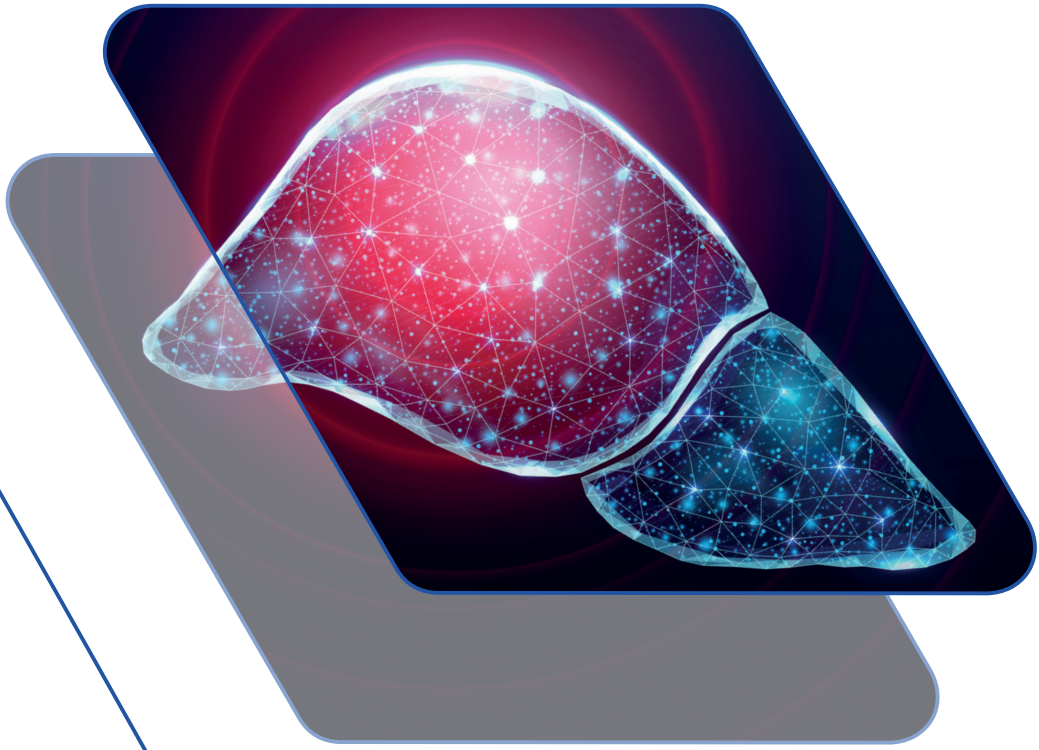
LEAP-2



USER GUIDE

LEAP-2 ELISA kit

A05038.96 wells

For laboratory research only.
Not for human or veterinary diagnostic use.
This assay was developed
& validated by Bertin Technologies

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96 wells
Storage: +4°C
Expiry date: stated on the package

This kit contains:

Designation	Colour of cap	Item #	Quantity per kit	Form
Mouse anti-Rabbit precoated 96-well Strip Plate	Blister with zip	A08100.1 ea	1	-
LEAP-2 Standard	Blue with red septum	A06038.1 ea	2	Lyophilised
LEAP-2 Quality Control	Green with red septum	A10038.1ea	2	Lyophilised
LEAP-2 Biotin Labelled	Gold	A22038.100 dtn	1	Lyophilised
LEAP-2 Antiserum	Red	A03038.100 dtn	1	Lyophilised
LEAP-2 Streptavidin-AChE Tracer	Green	A04038.100 dtn	1	Lyophilised
LEAP-2 ELISA Buffer	Blue	A07038.1 ea	1	Lyophilised
Wash Buffer	Silver	A17000.1 ea	1	Liquid
Tween 20	Transparent	A12000.1 ea	1	Liquid
Ellman's reagent_50	Black	A09000_50.100 dtn	2	Lyophilised
Technical Booklet	-	A11038.1 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 35 samples in duplicate.

1. 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

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.

2. 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 Technologies 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 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.

LEAP-2

LEAP-2 is part of the Liver-expressed antimicrobial peptides family (LEAP).
LEAP-2 is 40 amino acid peptide is from a 77 amino acid precursor wich is cleaved by furin-like endoprotease.
LEAP-2 peptide is rich in cysteine amino acid and contains two disulphides bonds formed by cysteine residues in relative 1-3 and 2-4 positions.
LEAP-2 is highly conserved among mammals and is expressed by the hepatocytes of the liver, by the small intestine, by the central nervous system (4).
Initially identified as an antimicrobial peptide serving as a part of innate immune system bacterial infection (4).
LEAP-2 is associated with inflammatory process link to patients with rheumatoid arthritis, an autoimmune disease (4).
The latest studies indicated that LEAP-2 has anorexigenic drive and is an antagonist of the ghrelin (5).
The plasmatic level of LEAP-2 is enhanced in obese patients (4).

3. PRINCIPLE OF THE ASSAY

The enzymatic immunoassay (ELISA) is based on the competition between unlabelled LEAP-2 and Biotin labelled LEAP-2 for limited specific rabbit anti-LEAP-antiserum sites.

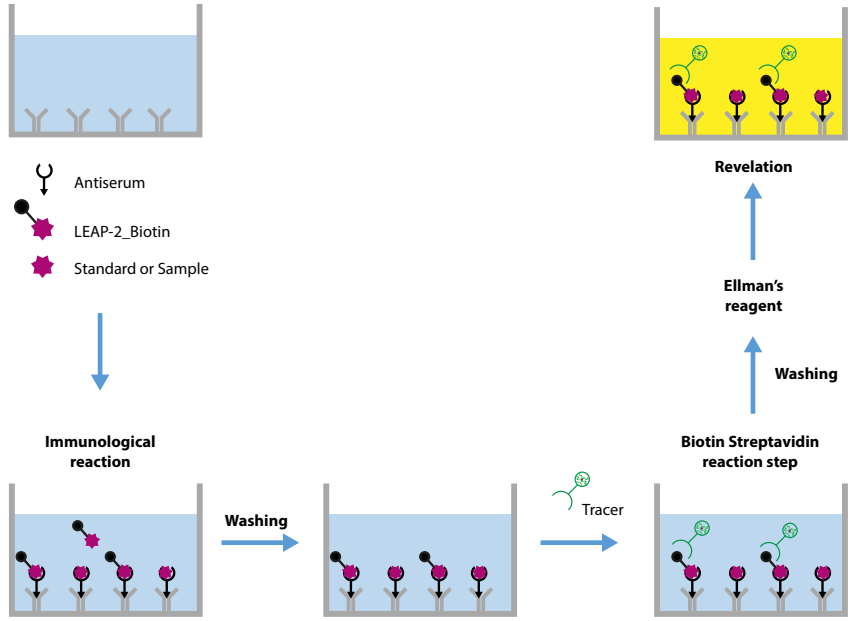
The complex rabbit antiserum – LEAP-2 (free LEAP-2 or Labelled) binds to the mouse monoclonal anti-rabbit antibody coated in the well.

After the washing step, the complex binds to the mouse monoclonal anti-rabbit antibody is revealed by Streptavidine_AChE (Tracer).

Finally, 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 that strongly absorbs at 414 nm.

The intensity of the color, determined by spectrophotometry, is proportional to the amount of tracer bound to the well and is inversely proportional to the amount of free LEAP-2 present in the well during the immunological incubation.
The principle of the assay is summarised below:



4. ASSAY CHARACTERISTICS

- **Validated for**
  Human species
- **IC50:** ≤0,2 ng/mL
- **Assay validation data:**
  ask Bertin Technologies (tech@bertin-bioreagent.com) or your local distributor for a copy of the following application notes:
  validation data with human samples

Initially identified as an antimicrobial peptide serving as a part of innate immune system bacterial infection (4).
LEAP-2 is associated with inflammatory process link to patients with rheumatoid arthritis, an autoimmune disease (4).
The latest studies indicated that LEAP-2 has anorexigenic drive and is an antagonist of the ghrelin (5).
The plasmatic level of LEAP-2 is enhanced in obese patients (4).

5. MATERIALS AND EQUIPMENT REQUIRED

In addition to standard laboratory equipment, the following materials are required:
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 Technologies (item #A07001.1L).

6. SAMPLE COLLECTION AND PREPARATION

This assay has been validated to measure LEAP-2 in buffer and in human plasma sampled on EDTA K3

- **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 or at -80°C prior the use with the assay.
- **Sample collection**

Blood samples are collected in tubes containing EDTA-K3.
Hemolysed plasma must be excluded of the study.

- **Sample collection**
  Plasma samples may be assayed directly without any extraction procedure after being diluted at least to 1:2 in LEAP-2 ELISA Buffer (100µL of plasma + 100µL of LEAP-2 ELISA Buffer) in order to avoid the matrix effect.

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 35 samples in duplicate according to suggested plate layout.
An additional vial of Standard and Quality Control 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.

- **LEAP-2 ELISA Buffer**
  Reconstitute the ELISA Buffer #A07038 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.
- **LEAP-2 Standard**
  Reconstitute the LEAP-2 Standard vial #A06038 with 1 mL of UltraPure water. 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 25 ng/mL. Prepare seven polypropylene tubes (for the seven other standards) and add 500 µL of LEAP-2 ELISA 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 LEAP-2 ELISA Buffer	Standard concentration
S1	-	-	25.0 ng/mL
S2	500 µL of S1	500 µL	12.5 ng/mL
S3	500 µL of S2	500 µL	6.3 ng/mL
S4	500 µL of S3	500 µL	3.1 ng/mL
S5	500 µL of S4	500 µL	1.6 ng/mL
S6	500 µL of S5	500 µL	0.8 ng/mL
S7	500 µL of S6	500 µL	0.4 ng/mL
S8	500 µL of S7	500 µL	0.2 ng/mL

Stability at 4°C: within the day

- **LEAP-2 Quality Control**
  Reconstitute the LEAP-2 #A10038 with 1 mL of UltraPure water. 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: within the day.

- **LEAP-2 Labelled Biotin**
  Reconstitute the LEAP-2 Labelled Biotin vial #A22038 with 5 mL of LEAP-2 ELISA Buffer. Allow LEAP-2 Labelled Biotin tracer to stand for 5 minutes or until it is completely dissolved. Mix tracer thoroughly by gentle inversions.

Stability at +4°C: 1 weeks.

- **LEAP-2 Antiserum**
  Reconstitute the LEAP-2 Antiserum vial #A03038 with 5 mL of LEAP-2 ELISA Buffer. Allow antiserum to stand for 5 minutes or until it is completely dissolved. Mix tracer thoroughly by gentle inversions.

Stability at +4°C: XX weeks.

- **LEAP-2 Streptavidin-AChE Tracer**
  Reconstitute the LEAP-2 Streptavidin-AChE Tracer vial #A04038 with 15 mL of LEAP-2 ELISA Buffer. Allow LEAP-2 Streptavidin-AChE Tracer to stand for 5 minutes or until it is completely dissolved. Mix tracer thoroughly by gentle inversions.

Stability at +4°C: 1 week.

- **Wash Buffer**
  Dilute 2 mL of concentrated Wash Buffer #A17000 with 800 mL of UltraPure water. Add 400 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 month.

- **Ellman's Reagent**
  5 minutes before use (development of the plate), reconstitute one vial of Ellman's Reagent #A09000_50 with 50 mL of UltraPure water. The tube content should be thoroughly mixed.

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

8. ASSAY PROCEDURE

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

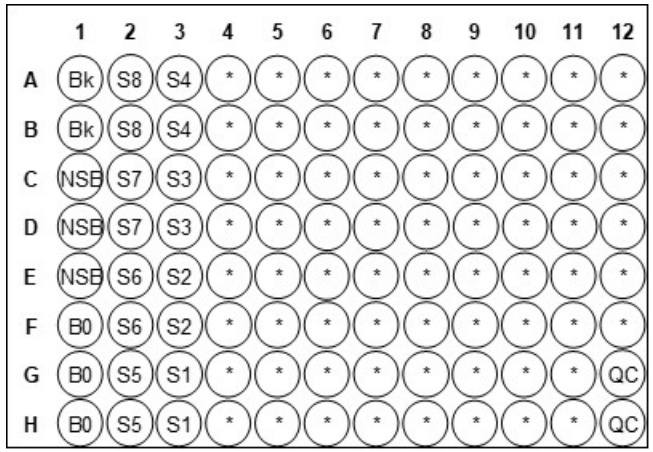
Stability at +4°C: 1 month.

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 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.

Bk : Blank
NSB : Non Specific Binding
QC : Quality Control
B0 : Maximum Binding
S1-S8 : Standards 1-8
* : Samples



Bk : Blank
NSB : Non Specific Binding
QC : Quality Control

B0 : Maximun Binding
S1-S8 : Standards 1-8
* : Samples or Quality Controls

- **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.

- **LEAP-2 ELISA Buffer**
  Dispense 100 µL to Non Specific Binding wells (NSB) wells and 50 µL to B0 well.

- **LEAP-2 Standard**
  Dispense 50 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.

- **LEAP-2 Quality Control and Sample**
  Dispense 50 µL in duplicate to appropriate wells. Highly concentrated samples may be diluted in LEAP-2 ELISA Buffer.

- **LEAP-2 Biotin Labelled**
  Dispense 50 µL to each well, except Blank (Bk) wells.

- **LEAP-2 Antiserum**
  Dispense 50 µL to each well except Blank (Bk) wells and Non Specific Binding (NSB)

- **Incubating the plate**
  Cover the plate with cover sheet and incubate over night at room temperature.

- **Washing the plate**
  Rinse each well 5 times with Wash Buffer (300 µL/well). Just before distributing reagents, remove the buffer from the wells by inverting the plate and shaking out the last drops on a paper towel developing and reading the plate

- **Pipetting the reagents**

- **LEAP-2 Streptavidin-AChE Tracer**
  Dispense 150 µL to each well, except Blank (Bk) wells.

- **Incubating the plate**
  Rinse each well 5 times with Wash Buffer (300 µL/well). Just before distributing reagents, remove the buffer from the wells by inverting the plate and shaking out the last drops on a paper towelveloping and reading the plate

- **Developing and reading the plate**
  - Empty the plate by inverting it. Rinse each well by adding 300 µL of Wash Buffer and repeat washing step 5 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 200µL of Ellman's reagent.
  - Cover the plate with cover sheet and incubate in the dark at room temperature for 60 minutes on an orbital microplate shaker at 300 rpm.
  - Read the plate at 405 nm or at 414 nm (yellow color) using spectrophotometer plate reader.

9. ASSAY PROCEDURE SUMMARY

Enzyme Immunoassay Protocol (volumes are in µL)					
	Blank	NSB	B0	Standard	Sample or QC
LEAP-2 ELISA Buffer	-	100	50	-	-
Standard	-	-	-	50	-
Sample or QC	-	-	-	-	50
LEAP-2 Labelled Biotin	-	50	50	50	50
LEAP-2 Antiserum	-	-	50	50	50
Cover plate, incubate over night at room temperature					
Wash strips 5 times with 300 µL/well Discard liquid from the wells & dry on absorbent paper					
LEAP-2 Streptavidin-AChE TracerEllman's reagent	-	150			
Cover plate, incubate 60 minutes at room temperature while shaking the plate using at 300 rpm on an orbital microplate					
Wash strips 5 times with 300 µL/well Discard liquid from the wells & dry on absorbent paper					
Ellman's reagent	200				
Read the plate at 405 nm or at 414 nm					

10. 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 of at least 25.0 ng/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. It is highly recommended to use this software if available on the device.
  For LEAP-2 ELISA kit the best curve-fitting is obtained with 4-parameter logistic fit (4PL). 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)

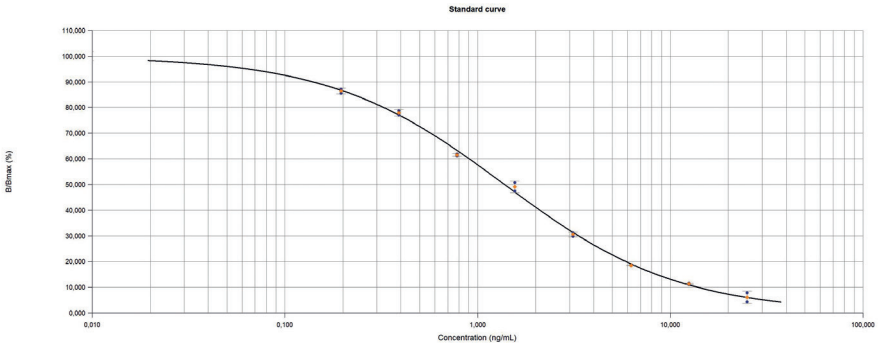
11. ACCEPTABLE RANGE

- NSB absorbance ≤ 0.03 A.U.
- IC 50 ≤ 2 ng/mL
- QC ±25% of the expected concentration (see the label of the QC vial)

12. 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: 60 minutes developing at room temperature, reading at 414 nm. A 4 parameter logistic fitting was used to determine the concentrations.

Standard	LEAP-2 ng/mL	Absorbance A.U.
S1	25.0	0.030
S2	12.5	0.055
S3	6.3	0.091
S4	3.1	0.150
S5	1.6	0.242
S6	0.8	0.302
S7	0.4	0.383
S8	0.2	0.424
Bmax	0.0	0.491
NSB	-	0.011



Typical standard curve

13. TROUBLESHOOTING

► **Absorbance values are too low:**
- one of the reagents was not properly dispensed,
- 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.

► **High dispersion of duplicates:**
- poor pipetting
- irregular plate washing.

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

14. BIBLIOGRAPHY

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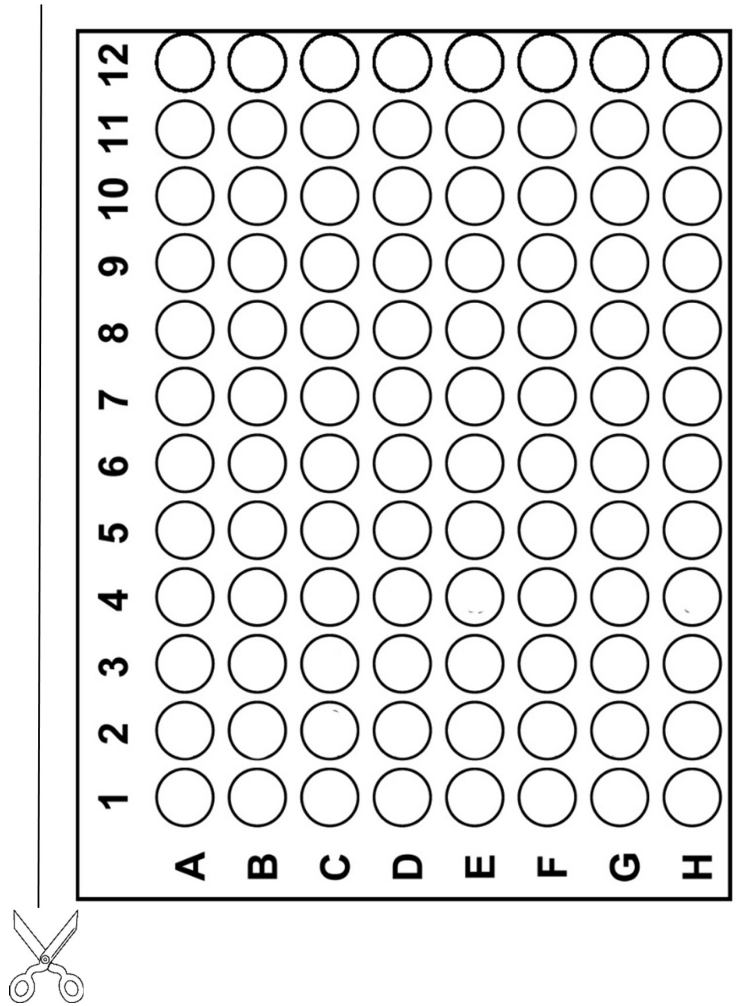

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Should you need help with a product, you can contact our technical support by emailing to tech@bertin-bioreagent.com
Should you need help with an order, you can contact our customer service by emailing to order-life@bertin.group

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Bertin Technologies also markets pre-analytical products, EIA kits, antibodies & biochemicals for:

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Diabetes
Obesity
Hypertension
Pain
Prion diseases

NOTE

NOTES

NOTE

Do not hesitate to contact our after-sales services for further information at

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