

POLYCLONAL ANTIBODY

Anti-monomeric Kusabira-Orange 2 pAb

Code No.	Quantity	Form
PM051M	100 μ L	Affinity Purified

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.

PM051M 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 PM051M for Fucci transgenic strain, B6.Cg-Tg(Fucci)596Bsi mice which express Fucci-G₁ Orange.

SOURCE: This antibody was purified from rabbit serum using affinity column. The rabbit was immunized with recombinant monomeric Kusabira-Orange 2.

FORMULATION: 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.

APPLICATIONS:

Western blotting: 1:1,000

Immunoprecipitation: 2 μ L/Sample

Immunohistochemistry: 1:500 (paraffin and frozen section)

Heat treatment is necessary.

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

Frozen section: 3 minutes

Paraffin section: 10 minutes

Immunocytochemistry: 1:500

Flow cytometry: Not tested

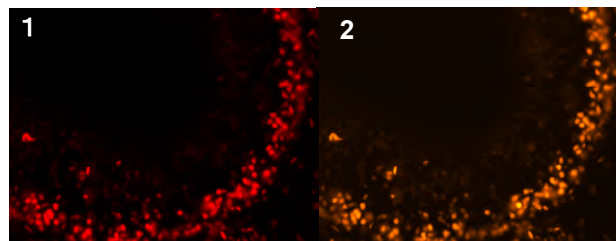
Detailed procedure is provided in the following **PROTOCOLS**.

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)



Immunohistochemical detection of mKO2 on frozen section of B6.Cg-Tg(Fucci)596Bsi mouse embryonic brain (E12) with PM051M (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.

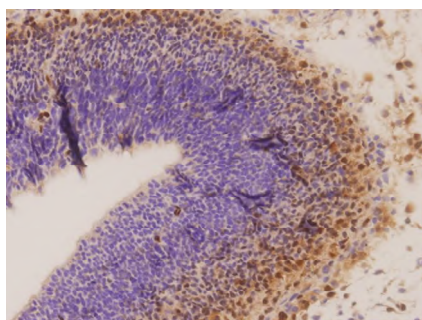
PROTOCOLS:

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**. Incubate the sections for 1 hour at room temperature.
- 6) Wash the slides 2 times in PBS-T for 5 minutes each.
- 7) Wipe gently around each section and cover tissues with 1:500 Alexa Fluor® 647 conjugated anti-rabbit IgG (Invitrogen; code no. A21245). Incubate for 30 minutes at room temperature. Wash as in step 6).
- 8) Wipe excess liquid off the slide but take care not to touch the section. Never leave the section to dry.
- 9) Promptly add mounting medium onto the slide, then put a cover slip on it.

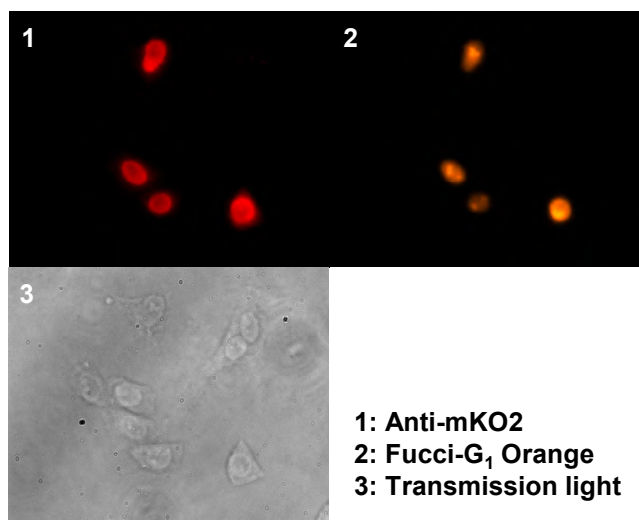


Immunohistochemical detection of mKO2 in paraffin embedded section of B6.Cg-Tg(Fucci)596Bsi mouse embryonic brain (E12) with PM051M.

Immunohistochemical staining for paraffin-embedded sections

- 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 3 times in PBS 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 1 mM EDTA (pH 8.0). Cover the beaker with plastic wrap, then process the slides for 10 minutes 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 1 mM EDTA (pH 8.0) 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 blocking buffer (20 mM HEPES, 1% BSA, 135 mM NaCl) 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 blocking buffer as suggested 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 Histostar™ (Rb) for Mouse tissue (MBL; code no. 8470). Incubate for 1 hour at room temperature. Wash as in step 9).
- 11) Visualize by reacting for 4 minutes with Histostar™ DAB Substrate Solution (MBL; code no. 8469) *DAB is a suspect carcinogen and must be handled with care. Always wear gloves.
- 12) Wash the slides in water for 5 minutes.
- 13) 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.
- 14) Dehydrate by immersing in Ethanol 3 times for 3 minutes each, followed by immersing in Xylene 3 times for 3 minutes each. Now ready for mounting.



**1: Anti-mKO2
 2: Fucci-G₁ Orange
 3: Transmission light**

Immunocytochemical detection of mKO2 in Fucci-G₁ Orange transfected HeLa with PM051M.

Fluorescence Microscope: Axiovert200

Filter set:

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

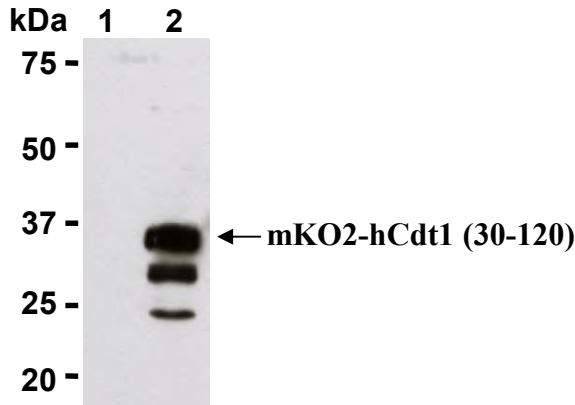
2: FSET-KOHQ (for mKO2)

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.
- 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 μ L of 1:500 Alexa Fluor[®] 647 conjugated anti-rabbit IgG (Invitrogen; code no. A21245) 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.



Western blot analysis in HeLa (1) and Fucci-HeLa (2) using PM051M.

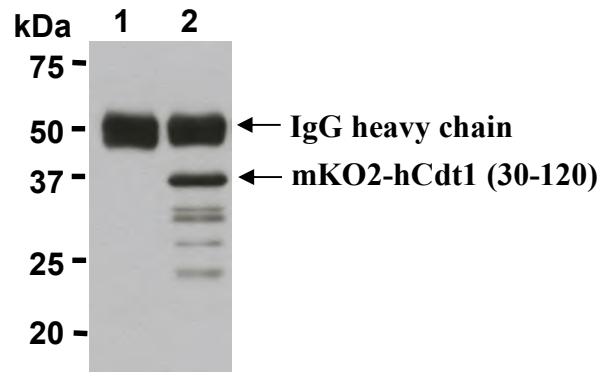
SDS-PAGE & Western Blotting

- 1) Wash cells (approximately 1×10^7 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 μ L 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% Methanol). 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 (Rabbit) pAb-HRP (MBL; code no. 458) 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.

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).

Use of **CoralHue[™] mKO** requires a license from MBL Co., Ltd. MBL grants non-profit research organizations the right to use the product for non-commercial research purposes. For commercial entities a commercial license is required. Patent Nos. JP4709653 and EP1700913. For more information, please contact to support@mbi.co.jp.

- 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 normal rabbit IgG (1) or PM051M (2). After immunoprecipitated with the antibody, immunocomplex was resolved on SDS-PAGE and immunoblotted with 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 60 minutes at 4°C.
- 5) Centrifuge the tube at 2,500 x g for 10 seconds and discard the supernatant.
- 6) Resuspend the agarose with cold Lysis buffer.
- 7) Centrifuge the tube at 2,500 x g for 10 seconds and discard the supernatant.
- 8) Repeat steps 6)-7) 2-4 times.
- 9) Resuspend the beads in 20 μ L of Laemmli's sample buffer, boil for 3-5 minutes, and centrifuge for 5 minutes. Use 20 μ L/lane for the SDS-PAGE analysis. (See **SDS-PAGE & Western blotting**.)

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