COSMO BIO CO., LTD.
Inspiration for Life Science

# Mouse/Rat CRF-HS ELISA

## Cat. No. YII- YK131 -EX

**FOR LABORATORY USE ONLY**

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## Contents



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

**YII-YK131-EX 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. We developed CRF-HS (high sensitivity) ELISA kit (YK131) 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. (Special 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**

- ▼ This 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-well plate of this kit consists of 12 8-wells strips, so that divided use by the strips is possible at user's option.
- ▼ 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 the components at 2-8°C.
  The kit is stable under the condition for 21 months from the date of manufacturing.
  The expiry date is stated on the package.

**Contents**

1) Antibody coated plate
2) Standard antigen
3) Labeled antibody solution
4) SA-HRP solution
5) Enzyme substrate solution (TMB)
6) Reaction stopping solution
7) Buffer solution
8) Washing solution (concentrated)
9) Adhesive sheet

## Ⅱ. Characteristics

This ELISA kit is used for quantitative determination of total mouse/rat CRF in its plasma and brain extract samples. The kit is characterized by its sensitive quantification and high specificity. In addition, it is not influenced by other constituents in samples. Standard antigen, CRF, of this kit is a highly purified synthetic product (purity: higher than 98%).

< Specificity >

This ELISA kit is highly specific to CRF. It shows no crossreactivity to urocortin (mouse/rat and human). 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, standard antigen or sample is added for the 1st step immunoreaction. After the 1st step incubation and plate washing, biotinylated rabbit anti mouse/rat CRF antibody is added as the 2nd step to form CRF antibody- antigen -biotinylated CRF antibody complex on the surface of the wells. After the 2nd step incubation and rinsing out excess biotinylated antibody, horseradish peroxidase (HRP) labeled streptoavidin (SA) is added for binding to biotinylated CRF antibody. Finally, HRP enzyme activity is determined by 3,3',5,5'-tetramethyl benzidine (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 |
| 2. | Standard antigen | 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) | HRP labeled SA |
| 5. | Enzyme substrate solution | Liquid | 1 bottle (12 mL) | 3,3',5,5'-tetramethyl benzidine (TMB) |
| 6. | Reaction 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 sheet | | 3 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. Polypropylene or glass test tubes for preparation of standard solution.
6. Graduated cylinder (1000 mL)
7. Distilled or deionized water

<Preparation of assay sample>

1. Extraction method of mouse or rat brain tissue
   Materials:
   Mouse or rat brain tissue
   Extraction solution： 10 mM PBS containing 0.2% Nonidet P-40 (NP40) pH 7.2
   Methods:

1) Mouse or rat brain tissue in a plastic tube is weighed and then homogenized (10,000 rpm/min) in 30–fold volume of extraction solution in an ice bath.
2) The homogenate is centrifuged (15,000 rpm/min, 20 min) at 4°C, and the supernatant is collected and should be used as soon as possible for measurement. If the sample are tested later, they should be divided into test tubes in small amount and frozen below -30°C until assay. The frozen samples should be warmed up to room temperature (20-30°C) before starting assay. If insoluble material observed in samples, it should be removed by centrifugation（3,000 rpm/min,15 min）at 4°C 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 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 CRF standard antigen (lyophilized mouse/rat CRF 2.5 ng/vial) with 1 mL of buffer solution, which affords 2.5 ng/mL standard solution. Dilute 0.2 mL of the standard solution with 0.2 mL of buffer solution, which yields 1.25 ng/mL standard solution. Repeat the dilution procedure to make each of 0.625, 0.313, 0.156 and 0.078 ng/mL standard solutions. Buffer solution itself is used as 0 ng/mL.
   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 1000 mL with distilled or deionized water.

3. The other reagents are ready for use.

< Procedure >

1. Bring all the reagents and samples to room temperature (20-30°C) at least 1 hour before starting assay.

2. Add 0.35mL/well of washing solution into the wells of the plate, and then aspirate the solution. 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. Fill 50µL of buffer solution first, and then add 50µL each of standard solutions (0, 0.078, 0.156, 0.313, 0.625, 1.25 and 2.5 ng/mL) or samples into wells.

4. Cover the plate with adhesive sheet and incubate it at room temperature for 4 hours. During incubation, the plate should be shaken with a microtiter plate shaker.

5. After incubation, take off the adhesive sheet, aspirate the solution in the wells and wash the wells 4 times with approximately 0.35 mL/well each 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. Pipette 100µL of labeled antibody solution into each of the wells.

7. Cover the plate with adhesive sheet and incubate it at room temperature for 2 hour. During incubation, the plate should be shaken with a microtiter plate shaker.

8. Take off the adhesive sheet, aspirate the solution in the wells and then wash the wells 4 times with approximately 0.35 mL/well each 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. Pipette 100µL of SA-HRP solution into each of the wells.

10. Cover the plate with adhesive sheet and incubate it at room temperature for 1 hour. During incubation, the plate should be shaken with a microtiter plate shaker.

11. Pipetting the required volume of enzyme substrate (TMB) solution into a vessel, and bring it to room temperature (20-30°C) under a light-proof condition for 1 hour before use. Store the rest of the TMB solution at 2-8°C for next use.

12. Take off the adhesive sheet, aspirate the solution in the wells and then wash the wells 4 times with approximately 0.35 mL/well each 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.

13. Add 100 µL of TMB solution into each of the wells, cover the plate with adhesive sheet and keep it for 30 minutes at room temperature under a light-proof condition.

14. Add 100 µL of reaction stopping solution into each of the wells.

15. Read optical absorbance of the solution in the wells at 450 nm. Calculate mean absorbance values of standard solutions and plot a standard curve on semi logarithmic graph paper (abscissa: concentration of standard antigen; ordinate: absorbance value). Use the average absorbance of each sample to determine the corresponding value by simple interpolation from the standard curve.

## Ⅴ. Notes

1. The brain extract supernatant should be used as soon as possible for assay. If the sample are tested later, they should be divided into test tubes in small amount and frozen below -30°C until assay. These samples should be bring back to room temperature (20-30°C) before starting assay. If insoluble material is observed in sample, they should be removed by centrifugation（3,000 rpm/min,15 min） at 4°C and the sample solution is submitted to assay immediately.

2. EDTA-2Na (1mg/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 below -30°C. Avoid repeated freezing and thawing of samples.

3. Standard antigen solution should be prepared immediately before use. This kit can be used dividedly in strips of the plate. In such a case, the rest of reconstituted reagents including standard solutions should be stored below -30°C.

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 precision of the assay. Pipette standard solutions or samples into each well of the plate precisely. Use clean test tubes and vessels in assay, and new tip must be used for each sample and standard solution to avoid cross contamination.

6. When concentration of CRF in sample is expected to exceed 2.5 ng/mL, the sample needs to be diluted with buffer solution to a proper concentration.

7. During incubation except the color reaction, the plate should be shaken gently with a microtiter plate shaker to promote immunoreaction

8. Perform all the determination in duplicate.

9. Read optical absorbance of reaction solution in the wells immediately after stopping color reaction.

10. For accurate quantification, plot a standard curve for each assay.

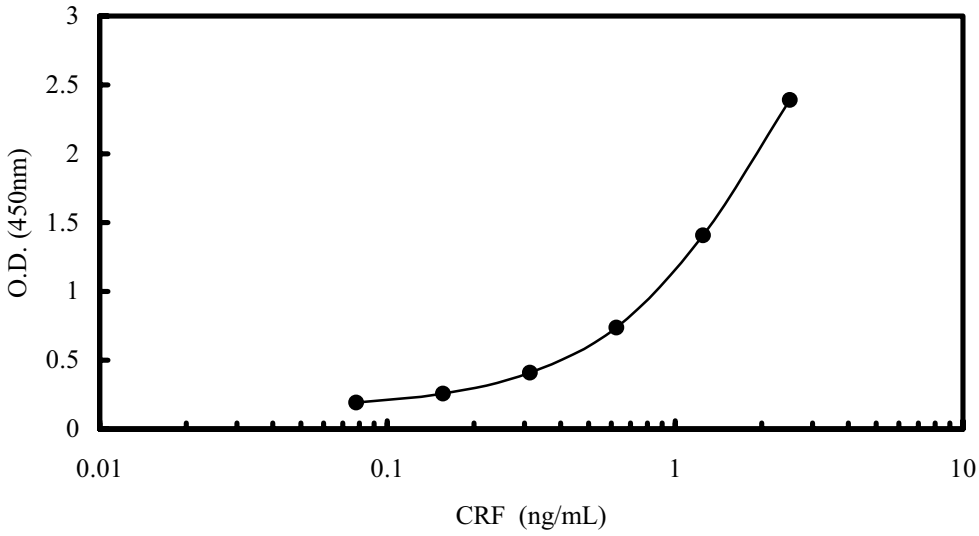
11. Protect reagents from strong light (e.g. direct sunlight) during assay and storage.

12. Satisfactory performance of assay is guaranteed only when reagents in combination pack with identical lot number are used.

13. Pipetting the required volume of enzyme substrate (TMB) solution into a vessel, and bring it to room temperature (20-30°C) under a light-proof condition 1 hour before use. Color reaction by TMB must be carried on under a light-proof condition.

## Ⅵ. Performance Characteristics

A 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

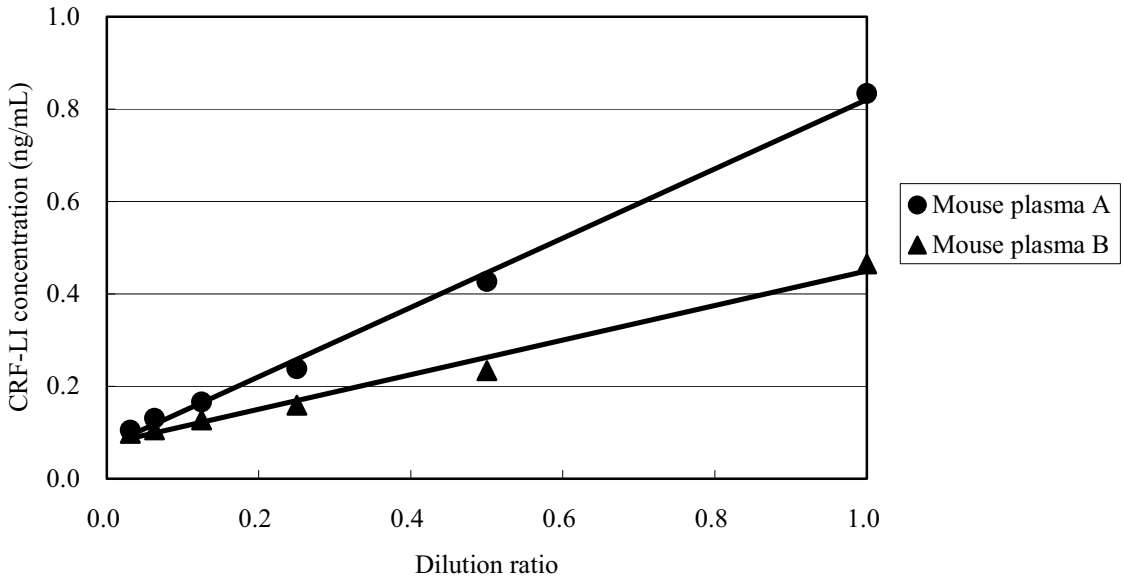
| Added CRF (ng/ml) | Observed (ng/ml) | Expected (ng/ml) | Recovery (%) |
|---|---|---|---|
| 0.0 | 0.00 | | |
| 0.1 | 0.11 | 0.10 | 110.00 |
| 0.3 | 0.23 | 0.30 | 76.67 |
| 1.0 | 0.68 | 1.00 | 68.00 |

Rat plasma D

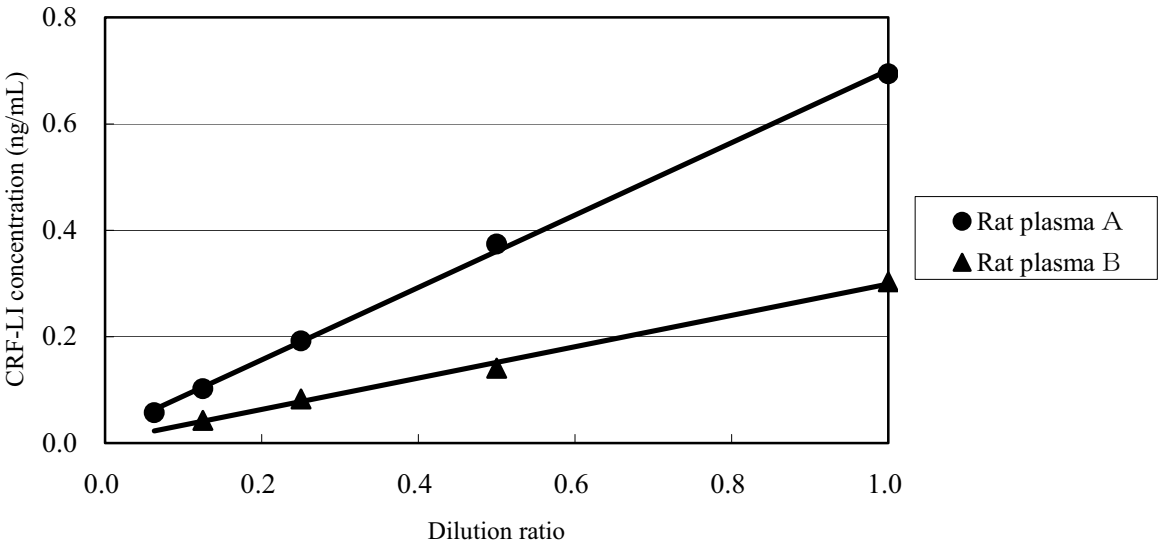
| Added CRF (ng/ml) | Observed (ng/ml) | Expected (ng/ml) | Recovery (%) |
|---|---|---|---|
| 0.0 | 0.00 | | |
| 0.1 | 0.09 | 0.10 | 90.00 |
| 0.3 | 0.21 | 0.30 | 70.00 |
| 1.0 | 0.68 | 1.00 | 68.00 |

<Dilution test>

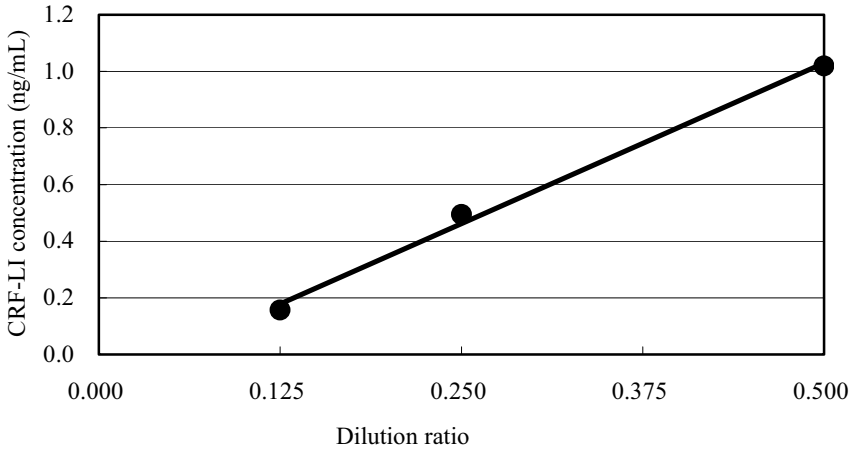
Mouse plasma



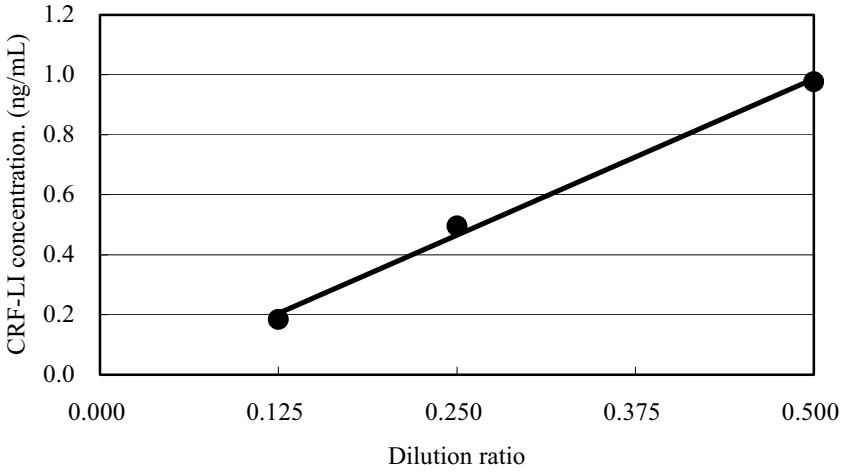

Rat plasma



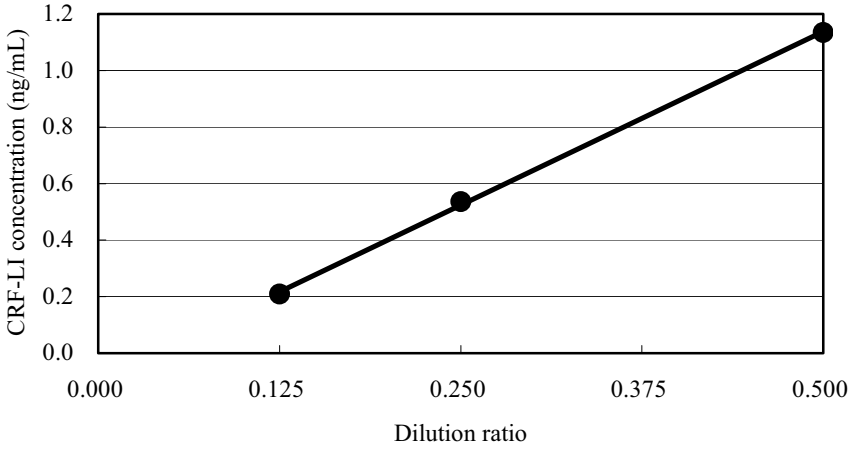
Mouse brain extract A (extracted with 30 fold volume of PBS containing 0.2%NP40)



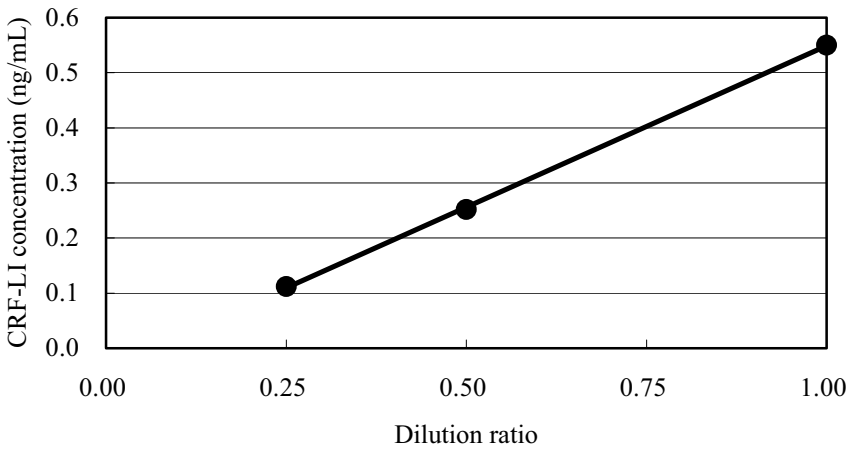

Mouse brain extract B (extracted with 30 fold volume of PBS containing 0.2%NP40)



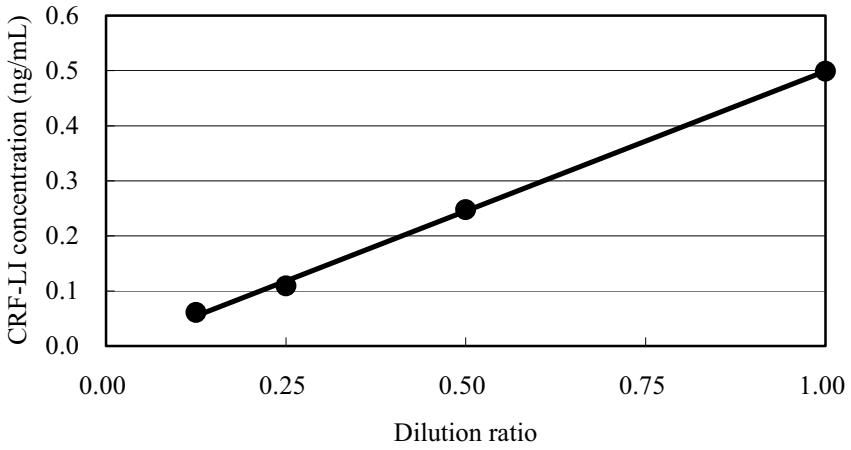

Mouse brain extract C (extracted with 30 fold volume of PBS containing 0.2%NP40)



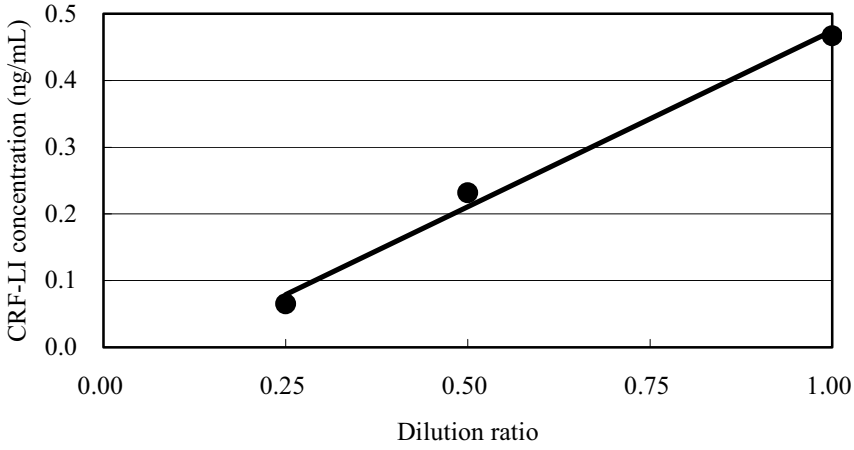
Rat brain extract A (extracted with 30 fold volume of PBS containing 0.2%NP40)




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 (Human) | 0.01 |
| Urocortin (Mouse, Rat) | 0.01 |
| Urocortin 2 (Mouse) | 0 |
| Urocortin 3 (Mouse) | 0 |
| PACAP27 | 0 |
| PACAP38 | 0 |
| VIP (Human, Porcine) | 0 |

< Precision and reproducibility >

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

## Ⅶ. Stability and Storage

| | |
|---|---|
| < Storage > | Store all the components at 2-8°C. |
| < Shelf life > | The kit is stable under the condition for 21 months from the date of manufacturing. The expiry date is stated on the package. |
| < Package > | For 96 tests per one kit. |

## Ⅷ. References

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