Chapter 20

Biosensors for Qualitative and Semiquantitative Analysis of Quorum Sensing Signal Molecules

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Abstract

Biosensors are biological tools that can be used to assay bacterial cultures for quorum sensing signal molecules (QSSMs) both qualitatively and semiquantitatively. QSSMs can be extracted from *Pseudomonas aeruginosa* cultures using organic solvents and tentatively identified via thin layer chromatography in combination with biosensor overlays. Alternatively, QSSMs can be quantified in spent culture supernatants or solvent extracts using biosensor-based spectrophotometric, luminescence, or fluorescence assays.

Key words *Pseudomonas aeruginosa*, Biosensor, Thin-layer chromatography (TLC), Quorum sensing, *N*-acylhomoserine lactones, *N*-butanoylhomoserine lactone, *N*-(3-oxo-dodecanoyl)homoserine lactone, *Pseudomonas* quinolone signal (PQS), 2-Heptyl-3-hydroxy-4(1*H*)-quinolone, 2-Heptyl-4-hydroxyquinoline (HHQ)

1 Introduction

Pseudomonas aeruginosa produces both *N*-acylhomoserine lactone (AHL) and 2-alkyl-4(1*H*)-quinolone (AQ) quorum sensing (QS) signal molecules (QSSMs) [1]. The primary AHLs are *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C_{12}-HSL) and *N*-butanoyl-homoserine lactone (C_4-HSL) produced via the *las* and *rhl* systems respectively (Fig. 1a, b). Among AQs, the major QSSMs are 2-heptyl-3-hydroxy-4(1*H*)-quinolone (PQS) and its immediate biosynthetic precursor, 2-heptyl-4-hydroxyquinoline (HHQ) (Fig. 1c, d). While the AHL and AQ-dependent QS systems collectively control the expression of a large number of both common and distinct sets of target genes, they are also interlinked. The *las* system positively regulates both the *rhl* and *pqs* systems while *rhl* negatively controls AQ-dependent signaling. For a review of QS in *P. aeruginosa*, see Williams and Cámara [1].

Given the importance of the AHL and AQ-dependent QS systems to the biology of *P. aeruginosa* and as both systems are potential targets for anti-infective agents that attenuate virulence [1],

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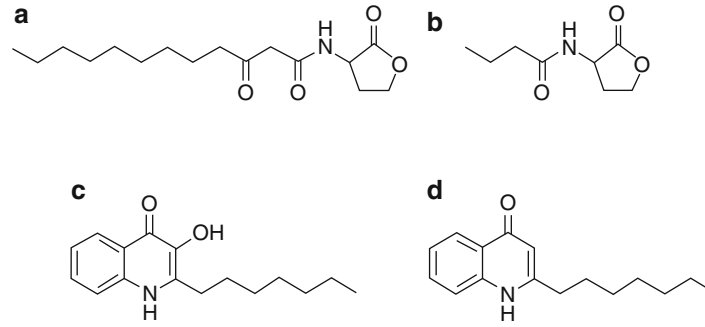

Fig. 1 Structures of the major QSSMs in *P. aeruginosa*. (**a**) *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C_{12}-HSL); (**b**) *N*-butanoyl homoserine lactone (C_4-HSL); (**c**) 2-heptyl-3-hydroxy-4(1*H*)-quinolone (the *Pseudomonas* quinolone signal; PQS); (**d**) 2-heptyl-4-hydroxyquinoline (HHQ)

a number of methods for AHL and AQ detection and quantification have been developed. These usually employ mass spectrometry (*see* Chapter 20; [2]) or biosensor-based analytical techniques [3–5]. Biosensors developed for both AHL and AQ detection have exploited the QSSM-dependent activation of a response regulator protein to drive the expression of a target promoter fused to a suitable reporter gene(s) [3–5]. Ideally such constructs should (a) be sufficiently sensitive to detect to submicromolar concentrations of a specific QSSM, (b) be highly selective and unambiguous, and (c) possess low background reporter output in the absence of QSSM. The advantages of such biosensors are that that they can be cheap to use, provide rapid results, and require little technical knowledge or expensive equipment. They can usefully provide qualitative data on the presence or absence of a specific QSSM and can also be used to give indirect quantitative data. The disadvantages of biosensors are that they do not always discriminate between closely related QSSMs although this can sometimes usefully be exploited in conjunction with thin layer chromatography (TLC) to profile the range of QSSMs present [3, 5]. In addition they frequently have higher detection thresholds than methods such as mass spectrometry and cannot be used to unequivocally identify a given QSSM as they may be activated by unrelated compounds [6] giving rise to false positive results.

AHL biosensors usually consist of a genetically modified organism engineered to contain a functional LuxR-type protein plus a cognate target promoter (e.g., a *luxI* synthase promoter) which positively drives the transcription of a reporter gene(s) such as *luxCDABE*, *lacZ*, or *gfp* [3, 7]. The outputs from such reporters include bioluminescence, β-galactosidase enzyme activity, fluorescence via green-fluorescent protein, and pigment production. A number of AHL biosensors are available and for an extensive review

of these, *see* Steindler and Venturi [7]. For example, for 3-oxo-C_{12}-HSL and C_4-HSL detection, Winson et al. [4] constructed the *Escherichia coli*-based bioreporter plasmids pSB1075 and pSB406 respectively in which LasR and RhlR drive the expression of *lasI′::luxCDABE* or *rhlI′::luxCDABE* in response to the cognate AHL. Similarly, biosensors for the AQs have also been constructed in which the functional *pqsR* (*mvfR*) gene product activates expression of the *pqsA* promoter coupled to *lux* or *lacZ* genes either in a *P. aeruginosa* AQ negative mutant or in *E. coli* [5, 8–10].

Since the same general biosensor methodology can be used for both AHL and AQ detection, their use will be illustrated in this chapter with reference to the AQs. The TLC and liquid assays outlined are essentially those originally described by Fletcher et al. [5, 9] and employ a *P. aeruginosa* biosensor strain rendered AQ negative through an in-frame, nonpolar, chromosomal deletion of *pqsA*, the first gene in the AQ biosynthetic operon and carrying a chromosomally integrated CTX::*pqsA′-luxCDABE* fusion. This biosensor strain produces light and pyocyanin in response to AQs that bind to PqsR and activate the *pqsA* promoter. These include PQS and HHQ as well as their C9 congeners, 2-nonyl-3-hydroxy-4(1*H*)-quinolone (C9-PQS) and 2-nonyl-4-hydroxyquinoline (NHQ) [5].

For qualitative analysis of both AHLs and AQs, cultures of interest are first extracted with an organic solvent such as ethyl acetate or dichloromethane. The QSSMs partition into the organic phase which can be removed and dried by evaporation to concentrate the extracted signal molecules. These can then be subjected to TLC using normal phase silica plates for AQs [5, 9], reversed-phase RP-18F254 plates for C_4-HSL [11], and RP-2F254 reversed-phase plates for 3-oxo-C_{12}-HSL [11] in conjunction with a mobile phase of dichloromethane-methanol (95:5 v/v) or methanol-water (60:40 v/v) or methanol-water (45:55 v/v) respectively. After chromatography the TLC plates are overlaid with a thin agar layer containing the relevant biosensor strain, incubated and examined for reporter output. Alternatively, for indirect semiquantitative analysis, culture supernatant extracts can be mixed with the biosensor strain and reporter output assayed using a spectrophotometer, luminometer, or fluorimeter depending on the nature of the readout. This works well for the AQs and 3-oxo-C_{12}-HSL although the latter may interfere with the quantification of C4-HSL using *E. coli*-based biosensors [12]. In addition it should also be noted that the AQ biosensor will only respond to AQs which function as QSSMs, i.e., those AQs which are capable of activating PqsR. Hence the assay will be an underestimate of the total concentration of all the AQs present since those such as the 2-alkyl-4-hydroxyquinoline-*N*-oxides which are produced at substantial levels are only weakly detected by the biosensor [2, 13].

2 Materials

2.1 Bacterial Cultures

1. PAO1. AQ-positive control.
2. PAO1Δ*pqsA*. AQ-negative control.
3. PAO1Δ*pqsH*. PQS-negative, HHQ-positive control.
4. PAO1Δ*pqsA* CTX::*pqsA′-luxCDABE*. AQ biosensor strain. Can be maintained in tetracycline 125 μg/ml.

All strains can be stored −80 °C in Luria-Bertani (LB) broth containing 25 % (v/v) glycerol, and revived by streaking onto LB agar containing the appropriate antibiotic and incubating at 37 °C overnight.

2.2 Growth Media

1. LB broth: tryptone (Bacto) 10 g/L, yeast extract (Oxoid) 5 g/L, sodium chloride 10 g/L in distilled water.
2. LB agar: as above but also containing technical agar No. 3 (Oxoid), 15 g/L.
3. Soft-top agar: agar technical no. 3 (Oxoid) 6.5 g/L, tryptone (Bacto) 10 g/L, sodium chloride 5 g/L in distilled water.

2.3 Reagents

1. Potassium dihydrogen phosphate solution: 5 % (w/v) KH_2PO_4 in distilled water.
2. Acidified ethyl acetate: 0.01 % (v/v) glacial acetic acid in ethyl acetate.
3. Normal phase 20 × 20 cm silica 60_{F254} TLC plates (Merck).
4. PQS and HHQ synthetic standards, 10 mM in methanol.
5. Dichloromethane-methanol mixture (95:5 v/v).

3 Methods

3.1 Growth of Bacterial Cultures for AQ Extraction

1. Streak out the test *P. aeruginosa* strain of interest and the control *P. aeruginosa* strains PAO1, PAO1Δ*pqsA*, and PAO1Δ*pqsH* onto fresh LB agar plates and incubate overnight at 37 °C.
2. Inoculate a single colony of each strain into 5 ml LB medium and grow overnight at 37 °C with shaking at 200 RPM.
3. Measure the optical density at 600 nm (OD_{600}) of each overnight culture and standardize the cultures to OD_{600} 1.0 by diluting with fresh LB (*see* **Note 1**).
4. Transfer 0.25 ml of the standardized cultures to 25 ml of LB medium in a 250 ml Erlenmeyer flask (*see* **Note 2**) and incubate at 37 °C with shaking at 200 RPM for 8 h (*see* **Note 3**).

3.2 Extraction of AQs from Bacterial Culture Supernatants

1. Transfer 10 ml of each culture to a 50 ml centrifuge tube and centrifuge at 10,000 × *g* for 10 min (*see* **Note 4**).

2. Filter the supernatants through sterile 0.2 μm filters (Minisart) into new centrifuge tubes to remove any unpelleted cells from the extraction mixtures. To use this filtered supernatant for quantitative analysis of total AQ production using a combined spectrophotometer/luminometer, proceed to steps in Subheading 3.5.
3. Add an equal volume of acidified ethyl acetate to the supernatants (10 ml) and vortex vigorously for 30 s to mix the two phases.
4. Allow the two phases to settle, and the tubes can be centrifuged at 10,000 × *g* for 10 min to hasten this process if desired. Transfer the top organic layer to a clean centrifuge tube. Repeat **steps 3** and **4** twice and pool the collected ethyl acetate organic phases for each strain.
5. Evaporate the extraction mixtures to dryness, for example, by transferring each sample to appropriate glassware and using a rotary evaporator or a centrifugal evaporator. The samples can also be allowed to air-dry in a fume hood if time is not an issue.
6. Add 0.5 ml of methanol to the evaporated samples, vortex for 30 s, and transfer to an appropriate receptacle, e.g., 2 ml glass sample vials or Eppendorf tubes. Repeat this step with two further additions of 0.5 ml methanol and pool the collected methanol for each strain.
7. Evaporate the extraction mixtures to dryness, e.g., under a stream of nitrogen gas or in a centrifugal evaporator. The samples can be stored at −20 °C until required before resuspension in methanol. To use these concentrated extracts for quantitative analysis of total AQ production using a combined spectrophotometer/luminometer, proceed to steps in Subheading 3.5.

3.3 Analysis of AQ Production via TLC

1. Preparation of TLC plates. Soak normal phase silica 20 × 20 cm 60_{F254} TLC plates (Merck) in a 5 % (w/v) solution of KH_2PO_4 for 30 min. Bake the plates at high temperature (70–100 °C) for 1 h, e.g., in a hybridization oven. The TLC plates can be stored for several weeks if kept clean and dry.
2. Draw a faint pencil line around 4 cm from the bottom of the activated silica TLC plates as a guide for spotting sample extracts. Spot 2 μl of each of the 10 mM standard solutions of synthetic PQS and HHQ in methanol onto the TLC plate. Extracted samples can be reconstituted in 50 μl of methanol and 5 μl of each can be spotted onto the TLC plate (*see* **Note 5**). Space each spot sufficiently far apart along the pencil line, beginning at least 2 cm from the edge of the TLC plate. Allow the spots to dry.
3. Place the spotted TLC plate into a developing tank and run using a mixture of dichloromethane-methanol (95:5 v/v) as the mobile phase. Remove the TLC plate when the solvent

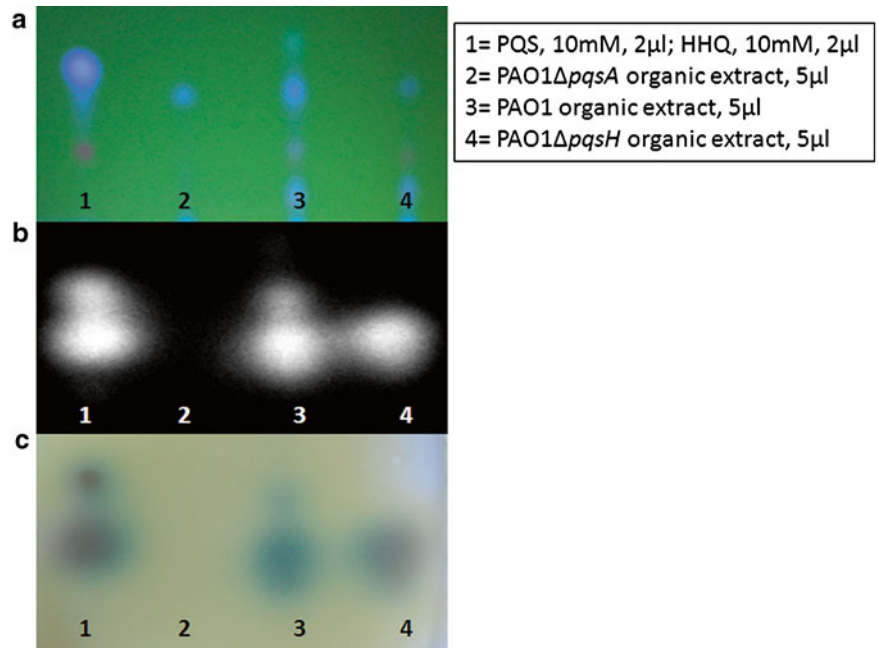

Fig. 2 TLC analysis of AQ production. (**a**) TLC plate showing PQS and HHQ standards together with organic solvent supernatant extracts of PAO1, PAO1Δ*pqsA* and PAO1Δ*pqsH*, visualized under UV light at 312 nm. (**b**) Overlay of the above TLC plate with AQ biosensor bacteria showing production of light in response to AQs, visualized using a luminograph photon camera; *upper spot*, PQS, *lower spot* HHQ. (**c**) Overlay of TLC plate with AQ biosensor bacteria showing production of the green pigment pyocyanin in response to AQs. *TLC lanes*: (1) PQS 10 mM, 2 µl and HHQ 10 mM, 2 µl, (2) PAO1Δ*pqsA* organic extract, 5 µl, (3) PAO1Δ*pqsH* organic extract, 5 µl, (4) PAO1 organic extract, 5 µl. The biosensor produces light and pyocyanin in response to PQS in the PAO1 wild type and to HHQ in the PAO1 wild type and the PAO1Δ*pqsH* mutant. The PAO1Δ*pqsA* mutant does not produce AQs so the biosensor is not activated

front approaches the top of the plate. The TLC plate can be photographed at this point, using a UV transilluminator at 312 nm to visualize the AQs (Fig. 2a).

4. Allow the TLC plate to dry and prepare for overlay of the biosensor by sticking autoclave tape to the underside edges of the TLC plate and then folding upward toward the silica surface so that the tape creates a well around the TLC plate. Place the TLC plate into an appropriate receptacle. This procedure is shown in Fig. 3 (*see* **Note 6**).

3.4 Overlay of TLC Plates with the Biosensor Strain

1. Streak out a 10 µl loop of the AQ biosensor PAO1Δ*pqsA* CTX::*pqsA′-luxCDABE* onto fresh LB agar plates containing 125 µg/ml tetracycline and grow overnight at 37 °C.
2. Inoculate a single colony of biosensor into 5 ml LB medium containing 125 µg/ml tetracycline and grow overnight at 37 °C with shaking at 200 RPM.
3. Melt 100 ml of soft top agar in a microwave and allow to cool to around 50 °C. Add 1 ml of the overnight culture of biosensor

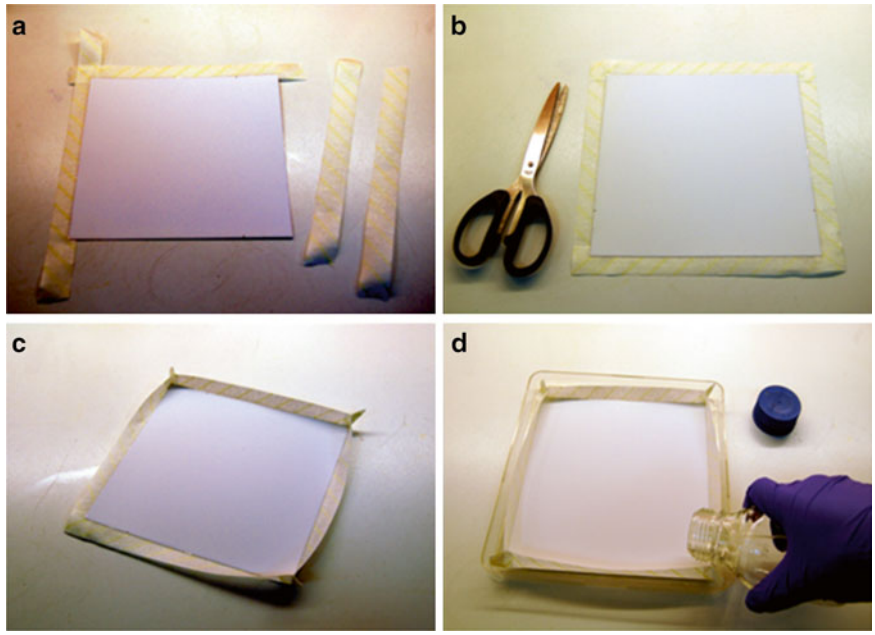

Fig. 3 Preparation of the TLC plate for biosensor overlay. (**a**) Attach autoclave tape to the underside edges of the TLC plate. (**b**) Trim the excess autoclave tape. (**c**) Pinch the autoclave tape at each corner of the TLC plate and then fold the tape upward toward the silica surface so that the tape creates a well around the TLC plate (**d**) Place the TLC plate into an appropriate container and slowly pour the molten soft top agar containing the biosensor bacteria onto the TLC plate

to the soft top agar and mix gently to avoid bubbles in the agar (*see* **Note 7**).

4. Pour the mixture slowly onto the TLC plate to minimize bubble formation. Make sure the agar is evenly spread (*see* **Note 8**).
5. Allow the agar to cool and set aseptically around a Bunsen flame before static incubation at 37 °C for 6–8 h.
6. Visualize the plates for light production using a luminograph photon camera (Fig. 2b) or overlay with X-ray film (e.g., Pierce CL-XPosure film). Alternatively, simply view production of the blue/green phenazine pigment pyocyanin by eye (Fig. 2c).

3.5 Analysis of AQ Production Using a Combined Spectrophotometer/ Luminometer Assay

1. Grow the AQ biosensor overnight as described in Subheading 3.4, measure the OD_{600}, and adjust with fresh LB medium to OD_{600} 1.0.
2. Further dilute this standardized biosensor culture with LB medium to give both 1 in 50 and 1 in 100 dilutions.
3. Sterilize a black, transparent-bottomed, 96-well plate, e.g., under strong UV light for 15 min.
4. For each test well, mix 100 μl of the test bacterial supernatant with 100 μl of the 1 in 50 dilution of the biosensor to give a final

culture dilution of 1 in 100 in the well. For a negative control, add 200 µl of the 1 in 100 dilution of the AQ biosensor alone. A positive control of a 1 in 100 dilution of the biosensor plus PQS or HHQ synthetic standard at a concentration of 5 µM can be added to wells, or alternatively 100 µl of *P. aeruginosa* culture supernatant plus 100 µl of the 1 in 50 dilution of the biosensor can also be added to the assay (*see* **Note 9**). The organic solvent culture extracts described earlier in Subheading 3.2 may also be analyzed by this method. Reconstitute the samples in 50 µl of methanol and dilute 5 µl in 100 µl of LB and add to 100 µl of 1 in 50 dilution of the AQ biosensor per well.

5. Monitor bioluminescence and OD_{600} at 37 °C using a combined spectrophotometer/luminometer, for example, the Infinite F200 controlled by the i-Control 1.9 Software (TECAN). The program measures OD and bioluminescence from all wells every 30 min for 24 h. Luminescence is recorded as relative light units (RLU) per unit of OD (Fig. 4). If an automated combined spectrophotometer/luminometer is unavailable, readings can be taken manually at defined time points by growing the bacterial cultures under specific conditions and measuring the OD and bioluminescence of culture samples using a spectrophotometer and tube luminometer (e.g., EG & G Junior), respectively.

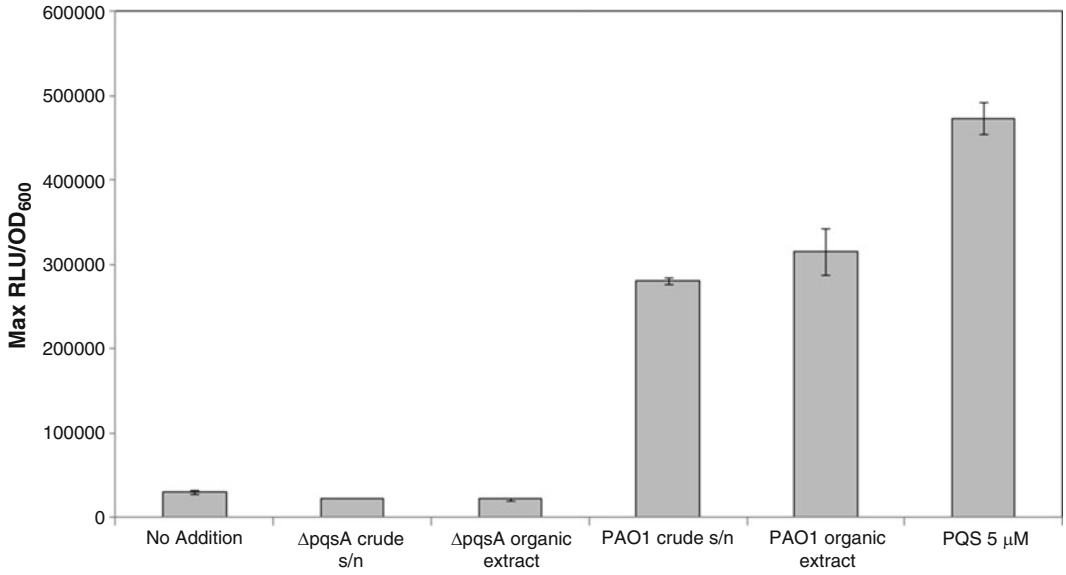

Fig. 4 Quantification of AQ production. Luminescence produced by the AQ biosensor, recorded as relative light units (RLU) per unit of OD by the Infinite F200 (TECAN), in response to the addition of crude supernatants and concentrated organic solvent extracts of PAO1 and PAO1Δ*pqsA*. In the absence of AQs, in the presence of crude supernatant or an organic solvent extract from the PAO1Δ*pqsA* mutant, the output of light is baseline. The AQ biosensor produces light in response to AQs present in the PAO1 wild-type supernatant and organic solvent extracts or to the PQS standard (5 µM)

4 Notes

1. This allows cultures to be compared with one another for AQ production by standardizing the initial inoculum. If direct comparison is not needed, this step can be omitted.
2. Growing the cultures in a small amount of medium in a large flask promotes good aeration, growth, and QSSM production.
3. The actual amount of time needed for growth depends on the growth characteristics of the strain but should be enough to provide the bacteria the opportunity to reach a high enough OD_{600} and produce AQs in sufficient quantity. A 1 in 100 dilution of OD_{600} 1.0 culture is inoculated here, but this can also be altered if necessary. For PAO1, a 10 ml extraction of culture after 8 h, grown to an approximate OD_{600} of 1.0–1.5, should produce enough AQs to be detected by the bioreporter. However, for other strains the exact amount of growth to gather enough AQs to be detected can vary and may have to be determined empirically.
4. The amount of culture to be extracted will depend on the purpose of the experiment and the AQ-producing characteristics of the strain. For those strains that do not produce a high quantity of AQs, more culture may have to be extracted. Accordingly, the specifics of the above method may have to be adjusted depending on the quantity of culture supernatant to be extracted.
5. The quantity of extract to be spotted can be altered as desired, depending on the quantity of AQs in the sample.
6. Make sure the autoclave tape is of decent quality and is pressed down firmly to form a tight seal around the bottom of the plate; otherwise leakage of agar may occur when the plate is overlaid with the biosensor.
7. Make sure that the agar has cooled sufficiently before adding the biosensor bacteria as too high a temperature will kill or harm the biosensor and attenuate growth. A temperature of 50 °C allows easy pouring of agar and gives sufficient time before the agar sets.
8. Do not delay too long or the agar will begin to solidify before pouring. If bubbles form, these can either be agitated to the edge of the plate before the agar sets or a Bunsen burner can be used to quickly and carefully flame the surface of the agar to remove the bubbles.
9. Test and control wells are usually performed in triplicate.

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