Rolling-Circle Amplification (RCA)

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INTRODUCTION

The rolling-circle amplification (RCA) reaction is an isothermal in vitro method for the hybridization-triggered enzymatic synthesis of hundreds to billions of linear copies of small, single-stranded, circular DNA probes. Thus, obtained long repeats of DNA sequences may serve as a signal amplifier for ultrasensitive detection of specific nucleic acids and other biologically important molecules in diagnostic genomics and proteomics. Depending on the goal and/or target molecule to be detected, RCA is performed using a variety of protocols and testing formats with either linear or exponentially branching kinetics. Because of their robustness and simplicity, the RCA-based assays hold a distinct position in the area of molecular diagnostics among other single-temperature amplification techniques.

RCA BASICS

RCA is based on the rolling replication of short, single-stranded DNA circles by certain DNA polymerases at constant temperature, the process discovered in the mid-1990s.[1,2] This reaction is initiated by the hybridization of a linear DNA single strand to a specific DNA minicircle, and it is widely used for diagnostic purposes in direct or indirect detection of different DNA/RNA, protein, and other biomarkers via a set of various biomolecular recognition events. A similar reaction was described for RNA polymerases as well,[3] but the RNA-generated process does not require any hybridization-dependent priming (or even promoter sequences). Therefore, the latter is only used to produce functional RNA sequences, such as RNA ladders and self-processing ribozymes.

Description of Method

Types and major features of RCA reactions

In its original formulation,[1,2] the RCA reaction involves numerous rounds of isothermal enzymatic synthesis in which DNA polymerase extends a circle-hybridized primer by continuously progressing around the circular DNA probe of several dozen nucleotides to replicate its sequence over and over again (Fig. 1A). This process is characterized by linear kinetics, easily yielding in one hour up to several thousands of sequence-complementary tandem repeats of an original DNA minicircle. These amplification products generally exhibit a wide, essentially continuous distribution over length and are normally seen in gel-electrophoretic images as a broad smear of high-molecular weight DNAs (Fig. 2, lane 2). The single-stranded nature of amplicons in case of linear RCA may be beneficial for subsequent manipulations with these DNAs towards their detection.[4]

A more complicated version, the double-primed RCA, called hyperbranched,[4] ramification[5] or cascade RCA,[6] operates with a pair of different primers. One primer is complementary, as in the linear RCA, to a DNA minicircle, whereas the other is targeted to the repeated, single-stranded DNA sequences of the primary RCA product.[4–6] Consequently, the double-primed RCA proceeds as a chain reaction with geometric kinetics featuring a ramifying cascade of multiple-hybridization, primer-extension, and strand-displacement events involving both primers (Fig. 1C). As a result, a discrete set of concatemeric double-stranded DNA (dsDNA) fragments is formed, yielding the distinct, ladder-type gel-electrophoretic bands (Fig. 2, lane 3).

Sometimes, this reaction produces amplicons so long that they cannot move through the gel during common gel electrophoresis (see lane 4 in Fig. 2). In terms of the degree of amplification, the geometric RCA is more potent, as compared with its linear alternative, yielding 10^9 or more copies of a circular sequence in about an hour.[4,6] Accordingly, it enables a bioanalyst to readily detect just a few probe molecules.[4–8] The double-primed RCA is usually performed at elevated temperatures by thermostable DNA polymerases, although in some cases it is performed by common polymerase enzymes at physiological or ambient temperatures.

Practical RCA formats

Generally, the RCA-based diagnostics can be classified into two groups: some of them operate with the preformed circular probes [Fig. 1D and E for the peptide nucleic acid (PNA)-assisted, nick-induced RCA and immuno-RCA,

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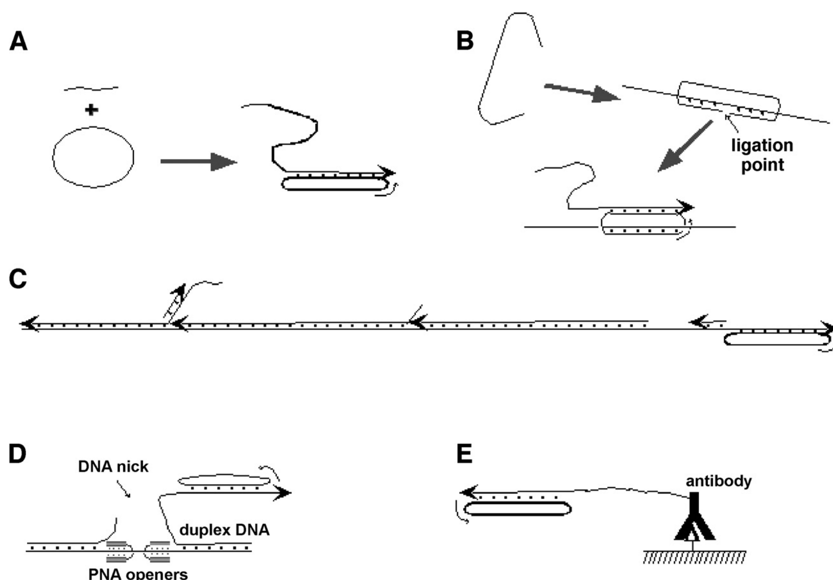

Fig. 1 Schematics of different RCA processes; the arrowhead symbolizes the DNA polymerase. Given a small, ≤100-nt size of DNA minicircles used in RCA and the strong rigidity of dsDNA fragments with these lengths, only part of the circular probe can be base-paired at any given time. Consequently, the geometry of RCA-generating complexes resembles a fiddlestick. (A) The RCA reaction carrying 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 (see schematics D, for example). For surface-attached targets, these products will be immobilized on the solid phase. (B) RCA-based diagnostics of probe amplification with an in situ circularized linear oligonucleotide probe and a target-unrelated primer (ligation–RCA/L–RCA). In some cases, the topological linkages between a DNA minicircle and the marker/target DNA site may affect the rolling replication. A circular probe should then be released from the DNA target following the hybridization. (C) Initial stages of the double-primed RCA.[4–6] In these reactions, the second primer, which is complementary to the original RCA product, is used. Here, the DNA polymerases capable of strand-displacement synthesis are necessary. (D) The RCA reaction, which proceeds on dsDNA if assisted by PNA openers and DNA nicking.[14] (E) In immuno-RCA, the 5′ end of a primer is attached to a reporter antibody, which selectively binds to a test analyte immobilized on a solid surface.[7]

as examples], whereas others involve the circularization of hybridized linear probes by ligation followed by RCA (L-RCA; Fig. 1B). In the latter case, the in situ assembled DNA minicircles, called padlocks and earrings,[9–13] provide the corresponding DNA/RNA diagnostic assay with a higher sequence specificity, which is warranted by a multiple (at least dual) probe–target recognition and is also owing to the fact that mismatches close to the ligation point severely interfere with the ligation process. Importantly, padlocks and earrings ensure a higher stability of hybridization complex because of additional topological stabilization through the probe–target concatenation.[13] All this improves the hybridization stringency, allows one to more efficiently distinguish single-base sequence variants, and results in a highly localized hybridization/amplification signal retaining the positional information.

Further localization of the RCA-generated signal to essentially single visible points can be reached by condensation of amplication products after their hybridization to labeled oligonucleotides, known as RCA-CACHET.[4] In this way, the RCA amplicons are tagged with fluorescent labels at multiple sites in the tandem RCA-amplified DNA sequence. Thus ‘‘decorated’’ amplicons can be compacted into tiny objects by cross-linking with multivalent proteins, such as streptavidin and antibodies, which bind to amplicon-incorporated tags. If necessary, further increase of the RCA-generated signal to a superexponential level can be achieved by combining the RCA and PCR reactions.[5] Until recently, the RCA reactions have been run only on the single-stranded DNA and RNA targets, but, with the aid of PNA openers, these reactions can now be performed with dsDNA.[12,14] In immuno-RCA assays, the attachment of a reporter antibody to an RCA primer makes it possible to extend the RCA-based diagnostics on the non-nucleic-acid analytes, including proteins, which can be detected with superior sensitivities, compared to conventional enzyme immunoassays in ELISA and microparticle formats.[7]

The RCA-based analyses can be executed both as homogeneous assays in solution and as heterogeneous ‘‘on surface’’ assays, including the microtiter plate and microarray approaches for high-throughput genomics and

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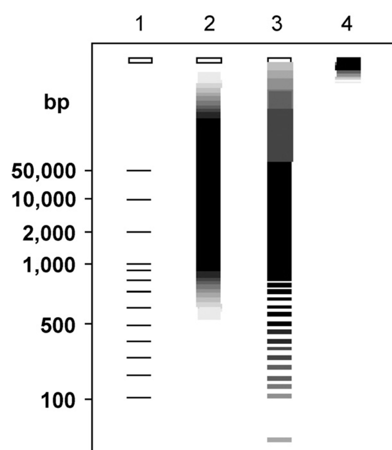

Fig. 2 Typical patterns of the RCA products generated with linear or geometric kinetics and resolved by gel electrophoresis (SYBR green or ethidium bromide staining). Usually, linear amplification is characterized by smearlike amplicons corresponding to essentially continuous distribution of single-stranded RCA products over length (lane 2). On the contrary, ladder-like amplicons are normally observed after geometric amplification representing linear concatemeric double-stranded copies of a circular template (lane 3). In some cases, most of the RCA products are so large that they cannot enter the gel (lane 4). Lane 1 corresponds to the size marker.

proteomics studies.[15,16] The RCA amplicons can be detected in several ways, such as fluorescence,[7] radiolabeling,[10] UV absorbance, and gel electrophoresis[4,12] by using either direct incorporation of various labels into the RCA products[10] or label-decorated amplicons.[4,7] Since it is not possible to even briefly describe this whole diverse range of RCA testing formats, the interested reader is referred to recent reviews[17,18] and corresponding original papers.

Specificity, sensitivity, and reproducibility

The RCA-based diagnostics exhibit an exceptional specificity for particular DNA/RNA sequences, as well as for marker molecules other than DNA/RNA, allowing the multiplex genotyping/detection of single-base mutations and specific antigens. Besides, RCA is an ultrasensitive method of detection: a variety of RCA formats permit essentially single-molecule counting of the DNA, RNA, or protein targets and some other analytes. Furthermore, RCA-based diagnostics are characterized by good reproducibility, with amplification errors being at a lower level compared to PCR. Consequently, such an exquisite sensitivity makes it possible to accurately and reliably quantify the gene copy number as well as to detect single-copy genes, discrete antigen–antibody complexes, and mRNA expression levels in individual cells.

Advantages and Limitations

RCA has several substantial advantages over other amplification techniques, which could be called ''the power of simplicity.''[17] Most importantly, RCA is an isothermal procedure and, therefore, has no need of special instrumentation to cycle the temperature, which is required with the widely used PCR-based diagnostics. This RCA feature significantly simplifies the automation and miniaturization of RCA-based diagnostics. In addition, RCA can be performed by a larger variety of DNA polymerases compared to PCR, which relies on only thermostable enzymes. Besides, RCA represents an inexpensive, more error-proof, and more sensitive (compared to PCR) analytical technology with a very wide dynamic range and higher multiplexity to serve as a potent alternative to the thermocycling diagnostic methods.

In comparison with other isothermal methods of signal, probe, or target amplification, such as transcription-based amplification, strand-displacement amplification, use of branched (or dendrimeric) probes, invasive signal amplification, or loop-mediated amplification, the RCA-based assays are less complicated and in many cases do not require any substantial preoptimization of an experimental protocol, thus being readily used by a beginner. RCA is the most flexible and adaptable amplification methodology featuring merely few drawbacks. The shortcoming of supersensitive RCA assays is that they require certain caution to avoid possible contamination/false positives. In some cases, release of probes from the hybridized targets and removal of the nontargeted DNA strands by additional treatment with endo- and exonucleases are also necessary to reach the requisite sensitivity of detection.[10,19]

CLINICALLY RELEVANT PILOT APPLICATIONS

The practical RCA potential to identify nucleic acid targets, antibodies, and antigens in clinical samples has recently been demonstrated in several feasibility studies. Specifically, the RCA-based protocol for an automated scoring of single nucleotide polymorphisms (SNPs) in a set of human genomic DNA samples with the nanogram sensitivity was developed.[15,20] Highly sensitive multiplex detection of hotspot somatic mutations present at very low abundance was also reported.[21] The RCA capability for pathogen diagnosis was convincingly proved by comparative study of RCA vs. PCR and ligase chain reaction (LCR) in detection of *Chlamydia trachomatis* in cervical specimens.[22] The workability of immuno-RCA for identification of allergen-specific immunoglobulins in samples from patients was shown in a microarray format.[23] In addition, RCA has been

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adapted to immunohistochemistry, flow cytometry, and in situ hybridization to significantly improve their sensitivity without compromising cellular and tissue morphology.[24,25] RCA-mediated multiplex profiling of cytokines on microarrays with femtomolar sensitivity offers an advantageous approach for proteomic surveys.[16] All these pilot applications establish the firm basis for future clinical use of RCA methodology in a range of diagnostic applications, enabling the integration of genomic and proteomic information into cell- and tissue-based tests.

CONCLUSION

The RCA methodology represents a powerful and simple procedure for signal amplification that may serve as universal platform for in vitro diagnosis of a variety of biomarkers based on either nucleic acid sequence or antigenicity. Consequently, this technology should soon provide the laboratory researchers and clinical diagnosticians with highly sensitive and efficient customary diagnostic assays to expedite and to facilitate testing of miscellaneous analytes.

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ARTICLE OF FURTHER INTEREST

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