Notes & Tips

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

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Received 7 May 2004
Available online 28 August 2004

Molecular diagnostics based on DNA amplification literally revolutionized biotechnology, health care, forensics, and biodefense \cite{1,2}. Recently emerged techniques that quantify the amplified DNA in real time have further advanced these fields \cite{3–5}. A key element in the real-time amplification methods are fluorogenic probes, with molecular beacons being among the most useful \cite{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),\textsuperscript{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 \cite{6}. In addition to DNA polymerase, DNA minicircle and a pair of primers required for exponentially branched RCA \cite{7}, our design employs a peptide nucleic acid (PNA) stemless molecular beacon \cite{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 \cite{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 \cite{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 \cite{8,9} should also promote its strand-displacement binding. The remarkable PNA biostability \cite{12} is also advantageous given certain problems with the degradation of DNA-based molecular beacons by the nuclease activity intrinsic in some DNA polymerases \cite{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.\textsuperscript{2} In this experiment, a 55-nt DNA minicircle was

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

\textsuperscript{2} Note in this connection that the real-time strand-displacement amplification \cite{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.
exponentially amplified by BstI DNA polymerase with the use of two 18–20mer primers; the resulting amplicons were digested by HpaII restriction enzyme and detected with an 18mer PNA beacon.¹ One can see that our assay allows reliable detection in a few minutes of just about 10⁷ 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 molecules. Nevertheless, the use of lasers, instead of microliters taken in our experiments, might result in 10³-fold sensitivity increase. Use of lasers, instead of the spectrofluorometer's xenon lamp used by us, should additionally enhance the fluorescence response by at least 10²-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 10⁷ molecules to 10⁶/10⁵ = 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., BstI or φ29 DNA polymerase with somewhat reduced overall rate).
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 condi-
tions where both efficient branched replication of a DNA 
minicircle and sensitive fluorescent detection of corre-
sponding amplicons take place simultaneously. The addi-
tion 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 diag-
nostic 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 sequences also helps to 
avoid circle-independent amplification artifacts occa-
sionally observed with the exponentially branched RCA 
[20]. Other convenient fluorogenic, amplicon-specific 
PNA probes can also be used to provide the assay flexi-
bility [25–27].

Acknowledgments

We thank J. Coull and M. Fiandaca (Applied Biosys-
tems) for providing us with the PNA beacon and G. 
Jones (Boston University) for providing access to the 
spectrofluorimeter in initial experiments. This work was 
supported by the Hamilton–Thorne Biosciences through 
a sponsored research agreement and by Boston Univer-
sity PIF award.

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