HO-1 (human), ELISA kit

For the detection and quantitation of human Heme Oxygenase-1 in cell lysates and tissue extracts.

Catalog Number: ADI-EKS-800

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A. INTRODUCTION

ASSAY DESIGN

The HO-1 (human), ELISA kit provides a method to detect and quantitate Heme Oxygenase (HO-1) in samples from human origins. This assay allows for reproducible, accurate and precise determination of HO-1 from cell lysates and tissue extracts. The assay is specific for human HO-1 and does not cross react with human HO-2 or HO-3, the other known heme oxygenase isoforms. This kit is not recommended for measuring rat or mouse HO-1.

The HO-1 (human), ELISA kit is a quantitative sandwich immunoassay. A mouse monoclonal antibody specific for HO-1 is pre-coated on the wells of the provided HO-1 Immunoassay Plate. HO-1 is captured by the immobilized antibody and is detected with a HO-1 specific, rabbit polyclonal antibody. The polyclonal antibody is subsequently bound by a horseradish peroxidase conjugated anti-rabbit IgG secondary antibody. The assay is developed with tetramethylbenzidine substrate and a blue color develops in proportion to the amount of captured HO-1. The color development is stopped with acid stop solution which converts the endpoint color to yellow. The intensity of the color is measured in a microplate reader at 450 nm. HO-1 concentrations from the sample are quantitated by interpolating absorbance readings from a standard curve generated with the calibrated HO-1 protein standard provided.

SCIENTIFIC OVERVIEW

Heme Oxygenase-1 (HO-1) also known as Hsp32, is the inducible isoform of heme oxygenase that catalyzes the NADPH, O2 and cytochrome P450 reductase dependent oxidation of heme to carbon monoxide, ferrous iron and biliverdin which is rapidly reduced to bilirubin. These products of the HO reaction have important physiological effects: carbon monoxide is a potent vasodilator and has been implicated to be a physiological regulator of cGMP and vascular tone; biliverdin and its product bilirubin are potent antioxidants; "free" iron increases oxidative stress and regulates the expression of many mRNAs (e.g., DCT-1, ferritin and transferring receptor) by affecting the conformation of iron regulatory protein (IRP)-1 and its binding to iron regulatory elements (IREs) in the 5'- or 3'- UTRs of the mRNAs. To date, three identified heme oxygenase isoforms are part of the HO system that catalyze heme into biliverdin and carbon monoxide. These are inducible HO-1 or Hsp32, constitutive HO-2 that is abundant in the brain and testis, and HO-3 which is related to HO-2 but is the product of a different gene. The HO system is the rate-limiting step in heme degradation and HO activity decreases the levels of heme which is a well known potent catalyst of lipid peroxidation and oxygen radical formation[1,2,3]. The expression of HO-1 is highly responsive to all types of stimuli that cause oxidative stress and it is up regulated during exposure to oxidants, UV-A irradiation and a series of agents including cytokines, hormones, heme and heavy metals[1,4]. HO-1 is a vital component of neuronal defense mechanisms and oxidative stress has been postulated to be the underlying basis for neuronal cell death in neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease[5]. The expression of HO-1 is normally very low in the brain but increases markedly after heat shock, ischemia or glutathione depletion[2,6,7]. Spatial distribution of HO-1 expression in AD brain is essentially identical to that of the pathogenic conformational changes of tau protein that is the major component of the intraneuronal lesion of AD, neurofibrillary tangles[8].

HO-1 expression and tau expression may be regulated by oxidative stresses in a coordinated manner and play a pivitol role in the cytoprotection of neuronal cells[9]. Plasma and cerebrospinal fluid HO-1 protein and lymphocyte HO-1 mRNA levels are decreased in subjects with sporadic AD relative to normal elderly controls suggesting that measurement of HO-1 may serve as a useful biological marker in early sporadic AD[10].

Oxidative stress in the heart caused by ischemia and reperfusion has been shown to lead to cardiomyocyte death. An absence of HO-1 has detrimental consequences whereas overexpression of HO-1 plays a protective role in hypoperfusion and ischemia/reperfusion-induced myocardial injury[11,12]. Under normal conditions, HO-1 is present at low levels in all organs except the spleen, but its expression is rapidly accelerated in response to pathophysiological conditions such as renal ischemia/reperfusion and cellular transformation[13]. HO-1 overexpression exerts beneficial cytoprotective effects in a number of transplantation models, including antigen-independent ischemia/reperfusion injury, acute and chronic allograft rejection and xenotransplantation[14,15].

The mechanisms by which HO-1 confers its protective effects are currently poorly understood but this area of investigation is active and rapidly evolving. The measurement of HO-1 in various cell types, tissues and bodily fluids may provide new insights into the physiological roles of HO-1 and may lead to monitoring HO-1 levels as a biomarker for therapeutic interventions or as an environmental biomarker in toxicology studies.

ASSAY PROCEDURE SUMMARY

1. Bring to room temperature: **Anti-HO-1 Immunoassay Plate**, **20X Wash Buffer**, **Sample Diluent**, **Antibody Diluent**, **HRP Conjugate Diluent**, **TMB Substrate** and **Stop Solution 2**.
2. Prepare **Recombinant HO-1 Standard** and samples in **Sample Diluent**.
3. Add 100 µL prepared standards and samples in duplicate to wells of **Anti-HO-1 Immunoassay Plate**. Cover immunoassay plate.
4. Incubate plate at room temperature for 30 minutes.
5. Wash wells 6X with 1X Wash Buffer.
6. Add 100 µL diluted **Anti-Human HO-1** to each well. Cover immunoassay plate.
7. Incubate plate at room temperature for 1 hour.
8. Wash wells 6X with 1X Wash Buffer.
9. Add 100 µL diluted **HRP Conjugate** to each well. Cover immunoassay plate.
10. Incubate plate at room temperature for 30 minutes.
11. Wash wells 6X with 1X Wash Buffer.
12. Add 100 µL **TMB Substrate** to each well.
13. Incubate at room temperature for 15 minutes (preferably in the dark).
14. Add 100 µL **Stop Solution 2** to each well.
15. Measure absorbance at 450nm.
16. Plot the HO-1 standard curve and calculate HO-1 sample concentrations.

B. MATERIALS

PRECAUTIONS

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- The activity of the **HRP Conjugate** *(part# 80-1507)* is affected by nucleophiles such as azide, cyanide, and hydroxylamine.

- The **Stop Solution 2** *(part# 80-0377)* is a 1 Normal (1N) solution of hydrochloric acid. This solution is corrosive; please use caution when handling.

Please read the complete kit insert before performing this assay.

MATERIALS PROVIDED

The HO-1 (human), ELISA kit contains the following components in sufficient quantities for 96 wells. These reagents are sufficient to assay one standard curve and 40 samples in duplicate or two standard curves and 32 samples in duplicate.

PART #	COMPONENT	SIZE	DESCRIPTION
80-1501	Anti-HO-1 Immunoassay Plate	96 well plate	12 x 8 removable strips and plate frame. Pre-coated plate with mouse monoclonal antibody specific for HO-1
80-1502	5X Extraction Reagent	10 mL	Concentrated buffer for preparation of cell and tissue extracts
80-1503	Recombinant HO-1 Standard	25 µL	5 µg/mL stock solution of Recombinant HO-1 protein
80-1504	Sample Diluent	50 mL	Buffer to dilute standards and samples
80-1287	20X Wash Buffer	100 mL	Concentrated solution of buffer and surfactant
80-1505	Anti-Human HO-1	25 µL	Rabbit polyclonal antibody specific for HO-1
80-1759	Antibody Diluent	11 mL	Buffer for dilution of Anti-Human HO-1
80-1507	HRP Conjugate	25 µL	Horseradish peroxidase conjugated to anti-rabbit IgG
80-1508	HRP Conjugate Diluent	11 mL	Buffer for dilution of Anti-Rabbit IgG: HRP Conjugate
80-0350	TMB Substrate	10 mL	Stabilized tetramethylbenzidine substrate
80-0377	Stop Solution 2	10 mL	Acid stop solution to stop color reaction

STORAGE OF MATERIALS

All reagents are stable as supplied at 4°C, except the **Recombinant HO-1 Standard**, which should be stored at -20°C. For optimum storage, the **Recombinant HO-1 Standard** should be aliquotted into smaller portions and stored at -20°C.

Unused wells of the **Anti-HO-1 Immunoassay Plate** should be resealed in the foil pouch provided and stored at 4°C until the kits expiry date.

MATERIALS REQUIRED BUT NOT PROVIDED

- Deionized or distilled water
- Precision pipettors capable of accurately delivering 1 to 1000 µL
- Disposable pipette tips
- 5, 10, 25 mL pipettes for reagent preparation
- 1L graduated cylinder
- Squirt bottle, manifold dispenser, or automated microtiter plate washer
- Disposable polypropylene tubes
- Microtiter plate reader capable of measuring absorbance at 450 nm
- Adhesive plate sealers or plastic wrap

C. PERFORMING THE ASSAY

CRITICAL ASSAY PARAMETERS AND NOTES

- The HO-1 (human), ELISA kit contains a pre-coated microtiter plate (**Anti-HO-1 Immunoassay Plate**) with removable wells to allow assaying on two separate occasions.

- A **5X Extraction Reagent** has been included in this assay. Use of other lysis or extraction buffers may interfere with the performance of the assay.

- Run both standards and samples in duplicate.

- Include a standard curve each time the assay is performed.

- The following kit components should be brought room temperature prior to use: **Anti-HO-1 Immunoassay Plate**, **Sample Diluent**, **Wash Buffer**, **Antibody Diluent, HRP Conjugate Diluent, TMB Substrate, Stop Solution 2.**

- Absorbance is a function of the incubation time. Therefore, prior to starting the assay it is recommended that all reagents are ready to use and all required strip-wells secured in the microtiter frame. This will ensure equal elapsed time for each pipetting step, without interruption.

- For each step in the procedure, total dispensing time for addition of reagents to the assay plate should not exceed 15 minutes.

- Mix all reagents and samples gently, yet thoroughly, prior to use. Avoid foaming of reagents.

- To avoid cross contamination, change disposable pipette tips between the addition of each standard, samples, and reagents. Use separate reagent troughs/reservoirs for each reagent.

PERFORMING THE ASSAY

- This assay requires pipetting of small volumes. To minimize imprecision caused by pipetting, ensure that pipettors are calibrated.

- Consistent, thorough washing of each well is critical. If using an automatic washer, check washing head before use. If washing manually, ensure all wells are completely filled at each wash. Air bubbles should be avoided.

- Exercise appropriate laboratory safety precautions when performing this assay.

- In this protocol, room temperature refers to 20-28°C. The room temperature should remain within this range throughout the assay.

NOTE: *The components in each kit lot # have been quality assured and warranted in this specific combination only; please do not mix them with components from other kit lot #s.*

SAMPLE PREPARATION

1. EXTRACTION OF SAMPLES

Suggested protocols for the preparation of cell lysates and tissue extracts samples may be found in Appendices I-II, respectively (pages 23 to 24). Investigators may use alternative methods of cell and tissue lysate preparation, however, it is recommended that the **5X Extraction Reagent** provided in this kit be diluted to 1X and used as the lysis buffer.
Use of alternative lysis buffers may contain components, which could interfere and compromise the performance of the assay, producing inaccurate results. For a complete list of known chemical compatibility within this assay, please refer to Appendix III (page 25).

2. DILUTION OF SAMPLES

Samples should be prepared as described in Appendix I-II. Cell and tissue lysates may be diluted 1:50 (v/v) in **Sample Diluent** as a suggested starting dilution only. Additional dilutions may be necessary to ensure that sample values are within the range of the standard curve. Users must determine the optimal sample dilutions for their particular experiments.

a) Dilute prepared samples (i.e. cell and tissue lysates) in **Sample Diluent**. Prepare at least 250 µL of diluted sample to permit assaying in duplicate.
b) Mix thoroughly.
c) Samples are now ready to be used in the Assay Procedure (see page 16). Samples may be left at room temperature while Reagents are being prepared (see page 12).

REAGENT PREPARATION

NOTE: *All reagents should be freshly prepared prior to use. Once prepared, reagents should be kept at room temperature for the duration of the assay.*

NOTE*: The preparation of the reagents is based on using the complete 1 X 96 well assay, unless otherwise noted. If only a portion of the immunoassay plate is to be used, please store all components as previously described (see page 8).*

1. TEMPERATURE OF REAGENTS

Bring the following reagents to room temperature prior to use:

- **Anti-HO-1 Immunoassay Plate** *(Part#: 80-1501)*
- **Sample Diluent** *(Part#: 80-1504)*
- **Wash Buffer** *(Part#: 80-1287)*
- **Antibody Diluent** *(Part#: 80-1759)*
- **HRP Conjugate Diluent** *(Part#: 80-1508)*
- **TMB Substrate** *(Part#: 80-0350)*
- **Stop Solution 2** *(Part#: 80-0377)*

2. RECOMBINANT HO-1 STANDARD *(Part#: 80-1503)*

NOTE*: The HO-1 Standard will withstand two freeze/thaw cycles to allow preparation of a second standard curve. However, to ensure product integrity, it is suggested that the HO-1 Standard be aliquotted into smaller portions and any remaining HO-1 Standard be discarded after the second use.*

The **HO-1 Standard** is used to generate a standard curve with 6 points, ranging from 0.78 – 25 ng/mL.

a) Centrifuge the **HO-1 Standard** vial before removing the cap. This process will assure that all of the standard is collected and available for use.
b) Label six (6) polypropylene tubes, each with one of the following standard values: 25 ng/mL, 12.5 ng/mL, 6.25 ng/mL, 3.125 ng/mL, 1.56 ng/mL, 0.78 ng/mL.
c) Add 500 µL of **Sample Diluent** to Tube #1.

d) Add 250 µL of **Sample Diluent** to Tube #2, 3, 4, 5, and 6.

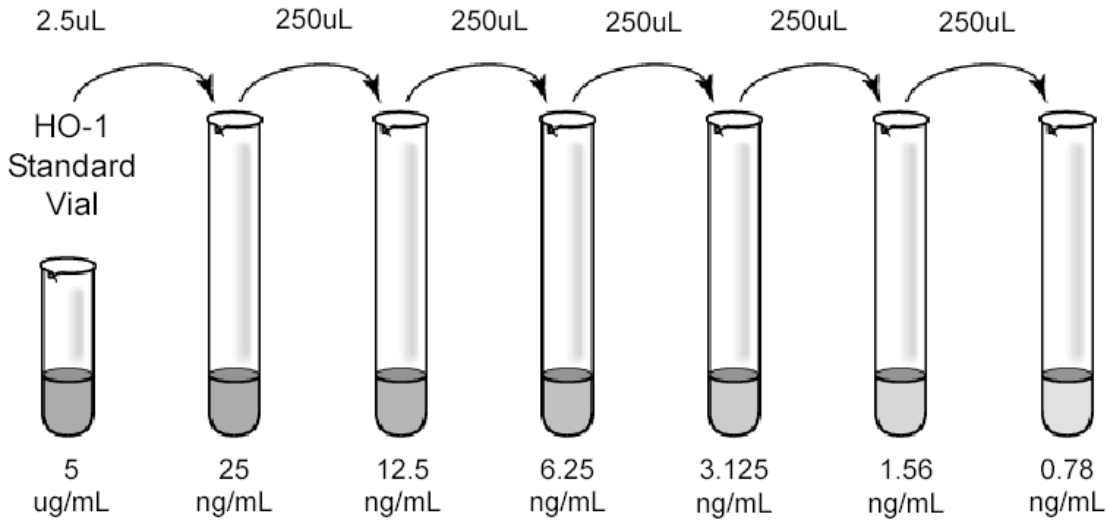
e) Add 2.5 µL of the **HO-1 Standard** stock solution (5 µg/mL) to Tube #1.

f) Mix thoroughly.

g) Transfer 250 µL from Tube#1 to Tube #2.

h) Mix thoroughly.

i) Similarly, complete the dilution series to generate the remaining standards (250 µL from Tube #2 to Tube #3, mix thoroughly, etc) up to and including Tube #6.



j) Finally, add 250 µL Sample Diluent to another 1.5 mL polypropylene tube (Tube # 7), which is the zero standard (0 ng/mL).

3. WASH BUFFER *(Part#: 80-1287)*

a) Bring the **20X Wash Buffer** to room temperature and swirl gently to dissolve any crystals that may have formed from storage.
b) Dilute the 100 mL of **20X Wash Buffer** with 1900 mL of deionized or distilled water. Once diluted, the 1X Wash Buffer is stable at room temperature for up to 4 weeks. For longer-term storage, the Wash Buffer should be stored at 4˚C.

***NOTE**: 100 mL of **20X Wash Buffer** has been provided in this kit, which is sufficient for the preparation of 2L of 1X Wash Buffer. The minimum required volume of 1X Wash Buffer is 520 mL (if the complete plate is used at once). However additional 1X Wash Buffer is supplied to allow for multiple assays or alternative washing techniques.*

4. ANTI-HUMAN HO-1 *(Part#: 80-1505)*

a) Centrifuge the vial before removing the cap to ensure maximum product recovery.
b) Dilute 22 µL of **Anti-Human HO-1** in 11 mL of **Antibody Diluent** in a polypropylene tube. If only using a portion of the plate, dilute only what is needed for number of wells used.
c) Mix gently by inversion.
d) Reagent is now ready to be used in the Assay Procedure (see page 16).
e) Do not re-use or store any remaining diluted **Anti-Human HO-1.**

5. ANTI-RABBIT IgG: HRP CONJUGATE *(Part#: 80-1507)*

a) Centrifuge the vial before removing the cap to ensure maximum product recovery.
b) Dilute 22 µL of the **HRP Conjugate** in 11 mL of the **HRP Conjugate Diluent** in a polypropylene tube. If only using a portion of the plate, dilute only what is needed for the number of wells used.
c) Mix gently by inversion.
d) Reagent is now ready to be used in the Assay Procedure (see page 17).
e) Do not re-use or store any remaining diluted **HRP Conjugate**.

ASSAY PROCEDURE

1. DETERMINE THE REQUIRED NUMBER OF WELLS

a) Refer to the HO-1 Plate Template on page 26 to determine the number of wells to be used.
b) Remove the **Anti-HO-1 Immunoassay Plate** from the packaging and note the color of the desiccant pack. Silica beads should be blue. Pink beads indicate that moisture is present and the performance of the plate may be compromised.
c) If less than 96 pre-coated microtiter wells are needed, remove the excess wells from the frame and return them to the foil pouch.
d) Reseal the pouch containing the unused wells and store at 4°C.

2. ADDITION OF STANDARDS AND SAMPLES

a) Add 100 µL (in duplicate) of each of the following to appropriate wells:
   - Prepared **HO-1 Standard** (Tube#1 through Tube #6)
   - Samples (previously prepared - see Sample Preparation, page 11)
   - Zero Standard (**Sample Diluent**, which represents 0 ng/mL)

b) Cover wells with an adhesive plate sealer or plastic wrap and incubate at room temperature for 30 minutes.

***NOTE**: For each step in the procedure, total dispensing time for the addition of the reagents and samples to the assay plate should not exceed 15 minutes.*

3. WASHING

a) Aspirate liquid from all wells.
b) Add 300 µL of 1X Wash Buffer to all wells, using a multi-channel pipette, manifold dispenser, automated microplate washer, or a squirt bottle.
c) Repeat the aspirating and washing 5 more times, for a total of 6 washes.
d) After the 6th addition of 1X Wash Buffer, aspirate the liquid from all wells. Invert the plate and carefully pat dry on clean paper towels.

4. ADDITION OF ANTI-HUMAN HO-1 ANTIBODY

(previously diluted, see page 14)

a) Add 100 µL of the previously diluted **Anti-Human HO-1** to each well, except the blank.
b) Cover wells with a fresh adhesive plate sealer (or plastic wrap) and incubate at room temperature for 1 hour.
c) Wash plate as described in Step #3.

5. ADDITION OF ANTI-RABBIT IgG: HRP CONJUGATE
(previously diluted, see page 15)

a) Add 100 µL of the previously diluted **HRP Conjugate** to each well, except the blank.
b) Cover wells with a fresh adhesive plate sealer (or plastic wrap) and incubate at room temperature for 30 minutes.
c) Wash plate as described in Step #3.

6. ADDITION OF TMB SUBSTRATE AND STOP SOLUTION

a) Add 100 µL of the **TMB Substrate** to the wells. Color development should be visible within 1 minute of addition to the plate.
b) Incubate the plate at room temperature for 15 minutes (preferably in the dark).
c) Add 100 µL of the **Stop Solution 2** to the wells in the same order that the **TMB Substrate** was added.

7. MEASURING ABSORBANCE

a) Set up the microplate reader according to the manufacturer's instructions.
b) Set wavelength at 450 nm.
c) Measure the absorbance. If the plate cannot be read immediately, it should be covered and kept at room temperature. The absorbance should be read within 30 minutes of adding the **Stop Solution 2**.

CALCULATION OF RESULTS - DETERMINATION OF HO-1 CONCENTRATIONS

1. Calculate the average of the duplicate absorbance measurements for each standard and sample.

2. Calculate the average of the duplicate absorbance measurements for the blank.

3. Subtract the average value obtained in Step#2 (blank) from the values obtained in Step#1 (standards and samples).

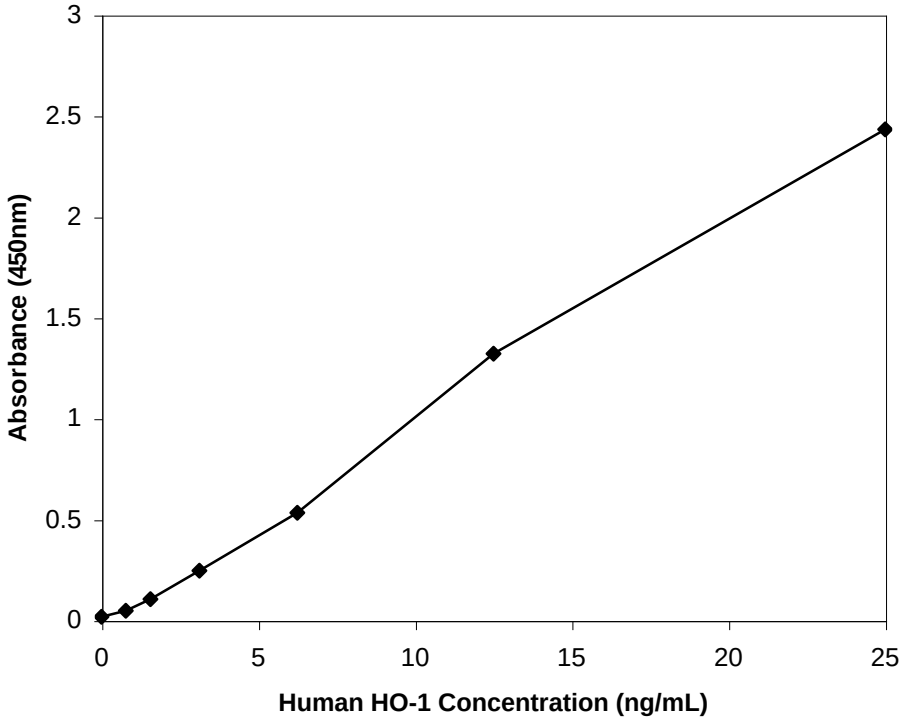
4. To generate the standard curve, plot the Recombinant HO-1 Standard concentrations (ng/mL) on the X-axis, and the absorbance measurements for the corresponding HO-1 standards on the Y-axis. Determine the best fit line.

5. Interpolate the sample concentrations from the standard curve and multiply by the dilution factor for the final sample HO-1 concentration. For example, if the sample was diluted 1:25 prior to assaying, the value generated from the standard curve must be multiplied by 25 to calculate the final sample HO-1 concentration.

NOTE: *Manufacturers of microplate readers usually offer accompanying software programs that will analyze data, plot standard curves and calculate sample concentrations. To set up the program for calculating the results, consult with the software instruction manual or contact the manufacturer of the microplate reader.*

D. ASSAY PERFORMANCE CHARACTERISTICS

TYPICAL HO-1 STANDARD CURVE



PERFORMANCE CHARACTERISTICS

1. SENSITIVITY

The sensitivity of the HO-1 (human), ELISA kit has been determined to be 0.78 ng/mL.
The standard curve has a range of 0.78-25 ng/mL.

2. PRECISION

a) Intra-Assay Precision (Within Run Precision)
To determine Intra-Assay Precision, three samples of known concentration were assayed thirty times on one plate.
The Intra-Assay Coefficient of variation of The HO-1 (human), ELISA kit has been determined to be <10%.

b) Inter-Assay Precision (Between Run Precision)
To determine Inter-Assay Precision, three samples of known concentration were assayed thirty times in three individual assays. The Inter-Assay Coefficient of variation of the HO-1 (human) ELISA kit has been determined to be <10%.

5. SPECIFICITY AND SPECIES REACTIVITY

The HO-1 (human), ELISA kit is specific for human HO-1. It does not cross react with human HO-2 or HO-3, the other known heme oxygenase isoforms. This kit is not recommended for measuring rat or mouse HO-1.

LIMITATIONS OF THE ASSAY

- This assay has been validated for use with cell lysates and tissue extracts. Other sample types or matrices (e.g. urine, cerebrospinal fluid, cell culture supernatant, etc.) may contain interfering factors that can compromise the performance of the assay, or produce inaccurate results.

- Although this assay has been validated for use with cell lysates and tissue extracts, some samples may contain higher levels of interfering factors that can produce anomalous results.

- If samples generate greater values than the highest standard, the samples should be re-assayed at a higher sample dilution. Similarly, if samples generate lower values than the lowest standard, the samples should be re-assayed at a lower sample dilution.

- The use of assay reagents not provided in this kit or amendments to the protocol can compromise the performance of this assay.

- The components in each kit lot number have been quality assured and warranted in this specific combination only; please do not mix them with components from other kit lot numbers.

E. REFERENCES

REFERENCES

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F. APPENDICES

APPENDIX I
PREPARATION OF CELL LYSATES

1. For adherent cell lines, aspirate the media and wash the cells three times with phosphate buffered saline. Harvest the cells using appropriate, established methods (e.g. scraping, trypsinization) and centrifuge cells to pellet.
2. For non-adherent cell lines, centrifuge cells to pellet, aspirate media and wash cells three times with phosphate buffered saline.
3. Aspirate the supernatant from the final wash.
4. If necessary, the cell pellet can be frozen at -70°C and processed at a later date.
5. Calculate the amount of 1X HO-1 Extraction Reagent that will be required. For every 1 X 10^6 to 1 X 10^7 cells, use 1ml of HO-1 Extraction Reagent.
6. Dilute an appropriate amount of 5X HO-1 Extraction Reagent with cold (4°C) deionized or distilled water to generate the required volume of 1X HO-1 Extraction Reagent. For example, if 5 mL of 1X HO-1 Extraction Reagent were required, dilute 1ml of the 5X HO-1 Extraction Reagent with 4 mL of cold deionized or distilled water.
7. Add protease inhibitors to the 1X HO-1 Extraction Reagent. Examples of appropriate protease inhibitors include 0.1mM PMSF, 1 µg/mL leupeptin, 1 µg/mL aprotinin, 1 µg/mL pepstatin. Alternatively, a protease inhibitor cocktail tablet can be added at a final 1X concentration. Protease inhibitor cocktail tablets are commercially available from a variety of scientific reagent vendors.
8. Resuspend the cell pellet with an appropriate volume of 1X HO-1 Extraction Reagent supplemented with protease inhibitors. Pipet up and down to break up the cell pellet until the cell suspension is homogeneous and no clumps are visible.
9. Incubate 30 minutes on ice with occasional mixing or alternatively, samples can be briefly sonicated.
10. Transfer extracts to polypropylene microcentrifuge tubes and centrifuge at 21,000 x g for 10 minutes in a 4°C refrigerated microfuge.
11. Transfer the supernatants to labeled polypropylene tubes. When collecting the supernatant, avoid disturbing the cell pellet. The supernatant collected is the cell lysate, which is now ready for analysis using the HO-1 ELISA kit. The resulting pellets can be discarded.
12. Alternatively, the cell lysates can be frozen at -70°C and assayed at a later date. It is recommended that a protein assay be performed and the lysates aliquotted to convenient amounts prior to storing at -70°C to avoid multiple freeze thaw cycles.

APPENDIX II
PREPARATION OF TISSUE EXTRACTS

1. Harvest tissue to be analyzed.
2. If necessary, tissues can be flash frozen, stored at -70°C and the extract prepared at a later time.
3. Calculate the amount of 1X HO-1 Extraction Reagent that will be required. For each ˜0.5 cm^3 piece of tissue, use 1mL of 1X HO-1 Extraction Reagent.
4. Dilute an appropriate amount of 5X HO-1 Extraction Reagent with cold (4°C) deionized or distilled water to generate the required volume of 1X HO-1 Extraction Reagent. For example, if 5 mL of 1X HO-1 Extraction Reagent were required, dilute 1 mL of the 5X HO-1 Extraction Reagent with 4 mL of cold deionized or distilled water.
5. Add protease inhibitors to the HO-1 Extraction Reagent. Examples of appropriate protease inhibitors include 0.1mM PMSF, 1 µg/mL leupeptin, 1 µg/mL aprotinin, 1 µg/mL pepstatin. Alternatively, a protease inhibitor cocktail tablet can be added at a final 1X concentration. Protease inhibitor cocktail tablets are commercially available from a variety of scientific reagent vendors.
6. Place the tissue in a mortar and add a sufficient volume of liquid nitrogen to cover the tissue.
7. Allow the liquid nitrogen to evaporate. The tissue should be thoroughly frozen.
8. Grind the frozen tissue to a powder with a pestle.
9. Add an appropriate volume of 1X HO-1 Extraction Reagent supplemented with protease inhibitors to the processed tissue.
10. Continue to homogenize the tissue with the pestle until the tissue suspension is homogeneous.
11. Transfer the extract to a polypropylene tube and centrifuge at 21,000 x g for 10 minutes in a 4°C refrigerated microfuge.
12. Transfer the supernatant to a labeled polypropylene tube. The supernatant collected is the tissue extract, which is now ready for analysis using the HO-1 ELISA kit. The resulting pellet can be discarded.
13. Alternatively, the tissue extracts can be frozen at -70°C and assayed at a later date. It is recommended that a protein determination assay be performed and the extracts aliquotted to convenient amounts prior to storing at -70°C to avoid multiple freeze thaw cycles.

APPENDIX III
CHEMICAL COMPATIBILITY LIMITS

Different chemicals may interfere with the HO-1 (human) ELISA kit. Although the effect of every chemical is not known, Enzo Life Sciences has tested the following chemicals to determine the level at which they may interfere with the kit.
The compatible limit is defined as the chemical concentration at which the measurement of HO-1 in a sample is inhibited by ≤10%.

CHEMICAL	COMPATIBLE LIMIT
Aprotinin	50 µg/mL
ß-mercaptoethanol	0.75 mM
CHAPS	1% (w/v)
Dithiothreitol (DTT)	0.2 mM
EDTA	5 mM
Glycerol	1% (v/v)
HEPES, pH 7.5	5 mM
Leupeptin	50 µg/mL
Magnesium Chloride (MgCl2)	5 mM
MOPS, pH 7.5	250 mM
NP-40	0.02% (v/v)
Pepstatin A	10 µg/mL
PMSF	0.5 mM
SDS	0.01% (w/v)
Sodium Azide (NaN3)	0.5% (w/v)
Sodium Deoxycholate	0.2% (w/v)
Sodium Chloride (NaCl)	200 mM
Sodium Phosphate, pH 7.2	50 mM
Tris, pH 7.5	250 mM
Triton-X100	<0.01% (v/v)
Tween-20	1% (v/v)

APPENDIX IV – Anti-HO-1 Immunoassay Plate Template

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	3.125 ng/mL										
B	Blank	3.125 ng/mL										
C	25 ng/mL	1.56 ng/mL										
D	25 ng/mL	1.56 ng/mL										
E	12.5 ng/mL	0.78 ng/mL										
F	12.5 ng/mL	0.78 ng/mL										
G	6.25 ng/mL	0 ng/mL										
H	6.25 ng/mL	0 ng/mL										

REFERENCE

1. Bring to room temperature: **Anti-HO-1 Immunoassay Plate**, **20X Wash Buffer**, **Sample Diluent**, **Antibody Diluent**, **HRP Conjugate Diluent**, **TMB Substrate** and **Stop Solution 2**.
2. Prepare **Recombinant HO-1 Standard** and samples in **Sample Diluent**.
3. Add 100 µL prepared standards and samples in duplicate to wells of **Anti-HO-1 Immunoassay Plate**. Cover immunoassay plate.
4. Incubate plate at room temperature for 30 minutes.
5. Wash wells 6X with 1X Wash Buffer.
6. Add 100 µL diluted **Anti-Human HO-1** to each well. Cover immunoassay plate.
7. Incubate plate at room temperature for 1 hour.
8. Wash wells 6X with 1X Wash Buffer.
9. Add 100 µL diluted **HRP Conjugate** to each well. Cover immunoassay plate.
10. Incubate plate at room temperature for 30 minutes.
11. Wash wells 6X with 1X Wash Buffer.
12. Add 100 µL **TMB Substrate** to each well.
13. Incubate at room temperature for 15 minutes (preferably in the dark).
14. Add 100 µL **Stop Solution 2** to each well.
15. Measure absorbance at 450nm.
16. Plot the HO-1 standard curve and calculate HO-1 sample concentrations.

NOTES

NOTES

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