INTRODUCTION

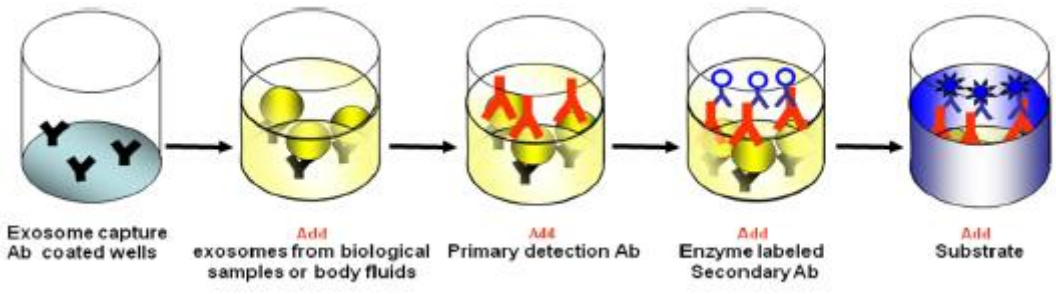
Exosomes are small endosome-derived lipid nanoparticles (50-120 nm) actively secreted by exocytosis by most living cells. Exosome relase occurs either constitutively or upon induction, under both normal and pathological conditions, in a dynamic, regulated and functionally relevant manner so that both 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). During their biogenesis exosomes incorporate a wide range of membranes and cytosolic proteins involved in many cellular functions. Exosome molecular composition reflect their endosomal origin as well as a parent cell type and condition. Exosomes shuttle also RNA molecules, mRNAs and miRNAs, both classes proved to be functional in target cell. Overall, exosomes contain a unique molecular fingerprint that reflects properties of a parent cell.

ExoTESTTM is a patented double sandwich ELISA assay for quantitative and qualitative analysis of exosomes. ExoTESTTM captures exosomes from biological samples by employing antibody specific for exosome surface protein(s) coated on a 96 well plate. Quantification and characterization of exosomal proteins is subsequently performed by using detection antibodies of interest. By employing different combinations of capture and detection antibodies ExoTESTTM can be customized for assessing multiple antigens in a total exosome population. For the samples of interest RNA (mRNA or miRNA) can be extracted and analyzed from captured exosomes.

ExoTESTTM therefore represent a fundamental novel, reliable, practical and easy solution for any comprehensive exosome analysis from different biological samples. Assay is analytically validated and provides ready-to-use reagents.

ASSAY SUMMARY

Standards and samples are pipetted into wells and exosomes present in the sample are bound to the wells by immobilized antibody. The wells are washed and primary detection antibody added. After washing of unbound primary antibody, HRP conjugated secondary antibody is pipetted to the wells. The wells are washed again and substrate solution (TMB or ECL) is added and color or light develops in proportion of exosomes bound.



Description	Quantity	Volume	Part #
EXOTEST™ precoated 96 well ELISA plate (12x8 wells ELISA strips) for exosome capture, transparent or white *	1 plate		PPT01-100 *PPW01-100
Mouse anti-human detection antibody for exosome identification (Anti -CD63, -CD9, -CD81)	1 vial	20µl	PA11, PA12, PA13
Exosome standard preparation for assay calibration, lyophilized exosomes purified from human cell line supernatant, 2x100µg	2 vials		PES100
Sample Buffer, 1x	1 bottle	15 ml	SB01
Washing Buffer, 25x	2 bottles	15 ml	WB01
HRP- conjugated anti mouse IgG antibody	1 vial	10 µl	SAH01
Substrate for chromogenic detection in buffered solution, ready to use	1 bottle	10 ml	SUC01
*Substrate for luminometric detection in buffered solution, Solution A	1 bottle	5 ml	SUL01A
*Substrate for luminometric detection in buffered solution, Solution B	1 bottle	5 ml	SUL01B
Stop solution, ready to use 1M Sulphuric Acid (H_2SO_4) (*attention – caustic agent*)	1 bottle	10 ml	STS01

ExoTEST KIT COMPOSITION

ExoTEST Basic kit for exosome capture and detection

Description	Quantity	Volume	Part #
EXOTEST™ precoated 96 well ELISA plate (12x8 wells ELISA strips) for exosome capture, transparent or white	1 plate		PPT01-100 PPW01-100
Mouse anti-human detection antibody for exosome identification (Anti -CD63, -CD9, -CD81)	1 vial	1µl	PA01, PA02, PA03
Exosome standard preparation for assay positive control, lyophilized exosomes purified from human cell line supernatant 2x30 µg	2 vials		PES30
Sample Buffer, 1x	1 bottle	15 ml	SB01
Washing Buffer, 25x	2 bottles	15 ml	WB01

ExoTEST Ready-to-use kit for exosome capture and quantification
**** components of kit for luminometric detection***

OTHER MATERIAL REQUIRED

- Single and/or pipettes with disposable tips 2-100 µl
- Polypropylene tubes
- Pipettes 1ml and 5 ml for reagent preparation
- Deionized water
- PBS
- Plate shaker
- Humidified chamber or incubator at 37 °C
- Disposable pipetting reservoirs
- Microplate reader
- Log-log graph paper or computer and software for ELISA data analysis

Additional material required for ExoTEST Basic kit users:

 Primary detection antibody of interest (anti human, recommended stock concentration 0,1-1 mg/ml)
 Secondary enzyme conjugated antibody matched to primary detection antibody
 ELISA development substrate

STORAGE INFORMATION

All reagents provided within the ExoTEST kits can be stored at +4°C for up to 6 months unsealed. Tested stability of precoated plates is equivalent to one year of shelf life maintaining here specified performance. Shipping is performed at wet ice. Please, refer to the expiration date indicated on the box.
DO NOT FREEZE!!!

OPENED OR RECONSTITUTED COMPONENTS	
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 1 month.
Exosome standard preparation	The remaining reconstituted standard stock solution should be aliquoted into polypropylene vials (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.
Mouse anti-human detection antibody for exosome identification	Store opened and diluted reagents at +4°C and use within one month.
Sample Buffer, 1x	
Washing Buffer, 25x	
HRP- conjugated anti mouse IgG antibody	
Substrate for chromogenic or luminometric detection in buffered solution, ready to use	
Stop solution, ready to use 1M Sulphuric Acid (H_2SO_4) (*attention – caustic agent*)	

ExoTEST PROTOCOL

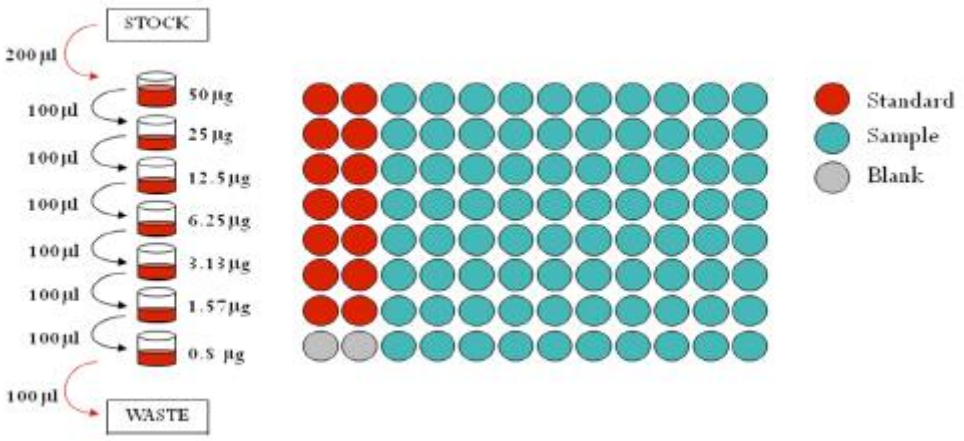
Sample preparation

ExoTEST assay is validated using exosomes purified from cell supernatants and human biological fluids. Recommended protocols for sample preparation are based on modified protocols from Thery et al 2006 and Zhou et al, 2006 and can be consulted at www.exotest.eu. ExoTEST was tested for analysis of purified exosomes as well as unfractioned biologic samples. Unfractioned samples are assayed without dilution upon preclearing as described in supporting protocols section.

Reagent preparation

All reagents should be reconstituted and diluted immediately prior to use and subsequently stored according the above suggestions. Do not mix reagents from different kits or lots. We do not guarantee assay performance if reagents and/or antibodies from different manufacturers should be used with this kit. The plate is ready for use as it is – do not wash.
1. Dilute the 25x Washing Buffer to 1x with deionized water. If crystals are observed dissolve them by warming up the solution before preparing a dilution.
2. Reconstitute the lyophilized exosome standard by adding to the vial indicated volume of deionized water (100 µL for calibration standard and 50 µL for positive control). Vortex reconstituted standard for 60 seconds in order to mix it thoroughly. For calibration standard add 100 µl of PBS as to obtain the 200 µL of 0,5µg/µL standard stock solution. For positive control add 50 µL of PBS to adjust volume to 100 µL sufficient for 1 well. Leave it at room temperature for 5-15 minutes. Prior to adding it to the plate wells vortex for additional 30 seconds and spin briefly in the top table centrifuge.
3. If purified exosome samples are analyzed, if necessarily, use PBS to adjust volume and concentration of a sample (overall volume/well is 100 µL).
4. In general, unfractioned samples are analyzed without dilutions (100 µl/well).
5. Detection Antibody should be diluted 500-fold in Sample Buffer.
6. HRP-conjugated Antibody should be diluted 1000-fold in Sample Buffer.
7. Substrate Solution and Stop Solution are ready-to use. If using substrate for luminometric detection mix Solutions A and B immediately prior to use.

Assay procedure
1. Unseal ELISA plate/strips.
2. Bring all reagents to room temperature before use and briefly vortex.
3. All standards, positive and negative controls, and samples should be run in duplicates. 9
4. Add 100 µl/well of standard dilutions or samples to appropriate wells. If necessary dilute samples in PBS.
5. Positive control is provided within the assay. Negative control is represented by Sample Buffer or PBS for purified exosomes analysis, and sample matrix (e.g. exosome depleted cell culture supernatant or plasma) for unfractioned samples analysis

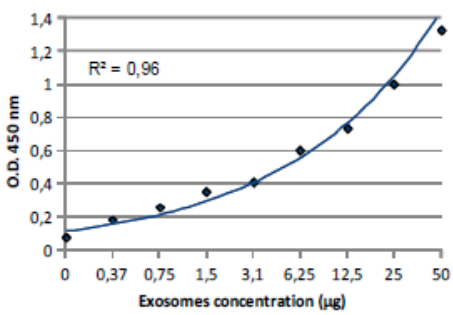


6. A calibration curve is required for samples quantification. Standard dilutions are prepared directly in the strips. Calibration standard is available in the kit or as a separate product. Use stock solution prepared as indicated above to perform six two-fold serial dilutions in PBS. In

this way standard concentrations in the wells will be 50 µg, 25 µg, 12.5 µg, 6.25 µg, 3.13 µg, 1.57 µg and 0.8 µg
7. Seal the plate with parafilm and incubate at room temperature with shaking for 20 minutes. Transfer plate to 37°C and incubate in humidified atmosphere over night.
8. Add 200 µl/well of 1x Washing Buffer and discard plate contents into the sink. Wash 3 times with 300 µl/well of Washing Buffer. For each of these three passages, discard the Washing Buffer into the sink and blot residual buffer by tapping plate upside down on absorbent paper. All subsequent washings should be performed in the same way.
9. Add 100 µl of mouse anti human exosome Detection Antibody solution (diluted in Sample Buffer as indicated above) to each well, seal the plate and incubate for two hours at 37 °C.
10. Wash the plate as in step 8.
11. Add 100 µl of rabbit anti mouse IgG HRP conjugated Secondary antibody solution (diluted as indicated above in Sample Buffer). Seal the plate and incubate for 1 hour at 37°C.
12. Wash the plate as in step 8 with last two washings for 1 minute with gentle shaking.
13. If using colorimetric detection, add 100 µl of Substrate Solution to each well and incubate for up to 15 minutes at room temperature in the dark. Be careful not to immerse metallic parts of a pipette into substrate solution. Also avoid making bubbles and, if formed, remove them gently with 10 a tip. Do not seal the plate and monitor the development of blue color. Intensity of color is proportional to exosomes concentration.
14. Stop the reaction by adding 100 µl of Stoping Solution to each well. The color should change from blue to yellow.
15. Read the absorbance at 450 nm within 10 minutes. If possible the absorbance should be read also at 570nm and this one subtracted from the absorbance at 450 nm.
16. If using chemiluminescent substrate, add 100 µl of Substrate Solution prepared freshly by mixing Solution A and B according to product sheet. Again, avoid making bubbles. Intense signal is produced immediately at room temperature.
17. Read emitted light in a luminometer.

Data analysis

Exosome standards are provided as positive assay controls or/and as assay calibrators. 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 corresponding amounts of exosomes. Calculate the mean absorbance for each set of duplicate standards, controls and samples. All OD or RLU values must be subtracted by negative controls (blanks) value before results interpretation. Plot the standard curve on log-log graph paper with standard concentrations on x-axis and absorbance on y-axis or use a curve fitting software. The regression curve coefficient should be above 0,95. The estimated sample concentration is reliable if within linear range of a curve, otherwise the samples must be diluted and test repeated. For diluted samples multiply the concentrations with an appropriate dilution factor.



Example of standard curve obtained with exosome standard preparation derived from human HBM-Colo1 cell line. This standard curve is for demonstration only. For quantification purposes a standard curve must be run with every assay.

Sensitivity

The minimum detectable amount of exosomes is 0,35 μg/well equivalent to sample concentration of 3,5 ng/μl. Detection limit is calculated as a mean of zero standard plus two standard deviations of a lowest dose from standard curve. The standard curve range covers 0-50 μg/well equivalent to sample concentration of 0-5 ng/μl. 11

Reproducibility

Intra Assay (within run) CV (%) < 10
Inter Assay (run-to-run) CV (%) < 13
Coefficient of variation (CV) is expressed as a percentage of a 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 absorbancies for the same samples on independent plates tested by different operators.

Specificity

HBM Kits used detection antibodies are specific for target proteins and do not show cross reactivity between themselves or other exosome surface proteins tested.

APPLICATIONS

EXOTEST® is a versatile assay in which precoated plates are combined with a range of specific detection antibodies that allow a range of applications (*RESEARCH USE ONLY*):
 Exosome capture and quantification - EXOTEST® ensures dose dependent exosome detection in any sample tested. The quantification can be both relative (comparison) and absolute as assay calibration enables to calculate approximate exosome quantity for analyzed sample.

 Exosome comprehensive profiling - By employing different combinations of capture and detection antibodies EXOTEST® can be customized for assessing multiple antigens in a total exosome population in the sample. Informative protein profile for the samples of interest can be combined with subsequent mRNA RNA (mRNA or miRNA) extraction and analysis from captured exosomes.

 Specific diagnostic applications - EXOTEST® allows quantitative assessment of total circulating exosomes and specific exosomal populations defined by antigens differentially expressed under normal and pathological conditions.

NOTES/TROUBLESHOOTING

PROBLEM	CAUSE	SOLUTION
Poor standard curve (irregular points)	Innacurate pipetting	Check pipettes and 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 with prolonged washings after incubation with secondary HRP conjugated antibody. Any residual drop of unbound HRP conjugate can alter absorbance readings
	Bubbles formed in the wells	Avoid making bubbles or remove them gently with a tip
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. Keep attention to reconstitute standards and prepare standard dilutions as suggested in data sheet
	Low signals in samples due to low exosome concentration	Check out the sample preparation protocols or contact our customers service at info@hansabiomed.eu
	Insufficient incubation times	Respect suggested incubation times and temperatures
	Inadequate reagents handling	Keep secondary antibody and substrate solution protected from light and avoid immersing metallic parts in substrate solution
Low reproducibility of dupplicates	Innacurate pipetting	Check pipettes and increase attention
	Improper standards and samples preparation	Ensure to mix thoroughly by vortexing and gentle pipetting before loading onto wells
High background	Insufficient plate washings	Review the data sheet suggestions for proper washings
	Wells dried out during incubation	Ensure to pipette appropriate reagent volumes in each well and seal the plate during incubations and perform 37°C incubations in humidified chamber or incubator
	Contaminated reagents	Store all opened reagents according to storage information provided within the data sheet. Use clean pipetting reservoirs. Prepare fresh washing buffer dilution