Chapter 13

Extraction and Analysis of RNA Isolated from Pure Bacteria-Derived Outer Membrane Vesicles

Janine Habier, Patrick May, Anna Heintz-Buschart, Anubrata Ghosal, Anke K. Wienecke-Baldacchino, Esther N.M. Nolte-'t Hoen, Paul Wilmes, and Joëlle V. Fritz

Abstract

Outer membrane vesicles (OMVs) are released by commensal as well as pathogenic Gram-negative bacteria. These vesicles contain numerous bacterial components, such as proteins, peptidoglycans, lipopolysaccharides, DNA, and RNA. To examine if OMV-associated RNA molecules are bacterial degradation products and/or are functionally active, it is necessary to extract RNA from pure OMVs for subsequent analysis. Therefore, we describe here an isolation method of ultrapure OMVs and the subsequent extraction of RNA and basic steps of RNA-Seq analysis. Bacterial culture, extracellular supernatant concentration, OMV purification, and the subsequent RNA extraction out of OMVs are described. Specific pitfalls within the protocol and RNA contamination sources are highlighted.

Key words Bacteria, RNA, Outer membrane vesicle (OMV), Gram-negative, Sequencing, Analysis, Extraction, Ultracentrifugation, Ultrafiltration, Density gradient

1 Introduction

The secretion of outer membrane vesicles (OMVs) is a common phenomenon apparent in many Gram-negative bacteria [1]. OMVs are spherical nanovesicles with an average diameter of 20–200 nm and are composed of LPS, protein, lipids, and nucleic acid [1]. The amount of secreted-OMVs as well as their content is dependent on the bacterial growth environment [2], and therefore OMVs can play a function in intra- and inter-species [3, 4] and even in inter-kingdom communication [5–8]. Interestingly, it has recently been shown that an OMV-associated small RNA molecule disseminated from *Pseudomonas aeruginosa* is able to trigger a functional response in its host target cell [9]. Thus, analogous to observations in eukaryotes it seems possible that OMVs not only protect bacterial extracellular RNA but also represent means of transfer of

Véronique Arluison and Claudio Valverde (eds.), *Bacterial Regulatory RNA: Methods and Protocols*, Methods in Molecular Biology, vol. 1737, https://doi.org/10.1007/978-1-4939-7634-8_13, © Springer Science+Business Media, LLC 2018

RNA molecules to recipient cells where they could be functionally active. Whether, this mechanism of inter-kingdom communication is restricted to *Pseudomonas aeruginosa* or if it is a common phenomenon in Gram-negative bacteria still remains unclear.

Bacterial extracellular RNA has recently been described for different bacterial species and environments [10–13]. Bacteria secrete products into the extracellular environment either through their secretory systems via continuous or discontinuous passages across the bacterial membrane or by the release of OMVs [1, 10, 14]. Another origin of extracellular bacterial RNA is the release of such molecules from decomposing or disrupted cells. As the extracellular RNA can thus originate from different sources, it is likely that the different fractions of RNA molecules also present distinct functions. To investigate this theory, it is necessary to analyze the different RNA subpopulations individually.

Therefore, we describe here a protocol on how to isolate non-OMV-associated and OMV-associated RNA. The protocol allows the extraction of total RNA, large RNA, or only small regulatory bacterial RNA molecules. We also highly recommend to control the percentage of dead bacteria in the bacterial culture of interest. Furthermore, it is important to note that OMV-associated RNA is only present in the extracellular environment in very low amounts. Consequently, extracellular RNA-Seq is prone to be enriched in RNA contamination sequences. Therefore, we highly recommend to include controls for contamination, such as extraction blanks.

2 Materials

It is worth noting that all the materials and methods described within this chapter have been applied and optimized for the culturing of *Salmonella enterica* subsp. *enterica* strain LT2 (henceforth referred to *Salmonella*), and thus culturing conditions and biomolecular extraction methods might need to be optimized for other Gram-negative species. Furthermore, we focus within this protocol on the extraction of small RNA out of OMVs, but in principle, this protocol can also be used to extract total RNA from OMVs or from the OMV-free extracellular environment.

All solutions should be prepared using ultrapure water (sensitivity of 18 MΩ-cm at 25 °C) and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise). All working areas should be cleaned as much as possible with an RNase decontamination solution, and certified RNase-free lab material should be used during the whole protocol execution. Precisely execute biosafety and standard operation procedures in order to follow all waste disposal regulations when disposing waste materials.

2.1 Minimal Bacterial Culture Media M9 (see Note 1)

1. M9 Salts 5×: 800 mL H_2O and add 64 g Na_2HPO_4-$7H_2O$, 15 g KH_2PO_4, 2.5 g NaCl, 5.0 g NH_4Cl. Stir until dissolved. Adjust to 1000 mL with distilled H_2O. Sterilize by autoclaving. 5× media should be kept at 4 °C until use.
2. 3000 mL of distilled autoclaved H_2O.
3. Autoclaved 50 mL 1 M $MgSO_4$: add 6.0183 g of $MgSO_4$ into 50 mL of distilled H_2O, dissolve, and autoclave (place at 4 °C until use).
4. Autoclaved 50 mL 1 M $CaCl_2$: add 5.549 g of $CaCl_2$ into 50 mL of distilled H_2O, dissolve, and autoclave (place at 4 °C until use).
5. M9 media 1×: add 600 mL of M9 salts 5×, 6 mL of 1 M $MgSO_4$, 300 μL of $CaCl_2$, 12 g of glucose in powder into an autoclaved 5 L Schott bottle. Adjust to 3000 mL with distilled autoclaved H_2O. Filter sterilization should be done using a steritop filter (0.2 μm). 1× M9 media should be prepared on the day of use.

2.2 Bacterial Live/Dead Staining (see Note 2 and Fig. 1)

1. LIVE/DEAD® BacLight™ Bacterial Viability Kit: SYTO 9 dye, 3.34 mM (Component A), 300 μL solution in DMSO, Propidium iodide, 20 mM (Component B), 300 μL solution in DMSO, BacLight mounting oil (Component C).

2.3 Ultrafiltration (see Note 3)

1. Tangential flow filtration device (Quixstand Benchtop System) using 100 kDa hollow fiber membrane and all the following solutions to wash the tangential flow device.
2. Phosphate-buffered saline, PBS 1×: heat to 50 °C.
3. Sterile distilled water, 5 L.

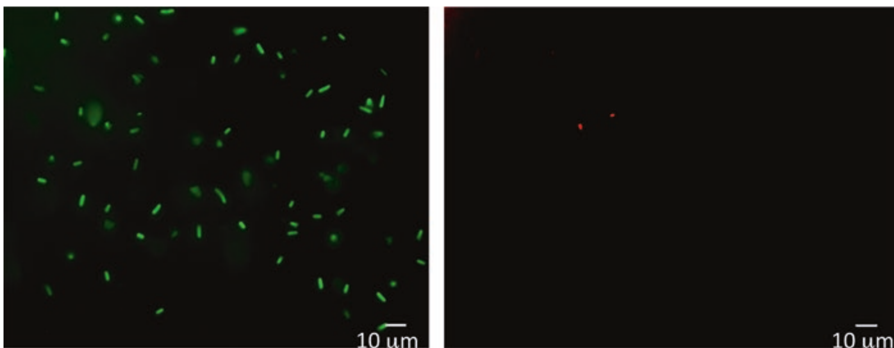

Fig. 1 Example of a Live/Dead stain for *Salmonella* grown in M9 minimal media (OD_{600}: 1). 1 mL of the *Salmonella* culture has been stained with a commercially available Live/Dead stain following the supplier's recommendations, whereby all *Salmonella* cells are stained in green (syto 9) and only the dead cells are stained in red (propidium iodide). The left image shows all the *Salmonella* cells present on the imaged area of the slide and the right image shows the dead cells within the same imaged area. Scale bar: 10 μm

4. NaOH–NaOCl solution (0.5 M–300 ppm): 10 g NaOH and 0.03% NaOCl in 500 mL sterile distilled water; heat to 50 °C.

2.4 Ultracentrifugation (see Note 4 and Fig. 2)

1. Precooled Beckman ultracentrifuge with a fixed angle rotor (90 Ti) and precooled thickwall, polypropylene, (10mL, 16 × 76mm) ultracentrifugation tubes.
2. Optiprep diluent buffer, ODB: 50 mM Hepes, 1.19 g into 500 mL of distilled water, and 150 mM NaCl, 4.24 g into 500 mL of distilled water, pH 6.8. Filter sterilization should be done using a steritop filter (0.2 μm) and the ODB should be stored at 4 °C until use.

2.5 Iodixanol Gradient Separation Protocol (see Fig. 3)

1. Optiprep diluent buffer (ODB): *see* Subheading 2.4.
2. OptiPrep™ Density Gradient Medium: Iodixanol solution.
3. Precooled Beckman ultracentrifuge with swinging bucket rotor (SW40 Ti) and precooled thinwall, polypropylene, (14 mL, 14 × 95 mm) ultracentrifugation tubes.

2.6 SDS-PAGE and Immunoblotting

1. 12% Bis-Tris selfcasted or precasted gels.
2. SDS-PAGE running buffer, 1×: 50 mM MOPS, 50 mM Tris Base, 0.1% SDS, 1 mM EDTA, pH 7.7: To prepare 500 mL of 20× MOPS SDS Running Buffer, dissolve 104.6 g of MOPS, 60.6 g of Tris Base, 10 g of SDS, and 3.72 g of EDTA in 400 mL ultrapure water; mix well and adjust the volume to

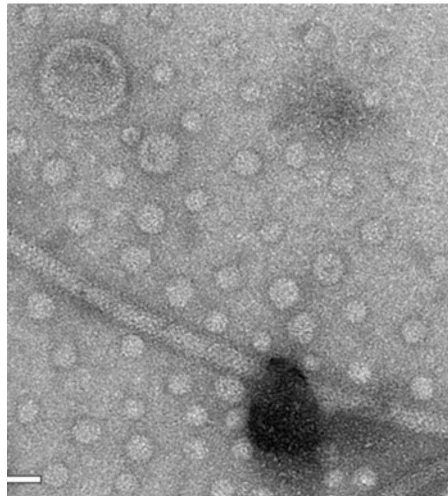

Fig. 2 Example of crude OMVs isolated from *Salmonella* grown in M9 media. An electron microscopy micrograph showing OMVs of sizes ranging from 20 to around 100 nm. The scale bar represents 50 nm. The long tubular structure in the middle of the micrograph represents flagellum, pilus, or fimbrium. The presence of these structures shows that crude OMVs are not pure and are still containing large protein complexes

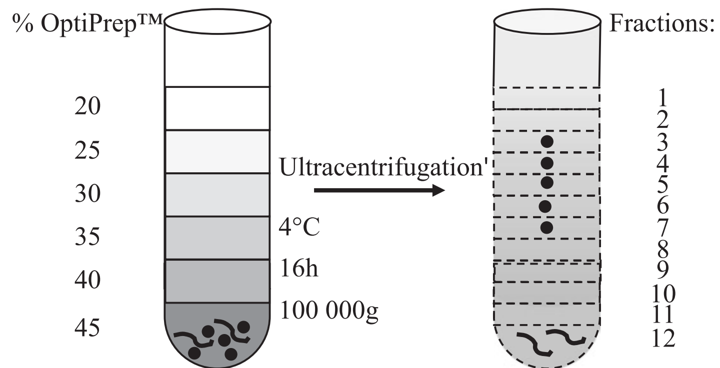

Fig. 3 Illustration of the bottom-up density gradient ultracentrifugation. Before ultracentrifugation, visible separations between the different density fractions can be observed (intact lines), whereas no clear visible layers are observable after ultracentrifugation (dashed lines). Crude OMVs are loaded in the bottom fraction and during ultracentrifugation vesicles migrate into the lower density fractions, while non-vesicular structures remain in the high density fraction. 1 mL fractions are taken from the top to the bottom and can then be further analyzed for the presence of vesicles

500 mL with ultrapure water; the buffer is stable for 6 months when stored at 4 °C. For electrophoresis, dilute this buffer to 1× with water. The pH of the 1× solution is 7.7. Do not use acid or base to adjust the pH.

3. Precision Plus Protein All Blue Standards (or any other protein ladder for polyacrylamide gels). For the lysis, denaturation and protease protection of the OMV samples: RIPA buffer, mini complete protease cocktail and Laemmli buffer with 10% β-mercaptoethanol.
4. Transfer Buffer: Mix 1 vol of 10× Tris/Glycine/SDS with 2 volume of methanol and 7 volume of ultrapure water. For 1 L: mix 100 mL of 10× Tris/Glycine/SDS, 200 mL methanol and 700 mL water. Store at 4 °C.
5. Blocking buffer: For 50 mL, mix 5 mL of 10× PBS and 1.5 g BSA and fill up to 50 mL with ultrapure water. Store at 4 °C.
6. Washing buffer: For 500 mL, mix 50 mL of 10× PBS and 2.5 mL of Tween-20. Store at 4 °C.
7. Polyvinylidene difluoride (PVDF) membrane and western blotting filter paper.
8. First and second antibody dilution buffer: For 10 mL, mix 1 mL 10× PBS, 0.3 g BSA, and 0.05 mL Tween-20. Prepare fresh for each SDS-PAGE.
9. First and secondary antibody. As an example, we use in the protocol as a first antibody a rabbit anti-*Salmonella typhi* outer

protein A and as a second antibody a goat anti-rabbit coupled to HRP (*see* **Note 5**).

2.7 Trichloroacetic Acid (TCA) Precipitation (see Note 6)

1. 20% of w/v TCA: for 100 mL: 20 g of TCA filled up to 100 mL with ultrapure water.
2. 80% ice-cold acetone: 40 mL of 100% acetone and 10 mL of sterile distilled water.

2.8 Small RNA Extraction (see Note 7)

1. Any commercially available kits allowing the extraction of small RNA.
2. 10 μg/mL lysozyme solution: add 1 mg of lysozyme to 1 mL of ultrapure water (1 mg/mL solution), mix, and dilute 100× to obtain a 10 μg/mL solution. The lysozyme solutions should be prepared just before the small RNA extraction and should not be stored for further use.
3. RNA clean & concentrator-5 kit (supplied with DNase I).
4. Agilent 2100 Bioanalyzer and Agilent Small RNA kit.

2.9 Small RNA Sequencing and Basic Analysis

1. To prepare a small RNA sequencing library different commercially available kits (NEB, Trueseq Illumina; or Perkin Elmer) can be used and the library can be sequenced using a single-end sequencing strategy on an Illumina Genome Analyzer, MiSeq, or NextSeq, where the maximum read length is set to 50 nt.
2. The data analysis can be performed on any computer having a command-line available (Linux/Mac) and Perl (http://www.perl.org) installed.
3. To process the data, the following software is needed: FastQC: for sequence data quality control [15], FASTX-Toolkit: for trimming and processing of sequence data [16], NovoAlign/NovoIndex: for mapping sequence data onto a reference genome [17, 18], HTSeq: Python tool to count RNA-Seq sequencing reads for genomic features [19], DESeq: R package for differential expression analysis [20, 21].

3 Methods

Carry out all experimental procedures at room temperature unless otherwise specified.

3.1 Bacterial Culturing

1. Inoculate *Salmonella* from a pure glycerol stock into 3 mL of M9 defined media (*see* **Note 1**) and grow this pre-culture for 6–8 h a day at 37 °C with a rotation of 200 rpm (*see* **Note 8**).
2. When the pre-culture reached an OD_{600} of around 1, inoculate 2 × 1.5 L of M9 media with the pre-culture by splitting it into

two. Incubate these cultures at 37 °C, overnight, with a rotation of 160 rpm. When the OD_{600} is between 0.5 and 1 (*see* **Note 9**), pursue with Subheading 3.2.

3.2 Live/Dead Staining and Crude OMV Isolation via Ultrafiltration and Ultracentrifugation

1. It is highly recommended to include a Live/Dead staining of the bacterial culture used for the isolation of outer membrane vesicle (OMV)-derived RNA (*see* **Notes 2** and **9**). For this staining, any commercially available kit can be used. If over 95% of the bacterial culture stain as viable cells (Fig. 1), it is recommended to pursue with the OMV-derived RNA isolation procedure.
2. Spin down the 3 L of bacterial culture for 30 min at 4700 × *g* at 4 °C. Take off the supernatant and place it into an autoclaved clean flask (the supernatant contains the OMVs). After having taken the desired amount of the bacterial culture for bacterial-derived RNA extraction (*see* **Note 10**), discard the remaining of the bacterial pellet as recommended by the biosafety rules appropriated for the bacterial type used. Filter the supernatant containing the OMVs through a 0.22 μm or 0.45 μm filter in order to eliminate the remaining bacteria that are still present in the supernatant. Use this filtered supernatant and concentrate it by using ultrafiltration.
3. Use a Quixstand Benchtop System (or any other tangential flow device) equipped with a 100 kDa hollow fiber membrane to concentrate the bacterial supernatant 50-fold following the supplier's recommendations (*see* **Note 11**). All supernatant that is not placed immediately into the tangential flow device should be kept at 4 °C until use. The concentrate (60 mL) should be filtered again through a 0.22 or 0.45 μm in order to be sure that no bacterial cells remain in the supernatant. Store the concentrate overnight at 4 °C or go on with ultracentrifugation.
4. Place the concentrate into precooled 10 mL ultracentrifuge tubes uniformly. Place the balanced tubes into a Beckman ultracentrifuge with a rotor 90 Ti (fixed angle rotor; the rotor and centrifuge should be precooled at 4 °C before use) and run with 150,000 × *g* for 2 h at 4 °C. After ultracentrifugation, the tubes should be turned upside down and the last drops should be absorbed on a tissue paper. The OMV pellets should be dissolved and pooled in 500 μL of ODB (depending on the amount of bacterial culture and the type of bacterial media used the crude OMV pellet will be visible or not; *see* **Notes 1** and **11**). The crude OMVs resuspended in buffer can be stored for 10 days at 4 °C. It is important to note that the pellet isolated at this step is highly enriched in OMVs but still contains larger protein complexes which may associate with RNA (*see* **Note 4** and Fig. 2) [10]. Therefore, we recommend to pro-

ceed with an iodixanol gradient separation in order to obtain pure OMVs. If, however, only a crude OMV isolation is desired, then the protocol can be stopped here and the pellet can be resuspended in 1× PBS or ODB. RNA extraction can be performed from crude OMVs if the absolute proof that a given RNA is associated with OMVs for follow-up experiments is not required. In that scenario, please skip Subheadings 3.3 and 3.4 and continue with Subheading 3.5.

3.3 Isolation of Pure OMVs via Iodixanol Gradient Separation (Fig. 3)

All the steps should be performed on ice.

1. Prepare the different OptiPrep™ density solutions by following the pipetting scheme shown in Table 1.
2. Follow a bottom-up approach to build up the density gradient within a 14 mL ultracentrifugation tube. For this, pipette the 2 mL 45% OptiPrep™-sample solution within the bottom of the tube and overlay carefully with the 2 mL 40% OptiPrep™-ODB solution. Continue to overlay with each of the 2 mL solutions prepared in **step 1** using the 35–20% OptiPrep™-ODB solution until obtaining a 12 mL density gradient where the different layers are visibly separated.
3. Place the ultracentrifuge tube containing the density gradient into a precooled bucket and place it into a precooled swinging bucket rotor (SW 40 TI). In the opposing bucket, place a balancing tube. Run the ultracentrifuge with 100,000 × *g*, for 16 h at 4 °C and importantly switch off the break of the ultracentrifuge so that the different density fractions that will form during ultracentrifugation will not be disturbed while the ultracentrifuge breaks. When running the ultracentrifuge at 100,000 × *g* without break it takes about 40 min to stop once the run has finished.

Table 1
Iodixanol gradient pipetting scheme for one tube

	OptiPrep™ (mL)	Sample (mL)
45% OptiPrep™-sample	1.5	0.5
	OptiPrep™ (mL)	ODB (mL)
40% OptiPrep™-ODB	1.3	0.7
35% OptiPrep™-ODB	1.2	0.8
30% OptiPrep™-ODB	1.0	1.0
25% OptiPrep™-ODB	0.8	1.2
20% OptiPrep™-ODB	0.7	1.3

4. Once the ultracentrifugation is finished, collect 1 mL fractions from top to the bottom of the gradient. Keep them separated and store on ice. From each fraction take 12.5 μL to check for the OMV presence using SDS-PAGE (*see* Subheading 3.4). Store the remaining 987.5 μL at 4 °C until use.

3.4 SDS-PAGE and Immunoblotting

To identify the iodixanol density fraction that contains pure OMVs, SDS-PAGE and immunoblotting can be used (*see* **Notes 5**, **12** and Fig. 4). In a pure OMV fraction, protein components of *Salmonella* flagella should not be detectable.

1. To prepare the SDS-PAGE sample, add to each 12.5 μL aliquot from the 12 individual iodixanol fractions, 5 μL of RIPA buffer and 2.8 μL of 7× complete mini protease. Incubate the samples on ice for 5 min. Then, add 7.5 μL of β-mercaptoethanol-containing 4× Laemmli buffer to each sample. Heat the samples for 5 min at 95 °C. Samples can now be stored at −20 °C or used for SDS-PAGE immediately.
2. Fill the gel running chamber with 1× SDS-PAGE running buffer and load the samples prepared in **step 1**, as well as an appropriated protein ladder on a 12% Bis-Tris gel. Run the SDS-PAGE with 200 V for 40 min. Stop migration, remove the gel, and proceed with western blotting.
3. Pre-soak PVDF membrane in methanol, then equilibrate in transfer buffer. Also, equilibrate 2 extra thick filter papers in transfer buffer. Prepare the western-blot sandwich as follows within a semidry blotter: filter paper–PVDF membrane–Bis-Tris gel–filter paper. Transfer with 25 V, 380 mA for an appropriated amount of time allowing the transfer of proteins with a molecular weight in the range of the protein of interest.

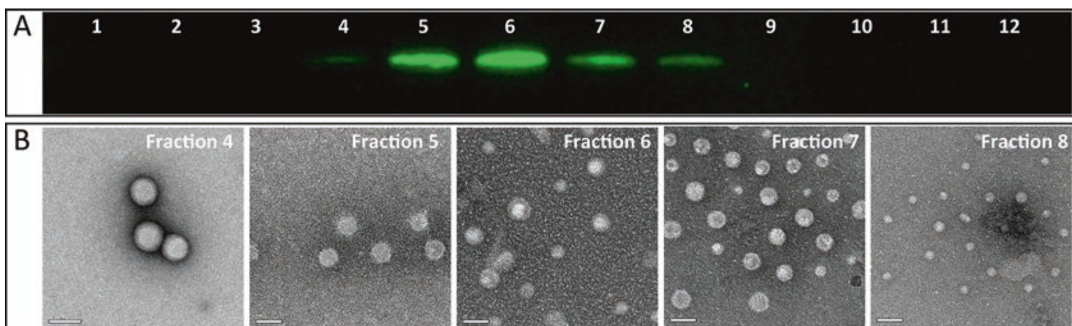

Fig. 4 Combination of electron microscopy and SDS-PAGE to identify iodixanol gradient fractions containing pure *Salmonella*-derived OMVs. (**a**) 10 μL out of the 1 mL fractions of all the individual 12 iodixanol gradient fractions had been analyzed for the presence of *Salmonella typhi* outer membrane protein A (OmpA) by SDS-PAGE. Fraction 1 being the lowest density fraction and fraction 12 being the highest density fraction. OmpA, an OMV-associated protein, is detected in fractions 4–8. (**b**) Electron micrographs showing that in fractions 4–8 spherical structures are present (OMVs have an average diameter of 20–200 nm and are bilayered). Scale bars are all 50 nm, except the scale bar in fraction 4 which represent 100 nm

4. Block the membrane with blocking buffer for 1 h. Wash the membrane 3 times for 5 min using the washing buffer. Incubate the membrane for 1 h with a first antibody directed against the protein of interest (we used antibody directed against *Salmonella typhi* outer membrane protein A: *see* **Notes 5** and **12**). Alternatively, the membrane can also be incubated with the primary antibody overnight at 4 °C. Wash the membrane 3 times for 5 min and incubate the membrane with an appropriated HRP-conjugated secondary antibody. Reveal and visualize the protein of interest by addition of peroxidase substrate and subsequent light detection by a CCD camera or photographic film. Identify the iodixanol fractions that contain OMVs and proceed with Subheading 3.5 with the 987.5 μL of these fractions set aside in Subheading 3.3 (**Note 13**).

3.5 Trichloroacetic Acid (TCA) Precipitation

TCA precipitation should be avoided if intact OMVs are required for subsequent analyses (*see* **Note 6**).

1. To each of the 987.5 μL OMV-containing fractions obtained in Subheading 3.3 and identified in Subheading 3.4, add 4 mL of ODB and 5 mL of 20% w/v TCA to achieve a final concentration of the TCA solution of 10% w/v. Vortex and incubate on ice for 30 min. Centrifuge for 30 min at 17136 × *g* at 4 °C. Discard the supernatant carefully and resuspend the pellet in 450 μL ice-cold 80% acetone. Centrifuge for 30 min at 10,000 rpm at 4 °C. Discard the supernatant carefully and dry the pellets (either let the pellets air-dry or use a vacuum pump). It is recommended to do for each OMV-containing fraction an individual TCA precipitation in order to dilute as much as possible the OptiPrep™ remaining in each fraction. When the pellets are dry, resuspend and pool them in 100 μL ODB. Keep the resuspended biomolecules at 4 °C and continue with Subheading 3.6.

3.6 Small RNA Extraction from Pure OMVs

1. Before isolation of RNA molecules from OMVs, the vesicles need to be lysed in order to release the RNA molecules. Add 10 μL of 10 μg/mL lysozyme solution, freshly prepared, to the precipitated OMV sample. Incubate for 5 min at room temperature.
2. Proceed with the extraction of RNA from the biomolecular fractions precipitated in Subheading 3.5 using either spin-column chromatography or phenol-chloroform extraction (*see* **Note 7**).
3. Isolated RNA can then further be concentrated using any commercial available clean-up and concentrator spin-columns that allow concentration of small molecules (<200 nt). Some concentrator columns can also be used to separate small and large RNA into individual fractions.

4. Finally, to digest any residual contaminant DNA within the isolated RNA sample, it is recommended to treat the extracted RNA with a DNAse.

3.7 Small RNA-Seq

1. All small RNA extracted out of OMVs (3 L *Salmonella* culture in M9 media) should be used to prepare a sequencing library (*see* **Note 1** and Fig. 5). Small RNA-Seq libraries can be prepared using commercially available kits and the library can be sequenced using a single-end sequencing strategy on an Illumina Genome Analyzer, MiSeq, or NextSeq, where the maximum read length is set to 50 nt. As OMV-derived small RNA samples are rather low yield samples, they are prone to contamination, and therefore it is highly recommended to include in the sequencing library a blank sample that has been processed exactly as the "real" samples, just that for this sample no bacteria had been inoculated in the growth media (*see* **Note 7**).
2. Optimal coverage and read depth are dependent on the experimental setup. A guide to determine recommended coverage and

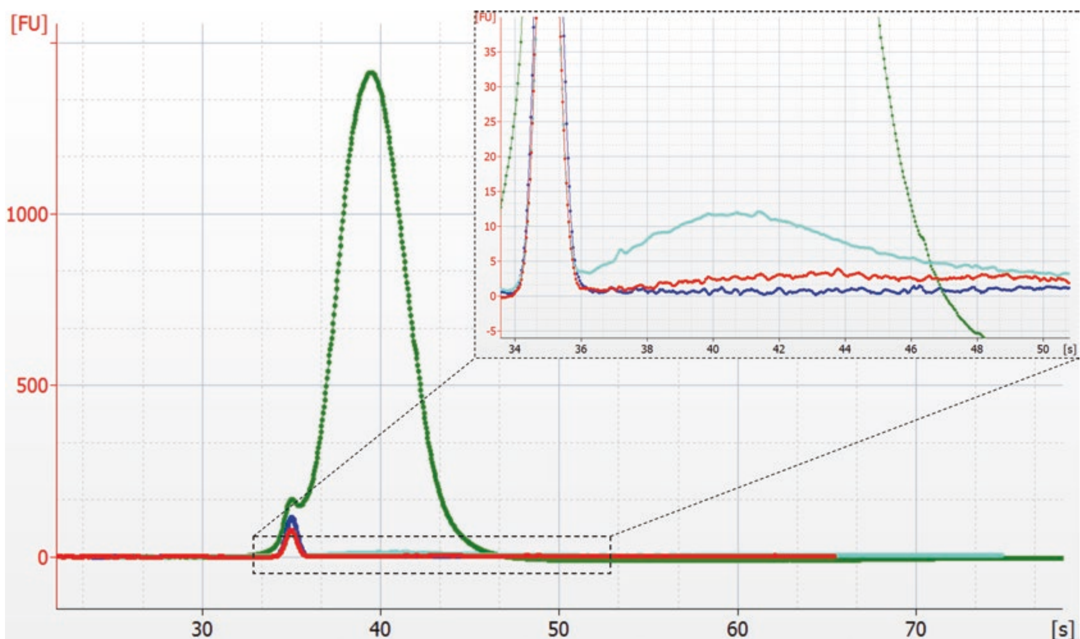

Fig. 5 Enriched broth media contains small RNA and the amount of small RNA released into OMVs varies drastically according to the growth environment of the OMV-secreting bacteria. Electropherograms of small RNA fractions isolated from density-gradient purified vesicles out of a rich broth media (light blue), of a *Salmonella* culture grown in a rich broth media (dark green) or of a *Salmonella* culture grown in a minimal chemically defined media (red). The electropherogram represented in dark blue shows the migration time and fluorescent unit of a water control sample. The OMV-secreting bacteria have been cultured to a similar OD_{600} within the chemically defined minimal media or the broth media and the supernatant volume used to isolate the small RNA has been identical for the three samples shown here

read depth is available in the following link: https://genohub.com/recommended-sequencing-coverage-by-application/.

3.8 Small RNA-Seq Analysis

1. For reference genome data acquisition, download bacterial genome sequences as well as known plasmid sequences in Fasta format and the genome annotation in Genbank (.gb), GTF (.gtf), or GFF3(.gff) format from the NCBI or EnsemblBacteria webpages. For *Salmonella*, the genome and plasmid sequences (in Fasta format) and annotations (in Genbank format) are available from NCBI, the genome accession AE006468 (https://www.ncbi.nlm.nih.gov/nuccore/AE006468) and the plasmid accession AE006471.1 (https://www.ncbi.nlm.nih.gov/nuccore/AE006471.1) or EnsemblBacteria (http://bacteria.ensembl.org/Salmonella_enterica_subsp_enterica_serovar_typhimurium_str_sl1344/Info/Index) (*see* **Note 14**).
2. For data preprocessing, FastQC [15], including visual inspection of QC reports, should be used to perform an initial quality check (QC) of Fastq (.fq) format files per lane. Fastq files should then be concatenated per condition and a second round of QC using FastQC, including visual inspection of the QC reports, should be performed and possible contamination/adapter sequences from the QC report (listed as enriched sequences) should be removed with the FastX-Toolkit [16]. The QC should be performed until FastQC detects no enriched adapter/contamination sequences anymore. Sequences are trimmed based on a Phred quality score threshold of 25 (nucleotides with lower quality are trimmed from the end of the sequence, and the Phred quality score of >25 has to apply for 100% of the bases in a sequence). Within small RNA *Salmonella* datasets, sequences with a length lower than 17 bp are prone for cross-mapping and therefore it is recommended to fix the minimum sequence length to 17 bp and to discard reads with a length < 17 bp. The remaining sequences are collapsed, so that identical sequences are represented by one sequence, but the information of the original read count is maintained in the read identifier.
3. For read mapping, a genome sequence index should be constructed with NovoIndex [17]. For example, for a *Salmonella* dataset, a *Salmonella* "genome" consists of the *Salmonella* genomic and plasmid sequence. Additional sequence indices should be built for genomes that are used for contamination filtration (e.g., human and/or yeast genomes). The mapping of trimmed sequence reads is performed with the following parameters using Novoalign [18] onto the *Salmonella* genome: the maximum acceptable mapping score of 60, which corresponds to 2 mismatches—no homopolymer filtering—a minimum length of 6 bp need to align—all mapping locations

(multiple) are reported, and the number of recorded mappings is limited to 51, which applies to the number of mappings with a score equal to the best alignment. When this limit is getting reached no further mappings are recorded and the search for this read is stopped.

4. Finally for annotation, successfully mapped reads are annotated for the genes of interest with a weighted score accounting for cross-mapping correction [22]. For example, annotation is done based on the *Salmonella* genome (AE006468) and plasmid (AE006471.1) NCBI Genbank, GTF or GFF3 files. The annotation for genetic features should comprise the assignment of different RNA types, which are “gene,” “CDS,” “tRNA,” “rRNA,” “ncRNA,” “tmRNA,” “unknown,” and, if available, some literature annotations for RNAs that could be available for other bacterial strains of interest. “CDS” refers here to the coding region of protein-coding genes. Reads are counted per genetic feature using the HTSeq tool [19] for each sample using any annotation file as input. If desired, differential expression analysis can then performed based on the count tables derived from HTseq using the R package DESeq [20, 21]. A detailed description can be found here: http://bioconductor.org/packages/release/bioc/vignettes/DESeq/inst/doc/DESeq.pdf.

4 Notes

1. For the bacterial culture, alternatively to chemically defined minimal media, enriched bacterial culture media, such as Lysogeny Broth (LB), can also be used. In that case, 1 L of culture is enough to obtain bioanalyzer-detectable amounts of OMV-associated RNA. However, it is important to note that non-bacteria-derived small RNAs are present in any broth media (Fig. 5) and appropriate controls should be taken into account. For example, for functional assays, small RNA extracted from only LB should also be tested for their functionality in comparison to the bacterial OMV-derived small RNA. For small RNA sequencing not only OMV-derived small RNA should be sequenced but also LB-derived RNA.

   It is also important to note that the amount of OMVs produced by bacteria [2, 23] and the amount of small RNA released into OMVs by bacteria does vary drastically depending on their growth environment (Fig. 5).

2. Any other bacterial viability measurement can be used. The Live/Dead staining of the cultured bacteria is only to assure that the extracellular small RNA is indeed extracted from OMVs that were actively released by intact bacteria and is not due to a contamination by small RNA released by dead bacteria.

3. Very low yield samples are always prone to contamination, and therefore it becomes essential to clean thoroughly the hollow fiber membrane and tubing from the ultrafiltration device. To get rid of nucleic acid contaminants, it is recommended to use NaOH–NaOCl (0.5 M–300 ppm) as a cleaning agent. To perform a more effective cleaning, the cleaning solution should be preheated to 50 °C before use. Clean ultrafiltration device in a closed loop for 1 h and rinse several times with water before concentration of the sample.
4. After ultrafiltration and ultracentrifugation of the bacteria-derived supernatant the recovered pellet is enriched in OMVs, but it does not contain pure OMVs (Fig. 2). If no pure OMV sample is required, the protocol can be stopped here and the OMVs can be resuspended in PBS 1×.
5. If no *Salmonella* strain is used to isolate bacteria-derived OMVs, then an antibody directed against an outer membrane protein of that given species might be used instead. It is important that electron microscopy analysis or any other technique allowing the detection of vesicles within a given sample correlates with the results of SDS-PAGE (Fig. 4 and also *see* **Note 12**).
6. If intact OMVs are required, then the TCA precipitation should be avoided and pure OMVs can alternatively be gained by following the subsequent method. Each 1 mL fraction collected after the density gradient centrifugation does still contain OptiPrep™, and to remove the latter, samples need to be diluted in ODB. Therefore, each 1 mL fraction should be transferred into a 10 mL ultracentrifugation tube and 8 mL of ODB should be added. The samples should then be ultracentrifuged using a fixed angle rotor (90 Ti) (150,000 × *g* for 3 h at 4 °C). After ultracentrifugation the supernatant should be decanted carefully in order to not perturb the pellet (not visible if minimal growth media has been used), containing pure OMVs. The pellets from the OMV-containing fractions (identified in Subheading 3.4) should be pooled and taken up in either ODB or PBS 1×.
7. RNA can be isolated from OMVs using phenol-chloroform extraction or silica-column based approaches. Phenol-chloroform extractions are more economic, and in combination with hot phenol provide higher lysis efficiency and therefore higher yields [3]. On the other hand, phenolic residues can interfere with downstream analyses, such as preparation of sequencing libraries, and the reagents are harmful. Silica-columns for the isolation of small RNAs are commercially available from a range of manufacturers and are applicable for the isolation of bacterial small RNAs. As with all reagents, care

has to be taken to control that kits are free of environmental small RNAs, like rRNA fragments, which can skew analyses, in particular when the number of RNA molecules in the samples is low. In contrast to mRNAs, which are hardly ever stable enough to contaminate lab-ware or reagents, small RNA can form stable structures, which may be further stabilized on silica matrices. Columns can be controlled by adding a defined spike—in mix of small RNAs with appropriate lysis/binding buffers, followed by washing and elution as usual. Eluates can be sequenced or analyzed by qPCR. We have observed several hundred thousands of contaminant RNA molecules per μl eluate in one line of commercial columns. In our experience microRNA enrichment columns of Norgen Biotek and Machery Nagel were free of contaminating RNAs.

8. Always prepare a non-inoculated media control tube (to compare the turbidity: bacterial growth in the inoculated tube, to have a blank for OD_{600} measurement and to see if the media per se has not been contaminated with another microorganism during the inoculation procedure).

9. An OD_{600} of 0.5–0.6 usually corresponds to the log or exponential growth phase during bacterial culture. During the log phase, bacteria are generally the most reproductive, and beyond these OD_{600} values, bacterial population stabilize and then decline. We observed that *Salmonella* culture grown in M9 media with an OD_{600} of 0.7–1 allows to isolate more OMVs compared to a culture with a lower OD_{600} value. It is however important to note that if OMV-derived RNAs are isolated from the supernatant of a bacterial culture with an OD_{600} above the exponential phase, it is crucial to check the viability of the culture before extraction of the RNA in order to exclude dead bacteria-derived RNA contamination in the OMV-resulting RNA preparation.

10. If RNA extraction is required from the bacterial pellet, it is recommended to snap-freeze a part of the bacterial pellet. To avoid overloading of the extraction columns, only roughly 10^9 bacteria should be loaded (for *Salmonella* an OD_{600} of 0.58 correspond approximately to 123×10^6 bacteria/mL). An appropriated bacterial pellet should be snap-frozen and stored at −80 °C until extractions will be done.

11. The yield of OMVs in the extracellular environment is very low, and thus to obtain a visible pellet after ultracentrifugation, a total volume of 5–7 L of bacterial culture should be used. However, depending on the amount of OMV needed, the volume of bacterial culture can be reduced [24]. It is also important to note that the amount of OMVs produced by bacteria is dependent on the environmental conditions that the bacteria encounter (*see* **Note 1** and Fig. 5).

12. In this protocol we suggest the use of SDS-PAGE to examine the presence of OMVs within the individual iodixanol gradient fractions. However, it is important to note that SDS-PAGE can only be used if specific antibodies directed against OMV-associated proteins are available. Moreover, SDS-PAGE should be combined at least once with another technique allowing for the detection of vesicles in order to correlate the presence of an OMV-associated protein within a given fraction with the presence of intact vesicles within that same fraction. Techniques that could be used are electron microscopy, single-particle tracking, atom force microscopy, and high-resolution flow cytometry [25–27]. We combined our SDS-PAGE experiment with an electron microscopy analysis (Fig. 4). Therefore, we collected 1 mL fractions from top to bottom of the iodixanol gradient and added in each fraction 8 mL of ODB buffer in order to dilute the iodixanol. We ultracentrifuged the 12 individual fractions for 2 h at 4 °C at 150,000 × *g*. After ultracentrifugation we discarded the supernatant and resuspended the pellet in 100 μL of ODB. For SDS-PAGE analysis we used 10 μL of each fractions and the remaining 90 μL had been fixed with 4% paraformaldehyde and analyzed by electron microscopy (EM). We detected by SDS-PAGE the *Salmonella typhi* outer membrane protein A (OmpA) in fractions 4–8 and by EM we observed vesicular structures in the exact same fractions. Interestingly, we could not observe any vesicles by EM in the high density fractions (fractions 10–12) and identically we could not detect any OmpA by SDS-PAGE in these same fractions indicating that no or almost no vesicles-free OmpA is present in our sample. Interestingly, we were also unable to observe any long tubular structure representing a flagella or pilus or fimbria in the fractions 4–8, indicating the purity of the vesicle preparation.
13. From the SDS-PAGE analysis not only OMV-containing iodixanol fractions can be visualized but also iodixanol fractions that are OMV-free (usually fractions 10–12).
14. As OMV-derived RNA samples are low yield samples, it is important to control contamination sources, and thus sequenced reads should be mapped against possible contamination genomes and sequences, e.g., the human (current version of the human genome (hg38) from the UCSC webpage (https://genome.ucsc.edu/)) or yeast genomes (SGD database; http://www.yeastgenome.org/) or the PhiX phage sequence (NCBI accession number: NC_001422.1; https://www.illumina.com/products/by-type/sequencing-kits/cluster-gen-sequencing-reagents/phix-control-v3.html) that is often used as a spike—in control for Illumina sequencing runs. Unmapped "bacterial" reads from the analyzed dataset are

mapped optionally against the human hg38 and yeast genome to check for sample contamination using NovoAlign with the same parameter as used for the mapping onto the genome of interest.

Acknowledgments

This work was supported by a CORE programme grant (CORE/14/BM/8066232) to J.V.F and by a Proof-of-Concept grant (PoC/13/02) to P.W, all funded by the Luxembourg National Research Fund (FNR). We are grateful to Dr. Jean-François Ménétret from the Department of Structural Biology and Genomics Institute of Genetics and of Molecular and Cellular Biology (IGBMC; France) for the acquisition of the electron microscopy images presented in this protocol. We also thank Alton Etheridge and David Galas (Pacific Northwest Diabetes Research Institute, Seattle, Washington 98122) for RNA-Seq. Bioinformatics analyses presented in this book chapter were carried out in part using the HPC facilities of the University of Luxembourg (http://hpc.uni.lu).

References

1. Mashburn-Warren LM, Whiteley M (2006) Special delivery: vesicle trafficking in prokaryotes. Mol Microbiol 61:839–846. https://doi.org/10.1111/j.1365-2958.2006.05272.x
2. Bonnington KE, Kuehn MJ (2016) Outer membrane vesicle production facilitates LPS remodeling and outer membrane maintenance in salmonella during environmental transitions. MBio 7:e01532-16. https://doi.org/10.1128/MBIO.01532-16
3. Kadurugamuwa JL, Beveridge TJ (1995) Virulence factors are released from Pseudomonas aeruginosa in association with membrane vesicles during normal growth and exposure to gentamicin: a novel mechanism of enzyme secretion. J Bacteriol 177:3998–4008
4. Mashburn LM, Whiteley M (2005) Membrane vesicles traffic signals and facilitate group activities in a prokaryote. Nature 437:422–425. https://doi.org/10.1038/nature03925
5. Kuehn MJ, Kesty NC (2005) Bacterial outer membrane vesicles and the host-pathogen interaction. Genes Dev 19:2645–2655. https://doi.org/10.1101/gad.1299905
6. Shen Y, Giardino Torchia ML, Lawson GW et al (2012) Outer membrane vesicles of a human commensal mediate immune regulation and disease protection. Cell Host Microbe 12:509–520. https://doi.org/10.1016/j.chom.2012.08.004
7. Park K-S, Choi K-H, Kim Y-S et al (2010) Outer membrane vesicles derived from Escherichia coli induce systemic inflammatory response syndrome. PLoS One 5:e11334. https://doi.org/10.1371/journal.pone.0011334
8. Bomberger JM, Maceachran DP, B a C et al (2009) Long-distance delivery of bacterial virulence factors by Pseudomonas aeruginosa outer membrane vesicles. PLoS Pathog 5:e1000382. https://doi.org/10.1371/journal.ppat.1000382
9. Koeppen K, Hampton TH, Jarek M et al (2016) A novel mechanism of host-pathogen interaction through sRNA in bacterial outer membrane vesicles. PLoS Pathog 12:1–22. https://doi.org/10.1371/journal.ppat.1005672
10. Ghosal A, Upadhyaya BB, Fritz JV et al (2015) The extracellular RNA complement of *Escherichia coli*. Microbiologyopen 4:252–266. https://doi.org/10.1002/mbo3.235
11. Biller SJ, Schubotz F, Roggensack SE et al (2014) Bacterial vesicles in marine ecosystems. Science 343:183–186
12. Sjöström AE, Sandblad L, Uhlin BE, Wai SN (2015) Membrane vesicle-mediated release of

bacterial RNA. Sci Rep 5:15329. https://doi.org/10.1038/srep15329

13. Blenkiron C, Simonov D, Muthukaruppan A et al (2016) Uropathogenic Escherichia coli releases extracellular vesicles that are associated with RNA. PLoS One. https://doi.org/10.1371/journal.pone.0160440
14. Tseng T-T, Tyler BM, Setubal JC (2009) Protein secretion systems in bacterial-host associations, and their description in the Gene Ontology. BMC Microbiol 9(Suppl 1):S2. https://doi.org/10.1186/1471-2180-9-S1-S2
15. Bioinformatics B (2014) FastQC: a quality control tool for high throughput sequence data. http://www.bioinformatics.babraham.ac.uk/projects/fastqc/
16. Lab H FASTX-Toolkit: FASTQ/A short-reads pre-processing tools. http://hannonlab.cshl.edu/fastx_toolkit/
17. NovoCraft (2008) NovoIndex. http://www.novocraft.com/documentation/novoindex/
18. NovoCraft (2009) NovoAlign. http://www.novocraft.com/products/novoalign/
19. Anders S, Pyl PT, Huber W (2015) Genome analysis HTSeq—a Python framework to work with high-throughput sequencing data. Bioinformatics 31:166–169. https://doi.org/10.1093/bioinformatics/btu638
20. Anders S, Huber W (2010) Differential expression analysis for sequence count data. Genome Biol 11:R106. https://doi.org/10.1186/gb-2010-11-10-r106
21. Love MI, Huber W, Anders S (2014) Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol 15:550. https://doi.org/10.1186/PREACCEPT-8897612761307401
22. de Hoon MJ, Taft RJ, Hashimoto T, Kanamori-Katayama M, Kawaji H, Kawano M, Kishima M, Lassmann T, Faulkner GJ, Mattick JS, Daub CO, Carninci P, Kawai J, Suzuki HHY (2010) Cross-mapping and the identification of editing sites in mature microRNAs in high-throughput sequencing libraries. Genome Res 20:257–264
23. Klimentová J, Stulík J (2015) Methods of isolation and purification of outer membrane vesicles from gram-negative bacteria. Microbiol Res 170:1–9. https://doi.org/10.1016/j.micres.2014.09.006
24. Youn Kim O, Sil Hong B, Park K-S, et al (2013) Preparation of outer membrane vesicle from Escherichia coli. 3. http://www.bio-protocol.org/e995
25. Gardiner C, Di Vizio D, Sahoo S et al (2016) Techniques used for the isolation and characterization of extracellular vesicles: results of a worldwide survey. J Extracell Vesicles 5:32945. https://doi.org/10.3402/jev.v5.32945
26. Maas SLN, de Vrij J, van der Vlist EJ et al (2015) Possibilities and limitations of current technologies for quantification of biological extracellular vesicles and synthetic mimics. J Control Release 200:87–96. https://doi.org/10.1016/j.jconrel.2014.12.041
27. Lötvall J, Hill AF, Hochberg F et al (2014) Minimal experimental requirements for definition of extracellular vesicles and their functions: a position statement from the International Society for Extracellular Vesicles. J Extracell Vesicles 3:26913. https://doi.org/10.3402/jev.v3.26913