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Western blot analysis V.2

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MATERIALS

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⊗ Purified Mouse Anti-Human HIF-1α Clone 54/HIF-1α **BD Biosciences Catalog #610959**

⊗ HIF-2 alpha/EPAS1 Antibody **Novus Biologicals Catalog #NB100-122**

⊗ HIF-1β/ARNT (D28F3) XP® Rabbit mAb **Cell Signaling Technology Catalog #5537**

⊗ Anti α-Tubulin Monoclonal Antibody **Fujifilm Wako Pure Chemical Catalog #017-25031**

⊗ Anti-Mouse IgG HRP-Linked Whole Ab Sheep **Ge Healthcare Catalog #NA931**

⊗ Anti-Rabbit IgG HRP-Linked Whole Ab Donkey **Ge Healthcare Catalog #NA934**

⊗ RIPA buffer **Nacalai Tesque Catalog #16488-34**

⊗ cOmplete™ Protease Inhibitor Cocktail **Roche Catalog #4693116001**

⊗ Blocking One **Nacalai Tesque Catalog #03953-95**

⊗ Chemi-Lumi One Super **Nacalai Tesque Catalog #02230-14**

⊗ DC™ Protein Assay Kit **BIO-RAD Catalog #500-0112**

Antibody dilutions

Primary antibodies

- Anti-HIF-1α 1:1000
- Anti-HIF-2α 1:1000
- Anti-HIF-1β 1:1000
- Anti-α-tubulin 1:2000

Secondary antibodies

- Anti-mouse-IgG 1:10,000
- Anti-rabbit-IgG 1:10,000

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Cell lysis

- 1 Wash the cells with ice-cold PBS and add ice-cold RIPA buffer with cOmplete™ Protease Inhibitor Cocktail.
- 2 Scrape cells and transfer the suspension to a 1.5 ml microcentrifuge tube.
- 3 Incubate the lysate on ice for 15 minutes.
- 4 Centrifuge at 10,000 x *g* for 5 minutes at 4°C and collect the supernatant in a new microcentrifuge tube.
- 5 Determine protein concentration by using DC™ Protein Assay Kit.

Separation of proteins by gel electrophoresis

- 6 Load 35 µg of total protein on a SDS-PAGE gel.

- 7 Run the gel for 1 h at 100 V.

Semi-dry membrane transfer

- 8 Transfer proteins onto a PVDF membrane using Trans-Blot Turbo™ Transfer System.

Western blotting

- 9 Place the blot in Blocking One and incubate with agitation for 20 minutes.
- 10 Incubate the blot with appropriate dilutions of primary antibody at 4°C overnight.
- 11 Place the blot in TBST and wash three times, 5 minutes each.
- 12 Incubate the blot with the species-matched horseradish peroxidase-conjugated secondary antibody for 1 hour.
- 13 Place the blot in TBST and wash three times, 5 minutes each.

Chemiluminescent Detection

14 Add Chemi-Lumi One Super to cover the blot and incubate for 1 minute.

15 Detect chemiluminescence using LAS-4000mini image analyzer.