**Basics and Advances in Recombinase Polymerase amplification for detection of Phytopathogens: An Alternative to PCR**

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**ABSTRACT**

The area of life sciences has been significantly impacted by the widespread use of nucleic acid amplification techniques, particularly since the development of the polymerase chain reaction (PCR) method in 1983 (Mullis *et al*., 1986). However, despite its extensive capabilities, the utilization of PCR has been limited to laboratory settings due to the necessity of a complex thermocycling equipment, hence restricting its practical implementation in resource-limited environments. Li *et al*. (2019) have proposed novel isothermal amplification methodologies that aim to transcend conventional laboratory limitations by enabling nucleic acid replication at consistent temperatures. Among the several techniques available, recombinase polymerase amplification (RPA) has emerged as a rapidly evolving technique, witnessing significant adoption and commercial growth, although being launched relatively late in comparison. The technique of RPA enables the amplification of nucleic acids in a manner that is both easily accessible and sensitive, even outside of traditional laboratory settings. This has significant implications for self-testing purposes. Recombinase polymerase amplification (RPA) is an isothermal amplification method known for its high sensitivity and selectivity. It operates within a temperature range of 37-42ºC and requires little sample preparation. RPA is capable of amplifying DNA target copies as low as 1-10 within a short time frame of less than 20 minutes (Piepenburg *et al*., 2006). The technique has been employed to enhance a range of targets, including as RNA, miRNA, single-stranded DNA (ssDNA), and double-stranded DNA (dsDNA), originating from a varied array of species and materials. There has been a noticeable rise in the number of papers documenting the utilization of RPA, with amplification being conducted in solution phase, solid phase, and bridge amplification format (Li *et al*., 2019). In addition, Lobato and O'Sullivan (2018) have reported effective integration of RPA with several detection methodologies, including end-point lateral flow strips and real-time fluorescence detection, among others.

**Introduction**

The main objective of the plant pathology is the management of disease, for this it is critical to detect the plant pathogens at their earlier stages. Traditionally, plant pathogens are detected on the basis of morpho-cultural characteristics, which is time consuming and laborious process with less sensitivity and specificity. In order to avoid this time consuming and laborious process various molecular approaches have been developed based on nucleic acid amplification. The process of nucleic acid amplification, which involves the realistic proliferation of genetic material, has become widely utilized in various fields of life sciences and biotechnology. These include but are not limited to genotyping, drug discovery, cancer research, food testing, genetic engineering, sequencing, lifestyle testing, synthetic biology, molecular archaeology, mutagenesis, wellness, cloning, pathogen detection, and forensic identification of crimes (Li *et al*., 2019). The inception of the transformative revolution may be traced back to the pioneering work of Kary Mullis, who introduced the polymerase chain reaction (PCR) in 1983 (Mullis *et al*., 1986). The authors of the study conducted by Li *et al*. (2014) propose novel isothermal amplification methodologies that aim to transcend conventional laboratory limitations by enabling nucleic acid replication at consistent temperatures. The most widely recognized techniques in the field include multiple displacement amplification (MDA), rolling circle amplification (RCA), signal-mediated amplification of ribonucleic acid (RNA) technology (SMART), strand displacement amplification (SDA), recombinase polymerase amplification (RPA), loop-mediated isothermal amplification (LAMP), nucleic acid sequence based amplification (NASBA, also referred to as transcription mediated amplification, TMA), and helicase-dependent amplification (HDA). Among the several techniques available, RPA has emerged as a rapidly advancing technique, gaining significant traction and commercial presence despite being launched relatively late in the field.

**Recombinase polymerase amplification**

Recombinase polymerase amplification (RPA) was first of all developed by Piepenberg *et al* in 2006. RPA is highly sensitive and specific isothermal amplification process operating at a constant temperature of 37-42˚C for less than 20 minutes (Lobato and Oˈ Sullivan, 2018). Piepenburg et al . in 2006 defined RPA as the “DNA detection by using recombination proteins”.

**RPA mechanism**

The RPA reaction starts by binding of forward and reverse primers with recombinase protein, forming recombinase-primer complex. This complex scans the double stranded target DNA in search of homologous/cognite sites on template DNA. Once it locates the homologous site, it invades the dsDNA and displaces one strand of DNA from other at a specific position and results in formation of D-loop. The displaced strand is stabilized by a protein known as single strand binding protein, to prevent the ejection of primers. After binding of primers to homologous sites, recombinase disassembles itself from the complex and starts new reaction with new primers. Bsu/Sau DNA polymerase binds with the 3ˈ OH group of primers to intiate the elongation. The parental DNA strands continue to separate as the polymerization continues, as a result exponential amplification occurs (Li *et al*., 2019).

**Reaction components of RPA**

The RPA process involves three essential proteins: the recombinase protein (T4 UvsX protein), the recombinase loading factor (T4 UvsY protein), and the single strand binding protein (T4 gp32). These proteins serve as substitutes for the conventional heat denaturation step in polymerase chain reaction (PCR). The proteins involved in the RPA reaction process, as described by Li *et al*. (2019), interact with several additional components such as DNA polymerase, crowding agent, energy/fuel components (e.g., adenosine triphosphate, ATP), and salt molecules. The table below provides information on the function and usual concentration of reaction components.

|  |  |  |
| --- | --- | --- |
| Reaction components | Typical concentration | Functions |
| T4 UvsX protein | 120 ng/μL | Pairing and strand transfer activity |
| T4 UvsY protein | 60 ng/μL | T4 UvsX single-stranded DNA-dependent ATPase activity is stimulated, and the critical concentration of T4 UvsX required for action is reduced.. |
| T4 gp32 | 600 ng/μL | Stablization of complementary strand |
| Bacillus subtilis DNA polymerase I (Bsu) or Staphylococcus aureus polymerase (Sau) | Bsu: 30 ng μL−1 ; Sau: 8.6 or 12.8 μg | Synthesis of new DNA templates. |
| Carbowax 20M (a high molecular weight polyethylene glycol (PEG) | PEG 35K (5%) | Enhance the catalytic activity of enzymes. |
| Phosphocreatine  Creatine kinase ,ATP | 50 ng μL−1  100 ng μL−1 | Energy source to recombinase and DNA polymerase to carryout the reaction. |
| Tris(hydroxymethyl) aminomethane(Tris) | 50 mM (pH 7.9) | In solution, consolidate and dissolve the DNA. |
| Potassium acetate  Magnesium acetate | 100 mM | Cofactor needed to support enzyme performance. When magnesium acetate is introduced, the RPA reaction begins. |

**RPA OPERATING PARAMETERS**

* **Primers:** Primers that are used in RPA should be 32-35 base-pairs in length (Mayboroda *et al*., 2015). Primers of longer length i.e., upto 45 bases can also be used, but they result in secondary structures and primer artefacts. It is recommended that GC content of primers and template should be 30-70% and 40-60% respectively. Long tracks of Guanine should be avoided at 5ˈend, however long tracks of guanine and cytidine at 3ˈ end improves the performance (Lobato and Oˈ Sullivan, 2018).
* **Temperature:**  It works at a constant temperature of 37-42˚C .The temperature is maintained through different apparatus like incubators, heating blocks, chemical heaters (Lillis *et al*., 2014), and there are also examples of RPA working at ambient temperature in warm areas (above 30ºC).
* **Incubation time:** The quantity of initial copies of DNA largely determines the reaction time, however it has been found that 20 minutes are adequate for the amplification.
* **Sample types:** The sample types that can be amplified through RPA arecDNA generated through reverse transcription of RNA or miRNA, double stranded DNA, methylated DNA, single stranded DNA (Wee *et al*., 2016).
* **Storage:** RPA reagents are available in the form of pellets and are sold as lyophilized kits. These can be stored in a freezer (<-15°C), fridge (2-8 °C) for at least one year and for 6 months at room temperature (Chandu *et al*., 2016).

**DETECTION METHODS FOR RPA AMPLICONS**

RPA amplicons have the capability to be identified using two methods: end point detection, which occurs after the amplification process, or real-time detection, which takes place during the amplification process. Various probes are employed for detection, based upon the specific method being utilized..

* **End point detection:** Various detection techniques can be employed subsequent to amplification in order to ascertain the presence or absence of certain nucleic acid sequences. Typically, the utilization of end point detection necessitates fewer instruments compared to real-time detection, resulting in reduced test expenses. Consequently, this approach may be more suited for environments with limited resources. The methods employed for end point detection encompass the following approaches:
* Lateral flow assay: The test for the identification of RPA amplicons is widely utilized. Typically, assay designs suitable with lateral flow strip detection include three distinct oligonucleotides, namely two primers and one probe, together with the TwistAmpTM nfo kit. The suggested probe in this context is typically 46-52 base pairs in length. It is changed at the 5' end with an antigenic label, often a Carboxyfluorescein group or FAM. Additionally, it is modified at the 3' end with a polymerase inhibiting group. Furthermore, there is an abasic site located between the 5' and 3' ends of the probe. A potential abasic site can refer to a tetrahydrofuran (THF) residue, which is alternatively known as a d-spacer. After the hybridization of the probe with the DNA template, the nfo nuclease cleaves the probe at the d-spacer region. Cleavage creates a -OH group in the probe, turning it into a primer. An opposing amplification primer with a biotin label at the 5' end is needed with the probe. The probe and two primers generate a dual-labeled amplicon, which is then detected using antibodies or streptavidin in a sandwich examination.
* Bridge flocculation assay: The basic principle of bridge flocculation assay is that, it involves use of long polymers which crosslink multiple particles and thus flocculate out of solution at specific buffer condition. It is an equipment free assay that provides a binary naked eye visual read out, suitable for low resource settings. RPA in combination with bridge flocculation assay was first of all demonstrated by Wee and his co-workers where RPA amplicons greater than 100 bp (from a plant pathogen) that resembled long polymers were precipitated onto a magnetic bead surface (at low buffer pH) (Wee et al., 2015). The resulted conjugated particles underwent flocculation due to cross-linking of magnetic beads via RPA amplicons. The detection is a sharp transition between solution phase and flocculate, and is therefore better as naked-eye visualization.
* Electrochemical detection: In this approach, electrochemically active chemicals are utilized to provide a signal corresponding to the amplified nucleic acids. In this experiment, one primer is fixed on a solid surface while the other is in solution alongside the template of DNA and RPA reagents. After the RPA reaction, streptavidin-conjugated horseradish peroxidase and electrochemically active 3,3',5,5'-tetramethylbenzidine are added. According to Xiao *et* *al*. (2015), a screen-printed carbon electrode can show the electrochemical pulse.
* Chemiluminescent detection: According to Farrell *et al*. (2010), chemiluminescent detection involves the conversion of chemical energy into the emission of visible light, which occurs as a consequence of either an oxidation or hydrolysis process. In this assay, two primers are employed, with one primer being immobilized on a solid surface, while the second primer remains unbound in the solution, alongside the template of DNA and RPA reagents. Following the RPA reaction, the subsequent step involves the introduction of streptavidin conjugated horseradish peroxidase, which is then followed by the addition of a chemically active compound known as Luminol. According to Kunze et al. (2016), the CCD (Charge Coupled Device) camera is capable of detecting the emitted light signal.
* **Real- time Detection:** Real time detection employs the use of various florescent probes to identify RPA amplicons. Two florescent probes are used, exo-probe and fpg-probe.
* Exo-probe: Exo probes are oligonucleotides that match target amplicons that is blocked at the 3’ to prevent probe elongation. The probe also has a dT-fluorophore and a dT-quencher flanking a tetrahydrofuran residue (dSpacer), which are separated by a maximum of 2-4 bases. The fluorophore signal is thus quenched when the single stranded DNA probe is in solution. However, when the Exo probe is annealed to a complementary DNA target, the DNA repair enzyme Exonuclease III, cleaves the probe at the dSpacer site, producing two probe fragments, separating the fluorophore from the quencher, and thus facilitating the generation of fluorescence (Lobato and Oˈ Sullivan, 2018).
* Fpg-probe: The Fpg probe, akin to the Exo probe, is an oligonucleotide possessing sequence similarity to the target amplicon. It is designed to prevent probe extension by blocking at the 3' end. Furthermore, the Fpg probe has both a quencher and a fluorophore, which are positioned at a distance of 4 to 5 nucleotides apart, with a maximum separation of 7 nucleotides. The quencher is at the probe's 5' end, and a dR-group links the fluorophore to an abasic nucleotide. Fluorophore signal is muted without a target. The Fpg enzyme cleaves the probe at the dR site when it is attached to the complementary DNA target, releasing the fluorophore and producing fluorescence (Lobato and O'Sullivan, 2018).

**RPA reaction kits**

All the RPA reagents are available in the form of kits and are commercialized by a company, TwistDx. This company provides the kits both in liquid form as well as lyophilized pellets. Details of the various kits are mentioned in Table 2.

**Table 2**

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| **Product name** | **Category** | **Nucleic acid detection** | **Detection method** |
| Twist-Amp® Basic | Lyophilised kit | DNA | Gel electrophoresis |
| Twist-Amp® Basic RT | Lyophilised kit | RNA | Gel electrophoresis |
| Twist-Amp® Liquid  Basic | Liquid kit | DNA | Gel electrophoresis |
| Twist-Amp® Liquid Basic RT | Liquid kit | RNA | Gel electrophoresis |
| TwistAmp® exo | Lyophilised kit | DNA | Real-time fluorogenic probe-based |
| TwistAmp® exo RT | Lyophilised kit | RNA | Real-time fluorogenic probe-based |
| TwistAmp® Liquid exo | Liquid kit | DNA | Real-time fluorogenic probe-based |
| TwistAmp® Liquid exo RT | Liquid kit | RNA | Real-time fluorogenic probe-based |
| TwistAmp® fpg | Lyophilised kit | DNA | Real-time and endpoint fluorogenic probe-based |
| TwistAmp® nfo | Lyophilised kit | DNA | Lateral flow strip |

**RPA devices and accessories**

TwistDx, a firm specializing in molecular biology, has produced customized devices and accessories specifically designed for use in recombinase polymerase amplification (RPA) reactions. These tailored components are distinct from the standard reaction kits offered by the company. These devices enable various functions such as incubation, dispensing, mixing, detection, power supply, and mobility.

* **Twirla portable incubator**: The Twirla portable incubator is a compact, battery-powered device that enables the conduction of up to six simultaneous recombinase polymerase amplification (RPA) operations and subsequent end-point detection.
* **T8-ISO Instrument**: The T8-ISO instrument is a device used for incubation and detection. It has the capability to incubate up to 8 recombinase polymerase amplification (RPA) processes, with each tube having 2 channels for fluorescence detection.
* **T16-ISO Instrument**: The T16-ISO Instrument is an enhanced iteration of the T8-ISO Instrument, designed to facilitate the incubation of up to 16 RPA reactions. This instrument is equipped with a 3 channel fluorescence detection system for each tube.
* **Milenia HybriDetect 1**: The Milenia HybriDetect 1 is a device designed for the purpose of lateral flow detection. The detection method relies on a sandwich assay approach, whereby gold nanoparticles are utilized as the tracer.
* **PCRD Nucleic Acid Detection**: The detection of nucleic acids using polymerase chain reaction with real-time detection (PCRD) is accomplished by a sandwich assay approach, wherein carbon nanoparticles are utilized as tracers. This detection process takes place within an open cartridge.
* **U-Star disposable nucleic acid lateral flow detection units**: This operates on the principle of a sandwich assay, utilizing carbon nanoparticles as tracers. This detection process takes place within a sealed cartridge.

**RPA in a suitcase for mobile laboratory**

The primary objective of RPA was to facilitate the decentralization of nucleic acid testing from laboratory environments to field or resource-limited settings. Wahed and his colleagues have designed a pair of standard luggage with dimensions of 56 cm × 45.5 cm × 26.5 cm. These suitcases have been specifically constructed for the purpose of conducting real-time RPA diagnostics, and they are equipped with all the essential chemicals and equipment required for this assay. According to Wahed et al. (2015), the reagents and equipment were securely positioned within the foam located at the bottom of the suitcase, serving as a means of cushioning against any shocks during transportation. Additionally, a solar panel and power pack were utilized to provide a reliable power supply. Furthermore, the storage box was designed to be replenished as necessary. In order to mitigate the risk of contamination, two transportable suitcase labs were designed with distinct workspaces for nucleic acid extraction and detection (see Figure 7). In addition to the conventional equipment, the extraction workspace is equipped with a heat block and a magnetic stand. Similarly, the detection suitcase is furnished with a tubescanner.

The extraction suitcase utilized a straightforward and efficient lysis process known as SpeedXtract (Qiagen, Hilden, Germany). The protocol was implemented in the following manner: A volume of 500 μl of the sample was subjected to incubation with 1500 μl of the enrichment buffer and 30 μl of the magnetic beads for a duration of 3 minutes at ambient temperature. The magnetic beads were isolated utilizing a magnetic stand, followed by the removal of the supernatant while ensuring little disturbance to the beads. Subsequently, the beads underwent two rounds of washing using 500 μl of enrichment buffer on each occasion. Subsequently, a volume of 100 μl of the lysis buffer was introduced into the mixture, which was then subjected to incubation at a temperature of 95 °C for a duration of 10 minutes. Subsequently, the beads were subjected to separation, and a volume of 5 μl of the resulting supernatant was utilized in the reaction of recombinase polymerase amplification (RPA). The whole duration required for the extraction process amounted to 20 minutes.

In the detection suitcase, a volume of 5 μl of the RPA primers and probe mix was introduced to the RPA lyophilized pellet (TwistAmpexo kits, TwistDx, Cambridge, UK) with concentrations of 420 nM and 120 nM, respectively. Next, a volume of 40 μl of rehydration buffer, which consisted of a concentration of 14 mM Mg acetate, was introduced. Subsequently, a volume of 5 μl of template was introduced. The tube was well mixed and thereafter inserted into the tubescanner (Twista, TwistDx, Cambridge, UK), where it was incubated at a temperature of 42 °C for a duration of 15 minutes. The fluorescence signals released were recorded at intervals of 20 seconds. Signal interpretation was performed using a hybrid approach that involved threshold analysis and first derivative analysis. According to Li et al. (2018), the overall duration of the RPA reaction, which encompasses both the reaction itself and the associated handling procedures, was 20 minutes.

**Detection of various pathogens through RPA**

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| Crop | Pathogen | Reference |
| Ground nut | *Cercospora arachidicola* | Ying Lin *et al.,* 2022 |
| Tobacco | *Ralstonia solanacearum* | Li *et.al.,* 2021 |
| Tomato | *Tomato yellow leaf curl virus* | Tzu-Ming Wang & Jing-Tang Yang, 2019 |
| Apple | *Candidatus phytoplasma mali* | Natallia Valasevich, 2017 |
| Cotton | *Verticilium dahliae* | Yuliang Ju *et al*., 2019 |
| Beans and Tomato | Tomato mottle virus, Bean golden yellow mosaic virus. | Londono et al., 2016 |
| Plum | Pulm pox virus | Zang et al., 2014 |

**Conclusion and future trends**

Since its initial publication in 2006, the isothermal amplification method known as RPA has had a significant surge in the number of publications, widespread recognition, and diverse applications. Since then, a significant portion of scholarly articles have focused on various applications of RPA. However, a rising number of papers have also provided specific methodologies to enhance RPA performance and broaden its scope of capabilities. User-friendliness distinguishes RPA from other isothermal amplification methods, remarkable sensitivity, specificity, ability to accommodate multiplexing, expedited amplification, and operation at a consistently low temperature. Additionally, it functions without requiring a pre-denaturation phase or the utilization of numerous primers. In spite of the existence of some recognized polymerase chain reaction (PCR) inhibitors or unrefined materials, RPA has the capability to amplify as little as 1-10 target copies within a time frame of less than 20 minutes. Moreover, RPA has the versatility to amplify both DNA and RNA targets in both liquid and solid states. RPA has demonstrated compatibility with a diverse array of detection methodologies.

Nevertheless, it is important to acknowledge that Robotic Process Automation (RPA) does possess certain limits. One prominent drawback is the exclusive availability of RPA kits from a single manufacturer, which might potentially influence pricing dynamics. Another limitation is the user's limited control over the kit composition. Although bespoke kits that exclude components like as polymerase or dNTPs are available, they tend to be costly.

Several crucial factors must be taken into account for future progress. Firstly, it is important to prioritize the development of sample preparation procedures that are suitable for field conditions. This includes focusing on concentration, extraction, and purification techniques. By doing so, it will greatly enhance the feasibility of conducting a comprehensive RPA assay in on-site or field settings. Furthermore, the development of portable and completely automated diagnostic instruments for RPA has significant relevance. This is crucial not only for enhancing diagnostic efficiency, but also for facilitating widespread use of RPA diagnostic methods. In addition, it is worth noting that real-time PCR operates under the control of a heat cycle, whereas RPA utilizes continuous amplification following the initiation of the reaction. Therefore, it would be advantageous to devise a technique or mechanism that enables precise control over the initiation of each RPA amplification cycle. This would greatly aid in obtaining dependable quantitative RPA results, as opposed to relying solely on absolute quantification through digital RPA. Additionally, RPA has 7.08% market share, which is much lower than that of PCR. Furthermore, RPA has a smaller proportion of the market than temperate nucleic acid enhancement methods like LAMP and SDA. In order to enhance its market presence, more research might be conducted to compare the clinical validity of robotic process automation (RPA) with existing polymerase chain reaction (PCR) tests. Furthermore, it is imperative to build realistic websites or software that can facilitate and enhance the process of designing RPA primers and probes. These tools should also have the capability to screen and identify the most optimum oligonucleotide pairings for the reaction. From a rational standpoint, it is unlikely that RPA will completely replace PCR in the foreseeable future. However, RPA possesses ability to serve as a flexible supplement to PCR. Currently, the technology of RPA is advancing in its development for use in clinical settings. However, it is currently in a transitional phase as it moves towards being utilized on-site or in the field. Given its rapid and ongoing advancement, it is anticipated that the technology of RPA would finally evolve into highly resilient mobile apps that cater to immediate needs.

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