Benchmark

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An easy method for quantification of anaerobic and microaerobic gene expression with fluorescent reporter proteins

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ABSTRACT

Fluorescent proteins, such as green fluorescent proteins, are invaluable tools for detecting and quantifying gene expression in high-throughput reporter gene assays. However, they introduce significant inaccuracies in studies involving microaerobiosis or anaerobiosis, as oxygen is required for the maturation of these proteins' chromophores. In this study, the authors highlight the errors incurred by using fluorescent proteins under limited oxygenation by comparing standard fluorescence-based reporter gene assays to quantitative real-time PCR data in the study of a complex oxygen-regulated gene network. Furthermore, a solution to perform quantification of anaerobic and microaerobic gene expression with fluorescent reporter proteins using a microplate reader with an oxygen control system and applying pulses of full oxygenation before fluorescence measurements is provided.

METHOD SUMMARY

Strains expressing fluorescent proteins to be compared in a reporter gene assay are grown in 96-well plates and incubated in a microplate reader with oxygen-control capabilities. Rather than determining fluorescence under microaerobic or anaerobic conditions, strains are first grown to the desired optical density under full oxygenation, followed by a long incubation under the oxygenation condition of choice and fluorescence is measured after a final pulse of 21% O_2 to allow for chromophore maturation.

KEYWORDS:

anaerobiosis ● fluorescent proteins ● gene expression ● GFP ● microaerobiosis ● reporter gene assay ● transcriptional fusion

Fluorescent proteins (FP) can be used to analyze protein expression, localization, movement and interactions, thereby facilitating the analysis of complex cellular processes and enabling biotechnological and biomedical applications. Specifically, FPs have become invaluable tools for detecting and quantifying gene expression in prokaryotes and eukaryotes. The use of FPs does not require exogenous substrates or cofactors, which constitutes a significant advantage over conventional reporter proteins (ß-galactosidase, chloramphenicol acetyltransferase etc.) which rely on substrate levels although both depend on energy reserves within cells.

However, the use of FPs for fluorescence microscopy, confocal microscopy or fluorescence-based reporter gene assays may introduce inaccuracies when studying anaerobic or microaerobic conditions. Conventional FPs such as green FP (GFP), DsRed, mCherry, mK-GO and so on require oxygen as a cofactor for the maturation of their respective chromophores. Once the FPs mature, O_2 is no longer required [1]. Anaerobiosis is not only the primary mode of growth for many bacterial species but also a mechanism for adaptation and virulence in many important pathogens. It is also a key factor in understanding bacterial biofilms [2]. It requires complex dedicated systems of metabolic shifts and gene regulation, which are often studied using reporter genes. Several researchers have developed various FP modifications at the protein level to increase their stability and fluorescence intensity, as well as to improve chromophore maturation, thus enhancing their performance under different environmental conditions [3]. Nevertheless, it remains paramount to establish a method to use FPs under anaerobic or microaerobic conditions without biasing the results [4].

It is important to note recent advances in the development of FPs that function under anaerobic conditions, notably HaloTag, SNAP-tag and FAST [5,6]. The activation of these proteins does not require oxygen; instead, they are activated by flavin mononucleotide or chloroalkane ligands, among others [5]. These FPs have been used in a variety of conditions, demonstrating their potential use in bacteria grown under anaerobic conditions [5,7,8]. However, one of the limitations of using this FP is the requirement for specific reading conditions that are not often available on flow cytometers or microscopes.

In this article, the authors describe an adaptation of traditional FP-based reporter gene assays aimed at obtaining gene expression data in bacteria grown under oxygenation conditions where FPs would present reduced or no activity. The Spark Multimode Microplate Reader (Tecan, Switzerland) platform, which combines high-throughput fluorescence detection and gas control to measure the expression of transcriptional fusions of anaerobically regulated promoters and FPs under known oxygen concentrations, was utilized. As a model, promoters of the three *Pseudomonas aeruginosa* (*P. aeruginosa*) ribonucleotide reductase (RNR) gene classes (Ia, II and III) were used. RNR is an essential enzyme that reduces the four ribonucleotides to their corresponding deoxyribonucleotides, thus playing a crucial role in cell DNA replication. This enzyme family is divided into three RNR classes depending on their enzyme activation system, cofactors and quaternary structure: class I only functions enzymatically under aerobic conditions, class III is strictly anaerobic and class II can work under either aerobic or anaerobic conditions [9,10]. Oxygen-dependent differential regulation of the three RNR classes of *P. aeruginosa* is well-known [9,11].

For this study, *E. coli* DH5α, as well as *P. aeruginosa* ATCC 15692 (CECT4122, hereafter PAO1 WT) and its isogenic *anr* chromosomal mutant (PW3874, hereafter Δ*anr*) were used [11]. Bacteria were grown under aerobic, microaerobic or anaerobic conditions in 96-well plates incubated in a Spark Multimode Microplate Reader (Tecan, Switzerland) with the Humidity Cassette accessory for humidity control (Tecan, Switzerland), incubated at 37°C with vigorous orbital shaking and ventilated for 20 s every 2 min using the Lid Lifter (Tecan, Switzerland), unless otherwise specified, for better exposure to the desired gas composition. *E. coli* DH5α was transformed with inducible expression vectors for FACS-optimized GFP, GFP*mut3* [12] (hereafter GFP, pUCP30T-*gfpmut3*) and far-red FP E2-Crimson [13], pUCP20T-*E2Crimson*), developed by Barbier *et al.* [14]. Strains containing these inducible FP expression plasmids were grown in Luria-Bertani (LB) (Sharlab, Spain) medium supplemented with 50 μg/ml gentamycin (Sigma, Spain) (pUCP30T-*gfpmut3*) and 100 μg/ml ampicillin (pUCP20T-*E2Crimson*). *P. aeruginosa* PAO1 was transformed with transcriptional fusions of GFP to the promoters of class Ia RNR (*nrdA*, P*nrdA*-pETS130), class II RNR (*nrdJ*, P*nrdJ*-pETS130) and class III RNR (*nrdD*, P*nrdD*-pETS130). Strains containing these plasmids were grown in LB medium supplemented with 150 μg/ml gentamycin. These transcriptional fusions were previously constructed in the current authors' laboratory and have been routinely used for measuring RNR gene expression [15–17]. Excitation and emission wavelengths were 485/535 nm for GFP and 580/680 nm for E2-Crimson. Fluorescence and OD_{550} were determined at a fixed Z-position of 30 mm on the Spark Multimode Microplate Reader (Tecan, Switzerland).

To illustrate the challenges faced when using FP reporters to study phenomena involving anaerobiosis or microaerobiosis, GFP transcriptional fusions were used to study the changes in gene expression of class Ia (P*nrdA*) and class II (P*nrdJ*) RNR promoters in full anaerobiosis, compared with aerobic conditions. Class II RNR expression has been well documented, with its gene expression being induced under microaerobic conditions and reaching its maximum level during strict anaerobiosis [11,18,19]. PAO1 P*nrdA*-pETS130 and PAO1 P*nrdJ*-pETS130 were grown in LB medium in 96-well plates incubated in the Spark Multimode Microplate Reader (Tecan, Switzerland) as previously detailed. Strains were inoculated at an initial OD_{550} of 0.05 and grown aerobically for 2.5 h and anaerobically for 4 h. OD_{550} and GFP fluorescence were monitored at the end of aerobic growth and every 20 min during anaerobiosis. No increase in GFP fluorescence could be detected (Figure 1A). To account for differences in basal expression as well as differences in bacterial growth, GFP relative fluorescence units were normalized divided by OD_{550} and then expressed as a fold-change compared with the aerobic measurement (Figure 1B), providing a clearer picture of the differences in GFP signal; however, no anaerobic induction of RNR expression could be detected. GFP produced under full anaerobiosis is expected to remain immature, obscuring the expected increase in gene expression. For the purpose of comparing and analyzing the trends and expression patterns among the various RNR classes, the AnaeroTrans system, a bioreactor setup previously described by the current authors' group, was employed [11]. PAO1 WT was sampled under aerobic, microaerobic and anaerobic conditions and quantitative real-time PCR (qRT-PCR) was used to determine the gene expression of class Ia (*nrdA*) and class II (*nrdJa*) RNR genes. All data analysis and normalization were performed as previously described [11]. As expected, *nrdJa* displayed microaerobic and anaerobic induction, peaking at x164 expression under full anaerobiosis (Figure 1C).

To assess the anaerobic conditions established by the microplate reader, we conducted a growth experiment using PAO1 WT and its isogenic mutant Δ*anr*, which is incapable of anaerobic growth [20]. Strains were inoculated at an OD_{550} of 0.05 and allowed to grow aerobically for 2.5 h, after which the O_2 concentration was set to the minimum (≤0.1%), and strains were incubated for 2 h, monitoring OD_{550} every 20 min. PAO1 WT continued growing, while Δ*anr* was unable to grow (Figure 1D).

While a setup like AnaeroTrans allows for the detection of changes in gene expression with the sensitivity that qRT-PCR provides, gene expression analysis using FP is often used as a straightforward and time-saving alternative. For that reason, we aimed to adapt traditional gene reporter assays to capture microaerobic and anaerobic changes in gene expression. Given that the *in vivo* half-life of most FP is orders of magnitude higher than their maturation time [21], we hypothesized that performing a first incubation under the desired O_2 concentration, followed by a pulse of 21% O_2, should allow for an amount of FP representative of the desired oxygenation condition, with full chromophore maturation, and without significantly affecting gene expression results.

We first tested this method using inducible expression vectors pUCP30T-*gfpmut3* and pUCP20T-*E2Crimson*. *E. coli* DH5α transformants carrying the expression vectors were grown aerobically in conical flasks up to an OD_{550} of 0.50; then, Isopropyl ß-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM to induce FP expression, and cultures were transferred to 96-well plates and incubated in the Spark Multimode Microplate Reader as detailed above. Strains were grown for 1 h aerobically, followed by 8 h of anaerobic growth. Finally, two pulses of 20 min of exposure to 21% O_2 were performed, measuring fluorescence after each pulse. During the oxygenation pulse, ventilation was increased to 20 s/min. As hypothesized, GFP and E2-Crimson produced

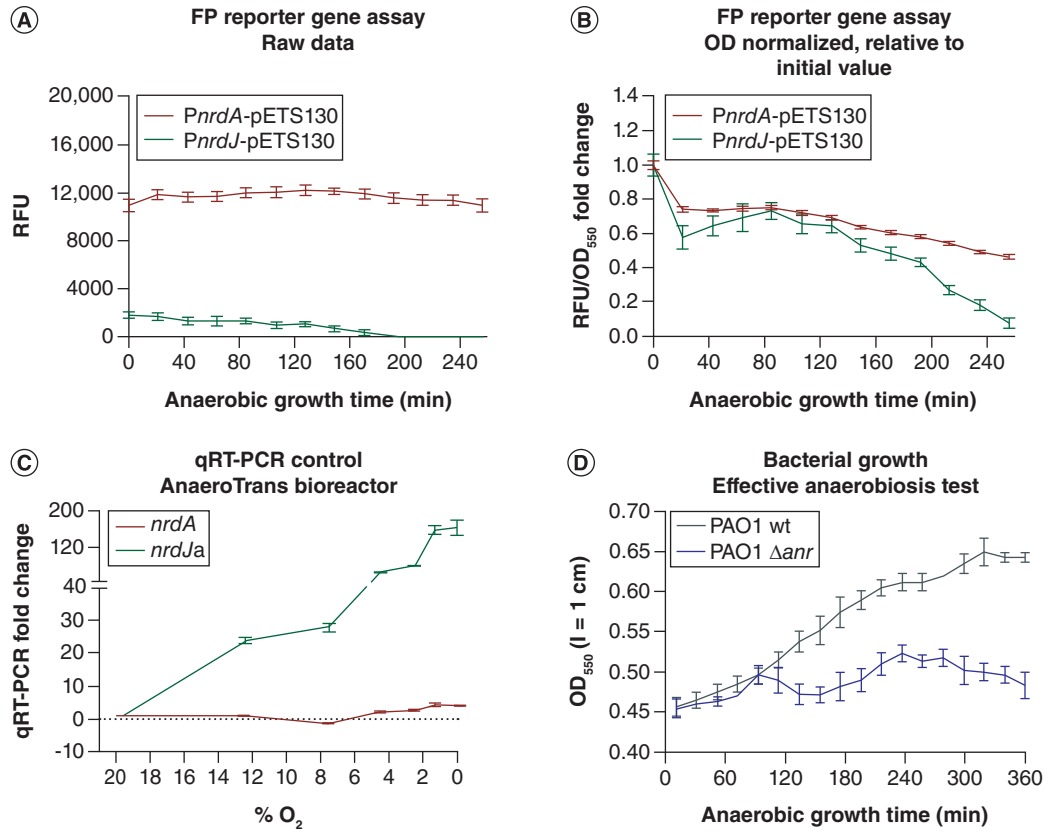

Figure 1. Failed determination of anaerobic gene expression using standard FP-based reporter gene assays compared with qRT-PCR. (A & B) GFP reporter gene assays for *P. aeruginosa* PAO1 promoters P*nrdA* and P*nrdJ* after full anaerobiosis was established, measured in **(A)** relative fluorescence units or **(B)** relative fluorescence units normalized by OD_{550} and expressed as fold-change relative to first measurement under aerobic conditions. **(C)** qRT-PCR relative quantitation ($\Delta\Delta C_t$) of *P. aeruginosa* PAO1 genes *nrdA* and *nrdJa* under microaerobic and anaerobic conditions (4 h), as previously determined using AnaeroTrans [11]. **(D)** Anaerobiosis test; bacterial growth during full anaerobiosis of *P. aeruginosa* PAO1 and its isogenic mutant Δ*anr*. OD_{550} values were converted to their equivalent for a path length of 1 cm (standard spectrophotometer cuvette) for reader's convenience. Error bars in all panels represent standard deviation of three technical replicates. Results presented are representative of same experiment repeated 2–3 times yielding identical results each time.

FP: Fluorescent protein; OD: Optical density; qRT-PCR: Quantitative retrotranscriptase PCR; RFU: Relative fluorescence units.

during anaerobic growth caused no significant increase in fluorescence (Figure 2A; anaero). However, after the oxygenation pulses, fluorescence increased to reflect all the GFP/E2-Crimson produced during the anaerobic period (Figure 2A; anaero + 40 min O_2).

The next goal was to reproduce this result with promoters that are naturally induced under anaerobic conditions. PAO1 transformants carrying transcriptional fusions of class Ia (P*nrdA*), class II (P*nrdJ*) and class III (P*nrdD*) RNR promoters to GFP were grown in LB medium in 96-well plates incubated in the Spark Multimode Microplate Reader as previously detailed. The same experiment as for the expression vectors was conducted, in this case, with 2.5 h of aerobic growth and 2.5 h of anaerobic growth before the O_2 pulses. Measuring GFP fluorescence after the anaerobic period showed no induction of P*nrdJ*, and only a moderate induction of P*nrdD*, attributable to residual O_2 present in the culture (Figure 2B; anaero). The O_2 exposure, however, activated GFP produced during anaerobic growth (Figure 2B; anaero + 40 min O_2), allowing the reporter gene assay to reflect the result observed in qRT-PCR data.

The next step was to establish how long the oxygenation pulse should take to achieve optimal activation of the FP without altering the gene expression data, using genes with known and different regulation patterns under changing O_2 concentrations. An analogous experiment to the previous one was performed, in which fluorescence and OD_{550} were measured every 10 min during O_2 exposure. As seen in Figure 2C, class III RNR (P*nrdD*) showed, as expected, the highest increase in fluorescence, peaking around 30 min of O_2 exposure and starting to decrease after 50 min, presumably due to the newly established aerobic conditions affecting gene expression. Class II RNR also showed an increase in fluorescence, although it kept growing with longer exposure time, as this class is also active in microaerobiosis. A total of 30 min of O_2 exposure was enough to properly capture the variation in gene expression that had occurred during anaerobiosis without introducing new, unintended variations. However, further analysis should be performed to evaluate the ratio of the total amount of GFP produced and the percentage that matured. A recent analysis of class III RNR (*nrdD*) gene expression con-

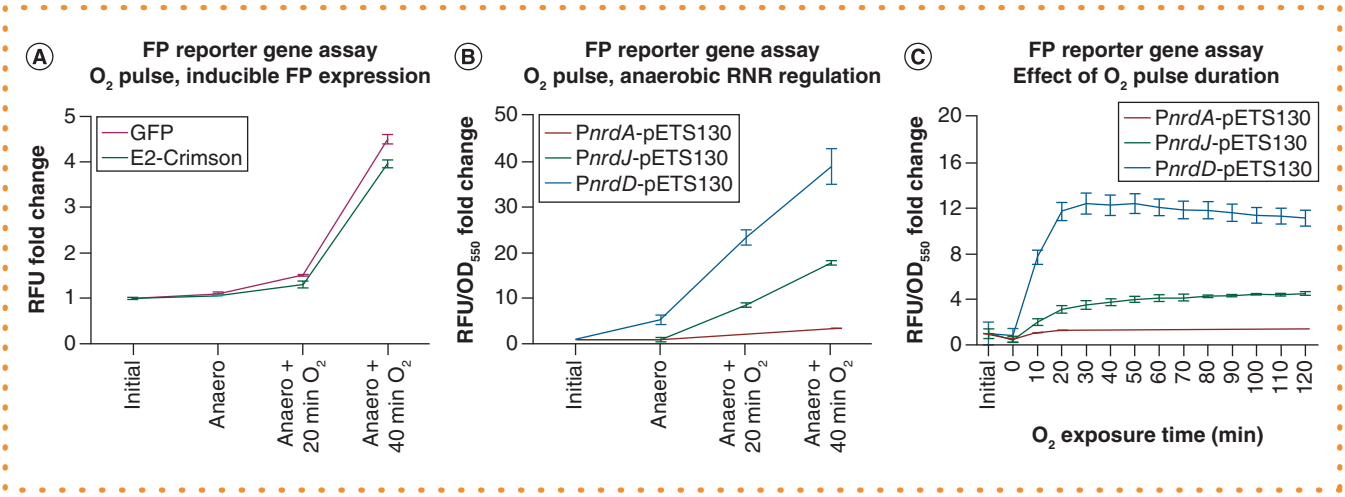

Figure 2. Development of method to capture anaerobic and microaerobic gene expression in FP-based reporter gene assays. (A) GFP reporter gene assay of *E. coli* DH5α carrying inducible GFP and E2-Crimson expression vectors. Fluorescence was determined after 1 h of aerobic growth (initial), 6 additional h of anaerobic growth (anaero) and previous treatment followed by 20 or 40 min of exposure to 21% O_2. **(B)** GFP reporter gene assay of *P. aeruginosa* PAO1 promoters P*nrdA*, P*nrdJ* and P*nrdD*. Fluorescence was determined after 2.5 h of aerobic growth (initial), after 2.5 additional h of anaerobic growth (anaero) and after previous treatment followed by 20 or 40 min of exposure to 21% O_2. **(C)** Effect of oxygen pulse duration on GFP reporter gene assay of *P. aeruginosa* PAO1 promoters P*nrdA*, P*nrdJ* and P*nrdD* determined after 2.5 h of aerobic growth (initial) and 2.5 h of anaerobic growth (anaero), followed by gradual exposure to 21% O_2. Fluorescence in all panels is represented as a fold-change relative to the "initial" measurement. Error bars in all panels represent standard deviation of 3–6 technical replicates. Results presented are representative of same experiment repeated 2–3 times yielding identical results each time.
FP: Fluorescent protein; GFP: Green fluorescent protein; OD: Optical density; RFU: Relative fluorescence units; RNR: Ribonucleotide reductase.

ducted under aerobic and anaerobic environments while measuring GFP fluorescence by flow cytometry [22] revealed a remarkably low fold change value (~1–2 times). This demonstrates the significance of the current studies.

Finally, to demonstrate the validity of this method, it was used to capture the whole differential gene expression experienced by the RNR network under gradients of oxygen concentration, such as those present across different layers of a biofilm. Even though chromophore maturation can still happen under microaerobic conditions, different oxygen concentrations introduce a bias that calls into question the validity of standard FP-based reporter gene assays. Here, PAO1 transformants carrying GFP transcriptional fusions of class Ia (P*nrdA*), class II (P*nrdJ*) and class III (P*nrdD*) RNR promoters were exposed to different oxygenation conditions for 5 h of growth (21%, 8.0%, 3.0% and 0.0% O_2), and then a pulse of 30 min of 21% O_2 was applied before measuring GFP fluorescence. The results obtained reflect the whole known pattern of microaerobic and anaerobic induction of the RNR network (Figure 3A): class I RNR (P*nrdA*) expression was not induced, except for a minor induction under full anaerobiosis caused by the reduction in growth speed; class II RNR (P*nrdJ*) expression was first activated under early microaerobic conditions (8.0% O_2), being further induced in late microaerobiosis (3% O_2) and anaerobiosis; finally, class III RNR (P*nrdD*) expression was not significantly activated in early microaerobiosis but presented the highest induction under anaerobic conditions. Although the order of magnitude for the fold-change was, as expected, lower than that of qRT-PCR, the resulting pattern of gene expression changes remarkably echoed that obtained using qRT-PCR of AnaeroTrans samples (Figure 3B [11]).

Conclusion

Despite the potential bias introduced by a partially immature population of FPs under microaerobic or anaerobic conditions, fluorescence-based reporter gene assays and other techniques using FPs are still commonly used in studies featuring limited oxygenation. Here, we demonstrated the significant bias that phenomenon can cause, as the whole anaerobic induction of class II RNR expression remained undetectable using a standard GFP reporter gene assay.

Furthermore, we provided a solution to perform quantification of anaerobic and microaerobic gene expression with fluorescent reporter proteins using a microplate reader with gas control capabilities, first performing a short aerobic growth until the desired optical density is obtained, followed by a long exposure to the oxygenation condition of choice and a final exposure to 21% O_2 to allow for FP maturation before measuring fluorescence.

One limitation of these techniques could be the time required for FP maturation, as protein synthesis can occur. Higher accuracy in GFP measurement under specific conditions can be achieved by inhibiting protein synthesis, for example, with the addition of an antibiotic before the oxygen pulse required for FP maturation. Under these conditions, we ensure that there is no further GFP production, thereby increasing the specificity of the GFP measurement. In addition, it would be interesting to evaluate the ratio of the total amount of

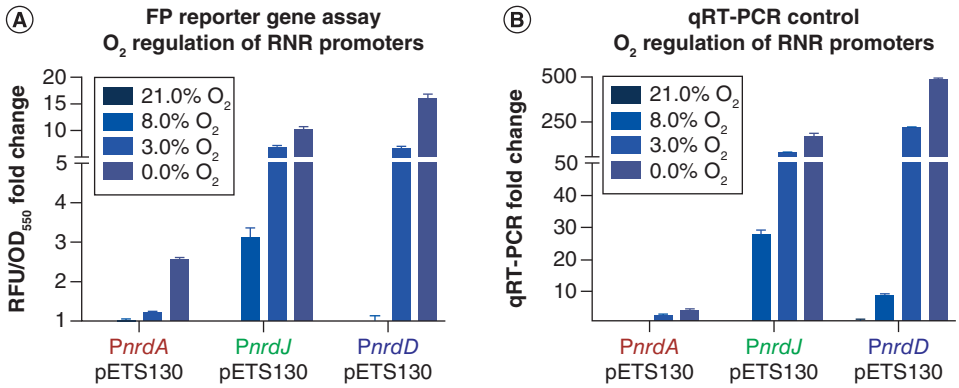

Figure 3. Successful determination of anaerobic and microaerobic gene expression using GFP-based reporter gene assays with oxygen pulses, compared with qRT-PCR. Microaerobic and anaerobic regulation of *P. aeruginosa* PAO1 promoters P*nrdA*, P*nrdJ* and P*nrdD* determined by **(A)** GFP reporter gene assay with 5 h of exposure to desired O_2 concentration followed by 30 min of exposure to 21% O_2 or **(B)** qRT-PCR relative quantitation ($\Delta\Delta C_t$) of AnaeroTrans samples as previously described [11]. Fold-change in both panels is relative to 21.0% average for each promoter. Error bars in both panels represent standard deviation of 2–3 technical replicates. Results presented are representative of same experiment repeated two to three times, yielding identical results each time.
FP: Fluorescent protein; GFP: Green fluorescent protein; OD: Optical density; qRT-PCR: Quantitative retrotranscriptase PCR; RFU: Relative fluorescence units; RNR: Ribonucleotide reductase.

GFP produced and the percentage that has matured, taking into account that this technique can be used for a variety of different types of FPs.

Using this method, we replicated qRT-PCR data that was otherwise irreproducible with reporter gene assays. The specific times for microaerobic/anaerobic growth and the 30-min oxygenation pulse were optimized for the specific setup of this study and may need to be modified. Particularly, the optimal oxygen pulse time is expected to be different for other bacterial strains, FPs or microplate readers and should be determined before applying this method to other setups.

Author contributions

L Pedraz performed the experiments. L Pedraz and E Torrents conceived and designed the experiments, analyzed the data, wrote the manuscript and edited and approved the final manuscript.

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Competing interests disclosure

The authors have no competing interests or relevant affiliations with any organization or entity with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

Writing disclosure

No writing assistance was utilized in the production of this manuscript.

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