

MONOCLONAL ANTIBODY

Anti-monomeric Kusabira-Orange 2 mAb

Code No.	Clone	Subclass	Quantity	Concentration
M168-3M	3B3	Mouse IgG1 κ	100 μ L	1 mg/mL

BACKGROUND: *CoralHue*[™] Kusabira-Orange (KO) has been cloned from the stony coral, whose Japanese name is “Kusabira-ishi”. Wild-type *CoralHue*[™] KO forms a brightly fluorescent dimer. *CoralHue*[™] KO has been carefully engineered to form a monomer, *CoralHue*[™] monomeric Kusabira Orange 1 (mKO1) that maintains the brilliance and pH stability of the parent protein. *CoralHue*[™] mKO2 is the mutant of mKO1 and has a feature of the rapid maturation. It absorbs light maximally at 551 nm and emits orange light at 565 nm. *CoralHue*[™] mKO2 can be used to label proteins or subcellular structures, or for reporter assay.

M168-3M is available for immunostaining of “Fucci-G₁ Orange” (Fucci; Fluorescent Ubiquitination-based Cell Cycle Indicator). Fucci-G₁ Orange encodes *CoralHue*[™] monomeric Kusabira-Orange2 (mKO2) fused to a part of human Cdt1 (hCdt1: Cdc10 dependent transcript 1). It is possible to use M168-3M for Fucci transgenic strain, B6.Cg-Tg(Fucci)596Bsi mice which express Fucci-G₁ Orange.

SOURCE: This antibody was purified from hybridoma (clone 3B3) supernatant using protein A agarose. This hybridoma was established by fusion of mouse myeloma cell P3U1 with C3H mouse lymphocyte immunized with recombinant monomeric Kusabira-Orange 2.

FORMULATION: 100 μ g IgG in 100 μ L 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 monomeric Kusabira-Orange 2 on Western blotting, Immunoprecipitation, Immunocytochemistry and Immunohistochemistry.

INTENDED USE:

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

REFERENCES:

- 1) Sakaue-Sawano, A., *et al.*, *Cell* **132**, 487-498 (2008)
- 2) Sakaue-Sawano, A., *et al.*, *Chem. Biol.* **15**, 1243-1248 (2008)

APPLICATIONS:

Western blotting; 1 μ g/mL for chemiluminescence detection system

Immunoprecipitation; 5 μ g/Sample

Immunohistochemistry; 5 μ g/mL (for frozen section)

Heat treatment is necessary.

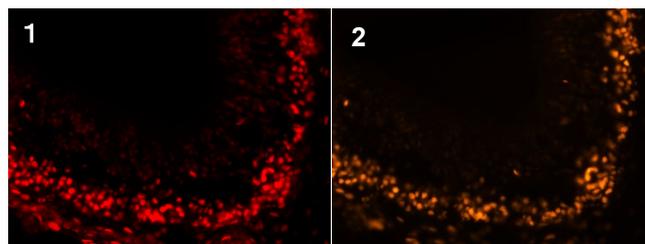
Microwave oven; 3 minutes at 500W in 1 mM EDTA (pH 8.0)

Immunocytochemistry; 5 μ g/mL

Flow cytometry; Not tested

Detailed procedure is provided in the following

PROTOCOLS.



Immunohistochemical detection of mKO2 on frozen section of B6.Cg-Tg(Fucci)596Bsi mouse embryonic brain (E12) with M168-3M (1) and Fucci-G₁ Orange own fluorescence (2).

Fluorescence Microscope: Axiovert200

Filter set:

1: Carl Zeiss Filter sets No.26 (for Alexa Fluor[®] 647)

2: FSET-KOHQ (for mKO2)

Lens: Plan-NEOFLUAR (Carl Zeiss), x20, NA=0.5

The descriptions of the following protocols are examples. Each user should determine the appropriate condition.

PROTOCOLS:

Immunohistochemical staining for frozen sections

For 4% paraform aldehyde fixed section

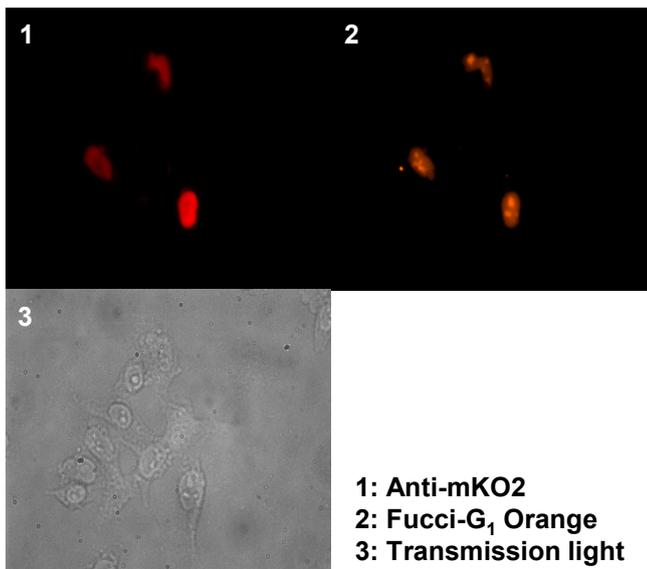
1) Wash the slides in PBS for 15 minutes.

2) Heat treatment

Heat treatment by Microwave:

Place the slides put on staining basket in 1 L beaker with 500 mL of 1 mM EDTA (pH 8.0). Cover the beaker with plastic wrap, then process the slides 3 minutes at 500 W with microwave oven. Let the slides cool down in the beaker at room temperature for about 40 minutes.

- 3) Immerse the slide in PBS containing 0.1% Tween 20 (PBS-T) for 30 minutes at room temperature.
- 4) Remove the slides from PBS-T, wipe gently around each section and cover tissues with blocking buffer (PBS containing 2% FCS, 0.1% Tween 20) for 5 minutes to block non-specific staining. Do not wash.
- 5) Tip off the blocking buffer, wipe gently around each section and cover tissues with primary antibody diluted with blocking buffer as suggested in the **APPLICATIONS**.
- 6) Incubate the sections for 1 hour at room temperature.
- 7) Wash the slides 2 times in PBS-T for 5 minutes each.
- 8) Wipe gently around each section and cover tissues with 1:500 Alexa Fluor® 647 F(ab')₂ Fragment of Goat Anti-Mouse IgG (Invitrogen; code no. A21237). Incubate for 30 minutes at room temperature. Wash as in step 7).
- 9) Wipe excess liquid off the slide but take care not to touch the section. Never leave the section to dry.
- 10) Promptly add mounting medium onto the slide, then put a cover slip on it.
- 5) Immerse the slide in PBS containing 0.1% Tween 20 for 30 minutes at room temperature.
- 6) Add the primary antibody diluted with PBS containing 0.1% Tween 20, 2% FCS as suggested in the **APPLICATIONS** onto the cells and incubate for 1 hour at room temperature. (Optimization of antibody concentration or incubation condition is recommended if necessary.)
- 7) Wash the glass slide 2 times with PBS containing 0.1% Tween 20 (PBS-T).
- 8) Add 200 µL of 1:500 Alexa Fluor® 647 F(ab')₂ Fragment of Goat Anti-Mouse IgG (Invitrogen; code no. A21237) diluted with PBS containing 0.1% Tween 20, 2% FCS onto the cells. Incubate for 30 minutes at room temperature. Keep out light by aluminum foil.
- 9) Wash the glass slide 2 times with PBS-T.
- 10) Wipe excess liquid off the slide but take care not to touch the cells. Never leave the cells to dry.
- 11) Promptly add mounting medium onto the slide, then put a cover slip on it.



Immunocytochemical detection of mKO2 in Fucci-G₁ Orange transfected HeLa with M168-3M.

Fluorescence Microscope: Axiovert200

Filter set

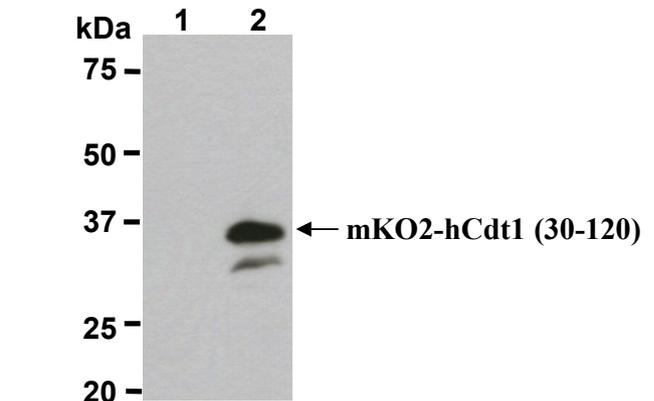
1: Carl Zeiss Filter sets No.26 (for Alexa Fluor® 647)

2: FSET-KOHQ (for mKO2)

Lens: LD ACHROPLAN (Carl Zeiss), x40, NA=0.6

Immunocytochemistry

- 1) Culture the cells in the appropriate condition on a glass slide. (for example, spread 1 x 10⁴ cells for one slide, then incubate in a CO₂ incubator for one night.)
- 2) Wash the glass slide 2 times with PBS.
- 3) Fix the cells by immersing the slide in PBS containing 4% paraformaldehyde for 10 minutes at room temperature.
- 4) Wash the glass slide 2 times with PBS.



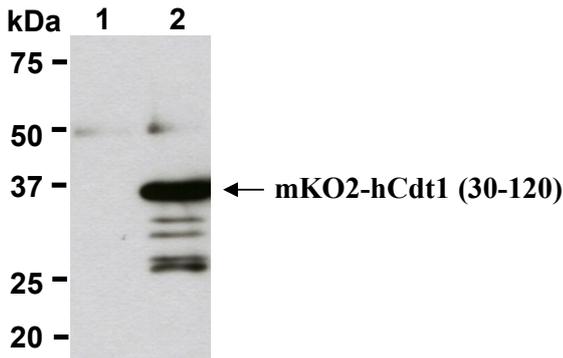
Western blot analysis in HeLa (1) and Fucci-HeLa (2) using M168-3M.

SDS-PAGE & Western Blotting

- 1) Wash cells (approximately 1 x 10⁷ cells) 3 times with PBS and resuspend them in 1 mL of Laemmli's sample buffer.
- 2) Boil the samples for 3 minutes and centrifuge. Load 10 µL of sample per lane on a 1-mm-thick SDS-polyacrylamide gel and carry out electrophoresis.
- 3) 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 manufacturer's manual for precise transfer procedure.
- 4) 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.
- 5) Incubate the membrane for 1 hour at room temperature with primary antibody diluted with 1% skimmed milk (in PBS, pH 7.2) as suggested in the **APPLICATIONS**. (The concentration of antibody will depend on the conditions.)
- 6) Wash the membrane with PBS-T [0.05% Tween-20 in PBS] (5 minutes x 3 times).
- 7) Incubate the membrane with 1:10,000 Anti-IgG (Mouse)

pAb-HRP (MBL; code no. 330) diluted with 1% skimmed milk (in PBS, pH 7.2) for 1 hour at room temperature.

- 8) Wash the membrane with PBS-T (5 minutes x 3 times).
- 9) Wipe excess buffer off the membrane, and incubate membrane with an appropriate chemiluminescence reagent for 1 minute.
- 10) Remove extra reagent from the membrane by dabbing with a paper towel, and seal it in plastic wrap.
- 11) Expose the membrane onto an X-ray film in a dark room for 3 minutes. Develop the film under usual settings. The conditions for exposure and development may vary.



Immunoprecipitation of mKO2 from Fucci-G₁ Orange transfected 293T with mouse IgG1 isotype control (1) or M168-3M (2). After immunoprecipitated with the antibody, immunocomplex was resolved on SDS-PAGE and immunoblotted with anti-mKO2 (MBL; code no. PM051M).

Immunoprecipitation

- 1) Wash cells (approximately 1×10^7 cells) 3 times with PBS and resuspend them in 1 mL of cold Lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% NP-40) containing protease inhibitors at appropriate concentrations. Incubate it at 4°C with rotating for 30 minutes; thereafter, briefly sonicate the mixture (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 fresh tube.
- 3) Add primary antibody as suggested in the **APPLICATIONS** into 200 μ L of the supernatant. Mix well and incubate with gentle agitation for 60-120 minutes at 4°C.
- 4) Add 20 μ L of 50% protein A agarose beads resuspended in the cold Lysis buffer. Mix well and incubate with gentle agitation for 1 hour 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 20 μ L of sample per lane on a 1-mm-thick SDS-polyacrylamide gel and carry out electrophoresis.
- 8) Blot the protein to a polyvinylidene difluoride (PVDF)

CoralHue™ mKO is a product of co-development with Dr. Atsushi Miyawaki at the Laboratory for Cell Function and Dynamics, the Brain Science Institute, and the Institute of Physical and Chemical Research (RIKEN).

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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 manufacturer'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 for 1 hour at room temperature with 1:1,000 Anti-monomeric Kusabira-Orange 2 pAb (MBL; code no. PM051M) as primary antibody diluted with 1% skimmed milk (in PBS, pH 7.2). (The concentration of antibody will depend on the conditions.)
- 11) Wash the membrane with PBS-T [0.05% Tween-20 in PBS] (5 minutes x 3 times).
- 12) Incubate the membrane with 1:10,000 Anti-IgG (Rabbit) pAb-HRP (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 3 times).
- 14) Wipe excess buffer off the membrane, and incubate membrane with an appropriate chemiluminescence reagent for 1 minute.
- 15) Remove extra reagent from the membrane by dabbing with a paper towel, and seal it in plastic wrap.
- 16) Expose the membrane onto an X-ray film in a dark room for 3 minutes. Develop the film under usual settings. The conditions for exposure and development may vary.

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