QRFP-26RFa (human)

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

QRFP-26RFa (human)
Enzyme Immunoassay kit
#A05037.96 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

#A11037
Version: 0117

Table of contents

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

This kit contains:

Designation	Colour of cap	Item #	Quantity	Form
Mouse anti-Rabbit precoated 96-well Strip Plate	Blister with zip	A08100.1 ea	1	-
QRFP-26RFa (human) Tracer	Green	A04037.100 dtn	1	Lyophilised
QRFP-26RFa (human) Antiserum	Red	A03037.100 dtn	1	Lyophilised
QRFP-26RFa (human) Standard	Blue with red septum	A06037.1 ea	2	Lyophilised
QRFP-26RFa (human) Quality Control	Green with red septum	A10037.1 ea	2	Lyophilised
EIA Buffer	Blue	A07000.1 ea	1	Lyophilised
Wash Buffer	Silver	A17000.1 ea	1	Liquid
Tween 20	Transparent	A12000.1 ea	1	Liquid
Ellman's Reagent 50	Black with red septum	A09000_50.100 dtn	2	Lyophilised
Technical Booklet	-	A11037	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 34 samples in duplicate.

If you want to use the kit in two times, we provide one additional vial of Standard, one of Quality Control and one of Ellman's Reagent.

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), 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.

QRFP-26RFa

The QRFP-26RFa is a neuropeptide of 26 amino-acids from the RFamide peptide family, neuropeptide with an arginine and an amidated phenylalanine-motif at its C-end ***[4]***.

QRFP-26RFa has been discovered in 2003 in European green frog brain ***[4]***. It has, since then, been characterised in many species such as human, rat and chicken ***[5]***.

The structural analysis of the peptide exhibits an amphipathic α-helix in its central domain ***[6]*** that plays a role in the interaction with its receptor called GPR103 ***[7]***.

The QRFP-26RFa is mainly produced in the hypothalamus and in some peripheral tissues like brainstem or the lateral horns of the spinal cord ***[8]***.

In vivo studies in mice demonstrated that the QRFP-26RFa is an orexigenic peptide by action on the NPY/pro-opiomelanocortin system ***[9]*** and by inhibition of the glucose-induced insulin secretion ***[10]***.

QRFP-26RFa has an action on the regulation of the high fat diet and on the lipolysis in adipocyte of obese individuals ***[11]***. In addition, some researchers study the roles of the 26RFa in sleep ***[12]*** and blood pressure ***[9]*** regulation.

Principle of the assay

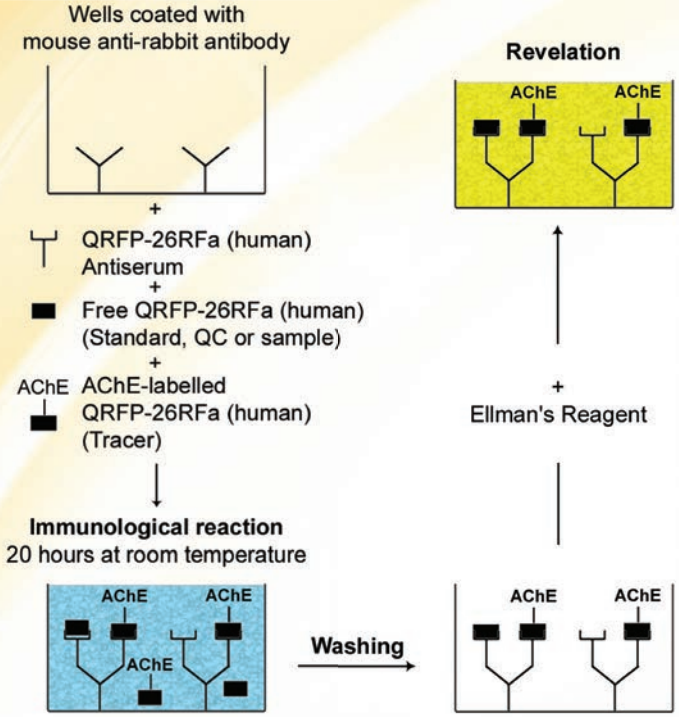
This Enzyme Immunometric Assay (EIA) is based on the competition between unlabelled (free) QRFP-26RFa (standard/QC/samples) and acetylcholinesterase (AChE) linked to QRFP-26RFa (Tracer) for limited specific rabbit anti-QRFP-26RFa antiserum sites.

The complex rabbit antiserum – QRFP-26RFa (free QRFP-26RFa or Tracer) binds to the mouse monoclonal anti-rabbit antibody coating in the well.

The plate is washed to remove any unbound reagent and Ellman's Reagent (enzymatic substrate for AChE and chromogen) is added to the wells.

AChE tracer acts on the Ellman's Reagent to form a yellow compound that strongly absorbs at 414 nm. The intensity of the colour, determined by spectrophotometry, is proportional to the amount of tracer bound to the well and is inversely proportional to the amount of free QRFP-26RFa present in the well during the immunological incubation.

The principle of the assay is summarised below:



Materials and equipment required

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

For sample preparation:

- Cartridge C18 SEP Pack 3cc (waters Ref WAT054945)
- Acetonitrile
- Trifluoroacetic acid (TFA)

For the assay:

- Precision micropipettes (20 to 1000 µL)
- Spectrophotometer plate reader (405 or 414 nm filter)
- Microplate washer (or washbottles)
- Orbital microplate shaker
- Multichannel pipette and disposable tips 30-300µL
- UltraPure Water #A07001.1L
- Polypropylene tubes

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 #A07001.1L may be purchased from Bertin Pharma.

Sample collection and preparation

This assay may be used to measure the QRFP-26RFa in plasma EDTA-K_3. For any other sample it is the responsibility of the user to check for potential interferences (see our web site or contact our technical support).

General precautions

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

Sample collection

Blood samples are collected in tubes containing EDTA-K_3. Then they are centrifuged at 3,500 rpm for 10 minutes at +4°C and supernatants are transfered in separate tubes.

Samples should be quickly assayed or stored at -20°C for later use.

Sample preparation

This part concerns the extraction for the measurement of QRFP-26RFa in plasma:

- Wash the cartridge twice with 1 mL of acetonitrile.
- Wash the cartridge three times with 1 mL of UltraPure water – 0.12 % TFA.

- Dilute sample at 1:2 with UltraPure Water – 0.12 % TFA: 500 µL sample + 500 µL UltraPure Water – 0.12 % TFA

- Pass 1 mL of diluted sample slowly (about 2 mL/minute) through the cartridge.

- Wash the cartridge twice with 1 mL of UltraPure water – 0.12 % TFA – 10 % acetonitrile.

- Discard the wash.

- Elute the QRFP-26RFa sample with 2 mL of UltraPure Water – 0.12 % TFA – 50 % acetonitrile, 1 mL at a time. First, load 1 mL of the solution onto the cartridge and wait until the solution goes through the cartridge. Then, load the second 1 mL.
  The 2 mL of solution must be collected in the same tube.

- Dry the sample by vacuum centrifugation.

- Reconstitute the QRFP-26RFa sample with EIA Buffer (¼ of the initial volume of the sample).

  Example : if the volume of the sample before dilution 1:2 is 500 µL, then reconstitute with 125 µL of EIA buffer.

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 34 samples in duplicate.

If you want to use the kit in two times, we provide one additional vial of Standard, one of Quality Control and one of Ellman's Reagent.

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

EIA Buffer

Reconstitute the vial #A07000 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

QRFP-26RFa (human) Standard

Reconstitute one Standard vial #A06037 with 1 mL of UltraPure Water. Allow it to stand 1 minute until completely dissolved and then mix thoroughly by gentle inversion. The concentration of the first standard (S1) is 12.50 ng/mL.

Prepare seven polypropylene tubes for the other standards (S2 to S8) and add 500 µL of EIA Buffer into each tube. Then prepare the standards by serial dilutions as follows:

Standard	Volume of Standard	Volume of Assay Buffer	Standard concentration
S1	-	-	12.50 ng/mL
S2	500 µL of S1	500 µL	6.25 ng/mL
S3	500 µL of S2	500 µL	3.13 ng/mL
S4	500 µL of S3	500 µL	1.56 ng/mL
S5	500 µL of S4	500 µL	0.78 ng/mL
S6	500 µL of S5	500 µL	0.39 ng/mL
S7	500 µL of S6	500 µL	0.20 ng/mL
S8	500 µL of S7	500 µL	0.10 ng/mL

Stability at 4°C: 1 week

▷ QRFP-26RFa (human) Quality Control

Reconstitute one QRFP-26RFa (human) Quality Control vial #A10037 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 week.

▷ QRFP-26RFa (human) Tracer

Reconstitute the QRFP-26RFa (human) Tracer vial #A04037 with 5 mL of EIA buffer. Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion.
Stability at +4°C: 1 week.

QRFP-26RFa (human) Antiserum

Reconstitute the QRFP-26RFa (human) Antiserum vial #A03037 with 5 mL of EIA Buffer. Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion.
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.
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

Assay procedure

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

Plate preparation

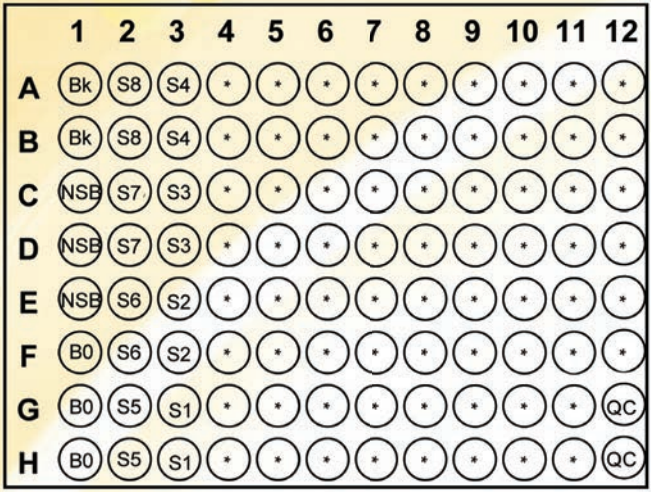
Prepare the Wash Buffer as indicated in the reagent preparation section.
Open the plate packet and select the sufficient strips for your assay and place the unused strips back in the packet.
Stability at +4°C: 1 month.

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.

Plate set-up

A plate set-up is suggested hereafter.
The content 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 : Maximun Binding S1-S8 : Standards 1-8
* / QC : Samples or Quality Controls

▷ Pipetting the reagents

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

Use different tips to pipet the buffers, standards, samples, tracer, antiserum 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.

> **EIA Buffer**
Dispense 100 µL to Non Specific Binding (NSB) wells and 50 µL to Maximum Binding (B0) wells.

> **QRFP-26RFa (human) 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.

> **QRFP-26RFa (human) Quality Control and Samples**
Dispense 50 µL in duplicate to appropriate wells. Highly concentrated samples may be diluted in EIA Buffer.

> **QRFP-26RFa (human) Tracer**
Dispense 50 µL to each well, **except** Blank (Bk) wells.

> **QRFP-26RFa (human) Antiserum**
Dispense 50 µL to each well, **except** Blank (Bk) wells and NSB wells.

▷ Incubating the plate

Cover the plate with the cover sheet and incubate 20 hours 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 5 times with 300 µL 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 200µL of Ellman's Reagent to each 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 the bottom of the plate with a paper towel, and make sure that no liquid has splashed outside the wells.

- Read the plate at a wavelength between 405 and 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 250 m.A.U. (blank subtracted).

Enzyme Immunoassay Protocol (volumes are in µL)						
Volume / Wells	Blank	NSB	B0	Standard	QC	Sample
Buffer	-	100	50	-	-	-
Standard	-	-	-	50	-	-
QC	-	-	-	-	50	-
Sample	-	-	-	-	-	50
Tracer	-	50				
Antiserum	-	-	50			
Cover plate, incubate 20 hours at room temperature						
Wash strips 5 times with 300 µL of Wash Buffer & 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						

Data analysis

Make sure that your plate reader has subtracted the absorbance readings of the blank wells (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 B0, standard, quality control and sample.
- Calculate the B/B0 (%) for each standard, QC and sample (*average absorbance of standards, QC or sample divided by average absorbance of B0*) & multiplied by 100.
- Using a semi-log graph paper for each standard point, plot the B/B0 (%) on y axis versus the concentration (ng/mL)on x axis. Draw a best-fit line through the points.
- To determine the concentration of your sample, the corresponding B/B0 (%) value has to be comprised between 20% and 80%. 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.
- **For samples extracted from plasma, the concentration must be divided by 4**.
- Most plate readers are supplied with a 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 +/- 40% of the expected concentration (see the label of QC vial)

Acceptable range

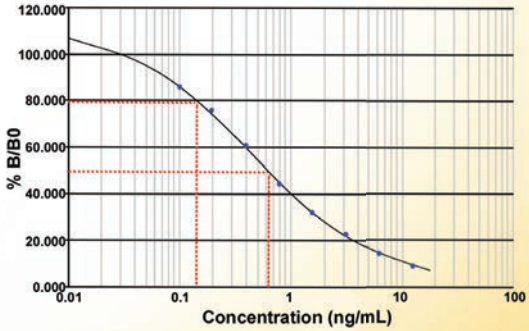
- B0 absorbance > 250 mAU blank subtracted.
- NSB absorbance < 20 mAU
- IC50: 0.47 - 0.78 ng/mL (*means at 0.62 ng/mL*)
- QC sample: ± 40% of the expected concentration (see the label of QC vial)

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 room temperature, reading at 414 nm. A 4-parameter 1/y fitting was used to determine the concentrations.

	[QRFP-26RFa (human)] concentration ng/mL	Absorbance mAU	B/B0 %
S1	12.50	33	9.1
S2	6.25	53	14.7
S3	3.13	82	22.8
S4	1.56	115	32.2
S5	0.78	174	45.4
S6	0.39	213	60.9
S7	0.20	273	76.2
S8	0.10	307	85.7
B0	0	0.358	100.0

Typical QRFP-26RFa standard curve



Assay validation and characteristics

The Enzyme Immunometric Assay of QRFP-26RFa (human) has been validated for its use in plasma EDTA-K_3.

For additional information regarding the validation of immunoassay for protein biomarkers in biological samples, please refer to bibliography ***[13, 14]***.

> The **IC 50** is the concentration corresponding to 50% of the maximum binding (B0) is the around 0.62 ng/mL.

> **Inter-assay variation** (n = 2) in EIA Buffer

	[QRFP-26RFa (human)] ng/mL	C.V %
QC 1	18.47	16.0
QC 2	1.87	5.8
QC 3	0.62	21.1

Quality Control samples (QC 1/QC 2/QC 3) are plasma EDTA-K_3 spiked at different concentrations with QRFP-26RFa peptide and stored at -20°C. Before each experiment, the quality controls are extracted as indicated in the section “Sample Preparation”.

The number of replicates (n) is equal to 2, for the three quality controls. The three validation levels were analysed along with the calibration curve for a total of 5 independent runs.

> Intra-assay variation (n = 10) in EIA Buffer

	[QRFP-26RFa (human)] ng/mL	C.V %
QC 1	19.32	6.4
QC 2	1.76	4.4
QC 3	0.50	10.2

Quality control samples (QC 1/QC 2/QC 3) are plasma EDTA-K_3 spiked at different concentrations with QRFP-26RFa peptide and stored at -20°C. Before the experiment the quality controls are extracted as indicated in the section “Sample Preparation”.

The number of replicates (n) is equal to 10 for the three quality controls. The three validation levels were analysed along with the calibration curve for a unique experiment.

> Cross-reactivity

QRFP-26RFa (mouse, rat)	10.0 %
QRFP-43RFa (human)	37.3 %
QRFP-43RFa (mouse, rat)	2.6 %

Matrix	Dilution (1/x)	Endogenous [QRFP-26 RFa (human)] measured concentration (ng/mL)	Spiked [QRFP-26 RFa (human)] (ng/mL)	Endogenous + Spiked [QRFP-26 RFa (human)] measured concentration (ng/mL)	Endogenous + Spiked [QRFP-26 RFa (human)] measured concentration X Dilution factor (ng/mL)	Accuracy (%)	CV (%)
1	1	0.08	2.00	-	-	-	-
	1		-	2.58	2.49	129.0	8.1
	2		-	1.33	2.67	133.4	
	4		-	0.65	2.60	129.8	
	8		-	0.28	2.24	110.8	
2	1	0.08	2.00	-	-	-	-
	1		-	2.09	2.09	104.3%	4.6
	2		-	1.16	2.32	116.2%	
	4		-	0.57	2.28	114.0%	
	8		-	0.28	2.24	112.0%	
3	1	0.10	2.00	-	-	-	-
	1		-	2.26	2.26	107.8%	8.6
	2		-	1.39	2.78	133.7%	
	4		-	0.61	2.44	117.7%	
	8		-	0.32	2.54	122.1%	

Single donor plasma are spiked with 2.00 ng/mL of QRFP-26RFa (human) and then extracted (see “Sample Preparation”). Each dilution is run in duplicate.

Troubleshooting

> **Absorbance values are too low**:
- organic contamination of water,
- one reagent has not been 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 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).

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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 bio-chemicals 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