Isothermal amplification

Recombinase Polymerase Amplification (RPA) Reaction for Target Detection and NGS Library Amplification

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Abstract

Purpose: Develop lyophilization-compatible reagents for the RPA (recombinase polymerase amplification) reaction. Develop guidelines for RPA based target detection and NGS library amplification.

Methods: RPA based amplification of DNA/RNA templates, under various conditions and using different detection methods, like end-point detection assay. RPA based library preparation of three bacterial genomes with GC content 33-67%.

Results: Lyo-ready RPA kit; stand-alone RPA proteins and RPA reaction buffer were developed. The kit shows high sensitivity and specificity for both DNA and RNA template amplification, robust amplification from samples containing inhibitors, notable multiplexing capabilities, and compatibility with different detection methods. The RPA proved to be efficient low temperature alternative for NGS library amplification.

Introduction

Recombinase Polymerase Amplification (RPA) is a novel isothermal DNA amplification technique that operates at a constant low temperature (37-45 ° C) with only two specific primers, offering a rapid and sensitive alternative to traditional temperature cycling requiring methods like PCR. High reaction temperatures not only require complex equipment but can damage fragile, scarce, fragmented samples. Therefore, RPA is cost, energy, and sample saving option for nucleic acid amplification. It can be adapted in resource-limited settings and decentralized diagnostics applications.

Here we present the development and performance testing of the reagents for RPA (recombinase polymerase amplification). We developed these RPA reagents in a format compatible with lyophilization. Additionally, the RPA reaction conditions were optimized and the composition of the RPA reaction buffer adapted to ensure consistent and reliable results across diverse sample types. Moreover, we demonstrated the use of RPA reaction as low temperature NGS library amplification option.

Materials and methods

Test Sample Preparation

Chikungunya inactivated virus suspension (MC014) was purchased from Vircell and virus RNA was purified using MagJET Viral DNA and RNA Kit. Genomic DNA from *M. pneumoniae* (15531D) and genomic RNA from Zika virus (VR-1843DQ) were purchased from ATCC. Adenovirus 41 DNA (MBC114-R) was purchased from Vircell. SARS-CoV-2 RNA (102019), Measles virus RNA (103009) and Influenza H1N1 RNA (103001) were purchased from Twist Bioscience. *S. aureus, E.coli,* and *P. aeruginosa* bacteria were locally cultivated, and their genomic DNA was purified using in-house methods.

Design of Target Specific (RT-)RPA Primers

(RT-)RPA primers were designed using Primer-BLAST tool. Primers' length varied in the range of 30-35 nt, GC content – 30-60%, and amplicon length – 150-450 bp.

(RT-)RPA Reaction Using End-point Detection

The RPA reaction mix consists of 1X RPA reaction buffer, 0.2 mM each dNTP, 0.3 µM forward and reverse primers, 0.03 mg/mL T4 UvsX protein, 0.03 mg/mL T4 UvsY protein, 0.4 mg/mL T4 gene 32 protein, 0.15 U/µL Bst DNA polymerase, 14 mM $MgCl_2$ and DNA template.

In case of RNA template, 2 U/µL SuperScript IV Reverse Transcriptase, 1.6 U/µL RNaseOUT Recombinant Ribonuclease Inhibitor and 0.1 U/µL RNase H are added additionally to the RT-RPA reaction mix.

(RT-)RPA reaction is performed at 42 ˚C for 20 min. After incubation samples were mixed with Loading Dye & SDS Solution, incubated at 65 ˚C for 10 min and diluted 30-fold. 20 µL of diluted sample is loaded on 2% E-Gel™ EX Agarose Gel and analyzed using E-Gel™ Power Snap Electrophoresis System.

NGS library preparation and data analysis

0.1ng genomic DNA with varying GC content (33% to 67%) from *S. aureus, E. coli* and *P. aeruginosa* bacteria were used. Fragmentation and adaptor ligation of the libraries was performed using Invitrogen™ Collibri™ ES DNA Library Prep Kits for Illumina Systems, followed by PCR amplification using Collibri™ Library Amplification Master Mix according to manufacturer's recommendations at 37 ° C using Invitrogen™ Lyo-ready RPA Kit. RPA reactions were optimized by adding DMSO to the samples with higher GC content. Average library size was determined using the Agilent™ 2100 Bioanalyzer™ instrument with the Agilent™ High Sensitivity DNA Kit. Library concentration was measured by qPCR using the Invitrogen™ Collibri™ Library Quantification Kit. The prepared libraries were sequenced on an Illumina™ MiSeq™ instrument.

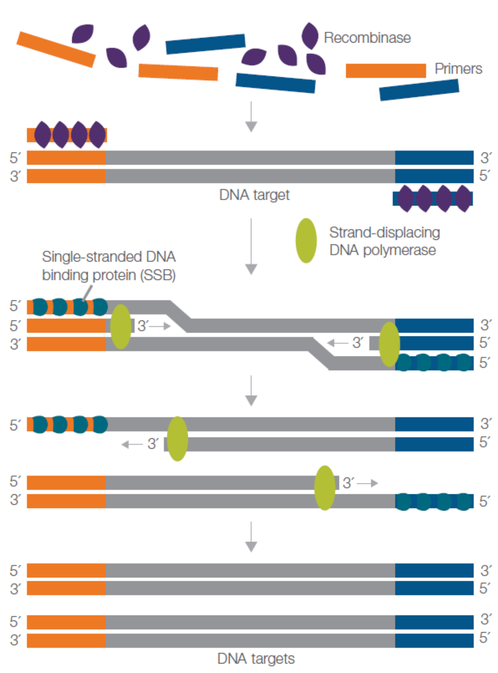
Adapter and quality trimming of paired-end reads was performed using BBDuk (BBMap suite). Trimmed reads were aligned to reference sequences using BWA-MEM (v0.7.15). Alignment metrics were assessed with Picard Collect Alignment Summary Metrics (v2.22.3) and Qualimap (v2.2.1). GC bias was evaluated using Picard CollectGCBiasMetrics.

Results

Overall Scheme of RPA Reaction

RPA technology includes 4 main proteins – recombinase UvsX, helper protein UvsY, single-stranded DNA binding (SSB) protein and strand-displacement DNA polymerase. UvsX and UvsY proteins form a recombinase complex, which binds the primers and search for homologous sequences in the target DNA, enabling the primers to invade the double-stranded DNA and bind to the complementary strand. SSB proteins stabilize the single-stranded DNA while DNA polymerase extends the primers thereby synthesizing the new DNA strand [3] (Figure 1).

Figure 1. RPA reaction scheme.



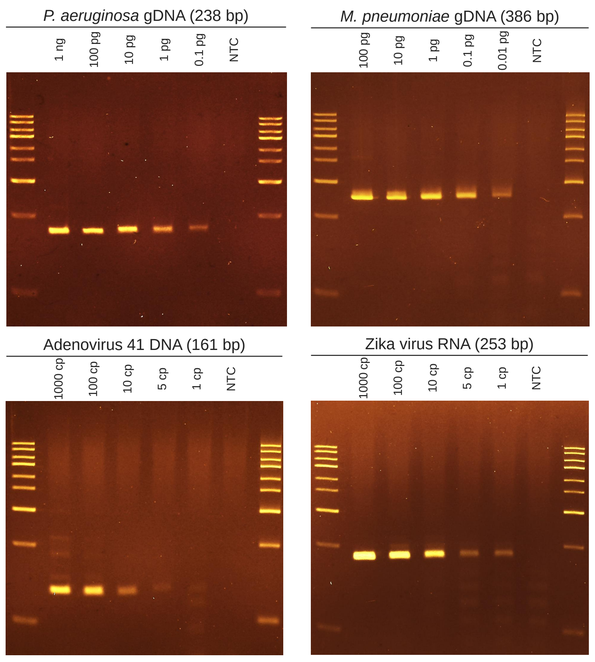
Our newly developed RPA Kit is compatible with lyophilization and includes all the essential components for the RPA reaction: Lyo-ready stand-alone proteins (T4 UvsX, T4 UvsY, T4 gene 32 proteins, Bst DNA polymerase) and 2X RPA Reaction Buffer (Figure 2). Stand-alone proteins are available in two concentration variants. Lower concentration reagents are ideal for direct RPA reaction setup while higher concentration proteins are flexible option for RPA reaction optimization and test developers.

Figure 2. Lyo-ready RPA Kit components.

High Sensitivity and Specificity of End-point (RT-)RPA Detection

Lyo-ready RPA kit can amplify targets from various types of template DNA or RNA with high specificity and sensitivity detecting as few as 1 copy. Targets of varying lengths (161-386 bp) were amplified from series dilutions of *P. aeruginosa* and *M. pneumoniae* genomic DNA, Adenovirus 41 DNA, and Zika virus RNA, and were analyzed on 2% E-gel (Figure 3).

Figure 3. End-point RPA and RT-RPA detection.



High Target Specificity

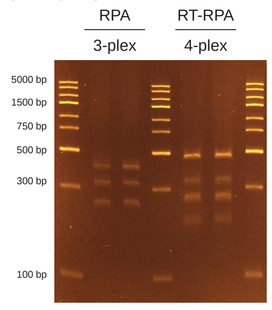
Five different DNA or RNA templates from various organisms were mixed together and using target specific primers each target was amplified specifically from the template that was surrounded with different unrelated molecules. The results showed that all targets can be successfully detected, and no cross-reaction occurred (Figure 4).

Figure 4. Target specificity of (RT-)RPA reaction.

Multiplexing Capabilities and Resistance to Inhibitors

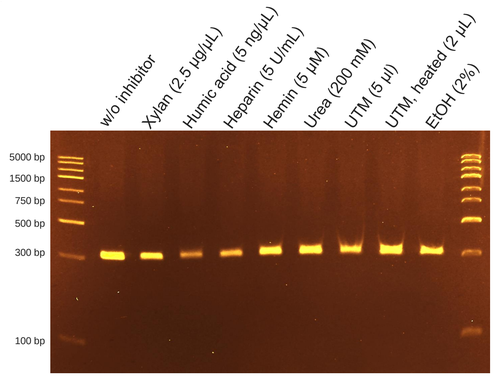
Lyo-ready RPA kit can successfully perform multiplex RPA reaction and amplify several different amplicons in the same reaction. Three targets were amplified from various DNA templates (376 bp from 1 ng of *S. aureus* genomic DNA, 305 bp from 1 ng of Human genomic DNA and 238 bp from 1 ng of *P. aeruginosa* genomic DNA) and four targets were amplified from various RNA templates (452 bp from 1000 copies of Measles virus RNA, 313 bp from 100X dilution of Chikungunya virus RNA, 253 bp from 1000 copies of Zika virus RNA and 192 bp from 1000 copies of SARS-CoV-2 RNA). All targets were detected with high specificity and without any non-specific products (Figure 5).

Figure 5. Multiplex (RT-)RPA.

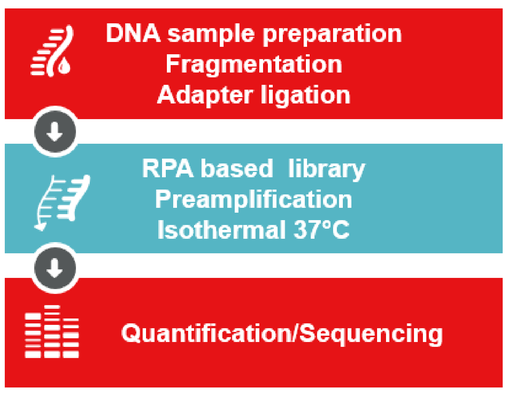


Lyo-ready RPA kit demonstrates superior tolerance to common inhibitors originating from reagents used in RNA/DNA extraction steps and transport media or transferred from biological samples, such as soil, plants, urine and blood. A 305 bp target from human genomic DNA was successfully amplified in an RPA reaction with an additional inhibitor present (Figure 6).

Figure 6. Resistance to inhibitors.



Efficient NGS library preparation using RPA



Efficient NGS library preparation

The RPA method can be used in NGS library preparation workflow as a low temperature library amplification alternative to PCR. The percentage of aligned reads and reads with ≥30X coverage were almost identical when compared between RPA and PCR library amplification. Also, simmilar GC coverage was obtained between both amplification methods, regardless of the genome GC content. These results show that the RPA method can be successfully applied to the amplification of library fragments as an alternative to PCR (Figure 7).

Figure 7. Normalized NGS coverage data after RPA reaction.

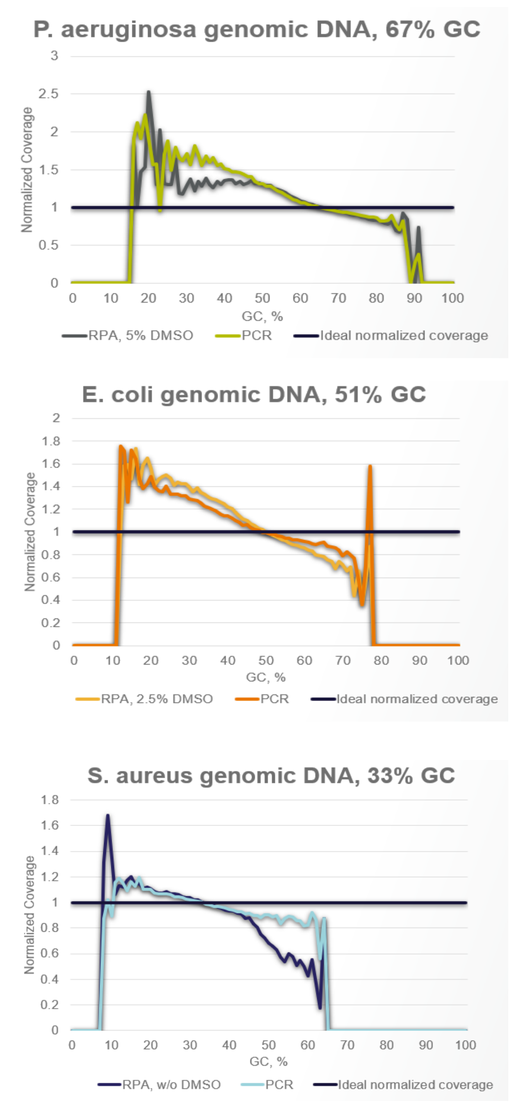


Figure 8. Library amplification method data comparison.

Organism	Genome Size	GC Content	Amplification Method	≥ 30X Coverage	Aligned Reads
S. aureus	2.8 Mb	33%	**RPA,** w/o DMSO	**98.0%**	**99.6%**
			PCR	**98.0%**	**99.4%**
E. coli	4.7 Mb	51%	**RPA,** 2.5% DMSO	**99.4%**	**95.6%**
			PCR	**99.5%**	**95.5%**
P. aeruginosa	6.3 Mb	67%	**RPA,** 5% DMSO	**98.7%**	**99.7%**
			PCR	**98.3%**	**96.1%**

Conclusions

- Optimized RPA reagents compatible with lyophilization, end-point and other detection methods were developed.
- Invitrogen™ Lyo-ready RPA Kit offers easy reaction setup, constant low temperature amplification, high sensitivity and specificity from both DNA and RNA templates, robust amplification in the presence of inhibitors, and multiplexing capabilities.
- RPA is fast (~20min) alternative to PCR in the NGS library preparation step that works for genomes with varying GC content.

References

1. Piepenburg O et al. DNA detection using recombination proteins. *PLoS Biol*. 4,7 (2006).

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