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TITLE:

Isolation of Total RNA from *Pseudomonas aeruginosa* Within Biofilms for Measuring Gene Expression

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SUMMARY:

This protocol presents a method to isolate RNA from *Pseudomonas aeruginosa* biofilms grown in chamber slides for high throughput sequencing.

ABSTRACT:

Pseudomonas aeruginosa is an opportunistic bacterial pathogen that causes infections in the airways of cystic fibrosis (CF) patients. *P. aeruginosa* is known for its ability to form biofilms that are protected by a matrix of exopolysaccharides. This matrix allows the microorganisms to be more resilient to external factors, including antibiotic treatment. One of the most common methods of biofilm growth for research is in microtiter plates or chambered slides. The advantage of these systems is that they allow for the testing of multiple growth conditions, but their disadvantage is that they produce limited amounts of biofilm for RNA extraction. The purpose of this article is to provide a detailed, step by step protocol on how to extract total RNA from small amounts of biofilm of sufficient quality and quantity for high throughput sequencing. This protocol allows for the study of gene expression within these biofilm systems.

INTRODUCTION:

Most chronic bacterial infections, such as pulmonary infections in cystic fibrosis (CF) patients and prosthesis related infections, are characterized by the growth of organisms within biofilms. Biofilms[1] are communities of bacteria encased in a matrix composed primarily of polysaccharides[2]. Bacteria within biofilms can be slow growing, metabolically dormant, and in anaerobic, hypoxic conditions. Biofilms are more resistant to antibiotics due to factors such as decreased antibiotic penetration, increased expression of drug efflux pumps, and decreased cell division[3]. For these and other reasons, they are of great research interest.

In order to accurately study persistent infections such as chronic *Pseudomonas aeruginosa* infections in CF patients, the growth conditions seen with biofilm formation need to be accurately reflected *in vitro*. A common, high throughput method is to grow them in chamber slides or microtiter plates and monitor biofilm formation by confocal microscopy[4]. It is known that a key regulator in the transition from a planktonic, or free-floating, to biofilm bacterial lifestyle is the secondary messenger, cyclic-di-GMP[5]. Increased cyclic-di-GMP levels increase the expression of specific genes that promote biofilm growth. Small non-coding regulatory RNAs and quorum sensing also play important roles in regulation of biofilm formation[5]. Measuring biofilm gene expression by sequencing extracted bacterial RNA can be challenging. *P. aeruginosa*, for example, produces three exopolysaccharides (Psl, Pel and alginate), which are produced in significant amounts in biofilms[5,6]. These polysaccharides can interfere with RNA extraction and purification leading to impure preparations containing low levels of bacterial mRNA[7]. Commercially available RNA extraction kits are able to produce high quality RNA from planktonic bacterial cultures but may not work as well with biofilm cultures[8-10]. There are a few commercial RNA extraction kits that claim to work for biofilms, one of which we use with this method.

In this manuscript, we describe the procedures for growing *P. aeruginosa* biofilms in chamber slides and extracting bacterial mRNA for high throughput sequencing[11,12]. Utilizing clinical isolates collected from sputum samples from CF patients, we demonstrate that these methods can be used for isolates with varying growth characteristics. In comparison to prior publications, this protocol is described in detail to enable better success in studying bacterial biofilm gene expression[10,13-16].

PROTOCOL:

The Research Ethics Board (REB) is required for the collection and processing of sputum samples from human subjects. This study was approved by the Hospital for Sick Children (REB#1000019444). Research Ethics Board (REB) is required to collect and store sputum samples from human subjects. These studies were approved by the Hospital for Sick Children REB#1000058579.

1. Biofilm formation

1.1. Grow *Pseudomonas aeruginosa* isolates obtained from the sputum samples of CF patients used in this study on Luria Broth (LB) agar plates in a 37 °C incubator overnight.

NOTE: Proper streaking technique is important for obtaining single bacterial colonies. Streaking while rotating the plate will dilute the bacteria cells sufficiently so that single colonies can grow.

1.1.1. Streak bacteria using an inoculation loop in a zig-zag pattern at the top end of a fresh LB plate until about ¼ of the plate is covered.

1.1.2. Rotate the plate about 60°. Take a new inoculation loop and pass it once through the streaked area and into a second, clean area of the plate, repeating the zig-zag pattern.

1.1.3. Repeat step 1.1.2 with a fresh loop into a third area of the plate. Replace the lid and invert the plate when placing in the incubator.

1.2. Use a sterile inoculating loop to pick a single bacterial colony from an agar plate (containing a single bacterial strain) and inoculate a culture tube filled with 5 mL of sterile LB media. Using a new loop each time, inoculate two additional culture tubes filled with 5 mL of LB media from the same plate.

1.3. Repeat the same inoculation procedure for the different bacterial strains. Grow the cultures overnight at 37 °C with shaking at 220 RPM.

NOTE: Each agar plate containing a single bacterial strain is used to inoculate 3 independent culture tubes. The three tubes represent biological replicates in triplicate for one strain and are treated as separate samples. This is different from technical replicates which would entail extracting RNA 3 times from a single culture tube.

1.3. Prepare 1:100 dilutions of the overnight cultures by transferring 50 µL of an overnight culture into a new culture tube containing 4.95 mL of new LB media.

1.4. Grow the diluted cultures for another 3 h at 37 °C with shaking at 220 RPM or until the OD_{600} is 0.1 or higher. Measure the cell densities on a spectrophotometer at OD_{600}.

1.5. In a new 1.7 mL microfuge tube, adjust theOD_{600} to 0.1 (early log phase) in a total volume of 1.5 mL with fresh LB.

1.6. Mix gently by inversion. Transfer 300 µL of each adjusted culture into 4 wells of an 8-well chamber slide to end up with 2 different samples per slide (**Figure 1**).

1.7. Place the slides, undisturbed, in a 37 °C incubator overnight for 24 h. To prevent evaporation, place the slides on the top of a damp paper towel in a small plastic tray.

2. Biofilm recovery

NOTE: Each glass slide contains eight separate wells. A single sample consists of four wells with biofilms that will be pooled[17]. This extraction protocol is for 1 sample (4 wells) where the biofilms

are recovered from 2 wells at a time. RNA extractions are performed using a commercial RNA extraction kit that includes a bead beating step and a column-based cleanup, with modifications. Follow the manufacturer's instructions for reagent preparation.

2.1. In a laminar flow hood, slowly remove the media from 2 out of 4 wells using a pipette tip. Tilt the slide at a 45° angle and pipet the media out from the bottom corner of the wells to prevent detachment of the biofilms.

2.2. Keeping the slide tilted, wash the planktonic cells off by gently pipetting 300 µL of RNase-free water into the bottom corner of the two emptied wells. Remove the water by gently pipetting it out, as described in step 2.1. Repeat the wash step removing as much water as possible.

2.3. Add 300 µL of an RNA protection reagent (see **Table of Materials**) to each of the two emptied wells with biofilms at their base. Place the chamber slide on a glass plate to prevent the wells from breaking, and then scrape the biofilms in the 2 wells with a small, nuclease free, sterile metal spatula to re-suspend the biofilm bacteria. Let sit until the biofilms are recovered from the 2 remaining untouched wells from the same sample.

NOTE: The addition of an RNA protection reagent ensures the stability of the biofilm samples in the scraped wells at ambient temperature while processing the remaining two wells of the same sample. The RNA protection reagent lyses cells and inactivates nucleases and infectious agents, resulting in preservation of the RNA.

2.4. To recover biofilms from the remaining 2 wells for a sample (reminder: one sample is comprised of 4 wells), remove the LB media from the 2 remaining new wells in the same way as described in step 2.1. Repeat step 2.2 to wash off the planktonic cells with RNase-free water from both new wells, as before.

2.5. Go back to the first 2 wells with scraped biofilms in protection reagent, generated at the end of step 2.3, and slowly pipet to mix the 300 µL of re-suspended biofilm from **one** scraped well, trying not to create too many bubbles.

2.5.1. Transfer all the contents from the well into **one** of the newly washed, emptied wells. Mix and transfer 300 µL of re-suspended biofilm from the second scraped well in the remaining, newly washed, emptied well.

NOTE: Instead of adding new RNA protection reagent to the 2 newly washed wells with biofilms, transfer the previously scraped biofilms already in protection reagent to these freshly washed wells. This will keep the combined sample volume low enough to meet the input requirements for the commercial RNA extraction kit. See **Figure 1** for a schematic.

2.6. Repeat scraping the biofilms in the new wells as in step 2.3, by placing the chamber slide on a glass plate and scraping the biofilms in the 2 new wells with a small, nuclease free, sterile metal spatula to re-suspend the biofilm bacteria.

2.7. Combine **all** the re-suspended biofilm from the 2 new wells into a single RNase-free, low-bind, 1.5 mL microcentrifuge tube. Measure the volume, which should be ~500 - 600 µL total.

NOTE: The combined biofilm suspension from this second pair of new wells will contain all of the biofilm material from the original 4 wells of a sample.

3. Total RNA isolation and quality assessment

NOTE: RNA extraction is performed using a commercial RNA extraction kit that claims to work on biofilms. The individual components are included in the **Table of Materials**, if possible. Explanations of the mechanisms behind each purification step are provided when possible.

3.1. Add enough RNA protection reagent to total 750 µL in the tube. Transfer the entire volume to a 2 mL bead beating lysis tube containing 0.1 and 0.5 mm beads (see **Table of Materials**). Beat for 2 ½ min in a bead beater at maximum speed.

NOTE: The combination of 0.1 mm and 0.5 mm high density beads plus high-speed mixing on a bead beater ensures thorough homogenization of microbial cell walls.

3.2. Centrifuge at 16,000 x *g* for 1 min to pellet the beads. Transfer the supernatant to a new microcentrifuge tube, minimizing the transfer of any beads, which will make step 3.3 easier. Measure the volume.

3.3. Add an equal volume of RNA lysis buffer (~450 µL) and mix well. Transfer up to 800 µL of sample, avoiding transfer of any beads, to a silica column in a collection tube and centrifuge at 16,000 x *g* for 30 s. Save the flow-through as it contains the RNA while DNA is bound to the column.

NOTE: The RNA lysis buffer contains guanidinium thiocyanate and the detergent N-lauroylsarcosine to lyse cells. Guanidinium thiocyanate is a chaotropic agent that also inactivates nucleases and, in the presence of silica, found in the spin column, promotes the binding of DNA to the silica[18]. The absence of ethanol allows for preferential binding of DNA and not RNA to the silica spin-column[19]. The purpose of step 3.3 is to bind and remove genomic DNA. We want to retain the RNA, which is contained in the flow-through portion.

3.4. If more sample remains, transfer the column to a new collection tube and reload with the rest of the sample. Centrifuge at 16,000 x *g* for 30 s. Keep the flow-through with the RNA and combine with the first aliquot.

3.5. Measure the combined flow-through volume and add an equal volume of 100 % ethanol and mix well. Transfer up to 800 µL of the solution to a new, second silica spin-column in a collection tube and centrifuge at 16,000 x *g* for 30 s. Discard the flow-through.

NOTE: The addition of ethanol to the chaotropic salt solution containing the RNA enhances the binding of RNA to the silica spin-column[19].

3.5. For solutions > 800 µL, reload the spin-column and centrifuge until the entire solution is spun through. Discard the flow-through after each spin.

3.6. Add 400 µL of wash buffer to the column and centrifuge at 16,000 x g for 30 s to remove some of the salts. Discard the flow-through.

3.7. Prepare the DNase I reaction mix according to the manufacturer's instructions and carry out the in-column DNase treatment to remove any residual DNA.

3.8. Resuspend the lyophilized DNase I in 275 µL of RNase-free water to make a 1 U/µL solution. Mix by gentle inversion.

3.9. Combine 5 µL of diluted DNase I with 75 µL of the provided DNase digestion buffer. Mix gently by inversion.

3.10. Add 80 µL of the prepared solution directly onto the column matrix. Incubate at room temperature for 20 min.

3.11. Add 400 µL of RNA prep buffer to the column and centrifuge at 16,000 x *g* for 30 s. Discard the flow-through.

3.12. Add 700 µL of RNA wash buffer to the column and repeat the centrifugation. Discard the flow-through.

3.13. Add 400 µL of RNA wash buffer and centrifuge the column for 2 min to completely remove any residual buffer.

NOTE: There are 2 different wash steps to remove impurities on the column. The prep buffer contains a weak chaotropic salt mixed with ethanol in order to remove residual proteins. Next, the wash buffer is used to perform ethanol washes to remove salts. Any remaining ethanol must be removed to allow efficient elution of the RNA[19,19].

3.14. Carefully transfer the column to a new, low-bind microcentrifuge tube.

3.15. Add 50 µL of RNase-free water directly to the column matrix and incubate for 5 min. Centrifuge at 16,000 x g for 1 min to elute the RNA.

3.16. For additional cleanup to remove PCR inhibitors, place a PCR inhibitor filter into a new collection tube. Add 600 µL of the provided inhibitor prep solution (see **Table of Materials**).

3.17. Centrifuge at 8,000 x g for 3 min to wash the filter. Transfer the washed filter into a new low-bind microcentrifuge tube.

3.18. Transfer the eluted RNA from step 3.13 into the washed filter, and centrifuge at 16,000 x g for 3 min.

NOTE: The RNA can be used immediately or stored at -80 °C.

3.19. Determine the concentration of the RNA using a high sensitivity fluorometric system[20].
NOTE: These systems allow for sensitive quantitation of a small amount of RNA in solution that is specific to the target of interest.

3.19.1. Assess the quality of the RNA using an automated electrophoresis system that can provide a RIN (RNA integrity number), which is a measure of RNA quality[21,22].

4. Ribosomal RNA depletion and high throughput sequencing

4.1. Submit RNAs to the Centre for the Analysis of Genome Evolution and Function (CAGEF) genome centre at the University of Toronto (Toronto, Canada) (https://www.cagef.utoronto.ca/) for bacterial rRNA depletion and RNA directional library preparation (see **Table of Materials**).

NOTE: Bacterial rRNA depletion targets the 5S, 16S and 23S rRNAs for removal[23].

4.2. Deplete rRNAs using a commercial rRNA bacterial depletion kit. Follow the protocol for inputting 10 ng - 1 µg intact or partially degraded total RNA.

4.3. Construct RNA sequencing libraries using an RNA directional library prep kit with different indexes attached to each library.

4.4. Pool equimolar amounts of each RNA library and perform high throughput sequencing with 100-base paired-end reads[24].

5. Quality assessment of sequencing reads

NOTE**:** Check the quality of the sequencing reads using the freely available program, FastQC[25], available through the free, open-source platform, Galaxy[27].

5.1. Go to https://usegalaxy.org/. Click on the **Login or Register** menu and log in with credentials or create an account.

5.2. Click on the **Upload Data** link at the top left of the page, under the Tools menu, and upload the fastq.gz sequencing files. Wait for the file names appear on the right side of the page, under the History panel.

5.3. Select FASTQ Quality Control under the Tools menu to reveal a list of programs. Select FastQC, which will populate the middle panel of the screen.

5.4. Under **Short read data from your current history**, select the uploaded fastq.gz files from the pull-down menu.

5.5. Select **Execute** to run the program.

5.6. View the results under the History panel (**Table 2**).

NOTE: For more detailed instructions on how to use Galaxy, visit the support page at https://galaxyproject.org/support/.

6. Mapping of sequencing reads

NOTE**:** Listed is a basic pipeline for adapter trimming and read mapping for RNA-seq data. Adapter sequences are trimmed from the reads using Trimmomatic[28]. The trimmed reads are mapped to the *P. aeruginosa* PAO1 reference genome (NC_002516.2), obtained from NCBI (https://www.ncbi.nlm.nih.gov/)[29], using BWA[30] and Samtools[31]. For simplicity, a pair of reads are called PA_1.fq and PA_2.fq; the adapter read file to be trimmed is called adapter.fa; and the PAO1 reference sequence is called PAO1.fasta. All of the tools are open source and run in a UNIX/LINUX environment. It is strongly advised you familiarize yourself with the fundamentals of UNIX/LINUX in order to execute these commands.

6.1. Open a window in UNIX/LINUX.

6.2. Install Java, Trimmomatic, BWA and Samtools.

6.3. Navigate into the folder where the file trimmomatic-0.39.jar resides.

6.4. Trim off any adapter sequences from the reads by typing the command:
Java -jar PA_1.fq PA_2.fq PA_1_paired.fq PA_1_unpaired.fq PA_2_paired.fq PA_2_unpaired.fq ILLUMINACLIP: adapters.fa

NOTE: Only adapter sequences are removed. Reads have not been trimmed for quality[32].

6.5. Move the PAO1.fasta reference file into the same folder.

6.6. Index the reference using BWA with the command:

Bwa index PA01.fasta

6.7. Map the paired reads to the reference genome by typing the following 4 commands. Type each command after the previous one has finished.

Bwa -mem PA01.fasta PA_1_paired.fq PA2_2_paired.fq > PA_R1R2_map.mem.sam
Samtools view -S -b PA_R1R2_map.mem.sam > PA_R1R2.bam
Samtools sort PA_R1R2.bam -o PA_R1R2_sorted.bam
Samtools index PA288_Rep1_R1R2_sorted.bam

6.8. View the mapping statistics by typing the command:

Samtools flagstat PA_R1R2_sorted.bam

NOTE: The 3rd line of the statistics reports the proportion of the reads that map to the reference genome.

6.9. Calculate the mean read depth and breadth of coverage with the following 2 commands[33], respectively:

samtools depth -a PA_R1R2_sorted.bam | awk '{c++;s+=$3}END{print s/c}'

*samtools depth -a PA288_Rep1_R1R2_sorted.bam | awk '{c++; if($3>0) total+=1}END{print (total/c)*100}'*

REPRESENTATIVE RESULTS:

The general overview of the method is shown in **Figure 1**. We previously used 8-well chamber slides to grow *P. aeruginosa* biofilms and expose them to antibiotics before then examining them via confocal microscopy at different time points[11,12]. This method can be used to extract total RNA directly from biofilms grown in this system in order to study gene expression changes post treatment. This protocol has been optimized for *P. aeruginosa* but can easily be adapted for other bacterial species.

It can be challenging to extract a sufficient quantity of good quality RNA from small amounts of biofilm for rRNA depletion and high throughput sequencing. Using this protocol, total RNA is successfully extracted from 17 different *P. aeruginosa* biofilm isolates in triplicate, totaling 51 separate samples. Quantities of extracted RNAs representing high and low yields are shown in **Table 1**. The RNAs range in concentration from 3.4 ng/µL (lowest) to 49.6 ng/µL (highest), with a mean concentration of 14 ng/µL and a median of 13.7 ng/µL. Intact RNA concentrations below 10 ng/µL are considered low abundance samples for rRNA depletion and Next Generation Sequencing, but low abundance RNA samples from biofilms can result in even poorer quality sequencing data compared to more concentrated samples[34-37]. The qualities of the RNAs are shown in **Table 1** by the RIN and in **Figure 2** by the corresponding RNA electropherograms for the low (PA565-3) and high (PA288-1) concentrated samples; the two remaining samples (PA375-3 and PA921-1) are representative of the majority of the samples. As seen in **Figure 2**, RNA extracted from these amounts of biofilm always contain some degraded RNA, which affects their RIN values. Therefore, visual confirmation of the 16S and 23S prokaryotic rRNA peaks is used to determine RNA quality when a RIN is not reported. Using these criteria, all 51 RNA samples are

chosen to proceed to rRNA depletion and sequencing, with the prediction that the sample of poorest quality, PA565-3, might fail. Out of the RNA samples submitted for sequencing, successful libraries are generated and sequenced for 49 samples, with PA565-3 failing, as predicted.

The number of sequencing reads generated for the high concentration sample with a good RIN (PA288-1) and the lower concentration sample without a RIN (PA375-3) are listed in **Table 2**. Basic statistics include the total number of reads, the read length, and the GC content. The summary statistics for both samples show a high number of generated reads and indicate that none are flagged as poor quality, suggesting good sequencing data. The average number of reads per sample is approximately 48 million, which is considered a good yield. A standard tool used to evaluate the quality of the raw sequencing data is FastQC[25]. This program is used to perform quality control checks on the raw sequencing files to determine whether the quality is sufficient for further analyses, or whether there are problems or biases in the data generated by the sequencer itself or from the input RNA libraries. **Figure 3** presents the quality control metrics for PA288-1 and PA375-3 sequencing data, representing a high-quality RNA sample and a typical, lower quality RNA sample, respectively. One of the more informative plots from FastQC is the Per Base Sequence Quality plot. Good sequencing data will show a high median quality score (>30) for each position in all the reads, with a drop in the mean quality score over the length of the read. A quality score of 30 represents an error rate of 1 in 1000, corresponding to a base call accuracy of 99.9 %. The vast majority of bases in **Figure 3** have mean quality scores ≥ 35 over the entire length of the read for both samples, which are indicative of exceptionally good quality sequencing data. This provides strong evidence that the RNA extraction protocol presented here is successful.

To show our method can recover *P. aeruginosa* transcripts, the high-quality sequencing reads for PA288-1 and PA375-3 are mapped to the *P. aeruginosa* PAO1 reference genome (NCBI NC_002516.2). The reads are only trimmed for sequencing adapter and not for quality, retaining all the bases prior to mapping[32]. The mapping statistics are presented in **Table 3**. The percentage of mapped reads is an important measure of sequencing accuracy. Simply put, the more reads that align to your reference sequence the better. For PA288-1 and PA375-3, 84% and 91% of the reads, respectively, map to the reference genome. The expected range for mapping standard RNA-seq reads falls between 70 – 90%, so these values are very good, especially when poor quality bases are not removed[38]. The mean read depth is a good indicator of the average number of reads that align at each base position in the reference sequence. The higher the depth at each base position, the more accurate the base call at each position. It is calculated by dividing the sum of the mapped read depths at every position in the reference genome by the total number of bases in the reference. PA288-1 and PA375-3 have mean read depths of 400 or greater, which is good for downstream gene expression analysis[38,40]. The breadth of coverage tells the percentage of the length of the reference genome that is covered by the sequencing[41]. The aligned reads from both PA288-1 and PA375-3 cover 96 % of the *P. aeruginosa* PAO1 reference genome. This suggests most of the *P. aeruginosa* genome is represented in the sequencing data and not just short stretches. The mapping statistics for PA288-1 and PA375-3 show this method can recover transcripts that align with good coverage and distribution to the *P. aeruginosa*

genome, further supporting a successful extraction protocol.

FIGURE AND TABLE LEGENDS:

Figure 1. Overview of the protocol. **A.** Schematic of experimental workflow. Planktonic cultures were grown overnight at 37 °C, diluted 1:100 with fresh media the next day and grown for another 3 h. Cultures were adjusted to an OD_{600} of 0.1 and 300 µL inoculated into 4 wells of an 8-chamber slide to generate biofilms. After 24 h, the biofilms were washed to remove planktonic cells; RNA protection reagent was added, and cells scraped off the wells. Total RNA was extracted, depleted of ribosomal RNAs and sequenced. **B.** Detailed workflow of biofilm growth in and removal from an 8-chamber slide. Two strains were grown per slide in the orientation shown on the left. The arrangement of each sample in four wells of the slide is shown. For each independent sample, wells 1 and 3 were processed first, ending with 300 µL of scraped cells in RNA protection reagent in each well. An example is depicted by the middle slide. Next, wells 2 and 4 were washed and the liquid removed. The re-suspended cells from wells 1 and 3 are transferred to wells 2 and 4, respectively, shown in the right hand slide. After scraping, the re-suspended cells in wells 2 and 4 are combined into a single microcentrifuge tube. This figure was created with BioRender.com.

Figure 2. Examples of RNA electropherograms from high to low quality extracted RNA samples. The 16S and 23S ribosomal peaks are labeled at the base of their peaks. Degraded RNA is represented by small-sized peaks, indicated by the arrows and a bumpy baseline above zero. Acceptable quality RNA samples are shown in panels **A, B** and **D**. Panel **C** shows an RNA sample of poor quality where the ribosomal peaks are missing and the concentration was of very low abundance, indicated by the scale on the Y-axis. FU, fluorescence units; nt, nucleotide.

Figure 3. **FastQC Per Base Sequence Quality plot for PA288-1 and PA375-3 sequence data**. **A.** The quality plot for the high-quality sample PA288-1. **B.** The quality plot for typical sample PA375-3. The plots show the aggregated quality score for each base position for all the reads in the file. The blue line represents the mean quality score at each base position. The red line within the yellow box represents the median quality score at each position, and the yellow box shows the quartile range for the 25^{th} to 75^{th} percentiles.

Table 1. Quality metrics of extracted RNAs from representative samples.

Table 2. PA288-1 and PA375-3 FastQC Summary Statistics

Table 3. PA288-1 and PA375-3 Mapping Statistics

DISCUSSION:

Total RNA is successfully extracted from 17 different bacterial biofilm samples in triplicate, yielding a total of 51 samples. The forty-nine RNA libraries are pooled and successfully sequenced. Overall, this validates our quality criteria with a 96 % success rate even though more than half the samples are considered to be low abundance and of sub-optimal quality[34-37].

Significance

This RNA extraction protocol is unique in its detailed explanation to extract RNA from limited amounts of biofilm that were grown in chamber slides. The growth of biofilms in an 8-well chamber slide is a useful system to study the effects of exogenously added factors to biofilms or effects of microbial interactions in biofilm formation by confocal microscopy[11,12,42]. In order to examine the expression of genes involved in biofilm formation in this system, we present a detailed method to extract intact RNA of sufficient quality and quantity for RNA sequencing. Other studies have reported successful RNA extraction from limited amounts of biofilm, but the majority grow biofilms on a larger surface area and often for 48 h instead of 24 h. They also lack sufficient detail to ensure success[4,14,16,43,44,46].

Furthermore, this protocol avoids the use of hazardous chemicals (such as phenol) or specialized equipment (such as a sonicator). The classic guanidinium thiocyanate phenol-chloroform extraction protocol[47] is not used for this system because, even though it yields approximately 2x more RNA compared to the commercial kit, in our hands it consistently results in fully degraded RNA, as assessed on an automated electrophoresis system. In addition, the use of a commercial extraction kit is shown to result in a user-friendly protocol that yields consistent results[14,16,45].

Critical Steps

There are a number of critical steps in this protocol that increase the probability of extracting RNA that can be successfully sequenced. First, it is important to inoculate at least 4 wells of the chamber slide with the same strain in order to obtain sufficient quantities of RNA to sequence. The biofilms from the 4 wells are pooled for a single extraction, which has the added advantage of reducing variability in the downstream gene expression analysis[17]. Extracting RNA from less than 4 wells often leads to yields that are too low to detect on a high sensitivity fluorometric system. Extracting RNA from 8 wells or an entire slide will yield higher quantities of RNA of similar quality to that obtained when using 4 wells, but one must consider if the additional time, effort, and cost for the extraction is worth the increased yield. Pipetting 300 µL of OD_{600} = 0.1 diluted culture to seed each well instead of the standard 200 µL improves the recovery of scraped biofilm material from each well. Gently washing the biofilm twice with nuclease-free water before applying the RNA protection reagent is critical to remove as many dead and/or planktonic cells as possible. The use of the RNA protective reagent is important to prevent RNA degradation due to the time required to process each well. Furthermore, instead of scraping with pipet tips, we prefer using metal weigh spatulas with a flat end that contacts a larger surface area than a pipet tip, and that are small enough to fit into a well. Scraping using a 1000 µL pipet tip will work but is less efficient and takes longer to scrape the entire biofilm compared to the metal spatula. In our experience, using metal spatulas saves time and effort. When scraping, make sure to place the chamber slide on top of the glass plate to prevent cracking the bottom of the wells. Sonication is not effective as it consistently results in highly degraded RNA due to too little sample volume and too much heat production. After collecting the biofilm material, the sample is input into a commercial kit for more reliable and reproducible RNA extractions. Finally, because of potential sources of variation in this method, it is very important to include experimental replication in addition to sample pooling[17,47]. This protocol describes triplicate biological replication of each

sample.

Limitations

There are a variety of techniques used to grow biofilms for study, the most common of which is formation in microtiter plates[4]. These plates are available with different sizes and numbers of wells. Chamber slides fall into this category. The ability to extract intact, pure RNA is important for all biofilm systems, but this system is limited by the low yields of RNA. The concentrations are low enough that their quality and quantity should be assessed by Qubit and Bioanalyzer instruments using high sensitivity RNA kits. A nanodrop can also be used, but it has more limited sensitivity compared to Qubit and cannot distinguish contaminating DNA from RNA[49]. If possible, a nanodrop should be used to get A_{260}/A_{280} and A_{260}/A_{230} ratios for purity. These ratios are useful, especially since RNA quality from this biofilm system is not as good as from larger amounts of starting material. This is most likely due to the low yield of RNA and presence of dead cells in the biofilms and/or RNase that may degrade RNA. Another limitation of this method is that it cannot separate different cell types within a heterogeneous biofilm but can only extract the cells as a whole population, potentially obscuring gene expression in any underrepresented subpopulations.

Potential Applications

The protocol presented here has been optimized for *P. aeruginosa* biofilms from clinical isolates grown in chamber slides. This method can be used to study changes in gene expression during biofilm formation under different growth conditions observed by confocal microscopy. The protocol can also be optimized for other biofilm generating bacterial species. With this method, it is also possible to extract genomic DNA since a dual extraction kit can be used. In this way, bacterial biofilm gene expression in clinical isolates causing infections in patients can be studied more accurately, leading to results that may guide future treatment strategies.

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DISCLOSURES:

The authors have no disclosures to declare.

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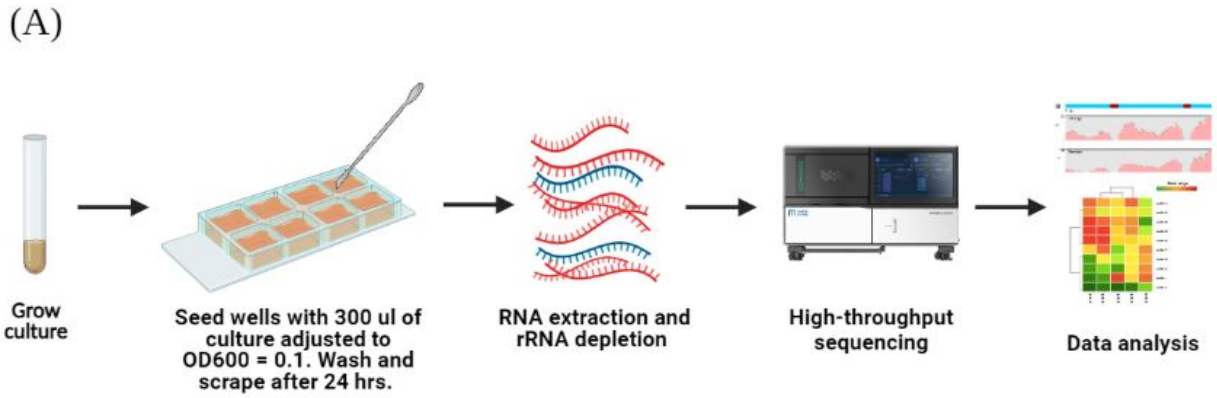


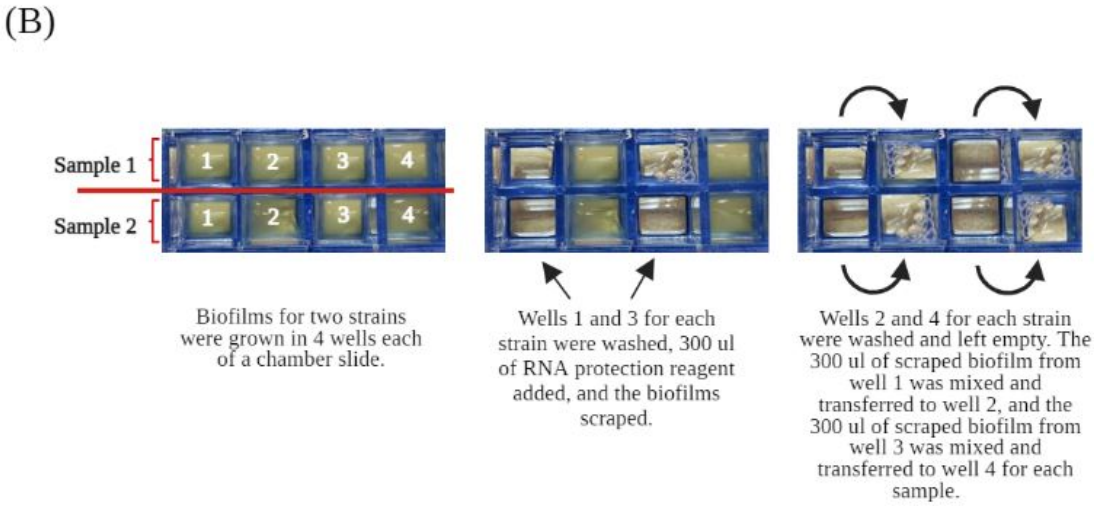


Figure 2

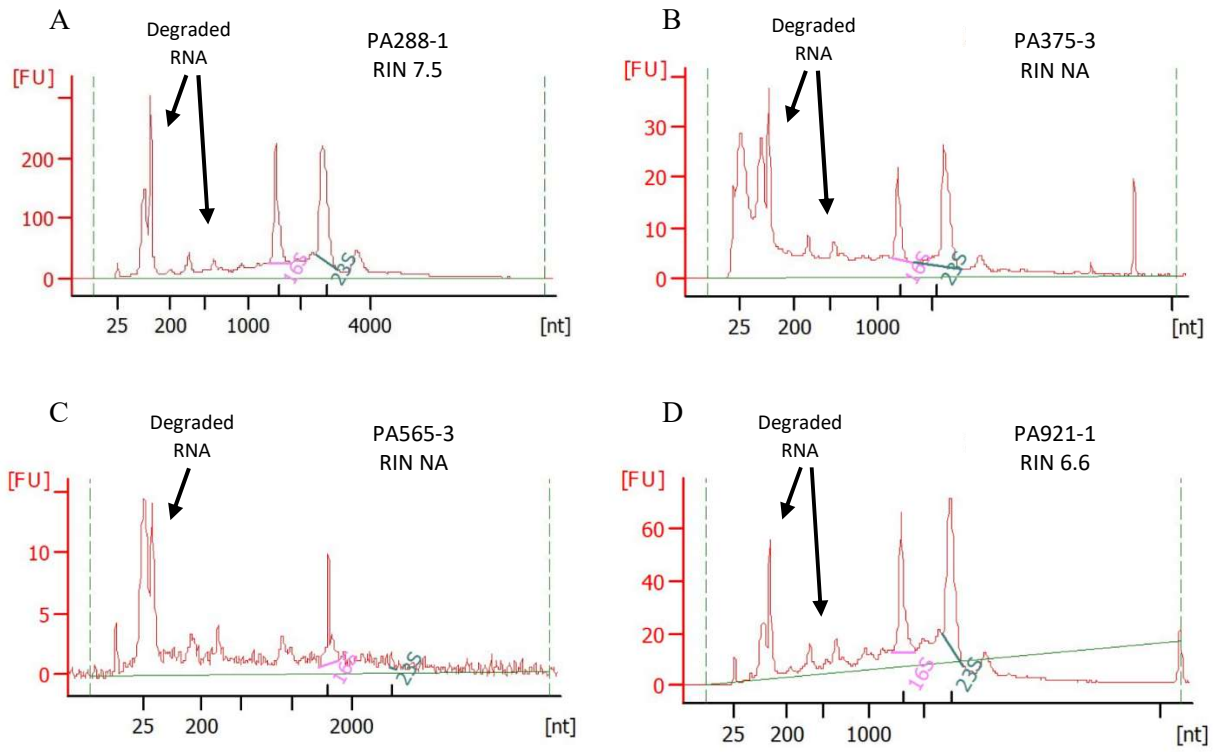
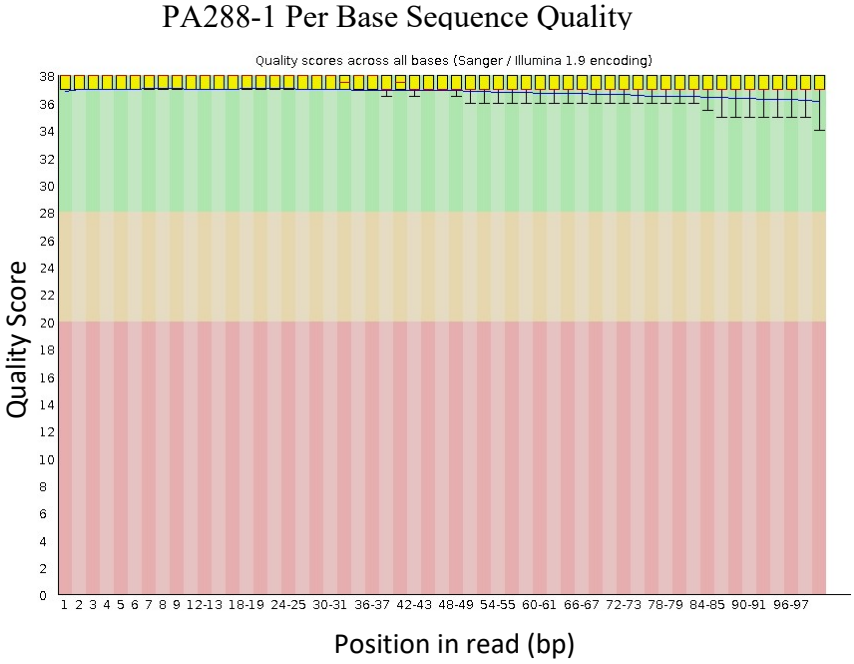


Figure 3

A.



B.

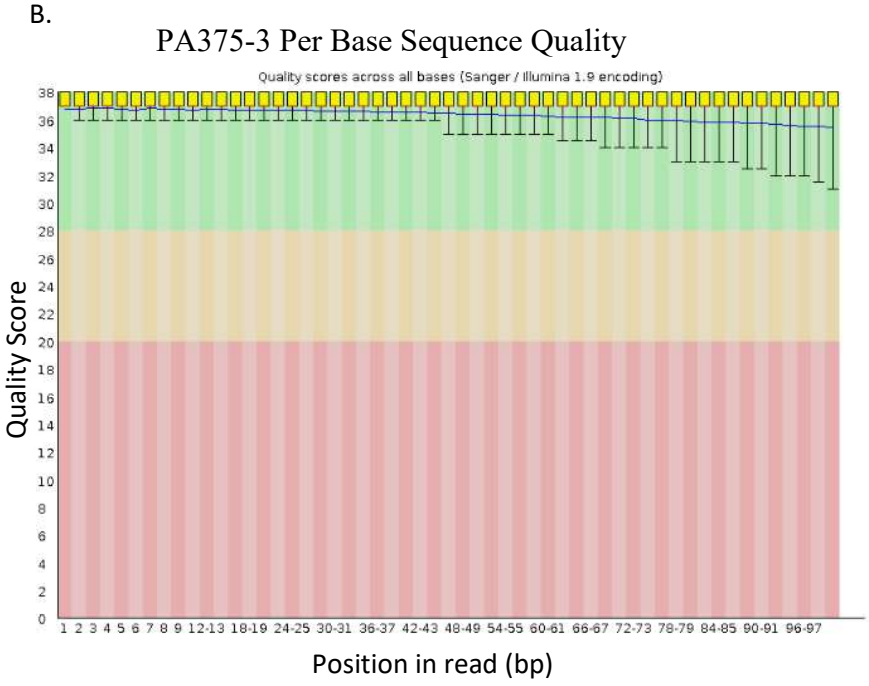


Table 1. Quality metrics of extracted RNAs from representative samples.

Isolate	Qubit	RIN	Sequenced
PA288-1	26 ng/µl	7.5	Yes
PA375-3	4.07 ng/µl	NA	Yes
PA565-3	3.4 ng/µl	NA	No
PA921-1	9.11 ng/µl	6.6	Yes

Table 2. PA288-1 and PA375-3 FastQC Summary Statistics

	PA288-1	PA375-3
Total Reads	85,957,720	31,849,575
Reads flagged as poor quality	0	0
Read length	100	100
%GC	61	60

Table 3. PA288-1 and PA375-3 Mapping Statistics

Isolate	% Reads Mapped to the Reference	Mean Read Depth	Breadth of Coverage
PA288-1	83.93%	404	96.68%
PA375-3	91.2%	578	96.97%

Dear Dr. Bajaj,
Review Editor, JoVE

Thank you for reviewing our revised manuscript (JoVE62755R1: Isolation of total RNA from *Pseudomonas aeruginosa* within biofilms for measuring gene expression) and sending three final comments that need to be addressed before a formal acceptance by JoVE. We have responded to each comment below in bold.

Thank you again for the thoughtful review of our work.

Sincerely and on behalf of all authors,
Pauline Wang

Editorial comments:

1. The editor has formatted the manuscript to match the journal's style. Please retain.

We thank the editor for formatting our manuscript to conform with the journal's style.

2. Please address specific comments marked in the manuscript.

E2.1. The title of the manuscript has remained unchanged. Instead, the highlighted protocol steps have been revised to reduce some of the biofilm inoculation steps and include the RNA extraction steps up to the repeated column washing/centrifugation steps. These steps follow the manufacturer's protocol and do not need to be repetitively shown. The last highlighted step is the elution of the RNA. The highlighted steps now include step 1.5 - 3.5 and step 3.15.

E2.2. An ethics statement has been included describing the approval for use of human sputum samples from CF patients.

E2.3. An acknowledgement has been added stating author contributions and a funding source for this research.

E2.4. The references have been reorganized such that the citations in the text follow the number order of the references.

3. Please let us know whether you will be producing your own video (where entire scripting, filming and production will be done by you and video will also be submitted for peer review) or you would want JoVE to produce the video (where after acceptance, we will script, you will film the video clips and will produce your video).

We are interested in the hybrid filming/production model where JoVE will script and produce the video, and we will film the video clips.