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RAT CGRP ENZYME IMMUNOASSAY KIT

catalogue # A05482

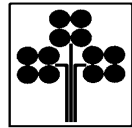
96 wells

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U.S. patent # 50 47 330
European patent # 89 139 552

**THE RAT CGRP
ENZYME IMMUNOASSAY HAS
BEEN DEVELOPED AND
VALIDATED BY SPI-BIO.**

*For research laboratory use only.
Not for human diagnostic use.*



Société de Pharmacologie et d'Immunologie - BIO

Parc d'Activités du Pas du Lac – Bertin Group
10 bis avenue Ampère
F-78180 – Montigny Le Bretonneux

FRANCE

☎: 33 (0)1 39 30 62 60

📠: 33 (0)1 39 30 62 99

E-Mail: sales@spibio.com

Web: www.spibio.com

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RAT CGRP EIA KIT

96 wells

Storage: -20 °C

Expiry date: stated on the package

This kit contains:

- ☞ A covered 96 well Microtiter plate, pre-coated with CGRP mouse monoclonal antibody, ready to use
- ☞ One vial of anti-CGRP tracer, lyophilised
- ☞ Two vials of rat CGRP standard, lyophilised
- ☞ One vial of EIA buffer, lyophilised
- ☞ One vial of concentrated Wash buffer, liquid
- ☞ One vial of tween 20, liquid
- ☞ Two vials of Quality Control sample, lyophilised
- ☞ Two vials of Ellman's reagent, lyophilised
- ☞ One instruction booklet
- ☞ One template sheet
- ☞ One well cover sheet

Each kit contains sufficient reagents for 96 wells. This allows for the construction of one standard curve in duplicate and the assay of 32 samples in duplicate.

PRECAUTIONS FOR USE

Users are recommended to read all instructions for use before starting work.

Each time a new pipet tip is used, aspirate a sample or reagent and dispense it back into the same vessel. Repeat this operation two or three times before distribution.

For research laboratory use only.

Not for diagnostic use.

Do not pipet liquids by mouth.

Do not use kit components beyond the expiration date.

Do not eat, drink or smoke in area in which kit reagents are handled.

Avoid splashing.

The total amount of reagents contains less than 100 µg of sodium azide. Flush the drains thoroughly to prevent the production of explosive metal azides.

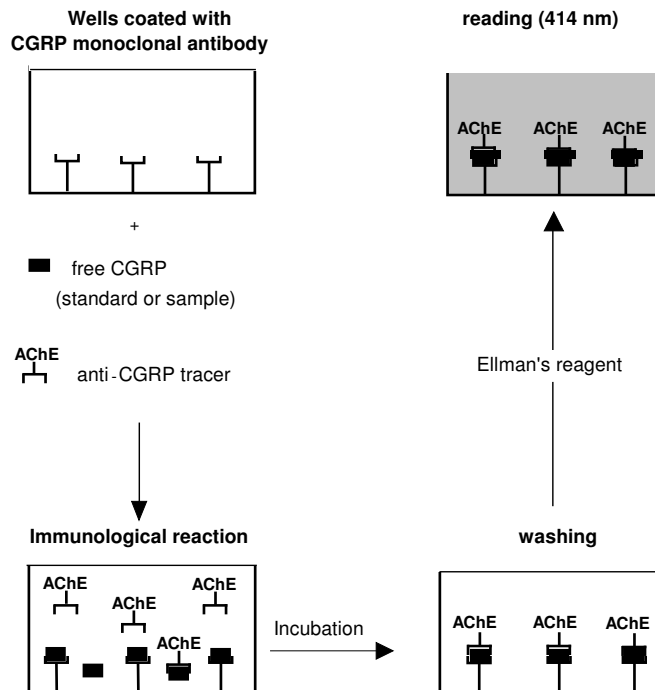
PRINCIPLE OF THE ASSAY

This Enzyme Immunometric Assay (EIA) is based on a double-antibody sandwich technique. The wells of the microtiter plate supplied with the kit are coated with a monoclonal antibody specific to CGRP. This antibody will bind any CGRP introduced in the wells (sample or standard). An acetylcholinesterase (AChE) - Fab' conjugate which binds selectively to a different epitope on the CGRP molecule, is also added to the wells. This allows the two antibodies to form a sandwich by binding on different parts of the rat CGRP molecule.

The sandwich is immobilised on the plate so the excess reagents may be washed away. The concentration of the rat CGRP is then determined by measuring the enzymatic activity of the AChE using the Ellman's Reagent. The AChE tracer acts on the Ellman's Reagent to form a yellow compound.

The intensity of the colour, which is determined by spectrophotometry, is proportional to the amount of the rat CGRP present in the well during the immunological incubation.

The principle of the assay is summarised below:



MATERIALS AND EQUIPMENT REQUIRED

In addition to standard laboratory equipment, the following material is required:

FOR SAMPLE PREPARATION

- ☞ C-18 reverse phase cartridges or Oasis® HLB Extraction cartridges
- ☞ Methanol
- ☞ Acetic acid
- ☞ Distilled or deionized water

FOR THE ASSAY

- ☞ Precision micropipettes (20 to 1000 µL)
- ☞ Spectrophotometer plate reader (405 or 414 nm filter)
- ☞ Microplate washer (or washbottles)
- ☞ Microplate shaker
- ☞ Distilled or deionized water
- ☞ Polypropylene tubes

SAMPLE PREPARATION

GENERAL PRECAUTIONS

- ☞ All samples must be free of organic solvents prior to assay.
- ☞ Samples should be assayed immediately after collection or should be stored at -20°C.

SAMPLE PREPARATION

Nervous tissues such as cerebrospinal fluid may be assayed directly if diluted more than 1:20 in EIA buffer. Other nervous tissues such as spinal cord may be assayed after extraction procedure. Basically, the procedure is to homogenize the tissue in 2 N acetic acid, heat at 90°C for ten minutes, centrifuge, dry the supernatant, and then dissolve in EIA buffer.



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Plasma and serum samples can be measured either after extraction (see protocol below), or without any extraction procedure. In this second case, CGRP standard and quality control need to be reconstituted with plasma or serum that is free of rat CGRP instead of the EIA buffer, as mentioned in reagent preparation section (CGRP standard and quality control). If you don't have plasma or serum that is free of rat CGRP, SPI-BIO can provide you with CGRP affinity sorbent containing anti-CGRP monoclonal antibody (the same as the one coated to the well). For further details, please feel free to contact our technical support staff by letter, phone (33 (0)1 39 30 62 60), fax (33 (0)1 39 30 32 99) or E-mail (sales@spibio.com).

Whole blood, as well as other heterogeneous mixtures such as lavage fluids and aspirates should be purified (see purification protocol below) before addition to the assay well.

EXTRACTION PROTOCOL

- ↳ Activate a 1 mL C-18 reverse phase cartridge or an Oasis® HLB Extraction cartridge (SPI-BIO # D30005 or Waters #WAT0944226-HLB-3cc) by first passing 5 mL methanol and then 10 mL distilled or deionized water through the cartridge. The reverse phase cartridge (RPC) may be stored with the water present.
- ↳ Dilute the sample 1:4 with 4 % acetic acid.
- ↳ Pass one mL of sample slowly (about 2 mL/minute) through the cartridge.
- ↳ Wash the cartridge with 10 mL 4% acetic acid.
- ↳ Prepare 3 mL of methanol:water with 4 % acetic acid (90:10, v/v). Elute the CGRP by passing the methanol:water solution through the cartridge one mL at a time. Be certain to pause between each mL of solution as the reproducibility of the recovery is increased by the care taken during this step.
- ↳ Dry the sample by vacuum centrifugation. Reconstitute the sample with a volume of EIA buffer equal to the original sample volume.
- ↳ Assay the aliquots of the sample and use the results to calculate the recovery.

RECOVERY AND CALCULATION

To determine the recovery, the sample may be split into two equal aliquots and one spiked with a known amount of CGRP (approximately equal to the expected amount in the sample).

The recovery will be determined after purification by comparing the concentration of the spiked and unspiked samples.

Either the original concentration of the sample or the recovery factor can be determined by solving the following equations simultaneously:

$z = \text{recovery factor}$

$X/a = \text{original concentration of the unspiked sample in a volume known (a)}$

$(X+Y)/b = \text{concentration of the spiked sample (pg/mL) after adding a known amount (Y) in a final volume (b)}$

The concentration of the unspiked and spiked samples determined by the EIA are respectively equal to $(X/a)z$ & $[(X+Y)/b]z$.

EXAMPLE

Volume of the unspiked sample: $a = 1 \text{ mL}$

Final volume of the spiked sample: $b = 2 \text{ mL}$

Concentration determined by EIA for the unspiked sample: $(X/a)z = 8 \text{ pg/mL}$

Concentration determined by EIA for the spiked sample: $[(X+Y)/b]z = 16 \text{ pg/mL}$

Quantity of spike: $Y = 30 \text{ pg in } 1 \text{ mL}$

$$Xz = 8 \Leftrightarrow z = 8/X$$

$$[(X+30)/2]z = 16 \Leftrightarrow [(X+30)]z = 32$$

thus,

$$[(X+30)]8/X = 32 \Leftrightarrow X+30 = 4X \Leftrightarrow 3X = 30 \Leftrightarrow X = 10$$

and

$$Xz = 8 \Leftrightarrow z = 0.8$$

NOTE

To minimise the calculations, the standard should be concentrated enough so that the addition of the standard does not alter the volume of the sample ($a = b$) to any great degree (i.e., the assumption is made that the volume is not changed by the addition of the standard).



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REAGENT PREPARATION

The coated microtiter plates and reagents are provided ready to use.

☞ EIA buffer

Reconstitute one vial with 50 mL of distilled or deionized water. Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion. Stability at 4°C: 1 month.

☞ CGRP standard

Reconstitute the vial with 1 mL of EIA buffer (for plasma sample see sample preparation section). Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion. The concentration of the first standard is 500 pg/mL. Prepare seven propylene tubes (for the seven other standards) and add 500 µL of EIA buffer into each tube. Add 500 µL of the first tube (containing the first standard) to the second tube. Continue this procedure for the other tubes. Thus, standard concentrations are: 500 (S1), 250 (S2), 125 (S3), 62.50 (S4), 31.25 (S5), 15.6 (S6), 7.81 (S7) and 3.91 pg/mL (S8), respectively. Stability at 4°C: 24 hours.

☞ Quality Control

Reconstitute one vial with 1 mL of EIA buffer (for plasma sample see sample preparation section). Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion. Stability at 4°C: 24 hours.

☞ Anti-CGRP-AChE tracer

Reconstitute one vial with 10 mL of EIA buffer. Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion. Stability at 4°C: 1 month.

☞ Wash buffer

Dilute 1 mL of concentrated Wash buffer to 400 mL with distilled or deionized water. Add 200 µL of tween 20 (Use a magnetic stirrer to mix the contents). Stability at 4°C: 1 week.

☞ Ellman's Reagent

Five minutes before use, reconstitute with 49 mL of distilled or deionized water and 1 mL of concentrated Wash buffer. The tube contents should be thoroughly mixed. Stability at 4°C and in the dark: 4 days.

ASSAY PROCEDURE

It is recommended to perform the assays in duplicate and to follow the instructions hereafter.

PLATE PREPARATION

Prepare the wash buffer as indicated in the reagent preparation section. Open the plate packet and select the sufficient strips for your assay and place the unused strips back in the packet (stored at 4°C). Rinse each well 5 times with the wash buffer (300 µL/well).

Just before distributing reagents and samples, remove the buffer from the wells by inverting the plate and shaking out the last drops.

DISTRIBUTION OF REAGENTS AND SAMPLES

A plate set-up is suggested on the following page. The contents of each well may be recorded on the sheet provided with the kit.

	1	2	3	4	5	6	7	8	9	10	11	12
A	B	NSB	S1	S5	*	*	*	*	*	*	*	*
B	B	NSB	S1	S5	*	*	*	*	*	*	*	*
C	B	NSB	S2	S6	*	*	*	*	*	*	*	*
D	B	NSB	S2	S6	*	*	*	*	*	*	*	*
E	B	NSB	S3	S7	*	*	*	*	*	*	*	*
F	B	NSB	S3	S7	*	*	*	*	*	*	*	*
G	B	NSB	S4	S8	*	*	*	*	*	*	*	*
H	B	NSB	S4	S8	*	*	*	*	*	*	*	*

B : Blank
 NSB : Non Specific Binding
 S1-S8 : Standards 1-8
 * : Samples or Quality Controls

PIPETTING THE REAGENTS

Note that the first column should be left empty for blanking Ellman's reagent. All samples and reagents must reach room temperature prior to performing the assay. Use different tips to pipet the buffer, standard, sample, tracer, antiserum and other reagents.

- ↳ EIA buffer:
Dispense 100 µL to Non Specific Binding (NSB) wells.
- ↳ Rat CGRP standard:
Dispense 100 µL of each of the eight standards (S1 to S8) in duplicate to appropriate wells. Start with the lowest concentration standard (S8) and equilibrate the tip in the next higher standard before pipetting.
- ↳ Quality Control and samples:
Dispense 100 µL in duplicate to appropriate wells. Highly concentrated samples may be diluted in EIA buffer.
- ↳ Anti-CGRP AChE tracer:
Dispense 100 µL to each well, except Blank (B) wells.

INCUBATING THE PLATE

Cover the plate with a plastic film and incubate for 16-20 hours at +4 °C.

DEVELOPING AND READING THE PLATE

Reconstitute Ellman's Reagent as indicated in reagent preparation section. Empty the plate by turning over and shaking. Wash each well three times with the wash buffer (300 µL/well), slightly shake them for 2 minutes (with the orbital shaker) and then rewash three times with the wash buffer (300 µL/well). Dispense 200 µL of Ellman's Reagent to the 96 wells. Incubate in the dark (plate covered with an aluminium sheet) at room temperature. Optimal development is obtained using an orbital shaker. The plate should be read between 405 and 414 nm (yellow colour) 30 to 60 minutes after adding the Ellman's reagent.



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Enzyme Immunoassay Protocol (Volume are in μ L)				
	Blank	Non Specific Binding	Standard	Sample
Buffer	-	100	-	-
Standard	-	-	100	-
Sample	-	-	-	100
Tracer	-	100	100	100
Cover the plate, incubate at 4°C for 16-20h				
Wash the strips 3 times, slightly shake them 2 min, rewash them 3 times and remove the liquid from the wells				
Ellman's reagent	200	200	200	200
Incubate the plate with an orbital shaker in the dark at room temperature				
Read the plate between 405 and 414 nm				

DATA ANALYSIS

Make sure that your plate reader has subtracted the absorbance readings of the blank well (absorbance of Ellman's reagent) from the absorbance readings of the rest of the plate. If not, do it now.

- ↳ Calculate the average absorbance for each NSB, standard and sample.
- ↳ Plot the absorbance for each standard (y axis) versus the concentration (x axis). Draw a best-fit line through the points.
- ↳ To determine the concentration of your samples, find the absorbance value of each sample on the y axis. Read the corresponding value on the x axis which is the concentration of your unknown sample.
- ↳ Samples with a concentration greater than 500 pg/mL should be re-assayed after dilution in EIA buffer.
- ↳ Most plate readers are supplied with curve-fitting software capable of graphing these data (linear curve fit or other). If you have this type of software, we recommend using it. Refer to it for further information.

TYPICAL DATA

EXAMPLE DATA

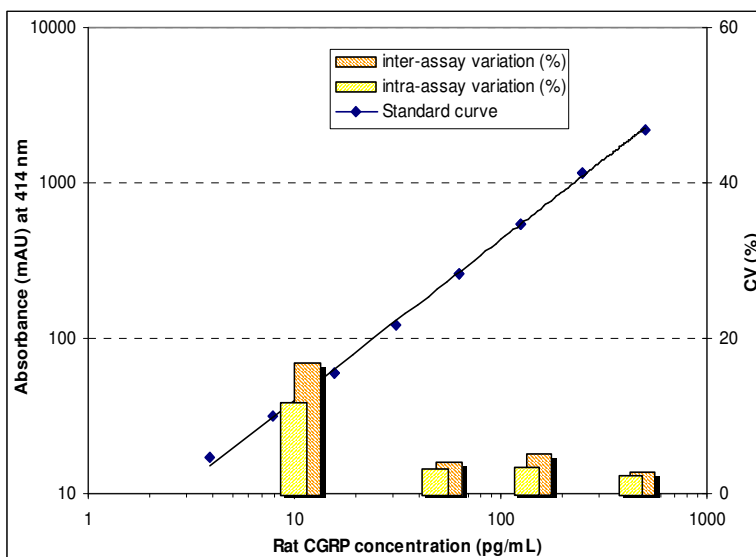
The following data are for demonstration purpose only. Your data may be different and still correct. These data were obtained using all reagents as supplied in this kit under the following conditions: 30 minutes developing at 20°C, reading at 414 nm. A spline fitting was used to determine the concentrations.

CGRP	mAU
Blank	4.0
Standard 500 pg/mL	2198
Standard 250 pg/mL	1168
Standard 125 pg/mL	548
Standard 62.5 pg/mL	260
Standard 31.25 pg/mL	123
Standard 15.63 pg/mL	59.5
Standard 7.81 pg/mL	32.0
Standard 3.91 pg/mL	17.0

ACCEPTABLE RANGE

- ↳ Limit of detection <1 pg/mL.
- ↳ QC sample: see the label on the vial.

RAT CGRP STANDARD CURVE



ASSAY VALIDATION AND CHARACTERISTICS

The Enzyme Immunometric Assay of rat CGRP has been validated for its use in buffer and in plasma (without any extraction, using a standard curve in plasma).

- ☞ The limit of detection, calculated as the concentration of CGRP corresponding to the NSB average (n = 8) plus three standard deviations is: 0.7 (in EIA buffer) & 2 pg/mL (in plasma).
- ☞ Quality Control samples intra-assay & inter-assay variations in EIA buffer (n = 25):

Intra-assay		Inter-assay	
Rat CGRP	C.V.	Rat CGRP	C.V.
400 pg/mL	6.3 %	400 pg/mL	6.3 %
150 pg/mL	3.4 %	150 pg/mL	4.3 %
50 pg/mL	2.7 %	50 pg/mL	9.3 %
10 pg/mL	2.7 %	10 pg/mL	15.5 %

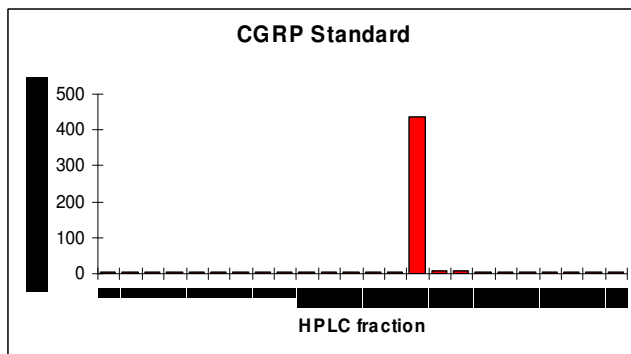
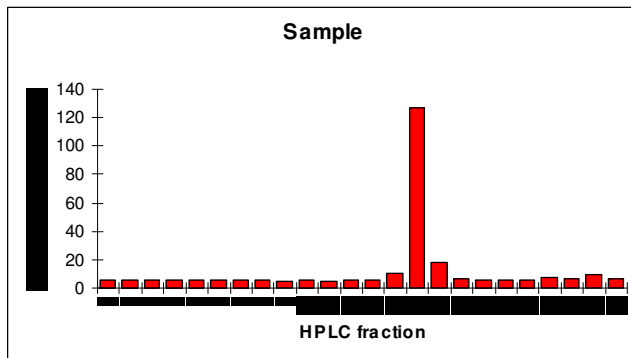
- ☞ Quality Control samples intra-assay & inter-assay variations (n=25) in plasma:

Intra-assay		Inter-assay	
Rat CGRP	C.V.	Rat CGRP	C.V.
400 pg/mL	2.5 %	400 pg/mL	2.9 %
150 pg/mL	2.9 %	150 pg/mL	4.2 %
50 pg/mL	2.9 %	50 pg/mL	3.7 %
10 pg/mL	11.5 %	10 pg/mL	16.4 %

- ☞ Limit of quantification in buffer & plasma: 10 pg/mL.

☞ Cross-reactivity:			
rat CGRP α & β :	100 %	CGRP (8-37):	<0.01 %
rat CGRP I & II:	100 %	Amylin:	<0.01 %
human CGRP α & β :	83 %	Calcitonin:	<0.01 %
human CGRP I & II:	83 %	Substance P:	<0.01 %

☞ Specificity: comparison of HPLC profiles of a CGRP standard and a sample.



ASSAY TROUBLE SHOOTING

- ☞ Absorbance values too low: incubation in wrong conditions (time or temperature) or reading time too short or CGRP standard, anti-CGRP tracer or Ellman's reagent have not been dispensed.
- ☞ NSB value too high: contamination of NSB wells with CGRP standard, or inefficient washing.
- ☞ High dispersion of duplicates: poor pipetting technique or irregular plate washing.
- ☞ Analyses of two dilutions of a biological sample do not agree: Interfering substances are present. Some samples must be purified prior to EIA analysis (see sample preparation section).

These are a few examples of trouble shooting that may occur. If you need further explanation, SPI-BIO will be happy to answer any questions or information about this assay. Please feel free to contact our technical support staff by letter, phone (33 (0)1 39 30 62 60), fax (33 (0)1 39 30 62 99) or E-mail (sales@spibio.com), and be sure to indicate the lot number of the kit (see outside of the box).

SPI-BIO offers a training workshop in EIA practice & theory. This workshop is given twice a year. For further information, please contact our Customer Relation Representative (33 (0)1 39 30 62 60).



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