Published in final edited form as:

Methods Mol Biol. 2011 ; 692: 207–217. doi:10.1007/978-1-60761-971-0_15.

Quantifying *Pseudomonas aeruginosa* Quinolones and Examining Their Interactions with Lipids

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Abstract

Pseudomonas aeruginosa produces a quorum sensing molecule termed the *Pseudomonas* Quinolone Signal (2-heptyl-3-hydroxy-4-quinolone; PQS) that regulates an array of genes involved in virulence. This chapter addresses four related techniques useful for detecting and quantifying PQS. First, extraction of PQS from complex mixtures (e.g. cell cultures) is described. Separation of PQS from extracts by Thin-Layer Chromatography (TLC) is used in combination with the natural fluorescence of the molecule for quantification. A second separation technique for the PQS precursor HHQ using High-Performance Liquid Chromatography (HPLC) is also described, and this assay exploits the molecule's characteristic absorbance for quantification. A third method for quantification of PQS from simple mixtures (e.g. enzyme assays) using fluorescence is outlined. Finally, a protocol for determining PQS interactions with membrane lipids through Fluorescence Resonance Energy Transfer (FRET) is presented. These techniques allow for quantification and characterization of PQS from diverse environments, a prerequisite to understanding the biological functions of QS molecules.

Keywords

Pseudomonas quinolone signal; Thin-layer chromatography; High-pressure liquid chromatography; Fluorescence resonance energy transfer

1. Introduction

The Gram-negative opportunistic pathogen *Pseudomonas aeruginosa* synthesizes several quorum sensing (QS) molecules, including two acylhomoserine lactones (3-oxo-dodecanoyl and butyryl homoserine lactone; AHLs), which are synthesized and sensed by the *las* and *rhl* QS systems, respectively (1). A third QS molecule is a hydrophobic quinolone (2-heptyl-3-hydroxy-4-quinolone) termed the *Pseudomonas* Quinolone Signal (PQS) (2). PQS, bound to its transcriptional regulator PqsR (MvfR), regulates an array of genes involved in the production of important virulence factors like hydrogen cyanide and pyocyanin (3). While the AHL autoinducers are soluble and can readily diffuse within the extracellular milieu, the hydrophobic PQS molecule is packaged into outer membrane vesicles (MV), and PQS is required for MV formation in *P. aeruginosa* (4). PQS has also been shown to stimulate MV formation in other Gram-negative bacteria (5). Additionally, other Gram-negative bacteria such as *Rhodobacter capsulatus* (6) and *Sinorhizobium meliloti* (7) produce hydrophobic quorum sensing signals that may also interact with outer membrane lipids.

PQS biosynthesis is affected by genetic and environmental factors. The *las* QS system with its aforementioned autoinducer 3-oxo-dodecanoyl homoserine lactone regulates transcription of *pqsH*, which encodes the terminal enzyme in the PQS biosynthetic pathway. PqsH

catalyzes the addition of the 3-hydroxyl group to the immediate PQS precursor molecule 2-heptyl-4-quinolone (HHQ) (8, 9). In addition, nutritional cues also affect PQS production. The amino acids phenylalanine and tyrosine significantly enhance PQS production (10), and it is hypothesized that this is due to flux through a precursor pathway shared by PQS production and aromatic amino acid biosynthesis (10).

The study of PQS as a QS molecule demands accurate determination of the concentration of signal present in both complex and simple mixtures. In this chapter, two methods for separating PQS and HHQ from complex mixtures, Thin-Layer Chromatography (TLC) and High-Performance Liquid Chromatography (HPLC), are described and used in conjunction with the natural fluorescence/absorbance of PQS and HHQ to determine their concentrations. We also describe a fluorescence assay for quantifying PQS in simple mixtures and the use of Fluorescence Resonance Energy Transfer (FRET) to determine interactions between PQS (or other hydrophobic signaling molecules) and membrane lipids. Through the use of these techniques, PQS and HHQ can be quantified from diverse environments including biological samples and whole bacterial cultures, and insights into interactions between quinolones and membrane lipids can be gained.

2. Materials

2.1. TLC for Separation and Quantification of PQS

1. Acidified ethyl acetate ACS grade (acidified with 0.15 mL/L glacial acetic acid).
2. Separation funnel.
3. Rotary evaporator.
4. Nitrogen gas and dry down apparatus.
5. Glass extraction vials (15 mL and 5 mL).
6. Monobasic potassium phosphate (KH_2PO_4) ACS grade.
7. 20 × 20 cm Silica 60 F₂₅₄ pre-coated plates for thin-layer chromatography.
8. Drying oven 100°C.
9. Methanol ACS grade.
10. Dichloromethane ACS grade.
11. Glass TLC chamber.
12. Fluorescence imager and spot densitometry analysis software (e.g. Syngene G:Box with GeneTools software).
13. 2 mM PQS standard (MW 259) in methanol.

2.2. HPLC for Separation and Quantification of HHQ

1. HPLC with Photo Diode Array (PDA) detector (e.g. Varian ProStar).
2. Reverse phase C8 column 140 × 4.5 mm 1 mL/min flow rate (Varian).
3. Acidified methanol HPLC grade (acidified with 1% glacial acetic acid by volume).
4. Acidified water HPLC grade (acidified with 1% glacial acetic acid by volume).
5. 2 mM HHQ standard (MW 243) in acidified methanol.

2.3. PQS Quantification from Simple Mixtures

1. Glass tubes or bottles (depending on sample volume).
2. Ethyl acetate (ACS grade acidified with 0.1 mL/L glacial acetic acid).
3. Nitrogen gas and dry down apparatus.
4. High-grade methanol minimal fluorescent impurities.
5. Fluorometer.

2.4. FRET to Detect PQS Interactions with Membrane Lipids

2.4.1. Preparation of Soluble Lipopolysaccharide and Phospholipids

1. Purified lipopolysaccharide (LPS).
2. Phosphatidylethanolamine (PE) from *E. coli* (Sigma) and phosphatidyl glycerol (PG) from phosphatidylcholine (Sigma).
3. Chloroform HPLC grade.
4. Methanol HPLC grade.
5. Ultrasonic Bath.

2.4.2. Labeling of LPS and Phospholipids with Donor and Acceptor Dyes

1. *Donor conjugated dye*: 4-Nitro-benz-2-oxa-phosphatidylethanolamine (NBD-PE), in chloroform (Molecular Probes).
2. *Acceptor conjugated dye*: Rhodamine-phosphatidylethanolamine (Rh-PE), in chloroform (Molecular Probes).
3. Chloroform.
4. Nitrogen gas.
5. 20 mM HEPES, pH 7.0.
6. Tip Sonicator.

2.4.3. FRET Measurements

1. Quinolones, in methanol.
2. Labeled LPS or phospholipids, 1 mM in HEPES buffer (see Subheading 3 for labeling protocol).
3. Fluorometer, heated at a constant 37°C with stirring.

3. Methods

3.1. TLC for Separation and Quantification of PQS

3.1.1. PQS Extraction

1. Using a sterile inoculation loop, streak relevant bacterial strains onto LB agar plates. Wild-type PA14 *P. aeruginosa* as well as *pqsA*[−] (no quinolone control) and *pqsH*[−] (no PQS control) mutants are recommended. Incubate at 37°C overnight.
2. The next day, inoculate 10 mL of LB or other desired medium from the plate and incubate at 37°C overnight.

3. The next day, determine the OD₆₀₀ of overnight cultures and inoculate 10 mL of LB or other desired medium to OD₆₀₀ = 0.05. Incubate at 37°C shaking (250 rpm) until the culture has reached OD₆₀₀ = 0.5 (mid-exponential phase).
4. Once the culture has reached mid-exponential phase pellet cells by spinning cultures at 7000 × g for 5 min. Re-suspend cells in 1 mL of sterile medium (see Note 1). Determine the OD₆₀₀ of the concentrated culture, and inoculate 55 mL of LB or other desired medium to OD₆₀₀ = 0.01. Incubate newly inoculated medium at 37°C shaking (250 rpm) until the culture desired density for extraction of PQS. In this example, a mid-exponential phase culture will be examined (OD₆₀₀ = 0.5).
5. Remove 50 mL of culture to a 500 mL flask washed with acidified ethyl acetate. Add 50 mL acidified ethyl acetate, vortex mixture for 30 s, and place mixture in a separation flask for 5–10 min or until aqueous and organic phases have completely separated.
6. Collect the clear organic phase top layer in a clean glass container.
7. Repeat steps 5 and 6 twice to fully extract PQS from 50 mL culture.
8. Use rotary evaporator to completely dry down ethyl acetate extract. Add 10 mL to rotary evaporator flask and twist to ensure that the entire flask contacts the ethyl acetate. Completely dry down the remaining ethyl acetate under a constant stream of nitrogen gas.
9. Extracts may be re-suspended in methanol for spotting on TLC plates (see next section) or stored in the dark until needed.

3.1.2. PQS Quantification with TLC

1. Prepare standards by diluting 2 mM PQS stock in methanol. As an example, the low range of standard visible on the plate is 100 ng; thus, standards in which 100, 200, 300, 400, and 500 ng/10 µL are spotted on the plate are an appropriate range.
2. Pre-heat the drying oven to 100°C.
3. Prepare TLC plate by incubating in 5% KH₂PO₄ solution at room temperature for 30 min. Transfer the plate to the preheated drying oven for a minimum of 1 h (see Note 2).
4. Concentrate PQS samples by completely drying down extracts and re-suspending them in 50 µL methanol. The extent to which samples should be concentrated depends on the range of PQS standards and the optical density of the culture extracted.
5. Prepare TLC chamber with 95:5 mixture of dichloromethane: methanol. Allow 10 min for solvent system to equilibrate within chamber.
6. Remove the plate from the oven and lightly draw a line in pencil 1–2 cm from the bottom of the plate. Sample and standard PQS will be spotted on this line; tick marks denoting the intended location of a spot may also be added (see Note 3).
7. Pre-run the marked plate by placing the plate in TLC chamber with equilibrated solvent. The solvent should not be above the line drawn in the previous step. Allow

¹If the effect of a specific medium on PQS production is of interest, washing the cell pellet twice and starving cells for 2 h in sterile buffer (e.g. phosphate buffered saline, PBS) is recommended before inoculation of 55 mL culture.

²TLC plates may be rocked during KH₂PO₄ incubation.

³TLC plates may be stored in a drying oven for longer than 1 h to prevent moisture from entering the plate before TLC run. The plates should be completely dry (directly out of oven) when spotting samples.

the solvent to migrate until the entire plate is covered. Allow the excess solvent to evaporate from the plate; pre-run plates should be returned to the drying oven until ready to be spotted.

8. Spot 10 μL of samples leaving at least 1 cm between the samples. Spot standards on either side of the samples. Spotting standards on either side of the samples (and between samples if space is available) allows for the standardization of any disproportionate illumination that may affect quantification.
9. Place the plate in TLC chamber and allow the solvent to migrate until the entire plate is covered. Be sure that the spotting solvents have completely evaporated before placing the plate in the TLC chamber (see Note 4).
10. Remove the plate from TLC chamber and image PQS spots using fluorescence imager. Excite the plate with UV light and observe fluorescent spots (see Note 5). Sample PQS should co-migrate with standard on the TLC plate (Fig. 1).
11. Perform spot densitometry analysis on imaged TLC. Plot fluorescence units against the amount of PQS standard to generate a standard curve. The standard curve should be linear, and PQS concentrations in samples can be calculated using the standard curve slope equation. Samples outside the standard curve must be diluted and re-run to be within the range of the standard.

3.2. HPLC for Separation and Quantification of HHQ

1. Re-suspend HHQ sample to be analyzed in 200 μL acidified methanol and remove to HPLC vials. HHQ samples may be obtained from the PQS extraction protocol detailed in the previous Subheading 3.1.
2. Prepare HHQ standards by diluting 2 mM stocks to 50, 100, 200, 300, 400, and 500 μM in acidified methanol. The final volume of each stock should be 200 μL , and the stocks should be removed to HPLC vials.
3. The mobile phase for the HPLC is acidified methanol and acidified water with the following program: 60% acidified methanol for 10 min, ramp up to 100% in 5 min, hold at 100% for 5 min, drop to 60% in 1 min, and hold at 60% for 3 min.
4. Analyze 50 μL of standards and samples sequentially over HPLC using the solvent program above.
5. Extract the chromatogram at 314 nm to view HHQ peak. HHQ elutes at 6 min (Fig. 2). Both HHQ and PQS may be viewed by extracting the chromatogram at 325 nm (see Note 6).
6. Plot peak absorbance values against the concentration of HHQ standard to generate a standard curve. The standard curve should be linear, and HHQ concentrations in samples can be calculated using the standard curve slope equation. Samples outside the standard curve must be diluted and re-run to be within the range of the standard.

3.3. PQS Quantification from Simple Mixtures

1. This method is suitable for detection and quantification of PQS in simple mixtures of known composition (i.e. enzymatic assays, defined media, etc.). It is important

⁴Sealing the TCL chamber with petroleum jelly can improve TLC quality.

⁵When visualizing the TLC in the fluorescence imager, exciting the plate from above with UV light tends to result in a brighter image. Exciting from above and below is ideal.

⁶PQS may also be separated and quantified by this protocol. To obtain a clear PQS peak it may be helpful to chelate iron from PQS samples before HPLC analysis.

that no ethyl acetate-extractible contaminant fluoresces at the wavelengths described.

2. The simple mixture containing PQS is extracted using three times the volume of acidified ethyl acetate. For example, add 3 mL acidified ethyl acetate to 1 mL of the mixture.
3. Agitate the extraction mixture. Several short bursts using a vortexer are recommended for small volumes; manual mixing may be required for larger volumes.
4. If the separation vessel can be accommodated by a centrifuge, a short low-speed spin (2 min at $2000 \times g$) will result in a sharp phase separation.
5. Recover as much as possible of the upper organic phase (the ethyl acetate) into a new glass vessel by pipetting. Record the recovered volume as this value will be important in back-calculating PQS concentration in the original mixture.
6. Place the recovered ethyl acetate sample under a stream of nitrogen gas until the liquid has completely evaporated.
7. Once all the samples have been evaporated, re-suspend each in a known volume of high-grade methanol. Once again record this volume for back-calculation.
8. Transfer the methanol samples into a black 96-well plate (or other appropriate sample holder required for use in the fluorometer) and place in the fluorometer. Excite at 342 nm and read fluorescence at 450 nm (Fig. 3). Plot fluorescence values against the concentration of PQS standards to generate a standard curve. The standard curve should be linear, and PQS concentrations in samples can be calculated using the standard curve slope equation. Samples outside the standard curve must be diluted and re-run to be within the range of the standard (see Note 7).

3.4. Preparation of Soluble LPS and Phospholipids

1. Weigh out LPS so that the final concentration is 1 mM in 1 mL.
2. Add 100 μ L of chloroform to the LPS and incubate at 90°C for 5 min.
3. Add 10 μ L of methanol and incubate at 90°C for 5 min.
4. Sonicate the LPS/chloroform/methanol mixture for 20 min in the ultrasonic bath.
5. Add 10 μ L methanol and incubate at 90°C for 5 min.
6. Repeat step 5 three times for a total volume of 150 μ L (50 μ L total methanol).
7. Sonicate the LPS/chloroform/methanol mixture for 15 min in the ultrasonic bath. After sonication the LPS should be in solution and appear clear. At this point the LPS is ready for labeling.
8. For the phospholipids PE and PG, separately weigh out for a final concentration of 1 mM in 1 mL final volume.
9. Re-suspend PE and PG separately in 150 μ L chloroform.
10. Sonicate the phospholipid/chloroform mixture in the ultrasonic bath for 20 min. The mixture should be clear and is now ready for labeling.

⁷The presence of HHQ in a simple mixture is not an issue in this protocol as fluorescence intensity from HHQ is 1000-fold less than PQS (Fig. 3b).

3.5. Labeling of LPS Aggregates and Phospholipid Liposomes

1. To the LPS/chloroform/methanol mixture add NBD-PE and Rh-PE (both in chloroform) to a final molar ratio of 100:1:1 LPS:NBD-PE:Rh-PE. Use the same molar ratio when labeling PE and PG.
2. Evaporate the solvent with a continuous stream of N₂ gas, until no liquid is present.
3. Add 1 mL of 20 mM HEPES, pH 7.0, to the labeled LPS or phospholipids.
4. Sonicate with the tip sonicator for around 5 min. Make sure that the LPS/phospholipids do not get too warm during sonication.
5. For preparing labeled LPS aggregates and phospholipid liposomes, LPS and phospholipids are temperature cycled for a total of four cycles. After sonication incubate the aqueous mixture at 4°C for 30 min and then transfer to a heat block set at 55°C for 30 min. Repeat the temperature cycling three more times.
6. Store at 4°C overnight. Labeled samples are then ready for FRET measurements and last about 2 weeks at 4°C.

3.6. FRET Measurements

1. Allow the fluorometer sample chamber to equilibrate to a constant 37°C with stirring.
2. Set the excitation wavelength to 470 nm and the emission wavelengths to 531 nm and 593 nm.
3. Dilute the labeled LPS aggregates or the phospholipid liposomes in 20 mM HEPES buffer, pH 7.0, so that the final concentration is 10 µM.
4. Add the labeled sample to the cuvette and let equilibrate at 37°C with stirring. The volume will vary depending on the size of your cuvette.
5. Adjust the emission intensities so that the ratio of the donor intensity and the acceptor intensities are 1. Record these intensities for 50 s to establish a baseline.
6. After 50 s add the appropriate amount of quinolone (or methanol as a control) at a final molar ratio of 1:10 LPS:PQS and record the intensities for an additional 250 s for a total of 300 s. The molar ratio of LPS:PQS may need to be adjusted. If intercalation has occurred, the intensity of the donor should increase, whereas the intensity of the acceptor should decrease.

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Fig. 1. Thin-Layer Chromatography separating PQS extracted from whole *P. aeruginosa* cultures (samples). PQS standards (100–500 ng) are shown on either side of the samples.

Fig. 2.
Representative HPLC chromatogram. The relevant portion of an HPLC chromatogram showing HHQ elutes at approximately 6 min.

Fig. 3. PQS absorption and emission spectra. The maximum absorbance for PQS (**a**) occurs at approximately 340 nm. The maximum emission of PQS occurs at approximately 450 nm. A *dotted line* indicates HHQ emission, which is 1000-fold less than PQS.