YK131 Mouse/Rat CRF-HS ELISA

FOR LABORATORY USE ONLY

YANAIHARA INSTITUTE INC.
2480-1 AWAKURA, FUJINOMIYA-SHI
SHIZUOKA, JAPAN 418-0011

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SCETI SCETI K.K.

Kasumigaseki place, 3-6-7, Kasumigaseki, Chiyoda-ku,
Tokyo 100-0013 Japan
https://research.sceti.co.jp/en **e-mail:export@sceti.co.jp**

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

YK131 Mouse/Rat CRF-HS ELISA Kit

Ⅰ. Introduction

Corticotropin releasing factor (CRF, also CRH) was initially isolated from ovine hypothalamus by Vale et al., in 1981, and identified as a novel neuropeptide comprising 41 amino acid residues with molecular weight 4758 [1]. Later human CRF[2] and rat CRF[3] were also isolated and identified. The mouse CRF peptide is identical at amino acid level to the rat and human CRF peptides[4]. CRF in anterior pituitary promotes the synthesis and secretion of ACTH, a main factor of hypothalamus-pituitary-adrenal (HPA) axis. In the rat and human, CRF distributes mainly in hypothalamus, but it was also found in spinal cord, stomach, spleen, duodenum, adrenal and placenta. In addition, immunochemical evidence supported the wide distribution of the peptide throughout the central nervous system (CNS) such as olfactory bulb, retina and central auditory system in the rat.

In mouse brain extracts, the highest concentrations of CRF-like immunoreactivity (CRF-LI) has been detected in median eminence and hypothalamus and also existing in amygdala, thalamus, frontal cortex, medulla/pons and cerebellum by radioimmunoassay[5]. However because of the wide distribution, it is still disputing about CRF whether its blood level can reflect only the function of HPA axis [6].

The relationships between CRF and stress, CRF and Alzheimer disease (AD) were attracted much attention recently. In fact the peptide was also suggested to regulate endocrine, autonomic and behavioral responses to stress, based on an experiment with acute and chronic stress rat models that showed endocrine function changes similar to those seen in patients with depression [6] CRF in serial cerebrospinal fluid（CSF）of patients with depression was strikingly reduced as compared to those of normal subjects [7,8] . The mean CRF and ACTH levels in the CSF of AD patients were significantly lower than those of healthy controls [9]. Only in the cortices of those with mild dementia, CRF was reduced significantly. Thus CRF was proposed to serve as a potential neurochemical marker of early dementia and possibly early AD [10].

A large proportion of CRF in human brain was shown to be in the form of complex with its binding protein (CRF-BP). CRF molecule in the complex is unavailable for activation of the CRF receptor. Accordingly reduction in total CRF do not necessarily predict reduction of bioactive free CRF, and the levels of total CRF and CRF in the form of complex (CRF/CRF-BP) were suggested to be the main factors determining the quantity of bioactive free CRF in human brain [11]. In AD there have been observed dramatic reduction in the content of free CRF in brain and thus displacement of CRF from CRF-BP was proposed as a possible treatment for AD [12] . In primary neuron culture, CRF exhibited protective effect against cell death induced by amyloid-beta peptide, suggesting that disturbances in HPA axis function can occur independently of alteration in CRF mRNA levels in AD brain and further suggesting an additional role for CRF in protecting neurons against cell death [13]. On the other hand, Yanaihara et al. demonstrated immunoreative CRF in various neuroendocrine tumors, and suggested that the blood level of the peptide might be used as a tumor marker [14] .

All these information urge crucial importance of the measurement of CRF in brain especially of experimental animals not only for analysis of the function of CRF in CNS, but also for research in the fields of stress response and AD. This time CRF-HS (high sensitivity) ELISA kit (YK131) was developed in our laboratory, which is highly specific and sensitive quantification of mouse/rat CRF. The kit can be used for measurement of CRF directly in mouse/rat plasma and their brain tissue extracts with high sensitivity (additional pretreatment of the brain tissue extract before assay is not necessary). It will

be a specifically useful and convenient tool for CRF researches.

YK131 Mouse/Rat CRF-HS ELISA Kit

- ▼ The assay kit can measure mouse/rat CRF within the range of 0.078-2.5 ng/mL.
- ▼ The assay is completed within 7.5 hr.
- ▼ With one assay kit, 41 samples can be measured in duplicate.
- ▼ Test sample: plasma and brain extracts
  Sample volume: 50 μL
- ▼ The 96-wells plate in kit is consisted by 8-wells strips, and the strips can be used separately.
- ▼ Precision and reproducibility
  Intra-assay CV(%)
  Mouse plasma 2.37-8.96, rat plasma 3.47-10.53
  Inter-assay CV(%)
  Mouse plasma 3.51-12.70, rat plasma 2.01-5.19
- ▼ Stability and storage
  Store all of the components at 2-8℃.
  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 antibody solution
4) SA-HRP solution
5) Enzyme substrate solution (TMB)
6) Stopping solution
7) Buffer solution
8) Washing solution (concentrated)
9) Adhesive foil

Ⅱ. Characteristics

This ELISA kit is used for quantitative determination of mouse/rat CRF in their plasma and brain extract samples. The kit is characterized by its sensitive quantification and high specificity. In addition, it has no influence by other components in samples. CRF standard is highly purified synthetic product.

< Specificity >

This ELISA kit has high specificity to CRF, and shows no crossreactivity to ACTH, urocortin 1, urocortin 2 (mouse) and urocortin 3 (mouse, rat). The detail data are presented on page 14.

< Assay principle >

This ELISA kit for determination of mouse/rat CRF is based on a sandwich enzyme immunoassay. To the wells of plate coated with highly purified antibody against mouse/rat CRF, standards or samples are added for the 1st step immunoreaction. After the 1st step incubation and plate washing, labeled antibody solution (biotinylated rabbit anti mouse/rat CRF antibody) is added as the 2nd step to form antibody - antigen - labeled antibody complex on the surface of the wells. After the 2nd step incubation and rinsing out excess labeled antibody, horseradish peroxidase (HRP) labeled streptoavidin (SA) is added for binding to labeled antibody. Finally, HRP enzyme activity is determined by 3,3',5,5'-Tetramethylbenzidine (TMB) and the concentration of mouse/rat CRF is calculated.

Ⅲ. Composition

	Component	Form	Quantity	Main Ingredient
1.	Antibody coated plate	microtiter plate	1 plate (96 wells)	Rabbit anti mouse/rat CRF antibody coated
2.	Standard	lyophilized	1 vial (2.5 ng)	Synthetic mouse/rat CRF (1-41)
3.	Labeled antibody solution	liquid	1 bottle (12 mL)	Biotinylated rabbit anti mouse/rat CRF antibody
4.	SA-HRP solution	liquid	1 bottle (12 mL)	Horseradish peroxidase labeled streptoavidin
5.	Enzyme substrate solution	liquid	1 bottle (12 mL)	3,3',5,5'-Tetramethylbenzidine (TMB)
6.	Stopping solution	liquid	1 bottle (12 mL)	1M H_2SO_4
7.	Buffer solution	liquid	1 bottle (20 mL)	Buffer containing a reaction accelerator
8.	Washing solution (concentrated)	liquid	1 bottle (50 mL)	Concentrated saline
9.	Adhesive foil		4 pieces	

Ⅳ. Method

< Equipment required >

1. Photometer for microtiter plate (plate reader) which can read extinction 2.5 at 450nm
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 (NP40)
   Methods:

1) Mouse and rat brain tissue in a plastic tube is weighed and then homogenized in 30–fold volume of extraction buffer in an ice bath.
2) The homogenate is centrifuged (18,360 x g, 20 min) at 4℃, and the supernatant is collected and should be used as soon as possible for measurement. If the samples are tested later, they should be divided into test tubes in small amount and frozen at or below -30℃ until assay. The frozen samples should be warmed up to room temperature (20-30℃) before starting assay. If insoluble material observed in samples, it should be removed by centrifugation (1,750 x g, 15 min) at 4℃ and the sample solution is submitted to assay immediately.
   *It is recommended that brain tissue extracts should be examined by dilution test in order to know suitable dilution ratio to be used , referring to **Ⅵ. Performance Characteristics** <Dilution test>.

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 CRF standard with 1 mL of buffer solution, which affords 2.5 ng/mL standard solution. The reconstituted standard solution (0.2 mL) is diluted with 0.2 mL of buffer solution that yields 1.25 ng/mL standard solution. Repeat the dilution procedure to make each standard solution of 0.625, 0.313, 0.156 and 0.078 ng/mL. Buffer solution itself is used as 0 ng/mL standard solution.
   If a sample concentration below 0.078 ng/mL is predicted, standard curve may be further set up a lower detection limit by using 0.039 ng/mL standard solution which can be prepared by 2-fold dilution of 0.078 ng/mL standard solution. In such case, however, assay precision may not be so excellent as that of the cases between 0.078 and 2.5 ng/mL.

2. Preparation of washing solution:
   Dilute 50 mL of washing solution (concentrated) to 1,000 mL with distilled or deionized water.

3. 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 50µL of buffer solution to the wells first, and then introduce 50µL of each of standard solutions (0, 0.078, 0.156, 0.313, 0.625, 1.25 and 2.5 ng/mL) or samples to the wells.

4. Cover the plate with adhesive foil and incubate it at room temperature for 4 hours. During the incubation, the plate should be shaken with a plate shaker (approximately 100 rpm).

5. After incubation, 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 100µL of labeled antibody 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. 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.

9. Add 100µL of SA-HRP solution to each of the wells.

10. Cover the plate with adhesive foil and incubate it at room temperature for 1 hour. During the incubation, the plate should be shaken with a plate shaker (approximately 100 rpm).

11. 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.

12. Add 100µL of Enzyme substrate solution (TMB) to each of the wells, cover the plate with adhesive foil and keep it for 30 minutes at room temperature in a dark place for color reaction (keep still, plate shaker not need).

13. Add 100 µL of stopping solution to each of the wells to stop color reaction.

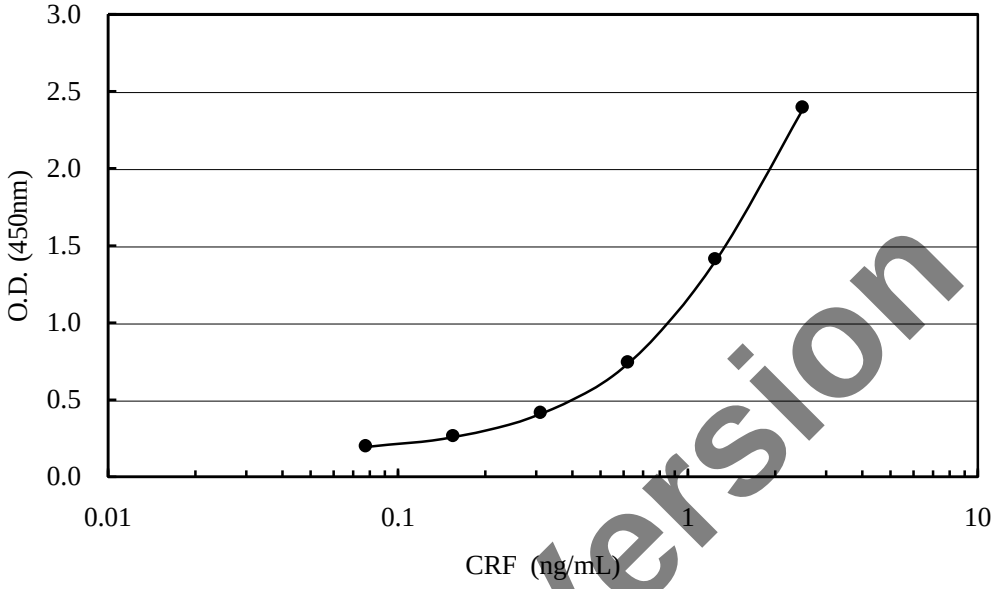
14. Read the optical absorbance of the solution in the wells at 450 nm. The dose-response curve of this assay fits best to a 4 (or 5)-parameter logistic equation. The results of unknown samples can be calculated with any computer program having a 4 (or 5)-parameter logistic function.
    Otherwise calculate 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. The brain extract supernatant should be used as soon as possible for assay. If the samples are tested later, they should be divided into test tubes in small amount and frozen at or below -30℃ until assay. These samples should be brought back to room temperature (20-30°C) before starting assay. If insoluble material is observed in sample, they should be removed by centrifugation（1,750 x g ,15 min） at 4℃ and the sample solution is submitted to assay immediately.
2. EDTA-2Na (1mg/mL) additive blood collection tube is recommended for the plasma collection. Plasma samples 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 -30°C. Avoid repeated freezing and thawing of samples.
3. Standard solutions should be prepared immediately before use. This kit can be used dividedly in strips of the plate. In such case, the rest of reconstituted reagent (standard) should be stored at or below -30°C（stable for 1 month）.
4. During storage of washing solution (concentrated) at 2-8°C, precipitates may be observed, however, they will be dissolved when diluted. Diluted washing solution is stable for 6 months at 2-8°C.
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 2.5 ng/mL, it needs to be diluted with buffer solution to proper concentration.
7. During the incubation except the color reaction, the 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.
13. Some reagents contain human serum (tested and found negative for HBsAG, HIV 1/2, HCV, HIV-1 AG or HIV-1 NAT, ALT and a test for Syphilis by FDA approved methods), care should be taken when handling.

Ⅵ. Performance Characteristics

Typical standard curve



<Analytical recovery>

<Mouse plasma A>

Added CRF (ng/ml)	Observed (ng/ml)	Expected (ng/ml)	Recovery (%)
0.0	0.06		
0.1	0.15	0.16	93.75
0.3	0.26	0.36	72.22
1.0	0.70	1.06	66.04

<Mouse plasma B>

Added CRF (ng/ml)	Observed (ng/ml)	Expected (ng/ml)	Recovery (%)
0.0	0.02		
0.1	0.11	0.12	91.67
0.3	0.26	0.32	81.25
1.0	0.64	1.02	62.75

<Mouse plasma C>

Added CRF (ng/ml)	Observed (ng/ml)	Expected (ng/ml)	Recovery (%)
0.0	0.04		
0.1	0.17	0.14	121.43
0.3	0.31	0.34	91.18
1.0	0.66	1.04	63.46

<Mouse plasma D>

Added CRF (ng/ml)	Observed (ng/ml)	Expected (ng/ml)	Recovery (%)
0.0	0.06		
0.1	0.17	0.16	106.25
0.3	0.30	0.36	83.33
1.0	0.71	1.06	66.98

<Rat plasma A>

Added CRF (ng/ml)	Observed (ng/ml)	Expected (ng/ml)	Recovery (%)
0.0	0.07		
0.1	0.16	0.17	94.12
0.3	0.30	0.37	81.08
1.0	0.84	1.07	78.50

<Rat plasma B>

Added CRF (ng/ml)	Observed (ng/ml)	Expected (ng/ml)	Recovery (%)
0.0	0.03		
0.1	0.14	0.13	107.69
0.3	0.35	0.33	106.06
1.0	0.85	1.03	82.52

<Rat plasma C>

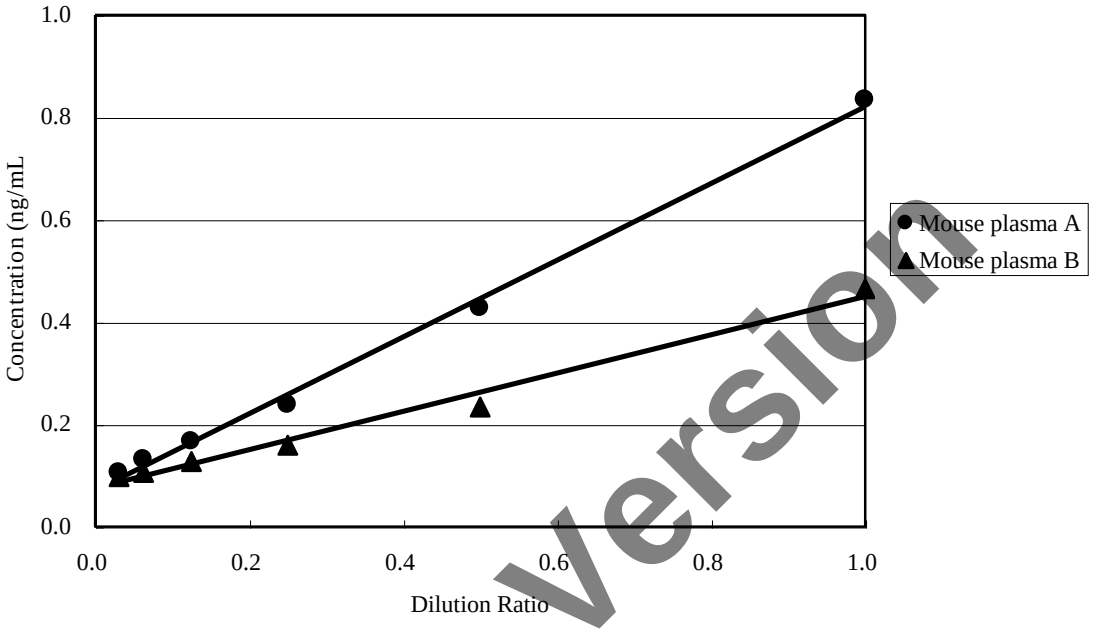
Added CRF (ng/ml)	Observed (ng/ml)	Expected (ng/ml)	Recovery (%)
0.0	0.00		
0.1	0.11	0.10	110.00
0.3	0.23	0.30	76.67
1.0	0.68	1.00	68.00

<Rat plasma D>

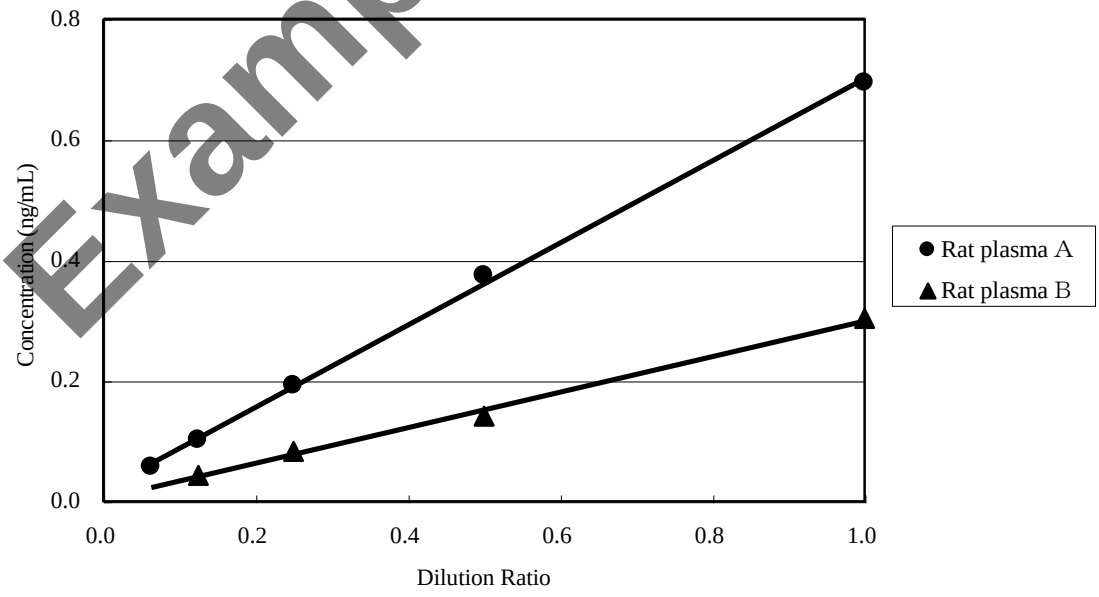
Added CRF (ng/ml)	Observed (ng/ml)	Expected (ng/ml)	Recovery (%)
0.0	0.00		
0.1	0.09	0.10	90.00
0.3	0.21	0.30	70.00
1.0	0.68	1.00	68.00

<Dilution test>

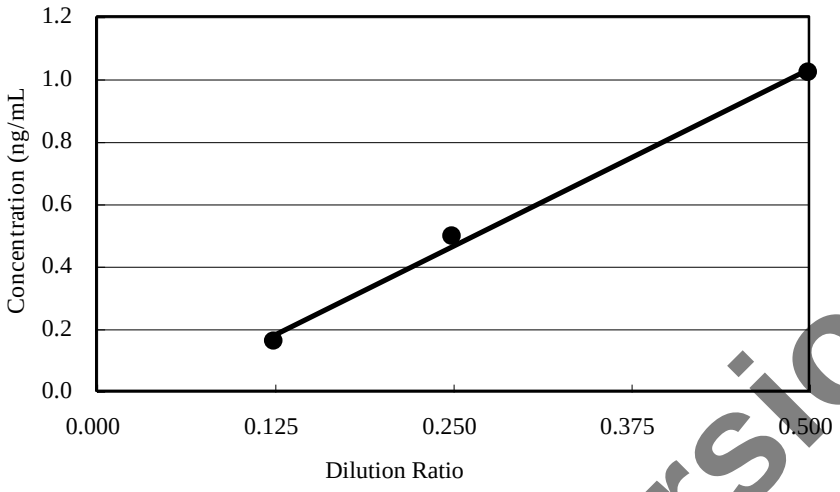
<Mouse plasma>



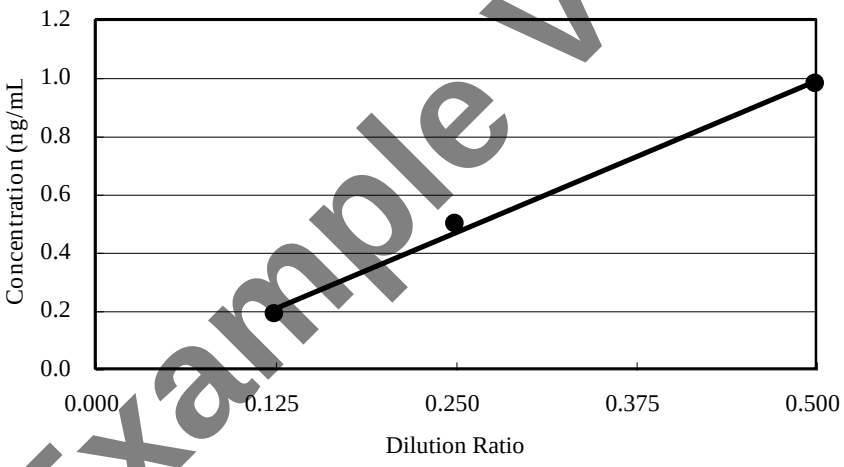
<Rat plasma>



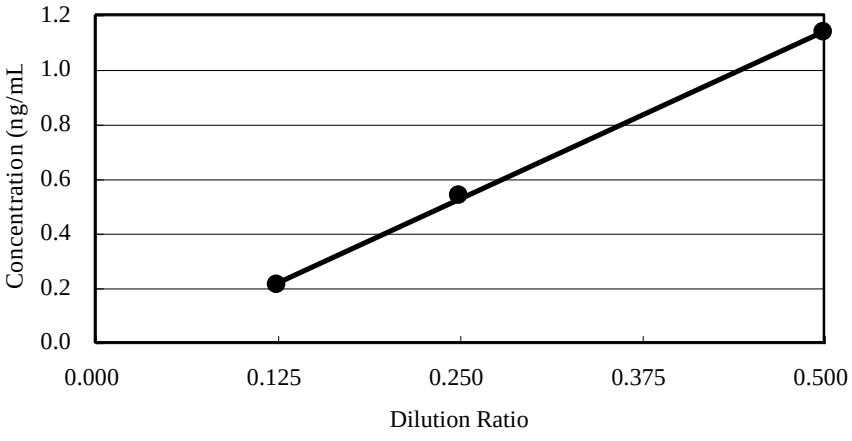
<Mouse brain extract A> (extracted with 30 fold volume of PBS containing 0.2%NP40)



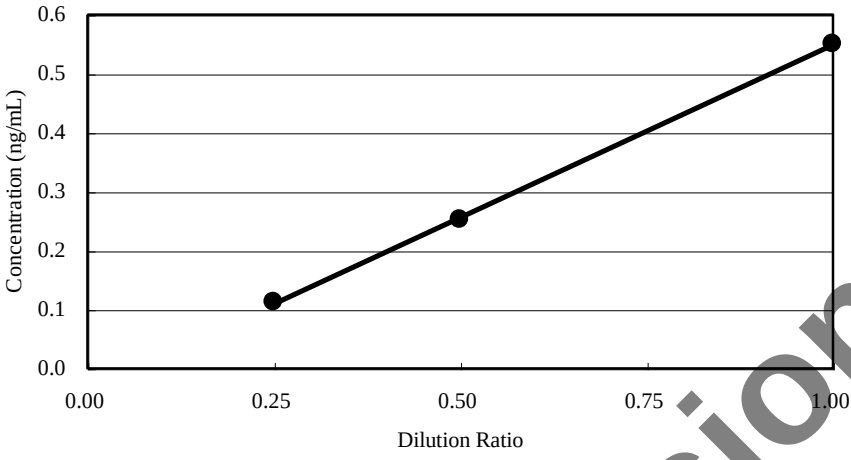
<Mouse brain extract B> (extracted with 30 fold volume of PBS containing 0.2%NP40)



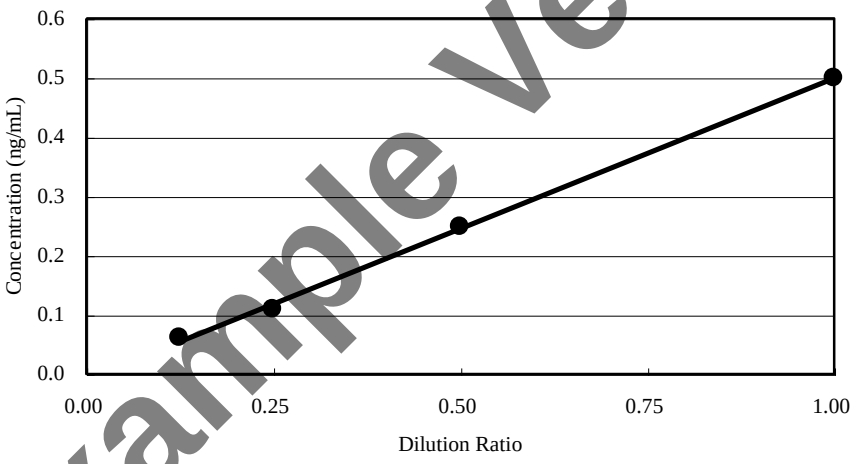
<Mouse brain extract C> (extracted with 30 fold volume of PBS containing 0.2%NP40)



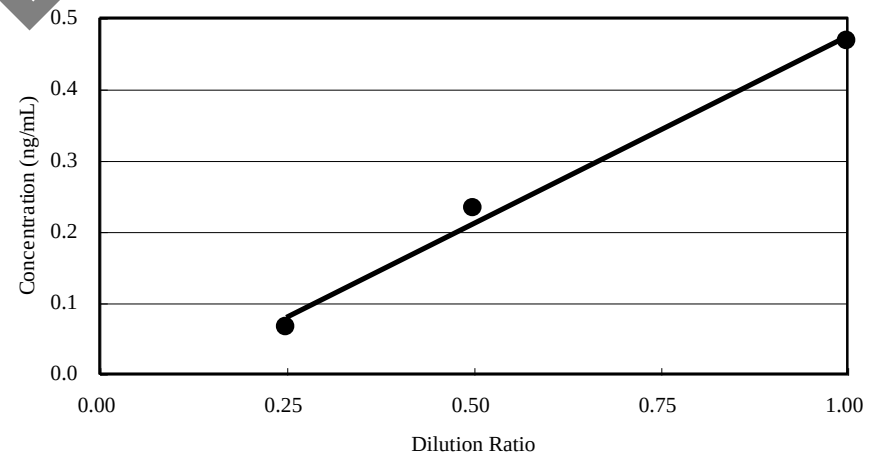
<Rat brain extract A> (extracted with 30 fold volume of PBS containing 0.2%NP40)



<Rat brain extract B> (extracted with 30 fold volume of PBS containing 0.2%NP40)



<Rat brain extract C> (extracted with 30 fold volume of PBS containing 0.2%NP40)



<Crossreactivity>

Related peptides	Crossreactivity（%）
CRF(1-41) (Mouse, Rat, Human)	100
CRF(17-41) (Mouse, Rat, Human)	0.1
ACTH (Human)	0.01
ACTH (Mouse, Rat)	0.01
Urocortin 1 (Human)	0.01
Urocortin 1 (Mouse, Rat)	0.01
Urocortin 2 (Mouse)	0
Urocortin 3 (Mouse, Rat)	0
PACAP27	0
PACAP38	0
VIP (Human, Porcine)	0

< Precision and reproducibility >

Test sample	Intra-assay CV（%）	Inter-assay CV（%）
Mouse plasma	2.37 -8.96	3.51-12.70
Rat plasma	3.47-10.53	2.01-5.19

<Assay range>
0.078 ~ 2.5 ng/mL

Ⅶ. Stability and Storage

< Storage > Store all of the components at 2-8°C.
< 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

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<Manufacturer>
Yanaihara Institute Inc.
2480-1 Awakura, Fujinomiya-shi
Shizuoka, Japan 418-0011
TEL: +81-544-22-2771 FAX: +81-544-22-2770
Website: http://www.yanaihara.co.jp E-mail: ask@yanaihara.co.jp
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