Instructions for Use

IBL HAMBURG
EN ISO 9001 certified by DNV

CatCombi ELISA

Cat.-No.	:	RE 592 42
Size	:	2 x 12 x 8 (Break Apart)
Storage	:	2 - 8 °C

Enzyme immunoassay (microtiter strips) for the quantitative determination of the catecholamines adrenalin and noradrenalin in plasma and urine

''For in-vitro diagnostic use only''

CatCombi (Adrenaline/Noradrenaline) Elisa Kit

For In Vitro Research Use Only

order through:

Research Diagnostics Inc
Pleasant Hill Road
Flanders NJ 07836

phone 973-584-7093
fax 973-584-0210
email: researchd@aol.com
web: http://www.researchd.com

Contents

1. Introduction

The IBL kit contains reagents for 96 determinations of adrenalin (epinephrine) in human plasma and urine as well as reagents for 96 determinations of noradrenalin (norepinephrine) in human plasma and urine.

The catecholamines adrenalin, noradrenalin and dopamine are synthesized in the adrenal medulla, the sympathetic nervous system and in the brain. As catecholamines and their metabolites metanephrine and normetanephrine are secreted in increasing amounts in a number of diseases, they may be used for diagnostic purposes.

In this context, diagnosis as well as the follow-up of tumor diseases of the nervous system are of special importance. This applies primarily to the pheochromocytoma, but also the neuroblastoma and the ganglioneuroma.

Malignant growth is described in 10% of pheochromocytomas. Furthermore, an increase of catecholamines and their metabolites metanephrine and normeta-nephrine can be observed in the carcinoid.

Because of the extraction step at the beginning of the assay, the customer is able to use all kinds of animal species material. It works for rats, rabbits, mice, goats, horses, dogs, pigs and others. The chemical structure of the catecholamines is identical in all animals.

2. Principle of the Test

The CatCombi ELISA kit provides materials for the quantitative measurement of derivatized adrenalin and noradrenalin in urine and plasma. In the sample preparation step, the catecholamines adrenalin and noradrenalin are specifically extracted by means of a cis-diol specific boronate-affinity gel and subsequently acylated.

After elution, there is an enzymatic derivatization step of the acylated adrenalin and acylated noradrenalin into N-acyl-Metanephrine and N-acyl-Normetanephrine.

In the ELISA the derivatized catecholamine is bound by a specific antibody immobilized on the surface of the microtiter plate. After a washing step, an antibody labelled with alkaline phosphatase (AP) binds to an additional epitope of the derivatized catecholamine to form a sandwich complex.

After washing off the unbound AP labelled antibodies an amplification system is used for detection.

For this sandwich ELISA the intensity of colour development is proportional to the catecholamine concentration in the sample and is measured at 490 nm. The measured ODs of the standards are used to generate a calibration curve against which the unknown samples are calculated.

3. Precautions

- The assay calibrators and controls are of human origin and have been tested and confirmed negative for HIV I/II, HBsAg and HCV by FDA approved procedures. All standards, however, should be treated as potential biohazards in use and for disposal.
- The assay reagents contain **sodium azide** which may be toxic if ingested. Sodium azide may react with copper and lead piping to form highly explosive salts. On disposal, flush with large quantities of water.
- The stop solution contains H_3PO_4. If it comes into contact with skin, wash thoroughly with water and seek medical attention. Since the H_3PO_4 used to terminate the colour reaction is corrosive, the instrumentation empoyed to dispense it should be thoroughly cleaned after use.
- This kit is for in vitro diagnostic use only.
- Never pipet by mouth and avoid contact of reagents and specimens with skin and mucous membranes. If contact occurs, wash with a germicidical soap and copious amounts of water.
- Do not smoke, eat, drink or apply cosmetics in areas where specimens or kit reagents are handled.

- Wear disposable latex gloves when handling specimens and reagents, and wash hands thoroughly afterwards. Microbial contamination of reagents or specimens may give false results.
- Handling should be in accordance with the procedures defined by an appropriate national biohazard safety guideline or regulation.
- Do not use reagents beyond expiry date.
- Chemicals and prepared or used reagents have to be treated as hazardous waste according the national biohazard safety guideline or regulation.
- In case of any damage of the kit or kit components, IBL have to be informed written, latest 1 week after receiving the kit.
- On request safety data sheets are at your´s disposal. The safety data sheets fits the demands of:
  - EU-Guideline 91/155 EWG
  - ISO-Standard 11014
  - ANSI-Standard
  - OSHA (US)

4. Storage and Stability

The CatCombi ELISA kit is shipped at ambient temperature and should be stored at 2-8°C by avoiding direct sunlight. Do not use components beyond the expiration date shown on the kit labels. Do not mix various lots of any kit component within an individual assay.
Once the foilbag of the coated microtiter strips has been broken, care should be taken to close it tightly again. The immuno reactivity of the coated microtiter strips is preserved up to the expiry date of the kit in the broken, but tightly closed bag.
Allow all reagents and required number of strips to reach room temperature prior to use.

5. Contents of the Testkit

5.1 **Acylation Reagent** — 1 vial
2.4 ml, ready for use,
contains dimethylformamide and ethanol.

5.2 **COMT** — 3 vials
lyophilized,
Catechol-O-methyltransferase from porcine liver with stabilizing additives.

5.3 **Coenzyme Solution** — 3 vials
1.25 ml, ready for use,
S-Adenosyl-L-methionine with stabilizing additives.

5.4 **Enzyme Buffer** — 2 vials
3 ml, ready for use, Tris-HCl buffer with stabilizers.

5.5 **Extraction Buffer** — 1 bottle
60 ml, pink, ready for use, contains stabilizers.

5.6 **Macrotiter Plate** — 2 plates
with 24 wells, coated with boronate-affinity gel.

5.7 **Noradrenalin Microtiter Strips** — 12 break apart strips
For the determination of Noradrenalin.
Blue colour,
each 8 wells, coated with antibody against N-acyl-normetanephrine for the determination of noradrenalin.

5.8 Adrenalin Microtiter Strips 12 break apart strips
For the determination of Adrenalin.
Red colour,
each 8 wells, coated with antibody against N-acyl-metanephrine for the determination of adrenalin.

5.9 Release Buffer 1 bottle
12 ml, yellow, ready for use.
0.1 M HCl with indicator.

5.10 Standard A - F
2.5 ml each, ready for use. 6 vials
The standards contain adrenalin and noradrenalin in the following concentrations:

Standard	**A**	**B**	**C**	**D**	**E**	**F**
Adrenalin ng/ml	0	1.5	5.0	15	50	150
Noradrenalin ng/ml	0	5.0	15	50	150	500

Conversion factors: Adrenalin µg/l x 5,458 = nmol/l
Noradrenalin µg/l x 5,911 = nmol/l
1 ng/ml = 1 µg/l

5.11 Control Urine, 1 and 2 2 vials
ready for use, 2.5 ml each.
For concentrations see quality control certificate.

5.12 Tris-Wash Buffer, Concentrate 1 bottle
50 ml,
Tris-HCl buffer with Tween and stabilizer;
dilute 1:10 with bidist. water.

5.13 Enzyme Conjugate, Concentrate — 2 vials

150 µl,
antibody conjugated with alkaline phosphatase
in Tris-HCl buffer with stabilizers,
dilute 1:101 with Wash Buffer.
NOTE: Prior to dilution with Wash Buffer
make sure that no liquid will remain in the stopper.

5.14 Amplification Reagent 1 — 2 vials

4.5 ml, ready to use,
containing ethanol dehydrogenase and diaphorase,
in buffer with stabilizers.

5.15 Amplification Reagent 2 — 2 vials

4.5 ml, ready to use,
containing iodonitrotetrazolium salt and ethanol
in buffer with stabilizers.

5.16 Amplification Reagent 3 — 2 vials

4.5 ml, ready to use,
containing NADPH
in buffer with stabilizers.

5.17 Amplification Stop Solution — 1 bottle

5 ml, ready for use,
contains 1 M phosphoric acid (H_3PO_4).
NOTICE: Avoid contact with this solution.
It may cause skin irritations and burns.

5.18 Adhesive Foil — 6 pieces

Material required but not provided

- Pipettes 10, 50, 100, 1000 µl Multipette Eppendorf or similar product. An eight-channel pipetting device such as Finnpipette (50-300 µl) is suitable for adding reagents to the wells. This avoids a drift during pipetting of substrate- and stop solution.
- Vortex mixer.
- Rotating mixer for thoroughly mixing of kit components.
- Orbital shaker, capable of shaking between 200-900 rpm, e. g. Microplate shaker EAS 2/4 from SLT: 500-900 rpm for **extraction procedure** and medium rotation (400 – 600 rpm) for incubation of **enzyme conjugate** and **substrate solution.**
- ELISA reader capable of reading absorbance at 490 nm.

6. Specimen Collection and Storage

EDTA Plasma: The usual precautions for venipuncture should be observed. Plasma may be stored at 4 - 8°C for up to 6 hours and should be frozen at -20°C or lower, if stored for longer periods. Do not use grossly hemolyzed, icteric or lipemic specimens. Repeated freezing and thawing must be avoided. Samples with concentrations higher than the highest standard have to be diluted with distilled water and reassayed again. For shipment of plasma samples they must be frozen.

Urine: The total volume of urine excreted during a 24-hours period should be collected and mixed in a single bottle containing 10 - 15 ml of 6 N hydrochloric acid as preservative. Avoid disposure to direct sun light. Urine samples which are not assayed immediately may be stored at -20°C or lower for at least 6 months. Samples with concentrations higher than the highest standard have to be diluted with 0.1 N HCl and reassayed again.

7. Preparation of Samples and Reagents

Preparation of Reagents

The contents of the kit can be divided into two separate runs. The volumes stated below are for one test procedure with 2x6 strips (96 determinations). If a larger number of strips is to be used, the volumes have to be changed accordingly.

7.1 Enzyme Solution (COMT)

The Enzyme Solution should be prepared (about 10 - 15 min.) just before use.

See Chapter Enzymatic Derivatization of extracted Standards, Controls, Plasma and Urines and Enzyme immunoassay. It is the same procedure for adrenalin and noradrenalin.

7.2 TRIS-Wash Buffer

20 ml of the Wash Buffer Concentrate have to be diluted 1:10 with bidistilled water up to 200 ml . This gives the ready for use Wash Buffer. Store at 2 - 8°C for up to 4 weeks.

7.3 Enzyme Conjugate

Dilute 110 µl of Enzyme Conjugate Concentrate in 11.0 ml of diluted Wash Buffer. Prepare freshly for each test run. Use only once.

7.4 Ready to use Amplification Reagent mixture (Substrate Solution)

Mix the Amplification Reagents 1, 2 and 3 in the ratio 1 : 1 : 1 immediately before use (no longer than 5 minutes). Example for 12 strips: each solution of 4.0 ml results in a total of 12.0 ml ready to use substrate solution. The mixture has to be nearly colourless. Use only once.

Sample Preparation – Extraction in the Macrotiterplate

Common sample preparation for Standards, Controls, Plasma and Urine

The yield of extraction is about 70%.

IMPORTANT: Instead of **10 µl Standards, Controls and Urines, 300 µl of Plasma** are extracted. Due to this the results for plasma have to be divided by 30 to obtain concentrations in ng/ml (see chapter Calculation of Results).

After removing assay reagents from the refrigerator, allow them to reach room temperature before pipetting. Unused reagents should be stored at 2 - 8°C.

The reagents provided with the kit are sufficient for single determinations for the sample preparation, i.g. extraction procedure, and duplicates for the ELISA. NOTE: For single determinations the individual kits Adrenalin ELISA, cat.-no. RE 59251 and Noradrenalin ELISA, cat.-no. RE 59261 can be used. Alternatively, additional reagents for single determinations are available separately upon request.

Step 1
Pipet **10 µl of Standards** (A - F), **10 µl of Kit-Controls** and each **of 10 µl of patient urine and 300 µl of patient plasma** into respective wells of the **macrotiter plate**. Add **300 µl of bidistilled water** to each well, except for the plasma samples.

Step 2
Pipet **1000 µl of Extraction Buffer (pink)** into each well. Cover the plate with adhesive foil and shake mix the plate for **30 min at room temperature** on an orbital shaker (rotation between 600 - 900 rpm; splashing of drops will not affect results).

Step 3
Remove adhesive foil and discard. Immediately empty the plate and eliminate residual fluids on cellulose pads.

Step 4
Pipet **2 ml of bidistilled water** into each well, cover the plate with new adhesive foil and shake mix for **5 min** on an orbital shaker at room temperature (rotation between 600 - 900 rpm; splashing of drops will not affect results).

Step 5
Remove adhesive foil and discard. Empty the plate and eliminate residual fluid on cellulose pads. A complete removal of fluid is necessary.

Step 6
Pipet **150 µl of Extraction Buffer** into each well. Immediately add **50 µl** of **Acylation Reagent** into each well and shake mix the plate without foil for **20 min** on an orbital shaker (medium rotation at **400 – 600 rpm).**

Step 7
Remove adhesive foil and discard. Empty the plate and eliminate residual fluid on cellulose pads. A complete removal of fluid is necessary.

Step 8
Pipet **2 ml of bidistilled water** into each well, cover the plate with new adhesive foil and shake mix for **5 min** on an orbital shaker at room temperature (rotation between **600 - 900 rpm**; splashing of drops will not affect results).

Step 9
Remove adhesive foil and discard. Empty the plate and eliminate residual fluid on cellulose pads. A complete removal of fluid is necessary.

Step 10
Pipet **250 µl of Release Buffer (yellow)** into each well and shake mix for **30 min** on an orbital shaker at room temperature (medium rotation **400 – 600 rpm**, without adhesive foil).

***NOTE*: Should it not be possible to perform the ELISA immediately after the extraction procedure, it is opportune to *cover the macrotiterplate* with foil and store it at 2-8°C overnight.**

8. Assay Procedure – Enzyme immunoassay

Enzymatic Derivatization of extracted Standards, Controls, Plasma and Urines and Enzyme immunoassay

IMPORTANT: Do not mix **Noradrenalin-Microtiter Strips (blue color)** with **Adrenalin-Microtiter Strips (red color).** This will lead to erroneous results! After the addition of extracted standards, controls and patient samples into the wells of the adrenalin and subsequently noradrenalin microtiter plate (see 8.2), both plates can be run together.

A summary of some possible pitfalls (critical steps) in this ELISA kit is given in the chapter 11., Limitations of Use.

A. NORADRENALIN-ELISA

Preparation of Enzyme Solution

Dissolve kit component COMT in 1.25 ml of bidistilled water. Thereafter, pipet 1.25 ml of Coenzyme Solution followed by 1.25 ml of Enzyme Buffer to the COMT to give a final volume of 3.75 ml of Enzyme Solution and use only once.

IMPORTANT: Enzyme Solution should be prepared freshly prior to ELISA (no longer than 15 minutes before!).

If only an aliquot of the COMT solution is needed for the run please take the actual needed volume from the COMT vial and mix thoroughly with Coenzyme and Enzyme Buffer.

The rest of the reconstituted COMT solution must be frozen **immediately in aliquots** at -20°C **without Coenzyme Solution** and **without Enzyme Buffer.** The COMT solution is stable under this conditions for 1-2 months. The enzyme solution may be turbid.

8.1 Pipet **25 µl** of **Enzyme Solution** into the appropriate wells of the **Noradrenalin Microtiter Strips (blue color).**

When pipetting 10 µl volume from the Macrotiter plate to Microtiter Strips, it is recommended to hold the Macrotiter plate in a sloping position.

8.2 Pipet immediately **10 µl of extracted standards**, **controls and patient samples** into the appropriate wells of the **Noradrenalin Microtiter Strips.** *By using of a white pad and gently shaking of the microtiter plate by hand a pink color will be visible.*

8.3 Seal the microtiter plate with the adhesive foil and incubate for **120 min at room temperature on a shaker (rotation between 400 – 600 rpm**).

8.4 Remove adhesive foil and discard. Empty the plate and eliminate residual fluid on cellulose pads. A complete removal of fluid is necessary.

8.5 Wash each well three times with **Wash Buffer** (the use of a washer is recommended). Remove the Wash Buffer carefully. Invert plate and remove any remaining liquid by tapping on clean blotting paper. Use 250-300 µl of Wash Buffer.

Note: Sensitivity and precision of this assay is markedly influenced by the correct performance of the washing procedure.

8.6 Pipet **each 100 µl** of diluted (1:101) **Enzyme Conjugate** into the wells.

8.7 Seal with the adhesive foil and incubate for **90 min at room temperature** on an orbital shaker (rotation between 400-600 rpm).

8.8 Remove adhesive foil and discard. Immediately empty the plate and eliminate residual fluid on cellulose pads. A complete removal of fluid is necessary.

8.9 Wash each well 3 x with **Wash Buffer** (see above).

When pipetting the Substrate and the Stop Solution, the application of an 8-channel pipette is useful. Pipetting should be carried out every 10 seconds per strip with the repetitive technique (positive displacement; see manual of pipettes manufacturer and also chapter 11.) to avoid formation of air bubbles.

8.10 Pipet **100 µl of ready to use Amplification Reagent mixture (Substrate Solution)** into each well.

8.11 Incubate at room temperature for **15 ± 2 min without foil** on an orbital shaker (rotation between 400-600 rpm).

8.12 Stop the substrate reaction by adding **50 µl Stop Solution** into each well**.**

8.13 Read the optical density at **490 nm** (reference wave length 600-650 nm) with
a microtiter plate reader within 60 min. after stopping. No air bubbles should be visible.

NOTICE: The incubation time of the substrate reaction (15 ± 2 min) is a critical step and must be kept carefully. In case of prolongation of the incubation time there will be elevated optical densities (ODs) and the standards E and F will have nearly the same value.

B. ADRENALIN-ELISA

Preparation of Enzyme Solution

For preparation of Enzyme Solution please refer to Noradrenalin-ELISA.

8.1 Pipet **50 µl** of **Enzyme Solution** into the appropriate wells of the **Adrenalin Microtiter Strips (red color).**

When pipetting 50 µl volume from the Macrotiter Plate to Microtiter Strips, it is recommended to hold the Macrotiter Plate in a sloping position.

8.2 Pipet immediately **50 µl of extracted standards**, **controls and patient samples** into the appropriate wells of the **Adrenalin Microtiter Strips.** *When adding the extracts the wells change into pink color.*

8.3 Seal the microtiter plate with the adhesive foil and incubate for **120 min at room temperature on a shaker (rotation between 400 – 600 rpm**).

8.4 Remove adhesive foil and discard. Empty the plate and eliminate residual fluid on cellulose pads. A complete removal of fluid is necessary.

8.5 Wash each well three times with **Wash Buffer** (the use of a washer is recommended). Remove the Wash Buffer carefully. Invert plate and remove any remaining liquid by tapping on clean blotting paper. Use 250-300 µl of Wash Buffer.

Note: Sensitivity and precision of this assay is markedly influenced by the correct performance of the washing procedure.

8.6 Pipet **each 100 µl** of diluted (1:101) **Enzyme Conjugate** into the wells.

8.7 Seal with the adhesive foil and incubate for **90 min at room temperature** on an orbital shaker (rotation between 400-600 rpm).

8.8 Remove adhesive foil and discard. Immediately empty the plate and eliminate residual fluid on cellulose pads. A complete removal of fluid is necessary.

8.9 Wash each well 3 x with **Wash Buffer** (see above).

When pipetting the Substrate and the Stop Solution, the application of an 8 channel pipette is useful. Pipetting should be carried out every 10 seconds per strip with the repetitive technique (positive displacement, see manual of pipettes manufacturer and also chapter 11.) to avoid formation of air bubbles.

8.10 Pipet **100 µl of ready to use Amplification Reagent mixture (Substrate Solution)** into each well.

8.11 Incubate at room temperature for **15 ± 2 min without foil** on an orbital shaker (rotation between 400-600 rpm).

8.12 Stop the substrate reaction by adding **50 µl Stop Solution** into each well**.**

8.13 Read the optical density at **490 nm** (reference wave length 600-650 nm) with
a microtiter plate reader within 60 min after stopping. No air bubbles should be visible.

NOTICE: The incubation time of the substrate reaction (15 ± 2 min) is a critical step and must be kept carefully. In case of prolongation of the incubation time there will be elevated

optical densities (ODs) and the standards E and F will have nearly the same value.

9. Calculation of Results

The concentrations of the standards (abscissa, logarithmic) are plotted against their corresponding optical density (ordinate, linear) on a semilogarithmic graph paper.

Alternatively, the optical density of each standard and sample can be related to the optical density of the highest standard F, expressed as the ratio OD/OD max, and plotted on the ordinate.

The standard curve and the calculation of the concentrations in the samples can also be done by a computer method. Computer programs using cubic spline method or 4 PL (4 Parameter Logistics) may be used.

Calculation of urines and kit-controls:

The concentrations can be read directly from the standard curve.

Calculation of plasma:

Due to the pipetting volume of 300 µl for plasma in comparison with 10 µl for the standards, the results have to be divided by 30. For units in pg/ml please multiply by 1000.

Example plasma calculation:

The result obtained from the standard curve is 3 ng/ml. Multiplication with the factor 1000/30 results in a concentration of 100 pg/ml.

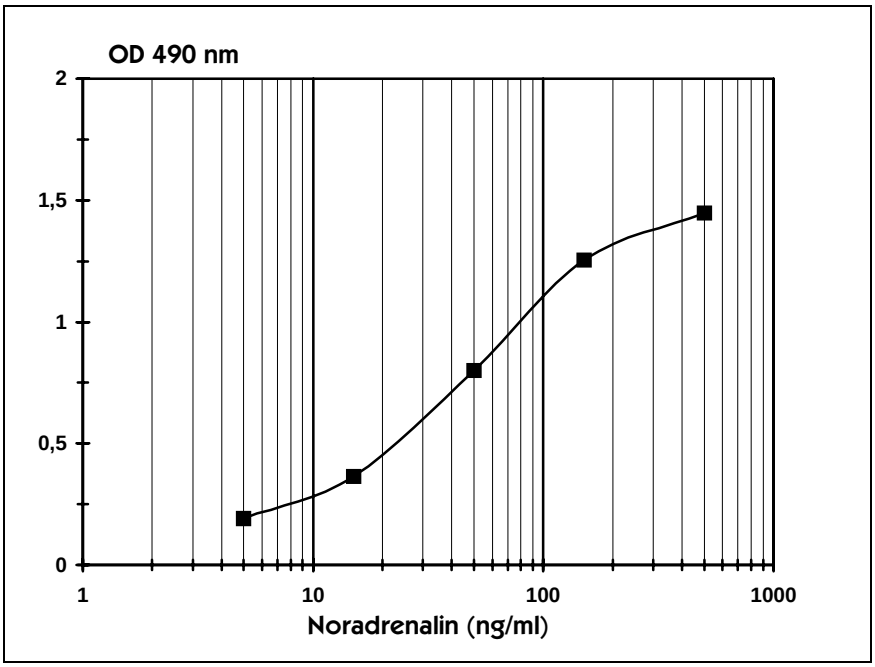
Typical example for Nordrenalin ELISA

Concentration (ng/ml)	OD 1	OD 2	Mean value OD	OD/OD max (%)	CV (%)
0.0	0.105	0.098	0.102	0.0	4.9
5.0	0.194	0.188	0.191	6.6	2.2
15	0.371	0.358	0.365	19.5	2.5
50	0.801	0.799	0.800	51.9	0.2
150	1.271	1.237	1.254	85.6	1.9
500	1.434	1.462	1.448	100	1.4

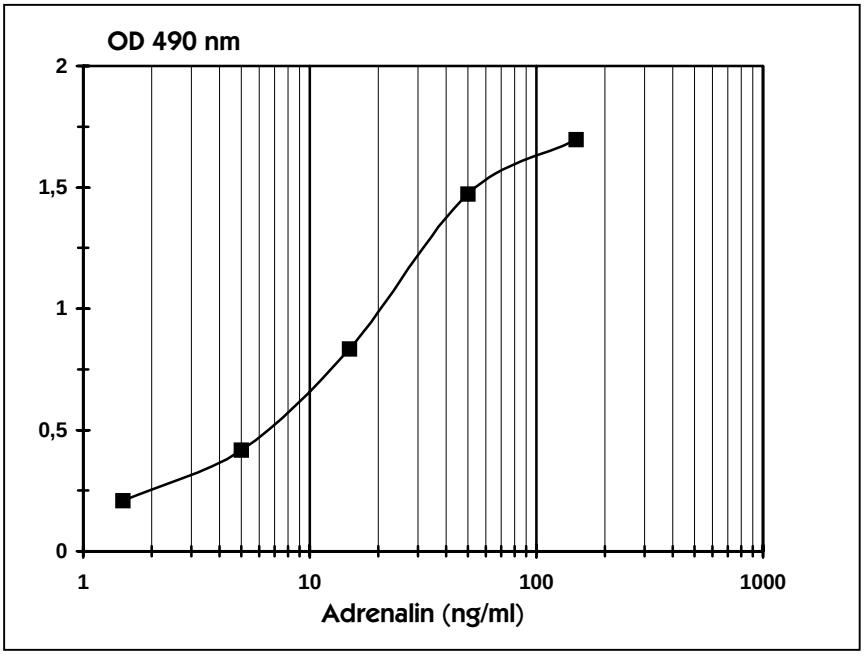
Typical example for Adrenalin ELISA

Concentration (ng/ml)	OD 1	OD 2	Mean value OD	OD/OD max (%)	CV (%)
0.0	0.122	0.118	0.120	0.0	2.4
1,5	0.204	0.213	0.209	5.6	3.1
5,0	0.413	0.422	0.418	18.9	1.5
15	0.848	0.815	0.832	45.1	2.8
50	1.484	1.460	1.472	85.8	1.2
150	1.701	1.692	1.697	100	0.4

Typical stand curve for the Noradrenalin ELISA



Typical standard curve for the Adrenalin ELISA



10. Assay Characteristics

10.1 NORADRENALIN

10.1.1 Expected values

A. In 24 h urine samples

It is recommended that each laboratory establishes its own range of normal noradrenalin values. All participants in the normal range study were apparently healthy subjects who were not taking any medications. The normal value range is assumed to be as 95%-percentile.

Noradrenalin:	up to 90 µg/d (535 nmol/d)

For conversion from µg/l to µg/d multiply the total volume of urine in litre excreted during 24 hours with the value obtained in µg/l (= ng/ml).

The conversion factor is: µg/d x 5.91 = nmol/d

B. In EDTA-Plasma

It is recommended that each laboratory establishes its own range of normal noradrenalin values. All participants in the normal range study were apparently healthy subjects who were not taking any medications. The normal value range is assumed to be as 95%-percentile.

Noradrenalin:	up to 600 pg/ml (3.55 nmol/l)

Note: The in vivo catecholamine release is influenced by several foods and drugs. Vitamin B, coffee and bananas as well as alpha-methyldopa, MAO inhibitors and medications related to hypertension should be discontinued for at least 72 hours prior to specimen collection.

10.1.2 Specificity-noradrenalin

The cross-reactivity of the antiserum has been measured against various compounds which were processed like patient samples:

Compound	Cross reactivity (%)
Noradrenalin	**100**
Normetanephrine	2
Adrenaline	0,46
Metanephrine	0,05
3 Methoxytyramine	<0,01
Dopamin	<0,01
Vanillic mandelic acid	<0,01
L-Tyrosine	<0,01
L-Dopa	<0,01
DL-Tryptophane	<0,01
DL-3-Methoxytyrosine	<0,01
4-Hydroxy 3 Methoxy-Phenylpyruvat acid	<0,01
4-Hydroxy 3 Methoxy-Phenyl glycol	<0,01
Caffeic acid	<0,005
Homovanillic acid	<0,005
Ferula acid	<0,005

10.1.3 Sensitivity-noradrenalin

The lowest detectable level that can be distinguished from the zero standard (defined as 2 x standard deviation of the zero calibrator) is 0.6 ng/ml for urine and 20 pg/ml for plasma.

10.1.4 Precision-noradrenalin

INTRA-assay variation
(all concentrations in ng/ml)

The intra-assay variation was measured with two different urines and two different plasma by 20-fold repetition.

	Mean	Standard Deviation	VK (%)	n
Urin 1	45.4	4.1	9.1	20
Urin 2	115	7.7	6.7	20
Plasma 1	0.305	0.020	6.5	20
Plasma 2	0.707	0.071	10.0	20

INTER-assay variation
(all concentrations in ng/ml)

The inter-assay variation was measured with three different urines and three different plasma by 20-fold repetition.

	Mean	Standard Deviation	VK (%)	n
Urin 1	21.5	2.45	11.4	20
Urin 2	44.7	4.88	10.9	20
Urin 3	102	12.2	12.1	20
Plasma 1	0.263	0.043	16.3	20
Plasma 2	0.527	0.086	16.4	20
Plasma 3	0.832	0.100	12.1	20

10.1.5 Linearity-noradrenalin

Patient urines were diluted with 0.1 M HCl before sample preparation and assayed. Patient plasma were diluted with distilled water before sample preparation and assayed. The calculated results are shown in pg/ml (related in % to the undiluted concentration) for plasma and in ng/ml for urine.

	Undiluted	1/2	1/4	1/8	1/16
Urine 1	255 (100%)	114 (89.5%)	60.9 (95%)	28.5 (89%)	13.7 (86%)
Urine 2	216 (100%)	101 (94%)	63.1 (117%)	27.9 (103%)	14.4 (107%)
Urine 3	183 (100%)	89.3 (98%)	48.9 (107%)	22.5 (98%)	11.5 (101%)
Plasma 1	5502 (100%)	2860 (104%)	979 (71%)	761 (111%)	401 (117%)
Plasma 2	5916 (100%)	2720 (92%)	1235 (83%)	653 (88%)	373 (101%)
Plasma 3	7336 (100%)	2782 (76%)	1305 (71%)	716 (78%)	401 (87%)

10.1.6 Recovery-noradrenalin

Three patient urines as well as three patient plasma samples were enriched with increasing amounts of noradrenalin and assayed (results for urine in ng/ml, für plasma in pg/ml).

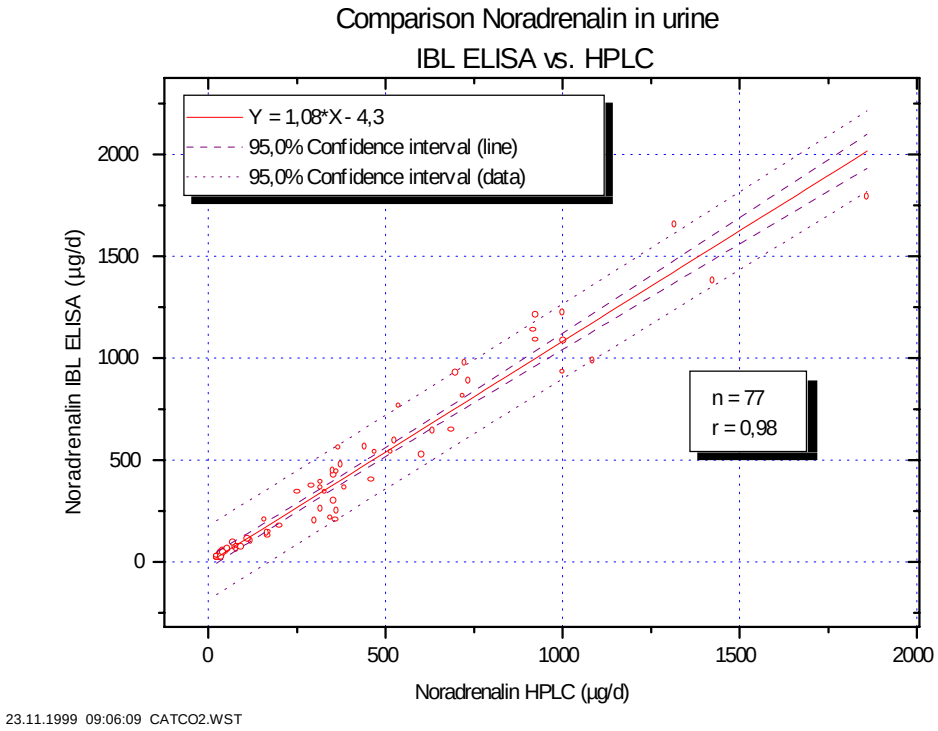
	Actual conc.	Amount added	Amount recovered	Amount expected	Recovery %
Urine 1	9.49	5	13.6	14.5	93.7
	9.49	15	21.4	24.5	87.5
	9.49	50	54.3	59.5	91.3
	9.49	150	139	159.5	87.6
Urine 2	15.4	5	18.5	20.4	90.7
	15.4	15	27.8	30.4	91.4
	15.4	50	62.3	65.4	95.3
	15.4	150	154	165	93.2
Urine 3	20.2	5	25.1	25.2	99.6
	20.2	15	35	35.2	99.4
	20.2	50	67	70.2	95.7
	20.2	150	155	170.2	91.2
Plasma 1	277	100	369	377	97.9
	277	300	437	577	75.7
	277	1000	1187	1277	93.0
	277	3000	3235	3277	98.7
Plasma 2	604	100	612	704	86.9
	604	300	786	904	86.9
	604	1000	1442	1604	98.9
	604	3000	3783	3604	105
Plasma 3	995	100	1007	1095	92.0
	995	300	1460	1295	112
	995	1000	2052	1995	102
	995	3000	4933	3995	123

10.1.7 Method Comparison Noradrenalin ELISA vs. HPLC

77 urine patient samples from the Quality Assurance Scheme UK NEQAS (Birmingham, UK) with noradrenalin values ranging from 17.4 to 1700 µg/d were assayed by the IBL ELISA procedure. The mean value of every single sample for all HPLC-methods was compared with the IBL ELISA result.

Linear regression gives the following statistics:

IBL ELISA = 1.08 HPLC – 4.3; r = 0.98 ; n = 77



10.2 ADRENALIN

10.2.1 Expected values

A. In 24 h urine samples

It is recommended that each laboratory establishes its own range of normal adrenalin values. All participants in the normal range study were apparently healthy subjects who were not taking any medications. The normal value range is assumed to be as 95%-percentile.

Aadrenalin: up to 20 µg/d (110 nmol/d)

For conversion from µg/l to µg/d multiply the total volume of urine in litre excreted during 24 hours with the value obtained in µg/l (= ng/ml).

The conversion factor is: µg/d x 5.46 = nmol/d

B. In EDTA-Plasma

It is recommended that each laboratory establishes its own range of normal adrenalin values. All participants in the normal range study were apparently healthy subjects who were not taking any medications. The normal value range is assumed to be as 95%-percentile.

Adrenalin: up to 125 pg/ml (0.69 nmol/l)

<u>Note:</u> The in vivo catecholamine release is influenced by several foods and drugs. Vitamin B, coffee and bananas as well as alpha-methyldopa, MAO inhibitors and medications related to hypertension should be discontinued for at least 72 hours prior to specimen collection.

10.2.2 Specificity-adrenalin

The cross reactivity of the antiserum has been measured against various compounds which were processed like patient samples:

	Cross reactivity(%)
Adrenalin	**100**
Metanephrine	2
Noradrenalin	0,13
DL 3-Methoxytyrosine	0,009
Normetanephrine	0,008
DL-3-Methoxytyramine	<0,005
Dopamine	<0,005
L-Dopa	<0,005
4-Hydroxy-3-methoxy-Phenylpyruvat acid	<0,005
4-Hydroxy-3-Methoxy Phenylglykole	<0,005
Vanillic mandelic acid	<0,005
L-Tyrosine	<0,005
DL-Tryptophane	<0,005
Caffeic acid	<0,005
Ferula acid	<0,005
Homovanillic acid	<0,005

10.2.3 Sensitivity-adrenalin

The lowest detectable level that can be distinguished from the zero standard (defined as 2 x standard deviation of the zero calibrator) is 0.3 ng/ml for urine and 10 pg/ml for plasma.

10.2.4 Precision-adrenalin

INTRA-assay variation
(all concentrations in ng/ml)

The intra-assay variation was measured with two different urines and two different plasma by 20-fold repetition.

	Mean	Standard Deviation	VK (%)	n
Urine 1	6.1	0.2	3.3	20
Urine 2	27.3	1.3	4.9	20
Plasma 1	0.159	0.007	4.2	20
Plasma 2	0.424	0.033	8.1	20

INTER-assay variation
(all concentrations in ng/ml)

The inter-assay variation was measured with three different urines and three different plasma by 20-fold repetition.

	Mean	Standard Deviation	VK (%)	n
Urine 1	5.2	0.82	15.8	20
Urine 2	7.9	0.77	9.7	20
Urine 3	25.7	4.07	15.8	20
Plasma 1	0.106	0.017	16.4	20
Plasma 2	0.187	0.029	15.5	20
Plasma 3	0.295	0.040	13.8	20

10.2.5 Linearity-adrenalin

Patient urines were diluted with 0.1 M HCl before sample preparation and assayed. Patient plasma were diluted with distilled water before sample preparation and assayed. The calculated results are shown in pg/ml (related in % to the undiluted concentration) for plasma and ng/ml for urine.

	Undiluted	1/2	1/4	1/8	1/16
Urine 1	126 (100%)	68.7 (109%)	28.3 (89%)	12.4 (79%)	6.3 (80%)
Urine 2	86.6 (100%)	42.2 (97%)	18.8 (87%)	8.8 (81%)	4.6 (85%)
Urine 3	57.4 (100%)	30.3 (105%)	14.7 (102%)	6.7 (94%)	3.6 (100%)
Plasma 1	3053 (100%)	1216 (80%)	531 (70%)	288 (76%)	192 (101%)
Plasma 2	2170 (100%)	906 (84%)	431 (80%)	250 (92%)	174 (128)
Plasma 3	2075 (100%)	1000 (96%)	506 (98%)	285 (110%)	156 (120%)

10.2.6 Recovery-adrenalin

Three patient urines as well as three patient plasma samples were enriched with increasing amounts of noradrenalin and assayed (results for urine in ng/ml, für plasma in pg/ml).

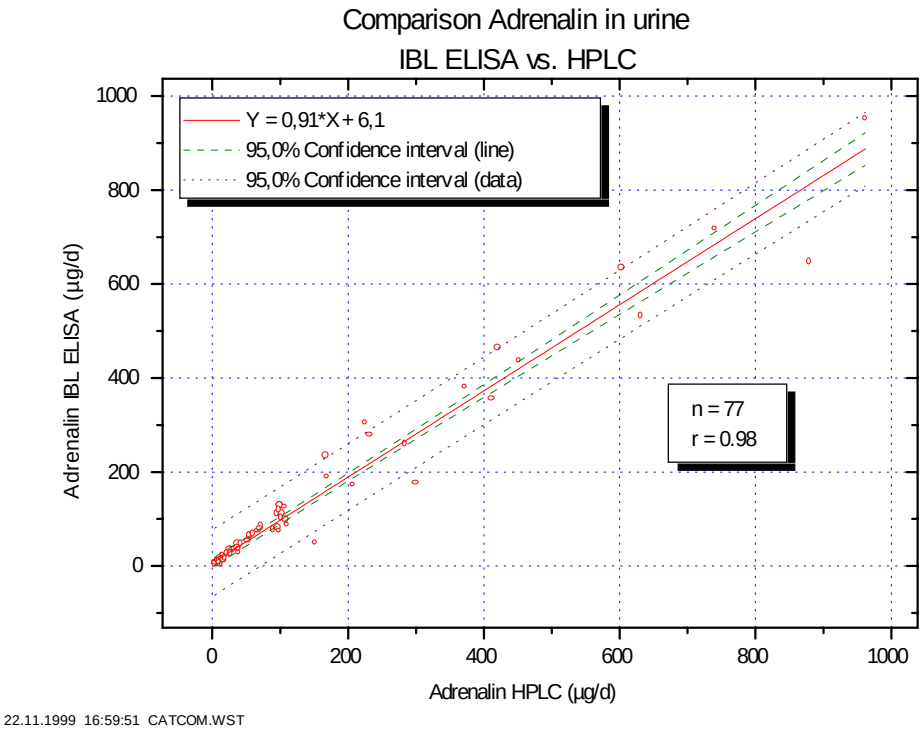
	Actual conc.	Amount added	Amount recovered	Amount expected	Recovery %
Urine 1	1.8	1.5	2.98	3.3	90.3
	1.8	5	5.95	6.8	87.5
	1.8	15	13.25	16.8	78.9
	1.8	50	48.27	51.8	96.5
Urine 2	2.17	1.5	3.59	3.67	97.8
	2.17	5	6.18	7.17	86.2
	2.17	15	14.2	17.17	82.8
	2.17	50	45.1	55.4	81.4
Urine 3	5.4	1.5	6.44	6.9	93.3
	5.4	5	9.94	10.4	95.6
	5.4	15	20.5	20.4	100.4
	5.4	50	58.7	55.4	105.9
Plasma 1	89	35	134	124	108
	89	116	177	205	86.3
	89	350	353	439	80.4
	89	1162	1212	1251	96.9
Plasma 2	173	35	203	208	97.6
	173	116	278	289	96.2
	173	350	476	523	91.0
	173	1162	1253	1335	93.9
Plasma 3	369	35	398	404	98.5
	369	116	475	485	97.9
	369	350	713	719	99.2
	369	1162	1272	1531	83.1

10.2.7 Method Comparison Adrenalin-ELISA vs. HPLC

77 urine patient samples from the Quality Assurance Scheme UK NEQAS (Birmingham, UK) with adrenalin values ranging from 4.4 to 980 µg/d were assayed by the IBL ELISA procedure. The mean value of every single sample for all HPLC-methods was compared with the IBL ELISA result.

Linear regression gives the following statistics:

IBL ELISA = 0.91 HPLC + 6.1; r = 0.98 ; n = 77



11. Limitations of Use

Reliable and reproducible results will be obtained when the assay procedure is carried out with a complete understanding of the package insert instructions and with adherence to good laboratory practice.

The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbances.

Samples with concentrations greater than the highest standard should be diluted and reassayed for correct values. As diluent for plasma we recommend to use distilled water and for urine 0.1 N HCl.

Like any analyte used for diagnostic purposes, results must be interpreted carefully with the clinical presentations and other supportive diagnostic tests.

Pitfalls (critical steps) in this ELISA procedure are:

- The sequence of pipetting steps has to be kept as given in the protocol: pipet at first the enzyme solution after this the extracted sample. If not kept in this sequence, the surface of the microtiter strips will be destroyed.
- Do not prepare the enzyme solution too early. The enzyme is unstable. The solution has to be prepared 10-15 minutes before its use.
- The ready to use amplification reagent mixture (substrate solution) has to be prepared immediately before its use. If this solution will be left at room temperature too long, a red color will develop.
- During pipetting of the substrate solution no air bubbles must be formed on the surface of the liquid. To avoid this, use the repetitive technique (positive displacement): *Depress the operating button all the way down to the second stop. Dip the tips just under the surface of the liquid in the reservoir (2-3 mm) and slowly release the operating button. Deliver the preset volume by gently depressing the operating button to the first stop. Some liquid will remain in the tip and should not be included in the delivery*. Air bubbles will lead to erroneous results (up to 20% higher or lower results). Air bubbles can be destroyed by using a tip.
- The substrate reaction is too long. Already after 18 minutes incubation time the ODs will increase dramatically and will result in the same extinction between standard E and F.

12. Quality control

It is recommended to use control samples according to state and federal regulations. The use of control samples is advised to assure the day to day validity of results. Use controls at both normal and pathological levels.

The controls and the corresponding results of the IBL QC-Laboratory are stated at the QC certificate added to the kit.

Employ appropriate statistical methods for analysing control values and trends. If the results of the assay does not comply with the established limits and repetition excludes errors in technique, patient sample should be considered invalid. Check the following areas:
Pipetting and timing devices, photometer, expiration dates of reagents, storage and incubation conditions, aspiration and washing methods

After checking the above mentioned items without finding any error contact your distributor or IBL directly.

13. Warranty

This test has been developed at the laboratories of IBL Hamburg.
Any modification of this test as well as exchange or mixture of any components from different lots might influence the results. In such cases there is no claim for damages.

14. References

1. Pomares FJ et al. Differences between sporadic and multiple endocrine neoplasia type 2A phaeochromocytoma. Clin. Endocrinol., **48**: 195-200 (1998)
2. Burton M. Pheochromocytoma. A triad of clinical manifestations marks this potentially fatal hypertensive disorder. AJN, **97**: 57 (1997)
3. McNeil AR et al. Urinary catecholamines and the diagnosis of phaeochromocytoma in Auckland. N.Z. Med. J., **110**: 331-333 (1997)
4. Lucon AM et al. Pheochromocytoma: Study of 50 cases. J. Urol., **157**: 1208-1212 (1997)
5. Peaston RT, Lennard TWJ, Lai LC. Overnight excretion of urinary catecholamines and metabolites in the detection of pheochromocytoma. J. Clin. Endocrinol. Metab., **80**: 1378-1384 (1996)
6. Weinkove C. Investigation and management of phaeochromocytoma. Proc. ACB National Meeting 1995: 19-21
7. Bouloux P-M and Fakeeh M. Investigation of phaeochromocytoma. Clin. Endocrin., **43**: 657-664 (1995)
8. Manger WM and Gifford, Jr. RW. Chap. 132: Pheochromocytoma: a clinical overview. In: Laragh JH, Brenner BM. Hypertension: Pathophysiology, Diagnosis, and Management, Second Edition. New York: Raven Press, 2225-2243 (1995)
9. Gerlo EAM and Sevens C. Urinary and plasma catecholamines and urinary catecholamine metabolites in pheochromocytoma: diagnostic value in 19 cases. Clin. Chem., **40**: 250-256 (1994)
10. Graham PE et al. Laboratory diagnosis of phaeochromocytoma: which analytes should we measure? Ann. Clin. Biochem., **30**: 129-134 (1993)
11. Smythe GA et al. Biochemical diagnosis of pheochromocytoma by simultaneous measurement of urinary excretion of epinephrine and norepinephrine. Clin. Chem., **38**: 486-492 (1992)

IBL HAMBURG	**CatCombi-ELISA** Short Instructions for use	**Cat.No.** **RE 592 42** Size: 12 x 8 Storage: 2- 8 °C

1. Preparation of Reagents (Volumes stated for 48 determinations each for adrenalin/noradrenalin.)

- **Enzyme Solution (COMT):** Dissolve the kit component **COMT** in **1.25 ml** of bidistilled water and mix the dissolved **COMT**. Then, pipet **1.25 ml** of **Coenzyme Solution** followed by **1.25 ml** of **Enzyme Buffer** to the mixed **COMT** vial to give a final volume of **3.75 ml** of **Enzyme Solution**. If necessary to use a greater volume than 3.75 ml, combine the contents of several vials and mix well before use. **Important**: Prepare freshly immediately before use!

- **Wash Buffer:** 20 ml of the Wash Buffer Concentrate have to be diluted with bidest. water to make up 200 ml. Store at 2-8° C.

- **Enzyme Conjugate:** Dilute 110 µl of Enzyme Conjugate Concentrate in 11.0 ml of ready for use Wash Buffer. Prepare freshly for each test run, use only once.

- **Ready to use Amplification Reagent-mixture**: Pipet 4.0 ml each of Amplification Reagenz 1,2 and 3; mix carefully. Prepare freshly for each test run, use only once.

2. Specimen Collection and Storage

Sample: spontaneous urine or 24-hour urine, collected in a bottle containing 10-15 ml of 6N HCl,
Storage: at –20 °C or lower for at least 6 month.
Sample: EDTA-Plasma,
Storage: up to 6 hours at 4-8°C, for longer period at –20°C or lower.

3. Assay Procedure

Allow reagents to reach room temperature. Unused reagents should be stored at 2-8°C.

A. Extraction (in Macrotiter Plate)

⇩	Pipet **10 µl** each **of Standards**, patient **urine** and **Controls**, pipet **300 µl of patient plasma**.
⇩	Add **300 µl of bidest. water** except to the wells filled with plasma.
⇩	Pipet **1000 µl of Extraktion Buffer**.
⌛	Cover and **shake** the plate for **30 min at room temperature** (600-900 rpm).
⇦	Empty plate and eliminate residual fluids.
⇩	Pipet **2 ml of bidest. water**, cover and **shake** for **5 min** (600-900 rpm).
⇦	Empty plate and eliminate residual fluids.
⇩	Pipet **150 µl of Extraction Buffer**.
⇩	Pipet **50 µl of Acylation Reagent**.
⌛	**Shake** plate uncovered for **20 min** (400-600 rpm).
⇦	Empty plate and eliminate residual fluids.
⇩	Pipet **2 ml of bidest. water**, cover and **shake** for **5 min** (600-900 rpm).
⇦	Empty plate and eliminate residual fluids.
⇩	Pipet **250 µl of Release Buffer**.
⌛	**Shake** plate uncovered for **30 min** (400-600 rpm).
Take extracts for immediate further procedure or cover plate with foil and put into refrigerator overnight.	

B. ELISA (in **red** Microtiter Plate for Adrenalin and **blue** Microtiter Plate for Noradrenalin)

		Noradrenalin	Adrenalin
⇩	Pipet **Enzyme Solution (COMT)**.	25 µl	50 µl
⇩	Shake **the Macrotiter Plate** briefly, pipet **Standards, patient samples and Controls.**	10 µl	50 µl
⌛	Cover the plate with foil and incubate for 120 min at room temperature on an orbital shaker (400-600 rpm).	120 min	120 min
≈	Wash 3 x with Wash Buffer	3 x	3 x
⇩	Pipet **diluted Enzyme Conjugate**.	100 µl	100 µl
⌛	Cover the plate with foil and incubate for 90 min at room temperature on an orbital shaker (400-600 rpm).	90 min	90 min
≈	Wash 3 x with Wash Buffer	3 x	3 x
⇩	Pipet **freshly prepared ready to use Amplification Reagent-mixture** by positive displacement (repetitive technique)#. **Avoid air bubbles**.	100 µl	100 µl
⌛	Incubate **15±2 min** at room temperature on an orbital shaker (400-600 rpm). ***Follow time schedule exactly***.	15±2 min	15±2 min
⇩	Pipet **Stop Solution by positive displacement**. **Avoid air bubbles**.	50 µl	50 µl
💻	Briefly mix contents, remove possible **air bubbles (use a tip)**, read the optical density at **490 nm** (reference wave lenght 600-650 nm) **within 60 min** after stopping.		

 # For further information of this pipetting technique see IBL full protocol or manual of pipetting manufacturer.

The concentrations of the standards (abscissa, logarithmic) are plotted against their corresponding optical density (ordinate, linear) on a semilogarithmic graph paper (standard curve).
The concentration of the urine samples and controls can be read directly from this standard curve. The values for the plasma samples have to be divided by factor 30 to get the correct concentration in ng/ml. These values have to be muliplied by factor 1000 to get the concentration in pg/ml.

4. Expected values

It is recommended that each laboratory establishes its own ranges of normal Adrenalin and Noradrenalin values. The following values may serve as a guide line:

Adrenalin in 24-hours urine: up to 20 µg/d (110 nmol/d)
Adrenalin in EDTA-Plasma: up to 125 pg/ml

Noradrenalin in 24-hours urine: up to 90 µg/d (535 nmol/d)
Noradrenalin in EDTA-Plasma: up to 600 pg/ml

Caution: For comprehensive information about the test procedure please refer to the detailed instructions for use available on the Internet

L 22 Dec 99 / K 01 Dec 99