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A mathematical model of recombinase polymerase amplification under continuously stirred conditions

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ABSTRACT

Growing interest surrounds isothermal PCR techniques which have great potential for miniaturization for mobile diagnostics. Particularly promising, Recombinase Polymerase Amplification (RPA), combines this advantage of isothermal PCR with simplicity and rapid amplification. A mathematical model is presented of Recombinase Polymerase Amplification (RPA) and compared to experimental data. This model identifies the rate limiting steps in the chemical process, the effects of stirring, and insights in to using RPA for quantitative measurement of initial DNA concentration. Experiments are shown in which DNA amplification occurs under conditions of Couette flow and conditions of rotational turbulent flow. Hand mixing has been shown to dramatically shorten amplification times but introduces unpredictable variability. In some cases, this variability manifests itself as human error induced false negatives, a serious problem for all potential applications. Mechanical stirring demonstrates similarly short delay times while retaining high repeatability and reduces the potential for human error.

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1. Introduction

Polymerase Chain Reaction (PCR) has revolutionized molecular biology and has proven itself as one of the fastest and most specific methods of disease detection. In many places around the world, the sparsity of hospitals and clinics make developing point of care procedures a necessity. For PCR, the requirement for bulky and expensive thermocyclers remains one of the main obstacles for point of care use. To address this issue, many are turning towards isothermal PCR for the answer. A technique that does not require thermocycling to denature double stranded DNA but instead only requires a thermal bath lends itself much more readily to point of care diagnostics.

Several isothermal techniques have emerged since the development of PCR. The most widely used are rolling-circle amplification, helicase-dependent amplification (HDA), Loop Mediated Isothermal Amplification (LAMP), and Recombinase Polymerase Amplification (RPA).

Amplification times for Rolling circle generally take 65 min [1]. In addition to this 65 min process, it also requires significant preparation steps beforehand that can significantly drive up the total time and overall complexity. The simplest and most efficient method for generating a suitable circular template, using a padlock probe, requires carefully designed oligomers and limits the amplification to small target regions [2].

Helicase-dependent amplification employs a helicase to unzip the double stranded DNA template to accommodate primer annealing. Helicase activity, however, requires bubbles to form naturally at AT rich regions [3]. The high GC content in Mycobacterium and other bacteria, in many cases, would inhibit this technique. Another drawback of HDA is that optimal amplification only occurs for short sequences of 80–120 bp [4].

Loop Mediated Isothermal Amplification (LAMP) has the benefit of being a simple method to perform from a kit but is deceptively complicated. It requires 4 primers that need to bind at 6 locations. The DNA regions around the primers must form loops without hairpins or other secondary structure so primer design is no trivial task [5]. Even though it does not require a high rate of thermocycling, it does require an initial denaturation step at 95° Celsius before amplifying at 65° C [6]. Amplifications with this method generally require 30–60 min [6].

A newly developed method called Recombinase Polymerase Amplification (RPA) shows great promise especially for point-of-care diagnostics. RPA uses recombinase to insert primers in to double stranded DNA rather than denaturing DNA using high temperature cycling [7]. This allows RPA to operate at a con-

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Nomenclature

G	Single stranded DNA binding protein (Gp32)
m	Number of Gp32 binding sites on a primer
R	Recombinase complex (UvsX.6*UvsY)
FG	Forward primer/Gp32 complex
n	Number of recombinase binding sites on a primer
H_3PO_4	Inorganic phosphate
FR'	Unstable forward primer/recombinase complex
FR	Stable forward primer/recombinase complex
FnR'	Forward primer complexed with unstable filament of n recombinase molecules
FnR	Forward primer complexed with unstable filament of n recombinase molecules
D	DNA template
FD	Forward primer/DNA complex
P	Polymerase
PFD	Polymerase/forward primer/DNA complex
B	Number of base pairs in template
np	Number of base pairs in primer
Sf	Empirical scaling factor to modify diffusion limited rate constants
$t_{\frac{1}{2}}$	Time required for DNA concentration to reach 50% of saturation
k_1	Equilibrium constant for Eq. (1) $10^5 M^{-1}$
k_{1f}	Forward rate constant for Eq. (1) $5 \times 10^8 \left(1/(M \times s)\right)$
k_{1r}	Reverse rate constant for Eq. (1) $5 \times 10^3 1/s$
k_{eq2a}	Equilibrium constant for Eq. (2a) $68 \times M^{-1}$
k_{eq2af}	Forward rate constant for Eq. (2a) $10^8 \left(1/(M \times s)\right)$
k_{eq2ar}	Reverse rate constant for Eq. (2a) $1.471/s$
k_{2b}	Rate constant for Eq. (2b) $47 \times 10^{-3} 1/s$
k_{eq2c}	Equilibrium constant for Eq. (2c) $3 \times 10^6 M^{-1}$
k_{eq2cf}	Forward rate constant for Eq. (2c) $10^8 \left(1/(M \times s)\right)$
k_{eq2cr}	Reverse rate constant for Eq. (2c) 33 1/s
k_{2d}	Rate constant for Eq. (2d) $4.6 \times 10^{-3} 1/s$
KM_{3a}	The Michaelis constant for Reactions (3) and (4a) $20.35 \times 10^{-6} M$
k_{3f}	Forward rate constant for Eq. (3) $10^8 \left(1/(M \times s)\right)$
k_{3r}	Reverse rate constant for Eq. (3) $59.37 \left(1/s\right)$
k_{4acat}	Forward rate constant for Eq. (4a) $4.22 \left(1/s\right)$
k_{4bcat}	Forward rate constant for Eq. (4b) $8.32 \left(1/s\right)$
k_{5f}	Forward rate constant for Eq. (5) $1.2 \times 10^7 \left(1/(M \times s)\right)$
k_{5r}	Reverse rate constant for Eq. (5) $0.06 \left(1/s\right)$
k_{6f}	Forward rate constant for Eq. (6) $87 \left(1/s\right)$
k_7	Forward rate constant for Eq. (7) $4.1 \left(1/(M \times s)\right)$
k_8	Forward rate constant for Eq. (8) $1.13 \left(1/(M \times s)\right)$

stant temperature of around degrees celsius—much lower than other isothermal techniques like LAMP [8]. Using the tubescanner from TwistDx, the instrumentation costs for RPA are significantly cheaper than those for either PCR or LAMP and include fluorometers for real time detection [8]. It only requires two primers that, with a length of 30–35 base pairs, are only modestly larger than PCR primers [9]. RPA also boasts shorter amplification times than LAMP. In combination with real time fluorescent measurements, a result can be determined in approximately 10 min [7]. Remarkably, the rapid rate of amplification does not come at the expense of reduced sensitivity. RPA can amplify as few as 10 initial copies of template—a detection threshold similar to that of traditional real time PCR [7]. While many detailed models have been presented for the other isothermal PCR methods mentioned, RPA differs from them in some key ways that warrant special attention. To compare the model with data, the experimental methodology must minimize the effect of poorly repeatable inputs. One example of this would be hand mixing.

The current methodology recommended by the supplier of RPA technology, Twistamp, requires two hand mixing steps. Our experience has shown that these mixing steps introduce a significant amount of variability in results. The tubes must be mixed both at the start of the process and after 4 min. The removal of the tube for mixing at 4 min creates a gap in data collection at a crucial point in the amplification process partly defeating the purpose of real time detection. To make matters worse, if the tubes are insufficiently mixed at 4 min, the samples will not amplify or amplification will be stunted. Another issue arises when, during the mixing step, droplets stick to the lids of the tubes artificially reducing the signal levels. These sources of human error make having a skilled operator imperative and confounds efforts to compare results obtained by different investigators. While these mixing steps are error prone and introduce significant randomness, without them the process takes several times as long to give a result. In this paper, we will demonstrate an automated stirring process that eliminates these sources of human error and simplifies the process to make it more easily employed in field applications. Automated stirring has been demonstrated before using lab-on-a-chip technology [10], but our method is more accessible to a wide range of users without buying specialty disposable instrumentation. Initially, our procedure used an ordinary benchtop drill press to stir the reaction mixture. The equipment was then scaled down to use instead a small RC motor. The only part of the set-up that needed replacement after each test was the drill bit/stir rod. Not only does this motor take up negligible space (about as much as a 1.5 ml tube) but its low cost also makes an array of 8, one for each tube, quite feasible even in low budget applications.

This stirring method, by removing the arbitrary element of hand shaking and moving the reaction in to a kinetically controlled regime, made the reaction more conducive to mathematical modeling. We herein present, to our knowledge, the first mathematical model specifically addressing the unique considerations of RPA.

2. Materials and methods

2.1. Amplicon design for real-time results

DNA was extracted from *Mycobacterium smegmatis* and *Escherichia coli* (as a negative control) using the phenol-chloroform method. The amplicon for the detection of the *Mycobacterium Smegmatis* DNA was designed using the sequence IS1096. The RPA amplicon was 116 base pairs long. SYBR green was used instead of designing a specific probe. The forward and reverse primers were:

IS1096F: 5′-CTCATCGAACATTCCCGCGAACACGTTCCGACCAG-3′
IS1096R: 5′-CTTGACGGTGTAGAGACGATCAGCTGCTTTCGC-3′

2.2. RPA conditions and detection

RPA was performed using the TwistAmp Basic kit, having a 50 μl volume. Reaction mixtures were formulated according to TwistDx Ltd. recommendations with one of notable change. Instead of using probes for detection, 5× concentrated SYBR Green was added to each reaction mixture. The DNA concentration was standardized at 0.1 ng/μl for all tests. Once all components were added, the tubes were vortexed and centrifuged; a solution of 14 mM Mg acetate was then pipetted into each reaction tube which was then immediately put into the tubescanner device. The reaction components were