# Challenging the proposed causes of the PCR plateau phase

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## ARTICLE INFO

## ABSTRACT

Despite the wide-spread use of the polymerase chain reaction (PCR) in various life-science applications, the causes of arrested amplicon generation in late cycles have not been confidently identified. This so-called plateau phase has been attributed to depletion or thermal break-down of primers or nucleotides, thermal inactivation of the DNA polymerase, and product accumulation resulting in competition between primer annealing and product re-hybridization as well as blocking of DNA polymerase by double-stranded amplicons. In the current study, we experimentally investigate the proposed limiting factors of PCR product formation. By applying robust and validated qPCR assays, we elucidate the impact of adding non-target and target amplicons to the reactions, mimicking the high amount of products in late PCR cycles. Further, the impact of increased primer concentrations and thermal stability of reagents are explored. Our results show that high amounts of non-target amplicons inhibit amplification by binding to the DNA polymerase, but that this effect is counteracted by addition of more DNA polymerase or prolonged annealing/extension times. Adding high amounts of target amplicons that also act as templates in the reaction is far less inhibitory to amplification, although a decrease in amplification rate is seen. When primer concentrations are increased, both amplification rates and end-product yields are elevated. Taken together, our results suggest that the main cause of PCR plateau formation is primer depletion and not product accumulation or degradation of reagents. We stress that a PCR plateau caused by primer depletion is assay-dependent, i.e. dependent on the primer design and primer characteristics such as the probability of primer-dimer formation. Our findings contribute to an improved understanding of the major parameters controlling the PCR dynamics at later cycles and the limitations of continued product formation, which in the end can facilitate PCR optimization.

## 1. Introduction

The polymerase chain reaction (PCR) is the cornerstone of contemporary nucleic acid analysis, enabling accurate detection, quantification and amplicon sequencing of genes or microbes of interest. The dynamic process of PCR involves highly specific interactions between primers and target DNA, and is well-described in different mathematical models and computerized simulations [1–4]. During a complex course of physical and biochemical reactions, the mass balance between template, primers, nucleotides, DNA polymerase, and product changes with every PCR cycle [5]. Consequently, the hybridization interactions between complementary DNA molecules that include genomic template, primers, and product are continuously shifting throughout early, middle and late cycles of PCR. At a certain point, the amount of available product and template molecules exceeds the number of DNA polymerase molecules, the latter now becoming the rate-limiting factor. This pushes amplification from the exponential phase into a linear state.

Eventually, PCR reaches its plateau phase where amplification ceases. The arrested product formation has been attributed to depletion or thermal break-down of primers or nucleotides, thermal inactivation of the DNA polymerase, and product accumulation, but the conclusions diverge between studies [1,2,6–9]. Understanding the causes of the plateau phase is highly relevant for applications where entering the plateau phase may introduce bias in relative abundance of amplicons, such as in preamplification, or increase the production of chimeras, such as in amplicon-based long-read sequencing [10–12].

Accumulation of PCR product that interacts with the DNA polymerase has been suggested as the main reason for the plateau phase [7], as it was shown that short double-stranded DNA fragments could block the DNA polymerase and hinder amplification [13]. This was further supported by computerized simulations predicting obstructed amplification with increased product re-hybridization and decreased effective polymerase concentration due to product accumulation [2,3]. However, Kainz [7] based their conclusions on results from addition of non-target

amplicons, i.e. amplicons different from the ones being produced in the reaction. A more accurate effect of products accumulating in the PCR would emanate from addition of high amounts of target amplicons, i.e. amplicons identical to the ones being produced in the reaction. Target amplicons will in contrast to non-target amplicons serve as abundant templates for PCR. This versatile effect of amplicon accumulation has not previously been investigated experimentally.

Studies aiming at investigating reagent depletion as a limiting factor of PCR product formation report conflicting results. The addition of nucleotides or primers after reaching the plateau phase has been shown not to re-start amplification [6,7], suggesting that neither of these are the limiting factor. On the contrary, others describe that initially increased primer concentrations [8] and addition of primers at the linear phase [2] led to increased product formation at late cycles.

In this study, we experimentally investigate the proposed limiting factors of PCR product formation, applying established qPCR assays. The hypothesis of thermal breakdown of reagents and inactivation of DNA polymerase is challenged by subjecting nucleotides, primers and DNA polymerase to extensive thermal cycling prior to the actual PCR analysis. The different effects when adding non-target and target amplicons in qPCR are highlighted, and finally the effect of increasing initial primer amounts is elucidated. Our findings contribute to a better understanding of the major parameters controlling the PCR dynamics at later cycles and the limitations of continued product formation. Such knowledge can facilitate the optimization of PCR, as more accurate thermal cycling programs and reagent amounts can be applied for the intended objective.

## 2. Material and methods

### 2.1. qPCR assays

The four qPCR assays used in this study were applied as follows. The *RB1* assay (targets the human *retinoblastoma-1* gene, generating a 156 bp amplicon) with 0.3 μM *RB1*_80F and *RB1*_235R primers and 0.2 μM *RB1*_MGB hydrolysis probe [14]; the *invA* assay (targets the *Salmonella enterica* invasion gene *invA*, generating a 88 bp amplicon) with 0.3 μM *invA*-F and *invA*-R primers and 0.2 μM *invA* hydrolysis probe [15]; the *CSF* assay (targets the human CSF1PO short tandem repeat locus, generating a 67 bp amplicon) with 0.4 μM *nuCSF*-F and *nuCSF*-R primers and 0.1 μM *nuCSF* hydrolysis probe [16]; the general bacteria assay (targets bacterial 16S rRNA gene, generating a 466 bp amplicon) with 0.3 μM Bact-F and Bact-R primers and 0.2 μM Bact hydrolysis probe [17]. All primers and probes were HPLC purified and purchased from Life Technologies (New York, NY, USA). When primer and polymerase concentrations were altered (*RB1* and *invA* assay 0.3–1.5 μM; polymerase 0.5–2 U), this is pointed out in the Result and discussion section. The *RB1* probe concentration was increased proportionally to the primer increase, except when *RB1* amplicons were added to the *RB1* assay, where the probe concentration was doubled in proportion to primers (0.4 μM probe for 0.3 μM primers and 0.8 μM probe for 0.6 μM primers).

### 2.2. DNA template

As DNA template for the *RB1* reactions, 2 ng Quantifiler Human DNA (QF DNA, Life Technologies) was used for all reactions except when non-target amplicons were added to the reactions. There, 25 ng of QF DNA template was applied for *RB1* and *CSF* reactions. For the *invA* reactions, 2 pg genomic *Salmonella enterica* DNA, extracted with GeneJET genomic DNA purification kit (Thermo Fisher Scientific, Waltham, MA, USA), was used. For the general bacteria assay reactions, 0.1 ng genomic *Staphylococcus aureus* DNA, extracted with a standard phenol/chloroform protocol [18], was used.

### 2.3. Generation of amplicons and genomic DNA

To elucidate the effect of product accumulation seen at the late PCR cycles, high amounts of non-target or target amplicons were added to the reactions. Amplicons used in these experiments were generated as follows: *Staphylococcus aureus* enterotoxin D (*SED*) amplicons used as non-target amplicons in the *RB1* assay, were produced using conventional PCR (ABI 9700 thermal cycler) with the following thermal cycling program: 95 °C for 5 min; 40 cycles of 95 °C for 30 s, 48 °C for 30 s, and 72 °C for 30 s; 72 °C for 5 min and cooling to 4 °C. The reaction master mix contained 1 U *Ex Taq* HS (Takara Bio Inc., Shiga, Japan), 1x *Ex Taq* buffer, 0.2 mM dNTP (Roche Diagnostics, Risch, Switzerland), 0.5 μM primers (*SED1* [19] and *ESD2* [20], generating a 331 bp amplicon), 5 ng template genomic *Staphylococcus aureus* DNA and SuperQ water up to 25 μL. *RB1* amplicons used as target amplicons in the *RB1* assay, were generated using conventional PCR with the following thermal cycling program: 95 °C for 5 min; 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s; 72 °C for 5 min and cooling to 4 °C. The reaction master mix contained 1 U *Ex Taq* HS, 1x *Ex Taq* buffer, 0.2 mM dNTP, 0.3 μM *RB1* primers (80 F and 235R), 25 ng template DNA (QF) and SuperQ water up to 25 μL. The resulting PCR product was applied to 1% agarose gel electrophoresis and subsequently purified with QIAquick Gel Extraction kit (Qiagen, Hilden, Germany) and GeneJet PCR purification kit (Thermo Fisher Scientific). Genomic *Staphylococcus aureus* DNA (2.8 Mbp) was extracted using a phenol/chloroform protocol and confirmed to be intact by applying the DNA to gel electrophoresis. All DNA/amplicon concentrations were determined using a BioDrop μLITE (BioDrop).

### 2.4. qPCR protocol and data analysis

All qPCR experiments were performed in technical triplicates on a LightCycler Nano instrument (Roche Diagnostics) with software v 1.1, using the following thermal cycling program: 95 °C for 1 min (*Taq* polymerase) or 2 min (*Ex Taq* HS); 45 cycles of 95 °C for 10 s, 60 °C for 20 s, and 72 °C for 30 s. For experiments where non-target (*SED*) and target (*RB1*) amplicons were added to the reactions, 45 and 60 PCR cycles were run, respectively. When 60 PCR cycles was applied in the experiments with increased primer concentration, this is pointed out in the Results and discussion section. In reactions with extended annealing and extension times, the PCR cycling protocol was changed to 95 °C for 10 s, 60 °C for 40 s, and 72 °C for 60 s (2x annealing/extension) or 95 °C for 10 s, 60 °C for 60 s, and 72 °C for 90 s (3x annealing/extension). The standard qPCR reaction master mix contained 1 U DNA polymerase, 1x polymerase-specific buffer, 0.2 mM dNTP and 4 mM MgCl$_2$ (Roche Diagnostics). Either *Taq* (Roche Diagnostics) or *Ex Taq* Hot Start polymerase was used together with their accompanying buffer (10x PCR buffer or 10x *Ex Taq* buffer). SuperQ water was added to generate a total reaction volume of 20 μL.

For experiments where target (*RB1*) amplicons were added to the PCR, maximum amplification rates were determined from the raw fluorescence data of the amplification curves, as the maximum value of the discrete derivative of fluorescence (F) as a function of cycle number (c) (dF/dc). Two-factor ANOVA with replication was applied to evaluate the statistical significance of differences in amplification rates and end-point fluorescence due to primer concentration and amount of added amplicons. Pearson's correlation coefficient (r) was determined to examine the strength of the linear relationship between end-point fluorescence and amount of added *RB1* amplicons, and the significance (p) assessed employing the t-distribution. Generation of the correct PCR product in the experiments where high amounts of target amplicons were added was confirmed by gel electrophoresis.

### 2.5. Thermal stability of PCR reagents

To investigate thermal inactivation of the DNA polymerase and

thermal break-down of primers and nucleotides, these reagents were subjected to PCR cycling (without target DNA) prior to the actual PCR analysis. Either dNTPs, *RB1* primers, *Taq* polymerase or *Ex Taq* HS were mixed with SuperQ water, buffer and up to 2.5 mM MgCl$_2$ and run in a standard *RB1* assay program for 30, 45 or 60 cycles, including a melt curve program starting at 60 °C with 0.1 °C/s temperature increase up to 97 °C. Then DNA template, the missing reagents (dNTP, primers and/ or DNA polymerase) and 1x EVAGreen dye (Biotium Inc., Hayward, CA, USA) were added to the reactions for subsequent qPCR analysis (45 cycles), including a melt curve analysis as described above. These experiments were performed applying EVAGreen dye to enable monitoring amplification of non-specific products through the melt curve analysis. Samples were analysed in triplicates for each reagent, and the results compared to controls (i.e. analyses with fresh reagents) by calculating ΔCq = Cq(analysis applying pre-cycled reagent) - Cq(control). Results are presented as ΔCq ± standard deviation. ΔCq values above zero thus indicate partially impaired amplification.

### 2.6. Product yield and theoretical calculations

The possible scenario of primer depletion as a limiting factor was assessed by increasing the concentration of *RB1* and *invA* primers from 0.3 μM to 1.5 μM. The resulting absolute product yield was determined after 45 or 60 cycles respectively, in technical triplicates using gel densitometry. After completed qPCR, one third of the reaction volume was applied in 1% agarose gel electrophoresis with 0.01% GelRed (Biotium Inc.) along with two different DNA mass ladders: Low DNA mass ladder (Invitrogen, Carlsbad, CA, USA) and Precision molecular mass ruler (Bio-Rad, Hercules, CA, USA). Gel pictures were acquired with BioRad imaging systems and densitometry analysis was performed using Quantity One 1-D analysis software (Bio-Rad). The total pixel density for each lane was determined by drawing a rectangle around the bands, and product concentrations were obtained by plotting the unknown samples against a standard curve with known DNA mass ladder concentrations (40–440 ng, $r^2 > 0.95$, n = 20). The resulting concentrations were multiplied by three to obtain the total product yield in the samples. The product formation is presented as absolute yield and as the percentage of consumed primers. qPCR assay data and calculations of theoretical maximum amplicon yields for the *RB1* and *invA* assays with regard to primer limitation, are described below.

Theoretical product yield (g), given that amplicon generation is limited by the primer amount:

$$C_{primer} \times V \times N_A \times MW_{amplicon} \times 1.660539 \times 10^{-24} \tag{1}$$

where $C_{primer}$ is the primer concentration, $0.3 \times 10^{-6}$–$1.5 \times 10^{-6}$ M; V is the reaction volume, $2.0 \times 10^{-5}$ L; $N_A$ is the Avogadro constant, $6.022 \times 10^{23}$; $MW_{amplicon}$ is the molecular weight of the amplicon in Dalton (Table 1); 1 Da equals $1.660539 \times 10^{-24}$ g.

Percentage of consumed primers:

$$\frac{Experimentally \quad determined \quad product \quad yield}{Maximum \quad theoretical \quad product \quad yield} \times 100 \tag{2}$$

where experimentally determined product yields are derived from the results in Table 6 (converted to grams).

Number of generated amplicons from theoretical or experimentally determined product yield:

$$\frac{Product \quad yield}{MW_{amplicon} \times 1.660539 \times 10^{-24}} \tag{3}$$

Additional information about the applied assays is found in Table 1.

**Table 1**

Molecular weight and size of the amplicons produced in the PCR assays *invA*, *RB1* and *SED*, and amplification efficiencies of the *invA* and *RB1* assays.

|  | *invA* qPCR assay | *RB1* qPCR assay | *SED* PCR assay |
|---|---|---|---|
| MW$_{amplicon}$ (Da) | 54407[a] | 96407[a] | 204481[a] |
| Amplicon size (bp) | 88 | 156 | 331 |
| Amplification efficiency (AE) | 1.04[b] | 1.04[c] | N/A |
| $r^2$ | 0.994[b] | 0.994[c] | N/A |
| Error | 0.442[b] | 0.247[c] | N/A |

[a] Calculated at http://www.bioinformatics.org/sms2/dna_mw.html, accessed 2018-09-27.
[b] Calculated from standard curve with 6 dilutions ranging from $5 \times 10^{-6}$–$5 \times 10^{-1}$ ng/μL.
[c] Calculated from standard curve with 5 dilutions ranging from 0.04 to 25 ng/μL.

## 3. Results and discussion

### 3.1. Thermal stability of PCR reagents

Early studies suggested thermal breakdown of reagents or polymerase inactivation as plausible causes of the PCR plateau phase [1]. Today, most reagents and DNA polymerases are very heat stable and some hot-start enzymes can require up to 40 min at high temperatures for complete activation [21]. However, heat stability may differ between different DNA polymerases [22] and supplied reagents. Thus we set out to investigate how PCR cycling affected the reagents used in our study. Initial pre-cycling of separate PCR reagents (nucleotides, primers, *Taq* and *Ex Taq* HS polymerase, but without template DNA) for up to 60 PCR cycles had no negative effect on subsequent qPCR analysis (Table 2). On the contrary, pre-cycling of *Ex Taq* HS polymerase increased amplification efficiency, giving significantly lower quantification cycle (Cq) values compared to without pre-cycling (Table 2). Pre-cycling of *Ex Taq* HS with 30 PCR cycles prior to PCR analysis, lowered the Cq values by around three cycles, while pre-cycling with 45 and 60 cycles lowered the Cq values by around one cycle (Table 2). This is likely an effect of enhanced enzyme activation, and indicates that the recommended initial heating step of two minutes where a monoclonal antibody is supposed to be released from the active site of *Ex Taq* HS, is insufficient. The results confirm that when applying high quality reagents and modern PCR instruments, thermal inactivation of polymerase or breakdown of nucleotides and primers can be dismissed as limiting factors of PCR.

### 3.2. Blocking of DNA polymerase by non-target amplicons

The high amount of amplicons in the late cycles of PCR has been proposed to block amplification due to the binding between DNA polymerase and short, blunt-end double-stranded (ds) DNA molecules [7]. By adding increasing amounts (0.5–2 μg; $1.47 \times 10^{12}$–$5.89 \times 10^{12}$) of non-target (*SED*) amplicons to the *RB1* qPCR assay, we could confirm that amplification was progressively hampered (Table 3). Applying *Taq* DNA polymerase (1 U), addition of 1.5 μg of *SED* amplicons caused complete amplification inhibition, whereas *Ex Taq* HS (1 U) generated detectable *RB1* products with the addition of up to 2 μg of *SED* amplicons. This difference suggests that blocking of DNA polymerase by double-stranded amplicons depends on polymerase type, possibly due to different affinities for binding to blunt-end dsDNA fragments. Such a difference between polymerase types was previously observed by Kainz [7].

Our results also show that the inhibitory effect of non-target amplicons was coupled to the target amplicon size. Amplification was less constrained when adding non-target amplicons to a PCR assay generating a shorter product (*CSF* assay, 67 bp) compared with the *RB1*

**Table 2**

Investigation of the possible thermal degradation or inactivation of reagents. DNA polymerase, nucleotides or primers were subjected to PCR cycling for 30, 45 or 60 cycles, prior to the actual PCR analysis. Data are presented as $\Delta$Cq $\pm$ standard deviation (n = 3). $\Delta$Cq = Cq(analysis with pre-cycled reagent) - Cq(control). $\Delta$Cq values above zero indicate partially impaired amplification.

| Nr. of PCR cycles performed to test thermal stability | *Ex Taq* HS | *Taq* polymerase | dNTPs | Primers |
|---|---|---|---|---|
| 30 PCR cycles | −3.05 ± 0.11 | −0.24 ± 0.21 | −0.01 ± 0.06 | 0.00 ± 0.03 |
| 45 PCR cycles | −1.81 ± 0.22 | 0.22 ± 0.05 | 0.02 ± 0.02 | 0.00 ± 0.04 |
| 60 PCR cycles | −1.75 ± 0.21 | −0.03 ± 0.04 | 0.05 ± 0.11 | 0.11 ± 0.11 |

assay (156 bp), and more constrained for an assay generating a larger product (General bacteria assay, 466 bp) (data not shown).

Increasing the polymerase amount and extending annealing/extension times strongly counteracted amplification inhibition caused by non-target amplicons. Adding 1.5 µg *SED* amplicons caused partial inhibition of 1 U *Ex Taq* HS polymerase, seen as delayed amplification (a Cq value shift corresponding to $\Delta$Cq = 8.79 ± 1.77). Increasing the polymerase amount to 2 U or applying three times longer annealing/extension time completely abolished this amplification inhibition (giving $\Delta$Cq = −0.40 ± 0.06 and 0.18 ± 0.36, respectively) (Table 3). Similar effects were seen when *Taq* polymerase was applied. Kainz (2000) also noted the positive effect of increasing the amount of polymerase, but concluded that the polymerase molecules are irreversibly inactivated by the products formed. With our results, we show that the interactions are dynamic and that successful amplification depends on the given extension time. When a *Taq* DNA polymerase molecule binds to a primer-template complex it can attach around 50–80 nucleotides before being released [23], a property called processivity. Given that the amplicon size of *RB1* is 156 bp, several binding events where the polymerase attaches to the primer-template complex are thus needed in each PCR cycle for the amplicon to be finalized. While a number of polymerase molecules are occupied by binding double-stranded PCR products, adding more polymerase molecules or reaction time creates a shift in the reaction kinetics towards higher availability of free polymerase molecules enabling the finalization of primer elongation within one cycle.

In the current study, we show that the nature of amplification disturbance by non-target PCR products is dynamic, as the polymerization activity simply is reduced due to less available DNA polymerase molecules in a given moment. Further, the impact of non-target amplicons is highly dependent on the type of DNA polymerase. The rationale for choosing to apply *Taq* and *Ex Taq* HS DNA polymerase in this study is that the former represents non-modified polymerases and the latter represents hot-start enzymes and has previously shown excellent performance compared to other variants, e.g. for PCR-inhibitory forensic samples [24].

### 3.3. Addition of non-target genomic DNA

To experimentally confirm that the inhibited amplification results from the presence of short dsDNA fragments and not simply from the presence of high amounts of dsDNA in general, we added up to 10 µg of non-target genomic (g)DNA (*S. aureus*) to the *RB1* qPCR assay. This had no negative effect on *Ex Taq* HS and amplification with *Taq* polymerase was only slightly hampered by the addition of 10 µg gDNA ($\Delta$Cq = 1.43 ± 0.88, Table 4). Adding 5 µg of gDNA had no effect on either DNA polymerase, confirming the previous notion that DNA polymerases are not expected to bind to large blunt-end dsDNA molecules [13]. In qPCR, the total DNA amount is usually in the picogram to nanogram range, and thus a negative impact of adding too much template DNA is unlikely. Yet, it is not unusual to observe failed amplification for crude DNA extracts with high DNA concentrations. This is however most likely due to a high amount of accompanying PCR inhibitors [25], and not due to the amount of DNA. Analysis of formalin-fixed and paraffin-embedded (FFPE) samples is a special case, where human DNA in the µg range may be needed to provide acceptable limits of detection due to DNA fragmentation. This may cause failed amplification, as the short DNA fragments inhibit DNA polymerase activity in a fashion similar to non-target amplicons [26]. Just as in our results above, the inhibitory effect of fragmented FFPE samples was alleviated by increased DNA polymerase concentrations and extended elongation times.

### 3.4. Addition of target amplicons

To highlight the different effects when adding target amplicons as opposed to non-target amplicons, 2.5 ng–3 µg ($1.25 \times 10^{10}$–$1.87 \times 10^{13}$) of *RB1* amplicons were added to the *RB1* PCR assay (Table 5). As a comparison, we determined the maximum *RB1* product yield to 1.06 ± 0.03 µg ($6.65 \times 10^{12}$ amplicons, Table 6). Thus, 3 µg is far more *RB1* amplicons than can be expected at the end of an *RB1* PCR reaction. The results showed that amplification occurred in all reactions, also when 3 µg of *RB1* amplicons was added (Fig. 1). This should be compared to the results above showing that 2 µg ($5.89 \times 10^{12}$) of non-target (*SED*) amplicons led to complete inhibition of amplification.

**Table 3**

The effect of adding different amounts of non-target (*SED*, 331 bp) amplicons to the *RB1* assay. The effect is shown for different polymerase types (*Ex Taq* HS or *Taq* polymerase), polymerase amounts (0.5 U, 1 U or 2 U) and annealing/extension times (1x, 2x or 3x). Data are presented as $\Delta$Cq $\pm$ standard deviation (n = 3). $\Delta$Cq = Cq(added non-target amplicons) – Cq(control). $\Delta$Cq values above zero indicate partially impaired amplification. ND – no detected amplification.

| qPCR parameters | | | Amount of non-target 331 bp amplicon | | | |
|---|---|---|---|---|---|---|
| DNA polymerase | Polymerase amount | Annealing/extension time | 0.5 µg | 1 µg | 1.5 µg | 2 µg |
| *Ex Taq* HS | 0.5 U | 1x | −0.05 ± 0.03 | – | ND | – |
| *Ex Taq* HS | 1 U | 1x | −0.44 ± 0.01 | 0.35 ± 1.06 | 8.79 ± 1.77 | ND |
| *Ex Taq* HS | 2 U | 1x | −0.11 ± 0.12 | – | 0.40 ± 0.06 | – |
| *Ex Taq* HS | 1 U | 2x | −0.33 ± 0 | – | 2.19 ± 0.84 | – |
| *Ex Taq* HS | 1 U | 3x | −0.12 ± 0.04 | – | 0.18 ± 0.36 | – |
| *Taq* | 0.5 U | 1x | 4.90 ± 0.36 | – | ND | – |
| *Taq* | 1 U | 1x | 0.58 ± 0.40 | 6.80 ± 0.26 | ND | ND |
| *Taq* | 2 U | 1x | −0.10 ± 0.02 | – | 0.22 ± 0.02 | – |
| *Taq* | 1 U | 2x | 0.25 ± 0.03 | – | 14.09 ± 2.20 | – |
| *Taq* | 1 U | 3x | 0.36 ± 0.03 | – | 0.97 ± 0.21 | – |

**Table 4**

Addition of non-target genomic DNA to a qPCR assay. The *RB1* assay was applied, with *Ex Taq* HS and *Taq* polymerase. Results are presented as ΔCq ± standard deviation (n = 3). ΔCq = Cq(addition of non-target gDNA) - Cq(control). ΔCq values above zero indicate partially impaired amplification.

| | Amount of non-target genomic DNA | | | | |
| --- | --- | --- | --- | --- | --- |
| | 0.5 µg | 1 µg | 2.5 µg | 5 µg | 10 µg |
| *Ex Taq* HS | −0.08 ± 0.02 | −0.16 ± 0.04 | −0.26 ± 0.01 | −0.38 ± 0.05 | −0.43 ± 0.08 |
| *Taq* polymerase | −0.27 ± 0.06 | −0.31 ± 0.04 | −0.23 ± 0.10 | 0.15 ± 0.16 | 1.43 ± 0.88 |

Clearly, the effect of adding target amplicons is significantly different from adding non-target amplicons to the PCR, as target amplicons also serve as abundant templates for amplification.

The detection of newly formed *RB1* products was immediate (Cq values around 1), when more than 10 ng ($6.25 \times 10^{10}$) of *RB1* amplicons was added (Fig. 1). Thus, around this many ($10^{10}$) cleaved hydrolysis probes are needed to generate a detectable fluorescence signal. To further assess the effect of increased amounts (2.5 ng–3 µg) of target amplicons, amplification rate and end-point fluorescence were studied. The amplification rate peaked when around 0.5 µg amplicons was added, then gradually declined as the amount of added amplicons increased (Table 5). This is seen by decreasing slopes in the amplification curves (Fig. 1). When the primer concentration was doubled (from 0.3 µM to 0.6 µM), increased amplification rates were observed (Table 5, Fig. 1). Analysis with two-factor ANOVA with replication showed that differences in amplification rates due to primer concentrations, added amplicon amounts and the interaction between these factors were statistically significant (≤0.0001). Doubled primer amounts also led to almost twice as high end-point fluorescence, indicating that the amount of new products increased proportionally to the primer concentration (Table 5, Fig. 1). These results suggest that the absolute number of primers is a limiting factor of amplification rate and continued product formation in late PCR cycles. In support of this, there was no correlation between the end-point fluorescence and the amount of added *RB1* amplicons (0.3 µM primers: r = −0.39, p = 0.21; 0.6 µM primers: r = −0.15, p = 0.63, Fig. 1). This demonstrates that irrespective of initial amplicon amount, the relative end-product yield was similar for a given primer concentration.

Our results indicate that primer depletion is the dominating limiting factor for continued product formation. High amounts of amplicons indeed slow down the reaction rate, likely due to DNA polymerase binding and to a mass balance favouring product re-hybridization over primer-template binding [27]. However, as long as sufficient primer amounts are available, the reaction continues and new products are formed. This is further investigated below.

### 3.5. Depletion of PCR reagents

Depletion of primers and nucleotides have previously been pointed out as limiting factors for PCR product formation. Whether primers or nucleotides would be exhausted first naturally depends on the initial concentrations as well as amplicon size. In qPCR, amplicons are typically < 200 bp and nucleotides are generally in excess compared to primers when applying the standard concentration of 0.2 mM. In conventional PCR however, nucleotide amount may become a limiting factor for large amplicons (kb size). In this study we assess qPCR assays with amplicon sizes of < 200 bp and thus focus on investigating primer depletion.

To evaluate the effect of primer depletion, the initial primer concentration for the two established qPCR assays, *invA* (88 bp amplicon) and *RB1* (156 bp amplicon), was increased up to five times the published/optimized concentrations of 0.3 µM [14,15]. The absolute product yield was determined for 45 or 60 PCR cycles, respectively. Applying 45 PCR cycles, increased primer concentrations gave steadily elevated amplicon yields for both assays (Table 6). However, the percentage of consumed primers gradually decreased as primer concentrations increased. This is due to continued product formation beyond cycle 45, as apparent from the higher product yield when applying 60 cycles (Table 6). The plateau phase is simply not reached at 45 cycles when the primer amount is above 0.3 µM. These results suggest that as long as sufficient primer amounts are available, more amplicons are produced in every cycle.

We applied *Taq* polymerase and *Ex Taq* HS in two concentrations (1 U and 2 U) with both qPCR assays to evaluate the role of the DNA polymerase in primer limited amplicon formation. *Ex Taq* HS gave a significantly higher product yield compared to *Taq* polymerase (Table 6), emphasizing that type and quality of the DNA polymerase has a great impact on the ability to use the available primers for product formation within the set thermal cycling program. Contrarily, doubling the amount of polymerase had no or little effect for either enzyme, implying that polymerase amount is generally not a limiting factor for product yield. Had blocking of DNA polymerase by double-stranded amplicons been the decisive factor causing the PCR plateau phase,

**Table 5**

Maximum amplification rates and end-point fluorescence levels for increasing amounts of target amplicons. Data presented as mean values ± standard deviation, for 0.3 or 0.6 µM primers (n = 3). Rfu = relative fluorescence units.

| Amount of template amplicons | Maximum amplification rate (dF/dc) | | End-point fluorescence (rfu) | |
| --- | --- | --- | --- | --- |
| | 0.3 µM primers | 0.6 µM primers | 0.3 µM primers | 0.6 µM primers |
| 0.0025 µg | 0.17 ± 0.013 | 0.20 ± 0.007 | 1.6 ± 0.11 | 2.4 ± 0.11 |
| 0.005 µg | 0.15 ± 0.007 | 0.22 ± 0.002 | 1.4 ± 0.071 | 2.6 ± 0.067 |
| 0.01 µg | 0.16 ± 0.012 | 0.19 ± 0.008 | 1.4 ± 0.096 | 2.2 ± 0.072 |
| 0.02 µg | 0.16 ± 0.011 | 0.15 ± 0.008 | 1.4 ± 0.101 | 1.6 ± 0.11 |
| 0.04 µg | 0.15 ± 0.006 | 0.16 ± 0.010 | 1.3 ± 0.040 | 1.8 ± 0.12 |
| 0.08 µg | 0.12 ± 0.006 | 0.18 ± 0.011 | 0.93 ± 0.029 | 2.0 ± 0.12 |
| 0.1 µg | 0.13 ± 0.006 | 0.25 ± 0.013 | 1.0 ± 0.035 | 2.7 ± 0.18 |
| 0.25 µg | 0.20 ± 0.007 | 0.24 ± 0.025 | 1.4 ± 0.056 | 2.4 ± 0.19 |
| 0.5 µg | 0.21 ± 0.005 | 0.30 ± 0.018 | 1.4 ± 0.072 | 3.0 ± 0.056 |
| 1 µg | 0.15 ± 0.009 | 0.19 ± 0.014 | 1.2 ± 0.044 | 2.2 ± 0.053 |
| 2 µg | 0.084 ± 0.007 | 0.13 ± 0.004 | 1.0 ± 0.043 | 2.0 ± 0.026 |
| 3 µg | 0.080 ± 0.005 | 0.11 ± 0.004 | 1.2 ± 0.039 | 2.1 ± 0.046 |

**Table 6**

Effect of initial primer concentration on product yield for the two tested assays. The effect of PCR cycle number (45 or 60 PCR cycles), DNA polymerase type (*Ex Taq* HS or *Taq* polymerase) and polymerase amount (0.5 U, 1 U or 2 U) is shown, as well as the impact of annealing/extension times (1x, 2x or 3x). In addition to product yield, the percentage of consumed primers is presented (n = 3).

| Amplicon size | PCR conditions | | | | Product yield ± S.D (ng) Percentage of consumed primers | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | DNA polymerase | Polymerase amount | Nr. of PCR cycles | Annealing/ extension time | 0.3 μM | 0.6 μM | 0.9 μM | 1.2 μM | 1.5 μM |
| *invA* assay 88 bp | *Ex Taq* HS | 1 U | 45 | 1x | 299 ± 19 92% | 563 ± 11 86% | 617 ± 40 63% | 748 ± 40 57% | 799 ± 10 49% |
| | *Ex Taq* HS | 1 U | 60 | 1x | 373 ± 25 114% | 685 ± 26 105% | 831 ± 46 85% | 1103 ± 120 85% | 1630 ± 11 100% |
| | *Ex Taq* HS | 2 U | 60 | 1x | 292 ± 13 90% | 632 ± 1 97% | – | 1060 ± 31 81% | – |
| | *Ex Taq* HS | 1 U | 60 | 3x | 285 ± 15 87% | 620 ± 11 95% | – | 1053 ± 45 81% | – |
| | *Taq* | 1 U | 60 | 1x | 301 ± 30 92% | 535 ± 30 82% | – | 608 ± 9 47% | – |
| | *Taq* | 2 U | 60 | 1x | 269 ± 21 82% | 538 ± 31 82% | – | 745 ± 10 57% | – |
| *RB1* assay 156 bp | *Ex Taq* HS | 1 U | 45 | 1x | 463 ± 44 80% | 749 ± 12 65% | 672 ± 58 39% | 663 ± 45 29% | 721 ± 13 25% |
| | *Ex Taq* HS | 1 U | 60 | 1x | 466 ± 45 81% | 841 ± 6 73% | 933 ± 24 54% | 1013 ± 45 44% | 1064 ± 27 37% |
| | *Ex Taq* HS | 2 U | 60 | 1x | 422 ± 38 73% | 709 ± 56 61% | – | 991 ± 86 43% | – |
| | *Ex Taq* HS | 1 U | 60 | 3x | 349 ± 35 60% | 605 ± 27 52% | – | 782 ± 68 34% | – |
| | *Taq* | 1 U | 60 | 1x | 261 ± 29 45% | 496 ± 10 43% | – | 573 ± 46 25% | – |
| | *Taq* | 2 U | 60 | 1x | 268 ± 33 46% | 510 ± 8 44% | – | 707 ± 41 31% | – |

increased amounts of polymerase would have been expected to give higher product yields. Note that increased amounts of polymerase, as well as prolonged annealing/extension time, partly counteracted the blocking effect of non-target amplicons (Table 3).

Applying 60 PCR cycles, the *invA* product yield was proportional to the increase in primer amount (Table 6). Thus, the maximum theoretical yield based on primer limitation was nearly reached. The percentage of used *RB1* primers, however, declined with higher primer concentrations. This reflects less efficient amplification for the *RB1 assay*, resulting in reduced generation of amplicons compared to the *invA* assay. Applying 60 PCR cycles, the number of produced *invA* amplicons was more than twice the number of *RB1* amplicons ($1.80 \times 10^{13}$ vs $6.65 \times 10^{12}$, with 1.5 μM primers), which further undermines the suggested role of product accumulation as the major limiting factor for continued amplification as the amount of polymerase was the same for both assays. Also, doubling the polymerase amount or extending the reaction time had no positive effect on *RB1* product yield for any of the primer concentrations (Table 6).

A possible explanation for the less efficient amplification of the *RB1* assay is primer-dimer formation. Increased initial primer concentrations generally lead to elevated risks of primer-dimer formation in PCR, and for *RB1* such products were visible on the gel for 1.2 and 1.5 μM primers (data not shown). No primer-dimer bands were seen with the *invA* assay at any primer concentration. Judging from theoretical thermodynamics calculations, the *RB1* primers should be less prone to form primer-dimers than *invA* primers. For *RB1* primers, delta G values for homo- and hetero-dimers are −5.2 kJ or higher, but as low as −9.8 kJ for the *invA* assay (OligoAnalyzer 3.1, Integrated DNA Technologies). This suggests that the risk for primer-dimer formation needs to be empirically assessed to get the true picture. Primer-dimer formation is a complicating factor in PCR kinetics, competing with production of true amplicons regarding primers, nucleotides and polymerase, thus lowering the yield and putting boundaries on the maximum production of true products. The role of primer-dimer generation in plateau phase formation was previously recognized by

Halford et al. [9].

Taken together, our results emphasize the significance of primer concentration rather than product accumulation as the major limiting factor of PCR amplification in late cycles. Our results contradict the results from Lee et al. [2], stating that although elevated levels of DNA polymerase and primers increase product yield, the plateau phase is inevitable due to product accumulation. However, in previous studies the continuation of amplification through the linear phase has not been pushed as far as in our experiments, where up to 60 cycles were applied before assessing product yield. We found that 45 PCR cycles was not enough to reach the plateau phase for increased primer concentrations, and consequently the full effect of elevated primer amounts was not seen until more PCR cycles were added. Our results have an impact on specific applications such as preamplification, where low amounts of primers are used and it is of importance to not reach the plateau phase where bias in relative abundance of template may be introduced [10]. It should also be noted that correct primer design has been described as the single most critical component of any PCR assay [28], and naturally the amplification kinetics is highly dependent on the primer characteristics.

## 4. Conclusions

In this study, we investigate the proposed causes of the PCR plateau phase. We highlight the different effects of adding target and non-target amplicons to PCR reactions, showing that the latter but not the former have a clear negative effect on amplification by blocking DNA polymerase activity. Thus, we demonstrate that polymerase blocking or product re-hybridization hindering primer annealing is not sufficient to arrest PCR amplification. Contrarily, we show that the PCR plateau level can be significantly delayed by increasing primer concentration up to five times the validated amount for two different qPCR assays. The ultimate bottleneck appears to be formation of unwanted products such as primer-dimers, which eventually disturb the proportionality between amount of primers added and true product formed. Increasing
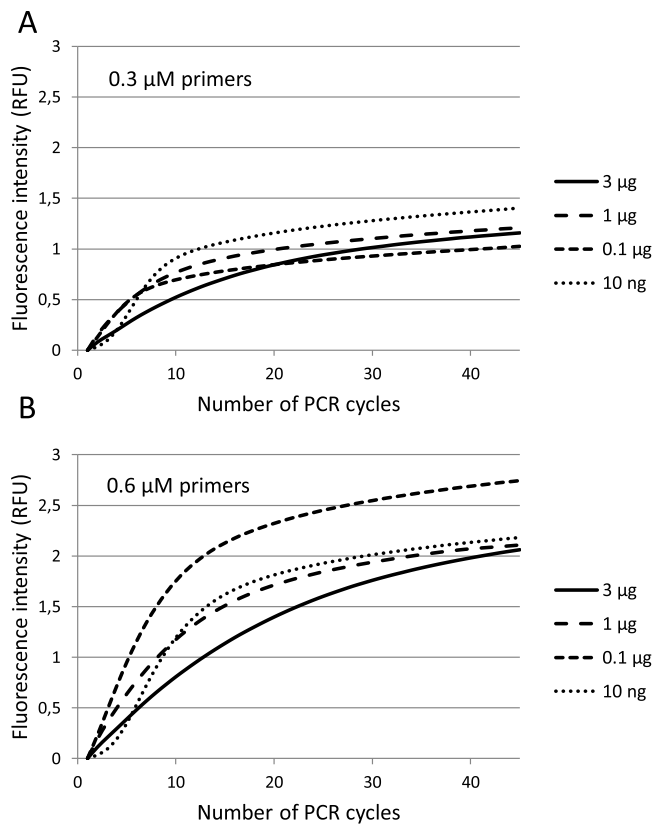
**Fig. 1.** Amplification curves generated in qPCR analysis with high amounts of target amplicons. The *RB1* qPCR assay was applied with A) 0.3 μM primers and B) 0.6 μg primers. The results for 10 ng, 0.1 μg, 1 μg and 3 μg of target amplicons are presented (n = 3, coefficient of variation from 0.04 to 0.08). The raw fluorescence data were adjusted for background fluorescence to a set-off at y = 0. Note that the end-point fluorescence, reflecting the number of generated amplicons, is almost twice as high when the primer concentration is doubled. RFU - relative fluorescence units.

polymerase concentrations on the other hand, generated minor or no improvements in product yield, depending on cycle settings, while the type of DNA polymerase was shown to affect product yield significantly. Applying reagents from dedicated manufacturers, thermal stability of DNA polymerase, primers and nucleotides should not limit amplification.

We stress that PCR plateau formation caused by primer depletion is assay-dependent, e.g. due to the probability of primer-dimer generation. With robust primers however, our results clearly refute that blocking of DNA polymerase by double-stranded amplicons or product re-hybridization would be the major cause of arrested amplification, and indeed point towards more trivial factors such as depletion of primers. We hope that our results will provide an improved understanding of PCR dynamics and limitations of continued product formation in late cycles, and in the end promote more fine-tuned PCR optimization for relevant applications.

## Competing interests

The authors declare no competing interests.

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