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Extracellular RNA Isolation from Biofilm Matrix of *Pseudomonas aeruginosa*

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[Abstract] Most bacteria in natural ecosystems form biofilms–a bacterial community, surrounded by a polymer matrix that consists mostly of exopolysaccharides, proteins, and nucleic acids. Extracellular RNA as a matrix component is involved in biofilm formation–the fact that was confirmed by direct detection of extracellular RNA in the biofilm matrix, and by an interruption of the biofilm's structure with RNases. Number of protocols describing isolation of RNA from biofilm matrix are limited and usually involve uncommon equipment and reagents. Here we describe simple method for extracellular RNA isolation from biofilm matrix using basic laboratory reagent and equipment. Key steps of the protocol include separation of matrix and bacterial cells with high ionic solution of NaCl, RNA precipitation with LiCl and clean up with option to use inexpensive column for plasmid DNA isolation rather than specialized RNA kits. Described protocol allows to isolate extracellular RNA suitable for further molecular biology procedures such as sequencing, RT-PCR and cloning in less than one day (excluding time for biofilm growing up).

Keywords: Biofilm, Matrix, Extracellular RNA, Cells RNA, Bacterial RNA, Extracellular nucleic acids, *Pseudomonas aeruginosa*

[Background] Biofilm matrix provides resistance to different influences (antibacterial drugs, disinfectants, mechanical forces) and creates environment for coordinated regulation of different processes (Svenningsen, 2018). RNA presents in extracellular biofilm matrix and forms the main cross-linking elastic copolymer of RNA-DNA (Seviour *et al.*, 2019). Treatment of biofilms with RNases resulted in significant loss of biofilm mass and underline importance of RNA for maintenance of biofilm integrity (Lee *et al.*, 2019). At the same time sources and roles of RNA in biofilm matrix remain poorly explored.

The use of solutions with high ionic strength allows separation of extracellular biofilm matrix without subjecting embedded cells to lysis (Chiba *et al.*, 2015). At the same time further workflow of RNA purification might be significantly affected with high salt concentration (*i.e.*, phase separation during phenol-chloroform extraction). Another important aspect of such method of separation includes relatively large volume of the dissolved biofilm matrix, so there is a need of efficient RNA precipitation for further purification steps. The method of RNA isolation from biofilm matrix described by Seviour *et al.* (2019) includes matrix separation with high ionic liquids, for example, 40% (v/v) 1-ethyl-3-479

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methylimidazolium acetate (EMIM Ac): 60% (v/v) N,N-dimethyl acetamide (DMAc) and Perchloric acid (70%) and further steps of RNA isolation by chromatographic separation, but these steps include some harsh conditions (*i.e.*, incubation at 55 °C for 16 h and long centrifugation steps). It is not easily applicable in molecular biology laboratories. We imply idea of using high ionic liquid from both studies, implement LiCl precipitation to concentrate and redissolve RNA and clean up with inexpensive silica columns adopted from (Yaffe *et al.*, 2012). Finally, we offer a simple, fast, and inexpensive method of extraction of extracellular RNA (exRNA), moreover intracellular cell RNA (cRNA) might be purified from the same biofilm sample. Protocol utilizes common laboratory reagents and equipment and enables to isolate extracellular RNA suitable for further procedures. Such a method may be applicable for studying the molecular mechanisms of biofilm formation and maintenance.

Materials and Reagents

1. 50 ml Falcon tubes (Corning, catalog number: CLS430829)
2. 15 ml Falcon tubes (Corning, catalog number: CLS430791)
3. 1.5 ml DNA LoBind tube (Eppendorf, catalog number: 0030108051)
4. QIAprep Spin Miniprep Columns (Qiagen, catalog number: 27115) or alternative kit-based RNA clean-up: RNeasy Plus Mini Kit (Qiagen, catalog number: 74134)
5. 2 ml collection tubes (Qiagen, catalog number:19201)
6. *Pseudomonas aeruginosa ExoS* strain (*i.e.*, PAO1 strain)
7. LB medium (BD Difco, catalog number: DF0446-07-5)
8. 96-98% ethanol molecular biology grade (keep at -20 °C)
9. Nuclease-free water (Sigma-Aldrich, catalog number: W4502)
10. Sodium Chloride (Sigma-Aldrich, catalog number: 71376)
11. Trizol® Reagent (Invitrogen, catalog number: 15596026)
12. Chloroform (Sigma-Aldrich, catalog number: 32211)
13. Lithium Chloride (Sigma-Aldrich, catalog number: L9650)
14. Sodium Acetate (Sigma-Aldrich, catalog number: S2889)
15. DNase I without RNase activity with 10x reaction buffer (Thermo Scientific, catalog number: EN0521)

Prepare following solutions before starting protocol:

16. 70% ethanol made with nuclease-free water (store at -20 °C)
17. 1.8 M NaCl solution in nuclease-free water (store at room temperature)
18. 8 M LiCl solution in nuclease-free water (store at room temperature)
19. 3 M NaOAc in nuclease-free water (store at room temperature)

Optional for RNA electrophoretic analysis:

20. Agarose (Promega, number catalog: V3121)

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21. TAE buffer, Molecular Biology Grade, 40x (Promega, catalog number: V4271)
22. UltraPure Ethidium Bromide 10 mg/ml (Thermo Scientific, catalog number: 15585011)

Equipment

1. Refrigerated centrifuge (Eppendorf, model: 5910R)
2. Dry Block Thermostat (Bio-San, model: BS-010412)
3. Basic Microbiological Incubators (Fisherbrand, catalog number: 15-015-2633)
4. Set of pipettes 100 to 1,000 µl; 20 to 200 µl; 1 to 10 µl (Thermo Scientific™ Finnpipette™ F2 catalog numbers: 4642090; 4642030; 4642080)

Optional equipment for RNA quality and quantity analysis:

5. NanoDrop 2000 for RNA quality analysis and A_{260}/A_{280} ratio estimation (Thermo Scientific; catalog number: ND 2000C)
6. UV-transilluminator (312 nm) for electrophoresis visualization (Vilber Lourmat, ECX-15.M catalog number: 2131 1502 1)
7. Electrophoresis System (Helicon, model: SE-1)

Procedure

Methods of both extracellular and cell RNA extraction from bacterial biofilm samples are described below. For applying each protocol, you have to perform following steps:
(A) growing biofilm and (B) separation of extracellular matrix from embedded cells.

A. Growing biofilm of *P. aeruginosa*
   1. Using aseptic techniques pick single colony of *P. aeruginosa* in 1 ml LB medium in 15 ml Falcon tube and grow overnight at 37 °C with shaking 210 rpm.
   2. Prepare bacterial suspension by diluting the overnight culture to OD_{600nm} = 0.1 with LB medium.
   3. Use up to 15 ml of bacterial suspension for growing biofilm in 50 ml Falcon tube at static condition for 18-20 h at 37 °C.

B. Separation of extracellular matrix from cells
   Optional: Remove planktonic cells by aspiration and washing with 0.9% NaCl.
   1. Pellet biofilm by centrifugation at 10,000 *x g*, 15 min, 4 °C.
   2. Carefully remove supernatant with pipette.
   3. Resuspend the pellet in 10 ml of 1.8 M NaCl.
   4. Gently mix by rotation until complete dissociation of extracellular matrix occurred (approximately 5 min). **Don't vortex!**
   5. Pellet bacterial cells by centrifugation at 13,500 *x g*, 15 min, 4 °C.

6. Transfer supernatant to a new tube and proceed to Procedure C–exRNA isolation.
   Note: Supernatant (dissolved biofilm matrix) contains extracellular RNA (exRNA).

Optional: Filter supernatant containing exRNA through 0.22 µm filter unit into a new tube.

7. Save bacterial pellet for cell's RNA (cRNA) isolation–resuspend bacterial pellet in Trizol® reagent and store at -20 °C. See Procedure D.
   Note: Cells pellet contains intracellular cell's RNA (cRNA).

C. exRNA purification

1. Add to the sample from Step B6 1/10 V of 8 M LiCl (*i.e.*, 1 ml = 1/10 V from 10 ml of supernatant) and incubate for at least 2 h at -20 °C
2. Centrifuge for 15 min at 13,000 *x g*, 4 °C.
3. Remove the supernatant by aspiration and add to the pellet 1 V (equal amount to supernatant volume in Step B6, *i.e.*, 10 ml = 1 V) of 70% ethanol.
4. Centrifuge for 15 min at 13,000 *x g*, 4 °C
5. Remove supernatant and let remaining ethanol evaporates with tube open lid for 5 min at 25 °C.
6. Dissolve the RNA pellet in 150 µl RNase- free water.
7. Add to RNA solution 1/10 V (15 µl) of 10x reaction buffer with $MgCl_2$ and 2 µl of DNase I.
8. Incubate at 37 °C for 30 min, deactivate DNase I by heating at 75 °C for 5 min or adding equal concentration EDTA to $MgCl_2$ concentration in the reaction buffer.
9. Proceed to RNA clean-up in Procedure E.
   Note: LiCl precipitates mostly RNA but in our experience samples from biofilm matrix need to be additionally treated with DNase I and cleaned-up. We suggest silica columns for DNA (plasmid DNA as well) purification as cheap alternative to commercially available kits for RNA clean-up. Alternatively, RNeasy Plus Mini Kit (or analogs) might be used according to manufacturing protocol.

D. cRNA purification using Trizol® Reagent

1. Resuspend the cell pellet from Step B7 in 5 ml Trizol®.
2. Add 1 ml of chloroform.
3. Incubate for 10 min at 25 °C.
4. Centrifuge for 15 min at 13,000 *x g* at 4 °C.
5. Transfer aqueous phase to a new tube and proceed to RNA clean-up Procedure **E.**

E. RNA clean-up using mini columns

1. Add to the sample 1 V of ice-cold 96-98% ethanol.
2. Transfer up to 750 µl of the solution to the mini spin column placed in a 2 ml collection tube.
3. Close the lid and centrifuge for 45 s at 8000 *x g*, 25 °C Discard the flow-through.
4. If volume of the sample solution after Step **E2** was more than 750 µl apply remaining portion of sample to the same column.

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5. Add 450 µl 3 M NaOAc. Repeat centrifugation Step (**E3**).
6. Add 320 µl 70%-ethanol. Repeat centrifugation Step (**E3**).
7. Remove ethanol residuals by centrifugation for 2 min at 10,000 *x g*, 25 °C.
8. Transfer the column to a new 1.5 ml collection tube. Add 50-80 µl RNase- free water directly to the center of spin column membrane and incubate for 2 min at 25 °C.
9. Elute RNA by centrifugation for 2 min at 8,000 *x g*, 25 °C.

Optional: Reapply eluate to the same column and repeat Steps E8-9.

10. Store RNA in 1.5 ml LoBind Eppendorf tubes at -80 °C.

Optional for cRNA: process the RNA-eluate with DNase I digestion according to manufacturing protocol.

Notes

1. Overall amount of exRNA obtained with this protocol is in a range 200-2,000 ng; amount of cRNA is several times higher.
2. Obtained samples can be analyzed by electrophoresis in 1-2%–agarose gel or by other methods depending of research goals. For representative picture of RNA gel-electrophoresis see Figure 1.

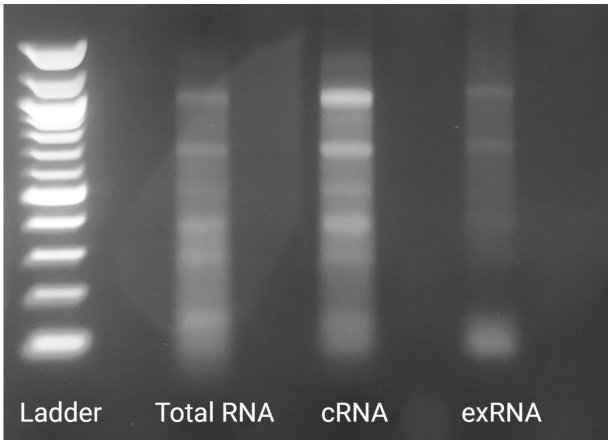

Figure 1. Representative picture of RNA isolation according to the protocol. Ladder–1 kb DNA ladder; total RNA–RNA was isolated from the total biofilm mass; cRNA–cells RNA; exRNA–extracellular RNA was isolated from biofilm matrix. Electrophoresis was performed in 2% agarose gel and stained with ethidium bromide. A_{260}/A_{280} ratio is 2.04; 2.01; 1.9 respectively.

3. We successfully applied this protocol for different *ExoS* strains and clinical isolates of *P. aeruginosa,* but for another strain protocol optimization should be performed. For adaptation of current protocol to other conditions (culture medium, incubation time, temperature, NaCl volume and concentration *etc*.) optimization should be also performed.

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Competing interests

The authors declare that they have no conflict of interest

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