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Significant enhancement of fluorescence on hybridization of a molecular beacon to a target DNA in the presence of a site-specific DNA nickase

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Abstract

We have developed a simple isothermal (55 °C) reaction that permits detection of DNA targets using only two components: a molecular beacon and a site-specific DNA nickase without deoxyribonucleotide triphosphates and primers. The loop sequence of the molecular beacon should contain a DNA nickase recognition site. The nickase–molecular beacon (NMB) combination permits a 100-fold increase in fluorescent signal. The applications of the NMB assay for enhancement of fluorescent signal in some isothermal methods are discussed.
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As first described, molecular beacons are synthetic DNA and RNA probes that fluoresce on hybridization [1]. A molecular beacon is a single-stranded oligonucleotide that has a fluorescent moiety at one end and a nonfluorescent quencher at the other end. The oligonucleotide contains a sequence complementary to a target that is flanked by short complementary sequences. In the absence of a specific single-stranded target sequence, the complementary sequences of the molecular beacon anneal to form a stem–loop structure that brings the two moieties into close proximity to each other, causing the fluorescence of the fluorophore to be quenched by fluorescence resonance energy transfer (FRET).[1] Hybridization of the loop to a target sequence leads to a separation of the fluorophore and the quencher from each other and fluorescence is not quenched.

Molecular beacons are increasingly used in many applications, including quantitative PCR, single nucleotide polymorphism (SNP) analysis, visualization of RNA expression in living cells, and detection of DNA binding proteins [2]. In all methods to detect specific nucleic acid sequences, a fluorescent signal is proportional to the amount of target sequence that is amplified during the reaction. In this article, we describe a new method whereby the amount of target remains constant throughout the reaction. In this case, the increase in fluorescent signal is due to cleavage of the molecular beacon loop by a site-specific DNA nickase.

Site-specific DNA nickases, like type II restriction endonucleases, recognize short-specific sequences on double-stranded DNA. However, in contrast to restriction endonucleases, nickases cleave only one strand. Today, four nickases have been isolated from natural sources [3], and DNA-nicking derivatives of some restriction endonucleases have been obtained by random mutagenesis [4], by selection from randomized expression library [5], and by catalytic

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[1] *Abbreviations used:* FRET, fluorescence resonance energy transfer; SNP, single nucleotide polymorphism; PNA, peptide nucleic acid; DTT, dithiothreitol; BSA, bovine serum albumin; NMB, nickase–molecular beacon; RCA, rolling-circle amplification; HRCA, hyperbranched rolling-circle amplification.

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site mutagenesis of the subunits of the heterodimeric restriction endonuclease BbvCI [6]. An artificial DNA-nicking system based on restriction enzymes assisted by peptide nucleic acid (PNA) openers has also been developed [7]. In the current work, we use DNA nickase Nb.BspD6I that recognizes the double-stranded 5′-GAGTC-3′/5′-GACTC-3′ site and cuts the GAGTC strand at the fourth nucleotide downstream from the site [8]. The nickase reveals an optimal activity at 55 °C.

Here we demonstrate that up to 100 molecular beacons can be cleaved by nickase on a single target molecule.

Materials and methods

Oligonucleotides

All oligodeoxyribonucleotides were obtained commercially from Eurogentec. The sequences of the oligonucleotides were as follows:

St5: 5′-FAM-GGCATTCTAGAGTCGACCTGCAGGCATGATGCC-BHQ1-3′
St7: 5′-FAM-GGCATCTTCTAGAGTCGACCTGCAGGCATGAGATGCC-BHQ1-3′
St9: 5′-FAM-GGCATCTATTCTAGAGTCGACCTGCAGGCATGATAGATGCC-BHQ1-3′
C5: 5′-GGCATCATGCCTGCAGGTCGACTCTAGAATGCC-3′
C7: 5′-GGCATCTCATGCCTGCAGGTCGACTCTAGAAGATGCC-3′
C9: 5′-GGCATCTATCATGCCTGCAGGTCGACTCTAGAATAGATGCC-3′

The oligonucleotides St5, St7, and St9 were molecular beacons with stem lengths of 5, 7, and 9 bp, respectively (stem arms are underlined in the text). All of the molecular beacons had the same loop sequence that was complementary to the polylinker region (6241–6263) of the phage M13mp19 DNA. The region contains the 5′-GACTC-3′ sequence. 6-Carboxyfluorescein was labeled at the 5′ end as the fluorophore, and a black hole quencher was labeled at the 3′ end as the quencher. The oligonucleotides C5, C7, and C9 were perfectly complementary to the oligonucleotides St5, St7, and St9, respectively.

DNA nickase

The N.BspD6I recombinant nickase was isolated from the TOP10F′ (pRARE/Nick) *Escherichia coli* strain containing two plasmids [9]. The plasmid pET28b/Nbsp carried the *nbsp* gene under the phage T7 promoter. The second plasmid, pRARE/Mssc, contained the DNA methyltransferase M.SscL1I gene inserted into plasmid pRARE. The M.SscL1I has been shown to protect bacterial DNA against hydrolysis by the nickase [8]. pRARE (Novagen) is a plasmid that contains genes of tRNA that rarely occur in *E. coli*. Purified nickase (>95%) was obtained using a phosphocellulose P11 (Whatman) column followed by hydroxyapatite HTP (Bio-Rad) and Ni-NTA agarose (Qiagen) columns and had activity of approximately 0.5×10^6 U/mg. The enzyme is commercially available from Bioline (Cat. No. BIO-27039, London, UK).

DNAs

The single-stranded phage M13mp19 DNA was isolated according to a standard procedure [10]. After quantitation with a UV spectrophotometer, the DNA was serially diluted in steps to a final concentration of 1–0.01 μM. Aliquots of 1 μl were used in the reactions. Thymus DNA was purchased from Sigma.

Nickase–molecular beacon assay

A premix for a number of reactions was prepared. Each reaction (20 μl) contained (final concentration) 1× nickase buffer (10 mM Tris–HCl [pH 7.8], 150 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol [DTT], 0.1 μg/μl bovine serum albumin [BSA]), 1 μl nickase (0.3 mg/ml), 1 μl ssM13mp19 DNA, and a molecular beacon at different concentrations (0.5–5 μM). The reactions were followed in a Rotor–Gene RG-3000 (Corbet Research) equipped with Rotor–Gene 6.0 for Windows.

Results and discussion

Previously, we showed that the nickase N.BstD6I can cleave linear nonfluorescent oligonucleotides repeatedly on a single target molecule provided that the target–oligonucleotide hybrid has a nickase recognition site [8]. In the current study, we used the same nickase in combination with molecular beacons, allowing registration of the cleavage process in real time.

Fig. 1 gives the concept view of the method. First, the loop of the molecular beacon is complementary to a target DNA and contains the 5′-GAGTC-3′ sequence. Consequently, the target should contain the 5′-GACTC-3′ sequence. This limitation is not severe because DNA sites featuring such sequence should occur quite frequently; statistically, one should occur every 200–300 bp of a random DNA sequence, on the average. Second, on hybridization of the loop to the target, the double-stranded nickase recognition site is formed and a nickase cuts the molecular beacon, producing two single-stranded fragments. Third, the two fragments are separated from the target if their melting temperatures are lower than the reaction temperature (55 °C). Consequently, the cleavage must give rise to an irreversible fluorescence enhancement. Fourth, the target released from the fragments is capable of adopting the next molecular beacon; thus, the cleavage process will be repeated many times on one target, providing accumulation of the fluorescence signal.

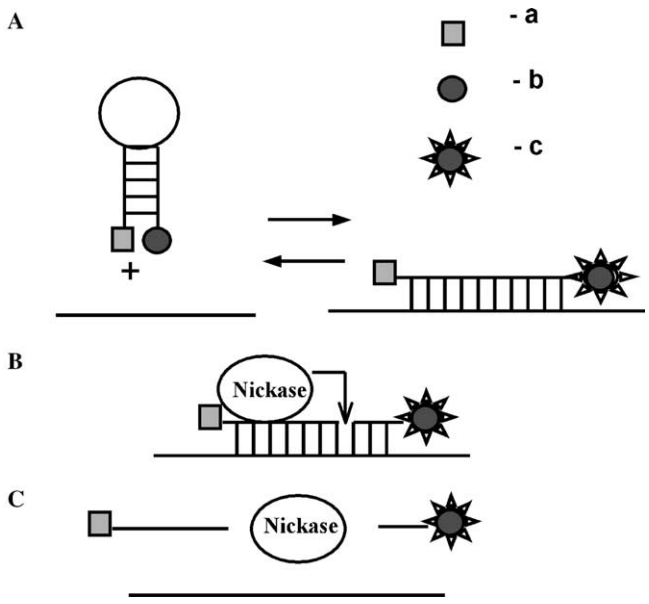

Fig. 1. Scheme demonstrating the use of a molecular beacon in hybridization analysis of nucleic acids with site-specific nickases. (A) Hybridization of the molecular beacon. (B) Cleavage of the molecular beacon by nickase. (C) Separation of short fragments and nickase. (a) fluorescence quencher; (b) fluorophore whose emission is absorbed by the quencher; (c) emitting fluorophore.

To test this principle, we used three molecular beacons with a loop length of 23 bases and stem lengths of 5, 7, and 9 bases. The length of the loop was chosen to ensure that the molecular beacon–target hybrid would be formed at the reaction temperature and that the fragments of the loop arising after cleavage by nickase would separate from the target.

Thermal denaturation profile studies were conducted to determine which of the molecular beacons is preferable for using in nickase–molecular beacon (NMB) assays (Fig. 2). The thermal transition profiles were in good accordance with the data reported by Tsourkas and coworkers [11]. The molecular beacon with the 5-base stem was unstable at 55 °C and had a great background. The molecular beacon with the 9-nucleotide stem was not completely melted even at 90 °C and produced the lowest signal on hybridization with complementary oligonucleotide. Therefore, for further study we chose the molecular beacon with the 7-base stem.

Fig. 3A shows the time curves of fluorescence intensity of St7 during cleavage by nickase. It can be seen that in the presence of a 50-nM target DNA, the fluorescence rose rapidly at first and then asymptotically approached a plateau. The fluorescence enhancement reflects restoration of the fluorescence of the fluorophore resulting from cleavage of the molecular beacon. All molecular beacons were cleaved after 25 min of reaction. However, the fluorescence intensity falls rapidly with target concentration, and its time dependence becomes linear. In the presence of 5 nM target, the intensity approaches a plateau in 16 h of incubation with nickase (data not shown). The sensitivity limit of the NMB assay is 0.5 nM. We found that changes in KCl concentrations (50–300 mM), in MgCl concentrations (5–20 mM), and in pH values (7.0–9.5) did not result in any increase in sensitivity of the NMB assay. The limiting step seems to be slow dissociation of nickase from the site. The fluorescence intensity increases with molecular beacon concentration. However, we could not use molecular beacon concentrations greater than 5 μM due to the upper measuring limit of the device. The addition of excess total calf thymus DNA did not significantly decrease the response (Fig. 3B). Therefore, the NMB assay can be used to enhance the fluorescence intensity of a target being in a complex mixture of DNAs.

We define the fluorescence enhancement factor as ratio of the fluorescence intensity (background subtracted) of the NMB assay after reaction to that (background subtracted) of the hybrid (target–molecular beacon). The background is fluorescence intensity of the molecular beacon alone. The enhancement factors were determined at different ratios of the molecular beacons to the constant amount of target (50 nM). These data are shown in Table 1. It can be seen that in the presence of a 100-fold excess of the molecular beacon, the enhancement factor of fluorescence reaches 100 after 60 min incubation with the nickase. Therefore, at least 100 molecular beacons can be cleaved on one target molecule during 60 min.

Our NMB assay can be used to increase the fluorescence in isothermal amplification of DNA at temperatures not

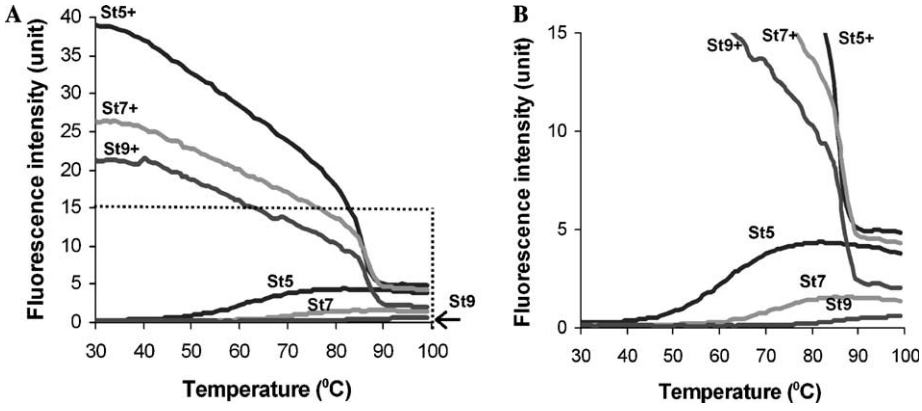

Fig. 2. (A) Melting curves of molecular beacons (0.5 μM in 1× nickase buffer) without any oligonucleotides or in the presence of 10-fold excess of oligonucleotides (+) perfectly complementary to each molecular beacon. The temperature profiles were obtained by sampling fluorescence after temperature increments of 1 °C held for 30 s. (B) Enlarged version of marked area in panel A.

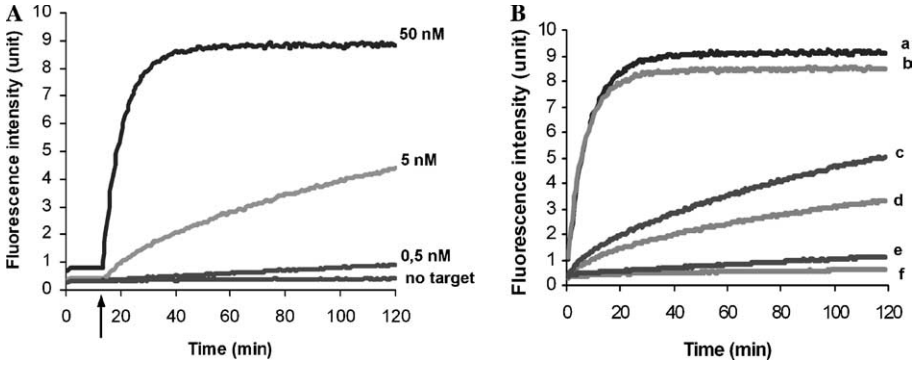

Fig. 3. Time course of cleavage of the molecular beacon St7 (0.5 μM) with the nickase. (A) The concentrations of target (ssDNA of M13mp19 phage) are denoted by numbers above the curves. The arrow marks the time of the addition of nickase. (B) Effect of calf thymus DNA on the NMB assay. (a,b) 50 nM of target; (c,d) 5 nM of target; (e,f) 0.5 nM of target; (a,c,e) without 200 μg/ml calf thymus DNA; (b,d,f) with 200 μg/ml calf thymus DNA.

Table 1
Enhancement factors of NBM assays

Concentration of beacon St7	Time of reaction (min)						
	5	10	20	30	40	60	120
0.5 μM	4.71	13.18	16.11	16.76	17.20	17.39	17.47
1 μM	20.09	28.61	38.38	42.94	44.74	46.27	47.48
2.5 μM	22.69	33.90	52.70	64.43	76.21	89.15	109.08
5 μM	20.34	31.48	51.80	67.48	81.39	104.18	106.80

exceeding 60 °C if the target molecule includes the GACTC sequence. In particular, it can be useful in real-time monitoring of the rolling-circle amplification (RCA) and the hyperbranched rolling-circle amplification (HRCA) [12,13]. The nucleotide sequence of a molecular beacon loop that includes a nickase site could be inserted in a padlock probe [14] outside the region complementary to the target. In this case, the same molecular beacon can be used for the detection of different targets. The combination of the nickase and beacon could also be used in the loop-mediated isothermal amplification of DNA [15]. In this case, the molecular beacon loop must be complementary to the amplified DNA loops having arbitrary sequences. Unfortunately, the nickase N.BspD6I does not cleave DNA–RNA hybrids [8]. Therefore, the NMB assay cannot be used for detection of RNA targets.

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References

[1] S. Tyagi, F.R. Kramer, Molecular beacons: probes that fluoresce upon hybridization, Nat. Biotechnol. 14 (1996) 303–308.
[2] N.E. Broude, Stem–loop oligonucleotides: a robust tool for molecular biology and biotechnology, Trends Biotechnol. 20 (2002) 249–256.
[3] R.J. Roberts, T. Vincze, J. Posfai, D. Macelis, REBASE: restriction enzymes and DNA methyltransferases, Nucleic Acids Res. 33 (2005) D230–D232.
[4] Z. Zhee, J.C. Samuelson, J. Zhou, A. Dove, S.-Y. Xu, Engineering strand-specific DNA-nicking enzymes from the type IIS endonuclease BsaI, BsmBI, and BsmAI, J. Mol. Biol. 337 (2004) 573–583.
[5] J.C. Samuelson, Z. Zhu, S.-Y. Xu, The isolation of strand-specific nicking endonucleases from randomized *Sap*I expression library, Nucleic Acids Res. 32 (2004) 3661–3671.
[6] D.F. Neiter, K.D. Lunnen, G.G. Wilson, Site-specific DNA-nicking mutants of the heterodimeric restriction endonuclease R.BbvCI, J. Mol. Biol. 348 (2005) 631–640.
[7] H. Kuhn, Y. Hu, M.D. Frank-Kamenetskii, V.V. Demidov, Artificial DNA-nicking system based on common restriction enzymes assisted by PNA openers, Biochemistry 42 (2003) 4985–4992.
[8] L.A. Zheleznaya, T.A. Perevyazova, E.N. Zheleznyakova, N.I. Matvienko, Some properties of site-specific nickase BspD6I and possibility of its use in hybridization analysis of DNA, Biochemistry (Moscow) 67 (2002) 498–502.
[9] E.A. Rogulin, T.A. Perevysova, L.A. Zheleznaya, N.I. Matvienko, Plasmid pRARE as a vector for cloning to construct a superproducer of the site-specific nickase N.BspD6I, Biochemistry (Moscow) 69 (2004) 1123–1127.
[10] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.
[11] A. Tsourkas, M.A. Behlke, S.D. Rose, G. Bao, Hybridization kinetics and thermodynamics of molecular beacons, Nucleic Acids Res. 31 (2003) 1319–1330.
[12] P.M. Lizardi, X. Huang, Z. Zhu, P. Bray-Ward, D.C. Thomas, D.C. Ward, Mutation detection and single-molecule counting using isothermal rolling-circle amplification, Nat. Genet. 19 (1998) 225–232.
[13] M. Nilsson, M. Gulberg, F. Dakl, K. Szuhai, A.K. Raap, Real-time monitoring of rolling-circle amplification using a modified beacon design, Nucleic Acids Res. 30 (2002) e66.
[14] J. Banar, M. Nilsson, A. Isaksson, M. Mendel-Hartvig, D.-O. Antson, U. Landegren, More keys to padlock probes: mechanisms for high-throughput nucleic acid analysis, Curr. Opin. Biotechnol. 12 (2001) 11–15.
[15] T. Notomi, H. Okayama, H. Masubuchi, T. Yonekawa, K. Watanabe, N. Amino, T. Hase, Loop-mediated isothermal amplification of DNA, Nucleic Acids Res. 28 (2000) e63.