5-HIAA ELISA

For the quantitative determination of 5-Hydroxyindolacetic Acid in urine samples.

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

Catalog Number: 17-5HIHU-E01
Size: 96 Wells
Version: 17.0 2022-05-17 – ALPCO 3.0

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1. Introduction

1.1. Intended Use

Enzyme immunoassay for the quantitative determination of 5-Hydroxyindolacetic Acid (5-HIAA) in urine.

1.2. Principle of the Assay

The quantitative determination of 5-HIAA follows the basic principles of a competitive enzyme immunoassay. First, 5-HIAA is derivatized by methylation. The subsequent competitive ELISA uses the microtiter plate format. The antigen is bound to the solid phase of the microtiter plate. The methylated analyte in the standards, controls, and samples and the solid phase bound analyte compete for a fixed number of antibody binding sites. After the system has reached 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, Warnings and Limitations

2.1. 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 protective 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 °C to 8 °C in the sealed foil pouch with desiccant and used in the frame provided.
7. Duplicate determination of sample is highly recommended to be able to identify potential pipetting errors.
8. Once the test has been started, all steps should be completed without interruption. Make sure that the required reagents, materials, and devices are prepared and ready at the appropriate time.
9. Incubation times do influence the results. All 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. For information on hazardous substances included in the kit please refer to Safety Data Sheet (SDS). The Safety Data Sheet for this product is made available directly on ALPCO's website.
17. Kit reagents must be regarded as hazardous waste and disposed according to national regulations.
18. In case of any severe damage to the test kit or components, ALPCO must be informed in writing within 1 week of receipt of the kit. Severely damaged single components must not be used for a test run. They must be stored properly until next steps are determined. If it is decided that they are no longer suitable for measurements, they must be disposed of in accordance with national regulations.

2.2. Limitations

Any inappropriate handling of samples or modification of this test might influence the results.

2.2.1. Interfering substances

24-hour urine
Please note the sample preparation! If the percentage of the final concentration of acid is too high, this will lead to incorrect results for urine samples.

2.2.2. Drug and food interferences

Foods generally rich in serotonin such as bananas, pineapple, plums, kiwi fruit, tomatoes, avocados, various nuts, and chocolate should be avoided a few days before sample collection. Drugs/substances such as imipramine, isoniazid, isocarboxazid, methyldopa, levodopa, MAO-inhibitors, general OTC medication, alcohol, paracetamol, diazepam, oprenolol, atenolol, phenothiazines, indomethican, naproxen, reserpine, glyceryl-guaiacolate have an influence of urinary 5-HIAA levels and should be discontinued a few days before sample collection. .

2.2.3. High-Dose-Hook effect

No hook effect was observed in this assay.

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, the reagents are stable for 2 months when stored at 2 – 8°C. Once the resealable pouch has been opened, care should be taken to close it tightly with desiccant again. Make sure that the Methylation Reagent is recapped immediately after pipetting.

4. Materials

4.1. Contents of the kit

FOILS	**Adhesive Foil** - Ready to use
Contents:	Adhesive Foils in a resealable pouch
Volume:	1 x 4 foils
REAC-PLATE	**Reaction Plate** - Ready to use
Contents:	1 x 96 well plate, empty in a resealable pouch
WASH-CONC 50x	**Wash Buffer Concentrate -** Concentrated 50x
Contents:	Buffer with a non-ionic detergent and physiological pH
Volume:	1 x 20 mL/vial, purple cap

CONJUGATE **Enzyme Conjugate** - Ready to use
Contents: Goat anti-rabbit immunoglobulins conjugated with peroxidase
Volume: 1 x 12 mL/vial, red cap

SUBSTRATE **Substrate** - Ready to use
Contents: Chromogenic substrate containing 3,3',5,5'-tetramethylbenzidine, substrate buffer and hydrogen peroxide
Volume: 1 x 12 mL/black vial, black cap

STOP-SOLN **Stop Solution** - Ready to use
Contents: 0.25 M sulfuric acid
Volume: 1 x 12 mL/vial, grey cap

H290 May be corrosive to metals.

SER 5-HIAA **Serotonin 5-HIAA Microtiter Strips** - Ready to use
Contents: 1 x 96 well (12x8) antigen pre-coated microwell plate in a resealable pouch with desiccant

5-HIAA-AS **5-HIAA Antiserum** - Ready to use
Contents: Rabbit anti-5-HIAA antibody, blue colored
Volume: 1 x 6 mL/vial, blue cap

Standards and **Controls** - Ready to use

Component	Color/Cap	Concentration mg/L 5-HIAA	Concentration mmol/L 5-HIAA	Volume/ vial
STANDARD A	white	0	0	4 mL
STANDARD B	yellow	0.5	2.63	4 mL
STANDARD C	orange	1.5	7.88	4 mL
STANDARD D	blue	5	26.3	4 mL
STANDARD E	grey	15	78.8	4 mL
STANDARD F	black	50	262.5	4 mL
CONTROL 1	green	Refer to QC Report for expected value and acceptable range.		4 mL
CONTROL 2	red			4 mL

Conversion: 5-HIAA (mg/L) x 5.25 = 5-HIAA (µmol/L)
Contents: Acidic buffer spiked with defined quantity of 5-HIAA

DILUENT **Diluent –** Ready to use
Contents: Acidic buffer with non-mercury preservatives
Volume: 1 x 22 mL/vial, white cap

ASSAY-BUFF **Assay Buffer –** Ready to use
Contents: TRIS containing buffer with non-mercury preservative
Volume: 2 x 55 mL/vial, green cap

METHYL-BUFF **Methylation Buffer** - Ready to use
Contents: Methanol and dimethyl sulfoxide

Volume:	1 x 11 mL/vial, brown cap
Hazards identification:	 H225 Highly flammable liquid and vapor. H331 Toxic if inhaled. H370 Causes damage to organs

METHYL-REAG	**Methylation Reagent** – Ready to use
Contents:	Methylation reagent in hexane
Volume:	1 x 2.25 mL, white cap
Hazards identification:	 H304 May be fatal if swallowed and enters airways H315 Causes skin irritation H225 Highly flammable liquid and vapor. H302 Harmful if swallowed. H370 Causes damage to organs. H330 Fatal if inhaled. H336 May cause drowsiness or dizziness. H350 May cause cancer. H361f Suspected of damaging fertility H373 Toxic to aquatic life with long lasting effects.

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

- Calibrated precision pipettes to dispense volumes between 20 - 300 µL; 1 mL
- 3.5 mL Polypropylene tubes and suitable rack
- Microtiter plate washing device (manual, semi-automated or automated)
- ELISA reader capable of reading absorbance at 450 nm and if possible 620 - 650 nm
- Microtiter plate shaker (recommended shaking amplitude 3 mm; approx. 600 rpm)
- Absorbent material (paper towel)
- Ventilated hood
- Water (deionized, distilled, or ultra-pure)
- Vortex mixer
- Timer

5. Sample Collection and Storage

24-hour urine sample is used for analysis. Over a defined period of 24 hours, all urine is collected in a bottle with acid (10-15 mL 6M hydrochloric acid) provided for stabilization and the total volume is noted for evaluation of the results. During the collection period, the collected sample must always be stored in a cool place protected from light (2-8°C). Storage for a short period of up to 7 days is at 2-8°C. Storage for a longer period up to 6 months is at -20°C.

Repeated freezing and thawing should be avoided. Avoid exposure to direct sunlight.

6. Assay Procedure

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

The binding of the antisera and the enzyme conjugate and the activity of the enzyme are temperature dependent. The higher the temperature, the higher the absorption values will be. Varying incubation times will have similar influences on the absorbance. The optimal temperature during the enzyme immunoassay is between 20 – 25 °C.

If a partial plate is run, unused wells in Reaction and Extraction plates should be covered to avoid contamination. After preparation, the used wells must be labeled to prevent double use.

⚠ *The use of a microtiter plate shaker with the following specifications is mandatory: shaking amplitude 3mm; approx. 600 rpm. Shaking with different settings might influence the results.*

⚠ *The Methylation Reagent is highly volatile. If possible, please pipette the Methylation Reagent with a repeater pipette and make sure that the vial is recapped immediately after pipetting.*

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: 2 months at 2 - 8 °C

Serotonin 5-HIAA 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. Predilution of the standards, controls, and samples

1. Pipette **50 µL** of **standards, controls,** and **urine samples** into the respective wells of the **Reaction Plate.**
2. Pipette **200 µL** of the **Diluent** into all wells.
3. Shake for **1 minute** at **Room Temperature** (20 - 25 °C) on a **shaker** (approx. 600 rpm). **20 µL** are needed for the **methylation.**

6.3. Methylation

1. Pipette **20 µL** of the **prediluted standards**, **controls,** and **urine samples** into the respective **Polypropylene Tubes.**
⚠ *The following steps 2-5 must be performed in a ventilated hood!*
2. Pipette **100 µL** of **Methylation Buffer** into all tubes.
3. Add **20 µL** of **Methylation Reagent** to each tube and **mix each tube immediately after addition of the Methylation Reagent.**
4. Cover all tubes and **methylate** for **20 minutes** at **Room Temperature** (approx. 20 °C).
5. Pipette **1000 µL** of **Assay Buffer** into all tubes. *After this step the use of a ventilated hood is not necessary anymore.*
⚠ **Proceed** with the **ELISA** (Section 6.4) **immediately** as the methylated standards, controls and samples are **only stable for 1 hour**!

6.4. 5-HIAA ELISA

1.	Pipette **25 µL** of the **methylated standards, controls,** and **samples** into the appropriate wells of the **5-HIAA Microtiter Strips.**
2.	Pipette **50 µL** of the **5-HIAA Antiserum** into all wells.
3.	Cover plate with **Adhesive Foil** and incubate for **1 hour** at **Room Temperature** (20 – 25°C) on a **shaker** (approx. 600 rpm)
4.	Remove the foil. Discard or aspirate the contents of the wells. Wash the plate **4 times** by adding **300 µL** of 1X working **Wash Buffer, discarding** the contents and **blotting dry each time** by tapping the inverted plate on absorbent material.
5.	Pipette **100 µL** of the **Enzyme Conjugate** into all wells.
6.	Cover plate with **Adhesive Foil** and incubate for **1 hour** at **Room Temperature** (20 – 25°C) on a **shaker** (approx. 600 rpm).
7.	Remove the foil. Discard or aspirate the contents of the wells. Wash the plate **4 times** by adding **300 µL** of 1X working **Wash Buffer, discarding** the contents and **blotting dry each time** by tapping the inverted plate on absorbent material.
8.	Pipette **100 µL** of the **Substrate** into all wells and incubate for **20 - 30 min** at **RT** (20 – 25°C) on a **shaker** (approx. 600 rpm). ***Avoid exposure to direct sunlight!***
9.	Add **100 µL** of the **Stop Solution** to each well and shake the microtiter plate briefly to ensure a homogeneous distribution of the solution.
10.	**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).

7. Calculation of results

Measuring range	5-HIAA
	0.4 – 50 mg/L

The standard curve is obtained by plotting the absorbance readings (calculate the mean absorbance) of the standards (linear, y-axis) against the corresponding standard concentrations (logarithmic, x-axis) using a concentration of 0.001 mg/l for Standard A. (This alignment is mandatory due to the logarithmic presentation of the data.) Use a non-linear regression for curve fitting (e.g. 4-parameter, marquardt).

This assay is a competitive assay. This means the OD-values decrease with increasing concentration 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 greater than the top standard concentration.

Urine samples and controls
The concentrations of the **urine samples** and the **controls** can be read directly from the standard curve. Samples found with concentrations higher than the highest standard (Standard F) should be diluted accordingly with deionized, distilled, or ultra-pure water and must be re-assayed.

The total amount of 5-HIAA excreted in urine in 24 hours is calculated as follows:
mg/24h = mg/L x L/24h

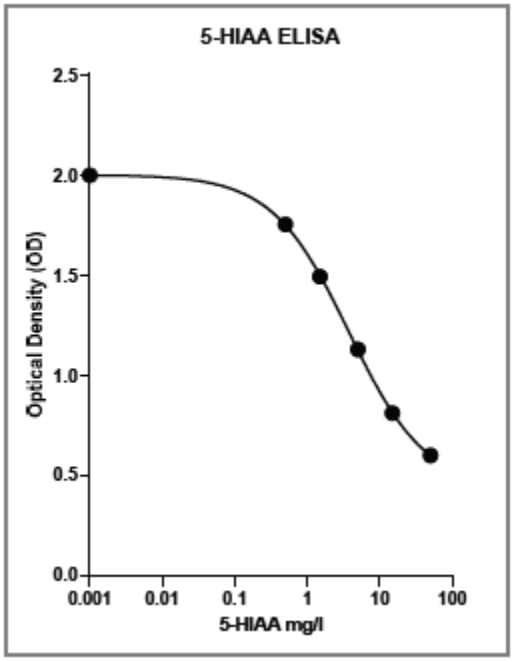
Conversion
5-HIAA (mg/L) x 5.25 = 5-HIAA (µmol/L)

7.1. Quality control

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

7.2. Typical standard curve

Example only, do not use for calculation!



8. Assay Characteristics

Analytical Sensitivity	**5-HIAA**
Limit of Blank (LOB)	0.16 mg/L
Limit of Detection (LOD)	0.23 mg/L
Limit of Quantification (LOQ)	0.40 mg/L

	Substance	**Cross-Reactivity (%)**
		5-HIAA
Analytical Specificity (Cross-Reactivity)	Serotonin	7.6
	5-Hydroxy-DL-Tryptophan	2.3
	Tryptamine	< 0.1
	Melatonin	< 0.1
	5-Methoxytryptamine	< 0.1
	DL-Vanillic mandelic acid	< 0.1
	Homovanillic Acid	< 0.1

Precision	
Intra-Assay (n=24)	**Inter-Assay (n=9)**

Sample	Range (mg/L)	CV (%)	Sample	Range (mg/L)	CV (%)
1	1.1 ± 0.15	13.3	1	11.3 ± 1.3	11.9
2	1.9 ± 0.18	9.3	2	4.8 ± 0.6	12.8
3	5.2 ± 0.48	9.0	3	3.1 ± 0.3	8.6
4	14.3 ± 1.2	8.7	4	7.3 ± 0.8	10.8
			5	19.0 ± 2.2	11.4

		Serial dilution up to	Range (%)	Mean (%)
Linearity	5-HIAA in Urine	1:10	98 - 112	105

		Range (mg/l)	Range (%)	Mean (%)
Recovery	5-HIAA in Urine	0.8 – 40.5	86 - 93	90

Method Comparison versus XLC-MS/MS	5-HIAA in urine	ELISA = 0.9749* (XLC-MS/MS) – 0.0868; r^2 = 0.98; n = 95

Inter-Lot					
	Sample	Reference Range (mg/l)	Mean ± SD (mg/l)	Mean ± SD (% Recovery)	CV (%)
5-HIAA in artificial matrix (n=-3)	1	3.0 – 7.0	4.9 ± 0.36	98.0 ± 7.2	7.4
	2	9.0 – 21.0	14.6 ± 1.3	97.3 ± 9.0	9.2

9. Metrological Traceability

The values assigned to the standards and controls of the 5-HIAA ELISA are traceable to SI units by calibrated weighting with quality-controlled analyte.

Standards and Controls	Uncertainty (%)
	1.2
5-HIAA ELISA	
Sample	Expanded Uncertainty (%) k = 2*
1	23.9
2	25.7
3	17.4
4	21.7
5	22.9

*This defines an interval about the measured result that includes the true value with a probability of 95%.

References/Literature

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