YK200 Mouse/Rat Urocortin 3 EIA

FOR LABORATORY USE ONLY

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Contents

- Please read all the package insert carefully before beginning the assay -

YK200　Mouse/Rat Urocortin 3 EIA Kit

Ⅰ. Introduction

Urocortin 3 (Ucn3) or stresscopin (SCP) is a new member of the corticotropin-releasing factor (CRF) peptide family identified in the mouse and human.[1)] The CRF family of neuropeptides includes mammalian peptides CRF, urocortin 1(Ucn1) and urocortin 2 (Ucn2) or stress-related peptide (SRP), as well as piscine urotensin 1 and frog sauvagine. Mouse and human Ucn3 share 90% identity in the 38-aa putative mature peptide.

In the human, Ucn1 immunoreactivity was marked in the medulla, whereas Ucn3 was immunostained mostly in the cortex. [2)] The receptors for Ucn1, Ucn2, Ucn3 and CRF are all expressed in human adrenal cortex and medulla[2)], therefore these peptides are expected to play important roles in physiological adrenal functions.[2)] Ucn3 was also detected by RIA in human heart 0.74-1.15 pmol/g wet weight, kidney 1.21 pmol /g wet weight, pituitary 2.72 pmol /g wet weight and brain tissues 1-2 pmol /g wet weight.[3)] Furthermore, immunoreactive Ucn3 was present in human plasma 51.8 pmol/L and urine 266 pmol/L obtained from healthy subjects.[3)] It was also detected in human rectum 15.4 pmol/g wet weight and sigmoid colon 6.5 pmol/g wet weight.[4)] These data suggest that Ucn3 regulates the cardiac and renal functions as a local factor and a circulating hormone and plays some physiological or pathological roles in the modulation of gastrointestinal functions during stressful conditions in different manners from those of Ucn1.[4)]

Pharmacological studies showed that Ucn3 is a high-affinity ligand for the type 2 CRF receptor (CRFR2). In the rat, Ucn3-positive neurons were found predominantly within the hypothalamus and medial amygdala.[5)] Ucn3 fibers were distributed mainly in the hypothalamus and limbic structures.[5)] These data support that Ucn3 is an endogenous ligand for CRFR2 in these areas. The results also suggest that Ucn3 is positioned to play a role in mediating physiological functions, including food intake and neuroendocrine regulation.[5)]

In the mouse, Ucn3 was expressed in pancreatic beta-cells and in a mouse beta cell line, MIN6. High potassium, forskolin or high glucose could stimulate Ucn3 secretion from these cells.[6)] Ucn3 injections to the rat resulted in significant increase of plasma insulin level.[6)] Ucn3 also stimulated glucagon and insulin release from isolated rat islets.[6)] Pancreatic Ucn3 acting through CRFR2 was suggested to be involved in the local regulation of glucagon and insulin secretion.[6)]

Treatment with Ucn3 (SCP) or Ucn2 (SRP) suppressed food intake, delayed gastric emptying and decreased heat-induced edema.[7)] Thus Ucn3 (SCP) and Ucn2 (SRP) might represent endogenous ligands for maintaining homeostasis after stress, and could allow the design of drugs to ameliorate stress-related diseases.[7)] The use of CRFR2 selective agonists, Ucn2 and Ucn3, to treat ischemic heart disease was proposed because of their potent cardioprotective effects in murine heart and their minimal impact on the hypothalamic stress axis.[8)]

Ucn1 is able to bind to two types of G-protein coupled receptors: CRFR1 and CRFR2, whereas Ucn3 (SCP) and Ucn2 (SRP) bind exclusively and with high affinity to CRFR2. [9)] Ucn3 (SCP) is expressed in rat cardiomyocytes and the levels of Ucn3 (SCP) and Ucn2 (SRP) were increased by hypoxic stress.[8)] All these three peptide were shown to have potent cardioprotective effects in cells exposed to hypoxia/reoxygenation. [9)]

We have already developed mouse/rat urocortin 1 EIA kit (YK210), mouse urocortin 2 EIA kit (YK190) and rat urocortin 2 EIA kit (YK191). This time, as a part of tools for urocortin research, our laboratory

developed mouse/rat urocortin 3 EIA kit (YK200), which is highly specific for mouse/rat urocortin 3 with almost no crossreaction to Ucn1 (mouse, rat), Ucn1 (human), Ucn2 (mouse), Ucn2 (rat), ACTH (mouse, rat), ACTH (human) and CRF (mouse, rat, human). The kit can be used for measurement of Ucn3 in mouse/rat plasma, serum and their brain tissue extracts with high sensitivity (The brain tissue extracts need to be treated with solid-phase extraction cartridges). It will be a specifically useful tool for Ucn3 researches.

YK200 Mouse/Rat Urocortin 3 EIA Kit

- ▼ The assay kit can measure mouse/rat urocortin 3 within the range of 0.41~100 ng/mL.
- ▼ The assay is completed within 16~18 hr.＋3 hr.
- ▼ With one assay kit, 41 samples can be measured in duplicate.
- ▼ Test sample: Mouse/rat plasma and serum, brain tissue extracts (The brain tissue extracts need to be treated with solid-phase extraction cartridges).
  Sample volume: 25 μL
- ▼ The 96-wells plate in kit is consisted by 8-wells strips, and the strips can be used separately.
- ▼ Stability and Storage
  Store all of the components at 2~8ºC.
  The kit is stable under the condition for 24 months from the date of manufacturing.
  The expiry date is stated on the label of kit.

Contents

1) Antibody coated plate
2) Standard
3) Labeled antigen
4) SA-HRP solution
5) Substrate buffer
6) OPD tablet
7) Stopping solution
8) Buffer solution
9) Washing solution (concentrated)
10) Adhesive foil

II. Characteristics

This EIA kit is used for quantitative determination of urocortin 3 in mouse/rat plasma, serum and their brain tissue extracts. The kit is characterized for sensitive quantification, high specificity and no influence with other components in samples. Mouse/rat urocortin 3 standard is highly purified synthetic product.

< Specificity >

This EIA kit has high specificity to mouse/rat urocortin 3 and shows no crossreactivity to urocortin 1 (mouse, rat), urocortin 1 (human), urocortin 2 (mouse), urocortin 2 (rat), ACTH (mouse, rat), ACTH (human) and CRF (mouse, rat, human).

< Assay principle >

This EIA kit for determination of mouse/rat urocortin 3 in samples is based on a competitive enzyme immunoassay using combination of highly specific antibody to mouse/rat urocortin 3 with biotin-avidin affinity system. To the wells of plate coated with rabbit anti mouse/rat urocortin 3 antibody, standards or samples and labeled antigen (biotinylated antigen) are added for competitive immunoreaction. After incubation and plate washing, horseradish peroxidase (HRP) labeled streptoavidin (SA) is added to form HRP labeled SA-labeled antigen-antibody complex on the surface of the wells. Finally, HRP enzyme activity is determined by o-Phenylenediamine dihydrochloride (OPD) and the concentration of mouse/rat urocortin 3 is calculated.

Ⅲ. Composition

	Component	Form	Quantity	Main Ingredient
1.	Antibody coated plate	microtiter plate	1 plate (96 wells)	Rabbit anti mouse /rat urocortin 3 antibody coated
2.	Standard	lyophilized	1 vial (100 ng)	Synthetic mouse/rat urocortin 3
3.	Labeled antigen	lyophilized	1 vial	Biotinylated mouse/rat urocortin 3
4.	SA-HRP solution	liquid	1 bottle (12mL)	Horseradish peroxidase labeled streptoavidin
5.	Substrate buffer	liquid	1 bottle (24 mL)	0.015% Hydrogen peroxide
6.	OPD tablet	tablet	2 tablets	o-Phenylenediamine dihydrochloride
7.	Stopping solution	liquid	1 bottle (12 mL)	1M H_2SO_4
8.	Buffer solution	liquid	1 bottle (15 mL)	Citrate buffer
9.	Washing solution (concentrated)	liquid	1 bottle (50 mL)	Concentrated saline
10.	Adhesive foil		3 pieces	

IV. Method

< Equipment required >

1. Photometer for microtiter plate (plate reader) which can read extinction 2.5 at 490 nm (or 492 nm)
2. Microtiter plate shaker
3. Washing device for microtiter plate and dispenser with aspiration system
4. Micropipettes, multi-channel pipettes for 8 wells or 12 wells and their tips
5. Glass test tubes for preparation of standard solution
6. Graduated cylinder (1,000 mL)
7. Distilled water or deionized water

<Preparation of assay sample>

1. Extraction method of mouse and rat brain tissue:
   Materials: Mouse and rat brain tissue
   Extraction buffer: 10 mM PBS (pH 7.2) containing 0.2% Nonidet P-40
   Extraction column: Oasis HLB 3 cc (60 mg) extraction cartridge (Part No.WAT094226, Waters)
   Extraction maniholde: Waters
   Elution buffer: Acetonitrile-0.075%TFA (80:20,vol/vol)
   Methods:
   1) Mouse and rat brain tissue is weighed and then homogenized in 15-fold volume of extraction buffer in an ice bath. The homogenate is centrifuged in plastic tubes (18,360 x g, 20 min) at 4ºC, and the supernatant is transferred into a glass tube in an ice bath.
   2) Methanol (6 mL) is applied onto an extraction column for conditioning, and then drained by aspiration (2 mL/min). The column is equilibrated twice with distilled water (3 mL each) and the supernatant above mentioned is applied onto the column with a pipette (for example 2 mL). The volume of the supernatant applied should be recorded. The column is aspirated slowly then washed twice with distilled water (3 mL each) and finally eluted with elution buffer (2 mL). The eluate is collected in a glass tube and dried in a centrifugal vaporizer. The mouse and rat brain extracts (dry residue) should be used as soon as possible after drying. If the dry residue is tested later, they should be stored at or below –30ºC until assay.
   3) The dry residue (sample for assay) is reconstituted with buffer solution in kit (75% volume of supernatant volume applied onto the column that recorded, for example 1.5 mL). The insoluble material should be removed by centrifugation（1,750 x g,15 min）at 4ºC and the sample solution is submitted to assay immediately.
2. Collection of mouse and rat plasma:
   EDTA-2Na (1 mg/mL) additive blood collection tube is recommended for plasma sample collection. It is strongly recommended that plasma samples should be used as soon as possible after collection. If the samples are tested later, they should be divided into test tubes in small amount and frozen at or below -30°C.

< Preparatory work >

1. Preparation of standard solution:
   Reconstitute the mouse/rat urocortin 3 standard with 1 mL of buffer solution, which affords 100 ng/mL standard solution. The reconstituted standard solution (0.1mL) is diluted with 0.2 mL of buffer solution that yields 33.3 ng/mL standard solution. Repeat the dilution procedure to make each standard solution of 11.1, 3.70, 1.23 and 0.41 ng/mL. Buffer solution itself is used as 0ng/mL standard solution.
   If a sample concentration below 0.41 ng/mL is predicted, standard curve may be further set up a lower detection limit by using 0.137 ng/mL standard solution which can be prepared by 3-fold dilution of 0.41 ng/mL standard solution. In such case, however, assay precision may not be so excellent as that of the cases between 0.41 and 100 ng/mL.
2. Preparation of labeled antigen:
   Reconstitute labeled antigen with 6 mL of distilled water.
3. Preparation of substrate solution:
   Dissolve one OPD tablet with 11 mL of substrate buffer. It should be prepared immediately before use.
4. Preparation of washing solution:
   Dilute 50 mL of washing solution (concentrated) to 1,000 mL with distilled or deionized water.
5. Other reagents are ready for use.

< Procedure >

1. Before starting the assay, bring all the reagents and samples to room temperature (20 ~ 30ºC).
2. Fill 0.35 mL/well of washing solution into the wells and aspirate the washing solution in the wells. Repeat this washing procedure further twice (total 3 times). Finally, invert the plate and tap it onto an absorbent surface, such as paper toweling, to ensure blotting free of most residual washing solution.
3. Add 25 μL of buffer solution to the wells first, then introduce 25 μL of each of standard solutions (0, 0.41, 1.23, 3.70, 11.1, 33.3 and 100 ng/mL) or samples and finally add 50 μL of labeled antigen to the wells. The total pipetting time of standard solutions and samples for a whole plate should not exceed 30 minutes.
4. Cover the plate with adhesive foil and incubate it at 4ºC for 16~18 hours (keep still, plate shaker not need).
5. After incubation, move the plate back to room temperature keeping for approximately 40 minutes (keep still, plate shaker not need) and take off the adhesive foil, aspirate and wash the wells 4 times with 0.35 mL/well of washing solution. Finally, invert the plate and tap it onto an absorbent surface, such as paper toweling, to ensure blotting free of most residual washing solution.
6. Add 100 μL of SA-HRP solution to each of the wells.
7. Cover the plate with adhesive foil and incubate it at room temperature for 2 hours. During the incubation, the plate should be shaken with a plate shaker (approximately 100 rpm).
8. Dissolve one OPD tablet with 11 mL of substrate buffer. It should be prepared immediately before use.
9. Take off the adhesive foil, aspirate and wash the wells 4 times with 0.35 mL/well of washing

solution. Finally, invert the plate and tap it onto an absorbent surface, such as paper toweling, to ensure blotting free of most residual washing solution.

10. Add 100 μL of substrate solution to each of the wells, cover the plate with adhesive foil and keep it for 20 minutes at room temperature (keep still, plate shaker not need).
11. Add 100 μL of stopping solution to each of the wells to stop color reaction.
12. Read the optical absorbance of the wells at 490 nm (or 492 nm). The dose-response curve of this assay fits best to a 4 (or 5)-parameter logistic equation. The results of unknown samples can be calculated with any computer program having a 4 (or 5)-parameter logistic function. Otherwise calculate mean absorbance values of wells containing standards and plot a standard curve on semi logarithmic graph paper (abscissa: concentration of standard; ordinate: absorbance values). Use the average absorbance of each sample to determine the corresponding value by simple interpolation from this standard curve.

V. Notes

1. Plasma and serum must be used as soon as possible after collection. If the samples are tested later, they should be divided into test tubes in small amount and frozen at or below -30ºC. Avoid repeated freezing and thawing of samples. EDTA-2Na (1 mg/mL) additive blood collection tube is recommended for the plasma. The mouse and rat brain extracts (dry residue) should be used as soon as possible after drying. If the dry residue is tested later, they should be stored at or below -30ºC until assay.

2. Standard, labeled antigen and substrate solution should be prepared immediately before use. This kit can be used dividedly in strips of the plate. In such case, reconstituted standard solution and labeled antigen should be divided into test tubes in small amount and stored at or below -30ºC (stable for 1 month).

3. The total pipetting time of standard solutions and samples for a whole plate should not exceed 30 minutes.

4. During storage of washing solution (concentrated) at 2~8ºC, precipitates may be observed, however they will be dissolved when diluted. Diluted washing solution is stable for 6 months at 2~8ºC.

5. Pipetting operations may affect the precision of the assay, so that pipette standard solutions or samples precisely into each well of plate. In addition, use clean test tubes or vessels in assay and use new tip for each standard or sample to avoid cross contamination.

6. When sample concentration exceeds 100 ng/mL, it needs to be diluted with buffer solution to proper concentration.

7. During the incubation with SA-HRP solution at room temperature, the assay plate should be shaken gently by a plate shaker to promote immunoreaction (approximately 100 rpm).

8. Perform all the determination in duplicate.

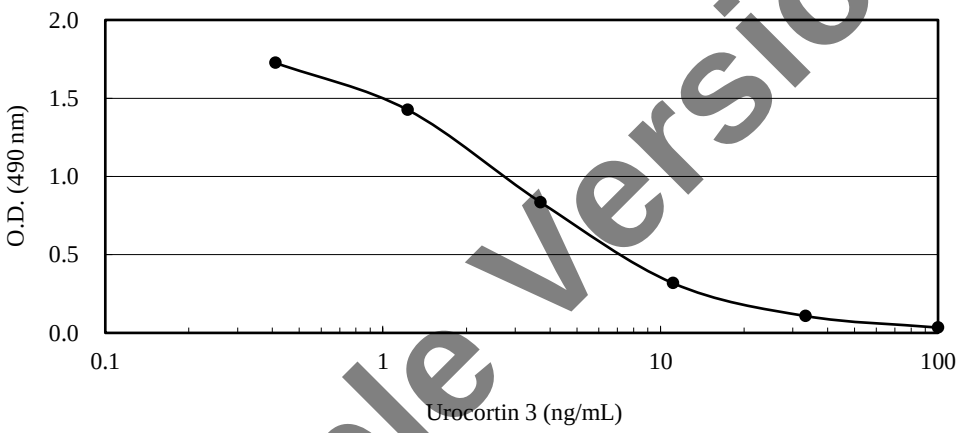
9. Read plate optical absorbance of reaction solution in wells as soon as possible after stop color reaction.

10. To quantitate accurately, always run a standard curve when testing samples.

11. Protect reagents from strong light (e.g. direct sunlight) during storage and assay.
12. Satisfactory performance of the test is guaranteed only when reagents are used from combination pack with identical lot number.

VI. Performance Characteristics

Typical standard curve



<Analytical recovery>

<Mouse plasma A>

Added Urocortin 3 (ng/ml)	Observed (ng/ml)	Expected (ng/ml)	Recovery (%)
0.0	0.38		
1.0	1.10	1.38	79.71
5.0	4.27	5.38	79.37
30.0	21.97	30.38	72.32
50.0	53.77	50.38	106.73

<Mouse plasma B>

Added Urocortin 3 (ng/ml)	Observed (ng/ml)	Expected (ng/ml)	Recovery (%)
0.0	0.26		
1.0	1.06	1.26	84.13

5.0	4.77	5.26	90.68
30.0	26.05	30.26	86.09
50.0	46.42	50.26	92.36

<Mouse plasma C>

Added Urocortin 3 (ng/ml)	Observed (ng/ml)	Expected (ng/ml)	Recovery (%)
0.0	0.31		
1.0	1.12	1.31	85.50
5.0	4.22	5.31	79.47
30.0	26.41	30.31	87.13
50.0	49.52	50.31	98.43

<Mouse plasma D>

Added Urocortin 3 (ng/ml)	Observed (ng/ml)	Expected (ng/ml)	Recovery (%)
0.0	0.34		
1.0	1.08	1.34	80.60
5.0	4.24	5.34	79.40
30.0	22.40	30.34	73.83
50.0	52.38	50.34	104.05

<Mouse serum A>

Added Urocortin 3 (ng/ml)	Observed (ng/ml)	Expected (ng/ml)	Recovery (%)
0.0	0.77		
1.0	1.32	1.77	74.58
5.0	5.63	5.77	97.57
30.0	25.91	30.77	84.21
50.0	45.58	50.77	89.78

<Mouse serum B>

Added Urocortin 3 (ng/ml)	Observed (ng/ml)	Expected (ng/ml)	Recovery (%)
0.0	0.40		
1.0	1.74	1.40	124.29
5.0	5.66	5.40	104.81
30.0	25.68	30.40	84.47
50.0	38.73	50.40	76.85

<Mouse serum C>

Added Urocortin 3 (ng/ml)	Observed (ng/ml)	Expected (ng/ml)	Recovery (%)
0.0	0.43		
1.0	1.31	1.43	91.61
5.0	5.55	5.43	102.21
30.0	27.46	30.43	90.24
50.0	35.78	50.43	70.95

<Mouse serum D>

Added Urocortin 3 (ng/ml)	Observed (ng/ml)	Expected (ng/ml)	Recovery (%)
0.0	0.46		
1.0	1.42	1.46	97.26
5.0	5.27	5.46	96.52
30.0	27.84	30.46	91.40
50.0	37.87	50.46	75.05

<Rat plasma A>

Added Urocortin 3 (ng/ml)	Observed (ng/ml)	Expected (ng/ml)	Recovery (%)
0.0	0.32		
1.0	1.48	1.32	112.12
5.0	4.90	5.32	92.11
30.0	27.43	30.32	90.47
50.0	52.62	50.32	104.57

<Rat plasma B>

Added Urocortin 3 (ng/ml)	Observed (ng/ml)	Expected (ng/ml)	Recovery (%)
0.0	0.58		
1.0	1.41	1.58	89.24
5.0	5.31	5.58	95.16
30.0	29.84	30.58	97.58
50.0	56.69	50.58	112.08

<Rat plasma C>

Added Urocortin 3 (ng/ml)	Observed (ng/ml)	Expected (ng/ml)	Recovery (%)
0.0	0.54		
1.0	1.56	1.54	101.30
5.0	4.88	5.54	88.09
30.0	30.88	30.54	101.11
50.0	64.49	50.54	127.60

<Rat plasma D>

Added Urocortin 3 (ng/ml)	Observed (ng/ml)	Expected (ng/ml)	Recovery (%)
0.0	0.79		
1.0	1.81	1.79	101.12
5.0	5.41	5.79	93.44
30.0	28.68	30.79	93.15
50.0	66.94	50.79	131.80

<Rat serum A>

Added Urocortin 3 (ng/ml)	Observed (ng/ml)	Expected (ng/ml)	Recovery (%)
0.0	0.17		
1.0	1.18	1.17	100.85
5.0	4.03	5.17	77.95
30.0	27.80	30.17	92.14
50.0	61.76	50.17	123.10

<Rat serum B>

Added Urocortin 3 (ng/ml)	Observed (ng/ml)	Expected (ng/ml)	Recovery (%)
0.0	0.21		
1.0	1.04	1.21	85.95
5.0	4.80	5.21	92.13
30.0	28.89	30.21	95.63
50.0	62.71	50.21	124.90

<Rat serum C>

Added Urocortin 3 (ng/ml)	Observed (ng/ml)	Expected (ng/ml)	Recovery (%)
0.0	0.27		
1.0	1.15	1.27	90.55
5.0	4.50	5.27	85.39
30.0	27.48	30.27	90.78
50.0	73.87	50.27	146.95

<Rat serum D>

Added Urocortin 3 (ng/ml)	Observed (ng/ml)	Expected (ng/ml)	Recovery (%)
0.0	0.14		
1.0	1.03	1.14	90.35
5.0	3.81	5.14	74.12
30.0	23.14	30.14	76.78
50.0	59.77	50.14	119.21

<Mouse brain>

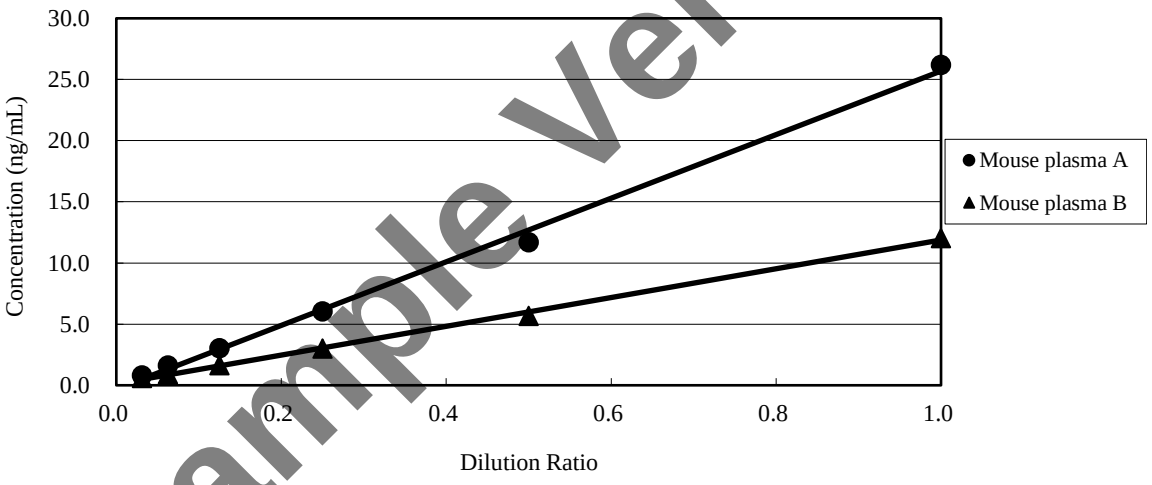
Added Urocortin 3 (ng/ml)	Observed (ng/ml)	Expected (ng/ml)	Recovery (%)
0.0		0.27	
1.0	0.80	0.77	103.90
5.0	4.95	5.27	93.93
30.0	31.17	30.27	102.97

<Rat brain>

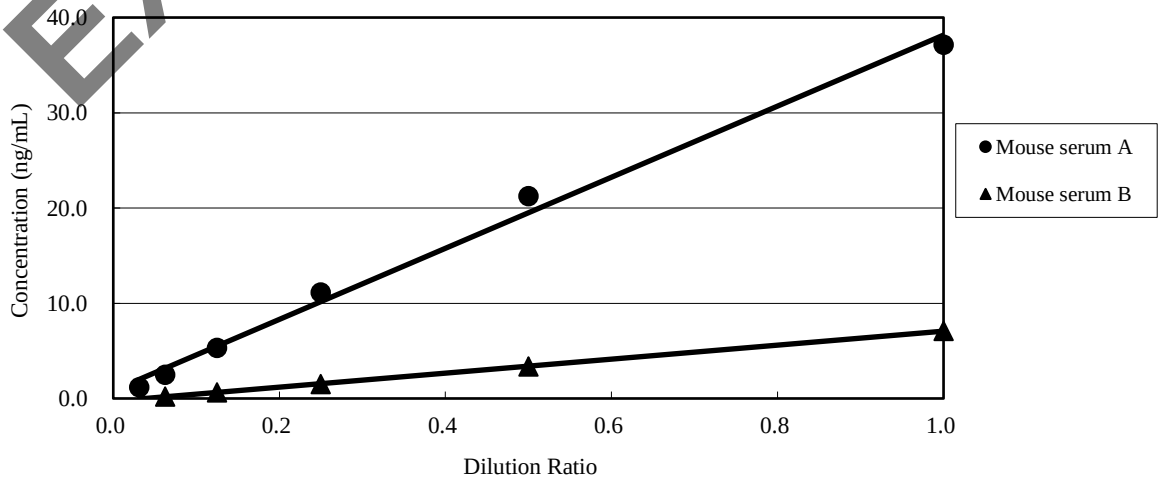
Added Urocortin 3 (ng/ml)	Observed (ng/ml)	Expected (ng/ml)	Recovery (%)
0.0	0.22		
1.0	0.69	0.72	95.83
5.0	4.24	5.22	81.23
30.0	26.93	30.22	89.11

< Dilution test >

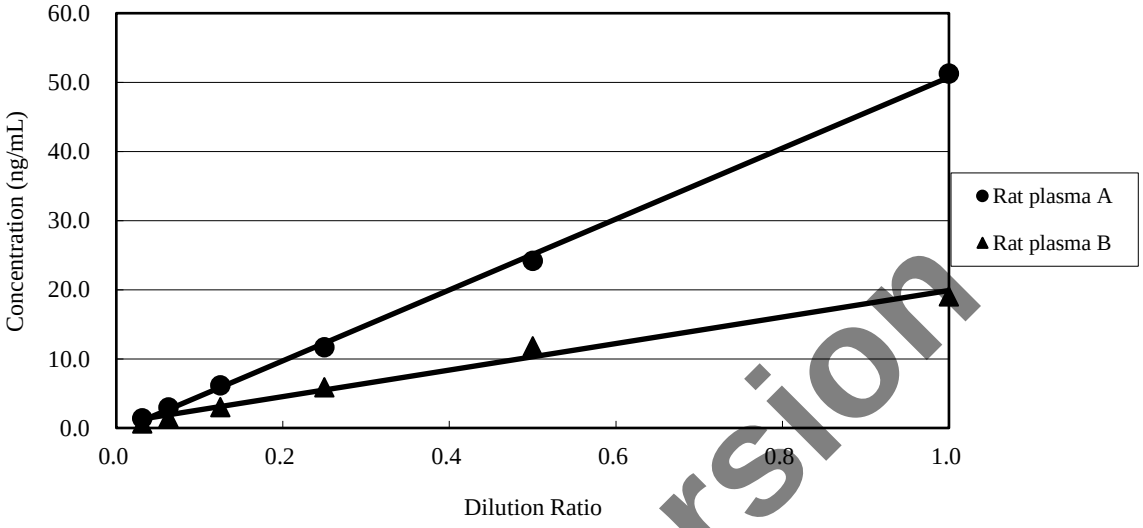
<Mouse plasma>



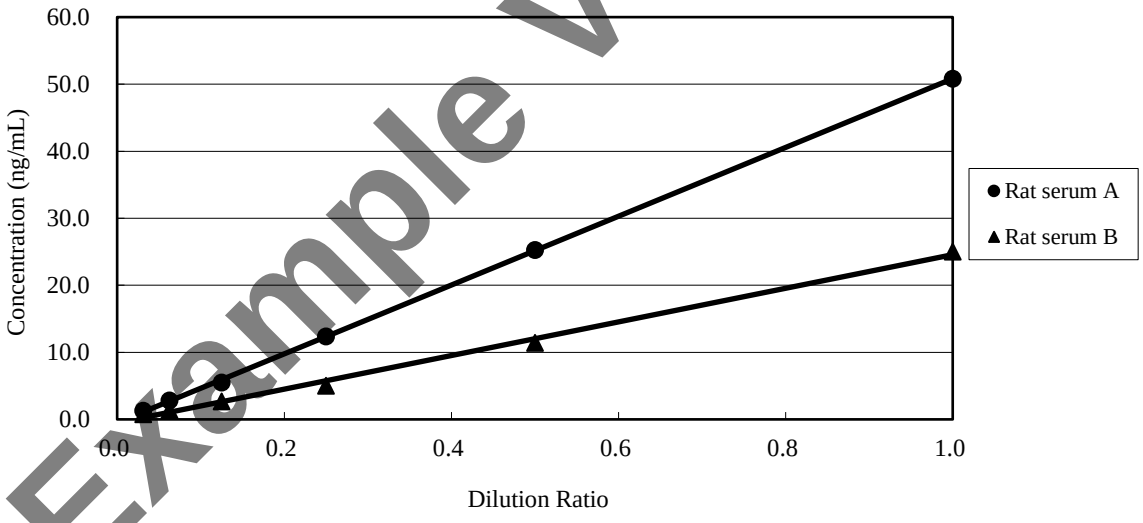
<Mouse serum>



<Rat plasma>



<Rat serum>



<Crossreactivity>

Related peptides	Crossreactivity (%)
Urocortin 3 (mouse, rat)	100
Urocortin 1 (mouse, rat)	0
Urocortin 1 (human)	0.04
Urocortin 2 (mouse)	0
Urocortin 2 (rat)	0
ACTH (mouse, rat)	0.03
ACTH (human)	0.03
CRF (mouse, rat, human)	0.01

<Precision and reproducibility>

Test sample	Intra-assay CV(%)	Inter-assay CV(%)
Mouse plasma	6.13~12.35	2.50~ 9.33
Mouse serum	5.10~13.58	5.69~10.24
Rat plasma	10.51~15.50	14.62~23.42
Rat serum	8.32~13.15	11.29~16.93

<Assay range>
0.41～100 ng/mL

VII. Stability and Storage

< Storage > Store all of the components at 2~8ºC.

< Shelf life > The kit is stable under the condition for 24 months from the date of manufacturing. The expiry date is stated on the label of kit.

< Package > For 96 tests per one kit including standards

VIII. References

1. Lewis K, Li C et al: (2001) Identification of urocortin III, an additional member of the corticotropin-releasing factor (CRF) family with high affinity for the CRF2 receptor. *Proc Natl Acad Sci USA*. ***98***, 7570-7575
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<Manufacturer>
Yanaihara Institute Inc.

Update at Dec. 1, 2010