented (Fig. 6), where authors combined ESDR with CRISPR/Cas9 guided cleaving system, 3D metal nanoflower, and differential pulse voltammetry measurement to detect *EGFR* exon 19 deletion [81].

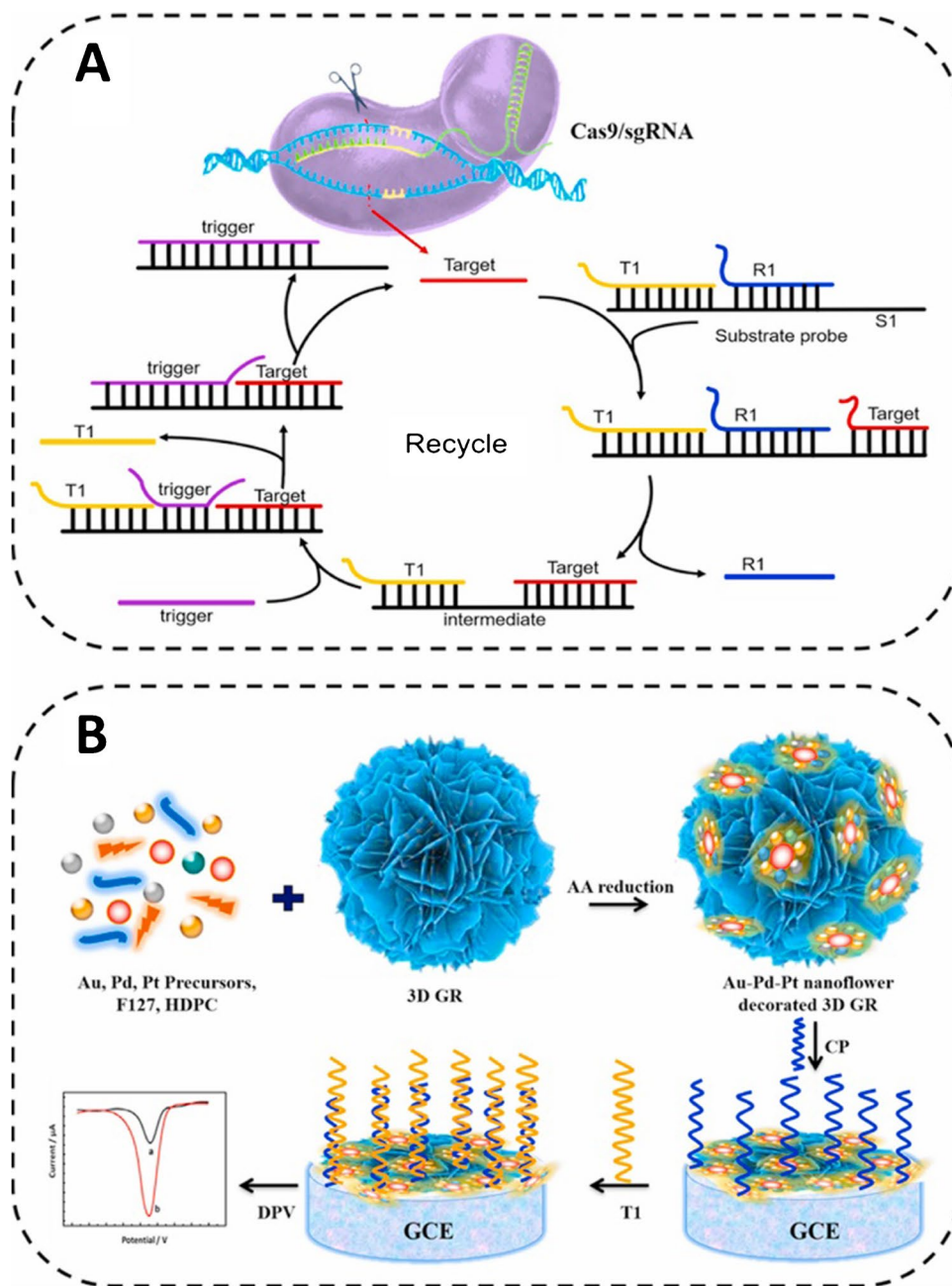
EC biosensors without enzymatic amplification

Although most papers describe some form of enzymatic amplification, several innovative enzyme-free electrochemical assays, where the amplification occurred in a different way, were reported [94, 96, 99]. These papers focused on mutations in circulating tumor DNA (ctDNA), which are DNA fragments shed from a tumor tissue into a bloodstream, enabling non-invasive sampling. In this case, enzyme-free

amplification assays have attracted attention due to their rapid response times and simple reporter systems [133]. For instance, Huang et al. developed nest hybridization chain reaction for the detection of E545K mutation in *PIK3CA* gene in ctDNA [99]. In that assay, authors introduced three different dumbbell-shaped DNA probes that formed a complex DNA nanostructure, which could hybridize to the target DNA on the gold electrode surface. Signal was generated via biotin-avidin system with the 3 pM limit of detection. Most importantly, the assay was directly applied to 72 clinical samples, including 25 pleural effusion samples from hepatocellular carcinoma patients, 23 serum samples from breast cancer patients, and 24 serums of healthy individuals, showing a great potential for ctDNA detection and cancer diagnosis.

We mentioned in a previous subsection that PNA blocking can be used to block amplification of a wild-type DNA, thus favoring mutated gene sequences to be preferentially amplified. Interestingly, PNA clamping can be also used without PCR or IAT. In an excellent paper by Das et al., authors applied this idea of PNA clamping to develop a rapid electrochemical assay that discriminates mutations in serum ctDNA, targeting seven most frequent mutations in *KRAS* gene and V600E mutation in *BRAF* gene [96]. In this assay, the authors designed multiple PNA clamps (one for each mutation), which served as sequence-selective clamps that hybridized to the wild type and other mutations (thus blocking them), and allowed only the chosen target mutated DNA sequence to remain unhybridized for subsequent hybridization with the PNA probe. The assay was tested on fourteen serum samples from lung cancer patients (to

Fig. 6 The principle of the CRISPR/Cas9-triggered entropy-driven strand displacement reaction (ESDR) based on a 3D graphene/AuPtPd nanoflower biosensor. (HDPC, hexadecylpyridinium chloride monohydrate; AA, L-ascorbic acid; CP, capture probe; GCE, glassy carbon electrode). Reprinted from [81] with permission from Elsevier



screen for *KRAS* mutation) and on seven serum samples from melanoma patients (for *BRAF* mutation). It could be used with unprocessed banked serum from cancer patients and produced results that were consistent with the PCR as a gold-standard method. With an ultralow detection limit of 1 fg/ μ L and 15 min diagnostics time, this assay is a perfect demonstration that electrochemistry can be coupled with clinical samples in sensitive and rapid format. Later on, the same research group introduced combinatorial probes for the detection of *EGFR* and *KRAS* gene mutations in patient serum [94]. Seven combinatorial probes were able to detect all of the 40 somatic mutations in the *EGFR* gene.

The application of PNA clamping was used to improve the specificity by blocking the wild-type sequences in the solution. The hybridization of mutated sequences to the probe on the gold nanostructured microelectrode was detected by an ultrasensitive non-enzymatic electrocatalytic system composed of $[\text{Ru}(\text{NH}_3)_6]^{3+}$ and $[\text{Fe}(\text{CN})_6]^{3-}$ (Fig. 7). Such approach allowed for a straightforward assay workflow, with minimized sample loss, and enabled the analysis of small samples. A possible downside of PNA clamping remains a limited number of companies that offer PNA synthesis, which inevitably leads to a high cost of PNA oligos.

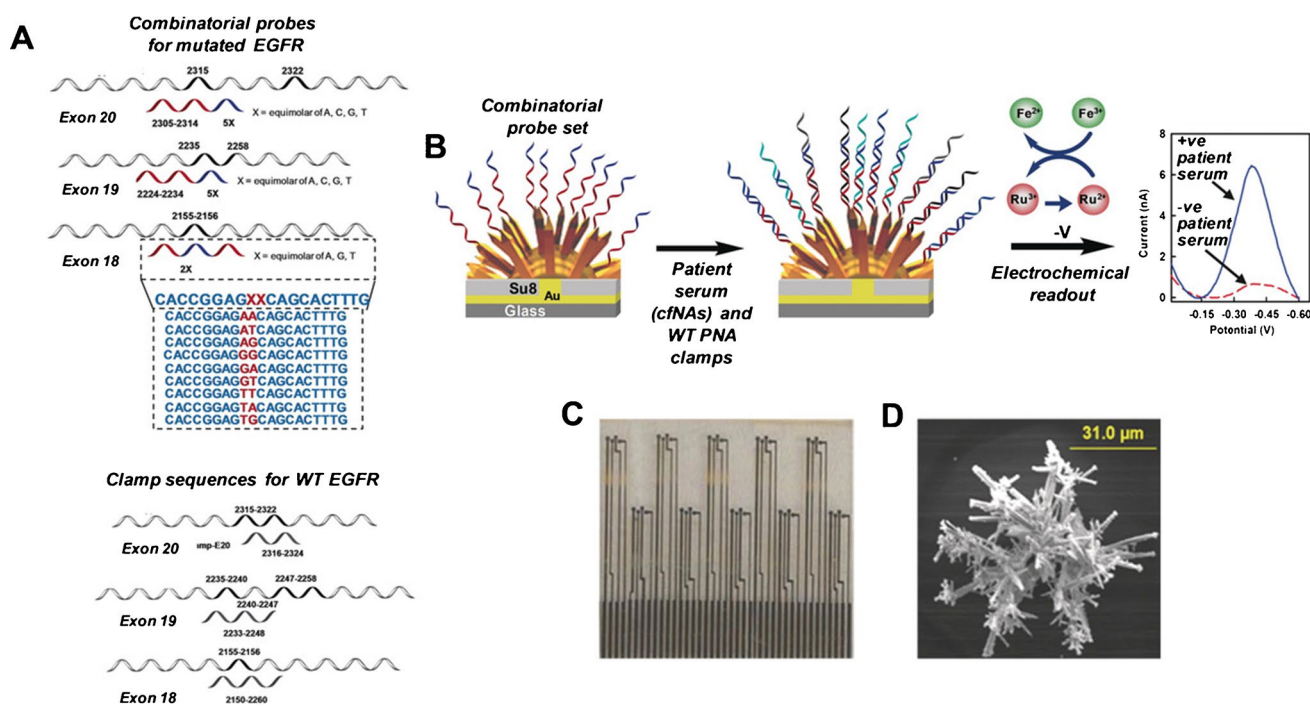


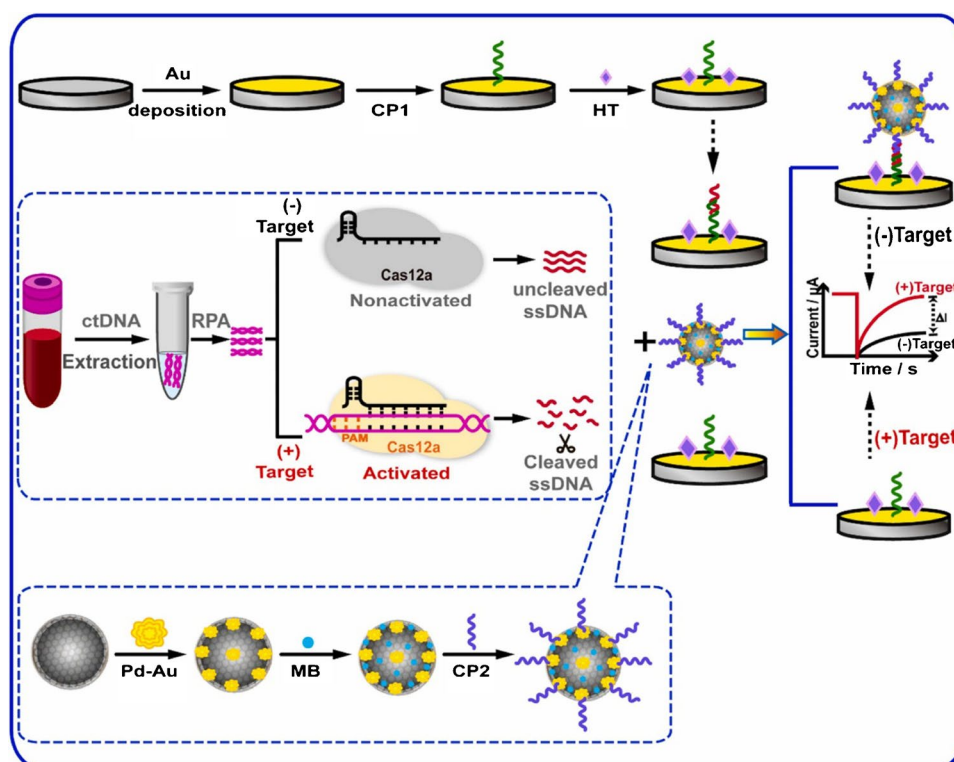
Fig. 7 Scheme of an EC assay for SNPs analysis of *EGFR* gene. Reprinted from [94] with permission from Wiley–VCH

Various types of *nanosstructures* are becoming popular tools when designing EC biosensors. For instance, a work by Wang et al. presented EC biosensor whereby intertwined three-dimensional DNA nanosheets were created by a covalent ligation of a triggering probe to the thiol-modified capture probe in a presence of wt sequence of *TP53*, followed by a hybridization chain reaction between two palindromic hairpin probes mediated by the triggering probe [100]. Although the assay was tested against single mismatches, it was not clearly specified which *TP53* mutation it targets. In addition to a commonly used recovery test in a spiked medium, authors also detected endogenous *TP53* gene extracted from breast cancer cell line MCF-7 after asymmetric PCR amplification, but no distinction between wt and mutated sequence was done in this case. In another work, Liu et al. developed an assay to search for a mutation in *TP53* gene by utilizing DNA-functionalized cadmium-doped metal organic framework (Cd-MOF-74) to initiate cascade amplification and target gene detection [101]. The detection limit of this assay was in a femtomolar range under enzyme-free conditions, but again, only recovery test in spiked human serum was conducted.

CRISPR/Cas system is an attractive technique that is increasingly used in nucleic acid detection, including highly specific sequence recognition with the ability to distinguish single-point mutations [134, 135]. The recognized sequence is usually cleaved which offers the possibility to use reporter-quencher technology with consequent fluorescence

measurement [136]. *CRISPR/Cas system* is often used for microRNA detection [137] or is coupled to various amplification technique such as PCR [71, 72], RPA [82], or ESDR (described above) [81]. Compared to optical techniques, EC methods are less frequently combined with the *CRISPR/Cas*, but this combination has a huge potential that should be undoubtedly exploited. For example, Liu et al. prepared a method (see Fig. 8) based on *CRISPR/Cas12a* for detecting *EGFR* L858R mutation (g.2573 T → G) in patients with non-small cell lung carcinoma (NSCLC) in ctDNA from plasma. They used RPA amplification of a mutated target sequence, followed by a *Cas12a* cleaving of the mutated strand. Unmutated (wild-type) amplicons remained uncleaved and were bound to a capture probe immobilized on the gold electrode surface. In the presence of an uncleaved amplicon, second capture probe was introduced that was immobilized on covalent organic frameworks covered with PdAu nanosheets and methylene blue to produce EC signal [82]. A very interesting study has been reported by Balderston et al. who discriminated single-point mutations in unamplified genomic DNA via *CRISPR-associated protein Cas9* immobilized on a graphene field-effect transistor [138]. Although not focused on a cancer diagnosis, proposed biosensor could discriminate between homozygous and heterozygous unamplified DNA samples from patients with sickle cell disease by targeting *HBB* gene. The principle utilized in this work is of great interest due to its high specificity; it was shown before that within the first few nucleotides in the so-called seed

Fig. 8 Principle of electrochemical biosensor for detection of EGFR L858R in ctDNA based on CRISPR/Cas12a system. Reprinted from [82] with permission from Elsevier



sequence, the Cas9 is most severely affected by mismatches which often results in the dissociation of Cas9 from DNA altogether [138]. Moreover, it could be easily extended also for DNA point mutations associated with cancer.

Outlook and conclusion

DNA point mutations in driver genes are closely linked to a carcinogenesis process. Therefore, their analysis became a vital part not only of a genetic screening to assess predisposition risks, but also of a successful cancer therapy where the information on the mutation may serve as a predictive biomarker. Many diverse analytical methods were developed for SNV detection, each having advantages and disadvantages that we discussed in separate section. Due to common drawbacks that these methods possess, especially costly instrumentation, long protocols, or need for a skilled personnel, biosensors as novel analytical tools have emerged that try to address these drawbacks. Electrochemical biosensors became a popular choice since they are inexpensive, simple, rapid, and easy to miniaturize and as such were successfully applied for detection of various cancer-related nucleic acid biomarkers [139, 140].

EC assays are considered as highly sensitive, but most of them still rely on an enzymatic amplification of DNA using either PCR or some isothermal alternative. Hence, disadvantages associated with amplification reactions,

especially high risk of contamination, are common for both standard methods and biosensors, and can be avoided only in amplification-free format (possibly leading to lower sensitivities). This should be taken into account when designing such biosensor. Although most EC biosensors now focus on achieving ultralow detection limits, we believe that when designing biosensors analyzing DNA point mutations, the limit of detection does not have to be as low as possible, but enough to detect DNA levels in clinical samples. It is more important to focus on reaching very good selectivity enabling reliable discrimination of a given point mutation.

A great obstacle for EC biosensors seems to be clinical sample analysis. Indeed, majority of studies do not demonstrate biosensor feasibility in samples from cancer patients (or at least in cancer cell lines). Instead, spiking of a human serum or other well-controlled media with short synthetic oligos to mimic real settings is a preferred option. Its usefulness is, however, questionable, since behavior of endogenous DNA molecules (either genomic DNA or ctDNA) may greatly differ to that of stable, short, purified DNA oligos. A reason for spiking is perhaps a lack of access to high-quality biospecimen successfully passing pre-analytical procedures such as those used in sample collection, processing, storage, and shipping that can significantly influence nucleic acid integrity. On the other hand, there are a number of academic and/or commercial biobanks either independent or associated into various organizations focused on biobanking such as a European

research infrastructure for biobanking (BBMRI-ERIC), International Society for Biological and Environmental Repositories (ISBER), or European, Middle Eastern, or African Society for Biopreservation and Biobanking (ESBB) offering human samples and associated data in standardized quality for research purposes. For example, the Directory (<https://directory.bbmri-eric.eu/>) provides a central listing of biobanks and their collections in the BBMRI-ERIC member states, and for researchers, it offers a means of finding samples and data they can subsequently request. Indeed, the access to human clinical samples does not represent an insurmountable obstacle, as demonstrated by EC works that successfully employed their assays into patient samples with satisfactory results [69, 70, 73, 75, 78, 141]. It would be highly beneficial if some EC strategy is applied to a larger cohort of patients (tens or even hundreds) and compared with a standard method of detection with rigorous statistics; this feature of EC assays is currently in its infancy [99, 124, 142], but would increase an impact of electrochemistry in clinical diagnostics.

Furthermore, most papers report analysis of only a single point mutation that is very frequently mutated (*KRAS*, *BRAF*, *TP53*, etc.). In some cases, this is sufficient and reasonable; on the other hand, a panel of SNVs where mutations (including rare ones) are analyzed in parallel could have much stronger value, and electrode chips and arrays with multiple electrodes could be easily adjusted for this purpose [143, 144].

Taken together, EC biosensors represent potentially useful alternatives in DNA point mutation analysis, and many of them have already shown impressive limits of detection or selectivity towards particular SNVs. We believe that if they are increasingly tested in real clinical samples and strictly compared to golden standards, they could eventually outcompete more expensive and laborious alternatives and become a reasonable choice for decentralized medicine at the point-of-care.

Author contribution Conceptualization: Martin Bartosik. Literature search: Katerina Ondraskova, Ravery Sebuyoya, and Ludmila Moranova. Writing—original draft preparation: Katerina Ondraskova, Ravery Sebuyoya, Ludmila Moranova, Jitka Holcakova, Petr Vonka, and Martin Bartosik. Writing—review and editing: Katerina Ondraskova, Ravery Sebuyoya, Ludmila Moranova, Jitka Holcakova, Petr Vonka, Roman Hrstka, and Martin Bartosik. Funding acquisition: Roman Hrstka and Martin Bartosik. Supervision: Martin Bartosik.

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Declarations

Conflict of interest The authors declare no competing interests.

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