Zip nucleic acids are potent hydrolysis probes for quantitative PCR

Clément Paris, Valérie Moreau, Gaëlle Deglane, Emilie Voirin, Patrick Erbacher and Nathalie Lenne-Samuel*

ABSTRACT

Zip nucleic acids (ZNAs) are oligonucleotides conjugated with cationic spermine units that increase affinity for their target. ZNAs were recently shown to enable specific and sensitive reactions when used as primers for polymerase chain reaction (PCR) and reverse-transcription. Here, we report their use as quantitative PCR hydrolysis probes. Ultraviolet duplex melting data demonstrate that attachment of cationic residues to the 3′ end of an oligonucleotide does not alter its ability to discriminate nucleotides nor the destabilization pattern relative to mismatch location in the oligonucleotide sequence. The stability increase provided by the cationic charges allows the use of short dual-labeled probes that significantly improve single-nucleotide polymorphism genotyping. Longer ZNA probes were shown to display reduced background fluorescence, therefore, generating greater sensitivity and signal level as compared to standard probes. ZNA probes thus provide broad flexibility in assay design and also represent an effective alternative to minor groove binder- and locked nucleic-acid-containing probes.

INTRODUCTION

The invention of polymerase chain reaction (PCR) in the 1980s has revolutionized research, diagnostics, medicine and forensics (1). With the development of quantitative PCR (qPCR), the technology has become quantitative and accurate (2). A target nucleic acid is detected by monitoring in real time the fluorescence generated either by an intercalating dye that quantifies the accumulation of double-stranded DNA or by a probe that hybridizes to the amplicon (3). Although more expensive, probe-based detection confers a higher specificity and provides the possibility to implement multiplex PCR or allelic discrimination. Among the numerous probe types available, dual-labeled probes are the most widely used in qPCR

(4,5). They consist of oligonucleotides with a fluorophore and a quencher dye attached at either end. Dual-labeled probe detection relies on the existence of distinct fluorescence emission levels associated with the free and hybridized states and it is mediated by Förster resonance energy transfer (FRET). Indeed, prior to hybridization, reporter and quencher can come close to each other due to the random-coil configuration of the oligonucleotide probe or can be kept in proximity through formation of a hairpin stem as in molecular beacons (5). The average distance between the dyes is of prime importance for detection sensitivity since it determines both quenching efficiency and background fluorescence of the probe. When the probe hybridizes to the target amplicon, helix formation maintains the reporter and the quencher at a large distance from each other with concomitant fluorescence increase. The maximal fluorescence intensity is reached when the fluorophore is cleaved from the probe by the 5′ nuclease activity of the polymerase (4,6). Dual-labeled probes undergoing this degradation process are referred to as 'hydrolysis probes'.

Reducing the probe length has become crucial to increase the discrimination in single-nucleotide genotyping, as well as to provide design flexibility for difficult targets. However, probe shortening decreases melting temperature (T_m), precluding efficient probe binding at PCR cycling temperatures. The introduction of chemical modifications that increase probe stability, such as a minor groove binder (MGB) moiety (7,8) peptide nucleic acid (PNA) backbones (9) or locked nucleic acid (LNA) analogs (10), has enabled efficient short PCR probes to be generated. Zip nucleic acids (ZNAs) are oligonucleotides conjugated with repeated cationic spermine units (11,12) that decrease electrostatic repulsions with target nucleic acid strands. The number of cationic units attached at any position of the oligonucleotide modulates the global charge of the molecule, thus raising the corresponding duplex T_m in a smooth, linear and predictable manner (13). We have recently reported the use of ZNAs as specific primers for PCR and reverse transcription (14). ZNAs enable fast and accurate detection of rare targets, and allow efficient PCR or reverse-transcription reactions

at high annealing temperatures, low primer concentrations, low magnesium and using fast protocols. Another valuable application for ZNA oligonucleotides could be as PCR probes, in particular, short hydrolysis probes. Here, we report that ZNAs are indeed potent short dual-labeled probes with enhanced discrimination level. Interestingly, we also found that cationic spermine unit conjugation improves the performances of longer probes as well, by decreasing the fluorescence background.

MATERIALS AND METHODS

Oligonucleotides

ZNA oligonucleotides were synthesized on a standard oligonucleotide synthesizer as previously described (11,12) and DMT-ON purified on reverse-phase Poly-Pak II cartridges (Glen Research, Sterling, VA, USA). Standard and ZNA dual-labeled probes were synthesized following the same procedure using BHQ1-CPG resin supports (Biosearch Technologies, Novato, CA, USA). 6-Carboxyfluorescein (6-FAM) phosphoramidite (Glen Research) was used to introduce the 5′-reporter dye. After ammonia deprotection at room temperature, probes were purified by anion-exchange HPLC under basic conditions as previously described (11,12). Dual-labeled probes and ZNA oligonucleotides were characterized by anion-exchange HPLC and ESI-TOF mass spectrometry.

Unmodified oligonucleotides were from Eurogentec (Seraing, Belgium). Primers were DMT-ON purified and oligonucleotides for T_m studies were purified by HPLC.

The TaqMan MGB probe was from Applied Biosystems (appliedbiosystems.com) and LNA probes were from Integrated DNA Technologies (Coralville, IA, USA).

T_m measurements

Melting temperature studies were carried out in 1-cm path-length quartz cells on a CARY 100 Bio UV/Visible spectrophotometer (Varian, France) by measuring the absorption at 260 nm. The samples were placed in a CARY Thermostatable Multicell Holder accessory and the temperature was regulated by a CARY Temperature controller by Peltier effect. Samples consisted of single-stranded oligonucleotides mixed in 1:1 molar ratio in a typical PCR buffer containing 3 mM $MgCl_2$, 50 mM KCl and 10 mM Tris–HCl (pH 8.5 at 25°C). The final oligonucleotide concentration was 2 μM. Samples were denatured by heating to 95°C at a rate of 1°C/min and maintained at this temperature for 20 min. Annealing was performed by cooling samples to 20°C at a cooling rate of 0.7°C/min and duplexes were maintained 15 min. Melting was performed by heating samples from 20 to 90°C at rate of 0.7°C/min and absorbance at 260 nm was recorded every 0.5 s. T_m values were determined by calculating the second derivative of the absorbance versus temperature curve and subsequent determination of the null. Measurements were repeated at least twice and the average deviation was ±0.2°C.

Genomic DNA extraction

Total DNA was isolated from A549 cells (lung carcinoma, ATCC CCL-185) which are wild-type for the factor V gene, and from factor V-deficient cells (GM14899, Coriell Institute, Camden, NJ, USA) containing the G1691A mutation, using the DNAeasy Blood and Tissue kit (Qiagen), according to the manufacturer's instructions. DNA purity and concentrations were assessed by agarose gel electrophoresis and UV spectrophotometry. Aliquots were stored at –20°C for long-term and +4°C for short-term use.

qPCR

All qPCR reactions were performed in a final volume of 10 μl in a Rotor-Gene 6000 instrument (corbettlifescience .com). Unless specified otherwise, the final reaction mixtures were composed of 2.5 μl Sensimix NoRef PCR kit (Quantace, London, UK), 3 mM $MgCl_2$, 10 ng of DNA, 200 nM of each primer and 200 nM probe. Salmon sperm DNA (Invitrogen.com) was used to spike diluted target genomic DNA and as negative control. Results shown are representative of at least three independent experiments.

All the studies were performed in accordance with Minimum Information for publication of quantitative real-time PCR Experiments (MIQE) guidelines (15).

RESULTS AND DISCUSSION

To address the performances of ZNA probes, we implemented a qPCR assay aiming to genotype the human blood coagulation factor V gene at position 1691. In this locus, the mutation G1691A, referred to as Factor V Leiden mutation, is a highly prevalent single-nucleotide polymorphism (SNP) that confers an increased genetic risk for venous thrombosis (16,17). Among numerous DNA tests developed so far to evaluate the predisposition of individuals to this hemostatic disorder, we have adapted a 5′ nuclease assay originally designed to be used with a short 17-base-long hydrolysis probe conjugated with an MGB (Table 1) (18). We synthesized a standard (unmodified) and a ZNA dual-labeled probe with the same sequence, replacing for the latter probe the MGB moiety by four cationic units. In order not to interfere with the exonuclease activity of the polymerase, the cationic units (Z units) were attached at the 3′ end. Prior to studying ZNA probe performance in qPCR, T_m measurements were carried out in order to investigate the impact of spermines on mismatch discrimination.

Mismatch discrimination

The mismatch discrimination ability of a probe relies upon the difference in melting temperatures (ΔT_m) between perfect-match and single-base-pair mismatched probe–target duplexes. The greater the difference, the more discriminant the probe is. Furthermore, the shorter the probe, the more a mismatch destabilizes the duplex. Consequently, the ΔT_m is higher when the probe size decreases. ΔT_m also depends on the nature of the

Table 1. Sequences of primers and hydrolysis probes (18)

Factor V gene	NCBI Accession number: NM_000130	
	5′–3′ sequences	Size (bases)
Primers		
Forward	GCCTCTGGGCTAATAGGACTACTT (exon 10)	24
Reverse	TTCTGAAAGGTTACTTCAAGGACAA (intron 10–11)	25
Dual-labeled probes	junction exon10-intron 10–11	
DNA -17	FAM-ACCTGTATTCCTCGCCT-BHQ1	17
ZNA -17	FAM-ACCTGTATTCCTCGCCT-Z_4-BHQ1	17
4LNA -17	FAM-ACCTG+TATTCC+T+C+GCCT-BHQ1	17
5LNA -17	FAM-ACCTG+TAT+TCC+T+C+GCCT-BHQ1	17
MGB-17	FAM-ACCTGTATTCCTCGCCT-MGBnfq	17
DNA -22	FAM-ACCTGTATTCCTCGCCTGTCCA–BHQ1	22
ZNA -22	FAM-ACCTGTATTCCTCGCCTGTCCA– Z_4- BHQ	22
DNA -33	FAM-ACCTGTATTCCTCGCCTGTCCAGGGATCTGCTC– BHQ1	33
ZNA -33	FAM-ACCTGTATTCCTCGCCTGTCCAGGGATCTGCTC–Z_4 BHQ1	33

Z_n denotes n spermine cationic units.
+N denotes LNA nucleotide.
The SNP position is underlined.

mismatch, the neighboring bases and the mismatch position along the probe sequence, the largest effect being generally achieved at central positions. To investigate whether the presence of spermine units affects these properties, the sequence corresponding to the probe used in the qPCR assay was chosen as a standard oligonucleotide and the corresponding ZNA was synthesized with four cationic units at the 3′ end. Melting temperatures of duplexes formed by the standard and ZNA oligonucleotides with the complementary oligonucleotide were measured in a PCR buffer containing Tris–HCl 10 mM pH 8.5, KCl 50 mM and $MgCl_2$ 3 mM. The matched ZNA duplex exhibited a 2.5°C T_m increase compared to the standard duplex. This rather limited T_m increase does not reflect the stabilization during PCR since cation conjugation has a much larger impact on T_m when the ZNA hybridizes to a target contained in a longer nucleic acid strand (13). In this situation, indeed cationic units counterbalance a much larger electrostatic repulsion presented by the target strand.

We introduced single-nucleotide changes all along the target oligonucleotide sequence, which led to mismatches at various positions in the probe–target duplex (Figure 1). T_m of mismatch duplexes, containing standard or ZNA oligonucleotide probes, were determined and ΔT_m were calculated by subtracting T_m values from the perfect-match duplex T_m. Within experimental error, data show identical ΔT_m values for both standard and ZNA probes for each mismatch position (Figure 1), demonstrating that ZNA retains full oligonucleotide discrimination capacity. This result strikingly contrasts with those obtained with MGB modification, which reduces ΔT_m except when the mismatch is within the MGB-binding region (8). This finding confers a strong advantage to ZNAs, making probe design much easier. In contrast to LNA modification, we did not observe that ZNA improves discrimination *per se*. However, LNA-containing probe design remains difficult due to T_m variation with sequence, position and number of modifications (19). In conclusion, these results clearly demonstrate that while the attachment of spermine units to oligonucleotides increases the T_m, the intrinsic oligonucleotide sequence recognition specificity remains unaffected.

Shorter probes for better discrimination

To evaluate ZNAs as probes, wild-type genomic DNA was serially diluted and amplifications were carried out under conditions allowing 5′ nuclease degradation using the 17-mer ZNA probe or the standard probe (Figure 2A). As shown by the amplification plot, the ZNA probe allowed sensitive and efficient detection, exhibiting higher signal levels and earlier C_q values than the 17-mer standard probe. Post-PCR melting analysis gave no variation in fluorescence signal with the temperature, and fluorescence accumulation over cycles was similar whether the acquisition was performed after the denaturing step (95°C) or the elongation/polymerization step (60°C) (data not shown). These two observations indicate that ZNA probe-based detection most probably occurs through a 5′ degradation mechanism (4,6).

The ZNA 17-mer probe performed much the same as a longer standard probe containing 22 bases (Figure 2B) for detection of the wild-type target. In contrast, due to its shorter length, the ZNA probe showed significantly improved SNP discrimination. Indeed, amplification of the genome containing the Leiden mutation was not detected by the ZNA probe, while the standard probe still displayed a fluorescence signal from the mutant sequence.

The short ZNA probe was then compared to MGB- and LNA-containing dual-labeled probes, maintaining sequence and length identical for all probes. LNA-modified oligonucleotide performances are highly dependent on the number of LNA substitutions, their positioning and the sequence context. The LNA probe design was performed by Integrated DNA Technologies (Coralville, IA, USA) according to Owczarzy and collaborators (19), which led to two probes comprising four and five LNA moieties which were tested. As shown in Figure 2C, ZNA and MGB probes behaved similarly and provided the best C_q improvement over the standard probe, while both LNA probes exhibited
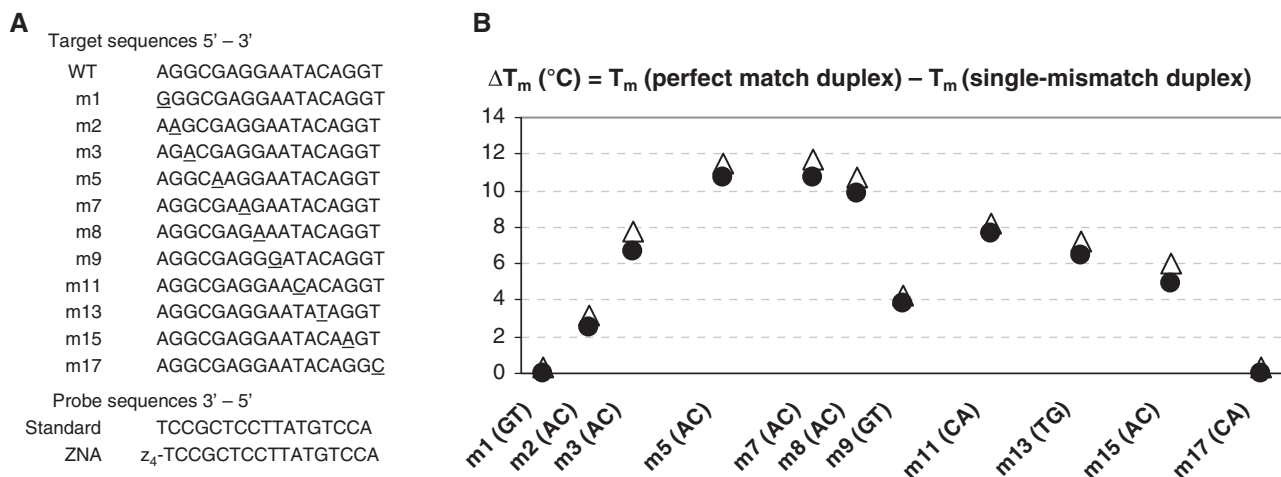
Figure 1. Identical mismatch discrimination of standard DNA and ZNA oligonucleotides for various mismatches located along the duplex. (**A**) Sequences of target and probe strands used in UV melting experiments. (**B**) ΔT_m of standard DNA (white triangles) and ZNA oligonucleotide (black circles) as a function of mismatch position.
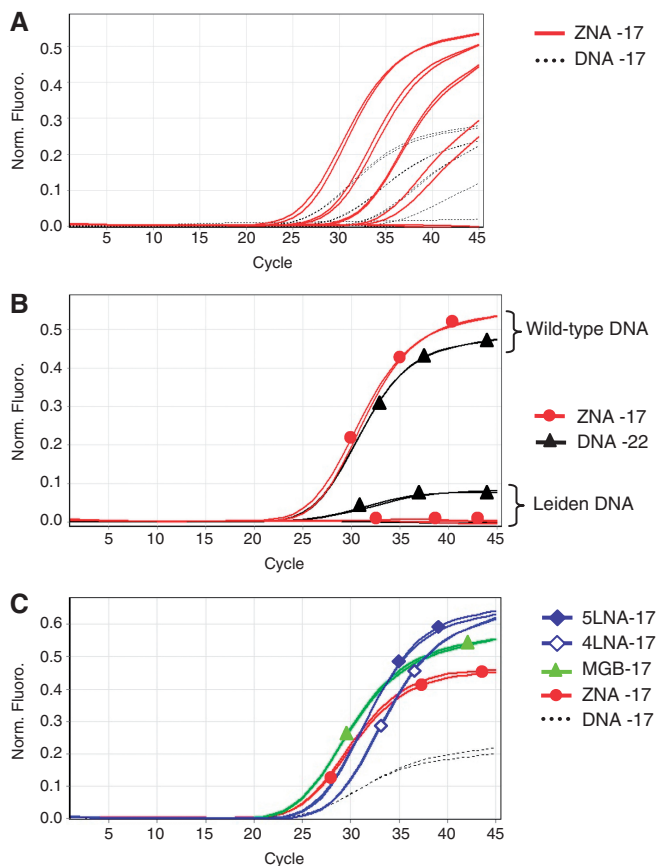

Figure 2. PCR detection and SNP discrimination with short ZNA dual-labeled probe, and comparison with other modified probes. (**A**) Serial dilution amplifications of target genomic DNA (10 ng to 10 pg) were detected using ZNA short hydrolysis probe (red lines) and its unmodified DNA counterpart (black dotted lines). (**B**) SNP discrimination. Amplification of 10 ng of wild-type and Leiden genomic DNA were detected using the short ZNA probe (red circle) and a longer unmodified DNA probe. The probes were respectively 17 and 22 bases in length. (**C**) Amplification of wild-type genomic DNA and detection using short unmodified, ZNA-, MGB- and LNA-containing probes. All probes have identical sequence.

slightly later C_q's. The comparison was also carried out using different commercial PCR mixes. We observed variations in relative performances of the modified probes depending on the PCR reagent, some being more favorable for one type of modification than another. Nevertheless, for all PCR mixes, ZNA-, MGB- and LNA-containing probes displayed improved performances over the standard probe (data not shown).

Improved quenching

Standard dual-labeled probes are typically 22–25-mer oligonucleotides in order to achieve a T_m value compatible with efficient PCR detection. We addressed the effect of the attachment of spermines on such probes that do not require any T_m increase by comparing 22- and 33-mer standard and ZNA probes. As previously observed for short probes, the conjugation of four cationic moieties at the 3′ end of the oligonucleotide provided earlier C_q's and higher signal levels than standard counterparts for both probes lengths (Figure 3). An examination of raw fluorescence data showed that standard probes exhibited a high background signal. This was expected since the FRET quenching decreases as the distance between fluorophore and quencher increases. Interestingly, both ZNA probes displayed significant reduction of the basal fluorescence emission, which may explain their better performances. Indeed, a lower fluorescence background allowed earlier detection and provided a higher signal-to-noise ratio after normalization by the instrument software. MGB conjugation has been reported to decrease the background fluorescence when adjacent to the fluorophore in 'Pleiades' probes (20), the heterocyclic MGB possibly acting as a quencher itself. Here, the greater quenching efficiency observed for the ZNA probe is presumably due to the oligocationic chain folding back toward the anionic oligonucleotide, thus leading to a shorter end-to-end distance than for the oligonucleotide where internal repulsions lead to an extended conformation (Figure 3C).
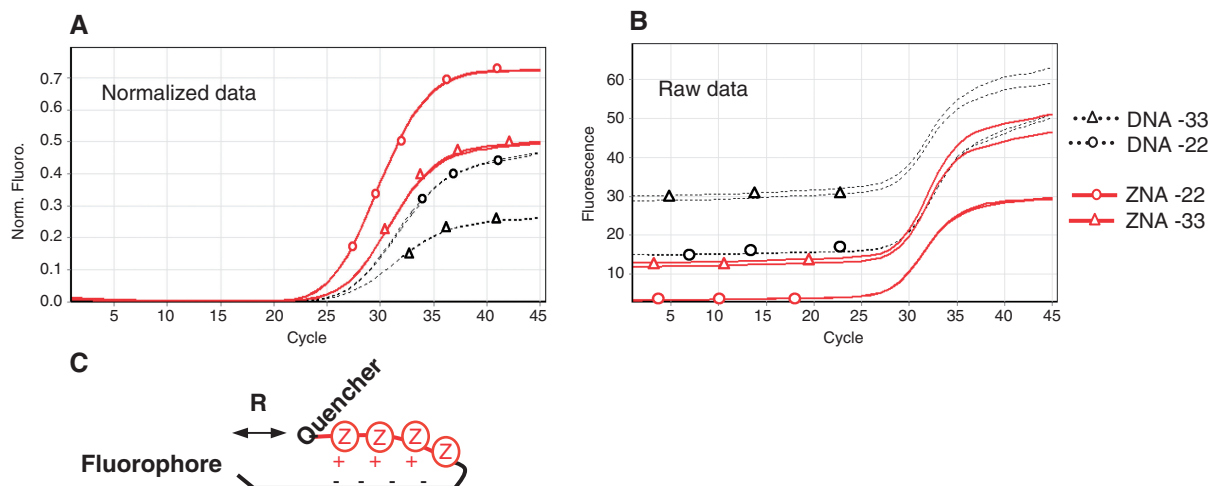
Figure 3. PCR detection with long ZNA dual-labeled probes. Amplifications of 10 ng of target genomic DNA were detected using ZNA hydrolysis probes (red) and their unmodified DNA counterpart (black dotted) containing 22 bases (circle) and 33 (triangle). (**A**) Normalized data by the instrument's software. (**B**) Raw fluorescence data. (**C**) Model for the greater quenching fluorescence of ZNA probes: the cationic charges of spermine units stabilize the probe in a coil conformation, reducing the distance R between the fluorophore and the quencher.
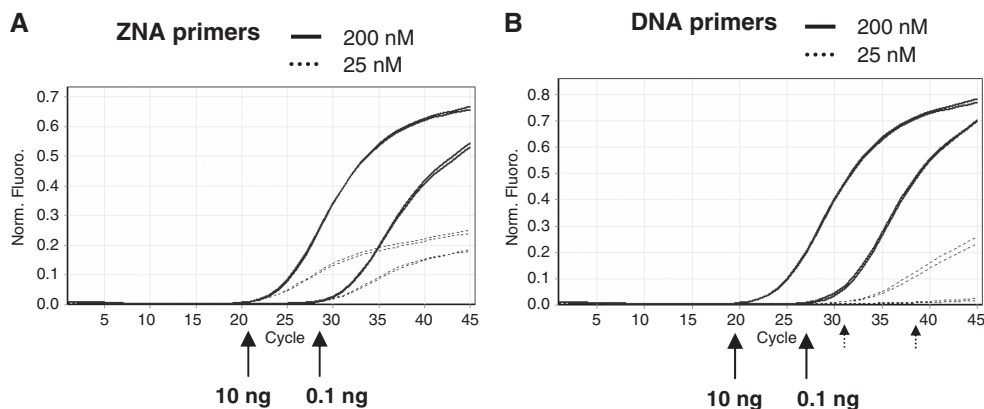

Figure 4. The 5′ nuclease assay using low concentration of ZNA and standard primers. Target genomic DNA (10 and 0.1 ng) was amplified with either ZNA primers (**A**) or standard primers (**B**). Reactions were performed with 200 nM (thin lines) and 25 nM (dotted lines) of primers, 200 nM of ZNA-22 hydrolysis probe and JumpStart Taq ReadyMix (Sigma–Aldrich).

Combining a ZNA probe with ZNA primers

We have recently shown that the use of ZNAs as primers improves PCR in AT-rich regions and provides greater flexibility in PCR and reverse-transcription conditions. Indeed, ZNA primers worked at higher annealing temperatures, in lower and constant magnesium concentration and under fast, time-saving protocols. The most striking advantage was the possibility to substantially decrease the ZNA primers concentration without affecting the C_q, dynamic range, sensitivity or efficiency. Lower primer concentrations allowed early PCR arrest as observed by a low plateau level (14). We suggested that such a property would be of interest for multiplex PCR where multiple target amplification reactions compete for the same, ultimately limiting reagents. Yet, simultaneous detection of several targets in a sample generally requires the use of probes. We therefore performed reactions using ZNAs both as primers and probes. ZNA primers with sequence identical to the standard primer pair were synthesized with four cationic units at their 5′ end and used in

conjunction with the 22-mer ZNA probe. As ZNA primers may generate nonspecific amplification products in the presence of high salt (14), reactions were carried out using a commercial kit optimized for probe-based PCR containing 1.5 mM $MgCl_2$. The $MgCl_2$ concentration was increased to 3 mM for the standard DNA primers. Standard DNA primers led to inefficient amplification at 25 nM concentrations as indicated by +10 cycles shift in C_q as compared to 200 nM (Figure 4). In contrast, ZNA primers, although showing different signal intensity, led to the same C_q value at 200 nM and 25 nM concentration, even with a diluted target. This result highlights the potential of ZNAs for use in multiplex PCR applications.

CONCLUSION

The data reported here show that ZNA probes are potent short hydrolysis probes. The presence of cationic residues increases the T_m without affecting the oligonucleotide

discrimination properties, making probe design for genotyping easy. In the present work, four spermine units have been conjugated between the oligonucleotide and the quencher at the 3′ end. Several other short hydrolysis probes were synthesized based on the same structure and, in all cases, assays were significantly improved (data not shown). Although more data have to be generated to determine the rules that will define the number of cationic moieties required for a given sequence, ZNA probes are easy to design and straightforward to synthesize. ZNA probes are also easy to use. Indeed, ZNA oligonucleotides were shown to exhibit an outstanding high affinity that may require increasing reaction stringency to maintain specificity (14). This is of prime importance for primers ensuring specific amplification. Here, we show that ZNA probes exhibit high performances under standard concentration conditions and PCR mixes. Finally, we also found that ZNAs improve long hydrolysis probes by decreasing the background fluorescence. This unexpected effect on quenching suggests that ZNAs may provide potent dual-labeled probe that do not function through 5′-nuclease degradation, a hypothesis that is currently under investigation.

REFERENCES

1. Saiki,R.K., Scharf,S., Faloona,F., Mullis,K.B., Horn,G.T., Erlich,H.A. and Arnheim,N. (1985) Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science*, **230**, 1350–1354.
2. Higuchi,R., Dollinger,G., Walsh,P.S. and Griffith,R. (1992) Simultaneous amplification and detection of specific DNA sequences. *Biotechnology*, **10**, 413–417.
3. Kubista,M., Andrade,J.M., Bengtsson,M., Forootan,A., Jonak,J., Lind,K., Sindelka,R., Sjoback,R., Sjogreen,B., Strombom,L. *et al.* (2006) The real-time polymerase chain reaction. *Mol. Aspects Med.*, **27**, 95–125.
4. Livak,K.J., Flood,S.J., Marmaro,J., Giusti,W. and Deetz,K. (1995) Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. *PCR Methods Appl.*, **4**, 357–362.
5. Tyagi,S. and Kramer,F.R. (1996) Molecular beacons: probes that fluoresce upon hybridization. *Nat. Biotechnol.*, **14**, 303–308.
6. Holland,P.M., Abramson,R.D., Watson,R. and Gelfand,D.H. (1991) Detection of specific polymerase chain reaction product by utilizing the 5′—3′ exonuclease activity of Thermus aquaticus DNA polymerase. *Proc. Natl Acad. Sci. USA*, **88**, 7276–7280.
7. Afonina,I.A., Reed,M.W., Lusby,E., Shishkina,I.G. and Belousov,Y.S. (2002) Minor groove binder-conjugated DNA probes for quantitative DNA detection by hybridization-triggered fluorescence. *Biotechniques*, **32**, 940–944, 946–949.
8. Kutyavin,I.V., Afonina,I.A., Mills,A., Gorn,V.V., Lukhtanov,E.A., Belousov,E.S., Singer,M.J., Walburger,D.K., Lokhov,S.G., Gall,A.A. *et al.* (2000) 3'-minor groove binder-DNA probes increase sequence specificity at PCR extension temperatures. *Nucleic Acids Res.*, **28**, 655–661.
9. Svanvik,N., Stahlberg,A., Sehlstedt,U., Sjoback,R. and Kubista,M. (2000) Detection of PCR products in real time using light-up probes. *Anal. Biochem.*, **287**, 179–182.
10. Ugozzoli,L.A., Latorra,D., Puckett,R., Arar,K. and Hamby,K. (2004) Real-time genotyping with oligonucleotide probes containing locked nucleic acids. *Anal. Biochem.*, **324**, 143–152.
11. Pons,B., Kotera,M., Zuber,G. and Behr,J.P. (2006) Online synthesis of diblock cationic oligonucleotides for enhanced hybridization to their complementary sequence. *Chembiochem.*, **7**, 1173–1176.
12. Voirin,E., Behr,J.P. and Kotera,M. (2007) Versatile synthesis of oligodeoxyribonucleotide-oligospermine conjugates. *Nat. Protoc.*, **2**, 1360–1367.
13. Noir,R., Kotera,M., Pons,B., Remy,J.S. and Behr,J.P. (2008) Oligonucleotide-oligospermine conjugates (zip nucleic acids): a convenient means of finely tuning hybridization temperatures. *J. Am. Chem. Soc.*, **130**, 13500–13505.
14. Moreau,V., Voirin,E., Paris,C., Kotera,M., Nothisen,M., Remy,J.S., Behr,J.P., Erbacher,P. and Lenne-Samuel,N. (2009) Zip nucleic acids: new high affinity oligonucleotides as potent primers for PCR and reverse transcription. *Nucleic Acids Res.*, **37**, e130.
15. Bustin,S.A., Benes,V., Garson,J.A., Hellemans,J., Huggett,J., Kubista,M., Mueller,R., Nolan,T., Pfaffl,M.W., Shipley,G.L. *et al.* (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem.*, **55**, 611–622.
16. Rees,D.C., Cox,M. and Clegg,J.B. (1995) World distribution of factor V Leiden. *Lancet*, **346**, 1133–1134.
17. Rosendaal,F.R., Koster,T., Vandenbroucke,J.P. and Reitsma,P.H. (1995) High risk of thrombosis in patients homozygous for factor V Leiden (activated protein C resistance). *Blood*, **85**, 1504–1508.
18. Luderer,R., Verheul,A. and Kortlandt,W. (2004) Rapid detection of the factor V Leiden mutation by real-time PCR with TaqMan minor groove binder probes. *Clin. Chem.*, **50**, 787–788.
19. You,Y., Moreira,B.G., Behlke,M.A. and Owczarzy,R. (2006) Design of LNA probes that improve mismatch discrimination. *Nucleic Acids Res.*, **34**, e60.
20. Lukhtanov,E.A., Lokhov,S.G., Gorn,V.V., Podyminogin,M.A. and Mahoney,W. (2007) Novel DNA probes with low background and high hybridization-triggered fluorescence. *Nucleic Acids Res.*, **35**, e30.