Evaluation of Recombinase Polymerase Amplification (RPA) for suitability in molecular diagnostic applications

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Introduction

With novel viruses arising on a routine basis globally, the need for fast, easy-to-use pathogen detection methods that can respond quickly to the demands of the infectious disease community is critical to tracking and containing new outbreaks. As a response, the use of isothermal amplification in point-of-care (POC) and field-based Molecular Diagnostics (MDx) testing platforms has grown dramatically over the past decade. One isothermal method ideally suited for these applications is Recombinase Polymerase Amplification (RPA). Here, we present research evaluating the sensitivity, inhibitor tolerance, and utility of the method in detecting both bacterial and viral pathogens.

RPA is an isothermal amplification method useful for single-target or low-multiplex assays. Through the use of T4 UvsX and T4 UvsY recombinases, T4 Gene 32 Protein (GP32), and a strand-displacing DNA polymerase such as *Bsu* DNA Polymerase, Large Fragment (*Bsu* DNAP), users can create an assay result that is capable of being visualized by multiple methods — such as gel electrophoresis, real-time fluorescence, or lateral flow strips. RPA has numerous advantages over Loop-Mediated Isothermal Amplification (LAMP), including simpler primer design, lower operating temperature, and faster overall reaction time.

Table 1: Comparison of RPA and LAMP methods

	RPA	LAMP
Primers needed	2	4 – 6
Initial heating	No	Yes
Incubation temp	37°C – 42°C	60°C – 65°C
Amplification time	10 – 20 min	20 – 60 min
Limit of detection	1 copy	5 copies

RPA Sensitivity

RPA is capable of working over a wide range of input amounts and can also be tuned for speed vs. sensitivity. To test speed, a reaction was optimized targeting a 168 bp synthetic target in a range of 100 to 1M copies. Results show the reaction achieving results in less than 10 minutes, with a consistently strong signal.

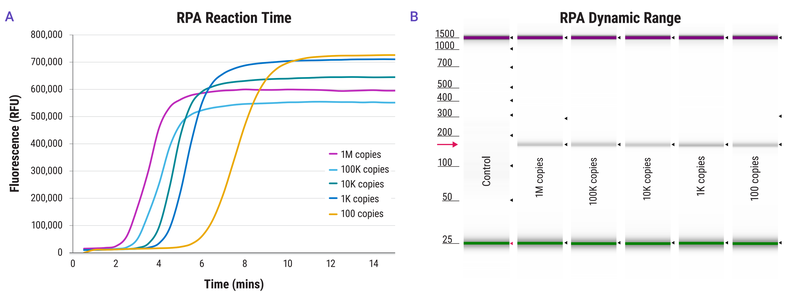

Figure 1. Rapid amplification with a strong signal across a broad template concentration range. Reactions containing a range of copy numbers of a 168 bp target were run for 30 min in RPA conditions optimized for speed of result. **(A)** Time to reach peak fluorescence was consistently <10 min, even for reactions containing as few as 100 target copies. **(B)** A consistently strong end-point signal was observed for all reactions between 100 and 1M copies.

To test sensitivity, conditions were optimized to detect as low as 0.1 pg (15 copies) of a *P. aeroginosa* genome using a SYBR-based real time detection method and a 177 bp target.

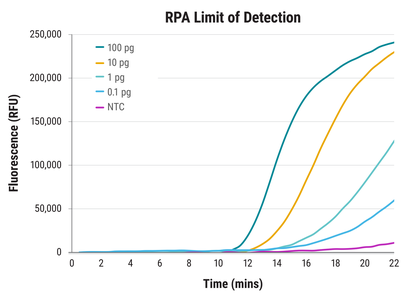

Figure 2. Limit of detection test demonstrates highly sensitive amplification. A 177 bp *P. aeroginosa* genomic DNA target was detected in a range of 100 pg (15,000 copies) to 0.1 pg (15 copies). Reactions were run at 45°C.

Inhibitor Tolerance

For assays developed for field or POC testing, direct amplification without nucleic acid purification saves precious time and reduces costs. However, this approach may result in a variety of amplification inhibitors being carried into the final reaction. The ability of RPA to amplify in the presence of a range of commonly encountered sample types is shown in Figure 3, along with inhibitors that can be found in extracted nucleic acids. Successful amplification of targets was detected while in the presence of blood, saliva, urine, universal transport media (UTM), EtOH, urea, heparin, humic acid, or hemin.

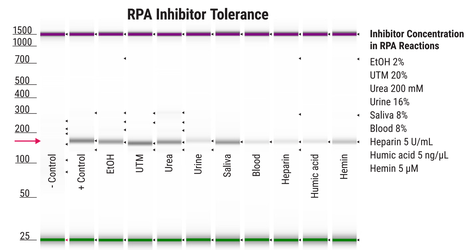

Figure 3. RPA amplification in the presence of inhibitors. A variety of common inhibitors were added to RPA reactions amplifying a 168 bp target. In all cases, RPA demonstrates strong tolerance to inhibitors routinely encountered in nucleic acid extraction methods or biological samples.

Multiplexing Capabilities

When an assay developer is creating POC or field-based MDx assays, it is advantageous to be able detect multiple targets in a single reaction. This enables a reduction in cost and time to result, and the inclusion of positive control reactions to ensure the assay is working as expected. To test multiplexing capability, triplex and quadruplex reactions were set up, amplifying DNA targets of different sizes. Amplification was consistently strong for all targets. Combined with readout methods such as lateral flow strips with multiple immobilized probes, this can enable fast, simple POC or field-based assays that require minimal or no instrumentation.

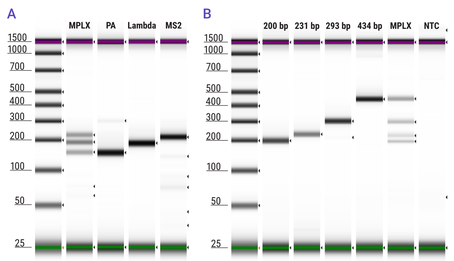

Figure 4. Multiplex amplification of DNA targets using RPA. (A) Triplex reaction of a 156 bp *P. aeruginosa*, 200 bp Lambda DNA, and 231 bp MS2 phage cDNA targets (MPLX) compared to the individual amplifications of each target (PA, Lambda and MS2). **(B)** Quadruplex reaction of 200, 231, 293, and 434 bp amplicons from MS2 phage cDNA (MPLX), compared to the individual amplifications of each target. Both multiplex reactions showed similar amplification of all targets with amplicons of the expected sizes.

Viral Detection with RT-RPA

Many POC MDx assay targets are RNA-based, so the ability to perform one-step RT-RPA at a single temperature is critical to successfully detecting viral pathogens — as is detecting multiple pathogens in a single reaction. By adding StellarScript® HT+ Reverse Transcriptase and optimizing the reaction buffer, a one-step RT-RPA reaction capable of multiplex amplification from RNA as the starting material was demonstrated.

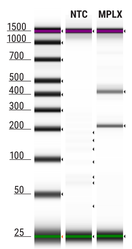

Figure 5. Multiplexed RT-RPA reaction. Duplex amplification of both 212 bp and 420 bp amplicons from MS2 RNA. All reactions were performed with the addition of 0.2 U of StellarScript HT+ RT in the RPA reaction, amplifying at 42°C for 30 min prior to detection on a TapeStation.

Enzyme Activity Comparisons

It has been shown that individual enzyme activity levels have significant effects on RPA reaction speed and final yield. Using a proprietary QC assay, T4 UvsX Recombinase activity levels were compared between enzymes sourced from Watchmaker and an alternative supplier. While both enzymes are sold on a concentration (mg/mL) basis, the activity levels showed significant differences. Higher activity of T4 UvsX recombinase can have a significant effect on the speed and sensitivity of an RPA assay; improving turnaround time and/or decreasing the amount of enzyme required.

In addition, some RPA protocols use *Bst* DNAP instead of *Bsu* DNAP. *Bst* requires a higher optimal operating temperature, which makes it less suited for POC and field-based assays. While the optimal temperature for RPA varies depending on assay design, it is useful to be able to run at lower temperatures to reduce the need for complex instrumentation or heat sources. At 37°C, *Bst* provides less than 10% of the polymerization activity of *Bsu*. The end result is a slower reaction time that takes longer to reach a final result, or lower levels of sensitivity.

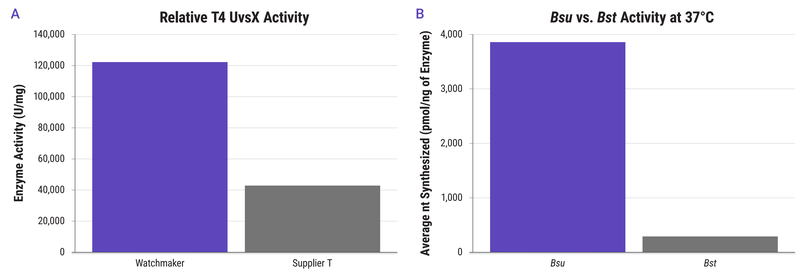

Figure 6. Relative activity of enzymes used for RPA. (A) Using a QC assay designed to measure activity of T4 UvsX Recombinase, enzymes from two manufacturers (Watchmaker and Supplier T) were compared. Supplier T demonstrated a 65% lower activity compared to Watchmaker, which can result in significant reduction in final amplification yield. This loss also highlights the need for individual enzyme activity QC assays to ensure reproducible results. **(B)** Using a DNA polymerase activity assay, both *Bsu* and *Bst* DNAPs were measured for speed of synthesis at 37°C. Bst is below optimal activity at this temperature, making it a less suitable polymerase for RPA when users desire to run at lower temperatures in field or POC settings.

Conclusions

- RPA is a highly sensitive method capable of detecting pathogens across a wide dynamic range, including copy numbers at or below clinically relevant limits of detection.
- The inhibitor tolerance of RPA makes it suitable for customer-developed field-based or POC assays where it is desirable to skip sample extraction or purification methods to achieve the fastest turnaround possible.
- With the addition of a suitable reverse transcriptase, RPA is efficient in detecting viral targets by RT-RPA.
- RPA is suitable for multiplex testing for either DNA or RNA targets, providing detection of at least four targets simultaneously.

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