KAMIYA BIOMEDICAL COMPANY

Mouse and Rat Urocortin 3 EIA

For the quantitative determination of Urocortin 3 in mouse and rat plasma, serum or brain tissue extracts.

Cat. No. KT-377

For Research Use Only.

PRODUCT INFORMATION

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INTENDED USE
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INTRODUCTION
Urocortin 3 (Ucn3) or stresscopin (SCP) is a new member of the corticotropin-releasing factor (CRF) peptide family identified in mice and humans. The CRF family of neuropeptides includes mammalian peptides CRF, Urocortin 1 (Ucn1) and Urocortin 2 (Ucn2) or Stress-Related Peptide (SRP), as well as pig Urotensin 1 and frog sauvagine. Mouse and human Ucn3 share 90% identity in the 38-aa putative mature peptide.

In humans, Ucn1 immunoreactivity was marked in the medulla, whereas Ucn3 was immunostained mostly in the cortex. The receptors for Ucn1, Ucn2, Ucn3 and CRF are all expressed in the human adrenal cortex and medulla, therefore these peptides are expected to play important roles in physiological adrenal functions. Ucn3 was also detected by RIA in the human heart at 0.74-1.15 pmol/g wet weight, kidney at 1.21 pmol/g wet weight, pituitary at 2.72 pmol/g wet weight and brain tissues at 1-2 pmol/g wet weight. Furthermore, immunoreactive Ucn3 was present in human plasma at 51.8 pmol/L and in urine at 266 pmol/L, obtained from healthy subjects. It was also detected in human rectum at 15.4 pmol/g wet weight and sigmoid colon at 6.5 pmol/g wet weight. This data suggests that Ucn3 regulates the cardiac and renal functions as a local factor and as 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.

Pharmacological studies showed that Ucn3 is a high-affinity ligand for the type 2 CRF Receptor (CRFR2). In rats, Ucn3-positive neurons were found predominantly within the hypothalamus and medial amygdala. Ucn3 fibers were distributed mainly in the hypothalamus and limbic structures. This data supports 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.

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

Treatment with Ucn3 (SCP) or Ucn2 (SRP) suppressed food intake, delayed gastric emptying and decreased heat-induced edema. 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. The use of CRFR2 selective agonists, Ucn2 and Ucn3, to treat ischemic heart disease was proposed because of their potent cardioprotective effects in mouse heart and their minimal impact on the hypothalamic stress axis.

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. Ucn3 (SCP) is expressed in rat cardiomyocytes and the levels of Ucn3 (SCP) and Ucn2 (SRP) were increased by hypoxic stress. All three peptides were shown to have potent cardio-protective effects in cells exposed to hypoxia/reoxygenation. This kit is highly specific for mouse/rat urocortin 3 with almost no cross-reaction 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).

PRINCIPLE
This EIA kit for the determination of mouse and rat Urocortin 3 is based on a competitive enzyme immunoassay using a combination of a highly specific antibody to mouse and rat Urocortin 3 and a biotin-avidin affinity system. The 96-well plate is coated with rabbit anti-mouse/rat Urocortin 3 antibody. Mouse/rat Urocortin 3 calibrator or samples and labeled antigen are added to the wells for a competitive immunoreaction. After incubation and washing, HRP-labeled streptoavidin (SA-HRP) is added to form HRP-labeled SA-labeled antigen-antibody complexes 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. This kit is characterized for sensitive quantification, high specificity and no influence with other components in samples. Mouse/rat urocortin 3 calibrator is highly purified synthetic product.

COMPONENTS

Component	Form	Quantity	Main Ingredient
1. Antibody-Coated Plate	MTP[*1]	1 plate (96-well)	Rabbit anti-mouse/rat Urocortin 3 antibody
2. Urocortin 3 Calibrator	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 (12 mL)	HRP-labeled streptoavidin
5. Substrate Buffer	Liquid	1 bottle (24 mL)	0.015% Hydrogen peroxide
6. OPD Tablet	Tablet	2 tablets	o-Phenylenediamine dihydrochloride
7. Stop Solution	Liquid	1 bottle (12 mL)	1 M H_2SO_4
8. Buffer Solution	Liquid	1 bottle (15 mL)	Citrate buffer
9. Wash Solution Concentrate	Liquid	1 bottle (50 mL)	Concentrated saline
10. Plate Seal		3 sheets	

MTP[*1] ..... Microtiter plate

MATERIALS REQUIRED BUT NOT PROVIDED

- Photometer for microtiter plate (plate reader), which can read absorbance up to 2.5 at 490 nm (or 492 nm)
- Microtiter plate shaker
- Washing device for microtiter plate and dispenser with aspiration system
- Micropipettes, multi-channel pipettes for 8 wells or 12 wells and their tips
- Glass test tubes for preparation of Calibrator Solutions
- Graduated cylinder (1,000 mL)
- Distilled water or de-ionized water

PRECAUTIONS

Protect reagents from strong light (e.g. direct sunlight) during storage and assay.

Satisfactory performance of the test is guaranteed only when reagents are used from a kit with identical lot number.

As pipetting operations may affect the precision of the assay, precisely pipette the prepared Calibrator Solutions or samples into corresponding wells. Use a new tip for each calibrator or sample to avoid cross-contamination. Use clean test tubes or vessels.

Always run a calibration curve when testing samples.

Test all samples in duplicate.

When a sample value exceeds 100 ng/mL, it must be diluted with Buffer Solution and re-assayed until the sample value is within the assay range.

Diluted Wash Solution is stable for 6 months when stored at 4°C. During storage of the Wash Solution Concentrate at 4°C, precipitates may be observed, however, they will dissolve when diluted.

The optical absorbance of reaction solution in wells should be read as soon as possible after stopping the color reaction.

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).

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

Calibrator, 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 calibrator solution and labeled antigen should be divided into test tubes in small amounts and stored at or below -30℃. (stable for 1 month)

Plasma and serum must be used as soon as possible after collection. If the samples are to be tested at a later time, they should be divided into test tubes in small amounts and frozen at or below –30℃. Avoid repeated freeze/thaw cycles. EDTA-2Na (1 mg/mL) additive blood collection tubes are recommended for plasma sample collection. 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 -30℃ until assay.

REAGENT PREPARATION

1. Preparation of Calibrator Solutions: Reconstitute the Urocortin 3 Calibrator with 1 mL of Buffer Solution, giving a 100 ng/mL Calibrator Solution after reconstitution. 0.1 mL of the reconstituted Calibrator Solution is diluted with 0.2 mL of Buffer Solution to yield a 33.3 ng/mL Calibrator Solution. Repeat the dilution procedure to make Calibrator Solutions at 11.1, 3.70, 1.23 and 0.41 ng/mL. Buffer Solution is used as the zero calibrator (0 ng/mL).

   Note: If a sample concentration below 0.41 ng/mL is predicted, the lower detection limit of the calibration curve can be extended by using a 0.137 ng/mL calibrator solution. This can be prepared by a 3-fold dilution of the 0.41 ng/mL calibrator solution. In this case, however, assay precision may not be as 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 in 11 mL of Substrate Buffer.

   Note: Substrate Solution must be prepared immediately before use.

4. Preparation of Wash Solution: Dilute 50 mL of Wash Solution Concentrate to 1,000 mL with distilled or de-ionized water.

5. Other reagents are ready for use.

STORAGE

Store kit at 4°C.

SPECIMEN COLLECTION AND HANDLING

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
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 4℃, 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 from above 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 elute is collected in a glass tube and dried in a centrifugal vaporizer. The mouse and rat brain extracts (dry residue) should be used a soon as possible after drying. If the dry residue is to be tested later, they should be stored at or below -30℃ until the assay.

3. The dry residue (sample for the assay) is reconstituted with buffer solution in the kit (75% volume of supernatant volume applied onto the column that was recorded, for example 1.5 mL). The insoluble material should be removed by centrifugation (1,750 x g, 15 min) at 4℃ and the sample solution is submitted to assay immediately.

2. Collection of mouse and rat plasma

EDTA-2Na (1 mg/mL) additive blood collection tubes are recommended for plasma sample collection. Plasma must be used as soon as possible after collection. If the samples are to be tested at a later time, they should be divided into test tubes in small amounts and frozen at or below –30℃.

ASSAY PROTOCOL

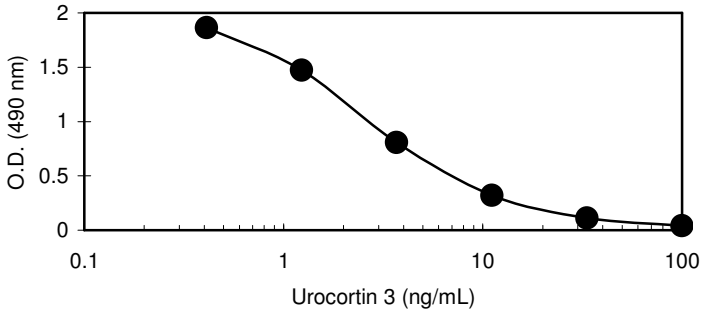
1. Warm the reagents and samples to room temperature (20 - 30℃) before beginning the test.
2. Add 0.35 mL/well of diluted Wash Solution into the wells and aspirate the Wash Solution in the wells. Repeat this washing procedure twice for a total of three wash steps. Finally, invert the plate and tap it onto an absorbent surface, such as paper towels, to ensure blotting free of most residual wash solution.
3. Add 25 µL of Buffer Solution into the wells, then add 25 µL of each of the prepared Calibrator Solutions (0, 0.41, 1.23, 3.70, 11.1, 33.3, 100 ng/mL) or samples into wells. Then add 50 µL of Labeled Antigen to all wells. The total pipetting time for this step should not exceed 30 minutes.
4. Cover the plate with the Plate Seal and incubate at 4℃ for 16-18 hours. Plate shaker not needed for this step.
5. After incubation, move plate back to room temperature and incubate for 40 minutes. Plate shaker not needed for this step. Remove the Plate Seal and aspirate the solution in the wells. Wash the wells four times with approximately 0.35 mL/well of diluted Wash Solution. Finally, invert the plate and tap it onto an absorbent surface, such as paper towels, to ensure blotting free of most residual wash solution.
6. Pipette 100 µL of SA-HRP Solution into each of the wells.
7. Cover the plate with a Plate Seal and incubate at room temperature for 2 hours. During the incubation, the plate should be shaken on a plate shaker (approximately 100 rpm).
8. Dissolve one OPD tablet in 11 mL of substrate buffer. It should be prepared immediately before use.
9. Remove the Plate Seal, aspirate and wash the wells four times with approximately 0.35 mL/well of diluted Wash Solution. Finally, invert the plate and tap it onto an absorbent surface, such as paper towels, to ensure blotting free of most residual wash solution.
10. Add 100 µL of Substrate Solution into the wells, cover the plate with a Plate Seal and incubate for 20 minutes at room temperature. Plate shaker not needed for this step.
11. Add 100 µL of Stop Solution into the wells to stop the reaction.
12. Read the optical absorbance of the wells at 490 nm (or 492 nm).

RESULTS

The dose-response curve of this assay best fits to a 4 (or 5)-parameter logistic equation. The result 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 the Calibrators and plot a calibration curve on semilogarithmic graph paper (abscissa: concentration of Calibrators; ordinate: absorbance values of Calibrators). Use the average absorbance of each sample to determine the corresponding value by simple interpolation from this calibration curve.

PERFORMANCE

Typical Calibration Curve (example only, a new calibration curve for each run must be established by the end-user)



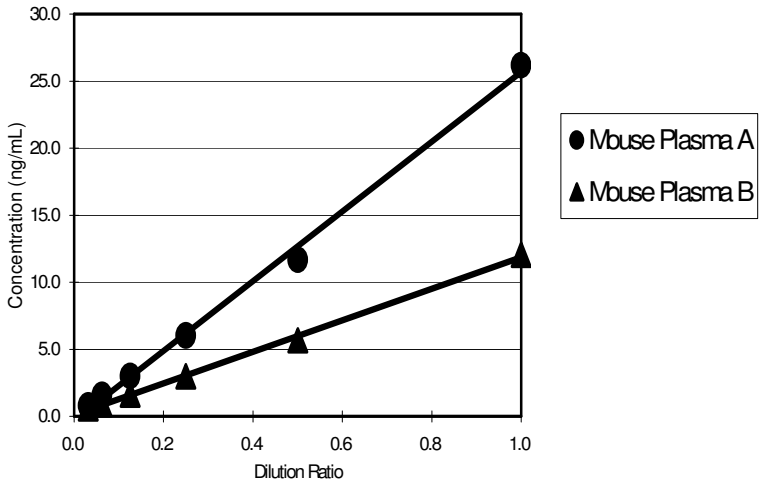
Analytical Recovery

	Urocortin 3 Added (ng/mL)	Observed (ng/mL)	Expected (ng/mL)	Recovery (%)
Mouse Plasma A	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	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	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	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	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	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	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	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

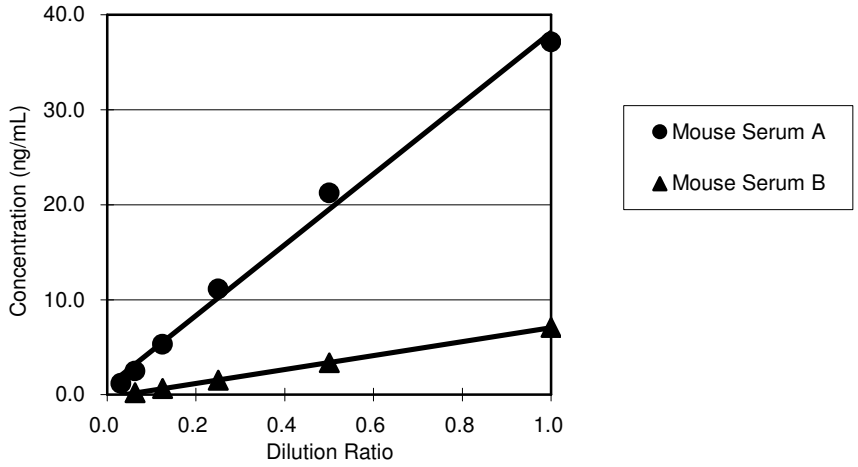
	Urocortin 3 Added (ng/mL)	Observed (ng/mL)	Expected (ng/mL)	Recovery (%)
Rat Plasma A	0.0	0.32		
	1.0	1.48	1.32	112.12
	5.0	4.90	5.32	92.11
	30.0	27.43	30.32	90.47
	50.0	52.62	50.32	104.57
Rat Plasma B	0.0	0.58		
	1.0	1.41	1.58	89.24
	5.0	5.31	5.58	95.16
	30.0	29.84	30.58	97.58
	50.0	56.69	50.58	112.08

Rat Plasma C	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	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	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	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	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	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	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	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

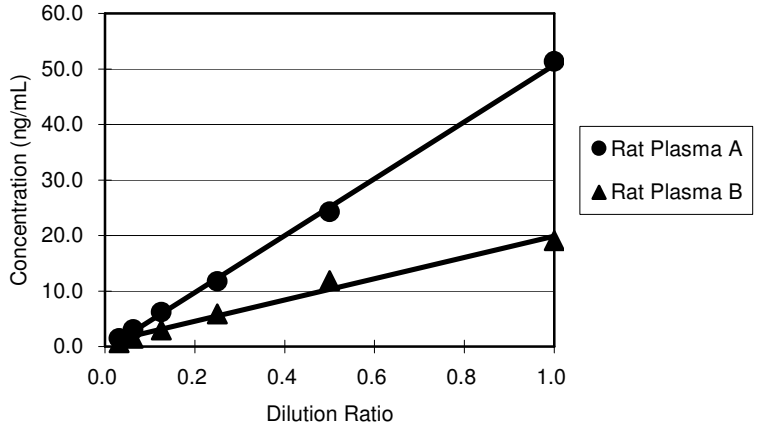
Dilution Test
Mouse Plasma



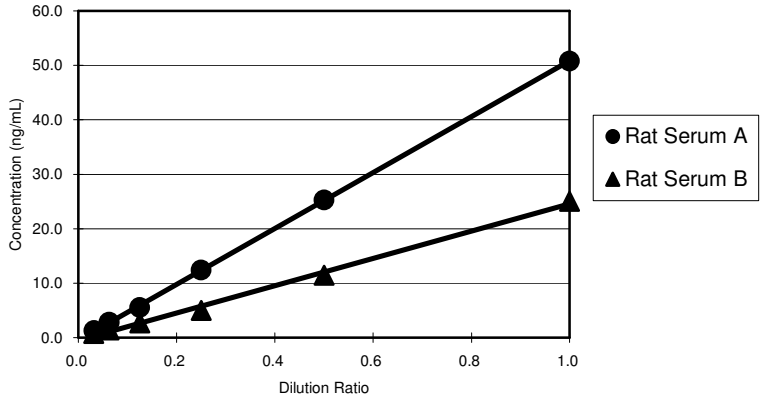
Mouse Serum



Rat Plasma



Rat Serum



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

Cross-Reactivity

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%

FOR RESEARCH USE ONLY

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