BioVendor Group

ENG

Instructions for Use:

MOUSE/RAT INSULIN ELISA

Catalogue number:

RA19004R

For research use only!

BioVendor R&D

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CONTENT

HISTORY OF CHANGES

Previous version	Current version
ENG.005.A	ENG.006.A
Product Data Sheet	Instructions for Use
The appearance of the document changed. (rebranding)	
History of changes added.	

1. MOUSE/RAT INSULIN ELISA

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

This kit contains:

REAGENTS	Quantity	Form
Antibody Coated Microtiter Strips (pre-coated with Goat anti-Guinea-Pig IgG, ready to use after thawing)	1	
Conjugate Solution (Mouse/Rat Insulin tracer)	1	lyophilized
Mouse/Rat Insulin Standard	2	lyophilized
Mouse/Rat Insulin antiserum	1	lyophilized
Quality Control sample	2	lyophilized
Dilution Buffer (EIA buffer)	1	lyophilized
Wash Solution Concentrate	1	liquid
Substrate Solution (Ellman´s reagent)	2	lyophilized
Tween 20	1	liquid
Cover Sheet	1	
Template 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 33 samples in duplicate.

2. PRECAUTION FOR USE

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

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

- For research laboratory use only.
- Not for diagnostic use.
- Do not pipet liquids by mouth.
- Do not use kit components beyond the expiration date.
- Do not eat, drink or smoke in area in which kit reagents are handled.
- Avoid splashing.

The QC samples provided in this kit have been prepared by diluting rat plasma (Sprague Dawley rat) in Dilution Buffer (EIA buffer). A sanitary control has been completed on Sprague Dawley rats following the Felasa Health Monitoring Recommendations. However, handle the CQ samples as a possible source of infection.
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.

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 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]***.

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 and can be read at 405-414 nm. AChE® offers several advantages compared to enzymes conventionally 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® allows a greater sensitivity than other labeling enzymes.

3.1.2 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.

3.1.3 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.

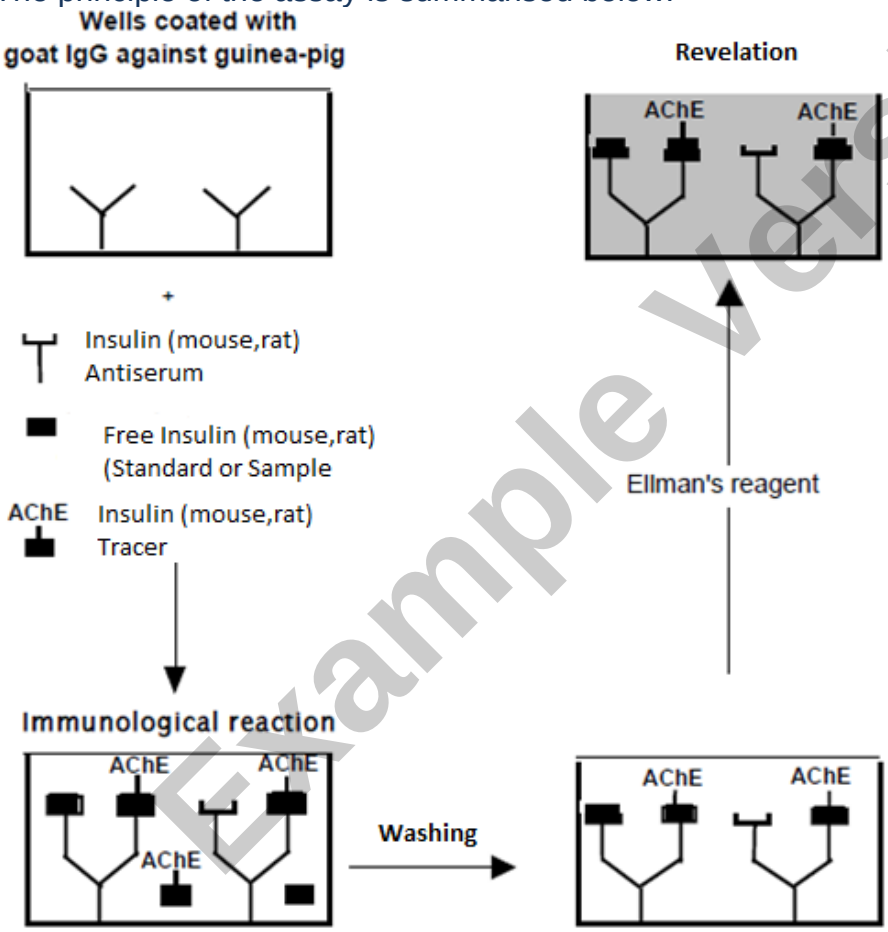
3.1.4 Versatility: AChE®

Is a completely stable enzyme, unlike peroxidase which is suicidal. Thus, if a plate is accidentally dropped after dispatch of the AChE® substrate solution (Ellman's reagent) or if it needs to be revealed again, one only needs to wash the plate, add fresh Substrate Solution (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.

4. PRINCIPLE OF THE ASSAY

This Enzyme Immunoassay (EIA) is based on the competition between unlabelled rat insulin and acetylcholinesterase (AChE) linked to rat insulin (tracer) for limited specific Guinea-Pig anti-rat insulin antiserum sites.
The complex Guinea-Pig antiserum-rat insulin (free insulin or tracer) binds to the Goat anti-Guinea-Pig antibody that is attached to the well.
The plate is then washed and Ellman's Reagent (enzymatic substrate for AChE and chromogen) is added to the wells.
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 tracer bound to the well and is inversely proportional to the amount of free rat insulin present in the well during the immunological incubation.

The principle of the assay is summarised below:



5. MATERIAL REQUIRED BUT NOT PROVIDED

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

For the assay:
- Precision micropipettes (20 to 1000 µL)
- Spectrophotometer plate reader (405 or 414 nm filter)
- Microplate washer (or wash-bottles)
- Microplate shaker
- Multichannel pipette and disposable tips 30-300 µL
- UltraPure water (cat. number S0001)
- 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.

Ulta pure water may be purchased from BioVendor (cat. number S0001)

6. SAMPLE COLLECTION AND PREPARATION

This assay may be used to measure insulin in mouse and rat plasma or serum sample. To do so, blood samples are collected in tubes containing heparin or EDTA. The samples are centrifuged at 1 600 g for 20 minutes.
Plasmas are collected and kept at -20°C until assay.
No prior extraction procedure is necessary to measure insulin in plasma samples. However, hemolysis interferes with the assay by degrading insulin. BioVendor has developed an inhibitor cocktail and a procedure presented hereafter to prevent hemolysis consequences. Users are recommended to follow it in such a case.

6.1 Inhibitor cocktail

Dilute 226.1 mg of tetrahydrated sodium salt of EDTA in 500 µL of distilled or deionized water and 65 mg of phenanthroline monohydrate in 500 µL of methanol. Mix them together.

6.2 Inhibitor buffer

Dilute 250 µL of the inhibitor cocktail in 25 mL of Dilution Buffer (EIA buffer) provided in the kit. Afterwards, prepare Insulin (mouse, rat) standards, quality control and samples as follows:

6.3 Insulin (mouse, rat) standard

Reconstitute the vial with 1 mL of UltraPure water. Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion. Then, add 10 µL of the inhibitor cocktail. Prepare seven propylene tubes for the other standards and add 500 µL of the inhibitor buffer in each tube. Add 500 µL of the first tube (containing the first standard) to the second tube. Continue this procedure for the other tubes.

6.4 Quality control

Reconstitute one vial with 1 mL of UltraPure water. Allow it to stand 5 minutes until completely dissolved and then add 10 µL of the inhibitor cocktail. Mix thoroughly by gentle inversion.

6.5 Sample

Prior dispatching, add 10 µL of inhibitor cocktail for 1 mL of plasma (or 5 µL for 500 µL, 2 µL for 200 µL, etc..). If necessary, dilute the sample with the inhibitor cocktail buffer.
If no hemolysis is observed in plasma sample, prepare the above-mentioned reagents as indicated in the next section: Reagent preparation.

6.6 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.

7. REAGENT PREPARATION

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

7.1 Dilution Buffer

Reconstitute one vial 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 Insulin (mouse, rat) Standard

Reconstitute the vial with 1 mL of UltraPure water. Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion. The concentration of the first standard is 10 ng/mL. Prepare seven propylene tubes for the other standards and add 500 µL of Dilution buffer (EIA Buffer) into each tube. Then prepare the standards by serial dilutions as follows:

Standard	Volume of Standard	Volume of Assay Buffer	Standard concentration ng/mL
S1	-	-	10
S2	500 µL of S1	500 µL	5
S3	500 µL of S2	500 µL	2.5
S4	500 µL of S3	500 µL	1.25
S5	500 µL of S4	500 µL	0.63
S6	500 µL of S5	500 µL	0.31
S7	500 µL of S6	500 µL	0.16
S8	500 µL of S7	500 µL	0.08

Stability at 4°C : 1 day.

7.3 Quality Control

Reconstitute one vial with 1 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 day.

7.4 Insulin (mouse, rat) Antiserum

Reconstitute one vial with 5 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 the concentrated Wash buffer with 400 mL of UltraPure water. Add 200 µL of tween 20 (Use a magnetic stirrer to mix the contents). Stability at +4°C: 1 week.

7.6 Conjugate Solution

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

7.7 Substrate Solution (Ellman's Reagent)

Five minutes before use (development of the plate), reconstitute with 50 mL of UltraPure water. The tube contents should be thoroughly mixed. Stability at +4°C and in the dark: 1 days.

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 packet and select the sufficient strips for your assay and place the unused strips back in the packet (stored at +4°C for 1 month maximum). Rinse each well five times with the wash buffer (300 µL/well).
Just before distributing reagents and samples, remove the buffer from the wells by inverting the plate and shaking out the last drops on a paper towel.

8.2 Distribution of reagents and samples

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

8.3 Pipetting the reagents

All samples and reagents must reach room temperature prior to performing the assay. Use different tips to pipet the buffer, standard, sample, tracer, antiserum and other reagents. Before pipetting, equilibrate the pipette tips in each reagent. Do not touch the liquid already in the well when expeling with the pipette tip.

	1	2	3	4	5	6	7	8	9	10	11	12
A	B	S1	S5	*	*	*	*	*	*	*	*	*
B	B	S1	S5	*	*	*	*	*	*	*	*	*
C	NSB	S2	S6	*	*	*	*	*	*	*	*	*
D	NSB	S2	S6	*	*	*	*	*	*	*	*	*
E	NSB	S3	S7	*	*	*	*	*	*	*	*	*
F	B_0	S3	S7	*	*	*	*	*	*	*	*	*
G	B_0	S4	S8	*	*	*	*	*	*	*	*	*
H	B_0	S4	S8	*	*	*	*	*	*	*	*	*

B : Blank

NSB : Non Specific Binding

S1-S8: Standards 1-8

B_0 : Maximum Binding

* : Samples or Quality contols

8.3.1 Dilution Buffer

Dispense 100 µL to Non Specific Binding (NSB) wells and 50 µL to Maximum Binding (Bo) wells.

8.3.2 Insulin (mouse, rat) Standards

Dispense 50 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.

8.3.3 Quality Control and samples

Dispense 50 µL in duplicate to appropriate wells. Highly concentrated samples may be diluted in Dilution Buffer (EIA buffer)

8.3.4 Insulin (mouse, rat) Antiserum

Dispense 50 µL to each well except Blank (B) wells and the Non Specific Binding (NSB) wells.

8.3.5 Conjugate Solution

Dispense 50 µL to each well, except Blank (B) wells.

8.4 Incubating the plate (second step)

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

8.5 Developing and reading the plate

Reconstitute Ellman's Reagent as indicated in reagent preparation section. Empty the plate by turning it over. Rinse each well five times with the wash buffer (300 µL). At the end of the last washing step, empty the plate and blot the plate on paper towel to discard any trace of liquid.
Add 200 µL of Ellman's Reagent to the 96 wells. Cover the plate with aluminium sheet and incubate in the dar kat 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 ouside the wells.
Read the plate at a wavelenght between 405 and 414 nm (yellow colour). After addition of Ellman´s reagent the absorbance has to be checked periodically (every 30 minutes) until the maximum absorbance (Bo) wells has reached 0.2-0.8 A.U. (blank subtracted).

Enzyme Immunoassay Protocole (volumes are in µL)						
	Blank	**NSB**	**BO**	**Standard**	**QC**	**Sample**
Buffer	-	100	50	-		
Standard	-			50	-	
Sample	-				50	
Tracer	-	50				
Antiserum	-	50				
Cover plate, incubate 16-20 hours at +4°C						
Wash strips 5 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						

9. 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, Bo, standards and samples.
- For each standard and sample calculate the B/Bo (%) on y axis versus the concentration on x axis. Draw best-fit line through the points.
- To determine the concentration of your samples, find the B/Bo (%) 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 10 ng/mL should be re-assayed after dilution in Dilution 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).

10. ACCEPTABLE RANGE

- Bo absorbance: > 200 mAU in the conditions indicated above.
- Ratio NSB absorbance / Bo absorbance: < 0.1. and NSB < 35 mAU.
- 50% B/Bo(%): < 1.6 ng/mL
- QC sample: ± 25% of the epected concentracion (see the label on QC vial)

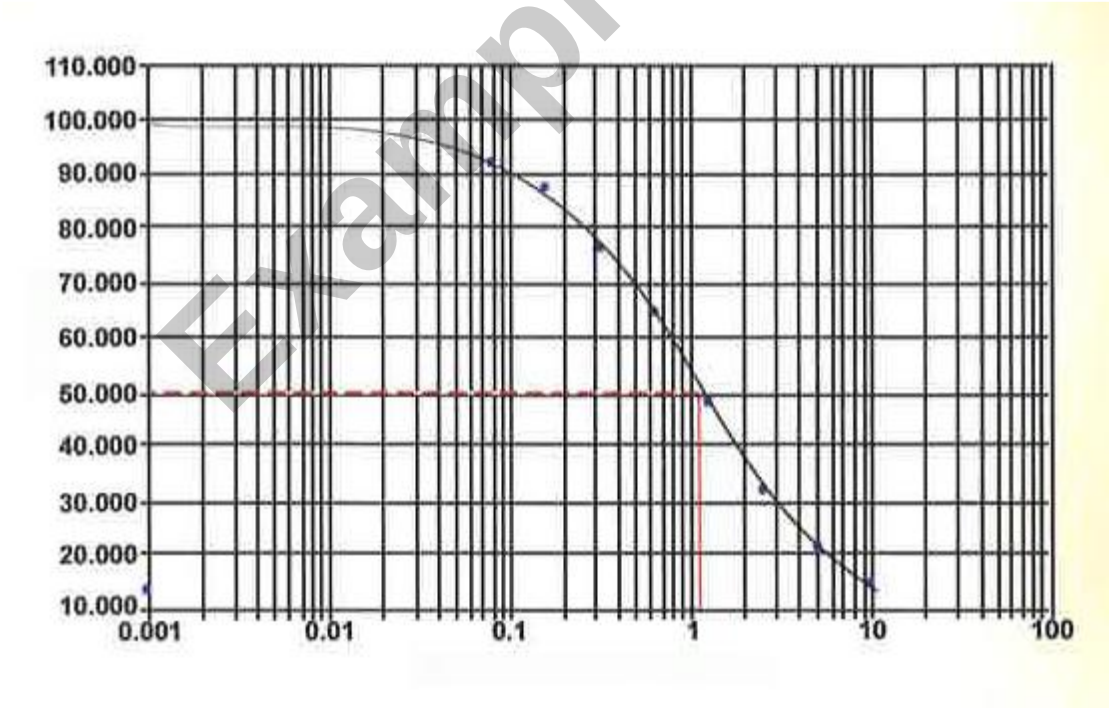
11. TYPICAL RESULTS

11.1 Example data

The following data are for demonstration purpose only. Your data may be different and still correct. These data were obtained using all reagents as supplied in this kit under the following conditions: 90 minutes developing at +20°C, reading at 414 nm. A logit/log curve fitting was used to determine the concentrations.

	MAU	B/Bo (%)
NSB	3	
Bo	453	100
Standard 10 ng/ml	64	13.6
Standard 5 ng/ml	90	19.3
Standard 2.5 ng/ml	126	27.3
Standard 1.25 ng/ml	171	37.3
Standard 0.63 ng/ml	235	51.6
Standard 0.31 ng/ml	293	64.4
Standard 0.16 ng/ml	352	77.6
Standard 0.08 ng/ml	384	84.7

11.2 Typical Insulin (mouse, rat) standard curve



12. ASSAY VALIDATION AND CHARACTERISTICS

12.1 Cross-reactivity:

Compound	%	Compound	%
Rat Insulin	100	Mouse Insulin	100
Hamster Insulin	100	Porcine Insulin	100
Human Insulin	100	Sheep Insulin	100

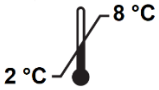
13. ASSAY TROUBLE SHOOTING

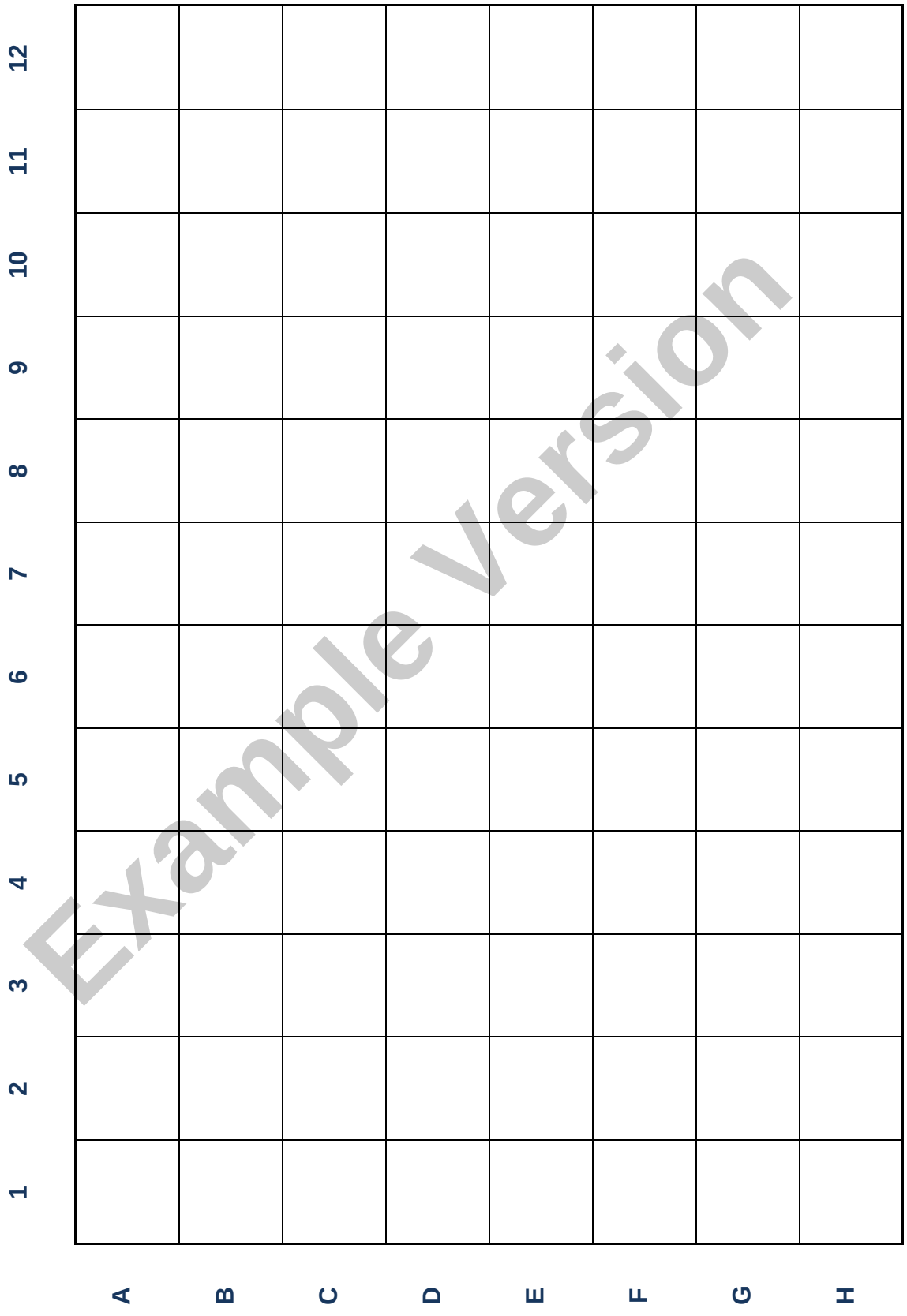
- Bo value is too low: incubation in wrong conditions (time or temperature) or reading time too short or Insulin (mouse,rat) -AChE tracer, Insulin (mouse, rat) antiserum or Ellman's reagent have not been dispensed.
- NSB value too high: contamination of NSB wells with Insulin (mouse rat) antiserum or inefficient washing.
- High dispersion of duplicates: poor pipetting technique or irregular plate washing.
- IC50 or QC concentrations not within the expected range: wrong preparation of standards.
- Analyses of two dilutions of a biological sample do not agree: Interfering substances are present. Sample must be purified prior to EIA analysis (excepting plasma samples).

14. REFERENCES

1. Grassi J. Pradelles P.
Compounds labelled by the acetylcholinesterase of *Electrophorus Electricus*. Its preparation process and its use as a tracer or marquer in enzymo-immunological determinations.
United States patent, N° 1,047,330. September 10, 1991
2. Grassi J. Pradelles P.
The use of Acetylcholinesterase as a Universal marker in Enzyme-Immunoassays. Proceeding of the Third International Meeting on Cholinesterases, American Chemical Society (1991)
3. Pradelles P. Grassi J, Maclouf J.
Enzyme Immunoassays of Eicosanoids Using Acetylcholinesterase. Methods in Enzymology (1990), vol. 187, 24-34

15. 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



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