

Cat. # MK124

For Research Use

TAKARA

**Rat Heme Oxygenase-1
EIA Kit**

Product Manual

v201608Da

Table of Contents

I.	Introduction	3
II.	Principle	4
III.	Components	5
IV.	Materials Required but not Provided	5
V.	Storage	5
VI.	Intended Use.....	5
VII.	Protocol.....	6
VIII.	Characteristics	9
IX.	Performance.....	10
X.	Reference Data about Measurement	12
XI.	Reference.....	18
XII.	Related Products	18
XIII.	Precautions.....	18

I. Introduction

When living organisms receive stress from the environment, their organs quickly show various responses as means of maintaining homeostasis. For example, when an organism is invaded by heavy metals such as cadmium, lead, and mercury or endotoxins or when it is exposed to heat shock, ultraviolet light, oxygen radicals, or hypoxic conditions, various proteins and enzymes are induced within its organs as response to stress and the defense mechanism is driven. One example of the response is heme oxygenase, an enzyme that acts on heme, which is a prosthetic group of heme proteins such as hemoglobin. Heme is degraded into bile pigments (biliverdin and bilirubin), carbon monoxide, and reduced iron (Fe^{2+}) by heme oxygenase (Figure 1). The bilirubin produced in this reaction exerts antiinflammatory effects through its strong radical scavenging activity, whereas carbon monoxide is known to exert effects on preservation of organ blood flow through its vasodilative effect, and on regulation of the spermatogenic functions of the testes when exposed to stress.

Heme oxygenase is known to exist as at least two isozymes (heme oxygenase-1 and heme oxygenase-2). While heme oxygenase-2 is a constitutive enzyme, heme oxygenase-1 (HO-1) is an enzyme whose expression is induced intracellularly in response to various type of stress as abovementioned. Thus, monitoring of heme oxygenase-1 is expected to be effective to detect stress. The Rat Heme Oxygenase-1 EIA Kit is a sandwich ELISA kit that uses 2 types of antibodies that recognize rat heme oxygenase-1 and provides a convenient and highly sensitive method for detecting heme oxygenase-1 expressed in response to stress induction.

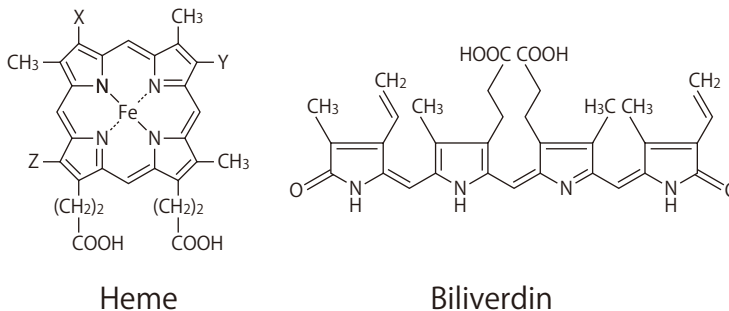
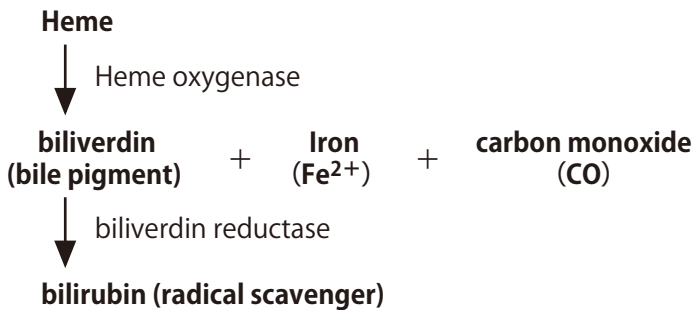
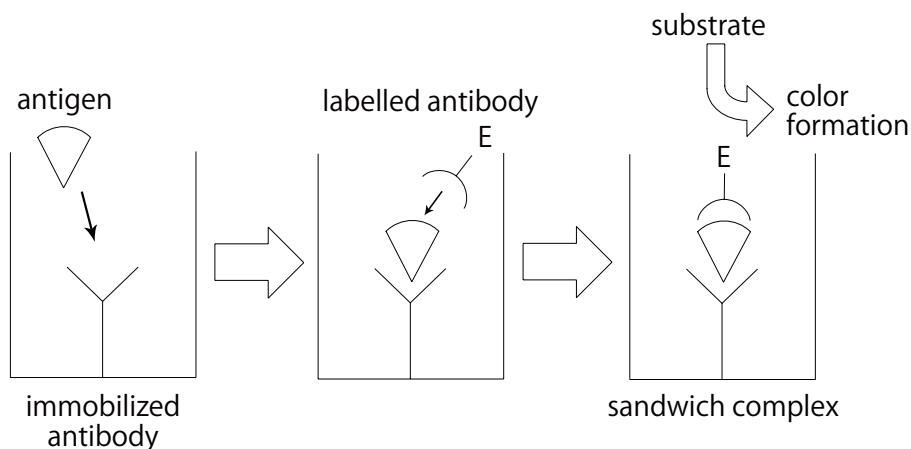


Figure 1. Reaction by heme oxygenase

II. Principle

The Rat Heme Oxygenase-1 EIA Kit is a solid phase EIA Kit based on a sandwich method that utilizes two rat monoclonal anti-heme oxygenase-1 (HO-1) antibodies to detect rat heme oxygenase-1 by two-step procedure. One of the rat monoclonal anti-heme oxygenase-1 is immobilized onto the microtiter plate and blocked against non-specific binding. Samples and standards are incubated in the microtiter plate. The second step is to wash the plate and add the second anti-heme oxygenase-1 labelled with peroxidase (POD). During this incubation, rat heme oxygenase-1 is bound to anti-heme oxygenase-1 (solid phase) on one side and tagged on the other by POD-anti-heme oxygenase-1. The reaction between POD and substrate (H_2O_2 and tetramethylbenzidine) results in color development with intensities proportional to the amount of rat heme oxygenase-1 present in samples and standards. The amount of rat heme oxygenase-1 can be quantitated by measuring the absorbance using an EIA plate reader. Accurate sample concentration of rat heme oxygenase-1 can be determined by comparing their specific absorbance with those obtained for the standards plotted on a standard curve.



III. Components

(1)	Antibody Coated Microtiterplate Anti-rat heme oxygenase-1 monoclonal antibody coated plate (96 wells: 8 well x 12 strips)	1 plate
(2)	Antibody-POD Conjugate (lyophilized) Peroxidase labeled monoclonal anti-rat heme oxygenase-1	For 11 ml
(3)	Standard (lyophilized) Rat heme oxygenase-1 (8 ng)	For 1 ml
(4)	Sample Diluent PBS containing 25% Block Ace and preservative	11 ml x 2
(5)	Substrate Solution 3,3',5,5'-tetramethylbenzidine solution	12 ml
(6)	Extraction Buffer 1% NP40 in PBS. Use for cell extraction	11 ml

IV. Materials Required but not Provided

- Wash and Stop solution for ELISA without Sulfuric Acid (Cat. #MK021)
Contains wash solution (10X PBS; 50 ml x 5 tubes, Tween 20; 3 ml) and reaction stop solution (60 ml).
 - * This product is a stop solution for peroxidase reactions without 1N sulfuric acid.
 - * 1N sulfuric acid can be used as a stop solution. Handle 1N sulfuric acid with caution.
- Pipette, micropipette and tips
- Microplate reader (capable of measuring absorbance of up to 3.5 when set to 450 nm)

V. Storage 4 °C**IV. Intended Use**

The Rat Heme Oxygenase-1 EIA Kit is an *in vitro* enzyme immunoassay (EIA) kit for quantitative determination of rat heme oxygenase-1 expressed in rat cell culture supernatant, rat blood, and various rat organs. Due to the nature of the antibodies, the kit cannot be used to quantitate human heme oxygenase-1. This kit is for research use only. It is not for use in diagnostic or therapeutic procedures.

VII. Protocol**1. Summary**

1. Add 100 μ l of Standard or sample to appropriate wells, and incubate 1 hours at room temperature (20 - 25°C).
2. Remove sample solution and wash the wells 3 times with 400 μ l of PBS containing 0.1% Tween20.
3. Add 100 μ l of Antibody-POD conjugate solution into wells and incubate at room temperature for 1 hour.
4. Remove sample solution and wash 4 times with 400 μ l of PBS per wells, aspirating thoroughly between washes.
5. Add 100 μ l of Substrate Solution to each well. Incubate 15 minutes at room temperature.
6. Add 100 μ l of Stop Solution to all wells. Mix gently.
7. Read at 450 nm as soon as possible.

2. Specimen collection and handling

Samples collection should be done just before the assay is performed. If sample needs to be stored before the assay, it should be stored frozen at -80°C for optimal results.

Rat cell culture supernatant, blood, and various organs are suitable for this assay. In case of blood sample, serum should be used.

All samples to be used in this assay should be treated by the same handling. Collection site is important for optimal result.

Dilution rate should be determined by the preliminary investigation. When rat serum or organ extracts are used as sample, dilute the sample with Sample Diluent by 2 - 8 times. The dilution rate can vary depending on the stress level.

When organs are used as sample, specimen should be homogenized Extraction Buffer after weighing. Centrifuge the homogenized solution, and the supernatant is used for the assay.

3. Preparation of solutions

Note : The following solutions should be prepared directly before use.

Solution 1. Antibody-POD Conjugate Solution*¹

Dissolve the contents of Vial 2 in 11 ml distilled water and mix gently followed by 10 minutes slowly rolling or occasional mixing, avoiding foam formation.

Solution 2. Standard Solution*²

Rehydrate Standard (3) with 1 ml distilled water. Slowly roll for approximately 10 minutes or let vials to stand and sporadically mix gently.

The Standard Solution contains 8 ng rat HO-1/ml. Prepare a dilution series of 4, 2, 1, 0.5, and 0.25 ng/ml by diluting the Standard Solution with Sample Diluent. Sample Diluent is directly used as 0 ng/ml.

Substrate Solution (5) should be brought to room temperature before assay. Confirm that the color of the solution has not changed into dark blue. When it reacts with metal ion, the color development can occur. Therefore caution should be paid not to contaminate with tapped water. When Substrate Solution is not all used up in one assay, dispensed in aliquots of required volume.

- * 1 : The reconstituted lyophilisate is stable for 1 week at 4°C and for 1 month when stored at -30°C. Do not repeat freeze-thaw cycles.
- * 2 : The reconstituted lyophilisate must be used on the same day when prepared. Standard before dilution (8 ng/ml) is stable for 1 month at -80°C. Freeze-thaw cycles should be minimized less than 3 cycles. Refer to the later information.

4. Procedure

Double determinations of all samples and standards should be performed. All of the Kit's content should be brought to room temperature before use. For thorough mixing, the microtiter plate can be gently agitated on a plate mixer or by mixing the plate sporadically by hand.

【 Enzyme immunoassay 】

- 1) Pipette 100 μ l sample and the dilution series of the Standard (Solution 2) into one well within 5 minutes. In this case, it is recommended to prepare sample on a different 96 well plate, and then quickly transfer on to the plate containing the Standard, using a 8-lane pipette. Mix, seal the microtiter plate (e.g. with a foil) and incubate 1 hour at room temperature (20 - 30°C). To obtain reliable assay result, it is recommended to prepare the dilution series of the Standard in both 1st and 12th rows in the plate.
- 2) Remove sample solution and wash the wells 3 times with ca. 400 μ l of PBS containing 0.1% Tween20; between the separate washing steps empty out the microtiter plate and vigorously tap onto paper towel, especially after the last washing.
- 3) Pipette 100 μ l of Antibody-POD Conjugate Solution (Solution 1) into one well, mix, seal the microtiter plate (e.g. with a foil) and incubate 1 hour at room temperature (20 - 25°C).
- 4) Remove sample solution and wash the wells 4 times as described above (It is especially important after this step to thoroughly empty out the remaining fluid before adding the substrate).
- 5) Add 100 μ l Substrate Solution (5) into each well and incubate at room temperature (20 - 25°C) for 15 minutes.
- 6) Add 100 μ l Stop Solution into each well in same order as for substrate. Tap plate gently to mix.
- 7) Measure the absorbance at 450 nm with a plate reader. The absorbance should be read as soon as possible after the completion of the assay. It may be read up to 1 hour after addition of Stop Solution if wells are protected from light at room temperature.

Note : It is important that Stop Solution is added to wells prior to reading at 450 nm. Addition of Stop Solution causes an increase in absorbance of the Substrate Solution and shift in absorbance spectrum.

5. Results

1. Standard curve

Record and average the absorbance at 450 nm for the duplicate standard wells. Plot the absorbance (vertical) versus the concentration of each rat HO-2 standard in ng/ml (horizontal) for the standards using optimal fitting curve.

2. Samples

Record and average the absorbance at 450 nm for the duplicate sample wells. Locate the average absorbance value on the vertical axis and follow a horizontal line intersecting the standard curve. At the point intersection, read the rat HO-1 concentration (ng/ml) from the horizontal axis.

VIII. Characteristics

1. Range of standard curve
Range : 0.125 - 8.0 ng/ml
Detection limit : 0.125 ng/ml
2. Specificity
This kit specifically measures rat HO-1 with no detectable cross reaction with rat HO-2.
It cannot be used for human, rabbit, guinea pig, or mouse sample.
3. Assay duration
Two and half hours after sample incubation.
4. Total assay capacity
96 assays
5. Assay capacity for test samples
If all assay wells (including standards and test samples) are run in duplicate, 40 samples can be run in duplicate per kit.
6. Test specimen type
Rat serum, organ extracts, cell culture supernatant.
7. Specimen volume required
If each test sample is run in duplicate, approximately 220 μ l is required. It is necessary to dilute blood sample of rat about 2 or 3 times.
8. Limitation
Since conditions may vary from assay to assay, a standard curve must be established for every run. Since cross contamination between reagents will invalidate the test, disposable pipette tips should be used.

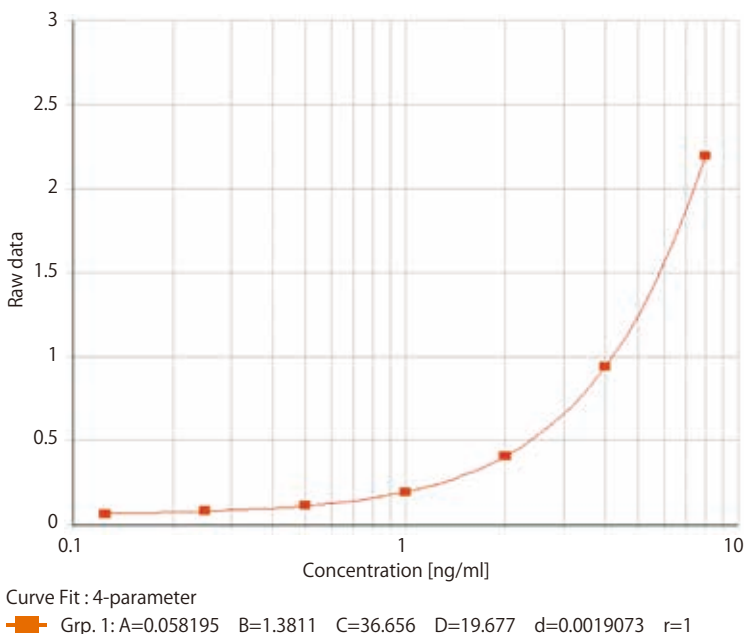
Thorough washing of the wells between incubations is required :
 - 1) Completely empty out the remaining fluid from the well before dispensing fresh wash solution.
 - 2) Use sufficient wash solution for each wash cycle (approximately 400 μ l).
 - 3) Do not allow wells to sit uncovered for extended periods between incubation steps.Only samples with absorbance values falling within the range of the standard curve should be assigned a rat HO-1 concentration from the curve.
9. Note
 - 1) When rat tissue extract or rat serum is used as assay sample, the expression of rat HO-1 largely varies depending on the degree of stress exposed to rat. Therefore, it is recommended to perform the assay using the double dilution, for example, so that the concentration can be obtained from the absorbance values falling within the range of the standard curve.
 - 2) It is recommended that a constant dilution rate be used for the continuous assay series. The values obtained with undiluted samples tend to be lower than those obtained with double dilution. This is due to the effects of the high concentrations of proteins present in serum and tissues.

IX. Performance

1. Typical Standard Curve

The following shows a typical standard curve of this kit as an example. The standard curve for calculation needs to be established in each assay.

Limit of detection: 0.125 ng/ml



HO-1 (ng/ml)	8.000	4.000	2.000	1.000	0.500	0.250	0.125	0.000
A450	2.194	0.938	0.404	0.193	0.112	0.081	0.062	0.050

2. Intra-assay precision (n=16)

Assay was carried out with 16 replicates of 3 samples containing different concentration of rat HO-1 positive samples derived from kidney and brain.

	Average concentration (ng/ml)	Standard deviation (ng/ml)	CV (%)
Sample A	5.646	0.117	2.07
Sample B	3.600	0.079	2.18
Sample C	0.726	0.018	2.52

3. Inter-assay precision (performance 3 times)

Assay to assay precision with one laboratory was evaluated in three independent experiment over three days using 4 different rat HO-1 positive samples derived from kidney and brain.

	Average concentration (ng/ml)	Standard deviation (ng/ml)	CV (%)
Sample A	11.901	0.280	2.35
Sample B	4.441	0.210	4.73
Sample C	2.907	0.016	3.65
Sample D	0.673	0.033	4.87

4. Recovery test

Two samples containing various concentration of rat HO-1 were mixed at the same ratio, and the measurement values and the calculation values were compared.

Sample A	Sample B	A+B/2 Actual measurement	A+B/2 Theoretical value	Recovery rate (%)
8.97	8.97	8.97	8.97	100
8.97	4.96	7.26	6.97	104
8.97	3.60	6.41	6.29	102
8.97	1.73	5.45	5.35	102
4.96	4.96	4.96	4.96	100
4.96	3.60	4.55	4.28	106
4.96	1.73	3.28	3.35	98
3.60	3.60	3.60	3.60	100
3.60	1.73	2.62	2.67	98
3.60	0.66	2.36	2.13	111
1.73	1.73	1.73	1.73	100
1.73	0.66	1.23	1.20	103
0.66	0.66	0.66	0.66	100
0.66	0.00	0.37	0.33	112

(unit: ng/ml)

X. Reference Data about Measurement**1. Antibody specificity**

The two antibodies used in this kit specifically react with rat heme oxygenase-1. They do not cross react with rat heme oxygenase-2. This kit cannot be used for the assay of heme oxygenase-1 of human, rabbit, guinea pig, or mouse.

Immobilized antibody : GTS-3⁴⁾

Labeled antibody : GTS-1^{1), 2), 3)}

2. Antigen stability

Assays were performed on 4 different concentrations of rat HO-1-positive samples after they were subjected to 10 cycles of freezing and thawing over the range of -80°C to +20°C. With each freeze-thaw cycle, 0.3 ml was sampled from the certain consistent volume of sample for the purpose of performing duplicate assay; the measurements were performed all at once after the 10th sampling was completed.

<Results>

The coefficient of variation was $\leq 3\%$ for all 4 concentrations, suggesting that the effects of freeze-thaw cycles on assay results are minimal.

	1	2	3	4	5
Sample A	1.361	1.374	1.372	1.345	1.375
Sample B	2.302	2.306	2.303	2.351	2.289
Sample C	2.668	2.666	2.627	2.634	2.600
Sample D	6.631	6.602	6.494	6.546	6.488
	6	7	8	9	10
	1.364	1.355	1.400	1.390	1.423
	2.346	2.283	2.343	2.339	2.437
	2.643	2.621	2.649	2.675	2.706
	6.431	6.411	6.402	6.556	6.521

The numbers at the upper row means the number of repeated freeze-thaw cycle.

The values are measured concentration on each sample.

	Average concentration (ng/ml)	Standard deviation (ng/ml)	CV (%)
Sample A	1.376	0.024	1.7
Sample B	2.330	0.055	2.4
Sample C	2.649	0.037	1.4
Sample D	6.508	0.090	1.4

3. Effects of hemolysis

The effects of hemolysis on sample assay were evaluated.

<Methods>

Rat spleens and livers were promptly extracted under ether anesthesia, and entire spleens and 1/5 portions of livers were ground separately using a stainless steel mesh in order to disperse the cells. The cells were collected as a pellet by centrifugation at 3,000 rpm using a desktop centrifuge, washed once using 10 ml of PBS, and recollected by centrifugation. After this wash was repeated twice, the 10 ml PBS suspension of rat spleen and liver cells was dispensed into two 15 ml centrifuge tubes in 5 ml per tube.

One tube was subjected to centrifugation, and the supernatant was removed. Five ml of a hemolytic agent containing ammonium chloride was added, and the tube was left at room temperature for 5 minutes in order to allow the erythrocytes present to burst. The cells were collected by spinning, washed with PBS once, and lysed in 1 ml of Extraction Buffer (hemolysis sample).

The other tube was subjected to centrifugation and the cells were collected. Then pellet was lysed in 1 ml of Extraction Buffer (untreated sample). Assays were performed on these samples using the kit.

<Results>

Assay result were Compared between the hemolysis sample from which the erythrocyte component was removed through hemolysis treatment, and the untreated sample. The results show there was no marked inhibition of the reactions due to hemolysis.

		5X	25X	125X	625X	Blank
Spleen	untreated	4.173	1.298	0.164	0.083	0.043
	Hemolyzed	4.000	1.536	0.220	0.082	0.044
Liver	untreated	1.782	0.243	0.073	0.053	0.044
	Hemolyzed	1.575	0.189	0.067	0.053	0.046

Absorbance at 450 nm

4. Effect of cadmium as a stressor

Cadmium exposure test was performed using rat cell culture.

<Method>

1. Cell culture

- 1) NRK49F (rat normal kidney cell) and 3Y1 (rat fibroblast) were added in each 1 ml into 24 well culture plate at the concentration of $5 - 10 \times 10^4$ cell/ml.
- 2) On the day after, 1 mM CdCl₂ was added to have the final concentration of 20 μ M. As the control, PBS was added instead of CdCl₂.
- 3) Three hours later, remove the culture supernatant of each one well from the CdCl₂-added group and PBS-added group, and wash with PBS. Then the cells were dispersed in 0.5 ml of 0.25% Trypsin, and the cell number was counted.

2. Method of cell collection and time course experiment

After exposing the cells to cadmium, cell culture supernatant was collected from each one well from the CdCl₂-added group and PBS-added group in the time course of 3 hours later, 8 hours later, 12 hours later, 22 hours later, 52 hours later, and 72 hours later. Then Extraction Buffer was added in 1 ml/well each well to prepare the cell extraction solution.

<Result>

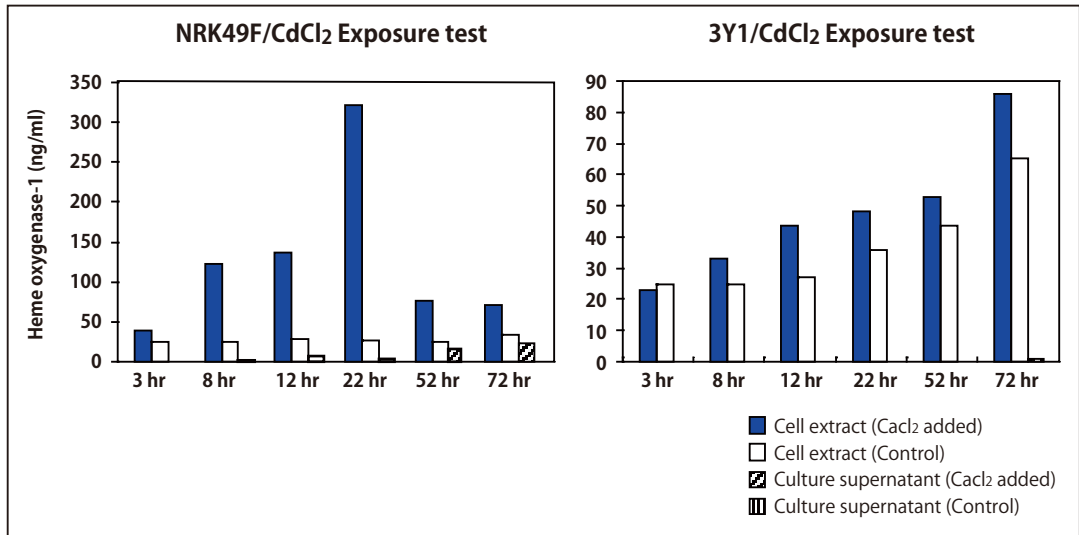
Table A. Cadmium exposure study using cultured cells. (HO-1 yield and cell number.)

[NRK49F]

		3 hr	8 hr	12 hr	22 hr	52 hr	72 hr
HO-1 in cell extract (ng/ml)	Addition CdCl ₂	38.70	123.30	137.37	321.30	76.98	71.01
	Contrast	25.07	25.07	27.97	26.06	25.27	33.69
HO-1 in supernatant (ng/ml)	Addition CdCl ₂	0.00	2.17	6.57	4.29	15.58	23.91
	Contrast	0.00	0.00	0.00	0.00	0.00	0.00
cell number/well	Addition CdCl ₂	90000					430000
	Contrast	90000					440000

[3Y1]

		3 hr	8 hr	12 hr	22 hr	52 hr	72 hr
HO-1 in cell extract (ng/ml)	Addition CdCl ₂	22.84	32.94	43.47	48.15	52.59	85.71
	Contrast	24.66	24.66	27.16	35.64	43.83	65.40
HO-1 in supernatant (ng/ml)	Addition CdCl ₂	0.00	0.00	0.00	0.00	0.00	1.05
	Contrast	0.00	0.00	0.00	0.00	0.00	0.00
cell number/well	Addition CdCl ₂	14000					550000
	Contrast	14000					630000



Graph B: Cadmium exposure test on cultured cells

Table A and Graph B show the concentrations of HO-1 found in each sample. Table A shows the numbers of cells present after 3 hours and 72 hours; there were no significant differences in the numbers of cells between the group to which cadmium was added and the PBS group (control), suggesting that the addition of cadmium (20 μM) does not inhibit cell proliferation. It was observed, however, that the amount of HO-1 produced increased with the addition of cadmium. In NRK49F cells, HO-1 was detected in the culture supernatant from 52 hours onward, and was suspected to have been released into the medium from the cells. The amount of HO-1 produced and the production patterns seen over time following the addition of cadmium were different for the 2 types of cells cultured. This suggests that different types of cells show different stress responses.

5. Amounts of cadmium added and heme oxygenase-1 production

The relationship between the amount of cadmium added and the HO-1 yield was studied using NRK49F cells.

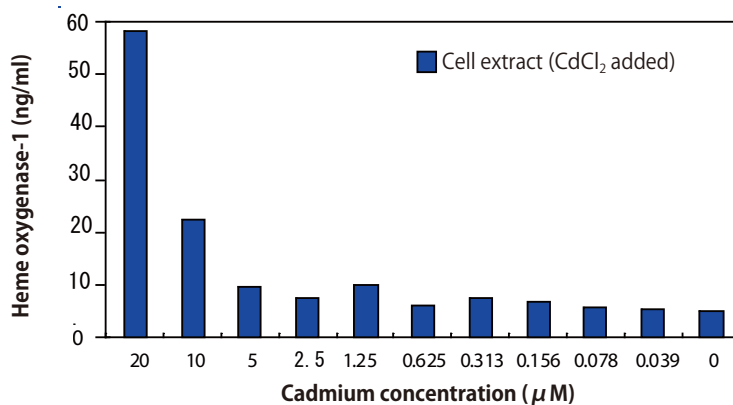
<Methods>

Two-fold serial dilutions of cadmium chloride were prepared, and NRK49F cells (culture volume : 1 ml/well) were cultured in the presence of various concentrations of cadmium chloride (final concentrations of 0 - 20 μ M). The culture supernatant was collected after 16 hours, and the cells were collected and extracted by adding the Extraction Buffer provided in the kit at 1 ml/well. The HO-1 concentration in the cell extract solution and the culture supernatant were measured using the kit.

The amount of CdCl₂ added, and the HO-1 yield of 16 hours after exposure (ng/ml)

		20 μ M	10 μ M	5 μ M	2.5 μ M	1.25 μ M	0.625 μ M
Cell extraction solution	CdCl ₂ Addition	57.900	22.164	9.621	7.476	9.941	6.135
culture supernatant	CdCl ₂ Addition	0.000	0.000	0.000	0.000	0.000	0.000

0.313 μ M	0.156 μ M	0.078 μ M	0.039 μ M	0 μ M
7.740	6.765	5.952	5.448	5.004
0.000	0.000	0.000	0.000	0.000



The above graph shows the assay result of HO-1 in cell extraction solution. There was no production of HO-1 in culture supernatant of 16 hrs. later of CaCl₂ addition.

6. Induction of heme oxygenase-1 in various rat organs by addition of cadmium

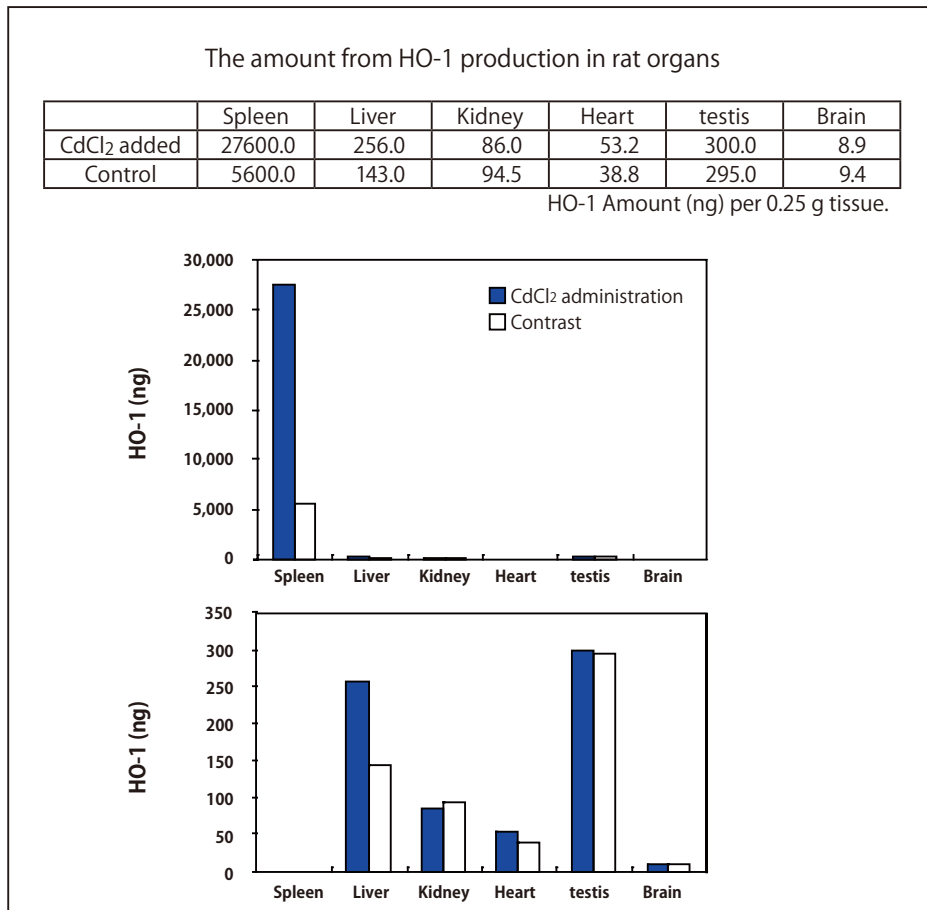
The changes in the amounts of HO-1 produced in various organs were studied using intraperitoneal administration of cadmium chloride as a stressor.

<Methods>

Cadmium chloride (20 μM/kg body weight) or PBS (control) was intraperitoneally administered into rats peritoneal, and various organs were collected after 16 hours under anesthesia. The Extraction Buffer provided in the kit was added to each organ (0.25 g wet organ weight/ml) and the organs were homogenized. The amounts of HO-1 in these homogenates were measured using the kit.

<Result>

The graph below shows that different organs shows different responses.



<Precautions in preparation of rat serum samples>

As the assay target (HO-1) is induced and expressed by stress; the ether anesthesia performed before injection can act as an additional stress factor and consequently may affect the data. In order to eliminate of this effect, control animals receiving anesthesia only should always be included in experiments. The blood collection method used should be thoroughly evaluated when performing blood concentration measurements. If heme oxygenase-1 produced from the tissues surrounding the blood collection site may be detected in measurements by mistake as enzymes present in the blood.

XI. References

- 1) Goda, N., *et al. J Clin Invest.* (1998) **101** (3), 604-612.
- 2) Hayashi, S., *et al. Circ Res.* (1999) **85**, 663-671.
- 3) Makino, N., *et al. Hepatology.* (2001) **33**, 32-42.
- 4) Ozawa, N., *et al. J Clin Invest.* (2002) **109** (4), 457-467.

XII. Related Products

Wash and Stop Solution for ELISA without Sulfuric Acid (Cat. #MK021)
Mouse Heme Oxygenase-1 EIA Kit (Cat. #MK125)

XIII. Precautions

- Do not mix reagents from different kit lots.
- Do not use reagents beyond expiration date on label.
- In order to avoid reagent contamination, use disposable pipette tips and/or pipettes.
- Sodium azide inactivates POD. Solutions containing sodium azide should not be used in this assay.
- Do not expose Substrate Solution to strong light during storage or incubation.
- Avoid contact of Substrate Solution and Stop Solution with skin or mucous membranes. If these reagents come into contact with skin, wash thoroughly with water. Do not pipette by mouth. Do not smoke, eat, or drink in area where specimens or kit reagents are handled. All blood fluids should be considered as potentially infectious.
- Avoid contact of Substrate Solution and Stop Solution with any metal surfaces. Disposable glassware or test tubes are recommended for handling the Substrate Solution. If non-disposable is used, it must be acid washed and thoroughly rinsed with distilled, deionized water.
- Do not use the Substrate Solution if its color is changed to thick blue.

NOTE : This product is for research use only. It is not intended for use in therapeutic or diagnostic procedures for humans or animals. Also, do not use this product as food, cosmetic, or household item, etc.

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