Rat HO-1 ELISA Kit

For the detection and quantitation of rat Heme Oxygenase-1 in cell lysates, tissue extracts, serum, and plasma.

Catalog Number: EKS-810A

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A. INTRODUCTION

ASSAY DESIGN

Assay Designs' Rat HO-1 ELISA (enzyme-linked immunosorbent assay) provides a method to detect and quantitate Heme Oxygenase (HO-1) in samples of rat origin. This assay allows for reproducible, accurate and precise determination of HO-1 from cell lysates, tissue extracts, serum, and plasma. The assay is specific for rat HO-1 and does not cross react with human or mouse HO-1.

Assay Designs' Rat HO-1 ELISA is a quantitative sandwich immunoassay. A mouse monoclonal antibody specific for HO-1 is pre-coated on the wells of the provided Anti-Rat 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 an anti-rabbit IgG antibody conjugated to horseradish peroxidase.

The assay is developed with tetramethylbenzidine substrate (TMB) and a blue color develops in proportion to the amount of captured HO-1. The color development is stopped with an acid stop solution which converts the endpoint color to yellow. The intensity of the color is measured in a microplate reader at 450nm. 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, O_2 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, however 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 heat shock, oxidants, UV-A irradiation and other 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. The highest concentration of HO-1 can be found in testes, brain, and liver. However expression in all organs is rapidly accelerated in response to pathophysiological conditions. Examples include 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 the appropriate reagents to room temperature: **Rat HO-1 Antibody**, **Rat HO-1 Conjugate**, **TMB Substrate, Anti-Rat HO-1 Immunoassay plate, Sample Diluent, Wash Buffer,** and **Stop Solution 2**.
2. Dilute **Rat HO-1 Standard** and samples in **Sample Diluent**.
3. Add 100µL prepared standards and samples in duplicate to wells of **Anti-Rat HO-1 Immunoassay Plate**. Cover immunoassay plate.
4. Incubate plate at room temperature for 1 hour.
5. Wash wells 4X with 1X Wash Buffer.
6. Add 100µL **Rat HO-1 Antibody** to each well. Cover immunoassay plate.
7. Incubate plate at room temperature for 1 hour.
8. Wash wells 4X with 1X Wash Buffer.
9. Add 100µL diluted **Rat HO-1 Conjugate** to each well. Cover immunoassay plate.
10. Incubate plate at room temperature for 30 minutes.
11. Wash wells 4X with 1X Wash Buffer.
12. Add 100µL **TMB Substrate** to each well.
13. Incubate at room temperature for 15 minutes (preferably in the dark).
14. Add 100µL **Stop Solution 2** to each well.
15. Measure absorbance at 450nm.
16. Plot the standard curve and calculate the Rat HO-1 sample concentrations.

B. MATERIALS

PRECAUTIONS

FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

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

- The activity of the **Rat HO-1 conjugate** *(part# 80-1602)* is affected by nucleophiles such as azide, cyanide and hydroxylamine.

Please read the complete kit insert before performing this assay.

MATERIALS PROVIDED

Assay Designs' Rat HO-1 ELISA kit contains the following components in sufficient quantities for 96 wells. These reagents are sufficient to assay one standard curve and 39 samples in duplicate or two standard curves and 30 samples in duplicate.

PART #	COMPONENT	SIZE	DESCRIPTION
80-1592	Anti-Rat HO-1 Immunoassay Plate	96 well plate	12 x 8 removable strips and plate frame; pre-coated with mouse monoclonal antibody specific for HO-1.
80-1502	5X Extraction Reagent 2	10mL	Concentrated buffer for preparation of cell and tissue extracts.
80-1588	Rat HO-1 Standard	25µL	5µg/mL stock solution of recombinant rat HO-1 protein.
80-1587	Sample Diluent	50mL	Buffer to dilute standards and samples.
80-1287	20X Wash Buffer	100mL	Concentrated solution of buffer and surfactant.
80-1589	Rat HO-1 Antibody	10mL	Rabbit polyclonal antibody specific for HO-1.
80-1602	Rat HO-1 Conjugate	10mL	Horseradish peroxidase conjugated anti-rabbit IgG.
80-0350	TMB Substrate	10mL	Stabilized tetramethylbenzidine substrate.
80-0377	Stop Solution 2	10mL	Acid stop solution to stop color reaction.

STORAGE OF MATERIALS

All reagents are stable as supplied at 4°C, except the **Rat HO-1 Standard**, which should be stored at -20°C. If assaying on two or more occasions, aliquot the standard into smaller portions and store at -20°C.

Unused wells of the **Anti-Rat HO-1 Immunoassay Plate** should be resealed, along with the accompanying desiccant pack, in the foil pouch provided and stored at 4°C until the kits expiration date.

MATERIALS REQUIRED BUT NOT PROVIDED

- Deionized or distilled water
- Precision pipettors capable of accurately delivering 1 to 1000µL
- Disposable pipette tips
- 5, 10, 25mL pipettes for reagent preparation
- 2L graduated cylinder
- Squirt bottle, manifold dispenser, or automated microtiter plate washer
- Disposable polypropylene tubes
- Microtiter plate reader capable of measuring absorbance at 450nm
- Adhesive plate sealers or plastic wrap

C. PERFORMING THE ASSAY

CRITICAL ASSAY PARAMETERS AND NOTES

- Assay Designs' Rat HO-1 ELISA kit contains a pre-coated microtiter plate (**Anti-Rat HO-1 Immunoassay Plate**) with removable wells to allow assaying on multiple 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.

- Absorbance is a function of the incubation time. Therefore, prior to starting the assay it is recommended that all reagents are read y 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, sample, and reagent. Use separate reagent troughs/reservoirs for each reagent.

- 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, tissue extracts, serum, and plasma samples may be found in Appendices I-IV, respectively (pages 23 to 25). Investigators may use alternative methods of preparation, however, it is recommended that the **5X Extraction Reagent** provided in this kit be diluted to 1X and used as the lysis buffer for cell lysates and tissue samples.

   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 V (page 26).

2. DILUTION OF SAMPLES

A minimum 1:2 dilution is required for **1X Extraction Reagent** to remove matrix interference of this buffer. Due to differences in sample types, number of cells, or total cellular protein concentration, samples may require greater dilution with **Sample Diluent** to remove interference or to be read within the range of the standard curve. Users must determine the optimal sample dilutions for their particular experiments. Below are examples of sample recoveries with several different types of samples. Recoveries are based on the values obtained where dilutions of the sample are linear.

Sample	# cells per mL of Lysis Buffer	Total cellular protein (mg/mL)	% Recovery	Recommended Dilution
Rat C6 cells	15 million	1.58	103	1:72
Heat -Shocked Rat C6 cells	15 million	1.77	95	1:144
Rat Liver microsomes	---	1.0	90	1:20
Rat serum*	---	---	96	1:10
Rat EDTA plasma*	---	---	93	1:5

* For optimal recovery, 0.5% NP-40 (non-ionic detergent) must be added to neat serum and plasma samples at least 10 minutes prior to dilution with sample diluent .

a) Dilute prepared samples (i.e. cell lysates, tissue extracts, serum, and plasma) in **Sample Diluent**. Prepare at least 300µL 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 15). Samples may be left at room temperature while reagents are being prepared.

REAGENT PREPARATION

NOTE: *Standards should be freshly prepared prior to use. Once prepared, reagents may 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 us e:

   - **Anti-Rat HO-1 Immunoassay Plate** *(Part#: 80-1592)*
   - **Sample Diluent** *(Part#: 80-1587)*
   - **Wash Buffer** *(Part#: 80-1287)*
   - **Rat HO-1 Antibody** *(Part#: 80-1589)*
   - **Rat HO-1 Conjugate** *(Part#: 80-1602)*
   - **TMB Substrate** *(Part#: 80-0350)*
   - **Stop Solution 2** *(Part#: 80-0377)*

2. RAT HO-1 STANDARD CURVE *(Part#: 80-1588)*

NOTE*: The HO-1 Standard will withstand multiple 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 **Rat HO-1 Standard** is used to generate a standard curve with eight points, ranging from 0.195 – 12.5ng/mL.

a) Briefly centrifuge the **Rat 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 seven polypropylene tubes, each with one of the following standard values: 12.5ng/mL, 6.25ng/mL, 3.125ng/mL, 1.56ng/mL, 0.78ng/mL, 0.39ng/mL , and 0.195ng/mL.

c) Add 1,995µL of **Sample Diluent** to Tube #1.

d) Add 500µL of **Sample Diluent** to Tubes #2, 3, 4, 5, 6, and 7.

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

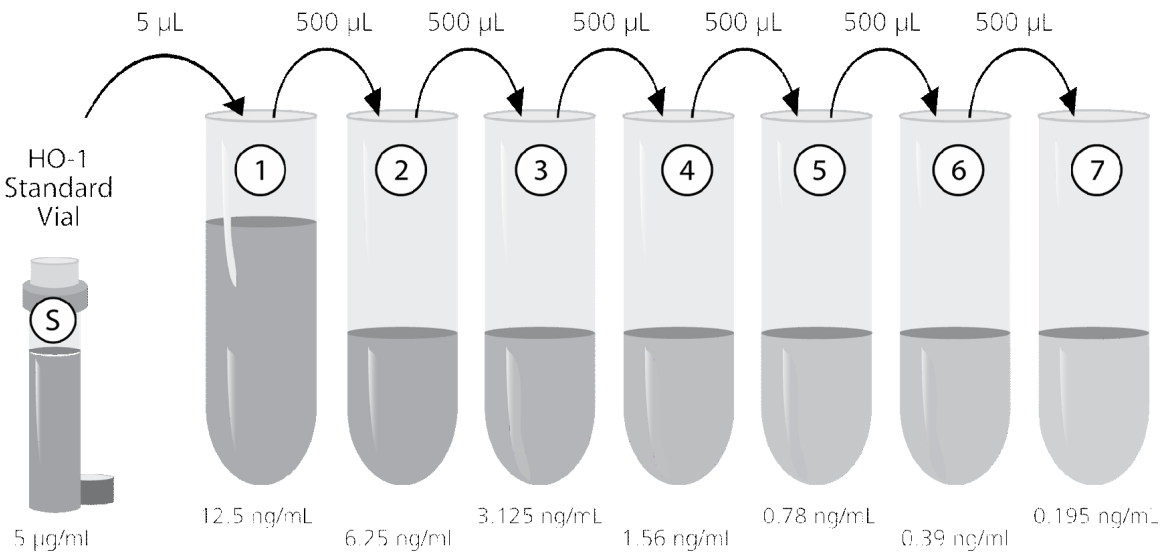
f) Mix thoroughly.

g) Transfer 500µL from Tube#1 to Tube #2.

h) Mix thoroughly.

i) Similarly, complete the dilution series to generate the remaining standards (500µL from Tube #2 to Tube #3, mix thoroughly, etc.), up to and including Tube #7.

j) Finally, add 500µL **Sample Diluent** to an eighth polypropylene tube, which is the zero standard (0ng/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 100mL of **20X Wash Buffer** with 1,900mL 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**: 100mL of **20X Wash Buffer** has been provided in this kit, which is sufficient for the preparation of 2L of 1X Wash Buffer. Additional 1X Wash Buffer is supplied to allow for multiple assays or alternative washing techniques.*

ASSAY PROCEDURE

1. DETERMINE THE REQUIRED NUMBER OF WELLS

a) Use the HO-1 Plate Template on page 26 to determine the number of wells to be used.
b) Remove the **Anti-Rat 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 100µL (in duplicate) of each of the following to appropriate wells:
   - Prepared **Rat HO-1 Standard Curve** (Tube#1-7)
   - Zero Standard (**Sample Diluent**, which represents 0ng/mL)
   - Samples (Previously prepared see Sample Preparation, page 11-12)

b) Cover wells with an adhesive plate sealer or plastic wrap and incubate at room temperature for 1 hour.

***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 300µL 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 3 more times, for a total of 4 washes.

d) After the 4th 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 RAT HO-1 ANTIBODY

a) Add 100µL of the **Rat HO-1 Antibody** 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

a) Add 100µL of the **Rat HO-1 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 100µL of the **TMB Substrate** to each well. 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 100µL of **Stop Solution 2** to each well 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 450nm.
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 **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.

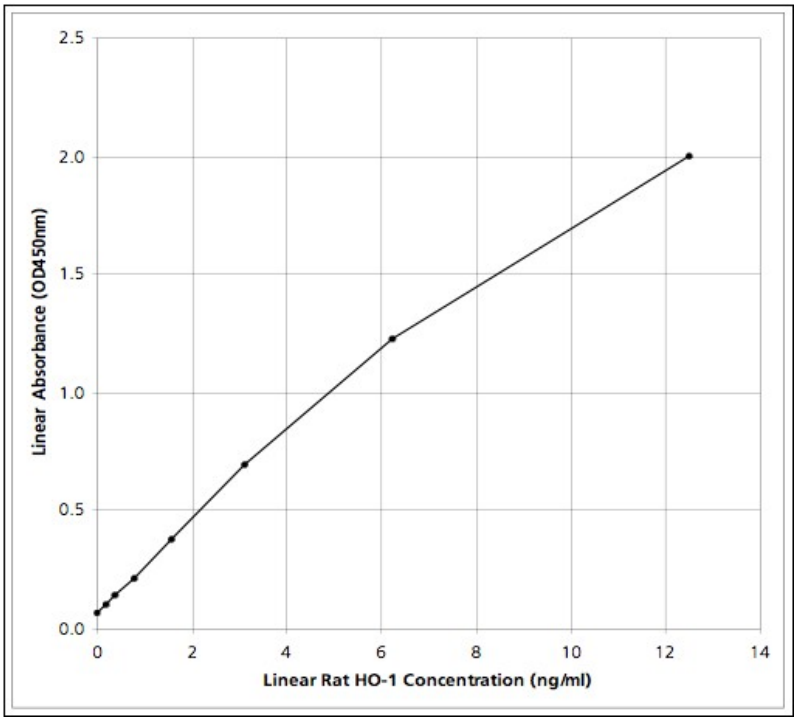
3. Subtract the average value obtained in Step #2 (blank) from the values obtained in Step#1 (standards and samples).

4. To generate the standard curve, plot Rat HO-1 (using linear scale) concentrations (ng/ml) on the X-axis, and the absorbance measurements for the corresponding Rat HO-1 standards on the Y-axis. Determine the best fit line.

5. Interpolate the sample concentrations (observed) from the standard curve and multiply by the dilution factor for the final Rat HO-1 sample 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 Rat HO-1 concentration.

NOTE: *Manufacturers of microplate readers usually offer accompanying sof tware 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.*

TYPICAL RAT HO-1 STANDARD CURVE



PERFORMANCE CHARACTERISTICS

1. SENSITIVITY

The sensitivity was calculated by first multiplying the concentration of the Low Standard (0.195 ng/mL) by two (2) standard deviations of the mean OD of twenty-four (24) replicates of 0 ng/mL Standard. This value was then divided by the difference between the mean OD of twenty-four (24) replicates of the low Standard and the mean OD of the twenty-four (24) replicates of 0 ng/mL Standard.

The sensitivity of Rat HO-1 ELISA has been determined to be 0.036 ng/mL.

The standard curve has a range of 0.195-12.5 ng/mL.

2. PRECISION

a) Intra-Assay Precision (Within Run Precision)
To determine Intra-Assay Precision, 3 samples of known concentration were assayed 24 times. The Intra-Assay Coefficient of Variation of Assay Designs' Rat HO-1 ELISA Kit has been determined to be < 4%.

b) Inter-Assay Precision (Between Run Precision)
To determine Inter-Assay Precision, 3 samples of known concentration were assayed 11 times in individual assays. The Inter-Assay Coefficient of Variation of Assay Designs' Rat HO-1 ELISA Kit has been determined to be < 10%.

3. LINEARITY

A buffer sample containing 10 ng/mL of recombinant Rat HO-1 was serially diluted 1:2 in **Sample Diluent** and measured in the assay. The results are shown in the table below.

Dilution	Expected (pg/mL)	Observed (pg/mL)	Recovery (%)
Neat	10	9.667	96.7
1:2	4.834	4.986	103.16
1:4	2.417	2.345	97.03
1:8	1.208	1.268	104.93
1:16	0.604	0.600	99.31
1:32	0.302	0.326	107.91

4. RECOVERY AND SAMPLE LINEARITY

Recovery was determined by running serial dilutions of relevant samples (cell lysate, tissue extract, serum, and plasma) in **Sample Diluent**. The observed concentration of each sample was interpolated from the standard curve and then multiplied by the dilution performed to give the final sample concentration. Linearity was calculated at each dilution (excluding the last dilution). Recoveries in which linearity fell between 85% and 115% were averaged to calculate the % recovery.

The % recoveries for cell lysates, tissue extracts, serum, and plasma were $\geq$ 90%.

5. SPECIFICITY AND SPECIES REACTIVITY

Assay Designs' Rat HO-1 ELISA is specific for Rat HO-1. Cross reactivity with Rat HO-2 is less than 0.02%.

This kit does not react with human or mouse HO-1. Human HO-1 can be measured using the Human HO-1 ELISA kit (Product EKS-800).

LIMITATIONS OF THE ASSAY

- This assay has been validated for use with cell lysates, tissue extracts, serum, and plasma. 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, tissue extracts, serum, and plasma, 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. Calculate the amount of **1X Extraction Reagent** that will be required. For every 1 X 10^6 to 1 X 10^7 cells, use 1ml of Extraction Reagent.
5. Dilute an appropriate amount of 5X Extraction Reagent with cold (4°C) deionized or distilled water to generate the required volume of **1X Extraction Reagent.** For example, if 5mL of **1X Extraction Reagent** were required, dilute 1ml of the 5X Extraction Reagent with 4mL of cold deionized or distilled water.
6. Add protease inhibitors to the **1X Extraction Reagent**. Examples of appropriate protease inhibitors include 0.1mM PMSF, 1µg/mL leupeptin, 1µg/mL aprotinin, 1µg/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.
7. Resuspend the cell pellet with an appropriate volume of **1X 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.
8. Incubate 30 minutes on ice with occasional mixing. Alternatively, samples can be briefly sonicated.
9. Transfer extracts to polypropylene microcentrifuge tubes and centrifuge at 21,000 x g for 10 minutes in a 4°C refrigerated microfuge.
10. 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.
11. 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 into smaller volumes 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 and stored at -70°C. The extract can be prepared at a later time.
3. Calculate the amount of **1X Extraction Reagent** that will be required. For each ~0.5cm^3 piece of tissue, use 1mL of **1X Extraction Reagent**.
4. Dilute an appropriate amount of 5X Extraction Reagent with cold (4°C) deionized or distilled water to generate the required volume of **1X Extraction Reagent**. For example, if 5mL of **1X Extraction Reagent** were required, dilute 1mL of the 5X Extraction Reagent with 4ml of cold deionized or distilled water.
5. Add protease inhibitors to the **1X Extraction Reagent**. Examples of appropriate protease inhibitors include 0.1mM PMSF, 1µg/mL leupeptin, 1µg/mL aprotinin, 1µg/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 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 into smaller volumes prior to storing at -70°C to avoid multiple freeze thaw cycles.

APPENDIX III
COLLECTION OF SERUM

1. Collect whole blood using established methods.

2. Allow samples to clot at room temperature for 30 minutes.

3. Centrifuge at 2,700 x g for 10 minutes, taking precautions to avoid hemolysis.

4. Remove serum. Transfer the serum to a labelled polypropylene tube. The serum collected is now ready for analysis.

5. Alternatively, the serum sample can be frozen at ≤ -20°C and assayed at a later date. It is recommended that the serum be aliquotted into smaller volumes prior to storing at ≤ -20°C to avoid multiple freeze thaw cycles.

APPENDIX IV
COLLECTION OF PLASMA

1. Using established methods, collect plasma using EDTA as an anticoagulant.

2. Within 30 minutes of collection, centrifuge at 1,000 x g for 15 minutes.

3. Remove plasma. Transfer the plasma to a labeled polypropylene tube. The plasma collected is now ready for analysis.

4. Alternatively, the plasma sample can be frozen at ≤-20°C and assayed at a later date. It is recommended that the plasma be aliquotted to convenient volumes prior to storing at ≤-20°C to avoid multiple freeze thaw cycles.

APPENDIX V
CHEMICAL COMPATIBILITY LIMITS

Different chemicals may interfere with the Assay Designs' Rat HO-1 ELISA kit. Although the effect of every chemical is not known, Assay Designs has tested the following chemicals to determine the levels 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	50µg/mL
β-mercaptoethanol	0.75 mM
CHAPS	1% (w/v)
Dithiothreitol (DTT)	1mM
EDTA	100mM
Glycerol	1% (v/v)
HEPES, pH 7.5	25mM
Leupeptin	50µg/mL
Magnesium Chloride ($MgCl_2$)	100mM
MOPS, pH 7.5	250mM
NP-40	5% (v/v)
Pepstatin A	50µg/mL
PMSF	5mM
SDS	0.01% (w/v)
Sodium Azide (NaN_3)	0.5% (w/v)
Sodium Deoxycholate	0.2% (w/v)
Sodium Chloride (NaCl)	500mM
Sodium Phosphate, pH 7.2	150mM
Tris, pH 7.5	250mM
Triton-X100	5% (v/v)
Tween-20	5% (v/v)

APPENDIX VI – Anti-Rat HO-1 Immunoassay Plate Template

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	3.125ng/mL	0.195ng/mL									
B	Blank	3.125ng/mL	0.195ng/mL									
C	0ng/mL	1.56ng/mL										
D	0ng/mL	1.56ng/mL										
E	12.5ng/mL	0.78ng/mL										
F	12.5ng/mL	0.78ng/mL										
G	6.25ng/mL	0.39ng/mL										
H	6.25ng/mL	0.39ng/mL										

Notes

REFERENCE

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

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