DHN-MA LIPID PEROXIDATION

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

DHN-MA Lipid Peroxidation Enzyme Immunoassay kit #A05033.96 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

#A11033
Version: 0217

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

This kit contains:

Designation	Colour of cap	Item #	Quantity per kit	Form
A covered 96 well Microtiter plate, pre-coated with mouse anti-rabbit IgG	Blister with zip	A08100.1 ea	1	-
DHN-MA Tracer	Green	A04033.100 dtn	1	Lyophilized
DHN-MA Antiserum	red	A03033.100 dtn	1	Lyophilized
DHN-MA Standard	Blue with red septum	A06033.1 ea	2	Lyophilized
DHN-MA Quality Control	Green with red septum	A10033.1 ea	2	Lyophilized
EIA Buffer	Blue	A07000.1 ea	1	Lyophilized
Wash buffer concentrated 400x	Silver	A17000.1 ea	1	Liquid
Tween 20	Transparent	A12000.1 ea	1	Liquid
Ellman's reagent	Black with red septum	A09000_50.100 dtn	2	Lyophilized
Technical booklet	-	A11033	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.

DHN-MA and lipid peroxidation

Reactive Oxygene Species (ROS) play an important role in pathogenicity of several diseases (cardiovascular diseases such as atherosclerosis, cerebral or heart ischemia-reperfusion injury, neurodegenerative diseases, diabetes, inflammation and cancer) but are also involved in cell signaling. Consequently there is an increasing need in assays to monitor those biomarkers.

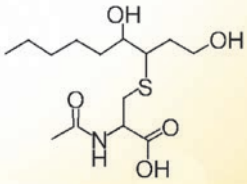
For decade, 8-Isoprostane (8-isoPGF2α, 8-isoprostaglandin F2 α, 8-epiPGF2α) has been used as the biomarker of lipid peroxidation. This biomarker is difficult to measure and needs an extraction whatever the method used. Also very popular are beta-cleavage products of polyunsaturated fatty acids (PUFA), such as alkanes, ketones or aldehydes.

Two well-known aldehydes formed during the lipid peroxidation process, namely malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE), have already been used as lipid peroxidation biomarkers for decades***[4]***.
MDA assays using thiobarbituric reaction are now named TBARS assay (ThioBarbituric Acid Reactive Substances assay) due to the lack of specificity of the reaction.
Measurement of HNE and protein/HNE adducts in tissues makes HNE more attractive in the field of clinical and experimental studies***[8, 9, 10]***.

Moreover, **1,4-dihydroxynonane mercapturic acid (DHN-MA)**, the major urinary metabolite of HNE, is present at a physiological level in rat and human urine. It was demonstrated that DHN-MA measured without extraction in rat urines treated with BrCCl3, which induces lipid peroxidation, mainly correlates with MDA and 8-Isoprostane, which were measured concomitantly***[7]***.

HNE and thus DHN-MA are generated from PUFA (alimentation), conclusions on lipid peroxidation should be considered accordingly***[5]***.

DHN-MA Structure



Principle of the assay

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

The complex rabbit antiserum - DHN-MA (free DHN-MA or Tracer) binds to the mouse monoclonal anti-rabbit antibody coated in the well.

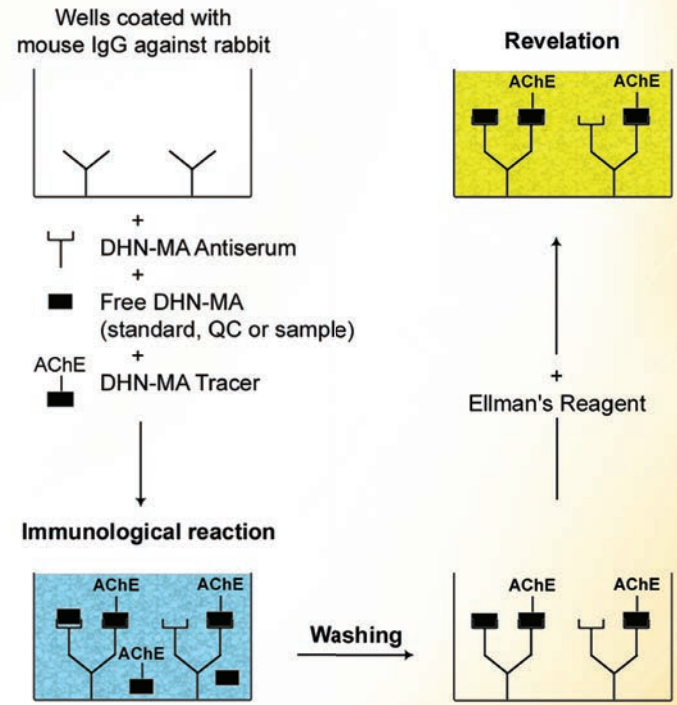
The plate is washed to remove any unbound reagent, and Ellman's Reagent (enzymatic substrate for AChE and chromogen) is added to the wells.

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 DHN-MA present in the well during the immunological incubation.

The kit has been developed and validated for urine samples. 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).

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 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

Urine samples collected in tubes are to be used immediately or stored at -20°C for long term storage.

Before use, urine samples must be centrifuged at 1500 g for 5 minutes at 4°C.

Samples are diluted in EIA buffer (recommended between 1/20 and 1/200). We advise to assay each sample in duplicate, in at least 3 dilutions. The urine sample minimal dilution is 1/5.

Avoid thawing samples more than three times.

Reagent preparation

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

DHN-MA Standard

Reconstitute the DHN-MA Standard vial #A06033 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 1000 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 pg/mL
S1	-	-	1000 pg/mL
S2	500 µL of S1	500 µL	500 pg/mL
S3	500 µL of S2	500 µL	250 pg/mL
S4	500 µL of S3	500 µL	125 pg/mL
S5	500 µL of S4	500 µL	62.5 pg/mL
S6	500 µL of S5	500 µL	31.3 pg/mL
S7	500 µL of S6	500 µL	15.7 pg/mL
S8	500 µL of S7	500 µL	7.8 pg/mL

Stability at 4°C: 48 hours

▷ Quality Control

Reconstitute the vial #A10033 with 1 mL of EIA Buffer. Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion.
Stability at +4°C: 48 hours

▷ DHN-MA Tracer

Reconstitute the vial #A04033 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

DHN-MA Antiserum

Reconstitute the vial #A03033 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.

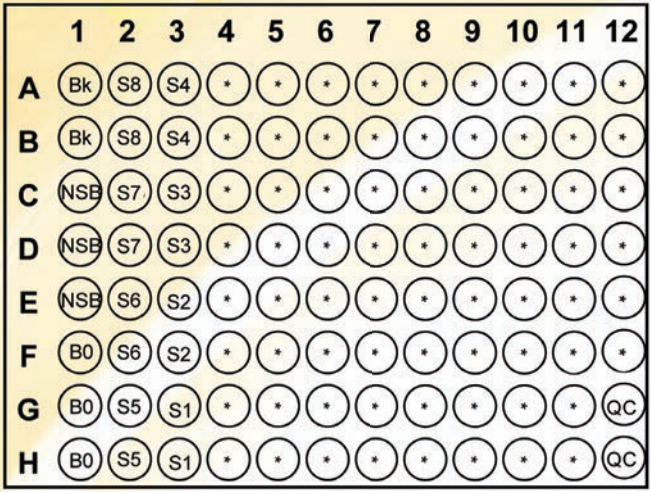
Rinse each well 4 times with the Wash Buffer 300 µL/well.

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

> **DHN-MA 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.

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

> **DHN-MA 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 over night 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 four 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 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 200 mAU 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 4 times & 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 well (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 1000 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

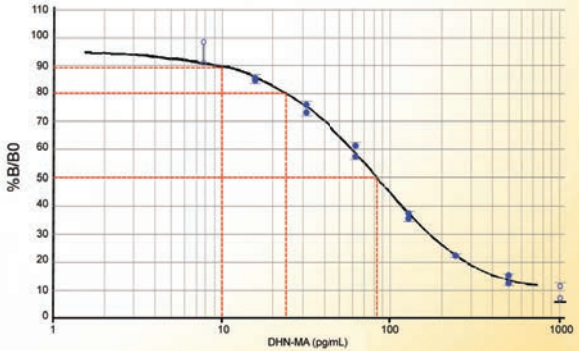
- B0 absorbance > 200 mAU blank subtracted in the conditions indicated above.
- NSB absorbance < 35 mAU
- IC50: 65 to 85 pg/mL (mean : 75 pg/mL).
- QC sample: ± 25% of the expected concentration (see the label of QC vial).

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: 120 minutes developing at +20°C, reading at 414 nm. A 5-parameter curve-fitting was used to determine the concentrations.

	DHN-MA pg/mL	mAU	B/B0 (%)
Standard S1	1000	41	8.9
Standard S2	500	63	13.7
Standard S3	250	102	22.4
Standard S4	125	172	37.7
Standard S5	62.5	270	59.2
Standard S6	31.3	342	74.8
Standard S7	15.7	392	85.8
Standard S8	7.8	433	94.8
B0	0	456	100

Typical DHN-MA standard curve



Assay validation and characteristics

The Enzyme Immunoassay of DHN-MA has been validated for its use in human urine.

For additional information regarding the validation of immunoassay for protein biomarkers in biological samples, please refer to bibliography ***[11, 12]***.

> The **Limit of Detection (LOD)** of DHN-MA corresponding to the B0 average minus three standard deviations is around 10 pg/mL.

> The **IC50** is the concentration in DHN-MA corresponding to 50 % of the maximum Binding is around 75 pg/mL.

> **Intra-assay variation** in EIA Buffer

QC level	QC1	QC2	QC3	QC4
QC dilution (1/x)	200	100	50	20
Mean of calculated concentration (pg/mL)	30.7	67.6	137	340
Mean concentration x dilution (pg/mL)	6133	6762	6842	6800
CV%	12.1%	6.6%	2.7%	2.7%

> **Inter-assay variation** in EIA Buffer

QC level	QC1	QC2	QC3	QC4
QC dilution (1/x)	200	100	50	20
Mean of calculated concentration (pg/mL)	31.9	60.8	131	332
Mean concentration x dilution (pg/mL)	6370	6083	6567	6633
CV%	17.9%	16.2%	17.7%	13.2%

Due to the endogenous presence of DHN-MA in urine, the intra-assay and inter-assay variations were performed on a pool of human urines at four dilutions (1/20, 1/50, 1/100 and 1/200) corresponding for each at a level of QC.

For the intra-assay validation, the number of replicates (n) is equal to 6 for each level of QC. The four validation levels were analysed along with the calibration curve for a unique experiment.

For the inter-assay validation, the number of replicates (n) is equal to 6 for each level of QC. The four validation levels were analysed along with the calibration curve for a total of 6 independent runs.

> Matrix variability

Matrix	1	2	3
Mean endogenous conc. x dil factor* (pg/mL)	2033	2193	2733
CV% (n=3)	17.6%	13.7%	10.2%
Mean spiked$ matrix con. x dil factor* (pg/mL)	3120	1260	1737
CV% (n=3)	2.6%	9.6%	10.5%
Back calculated spiked concentration$ (pg/mL)	1087	933	997
Back calculated spiked concentration$ (recovery)	8.67%	-6.67%	-0.33%

* Matrix dilution factor: 1/100

$ Matrix spiked with DHN-MA 1000 pg/mL

Three individual urine samples were spiked or not with DHN-MA 1000 pg/mL. Each sample (spiked or not) was evaluated 100 fold diluted in triplicate and analysed against a calibration curve.

> Cross-reactivity*[6]*

DHN-MA (racemic)	100%
MA(N-acetyl-cysteine)	< 0.1%
3-methylindole-MA	< 0.1%
4-HNE hemiacetal-MA	< 0.1%
HNA-lactone-MA	< 0.1%
HNA-MA	30%
butan-1-ol-MA	< 0.1%
1-hexanol-MA	< 0.1%
1-nonanol-MA	< 0.1%
DHN	< 0.1%
HNA	7%
1,4-dihydroxyoctane MA	< 0.1%
1,4-hydroxyundecane MA	< 0.1%
1,3,4-trihydroxynonane MA	< 0.1%
1,4-dihydroxyhexane MA	0.6%

> Linearity

Matrix	Endogenous calculated conc. (pg/mL)	Spiked DHN-MA (pg/mL)	Dilution (1/x)	Mean calculated conc. (pg/mL) n=3	Mean conc. X dil (pg/mL) n=3	CV%	Back calculated spiked conc. (pg/mL)	Back calculated spiked conc. %RE
1	7 096	300 000	50	ND	ND	ND	ND	ND
			100	ND	ND	ND	ND	ND
			200	ND	ND	ND	ND	ND
			500	504	252 167	4,89%	245 071	-18,3%
			1000	239	239 333	3,60%	232 237	-22,6%
			2000	123	246 000	5,86%	238 904	-20,4%
2	5 432	300 000	50	ND	ND	ND	ND	ND
			100	ND	ND	ND	ND	ND
			200	1 260	252 067	3,39%	246 635	-17,8%
			500	548	274 167	9,30%	268 735	-10,4%
			1000	261	260 667	15,70 %	255 235	-14,9%
			2000	133	266 667	5,43%	261 235	-12,9%

ND: outside range of standard curve

Two individual human urine samples were spiked or not with DHN-MA 300 ng/mL.
Each sample (spiked or not) was evaluated at 6 serial dilutions in order to be detected for at least 3 dilutions in the range of the standard curve.
Each dilution was tested in triplicate and analysed against a calibration curve.

> Parallellism

Matrix	Dilution factor (1/x)	Back calculated DHN-MA (pg/mL)	DHN-MA corrected dilution factor (pg/mL)	%CV
1	5	488	2440	15.1%
	10	253	2530	
	20	108	2160	
	50	35.6	1780	
	100	ND	ND	
	200	ND	ND	
	5	605	3025	18.8%
	10	248	2480	
	20	125	2500	
	50	37.7	1885	
	100	ND	ND	
	200	ND	ND	
	5	661	3305	19.8%
	10	263	2630	
	20	107	2140	
	50	46.2	2310	
	100	ND	ND	
	200	ND	ND	

ND: outside range of standard curve

Matrix	Dilution factor (1/x)	Back calculated DHN-MA (pg/mL)	DHN-MA corrected dilution factor (pg/mL)	%CV
2	5	ND	ND	11.7%
	10	586	5860	
	20	318	6360	
	50	137	6850	
	100	69.1	6910	
	200	40	8000	
	5	1305	6525	4.8%
	10	700	7000	
	20	326	6520	
	50	147	7350	
	100	71.1	7110	
	200	34.9	6980	
	5	ND	ND	5.8%
	10	659	6590	
	20	326	6520	
	50	148	7400	
	100	72.9	7290	
	200	35.3	7060	

ND: outside range of standard curve

Matrix	Dilution factor (1/x)	Back calculated DHN-MA (pg/mL)	DHN-MA corrected dilution factor (pg/mL)	%CV
3	5	360	1800	1.5%
	10	177	1770	
	20	91.1	1822	
	50	ND	ND	
	100	ND	ND	
	200	ND	ND	
	5	306	1530	25.1%
	10	160	1600	
	20	82.3	1646	
	50	50	2500	
	100	ND	ND	
	200	ND	ND	
	5	352	1760	7.7%
	10	182	1820	
	20	80.2	1604	
	50	38.7	1935	
	100	ND	ND	
	200	ND	ND	

ND: outside range of standard curve

Three individual human urine samples were diluted between 1:5 and 1:200 by serial dilution in order to be detected for at least 3 dilutions in the range of the standard curve.
Each dilution was tested in triplicate and analysed against a calibration curve.

> **Stability tests** (freezing, thawing and 24 hours at +4°C or 20-25°C)

QC level	QC condition	Mean concentration (pg/mL) n=3	CV%
LQ	Run QC	57.3	1.7%
	Freeze stability (3 cycles)	67.1	8.0%
	24h at RT stability	58.9	0.6%
	24h at +4°C stability	53.2	3.7%
HQ	Run QC	345.3	7.4%
	Freeze stability (3 cycles)	358.7	1.6%
	24h at RT stability	345.7	3.4%
	24h at +4°C stability	330.3	1.3%

The stability tests were performed on a pool of human urines at two dilutions (1/50 and 1/200) corresponding to Low QC and High QC respectively.

QCs were prepared, frozen at -20°C, and then stored 24h at room temperature or +4°C, or frozen/thawed 3 times. QCs were then analysed against the calibration curve.

> Long term stability tests

QC level	QC condition	Mean concentration (pg/mL) n=3	CV%
LQ	Run QC	62.5	0.96%
	1 month stability	60.2	4.07%
HQ	Run QC	333	0.35%
	1 month stability	335	2.37%

QC level	QC condition	Mean concentration (pg/mL) n=3	CV%
LQ	Run QC	68.0	7.39%
	3 months stability	72.2	14.30%
HQ	Run QC	353	15.80%
	3 months stability	326	1.69%

QC level	QC condition	Mean concentration (pg/mL) n=3	CV%
LQ	Run QC	66.9	4.01%
	6 months stability	73.8	3.42%
HQ	Run QC	324	2.94%
	6 months stability	359	10.70%

The long term stability tests were performed on a pool of human urines at two dilutions (1/50 and 1/200) corresponding to Low QC and High QC respectively.

QCs were prepared then frozen at -20°C before to be assayed at different times. QCs were then analysed against the calibration curve.

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).

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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.

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For further information, please send your request to
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