

Establishment of Optimal Housekeeping Genes for Urinary Extracellular Vesicle Based Biomarker Development: A Step Towards Non-Invasive Diagnostics.

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Research

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Abstract

Background

Recent years have witnessed a growing interest in employing urine as a clinical source of renal pathology biomarkers. Urinary extracellular vesicles (UEVs) hold cellular RNAs, including small RNA and micro RNA. Quantitative polymerase chain reaction (qPCR) is one of the most sensitive methods for evaluating gene expression, which depends on comparative analysis with reference/house-keeping genes. However, reliable interpretation of UEVs gene expression data is biased due to the lack of reported ideal house-keeping reference-genes in UEVs.

Methods

UEVs were isolated from 40 healthy human controls using Polyethylene glycol (P.E.G. Mn6000) based precipitation. UEVs were characterized by biophysical and biochemical assays. At the molecular biology level, the expression and stability of five commonly used housekeeping genes B2M, RPL13A, PPIA, HMBS, and GAPDH, were considered for comparing and finding out ideal reference gene. Data were analyzed using four practical algorithmic approaches, including Norm Finder, GeNorm, Best Keeper, and the Delta Ct for reference gene evaluation integrated with RefFinder. The final ranking of stable genes is derived from the weighted geometric means of all the above algorithms.

Results

12% PEG isolated UEVs were round and cup-shaped, ranging from 30 - 100nm, as per electron microscopy, nanoparticle-tracking-analysis, and dynamic-light-scattering profile. The functional purity of UEVs was determined with their acetylcholine esterase and Dipeptidyl peptidase-IV activity. RefFinder established the stability index of housekeeping genes. B2M and RPL13A genes were identified as stable genes with a mean stability score of 1.5 (Genorm) and below 1 (Norm finder), indicating a reduced gene expression variation.

Conclusions

The comprehensive ranking analysis identified B2M and RPL13A as optimal reference genes for UEVs based gene expression studies.

Background

Early and precise diagnosis of renal disease translates into improved treatment outcomes. Currently, renal biopsy is the gold standard for identifying renal pathologies. However, it is expensive, invasive, and is usually conducted at the terminal stages to confirm renal dysfunction (1). Therefore, new biomarker strategies aiding in early, accurate identification of renal disease are of prime importance in the refinement of renal pathology. Although mRNA profiling is an attractive means for biomarker identification, mRNA analysis through urine is challenging as free-floating RNA in urine is often degraded by urinary ribonucleases (2), resulting in biased interpretation. Recent studies have highlighted the importance of microvesicles, including exosomes, as important source material of RNA species, including mRNA, miRNA, and other small RNA. Extracellular vesicle membrane protects RNA in the exosome pool from degradation by urinary ribonucleases (2, 3). In this context, UEVs are especially gaining importance as ideal diagnostic tools, forming the basis for liquid biopsies (4, 5).

Urinary exosomes are an essential category of extracellular vesicles secreted by the kidney and urinary tract and are in size range of 30–150nm (6). They maintain homeostasis and mediate intra and intercellular communication between cells by shuttling proteins, RNAs, lipids, and other biomolecules (7). Their cargo represents the cells' physiological state' in real-time, framing the reason for building up ideal non-invasive diagnostics. Precipitation, size exclusion chromatography, filtration, and differential centrifugation are conventional UEVs isolation methods (8–10). Although ultracentrifugation (UC) is the best quality level for isolating EVs, challenges like time-consuming protocols, lower vesicular yields, and the need for large amounts of starting material forestalled UC to be adopted in a clinical setting. Therefore, a simple, cost-effective alternative method of isolation of UEVs is warranted. Exosome precipitation is the standard protocol adopted by many commercial exosome isolation kits, including Exo Quick (SBI), Total Exosome isolation (Invitrogen), etc. The above kits are expensive for accommodating large sample volumes and for longitudinal research studies. In the current study, we adapted the Polyethylene Glycol (PEG)-based isolation method, widely used in viral particle isolation from various biofluids (11). We are uniformly using the term "UEVs" throughout our description for describing vesicles isolated from urine.

Reverse transcription-quantitative polymerase chain reaction (qPCR) is the most common and standard technique used in gene-expression studies. qPCR findings' primary requisite is the normalization of the data, which depends on endogenous gene expression. Genes that are constitutively active, endogenously abundant, and stable across different conditions are considered reference genes or housekeeping genes. Exosome secretion is a random event, and RNA distribution processes into exosomes are not fully elucidated, making normalization an essential requisite for UEVs based gene expression studies. Also, endogenous reference genes are currently not known for UEVs (12, 13). As the field of Urinary exosome diagnostics is developing rapidly, and a urinary exosome-based gene expression test for detecting prostate cancer is already in the market (14), the need for identifying ideal housekeeping genes in UEVs is the need of the hour (15).

B2M, *L13A*, *GAPDH*, *PPIA*, and *HMBS* are used as reference genes because of their role in cellular process, cell structure integrity, and primary metabolism. Normfinder, Genorm, Bestkeeper, and Delta Ct are the standard algorithms used in transcript normalization and reference gene evaluation. Normfinder is a model-based approach that considers the inter and intragroup variability of genes when ranking their stability profile (16). Genorm measures pairwise standard deviation for all genes, eliminates the least stable genes, and retains the most stable genes (17). Best keeper generates a stability index based on their quantification cycle (C_q) values and amplification efficiencies, followed by a pairwise correlation to rank each candidate in the list (18). For each

pairwise comparison, the Delta Ct method assigns stability values based on C_q standard deviation differences (19). RefFinder (<https://www.heartcure.com.au/reffinder/>); a web-based tool that evaluates and screens reference genes from experimental data sets integrating the weighted average from the above four algorithms. This study established an optimal UEVs isolation protocol and identified the most stable housekeeping genes among the five conventional reference genes from the gene pool of UEVs.

Materials And Methods

Urine collection and processing

Urine samples from forty healthy individuals (aged 20–72 years) were collected from Apollo Hospital, Hyderabad. Demographic characteristics are represented in Table 1.

Table 1
– Healthy control demographics

Parameter	value
Sample Size	40
Age	47.2 ± 13.4
Gender	
Male	35
Female	5

Table 1: Urine samples were collected from 40 healthy individuals whose age is represented as Mean ± SD.

Urine was collected in collection containers, and 4.2ml protease inhibitor (1mg/ml Leupeptin (G Biosciences, MO, USA), 10mM Sodium Azide, 50mM PMSF (both from Sigma, MO, USA) per 50ml of urine was added. Before proceeding with UEV isolation, all urine samples were subjected to urine tests using the urinalysis strips (Siemens Multistix 10 SG, Siemens, Munich, Germany). The urinalysis strips were dipped into the urine containing tube, and excess urine was drained. The colour changes on the strips were observed and quantified using Siemens instrument CLINITEK Advantus for the parameters shown in Table 2.

Table 2
– Summary of urine analysis of healthy individuals

Samples (40)	Leukocyte	Nitrite	Urobilinogen (mg/dl)	Protein (mg/dl)	pH	Blood	Specific Gravity	Ketone (mg/dl)	Bilirubin	Glucose (mg/dl)	Colour	Clarity
Observed	Negative	Negative	0.2	Negative	5.5–7.5	Negative	1-1.03	Negative	Negative	Negative	Yellow	Clear
Reference range	Negative	Negative	0.2–3.2	Negative	5–8.5	Negative	1-1.03	Negative	Negative	Negative		

The collected urine samples were immediately processed for UEV isolation using the modified salt precipitation method described previously (5). Briefly, 30–50 ml of first void urine samples were centrifuged at 1800*g for 10 min at 4°C to clarify the cell and cellular debris. The cell pellet was washed with 1x PBS (Hyclone GE Healthcare, IL, USA) and resuspended in RNA later solution (Sigma) for RNA isolation. The supernatant was carefully transferred to a fresh tube containing 20mM Tris-EDTA (Sigma) pH 6.8, and the pH was adjusted to 4. The supernatant mixture was vortexed for 90sec and further centrifuged at 8000*g for 15min at 4°C. After the centrifugation step, the supernatant was collected in a fresh tube, an equal volume of 2x PEG solution was added (final 12% PEG in 0.5M NaCl (Sisco Research Laboratories (SRL), Mumbai, India) and mixed thoroughly. The UEV pellet obtained was then treated with 500µl of 100mM DTT (Sigma) and incubated for 10 min at 37°C for Tamm-Horsfall Glycoprotein (THP) removal. Subsequently, the DTT treated pellet was centrifuged at 17000*g for 15 min at 4°C with zero brakes. The supernatant obtained here was added to the previously derived PEG- supernatant mixture and incubated overnight at 4°C. The next day, the PEG-supernatant mixture was centrifuged at 10,000*g for 60 min at 4°C. Finally, the pellet containing UEVs was resuspended in 1x PBS and stored at -80°C for further use.

Urinary Extracellular Vesicle Characterisation

The isolated UEVs were subjected to biophysical and biochemical characterization to ensure the quality of isolated vesicles.

Morphological characterization

TEM Analysis

Transmission electron microscopic analysis was performed to examine the size and confirm the presence of intact exosomes and size determination. Briefly, UEV samples were fixed with 1% glutaraldehyde for 5 min on the 400 mesh copper grids (FCF400-Cu, Electron Microscopy Sciences, Hatfield, PA).

The grid was washed twice with water, and further stained with 2% uranyl acetate, and then air-dried at room temperature. Images of the isolated UEVs were captured using a transmission electron microscope (JEM-2100, JEOL Ltd. Tokyo, Japan).

Nanoparticle Tracking Analysis

Nanoparticle tracking analysis is a powerful technique that utilizes laser light scattering microscopy combined with Brownian motion to determine the size, concentration, and morphology of extracellular vesicles [45]. We performed NTA on the isolated UEVs using NanoSight LM10 instrument (NanoSight, Amesbury, UK), where the laser light-scattering was measured at 488 nm, and the relative concentration and size was determined.

Dynamic Light Scattering

The size of UEVs was further determined using the dynamic light scattering (DLS) technique that measures the size based on the Brownian motion of the dispersed particle as described previously[46]. 3ml homogenous suspension of UEVs (diluted 1:100 in 1X PBS) was transferred to a cuvette, and the hydrodynamic diameter of exosomes was measured in a DLS instrument (Nicomp Z3000, Entegris, MA, USA).

Biochemical Characterisation

Protein estimation and SDS-PAGE analysis

The total protein content in isolated UEVs was quantified using a bicinchoninic (BCA) protein assay kit (G-Biosciences) following the manufacturer's instructions. Samples were stored at -80°C until further analysis. For SDS-PAGE, 10µg of UEV protein was lysed using RIPA lysis and extraction buffer (G-Biosciences) including protease inhibitor cocktail (Roche, Basel, Switzerland) followed by incubation at 4°C for 15min. The lysed protein samples were then mixed with reducing sample buffer and denatured for 10 mins at 70°C. The exosomal proteins were resolved on 10% SDS PAGE for 1.5 h at 120 V, and the gels were stained using 2% silver nitrate.

Lipid Estimation

Total lipid content of the UEVs was quantified using Phospho Vanillin Assay, as reported previously (35). Briefly, 200µl of 96% H₂SO₄ was added to 40µl of the lipid standard, DOPC (1,2-Dioleoyl-sn-glycero-3-phosphocholine), or the UEV samples and evaporated at 90°C on a dry bath for 20 min. After the tubes were cooled to RT, 120µl of Phosphovanillin reagent (200µg vanillin in 17% H₃PO₄) was added. The reaction is allowed to occur by incubating for 1 h at 37°C, and then absorbance was recorded at 540nm.

AChE activity

Acetylcholinesterase (AChE) activity assay was carried out to confirm the presence of acetylcholinesterase (Sigma), which is considered as a marker enzyme for exosomes[21]. 20µl UEV fraction was added to 96-well flat-bottomed microplate. Acetylthiocholine iodide (final 1.25mM) and 5', 5'-dithio-bis (2-nitrobenzoic acid) (final 0.1mM) (both from Sigma) was then added to each well at a final volume of 300µl. The absorbance was recorded at 412nm every 5 min for 30 min. The amount of AChE activity in the exosome fraction was determined from the AChE enzyme's standard curve.

Dipeptidyl peptidase-IV activity

Dipeptidyl peptidase-IV (DPPIV), a membrane-associated peptidase, is associated with kidney diseases and is secreted by tubular epithelial cells in the kidney. Hence, it is considered to be a component of urinary microvesicles in the urine. We used a colorimetric assay previously used to determine serum DPPIV activity by quantifying DPPIV in UEVs as additional criteria to assess exosome quality[31]. Briefly, 10µl of UEV sample was mixed with 50µl of 71mmol/l glycine/NaOH (pH 8.3) buffer in 96 well plate. 50µl of 0.5mg/ml substrate Gly-Pro-p-nitroanilide (Sigma) was added to all wells, including blank wells, and incubated for 60 min at 37°C. The substrate is cleaved by DPPIV in the sample which releases free 4-nitroaniline, a chromogenic, whose absorbance is measured at 405nm in a plate reader. DPPIV activity in the sample was calculated against the standard plot of p-nitroaniline standard (Sigma).

Antibody Array

Exo-Check exosome antibody array (SBI, Systems Biosciences, USA) was performed according to the manufacturer's instructions. The array contains 8 antibodies for known exosome markers including (CD63, CD81, ALIX, FLOT1, ICAM1, EpCam, AnXA5, and TSG101) and 4 controls, including two positive controls, blank and gm130 cis-Golgi marker which monitors for any cellular contamination. Briefly, 100µg of UEV protein was treated with lysis buffer; 1µl of Labelling reagent was then added and incubated at RT for 30mins with constant mixing. The column provided in the kit was equilibrated with column buffer before the sample was eluted. The eluted lysate was mixed with blocking buffer, transferred on to membrane, and incubated at 4°C overnight on a rocker. The next day, the membrane was washed thrice (5 mins each) with washing buffer and incubated with detection buffer for 30mins at RT on a shaker. The membrane was washed thrice again with washing buffer before development. Super Signal West Femto Maximum sensitivity substrate (Thermo Fisher Scientific, US) was used for chemiluminescence detection of the membrane in the imaging system (ChemiDoc, BioRad, USA).

RNA isolation and quantification

Total RNA from UEV was isolated using TRIzol™ LS Reagent (Invitrogen, California, USA) following the manufacturer's instructions. Briefly, the exosome sample was mixed with Trizol Reagent (1:3) and incubated for 5 min at RT. Chloroform was added (1:5) to the exosomal mixture and incubated for 15 min at RT, followed by centrifugation at 12000g for 15 min at 4°C. The aqueous phase was carefully removed and transferred to a fresh tube. To all the tubes equal volume of isopropanol, 500µg/ml Glycogen (GlycoBlue™ co-precipitant Invitrogen), and 0.5µg yeast tRNA (Invitrogen) was added and incubated overnight at -20°C. The samples were then centrifuged at 12000g for 15 min at 4°C, followed by an ethanol wash. Finally, the air-dried RNA pellet was

resolved in nuclease-free water and stored at -80°C for further analysis. The RNA content was determined using Qubit™ RNA HS Assay Kit (Invitrogen). Additionally, the UEV RNA quality and quantity were analysed in Agilent Bioanalyzer 2.1 instrument using RNA Pico kit for high sensitivity analysis of total RNA and mRNA samples (Agilent Technologies, California, USA).

Reverse Transcription

Exosomal RNA was reverse transcribed with High capacity cDNA Reverse Transcription kit (Invitrogen) using 10µL exosomal RNA and 10µl cDNA master mix prepared according to the manufacturer's instruction. cDNA was pre-amplified using Sapphire Amp fast PCR master mix (Takara Bio Inc. Shiga Prefecture, Japan) Pre-amplification with pooled primers was carried out for 20 cycles at 95°C for 5 min, 95°C for 1 min, 60°C for 30 sec, 72°C for 1 min, and 72°C for 5 min and hold 4°C. The pre-amplified transcript was then used as a template for PCR and RT PCR analysis.

Endogenous gene expression in Urinary EV

In the present study, we selected 5 reference genes, namely *GAPDH*, *B2M*, *RPL13A*, *PPIA*, and *HMBS*, for normalization of quantitative real time PCR in UEV samples from healthy individuals. All primer sequences were custom synthesized by the supplier (Bio serve, India), a detail of which is listed in Table 3. The pre-amplified samples mentioned in the above section were diluted 1:5 with nuclease-free water, and 1µl of the diluted product was used as a template for semi-quantitative as well as quantitative PCR. Briefly, a 10µl reaction was performed in Applied Biosystem 7500 Real-Time System using 5µl TB Green Premix Ex Taq (Takara Bio Inc.), 0.1µl 10µM primer pair, 1µl template cDNA, and 3.8µl nuclease-free water. Real-time PCR was conducted for 30s at 95°C, followed by 35 cycles of 5s at 95°C and 30s at 60°C. All samples were evaluated in duplicates, and for every gene analyzed, a non-template control (NTC) was also included. PCR product purity was monitored from melting curve analysis and 2.0% agarose gel electrophoresis.

Table 3 - Primer pair sequences of the candidate reference genes used in this study.

Gene	Forward Primer	Reverse primer
B2M (Beta-2-Microglobulin)	5'-CCTGCCGTGTGAACCATGTGA-3'	5'-GCTGCTTACATGTCTCGATCCCA-3'
GAPDH (Glyceraldehyde-3-phosphate dehydrogenase)	5'-CCACTAGGCGCTCACTGTTCT-3'	5'-GACCAAATCCGTTGACTCCGAC-3'
RPL13A (Ribosomal protein L13a)	5'-TCCTTTCCGCTCGGCTGTTT-3'	5'-GGGCCTTACGCTCGGGAT-3'
PPIA (peptidylprolyl isomerase A)	5'-CCGCCGAGGAAAACCGTGTA-3'	5'-TGCAAACAGCTCAAAGGAGACG-3'
HMBS (hydroxymethylbilane synthase)	5'-CAGCCTACTTTCCAAGCGGA-3'	5'-CCTGTGGTGGACATAGCAATGA-3'

Normalization of housekeeping genes

To analyze the candidate gene expression stability, normalization analysis was performed using online available software RefFinder. RefFinder determines the best stably expressed candidate reference gene by generating a comprehensive ranking for each gene based on the combined expression stability data from four statistical algorithms- Genorm, Normfinder, Bestkeeper, and Delta Ct. These mathematical algorithms use threshold cycle (Ct) values to calculate expression stability of each candidate reference gene. Precisely, Normfinder implements an ANOVA based algorithm to determine inter and intragroup variations in the reference gene expression, which is represented in terms of stability value. The gene expression stability score in Genorm is determined by pairwise comparison, while Bestkeeper applies pairwise correlation analysis. Delta Ct method calculates variations in delta Ct by comparing relative transcription of pairs of genes. Ranking of genes was done based on the stability score, where genes with the lowest stability score were considered more stable.

Statistics

For the comparative analysis of exosomal characteristics using the PEG vs. Kit method, a paired student's t-test was done to test the statistical significance using GraphPad software version 8.3 was used. Only $P < 0.05$ was considered statistically significant. All experimental data are shown as mean \pm SEM unless otherwise mentioned. Gene stability analysis of housekeeping genes was performed in RefFinder. The stability value or SD data obtained using different algorithms were used for the ranking of genes.

Results

Urinary EV isolation from healthy subjects

UEVs were isolated from forty healthy individuals using the PEG-based precipitation method described previously⁽⁸⁾ with modifications. For reference, we performed a comparative analysis of the UEVs isolated using a commercially available kit (n=11). An overview of the experimental procedure is shown in Figure 1. We further determined the characteristic features of the isolated UEVs for their biochemical, biophysical, and molecular characteristics.

Optimization of the exosome isolation procedure

Initially, we had optimized the UEV isolation method by altering PEG concentrations, i.e. (6%, 12%, 24%). The total protein content and yield were maximum using 12% PEG compared to 6% or 24% (Supplementary Figure 1). To confirm the purity of the isolated EVs, we have compared their biochemical properties with those isolated, using the standard commercially available kit as a reference. Quantitative analysis of the UEV protein and lipid content showed moderate differences in the yield, Figure 2A-B. The ratio of protein to lipid content, a commonly used parameter to assess the quality of vesicles (20), was comparable (Figure 2C). Also, acetylcholine esterase (AChE) enzyme activity was quantified in the UEVs to assess the purity of isolation as previously

reported (21). AChE enzyme activity of the PEG-isolated UEVs was slightly lower (1.7-fold) when compared to the Kit – method (Figure 2D). As the biochemical properties of the vesicle-derived using both methods were comparable, we chose to isolate using the PEG-based method further.

Qualitative assessment of Urinary EVs

Following UEVs isolation, THP contaminant proteins were removed by treating the fraction with reducing agent DTT(22). SDS-PAGE analysis confirmed the reduction of THP protein in the DTT treated fraction compared to the untreated fraction, Figure 3A. THP removal from the UEV fractions enhanced the yield by 1.5-fold when compared to the unremoved fractions (Figure 3B, n=3). Hence, 12% PEG-based isolation following DTT treatment was considered optimal for UEVs isolation. Following this procedure, UEVs were isolated from healthy individuals (n=12) and were characterized as described in the methods section. The exosomes' average total protein and lipid content was 646µg/ml and 1240µg/ml, respectively, as shown in Figure 3C. A semi-quantitative exosome array was performed, Figure 3D, results showed intense positive staining for various genes including in the exosome array – Intercellular Adhesion Molecule 1 (ICAM), Tumor Susceptibility Gene 101 (TSG101), and tetraspanins CD63 and CD81. A weak signal was observed for Flotillin 1 (FLOT1) and programmed cell death 6 interacting protein (ALIX) and Annexin A5 (ANXA5), while no signal was detected for Epithelial Cell Adhesion Molecule (EpCAM). cis-Golgi matrix protein (GM130) was employed as a cellular protein contamination spot and negative control (blank spot). The quality of UEVs was assessed from the ratio of protein to lipid content (P/L), which ranged from 0.3-1.6, Figure 3E, indicating the integrity of extracellular vesicles. The average total UEV yield was 2.45 mg per 100 ml of urine. The average AChE activity was 550mU per mg of protein, confirming the purity, as shown in Figure 3F. Furthermore, DDPIV activity in the isolate was quantified in Figure 3G to verify that the activity was indeed in the microvesicular components of urine(22). These observations confirmed the quality of UEVs isolated from urine.

Morphology and size distribution of Urinary Exosomes

UEVs appeared round cup-shaped when visualized under the transmission electron microscope (Figure 4 A). Nanoparticle tracking (NTA) analysis revealed the size distribution profile of UEV with a mean particle size of 59 ± 22 nm (Figure 4 B). The concentration of the UEV particles was 3.19×10^8 particles per ml as quantified by NTA. Also, the size was determined from the intensity distribution calculated by DLS (Figure 4 C). Results of DLS measurements are shown as a number weighted distribution curve. At least three independent aliquots were measured in triplicate. The average size of the UEVs was 78 ± 56 nm based on the number distribution. Thus, using a combination of biophysical parameters, we verified that our isolated UEV fractions were in the reported size range and morphology of exosomes (23).

Selection of optimal housekeeping genes

Urinary EV RNA was extracted and quantified using the Agilent Pico RNA detection kit. The RNA was quantified to be 137pg/µl, n=6. The quality of isolated RNA was assessed against a standard marker; a sharp 5S peak was identified in between 25-200 nucleotides in RNA from UEV fractions, which confirmed the presence of exosome RNA, as shown in Figure 5A. There were no detectable 18S or 28S peaks. An overlay of urinary cell pellet RNA with the exosomal RNA shows degraded cellular RNA in the pellet RNA (24).

RNA was extracted from UEVs isolated from forty healthy urine samples, and the expression levels of five selected reference genes were quantified using qPCR. The expression levels of all genes were normally distributed, as shown in Figure 5B, with an average Ct value of 10.26 ± 1.33 . To identify the most stably expressed housekeeping genes, we have normalized the gene expression levels using the online software RefFinder. Each gene's stability score was determined using different tools included in the software such as Genorm, Normfinder, Bestkeeper, and the comparative delta Ct method to minimize the error associated with a single software-based evaluation. As different statistical algorithms were employed to evaluate the stability score, the ranking of genes slightly varied in different tools (details listed in Table 5).

Nevertheless, *B2M* and *RPL13A* were identified to be consistently stable with a lower mean stability score below 1.5 (Genorm) and below 1 (Normfinder), indicating a reduced variation of the expressed genes shown in Figure 5C. The recommended comprehensive ranking values for the average gene expression data (n=40) showed a similar trend with *B2M* and *RPL13A* in top-ranking positions (Figure 5D). The above two genes may be used as the most stable housekeeping genes for accurate gene expression analysis, particularly in urine samples.

Table 5 – Gene stability analysis of housekeeping genes

Gene Name	Accession Number	Genorm		Normfinder		Delta Ct		Bestkeeper			Comprehensive Ranking		
		Stability value	Rank	Stability value	Rank	Avg of	Rank	Std dev [+/- CP]	Rank	[r]	P-value	Geomean of ranking values	Rank
B2M	NM_004048.3	1.1	1	0.5	1	1.7	1	1.7	2	0.94		1.2	1
RPL13A	NM_000977.3	1.1	1	1.0	3	1.9	2	1.5	1	0.90	0.001	1.6	2
PPIA	NM_021130.5	1.3	3	1.3	4	2.0	4	1.8	3	0.94		3.5	4
HMBS	NM_000190.4	1.4	4	0.9	2	1.9	3	2.2	5	0.94	0.001	3.3	3
GAPDH	NM_001282587.2	2.2	5	3.3	5	3.4	5	2.2	4	0.43	0.006	4.7	5

Discussion

UEVs constitute a significant subset of extracellular vesicles derived from the kidney and urogenital tract (25, 26). They hold a concentrated source of biomarkers (RNA, protein, and lipid), protected from urinary ribonucleases and proteases degradation(2). Easy access and non-invasiveness have made urine an ideal source for biomarker analysis. This study established a clinically adaptable protocol for UEVs isolation and characterization and defined a normalization strategy for UEVs based gene expression studies.

Several techniques, including ultracentrifugation, ultra-filtration, size exclusion chromatography, and precipitation, are currently used for the isolation of UEVs (23). In this study, owing to the lower cost and easy adaptability of PEG-based precipitation of UEVs in clinical laboratories, we optimized this procedure with modifications to isolate EVs from urine (8). Comparison of biochemical properties like total protein, lipid and P/L ratio, and acetylcholine esterase between exosomal preparations obtained by PEG-based precipitation and commercial kit showed similar yields. Therefore, our results emphasize that PEG-based UEVs precipitation can be used in clinical labs for microvesicle-based downstream applications.

Reduced vesicle load and the presence of contaminant protein Tamm-horse fall protein (THP) or uromodulin in the urine leads to reduced UEVs yields. THP is a protein that is abundantly found in urine under physiological conditions. It is known to oligomerize into long polymers and compromises the vesicular yield at lower temperatures by forming a mesh that entraps the vesicles (22). It is also often a common contaminant that co-precipitates out along with exosomes(27). A previous study emphasized employing a DTT treatment step to release THP-entrapped exosomes(28). Similarly, we observed a decrease of THP protein density and an increase in the microvesicular yield after treating the fraction with dithiothreitol (DTT), suggesting a dissociation of THP mesh entrapping UEVs, as reported earlier (8).

Protein/Lipid ratio represents pure exosome preparations and can also be used as one of the quality control measures for distinguishing soluble proteins and protein aggregates in the EVs preparations (20). Acetyl-CoA choline esterase (AChE) was shown to be concentrated in exosomes and can thus be used to measure the quality of exosome preparations. (21, 23, 29). Thus, to characterize our isolated UEV fractions' purity, we measured both the total protein content and lipid content by using BCA kit and Sulpho-phospho-vanilin assay, respectively (30). Dipeptidyl peptidase-IV (DPP IV) is another pan membrane-associated peptidase, widely expressed in tissues and is also reported in the kidney cortex and the brush border and microvillus fraction(31). Both AChE and DPP IV are used to measure the bioactivity, purity, and functional activity of exosome preparations (32). The protein determination of the exosome antibody array confirmed the purity of vesicular preparation (33, 34). Tetraspanins CD63 and CD81, along with TSG101 and ALIX are pan exosome markers present on urinary exosomes (35, 36). Generally, the majority of vesicles present in urine are believed to be exosomes (37).

Biophysical characterization involving DLS, NTA, and EM estimates quality check of exosome preparations(38). In this study, we also used Transmission Electron Microscopy to characterize the morphological structure of the UEV fraction isolated. The size profile by DLS and NTA were in accord with the performance of exosomes(3). Thus, the above three methods estimate size, shape, morphology and agree with the published literature(3).

The need for ensuring quality assurance measures for qPCR is well acknowledged. MIQE guidelines provide an operational framework for qPCR-based gene expression studies (39). The above guidelines enable an efficient design of qPCR experiments, ascertain technical quality of gene expression studies, and reliable replication of experiments. For accurate interpretation of qPCR results, data normalization is a prerequisite step (19). Currently, there is a deficit of reliable housekeeping genes for conducting UEVs gene expression studies. Lack of ideal housekeeping genes leads to misinterpretation of final results.

Candidate reference genes analyzed showed a narrow Cq range among all experimental series ranging from 5 to 15. According to Genorm, the candidate genes with the lowest stability values are B2M (1.1) and RP13A (1.1) in the study. Based on Normfinder analysis, B2M (0.5) and RP13A (1.0) were top stable candidates. Genes with stable expression are indicated by low average expression stability values by Normfinder (40). B2M (1.7) and RP13A (1.5) can be considered as stable gene candidates. Best keeper calculates the CP standard deviation (SD) and coefficient of variation (CV) for each gene(41). Genes with SD values <1 are considered stable and are categorized as reference genes.

RefFinder integrates the above four programs' outcomes and derives the most stable gene profile(42). RefFinder analysis reduced the individual bias of each software and yielded a cumulative score. Absolute ranking values of B2M (1.2) and RP13A (1.6) defined them as universal housekeeping genes in UEVs gene expression studies. Following the recent guidelines, two or more genes have to be used at arriving at a normalized score(43).

Taken together, we have developed an easily adoptable lab protocol for isolation and characterization of UEVs. We established a quality control profile to characterize the urinary EVs preparations at the biochemical, biophysical, and molecular levels. To our knowledge, this is the first study reporting stable housekeeping genes in UEVs. The optimal reference genes derived from this study could provide meaningful choices for target gene expression and functional studies based on UEVs, thereby strengthening UEVs applications, including diagnostics and biomarker studies.

Abbreviations

UEVs – Urinary Extracellular Vesicles, DN – Diabetic nephropathy, B2M – Beta 2 microglobulin, RPL 13A – ribosomal protein L13, GAPDH- Glyceraldehyde 3-phosphate dehydrogenase, PPIA – Peptidylprolyl isomerase A, HMBS – Hydroxymethylbilane synthase, NTA - Nanoparticle tracking analysis, DLS – Dynamic light scattering.

Declarations

Ethics approval: The study was approved by Apollo Hospital Ethics Committee, Protocol no. CMBRC/2017/004.

Consent for publication: Not applicable

Availability of data and materials: The data supporting the findings of present study are available upon request.

Competing interest: There are no competing interests to declare. M.V.S. is a cofounder of Urvogelbio Private Ltd and owns equity in Urvogelbio Private Ltd and has no direct conflict in reference to this publication.

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Author contributions: Conceptualizations, Funding acquisition, Supervision, MVS; methodology, ADS, RK; software; ADS,RK, RS, formal analysis, ADS , RS and RK; investigation, SSM and SM, resources, MVS, ADS, SP; data curation, ADS, RK, SBA, SP; writing and original draft preparation, ADS; writing and review and editing, RS, MVS.

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References

1. Dhaun N, Bellamy CO, Cattran DC, Kluth DC; Utility of renal biopsy in the clinical management of renal disease. *Kidney Int* 2014; 85(5):1039-48.
2. Sugiyama RH, Blank A, Dekker CA; Multiple ribonucleases of human urine. *Biochemistry* 1981; 20(8):2268-2274.
3. Merchant ML, Rood IM, Deegens J.K.J., Klein JB; Isolation and characterization of urinary extracellular vesicles: implications for biomarker discovery. *Nature reviews. Nephrology* 2017; 13(12):731-749.
4. Feng Y, Lv LL, Wu WJ, et al.; Urinary Exosomes and Exosomal CCL2 mRNA as Biomarkers of Active Histologic Injury in IgA Nephropathy. *Am J Pathol* 2018; 188(11):2542-2552.
5. Gudehithlu KP, Hart P, Joshi A, et al.; Urine exosomal ceruloplasmin: a potential early biomarker of underlying kidney disease. *Clin Exp Nephrol* 2019; 23(8):1013-1021.
6. Karpman D, Ståhl A-L, Arvidsson I; Extracellular vesicles in renal disease. *Nature reviews. Nephrology* 2017; 13(9):545-562.
7. Lv LL, Feng Y, Tang TT, Liu BC; New insight into the role of extracellular vesicles in kidney disease. *J Cell Mol Med* 2019; 23(2):731-739.
8. Kanchi Ravi R, Khosroheidari M, DiStefano JK; A modified precipitation method to isolate urinary exosomes. *J Vis Exp* 2015(95):51158.
9. He L, Zhu D, Wang J, Wu X; A highly efficient method for isolating urinary exosomes. *Int J Mol Med* 2019; 43(1):83-90.
10. Musante L, Tataruch D, Gu D, et al.; A simplified method to recover urinary vesicles for clinical applications, and sample banking. *Sci Rep* 2014; 4:7532.
11. Sugimura T, Tanaka Y; The use of polyethylene glycol in concentration and purification of several bovine viruses. *Natl Inst Anim Health Q (Tokyo)* 1978; 18(2):53-7.
12. Gunasekaran PM, Luther JM, Byrd JB; For what factors should we normalize urinary extracellular mRNA biomarkers? *Biomol Detect Quantif* 2019; 17:100090.
13. Perez LJ, Rios L, Trivedi P, et al.; Validation of optimal reference genes for quantitative real time PCR in muscle and adipose tissue for obesity and diabetes research. 2017; 7(1):3612.
14. McKiernan J, Donovan MJ, Margolis E, et al.; A Prospective Adaptive Utility Trial to Validate Performance of a Novel Urine Exosome Gene Expression Assay to Predict High-grade Prostate Cancer in Patients with Prostate-specific Antigen 2-10ng/ml at Initial Biopsy. *European urology* 2018; 74(6):731-738.
15. Alberts B. Introduction to pathogens: *Molecular Biology of the cell*. Garland Science, New York: 2002.
16. Andersen CL, Jensen JL, Orntoft TF; Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res* 2004; 64(15):5245-50.
17. Vandesompele J, De Preter K, Pattyn F, et al.; Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 2002; 3(7):Research0034.
18. Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP; Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper-Excel-based tool using pairwise correlations. *Biotechnol Lett* 2004; 26(6):509-15.
19. Silver N, Best S, Jiang J, Thein SL; Selection of housekeeping genes for gene expression studies in human reticulocytes using real-time PCR. *BMC Mol Biol* 2006; 7:33.
20. Osteikoetxea X, Balogh A, Szabo-Taylor K, et al.; Improved characterization of EV preparations based on protein to lipid ratio and lipid properties. *PLoS One* 2015; 10(3):e0121184.
21. Savina A, Vidal M, Colombo MI; The exosome pathway in K562 cells is regulated by Rab11. *J Cell Sci* 2002; 115(Pt 12):2505-15.

22. Fernandez-Llama P, Khositseth S, Gonzales PA, Star RA, Pisitkun T, Knepper MA; Tamm-Horsfall protein and urinary exosome isolation. *Kidney Int* 2010; 77(8):736-42.
23. Gheini AH, Vogeli M, Baumgartner U, Vassella E, Draeger A, Burkhard FC; Improved isolation strategies to increase the yield and purity of human urinary exosomes for biomarker discovery. 2018; 8(1):3945.
24. Garcia-Contreras M, Shah SH, Tamayo A, et al.; Plasma-derived exosome characterization reveals a distinct microRNA signature in long duration Type 1 diabetes. *Scientific reports* 2017; 7(1):5998-5998.
25. Williams TL, Bastos C, Faria N, Karet Frankl FE; Making urinary extracellular vesicles a clinically tractable source of biomarkers for inherited tubulopathies using a small volume precipitation method: proof of concept. *Journal of nephrology* 2019;10.1007/s40620-019-00653-8.
26. Braun F, Müller R-U; Urinary extracellular vesicles as a source of biomarkers reflecting renal cellular biology in human disease. *Methods in cell biology* 2019; 154:43-65.
27. Chen CY, Hogan MC, Ward CJ; Purification of exosome-like vesicles from urine. *Methods in enzymology* 2013; 524:225-241.
28. Wachalska M, Koppers-Lalic D, van Eijndhoven M, et al.; Protein Complexes in Urine Interfere with Extracellular Vesicle Biomarker Studies. *Journal of circulating biomarkers* 2016; 5:4-4.
29. Niu Z, Pang R.T.K., Liu W, Li Q, Cheng R, Yeung W.S.B.; Polymer-based precipitation preserves biological activities of extracellular vesicles from an endometrial cell line. *PloS one* 2017; 12(10):e0186534-e0186534.
30. Visnovitz T, Osteikoetxea X, Sodar BW, Mihaly J, Lorincz P; An improved 96 well plate format lipid quantification assay for standardisation of experiments with extracellular vesicles. 2019; 8(1):1565263.
31. Sun AL, Deng JT, Guan GJ, et al.; Dipeptidyl peptidase-IV is a potential molecular biomarker in diabetic kidney disease. *Diab Vasc Dis Res* 2012; 9(4):301-8.
32. Spanu S, van Roeyen CR, Denecke B, Floege J, Muhlfeld AS; Urinary exosomes: a novel means to non-invasively assess changes in renal gene and protein expression. *PLoS One* 2014; 9(10):e109631.
33. Théry C, Zitvogel L, Amigorena S; Exosomes: composition, biogenesis and function. *Nature reviews. Immunology* 2002; 2(8):569-579.
34. Baranyai T, Herczeg K, Onódi Z, et al.; Isolation of Exosomes from Blood Plasma: Qualitative and Quantitative Comparison of Ultracentrifugation and Size Exclusion Chromatography Methods. *PloS one* 2015; 10(12):e0145686-e0145686.
35. Gheini AH, Vögeli M, Baumgartner U, et al.; Improved isolation strategies to increase the yield and purity of human urinary exosomes for biomarker discovery. *Scientific reports* 2018; 8(1):3945-3945.
36. Alvarez ML, Khosroheidari M, Kanchi Ravi R, DiStefano JK; Comparison of protein, microRNA, and mRNA yields using different methods of urinary exosome isolation for the discovery of kidney disease biomarkers. *Kidney international* 2012; 82(9):1024-1032.
37. Pisitkun T, Shen R-F, Knepper MA; Identification and proteomic profiling of exosomes in human urine. *Proceedings of the National Academy of Sciences of the United States of America* 2004; 101(36):13368-13373.
38. Li M, Zeringer E, Barta T, Schageman J, Cheng A, Vlassov AV; Analysis of the RNA content of the exosomes derived from blood serum and urine and its potential as biomarkers. *Philos Trans R Soc Lond B Biol Sci* 2014; 369(1652).
39. Bustin SA, Benes V, Garson JA, et al.; The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem* 2009; 55(4):611-22.
40. De Spiegelaere W, Dern-Wieloch J, Weigel R, et al.; Reference gene validation for RT-qPCR, a note on different available software packages. *PloS one* 2015; 10(3):e0122515-e0122515.
41. Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP; Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper–Excel-based tool using pairwise correlations. *Biotechnology letters* 2004; 26(6):509-515.
42. He S, An T, A R, Liu S; Validation of Reliable Reference Genes for RT-qPCR Studies of Target Gene Expression in *Colletotrichum camelliae* During Spore Germination and Mycelial Growth and Interaction With Host Plants. *Frontiers in Microbiology* 2019; 10(2055).
43. Jain N, Vergish S, Khurana JP; Validation of house-keeping genes for normalization of gene expression data during diurnal/circadian studies in rice by RT-qPCR. *Scientific reports* 2018; 8(1):3203-3203.
44. Théry C, Witwer KW, Aikawa E, et al.; Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *Journal of extracellular vesicles* 2018; 7(1):1535750-1535750.
45. Oosthuyzen W, Sime NE, Ivy JR, et al.; Quantification of human urinary exosomes by nanoparticle tracking analysis. *J Physiol* 2013; 591(23):5833-42.
46. Kesimer M, Gupta R; Physical characterization and profiling of airway epithelial derived exosomes using light scattering. *Methods* 2015; 87:59-63.

Tables

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Figures

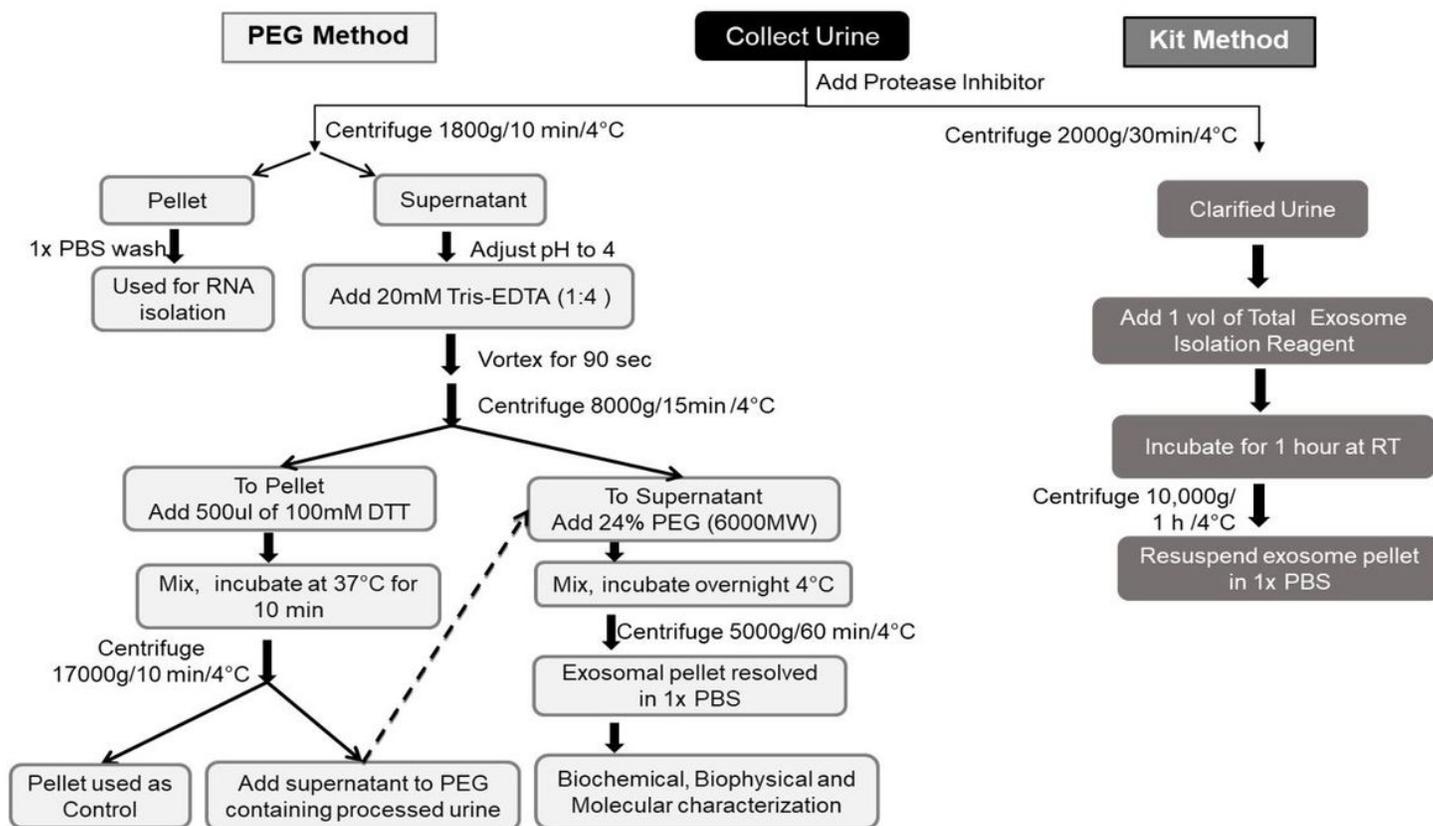


Figure 1

Schematic representation of the Urinary EVs Isolation procedure using PEG and commercially available kit method.

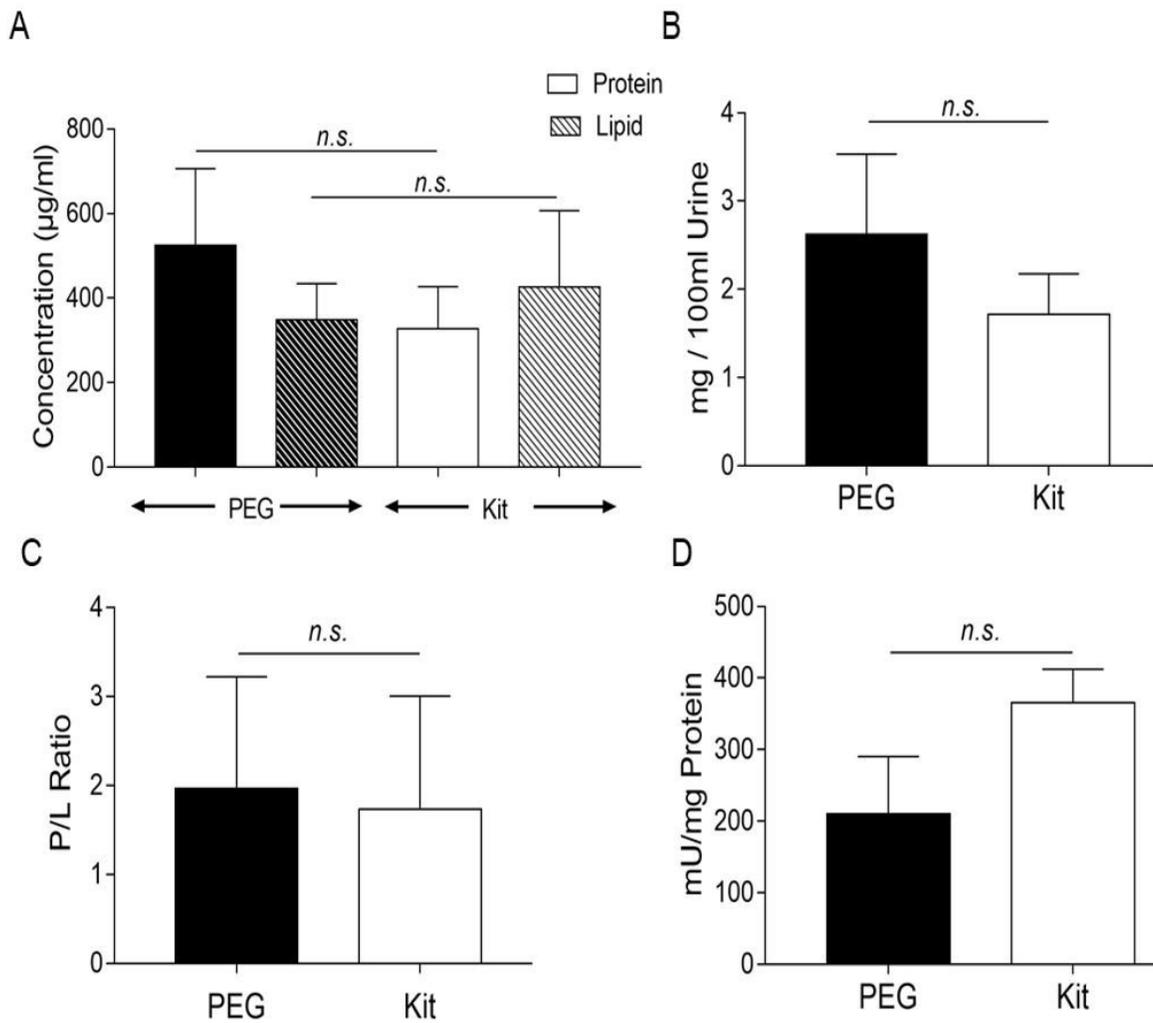


Figure 2
 Comparison of PEG and Kit method of UEVs isolation (A) Total protein and lipid content of the UEVs isolated using PEG method compared to the Kit method are shown as bar plots. (B) Bar plots indicate the UEV yield per 100ml of urine. (C) The ratio of Protein to Lipid contents are shown. (D) Acetylcholinesterase activity assay demonstrated the quality of the isolated UEV using both methods. All data are represented as mean ± SEM, n=3.

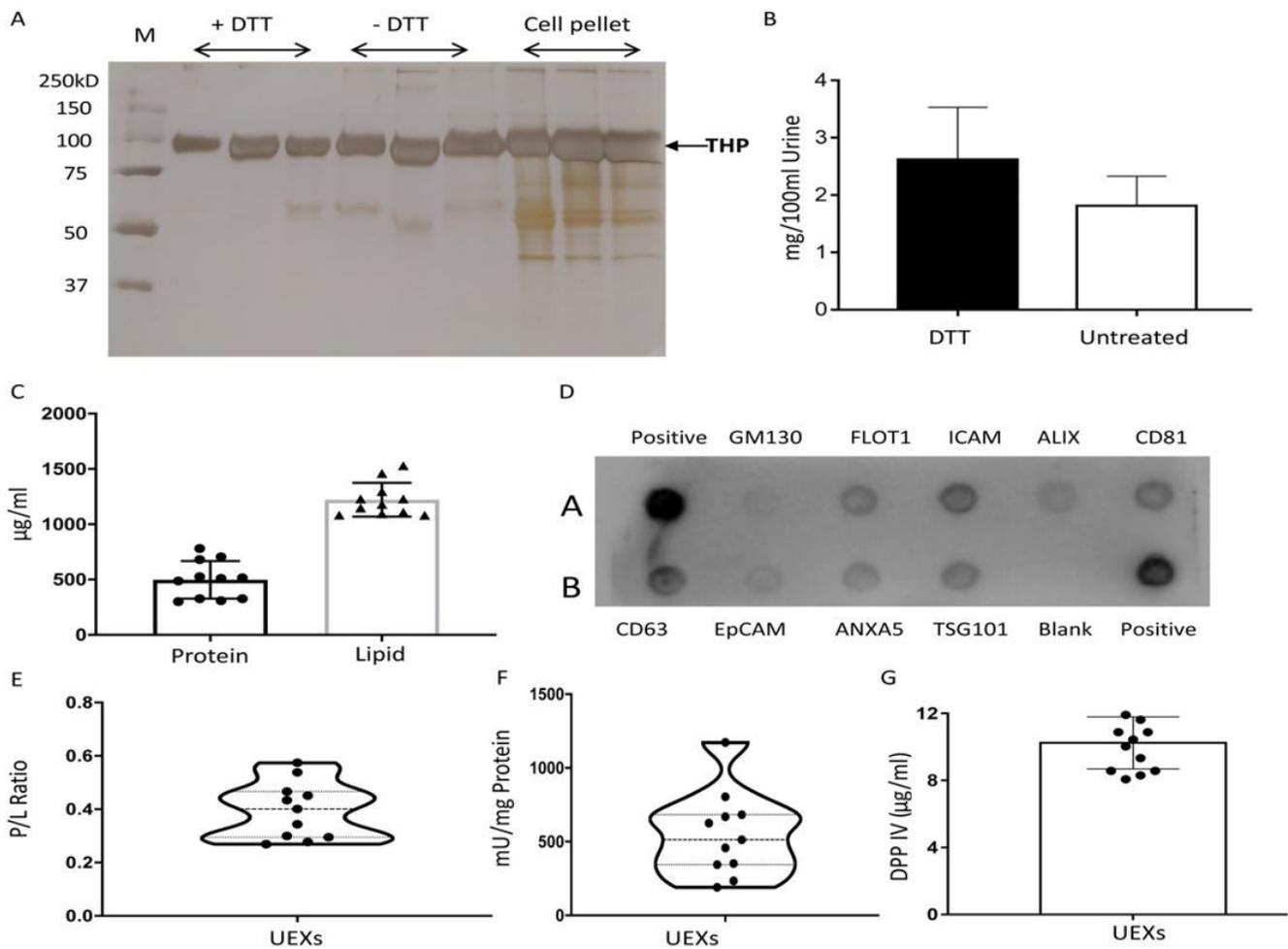


Figure 3

Biochemical characteristics of the UEVs. (A) SDS-PAGE analysis illustrates the abundance of THP protein in the urinary cell pellet compared to UEV with or without DTT treatment (n=3). (B) DTT treatment of the UEV protein aggregate dissociates and makes exosome protein available. (C) Protein and lipid content of the UEVs derived from healthy individuals (D) Western blot array of UEV fraction proteins confirming presence of exosome in the isolate (E) Protein to Lipid ratio of the UEVs (F) Acetylcholinesterase assay of UEVs ensures the purity of the exosomes. (G) Dipeptidyl peptidase IV activity demonstrates the isolate as the microvesicular component of urine. Data illustrated in B-E are represented as mean \pm SEM, n=3.

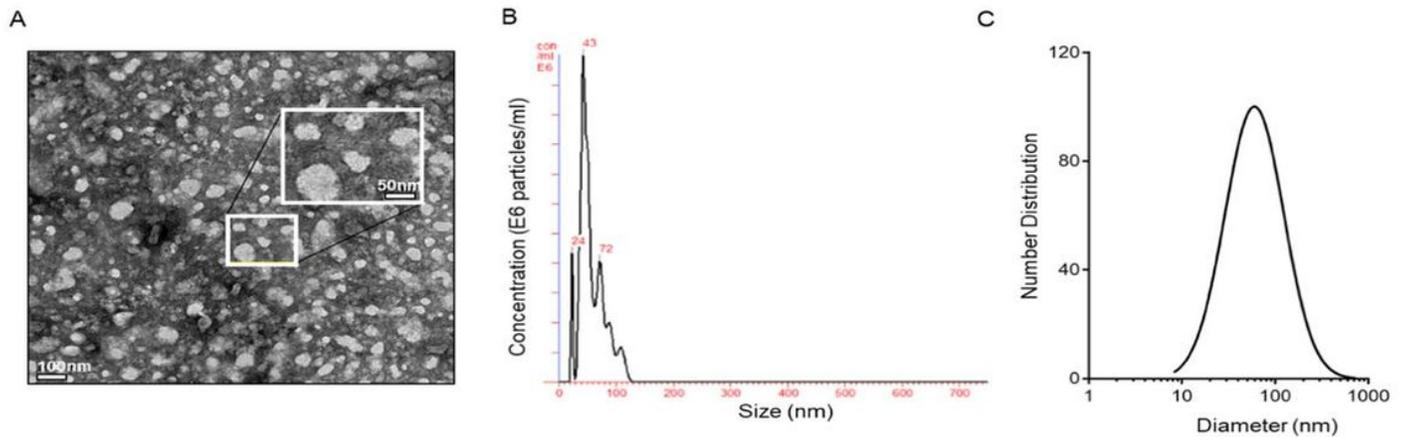


Figure 4

Morphology and size distribution of the UEVs. (A) Representative TEM images of the isolated UEVs are shown. The scale bar represents 100nm; inset 50 nm. (B) The size distribution profile generated by NTA revealed the size of UEVs ranging between 30-130nm. (C) Size distribution of exosomes measured by DLS is shown as a number distribution curve with a mean particle diameter of 78 ± 56 nm. Three measurements were taken for at least 3 independent urine samples and performed in triplicates.

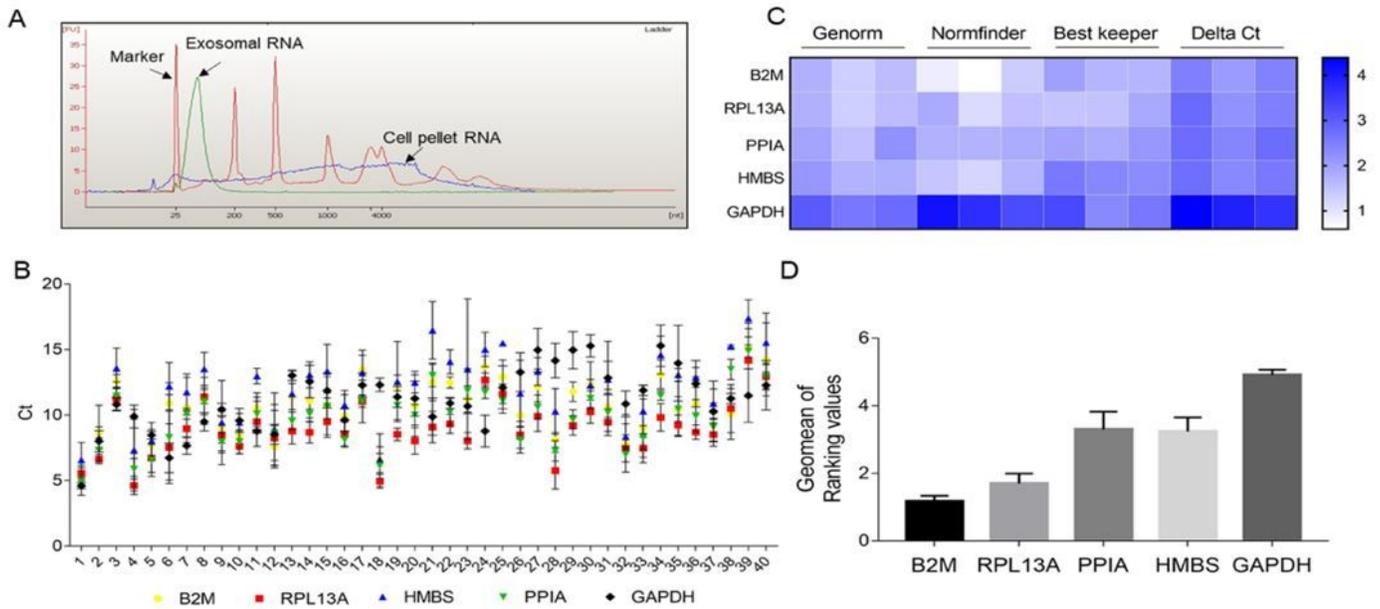


Figure 5
 Identification of optimal reference gene (A) The quality of RNA from UEVs was assessed using Bioanalyzer. A sharp 5S RNA peak appeared between 25-200nt (marked in green), while no detectable 18S and 28SRNA peaks were observed. (B) Gene expression profiles of five candidate reference genes in UEVs are shown as cycle threshold (Ct) values. Data are shown as SEM, n=40. (C) Heatmap represents the normalized gene stability (n=40) using different algorithms present in RefFinder. Most stable genes are illustrated in dark and least stable genes in light blue shades. (D) Bar plot illustrates the overall comprehensive ranking of genes based on the geomean ranking values (n=3).

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