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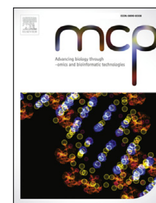

Original research article

# 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 min 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 min 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 μL to 5 μL 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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## 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 min [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]. Crowing 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

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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 min [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 Twirla™, 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 μL were prepared according the manufacturer's instructions with the sequential addition of rehydration buffer (37.5 μL), nuclease-free water and then DNA template (to a final volume of 47.5 μL) to the reagent pellet. A further 25 μL 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 μL final reaction volumes, single 50 μL RPA reactions were prepared as described and stored on ice to prevent amplification; aliquots of 5 μL were then transferred into 200 μL 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 μL or 5 μL 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 μL reactions in were incubated in either a Twista® real time reactor or in a Twirla™ mixing incubator (TwistDx Ltd., Cambridge, United Kingdom). The 50 μL reactions incubated in the Twista® were heated at 39 °C for 20 min and reactions were assessed in the absence or inclusion of a mix step after 5 min. Further 50 μL reactions were incubated in the Twirla™ for 20 min at 39 °C with a 1 s mix every minute. Finally, 5 μL reactions were incubated at 39 °C in the Twista® device for 20 min without a shake step.

### *2.3. Immunochromatographic strip detection*

After incubation, the RPA nfo reactions were immediately placed on ice before addition of 5 μL 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 (Gieβen, 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

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