



PNA Clamp Technique for Detecting a Ki-ras2 Mutation Using the LightCycler Instrument



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Literature indicates that point mutations in codon 12 of the Ki-ras2 gene are associated with colon cancer [1]. The detection of a point mutation in the high background of wild-type cells is very difficult, which represents a problem for many research projects focused on processes that take place during cancerogenesis. Therefore, a quick and easy, yet reliable method of detecting single point mutations is preferred. This article shows an application example for Peptide Nucleic Acid (PNA) oligomers in combination with Hybridization Probes on the LightCycler Instrument. Here, PNAs suppress the codon 12 wild-type PCR product, as they bind to nucleic acids with higher stringency, compared to deoxyribonucleotides, and do not serve as primers for Taq polymerase. Due to these properties, PNAs can be used to detect single point mutations in research samples in a high background of wild-type sequences.

Introduction

According to the literature, mutations in the Ki-ras2 gene have been implicated in approximately 50% of colon adenocarcinomas [1]. The gene codes for a 21 kDa

GTP-binding protein which controls the mechanisms of cell growth and differentiation [2]. The Ki-ras2 gene is converted to an active oncogene by point mutations in codons 12, 13, or 61, in a region that may be involved in GTP binding. Since sample material normally contains different amounts of unaffected wild-type cells, the detection of these point mutations represents a problem in many research applications. In Minimal Residual Disease (MRD), only a few cells exist that must be detected. For example, analyzing the codon 12 Ki-ras2 point mutation by artificial restriction fragment length polymorphism (aRFLP) is very time-consuming [3-5]. Therefore, the application of a PNA oligomer, in combination with LightCycler Hybridization Probes, is a quick and convenient way to detect point mutations in research samples. PNA binds the complementary sequences tighter than DNA or RNA, and cannot be extended by Taq DNA polymerase. Originally, Thiede *et al.* described a method to prevent PCR amplification of wild-type Ki-ras2 chromosomal DNA using a wild type-specific PNA oligomer [6]. Recently, this method was modified for use with the LightCycler Instrument in combination with Hybridization Probes [7]. Now, PNA binds to the amplified Ki-ras2 sequence and lowers the amplification of the complementary wild-type sequence by competing with the mutation-specific hybridization probe primer (sensor) for the wild-type sequence (Figure 1). In this configuration, only the mutant-specific signal is obtained, allowing the identification of the mutation present in the sample. Here, the use of PNA clamping in combination with Hybridization Probes in

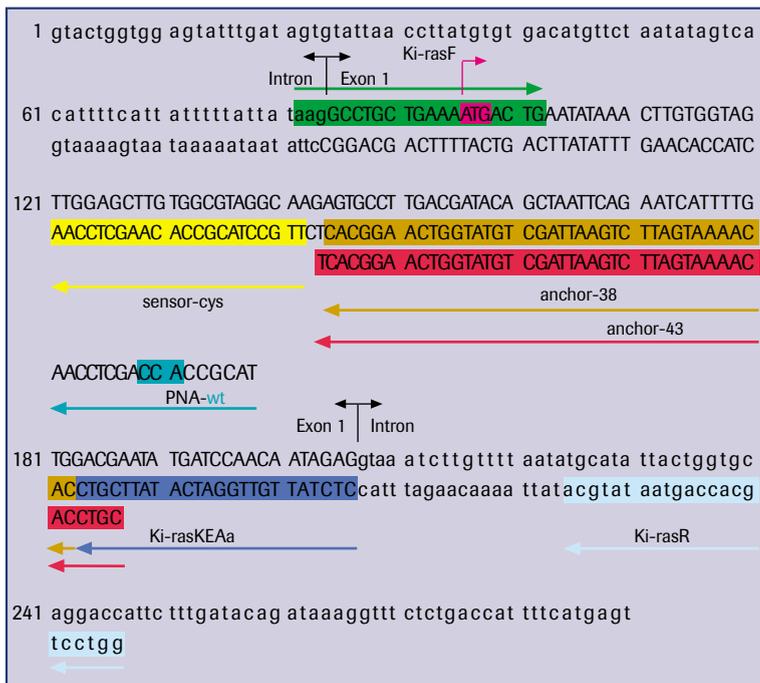


Figure 1: Sequence and position of the Ki-ras2 target sequence (GenBank Accession # L00045) are shown. Exon sequences are typed in upper-case letters, intron sequences in lower-case letters. The PCR primers sense (green) and antisense (blue), as well as the Hybridization Probes sensor (yellow), anchor (red), the PNA oligomer, and intron/exon boundaries are indicated.

the LightCycler Instrument to detect the codon 12 point mutation in the Ki-ras2 gene and mRNA is described.

Materials and Methods

Isolation of DNA / total RNA and reverse transcription

Chromosomal DNA and RNA from biopsy research samples and from the cell line SW480 differing from wild-type Ki-ras2 at codon 12, but not at codon 13 [8–9], and chromosomal DNA from stool were isolated using commercial kits. cDNA synthesis from total RNA was performed using a two-step protocol in a 25- μ l reaction volume, total RNA, and 2 μ l 10x random hexamers. The reaction was incubated for 60 minutes at 42 °C [5].

PCR amplifications

PCR was performed with the LightCycler FastStart DNA Master Hybridization Probes mix using 3 mM MgCl₂, and 25% of the reaction volume of DNA or cDNA in a dilution of 1:10. For parallel analysis of DNA and cDNA, primer pairs Ki-rasF and Ki-rasKEAa (generating a 125-bp PCR fragment), in combination with anchor-38, were used. For DNA analysis only, primer pairs Ki-rasF and Ki-rasR (165 bp), in combination with anchor-43, were used.

The PCR primers were: Ki-rasF 5'-AAG GCC TGC TGA AAA TGA CTG -3' (forward), Ki-rasKEAa 5'-CTC TAT TGT TGG ATC ATA TTC GTC -3' (reverse), and Ki-rasR 5'-GGT CCT GCA CCA GTA ATA TGC A -3' (reverse). One of the Hybridization Probes was labeled at the 5'-end with the LightCycler Red 705 fluorophore 5'-RED705-TTG CCT ACG CCA CAA GCT CCA A (sensor; complementary to the codon 12 cystein mutant), the other at the 3'-end with Fluorescein 5'-CAC AAA ATG ATT CTG AAT TAG CTG TAT CGT CAA GGC AC-F1 (anchor-38) or 5'-CGT CCA CAA AAT GAT TCT GAA TTA GCT GTA TCG TCA AGG CAC T-F1 (anchor-43). Both anchor and sensor oligonucleotides were used at a final concentration of 0.2 μ M, whereas PCR primers were 0.3 μ M. The PNA oligomer (PNA-wt) NH-TACGCCACCAGCTCC-CONH was added in a concentration of 0.7 μ M. All primers and the PNA oligomer were synthesized by TIB Molbiol, Berlin, Germany. The amplifications of the 125 (165)-bp PCR fragments were performed in the LightCycler Instrument running 59 cycles of 2(3) seconds at 95 °C, 10 (15) seconds at 56 (60) °C and 10 (15) seconds at 72 °C, starting with a 10-minute denaturation/activation at 95 °C. Melting-curve analysis was performed by a 0 (20)-second denaturation at 95 °C, a hybridization for 20 seconds at 52 (40) °C with continuous increasing of the temperature from 52 (40) °C to 95 °C (with 0.1 [0.3] °C/second). Fluorescence was detected in channel F3.

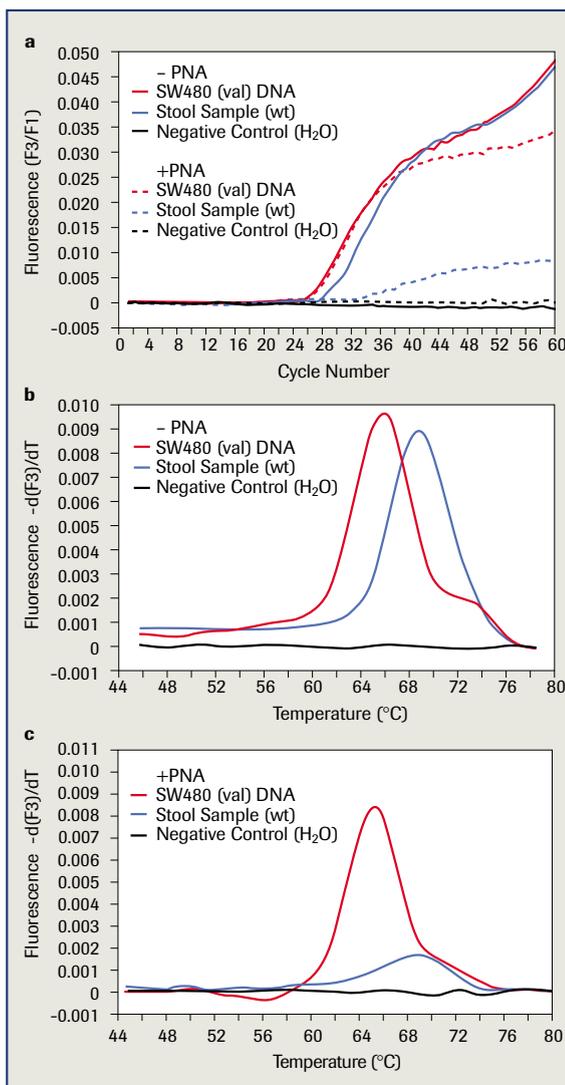


Figure 2: Data analysis of the LightCycler PCR of wild-type (wt) and valin mutant (val) DNA. The amplification curve from DNA preparations, which were amplified without (b) and with (c) the 15-mer PNA oligomer are shown in (a). For the melting-curve analysis (b, c), the first negative derivative of the fluorescence (-dF3/dT) was plotted as a function of temperature (°C; T_m). Blue is the wild-type DNA derived from the stool of subject 1. Red is the codon-12 val variant DNA sample derived from the tumor cell line SW480, and black is the negative control (H₂O).

Results and Applications

Detection of the mutant codon 12 Ki-ras2 chromosomal DNA

Hybridization Probes are sensitive when monitoring single-base sequence variations, using the specific melting temperature of the sensor probe that is specific for the codon-12 cystein variant of Ki-ras2. The results of a specific PCR analysis from wild-type and valin mutant

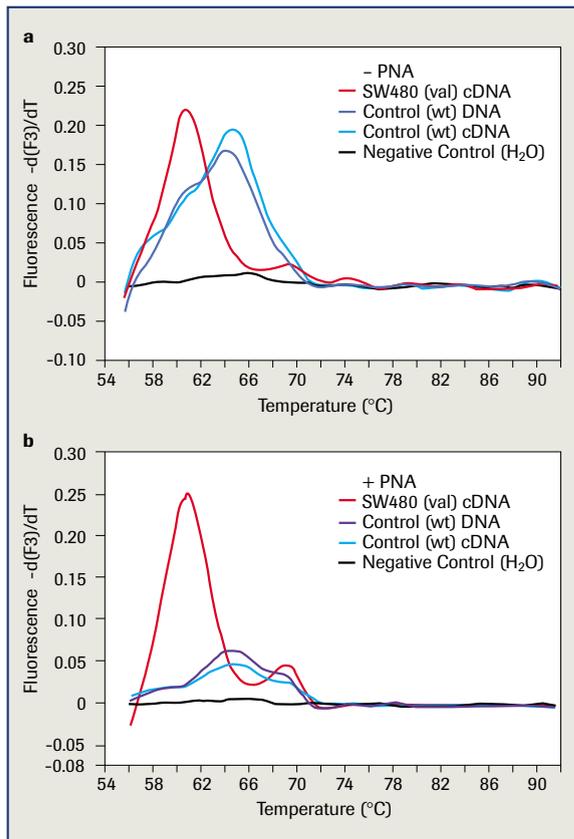


Figure 3: Data analysis of the LightCycler PCR of wild-type (wt) and valin (val) mutant cDNA, where the first negative derivative of the fluorescence ($-dF3/dT$) was plotted as a function of temperature ($^{\circ}\text{C}$; T_m). Reverse transcribed total RNA preparations from codon 12 wt and val mutants were amplified without (a) and with (b) PNA.

DNA is exemplified by the amplification of the 165-bp fragment, and shown in Figure 2. The PCRs (with and without PNA) result in specific amplification curves (Figure 2a). The melting-curve analysis after 59 cycles (without PNA) is shown in Figure 2b, where the first negative derivative of the fluorescence ($-dF/dT$) was plotted as a function of temperature ($^{\circ}\text{C}$; T_m). Analysis of mutant DNA resulted in a melting temperature of 66°C. Wild-type samples showed a melting temperature of 68.5°C. The function of the PNA oligomer is shown in Figure 2c. The amplification with 0.7 μM PNA leads to a suppression of the codon 12 wild-type amplicon resulting in a significant shift to a higher crossing point (Figure 2a), but not a complete inhibition. This result was additionally confirmed by agarose-gel electrophoresis (data not shown).

Detection of mutant codon 12 Ki-ras2 mRNA

The results of a specific Ki-ras2 RT-PCR, which illustrate the generation of the 125-bp PCR fragment from wild-type and valin mutant mRNA, is shown in Figure 3.

Figure 3a depicts the melting-curve analysis after 59 cycles without PNA. Here, analysis of mutant mRNA and DNA showed a melting temperature of about 61°C and wild-type samples of about 65°C. Again, 0.7 μM PNA suppressed the amplification of codon-12 wild-type DNA and cDNA at a melting temperature of 65°C (Figure 3b). Supplementary to the work of Landt et al. [7], here the mRNA analysis of an expressed gene was included in the PNA-clamp assay.

The described method allows specific detection of minor mutated mRNA and DNA sequences within a wild-type background. This has been demonstrated for serial dilutions of the mutant SW480 cell line spiked into a wild-type background, as well as for MRD samples (data not shown). The method combines the PNA-mediated PCR clamping approach with the sequence-sensitive identification, using Hybridization Probes specific for a sequence containing only a single base pair variation. Using the LightCycler Instrument, the entire assay can be performed within 70 minutes.

Summary

An application example of PNA oligomers, in combination with Hybridization Probes, using the LightCycler Instrument, is shown. PNA can suppress a specific PCR product, bind to nucleic acids with higher stringency and specificity in comparison to deoxyribonucleotides, and does not serve as a primer for Taq DNA polymerase. Due to these properties, PNAs can be used to detect single-point mutations in research samples within a high background of wild-type DNA and mRNA sequences.

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