Histamine

A brand name of

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

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

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

Histamine
Enzyme Immunoassay kit
A05890.96 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

Version: 0117
Ref. #A11890

Table of contents

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

Designation	Colour of cap	Item #	Quantity per kit	Form
Histamine precoated 96-well Strip Plate	Blister with zip	A08890.1 ea	1	-
Histamine Tracer	Green	A04890.100 dtn	1	Lyophilised
Histamine Standard	Transparent	A06890.1 ea	2	Liquid
Derivatization Reagent	White with red septum	A15890.1 ea	2	Powder
Derivatization Buffer	Silver / Pink	A16890.1 ea	1	Liquid
Histamine EIA Buffer	Silver / Blue	A07890.1 ea	1	Lyophilised
Wash Buffer	Silver	A17000.1 ea	1	Liquid
Tween 20	Transparent	A12000.1 ea	1	Liquid
Histamine Quality Control	Transparent	A10890.1 ea	2	Liquid
Ellman's Reagent 50	Black with red septum	A09000_50. 100 dtn	2	Lyophilised
Technical Booklet	-	A11890	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.

If you want to use the kit in two times, we provide one additional vial of Standard, one of Derivatization Reagent, 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.

Histamine

Histamine is a natural amine present in many animal and human tissues. It was considered to be the mediator of immediate allergy.

Histamine plays a role in various physiological processes, such as control of gastric acid secretion, neurotransmission and modulation of inflammatory and immunological reactions.

Principle of the assay

This Enzyme Immunoassay (EIA) is based on the competition between unlabelled (free) Histamine (standards / QC / samples) and acetylcholinesterase (AChE) linked to Histamine (Tracer) for limited specific mouse anti-Histamine antibody sites.

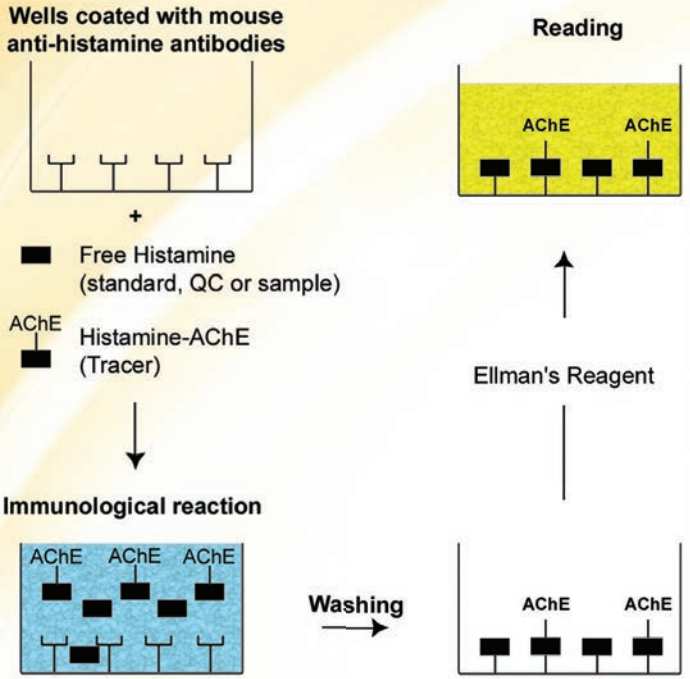
As a former step of this assay, Histamine is derivatized to increase the affinity of Histamine to the antibody and consequently increase the sensitivity of the assay.

Tracer and free Histamine are incubated in wells which have been precoated with a mouse anti-Histamine antibody. The plate is washed to remove any unbound reagent, then 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 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 Histamine 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:

- Anhydrous N,N-Dimethylformamide (DMF)(*) (if not available, DMS0)
- Precision micropipettes (20 to 1000 µL)
- Multichannel pipette and disposable tips 30-300µL
- Spectrophotometer plate reader (405 or 414 nm filter)
- Microplate washer (or wash bottles)
- UltraPure water #A07001.1L
- Orbital microplate shaker
- Polypropylene tubes (**no glass tubes**)
- For plasma sample: EDTA tubes
- For solid and liquid biological samples: HCl04 & NaoH

(*) Make sure that the bottle of DMF has been opened for a short period of time. This point is important to get a good derivatization rate.

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 and preparation

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.

This assay may be used to measure Histamine in samples such as plasma, urine, culture supernatants as well as liquid (e.g. broncho-alveolar lavage fluids) or solid (brain, nervous tissues) biological samples after extraction. Please refer to the appropriate paragraph for your samples preparation protocol.

Plasma

Collect blood samples in tubes containing EDTA. Centrifuge the samples at 1,600 g for 20 minutes. Collect plasma and keep at -20°C until assay. Thaw the sample on the day of the assay, vortex and centrifuge it at 1,600 g for 20 minutes to eliminate the fibrin.

No prior extraction procedure is necessary to measure Histamine in plasma samples. If necessary, plasma samples may be diluted in Histamine EIA buffer before derivatization (see below).

Urine or culture supernatants

Collect samples in polypropylene tubes. Store the samples at -20°C until assay. No prior extraction is necessary to measure Histamine in such samples.

Liquid or solid biological samples

> For solid samples, we recommend addition of $HClO_4$ which will precipitate the big proteins, while Histamine will remain in solution. Samples must be extracted at room temperature with 0.1M Perchloric Acid final concentration (10µL/mg of tissue).

Homogenize and then centrifuge at 10,000 g for 5 minutes. Collect the supernatant.

> Liquid samples can be stored at -20°C just after collection in polypropylene vials. Before assaying, you have to filter liquid samples through a 0.22 µm filter in a tube containing $HClO_4$ 1M to get a final $HClO_4$ concentration of 0.1 M. (for example: your tube must contain 20 µL $HClO_4$ 1M for 200 µL of sample).

Homogenize and then centrifuge at 10,000g for 5 minutes. Collect the supernatant.

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.

If you want to use the kit in two times, we provide one additional vial of Standard, one of Derivatization Reagent, 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.

Histamine EIA Buffer

Reconstitute the vial #A07890 with 25 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.

Histamine Standard

The Assay Buffer used to prepare the standards depends on the sample to be assayed:

- for plasma or urine samples, prepare the Standards using the Histamine EIA Buffer,
- for culture supernatant samples, prepare the Standards using the same culture medium as for the sample,
- for extracted liquid or solid samples, prepare the Standards in 0.1M $HClO_4$.

Reconstitute one Histamine Standard vial #A06890 with 900 µL of Assay Buffer. Mix carefully 3 times with the pipet tip.

The obtained solution, called S0, has a concentration of 500 nM.

Take 8 polypropylene tubes (for the eight standards S1 to S8) and prepare the standards by serial dilutions as follow:

Standard	Volume of Standard	Volume of Assay Buffer	Standard concentration nM
S1	100 µL of S0	900 µL	50 nM
S2	500 µL of S1	500 µL	25 nM
S3	500 µL of S2	500 µL	12.5 nM
S4	500 µL of S3	500 µL	6.25 nM
S5	500 µL of S4	500 µL	3.13 nM
S6	500 µL of S5	500 µL	1.56 nM
S7	500 µL of S6	500 µL	0.78 nM
S8	500 µL of S7	500 µL	0.39 nM

Stability at +4°C: 24 hours.

▷ Histamine Quality Control

Reconstitute one Histamine Quality Control vial # A10890, with 900 µL of assay medium, as for the standard. Then dilute 100 µL of QC in 900 µL of assay medium. The final concentration of this QC is labelled on the vial.
Stability at 4°C: 24 hours.

Derivatization reagent

Before use, reconstitute the vial #A15890 with 1 mL of N-N-dimethylformamide (DMF). The kit was validated with fresh DMF; if not available, one can use 1 mL DMSO. Vortex the contents until completely dissolved. This reagent can not be stored. Eliminate the remaining volume.

Histamine-AChE Tracer

Reconstitute the Tracer vial #A04890 with 10 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.

Wash Buffer

Dilute 2 mL of the concentrated Wash buffer #A17000 with 800 mL UltraPure water. Add 400 µL of tween 20 #A12000. Use a magnetic stirrer to mix the contents.

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 contents should be thoroughly mixed.

Stability at 4°C and in the dark: 24 hours.

Assay procedure

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

Before derivatization, highly concentrated samples may be diluted in Histamine EIA buffer (plasma, urine) or in assay medium (cell culture medium, $HClO_4$, ...).

The assay procedure depends of the sample to be assayed.

Sample derivatization

Histamine EIA buffer, plasma, urine, culture supernatant:

> In a polypropylene tube, distribute:
  - 200 µL of standard, quality control or sample
  - 50 µL of derivatization buffer

> In two polypropylene tubes that will allow evaluation of maximum binding (B0), distribute:
  - 200 µL of assay medium
  - 50 µL of derivatization buffer

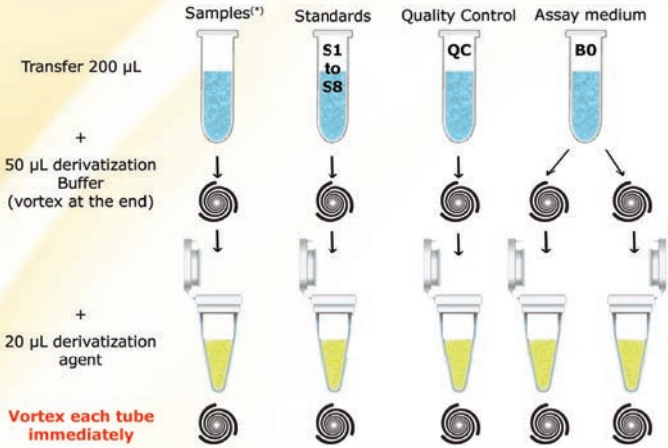
  Vortex all the tubes. Add 20 µL of the derivatization reagent to each polypropylene tube and **vortex each tube immediately**.

Liquid or solid biological sample :

> In a polypropylene tube, distribute:
  - 200 µL of standard, quality control or sample
  - 20 µL of 1.5M NaOH
  - 50 µL of derivatization buffer

> In two polypropylene tubes that will allow evaluation of maximum binding (B0), distribute:
  - 200 µL of assay medium
  - 20 µL of 1.5M NaOH
  - 50 µL of derivatization buffer

  Vortex all the tubes. Add 20 µL of the derivatization reagent to each polypropylene tube and **vortex each tube immediately**.



(*) $HClO_4$ extracted supernatant from liquid / solid samples should be neutralised with 20 µL of NaOH 1.5M

Plate preparation

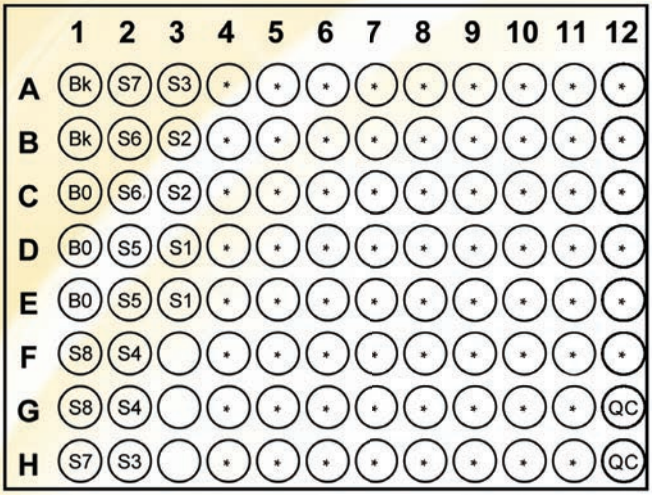
Prepare the Wash Buffer as indicated in the reagent preparation section.
Open the plate packet and select the sufficient strips for your assay and place the unused strips back in the packet, stored at 4°C.
Stability at +4°C: 1 month.

Rinse each well 5 times with 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
S1-S8: Standards 1-8
*: Samples or Quality Controls

Pipetting the reagents

All samples and reagents must reach room temperature prior to performing the assay. Use different tips to pipette buffer, standard, sample, tracer 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.

> **Assay medium**
Dispense 100 µL to Maximum Binding (B0) wells.

> **Histamine standard**
Dispense 100 µL of each of the eight derivatized 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.

> **Histamine Quality Control and Samples**
Dispense 100 µL in duplicate to appropriate wells.

> **Histamine AChE Tracer**
Dispense 100 µL to each well, **except** Blank (Bk) wells.

▷ Incubating the plate

Cover the plate with the cover sheet and incubate 24 hours at +4°C.

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 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 200µL of Ellman's Reagent to each well. Cover the plate with an aluminum sheet and incubate in the dark at room temperature on 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 0.2-0.8 A.U. (blank subtracted).

Enzyme Immunoassay Protocol (volumes are in µL)			
Steps	Blank	Maximum binding	Standard, QC & sample
Derivatization	-	200 µL of assay medium	200 µL of standard, QC or sample
	-	20 µL of 1.5M NaOH if assay medium is $HClO_4$	
	-	50 µL of derivatization buffer	
	Vortex all tubes		
	-	20 µL of derivatization agent	
Wash the plate 5 times			
Distribution of reagents	-	100 µL of derivatised solution	
	-	100 µL of Tracer	
Cover plate, incubate at +4°C for 24 hours			
Wash strips 5 times & remove the liquid from the wells			
Developing	200 µL of Ellman's Reagent		
Incubate with an orbital shaker in the dark at room temperature			
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 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.
- For each standard, using a semi-log graph paper, plot the B/B0 (%) on y axis versus the concentration 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 fall within the linear range of the standard curve (usually 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.
- Samples which concentration determined on standard curve is greater than 50 nM should be re-assayed after appropriate dilution in Assay Buffer prior to the derivatization step.
- Most plate readers are supplied with 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 +/- 25% of the expected concentration (see the label of QC vial).

Acceptable range

- B0 absorbance > 200 mA.U.
- IC50: 3,0 - 4,5 nM
- QC sample: ± 25% of the expected concentration (see the label of QC vial)

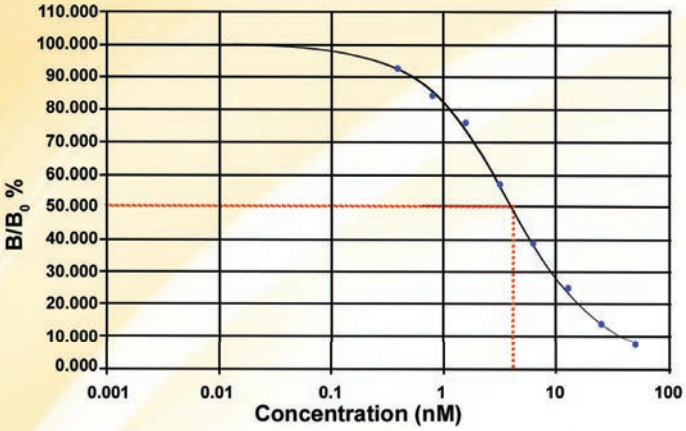
Typical results

The following data are for demonstration purposes only. Your data may be different but still be correct.

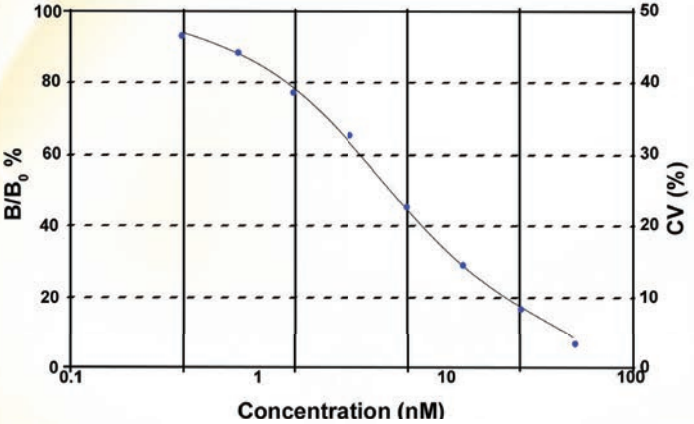
These data were obtained using all reagents as supplied in this kit under the following conditions: 1 hour developping at 20°C, reading at 414 nm. A 4-parameter curve fitting was used to determine the concentrations.

Standard or QC	Histamine EIA buffer		Cell culture medium (RPMI)		Perchloric acid ($HClO_4$)	
	mA.U.	B/B0 ()	mA.U.	B/B0 ()	mA.U.	B/B0 ()
B0	515	100,0	514	100,0	321	100,0
50 nM	30	5,8	45	8,8	21	6,5
25 nM	65	12,6	83	16,1	52	16,2
12.5 nM	124	24,1	148	28,8	92	28,7
6.25 nM	215	41,7	231	44,9	145	45,2
3.13 nM	329	63,9	323	62,8	210	65,4
1.56 nM	446	86,6	399	77,6	248	77,3
0.78 nM	499	96,9	414	80,5	285	88,8
0.39 nM	515	100,0	453	88,1	300	93,5
QC 4 nM	249	48,3	-	48,4	-	77,6

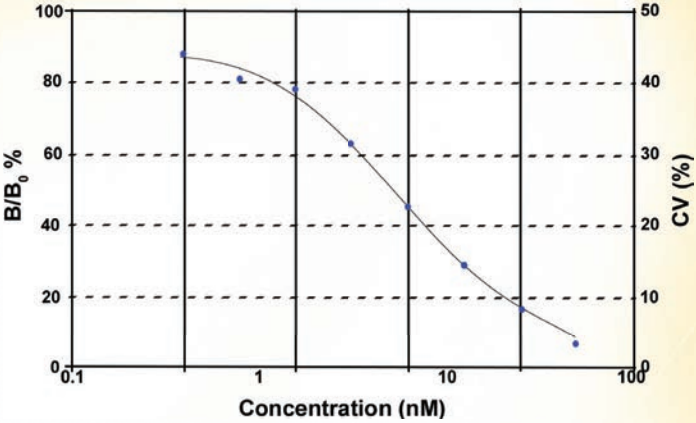
Typical Histamine standard curve in EIA Buffer



Standard curve in perchloric acid ($HClO_4$)



Standard curve in cell culture medium (RPMI)



Assay validation and characteristics

For additional information regarding the validation of immunoassay for protein biomarkers in biological samples, please refer to bibliography ***[4, 5]***.

> The **Limit of Detection** (LOD) calculated as the concentration of Histamine corresponding to the B0 average minus three standard deviation is 0.5 nM.

> **Intra & Inter-assay variation**

	Plasma QC (2.2 nM)	Plasma QC (8.6 nM)	Plasma QC (33.6 nM)
Mean value	-	7.8	32.6
Number of values	30	30	30
Intra-assay coeff. of variation (%)	13.6	7.4	15.1
Inter-assay coeff. of variation (%)	19.4	8.0	15.8
Recovery (%)	-	90.7	97.1
Confidence interval	-	90.71 ± 2.74	97.08 ± 5.78

	Buffer QC (1 nM)	Buffer QC (5 nM)	Buffer QC (30 nM)
Mean value	1.2	5.2	32.8
Number of values	30	30	30
Intra-assay coeff. of variation (%)	16.3	9.8	21.4
Inter-assay coeff. of variation (%)	28.9	11.6	21.4
Recovery (%)	123.2	103.4	109.5
Confidence interval	123.15 ± 13.39	103.41 ± 4.51	109.47 ± 8.80

	Cell culture medium QC (5 nM)	$HClO_4$ QC (5 nM)
Mean value	4.58	5.47
Number of values	20	20
Intra-assay coeff. of variation (%)	7.07%	6.43%
Inter-assay coeff. of variation (%)	5.20%	3.97%
Recovery (%)	91.60%	109.40%

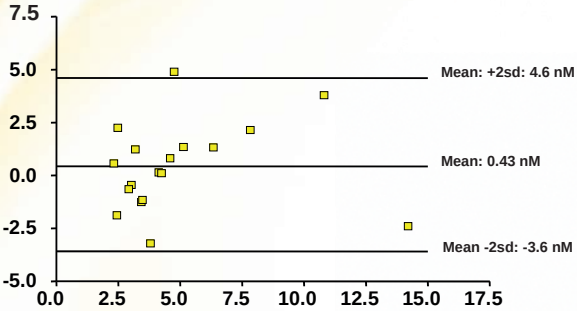
> Dilution test

Day	Dilution factor	Histamine measured	Corrected concentrations	Recovery (%)	Mean
1	1/1	30.79	30.79	94.39	136.41
	1/5	9.40	47.00	144.08	
	1/10	5.08	50.80	155.73	
	1/20	2.47	49.40	151.44	
2	1/1	32.15	32.15	98.56	113.65
	1/5	8.59	43.00	131.82	
	1/10	3.45	34.56	105.95	
	1/20	1.92	38.58	118.27	
3	1/1	31.94	31.94	97.92	130.08
	1/5	8.77	43.83	134.37	
	1/10	4.43	44.31	135.84	
	1/20	2.48	49.65	152.21	
4	1/1	26.56	26.56	81.42	115.24
	1/5	8.17	40.85	125.23	
	1/10	3.86	38.58	118.27	
	1/20	2.22	44.38	136.05	
5	1/1	22.15	22.15	67.90	107.72
	1/5	7.10	35.50	108.83	
	1/10	3.75	37.50	114.96	
	1/20	2.27	45.40	139.18	

> Recovery test

	Histamine added (nM)	Histamine measured (nM)	Recovery (%)
Histamine EIA buffer	5	5.2	103
Cell culture medium	5	5.04	101
Cell culture medium & additives	5	4.58	91.6
$HClO_4$	5	5.47	109

> Comparison with a reference method on 18 samples



> Stability test (freezing / thawing)

	Plasma QC1		Plasma QC2	
	32.6 nM	Recovery (%)	7.8 nM	Recovery (%)
1 cycle	26.69	81.9	8.49	108.9
2 cycles	29.42	90.3	9.63	123.5
3 cycles	30.76	94.4	8.22	105.4
4 cycles	24.26	74.4	6.73	86.3
5 cycles	-	-	7.78	99.7
Mean	27.78	-	8.17	-
Standard deviation	2.90	-	1.06	-
CV (%)	10.42	-	12.93	-

	Buffer QC1		Buffer QC2		Buffer QC3	
	30 nM	Recovery (%)	5 nM	Recovery (%)	1 nM	Recovery (%)
1 cycle	36.67	122.2	6.88	137.6	1.36	136.0
2 cycles	29.63	98.8	4.17	83.4	1.29	129.0
3 cycles	34.30	114.3	5.62	112.4	1.83	183.0
4 cycles	29.83	99.4	5.69	113.8	1.31	131.0
5 cycles	30.17	100.6	6.94	138.8	1.84	184.0
Mean	32.12	-	5.86	-	1.53	
Standard deviation	3.19	-	1.13	-	0.28	
CV (%)	9.93	-	19.6	-	18.56	

> Cross-reactivity

Histamine	100%
Histidine	<0,01%
1-methylHistamine	0.01%
3-methylHistamine	0.038%
Serotonin	<0.01%

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,
- DMF used for the derivatisation was not fresh.

> **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.

> **IC_{50} 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 (except plasma samples).

> **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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