

Isolation and Characterization of Exosomes from Mouse Feces

Chunhua Yang^{1, *}, Mingzhen Zhang¹, Junsik Sung¹, Lixin Wang^{1, 2}, Yunjin Jung^{1, 3} and Didier Merlin^{1, 2}

¹Institute for Biomedical Sciences, Center for Diagnostics and Therapeutics, Digestive Disease Research Group, Georgia State University, Atlanta, Georgia, 30302, United States; ²Atlanta Veterans Affairs Medical Center, Decatur, Georgia, 30033, United States; ³College of Pharmacy, Pusan National University, Busan, Republic of Korea

*For correspondence: cyang16@gsu.edu

[Abstract] Exosomes secreted by colonic epithelial cells are present in feces and contain valuable epigenetic information, such as miRNAs, proteins, and metabolites. An in-depth study of this information is conducive to the diagnosis or treatment of relevant diseases. A crucial prerequisite of such a study is to establish an efficient isolation method, through which we can obtain a relatively more significant amount of exosomes from feces. This protocol is designed to effectively isolate a large number of exosomes from contaminants and other particles in feces by a combined method with fast filtration and sucrose density gradient ultracentrifugation. Exosomes generated by this method are suitable for further RNA, protein, and lipid analysis.

Keywords: Colonic epithelial cells, Fecal exosomes, Isolation, Ultracentrifugation, Characterization

[Background] Colonic exosomes are secreted into the lumen by colonic epithelial cells, and they transit along the large intestine and present in feces. The lipid bilayer structure of these exosomes can prevent the degradation of encapsulated biomolecules (such as miRNAs) from complex conditions (as in feces) (Koga *et al.*, 2011; Deng *et al.*, 2013). This protective function of exosomes is especially useful, as these protected contents can be used to diagnose the diseases, such as colitis and colonic cancer. Importantly, reengineered exosomes can also efficiently deliver therapeutic biomolecules to some specific disease targets without generating immune toxicity to the host (Sun *et al.*, 2010; Johnsen *et al.*, 2014; Wang *et al.*, 2016; Kim and Kim, 2018).

To date, exosomes have been successfully isolated from blood (Wu *et al.*, 2017), urine (Knepper and Pisitkun, 2007; Motamedinia *et al.*, 2016), cultured cell (Yeo *et al.*, 2013), and tissue samples (Gallart-Palau *et al.*, 2016; Vella *et al.*, 2017). However, the isolation and characterization of exosomes from feces remain understudied, mainly because of their low abundance and the complexity of the biomatrix. Although few studies reported using CD63 conjugated immunomagnetic beads to isolate of exosome from feces (Koga *et al.*, 2011), this method may not be able to capture a large number of exosomes for specific diagnostic studies, such as the metabolomics analysis, not to mention furthering the drug delivery studies. Contrarily, our protocol enables the production of a relatively large amount of exosomes and is suitable for further RNA, protein, and lipid analysis (Yang *et al.*, 2019).

Materials and Reagents

1. Powder-free gloves (Denville Scientific, catalog number: G4162)
2. Disposable spatulas (VWR, catalog number: 80081-194)
3. 50 ml conical tubes (Denville Scientific, catalog number: C1062-P (1000799))
4. Sterilized glass funnel, 50 mm (Pyrex)
5. Scissor
6. Metal forceps
7. Filter paper, Medium/Fast flow (VWR®, Grade 417, catalog number: 28313-068)
8. 50 ml vacuum-driven filtration system (Steriflip®, Millipore, 0.22 µm pore size, catalog number: SCGP00525)
9. BioLite 96-well plate (Thermo Fisher Scientific, catalog number: 12-556-008)
10. Nitrocellulose membranes/filter paper (Bio-Rad, catalog number: 1620215)
11. Pipettes (Eppendorf, 2.5, 10, 20, 100, 200, and 1,000 µl)
12. Formvar®-coated copper grids (Electron Microscopy Sciences, catalog number: FCF300-CU-SC)
13. Mica sheet (Electron Microscopy Sciences, catalog number: 71855-15)
14. Regular pipette tips
15. Paper towels
16. 130 oz paper buckets with 215 mm lids (Karat, catalog number: C-FB130W)
17. Basic mouse cage assembly includes an internal water bottle (Optimice®, catalog number: C79100 M/P/S)
18. Anti-CD63, rat anti-mouse (BD Biosciences, catalog number: 564221)
19. Bovine serum albumin (Sigma-Aldrich, catalog number: A2153-10G)
20. Pre-stained protein standards, 10-250 KD (Precision Plus Protein™, Bio-Rad, catalog number: 1610373)
21. Non-fat milk (Sigma-Aldrich, catalog number: M7409-1BTL)
22. Sodium dodecyl sulfate (SDS) powder (BIO-RAD, catalog number: 1610301)
23. Glycine (Sigma-Aldrich, catalog number: 50046-50G)
24. Roche protease inhibitor cocktail, tablets (Sigma-Aldrich, catalog number: 4693159001)
25. Sucrose (Calbiochem®, Millipore, catalog number: 573113-1KG)
26. Ponceau S solution (Sigma-Aldrich, catalog number: P7170-1L)
27. DC™ Protein assay reagent kit II (Bio-Rad, catalog number: 500-0002)
28. Phosphate-Buffered Saline (PBS) (Corning, catalog number: 21-040-CV)
29. RIPA Lysis Buffer, 10x (Sigma-Aldrich, catalog number: 20-188)
30. Laemmli buffer, 2x (Bio-Rad, catalog number: 1610737)
31. 4-20% pre-casted gels (Bio-Rad, catalog number: 4561094)
32. ECL Prime Western blotting detection reagent (GE Healthcare Life Sciences, catalog number: RPN 2232)

33. Tris Base (Fisher Scientific, catalog number: BP152-500)
34. Methanol (Sigma-Aldrich, catalog number: 34860-2L-R)
35. Uranyl Acetate powder (Electron Microscopy Sciences, catalog number: 22400)
36. Running buffer for Western Blotting (see Recipes)
37. Transfer buffer for Western Blotting (see Recipes)
38. 1% Uranyl Acetate solution (see Recipes)

Equipment

1. Optima™ L-90K ultracentrifuge (Beckman Coulter, catalog number: 969349)
2. Fixed angle type 45 Ti rotor (Beckman Coulter, Brea, CA, catalog number: 339160)
3. 70 ml Centrifuge bottles (Beckman Coulter, catalog number: 355655)
4. SW 41 Ti rotor, swinging bucket, titanium, 6 x 13.2 ml, 41,000 rpm, 288,000 x *g* (Beckman Coulter, catalog number: 331362)
5. Thin wall, Ultra-Clear™ 13.2 ml, 14 x 89 mm ultracentrifuge tubes (Beckman Coulter, catalog number: 344059)
6. Ultrasonic bath (Branson, catalog number: 3510MTH)
7. Transmission electron microscope (TEM, LEO 906E, Carl Zeiss, Germany)
8. Atomic force microscopy (AFM, Bruker Nano Surfaces, Santa Barbara, CA)
9. Electronic balance, readability: 0.01 mg (OHAUS, model: EX125)
10. Particle size analyzer (Brookhaven Instruments Corporation, model: 90PLUS)
11. Semi-micro Cuvettes, Clear, 1.5 ml (Sigma, Catalog number: BR759115)
12. Zetasizer (Malvern, Nano series, model: Nano-ZS90)
13. Disposable Capillary Cell (Malvern, Catalog number: DTS 1070 or DTS 1061)
14. Fluorometric microplate reader (BioTek, model: Synergy 2)
15. Western blot apparatus (BioRad, PowerPac® Basic power, and Mini-Protein® Electrophoresis chamber)
16. Milli-Q water purification system (Millipore, model: Advantage A10)
17. Ice bucket
18. Timer

Software

1. 90 Plus Particle Sizing Software (Brookhaven Instruments Corporation)
2. Malvern Zetasizer

Procedure

- A. Preparing the feces sample (selection of the mouse strain depends on the animal model of interest, in our case, we use C57BL/6, as DSS induced acute colitis in C57BL/6 is a well-established model)
1. Put 20 C57BL/6 mice to autoclaved mouse cages (without bedding, 5 per cage or one mouse per cage, depends on the experiment) or clean disposable paper boxes (5 per box or one mouse per box, depends on the experiment).
 2. After 2 to 3 h, collect all the feces.
 3. Store the feces to -20 °C fridge if not use immediately. The fecal pellets can be stored for one week.
- B. Isolating the total exosomes
1. Weigh the feces, add to a clean 50-ml conical tube (1.0 gram of feces in each tube, roughly 50 to 75 fecal pellets.)
 2. Take another clean 50-ml conical tube, put five protease inhibitor cocktail tablets, add 50 ml ice-cold PBS, shake until the tablets are dissolved.
 3. Add 25 ml PBS solution prepared in Step B2 to the 50-ml conical tube (contains 1.0-gram feces.)
 4. Put the tube in the 4 °C fridge for 30 min.
 5. Take out the tube, vortex for 5 min at maximum speed.

Note: A simplified workflow for isolating exosomes from feces is shown in Figure 1. All the feces should be broken up into small particles (Figure 2).

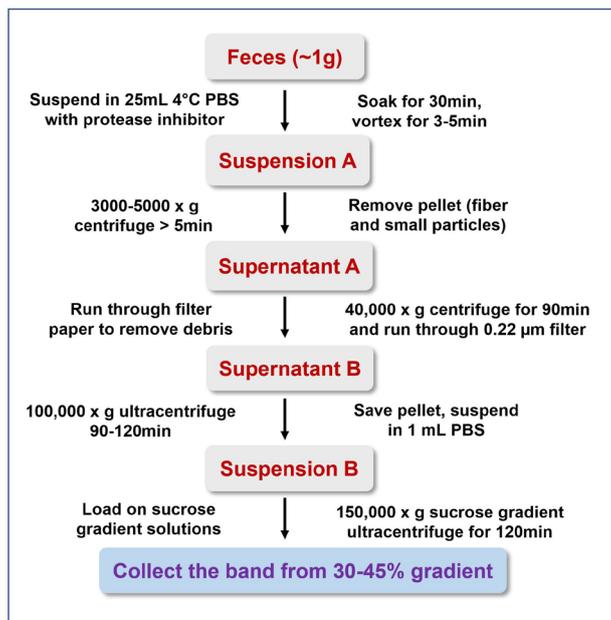


Figure 1. Simplified workflow for isolation exosomes from mouse feces

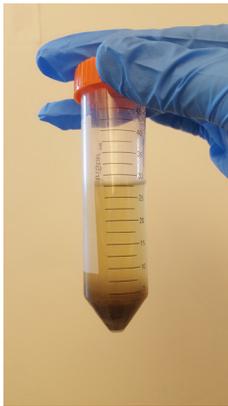


Figure 2. Fecal suspension after vortex

6. Centrifuge at 3,000 x *g* for 10 min at 4 °C, collect the supernatant.
7. Add 25 ml PBS solution prepared in Step B2 to the remains of the 50-ml conical tube.
8. Repeat Step B6.
9. Pool the supernatant.
10. Centrifuge at 3,000 x *g* for 30 min at 4 °C, collect the supernatant.
11. Set up a filtration device with a sterilized glass funnel, run the sample through filter paper to remove debris (Figure 3).



Figure 3. Paper filtration to remove the fecal debris

12. Centrifuge at 40,000 x *g* for 1.5 h at 4 °C, collect the supernatant.



Figure 4. Vacuum filtration to remove the microbes

13. Run through a 0.22 μm filter to remove microbes (Figure 4).
14. Centrifuge at 150,000 $\times g$ for 2.5 h at 4 °C, discard the supernatant, and keep the visible pellet.
15. Wash the pellet three times with 1-2 ml PBS, centrifuge at 150,000 $\times g$ for 10-15 mins (at 4 °C) between each wash and discard the first and second time's PBS, save the last time washing PBS as the exosome blank solution for protein quantitation.
16. Save the pellet (Figure 5).



Figure 5. Exosome pellet after ultracentrifugation

17. Resuspend the pellet in PBS (1 ml PBS per 1 g of feces sample).
18. Store the suspension in the 4 °C fridge before characterization.

Note: Store the samples at -80 °C if you do not test the sample in 2 days.

- C. Subfractionation of total exosome (sucrose density gradient ultracentrifugation) to isolate colonic exosomes from microbiota-derived extracellular vesicles

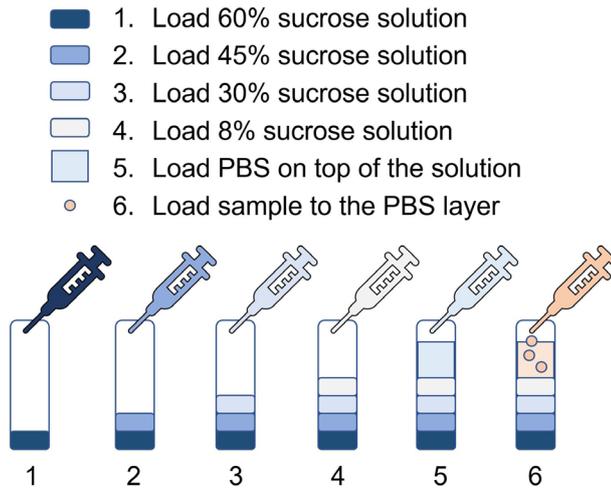


Figure 6. Setting up of the sucrose gradient

1. Make 8%, 30%, 45%, 60% (w/v) sucrose solution in double distilled water and filtrate the solution through 0.22 μm filter.

Note: Filter all the 8%, 30%, 45%, 60% sucrose solutions through 0.22 μm membrane to remove small particles before sample loading, small particles will contaminate the exosomes when doing ultracentrifugation.

2. Load the centrifuge tubes (14 x 89 mm) with sucrose solution, from 60% to 8%, with 1.5 ml of 60%, 1.5 ml of 45%, 1.5 ml of 30%, 1 ml of 8%, then 4.5 ml of PBS (Figure 6).
3. Label the sample with dye (Ponceau S Solution, 1.0 ml sample with 10 μl dye).
4. Load labeled sample to the PBS layer.
5. Centrifuge at 150,000 x g for 3 h at 4 $^{\circ}\text{C}$ (using SW41 rotor).
6. Remove the 8-30% layer by pipetting, use a glass pipette to collect the layer with dye (30-45% layer, Figure 7), dilute the collected solution with PBS to 10 times volume.

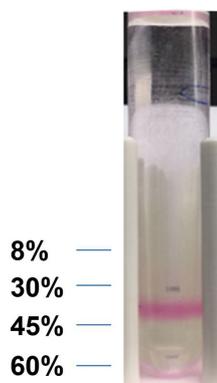


Figure 7. Labeled band of exosome

7. Centrifuge at 150,000 x g for 2 h at 4 °C, save the pellet.
8. Re-suspend the pellet in 5 ml PBS.
9. Store the suspension in the 4 °C fridge before characterization (see Note 1).

Characterization

D. Particle size and surface zeta potential

Note: Turn on the instrument (Particle size analyzer and Zetasizer) for at least 15 min before the size and zeta potential measurement.

1. Particle size measurement: Take 0.5 ml exosome solution, dilute with 2.0 ml PBS, pipette into a cuvette (avoid generating bubbles), insert cuvette to the chamber of particle size analyzer. Read the size of the particles by the 90 Plus Particle Sizing Software. The average size of the hydrated exosome is about 155 nm (152.8 to 157.9 nm in this test, see Note 2) in diameter (Figure 8).
2. Particle surface zeta potential measurement: Take a 0.8 ml diluted solution (prepared for particle size measurement in Step D1), slowly pipette into a disposable capillary cell, cover the cell, insert the cell to the chamber of zeta analyzer, and record the value. The Z-potential of the sample is about -11.2 mV (-10.2 to -12.2 mv in this test) at neutral pH (Figure 9, software: Malvern Zetasizer, see Note 2).

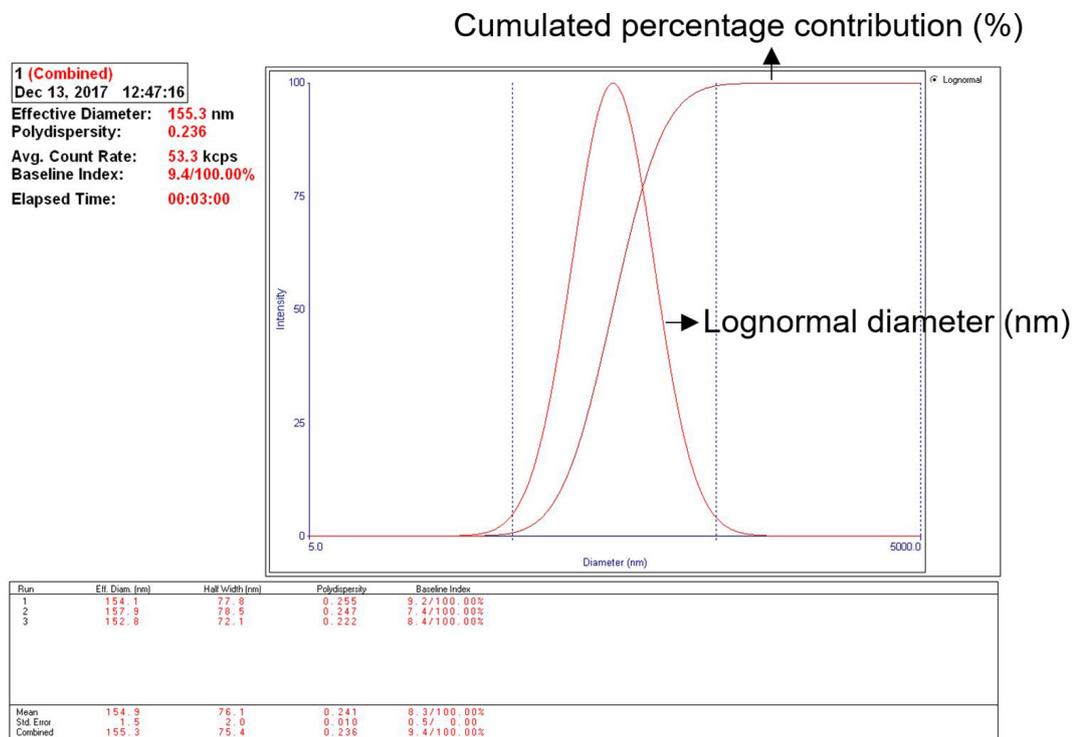


Figure 8. Particle size measurement of the hydrated fecal exosome

Sample Name: 171214exosome01 1
SOP Name: mansettings.nano
File Name: 10-6-2017.dts
Record Number: 111
Date and Time: Thursday, December 14, 2017 2:34:38 PM
Dispersant Name: Water
Dispersant RI: 1.330
Viscosity (cP): 0.8872
Dispersant Dielectric Constant: 78.5

Temperature (°C): 25.0
Count Rate (kcps): 92.9
Cell Description: Clear disposable zeta cell
Zeta Runs: 19
Measurement Position (mm): 2.00
Attenuator: 11

	Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV): -11.2	Peak 1: 0.00	0.0	0.00
Zeta Deviation (mV): 0.00	Peak 2: 0.00	0.0	0.00
Conductivity (mS/cm): 16.8	Peak 3: 0.00	0.0	0.00

Result quality : Good

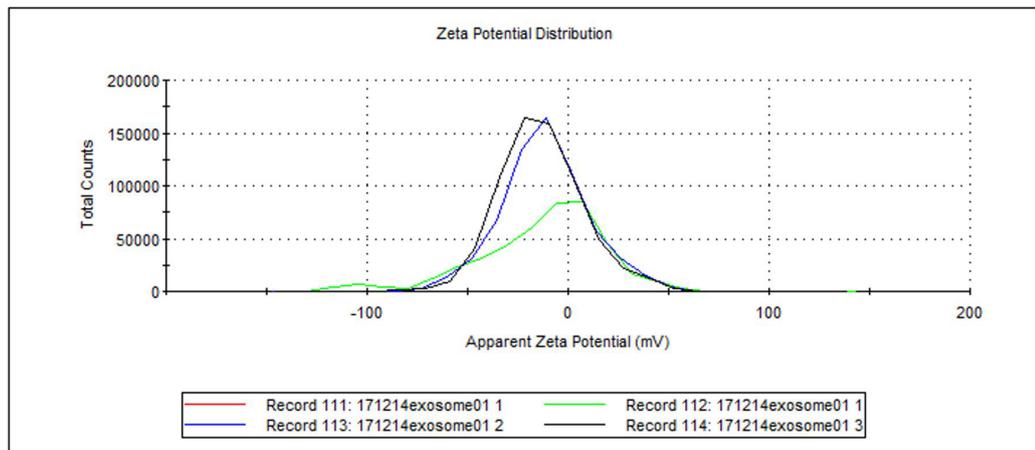


Figure 9. Zeta potential test of fecal exosomes

E. Protein concentration

1. Use the DC™ Protein assay reagent kit. Add 20 µl of reagent S to each ml of reagent A.
2. Prepare a protein standard curve containing 0.25, 0.50, 0.75, 1.0, 1.25, 1.50 mg/ml BSA in 1x PBS.
3. Pipet 5 µl of standards and samples into a clean 96-well plate.
4. Add 25 µl of reagent A' (1 ml reagent A and 20 µl of reagent S) into each well.
5. Add 200 µl reagent B into each well. Mix by pipetting up and down three times.
6. Wait for 15 min at room temperature.
7. Read the absorbance at 750 nm by a microplate reader.
8. Draw the standard curve and calculate the protein concentration.

Note: Expected protein concentration is between 0.25 to 1.5 mg/ml for exosomes isolated from 1g of feces as starting material. If the concentration is beyond the linear range (0.25 to 1.5 mg/ml, see Figure 13 and Table 1), dilute or concentrate the sample and redo the test.

F. CD-63 protein detection (Western Blotting method)

1. Add 100 μ l RIPA buffer (10x) to 900 μ l icy cold PBS (containing protease inhibitor cocktail (Roche)) to make a 1 ml solution A.
2. Take 20 μ g fecal nanovesicles (20 μ l from 1 mg/ml solution), add 100 μ l icy cold solution A.
3. Sonicate the sample for 3 times at 42 kHz, room temperature, 10-15 s each time.
4. Spin at 16,000 \times *g* for 20 min at 4 $^{\circ}$ C.
5. Take the supernatant, add the same volume of Laemmli buffer (2x).
6. Boil the samples at 95 $^{\circ}$ C for 5 min.
7. Centrifuge at 16,000 \times *g* in a microcentrifuge for 1 min.
8. Load the sample on 4-20% pre-casted gel.
9. Add running buffer (Recipe 1), run the gel for 5 min at 50 v.
10. Increase the voltage to 100 v, run the gel for 1 h.
11. Take out the gel, add transfer buffer (Recipe 2), adjust the voltage to 100 v, and transfer to nitrocellulose membranes (Chankova *et al.*, 2013).
12. Take out the nitrocellulose membrane, block with 5% non-fat milk at room temperature for 1 h.
13. Add primary antibody (Anti-CD63 1:200 dilution in PBS, and Anti-beta-actin, 1:200 dilution in PBS), incubate for 2 h at room temperature.
14. Wash 3-5 times with PBS for 5 mins, add the secondary HRP-conjugated antibody and incubate for 2 h at room temperature.
15. Detect the chemiluminescence bands with ECL Plus reagent. A representative western blotting picture is shown in Figure 10.

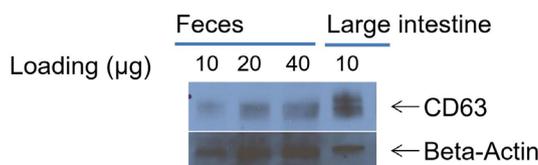


Figure 10. Western blot band of CD63 (use lysed mouse large intestine sample as control)

G. Transmission electron microscopy (TEM)

1. Pipette 1 μ g (1 μ l of 1mg/ml) fecal exosome solution in 10 μ l distilled water.
2. Deposit 5 to 10 μ l sample onto the surface of Formvar[®]-coated copper grids.
3. Add 5 μ l 1% uranyl acetate (Recipe 3) for 15 s.
4. Dry the sample at room temperature for at least 30 mins.
5. Test the sample by the transmission electron microscopy. A representative TEM picture of the fecal exosome is shown in Figure 11.

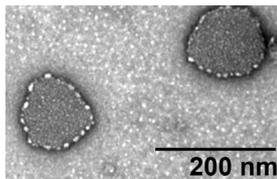


Figure 11. TEM graph of the isolated fecal exosome

H. Atomic force microscopy (AFM)

1. Pipette 0.25 μg (0.5 μl of 0.5 mg/ml) fecal exosome solution in 10 μl PBS.
2. Deposit the 5 to 10 μl sample onto a mica sheet.
3. Dry the sample at room temperature for about 2 h.
4. Add 20 μl distilled water to the mica sheet.
5. Dry the sample at room temperature for another 2 h.
6. Wait until the sample is relatively flat.
7. Scan the sample with an area about 4 x 4 μm or a smaller area with 2-50 nm in height. A typical AFM graph of the fecal exosome is shown in Figure 12.

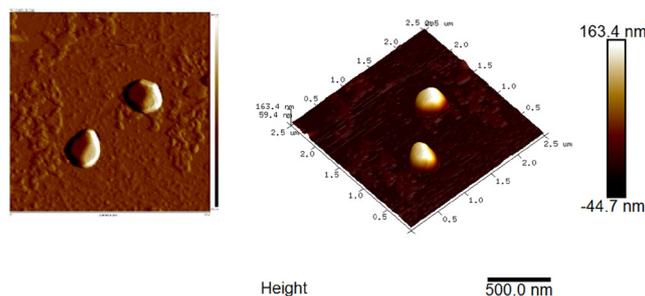


Figure 12. AFM graph of the isolated fecal exosome

Data analysis

Table 1 is a typical data analysis for the exosome sample's protein concentration test; quantification is based on the standard curve of BSA samples; this is as described in "Section E. Protein concentration".

1. Subtract the averaged blank PBS value from the standards values, (or select the "subtract blank value function" in the plate reader's software and take the value directly).
2. Take the average value of adjusted standards and make a regression equation in Microsoft Excel, calculate the correlation coefficient (R or R square), make sure the correlation coefficient is above 0.95 (Figure 13).
3. Adjust the exosome sample's value by subtracting the averaged exosome blank's (PBS) value. Take the average of the adjusted exosome sample's values, and substitute into the regression equation as "y" (OD 750), calculate the value of "x", which is the protein concentration.

4. Last, normalize the protein concentration as “mg per gram of feces” by multiplying the dilution factor. (2 x dilution means 1 g of feces in 2 ml PBS, 0.5x dilution means 1 g of feces in 0.5 ml PBS. See Note 1).

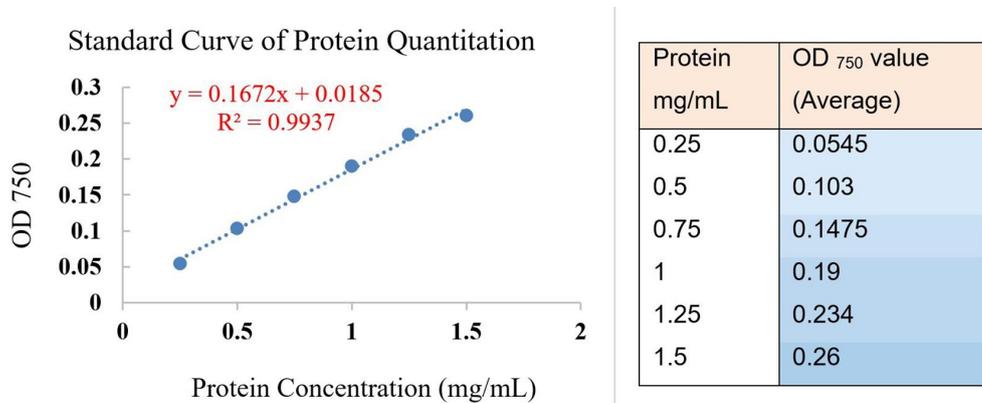


Figure 13. Standard curve and regression equation for DC™ protein concentration test

Table 1. Protein quantitation of fecal exosome samples

Standards (BSA)	Concentration (mg/ml)					
	0.25	0.5	0.75	1.0	1.25	1.5
	OD₇₅₀ value					
Standards 1	0.115	0.166	0.208	0.257	0.297	0.328
Standards 2	0.125	0.171	0.219	0.254	0.303	0.324
Blank (PBS)	0.065	0.066	0.067	0.067	0.065	0.065
Blank (Average)	0.066					
Standards 1 minus Blank	0.050	0.101	0.142	0.191	0.231	0.262
Standards 2 minus Blank	0.059	0.105	0.153	0.189	0.237	0.258
Standards minus Blank (Average)	0.055	0.103	0.148	0.190	0.234	0.260
Standard curve	Y = 0.1672x + 0.0185 (R² = 0.9937)					
Exosome Blank*	0.072	0.076		0.075		
Blank (Average)	0.074					
Exosome sample 1	0.219	0.223		0.212		
Sample 1 minus Blank	0.145	0.149		0.138		
Sample 1 concentration	0.750 mg/ml					
Normalized concentration (2x dilution)	1.500 mg/g					
Exosome sample 2	0.188	0.201		0.202		
Sample 2 minus Blank	0.114	0.127		0.128		
Sample 2 concentration	0.630 mg/ml					
Normalized concentration (2x dilution)	1.260 mg/g					
Exosome sample 3	0.178	0.178		0.176		
Sample 3 minus Blank	0.104	0.104		0.102		
Sample 3 concentration	0.510 mg/ml					
Normalized concentration (0.5x dilution)	0.26 mg/g					

Notes

1. The yield of total exosome before sucrose density gradient ultracentrifugation is about 0.8-2.0 mg per gram of feces, the yield of exosome sub-fraction from 30-45% sucrose density is around 0.1-0.3 mg per gram of feces. The quantitation is performed by the protein quantitation method.
2. The size of isolated fecal exosomes may vary from 125 to 170 nm; Surface zeta potential range may vary from -14.0 to -9.0 mv.

Recipes

1. Running buffer for Western Blotting (pH 8.3, store in 4 °C, shelf life: 3 months)
25 mM Tris
190 mM glycine
0.1% w/v SDS in distilled water
2. Transfer buffer for Western Blotting (pH 8.3, store in 4 °C, shelf life: 3 months)
25 mM Tris
190 mM glycine
20 % v/v methanol in distilled water
3. 1% Uranyl Acetate solution (pH 4.5, store in 4 °C, shelf life: 3 months)
 - a. Use prepared 4% Uranyl Acetate stock solution: 4 grams Uranyl Acetate powder dissolved in 100 ml preheated milli-Q filtered water (50-60 °C)
 - b. Mix 1 ml 4% stock solution with 3 ml of Milli-Q filtered water

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Competing interests

The authors declare no conflicts of interest within the work.

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