

## Rat Heme Oxygenase-1 EIA Kit (precoated) **Manual**

TaKaRa announces development of a new rat heme oxygenase-1 EIA kit (precoated) for assay of the stress marker enzyme rat heme oxygenase-1.

This kit is a sandwich type ELISA kit using a combination of two types of anti-rat heme oxygenase-1 monoclonal antibodies (GTS-1 and GTS-3). The kit provides convenient assay of rat heme oxygenase-1 in rat serum and tissue as well as in rat culture cells and supernatants (does not assay human antigens). The entire procedure can be performed in 2 hours 30 minutes.

### Heme Oxygenase

Heme oxygenase is an enzyme that degrades heme, particularly the prosthetic group of heme proteins such as hemoglobin, into bile pigments (biliverdin, bilirubin), carbon monoxide, and reduced iron ( $Fe^{2+}$ ). Heme oxygenase is known to exist as at least two isozymes (heme oxygenase-1 and heme oxygenase-2). Heme oxygenase-2 is a constitutive enzyme, whereas heme oxygenase-1 (HO-1) is an enzyme whose expression is induced intracellularly in response to various type of

stress (eg, heavy metals, endotoxins, ultraviolet radiation, heat shock, activated oxygen species, hypoxemia). Bilirubin, produced via degradation of heme by heme oxygenase, has antiinflammatory effects by virtue of action as a potent radical scavenger. In addition, simultaneously produced carbon monoxide stimulates vasodilation and thus helps to maintain organ bloodflow.

### Antibodies Used in Kit

This kit utilizes two monoclonal antibodies (GTS-1 and GTS-3) developed by Professor Makoto Suematsu (Department of Biochemistry, School of Medicine, Keio University) as anti-heme oxygenase-1 antibodies. Both antibodies react to rat and human antigens (see BIO VIEW 7). These antibodies are derived from mouse hybridomas produced using as the immunogen the microsomal fraction of transformed cells (WR19LrHO-1), which express rat heme oxygenase-1. The combination of the two types of antibodies was used to construct a sandwich type ELISA for assay of rat heme oxygenase-1. Because of the nature of the antibodies, the assay is not suitable for human antigens.

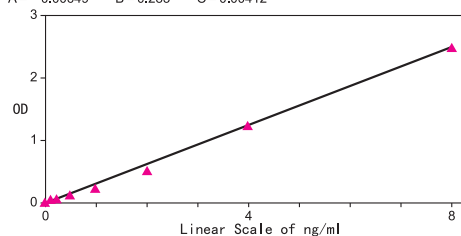
### Kit Contents (for 96 reactions)

Anti-rat HO- F1 monoclonal antibody plate	96 well (8 well x 12 strips)
Peroxidase labeled anti-rat HO-1 antibody (lyophilized)	11 ml at use
Reference product (rat HO-1, lyophilized)	5 ml at use
Sample diluent	11 ml x 2
Substrate solution (TMBZ: 3,3',5,5'-tetramethylbenzidine)	12 ml
Cell extraction buffer	11 ml

### Procedure

- Add 100  $\mu$ l of sample to antibody plate
- ↓ React for 1 h at 20 - 30°C (room temperature)
- Wash 3 times
- ↓ Add 100  $\mu$ l of POD labeled antibody
- ↓ React for 1 h at 20 - 30°C (room temperature)
- Wash 4 times
- ↓ Add 100  $\mu$ l of substrate solution
- ↓ React for 15 min at 20 - 30°C (room temperature)
- Stop reaction and measure absorbance at 450 nm

Curve Fit: Quadratic  
 $y = A + B \cdot x + C \cdot x^2$   
 A = -0.00649 B = 0.283 C = 0.00412  
 Corr. Coeff: 0.999



HO-1 (ng/ml)	8,000	4,000	2,000	1,000	0,500	0,250	0,125	0,000
$A_{450}$	2,512	1,263	0,522	0,222	0,114	0,071	0,039	

Figure 1. Assay range and detection sensitivity

## Performance

(1) Assay range and detection sensitivity (Fig. 1)

(2) Reproducibility

Simultaneous reproducibility: 3 concentrations,  
n = 16, CV = 2.3 - 6.1%

Interday reproducibility: 3 concentrations over 3 days, CV ≤ 8%

(3) Recovery rate: 88 - 111%

## Specificity

There is specific activity against rat heme oxygenase-1; there is no reactivity against heme oxygenase-2.

The kit is not suitable for assay of human, rabbit, guinea pig, or mouse antigen samples.

## Assay Precautions

1) When using rat tissue extracts or rat serum as assay samples, the expression of heme oxygenase-1 can vary considerably with the degree of stress. Prepare these samples as a twofold dilution series and determine the concentrations where they fall within the calibration range.

2) Assay values of stock solutions used as samples tend to be lower than twofold dilution series of samples due to the effects of high protein concentrations in serum or tissue. In a continuing series of experiments, we recommend always performing assays using the same dilution factor.

3) Injection even with ether anesthesia can be a cause of considerable stress. To exclude these effects, always perform a concurrent assay in an anesthetized control animal.

## Example 1: Effects of Hemolysis on Assay

This experiment evaluated whether hemolysis had any effect on assay of samples.

### [Method]

The spleen and liver were rapidly resected from a rat under ether anesthesia. The entire spleen and 1/5 of the liver were each ground with a stainless steel mesh, and the cells were dispersed. These were centrifuged (table top centrifuge, 3000 rpm). The cell sediments were collected, suspended and washed once with 10 ml of PBS, recentrifuged, then the cells were collected. This washing procedure was repeated a total of two times. Then, 5 ml each of the PBS suspensions (10 ml) of the rat spleen and liver cells were dispensed into two 15 ml centrifuge tubes.

One tube of each cell suspension was centrifuged, the supernatant was discarded, and 5 ml of a hemolytic agent containing ammonium chloride was added. These were allowed to stand for 5 min at room temperature to burst the erythrocytes. The samples were centrifuged and the cells were collected. The cells were washed once with PBS and then suspended by adding 1 ml of cell extract buffer (hemolyzed samples).

The other tube of each cell suspension was centrifuged only and the cells were collected.

The cells were suspended by adding 1 ml of cell extract buffer (untreated samples).

The above samples were assayed using this kit.

### [Results]

As shown in Table 1, there was no marked inhibition of the reactions due to hemolysis.

Table 1

Sample dilution factor		x 5	x 25	x 125	x 625	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

\* The results are A450 values.

## Example 2: Effect of Cadmium as a Stressor (Cadmium Exposure Test with Cultured Rat Cells)

### [Method]

This experiment was performed using two types of cultured cells: NRK49F cells (normal rat kidney cells) and 3Y1 cells (rat

fibroblasts).

The cells were suspended in medium, 1 ml each was added to a well of a 24-well plate, and cadmium chloride was added to a final concentration of 20 μM (PBS was used as the control). The cells were

The cells were cultured for 72 hours. The culture supernatants in the wells were collected over time, and the cells were collected and extracted. Cell collection and extraction was performed, after removing the supernatants from the wells, by adding 1 ml of cell extract buffer (supplied with the kit) to the wells and gently pipetting. Each sample was stored at -20°C until assay.

The samples for each time point (supernatants and cell extracts) were all collected, and heme oxygenase-1 production in each sample was assayed at one time using the kit.

### [Results]

Table 2 shows the cell counts at the time cadmium (or PBS) was added and 72 hours later. The measured values of heme oxygenase-1 in each sample are depicted in Fig. 2.

As shown in Table 2, there were no marked differences in cell counts between the cadmium group and PBS group (control). These results indicated the lack of growth inhibition by cadmium (20 µM).

As depicted in Fig. 2, treatment with cadmium increased the production of heme oxygenase-1. However, the amount of heme oxygenase-1 produced in response to cadmium and the pattern of production over time differed between the two types of cultured cells. The results showed a difference in the stress response between the cell types.

In addition, for the NRK49F cells, heme oxygenase-1 was detected in the culture supernatants 52 hours and later after the addition of cadmium. This finding suggested release from the cells into the culture medium.

Table 2. Cell counts when cadmium added and 72 hours later.

	Cadmium added (0 hr)	After 72 hours	
		Cadmium group	PBS group (control)
NRK49F	9 x 10 <sup>4</sup>	43 x 10 <sup>4</sup>	44 x 10 <sup>4</sup>
3Y1	1.4 x 10 <sup>4</sup>	55 x 10 <sup>4</sup>	63 x 10 <sup>4</sup>

\* Values are cell counts in one well (1 ml of medium).

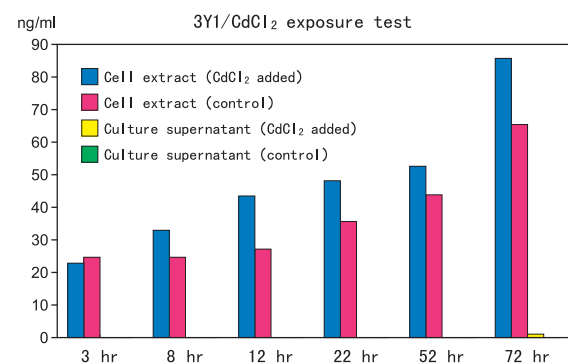
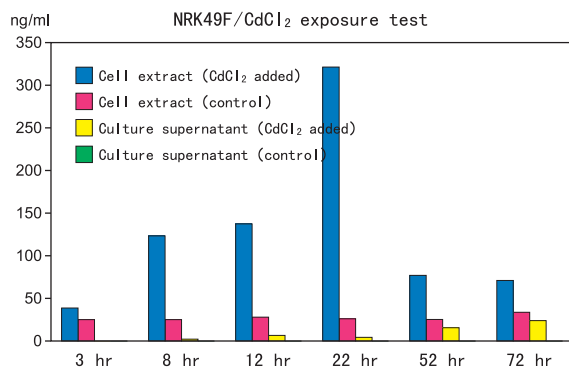


Figure 2. Cadmium exposure test on cultured cells. The vertical axis denotes values of heme oxygenase-1 production (ng/ml).

### Example 3: Concentration of Cadmium and Production of Heme Oxygenase-1

This experiment was performed with NRK49F cells (normal rat kidney cells) to evaluate the relation between the added concentration of cadmium and production of heme oxygenase-1.

A twofold dilution series of cadmium chloride was prepared. The cadmium chloride was added at final concentrations of 0 - 20  $\mu$ M and incubated with the NRK49F cells (1 ml of medium per well). After 16 hours, the culture supernatant was collected, and the cells were collected and extracted by adding 1 ml of cell extract buffer supplied with the kit to each well. The kit was used to assay heme oxygenase-1 in the cell extracts and supernatants.

#### [Results]

Fig. 3 shows the assay results of heme oxygenase-1 in the cell extracts. There was no detection of heme oxygenase-1 in the culture supernatants after 16 hours.

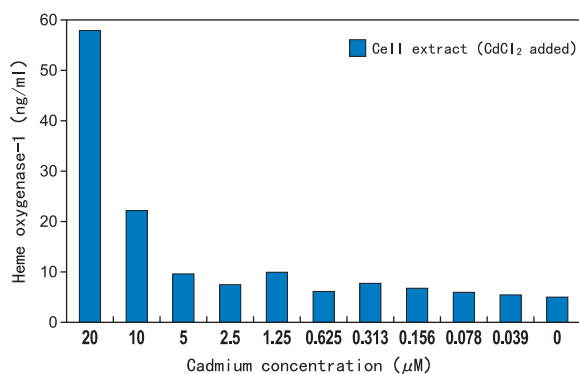


Figure 3. Relation of heme oxygenase-1 production to cadmium concentration in NRK49F cells

### Example 4: Induction of Heme Oxygenase-1 in Various Rat Organs by Cadmium Administration

This experiment evaluated changes in production of heme oxygenase-1 in various organs after intraperitoneal administration of cadmium chloride as a stressor to rats.

#### [Method]

Cadmium chloride (20  $\mu$ mol/kg body weight) or PBS (control) was administered intraperitoneally to rats. After 16 hours, the animals were anesthetized, and various organs were resected. The cell extraction buffer supplied with the kit was added to each organ (0.25 mg wet tissue weight/ml), and the organs were homogenized. The kit was used to assay heme oxygenase-1 in the homogenates.

#### [Results]

Fig. 4 depicts the assay results.

The results indicate a difference in stress responses of each organ.

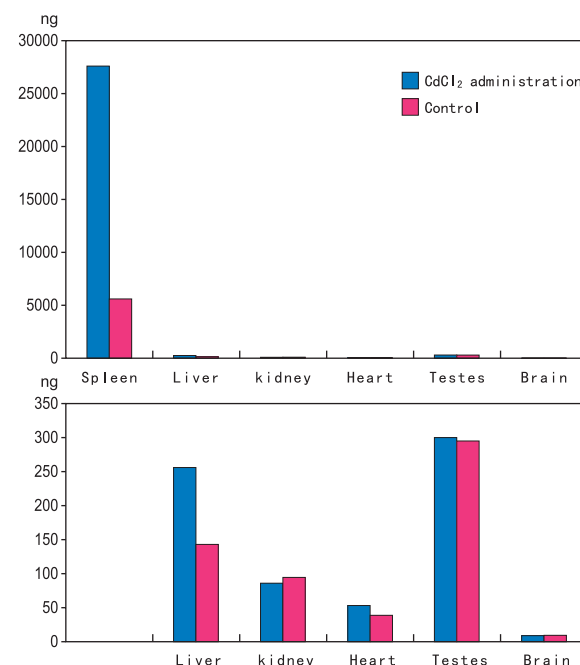


Figure 4. Heme oxygenase-1 in rat organs  
The vertical axis shows the values for heme oxygenase-1 (ng) per 0.25 mg of tissue (wet weight).