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Hydroxylated-HIF-1alpha (Pro402) and Total HIF-1alpha ELISA Kit

Catalog #: PTE-HIF1A-P402-T

User Manual
Last Revised: May 17, 2024

Introduction

The RayBio® Hydroxylated-HIF-1alpha (Pro402) and Total HIF-1alpha ELISA Kit is an in vitro enzyme-linked immunosorbent assay for the measurement of human hydroxylated-HIF-1alpha and HIF-1alpha. An anti-pan HIF-1alpha antibody has been coated onto a 96-well plate. Samples are pipetted into the wells and HIF-1alpha present in a sample is bound to the wells by the immobilized antibody. The wells are washed, and rabbit anti-Hydroxylated-HIF-1alpha (Pro402) antibody is used to detect Hydroxylated HIF-1alpha or biotinylated anti-HIF-1alpha antibody is used to detect HIF-1alpha. After washing away unbound antibody, HRP-conjugated anti-rabbit IgG or HRP-conjugated streptavidin is pipetted to the wells. The wells are again washed, a TMB substrate solution is added to the wells and color develops in proportion to the amount of Hydroxylated HIF-1alpha bound or HIF-1alpha bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

Storage / Stability

The entire kit may be stored at -20°C for up to 6 months from the date of shipment. Avoid repeated freeze-thaw cycles. For prepared reagent storage, see kit contents on the next page.

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Kit Components

Name	Catalog #	Size / Qty	Description	Storage / Stability After Preparation
Anti-pan-HIF-1alpha Microplate	PTE-HIF1A-P402-A	96 wells	Microplate coated with anti-pan-HIF-1alpha antibody.	1 month at -20°C*
Positive Control	HMGS001-1	1 vial	HeLa cell lysate	1 week at -80°C
Hydroxylated-HIF-1alpha (Pro402) Detection Antibody	PTE-HIF1A-P402-C1	1 vial	Rabbit anti-Hydroxylated-HIF-1alpha (Pro402) antibody. 1 vial is enough to assay half the microplate.	5 days at 4°C
HIF-1alpha Detection Antibody	PTE-HIF1A-P402-T-C2	1 vial	Biotinylated anti-HIF-1alpha antibody. 1 vial is enough to assay half the microplate.	5 days at 4°C
HRP-conjugated Anti-rabbit IgG	PEL-ITEMD1	1 vial	1 vial (25 µl) 1000x concentrated HRP-conjugated anti-rabbit IgG	Do not store and reuse.
HRP-conjugated Streptavidin	PEL-ITEMG	1 vial	1 vial (200 µl) 300x concentrated HRP-conjugated Streptavidin.	Do not store and reuse.
Wash Buffer	EL-ITEMB	25 ml	20X concentrated wash buffer	1 month at 4°C
Assay Diluent B	EL-ITEME	15 ml	5X concentrated assay diluent	1 month at 4°C
Lysis Buffer	EL-Lysis	5 ml	2X cell lysate buffer	1 month at 4°C
TMB One-Step Substrate Reagent	EL-TMB	12 ml	3,3,5,5'-tetramethylbenzidine (TMB) in buffer solution	N/A
Stop Solution	EL-STOP	8 ml	0.2 M sulfuric acid	N/A

*Return unused wells to the pouch containing desiccant pack, reseal along entire edge.

Additional Materials Required

- Microplate reader capable of measuring absorbance at 450 nm
- Protease and Phosphatase inhibitors.
- Precision pipettes to deliver 2 µl to 1 ml volumes

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- Adjustable 1-25 ml pipettes for reagent preparation
- 100 ml and 1 liter graduated cylinders
- Log-log graph paper or computer and software for ELISA data analysis.
- Absorbent paper
- Tubes to prepare positive control or sample dilutions
- Distilled or deionized water
- Shaker

Sample Preparation

For the initial experiment, we recommend a serial dilution, such as a 5-fold to 50-fold dilution, for your cell lysates with prepared Assay Diluent (see Reagent Preparation step 2) before use.

Note: The fold dilution of sample used depends on the abundance of phosphorylated proteins and should be determined empirically. More of the sample can be used if signals are too weak. If signals are too strong, the sample can be diluted further.

Reagent Preparation

1. Bring all reagents and samples to room temperature (18 - 25ºC) before use.
2. Assay Diluent B should be diluted 5-fold with deionized or distilled water before use.
3. Lysis Buffer should be diluted 2-fold with deionized or distilled water (for cell lysate and tissue lysate). We also recommend the addition of protease and phosphatase inhibitors (not included) to the lysis buffer prior to use.
4. Preparation of Positive Control: Briefly spin the Positive Control Vial. Add 400 µl of prepared 1X Assay Diluent to prepare Positive Control solution. Gently mix the powder to allow it to dissolve thoroughly.
5. If the Wash Buffer concentrate contains visible crystals, warm to room temperature and mix gently until dissolved. Dilute 20 ml of Wash Buffer concentrate into deionized or distilled water to yield 400 ml of 1X Wash Buffer.
6. Preparation of Detection Antibody: Briefly spin the detection antibody vial. Add 100 µl of 1X Assay Diluent into the vial to prepare a detection antibody concentrate. Pipette up and down to mix gently (the concentrate can be stored at 4°C for 5 days or at -80°C for one month). The concentrate should then be diluted 55- fold with 1X Assay Diluent and used in step 5 of the Assay Procedure.
7. Preparation of HRP-conjugated Anti-rabbit IgG or HRP-conjugated Streptavidin: Briefly spin the vial of HRP-conjugated Anti-rabbit IgG concentrate or HRP-conjugated Streptavidin before use. HRP-conjugated Anti-rabbit IgG should be diluted 1000-fold

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with 1X Assay Diluent. HRP-conjugated Streptavidin concentrate should be diluted 300-fold with 1x Assay Diluent and used in step 7 of the Assay Procedure.

Assay Procedure

1. Bring all reagents and samples to room temperature (18 - 25ºC) before use. It is recommended to run all positive control and samples in at least duplicate.
2. Label removable 8-well strips as appropriate for your experiment.
3. Add 100 µl sample and Positive Control (see Reagent Preparation step 4) into appropriate wells. Cover the wells and incubate for 2.5 hours at room temperature or overnight at 4ºC with gentle shaking.
4. Discard the solution and wash 4 times with 1X Wash Solution. Wash by filling each well with Wash Buffer (300 µl) using a multi-channel pipette or auto-washer. Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
5. Add 100 µl of prepared 1X detection antibody, anti-Hydroxylated-HIF-1alpha (Pro402) or anti-HIF-1alpha (see Reagent Preparation step 6) to each well. Incubate for 1 hour at room temperature with gentle shaking.
6. Discard the solution. Repeat the wash as in step 4.
7. Add 100 µl of prepared HRP-conjugated Anti-rabbit IgG solution or HRP-conjugated Streptavidin (see Reagent Preparation step 7) to each well. Incubate for 1 hour at room temperature with gentle shaking.
8. Discard the solution. Repeat the wash as in step 4.
9. Add 100 µl of TMB One-Step Substrate Reagent to each well. Incubate for 30 minutes at room temperature in the dark with gentle shaking.
10. Add 50 µl of Stop Solution to each well. Read at 450 nm immediately.

Assay Procedure Summary

1. Prepare all reagents, samples, and Positive Control as instructed.
2. Add 100 µl sample and Positive Control to each well. Incubate 2.5 hours at room temperature or overnight at 4ºC with gentle shaking.

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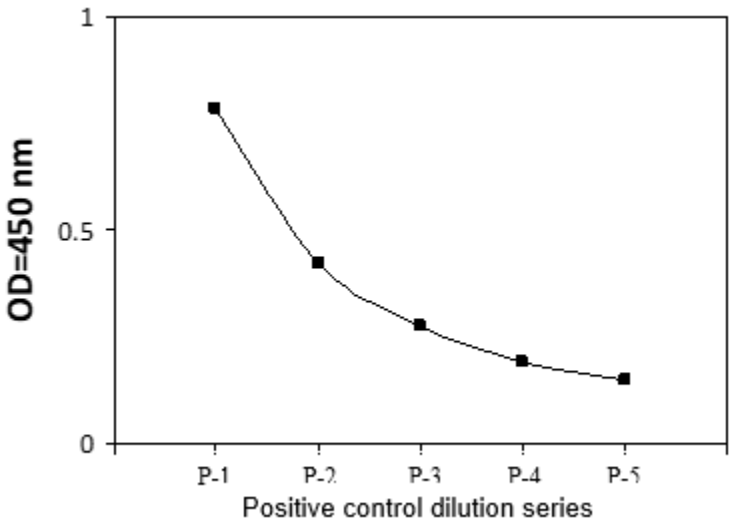
3. Add 100 µl prepared detection antibody to each well. Incubate for 1 hour at room temperature with gentle shaking.
4. Add 100 µl prepared HRP-conjugated Anti-rabbit IgG or HRP-conjugated Streptavidin solution. Incubate for 1 hour at room temperature with gentle shaking.
5. Add 100 µl TMB One-Step Substrate Reagent to each well. Incubate 30 minutes at room temperature.
6. Add 50 µl Stop Solution to each well. Read at 450 nm immediately.

Typical Data

Calculate the mean absorbance for each set of duplicate positive controls, and samples, and then subtract the average zero (blank) optical density.

A. Positive Control

HeLa cells were treated with MG132. Solubilize cells at 4 x 10^7 cells/ml in Cell Lysate Buffer. Serial dilutions of lysates were analyzed in this ELISA.



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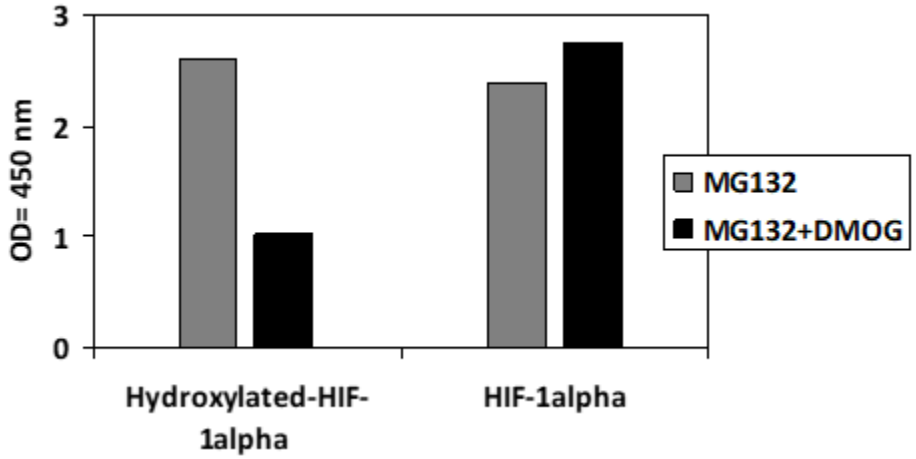
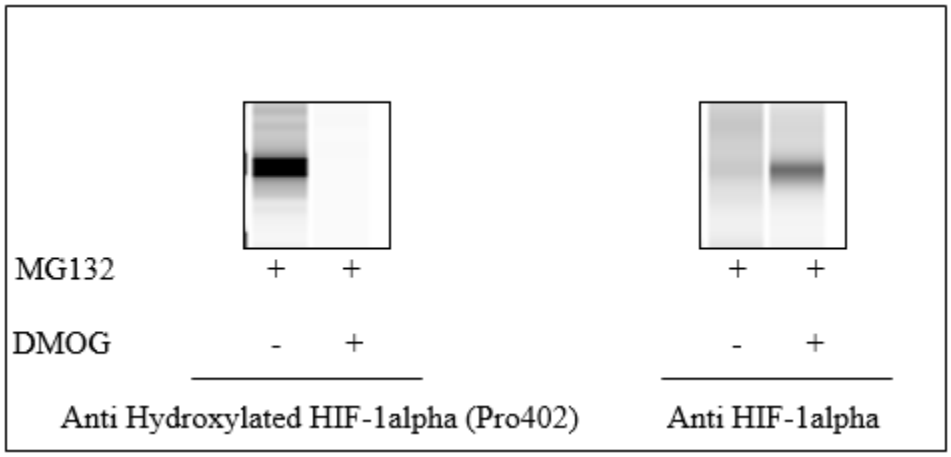
B. MG132 and DMOG Inhibition of HeLa Cell Line

HeLa cells were treated or untreated with MG132 and DMOG. Cell lysates were analyzed using ELISA and Western Blot.

I. ELISA



II. Western-Blot Analysis



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Troubleshooting Guide

Problem	*Cause*	*Solution*
Low signal in samples	• Sample concentration is too low • Improper preparation of detection antibody • Too brief incubation times • Inadequate reagent volumes or improper dilution	• Increase sample concentration Briefly spin down vials before opening. Dissolve the powder thoroughly. • Ensure sufficient incubation time; assay procedure step 3 may be done overnight Check pipettes and ensure correct preparation
High signal in samples	• Sample concentration is too high	• Reduce sample concentration
Large CV	• Inaccurate pipetting Air bubbles in wells	• Check pipettes • Remove bubbles in wells
High background	• Plate is insufficiently washed • Contaminated wash buffer	• Review the manual for proper wash. If using a plate washer, ensure that all ports are unobstructed. • Make fresh wash buffer
Low sensitivity	• Improper storage of the ELISA kit • Stop solution • Improper primary or secondary antibody dilution	• Store your positive control at <- 70ºC after reconstitution, others at 4ºC. Keep substrate solution protected from light. • Add stop solution to each well before reading plate • Ensure correct dilution