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УСЛОВИЯ ЭФФЕКТИВНОГО ПОДАВЛЕНИЯ ПЦР С ПОМОЩЬЮ LNA-ОЛИГОНУКЛЕОТИДОВ ДЛЯ ПРОСТОЙ И ВЫСОКОЧУВСТВИТЕЛЬНОЙ ДЕТЕКЦИИ СОМАТИЧЕСКИХ МУТАЦИЙ

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Аннотация

Специфическое блокирование амплификации аллеля дикого типа в ПЦР с помощью олигонуклеотидов, модифицированных по остатку рибозы (закрытые нуклеиновые кислоты, locked nucleic acids, LNA), используется для высокочувствительной детекции соматических мутаций в опухолях. Описаны различные версии метода анализа мутаций с использованием LNA-олигонуклеотидов как с дополнительной модификацией фосфотиоатными группами, так и без таких групп, при этом использовались различные ДНК полимеразы. В работе проведен анализ оптимальных условий для успешного специфического блокирования ПЦР с помощью LNA-олигонуклеотидов при анализе мутаций в генах KRAS и BRAF. Мы обнаружили, что фосфотиоатная защита на 5'-конце олигонуклеотидов не влияет на эффективность блокирования аллеля дикого типа. Выявлено, что для большинства последовательностей эффективное блокирование наблюдается при проведении шага отжига и элонгации ПЦР при температуре на 20–25°C ниже температуры плавления LNA-олигонуклеотида. При таких условиях реакции возможна простая и высокочувствительная детекция мутаций в генах KRAS и BRAF с использованием как секвенирования по Сэнгеру, так и ПЦР в реальном времени с Taqman зондами.

Ключевые слова: запертая нуклеиновая кислота, зажим PCR, мутации, KRAS, BRAF, ДНК-полимераза Taq.

REQUIREMENTS FOR EFFICIENT PCR CLAMPING BY LOCKED NUCLEIC ACID OLIGONUCLEOTIES FOR SIMPLE AND SENSITIVE DETECTION OF SOMATIC MUTATIONS

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Abstract

PCR clamping/wild-type blocking PCR with non-extendable locked nucleic acid (LNA) oligonucleotides is used for sensitive detection of somatic mutations in tumors. Various versions of the technique use different DNA polymerases and LNA oligonucleotides with and without additional phosphorothioate modifications. Here we

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studied requirements for successful PCR clamping with LNA oligonucleotides and Taq DNA polymerase for analysis of mutations in KRAS and BRAF genes by means of real-time PCR and Sanger sequencing. We found that addition of phosphorothioate linkages at the 5'-end of LNA oligonucleotide to protect from 5'-exonuclease activity of Taq DNA polymerase did not improve clamping. For most target sequences, efficient clamping was observed at melting temperature of LNA oligonucleotide 20-25°C above annealing/extension temperature of the PCR with a 2-step protocol. Under such conditions, simple and sensitive detection of mutations in KRAS and BRAF genes was feasible using real-time PCR with TaqMan probes or Sanger sequencing.

Keywords: locked nucleic acid, PCR clamp, mutations, KRAS, BRAF, Taq DNA polymerase.

Introduction

Activating somatic mutations in certain genes (e.g. *BRAF, KRAS, EGFR*) are used to guide the choice of cancer therapy. The large excess of wild-type DNA often complicates detection of somatic mutations in tumor tissue. To detect small amount of a mutant allele within a large excess of wild-type DNA, a number of methods based on PCR, Sanger sequencing, pyrosequencing, mass spectrometry and next generation sequencing (NGS) were developed. PCR clamping or wild-type blocking (WTB) PCR with locked nucleic acid (LNA) oligonucleotide can be used for the sensitive detection of somatic mutations [1-4]. LNAs are nucleic acids with 2'-O-4'-C methylene bridge [5]. LNA modifications in an oligonucleotide increase melting temperature (Tm) and the specificity of binding to the target [5-7]. In WTB PCR non-extendable oligonucleotide (PCR clamp) corresponding to the wild-type sequence of expected mutation is added to PCR. Binding of PCR clamp to a target DNA inhibits amplification of the wild-type allele and results in the selective amplification of mutant allele(s) that can be detected by Sanger sequencing or by other methods.

It was proposed that clamping oligonucleotide can be degraded by the 5'-exonuclease activity of Taq DNA polymerase, and for efficient PCR clamping, the Stoffel fragment of DNA polymerase without 5'-exonuclease activity is required [1, 8]. Alternatively, some studies used Taq DNA polymerase and LNA oligonucleotides protected at the 5'-end with phosphorothioate modifications or employed Pfu DNA polymerase and its variants without 5'-exonuclease activity [9-11]. However, successful PCR clamping was also reported with regular Taq DNA polymerase and unprotected LNA oligonucleotides [2-4]. Thus, the need of PCR clamping to protect the 5'-end of LNA oligonucleotide and the need of DNA polymerase defective in 5'-exonuclease activity remains obscure. Here we studied the requirements for efficient PCR clamping with LNA oligonucleotides and Taq DNA polymerase for the simple and sensitive detection of somatic mutations in tumors.

Materials and Methods

LNA oligonucleotides

We designed PCR clamps KLNA1 (5'-GCCTACG+C+CA+C+CAGCTCCTT-p-3'; melting temperature (Tm) 80°C) and KLNA1S (5'-$G_SC_SC_S$TACG+C+CA+C+CAGCTCCTT-p-3'; Tm 80°C) corresponding to wild-type anti-sense KRAS sequence with codons 12 and 13. The clamps had identical nucleotide sequence but KLNA1S had phosphorothioate linkages for the first three internucleotide bonds at the 5'-end. For BRAF sequence including codon V600, we used PCR clamp BLNA2 (5'+G+C+T+A+C+A+G+T+G+AGGG-p-3'; Tm 77°C) [1]. Tm of LNA oligonucleotides was calculated by the on-line tool [12]. In the oligonucleotides LNA positions are preceded with "+" sign, while phosphorothioate linkages are designated by "S". Additionally LNA oligonucleotides carried at the 3'-end two or three nucleotide mismatches (underlined) and phosphate to block an extension. Oligonucleotides were synthesized by «DNK-sintez» (Moscow, Russia).

Wild-type and mutant human DNA standards

Recombinant plasmids with mutations KRAS-G12D (c.35G>A) and BRAF-V600E (c.1799T>A) were constructed by site-directed mutagenesis and mutations were confirmed by Sanger sequencing. Human placenta DNA without KRAS and BRAF mutation (Biolink, Russia) was diluted to 2 ng/µl in TE buffer (10 mmol/L Tris-HCl, pH 8.0; 1mmol/L EDTA) and used as wild-type DNA standard. Concentration of wild-type and mutant DNA was determined by real-time PCR. Wild-type DNA standard was spiked with the KRAS-G12D or BRAF-V600E plasmid DNA to prepare mutant DNA standards with different ratios of mutant/normal allele.

Real-time PCR

Real-time PCR KRAS was done in 25 µl mixture containing 1× buffer for Taq DNA polymerase (Medigen, Russia), 1.5 mmol/L $MgCl_2$, 0.2 mmol/L each of dATP, dGTP, dTTP, dCTP (Medigen, Russia), 0.5 µmol/L of forward primer KF1 (5'-GGCTGGTGGAGTATTTGATAGTGT-3'), 0.5 µmol/L reverse primer KR1 (5'-GGACAAGATTTACCTCTATTGTTGG-3'), 0.5 µmol/L TaqMan probe KTM6-2 (5'-FAM-CCTGC(dT-BHQ1)GAAAATGACTGAATATAAACTTGTGG-p-3'), and 1U of SuperHot Taq DNA polymerase (Bioron, Germany). SuperHot DNA polymerase is recombinant Taq DNA polymerase with anti-Taq Ab for hot-start. PCR clamps KLNA1 or KLNA1S were added to 2.0 µmol/L when required. Real-time PCR was performed in iCycler iQ5 or CFX96 thermocyclers (Bio-Rad) using

2-step protocol: 1 cycle 95 ºC for 3 min; followed by 50 cycles 95 ºC for 15 sec and 55 ºC for 90 sec with optical reading at 55 ºC. Difference in Ct values (dCt) in reactions with and without PCR clamp was calculated as the following: $dCt = Ct_{+LNA} - Ct_{-LNA}$, where Ct_{+LNA} – is the Ct of the DNA in PCR with clamp, Ct_{-LNA} – is the Ct of the DNA in PCR without clamp.

Unless otherwise indicated real-time PCR BRAF was done as described for KRAS with 2-step protocol but using forward primer BF2-2 (5'- AATGCTTGCTCTGATAGGAAAATG-3'), reverse primer BR2-3 (5'-AGTGGAAAAATAGCCTCAATTCTTA-3'), TaqMan probe BTM5-2 (5'-FAM-ATGAAGACC(dT-BHQ1) CACAGTAAAAATAGGTGATTTTGG-p-3') and PCR clamp BLNA2. When indicated the following 3-step protocol was used: 1 cycle 95 ºC for 3 min followed by 50 cycles 95 ºC for 15 sec, 55 ºC for 30 sec (with optic reading) and 72 ºC for 20 sec.

Sanger sequencing after PCR clamp

After PCR with clamp oligonucleotide amount of DNA may not be sufficient for Sanger sequencing. Therefore, DNA was amplified by nested PCR in two rounds. In the first round, reaction was done with PCR clamp to select for mutant allele essentially as described for the real-time PCR with primers KF1, KR1 and PCR clamps KLNA1 or KLNA1S without TaqMan probe; the following PCR protocol was used: 1 cycle 95 ºC for 3 min; followed by 25 cycles 95 ºC for 15 sec and 55 ºC for 90 sec. Product of the first round was diluted 1:50 in water and 5µl of diluted DNA was used in the second round without PCR clamp to obtain sufficient amount of mutation-enriched DNA for Sanger sequencing. In the second round, forward primer KF2 (5'-GCGTGTATTAACCTTATGTGTGACA-3') and reverse primer KR2 (5'-GGCAAGATTTACCTCTATTGTTGGA-3') were used with the following protocol: 1 cycle 95 ºC for 3 min; followed by 20 cycles 95 ºC for 15 sec, 60 ºC for 20 sec and 72 ºC for 20 sec. After the second round the DNA was purified on AMpure XP magnetic beads (Beckman Coulter) and sequenced using BigDye1.1 Kit (Applied Biosystems) with KF2 and KR2 primers.

Results

Protection of the 5'-end of LNA oligonucleotide is not required for efficient PCR clamp with Taq DNA polymerase.

We used initially WTB PCR to detect mutations at KRAS codons 12 and 13. These two codons have sequence GGTGGC with guanine nucleotides being mutation hot-spots in different cancers. We used sequence of the anti-sense strand of the KRAS gene for the clamp design to avoid LNA modification of guanine nucleotides that negatively impacts mismatch discrimination [6]. We designed clamp oligonucleotides KLNA1 and KLNA1S that had identical nucleotide sequence but the latter had phosphorothioate linkages for the first three internucleotide bonds at the 5'-end to protect from digestion by the 5'-exonuclease activity of Taq DNA polymerase. We tested both PCR clamps for the detection of mutation KRAS-G12D by real-time PCR and Sanger sequencing.

In real-time PCR both PCR clamps inhibited amplification of WT human KRAS DNA as evidenced by large increase in Ct (Figure 1A). As a quantitative parameter of performance of a PCR clamp we used

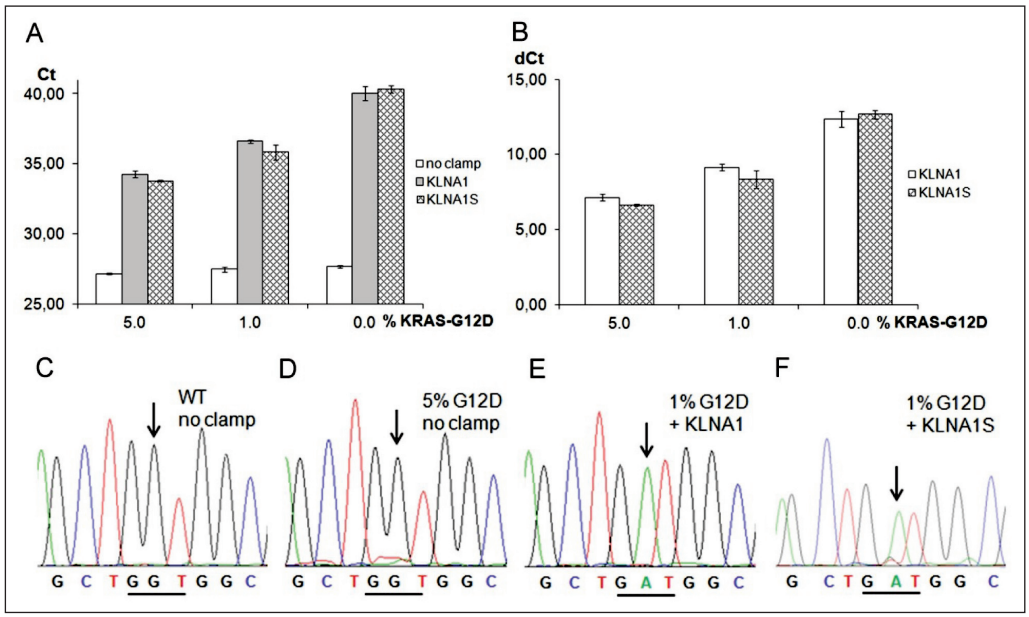

Figure 1. Detection of KRAS mutation G12D using Taq DNA polymerase and different PCR clamps. Ten nanogram human placenta DNA with wild-type KRAS or the same DNA spiked with 1% or 5% DNA copies KRAS-G12D was tested. PCR was done with and without PCR clamp KLNA1 without phosphorothioate linkages, or KLNA1S with phosphorothioate linkages. A, B - real-time PCR, plot of Ct and dCt, accordingly. C- F - Sanger sequencing after PCR without clamps (C, D) or with clamps KLNA1 (E) or KLNA1S (F). Codon 12 is underlined, nucleotide position corresponding to wild-type and mutation p.G12D (c.35G>A) is indicated by an arrow

the difference in Ct values (dCt) in reactions with and without PCR clamp. DNA samples spiked with KRAS-G12D plasmid had smaller dCt compared to wild-type, so that 1% mutant allele was readily distinguished (Figure 1B). Importantly, Ct as well as dCt values were similar in PCR with either clamp indicating similar performance of LNA oligonucleotides with and without phosphorothioate linkages.

Sanger sequencing of amplicons after PCR with either oligonucleotide clamp showed similar detection of 1% mutant allele (Figure 1D, F). Of note, 5% mutant allele was not visible by Sanger sequencing without PCR clamp (Figure 1E) and reliable detection required 20% or more mutant allele (data not shown).

These real-time PCR and Sanger sequencing data indicated that protection from 5'-exonuclease activity of Taq DNA polymerase did not improve PCR clamping by LNA oligonucleotides.

Efficient PCR block is observed at an annealing/extension temperature of 20-25 degrees below calculated Tm of the PCR clamp.

The strength of oligonucleotide binding to the template, and consequently efficiency of clamping should increase with higher Tm of LNA oligonucleotide and lower annealing/extension temperature (Tann/ext) during PCR. We investigated optimal Tann/ext for a PCR clamp with certain calculated Tm. Increase in Tann/ext from 54.6 to 62.8°C resulted in decrease in dCt of the wild-type DNA, while dCt of DNA with 5% allele KRAS-G12D was not appreciably changed (Figure 2). These data indicated less efficient PCR clamp and drop in discriminating power between wild-type and mutant alleles with increased Tann/ext. Further increase of Tann/ext to 65°C resulted in poor PCR performance, which was manifested in drop in fluorescence and large increase in Ct (data not shown).

Two-step PCR protocol is preferred for effective clamping.

Different studies use LNA-based PCR clamps either in 2-step PCR with 60-65°C annealing/extension temperature [2, 3, 10] or in classic 3-step PCR protocol with 56-64°C annealing and 72°C extension temperature [1, 4, 9, 11]. We speculated

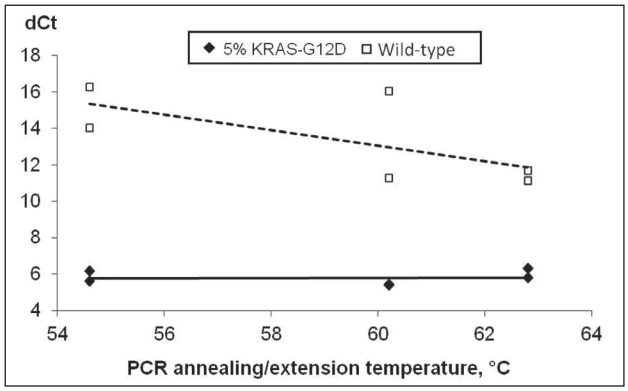

Figure 2. Performance of PCR clamp at different annealing/extension temperatures (Tann/ext). Real-time PCR of 5% KRAS-G12D and wild-type DNA with and without PCR clamp KLNA1. PCR was done using 2-step protocol with Tann/ext at 54.6; 60.2; or 62.8°C. Difference of Ct for reactions with and without clamp (dCt) and linear approximation of dCt is shown for 5% KRAS-G12D (solid line) and wild-type DNA (broken line)

that extension at 72°C can cause weaker binding of LNA oligonucleotide to the target and compromise clamping. Importantly, we described PCR clamp for BRAF-V600 that did not block amplification of the wild-type DNA in 3-step PCR protocol with Taq DNA polymerase [1]. We tested this PCR clamp in 2-step and 3-step PCR protocols. The amplification of wild type DNA was effectively blocked by PCR clamp that was manifested in large increase in Ct of the DNA if 2-step PCR protocol was applied with 55°C Tann/ext. Under such conditions DNA spiked with 1% BRAF-V600E allele was readily detected (Figure 3A). However, in agreement with Dominguez and Kolodney [1] the clamp did not block PCR with Taq DNA polymerase in 3-step PCR protocol (Figure 3B). These data showed that 2-step PCR protocol with 55 ºC Tann/ext was preferred for efficient clamping.

Furthermore, we designed and tested additional LNA oligonucleotides as PCR clamps to different loci in EGFR, KRAS and NRAS genes (data not shown). PCR was done using 2-step protocol with 55°C annealing temperature. We found that LNA oligonucleotides with Tm 72-73°C were poor blockers; in contrast, PCR was efficiently blocked by LNA

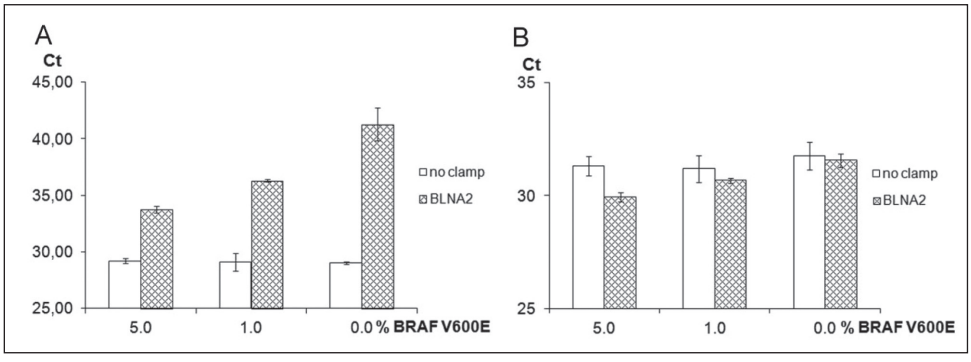

Figure 3. Detection of BRAF mutation V600E by PCR clamping using 2-step and 3-step protocol. Real-time PCR BRAF using 2-step protocol with Tann/ext 55°C (A), or 3-step protocol with 55°C annealing and 72°C extension (B). Ct of wild-type DNA and 1% or 5% BRAF-V600E is shown in reactions with and without PCR clamp BLNA2

oligonucleotides that had Tm about 80°C (range 76-80°C). The only exception was PCR clamp for sequence of NRAS including codons 12 and 13. In this case LNA oligonucleotide with Tm 80°C did not block PCR. Importantly, another oligonucleotide for the same sequence with additional LNA positions that increased Tm to 92°C blocked PCR of wild-type NRAS DNA and allowed detection of 1% NRAS-G12D (data not shown). Taken together these data indicated that for efficient clamping the Tm of oligonucleotide clamp should be at least 20°C above Tann/ext.

Discussion

WTB PCR using LNA oligonucleotides is a simple sensitive method for the detection of somatic mutations in tumors. Nafa et al. [13] reviewed the method; however, several important parameters of the technique remained unclear. In this study we evaluated requirements for effective PCR clamping by LNA oligonucleotides.

We found that phosphorothioate linkages in LNA oligonucleotide did not improve PCR clamping, indicating that protection of the clamp from 5'-exonuclease activity of the Taq DNA polymerase was not required. Interestingly, Stoffel fragment that lacks 5'-3' exonuclease activity was much more sensitive to PCR clamp in comparison to Taq DNA polymerase [1]. Our observation that phosphorothioate modifications of LNA oligonucleotide did not improve clamping indicated that the 5'-exonuclease activity of Taq DNA polymerase was not likely the reason for difference in sensitivity of these enzymes to PCR clamp. Most plausible explanation is weaker binding to the DNA template of Stoffel fragment in comparison to Taq DNA polymerase, which is manifested in about 10-fold difference in the binding constants of these enzymes to the DNA [14].

Tm of LNA oligonucleotide and annealing/extension temperature during PCR were critical parameters that had strong impact on clamping. There was a poor clamping if Tm of LNA oligonucleotide was 20°C lower than the annealing/extension temperature of the PCR. This observation provides simple and efficient rationale for design and optimization of WTB PCR with PCR clamps based on LNA oligonucleotides. Software to determine Tm of LNA oligonucleotides is available at the IDT and the Exiqon company sites [12, 15]. However, we noted that for different LNA oligonucleotides, the calculated Tm was 2.5-19.5°C lower using IDT software compared to the Exiqon one. We used Exiqon tool, however calculated Tm using either program should be used with caution for predicting real Tm for the design of PCR protocol. We think that poor clamping by some LNA oligonucleotides with the calculated Tm of about 80°C was the result of an error in the calculated Tm. In all cases when LNA oligonucleotide was not blocking PCR, adding more LNA positions to increase Tm incrementally by 5-12°C was sufficient for the effective PCR clamping.

Gel-electrophoresis, Sanger sequencing or real-time PCR can be used to evaluate performance of a PCR clamp. However, in our hands impact of PCR clamp on intensity of PCR bands sometimes was not clearly visible after gel-electrophoresis of PCR products. Importantly, real-time PCR and Sanger sequencing were in agreement with each other and provided more accurate estimate of performance of a PCR clamp.

Both real-time PCR and Sanger sequencing can be used to detect mutations by PCR clamping. In comparison to Sanger sequencing, real-time PCR had faster turnaround time and was less expensive, however exact genotyping was not possible. A limitation of Sanger sequencing after PCR clamping were occasional mutation artifacts on wild-type DNA. The problem was previously reported [4, 10] and is believed to result from mistakes of Taq DNA polymerase. The problem can be solved by the use of DNA polymerase with proofreading activity instead of Taq DNA polymerase [10]. Other potential options are a hi-fidelity version of Taq DNA polymerase [16], or use of less PCR cycles with PCR clamp.

In conclusion, our data showed efficient PCR clamping by LNA oligonucleotides with calculated Tm of at least 20°C above the annealing/extension temperature in a 2-step PCR protocol. Enrichment for target mutations was confirmed by the Sanger sequencing of PCR products. Importantly, Taq DNA polymerase and LNA oligonucleotides without phosphorothioate modifications were used without compromise in the efficiency of PCR clamp. This allows rational design of inexpensive, simple and sensitive assays for clinically actionable mutations in convenient format of real-time PCR with TaqMan probes.

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V.A. Shamanin and S.P. Kovalenko owe shares of the Biolink Ltd.
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