A Two Stage Model for Quantitative PCR

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Abstract

In this paper we develop a suite of deterministic models for the reactions of quantitative PCR based on the law of mass action. The models are created by adding more reaction species to a base model (the logistic equation). Qualitative analysis is preformed at each stage and parameters are estimated by fitting each model to data from Roche LightCycler (TM) runs.

1 Introduction

The Polymerase Chain Reaction (PCR), a technique for the enzymatic amplification of specific target segments of DNA, has revolutionized molecular biological approaches involving genomic material. This, in turn, has impacted research in human genetics, disease diagnosis, cancer detection, evolutionary and developmental biology, and pathogen detection, to name a few. The company Idaho Technology, Inc. has capitalized on the invention of fluorescent probe techniques to create fast, accurate devices for quantitiative PCR. Quantitative PCR is a method where the amount of amplified DNA (or amplicon) is tracked throughout the reaction and the initial amount of sample DNA can then be estimated. Understanding the important parts of a complex reaction that is repeated tens of times, is critical in improving the design of these processes in the laboratory, and to date theoretical studies of quantitative PCR are limited. In this paper we present a suite of deterministic models for quantitative PCR, with parameters estimated from data provided from Roche LightCycler (TM) PCR runs. Determining the critical features of the model through construction of increasingly complex descriptions of the reaction is the overall goal of the project.

In PCR a reaction mixture containing a few copies of the target double-stranded DNA is first heated to separate the DNA into single strands. It is then rapidly cooled and held at a lower temperature briefly so that PCR primers (short single strands of DNA that have been designed for this purpose) anneal specifically to the template DNA. The enzyme Taq Polymerase recognizes these primer-template pairs and synthesizes a new strand of DNA, starting at the end of the annealed primer. In this way, a complementary strand is made from each strand of the original double-stranded DNA molecule. Under ideal reaction conditions the number of copies of this stretch of DNA in the sample is doubled in each heating-cooling cycle.

Instruments that perform real-time PCR usually detect the amplified DNA using fluorescent probes that are added to the PCR reagents before temperature cycling. These probes bind to the DNA and generally fluoresce more when bound than when free. When there is a sufficient quantity of DNA present in the sample (for example, after many temperature cycles), this change in fluorescence is detected using a fluorimeter. If the fluorescent signal of a sample rises above a background level, a sizable amount of DNA has been synthesized, indicating that the specific DNA was initially present.

Current methods for DNA quantification (for more information see the following references: Morrison et al. (1998), Wittwer et al. (1994, 1997), Weiss and Von Haeseler (1997), Sun (1995), Sun et al. (1996)) with PCR are based on comparing a set of successively diluted standards against unknown samples. The methods utilize the concentrations of the standards in a dilution series to determine the concentration of the unknown. The amount of DNA in successively diluted standards is typically decreased by factors of 2 or 10, and anywhere from three to ten standards are used. Figure 1 shows a set of six standards containing between one and 1,000,000 copies of initial DNA template. The fluorescence curve that crosses the threshold value at the smallest cycle number initially had 1,000,000 copies of DNA, the next curve to cross the threshold had 100,000 copies of DNA initially, and so on. Notice that the curve that does not cross the threshold is the control no template sample. Also plotted on this graph are two sets of replicates with unknown initial quantities of DNA template.

A quick estimate of the order of magnitude of the number of copies of DNA initially in the unknown samples can be found by simply comparing the amplification curves of the samples and diluted

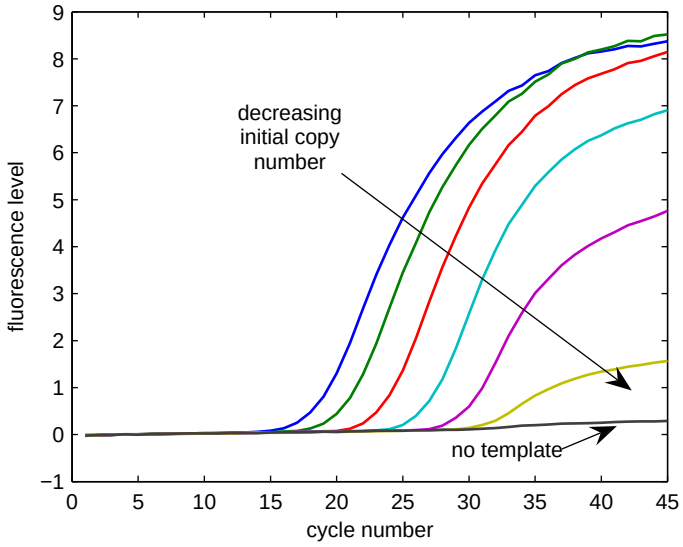

Figure 1: Fluorescence level vs. cycle number during PCR Roche Lightcycler run. Different lines are standard dilutions for quantification purposes, from 10^6 copies down to 10^1 and no template as a control. Also run simultaneously are two samples of unknown concentration, five replicates each.

standards. Current methods produce more precise estimates using a mathematical model of PCR that assumes the product grows exponentially:

$$C_{n+1} = C_n + EC_n = (1 + E)C_n = (1 + E)^n C_0. \tag{1}$$

In this model, C represents the number of copies of DNA, n represents the cycle number and E represents the efficiency of the PCR. E can be thought of as the percentage of existing DNA that is replicated in a cycle. Dilution standards have known values for C_0, and data from these samples can be used to calculate the efficiency E. Given the efficiency, the initial copy number C_0 can be estimated for each unknown sample.

This model is accurate for a small number of cycles, but grows less and less accurate as the number of DNA copies grows. Unfortunately, the fluorescence signal can be distinguished from the noise only later in the experiment, precisely as the model becomes less accurate. The simplest mistake in the model is the assumption that the efficiency does not change with cycle number, and that the number of copies of DNA always grows. In reality, PCR products saturate the reaction and resources are exhausted, slowing and eventually stopping DNA synthesis.

Quantification software based on the exponential growth model uses cycle-by-cycle fluorescence measurements to determine a quantity known as the cycle threshold (sometimes abbreviated CT). This threshold is a measure of the smallest fractional cycle number where the fluorescence of the sample is greater than the background. CTs are difficult to determine because the background fluorescence can drift up or down and the point where the fluorescence crosses the threshold is very dependent on noise in the data. Furthermore, most existing software requires users to set the threshold manually. The best automated method for finding CT doesn't associate CT with a

threshold, but instead determines CT with the cycle where the second derivative of the fluorescence curve is maximized. It is thought that this point is the cycle value where the fluorescence first exceeds the background, i.e. by "accelerating" out of the background. However, this method has two potential numerical problems. First, differentiation of data is a numerically ill-conditioned problem; and second, the data must be approximated by a continuous function to find fractional values for CT. Therefore, with either the thresholding algorithm or the second derivative algorithm, it is unlikely CT can be calculated robustly. The goal of this calculation is to compute values C_0, and unfortunately, even small errors in CT lead to large errors in value of C_0 because PCR increases the number of copies exponentially.

The amplification curves suggest that a more natural model for the PCR reaction would be logistic, which proceeds to saturation as a resource is depleted. Current IT software fits the data to such a logistic map, and uses the result to estimate initial copy number of the template. For the purpose of this estimation both the exponential growth model and the logistic model are sufficient in many cases, and have the advantage of a limited number of free parameters, requiring a minimum amount parameter estimation. However, for the long range goal of developing a more complete model of the reaction that can lead to innovation in process design, we must look beyond these one dimensional approximations. We also see that the data deviate from the logistic model in a consistent way for all the amplification curves, suggesting that the simplifications leading to it eliminate some critical features of the dynamics.

To our knowledge no deterministic model of the reactions of PCR that does not include assumptions about the kind of enzyme kinetics involved (i.e. Michaelis-Menten) are present in the literature. Stochastic models for estimating reaction efficiency and specificity can be found however, for instance, in Sun (1995) a model for distributions of mutations and estimation of mutation rates during PCR is developed, using the theory of branching processes. Another such model is reported in Weiss and Von Haeseler (1995), where the accumulation of new molecules during PCR is treated as a randomly bifurcating tree to estimate overall error rates for the reaction. In Schnell and Mendoze (1997), the reaction efficiency of quantitative competitive PCR (QC-PCR: a target and a competitor template are amplified simultaneously to provide an internal standard for identifying the initial target template amount) is computed using Michaelis-Menten type kinetics.

Stolvitzky and Cecchi (1996) address the validity of assuming a constant efficiency during a PCR reaction by deriving the probability of replication during successive cycles as a function of physical parameters. In the same vein, Velikanov and Kapral (1999) report on a probabilistic model of the kinetics of PCR using microscopic Markov processes. The result is an exact solution for the distribution of lengths of synthesized DNA strands, and an optimization procedure is applied to determine control parameters that maximized the yield of the target sequence. Most recently, in a 2004 publication Whitney et al. describe a stochastic model for competitive interactions during PCR to compute product distributions at the completion of regular PCR. The calculated yield is compared to experimental values from the amplification of three different size amplicons, with good results.

In the following sections we describe a hierachy of models built by including more biochemistry into each sucessive level of approximation. We analyze qualitatively and numerically the solutions to the models under typical operating conditions, and perform parameter estimation with data provided by Idaho Technology. Finally the advantages and disadvantages of including more details of the reactions into the model are discussed.

2 The Reactions of PCR

Table I: List of Variables and Notation Used in PCR Models

Variable name	quantity
C	copy number
E	exponential efficiency of reaction
S, s	single stranded DNA (ssDNA), $s = [S]$
P, p	primer molecule, $p = [P]$
S', s'	primed ssDNA, $s' = [S']$
Q, q	Taq molecule, $q = [Q]$
C, c	enzyme complex, $c = [C]$
N, n	nucleotide sequence for the extension, $n = [N]$
D, d	double stranded DNA (dsDNA), $d = [D]$
k_{-1}, k_1	forward and backward reaction rates for annealing
k_{-2}, k_2	forward and backward reaction rates for complex formation
k_{-3}, k_3	forward and backward reaction rates for extension
ϵ	logistic map growth parameter
δ	logistic model growth parameter
$\mathcal{K}$	carrying capacity of the logistic map
$\Gamma(d_i)$	growth parameter function for Taq model
e, α	parameters in $\Gamma(d_i)$
W_i	estimation of $\Gamma(d_i)$ from experimental data
Y_i	logarithmic regression variable
Δt	time step in discrete version of logistic model
t_1	time in stage I of two stage model without Taq dynamics
t_2	time in stage II of two stage model without Taq dynamics
τ_I	rescaled time in stage I of two stage model without Taq dynamics
τ_{II}	rescaled time in stage II of two stage model without Taq dynamics
K, K_n, K_d, K_s	conserved quantities in the two stage models
KK	normalization parameter used for experimental data
β, γ	rescaled reaction rates in the two stage model with Taq dynamics
s'^I, s'^{II}	s' in stage I and stage II respectively
$\bar{x}$	fixed point of the x variable
t_I, t_{II}	time in stage I and stage II in model with Taq dynamics

The PCR reaction proceeds through repeated cycles of dissociation, annealing and extention by Taq polymerase. During dissociation the sample is heated to approximately 90 degrees C where the template's DNA nucleotide base pairs unbind and the strand essentially unzips to form two half-strands (single stranded DNA). The sample is then cooled to a temperature where the primer reaction is optimal (about 60 degrees C), during which primer molecules, themselves sequences of single stranded DNA that have been designed to adhere to either end of the target sequence of the template, bind on. Then the sample is heated again to a temperature where Taq enzyme adds base pairs on the bracketed sequence to form a new double-stranded piece of DNA. The annealing/extension can done in one or two distinct steps, either with a continuous ramp-up to the Taq operating temperature (during which time the primers anneal) or with a lower temperature annealing stage followed by a higher temperature extension phase. We model the latter, but the

model itself could easily be adapted for the one-step scenario.

These three phases, dissociation, annealing, and extension are repeated typically 30-40 times to yield exponentially growing numbers of the target sequence, assuming the reaction runs as designed. Factors influencing the success of the reaction (is there a product?) are competition from contaminants in the reaction mixture, primers that bind to themselves or other primer molecules (primer-dimers), or primers that can extend pieces of the template other than the target, to name a few. Naturally the reaction saturates, see fig. 1, which is assumed to occur by complete depletion of primer molecules, since they are incorporated into the extended strands. The nucleotides in the mixture could also be used up, but typically they are present in great numbers to prevent this from occurring. Primers are synthesized molecules and are therefore much more costly than nucleotides. Also, the initial amount of DNA to be amplified can not be either too large or too small. If it is too large the number of primers is not sufficient to completely prime the molecules, and if too small it can lose out to the competing amplification of undesired sequences.

The reaction equations for these phases can be written as follows.
Dissociation:

$$D \rightarrow 2S$$

Annealing:

$$S + P \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} S'$$

Extension:

$$S' + Q \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} C$$

$$C + N \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} D + Q$$

Here D is double-stranded DNA, S is single-stranded DNA, P is primer, S', primed single-stranded DNA, Q, Taq polymerase, C, complex of primed single-stranded DNA, P', and Taq, N, nucleotides. The plus/minus k's represent the forward and backward reaction rate respectively. Ideally the reactions form a cascade, the product of one reaction continues into the next reaction and the final double-stranded DNA cycles back to the dissociation phase. In reality the reactions occur simultaneously, with highest frequency at their optimal temperature. For our purpose we will treat the phases as distinct and cascade the output of one phase to the input of the next. We also assume that the back reactions are negligible compared to the forward reactions in all but the creation of the enzyme complex, e.g. $k_{-1} = k_{-3} = 0$.

The law of mass action can be invoked to create differential equations for the concentrations of the above reactants, and we use lower case letters to indicate these concentrations, e.g. $[S] = s$, $[D] = d$, etc. We assume that the resource, nucleotides, is present in chunks of appropriate sequences of base pairs for the segment of DNA being extended. That is, we will assume that the extension happens all at once, not one base pair at a time. For the annealing reaction we have:

$$\frac{ds}{dt} = -k_1 sp \tag{2}$$

$$\frac{dp}{dt} = -k_1 sp \tag{3}$$

$$\frac{ds'}{dt} = +k_1 sp. \tag{4}$$

And for the extension phase the equations are:

$$\frac{ds'}{dt} = -k_2 s'q + k_{-2}c \tag{5}$$

$$\frac{dq}{dt} = -k_2 s'q + k_{-2}c + k_3 cn \tag{6}$$

$$\frac{dc}{dt} = k_2 s'q - k_{-2}c - k_3 cn \tag{7}$$

$$\frac{dn}{dt} = -k_3 cn \tag{8}$$

$$\frac{dd}{dt} = k_3 cn. \tag{9}$$

The exponential model (1) is a first level of approximation to the growth of double-stranded DNA created in these reactions, and the next level is simplification to a logistic map. This can be arrived at in a straightforward manner from these reaction equations by first ignoring the enzyme (Taq) dynamics. Working with the set of equations that result if the Taq dynamics is ignored we have: Annealing (as above):

$$\frac{ds}{dt} = -k_1 sp$$

$$\frac{dp}{dt} = -k_1 sp$$

$$\frac{ds'}{dt} = +k_1 sp$$

Extension:

$$\frac{ds'}{dt} = -k_3 s'n \tag{10}$$

$$\frac{dn}{dt} = -k_3 s'n \tag{11}$$

$$\frac{dd}{dt} = k_3 s'n \tag{12}$$

If the annealing stage is assumed to acheive 100% priming, the output of the first three equations is $s' = s$, the initial condition into the extension phase. In the extension phase there is a conserved quantity, namely $d + n$. Allowing $d + n = \mathcal{K} = d(0) + n(0)$ and setting $n = \mathcal{K} - d$ the extension phase can be reduced to two equations:

$$\frac{ds'}{dt} = -k_3 s'(\mathcal{K} - d)$$

$$\frac{dd}{dt} = k_3 s'(\mathcal{K} - d).$$

Taking an Euler step approximation to the ODEs in the extension phase leads to:

$$\Delta s' = -k_3 \Delta t s'(\mathcal{K} - d)$$

$$\Delta d = k_3 \Delta t s'(\mathcal{K} - d).$$

If the time step Δt is taken to be the time in the extension phase the result is a map for amounts of s', d, and n from the beginning of the extension phase to the end. For the i-th cycle:

$$\Delta s' = s'_{i+1} - s'_i = -\epsilon s'_i(\mathcal{K} - d_i)$$

$$\Delta d = d_{i+1} - d_i = \epsilon s'_i(\mathcal{K} - d_i).$$

where $\epsilon = \Delta t k_3$. With perfect priming $s'_i = s_i$, and with perfect dissociation $s_i = 2d_i$, the last equation becomes

$$d_{i+1} = d_i + \epsilon 2 d_i(\mathcal{K} - d_i), \tag{13}$$

which is a logistic map for d_i. The limiting factor in this derivation is the nucleotides, n, but more realistically the primers are limiting. A similar derivation can be carried out where the extension phase is perfect and the annealing phase is the limiting step, in which case the equation is the same, but the limiting factor is the primer.

To test the assumption of logistic data, and we fit a dilution series from a Roche LightCycler (TM) run to (13). The run was typical for these quantification experiments: it had 45 cycles, each consisting of a brief melt stage at 95 degrees C, a 10 second annealing stage at 55 degrees C, and a 30 second extension stage at 72 degrees C. The fluorescence aquisition occurred at the end of the annealing stage, and used a FRET (fluorescence resonance energy transfer) probe system. FRET probes are a pair of oglionucleotides labeled with fluorescent dyes. The pair are designed to hybridize to adjacent regions on the target DNA, and the marker dyes of each probe can only interact when they are in close proximity and bound to the target. The fluorophores are chosen so that the emission spectrum of one overlaps with the excitation spectrum of the other. The donor fluorophore is excited by a light source, transfers its energy to an acceptor fluorophore, which then emits light of a longer wavelength. This light is then detected during the fluorescence acquisition.

The parameter estimation was done in MATLAB using least squares to first compute K and γ, and a simplex search method based scheme (MATLAB's *fminsearch*) to find the initial fluorescence level. The objective function used in the nonlinear optimization was the two-norm of the difference between the model time series and the data. Percentage error was computed by dividing the final value of the objective function by the two-norm of the model time series.

Table II: Parameter Estimation for the Logistic Model (13)

	γ	K	d_0	% error
run 1	0.0185	8.1473	2.9×10^{-2}	5.15
run 2	0.0189	8.2832	1.6×10^{-2}	5.8
run 3	0.0214	7.9001	4.2×10^{-3}	5.15
run 4	0.0296	6.678	5.5×10^{-4}	4.9
run 5	0.0457	4.5959	1.02×10^{-5}	5.3
run 6	0.1273	1.5445	4.12×10^{-5}	5.82

The results of the parameter estimation for the dilution series are present in figure 3 and Table II. We see that the model is more than adequate for predicting initial copy number, given the current practice of running standards simultaneously with samples to generate a map between initial copy number and fluorescence level. The drift in the growth constant for decreasing copy number indicates that some aspect of the dynamics is not captured by this map, and for the lowest copy number run (number 6) we see that the initial amount estimated is off by an order of magnitude. In this case competition from other reactions is thought to be the culprit, but in all cases the map is a gross simplification to the actual dynamics. It clearly overestimates the growth for earlier cycles, and approaches saturation more quickly at later cycles. To verify this intuition quantitatively we preformed a version of logarithmic regression on the first five runs of the dilutions series (see figure 2). The basis for this regression is the separation of variables solution to the logistic differential equation:

$$\frac{dy}{dt} = \delta y(K - y),$$

namely

$$\ln(\frac{y}{K-y}) = K\delta t + \ln(\frac{y_0}{K-y_0}).$$

If y follows logarithmic growth the variable $Y = \ln(\frac{y}{K-y})$ will depend linearly on time t. Plots of the discretely sampled variable Y_i vs. cycle number i for standard data set are shown in figure 4, where it is obvious that they are not well-estimated by a linear function of i. There are several straight line regions in these graphs, corresponding to a) low cycle number noise, b) a region where the initial exponential growth occurs, and c) a third region where saturation happens, with a slope less than that seen in the second region. A linear fit of this data would have an intermediate slope causing an overestimate or an underestimate of the data, depending on the cycle number. This is apparent in figure 3, where it also is clear that the data approach the saturation level more slowly than logistic growth would warrant. One explanation of this is reduced efficiency of Taq polymerase when the quantity of molecules to extend becomes very large. This suggests a growth parameter $\Gamma(d_i)$ that varies with the amount of amplicon, d, so that

$$d_{i+1} = d_i + \Gamma(d_i)d_i(K - d_i),$$

where $\Gamma(d_i)$ is a decreasing function of d_i. The shape of this function can be estimated by plotting the variable found by solving the above equation for Γ:

$$W_i = \frac{d_{i+1} - d_i}{d_i(K - d_i)} = \Gamma(d_i).$$

That is, the graph of W_i vs. d_i will give an idea of $\Gamma(d_i)$. An example is shown in figure 5. The function Γ appears to be inversely proportional to d_i, so we fit a new map with $\Gamma(d_i) = \frac{e}{1+\alpha d_i}$, so

$$d_{i+1} = d_i + \frac{ed_i}{1 + \alpha d_i}(K - d_i). \tag{14}$$

The results are shown in figure 6, and in Table III. The error presented there is the mean square error.

Table III: Parameter Estimation of Taq Model (14).

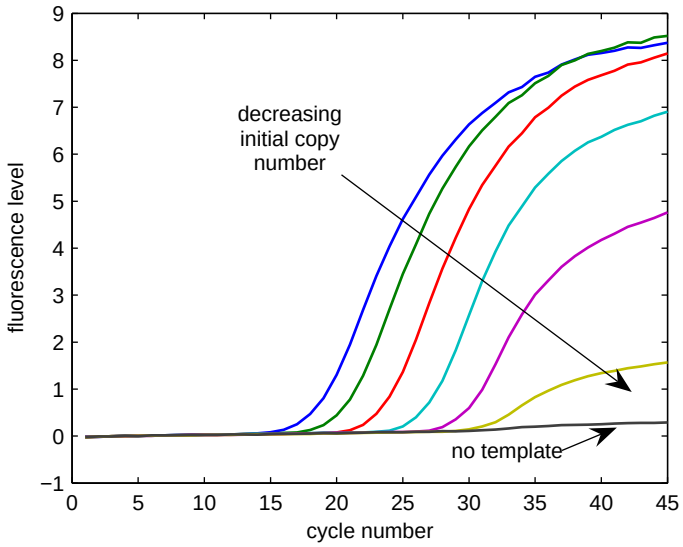

Figure 2: Fluorescence amplification data from an Roche Light-Cycler run. Five standard dilution series

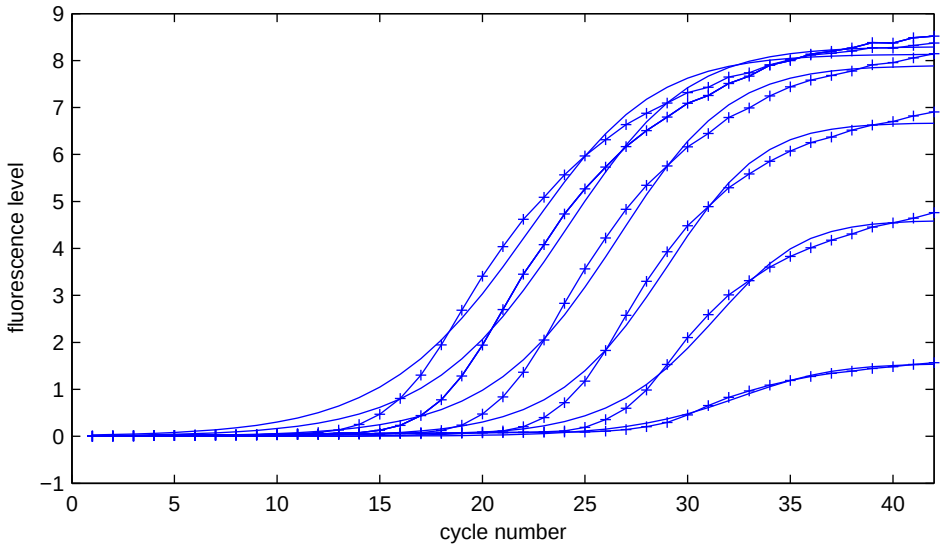

Figure 3: Fitting dilution series data with a logistic map. See text for parameter values. Solid line- model, (+++++)- data.

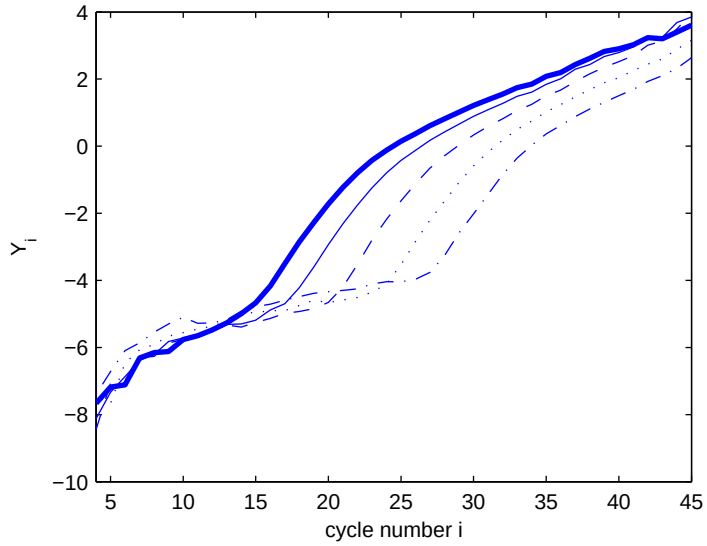

Figure 4: Logarithmic regression curves for five standard dilution series.

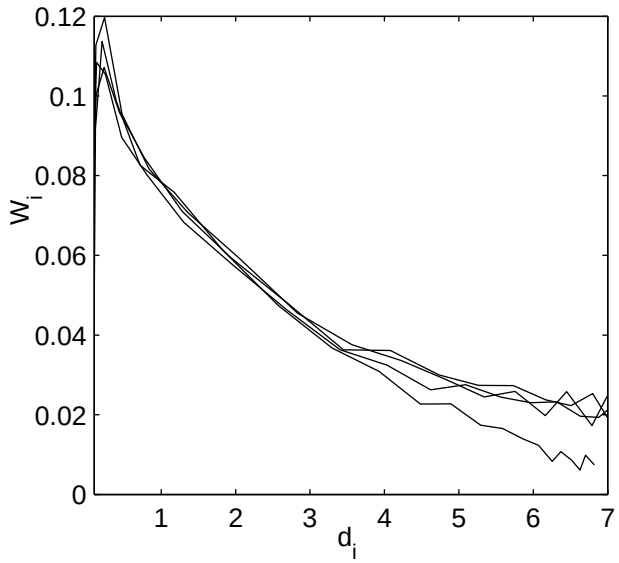

Figure 5: Plotting W_i vs. d_i to estimate $\Gamma(d_i)$ for the first four dilution series runs.

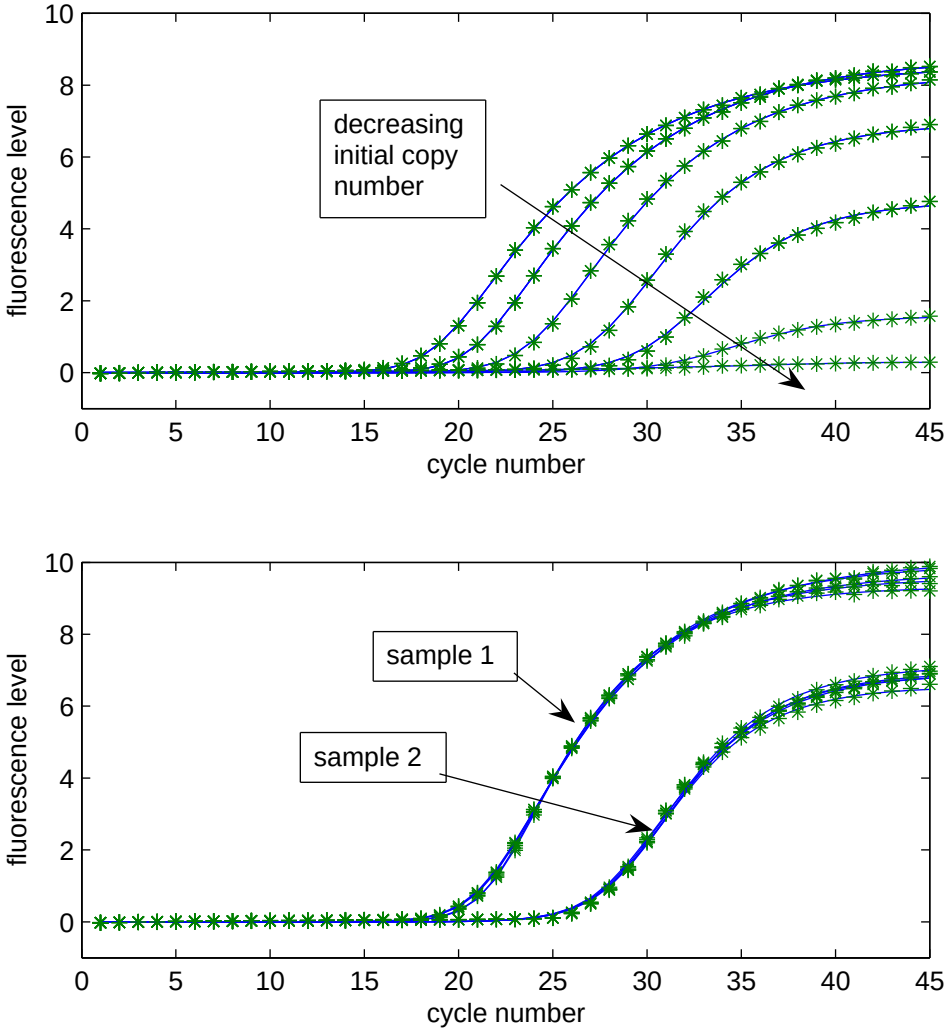

Figure 6: Standards data fitted with the Taq model (14). See Table II for parameter and error information.

	error	e	K	α	d_0
run 1	0.1894	0.1481	8.5579	0.9148	4.91×10^{-7}
run 2	0.2055	0.1473	8.7559	0.8907	7.31×10^{-8}
run 3	0.2587	0.1227	8.3295	0.6096	1.20×10^{-7}
run 4	0.3639	0.1237	6.9411	0.4158	1.06×10^{-7}
run 5	0.3945	0.1592	4.7559	0.4111	7.53×10^{-8}
run 6	0.2394	0.3721	1.5740	0.6636	3.02×10^{-7}
sample 1a	0.2321	0.1413	9.3277	0.5918	3.98×10^{-8}
sample 1b	0.2377	0.1546	9.5827	0.7381	1.04×10^{-8}
sample 1c	0.2189	0.1231	9.7042	0.5957	1.57×10^{-7}
sample 1d	0.2582	0.1202	9.9448	0.6035	1.60×10^{-7}
sample 1e	0.2610	0.1196	10.0509	0.6391	1.62×10^{-7}
sample 2a	0.4427	0.1297	6.5511	0.3543	8.28×10^{-8}
sample 2b	0.4203	0.1222	6.8775	0.3390	9.03×10^{-8}
sample 2c	0.4328	0.1214	6.9499	0.3581	9.08×10^{-8}
sample 2d	0.4223	0.1173	7.1260	0.3541	1.07×10^{-8}
sample 2e	0.4270	0.1267	6.9205	0.3514	6.42×10^{-8}

The growth coefficient for this model (e), and the value for α are more consistent than for the regular logistic model, though the variation is more pronounced in smaller copy number runs, and suffers the same overcalculation of the initial fluorescence in run 6. (Also in run 6 the model coefficients are significantly different from the other runs). We also fitted the replicates of the unknown samples with reasonably consistent results, though it is clear there is a trade-off between values such as the growth constant e and the initial fluorescence, indicating hidden dependencies in the parameters that cannot be differentiated with this sort of data.

This could be the end of the story, but an empirically determined rate function is not as satisfactory as a model that captures the behavior built-up directly from the reaction equations. In the next section we construct such a model and parameterize it with the data.

3 The Two Stage Model

We consider here two versions of a two stage model, one that includes the dynamics of the Taq enzyme, and one that does not. The latter is much simpler and can be solved analytically, so we present it first. Both make the assumption of complete dissociation, so that the amount of ssDNA entering the annealing phase is equal to twice the amount of dsDNA from the previous extension phase, plus whatever ssDNA was leftover from the previous annealing phase. This eliminates the need for the equation that describes dissociation (or the "melt") phase of the reaction. We next assume that the annealing phase happens distinct from the extension phase and call this stage I. The equations for stage I are (2)-(4). The extension phase we name stage II, and with Taq dynamics the equations are (5)-(9). Without the Taq dynamics the equations are given by (10)-(12).

3.1 Two Stage Model without Taq Dynamics

The two stages in both versions are linked through their initial conditions, and in both the initial amount of primed ssDNA in stage II is equal to the amount created in stage I, while the initial amount of nucleotide in stage II is whatever was left over from the previous cycle of stage II. For the model without Taq dynamics, upon completion of stage II any unextended primed ssDNA will break-up in dissociation, and thus the initial amount of ssDNA in stage I is that plus the amount left-over from the previous stage I, plus twice the amount of double stranded DNA created in stage II. The initial amount of primer in stage I is also the sum of the dissociated amount from stage II and the amount leftover from the previous stage I. The initial amount of double-stranded DNA in stage II is assumed to be zero at the start of every cycle. Written as equations these initial conditions are:

Stage I:

$$s(0) = s(t_{end}, \text{previous stage I}) + s'(t_{end}, \text{previous stage II}) + 2d(t_{end}, \text{previous stage II}); \quad (15)$$

$$p(0) = s'(t_{end}, \text{previous stage II}) + p(t_{end}, \text{previous stage I}); \;\; s'(0) = 0.0; \quad (16)$$

Stage II:

$$s'(0) = s'(t_{end}, \text{ stage I}); \quad n(0) = n(t_{end}, \text{previous stage II}); \;\; d(0) = 0.0. \quad (17)$$

There are two conserved quantities in the equations for both stage I and stage II, so the systems can be reduced to one equation each and solved analytically. For stage I we use the conserved quantities: $p(t) - s(t) = K = p(0) - s(0)$ and $s(t) + s'(t) = K_s = s(0) - s'(0) = s(0)$, (since $s'(0) = 0$) so that the resulting ODE for $p(t)$ is

$$\frac{dp}{dt} = k_1(K - p)p,$$

with the solution

$$p(t) = \frac{K}{1 - \frac{s(0)}{p(0)} \exp(-Kk_1 t)}.$$

and the other variables are found from the conserved quantities:

$$s(t) = p(t) - K = p(t) - p(0) + s(0),$$

$$s'(t) = K_s - s(t) = s(0) - s(t).$$

It is expedient to rescale the dependent variables in the original ODEs by the amount of one quantity at the beginning of the reaction, and since the nucleotides are present in excess we call that N_0 and define new variables: $\hat{p} = \frac{p}{N_0}, \hat{s} = \frac{s}{N_0}, \hat{s'} = \frac{s'}{N_0}, \hat{n} = \frac{n}{N_0}, \hat{d} = \frac{d}{N_0}$. We also rescale time by define a new time $\hat{t_1} = \hat{k_1} t$, where $\hat{k}_1 = k_1 N_0$.

The solution is then

$$\hat{p}(\hat{t}_1) = \frac{\hat{K}}{1 - \frac{\hat{s}(0)}{\hat{p}(0)} \exp(-\hat{K}\hat{t}_1)},$$

$$\hat{s}(\hat{t}_1) = \hat{p}(\hat{t}_1) - \hat{K} = \hat{p}(\hat{t}_1) - \hat{p}(0) + \hat{s}(0),$$

$$\hat{s}'(\hat{t}_1) = \hat{K}_s - \hat{s}(\hat{t}_1) = \hat{s}(0) - \hat{s}(\hat{t}_1).$$

where $\hat{K} = \hat{p}(0) - \hat{s}(0)$, $\hat{K}_s = \hat{s}(0)$.

The quantities in stage II can be computed in an identical manner, and with the choice of conserved quantities $s'(t) + d(t) = K_d = s'(0) + d(0) = s'(0)$, $n(t) - s'(t) = K_n = n(0) - s'(0)$ the solution is

$$n(t) = \frac{K_n}{1 - \frac{s'(0)}{n(0)} \exp(-K_n k_2 t)},$$

and the other variables are found from the conserved quantities:

$$s'(t) = n(t) - K_n = n(t) - n(0) + s'(0),$$

$$d(t) = K_d - s'(t) = s'(0) - (n(t) - n(0) + s'(0)) = n(0) - n(t).$$

Rescaling again by the amount of nucleotide at the beginning of the first cycle, N_0, and defining $\hat{t}_2 = \hat{k}_2 t$, (where $\hat{k}_2 = k_2 N_0$) results in

$$\hat{n}(\hat{t_2}) = \frac{\hat{K}_n}{1 - \frac{\hat{s}'(0)}{\hat{n}(0)} \exp(-\hat{K}_n \hat{t_2})}$$

$$\hat{s}'(\hat{t_2}) = \hat{n}(\hat{t_2}) - \hat{K}_n = \hat{n}(\hat{t_2}) - \hat{n}(0) + \hat{s}'(0)$$

$$\hat{d}(\hat{t_2}) = \hat{K}_d - \hat{s}'(\hat{t_2}) = \hat{n}(0) - \hat{n}(\hat{t_2})$$

where $\hat{K}_d = \hat{s}'(0)$, $\hat{K}_n = \hat{n}(0) - \hat{s}'(0)$. The initial conditions can be written in terms of the rescaled variables, they are identical in form to (16) and (17), with $\hat{X}$ replacing X, for each variable. From this point forward we rename $\hat{X} = X$ to simplify the notation, while keeping in mind how the rescaling changes the initial conditions.

A map from one cycle to the next can be constructed from these solutions and the initial conditions (16) and (17). To distinguish between the concentration of primed single stranded DNA (ssDNA') in the first and second stage we designate them s'^I and s'^{II}. Let the final time for each stage be fixed at τ_I and τ_{II} respectively. The map for stage I is then:

$$p_i(\tau_I) = \frac{K_i}{1 - \frac{s_i(0)}{p_i(0)} \exp(-K_i \tau_I)},$$

where $K_i = p_i(0) - s_i(0)$, and

$$s_i(\tau_I) = p_i(\tau_I) - K = p_i(\tau_I) - p_i(0) + s_i(0),$$

$$s_i'^I(\tau_I) = K_s - s_i(\tau_I) = s_i(0) - s_i(\tau_I),$$

and for stage II:

$$n_i(\tau_{II}) = \frac{K_n}{1 - \frac{s_i'^{II}(0)}{n_i(0)} \exp(-K_n \tau_{II})},$$

where $K_n = n_i(0) - s_i'^{II}(0)$, and

$$s_i'(\tau_{II}) = n_i(\tau_{II}) - K_n = n_i(\tau_{II}) - n_i(0),$$

$$d_i(\tau_{II}) = K_d - s_i'^{II}(\tau_{II}) = n_i(0) - n_i(\tau_{II}).$$

The initial conditions for the ith cycle are:

$$\begin{gathered} s_i(0) = s_{i-1}(\tau_I) + s_{i-1}'^{II}(\tau_{II}) + 2d_{i-1}(\tau_{II}), \\ p_i(0) = p_{i-1}(\tau_I) + s'^{II}(\tau_{II}), \\ s'^{I}(0) = 0.0, \\ s_i'^{II}(0) = s_i'^{I}(\tau_I), \\ n_i(0) = n_{i-1}(\tau_{II}), \\ d_i(0) = 0.0. \end{gathered}$$

While a closed form version of the map can be written down, it is not particularly illuminating, simulations must be performed to uncover the behavior of the solutions. To do this parameters must be estimated or fit from the data, these are τ_I, τ_{II}, and the initial concentrations of primer and ssDNA, $p_1(0), s_1(0)$, relative to the initial concentration of nucleotides, N_0. The model and data must both saturate at the same level, and a scaling quantity for the data values was fit for each curve, which we called KK. The parameter estimation was done as in the previous section, with the same set of data. The percentage error in each fit was computed by dividing the final value of the objective function by the two-norm of the model time series vector.

For the dilution series all these parameters were fit, see figure 7 and Table IVa. The KK value found is consistent with the variation of the saturation value for each run, and the for each run τ_1 is greater than τ_2, indicating that annealing is slower than extension. The initial quantity $s_1(0)$ decreases roughly by a factor of 10 for each run, also consistent with the dilution series. The initial amount of primer in each run is close to 1.0, indicating the amount of primer must be close to the amount of nucleotide in a run to match the data, which is not consistent with the information we have about the experiment, which is that the nucleotides (measured in blocks of the template sequence to be amplified) to primer ratio is about 4-to-1. We return to this issue when we examine the model including Taq dynamics. The 6th run has a much larger error than the others, indicating that other factors come into play in the dynamics of very small copy number runs, such as competition from other species (e.g. primer-dimers).

We then fit the model to the runs with unknown initial concentrations, of which there are two, each with five replicates. For the these 10 runs $\tau_I, \tau_{II}, p_1(0)$ were fixed at values from the dilution run nearest in CT (for the sample 1 this was run 2, for sample 2, run 4). The results from this exercise are presented in Table IVb.

Table IVa: Parameter Estimation of the Two Stage Model without Taq Dynamics-Dilution Series

	KK	τ_I	τ_{II}	$s_1(0)$	$p_1(0)$	% error
run 1	8.989	1.525	0.829	1.84e-04	0.9935	10.1
run 2	9.169	1.551	0.853	7.23e-05	0.9933	9.8
run 3	8.768	1.597	0.916	1.67e-05	0.9930	9.4
run 4	7.390	1.466	1.216	1.70e-06	0.9978	8.0
run 5	4.902	1.454	1.598	1.68e-07	0.9636	10.4
run 6	1.645	4.787	0.939	8.00e-8	0.991	19.38

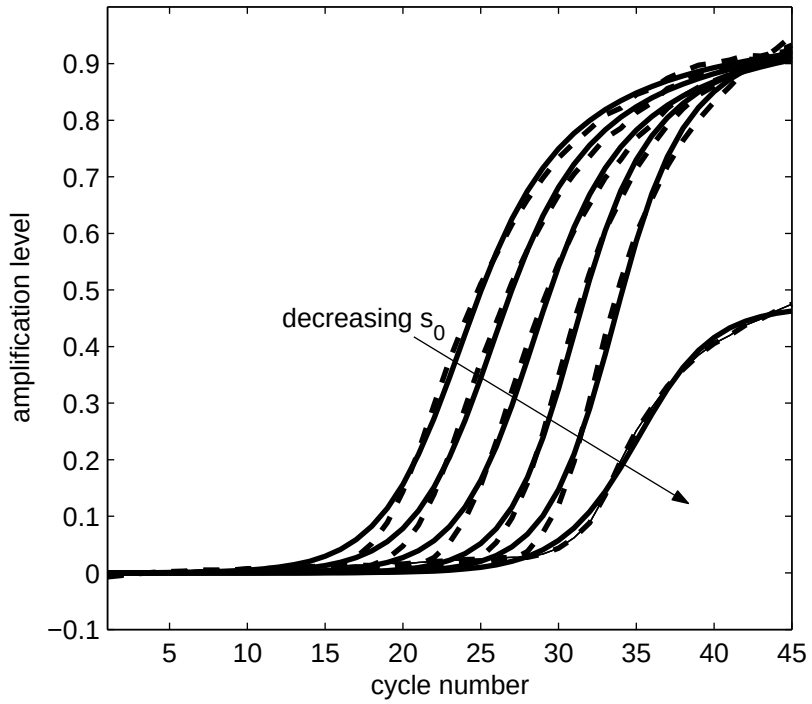

Figure 7: Fitting dilution series data to the two stage model without Taq dynamics. For parameter values see Table IVa.

Table IVb: Parameter Estimation of Two Stage Model w/out Taq Dyn.-Unknown Samples

	$s_1(0)$	KK	% error
sample 1a	8.019e-05	10.35	14.7
sample 1b	7.87e-05	10.482	13.0
sample 1c	7.67e-05	10.518	11.2
sample 1d	7.57e-05	10.713	10.9
sample 1e	7.42e-05	10.722	10.7
sample 2a	1.51e-06	7.158	9.4
sample 2b	1.40e-06	7.490	8.6
sample 2c	1.40e-06	7.518	8.6
sample 2d	1.39e-06	7.688	8.5
sample 2e	1.50e-06	7.430	8.8

3.2 Two Stage Model with Taq Dynamics

For this model the equations for stage I remain as (2)-(4), while the stage II equations now include both Taq and complex concentrations and are given by (10)-(12). As in the simpler model the initial amount of primed ssDNA in stage II is equal to the amount created in stage I, while the initial amount of nucleotide in stage II is whatever was left over from the previous cycle of stage II. Upon completion of stage II any unextended complex will break-up during dissociation, as will any primed ssDNA. Thus the initial amount of complex in stage II will be zero, and the amount of Taq enzyme will be the original amount from the beginning of the PCR reaction (Q). Stage

I starts with no primed ssDNA, it is assumed to dissociate during the melt phase. The primer initial condition is the amount of unused primer from the previous cycle, plus the amount created during the dissociation of the complex during the melt phase. The initial amount of ssDNA will be that left from the previous annealing phase plus an amount equal to amount of complex left in stage II that dissociates, plus the ssDNA that results from the dissociation of the dsDNA created in the previous stage II, which is double the amount of dsDNA. In terms of equations these initial conditions can be stated:

$$s'(0) = s'(t_{end},\ \text{stage I});\quad q(0) = Q;\quad c(0) = 0.0;\quad n(0) = n(t_{end}, \text{previous stage II});\quad d(0) = 0.0. \tag{18}$$

and for stage I:

$$s(0) = s(t_{end}, \text{previous stage I}) + c(t_{end}, \text{previous stage II}) + 2d(t_{end}, \text{previous stage II}); \tag{19}$$
$$p(0) = c(t_{end}, \text{previous stage II}) + s'(t_{end}, \text{previous stage II}) + p(t_{end}, \text{previous stage I});$$
$$s'(0) = 0.0.$$

A map for the reaction is created by integrating the ODEs in each stage and using the initial condition rules to link one stage to the other. However, insight can be gained by analyzing the dynamics of each stage separately and forming some special limiting cases for this map.

3.2.1 Dynamics of Stage I

The equations for stage I are again completely integrable, because of two conserved quantities, $s + s' = K_s = s'(0) + s(0) = s(0)$, and $p - s = K = p(0) - s(0)$. Simplifying the equation for p using these quantities yields

$$\frac{dp}{dt} = k_1(K - p)p. \tag{20}$$

Rescaling time, $\tilde{t} = p(0)k_1 t$, $\frac{dp}{d\tilde{t}} = \dot{p}$, and the molecular concentration of all three quantities by $p(0)$, i.e. $\hat{p} = \frac{p}{p(0)}, \hat{s} = \frac{s}{p(0)}, \hat{s'} = \frac{s'}{p(0)}$, and $\hat{K} = \frac{p(0)-s(0)}{p(0)} = 1 - \hat{s}(0)$, results in

$$\dot{\hat{p}} = (\hat{K} - \hat{p})\hat{p},$$

which has the solution

$$\hat{p}(\tilde{t}) = \frac{\hat{K}}{1 - \hat{s}(0)e^{-\hat{K}\tilde{t}}}, \tag{21}$$

so the remaining quantities can be computed

$$\hat{s}(\tilde{t}) = \hat{p}(\tilde{t}) - \hat{K} = \hat{p}(\tilde{t}) + \hat{s}(0) - 1,$$

and

$$\hat{s'}(\tilde{t}) = \hat{K}_s - \hat{s}(\tilde{t}) = 1 - \hat{p}(\tilde{t}).$$

Note that in the limit as $\tilde{t} \to \infty$, $\hat{p}(\tilde{t}) \to \hat{K}$ if $\hat{K} > 0$, e.g. $1 > \hat{s}(0)$, more initial primer than ssDNA, and $\hat{p}(\tilde{t}) \to 0$ if $\hat{K} < 0$, i.e., there is more ssDNA to begin with than primer. There is a transcritical bifurcation at $\hat{K} = 0$, $\hat{s}(0) = 1$, where the two fixed points for the system (20), $\bar{p} = K$ and $\bar{p} = 0$, exchange stability.

3.2.2 Dynamics of Stage II

The stage II ODEs can be simplified by rescaling time to remove one rate constant. The dimensionless time chosen, τ, is $k_{-2}t$ and the new system is

$$\dot{s'} = -\gamma s' q + c \tag{22}$$

$$\dot{q} = -\gamma s' q + c + \beta c n \tag{23}$$

$$\dot{c} = \gamma s' q - c - \beta c n \tag{24}$$

$$\dot{n} = -\beta c n \tag{25}$$

$$\dot{d} = \beta c n \tag{26}$$

where $\frac{df}{d\tau} = \dot{f}, \beta = \frac{k_3}{k_{-2}}, \gamma = \frac{k_2}{k_{-2}}$.

The initial conditions are (as stated previously):

$$s'(0) = s'(t_{end}, \text{stage I});\ \ q(0) = Q;\ \ c(0) = 0.0;\ \ n(0) = n(t_{end}, \text{previous stage II}),\ \ d(0) = 0.0.$$

The molecular quantities in the above equations can themselves be scaled, we choose here to scale by the initial amount of nucleotides available at the start of the cycle, $N_0 = n(0)$. The rescaled equations have the same form, with rescaled parameters $\gamma = \gamma/N_0$ and $\beta = \beta/N_0$ (meaning we adopt the notation $\hat{X} = \frac{X}{N_0}$ for each quantity, and then discard the hat for simplicity). The corresponding initial conditions are

$$s'(0) = \frac{s'(t_{end}, \text{stage I})}{N_0};\ \ q(0) = \frac{Q}{N_0};\ \ c(0) = 0.0;\ \ n(0) = 1,\ \ d(0) = 0.0.$$

The equations (22)-(26), have three conserved quantities (e.g. $n+d, q+c, s+c+d$), so the dynamics can be reduced from a five to a two-dimensional system. Since initially there will be no c or d, the these conserved quantities can be written $s' + c + d = s'(0),\ \ q + c = q(0),\ \ n + d = 1$. The two dimensional system that results from incorporating the conserved quantities is:

$$\dot{q} = -\gamma(s'(0) - q(0) - 1 + q + n)q + q(0) - q + \beta(q(0) - q)n$$

$$\dot{n} = -\beta(q(0) - q)n.$$

From this we can more readily determine fixed points and analyze their stability. Two of the three fixed points for this system are physically relevant, and are given by

$$\text{f.p. } 1 = (\bar{s'} = 0,\ \ \bar{q} = q(0),\ \ \bar{c} = 0,\ \ \bar{n} = 1 - s'(0),\ \ \bar{d} = s'(0)).$$

and

$$\text{f.p. } 2 = (\bar{q} = \frac{1}{2\gamma}(\gamma(q(0) + 1 - s'(0)) - 1 + \sqrt{(-\gamma(q(0) + 1 - s'(0)) + 1)^2 + 4\gamma q(0)})),$$

$$\bar{s'} = s'(0) - q(0) - 1 + \bar{q},\ \ \bar{c} = q(0) - \bar{q},\ \ \bar{n} = 0,\ \ \bar{d} = 1).$$

The third fixed point has the q-coordinate:

$$\bar{q} = \frac{1}{2\gamma}(\gamma(q(0) + 1 - s'(0)) - 1 - \sqrt{(-\gamma(q(0) + 1 - s'(0)) + 1)^2 + 4\gamma q(0)}))$$

which can be shown to be always negative, and so physically irrelevant. For a proof of this fact, and the complete stability analysis, see appendix I. Here we state the main result only: There is a transcritical bifurcation when $s'(0) = 1 = n(0)$, here f.p. 1 and f.p. 2 are identical and exchange stability. For $n(0) > s'(0)$ f.p. 1 is attracting and has non-negative coordinate values. For $n(0) > s'(0)$ f.p. 2 is attracting with non-negative coordinate values. The reaction will typically begin with more nucleotides than primed ssDNA, so the long-time behavior is represented by f.p. 1, which has final dsDNA value equal to the amount of primed ssDNA (all primed ssDNA is extended). If the initial amount of primed ssDNA exceeds the amount nucleotide at the start of that cycle, the second fixed point becomes attracting and non-negative, and in this case the long term dsDNA amount will be equal to the initial amount of nucleotide. Hence the limiting value of dsDNA switches from the initial amount of primed ssDNA to the initial amount of nucleotide as primer becomes limiting, as would be anticipated.

3.3 Limiting Cases

Assuming both stage I and stage II ODEs reach steady state, a map can be constructed by linking the attracting fixed points through the initial conditions. Since the rescaling of each stage used in the previous subsection is different, (and changes with each cycle) it is best to return to the unscaled quantities to create the map. The fixed points for stage II in terms of the unscaled quantities are the same, with the 1.0's replaced by $n(0)$'s. The bifurcation occurs when $s'(0) = n(0)$, as was outlined previously.

The attracting fixed point for stage I depends on the initial values of primer and ssDNA, we will write it $(\bar{p},\ \bar{s},\ \bar{s}'^{II} = p(0) - s(0),\ 0,\ s(0))$ if $p(0) > s(0)$ (there is enough primer to prime all the ssDNA). If $p(0) < s(0)$ it is $(\bar{p},\ \bar{s},\ \bar{s}'^{II} = 0,\ p(0) - s(0),\ p(0))$: the limiting amount is primer. The attracting fixed point for stage II depends on the relative size of $n(0)$ and $s'(0)$, the latter being equal to $\bar{s}'$ from the previous stage I. The initial amount of nucleotide will be determined by how much remains from the previous cycle. In a similar manner the rest of the initial conditions are determined by the fixed points from the previous cycle, this dependance is detailed below.

For stage I the initial amount of primer is the sum of what is left over from the previous stage I (cycle $i-1$), and the primer released from the dissociation of the complex and the unextended primed ssDNA from the previous stage II:

$$p_i(0) = \bar{p}_{i-1} + \bar{c}_{i-1} + \bar{s}'^{II}_{i-1}.$$

The initial amount of ssDNA will be twice the amount of dsDNA created in the previous cycle, plus the ssDNA released from the dissociation of the complex and the unextended primed ssDNA from the previous stage II, plus the amount of ssDNA not primed in the previous stage I:

$$s_i(0) = 2\bar{d}_{i-1} + \bar{c}_{i-1} + \bar{s}'^{II}_{i-1} + \bar{s}_{i-1}.$$

At the start of stage I there is no primed ssDNA: $s'^{I}_i(0) = 0$.

In stage II the initial amount of primed ssDNA is equal to the amount coming out of stage I, $\bar{s}'^{I}_i$, the initial amount of Taq is a constant, Q, the complex has been completely dissociated during the melt phase, along with any dsDNA. The amount of nucleotide is equal to the amount left over from the previous stage II:

$$s'^{II}(0) = \bar{s}'^{I}_i;\ \ q_i(0) = Q;\ \ c_i(0) = 0.0;\ \ n_i(0) = \bar{n}_{i-1};\ \ d_i(0) = 0.0.$$

There will be four distinct cases of the map depending on the relative size of $n(0), s'^{II}(0)$ and $p(0), s(0)$. Here we limit the analysis to a physically realistic scenario: initially both primer and nucleotide dominate, but initial primer amount is less than initial nucleotide amount. In the course of creating new amplicon both primer and nucleotide amounts decrease, and since they are used in the same proportion the primers will be exhausted before the nucleotides. This will cause a shift to another case of the map, and the remaining two iterations will complete the process, since no more primer will be available to make the extension possible. The equations for this scenario are presented next.

First assume $p_i(0) > s_i(0)$ and $n_i(0) > s - I'^{II}(0)$, so the stage I fixed point is (for the ith cycle)

$$\bar{p}_i = p(0)_i - s(0)_i; \;\; \bar{s}_i = 0; \;\; \bar{s}_i'^{I} = s_i(0).$$

Then assume $n_i(0) > s_i'^{II}(0)$ so that the fixed point for stage II is

$$\bar{s}_i'^{II} = \bar{c}_i = 0, \;\; \bar{q}_i = q(0).$$

$$\bar{n}_i = n(0)_i - s_i'^{II}(0),$$
$$\bar{d}_i = s_i'^{II}(0).$$

Now, the initial conditions for cycle i are determined by the fixed points from cycle $i-1$ in this manner:

$$p(0)_i = \bar{p}_{i-1} + \bar{c}_{i-1} + \bar{s}_{i-1}'^{II} = \bar{p}_{i-1}$$
$$s(0)_i = 2\bar{d}_{i-1} + \bar{c}_{i-1} + \bar{s}_{i-1}'^{II} + \bar{s}_{i-1} = 2d_{i-1}.$$

Substituting these into the fixed point for stage I yields:

$$\bar{p}_i = \bar{p}_{i-1} - 2\bar{d}_{i-1}; \;\; \bar{s}_i = 0; \;\; \bar{s}'^{I} = 2\bar{d}_{i-1}.$$

The initial conditions for stage II in terms of fixed points for the previous cycle are

$$n(0)_i = \bar{n}_{i-1}; \;\; s'^{II}(0)_i = \bar{s}_i'^{I} = 2\bar{d}_{i-1}.$$

And so the stage II fixed point is then

$$\bar{s}_i'^{II} = \bar{c}_i = 0, \;\; \bar{q}_i = Q$$

$$\bar{n}_i = \bar{n}_{i-1} - 2\bar{d}_{i-1}$$
$$\bar{d}_i = 2\bar{d}_{i-1}$$

This is simple doubling of the double-stranded DNA, and will proceed until the amount of primer ($p(0)$) at the beginning of stage I is less than the amount of single-stranded DNA ($s(0)$). At this cycle (call it N) the attracting fixed point switches in stage I and a new map is created that is valid for exactly one cycle. The new stage I fixed point is:

$$\bar{p}_N = 0$$

$$\bar{s}_N = s_N(0) - p_N(0) = 2\bar{d}_{N-1} - \bar{p}_{N-1}$$
$$\bar{s}_N'^{I} = p_N(0) = \bar{p}_{N-1}$$

In stage II the amount of nucleotide is depleted by an amount equal to the amount of $\bar{s}'^{I}$ created, which equals $\bar{p}_{N-1}$. The amount of double stranded DNA will be equal to that amount as well, so the map for stage II is:

$$\bar{s}'^{II}_N = \bar{c}_N = 0, \;\; \bar{q}_N = q(0)$$

$$\bar{n}_N = \bar{n}_{N-1} - \bar{p}_{N-1}$$

$$\bar{d}_N = \bar{p}_{N-1}$$

For the next cycle, $N+1$, there is no primer left so the duplication ends. The fixed point values in this cycle are:

$$\bar{p}_{N+1} = 0$$

$$\bar{s}_{N+1} = 2\bar{d}_N + \bar{s}_N = 2\bar{p}_{N-1} + 2\bar{d}_{N-1} - \bar{p}_{N-1} = \bar{p}_{N-1} + 2\bar{d}_{N-1} = s_{\text{final}}$$

$$\bar{s}'^{I}_{N+1} = 0.$$

For stage II:

$$\bar{n}_{N+1} = \bar{n}_N - \bar{p}_N = \bar{n}_{N-1} - \bar{p}_{N-1} = n_{\text{final}}$$

$$\bar{d}_{N+1} = 0$$

It is straight-forward to show that this is a fixed point for the map from one cycle to the next, once this stage is reached the final value of extended DNA is fixed. It lives in the reaction as single stranded DNA until the mixture is allowed to "finish off" and the strands reanneal (finishing off more technically refers to the stage in which the extension is allowed to run to completion, and all the primed single-stranded DNA molecules have been turned into double stranded DNA, which happens in cycle N).

This map, created for the limiting case of infinite time for each stage in each cycle, converges on the model of simple doubling until the reaction limiting species (either p or n) is exhausted. Then in one cycle the reaction finishes off and reaches a fixed point. Clearly this does not capture the sigmoidal growth curve or the variation away from it. The next step is to allow the extension in stage II to reach the asymptotic fixed point determined by initial conditions for each cycle, but to use the exact solution of the stage I equations, with the run time left as a parameter, to determine the values at the end of stage I. We construct this map next.

The exact solution for the stage I variables is as follows:

$$p_i(T_I) = \frac{K}{1 + \frac{s_i(0)}{p_i(0)} e^{-KT_I}} = f_{T_I}(p_i(0), s_i(0)), \tag{27}$$

where $K = p_i(0) - s_i(0)$. The unprimed and primed ssDNA depend on p through the conserved quantities:

$$s_i(T_I) = p_i(T_I) + s_i(0) - p_i(0) \tag{28}$$

$$s'^{I}_i = p_i(0) - p_i(T_I). \tag{29}$$

The stage II fixed point for $n(0) > s'^{II}(0) > 0$ will be:

$$\bar{s}'^{II}_i = \bar{c}_i = 0, \;\; \bar{q}_i = Q$$

$$\bar{n}_i = n_i(0) - s'^{II}_i(0) = n_i(0) - s'^{I}_i(T_I)$$

$$\bar{d}_i = s'^{II}(0) = s'^{I}_i(T_I) = p_i(0) - p_i(T_I).$$

The initial conditions for the next stage I are then

$$p_{i+1}(0) = p_i(T_I) + \bar{c}_i + \bar{s}_i'^{II} = p_i(T_I), \tag{30}$$

$$s_{i+1}(0) = 2\bar{d}_i + \bar{c}_i + \bar{s}_i'^{II} + s_i(T_I) = 2(p_i(0) - p_i(T_I)) + p_i(T_I) + s_i(0) - p_i(0) = p_i(0) - p_i(T_I) + s_i(0), \tag{31}$$

and

$$s'_{i+1}(0) = 0. \tag{32}$$

Note that at this point the stage I initial conditions, in which the map is cast, depend only on the previous stage I values, the stage I map runs independently of stage II. The stage II fixed point can be determined directly from the stage I variables, and the only one of interest is the amount of nucleotide, for when that is exhausted the reaction will stop. The equation for nucleotide, n, is:

$$n_{i+1}(0) = \bar{n}_i = n_i(0) - s_i'^{I}(T_I) = n_i(0) - (p_i(0) - p_i(T_I)). \tag{33}$$

There are two possibilities for the completion of the reaction: either primer runs out or the nucleotides. In the case that primer runs out first, we look at the limit as $p_i(0) \to 0$, so that $p_i(T_i) \to 0$ and $s_i(T_I) \to s_i(0)$ and $s'_i(T_I) \to 0$. In stage II no complex will be formed, or double-stranded DNA created, as there is no primed ssDNA available at the start of the reaction: $s_i'^{II}(0) = s'_i(T_I) = 0$. The fixed point for the nucleotide is thus the value of the nucleotide at the beginning of the cycle, $\bar{n}_i = n_i(0) - s_i'^{I}(T_I) = n_i(0)$, and the map for n_i is at a fixed point, $n_{i+1}(0) = n_i(0)$.

If the resource is the limiting factor, rather than primer, in the second to last cycle ($(r-1)$-cycle) we have $n_{r-1}(0) < s_{r-1}'^{II}(0)$. This sends stage II variables to f.p. 2. In the final stage I the initial conditions are then

$$p_r(0) = p_{r-1}(T_I) + \bar{c}_{r-1} + \bar{s}_{r-1}'^{II}$$
$$s_r(0) = 2\bar{d}_{r-1} + \bar{c}_{r-1} + \bar{s}_{r-1}'^{II} + s_{r-1}(T_I),$$

with f.p. 2 values. At the end of stage I the function values are:

$$p_r(T_I) = f_{T_I}; \;\; s_r(T_I) = f_{T_I} + s_r(0) - p_r(0); \;\; s_r'^{I}(T_I) = p_r(0) - f_{T_I}.$$

The initial condition for $s_r'^{II}(0) = s_r'^{I}(T_I) = p_r(0) - f_{T_I}$, but only complex can be formed in the final stage II, since the resources have been exhausted, $n_r(0) = 0$. During the dissociation phase the complex breaks up, and the initial quantities for stage I are $p_{r+1}(0) = p_r(0)$; $s_{r+1}(0) = s_r(0)$. The reaction has reached a fixed point with ($\bar{p} = p_r(0)$, $\bar{s} = s_r(0)$, $\bar{n} = 0$).

To illustrate the dynamics of this map we plot n_i, s_i and p_i in figure 8, called N_i, S_i and P_i in the figure caption. Six runs were performed with varying stage I integration time: ($t_I = 0.5, 0.75, 1.0, 1.5, 2.0$). The initial conditions are $n_0 = 1.0; p_0 = 0.25; s_0 = 0.001$, i.e., the case in which primer is limiting. To further analyze the effect of varying integration time we plot the logarithmic regression of s, the quantity $Y_i = \ln(|\frac{s_i}{p_0 - s_i}|)$, in figure 9. Here we see that in the limit of shorter integration times the growth is more nearly logistic, and for longer integration times it deviates from logistic by being concave up, rather than concave down, as seen in the amplification data (figure 4). Including variation in stage I integration time is clearly not enough to capture the correct non-sigmoidal behavior of the growth curves, some other part of the reaction dynamics must explain the accentuated slowing of growth during the latter half of the reaction. This leads us to integrating and fitting parameters on the full model, eq.s (2)-(4), and (5)-(9).

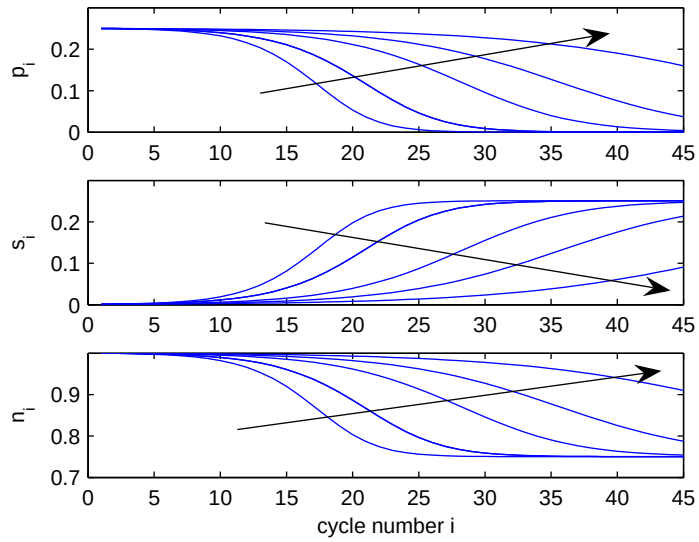

Figure 8: Integration of map with varying stage I integration time. Arrows point in the direction of decreasing stage I integration time.

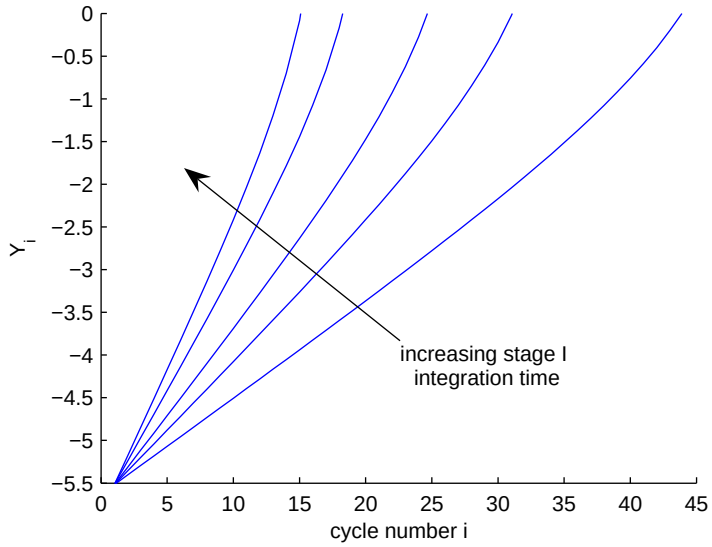

Figure 9: Logarithmic regression variable Y_n from the two-stage map with varying stage I integration time.

3.4 Parameterizing the Full Two Stage Model with PCR Data

We now investigate the parameterization of the model with arbitrary time in stage I and in stage II. The reactions in the annealing phase (stage I) are the same those presented in equations (20),(21), and the linking initial conditions are (19). The stage II ODEs are given in equations (22)-(26) with rescaled parameters and initial conditions:

$$s'(0) = \frac{s'(t_{end}, \text{stage I})}{N_0}; \;\; q(0) = \frac{Q}{N_0}; \;\; c(0) = 0.0; \;\; n(0) = 1.0, \;\; d(0) = 0.0.$$

Integrating the complete model occurs in phases, first computing the solution of stage I: eqs. (21), then the value of the stage I variables at T_I are used as initial conditions (18) for the stage II integration: (5)-(9). The following dissociation phase breaks up existing complex into ssDNA, primer and Taq, and dsDNA into twice as many ssDNA strands, and these are used as initial conditions for the next stage I (see (19)). Examples of runs with varying amounts of initial template are shown in figure 10.

The parameter estimation was done using the same quantitative PCR data set in the previous sections, again using the Matlab function *fminsearch* to minimize the mean square error between the amplification data and the simulated time series. In performing the parameterization we used the value determined for the initial s level from fitting the two stage model without Taq dynamics. We fit the normalization constant for the data, KK, the two reaction coefficients, β and γ, and the reaction times t_I and t_{II}. That leaves the initial amount of primer, $p_1(0)$, and of taq, Q, relative to the initial amount of nucleotides. From the results of many parameterization runs we determined that the best fit was obtained when $p_1(0) = 1.0$, which is not what is indicated by IT protocol, where a standard reaction set-up has 0.5 micromole of each primer and 0.8 millimole of dNTPs, the base pairs (BP) used in extension. Given an amplicon of 200 BPs this means about 4 micromole of completed segments, or 2 micromole of each complementary segment. The ratio of primers to nucleotides is about four to one, then, so we should set the initial primer amount to 0.25. This never achieved the same goodness-of-fit that the runs with higher initial amounts of primer did. On the other hand, the parameterization was relatively insensitive to the initial amount of Taq polymerase, which we set at 1.0. See Tables Va,b for the parameterization results.

In figure 11 a) we show a comparison of data to model with parameters found by the algorithm, for the dilution series. The logarithmic regression variable, $Y_i = log(\frac{y_i}{KK - y_i})$ was plotted for model and the same data in figure 11 b).

Table Va: Parameter Estimation: Two Stage Model with Taq Dynamics-Dilution Series

	KK	$s_1(0)$	β	γ	t_I	t_{II}	% error
run 1	9.126	1.8e-04	4.0267	0.8650	2.2137	1.1521	2.57
run 2	9.3252	7.23e-05	5.4532	1.0404	2.2440	0.9242	2.62
run 3	8.9122	1.67e-05	4.8744	0.9028	2.2876	1.1484	2.66
run 4	7.5077	1.70e-06	4.6310	0.9777	2.5459	1.2457	2.43
run 5	5.0893	1.68e-07	4.3792	0.9423	2.0108	1.6888	3.05
run 6	1.6993	8.00e-08	5.9758	0.6248	1.9002	2.3773	5.52

We then performed the parameter estimation with data from the replicates with unknown initial

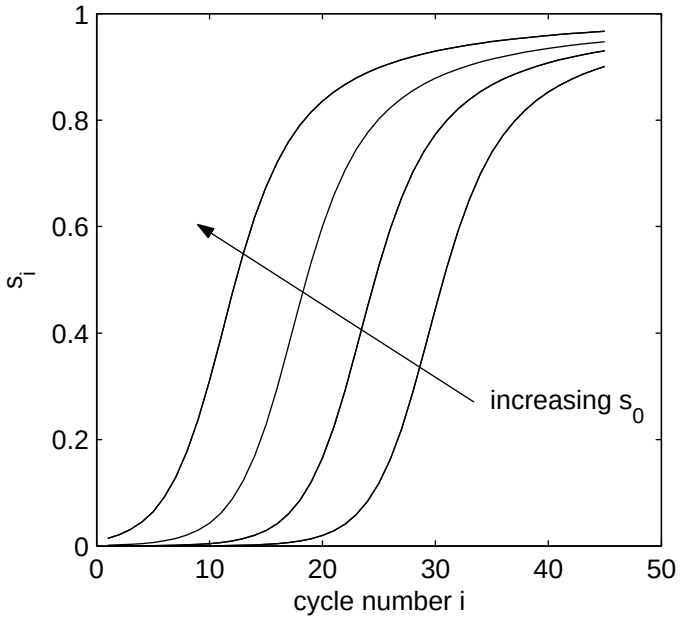

Figure 10: Integration of the coupled ODE model, with varying initial template amount, $s_0(0)$.

concentrations, the results are presented in Table Vb.

Table Vb: Parameter Estimation: Two Stage Model with Taq Dynamics-Unknown Samples

	KK	$s_1(0)$	β	γ	t_I	t_{II}	% error
sample 1a	10.13	8.02e-05	0.8133	1.5316	2.5651	1.8070	3.42
sample 1b	10.49	7.87e-05	1.7851	0.7927	3.3509	1.6211	3.32
sample 1c	10.42	7.67e-05	3.1262	2.2279	4.3212	0.6089	2.86
sample 1d	10.73	7.57e-05	4.5382	1.3121	4.2464	0.7309	2.86
sample 1e	10.82	7.42e-05	5.4529	1.3825	3.1713	0.6814	2.90
sample 2a	7.18	1.51e-06	7.6983	1.2537	4.7358	0.7902	2.76
sample 2b	7.54	1.40e-06	4.6407	0.7659	4.2093	1.3862	2.60
sample 2c	7.60	1.40e-06	5.4337	0.9113	3.6034	1.1658	2.67
sample 2d	7.78	1.39e-06	6.7849	0.8755	3.4071	1.1475	2.64
sample 2e	7.50	1.50e-06	4.7158	0.7127	3.6256	1.4914	2.73

Comparing the fitted parameters for the different replicates in the unknown sample runs points out an obvious flaw with this parameterization. There is clearly more than one parameter set with an equally good fit to the data, meaning hidden dependencies in the parameters that simple rescaling cannot uncover. Determining these dependencies through alternative rescalings and singular perturbation analysis, and through other parameter estimation techniques is the subject of ongoing research.

Turning to the problem of realistic initial primer concentration, we found that for values much less than 1.0 the model did not capture the non-logistic behavior of the data. This can be seen in figure 11 b), where the curves have a concave down portion near the end of the run, indicating the slowing of growth of the amplicon. In figure 12 we illustrate this by graphing the logarith-

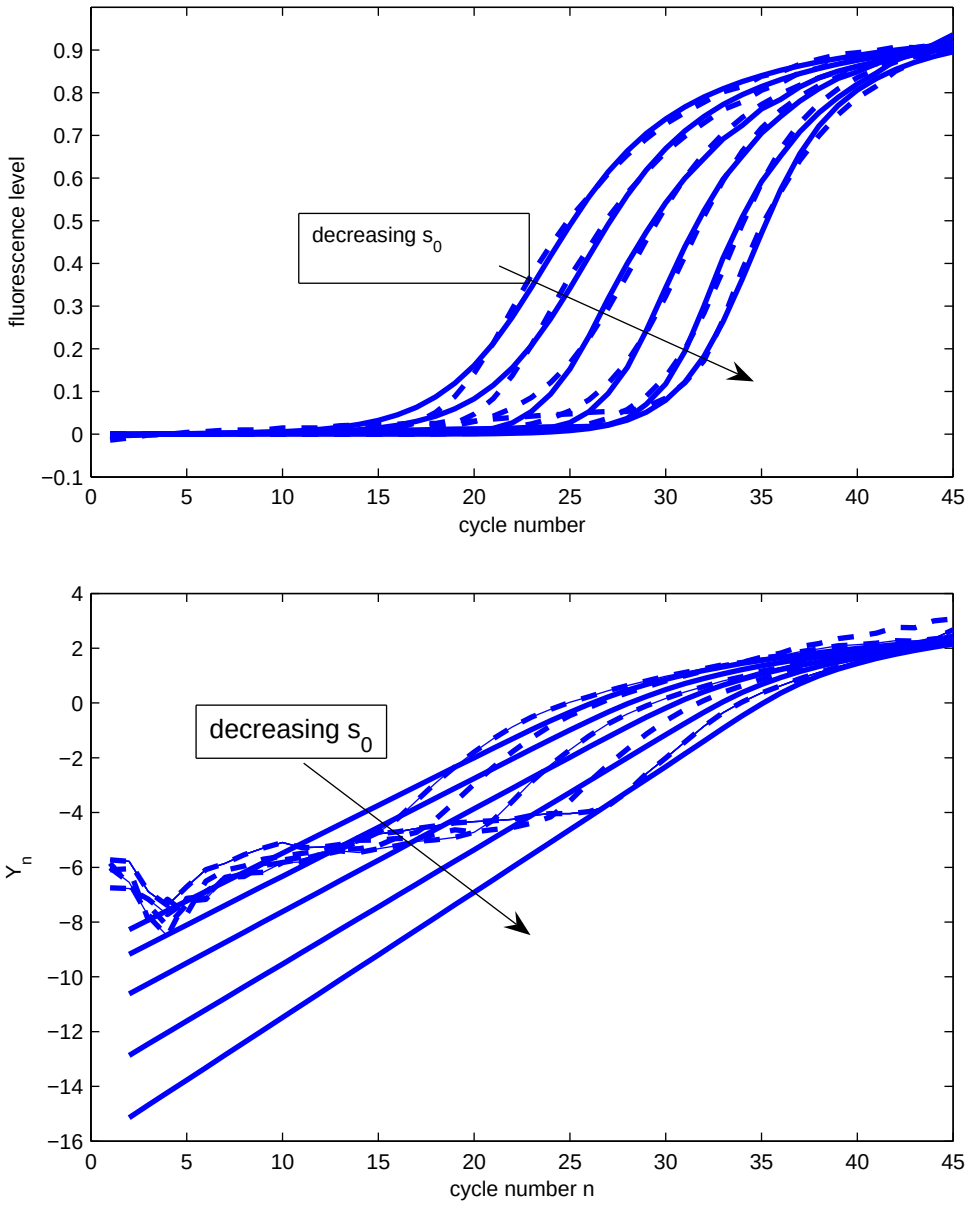

Figure 11: Comparison of two stage model with Taq dynamics to data. a) amplification curves, b) logarithmic regression curves. The data is represented with dashed lines. See the text for parameter values.

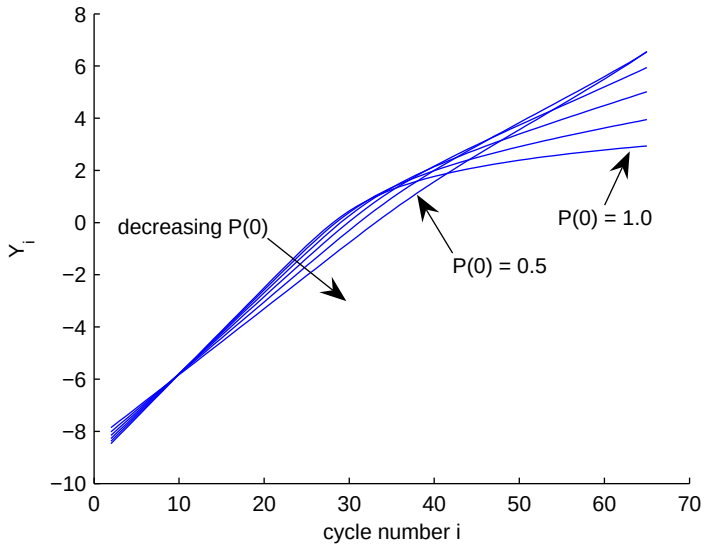

Figure 12: Integration of the two stage model with Taq Dynamics, with varying initial primer amount, $p_0(0)$.

mic regression variable for differing values of initial primer, with all other parameters fixed. In performing parameter estimation, and taking lower initial primer concentration, we found that we could not overcome the logistic-type behavior by varying other reaction parameters. A heuristic explanation for this is that the nucleotides need to be running out as well as primer in this model to get the slower than logistic growth at the end of the run. The equation for $\dot{n}$ (from the two dimension reduction) is

$$\dot{n} = -\beta(q(0) - q)n,$$

so the rate of loss of n is proportional to n. The amount of dsDNA created is equal to $n(0) - n(t)$ at the end of the cycle, so slower loss of n means slower growth of d and hence s. If the initial primer concentration is not close to that of the initial nucleotide concentration the quantity n (which is depleted necessarily at the same rate as p) will not become small, and the rate of creation of s will not slow accordingly.

4 Discussion and Conclusions

In this paper we have analyzed a sequence of models for the reactions of PCR. Exponential growth, the first order approximation of simple doubling of the DNA strands is replaced by a logistic model, which captures the sigmoidal nature of the amplification curves. Both these models are in common use in current devices. We postulated a variation on the logistic model where the efficiency decreases in time, and were able to fit the data with good results. This, however, is less satisfactory than a model built directly from the reactions that captures the decrease in efficiency as cycle number increases. We then built two such models, one that does not include the enzyme Taq directly, and a second which does.

The model that did not include Taq dynamics is solvable analytically, at least for each stage. A

map is created by linking the closed form solutions by their initial conditions. It was found that the data could be well estimated by reasonable parameter values if the initial amount of primer was taken close to that of the initial amount of nucleotide. This is not the protocol followed in the experiment, however, where for a 200 BP sequence the primer:nucleotide ratio is about 1:4. It appears that the amount of nucleotides in the reaction must decrease significantly by the end of the reaction in order to obtain a decrease in overall reaction efficiency. For this to happen the initial concentration of primer must about that of the nucleotide. From these observations we concluded that the model without Taq dynamics did not capture the full behavior of the amplification curves.

The second model is built on reactions that include the formation of a complex of primed ssDNA and the Taq enzyme. The equations of stage I could still be solved analytically, but this was not possible for stage II. Instead limiting cases of long integration time in stage I or stage II or both were considered, and by analyzing the amplification curves created by these maps we concluded that none of the limiting cases, including variable annealing and long time extension, would create the desired behavior at the end of the reaction. If the extension stage of the reaction was very fast compared to the annealing phase, you might expect to capture the qualitative behavior with this last case.

As none of the limiting cases created the qualitative end-of-reaction behavior we thought the data demonstrate so clearly, we proceeded to parameterize the full equations for the two stage model with Taq dynamics. With a map created from solutions to these we were able to find parameters that captured the decreased efficiency at the end of the reaction, but only if initial primer concentration was again roughly the same size as the initial concentration of nucleotides. The extension phase of the reaction would need to slow according to fit this aspect of the behavior. Also, multiple sets of parameters were found to fit the same amplification run, indicating hidden dependencies in the parameters that simple rescaling does not uncover.

While we were not able to completely explain the reduced efficiency seen in the data with our suite of models, we were able to determine what portions of the model were important in capturing its non-logistic character. Competing reactions at higher cycle numbers most certainly will have an effect on the efficiency, especially with the lower initial copy number runs. Future work will include analyzing these dependencies both numerically and analytically, and using the two stage model to seek reaction protocols that minimize time to almost complete creation of amplicon, and maximize yield for a fixed total cycle time.

Acknowledgements

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Appendix: Linear stability analysis for stage II with Taq Dynamics

This stability analysis uses the two dimensional system that takes advantage of the conserved quantities, $s' + c + d = s'(0)$, $q + c = q(0)$, and $n + d = 1$.

$$\dot{q} = -\gamma(s'(0) - q(0) - 1 + q + n)q + q(0) - q + \beta(q(0) - q)n$$

$$\dot{n} = -\beta(q(0) - q)n.$$

The fixed points

Solving $\dot{q} = 0$ and $\dot{n} = 0$ simultaneously gives three fixed points.

$$f.p.1 = (\bar{q} = q(0), \bar{n} = 1 - s'(0))$$

$$f.p.2 = (\bar{q} = \frac{1}{2\gamma}(\gamma(q(0) + 1 - s'(0)) - 1 + \sqrt{(-\gamma(q(0) + 1 - s'(0)) + 1)^2 + 4\gamma q(0)}),$$

$$\bar{n} = 0)$$

The third fixed point has

$$\bar{q} = \frac{1}{2\gamma}(\gamma(q(0) + 1 - s'(0)) - 1 - \sqrt{(-\gamma(q(0) + 1 - s'(0)) + 1)^2 + 4\gamma q(0)}),$$

which is always negative. Proof:

$\bar{q}$ is a root of the polynomial $\gamma q^2 + (-\gamma(q(0)+1-s'(0))+1)q - q(0)$. Let $b = -\gamma(q(0)+1-s'(0))+1$. Then

$$\sqrt{b^2 + 4\gamma q(0)} > b,$$

therefore,

$$-b - \sqrt{b^2 + 4\gamma q(0)} < 0.$$

Since f.p.3 is not of physical importance, we will only analyze the stability of f.p. 1 and f.p. 2.

Stability of f.p.1

The Jacobian for the reduced system is

$$\begin{bmatrix} -\gamma\, q - \gamma\ (s'(0) - q(0) - 1 + q + n) - 1 - \beta\, n & -\gamma\, q + \beta\ (q(0) - q) \\ \beta\, n & -\beta\ (q(0) - q) \end{bmatrix}$$

Let $s'(0), q(0) > 0, c(0) = 0, n(0) = 1$, and $d(0) = 0$. Also let $\gamma, \beta > 0$. For f.p. 1, the Jacobian becomes

$$\begin{bmatrix} -\gamma\, q(0) - 1 - \beta\ (1 - s'(0)) & -\gamma\, q(0) \\ \beta\ (1 - s'(0)) & 0 \end{bmatrix}$$

The eigenvalues are,

$$1/2(-\gamma q(0) - 1 - \beta(1 - s'(0)) \pm \sqrt{(\gamma q(0) + 1 + \beta(1 - s'(0)))^2 - 4\gamma q(0)\beta(1 - s'(0))}).$$

The location of the first fixed point and the sign of the nonzero eigenvalues depend on the relationship between $n(0) = 1$ and $s'(0)$.

Case1: $1 > s'(0) \Rightarrow 1 - s'(0) > 0$ Both coordinates of the first fixed point are non-negative. The eigenvalues are real and negative. They are real because the discriminant is greater than zero. Proof:

$$(\gamma q(0) + 1 + \beta(1 - s'(0)))^2 - 4\gamma q(0)\beta(1 - s'(0))$$
$$= \gamma^2 q(0)^2 + 2\gamma q(0) + 2\gamma q(0)\beta(1 - s'(0)) + 2\beta(1 - s'(0)) + 1 + \beta^2(1 - s'(0))^2 - 4\gamma q(0)\beta(1 - s'(0))$$
$$= \gamma^2 q(0)^2 - 2\gamma q(0)\beta(1 - s'(0)) + \beta^2(1 - s'(0))^2 + 2\gamma q(0) + 2\beta(1 - s'(0)) + 1$$
$$= (\gamma q(0) - \beta(1 - s'(0)))^2 + 2\gamma q(0) + 2\beta(1 - s'(0)) + 1 > 0.$$

They are both negative. Proof:

Let$b = \gamma q(0) + 1 + \beta(1 - s'(0))$. Then $b^2 > 4\gamma q(0)\beta(1 - s'(0))$ as shown above.

$$\sqrt{b^2 - 4\gamma q(0)\beta(1 - s'(0))} < b,$$

therefore,

$$\frac{-b + \sqrt{b_2 - 4\gamma q(0)\beta(1 - s'(0))}}{2}$$

and

$$\frac{-b - \sqrt{b_2 - 4\gamma q(0)\beta(1 - s'(0))}}{2}$$

are both < 0.

Case 2: $n(0) = 1 = s'(0) \Rightarrow 1 - s'(0) = 0$. The first fixed point becomes $(q(0), 0)$ which still is nonnegative. The eigenvalues become:

$$\frac{1}{2}(-\gamma q(0) - 1 + \sqrt{(\gamma q(0) + 1)^2}) = \frac{1}{2}(-\gamma q(0) - 1 + \gamma q(0) + 1) = 0$$

and

$$\frac{1}{2}(-\gamma q(0) - 1 - \sqrt{(\gamma q(0) + 1)^2}) = \frac{1}{2}(-2\gamma q(0) - 2) = -\gamma q(0) - 1.$$

For this case, there is only one nonzero eigenvalue, $-\gamma q(0) - 1$. $-\gamma q(0) - 1 < 0$, so this eigenvalue is negative.

Case3: $n(0) = 1 < s'(0) \Rightarrow 1 - s'(0) < 0$. The coordinate, $\bar{n} = 1 - s'(0)$, of the first fixed point is now negative. The eigenvalues,

$$1/2(-\gamma q(0) - 1 - \beta(1 - s'(0)) \pm \sqrt{(\gamma q(0) + 1 + \beta(1 - s'(0)))^2 - 4\gamma q(0)\beta(1 - s'(0))}),$$

are real. Proof:

Let $\alpha > 0$ and let $1 - s'(0) = -\alpha$. Let

$$b = \gamma q(0) + 1 + \beta(-\alpha) = \gamma q(0) + 1 - \beta\alpha.$$

$$b^2 - 4\gamma q(0)\beta(-\alpha) = b^2 + 4\gamma q(0)\beta\alpha,$$

therefore

$$b^2 + 4\gamma q(0)\beta\alpha > 0.$$

One of the nonzero eigenvalues is positive and the other is negative. Proof:

$b^2 + 4\gamma q(0)\beta\alpha > 0$, so $\sqrt{b^2 + 4\gamma q(0)\beta\alpha} > b$. Then

$$\frac{-b + \sqrt{b^2 + 4\gamma q(0)\beta\alpha}}{2} > 0$$

and

$$\frac{-b - \sqrt{b^2 + 4\gamma q(0)\beta\alpha}}{2} < 0.$$

Stability of f.p.2

For f.p.2, the Jacobian becomes

$$\begin{bmatrix} -\gamma\,\bar{q} - \gamma\,(s'(0) - q(0) - 1 + \bar{q}) - 1 & -\gamma\,\bar{q} + \beta\,(q(0) - \bar{q}) \\ 0 & -\beta\,(q(0) - \bar{q}) \end{bmatrix}$$

where $\bar{q} = \frac{1}{2\gamma}(\gamma(q(0) + 1 - s'(0)) - 1 + \sqrt{(-\gamma(q(0) + 1 - s'(0)) + 1)^2 + 4\gamma q(0)})$. The eigenvalues are

$$\frac{\beta}{2\gamma}(-\gamma q(0) + \gamma(1 - s'(0)) - 1 + \sqrt{(\gamma q(0) - \gamma(1 - s'(0)) + 1)^2 + 4\gamma^2 q(0)(1 - s'(0))},$$

$$-\sqrt{(\gamma q(0) - \gamma(1 - s'(0)) + 1)^2 + 4\gamma^2 q(0)(1 - s'(0))}.$$

Case1: $1 - s'(0) > 0$ For this case $\bar{q}$ is positive. Proof:

Let $b = -\gamma(q(0) + 1 - s'(0)) + 1$. $b^2 + 4\gamma q(0) > 0$, so $\sqrt{b^2 + 4\gamma q(0)} > b$. Therefore,

$$\frac{-b + \sqrt{b^2 + 4\gamma q(0))}}{2\gamma} > 0.$$

The eigenvalues are both real. Proof:

$$(\gamma q(0) - \gamma(1 - s'(0)) + 1)^2 > 0.$$

Also, for $1 - s'(0) > 0$, $4\gamma^2 q(0)(1 - s'(0)) > 0$. Therefore, the discriminant is positive. The eigenvalue

$$-\sqrt{(\gamma q(0) - \gamma(1 - s'(0)) + 1)^2 + 4\gamma^2 q(0)(1 - s'(0))}$$

is clearly negative. The other eigenvalue is positive for this case. Proof:

Let $b = \gamma q(0) - \gamma(1 - s'(0)) + 1$.

$$b < \sqrt{b^2 + 4\gamma^2 q(0)(1 - s'(0))}$$

therefore,

$$-b + \sqrt{b^2 + 4\gamma^2 q(0)(1 - s'(0))} > 0$$

Case 2: $1 - s'(0) = 0$. For this case, $f.p.1 = f.p.2$. Proof:

$$\bar{q} = \frac{1}{2\gamma}(\gamma q(0) - 1 + \sqrt{(-\gamma q(0) + 1)^2 + 4\gamma q(0)})$$

$$= \frac{1}{2\gamma}(\gamma q(0) - 1 + \sqrt{-\gamma^2 q(0)^2 - 2\gamma q(0) + 1 + 4\gamma q(0)})$$

$$= \frac{1}{2\gamma}(\gamma q(0) - 1 + \sqrt{-\gamma^2 q(0)^2 + 2\gamma q(0) + 1}$$

$$= \frac{1}{2\gamma}(\gamma q(0) - 1 + \sqrt{(\gamma q(0) + 1)^2}$$

$$= \frac{1}{2\gamma}(\gamma q(0) - 1 + \gamma q(0) + 1) = \frac{1}{2\gamma}(2\gamma q(0))$$

$$= q(0).$$

$f.p.2$ becomes $\bar{q} = (q(0), \bar{n} = 0)$ which equals $f.p.1$.

Case 3: $1 - s'(0) < 0$. The eigenvalues remain real for this case, but the one that was positive in Case 1 becomes negative. Proof:

Let $-\alpha = 1 - s'(0)$, $\alpha > 0$, and let $b = \gamma q(0) + \gamma\alpha + 1$. Then

$$b > \sqrt{b^2 - 4\gamma^2 q(0)\alpha}$$

therefore,

$$-b + \sqrt{b^2 - 4\gamma^2 q(0)\alpha} < 0$$

Conclusion

For Case 1, f.p.1 is a sink, but becomes a saddle as s'(0) becomes larger than n(0)(for Case 3). The opposite occurs for f.p.2. It starts out as a saddle (for Case 1) and becomes a sink (for Case 3). This indicates a transcritical bifurcation when the initial amount of nucleotides equals the initial amount of primed single-strand DNA.

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