Use of a PNA probe to block DNA-mediated PCR product formation in prokaryotic RT-PCR

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A novel method eliminating DNA-mediated PCR product formation in reverse transcription PCR (RT-PCR) amplification of specific RNA sequences is described. The method exploits the higher melting temperature values of peptide nucleic acid (PNA)/DNA duplexes compared with DNA/DNA duplexes by binding a sequence-specific PNA probe to a genomic sequence immediately overlapping one of the PCR-primer attachment sites within the sequence of interest. Hybridization of the blocking probe precludes primer attachment to DNA without affecting attachment of the same primer to the reverse transcription-generated cDNA sequence. A four-step PCR cycle is used that allows the PNA probe to hybridize to the DNA strand at a higher temperature just prior to the primer annealing step.

INTRODUCTION

When analyzing RNA by reverse transcription PCR (RT-PCR) procedures, the elimination of contaminating DNA is of fundamental importance. Otherwise, the products obtained in PCR might originate from genomic DNA rather than specifically from cDNA produced from messenger RNA (mRNA). Removal of contaminating DNA is commonly achieved by treating samples with RNase-free DNase. However, DNase treatment is not well suited to a number of applications. The purpose of this study was to develop a new method to eliminate DNA-mediated product formation in RT-PCR by means of a probe specifically blocking amplification of contaminating DNA without affecting amplification of cDNA. Unlike eukaryotic mRNA, a poly(A) tail is not added to the 3' end of bacterial mRNAs. This makes the design of RT-PCR primers that target mRNA without targeting the corresponding DNA sequence impossible. However, unlike the corresponding chromosomal DNA sequence, the cDNA has two defined ends, one corresponding to either the RNA 5' end at the transcription initiation site or an RNA 5' end generated by RNA processing and a 3' end

generated by the primer used in the reverse transcription procedure. By contrast, the corresponding chromosomal DNA sequences continue beyond these points and have ambiguous ends based on the fragment length of each molecule. Herein, we demonstrate that sequence-specific blocking probes (1) can be designed, targeting the region immediately upstream of and proceeding through, these cDNA end-sequences. Selective inhibition of PCR amplification of the chromosomal DNA is achieved by introducing a pre-primer annealing step at a temperature sufficiently high to allow a peptide

nucleic acid (PNA) blocking probe to anneal to the genomic DNA but not to cDNA. The PNA probe will hereafter block attachment of a cDNA-targeted primer to the genomic DNA sequence, when the temperature is lowered to the primer annealing temperature. Under these conditions, the blocking probe will not affect attachment of the PCR primers to the cDNA. The blocking probe thereby prevents PCR amplification of genomic DNA without affecting amplification of RNA-derived cDNA (Figure 1).

For this approach to work, probes should meet the following requirements: (i) the probe should not itself function as a primer; (ii) the probe should exhibit a higher melting temperature (T_m) value than the corresponding PCR primer; and (iii) the probe should, when attached to the target sequence, efficiently prevent primer attachment. PNA bind tightly to complementary sequences of DNA, and the thermal stability of PNA/DNA duplexes at physiological ionic strength is generally higher per base pair than the corresponding DNA/DNA duplex (2,3). Further, PNA does not function as a primer for DNA polymerase and was therefore used in the present study. Probes consisting of PNA have previously been used successfully to eliminate PCR product formation in a sequence-specific manner (1,3-5). However, all probes previously used in attempts to block specific PCRs have been placed within the PCR-amplified sequence, inhibiting a fragment of differing sequence from the PCR

Figure 1. Peptide nucleic acid (PNA) inhibitor probe principle. After attachment of the probe to the genomic DNA sequence in the PCR pre-primer annealing step, PCR primers only anneal to the RNA-derived cDNA in the subsequent primer annealing step. Thereby selective amplification of cDNA will be achieved.

target. This strategy is not suited for the present purpose.

To our knowledge, this study represents the first attempt to block a specific PCR by placing a probe partly outside the sequence of interest. We show that the PNA probe inhibition assay is reproducible and that amplification of cDNA templates is not inhibited.

MATERIALS AND METHODS

Strains and Culture Conditions

Three bacterial strains, *Escherichia coli* SP308/pKJK10 (6), *Pseudomonas putida* OUS82 UCB55 (7), and *Salmonella typhimurium* LT2 (8) were used in this study. They were grown in appropriate media facilitating expression of the relevant genes.

DNA Extractions

DNA was extracted from pure cultures using commercial kits [Fast Soil DNA purification kit (BIO101, Vista, CA, USA) or UltraClean™ Soil DNA Extraction kit (MO-BIO, Solana Beach, CA, USA)] according to the manufacturer's instructions except that soil samples were replaced by pure culture samples.

RNA Extractions

Disposables and reagents were either certified RNase-free, or diethyl pyrocarbonate (DEPC)-treated (0.1%; Sigma, St. Louis, MO, USA) was added prior to autoclaving. RNA was extracted from approximately 10⁷ cells (200 µL, A₆₀₀ = 0.1) *P. putida* OUS82 UCB55 overnight culture; from 300 µL *S. typhimurium* overnight culture; and from 300 µL *E. coli* SP308 cells either grown to stationary phase. Total RNA was extracted using the FastRNA® Pro Blue kit (BIO101) in accordance with the manufacturer's instructions.

Optional DNase Treatment

DNase treatment of *P. putida* and *E. coli* RNA preparations was performed using the amplification-grade DNase I kit (Sigma), and DNase treatment

for *S. typhimurium* RNA preparations was performed using RQ1 RNase-free DNase (Promega, Madison, WI, USA); in both cases in accordance with the manufacturer's instructions.

Primers and Blocking Probes

The primers and blocking probes used in this study are shown in Table 1. All primers and PNA probes were custom-synthesized commercially and of high purity salt-free (HPSF®) purity.

Two inhibition strategies were assayed. First, a terminal inhibition, where the probe and the forward primer were targeted at the 3' end of the cDNA corresponding to the 3' end of the mRNA, was designed. Primer *gfpmb1-Fx* was designed to target the leading strand sequence corresponding to the expected start sequence of the reverse-transcribed *gfp* messenger 10 bases from the Pribnow box. Primer *gfpmb2-R* was placed 770 bases downstream of *gfpmb1-Fx* within the *gfp* gene. A blocking probe, PNAGFP1, was designed targeting the leading strand region immediately upstream of, and just overlapping, the *gfpmb1-Fx* target. A mismatch was introduced in the region of the probe overlapping the *gfp* cDNA sequence, because a PNA with the exact sequence could not be synthesized by our supplier. By this placement of the mismatch, the expected T_m value of the PNA/cDNA hybrid (1 mismatch/10 nucleotides) was lowered, while the expected T_m

of the PNA/genomic DNA hybrid was affected to less extent (1 mismatch/18 nucleotides). An A to G base change was chosen, as a purine transition substitution was expected to have the least impact on T_m (3).

In the second approach, internal inhibition, the probe PNA16S1-reverse was designed to target the region amplified by the reverse primer used in the RT-PCR. The primers PRBA338F and 511R2 were used to amplify ribosomal RNA (rRNA) from *E. coli*. A 16S rRNA encoding gene (in this case from *E. coli*) was selected because it is so widely targeted in phylogenetic and community-level analyses of microbial systems. This approach requires a two-step RT-PCR procedure, as having the PNA probe present in the reverse transcription step would inhibit cDNA synthesis. However, the general applicability of the developed method is greatly enhanced using the internal inhibition approach, as it liberates the researcher from design restrictions arising due to the transcription initiation-specific or termination signal-specific probe placement when the terminal PNA approach is used.

Primers *invAF2* and *invAR1* were designed to target the *invA* gene from *Salmonella* sp. involved in the invasiveness of *Salmonella* and has also been shown to be a diagnostic target for most *Salmonella* (9). PNAInvA1-reverse is an internal-PNA probe designed to block DNA-based synthesis from the *invAR1* primer.

Table 1. Primers and Probes Used in this Study

Primer/Probe	Sequence
<i>gfpmb1-Fx</i>	5'-AATTGTGAGCGGATAACA-3'
<i>gfpmb2-R</i>	5'-AAGCTTATTTGTATAGTTCATCCATGC-3'
PRBA338F	5'-ACTCCTACGGGAGGCAGCAG-3'
511R2	5'-GCTGGCACGGAG-3'
<i>invAF2</i>	5'-ACAGTGCTCGTTTACGAC-3'
<i>invAR1</i>	5'-ACTGGTACTGATCGATAA-3'
PNAGFP1	5'-H-ACTAGATT CG ATTGTGAG-NH ₂ -3'
PNA16S1-reverse	5'-H-GCGGCTGCTGG-NH ₂ -3'
PNAInvA1-reverse	5'-H-AGACGA/CCTGGTACT-NH ₂ -3'

Boldface shows mismatched base in PNAGFP1.

Reverse Transcription

gfp-targeted reverse transcription was performed using the GeneAmp® Gold RNA PCR Reagent kit (Applied Biosystems, Foster City, CA, USA) in accordance with the manufacturer's instructions. One microliter template RNA was reverse-transcribed in 20 µL reaction volume for 15 min at 45°C. *Salmonella* RNA was reverse-transcribed using SuperScript™ II (Promega) according to the manufacturer's instructions using 5 µL RNA as template. Reverse transcription of *P. putida* and *E. coli* RNA was performed using ImProm-II™ Reverse Transcriptase (Promega) in accordance with the manufacturer's instructions.

PNA Probe Pre-PCR Preparation

PNAGFP1, PNA16S1-reverse, and PNAInvAR1-reverse stock solutions (100 µM) were vortex mixed and then heated to 50°C for 10 min prior to addition to PCR mixtures.

PCR Conditions

gfp-targeted PCR was performed in a final volume of 25 µL, containing 17.5 µL or, when blocking probe was added, 15.5 µL DNase/RNase-free water (BIO101), 2.5 µL 10× AmpliTaq® PCR buffer (Applied Biosystems), 0.125 µL 12.5 pmol each primer, 2.5 µL 25 µg DNase-free bovine serum albumin (BSA; GE Healthcare, Uppsala, Sweden), 1 µL GeneAmp 10 mM dNTP mixture (Applied Biosystems), 0.125 µL 0.6 U AmpliTaq polymerase (Applied Biosystems), and 1 µL DNA. Blocking probe (PNAGFP1 or DNAGFP1) was added to a final concentration of 8.0 µM. Cycling conditions were 10 min at 95°C (initial denaturation), then 35 cycles of 30 s at 92°C (denaturation), 30 s at 63°C (blocking probe annealing), 30 s at 57°C (PCR primer annealing), and 1 min at 72°C (primer extension), and then 6 min of final extension at 72°C and final cooling at 4°C.

For rRNA-targeted PCR, conditions were essentially the same except that 5% of the recovered RNA (5 µL) was added as template, the PNA16S1-reverse blocking probe, and PRBA338F

and 511R2 primers were used, and the cycling conditions were 10 min at 95°C, then 35 cycles of 30 s at 95°C, 30 s at 58°C, 30 s at 53°C, and 1 min at 72°C, and then followed by 6 min at 72°C and final cooling at 4°C.

PCR of cDNA was performed as described above except that 3 µL of the cDNA product were used in a 50-µL reaction containing 29.7 µL or, when blocking probe was added, 25.7 µL RNase-free water, 5 µL 10× AmpliTaq PCR buffer, 3.2 µL 25 mM MgCl₂, 3.7 µL 10 mM dNTP mixture, 0.25 µL 25 pmol each primer, 0.5 µL 2.5 U AmpliTaq Gold® DNA polymerase (Applied Biosystems), and, where appropriate, 4.0 µL 400 pmol PNAGFP1. PCR cycling conditions were as indicated above for RNA-based amplification.

Real-Time PCR of *S. typhimurium* *invA* cDNA

PCRs were prepared in a total volume of 25 µL by combining 13.25 µL distilled water, 2.5 µL 10× reaction buffer (Bio-Rad Laboratories, Hercules, CA, USA) 0.75 µL MgCl₂, 1 µL 5mM dNTP mixture (Invitrogen, Carlsbad, CA, USA), 0.5 µL 10 pmol

InvAF2 and InvAR1 primers, 0.25 µL 1/1000 diluted SYBR® Green (BioWhittaker, Rockland, ME, USA), and 0.25 µL iTaq™ polymerase (Bio-Rad Laboratories). PCR conditions in the iCycler® (Bio-Rad Laboratories) were 10 min at 95°C, then 35 cycles of 30 s at 95°C, 30 s at 67°C, 30 s at 55°C, and 30 s at 72°C, then followed by a melting program with a 0.5°C increase/min to 95°C between each measurement using FAM 490 filters, followed by cooling to 4°C.

RESULTS AND DISCUSSION

Efficiency of PNAGFP1 Blocking

To test the effectiveness of the terminal and internal PNA blocking strategy, PCR assays were performed on duplicate samples of total RNA from exponentially growing *P. putida* OUS82 UCB55 cells constitutively transcribing the *gfp* gene (Figure 2A) and exponentially growing *E. coli* SP308/pKJK10 (Figure 2B), respectively. The samples were each divided in two pairs. One pair of samples was reverse-transcribed immediately, while the other pair was treated with DNase prior to reverse transcription. Reverse transcriptase was excluded from one of the samples of each pair, and PCR was performed with and without the PNA probe for all samples.

In the terminal assay, the presence of contaminating DNA in the RNA extracts was indicated by production of ample PCR product with relative fluorescence intensity (RFI) equal to 2.32 in the sample that was not DNase-treated nor reverse-transcribed prior to PCR amplification (Figure 2A, lane 7). In this experiment, DNase treatment was shown to efficiently destroy contaminating DNA, since essentially no PCR product was produced from samples that were DNase-treated but not reverse-transcribed (Figure 2A, lanes 3 and 4; RFI = 0.01 and 0.06, respectively). A similar elimination of genomic DNA-derived PCR product was observed using the PNA blocking probe (Figure 2A, lane 8; RFI = 0.15). By comparing lanes 1, 2, and 6, it seems evident that the PNA probe had a beneficial effect on overall PCR

Figure 2. Ethidium bromide-stained reverse transcription PCR (RT-PCR) product bands using DNase treated (lanes 1–4) and untreated (lanes 5–8) total RNA extracts from (A) *Pseudomonas putida* OUS82 UCB55 and (B) exponentially growing cultures of *Escherichia coli* SP308/pKJK10. Reverse transcription enzyme was excluded from reactions 3, 4, 7, and 8, while in reaction 2, 4, 6, and 8, addition of the corresponding peptide nucleic acid (PNA) blocking probe [PNAGFP1 (8.0 µM final concentration) and PNA16S1-reverse (2.0 µM final concentration), respectively] to PCRs was performed. M, 100-bp ladder (Promega).

Figure 3. Representative bands originating from real-time PCR presented in Table 2. Lane 1, nucleic acid extract, with no reverse transcriptase treatment but with addition of PNAInvA1-reverse; lane 2, like lane 1, but with no peptide nucleic acid (PNA) added; lane 3, DNase-treated and reverse-transcribed with PNA; lane 4, like lane 3, but with no PNA addition; lane 5, reverse-transcribed and PCR with PNA probe; lane 6, like lane 5, but without PNA addition; lane 7, 100-bp ladder; lane 8, negative control.

performance (Figure 2A, lanes 1 and 2; RFI = 1.00 and 1.42, respectively). Collectively, these data indicate that the terminal PNA probe blocking approach eliminates the need for DNase treatment by physically blocking PCR primer binding to contaminating genomic DNA while enhancing PCR product formation through some as yet undefined and probably nonspecific mechanism. It has previously been shown that addition of nonspecific oligonucleotides (transfer RNA or tRNA) to PCR and RT-PCRs can increase product formation, presumably by loosely binding to excess primers during initial amplification cycles (10). The enhanced PCR performance observed in the current study after

addition of PNAGFP1 may be caused by a similar effect. A similar terminal blocking system using a 34-mer 3' capped DNA probe as a blocking probe was not found to work efficiently (data not shown).

In the internal blocking assay, contaminating rRNA gene-encoding genomic DNA was present in both RNA extractions, as evidenced by production of ample PCR product where DNase and blocking probe were not used, even when the reverse transcriptase step was omitted (Figure 2B, lanes 5 and 7). DNase treatment effectively removed contaminating genomic DNA, preventing formation of PCR product where reverse transcription was omitted (Figure 2B, lanes 3 and 4). PNA blocking also eliminated PCR amplification from contaminating genomic DNA, even where DNase treatment was not performed (Figure 2B, lane 8). These results also indicate that internal PNA blocking approach is as effective as DNase treatment and thus can be applied where DNase treatment is impractical.

Application of PNA Blocking to Structural Genes of Diagnostic Interest

PNAInvA1-reverse block the formation of DNA-based PCR product in quantification of mRNA from the *invA* gene in *S. typhimurium*. To demonstrate the generality of the PNA blocking approach and extend its utility to quantification of expression of structural genes of diagnostic interest, we next applied the internal blocking approach to measure *invA* gene expression in *S. typhimurium*. To accomplish this, blocking of genomic DNA-derived

invA PCR product formation was first optimized using primers *invAF2* and *invAR1* and the blocking probe PNAInvA1-reverse (Table 1) using blocking primer annealing temperatures between 60° and 67°C and PCR primer annealing temperatures between 52° and 67°C with genomic DNA from *S. typhimurium* as template. The final cycling protocol was selected based on near-optimal PCR product formation in the absence of PNAInvA1-reverse and no detectable product in the presence of PNAInvA1-reverse (Figure 3, lane 1). In Figure 3, real-time RT-PCR amplification of cDNA from the *invA* gene of *S. typhimurium* without PCR signal from the corresponding genomic DNA was accomplished using the PNA inhibitor probe PNAInvA1-reverse (Figure 3, lane 4). Real-time PCR analysis of the triplicate samples from DNase-treated samples with or without the addition of PNA probe provided comparable cycle threshold values (Table 2), indicating that the blocking probe does not inhibit the PCR process when true cDNA is the template.

Running extended numbers of amplification cycles for *Salmonella* sp. *invA* detection in certain cases resulted in signal arising from insufficiently suppressed DNA. Thus, the amount of PNA added must be directly determined for each detection design, since nonspecific binding of the PNA eventually could result in low concentrations of available PNA for hybridization and blocking in latter PCR cycles and subsequent generation of PCR product based on DNA. We have not optimized the needed concentration of PNA probe for excessively extended numbers of cycles in PCR, but in general, a lower number of target genes in a small amount of background DNA requires less blocking probe than would a large number of target genes in a rich and complex background of nontarget DNA.

Here we have suggested an alternative approach to eliminate formation of DNA-mediated RT-PCR products. We applied blocking probes with success in three different systems, *gfp* mRNA, 16S rRNA, and *invA* mRNA. Strong blocking efficiency was found in all three systems. Similar PNA blocking systems could easily be developed for

Table 2. Cycle Threshold Values for Real-Time PCR Amplification of *Salmonella typhimurium* *invA* Gene

±PNA	+PNA			-PNA		
Replicate	1	2	3	1	2	3
Direct nucleic acid extract (RNA and DNA)	N.A.	N.A.	N.A.	26.9	26.9	27.9
Reverse-transcribed after DNase treatment	29.3	29.7	28.9	28.5	28.8	29.7
Reverse-transcribed no DNase treatment	29.5	29.9	28.9	28.8	29.2	28.7

PNA, peptide nucleic acid; N.A., not applicable.

other RNA sequences, and the general applicability of the technique was demonstrated using both terminal and internal blocking strategies in three different systems. Additionally, the approach described herein could find application for the development of improved viral detection methods for RNA or DNA viruses based on strand-specific RT-PCR procedures.

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COMPETING INTERESTS STATEMENT

M.B. and C.S.J. are co-inventors of "Method of Selective Detection of a Target Nucleic Acid," European patent no. 04738977.0-240, assigned to the Geological Survey of Greenland and Denmark. W.E.H. and S.J.S declare no competing interests.

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