

Anti-Podoplanin (Human) mAb

CODE No.	D320-3
CLONALITY	Monoclonal
CLONE	NZ-1.2
ISOTYPE	Rat IgG2a κ
QUANTITY	100 μ L, 1 mg/mL
SOURCE	Purified IgG from hybridoma supernatant
FORMULATION	PBS containing 50% Glycerol (pH 7.2). No preservative is contained.
STORAGE	This antibody solution is stable for one year from the date of purchase when stored at -20°C.

APPLICATIONS-CONFIRMED

<u>Western blotting</u>	0.1-1 μ g/mL
<u>Immunoprecipitation</u>	0.5-1 μ g/200 μ L of cell extract from 1×10^6 cells
<u>Immunohistochemistry</u>	0.25-1 μ g/mL
<u>Flow cytometry</u>	0.1 μ g/mL

APPLICATIONS-UNDER EVALUATION

Immunocytochemistry

SPECIES CROSS REACTIVITY on WB

Species	Human	Mouse	Rat	Hamster
Cells	Transfectant, H226, U87MG	Transfectant, Colon-26, B16-F10	Transfectant	Transfectant
Reactivity	+	-	-	-

Entrez Gene ID 10630 (Human)

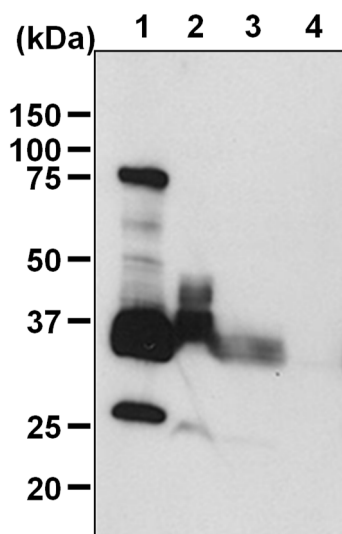
- REFERENCES**
- 1) Kaji, C., *et al.*, *Acta. Histochem. Cytochem.* **45**, 227-237 (2012)
 - 2) Kato, Y., *et al.*, *Biochem. Biophys. Res. Commun.* **349**, 1301-1307 (2006)
 - 3) Kaneko, M. K., *et al.*, *FEBS Lett.* **581**, 331-336 (2007)
 - 4) Kato, Y., *et al.*, *Cancer. Sci.* **99**, 54-61 (2008)
 - 5) Ogasawara, S., *et al.*, *Hybridoma* **27**, 259-267 (2008)
 - 6) Kato, Y., *et al.*, *Nucl. Med. Biol.* **37**, 785-794 (2010)

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SDS-PAGE & Western blotting

- 1) Wash 1×10^7 cells 3 times with PBS and suspend them in 1 mL of Laemmli's sample buffer, then sonicate briefly (up to 10 sec.).
- 2) Centrifuge the tube at $12,000 \times g$ for 5 min. at 4°C and transfer the supernatant to another tube.
- 3) Boil the samples for 3 min. and centrifuge. Load $10 \mu\text{L}$ of the sample per lane in a 1-mm-thick SDS-polyacrylamide gel (12.5% acrylamide) for electrophoresis.
- 4) Blot the protein to a polyvinylidene difluoride (PVDF) membrane at $1 \text{ mA}/\text{cm}^2$ for 1 hr. in a semi-dry transfer system (Transfer Buffer: 25 mM Tris, 190 mM glycine, 20% methanol). See the manufacturer's manual for precise transfer procedure.
- 5) To reduce nonspecific binding, soak the membrane in 10% skimmed milk (in PBS, pH 7.2) for 1 hr. at room temperature.
- 6) Incubate the membrane with primary antibody diluted with 1% skimmed milk (in PBS, pH 7.2) as suggested in the **APPLICATIONS** for 1 hr. at room temperature. (The concentration of antibody will depend on the conditions.)
- 7) Wash the membrane with PBS-T (0.05% Tween-20 in PBS) (5 min. x 3).
- 8) Incubate the membrane with an HRP conjugated anti-rat IgG antibody diluted with 1% skimmed milk (in PBS, pH 7.2) for 1 hr. at room temperature.
- 9) Wash the membrane with PBS-T (5 min. x 3).
- 10) Wipe excess buffer on the membrane, and then incubate it with appropriate chemiluminescence reagent for 1 min. Remove extra reagent from the membrane by dabbing with paper towel, and seal it in plastic wrap.
- 11) Expose to an X-ray film in a dark room for 3 min. Develop the film as usual. The condition for exposure and development may vary.

(Positive controls for Western blotting; transfectant, H226, U87MG)



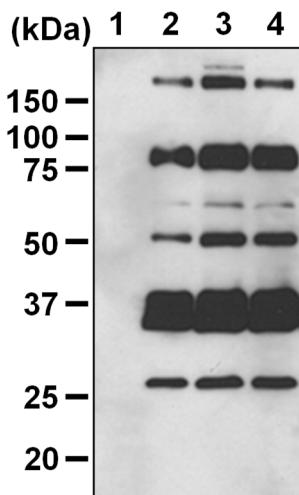
Western blot analysis of Podoplanin

Lane 1: human Podoplanin/CHO
Lane 2: H226
Lane 3: U87MG
Lane 4: H522 (negative control)
Immunoblotted with D320-3

Immunoprecipitation

- 1) Wash 5×10^6 cells twice with PBS and resuspend them with 1 mL of ice-cold Lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.05% NP-40] containing appropriate protease inhibitors, then sonicate briefly (up to 15 sec.). Then, incubate for 15 min. on ice.
- 2) Centrifuge the tube at 12,000 x g for 5 min. at 4°C and transfer the supernatant to another tube.
- 3) Mix 20 μ L of 50% protein G agarose beads slurry resuspended in 250 μ L of IP buffer [10 mM Tris-HCl (pH 8.0), 500 mM NaCl, 0.1% NP-40] with primary antibody as suggested in the **APPLICATIONS**. Incubate with gently agitation for 1 hr. at room temperature. (The amount of antibody will depend on the conditions.)
- 4) Centrifuge the tube at 2,500 x g for 10 seconds and discard the supernatant.
- 5) Resuspend the agarose with 1 mL of IP buffer.
- 6) Centrifuge the tube at 2,500 x g for 10 seconds and discard the supernatant.
- 7) Repeat steps 5)-6) twice.
- 8) Add 200 μ L of cell lysate (prepared sample from step 2)), then incubate with gentle agitation for 1 hr. at room temperature.
- 9) Centrifuge the tube at 2,500 x g for 10 seconds and discard the supernatant.
- 10) Resuspend the agarose with 1 mL of Lysis buffer.
- 11) Centrifuge the tube at 2,500 x g for 10 seconds and discard the supernatant.
- 12) Repeat steps 10)-11) 5 times.
- 13) Resuspend the beads in 20 μ L of Laemmli's sample buffer, boil for 3 min. and centrifuge.
- 14) Load 10 μ L of the sample per lane in a 1-mm-thick SDS-polyacrylamide gel (12.5% acrylamide) for electrophoresis.
- 15) Blot the protein to a polyvinylidene difluoride (PVDF) membrane at 1 mA/cm² for 1 hr. in a semi-dry transfer system (Transfer Buffer: 25 mM Tris, 190 mM glycine, 20% methanol). See the manufacturer's manual for precise transfer procedure.
- 16) To reduce nonspecific binding, soak the membrane in 10% skimmed milk (in PBS, pH 7.2) overnight at 4°C.
- 17) Incubate the membrane with primary antibody diluted with 1% skimmed milk (in PBS, pH 7.2) as suggested in the **APPLICATIONS** for 1 hr. at room temperature. (The concentration of antibody will depend on the conditions.)
- 18) Wash the membrane with PBS-T (0.05% Tween-20 in PBS) (5 min. x 3).
- 19) Incubate the membrane with an HRP conjugated anti-rat IgG antibody diluted with 1% skimmed milk (in PBS, pH 7.2) for 1 hr. at room temperature.
- 20) Wash the membrane with PBS-T (5 min. x 3).
- 21) Wipe excess buffer on the membrane, and then incubate it with appropriate chemiluminescence reagent for 1 min.
- 22) Remove extra reagent from the membrane by dabbing with paper towel, and seal it in plastic wrap.
- 23) Expose to an X-ray film in a dark room for 3 min. Develop the film as usual. The condition for exposure and development may vary.

(Positive control for Immunoprecipitation; transfectant)



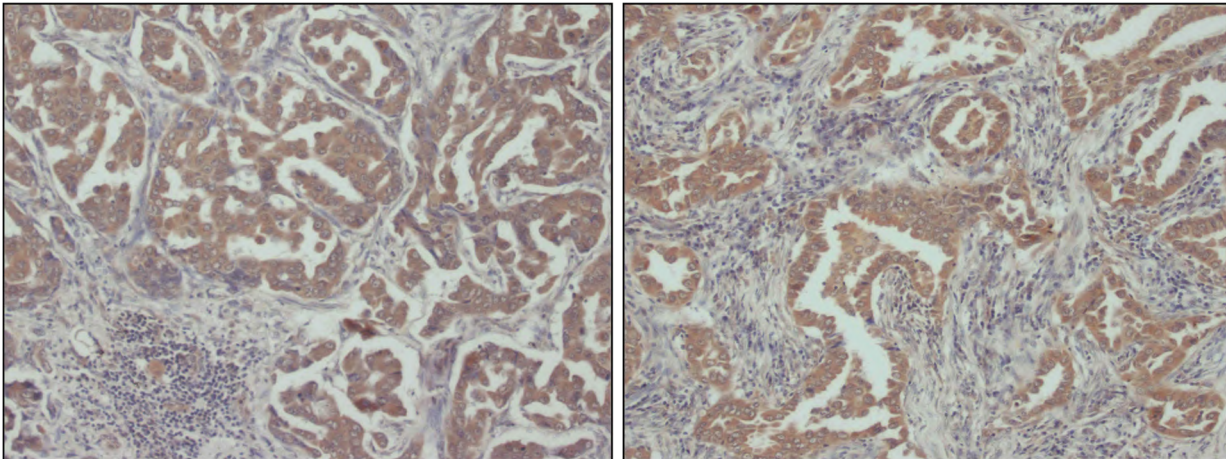
Immunoprecipitation of Podoplanin from transfectant

- Lane 1: IP with isotype control (M081-3, 1 μ g)
Lane 2: IP with D320-3, 0.5 μ g
Lane 3: IP with D320-3, 1 μ g
Lane 4: human Podoplanin/CHO, whole cell lysate
Immunoblotted with D320-3

Immunohistochemical staining for paraffin-embedded sections

- 1) Deparaffinize the sections with Xylene 3 times for 3 min. each.
- 2) Wash the slides with Ethanol 3 times for 3 min. each.
- 3) Wash the slides with PBS 3 times for 3 min. each.
- 4) Remove the slides from the PBS and cover each section with 3% H₂O₂ in PBS for 10 min. at room temperature to block endogenous peroxidase activity.
- 5) Wash the slides with PBS 3 times for 5 min. each.
- 6) Remove the slides from PBS, wipe gently around each section and cover tissues with blocking buffer [20 mM HEPES (pH 7.2), 1% BSA, 135 mM NaCl] for 5 min. at room temperature to block non-specific staining. Do not wash.
- 7) Tip off the blocking buffer, wipe gently around each section and cover tissues with primary antibody diluted with 1% BSA/PBS as suggested in the **APPLICATIONS** for 1 hr. at room temperature. (The concentration of antibody will depend on the conditions.)
- 8) Wash the slides 3 times in PBS for 5 min. each.
- 9) Wipe gently around each section and cover tissues with a biotin conjugated anti-rat IgG antibody. Incubate at room temperature.
- 10) Wash the slides 3 times in PBS for 5 min. each.
- 11) Wipe gently around each section and cover tissues with an HRP conjugated streptavidin. Incubate at room temperature.
- 12) Wash the slides 3 times in PBS for 5 min. each.
- 13) Visualize by reacting for 10 min. with DAB substrate solution (MBL, code no. 8469). *DAB is a suspect carcinogen and must be handled with care. Always wear gloves.
- 14) Wash the slides in water for 5 min.
- 15) Counter stain in hematoxylin for 1 min., wash the slides 3 times in water for 5 min. each, and then immerse the slides in PBS for 5 min. Dehydrate by immersing in Ethanol 3 times for 3 min. each, followed by immersing in Xylene 3 times for 3 min. each.
- 16) Now ready for mounting.

(Positive control for Immunohistochemistry; human lung cancer)



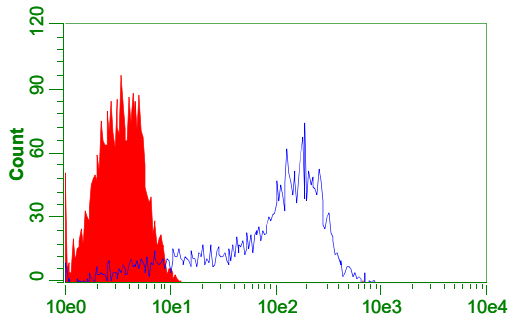
Immunohistochemical detection of Podoplanin in human lung cancer

Immunohistochemical staining with D320-3

Flow cytometric analysis

- 1) Wash the cells (5×10^5 cells/sample) 3 times with 1 mL of washing buffer (PBS containing 2% fetal calf serum).
- 2) Add 100 μ L of 4% paraformaldehyde/PBS to the cell pellet after tapping. Mix well, then fix the cells for 10 min. at room temperature.
- 3) Wash the cells twice with 1 mL of washing buffer.
- 4) Add 10 μ L of Clear Back (human Fc receptor blocking reagent, MBL, code no. MTG-001) to the cell pellet after tapping. Mix well and incubate for 5 min. at room temperature.
- 5) Add 40 μ L of the primary antibody at the concentration as suggested in the **APPLICATIONS** diluted in the washing buffer. Mix well and incubate for 30 min. at room temperature.
- 6) Wash the cells once with 1 mL of washing buffer.
- 7) Add 40 μ L of 1:400 anti-IgG (Rat)-Alexa Fluor[®] 488 (Thermo Fisher Scientific, code no. A11006) diluted with the washing buffer. Mix well and incubate for 20 min. at room temperature.
- 8) Wash the cells once with 1 mL of washing buffer.
- 9) Resuspend the cells with 500 μ L of the washing buffer and analyze by a flow cytometer.

(Positive control for Flow cytometry; transfectant)



***Flow cytometric detection of Podoplanin
in transfectant***

Open: D320-3

Closed: isotype control (M081-3)