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Factors influencing Recombinase Polymerase Amplification (RPA) assay outcomes at point of care

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Abstract

Recombinase Polymerase Amplification (RPA) can be used to detect pathogen-specific DNA or RNA in under 20 minutes without the need for complex instrumentation. These properties enable its potential use in resource limited settings. However, there are concerns that deviations from the manufacturer's protocol and/or storage conditions could influence its performance in low resource settings. RPA amplification relies upon viscous crowding agents for optimal nucleic acid amplification, and thus an interval mixing step after 3–6 minutes of incubation is recommended to distribute amplicons and improve performance. In this study we used a HIV-1 RPA assay to evaluate the effects of this mixing step on assay performance. A lack of mixing led to a longer time to amplification and inferior detection signal, compromising the sensitivity of the assay. However lowering the assay volume from 50 μ L to 5 μ L showed similar sensitivity with or without mixing. We present the first peer-reviewed study that assesses long term stability of RPA reagents without a cold chain. Reagents stored at -20°C , and 25°C for up to 12 weeks were able to detect 10 HIV-1 DNA copies. Reagents stored at 45°C for up to 3 weeks were able to detect 10 HIV-1 DNA copies, with reduced sensitivity only after >3 weeks at 45°C . Together our results show that reducing reaction volumes bypassed the need for the mixing step and that RPA reagents were stable even when stored for 3 weeks at very high temperatures.

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Keywords

Recombinase polymerase amplification; sensitivity; reagent stability; cold chain; low resource settings

1. Introduction

Recombinase polymerase amplification (RPA) is an isothermal amplification method that can rapidly detect nucleic acids without complex laboratory equipment [1]. Numerous RPA-based assays have been developed that can detect very low concentrations of pathogen-specific DNA and RNA in under 20 minutes [1–5], which is significantly more rapid than other isothermal assays [6–9] or PCR. RPA employs a number of biochemical mechanisms to allow for this rapid and sensitive amplification. It utilizes a recombinase to facilitate the insertion and binding of oligonucleotide primers to their complementary sequence within a double-stranded DNA molecule [1]. Opposing primers allow for the exponential amplification of a defined region of DNA in a manner similar to PCR. An oligonucleotide probe with a specific abasic nucleotide analogue is recognized and cleaved by endonuclease IV (nfo) or exonuclease III (exo) but only when the probe is bound to its complementary target sequence. This allows detection of amplification with Immunochromatographic strips (ICS) via nfo or real time fluorescent detection using exo probes [1].

To facilitate amplification, a high molecular weight polyethylene glycol (PEG) is used as a component of the RPA formulation [10]. Crowding agent agents have been shown to modulate the efficiency of different biochemical processes, including an enhancement of enzyme catalytic activity [11–13]. When used as an additive in PCR and RT-PCR, crowding agents have been shown to improve efficiency, specificity and sensitivity [14;15]. Crowding agents are viscous and RPA utilizes relatively low temperatures (37–42°C) for amplification, reducing the mixing effects of thermal convection within the reaction. Both phenomena may combine to cause the localized depletion of reagents in areas of high RPA activity within a reaction, therefore restraining the amplification cascade [16]. Therefore, the TwistDx protocol includes a vigorous mixing step after 4–5 minutes [16]. This ‘mix step’ is highly recommended in reactions with low numbers of target sequences and the optimum time to mix can vary depending on a number of factors including the length of the target amplicon. In a recent study, Kalsi *et al.* demonstrated that continuous mixing of microdroplets from an RPA exo (fluorescence based) assay led to a faster time to amplification, increased fluorescence and improved sensitivity [5]. However, the effect of mixing during RPA has not been examined with RPA assays that uses nfo chemistry to detect amplification on ICS. A number of devices specifically designed to be used with RPA are available, including the Twista® and the Twirla™. The Twista® is a small real time fluorescence reader that can be used to incubate RPA reactions at their optimal temperature and includes a “remind to mix” alarm that sounds continuously after a specified time period, indicating that the user should remove the strip, shake vigorously and return to the device. The Twirla™ is a standalone battery-powered incubator with a magnetic stirrer that mixes the assay at user-specified time intervals when a steel bead is added to the reaction mixture, removing the need for the user to remember to manually shake the reactions.

The ability to amplify low concentrations of DNA and RNA in a short time without complex instrumentation make RPA a tool suitable for use at point of care in resource limited settings (RLS). However, some of the challenges that nucleic acid based diagnostics face in RLS may be of concern with RPA. First, the necessity of an interval mix step may be difficult to implement consistently. Incubating devices used to heat the assay generally do not have the capacity to shake the reactions automatically, and thus manual intervention is required. Inconsistent adherence to the shake step could lead to user-dependent variability in assay results. Secondly, the storage of RPA reagents outside of a cold chain may affect their performance. To date, we are not aware of published assessment of RPA stability. The manufacturer's instructions recommend storing RPA reagents at -20°C , which is not possible in many LRS [17;18]. In one study on the characteristics of peripheral microscopy centers in 22 countries, it was found that only 18% had an uninterrupted power supply and only 27% had refrigerators [19]. Thus, it is important to assess whether RPA reagents are stable and can produce consistent results after exposure in storage at a wide range of ambient temperatures for long periods of time.

We previously described the development of an RPA assay for rapid diagnosis of HIV-1 in infants that showed high sensitivity and specificity across diverse subtypes of HIV-1 [2;20]. In the present study we investigated the impact of the recommended "mix" step on the performance of low copy detection with our HIV-1 assay. Our results indicate that while mixing is a necessary step for optimum RPA performance, reducing the assay volume can obviate the need for mixing. Furthermore, we evaluated the stability of RPA reagents outside of cold chain conditions by storing them at -20 , 25 and 45°C for up to 12 weeks. Reactions stored at -20°C and 25°C retained the ability to detect low HIV DNA concentrations for 3 months, while reagents stored at 45°C performed optimally for up to 3.5 weeks.

2. Materials and methods

2.1. Reagents and genetic material

TwistAmp exo and nfo RPA reactions were supplied by TwistDx Ltd., Cambridge, United Kingdom. Oligonucleotide primers were purchased from Integrated DNA Technologies (IDT, Coralville, USA) and oligonucleotide probes from Biosearch Technologies (Novato, USA). HIV-specific primers and probe previously described [2] were modified here for optimized performance, and the newly designed HIV *pol* primers and probe are referred to as the Twist Alpha HIV-1 assay [2]. For reactions incubated in the TwirlaTM, a steel ball bearing was added prior to the addition of magnesium acetate. HIV-1 proviral target DNA was from the ACH-2 cell line that contains a single full-length integrated copy of HIV-1 *Bru* (subtype B; GenBank accession number K02013.1) DNA per cell [21] and was prepared and quantified as previously described [2]. Purified human genomic was procured from Promega Corp. (Madison, USA). Individual reactions with final volumes of $50\text{ }\mu\text{L}$ were prepared according the manufacturer's instructions with the sequential addition of rehydration buffer ($37.5\text{ }\mu\text{L}$), nuclease-free water and then DNA template (to a final volume of $47.5\text{ }\mu\text{L}$) to the reagent pellet. A further $2.5\text{ }\mu\text{L}$ volume of magnesium acetate was added to the lid of each reaction tube, the tubes were then sealed and mixed, via brief vortexing, and spun down before being immediately placed into the incubation source. To create $5\text{ }\mu\text{L}$ final reaction

volumes, single 50 μ L RPA reactions were prepared as described and stored on ice to prevent amplification; aliquots of 5 μ L were then transferred into 200 μ L PCR tubes and then placed into the incubator.

2.2. Mixing of RPA reactions

Twist Alpha HIV-1 RPA reactions containing 100, 50, 25, 10, 5 and 1 HIV-1 copies per either 50 μ L or 5 μ L reaction volumes were prepared. Negative template controls (NTC) consisted of human genomic DNA (gDNA) used in the diluent or nuclease free water. Replicates of eight 50 μ L reactions in were incubated in either a Twista[®] real time reactor or in a Twirla[™] mixing incubator (TwistDx Ltd., Cambridge, United Kingdom). The 50 μ L reactions incubated in the Twista[®] were heated at 39°C for 20 minutes and reactions were assessed in the absence or inclusion of a mix step after 5 minutes. Further 50 μ L reactions were incubated in the Twirla[™] for 20 minutes at 39°C with a 1 second mix every minute. Finally, 5 μ L reactions were incubated at 39°C in the Twista[®] device for 20 minutes without a shake step.

2.3. Immunochromatographic strip detection

After incubation, the RPA nfo reactions were immediately placed on ice before addition of 5 μ L EDTA (250 mM) to terminate each reaction. The detection of hapten-labeled RPA amplicons was assessed with immunochromatographic strips (ICS) purchased from either Milenia Biotech GmbH (Giesen, Germany) or Ustar Biotechnologies (Hangzhou, China). The intensity of the test lines on the ICS were analyzed using ImageJ (NIH) [22] by measuring average pixel intensity across the length of the strip.

2.4. Stability study

Customized nfo reagents with the Twist Alpha HIV-1 primers included in the lyophilized reaction pellets (primer-in) were procured from TwistDx Ltd., Cambridge, United Kingdom. Reagents were then incubated at -20 °C, 25 °C and 45 °C for a 12 week period, with a strip of 8 reaction tubes from each incubation temperature tested at twice-weekly intervals for the first 4 weeks, and every 2 weeks thereafter. RPA reagents were assessed for their ability to amplify 40, 20, 10 or 0 copies of HIV-1 DNA in replicates of two from each storage temperature. As a comparator, primer-free RPA reagent, where the primers were added to the RPA rehydration buffer prior to incubation were also stored at -20 °C, 25 °C and 45 °C and tested in parallel to the primer-in reagents. Temperature data loggers (LogTag, Auckland, New Zealand) were placed in each environmental chamber to confirm that temperatures as specified and to indicate any significant and sustained fluctuation ($\pm$ 1 °C) in temperature during the study.

3. Results

3.1. RPA requires mixing or reduced reaction volumes to detect low copy numbers of target DNA

In order to assess the impact of the mixing step on RPA, we used the Twist Alpha HIV-1 RPA assay on 100, 50, 25, 10, 5, 1 and 0 copies of HIV-1 DNA. Initial testing used the exo format for fluorescent detection to compare the recommended mix step at 5 minutes (Figure

1A) to RPA without mixing (Figure 1B). Reactions without mixing resulted in an atypical amplification curve with significantly lower fluorescence and a slower time to amplification at all HIV-1 DNA copy numbers tested (Figure 1).

Subsequent testing was performed using the Twist Alpha HIV-1 assay with nfo probes for ICS detection. Mixing was either every minute during incubation in the Twirla, after 5 minutes in the Twista, or no mixing was carried out at all. Reactions that were mixed either every minute, or only once after 4 minutes, were positive in 8/8 (100%) replicate RPA reactions containing 100, 50, 25 and 10 copies of HIV-1 DNA (Table 1). In contrast without mixing, reactions with 100 or 50 copies also amplified in all replicates, but only 6/8 (75%) or 5/8 (62.5%) of the replicates containing 25 or 10 copies, respectively, gave a positive result. In addition, the lack of a mixing step resulted in a still lower proportion of positives in reactions with only 5 or 1 copies of HIV-1 DNA as compared to the parallel low copy reactions that were mixed during incubation (Table 1).

In addition, RPA reaction volumes were reduced to 5 μ L and the mix step was omitted. Results of the 5 μ L reactions without mixing were similar to the results of 50 μ L reactions with mixing: 8/8 (100%) replicates positive in all reactions containing 10 HIV-1 copies, and a similar proportion of positives noted in reactions with 5 or 1 HIV-1 copies (Table 1).

We next analyzed and quantified the test stripe on each ICS using ImageJ (Figure 2). This detailed examination of ICS images indicated that the intensity of the test stripe was also affected by the inclusion of the shake step and the final reaction volume. At all levels of HIV-1 DNA copies tested, the median pixel density on the ICS was greatest in the reactions mixed every minute in the Twirla. The reactions mixed only at 5 minutes had the 2nd highest pixel density, followed by the 5 μ L reactions without mixing, while the 50 μ L reactions without shaking resulted in the lowest intensity bands (Figure 2).

3.2. RPA reagents are stable despite storage at high temperatures

Reduced infrastructure and lack of refrigeration equipment in many facilities in RLS may be problematic for temperature sensitive reagents, and this is a key barrier to point of care testing. Therefore, we sought to determine the ability of the HIV-1 RPA reagents to detect 40, 20 or 10 copies of HIV-1 DNA following storage for up to 12 weeks at 25°C or 45°C compared to storage as recommended at -20°C. As expected, all assays performed on sample with low copy HIV-1 DNA using reagents stored at -20°C remained were positive throughout the 12 weeks of storage and testing (Table 2). Reagents stored at 25°C showed similar stability, with all HIV+ samples detected throughout the 12 weeks of storage (Table 2). More remarkably, assays stored for up to 3 weeks at 45°C were able to accurately detect low copies of HIV-1 DNA in all tests, suggesting storage at high temperature for short periods of time does not cause loss of sensitivity. However, after 3.5 weeks at 45°C the “primer-in” strips (primers included in the lyophilized RPA pellet) did not detect 10 HIV-1 DNA copies in any replicates, with additional loss of sensitivity to detect 20 or 40 HIV-1 DNA copies after 4 weeks at 45°C. Concurrently, “primer-free” reagents (whereby the primers were prepared and stored at 45 °C in a liquid reaction master-mix with the rehydration buffer as opposed by being incorporated into the lyophilized RPA reagent pellet) were also incubated at 45°C. Primer-free reagents appeared to be more stable when stored at

45°C, with all HIV+ samples detected through the first 6 weeks of storage and testing, with a loss in ability to detect 10 HIV DNA copies after storage at 45°C for 8 weeks (Table 2). No false positive tests were recorded, indicating that prolonged storage under elevated temperatures does not cause a loss in specificity.

4. Discussion

RPA has been suggested as an ideal alternative to PCR for point-of-care diagnostics in resource limited settings because it does not rely on complex laboratory equipment and has a rapid time to detection [20;23]. However, concerns remain that user-dependent steps in the protocol and reagent storage at ambient temperatures may negatively affect test results. The requirement for a user dependent mixing step during incubation of RPA is cumbersome and may be difficult to consistently implement in the field. In this study we demonstrate that adherence to this mix step is vital to the assay performance, particularly in samples with very low concentrations of target sequence. When mixing was not performed during incubation in the Twista, both the intensity of detection stripes on ICS and real time fluorescent signal were noticeably impacted, which has implications for use of RPA for both qualitative and quantitative assays [24].

As a solution, we utilized an incubator with automatic mixing capabilities, the Twirla™ device, and note that this avoids reliance on the user to apply the essential mixing step. However, during initial trials performed with continuous mixing in the Twirla, the sensitivity of our assay was compromised (data not shown), and thus the ideal frequency of mixing may be assay dependent and should be determined prior to use. The Twirla cannot be used for fluorescence real time detection, limiting its use to end point detection, however TwistDx have recently released a new product, the T-8 isothermal device which combines the capabilities of the Twirla™ and the Twista® for assay incubation, automated mixing and real time detection of fluorescence-based real time RPA reactions. The same product can also be used to incubate nfo-based RPA reactions using end point analysis via ICS.

Alternatively, low volume (5 µL) RPA reactions appeared to bypass the need for mixing. We hypothesize that this is due an increase in the concentration of target DNA relative to the core RPA reagents in the smaller volume, leading to increased interactions with the reagents required for amplification. Using low volume RPA reactions in a diagnostic capability will be very dependent on target copy number in the greatly reduced sample volume that can be added. Therefore this approach is limited to highly sensitivity assays or samples with a high copy number of targets. Furthermore, while not assessed here, others have noted high concentrations of genomic DNA can negatively affect RPA performance [25], an effect which may be compounded in low volume assays.

The stability of RPA reagents after storage at temperatures of 25°C and 45°C for up to 12 weeks was also assessed. We found that storage at 25°C for up to 12 weeks and 45°C for up to 3 weeks had no detectable impact on the performance of the assay. However reagents stored at 45°C for more than 3 weeks had reduced sensitivity. The primer-free reactions were stable for longer periods of time at 45°C compared to the primer-in reactions, suggesting decay of the primers and/or probe. We hypothesize that this may be due to residual nuclease

activity at high temperature in the lyophilized pellets gradually digesting the pool of oligonucleotides to suboptimal amounts for RPA.. TwistDx are now investigating methods to better stabilize the oligonucleotides within lyophilized reaction pellets. While storage at 45°C did have a negative effect on RPA performance over the 12 week period, the reagents are not expected to be stored at these temperatures for long extended periods. In a previous study we noted that daily mean afternoon ambient temperatures in sub-Saharan Africa are rarely above 43°C, and that many regions rarely exceed temperatures of 30°C [20]. These regions are also subject to high humidity levels, however, as RPA reagents are stored in water impermeable heat-sealed aluminum foil pouches with desiccant, we suspect that humidity levels will not influence the long term stability of RPA reagents.

In conclusion, we have demonstrated that while RPA requires mixing during incubation, the dependence on the user to perform this mixing step at a specified time can be bypassed by either using incubators with automatic mixing capabilities or by reducing the reaction volume. These alternatives may improve the reliability of test results from RPA assays performed at the point of care. In addition, we demonstrate the first stability study of storing RPA reagents at elevated temperatures. Our findings suggest that RPA reagents do not require cold chain storage for short term transport at up to 45°C and will perform satisfactorily even if exposed to elevated temperatures for short periods of time. These results overall provide further evidence of the potential for RPA-based assays to be used as diagnostic tools in RLS.

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Highlights

- Mix step during RPA incubation is vital for optimum assay performance
- Lower reaction volumes remove the need for mixing during incubation
- RPA reagents are stable at 45°C for 3 weeks and at 25°C for 3 months

Fig. 1.
The effect of mixing on RPA exo assays for the fluorescent detection of HIV-1 DNA at 100, 50, 25, 10, 5, 1 and 0 copies of HIV-1 DNA. A: Reactions mixed at 5 minutes. B: Reactions left unmixed.

Fig. 2.

The median pixel density measured from positive test lines of ICS using RPA HIV-1 reactions incubated with shaking or without shaking. Key: ◆ 50 µL Reactions incubated in the Twirla™, ■ 50 µL Reactions incubated in the Twista® with a mix at 5 minutes, ▲ 50 µL reactions incubated in the Twista® with no mix step, × 5 µL reactions incubated in the Twista® with no mix step.

Table 1

The effects of various interval mixing approaches and total reaction volumes on the limit of detection of HIV-1 DNA by an HIV-1 RPA assay. Each reaction was incubated for 20 mins at 39°C as follows: A) 50 µL Reactions incubated in the Twirla™ with a mix every one minute, B) 50 µL Reactions incubated in the Twista® with a mix at 5 minutes, C) 50 µL reactions incubated in the Twista® with no mix step, D) 5 µL reactions incubated in the Twista® with no mix step.

	A	B	C	D
Reaction volume	50 µL	50 µL	50 µL	5 µL
Mix times	Every 1 min.	Mix at 5 min.	No mix	No mix
Copy #	# positive/# tested	# positive/# tested	# positive/# tested	# positive/# tested
100	8/8	8/8	8/8	8/8
50	8/8	8/8	8/8	8/8
25	8/8	8/8	6/8	8/8
10	8/8	8/8	5/8	8/8
5	7/8	6/8	3/8	7/8
1	5/8	2/8	2/8	4/8
gDNA	0/8	0/8	0/8	0/8
NTC	0/8	0/8	0/8	0/8

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Table 2

A comparison of the performance of HIV-1 RPA assays for the amplification and detection of HIV-1 proviral DNA after storage at temperatures of -20 °C, 25 °C and 45 °C.

Storage	-20°C				25°C "Primer In"				45°C "Primer In"				45°C "primer free"			
	Control				Room temperature				Extreme Temperature				Extreme temperature			
Week	40 copy	20 copy	10 copy	NTC	40 copy	20 copy	10 copy	NTC	40 copy	20 copy	10 copy	NTC	40 copy	20 copy	10 copy	NTC
0	4/4	4/4	4/4	0/4	-	-	-	-	-	-	-	-	-	-	-	-
0.5	-	-	-	-	-	-	-	-	2/2	2/2	2/2	0/2	2/2	2/2	2/2	0/2
1	2/2	2/2	2/2	0/2	2/2	2/2	2/2	0/2	2/2	2/2	2/2	0/2	2/2	2/2	2/2	0/2
1.5	-	-	-	-	-	-	-	-	2/2	2/2	2/2	0/2	2/2	2/2	2/2	0/2
2	2/2	2/2	2/2	0/2	2/2	2/2	2/2	0/2	2/2	2/2	2/2	0/2	2/2	2/2	2/2	0/2
2.5	-	-	-	-	-	-	-	-	2/2	2/2	2/2	0/2	2/2	2/2	2/2	0/2
3	2/2	2/2	2/2	0/2	2/2	2/2	2/2	0/2	2/2	2/2	2/2	0/2	2/2	2/2	2/2	0/2
3.5	-	-	-	-	-	-	-	-	2/2	2/2	1/2	0/2	2/2	2/2	2/2	0/2
4	2/2	2/2	2/2	0/2	2/2	2/2	2/2	0/2	2/2	0/2	0/2	0/2	2/2	2/2	2/2	0/2
6	2/2	2/2	2/2	0/2	2/2	2/2	2/2	0/2	1/2	0/2	0/2	0/2	2/2	2/2	2/2	0/2
8	2/2	2/2	2/2	0/2	2/2	2/2	2/2	0/2	1/2	0/2	0/2	0/2	2/2	2/2	1/2	0/2
10	2/2	2/2	2/2	0/2	2/2	2/2	2/2	0/2	0/2	0/2	0/2	0/2	2/2	2/2	0/2	0/2
12	2/2	2/2	2/2	0/2	2/2	2/2	2/2	0/2	0/2	0/2	0/2	0/2	2/2	1/2	0/2	0/2