A stoichiometric and pseudo kinetic model of loop mediated isothermal amplification

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Abstract
Loop mediated isothermal amplification (LAMP) is one of the most popular isothermal DNA amplification techniques for research and commercial applications. The LAMP mechanism is powered by strategic primer design and a strand displacement polymerase, generating products that fold over, creating loops. LAMP leads to generation of products of increasing length over time. These products containing multiple loops are conventionally called cauliflower structures. Existing literature on LAMP provides extremely limited understanding of progression of cascades of reactions involved in the reaction and it is believed that cauliflower structures of increasing length constitute a majority of the product formed in LAMP. This study presents a first of its kind stoichiometric and pseudo kinetic model to comprehend LAMP reactions in deeper depth by (i) classifying LAMP reaction products into uniquely identifiable categories, (ii) generating a condensed reaction network to depict millions of interconnected reactions occurring during LAMP, and (iii) elucidating the pathways for amplicon generation. Despite the inherent limitations of conventional stoichiometric modelling for polymerization type reactions (the network rapidly becomes too large and intractable), our model provides new theoretical understanding of the LAMP reaction pathway. The model shows that while longer length products are formed, it is the smaller length recycle amplicons that contribute more towards the exponential increase in the amount of double stranded DNA. Prediction of concentration of different types of LAMP amplicons will also contribute substantially towards informing design of probe-based assays.

1. Introduction
Loop-mediated isothermal amplification (LAMP) has been identified as a powerful isothermal nucleic acid amplification (NAA) technique over the past few decades. It has been a very popular choice among researchers developing isothermal NAA based technologies[1–4] and has also been recognized as a robust NAA technique for tuberculosis diagnosis by the World Health Organization[5]. A literature search conducted on Web of Science revealed that over the last 5 years, LAMP has been the most frequently used technique for isothermal NAA with ~4x more publications compared to the second most popular technique (ESI Fig. S1). It has emerged as a popular technique to enable PCR-free molecular diagnosis of COVID19[6,7].

The mechanism for LAMP has been explained in detail by Notomi et al.[8] and some online animations are also available [9,10]. LAMP, as invented in 2000 by Notomi et al.[8], included four primers – a pair of inner primers (forward inner primer, FIP, and backward inner primer, BIP) and a pair of outer primers (forward outer primer, F3, and backward outer primer, B3). These four primers span six different regions on the target DNA conventionally marked as F1c, F2c, F3c, B1, B2, and B3 on the sense strand of the target DNA as marked in LAMP tree structure (Fig. 1). Briefly, in general the reaction operates at a temperature in the range of 60-

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**New abbreviations used in this manuscript**

1. SL (Single Loop)
2. T (Terminated)
3. PDS (Partially Double Stranded)
4. cPDS (child Partially Double Stranded)
5. SS (Single Stranded)
6. SSD (Single Stranded Dumbbell)
7. SPK (Stoichiometric and Pseudo Kinetic)
8. c-SPK (compressed Stoichiometric and Pseudo Kinetic)
9. PBE (Primer Based Extension)
10. CB (Color of Band)
65°C at which the DNA target is spontaneously breathing at multiple locations. This creates regions of weak or broken hydrogen bonds between the two strands of template DNA, creating single-stranded pockets where primers can anneal. FIP partially anneals to its complementary sequence (F2c) on the sense strand and this is followed by extension using a strand displacement polymerase (Fig. 1 (i)). F3 anneals to its complementary sequence (F3c) and extension by the polymerase displaces the newly synthesized strand (Fig. 1 (i)). BIP then anneals to B2c on the newly synthesized strand and is extended by the polymerase (Fig 1 (ii)). B3 displaces this strand (Fig. 1 (ii)) and a dumbbell structure (Fig 1 (iii)) is created along with a double-stranded product. The concentration of inner primers in LAMP reactions is usually four to eight times the concentration of outer primers. This ratio of inner and outer primers’ concentration plays a major role in driving LAMP reactions towards formation of the dumbbell structure, which acts as the template required for initiating the exponential phase (Fig. 1 (iv)) of LAMP reactions. In 2002, Nagamine et. al[11] introduced a pair of loop primers in addition to the existing four LAMP primers to accelerate the kinetics and increase the yield of LAMP reactions.
One of the most noteworthy features of LAMP is the formation of increasing length amplicons, popularly called as cauliflower structures. The mechanism of LAMP, governed by primer sequence design and absence of exonuclease activity in the strand displacement polymerase, results in extension of existing amplicons over time to form products of larger and larger length. This is demonstrated by gel electrophoresis analysis of LAMP amplicons displaying ladder-like patterns containing products ranging from less than 100 base pairs up until the wells in agarose gels. This is in contrast to the gold standard polymerase chain reaction (PCR), in which the amplicon size is known a priori and it is straightforward to estimate the concentration and
structure of amplicons formed at the end of the reaction, i.e. when all primers are utilized. Such estimations are not possible for LAMP as LAMP amplicons are not always completely double-stranded and linear. They are rather a combination of intricately coiled single and double-stranded segments, which is confirmed by smudges throughout the gel lanes apart from a few crisp bands at certain specific lengths. This extraordinary element of LAMP reactions creates multiple primer annealing sites on longer length amplicons. The inner primers and loop primers can anneal at numerous locations on each amplicon generating an extremely heterogeneous soup of products. This complex mechanism has limited the theoretical understanding of LAMP reaction pathways.

Most of the available literature on LAMP mechanism depicts a few steps into the exponential stage[12]. There have been theoretical studies to understand the kinetics and mechanisms of PCR [13] and isothermal NAA techniques like isothermal strand displacement amplification (iSDA)[14]. But to the best of our knowledge, detailed studies focussing on mechanistic understanding of generation of amplicons in LAMP do not exist. Extremely limited literature existing on theoretical knowledge about LAMP includes development of a method for better quantification of rise times for LAMP reactions[12], demonstration of the effect of internal primer-template mismatches on LAMP efficiency[15], and observation of the impact of primer dimers and self-amplifying hairpins on LAMP reactions and proposing a thermodynamic parameter to predict non-specific amplification for different sets of LAMP primers[16]. A recent study presented a mathematical model to reduce false-positive diagnosis by LAMP by predicting the expected size of amplicons and comparing it with the results obtained from electropherograms [17]. The authors presented a new method to analyse the increasing length of LAMP amplicons. But they restricted their analysis to only one class of amplicons termed as ‘stem and loop structures’, which as they acknowledge, does not include all different types of amplicons produced during LAMP. Further, their analysis was restricted to products of less than 1500 bp (a maximum of 7 stem and loop structures), whereas LAMP produces amplicons of several thousand base pairs in length. Nonetheless, this study demonstrated the utility of developing a detailed understanding of LAMP mechanism and predicting the sizes of amplicons generated by LAMP.

In this study, we present the first attempt at developing a stoichiometric and pseudo kinetic (SPK) model to provide deeper insights into the cascades of reactions occurring in LAMP with four primers (FIP, BIP, F3 and B3). This was accomplished by first recognizing that the very large number of LAMP amplicons can be categorized into four exclusive structural groups and the complex LAMP reaction mechanism can be condensed into a compact reaction network between these four groups. The different pathways of the condensed reaction network were modelled to develop an SPK model. Given the hundreds of distinct length LAMP products and the stochasticity in their formation, it was not possible to develop a comprehensive kinetic model of LAMP governed by the law of mass action. We therefore developed a “pseudo” kinetic model, i.e. the only time scale in the model emerges from the rate of nucleotide incorporation by the polymerase. The extension rates are considered to be independent of the concentration of free enzyme, nucleotides, and primers. This assumption, in fact, holds true in early stages of the LAMP reaction when there are no limiting reagents. This model presents unprecedented insights into the structures of LAMP amplicons and reaction pathways. It successfully predicts rise times of real-time LAMP amplification curves in close agreement with experimentally measured rise times. The model can be used to predict the concentration profiles of the different amplicon types generated during LAMP, which can inform design of downstream detection strategies.
2. Results and discussion

2.1 Classification of LAMP amplicons

Analysis of the LAMP tree structure (Fig. 1) revealed that there are only two pathways by which a reactant DNA molecule can generate further products: a) primer-based extension, and b) self-extension. Primer-based extension involves the annealing of inner primers (FIP or BIP) to their complementary region on the reactant DNA molecule, followed by extension by DNA polymerase. Self-extension is facilitated by creation of patches of complementary regions within a single DNA molecule. DNA strands loop around to anneal with their complementary sequences and the free 3’ ends are extended by DNA polymerase. Each amplicon formed during LAMP can adopt either of these two strategies for generating subsequent products. The type of reactant and the choice of pathway determines the type of product formed.

Careful investigation of literature brought to our attention that a method to classify LAMP amplicons according to their structure does not exist. This motivated us to study all possible structures of LAMP amplicons; we concluded that all LAMP amplicons may be categorized into four structural categories. Fig 2 shows representative structures for each category of amplicons and the unique identifiers for each category are explained below:

(i) Single-loop (SL) amplicon (Fig 2A): This amplicon type contains only one single-stranded loop and the remaining part of the amplicon is double-stranded. The single loop of the SL amplicon acts as a primer annealing site.

(ii) Terminated (T) amplicon (Fig 2B): This amplicon type is entirely double-stranded. We assume that terminated amplicons do not participate in further reactions.

(iii) Single-stranded (SS) amplicon (Fig 2C): This amplicon type is predominantly single-stranded with short, discontinuous double-stranded sections restricted to B1/B1c and F1/F1c sequences. SS amplicons consist of multiple single-stranded loops that could be sites for potential annealing of primers. The 3’ end of SS amplicons can be extended by the polymerase via the self-extension pathway.

(iv) Partially double-stranded (PDS) amplicons (Fig 2D): This amplicon type has a combination of single-stranded and double-stranded regions. These amplicons consist of single-stranded regions including multiple loops that could be potential sites for primer annealing along with a continuous stretch of double-stranded section. The 3’ end of PDS amplicons can be extended by the polymerase via the self-extension pathway.

Figure 2. Classification of LAMP amplicons into four categories. (A) Single loop amplicon (SL), (B) Terminated amplicon (T), (C) Single-stranded amplicon (SS), and (D) Partially double-stranded amplicon (PDS).
2.2 Condensed reaction network
Another novel feature of this study is creation of a compact representation of the complex LAMP reaction network. We investigated each reaction pathway in detail and found that the expanding reaction network could be expressed as a simplified network of reactions between the four amplicon categories. In order to understand the mechanisms of amplicon generation as captured by the condensed reaction network, consider an illustration for a 5-loop long SS amplicon (Fig. 3 SS(i)). The length of SS was chosen to ensure that this reactant SS amplicon and the products formed from it could follow all possible subsequent pathways. The fate of each type of amplicon is discussed next.

2.2.1 The SS amplicon: The SS amplicon has three possible fates:
I) The 3’ end of SS(i) amplicon can undergo self-extension to generate the corresponding SL amplicon (Fig. 3 SL(i)).
II) A primer can anneal at its 3’ end to generate a T amplicon (Fig. 3 T(i)); and
III) A primer can anneal to any loop other than the 3’ terminal loop to generate a PDS amplicon (Fig. 3 PDS (i)). For an SS amplicon to form a PDS amplicon, it should have at least three single loops. An SS with only two single loops will be the dumbbell structure that can only form an SL and a T amplicon.

2.2.2 The SL amplicon: The SL amplicon (Fig. 3 SL(i)) has a single fate; it can only undergo primer annealing at a single location to generate a PDS amplicon (Fig. 3 PDS(iii)) in which a part of the double-stranded region of the SL opens up to become single-stranded while primer extension creates another section of double-stranded DNA.
Figure 3. Illustration of all LAMP reaction pathways using amplicon structures. Roman numerals I, II and III represent the different types of reactions each type of amplicon can undergo. PBE refers to primer-based extension.

2.2.3 The PDS amplicon: PDS is the most versatile amplicon type as it can form all other types of amplicons. Like SS, it also has three fates:

I) Self-extension of 3’ end of PDS amplicon leads to generation of an SL amplicon (Fig. 3, SL(i) from PDS(i) and SL(ii) from PDS(ii)) along with displacement of an SS amplicon (Fig. 3, SS(ii) from PDS(i), SS(iii) from PDS(ii) and SS(iv) from PDS(iii)). An interesting feature we discovered was that SS(iii) is an exact complement of SS(i) and this helped us create the SS cycle for the SPK model, as will be explained in subsequent sections.

II) Primer annealing to the 3’ end of a PDS leads to formation of T (Fig. 3, T(ii) from PDS(ii)) along with displacement of an SS;

III) Primer annealing at an internal site of a PDS leads to the formation of a sub-class of PDS products that we refer to as child PDS (Fig. 3, cPDS from PDS(ii)) amplicon along with displacement of an SS. For a PDS amplicon to form a cPDS amplicon, it requires
at least two single loops and a primer annealing site at any loop except the one at the terminal 3’ end. Primer annealing to the terminal single loop at the 3’ end of a PDS amplicon will generate a T amplicon and not cPDS.

The abovementioned understanding of the evolution of amplicons with time to generate different categories of products was used to generate a condensed reaction network for LAMP (Fig.4). SS amplicons can undergo three types of reactions: self-extension (SS_I) to form an SL, primer-based extension (SS_II) to form a T, and primer-based extension (SS_III) to form a PDS. SL amplicons only undergo primer-based extension (SL_I), resulting in formation of PDS amplicons. PDS amplicons can further undergo three types of reactions: self-extension (PDS_I) to form SL amplicons, primer-based extension (PDS_II) to form T, and primer-based extension (PDS_III) to form cPDS amplicons. An SS amplicon is generated by strand displacement whenever a PDS amplicon forms any other product. The suffix I, II and III used in Fig. 4 for different amplicon types are corresponding to the labelling of reaction pathways in Fig. 3 to aid the reader in better understanding of the condensed reaction network.

![Figure 4. Condensed reaction network for LAMP amplification pathways.](image)

**Figure 4.** Condensed reaction network for LAMP amplification pathways. Roman numerals I, II and III represent formation of different amplicon types from a particular starting amplicon type.

### 2.3 Brute force stoichiometric and pseudo kinetic model

The first version of the SPK model was a brute force model that tracked each amplicon molecule individually. It generated all possible products starting from the dumbbell structure, resulting in a growing reaction matrix over time (Fig. 5). Each row of the matrix in Fig. 5 represents one generation. All possible products from reactants in the current row (generation) are shown in the subsequent row. The dumbbell structure is represented by a first-generation SS in the first row (Fig. 5). The dumbbell can only produce two products of type SL and T, which appear in row 2. For ease of tracking, suffixes a, b and c are added to amplicon names depending on whether they are produced from an SS, SL or PDS parent amplicon, respectively. Therefore, the names of amplicons in row 2 are Sl_a and Ta (generated from an SS). Because
Ta does not participate in further reactions and SLa can only generate one product, row 3 only contains a PDSb (generated from an SL). PDSb, in turn, can generate three types of products that show up in row 4 – SS, SLb, and Tb. Note that the SS amplicons are not provided a suffix because they are only formed from PDS amplicons. Now at this stage, there are two amplicons in row 4 that can generate subsequent products. The products formed from these two are separated by a blank cell in row 5. Generation matrix in Fig.5 (for only 25 seconds of reaction time) demonstrates the rapid rate at which amplicons are generated in LAMP. Because this version of the model tracked each amplicon and stored the corresponding data, we faced processing speed and storage issues for reaction time of greater than two minutes. Although the brute force model was computationally too expensive for modelling reactions over meaningful time scales, the data on patterns for amplicon generation obtained from this model helped us understand the reaction pathways better, design methods to circumvent the need of analysing each amplicon individually, and build the current version of the SPK model. Detailed description of the brute force model has been provided in ESI Note S1 and Fig. S2.

**Figure 5. Generation matrix for a reaction time of 25 seconds using the brute force model.** Amplicon types have been color coded as green for SS, blue for SL, red for T and purple for PDS. Suffix a, b and c help in identifying the type of parent amplicon (SS, SL or PDS, respectively) from which product amplicon is generated. SLa: SL from an SS amplicon, SLb: SL from a PDS amplicon, PDSa: PDS from an SS amplicon, PDSb: PDS from an SL amplicon and PDSc: PDS from another larger PDS amplicon. Ta: T from an SS amplicon, Tb: T from a PDS amplicon. Blank cells separate amplicons generated from different parent amplicons.

### 2.4 Compressed stoichiometric and pseudo kinetic (c-SPK) model for LAMP

Inferences from the brute force model helped us generate a more refined model which replicated the condensed reaction network better and obviated the need to analyze each amplicon individually. Flow diagram in Fig. 6 explains the crux of the c-SPK model and the assumptions made for constructing this model are explained in ESI Note S2. Starting from the sense-strand dumbbell (SSD) structure, which is a special case of an SS amplicon containing only two loops (and has only two fates as opposed to three; see 2.2.1), we followed all amplicon generation pathways. The first feature of this network is the sense-strand dumbbell cycle (Fig. 6, shown in pink) which handles generation of all SSD amplicons throughout the reaction. We found that SSD is generated at all times during LAMP and each of those SSD molecules act as templates for initiating exponential amplification. In fact, at any stage of the reaction, there are cyclic reaction networks that regenerate products of equal length while simultaneously producing larger products. The larger products participate in additional cycles of equal size product generation while simultaneously generating even larger products, and so on. Once an
SSD self-extends it forms an equal length SL amplicon, which has only one fate and extends via primer-based extension to form a longer length PDS (orange colored PDS in SSD cycle). Because this PDS is not the same length as SSD and SL that participated in forming it, this PDS is fed to the SL-PDS highway in sister network 1(Fig. 6, shown in orange). But when this PDS self-extends to form a longer length SL (orange arrow leading into sister network 1), an SS amplicon (SSDc) is displaced which is complementary and equal in length to SSD. This SSD again generates an equal length SL (SLc) followed by a larger length PDS. The second, larger length PDS is handled by SL-PDS highway in sister network 2. The SS displaced this time is identical to the SSD with which the SSD cycle began and hence this cycle continues over time. Since the first two PDS amplicons formed during SSD cycle form at different points in time, they are handled by two different sister networks to conveniently track the time of formation of all subsequent amplicon types (see ESI Note S3, Note S4 and Fig. S4, S5, S6, S7 and S8 for definition of length of each amplicon type and calculation of number of nucleotides added to form new amplicons in LAMP).

The SL-PDS highway handles increasing length amplicons and whenever a new, larger length PDS is generated, it is fed to the SL-PDS highway. Let us start by following the SL that formed from the PDS in the SSD cycle (SL on the top right corner of Sister Network 1). This SL forms a PDS, which self-extends to form a larger length SL than its parent SL amplicon, displacing an SS amplicon in the process. The SS amplicon is sent to the corresponding SS cycle (Fig. 6, shown in blue) which operates similar to the SSD cycle. Note that the SS formed now has three fates as opposed to the SSD, which only had two. While the SSD cycle exclusively handles SSD amplicons, SS cycles handle all different sized SS amplicons generated during LAMP.

The last pathway is the PDS linker (Fig. 6, shown in green). As explained earlier, SS amplicons can form PDS amplicons and PDS amplicons can form cPDS amplicons. These product PDS amplicons from SS and PDS contain fewer single-stranded loops than their parent amplicon, depending on location of primer annealing. These product PDS amplicons are handled by the PDS linker pathway and can self-extend to form an SL amplicon which is fed to the SL-PDS highway and the displaced SS amplicon creates a new SS cycle. Even though the product PDS amplicons can further form PDS amplicons depending on the number of single-stranded loops in the amplicon, we neglect these scenarios due to a very low contribution of this pathway to the total number of amplicons generated (ESI Note S2, point number 11). T amplicons (Fig. 6, shown in light blue) are formed at different stages of the reaction network; we assume that T amplicons do not participate in any further reactions.
Figure 6. Flow diagram for compressed stoichiometric and kinetic model. Sense strand dumbbell cycle, SS cycle, SL-PDS highway, and PDS linker capture all the possible reaction pathways for LAMP.

Functions were written to individually model the SS cycle, SL-PDS highway and PDS linker in MATLAB. Data pertaining to an amplicon such as its structure, time of formation (as determined by the rate of nucleotide incorporation), and number of copies were stored in a MATLAB data structure called cell array. A function takes in two cell arrays (titled as data packages, Fig. S9, S10 and S11), one each from sister network 1 and sister network 2. As each function progresses through its respective pathway it performs the process of determining the number of nucleotides added to a reactant to form products (ESI Note S4), time of formation and number of copies (ESI Note S5) of the product amplicon formed from the reactant amplicon. The function eventually reaches a stage when the time of formation of the product is greater than a parameter, \( t_R \), the total reaction time. At this point, the abovementioned amplicon details are directed to a matrix which stores details of all the amplicons formed at the end of the reaction time from different functions (SS cycle, SL-PDS highway, PDS linker). A summation of the copies of the latest formed amplicons stored in the matrix provides the total number of copies of products produced for a given reaction time. Similarly, summation of different amplicon types gives the total number of copies of SS, SL and PDS products at the end of reaction time. The program is run for successively increasing values of the parameter, \( t_R \) (reaction time) for both the sense strand dumbbell and the anti-sense strand dumbbell for different reaction times. The total copies of amplicons generated for both sense and anti-sense strand is added to generate the total copies versus reaction time curves. This framework has
been qualitatively validated in the following section to demonstrate that c-SPK model generates results in agreement with experimentally observed results.

### 2.5 c-SPK model captures characteristic features of LAMP

An important capability of the model is to generate the concentration profile of amplicons and predict rise time (equivalent to threshold cycle (Ct) in PCR) for LAMP reactions. In order to achieve this, the c-SPK model was run for different reaction times starting with 10 and 100 copies of double-stranded DNA to obtain the total copies of amplicons (ESI Note S5) generated for respective reaction times. Because one copy of ds-DNA gives rise to a sense strand and an anti-sense strand dumbbell, and the c-SPK model took starting concentration of the dumbbell as a user-defined input, the starting concentration of the dumbbell was taken as 20 and 200 copies, respectively. Due to limitations in computational power, c-SPK model could be run for limited reaction times (14 minutes for 20 starting copies and 9 minutes for 200 starting copies) (ESI Fig. S15). In depth description of the computational program and infrastructure used to solve the model is provided in the Methods section and in ESI Note S6. To extrapolate and generate results for greater reaction times, the different reaction time snapshots of concentration profiles were curve fitted using a slight modification of the generalized Richard’s function (Equation 1). The Richard’s function was successfully used earlier by Subramanian and Gomez [12] for fitting real-time LAMP curves.

\[
y(t) = \frac{k}{1 + \exp((-b) \times (t - m))}
\]

*Equation 1*

where,

- \(y(t)\) represents the concentration of amplicons at time ‘\(t\)’
- \(k\) represents concentration of amplicons at infinite time
- \(b\) represents the maximum slope of the amplification curve, which occurs at \(t = m\), and
- \(m\) represents the time at which the growth rate is maximum

Plots for total amplicons versus time, \(t\), obtained by solving the cPDS model for different values of parameter, total reaction time \((t_r)\), were curve-fitted to Richard’s function (ESI Fig. S16), and values of Richard’s function parameters \(k\), \(b\) and \(m\) were obtained at each value of \(t_r\). The values of \(k\), \(b\) and \(m\) for 20 (ESI Table S1) and 200 (ESI Table S2) starting copies of the template were then analyzed to obtain time-dependent equations for them (ESI Fig. S17). These equations were then used to develop the methodology for prediction of amplicon concentration-time profiles. ESI Note S6 provides detailed description on curve fitting of model results, creation of time-dependent equations for extrapolation of Richard’s parameters \((k, b \text{ and } m)\), development of a new technique to compensate the assumption of zeroth order kinetics and introduce the plateau phase into the model generated curves, and shows the results for concentration profiles of amplicons obtained from c-SPK model results. Despite a few simplifying assumptions, rise time predictions from c-SPK model were comparable with experimental results. Rise times predicted by c-SPK model were 96.89 and 81.66 minutes for 20 and 200 starting copies of the dumbbell, respectively, while the corresponding experimental rise times were 103.76 ± 7.79 minutes and 81.45 ± 2.21 minutes, respectively (ESI Fig. S18).

An even more powerful result from the c-SPK model is prediction of number of copies formed for the different categories of LAMP amplicons (Fig. 7A and 7B). Because the model identifies different classes of amplicons, it provides amplicon counts for each category, which has been impossible to estimate till date. Following the strategies developed in the previous section, the number of amplicons generated under each category were calculated for various reaction times.
and starting dumbbell concentrations. Results show that at 8 minutes of reaction time, PDS and SL amplicons form a greater percentage of total amplicons (roughly 85%) while SS and T amplicons constitute the remaining minor fraction (roughly 15%). While these results are for only 8 min reaction time, it is safe to assume that these fractions will be maintained throughout the course of the reaction. These results will empower decisions for downstream detection techniques, for instance design of different types of probe-based detection strategies for LAMP amplicons.

**Figure 7. Results from the c-SPK model.** (A) Plots for total copies of PDS, SL and SS amplicons versus time for (A) 20 and (B) 200 starting copies of dumbbell for a reaction time of eight minutes. (C) (i) Simulated gel depicting ladder like pattern associated with LAMP amplicons obtained from the model for a reaction time of seven minutes with 200 starting copies of the dumbbell. (ii) Experimental gel electrophoresis image for LAMP amplicons obtained at the end of 120 minutes reaction starting with 10 copies of double-stranded gDNA. (D) Length pyramid depicting the number of copies formed for different ranges of length of LAMP amplicons.

c-SPK model also captures the distinctive feature of ladder-like appearance of LAMP amplicons on gels. All amplicons generated within a reaction time of 7 minutes and 200 starting copies of the dumbbell were considered to create a simulated gel (Fig. 7C (i)). Since LAMP amplicons are transient in nature (keep on forming and being utilized in reaction), reaction time of seven minutes was chosen as it was found to be representative of presence of varied length...
amplicons. The simulated gel captured the ladder-like appearance of LAMP amplicons as seen in the experimental gels (Fig. 7C(iii)) for LAMP amplicons with 4 primers and a reaction time of 120 minutes. Amplicon bands of different sizes were observed, and their positions were similar to positions of bands in experimental gel. Although a note of caution here would be that while LAMP amplicons are assumed as rigid molecules for length calculations in c-SPK model, in reality LAMP amplicons are cauliflower structures that migrate through the gels at a different rate than fully double stranded DNA. This would explain why the location of bands in experimental gels does not exactly match their linear length. Another significant inference obtained from the c-SPK model is that while longer length amplicons are formed due to the extension of cauliflower structures, it is the shorter length amplicons that form a major proportion of LAMP products. At the end of 7 minutes, starting with 200 copies (Fig. 7D), the number of amplicons was highest for 0 to 5000 nucleotides long amplicons followed by 5000 to 10000 nucleotides long amplicons followed by 10000 – 15000 nucleotides long amplicons. A drastic reduction in the total number of amplicons was observed for amplicons in the category of 15000 to 20000 nucleotides and 20000 to 25000 nucleotides long amplicons. This can be explained by the fact that the shorter length SS amplicons are formed faster, which can keep giving rise to the same sized products via the SS cycle (Fig. 6) and larger length amplicons via the SL-PDS highway (Fig. 6).

3. Conclusion
This work provides the first framework to: (i) classify LAMP reaction products into uniquely identifiable categories, (ii) generate a condensed reaction network to depict the complex cascade of reactions taking place in LAMP, and (iii) understand the amplicon generation pathways involved in LAMP in depth. While this model incorporates many simplifying assumptions and is not an absolute replica of real LAMP reactions, our attempt is to open the field for deeper theoretical understanding of the LAMP reaction network. Improved comprehension of LAMP mechanism would aid in designing robust probe-based detection strategies to replace the traditional whole DNA detection methods fraught with false positive results [18]. Future work in our group will focus on developing sensitive lateral flow detection strategies of LAMP amplicons guided by the results of this model. We would also like to invite researchers from fields of polymer science, population balance modelling, and other related areas to extend application of principles from their fields to understand the growth of complex DNA molecules in LAMP.

4. Materials and methods
4.1 Experimental Methods
The LAMP assay used for comparison of experimental and c-SPK model data was designed for the hspX gene of Mycobacterium tuberculosis. The primers for the LAMP assay were taken from a published study [19], but only the inner primers (FIP and BIP) and the outer primers (F3 and B3) were used for this work. 12.5 µl real-time LAMP reactions were conducted in a Quant Studio 3 (Applied Biosystems) PCR machine with the following composition: 1.6 µM forward inner primer (FIP) and backward inner primer (BIP), 0.2 µM forward outer primer (F3) and backward outer primer (B3), 1X LAMP reaction buffer, 8mM MgSO₄, 0.9M Betaine (B0300-1VL, Sigma-Aldrich), 2.5 µl dNTP mix (10 mM each, N0447S, New England Biolabs (NEB)), 4 units of Bst 2.0 WarmStart® DNA polymerase (M0538S, NEB), 1 µl of the target DNA, 3X SYBR Safe I (Invitrogen, P/NS33102) and DEPC water to adjust the final volume to 12.5 µl. LAMP products were also analyzed using gel electrophoresis by running them in 2% agarose gels (115V) stained with ethidium bromide and the gels were imaged in a custom-made gel imager. Richard’s equation was used to curve fit experimental real-time amplification curves for each sample to obtain Richard parameters m and b. Time to positive, Tp, was
calculated based on the method developed by Subramanian and Gomez[12] using the below formula:

\[ Tp = m - (2/b) \]

Triplicates were run for both 10 and 100 starting copies of the template. \( Tp \) values for replicates were taken to calculate the average and standard deviation for \( Tp \) for 10 and 100 starting copies of template, respectively.

4.2 Computational methods

4.2.1 Defining characteristic features of LAMP amplicons

Because increasing length amplicons are produced over time, each category of amplicons has many variants depending on the length of the amplicon. Therefore, it became imperative to define a method of identification for these different types of amplicons. The structure and length of an amplicon were used as its unique identifiers. A detailed description of the representation style used to depict a particular category of amplicons and formulae used to calculate the length of amplicons from each category is provided in ESI Note S3. The procedure used to calculate the number of nucleotides added to reactant amplicons to form the corresponding product amplicons is explained in ESI Note S4.

4.2.2 Compressed stoichiometric and pseudo kinetic (c-SPK) model for LAMP

Drawbacks of the brute force model were overcome by developing the c-SPK model. The set of assumptions involved in this model are provided in ESI Note S2. This model was coded in MATLAB using SimBiology and Parallel Computing toolboxes. The program was run on a cluster having MATLAB version R2017a. We recommend having a cluster with minimum 50 GB RAM to run the program. The LAMP mechanism was divided into loop, series and parallel reactions which handle the SS, SL and PDS reactants, respectively, and they were named as SS loop, SL-PDS highway and PDS linker (Fig. 6) functions respectively. Each reaction function evaluates the reactant and generates the structures, number of copies and time of formation of the product. These details are then fed into the other reaction functions to generate products from the new reactants. The program has a main loop that goes through forty SS loop functions simultaneously followed by forty SL highway functions simultaneously followed by forty PDS functions simultaneously. As an example, consider a PDS product amplicon formed from an SS in the SS cycle and a cPDS product amplicon formed from a PDS in the SL-PDS highway. These product PDS amplicons are not evaluated in the SS cycle and SL-PDS highway functions. Instead, all the PDS product amplicons formed from SS and PDS amplicons are gathered and stored in a common PDS array. Then, forty PDS linker functions parse forty PDS cell arrays from this common PDS array. The program was run on a twenty-core cluster, each core able to run 2 computational threads parallelly. Hence, forty computational threads ran parallelly. Users having access to larger number of cores can modify the program to utilize the full potential of the available hardware. Each of these forty functions run on separate computational threads using the parfor function defined in the Parallel Computing toolbox. This allows to run the model for greater reaction times. Using these reaction constructs and concepts to determine the number of copies and time of formation of amplicons, LAMP reaction network was decentralized and solved in small chunks. However, the run times for this model were prohibitive beyond 14 minutes of reaction time for 20 starting copies of template and 9 minutes of reaction time for 200 starting copies of template because of the long run times of the program.
4.2.3 Simulated gel
A matrix named lastAmpliconsLen contains the number of copies of each length of amplicon formed at the end of reaction time. The following steps were followed to create the simulated gel:

1. Amplicons that have lengths less than or equal to 1000 nucleotides are chosen from the lastAmpliconsLen matrix because the experimental gel ladder ranged from 100 to 1000 base pairs.
2. A MATLAB script was written to create the simulated gel image.
3. The ladder in the simulated gel was created by emulating the distances between the bands and the intensities of the bands in the ladder in the experimental gel.
4. The bands in the adjacent lane were created by parsing the lastAmpliconsLen matrix and extracting the lengths of amplicons lying between 100 to 1000.
5. Each length is assigned a rectangle of appropriate width and height and is then positioned according to the ladder. The face color of the rectangles is set to white to show them as white bands against a black background.

Conflict of interest
None to declare

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A stoichiometric and pseudo kinetic model of loop mediated isothermal amplification

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SUPPLEMENTARY INFORMATION

Supplementary Figure S1. Publications on different isothermal DNA amplification techniques.

Supplementary Figure S1. Publications on different isothermal DNA amplification techniques. LAMP was found to be the most popular nucleic acid amplification technique in the research community for the last five years. Numbers as on 11th February 2020.

A literature search was conducted on Web of Science using the title of various isothermal amplification techniques as keywords and article as the document type filter. Names of the following isothermal amplification techniques were used as keywords: loop-mediated isothermal amplification (LAMP), rolling circle amplification (RCA), recombinase polymerase amplification (RPA), strand displacement amplification (SDA), exponential amplification reaction (EXPAR), nucleic acid sequence-based amplification (NASBA), and helicase dependent amplification (HDA). This revealed that over the last 5 years, LAMP has been the most frequently used technique for isothermal NAA with ~4x more publications compared to the second most popular technique.
ESI Note S1 Brute force stoichiometric and pseudo kinetic (SPK) model

The first version of the SPK model was a brute force model that tracked each and every amplicon generated in LAMP. It determined and stored data for number of copies, length, sequence, formation time, parent amplicon and child amplicons for every amplicon. Formation time was based on the extension rate of the enzyme alone and had no dependence on concentrations of any species, i.e. assuming zeroth order kinetics. Using this program, the exact sequence of any species in any cell from this matrix can be extracted. All other assumptions made for developing the brute force model are provided in ESI Note S2. The model was programmed in MATLAB using the SimBiology toolbox and a detailed block diagram explaining flow of the computational program is provided in ESI Fig S2. Because this version of the model tracked each amplicon and stored the corresponding data, it required high computational power and data storage capacity. This led to a major roadblock as we couldn’t run the program of reaction time of more than two minutes.

Computational program for Brute force stoichiometric and pseudo kinetic model

This model is based on object-oriented methodology and individual class files were written for SS, SL and PDS reactants respectively. Objects are created using the constructor function and the class functions use these objects to create the product amplicon data. The product amplicon data along with the reactant data is stored in another set of objects and these objects are stored in an object array. There are separate object arrays for each reactant amplicon (SS, SL and PDS). After the program has finished running, information about each amplicon produced in the reaction network can be accessed through the corresponding object array. The data in the object arrays is structured into two sections. The first section contains data for the parent of the reactant amplicon and the
reactant amplicon’s formation time, length, sequence and the number of copies. The second section contains information about the sequence of the product amplicon, the nucleotides used by the reactant amplicon to form the product and the number of copies of the product. The program only requires sequence of the dumbbell to initiate the calculations and it builds the entire reaction network independently.

An important point to note is that the program calculates the reaction network for only one dumbbell produced from the target strand. The program is first run for the sense strand dumbbell and then for the anti-sense strand dumbbell to cover the entire reaction network. The brute force model builds the reaction network generation by generation. The current generation is defined as a row vector which contains the reactant amplicons and the next generation is another row vector which contains the products of the reactants. The program goes through each entry in the current generation and the products formed from each entry are stored in the next generation amplicon array. After it goes through each entry in the current generation, it then appends the next generation to the bottom of the reaction network matrix and makes it the current generation, repeating the whole process. The reaction network keeps expanding until formation time of amplicons is greater than reaction time. The brute force model analyzed each amplicon individually, determined and stored its number of copies, length, sequence, formation time, parent amplicon and child amplicons. A detailed block diagram explaining the computational program flow is provided below:
Supplementary Figure S2. Block diagram for the logic used for creating brute force stoichiometric and pseudo-kinetic model.

ESI Note S2. Assumptions for developing Brute force and compressed stoichiometric and pseudo kinetic (SPK) model

1. The concentration of nucleotides and primers remains constant throughout the reaction.
2. Time taken to form a product amplicon from a reactant amplicon is taken to depend only on the extension time obtained from the nucleotide incorporation rate of the enzyme. This
assumption is based on the fact that the time scale for primer annealing and enzyme attachment to template is of the order of nanoseconds while the extension time is of the order of seconds.

3. Probability of formation of different amplicon types is constant with respect to reaction time.

4. Primers are equally likely to anneal at all primer annealing sites present in an amplicon. This implies that there is equal probability of formation of partially double stranded (PDS) and terminated (T) amplicons from a single-stranded (SS) or PDS amplicon.

5. Once formed, T amplicons do not participate in any further reactions.

6. Nucleotide incorporation rate of the enzyme (Bst 2.0) is taken to be constant for entire duration of the reaction. In reality, nucleotide incorporation rate should decrease over time owing to decreasing concentrations of primers and nucleotides in the reaction mix and increasing length of the amplicons.

7. Formation time of an SS amplicon formed from a PDS amplicon via different reaction pathways is taken to be the same. Furthermore, SS formation time is taken to be equal to the time it takes to generate a single-loop (SL) amplicon from a PDS.

Consider a PDS amplicon (Fig S3, PDS) to which an FIP (5’F1c –F2) can anneal at F2c loop to produce a child PDS (cPDS) and a BIP (5’ B1c-B2) can anneal at the terminal B2c loop to produce T. The 3’ end of the single-stranded section of PDS amplicon can also self-anneal to produce an SL. In all these cases an SS is formed via strand displacement, but the time taken for strand displacement is different for each case. It depends on number of nucleotides to be added to form the corresponding products. Time taken for strand displacement of SS for different product types varies as: formation time for T > formation
time for SL > formation time for child PDS. If there were ‘n’ primer annealing locations on a PDS which could lead to formation of child PDS and T, there would be an SS displaced at ‘n’ different times and the program would require ‘n’ separate variables to store SS formation times. This increases the memory space requirement of the program and slows down its execution speed. SL formation pathway was chosen to define SS formation time because we found that probability of formation of an SL amplicon is considerably larger than that for T and child PDS. This can be explained from the fact that the first step in SL formation reaction is enzyme binding while for child PDS and T it is primer annealing. Since the forward binding constant for enzyme attachment is two orders of magnitude greater than forward binding constant for primer annealing, a larger fraction of reactant copies is transformed to SL.

Supplementary Figure S3. Formation time of single-stranded (SS) amplicon.
SS amplicons can be formed by strand displacement when a partially double stranded (PDS) amplicon forms a terminated (T), child PDS or single-loop (SL) amplicon. The number of nucleotides incorporated for the three pathways is different, leading to different formation times for the same length SS amplicon.

8. All amplicons are assumed to be rigid molecules such that they do not coil up to have a shorter length than their actual length.

9. The double-stranded segments of different amplicon types are considered not to breathe as that would generate even greater number of primer annealing sites and increase the complexity of the model.

10. For determination of the last amplicon, the formation time of the product is compared with the total reaction time. If the formation time of the product exceeds the reaction time, then the corresponding reactant has to be the last amplicon. In case of SL reacting to form PDS it is easy to determine if SL is the last reactant because an SL can produce only a PDS amplicon. But in cases like SS and PDS where parallel reactions occur producing multiple products, only the SL product amplicon is considered to determine if the last amplicon is a SS or PDS reactant. Since majority of the SS and PDS reactants are converted to SL and because it takes the longest time to generate, if formation time for SL exceeds reaction time, we consider the SS or PDS to be the last amplicon.

11. PDS products produced from an SS and child PDS product produced from a PDS do not form any further PDS amplicons in the c-SPK model. For these PDS product amplicons produced from an SS or PDS to further form PDS amplicons, primer annealing is required and as explained in point 7, self-extension is more favorable than primer annealing. We
also did theoretical calculations and found that the number of amplicons formed via the two abovementioned pathways would be negligible.

12. The extension process was assumed to be a single step process in the c-SPK model. Extension proceeds by incorporation of oligomers having length equal to the number of nucleotides to be added to the reactant for each extension step.

ESI Note S3. Length of different LAMP amplicon types

It was observed that the structure of dumbbell and following all LAMP amplicons only comprised of F1, F2, B1, B2 and their complementary patches. To make the representation of sequences simpler, the repeating patches F1c-F2c-F1 and F1c-F2-F1 are represented by F patches (Fig. S4A), and patches B1c-B2c-B1 and B1c-B2-B1 are represented by B patches (Fig. S4A). It is these ‘F’ and ‘B’ patches which are repeatedly added to newly forming amplicons in the exponential phase of LAMP reaction. The nucleotides present between the ‘F’ and ‘B’ patches are represented by ‘D’ patches (Fig. S4A). The regions that form F and B patches are obtained from the target DNA sequence and the number of nucleotides in each patch can be calculated from the sequence of the target DNA. Fig. S4B shows the number of nucleotides present in the target sequence considered for our model and the total number of nucleotides present in the F, B and D patches in the dumbbell formed from this target are calculated as follows:

\[
\text{Number of nucleotides in } B \text{ patch} = 22 + 23 + 9 + 19 + 22 = 95
\]

\[
\text{Number of nucleotides in } F \text{ patch} = 19 + 19 + 20 + 1 + 19 = 78
\]

\[
\text{Number of nucleotides in } D \text{ patch} = 33
\]

\[
\text{Total number of nucleotides in dumbbell} = 206
\]
Since the compressed stoichiometric and pseudo kinetic (c-SPK) model is developed using MATLAB, we came up with a matrix-based representation for length of different types of amplicons (SS, SL, PDS and T) generated in LAMP. These matrices, called patch matrices, store sequence of patches present in the amplicons. The patch matrices are utilized to identify primer annealing sites present on amplicons and to calculate length of amplicons and number of nucleotides added to a reactant to form corresponding product. The D patch does not possess sites for primer annealing and hence is not stored in patch matrices. The patch matrices for SS (Fig. S5A) and SL amplicons (Fig. S5B) are denoted by ss (Fig. S5A) and ds (Fig. S5B) respectively. The PDS structure (Fig. S5C) is divided into 3 parts and each part is stored in different patch matrices; namely es, ls and ss. The ss patch matrix of PDS stores the sequence of the SS amplicon.
that will be generated by strand displacement when a PDS undergoes self-extension or primer annealing. The es patch matrix of PDS stores the strand of PDS that is annealed to ss as well as the part containing single loops where primer can anneal and form child PDS or T. The ls patch matrix stores only the single-loop region sequence where a primer can anneal to form a child PDS or T. For all patch matrices except ds, the 3’ end is located at the end of the matrix whereas for ‘ds’ patch matrix it is located at the beginning of the second row (Fig. S5 B).

Supplementary Figure S5. Matrix based representation for calculating length of different categories of LAMP amplicons. (A) SS amplicon (B) SL amplicon and (C) PDS amplicon

The program calculates number of columns present in patch matrices by using the length function inbuilt in MATLAB and that is defined as the length of a patch matrix. Length of an SS, SL and PDS amplicon is represented by length of ss, ds and es patch matrix, respectively. Length of T amplicon is calculated using length of the SS or PDS reactant which formed the T amplicon,
because to form a T amplicon one has to create a complement of the entire ss length in case of SS reactant or the entire es length in case of PDS amplicon. After analyzing the trend in patch sequences, we found that if length of patch matrix is even then there should be an equal number of ‘B’ and ‘F’ patches and if length is odd then there should be an excess of either ‘B’ or ‘F’ patch depending on whether the end of the patch matrix has a ‘B’ or ‘F’ patch, respectively. The formulae used for calculating the number of ‘B’, ‘F’ and ‘D’ patches in the patch matrix are defined below. Multiplying the number of patches with the number of nucleotides present in each patch provides the total number of nucleotides present in an amplicon.

Case I: Length of patch matrix is even:

\[
\text{Number of 'B' patches in patch matrix} = \frac{\text{length of patch matrix}}{2} \quad - \text{Eqn 1}
\]

\[
\text{Number of 'F' patches in patch matrix} = \frac{\text{length of patch matrix}}{2} \quad - \text{Eqn 2}
\]

\[
\text{Number of 'D' patches in the patch matrix} = (\text{Eqn 1}) + (\text{Eqn 2}) - 1 \quad - \text{Eqn 3}
\]

The total number of nucleotides in the amplicon, equivalent to length of the amplicon is calculated as follows:

\[
\text{Length} = (\text{Eqn 1}) \times (B \text{ patch nucleotides}) + (\text{Eqn 2}) \times (F \text{ patch nucleotides}) + (\text{Eqn 3}) \times (D \text{ patch nucleotides})
\]

Case II: Length of patch matrix is odd:

(a) Length of patch matrix is odd and 3’ end has a ‘B’ patch

\[
\text{Number of 'B' patches in the patch matrix} = \text{ceiling} \left( \frac{\text{length of patch matrix}}{2} \right) \quad - \text{Eqn 4}
\]

\[
\text{Number of 'F' patches in the patch matrix} = (\text{length of patch matrix}) - (\text{Eqn 4}) \quad - \text{Eqn 5}
\]

\[
\text{Number of 'D' patches in the patch matrix} = (\text{Eqn 4}) + (\text{Eqn 5}) - 1 \quad - \text{Eqn 6}
\]
The total number of nucleotides in the amplicon, equivalent to length of the amplicon is calculated as follows:

\[
\text{Length} = (\text{Eqn 4}) \times (B \text{ patch nucleotides}) + (\text{Eqn 5}) \times (F \text{ patch nucleotides}) + (\text{Eqn 6}) \\
\quad \times (D \text{ patch nucleotides})
\]

(b) Length of patch matrix is odd and 3’ end has an ‘F’ patch

Number of ‘F’ patches in the patch matrix = ceiling\left(\frac{\text{length of patch matrix}}{2}\right) \quad - \text{Eqn 7}

Number of ‘B’ patches in the patch matrix = (\text{length of patch matrix}) - (\text{Eqn 7}) \quad - \text{Eqn 8}

Number of ‘D’ patches in the patch matrix = (\text{Eqn 7}) + (\text{Eqn 8}) - 1 \quad - \text{Eqn 9}

The total number of nucleotides in the amplicon, equivalent to length of the amplicon is calculated as follows:

\[
\text{Length} = (\text{Eqn 8}) \times (B \text{ patch nucleotides}) + (\text{Eqn 7}) \times (F \text{ patch nucleotides}) + (\text{Eqn 9}) \\
\quad \times (D \text{ patch nucleotides})
\]

**ESI Note S4. Calculation of number of nucleotides added to form new amplicons in LAMP**

(i) **Number of nucleotides added to SL to form PDS**

Fig. S6 demonstrates the formation of a PDS amplicon from SL. The number of ‘B’ and ‘F’ patches added can be calculated using the length of the ds patch matrix of reactant SL. The number of nucleotides added to an SL to form PDS can be calculated by multiplying the number of patches with the number of nucleotides in each patch.
Supplementary Figure S6. Formation of a PDS amplicon from an SL amplicon.

(ii) Number of nucleotides added to SS to form T

Fig. S7A demonstrates formation of T from SS via primer annealing and extension. The same number of ‘B’, ‘F’ and ‘D’ patches, as present in ss patch matrix, are added to SS to form T amplicon. The number of nucleotides added to an SS to form T can be calculated by multiplying the number of patches with the number of nucleotides in each patch.

(iii) Number of nucleotides added to SS to form SL

Fig. S7B demonstrates formation of SL from SS via self-extension. The following set of equations describe the calculations done for finding the number of nucleotides added to an SS to form SL, depending on the nature of sequence of parent SS.

If the 3’ end of SS is a ‘B’ patch:

- B patches added = (No. of B patches in SS) − 1
  \[ − \text{Eqn 10} \]

- F patches added = (No. of F patches in SS)
D patches added = (No. of B patches in SS + No. of F patches in SS) − 1 \quad – \text{Eqn 12}

If the 3’ end of SS is an ‘F’ patch:

B patches added = (No. of B patches in SS) \quad – \text{Eqn 13}

F patches added = (No. of F patches in SS) − 1 \quad – \text{Eqn 14}

D patches added = (No. of B patches in SS + No. of F patches in SS) − 1 \quad – \text{Eqn 15}

(iv) Number of nucleotides added to SS to form PDS

Fig. S7C demonstrates formation of PDS from SS via primer-annealing and extension. The location(s) of primer binding sites (F2c or B2c) are determined for the parent SS amplicon using the find function in MATLAB. The primer binding site at the 3’ end is not considered as it leads to formation of a T amplicon and not PDS. As shown in Fig. S7C, if the primer anneals to the second loop (F2c) from right of the SS amplicon, then the length of the SS patch matrix of PDS product formed gives the number of patches added to SS to form PDS. If the number of patches in the ss matrix of PDS product is even, then there will be an equal number of ‘B’ and ‘F’ patches added. However, if the number of patches is odd then there will be an excess of either ‘B’ or ‘F’ patches added depending on location of primer annealing. This analysis is repeated for each of the primer annealing sites on the SS amplicon.
(v) Number of nucleotides added to PDS to form T

Fig. S8A demonstrates formation of T from PDS via primer annealing and extension. The new strand generated to create the T amplicon encompasses the entire es strand of the PDS amplicons. The number of ‘B’ and ‘F’ patches in T will be same as in the es patch matrix of PDS. The number of D patches can be calculated accordingly.

(vi) Number of nucleotides added to PDS to form SL

Fig. S8B demonstrates formation of SL from PDS via self-extension. The following set of equations describe the calculations done for finding the number of nucleotides added to a PDS to form SL, depending on the nature of sequence of parent PDS:

If the 3’ end of es strand is a ‘B’ patch:

B patches added = (No. of B patches in es of PDS) − 1  — Eqn 16

F patches added = (No. of F patches in es of PDS)  — Eqn 17
D patches added = ( No. B patches in es of PDS + No. F patches in es of PDS ) – 1 – Eqn 18

If the 3’ end of es strand is an F patch then,

B patches added = (No. of B patches in es of PDS) – Eqn 19

F patches added = (No. of F patches in es of PDS) – 1 – Eqn 20

D patches added = (No. B patches in es of PDS + No. F patches in es of PDS ) – 1 – Eqn 21

(vii) Number of nucleotides added to PDS to form child PDS

A child PDS is the product PDS amplicon produced from a parent PDS amplicon. Fig. S8C demonstrates formation of child PDS from parent PDS via primer annealing and extension. Length of the ss patch matrix of child PDS product gives the number of patches added to form the child PDS. The location(s) of primer binding sites (F2c or B2c) are determined for the ss patch matrix of PDS amplicon using the find function in MATLAB. The primer binding site at the 3’ end is not considered as it leads to formation of a T amplicon and not child PDS. As shown in Fig. 8C, if the primer anneals to the second loop (F2c) from right of the PDS amplicon, then the length of the ss patch matrix of child PDS product formed gives the number of patches added to parent PDS to form child PDS amplicon. If the length of ss patch matrix of child PDS is even, then there will be equal number of ‘B’ and ‘F’ patches added. If the length of ss patch matrix of child PDS is odd, then there will be an excess of either ‘B’ or ‘F’ patches added depending on the location of primer annealing.
Supplementary Figure S8. Formation of different amplicon types from PDS amplicon. (A) Formation of T from PDS, (B) Formation of SL from PDS, and (C) Formation of child PDS (cPDS) from PDS.

ESI Note S5. Formulae for calculating the number of copies of product formed from a reactant

(i) Formation of SL from SS

Number of copies of SL = round((probSL) * (number of copies of SS))

(ii) Formation of PDS and T from SS
Consider ‘n’ primer annealing sites available on SS out of which n-1 can form ‘n – 1’ different types of PDS amplicons and the terminal primer annealing site located at the 3’ end forms a T amplicon.

Number of copies of T and each PDS formed from an SS

= round(number of copies of SS – number of copies of SL formed from SS

number of primer annealing sites)

(iii) Formation of SS from PDS

Number of copies of SS = number of copies of PDS
(iv) **Formation of SL from PDS**

Number of copies of SL = round((probSL) * (number of copies of PDS))

(v) **Formation of T and child PDS from PDS**

Consider ‘n’ primer annealing sites available on PDS out of which n-1 can form ‘n – 1’ different types of child PDS and the terminal primer annealing site located at the 3’ end forms a T amplicon.

Number of copies of T and each child PDS formed from PDS

= round\left(\frac{\text{number of copies of SS} - \text{number of copies of SL formed from PDS}}{\text{number of primer annealing sites}}\right)
Supplementary Figure S9. Block diagram representing computational program flow for SSD and SS cycle.
Supplementary Figure S10. Block diagram for SL-PDS highway

Unpack formation time, copies vector and reactant sequence from the SL data package

Create cells that will store product data. These cells store patch sequences for SS and child PDS produced from PDS

Create matrices to store time and copies vector

The first SL in SL highway function will obtain its data from the SL package while all subsequent SL’s will obtain data their data from the PDS formed in the function. If the SL is formed within reaction time send its number of copies to final data storage matrix (dataMat)

Determine length of SL reactant and nucleotides added to SL to form PDS. Calculate PDS formation time based on number of nucleotides added to SL

First entry in PDS formation time vector greater than reaction time?

Yes

No

First entry in SL formation time vector greater than rxn time?

Yes

No

Scan each entry in the pds time vector. If formation time is within reaction time, send the number of copies of PDS formed into dataMat. Else send the data of SL (parent of PDS) into lastAmpliconsLen.

Generate patch sequence for PDS formed and determine length of PDS, which now becomes reactant to form further products. Calculate the number of nucleotides added to PDS to form SL and T and create corresponding formation time vectors. The time taken for strand displacement to occur to form SS is taken to be equal to the time it takes for PDS to form SL. Hence, SS formation time vector is the same as SL formation time vector.

Check if any primer binding sites are available on PDS and calculate how many child PDS can be produced

Create the SS, child PDS, SL and T copies vector

Create child PDS patch sequences only if the number of copies of PDS is greater than zero. Create child PDS formation time vector and store it with copies vector in a matrix. Store child PDS patch sequence in a cell. This child PDS will be used to generate subsequent product patch sequences

Send SS copies to dataMat and store its patch sequence, formation time and copies vector in respective cells and matrices. If SS has formation time greater than reaction time, then PDS is the last amplicon and store its data in lastAmpliconsLen

Scan formation time array of T and send data to dataMat if formation time is less than reaction time

Store all product data cells and matrices in one cell called as the dataFun which will be the output from function SL Highway

Is sister SL data package present?

Yes

No

Exit

Supplementary Figure S10. Block diagram representing computational program flow for SL-PDS highway.
Supplementary Figure S11. Block diagram for PDS linker

Unpack formation time, copies vector and reactant sequence from the PDS data package

Calculate the number of ‘B’ and ‘F’ patches and length of PDS reactant. Create cells that will store the patch sequence for SS and SL products generated from PDS. T amplicons are terminal and patch seq is not generated for them

Determine number of nucleotides added to PDS to form SL and T

Create SS, SL and T formation time vectors to store product formation times

Create the SS and SL patch sequence. Check and determine number of primer annealing sites

Obtain copies of SS, SL and T and store in their respective copies vector

Scan SS, SL and T formation time vectors for formation times less than reaction time and send number of copies of SS, SL and T into final data storage matrix (dataMat) else if a formation time greater than reaction time appears in the vector then the PDS becomes the last amplicon and its data is stored in the lastAmpliconsLen matrix

Store the SS and SL patch sequences in their respective cells and create a matrix to store the SS and SL copies and formation time vectors

Store all the product data in PDS dataFun which is the output from function childPDS

Is sister PDS package present?

Yes

No

Supplementary Figure 11. Block diagram representing computational program flow for PDS linker.
Enzyme concentrations are conventionally reported in units/ml while we required enzyme concentrations in molarity to solve the kinetic model for calculating probability of formation of different types of LAMP amplicons. In order to solve the kinetic model for conversion of enzyme units into molarity, the starting concentration of the reactants (SS and nucleotides) was estimated such that reaction is only sensitive to the enzyme concentration. This was done by choosing a relatively high enzyme concentration (70 nM) and steadily increasing the concentrations of both SS and nucleotides. The color-coded curves in Fig. S16 correspond to different SS and N concentrations, varying from $1.6 \times 10^{-5}$M to $10 \times 10^{-5}$M. We found that concentration of SS and N above $4 \times 10^{-5}$M resulted in negligible change in the rate of generation of SL. Hence, starting concentration of SS and N was taken as $10 \times 10^{-5}$ M.
Supplementary Figure S13. Conversion of enzyme units into molarity. (A) Schematic for conversion of SS into SL. (B) Set of equations used for solving the kinetic model. (C) Results for iteration of enzyme concentration. The kinetic model was solved for a reaction time of 30 minutes with initial concentration of SS and nucleotides equal to $10^{-4}$ M each. E – enzyme, N – nucleotides, $k_e$ and $k_{e1}$ – enzyme binding and unbinding rate constants, $k_n$ and $k_{n1}$ – nucleotide binding and unbinding rate constant and $k_{cat}$ - catalytic rate constant of the enzyme.
In order to be consistent with the concentration units of different species involved in LAMP reactions, the conventional representation of enzyme concentration in enzyme units was converted into molarity. This kinetic model was created using the Simbiology toolbox of MATLAB. The product catalogue for Bst 2.0 (M0538S, NEB) specifies that one unit of the enzyme is defined as the amount that incorporates 25nmoles of dNTPs in 30 minutes at 65°C. For the considered amplification from SS to SL, it requires addition of 128 nucleotides to form the product. It was assumed that all dntps are incorporated in a single extension step and the species dntps was taken to be a collection of 128-mers. As explained in the previous section, the concentration of SS reactant amplicons and nucleotides was kept in excess to ensure that only the enzyme concentration impacted reaction kinetics. A range of enzyme concentration (70nM to 30nM) was iterated to find the enzyme concentration that enabled conversion of reactant equivalent to the activity of one unit of the enzyme. One unit of enzyme (Bst 2.0, NEB) incorporates 25nmoles of dNTPs in 30 minutes at 65°C. Ode15s solver was used for solving the differential equations corresponding to the above set of reactions. List of parameters required to solve this kinetic model is provided in ESI Table S3. It was found that 31nM of the enzyme could incorporate the same number of nucleotides as 1 unit of enzyme under the reaction conditions as specified by the enzyme manufacturer.

**Supplementary Table S3. List of the parameters used for converting enzyme units into molarity**

| S. No. | Parameter name                  | Value  | Unit             |
|--------|--------------------------------|--------|------------------|
| 1      | Volume                         | 12.5E-6| L                |
| 2      | Enzyme binding rate constant (ke)| 1E7    | Molarity⁻¹s⁻¹    |
| 3      | Enzyme unbinding rate constant (ke1)| 1E-5   | s⁻¹              |
| 4      | Nucleotide binding rate constant (kn)| 5E5    | Molarity⁻¹s⁻¹    |
| 5      | Nucleotide unbinding rate constant (kn1)| 1E-4   | s⁻¹              |
Supplementary Figure S14. Probability of formation of different types of LAMP amplicons

(A) Schematic for the two possible pathways of self-extension and primer annealing. (B) Set of equations solved for the kinetic model for calculating the probabilities. The probability for following pathway 1 was found to be 0.674 and 0.326 for pathway 2. E – enzyme, N – nucleotides, P - primer, ke and ke1 – enzyme binding and unbinding rate constants, kn and kn1 – nucleotide binding and unbinding rate constant, kp and kp1 – primer binding and unbinding rate constant, and kcat - catalytic rate constant of the enzyme.

A simple parallel reaction of SS giving rise to SL and T was written in SimBiology toolbox and solved using ode15s solver in MATLAB. The number of dntps added to SS to form SL and T is 128 and 206 respectively. Hence, the species dNTP is assumed to be a collection of 167-mers (average of 128 and 206). The equations were solved till steady state and the amount of SL and T produced were used to calculate the probability of formation of an SL or T amplicon for the entire reaction network. Since the mechanism for formation of T and PDS involves primer-based extension, the probability of formation of PDS was taken to be same as the probability of formation of T. List of parameters required to solve this kinetic model is provided in ESI Table S4. Starting
with $1.33 \times 10^{-8}$ nM (100 copies) of SS, $1.19 \times 10^4$ nM of nucleotides, 1200 nM of primer (FIP) and 124 nM of enzyme (equivalent to 4 units used in LAMP reactions), the model was allowed to reach steady state, at which point $8.96 \times 10^{-9}$ nM of SL and $4.34 \times 10^{-9}$ nM of T was produced. The probability for formation of SL and T were hence calculated to be 0.674 and 0.326.

**Supplementary Table S4. List of parameters used to calculate probability of formation of different amplicon types**

| S. No | Parameter name                        | Value                     | Unit     | Reference          |
|-------|---------------------------------------|---------------------------|----------|--------------------|
| 1     | Volume                                | 12.5E-6                   | Liter    | As used in experiments |
| 2     | Starting conc of dumbbell             | 1.33E-8 (100 copies)      | nM       | As used in experiments |
| 3     | Starting conc of Primers              | 1200                      | nM       | As used in experiments |
| 4     | Starting conc of Enzyme               | 124 (1 unit = 31 nM)      | nM       | Calculated          |
| 5     | Starting conc of Nucleotide           | 2E6/167                   | nM       | As used in the experiments |
| 6     | Primer binding rate constant (kp)     | 5E5                       | Molarity$^{-1}$ s$^{-1}$ | Mehra et al. |
| 7     | Primer unbinding rate constant (kp1)  | 1E-4                      | s$^{-1}$ | Mehra et al.       |
| 8     | Enzyme binding rate constant (ke)     | 1E7                       | Molarity$^{-1}$ s$^{-1}$ | Andreas et al |
| 9     | Enzyme Unbinding rate constant (ke1)  | 1E-5                      | s$^{-1}$ | Andreas et al.     |
| 10    | Nucleotide binding rate constant (kn) | 5E5                       | Molarity$^{-1}$ s$^{-1}$ | Mehra et al |
| 11    | Nucleotide unbinding rate constant (kn1) | 1E-4                  | s$^{-1}$ | Mehra et al.       |
| 12    | Catalytic rate constant of polymerase (kcat) | 0.283                | s$^{-1}$ | Montagne et al     |
ESI Note S6 Curve fitting and extrapolation of results obtained from c-SPK model

While this model is more compact, highly parallelized and optimized, the c-SPK model also ran into issues with long processing times owing to the large number of amplicons generated in LAMP. The run times for this model were prohibitive beyond 14 minutes of reaction time for 20 starting copies of template and beyond 9 minutes of reaction time for 200 starting copies of template. While in experimental LAMP reactions all the reaction pathways occur simultaneously, in the c-SPK model only forty reactions run simultaneously. The processor has a fixed processing speed and with each iteration more data gets stored in the RAM. This leads to exponentially increasing run times with increasing reaction time (ESI Fig S15).

![Graph of extrapolated curve fitting results](https://via.placeholder.com/150)

Supplementary Figure S15. Time required to run the c-SPK model for different reaction times. The run times increased exponentially with increasing reaction times. (A) 20 and (B) 200 starting copies of the dumbbell.

To overcome this hurdle and extend the model predictions to greater reaction times, a slight modification of the generalized Richard’s function (Equation 1) was adopted to curve fit the amplicon concentration profiles:

\[
y(t) = \frac{k}{1 + \exp\left(-b \times (t - m)\right)} \quad Equation 1
\]
where,

\( y(t) \) represents the concentration of amplicons at time ‘\( t \)’

\( k \) represents concentration of amplicons at infinite time

\( b \) represents the maximum slope of the amplification curve, which occurs at \( t = m \), and

\( m \) represents the time at which the growth rate is maximum

Because the reaction volume is taken to be constant for all model calculations, the model results are reported directly in terms of copies of amplicons generated. The c-SPK model was run with fixed increments of time intervals for a range of reaction times starting from 1 minute till 14 minutes and 1 minute till 9 minutes for 10 and 100 starting copies of SSD, respectively. An equal number of anti-sense single strand dumbbells (ASSD) were also considered as the template for LAMP experiments was double-stranded DNA which would lead to formation of both SSD and ASSD. Hence, the total starting number of copies of the template for model calculations was 20 and 200 copies.

The total number of amplicons generated were plotted against time for different durations of total reaction time. The formulae used for copy number calculations are explained in the previous section (ESI Note S5). Equation 1 was used for curve fitting the total amplicons plots for different reaction times. The coefficient of determination (\( R^2 \)) and normalized root mean square error (NRMSE) were used to decide goodness of the fits. Fig. S16 shows representative curves for total amplicons generated in 2.5, 5 and 12 minutes of reaction time (Fig. S16A-C) with 20 starting copies of the dumbbell and 2.5, 5, and 7 minutes of reaction time (Fig. S16D-F) with 200 starting copies of the dumbbell. As was expected, the curve fitting parameters \( k, b, \) and \( m \) were found to
be strong functions of time. The values of $k$, $b$ and $m$ for different reaction times and starting concentration of dumbbell are provided in ESI Table S1 and Table S2.

**Supplementary Figure S16.** Representative snapshots of sigmoidal fits for total amplicons versus time as predicted by the c-SPK model.

**Supplementary Table S1** Variation of curve fitting parameters $k$, $b$ and $m$ for different reaction times starting with 20 copies of dumbbell.

| Reaction time | $k$     | $b$     | $m$     |
|--------------|---------|---------|---------|
| 2.5          | 2.68E+04 | 2.309   | 2.706   |
| 3            | 5.50E+04 | 2.048   | 3.218   |
| 3.5          | 1.20E+05 | 1.748   | 3.869   |
| 4            | 2.14E+05 | 1.566   | 4.415   |
| 4.5          | 3.19E+05 | 1.479   | 4.8     |
| 5            | 5.40E+05 | 1.336   | 5.384   |
| 6            | 1.19E+06 | 1.177   | 6.328   |
| 7            | 2.79E+06 | 1.022   | 7.533   |
Supplementary Table S2 Variation of curve fitting parameters $k$, $b$ and $m$ for different reaction times starting with 200 copies of dumbbell.

| Reaction time | $k$    | $b$    | $m$    |
|---------------|--------|--------|--------|
| 2.5           | $3.10\times10^5$ | 2.237  | 3.063  |
| 3             | $5.07\times10^5$ | 2.144  | 3.366  |
| 3.5           | $1.00\times10^6$ | 1.944  | 3.859  |
| 4             | $1.97\times10^6$ | 1.732  | 4.419  |
| 4.5           | $3.37\times10^6$ | 1.592  | 4.902  |
| 5             | $5.27\times10^6$ | 1.485  | 5.336  |
| 6             | $1.24\times10^7$ | 1.298  | 6.256  |
| 7             | $2.98\times10^7$ | 1.126  | 7.386  |
| 8             | $6.42\times10^7$ | 0.998  | 8.5    |

Patterns were then identified in time evolution of the curve fitting parameters present in Richard’s equation (equation 1). Correlations were developed to express them as functions of time for both 20 (Fig. 17A-C) and 200 (Fig. 17D-F) starting copies of dumbbell. As defined in Richard’s equation (equation 1), parameter ‘$k$’ represents concentration of amplicons at infinite time. For c-SPK model results, since reaction volume is taken to be constant, ‘$k$’ will represent total amplicons generated at the end of the reaction time. A power fit for ‘$k$’ captured the trend for the model output values the best. It also generated realistic number of total amplicons at the end of reaction time when theoretically compared with number of nucleotides added in a real LAMP reaction, considering an average size for LAMP amplicons to obtain a theoretical ballpark. The LAMP reaction slows down over time due to reducing reactant concentrations and formation of longer length amplicons. Parameters ‘$b$’ and ‘$m$’ play a crucial role in mathematically capturing the slowing down of reaction kinetics. We chose a decreasing power fit for parameter ‘$b$’ and an
increasing power fit for parameter ‘m’ to best capture the slowing down of reaction kinetics during the later stages of the reaction.

Supplementary Figure S17. Time-dependence of Richard’s equation parameters.

Supplementary Figure S17. Time-dependence of Richard’s equation parameters. A-C: Curve fitting for parameters k (A), b (B) and m (C), with 20 starting copies of the dumbbell, respectively. D-F: Curve fitting for parameters k (D), b (E) and m (F), with 200 starting copies of the dumbbell, respectively.

Extrapolating Richard’s equation parameters to generate concentration profiles for longer reaction times

As stated in assumptions (ESI NoteS2), time scale in the model comes only from nucleotide incorporation rate of enzyme (zeroth order kinetics) and the reduction of concentration of reactants does not factor into the rate due to absence of law of mass action kinetics. Accordingly, in order to incorporate the slowing down of reaction kinetics with time in the c-SPK model, the strategy was to estimate a reaction-time beyond which the maximum slope of the amplification curve would
no longer increase, i.e. estimate a cut off value for $m$. For short reaction times, $m$ would increase with reaction time according to the power fit in Fig. 17 (C,F) until a critical reaction time, $t_c$, beyond which $m$ would be kept fixed. Analogously, because $b$ is the maximum slope of the amplification curve, it would also be saturated for reaction times beyond $t_c$. In order to determine the saturation values of $m$ and $b$, the following strategy was designed:

1. $k$, $m$ and $b$ were calculated using their time dependent equations for 1 to 120 minutes, with an increment step of 1 minute.

2. These values were substituted in equation 1 for each minute to calculate the total number of copies ($y_i$) at every minute till 120 minutes.

3. In order to find the cut-off value for parameter $m$, time $t$ was substituted equal to $m$ in Equation 1, resulting in:

$$y(t) = \frac{k}{2} \quad - \text{Equation 2}$$

4. Equation 2 was used to calculate the total number of copies ($y_m$) at $t = m$ by substituting the value of $k$ at 120 minutes, which is the saturation value at the end of 120 minutes, total reaction time considered for this illustration.

5. The value of $y_m$ was then compared to all $y_i$s to back calculate the time at which the two values were equal. The time value thus obtained is designated as the cut-off value for $m$ for the 120 minutes reaction curve.

6. The $b$ value corresponding to the cut-off value of $m$ is defined as the cut off value of $b$ for the 120 minutes reaction curve.

Total amplicon plots were generated for 20 (Fig. S18A) and 200 (Fig. S18C) starting copies of dumbbell using Equation 1 and the trends were compared with the corresponding experimental
curves with 10 (Fig. S18B) and 100 (Fig. S18) starting copies of the double stranded genomic DNA template. For calculating the model predicted $y_i$ values, parameter ‘$k$’ was updated at each minute up to 120 minutes while parameters ‘$m$’ and ‘$b$’ were updated till time ‘$t$’=‘$m$’ minutes, beyond which their values were frozen to slow down the reaction kinetics. The rise times for the model-generated (Fig. S18A and 18C) and experimental curves (Fig. S18B and S18D) were calculated using the strategy developed by Subramanian and Gomez[1]. Briefly, the time to positive was derived as:

$$t_p = m - (2/b)$$

where,

$t_p$ = Rise time or time to positive

$m$ and $b$ are parameters from Richard’s equation
Supplementary Figure S18. Comparison of c-SPK model results with experimental results.

Supplementary Figure S18. Comparison of c-SPK model results with experimental results. Time dependent equations for extrapolation of $k$, $b$ and $m$ were used to generate the final amplification curves for a reaction time of 120 minutes. (A) Concentration profile of amplicons with 20 starting copies of the dumbbell (B) Real-time amplification curves for LAMP experiments with 10 starting copies of the target (double stranded *Mtb* gDNA) (C) Concentration profile of amplicons with 200 starting copies of the dumbbell. (D) Real-time amplification curves for LAMP experiments with 100 starting copies of the target (double stranded *Mtb* gDNA). BS – Background subtracted.

The model generated curves constitute of total number of amplicons plotted against reaction time while the experimental curves provide a fluorescence read-out with time, normalized between 0 to 1. Multiple strategies were tested to create a calibration curve for DNA concentration versus fluorescence in order to convert the model generated results to fluorescence read outs. But it was
found that the linear dynamic range of fluorescence read-outs was very narrow with respect to the range of DNA concentration being generated in LAMP. Furthermore, the calibration curve used by Subramanian and Gomez[1] contains only two points and considers a linear behaviour in that range. With millions of copies of amplicons being generated in LAMP, we were unsuccessful in finding a way to correlate amplicon numbers with corresponding fluorescence. Despite that, it was observed that rise time predictions were quite comparable. Rise times predicted by c-SPK model were 96.89 minutes and 81.66 minutes for 20 and 200 starting copies of the dumbbell, respectively, while the corresponding experimental rise times were 103.76 ± 7.79 minutes and 81.45 ± 2.21 minutes, respectively.

References

[1] Subramanian S, Gomez RD. An empirical approach for quantifying loop-mediated isothermal amplification (LAMP) using Escherichia coli as a model system. PLoS One 2014;9:1–10. https://doi.org/10.1371/journal.pone.0100596.