Mouse/Rat CGRP ELISA Kit

Enzyme Immunoassay for the quantification of CGRP in Mouse, Rat plasma samples

Catalog number: ARG81358

TABLE OF CONTENTS

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INTRODUCTION

CGRP gene encodes the peptide hormones calcitonin, calcitonin gene-related peptide and katacalcin by tissue-specific alternative RNA splicing of the gene transcripts and cleavage of inactive precursor proteins. Calcitonin is involved in calcium regulation and acts to regulate phosphorus metabolism. Calcitonin gene-related peptide functions as a vasodilator and as an antimicrobial peptide while katacalcin is a calcium-lowering peptide. Multiple transcript variants encoding different isoforms have been found for this gene. [provided by RefSeq, Aug 2014]

CGRP induces vasodilation. It dilates a variety of vessels including the coronary, cerebral and systemic vasculature. Its abundance in the CNS also points toward a neurotransmitter or neuromodulator role. It also elevates platelet cAMP. [UniProt]

PRINCIPLE OF THE ASSAY

This is an Enzyme Immunoassay for the quantification CGRP in Mouse and Rat plasma samples. This assay employs the competitive quantitative enzyme immunoassay technique. A secondary antibody has been pre-coated onto a microtiter plate. The secondary antibody can bind to the Fc fragment of the primary antibody which recognizes CGRP. The primary antibodies in the kit will be competitively bound by both biotinylated CGRP peptides and CGRP peptides in standards or targeted CGRP peptides in samples. The wells are washed and then incubated with Streptavidin-HRP reagent. The biotinylated peptide interacts with streptavidin-horseradish peroxidase to form a complex. After washing away any unbound Streptavidin-HRP reagent, a substrate solution (TMB) is added to the wells and color develops in inverse-proportion

to the amount of CGRP present in the samples. The color development is stopped by the addition of STOP solution and the intensity of the color is measured at a wavelength of 450nm±2 nm. The concentration of CGRP in the sample is then determined by comparing the O.D of samples to the standard curve.

MATERIALS PROVIDED & STORAGE INFORMATION

Store the unopened kit at 2-8 °C. Use the kit before expiration date.

Component	Quantity	Storage information
Secondary antibody coated microplate	12 x 8 wells	4°C
20X Wash Buffer	50 ml	4°C
Primary antibody	1 vial	4°C
Biotinylated peptide	1 vial	4°C
Standard	1 vial	4°C
1000X Streptavidin-HRP conjugate	30 µl	4°C
TMB substrate	12 ml (Ready-to-use)	4°C (Protect from light)
STOP solution (2N HCl)	15 ml (Ready-to-use)	4°C
Positive Controls	2 vial	4°C
Plate sealer	3 pieces	Room Temperature

MATERIALS REQUIRED BUT NOT PROVIDED

- Microplate reader capable of measuring absorbance at 450nm
- Pipettes and pipette tips
- Deionized or distilled water
- Microplate shaker (300-400rpm)

- Automated microplate washer (optional)

TECHNICAL HINTS AND PRECAUTIONS

- Wear protective gloves, clothing, eye, and face protection especially while handling blood or body fluid samples.
- Store the kit at 4°C at all times.
- Before opening any Eppendorf tubes for reconstitution, briefly centrifuge at ~3,000rpm for 5 seconds to ensure that all the lypholized material is at the bottom of the tube.
- If crystals are observed in the 20X Wash buffer, warm to RT or 37°C until the crystals are completely dissolved.
- Ensure complete reconstitution and dilution of reagents prior to use.
- Unused microplate strips should be placed back in the foil pouch with a desiccant and stored at 4°C. Do not allow moisture to enter the wells.
- All reagents should be mixed by gentle inversion or swirling prior to use. Do not induce foaming.
- Before using the kit, spin tubes and bring down all components to the bottom of tubes.
- Each time a new tip is used, make sure the tip is secure and free of air bubbles. For better intra-assay variation, aspirate and expel a reagent or sample back into the container a few times prior to loading.
- Avoid submerging the whole tip into reagents because droplets can accumulate at the end of the tip causing an excess of reagent to be loaded into the well. This can lead to poor results.
- For optimal results, an orbital plate shaker capable of 300-400 rpm is recommended for all incubations.

- It is highly recommended that the standards, samples and controls be assayed in duplicates.

SAMPLE COLLECTION & STORAGE INFORMATION

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 minutes at 1600 x g at 4°C. Collect serum and assay immediately or aliquot and store samples at ≤ -80 °C up to 1 month. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA as an anticoagulant. Centrifuge for 15 minutes at 4°C at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -80 °C up to 1 month. Avoid repeated freeze-thaw cycles.

Note: We recommended add Aprotinin (enzyme inhibitor) for ALL sample collection to prevent sample degradation. 0.6 TIU or 100 µl of Aprotinin per mL of sample solution.

Peptide extraction –

1. It is recommended through peptide extraction protocol.
2. Mix an equal amount of Binding Buffer (1% trifluoroacetic acid (TFA), HPLC grade is recommended) with the plasma or serum samples and vortex the mixture. Centrifuge at 6,000-17,000 × g for 20 minutes at 4°C. Collect the supernatant.
3. Slowly equilibrate a C18 column (SEP-COLUMN containing 200 mg of C18) by washing the C18 column with 1 ml Elution Buffer (60% acetonitrile, 1% TFA, and 39% distilled water, HPLC grade of acetonitrile and TFA are

recommended).

4. Wash the C18 column with 3 ml of Binding Buffer three times.
5. Load the sample/Binding Buffer mixture solution from step 1 into the washed C18 column from step 3.
6. Wash the column slowly with Binding Buffer (3 ml, twice) and discard the wash.
7. Elute the peptide slowly with Elution Buffer (3 ml, once) and collect eluant into a polystyrene tube.

   Note: From steps 5-7, no pressure should be applied to the column.
8. Evaporate eluant to dryness in a centrifugal concentrator or by a suitable substitute method to dryness the eluant. (Freeze-dry the resulting water/TFA solution to dryness)
9. Keep the dried extract at -20°C and perform the assay as soon as possible.
10. Use 1x assay buffer to reconstitute the dried extract. If the peptide value does not fall within the range of detection, dilute or concentrate the sample accordingly.

Note: For normal subject extracted from 1 ml original plasma/serum, use 125 µl 1x Wash buffer to reconstitute the dried extract. Aliquot 50 µl into two designated assay wells (25 µl is left over). The concentration factor in this case is 8. (1 ml / 125 ul = 8). The original plasma peptide concentration level is 1/8 of the level of final extracted plasma.

e.g. If the level of the final extracted plasma is 100 pg/ml, then the total level of peptide in the original plasma = (100 pg/ml) / 8 = 12.5 pg/ml. After performing assay, if the peptide value exceeds or does not fall in the range of detection, dilute or concentrate the samples accordingly.

Drying Sample After Extraction:

A combination of a centrifugal concentrator (i.e. Speedvac) and a lyophilizer (freeze-dryer) produces the best results for drying the sample after extraction. First, use a Speedvac to dry sample for approximately 15 minutes to remove the organic layer. Then snap-freeze the remaining sample, and freeze-dry overnight using a lyophilizer. This two-step procedure produces a more consistent fluffy powder that is easier to rehydrate than a sample dried only with a centrifugal concentrator. However, if a centrifugal concentrator is not accessible, freeze-drying overnight using a lyophilizer will be sufficient.

REAGENT PREPARATION

- 1X Wash Buffer: Dilute 20X Wash Buffer into distilled water to yield 1X Wash buffer. Keep 1X Wash Buffer at 4°C. If crystals appear in 20X Wash Buffer, warm the buffer in warm water bath (not higher than 50°C) for 30 minutes or until crystals disappear. Mix well before use.
- Primary antibody: Reconstitute the Primary antibody vial with 5 ml of 1X Wash Buffer. Allow it to sit for 5 minutes to completely dissolve, mix well before use. Keep rehydrated solution at 4°C.
- Biotinylated peptide: Reconstitute the Biotinylated peptide vial with 5 ml of 1X Wash Buffer. Allow it to sit for 5 minutes to completely dissolve, mix well before use. Keep rehydrated solution at 4°C.
- Positive control: Centrifuge and reconstitute the Positive control vial with 200 µl of 1X Wash Buffer. Allow it to sit for 5 minutes to completely dissolve, mix well before use. Keep rehydrated solution at 4°C.

(acceptable range dependent on lot#)

- **1X Streptavidin-HRP conjugate:** Centrifuge 1000X Streptavidin-HRP conjugate briefly and add 12µl of Streptavidin-HRP to 12ml 1X wash buffer to make Streptavidin-HRP solution. Vortex thoroughly.
- **Standard peptide**: Centrifuge and dilute the standard with 1 ml of 1X Wash buffer and vortex. The concentration of this stock solution is 1000 ng/ml. Allow the solution to sit for at least 10 minutes at room temperature to completely dissolve. Dilute peptide standard solutions with 1X Wash buffer to 100 ng/ml, 10 ng/ml, 1 ng/ml, 0.1 ng/ml, 0.01 ng/ml as follows:

The example of the dilution of standards

Standard No.	Standard Conc. (ng/ml)	1X Wash Buffer (µl)	Standard (µl)
Stock	1000	-	-
S1	100	900	100 µl of Stock
S2	10	900	100 µl of S1
S3	1	900	100 µl of S2
S4	0.1	900	100 µl of S3
S5	0.01	900	100 µl of S4
S0 (Total binding)	0	900	0

ASSAY PROCEDURE

All materials should be equilibrated to room temperature (RT, 20-23°C) 30 minutes before use. Standards, samples and blank should be assayed in duplicates.

1. Remove excess microtiter strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal it.
2. Add **50 µl of 1X Wash Buffer** as Total Binding (S0, zero standard). Two empty wells should be left as blank.
3. Add **50 µl of prediluted peptide standards** (add from S5 to S1), **50 µl positive controls** or **50 µl samples** into corresponding wells. It is advisable to assay each condition in duplicates.
4. Add **25 µl of primary antibody** into each well **except the Blank wells**.
5. Add **25 µl of Biotinylated peptide** into each well **except the Blank wells**. It is not recommended to use a multi-channel pipette to load the primary antibody and biotinylated peptide.
6. Seal the microtiter plate with plate sealer. Incubate for **2 hours at RT.** Orbital shaking at 300-400 rpm is recommended.
7. Mix and centrifuge Streptavidin-HRP vial (3,000-5,000 rpm for 5 seconds) before use. Pipette 12 µl of Streptavidin-HRP into 12 ml of 1X Wash Buffer. Vortex thoroughly. Prepare freshly.
8. Remove sealer from plate.
9. Aspirate each well and wash, repeating the process 3 times for a **total 4 washes**. Wash by filling each well with **1× Wash Buffer (350 µl)** using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each is essential to good performance. After the last wash,

remove any remaining Wash Buffer by aspirating, decanting or blotting against clean paper towels.

10. Add **100 µl of diluted (1X) Streptavidin-HRP** solution into **each well**.
11. Reseal the plate with sealer. Incubate for **1 hour at RT.** Orbital shaking at 300-400 rpm is recommended.
12. Remove sealer from plate. **Wash** as according to step 9.
13. Add **100 µl of TMB substrate** solution into each well.
14. Reseal the plate with sealer. Incubate for **1 hour at RT in dark** Orbital shaking at 300-400 rpm is recommended.
15. Remove sealer from plate. (**DO NOT** wash or discard the contents of the wells)
1. Add **100 µl of 2N HCl** into all wells to stop the reaction. Gently tap the plate to ensure thorough mixing. The color of the solution should change from blue to yellow.
2. Read the OD with a microplate reader at 450 nm immediately. It is recommended that the wells be read within 20 minutes after adding the Stop Solution.

CALCULATION OF RESULTS

1. Calculate the average absorbance values for each set of standards, controls and patient samples.

2. Using log-log, semi-log or linear graph paper, construct a standard curve by plotting the mean absorbance obtained from each standard against its concentration with absorbance value on the vertical (Y) axis and concentration on the horizontal (X) axis.

3. Using the mean absorbance value for each sample determine the

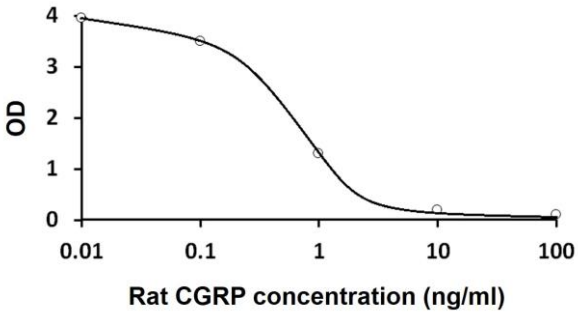
corresponding concentration from the standard curve.

4. Automated method: The results in the IFU have been calculated automatically using a 4 PL (4 Parameter Logistics) curve fit. 4 Parameter Logistics is the preferred method. Other data reduction functions may give slightly different results.

5. If samples have been diluted prior to the assay, the measured concentration must be multiplied by their respective dilution factors.

EXAMPLE OF TYPICAL STANDARD CURVE

The following data is for demonstration only and cannot be used in place of data generations at the time of assay.



QUALITY ASSURANCE

Sensitivity

The standard of CGRP ranged from 0-100 ng/ml.

The mean MDD was 0.16 ng/ml.

Linear Range

0.16- 1.81 ng/ml

Precision:

Intra-assay: < 10%

Inter-assay: < 15%

Cross Reactivity

The cross reactivity ratio of the tested peptide as the table:

Peptide	Cross Reactivity (%)
CGRP (Rat)	100
CGRP (Human)	15.1
CGRP II (Rat)	78.6
CGRP II (Human)	20.1
Amylin Amide (Rat)	<0.01
Amylin (Human)	<0.001
Calcitonin (Rat)	0
Somatostatin-14	0