Purification of Bacterial RNA from Infected Macrophages

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[Abstract] Studying the transcriptome of bacterial pathogens during infection is a very informative and effective tool for discovering genes that contribute to successful infection. However, isolating bacterial RNA from infected cells or tissues is a challenging process due to the much higher amounts of host RNA in the lysates of infected cells. We have optimized a method for isolating RNA of *Listeria monocytogenes* (*L. monocytogenes*) bacteria infecting bone marrow derived macrophage cells (BMDM). After infection, we lyse the cells and filter the lysates through 0.45 µm filters to discard most of the host proteins and RNA. Next, we resuspend the bacteria and extract RNA following DNase treatment. The extracted RNA is suitable for gene expression analysis by real-time PCR or microarray. We have successfully employed this protocol in our studies of *Listeria monocytogenes* gene regulation during infection *in vitro* (Lobel *et al.*, 2015; Lobel *et al.*, 2012; Kaplan Zeevi *et al.*, 2013; Rabinovich *et al.*, 2012).

Materials and Reagents

1. Cell scrapers (Thermo Fisher Scientific, Nunc™, catalog number: 179693)
2. MF-Millipore filters (Merck Millipore Corporation, catalog number: HAWP04700)
3. Cell culture dishes (Greiner Bio One International GmbH, catalog number: 639160)
4. Eppendorf tubes (Corning Inc., Axygen, catalog number: MCT175C)
5. Pipettes sterile (25 ml) (Greiner Bio One International GmbH)
6. Falcon tubes (50 ml) (Greiner Bio One International GmbH)
7. *Listeria monocytogenes* 10403S (Daniel Portnoy's lab stock) (Becavin *et al.,* 2014)
8. Bone marrow (from C57B/6 female mice; ordered from Harlan labs Israel)
9. Liquid nitrogen
10. RNase-free water (Thermo Fisher Scientific, Invitrogen™, catalog number: 10977015)
11. DMEM (Thermo Fisher Scientific, Gibco™, catalog number: 41965039)
12. L-Glutamine (200 mM) (Thermo Fisher Scientific, Gibco™, catalog number: 25030081)
13. Sodium pyruvate (Thermo Fisher Scientific, Gibco™, catalog number: 11360088)
14. 2-Mercaptoethanol (Thermo Fisher Scientific, Gibco™, catalog number: 31350010)
15. Penicillin-Streptomycin (10,000 U/ml) (Thermo Fisher Scientific, Gibco™, catalog number: 15140122)

16. Gentamicin (Sigma-Aldrich, catalog number: G1397)
17. Fetal Bovine Serum (FBS) (Thermo Fisher Scientific, Gibco™, catalog number: 10270106)
18. Dulbecco's Phosphate Buffered Saline (Sigma-Aldrich, catalog number: D8537)
19. Brain Heart Infusion (BHI) (Merck Millipore Corporation, catalog number: 1104930500)
20. Phenol saturated (pH 4.3) (Thermo Fisher Scientific, catalog number: BP1751I-400)
21. Chloroform (Thermo Fisher Scientific, catalog number: BP1145-1)
22. Isoamyl alcohol (Sigma-Aldrich, catalog number: W205702)
23. Sodium acetate Anhydrous (Sigma-Aldrich, catalog number: W302406)
24. Ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich, catalog number: EDS)
25. DNase I, RNase-free (supplied with MnCl2) (1 U/µl) (Fermentas, catalog number: EN0521)
    Note: Currently, it is "Thermo Fisher Scientific, catalog number: EN0521".
26. 10% Sodium dodecyl sulfate (SDS) (Sigma-Aldrich, catalog number: L4522)
27. Ethanol absolute (Merck Millipore Corporation, catalog number: 1070174000)
28. M-CSF (L-929 conditioned medium) (Englen *et al.*, 1995)
    Note: Alternatively M-CSF can be bought from Sigma (Sigma-Aldrich, catalog number: SRP3221).
29. BMDM + PS media (filter sterilized) (see Recipes)
30. BMDM no PS media (filter sterilized) (see Recipes)
31. AE buffer (see Recipes)
32. Phenol-chloroform-IAA (see Recipes)
33. Chloroform-IAA (see Recipes)

Equipment

1. CO_2 forced-air incubator (Thermo Fisher Scientific, model: 3111)
2. Kontes glass holder (Thermo Fisher Scientific, catalog number: K953755-0045)
3. Speed-Vac Concentrator (Thermo Fisher Scientific, catalog number: SPD131DDA)
4. Vortex-Genie 2 (Scientific Industries, model: G560E)
5. Nanodrop 1000 (Thermo Fisher Scientific)
6. 30 °C incubator (Thermo Fisher Scientific)
7. 65 °C heat block (Thermo Fisher Scientific)
8. 4 °C table centrifuge (Thermo Fisher Scientific, Eppendorf™, model: 5417R)

Procedure

A. Infection

Day 1:

1. Seed 2.0 x 10^7 bone marrow derived macrophages (BMDM) in 145 mm dishes with 30 ml BMDM + PS media, 3 plates for each bacterial strain being analyzed.
2. Pick a bacterial colony from a BHI plate and inoculate into 10 ml of BHI medium (repeat for each sample), incubate at 30 °C incubator, slanted without shaking overnight.

Day 2:

1. Pre-warm BMDM no PS medium and PBS at 37 °C.
2. Wash macrophage monolayer twice with 25 ml of warmed PBS to remove the antibiotics.
3. Add 30 ml fresh medium.
4. Wash 1.5 ml of overnight bacterial cultures twice with PBS.
5. Infect macrophages at MOI of 90:1 (in favor for the bacteria) for wild-type *L. monocytogenes* 10403S. When mutants defected in intracellular growth are used, consider increasing the MOI.
6. Infect each plate 15 min apart (same as in step A5).
7. Following 0.5 h incubation (in respect to each sample infection time), wash monolayers twice with 30 ml PBS. Add 30 ml pre-warmed BMDM no PS medium.
8. At 1 h post infection, add 30 µl of gentamicin (1:1,000) to kill extracellular bacteria.
9. Prepare filter apparatus and ice-cold RNase-free water.
10. At 6 h post infection, harvest the bacteria. Treat each plate individually.
    a. Wash monolayers with PBS once.
    b. Add 20 ml of ice-cold RNase-free water.
    c. Using a cell scraper, scrape and lyse cells by pushing the liquid in front of the scraper.
    d. Collect by pipetting the lysed monolayer in a 50ml conical tube. Vortex for 30 sec. Centrifuge 2,000 rpm (720 *x g*) /3 min/ 4 °C.
    e. Pass supernatant through filter apparatus. Using tweezers, put the filter membrane in 15 ml conical tubes, loosely rolled.
    f. Immediately freeze filters by dipping the 15 ml tubes in liquid nitrogen.
    g. Prepare the filter apparatus for the next sample and repeat.

B. Nucleic acids extraction

Day 3:

1. Prepare 1:1 mix of acidified phenol:chloroform, 400 µl for each sample. Mix in a separate tube, and then expel the resulting aqueous layer aspirated off.
2. Thaw your filter-containing tubes on ice. Insert each tube fully into ice.
3. To each filter-containing tube add 650 µl AE buffer. Add buffer to all tubes.
4. Working quickly, vigorously vortex your filter containing tube so that the filter whisks to the periphery of the tube and the buffer fully washes over the filter. It may be necessary to additionally vortex the tube while inverted to fully wash the bacteria off

the filter. Always keep tube cold by placing it back on ice. Repeat for all tubes. It may be useful to spin-down the suspension shortly (1 min/750 rpm/60 *x g*) at the end of the process.

5. Transfer the bacteria-containing buffer to the 1.5 ml Eppendorf tube containing 40 µl of 10% SDS and 400 µl of phenol/chloroform mix. Repeat for all tubes. It may be necessary to spin down tubes shortly (1 min/750 rpm) to get residual buffer from the filter.
6. Place all of your tubes in the multi-tube vortex device, and vortex at full speed for 10 min.
7. Incubate the tubes at 65 °C heat block for 10 min.
8. Centrifuge at maximum speed (14,000 RPM / 20,817 *x g*) for 5 min.
9. Transfer the aqueous layer (about 400 µl) from each tube to the 1.5 ml tube containing 40 µl 3 M sodium acetate (pH 5.2) and 1.0 ml 100% ethanol. Vortex each tube thoroughly.
10. Incubate your sample at -80 °C 1 h.
11. Centrifuge at 4 °C for 20 min at maximum speed (14,000 RPM / 20,817 *x g*).
12. Carefully aspirate off the ethanol from each tube. Add 500 µl cold 70% ethanol to each sample and vortex thoroughly.
13. Centrifuge at 4 °C for 20 min at maximum speed.
14. Carefully aspirate off the ethanol from each tube. Dry your samples for approximately 2 min in a speed-Vac device without heating. It is critical not to over-dry your samples.
15. To each sample, add 25 µl RNase-free water. Incubate at room temperature for 20 min. Carefully vortex and spin-down.
16. Analyze the RNA concentration in the samples using the NanoDrop. Expect to extract about 0.5-1 µg/plate.
17. You can unite the technical replicates at this stage.

C. DNase treatment

1. Set reaction:

RNA	up to 2 µg (max 44 µl)
10x DNase buffer	5 µl
H_2O	complete to 50 µl
DNase	1 µl
Total	50 µl

2. Incubate at 37 °C for 45 min.
3. Add 450 µl RNase-free water and 500 µl of Phenol-Chloroform-IAA mix, separate phases by 2 min centrifugation at maximal speed.
4. Transfer the aqueous layer to a new tube and add 500 µl chloroform-IAA mix, vortex and separate phases by 2 min centrifugation at maximal speed.

5. Transfer the aqueous layer to a new tube and add 1 ml of ethanol and 50 µl of 3 M sodium acetate (pH 5.2). Vortex.
6. Incubate for 1 h at -80 °C.
7. Centrifuge at 4 °C for 20 min at maximal speed.
8. Carefully aspirate off the ethanol from each tube. Add 500 µl cold 75% ethanol to each sample and vortex thoroughly.
9. Centrifuge at 4 °C for 20 min at maximum speed.
10. Carefully aspirate off the ethanol from each tube. In a speed-Vac without heating, dry your samples for approximately 2 min. It is critical not to over-dry your samples.
11. Add 12 µl of RNase-free water, incubate for 2 min at room temperature, vortex, and spin-down. Keep the RNA on ice.
12. Analyze the RNA concentration in the samples using the NanoDrop. Expect to extract about 100 ng/sample.

Representative data

We provide representative data of induction of the virulence gene *hly* of *L. monocytogenes* during intracellular growth in macrophages compared to growth in BHI medium (Figure 1). mRNA was purified from intracellular bacteria 6 hours post infection at M.O.I of 90:1 and from bacteria grown to mid log (OD_{600} = 0.5) in BHI medium.

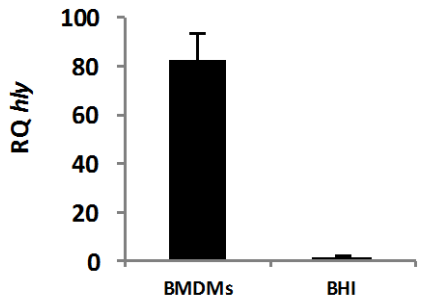

Figure 1. Transcription of *hly* during growth of *L. monocytogenes* in BMDMs (6 h.p.i.) and BHI lab medium. Error bars represent 95% confidence interval. Results are representative of 3 repeats (N=3). RQ: Relative quantity.

Notes

1. Check the morphology of the infected cells before and after infection. The cells should be viable during the infection process.
2. Be very careful when handling the membranes-use tweezers and touch only the edges.

3. When passing the samples through the filter apparatus, first filter RNase-free water to verify that the vacuum is working and that there is no blockage in the system.
4. You should observe a reduction in nucleic acid concentration following DNase I treatment. If you don't observe a reduction, consider another round of DNase I treatment.

Recipes

1. BMDM+PS media (filter sterilized)

DMEM	250 ml
FBS (inactivated 30 min at 54 °C)	100 ml
M-CSF (L-929 conditioned medium) (Englen *et al.*, 1995)	150 ml
Glutamine	5 ml
Sodium pyruvate	5 ml
β-Mercaptoethanol	0.5 ml
Pen/Strep	5 ml

2. BMDM no PS media (filter sterilized)

DMEM	255 ml
FBS (inactivated 30 min at 54 °C)	100 ml
M-CSF (L-929 conditioned medium) (Englen *et al.*, 1995)	150 ml
Glutamine	5 ml
Sodium pyruvate	5 ml
β-Mercaptoethanol	0.5 ml

3. AE buffer
   50 mM NaOAc (pH 5.2)
   10 mM EDTA
   RNase-free water
4. Phenol-chloroform-IAA
   Phenol 25 ml
   Chloroform 24 ml
   Isoamyl alcohol 1 ml
5. Chloroform-IAA
   Chloroform 24 ml
   Isoamyl alcohol 1 ml

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