Example Version

CGRP
(rat)

For laboratory research only. Not for human or veterinary diagnostic use.

Bertin Bioreagent also markets pre-analytical products, ELISA kits, antibodies & biochemicals for:

- **Inflammation**
- **Oxidative injury**
- **Endocrinology**
- **Diabetes**
- **Obesity**
- **Hypertension**
- **Pain**
- **Prion diseases**

Example Version

Do not hesitate to contact our after-sales services for further information at tech@bertin-bioreagent.com

CGRP (rat)

ELISA kit

#A05482.96 wells

Example Version

For research laboratory use only
Not for human diagnostic use

This assay was developed
& validated by Bertin Bioreagent

Fabriqué en France
Made in France

#A11482

Version: 0122

Example Version

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Example version

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	A05481.1 ea	1	-
CGRP (rat) Tracer	Green	A04482.100 dtn	1	Lyophilised
CGRP (rat) Standard	Blue with red septum	A06482.1 ea	2	Lyophilised
CGRP (rat) Quality Control	Green with red septum	A10482.1ea	2	Lyophilised
ELISA Buffer	Blue	A07000.1 ea	1	Lyophilised
Wash Buffer	Silver	A17000.1 ea	1	Liquid
Tween 20	Transparent	A12000.1 ea	1	Liquid
Ellman's reagent 49+1	Black with red septum	A09000_49+1.100 dtn	2	Lyophilised
Technical Booklet	-	A11482.1 ea	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.

Example Version

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 where kit reagents are handled
- Avoid splashing

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

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

Temperature

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

Example version

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 it is capable of providing a rapid catalytic turnover during the generation of the electrochemical discharges. The use of AChE as enzymatic label for EIA is patented by the French academic research Institute CEA ***[1, 2, 3]***, and Bertin Bioreagent has expertise to develop and produce EIA/ELISA 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 in color and can be read at 405-414 nm using a spectrophotometer. AChE offers several advantages over other commonly used enzymes 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 Horse Radish Peroxidase (HRP) or alkaline phosphatase. AChE provides greater sensitivity than other labeling enzymes.
- **Low background**: Non-enzymatic hydrolysis of acetylthiocholine in buffer is essentially absent. Thus, AChE ensures a very low background and an increased signal/noise ratio compared to other

Example version

substrate of enzymes that are inherently unstable.

- **Wide dynamic range:** AChE is a stable enzyme and its activity remains constant for many hours. Unlike other enzymes, AChE has substrate that is not suicidal which permits simultaneous assays of high and low concentration samples.
- **Versatility:** AChE is a completely stable enzyme, unlike peroxidase which is suicidal. The accidentally dropped plate containing AChE substrate (Ellman's reagent) does not need to be discarded and experiment can be continued by adding washing buffer and fresh Ellman's reagent into the plate wells. As an option Otherwise, plate can be stored at +4°C containing washing buffer 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 ***[4, 5]***.

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.

Example Version

Principle of the assay

The enzymatic immunoassay (EIA/ELISA) is based on a sandwich technique. Wells of supplied plate are coated with a monoclonal antibody specific to CGRP.

CGRP introduced into the wells (standard or sample) is bound by the monoclonal antibody coated on the plate. Then an acetylcholinesterase (AChE) - Fab' conjugate, which binds selectively to a different epitope on CGRP, 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.

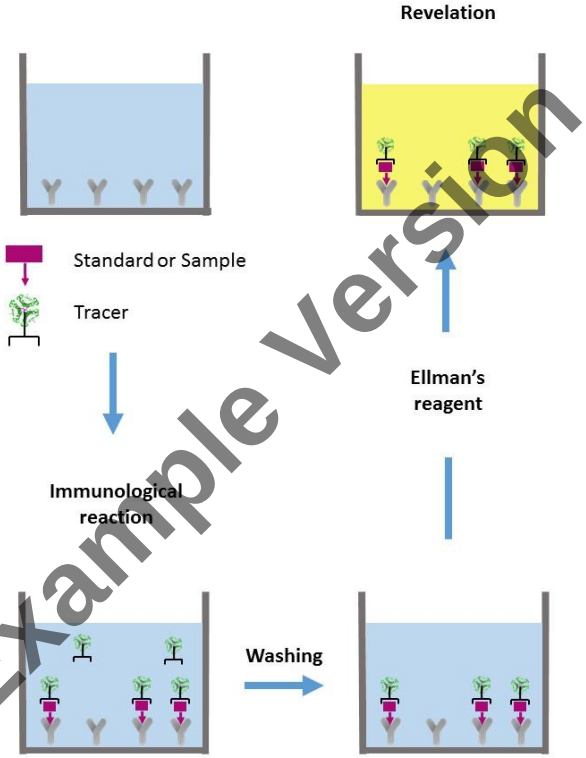
The sandwich is immobilised on the plate so the excess reagents may be washed away.

The concentration of rat CGRP is determined by measuring the enzymatic activity of immobilized Tracer using Ellman's reagent. AChE tracer acts on Ellman's Reagent to form a yellow compound that strongly absorbs at 405 nm or at 414 nm.

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

Example version

The principle of the assay is summarised below:



Example Version

Assay validation and characteristics

Validated for use:

- in buffer
- in plasma without extraction (using a standard curve in CGRP free plasma) ***[6]***.

For additional information regarding the validation of immunoassay for protein biomarkers in biological samples, please refer to bibliography ***[7,8]***

Limit of detection (LOD):

0.7 pg/mL (in ELISA Buffer) and 2 pg/mL (in plasma), calculated as the concentration of CGRP corresponding to the NSB average (n=8) plus three standard deviations.

Intra-assay & inter-assay variation:

Quality control samples intra-assay and inter-assay variations in ELISA buffer (n=25)

Intra-assay		Inter-assay	
CGRP (rat)	**C.V**	**CGRP (rat)**	**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 %

Example Version

Quality control samples intra-assay and inter-assay variations in plasma (n= 25)

Intra-assay		Inter-assay	
CGRP (rat)	**C.V**	**CGRP (rat)**	**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	16.5 %

> ***Cross-reactivity***

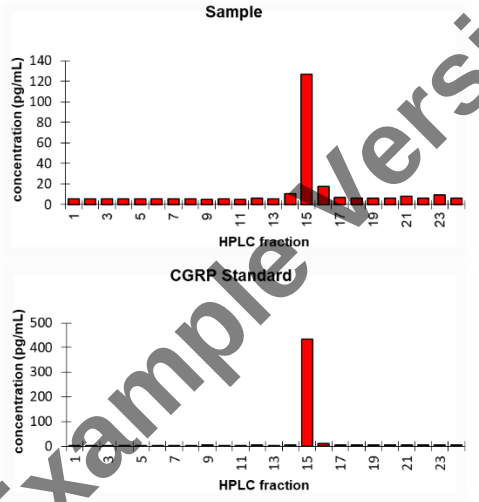
Cross reactivity was tested in ELISA buffer. For each tested molecule, a standard curve was prepared with identical concentration to the standard range and was assayed with the kit.

CGRP-α/β (rat)	100 %	CGRP (8-37)	<0.01 %
CGRP-I/II (rat)	100 %	Amylin	<0.01 %
CGRP-α/β (human)	83 %	Calcitonin	<0.01 %
CGRP-I/II (human)	83 %	Substance P	<0.01 %

Example Version

> *Specificity*

Comparison of HPLC profiles of a CGRP standard and a sample



Example Version

Materials and equipment required

In addition to standard laboratory equipment, the following materials are required:

For the sample preparation (not necessary for all types of samples):

- 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 nm or 414 nm filter)
- Microplate washer (or wash bottles)
- Orbital microplate shaker
- Multichannel pipette and disposable tips 30-300µL
- UltraPure water #A07001.1L
- Polypropylene tubes

Water used to prepare all ELISA reagents and buffers must be UltraPure (deionized & free from organic contaminant traces).
Do not use distilled water, HPLC-grade water or sterile water.

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

Example Version

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 tissue samples

Nervous tissues such as cerebrospinal fluid may be assayed directly if diluted more than 1/20 in ELISA buffer. Other nervous tissues such as spinal cord may be assayed after extraction procedure.
Basically, the procedure [6] is to homogenize the tissue in 2N acetic acid (1 mg tissue in 4mL acid), heat at 90°C for ten minutes, centrifuge, freeze-dry the supernatant (if freeze-drying is not possible a vacuum centrifugation with controlled temperature (+4°C) can be used.), and store under lyophilized form. Just before assay, reconstitute with ELISA buffer.

Plasma and serum

Plasma and serum samples should be measured according to one of these two methods:

- Either without an extraction procedure (option 1, left side of the scheme below); in which case the QC and Standards should be reconstituted with CGRP-free plasma/serum.

Example Version

- or with an extraction procedure (option 2, right side of the scheme below); in which case the QC and Standards should be reconstituted in ELISA buffer.

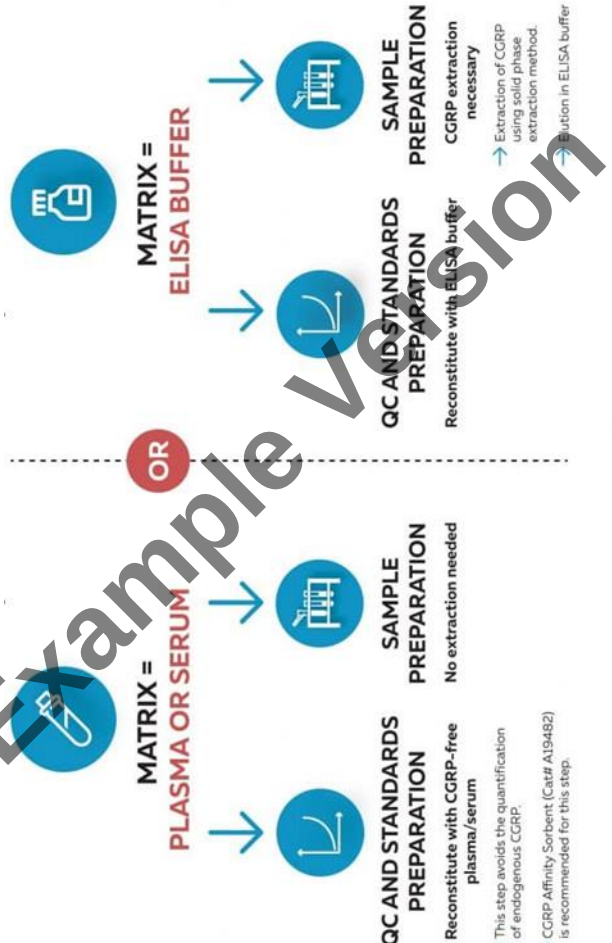
> *Option 1: Without extraction procedure*

In this first option, the common matrix for samples, Standard and QC is plasma or serum. Therefore, CGRP Standard and Quality Control have to be reconstituted with plasma or serum that is free from CGRP (human), instead of the ELISA buffer as mentioned in reagent preparation section (CGRP Standard and Quality Control). The dilutions of the CGRP Standard should also be prepared with plasma or serum that is free from CGRP (human).
If you don't have plasma or serum that is free of CGRP (human), Bertin Bioreagent offers CGRP Affinity Sorbent containing anti-CGRP monoclonal antibody (the same as the one coated on the wells) as item Cat# A19482. To prepare CGRP-free plasma, use this affinity sorbent Cat# A19482 with a pool of 2 or 3 different sources of plasma or serum.

> *Option 2: With extraction procedure*

In this option, Standards QC and samples are assayed in ELISA buffer as a matrix. Please refer to the extraction protocol below to process your samples before the ELISA assay.

Example version



Example Version

Other samples

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 wells.

As CGRP has got a short half-life in blood, it is advised to add inhibitors at the time of the sample collection, to prevent any degradation of CGRP by blood proteases. Here is an example of composition of protease inhibitor cocktail which can be used: Leupeptine 20 µg/mL, Benzamindine 0.3 mg/mL, Pepstatin 2.5 µg/mL, Chymostatin 20 µg/mL, EDTA $6x10^{-3}$ M, PHMB 0.36 mg/mL.

Extraction protocol

Extraction steps:

- Activate a 1 mL C-18 reverse phase cartridge or an Oasis® HLB Extraction cartridge Waters (cat# WAT094226-HLB-3cc) by first passing 5 mL of methanol and then 10 mL UltraPure water through the cartridge. The reverse phase cartridge (RPC) may be stored with the water present.
- Dilute the sample at 1/4 with 4% acetic acid (e.g. 250 µl of sample + 750 µl of 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: 4% acetic acid aqueous solution (90:10, v/v). Elute the CGRP by passing the methanol: water solution through the cartridge

Example version

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 with a temperature controlled device (+4°C). If done at room temperature, the vacuum centrifugation (Speed Vac) shouldn't last too long (one hour or less). Reconstitute the sample with a volume of ELISA Buffer equal to the original sample volume.
- Assay the aliquots of the sample and use the results to calculate the recovery.

> *Extraction recovery and calculation*

When an extraction process is done, it is recommended to calculate a recovery rate which is the percentage of analyte recovered after the extraction.

To determine the recovery rate, a sample needs to be split into two aliquots before extraction:

- One aliquot that is unspiked. The concentration of analyte in this aliquot is equal to the concentration of analyte in the original sample.
- One Aliquot which will be spiked with a known amount of analyte.

The extraction protocol will be applied to both, unspiked and spiked, aliquots and then the level of analyte in each aliquot will be determined by ELISA, allowing for the calculation of the recovery rate. For an example of the calculation, please read the Appendix section.

Example Version

Reagent preparation

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 according to suggested plate layout.

An additional vial of Standard, Quality Control and Ellman's reagent are provided in case you need to perform 2 assays with the kit.

All reagents must be brought to room temperature (around +20°C) prior the use in assay.

ELISA Buffer

Reconstitute the ELISA Buffer #A07000 with 50 mL of UltraPure water. Allow buffer to stand for 5 minutes or until it is completely dissolved. Mix buffer thoroughly by gentle inversions.
Stability at 4°C: 1 month.

CGRP (rat) Standard

Reconstitute the CGRP (rat) Standard vial #A06482 with 1 mL of ELISA Buffer (For plasma and serum samples without extraction, CGRP Standard need to be reconstituted with CGRP free plasma and serum (see sample preparation step) The dilutions of CGRP standard should also be prepared with CGRP free plasma or serum).

Allow standard to stand for 5 minutes or until it is completely

Example Version

dissolved. Mix standard thoroughly by gentle inversions.

The concentration of the first standard (S1) is 500 pg/mL. Prepare seven polypropylene tubes (for the seven other standards) and add 500 µL of ELISA Buffer of CGRP-free serum/plasma into each tube. Then prepare the standards by serial dilutions as indicated in following table. Mix each tube thoroughly before the next transfer.

Standard	Volume of Standard	Volume of ELISA Buffer or CGRP-free serum/plasma	Standard concentration
S1	-	-	500 pg/mL
S2	500 µL of S1	500 µL	250 pg/mL
S3	500 µL of S2	500 µL	125 pg/mL
S4	500 µL of S3	500 µL	62.5 pg/mL
S5	500 µL of S4	500 µL	31.25 pg/mL
S6	500 µL of S5	500 µL	15.53 pg/mL
S7	500 µL of S6	500 µL	7.81 pg/mL
S8	500 µL of S7	500 µL	3.91 pg/mL

Stability at 4°C: 24 hours

CGRP (rat) Quality Control

Reconstitute one QC vial #A10482 with 1 mL of ELISA Buffer (For plasma and serum samples without extraction, CGRP Quality Control need to be reconstituted with CGRP free plasma and serum (see sample preparation step)).
Allow it to stand for 5 minutes or until it is completely dissolved. Mix quality control thoroughly by gentle inversions.
Stability at 4°C: 24 hours

Example Version

CGRP (rat) Tracer

Reconstitute the Tracer vial #A04482 with 10 mL of ELISA Buffer. Allow tracer to stand for 5 minutes or until it is completely dissolved. Mix tracer thoroughly by gentle inversions.
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. Note that concentrated wash buffer is also used for Ellman's reagent preparation.
Stability at +4°C: 1 week.

Ellman's Reagent

5 minutes before use (development of the plate), reconstitute one vial of Elman's Reagent #A09000_49+1 with 49 mL of UltraPure water and 1 mL of **concentrated** Wash Buffer#A17000. The tube content should be thoroughly mixed.

Stability a +4°C and in the dark: 24 hours

Assay procedure

It is recommended to measure the samples in duplicate following the instruction below.

Example Version

Plate preparation

Prepare the Wash Buffer as indicated in the reagent preparation section.

Open the plate pouch and select enough strips for your assay. Place unused strips back in the pouch.

Stability at +4°C: 1 month.

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

Just before distributing the reagents and samples, remove the buffer from the wells by inverting the plate and blot the last drops by tapping it on paper towels.

Plate set-up

A plate set-up is suggested hereafter. The contents of each well may be recorded on the template sheet provided at the end of this technical booklet.

Example Version

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

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

Example version

Pipetting the reagents

Samples and reagents must reach room temperature prior performing the assay.

Use new tips to pipet buffers, standards, samples, antibody and other reagents.

Before pipetting, equilibrate the pipette tips in each reagent. Do not touch the liquid already in the well when expelling with the pipette tip.

> ***ELISA Buffer***

Dispense 100 µL to Non Specific Binding wells (NSB) wells.

> ***CGRP (rat) Standard***

Dispense 100 µL of each of the eight standards (S8 to S1) in duplicate to appropriate wells.

Start with the lowest concentration standard (S8) and equilibrate the tip in the next higher standard before pipetting.

> ***CGRP (rat) Quality Control and Sample***

Dispense 100 µL in duplicate to appropriate wells. Highly concentrated samples may be diluted in ELISA Buffer.

> ***CGRP (rat) Tracer***

Dispense 100 µL to each well, <u>except Blank (Bk) wells.</u>

▷ Incubating the plate

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

▷ 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 3 times with 300 µL of Wash Buffer. The 3rd time,

Example Version

slightly shake during 2 minutes. Then wash 3 times. 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 well.
- Cover the plate with cover 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 been projected outside the wells.
- Read the plate at 405 nm or at 414 nm (yellow color) using spectrophotometer plate reader. **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).**

Assay procedure summary

Enzyme Immunoassay Protocol (volumes are in µL)				
	Blank	NSB	Standard	Sample or QC
ELISA Buffer or CGRP-free serum/plasma	-	100	-	-
Standard	-	-	100	-
Sample or QC	-	-	-	100
Tracer	-	100	100	100
Cover plate, incubate **16-20 hours** at +4°C				
Wash strips 3 times, slightly shake during 2 min, then wash 3 times & Discard liquid from the wells & dry on absorbent paper				
Ellman's reagent	200			
Incubate with an orbital shaker in the dark at RT				
Read the plate at 405 nm or at 414 nm				

Example version

Data analysis

Make sure that your plate reader has subtracted the absorbance readings of the blank wells from the absorbance readings of the rest of the plate.

- Calculate the average absorbance for each NSB, standards, QC and samples.
- For each standard, plot the absorbance (y axis) versus the concentration (x axis) graph. Draw a best-fit line through the points.
- To determine the concentration of 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 unknown samples.
- Samples with a concentration greater than 500 pg/mL must be re-assayed after dilution in ELISA Buffer.
- Most plate readers come with a curve-fitting software pre-installed that is capable of generating graphs (4-parameter logistic fit 4PL). It is highly recommended to use this software if available on the device. Refer to it for further information.

2 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 the QC vial)

Example Version

Acceptable range

- NSB absorbance < 0.06 A.U.
- Limit of detection < 10 pg/mL
- QC ±25% of the expected concentration (see the label of the QC vial)

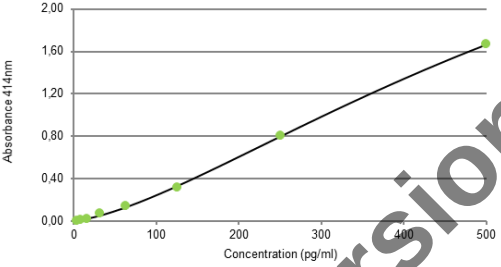
Typical results

The following data are for demonstration purpose only. Your data may be different and still correct.
The data was 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.

Standard	CGRP (pg/mL)	Absorbance A.U.
S1	500	1.669
S2	250	0.808
S3	125	0.319
S4	62.5	0.144
S5	31.25	0.071
S6	15.63	0.024
S7	7.81	0.013
S8	3.91	0.006

Example Version

Typical CGRP (rat) standard curve



Troubleshooting

> ***Absorbance values are too low:***

- one of the reagents was not properly dispensed,
- organic contamination of water,
- incorrect preparation,
- assay performed before reagents reached room temperature,
- reading time not long enough.

> ***High signal and background in all wells:***

- inefficient washing,
- overdeveloping (incubation time should be reduced),
- high ambient temperature.

Example Version

> ***High dispersion of duplicates:***

- poor pipetting
- irregular plate washing.

> ***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.

These are a few examples of troubleshooting that may occur. If further information or explanation is needed, please contact Bertin Bioreagent Technical Support by phone on +33 (0)139 306 036, fax +33 (0)139 306 299 or by E-mail tech@bertin-bioreagent.com. Please have batch number of the kit (see outside the box) ready to provide to the technical support.

Bertin Bioreagent offers EIA Training kit #B05005. Feel free to contact our Technical Support. We are always happy to hearing from you.

Example version

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Example Version

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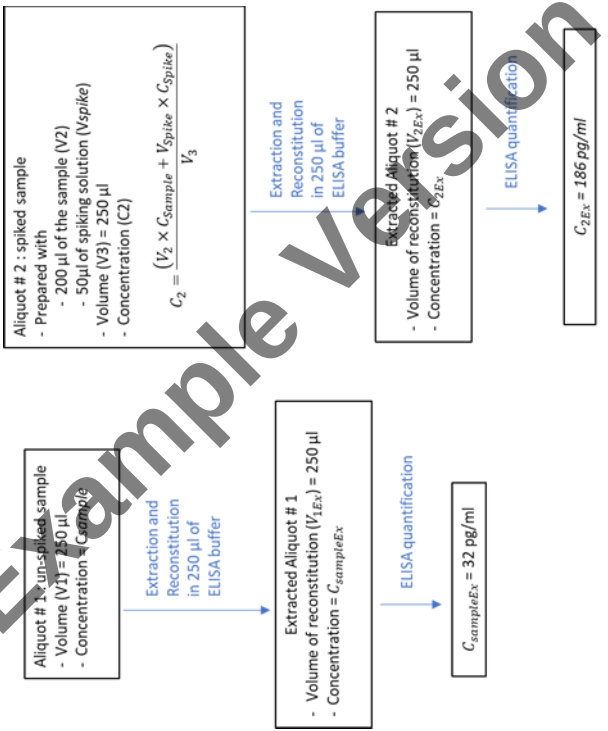
Example Version

Appendix: CGRP Extraction Method: Calculating the recovery rate

The following example depicts the calculation of the recovery rate after extraction of a sample with an unknown CGRP concentration (Csample) and a spike solution with a concentration Cspike of 1000 pg/ml
After extraction, the recovery rate (R) is calculated by comparing the measured levels of analyte before and after extraction.



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By definition: $$R = \frac{C_{sampleEx} \times V_{1Ex}}{C_{sample} \times V_1} = \frac{C_{2Ex} \times V_{2Ex}}{C_2 \times V_2}$$

As $V_{1Ex} = V_1$, then $$C_{sample} = \frac{C_{sampleEx}}{R}$$

As $V_{2Ex} = V_2$, then $$C_{2Ex} = R \times C_2$$

$$C_{2Ex} = R \times \frac{(V_3 \times C_{Sample} + V_{Spike} \times C_{Spike})}{V_2}$$

$$C_{2Ex} = R \times \frac{\left(V_3 \times \frac{C_{sampleEx}}{R} + V_{Spike} \times C_{Spike}\right)}{V_2}$$

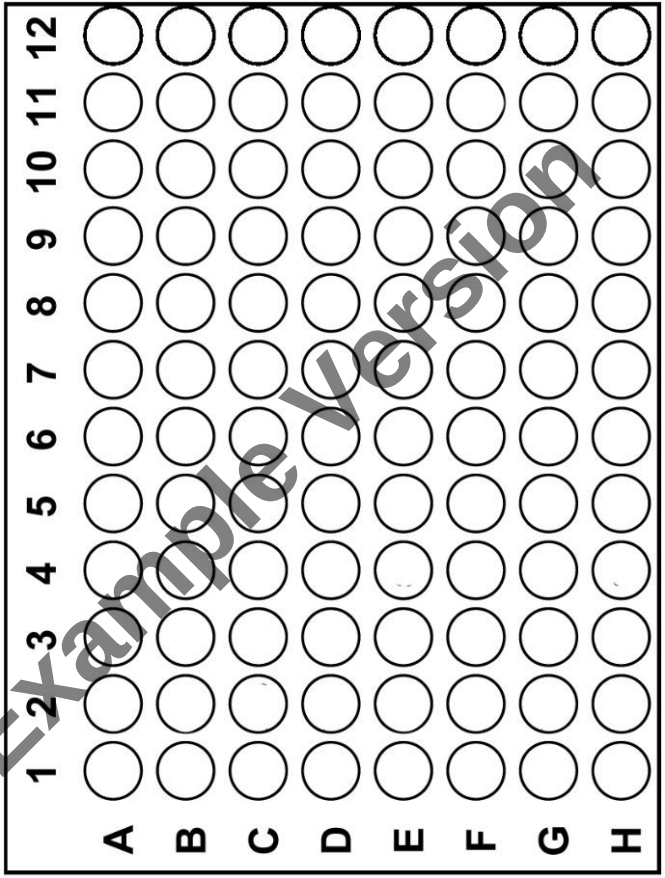
Thus $$\boxed{R = \frac{C_{2Ex} \times V_2 - C_{sampleEx} \times V_3}{V_{Spike} \times C_{Spike}}}$$

$$R = \frac{186 \times 250 - 32 \times 200}{50 \times 1000} = 0.8$$

And finally: $$\boxed{C_{sample} = \frac{C_{sampleEx}}{R} = \frac{32}{0.8} = 40\ pg/ml}$$

N.B. : To minimize the calculations, the spiking solution should be concentrated enough so that its addition does not alter the volume of the spiked aliquot to any great degree

Example Version



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