Tuning PCR Specificity by Chemically Modified Primer Probes**

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Dedicated to Professor Peter Welzel
on the occasion of his 65th birthday

Since the publication of the first draft of the human genome sequence in 2001, the discovery of genomic dissimilarities such as single nucleotide polymorphisms (SNPs) between different individuals has been a main focus of many research...
efforts.[3] This is an specially intriguing issue since it is becoming increasingly apparent that variable effects of drugs on different patients or predisposition for various diseases can often be linked to variations in the genome sequence.[1] In the future, the exact knowledge of medicinally relevant nucleotide variations may allow any therapy to be adapted to the genetic equipment of the individual and would abolish treatment with drugs that are ineffective or cause severe side effects.[3] Methods that allow time and cost efficient verification of nucleotide variations should lead to further advances in pharmacogenetics.[1,2]

Many methods for the detection of nucleotide variations in genes have been described to date.[2] Each method exhibits advantages and disadvantages, and thus no methodology has prevailed so far. Most known methods are applied after amplification of the target genome sequence through the polymerase chain reaction (PCR). Thus, after isolation of the genetic material, the sequence of interest has to be amplified by PCR prior to the analytical step determining nucleotide variation.[3] Methods for the direct detection of nucleotide variations through PCR are rare. Allele specific amplification (ASA) determines nucleotide variations through either the presence or absence of a DNA product obtained through PCR amplification.[3] The principle of ASA is based on the formation of matched or mismatched primer template complexes through allele specific primer probes. From matched 3’ primer termini PCR amplification by a DNA polymerase proceeds, while a mismatch should obliterate amplification. Nevertheless, there have been many reports indicating the low selectivity of this approach, thus necessitating further tedious time and cost intensive optimizations.[3] Therefore, any means of increasing the selectivity of allele specific PCR amplification should have significant impact on the reliability and robustness of direct SNP analysis by PCR.

Recently, we reported that nucleotide insertion selectivity of a DNA polymerase can be increased by chemical means by using 4’ alkylated nucleotides.[3] The supplemental small modifications are believed to cause less flexibility within the DNA polymerase substrate complex and thus decrease the tolerance for formation of geometrically altered nascent nucleotide pairs.[5, 6] Based on this model, we speculated that the flexibility at the 3’ primer end can be decreased as well through these modifications, resulting in lower DNA polymerase propensity for extension of geometrically altered mismatched primer template complexes. Such a system could in turn be the basis for the development of a novel highly allele specific PCR approach. Here we show that this concept is indeed applicable and report on a significant increase of the specificity of PCR by the use of chemically modified primer probes. Our results represent a major advancement in the development of robust methods for the detection of nucleotide variation through PCR.

We first synthesized DNA strands bearing 4’ modified thymidine residues at the 3’ terminal position and subsequently used these DNA strands as primers in primer extension assays.[6, 7] First, we screened several thermostable DNA polymerases in their ability to extend from 4’ modified primer ends, which are either bound to the template according to the Watson Crick rule or which cause a single mismatch through a mutated template nucleotide (Figure 1).[8] To mimic a cycle in PCR we employed reaction conditions commonly used in standard PCR protocols and used reaction buffers as recommended by the supplier.[7] Screening several commer-

![Figure 1. Effects of 4’ modifications on the primer extension catalyzed by Vent.](Image)

A) Section of the primer template sequences employed. B) Results of primer extension reactions obtained when canonical (X = A) or noncanonical (X = G, C, or T) primer ends were employed. The respective template nucleobases and 4’ substituents are depicted in the figure. Lanes 1, 6, 11, 16: primer only.[7]

A) Primer Template

B) Results of primer extension reactions obtained when canonical (X = A) or noncanonical (X = G, C, or T) primer ends were employed. The respective template nucleobases and 4’ substituents are depicted in the figure. Lanes 1, 6, 11, 16: primer only.[7]
observed effects on mismatch discrimination are exclusively linked to thymidine residues. Following a published procedure for the synthesis of 1,[9] we synthesized the modified solid support 2 bearing adequately protected 4′-vinylated 2′-deoxycytidine (Figure 2A).[10]

The required 4′ modified primer probes were synthesized by means of automated DNA synthesis by employing the modified solid supports. Subsequent primer extension studies with 4′-vinylated primer and Vent− conducted under identical conditions as above led to results similar to those obtained with 4′ modified thymidines. The 4′ vinylated 2′ deoxycytidine residues likewise show significant beneficial effects on the apparent mismatch discrimination by enzymatic primer extension.

Encouraged by these promising findings we next explored whether the results of the primer extension can be further amplified by means of automated DNA synthesis by employing the modified primer only.[7] The respective template nucleobases and 4′ substituents are depicted in the figure. Lanes 1 and 6: primer only.[7]

Figure 2. Synthesis of probes containing 4′-vinylated 2′ deoxycytidine and their effects on primer extension. A) Synthesis of solid supports bearing 4′ modified 2′ deoxycytidines. Reagents and conditions: a) TBAF, THF, 25°C, 4 h; b) DMTrCl, cat. DMAP, pyridine, 25°C, 14 h; then nitrophenol, 25°C, 14 h, then piperidine, 25°C, 5 min, then acetic anhydride, DMAP, pyridine, 25°C, 14 h. C= tert butyldimethylsilyl; TBDPS = tert butylidiphenylsilyl, DMTr = 4′ dimethoxytrityl; DMAP = dimethylaminopyridine; LCAA CPG = long chain alkyl amine modified controlled pore glass; EDC = 1(3 dimethylamino propyl) 3 ethykarbodimide hydrochloride. B) Section of the primer template sequences employed. C) Results of primer extension reactions obtained when canonical (X = G) or noncanonical (X = A, C, or T, respectively) primer ends were employed. The respective template nucleobases and 4′ substituents are depicted in the figure. Lanes 1 and 6: primer only.[7]

To gain detailed quantitative insight into this process we employed radioactively labeled primer probes and analyzed the ratio of product amplification after every cycle.[7] The obtained results clearly show the highly beneficial effects of our modified primer probes on PCR amplification selectivity (Figure 4). For instance, 4′ vinylated primer termini are proficiently elongated in matched cases with somewhat lower efficiency than the unmodified probes. Nevertheless, single mismatches at the primer end prevent efficient amplification significantly more pronounced in the 4′ modified cases resulting in a more selective PCR amplification with respect to a single nucleotide variation at the primer end.

Figure 3. Effects of modified primer probes on the selectivity of PCR amplification. A) Section of sequences employed. PyR = pyrimidine residues bearing 4′ hydrogen atom or 4′ vinyl modifications; X = respective template nucleobase as depicted in B) and C). B) Results obtained with primer probes bearing 4′ vinylated thymidine residues. C) Results obtained with primer probes bearing 4′ vinylated 2′ deoxycytidine residues. M = size marker; Lanes 1, 10: Experiments conducted in the absence of template and in each case with unmodified primers or 4′ modified primers.[7]

In summary, we report on effects of 4′ modified primer probes on single mismatch discrimination in allele specific PCR amplification. We found that in all investigated single mismatches, significantly higher amplification selectivity is observed by use of 4′ vinylated primer probes and Vent−DNA polymerase compared to cases were unmodified primers were used. This study reveals that small chemical modifications of DNA can trigger distinct enzymatic responses.
es that are not easily accessible with unmodified substrates. Considering recent developments in the use of real time quantitative PCR as an analytical tool for detecting variations in nucleotides,[11] these findings should have significant impact on the development of reliable and robust methods.


[7] Detailed experimental procedures as well as DNA sequences applied are provided in the Supporting Information.


