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Genotyping—From Genomic DNA to Genotype in a Single Tube

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Nucleotide variations in the human genome, such as single-nucleotide polymorphisms, have been researched more intensively since it became apparent that these deviations are linked to various diseases and also several side effects of drugs. The investigation of genomic DNA in the laboratory requires routine methods that are time-, labour-, and cost-effective. These criteria are fulfilled by so-called closed-tube methods, which are applied directly to isolated genomic DNA without any preamplification.

1. Introduction

Within the entire human genome, which comprises approximately three billion base pairs, individuals differ in approximately 0.1 % of their nucleotide sequence.[1,2] The most common of these three million sequence variations are single nucleotide polymorphisms (SNPs).[3–5] SNPs are defined as sites in the genome at which the less common variant has a frequency of at least 1 % in the population. There is a direct link between some of these dissimilarities and certain diseases, a topic dealt with in pharmacogenomics. Additionally, different effects of drugs on different patients can also be linked to SNPs.[3,5,6] Thus, considerable efforts are focused on finding new SNPs and elucidating their connection to certain phenotypes. To date, nearly 1.8 million SNPs have been discovered and characterized through a variety of methods. First, methods are needed to identify unknown nucleotide variations so that their medicinal relevance can be investigated. Once the exact sequence is known, other methods are required for high throughput screening of populations in the search for known SNPs or to analyze individuals for SNP patterns. Thus, time-, labor-, and cost-effective analytical methods are clearly essential to allow routine studies of such nucleotide variations in the laboratory.[7–15] The efficacy of these methods is decisive for further advances in pharmacogenetics, which may, in the future, allow a therapy that is tailored to the genetic constitution of the individual. Treatments with drugs that are ineffective or cause severe side effects due to the genetic basis of the individual may be avoided.

Many methods are known for SNP diagnosis, but none has prevailed so far.[8–15] An important criterion for progress in this field is that the number of working steps must be kept to a minimum. However, most known methods require preamplification of the genomic target sequence through the polymerase chain reaction (PCR) prior to the actual analytical detection step. Hence, these methods usually involve at least two steps.[7–14] Methods that allow genotyping in one step directly from genomic DNA without preamplification would be ideal. In this Minireview, we focus on procedures of the latter type and discuss methods that link the amplification step with analysis in a closed tube reaction. Furthermore, we focus on applications that exploit the properties of many enzymes to preferentially process canonically paired (according to the Watson Crick rule) DNA constructs. For a general overview of methods applied in DNA diagnostics, we refer to several excellent reviews published recently.[7–15]

2. Sequence-Specific Invasive Cleavage of Oligonucleotide Probes: The Invader Assay

The invader assay can be divided into two reactions.[16,17] First, two oligonucleotides termed “signal probe” and “invasive probe” simultaneously anneal to a genomic DNA target sequence. The sequences of both probes are designed to form a characteristic structure when they bind to the target sequence (see Scheme 1 a).

The signal probe consists of two regions: one is complementary to the target and the other consists of a 5′ arm (Flap) and is not complementary to the target and invasive probes. The formed structure is recognized by certain 5′ 3′ nucleases,

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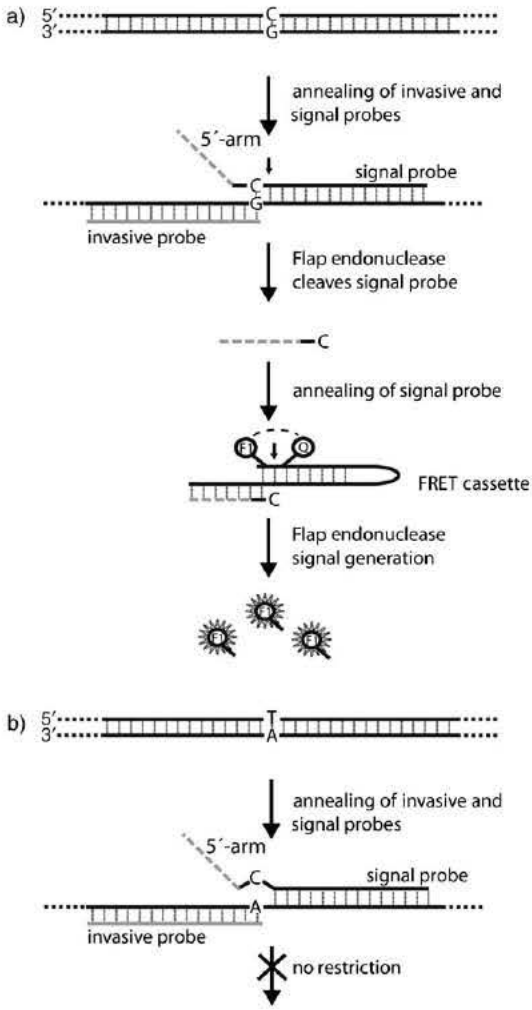

Scheme 1. Genotyping by means of the invader assay. Through annealing of two oligonucleotide probes (signal and invasive probe) a triplex structure is formed at the position of interest. a) In the case of a perfectly matched triplex, the Flap endonuclease is able to cleave the signal probe at the indicated position and the 5′ arm of the signal probe is released. The released 5′ arm anneals sequence specifically to a FRET cassette and catalyzes its restriction by the Flap endonuclease. This restriction cause a spatial separation between the fluorophore (F1) and the quencher molecule (Q), and a fluorescent signal is generated. b) If a noncanonical triplex is formed owing to the presence of a single nucleotide variation, efficient restriction of the formed triplex structure is prevented, and the FRET cassette remains intact, thus causing quenching of fluorescence.

for example, Flap endonuclease I of *Archaeoglobus fulgidus* or DNA polymerase of *Thermus aquaticus* (which exhibits 5′ 3′ exonuclease activity). The signal probe is cleaved at the indicated position to release an oligonucleotide,[18] which is composed of the 5′ arm and one nucleotide derived from its target specific region. Remarkably, the cleavage rate is significantly enhanced by the presence of the invasive probe.[18,19] Furthermore, it is crucial to the cleavage process that the signal probe and target sequence are fully complementary at the SNP site. The discrimination between single nucleotide variations in the invader assay derives from the ability of the enzyme to cleave canonical target signal probe complexes selectively. Single nucleotide variations within the target site (e.g. because of a single nucleotide polymorphism) result in a mismatched complex that is cleaved significantly less efficiently (Scheme 1 b). If reactions are performed at elevated temperatures with an excess of the signal probe, the generated fluorescence signal will be amplified owing to the dissociation of the cleaved signal probe from the target and repeated annealing of a new and uncleaved signal probe.[19]

The signal can be amplified further by using a subsequent reaction step. In this second reaction, the cleaved strand anneals to a fluorescence resonance energy transfer (FRET) cassette and invades a short hairpin, which has a fluorophore and quencher pair at its 5′ end. Cleavage of the hairpin construct at the indicated position is again executed by the 5′ 3′ exonuclease function of the enzymes mentioned above. The cleavage liberates the dye and generates a fluorescence signal owing to the separation of the fluorophore and the quencher. Therefore, the cleaved signal probe catalyzes the cleavage of a FRET cassette and releases a fluorophore in the second step of the invader assay.[20] In practice, both reactions are carried out in the same reaction tube, and an overall signal amplification of up to 10^7 fold within 4 h has been reported.[20] Direct SNP analysis can be performed from samples containing 10 100 ng of human genomic DNA.[17]

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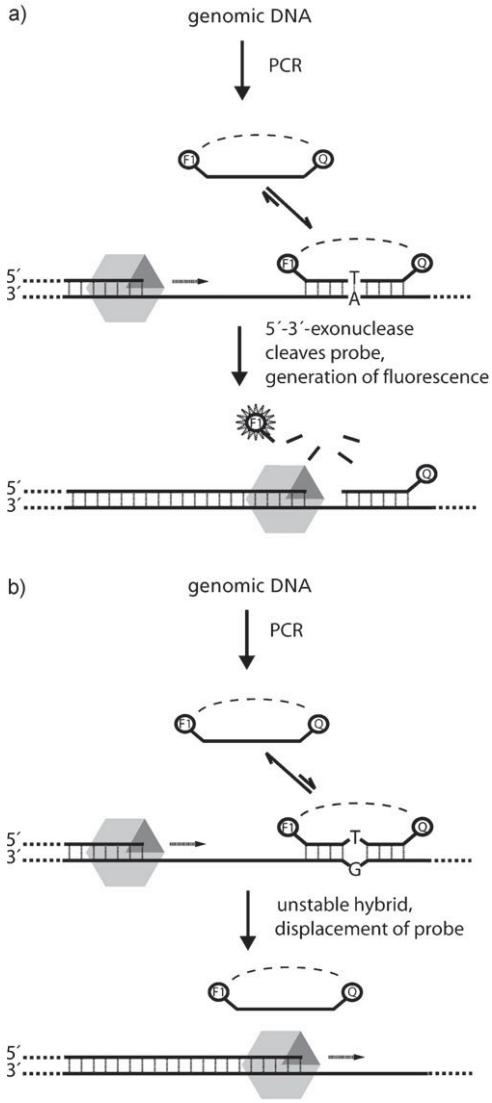
Andreas Marx studied Chemistry in Freiburg, Sussex, and Bochum, where he completed his Diplom with Peter Welzel in 1994. He obtained his PhD in 1997 with Bernd Giese at Basel University. After postdoctoral studies with Hisashi Yamamoto at Nagoya University as a EU/JSPS fellow, he started his independent research on the chemical biology of nucleic acids associated with Michael Famulok at Bonn University and obtained his habilitation in organic chemistry and biochemistry in 2003. Since January 2004 he has held the Chair of Organic Chemistry Cellular Chemistry at Konstanz University.

3. Genotyping through the 5′-3′-Exonuclease Activity of DNA Polymerases: The TaqMan Assay

The 5′ 3′ nuclease proficiency of DNA polymerase of *Thermus aquaticus* is exploited in the TaqMan assay.[21] The characteristic steps of this approach are the DNA synthesis by DNA polymerase, partial strand displacement, and cleavage of a FRET DNA probe by the enzyme to generate a fluorescence signal through separation of the fluorophore and quencher (Scheme 2).[21 24]



Scheme 2. The TaqMan assay. a) The target sequence is amplified through a PCR by using *Taq* DNA polymerase that exhibits 5′ 3′ nuclease activity. The probe sequence is complementary to one allelic variant. b) In the case of the other allele, a mispaired complex is formed. The perfectly matched hybrid (a) is thermodynamically stable, whereas the mispaired hybrid (b) is thermodynamically unstable at the applied temperature. As strand extension proceeds, the 5′ 3′ nuclease activity of *Taq* DNA polymerase cleaves canonically bound probes and releases the fluorophore, thus resulting in the generation of a signal. In the case of noncanonical probe target sequences, the displacement of a portion of the probe by the DNA polymerase lowers the thermodynamic stability even further, and the probe is released. Thus, cleavage of the probe is prevented and hence no signal is generated.

Several factors contribute to the capability of this approach to discriminate between single nucleotide variations. First, probes with complementary sequences form duplexes with the target site that are inherently more stable than those that contain mismatches.[22] Accordingly, judicious tuning of the annealing and extension temperatures in the PCR favors formation of a perfectly matched duplex over a mismatched duplex. Duplex formation is mandatory for the 5′ 3′ nuclease action of the DNA polymerase. Recognition and cleavage by an enzyme occur at the bifurcated end of a duplex. Therefore one to three nucleotides at the 5′ end of the probe must be displaced prior to cleavage of the probe. This further destabilizes a mismatched probe and promotes its dissociation, thereby preventing cleavage and signal formation. A crucial parameter for the success of this approach is the design of the probe. The probe must be long enough to build stable duplexes at the extension temperature ($\approx 70\,^{\circ}C$), but must be short enough to discriminate sufficiently between single nucleotide variations within the target site. It has been shown that probes that are conjugated with a minor groove binder have higher binding affinity to a complementary sequence and can still be processed by enzymes (Figure 1).[22,25,26] This allows application of shortened fluorogenic probes, which have better sequence selectivity.

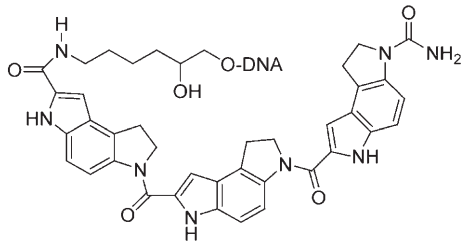
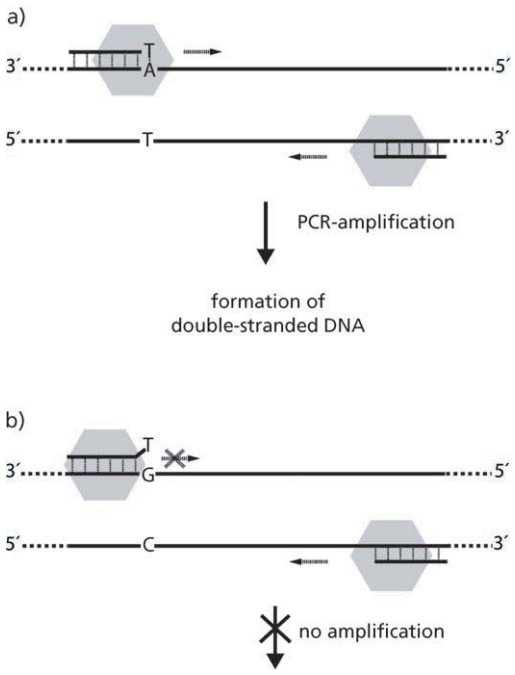


Figure 1. Structure of the DNA minor groove binder conjugated to DNA.

The entire process is carried out in a closed tube and does not require further post PCR handling and processing steps. Additionally, the analysis can be conducted and monitored in real time. The assay is sensitive and only minute amounts of genomic DNA (2 20 ng) are required.[22]

4. Genotyping through DNA Polymerase Selectivity: Allele-Specific Amplification

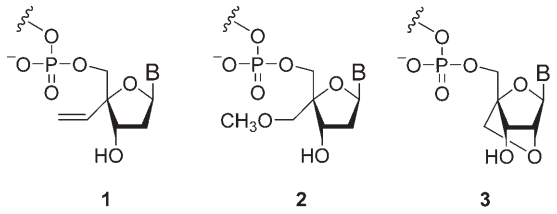
A conceptually simple approach to detect allele variations is realized in allele specific amplification (ASA). ASA is used to determine nucleotide variations through either the presence or absence of a DNA product obtained by PCR amplification. The principle of ASA is based on the formation of matched or mismatched primer template complexes through allele specific primer probes. PCR amplification by a DNA polymerase proceeds from matched 3′ primer termini, whereas a mismatched primer terminus inhibits amplification (Scheme 3).[27 34]



Scheme 3. Allele specific amplification. The PCR primer pair is designed to amplify a part of the genomic DNA of interest. A 3′ end of a primer strand is positioned opposite to the SNP position, resulting in a matched (a) or a mismatched (b) complex, depending on the nature of the target nucleotide. In the case of the matched hybrid, the DNA polymerase elongates the primer which results in the exponential amplification of the PCR product. The elongation of the mismatched primer is hampered, and thus the formation of the PCR product is prevented.

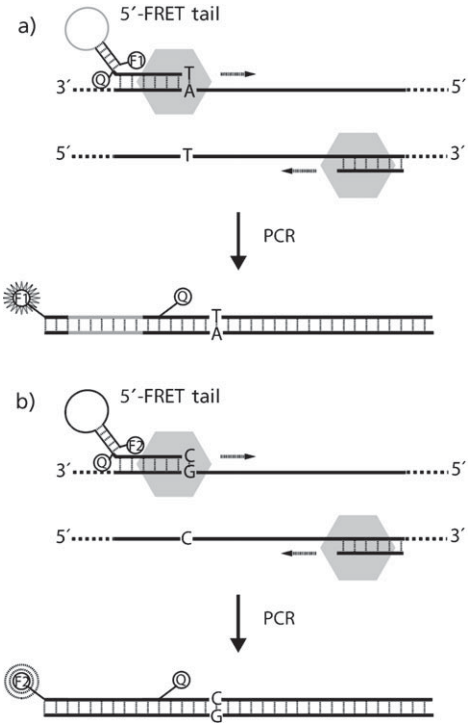
Ideally, the extension of a mismatched primer end by the DNA polymerase should be discriminated. In the case of a mismatch, exponential product formation by PCR is prevented, in contrast to the situation when both primers are complementary to the target site. Product formation can be detected by gel chromatography or monitored in real time by using a double stranded DNA specific dye (e.g. SybrGreen I), which shows fluorescence after binding to the PCR product in a sequence independent manner.[35] The main advantage of this approach is that no relatively expensive fluorescence probes are required. However, only the formation of double stranded DNA is detected without any sequence specificity and therefore false signals are generated from unspecific amplification, for example, the formation of primer dimers. To prevent the detection of false amplificates, sequence specific FRET probes (e.g. molecular beacons) can be employed.[36] Such probes bind sequence specifically to the amplificate and thus link signal generation to amplification of the desired sequence. In many cases, however, the method still requires tedious costly and time consuming optimization. Accordingly, any factors that increase the selectivity and reliability of the allele specific PCR approach should have a significant impact on the reliability and robustness of direct SNP analysis by ASA.

Recently, it was reported that the selectivity of allele specific PCR can be significantly increased through the employment of chemically modified primer probes.[37 41] Significantly higher amplification selectivity is observed by application of primer probes that bear small 4′ C modifications such as vinyl (**1**) or methoxymethylene (**2**) groups at the 3′ end. A commercially available 3′ 5′ exonuclease deficient variant of a DNA polymerase from *Thermococcus litoralis* (Vent(exo−) DNA polymerase) was used for amplification (Scheme 4).[37 39]

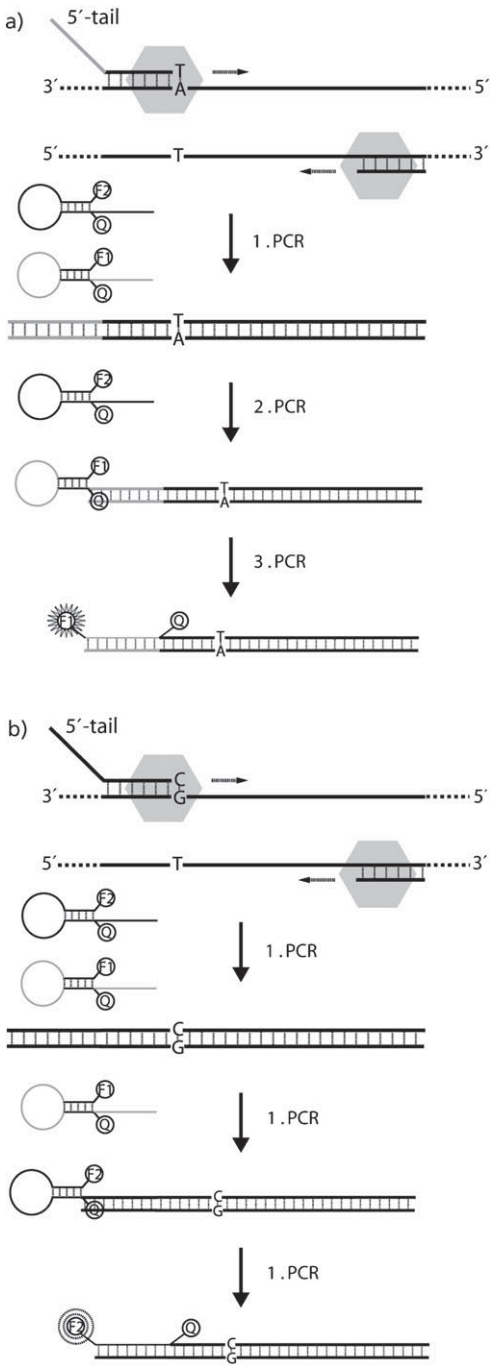


Scheme 4. Structures of modified primer strands employed in allele specific PCR. B nucleobase.

As already mentioned, the identification of single nucleotide variations within genes is feasible in real time. This makes real time PCR a rapid and convenient tool for the identification and analysis of allele variations. Interestingly,



Scheme 5. Multiplex allele specific amplification. Each allele specific primer is coupled to a specific oligonucleotide that is not complementary to the target tail sequence and forms a hairpin structure. Each stemloop bears a fluorophore quencher FRET pair. During PCR the primer strands are elongated in an allele specific manner, and the loop sequence is linearized. This leads to a separation of the fluorophore and quencher, and a fluorescent signal is generated. The wavelength of the construct is therefore coupled to the allelic variant, as shown for genotype A (signal F1 in (a)) and genotype G (signal F2 in (b)) as examples.



Scheme 6. Multiplex allele specific amplification. Each allele specific primer bears a specific tail sequence that is not complementary to the target sequence. During PCR, the allele specific primer is elongated, and the tail complementary sequence is added to the PCR product. An allele specific primer bearing a priming and a stem loop structure hy bridizes to this sequence and is therefore coupled to the PCR product. During the proceeding PCR, the stem loop is linearized and results in a fluorescence signal. The wavelength of the construct is therefore cou pled to the allelic variant as shown for genotype A (signal F1 in (a)) and genotype G (signal F2 in (b)).

modified building blocks that are bridged with a 2′ *O* 4′ *C* methylene bridge (**3**) at the 3′ end of primer probes exhibit similar features (Scheme 4).[42]

So called multiplex reactions in which two allele variants are differentiated in a single reaction tube are realized by adding a specific 5′ tail sequences to each allele specific primer. The signal generation is thereby directly linked to the allele specific primer in the PCR amplification. Two hairpin FRET structures bearing different fluorophores (which emit at different wavelengths) are added as an overhang sequence to the discriminating primer (Scheme 5).[36] During PCR, the overhang becomes double stranded and the hairpin is opened, thus resulting in an allele specific fluorescence signal.

In another approach, a specific tail complementary se quence is added which can be detected by the use of overhang primers as described above.[43] This method has the advantage that the primers that bear the overhang FRET cassette are independent of the target sequence and can therefore be used in a universal manner (Scheme 6). The same pair of FRET primers can be used for each sequence, and only the simple, allele specific primers are varied. Allele discrimination was achieved with 40 ng of genomic DNA.

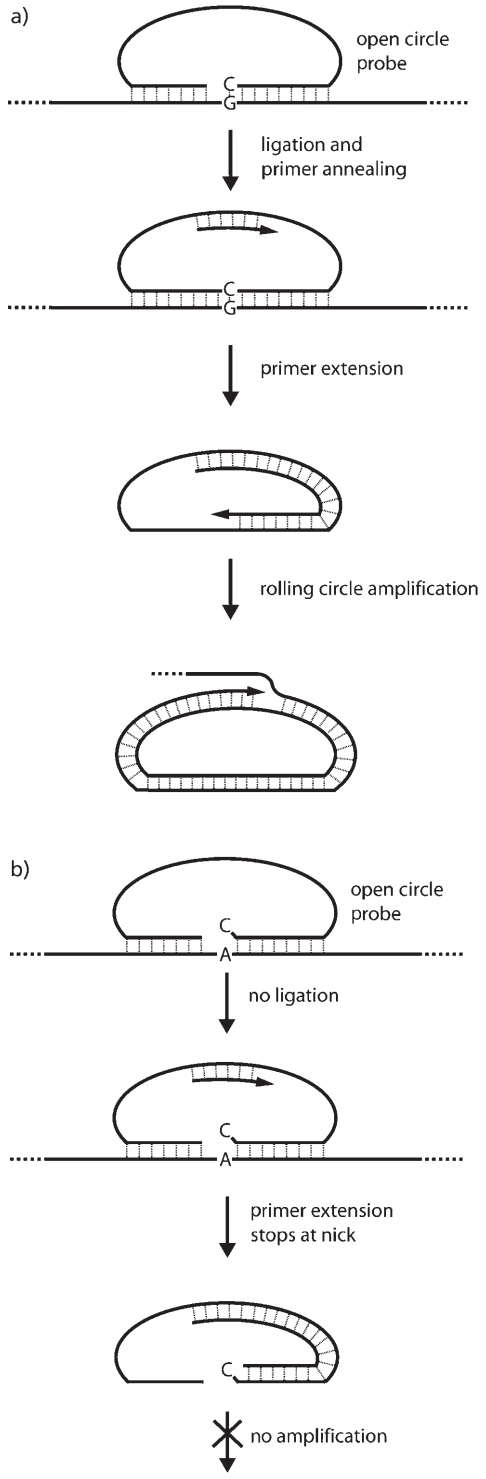
The ability of the enzyme to discriminate between mismatched primer ends was increased by mutating the DNA polymerase. A *Taq* DNA polymerase variant was identified that exhibits significantly higher single nucleotide discrimination than the wild type enzyme in allele specific PCR.[44] This goal was accomplished through a combinatorial approach by randomization of a gene cassette of a DNA polymerase and subsequent screening for variants with higher mismatch discriminating properties.

DNA diagnostic methods that do not employ labeled probes are highly promising candidates for further develop ments in terms of selectivity and robustness, as costly fluorescent probes could be replaced by standard primer strands that are required for PCR amplification anyway.

5. Isothermal Genotyping by Circularized Oligonucleotide Probes: Padlock Probes and Rolling Circle Amplification (RCA)

Padlock probes are circularized single stranded oligonu cleotides made up of 70 110 nucleobases. They are designed in an open circle form with two target complementary regions at each end (see Scheme 7).[45]

In the presence of a complementary target sequence, the two ends of the open circle oligonucleotides are covalently connected by a DNA ligase to form circularized padlock probes. It is possible to degrade open probes that are not ligated as a result of a noncanonical target sequence by treatment with exonucleases, whereas closed circles are resistant and remain intact. In many applications, the formation of circularized probes is followed by isothermal amplification by using a primer derived from a central region of the padlock probe. This results in hundreds of tandemly linked copies of the padlock probe. For this copy process, termed rolling circle amplification (RCA),[46] a DNA poly merase with strand displacing properties is required. Typi cally, DNA polymerase *Φ*29 is used, but there are also applications in which thermostable DNA polymerases are employed.[47,48]

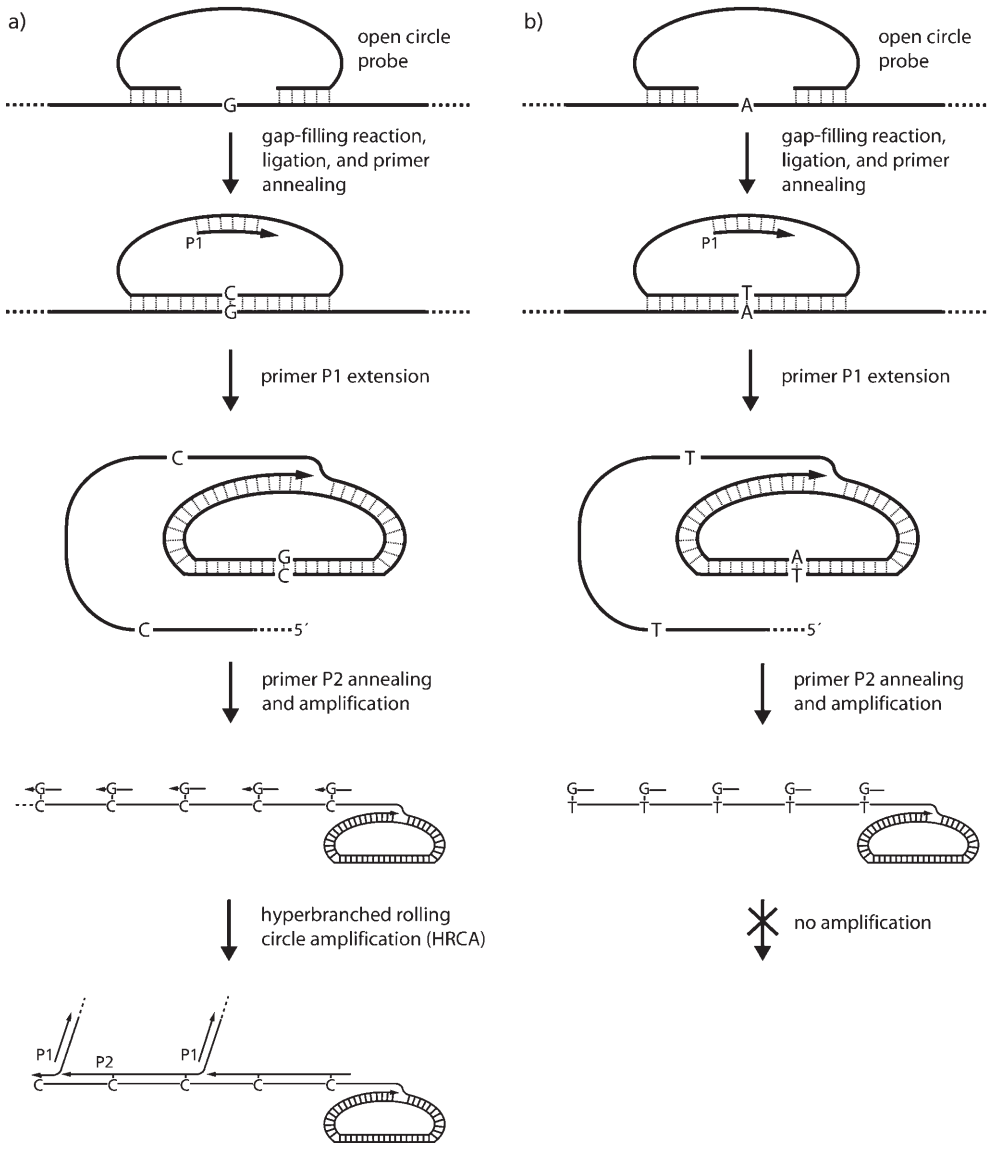


Scheme 7. Rolling circle amplification (RCA). a) In the first step, the open circle probe (OCP) anneals to the target sequence with both of its target complementary regions. Both ends lie adjacent to each other and form a nick. In the case of one allelic variant the 3′ end forms a perfectly matched hybrid (a). In the other case, a mismatch is formed (b). The perfectly matched ends are ligated by a DNA ligase and a cir cular molecule is generated. In the case of the mismatched complex, ligation cannot take place. The reaction mixture also contains an oligo nucleotide that is complementary to a part of the OCP and that can serve as a primer. A DNA polymerase elongates this primer and stops at the nick site of the OCP in the case of a nonligated molecule (b). In contrast, if the probe is circularized by the DNA ligase, the elongation can proceed (a). After one round of amplification, the already hybri dized oligonucleotide sequence is removed by the DNA polymerase strand. This results in thousands of tandem repeats of the comple mentary sequence of the closed circle.

The most common applications of padlock probes in allele diagnostics make use of the mismatch discriminating proper ties of various enzymes, often in combination with hybrid ization assays. In the DNA ligase dependent method, the open circle probe is designed to form either a perfectly matched or a single mismatched 3′ complementary region according to the target sequence.[46] The mismatch discrim inating properties of the DNA ligase applied allow circular ization of the probe in the former case. In contrast, no ligation occurs in the second case owing to the mismatched primer target complex.[49] Additionally, the 5′ complementary part of the open circle probes (OCP) is designed to hybridize tightly with the target sequence. On the other hand, the 3′ region is in an association dissociation equilibrium with the target be cause of a calculated dissociation temperature that is 10 °C lower than the reaction temperature of 60 °C.[50] Accordingly the specificity of the ligation reaction is enhanced owing to the favorable hybridization of the perfectly matched OCP. After ligation, linear isothermal RCA can be used to determine whether the probe was circularized or not.

An additional application of padlock probe ligation followed by RCA makes use of the mismatch discriminating properties of DNA polymerases.[47] The OCP is designed to form a gap of typically seven nucleotides between the target complementary parts (Scheme 8). This gap includes the sequence context surrounding the SNP site to be studied. In a first step, a DNA polymerase is used to fill the gap by copying the sequence context into the padlock probe. After the gap filling reaction, a DNA ligase is used to circularize the probe. Subsequently, the circular padlock is amplified by RCA. The linear single stranded product bears hundreds of tandemly linked repeated copies of the padlock probe, including the incorporated sequence context. These sequen ces are hybridized to an allele specific primer (P2) and form either a perfectly matched (Scheme 8a) or mismatched (Scheme 8b) 3′ end.

Afterwards, the mismatch discriminating properties of a DNA polymerase such as Vent(exo−) are used in an allele specific amplification to distinguish the allelic variants. The products of this second amplification represent tandem repeats of the original padlock probe. These sequences bear multiple binding sites for the first RCA primer (P1). The extension of primers bound to these sites generates a continuous pattern of double stranded DNA with branches and is therefore called hyperbranching RCA (HRCA). In contrast to allele specific PCR, mispriming events will only incorporate the incorrect base in the primer itself so that nonspecific priming is a nonpropagating event in HRCA. By this method up to 10^9 copies of each circle can be generated in 90 min.[47]



Scheme 8. Hyperbranching rolling circle amplification (HRCA). The two ends of the open circle probe hybridize to the target strand in such a way that a short gap is formed. In a first gap filling reaction, a DNA polymerase closes the gap by copying a part of the target sequence into the probe. Subsequently, a ligase circularizes the probe and RCA can proceed, starting from the primer P1. The single stranded product includes repetitions of the short incorporated sequence containing the SNP site. A second oligonucleotide with a 3′ end is complementary to one allelic variant at the SNP site and serves as a primer (P2) in a second reaction. It can only be elongated when it is perfectly matched (a), whereas no extension pro ceeds in the mismatched case (b). The product of this extension consists of the original circle probe sequence. Thus the first primer P1 can bind to these new synthesized products, and the target sequence is amplified. The result is the amplification of a highly branched DNA product.

In 2001, Qi et al. introduced a procedure to perform DNA probe ligation and RCA under identical buffer conditions which enabled closed tube or closed microplate formats.[48]

6. Outlook

Currently, all known methods exhibit advantages and disadvantages, and none of the described procedures has prevailed so far. Thus, improvements are needed to facilitate general application in daily practice.

To ensure allelic discrimination in a more robust and selective manner, methods that use enzymes in the discriminating step might be further optimized by mutagenic engineering the respective enzymes. The high costs associated with the assays are still a matter of concern and prevent general application. These costs could be reduced by the development of methods that do not make use of probes with covalently linked dyes, as their synthesis is time and cost intensive.

The development of cheaper and faster closed tube detection methods should put the aim of adopting SNP detection for general use in clinical practice within reach.

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