Plasma Exosome Endothelial Inflammation Kit

Appendix 2 - IP of Human Endothelial-derived Exosomes (EDEs) from PPP

1. ISOLATION OF EDEs

Equipment & Consumables:

Equipment Name	Manufacturer & Catalog Number	Product Link
Eppendorf Research Plus Pipette: 1-10µl,10-100µl, 200µl, 1000µl (calibrated <6months)		
1.5 ml Eppendorf tubes	ThermoFisher Scientific 05408129	Eppendorf Tubes
Tube Revolver 120V/US PLUG, speed set to 10 rpm	ThermoFisher Scientific 11676341	Tube revolver
15 ml conical tubes	ThermoFisher Scientific 339650	15ml tube
50 ml conical tubes	ThermoFisher Scientific 339652	50ml tube
Plate/tube shaker		
Benchtop cooling centrifuge		
Vortex		
VersaMax ELISA (Absorbance Microplate Reader): Molecular Devices		
Ice Tray/ Bucket		
Tube rack		

Product Name	Manufacturer & Catalog Number	Product Link	Notes
Thromboplastin-D	ThermoFisher Scientific 100357	Thromboplastin-D	Sites must request quote from ThermoFisher to purchase
Dulbecco's balanced salt solution	ThermoFisher Scientific 14190136	DPBS, no calcium, no magnesium	
100x Protease inhibitor cocktail	Sigma-Aldrich/ MilliPore-SIGMA P8340-1ML	Protease inhibitor	
100x Halt Protease/Phosphatase inhibitor cocktail	Fisher Scientific 78441	Phosphatase inhibitor	
ExoQuick solution	System Biosciences Inc. EXOQ20A-1	ExoQuick solution	Request quote by email: Andre Lubarsky. Mention the MarkVCID to receive a 25% discount
100 ml Glacial acetic acid	Sigma-Aldrich A6283-100ML	Acetic acid	
Distilled water	ThermoFisher Scientific 15230001	Distilled Water	
Mouse anti-human CD31 biotinylated antibody (clone MEM-05)	ThermoFisher Scientific MA1-19510	CD31 Monoclonal Antibody (MEM-05), Biotin	
Bovine serum albumin (BSA)	ThermoFisher Scientific 37525	BSA (10X) in PBS	
Streptavidin-Plus UltraLink resin	ThermoFisher Scientific 53116 or 53117	Streptavidin Resin 2ml OR Streptavidin Resin 5ml	
M-PER mammalian protein extraction reagent	ThermoFisher Scientific 78501	M-PER Mammalian Protein Extraction Reagent	
Goat anti-human CD146 biotinylated antibody (Novus)	Novus Biologicals NBP2-47777B	CD146/MCAM	
UltraPure 1M Tris-HCl (pH 8.0)	ThermoFisher Scientific 15568025	Tris-HCl, pH 8.0	
ELISA kit CD81	Cusabio P60033	Human CD81 antigen(CD81) ELISA kit	
ELISA kit for C3b	Abcam ab195461	Human Complement C3b ELISA Kit (ab195461)	
ELISA kit for Bb	Quidel-Microvue A027	MicroVue Bb Plus EIA	

A. Isolation of All Exosomal subtypes from plasma

1. Centrifuge the thawed PPP samples at 1000xg for 10 min at 4˚C.

2. Transfer 250 µl PPP supernatants to 1.5 ml Eppendorf tubes containing 75 µl of Thromboplastin-D and incubate for 60 min at room temperature.

3. Add 175 µl DBS containing 3X protease inhibitor cocktail and 3X protease/phosphatase inhibitor cocktail (164.5 µl water + 5.25 µl Protease inhibitor cocktail + 5.25 µl Halt Protease/Phosphatase inhibitor cocktail) to each tube vortex for 10 seconds and centrifuge at 3000xg for 20 min at 4°C.

4. Aliquot 126 µl ExoQuick exosome precipitation solution into fresh tubes (chilled on ice).

5. Transfer sample supernatants to tubes containing ExoQuick, mix by inversion six times, and incubate for 60 min at 4˚C. *This incubation time must be exactly 60 min.

6. After the incubation centrifuge the samples at 1500xg for 30 min at 4°C and discard the supernatants.

7. Each pellet is resuspended in 350 µl distilled water containing 1X protease and phosphatase inhibitor cocktails (343 µl water + 3.5 µl Protease inhibitor cocktail + 3.5 µl Halt Protease/Phosphatase inhibitor cocktail) for the immunochemical enrichment of endothelial exosomes. Vortex the sample for 20 s and rotate at ~10 rpm 4˚C for 2H. Vortex the sample for 30 s to further encourage pellet resuspension and then rotate at ~10 rpm overnight at 4˚C if the pellet is not resuspended. *This step is frequently performed over night to ensure maximum resuspension of the exosome pellet. (With optimal resuspension, the solution appears semi-opaque with no obvious pellet.)

B. Immuno-precipitation of EDE

Make a master **CD31 antibody solution**: [2 µl CD31 + 15 µl 10% BSA + 33 µl DBS] per sample. ***** Use 2 µg/µl antibody for the isolations. The current lot is 1 µg/µl, so 2 µg = 2 µl. Check the lots and recalculate the amount of antibody as needed.

8. Vortex the sample as needed to ensure that the exosomes appear resuspended followed by centrifugation at 400xg for 5 min to precipitate any insoluble material.

   **** Freeze 10 µl of total exosomal prep at -80°C to be shipped to UCSF for inter-site reliability testing**

9. Transfer the supernatants to a fresh microcentrifuge and add 50 µl CD31 antibody solution to each exosome suspension before mixing for 1H at room temperature using a rotator.

Make a master **Streptavidin solution**: [10 µl Streptavidin-Plus UltraLink resin + 12 µl 10% BSA + 18 µl DBS] per sample.

10. Add 40 µl of the Streptavidin solution* to each exosome suspension and mix on rotator for 1H at room temperature. *Pipet Streptavidin solution up and down prior to taking 40 µl to ensure that equal amount of resin gets added to each tube.

11. Centrifuge samples at 4°C at 600xg for 10 min and remove the supernatant.

Make a stock solution of **0.05 M acetic acid**: slowly add 86 µl of glacial acetic acid to 7.5 ml deionized water. Adjust the final volume of the solution to 30 ml by adding 22.414 µl of deionized water. Stock solution can be stored at 4°C.

12. Resuspend each pellet in 100 µl cold 0.05M acetic acid and vortex for 10 seconds. After a 10 min stand at 4°C, the samples are centrifuged at 4°C at 4000xg for 10 min.

Make a master of **DBS + Tri-HCl solution**: [265 µl DBS + 10 µl 1M Tris-HCl + 25 µl 10% BSA] per sample.

13. Transfer the supernatants to new, prechilled 1.5 ml Eppendorf tubes containing 300 µl of the DBS + Tri-HCl solution and vortex for 10 seconds to mix.

    **** Freeze 10 µl of EDE (intermediate prep) at -80°C to be shipped to UCSF for inter-site reliability testing**

Make a master **CD146/MCAM antibody solution**: [3.08 µl CD146/MCAM + 15 µl 10% BSA + 31.92 µl DBS] per sample. * Use 2 µg/µl antibody for the isolations. The current lot is 0.65 µg/µl, so 2 µg = 3.08 µl. Check the lots and recalculate the amount of antibody as needed.

14. Add 50 µl CD146/MCAM antibody solution to each exosome suspension and mix on rotator at room temperature for 1H

Make a master **Streptavidin solution**: [10 µl Streptavidin-Plus UltraLink resin + 12 µl 10% BSA + 18 µl DBS] per sample.

15. Add 40 µl of the Streptavidin solution* to each exosome suspension and mix for 1H at room temperature using a rotator. *Pipet Streptavidin solution up and down prior to taking 40µl to ensure that equal amount of resin gets added to each tube.

16. Centrifuge the samples at 4°C at 600xg for 10 min and remove the supernatant.

17. Resuspend each pellet in 100 µl cold 0.05 M acetic acid and vortex for 10 seconds. After a 10 min stand at 4°C, the samples are centrifuged using 4000xg for 10 min at 4°C.

Make a master BSA **+ Tris-HCl solution**: [10 µl 1M Tris-HCl + 25 µl 10% BSA] per sample.

18. Transfer the supernatants to new, prechilled 1.5 ml Eppendorf tubes containing 35 µl of the BSA + Tris-HCl solution and vortex the sample for 10 seconds to mix.

    **** Freeze 10µl of EDE (final prep) at -80°C to be shipped to UCSF for inter-site reliability testing**

Make a master **MPER solution**: [357.7 µl of M-PER + 3.65 µl protease inhibitor + 3.65 µl phosphatase inhibitor] per sample.

19. Add 365 µl of M-PER solution to each sample to lyse the exosomes and extract the EDE proteomic cargo. Perform two freeze thaw cycles, freezing at -20˚C and thawing on ice, respectively, with a 10 second vortex step in between. Aliquot out the EDE lysates and place at -80˚C for long-term storage.

    **** Freeze 100 µl of EDE lysate at -80°C to be shipped to UCSF for inter-site reliability testing**

20. EDE proteins are quantified by ELISA kits for CD81, C3b, and Bb (per manufacturer's protocol).

Questions/Comments?
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