Store at -20°C

Exosomal Marker Antibody Sampler Kit

#74220

1 Kit (8 x 20 µl)

Support: +1-978-867-2388 (U.S.)
www.cellsignal.com/support

Orders: 877-616-2355 (U.S.)
orders@cellsignal.com

New 02/16

For Research Use Only. Not For Use In Diagnostic Procedures.

Products Included	Product #	Quantity	Mol. Wt.	Isotype
Alix (3A9) Mouse mAb	2171	20 µl	95 kDa	Mouse IgG1
Annexin V Antibody	8555	20 µl	30 kDa	Rabbit
CD54/ICAM-1 Antibody	4915	20 µl	89, 92 kDa	Rabbit
CD9 (D8O1A) Rabbit mAb	13174	20 µl	22, 24, 35 kDa	Rabbit IgG
GM130 (D6B1) XP® Rabbit mAb	12480	20 µl	130 kDa	Rabbit IgG
EpCAM (D183) Rabbit mAb	2626	20 µl	40 kDa	Rabbit IgG
HSP70 (D69) Antibody	4876	20 µl	70 kDa	Rabbit
Flotillin-1 (D2V7J) XP® Rabbit mAb	18634	20 µl	49 kDa	Rabbit IgG
Anti-rabbit IgG, HRP-linked Antibody	7074	100 µl		Goat
Anti-mouse IgG, HRP-linked Antibody	7076	100 µl		Goat

See www.cellsignal.com for individual component applications, species cross-reactivity, dilutions and additional application protocols.

Storage: Supplied in 10 mM sodium HEPES (pH 7.5), 150 mM NaCl, 100 µg/ml BSA, 50% glycerol and less than 0.02% sodium azide. Store at –20°C. *Do not aliquot the antibodies.*

Recommended Antibody Dilutions:
Western blotting 1:1000

Please visit www.cellsignal.com for a complete listing of recommended companion products.

Description: The Exosomal Marker Antibody Sampler Kit provides an economical means to evaluate the presence of exosomal markers. The kit includes enough primary antibody to perform two western blot experiments for each target.

Background: Exosomes are small membrane-bound vesicles that in recent years have emerged as important molecules for inter-cellular communication. Exosomes are produced during both normal and pathophysiological conditions, and cancer cells have been shown to secrete exosomes in greater amounts than normal cells (reviewed in 1). The exosomal markers contained in this kit are Alix, Annexin V, ICAM-1, CD9, GM130, EpCAM, flotillin, and HSP70.

Alix, a cytosolic scaffold protein, regulates many cellular processes including endocytic membrane trafficking, cell adhesion through interactions with ESCRT (endosomal sorting complex required for transport) proteins, endophilins, and CIN85 (Cbl-Interacting protein of 85 kDa) (2, 3).

Annexin V is a ~30 kDa protein that binds to phospho-lipids in a calcium-dependent manner (4). All annexins contain a putative PKC binding site, but only annexin V has been identified as an inhibitor of this pathway (5).

Intracellular cell adhesion molecule-1 (CD54 or ICAM-1) is a cell surface glycoprotein that belongs to the immunoglobulin superfamily (IgSF) of adhesion molecules. CD54 is expressed at low levels in diverse cell types, and is induced by cytokines (TNF-alpha, interleukin-1) and bacterial lipopolysaccharides (6). Apical localization on endothelial cells (or basolateral localization on epithelial cells) is a prerequisite for leukocyte trafficking through the endothelial (or epithelial) barrier (6).

The CD9 antigen belongs to the tetraspanin family of cell surface glycoproteins. Tetraspanins interact with a variety of cell surface proteins and intracellular signaling molecules in specialized tetraspanin-enriched microdomains (TEMs), where they mediate a range of processes including adhesion, motility, membrane organization, and signal transduction (7). Additional research identified CD9 as an abundant component of exosomes, and may play a role in the fusion of these secreted membrane vesicles with recipient cells (8).

GM130 is required for membrane fusion events that mediate ribbon formation during Golgi assembly (9). The Golgi apparatus functions in the modification, organization, and transport of proteins and membrane targeted to other parts of the cell, such as the plasma membrane, lysosomes, and endosomes. This regulated transport is important for appropriate protein localization, secretion, and signal transduction (reviewed in 10).

Epithelial cell adhesion and activation molecule (EpCAM/CD326) is a transmembrane glycoprotein that mediates calcium-independent, hemophilic adhesions on the basolateral surface of most epithelial cells (11). One of the first tumor-associated antigens discovered, EpCAM has long been a marker of epithelial and tumor tissue. Research studies have shown that EpCAM is highly expressed in cancer cells and can be used as a biomarker for the detection of tumor-derived exosomes (reviewed in 1, 12, 13).

Flotillins belong to a famiy of lipid raft-associated integral membrane proteins that are ubiquitously expressed and located to lipid rafts on the cell plasma membrane where they support signal transduction and regulate lipid raft motility and localization (14-17). In addition to its colocalization with lipid rafts on the plasma membrane, flotillin-1 also has been found at compartments of the endocytic and autophagosomal pathways, such as recycling/ late endosomes, the Golgi complex, as well as the nucleus (18, 19).

HSP70 is a molecular chaperone expressed constitutively under normal conditions to maintain protein homeostasis and is induced upon environmental stress (20). HSP70 is able to interact with unfolded proteins to prevent irreversible aggregation and catalyze the refolding of their substrates in an ATP and co-chaperone dependent manner (21). An immune response is elicited upon excretion of heat shock proteins from tumor exosomes (reviewed in 1).

Specificity/Sensitivity: All antibodies provided in the kit detect endogenous levels of the respecitve target protein. Additionally, the Annexin V Antibody is not predicted to cross-react with other annexin family members and the CD54/ICAM-1 Antibody does not cross-react with other IgSF adhesion molecules. The GM130 antibody may cross-react with a protein of unknown origin at 30 kDa.

Source/Purification: Monoclonal antibodies are produced by immunizing animals with full-length recombinant human Alix protein, a synthetic peptide corresponding to residues surrounding Val178 of human CD9 protein, residues surrounding Thr195 of human GM130 protein, residues near the amino terminus of human EpCAM protein and residues surrounding Ile368 of human flotillin-1 protein. Polyclonal antibodies are produced by immunizing animals with a synthetic peptide corresponding to residues near the amino terminus of human annexin V protein, residues of human CD54 (ICAM-1) protein, and residues surrounding Asp69 of human HSP70. Polyclonal antibodies are purified by protein A and peptide affinity chromatography.

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Applications: **W**—Western **IP**—Immunoprecipitation **IHC**—Immunohistochemistry **ChIP**—Chromatin Immunoprecipitation **IF**—Immunofluorescence **F**—Flow cytometry **E-P**—ELISA-Peptide **Species Cross-Reactivity:** **H**—human **M**—mouse **R**—rat **Hm**—hamster **Mk**—monkey **Mi**—mink **C**—chicken **Dm**—D. melanogaster **X**—Xenopus **Z**—zebrafish **B**—bovine **Dg**—dog **Pg**—pig **Sc**—S. cerevisiae **Ce**—C. elegans **Hr**—Horse **All**—all species expected Species enclosed in parentheses are predicted to react based on 100% homology.

Western Immunoblotting Protocol

For western blots, incubate membrane with diluted primary antibody in either 5% w/v BSA or nonfat dry milk, 1X TBS, 0.1% Tween® 20 at 4°C with gentle shaking, overnight.
NOTE: Please refer to primary antibody datasheet or product webpage for recommended primary antibody dilution buffer and recommended antibody dilution.

A. Solutions and Reagents

NOTE: Prepare solutions with reverse osmosis deionized (RODI) or equivalent grade water.

1. **20X Phosphate Buffered Saline (PBS):** (#9808) To prepare 1 L 1X PBS: add 50 ml 20X PBS to 950 ml dH_2O, mix.
2. **10X Tris Buffered Saline (TBS):** (#12498) To prepare 1 L 1X TBS: add 100 ml 10X to 900 ml dH_2O, mix.
3. **1X SDS Sample Buffer:** Blue Loading Pack (#7722) or Red Loading Pack (#7723) Prepare fresh 3X reducing loading buffer by adding 1/10 volume 30X DTT to 1 volume of 3X SDS loading buffer. Dilute to 1X with dH_2O.
4. **10X Tris-Glycine SDS Running Buffer:** (#4050) To prepare 1 L 1X running buffer: add 100 ml 10X running buffer to 900 ml dH_2O, mix.
5. **10X Tris-Glycine Transfer Buffer:** (#12539) To prepare 1 L 1X transfer buffer: add 100 ml 10X transfer buffer to 200 ml methanol + 700 ml dH_2O, mix.
6. **10X Tris Buffered Saline with Tween® 20 (TBST):** (#9997) To prepare 1 L 1X TBST: add 100 ml 10X TBST to 900 ml dH_2O, mix.
7. **Nonfat Dry Milk:** (#9999)
8. **Blocking Buffer:** 1X TBST with 5% w/v nonfat dry milk; for 150 ml, add 7.5 g nonfat dry milk to 150 ml 1X TBST and mix well.
9. **Wash Buffer:** (#9997) 1X TBST
10. **Bovine Serum Albumin (BSA):** (#9998)
11. **Primary Antibody Dilution Buffer:** 1X TBST with 5% BSA or 5% nonfat dry milk as indicated on primary antibody datasheet; for 20 ml, add 1.0 g BSA or nonfat dry milk to 20 ml 1X TBST and mix well.
12. **Biotinylated Protein Ladder Detection Pack:** (#7727)
13. **Prestained Protein Marker, Broad Range (Premixed Format):** (#7720)
14. **Blotting Membrane and Paper:** (#12369) This protocol has been optimized for nitrocellulose membranes. Pore size 0.2 µm is generally recommended.
15. **Secondary Antibody Conjugated to HRP:** anti-rabbit (#7074); anti-mouse (#7076)
16. **Detection Reagent:** LumiGLO® chemiluminescent reagent and peroxide (#7003) or SignalFire™ ECL Reagent (#6883)

B. Protein Blotting

A general protocol for sample preparation.

1. Treat cells by adding fresh media containing regulator for desired time.
2. Aspirate media from cultures; wash cells with 1X PBS; aspirate.
3. Lyse cells by adding 1X SDS sample buffer (100 µl per well of 6-well plate or 500 µl for a 10 cm diameter plate). Immediately scrape the cells off the plate and transfer the extract to a microcentrifuge tube. Keep on ice.
4. Sonicate for 10–15 sec to complete cell lysis and shear DNA (to reduce sample viscosity).
5. Heat a 20 µl sample to 95–100°C for 5 min; cool on ice.
6. Microcentrifuge for 5 min.
7. Load 20 µl onto SDS-PAGE gel (10 cm x 10 cm). **NOTE:** Loading of prestained molecular weight markers (#7720, 10 µl/lane) to verify electrotransfer and biotinylated protein ladder (#7727, 10 µl/lane) to determine molecular weights are recommended.
8. Electrotransfer to nitrocellulose membrane (#12369).

C. Membrane Blocking and Antibody Incubations

NOTE: Volumes are for 10 cm x 10 cm (100 cm^2) of membrane; for different sized membranes, adjust volumes accordingly.

I. Membrane Blocking

1. (Optional) After transfer, wash nitrocellulose membrane with 25 ml TBS for 5 min at room temperature.
2. Incubate membrane in 25 ml of blocking buffer for 1 hr at room temperature.
3. Wash three times for 5 min each with 15 ml of TBST.

II. Primary Antibody Incubation

1. Incubate membrane and primary antibody (at the appropriate dilution and diluent as recommended in the product datasheet) in 10 ml primary antibody dilution buffer with gentle agitation overnight at 4°C.
2. Wash three times for 5 min each with 15 ml of TBST.
3. Incubate membrane with the species appropriate HRP-conjugated secondary antibody (#7074 or #7076 at 1:2000) and anti-biotin, HRP-linked Antibody (#7075 at 1:1000–1:3000) to detect biotinylated protein markers in 10 ml of blocking buffer with gentle agitation for 1 hr at room temperature.
4. Wash three times for 5 min each with 15 ml of TBST.
5. Proceed with detection (Section D).

D. Detection of Proteins

1. Incubate membrane with 10 ml LumiGLO® (0.5 ml 20X LumiGLO® #7003, 0.5 ml 20X peroxide, and 9.0 ml purified water) or 10 ml SignalFire™ #6883 (5 ml Reagent A, 5 ml Reagent B) with gentle agitation for 1 min at room temperature.
2. Drain membrane of excess developing solution (do not let dry), wrap in plastic wrap and expose to x-ray film. An initial 10 sec exposure should indicate the proper exposure time. **NOTE:** Due to the kinetics of the detection reaction, signal is most intense immediately following incubation and declines over the following 2 hr.