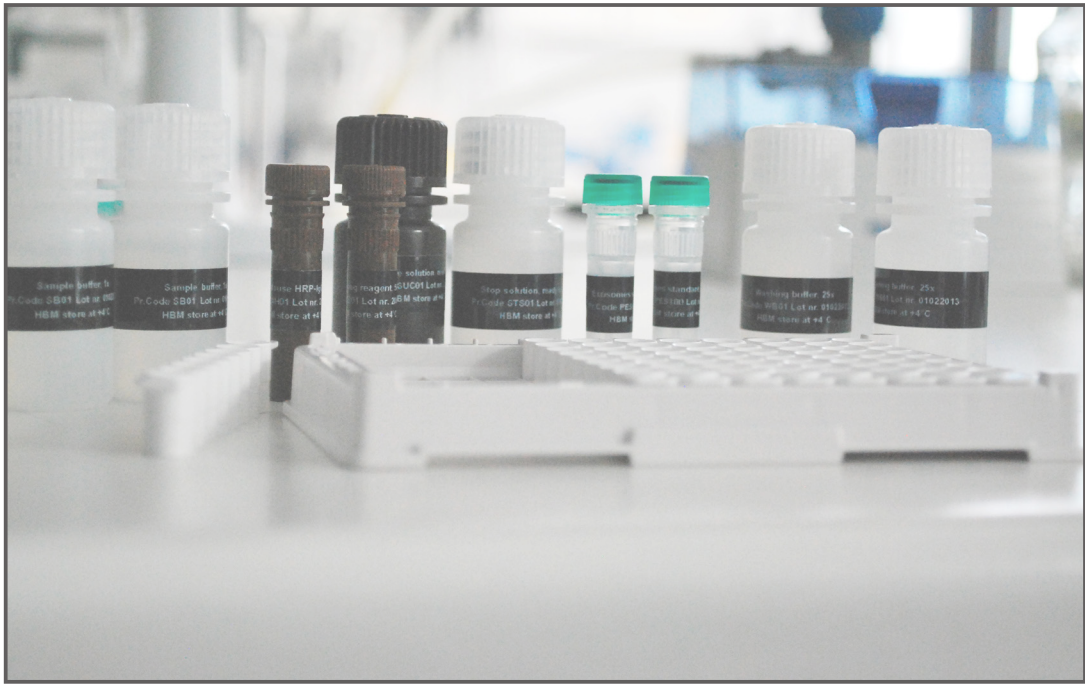
Product Insert

HansaBioMed



ExoTEST™
Ready to Use Kit for Exosome quantification

HBM-RTK-POF/TP ExoTEST™ Ready To Use Kit for Overall Exosome capture and quantification from Plasma (transparent plate for colorimetric readings)

HansaBioMed

This product is for research use only.
It is highly recommended to read this users guide in its entirety prior to using this product.
Do not use this kit or its components beyond the indicated expiration date.

Content

PRODUCT INFORMATION

PRODUCT DESCRIPTION

Overview

ExoTEST™ Ready to Use Kit is the ideal platform for capturing and the quantifying exosomes from biological samples.

ExoTEST™ Ready to Use Kit features ELISA plate(s) pre-coated with primary antibodies detection directed against exosome-specific markers, namely α-CD9. Secondary antibodies and substrate reagents are included in the kit.

Exosome standards for assay calibration are also included in the kit. Exosome standards are purified by ultracentrifugation and microfiltration, quantified for overall protein content and exosome number and then lyophilized for long-term storage.

HansaBioMed will also help you customizing your own kit by choosing among a wide variety of reagents available in our catalog.

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 been isolated from diverse cell lines (hematopoietic cells, tumor lines, primary cultures, virus infected cells) as well as from biological fluids in particular blood (e.g. serum and plasma from cancer patients) and other body fluids (bronchoalveolar lavage fluid, pleural effusions, synovial fluid, urine, amniotic fluid, semen, saliva etc).

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

About ExoTEST™ Ready to Use Kit

ExoTEST™ has been developed to fill the need for a reliable method to quantify exosomes and examine their biomarkers.

Our multiwell plates are standard 96-well format where assays can be conducted as singletons and/or multiple wells. This enables optimization of a wide range of sandwich ELISA assays or other downstream applications, such as RNA extraction and proteomic analyses. Transparent, white and black plates are available depending on the downstream detection approach (colorimetric, luminometric or fluorimetric).

HansaBioMed offers several kits for exosome capture and quantification depending on the kind of exosomes targeted and/or the biological sample tested.

PRODUCT CONTENT

Common 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	2 bottles (2 X 15 ml)
Substrate chromogenic solutions	Substrate for chromogenic detection in buffered solution, ready to use	1 bottle (10 ml)
Stop solution	1M Sulphuric Acid (H_2SO_4) stop solution, ready to use. (Attention–caustic agent)	1 bottle (10 ml)
Immunoplate	Immunoplate for overall exosome capture from biological fluids, 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 Exosomes from plasma of healthy donors	2 vials (2 X 100 µg)
Primary Antibody	α-CD9 mouse antibody	1 vial (20 µl)
HRP conjugated	HRP-conjugated anti-mouse Ig antibody	1 vial (5 µl)

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 ExoTEST™ Ready To Use Kits can be stored at +4°C for up to 24 months, if unopened.

DO NOT FREEZE!

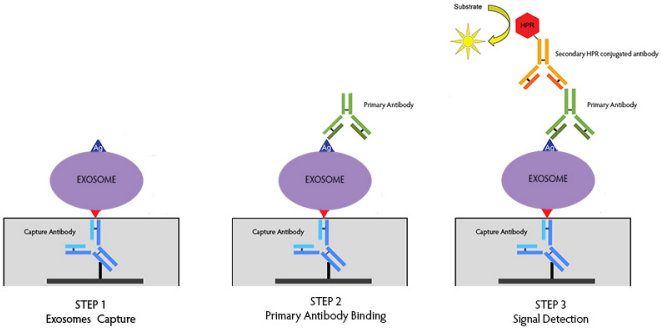
The plate is packed in an opaque aluminium pouch which complies to food and pharmaceutical regulation. Easy opening and re-seasable by zip closure.

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	
Anti-mouse Ig antibody	
Substrate for chromogenic or luminometric detection in buffered solution, ready to use	
Stop solution, caustic reagent	

PROCEDURE

Overview

ExoTEST™ 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.



ExoTEST™ Ready to Use Kit principle

STEP A: Plasma sample preparation

1. 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.
   i. 10' at 300 g (save supernatant; discard pellet).
   ii. 20' at 1200 g (save supernatant; discard pellet).
   iii. 30' at 10 000 g (save supernatant; discard pellet).

**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 Incubation

1. Reconstituted Exosome Standard for calibration curve
   i. Take the two vials which contain lyophilized exosomes from plasma (PEP100).
   ii. Reconstitute lyophilized exosome standard by adding 100 µl of deionized water in each vial.
   iii. Pipette the solution up and down 10-15 times, avoiding bubbles.
   iv. 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.
   v. Pipette the solution up and down 10 times, again avoiding bubbles.
   vi. Briefly centrifuge again.
   vii. Add 100 µl of PBS 1X to reach a final volume of 200 µl per vial.

2. Calibration curve:
    i. Add 200 µl of Reconstituted exosome stock solution to wells A1 and A2 (2 wells only).
    ii. Add 100 µl of 1x PBS to wells B1 to H2 (14 wells).
    iii. Serial Dilution (stop at G1 and G2).
    iv. Transfer 100 µl of A1 into B1 and mix.
    v. Transfer 100 µl of B1 into C1 and mix.
    vi. Transfer 100 µl of C1 into D1 and mix.
    vii. Transfer 100 µl of D1 into E1 and mix.
    viii. Transfer 100 µl of E1 into F1 and mix.
    ix. Transfer 100 µl of F1 into G1 and mix.
    x. Discard 100 µl from G1 to result in a final volume of 100 µl.
    xi. NOTE: Leave H1 (and H2) as 1x PBS for negative controls.
    xii. Repeat serial dilution for A2 to G2.

3. Sample loading: Add 100 µl of samples (or standard dilutions) to wells A3 to H12 (resuspend in 1x PBS if <100 µl).
4. Seal the plate with parafilm and incubate at room temperature while shaking for 30' (2-3 rotations per second).
5. Transfer the plate to +4°C and incubate overnight (12h-20h).

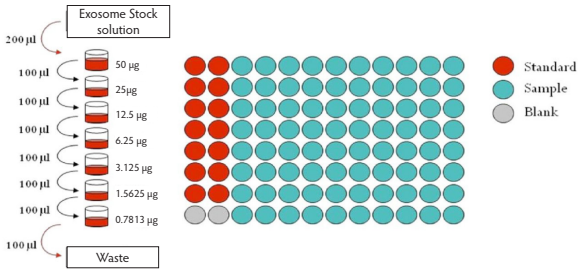
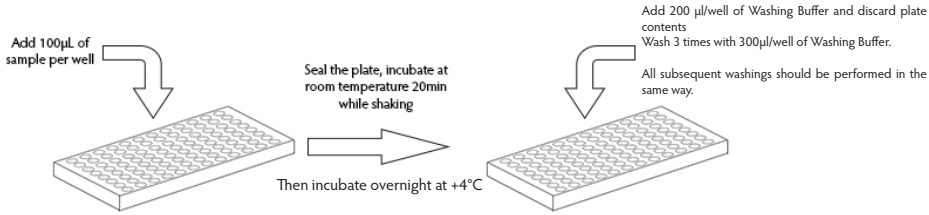


Illustration of the two-fold serial dilutions required for calibration curve
Add 100 ul of PBS 1X to reconstituted exosome standard to obtain a volume of 200 ul, then transfer in the first well of ELISA strip. Add 100 ul of PBS 1X in the other wells of the strip used for standard curve and prepare exosome dilutions as indicated in figure.

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.

1. Prepare 1X Washing Buffer by adding 30 ml of 25X to 720 ml of deionized water for a final volume of 750 ml.
2. Add 200 µl/well of Washing Buffer and discard plate contents by pouring out. No Touch.



3. Wash 3 times with 300 µl/well of washing buffer. After each addition, pour off wash. No Touch.

STEP D: Primary antibodies binding

1. Prepare primary antibody solution. Dilute detection antibody 500-times by adding and mixing sample buffer with the antibody. Mix 20 µl detection antibody with 10 ml sample buffer.
2. Add 100 µl of diluted detection antibody solution to each well.
3. Seal the plate with parafilm and incubate at room temperature while shaking for 20' (2-3 rotations per second). Then incubate for 2 hours at +4°C.
4. Wash the plate (follow STEP C: Wash the plate).
5. Proceed to STEP E: Signal Detection.

STEP E: Signal Detection

1. Secondary antibody should be diluted 2000-times by adding and mixing in sample buffer. Mix 5 µl of secondary antibody with 10 ml sample buffer.
2. Add 100 µl of secondary antibody solution to each well.
3. Seal the plate with parafilm and incubate at room temperature while shaking for 20' (2-3 rotations per second). Then incubate for 1 hour at +4°C.
4. Wash the plate (follow STEP C: Wash the plate).
5. Add 100 µl of substrate solution to each well and incubate uncovered at room temperature in the dark for 5-10'. Monitor until a blue color is visible.

NOTE:

i. Be careful not to immerse metallic components of a pipette into substrate solution.

ii. 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.

iii. Many plate readers do not deliver accurate results when the OD is above 3.

6. Stop the reaction by adding 100 µl of stopping solution to each well. The color will change from blue to yellow.
7. 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.

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, RLU or fluorescence emissions 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 factor.

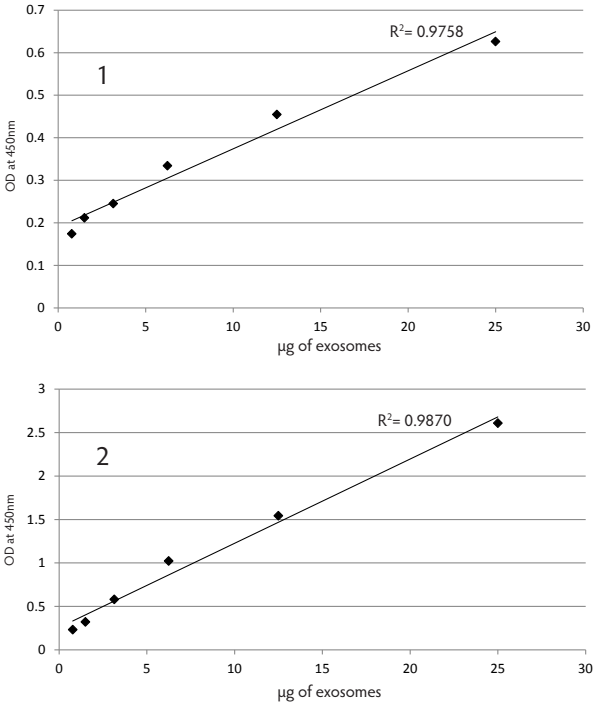


Figure 1: CD9 Titration of COLO1 (1) and plasma (2) exosomes. Example of standard curves obtained.
Exosome standard preparation derived from COLO1 Cell Culture Supernatant (1) and plasma of healthy donors (2). This standard curve is for demonstration only. For quantification purposes a standard curve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

SENSITIVITY

The sensitivity of the ExoTEST™ was compared to Western blot. The data reported in figure 2 and 3 demonstrate that the sensitivity of the ExoTEST™ 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.

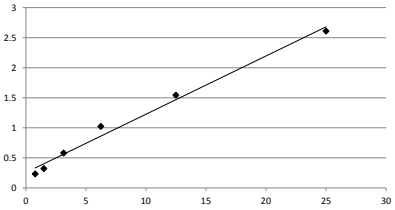


Figure 2. CD9 titration of plasma healthy donor exosome standards (HBM-PEP100)

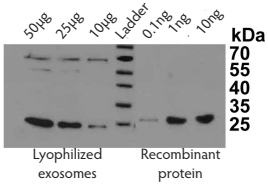


Figure 3. CD9 marker detection by Western Blotting on lyophilized exosomes from cell culture supernatant and recombinant exosomal proteins.

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.

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 left on too long	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).
Reaction not stopped	Colour will keep developing if the substrate reaction is not stopped.
Plate left too long before reading on the plate reader	Colour will keep developing (though at a slower rate if stop solution has been added).
Contaminants from laboratory glassware	Ensure reagents are fresh and prepared in clean glassware.

Problem/Possible Cause	Suggested Solution
Positive results 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.
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 colour reaction and stabilizes the final colour reaction.

Problem/Cause	Suggested Solution
High absorbance values for samples and/or positive control - absorbance does not go down as the sample is diluted down the plate).	
	The concentration of samples or positive control is too high and out of range. Re-assess the assay you are using OR reduce the concentration of samples and control by making a dilution. Take the dilution into account when calculating the resulting concentrations.
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.
Colour 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.

Problem/Cause	Suggested Solution
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; wash for longer time after incubation with HRP-conjugated secondary antibody.
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 at info@hansabiomed.eu.
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.

APPENDIX

RELATED PRODUCTS

Overview

As the first company entirely dedicated to the field of exosomes, we offer a panel of Kits and Reagents for Exosome Research.
Ordering information on a variety of reagents and apparatus available from HansaBioMed is provided below. For more information, visit our website at www.exotest.eu.

Products	Quantity	Catalog Number
Exosome Total RNA Extraction Kit (Immunobeads, 10 or 20 Reactions)	Ready to Use Kit	HBM-RNA-BOF-##/#
Exosome Total RNA Extraction Kit (Immunoplate)	Ready to Use Kit	HBM-RNA-POF/#
Tumor-derived Exosome Total RNA Extraction Kit (Immunobeads, 10 or 20 Reactions)	Ready to Use Kit	HBM-RNA-BTF-##/#
Tumor-derived Exosome Total RNA Extraction Kit (Immunoplate)	Ready to Use Kit	HBM-RNA-PTF/#
Immunoplates for Overall Exosome capture from Biological fluids	96 wells immunoplate	HBM-POF-##/##
Immunoplates for Overall Exosome capture from human Serum	96 wells immunoplate	HBM-POS-##/##
Immunoplates for Overall Exosome capture from Cell culture supernatant	96 wells immunoplate	HBM-POC-##/##
Immunoplates for Tumor-derived Exosome capture and enrichment from Biological fluids	96 wells immunoplate	HBM-PTF-##/##
Immunoplates for Neural-derived Exosome capture and enrichment from Biological fluids	96 wells immunoplate	HBM-PNF-##/##
Immunoplates for Glial-derived Exosome capture and enrichment from Biological fluids	96 wells immunoplate	HBM-PGF-##/##
Immunoplates for Monocytes- and Platelets-derived Exosome capture and enrichment from Plasma samples	96 wells immunoplate	HBM-PPP-##/##
Immunobeads for Overall Exosome capture from Biological fluids - 0.4, 1 or 4 microns immunobeads size - Simple or Covalent coating	10 or 20 reactions	HBM-BOLF-##/##-#
Immunobeads for Overall Exosome capture from Cell culture supernatant - 0.4, 1 or 4 microns immunobeads size - Simple or Covalent coating	10 or 20 reactions	HBM-BOLC-##/##-#
Immunobeads for Tumor-derived Exosome capture and enrichment from Biological fluids - 0.4, 1 or 4 microns immunobeads size - Simple or Covalent coating	10 or 20 reactions	HBM-BTLF-##/##-#
Exosome Binding Antibodies		Check Ab available on www.exotest.eu

TECHNICAL SUPPORT

Get support

For the latest services and support information for all locations, go to www.exotest.eu
At the website, you can:

- Search for user documents, handbooks, certificates of analysis, citations, and other product support documents
- Access telephone and fax numbers to contact Technical Support and Sales facilities

Or contact us at info@exotest.eu

Material Safety Data Sheet (MSDS)

Material Safety Data Sheets (MSDSs) are available at
www.exotest.eu/index.php/documentation

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Research Use

All products are sold for research or laboratory use only and are not intended to be administered to humans or used for medical diagnostics.

For the latest product and technical information, visit hansabiomed.eu and exotest.eu

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