Gentaur Europe BVBA Voortstraat 49, 1910 Kampenhout BELGIUM
Tel 0032 16 58 90 45 info@gentaur.com

ExoQuant™ Overall Exosome Capture and Quantification Assay Kit (human urine, colorimetric)

(Catalog # K1202-100; 100 assays (96 well transparent immunoplate for colorimetric readings; Store at 4°C))

I. Introduction:

Exosomes are small endosome derived lipid nanoparticles (50-120 nm) actively secreted by exocytosis by most living cells. Exosome release occurs either constitutively or upon induction, under both normal and pathological conditions, in a dynamic, regulated and functionally relevant manner. Both the amount and molecular composition of released exosomes depend on the state of a parent cell. Exosomes have been isolated from diverse cell lines (hematopoietic cells, tumor lines, primary cultures, and virus infected cells) as well as from biological fluids in particular blood (e.g. serum and plasma from cancer patients) and other body fluids (broncho alveolar lavage fluid, pleural effusions, synovial fluid, urine, amniotic fluid, semen, saliva etc). Exosomes have pleiotropic physiological and pathological functions and have an emerging role in diverse pathological conditions such as cancer, infectious and neurodegenerative diseases.

ExoQuant™ Kit is a patented double sandwich enzyme-linked immunoassay for quantitative and qualitative analysis of exosomes. It is a successful platform for exosomes quantification and characterization from small amount of human biological fluids (plasma, serum, urine, saliva) or cell media. It may be exploited to identify exosomes released by cancer cells in the plasma and urine of tumor patients in various disease conditions. ExoQuant™ consists of ELISA plates pre-coated with proprietary pan-exosome antibodies enabling specific capture of exosomes from different biological samples, including cell culture supernatants and human biological fluids. Quantification and characterization of exosomal proteins is subsequently performed using appropriate detection antibodies against exosome associated antigens that can be for either generic or cell/tissue-specific exosomes. The kit contains Kit contains Lyophilized Exosome Standards for assay calibration. Lyophilized Exosome Standards, characterized for protein content and particle number allow the quantification of unknown sample by a standard calibration curve. Transparent and white plates are available depending on the downstream detection approach (colorimetric and luminometric respectively).

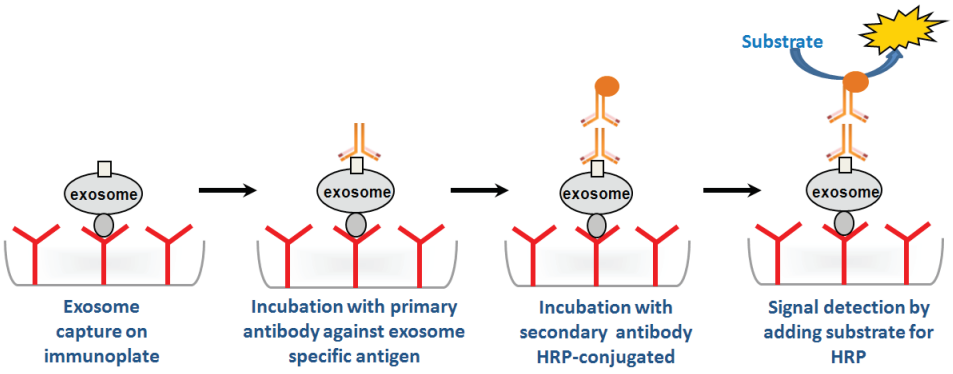


Figure 1. ExoQuant™ Exosome Capture and Quantification Assay Kit Principle.

II. Application:

- ExoQuant™ Kits ensure dose-dependent exosome detection in a variety of samples. No initial exosome purification required. The quantification can be both relative and absolute as the assay calibration enables the calculation of the approximate exosome quantity for each sample.
- Exosome comprehensive profiling.
- Pre-clinical research on non-invasive biomarkers for detection and monitoring of a number of pathological conditions (inflammation, cancer, neurodegeneration, etc). User friendly and suitable for multiple marker analyses.
- Up to 100 µl of biological sample can be loaded per well. Concentrated urine samples are recommended prior to capture.

III. Sample Type:

- Human biological fluids: Urine.

IV. Kit Contents (for overall exosomes capture and quantification from human urine):

Components	Description	K1202-100	Part Number
Sample buffer (1X)	Buffer for antibody dilution and incubation	2 bottles (2 x 10 ml)	K1202-100-1
Washing buffer (25X)	Buffer for washing plate	2 bottles (2 x 15 ml)	K1202-100-2
Primary antibody	α-CD9 mouse antibody	1 vial (20 µl)	K1202-100-3
HRP-conjugated	HRP-conjugated secondary antibody	1 vial (5 µl)	K1202-100-4
Substrate chromogenic solutions	Substrate for chromogenic detection	1 bottle (10 ml)	K1202-100-5
Stop solution	1M Sulphuric Acid (H_2SO_4)	1 bottle (10 ml)	K1202-100-6
Exosome Standards	Lyophilized exosomes from healthy donors (Urine)	2 vials (2 x 100 µg)	K1202-100-7
Immunoplate (Transparent)	Standard multiwell plates 96-well format where assays can be conducted as singletons and/or multiple wells	1 plate	K1202-100-8

V. User Supplied Reagents and Equipment:

- Single-use and/or pipettes with disposable tips 2-100 µl
- Polypropylene tubes
- Pipettes 1 ml and 5 ml for reagent preparation
- Deionized water
- PBS
- Plate shaker
- Humidified chamber or incubator at 37°C
- Disposable pipetting reservoirs
- Microplate reader
- ELISA sealing film or parafilm

VI. Shipment and Storage:

All the reagents are shipped and stored at 4°C for up to 24 months, if unopened. Briefly centrifuge small vials prior to opening. DO NOT FREEZE!

VII. Reagent Preparation and Storage Conditions:

- Dilute the 25X Washing Buffer to 1X with deionized water. If crystals are observed, dissolve them by warming up the vial at 37°C the solution before preparing a dilution.
- Reconstitute lyophilized exosome standard by adding 100 µl of deionized water, pipette the solution up and down 10-15 times, avoiding bubbles. Vortex the reconstituted standard for 60 sec. Briefly centrifuge the tubes containing standards to ensure that the solution is collected at the bottom of the tube. Pipette the solution up and down 10 times, again avoiding any bubbles.
- If purified exosome samples are analyzed, use 1X PBS to adjust the volume and concentration of samples (overall volume/well is 100 µl).
- In general, unfractionated samples are analyzed without dilutions (100 µl/well). If OD values observed, are beyond the range of standard curve. Dilute the samples using 1X PBS.
- Detection antibody should be diluted to 500-fold in sample buffer.
- HRP-conjugated secondary antibodies should be diluted to 2000-fold in sample buffer.
- Substrate solution for colorimetric readings and stop solution are ready to use.
- The plate is packed in an opaque aluminum pouch which complies with the food and pharmaceutical regulation. The pouch is easy to open and is re-sealable by zip closure.
- ELISA strips: Unused strips should be placed back in the foil pouch with the included desiccant pack, resealed and stored at 4°C for up to one month.
- Exosome standards: The remaining reconstituted standard stock solution should be aliquoted into polypropylene vials (preferably low binding) and stored at -20°C for up to one month or at -80°C for up to six months. Strictly avoid repeated freeze-and-thaw cycles.
- Store opened and diluted reagents at 4°C up to 24 months if unopened. After opening, use within one month.

VIII. ExoQuant™ Assay Protocol:

1. Human Urine Sample Preparation:

a) Centrifuge at 16,000g for 20 min at room temperature.
b) Filter by using 0.45 µm filter.

Concentrate urine samples using a spin concentrator (15-20 times) *. **The quantity of exosomes could vary between samples. A larger starting amount of sample should be used if the signal is weak.*

2. Reconstituted Exosome Standard for calibration curve: Bring all the reagents to room temperature and briefly vortex the tubes before use. The positive control is represented by the highest concentration of exosome standards. The negative control is represented by sample buffer or 1X PBS for analysis of purified exosomes and sample matrix (e.g. exosome depleted cell culture supernatant or plasma) for unfractionated samples.

a) Reconstitute lyophilized exosome standard by adding 100 µl of deionized water in each vial.
b) Pipette the solution up and down 10-15 times, avoiding bubbles.
c) Vortex the reconstituted standard for 60 sec. Briefly centrifuge the tubes to spin down the drops and ensure that the solution is collected at the bottom of the tube.
d) Pipette the solution up and down 10 times, again avoiding bubbles.
e) Briefly centrifuge again. Add 100 µl of 1X PBS to reach a final volume of 200 µl per vial.

3. Standard Curve Preparation: Standard dilutions are prepared directly in the strips. Please use the exosome stock solution prepared as indicated above to perform six two-fold serial dilutions using 1X PBS (see figure below). The standard concentrations in the wells will be represented as 50 µg, 25 µg, 12.5 µg, 6.25 µg, 3.125 µg, 1.5625 µg and 0.78125 µg respectively.

a) Add 200 µl of reconstituted exosome solution to wells A1 and A2 (2 wells only).
b) Add 100 µl of 1X PBS to wells B1 to H2 (14 wells).
c) Serial dilution (stop at G1 and G2).
d) Transfer 100 µl of A1 into B1 and mix.
e) Transfer 100 µl of B1 into C1 and mix.
f) Transfer 100 µl of C1 into D1 and mix.
g) Transfer 100 µl of D1 into E1 and mix.
h) Transfer 100 µl of E1 into F1 and mix.
i) Transfer 100 µl of F1 into G1 and mix.

j) Discard 100 µl from G1 to result in a final volume of 100 µl.
k) NOTE: Leave H1 (and H2) as 1x PBS for negative controls.
l) Repeat serial dilution for A2 to G2.

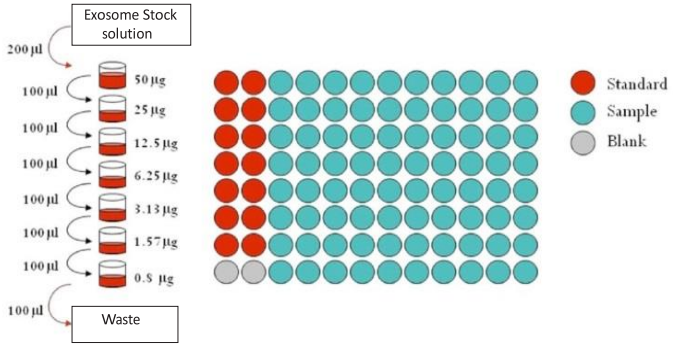
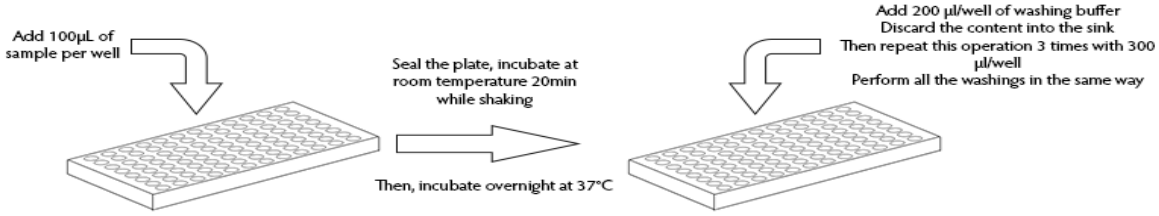


Figure 2. Illustration of the two-fold serial dilution required for the calibration curve. Add 100 µl of 1X PBS to reconstituted exosome standard to obtain a volume of 200 µl. Then transfer in the first well of ELISA strip. Add 100 µl of 1X PBS in the other wells of the strip used for standard curve and prepare exosome dilutions as shown in the Figure 2.

4. **Exosome binding**: Add 100 µl of prepared samples to wells A3 to H12 (add PBS 1X if the volume is less than 100 µl). Seal the plate with a parafilm and incubate at room temperature while shaking for 30 min (2-3 rotations per sec). Transfer the plate to +37°C and incubate overnight (12 hr-20 hr) in humid chamber **(for human urine samples).**
5. Wash the plates as shown below. Add 200 µl/well of Washing Buffer and discard the plate contents by pouring out. No Touch. Wash three times with 300 µl/well of Washing Buffer. After each addition, pour off wash. No Touch. All subsequent washings should be performed in the same manner.



6. **Primary antibody binding:** Wash the plate as indicated above. Then add 100 µl of mouse anti-human exosome Detection Antibody solution (diluted in Sample Buffer at 1:500 dilution) to each well. Seal the plate with parafilm and incubate at room temperature while shaking for 15 min (2-3 rotations per sec). Then incubate for 2 hr at 37°C in humid chamber **(for human urine samples).**
7. Wash the plate as indicated above. Add 100 µl of rabbit anti-mouse IgG HRP-conjugated secondary antibody solution to each well (diluted to 1:2000 dilutions in 1X Sample Buffer). Seal the plate with parafilm and incubate at room temperature while shaking for 15 min (2-3 rotations per sec). Then incubate for 1 hr at 37°C in a humid chamber **(for human urine samples).** Wash the plate as indicated above.
8. **For Colorimetric detection:** Add 100 µl of Substrate Chromogenic Solution to each well and incubate at room temperature in the dark for 5-10 min. Be careful not to immerse the metallic components of a pipette into the substrate solution. Also avoid making bubbles and, if formed, remove them gently with a pipette tip. Do not seal the plate and monitor till a blue color is visible. Intensity of the color is proportional to the exosome concentration only within a certain dynamic range. Many plate readers do not deliver accurate results when the OD is above 3. Stop the reaction by adding 100 µl of Stopping Solution to each well. The color will change from blue to yellow. Read the absorbance at 450 nm within 10 min. If possible, the absorbance should also be read at 570 nm and the measurement should be subtracted from the measurement at absorbance 450 nm.
9. **Calculation:** Exosome standards are provided as assay calibrators and also as the positive control. It is important to note that the origin of purified standard exosomes may change the proportion of common exosomal proteins such as CD9. The amount of proteins on their membrane might differ slightly from the amount on the sample exosomes. The standard curve is used to determine the amount of exosomes in an unknown sample. Figure 3, is an example of standard curves obtained. The curve is obtained by plotting the average readings for different standard concentrations against the corresponding amounts of exosomes. Calculate the mean absorbance for each set of duplicate standards, controls and samples. The values of the negative controls (blanks) must be subtracted from all OD values before the results can be interpreted. The regression curve coefficient should be above 0.95. The estimated sample concentration is reliable if within the linear range of the curve, otherwise the samples must be diluted and the test repeated. For diluted samples, multiply the concentrations with the appropriate dilution factors.

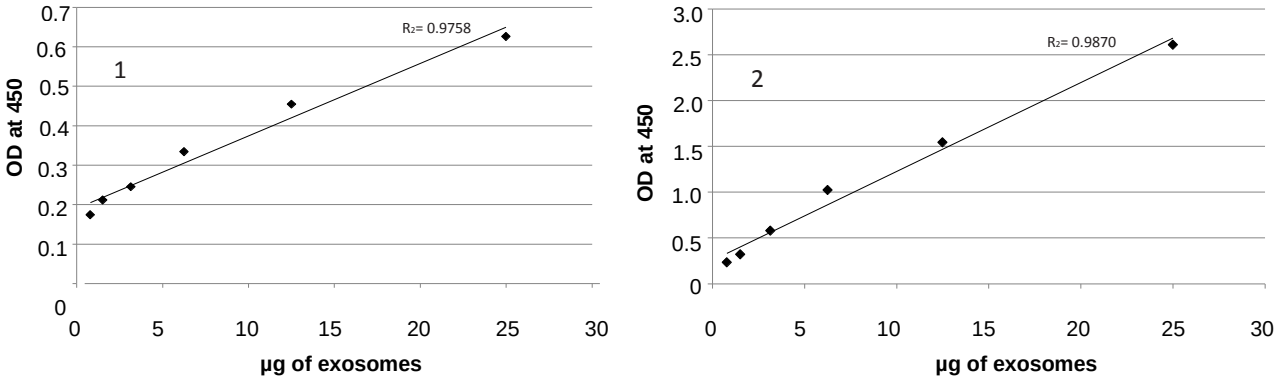


Figure 3. CD9 Titration of COLO1 (1) and plasma (2) exosomes. Example of standard curves obtained. Exosome standard preparation derived from (1) COLO1 Cell Culture Supernatant and (2) Plasma of healthy donors. This standard curve is for demonstration only. For quantification purposes, a standard curve must be obtained with every assay.

10. **Sensitivity:** The sensitivity of the ExoQuant™ was compared to Western blot. The data reported in Figure 4 and 5 demonstrate that the sensitivity of the ExoQuant™ is higher than that of Western blotting. Figure 5 shows that 10 µg of lyophilized exosomes is equivalent to 0.1 ng of recombinant exosomal protein (CD9). Since the standard curve's lower concentration is 0.39 µg of lyophilized exosomes (Figure 4), the sensitivity of our test is around 39 pg of protein equivalent.

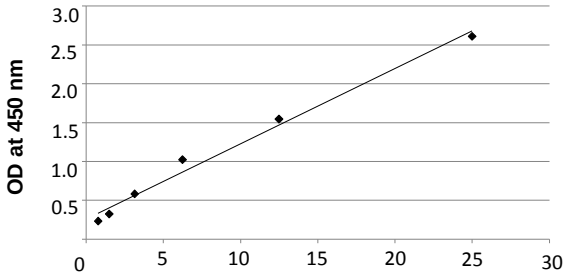


Figure 4. CD9 titration of plasma healthy donor exosome standards.

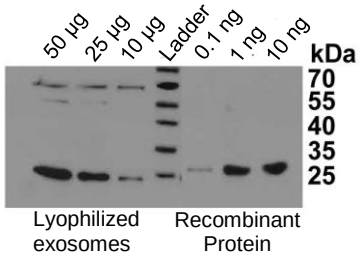


Figure 5. CD9 marker detection by Western Blotting on lyophilized exosomes from human plasma and the recombinant CD9 protein.

11. **Reproducibility:** Intra Assay (within run) CV (%) < 10 Inter Assay (interplate run) CV (%) < 13. Coefficient of variation (CV) is expressed as a percentage of variance to the mean calculated for intra assay reproducibility evaluation by assessing at least 4 replicates of three different standard preparations containing different exosome concentrations. Subsequently, assay to-assay reproducibility was assessed by comparing mean absorbance for the same samples on independent plates tested by different operators.

IX. Related Products:

Products/Catalog Number
ExoQuant™ Overall Exosome Capture and Quantification Assay Kit (human plasma, Colorimetric) # K1201-100
ExoQuant™ Overall Exosome Capture and Quantification Assay Kit (human urine, Colorimetric) # K1202-100
ExoQuant™ Overall Exosome Capture and Quantification Assay Kit (human serum, Colorimetric) # K1203-100
ExoQuant™ Tumor-derived Exosome enrichment and Quantification Assay Kit (biological fluids/cell media, Colorimetric) # K1204-100
ExoQuant™ Overall Exosome Capture and Quantification Assay Kit (Cell media, Colorimetric) # K1205-100
ExoQuant™ Overall Exosome Capture and Quantification Assay Kit (human plasma, Luminometric) # K1206-100
ExoQuant™ Overall Exosome Capture and Quantification Assay Kit (human urine, Luminometric) # K1207-100
ExoQuant™ Overall Exosome Capture and Quantification Assay Kit (human serum, Luminometric) # K1208-100
ExoQuant™ Tumor-derived Exosome enrichment and Quantification Assay Kit (biological fluids/cell media, Luminometric) # K1209-100
ExoQuant™ Overall Exosome Capture and Quantification Assay Kit (cell media, Luminometric) # K1210-100

X. General Troubleshooting Guide:

Problems	Cause	Solution
High background across entire plate	. Substrate incubation carried out in the light . Incubation temperature too high . Secondary antibody too concentrated or left on too long . Substrate solution or stop solution is not fresh . Contaminants from laboratory glassware	. Substrate incubation should be carried out in the dark . Antibodies have optimum binding activity at the correct temperature. Ensure that the incubations are carried out at the correct temperature and that incubators are set at the correct temperature and is working. The Incubation temperature may require some optimization . Check dilution of the secondary antibody. Use it at the recommended dilution. Stop the reaction using stop buffer as soon as the plate has developed enough for the absorbance readings . Use fresh substrate solution. Stop solution should be clear (if it has gone yellow, this is a sign of contamination and it should be replaced) . Ensure reagents are fresh and prepared in clean glassware
Positive results in negative control	. Contamination of reagents/samples . Insufficient washing of plates	. May be contamination of reagents or samples. Avoid cross contamination between different wells. Use fresh reagents and pipette carefully . Ensure wells are washed adequately soak the wells with wash buffer and tap plate on absorbent paper after every wash
Low absorbance values	. Target protein not present in sample/Low level of target protein in sample . Insufficient primary or secondary antibody . Substrate solutions not fresh or incorrectly combined . Reagents not fresh or not at the correct pH . Incubation time not long enough . Incubation temperature too low . Stop solution not added	. Check the expression profile of the target protein to ensure that it is present in your samples. If the quantity of target protein is very low, increase the amount of sample used, or try to concentrate. Ensure you are using a positive control within the detection range of the assay . Check if the recommended amount of antibody is being used. The concentration of antibody may require increasing for the optimization of results . Prepare the substrate solutions immediately before use. Ensure that the stock solutions are in date and have been stored correctly, and are being used at the correct concentration. Ensure the reagents are used as directed at the correct concentration . Ensure reagents have been prepared correctly and are within the expiry date . Ensure that you are incubating the antibody for the recommended amount of time, if an incubation time is suggested. The incubation time may need to be increased for optimization of results. Longer incubation time may be required . Antibodies have optimum binding activity at the correct temperature. Ensure the incubations are carried out at the correct temperature and that incubators are set at the correct temperature and is working. Incubation temperature may require some optimization. Ensure that all reagents are at room temperature before proceeding . Addition of stop solution increases the intensity of the color reaction and stabilizes the final color reaction

Inconsistent absorbance across the plate	. Plates stacked during incubations . Pipetting inconsistent . Antibody dilutions/Reagents not well mixed . Wells allowed to dry out . Inadequate washing . Bottom of the plate is dirty affecting absorbance readings	. Stacking of plates does not allow distribution of temperature across the wells of the plates. Avoid stacking . Ensure pipettes are working correctly and are calibrated. Ensure pipette tips are pushed on far enough to create a good seal. Take particular care when diluting down the plate and watch to make sure the pipette tips are all picking up and releasing the correct amount of the liquid. This will greatly affect the consistency of results between duplicate wells . To ensure a correct dilution of samples/standards across wells, ensure that all the reagents and samples are mixed before pipetting onto the plate . Ensure that plates are well sealed with film when incubating. Place a humidifying water tray (bottled clean/sterile water) on the bottom of the incubator . This will lead to some wells not being washed well as others, leaving inside different amounts of unbound antibody, which will give inconsistent results . Clean the bottom of the plate carefully before re-reading the plate
Color developing slowly	. Plates are not at the correct temperature Secondary . Secondary Antibody too weak staining . Contamination of solutions	. Ensure plates are at room temperature and that the reagents are at room temperature before use . Prepare the substrate solutions immediately before use. Ensure that the stock solutions are in date and have been stored correctly, and are being used at the correct concentration. Ensure the reagents are used as directed . Presence of contaminants, such as sodium azide and peroxides can affect the substrate reaction. Avoid using reagents containing these preservatives
Poor Standard Curve	. Inaccurate pipetting . Improper standards and samples preparation . Improper washings	. Check pipettes and increase attention . Ensure to reconstitute standards in a proper buffer and mixing thoroughly by vortexing and gentle pipetting. . Wash thoroughly as recommended in the assay procedure. Wash for longer time after incubation with the HRP-conjugated secondary antibody
Low signals	. Low signals in standards due to improper storage or preparation . Low signals in samples due to low exosome concentration	. Ensure to store reconstituted standards at -20°C and strictly avoid thaw and freeze cycles. Assure that standards are properly reconstituted and standard dilutions are prepared as suggested in the data sheet . Check out the sample preparation protocols
Low reproducibility of duplicates	. Inaccurate pipetting . Improper standards and samples preparation	. Check the pipettes and be careful with pipetting . Ensure to mix thoroughly by vortexing and gentle pipetting before loading onto wells

FOR RESEARCH USE ONLY! Not to be used on humans.