Sensitive Nucleic Acid Detection by Primer Generation-Rolling Circle Amplification

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Introduction

A simple isothermal nucleic acid amplification method, primer generation-rolling circle amplification (PG-RCA), was developed to detect specific nucleic acid sequences in DNA samples.[1] This amplification method is achieved at constant temperature simply by mixing a circular DNA probe (generated using EPICENTRE's CircLigase™ ssDNA Ligase), DNA polymerase, and a nicking enzyme. As compared to conventional nucleic acid amplification methods, PG-RCA is free from the design and usage of exogenous primers, since "primers" are generated during the reaction. Previously, we demonstrated that PG-RCA can detect as little as 0.163 pg (~60 molecules) of genomic DNA from *Listeria monocytogenes* and distinguish clearly *L. monocytogenes* genomic DNA from other bacterial DNA.[1] In this report, we describe further optimization of PG-RCA assay conditions to enhance assay speed (previously compromised to improve assay sensitivity), and to expand the same assay scheme to detection of RNA samples.

Methods

Materials

CircLigase ssDNA Ligase, Hybridase™ Thermostable RNase H, and Exonuclease I were purchased from EPICENTRE Biotechnologies. *Bst* DNA polymerase, *Nb.Bsm* I, and Exonuclease III were purchased from New England Biolabs. Oligonucleotides were purchased from Integrated DNA Technologies or Sigma Genosys.

Preparation of circular probes

Circular probe was prepared by self-ligation of 20 µM 5′-phosphorylated circular probe precursor (5′-pGCTG TGCTCAAGGTGTGTGAATGCTGTGCTCAAGGTG TGTGAATGCTGTGCTCAAGGTGTGTGAAT-3′). The self-ligation reaction was conducted at 60°C for 3 hours in a 40-µl reaction containing 50 mM MOPS buffer (pH 7.5), 10 mM KCl, 5 mM $MgCl_2$, 2.5 mM $MnCl_2$, 50 µM ATP, 1 mM DTT, and 200 U CircLigase ssDNA Ligase. Circularized probes were treated with 10 U Exonuclease I and 100 U Exonuclease III at 37°C overnight; probes were subsequently purified by electrophoresis in 20% polyacrylamide/7 M urea gels.

PG-RCA

PG-RCA was performed at 60°C in a 10-µl reaction containing 20 mM Tris-HCl (pH 8.8), 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 6 mM $MgSO_4$, 0.4 µM each dNTP, 0.1 % Triton® X-100, 10,000-fold diluted SYBR® Green I (Invitrogen), 7.5 nM circular probe, 0.1 U *Bst* DNA polymerase large fragment, 1 U *Nb.Bsm* I and varying amounts of sample DNA. For detection of sample RNA, 0.00016 U Hybridase Thermostable RNase H was added to the above reaction mixture. Sample nucleic acids used in this study were synthetic DNA (5′-CACACACCTTGAGCACAGCATTCACACA CCAAAAA-3′) and RNA (5′-CACACACCUUGAGCAC AGCAUUCACACACCAAAAA-3′) molecules with complementary internal sequences to the circular probe. For real-time detection, the fluorescence intensity of each reaction was monitored in a MyiQ™ real-time PCR instrument (Bio-Rad) with a SYBR® Green filter set. Threshold time (T_T) was estimated from the reaction time when fluorescence intensity of the reaction exceeded an arbitrary threshold, which was set right above the background fluorescence intensity.

Results

Principle of PG-RCA and probe design

The PG-RCA method enables sensitive detection of sample DNA using circular DNA probes as signal amplification tools.[1] Designing circular probes is fairly easy, as they are required to carry only two sequences for signal amplification: a hybridization sequence complementary to sample DNA, and a complementary nicking site. PG-RCA initiates from hybridization of a circular probe to sample DNA. A nicking enzyme recognizes the duplex structure and cleaves the sample DNA at the nicking site, which triggers a cascade reaction of linear rolling circle amplification (LRCA) and nicking reactions (Fig. 1). LRCA is designed to produce long, concatenated copies of the circular probe sequence, while the nicking reaction generates multiple "primers" for the circular probe from the LRCA product. Therefore, these reactions continuously initiate each other. Also, as multiple reaction cycles can be initiated from a single cycle, the PG-RCA reaction accumulates LRCA products and "primers" in an exponential manner over time.

Fairly pure circular probes for PG-RCA can be prepared easily by circularizing 5′-phosphorylated oligonucleotides intramolecularly using CircLigase ssDNA Ligase (Fig. 2). Because of the extraordinary sensitivity of PG-RCA, the use of splint oligonucleotides for preparation of circular

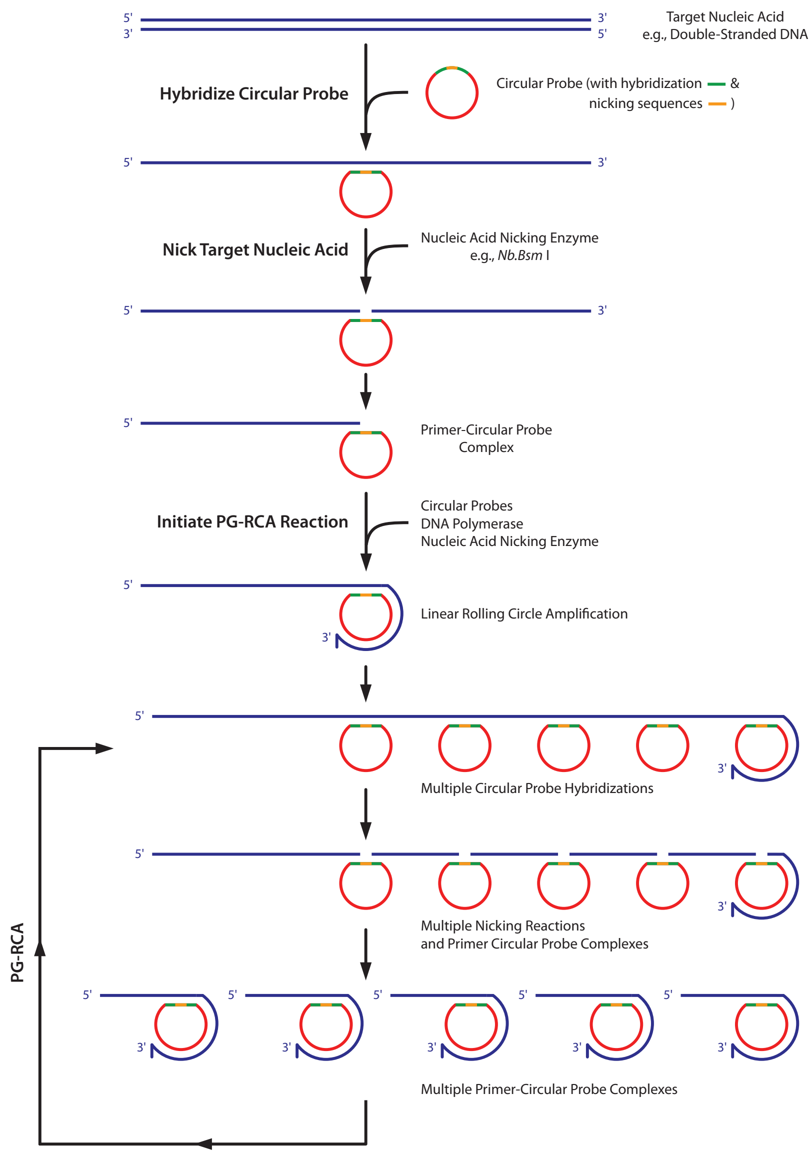

Figure 1. Schematic overview of the Primer Generation-Rolling Circle Amplification (PG-RCA) technique.

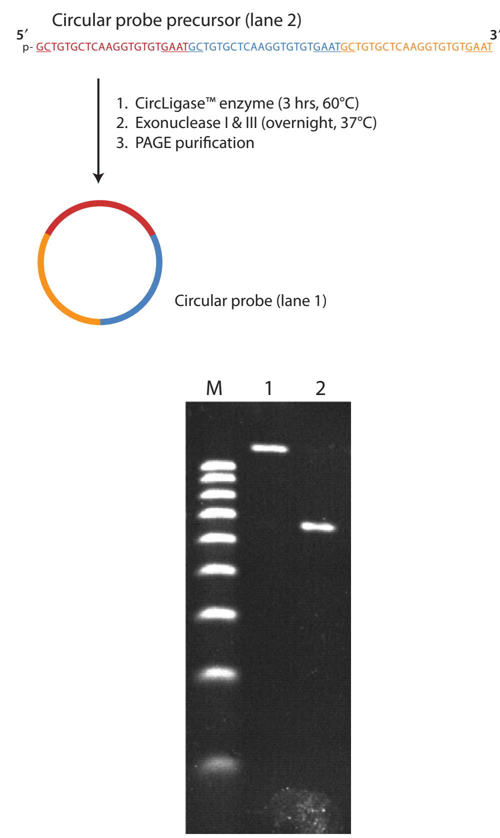

Figure 2. Preparation of circular probe. Circular probe contains three repeats (colored) of the hybridization sequence to sample nucleic acid with 8-base overlaps and a nicking site for *Nb.Bsm* I (underlined). A 5′-phosphorylated oligonucleotide was circularized using CircLigase™ ssDNA Ligase, followed by exonuclease treatment and purification by denaturing polyacrylamide gel electrophoresis (15% PAGE/7 M urea). This analysis confirmed the purity of the circular probe: lane M, 20- to 100-base ladder; lane 1, circular probe; lane 2, circular probe precursor.

probes is not recommended. Splint oligonucleotides include complementary sequences to the circular probes, and may cause background signals and lower the assay sensitivity unless they are removed completely and/or designed carefully.

PG-RCA of RNA samples

Bst DNA polymerase can also initiate polymerization from the 3′ ends of RNA. In order to initiate PG-RCA from RNA samples efficiently, Hybridase Thermostable RNase H enzyme was included in the reaction to cleave sample RNA upon hybridization with circular probes (similar to the nicking reaction). Once the sample RNA is cleaved by RNase H, a cascade reaction of PG-RCA will begin. Without RNase H, PG-RCA did not detect sample RNA efficiently because the nicking enzyme is not able to cleave RNA strands in RNA-DNA duplexes (i.e., duplexes formed between sample RNA and circular probe). The optimum concentration of Hybridase Thermostable RNase H for RNA detection was extremely low (0.00016 units per reaction); higher concentrations decreased the assay sensitivity, because RNase H digests sample RNA completely on the circular probe before *Bst* DNA polymerase recognizes the cleaved 3′ ends (Fig. 3). Real-time PG-RCA analysis indicates that the assay can detect as low as 25.3 zmol (1.5 x 10^4 molecules) of sample RNA (Fig. 4). The detection limit of RNA is two orders of magnitude lower than that of DNA, because *Bst* DNA polymerase initiates LRCA from RNA 3′ ends approximately 100 times less efficiently than from DNA 3′ ends (unpublished data).

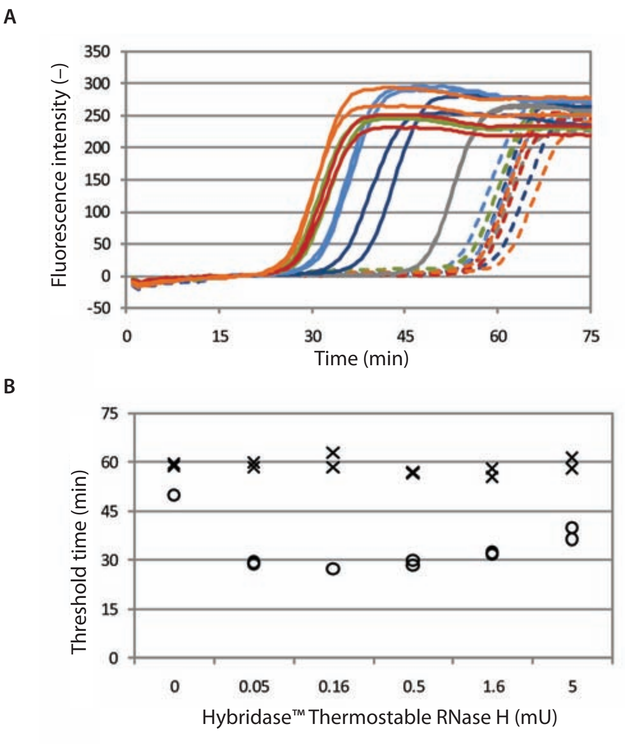

Figure 3. The concentration of Hybridase™ Thermostable RNase H was optimized for PG-RCA analysis of sample RNA. A, Positive control (500 amol sample RNA, solid lines) and negative control (no sample RNA, perforated lines) were analyzed by real-time PG-RCA supplemented with 0 mU (gray), 0.05 mU (red), 0.16 mU (orange), 0.5 mU (green), 1.6 mU (light blue) or 5 mU (blue) Hybridase Thermostable RNase H (n = 2). B, Threshold time (T_T) of the positive (o) and negative (×) control reactions were compared to obtain the optimum concentration of Hybridase Thermostable RNase H.

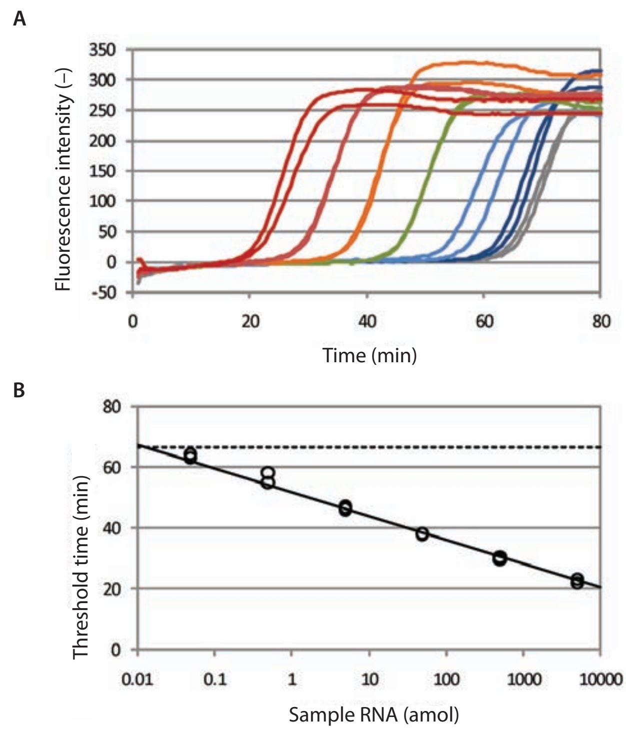

Figure 4. RNA detection by PG-RCA. A, Fluorescence intensity of the PG-RCA reaction was monitored in real time. Sample RNA for each reaction was prepared by 10-fold serial dilution from 5,000 to 0.05 amol (1 amol = 10^{-18} mol), and the respective signal amplification curves are indicated by colored lines (dark red, red, orange, green, light blue and blue, respectively) (n = 2). Negative controls are indicated by gray lines (n = 2). B, Threshold time T_T was plotted against the sample RNA concentration (S) of the reaction. The solid line indicates linear least-squares fitting between 5,000 and 0.05 amol of sample RNA [$T_T = -8.37 \log_{10}(S) + 53.0$], $R^2 = 0.995$. The dotted line indicates average T_T value of the negative controls. The detection limit was 0.0253 amol (25.3 zmol, or 1.52 x 10^4 molecules) of RNA, determined by calculation from the intersection of both lines.

Conclusions

PG-RCA is useful for specific and sensitive detection of nucleic acid sequences in sample DNA and RNA under isothermal conditions. Compared to PCR, precise control of thermal cycling is not required, which is advantageous in point-of-care molecular diagnostics and high-throughput systems. The method also does not require specially designed exogenous primers, making it easier to use than PCR-based amplification. The circular probes required can be easily generated using CircLigase ssDNA Ligase, and the only requirement is the inclusion of hybridization and nicking sequences.

Reference

1. Murakami, T. *et al.* (2009) *Nucleic Acids Res.* **37**, e19.

Note: *EPICENTRE recently introduced CircLigase™ II ssDNA Ligase, an enzyme formulation that requires different reaction conditions compared to the original CircLigase enzyme.*

Cat. #	Conc.	Quantity
CircLigase™ II ssDNA Ligase		
CL9021K		1,000 Units
CL9025K		5,000 Units
Hybridase™ Thermostable RNase H		
H39100	5 U/µl	100 Units
H39500	5 U/µl	500 Units
Exonuclease I, *E. coli*		
X40501K	20 U/µl	1,000 Units
X40505K	20 U/µl	5,000 Units
X40520K	20 U/µl	20,000 Units
Exonuclease III, *E. coli*		
EX4405K	200 U/µl	5,000 Units
EX4425K	200 U/µl	25,000 Units