



## MONOCLONAL ANTIBODY

For research use only, Not for diagnostic use.

Catalog No. LKG-M001

**Anti Human CD44 v9 [ Clone : RV3]****BACKGROUND**

CD44 is a single-pass type I transmembrane protein and functions as a cellular adhesion molecule for hyaluronic acid, a major component of the extracellular matrix. It exists in numerous isoforms that are generated through alternative splicing of CD44 precursor mRNA. Whereas the standard isoform of CD44 (CD44 s) is expressed predominantly in hematopoietic cells and normal epithelial cell subsets, CD44 v (variant) isoforms, which contain additional insertions in the membrane-proximal extracellular region, are highly expressed in epithelial-type carcinomas<sup>5</sup>. Moreover, CD44 is reported as cell surface marker for **cancer stem cells (CSCs)** derived from solid tumors including breast, prostate, colon, head and neck and pancreatic cancer. Expression of CD44, especially variant isoforms (CD44 v8-10), contributes to reactive oxygen species (ROS) defense through upregulation of the synthesis of reduced glutathione (GSH), the primary intracellular antioxidant. CD44 v8-10 interacts with and stabilizes xCT, a subunit of the cystine-glutamate transporter xc(-), and thereby promotes cystine uptake for GSH synthesis. The ability to avoid the consequences of exposure to high levels of ROS is required for cancer cell survival and propagation *in vivo*. CSCs, in which defense against ROS is enhanced by CD44v8-10 are thus thought to drive tumor growth, chemoresistance and metastasis<sup>1-4</sup>.

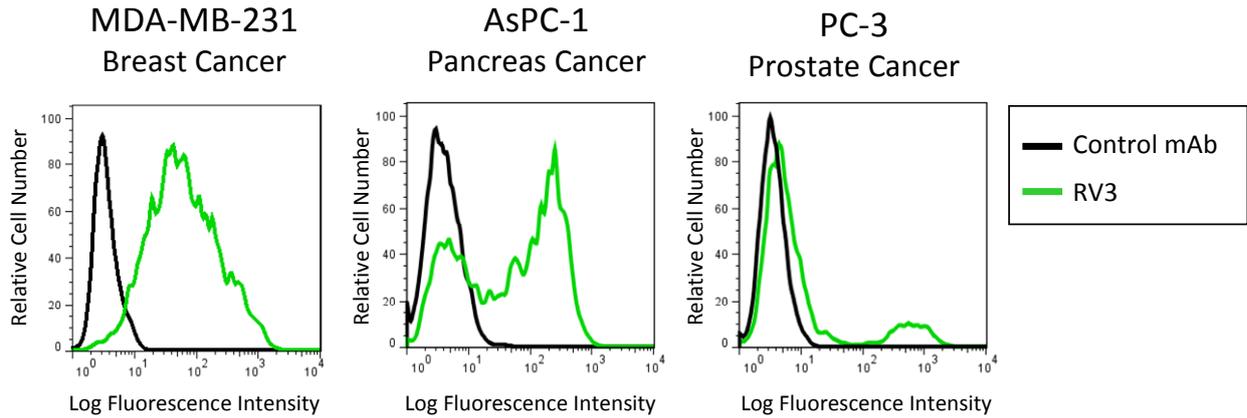
**Clone RV3**, is a monoclonal antibody specific for **human CD44 v9** can be used for FCM, WB, IHC, ICC, IP, and ELISA assay, and importantly, for the enrichment of CSCs using a cell sorter. RV3 can be applied towards understanding a variety of molecular mechanisms and towards the development of new medicines against cancer stem cells using *in vitro* cell-based assays such as "*in vitro* sphere formation assay" and "*in vivo* lung metastasis assay".

<b>Product type</b>	Primary antibody
<b>Immunogen</b>	Human CD44 v8-10 transfected cell
<b>Rased in</b>	Rat
<b>Myeloma</b>	X63-Ag8-653
<b>Clone number</b>	RV3
<b>Isotype</b>	IgG2a
<b>Source</b>	Ascites
<b>Purification</b>	Affinity purified by Protein G
<b>Buffer</b>	Phosphate buffered saline (PBS)* *NOTE: PBS doesn't contain preservative. Preservative is added based on the research purpose.
<b>Concentration</b>	1 mg / mL
<b>Volume</b>	100 uL (100 ug)
<b>Label</b>	Unlabeled
<b>Specificity</b>	Human CD44 v9
<b>Cross reactivity</b>	Other species is not tested.
<b>Storage</b>	Store cold (2 to 8 °C)

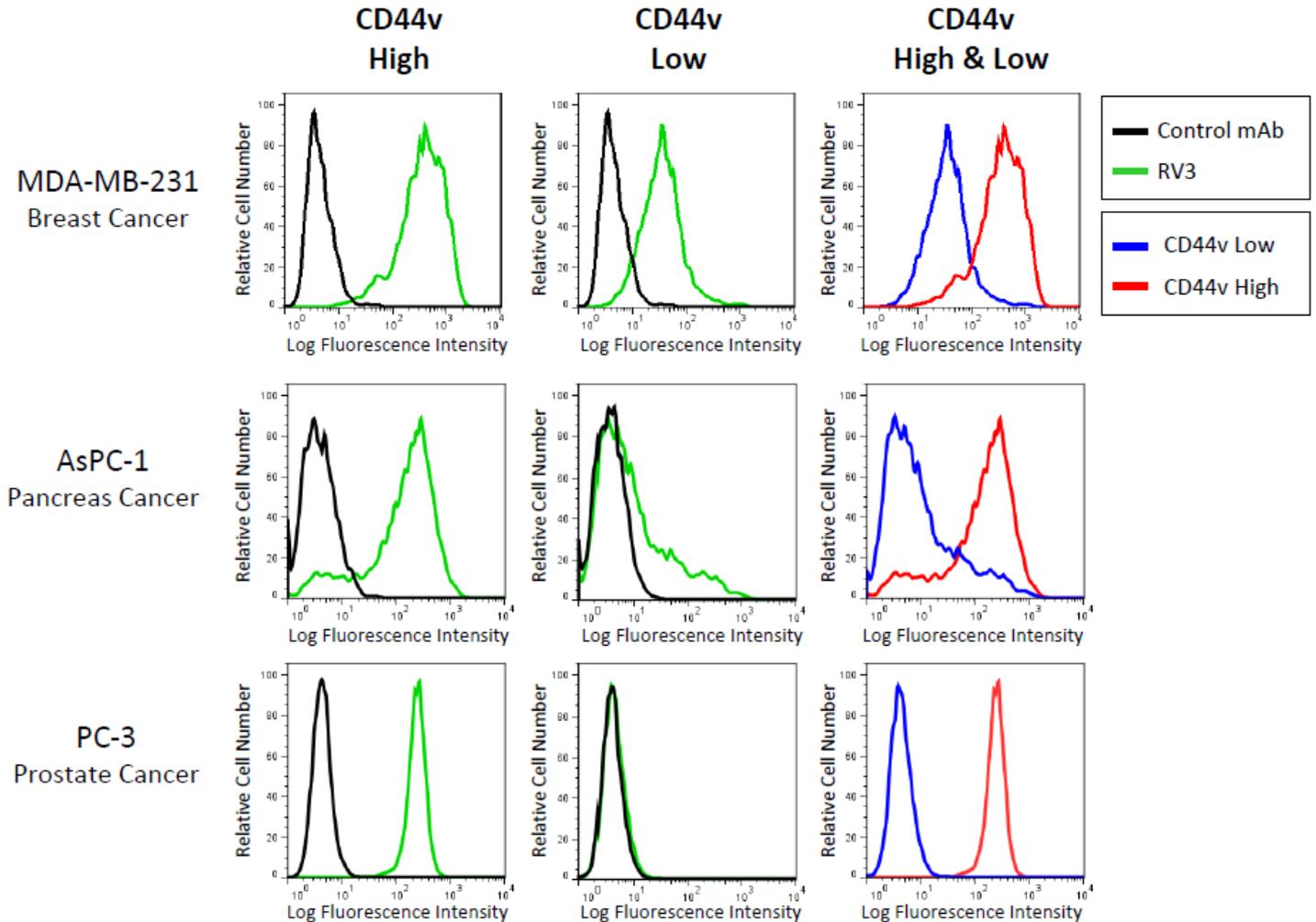
<b>Application notes</b>	<ul style="list-style-type: none"><li>• <b>Flow cytometry:</b> 1-10µg/mL</li><li>• <b>Immunohistochemistry:</b> 0.2µg/mL (Paraffin section)</li><li>• <b>Immunofluorescence:</b> 3µg/mL</li><li>• <b>Western blotting:</b> 1µg/mL</li><li>• <b>Immunoprecipitation:</b> 10µg/mL</li><li>• <b>ELISA:</b> 5µg/mL</li></ul>
<b>Recommended dilutions</b>	Other applications have not been tested. Optimal dilutions/concentrations should be determined by the end user. Detailed procedure is provided in the following <b>PROTOCOLS</b> .

<b>References</b>	1) Nagano O., <i>et al.</i> , Oncogene. 2013 Jan 21., 1-8. PMID: <a href="#">23334333</a> 2) Ishimoto T., <i>et al.</i> , Cancer Cell. 2011 Mar 8;19(3):387-400. PMID : <a href="#">21397861</a> 3) Yae T., <i>et al.</i> , Nat Commun. 2012 Jun 6;3:883. PMID: <a href="#">22673910</a> 4) Tsugawa H., <i>et al.</i> , Cell Host Microbe. 2012 Dec 13;12(6):764-77. PMID: <a href="#">23245321</a> 5) Tanabe KK., <i>et al.</i> , Lancet. 1993 Mar 20;341(8847):725-6. PMID: <a href="#">8095628</a>
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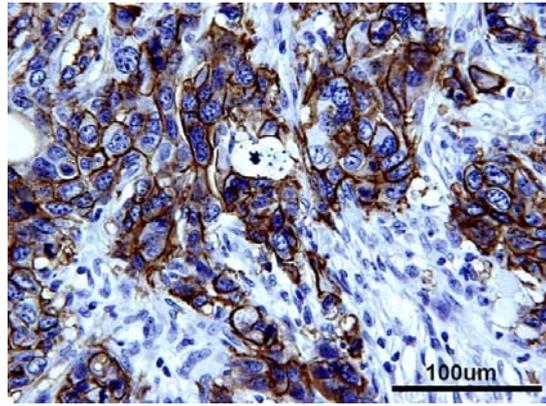
**ANTIBODY CHARACTERIZATION**



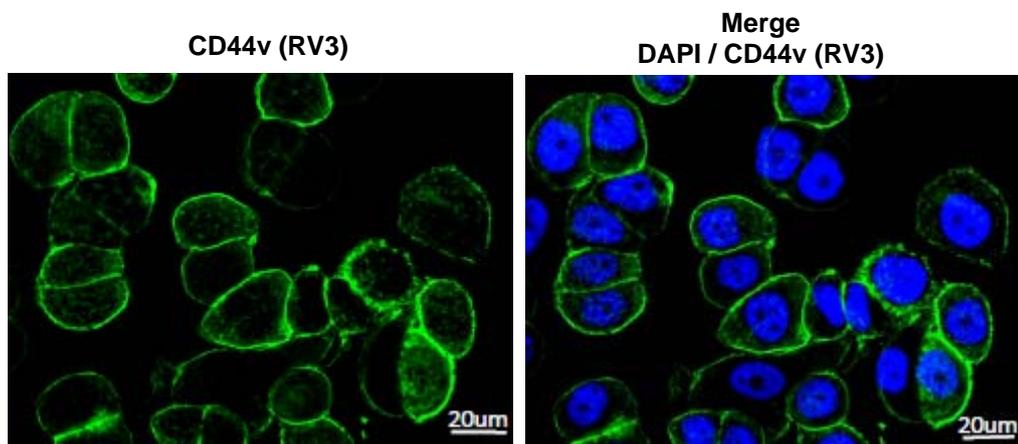
**Fig.1** Flow cytometry analysis of CD44 v in Human cancer cell line with anti-CD44 v9 (RV3, 3µg/mL) antibody and PE-labeled anti Rat IgG antibody.



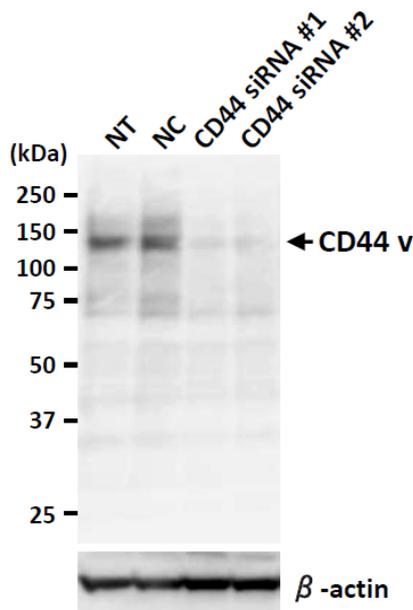
**Fig.2** Flow cytometry Cell Sorting of CD44 v expression level in Human cancer cell line with anti-CD44 v9 (RV3) antibody and PE-labeled anti Rat IgG antibody. Two kinds of subpopulations “CD44v<sup>+</sup>(High) and CD44v<sup>-</sup>(Low)” were isolated.



**Fig.3** Immunohistochemistry staining Breast Invasive Ductal Carcinoma with anti-CD44 v9 antibody (clone RV3, 0.2µg/mL).



**Fig.4** Immunofluorescence staining of CD44 v (green) in MDA-MB-468 cells with anti-CD44 v9 antibody (RV3, 3µg/mL).



**Fig.4** Western blot analysis of CD44 v in BT20 cell lysate with anti-CD44 v9 antibody(RV3, 1µg/mL). CD44 knockdown samples (CD44 siRNA #1, #2) were not detected.  
NT: no treated    NC: non-targeting siRNA treated sample

## PROTOCOLS:

### Flow cytometry protocol (Cell Analysis)

#### A. Cell Preparation

1. Remove cells from incubator.
2. Discard culture medium.
3. Briefly rinse the cell layer with PBS.
4. Add 0.25% trypsin-EDTA solution to dish. Return the dish to the incubator and incubate for 2-10 minutes or until cells are detached.
5. Resuspend cells in complete growth medium to inactivate the trypsin.

#### B. Staining

1. Aliquot  $1 \times 10^5$  cells into each assay tube.
2. Add 150  $\mu$ l 0.2 % BSA in PBS to each tube and rinse by centrifugation.
3. Add 50  $\mu$ l diluted primary antibody (3  $\mu$ g/ml RV3 in 0.2 % BSA in PBS) to the assay tubes.
4. Incubate 45 minutes at 4 °C.
5. Add 100  $\mu$ l 0.1 % BSA in PBS to each tube and wash by centrifugation.
6. Wash two times in 150  $\mu$ l 0.1 % BSA in PBS by centrifugation.
7. Resuspend cells in 50  $\mu$ l PE-labeled secondary antibody solution (Jackson Immuno Research 712-116-153), diluted 1:200 in 0.1 % BSA in PBS.
8. Incubate 30 minutes at 4 °C in the dark.
9. Add 100  $\mu$ l 0.1 % BSA in PBS to each tube and wash by centrifugation.
10. Wash two times in 150  $\mu$ l 0.1 % BSA in PBS by centrifugation.
11. Resuspend cells in 100  $\mu$ l PBS.
12. Add 100  $\mu$ l Propidium Iodide (SIGMA, P4864), diluted 1:500 in PBS, to stain dead cells.
13. Analyze using flow cytometry

### Flow cytometry protocol (Cell Sorting)

#### A. Cell Preparation

1. Prepare cultured cells for sorting based on the ratio of the CD44v expression cells.
2. Remove cells from incubator.
3. Discard culture medium.
4. Briefly rinse the cell layer with PBS.
5. Add 0.25% trypsin-EDTA solution to dish. Return the dish to the incubator and incubate for 2-10 minutes or until cells are detached.
6. Resuspend the cells in complete growth medium to inactivate the trypsin.

#### B. Staining (for $1 \times 10^7$ cells)

1. Aliquot  $1 \times 10^7$  cells into 15ml tube.
2. Add 10 ml 0.2 % BSA in PBS to the tube and rinse by centrifugation.
3. Add 5 ml diluted primary antibody (3  $\mu$ g/ml RV3 in 0.2 % BSA in PBS) to the tube.
4. Incubate with gentle agitation 45 minutes at 4 °C.
5. Wash three times in 10 ml 0.1 % BSA in PBS by centrifugation.
6. Resuspend cells in 5 ml PE-labeled secondary antibody solution (Jackson Immuno Research 712-116-153), diluted 1:200 in 0.1 % BSA in PBS.
7. Incubate 45 minutes at 4 °C in the dark.
8. Wash three times in 10 ml 0.1 % BSA in PBS by centrifugation.
9. Resuspend cells in 5 ml PBS.
10. Add 5 ml Propidium Iodide (SIGMA, P4864) diluted 1:500 in PBS, to stain dead cells.
11. Sort CD44v high and low expression cells using a cell sorter.
12. Wash the sorted cells in 5 ml complete growth medium (added antibiotic drug) three times by centrifugation.
13. Culture the sorted cells and scale up.
  - Note the passage number and analyze the cell population periodically using flow cytometry.
14. If desired, sort the cells again, they would be high-enrichment.

## **Immunohistochemistry Protocol (Paraffin)**

### **A. Deparaffinization / Rehydration**

1. Deparaffinize/hydrate
  - a. Incubate sections in xylene three times for 5 minutes each.
  - b. Incubate sections in 100% ethanol for 10 seconds.
  - c. Incubate sections in 90% ethanol for 10 seconds.
  - d. Incubate sections in 70% ethanol for 10 seconds.
2. Wash sections in dH<sub>2</sub>O for 5 minutes.

### **B. Antigen Unmasking**

1. Immerse sections in 10 mM citrate buffer (pH 6.0) for 7 minutes at 100 °C in microwave.
2. Cool slides on bench top for 30 minutes.
3. Wash sections in TBS-T buffer for 5 minutes.

### **C. Staining**

1. Incubate sections in 0.3% hydrogen peroxide in Methanol for 15 minutes to block endogenous peroxidase.
2. Wash sections in TBS-T buffer three times for 5 minutes each.
3. Cover sections with 300 µl blocking solution (10 % normal rabbit serum in TBS) for 30 minutes at room temperature.
4. Remove excess blocking solution and add 200 µl diluted primary antibody to each section (0.2µg/ml RV3 in 1.5 % normal rabbit serum in TBS). Incubate 1 hour at room temperature.
5. Remove antibody solution and wash sections in TBS-T buffer three times for 5 minutes each.
6. Add 200 µl biotinylated secondary antibody (Dako, E0468), diluted 1:200 in 1.5 % normal rabbit serum in TBS, to each section.
7. Incubate 30 minutes at room temperature.
8. Remove antibody solution and wash sections in TBS-T buffer three times for 5 minutes each.
9. Add 200 µl VECTASTAIN ABC Reagent (VECTOR LABORATORIES, PK-6100) to each section. Incubate 30 minutes at room temperature.
10. Wash sections in TBS-T buffer three times for 5 minutes each.
11. Add 200 µl peroxidase substrate reagent (PIERCE, 34065) to each section and incubate until desired stain intensity develops (about 1 minute).
12. Wash sections in TBS-T buffer three times for 5 minutes each.
13. Wash sections in dH<sub>2</sub>O two times for 5 minutes each.
14. Counterstain sections in hematoxylin per manufacturer's instructions.
15. Dehydrate sections:
  - a. Incubate sections in 70% ethanol for 10 seconds.
  - b. Incubate sections in 90% ethanol for 10 seconds.
  - c. Incubate sections in 100% ethanol for 10 seconds.
  - d. Incubate sections in xylene three washes for 5 minutes each.
16. Mount coverslips.

## **Immunofluorescence Protocol**

### **A. Cell Preparation**

1. Grow cultured cells on Cell Chamber Slide.
2. Remove cells from incubator.
3. Discard medium and rinse briefly with PBS.
4. Fix in 4% paraformaldehyde in PBS for 15 minutes at room temperature.
5. Remove fixative and rinse three times in PBS for 3 minutes each.

### **B. Staining**

1. Block sample in 3% BSA in PBS for 30 minutes at room temperature.
2. Remove excess blocking solution and apply diluted primary antibody (3 µg/ml RV3 in 0.2 % BSA in PBS).
3. Incubate 1 hour at room temperature.
4. Wash three times in PBS for 3 minutes each.
5. Apply Alexa Fluor 488 anti-rat IgG secondary antibody solution (Invitrogen, A11006), diluted 1:400 in 0.2 % BSA in PBS.
6. Incubate 1 hour at room temperature in the dark.
7. Wash three times in PBS for 3 minutes each.
8. Mount the coverslip using an anti-fade mounting reagent with DAPI (Invitrogen, S36939).
9. Seal slide by painting around edges of coverslip with nail polish.
10. Store slides flat at 4 °C protected from light until until examined.

## Western blot protocol

1. Load 20 µl cell lysate samples (40µg/lane) onto SDS-PAGE gel (ATTO E-T10L).
2. Electrotransfer to PVDF membrane.
3. Block membrane in 5% Skim milk in PBS-T for 1 hour at room temperature.
4. Incubate membrane with 1 µg/ml RV3 primary antibody in Can Get Signal Solution I (TOYOBO, NKB201) with gentle agitation overnight at 4°C.
5. Wash membrane in PBS-T three times for 5 minutes each.
6. Incubate membrane with HRP-conjugated anti-rat IgG secondary antibody (GE Healthcare NA9350V), diluted 1:20000 in Can Get Signal Solution II (TOYOBO, NKB301), with gentle agitation 30 minutes at 37 °C.
7. Wash membrane in PBS-T three times for 5 minutes each.
8. Incubate membrane in detection mix (Thermo SuperSignal West Dura, 34075) with gentle agitation 1 minute at room temperature.
9. Detect using an imager.

## **RELATED PRODUCT:**

Product Name	Clone	Application	Quantity	Maker	Cat#
Anti Human CD44 v9 Monoclonal Antibody	RV3	FCM / IHC / IF / WB / IP / ELISA	100 ug / 100 uL	CAC	LKG-M001
Anti Mouse CD44 v10-e16 Monoclonal Antibody	RM1	FCM	100 ug / 200 uL	CAC	LKG-M002

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