A Bioassay Protocol for Quorum Sensing Studies Using *Vibrio campbellii*

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[Abstract] Quorum Sensing (QS), or bacterial cell-to-cell communication, is a finely-tuned mechanism that regulates gene expression on a population density-dependent manner through the production, secretion and reception of extracellular signaling molecules termed autoinducers (AIs). Given that QS plays an important role in bacterial biofilm formation and virulence factor production in many pathogenic strains, QS disruptors have become a hot topic in current antimicrobial research. There are several reporter strains exhibiting QS-regulated phenotypes that have been engineered for the identification of QS inhibitors, including, for example, pigment production (González and Keshavan, 2006; Steindler and Venturi, 2007), *gfp*, *lacZ* or *lux* reporter gene fusions (González and Keshavan, 2006; Steindler and Venturi, 2007), or lethal gene fusions downstream QS-controlled promoters (Weiland-Bräuer *et al*., 2015). With three parallel QS circuits, the bioluminescent marine bacterium *Vibrio campbellii* (formerly *harveyi,* Lin *et al*., 2010) constitutes a complex Gram-negative model for which an extensive body of knowledge exists, including an array of mutant biosensors. In *V. campbellii*, bioluminescence is regulated by QS. However, bioluminescence is the result of complex biochemical networks that converge with cell respiration and fatty acid metabolism. It is also an energy-demanding reaction that strongly depends on the overall metabolic state of the bacterium, consuming up to 1/5 of the cell resources (Munn, 2011). Thus, disruption of QS-controlled phenotypes might be the result of toxic side effects or interference with the above-mentioned biochemical pathways rather than QS signaling. Therefore, adequate control experiments should be included. The protocol described herein provides a method and workflow for the identification of putative QS-disrupting compounds in *Vibrio*. It can also be easily adapted for other QS studies (*e.g.,* detection of AI molecules).

Materials and Reagents

1. White, clear-bottom 96-well plates (Sigma-Aldrich, Corning® Costar®, catalog number: 3610)
2. Sterile sealing membrane (Sigma-Aldrich, Breathe-Easy®, catalog number: Z380059)
3. 15-ml tubes (VWR international, catalog number: 525-0150)
4. 96-well plate (Thermo Fisher Scientific, Nunc™ MicroWell™, catalog number: 167008)
5. Cryovials (optional) (Cryoinstant) (Deltalab, catalog number: 409113/6)
6. Disposable 1 mm path length cuvettes (Sigma-Aldrich, Brand®, catalog number: Z637092)

7. *Vibrio campbellii* strain
8. Dimethylsulfoxide (DMSO) (Sigma-Aldrich, catalog number: D8418)
9. NaCl (Sigma-Aldrich, catalog number: S7653)
10. $MgSO_4$ (Sigma-Aldrich, catalog number: M7506)
11. Protein Hydrolysate Amicase (Sigma-Aldrich, catalog number: 82514)
12. DI water
13. NaOH (Sigma-Aldrich, catalog number: S5881)
14. Glycerol
15. Potassium phosphate monobasic (Sigma-Aldrich, catalog number: P5655)
16. Potassium phosphate dibasic (Sigma-Aldrich, catalog number: 1551128)
17. L-arginine (Sigma-Aldrich, catalog number: A5006)
18. Kanamycin (Sigma-Aldrich, catalog number: K1377)
19. Cinnamaldehyde (Sigma-Aldrich, catalog number: W228613)
20. Marine Agar (MA) (Conda, catalog number: 1217)
21. Autoinducer Bioassay (AB) medium (see Recipes)

Equipment

1. EnSpire® Multimode Plate Reader (PerkinElmer, model: 2300-0000)
2. Tube rotator (Grant-bio, model: PTR35)
3. Spectrophotometer (Beckman Coulter, model: DU 530)

Software

1. GraphPad Prism (GraphPad Software, Inc.)
2. Microsoft Excel (Microsoft Corporation)

Procedure

1. Streak a MA plate with the cryopreserved *V. campbellii* strain of interest. Incubate for single colonies at 30 °C for 24-36 h. If required, supplement the plates with suitable antibiotics (*e.g.*, kanamycin, see flow chart in Figure 4 for more details).
   Note: We generally prepare two bacterial stocks: the first in commercial cryovials with beads for storage in our strain repository, and the second in 25-30% glycerol to be used as a working stock. Both are preserved at -80 °C. Even if we have not experienced any issue, we use this copy of the stock since repeated freezing and thawing can damage the cells, particularly if they are used routinely.
   For one streak we just use a 1-µl inoculating loop or any other sterile material. We start the assays from single colonies after 24-36 h incubation. This agar plate can be preserved at 4-5 °C

for at least 1 week without loss of reproducibility. Alternatively, cultures can be prepared directly from the stocks; in that case, we recommend using 10 µl for inoculating 3 ml of AB medium (see next step).

2. Prepare a culture of the *V. campbellii* strain of interest by inoculating 3 ml of AB medium in a 15-ml tube with a fresh colony. Incubate overnight in a tube rotator (40 rpm) at 30 °C to an OD_{600} of around 0.5 (1 mm path length).
3. Distribute 100 µl of a 2x dilution of the test compounds in AB medium into the wells of a white, clear-bottom 96-well plate. Serial dilutions, if required, can be prepared in the plate.
   Note: We usually prepare 80 mM stocks of the test products in DMSO. Using a cut-off dose of 200 µM yields a maximum solvent concentration of 0.25% v/v that has not significant effect on bacterial growth and luminescence.
   No-treatment controls and solvent controls are included in each plate, as well as 100 µM cinnamaldehyde (positive control) and water (negative control) (Figure 1).

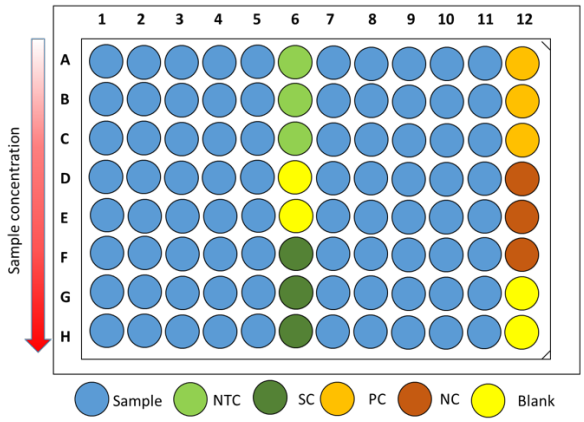


Figure 1. Plate distribution showing sample wells (x80), No-Treatment Control wells (NTC, x3), Solvent Control wells (SC, x3), Positive Control wells (PC, x3), Negative Control wells (NC, x3) and Blank wells (x4). Serial two-fold dilutions of the test compounds, if required, are proposed from rows A to H.

4. Dilute the overnight *V. campbellii* culture 1:50 in fresh AB medium. This gives an inoculum of approximately 1-2 x 10^7 CFU ml^{-1} that will be further diluted 1:2 (see next step).
   Note: When establishing this protocol in the laboratory, we recommend verifying the cell density of the inoculum as described by Harrison et al., 2010. Briefly, 180 µl of AB medium are distributed into four columns of a sterile 96-well plate, from row A to H. Twenty µl of inoculum are pipetted into the A-row wells and serially diluted (-1 to -8 dilutions). Using a multichannel pipette, 10 µl of the dilutions are spotted onto the surface of a MA plate. Up to four replicates of each dilution can be easily spotted onto a standard 90-100 mm agar plate. The spots are dried inside a biosafety cabinet and the plates incubated for 24 h at 30 °C for CFU count. For an accurate determination of the cell density, take into consideration the lowest concentration in

which individual colonies can be counted. The CFU per ml will be = $\frac{\sum_{i=1}^{4} n_i}{4}$ *x 10* $^{(d+2)}$, *where n represents the colony count in each of the 4 replicates, and d the corresponding dilution. The actual cell density in the assay will be half of this calculation.*

5. Distribute 100 µl of the diluted bacterial culture into the wells.
6. Seal the plate with a sterile, breathable and transparent sealing film (Figure 2).
   Note: Use a brayer for carefully sealing the plate. Do not remove the external protective cover until the plate is properly sealed in order to avoid contamination.

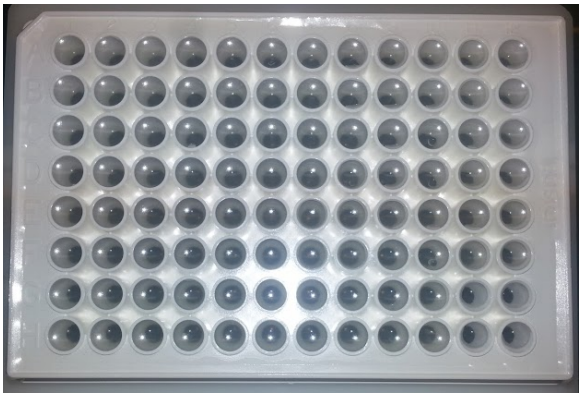


Figure 2. **Aspect of a sealed, white, clear-bottom 96-well plate**

7. Incubate the plate for 18 h at 30 °C and internal orbital shaking (150 rpm). Take OD_{600} and luminescence readings every 15 min.
   Note: Plate dimension, measurement height and Z' optimizations are advised when using EnSpire® Multimode Plate Reader. Similarly, flatfield and crosstalk corrections optimize the readings. We do not recommend using a distance between plate and detector lower than 0.6 mm (otherwise, contaminations might occur due to damage of the sealing film). Activate the condensation prevention option for sealed plates (upper heater temperature 2 °C warmer than lower heater temperature).

Data analysis

1. Subtract the background values for OD_{600} and luminescence (Microsoft Excel)
   Note: In luminescence readings, cross-talk differs slightly in function of the position of the wells. Thus, central wells are more susceptible to well-to-well light cross-talk. A tentative plate distribution that takes into account this effect is shown in Figure 1. Optimization of measurement parameters diminishes this phenomenon, although it takes place even with proper optimization. However, luminescence readings in blank wells are always at least two orders of magnitude lower than those recorded for sample wells and consequently, cross-talking exerts a negligible effect with our experimental settings.

2. Using a scientific graphing software (*e.g.,* GraphPad Prism), represent the time-course curves for bacterial growth and bioluminescence (Figure 3). Determine the area below the curves and calculate the relative inhibition of the treatments with respect to the controls for each variable.

   Note: It could be useful to normalize the luminescence data to the OD_{600} for each dose or treatment, i.e., $NL = \frac{AULC}{AUGC}$ where NL = Normalized Luminescence, AULC = Area Under Luminescence Curve and AUGC = Area Under Growth Curve.

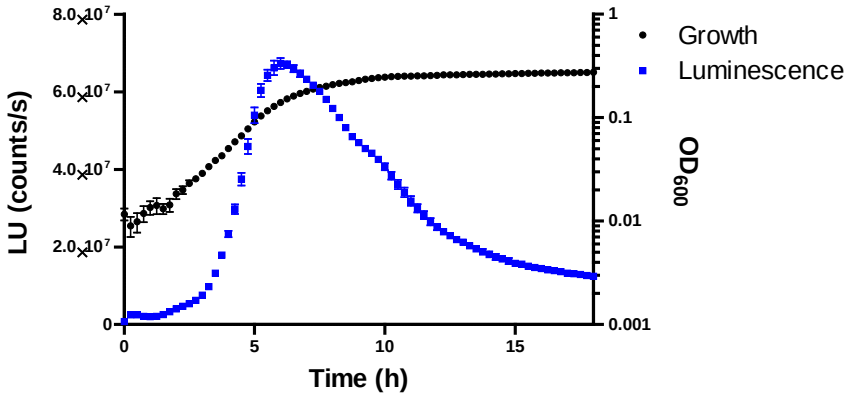
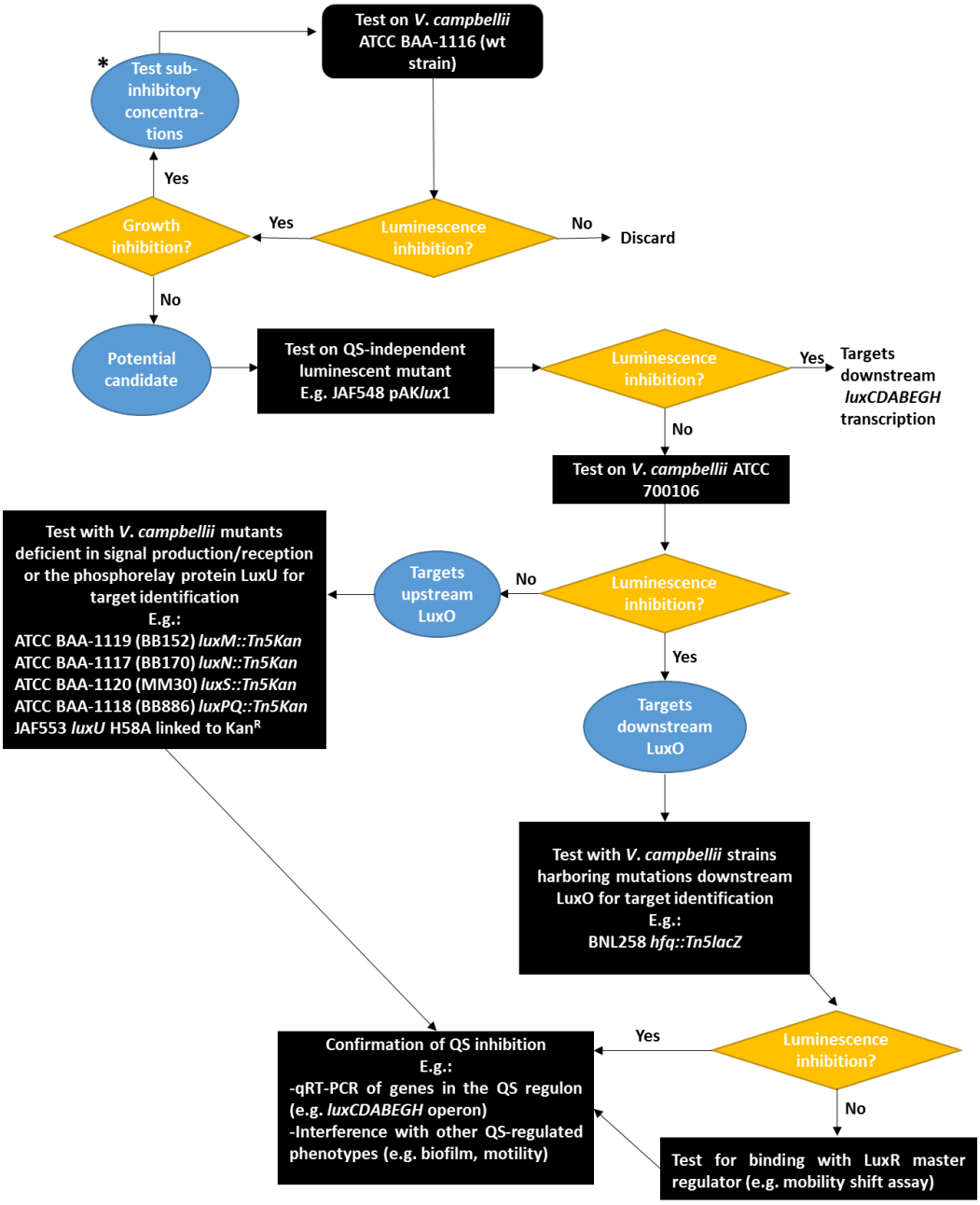


Figure 3. Chart displaying a typical NTC growth curve (black squares) and, overlapped, the corresponding luminescence curve (blue squares) over an assay period of 18 h. Data represent the mean and standard deviation of three replicates.

3. With these data, calculate the corresponding IC_{50} values using non-linear regression (GraphPad Prism).
4. Proposed workflow for the discovery of Quorum Sensing Inhibitors (Figure 4).



*We have observed no growth defects in *V. campbellii* QS mutants. Therefore, this checkpoint is only presented for the wt strain.

Figure 4. Flowchart for testing and characterizing the activity of putative Quorum Sensing Inhibitors using *Vibrio campbellii* QS mutants

Recipes

1. AB Medium
   17.5 g NaCl
   12.3 g $MgSO_4$

2.0 g casamino acids
970 ml DI water
Dissolve the ingredients and bring the pH of the solution to 7.5 with 3 N NaOH
Autoclave at 121 °C and after completely cooling down, add the following ingredients from separate filter-sterilized stocks:
10 ml 1 M potassium phosphate (pH 7.0)
10 ml 0.1 M L-arginine
10 ml glycerol

Acknowledgments

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References

1. González, J. E., Keshavan, N. D. (2006). Messing with bacterial Quorum Sensing. *Microbiol Mol Biol Rev* 70(4): 859-875.
2. Harrison, J. J., Stremick, C. A., Turner, R. J., Allan, N. D., Olson, M. E. and Ceri, H. (2010). Microtiter susceptibility testing of microbes growing on peg lids: a miniaturized biofilm model for high-throughput screening. *Nat Protoc* 5(7): 1236-1254.
3. Lin, B., Wang, Z., Malanoski, A. P., O'Grady, E. A., Wimpee, C. F., Vuddhakul, V., Alves Jr, N., Thompson, F. L., Gomez-Gil, B. and Vora, G. J. (2010). Comparative genomic analyses identify the *Vibrio harveyi* genome sequenced strains BAA-1116 and HY01 as *Vibrio campbellii*. *Environ Microbiol Rep* 2(1): 81-89.
4. Munn, C. (2011). Marine Microbiology. 2nd ed. *New York: Garland Science*: 105.
5. Martín-Rodríguez A. J., Babarro J. M. F., Lahoz F., Sansón M., Martín V. S., Norte M., Fernández J. J. (2015). From broad-spectrum biocides to Quorum Sensing disruptors and mussel repellents: antifouling profile of alkyl triphenylphosphonium salts. *PLoS One* 10(4): e0123652.
6. Martín-Rodríguez A. J., Ticona J. C., Jiménez I. A., Flores N., Fernández J. J., Bazzocchi I. (2015). Flavonoids from *Piper delineatum* modulate Quorum-Sensing regulated phenotypes in *Vibrio harveyi*. *Phytochemistry* 117: 98-106.
7. Steindler, L., Venturi, V. (2007). Detection of Quorum-Sensing *N*-acyl homoserine lactone signal molecules by bacterial biosensors. *FEMS Microbiol Lett* 266(1): 1-9.
8. Weiland-Bräuer N., Pinnow, N., Schmitz R. A. (2015). Novel reporter for identification of interference with acyl homoserine lactone and autoinducer-2 Quorum Sensing. *J Bacteriol* 81(4): 1477-1489.

