# ANTHRAX EDEMA FACTOR

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European patent # 89 139 552
U.S. patent # 50 47 330

# Anthrax Edema Factor
# Assay kit
# #A05230.96 wells

For research laboratory use only
Not for human diagnostic use

This assay has been developed & validated
by Bertin Pharma

Fabriqué en France
Made in France

#A11230
Version: 0117

# Table of contents

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

This kit contains:

> For Adenylyl cyclase EF assay:

| Designation | Colour of cap | Item # | Quantity per kit | Form |
|---|---|---|---|---|
| Assay Buffer | Grey & red | A17230.1 ea | 1 | Lyophilized |
| Anthrax EF Standard | Blue with red septum | A06230.1 ea | 2 | Lyophilized |
| Adenylyl Cyclase Reagent | Black with red septum | A22230.100 dtn | 2 | Lyophilized |
| Sodium Periodate | Black with white septum | A14230.100 dtn | 2 | Powder |
| Rhamnose | Red with white septum | A15230.100 dtn | 2 | Powder |

> For cAMP competitive immunoassay :

| Designation | Colour of cap | Item # | Quantity per kit | Form |
|---|---|---|---|---|
| Mouse anti-Rabbit pre-coated 96 Strip Plate | blister with zip | A08100.1 ea | 1 | - |
| Potassium Hydroxide | gold | A23230.100 dtn | 1 | Solid |
| Acetic Anhydride | blue with white septum | A16230.100 dtn | 1 | Liquid |
| cAMP Tracer | green | A04230.100 dtn | 1 | Lyophilized |
| cAMP Antiserum | red | A03230.100 dtn | 1 | Lyophilized |
| EIA Buffer | blue | A07000.1 ea | 1 | Lyophilized |
| Wash Buffer - concentrated 400x | silver | A17000.1 ea | 1 | Liquid |
| Tween20 | plastic | A12000.1 ea | 1 | Liquid |
| Ellman's reagent 50 | black with red septum | A09000_50.100 dtn | 2 | Lyophilized |
| Technical booklet | - | A11230 | 1 | - |
| 96 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.

## Precaution for use

**Users are recommended to 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

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.

# 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 is 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]***, and Bertin Pharma, formerly known as SPI-Bio, has expertise to develop and produce EIA 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 and can be read at 405-414 nm. AChE® offers several advantages compared to enzymes conventionally 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 Horseradish Peroxidase (HRP) or alkaline phosphatase. AChE® allows a greater sensitivity than other labeling enzymes.
- **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.

- **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.
- **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 (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.

## Anthrax

Anthrax is an infectious disease due to *Bacillus anthracis*, a gram-positive, spore-forming bacterium ***[4]***.

Among bioterrorist agents, anthrax is considered as one of the most potent and dangerous ***[5]*** bio-agent and has been classified in the A list of the Centers of Disease Control ***[6]***.

The pathogenic power of this bacteria is due to an exotoxin, which is secreted into the host's system when three nontoxic proteins link together to form a toxin complex. The three parts of the pathogen complex are protective antigen (PA), lethal factor (LF) and edema factor (EF).

At molecular level, the PA binds to cell receptors where it is cleaved into PA20 and PA63 ***[7]***. PA63 can competitively bind EF or LF or both.

The PA-LF complex forms the lethal toxin (LTx) and the PA-EF complex forms the edema toxin (ETx). Once ETx is endocytosed, EF is released in cytosole. EF is a calmoduline and $Ca^{2+}$ dependent adenylyl cyclase which catalyses the conversion of cytosolic adenosine triphosphate into cyclic adenosine monophosphate (cAMP) leading to edema ***[8]***.

## Principle of the assay

The principle of the test uses the natural capacity of EF to convert ATP into cAMP.

Standard or sample containing EF are incubated with calmoduline and ATP, which is converted to cAMP. After oxidation and derivatization, cAMP is monitored by competitive immunoassay ***[9]***.

The latter is based on the competition between unlabeled cAMP and tracer (cAMP linked to AChE) for a limited amount of cAMP antiserum binding sites.

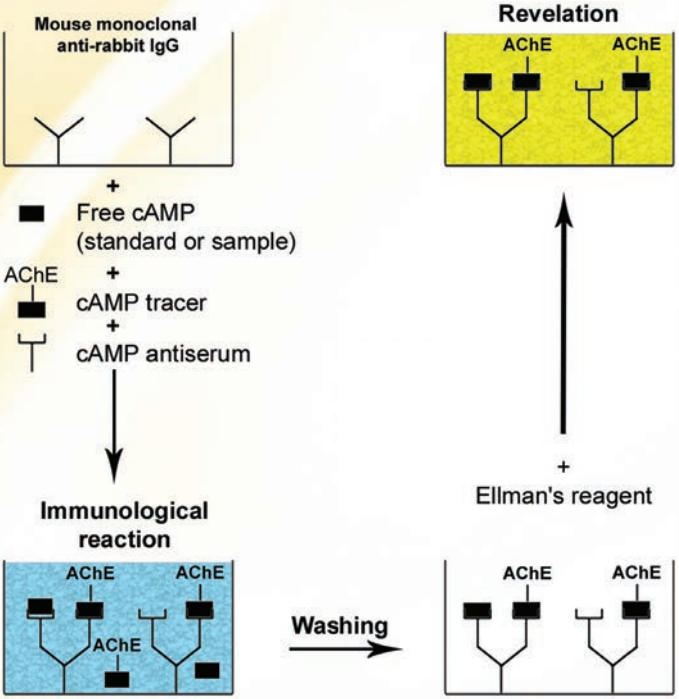
The complex rabbit antiserum-cAMP (unlabeled cAMP or tracer) binds to the mouse monoclonal anti-rabbit antibody coated on the well.
The plate is then washed and Ellman's Reagent (enzymatic substrate for the AChE and chromogen) is added to the wells. AChE tracer acts on Ellman's Reagent to form a yellow compound.

The intensity of the colour, which is determined by spectrophotometer, is proportional to the amount of tracer bound to the well, and inversely proportional to the amount of unlabeled cAMP present in the well during the immunological incubation.

The principle of the assay is summarized below:

## Adenylyl cyclase EF assay:


## cAMP competitive immunoassay:



## Materials and equipment required

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

- Precision micropipettes (5 to 1000 µL)
- Spectrophotometer plate reader (405 or 414 nm filter)
- Microtitration washer (or washbottles)
- Magnetic stirring bar
- Heating and shaking tubes incubator or water bath
- Shaker, orbital or not
- Multichannel pipette 100 µL and disposable tips
- UltraPure water
- Polypropylene micro tubes (44 at least)
- Plasma free of Edema Factor, obtained from healthy subject

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

Otherwise, organic contamination can significantly affect the enzymatic activity of the tracer Acetylcholinesterase (AChE).
Do not use distilled water, HPLC-grade water or sterile water.
UltraPure water may be purchased from Bertin Pharma : item #A07001.1L

## Sample collection & preparation

This assay has been validated to measure the EF in human plasma samples. Blood was collected in citrate primary tubes.

### General precautions

- All samples must be free of organic solvents prior to the assay.
- Samples should be assayed immediately after collection or should be stored at -20°C.

### Sample preparation

- No pre-analytical phase is required.

## Reagent preparation

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

### Adenylyl Cyclase EF Assay

- Assay Buffer
  Reconstitute the vial #A17230 with 10 mL of UltraPure water.
  *Stability at +4°C: 1 week*

- Anthrax EF Standards
  Reconstitute the EF Standard vial #A06230 with 500 µL of UltraPure water. The concentration of this solution **S1** is 1000 pg/mL. Then take 8 polypropylene micro tubes and prepare the standards by serial dilutions as follow.
  *Use just after reconstitution*

| Standard | Volume of Standard | Volume of Assay Buffer | Standard concentration pg/mL |
|---|---|---|---|
| S2 | 200 µL of S1 | 200 µL | 500 pg/mL |
| S3 | 200 µL of S2 | 200 µL | 250 pg/mL |
| S4 | 200 µL of S3 | 200 µL | 125 pg/mL |
| S5 | 200 µL of S4 | 200 µL | 62.5 pg/mL |
| S6 | 200 µL of S5 | 200 µL | 31.2 pg/mL |
| S7 | 200 µL of S6 | 200 µL | 15.6 pg/mL |
| S8 | 200 µL of S7 | 200 µL | 7.8 pg/mL |
| S9 (B0) | - | 200 µL | 0 pg/mL |

> Adenylyl Cyclase Reagent
Reconstitute one vial #A22230 with 1.5 mL of UltraPure water.
*Use just after reconstitution*

> Sodium Periodate
Reconstitute one vial #A14230 with 4.5 mL of UltraPure water.
*Use just after reconstitution*

> Rhamnose
Reconstitute one vial #A15230 with 2.5 mL of UltraPure water.
*Use just after reconstitution*

> Blank Matrix
Choose and dilute at 1/10 a plasma free of Edema Factor in Assay Buffer.

## cAMP competitive immunoassay

> Potassium Hydroxide
**30 minutes before use,** reconstitute the vial #A23230 with 5 mL of UltraPure water.
*Stability at room temperature: 1 month*

This step is exothermic, the water must be added carefully

> EIA Buffer
Reconstitute the vial #A07000 with 50 mL of UltraPure water.
*Stability at +4°C: 1 month*

> cAMP Tracer
Reconstitute the vial #A04230 with 5 mL of EIA Buffer.
*Stability at +4°C: 1 week*

> cAMP Antiserum
Reconstitute the vial #A03230 with 5 mL of EIA Buffer.
*Stability at +4°C: 1 week*

> Wash Buffer
Dilute 1 mL of concentrated Wash Buffer #A17000 with 400 mL UltraPure water. Add 200 µL of Tween20 #A12000. Use a magnetic stirring bar to mix the content.
*Stability at +4°C: 1 week*

> 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 at +4°C and in the dark: 24 hours*

## Adenylyl Cyclase EF Assay procedure

For this part of the assay, use reagents just after reconstitution (see reagent preparation section).

### Standard

- Dispense 5 µL of each standard S1 to S9 in a polypropylene micro tube. Start with the lowest concentration standard and equilibrate the tip in the next higher standard before pipetting.
- Dispense 50 µL of blank matrix into each standards and QC tube.

### Sample tubes

- Dispense 5 µL of each sample in a polypropylene tube (sample 1 to sample 34).
  Highly concentrated samples must be diluted with the Assay Buffer in order to be included into the standard concentration range.
- Dispense 50 µL of Assay Buffer into each sample tube.

### Adenylyl Cyclase Reagent

- Dispense 50 µL of Adenylyl Cyclase Reagent in each tube (standards, samples).
- Incubate 30 minutes at +30°C under gentle shaking at 500 rpm.

## Oxidation Step

> Add 45 µL of Sodium Periodate to each tube. Gently mix and incubate at +37°C during 5 minutes.

## Neutralization Step

> Add 20 µL of Rhamnose in each tube. Gently mix and incubate at +37°C during 20 minutes.

## Acetylation step

**This step must be performed tube by tube**

All the samples and standards must be acetylated as described below:

> Add 33 µL of reconstituted potassium hydroxide and immediately after add 8 µL of Acetic Anhydride #A16230.
> Vortex for 15 seconds.
> Add 8 µL of potassium hydroxide
> Vortex for 15 seconds.

Flakes or small particles may appear, please centrifuge 30 seconds at 1 500 rpm before pipetting.

Tubes can stay at room temperature. **You must run the cAMP competitive immunoassay immediately after this step.**

| Adenylyl Cyclase EF Assay protocol (volumes are in µL) | | |
|---|---|---|
| | Standard (S1-S9) | Sample |
| Standard | 5 | - |
| Sample | - | 5 |
| EF assay buffer | - | 50 |
| Blank matrix | 50 | - |
| Adenylyl cyclase reagent | 50 | |
| Incubate for 30' at +30°C under gentle shaking at 500 rpm | | |
| Sodium periodate | 45 | |
| Incubate for 5' at +37°C | | |
| Rhamnose | 20 | |
| Incubate for 20' at +37°C | | |
| Potassium hydroxide | 33 | |
| Acetic anhydride | 8 | |
| | Vortex 15'' | |
| Potassium hydroxide | 8 | |
| | Vortex 15'' | |

Tube by tube

## cAMP competitive immunoassay procedure

### Plate preparation

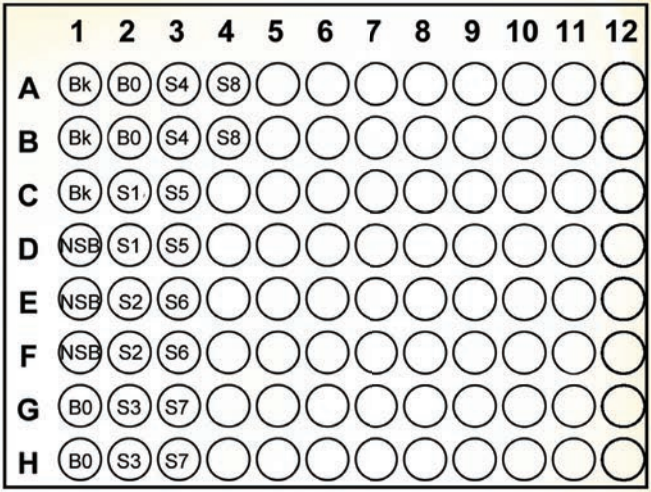
Prepare the Wash Buffer as indicated in the reagent preparation section. Open the plate packet and select the sufficient strips for your assay. Place the unused strips back in the packet, store at +4°C for one month maximum.

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.

### 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

B0: Maximum binding = S9 S1-S8 : Standards 1-8

## ▷ Pipetting the reagents

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

Use different tips to pipette the buffer, standards, samples, tracer, antiserum and other reagents.

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

Respect the order mentioned below when distributing the reagents into plate's wells:

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

- cAMP Tracer
  Dispense 50 µL to each well, **except** Blank (Bk) wells.

- Standards S1-S9
  Dispense 50 µL of each acetylated standard tubes (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. Dispense 50 µL of acetylated tube S9 (B0) into the 4 B0 wells.

- Samplesr
  Dispense 50 µL of each samples, in duplicate, to appropriate wells.

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

## ▷ Incubating the plate

Cover the plate with the cover sheet and incubate the plate for 2h30 at room temperature.

## Developing and reading the plate

- Reconstitute Ellman's reagent as mentioned in the Reagent preparation section.
- Empty the plate by turning over. Rinse each well five times with 300 µL of Wash Buffer. 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 96 well. Cover the plate with an aluminium sheet and incubate in the dark at room temperature. Optimal development is obtained using an orbital shaker.
- Wipe carefully the bottom of the plate with a paper towel, and make sure that no liquid has splashed outside the wells.
- Read the plate at the wavelength of 405 or 414nm (yellow colour).

  **After addition of Ellman's reagent, the absorbance has to be checked periodically, every 30 minutes, until the maximum absorbance (B0 wells) has reached a minimum of 0.3 A.U., blank subtracted.**

| cAMP competitive Immunoassay Protocole (volumes are in µL) | | | | |
|---|---|---|---|---|
| Volume / Wells | Blank | NSB | S1-S9 (B0) | Sample |
| EIA Buffer | - | 100 | - | - |
| Tracer | - | 50 | 50 | 50 |
| Standard | - | - | 50 | - |
| Sample | - | - | - | 50 |
| Antiserum | - | - | 50 | 50 |
| Cover the plate, incubate 2h30 at RT | | | | |
| Wash 5 times - Discard liquid | | | | |
| Ellman's reagent | 200 | | | |
| Incubate the plate on orbital shaker in the dark at RT | | | | |
| Read the plate between 405 and 414 nm | | | | |

## 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 not, do it now.

- Calculate the average absorbance for each NSB, B0, standards and samples.
- Calculate the B/B0 (%) for each standard and sample: (average absorbance of standards or sample - average absorbance of Bk) divided by (average absorbance of B0 - average absorbance of Bk) & multiplied by 100.
- Using a semi-log graph paper for each standard point, plot the B/B0 (%) on y axis versus the concentration (pg/mL) on x axis. Draw a best-fit line through the points.
- To determine the concentration of your samples, find the B/B0 (%) value 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 1000 pg/mL should be re-assayed after dilution in assay buffer.
- Most plate readers are supplied with a curve-fitting software capable of graphing this type of data: 4-parameter logistic fit (4PL). If you have this type of software, we recommend using it. Refer to it for further information.

## Acceptable range

- B0 absorbance > 300 mA.U.
- IC50: 42.7 to 71.2 pg/mL.

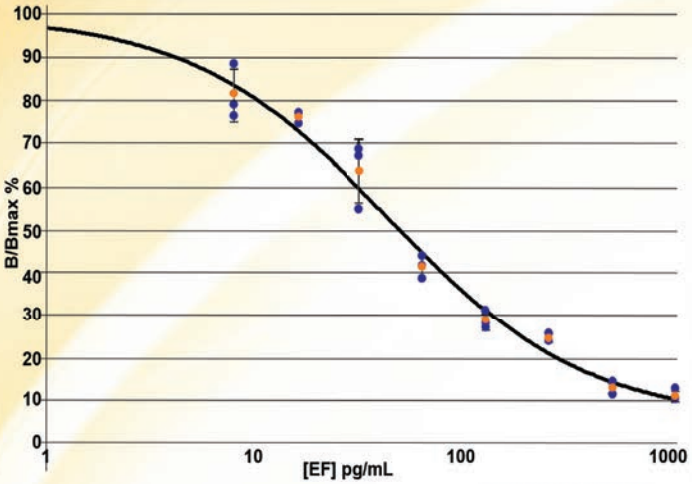
## Typical results

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: 1 hour developing at room temperature.

A 4PL curve fitting was used to determine the concentrations.

| Standards | [EF] pg/mL | mAU | B/B0 (%) |
|---|---|---|---|
| NSB | N/A | 6 | - |
| S9 (B0) | 0 | 452.5 | 100 |
| S8 | 7.8 | 367 | 81 |
| S7 | 15.6 | 342 | 76 |
| S6 | 31.2 | 287 | 63 |
| S5 | 62.5 | 188 | 42 |
| S4 | 125 | 128 | 28 |
| S3 | 250 | 111 | 24 |
| S2 | 500 | 57 | 13 |
| S1 | 1000 | 49 | 11 |

## Typical Anthrax Edema factor standard curve



## Assay validation and characteristics

IC 50 is the concentration of EF corresponding to 50% of the maximum binding. It is around 57 pg/mL.

For additional information regarding the validation of imunoassay for protein biomarkers in biological samples, please refer to bibliography ***[10, 11]***.

### > Intra-assay variations

| Intra-assay variation | | |
|---|---|---|
| **QC** | Mean of observed concentrations (pg/mL) | Intra-assay (CV%) |
| **QC n°1** | 143 | 33,46 |
| **QC n°2** | 47 | 25,68 |

For the intra-assay validation, the number of replicates (n) is equal to 10 for each levels of QC. The 2 validation levels were analyzed along with the calibration curve for a unique experiment.

### > Inter-assay variations

| Inter-assay variation | | |
|---|---|---|
| **QC** | Mean of observed concentrations (pg/mL) | Inter-assay (CV%) |
| **QC n°1** | 103 | 35,75 |
| **QC n°2** | 46 | 16,06 |

For the inter-assay validation, the number of replicates (n) is equal to 3 for each levels of QC.

The 2 validation levels were analyzed along with the calibration curve for a total of 5 independant runs.

The intra-assay and the inter-assay variations were studied on human plasma spiked with EF at each levels of QC.

## 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 technique,
- 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 you need further explanation, Bertin Pharma will be happy to assist you. Feel free to contact our technical support staff by phone (+33 (0)139 306 036), fax (+33 (0)139 306 299) or E-mail (bioreagent@bertinpharma.com), and be sure to indicate the batch number of the kit (see outside the box).

Bertin Pharma proposes EIA Training kit #B05005 and EIA workshop upon request. For further information, please contact our Marketing Department by phone (+33 (0)139 306 260) or E-mail (marketing@bertinpharma.com).

## Bibliography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Bertin Pharma, over the last decades, has been developing and marketing over 100 biomarker assays, pre-analytical products, kits, antibodies and biochemicals thanks to its innovative work in research and development.
Our core areas are orientated to inflammation, oxidative injury, endocrinology, diabetes, obesity, hypertension, neurodegenerative diseases, HIV, prion diseases, pharmacokinetics and metabolism.

Bertin Pharma is active worldwide either with direct sales or through our qualified and trained international distribution network from the United States to Japan.

We are able to provide you with local technical support to use at ease our products.

For further information, please send your request to
bioreagent@bertinpharma.com

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care. innovate. deliver.

Parc d'activités du Pas du Lac - 10 bis avenue Ampère
F-78180 Montigny-le-Bretonneux - France
Tel: +33 (0)139 306 036 - Fax: +33 (0)139 306 299
bioreagent@bertinpharma.com - www.bertinpharma.com