Optimization of Recombinase Polymerase Amplification (RPA) highlights the importance of enzyme consistency for reproducible results

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Introduction

With novel viruses arising on a routine basis globally, the need for fast, easy-to-use diagnostic 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 diagnostic (MDx) testing platforms has grown dramatically over the past decade. An increasingly popular isothermal method is Recombinase Polymerase Amplification (RPA); however, users of the method have reported inconsistent amplification and fluctuating positive signal response times. Here, we present research evaluating the roles of individual enzymes in amplification efficiency, reproducibility, and stability.

RPA as an MDx Method

Recombinase Polymerase Amplification is an isothermal amplification method useful for single-target or small 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.

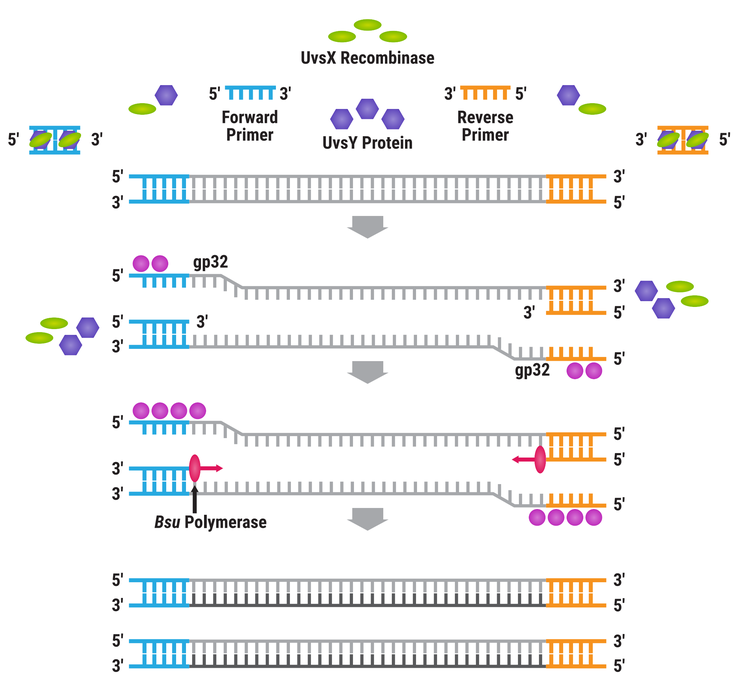

Figure 1. Molecular representation of RPA.[1] T4 UvsX recombinase (green ovals) and UvsY protein (purple hexagons) bind to amplification primers, forming a complex that is complementary to sequences in double-stranded DNA. T4 Gene 32 Protein (pink dots) acts as a single-stranded binding protein and stabilizes the unwound DNA strand, allowing *Bsu* DNA Polymerase, Large Fragment (red ovals) to initiate strand-displacing amplification. The repetition of the cycle leads to exponential amplification.

Table 1. Advantages of RPA over other isothermal methods[2]

Isothermal Method	Target	Primers Required per Target	Initial Heating	Incubation Temp (°C)	Amplification Time (Min)	Limit of Detection (Copies)	Multiplexing
RPA	DNA/RNA	2	No	37 – 42	20 – 40	1	Yes
LAMP	DNA/RNA	4 – 6	Yes	60 – 65	60	5	Yes
NASBA	RNA	2	No	41	60 – 180	1	Yes
SDA	DNA	4	Yes	30 – 55	60 – 120	10	Yes
RCA	DNA/RNA	1	Yes	30 – 65	60 – 240	10	No
HDA	DNA	2	No	65	30 – 120	1	Yes

- RPA has one of the lowest optimal operating temperature ranges, enabling easier use with low-cost heat sources. Some users have reported being able to successfully run RPA as low as 25°C.
- Only two primers are required per target, similar to traditional PCR primers, compared to the more complex 4 – 6 primers per target required for loop-mediated isothermal amplification (LAMP).
- RPA also provides the fastest overall time to result at an average of 20 – 40 minutes, with users known to have pushed this down to as little as 10 minutes.
- The ability to multiplex, achieve high sensitivity (i.e., low copy number limit of detection), and connect to a range of readout modalities provides the assay developers with flexibility in method design.
- RPA is amenable to creating reaction mixes in lyophilized format, reducing cold-chain storage concerns, extending shelf-life, and providing compatibility with point-of-care (POC) or field-based detection systems.

RPA Challenges: Consistency and Stability

For all the advantages that RPA presents, challenges do exist. The issues primarily stem from reproducibility of the reactions over time. Here, we theorize that this sub-optimal reproducibility is not due to the method itself; instead, the challenges are due to poor consistency and quality of the enzymes sourced and utilized in creating the assays.

MDx developers using RPA routinely want to optimize their individual assays to achieve the highest sensitivity and specificity depending on their target and readout method. This necessitates the ability to source the individual reagents and enzymes so that the developer can mix these components in ratios that allow these goals to be met. However, there are only a few sources for some of the key enzymes, especially T4 UvsX and T4 UvsY, and users have reported multiple issues with these enzymes. Of primary interest among these issues are stability (both in shelf-life and freeze/thaw cycles) and lot-to-lot reproducibility. Some users have reported needing to recalibrate their assays with every new lot of T4 UvsX — even when purchased from the same supplier — making the creation of a stable, reproducible assay very difficult.

Impact of Enzyme Quality on RPA Efficiency and Specificity

Recognizing the potential effects of individual enzyme quality and activity on RPA as a method, this poster attempts to break down the contribution of the T4 UvsX, T4 UvsY, T4 GP32, and *Bsu* DNAP enzymes by systematically spiking in damaged enzymes in a controlled manner to determine the downstream effects on final results.

To approximate the effects of inactive enzyme, concentration, or activity variability of individual enzymes on RPA, we heat-killed each of the four key enzymes and added them to the reaction at known ratios compared to active enzyme. Each heat-killed enzyme was spiked in at ranges from 10 – 50% of the total enzyme volume normally used in a control RPA reaction. The control reaction utilizes a synthetic 168 bp target at 1M copies input.

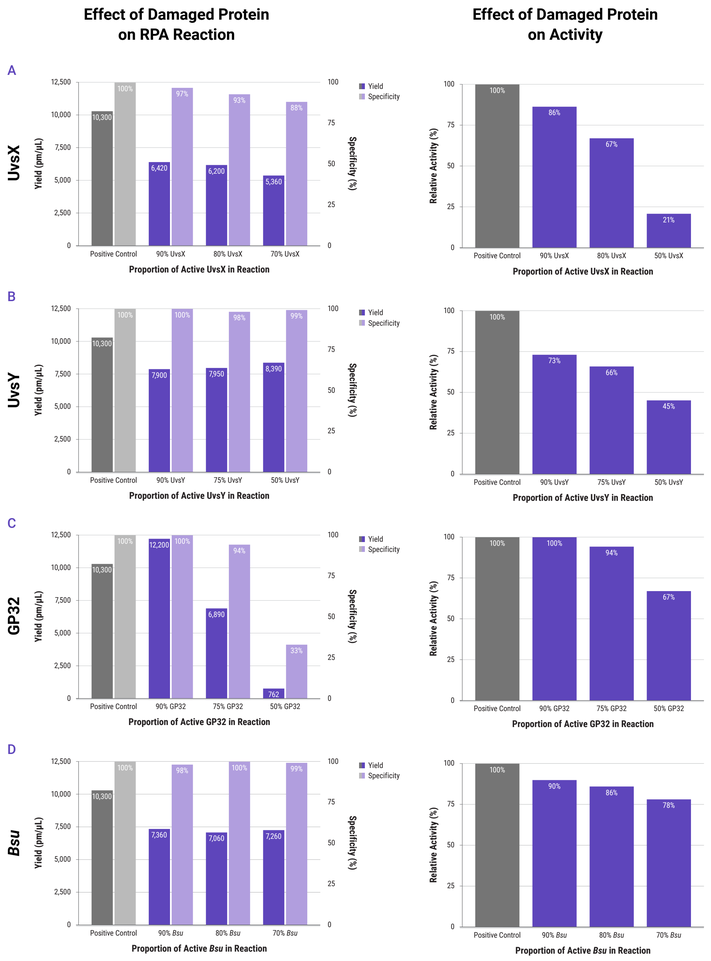

Figure 2. Effects on yield, specificity, and enzyme activity of changes in enzyme input. For each of the four key RPA enzymes, the ratio of heat-killed to active enzyme was varied to simulate the effects on the final assay of variable quantity or quality. Product was analyzed via TapeStation to measure total yield (left panels/left Y-axes) and % specificity (left panels/right Y-axes). Enzyme activity was measured via Watchmaker's proprietary activity assays (right panels). Effects varied between enzymes, with reduced amounts of T4 UvsX having the greatest effect on total yield, and the second greatest effect on specificity. T4 GP32 had the third-highest effect on yield, but showed the largest effect on specificity. Variance in *Bsu* DNAP inputs had the least overall effects on both yield and specificity.

References

1. Schematic recreated from James, *et al.*, *Diagnostics* 2020, 10, 399
2. Table recreated from Lobato, *et al.*, *Trends in Analytical Chemistry* 2018, 98, 19 – 35

Freeze/Thaw Stability

As previously stated, the suite of RPA enzymes can be unstable, especially for glycerol-free formulations required for downstream lyophilization. Notably, some suppliers of T4 UvsX and T4 UvsY state that these enzymes should be used immediately upon thawing as significant activity loss will occur if refreezing is attempted. To combat this issue, we have formulated glycerol-free storage buffers for all four RPA enzymes that not only allow lyophilization, but also survive multiple freeze/thaw cycles without loss of activity.

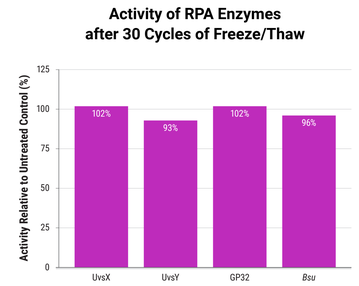

Figure 3. Freeze/thaw stability for RPA enzymes. Each of the four key RPA enzymes was stored in a glycerol-free, lyophilization-friendly buffer and subjected to 30 freeze/thaw cycles, going from -80°C to room temperature. Percent remaining activity was measured relative to untreated control using individual enzyme activity assays developed by Watchmaker Genomics.

QC Development

In order to control concentration and activity of the RPA enzymes, we developed new, proprietary QC assays or expanded existing methods to ensure consistent enzyme production prior to input into RPA assays.

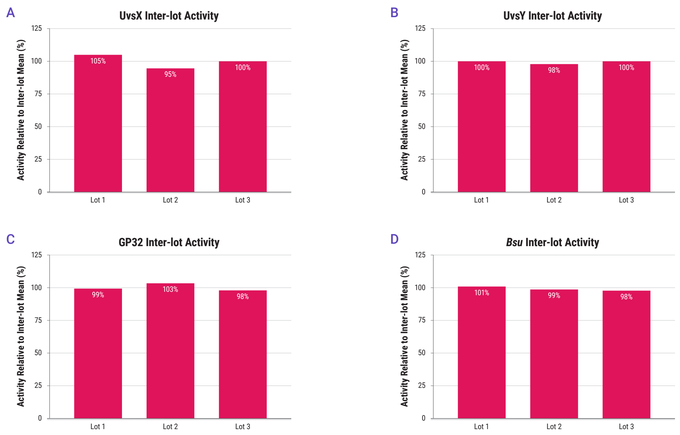

Figure 4. RPA enzyme activity lot-to-lot consistency. Using in-house developed QC assays, individual enzyme activity levels were measured for multiple lots. Each enzyme showed a high level of consistency, demonstrating robust production processes.

Conclusions

- RPA results, especially in quantitative assays, can vary due to inadequate control of enzyme quality, concentration, or activity
- Variability in T4 UvsX and T4 GP32 appear to have the greatest effects on yield and specificity of target amplification
- The solution to these issues is to create more stable versions of the enzymes RPA depends on as a method and establish strong manufacturing QC methods that ensure reproducible activity or concentration of each enzyme
- By utilizing RPA enzymes with individual activity QC prior to input into assays, MDx developers can remove a primary source of variation in their assays and enable better lot-to-lot consistency in their final method
- Watchmaker Genomics has developed stable RPA enzymes with first of their kind individual activity QC methods to deliver the reproducibility needed for assay optimization