bertin BIOREAGENT

20-HYDROXYECDYSONE

For laboratory research only. Not for human or veterinary diagnostic use.

Bertin Bioreagent also markets pre-analytical products, ELISA kits, antibodies & biochemicals for:

- **Inflammation**
- **Oxidative injury**
- **Endocrinology**
- **Diabetes**
- **Obesity**
- **Hypertension**
- **Pain**
- **Prion diseases**

Do not hesitate to contact our after-sales services for further information at tech@bertin-bioreagent.com

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

20-Hydroxyecdysone
Enzyme Immunoassay kit
#A05120.96 wells

For research laboratory use only
Not for human diagnostic use

This assay has been developed & validated
by Bertin Bioreagent

Fabriqué en France
Made in France

#A11120
Version: 0119

Table of contents

96 wells
Storage: -20°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	-
20-Hydroxyecdysone Tracer	Green	A04120.100 dtn	1	Lyophilized
20-Hydroxyecdysone Antiserum	Red	A03120.100 dtn	1	Lyophilized
20-Hydroxyecdysone Standard	Blue with red septum	A06120.1 ea	2	Lyophilized
20-Hydroxyecdysone Quality Control	Green with red septum	A10120.1 ea	2	Lyophilized
EIA Buffer	Blue	A07000.1 ea	1	Lyophilized
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	Lyophilized
Technical booklet	-	A11120.1 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 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 the 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 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]***, and Bertin Bioreagent, 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 Horse Radish 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), 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 while waiting for technical advice from the Bioreagent Department.

20-Hydroxyecdysone

Ecdysone is a steroid hormone of the ecdysteroids family which is found in animals (vertebrates and invertebrates) and in plants. There exist different polyhydroxylated forms of ecdysone, the major one being the 20-Hydroxyecdysone.

20-Hydroxyecdysone (20E) is known as the insect moulting hormone ***[20]***. Indeed, in interaction with the juvenile hormone (JH), 20E plays a key role in growth, development, metamorphosis and reproduction ***[4, 5]*** of the Arthropods (i.e. invertebrates such as insects, crustaceans, arachnids, etc.) ***[6, 7, 8]***.

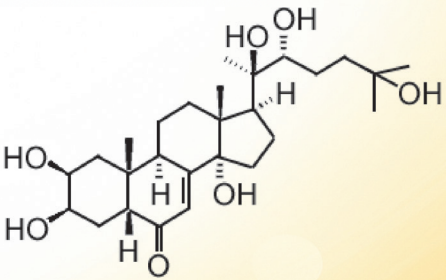
20E regulates target gene transcription by binding a heterodimeric nuclear receptor composed of the ecdysone receptor (EcR) and Ultraspiracle (USP) ***[9, 10, 11]***.

Various plants synthesize plant-derived ecdysteroids. However, the role of these phytoecdysteroids (PEs) is not currently well-known. 20E is widespread in plants and represents the main PE. These hormones may have a potential role in plant defense against herbivore pest insects and nematodes ***[12, 13]***.

Little is known about the function of ecdysteroids in the vertebrates. There are several reports which indicate an effect of the phycoecdysteroid 20E on muscle growth and fat loss in mammals. Some dietary supplements are marketed to enhance physical performances of athletes, and particularly of bodybuilders. Besides, researchers are currently studying the effect of 20E in the treatment of obesity and diabetes ***[14, 15]***. And there are on-going investigations on the possible interest of 20E in the prevention and the treatment of diseases such as osteoporosis and osteoarthritis ***[16, 17]***.

Synonyms: 20E, insect molting hormone, 20-OH ecdysone, β-ecdysone, β-ecdysterone, crustecdysterone, ecdysterone, 20-β-hydroxyecdysterone.

20-Hydroxyecdysone Structure



Principle of the assay

This Enzyme Immunoassay (EIA) is based on the competition between unlabelled 20-Hydroxyecdysone and acetylcholinesterase (AChE®)-labelled 20-Hydroxyecdysone (Tracer) for limited specific rabbit anti-20-hydroxyecdysone antiserum sites.

The complex rabbit antiserum – 20-Hydroxyecdysone (free 20E or Tracer) binds to the mouse monoclonal anti-rabbit antibody coated in the well.

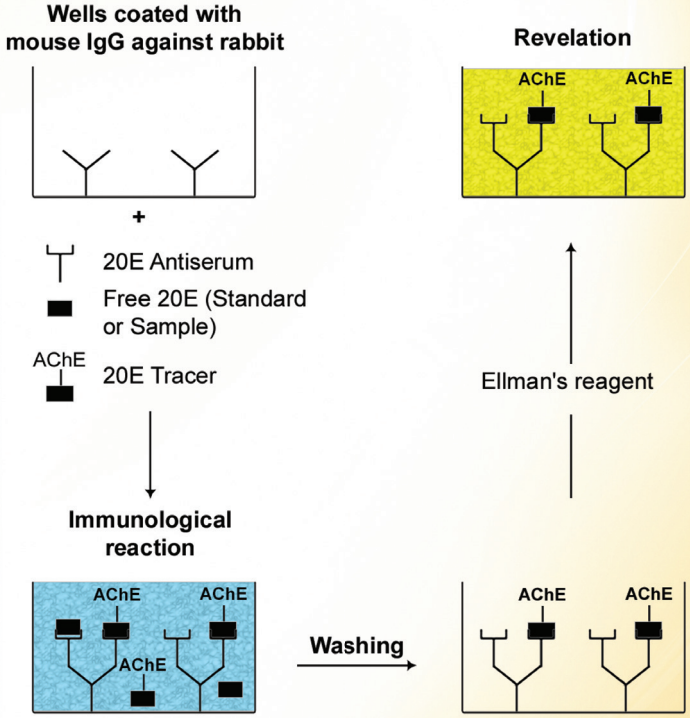
The plate is washed to remove any unbound reagent, and Ellman's reagent (enzymatic substrate for AChE and chromogen) is added to the wells.

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 20E present in the well during the immunological incubation.

The kit has been validated by Bertin Bioreagent in EIA buffer, and has not been tested on biological samples. However, several scientists used immunoassay technics to quantify 20-hydroxyecdysone in biological samples like haemolymph ***[18, 20, 21, 22]***.

The principle of the assay is summarised below:



Materials and equipment required

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

- Precision micropipettes (20 to 1000 µL)
- Multichannel pipette 100 µL or 200 µL and disposable tips
- Spectrophotometer plate reader (405 or 414 nm filter)
- Microplate washer (or washbottles)
- Microplate shaker
- Magnetic stirring bar
- UltraPure water
- Polypropylene tubes

Water used to prepare all EIA reagents and buffers must be UltraPure, 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.

- UltraPure water may be purchased from Bertin Bioreagent (item #A07001.1L)

Sample collection and preparation

The kit has been validated in EIA buffer, and has not been tested on biological samples. However, several scientists used immunoassay technics to quantify 20-hydroxyecdysone in biological samples like haemolymph ***[18, 20, 21, 22]***.

It is the responsibility of the user to find the proper dilution according to the type of biological material and to check for potential interferences (see our website or contact our technical support).

We suggest the following protocol to prepare biological samples ***[18, 19]*** :

- Extraction of the 20-Hydroxyecdysone (which is hydrophobic) with methanol
- Centrifugation and collection of the supernatant
- Evaporation until completely dry
- Dilution in 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

20-hydroxyecdysone Standard

Reconstitute the 20-hydroxyecdysone Standard vial #A06120 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 this first standard **S1** is 5000 pg/mL. Prepare seven propylene tubes for the other standards 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	-	-	5 000 pg/mL
S2	500 µL of S1	500 µL	2 500 pg/mL
S3	500 µL of S2	500 µL	1 250 pg/mL
S4	500 µL of S3	500 µL	625 pg/mL
S5	500 µL of S4	500 µL	312.5 pg/mL
S6	500 µL of S5	500 µL	156.3 pg/mL
S7	500 µL of S6	500 µL	78.1 pg/mL
S8	500 µL of S7	500 µL	39.1 pg/mL

Stability at 4°C: 1 week

▷ 20-hydroxyecdysone Quality Control

Reconstitute the vial #A10120 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

▷ 20-hydroxyecdysone Tracer

Reconstitute the vial #A04120 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

20-hydroxyecdysone Antiserum

Reconstitute the vial #A03120 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 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

Assay procedure

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

Plate preparation

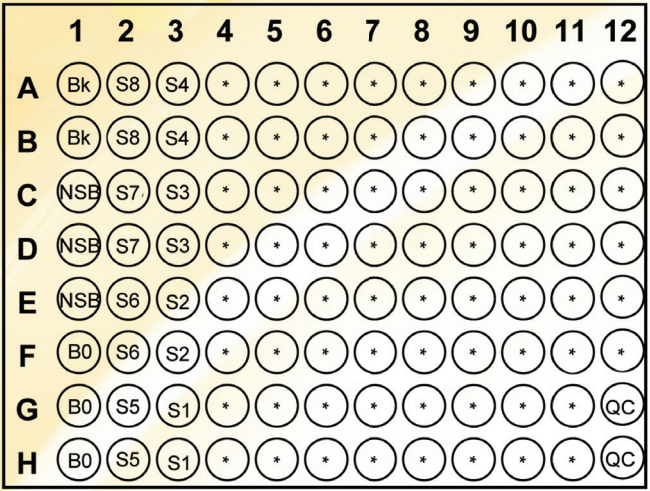
Prepare the Wash Buffer as indicated in the reagent preparation section.
Open the plate packet and select the sufficient strips for your assay and place the unused strips back in the packet, store at +4°C for 1 month maximum.

Rinse each well 4 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
B0: Maximum Binding
NSB: Non Specific Binding
S1-S8: Standards 1-8
QC: Quality Control
*: Samples

▷ Pipetting the reagents

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

Use different tips to pipette 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.

> **EIA Buffer**

Dispense 100 µL to NSB wells and 50 µL to B0 wells.

> **20-hydroxyecdysone 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.

> **Quality Control and samples**

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

> **20-hydroxyecdysone Tracer**

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

> **20-hydroxyecdysone 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 overnight at +4°C.

Developing and reading the plate

- Reconstitute Ellman's reagent as mentioned in the Reagent preparation section.

- Empty the plate by turning it over. Rinse each well five 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 96 well. Cover the plate with an aluminium foil sheet and incubate in the dark at room temperature. Optimal development is obtained using an orbital shaker.

- Gently 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 0.200 A.U. blank subtracted.

Enzyme Immunoassay Protocole (volumes are in µL)						
Volume / Wells	Blank	NSB	B0	Standard	QC	Sample
EIA Buffer	-	100	50	-	-	-
Standard	-	-	-	50	-	-
QC	-	-	-	-	50	-
Sample	-	-	-	-	-	50
Tracer	-	50				
Antiserum	-	-	50			
Cover plate, incubate overnight at +4°C						
Wash plate 5 times & discard liquid from the wells						
Ellman's reagent	200					
Incubate with an orbital shaker at 400 rpm 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 not, do it now.

- Substract the average absorbance of NSB for each B0, standards, quality control and samples.
- 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 (pg/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.
- Diluted samples which concentration determined on standard curve is greater than 5000 pg/mL should be re-assayed after appropriated dilution in EIA buffer.

> Most plate readers are supplied with curve-fitting software capable of graphing these data (4-parameter or 5-parameter logistic fit). 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 (written on the label of the QC vial)

Acceptable range

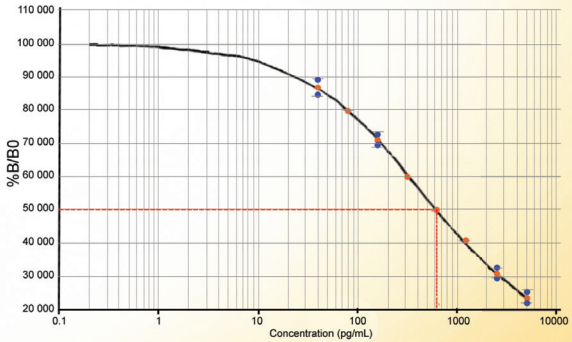
- B0 absorbance > 200 mAU blank subtracted in the conditions indicated above.
- NSB absorbance < 35 mAU
- IC50: <1000 pg/mL
- QC sample: ± 25% of the expected concentration (see the label of QC vial).

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: 90 minutes developing at +20°C, reading at 414 nm. A 4-PL fitting was used to determine the concentrations.

	20-Hydroxyecdysone pg/mL	mAU	B/B0 (%)
Standard S1	5000	120	23.2
Standard S2	2500	160	31.0
Standard S3	1250	211	40.8
Standard S4	625	256	49.5
Standard S5	312.5	310	59.9
Standard S6	156.3	369	71.3
Standard S7	78.1	413	79.7
Standard S8	39.1	451	87.1
B0	0.0	518	100.0

Typical 20-hydroxyecdysone standard curve



Assay validation and characteristics

The Enzyme Immunoassay of 20-Hydroxyecdysone has been validated in EIA Buffer only.
For additional information regarding the validation of immunoassay for protein biomarkers in biological samples, please refer to bibliography ***[24, 25]***.

> The **Limit of Detection (LOD)** of 20-Hydroxyecdysone corresponding to the B0 average minus three standard deviations is around 31 pg/mL.

> The **IC50** is the concentration in 20-Hydroxyecdysone corresponding to 50 % of the maximum Binding is less than 1000 pg/mL.

> **Cross-reactivity *[18]***

20-hydroxy-ecdysone	100%
Ecdysone	100%
2-deoxy-20-hydroxy-ecdysone	88%
Polypodine B	70%
2-deoxy-ecdysone	63%
Ponasterone A	43%
Cyasterone	5%
Podecdysone C	4.5%
Makisterone A	4%
26-hydroxy-ecsydone	1.4%
Muristerone A	1.2%
Kaladasterone	1%
22-epi-ecdysone	< 0.1%
Posterone	< 0.1%

Troubleshooting

- **Absorbance values are too low**: organic contamination of water, incubation in wrong conditions (time or temperature), reading time not long enough, 20-Hydroxyecdysone Tracer has not been dispensed.

- **High signal and background in all wells**: inefficient washing or overdeveloping (incubation time should be reduced) or high ambiant temperature.

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

These are a few examples of trouble shooting that may occur. If you need further explanation, Bertin Bioreagent 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 (tech@bertin-bioreagent.com), and be sure to indicate the batch number of the kit (see outside the box).

Bertin Bioreagent proposes EIA Training kit #B05005.2x96 wells and EIA workshop upon request. For further information, please contact our Marketing Dpt by phone (+33 (0)139 306 260) or E-mail (tech@bertin-bioreagent.com).

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With 30 years of experience, Bertin Bioreagent develops and sells best-in-class kits and products for life science research labs. Our scientist team innovate each day to tailor biomarker assays, pre-analytical products, kits, antibodies and biochemicals that are ready to use, fully validated with a strict quality control.
We strive to address a broad range of research interest: inflammation, oxidative injury, endocrinology, diabetes, obesity, hypertension, pain, prion diseases.

Bertin Bioreagent has also a long expertise in developing customized solutions adapted to your need. Feel free to contact us for your special projects!

To offer a complete solution to researchers, Bertin Instruments offers a range of unique laboratory equipment from Air Sample collection, Sample Homogenisation and Digital Imaging.

Our products are available worldwide through us directly or via our distributor network. Our sales team is active on all continents and will be delighted to answer all your commercial questions.

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