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Review Article

Rolling Circle Amplification as Isothermal Gene Amplification in Molecular Diagnostics

Nam-In Goo[1] **& Dong-Eun Kim**[1,*]

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Abstract Rolling circle amplification (RCA) developed in the mid-1990s has been widely used as an efficient isothermal DNA amplification process for molecular diagnosis. This enzymatic process amplifies target DNA sequences with high fidelity and specificity by using the strand displacing DNA polymerases. The product of RCA is long single-stranded DNA that contains tandem repeat of target sequence. Isothermal reaction amplification condition of RCA has an advantage over conventional polymerase chain reaction, because no temperature cycling devices are needed for RCA. Thus, RCA is suitable tool for point-of-care detection of target nucleic acids as well as facile detection of target genes. Combined with various detection methods, RCA could amplify and detect femtomolar scale of target nucleic acids with a specificity of one or two base discrimination. Herein, RCA technology is reviewed with an emphasis on molecular diagnosis of microRNAs, infectious pathogens, and point mutations.

Keywords: Rolling circle amplification, Isothermal DNA amplification, Molecular diagnostics, Micro RNA, Single Nucleotide Polymorphisms

Introduction

Rolling circle amplification (RCA) was introduced in the middle of 1990s as a new isothermal DNA amplification method[1,2]. As shown in Figure 1A, the amplification of DNA is based on circular template and strand-displacing DNA polymerases, such as phi 29 DNA polymerase[3,4]. Especially, phi 29 DNA polymerase harboring 3′ to 5′ exonuclease activity for proofreading contributes to high fidelity of DNA polymerization. As product of RCA, long single-stranded DNA (ssDNA) products containing tandemly repeating sequences complementary to the circular target DNA are generated[5], which can be often observed as long stretch of ssDNA under atomic force microscopy (AFM, Figure 1A).

Ligation-RCA (L-RCA) is one of RCA variations, which is based on padlock probe[6,7], a linear single-stranded template DNA for RCA reaction (Figure 1B). The 5′- and 3′-end of padlock probes are designed to be hybridized with target nucleotides such as genomic DNA or microRNA (miRNA). The only padlock probe that is hybridized with its target nucleotides is eligible for ligation reaction to form circular DNA templates for subsequent amplification process. Addition of deoxynucleotide triphosphates (dNTPs), primer DNA, and phi 29 DNA polymerase to the circular template DNA can initiate polymerization of target DNA sequence. In addition, branched RCA (BRCA), or hyperbranched RCA (HRCA) has been developed as expanded variations of RCA (Figure 1C), which amplify DNA exponentially with forward and reverse primers[8]. HRCA products are double-stranded DNAs (dsDNA), whereas the ordinary RCA products are long ssDNAs. Thus, dsDNA-specific intercalating fluorescent dyes such as SYBR Green (SG) has been often used for the detection of the HRCA products[9].

RCA has an obvious advantage over polymerase chain reaction (PCR); unlike PCR, RCA is performed at an isothermal reaction condition at room tempera-

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[1] Department of Bioscience and Biotechnology, Konkuk University, 120 Neungdong-ro, Gwangjin-gu, Seoul 05029, Korea
*Correspondence and requests for materials should be addressed to D.-E. Kim (✉ kimde@konkuk.ac.kr)

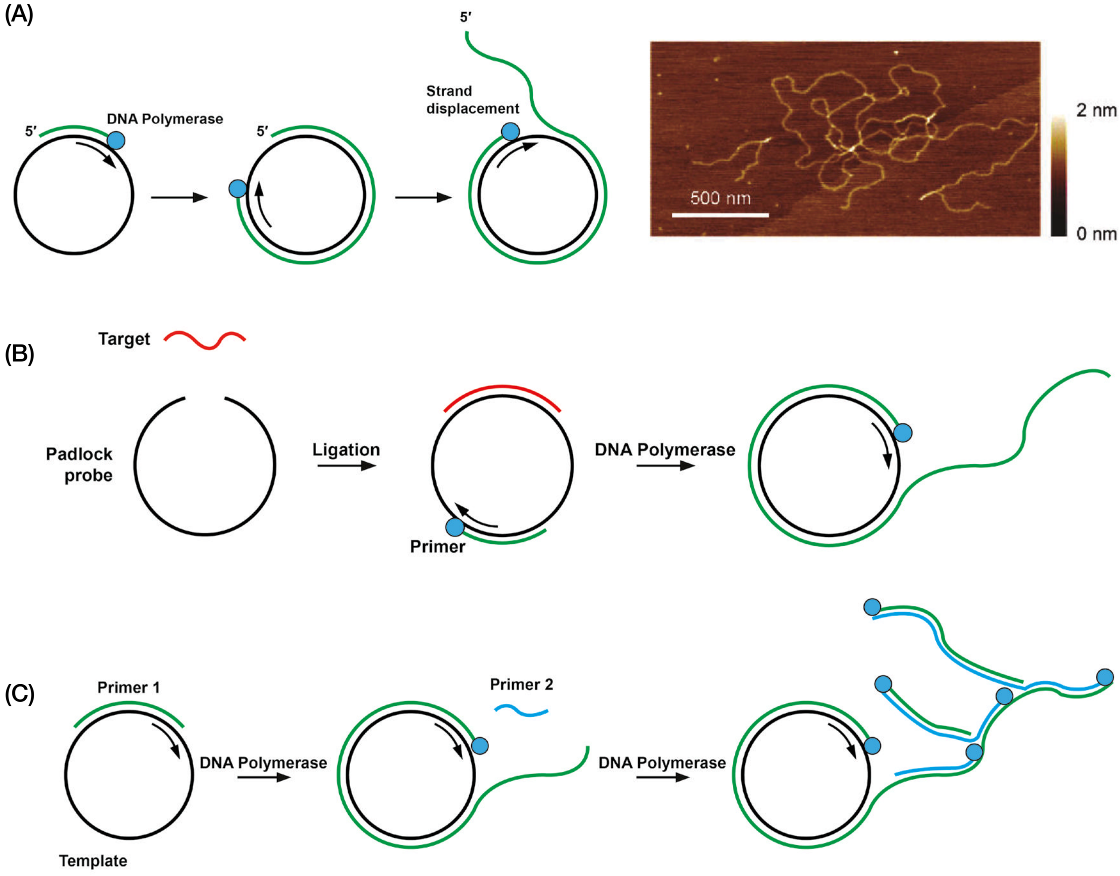

Figure 1. (A) Principle of RCA (left) and AFM image of RCA product (right). The primer DNA is extended by DNA polymerase. The DNA polymerase unwinds dsDNA (strand displacement) and synthesizes long ssDNA with high processivity and fidelity. The AFM image shows that the length of RCA product varies at least hundred nanometers to few micrometers. AFM image was reproduced from Ref. 5 with a permission. (B) Schematic illustration of L-RCA. In the presence of target nucleotide, the padlock probe hybridizes with the target and both ends of the padlock probe are ligated to form circular template DNA by DNA ligase. DNA polymerization is then initiated from 3′-end of RCA primer by DNA polymerase. (C) Schematic representation of HRCA. During the extension of the first primer (primer 1), addition of the second primer (primer 2), which is complementary to the first RCA product, initiates synthesis of complementary RCA product, resulting in formation of double-stranded RCA product.

ture or at 30°C. Since RCA does not need thermal cycling for amplification of DNA, no additional devices such as thermal cyclers, are needed for amplification of DNA through RCA. Thus, RCA as isothermal amplification of target gene is regarded as a suitable tool for point-of-care detection of target genes. In addition, RCA can amplify target DNA sequences with high specificity and sensitivity; 1 copy of target DNA in 100,000 copies of non-target DNAs can be amplified by RCA[10]. High sensitivity and specificity makes RCA as a feasible tool for detection of single nucleotide polymorphisms (SNPs)[8,11-17], microRNAs (miRNA)[9,18-29], bacterial[30-34], and viral nucleic acids[35-39]. Amplified DNA products can be detected with diverse methods including fluorescence measurement[8], colorimetric assay[32,37], enzymatic luminescence assay[20,21], or electric signals[33]. Herein, we reviewed recent advances in molecular diagnosis methods based on RCA for specific detection of target nucleotides such as miRNAs and infectious pathogens, and point mutations causing SNP.

RCA in Detection of Micro RNAs (miRNAs)

MicroRNAs (miRNAs) are non-coding single-stranded RNAs (ssRNA) that have ~25 nucleotides in length. MiRNAs anneal to 3′-untranslated region (3′-UTR) of target mRNA and induce degradation of the mRNA, leading to inhibition of the target gene translation, as a post-transcriptional regulation of target gene[40]. It has been known that miRNAs are closely associated with various cell metabolisms such as cell differentiation, proliferation, fat metabolism, and cell death[41]. Of importance, some miRNAs are significantly overexpressed in cancerous tissues compared with normal tissues[42,43]. Therefore, detection and quantification of miRNAs in clinical samples are important for diagnosis of certain cancer.

The sensitivity and target specificity of RCA made it suitable for detection and quantification of miRNA. In 2006, Jonstrup *et al*. reported padlock probe and L-RCA based miRNA detection method[18]. They used padlock probes targeting several miRNAs and the miRNAs are used as both target molecule and primer of DNA polymerization. The resulting RCA products are detected by autoradiography. Compared with Northern blot, a commonly-used RNA detection method which require micrograms of RNA samples for detection, RCA detected target miRNAs in nanograms of total RNA sample. In addition to L-RCA, BRCA was also utilized for detection of miRNAs; Cheng *et al*. reported a BRCA-based fluorescence detection of let-7a miRNA[9]. For fluorescence detection of BRCA product, SYBR Green I (SG I) dye, which intercalates into double-stranded DNA (dsDNA) and shows green fluorescence, was utilized in this method (Figure 2A). The BRCA-based miRNA detection could detect 10 fM of miRNA and discriminate let-7a from let-7b and let-7c, which differ in one or two bases in sequence.

In addition to fluorescence detection of miRNA using fluorescent dye, indirect detection of RCA product using luminescence assay coupled with other enzymatic reactions was adopted for detection of miRNAs. Mashimo *et al*. reported miRNA detection based on BRCA coupled with bioluminescent (BL) pyrophosphate assay (Figure 2B)[20]. The BL assay utilizes target miRNA molecule as primer of the DNA polymerization reaction. During the synthesis of RCA product, inorganic pyrophosphates (PPi) are released when dNTPs are incorporated. Adenylyl transferase then converts released PPi to ATP, which provides chemical energy for firefly luciferase to generate light. Target RNA could be detected up to 0.1 fM through this method, and the luminescence showed linearity as a function of target miRNA amount. Sun *et al*. also developed miRNA quantification system using RCA coupled with enzymatic luminescence assay (Figure 2C)[21]. In this assay, 2′-deoxyadenosine-5′-O-(1-thiotriphosphate) (dATPαS) was used as a replacement of dATP for generation of adenosine 5′-phosphosulfate (APS) and PPi during the DNA polymerization. The released APS reacts with the PPi to form ATP by the catalytic activity of ATP-sulfurylase, and the generated ATP provides energy for subsequent reaction of firefly luciferase to generate luminescence. This method claims to detect 0.01 pg of target miRNA and discriminate let-7d miRNA from other let-7 miRNA families (let-7a, let-7b, let-7c, and let-7e).

In place of using padlock probe based RCA for detection of miRNA, some researchers used hybridization of miRNA to dumbbell[19] or hairpin-shaped probe[22]. Zhou *et al*. used dumbbell probe based RCA (D-RCA) and SG I for fluorescence detection of miRNA (Figure 3A)[19]. The dumbbell probe is composed of 3 domains: miRNA-binding domain (MBD), SYBR green I binding domain (SGBD), and loop domain. Once the targeting sequences of MBD hybridize with target miRNA, closed template for RCA forms and DNA polymerization occurs in the presence of phi 29 polymerase and miRNA as primer. The RCA product forms repeating SGBDs and the SG I dye intercalates into double-stranded region of the SGBD, which generates detectable green fluorescence. Detection limit of this method was calculated as low as 1 fM. Rather than the conventional padlock probe DNA for L-RCA, Li *et al*. adopted hairpin-shaped probe mediated RCA (HP-RCA) for detection of miRNA (Figure 3B)[22]. The hairpin probe contains miRNA binding domain, which targets miR-486-5p, and circular template binding domain, which also acts as primer for DNA polymerization. In the presence of target miRNA, the hairpin structure of the hairpin probe melts and the target miRNA binds to the probe, which exposes recognition site of circular template. The exposed probe sequence then binds with circular template to initiate polymerization of RCA product. The amplified DNA products are detected by SYBR Green II dye, which can stain ssDNA to emit green fluorescence. Detection limit of this method was calculated as low as 10 fM of target miRNA and could discriminate closely related miRNA families.

Very recently, our group also reported a fluorometric system for the detection of miRNA using L-RCA, graphene oxide (GO), and fluorescent peptide nucleic acid (F-PNA) probe[29]. This assay method is featured by unique properties of GO such as high affinity to single-stranded nucleic acids and quenching of nearby fluorescence via long-range energy transfer. As shown in Figure 3C, the padlock probe DNA complementary to a target miRNA was specifically ligated to form circular DNA and then used as the template for RCA. F-PNAs complementary to the target miRNA were annealed to multiple sites of the amplified single-stranded RCA product (RCAP) containing multiple target miRNA sequences. This F-PNA/RCAP duplex is less adsorbed onto the GO monolayer, thus attenuating the quenching of F-PNA fluorescence by GO. High sen-

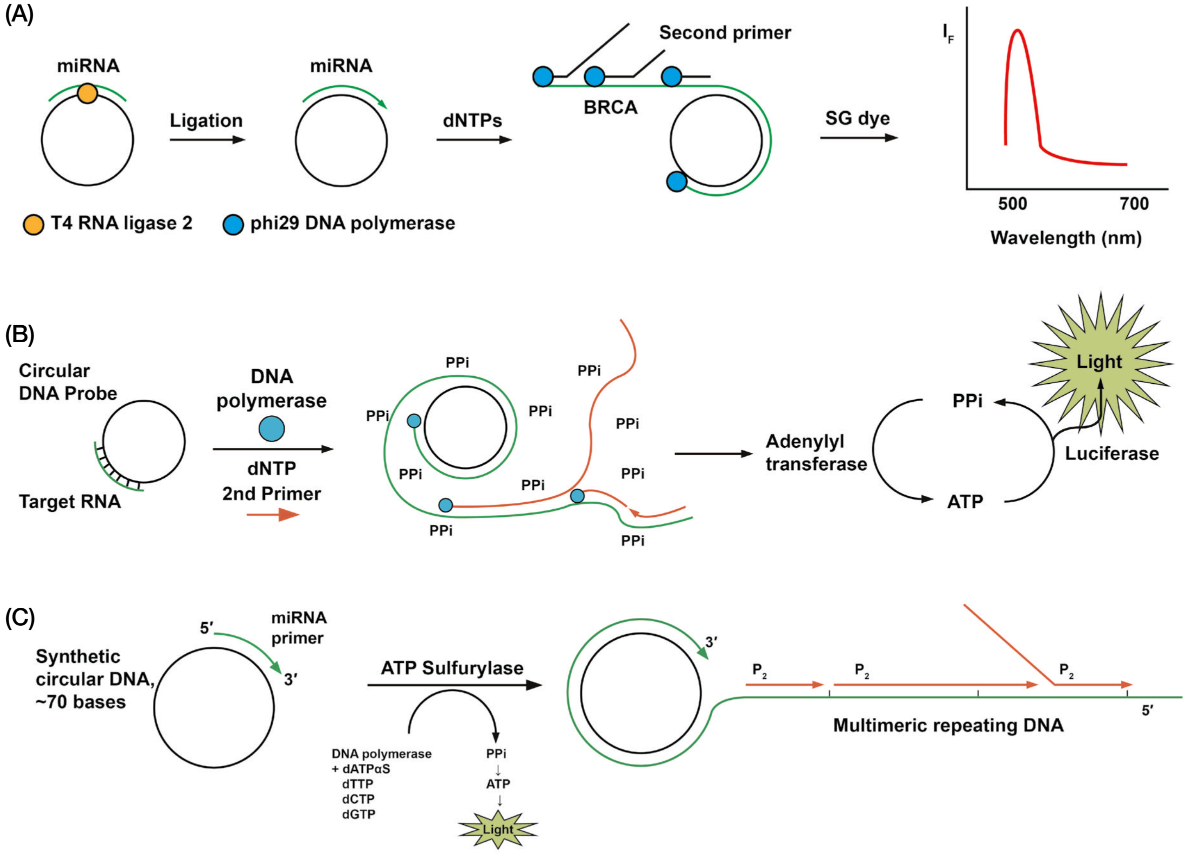

Figure 2. (A) Schematic representation of ligation-BRCA based miRNA detection using SYBR Green dye. BRCA was performed in the presence of miRNA target, which is also served as primer of BRCA reaction. The amplified DNA sequences could be detected by measuring of fluorescence of dsDNA-intercalated SYBR Green I. Image was adapted from Ref. 9 with permission. (B) Schematic illustration of miRNA detection using BL assay coupled BRCA. The circular DNA probe hybridizes with the target RNA and the DNA polymerization initiates from the 3′-end of the target RNA by phi 29 DNA polymerase. The 2nd primer then binds to the RCA product extended from RNA primer, which initiates polymerization of complementary RCA products. Inorganic pyrophosphates (PPi) are released from dNTPs and PPi are converted to ATP by adenylyl transferase. Generated ATPs provide energy for luciferase reaction which emits bioluminescence. Figure was reproduced from Ref. 20. (C) miRNA detection using BRCA combined with luminescence assay. Circular DNA template binds to miRNA and the miRNA acts as primer of DNA synthesis. DNA polymerization was proceeded in the presence of dATPαS and other dNTPs. As the DNA polymerized, more PPi are released and APS are converted to ATP by reaction of ATP sulfurylase. The ATP provides energy for luciferase reaction to generate light. Figure was reproduced from Ref. 21.

sitivity (i.e. LOD of pM range) and selectivity of the assay for miRNAs allows the efficient detection of multiple miRNAs in a mixture, using a simple 96-well format that can be completed within an hour.

RCA in Detection of Infectious Pathogens

Infectious diseases are still major cause of death in developing countries and causing millions of deaths every year. Thus, detection of infectious pathogens such as bacteria and viruses is important for early diagnosis and prevention of disease spreading. RCA has been developed for detection of pathogens due to its sensitivity, simplicity, and specificity. More importantly, RCA is advantageous in point-of-care diagnosis because RCA is an isothermal DNA amplification method and no sophisticated device is needed. For detection of bacteria, research groups targeted genomic DNAs of target bacterium.

Gomez *et al*. developed a colorimetric detection method of bacterial pathogens using HRCA combined with nicking endonuclease, which they called exponential linear RCA (ELRCA) (Figure 4)[32]. They used bis-PNA openers to expose target genomic DNA sequence for padlock probe binding. The exponential amplifica-

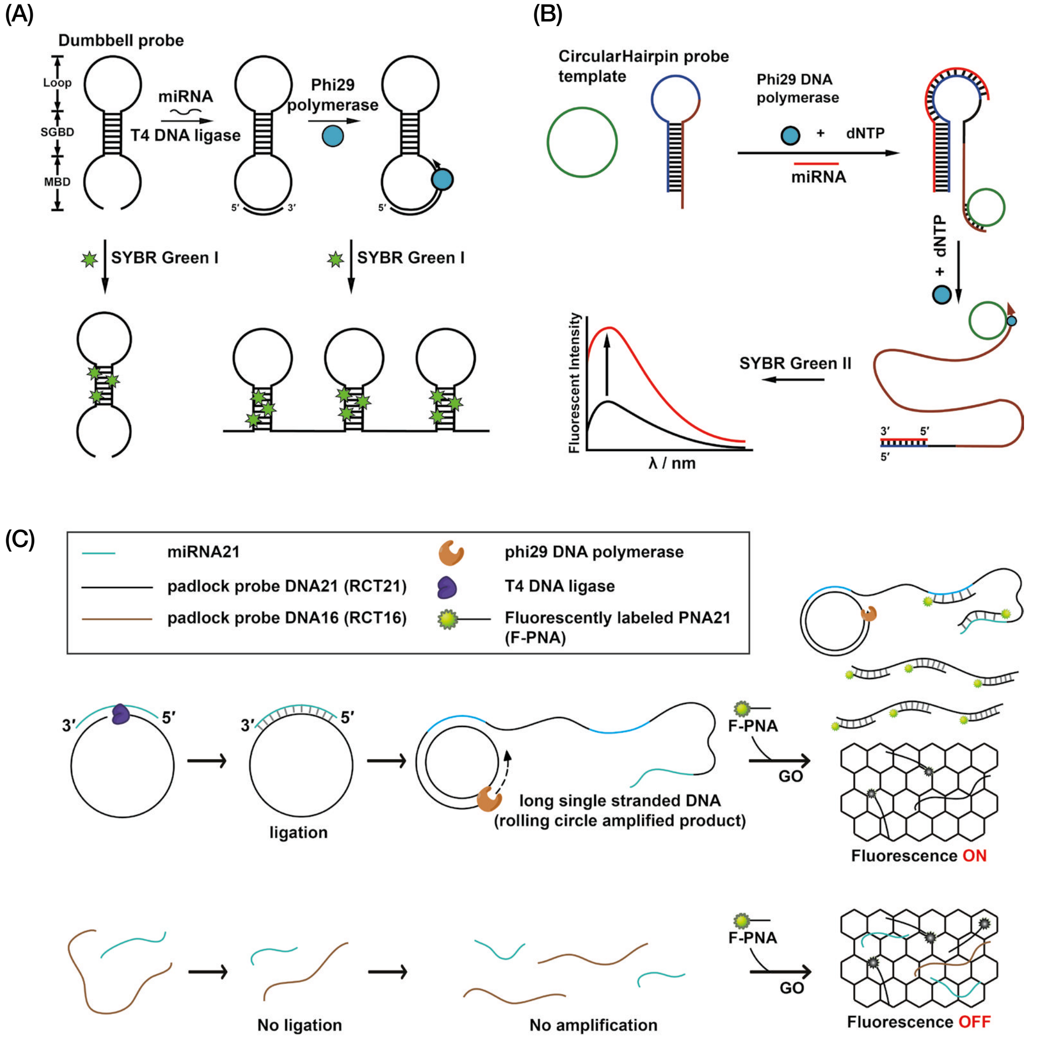

Figure 3. (A) miRNA detection using dumbbell probe RCA (D-RCA) and SYBR Green I (SG I). The dumbbell probe is comprised with three domains: Loop domain, SYBR Green I binding domain (SGBD), and miRNA binding domain (MBD). Ligation of the dumbbell probe can be initiated by the binding of target miRNA to the MBD. The bound miRNA serves as primer of DNA polymerization by phi 29 DNA polymerase, which generates multiple copies of the SGBD. SG I intercalates into double-stranded region of the SGBD and emits green fluorescence, which can be detected at 530 nm. Image was reproduced from Ref. 19 with permission. (B) Schematic illustration of miRNA detection using hairpin probe-based RCA (HP-RCA). The hairpin probe contains miRNA targeting sequence and circular template binding sequence. The hybridization of target miRNA with the hairpin probe exposes circular template binding sequence. The 3′-end of the hairpin probe act as primer of RCA. Thus, binding of the circular template to the hairpin probe can initiate the synthesis of RCA product. The RCA product can be detected by the fluorescence at 512 nm of fluorescent dye, SYBR Green II, which can stain ssDNA. Figure was reproduced from Ref. 22. (C) miRNA detection system using L-RCA, GO, and F-PNA. In the presence of target miRNA, padlock probe was ligated to form circular DNA template. Subsequently, synthesis of RCA product was initiated from 3′-end of the miRNA, which serves as both target and primer. Hybridization of F-PNA to RCA product inhibited binding of RCA product on the GO surface, which prevents fluorescence quenching. Image was reproduced from Ref. 29 with permission.

tion of DNA is proceeded by HRCA. By the activity of nicking endonuclease, multiple gaps are generated on the forward strand. Polymerization can be initiated from the gap; thus displacement of nicked pieces can occur because of the strand-displacing activity of the DNA polymerase. The ssDNA pieces fold to form G-quadruplex structures, which show catalytic activity of oxidization of 2,2′-azino-bis (3-ethyl-benzthiazoline)-6-sulfonic acid (ABTS) to ABTS- in the presence of Hemin and H_2O_2. The reaction progress was monitored by measuring absorbance at 412 nm. This method was able to detect bacterial genomic DNA as low as

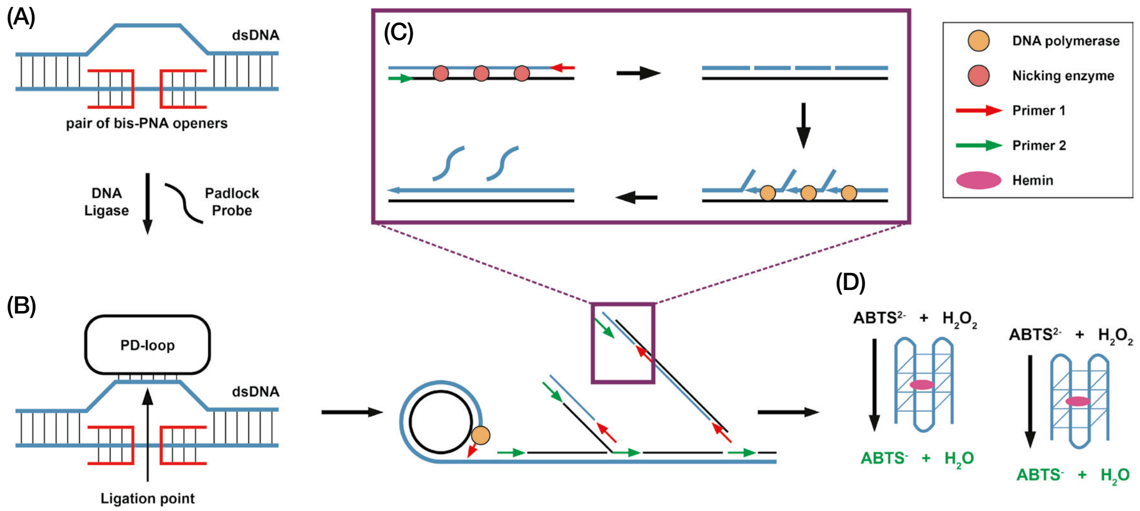

Figure 4. Detection of bacterial genomic DNA using exponential linear RCA (ELRCA). (A) The bis-PNA openers hold one strand of double-stranded genomic DNA, thus expose the target sequence of padlock probe. (B) Ligation of the padlock probe occurs to form PD-loop. In the presence of primer 1, 2, and DNA polymerase, HRCA occurs for exponential amplification of DNA. (C) The Nicking enzyme cleaves the forward strand (Inset box in panel B). By the extension of the forward strand, displacement of ssDNA fragments occur, which fold into G-quadruplex. (D) The G-quadruplex can oxidize ABTS^{2-} to ABTS^{-} in the presence of hemin and H_2O_2. The generation of ABTS- is monitored by the absorbance change at 412 nm. Figure was adapted with modification from Ref. 32.

femtomolar concentration, and discriminate sequence of the target genomic DNA specifically.

Schopf *et al*. reported a RCA based detection method targeting genomic DNA of *Mycobacterium tuberculosis* (Figure 5A)[30]. They used capture probe that is immobilized on surface of sepharose beads to capture both single-strands of target double-stranded after heat denaturation. The genomic DNA of *M. tuberculosis* is fragmented via restriction enzyme reaction and heated for denaturation. The denatured genomic DNA then captured on the surface of the sepharose beads by capture probe. The captured genomic DNA serves as both target DNA for padlock probe and primer for DNA polymerization. The sensitivity of this method was calculated as low as 4.25 fM of target dsDNA and 10,000 colony forming units per milliliter (cfu/mL) for *M. tuberculosis*. genomic DNA. Xiang *et al*. applied L-RCA combined with surface plasmon resonance (SPR) biosensor and gold nanoparticles (AuNPs) for detection of bacterial genomic DNA (Figure 5B)[33]. SPR biosensor monitors the change of refractive index, which is caused by the hybridization of RCA products and capture probes immobilized on the surface of AuNPs. The limit of detection was as low as 10 pM of target DNA, and this method could specifically detect 42,000 cfu/mL (5 pg/μL) of *M. tuberculosis* genomic DNA and 37,000 cfu/mL (2 pg/μL) of *Mycobacterium avium* genomic DNA.

RCA has been also used for detection of viruses, which is based on the detection of genomic DNA or RNA of target viruses. Wang *et al*. designed a detection method using HRCA targeting severe acute respiratory syndrome coronavirus (SARS-CoV) RNA[35]. They tested liquid-phase RCA and solid-phase RCA, which proceeds in a reaction buffer and on surface of magnetic bead coated with oligo (dT), respectively. Gel electrophoresis was performed for analysis of the RCA products, and both methods could detect single-copy of SARS-CoV RNA. However, accurate quantification of the target RNA was not attained because the detection of the RCA products was based on gel electrophoretic signal, which is insufficient for quantification of the samples. To overcome the lack of quantification, Hamidi *et al*. developed real-time monitoring methods of H5N1 influenza virus RNA using HRCA combined with colorimetric[37] or fluorometric[38] assay. For fluorescence detection of H5N1 RNA, cDNA synthesis was performed and subsequent HRCA was proceeded by the phi 29 polymerase. SG I was adopted to generate fluorescence signal in the presence of HRCA product. The limit of detection was calculated as low as 9 fM of target, and the signal could be obtained within 3 h. The colorimetric assay was based on characteristics of Hydroxy Naphthol Blue (HNB), which is known as metal chelator. HNB has sky blue color at pH 8.8 with absorption peak at 650 nm. In the presence of metal

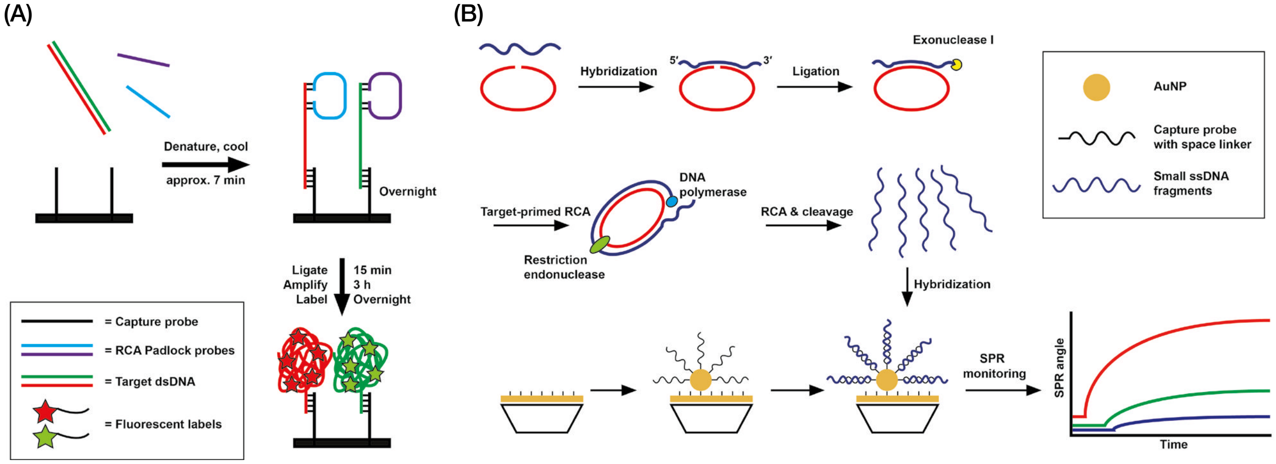

Figure 5. (A) Schematic illustration of fluorescence detection of *M. tuberculosis* genomic DNA using capture probe and RCA. The capture probes are immobilized on the surface of sepharose beads which targets restriction fragment of *M. tuberculosis* genomic DNA. Double-stranded genomic DNA fragments are heat-denatured and the capture probes hold each strand of the DNA fragments. Padlock probes subsequently bind to the 3′-end of the captured DNA fragments, which allow the fragments serve as primers of DNA polymerization. RCA was performed and the RCA products formed small DNA clusters, which were detected by FAM or Cy3 labeled oligonucleotide probes. Figure was reproduced from Ref. 30. (B) Detection of bacterial genomic DNA using RCA and SPR biosensor. Padlock probe hybridizes with the target DNA, which initiates ligation by *E. coli* DNA ligase. Exonuclease I degrades single-stranded DNA from 3′ to 5′ direction, and unhybridized target DNA sequences are degraded. The 3′-end of the hybridized target DNA serves as primer of DNA amplification. The amplified DNAs are captured by the capture probes, which are immobilized on the surface of AuNPs. The SPR biosensor detects refraction change by the amplification and hybridization of RCA products at 650 nm and generates electric signal for detection of the amplified DNA. Figure was adapted from Ref. 33.

ions such as Mg^{2+}, HNB captures Mg^{2+} to form HNB-Mg complex and the absorption peak shifts to 530 nm, which appears in dark blue color. During the polymerization of DNA by *Bst* DNA polymerase, PPi is released and chelates Mg^{2+} in reaction buffer to form PPi-Mg complex. As RCA proceeds, more PPi-Mg complexes are generated and Mg^{2+} ions are released from HNB-Mg complexes. Thus, the absorption peak shifts from 530 nm to 650 nm and the color change can be observed via naked eye. By measuring the absorption at 650 nm, the presence of H5N1 influenza virus in sample can be detected. The limit of detection was calculated as low as 28 fM of target and could detect H5N1 virus in real samples.

RCA in Detection of Single Nucleotide Change in Genes

Single nucleotide polymorphisms (SNPs) are alteration of single base in specific position of a gene sequence, which make individual difference of disease susceptibility[44,45] and drug response[46]. In addition, SNPs are known to be cause of cancers[47] and genetic diseases[48,49]. Thus, sensitive detection of SNPs is crucial for diagnosis of multiple diseases. RCA has been widely adopted in detection of SNPs because of its high sensitivity and specificity. The first SNP analysis using RCA was reported by Lizardi *et al*. in 1998[8]. Their RCA strategy was designed for detection of CFTR G542X gene causing cystic fibrosis. Oligonucleotide probe was immobilized on glass surface and the probe was ligated with RCA primer depending on the sequence of the target DNA. Amplification of DNA sequence occurred selectively in the presence of circularized template DNA, and the amplified DNA was monitored by fluorophore-labeled probe. Using a similar RCA method but different report system, Zhang *et al*. designed electrochemical label-free SNP detection system using RCA[17]. They immobilized capture probe, which captures RCA products, on the gold electrode and used methylene blue as signal molecule or detection of RCA products. This method can detect 40 amol of mutant strand and distinguish 1 target mutant from 5,000 non-target wild type DNAs. The SNP detection methods described above are using immobilized oligonucleotides as amplification and signal detection platform. Those methods are expected to be utilized for microarray-type SNP assay system.

Molecular beacons are oligonucleotide probes containing both fluorophore and quencher. Without target nucleic acids, the fluorescence of molecular beacons

Table 1. Summary of RCA-based diagnostic methods.

Target	Method	Detection Signal	LOD	Year	Reference
miRNA	BRCA-based fluorescence detection	Fluorescence	10 fM	2009	9
	L-RCA	Autoradiography	few ng	2006	18
	D-RCA	Fluorescence	1 fM	2010	19
	BRCA coupled with bioluminescent pyrophosphate assay	Bioluminescence	0.1 fM	2011	20
	RCA coupled with enzymatic luminescence assay	Luminescence	0.01 pg	2012	21
	HP-RCA	Fluorescence	10 fM	2013	22
	L-RCA, GO, and PNA based miRNA detection	Fluorescence	0.7 pM	2016	29
Pathogen*	Immobilized capture probe-based RCA	Fluorescence	4.25 fM	2011	30
	ELRCA	Absorbance at 412 nm	few fM	2014	32
	L-RCA combined SPR biosensor and AuNPs system	Electric signal	10 pM	2015	33
	HRCA	Gel electrophoresis		2005	35
	HRCA combined with colorimetric assay	Absorbance at 650 nm	28 fM	2015	37
	HRCA combined with fluorescence assay	Fluorescence	9 fM	2015	38
SNP**	L-RCA	Fluorescence	N/D***	1998	8
	BRCA with molecular beacon primer	Fluorescence	N/D	2001	12
	BRCA with molecular beacon primer	FlN/D	N/D	2002	16
	L-RCA	Electric signal	40 amol	2009	17

*: Bacterial, viral nucleic acids, **: Single Nucleotide Polymorphism (point mutation), ***: Not determined

is quenched because hairpin structure of the probes places fluorophore and quencher at near distance. Fluorescence of molecular beacons is recovered when the probes specifically hybridized with their target nucleic acids. Based on the specificity of the molecular beacons, they are widely used as fluorescent probe for SNP genotyping using RCA. Faruqi *et al*. utilized molecular beacon to develop solution-based high-throughput SNP genotyping of human genomic DNA using RCA[12]. They used two sets of primers, forward primer containing molecular beacon with hairpin structure, and reverse primer for exponential amplification of DNA. The forward primer binds to RCA template and the reverse primer binds to elongated forward primer. Elongation of the reverse primer denatures hairpin structure of the forward primer, which recovers quenched fluorescence of the molecular beacon. They tested 10 SNPs in 2 sets of 96 different DNA samples and achieved average 93% of accuracy of genotyping. Pickering *et al*. also used reverse primer containing molecular beacon for SNP genotyping[16]. They chose 3 genes from human genome and amplified target genes by PCR for genotyping. The genotyping using RCA and molecular beacon showed near 100% accuracy, only 4 are failed to detect signal from total 192 samples.

Summary

Success of molecular diagnostics are heavily dependent upon the target specificity and sensitivity of the detection method as well as target gene amplification. Rolling circle amplification (RCA) is an isothermal DNA amplification method that is based on circular template and high fidelity of strand-displacing DNA polymerases, such as phi 29 or *Bst* DNA polymerase. RCA and its variations were combined with fluorescence detection, colorimetric assay, enzymatic luminescence assay, or electric signal for the detection of target nucleic acids, such as human or bacterial genomic DNAs, miRNAs, and viral RNAs. The detection sensitivity of the RCA based methods are as low as femtomolar scale of target nucleic acids. Furthermore, those methods could discriminate the sequence of the target nucleic acids, even one or two bases could be discriminated by RCA. Therefore, RCA is expected to be an excellent point-of-care diagnostic tool for SNPs, miRNAs, and viral pathogens.

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