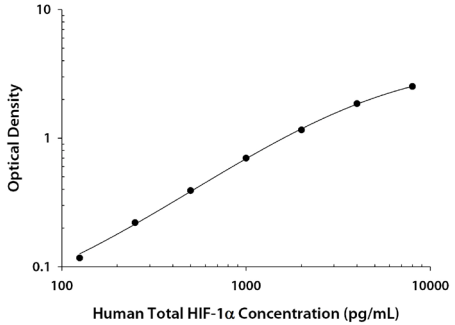
CALCULATION OF RESULTS

Average the duplicate readings for each standard and sample, then subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the human/mouse HIF-1α concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

TYPICAL DATA

A standard curve should be generated for each set of samples assayed. The graph below represents typical data generated when using the Human/Mouse Total HIF-1α/HIF1A DuoSet IC ELISA. The standard curve was calculated using a computer generated 4-PL curve-fit. This standard curve is for demonstration purposes only.



CALIBRATION

The Human/Mouse Total HIF-1α/HIF1A DuoSet IC ELISA is calibrated against a highly purified *E. coli*-expressed recombinant human HIF-1α produced at R&D Systems®. Samples containing natural HIF-1α showed linear dilution parallel to the standard curve obtained using the Human/Mouse Total HIF-1α Standard. These results indicate that O.D. values from this DuoSet IC ELISA can be used to determine the concentration of human/mouse HIF-1α in natural samples.

SPECIFICITY

The Human/Mouse Total HIF-1α/HIF1A DuoSet IC ELISA specifically recognizes HIF-1α. Specificity was demonstrated by Western Blot analysis of the protein bound by the capture antibody supplied in the kit.

To further determine specificity, recombinant human HIF-2α (amino acid residues 543-870) was assayed at 50 ng/mL and did not cross-react or interfere in the assay.

TECHNICAL HINTS & LIMITATIONS

- This DuoSet IC ELISA should not be used beyond the expiration date on the kit label.
- Individual results may vary due to differences in technique, plasticware, and water sources.
- It is important that the diluents selected for reconstitution and for dilution of the samples and standard reflect the environment of the samples being measured. The diluents suggested in this protocol should be suitable for most cell lysates.
- The type of enzyme and substrate and the concentrations of capture/detection antibodies used can be varied to create an immunoassay with a different sensitivity and dynamic range. A basic understanding of immunoassay development is required for the successful use of these reagents in immunoassays.
- A thorough and consistent wash technique is essential for proper assay performance. Wash Buffer should be dispensed forcefully and removed completely from the wells by aspiration or decanting. Remove any remaining Wash Buffer by inverting the plate and blotting it against clean paper towels.
- Use a fresh reagent reservoir and pipette tips for each step.
- It is recommended that all standards and samples be assayed in duplicate.
- Avoid microbial contamination of reagents and buffers. This may interfere with the sensitivity of the assay. Buffers containing protein should be made under aseptic conditions and stored at 2-8 °C or be prepared fresh daily.

PRECAUTIONS

The Stop Solution recommended for use with this kit is an acid solution.

Some components in this kit contain a preservative which may cause an allergic skin reaction. Avoid breathing mist.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the SDS on our website prior to use.

DuoSet™ IC ELISA
INTRACELLULAR

Human/Mouse Total HIF-1α/HIF1A

Catalog Number: DYC1935-2 (2 plates)
DYC1935-5 (5 plates)
DYC1935E (15 plates)

INTENDED USE

For the development of sandwich ELISAs to measure human and mouse Hypoxia-Inducible Transcription Factor 1 Alpha (HIF-1α) in cell lysates.

PRINCIPLE OF THE ASSAY

This DuoSet IC ELISA contains the basic components required for the development of sandwich ELISAs to measure human and mouse HIF-1α in cell lysates. An immobilized capture antibody specifically binds human/mouse HIF-1α . After washing away unbound material, a biotinylated detection antibody specific for human/mouse HIF-1α is used to detect the protein, utilizing a standard Streptavidin-HRP format.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

DESCRIPTION	PART #	CATALOG # DYC1935-2	CATALOG # DYC1935-5	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human/Mouse Total HIF-1α Capture Antibody	841689	1 vial	2 vials	Store for up to 1 month at 2-8 °C or aliquot and store at ≤ -20 °C for up to 3 months in a manual defrost freezer.*
Human/Mouse Total HIF-1α Detection Antibody	841690	1 vial	2 vials	
Streptavidin-HRP A	890803	1 vial	1 vial	Store for up to 3 months at 2-8 °C.* **DO NOT FREEZE.**
Human/Mouse Total HIF-1α Standard	841691	3 vials	5 vials	**Use within one hour of reconstitution. Use a fresh standard for each assay.**

* Provided this is within the expiration date of the kit.

DYC1935-2 contains sufficient materials to run ELISAs on at least two 96 well plates.†
DYC1935-5 contains sufficient materials to run ELISAs on at least five 96 well plates.†

This kit is also available in an Economy Pack (R&D Systems®, Catalog # DYC1935E). Economy Packs contain sufficient materials to run ELISAs on 15 microplates.† Specific vial counts of each component may vary. Refer to the literature accompanying your order for specific vial counts.

† Provided the following conditions are met:

- The reagents are prepared as described in this package insert.
- The assay is run as described in the General ELISA Protocol.
- The recommended microplates, buffers, diluents, substrates, and solutions are used.

OTHER MATERIALS REQUIRED

- Aprotinin (Tocris™ # 4139)
- Leupeptin (Tocris # 1167)
- Pepstatin (Tocris # 1190)
- Glycerol
- Magnesium Chloride ($MgCl_2$)
- β-glycerophosphate (Sigma # 50020)
- Sodium Fluoride (NaF) (Sigma # 201154)
- Triton™ X-100 (Sigma # T9284)
- Pipettes and pipette tips
- Deionized or distilled water
- 96 well microplates (R&D Systems, Catalog # DY990)
- Plate sealers (R&D Systems, Catalog # DY992)
- Squirt bottle, manifold dispenser, or automated microplate washer

SOLUTIONS REQUIRED

PBS - 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2-7.4, 0.2 µm filtered (R&D Systems, Catalog # DY006)

Wash Buffer - 0.05% Tween® 20 in PBS, pH 7.2-7.4 (R&D Systems, Catalog # WA126)

Reagent Diluent - 5% BSA* in Wash Buffer

Lysis Buffer #11 - 50 mM Tris (pH 7.4), 300 mM NaCl, 10% (w/v) Glycerol, 3 mM EDTA, 1 mM $MgCl_2$, 20 mM β-glycerophosphate, 25 mM NaF, 1% Triton X-100, 25 µg/mL Leupeptin, 25 µg/mL Pepstatin, and 3 µg/mL Aprotinin

Substrate Solution: ELISA TMB Substrate (R&D Systems, Catalog # DY999B)

Stop Solution - 2N H_2SO_4 (R&D Systems, Catalog # DY994)

*The use of Millipore Bovine Serum Albumin, Fraction V, Protease free (Catalog # 82-045) is recommended. All buffers containing BSA must be stored at 2-8 °C.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Human/Mouse Total HIF-1α Capture Antibody (Part 841689) - Each vial contains 720 µg/mL of mouse anti-human HIF-1α antibody when reconstituted with 200 µL of PBS.

Human/Mouse Total HIF-1α Detection Antibody (Part 841690) Each vial contains 3.6 µg/mL of biotinylated goat anti-human HIF-1α antibody when reconstituted with 1.0 mL of Reagent Diluent. Immediately before use, dilute the detection antibody to a working concentration of 100 ng/mL in Reagent Diluent. Prepare only as much detection antibody as required to run each assay.

Human/Mouse Total HIF-1α Standard (Part 841691) - **Reconstitute with a recommended volume of 500 µL of Reagent Diluent to produce a stock solution. Refer to the vial label for the concentration of recombinant human HIF-1α.** A seven point standard curve using 2-fold serial dilutions and a high standard of 8000 pg/mL is recommended.

Streptavidin-HRP A (Part 890803) - 1 mL of Streptavidin conjugated to horseradish-peroxidase. Immediately before use, dilute the Streptavidin-HRP A to the working concentration specified on the vial using Reagent Diluent.

PREPARATION OF SAMPLES

Cell Lysates - Rinse cells two times with PBS, making sure to remove any remaining PBS after the second rinse. Solubilize cells at 1 x 10^7 cells/mL in Lysis Buffer #11 and allow samples to sit on ice for 15 minutes. Assay immediately or store at ≤ -70 °C. Before use, centrifuge samples at 2000 x g for 5 minutes, and transfer the supernatant to a clean test tube. Sample protein concentration may be quantified using a total protein assay. If needed, further dilutions should be made in Reagent Diluent.

GENERAL ELISA PROTOCOL

Plate Preparation

1. Dilute the capture antibody to a working concentration of 4 µg/mL in PBS, without carrier protein. Immediately coat a 96 well microplate with 100 µL per well of the diluted capture antibody. Seal the plate and incubate overnight at room temperature.
2. Aspirate each well and wash with Wash Buffer, repeating the process two times for a total of 3 washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining Wash Buffer by aspirating or by inverting the plate and blotting it against clean paper towels.
3. Block plates by adding 300 µL of Reagent Diluent to each well. Incubate at room temperature for 1-2 hours.
4. Repeat the aspiration/wash as in step 2. The plates are now ready for sample addition.

Assay Procedure

1. Add 100 µL of sample or standard in Reagent Diluent per well. Use Reagent Diluent as the zero standard. Cover with a plate sealer and incubate 2 hours at room temperature.
   Note: *A seven point standard curve using 2-fold serial dilutions and a high standard of 8000 pg/mL is recommended.*
2. Repeat the aspiration/wash as in step 2 of the Plate Preparation.
3. Add 100 µL of the diluted detection antibody to each well. Cover with a new plate sealer and incubate 2 hours at room temperature.
4. Repeat the aspiration/wash as in step 2 of the Plate Preparation.
5. Add 100 µL of the diluted Streptavidin-HRP A to each well. Incubate for 20 minutes at room temperature. Avoid placing the plate in direct light.
6. Repeat the aspiration/wash as in step 2 of the Plate Preparation.
7. Add 100 µL of Substrate Solution to each well. Incubate for 20 minutes at room temperature. Avoid placing the plate in direct light.
8. Add 50 µL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.
9. Determine the optical density of each well immediately, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.