Published in final edited form as:

Methods Mol Biol. 2016 ; 1354: 311–326. doi:10.1007/978-1-4939-3046-3_21.

Proteomic characterization of exosomes from HIV-1-infected cells

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Summary

Proteomics has increasingly become an invaluable tool to characterize proteomes from various subcellular compartments. Here, we describe a quantitative proteomics method using the technique of Stable Isotope Labeling by Amino acids in Cell culture (SILAC) to analyze the effects of HIV infection on host exosomal proteomes.

The procedure, described below, involves differential isotope labeling of cells, exosome purification, mass spectrometric quantification, and various bioinformatic analyses/verifications. Although this chapter focuses on analyzing the effects of HIV-1 infection on the exosomal proteome, the protocol can easily be adapted to other subcellular compartments under different stress conditions.

Keywords

HIV-1; Exosome; Proteomics; Mass spectrometry; SILAC

1. Introduction

HIV-1 needs to bud from the host plasma membrane in order to complete its life cycle [1-2]. Exosomes, 30-100 nm vesicles secreted by a wide range of cell types [3-5], have been shown to play crucial roles during this process [6-7]. As exosomes are largely composed of various proteins [8], characterizing these proteins may allow us to better understand how HIV-1 influences exosomal cargo and the host secretion machinery.

Numerous quantitative proteomics methods have been developed in recent years. These include the commonly used metabolic labeling based SILAC technique [9]. Other examples include chemical labeling based iTRAQ (isobaric Tags for Relative and Absolute Quantification), an isobaric labeling method that can be used to determine the amount of proteins from multiple sources in a single experiment [10]. A label-free MRM (Multiple Reaction Monitoring) method has been commonly used for the targeted quantitation of proteins/peptides in biological samples [11-12]. However, a detailed comparison (Table 1)

shows that SILAC is an excellent choice for HIV-1/exosomal proteome study in cultured cells. For a majority of biomedical laboratories, the whole procedure is quite straightforward and the cost is relatively low. The principle of SILAC labeling is based on metabolic incorporation of given non-radioactive isotopic forms of an amino acid in the culture media into cellular proteins. Typically, SILAC experiments start with two cell populations: one is labeled in the culture medium composed of heavy isotopic $^{13}\text{C}_6$ L-lysine and/or $^{13}\text{C}_6^{15}\text{N}_4$ L-arginine for 6 doublings, while the other is maintained in the same medium but with normal L-lysine and/or L-arginine for the same period. Protein is extracted from the two cellular populations respectively, 1:1 mixed and subjected to mass spectrometry. The relative intensities of mass spectrometric peak(s) generated from a protein reflect its relative abundance [13]. As seen in Figure 1, we adapted SILAC based proteomics to determine the effects of HIV-1 infection on host exosomal proteome using the following procedure. Initially, HIV-1 uninfected and infected cells are differentially isotope labeled. Then, the labeled exosomes are purified and subjected to total protein extraction. Next, liquid chromatography-tandem mass spectrometry is employed to analyze the exosomal proteome. Finally, the resulting mass spectrometry data and potential candidates are subjected to statistical, bioinformatics analyses as well as vigorous biochemical verification.

2. Materials

Prepare solutions using ultrapure water and store reagents according to their specific requirements as stated below. Strictly follow all waste disposal regulations when disposing of waste materials, especially HIV-1 infected samples.

1. Cells: In order to be effectively labeled by SILAC, the cells chosen need to be in an actively proliferative stage (see **Note 1**). Depending on specific experimental settings, cells are also often required to be susceptible to HIV-1 infection. Some examples of cell lines that meet these two criteria are: HeLa T4⁺ cells a human cervical epithelial carcinoma modified to stably express CD4⁺; H9 cells and CCRF-CEM cells, both suspension cell lines derived from acute lymphoblastic leukemia.
2. Reagents for checking cell survival and proliferation rate: Trypan blue and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay kit are commercially available.
3. Dialyzed fetal bovine serum (FBS): (see **Note 2**).
4. Medium: Standard DMEM, RPMI or other medium of choice deficient in Arginine and Lysine. Please refer to <http://www.atcc.org/> for detailed composition. Store it at 4°C.
5. Amino acids: Normal light L-Lysine and L-Arginine; Heavy isotopic Lysine ($^{13}\text{C}_6$ L-lysine) and Arginine ($^{13}\text{C}_6^{15}\text{N}_4$ L-arginine) (see **Note 3**). All amino acids are commercially available.
6. Facilities for HIV-1 related research: Class II biological safety cabinets.

7. Refrigerated ultracentrifuge or super-speed centrifuge: It is required that the centrifuge chosen can reach at least 100,000g (see **Note 4**).
8. Centrifuge tubes: Tubes that can withstand ultracentrifugation and compatible with rotors.
9. Phosphate-buffered saline (PBS): 144.0 mg/L of Potassium Phosphate monobasic (KH_2PO_4), 795.0 mg/L of Sodium Phosphate dibasic ($\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$) and 9.0 g/L of Sodium Chloride (NaCl) pH7.4. Store at 4°C.
10. RIPA (radio immuno precipitation assay) buffer : 50 mM Tris, 150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate and 0.1% SDS (sodium dodecyl sulphate), pH 8.0.
11. Protease inhibitor cocktails: Commercially available.
12. Microcentrifuge shaker: A shaker with speed and temperature control is recommended.
13. Platform rocker with circular motion:
14. Benchtop microcentrifuge: Centrifuge with cooling feature is recommended.
15. Spectrophotometer: Fluorometer can be an alternative.
16. SDS-PAGE (Polyacrylamide gel electrophoresis) gel running apparatus and a quality power supply: (see **Note 5**).
17. Gel running reagents: Refer to specific literature for detailed procedures on how to prepare gel casting reagents, running buffers and sample buffers. Alternatively, precast gels and proprietary reagents can be purchased.
18. Gel staining and de-staining reagents: Staining solution: 15% methanol, 10% acetic acid and 2 g Coomassie Brilliant Blue in water; Destaining solution: 15% methanol and 10% glacial acetic acid in water.
19. Gel wash buffer: 25 mM ammonium bicarbonate (NH_4HCO_3) prepared in HPLC grade water; 25 mM ammonium bicarbonate (NH_4HCO_3) dissolved in 50% acetonitrile (1:1 ACN/ H_2O).
20. Reduction and alkylation reagents: 10 mM Dithiothreitol (DTT) in 25 mM NH_4HCO_3 and 55 mM iodoacetamide in 25 mM NH_4HCO_3 . Prepare freshly.
21. Digestion buffer: 12.5 ng/ μL mass spectrometry grade modified trypsin dissolved in freshly prepared 25 mM NH_4HCO_3 .
22. Peptide recovery buffer: 5% formic acid in 50% CAN.
23. Vacuum concentrators

24. HPLC buffers: Buffer A: 0.9% acetonitrile and 0.1% formic acid in HPLC grade water; Buffer B: 100% acetonitrile.
25. Nanoflow HPLC (High-performance liquid chromatography) and Mass spectrometer: Contact proteomics core facilities in your institution or send your samples to outside institutions that have experience in SILAC based quantitative proteomic analysis (see **Note 6**).
26. Protein/peptide identification software: Open source Andromeda available at <http://www.andromeda-search.org/> or Mascot, commercially available from MatrixScience.
27. Quantitation software: MAXQuant, freely available at <http://www.maxquant.org/> or other software from mass spectrometer instrument vendors.
28. Western blotting reagents: Related buffer and reagents are commercially available or can be prepared in the lab. Refer to western blotting specific manual for details.
29. Software and databases: ImageStudio® lite can be downloaded from http://www.licor.com/bio/products/software/image_studio_lite/ with registration; Silacratioanalyser is available at <http://proteome.moffitt.org/proteomics/silacratioanalyser/silacratioanalyser.jnlp>; STRAP (Software Tool for Rapid Annotation of Proteins) can be downloaded from <http://www.bumc.bu.edu/cardiovascularproteomics/cpctools/strap/>. Exosome database can be accessed at <http://www.exocarta.org>; the URL of the HIV-1 Human Interaction Database is <http://www.ncbi.nlm.nih.gov/genome/viruses/retroviruses/hiv-1/interactions/>; DAVID (Database for Annotation, Visualization and Integrated Discovery) can be accessed at <http://david.abcc.ncifcrf.gov>; the URL of STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) is <http://string-db.org/>.

3. Methods

1. Cell culture (labeling) and HIV-1 infection

SILAC medium should be freshly prepared (follow manufacturer's manual closely) before use. Medium can be stored at 4°C for up to about three months. To assure healthy and highly proliferative cells, the use of trypan blue staining (to measure cell viability) and MTT assay (to check cellular proliferation) are recommended before conducting actual metabolic labeling.

1. Seed and culture two batches of cells in 10 cm cell culture dishes in parallel. One is in “heavy” labeling medium, which contains 10% dialyzed FBS supplemented with 100 mg/L $^{13}\text{C}_6$ L-lysine and 100 mg/L $^{13}\text{C}_6^{15}\text{N}_4$ L-arginine, the other one is in “light” medium (containing 10% dialyzed FBS supplemented with 100 mg/L L-Lysine and 100 mg/L L-Arginine) (see **Note 7**).

2. Expand cells in the heavy medium for six doublings to achieve complete (>99%) labeling of cellular proteins with heavy amino acids, while independently maintain cells in light medium for the same number of doublings (see **Note 8**).
3. Change media regularly (depending on specific cell type, normally every two to three days) during the labeling period.
4. After complete labeling, infect heavy cells with HIV-1 (e.g., NL4-3 HIV-1 strain, see **Note 9**) by following standard HIV infection protocol [14]. During the same period, maintain light cells in parallel without infection.
5. Harvest both light (uninfected) and heavy (infected) supernatants independently at the end of infection (see **Note 10**).

2 Exosome isolation

Using sequential ultrahigh centrifugation, exosomal fractions are independently enriched from culture supernatants from both infected and uninfected cells [15]. All of the followings steps are performed at 4°C (Figure 2).

1. Collect culture supernatants, not cells, into proper sized centrifuge tubes and centrifuge at 300g for 10 minutes to remove residual cells.
2. Transfer the cleared supernatants to new centrifuge tubes and centrifuge at 2,000×g for 10 minutes to remove dead cells. Pipet supernatants carefully, do not touch pellets as they can contaminate supernatants.
3. Transfer the supernatants to ultracentrifugation tubes and centrifuge (make sure tubes are well-balanced) at 10,000g for 30 minutes to remove cell debris. Similar to step 2, do not touch pellets while collecting supernatants
4. Transfer the resulting supernatants to fresh ultracentrifugation tubes and centrifuge at 100,000g for 70 minutes. Remove the supernatants completely and discard. Unlike previous steps, starting from this step, remove supernatants and save pellets.
5. Resuspend the pellets, containing exosomes, in 5 ml fresh PBS, transfer the suspensions to clean ultracentrifugation tubes and centrifuge again at 100,000g for 70 minutes.
6. Remove the supernatants and discard, resuspend the resulting pellets, in 50 µl PBS (see **Note 11**) for long term storage (at - 80°C freezer) or proceed directly to protein extraction.

3. Protein extraction and preparation

1. Add 50-200 µl of RIPA buffer supplemented with protease inhibitor cocktails to the tubes containing the purified exosomal pellets. Vortex the tubes vigorously to completely dissolve the pellets.

2. Transfer the dissolved protein solutions to new 1.5ml microcentrifuge tubes. Shake the tubes vigorously (e.g. 400 rpm) for 5 minutes on a microcentrifuge shaker.
3. Centrifuge the mixtures for 10 minutes at 13,000g.
4. Transfer the resulting supernatants, which contain extracted exosomal proteins, to new microcentrifuge tubes for storage or continue to the next step.
5. Quantify protein concentration from the light (uninfected) and heavy (infected) exosomal samples respectively by employing a standard protein quantification assay, such as bicinchoninic acid (BCA) or Bradford based method.
6. Based on the concentrations determined from previous step, calculate and mix the right volumes of light and heavy protein extracts to form a mixture that contains equal amounts (1:1) of total protein from light and heavy samples. Load the single mixture on an SDS-PAGE gel and run electrophoresis. 2-10 μ g of total protein per gel lane is recommended.
7. Transfer the finished gel to a square petri dish and stain it with Coomassie blue for 1 hour with gentle orbital shaking.
8. Destain gel for a few hours to overnight.
9. Cut the entire sample lane from the de-stained gel into ten to fifteen equal gel pieces with a clean razor blade. Further slice each gel piece into smaller (e.g. 1 x 1 mm) cubes and transfer them into a new 1.5 ml microcentrifuge tube.
10. In each tube, add enough 25 mM NH_4HCO_3 in 50% ACN to fully immerse the gel cubes and vortex for 10 minutes. Remove the supernatants and discard. Repeat this step twice.
11. Dry the gel cubes in a vacuum concentrators for 20 minutes.
12. Rehydrate gel cubes with enough 10 mM DTT, vortex and centrifuge briefly. Allow reaction to proceed at 56°C for 1 hour.
13. Remove the supernatant. Add enough 55 mM iodoacetamide to fully immerse the gel cubes. Vortex and spin briefly. Incubate in the dark at room temperature for 45 minutes.
14. Remove the supernatant. Add enough 25 mM NH_4HCO_3 to immerse the gel pieces. Vortex and centrifuge briefly.
15. Remove the supernatant. Add 25 mM NH_4HCO_3 in 50% ACN to immerse the gel pieces. Vortex and centrifuge briefly.
16. Repeat step 14 to 15 once.
17. Dry the gel cubes in a vacuum concentrators.

18. Add 25 μ l trypsin in 25 mM NH_4HCO_3 to the dried gel cubes and incubate at 4 °C for 30 minutes. Remove excess solution and discard. Add a minimum amount of 25 mM NH_4HCO_3 without trypsin to keep gel pieces immersed throughout digestion. Incubate overnight at 37°C.
19. To recover peptides from the gel: Spin down briefly and transfer extract to a new tube; Add 30 μ l of 5% formic acid in 50% ACN to the original gel cubes, vortex for 30 minutes and spin. Transfer the supernatant and combine with the extract obtained initially.
20. Dry down the combined peptide extracts by vacuum concentrator to less than 5 μ l and resuspend with 20 μ l HPLC buffer A before proceeding to next step.

4. Mass spectrometry identification and quantitation

Duplicate runs of samples are required, triplicates are recommended to minimize variation and to achieve a high fidelity of proteomic analysis. Your mass spectrometry core facilities are likely to perform all the steps in this section for you. We list brief steps here as a reference.

1. Apply the resulting peptide mixtures from each gel piece to microcapillary reversed phase liquid chromatography tandem mass spectrometry (LC-MS/MS) separately.
2. Perform HPLC using a nanoflow HPLC with a self-packed 75 μ m id x 15 cm C^{18} column in buffer A. The LC gradient is produced over 60 minutes from 2% to 38% HPLC buffer B.
3. Set mass spectrometer (MS) at following settings: Operate in data dependent acquisition (DDA) / positive ion mode; Acquire the full scan range between 390 m/z to 1500 m/z at 30,000 resolution using a Top5 DDA method via collision-induced dissociation (CID); Exclude precursor ions for a duration of 2 minutes; Use Nano-ESI spray voltage of +3kV without sheath or auxiliary flow gas; Set the ion count threshold for MS/MS selection at 700 counts; Use default settings for activation time and Q values.
4. Search the generated MS/MS spectra against the reversed and concatenated non-redundant Human IPI database by using the program Mascot with the following parameters: use the fixed modification of carbamidomethyl Cys and variable modifications of oxidation of Met and acetylation of protein N-terminus; allow two missed cleavages and Trypsin with Pro restriction; require a match of at least 6 amino acid residues; Set MS tolerance at 30 ppm and MS/MS tolerance at 0.08 Dalton; Allow the peptide and protein false discovery rate (FDR) 1%.
5. Achieve protein quantitation by MaxQuant software [16], which is set to require at least 2 peptides per protein (1 razor and 1 unique) for

quantitation. Normalized SILAC ratios are used for all subsequent interpretation.

Various information, including protein IDs, names, descriptions and heavy/light ratios, of the identified proteins can be exported and saved as spreadsheet format for detailed downstream analysis.

5. Western blotting verification

Western blotting is recommended to verify protein quantification as determined by mass spectrometry. Standard western blotting procedures should be followed. Please refer to specific protocols for details. It is important to ensure that equal amounts of protein (can use aforementioned protein quantification method) are loaded from the control and experimental arms. In order to obtain robust quantitative verification, it is also recommended to develop blots with a digital imaging system and to analyze acquired image with analysis software (e.g. ImageStudio®).

6. Proteomic data analysis

The data quality assessment, data pretreatment and calculation steps are carried out for each mass spectrometric replicate independently before merging data from all replicates for final inspection (Figure 3).

- i. Data quality assessment: 1. Log2 transform heavy/light ratios of all quantified proteins; 2. Group the transformed ratios into multiple (40-100) ratio bins; 3. Use statistical software to plot the number of detected ratios per bin. The resulting histogram of SILAC ratio distribution should follow normal distribution (see **Note 12**) [17].
- ii. Data pretreatment: To ensure greater confidence in the accuracy of the mass spectrometry-derived peptide (protein) ratios, a given protein needs at least two quantifiable peptides in order to be included in candidate selection (see **Note 13**).
- iii. Determination of significant threshold: To determine significantly up- and down-regulated protein candidates, conservative cut-off values are calculated as followings: 1. Log2 transform the original SILAC protein ratios; 2. Calculate the median and standard deviation (σ) of the transformed ratios by using a statistical or spreadsheet software; 3. Calculate the cut-off of value ($\text{median} \pm 2\sigma$) in log space initially and then transform back into linear space. Candidates having ratios above median $+2\sigma$ or less than median -2σ are considered to be significant. In most cases, a small percent of candidates on both extreme ends will be assigned as significant.
- iv. Data comparison and inspection: The resulting significant candidates are compared across all replicates. A three-step workflow is carried out to identify potential candidates: 1. Only select the candidate(s) consistently shown in all replicates; 2. For each candidate, manually inspect all

identification and quantitation, remove candidates that do not have the same direction among the replicates (up or down-regulated) and/or similar expression ratios. 3. Finalize data for the selected candidates by merging their data from all replicates.

7. Bioinformatics verification and characterization of HIV-1 Impacted Exosomal Protein (HIEP) candidates

To take advantage of abundant (genomic, proteomic and bioinformatic) existing information for almost every single protein, data mining and bioinformatics analysis are carried out to gain insights into HIEP candidates (Figure 4). Several relevant databases and software programs are described below.

- a. Validation against exosome database: To further verify a given candidate protein's assignment to the exosomal compartment, search HIEP candidates using their name or UniProt IDs against the current exosome database, which is a manually curated database that is integrated from both published and unpublished exosomal studies, by using “query” function [18-19] (see **Note 14**).
- b. Examination against HIV-1/host proteins database: To find potential known associations between HIV-1 and HIEP candidates, search the HIEP candidates (using the search boxes) against the HIV-1 and Human Protein Interaction Database [20] in order to identify proteins shared by the database and HIEP candidates (see **Note 15**).
- c. Gene Ontology (GO) characterization: To get representation of GO terms: 1. Save UniProt IDs of HIEP candidates on txt file; 2. Import the txt file to STRAP software [21] and run the software to search against UniprotKB, EBI and GO databases; 3. Visualize GO analysis results (e.g. in the form of pie chart) through a spreadsheet software by using analyzed data from STRAP (see **Note 16**).
- d. GO term enrichment analysis: To find statistically significant overrepresented gene ontology (GO) terms: 1. Submit the HIEP candidate list to DAVID via its web interface by using “Functional Annotation Tool” [22]; 2. On the page of “Annotation Summary Results”, choose the “Gene_Ontology” category, the enriched GO terms, p-value and other associated parameters will be reported there (see **Note 17**).
- e. Interaction and pathway analysis: To help downstream biochemical analysis and pathway discovery, it is worthwhile to find the known and predicted associations of HIEP candidates beforehand. A representative tool, STRING is employed to provide direct (physical) and indirect (functional) associations [23]. This process is done by inputting protein ID or sequence in designated search box, choosing the right species and searching the database. The top ten known partners associated with each exosomal candidate will be shown as a part of the results (see **Note 18**).

With all above mentioned analyses, a wealth of information of HIEP candidates will be analyzed. The most significant candidate(s) will be selected and subjected to downstream molecular and biochemical analysis.

4. Notes

1. For SILAC labeling, try to avoid using cells that have been propagated for an extended period. It is preferable to use early passage cells to achieve high labeling efficiency.
2. Dialyzed fetal bovine serum (FBS), not regular FBS, needs to be added to SILAC media to make the complete labeling medium. The Dialyzed FBS contains no or few free amino acids and peptides, which may interfere with SILAC labeling.
3. Usually, a SILAC labeling kit comes with only a single heavy isotope $^{13}\text{C}_6$ L- lysine. In order to improve coverage in mass spectrometry analysis, double “heavy” isotope labeling using $^{13}\text{C}_6$ L- lysine and $^{13}\text{C}_6^{15}\text{N}_4$ L- arginine is recommended. In which case, “heavy” $^{13}\text{C}_6^{15}\text{N}_4$ L-arginine should be obtained and added to labeling media (final concentration at 100 mg/L) accordingly.
4. The classical way of isolating exosomes involves ultra high-speed centrifugation. Currently, there are a few commercially available reagents that permit exosome isolation without ultra high-speed centrifugation and related equipment. These alternative methods maybe carried out at the reader's discretion.
5. An alternative way to perform trypsin digestion is to do it directly in the protein extract without running a gel. However, conditions, such as buffer and digestion time, will have to be optimized for complete digestion.
6. Each proteomics core may have their own specific procedures and requirements for sample preparation and mass spectrometry analysis; consult them first to determine the best strategy and procedures.
7. Many factors, such as the sensitivity of mass spectrometer, goal of analysis (whole proteome or post-translational modification) and mass per cell, affect the minimum amount of protein (thereby minimum number of cells) needed for downstream analyses. We found 10 million cells can be a good starting point to determine the optimal number of cells for proteomic analysis.
8. For a specific cell type, the exact cell doubling time needs to be determined. Fast growing cells obviously need less time to be completely labeled than slow growing cells.
9. HIV-1 Infection should be monitored. One way of doing this is to via p24 quantification in culture supernatants

10. For adherent cells, the supernatants can be used directly for exosome isolation. For suspension cells, a pre-clearing step of centrifugation at 200g for 10 minutes is needed to remove excess cells.
11. As HIV-1 virions are similar in size to exosomes, the purified exosomes from HIV-1 infected samples may be contaminated with HIV-1 virions. In proteomic screen, potential viral contaminants can be filtered out by limiting search database only to human. However, if further downstream experiments are expected, readers should employ established methodologies separate HIV-1 virions from exosomes, if necessary [24].
12. If the SILAC ratio distribution is heavily skewed in one direction, the reader needs to determine the causes before continuing data analysis. Possible cause(s) may be due to unequal mix of “light” and “heavy” samples.
13. Although some proteins may have multiple (2) identified peptides, however, not all peptides come with heavy/light (SILAC) ratio values. Those proteins are recommended to be excluded if they have one or no peptide bearing SILAC values.
14. One way of verifying the success of exosomal purification and proteomic identification is to search the top 25 exosomal marker proteins listed at ExoCarta against HIEP candidates. A successful experiment usually finds a majority of the 25 exosome marker proteins in the SILAC list.
15. The database contains extensive HIV-1/host protein interactions that have been reported. The detailed interactions between HIEP candidates and HIV-1 are recommended to be mined.
16. There are many other (either standalone or web-based) GO term analysis software (e.g. GoMiner® [25] or PANTHER (Protein ANalysis THrough Evolutionary Relationships) [26]) available. It is up to reader's judgment to choose which software suits their needs.
17. Another alternative tool to DAVID is GORILLA (Gene Ontology enRIchment anaLysis and visuaLizAtion, <http://cbl-gorilla.cs.technion.ac.il/>) [27].
18. There are also quite a few software can do pathway analysis. A proprietary Ingenuity IPA from QiaGen and a public accessible ConsensusPathDB (<http://cpdb.molgen.mpg.de/>) [28] are good examples.

Acknowledgments

This work was supported by an ARRA supplement to the Lifespan/Tufts/Brown CFAR, P30AI042853-13S1, NIH P20GM103421, P01AA019072, R01HD072693 to BR. This work was also supported by Lifespan Pilot Research Fund (#701-5857), Rhode Island Foundation Medical Research Grant (#20133969) and NIH COBRE URI/RIH Pilot Research Grant (P20GM104317) to ML.

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Figure 1.

Overall experimental schema for SILAC labeling. Ready to be labeled cells are divided into two batches. One batch is expanded in “heavy” medium for 6 doublings to achieve complete labeling. The other batch is independently maintained for the same periods. When the cells are completely labeled, the heavy cells are infected with HIV-1, while light cells receive no infection. At the end of infection, heavy (infected) and light (uninfected) are subjected to exosome isolation in parallel.

Figure 2.

Flow chart of exosome purification procedure based on differential ultracentrifugation. For each step, samples need to be processed are listed in the middle of chart. Fractions to be discarded in each step are indicated on the left. The speed and length of each centrifugation are shown on the right. Need to note, for the first three centrifugations, pellets are discarded and the supernatants are saved for the next step. However, for the last two ultracentrifugation steps, pellets are saved and supernatants are discarded.

Figure 3.

Flow chart for determining significant candidates from proteomic analysis. Four successive steps of analysis and verification are implemented to ensure finding reliable, significant candidates. First step of plotting SILAC ratio histogram is to ensure the global data integrity. Second step is to make whole dataset less noisy by removing less ideal candidates. By employing unbiased statistical tools, the third step defines the significant thresholds for potential candidates. The last step finalizes the list of significant candidates by merging and analyzing the replicate data for each candidate from previous step.

Figure 4.

Overall bioinformatics analysis procedures for HIEP candidates. To gain insights into HIEP candidates, data mining and bioinformatics analysis are implemented. Current exosome database and HIV-1 /host interactome database are mined to verify the exosomal origins and the known interactions with HIV-1. Gene ontology characterization and enrichment analysis are performed to narrow down research scope and point out potential direction. Interaction and pathway analysis are also carried out to find potential binding or interacting partners.

Table 1

The comparison among three commonly used quantitative proteomics methods. The differences of SILAC, iTRAQ and MRM are compared in the category of labeling principle, sample application scope, experimental cost, easiness of doing analysis, ability to detect small changes and technical variability

	Labeling	Samples	Cost	Analysis	Small change measurement	Technical variability
SILAC	Metabolic	Mainly for cultured cells	Relatively low	Relatively straightforward	Reliable	Low
iTRAQ	Chemical	Wide ranges of samples	Moderate	Moderate	Relatively reliable	Relatively low
MRM	No labeling	Mainly for clinical samples	Relatively high	Time-consuming	Less reliable	Relatively high