A Viability Quantitative PCR Dilemma: Are Longer Amplicons Better?

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ABSTRACT

The development of viability quantitative PCR (v-qPCR) has allowed for a more accurate assessment of the viability of a microbial sample by limiting the amplification of DNA from dead cells. Although valuable, v-qPCR is not infallible. One of the most limiting factors for accurate live/dead distinction is the length of the qPCR amplicon used. However, no consensus or guidelines exist for selecting and designing amplicon lengths for optimal results. In this study, a wide range of incrementally increasing amplicon lengths (68 to 906 base pairs [bp]) was used on live and killed cells of nine bacterial species treated with a viability dye (propidium monoazide [PMA]). Increasing amplicon lengths up to approximately 200 bp resulted in increasing quantification cycle ($C_q$) differences between live and killed cells while maintaining a good qPCR efficiency. Longer amplicon lengths, up to approximately 400 bp, further increased the $C_q$ difference but at the cost of qPCR efficiency. Above 400 bp, no valuable increase in $C_q$ differences was observed.

IMPORTANTCE

Viability quantitative PCR (v-qPCR) has evolved into a valuable, mainstream technique for determining the number of viable microorganisms in samples by qPCR. Amplicon length is known to be positively correlated with the ability to distinguish between live and dead bacteria but is negatively correlated with qPCR efficiency. This trade-off is often not taken into account and might have an impact on the accuracy of v-qPCR data. Currently, there is no consensus on the optimal amplicon length. This paper provides methods to determine the optimal amplicon length and suggests an amplicon length range for optimal v-qPCR, taking into consideration the trade-off between qPCR efficiency and live/dead distinction.

KEYWORDS viability, vitality, v-qPCR, propidium monoazide, PMA, qPCR efficiency, biofilm, microbiome

The accurate detection and quantification of specific bacterial cells are of prime importance for many in vitro experiments as well as in in vivo settings. DNA-based techniques such as quantitative PCR (qPCR) are routinely used for this purpose. qPCR offers several advantages over culturing techniques, especially in complex multispecies ecologies such as the oral cavity (1), the gut (2), and environmental samples (3). However, one major limitation is that qPCR, as such, is unable to distinguish DNA originating from live or dead bacterial cells. The development of live/dead viability qPCR (v-qPCR) techniques that theoretically quantify only living bacteria has provided a solution (4, 5). The v-qPCR technique relies on the use of membrane-impermeable DNA dyes such as ethidium monoazide (EMA) or propidium monoazide (PMA). In dead cells with a compromised membrane, the dye will diffuse through the cell membrane and intercalate with the cell’s DNA. Subsequent photoactivation of the azide group...
irreversibly binds the dye to the DNA. The dye-bound DNA will be excluded through precipitation of the DNA and blocking of DNA polymerase function during qPCR. Since viable cells maintain their membrane integrity, the dye theoretically will not enter these viable cells and will not block the DNA amplification. This results in the specific amplification and quantification of the DNA from the live cells with uncompromised cell membranes without the amplification and quantification of the DNA from dead cells with compromised cell membranes.

Although the technique is well established, two major limitations of its efficacy must be kept in mind. First, although viability dyes are supposed to exclusively enter membrane-compromised prokaryotic cells, EMA is known to also enter cells with intact membranes, affecting the viable cells’ DNA. PMA is less likely to pass through intact membranes, providing a more accurate determination of viable cells of both pro- and eukaryotic genera (6). However, the membrane properties of some microbial species limit dye entry (e.g., double membranes of Gram-negative bacteria). Therefore, some v-qPCR protocols utilize a pretreatment with a gentle detergent to facilitate the entry of the dye into the dead cells (7–9). However, there is a considerable risk that such pretreatment also affects the membrane integrity of viable cells, allowing PMA to enter live cells and thus possibly resulting in a biased result.

Second, a multitude of factors limits the efficacy of the technique to exclude DNA from dead cells, overestimating the viability of the sample. These factors include the dye/cell concentration, incubation time and temperature, photolysis time, wavelength, presence of facilitating agents, cell membrane and wall properties, type of qPCR assay and materials (primer properties, polymerase, and free DNA, etc.), and length of the DNA sequence amplified, also known as the amplicon (4, 5, 10). Because of this, a total signal neutralization of dead cells (i.e., no detectable amplifications) remains one of the greatest challenges to be resolved for v-qPCR (10). Several of the above-mentioned factors are in direct competition with each other, resulting in the requirement for evaluating and optimizing which trade-offs are acceptable for the goals of the developed v-qPCR assay.

Many studies developing a v-qPCR assay describe a detailed optimization of the dye treatment protocol, while a crucial component for accurate v-qPCR, the amplicon length, is often overlooked or only briefly assessed. Highly efficient qPCRs employ amplicons shorter than 200 bp because the chance of polymerization errors is lower, enabling quick, accurate, and efficient quantification (11–13). Many v-qPCRs are adapted from very efficient qPCR assays that are highly specific for the target DNA, which results typically in v-qPCR assays using the recommended short amplicon lengths. However, this poses an important predicament for v-qPCR: increasing the length of an amplicon increases the probability of sufficient dye-DNA binding events having occurred, which are required to block polymerase function and, thus, PCR amplification of the dead cells’ DNA (4). The efficiency of the qPCR amplicon is therefore in direct trade-off with the reduction of the false-positive signal from membrane-compromised, dead cells. Despite this important trade-off, it is currently unclear which amplicon lengths should be used in v-qPCR. Several groups have used a variety of amplicons, ranging from below 100 bp to upwards of 1,000 bp, with various results (8, 14–19).

Since there is no consensus on the minimal or optimal amplicon length and since there is no clear way to determine this for a v-qPCR assay, this study aimed to evaluate different amplicon lengths, ranging from 63 bp up to 906 bp, and provide a method to calculate a working range and optimal amplicon lengths for v-qPCR assays of eight oral bacteria and the model organism *Escherichia coli*.

**RESULTS**

To determine the effect of only the amplicon length on the distinction between live and killed cells, the factors influencing viability qPCR efficacy were tested in preliminary experiments to determine conditions under which each factor was either kept constant (temperature, treatment solution, cell concentrations, and qPCR conditions),
provided in excess (PMA concentration, incubation, and photolysis duration), or deliberately tested (different species and biological and technical replicates) without affecting the viability of live cells or the qPCR assay (see Fig. S1 in the supplemental material).

**Live/dead distinction of *A. actinomycetemcomitans***. Using incrementally increasing amplicon lengths of the 32 primer sets on live and completely killed *Aggregatibacter actinomycetemcomitans* cells, the effect of amplicon length on live/dead distinction using probe-based qPCR (Fig. 1A), dye-based qPCR (Fig. 1B), the differences between live and killed samples (quantification cycle difference (ΔCq)), and the derivative of ΔCq’s logistic fit with respect to amplicon length (d(ΔCq)/d(bp)) (Fig. 1C and D) were evaluated. For both types of qPCR, in all live samples, slight increases in Cq values with amplicon length together with increased variation between replicates were observed. In contrast, the killed samples showed drastically increased Cq values with increased amplicon lengths and also showed increased variation between the replicates. Signal neutralization (Cq equal to 45) started to occur for amplicons of 300 bp and longer but did not evolve to consistent signal neutralization at longer amplicon lengths for each replicate (Fig. 1A and B), except once with the 838-bp amplicon.

Live/dead distinction increased from a ΔCq of below 5 for amplicons shorter than 100 bp up to a ΔCq of 20 or higher for amplicons longer than 300 bp, after which it remained largely constant (Fig. 1C and D). This transition region of amplicon lengths (where the increase in ΔCq started to plateau) was marked as the maximum of the working range. This maximum was calculated from 5% of the maximum of d(ΔCq)/d(bp), which describes most of the change in ΔCq. At these amplicon lengths, 98.5% of the maximal ΔCq was achieved (Table 1 and Table S5) while keeping the amplicon length as short as possible (around 400 bp). The second limit, the minimum of the working range, was calculated from the inflection point of d(ΔCq)/d(bp) after the maximum. At this point, around 79% of the maximum ΔCq is reached (Table 1 and Table S5) while having amplicons of around 200 bp (Fig. 1C and D and Table 1).

**Translation to v-qPCR application.** To illustrate the effect of amplicon length on live/dead distinction, two amplicons with low specificity for the 16S rRNA genes of other oral bacteria (20, 21) were tested on the live and completely killed samples of *A. actinomycetemcomitans* and quantified with plasmid standards for each amplicon (Fig. 1E). For both amplicon lengths, similar numbers of *A. actinomycetemcomitans* bacteria were detected in the live samples and confirmed with the plating of serial dilutions on agar. While the 80-bp amplicon still resulted in the detection of >6 log genome equivalents/ml [log(Geq/ml)] in the killed samples [only 1.95-log(Geq/ml) live/dead distinction], the 237-bp amplicon significantly increased the live/dead distinction to 5.42 log (Geq/ml) (Fig. 1E), while the qPCR efficiency was reduced by only 4.5% (Fig. 1G). A 10-fold dilution series of these samples was made and submitted to v-qPCR to investigate if the DNA concentration affected the v-qPCR outcome (Fig. 1F). For the 80-bp amplicon, consistent detection of killed cells was observed, as was a consistent difference between the numbers of live and dead cells. For the 237-bp amplicon, v-qPCR gave consistently lower live-cell numbers for the live sample. The dilution series of the killed sample remained almost constant at approximately 500 Geq/ml. Occasionally, these killed samples gave no signal at this concentration. While this occurred only at the highest dilution for the 80-bp amplicon, this already occurred at lower dilutions for the 237-bp amplicon. The Cq values of 500 Geq/ml approximate the Cq value plateau reached for amplicons longer than 300 bp (Fig. 1A and B).

**Efficiency and sensitivity trade-offs.** Of the 32 primer sets for *A. actinomycetemcomitans*, 14 sets were selected to evaluate the efficiency with 10-fold dilution series of the three independent replicates instead of aliquots taken from each replicate to account for biological variability rather than only technical variability (Fig. 1G and Table S1). By design, the amplification time was not extended according to the increase in the length of the amplicons, contributing to an expected reduction in efficiency and the associated sensitivity. This reduction in efficiency remained acceptable for amplicons...
Aggregatibacter actinomycetemcomitans

FIG 1 Effect of amplicon length on live/dead distinction of the v-qPCR assay. (A and B) Probe-based (A) and dye-based (B) qPCR with 32 different amplicon lengths designed for the 16S rRNA of *A. actinomycetemcomitans* and tested on three independent, biological replicates. Closed triangles, (Continued on next page)
The intercept of this lowest limit and the per bacterium length (Fig. 1A to D). Amplicons longer than 200 bp, and standard deviations further increased with amplicon between independent replicates in both live and killed samples were also observed for increases in variability between replicates (Fig. 1G). Increases in standard deviations efficiency, 93.5%, of the 80-bp amplicon) but drastically decreased past 300 bp and showed diminishing returns afterward. (Fig. 1H, red vertical). Using a common qPCR efficiency method can be used to calculate the maximum determined to be 242 bp (extrapolated from the minimum and maximal data points), was determined to be 242 bp (extrapolated from the Cq of 16.08 (79% of the maximum Cq) and an efficiency of 86.79% (Fig. 1H, red slope). Alternatively, the ROC-like method can be used to calculate the Cq with an established lowest qPCR efficiency limit (Fig. 1H, red vertical). Using a common qPCR efficiency limit of 90%, from the intercept of this lowest limit and the fit of the ROC curve, the Cq was calculated to be 13.57 and extrapolated from the Cq fit to an amplicon length of 188 bp.

**Confirmation in other bacterial species.** To confirm the results observed for A. actinomycetemcomitans, six primer sets with similar properties and amplicon lengths of around 100, 150, 200, 300, 400, and 500 bp were designed and tested on live and killed samples of an additional eight bacterial species (Fig. 2 and Table S2). The species examined in this study were chosen due to the in-house availability of the strains and their genomic sequences. Similar to A. actinomycetemcomitans, the live/dead distinction of the eight bacteria drastically improved with amplicon lengths of up to 200 to 300 bp and showed diminishing returns afterward.

**FIG 1** Legend (Continued)

Live samples; open triangles, killed samples. (C and D) Live/dead distinction from panels A and B, respectively, expressed as ΔCq. Red curve, derivative of the ΔCq curve (d[ΔCq]/dt[bp]); red diamonds, amplicon lengths corresponding to the inflection point and 5% of the maximum from the d(ΔCq)/dt[bp] curve. (E) Quantification of A. actinomycetemcomitans live and killed samples with either the 80-bp or 237-bp amplicon and their respective plasmid standards. Data are expressed as log10 genome equivalents (Geg) per milliliter. *, statistically significant difference (P < 0.05); n.s., no significant difference. (F) Tenfold dilution series of the sample in panel E with the concentrations determined from enumeration of the untreated samples on agar. The plate count error was only measured and displayed for the highest live concentration (x axis error). Closed triangles, live with the 80-bp amplicon; open triangles, killed with the 80-bp amplicon; closed circles, live with the 237-bp amplicon; open circles, killed with the 237-bp amplicon. If no detection occurred for one or more samples, this is indicated by their respective symbols on the x axis at that dilution. (G) qPCR efficiency deviation and standard deviation of the qPCR efficiency from 14 selected amplicon lengths tested on the 10-fold dilution series of the replicates (see Table S2 in the supplemental material). (H) ROC-like method (ΔCq [of probe qPCR] versus 100 – efficiency of each amplicon length tested for qPCR efficiency). Selected ΔCq’s for minimal qPCR efficiency were set at a 90% efficiency (red vertical), and the optimized qPCR efficiency versus ΔCq (red slope) is indicated as encircled points on the ROC curve. Details on the fitted functions are available in Tables S4 to S8 in the supplemental material.

### Table 1

<table>
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<th>Bacterium</th>
<th>Min amplicon length (bp)</th>
<th>ΔCq of min</th>
<th>Max amplicon length (bp)</th>
<th>ΔCq of max</th>
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<td>S. salivarius</td>
<td>157</td>
<td>17.30</td>
<td>271</td>
<td>21.65</td>
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</table>

*Minimum and maximum amplicon lengths were determined from the infection point after the maximum and 5% of the maximum of d(ΔCq)/dt[bp], respectively, as described in Materials and Methods and marked as red diamonds in Fig. 1C and D and Fig. 2.
FIG 2 Confirmation of live/dead distinction of six amplicon lengths for eight bacterial species. Per bacterium, the top graphs show $C_q$'s of live and killed samples (closed triangles, live samples; open triangles, killed samples), and the bottom graph shows $\Delta C_q$ and $d(\Delta C_q)/d(bp)$. Details on the fitted functions are available in Tables S4 to S8 in the supplemental material.
While *A. actinomycetemcomitans*, *Prevotella intermedia*, and *Porphyromonas gingivalis* still showed benefits for amplicons of up to 400 bp (Fig. 1C and D and Fig. 2), the difference in live/dead distinction between the 300-bp and 400-bp amplicons for the other six bacteria was negligible (Fig. 2). Minimal and maximal amplicon lengths generally were similar to each other, with most variation being on maximal amplicon length (Table 1). However, *Fusobacterium nucleatum* and *Streptococcus salivarius* allowed shorter-than-average amplicons to be used to obtain the same \( \Delta C_q \) (Table 1).

**DISCUSSION**

The discovery and development of dyes that specifically enter dead cells have allowed for techniques like v-qPCR that give a more accurate representation of the bacterial cell viability of a sample. However, the efficacy of the technique can be severely limited if suboptimal qPCR amplicons are used. Despite this known limitation, no clear consensus or guidelines exist on which amplicon length has to be used for optimal efficacy. This study aimed to determine a working range of amplicon lengths that provides substantial live/dead distinction using v-qPCR while retaining a sufficiently high qPCR efficiency, thereby allowing for an accurate and specific determination of the number of viable cells. Depending on the examined bacterial species, on average, a minimum amplicon length of 200 bp was determined to provide sufficient distinction between living and killed samples. Nonviability qPCR guidelines generally recommend amplicon lengths below 150 to 200 bp for optimal qPCR efficiency. As shown here, amplicon lengths under 200 bp had lower live/dead distinction than amplicon lengths of between 200 and 300 bp. Amplicon lengths below 200 bp are consequently not recommended to be used for v-qPCR. Increasing the amplicon length above 300 bp was beneficial for the live/dead distinction in some species but reduced qPCR efficiency and sensitivity. For all bacteria, while there were variations in the calculated maximum amplicon length, amplicons longer than 400 bp generally showed no further improvement in live/dead distinction, making these lengths undesirable for v-qPCR. Under optimal conditions for viability treatment, the results and conclusions of this study are likely to translate to other bacteria and eukaryotes since the effect of amplicon length on v-qPCR is based on qPCR chemistry rather than the source of the DNA. If the viability treatment is suboptimal, fewer dye-DNA binding events will have occurred, necessitating the use of longer amplicons. The observed variation in optimal amplicon lengths is likely due to how each species reacts slightly differently to the dye treatment protocol, allowing for shorter or requiring longer amplicons. Still, these results and this approach are still ideally further confirmed in other bacterial and eukaryotic genera and with other qPCR chemistries and validated for each v-qPCR setup with different organisms and protocols. The presented \( d(\Delta C_q)/d(bp) \) or receiver operating characteristic methods can be used to calculate an optimal amplicon length or working range.

**Importance of amplicon length.** According to our estimations (see Table S1 in the supplemental material), for the concentration of PMA applied to the *A. actinomycetemcomitans* samples, an excess of 151 PMA molecules per nucleotide was supplied during dye treatment. However, only at amplicon lengths of around 400 bp (the quasimaximum) does the v-qPCR signal remain consistently low. This implies that per 400 bp, only two PMA molecules (one for each strand of the target) were bound, inhibiting target amplification. While losses are expected, only 1 in 30,200 molecules binding their nucleotide is a severe discrepancy. This apparent DNA-dye discrepancy has led to several groups developing pretreatment protocols to improve dye entry and DNA binding, with various results. Using the methods presented here, the extent of the DNA-dye discrepancy was evaluated, and its influence on the experimental outcome can be mediated or subverted within the selection of amplicon lengths.

It was previously shown that very long amplicons for qPCR (1,490 bp and 2,840 bp) (completely) reduce the signal from killed cells treated with EMA. The use of these long amplicons allowed for a significant signal reduction in killed samples (\( \Delta C_q \geq 15 \))...
with low concentrations of EMA (10 µg/ml), while it avoided affecting detections from live samples caused by increased EMA concentrations (19). The use of PMA largely avoids the reduction in the detection of live cells and has also been observed to yield a significantly reduced signal when using longer amplicons (618 bp and 724 bp, with ΔCq values of ≥13 for both) as opposed to shorter amplicons (140 bp, with a ΔCq of ≤5, and 329 bp, with a ΔCq of 7 to 12) (17). In the current study, further increasing the concentration of PMA (from 10 µM to 100 µM) and using an altered PMA formulation (PMAxx), live/dead distinction at a ΔCq value of ≥15 can be obtained with amplicon lengths of 200 to 300 bp (Fig. 1A to D and G) while not affecting the detection of live cells.

Several groups have used amplicon lengths shorter than 200 bp (8, 14, 15), which might have benefitted greatly from slight increases in amplicon length. As shown in this study when quantifying *A. actinomycetemcomitans*, if a viability experiment is analyzed using a short amplicon, the decreases in bacterial numbers are unlikely to be an accurate depiction of the reality (Fig. 1E). If a short amplicon is used to evaluate the efficacy of a novel treatment (e.g., the antimicrobial effect of a mouth rinse on oral biofilms), the result might be underestimated or nonsignificant. False-negative results like these can be avoided and even reassessed if the samples are (re)analyzed using longer amplicons.

The sensitivity of qPCR allows for more accurate detections of low-biomass samples, which is not possible with similar techniques such as flow cytometry using viability staining (5). However, the accuracy of v-qPCR in reducing detections of killed samples was limited below 500 Geq/ml (Fig. 1F) or at staining (5). However, the accuracy of v-qPCR in reducing detections of killed samples which is not possible with similar techniques such as amplicons. These can be avoided and even reassessed if the samples are (re)analyzed using longer amplifiers.

The proposed upper limit is the quasimaximum of $D_{Cq}$, if a viability experiment is analyzed using a short amplicon, the decreases in bacterial numbers are unlikely to be an accurate depiction of the reality (Fig. 1E). If a short amplicon is used to evaluate the efficacy of a novel treatment (e.g., the antimicrobial effect of a mouth rinse on oral biofilms), the result might be underestimated or nonsignificant. False-negative results like these can be avoided and even reassessed if the samples are (re)analyzed using longer amplifiers.

**Working range of amplicon lengths from $d(\Delta C_q)/d(bp)$.** To objectively determine the working range of amplicon lengths, from the $d(\Delta C_q)/d(bp)$ curve, the inflection points after the maximum and 5% of the maximum were chosen as the minimal and maximal amplicon lengths, respectively. Amplicon lengths below the lower limit of the working range have limited $D_{Cq}$ and, thus, poor live/dead distinction. The lower limit of each bacterium has 79.9% of the $D_{Cq}$ of the upper limit (Table 1) while maintaining an amplicon length as short as possible for a minimal reduction in qPCR efficiency. Increasing the amplicon length past the lower limit up to the upper limit yields an additional 20.1% increase in $D_{Cq}$ (Table 1), at the cost of qPCR efficiency. Since the upper limit was calculated from 5% of the maximum of $d(\Delta C_q)/d(bp)$, the change in $D_{Cq}$ of any amplicon longer than the upper limit was negligible (<2% increase in $D_{Cq}$). The proposed upper limit is the quasimaximum of $D_{Cq}$, after which longer amplicons do not supply added benefit while maintaining a reduction in qPCR efficiency.

While most qPCR polymerases and kits are developed with short amplicons and quick qPCRs in mind, these are suboptimal for v-qPCR, as shown in our study. The development and use of “long-amplicon v-PCR kits” have been suggested to counteract these observed limitations (4). However, little to no progress has been made on developing these kits to our knowledge. The development of new, high-fidelity DNA polymerases, which provide a low error rate and robust DNA replication, together with chemical PCR enhancers could resolve this reduced qPCR efficiency, reducing the trade-off caused by the longer amplicons required for v-qPCR. If such kits are developed, the amplicon lengths can be reevaluated with a likely shift of the optima toward longer amplicons.

**Effect of reduced qPCR efficiency.** While the reduction in qPCR efficiency was limited, this reduction is still likely to influence the sensitivity and accuracy of the v-qPCR assays examined. The decrease in efficiency can also affect the live/dead distinction since efficiency is determined by the slope of 10-fold dilutions of the template. As an example, if a $D_{Cq}$ of 20 is obtained with a 100% efficient qPCR (slope of −3.3), this translates to a 6-log reduction in detections. A 75% efficiency qPCR (slope of −4.1)
obtained a <5-log reduction, a 50% efficiency qPCR (slope of −5.6) returns a 4-log reduction, and so forth. Selection of the shortest possible amplicon with the highest qPCR efficiency and a (close to) optimal $\Delta C_q$ for live/dead distinction is preferable. Reductions in efficiency can be counteracted with a live or plasmid standard since each target is subject to the same efficiency. However, both the standard and the samples will be subject to the increase in variability caused by reduced qPCR efficiency (Fig. 1G), possibly reducing the accuracy of predictions made. As an alternative to the $D_{\Delta C_q}/d(bp)$ method, an optimal amplicon length can be calculated by plotting the $\Delta C_q$ and the inverse of the efficiency in an ROC-like method (Fig. 1H).

With the ROC-like method, a minimal qPCR efficiency can be selected, e.g., no lower than 90% efficiency, and the optimal amplicon for that efficiency can be extrapolated from the obtained $\Delta C_q$. Alternatively, using this method, both $\Delta C_q$ and efficiency can be maximized simultaneously, giving an optimized point with a balance between both factors. The $D_{\Delta C_q}/d(bp)$ method attempts to keep the efficiency as high as possible by selecting an amplicon as short as possible with approximately 80% of the total $\Delta C_q$ change. Whereas the ROC-like method compares $\Delta C_q$ and qPCR efficiency directly, the $D_{\Delta C_q}/d(bp)$ method serves as an approximation. With the $D_{\Delta C_q}/d(bp)$ method, the lower range for A. actinomycetemcomitans was calculated to be 224 bp, relatively similar to the 242-bp optimum of the ROC-like method and with similar $\Delta C_q$’s (16.05 and 16.08, respectively).

Although the ROC-like method provides a direct link between $\Delta C_q$ and qPCR efficiency and is thus preferable, the amounts of amplicon lengths and qPCRs required are multiple times larger than with the $D_{\Delta C_q}/d(bp)$ method. Depending on the level of detail or goals desired for designing a v-qPCR, either method can be employed to calculate suitable amplicon lengths.

**Conclusion.** In this study, v-qPCR amplicon lengths of between 200 and 400 bp were shown to provide a satisfactory live/dead distinction at a relatively low cost to the qPCR efficiency. A selection within this working range can provide either a sufficient live/dead distinction with a minimal reduction in qPCR efficiency (lower limit) or a quasimaximal live/dead distinction. Further increasing the amplicon length can prove beneficial in some assays but is ideally examined in detail with the presented $D_{\Delta C_q}/d(bp)$ or ROC-like method to determine an optimal amplicon length for the v-qPCR assay with as little a trade-off as possible. In summary, amplicons longer than those in traditional qPCR recommendations clearly improve live/dead distinction for v-qPCR, but extreme amplicon lengths with their reduced qPCR efficiency are not desirable.

**MATERIALS AND METHODS**

**Bacterial strains, media, and culture conditions.** *Aggregatibacter actinomycetemcomitans* ATCC 43718, *Prevotella intermedia* ATCC 25611, *Porphyromonas gingivalis* ATCC 33277, *Fusobacterium nucleatum* DSM 20482, *Streptococcus mutans* DSM 20523, *Streptococcus gordonii* ATCC 49818, *Streptococcus oralis* DSM 20627, *Streptococcus salivarius* TOVE-R, and *Escherichia coli* DH5α were grown on blood agar base (Oxoid, Basingstoke, UK) supplemented with 5 mg/ml hemin (Sigma, St. Louis, MO, USA), 1 mg/ml menadione (Calbiochem-Novabiochem, La Jolla, CA, USA), and 5% sterile horse blood (E&O Laboratories, Bonnybridge, Scotland). Liquid cultures were grown in brain heart infusion (BHI) broth (Difco, Detroit, MI, USA) without agitation to allow dead cells to sediment. This sediment was avoided to ensure that the live cultures contain as few dead cells as possible. Bacteria were grown at 37°C under aerobic (5% CO2) (all except *P. intermedia, P. gingivalis, and F. nucleatum*) or anaerobic (80% N2, 10% H2, and 10% CO2) conditions. The optical density at 600 nm (OD600) was measured with a spectrophotometer (GeneQuant spectrophotometer).

**Bacterial growth and killing.** For each bacterium, three independent biological replicates were grown on three separate days. From *A. actinomycetemcomitans* cultures grown overnight (16 h), a subculture in fresh BHI broth was grown for 4 h before adjusting the culture to an OD600 of 0.5. For the other bacteria, the culture grown overnight was directly adjusted to an OD600 of 0.5. All bacteria were pelleted at 6,000 × g for 5 min and resuspended in an equal volume phosphate-buffered saline (PBS) (8% NaCl, 0.2% KCl, 3.63% NaHPO4·12H2O, 0.2% KH2PO4 [pH 7.4]). These samples were equally divided into a “live” sample (kept at room temperature) and a “killed” sample (heat block set at 95°C for 15 min) before treatment with the viability dye. Killing of the bacteria was confirmed by plating of 200 μl of the killed samples on blood agar, followed by incubation under their preferred growth conditions for at least 1 week. Live bacteria were enumerated by plating 10-fold serial dilutions of the live samples, followed by incubation for 24 to 48 h before calculating CFU and calculating the original undiluted sample concentration (CFU per milliliter).
PMA treatment and DNA extraction. Live and killed samples were transferred to a 96-well plate (90 μl/well), followed by the addition of PMAx (Biotium, Hayward, CA, USA) (10 μl/well) with a final concentration of 100 μM. Samples were incubated in the dark for 10 min, followed by photoactivation of the dye for 15 min using Glo-Plate blue (Biotium, Hayward, CA, USA). After photoysis, samples were centrifuged for 10 min at 6,000 × g, after which the supernatants were discarded. DNA from the pelleted cells was extracted using the QiAamp DNA minikit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. DNA elution was performed with the maximal volume of the kit’s elution buffer (200 μl) to ensure maximal DNA release from the column. For all DNA dilutions, Milli-Q water (Merck, Darmstadt, Germany) was used.

qPCR and qPCR efficiency. All qPCR assays were performed with a CFX96 real-time system (Bio-Rad, Hercules, CA, USA) and its associated software CFX Manager (version 3.1). Probe-based TaqMan qPCR was performed using 12.5 μl of TaqMan Rox probe master mix dTTBP blue (Eurogentec, Seraing, Belgium), 1 μl of primers and probe (for concentrations, see Tables S2 and S3 in the supplemental material), 5 μl of the template, and 4.5 μl of Milli-Q water. Dye-based SYBR green qPCR (for A. actinomycetemcomitans and E. coli) was performed using 12.5 μl of TaqMan SYBR master mix dTTBP blue (Eurogentec, Seraing, Belgium), 1 μl of primers (for concentrations, see Tables S2 and S3 in the supplemental material), and 4.5 μl of Milli-Q water. Cycle conditions for both dye- and probe-based qPCRs were an initial step at 50°C for 2 min and 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. Primers (IDT, Haasrode, Belgium) and hydrolysis/TaqMan probes (DD probes, 5′-FAM [6-carboxyfluorescein] and 3′-TAMRA [6-carboxytetramethylrhodamine]; Eurogentec, Seraing, Belgium) (Tables S2 and S3) were selected from the literature or designed using genome sequences sequenced from the specific strains. In the case study of A. actinomycetemcomitans, 32 primer sets were designed to incrementally increase the amplicon length by moving either the forward or reverse primer further away from the probe while keeping the probe and the complementary primer unchanged (Table S2 and Fig. S2) and tested in parallel on live and completely killed samples to evaluate probe- and dye-based qPCR chemistries and the associated qPCR efficiencies (only for probe-based qPCR). For the eight other bacteria, amplicon lengths of around 100, 150, 200, 300, 400, and 500 bp were chosen, analogous to the A. actinomycetemcomitans lengths of interest (Table S3). All primers were designed to obtain incrementally increasing amplicon lengths while maintaining the same melting temperature (maximum ± 2°C), percent GC content (GC%) (between 40 and 60%), and primer length (18 to 30 bp) with no secondary structures (analyzed with the OligoAnalyzer tool [IDT] and OligoEvaluator [Sigma-Aldrich]). Primers were also checked by performing a BLAST comparison to reference sequences (BLASTN; NIH). For the 80- and 237-bp amplicons of A. actinomycetemcomitans, plasmid standards were created with the amplicon inserted into a pGEM-T Easy plasmid (Promega, Madison, WI, USA) to quantify the number of 16S rRNA copies, and the number of cells was expressed as genome equivalents (Geq) per milliliter (6 copies per genome). From the three independent replicates of A. actinomycetemcomitans, a 10-fold dilution series was made from the original samples and tested with 14 of the 32 primer sets (Table 1 and Table S2). From the slopes of the dilution series, the qPCR efficiency was calculated as $E = 10^{-1/Slope} - 1$ (22).

Models and statistical analysis. Linear and nonlinear (logistic, exponential, and rational) data sets were fitted to a predefined model in MATLAB R2018b (MathWorks, Inc., MA, USA). All parameters and their 95% confidence intervals were estimated using the embedded optimization routines fmincon, lsqcurvefit for linear and nonlinear regression, respectively. Model functions that captured the main trends were selected. Based on the derivative of the sigmoidal function for $\Delta C_q$, the working range of amplicon lengths was determined, starting with the amplicon length at the inflection point of $d(\Delta C_q)/d(bp)$ (minimum amplicon length) and ending at 5% of the maximum value of this derivative (maximum amplicon length). For the receiver operating characteristic (ROC)-like analysis, the optimal combination of operating characteristics ($\Delta C_q$ and efficiency) was selected as the point where the perpendicular distance between the linear base curve (connecting the minimum and maximum data points) and the nonlinear regression curve is maximal. The amplicon length with the lowest qPCR efficiency limit was calculated from the intercept of a vertical linear function (90%, or $x = 10$) and the nonlinear regression curve. $\Delta C_q$ values obtained were extrapolated to amplicon lengths from the $\Delta C_q$ sigmoidal function for probe qPCR. Model equations and parameters are available in Tables S4 to S8 in the supplemental material. Significance between live and killed samples and between 80-bp and 237-bp amplicons was evaluated with a pairwise t test with Bonferroni correction in R (version 3.6.0 [https://www.R-project.org/]).

SUPPLEMENTAL MATERIAL
Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

REFERENCES


