





- Vortex mixer

## 5. **Sample collection and storage**

Storage: up to 6 hours at 2 – 8 °C; for longer periods (up to 6 months) at - 20°C or – 80 °C.

*Advice for the preservation of the biological sample:* to prevent catecholamine degradation add EDTA (final concentration 1mM) and sodium metabisulfite (final concentration 4 mM) to the sample.

## 6. **Test procedure**

Allow reagents and samples to reach room temperature. Duplicate determinations are recommended.

### 6.1 **Preparation of reagents**

#### **Wash Buffer**

Dilute the 20 mL Wash Buffer Concentrate with distilled water to a final volume of 500 mL. Store the diluted Wash Buffer Concentrate (Wash Buffer) at 2 – 8 °C. Shelf life: please refer to the expiry date indicated on the kit.

#### **Acylation Solution**

The Acylation Concentrate has to be diluted 1 + 60 with Acylation-Diluent in a glass or polypropylene-vial.

<b>Acylation Concentrate</b>	10 µL	20 µL	25 µL	50 µL
<b>Acylation-Diluent</b>	600 µL	1.2 mL	1.5 mL	3 mL

⚠ *The Acylation Solution has to be prepared freshly prior to the assay (not longer than 60 minutes in advance). Discard after use!*

#### **Enzyme Solution**

Reconstitute the content of the vial labelled 'Enzyme' with 1 mL distilled water and mix thoroughly. Add 0.3 mL of Coenzyme followed by 0.7 mL of Adjustment Buffer. The total volume of the Enzyme Solution is 2.0 mL.

⚠ *The Enzyme Solution has to be prepared freshly prior to the assay (not longer than 10 - 15 minutes in advance). Discard after use!*

### 6.2 **Sample preparation**

The Adrenaline Research ELISA is a flexible test system for various biological sample types and volumes. It is not possible to give a general advice how to prepare the samples. However, the following basics should help the researcher to fit the protocol to his specific needs.

- Avoid excess of acid: excess of acid might exceed the buffer capacity of the extraction buffer. A pH > 7.0 during the extraction is mandatory.
- Prevent catecholamine degradation by adding preservatives to the sample (see 5. Sample collection and Storage).
- Avoid chaotropic chemicals like perchloric acid. The high salt content might reduce the recovery of Adrenaline. If your samples already contain high amounts of perchloric acid, neutralize the sample prior to the extraction step.
- Tissue samples can be homogenised in 0.01 N HCl in the presence of EDTA and sodium metabisulfite. Under these conditions, Adrenaline is positively charged which reduces binding to proteins and optimizes solubility.
- Avoid samples that contain substances with a cis-diol structure. These will reduce the recovery of the Adrenaline.
- It is advisable to perform a "Proof of Principle" to determine the recovery of the Adrenaline in your samples. Prepare a stock solution of Adrenaline. Add small amounts (to change the native sample matrix as less as possible) of the stock solutions to the sample matrix and check the recovery.
- The used sample volume determines the sensitivity of this test. Determine the sample volume needed to determine the Adrenaline in your sample by testing different amounts of sample volume.

*If you need any support in establishing a protocol for your specific purposes, do not hesitate to contact the manufacturer directly!*

### 6.3 Extraction and acylation

The Adrenaline Research ELISA offers a flexible test system for various biological sample types and volumes. Step 1 of the extraction procedure depends on the sample volume:

- in case you have sample volumes between 1 – 100 µL follow **1.1**
- in case you have sample volumes between 100 – 500 µL follow **1.2**
- in case you have sample volumes between 500 – 750 µL follow **1.3**



**Within a run it is only possible to measure samples with the same volume!**

1.	1.1 Sample volume 1 – 100 µL	1.2 Sample volume 100 – 500 µL	1.3 Sample volume 500 – 750 µL
	Pipette into the respective wells of the Extraction Plate: <b>10 µL standards, 10 µL controls and 1 – 100 µL of the sample.</b> Fill up each well with distilled water to a <b>final volume</b> of 100 µl (e.g. 10 µl standard plus 90 µl dist. water).	Pipette into the respective wells of the Extraction Plate: <b>10 µL standards, 10 µL controls and 100 – 500 µL of the sample.</b> Fill up each well with distilled water to a <b>final volume</b> of 500 µl (e.g. 10 µl standard plus 490 µl dist. water).	Pipette into the respective wells of the Extraction Plate: <b>10 µL of Standards, 10 µL of controls and 500 – 750 µL of sample.</b> Fill up each well with distilled water to a <b>final volume</b> of 750 µl (e.g. 10 µl standard plus 740 µl dist. water).
2.	Pipette <b>50 µL</b> of <b>Assay Buffer</b> into all wells.		
3.	Pipette <b>50 µL</b> of <b>Extraction Buffer</b> into all wells		
4.	Cover the plate with adhesive foil. Incubate <b>60 min</b> at <b>RT</b> (20-25°C) on a <b>shaker</b> (approx. 600 rpm).		
8.	Remove the foil and empty the plate. Blot dry by tapping the inverted plate on absorbent material.		
6.	Pipette <b>1 mL</b> of <b>Wash Buffer</b> into all wells. Cover the plate with adhesive foil.		
7.	Shake <b>5 min</b> at <b>RT</b> (20-25°C) on a <b>shaker</b> (approx. 600 rpm).		
8.	Remove the foil and empty the plate. Blot dry by tapping the inverted plate on absorbent material.		
9.	<b>Wash one more time</b> as described (step 6, 7 and 8)!		
10.	Pipette <b>150 µL</b> of <b>Acylation Buffer</b> into all wells.		
11.	Pipette <b>25 µL</b> of <b>Acylation Solution</b> (refer to 6.1) into all wells.		
12.	Incubate <b>20 min</b> at <b>RT</b> (20-25°C) on a shaker (approx. 600 rpm).		
13.	Empty the plate and blot dry by tapping the inverted plate on absorbent material.		
14.	Pipette <b>1 mL</b> of <b>Wash Buffer</b> into all wells. Cover plate with adhesive foil.		
15.	Shake <b>5 min</b> at <b>RT</b> (20-25°C) on a shaker (approx. 600 rpm).		
16.	Remove the foil and empty the plate. Blot dry by tapping the inverted plate on absorbent material.		
17.	<b>Wash one more time</b> as described (step 14, 15, 16).		
18.	Pipette <b>100 µL</b> of <b>Hydrochloric Acid</b> into all wells.		
19.	Cover plate with adhesive foil. Incubate <b>10 min</b> at <b>RT</b> (20-25°C) on an o shaker (approx. 600 rpm).		
	<b>Do not decant the supernatant thereafter!</b>		
	<b>90 µL of the supernatant is needed for the subsequent enzymatic conversion</b>		

### 6.4 Enzymatic Conversion

1.	Pipette <b>90 µL</b> of the <b>extracted standards, controls and samples</b> into the respective wells of the <b>Microtiter Plate</b> .		
2.	Add <b>25 µL</b> of <b>Enzyme Solution</b> (refer to 6.1) to all wells.		
3.	Cover plate with <b>Adhesive Foil</b> . Incubate <b>1 min</b> at <b>RT (20-25°C)</b> on a shaker to mix.		
4.	Incubate for <b>2 hours</b> at <b>37°C</b> . The following volumes of the supernatants are needed for the subsequent ELISA: <table border="1" style="margin-left: 20px;"> <tr> <td style="padding: 2px;"><b>Adrenaline</b></td> <td style="padding: 2px;"><b>100 µL</b></td> </tr> </table>	<b>Adrenaline</b>	<b>100 µL</b>
<b>Adrenaline</b>	<b>100 µL</b>		

## 6.5 Adrenaline ELISA

1.	Pipette <b>100 µL</b> of <b>standards, controls</b> and <b>samples</b> from the <b>Microtiter Plate</b> (refer to 6.4) into the respective pre-coated <b>Adrenaline Microtiter Strips</b> .
2.	Pipette <b>50 µL</b> of the <b>Adrenaline Antiserum</b> into all wells.
3.	Cover the plate with <b>Adhesive Foil</b> . Incubate for <b>1 min</b> at <b>RT</b> (20-25°C) on a <b>shaker</b> .
4.	Incubate for <b>15 – 20 hours</b> (overnight) at <b>2 – 8 °C</b> .
5.	Remove the foil and discard or aspirate the contents of the wells and <b>wash</b> each well <b>4 times</b> thoroughly with 300 µL <b>Wash Buffer</b> . Blot dry by tapping the inverted plate on absorbent material.
6.	Pipette <b>100 µL</b> of <b>Enzyme Conjugate</b> into all wells.
7.	Cover the plate with <b>Adhesive Foil</b> and incubate <b>30 min</b> at <b>RT</b> (20-25°C) on a <b>shaker</b> (approx. 600 rpm).
8.	Remove the foil and discard or aspirate the contents of the wells and <b>wash</b> each well <b>4 times</b> thoroughly with 300 µL <b>Wash Buffer</b> . Blot dry by tapping the inverted plate on absorbent material.
9.	Pipette <b>100 µL</b> of <b>Substrate</b> into all wells.
10.	Incubate <b>20-30 min</b> at <b>RT</b> (20-25°C) on a <b>shaker</b> (approx. 600 rpm). ⚠ <b>Avoid exposure to direct sun light!</b>
11.	Pipette <b>100 µL</b> of <b>Stop Solution</b> into all wells.
12.	<b>Read</b> the absorbance of the solution in the wells within 10 minutes, using a microplate reader set to <b>450 nm</b> and a reference wavelength between 620 nm and 650 nm.

## 7. Calculation of results

The calibration curve from which the concentrations in the samples can be read off is obtained by plotting the absorbance readings (calculate the mean absorbance) measured for the standards (linear, y-axis) against the corresponding concentrations (logarithmic, x-axis).

The use of a non-linear regression for curve fitting (e.g. spline, 4- parameter, akima) is recommended.

### The standards refer to:

Standard	Concentration of the standards (ng/mL)					
	A	B	C	D	E	F
Adrenaline	0	0.45	1.5	4.5	15	45

⚠ The concentrations of the samples taken from the standard curve have to be multiplied by a correction factor.

$$\text{Correction factor} = \frac{10 \mu\text{L (volume of standards extracted)}}{\text{sample volume } (\mu\text{L}) \text{ extracted}}$$

**Example:** 750µL of the sample is extracted and the concentration taken from the standard curve is 0.45 ng/mL Adrenaline.

Correction factor = 10/750 = 0.013

Concentration of the sample = 0.45 ng/mL x 0.013 = 0.006ng/mL = 6 pg/mL Adrenaline

### 7.1 Quality control

It is recommended to use control samples according to state and federal regulations. Use controls at both normal and pathological levels. The kit or other commercial controls should fall within established confidence limits. The confidence limits of the kit controls are indicated on the QC Report.

### 7.2 Calibration

The binding of the antisera and the enzyme conjugates and the activity of the enzyme used are temperature dependent, and the extinction values may vary if a thermostat is not used. The higher the temperature, the higher the extinction values will be. The extinction values also depend on the incubation times. The optimal temperature during the Enzyme Immunoassay is between 20-25°C.

⚠ In case of overflow, read the absorbance of the solution in the wells within 10 minutes, using a microplate reader set to 405 nm

## 8. Assay characteristics

Analytical Specificity (Cross Reactivity)	Substance	Cross Reactivity (%)
	Derivatized Adrenaline	100
	Derivatized Noradrenaline	0.20
	Derivatized Dopamine	< 0.0007
	Metanephrine	0.64
	Normetanephrine	0.0009
	3-Methoxytyramine	< 0.0007
	3-Methoxy-4-hydroxyphenylglycol	0.03
	Tyramine	< 0.0007
	Phenylalanine, Caffeinic acid, L-Dopa, Homovanillic acid, Tyrosine, 3-Methoxy-4-hydroxymandelic acid	< 0.0007

Analytical Sensitivity (Limit of Detection)	Adrenaline
	0,3 ng/mL x C*

C\* = Correction factor (refer to 7.)

Precision				
Intra-Assay Human EDTA-Plasma				
	Sample	Mean ± 3 SD (pg/mL)	SD (pg/mL)	CV (%)
Adrenaline	high	1329.3 ± 372.6	124.2	9.3
	medium	412.1 ± 129.6	43.2	10.5
	low	37.9 ± 19.5	6.5	17.1
Intra-Assay Cell Culture Medium (RPMI)				
	Sample	Mean ± 3 SD (pg/mL)	SD (pg/mL)	CV (%)
Adrenaline	high	1649.6 ± 555.0	185	11.2
	medium	526.2 ± 186.6	62.2	11.8
	low	38.7 ± 18.9	6.3	16.3

Recovery	Mean (%)	Range (%)	SD (%)	CV (%)
Adrenaline				
Human EDTA-Plasma	104.0	89.4 – 128.3	13.1	12.6
Cell Culture Medium	95.5	81.6 – 109.6	8.3	8.7



