Bi-CAT (Research) ELISA

For quantitative determination of adrenaline (epinephrine) and noradrenaline (norepinephrine) in various biological samples.

For Research Use Only. Not For Use In Diagnostic Procedures.

Catalog Number: 17-BCTHU-E02-RES
Size: 96 Wells
Version: 15.0 - ALPCO 3.0

1. Intended Use and Principle of the Assay

The Bi-Cat Research ELISA is an enzyme immunoassay for the quantitative determination of adrenaline (epinephrine) and noradrenaline (norepinephrine). The kit is a flexible test system suitable for various biological sample types and volumes.

Adrenaline (epinephrine) and noradrenaline (norepinephrine) are extracted by using a cis-diol-specific affinity gel, acylated, and then converted enzymatically.

The competitive ELISA kit uses the microtiter plate format. The antigen is bound to the solid phase of the microtiter plate. The derivatized standards, controls, and samples and the solid phase bound analytes compete for a fixed number of antibody binding sites. After the system is in equilibrium, free antigen and free antigen-antibody complexes are removed by washing. The antibody bound to the solid phase is detected by an anti-rabbit IgG-peroxidase conjugate using TMB as a substrate. The reaction is monitored at 450 nm.

Quantification of unknown samples is achieved by comparing their absorbance with a standard curve prepared with known standard concentrations.

2. Procedural Cautions, Guidelines and Warnings

(1) This kit is intended for professional use only. Users should have a thorough understanding of this protocol for the successful use of this kit. Only the test instruction provided with the kit is valid and must be used to run the assay. Reliable performance will only be attained by strict and careful adherence to the instructions provided.
(2) The principles of Good Laboratory Practice (GLP) must be followed.
(3) To reduce exposure to potentially harmful substances, wear lab coats, disposable gloves, and protective glasses where necessary.
(4) All kit reagents and samples should be brought to room temperature and mixed gently but thoroughly before use. Avoid repeated freezing and thawing of reagents and samples.
(5) For dilution or reconstitution purposes, use deionized, distilled, or ultra-pure water.
(6) The microplate contains snap-off strips. Unused wells must be stored at 2 - 8 °C in the sealed foil pouch with desiccant and used in the frame provided. Strips which are removed from the frame for usage should be marked accordingly to avoid mix-up.
(7) Duplicate sample measurement is highly recommended to be able to identify any pipetting errors.
(8) Once the test has been started, all steps must be completed without interruption. Make sure that the required reagents, materials and devices are prepared and ready at the appropriate time.
(9) Incubation times influence results. Wells should be handled in the same order and time intervals.
(10) To avoid cross-contamination of reagents, use new disposable pipette tips for dispensing each reagent, sample, standard, and control.
(11) A standard curve must be established for each run.
(12) The controls should be included in each run and fall within established confidence limits. The confidence limits are listed in the QC-Report.
(13) Do not mix kit components with different lot numbers within a test and do not use reagents beyond expiry date as shown on the kit labels.
(14) Avoid contact with Stop Solution containing 0.25 M H_2SO_4. It may cause skin irritation and burns. In case of contact with eyes or skin, rinse off immediately with water.
(15) TMB substrate has an irritant effect on skin and mucosa. In case of possible contact, wash eyes with an abundant volume of water and skin with soap and abundant water. Wash contaminated objects before reusing them.
(16) Refer to Safety Data Sheet (SDS) for information on hazardous substances included in the kit. The Safety Data Sheet for this kit is made available directly on ALPCO's the website or upon request.
(17) Kit reagents must be regarded as hazardous waste and disposed according to national regulations.

3. Storage and stability

Store the unopened reagents at 2 - 8 °C until expiration date. Do not use components beyond the expiry date indicated on the kit labels. Once opened reagents are stable for 2 month when stored at 2–8 °C. Once the resealable pouch has been opened, care should be taken to close it tightly with desiccant again.

4. Materials

4.1 Content of the kit

96 **Microtiter Plate** - Ready to use
Content: 1 x 96 wells, empty in a resealable pouch

FOILS **Adhesive Foil** - Ready to use
Content: Adhesive Foils in a resealable pouch
Volume: 2 x 4 foils

WASH-CONC 50x **Wash Buffer Concentrate -** Concentrated 50x
Content: Buffer with a non-ionic detergent and physiological pH
Volume: 2 x 20 mL/vial, light purple cap

CONJUGATE **Enzyme Conjugate** - Ready to use
Content: Goat anti-rabbit immunoglobulins, conjugated with peroxidase
Volume: 2 x 12 mL/vial, red cap

SUBSTRATE **Substrate** - Ready to use
Content: Chromogenic substrate containing tetramethylbenzidine, substrate buffer and hydrogen peroxide
Volume: 2 x 12 mL/black vial, black cap

STOP-SOLN **Stop Solution** - Ready to use
Content: 0.25 M sulfuric acid
Volume: 2 x 12 mL/vial, light grey cap
Hazards Identification:

H290 May be corrosive to metals.

ADR MN **Adrenaline Microtiter Strips**- Ready to use
Content: 1 x 96 well (12x8) antigen precoated microwell plate in a resealable blue pouch with desiccant

NAD NMN **Noradrenaline Microtiter Strips**- Ready to use
Content: 1 x 96 well (12x8) antigen precoated microwell plate in a resealable yellow pouch with desiccant

ADR-AS **Adrenaline Antiserum** - Ready to use
Content: Rabbit anti-adrenaline antibody, blue colored
Volume: 1 x 6 mL/vial, blue cap

NAD-AS **Noradrenaline Antiserum** - Ready to use
Content: Rabbit anti-noradrenaline antibody, yellow colored
Volume: 1 x 6 mL/vial, yellow cap

ADJUST-BUFF **Adjustment Buffer** - Ready to use

Content: TRIS buffer
Volume: 1 x 4 mL/vial, green cap

TE-BUFF **TE Buffer** - Ready to use
Content: TRIS-EDTA buffer
Volume: 1 x 4 mL/vial, brown cap

EXTRACT-PLATE 48 **Extraction Plate** - Ready to use
Content: 2 x 48 well plates coated with boronate affinity gel in a resealable pouch

HCL **Hydrochloric Acid** - Ready to use
Content: 0.025 M Hydrochloric Acid, yellow colored
Volume: 1 x 20 mL/vial, dark green cap

Standards and **Controls** - Ready to use

Cat. no.	Component	Color/Cap	Concentration ng/mL		Concentration nmol/L		Volume/ Vial
			ADR	NAD	ADR	NAD	
	STANDARD A	white	0	0	0	0	4 mL
	STANDARD B	light yellow	0.5	0.2	2.7	1.2	4 mL
	STANDARD C	orange	1.5	0.6	8.2	3.5	4 mL
	STANDARD D	dark blue	5	2	27	12	4 mL
	STANDARD E	light grey	20	8	109	47	4 mL
	STANDARD F	black	80	32	437	189	4 mL
	CONTROL 1	light green	Refer to QC-Report for expected value and				4 mL
	CONTROL 2	dark red	acceptable range!				4 mL

Conversion: Adrenaline (ng/mL) x 5.46 = Adrenaline (nmol/L)
Noradrenaline (ng/mL) x 5.91 = Noradrenaline (nmol/L)
Content: Acidic buffer with non-mercury stabilizer, spiked with defined quantity of adrenaline and noradrenaline

ACYL-BUFF **Acylation Buffer** - Ready to use
Content: Buffer with light alkaline pH for the acylation
Volume: 1 x 20 mL/vial, white cap

ACYL-REAG **Acylation Reagent** - Ready to use
Content: Acylation reagent in DMF and DMSO
Volume: 1 x 3 mL/vial, light red cap
Hazards identification:

H226 Flammable liquid and vapour.
H360D May damage the unborn child.
H312 + H332 Harmful in contact with skin or inhaled.
H319 Causes serious eye irritation.

COENZYME **Coenzyme** - Ready to use
Content: S-adenosyl-L-methionine
Volume: 1 x 4 mL/vial, purple cap

ENZYME **Enzyme** - Lyophilized
Content: Catechol-O-methyltransferase
Volume: 4 vials, pink cap

4.2 Additional materials and equipment required but not provided in the kit

- Calibrated precision pipettes to dispense volumes between 1 – 750 µL; 1 mL
- Microtiter plate washing device (manual, semi-automated or automated)
- ELISA reader capable of reading absorbance at 450 nm and if possible 620 – 650 nm
- Shaker (shaking amplitude 3 mm; approx. 600 rpm)
- Temperature controlled incubator (37 °C) or similar heating device
- Absorbent material (paper towel)
- Water (deionized, distilled, or ultra-pure)
- Vortex mixer
- Timer

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 1 mM) and sodium metabisulfite (final concentration 4 mM) to the sample.

6. Test procedure

Allow reagents and samples to reach room temperature and mix thoroughly by gentle inversion before use. Duplicate measurements are recommended. It is also recommended to number the strips of the microwell plate before usage to avoid any mix up.

The binding of the antiserum and the enzyme conjugate and the activity of the enzyme are temperature dependent, and the absorbance may vary if a thermostat is not used. The higher the temperature, the higher the absorbance will be. Varying incubation times will have a similar influence on the absorbance. 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 405nm.

6.1 Preparation of reagents

Wash Buffer
Dilute the 20 mL Wash Buffer Concentrate with water (deionized, distilled, or ultra-pure) to a final volume of 1000 mL.
Storage: 1 month at 2 – 8 °C

Enzyme Solution
Reconstitute the content of the vial labelled ‘Enzyme’ with 1 mL water (deionized, distilled, or ultra-pure) 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 must be prepared fresh prior to the assay (not longer than 10 to 15 minutes in advance). Discard after use!*

Adrenaline Microtiter Strips and Noradrenaline Microtiter Strips
In rare cases, residues of the blocking and stabilizing reagent can be seen in the wells as small, white dots or lines. These residues do not influence the quality of the product.

6.2 Sample preparation

The Bi-CAT (Epinephrine & Norepinephrine) Research ELISA is a flexible test system for various biological sample types and volumes. It is not possible to give general advice on how to prepare the samples. However, the following basics should help the researcher adjust 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 is mandatory during the extraction.
- Prevent catecholamine degradation by adding preservatives to the sample (see Section 5. Sample collection and storage).
- Avoid chaotropic chemicals like perchloric acid. High salt content may reduce catecholamine recovery. Neutralize samples containing high levels of perchloric acid prior to the extraction step.
- Tissue samples can be homogenized in 0.01 N HCl with EDTA and sodium metabisulfite. Under these conditions, catecholamines are 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 catecholamines.
- It is recommended to perform a "Proof of Principle" to determine the recovery of the catecholamines in the samples. Add small amounts of concentrated stocks of adrenaline and noradrenaline (to change the native sample matrix as little as possible) to the sample matrix and check the recovery.
- The used sample volume determines the test sensitivity. Determine the sample volume needed to determine the catecholamines in your sample by testing different amounts of sample volume.

If support is needed establishing a protocol, do not hesitate to contact ALPCO directly!

6.3 Extraction and acylation

The Bi-CAT (Epinephrine & Norepinephrine) 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:

- if sample volumes are between 1 – 100 µL follow **1.1**
- if sample volumes are between 100 – 500 µL follow **1.2**
- if sample volumes are 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: **20 µL standards, 20 µL controls and 1 – 100 µL sample**. Fill up each well with water (deionized, distilled, or ultra-pure) to a **final volume** of 100 µL [e.g. 20 µL standard plus 80 µL water (deionized, distilled, or ultra-pure)].	Pipette into the respective wells of the Extraction Plate: **20 µL standards, 20 µL controls and 100 – 500 µL sample**. Fill up each well with water (deionized, distilled, or ultra-pure) to a **final volume** of 500 µL [e.g. 20 µL standard plus 480 µL water (deionized, distilled, or ultra-pure)].	Pipette into the respective wells of the Extraction Plate: **20 µL of standards, 20 µL controls and 500 – 750 µL sample**. Fill up each well with water (deionized, distilled, or ultra-pure) to a **final volume** of 750 µL [e.g. 20 µL standard plus 730 µL water (deionized, distilled, or ultra-pure)].
2.	Pipette **25 µL** of **TE Buffer** into all wells		
3.	Cover the plate with **Adhesive Foil**. Shake **60 min** at **RT** (20 – 25 °C) on a **shaker** (approx. 600 rpm).		
4.	Remove the foil and empty the plate. Blot dry by tapping the inverted plate on absorbent material.		
5.	Pipette **1 mL** of **1X Working Wash Buffer** into all wells.		
6.	Shake **5 min** at **RT** (20 – 25 °C) on a **shaker** (approx. 600 rpm).		
7.	Blot dry by tapping the inverted plate on absorbent material.		
8.	**Wash one more time** as described (step 5, 6 and 7).		

9. Pipette **150 µL** of **Acylation Buffer** into all wells.
10. Pipette **25 µL** of **Acylation Reagent** into all wells.
11. Shake **20 min** at **RT** (20 – 25 °C) on a **shaker** (approx. 600 rpm).
12. Empty the plate and blot dry by tapping the inverted plate on absorbent material.
13. Pipette **1 mL** of 1x Working **Wash Buffer** into all wells.
14. Shake **5 min** at **RT** (20 – 25 °C) on a **shaker** (approx. 600 rpm).
15. Blot dry by tapping the inverted plate on absorbent material.
16. Wash one more time as described (step 13, 14, 15).
17. Pipette **150 µL** of **Hydrochloric Acid** into all wells.
18. Cover plate with **Adhesive Foil**. Shake **10 min** at **RT** (20 – 25 °C) on a **shaker** (approx. 600 rpm). ***Do not decant the supernatant thereafter!*** **140 µL of the supernatant is needed for the subsequent enzymatic conversion**

6.4 Enzymatic Conversion

1.Pipette **140 µL** of the **extracted standards, controls,** and **samples** into the respective **Microtiter Plate** wells**.**
2.Add **50 µL** of **Enzyme Solution** (refer to 6.1*)* to all wells.
3.Cover plate with **Adhesive Foil**. Shake **1 min** at **RT** (20 – 25 °C) on a **shaker** (approx. 600 rpm).
4.Incubate for **2 hours** at **37°C.** The following volumes of the supernatants are needed for the subsequent ELISA: **Adrenaline 90 µL** **Noradrenaline 90 µL**

6.5 Adrenaline and Noradrenaline ELISA

1. Pipette **90 µL** of **standards**, **controls** and **samples** from the **Enzyme Plate** (refer to 6.4) into the respective pre-coated **Microtiter Strips (*1)**.
2. Pipette **50 µL** of the respective **Antiserum (*2)** into all wells.
3. Cover the plate with **Adhesive Foil**. Shake **1 min** at **RT** (20 – 25 °C) on a **shaker** (approx. 600 rpm).
4. Incubate for **15 – 20 hours** (overnight) at **2 – 8 °C**.
5. Remove the foil. Discard or aspirate the content of the wells. Wash the plate **4 times** by adding **300 µL** of **1x Working Wash Buffer**, **discarding** the content and **blotting dry each time** by tapping the inverted plate on absorbent material.
6. Pipette **100 µL** of **Enzyme Conjugate** into all wells.
7. Cover the plate with **Adhesive Foil**. Incubate **30 min** at **RT** (20 – 25 °C) on a **shaker** (approx. 600 rpm).
8. Remove the foil. Discard or aspirate well contents. Wash the plate **4 times** by adding **300 µL** of **1x Working Wash Buffer**, **discarding** contents and **blotting dry each time** by tapping the inverted plate on absorbent material.
9. Pipette **100 µL** of **Substrate** into all wells.
10. Incubate **20 - 30 min** at **RT** (20 – 25 °C) on a **shaker** (approx. 600 rpm). ***Avoid exposure to direct sunlight!***
11. Pipette **100 µL** of **Stop Solution** into all wells.
12. Read the absorbance of the solution in the wells within 10 minutes, using a microplate reader set to **450 nm** (if available a reference wavelength between 620 nm and 650 nm is recommended).

(*1): **Adrenaline Microtiter Strips, Noradrenaline Microtiter Strips**

(*2): **Adrenaline Antiserum, Noradrenaline Antiserum**

7. Calculation of results

The standard 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 standard concentrations (logarithmic, x-axis).
Use a non-linear regression for curve fitting (e.g. 4-parameter, spline, akima).

This assay is a competitive assay. This means: the OD-values are decreasing with increasing concentrations of the analyte. OD-values found below the standard curve correspond to high concentrations of the analyte in the sample and must be reported as positive.

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

$$\text{Correction factor} = \frac{\text{20 µL (volume of standards extracted)}}{\text{sample volume (µL) extracted}}$$

Example:
750 µL of the sample is extracted. The concentration taken from the standard curve is 0.15 ng/mL noradrenaline.

Correction factor = 20/750 = 0.027
Concentration of the sample = 0.15 ng/mL x 0.027 = 0.004 ng/mL = 4 pg/mL noradrenaline

Conversion

Adrenaline (ng/mL) x 5.46 = Adrenaline (nmol/L)
Noradrenaline (ng/mL) x 5.91 = Noradrenaline (nmol/L)

7.1 Quality control

The confidence limits of the kit controls are indicated on the QC-Report.

8. Assay characteristics

	Substance	Cross-Reactivity (%)	
		Noradrenaline	Adrenaline
Analytical Specificity (Cross-Reactivity)	Derivatized Adrenaline	0.14	100
	Derivatized Noradrenaline	100	0.20
	Derivatized Dopamine	0.2	< 0.0007
	Metanephrine	< 0.003	0.64
	Normetanephrine	0.48	0.0009
	3-Methoxytyramine	< 0.003	< 0.0007
	3-Methoxy-4-hydroxyphenylglycol	0.01	0.03
	Tyramine	< 0.003	< 0.0007
	Phenylalanine, Caffeinic acid, L-Dopa, Homovanillic acid, Tyrosine, 3-Methoxy-4-hydroxymandelic acid	< 0.003	< 0.0007

Sensitivity (Limit of Detection)	**Adrenaline**	**Noradrenaline**
	0.25 ng/mL x C*	0.1 ng/mL x C*

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

Analytical Sensitivity (750 µL undiluted sample)	**Adrenaline**	**Noradrenaline**
	6.6 pg/mL	2.6 pg/mL

Functional Sensitivity (750 µL undiluted sample)	Adrenaline	Noradrenaline
	10 pg/mL	4 pg/mL

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
Noradrenaline	high	1377.4 ± 483.6	161.2	11.7
	medium	502.6 ± 126.9	42.3	8.4
	low	32.7 ± 15.3	5.1	15.6
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
Noradrenaline	high	2027.8 ± 712.5	237.5	11.7
	medium	716.5 ± 179.7	59.9	8.4
	low	46.0 ± 16.8	5.6	12.2

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
Noradrenaline				
Human EDTA Plasma	116.5	104.8 – 125.6	8.0	6.9
Cell Culture Medium	96.7	70.6 – 124.7	17.1	17.7