Supplementary information

Supp 1. Melting curve analysis of interaction between RNA50 and 3WJ probes

A. Melting curve analysis was conducted in 10 µL of PG-RCA buffer (20 mM Tris-HCl buffer (pH 8.8), 10 mM (NH₄)₂SO₄, 10 mM KCl, 6 mM MgSO₄, 0.1% Triton X-100 and 0.01% SYBR Green I (Invitrogen)) in MyiQ real-time PCR instrument (Bio-rad) with a SYBR Green filter set. 1 µM RNA50, 1 µM 3WJ primer P1 and 1 µM 3WJ template T1b were mixed in different combinations (RNA50/P1/T1b (blue), P1/T1b (red), RNA50/P1 (green) and RNA50/T1b (purple)) and incubated for complex formation following heat denaturation (80°C for 3 min, followed by 37°C for 30 min). Temperature was increased from 37°C to 80°C at 0.1°C/10 s and fluorescent intensity of each sample was monitored and plotted against temperature. B. The first negative derivative plot (-dF/dT). From the melting peak, melting temperature of each sample was estimated: 70.3°C for RNA50/P1/T1b (blue), N/A for P1/T1b (red), 69.0°C for RNA50/P1 (green) and 69.8°C for RNA50/T1b (purple).
Supp 2. Signal primer generation from 3WJ structure

A. Schematic mechanism of ligation-qPCR used to detect signal primer. B. Ligation-qPCR detection of signal primer generated from 3WJ structure. Positive control (250 amol RNA50, ○) and negative control (no RNA, ×) were incubated in 5 µL of PG-RCA buffer containing 1 nM each of 3WJ probes P1 and T1b for 3WJ complex formation (80°C for 5 min, followed by 37°C for 90 min), and then incubated for signal primer generation after mixing 5 µL of PG-RCA buffer containing 0.8 µM each dNTP, 0.05 U Vent (exo-) DNA polymerase and 1 U Nb.BsmI (60°C for 0 to 180 min). The reaction was terminated by adding 10 µL of 10 mM EDTA solution and stored at -20°C. Ligation-qPCR was conducted as follows. 2 µL of the above reaction product was mixed with 2 µL of 1.56 nM each of ligation probes (CCTAAGACGCAATCGAAAAGAAATGCTCAAGG and p-TGTGTGGAATGTCTGCTCAGGTGATGTAGAACTA) and incubated at 20°C for 30 min after initial heat denaturation at 95°C for 2 min. 5 µL of 2x iQ SYBR Green Supermix (Bio-rad) and 1 µL of the solution containing 5 µM each of PCR primers
(CCTAAGACGCAATCGAAAGAAA and TAGTTCTACATCACCTGAGACAGA), 80 µM ATP and 0.1 U T4 DNA ligase were further mixed and ligation-qPCR was conducted in MyiQ real-time PCR instrument (Bio-rad) with the following condition: 20°C 5 min, 95°C 5 min, 50 cycles of 95°C 10 s, 60°C 30 s and 72°C 30 s. Threshold cycle (C_T) of each reaction was calculated following iQ5 Optical System Software (Bio-rad).
Supp 3.  **Background signal primer generation from 3WJ template**

PG-RCA sensitivity using synthetic signal primer (DNA oligonucleotide) was compared in the absence or presence of 3WJ primer P1, template T1b or both probes to investigate whether 3WJ probes affect signal primer detection by PG-RCA (Figure S1). The presence of 3WJ probes did not affect $T_T$ values of 5 or 50 amol signal primer meaningfully, therefore it is suggested that 3WJ probes do not inhibit PG-RCA reaction itself. However, the presence of 3WJ template significantly decreased $T_T$ values of 0.5 amol signal primer and negative controls comparing with the absence of 3WJ template, suggesting that 3WJ template causes the increase of background amplification.

Calculated detection limits were around 0.4 zmol signal primer in the absence of 3WJ template, meaning that the background amplification of PG-RCA was as strong as 0.4 zmol signal primer in this study, which is in similar range to what we observed before (16). On the other hand, the detection limits deteriorated to around 40 zmol signal primer in the presence of 3WJ template, therefore the background amplification caused by 3WJ template is as strong as 40 zmol signal primer or 100 times higher than that of PG-RCA itself. From the discrepancy between the background amplification intensities, it can be deduced that the mechanism of this new background amplification associated with 3WJ template may be different from that of PG-RCA.
One possible explanation of this background amplification is that complementary strand of 3WJ template was synthesized and signal primer was generated independent of target RNA or 3WJ primer. Considering that 40 zmol signal primer was generated from 10 fmol 3WJ template used in this study, the reaction efficiency of this background signal generation is only 0.0004%, and it is very inefficient as regular enzymatic reaction but seems to be efficient enough to deteriorate PG-RCA sensitivity. In order to confirm the above speculation, 3WJ template T1b was incubated with DNA polymerase and nicking enzyme in two different conditions: (a) 0.4 µM dNTP, 0.05 U Vent(exo-) DNA polymerase and 1U Nb.BsmI, which is the PG-RCA reaction condition without circular probe, and (b) 40 µM dNTP, 0.4 U Vent(exo-) DNA polymerase and 1U Nb.BsmI, which contains higher concentrations of dNTP and DNA polymerase, and the reaction products were analyzed using signal primer specific padlock probe and qPCR for quantification (Figure S2A). Interestingly, Ct values of qPCR decreased along with the incubation in both incubation conditions, and the following melting curve analysis confirmed the amplification of the circularized padlock probe (Figure S2B). On the other hands, when negative controls (no oligonucleotide) were incubated in conditions (a) and (b), no change of $C_T$ values was observed and no amplification of circularized padlock probe was observed
(Figure S2). These data suggest that signal primer was generated from 3WJ template independent of target RNA or 3WJ primer.

Figure S1. Background signal primer generation associated with 3WJ template. By real-time PG-RCA analysis, 0.5, 5 and 50 amol signal primer (DNA oligonucleotide, CATTACACACCTTGAGCA) was analyzed in the absence (a) or presence of 1 nM 3WJ primer P1 (b), 1 nM 3WJ template T1b (c) or 1 nM each of both probes (d). PG-RCA was conducted at 60°C in 10 µL of PG-RCA buffer containing 0.4 µM each dNTP, 7.5 nM circular probe, 0.05 U Vent (exo-) DNA polymerase and 1 U Nb.BsmI. The fluorescent intensity of each reaction was monitored in real time and its threshold time ($T_T$) was analyzed. Perforated lines indicate average $T_T$ values of the negative controls. Calculated detection limit in each condition was 0.48 zmol (a), 0.44 zmol (b), 37 zmol (c) or 44 zmol signal primer (d).
Figure S2. Detection of background signal primer generation associated with 3WJ template.

A. 1 nM 3WJ template T1b (○, ●) or negative control (no oligonucleotide) (×, +) was incubated in 10 µL of PG-RCA buffer containing (a) 0.4 µM dNTP, 0.05 U Vent(exo-) DNA polymerase and 1 U Nb.BsmI (○, ×) or (b) 40 µM dNTP, 0.4 U Vent(exo-) DNA polymerase and 1 U Nb.BsmI (●, +), at 60°C for 0, 60, 120 and 180 min. The reaction was terminated by adding 10 µL of 10 mM EDTA solution and stored at -20°C. Signal primer generated in the above reaction was detected and quantified by signal primer specific padlock probe and real-time PCR (qPCR) as follows. 1 µL of the above reaction product was mixed with 1 µL of 4 nM signal primer specific padlock probe (p-GTGTGAATGTCTCTCAGGTGATGTAGAAGACGCCAATCGAAAAGAAAAACCTCAAGGT) and incubated at 20°C for 20 min after initial heat denaturation at 95°C for 3 min. Circularization of padlock probe was conducted at 20°C for 10 min by adding 2 µL of 2x T4 DNA ligase buffer containing 1 mM ATP and 0.04 U T4 DNA ligase, and terminated by incubating at 95°C for 10 min. Non-circularized padlock probe was removed by exonuclease treatment at 37°C for 30 min by adding 2 µL of 1x T4 DNA ligase buffer containing 1 U exonuclease I and 10 U exonuclease III, and terminated by incubating at 80°C for 15 min.
Circularized padlock probe in the above reaction mixtures was quantified by qPCR. qPCR was conducted in 10 µL of 1x iQ SYBR Green Supermix (Bio-rad) containing 500 nM each of PCR primers (CCTAAGACGCAATCGAAAAGAAA and TAGTTCTACATCACTGAGACAGA) in MyiQ real-time PCR instrument (Bio-rad) with the following condition: 95°C 5 min, 50 cycles of 95°C 10 s, 60°C 30 s and 72°C 30 s. Threshold cycle (C_T) of each reaction was calculated following iQ5 Optical System Software (Bio-rad).

B. Melting curve analysis of qPCR products in A. Fluorescent intensity of each sample was monitored while temperature was increased from 60°C to 90°C at 0.5°C/30 s, and its first negative derivative (-dF/dT) was plotted against temperature. Samples: 3WJ template incubated in condition (a) (orange) or (b) (red). Negative control (no oligonucleotide) incubated in condition (a) (light blue) or (b) (blue).
Supp 4. 3WJ structure formation mechanism

A. Using mfold program, the melting temperatures of the 7 base long complementary sequences (CATAATC(T)\textsubscript{n}GATTATG) (blue) and 13 base long complementary sequences (CTTTTTCCATAATC(T)\textsubscript{n}GATTATGAAAAAG) (red) were calculated. The former sequences can be considered as 3WJ probes before primer extension, and the latter is after primer extension. ‘n’ is a linker length between 0 and 100 bases long. B. Proposed mechanism of 3WJ structure formation.