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NOVEL ISOTHERMAL NUCLEIC ACID AMPLIFICATION METHOD: RAMIFICATION AMPLIFYING METHOD
(A NARRATIVE DESCRIPTION)

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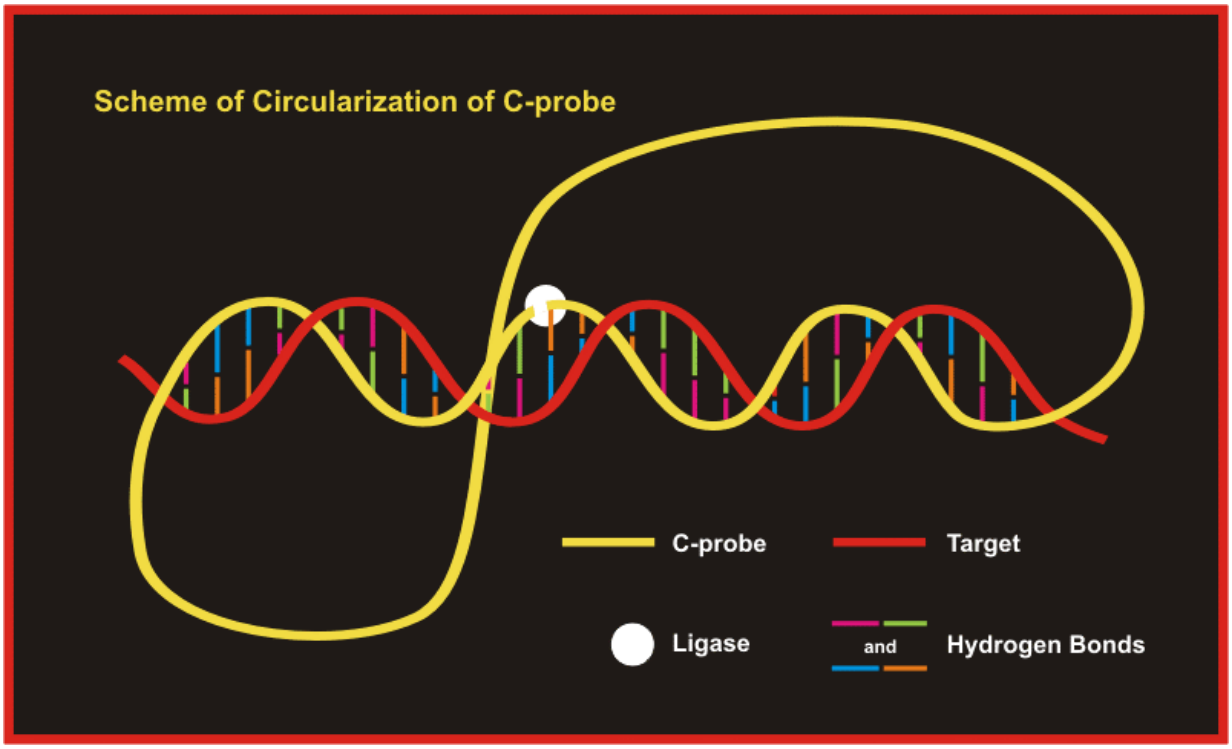
Many techniques have been developed to meet the demand of rapid and accurate detection of abnormal genes or infectious pathogens. Nucleic acid amplification techniques, including polymerase chain reaction (PCR), ligase chain reaction (LCR), strand displacement amplification (SDA), and transcription-based amplification (TBA) are powerful tools for the detection of minute pathogenic sequences. However, all of these techniques suffer many problems. For example, an expensive thermocycler is required for amplification (PCR and LCR). Another example is that amplification templates differ with different target sequences (especially PCR and SDA), thus, amplification conditions vary with each test (that is, no single optimal condition). This is especially critical for the quantitation of target DNA/RNA by competitive PCR and for the simultaneous detection of multiple targets (multiplex amplification). Furthermore, *in situ* amplification and detection is extremely difficult with this technique because problems occur with heat distortion of morphology, diffusion of small amplicons, and poor reproducibility.

Ramification Amplification Method

We are developing an alternative amplification scheme that avoids problems related to thermocycling and to make assays easier and more practical for the clinical laboratory. Our approach is to combine magnetic bead technology for isolation of target nucleic acids from specimens with the target-dependent ligation to generate functional probe and the isothermal probe amplification to achieve a simple, practical amplification technique. We termed this technique ramification-extension amplification (RAM) because the amplification power is derived from primer extension, strand displacement, and multiple ramification (branching) points (Zhang et al. Gene 211: 277-285, 1998).

Target nucleic acids (DNA or RNA) are isolated through DNA capture probes which contain a 3' sequence complementary to a target and a biotin moiety at their 5' ends capable of interacting with streptavidin coated paramagnetic beads (Figure 1). After lysis of cells in GTC, which inactivates pathogens, hybridization of probe to target can be accomplished without prior isolation of target nucleic acids, therefore, eliminating the potential for infection of laboratory personnel and reducing length of assay. Capture of the target and its associated probe onto a magnetic bead allows for stringent washes to remove non-target DNA and RNA, as well as inhibitors that would inhibit subsequent enzymatic reaction. The capture probe can be sequenced specific, for capturing particular targets, or generic (oligo(dT)), to capture all cellular mRNA. Therefore, the capture probe can selectively isolate DNA or RNA, making the assay more specific. Furthermore, magnetic bead technology allows for simple, high throughput process of clinical specimens (Hsuih et al, J Clinical Microbiol, 34:501-507, 1996).

The amplification is achieved by utilizing a synthetic circular DNA probe (C-probe) (Figure 1). This probe contains three regions: a 5' region (25 nucleotides); a 3' region (25 nucleotides) complementary to the target sequence at an adjacent position; and a linker region (59 nucleotides) that is composed of a generic sequence for RAM primer to bind. Once the C-probe binds to the target, the 5' and 3' regions are brought to juxtaposition. A covalent circular molecule (ccDNA) can then be generated by incubating with a DNA ligase (T4 DNA ligase or *Taq* DNA ligase). Introducing ligation into the RAM system increases the assay specificity since no ligation could occur unless both 3' and 5'ends hybridize to the target in a perfect alignment (Hsuih et al, J Clinical Microbiol, 34:501-507, 1996). This stringent requirement makes detection of a single nucleotide mutation possible.

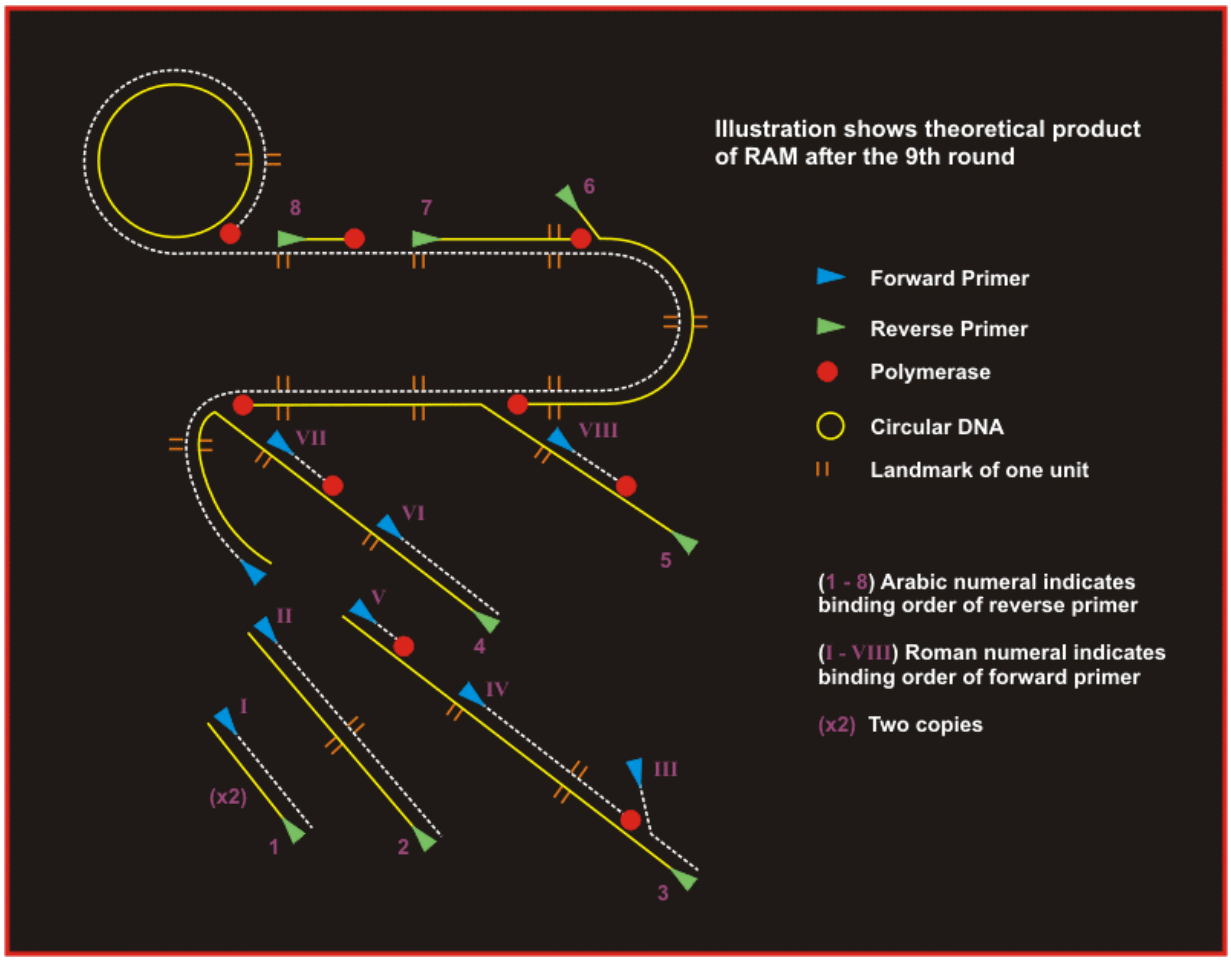


After ligation, multiple forward primers (F-primers), reverse primers (R-primers), and DNA polymerase are added directly to the reaction. F-primers bind to the linker region of ligated C-probe (Figure 1) and can be extended by phi 29 DNA polymerase. Once the DNA polymerase reaches to the downstream F-primer binding sites, the primers and their extended sequences are displaced by the polymerase. Therefore, multiple long ssDNAs, each about 8 Kb in length and containing about 80 units (100 bases per unit), can be produced by continuously rolling over the ligated circular DNA. Multiple reverse primers (R-primers), which are identical to the linker region of the C-probe, bind to the growing ssDNAs. While extending from each R-primer, the earlier bound downstream R-primer with its extended sequence is displaced from the template by the sequences extended from upstream primer. The displaced ssDNA can then serve as a template for further primer extension reaction; thus, a multiple ramification molecule is produced. Since the displaced DNAs are single stranded, RAM primers can bind to them at a consistent temperature, obviating use of a thermocycler. The reaction comes to an end when all ssDNA become dsDNA where no new primer can bind to it.

If one F-primer and one R-primer are included in the RAM reaction, exponential growth occurs. A mathematical formula is developed to express the reaction:

$$2^U$$

where **U** stands for the number of rounds of extension made. This formula indicates that the ramification-extension amplifies in an exponential fashion. Thus, a million-fold amplification of the circular probe sequence can be achieved in 1 hour under an isothermal condition (the extension rate for DNA polymerase is about 400-1,000 nucleotides/minute). A limiting factor for this reaction is the length of initial ssDNA generated from the ccDNA. In order to achieve million-fold amplification, initial ssDNA should be 20 units in length (about 2,000 bases). For billion-fold amplification, a 30-unit ssDNA (about 3,000 bases) is required. This formula shows that RAM has the same amplification power as other amplification techniques, such as PCR. In summary, the essence of the RAM assay is that it combines the specificity of ligation-dependent gene detection and the powerful nucleic acid amplification technique. This combination compensates for the inadequate sensitivity of the direct ligation-dependent detection method and the low specificity of the LCR.



Advantages

This system has many advantages over above-mentioned techniques.

1. This system utilizes the extension and displacement properties of DNA polymerase. Therefore, one enzyme can carry out both extension and ramification. Million-fold amplification can be achieved in a short period under an isothermal condition (either 37°C or 60°C depending on which enzyme is used).
2. Generic extension primers are used for all target nucleic acids (i.e., all infectious agents or mutated mRNAs); the primer-binding sequence (linker region) is identical in all circular probes independent of hybridization region (5' and 3' regions). As a consequence, detection of multiple infectious agents from the same specimen becomes possible.
3. Because primer extension and ramification generates larger polymers (>8,000 bp), it does not require thermal cycling. It is an ideal method for *in situ* amplification. RAM provides intra-cell localization and cell morphology is preserved during *in situ* amplification and detection of DNA or RNA (Park et al. Am J Pathol 149:1485-1491, 1996).
4. All three steps (target capture, RAM reaction and detection) can be carried out on same solid support, such as dipstick or latex beads. Therefore, simple assay formats can be developed for field tests, doctors' offices, or clinical diagnostic laboratories.

In addition, this system also offers:

1. With an introduction of the magnetic (or micro-plate) separation technique, isolation of the target template becomes easier and faster; isolation, amplification, and detection can be carried out in the same vessel. All noxious chemicals and inhibitors will be removed by extensive washes (Hsuih et al, J Clinical Microbiol, 34:501-507, 1996).
2. Introducing ligation into the system adds another level of specificity, i.e., only properly aligned C-probes will be ligated. This property is especially useful for diagnosing single base mutations, such as in sickle cell anemia or single mutations in an oncogene.
3. Its use can be extended by combination with other technologies, such as DNA chips (amplification on solid surface) and antibodies to detect antigens.
4. Since DNA ligase can work on both DNA-DNA hybrids and DNA-RNA hybrids, both DNA and RNA targets can be detected. In addition, no reverse transcription is required in this system, thus avoiding RNA structurally related problems.

Application of the Invention

This system is a novel nucleic acid amplification system with many unique features. It can be readily used in clinical laboratories for the detection of genes and infectious agents in various areas, such as hematology, oncology, infectious disease, pathology, forensics, blood banks, and genetic disease. In addition, it has great potential for use in field tests and doctors' offices due to its simple and isothermal amplification format.