User Guide

MILLIPLEX® Human Exosome Characterization Magnetic Bead Panel

96-Well Plate Assay

HEXSM-170K, HEXSM-170K-PMX, HEXSM-170K-BKPMX

MERCK

Introduction

Cells release extracellular vesicles (EV), including exosomes, as part of normal physiological processes including removal of waste and regulation of intercellular communication. Furthermore, increased exosome release has been implicated in the progression of various disease states and other abnormalities such as cancer and neurodegeneration. Exosome identification, classification, and differentiation from other extracellular vesicles is often complicated due to the variability in isolation and preparation protocols, exosome biogenesis, as well as exosome source. Thus, development of additional exosome characterization tools is beneficial to the exosome research field. Commonly associated exosome-enriched markers include tetraspanins CD9, CD63, and CD81, cargo sorting-associated markers TSG101 and Flotillin-1, and biogenesis marker syntenin-1. Other markers, including Argonaute-2 (Ago2), GAPDH, and Calreticulin, may or may not be found in exosome-enriched samples, but this can vary depending on source, isolation method, and/or EV biogenesis mechanism. The Human Exosome Characterization panel provides a novel tool for researchers to study exosomal makers and content in lysed exosome samples.

The MILLIPLEX® portfolio offers the broadest selection of analytes across a wide range of disease states and species. Once the analytes of interest have been identified, you can rely on the quality that we build into each kit to produce results you can trust. In addition to the assay characteristics listed in the protocol, other performance criteria evaluated during the verification process include: cross-reactivity, dilution linearity, kit stability, and sample behavior (for example detectability and stability).

Each MILLIPLEX® panel and kit includes:

- Quality controls (QCs) provided to qualify assay performance
- Comparison of standard (calibrator) and QC lots to a reference lot to ensure lot-to-lot consistency
- Detection antibody cocktails designed to yield consistent analyte profiles within panel

In addition, each kit meets stringent manufacturing criteria to ensure batch-to-batch reproducibility. Coupled with the Luminex® xMAP® platform in a magnetic bead format, you receive the advantage of ideal speed and sensitivity, allowing quantitative multiplex detection of dozens of analytes simultaneously, which can dramatically improve productivity.

MILLIPLEX® Human Exosome Characterization Magnetic Bead Panel is part of the most versatile system available for exosome research. From our single to multiplex biomarker solutions, we partner with you to design, develop, analytically verify, and build the most comprehensive library available for protein detection and quantitation.

This MILLIPLEX® kit offers you:

- The ability to select a 9-plex or premixed option.
- The ability to choose any combination of analytes from our panel of 9 analytes to design a custom kit that better meets your needs. Ago2 is provided as a static analyte in all kits.
- A convenient “all-in-one” box format that gives you the assurance that you will have all the necessary reagents you need to run your assay.

MILLIPLEX® Human Exosome Characterization Magnetic Bead Panel is a 9-plex kit to be used for the simultaneous quantification of any or all of the following analytes in exosomes isolated from serum, plasma and tissue culture supernatants: Argonaute-2 (Ago2), Calreticulin (CALR), CD9, CD63, CD81, Flotillin-1 (FLOT-1), GAPDH, Syntenin-1 (Syn-1), and TSG101.

For Research Use Only. Not for Use in Diagnostic Procedures.

Please read entire protocol before use.

It is important to use same assay incubation conditions throughout your study.

Principle

MILLIPLEX® kits are based on the Luminex® xMAP® technology — one of the fastest growing and most respected multiplex technologies offering applications throughout the life sciences and capable of performing a variety of bioassays including immunoassays on the surface of fluorescent-coded magnetic beads known as MagPlex® microspheres.

- Luminex® uses proprietary techniques to internally color-code microspheres with two fluorescent dyes. Through precise concentrations of these dyes, distinctly colored bead sets of 500 5.6 µm polystyrene microspheres or 80 6.45 µm magnetic microspheres can be created, each of which is coated with a specific capture antibody.
- After an analyte from a test sample is captured by the bead, a biotinylated detection antibody is introduced.
- The reaction mixture is then incubated with Streptavidin-PE conjugate, the reporter molecule, to complete the reaction on the surface of each microsphere.
- The following Luminex® instruments can be used to acquire and analyze data using two detection methods:
    - The Luminex® analyzers Luminex® 200™, FLEXMAP 3D®, and xMAP® INTELLIFLEX are flow cytometry-based instruments that integrate key xMAP® detection components, such as lasers, optics, advanced fluidics, and high-speed digital signal processors.
    - The Luminex® analyzer, MAGPIX®, is a CCD-based instrument that integrates key xMAP® capture and detection components with the speed and efficiency of magnetic beads.
- Each individual microsphere is identified, and the result of its bioassay is quantified based on fluorescent reporter signals. We combine the streamlined data acquisition power of Luminex® xPONENT® acquisition software with sophisticated analysis capabilities of MILLIPLEX® Analyst 5.1, integrating data acquisition and analysis seamlessly with all Luminex® instruments.

- xMAP® INTELLIFLEX runs on INTELLIFLEX software for instrument control, run setup and generating high quality data with flexible output options. Data can be exported in xPONENT® style CSV files for compatibility with many existing analytical applications, or in the new, customizable INTELLIFLEX file format. The INTELLIFLEX file format is intended for flexibility and simplicity, allowing the user to freely select which data points to include and to reduce the time to analysis.

The capability of adding multiple conjugated beads to each sample results in the ability to obtain multiple results from each sample. Open-architecture xMAP® technology enables multiplexing of many types of bioassays reducing time, labor, and costs over traditional methods.

Storage Conditions Upon Receipt

- Recommended storage for kit components is 2-8 °C.
- For long-term storage, freeze reconstituted standards and controls at ≤ -20 °C. Avoid multiple (> 2) freeze/thaw cycles.
- DO NOT FREEZE Antibody-Immobilized Beads, Detection Antibody, and Streptavidin-Phycoerythrin.

Reagents Supplied

Products can be ordered from SigmaAldrich.com using the catalogue numbers following the product name. Products are available from other sources if indicated.

Reagents	Volume	Quantity	Catalogue No.
Human Exosome Characterization Panel Standard	1 vial	Lyophilized	HEXSM-8170
Human Exosome Characterization Panel Quality Controls 1 and 2	1 vial each	Lyophilized	HEXSM-6170
MCF7: IGF-1 Cell Lysate	1 vial	Lyophilized	47-216
Set of one 96-Well Plate with 2 sealers	1 plate 2 sealers	-	-
Assay Buffer	1 bottle	30 mL	L-AB
Bead Diluent	1 bottle	3.2 mL	LBD
10X Wash Buffer	1 bottle	60 mL	L-WB
Human Exosome Characterization Panel Detection Antibodies	1 bottle	5.5 mL	HEXSM-1170
Streptavidin-Phycoerythrin	1 bottle	5.5 mL	L-SAPE19
Mixing Bottle *(not provided with premixed panel)*	1 bottle	-	-

Human Exosome Characterization Panel Antibody-Immobilized Premixed Magnetic Beads

Premixed 9-plex Beads	3.5 mL	1 bottle	HEXSMPMX9-MAG

Included Human Exosome Characterization Panel Antibody-Immobilized Beads are dependent on customizable selection of analytes within the panel.
Note: The Ago-2 bead is provided as a static kit component (see below).

Human Exosome Characterization Panel Antibody-Immobilized Magnetic Beads

Bead/Analyte Name	Luminex® Magnetic Bead Region	Available	9-Plex Magnetic Premixed Beads	Catalogue No.
Anti-Human Ago-2 Bead*	12	✔	✔	HAG02-MAG
Anti-Human CALR Bead	14	✔	✔	HCALR-MAG
Anti-Human CD9 Bead	22	✔	✔	HCD9-MAG
Anti-Human CD63 Bead	33	✔	✔	HCD63-MAG
Anti-Human CD81 Bead	54	✔	✔	HCD81-MAG
Anti-Human GAPDH Bead	66	✔	✔	HGAPDH-MAG
Anti-Human Syn-1 Bead	72	✔	✔	HSYN1-MAG
Anti-Human Flot-1 Bead	74	✔	✔	HFLOT1-MAG
Anti-Human TSG101 Bead	77	✔	✔	HTSG101-MAG

* The Ago-2 bead is provided as a static kit component and not part of the configurable kit format.

Materials Required (Not provided)

Reagents

MAGPIX® Drive Fluid PLUS (40-50030), xMAP® Sheath Fluid PLUS (40-50021), or xMAP® Sheath Concentrate PLUS (40-50023).

Instrumentation/Materials

- Adjustable Pipettes with Tips capable of delivering 25 µL to 1000 µL
- Multichannel Pipettes capable of delivering 5 µL to 50 µL, or 25 µL to 200 µL
- Reagent Reservoirs
- Polypropylene Microfuge Tubes
- Rubber Bands
- Aluminum Foil
- Absorbent Pads
- Laboratory Vortex Mixer
- Sonicator (Branson Ultrasonic Cleaner, Model No. B200 or equivalent)
- Titer Plate Shaker (VWR® Microplate Shaker, 12620-926 or equivalent)
- Luminex® 200™, HTS, FLEXMAP 3D®, MAGPIX® with xPONENT® software or xMAP® INTELLIFLEX with INTELLIFLEX software by Luminex® Corporation.
- Automatic Plate Washer for magnetic beads (BioTek® 405 LS and 405 TS, 40-094, 40-095, 40-096, 40-097 or equivalent) or Handheld Magnetic Separation Block (40-285 or equivalent).

  Note: If a plate washer or handheld magnetic separation block for magnetic beads is not available, one can use a microtiter filter plate (MX-PLATE) to run the assay using a Vacuum Filtration Unit (Vacuum Manifold, MSVMHTS00 or equivalent with Vacuum Pump, WP6111560 or equivalent).

Safety Precautions

- All blood components and biological materials should be handled as potentially hazardous. Follow universal precautions as established by the Centers for Disease Control and Prevention and by the Occupational Safety and Health Administration when handling and disposing of infectious agents.
- Sodium azide or ProClin™ has been added to some reagents as a preservative. Although the concentrations are low, Sodium azide and ProClin™ may react with lead and copper plumbing to form highly explosive metal azides. Dispose of unused contents and waste in accordance with international, federal, state, and local regulations.

Symbol Definitions

Ingredient	Catalogue No.	Label	
Human Exosome Characterization Panel Standard	HEXSM-8170		**Danger.** Harmful if swallowed or if inhaled. Toxic in contact with skin. Causes serious eye damage. May cause damage to organs Respiratory Tract through prolonged or repeated exposure. May cause damage to organs Brain through prolonged or repeated exposure if swallowed. Harmful to aquatic life with long lasting effects. Do not breathe dust. Wash skin thoroughly after handling. Do not eat, drink or smoke when using this product. Use only outdoors or in a well-ventilated area. Avoid release to the environment. Wear protective gloves/ protective clothing/ eye protection/ face protection. IF SWALLOWED: Call a POISON CENTER/ doctor if you feel unwell. Rinse mouth. IF ON SKIN: Wash with plenty of water. Call a POISON CENTER/ doctor if you feel unwell. IF INHALED: Remove person to fresh air and keep comfortable for breathing. Call a POISON CENTER/ doctor if you feel unwell. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. Get medical advice/ attention if you feel unwell. Take off contaminated clothing and wash before reuse. Store locked up. Dispose of contents/ container to an approved waste disposal plant.

For research use only. Not for use in diagnostic procedures.

Ingredient	Catalogue No.	Label	
Human Exosome Characterization Panel Quality Control	HEXSM-6170		**Danger.** Harmful if swallowed or if inhaled. Toxic in contact with skin. Causes serious eye damage. May cause damage to organs Respiratory Tract through prolonged or repeated exposure. May cause damage to organs Brain through prolonged or repeated exposure if swallowed. Harmful to aquatic life with long lasting effects. Do not breathe dust. Wash skin thoroughly after handling. Do not eat, drink or smoke when using this product. Use only outdoors or in a well-ventilated area. Avoid release to the environment. Wear protective gloves/ protective clothing/ eye protection/ face protection. IF SWALLOWED: Call a POISON CENTER/ doctor if you feel unwell. Rinse mouth. IF ON SKIN: Wash with plenty of water. Call a POISON CENTER/ doctor if you feel unwell. IF INHALED: Remove person to fresh air and keep comfortable for breathing. Call a POISON CENTER/ doctor if you feel unwell. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. Get medical advice/ attention if you feel unwell. Take off contaminated clothing and wash before reuse. Store locked up. Dispose of contents/ container to an approved waste disposal plant.
Human Exosome Characterization Panel Detection Antibodies	HEXSM-1170		**Warning.** Causes serious eye irritation. May cause damage to organs Respiratory Tract through prolonged or repeated exposure. Do not breathe mist or vapours. Wash skin thoroughly after handling. Wear eye protection/ face protection. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. Get medical advice/ attention if you feel unwell. If eye irritation persists: Get medical advice/ attention. Dispose of contents/ container to an approved waste disposal plant.

Ingredient	Catalogue No.	Label	
MCF7: IGF-1 Cell Lysate	47-216		**Danger.** Harmful if swallowed. Causes skin irritation. Causes serious eye damage. Very toxic to aquatic life. Toxic to aquatic life with long lasting effects. Avoid release to the environment. IF SWALLOWED: Call a POISON CENTER/ doctor if you feel unwell. Collect spillage. Dispose of contents/ container to an approved waste disposal plant.
10X Wash Buffer	L-WB		**Warning.** May cause an allergic skin reaction. Wear protective gloves. IF ON SKIN: Wash with plenty of soap and water.
Streptavidin-Phycoerythrin	L-SAPE19		**Warning:** Causes serious eye irritation. May cause damage to organs Respiratory Tract through prolonged or repeated exposure. Do not breathe mist or vapours. Wash skin thoroughly after handling. Wear eye protection/ face protection. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. Get medical advice/ attention if you feel unwell. If eye irritation persists: Get medical advice/ attention. Dispose of contents/ container to an approved waste disposal plant.

Technical Guidelines

To obtain reliable and reproducible results, the operator should carefully read this entire manual and fully understand all aspects of each assay step before running the assay. The following notes should be reviewed and understood before the assay is set up.

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- Do not use beyond the expiration date on the label.
- Do not mix or substitute reagents with those from other lots or sources.
- The Antibody-Immobilized Beads are light sensitive and must be protected from light at all times. Cover the assay plate containing beads with opaque plate lid or aluminum foil during all incubation steps.
- It is important to allow all reagents to warm to room temperature (20-25 °C) before use in the assay.
- Incomplete washing can adversely affect the assay outcome. All washing must be performed with the Wash Buffer provided.
- The standards prepared by serial dilution must be used within 1 hour of preparation. Discard any unused standards except the standard stock which may be stored at ≤ -20 °C for 1 month and at ≤ -80 °C for greater than one month.
- If samples fall outside the dynamic range of the assay, further dilute the samples with the appropriate diluent and repeat the assay.
- Any unused mixed Antibody-Immobilized Beads may be stored in the Mixing Bottle at 2-8 °C for up to one month.
- During the preparation of the standard curve, make certain to mix the higher concentration well before making the next dilution. Use a new tip with each dilution.
- The plate should be read immediately after the assay is finished. If, however, the plate cannot be read immediately, seal the plate, cover with aluminum foil or an opaque lid, and store the plate at 2-8 °C for up to 24 hours. Prior to reading, agitate the plate on the plate shaker at room temperature for 10 minutes. Delay in reading a plate may result in decreased sensitivity for some analytes.
- The titer plate shaker should be set at a speed to provide maximum orbital mixing without splashing of liquid outside the wells. For the recommended plate shaker, this would be a setting of 5-7 which is approximately 500-800 rpm.
- Ensure that the needle probe is clean. This may be achieved by sonication and/or alcohol flushes.

- When reading the assay on Luminex® 200™, adjust probe height according to the protocols recommended by Luminex® to the kit solid plate or to the recommended filter plates using 3 alignment discs. When reading the assay on MAGPIX®, adjust probe height according to the protocols recommended by Luminex® to the kit solid plate or to the recommended filter plates using 2 alignment discs. When reading the assay on FLEXMAP 3D®, adjust probe height according to the protocols recommended by Luminex® to the kit solid plate using 1 alignment disc.
    - For FLEXMAP 3D® when using the solid plate in the kit, the final resuspension should be with 100 µL Sheath Fluid PLUS in each well and 50 µL should be aspirated.
    - For xMAP® INTELLIFLEX, adjust probe height based on the type of plate you are using, place an alignment disk or an alignment sphere in the well according to the protocol recommended by Luminex®.
    - For cell/tissue homogenate, the final cell or tissue homogenate should be prepared in a buffer that has a neutral pH, contains minimal detergents or strong denaturing detergents, and has an ionic strength close to physiological concentration. Avoid debris, lipids, and cell/tissue aggregates. Centrifuge samples before use.
- Vortex all reagents well before adding to plate.

Sample Collection and Storage

Preparation of Serum Samples

- Allow the blood to clot for at least 30 minutes before centrifugation for 10 minutes at 1000 *x g*. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C.
- Avoid multiple (> 2) freeze/thaw cycles.
- When using frozen samples, it is recommended to thaw the samples completely, mix well by vortexing and centrifuge prior to use in the assay to remove particulates.
- Proceed to exosome isolation procedure.

Preparation of Plasma Samples

Plasma collection using EDTA as an anti-coagulant is recommended. Centrifuge for 10 minutes at 1000 *x g* within 30 minutes of blood collection. Remove plasma and assay immediately or aliquot and store samples at ≤ -20 °C.

- Avoid multiple (> 2) freeze/thaw cycles.
- When using frozen samples, it is recommended to thaw the samples completely, mix well by vortexing and centrifuge prior to use in the assay to remove particulates.
- Proceed to exosome isolation procedure.

Preparation of Tissue Culture Supernatant

- Centrifuge the sample to remove debris and assay immediately or aliquot and store samples at ≤ -20 °C.
- Avoid multiple (> 2) freeze/thaw cycles.
- Proceed to exosome isolation procedure.

Preparation of Exosomes Isolated from Serum/Plasma or Tissue Culture Supernatant

- Following sample collection and preparation, exosomes can now be isolated and lysed according to your lab's established protocol. We recommend Cell Signaling Lysis Buffer (43-040). Each lab may use their preferred lysis buffer but must predetermine that the assay will not be negatively affected by lysis buffer. Samples may be further diluted in Assay Buffer, if needed, as sample concentration will determine resuspension volume and if any dilution is necessary prior to running in the assay.
- The suggested working range of protein concentration for the assay is 1 to 25 µg of total protein/well (25 µL/well at 40 to 1000 µg/mL). A total protein amount of 10 µg/well is generally a good starting point for lysed exosome samples for which target protein expression levels are unknown.

Note:

- A maximum of 25 µL per well of diluted or neat isolated exosomes can be used.
- All samples must be stored in polypropylene tubes. DO NOT STORE SAMPLES IN GLASS.
- Avoid debris, lipids and cells when using samples with gross hemolysis or lipemia.
- Care must be taken when using heparin as an anti-coagulant since an excess of heparin will provide falsely high values. Use no more than 10 IU heparin per mL of blood collected.

Preparation of Reagents for Immunoassay

Preparation of Antibody-Immobilized Beads

If **premixed beads** are used, sonicate the premixed bead bottle 30 seconds and then vortex for 1 minute before use.

For **individual vials of beads**, sonicate each antibody-bead vial for 30 seconds; vortex for 1 minute. Add 150 µL from each antibody-bead vial to the Mixing Bottle and bring final volume to 3.0 mL with bead diluent. Vortex the mixed beads well. Unused portion may be stored at 2-8 °C for up to one month.

Note: Due to the composition of magnetic beads, you may notice a slight color in the bead solution or variability between certain analyte beads. This does not affect the performance of the beads or the kit.

Example 1: When using 5 antibody-immobilized beads, add 150 µL from each of the 5 bead vials to the Mixing Bottle. Then add 2.25 mL bead diluent.

Example 2: When using 2 antibody-immobilized beads, add 150 µL from each of the 2 bead vials to the Mixing Bottle. Then add 2.70 mL bead diluent.

Preparation of Quality Controls

Before use, reconstitute Quality Control 1 and Quality Control 2 with 250 µL deionized water. Invert the vial several times to mix and vortex. Allow the vial to sit for 5-10 minutes. Transfer the reconstituted Quality Control 1 and Quality Control 2 into two polypropylene microfuge tubes. Unused portion may be stored at ≤ -20 °C for up to one month.

Preparation of Wash Buffer

Bring the 10X Wash Buffer to room temperature and mix to bring all salts into solution. Dilute 60 mL of 10X Wash Buffer with 540 mL deionized water. Store the unused portion at 2-8 °C for up to one month.

Preparation of MILLIPLEX® Cell Lysate Control

MILLIPLEX® MCF7 Cell Lysate: IGF-1 (47-216) is provided as a lyophilized stock of cell lysate prepared from stimulated MCF-7 cells and is used as a positive control for Argonaute-2.

Reconstitute the lyophilized cell lysate in 100 µL of ultrapure water. Invert the vial several times to mix. Vortex the vial for 10 seconds. Allow the vial to sit for 5-10 minutes. Add 150 µL of Assay Buffer to the cell lysate vial and vortex well. Unused lysate may be stored in its original container at ≤ -20 °C for up to one month.

Preparation of Human Exosome Characterization Panel Standard

Lyophilized standard and quality controls include all analytes except for Argonaute-2 ("non-canonical exosome marker"; typically absent from classical exosomes control).

1. Prior to use, reconstitute the Human Exosome Characterization Panel Standard with 250 µL deionized water. Refer to table below for analyte concentrations. Invert the vial several times to mix. Vortex the vial for 10 seconds. Allow the vial to sit for 5-10 minutes. Transfer the reconstituted standard to a polypropylene microfuge tube. This will be used as Standard 7; the unused portion may be stored at ≤ -20 °C for up to one month.

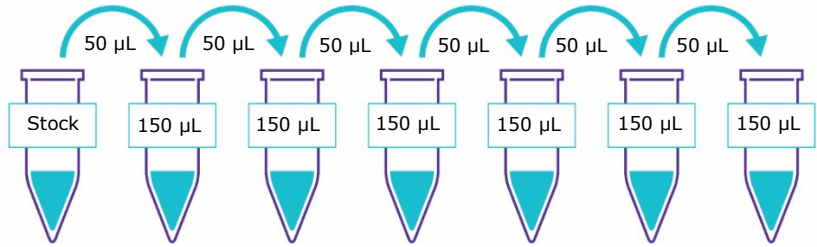
2. Preparation of Working Standards

Label 6 polypropylene microfuge tubes Standard 1 through Standard 6. Add 150 µL of Assay Buffer to each of the 6 tubes. Prepare serial dilutions by adding 50 µL of the reconstituted standard to the Standard 6 tube, mix well and transfer 50 µL of Standard 6 to the Standard 5 tube, mix well and transfer 50 µL of Standard 5 to the Standard 4 tube, mix well and transfer 50 µL of Standard 4 to the Standard 3 tube, mix well and transfer 50 µL of Standard 3 to the Standard 2 tube, mix well and transfer 50 µL of Standard 2 to the Standard 1 tube and mix well. The 0 [units] standard (Background) will be Assay Buffer.

Standard No.	Add Deionized Water (µL)	Add Standard (volume)
Standard 7	250 µL	0

Standard No.	Add Assay Buffer (µL)	Add Standard (volume)
Standard 6	150 µL	50 µL of Standard 7
Standard 5	150 µL	50 µL of Standard 6
Standard 4	150 µL	50 µL of Standard 5
Standard 3	150 µL	50 µL of Standard 4
Standard 2	150 µL	50 µL of Standard 3
Standard 1	150 µL	50 µL of Standard 2

Preparation of Standards



Reconstituted Standard 7 | Standard 6 | Standard 5 | Standard 4 | Standard 3 | Standard 2 | Standard 1

Standard	Calreticulin, GAPDH (pg/mL)	CD9 (ng/mL)	CD63 (pg/mL)
Standard 1	24	0.4	122
Standard 2	98	1.5	488
Standard 3	391	5.9	1,953
Standard 4	1,563	23	7,813
Standard 5	6,250	94	31,250
Standard 6	25,000	375	125,000
Standard 7	100,000	1,500	500,000

Standard	CD81 (ng/mL)	Syntenin-1 (ng/mL)	Flotillin-1 (ng/mL)	TSG101 (pg/mL)
Standard 1	0.5	0.9	0.6	49
Standard 2	2.0	3.4	2.4	195
Standard 3	7.8	14	9.8	781
Standard 4	31	55	39	3,125
Standard 5	125	219	156	12,500
Standard 6	500	875	625	50,000
Standard 7	2,000	3,500	2,500	200,000

Immunoassay Procedure

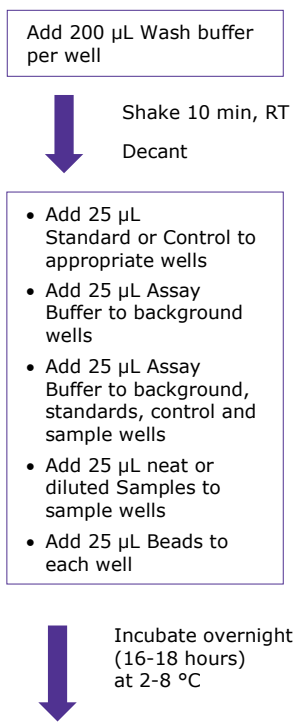
- Prior to beginning this assay, it is imperative to read this protocol completely and to thoroughly understand the Technical Guidelines.
- Allow all reagents to warm to room temperature (20-25 °C) before use in the assay.
- Diagram the placement of Standards 0 (Background), Standard 1 through 7, Controls 1 and 2, MCF7 + IGF Lysate Control and Samples on Well Map Worksheet in a vertical configuration. It is recommended to run the assay in duplicate.

  Note: Most instruments will only read the 96-well plate vertically by default.

- If using a filter plate, always set the filter plate on a plate holder during reagent dispensing and incubation steps so that the bottom of the plate does not touch any surface.

1. Add 200 µL of Wash Buffer into each well of the plate. Seal and mix on a plate shaker for 10 minutes at room temperature (20-25 °C).
2. Decant Wash Buffer and remove the residual amount from all wells by inverting the plate and tapping it smartly onto absorbent towels several times.
3. Add 25 µL of each Standard or Control into the appropriate wells. Add 25 uL prepared MCF7 + IGF Lysate into the appropriate wells. Assay Buffer should be used for Background.
4. Add 25 µL of Assay Buffer to the background, standards, control, MCF7 + IGF Lysate and sample wells.
5. Add 25 µL of neat or diluted prepared Sample into the appropriate wells.
6. Vortex Mixing Bottle and add 25 µL of the Mixed or Premixed Beads to each well.

   Note: During addition of Beads, shake bead bottle intermittently to avoid settling.
7. Seal the plate with a plate sealer. Wrap the plate with foil and incubate with agitation on a plate shaker overnight (16-18 hours) at 2-8 °C.



8. Gently remove well contents and wash plate 3 times following instructions listed in the **PLATE WASHING** section.
9. Add 50 µL of Detection Antibodies into each well.

   Note: Allow the Detection Antibodies to warm to room temperature prior to addition.
10. Seal, cover with foil and incubate with agitation on a plate shaker for 1 hour at room temperature (20-25 °C). **DO NOT ASPIRATE AFTER INCUBATION.**
11. Add 50 µL Streptavidin-Phycoerythrin to each well containing the 50 µL of Detection Antibodies.
12. Seal, cover with foil and incubate with agitation on a plate shaker for 30 minutes at room temperature (20-25 °C).
13. Gently remove well contents and wash plate 3 times following instructions listed in the **PLATE WASHING** section.
14. Add 100 µL of Sheath Fluid PLUS (or Drive Fluid PLUS if using MAGPIX®) to all wells. Resuspend the beads on a plate shaker for 5 minutes.
15. Run plate on Luminex® 200™, HTS, FLEXMAP 3D®, MAGPIX® with xPONENT® software or xMAP® INTELLIFLEX with INTELLIFLEX Software.
16. Save and analyze the Median Fluorescent Intensity (MFI) data using a 5-parameter logistic or spline curve-fitting method for calculating analyte concentrations in samples.

Plate Washing

If using a solid plate, use either a handheld magnet or magnetic plate washer.

Solid Plate

- Handheld magnet (40-285)

  Rest plate on magnet for 60 seconds to allow complete settling of magnetic beads. Remove well contents by gently decanting the plate in an appropriate waste receptacle and gently tapping on absorbent pads to remove residual liquid. Wash plate with 200 µL of Wash Buffer by removing plate from magnet, adding Wash Buffer, shaking for 30 seconds, reattaching to magnet, letting beads settle for 60 seconds and removing well contents as previously described after each wash. Repeat wash steps as recommended in Assay Procedure.

- Magnetic plate washer (40-094, 40-095, 40-096 and 40-097)

  Please refer to specific automatic plate washer manual for appropriate equipment settings. Please note that after the final aspiration, there will be approximately 25 µL of residual wash buffer in each well. This is expected when using the BioTek® plate washer and this volume does not need to be aspirated from the plate.

If using an automatic plate washer other than BioTek® 405 LS or 405 TS, please refer to the manufacturer's recommendations for programming instructions.

Filter Plate (MX-PLATE)

If using a filter plate, use a vacuum filtration manifold to remove well contents. Wash plate with 200 µL/well of Wash Buffer, removing Wash Buffer by vacuum filtration after each wash. Repeat wash steps as recommended in the Assay Procedure.

Equipment Settings

Luminex® 200™, HTS, FLEXMAP 3D®, MAGPIX® with xPONENT® software and xMAP® INTELLIFLEX with INTELLIFLEX software:

These specifications are for the above listed instruments and software. Luminex® instruments with other software (for example MasterPlex®, StarStation, LiquiChip, Bio-Plex® Manager™, LABScan™100) would need to follow instrument instructions for gate settings and additional specifications from the vendors for reading Luminex® magnetic beads.

For magnetic bead assays, each instrument must be calibrated, and performance verified with the indicated calibration and verification kits.

Instrument	Calibration Kit	Verification Kit
Luminex® 200™ and HTS	xPONENT® 3.1 compatible Calibration Kit (LX2R-CAL-K25)	Performance Verification Kit (LX2R-PVER-K25)
FLEXMAP 3D®	FLEXMAP 3D® Calibrator Kit (F3D-CAL-K25)	FLEXMAP 3D® Performance Verification Kit (F3D-PVER-K25)
xMAP® INTELLIFLEX	xMAP® INTELLIFLEX Calibration Kit (IFX-CAL-K20)	xMAP® INTELLIFLEX Performance Verification Kit (IFX-PVER-K20)
MAGPIX®	MAGPIX® Calibration Kit (MPX-CAL-K25)	MAGPIX® Performance Verification Kit (MPX-PVER-K25)

Note: When setting up a Protocol using the xPONENT® software, you must select MagPlex® as the Bead Type in the Acquisition settings.

Note: These assays cannot be run on any instruments using Luminex® IS 2.3 or Luminex® 1.7 software.

The Luminex® probe height must be adjusted to the plate provided in the kit. Please use (MAG-PLATE), if additional plates are required for this purpose.

Events	50, per bead	
Sample Size	50 µL	
Gate Settings	8,000 to 15,000	
Reporter Gain	Default (low PMT)	
Time Out	60 seconds	
Bead Set	Customizable 9-plex Beads	
	Ago-2*	12
	CALR	14
	CD9	22
	CD63	33
	CD81	54
	GAPDH	66
	Syntenin-1	72
	Flotillin-1	74
	TSG101	77

*The Ago-2 bead is provided as a static kit component and not part of the configurable kit format.

Quality Controls

The ranges for each analyte in Quality Control 1 and 2 are provided on the card insert or can be located at our website SigmaAldrich.com using the catalogue number as the keyword.

Assay Characteristics

Cross-Reactivity

There was no or negligible cross-reactivity between the antibodies for an analyte and any of the other analytes in this panel, except Syntenin-1 and Flotillin-1 antibodies had minimal cross reactivity with GAPDH standard.

Assay Sensitivities (minimum detectable concentrations)

Minimum Detectable Concentration (MinDC) is calculated using MILLIPLEX® Analyst 5.1. It measures the true limits of detection for an assay by mathematically determining what the empirical MinDC would be if an infinite number of standard concentrations were run for the assay under the same conditions.

	Overnight Protocol (n = 12 Assays)	
Analyte	**MinDC**	**MinDC+2SD**
Ago2	N/A	N/A
CALR	13.36 pg/mL	22.99 pg/mL
CD9	0.22 ng/mL	0.37 ng/mL
CD63	98.71 pg/mL	134.67 pg/mL
CD81	0.37 ng/mL	0.58 ng/mL
GAPDH	18.71 pg/mL	29.40 pg/mL
Syntenin-1	0.42 ng/mL	0.79 ng/mL
Flotillin-1	2.82 ng/mL	10.38 ng/mL
TSG101	29.54 pg/mL	59.43 pg/mL

Precision

Intra-assay precision is generated from the mean of the %CVs from 8 reportable results across two different concentrations of analytes in a single assay. Inter-assay precision is generated from the mean of the % CVs across two different concentrations of analytes across 11 different assays.

	Overnight Protocol	
Analyte	**Intra-assay %CV**	**Inter-assay %CV**
Ago2	< 10%	< 20%
CALR	< 10%	< 20%
CD9	< 10%	< 20%
CD63	< 10%	< 20%
CD81	< 10%	< 20%
GAPDH	< 10%	< 20%
Syntenin-1	< 10%	< 20%
Flotillin-1	< 10%	< 20%
TSG101	< 10%	< 20%

Troubleshooting

Problem	Probable Cause	Solution
Insufficient bead count	Plate washer aspirate height set too low	Adjust aspiration height according to manufacturers' instructions.
	Bead mix prepared inappropriately	Sonicate bead vials and vortex just prior to adding to bead mix bottle according to protocol. Agitate bead mix intermittently in reservoir while pipetting this into the plate.
	Samples cause interference due to particulate matter or viscosity	See above. Also sample probe may need to be cleaned with alcohol flushes, back flushes, and washes; or, if needed, probe should be removed and sonicated.
	Probe height not adjusted correctly	When reading the assay on Luminex® 200™, adjust probe height to the kit solid plate or to the recommended filter plates using 3 alignment discs. When reading the assay on MAGPIX®, adjust probe height to the kit solid plate or to the recommended filter plates using 2 alignment discs. When reading the assay on FLEXMAP 3D®, adjust probe height to the kit solid plate using 1 alignment disc. For FLEXMAP 3D® when using the solid plate in the kit, the final resuspension should be with 150 µL Sheath Fluid PLUS in each well and 75 µL should be aspirated.
		When reading the assay on xMAP® INTELLIFLEX, adjust probe height based on the type of plate you are using, place an alignment disk or an alignment sphere in the well according to the protocol recommended by Luminex®.

Problem	Probable Cause	Solution
Background is too high	Background wells were contaminated	Avoid cross-well contamination by using sealer appropriately and pipetting with multichannel pipettes without touching reagent in plate.
	Matrix used has endogenous analyte or interference	Check matrix ingredients for cross-reacting components (for example, interleukin modified tissue culture medium).
	Insufficient washes	Increase number of washes.
Beads not in region or gate	Luminex® instrument not calibrated correctly or recently	Calibrate Luminex® instrument based on manufacturer's instructions, at least once a week or if temperature has changed by >3 °C.
	Gate settings not adjusted correctly	Some Luminex® instruments (for example Bio-Plex®) require different gate settings than those described in the kit protocol. Use instrument default settings.
	Wrong bead regions in protocol template	Check kit protocol for correct bead regions or analyte selection.
	Incorrect sample type used	Samples containing organic solvents or if highly viscous should be diluted or dialyzed as required.
	Instrument not washed or primed	Prime the Luminex® instrument 4 times to rid it of air bubbles, wash 4 times with Sheath Fluid PLUS or water if there is any remnant alcohol or sanitizing liquid.
	Beads were exposed to light	Keep plate and bead mix covered with dark lid or aluminum foil during all incubation steps.

Problem	Probable Cause	Solution
Signal for whole plate is same as background	Incorrect or no Detection Antibody was added	Add appropriate Detection Antibody and continue.
	Streptavidin-Phycoerythrin was not added	Add Streptavidin-Phycoerythrin according to protocol. If Detection Antibody has already been removed, sensitivity may be low.
Low signal for standard curve	Detection Antibody may have been removed prior to adding Streptavidin-Phycoerythrin	May need to repeat assay if desired sensitivity not achieved.
	Incubations done at inappropriate temperatures, timings, or agitation	Assay conditions need to be checked.
Signals too high, standard curves are saturated	Calibration target value set too high	With some Luminex® instruments (for example, Bio-Plex®) default target setting for RP1 calibrator is set at high PMT. Use low target value for calibration and reanalyze plate.
	Plate incubation was too long with standard curve and samples	Use shorter incubation time.
Sample readings are out of range	Samples contain no or below detectable levels of analyte	If below detectable levels, it may be possible to use higher sample volume. Check with technical support for appropriate protocol modifications.
	Samples contain analyte concentrations higher than highest standard point	Samples may require dilution and reanalysis for just that particular analyte.
	Standard curve was saturated at higher end of curve	See above.

Problem	Probable Cause	Solution
High variation in samples and/or standards	Multichannel pipette may not be calibrated	Calibrate pipettes.
	Plate washing was not uniform	Confirm all reagents are removed completely in all wash steps.
	Samples may have high particulate matter or other interfering substances	See above.
	Plate agitation was insufficient	Plate should be agitated during all incubation steps using an orbital plate shaker at a speed where beads are in constant motion without causing splashing.
	Cross-well contamination	Check when reusing plate sealer that no reagent has touched sealer. Care should be taken when using same pipette tips that are used for reagent additions and that pipette tip does not touch reagent in plate.

For Filter Plates Only

Problem	Probable Cause	Solution
Filter plate will not vacuum	Vacuum pressure is insufficient	Increase vacuum pressure such that 0.2 mL buffer can be suctioned in 3-5 seconds.
	Samples have insoluble particles	Centrifuge samples just prior to assay set-up and use supernatant.
	High lipid concentration	After centrifugation, remove lipid layer and use supernatant.
Plate leaked	Vacuum pressure too high	Adjust vacuum pressure such that 0.2 mL buffer can be suctioned in 3-5 seconds. May need to transfer contents to a new (blocked) plate and continue.
	Plate set directly on table or absorbent towels during incubations or reagent additions	Set plate on plate holder or raised edge so bottom of filter is not touching any surface.
	Insufficient blotting of filter plate bottom causing wicking	Blot the bottom of the filter plate well with absorbent towels after each wash step.
	Pipette touching plate filter during additions	Pipette to the side of plate.
	Probe height not adjusted correctly	Adjust probe to 3 alignment discs in well H6.
	Sample too viscous	May need to dilute sample.

Product Ordering

Order products online at SigmaAldrich.com.

Replacement Reagents	Catalogue Number
Human Exosome Characterization Panel Standard	HEXSM-8170
Human Exosome Characterization Panel Quality Control 1 and 2	HEXSM-6170
MCF7: IGF-1 Cell Lysate	47-216
Bead Diluent	LBD
Human Exosome Characterization Panel Detection Antibodies	HEXSM-1170
Streptavidin-Phycoerythrin	L-SAPE19
Assay Buffer	L-AB
Set of two 96-Well plates with sealers	MAG-PLATE
10X Wash Buffer	L-WB
Premixed 9-plex Beads	HEXSMPMX9-MAG

Antibody-Immobilized Magnetic Beads

Bead/Analyte Name	Bead No.	Catalogue Number
Anti-Human Ago-2 Bead	12	HAG02-MAG
Anti-Human CALR Bead	14	HCALR-MAG
Anti-Human CD9 Bead	22	HCD9-MAG
Anti-Human CD63 Bead	33	HCD63-MAG
Anti-Human CD81 Bead	54	HCD81-MAG
Anti-Human GAPDH Bead	66	HGAPDH-MAG
Anti-Human Syn-1 Bead	72	HSYN1-MAG
Anti-Human Flot-1 Bead	74	HFLOT1-MAG
Anti-Human TSG101 Bead	77	HTSG101-MAG

Well Map

	1	2	3	4	5	6	7	8	9	10	11	12
A	0 Standard (Background)	Standard 4	QC-1 Control	Etc.								
B	0 Standard (Background	Standard 4	QC-1 Control									
C	Standard 1	Standard 5	QC-2 Control									
D	Standard 1	Standard 5	QC-2 Control									
E	Standard 2	Standard 6	47-216 Lysate Control									
F	Standard 2	Standard 6	47-216 Lysate Control									
G	Standard 3	Standard 7	Sample 1									
H	Standard 3	Standard 7	Sample 1									

Notice

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