BioVendor Group

ENG

Instructions for use:

RAT CGRP ELISA

Catalogue number:

RA19022R

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

CONTENT

HISTORY OF CHANGES

Previous version	Current Version
ENG.003.A	ENG.004.A
Full text of Chapter 6.3. Picture added.	
6.5.1. point 2: example added	
Full text of Chapter 6.5.2.	
Chapter 7.2. Sentence: “The dilutions of CGRP standard should also be prepared with CGRP free plasma or serum“ added.	
Chapter 8.5. -point 5 added -table “Enzyme Immunoassay Protocol” changed	
Chapter 13. added	

1. RAT CGRP ELISA

96 wells

Storage: -20°C

Expiry date: stated on the package

This kit contains:

Reagents	Colour code	Quantity	Form
Antibody Coated Microtiter Strips	Blister with zip	1	-
Conjugate Solution (Rat Tracer)	Green	1	lyophilized
Rat CGRP Standard	Blue with red septum	2	lyophilized
Quality Control	Green with red septum	2	lyophilized
Dilution Buffer (EIA buffer)	Blue	1	lyophilized
Wash Buffer	Silver	1	liquid
Substrate Solution (Ellman´s Reagent)	Black with red septum	2	lyophilized
Tween 20	Transparent	1	liquid
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. PRECAUTIONS 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 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), that is around 20°C. Working at 25°C or more affects the assay and decreases its efficiency.

3. BACKGROUND

3.1 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's capable of providing a rapid catalytic turnover during the generation ofthe electrochemical discharges.

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 color and can be read at 405-414 nm using a spectrophotometer. AChE offers several advantages over other commonly used enzymes used in EIAs:

3.1.1 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 phosphate. AChE provides a greater sensitivity than other labeling enzymes.

3.1.2 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 which is inherently unstable.

3.1.3 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

3.1.4 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.

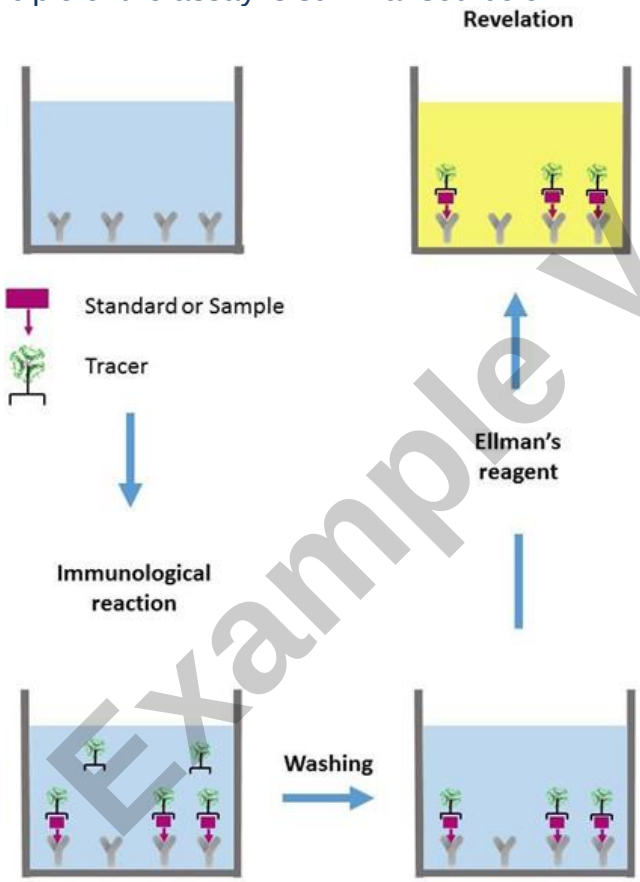
3.2 CGRP

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.

4. 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 Substrate Solution (Ellman's reagent). AChE tracer acts on Substrate Solution (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
The principle of the assay is summarised below:



5. MATERIALS AND EQUIPMENT REQUIRED

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

5.1 For the sample preparation

(not necessary for all types of samples):

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

5.2 For the assay

- Multichannel pipette and disposable tips 30-300µL
- Precision micropipettes (20 to 1000 µL)
- Spectrophotometer plate reader (405 or 414 nm filter)
- Microplate washer (or washbottles)
- Orbital Microplate shaker
- UltraPure water (item number #S0001)
- Polypropylene tubes

Water used to prepare all EIA 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.

6. SAMPLE PREPARATION

6.1 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.

6.2 Nervous tissues

Nervous tissues such as cerebrospinal fluid may be assayed directly if diluted more than 1:20 in Dilution 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 Dilution Buffer.

6.3 Plasma or serum

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

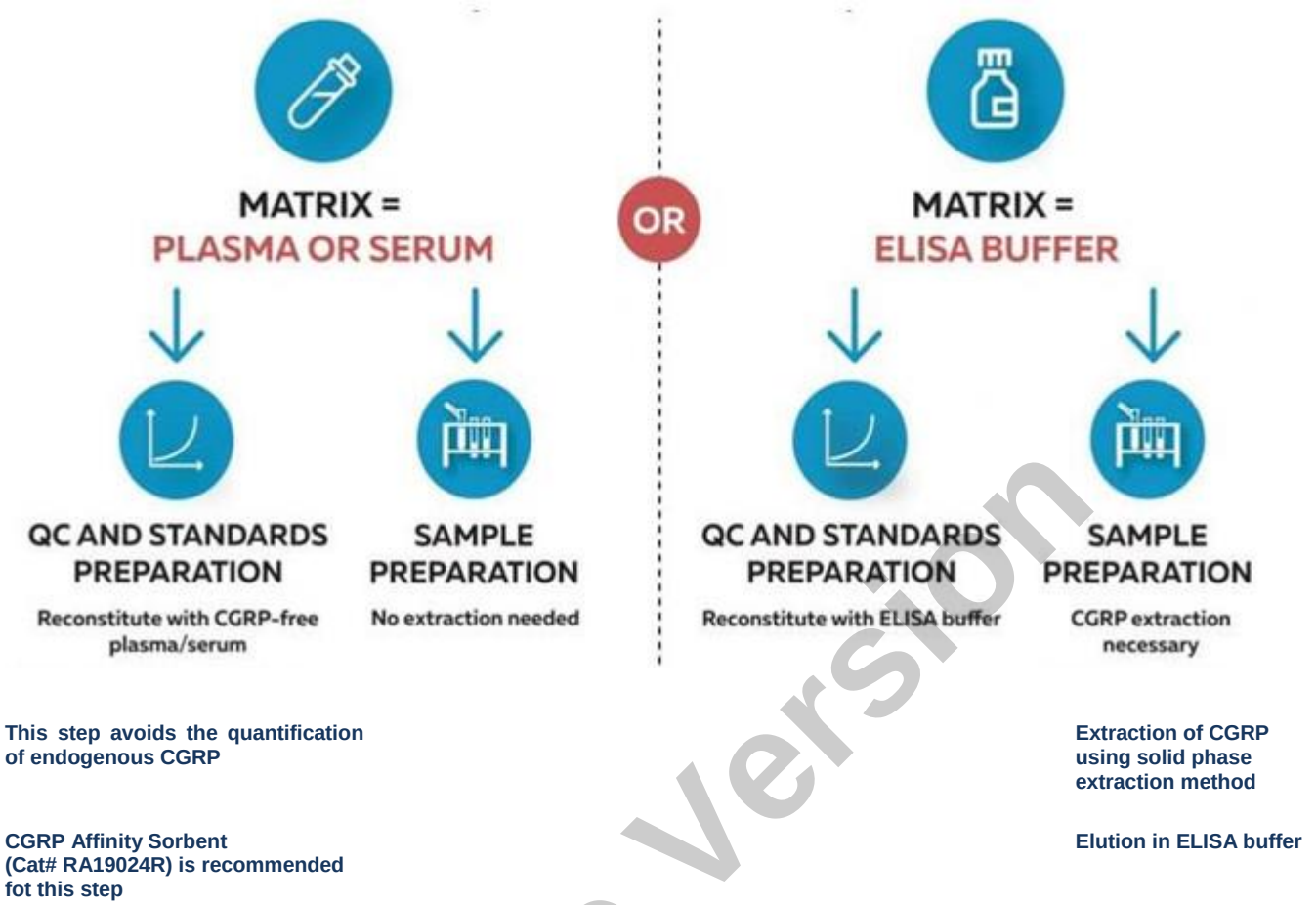
– Either without an extraction procedure (6.3.1, left side of the scheme below); in which case the QC and Standards should be reconstituted with CGRP-free plasma/serum.
– or with an extraction procedure (6.3.2., right side of the scheme below); in which case the QC and Standards should be reconstituted in ELISA buffer.

6.3.1 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), 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.

6.3.2 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.



6.4 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.

6.5 Extraction Protocol

6.5.1 Extraction steps:

- Activate a 1 mL C-18 reverse phase cartridge or an Oasis® HLB Extraction cartridge Waters 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
- 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.

6.5.2 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.

7. 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 Substrate Solution 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.

7.1 Dilution Buffer

Reconstitute the vial of Dilution Buffer 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

7.2 Rat CGRP Standard

Reconstitute the Standard vial 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 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 Dilution Buffer	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

7.3 Rat CGRP Quality Control

Reconstitute one QC vial 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 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

7.4 Rat CGRP Conjugate Solution

Reconstitute the 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

7.5 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 (Ellman's reagent) preparation.
Stability at 4°C: 1 week

7.6 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

8. ASSAY PROCEDURE

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

8.1 Plate preparation

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

Open the plate pouch and select the sufficient strips for your assay and place the 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 shaking out the last drops on a paper towel.

8.2 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

8.3 Pipetting the reagents

All samples and reagents must reach room temperature prior to performing the assay.
Use new tips to pipette the buffer, standard, sample, conjugate, antiserum 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.

8.3.1 Dilution Buffer

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

8.3.2 Rat 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.

8.3.3 Rat CGRP Quality Control and Samples

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

8.3.4 Conjugate Solution

Dispense 100 µL to each well, **except** Blank (Bk) wells.

8.4 Incubating the plate

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

8.5 Developing and reading the plate

- Reconstitute Substrate Solution 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, slightly shake them 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 Substrate Solution to each well.
- Cover the plate with an 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 414 nm (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).**

Enzyme Immunoassay Protocol (volumes are in µL)				
Volume/ Wells	Blank	NSB	Standard	Sample or QC
Dilution Buffer or CGRP-free serum/plasma	-	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 during 2 min, then wash 3 times & Discard liquid from the wells & dry on absorbent paper				
Substrate Sol.	200			
Incubate with an orbital shaker in the dark at RT				
Read the plate between 405 and 414 nm				

9. DATA ANALYSIS

Make sure that your plate reader has subtracted the absorbance readings of the blank well from the absorbance readings of the rest of the plate. If not, do it now.

- Calculate the average absorbance for each NSB, standards, QC and samples.
- 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 500 pg/mL should be re-assayed after dilution in Dilution Buffer.
- Most plate readers are supplied with curve-fitting software capable of graphing these data (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 CoA).

10. ACCEPTABLE RANGE

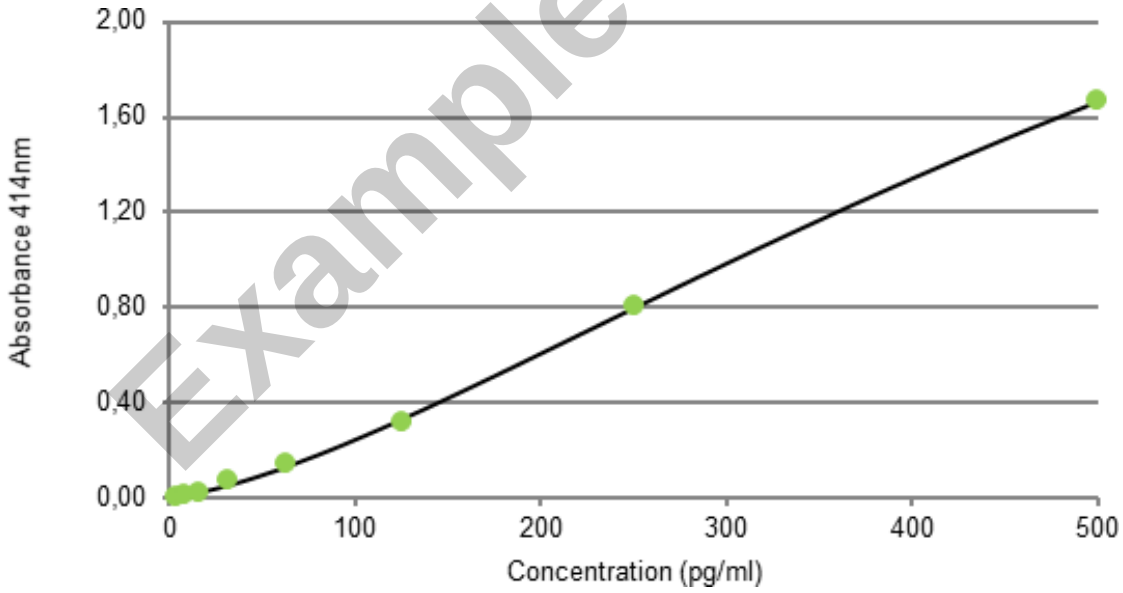
- Non Specific Binding (NSB) absorbance < 0,06 A.U.
- Limit of detection < 10 pg/mL
- Quality Contol: ± 25% of the expected concentration (see the CoA)

11. 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: 30 minutes developing at 20°C, reading at 414 nm. A spline fitting was used to determine the concentrations.

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



Typical CGRP (rat) standard curve

12. ASSAY VALIDATION AND CHARACTERISTICS

The Enzyme Immunometric assay of CGRP (rat) has been validated for its use in buffer and 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]**.

– The **Limit of Detection** (LOD), calculated as the concentration of CGRP corresponding to the NSB average (n = 8) plus three standard deviations, is: 0.7 (in Dilution Buffer) and 2 pg/mL (in plasma).

– Quality Control **samples intra-assay & inter-assay variations in Dilution 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 in plasma (n=25)

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	16.5 %

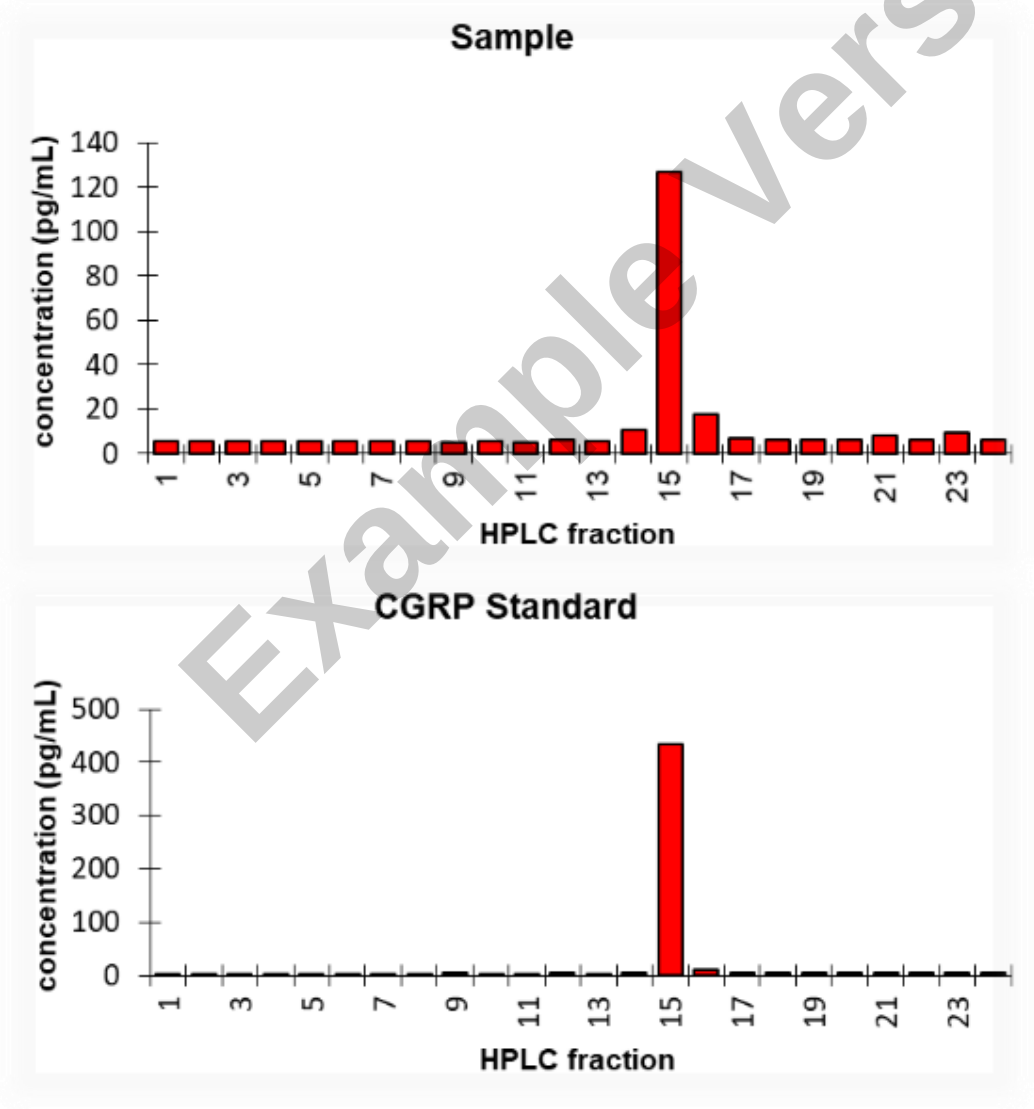
– **Cross-reactivity**

Cross reactivity was tested in Dilution 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 %

– **Specifity**

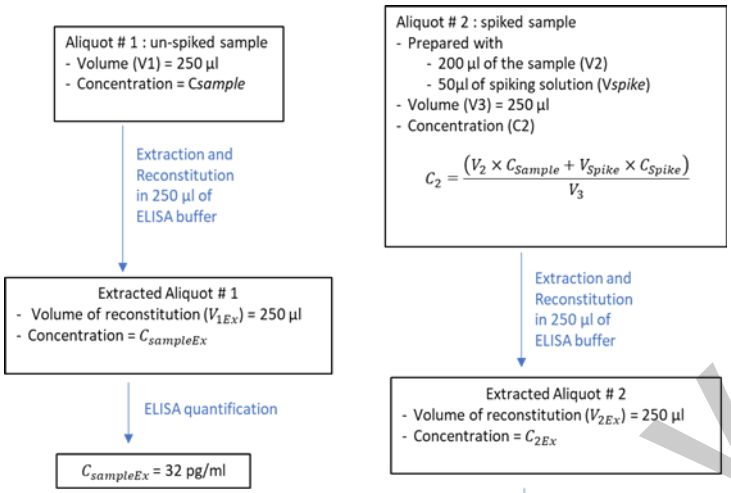
Comparison of HPLC profiles of a CGRP standard and a sample.



13. CGRP EXTRACTION METHOD

13.1 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.



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

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

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

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

14. ASSAY TROUBLESHOOTING

14.1 Absorbance values 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.

14.2 High signal and background in all wells:

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

14.3 High dispersion of duplicates:

– poor pipetting technique,
– irregular plate washing.

14.4 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 Bioreagent Department.

These are a few examples of troubleshooting that may occur.

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Additional readings

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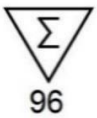
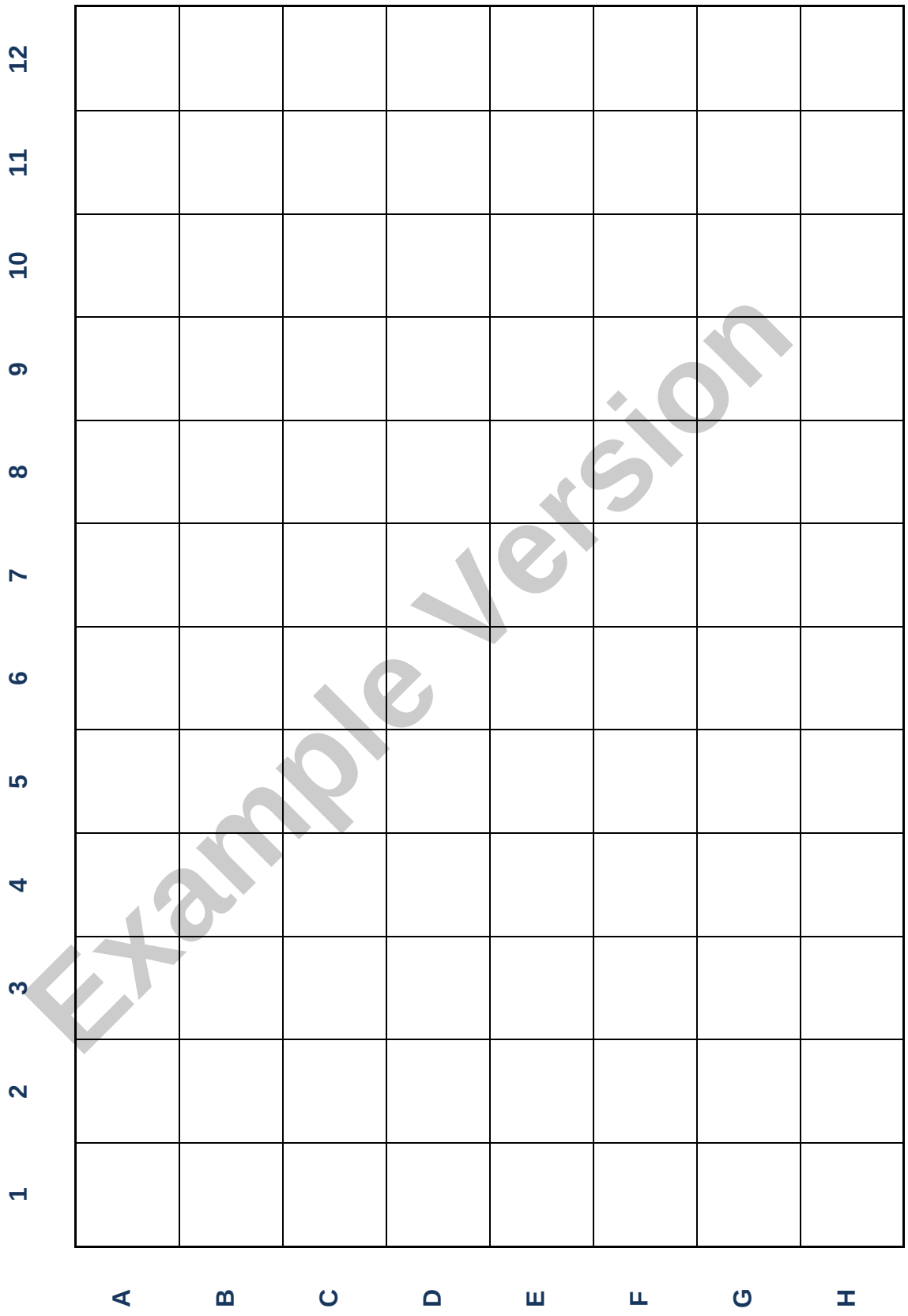
16. EXPLANATION OF SYMBOLS

	Catalogue number
	Batch code
	Caution
	Use by date
	Temperature limit
	Manufacturer
	Read electronic instructions for use - eIFU
	The content is sufficient for 96 tests
	Biological risks



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