

Notes & Tips

Real-time monitoring of branched rolling-circle DNA amplification with peptide nucleic acid beacon

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Molecular diagnostics based on DNA amplification literally revolutionized biotechnology, health care, forensics, and biodefense [1,2]. Recently emerged techniques that quantify the amplified DNA in real time have further advanced these fields [3–5]. A key element in the real-time amplification methods are fluorogenic probes, with molecular beacons being among the most useful [4]. Despite an assortment of detection formats, highly potent real-time monitoring of exponential DNA amplification at a single temperature using molecular beacons has not yet been developed. We present here an innovative approach to this important goal.

The approach is based on the rolling-circle DNA amplification (RCA),¹ an increasingly popular method for molecular diagnostics due to its simplicity and high specificity in the detection of nucleic acids, proteins, and other biomarkers [6]. In addition to DNA polymerase, DNA minicircle and a pair of primers required for exponentially branched RCA [7], our design employs a peptide nucleic acid (PNA) stemless molecular beacon [8,9] and a restriction enzyme, as shown in Fig. 1. Branched RCA reaction yields long double-stranded DNA amplicons generally inaccessible for probe hybridization. Given the unique duplex-invading ability of PNA oligomers [10,11], we assumed that a PNA beacon could rapidly bind the RCA products by strand displacement provided that these products are converted into short

DNA duplexes to facilitate the PNA-to-DNA invasion process from the duplex termini.

Short DNA duplexes are compulsory components in our design because PNA invasion into DNA sites located inside long DNA duplexes is known to be strongly inhibited by high concentrations of salts [11] usually required by DNA polymerase. The conversion of long DNA amplicons into smaller fragments can be done by a restriction enzyme if a DNA minicircle carries a pair of enzyme recognition sequences flanking the PNA-binding site in the resulting duplex amplicon. The lack of a stabilizing stem in a PNA beacon [8,9] should also promote its strand-displacement binding. The remarkable PNA biostability [12] is also advantageous given certain problems with the degradation of DNA-based molecular beacons by the nuclease activity intrinsic in some DNA polymerases [13]. The entire assay we envisioned should work in the closed tube in real time provided that all necessary components were in place and conditions were compatible with all of the processes involved.

The proof-of-concept experiment shown in Fig. 2 demonstrates the functionality of a new assay that is rooted in a strikingly concerted performance of the molecular quintet: DNA minicircle and a pair of primers, two DNA-processing enzymes, and PNA beacon.² In this experiment, a 55-nt DNA minicircle was

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¹ Abbreviations used: RCA, rolling-circle amplification; PNA, peptide nucleic acid; SNP, single-nucleotide polymorphism.

² Note in this connection that the real-time strand-displacement amplification [5] has a very close design (although based on different principles) in that it also successfully uses the concerted performance of DNA polymerase, restriction enzyme, and fluorogenic primers/probes.

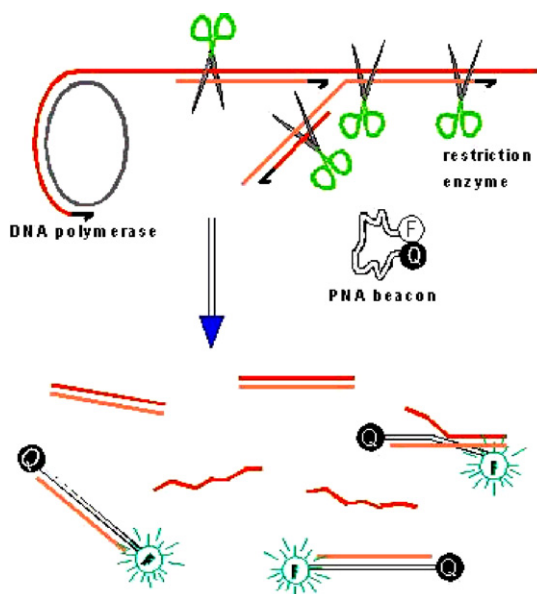


Fig. 1. Schematic representation of the real-time branched RCA assay. A pair of primers (they correspond to the complementary DNA strands in RCA amplicons shown in dark and light red) provides for the replication branching; a strand-displacing DNA polymerase is required here. In PNA beacon, F and Q are fluorophore and quencher, respectively.

exponentially amplified by *Bst*I DNA polymerase with the use of two 18–20mer primers; the resulting amplicons were digested by *Hpa*II restriction enzyme and detected with an 18mer PNA beacon.³ One can see that our assay allows reliable detection in a few minutes of just about 10^7 DNA minicircles (i.e., ~ 15 amol). Therefore, it features close to attomolar sensitivity, which is superior to the sensitivity of real-time detection with linear RCA using conventional DNA beacons [13].

We expect the sensitivity of our assay to be substantially improved by further optimization. Most important factors here could be the relative and absolute amounts of DNA polymerase and restriction enzyme because their concerted interplay is essential for higher amplification. Smaller reaction volumes and more powerful light sources (similar to laser detectors in automated sequencing machines) may also considerably reduce the number of input analytes in real-time quantitation. Indeed, use of nanoliters in real-time nucleic acid diagnostics [15], instead of microliters taken in our experiments, might result in 10^3 -fold sensitivity increase. Use of lasers, instead of the spectrofluorometer's xenon lamp used by

³ Although a DNA minicircle carries restriction enzyme recognition sequences, we presumed that these sites would be poor substrates for restriction enzymes both within a single-stranded probe circle and in its complex with a complementary DNA strand because the resulting duplex is short (~ 25 bp) and the complex is actually triple-stranded [6]. Still, the choice of restriction enzyme may be critical for the assay performance. We have found that though *Msp*I endonuclease, the *Hpa*II isoschizomer, is also suitable for the assay, (although it works however with somewhat reduced overall rate).

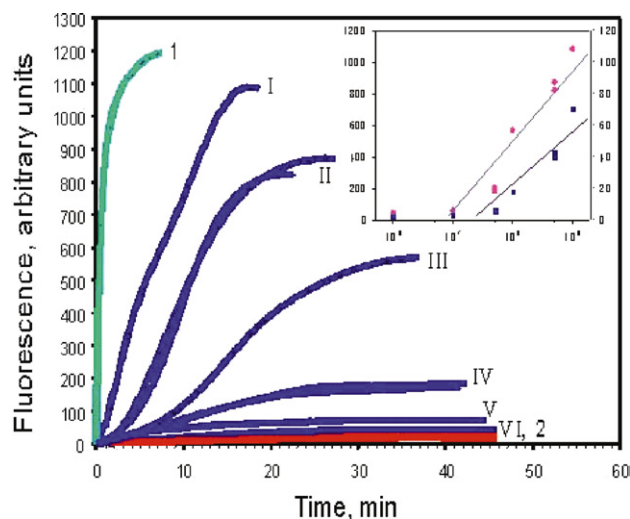


Fig. 2. Real-time kinetics of branched RCA reactions. Input numbers of DNA minicircles are 10^9 , 5×10^8 , 10^8 , 5×10^7 , 10^7 , and 10^6 (curves I–VI, respectively), estimated for approximately $1 \mu\text{l}$ fluorescence-generating illuminated portion of the reaction volume. Curves II and IV were obtained in duplicates to demonstrate good reproducibility of the assay. Curves 1 and 2 are positive and negative controls showing the corresponding time courses of the PNA beacon hybridization with the single-stranded DNA target ($1 \mu\text{M}$) and the undigested double-stranded RCA amplicons (10^9 probe circles; no signal is observed without restriction enzyme). Inset: real-time quantitation of probe circles using initial rates of fluorescent responses (right: fluorescence units [FU]/min) and their maximal/saturation values (left: FU) calculated from the data of the main graph. Experimental conditions: 10^6 – 10^9 DNA minicircles/ μl | 4 U *Bst*I DNA polymerase (large fragment) | 40 U *Hpa*II restriction enzyme | $0.4 \mu\text{M}$ of primers (each) | $0.15 \mu\text{M}$ of PNA beacon | reaction buffer (ThermoPol; pH 8.8): 20 mM Tris–HCl, 10 mM KCl, 10 mM $(\text{NH}_4)_2\text{SO}_4$, 2 mM MgSO_4 , 0.1% Triton X-100 | 45°C | 200 μl reaction volume. DNA minicircle, 5'*TGTACTACTG**GTCTCCGGCTGAAGTGGCAACAGACC**GGTGAGACCGTTACGCTCA* (written here as a linear sequence with the *Hpa*II sites shown in italic and a part corresponding to PNA beacon being underlined) was obtained by ligation with splint oligonucleotide 5'*CAGTAGTACATGAGCGTAAC* and T4 DNA ligase (similar to that described in [14]). Primers: 5'*GAGCGTAACGGTCTCACC* and 5'*CTCATGTACTACTGGTCTCC*. PNA beacon (gift from M. Fiandaca): Flu–Glu–TCTGTTGCCACTTCAGCC–Lys–Lys[DABCYL] (DABCYL: 4-[4-dimethylaminophenylazo]benzoic acid, the quencher). Fluorescence was monitored on a Hitachi F-2500 spectrofluorometer (493 nm excitation and 527 nm emission) using a closed thermostatted semi-micro quartz cell. The assay was performed at 45°C because a faster response was observed under this condition than at room temperature or 37°C .

us, should additionally enhance the fluorescence response by at least 10^2 -fold. As a result, just a combination of these two rather simple technical improvements may, in essence, decrease the number of input molecules from the currently required 10^7 molecules to $10^7/10^5 = 100$ molecules.

In our design, the PNA beacon is complementary to a part of the DNA minicircle and, therefore, can hybridize to it, imposing an obstacle for DNA polymerase movement. However, we assume that DNA polymerases with robust strand-displacing ability (e.g., *Bst*I or $\phi 29$ DNA

polymerases) can readily displace PNA beacon during rolling replication, and our positive results prove this assumption. Still, the PNA beacon binding to the DNA minicircle may limit the assay sensitivity, but pseudo-complementary modifications of nucleobases [16–18] within DNA minicircles and PNA beacons can then be used to completely avoid the unwanted PNA–DNA complexes.

The workability of our assay is indeed remarkable given that a more straightforward and seemingly simpler design employing the triplex-forming DNA beacons [19] failed. In our pilot study, we could not find the conditions where both efficient branched replication of a DNA minicircle and sensitive fluorescent detection of corresponding amplicons take place simultaneously. The addition of one more enzyme, the DNA ligase, to our biomolecular ensemble will allow the extension of this approach to analysis of single-nucleotide polymorphisms (SNPs) [20] and RNA targets. We believe that our approach can also be employed as signal amplifier in immuno-RCA diagnostics [21], further extending its applicability to important nonnucleic acid analytes, including environmental pollutants, allergens, and toxins.

In conclusion, proof of principle established in this study opens up new vistas in the development of real-time RCA technology. This novel *in vitro* diagnostic tool promises expedient homogeneous systems for isothermal wide-range quantitation of various analytes and makes such systems more favorable for automation. Because the real-time RCA monitoring allows a closed-tube diagnostic format, it also minimizes the risk of false-positive results via cross-contamination, especially in case of very small probe inputs. Use of PNA beacons as hybridization probes to specific amplicon sequences⁴ also helps to avoid circle-independent amplification artifacts occasionally observed with the exponentially branched RCA [20]. Other convenient fluorogenic, amplicon-specific PNA probes can also be used to provide the assay flexibility [25–27].

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⁴ Detection of RCA amplicons in real time is readily possible with the use of nonspecific profluorescent DNA-binding dyes [22,23]. However, in this approach, the detection specificity often can be compromised, and multiplex analyses are not possible. Alternatively, a fluorogenic primer (or a pair of them) can be employed in RCA reactions, becoming brightly fluorescent after incorporation into the RCA amplicon [20,24]. The latter approach is an analyte/amplicon-specific one and can be multiplex [20]. Yet no more than two fluorophores can be incorporated per amplicon in this way, possibly limiting the real-time detection sensitivity. Modified structured primers may also have reduced RCA-priming efficiency.

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