ELISA PRODUCT INFORMATION & MANUAL

Human Exosome ELISA Kit (Colorimetric)

NBP3-11770 (Plasma)

NBP3-11771 (Serum)

NBP3-11772 (Cell Media)

NBP3-11773 (Tumor-derived)

NBP3-14800 (Urine)

Enzyme-linked Immunosorbent Assay for quantitative detection. For research use only. Not for diagnostic or therapeutic procedures.

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PRODUCT DESCRIPTION

Product overview

Human Exosome ELISA Kits are a patented double sandwich ELISA assay for quantitative and qualitative analysis of exosomes. In particular, Human Exosome ELISA Kit is a successful platform for exosomes quantification and characterization from small amount of human biological fluids or cell media and it may be exploited to identify exosomes released by cancer cells in the plasma and urine of tumor patients in various disease conditions.

Human Exosome ELISA Kits consits 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. Lyophilized Exosome Standards, characterized for protein content and particle number (NTA) allow the quantification of unknown sample by a standard calibration curve.

About Exosomes

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 amount and molecular composition of released exosomes depend on the state of a parent cell.

Exosomes have pleiotropic physiological and pathological functions and an emerging role in diverse pathological conditions such as cancer, infectious and neurodegenerative diseases.

PRODUCT CONTENT

Reagents provide in each kit:

Description	Component		Amount
Sample buffer 1X	Buffer for antibody dilution and incubation		2 bottles (2 X 10 ml)
Washing buffer 25X	Buffer for washing plate		1 bottle (20 ml)
Detection solutions	Colorimetric kit	Substrate solution + Stop solution	2 bottles (2 x 10 ml)
	Luminometric Kit	Substrate solutions A and B	2 bottles (2 x 10 ml)
Immunoplate	Immunoplate standard multiwell plates 96-well format where assays can be conducted as singletons and/or multiple wells		1 plate
Exosome Standards for assay calibration	Lyophilized Exosome Standards in according with kit purchased		2 vials (2 X 100 µg)
Primary Antibody	Monoclonal Anti-Human CD9 (unconjugated or biotin conjugated)		1 vial (20 µl)
HRP conjugated	HRP-conjugated secondary antibody (5 µl) or streptavidin (2 µl)		1 vial (5 µl) or 1 vial (2 µl)
* For TEST kit the amount of reagents is limited to 24 well assay			

Other material required

- 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

STORAGE INFORMATION

All reagents provided within the Human Exosome ELISA Kit Ready To Use Kits can be stored at +4°C for up to 24 months, if unopened.

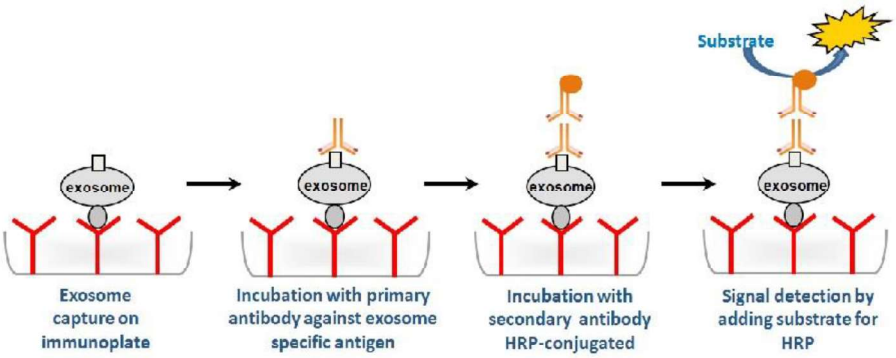
DO NOT FREEZE!

Open and reconstituted components	
ELISA stripes	Unused stripes should be placed back in the foil pouch with the included desiccant pack, resealed and stored at +4°C for up to six months.
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.
Primary detection antibody	Store opened and diluted reagents at +4°C up to 24 months if unopened. After opening, use within one month.
Sample buffer 1X	
Washing buffer 25X	
HRP conjugated	
Substrate for chromogenic or luminometric detection in buffered solution, ready to use	
Stop solution (only in colorimetric kit)	

Human Exosome ELISA Kit Ready to Use Kit Procedure

Overview

Human Exosome ELISA Kit Ready to Use Kit has been designed to capture exosomes onto ELISA plate and quantify them by colorimetric, luminometric or fluorimetric detection from any biological sample.



WARNING! Do not use with plasma heparin samples!

STEP A: Sample preparation

1- Human plasma and serum

Prepare samples by 3 centrifugation steps to eliminate red blood cells and cellular debris. After each step, transfer the supernatant to a new tube and discard the pellet.

- 10' at 300 g at 4°C (save supernatant; discard pellet).
- 20' at 1200 g at 4°C (save supernatant; discard pellet).
- 30' at 10 000 g at 4°C (save supernatant; discard pellet).
- Human plasma can be diluted 1/1 in PBS 1X prior to load onto ELISA plates. Human serum has to be used without dilution.

2- Cell culture medium

Prepare cell supernatants by 3 centrifugation steps:

- 10' at 300 g at 4°C (save supernatant; discard pellet).
- 20' at 1600 g at 4°C (save supernatant; discard pellet).
- 30' at 10000 g at 4°C (save supernatant; discard pellet).
- Concentrate cell supernatant 10-20 fold in spin concentrator*.

3- Human urine

- Centrifuge at 16 000 g for 20' at room temperature.
- Filter by using 0.45 µm filter.
- Concentrate urine samples by spin concentrator 15-20 times*.

**The quantity of exosomes could vary between samples. Concentration factors are given for information purposes only, a larger starting amount of sample should be used if the signal is weak.*

STEP B: Exosome Standard reconstitution and calibration curve

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

- **Calibration curve:**
  - Add 200 µl of reconstituted exosome solution to wells A1 and A2 (2 wells only).
  - Add 100 µl of 1x PBS to wells B1 to H2 (14 wells).
  - Serial dilution (stop at G1 and G2).
  - Transfer 100 µl of A1 into B1 and mix.
  - Transfer 100 µl of B1 into C1 and mix.
  - Transfer 100 µl of C1 into D1 and mix.
  - Transfer 100 µl of D1 into E1 and mix.
  - Transfer 100 µl of E1 into F1 and mix.
  - Transfer 100 µl of F1 into G1 and mix.
  - Discard 100 µl from G1 to result in a final volume of 100 µl.
  - NOTE: Leave H1 (and H2) as 1x PBS for negative controls.
  - Repeat serial dilution for A2 to G2.

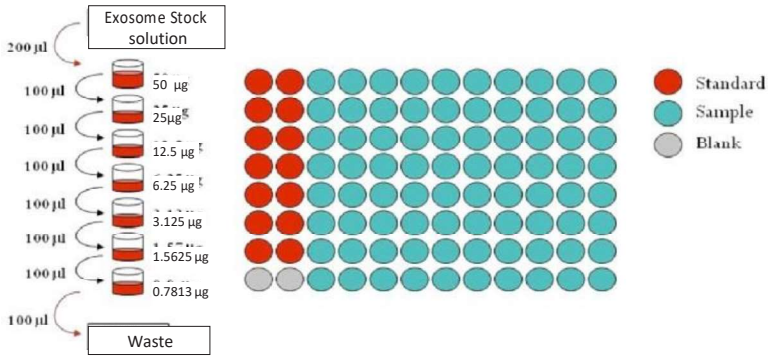


Illustration of the two-fold serial dilutions required for calibration curve

Add 100 µl of PBS 1X to reconstituted exosome standard to obtain a volume of 200 µl, then transfer in the first well of ELISA strip. Add 100 µl of PBS 1X in the other wells of the strip used for standard curve and prepare exosome dilutions as indicated in figure.

3. Sample loading

- Add 100 µl of prepared samples to wells A3 to H12 (add PBS 1X if the volume is minor than 100 µl).
- Seal the plate with parafilm and incubate at room temperature while shaking for 30' (2-3 rotations per second).

4. Sample incubation for exosome binding

- **For human plasma and serum**
- Transfer the plate to +4°C and incubate overnight (12h-20h).

- **For human urine and cell culture medium**
- Transfer the plate to +37°C and incubate overnight (12h-20h) in humid chamber.

STEP C: Wash the plate

NOTE: Make sure to never touch the bottom or sides of the wells or you will scrape off your samples/standards. As a reminder "No Touch" is placed on that line.

- Prepare 1X Washing Buffer by adding 20 ml of 25X to 480 ml of deionized water for a final volume of 500 ml.
- Add 200 µl/well of Washing Buffer and discard plate contents by pouring out. No Touch.
- Wash 3 times with 300 µl/well of Washing Buffer. After each addition, pour off wash. No Touch.

STEP D: Antibody binding

- Prepare primary antibody solution. Dilute detection antibody 1/500 in Sample Buffer 1X by mixing 20 µl of antibody with 10 ml of Sample buffer.
- Add 100 µl of diluted antibody solution to each well.

For human plasma and serum:

- Seal the plate with parafilm and incubate at room temperature while shaking for 15' (2-3 rotations per second). Then incubate for 2 hours at 4° C.

For human urine and cell culture medium:

- Seal the plate with parafilm and incubate at room temperature while shaking for 15' (2-3 rotations per second). Then incubate for 2 hours at 37°C in humid chamber.

- Wash the plate (follow STEP C: Wash the plate).
- HPR-Conjugated (anti-Mouse-HRP or Streptavidin-HRP) must be diluted in Sample Buffer 1X as indicated below.

  Anti-Mouse-HRP: Dilute 1/2000 by mixing 5 µl of anti-Mouse-HRP to 10 ml of Sample Buffer 1X.

  Streptavidin-HRP: Dilute 1/5000 by mixing 2 µl of Streptavidin-HRP to 10 ml of Sample Buffer 1X.

- Add 100 µl of diluted HRP-conjugated to each well.

For human plasma and serum:

- Seal the plate with parafilm and incubate at room temperature while shaking for 15' (2-3 rotations per second). Then incubate for 1 hours at 4° C.

For human urine and cell culture medium:

- Seal the plate with parafilm and incubate at room temperature while shaking for 15' (2-3 rotations per second). Then incubate for 1 hours at 37°C in humid chamber.

- Wash the plate (follow STEP C: Wash the plate).

STEP E: Signal Detection

Detection for colorimetric reading

- Add 100 µl of Substrate Chromogenic Solution to each well and incubate uncovered at room temperature in the dark for 5-10'. Monitor until a blue color is visible.
- Stop the reaction by adding 100 µl of Stop Solution to each well. The color will change from blue to yellow.
- Read the absorbance at 450 nm within 10 minutes. If possible, the absorbance should also be read at 570 nm and the measurement should be subtracted from the measurement at absorbance 450 nm.

Detection for luminometric reading

- Mix 5 ml of luminometric Solution A with 5 ml of luminometric Solution B.
- Add 100 µl of Substrate Solution A+B per well.
- Read immediately in a luminometer at 425 nm.

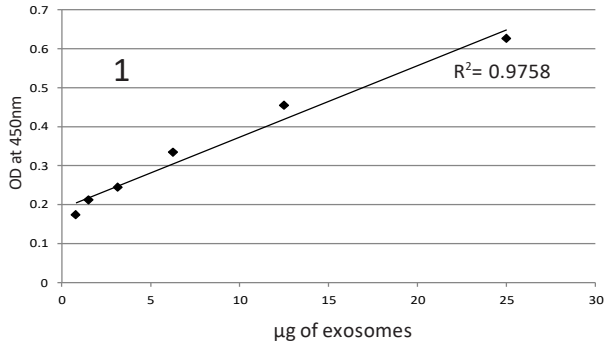
NOTE:

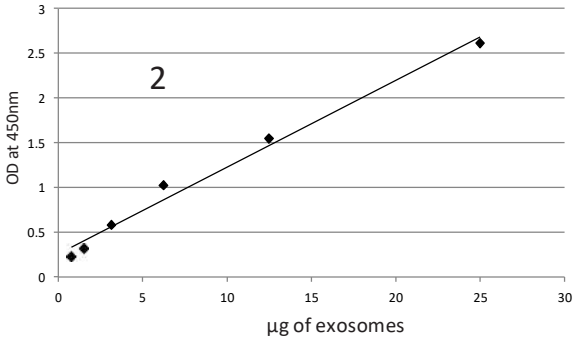
- Be careful not to immerse metallic components of a pipette into substrate solution.
- Avoid making bubbles and, if formed, remove them gently with a pipette tip. Intensity of color is proportional to the exosome concentration only within a certain dynamic range.
- Many plate readers do not deliver accurate results when the OD is above 3.

DATA ANALYSIS

Exosome standards are provided as assay calibrators and also as positive control. And it is important to note that the origin of purified standard exosomes may change the proportion of common exosomal proteins such as CD9 or CD63.
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. 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 and RLU.

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 factor.

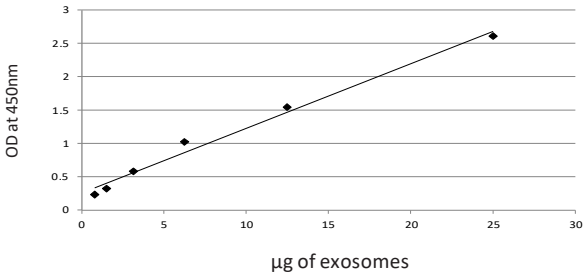




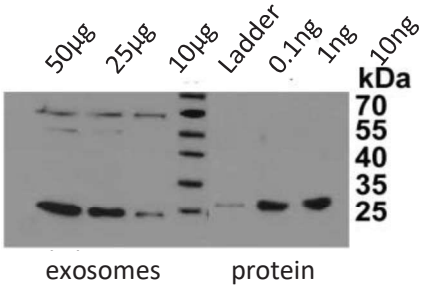
1: Example of standard curves: CD9 Titration of COLO1 (1) and plasma (2) Exosome Standards. This standard curve is for demonstration only. For quantification purposes a standard curve must be obtained with every assay.

SENSITIVITY

The sensitivity of the Human Exosome ELISA Kit was compared to Western blot. The data reported in figure 2 and 3 demonstrate that the sensitivity of the Human Exosome ELISA Kit is higher than that of Western blotting. Figure 3 shows that 10 µg of lyophilized exosomes are equivalent to 0.1 ng of recombinant exosomal protein. Since the standard curve's lower concentration is 0.39 µg of lyophilized exosomes (fig. 2), the sensitivity of our test is around 39 pg of protein equivalent.



2. CD9 titration of plasma healthy donor exosome standards.



3. CD9 exosome marker detection by Western Blotting on lyophilized exosomes from human plasma (NBP3-11686) and recombinant CD9 protein.

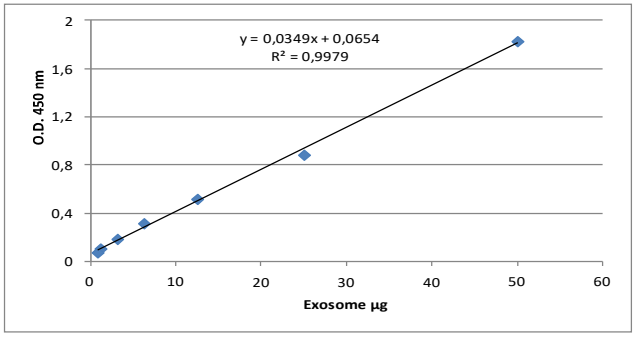
REPRODUCIBILITY

Intra assay (within run) CV (%) < 10
Inter assay (interplate run) CV (%) < 13

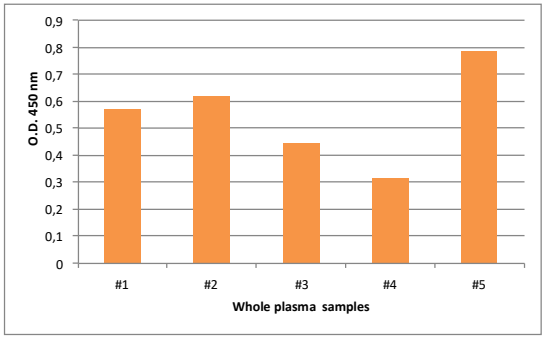
Coefficient of variation (CV) is expressed as a percentage of variance to the mean calculated for intra assay reproducibility evaluation by assessing at least 4 replicates of 3 different standard preparations containing different exosome concentrations. Subsequently, assay-to-assay reproducibility was assessed by comparing mean absorbances for the same samples on independent plates tested by different operators.

The Exosome ELISA Kit (Colorimetric) is a sensitive method for exosome quantification in humanbiofluids

We report an example of exosome quantification performed in 5 unknown plasma samples from healthy donors using the Human Exosome ELISA Kit Ready to use kit for Overall Exosome quantification from human plasma. Following Lyophilized Standards and unknown samples binding onto the ELISA plate, test is run in according to the kit protocol and exosome detection is performed with anti-CD9 antibody.



4. Standard curve obtained with Lyophilized Exosome Standards from human plasma healthy donors with anti- CD9 antibody.



5. CD9 titration of exosomes in 5 different whole plasma from healthy donor samples.

Exosome quantification is finally performed calculating the quantity of exosomes (expressed in µg) into the 5 unknown samples through the equation of the standard curve (Fig 4). The particles number contained in 100 µl of plasma is calculated from quantity of exosomes (expressed in µg) in according to the particle's concentration (number of particles/ml) indicated in the label of the Lyophilized Exosome Standards (NTA: 3x10^11 particles/ml)

Plasma sample	O.D. 450 nm	Exosome µg	Particle number in 100 µl of plasma
#1	0.5673	12.869	3.86x10^9
#2	0.6194	14.205	4.26x10^9
#3	0.4425	9.6692	2.90x10^9
#4	0.3100	6.2717	1.88x10^9
#5	0.7853	18.458	5.54x10^9

TROUBLESHOOTING

Problem/Possible Cause	Suggested Solution
High background across entire plate	
Substrate incubation carried out in the light	Substrate incubation should be carried out in the dark.
Incubation temperature too high	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 working. Incubation temperature may require some optimization.
Secondary antibody too concentrate or too long incubation	Check dilution of secondary antibody, use it at the recommended dilution. Stop the reaction using stop buffer as soon as the plate has developed enough for absorbance readings.
Substrate solution or stop solution is not fresh	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).
Contaminants from laboratory glassware	Ensure reagents are fresh and prepared in clean glassware.
Positive signal in negative control	
Contamination of reagents/ samples	May be contamination of reagents or samples, avoid cross contamination between different wells. Use fresh reagents and pipette carefully.
Insufficient washing of plates	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	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.
Insufficient primary or secondary antibody	Check the recommended amount of antibody is being used. The concentration of antibody may require increasing for optimization of results.
Substrate solutions not fresh or incorrectly combined	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.
Reagents not fresh or not at the correct pH	Ensure reagents have been prepared correctly and are in date.

TROUBLESHOOTING

Problem/Possible Cause	Suggested Solution
Incubation time not long enough	Ensure you are incubating the antibody for the recommended amount of time, if an incubation time is suggested. The incubation time may require increasing for optimization of results. Longer incubation time may be required.
Incubation temperature too low	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 working. Incubation temperature may require some optimization. Ensure all reagents are at room temperature before proceeding.
Stop solution not added	Addition of stop solution increases the intensity of color reaction andstabilizes the final color reaction.
Inconsistent absorbances across the plate	
Plates stacked during incubations	Stacking of plates does not allow distribution of temperature across the wells of the plates. Avoid stacking.
Pipetting inconsistent	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 liquid. This will greatly affect consistency of results between duplicates.
Antibody dilutions/Reagents not well mixed	To ensure a correct dilution of samples/standards across wells, ensure all reagents and samples are mixed before pipetting onto the plate.
Wells allowed to dry out	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.
Inadequate washing	This will lead to some wells not being washed as well as others, leaving inside different amounts of unbound antibody, which will give inconsistent results.
Bottom of the plate is dirty affecting absorbance readings	Clean the bottom of the plate carefully before re-reading the plate.
Color developing slowly	
Plates are not at the correct temperature	Ensure plates are at room temperature and that the reagents are at room temperature before use.
Secondary Antibody too weak staining	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.
Contamination of solutions	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	Check pipettes and pipette with increase attention.
Improper standard preparation	Ensure to reconstitute standards in a proper buffer and mixing thoroughly by vortexing and gentle pipetting.
Improper washings	Wash thoroughly as recommended in the assay procedure; add one wash step after incubation with HRP-conjugated.

TROUBLESHOOTING

Problem/Cause	Suggested Solution
Low signals	
Low signals in standards due to improper storage or preparation	Ensure to store reconstituted standards at -20°C and strictly avoid thaw and freeze cycles. Assure that standards are properly reconstituted and standard dilutions prepared as suggested in data sheet.
Low signals in samples due to low exosome concentration	Check out sample preparation protocols or contact our customer service
Low reproducibility of duplicates	
Inaccurate pipetting	Check pipettes and increase attention.
Improper standards and samples preparation	Ensure to mix thoroughly by vortexing and gentle pipetting before loading onto wells.