Electronic supplementary information

Highly specific DNA detection from massive background nucleic acids based on rolling circle amplification of target dsDNA

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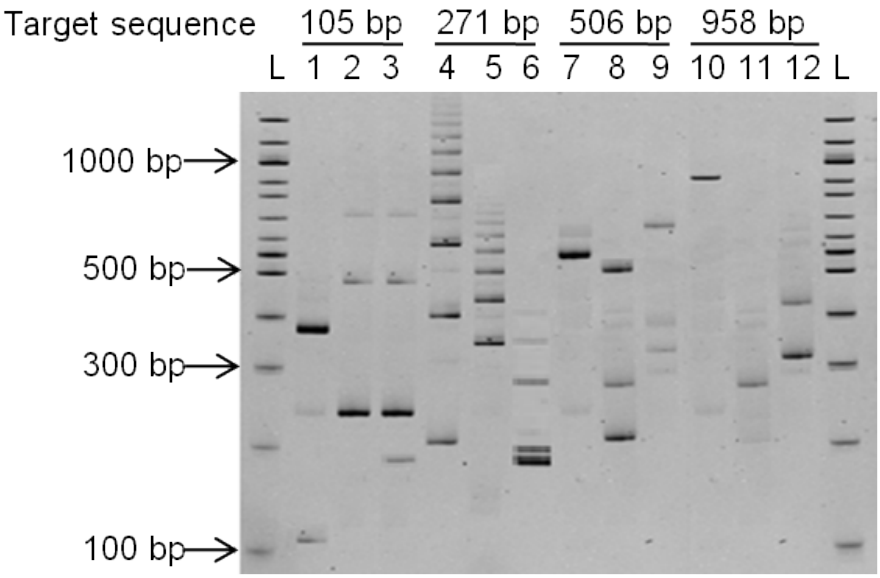

Fig. S1 Detection of target sequences with different length by the RCA protocol without using SMB. Four target sequences (varies in length) were amplified by TC-RCA and analyzed on 8.0% PAGE. The copy number of the target sequences was 6×10^2 in lane 1, 4, 7, 10; 60 in lane 2, 5, 8, 11 and 0 (negative control) in lane 3, 6, 9, 12. Lane L was a 100 bp DNA ladder.

Comparing to Fig. 4, the data indicated that the SMB (streptavidin magnetic beads) were essential for SMB-assisted TC-RCA to perform with desirable specificity.

Experiment: All the procedures were performed according to the steps described for SMB-assisted TC-RCA, except using SMB to immobilize target amplicons to eliminate background nucleic acids. The assay was performed according to the following steps: 1) Digestion of sample DNA by TspR I; 2) Circularization of target DNA by Ligation; 3) Hyper-branched RCA amplification; 4) Digestion of RCA products by TspR I.

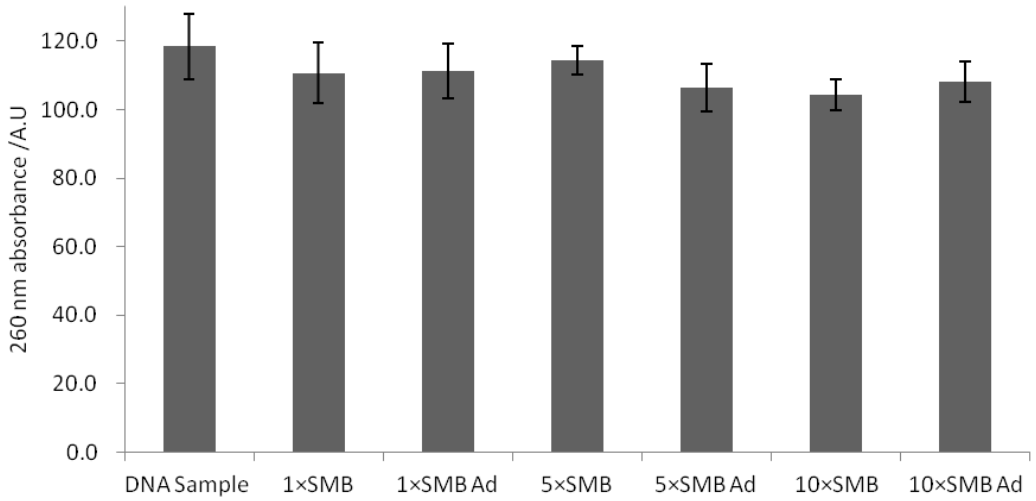

Fig. S2 Investigation of the non-specific adsorption of DNAs. The subscript "1×SMB" indicated the added SMB had the same amount as used in SMB-assisted TC-RCA, and "5×SMB" or "10×SMB" denoted five or ten folds of SMB used in SMB-assisted TC-RCA, respectively. "SMB Ad" representsed the added magnetic beads were loaded with biotinylated adaptor with the same amount as used in SMB-assisted TC-RCA. Statistical analysis (analysis of variance) showed that there was no significant difference between the ultraviolet absorption of DNA sample and DNA with SMB.

The experimental data showed that the non-specific adsorption of DNAs to the magnetic beads barely happened in SMB-assisted TC-RCA. Even if the SMB were loaded with adaptor, the adsorption of DNAs was tiny. Therefore, non-specific adsorption of DNAs to magnetic beads was negligible in our protocol.

Experiment: Oyster genomic DNA (ca.110 ng/μL) was used as DNA sample in 10 μL volume containing various amounts of streptavidin magnetic beads (SMB). After addition of SMB with or without adaptor, the solution was kept at room temperature for 20 min. Then SMB were separated and aliquot of 2.0 μL solution was subject to ultraviolet adsorption measurement by Nanodrop 2000.

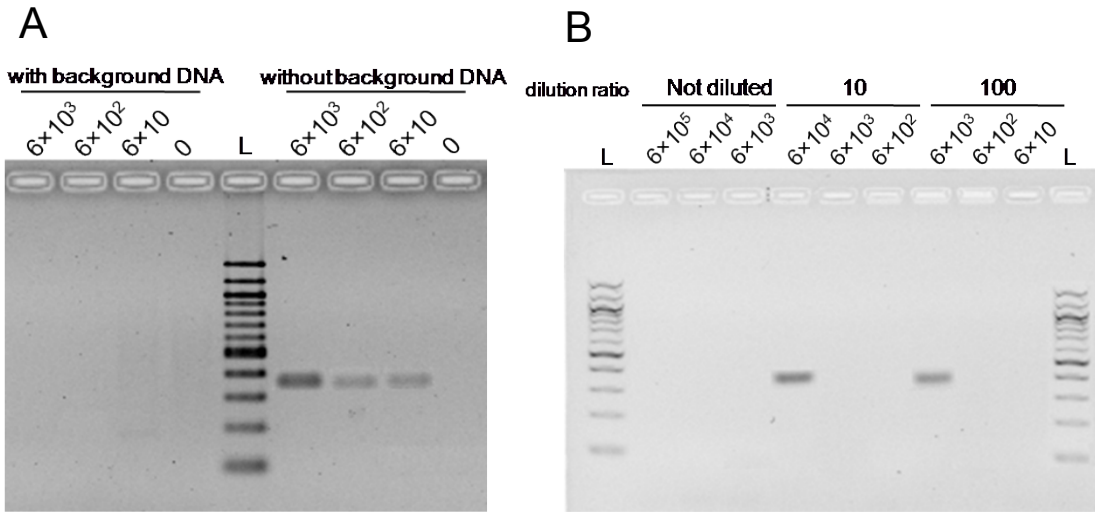

Fig. S3 Effect of background DNA on PCR for *Vibrio Parahaemolyticus* detection. (A) The effect of background DNA on PCR detection. Oyster genomic DNA (600 ng) was added as background DNA. The products were analyzed on 1.5% agarose gel. Lane L was loaded with a 100 bp DNA ladder (100, 200, 300, 400, 500/517, 600, 700, 800, 900, 1000, 1200 and 1517 bp from the bottom). (B) Gradient dilution for PCR to detect target sequence in the presence of background DNA. The results were analyzed on 1.5% agarose gel. Target sequence was incorporated with 600 ng oyster genomic DNA. Lane L was a 100 bp DNA ladder as detailed in Figure S3A.

The result showed that routine PCR was not capable of detecting target sequence in the presence of massive background DNA. Gradient dilution for PCR to detect the target sequence in the presence of massive background DNA was not applicable for the possibility of causing false negative results.

Experiment: For detecting target sequence (pUC-18) from massive background DNA, the primers were selected and the conditions were optimized. The PCR procedure was as follow: initial denature: 94°C for 2 min, denature: 94°C for 15 s, annealing: 49°C for 30s, primer elongation: 68°C for 60 s. 30 cycles was employed. Final extension: 68°C for another 5 min and terminated at 4°C. 20 μL PCR reaction system (pH=8.3) consisted of 2.0 μL template, 20 mM Tris-HCl (pH 8.8), 10 mM $(NH4)_2SO_4$, 2.0 mM $MgSO_4$, 1.0 mg/mL BSA, 1.0% Triton X-100, 0.25 mM dNTPs, 0.2 μM primers and 1.25 U Pfu DNA polymerase. Forward primer: 5'-ATGACCAAAATCCCTTAACG-3', reverse primer: 5'-GTCTTGAGTCCAACCCGG-3'. Length of the product was 358 bp.

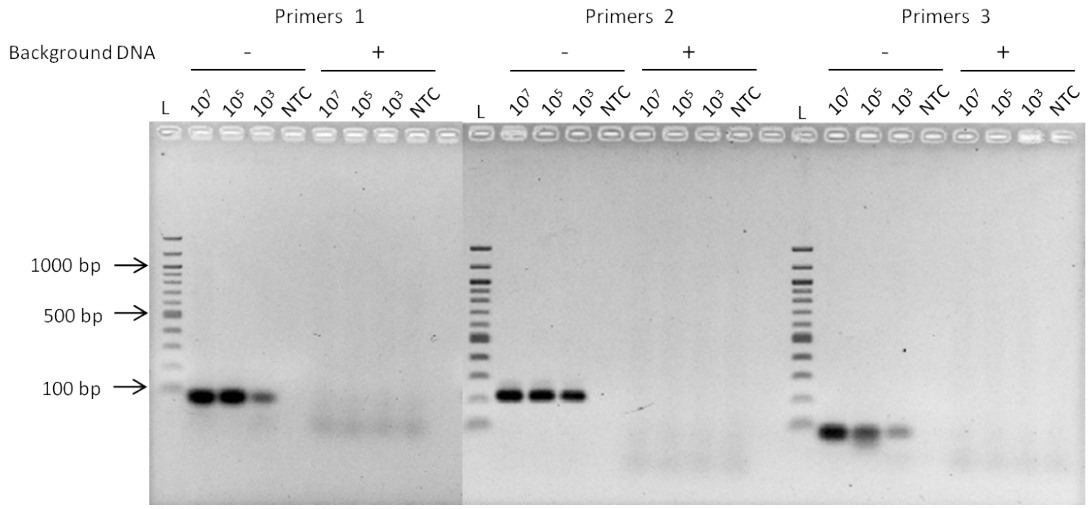

Fig. S4 Effect of background DNA on PCR for *Vibrio Parahaemolyticus* detection. Amplification products were analyzed on 1.5% agarose gel and 600 ng oyster genomic DNA was added as background DNA. Lane L was loaded with a 100 bp DNA ladder (100, 200, 300, 400, 500/517, 600, 700, 800, 900, 1000, 1200 and 1517 bp from the bottom).

The result indicated that the routine PCR was not capable of detecting specific gene sequence of *Vibrio Parahaemolyticus* in the presence of oyster genomic DNA.

Experiment: We studied the detection of target sequences from massive background DNA by PCR. The PCR procedure was as follow: initial denature: 94°C for 2 min, denature: 94°C for 15 s, annealing: 58°C for 30s, primer elongation: 72°C for 45 s. 35 cycles was employed. Final extension: 72°C for another 5 min and terminated at 4°C. 10 μL PCR reaction system (pH=8.3) consisted of 2.0 μL template, 20 mM Tris-HCl (pH 8.8), 10 mM $(NH4)_2SO_4$, 2.0 mM $MgSO_4$,1.0 mg/mL BSA, 1.0% Triton X-100, 0.25 mM dNTPs, 0.2 μM primers and 1.25 U Pfu DNA polymerase. Primer 1: 5'-TTTCCGTGCGGCAATAACAC-3' (forward), 5'-TGCCGTTTTCGCTTTCTTCG-3' (reverse), length of product: 76 bp. Primer 2: 5'-AATCGGCGATGGGTGAGAAG-3' (forward), 5'-CGAGTCATTTCACGCGCTTT-3' (reverse), length of product: 130 bp. Primer 3: 5'-ACCAGGCAAACTTGCAGACT-3' (forward), 5'- GCAGAGTCACCCTCCACAAT-3' (reverse), length of product: 75 bp.

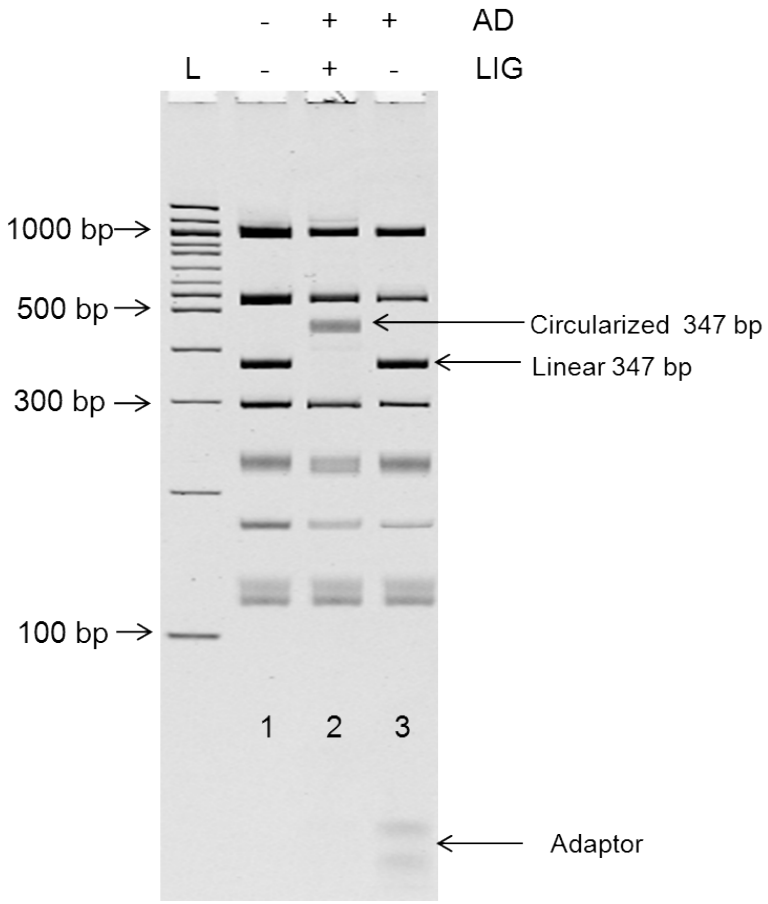

Fig. S5 Specific circularization of the 347 bp target sequence by 347 bp adaptor. The 347 bp sequence was circularized by 347 bp adaptor with the help of Taq DNA ligase in the presence of pUC-18 digested sequence**.** Ligation products were analyzed on 8 % PAGE gel. Lane 1 was loaded with TspR I digested pUC-18 fragments (105, 109, 149, 201, 271, 347, 506 and 958 bp from the bottom to top). Lane was loaded with the ligation products of specific circularization. Lane 3 was the ligase missing control. AD and LIG indicated specific adaptor and Taq DNA ligase, respectively.

Table S1 Overhang sequence of the digested fragments

Name	Sequence of two overhangs of each fragment (5' →3')	Length
105 bp	CCCAGTGCT / CTCACTGAT	9 nt
109 bp	CTGTCACTC / GGCAGTGAG	9 nt
149 bp	ATCAGTGAG / TCCACTGAG	9 nt
201 bp	ATCAGTGCG / GGCACTGGC	9 nt
271 bp	CTCAGTGGA / ACCAGTGGC	9 nt
506 bp	GCCACTGGC / CTCAGTGAG	9 nt
958 bp	CGCAGTGCC / TGCAGTGCT	9 nt
347 bp	CGCAGTGTT / AGCACTGGG	9 nt
347 bp adaptor	AACACTGGC / CCCAGTGCT	9 nt

The underlined portion of overhang sequence indicated the mismatched bases paring to the 347 bp adaptor.

The result demonstrated the specificity of target circularization. The 347 bp adaptor was able to recognize 347 bp target sequence specifically other than other sequences from pUC-18 plasmid by TspR I digestion. All other digested sequences have mismatched sticky ends (Table S1) toward that of the 347 bp adaptor. For 201 bp and 958 bp, there were only two mismatched base pairs in overhang region, but they failed to be ligated. This indicated the high specificity of target circularization.

Experiment: To digest pUC-18 plasmid, 1.0 μL of pUC-18 plasmid (300 nM) was mixed with 1.0 μL Fast Digest TspR I in 10 μL 1×Fast Digest buffer (Thermo Scientific, the buffer composition was not disclosed). The mixture was incubated at 65°C for 10 min. Then all 10 μL of digested product was introduced into 20 μL of 1×Taq ligation buffer containing 100 nM specific adaptors, 30 nM pUC-18 digested products, 10 mM Tris-HCl, 25 mM KAc, 10 mM $Mg(Ac)_2$, 10 mM DTT, 1.0 mM NAD (pH 7.8), 0.1% Triton. Before Taq DNA ligase was added, the mixture was incubated at 50°C for 5 min, then 2.0 U Taq DNA ligase was added. The mixture was incubated at 45°C for 2 h. The ligation reaction was terminated by adding 6×loading buffer and analyzed on 8% PAGE gel.

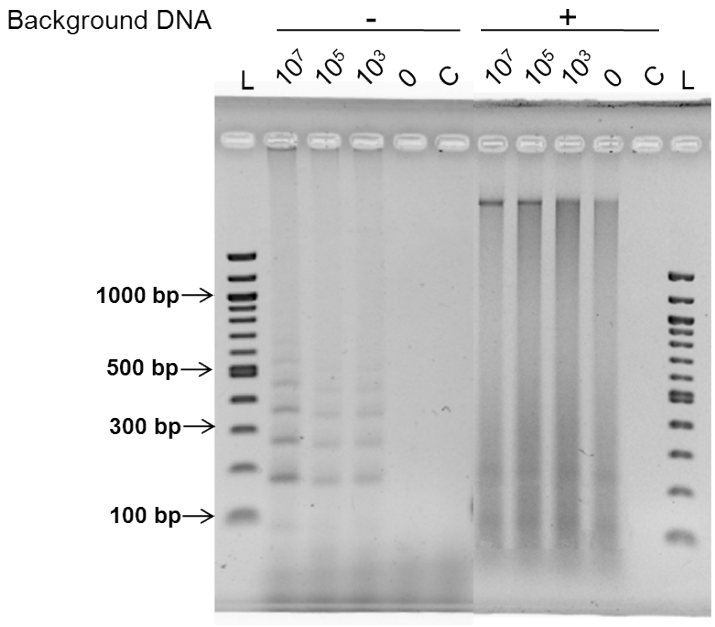

Fig. S6 Detection of the 347 bp target sequence using Padlock-RCA. Lane L was loaded with a 100 bp DNA ladder. Lane C indicated the control in the absence of ligase. Amplification products were analyzed on 1.5% agarose gel and 600 ng oyster genomic DNA was added as background DNA.

Table S2 Padlock probe with corresponding RCA primers for 347 bp sequence detection

Name	Sequence (5' →3')	Length
Pl probe-347	p-*TGGTATGGCTTCATTCAGCTC*AGTACGCTGATATTCGTGTCAA GATCTAACGCGTAATGACCTTAGTTACGGTCACGCTCGTCGTT	86 nt
347 Primer 1	ACGCGTAATGACCTTAGTTACG	22 nt
347 Primer 2	ATCTTGACACGAATATCAGCGT	22 nt
Target sequence	*GAGCTGAATGAAGCCATACCA*AACGACGAGCGTGAC	36 nt

The underlined and italic regions of the probe sequences were target-binding sites, while the complementary regions in target sequence were marked with the corresponding fonts.

The result demonstrated that padlock-RCA approach, which amplified circularizable probe other than target sequence, was not capable of detecting sequence of interest in the presence of massive background DNA.

Experiment: To detect 347 bp target sequence in the presence of massive background DNA, a padlock-RCA was used. The first step was a ligation reaction, wherein the probe specifically bound the target template and become circularized by DNA ligase. Probe (0.2 fmol, 10^8 copies in ligation reaction) was mixed with DNA template (various in copy number) in 1×T4 DNA ligase buffer containing 50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT, 1.0 mM ATP (pH 7.8) in 10 μL volume. 600 ng oyster genomic DNA was used as background

nucleic acids. Before T4 DNA ligase was added, the mixture was incubated at 95°C for 5 min, and slowly cooled down to 37°C. Then 5 U T4 DNA ligase (New England Biolabs) was added and the mixture was incubated at 37°C for 30 min followed by inactivation of the ligase at 65°C for 10 min.

After ligation, Vent (exo-) DNA polymerase (New England Biolabs) was chosen to perform hyper-branched-RCA in 20 μL 1×Thermopol buffer containing 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl, 2 mM $MgSO_4$, 0.1% Triton X-100, 2 U Vent (exo-) DNA polymerase, 400 μM dNTPs, 50 pmol of each RCA primer, and 10 μL of the original ligation mix. Circularized probes were amplified by incubation at 65°C for 90 min and analyzed on a 1.5% agarose gel under UV illumination.

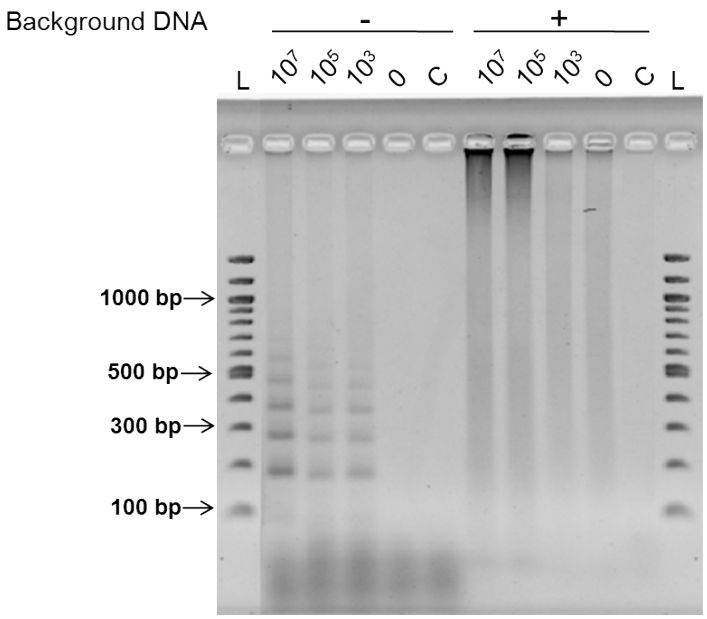

Fig. S7 Detection of *Vibrio parahaemolyticus* DNA by Padlock-RCA. Lane L was loaded with a 100 bp DNA ladder. Lane C indicated ligase missing control during ligation reaction. Amplification products were analyzed on a 1.5% agarose gel and 600 ng oyster genomic DNA was added as the background DNA.

Table S3 Padlock probe with corresponding RCA primers for ***Vibrio parahaemolyticus*** gene detection

Name	Sequence (5' →3')	Length
Pl probe-GyrB	p-*AAAGCTGTTTAGCGTACGTG*TTTAGGTTACTGCGATTAGCAC AAGCACCAAGAGCAACTACACGAATTGCCTTCTTTATCCAT	83 nt
GyrB Primer 1	ACCAAGAGCAACTACACGAATT	22 nt
GyrB Primer 2	TGTGCTAATCGCAGTAACCTAA	22 nt
Target sequence	*ACACGTACGCTAAACAGCTTT*ATGGATAAAGAAGGC	36 nt

The underlined and italic regions of the probe sequences were target-binding sites, while the complementary regions in target sequence were marked with the corresponding fonts.

A padlock probe that specifically target GyrB gene sequence and two primers initiating hyper-branched RCA were designed to compare the method with SMB-assisted TC-RCA. The padlock-RCA method failed to amplify the target sequence, and the NTC (none template control, 0 copy) delivered unexpected products, indicating that the method cannot be applied to detect bacterial DNA in the presence of background DNA.

Experiment: To detect the GyrB gene of ***Vibrio parahaemolyticus*** in the presence of massive background DNA, a padlock-RCA protocol was used. The first step was a ligation reaction, wherein the probe specifically bound the target sequence and become circularized by DNA

ligase. Probe (0.2 fmol, 10^8 copies in ligation reaction) was mixed with DNA template (various in copy number) in 1× T4 DNA ligase buffer containing 50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT, 1.0 mM ATP (pH 7.8) in 10 μL volume. 600 ng oyster genomic DNA was used as background nucleic acids. Before T4 DNA ligase was added, the mixture was incubated at 95°C for 5 min, and slowly cooled down to 37°C. Then 5 U T4 DNA ligase (New England Biolabs) was added and the mixture was incubated at 37°C for 30 min followed by inactivation of the ligase at 65°C for 10 min.

After ligation, Vent (exo-) DNA polymerase (New England Biolabs) was chosen to perform hyper-branched-RCA in 20 μL 1×Thermopol buffer containing 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl, 2 mM $MgSO_4$, 0.1% Triton X-100, 2 U Vent (exo-) DNA polymerase, 400 μM dNTPs, 50 pmol of each RCA primer, and 10 μL of the original ligation mix. Circularized probes were amplified by incubation at 65°C for 90 min and analyzed on a 1.5% agarose gel under UV illumination.