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Sensitive RNA detection by combining three-way junction formation and primer generation-rolling circle amplification

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ABSTRACT

Recently, we developed a simple isothermal nucleic acid amplification reaction, primer generation-rolling circle amplification (PG-RCA), to detect specific DNA sequences with great sensitivity and large dynamic range. In this paper, we combined PG-RCA with a three-way junction (3WJ) formation, and detected specific RNA molecules with high sensitivity and specificity in a one-step and isothermal reaction format. In the presence of target RNA, 3WJ probes (primer and template) are designed to form a 3WJ structure, from which multiple signal primers for the following PG-RCA can be generated by repeating primer extension, nicking and signal primer dissociation. Although this signal primer generation is a linear amplification process, the PG-RCA exponentially can amplify these signal primers and thus even a very small amount of RNA specimen can be detected. After optimizing the structures of 3WJ probes, the detection limit of this assay was 15.9 zmol (9.55×10^3 molecules) of synthetic RNA or 143 zmol (8.6×10^4 molecules) of *in vitro* transcribed human CD4 mRNA. Further, the applicability of this assay to detect CD4 mRNA in a human mRNA sample was demonstrated.

INTRODUCTION

Several mRNA quantification technologies have been available including northern blot analysis, ribonuclease protection assay, reverse transcriptase polymerase chain reaction (RT-PCR) and DNA microarray (1–5). Currently, real-time RT-PCR analysis is a gold standard for RNA quantification and detection in the fields of fundamental research, drug discovery and molecular diagnostics, and it is especially useful for quantifying small to medium numbers of RNA targets with large dynamic range and high sensitivity.

However, RT-PCR usually requires two separate reactions, RT and PCR, in different buffers and temperatures; therefore, it often causes long hands-on time for reaction preparation and risk of cross-contamination among samples. Although several one-step RT-PCR reagents are commercially available and allow both RT and PCR in a single tube without sample dilution or transfer between the reactions, two-step RT-PCR is generally more sensitive and specific than the one-step reaction format. One of the major problems to use the one-step reaction format is primer dimerization or mis-priming formation during the RT step, which deteriorates the sensitivity and accuracy of the following PCR, especially problematic in real-time PCR using SYBR Green (6).

Rolling circle amplification (RCA) is a powerful isothermal nucleic acid amplification technology utilizing circular single-stranded DNA probes. Among several modes of RCA proposed (7–11), hyper-branched RCA (also known as ramification or cascade RCA) shows exponential signal amplification with great sensitivity (9,10). In order to detect nucleic acid sequences, circular probes can be obtained through circularization of padlock probes in a separate ligation reaction; however, it is difficult to conduct both reactions simultaneously (12). Also, ligation reaction of padlock probes on RNA templates is not as efficient as on DNA templates; therefore it may be difficult to utilize conventional RCA for RNA detection yet (13–15).

Recently, we developed a novel mode of RCA, dubbed primer generation-rolling circle amplification (PG-RCA), in which specific sample DNA are detected with high sensitivity and wide dynamic range (16). By simple design of circular probes and addition of a nicking enzyme, conventional linear RCA was successfully converted to an exponential amplification mode without complicated topological factors. One of the distinctive advantages

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over conventional nucleic acid amplification technologies is being free from troublesome design and usage of exogenous primers since 'primers' are generated successively during the reaction. However, PG-RCA can detect only DNA sequences that carry nicking sites used in PG-RCA because PG-RCA reaction cycle can be initiated only from specific 3′ end sequences. Also, the same assay scheme does not work to detect RNA because nicking enzymes do not efficiently cleave RNA strands in RNA/DNA duplexes. Recently, we demonstrated that supplementation of a very low concentration of thermostable RNaseH to a PG-RCA reaction allows RNA detection in the same assay scheme by cleaving target RNA upon hybridization with circular probes and initiating the reaction cycle from the cleaved RNA strands (17). However, the sequence recognition of RNaseH is not sufficiently strict; therefore, many non-specific amplifications are expected to occur when this reaction format is applied to biological samples.

In this paper, target RNA specimens are detected with high sensitivity and specificity by combining PG-RCA technology with a three-way junction (3WJ) formation. 3WJ probes (primer and template) are designed to form a 3WJ structure on target RNA, and DNA primers (signal primers) for the following PG-RCA reaction are generated from the 3WJ structure by repeating primer extension, nicking and signal primer dissociation. These signal primers are, in turn, exponentially amplified *in situ* by PG-RCA. As a result, the target RNA sequence is precisely and effectively detected in a one-step and isothermal reaction format.

MATERIALS AND METHODS

Reagents

Oligonucleotides were purchased from Integrated DNA Technologies, Trilink biotechnologies or Sigma Genosys. Vent (exo-) DNA polymerase, Nb.BsmI and exonuclease III were purchased from New England Biolabs. Circligase ssDNA ligase, T4 DNA ligase and exonuclease I were from Epicentre Biotechnologies. Rat spleen mRNA and human spleen mRNA were from Clontech Laboratories. Preparation of *in vitro* transcribed CD4 mRNA (742 bases long) was described elsewhere (18).

Polyacrylamide gel analysis of 3WJ primer extension and signal primer generation

Two-hundred nanomoles each of RNA50, 3WJ primer P1 and template T1b were mixed in different combinations in 10 µl of PG-RCA buffer [20 mM Tris–HCl buffer (pH 8.8), 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 6 mM $MgSO_4$, 0.1% Triton X-100 and 0.01% SYBR Green I (Invitrogen)], and incubated at 37°C for 30 min after initial denaturation at 80°C for 3 min. To initiate a PG-RCA reaction, 10 µl of PG-RCA buffer containing 80 µM dNTP, 0.4 U Vent(exo-) DNA polymerase and 2 U Nb.BsmI, was mixed to the samples and incubated at 60°C for 0, 15 and 30 min. The reaction products were analyzed by 15% denaturing polyacrylamide gel with SYBR gold staining (Invitrogen).

RNA detection by 3WJ probes and PG-RCA

Circular probe was prepared by self-ligation of a 5′-phosphoriated circular probe precursor (Table 1) as described elsewhere (16,17). Sample RNA and 1 nM each of 3WJ probes were mixed in 5 µl of PG-RCA buffer and incubated at 37°C for 90 min after initial denaturation at 80°C for 5 min. PG-RCA reaction was conducted at 60°C by adding 5 µl of PG-RCA buffer containing 0.8 µM each dNTP, 15 nM circular probe, 0.05 U Vent (exo-) DNA polymerase and 1 U Nb.BsmI to the RNA samples. The fluorescent intensity of each reaction was monitored in MyiQ real-time PCR instrument (Bio-rad) with a SYBR green filter set. Threshold time (T_T) was estimated from the reaction time when the fluorescent intensity of the reaction exceeds an arbitrary threshold, which was set right above the background fluorescent intensity.

RESULTS

General assay scheme of RNA detection using 3WJ probes and PG-RCA

We recently developed a novel isothermal nucleic acid amplification reaction, PG-RCA to detect DNA (16). It may be possible to apply this technology to RNA detection using reverse transcription to convert RNA to cDNA before PG-RCA detection. However, in order to detect RNA in a one-step reaction using PG-RCA, we utilized 3WJ probes in this manuscript. 3WJ probes, which comprise of 3WJ primer and 3WJ template, are designed to have 7-base-long complementary sequences each other. At the reaction temperature of PG-RCA (60°C), 3WJ probes do not associate with each other because the melting temperature of the complementary sequence is set well below the reaction temperature. However, when target RNA exists, 3WJ probes can hybridize stably to the RNA in close proximity; therefore, the short complementary sequences of 3WJ probes can form a double-strand structure even at the reaction temperature, resulting in a 3WJ structure among the target RNA and 3WJ probes (Figure 1A).

5′ end sequence of 3WJ template is designed to contain a nicking site and a complementary sequence of DNA primer for circular probe (signal primer). Therefore, under the PG-RCA reaction condition containing DNA polymerase and nicking enzyme, a reaction cycle of primer extension, nicking reaction and signal primer dissociation can generate signal primers continuously from the 3WJ structure (Figure 1B). Although this signal primer generation is inherently linear, the following PG-RCA reaction can detect very low number of generated signal primers through its exponential amplification mechanism (Figure 1C). In this way, target RNA can be detected by PG-RCA using 3WJ probes in a one-step reaction format.

Design of 3WJ probes to detect human CD4 mRNA

3WJ probes P1 and T1b were designed to target human CD4 mRNA for proof of concept (Table 1). Target RNA used for demonstration and reaction optimization

Table 1. Oligonucleotide sequences[a–e]

Oligonucleotide	Sequence (5′ to 3′)
Circular probe	
Circular probe precursor	p-**GC**TGTGCTCAAGGTGTGT**GAATGC**TGTGCTCAAGGTGTGT**GAATGC**TGTGCTCAAGGTGTG**TGAAT**
Mock gene	
RNA50	UCUUCCUACCCUUCCUCACCACUUCCCUCAGUCCCAACUCCAAAAAAAAA
3WJ probe for RNA50 and Human CD4 mRNA	
3WJ primer PI	GGAGTTGGGACTGAGGGTTGATTATG
3WJ template T1a	TGCTCAAGGTGTGT**GAATGC**TTTTTCATAATCGTGGTGAGGAAGGGTAGGAAGA
3WJ template T1b	TGCTCAAGGTGTGT**GAATGC**TTTTTCATAATCGTGGTGAGGAAGGGTAGGAAGATTTTT
3WJ template T1c	TGCTCAAGGTGTGT**GAATGC**TTTTTCATAATCGTGGTGAGGAAGGGTAGGAAGATTTTTp
3WJ template T1d	TGCTCAAGGTGTGT**GAATGC**TTTTTCATAATCxGTGGTGAGGAAGGGTAGGAAGATTTTTp

[a]'p' indicates a 5′ or 3′ phosphate modification.
[b]Bold characters indicate the recognition sequence of Nb.BsmI.
[c]Solid underlines indicate hybridization sequences between three-way junction probes.
[d]Perforated underlines indicate the template sequences for signal primer generation.
[e]'x' indicates a C3 linker.

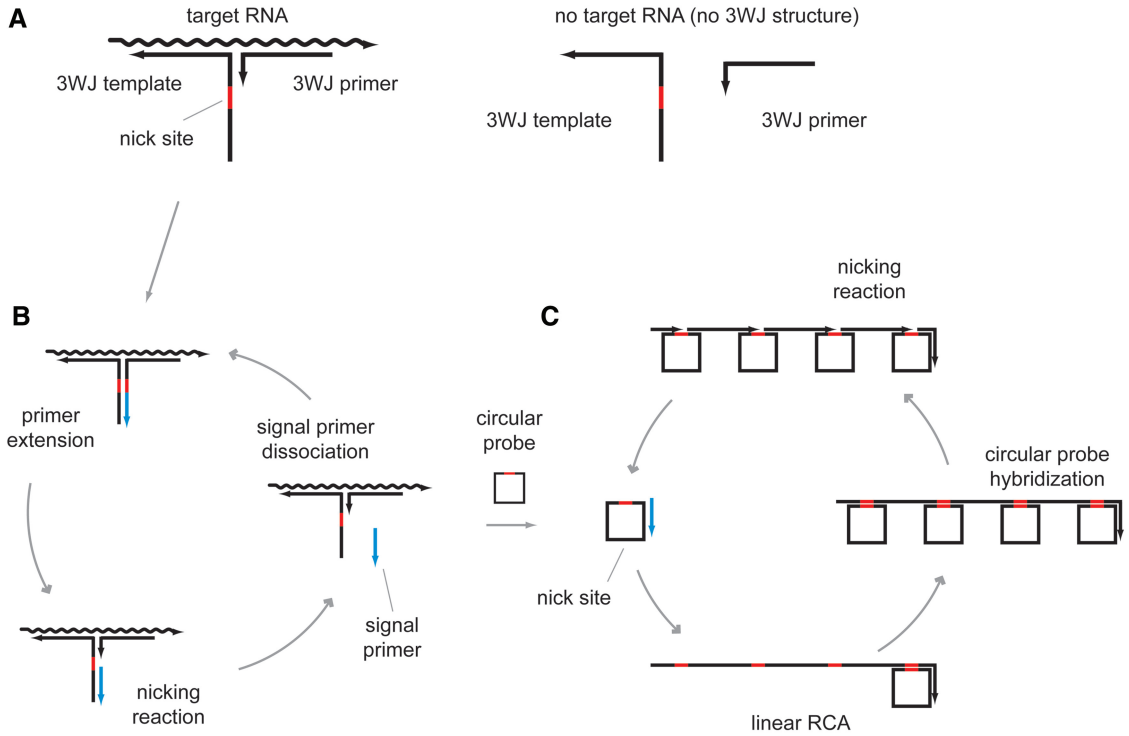

Figure 1. RNA detection mechanism by three-way junction probe and primer generation-rolling circle amplification. (**A**) Three-way junction (3WJ) probes (primer and template) are designed to form a 3WJ structure on target RNA, however they do not interact each other without target RNA because their complementary sequence is only 6–8 bases. (**B**) Addition of DNA polymerase and nicking enzyme initiates a reaction cycle of primer extension, nicking reaction and signal primer generation under an isothermal condition to generate signal primers. (**C**) The generated signal primers can be detected by primer generation-rolling circle amplification.

is a 50-base-long synthetic RNA that carries CD4 mRNA sequence (RNA50). Using these probes and RNA, each step of RNA detection scheme in Figure 1 was investigated.

First, we confirmed the complex formation among RNA50 and 3WJ probes in Figure 1A by melting curve analysis using SYBR Green I dye (Supplementary information 1). When RNA50 and 3WJ probes are mixed, strong fluorescent intensity was observed between 40 and 70°C by forming stable double strands, and decreased rapidly as temperature increased above 70°C by dissociation of the complex structure. Observed melting temperature of the complex structure was 70.3°C, suggesting that RNA50 and 3WJ probes can form stable complex structures at the reaction temperature of PG-RCA (60°C). Similar melting curves were observed between RNA50 and either of 3WJ probes, and observed melting temperatures were 69.0°C for 3WJ primer and 69.8°C for 3WJ template. On the other hand, when only 3WJ probes were mixed, much weaker fluorescent intensity was

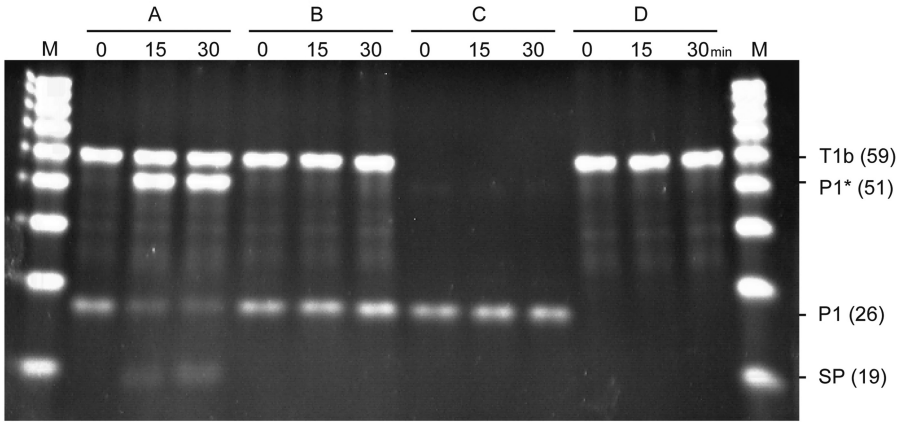

Figure 2. Confirmation of 3WJ primer extension and signal primer generation products. 3WJ primer P1, 3WJ template T1b and RNA50 were mixed in different combinations [(**A**): P1/T1b/RNA50, (**B**): P1/T1b, (**C**): P1/RNA50 and (**D**): T1b/RNA50] and incubated to form a 3WJ structure. Signal generation reaction was conducted at 60°C for 0, 15 and 30 min by adding an enzyme mix [0.2 U Vent(exo-) DNA polymerase and 1 U Nb.BsmI] to the samples. The reaction products were analyzed on 15% denaturing polyacrylamide gel. P1* indicates a reaction product through primer extension of 3WJ primer and SP indicates signal primer generated from 3WJ structure. The numbers in parentheses indicate the length or expected length of each probe or reaction product. Lane M is 20–100 nt oligonucleotide marker.

observed between 40 and 70°C, suggesting that no double strand was formed without RNA50.

To confirm that 3WJ probes form a 3WJ structure only when target RNA exists and signal primers are generated through a reaction cycle of primer extension, nicking reaction and signal primer dissociation (Figure 1A and B), a mixture of RNA50 3WJ probes P1 and T1b was incubated at 60°C with DNA polymerase and nicking enzyme (40 μM dNTP, 0.2 U/10 μl Vent(exo-) DNA polymerase and 1 U/10 μl Nb.BsmI) after complex structure formation and analyzed on 15% denaturing polyacrylamide gel (Figure 2). Within 15 min, the majority of 3WJ primer disappeared and an ~50-base-long product was observed because DNA polymerase extended the 3′ end of 3WJ primer on the 3WJ template and converted the 3WJ primer to a 51-base-long product, which is expected from the 3WJ probe sequences. Also, an ~20-base-long product was accumulated along with the reaction, which is assumed to be signal primer (19 base long expected from the 3WJ probe sequences). On the other hand, when only 3WJ probes were incubated with DNA polymerase and nicking enzyme, both of the 50 and 20-base-long products were not observed, suggesting that, without target RNA, 3WJ probes do not interact with each other and 3WJ primer extension and signal primer generation do not occur. Similarly, when RNA50 and either of 3WJ probes were incubated with the enzymes, no new products were observed.

In order to detect RNA in a single-step reaction format, signal primer generation is designed to work concurrently with PG-RCA. PG-RCA utilizes a very limited amount of dNTP and DNA polymerase to improve the assay sensitivity by suppressing the background amplification of PG-RCA as described before (16). Therefore, the signal primer generation reaction was further investigated under the PG-RCA reaction condition [0.4 μM dNTP, 0.05 U Vent(exo-) DNA polymerase and 1U Nb.BsmI] by ligation-qPCR (Supplementary Information 2). Positive control (250 amol RNA50) and negative control (no RNA) samples were incubated with 3WJ probes P1 and T1b for complex formation, and then incubated under the PG-RCA reaction condition without circular probe for signal primer generation. Using signal primer-specific ligation-qPCR, which was developed to detect signal primers in the presence of excess complementary sequence (3WJ template), it was confirmed that C_T value of the positive control decreased along with the signal primer generation reaction. Using a standard curve prepared using synthetic signal primer, the signal primer generation speed was estimated at 0.69 amol/min for the positive control. On the other hand, C_T value of the negative control did not change meaningfully along with the reaction.

These data indicate that when target RNA exists, a 3WJ structure is formed among target RNA and 3WJ probes at 60°C and DNA polymerase and nicking enzyme catalyze a signal primer generation reaction; however, without target RNA, 3WJ probes do not associate with each other and 3WJ primer extension or signal primer generation does not occur.

Finally, we investigated RNA50 detection by 3WJ probes and real-time PG-RCA concurrently (Figure 1A–C); 0–250 amol RNA50 was incubated with 3WJ probes P1 and T1b after initial denaturation, and then real-time PG-RCA was conducted by adding a PG-RCA reaction mix (Figure 3). Typical PG-RCA amplification curves were observed and synchronized very well among triplicate experiments containing the same amount of RNA50 as well as in our previous study for DNA detection (16). Furthermore, the abrupt signal increases appeared earlier when the reaction contained a larger amount of RNA50. Threshold time (T_T: the time when the fluorescent intensity exceeds an arbitrary threshold) was defined in analogy to threshold cycle (C_T) in real-time PCR analysis (19), and plotted against the amount of RNA50 on a log scale (Figure 3B). We obtained a good linearity between

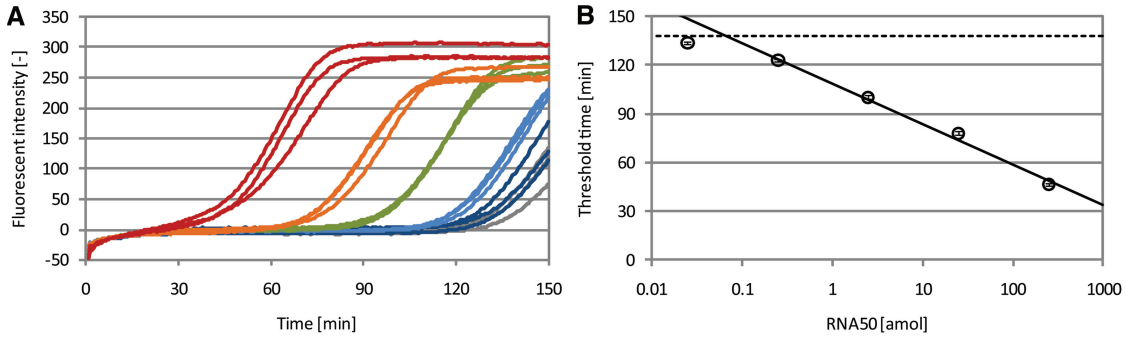

Figure 3. Detection of RNA50 by 3WJ probe and real-time PG-RCA. (**A**) Different concentrations of RNA50 were analyzed using 3WJ probe P1 and T1b at 37°C for 90 min following initial heat denaturation at 80°C for 5 min. PG-RCA was conducted at 60°C by adding a PG-RCA reaction mixture to the RNA samples and the fluorescent intensities of each reaction were monitored in real time. The RNA50 concentration in each reaction was prepared by 10-fold serial dilution from 250 amol to 25 zmol, and their signal amplification curves are indicated by colored lines (red, orange, green, light blue and blue, respectively). Negative controls are indicated by gray lines. (**B**) Threshold time T_T (the reaction time when fluorescent intensity of each reaction exceeds an arbitrary threshold) was plotted against the RNA50 concentration (S) of the reaction. The solid line indicates linear least squares fitting between 250 zmol and 250 amol RNA50 and its formulation is $T_T = -25.2 \log_{10}(S) + 110$ ($R^2 = 0.989$). The perforated line indicates average T_T value of the negative controls. Detection limit is 76.5 zmol (4.59×10^4 molecules) of RNA50 by calculation from the intersection of both lines.

250 zmol and 500 amol RNA50 in linear least squares fitting ($R^2 = 0.989$) and detection limit of PG-RCA was obtained as 76.5 zmol (4.59×10^4 molecules) of RNA50 by calculating from the intersection of the fitting and average T_T value of the negative controls. These data indicate that the whole process of 3WJ structure formation, signal primer generation and PG-RCA can proceed quantitatively corresponding to the amount of RNA50; therefore it is possible to apply this assay to RNA quantification as well.

Suppression of background amplification using modified 3WJ templates

We observed a background signal amplification without target RNA, which is as strong as 76.5 zmol RNA50 (the detection limit described above); however, it seemed to be different from that of PG-RCA observed before because the background signal intensities differ by approximately two to three orders (Supplementary information 3) (16). It was confirmed that signal primers were generated from 3WJ template (T1b) independent of target RNA or 3WJ primer, and increased the background signal amplification of PG-RCA (Supplementary information 3). Therefore, two modified 3WJ templates were designed to minimize this false signal primer generation (Table 1). The first modified 3WJ template carries a 3′-phosphate poly(dT) in order to prevent 3′ end extension (T1c), assuming that the complementary strand of 3WJ template was synthesized through hairpin/dimer formation of its 3′ end sequence. In addition to the 3′-phosphate poly(dT) modification, the second one carries an internal C3 linker in order to stop complementary strand synthesis before DNA polymerase reaches the template region for signal primer generation (T1d), assuming that the complementary strand synthesis of 3WJ template is initiated by *ab initio* DNA polymerization, which was suggested to cause the background amplification of PG-RCA in our previous study (16). In order not to affect 3WJ formation, the internal C3 linker was introduced at the 3WJ junction to form a bulge loop.

Those modified 3WJ templates were compared with 3WJ templates without a 3′-poly(dT) (T1a) and with a 3′-poly(T) (T1b) by real-time PG-RCA analysis of positive control (50 amol RNA50) and negative control (no RNA) samples (Figure 4). T_T values of the positive controls were not different meaningfully among the 3WJ templates, suggesting that those modifications may not affect 3WJ structure formation significantly. Only T1d gives slightly larger T_T value of the positive control than the others maybe because the internal C3 linker modification may to some extent affect 3WJ formation. On the other hand, T_T values of the negative controls increased significantly by introducing a poly(dT) tail (T1a→T1b), a 3′ phosphate (T1b→T1c) and an internal C3 linker (T1c→T1d). Apparently, each of those modifications contributes to the suppression of the background signal primer generation associated with 3WJ template.

In order to further evaluate the modified 3WJ templates T1c and T1d, 0–500 amol RNA50 were analyzed by real-time PG-RCA and detection limits were calculated for both 3WJ templates (Figure 5). In comparison with T1b (Figure 3), T_T values of the negative controls increased and the background signal amplifications were successfully suppressed for both of T1c and T1d, being consistent with the results in Figure 4. The obtained detection limit was 15.9 zmol (9.55×10^3 molecules) RNA50 for T1c and 18.2 zmol (1.09×10^4 molecules) RNA50 for T1d. Although T1d suppressed the background signal amplification more than T1c, T_T values of the RNA50 positive samples for T1d were always slightly larger than those for T1c, therefore T1c indicated a lower detection limit-than T1d.

Human CD4 mRNA detection by 3WJ probes and PG-RCA

The applicability of this RNA detection method to real mRNA samples was investigated. First, it was investigated whether excess mRNA affects RNA detection by 3WJ probes and PG-RCA. Positive control (50 amol RNA50)

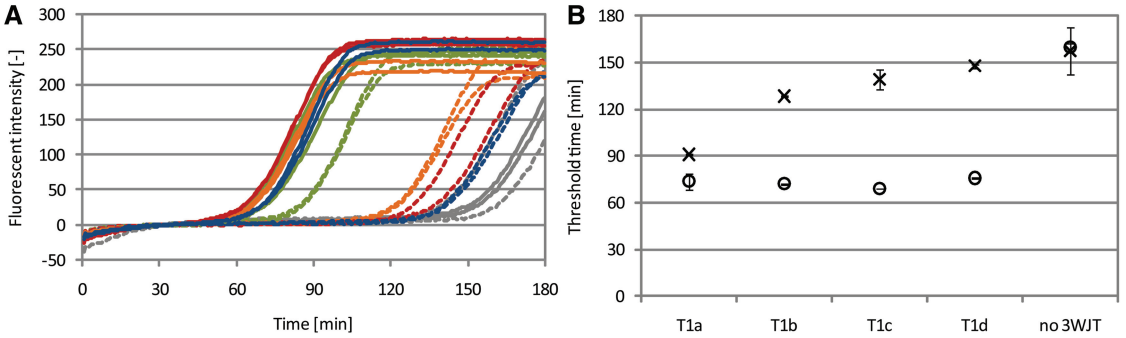

Figure 4. Suppression of background amplification using modified 3WJ templates. (**A**) Performance of modified 3WJ templates were compared by analyzing 50 amol RNA50 (solid lines) and negative control (perforated lines) with 3WJ probe and real-time PG-RCA. 3WJ templates used in this experiment are 3WJ template T1a (green), T1b (orange), T1c (red) and T1d (blue) in comparison with no 3WJ template (gray). (**B**) Threshold time T_T of the positive (open circle) and negative (cross) control experiments in (A) were analyzed and compared.

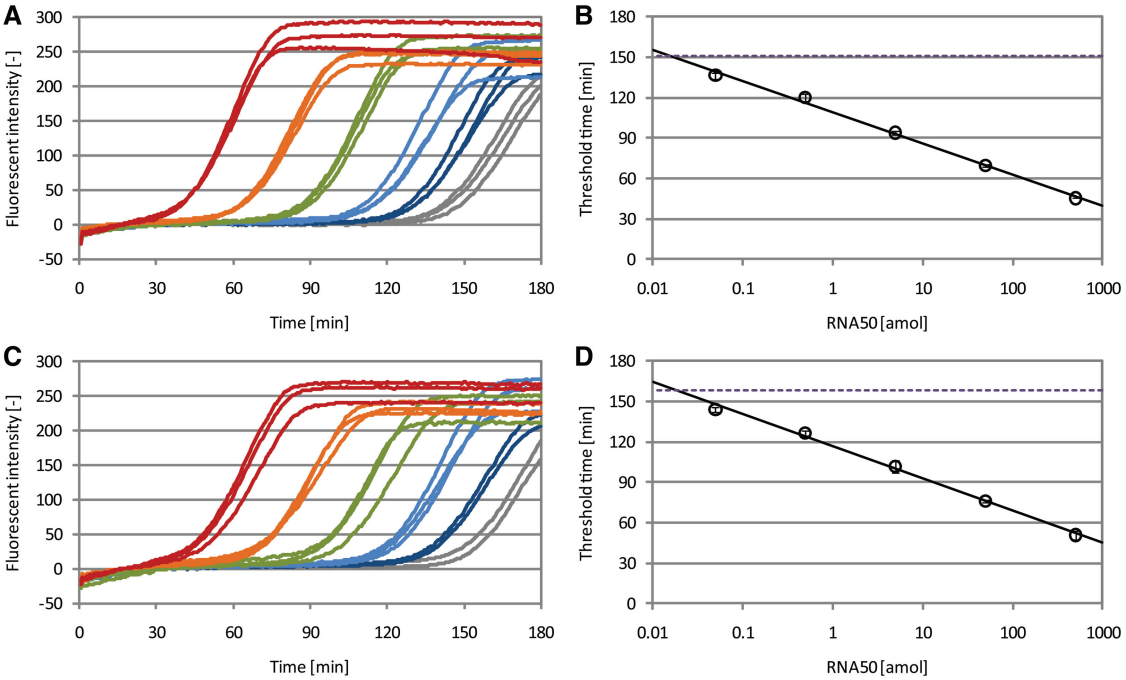

Figure 5. RNA detection by modified 3WJ templates. (**A** and **C**) Different concentrations of RNA50 were analyzed by the modified 3WJ probes (in Table 1). The reaction condition is the same as in Figure 3 except using modified 3WJ template T1c (A and B) or T1d (C and D). The RNA50 concentration in each reaction was prepared by 10-fold serial dilution from 500 amol to 50 zmol, and their signal amplification curves are indicated by colored lines (red, orange, green, light blue and blue, respectively). Negative controls are indicated by gray lines. (**B** and **D**). Threshold time T_T was plotted against the RNA50 concentration (S) of the reaction. Solid lines indicate linear least squares fitting between 50 zmol and 500 amol RNA50 and their formulations are $T_T = -23.2 \log_{10}(S) + 109$ ($R^2 = 0.993$) and $T_T = -23.9 \log_{10}(S) + 117$ ($R^2 = 0.991$), respectively. Perforated lines indicate average T_T values of the negative controls. Detection limits are 15.9 zmol (9.55×10^3 molecules) of RNA50 for T1c (B) and 18.2 zmol (1.09×10^4 molecules) of RNA50 for T1d (D) by calculation from the intersections of both lines.

and negative control (no RNA50) were analyzed in the presence of 1–4000 pg rat spleen mRNA (Figure 6). No mRNA similar to RNA50 is expressed in rat mRNA according to the BLAST program (http://blast.ncbi.nlm.nih.gov); therefore, it was expected that 3WJ probes P1 and T1c do not associate with rat mRNA and initiate PG-RCA reaction cycle. Indeed, T_T values of the positive and negative controls were not affected in the presence of 1–1000 pg rat mRNA, suggesting that sequence recognition by the 3WJ probes is very specific to target RNA. Even in the presence of more than 1000 pg rat mRNA, T_T value of the positive control was significantly lower than that of the negative control, therefore specificity of the 3WJ probes still remains the same; however, both T_T values were larger than in the presence of <1000 pg rat mRNA. Since similar increase of T_T values were observed when sample DNA was analyzed by PG-RCA in the presence of excess mRNA, total RNA or rRNA (data not shown), the excess rat mRNA may affect the PG-RCA reaction speed by inhibiting hybridization between circular probe and linear RCA product, but not 3WJ structure formation.

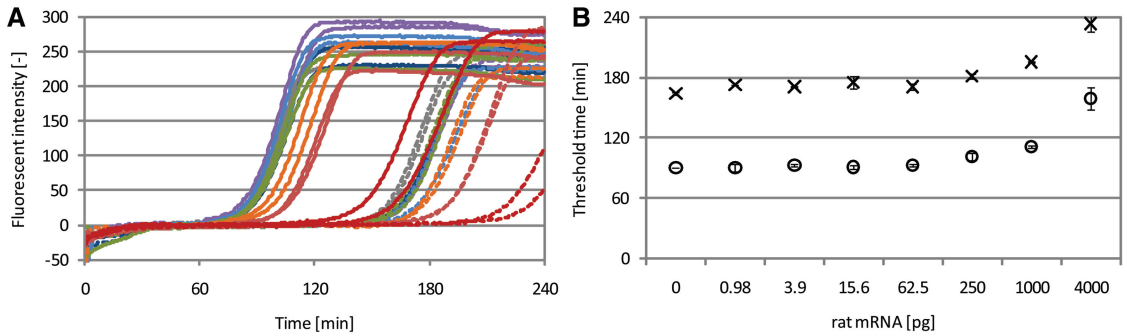

Figure 6. RNA detection in the presence of excess amounts of unrelated mRNA. (**A**) 50 amol RNA50 (solid lines) and negative control (perforated lines) were analyzed by 3WJ probe P1 and T1c in the presence of 0–4000 pg rat spleen mRNA. (**B**) Threshold time T_T of the positive (open circle) and negative (cross) control experiments in (A) were analyzed and compared.

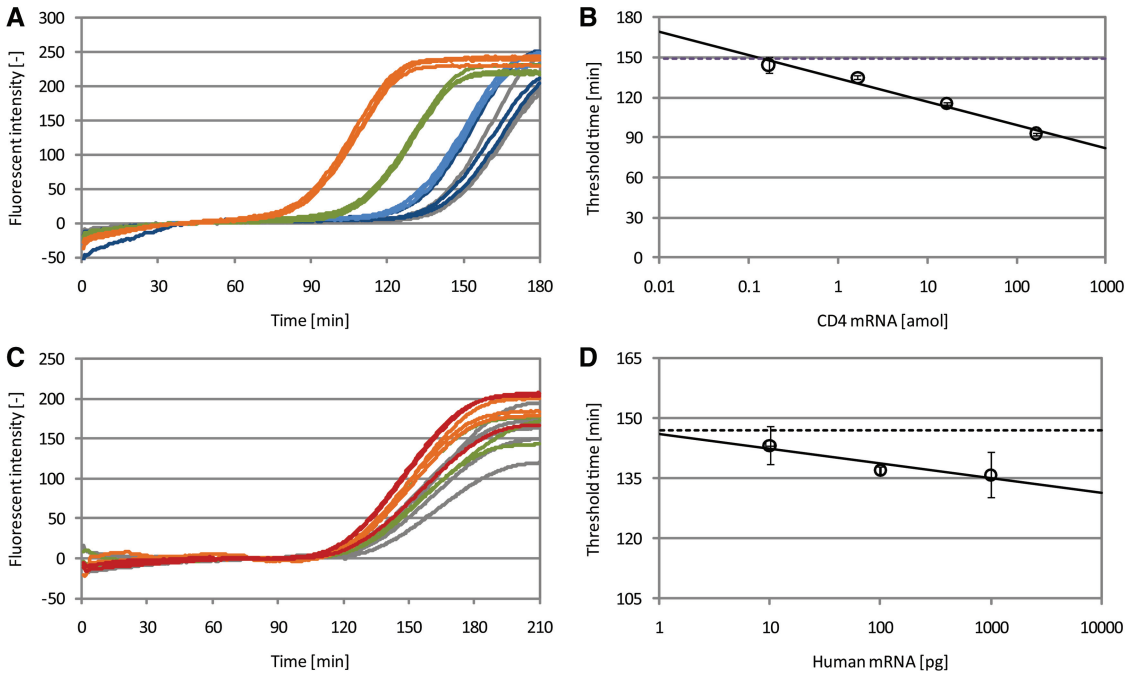

Figure 7. Human CD4 mRNA detection. (**A**) Different concentrations of *in vitro* transcribed human CD4 mRNA were analyzed using 3WJ probe P1 and T1c. The CD4 mRNA concentration in each reaction was prepared by 10-fold serial dilution from 250 amol to 250 zmol, and their signal amplification curves in real-time PG-RCA are indicated by colored lines (orange, green, light blue and blue, respectively). Negative controls are indicated by gray lines. (**B**) Threshold time T_T was plotted against the CD4 mRNA concentration (S) of the reaction. The solid line indicates linear least squares fitting between 250 zmol and 250 amol CD4 mRNA and its formulation is $T_T = -17.3 \log_{10}(S) + 134$ ($R^2 = 0.953$). The perforated line indicates average T_T value of the negative controls. Detection limit is 143 zmol (8.60×10^4 molecules) of RNA50 by calculation from the intersection of both lines. (**C**) CD4 mRNA expressed in human mRNA was analyzed using 3WJ probe P1 and T1c. Human mRNA used in this experiment was 0 (gray), 10 (green), 100 (orange) and 1000 pg (red). (**D**) Threshold time T_T was plotted against the human mRNA concentration (S) of the reaction. The solid line indicates linear least squares fitting between 10 and 1000 pg human mRNA and its formulation is $T_T = -3.67 \log_{10}(S) + 146$ ($R^2 = 0.396$). The perforated line indicates average T_T value of the negative controls.

Detection of 742-base-long *in vitro* transcribed human CD4 mRNA, which is expected to take complicated secondary structure according to the mfold program (20), was investigated by 3WJ probes and PG-RCA (Figure 7A and B). RNA50 is a mock gene of human CD4 mRNA, therefore 3WJ probes P1 and T1c can be applied to detect CD4 mRNA (0–250 amol) without any modification. A good dose–response data were obtained between 250 zmol and 250 amol. The obtained detection limit was 143 zmol (8.60×10^4 molecules) CD4 mRNA, which was slightly lower than that of RNA50 (Figure 5A and B), probably because of the complicated secondary structure of CD4 mRNA.

Lastly, detection of CD4 mRNA naturally expressed in human mRNA was investigated (Figure 7C and D). Human mRNA (0–1000 pg) was analyzed using 3WJ probes P1 and T1c, and it was confirmed that T_T values of human mRNA samples were meaningfully lower than

that of the negative controls, suggesting that CD4 mRNA naturally expressed in human mRNA was successfully detected. Unfortunately, its dose–response trend was not as linear as those of RNA50 or *in vitro* transcribed CD4. This may be because CD4 gene expressed in human mRNA is more than 3000 base long and expected to have much more complicated RNA structure than RNA50 and *in vitro* transcribed CD4 (742 base long), therefore 3WJ formation might be more difficult. It is also possible that the mRNA amounts in the reaction might affect the PG-RCA reaction speed as observed in Figure 6 as well.

DISCUSSION

RNA detection by 3WJ probes and PG-RCA

In this manuscript, we introduced 3WJ probes to detect desired RNA sequences by PG-RCA. 3WJ probes (3WJ primer and template) hybridize to target RNA in close proximity and form a 3WJ structure with target RNA. Under the reaction condition of PG-RCA, signal primers can be generated from the 3WJ structure to initiate the reaction cycle of PG-RCA. We demonstrated that a combination of 3WJ probes and PG-RCA allows detection of specific RNA sequences using 50-base-long synthetic RNA (RNA50) and *in vitro* transcribed CD4 mRNA with the sensitivities of 15.9 zmol and 143 zmol, respectively.

Using 3WJ probes, it is possible to detect any RNA sequences by changing the hybridization sequences of 3WJ probes to target RNA. Also, 3WJ probes can recognize not only RNA but also DNA in the same manner, therefore the limitation of PG-RCA in DNA detection described before is not an issue any more, where target DNA requires a nicking sequence (16). Unlike other nucleic acid detection methods, this method does not use target nucleic acids as templates for signal amplification; therefore, it may be possible to detect even heavily damaged nucleic acids as long as 3WJ probes can hybridize to the target sequences.

3WJ probes can recognize target RNA very specifically even in the presence of excess non-target mRNA. Such specificity is necessary to detect a low copy number of target RNA because non-target mRNA such as housekeeping genes is expected to exist in excess in biological samples. Each of 3WJ probes may not recognize target RNA specifically and may hybridize to non-target RNA or even to non-target regions of the target RNA because these probes are regular DNA probes without any nucleotide modifications and hybridization condition is not stringent. However, non-specific bound 3WJ probes do not initiate signal primer generation or PG-RCA because it is very unlikely for both probes to be in close proximity or interact with each other on non-target sequences. Instead, only target RNA promotes both 3WJ probes to be in close proximity for signal primer generation and PG-RCA. This recognition mechanism can be categorized as proximity assay as well as ligation-PCR, proximity ligation assay, open sandwich ELISA or SMART assay (21–24).

3WJ structure formation mechanism

It was confirmed that 3WJ probes and target RNA form stable double strands at 60°C by melting curve analysis; however, we were not able to detect a melting peak that can be assigned to the 7-base-long complementary sequences of 3WJ probes (Supplementary information 1), which may require alternative analytical method with higher resolution such as high-resolution melting curve analysis (24). Since primer extension and signal primer generation were observed, we concluded that a 3WJ structure is formed at the reaction temperature (Figure 2).

In order to discuss the stability of the 3WJ structure, the melting temperatures of the complementary sequences of 3WJ probes before (7 bases long) and after primer extension (13 bases long) were calculated assuming these sequences are connected by 0–100-base-long poly(dT) linker using the mfold program (Supplementary information 4) (21). When target RNA exists, the 7 base sequences are in close proximity due to the stable double-strand formation between 3WJ probes and target RNA, therefore it can be considered that the complementary sequences are connected by a short linker in order to predict the melting temperature. When the linker length is 4 bases long, the melting temperature reaches maximum (56.5°C), which is slightly below the reaction temperature of PG-RCA. However, when the complementary sequences are extended to 13 bases long by primer extension, the melting temperature becomes 66.9°C or above the reaction temperature. Therefore, it is suggested that the 7 base complementary sequences of 3WJ probes may not form a stable double strand at 60°C, but probably in equilibrium between dissociation and hybridization (Supplementary information 4). Only after primer extension, the 3WJ structure may be stabilized by the extended complementary sequences. Considering this 3WJ structure formation mechanism, the distance between the hybridization sites between 3WJ probes and target RNA may play a key role to form a stable 3WJ structure. Indeed, the 3WJ probes used in this study were designed to hybridize to the target RNA with a distance of 2 bases between them; however, the 3WJ probes designed to hybridize without any space failed to detect RNA under the same reaction condition (data not shown). On the other hand, when target RNA does not exist, 3WJ probes are not in close proximity, therefore it can be considered that the 7-base-long sequences are connected by an infinite linker. When the linker length is more than 80 bases long, the melting temperature of the 7-base-long sequences is predicated to be <30°C, which is well below the reaction temperature of PG-RCA. Therefore, without target RNA, 3WJ probes may not interact with each other at all.

Suppression of background amplification and improvement of assay sensitivity

The sensitivity we obtained for RNA detection in this study was 15.9 zmol RNA50. Since PG-RCA is able to detect DNA with greater sensitivity (84.5 ymol synthetic DNA) as described before (16), the sensitivity of RNA detection can be improved further. In this study, the background signal amplification associated with 3WJ template

was observed, which was estimated almost 100 times stronger than that of PG-RCA itself, and successfully suppressed by introducing chemical modifications such as a 3′-phosphate poly(dT) tail and an internal C3 linker to 3WJ template. Therefore, inhibition of complementary strand synthesis by modified 3WJ template may be one of the ways to improve the sensitivity of RNA detection further. For example, 3WJ template with non-natural nucleotide modifications such as peptide nucleic acid (PNA) and locked nucleic acid (LNA) may be useful to prevent complementary strand synthesis further as DNA polymerase does not polymerize on these modified nucleotides in addition to the improvement of hybridization specificity.

Also, the signal generation reaction efficiency may affect the assay sensitivity as well. Under the PG-RCA reaction condition, the signal primer was generated at 0.69 amol/min from 250 amol RNA50, or only about 0.33 molecules of signal primer per each RNA50 molecule were generated in a 2-h reaction. This means that the reaction efficiency of signal primer generation has some space for further improvement by optimizing the reaction condition. For example, Bst DNA polymerase is known to have stronger strand displacement activity than Vent(exo-) DNA polymerase used in this study; therefore, the use of Bst DNA polymerase may improve signal primer generation efficiency further by dissociating the signal primer from 3WJ template more actively. Since we recently confirmed that the use of Bst DNA polymerase accelerates PG-RCA reaction speed without compromising the sensitivity of DNA detection (unpublished data), it may be possible to improve not only sensitivity but also reaction speed for RNA detection although the reaction condition requires further optimization for this enzyme.

CONCLUSION

In this manuscript, we presented a novel RNA detection method using 3WJ probes and PG-RCA in a one-step and isothermal reaction format. The use of 3WJ probes expanded the applicability of PG-RCA to detection of desired RNA sequences without additional enzymes or a complicated protocol, and thus both versatility and feasibility in RNA assay are sufficiently fulfilled. This assay method is especially advantageous to judge whether target RNA is, to notable extent, expressed in clinical samples in molecular diagnostics. Furthermore, detection of target DNA in cell extracts is also facilitated by this method, since no nicking sequence is required in the DNA sample and thus one of the most critical limitations of PG-RCA can be solved. Therefore, the combination of 3WJ probes and PG-RCA can be a useful nucleic acid detection technology in the field of molecular diagnostics.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Data 1–4.

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