

MONOCLONAL ANTIBODY

Anti-Cyclin D1

Code No.	Clone	Subclass	Quantity	Concentration
MD-17-3	5D4	Mouse IgG2a	100 µg	1 mg/mL

BACKGROUND: Cyclin D1, also known as Bcl-1, CCND1, or PRAD-1, is a 36 kDa nuclear protein that plays a key regulatory role during the G₁ phase of the cell cycle by regulating the activity of Cdk proteins. Cyclin D1 forms a complex with Cdk4, then binds to and phosphorylates Rb protein, triggering cells to progress from G₀/G₁ to S and thus driving cellular proliferation. Cyclin D1 protein overexpression is found in the majority of human breast cancers and is usually linked with poor prognosis. Overexpression of cyclin D1 is a well-established criterion for the diagnosis of Mantle Cell Lymphoma, and also has been used to distinguish malignant breast carcinomas from premalignant breast lesions.

SOURCE: This antibody was purified from hybridoma (clone 5D4) supernatant using protein A agarose. This hybridoma was established by fusion of mouse myeloma cell PAI with Balb/c mouse splenocyte immunized with the recombinant human PRAD1/Cyclin D1.

FORMULATION: 100 µg IgG in 100 µL volume of 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.

REACTIVITY: This antibody reacts with human Cyclin D1 (36 kDa) on Western blotting, Immunoprecipitation, Immunohistochemistry and Flow cytometry. This clone 5D4 recognizes human Cyclin D1, D2 and mouse Cyclin D1, D2, but not human and mouse Cyclin D3.

APPLICATIONS:

Western blotting: 1 µg/mL for chemiluminescence detection system

Immunoprecipitation: 1-10 µg/200 µg of cell extract from 5x10⁶ cells

Immunocytochemistry: Not recommended

Immunohistochemistry: 1-10 µg/mL

Heat treatment is necessary for paraffin embedded sections.

Microwave oven; 2 times for 10 minutes each in 10 mM citrate buffer (pH 6.5)

Flow cytometry: 10 µg/mL (final concentration)

Detailed procedure is provided in the following **PROTOCOLS.**

INTENDED USE:

For Research Use Only. Not for use in diagnostic procedures.

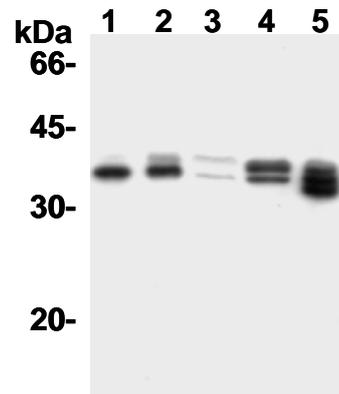
SPECIES CROSS REACTIVITY:

Species	Human	Mouse	Rat
Cells	ZR-75-1, MCF7, HeLa	WR19L, NIH/3T3	Not Tested
Reactivity on WB	+	+	

REFERENCES:

- 1) Tokugawa, T., *et al.*, *Cancer Res.* **62**, 4938-4944 (2002)
- 2) Inagaki, H., *et al.*, *Am. J. Pathol.* **160**, 1435-1443 (2002)
- 3) Kumazawa, Y., *et al.*, *Mol. Pathol.* **55**, 61-64 (2002)

Clone 5D4 is used in these references.



Western blot analysis of Cyclin D1 expression in ZR-75-1 (1), MCF7 (2), HeLa (3), WR19L (4) and NIH/3T3 (5) using MD-17-3.

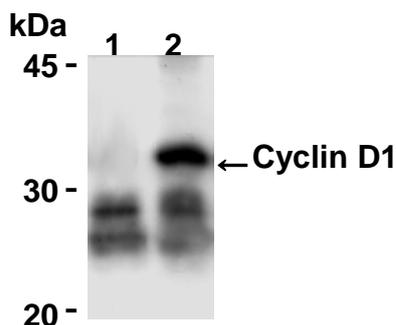
PROTOCOLS:

SDS-PAGE & Western Blotting

- 1) Wash the cells 3 times with PBS and suspend with 10 volume of cold Lysis buffer (50 mM Tris-HCl, pH 7.2, 250 mM NaCl, 0.1% NP-40, 2 mM EDTA, 10% glycerol) containing appropriate protease inhibitors. Incubate it at 4°C with rotating for 30 minutes, then sonicate briefly (up to 10 seconds).
- 2) Centrifuge the tube at 12,000 x g for 10 minutes at 4°C and transfer the supernatant to another tube. Measure the protein concentration of the supernatant and add the cold Lysis buffer to make 8 mg/mL solution.

- 3) Mix the sample with equal volume of Laemmli's sample buffer.
- 4) Boil the samples for 3 minutes and centrifuge. Load 10 μ L of the sample per lane in a 1 mm thick SDS-polyacrylamide gel for electrophoresis.
- 5) Blot the protein to a polyvinylidene difluoride (PVDF) membrane at 1 mA/cm² for 1 hour in a semi-dry transfer system (Transfer Buffer: 25 mM Tris, 190 mM glycine, 20% MeOH). See the manufacture's manual for precise transfer procedure.
- 6) To reduce nonspecific binding, soak the membrane in 10% skimmed milk (in PBS, pH 7.2) for 1 hour at room temperature, or overnight at 4°C.
- 7) Incubate the membrane with primary antibody diluted with PBS, pH 7.2 containing 1% skimmed milk as suggest in the **APPLICATIONS** for 1 hour at room temperature. (The concentration of antibody will depend on condition.)
- 8) Wash the membrane with PBS-T [0.05% Tween-20 in PBS] (5 minutes x 3 times).
- 9) Incubate the membrane with the 1:10,000 HRP-conjugated anti-mouse IgG (MBL; code no. 330) diluted with 1% skimmed milk (in PBS, pH 7.2) for 1 hour at room temperature.
- 10) Wash the membrane with PBS-T (10 minutes x 3 times).
- 11) Wipe excess buffer on the membrane, then incubate it with appropriate chemiluminescence reagent for 1 minute.
- 12) Remove extra reagent from the membrane by dabbing with paper towel, and seal it in plastic wrap.
- 13) Expose to an X-ray film in a dark room for 3 minutes.
- 14) Develop the film as usual. The condition for exposure and development may vary.

(Positive controls for Western blotting; ZR-75-1, MCF7, HeLa, WR19L, NIH/3T3)

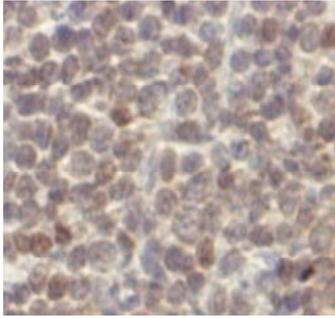


Immunoprecipitation of Cyclin D1 from ZR-75-1 cells with Mouse IgG2a (1) or MD-17-3 (2). After immunoprecipitated with the antibody, immunocomplex was resolved on SDS-PAGE and immunoblotted with 553.

Immunoprecipitation

- 1) Wash the cells 3 times with PBS and suspend with 10 volume of cold Lysis buffer (50 mM Tris-HCl pH 7.2, 250 mM NaCl, 0.1% NP-40, 2 mM EDTA, 10% glycerol) containing appropriate protease inhibitors. Incubate it at 4°C with rotating for 30 minutes, then sonicate briefly (up to 10 seconds).
- 2) Centrifuge the tube at 12,000 x g for 10 minutes at 4°C and transfer the supernatant to another tube.
- 3) Add primary antibody as suggest in the **APPLICATIONS** into 200 μ L of the supernatant. Mix well and incubate with gentle agitation for 30-120 minutes at 4°C.
- 4) Add 20 μ L of 50% protein A agarose resuspended in the cold Lysis buffer. Mix well and incubate with gentle agitation for 60 minutes at 4°C.
- 5) Wash the beads 3-5 times with the cold Lysis buffer (centrifuge the tube at 2,500 x g for 10 seconds).
- 6) Resuspend the beads in 20 μ L of Laemmli's sample buffer, boil for 3-5 minutes, and centrifuge for 5 minutes.
- 7) Load 10 μ L of the sample per lane in a 1 mm thick SDS-polyacrylamide gel for electrophoresis.
- 8) Blot the protein to a polyvinylidene difluoride (PVDF) membrane at 1 mA/cm² for 1 hour in a semi-dry transfer system (Transfer Buffer: 25 mM Tris, 190 mM glycine, 20% MeOH). See the manufacture's manual for precise transfer procedure.
- 9) To reduce nonspecific binding, soak the membrane in 10% skimmed milk (in PBS, pH 7.2) for 1 hour at room temperature, or overnight at 4°C.
- 10) Incubate the membrane with 0.1 μ g/mL of polyclonal antibody Anti-Cyclin D1 (MBL; code no. 553) as primary antibody diluted with PBS, pH 7.2 containing 1% skimmed milk for 1 hour at room temperature. (The concentration of antibody will depend on condition.)
- 11) Wash the membrane with PBS-T [0.05% Tween-20 in PBS] (5 minutes x 3 times).
- 12) Incubate the membrane with the 1:10,000 HRP-conjugated anti-rabbit IgG (MBL; code no. 458) diluted with 1% skimmed milk (in PBS, pH 7.2) for 1 hour at room temperature.
- 13) Wash the membrane with PBS-T (5 minutes x 6 times).
- 14) Wipe excess buffer on the membrane, then incubate it with appropriate chemiluminescence reagent for 1 minute. Remove extra reagent from the membrane by dabbing with paper towel, and seal it in plastic wrap.
- 15) Expose to an X-ray film in a dark room for 5 minutes. Develop the film as usual. The condition for exposure and development may vary.

(Positive control for Immunoprecipitation; ZR-75-1)



Immunohistochemical detection of Cyclin D1 on paraffin embedded section of Human Mantle Cell Lymphoma with MD-17-3.

Immunohistochemical staining for paraffin-embedded sections: SAB method

- 1) Deparaffinize the sections with Xylene 3 times for 3-5 minutes each.
- 2) Wash the slides with Ethanol 3 times for 3-5 minutes each.
- 3) Wash the slides with PBS 3 times for 3-5 minutes each.
- 4) Heat treatment

Heat treatment by Microwave:

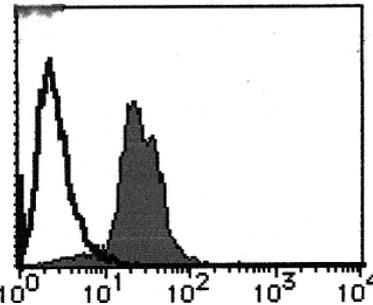
Place the slides put on staining basket in 500 mL beaker with 500 mL of 10 mM citrate buffer (pH 6.5). Cover the beaker with plastic wrap, then process the slides 2 times for 10 minutes each at 500 W with microwave oven. Let the slides cool down in the beaker at room temperature for about 40 minutes.

- 5) Remove the slides from the citrate buffer and cover each section with 3% H₂O₂ for 10 minutes at room temperature to block endogenous peroxidase activity. Wash 3 times in PBS for 5 minutes each.
- 6) Remove the slides from PBS, wipe gently around each section and cover tissues with Protein Blocking Agent (Ultratech HRP Kit; MBL, code no. IM-2391) for 5 minutes 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 PBS containing 1% BSA as suggest in the **APPLICATIONS**.
- 8) Incubate the sections for 1 hour at room temperature.
- 9) Wash the slides 3 times in PBS for 5 minutes each.
- 10) Wipe gently around each section and cover tissues with Polyvalent Biotinylated Antibody (Ultratech HRP Kit). Incubate for 15 minutes at room temperature. Wash as in step 9).
- 11) Wipe gently around each section and cover tissues with Streptavidin-Peroxidase (Ultratech HRP Kit). Incubate for 15 minutes at room temperature. Wash as in step 9).
- 12) Visualize by reacting for 10-20 minutes with substrate solution containing 7.5 mg DAB, 40 μ L of 30% H₂O₂ in 150 mL PBS. *DAB is a suspect carcinogen and must be handled with care. Always wear gloves.
- 13) Wash the slides in water for 5 minutes.
- 14) Counter stain in hematoxylin for 1 minute, wash the slides 3 times in water for 5 minutes each, and then immerse the

slides in PBS for 5 minutes. Dehydrate by immersing in Ethanol 3 times for 3 minutes each, followed by immersing in Xylene 3 times for 3 minutes each.

- 15) Now ready for mounting.

(Positive controls for Immunohistochemistry; ZR-75-1, mantle cell lymphoma)



Flow cytometric analysis of Cyclin D1 expression in HeLa cells. Open histogram indicates the reaction of isotypic control to the cells. Shaded histogram indicates the reaction of MD-17-3 to the cells.

Flow cytometric analysis for cells

We usually use Fisher tubes or equivalents as reaction tubes for all step described below.

- 1) Wash the cells 3 times with washing buffer [PBS containing 2% fetal calf serum (FCS) and 0.1% NaN₃].
- 2) Add 200 μ L of 4% paraformaldehyde (PFA)/PBS to the cell pellet after tapping. Mix well, then fix the cells for 15 minutes at 4°C.
- 3) Wash the cells 3 times with washing buffer.
- 4) Add 200 μ L of 70% ethanol to the cell pellet after tapping. Mix well, then permeablize the cells for 30 minutes at -20°C.
- 5) Wash the cells 3 times with washing buffer.
- 6) Add 20 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 minutes at room temperature.
- 7) Add 40 μ L of the primary antibody at the concentration of as suggest in the **APPLICATIONS** diluted in the washing buffer. Mix well and incubate for 30 minutes at room temperature.
- 8) Add 1 mL of the washing buffer followed by centrifugation at 500 x g for 1 minute at room temperature. Remove supernatant by careful aspiration.
- 9) Add 30 μ L of 1:100 FITC conjugated anti-mouse IgG (MBL; code no. 238) diluted with the washing buffer. Mix well and incubate for 15 minutes at room temperature.
- 10) Add 1 mL of the washing buffer followed by centrifugation at 500 x g for 1 minute at room temperature. Remove supernatant by careful aspiration.
- 11) Resuspend the cells with 500 μ L of the washing buffer and analyze by a flow cytometer.

(Positive control for Flow cytometry; HeLa)

RELATED PRODUCTS:

K0162-3 anti-Cyclin A (E23.1)
 K0163-3 anti-Cyclin A (E67.1)
 K0163-6 biotin labeled anti-Cyclin A (E67.1)
 K0128-3 anti-Cyclin B1 (V152)
 K0164-3 anti-Cyclin B1 (V92.1)
 K0189-3 anti-Cyclin B2 (X121.10)
 553 anti-Cyclin D1 (polyclonal)
 MD-17-3 anti-Cyclin D1 (5D4)
 MD-17-3H anti-Cyclin D1 (5D4)
 K0062-3 anti-Cyclin D1 (DCS-6)
 K0063-3 anti-Cyclin D2 (DCS-3)
 K0064-3 anti-Cyclin D2 (DCS-5)
 K0013-3 anti-Cyclin D3 (DCS-22)
 K0172-3 anti-Cyclin E (HE12)
 K0173-3 anti-Cyclin E (HE172)
 MT-19-3 anti-Cdc2Hs (5F6)
 K0069-3 anti-CDC6 (DCS-180)
 K0070-3 anti-CDC7 (DCS-342)
 CY-M1021 anti-Phospho-Cdc7 (Thr376)
 K0140-3 anti-Cdc20 (AR12)
 K0071-3 anti-CDC25A (DCS-120)
 K0072-3 anti-CDC25A (DCS-121)
 K0073-3 anti-CDC25A (DCS-124)
 CY-1352 CycLex[®] Cdc25A Protein Phosphatase
 Fluorometric Assay Kit
 CY-E1352 Recombinant Cdc25A (Catalytic Domain)
 CY-1353 CycLex[®] Cdc25B Protein Phosphatase
 Fluorometric Assay Kit
 CY-E1353 Recombinant Cdc25B (Catalytic Domain)
 K0075-3 anti-CDC25C (DCS-193)
 K0200-3 anti-Cdc25C (TC14)
 CY-M1018 anti-Phospho-Cdc25C Ser216 (TK-1F1)
 CY-1354 CycLex[®] Cdc25C Protein Phosphatase
 Fluorometric Assay Kit
 CY-E1354 Recombinant Cdc25C (Catalytic Domain)
 CY-1355 CycLex[®] Cdc25 Combo Protein Phosphatase
 Fluorometric Assay Kit
 K0141-3 anti-CDC27 (AF3.1)
 K0150-3 anti-CDCP1 (CUB1)
 K0150-4 FITC labeled anti-CDCP1 (CUB1)
 MK-13-3 anti-Cdk2 (8A12)
 K0065-3 anti-Cdk4 (DCS-156)
 K0066-3 anti-Cdk6 (DCS-83)
 K0067-3 anti-Cdk6 (DCS-130)
 K0068-3 anti-Cdk7 (DCS-MO1)
 K0077-3 anti-p16^{INK4a} (DCS-50)
 M124-3 anti-p15^{INK4b} (1F6)
 K0079-3 anti-p18^{INK4c} (DCS-118)
 K0080-3 anti-p19^{INK4d} (DCS-100)
 K0081-3 anti-p21^{WAF/CIP1} (DCS-60)
 K0082-3 anti-p27^{Kip2} (DCS-72)
 K0083-3 anti-p57^{Kip2} (DCS-230)
 K0084-3 anti-p14^{ARF} (DCS-240)
 K0085-3 anti-Cdh1 (DCS-266)
 K0086-3 anti-Chk1 (DCS-310)

K0087-3 anti-Chk2 (DCS-270)
 K0088-3 anti-Chk2 (DCS-273)
 K0094-3 anti-E2F-4 (TFE42)
 K0095-3 anti-DP-1 (TFD10)
 M043-3 anti-DJ-1 (3E8)
 M069-3 anti-Mcm2 (4B8)
 M038-3 anti-Mcm3 (3A2)
 M049-3 anti-Mcm7 (4B4)
 M050-3 anti-RCC1 (3D11)
 K0181-3 anti-p53 (DO-1)
 K0059-3 anti-p53 (FPS315)
 K0060-3 anti-p53 (FPS392)
 K0181-3 anti-p53 (DO-1)
 D240-3 anti-phospho-p53 (Ser46)(#36)
 D242-3 anti-phospho-p52 (Ser315)(#18)
 K0059-3 anti-phospho-p53 Ser315 (FPS315)
 K0060-3 anti-phospho-p53 Ser392 (FPS392)
 CY-M1022 anti-phospho-p53 Ser46 (TK-4D4)
 CY-M1029 anti-Acetylated Histone/p53-Lys382 (TM-5C5)
 CY-7049 CycLex[®] Total p53 ELISA Kit
 CY-7050 CycLex[®] Phospho-p53 Ser46 ELISA Kit
 CY-7051 CycLex[®] Phospho-p53 Ser392 ELISA Kit
 MK-15-1 anti-RB (3H9)
 M045-3 anti-phospho RB (2C4)
 555 anti-Phospho RB (Ser 780) (Polyclonal)
 K0091-3 anti-RB2 (DCS-211)
 D081-1 anti-DNA Topoisomerase II α (8D2)
 M025-3 anti-Phospho DNA Topoisomerase II α (3D4)
 M052-3 anti-DNA Topoisomerase II $\alpha\beta$ (AK5)
 M055-3 anti-ORC2 (3B7)
 M057-3 anti-GAK (1C2)
 M019-3 anti-Nucleolin (4E2)
 PM006-3 anti-Phospho Histone H3 (Polyclonal)
 PM026 anti-ATM (polyclonal)