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Symmetric vs asymmetric PCR and molecular beacon probe in the detection of a target gene of adenovirus

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A DNA fragment (307 bp) from the conserved region of an adenovirus gene (hexon) was amplified by symmetric and by asymmetric polymerase chain reaction (PCR). Two amplifications, one in the absence other in the presence of a molecular beacon probe were conducted by both symmetric and asymmetric PCR. The probe sequence was complementary to an internal segment of the amplified fragment. The product amplified in the absence and presence of the probe was detected by agarose gel and fluorescence analysis, respectively. A symmetric PCR results in exponentially grown double stranded DNA. An asymmetric PCR generates one of the strands by linear amplification and a fraction of its total product as double-stranded DNA limited by the concentration ratio of the primers used. Thus asymmetric PCR provided lower intensity signal hence less sensitivity than symmetric PCR by agarose gel analysis as expected. However, signal from a beacon probe based PCR assay is generated only from the probe fraction that hybridizes successfully competing against the strand complementary to the target strand of the product generated by PCR. The symmetric PCR has so far been used for the molecular beacon based fluorescent signal detection. The present study compared the level of fluorescent signal detectable from a symmetric PCR with that from an asymmetric PCR. The fluorescent data analysis demonstrated that a significant higher level of fluorescent signal hence higher sensitivity of detection is obtainable using asymmetric PCR than symmetric PCR performed in presence of the molecular beacon probe.
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KEYWORDS: adenovirus, asymmetric PCR, symmetric PCR, fluorescent signal.

INTRODUCTION

Molecular beacon is a probe system that has a stem-loop structure, the loop region is a single-stranded probe sequence and the stem is formed of two short complementary sequences unrelated to the target.[1] A fluorophore is conjugated to the end of one stem strand and a quencher to the end of the other. In this conformation of the probe, the fluorophore is in close proximity of the quencher and thus the fluorescence emitted by the fluorophore in response to an excitation light is adsorbed by the quencher and the probe does not fluoresce. It provides fluorescent signal only upon hybridization with its target, when the probe undergoes a conformational change, and the fluorophore and quencher are separated by a distance

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equal to the stretch of nucleotide sequence between them (illustrated in Fig. 1).

The functional property of molecular beacon probe has been applied for detection of PCR amplified fragments.[1,2] The qualitative and quantitative detection of the amplified targets are possible even during real time in a PCR[3,4] by bringing the probe-target solution first to a high denaturing temperature and then allowing the temperature of reaction to drop gradually. As the temperature favourable for target-probe (loop sequence) hybrid formation is reached, the probe interacts with target and produce fluorescence. In addition what makes the beacon probe system unique is that the unhybridized fraction of the probe regains its original conformation fast, when a suitable temperature lower than the melting temperature of the stem is reached. Then the unhybridized probe does not contribute any fluorescence. Thus, with a suitable temperature cycling a PCR in the presence of a beacon probe and subsequent fluorescence analysis will allow the detection of a target immediately after it is amplified, requiring no additional steps. However, with a double-stranded PCR amplified fragment, only the probe fraction that will successfully compete against the strand complementary to the target strand in the hybridization reaction, will fluoresce. Therefore, the detection signal and/or the sensitivity of detection of a molecular beacon based PCR assay is expected to be less than if only the single stranded targets were present instead of double-stranded DNA in a PCR.

The single-stranded target strand along with the double-stranded DNA can be generated by an asymmetric (when one primer is used at higher concentration than the other) PCR.[5] However, the conditions of asymmetric PCR lead to linear amplification of one of the strands and the amount of amplified product is expected to be less than that obtainable by a conventional symmetric PCR. Therefore it is important to examine whether or not the asymmetric PCR overall could be an advantage for providing increased sensitivity of fluorescence signal detection in a PCR in presence of a molecular beacon. PCR based assays are being applied ever increasingly for rapid detection of microbial agents in a specimen from patients[6–8] or from environment.[9] Improved detection signal is always desired in an assay protocol, and can be useful to avoid false-negative[10] for some specimen containing low tighter microbial agent in question. This paper, is a study on intensity of signal detected for the product of symmetric versus asymmetric PCR in presence of a molecular beacon probe, of a target gene of adenovirus an important human pathogen[11] and an environmental pollutant worldwide.[12,13]

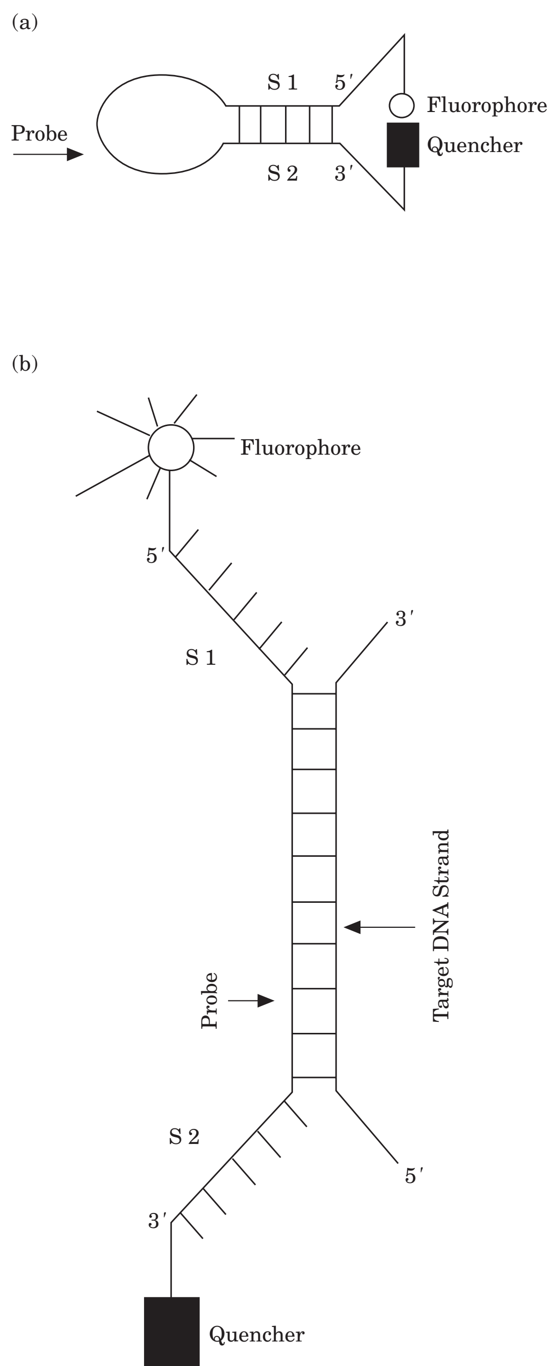

Fig. 1. (a) Schematic diagram of the probe. S1 and S2 are complementary strands of the stem structure. A fluorophore and a quencher are conjugated to the end of S1 and S2, respectively. The probe sequence is within the loop and is much larger than the stem structure. When such a probe finds its complementary target DNA strand, S1 and S2 are forced apart. (b) The probe-target hybrid is formed and in this conformation of the probe, the quencher (attached to the end of S2) can not adsorb the fluorescence emitted by the fluorophore (attached to the end of S1) and the hybridized probe–target combination will emit fluorescence in response to an excitation light.

MATERIALS AND METHODS

Stock virus and isolation of nucleic acid

A stock of adenovirus type 7 obtained from the Department of Public Health Laboratory (PHL) San Diego was used. The virus stock was 10-fold serially diluted in the transport medium (Veal infusion broth) and the nucleic acid was isolated from each diluted stock virus sample using QIamp kit (Quiagen Inc, Chatsworth, CA, USA) following the manufacturer's suggested protocol.

Probe design and preparation

A molecular beacon probe (5′ FAM GCGCTG AACAAGTTTAGAAACCCCACGGTG CAGCGC DACYL3′) was designed with a 24 nucleotide long loop sequence (not underlined) corresponding to an internal sequence of the fragment to be amplified. The six nucleotides (underlined) sequences formed the stem structure. DABCYL and 5′-6-Fluorescein (FAM) were used as the quencher and fluorophore and were coupled to 3′ and 5′ ends of the stem structure, respectively. The coupling reactions and purification of the beacon were performed by a commercial company (Midland Certified Reagent, Midland, Houston, TX, USA). A stock solution of the probe was prepared in 10 mM Tris (pH 8·0) 1 mM $MgCl_2$ and stored at −70°C.

Symmetric and asymmetric PCR amplifications

The sense (5′TGATGCCGCAGTGGTCTTAC ATGCAC3′) and antisense (5′TCCAGCAGCC CGCGGATGTCAAAGTA3′) primers derived from the conserved region of hexon gene, were identical to those used in previously reported studies[8,10] and were purchased from Life Technologies, Inc; Gaithesburg, MD, USA. The sense and antisense primers spanned bases 241–266 and 522–547, respectively, in the hexon gene sequence. The 100 µl mixture for symmetric PCR either in the absence or presence of 0·125 µM of molecular beacon probe, contained 200 µM each of four dNTPs (dATP, dGTP, dCTP, and dUTP), 0·5 U of Uracil N glycosylase (UNG), 50 pmol each of sense and antisense primers, 2·5 mM $MgCl_2$, 2·5 units of *Taq* DNA polymerase and 20 µl of the isolated nucleic acid in 1 × PCR buffer (10 mM Tris (pH 8·3), 50 mM KCl). *Taq* DNA polymerase, UNG and dNTPs were all purchased from Perkin Elmer, Cetus. The digestion of any carry over contaminant by UNG and subsequent UNG inactivation were conducted by a precycle incubation at 20°C for 10 min and at 95°C for 5 min, respectively. PCR amplification was performed using 40 cycles. Each PCR cycle consisted of 94°C for 10 s and 68°C for 1 min (for annealing and primer extension). The reaction was subjected to one cycle of post-amplification consisting of 7 min at 72°C, 5 min at 95°C and then ramping down to room temperature (20°C) within a time-period of 20 min.

The 100 µl mixture for asymmetric PCR contained all the reaction components in identical amount as that in symmetric PCR except that sense primer amount either was varied, or was 1 pmol. Also the asymmetric reactions were subjected to identical precycling, cycling, and post-cycling conditions as those used in symmetric PCR. Two identical PCR amplifications using symmetric or asymmetric conditions (with the sense primer concentration of 1 pmol) were performed for each diluted sample, one in the presence, other in the absence of molecular beacon.

Agarose gel electrophoresis

The product of the symmetric and asymmetric PCR performed in the absence of molecular beacon was analysed by agarose gel electrophoresis. Electrophoresis was conducted in TBE buffer (89 mM Trisborate, 2 mM EDTA, (pH 8·3) in a 2% (w/v) agarose gel containing 0·5 µg/ml of ethidium bromide. Ten microlitres of final PCR product was loaded on the gel. After electrophoresis, the DNA bands were visualized through an U.V. transilluminator.

Fluorescence measurement

All fluorescence measurements were conducted in the model SFM-25 Spectrofluorometer (KONTRON). The fluorescence emission spectrum was analysed using the excitation maxima for FAM at 491 nm. 50 µl out of 100 µl product of symmetric or asymmetric PCR in presence of beacon probe, was diluted to 1 ml with PCR buffer immediately after the completion of post PCR cycle and analysed for fluorescence signal at room temperature. The fluorescence emission was measured at 515 nm with excitation at 491 nm.

RESULTS

Molecular beacon probe characterization

One ml of 50 nM probe was heated to 95°C for 10 min in the absence and presence of 200 nM complementary oligonucleotide target and allowed to cool down to room temperature. Samples were then

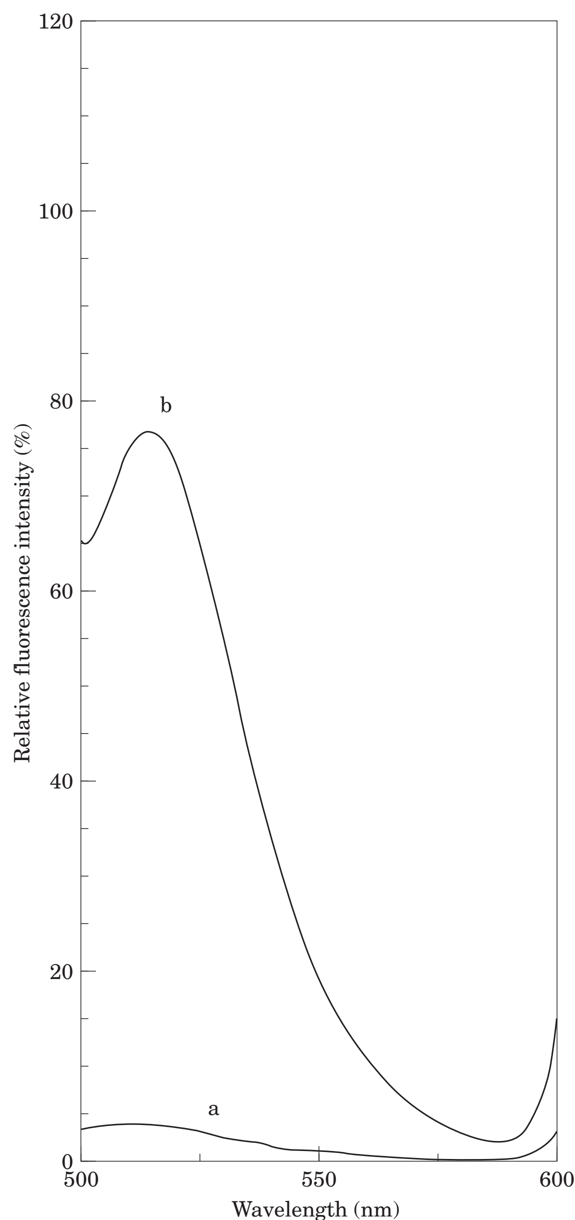

Fig. 2. Fluorescence emission spectrum analysis. 50 nM of beacon probe were analysed for emission spectrum (a) before hybridization and (b) after hybridization, with 200 nM of complementary oligonucleotide, using excitation at 491 nm. The spectrum analysis was performed in the model SFM-25 spectrofluorometer (KONTRON).

analysed, for fluorescence emission spectrum. Fig. 2 shows the characteristic emission spectrum of the hybridized (curve b) and unhybridized (curve a) samples. The curve (a) was retraced when hybridization was allowed with an oligonucleotide the sequence of which was unrelated to target. Fluorescence intensity measured at emission maxima at 515 nm, increased about 20-fold on hybridization with the complementary target. When the probe-target hybrid was digested to nucleotide by treating with deoxyribonuclease, resulting in complete separation of fluorophore from the quencher, a similar level of increase in fluorescence was observed (data not shown). This verified that all the probes were hybridized with the target, the increase in fluorescence occurred by specific hybridization and that the signal to noise ratio was expected to be 20:1 for this probe preparation. Also the efficiency of quenching (E_{ff}) of light emitted from fluorophore by the quencher in this probe preparation was found to be >96% [$E_{ff}=\{1-(F_{uh}-F_b)/(F_{ch}-F_b)\}\times 100$, where F_{uh} and F_{ch} were the fluorescence intensity of unhybridized and completely hybridized probe, respectively. F_b was the background fluorescence of buffer only].

Optimization of antisense to sense primer ratio for asymmetric PCR

Fig. 3a shows analysis of the amplified product of nucleic acid isolated from the stock virus, when PCR was conducted using different pmol ratio of antisense to sense primer. Double-stranded DNA of expected size (307 bp) was produced and the DNA band intensity decreased as the concentration of sense primer decreased. Only at 1 and 0·5 pmol of sense primer concentrations, additional product of lower molecular weight presumably the single-stranded target strand was detected. Southern hybridization of the gel (not shown), with an oligonucleotide probe complementary to the target strand showed signal for both upper and lower bands. On subsequent re-probing with an oligonucleotide made identical to that in target strand, hybridization signal only for upper band was obtained. This hybridization data verified that the lower-molecular weight band was indeed single-stranded target DNA and was neither the strand complementary to the target strand nor was any non-specific product. However, at sense primer concentration of 1 pmol, the intensity of the single-stranded band was higher than that at 0·5 pmol. At sense primer higher than 1 pmol and lower than 0·5 pmol, single-stranded DNA bands were not detected by ethidium staining. Therefore optimum pmol ratio of antisense to sense primer was determined to be 50:1 for asymmetric PCR generating the single-stranded target DNA fragment for the specific template primer combination used.

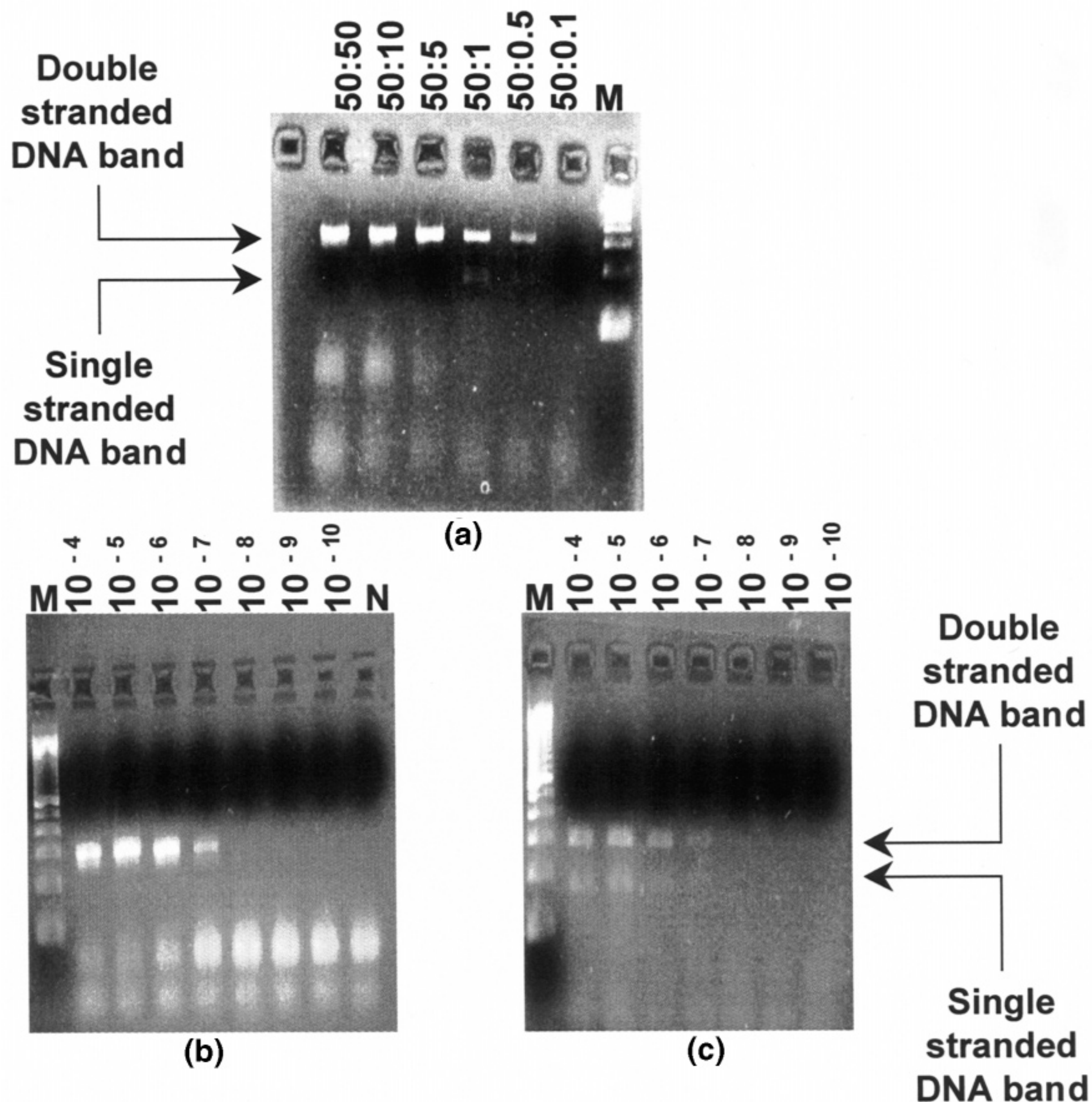

Fig. 3. Agarose gel analysis. The product of PCR using different ratio of antisense:sense primer with (a) approximately 250 ng nucleic acid purified from adenovirus stock; (b) symmetric PCR using nucleic acid from serial diluted adenovirus stock and (c) optimized asymmetric PCR nucleic acid from serial diluted adenovirus stock. The antisense:sense primer ratio and the dilutions of virus are as indicated on top of lanes. M, 100 bp molecular size marker. N, amplification of a negative control template DNA (PAW 109 plasmid DNA).

Agarose gel analysis of symmetric and asymmetric PCR

The analysis of amplified product by symmetric and asymmetric PCR for nucleic acid isolated from serially diluted virus are shown in Fig. 3b and c, respectively. The 307-bp double-stranded DNA fragments were distinctly detected up to 10^{-7} dilution of the virus stock by symmetric PCR amplification. Whereas both double- and single-stranded DNA fragments were detected up to 10^{-7} dilution of the virus by asymmetric PCR, however the band intensities of double-stranded fragment were significantly lower than those obtained by symmetric PCR. This should be due to the fact, that efficiency of generation of double-stranded fragment by asymmetric PCR is lower than that by conventional symmetric PCR. The accumulation of single stranded DNA amount by an optimized asymmetric PCR exceeds that of double stranded DNA.[4] However the fluorescence emission from ethidium bromide stained single-stranded DNA being inherently less efficient compared to double-stranded DNA, the single-stranded band intensities were not as prominent. Thus, although detection limit of both

Table 1. Relative fluorescence signal from symmetric and asymmetric PCR performed in presence of the molecular beacon probe

Dilution of virus stock	Increase in relative fluorescence signal amplified by		Percent signal intensity increase in asymmetric PCR $(\delta F_{as} - \delta F_s)/\delta F_s \times 100$
	Symmetric PCR (δF_s)	Asymmetric PCR (δF_{as})	
10^{-4}	20·5	30·3	47·8
10^{-5}	20·0	31·5	57·5
10^{-6}	16·9	18·6	10·5
10^{-7}	13·3	14·1	6·1
10^{-8}	9·4	10·0	6·3
10^{-9}	0·6	0·8	—
10^{-10}	0·5	0·6	—
N	0·6	0·5	—

Measurement of fluorescence emission was at 515 nm, with excitation at 491 nm. The fluorescence intensity of a 5 nM probe solution completely hybridized with excess complementary oligonucleotide, was calibrated as 100. All fluorescence values were obtained relative to this calibration.
Fluorescence background of buffer was 0·5. Fluorescence of probe solution without any target DNA was 17·7.
δF_s or δF_{as}, Fluorescence of hybridized sample DNA—Fluorescence of probe solution only (17·7).
N, Negative control DNA (PAW 109 plasmid DNA) sequence of which is unrelated to viral DNA.

symmetric and asymmetric PCR was the same (10^{-7} dilution), the symmetric PCR provided the expected higher-intensity DNA bands, hence higher sensitivity of detection than asymmetric PCR when analysed by electrophoresis in an ethidium bromide stained agarose gel.

Fluorescence analysis of symmetric and asymmetric PCR

The data obtained from fluorescence analysis of post PCR samples for both symmetric and asymmetric PCR using nucleic acid from serial diluted virus stock is provided in Table 1. The fluorescent signal over background was detected up to 10^{-8} diluted virus stock by both symmetric and asymmetric PCR. In contrast, the fluorescence from higher dilution virus samples were very close (for 10^{-9} dilution) or the same as that from the negative control DNA sample (background signal). Therefore, the signal detected for the samples was due to the presence of specific target. This detection limit (10^{-8} dilution) is 10-fold higher than that obtained by agarose gel analysis of unhybridized PCR product. Most importantly, fluorescence data analysis further shows that the intensity of fluorescent signal detected from asymmetric PCR was significantly higher than that from symmetric PCR in presence of beacon, for each dilution (up to 10^{-8} dilution) of the virus stock. Depending on the dilution of the virus sample, the fluorescence signal from asymmetric PCR was higher (6–57%) than that from the symmetric PCR (Table 1, column 4). Thus, for increased signal intensity and hence for increased sensitivity of detection by fluorescence analysis of a PCR performed in the presence of a molecular beacon, an optimized asymmetric PCR is preferable over symmetric PCR.

DISCUSSION AND CONCLUSIONS

The sensitivity of detection by fluorescence analysis of the product of a PCR in presence of a molecular beacon probe, depends on the amount of the probe that hybridizes to its target strand. This in turn depends on the amount of available single-stranded target and on the competition between probe and the strand complementary to the target strand of the double-stranded DNA, during hybridization. The present paper demonstrates that effective amount of target strand capable of hybridizing to the beacon probe would be generated more by asymmetric PCR than by symmetric PCR. Molecular-beacon based assay using symmetric PCR has been documented before.[1–4] The present study describes the first application and possible advantages of asymmetric PCR in a molecular beacon based detection of the signal from an assay. An assay protocol that can provide improved level of signal and/or higher sensitivity of detection is of much importance particularly for application in clinical specimens. The use of asymmetric instead of symmetric PCR should provide such desired improvements.

Amplicons (the amplified fragments) being always larger in size than the probe, will have melting temperature higher than that of probe-target. Therefore, the target strand of the amplicon will start re-annealing

to its complementary strand at temperature higher than that favorable for target-probe hybridization in the cooling-down phase during post-PCR, leaving only a fraction of the target strand for the probe at temperature lower than the melting temperature of probe-target. In addition, the complexity of sequence such as unique vs. repetitive region of the amplified DNA fragment can make a difference in the rate of re-annealing of its complementary strands. Report on detection of the amplified fragment using liquid-phase hybridization with beacon probes such as the present one is limited.[1,4] In previous reports, fragment size detected using symmetric PCR and molecular beacon was only 81 bp.[1] Although there is no reported data, it is quite possible that beacon probe based detection is efficient only for such short double-stranded amplicons generated during a symmetric PCR and that could be a potential limitation of the use of symmetric PCR with beacon probe. In many PCR based diagnostic protocol however, larger amplicon sizes in the range of 200–400 bp have been routinely used.[6–10, 14] Here, an amplicon within such a size range (307 bp) was detected by a molecular beacon based liquid-phase hybridization, and the asymmetric PCR provided significant higher detection signal and hence higher sensitivity than symmetric PCR.

At 10^{-9} dilution of the virus, the signal from symmetric PCR was within the background level whereas in asymmetric PCR the signal was higher than that from background but not appreciable enough. So the signal intensity difference between asymmetric and symmetric PCR was not given any emphasis for 10^{-9} dilution (Table 1). The percent difference in signal intensity between asymmetric and symmetric PCR was less for higher diluted (10^{-5} to 10^{-8}) sample (Table 1) possibly because of lower rate of self annealing of target and complementary strand due to the lower concentration of symmetric PCR product generated from lower amount of template DNA. At 10^{-4} dilution the amount of DNA synthesis was not appreciably higher than that at 10^{-5} dilution by both symmetric and asymmetric PCR (Fig. 3(b) and (c), Table 1). This might be due to partial inhibition of *Taq* DNA polymerase activity by inhibitors often present in clinical samples and isolates and that gets co-purified with nucleic acid.[14,15]

Hybridization of an internal probe such as the one used here provides an additional level of specificity of detection over that by agarose gel analysis and ethidium bromide staining. This is because of the fact that a non-specific amplified fragment is improbable to contain sequence specific for hybridization to the beacon probe and produce fluorescence. The beacon probe based detection further increased the detection limit 10-fold by symmetric PCR and possibly more than 10-fold by asymmetric PCR as compared to that by an agarose gel analysis of a conventional symmetric PCR (Table 1 and Fig. 3(b)). This comparative data may help clinical laboratorians to consider adopting the hybridization probe based detection protocol over that of the agarose gel analysis for certain class of specimen where a 10-fold difference in sensitivity of assay might help avoiding false-negative for a significant number of specimens.

The quality and performance efficiency of a molecular beacon probe depends on the signal to noise ratio, and on the efficiency of quenching. The signal to noise ratio (20:1) and the efficiency of quenching (>96%) for the probe preparation used in the present study were comparable to those used in other studies.[2] The molecular beacon based technology offers a truly homogeneous assay in which amplification and detection of fluorescent signal emitted from the beacon probe hybridized to the amplified DNA can occur in one tube. This makes the assay method easy to perform, and less time consuming by reducing the number of steps compared to blotting and probing methods.[7,8] In principle and in practice the risk of carry-over contamination is also considerably minimized by the advantage of performing the entire method in a hermetically closed tube. In a molecular beacon based assay, asymmetric PCR on one hand can potentially increase the sensitivity of detection and on the other reduces the cost of an assay by reducing the usage of the amount of one of the primers. In a high throughput diagnostic assay laboratory asymmetric PCR could be of economic advantage over symmetric PCR. Thus the use of an optimized asymmetric amplification condition in conjunction with a well designed and characterized beacon probe will provide all the practical advantages desirable.

In conclusion, detection with high sensitivity is possible by both symmetric and asymmetric PCR in presence of a molecular beacon and subsequent fluorescence analysis. However, asymmetric PCR can result in higher sensitivity than symmetric PCR by providing higher fluorescent signal due to the presence of target strand alone in higher proportion in the product that can hybridize non-competitively with the probe. Therefore, in a molecular beacon based PCR assay method, asymmetric PCR could be a preferred choice compared to symmetric PCR for the detection of a target gene of a microbial agent in question.

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