



## Instructions for Use

# Noradrenaline High Sensitive ELISA

(Highly sensitive and for small sample volume)

Enzyme Immunoassay for the  
Quantitative Determination of  
**Noradrenaline**

**RUO**

For Research Use Only  
Not for use in diagnostic procedures

**REF** EA633/96



96



2 – 8 °C



DLD Gesellschaft für Diagnostika und medizinische Geräte mbH  
Adlerhorst 15 • 22459 Hamburg • Telefon: 040/ 555 87 10 • Fax: 040/ 555 87 111  
Internet: <http://www.dld-diagnostika.de> • E-Mail: [contact@dld-diagnostika.de](mailto:contact@dld-diagnostika.de)

## Table of Contents

|    |  |    |
|----|--|----|
| 1  | Introduction and Principle of the Test ..... | 3  |
| 2  | Precautions .....                            | 3  |
| 3  | Storage and Stability .....                  | 4  |
| 4  | Contents of the Kit .....                    | 4  |
| 5  | Sample Collection and Storage.....           | 7  |
| 6  | Preparation of Reagents and Samples.....     | 10 |
| 7  | Test Procedure .....                         | 13 |
| 8  | Calculation of Results .....                 | 14 |
| 9  | Assay Characteristics .....                  | 16 |
| 10 | Changes to declare.....                      | 18 |
|    | Pipetting Scheme Sample Preparation .....    | 19 |
|    | Pipetting Scheme ELISA.....                  | 20 |

## Symbols

|             |                   |  |
|-------------|-------------------|--|
| <b>RUO</b>  | Research Use Only |  |
| <b>CONT</b> | Content           |  |
| <b>LOT</b>  | Lot Number        |  |
|             | Manufactured by   |  |
| <b>REF</b>  | Catalogue Number  |  |
|             |                   |  |
|             |                   |  |
|             |                   |  |
|             |                   |  |

The symbols of the components of the kit are described in chapter 4 Contents of the test kit.

## 1 Introduction and Principle of the Test

The Noradrenaline High Sensitive ELISA provides materials for the quantitative measurement of noradrenaline in low concentrated samples and for small sample volumes. Noradrenaline is extracted using a cis-diol-specific affinity gel and acylated to N-acylnoradrenaline and then converted enzymatically into N-acylnormetanephrine.

The competitive Noradrenaline High Sensitive ELISA kit uses the microtiter plate format with Noradrenaline bound to the solid phase of the microtiter plate. Acylated catecholamine from the sample and solid phase bound catecholamine compete for a fixed number of antiserum binding sites. When the system is in equilibrium, free antigen and free antigen-antiserum complexes are removed by washing. The antibody bound to the solid phase catecholamine is detected by anti-rabbit IgG peroxidase conjugate. The peroxidase-induced conversion of the TMB substrate is monitored at 450 nm. The amount of antibody bound to the solid phase catecholamine is inversely proportional to the catecholamine concentration of the sample.

## 2 Precautions

- For research use only. Not for use in diagnostic procedures.
- Before carrying out this test, the instructions for use, as included in the kit, should be completely read and the content understood.
- Material of animal origin used in the preparation of the kit has been obtained from animals certified as healthy; however, these materials should be handled as potentially infectious.
- Individual components of different lots and test kits should not be interchanged. The expiration dates and storage conditions indicated on the packaging and labels of individual components must be observed.
- When handling reagents, controls, and samples, follow established laboratory safety guidelines and Good Laboratory Practice.
- Wear protection, such as lab coat, appropriate gloves and eye protection.
- Some of the components of this test kit are subject to labeling. These components bear the corresponding hazard symbol on their label. For further information, please see Chapter 4 Contents of the test kit and the relevant safety data sheets.

Avoid any actions that could result in ingestion, inhalation or injection of the reagents. Never pipette by mouth.

- Avoid contact with individual reagents.
- Some components contain small amounts of sodium azide as a preservative. Avoid the build-up of heavy metal azides in the drainage system by flushing thoroughly with water.
- Dispose of waste according to state and local environmental protection regulations.
- Broken glass can cause injury. Be careful with glass containers.

### 3 Storage and Stability

This kit is shipped at ambient temperature. Upon arrival, store the kit at 2 - 8 °C. Once opened, the kit is stable until its expiry date. For stability of prepared reagents refer to 6.1.

Bring all reagents to room temperature before use and refrigerate immediately after use.

### 4 Contents of the Kit

#### 4.1 Reagents for Sample Preparation

|  |                      |          |
|--|----------------------|----------|
| <b>Extraction Plate</b><br>48 wells<br>coated with boronate affinity gel                   | <b>EX-PLATE</b>      | 2 plates |
| <b>Extraction Buffer</b><br>6 ml, ready for use  | <b>EX-BUFF</b>       | 2 vials  |
| <b>HCl</b><br>21 ml, ready for use<br>0.025 M HCl  | <b>HCL</b>           | 1 vial   |
| <b>Control 1 &amp; 2</b><br>4 ml each, ready for use<br>Concentrations: see QC Certificate | <b>CON 1 &amp; 2</b> | 2 vials  |

**Standards (A - F)****CAL A - F**

6 vials

4 ml each, ready for use

Concentrations:

| Standard               | A | A/B* | B    | C   | D   | E    | F   |
|------------------------|---|------|------|-----|-----|------|-----|
| Noradrenaline (ng/ml)  | 0 | 0.05 | 0.15 | 0.5 | 1.5 | 5    | 25  |
| Noradrenaline (nmol/l) | 0 | 0.30 | 0.89 | 3.0 | 8.9 | 29.6 | 148 |

\*prepare Standard A/B just prior to assay, see 6.1.3

**Acylation Reagent****ACYL-REAG**

1 vial

6 ml, ready for use

Contains DMSO and DMF (please note that solvent reacts with many plastic materials including plastic trays; it does not react with normal pipette tips and with glass devices).

Danger  
Warning**Acylation Buffer****ACYL-BUFF**

1 vial

20 ml, ready for use

color coded purple

**Enzyme****ENZYME**

3 vials

2 ml each, lyophilized

Catechol-O-methyltransferase

**Coenzyme****COENZYME**

1 vial

1 ml, ready for use

S-adenosyl-L-methionine

**Enzyme Buffer****ENZYME-BUFF**

1 vial

3.5 ml, ready for use



Warning

**Enzyme Plate****ENZYME-PLATE**

1 piece

96 wells, break apart

**Sample Stabilizer****STABILIZER**

1 vial

20 ml, ready for use



Warning

## 4.2 Reagents for ELISA

|  |                   |           |
|--|-------------------|-----------|
| <b>Noradrenaline-Antiserum</b><br>2.5 ml, ready for use, rabbit<br>color coded yellow              | <b>AS-NAD</b>     | 1 vial    |
|  | !                 | Warning   |
| <b>MT-STRIPS</b><br>8 wells each, break apart, precoated with<br>Noradrenaline (12 strips)         | <b>STRIPS-NAD</b> | 12 strips |
| <b>Enzyme Conjugate</b><br>12 ml each, ready for use,<br>Anti-rabbit IgG Peroxidase                | <b>CONJ</b>       | 1 vial    |
|  | !                 | Warning   |
| <b>Wash Buffer</b><br>20 ml, concentrate<br>Dilute content with dist. water to 500 ml total volume | <b>WASH</b>       | 2 bottles |
| <b>Substrate</b><br>12 ml TMB solution, ready for use  | <b>SUB</b>        | 1 vial    |
| <b>Stop Solution</b><br>12 ml, ready for use<br>Contains 0.3 M sulphuric acid                      | <b>STOP</b>       | 1 vial    |
| <b>Adhesive Foil</b><br>Ready for use  | <b>FOIL</b>       | 10 pieces |

Additional materials and equipment required but not provided:

- Pipettes for pipetting 20, 50, 100, 150, 175, 280 µl
- Repeating dispenser for 20, 25, 50, 100, 150 and 1 ml
- Orbital shaker
- Multichannel pipette or Microplate washing device
- Microplate photometer
- Distilled water
- Paper towels, pipette tips, stopwatch
- Heating cabinet with 37 °C (optional)

## 5 Sample Collection and Storage

### 5.1 Plasma

EDTA plasma samples are required for the assay. Physical and psychical stress usually causes a high increase of the catecholamine concentration. Therefore, it is recommended to let the patient rest for 20 to 30 minutes after the venipuncture and before collecting the blood sample.

Haemolytic and especially lipemic samples should not be used for the assay, otherwise false low values will be obtained with such samples.

Immediately after collection the plasma samples should be centrifuged (preferable at 2 – 8 °C) and frozen. The samples are stable up to 1 week at -20 °C.

To improve the stability each sample should be enriched with the Sample Stabilizer **STABILIZER** before freezing (add 20% of the sample volume), e.g.:

| Sample volume | + Stabilizer volume | = Total volume |
|---------------|---------------------|----------------|
| 20 µl         | 4 µl                | 24 µl          |
| 50 µl         | 10 µl               | 60 µl          |
| 100 µl        | 20 µl               | 120 µl         |
| 200 µl        | 40 µl               | 240 µl         |
| 300 µl        | 60 µl               | 360 µl         |
| 500 µl        | 100 µl              | 600 µl         |

Note: Multiply the achieved result of the sample by the factor 1.2.

## 5.2 Cell culture samples and various biological samples

The stability of such samples depends on the sample type and the way of collection. Therefore, a general procedure for collection and storage is not possible. However, it is recommended to freeze the samples immediately after collection. The samples should be stable at -20 °C for up to 1 week.

To improve the stability each sample should be enriched with the Sample Stabilizer **STABILIZER** before freezing (10% of the sample volume), e.g.:

| Sample volume | + Stabilizer volume | = Total volume |
|---------------|---------------------|----------------|
| 20 µl         | 2 µl                | 22 µl          |
| 50 µl         | 5 µl                | 55 µl          |
| 100 µl        | 10 µl               | 110 µl         |
| 200 µl        | 20 µl               | 220 µl         |
| 300 µl        | 30 µl               | 330 µl         |
| 500 µl        | 50 µl               | 550 µl         |

Note: Multiply the achieved result of the sample by the factor 1.1.

**Acidified samples, which have a pH value of 5 or less must not be enriched with the Sample Stabilizer and have to be frozen immediately after collection.**

### 5.3 Tissue samples

Tissue samples can be homogenized in Sample Stabilizer **STABILIZER** diluted 1:20 (e.g. 19 ml dist. water + 1 ml Sample Stabilizer **STABILIZER**).

For all sample types, the following basic principles should be followed:

- Avoid excess of acid. This might exceed the buffer capacity of the extraction buffer. After adding the extraction buffer **EX-BUFF** a pH value of 7 or above is mandatory. If the pH value is below 7 it is necessary to repeatedly add 50 µl of Extraction Buffer until the pH value is at or above 7.  
Acidified samples, which have a pH value of 5 or less must not be enriched with the Sample Stabilizer.
- Avoid substances in the samples with a cis-diol-structure (boric acid, sorbitol, mannitol, etc.). These substances reduce the recovery of extraction.

## 6 Preparation of Reagents and Samples

### 6.1 Preparation of Reagents

#### 6.1.1 Wash Buffer

Dilute the contents (20 ml) of the bottle **WASH** (25x) with distilled water to a total volume of 500 ml, mix briefly.

For further use the diluted wash buffer must be stored at 2-8 °C for a maximum period of 4 weeks.

If the kit is used in several runs, only the amount of wash buffer required for that run should be prepared.

#### 6.1.2 Enzyme Mix

NOTE: The enzyme mix has to be prepared freshly prior to the assay (not longer than 10 - 15 minutes in advance). After use the reagent has to be discarded.

Reconstitute the content of one vial labelled **ENZYME** with 2 ml distilled water.

Add 0.3 ml **COENZYME** and 0.7 ml **ENZYME-BUFF**, mix thoroughly (total volume: 3 ml).

The two additional bottles of **ENZYME** allow a second and a third run of the test. If the whole kit is to be used in one run, it is sufficient to prepare one vial of enzyme mix.

#### 6.1.3 Standard A/B

Prepare Standard A/B by mixing 400 µl Standard A **CAL A** and 200 µl Standard B **CAL B** in suitable polypropylene tubes or Eppendorf cups; prepare just prior to assay.

All other reagents are ready for use.

## 6.2 Preparation of Samples

Allow reagents to equilibrate to room temperature. Determinations in duplicates are recommended. Each 20 µl of Standards and Control 1 & 2 are extracted. Each 1 µl - 300 µl of samples are extracted (alternatively: > 300 µl up to 500 µl).

1. Pipette **20 µl** of each Standard **CAL A**, **CAL A/B** (see 6.1.3), **CAL B** - **CAL F**, **20 µl** of each Control **CON 1** & **CON 2** and **1 µl - 300 µl of each Sample** into the respective wells of the extraction plate **EX-PLATE**.

Within a run the final volume has to be the same in all wells: 300 µl or 500 µl.

Correction of volume:

Pipette 280 µl of distilled water into the wells of the standards and controls (final volume: 300 µl). Pipette as much distilled water into the wells of the samples to obtain a final volume of 300 µl, e.g. 100 µl sample + 200 µl distilled water.

For sample volumes > 300 µl up to 500 µl: fill up all wells of the standards, controls and eventually samples to 500 µl with distilled water.

2. Pipette **100 µl Extraction Buffer** **EX-BUFF** into each well.
3. Cover the plate with Adhesive Foil **FOIL** and incubate for 60 minutes at room temperature on an orbital shaker (high shaking rate).
4. Decant the plate and remove residual liquid by tapping the inverted plate on a paper towel.
5. Pipette **1 ml prepared Wash Buffer** **WASH** (see 6.1.1) into each well and incubate for 5 minutes at room temperature on an orbital shaker (slow shaking rate).
6. Decant the plate and remove residual liquid by tapping the inverted plate on a paper towel.
7. Pipette **150 µl Acylation Buffer** **ACYL-BUFF** into each well.
8. Add **50 µl Acylation Reagent** **ACYL-REAG** into each well and continue with the next step, immediately.

(please note, that solvent reacts with many plastic materials including plastic trays; it does not react with normal pipette tips or glass devices)

9. Incubate for 20 minutes at room temperature on an orbital shaker (medium shaking rate).
10. Decant the plate and remove residual liquid by tapping the inverted plate on a paper towel.
11. Pipette **1 ml prepared Wash Buffer** **WASH** (see 6.1.1) into each well and incubate for 5 minutes at room temperature on an orbital shaker (slow shaking rate).
12. Decant the plate and remove residual liquid by tapping the inverted plate on a paper towel.
13. Repeat the wash steps 11. and 12.
14. Pipette **125 µl HCl** **HCl** (0.025 M) for elution into each well.
15. Cover the plate with Adhesive Foil **FOIL** and incubate for 20 minutes at room temperature on an orbital shaker (medium shaking rate).  
**Caution: Do not decant the supernatant.**
16. Transfer **100 µl from each** well of the extraction plate **EX-PLATE** into the respective wells of the enzyme plate **ENZYME-PLATE**.  
(do not discard extraction plate, seal and keep for second or third assay run)
17. Add **20 µl of freshly prepared Enzyme Mix** (s. 6.1.2) into each well. Color changes to red.
18. Cover the plate with Adhesive Foil **FOIL** and incubate for 1 minute at room temperature on an orbital shaker (medium shaking rate).
19. Incubate the plate for 90 minutes at 37°C without shaking.  
(Alternatively: 120 minutes at room temperature (20 - 25°C) on an orbital shaker at medium shaking rate).  
**Caution: Do not decant the supernatant.**  
**100 µl of the supernatant are used in the ELISA.**

## 7 Test Procedure

1. Transfer **100 µl** each of prepared Standards, Controls and Samples from the enzyme plate **ENZYME-PLATE** into the respective wells of the MT-Strips **STRIPS-NAD**.  
(do not discard enzyme plate, seal and keep for second or third assay run)
2. Add **20 µl** **Noradrenaline-Antiserum** **NAD-DA** (color coded yellow) into each well.
3. Cover the plate with Adhesive Foil **FOIL**, shake briefly and incubate for 15-20 hours (overnight) at 2-6 °C.
4. Discard or aspirate the contents of the wells and wash thoroughly with **250 µl** prepared Wash Buffer **WASH** (see 6.1.1) per well. Remove residual liquid by tapping the inverted plate on clean absorbent paper. Repeat the washing procedure 3 times.
5. Pipette **100 µl** **POD-Conjugate** **CONJ** into each well.
6. Incubate for 60 minutes at room temperature on an orbital shaker (medium shaking rate).
7. Wash according to step 4.
8. Pipette **100 µl** **Substrate** **SUB** into each well.
9. Incubate for 35 to 45 minutes at room temperature (20-25°C) on an orbital shaker (medium shaking rate). Avoid exposure to direct sun light.
10. Add **100 µl** **Stop Solution** **STOP** into each well. Shake for 10 seconds on an orbital shaker.
11. Read the optical density at 450 nm (reference wavelength between 570 nm and 650 nm) in a microplate photometer within 15 minutes.

## 8 Calculation of Results

The concentration of the standards (x-axis, logarithmic) are plotted against their corresponding optical density (y-axis, linear). Alternatively, the optical density of each standard and sample can be related to the optical density of the zero standard, expressed as the ratio  $OD/OD_{max}$ , and then plotted on the y-axis.

A good fit is provided with 4 Parameter Logistic (alternatively Log-Logit or Cubic Spline).

The concentration of the controls can be read off the standard curve directly without any further conversion.

The read concentrations of the samples have to be divided by a correction factor due to the use of 1  $\mu$ l – 300  $\mu$ l sample volume in relation to 20  $\mu$ l standard.

$$\text{Correction factor} = \frac{\text{Sample volume for extraction} [\mu\text{l}]}{20 \mu\text{l} \text{ (Standard volume)}}$$

Example:

300  $\mu$ l sample was extracted and the concentration read off from the standard curve is 0.6 ng/ml.

Correction factor = 300  $\mu$ l / 20  $\mu$ l = 15

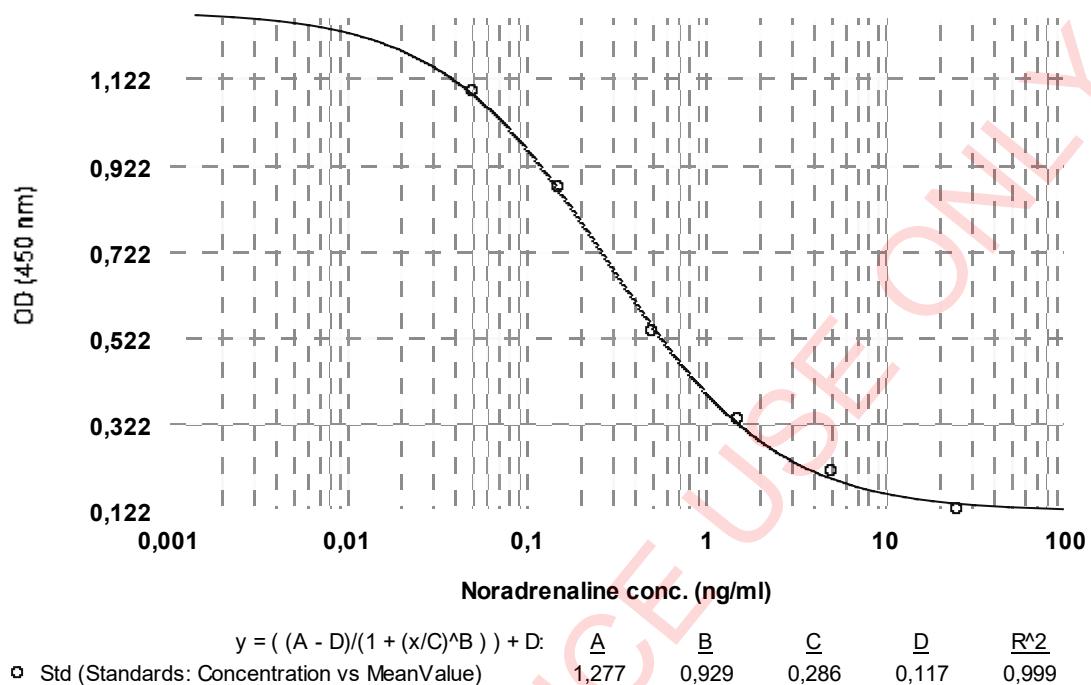
Concentration of the sample = 0.6 ng/ml / 15 = 0.040 ng/ml = 40 pg/ml

Conversion into pmol/l:

Noradrenaline: 1 pg/ml = 5.91 pmol/l

Should the samples have been enriched with Sample Stabilizer, multiply the result with the appropriate dilution factor indicated in section 5.

Below a typical example of a standard curve with the Noradrenaline High Sensitive ELISA is shown:



Quality Control: Test results are valid only if the kit controls are within the ranges specified on the Quality Control Certificate. Otherwise, the test should be repeated.

## 9 Assay Characteristics

### 9.1 Analytical Sensitivity

The lower limit of detection was determined by taking the 2-fold standard deviation of the absorbance of the Zero Reference and reading the corresponding value from the standard curve. The sensitivity depends on the sample volume and can be calculated with the corresponding correction factor (see 8. Calculation of Results)

| Noradrenaline  |   |
|--|---|
| <b>Sensitivity:</b>                                      | $\frac{15 \text{ pg/ml (89 pmol/l)}}{\text{Correction factor}}$ |
| <b>Example for 300 µl Sample (Correction factor 15):</b> | $\frac{15 \text{ pg/ml}}{15} = 1.0 \text{ pg/ml (5.9 pmol/l)}$  |

### 9.2 Analytical Specificity (Cross Reactivity)

Structural related components were tested for possible interference:

| Substance          | Cross Reactivity (%)<br>Noradrenaline-Ab |
|--------------------|--|
| Noradrenaline      | 100                                      |
| Dopamine           | 0.092                                    |
| Adrenaline         | < 0.012                                  |
| Metanephrine       | < 0.012                                  |
| Normetanephrine    | 0.16                                     |
| 3-Methoxytyramine  | < 0.012                                  |
| L-Dopa             | < 0.005                                  |
| Tyramine           | < 0.005                                  |
| Tyrosine           | < 0.005                                  |
| Homovanillic acid  | < 0.005                                  |
| Vanilmandelic acid | < 0.005                                  |

### 9.3 Recovery

Increasing amounts of noradrenaline were added to an EDTA plasma sample and to a cell culture medium (RPMI 1640). Each spiked sample was assayed. The analytical recovery was estimated at different concentrations by using the theoretically expected and the actually measured values.

Concentrations in pg/ml

| EDTA-Plasma |          |          |            | Cell culture medium |          |          |            |
|-------------|----------|----------|------------|---------------------|----------|----------|------------|
| added       | measured | expected | % recovery | added               | measured | expected | % recovery |
| 0.0         | 4.5      |          |            | 0.0                 | 1.1      |          |            |
| 6.7         | 10.4     | 11.2     | 92         | 6.7                 | 7.1      | 7.8      | 91         |
| 10.9        | 14.5     | 15.4     | 94         | 10.9                | 13.2     | 12.0     | 110        |
| 15.2        | 20.4     | 19.7     | 104        | 15.2                | 15.4     | 16.3     | 95         |
| 22.4        | 27.5     | 26.9     | 102        | 22.4                | 22.7     | 23.5     | 97         |
| 45.5        | 45.5     | 50.0     | 91         | 45.5                | 48.9     | 46.6     | 105        |
| 67.2        | 58.9     | 71.7     | 82         | 67.2                | 59.1     | 68.3     | 87         |
| 88.2        | 80.3     | 92.8     | 87         | 88.2                | 82.9     | 89.4     | 93         |
| 151.5       | 131.7    | 156.0    | 84         | 151.5               | 108.7    | 152.6    | 71         |
| 294.1       | 246.0    | 298.7    | 82         | 294.1               | 246.6    | 295.3    | 84         |
|             | mean:    | 91       |            |                     | mean:    | 92       |            |

### 9.4 Reproducibility

Intra-Assay

The reproducibility of the ELISA method was investigated by determining the intra-assay-coefficients of variation (cv) by repeated measurements for EDTA-Plasma and cell culture medium (RPMI 1640) with different concentrations.

Concentrations in pg/ml

| Sample              | N = | mean value | sd   | cv (%) |
|---------------------|-----|------------|------|--------|
| EDTA-Plasma         | 16  | 113.1      | 7.57 | 6.7    |
| Cell culture medium | 16  | 21.9       | 1.07 | 4.9    |

## **9.5 Calibration**

The calibration is carried out by weighing the pure substance.

## **9.6 Limitations of the method**

Results are for research purposes only.

Samples measured above the highest standard must be diluted and re-determined. Values of diluted samples must be multiplied by the appropriate dilution factor.

## **9.7 Interferences**

See Sample Collection and Storage.

## **10 Changes to declare**

Version \_11: Changes are highlighted in gray.

Version \_10: Hazard symbol “Warning” was added to CONJ and AS-NAD in section 4. Further additions/changes are highlighted in gray.

Version 9: Hazard symbol “Warning” was removed from POD Conjugate in section 4, as no longer required.

Version 8: “Urine” was removed from title of section 5.2.

Version 7: IFU has been re-formatted. Parts of sections 5, 6, 7 and 8 and pipetting schemes have been rephrased to provide greater clarity.

No changes have been made to components or execution of protocols.

**Pipetting Scheme Sample Preparation**

|                    |    | Standards | Controls | Samples        |
|--------------------|----|-----------|----------|----------------|
| CAL A , A/B, B – F | µl | 20        |          |                |
| CON 1 & 2          | µl |           | 20       |                |
| Samples            | µl |           |          | 1 - 300        |
| Dist. Water        | µl | 280       | 280      | Fill up to 300 |
| EX-BUFF            | µl | 100       | 100      | 100            |

Cover the plate with FOIL; Shake for 60 minutes at room temperature  
 Decant plate and tap out residual liquid

|      |    |      |      |      |
|------|----|------|------|------|
| WASH | µl | 1000 | 1000 | 1000 |
|------|----|------|------|------|

Shake for 5 minutes at room temperature (slow shaking rate)  
 Decant plate and tap out residual liquid

|           |    |     |     |     |
|-----------|----|-----|-----|-----|
| ACYL-BUFF | µl | 150 | 150 | 150 |
| ACYL-REAG | µl | 50  | 50  | 50  |

**Immediately:** Shake for 20 minutes at room temperature  
 Decant plate and tap out residual liquid

|      |    |      |      |      |
|------|----|------|------|------|
| WASH | µl | 1000 | 1000 | 1000 |
|------|----|------|------|------|

Shake for 5 minutes at room temperature (slow shaking rate)  
 Decant plate and tap out residual liquid  
 Repeat wash step 1x, **Decant plate and tap out residual liquid**

|     |    |     |     |     |
|-----|----|-----|-----|-----|
| HCl | µl | 125 | 125 | 125 |
|-----|----|-----|-----|-----|

Cover the plate with FOIL; Shake for 20 minutes at room temperature  
**Caution: Do not decant the supernatant**

Use ENZYME-PLATE

|  |    | Acyl. Standards | Acyl. Controls | Acyl. Samples |
|--|----|-----------------|----------------|---------------|
| Transfer from EX-PLATE to ENZYME-PLATE | µl | 100             | 100            | 100           |

|                    |    |    |    |    |
|--------------------|----|----|----|----|
| Enzyme mix (fresh) | µl | 20 | 20 | 20 |
|--------------------|----|----|----|----|

Cover the plate with FOIL; Shake for 1 minute at room temperature  
 Incubate for 90 minutes at 37°C  
**Caution: Do not decant the supernatant**  
 Add 100 µl each to the ELISA

**Pipetting Scheme ELISA**

|  |    | Prepared Standards | Prepared Controls | Prepared Samples |
|--|----|--------------------|-------------------|------------------|
| Transfer from ENZYME-PLATE to STRIPS-NAD | µl | 100                | 100               | 100              |
| AS-NAD                                   | µl | 20                 | 20                | 20               |

Cover the plates with FOIL and shake briefly  
 Incubate for 15 - 20 hours (overnight) at 2 - 6 °C  
 Decant or aspirate plate

|      |    |     |     |     |
|------|----|-----|-----|-----|
| WASH | µl | 250 | 250 | 250 |
|------|----|-----|-----|-----|

Decant or aspirate plate  
 Repeat wash step 3 x, decant plate and tap out residual liquid

|      |    |     |     |     |
|------|----|-----|-----|-----|
| CONJ | µl | 100 | 100 | 100 |
|------|----|-----|-----|-----|

Shake for 60 minutes at room temperature (medium shaking rate)  
 Discard or aspirate plate

|      |    |     |     |     |
|------|----|-----|-----|-----|
| WASH | µl | 250 | 250 | 250 |
|------|----|-----|-----|-----|

Decant or aspirate plate  
 Repeat wash step 3 x, decant plate and tap out residual liquid

|     |    |     |     |     |
|-----|----|-----|-----|-----|
| SUB | µl | 100 | 100 | 100 |
|-----|----|-----|-----|-----|

Shake for  $40 \pm 5$  minutes at room temperature (medium shaking rate)

|      |    |     |     |     |
|------|----|-----|-----|-----|
| STOP | µl | 100 | 100 | 100 |
|------|----|-----|-----|-----|

Shake for 10 seconds  
 Read absorbance at 450 nm within 15 minutes  
 (Ref. 570 nm to 650 nm)