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**KEYWORDS:**  
circular probes, DNA diagnostics,  
PNA openers, strand  
displacement, rolling-circle  
amplification, topological  
labeling, single nucleotide  
polymorphisms

# Rolling-circle amplification in DNA diagnostics: the power of simplicity

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**Due to its robustness and simplicity, the rolling replication of circular DNA probes holds a distinct position in DNA diagnostics among other isothermal methods of target, probe or signal amplification. Major rolling-circle amplification approaches to DNA detection via posthybridization probe/signal turn-by-turn enhancement are briefly overviewed here with an emphasis on the new concepts and latest progress in the field, including the single-molecule and single-mutation detection assays as exemplary applications. Underlying mechanisms, current controversies and principal advantages of rolling-circle amplification are also considered. Possible future directions for the further advancement of this diagnostic methodology are outlined.**

*Expert Rev. Mol. Diagn.* 2(6), 542–548 (2002)

'The power of the world is always works in circles.'

Black Elk (Hehaka Sapa), a Holy Man of the Oglala Sioux American Indians

In the mid1990s, it was discovered that certain polymerase enzymes are able to effectively perform the rolling nucleic acid synthesis, most often termed rolling-circle amplification (RCA), using single-stranded (ss) DNA minicircles as templates (FIGURE 1A,B) [1–3]. In the case of DNA polymerases, the realization of this potential has soon led to the robust strategy for probe/signal amplification in DNA diagnostics [4–7]. It turned out that the RCA methodology may provide the laboratory researchers and clinical diagnosticians with highly sensitive and efficient contamination-resistant diagnostic assays that are capable of high multiplexity and can be used in a variety of testing formats. That is why the RCA-based approaches have recently been attracting attention of diagnostics-oriented biotech companies and research centers (TABLE 1).

The idea of RCA is based on the fundamental property of a circle, i.e. the endlessness of a circular line and topologically similar closed objects (FIGURE 2). For a ring of DNA copied by DNA polymerase, this feature of circles means that the polymerase enzyme, if initiated by a primer at some point on circular DNA, will

recurrently synthesize the linear replica of the DNA minicircle until the process is terminated by some reasons. Hence, the basic RCA 'recipe' for DNA diagnostics is quite simple: just extend the primer (could be a target or a secondary probe) hybridized to a DNA minicircle (primary probe) by its multiply repetitive replication (FIGURE 1A–C). As a result, long DNA repeats will be formed, that can readily be detected. Importantly, RCA has no need in special instrumentation to cycle the temperature, as it is required with the widely used PCR-based DNA diagnostics. Accordingly, more than billion-fold amplification can be achieved with certain RCA protocols at a single temperature in as little as 1–2 h, thus insuring ultrasensitive detection of specific DNA markers.

Due to its robustness and simplicity, RCA holds a distinct position in DNA diagnostics among other single-temperature amplification techniques. As compared with RCA, all other isothermal methods of signal, probe, or target DNA amplification, such as transcription-based system [8], strand-displacement approach [9,10], use of branched (or dendrimeric) probes [11,12], invasive-cleavage

reactions [13,14] or loop-mediated techniques [15,16], are rather complicated and in most cases they require prior assay optimization. On the contrary, the RCA-based assays do not normally require any substantial preoptimization of an experimental protocol and can readily be employed by a beginner. As a consequence, the RCA-based methods have been successfully used by several groups for DNA diagnostic purposes.

With dozens of publications on RCA, it is clearly beyond the scope of this review to fully cover all of materials published during almost a decade since RCA discovery. Instead, the focus will be placed on the major RCA approaches to DNA detection via posthybridization probe/signal amplification, giving emphasis to the new concepts and latest progress toward RCA diagnostic applications, outlining current controversies in the field and critically discussing basic RCA mechanisms. For a more comprehensive coverage of the RCA technology and related topics, see other reviews dealing with this subject [17–25].

### Basics of RCA in DNA diagnostics

There are two major priming approaches for RCA in DNA diagnostics: one uses a single primer, whereas the other employs a pair of primers. The single-primed, or linear RCA (FIGURE 1A–C) results in the monotonous rolling out of long, repeated sequences of DNA with a gradual accumulation of RCA products. These products generally exhibit a wide,

essentially continuous distribution over length and therefore cannot be resolved by gel electrophoresis yielding a broad smear of the high molecular weight DNAs (FIGURE 3B). As expected, they are mostly represented by ssDNAs commonly consisting of  $10^2$ – $10^3$  repeats of the complementary to minicircle sequence [3,4,32,33]. However, in some single-primed RCA reactions, dsDNAs predominate over ssDNAs and higher than usual, up to  $10^5$ -fold degree of amplification is observed [1,31,33]. Such a pseudolinear RCA could be a result of the self-primed ‘fold-back’ replicative DNA synthesis [1,3]. The single-stranded nature of products in case of linear RCA may be beneficial for certain subsequent manipulations with these DNAs and/or for their detection [4,33].

The double-primed, or geometric RCA (also called as hyperbranched [4], ramification [5], or cascade RCA [7]) operates with a pair of primers. One primer is complementary, as in the linear RCA, to a DNA minicircle, while the other is targeted to the repeated ssDNA sequences of the original RCA product [4,5,7]. Consequently, the geometric RCA proceeds as a turn-by-turn cascade of multiple-hybridization, primer-extension and strand-displacement events involving both primers (FIGURE 1D). As a result, a discrete set of concatemeric dsDNA fragments is formed yielding the distinct, ladder-type bands during gel electrophoresis (FIGURE 3C). In terms of the degree of amplification, the geometric RCA is much more potent, as compared with its linear alternative, yielding  $10^9$  or more copies of a circular sequence in 1 h [4,7,31]. Accordingly, it has the power to readily detect as little as ten probe molecules [4,7,34,35].

A number of DNA polymerases, including the thermophilic ones, are able to effectively perform the RCA reactions. Examples are  $\phi$ 29 DNA polymerase [4,6,33], Sequenase™ (Amersham Biosciences, USA) [1,3,31], Klenow [1,3,31,32] and Vent™ exo- [4,31] enzymes, and the large fragment of Bst DNA polymerase [4,7,34]. Some of these enzymes, like  $\phi$ 29 DNA polymerase and Sequenase, are most effective for performing the linear RCA, whereas others (e.g., Vent exo- DNA polymerase) are preferable in the geometric RCA. The RCA efficiency is normally increased by the addition of proteins that bind ssDNA – phage T4 gene-32 protein and *Escherichia coli* SSB protein are most used for this [4].

From the structural-topological viewpoint, several features of RCA reactions are unusual thus raising various questions [1,3,6,31,32]. The most important are:

- What is the actual shape of a circular template during the RCA processes?
- Do the RCA-capable polymerases have special abilities, such as the strand-displacement or duplex-unwinding (helicase) activity, allowing them to perform the rolling synthesis?
- To what extent could the topological and spatial constraints affect the RCA efficiency?
- Is there any sterical limit imposed on these processes by the reaction participants?

**Table 1.** Some biotech companies and research centers dealing with RCA diagnostics.

|                                        | RCA-related activity                                          | Websites                    |
|----------------------------------------|---------------------------------------------------------------|-----------------------------|
| Molecular Staging                      | RCA-based gene tests and immunoassays                         | www.molecularstaging.com    |
| Amersham Biosciences                   | SNP scoring and sequencing template preparation               | www.amershambiosciences.com |
| Hamilton Thorne Biosciences            | Single-cell analysis systems SNPs and gene expression studies | www.hamiltonthorne.com      |
| Cytogen                                | Prostate cancer tests                                         | www.cytogen.com             |
| Lawrence Livermore National Laboratory | RCA-based DNA/RNA clinical diagnostics                        | www.llnl.gov                |
| John Innes Centre                      | Ligation-based RCA diagnostics                                | www.jic.bbsrc.ac.uk         |
| CRC for Diagnostics                    | RCA-based SNP diagnostics                                     | www.crc.gov.au              |

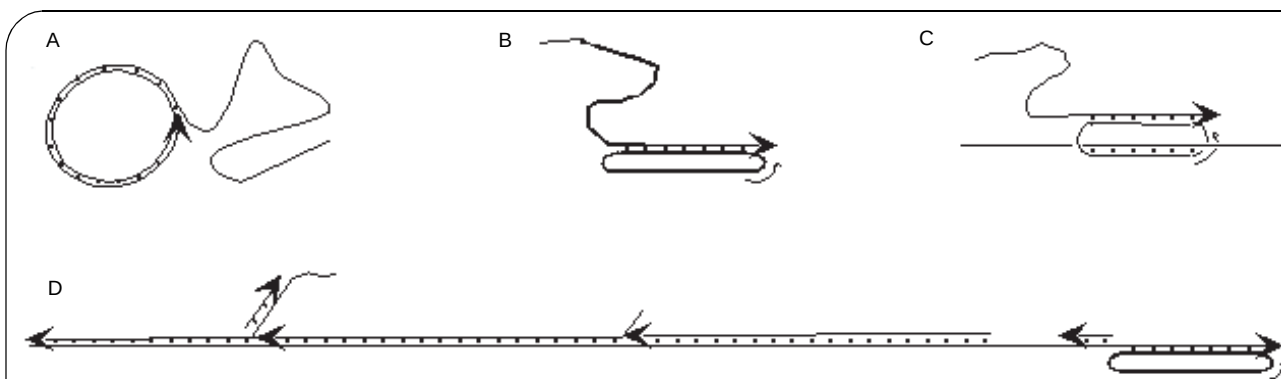


**Figure 1. Schematics of the RCA processes with an arrowhead symbolizing the DNA polymerase. (A)** Traditional phenomenological view of RCA going on a free DNA minicircle with the use of a single primer. If the DNA target is used to prime the RCA reaction, amplification products are fixedly linked to target molecules. For surface-attached targets, these products will be immobilized on solid-phase. **(B)** More realistic representation of the RCA-generating complex that resembles here a bow with a string. **(C)** RCA-based diagnostics of probe amplification with a target-unrelated primer. In some cases, the pseudo- or true topological linkages between a DNA minicircle and the marker/target DNA site may affect the rolling replication [6]. A circular probe should then be released from the DNA target following the hybridization. **(D)** Simplified (not in scale) representation of initial stages of the double-primed RCA (for more detailed schematics see [4,23]). In these reactions, the second primer is used, which is complementary to the original RCA product; DNA polymerases capable of strand-displacement synthesis are also necessary.

Few mechanistic studies were undertaken to shed a light on these problems [1,3,6,31,32,36]. It has been shown that particular RCA-active polymerases (e.g., Klenow enzyme) can effectively work on very small, down to 13 nt circular DNAs while other enzymes (like Sequenase) require relatively large,  $\geq 30$  nt DNA minicircles [3,32]. Our recent study also proved that certain DNA polymerases can efficiently carry out the replicative synthesis under true topological constraints allowing the detection of several hundred copies of a dsDNA marker without DNA denaturation and/or without the release of circular probes from the DNA target (FIGURE 3) [31]. This latest finding resolves the controversy on the possibility of the topologically-constrained RCA that is important for the use of circular probes in DNA diagnostics [4-7,19,23]. Furthermore, it provides the emerging field of RCA-based DNA diagnostics with the innovative DNA labeling system that offers enhanced hybridization specificity and could then be advantageous for the DNA mutation analysis.

FIGURE 1 presents one more, still unresolved RCA controversy in the correct description of the RCA mechanism. The commonly-accepted scheme of RCA process is shown in FIGURE 1A. It is widely used in phenomenological speculations assuming the continuous displacement of a newly synthesized DNA strand. However, given a small,  $\leq 100$  nt size of DNA minicircles used in RCA and the strong rigidity of dsDNA fragments significantly shorter than 150–200 bp [37-39], it is more reasonable to assume that the real geometry of the RCA complex resembles a bow with a string (FIGURE 1B) [1,3,32]. This model postulates that the RCA proceeds via the spontaneous fluctuational openings of a few end-located base pairs, thus allowing further rotation of DNA minicircles. Besides, no strand-displacement activity of the polymerase enzyme is required here. It is noteworthy after all that the geometric RCA (FIGURE 1D) does require the DNA strand displacement.

Yet, the mechanism of these reactions has not been studied in full and some of the key questions remain unanswered. Further experiments are necessary to completely clarify all unusual features of RCA reactions. Nevertheless, the practical potential inherent in RCA has already stimulated its pilot applications in DNA diagnostics, some of which are briefly overviewed below with an emphasis on the new concepts and latest progress in the field.

#### Recent developments & exemplary applications

The RCA robustness can be clearly seen in the recent pilot diagnostic applications of this methodology. First of all, it was convincingly demonstrated that the RCA-sensing capabilities could be optimized to readily detect down to a few target-specific circularized probes in a test sample both in solution and on the solid phase [4,7,34,35,40]. As a result, the RCA technology is well-suited to single-cell monitoring of rare DNA analytes [34,35], allows the unambiguous identification of DNA markers on excessive background of unrelated material [7] and can be exploited to visualize the single-molecule recognition events [4,41]. Furthermore, the RCA reactions exhibit an exceptional sequence specificity that is favorable for genotyping or mutation detection.

Single-base changes are the predominant form of genetic variations in humans (termed as single nucleotide polymorphisms [SNPs]) and their analysis has recently attracted a great deal of attention. It appears that the ligation-dependent RCA-based DNA diagnostics have the requisite single-base specificity to be a valuable tool for detection of point mutations [4,7,31,33,42].

Accordingly, it was reported that RCA allows the detection of single-base mutations in cytological preparations, with an efficiency of up to 90% if cells were pretreated by restriction enzymes and exonucleases [43]. In another recent study, it was shown that a combination of RCA with PCR

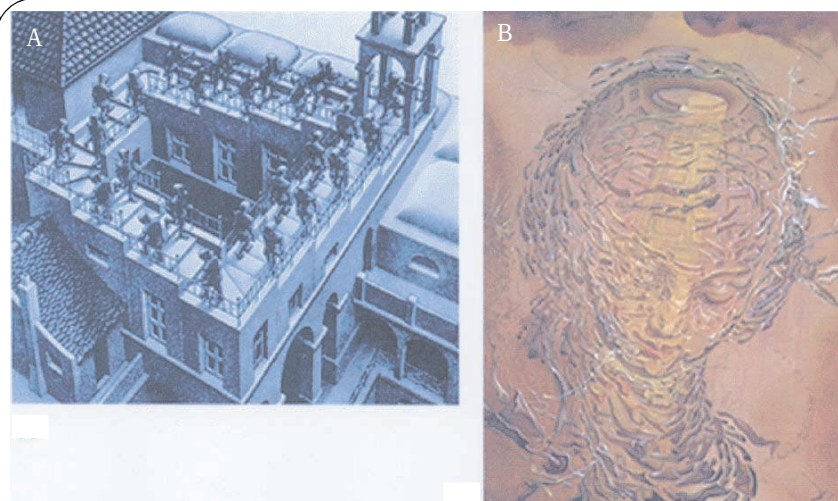


**Figure 2. The infinity of ring-shaped objects** inspired not only those scientists who came up with the RCA idea, but also some artists like Maurits Cornelis Escher and Salvador Dalí. Their paintings may serve as intelligible illustrations of RCA principles. **(A)** Escher's 'Ascending and Descending' (fragment; 1960) shows the closed staircase going on endlessly up or down, depending on a viewpoint. Starting at some place on these stairs, one will interminably turn around similar to the RCA-performing enzyme that reads a circle of DNA over and over again. **(B)** Dalí's 'Exploding Raphaelesque Head' (1951) portrays the hollow spherical object resembling a female head that blows up into the numberless similar fragments rotating around some central point. These fragments are analogous to the RCA products consisting of multiply repeated DNA sequences.

and LDR (ligation detection reaction) makes it possible to discriminatory analyse the low-abundance hotspot somatic mutations on universal multiplex oligonucleotide microarrays [44]. The authors of this work note that such a system is readily adaptable to high-throughput robotization. RCA was also used to simultaneously discriminate and multicolor visualize wild type and mutant alleles for the number of disease-causing genes in stretched DNA fibers or directly in cultured cells [45]. These evaluation studies demonstrate that RCA can successfully be applied *in situ* within cell-based assays and clinical diagnostics not only for the analysis of somatic mutations, but also for direct physical haplotyping.

To this end, it was demonstrated that RCA provides the means for cost-effective, fully automated and high-throughput SNP genotyping [46–49]. The integration of high-fidelity ligation of allele-specific circular probes with subsequent ultrasensitive FRET-based RCA detection allows to simultaneously genotype ten SNPs directly from 1 ng of genomic DNA in a set of approximately 200 samples [48]. Computerization of probe design and data analysis eliminates the problems with nonspecific background signal related to primer artifacts or to the amount of probe used in the assay [49]. These developments promise to further speed up the SNP analysis and to make it more accurate.

Potentially, the peptide nucleic acid (PNA)-assisted topological DNA labeling shown in FIGURE 3 should provide such analyses with much higher selectivity than the conventional padlock-based techniques [31]. Indeed, the multiple-probe coincidence hybridization intrinsic in this approach accompanying

with a 'check-over' by a DNA ligase essentially warrant zero tolerance of that novel DNA labeling scheme to the single-base, probe-target mismatches [26,27]. Moreover, the SNP screening can now be performed without DNA denaturation. In addition, the PNA-assisted assay has one more substantial advantage: the probe is much more stably bound to the target site and its movement along the target molecule is totally restricted, thus keeping the circular probe at a precise position on the DNA double helix [29].

#### Expert opinion & five-year view

Until recently, PCR was the primary technique of DNA amplification in the area of DNA diagnostics and it still remains one of the most useful methods for DNA analysis. Nevertheless, the PCR-based diagnostics have some substantial drawbacks, including high cost, low multiplexity, insufficient sensitivity in the single-mutation detection, proneness to amplification errors due to cross-contamination, problems with the *in situ* applica-

tions and finally, the need for high-precision temperature cycling. All these PCR limitations strongly justify a quest for robust isothermal alternatives.

As shown above, RCA is a very efficient single-temperature DNA amplification technique, being essentially free of all aforementioned shortcomings. The RCA methodology has several advantages that are summarized in the Key issues box and could be called 'the power of simplicity'. There is no doubt that these advantages should help RCA to successfully compete with its widely-used nonisothermal predecessor (PCR) for the number of diagnostic applications. For example, I expect that RCA will become the method of choice for scoring SNPs and to identify biomarkers associated with certain somatic disorders. Besides, it may be of practical use for reliable pathogen detection at the molecular level thus helping to combat the germ-related diseases and preventing biothreat. In this connection, it is very likely that the PNA-assisted topological labeling of dsDNA by earring-like probes will be adapted for both RCA-based genotyping and pathogen detection as a potent diagnostic approach featuring the exquisite sequence specificity [31].

Although the following topics have not been considered in this review, I also expect that RCA could be very effective in amplifying large stretches of DNA, including recombinant plasmids [50], as well as in protein functional analysis and expression profiling [51]. Accordingly, I believe that the RCA-based diagnostics will soon solidly enter both research and clinical laboratories, becoming the routine molecular biological assays. Furthermore, I am hopeful that the new RCA-related approaches to gene therapy could be used to efficiently shut down disease-causing genes [52].

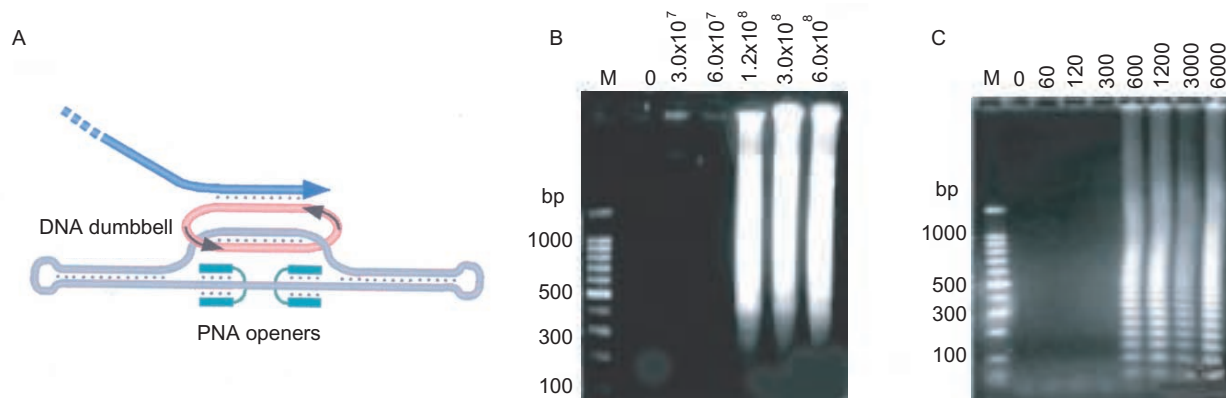


**Figure 3.** RCA in the truly topologically linked setting. (A) The DNA catenane used in this study. It is comprised of the linked to each other DNA minicircle (dubbed earring probe [26,27]) and large DNA dumbbell [28]. The assembly is facilitated by two bis-PNAs (PNA openers [29,30]). Shown in blue is a primer (and the corresponding RCA product) hybridized to the earring probe. For simplicity, only the single-primed RCA is presented here. (B,C) Analysis of RCA products by nondenaturing agarose gel electrophoresis. The single-primed pseudolinear RCA (B) was performed by Sequenase 2.0™ at 37°C. The double primed geometric RCA (C) was performed by Vent exo<sup>-</sup> polymerase enzyme at 62°C. A conservative site from the HIV-1 *nef* gene served as a target for the PNA-assisted labeling of duplex DNA with a circular probe. The RCA products were generated starting from different initial inputs of catenated construct given in number of molecules above the corresponding lanes. Lanes M correspond to a 100 bp size marker. For experimental details see [31]. M: Molecular weight marker; PNA: Peptide nucleic acid; RCA: Rolling-circle amplification.

To fulfill this promise, persistence in basic studies of constrained RCA reactions is desirable to fully unravel their mechanisms and to reveal the key factors affecting the efficiency of these processes. A search for more robust, in terms of fidelity, processivity and wider range of working conditions, the RCA-active polymerase enzymes is also needed. All this should open ways for design of new potent RCA-based DNA diagnostics and will allow us to understand the mechanism of rolling and strand-displacement nucleic acid syntheses in general.

#### Acknowledgments

Heiko Kuhn offered helpful comments to the proof of this manuscript. Supported by the Provost's Innovation Fund (PIF) and Special Program for Research Initiation Grants (SPRInG) from Boston University (MA, USA) and the National Cancer Institute (MD, USA) (grant #CA89833).

#### Information resources

- Molecular staging (Viewed October 2002) [www.molecularstaging.com](http://www.molecularstaging.com)
- Hamilton Thorne Biosciences (Viewed October 2002) [www.hamiltonthorne.com](http://www.hamiltonthorne.com)

#### Key issues

- Rolling-circle amplification (RCA) technology enables the amplification of the probe DNA sequences more than  $10^9$ -fold both in solution and on the solid phase at a single temperature. It has the ability to readily detect down to a few target-specific circularized probes in a test sample.
- The capacity of RCA to yield the surface-bound amplification products offers significant advantages to *in situ* or microarray hybridization assays.
- RCA reactions exhibit an excellent sequence specificity that is favorable for genotyping or mutation detection and allows to unambiguously identify DNA markers on the excessive unrelated background.
- If combined with the peptide nucleic acid (PNA)-assisted topological labeling of dsDNA by earring-like probes, RCA has the potential for a highly localized isothermal detection of the designated sites on essentially intact duplex DNA with even superior specificity.
- The simplicity and efficiency of RCA technology, along with the ease and accuracy of quantitation, makes it amenable for miniaturization and automation in high-throughput analysis.
- As compared with PCR, the RCA-based DNA diagnostics are capable of higher multiplexity and they are less prone to amplification errors, thus allowing contamination-resistant detection of target molecules in a variety of testing formats.

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