BioVendor Group

ENG

Product Data Sheet:

HUMAN CGRP ELISA

Catalogue number:

RA19021R

For research use only!

BioVendor R&D

BioVendor – Laboratorní medicína a.s.
Karásek 1767/1, 621 00 Brno, Czech Republic
+420 549 124 185
info@biovendor.com
sales@biovendor.com
www.biovendor.com

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1. HUMAN CGRP ELISA

96 wells

Storage: -20°C

Expiry date: stated on the package

This kit contains:

Reagents	Colour of cap	Quantity	Form
Antibody Coated Microtiter Strips	blister with zip	1	-
Conjugate Solution (Human CGRP Tracer)	green	1	lyophilized
Human CGRP Standard	blue with red septum	2	lyophilized
Quality Control	transparent	2	lyophilized
Dilution Buffer (EIA buffer)	green with red septum	1	lyophilized
Wash Solution	blue	1	liquid
Substrate Solution (Ellman´s Reagent 49+1)	black with red septum	2	lyophilized
Tween 20	silver	1	liquid
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.

2. 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), which is around 20°C. Working at 25°C or more affects the assay and decreases its efficiency.

3. 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].

AChE assays are revealed with Substrate Solution (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 colour and can be read at 405-414 nm using 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 a 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 substrate of enzymes that are inherently unstable.

Wide dynamic range

AChE is a stable enzyme and its activity remains constant for many hours as. 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 (Substrate solution) does not need to be discarded and experiment can be continued by adding washing buffer and fresh Substrate Solution into the plate wells.

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.

4. PRINCIPLE OF THE ASSAY

This Enzyme Immunometric Assay (EIA/ELISA) is based on a sandwich technique. Wells of supplied plate are coated with a monoclonal antibody specific of CGRP.

CGRP introduced into the wells (standard or sample) is bound by the monoclonal antibody coated on the plate and is then detected by an acetylcholinesterase (AChE) - Fab' conjugate also specific of CGRP.

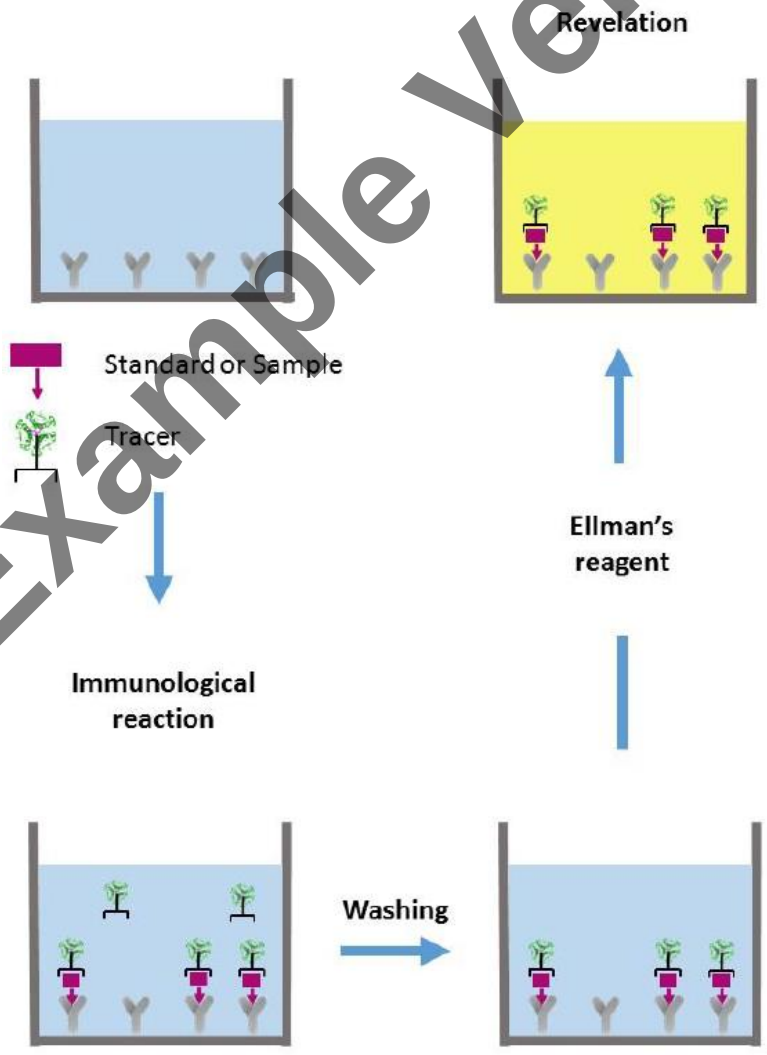
The two antibodies then form a sandwich by binding on different epitopes of the CGRP.

The sandwich is immobilised on the plate where excess reagents are washed away.

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

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 reaction.

The principle of the assay is summarised below:



5. ASSAY VALIDATION AND CHARACTERISTICS

Validated for use:

- in buffer
- in human plasma (without extraction, using a standard curve in plasma).

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

Assay validation data: ask BioVendor (info@biovendor.com) or your local distributor for a copy of the validation data in EIA buffer.

Limit of detection (LOD)

0.7 pg/mL (in EIA 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 variation:

Quality Control sample intra-assay variations in ELISA 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 %

Inter-assay variation:

Quality Control sample inter-assay variations in ELISA buffer (n=25)

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	-

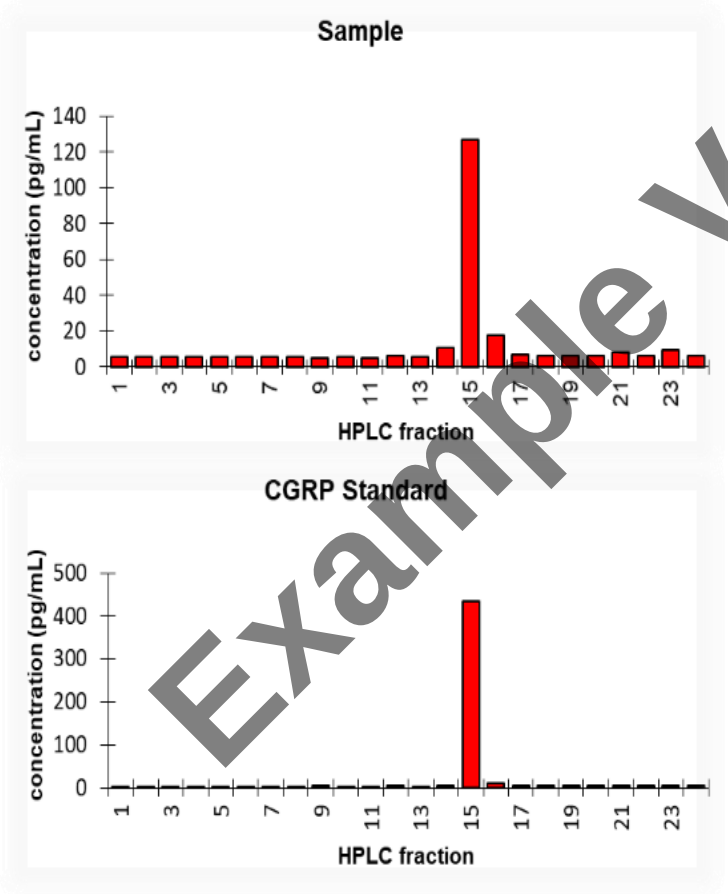
Cross-reactivity

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.

Human CGRP-α/β	100 %	CGRP (8-37)	< 0.01 %
Human CGRP-I/II	100 %	Amylin	< 0.01 %
Rat CGRP-α/β	120 %	Calcitonin	< 0.01 %
Rat CGRP-I/II	120 %	Substance P	< 0.01 %

Specifity

Comparison of HPLC profiles of a CGRP standard and a sample



6. MATERIALS AND EQUIPMENT REQUIRED

In addition to standard laboratory equipment, the following material is 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)
- Multichannel pipette and disposable tips 30-300µL
- Orbital microplate shaker
- Microplate washer (or wash bottles)
- Spectrophotometer plate reader (405 or 414 nm filter)
- UltraPure water (item number #S0001)
- Polypropylene tubes

Water used to prepare all ELISA reagents and buffers must be UltraPure (deionized & free from organic contaminants traces).

Do not use distilled water, HPLC-grade water or sterile water.

UltraPure water may be purchased from BioVendor #S0001.

7. 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 or at -80°C prior the use with the assay.

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 samples

Plasma and serum samples should be measured either after extraction (option 1) or without extraction procedure (option 2):

Option 1: With extraction of sample step

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.

Option 2: Without extraction of sample step

In this second option, standards and QC should be assayed in the same matrix as the sample. 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).
If you don't have plasma or serum that is free of CGRP (human), BioVendor offers CGRP affinity sorbent containing anti-CGRP monoclonal antibody (the same as the one coated on the wells) as item cat# RA19024R. To prepare CGRP-free plasma, use this affinity sorbent cat# RA19024R with a pool of 2 or 3 different sources of plasma or serum.

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 3 cc Vac Cartridge from Waters by first passing 5 mL 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.
- 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 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

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 of calculation

- 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 hence z = 8/X

[(X+30)/2]z = 16 hence [(X+30)]z = 32

thus,

[(X+30)]8/X = 32

X+30 = 4X

3X = 30

X = 10

And Xz = 8, so z = 0.8

Note

To minimize 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., assumption is made that the volume is not changed by the addition of the standard).

8. 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 and 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.

Dilution Buffer

Reconstitute the Dilution Buffer with 50 mL of UltraPure water. Allow buffer to stand 5 minutes or until it is completely dissolved. Mix buffer thoroughly by gentle inversions.

Stability at 4°C: 1 month

Human CGRP Standard

Reconstitute the Human CGRP Standard vial with 1 mL of Dilution Buffer. (For plasma and serum samples without extraction, CGRP Standard need to be reconstituted with CGRP free plasma and serum (see sample preparation step))

Allow standard to stand for 5 minutes or until it is completely dissolved. Mix standard thoroughly by gentle inversions.

The concentration of the first standard (S1) is 1000 pg/mL.

Prepare seven propylene tubes (for the other standards) and add 500 µL of Dilution Buffer 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 Dilution 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

Human CGRP Quality Control

Reconstitute one vial of Quality Control with 1 mL of Dilution 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 Quality Control to stand for 5 minutes or until it is completely dissolved. Mix Quality Control thoroughly by gentle inversions.

Stability at 4°C: 24 hours

Human CGRP Conjugate Solution

Reconstitute one vial of Conjugate Solution with 10 mL of Dilution 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 with 400 mL of UltraPure water. Add 200 µL of Tween 20. Use a magnetic stirring bar to mix the content. Note that concentrated wash buffer is also used for Substrate Solution preparation.

Stability at 4°C: 1 week

Substrate Solution (Ellman's Reagent)

5 minutes before use (development of the plate), reconstitute one vial of Substrate Solution with 49 mL of UltraPure water and 1 mL of **concentrated** Wash Buffer. The tube content should be thoroughly mixed.

Stability at 4°C and in the dark: 24 hours

9. ASSAY PROCEDURE

It is recommended to measure the samples in duplicate followin the instructions below.

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 tappig 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.

	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

Pipetting the reagents

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

Use new tips to pipette the 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 expeling with the pipette tip.

Dilution Buffer

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

Human CGRP 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.

Human CGRP Quality Control and Sample

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

Conjugate Solution

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.

Developing and reading the plate

- Reconstitute Substrate Solution as mentioned in the Reagent preparation section.
- Empty the plate by inverting it. Rinse each well by adding 300 µL of Wash Buffer. Wash strips 3 times, slightly shake them during 2 minutes, then wash 3 times & discard liquid from the wells. 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 Substrate Solution.
- 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 a wavelength between 405 and 414nm (yellow colour).**

  After addition of Substrate Solution, 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).

10. ASSAY PROCEDURE SUMMARY

Enzyme Immunoassay Protocol (volumes are in µL)				
	Blank	NSB	Standard	Sample or QC
Dilution Buffer	-	100	-	-
Standard	-	-	100	-
Sample or QC	-	-	-	100
Conjugate Sol.	-	100	100	100
Cover plate, incubate **16-20 hours** at 4°C				
Wash strips 3 times, slightly shake them during 2 min, than wash 3 times & discard liquid from the wells & dry on absorbent paper				
Substrate Sol. Solsolution	200			
Cover plate, incubate at room temperature in the dark using an orbital microplate				
Read the plate at 405 or 414 nm				

11. 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 1000 pg/mL must be re-assayed after dilution in Dilution Buffer.
- Most plate readers come with curve-fitting software pre-installed that is capable of generating graphs (logit/log or 4-parameter logistic fit 4PL). It is highly recommend to use this software if available on the device. 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 Quality Control Sheet).

12. ACCEPTABLE RANGE

- Non Specific Binding (NSB) absorbace < 0.06 A.U.
- Limit of detection < 10 pg/mL
- Quality Contol: ± 25% of the expected concentration (see the Quality Control Sheet)

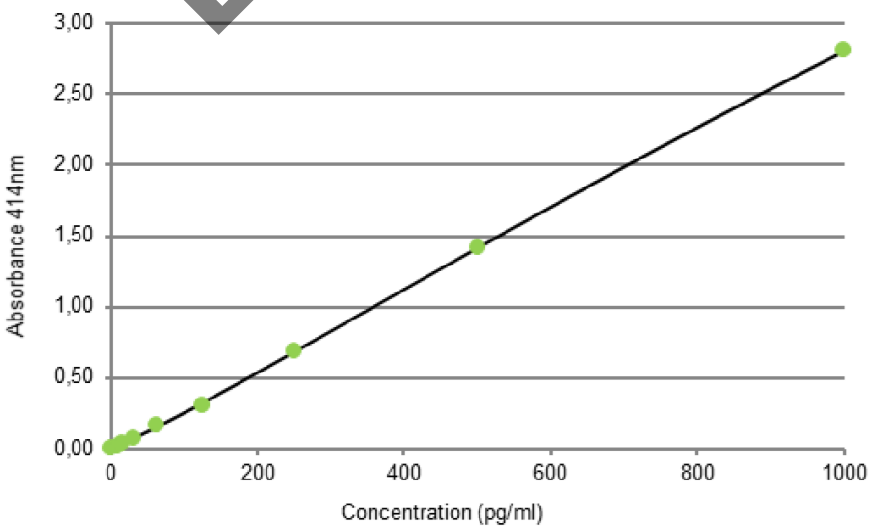
13. TYPICAL RESULTS

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 RT, reading at 414 nm. A spline line fitting was used to determine the concentrations.

Standard	CGRP (pg/mL)	A.U.
S1	1000	2.808
S2	500	1.42
S3	250	0.686
S4	125	0.311
S5	62.5	0.167
S6	31.25	0.0765
S7	15.63	0.0405
S8	7.81	0.018
NSB	0	0.002

Typical Standard curve for Human CGRP ELISA



14. ASSAY TROUBLESHOOTING

Absorbance values are too low:

- organic contamination of water,
- one reagent has not been dispensed,
- incorrect preparation/dilution,
- 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.

High dispersion of duplicates:

- poor pipetting,
- irregular plate washing.

If a plate is accidentally dropped after dispatch of the AChE (Substrate Solution) or if it needs to be revealed again:

- one only needs to wash the plate, add fresh Substrate Solution 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 BioVendor.

These are a few examples of troubleshooting that may occur.

If further information or explanation is needed, please contact BioVendor.

15. BIBLIOGRAPHY

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Additional readings

List of publications quoting the use of this kit

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BioVendor R&D®

BioVendor – Laboratorní medicína a.s.
Karásek 1767/1, 621 00 Brno, Czech Republic
+420 549 124 185
info@biovendor.com
sales@biovendor.com
www.biovendor.com