CGRP (human)

A brand name of

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

CGRP (human)
Enzyme Immunoassay kit
#A05481.96 wells

For research laboratory use only
Not for human diagnostic use

This assay has been developed & validated
by Bertin Pharma

Fabriqué en France
Made in France

#A11481
Version: 0116

Table of contents

96 wells
Storage: -20°C
Expiry date: stated on the package

This kit contains:

Designation	Colour of cap	Item #	Quantity per kit	Form
CGRP precoated 96-well Strip Plate	blister with zip	A08481.1 ea	1	-
CGRP (human) Tracer	green	A04481.100 dtn	1	Lyophilised
CGRP (human) Standard	blue with septum	A06481.1 ea	2	Lyophilised
EIA Buffer	blue	A07000.1 ea	1	Lyophilised
Wash Buffer	silver	A17000.1 ea	1	Liquid
Tween 20	transparent	A12000.1 ea	1	Liquid
CGRP (human) Quality Control	green with septum	A10481.1 ea	2	Lyophilised
Ellman's Reagent_49+1	black with septum	A09000_49+1.100 dtn	2	Lyophilised
Technical Booklet	-	A11481. 1ea	1	-
Well cover sheet	-	-	1	-

Each kit contains sufficient reagents for 96 wells. This allows for the construction of one standard curve in duplicate and the assay of 36 samples in duplicate

Precaution for use

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

Each time a new pipette tip is used, aspirate a sample or reagent and expel it back into the same vessel. Repeat this operation two or three times before distribution in order to equilibrate the pipette tip.

- For research laboratory use only
- Not for human 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.

Wearing gloves, laboratory coat and glasses is recommended when assaying kit materials and samples.

Temperature

Unless otherwise specified, all the experiments are done at room temperature (RT), that is around +20°C. Working at +25°C or more affects the assay and decreases its efficiency.

Background

Acetylcholinesterase AChE® Technology

Acetylcholinesterase (AChE®), the enzymatic label for EIA, has the fastest turnover rate of any enzymatic label. This specific AChE is extracted from the electric organ of the electric eel, *Electrophorus electricus*, and is capable of massive catalytic turnover during the generation of the electrochemical discharges. The use of AChE as enzymatic label for EIA has been patented by the French academic research Institute CEA ***[1, 2, 3]***, and Bertin Pharma, formerly known as SPI-Bio, has expertise to develop and produce EIA kits using this technology.

AChE® assays are revealed with Ellman's Reagent, which contains acetylthiocholine as a substrate. The final product of the enzymatic reaction (5-thio-2-nitrobenzoic acid), is bright yellow and can be read at 405-414 nm. AChE® offers several advantages compared to enzymes conventionally used in EIAs:

- **Kinetic superiority and high sensitivity**: AChE® shows true first-order kinetics with a turnover of 64,000 sec^{-1}. That is nearly 3 times faster than Horseradish Peroxidase (HRP) or alkaline phosphatase. AChE® allows a greater sensitivity than other labeling enzymes.
- **Low background**: non-enzymatic hydrolysis of acetylthiocholine in buffer is essentially absent. So, AChE® allows a very low background and an increased signal/noise ratio compared to other substrate of enzymes which is inherently unstable.

- **Wide dynamic range**: AChE® is a stable enzyme and its activity remains constant for many hours as, unlike other enzymes, its substrate is not suicidal. This permits simultaneous assays of high diluted and very concentrated samples.
- **Versatility:** AChE® is a completely stable enzyme, unlike peroxidase which is suicidal. Thus, if a plate is accidentally dropped after dispatch of the AChE® substrate (Ellman's Reagent) or if it needs to be revealed again, one only needs to wash the plate, add fresh Ellman's Reagent and proceed with a new development. Otherwise, the plate can be stored at +4°C with wash buffer in wells while waiting for technical advice from the Bioreagent Department.

CGRP

Calcitonin Gene Related Peptide (CGRP) is a potent vasodilator, and also elicits a number of other biological effects.

Average plasma levels of CGRP have been reported to be from 0.8 pmol/L to 71 pmol/L (3 pg/mL to 269 pg/mL) in normal subjects. Increases in circulating CGRP levels have been noticed during hemodialysis, pregnancy, exacerbation of asthma and in cases of medullary thyroid carcinoma.

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 of 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.

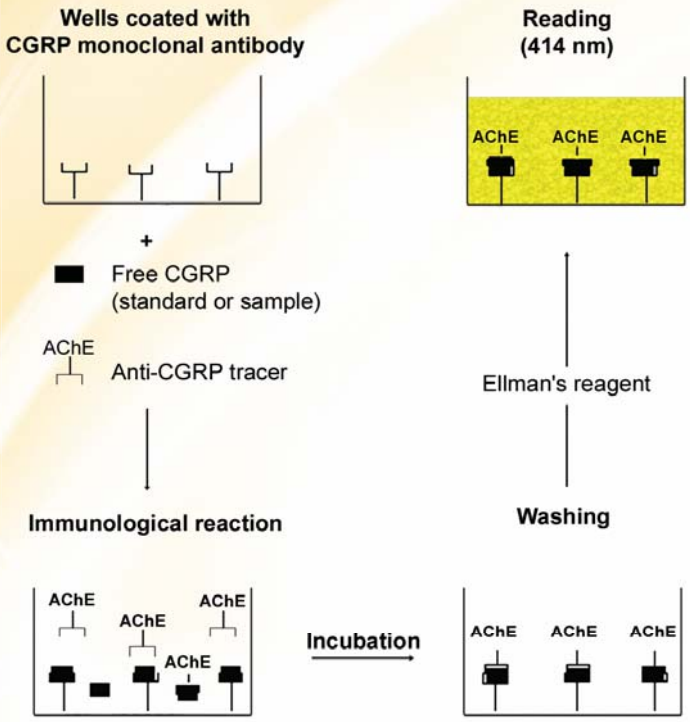
This allows the two antibodies to form a sandwich by binding on different parts of the human CGRP molecule.

The sandwich is immobilised on the plate so the excess reagents may be washed away.

The concentration of human CGRP is then determined by measuring the enzymatic activity of the AChE using 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 human 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 the sample preparation:

- C-18 reverse phase cartridges or Oasis® HLB Extraction cartridges
- Methanol
- Acetic acid

For the assay:

- Precision micropipettes (20 to 1000 µL)
- Spectrophotometer plate reader (405 or 414 nm filter)
- Microplate washer (or washbottles)
- Orbitral microplate shaker
- UltraPure water (item number #A07001.1L)
- Polypropylene tubes

Water used to prepare all EIA reagents and buffers must be Ultra Pure, deionized & free from organic contaminants traces.
Otherwise, organic contamination can significantly affect the enzymatic activity of the tracer Acetylcholinesterase.
Do not use distilled water, HPLC-grade water or sterile water.

- UltraPure water may be purchased from Bertin Pharma (item #A07001.1L).

Sample preparation

General precautions

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

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 2N acetic acid, heat at 90°C for ten minutes, centrifuge, dry the supernatant, and then dissolve in EIA Buffer.

Plasma and serum samples should be measured after extraction (see extraction protocol below), or without any extraction procedure.

Whole blood, as well as other heterogeneous mixtures such as lavage fluids and aspirates should be purified (see extraction 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 (Bertin Pharma #D30005 or Waters item #WAT094226-HLB-3cc) by first passing 5 mL of methanol and then 10 mL of UltraPure water through the cartridge. The reverse phase cartridge (RPC) may be stored with the water still present.
- Dilute the sample 1:4 with 4% acetic acid.
- Pass 1 mL of sample slowly (about 2 mL/minute) through the cartridge.
- Wash the cartridge with 10 mL of 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 1 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 = recovery factor
X/a = original concentration of the unspiked sample in a volume known (a)
(X+Y)/b = 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 and [(X+Y)/b]z.

Example

- Volume of the unspiked sample: a = 1mL
- Final volume of the spiked sample: b = 2 mL
- Concentration determined by EIA for the unspiked sample: (X/a)z = 8 pg/mL
- Concentration determined by EIA for the spiked sample: [(X+Y)/b]z = 16 pg/mL
- Quantity of spike: Y = 30 pg in 1mL

$Xz = 8 \Leftrightarrow z = 8/X$
$[(X+30)/2]z = 16 \Leftrightarrow [(X+30)]z = 32$
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).

Reagent preparation

All reagents need to be brought to room temperature, around +20°C, prior to the assay.

EIA Buffer

Reconstitute the vial #A07000 with 50 mL of UltraPure water. Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion.
Stability at 4°C: 1 month

CGRP (human) Standard

Reconstitute the Standard vial (item #A06481) with 1 mL of EIA Buffer. Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion. The concentration of the first standard (S1) is 1000 pg/mL.

Prepare seven propylene tubes for the other standards and add 500 µL of EIA Buffer into each tube. Then prepare the standards by serial dilutions as follows:

Standard	Volume of Standard	Volume of Assay Buffer	Standard concentration
S1	-	-	1000 pg/mL
S2	500 µL of S1	500 µL	500 pg/mL
S3	500 µL of S2	500 µL	250 pg/mL
S4	500 µL of S3	500 µL	125 pg/mL
S5	500 µL of S4	500 µL	62.5 pg/mL
S6	500 µL of S5	500 µL	31.25 pg/mL
S7	500 µL of S6	500 µL	15.53 pg/mL
S8	500 µL of S7	500 µL	7.81 pg/mL

Stability at 4°C: 24 hours

▷ CGRP (human) Quality Control

Reconstitute one vial with 1 mL of EIA Buffer. Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion.
Stability at +4°C: 24 hours.

▷ CGRP (human) 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 #A17000 with 400 mL of UltraPure water. Add 200 µL of Tween 20 #A12000. Use a magnetic stirring bar to mix the content.
Stability at +4°C: 1 week

Ellman's Reagent

5 minutes before use (development of the plate), reconstitute one vial of Ellman's Reagent #A09000_49+1 with 49 mL of UltraPure water and 1 mL of Wash Buffer #A17000. The tube content should be thoroughly mixed.
Stability at +4°C and in the dark: 24 hours

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, store at +4°C for 1 month maximum.

Rinse each well 5 times with the Wash Buffer (300 µL/well).

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

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 template sheet provided at the end of this technical booklet.

Pipetting the reagents

All samples and reagents must reach room temperature prior to performing the assay.

Use different tips to pipette the buffer, standard, sample, tracer and other reagents.
Before pipetting, equilibrate the pipette tips in each reagent.
Do not touch the liquid already in the well when expeling with the pipette tip.

> **EIA Buffer**
Dispense 100 µL to Non Specific Binding (NSB) wells.

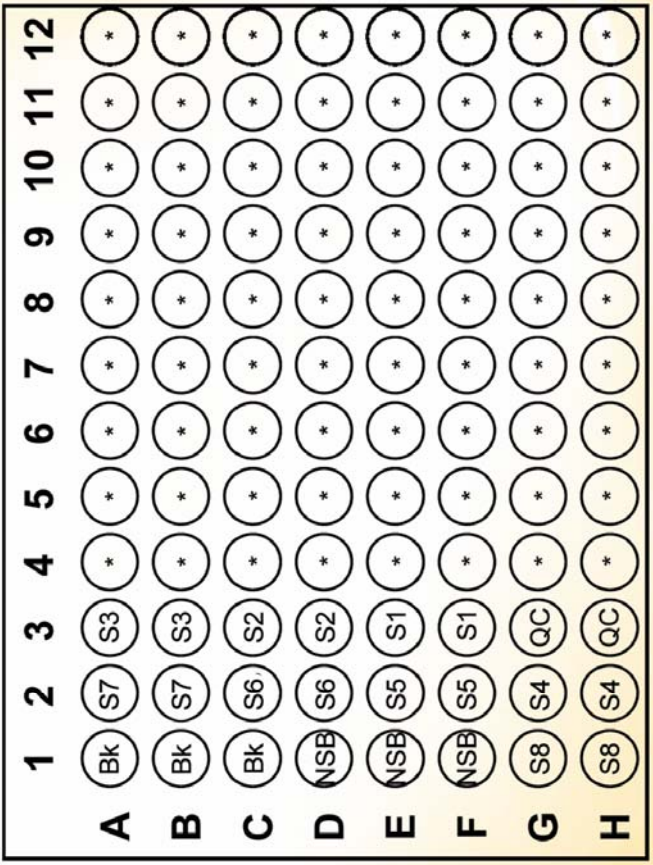
> **CGRP (human) Standard**
Dispense 100 µL of each of the eight standards S1 to S8 in duplicate to appropriate wells.
Start with the lowest concentration standard S8 and equilibrate the tip in the next higher standard before pipetting.

> **CGRP (human) Quality Control and samples**
Dispense 100 µL in duplicate to appropriate wells. Highly concentrated samples may be diluted in EIA Buffer.

> **CGRP (human) Tracer**
Dispense 100 µL to each well, **except** Blank (Bk) wells.

▷ Incubating the plate

Cover the plate with the cover sheet and incubate for 16-20 hours at +4°C.



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

Developing and reading the plate

- Reconstitute Ellman's Reagent as mentioned in the Reagent preparation section.
- Empty the plate by turning it over. Rinse each well five times with 300 µL of Wash Buffer. At the end of the last washing step, empty the plate and blot the plate on a paper towel to discard any trace of liquid.
- Add 200 µL of Ellman's Reagent to each 96 well. Cover the plate with aluminium sheet and incubate in the dark at room temperature. Optimal development is obtained using an orbital shaker.
- Wipe the bottom of the plate with a paper towel, and make sure that no liquid has splashed outside the wells.
- **Read the plate at a wavelength between 405 and 414nm (**yellow colour**). After addition of Ellman's Reagent, the absorbance has to be checked periodically (**every 30 minutes**) until the maximum absorbance has reached a minimum of 0.5 A.U. (**blank subtracted**).**

Enzyme Immunoassay Protocol (volumes are in µL)				
	Blank	NSB	Standard	Sample
Buffer	-	100	-	-
Standard	-	-	100	-
Sample	-	-	-	100
Tracer	-	100	100	100
Cover plate, incubate 16-20 hours at +4°C				
Wash strips 3 times, shlightly shake them 2 min., rewash 3 times & discard liquid from the wells				
Ellman's Reagent	200			
Incubate with an orbital shaker in the dark at RT				
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 alone) from the absorbance readings of the rest of the plate. If it is not the case, please do it.

- Calculate the average absorbance for each NSB, standard and sample.
- For each standard, plot the absorbance on *y* axis versus the concentration on *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 1000 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 (logit/log or 4-parameter logistic fit 4PL). If you have this type of software, we recommend using it. Refer to it for further information.

Two vials of Quality Control are provided with this kit.
Your standard curve is validated only if the calculated concentration of the Quality Control obtained with the assay is +/- 25% of the expected concentration (see the label of QC vial**)**

Acceptable range

- Non-Specific Binding: <60 mAU
- Limit of detection: <10 pg/mL.
- Quality control: +/-25% of the expected concentration (see the label of QC vial)

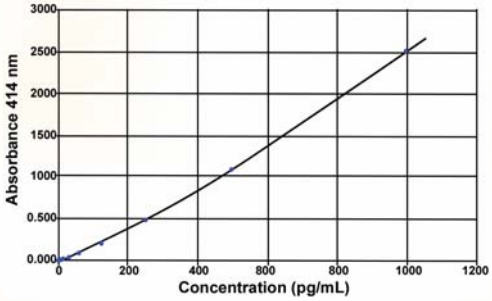
Typical results

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: 60 minutes developing at +20°C, reading at 414 nm. A spline fitting was used to determine the concentrations.

CGRP	mAU
Blank	2.0
Standard 1000 pg/mL	2808
Standard 500 pg/mL	1420
Standard 250 pg/mL	686
Standard 125 pg/mL	311
Standard 62.5 pg/mL	167
Standard 31.25 pg/mL	76.5
Standard 15.63 pg/mL	40.5
Standard 7.81 pg/mL	18.0

Typical CGRP (human) standard curve



Assay validation and characteristics

The Enzyme Immunometric assay of CGRP (human) has been validated for its use in buffer and plasma (without 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) and 2 pg/mL (in plasma).

> Quality Control samples intra-assay variations in EIA Buffer (n = 25)

CGRP (human)	C.V.	CGRP (human)	C.V.
7.81 pg/mL	> 25 %	125 pg/mL	3.3 %
15.6 pg/mL	20.6 %	250 pg/mL	3.0 %
31.3 pg/mL	7.0 %	500 pg/mL	2.7 %
62.5 pg/mL	5.2 %	1000 pg/mL	3.4 %

> Quality control samples inter-assay variations (n=25) in EIA Buffer

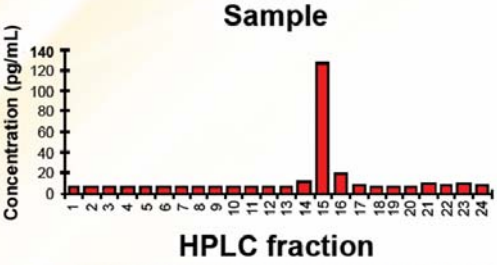
CGRP (human)	C.V.	CGRP (human)	C.V.
7.81 pg/mL	16.6 %	125 pg/mL	2.8 %
15.6 pg/mL	12.2 %	250 pg/mL	0.7 %
31.3 pg/mL	6.3 %	500 pg/mL	4.1 %
62.5 pg/mL	4.3 %	1000 pg/mL	-

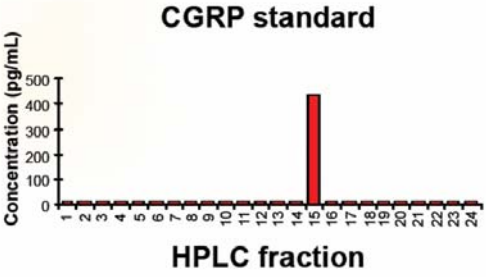
> Cross-reactivity

Intra-assay		Inter-assay	
CGRP-I/II (human)	100 %	CGRP (8-37)	<0.01 %
CGRP-α/β (human)	100 %	Amylin	<0.01 %
CGRP-I/II (rat)	120 %	Calcitonin	<0.01 %
CGRP-α/β (rat)	120 %	Substance P	<0.01 %

> Specificity

Comparison of HPLC profiles of a CGRP standard and a sample.





Assay troubleshooting

- **Absorbance values are too low**: organic contamination of water, or one reagent has not been dispensed, or incorrect preparation, or assay performed before reagents reached room temperature, or reading time not long enough.

- **High signal and background in all wells**: Inefficient washing or overdeveloping (incubation time should be reduced) or high ambient temperature.

- **High dispersion of duplicates**: Poor pipetting technique or irregular plate washing.

These are a few examples of troubleshooting that may occur. If you need further explanation, Bertin Pharma will be happy to assist you. Feel free to contact our technical support staff by phone (+33 (0)139 306 036), fax (+33 (0)139 306 299) or E-mail (bioreagent@bertinpharma.com), and be sure to indicate the batch number of the kit (see outside the box).

Bertin Pharma proposes EIA Training kit #B05005 and EIA workshop upon request. For further information, please contact our Marketing Department by phone (+33 (0)139 306 260) or E-mail (marketing@bertinpharma.com).

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Bertin Pharma, over the last decades, has been developing and marketing over 100 biomarker assays, pre-analytical products, kits, antibodies and bio-chemicals thanks to its innovative work in research and development.
Our core areas are orientated to inflammation, oxidative injury, endocrinology, diabetes, obesity, hypertension, neurodegenerative diseases, HIV, prion diseases, pharmacokinetics and metabolism.

Bertin Pharma is active worldwide either with direct sales or through our qualified and trained international distribution network from the United States to Japan.

We are able to provide you with local technical support to use at ease our products.

For further information, please send your request to
bioreagent@bertinpharma.com

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