CHAPTER 9

DNA HYBRIDIZATION IN SOLUTION FOR MUTATION DETECTION

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Abstract: This group of methods is aimed at the identification of single nucleotide-scale differences between the comparing DNA samples. Mutation and polymorphism detection is of increasing importance in the field of molecular genetics because the study of mutations reveals the normal functions of genes, proteins, noncoding RNAs, the causes of many malignancies, and the variability of responses among individuals. A plethora of single nucleotide polymorphisms (SNPs) are not deleterious by themselves, but are linked to phenotypes associated with diseases and drug responses, thus providing a great opportunity for their use in large-scale association and population studies. Millions of SNPs have been identified in recent years. However, this figure seems negligible compared to the real number of SNPs and other mutations presented in the genomes. Many mutation discovery methods quickly and effectively indicate the presence of a mutation in a sample region, but fail to resolve its characterization and localization; another family of methods permits precise mutation mapping, but in a greatly more laborious and expensive way. The group of novel approaches for mutation detection, which combines high performance, cost-efficiency, reliability, and detailed mutation characterization, will be reviewed in this chapter.

Keywords: Mutation detection, chemical cleavage, chemical modification of mispaired nucleotides, hydroxylamine, osmium tetroxide, potassium permanganate, nuclease-based mutation scanning, resolvase-like endonucleases, artificial nucleases, single-stranded DNA specific nucleases, T4 endonuclease VII, T7 endonuclease I, CEL I, Surveyor, endonuclease V, enzymatic mismatch cleavage (EMC), RNase cleavage of mismatched nucleotides, single-base extension (SBE), duplex-specific nuclease preference (DSNP), allele-specific PCR, allele-specific competitive blocker–polymerase chain reaction (ACB–PCR), LigAmp, MutS, glycosylase mediated polymorphism detection, physical isolation of imperfectly matched DNA, single-strand conformational polymorphism (SSCP), enzyme-amplified electronic transduction, QCM.

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Abbreviations: ACB–PCR, allele-specific competitive blocker–polymerase chain reaction; dNTP, deoxyribonucleotidetriphosphate; DSN, duplex-specific nuclease; DSNP, duplex-specific nuclease preference; EMC, enzymatic mismatch cleavage; FRET, fluorescent resonance energy transfer; MALDI, matrix-assisted laser desorption ionization; PCR, polymerase chain reaction; QCM, quartz crystal microbalance; RT-PCR, reverse transcription polymerase chain reaction; SNP, single nucleotide polymorphisms; SPR, surface plasmon resonance; SSCP, single-strand conformational polymorphism.

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1. INTRODUCTION

Unlike subtractive hybridization, which generally deals with finding relatively long differential DNA fragments, this group of methods is aimed at the identification of very small, single nucleotide-scale differences between the comparing DNA samples. Mutation and polymorphism detection is of increasing importance in the field of molecular genetics. Mutations are believed to contribute strongly to the genetic variability in living beings, in particular their disease or drug side-effect predispositions. The study of mutations reveals the normal functions of genes, proteins, noncoding RNAs, the causes of many malignancies, and the variability of responses among individuals. Recent mutations that have not yet become polymorphisms are often deleterious and pertinent to the disease history of afflicted individuals. Small insertions or deletions of nucleotides are common polymorphic variations in the human genome and mutation-induced sequence variations are playing an important role in the development of cancer, among others. A plethora of single nucleotide polymorphisms (SNPs) are not deleterious by themselves, but are linked to phenotypes associated with diseases and drug responses, thus providing a great opportunity for their use in large-scale association and population studies. Moreover, SNPs are increasingly recognized as important diagnostic markers for the detection of drug-resistant strains of hazardous microorganisms like bacterium *Bacillus anthracis* and for differentiation of virulent strains from their nonvirulent counterparts. From this, it is clear that SNP and mutation discovery is of great interest in today's life sciences.

Millions of SNPs have been identified in recent years. However, this figure seems negligible compared to the real number of SNPs and other mutations presented in the genomes. Therefore, detection of mutations, what requires the ability to detect differences in DNA structure with single nucleotide specificity, in a cheap, 100% effective manner is one of the major objectives in modern molecular genetics. However, this ideal is a little way off, and many methods are used, each with their own particular advantages and disadvantages. The ideal method would detect mutations in large fragments of DNA and position them to single base-pair (bp) accuracy and would be sensitive, precise, and robust. Currently, the need in mutation detection is reflected by the plethora of chemical, enzymatic, bioinformatical, and physically based techniques. Many mutation discovery methods quickly and effectively indicate the presence of a mutation in a sample region, but fail to resolve its characterization and localization; another family of methods permits precise mutation mapping, but in a greatly more laborious and expensive way. The group of novel approaches for mutation detection, which combines together high performance, cost efficiency, reliability, and detailed mutation characterization, will be reviewed in this chapter.

At present, mutation discovery is often performed utilizing Sanger Sequencing, which is often thought of as the "gold standard" for mutation detection. This perception is distorted due to the fact that this is the *only* method of mutation identification but this does not mean it is the best for mutation detection. The fact that many scanning methods detect 5–10% of mutant molecules in a wild-type environment immediately indicates these methods are advantageous over sequencing, at least for some purposes. Using bioinformatical approaches, discovery of a great number of mutations (mostly SNPs) was recently performed.

However, these methodologies require prior knowledge of target sequences, normally obtained through DNA sequencing, and mutation recovery in such case is usually performed by multiple sequence alignment of publicly available sequence data. Recent studies indicate that only a small percentage of mutations can be discovered using this approach and, in particular, that SNPs with low frequency are often missed. It is a serious problem for the software to detect mutations when four-color fluorescence is used when mutations are 50% (heterozygous) or less in a sample (mixed genomic samples, mitochondrial mutations, and tumor samples). These rare base substitutions within populations of DNA molecules are valuable tools for studying the DNA-damaging effects of chemicals and for pool screening for disease-associated polymorphisms.

It is clear now that high throughput methods for detecting these variations are needed for in-population screening for complex genetic diseases in which extended genomic loci, large genes, and/or several genes may be affected. Obviously, automation of the mutation detection analysis is desirable. In this respect, the idea that direct quantitation of SNPs from DNA sequencing raw data will save time and money for large amount sample analysis may seem advantageous. However, this high throughput sequencing is limited to a small

number of samples, and each mutation detected in such a way, which is not considered sequencing error needs experimental confirmation and, thus, cloning and resequencing in several replicates. Therefore, such high-throughput SNP typing techniques require expensive and dedicated instruments, which render them out of reach for many laboratories. Moreover, most part of mutations is missed (few alleles analyzed). Probably, this problem will be solved by further advances in pyrosequencing-related approaches (Guo et al. 2003; Qiu et al. 2003), but the current state of the art requires recruitment of other techniques for effective large-scale mutation scanning. To meet the need of these studies, several groups of approaches have been developed. All of them are based on the rationale that mutation containing DNA molecule will form mismatches at the mutation site when hybridized to the reference wild-type DNA (Figure 1).

Thus, when mutant and wild-type DNAs are hybridized together, two complementary mismatches are formed. For example, with a T→C substitution, T–G and C–A mismatches are formed in the two heteroduplexes. Therefore, the detection and correct position of such mismatches is the key for mutation recovery. Such mispaired nucleotides can be identified directly or indirectly using very different chemical, enzymatic, or physical approaches, which offer excellent detection efficiencies coupled with high throughput and low unit cost. It should be noted that definition of the mutational change obviously requires a sequencing step, at least to confirm the results. But in this case sequencing is targeted, not a "fishing expedition", when the region, where mutation occurred, is unknown, and plenty of sequencing work is absolutely required. As a result, these methods are able to cut the costs of detecting a mutation one order of magnitude or more. Richard Cotton, one of the leading scientists in this field, says: "There are a handful of scanning methods, each having their own problems. . . . All of these methods are being improved constantly by their creators and users, thus the life

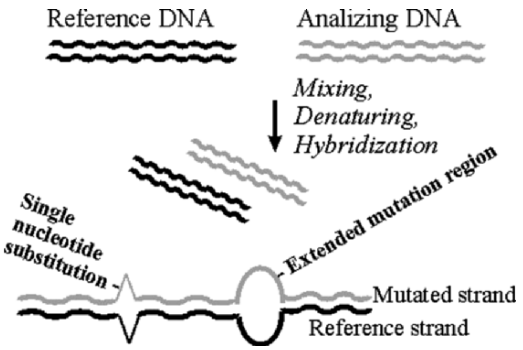

Figure 1. When hybridized to reference wild-type DNA, mutated DNAs form heteroduplexes with one or more mispaired nucleotides. These mismatch sites are targets for the majority of hybridization-based mutation detection techniques.

of those needing to detect mutations is slowly improving without the magic universal method being visible in the near future" (Cotton 1999).

The majority of such mutation detection methods are polymerase chain reaction (PCR)-based techniques dependent on the formation of heteroduplexes between wild-type and mutant strands of DNA. Briefly, chemical approaches utilize chemical cleavage or modification of the mispaired nucleotides, enzymatic ones employ enzymatic recognition of the mismatch (with further binding, cleavage, modification, or ligation of the DNA at the mispaired nucleotide(s)), whereas physical methods look for a physical difference between the mutant strand and wild-type strands of DNA, being based either on physical isolation of imperfectly matched DNA hybrids (like electrophoretic separation), or on finding differences in mismatched versus perfect DNA hybrid physical peculiarities. All these approaches utilize nucleic acids hybridization in solution and, therefore, will be described below in more detail, in comparison with each other and with direct sequencing-based approaches.

2. CHEMICAL APPROACHES

Chemical approaches for mutation detection (reviewed by Cotton (1999) and Taylor (1999)) are based on the chemical modification of mispaired nucleotides with the subsequent cleavage exactly at the position of a mismatch. Chemical cleavage, which is one of few methods capable of detecting nearly all single base mismatches, was developed in 1988 by Cotton et al. (1988) and has been widely used in research and diagnosis of many inherited diseases.

This approach, outlined in Figure 2, utilizes PCR amplification of a genomic locus of interest (alternatively, cDNA region) using as the templates reference wild-type DNA and a sample DNA that may have mutations in this region (T→C substitution in the figure). Following mixing, denaturing, and hybridizing PCR fragments, these are treated with chemical reagents, which modify mispaired nucleotides. The chemicals modify preferentially at mismatched T bases and mismatched C bases. For C bases modification, hydroxylamine (NH_2OH) is used, whereas mispaired T was originally modifying with osmium tetroxide (OsO_4), which is now replaced by potassium permanganate ($KMnO_4$) solution with a coadditive triethylammonium chloride due to high toxicity of the former (Roberts et al. 1997). Thus, it can be seen that if all four strands are labeled this mutation (as represented by the two heteroduplexes) has two chances of being detected. It should be noted that this second chance can become vital as in approximately one third of all T-G mismatches the T is unreactive, presumably due to sequence context effects (Cotton 1999). Modified DNAs are further simultaneously cleaved by piperidine, purified and analyzed either on sequencing gels or using capillary electrophoresis.

The main advantages of this approach include nearly 100% efficiency in detecting mutations in the DNA scanned and the possibility to precisely locate mutation (Figure 2) when the reference and sample DNAs are differentially

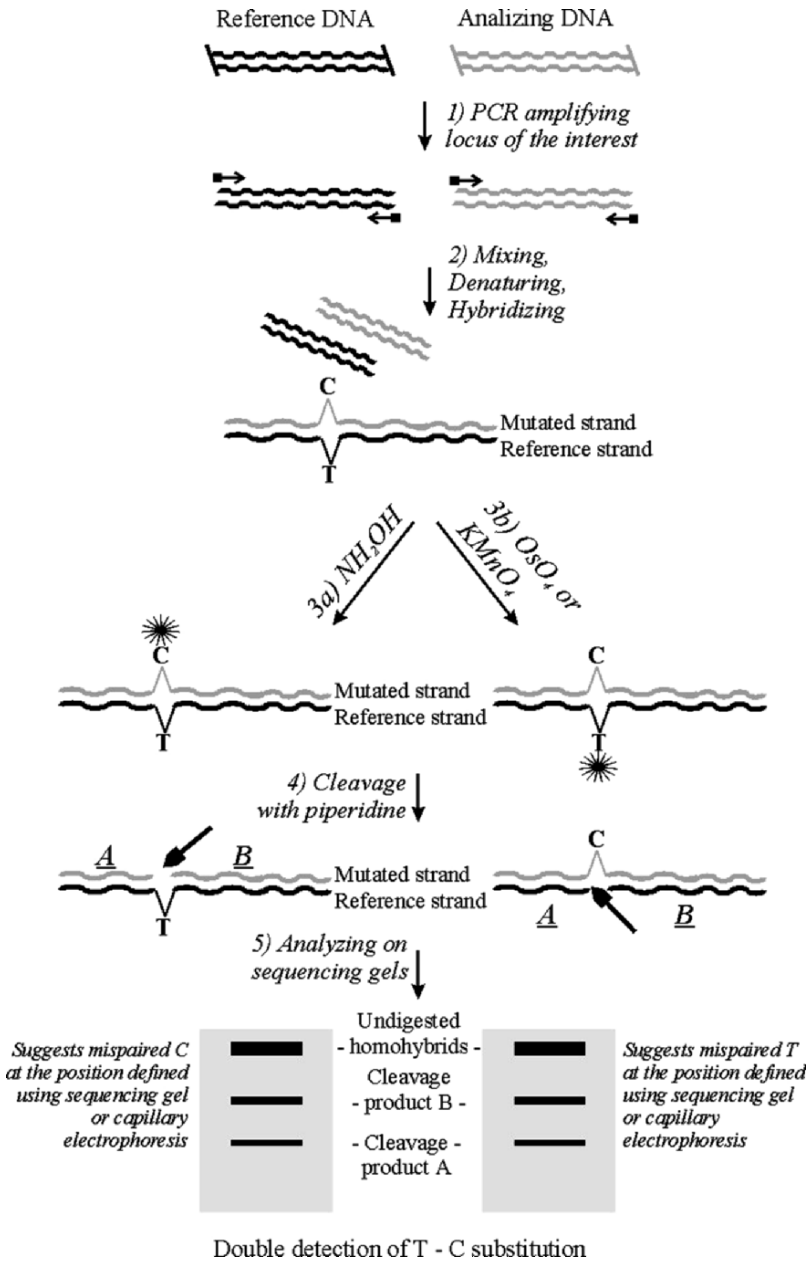

Figure 2. Outline of the chemical cleavage approach for mutation detection. In this example, mutated sample differs from the reference wild-type DNA in a single T→C substitution. Unlike enzymatic approaches, the chemical cleavage enables double detection of such types of mutations, thus significantly increasing sensitivity of the assay.

labeled (e.g. by fluorescent reagents). The most serious shortcomings of the method in its initial form include the multiple manipulations and the fact that toxic chemicals are required. This technique was greatly improved when chemical cleavage on solid support became practice: first, using biotinylated PCR primers, which made it possible to attach the DNA to streptavidin-coated beads (Rowley et al. 1995), and, second, utilizing nonspecific DNA binding to commercially available silica solid support (Bui et al. 2003). Operating with solid phase-bound DNA provides a great advantage over having to perform multiple ethanol washes and makes the protocol much simpler. Among the two variants mentioned above, the latter seems advantageous, as it somewhat economizes cost of an experiment and increases the efficacy of the whole procedure, as all DNA strands can be labeled. Another important improvement of this method is multiplexing (Rowley et al. 1995), when several analyzing DNAs may be run in the same gel lane or capillary (e.g. by using different fluorophores labeling different analyzing DNAs).

This method is currently in use in many laboratories, and many successful applications of chemical cleavage for mutation detection can be mentioned. For example, in 2004 this method was used for the recovery of mutations in the tumor suppressor p53 gene *TP53* in a group of 89 breast cancer patients, and three previously unknown mutations in protein coding sequence have been established (Lambrinakos et al. 2004). Another example was the first prenatal diagnosis performed on chorionic villi biopsy of a pregnant woman affected by a severe form of autosomal dominant transmitted retinitis pigmentosa, due to the Arg135Trp substitution in rhodopsin (Tessitore et al. 2002).

However, the chemical cleavage method is far less popular than its most serious competitor, enzymatic recognition-based approach, probably, due to its labor intensity and use of hazardous chemicals. As to sensitivity and accuracy of mutation detection, in the experiments by Deeble et al. (1997), chemical cleavage was found substantially more reliable and advantageous than producing higher background enzymatic cleavage approach.

3. ENZYMATIC APPROACHES

This group of methods is significantly more diverse than chemical cleavage-based techniques. Enzymatic approaches can be classified into three major groups: (1) those using nuclease cleavage of mispaired nucleotides in heteroduplexes (reviewed by Yeung et al. 2005)), (2) allele-specific PCR-based, and (3) methods utilizing mismatched DNA specific biding by some proteins. The first group of methods is the most widely used due to its relative simplicity, good reproducibility of the results, and the ability to find and precisely locate unknown mutations, thus permitting performing large-scale (even genome wide for some modifications) mutation scanning.

3.1 Nuclease-Based Mutation Scanning

A great variety of nucleases with very different specificities has been identified in the living organisms. Some of them have already found their application(s) in molecular biology and biomedicine, some did not find yet (e.g. the recently isolated human nuclease with an exotic function of tetraplex DNA cleavage (Sun et al. 2001)). Nuclease-based methods for mutation recovery utilize a simple rationale that some nucleases are able to bind and to preferentially digest double-stranded DNA at the mismatched nucleotide positions. Depending on the nature of the nuclease used, these techniques can be subdivided into (1) those using resolvase-like endonucleases, (2) those employing restriction endonucleases, single-stranded DNA specific nucleases and RNases, and (3) those based on the action of artificial nucleases.

3.1.1 Resolvase-like endonucleases

Bacteriophage resolvases T7 endonuclease I and T4 endonuclease VII are able to produce breaks in the double-stranded DNA duplexes at the mispaired nucleotide positions. It was in 1995, when three independently working research teams, Mashal et al. (1995), Birkenkamp and Kemper (1995), and Youil et al. (1995) simultaneously published the same idea of treatment of duplexes reference DNA-analyzing DNA with bacteriophage resolvases for the mutation recovery. The resolvases are an important group of enzymes that are responsible for catalyzing the resolution of branched DNA intermediates that form during genetic recombination. Their mode of action is directed by bends, kinks, or DNA deviations. These enzymes have their effect close to the actual site of DNA distortion (Birkenkamp and Kemper 1995). T4 endonuclease VII, the product of gene 49 of the bacteriophage T4, was the first enzyme shown to resolve Holliday structures. It has also been shown to recognize cruciforms and loops. It may also be involved in very short patch repair. Its cleavage characteristics involve it cleaving 3′ and within 6 nt from the point of DNA perturbation-causing double-stranded breakage (Youil et al. 1995).

As early as in 1990, in model experiments with synthetic oligonucleotides, T4 endonuclease VII has been shown to cleave single base-pair mismatches by Kosak and Kemper (1990). More recently, Youil and colleagues demonstrated that single mismatched nucleotide cleavage by T4 endonuclease VII depends greatly on the mispaired nucleotides: G-A and G-G mismatches are processed less effective than other types of mispaired nucleotides (Youil et al. 1995). However, as shown by Mashal et al. (1995), the use of a combination of T4 endonuclease VII with T7 endonuclease I provides very efficient digestion of all possible single nucleotide mismatches and of short (few nucleotide long) loops. More recently, cleavage with T4 endonuclease VII was used for the identification of variable number of short tandem repeats polymorphisms (Surdi et al. 1999). It should be also kept in mind that some background cleavage may occur, probably, due to unexpected secondary structures in DNA heteroduplexes (for instance,

mispaired duplex cleavage specificity using this approach was ~80%, as measured by Inganas et al. (2000)). To solve this problem, Golz and colleagues have developed improved reaction conditions which can increase the selectivity of the enzyme for mismatches up to 500-fold, as demonstrated with a mutation in a 247 nt long fragment from exon 7 of human gene for p53 protein. The new conditions involve replacement of Tris/HCl buffer by phosphate buffer and change from pH 8.0 to pH 6.5. To achieve the best results, the authors recommend trying various concentrations of phosphate ions to meet individual requirements of the substrate (Golz et al. 1998b).

The basic protocols for mutation scanning using resolvases are simple and quite similar to those utilizing chemical cleavage (Figure 3; reviewed by Babon et al. (2003)). Genomic region of interest (usually 0.1–2 kb long, but in some cases up to 4 kb long, according to Del Tito et al. (1998)) is amplified with unique primers from reference and sample DNA templates, PCR products are mixed, melted, and hybridized. When the hybridizing molecules are too long, correct heteroduplex formation may be problematic and DNA fragmentation is recommended (Smith et al. 2000). Duplexes are further treated with endonuclease(s) and analyzed on sequencing gels, capillary electrophoresis, or using chromatography. The size of the digestion products indicates the location of the mutation, which is then confirmed and characterized by sequencing. As for chemical cleavage, fluorescent labeling (Del Tito et al. 1998) and multiplexing (Schmalzing et al. 2000; Shi et al. 2006) were recruited to improve the robustness of this technique.

Also, at least two new enzymes of unknown natural function mimicking resolvase activities became known: CEL I nuclease from celery (Oleykowski et al. 1998) and closely related Surveyor nuclease (Qiu et al. 2004; Mitani et al. 2006). CEL I produces single-strand breaks, whereas Surveyor nuclease cleaves with high specificity at the 3′-side of any mismatch site in both DNA strands, including all base substitutions and insertion/deletions up to at least 12 nucleotides (however, being taken in 100-fold excess, at a 1:1 to 1:4 w/w enzyme/DNA ratio, CEL I produces double-strand breaks as well (Sokurenko et al. 2001)). The use of CEL I and Surveyor nucleases is advantageous, as they can detect 100% of the sequence variants present, including deletions, insertions, and missense alterations, without having to finely tune buffer conditions (Oleykowski et al. 1998; Comai et al. 2004).

As compared with chemical cleavage, resolvases-based approach became far more popular, probably, due to its simplicity, improved safety for the researchers, and commercial availability of some mismatch cleavage enzymes and even of special kits for mutation scanning. For example, T7 endonuclease I can be purchased from New England Biolabs (USA), and another mismatch sensitive enzyme, Surveyor nuclease, is produced by Transgenomic (USA).

The efficacy of this approach has been demonstrated in a number of successful applications. For example, a number of diagnostic mutations in the cationic trypsinogen gene was found in a group of 29 hereditary pancreatitis patients

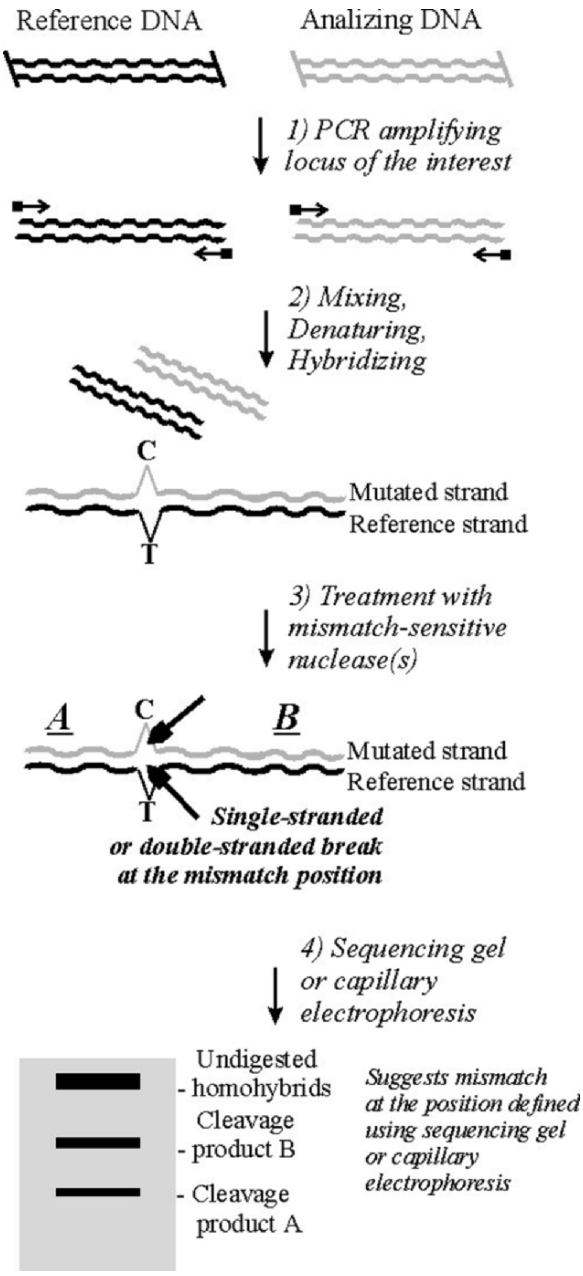

Figure 3. Generalized scheme for the enzymatic hybridization-based mutation detection approaches.

(Ford and Whitcomb 1999), the analysis of 178 colorectal cancer samples identified deleterious mutations in p53 coding sequence in 51 of them (Inganas et al. 2000); method was fruitful for the large-scale detection of mutations in two mitochondrial tRNA genes even when they were present at levels as low as 3% in DNA samples derived from patients with respiratory chain defects (Bannwarth et al. 2005); in very good agreement with these results, method was capable to detect mutations in *p53* gene when 20:1 ratio of normal versus mutated DNA was analyzed (Del Tito et al. 1998).

When the large and complex gene for fibrillin 1 (*FBN1*) was scanned in a cohort of six patients diagnosed with connective tissue disorders (four of them being diagnosed with classic Marfan syndrome), two causative mutations that result in premature translation termination, that were missed by other methods, have been identified (Youil et al. 2000). Similarly, 10 multidrug-resistant and 10 drug-susceptible clinical isolates of *Mycobacterium tuberculosis* were quickly investigated for point mutations in drug-resistant genes, *katG*, *rpoB*, *embB*, *gyrA*, *pncA*, and *rpsL* genes, which are known to be responsible for antibiotic resistance (Shi et al. 2004). Also, the use of mismatch sensitive nucleases (Surveyor nuclease in this example) provides an alternative to a laborious standard selection of desired clones from site-directed mutagenesis and PCR-based cloning methods without the necessity of sequencing DNAs purified from multiple clones. This approach was used to identify error-free clones of three genes from celery cDNA (Qiu et al. 2005).

Another application of the same idea is the removal of mismatched bases from synthetic genes by enzymatic mismatch cleavage (EMC) (Fuhrmann et al. 2005). The success of long polynucleotide *de novo* synthesis is largely dependent on the quality and purity of the oligonucleotides used. Generally, the primary product of any synthesis reaction is directly cloned, and clones with correct products have to be identified. Using mismatch sensitive nucleases like T7 endonuclease I, T4 endonuclease VII, and *Escherichia coli* endonuclease V, a novel strategy has been established for removing undesired sequence variants from primary gene synthesis products. As a model, a synthetic polynucleotide encoding the bacterial chloramphenicol-acetyltransferase (*cat*) was synthesized using different methods for one-step polynucleotide synthesis based on ligation of oligonucleotides. The influence of EMC as an error correction step on the frequency of correct products was analyzed by functional cloning of the synthetic *cat* and comparing the error rate with that of untreated products. Significant reduction of all mutation types was observed. The treatment with nucleases was successful especially in the removal of deletions and insertions from the primary ligation products.

The classical mismatch sensitive nuclease cleavage protocol has greatly evolved now, thus giving rise to a variety of new experimental techniques. First, a genome-wide approach published by Sokurenko et al. (2001), that is suitable for comparison of small genomes like bacterial DNAs (Figure 4). The method has six stages: (1) mixing two compared genomes, (2) complete restriction

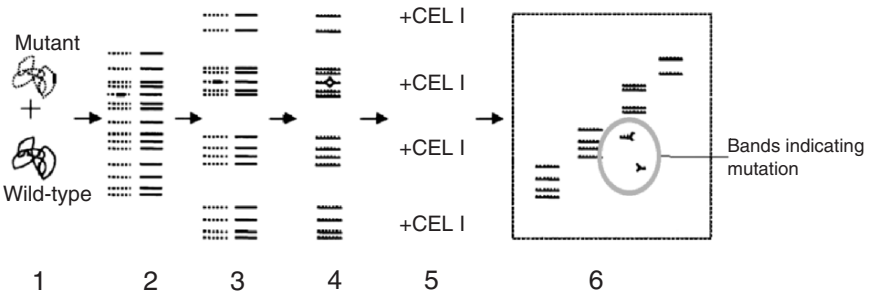

Figure 4. Scheme for the large-scale genomic SNP screening in bacterial genomes or genomic contigs of comparable lengths. Stage 1: total genomic DNA from two bacterial strains is purified and combined (a possible polymorphic site is indicated as a solid bar). Stage 2: complete endonuclease restriction of the combined genomic DNA. Stage 3: size-fractionation of the restricted DNA fragments. Stage 4: DNA heteroduplex formation by heat denaturation and reannealing of the fractionated DNA fragments (the mutation-induced mispaired region is shown as an open square). Stage 5: CEL 1 treatment of the reannealed DNA fragments. Stage 6: agarose gel analysis of CEL 1-treated DNA fractions (the mismatch-cleaved fragments are indicated as shortened fragments with halved squares on the ends).

endonuclease digestion of the combined genomic DNA, (3) size-fractionation of the restricted DNA fragments, (4) DNA heteroduplex formation by heat denaturation and reannealing of the fractionated DNA, (5) CEL I treatment of the reannealed DNA fragments and, finally, (6) agarose gel analysis of CEL I-treated DNA fractions. If a mutation occurs, two new lower molecular weight bands appear on gel that can be isolated and sequenced to locate the mutation. Using this approach, the authors managed to detect various simple mutations directly in the genomic DNA of isogenic pairs of recombinant *Pseudomonas aeruginosa*, *E. coli*, and *Salmonella* isolates. Also, by using a cosmid DNA library and genomic fractions as hybridization probes, they compared total genomic DNA of two clinical *P. aeruginosa* clones isolated from the same patient, but exhibiting divergent phenotypes. This multistep method, although efficient in hands of Sokurenko and colleagues did not become popular, probably, due to its labor intensity.

Another method, recently published by Huang et al. (2002), is the elegant modification of the standard enzymatic mismatch digestion protocol, aimed to increase the cleavage specificity and thus to decrease background signaling, which sometimes makes mutation detection problematic. The authors have developed a mutation scanning method that combines thermostable bacterial Endonuclease V (Endo V) and DNA ligase (Figure 5). Variant and wild-type PCR amplicons are generated using fluorescently labeled primers, and heteroduplexed. *Thermotoga maritima* EndoV recognizes and primarily cleaves heteroduplex DNA one base 3′ to the mismatch, as well as nicking matched DNA at low levels. *Thermus* sp. DNA ligase reseals the background nicks to create a highly sensitive and specific assay.

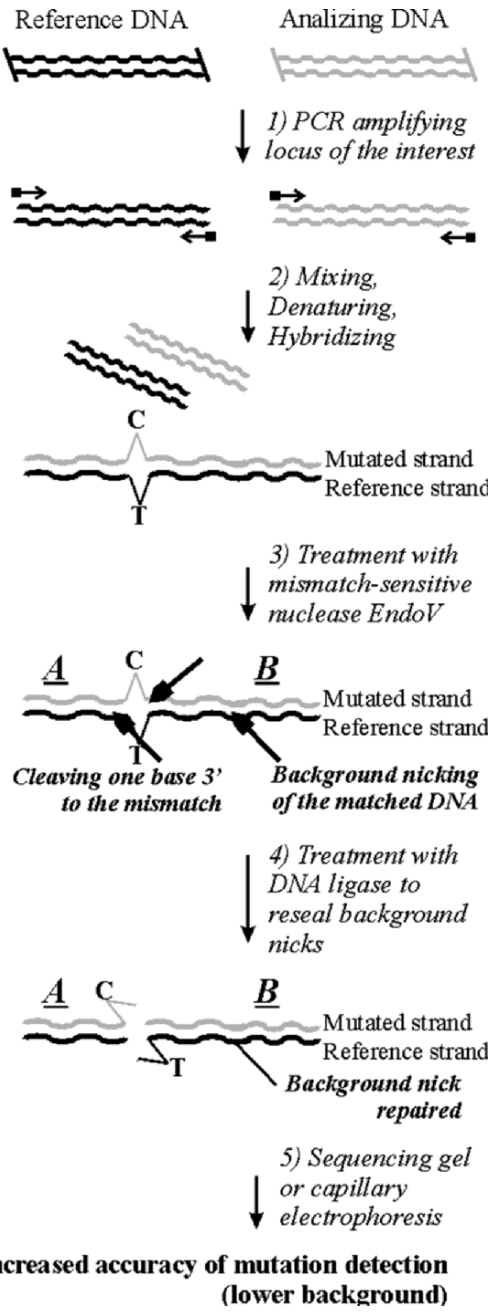

Figure 5. Enzymatic mutation detection technique of an increased accuracy, mediated by DNA ligase repair of background nicks. At stage 4, DNA ligase reseals background nicks produced by mismatch sensitive nuclease(s) employed at the previous step. Note that true mismatches remain unrepaired.

The fragment mobility on a DNA sequencing gel reveals the approximate position of the mutation. This method identified 31/35 and 8/8 unique point mutations and insertions/deletions, respectively, in the *p53*, *VHL*, *K-ras*, *APC*, *BRCA1*, and *BRCA2* genes. The technique has the sensitivity to detect unknown mutations diluted 1:20 with wild-type DNA, This method is well suited for scanning low-frequency mutations in pooled samples and for analyzing tumor DNA containing a minority of the unknown mutation.

3.1.2 Restriction endonucleases, single-stranded DNA-specific nucleases and RNases.

Not only mismatch specific nucleases may be employed for enzymatic mutation detection. RNase cleavage of mismatched nucleotides in single-stranded RNA probes hybridized to reference DNA sequences became a practice over 20 years ago (reviewed by Goldrick (2001)) The original methods relied on RNase A for mismatch cleavage; however, this enzyme fails to cleave many mismatches and more recently it was replaced by other enzymes like RNase 1 and RNase T1 to cleave mismatches in duplex RNA targets. The detection is improved, when these enzymes are used in conjunction with nucleic acid intercalating dyes. This method is being used to detect mutations and SNPs in a wide variety of genes involved in human genetic disease and cancer, as well as in disease-related viral and bacterial genes (Goldrick 2001). The most serious drawback of this method is that large amount of RNA probe is needed; moreover, nonspecific RNA degradation frequently makes such an analysis problematic.

Recently, two research groups proposed using single-strand-specific nucleases for DNA heterohybrids processing. Chalaya et al. (2004) used mung bean nuclease for cleavage of the loop regions produced during heterohybrid formation. In the conditions used by the authors, the enzyme did efficiently cleave the extended mispaired DNA (but not single nucleotide mismatches) and displayed perfect results in mismatched DNA cleavage when used together with Surveyor nuclease. Simultaneously, Till and coauthors used the same enzyme and another single-stranded DNA-specific nuclease S1 from Aspergillus to detect mutations as small as single nucleotide substitutions (Till et al. 2004). Surprisingly, in suboptimal conditions (higher pH, temperature, and divalent cation concentrations) these nucleases were able to specifically cleave nearly all single-stranded mismatches tested. These intriguing results imply that plenty of other single-stranded DNA-specific nucleases belonging to S1 and mung bean families theoretically might be used for effective mutation detection. Overall, the use of single-stranded specific DNases provides an advantage of cleaving not only single nucleotide mismatches, but also more extended loop regions, which are frequently ignored by resolvases.

Restriction endonucleases can be used for mutation detection as well. An interesting approach for high throughput screening of known SNPs in the analyzing samples was recently published by Che and Chen (2004). This method uses a type II restriction endonuclease to create extendable ends at target

polymorphic sites and uses single-base extension (SBE) to discriminate alleles (Figure 6). A restriction site is engineered in one of the two PCR primers so that the restriction enzyme cuts immediately downstream of the targeted SNP site. The digestion of the PCR products generates a 5′-overhang structure at the targeted polymorphic site. This 5′-overhang structure then serves as a template for SBE reaction to generate allele-specific products using fluorescent dye-terminator nucleotides.

Following the SBE, the allele-specific products with different sizes can be resolved by DNA sequencers. Through primer design, one can create a series of PCR products that vary in size and contain only one restriction enzyme recognition site. This allows loading of many PCR products in a single capillary/lane. This method, restriction-enzyme-mediated SBE, was demonstrated by typing multiple SNPs simultaneously for 44 DNA samples. By multiplexing PCR and pooling multiplexed reactions together, this method has the potential to score 50–100 SNPs/capillary/run if the sizes of PCR products are arranged at every 5–10 bases from 100 to 600 base range.

If one is interested in investigating a particular point mutation that disrupts a wild-type restriction site, than a variety of simple, cost efficient, and sensitive techniques utilizing heteroduplex treatment with restriction endonucleases is available (e.g. Zhu et al. 2004). The shortcoming that is shared by all restriction endonuclease-based mutation screening methods is that they cannot identify new, previously unknown mutations. Therefore, their area of application is the studying of already established mutations and polymorphisms.

The last method reviewed in this section will be the novel technique recently proposed by Shagin et al. (2002) for SNP detection. The authors have characterized a novel nuclease from the Kamchatka crab, designated duplex-specific nuclease (DSN). DSN displays a strong preference for cleaving double-stranded DNA and DNA in DNA–RNA hybrid duplexes, compared to single-stranded DNA. Moreover, the cleavage rate of short, perfectly matched DNA duplexes by this enzyme is essentially higher than that for nonperfectly matched duplexes of the same length. Thus, DSN differentiates between one-nucleotide variations in DNA. The authors developed a novel assay for SNP detection based on this unique property, termed "duplex-specific nuclease preference (DSNP)". In this assay, the DNA region containing the SNP site is amplified and the PCR product is mixed with signal probes (mutation or wild-type-specific short oligonucleotides labeled by fluorescent resonance energy transfer (FRET)) and DSN (Figure 7).

During incubation, only perfectly matched duplexes between the DNA template and signal probe are cleaved by DSN to generate sequence-specific fluorescence. The use of FRET-labeled signal probes coupled with the specificity of DSN presents a simple and efficient method for detecting SNPs. In model experiments, the authors have demonstrated the robustness of this assay for the typing of SNPs in methyltetrahydrofolate reductase, prothrombin, and *p53* genes on homozygous and heterozygous genomic DNA. Now mutation detection

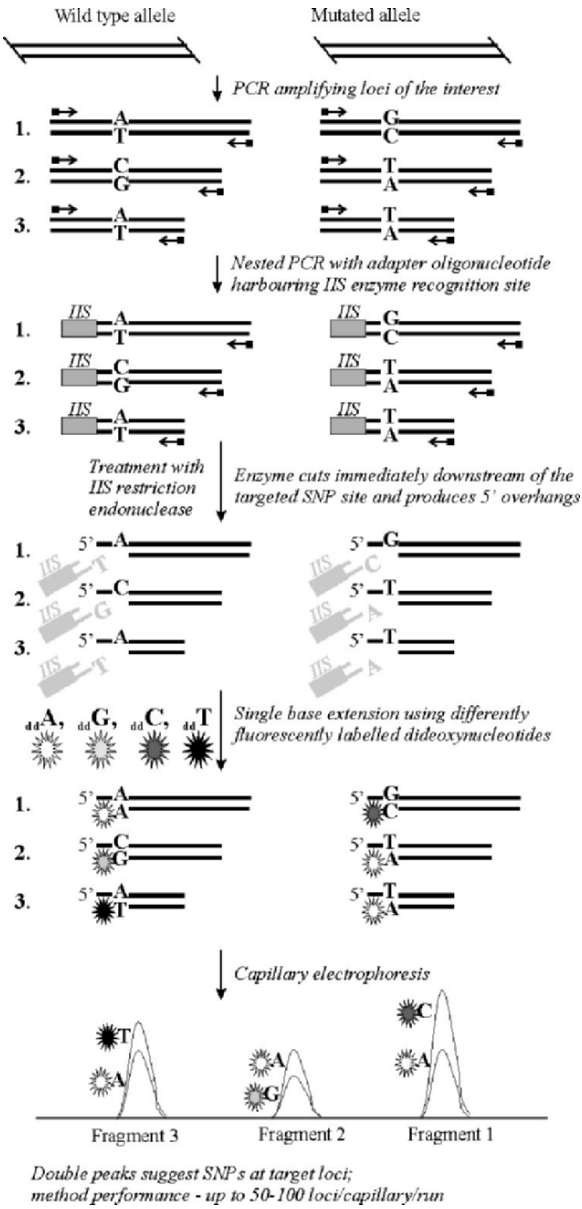

Figure 6. Single-base extension (SBE) technique for multiplex mutation detection. Theoretically, up to 50–100 potent mutation sites may be screened in a single experiment. A restriction site for type II restriction endonuclease is engineered in one of the two PCR primers so that the restriction enzyme cuts immediately downstream of the targeted SNP site. PCR product digestion generates a 5′-overhang structure at the targeted polymorphic site. This 5′-overhang then serves as a template for single base extension reaction to generate allele-specific products using fluorescent dye-terminator nucleotides (e.g. dideoxyribonucleotides). Differentially labeled DNA strands of different lengths can be further resolved in sequencing gels or using capillary electrophoresis.

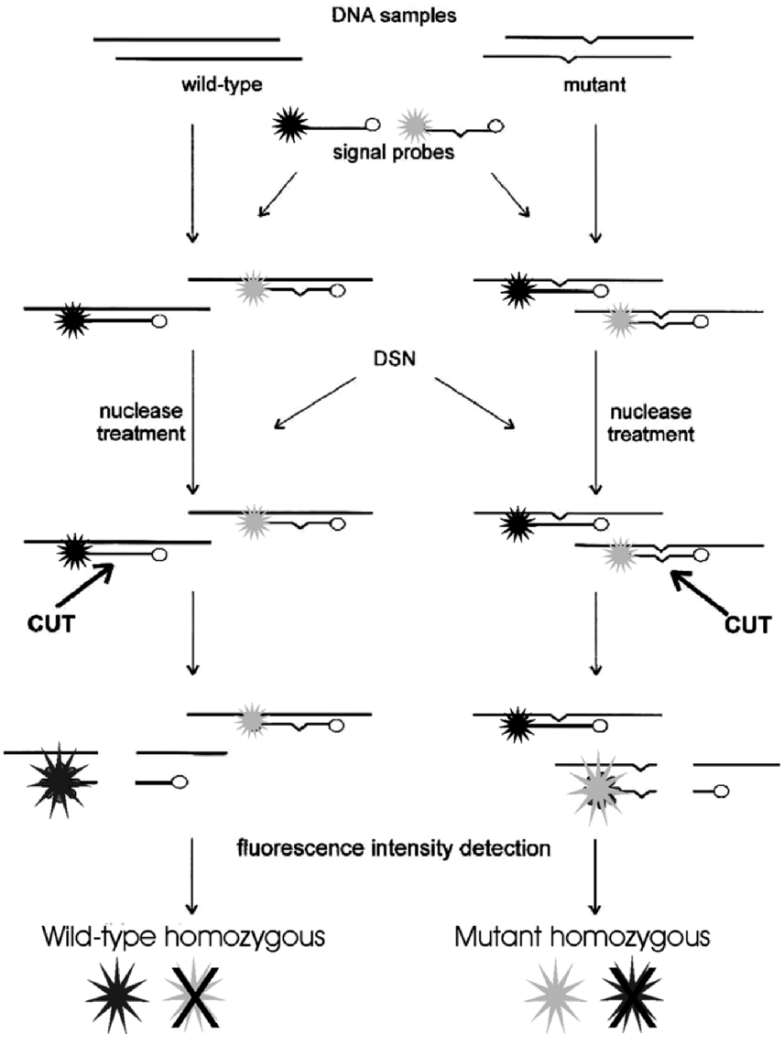

Figure 7. Scheme of the DSNP assay. The black star represents the first fluorescent donor, the gray star the second fluorescent donor, and the open circle the fluorescent quencher.

using this approach is available as a custom service from Eurogene, Inc. The limitation of this promising technique is that no new mutations can be detected.

3.1.3 Artificial endonucleases

The last group of nuclease-based methods for mutation detection involves treatment of the hybridized DNA with artificial nucleases engineered to recognize and to specifically cleave the desired sequence motif. Depending on the further signal detection technique, mutation to be quantified creates or, on the contrary,

disrupts such a recognition sequence. Treatment with the nuclease produces a signal evidencing presence or absence of mutation at the position of interest. For example, PCR-amplified genomic locus to be screened for SNPs is hybridized to a signal probe (mutation-specific short oligonucleotide labeled by fluorophore and quencher at the opposite ends). The hybrids are treated with the nuclease, which cleaves duplexes in the presence of mutation, and screened for fluorescence. When quencher and flurophore are localized close to each other, no fluorescence can be detected (as in the case of undigested duplexes), whereas when duplex is cleaved, the distance between fluorophore and quencher is big enough to allow fluorescence emission.

In principle, the rationale of this approach is quite similar to that of restriction endonuclease-based mutation detection, but the current approach theoretically has an advantage of being able to cut DNA at any desired nucleotide position, as nuclease specificity may be engineered. Such artificial nucleases may be proteins or nucleic acid enzymes – ribozymes and DNAzymes (RNA or DNA molecules able to catalyze single- or double-stranded nucleic acid cleavage exactly at the target site). Artificial nucleases of a protein origin are the chimeras comprised of at least two domains, one of which is a nonspecific DNA cleavage domain and another one is a DNA-binding domain. DNA cleavage domain can be engineered from the endonuclease *Fok* I (Lloyd et al. 2005) or can it be an analogue of the metal-binding loop (12 amino acid residues), peptide P1, which has been reported to exhibit a strong binding affinity for a lanthanide ion and DNA cleavage ability in the presence of Ce(IV) (Nakatsukasa et al. 2005).

Specific DNA binding is provided by several Cys2His2 zinc-fingers that are engineered to bind to specific DNA sequences. The Fok I domain must dimerize to cut DNA, and the zinc-finger pairs function most efficiently when their binding sites are separated by precisely 6 bp. When the metal-binding loop is used, no dimerization is needed, as the problem is solved by connecting two distinct zinc finger proteins with this functional linker possessing DNA cleavage activity. To the date, this modern approach of engineering proteins with targeted nuclease activity has been applied only to the tasks of site-specific mutagenesis and *in vivo* gene targeting in eukaryotes like drosophila and higher plants, and the engineering of nuclease specificity is not trivial. However, further progress in this field may be helpful for recruiting these enzymes in mutation detection.

Unlike the former technique, nuclease ribozymes or DNAzymes may be widely used for mutation recovery. These are RNA enzymes with the targeted DNA or RNA cleavage specificity. The latter is provided by a Watson–Crick base pairing, thus making engineering sequence-specific nuclease a much easier task than in the case of artificial nucleases of protein origin. In addition to target binding motif, ribozyme harbors also a DNA/RNA cleavage domain. Being hybridized with the analyzing DNA or RNA, ribozyme binds to the target site and, if it matches perfectly, cuts the target strand, what can be detected as described above in this section. This promising technique works well in the hands of several research groups (Fiammengo and Jaschke 2005) and has bright perspectives for the future.

3.2 Allele-Specific PCR-Based Approaches

Allele-specific PCR is based on polymerase extension from primers that contain a 3′-end base that is complementary to a specific mutation (Figure 8) and inhibition of extension with wild-type DNA due to a 3′-end mismatch. Using a mutant-specific PCR primer with more artificially introduced 3′-terminal mismatches somewhat adds specificity to this amplification of an allele that differs from the wild-type by a single base pair (Parsons et al. 2005). The presence of a PCR product suggests mutation in the analyzing locus at least in one allele, or at least in one sample (when mixed DNA probes are amplified). Taq polymerase is commonly used for this assay, but because of the high rate of nucleotide extension from primer 3′-base mismatches documented for this enzyme, high sensitivity is difficult to achieve and using other polymerases may be advantageous (Gale and Tafoya 2004). If the enzyme having 3′ → 5′ exonuclease "proofreading" activity is used, than primers must be modified (e.g. by phosphothioate) on the 3′-end to block removal of the critical 3′-mutation-specific base by the polymerase. The protocol can be modified to include a stage of real-time PCR in the procedure.

Following PCR or RT–PCR of a gene segment that may contain allele-specific differences, 100 pg amplified product may be used for a real-time PCR with allele-specific primers and SYBR Green. The use of HEPES buffer at a pH of 6.95 together with AmpliTaq DNA polymerase results in a threshold difference between the correct template and the mismatched template is as many as 20 cycles, depending on the mismatch (Shively et al. 2003). The assay is sensitive, as it permits specific, low-level detection (25 fg DNA) of the SNP, even in the presence of a wild-type allele taken in a 20,000-fold excess (Easterday et al. 2005). Such an approach has been used for many applications, among others, for the

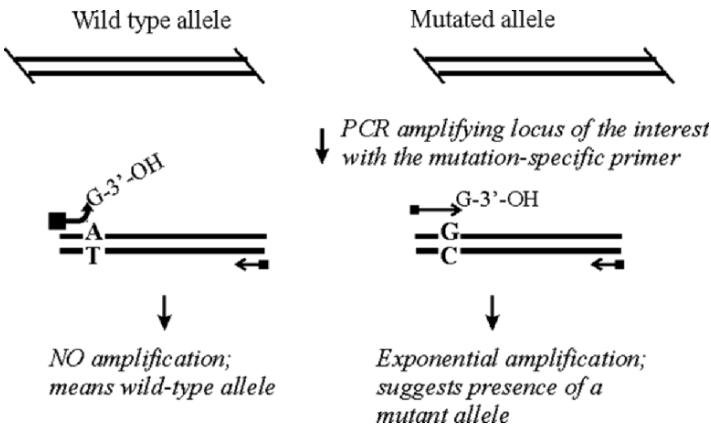

Figure 8. Schematic representation of the allele-specific PCR. PCR primer(s) is (are) generated to selectively amplify one of the two or more alleles, which is provided by 3'-terminal base complementary to a specific mutation or SNP site.

detection of particular strains of viruses and bacteria in environmental samples (Easterday et al. 2005).

The modification of this method termed allele-specific competitive blocker–polymerase chain reaction (ACB–PCR) includes a blocker primer to reduce the amount of background signal generated from the abundant wild-type template. The nonextendable blocker primer preferentially anneals to the wild-type DNA sequence, thereby excluding the annealing of the extendable mutant-specific primer to the wild-type sequence. Inclusion of single-strand DNA binding protein in the ACB–PCR reaction and use of the Stoffel fragment of Taq DNA polymerase both significantly increase allele discrimination. ACB–PCR can detect a base-pair substitution in the presence of a 105-fold excess of wild-type DNA (Parsons et al. 2005). In another variation called LigAmp, two oligonucleotides are hybridized adjacently to a DNA template. One oligonucleotide matches the target sequence and contains adapter sequence for further PCR amplification. If the target sequence is present, the oligonucleotides are ligated together and detected using real-time PCR (Shi et al. 2004).

These methods work well for the qualitative (not quantitative) identification of known SNPs, but are impossible for finding new mutations. In addition, direct PCR assays can be used for the identification of relatively long (more than 15 bp long) insertion/deletion polymorphisms. To this end, primers are designed to flank the site of possible insertion/deletion. PCR fragments of a size greater than expected suggest an insertion in the analyzing locus, whereas shorter PCR products evidence deletion. Using different fluorescent labels, such an approach can be multiplexed, thus allowing analyzing several loci in a single experiment (Diebold et al. 2005).

3.3 Other Enzymatic Approaches for Mutation Scanning

Not only nucleases, but also proteins that specifically bind mispaired nucleotides can be recruited for the mutation analysis. For example, MutS protein is a mismatch binding protein that recognizes mispaired and unpaired base(s) in DNA. Immobilized mismatch binding protein can bind DNA heteroduplexes while allowing homoduplexes to be washed away, thus enriching for rare mutations. In model experiments, unlabeled and fluorescent-labeled oligonucleotides, either perfectly complementary or with single nucleotide mismatches or deletions, were combined to form homo- or heteroduplexes that were further mixed at low ratios of hetero- to homoduplexes and exposed to MutS. Using a capillary DNA sequencer, 29-fold enrichment was detected for oligonucleotides with a single base deletion, whereas a rather modest figure of twofold enrichment was seen for single mismatch oligonucleotides (Baum et al. 2005).

Alternatively, MutS protein chip is available for rapid screening of single-nucleotide polymorphisms. The specific binding of dye-labeled MutS protein with surface-bound DNA or dye-labeled DNA with surface-bound MutS protein is revealed by the obtained fluorescence images (Behrensdorf et al. 2002;

Bi et al. 2003). By measuring the distance from the MutS binding site to DNA ends, one can locate the position of a mutation site. Being a label-free, surface-sensitive technique, quartz crystal microbalance (QCM) (Su et al. 2004) and surface plasmon resonance (SPR) devices have been used to study MutS interactions with the mismatches.

QCM detection principle provides additional information about the structural and viscoelastic properties of adsorbed molecules. The measured motional resistance changes per coupled MutS unit mass (deltaR/deltaf) are found to be indicative of the viscoelastic or structural properties of the bound protein, corresponding to different binding mechanisms. In addition, the deltaR/deltaf values vary remarkably when the MutS protein binds at different distances away from the QCM surface. Thus, these values can be used as a "fingerprint" for MutS mismatch recognition and also used to quantitatively locate the mutation site. However, the robustness of such an approach has not yet been demonstrated in experimental high-throughput screens.

Another mismatch binding protein that can be used for the polymorphism recovery is the cleavage-deficient mutant endonuclease VII that can only attach to mispaired nucleotides (Golz et al. 1998a). The protein can be immobilized on a solid-phase carrier (sepharose) via special tags, thus providing an efficient way to enrich in heteroduplex DNA in several repeated rounds of binding steps (Golz and Kemper 1999). In model experiments, the content of heteroduplex DNAs among the total DNA bound increased from ~10% to 45% after the third cycle of binding with no further increase in additional cycles. This method allows identification of previously unknown mutations, but is not very sensitive, as it allows reliable detection of heteroduplexes in samples with a heteroduplex content starting from 10% (Golz and Kemper 1999).

The technique termed "Glycosylase mediated polymorphism detection" utilizes quite different rationale of using specific glycosylases to detect mimatches (Vaughan and McCarthy 1999; O'Donnell et al. 2001). To this end, target DNA is PCR amplified using three normal dNTPs and a fourth modified dNTP, whose base is a substrate for a specific DNA-glycosylase once incorporated into the DNA (e.g. dUTP). Primers for this amplification are designed so that during extension, the position of the first modified nucleotide (uracil) incorporated into the extended primers differs depending on whether a mutation is present or absent. Subsequent glycosylase excision of the uracil residues followed by chemical or enzymatic cleavage of the apyrimidinic sites allows detection of the mutation in the amplified fragment as a fragment length polymorphism. The technique was shown to be robust and sensitive tool in several applications. However, been published for the first time in 1998 by Patrick Vaughan and Tommie McCarthy (Vaughan and McCarthy 1998), this method never went beyond the use by the same research team.

The last technique described in this section, recently proposed by Li et al., is based on a point mutation detection using high-fidelity DNA ligase (Li et al. 2005). Its protocol has three major steps (Figure 9). First, a hybridization of the target

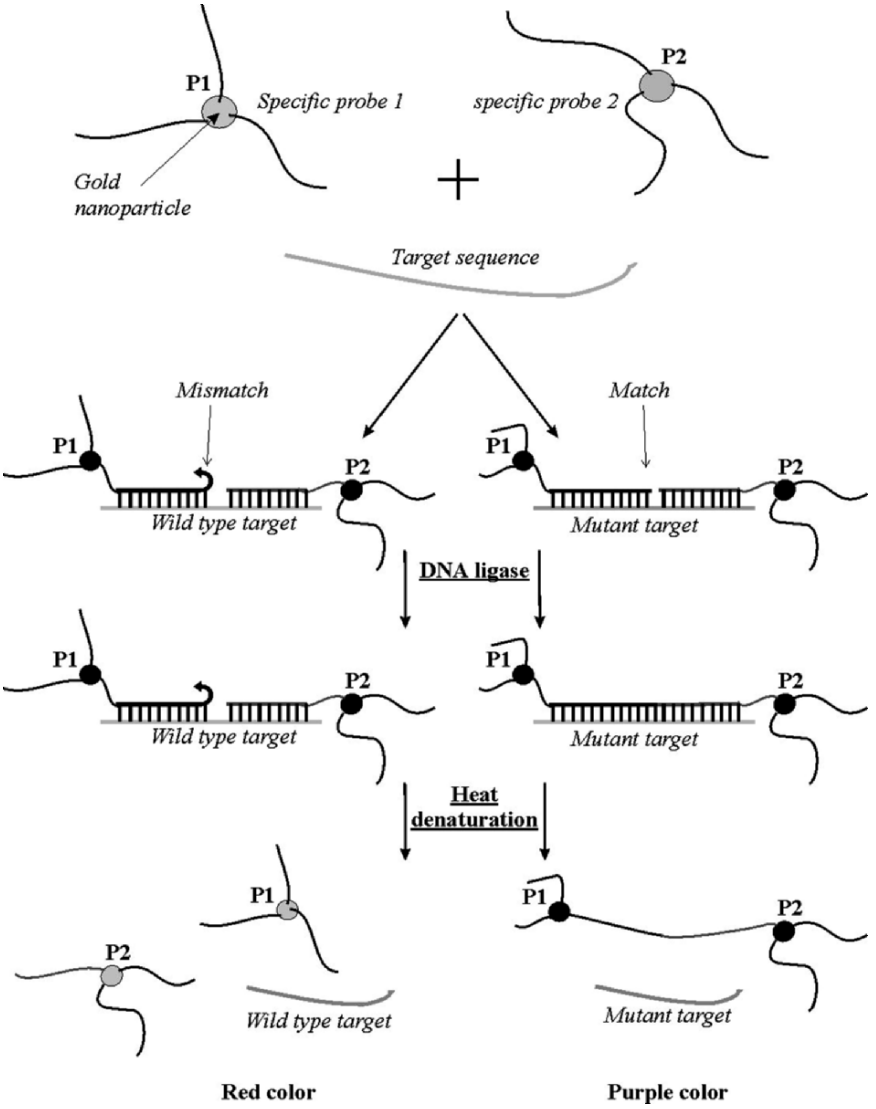

Figure 9. Point mutation detection using high fidelity DNA ligase and two mutation-specific DNA probes, labeled with gold nanoparticles. When the target mutation presents in the analyzing DNA mixture, both probes hybridize perfectly to the target strand, and further ligation step covalently binds both (or more) labeled probes. After heat denaturation the color of covalently attached gold nanoparticle aggregates does not revert to red, in contrast to those corresponding to a wild-type allele.

DNA strand (wild-type or mutant) with two gold nanoparticle-tagged probes occurs. Hybridization results in the formation of an extended polymeric gold nanoparticle–polynucleotide aggregate, turning the solution color from red to purple. At the next stage, high-fidelity ligase is added (Tth DNA ligase in this study) that covalently binds perfectly matched probes while no ligation occurs between mismatched ones. Finally, the mixture is heated to denature double-stranded DNA hybrids. In the case of no ligation occurred, the color reverts to red (as nanoparticles are not bound anymore), whereas in the opposite case the color remains purple. This approach efficacy was demonstrated by scoring single nucleotide mutations in the human oncogene *K-ras* (Li et al. 2005).

4. PHYSICAL APPROACHES

This group of methods look for a physical difference between the mutant strand and wild-type strands of DNA, being based either on physical isolation of imperfectly matched DNA hybrids (like electrophoretic separation), or on finding differences in mismatched versus perfect DNA hybrid physical peculiarities.

One of these approaches is quite similar to well-known protein fingerprinting utilizing the protein molecule digestion with a proteolytic enzyme (usually trypsinization), followed by mass spectrometry analysis of the resulting short peptides. If any protein modification or amino acid substitution occurs, than the position(s) and intensity(s) of the corresponding peaks on the mass specter are changed. Further discovery and development of matrix-assisted laser desorption ionization (MALDI) technique produced revolution in proteomics by making it possible to precisely define peptide amino acid compositions and based on this knowledge to unambiguously identify the whole proteins, even analyzed in complex mixtures. The rationale for this identification is that the number of theoretically possible variants of such peptides for each protein is limited and unique.

Similarly, nucleic acid molecule digested with base-specific enzymes (e.g. uracil DNA glycosylase, RNases A, or T1) or chemicals (see above) yields a set of short oligonucleotides, which can be analyzed using mass spectrometry. If a mutation occurs, then base composition of the respective oligonucleotide will be different from that of the reference sequence, resulting in changes in the resulting mass specter (Bocker 2003). Following analysis with the specialized software, these changes transform to the knowledge regarding (1) type of the mutation (if any) and (2) its localization within the nucleic acid molecule under study. The limitation of such an approach is that not all nucleic acid sequences may be analyzed in such a way: to be informative for mass-specter analysis, small oligonucleotides cannot be too short, but at the same time their length should not exceed 25 nt. To meet these criteria, parallel experiments with treatment using different base-specific reagents are recommended.

Another approach, proposed by Maruyama et al. (2003), is based on the observation that DNA intercalators (including fluorescent dye SYBR Green I

used in this study) bind specifically with a duplex DNA. The fluorescence intensity of mismatched oligonucleotides decreases relatively to perfectly matched oligonucleotides. For example, for 40 bp long duplex containing one mismatched pair of nucleotides the fluorescence decreased by more than 13%. Such an assay can distinguish various types of single-base mismatches, the sensitivity being improved in the presence of 20% formamide. This detection method requires only a normal fluorescence spectrophotometer, an inexpensive dye and just 50 pmol of sample DNA.

Undoubtedly, the most widely used physical approach for mutation detection is the *single-strand conformational polymorphism* (SSCP) (e.g. Maekawa et al. 2004). This simple and cost-efficient technique assists in choosing only the DNA fragments of interest with expected mutation. The principle of detection of small changes in DNA sequences is based on the changes in single-strand DNA conformations created by mutations. The electrophoretic mobility of the respective DNAs differs so that SSCP detects such changes, sometimes in a sequence-dependent manner. The limitations faced in SSCP range from the routine polyacrylamide gel electrophoresis problems to the problems of resolving mutant DNA bands. Both could be solved by controlling electrophoresis conditions and by varying physical and environmental conditions like pH, temperature, voltage, gel type and percentage, addition of additives and denaturants (Gupta et al. 2005).

Microchips, which offer an advantage of screening a wide number of polymorphisms in a single experiment (Maekawa et al. 2004) employ hybridization of the sample probe with short oligonucleotides arrayed on a chip. The hybridization temperature and conditions are optimized so that the sequence variants differing from the arrayed oligonucleotides in one nucleotide or more do not hybridize with the chip. This expensive approach has been extensively used for many applications, including SNP detection in the genomes of eukaryotes, bacteria, and mitochondria (Maitra et al. 2004).

Another promising approach, recently developed by Patolsky and colleagues, termed "enzyme-amplified electronic transduction" (Patolsky et al. 2001), made it possible to perform a quantitative analysis of mutations with no PCR preamplification, with the lower limit of sensitivity for the detection of the mutant DNA as low as 1×10^{-14} mol/ml. The authors designed a thiolated sensing oligonucleotide, complementary to the target DNA as far as one base before the mutation site (Figure 10). After hybridizing of the target DNA, normal or mutant, with the sensing oligonucleotide, the resulting assembly is reacted with the biotinylated nucleotide, complementary to the mutation site, in the presence of DNA polymerase. The labeled nucleotide is coupled only to the double-stranded assembly that includes the mutant site. Subsequent binding of avidin–alkaline phosphatase and the biocatalyzed precipitation of an insoluble product on the transducer, provide means to confirm and amplify signal suggesting detection of the mutant allele. Faradaic impedance spectroscopy and microgravimetric QCM analyses were further employed by the

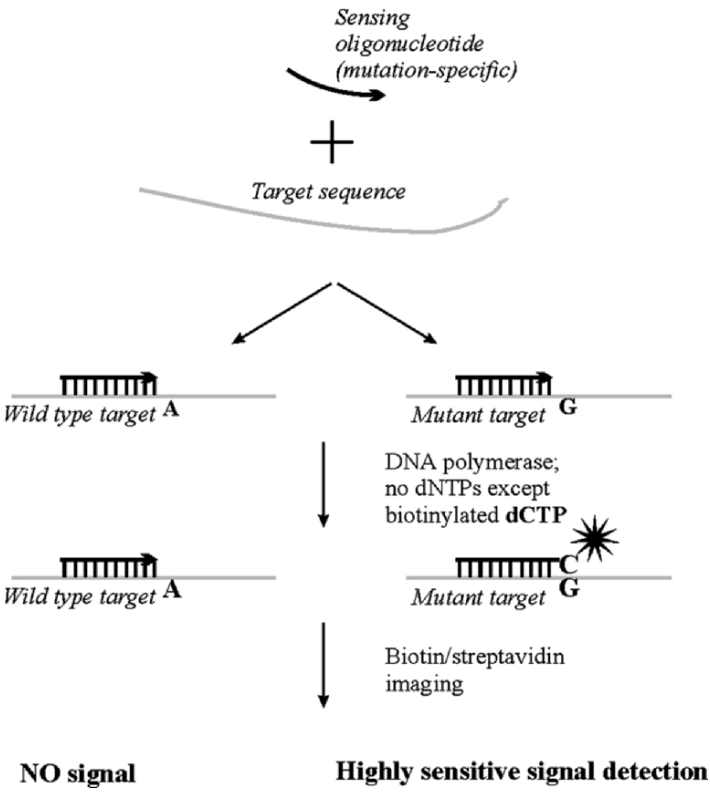

Figure 10. Schematic representation of the enzyme-amplified electronic transduction assay principle (see text).

authors for extremely sensitive and accurate electronic detection of SNPs (Patolsky et al. 2001).

5. BIOINFORMATICAL APPROACHES

These techniques mainly rely on the available sequencing data and deal either with treatment of the information available from databases (Vilella et al. 2005) or with the recognition of polymorphisms from "raw" primary PCR – sequencing data. In the latter case, when more than one allele is PCR-amplified, nucleotide substitutions can be recognized as double peaks on sequencing capillary electrophoregrams (Qiu et al. 2003; Lee and Vega 2004). Another important application is the detection of new bands appeared upon chemical or enzymatic cleavage of the mispaired DNA and the effective discrimination of target against background signaling (Zerr and Henikoff 2005). Overall, bioinformatical techniques provide algorithms or ready-to-use software (Unneberg et al. 2005) to the research community.

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