Isothermal Target and Signaling Probe Amplification Method, Based on a Combination of an Isothermal Chain Amplification Technique and a Fluorescence Resonance Energy Transfer Cycling Probe Technology

Cheulhee Jung,† Ji Won Chung,‡ Un Ok Kim,‡ Min Hwan Kim,‡ and Hyun Gyu Park*,†

Department of Chemical and Biomolecular Engineering, KAIST, 335 Gwahak-ro, Yuseong-gu, Daejeon, Republic of Korea, and RapleGene Inc., A-207 Kumkang Penterium IT Tower, 333-7 Sangdaewon-Dong, Joongwon-Gu, Sungnam, Kyunggi, Republic of Korea

An iTPA (isothermal target and signaling probe amplification) method for the quantitative detection of nucleic acids, based on a combination of novel ICA (isothermal chain amplification) and fluorescence resonance energy transfer cycling probe technology (FRET CPT), is described. In the new ICA method, which relies on the strand displacement activity of DNA polymerase and the RNA degrading activity of RNase H, two displacement events occur in the presence of four specially designed primers. This phenomenon leads to powerful amplification of target DNA. Since the amplification is initiated only after hybridization of the four primers, the ICA method leads to high specificity for the target sequence. As part of the new ICA method, iTPA is achieved by incorporating FRET CPT to generate multiple fluorescence signals from a single target molecule. Using the resulting dual target and signaling probe amplification system, even a single copy level of a target gene can be successfully detected and quantified under isothermal conditions.

The invention of the PCR technique has made a tremendous impact on the areas of biological research and diagnostics since it can be used to generate quantities of nucleic acids needed for detection and measurements.1–6 However, the need for a temperature cycling instrument limits applications of PCR in point-of-care testing (POCT) environments.7 In order to overcome this limitation, several isothermal nucleic acid amplification methods have been developed. The techniques can be classified into two main types according to whether they employ enzyme-based denaturation methods or self-denaturation methods based on specially designed primers or probes. In a typical enzyme-based method, RNA degrading enzymes are used to initiate the displacement event by degrading the RNA region of a DNA/RNA heteroduplex. In the cases of transcription-mediated amplification (TMA)8 and nucleic acid sequence-based amplification (NASBA),9 RNA regions are completely digested by RNase H, generating single stranded DNA that can then be annealed with another primer. In contrast, only partial RNA regions are cleaved to induce the displacement of one DNA strand in the single primer isothermal amplification (SPIA)10 and isothermal chimeric primer-initiated amplification (ICAN)11 methods. The nicking activities of restriction endonucleases are also used to initiate isothermal displacement of one DNA strand by cleaving the nicking site of a previously extended double stranded DNA, which is followed by DNA extension like in the case of strand displacement amplification (SDA).12,13 This strategy relies on the use of a primer that contains a specific restriction endonuclease (RE) recognition site and a modified base, which enables cleavage of only one DNA strand at the nicking site. In addition to nucleases, helicase enzymes can be employed to regenerate single stranded templates for primer hybridization and subsequent extension. These enzymes operate by unwinding double stranded DNA as in helicase-dependent amplification (HDA).14

In addition to utilizing enzymes to promote denaturation, an autocycling denaturation process that does not rely on extra enzymatic activities can take place when specially designed

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* To whom correspondence should be addressed. E-mail: hgpark@kaist.ac.kr.
† KAIST.
‡ RapleGene Inc.

primers or probes are used. For example, rolling circle amplification (RCA) employs a 5’ to 3’ exonuclease-deficient DNA polymerase to extend a primer on a circular template. This generates tandemly linked copies of the complementary sequence of the template.\textsuperscript{15,16} Loop-mediated amplification (LAMP) also employs a 5’ to 3’ exonuclease-deficient DNA polymerase and a set of four primers that recognize sequences in the target DNA. Extension for amplifying target DNA under isothermal conditions. This template.\textsuperscript{15,16} Loop-mediated amplification (LAMP) also employs merase to extend a primer on a circular template. This generates 5’ to 3’ amplification. This is possible for the detection of target DNA sequences when the cycling probe technology (CPT)\textsuperscript{24–26} is employed. In CPT, a DNA–RNA–DNA chimeric probe is hybridized with the target DNA, and the RNA region of the duplex is specifically cleaved by RNase H. The cleaved probe fragment is dissociated from the target DNA and another intact probe is again hybridized and then cleaved. In the cycling events, a single target DNA molecule results in a large number of cleaved probe fragments, which can be designed to generate signals (e.g., fluorescence). Furthermore, the concentration of the cleaved probe is linearly dependent on time. Consequently, the original amount of the target DNA can be quantitatively determined by monitoring the amount of the amplified DNA in real-time.

In the study described below, a novel and quantitative DNA detection system has been developed using a combination of ICA and fluorescence resonance energy transfer (FRET) CPT, which we have termed isothermal target and signaling probe amplification (iTPA). By simultaneously utilizing the dual amplification powers of the target DNA and FRET probes, we have demonstrated that iTPA can be used to quantitatively detect a model Chlamydia trachomatis gene down to single copy level.

MATERIALS AND METHODS

Materials. Ultrapuré, DNase/RNase-free distilled water was purchased from Invitrogen. Outer primers were synthesized by Genotech, and inner primers and the FRET probe, with a donor of carboxyfluorescein (FAM) at the 5’ end and the quencher of 4-(4-(dimethylamino)phenyl)azo)benzoic acid (DABCYL) at the 3’ end, were synthesized in Integrated DNA Technologies (IDT).

In order to overcome these problems, we developed a new, highly sensitive and specific isothermal chain amplification system (ICA), which can be regarded as an improved version of a combination of LAMP (the outer and inner primers concept) and ICAN (the DNA–RNA chimeric primers and RNase H concept) for amplifying target DNA under isothermal conditions. This combination generates two separate, autocycling amplification routes, leading to high sensitivity, and the use of outer primers increases the specificity.

Another major effort underway is aimed at the development of nucleic acid amplification methods that enable real-time detection of DNA products. At the current time, several sequence-specific probes have been designed to achieve real-time signal amplification. For example, molecular beacons, adjacent hybridization probes, and 5’-nucleic probes (TaqMan probes) are capable of bringing about real-time amplification.\textsuperscript{20} In these strategies, however, amplified target DNA is used only once to generate a corresponding signal in each cycle of template DNA amplification. Although it has been demonstrated that coupling these methods to PCR enables achievement of very low (<10 copies) limits of detection in clinical diagnostic assays,\textsuperscript{21–23} the 1:1 stoichiometric ratio in signaling might be a limitation to the further enhancement of the sensitivity. Therefore, a great incentive exists for the development of new strategies that enable their own signal amplification mechanism.

Unique and rapid isothermal probe amplification is possible for the detection of target DNA sequences when the cycling probe technology (CPT)\textsuperscript{24–26} is employed. In CPT, a DNA–RNA–DNA chimeric probe is hybridized with the target DNA, and the RNA region of the duplex is specifically cleaved by RNase H. The cleaved probe fragment is dissociated from the target DNA and another intact probe is again hybridized and then cleaved. In the cycling events, a single target DNA molecule results in a large number of cleaved probe fragments, which can be designed to generate signals (e.g., fluorescence). Furthermore, the concentration of the cleaved probe is linearly dependent on time. Consequently, the original amount of the target DNA can be quantitatively determined by monitoring the amount of the amplified DNA in real-time.

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Table 1. Oligonucleotide Sequences Used in This Study

<table>
<thead>
<tr>
<th>primer sequence (5′-3′)</th>
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<tr>
<td>outer F primer</td>
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<td>inner F primer</td>
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<td>inner R primer</td>
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<td>FRET probe</td>
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(Table 1). The T4 gene 32 protein was purchased from USB Corporation. The RNase inhibitor was purchased from iNtRON Biotechnology. Hybridase Thermostable RNase H was purchased from EPICENTRE. Bst DNA polymerase was purchased from New England BioLabs. dNTP set was purchased from Fermentas. Other reagents were purchased from Sigma-Aldrich.

Preparation of *C. trachomatis* Plasmid and Human Genomic DNA. An 1130 base pair fragment of *C. trachomatis* cryptic plastid was amplified using a PCR process and cloned using GeneJET PCR cloning kit (Fermentas). Briefly, the DNA of *C. trachomatis* strain (ATCC VR-887) was prepared using a G-spin column (Aldrich). Fermentas. Other reagents were purchased from Sigma-Aldrich.

ICA Reaction for Amplifying a Target Sequence. ICA was carried out in a total 20 µL of reaction mixture containing 5 mM Tris–Cl (pH 8.5), 10 mM (NH₄)₂SO₄, 10 mM KCl, 28 mM MgSO₄, 2.5 mM of each dNTP, 0.5 mM DTT, 0.1 µg BSA, 0.15 µM outer primer set, 2.25 µM inner primer set, and the specified amounts of double stranded target DNA. The reaction mixture was denatured for 5 min at 95 °C and cooled for 5 min at 60 °C. T4 gene 32 protein (0.3 µg), 6 U RNase inhibitor, 10 U RNase H, and 5 U Bst DNA polymerase were added to the reaction mixture, which was then subjected to isothermal amplification for 40 min at 60 °C.

Quantitative Analysis of iTPA (ICA with FRET CPT). iTPA was performed in a 20 µL reaction mixture containing 5 mM Tris–Cl (pH 8.5), 10 mM (NH₄)₂SO₄, 10 mM KCl, 20 mM MgSO₄, 2.5 mM of each dNTP, 5 mM DTT, 0.1 µg BSA, 0.15 µM outer primer set, 2.25 µM inner primer set, and the specified amounts of double stranded target DNA. The reaction mixture was denatured for 5 min at 95 °C and cooled for 5 min at 60 °C, and 1 µL of 2 µM FRET probe (IDT), 0.3 µg of T4 gene 32 protein, 6 U RNase inhibitor, 10 U RNase H, and 5 U Bst DNA polymerase were added. The mixture was subjected to isothermal amplification for 90 min at 60 °C. Six reaction samples including different template copy numbers (1 × 10⁴, 1 × 10⁵, 1 × 10⁶, 10, 1, and 0) were subjected to iTPA, and the amplified products were diluted with deionized water to make a volume of 60 µL. The emission intensities of solutions containing the amplified iTPA products were determined at 518 nm using an excitation wavelength of 480 nm on a RF-5301 spectrophotometer (Shimadzu).

RESULTS AND DISCUSSION

Mechanism of the Novel ICA Method. The isothermal autocycling DNA synthesis method, developed in this investigation, consists of RNase H, DNA polymerase, two outer primers, and two specially designed inner primers. The essential issue in isothermal amplification methods is the achievement of denaturation of double stranded DNA under isothermal conditions. The strategy devised for this purpose relies on two main displacement events that are mediated by the RNA degrading activity of RNase H within a RNA/DNA heteroduplex and the strand displacement activity of DNA polymerase during DNA extension. Since the two distinct displacement events are designed to take place simultaneously and lead to two different amplification routes, they can produce remarkably high amplification efficiencies even under isothermal conditions. The detailed mechanism and reaction steps of ICA are illustrated in Figure 1. The first step in the scheme involves denaturation of the target DNA mixed with an outer primer and a DNA–RNA–DNA chimeric inner primer set. The mixture is then cooled to an isothermal amplification temperature, allowing the outer and inner primer set to be annealed to the target DNA. This is followed by the addition of a mixture of the required enzymes. The inner primer set is designed to be complementary to the more upstream region of the target template than the outer primer set. Therefore, both the inner and outer primers anneal to the target template simultaneously followed by their extension by DNA polymerase. During extension of the outer primer, the strand extended from the inner primer is displaced by the action of the DNA polymerase working with the outer primer. As a result, a set of double stranded DNA is obtained from the extension of outer primers along with a displaced single stranded DNA extended from inner primers. The displaced single stranded DNA can act as a new template to which new outer and inner primers can hybridize. Again, the strand extended from the inner primer is displaced by the extension of the outer primer. The double stranded DNA resulting from the extension of the outer primer follows case A while the released single stranded DNA follows case B shown in Figure 1. In case A, the RNA region at the 5′ end of the inner primer-containing strand of the double stranded DNA, formed in the previous step, is degraded by RNase H. This event results in production of two DNA segments that are separated by
the base length of the removed RNA sequence. The shorter DNA fragment at the 5′ end can act as a new primer and, as such, it is extended by DNA polymerase displacing the longer DNA fragment separately located at the 3′ end. As the primer annealing, extension, and strand displacement steps progress, specific DNA/RNA heteroduplex forms are generated, as depicted in the red-dotted box in Figure 1. As represented by case B, simultaneously, the inner primers are annealed to the inner primer-containing single stranded DNA generated in the previous step. The annealed primers are extended to form the same DNA/RNA heteroduplex structures as those shown in the red-dotted box. In a way, represented by the last part, an auto cycling route, which includes RNA digestion, DNA extension, strand displacement, and primer annealing, followed by the extension, is derived from the heteroduplex forms shown in the red dotted boxes for both cases A and B. Through these automatically repeated cycles, highly enhanced amplification of double stranded DNA, including the target region, can occur. In the rationally designed, isothermal autoamplification system, three differently sized amplification products are generated. The main product is double stranded DNA including either the forward inner primer or the reverse inner primer (blue dotted box in the Figure 1). Besides the main amplification product, two other double stranded DNAs are also expected to form, one that includes both the forward and the reverse inner primers (green dotted box in the Figure 1) and the other that is composed of only the 3′ DNA region inside both the forward and reverse inner primers (pink dotted box in the Figure 1).
Construction of ICA Amplification Using *C. trachomatis* as a Model Gene. In order to confirm the operation of the envisioned ICA mechanism, *C. trachomatis* plasmid was employed as a model target gene. Amplification of the gene, accomplished using four specially designed primers, was carried out isothermally at 60 °C for 40 min. Product formation was confirmed using agarose gel electrophoresis followed by DNA sequencing. The ICA reaction was found to successfully produce electrophoresis bands associated with the three expected segments consisting of 88 bp, 106 bp, and 124 bp (lane 5 of Figure 2).

The necessity, for all of the components involved in the ICA process, was probed by carrying out the same amplification reactions in which individual components are omitted one at a time. When the target DNA (*C. trachomatis* plasmid), RNaseH, inner primers, or outer primers are absent, no amplified target DNA products could be detected using electrophoretic analysis (Figure 2, lanes 1–4). Interestingly, in cases where the target DNA or outer primers are omitted, some bands corresponding to primer dimers are observed (Figure 2, lanes 1 and 4). The mechanism for formation of the side products is not fully understood, but a plausible pathway is presented in Figure S1 (Supporting Information). As the relatively long length of inner primers makes the nonspecific annealing of primers possible at 60 °C, some of the inner primers tend to bind partially to each other and then form a DNA/RNA heteroduplex through the extension process. After a DNA/RNA heteroduplex is formed, the RNA region of the heteroduplex will be digested by RNase H, initiating the nonspecific amplification process. Although the amount of the initially formed DNA/RNA heteroduplex is small, through repeated amplification cycles presented in Figure S1 (Supporting Information), three kinds of nonspecific bands will be generated in substantial quantities. Only in the case where all four components are present in the reaction mixture are bands corresponding to the expected amplification products observed (lane 5 of Figure 2). This finding indicates that all four components are essential for operation of the ICA method.

The presence of DNA polymerase is crucial in order to obtain efficient amplification. Besides its general DNA extension activity, the DNA polymerase used in the present study has displacement activity that separates one DNA strand from the cDNA strand. Studies with Bst DNA polymerase, Bca DNA polymerase, exo(-) vent DNA polymerase, exo(-) Deep vent DNA polymerase, exo(-) Pfu DNA polymerase, and phi29 DNA polymerase demonstrated that Bst DNA polymerase displays the best amplification efficiency in the ICA system. In order to initiate strand displacement, the RNA region of RNA/DNA heteroduplex formed by the DNA–RNA–DNA chimeric primer should be specifically degraded in our ICA system while single stranded RNA should not be degraded. Therefore, RNase H, which brings about selective digestion of duplex RNA, is used along with an RNase inhibitor to prevent hydrolysis of single stranded RNA. Hybridization of four primers to the target DNA needs to take place at the same temperature for the efficient operation of the new ICA method. For this reason and owing to the fact that the optimal temperature range of Bst DNA polymerase is 60–65 °C, the Tm values of the four primers were designed to have a range of 55–65 °C and the optimal temperature of the ICA reaction was determined to be 60 °C by experimenting with various conditions.

**Sensitivity and Specificity of ICA.** In order to evaluate sensitivity, amplifications of different copies of target DNA from 1 × 10^6 to 1 × 10^2 were examined. The results show that 1000 copies of the *C. trachomatis* were amplified, generating the expected three sized products in 40 min, which can be unambiguously differentiated from the negative control. Moreover, in the case of even 10 copies, trace amounts of the specific amplification products were produced, in addition to nonspecific products shown in Figure 3. This sensitivity is due to the simultaneous operation of two different amplification routes (case A and case B), which derive from the unique displacement features of the ICA system in which the strand extended from the inner primer is displaced by the strands extended from either outer primers or a shorter DNA segment of the inner primer at the 5′ end previously cleaved by RNase H (Figure 1). As the initial copy number is lowered, the amount of the ICA product decreases and other smaller sized products are observed to form. The byproducts correspond to dimers of primers generated when the amount of the template is low. Nevertheless, using a negative control, the target-specific amplification products can be distinguished from the nontarget specific primer dimers.

Another significant merit of the new ICA method is a high specificity that originates from the use of additional outer primers, which simultaneously recognize the specific target sequence along with inner primers. Only when both inner and outer primers are specifically annealed to the target sequence, the ICA system operates to generate three different sized amplification products. This is an advantageous feature since it ensures that specific amplification takes place. Even when a background of host
genomic DNA is present in the reaction mixture, the ICA method is still able to amplify the target DNA with the same efficiency and specificity as when host genomic DNA is absent (Figure 4). The presence of 50 ng of human genomic DNA, intentionally added to the ICA reaction mixture containing 100 copies of the C. trachomatis plasmid, had no adverse effect on either the amplification efficiency or the specificity.

It should also be noted that the reaction occurring in the new ICA system results in formation of three differently sized products through amplification by two simultaneously operating routes. This unique feature contributes to both the sensitivity and specificity of the method. Since all three products contain the specific target DNA sequence, they can be used for analysis of the target sequence, consequently resulting in highly enhanced sensitivity. Furthermore, since their sizes are based on the sequences of the inner and outer primers, the three products can be used to ensure that specific amplification of the target sequence has taken place.

Quantitative Analysis of iTPA (ICA Combined with FRET CPT). Finally, we have developed a quantitative analysis system incorporating the new ICA method into FRET CPT, which we have termed iTPA (isothermal target and signaling probe amplification). This system can simultaneously amplify target nucleic acids and generate fluorescence signals by cleaving the RNA region of the DNA–RNA–DNA FRET probe. During isothermal amplification of the target nucleic acids, the amplified DNA is hybridized with the DNA–RNA–DNA FRET probe to form an RNA/DNA het-

Figure 4. ICA in the presence of irrelevant genomic DNA. Lane M, 50 bp ladder size markers; lanes 1–4, ICA carried out in the absence of human genomic DNA; lanes 5–8, ICA carried out in the presence of 50 ng of human genomic DNA.

Figure 5. Isothermal quantification of a target gene by iTPA. (a) Overview of FRET CPT mechanism, a part of iTPA generating fluorescent signal. (b) Linear regression analysis of a plot of the percentage of relative fluorescence intensity increase of the FRET probe versus the log of the initial copy number.
eroduplex. The RNA region of the FRET probe in the duplex is cleaved using the same RNase H that is employed in the ICA step. The cleaved FRET probe is separated from the target DNA, which leads to generation of fluorescence signals. Next, the released target DNA can be hybridized with another intact FRET probe, followed again by RNase H cleavage and subsequent separation from the target DNA. The repeated cycles result in FRET probe amplification because a single target molecule can generate multiple FRET signals (Figure 5a). Therefore, iTPA possesses the dual amplification power, in terms of both the target and probe, that leads to a remarkably enhanced sensitivity under isothermal conditions. Furthermore, end-point quantification of target nucleic acids can be made by measuring the fluorescence intensities of reaction mixtures directly.

By detecting fluorescent signals generated from the FRET probe, the iTPA method was shown to be applicable to quantitative analysis of target amplification. The fluorescence intensities of the iTPA product, determined at 518 nm with and without the target plasmid (negative control), are defined as \( f \) and \( f_0 \), respectively. The difference in fluorescence between \( f \) and \( f_0 \) is defined as \( \Delta f \). A standard graph is obtained by plotting \( F \) and log \( C \), where \( F \) is the relative fluorescence increase percentage of the FRET probe defined as (\( \Delta f / f_0 \)) \( \times \) 100 and \( C \) is the copy number of the target DNA. To verify the reliability of the quantitative nature of iTPA, three duplicate assays were performed. The \( F \) values of the independently repeated assays for each copy number were averaged and standard deviations were calculated and expressed as error bars in Figure 5b (Table S1, Supporting Information).

First, a standard curve was constructed by plotting the relative fluorescence increase percentage (\( F \)) versus concentrations of the C. trachomatis plasmid obtained by serial dilution to bring about \( 1 \times 10^4, 1 \times 10^3, 1 \times 10^2, 10, 1, \) and 0 copy numbers. As shown in Figure 5b, the standard curve displays a linear response with a high \( R \) value (0.97). Furthermore, the \( R \) values obtained from three independent assays are >0.96, confirming high quantitative reliability of the iTPA method (Table S2, Supporting Information). Using the standard curve, the initial concentration of any unknown sample can be determined once its fluorescence intensity is measured. As clearly shown in Figure 5b, even a single copy of a target plasmid determined based on the serial dilution method can be distinguished from a negative control. This remarkably high sensitivity is assumed to be associated with the simultaneous, dual amplification of target and probe that results from the combined use of ICA and FRET CPT. More significantly, this iTPA method could be further developed to quantitatively analyze target DNA in real-time.

CONCLUSION

In this investigation, we have developed a powerful isothermal amplification method (ICA) as part of a quantitative amplification technology (iTPA) that combines ICA and FRET CPT techniques. By creating a rationally designed amplification mechanism, ICA amplifies target DNA in a specific manner to detect up to 100 copies under isothermal conditions. To achieve the iTPA, dual amplification of both the target and the FRET probe is efficiently utilized to bring about high sensitivity. The observations made in this study clearly demonstrate that even a single copy level of target DNA can be reliably quantified. Since the ICA and iTPA methods we have developed are conveniently performed, requiring only a simple water bath or heat block, they have great potential in applications to hand-held or point-of-care-testing (POCT) diagnostic devices.

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SUPPORTING INFORMATION AVAILABLE

Tables showing the calculated values used for a linear plot of the percentage of fluorescence increase of the FRET probe versus the log of the initial copy number and regression coefficients in three independent assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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