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Instructions for use

Citrulline ELISA

REF **BA E-2800**

2°C – 8°C

Σ 96

RUO For research use only – Not for use in diagnostic procedures

Citrulline ELISA

1. Introduction

1.1 Intended use and principle of the test

Enzyme Immunoassay for the quantitative determination of Citrulline in urine, serum, plasma and various biological samples.

The samples are first cleaned up by an extraction procedure. After derivatization Citrulline is quantitatively determined by ELISA. The competitive ELISA 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 analyte 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 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 reference curve prepared with known standards.

2. Procedural cautions, guidelines, warnings and limitations

(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 has to be used to run the assay. Reliable performance will only be attained by strict and careful adherence to the instructions provided.
(2) This assay was validated for a certain type of sample as indicated in *Intended Use* (please refer to Chapter 1). Any off-label use of this kit is in the responsibility of the user and the manufacturer cannot be held liable.
(3) The principles of Good Laboratory Practice (GLP) have to be followed.
(4) In order to reduce exposure to potentially harmful substances, wear lab coats, disposable protective gloves and protective glasses where necessary.
(5) All kit reagents and specimens should be brought to room temperature and mixed gently but thoroughly before use. Avoid repeated freezing and thawing of reagents and specimens.
(6) For dilution or reconstitution purposes, use deionized, distilled, or ultra-pure water.
(7) 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.
(8) Duplicate determination of sample is highly recommended to be able to identify potential pipetting errors.
(9) Once the test has been started, all steps should be completed without interruption. Make sure that the required reagents, materials and devices are prepared ready at the appropriate time.
(10) Incubation times do influence the results. All wells should be handled in the same order and time intervals.
(11) To avoid cross-contamination of reagents, use new disposable pipette tips for dispensing each reagent, sample, standard and control.
(12) A standard curve must be established for each run.
(13) The controls should be included in each run and fall within established confidence limits. The confidence limits are listed in the QC-Report provided with the kit.
(14) 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.
(15) 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.
(16) 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.
(17) For information on hazardous substances included in the kit please refer to Material Safety Data Sheet (MSDS). The Material Safety Data Sheet for this product is made available directly on the website of the manufacturer or upon request.
(18) The expected reference values reported in this test instruction are only indicative. It is recommended that each laboratory establishes its own reference intervals.
(19) Kit reagents must be regarded as hazardous waste and disposed of according to national regulations.

2.2 Limitations

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

2.2.1 Interfering substances

Serum/Plasma

Samples containing precipitates or fibrin strands or which are haemolytic or lipemic might cause inaccurate results.

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 the urine samples.

2.2.2 Drug interferences

There are no known substances (drugs) which ingestion interferes with the measurement of citrulline level in the sample.

2.2.3 High-Dose-Hook effect

No hook effect was observed in this test.

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 1 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

BA D-0090 FOILS **Adhesive Foil** - Ready to use
Contents: Adhesive Foils in a resealable pouch
Volume: 1 x 4 foils

BA D-0033 48 **Macrotiter Plate** - Ready to use
Contents: 2 x 48 well plate, empty in a resealable pouch

BA D-2442 EXTRACT-PLATE 48 **Extraction Plate** - Ready to use
Contents: 2 x 48 well plate, precoated with cation exchanger in a resealable pouch

BA E-0030 WASH-CONC 50x **Wash Buffer Concentrate -** Concentrated 50x
Contents: Buffer with a non-ionic detergent and physiological pH
Volume: 1 x 20 ml/vial, light purple cap

BA E-0040 CONJUGATE **Enzyme Conjugate** - Ready to use
Contents: Goat anti-rabbit immunoglobulins conjugated with peroxidase
Volume: 1 x 12 ml/vial, red cap

BA E-0055 SUBSTRATE **Substrate** - Ready to use
Contents: Chromogenic substrate containing tetramethylbenzidine, substrate buffer and hydrogen peroxide
Volume: 1 x 12 ml/black vial, black cap

BA E-0080 STOP-SOLN **Stop Solution** - Ready to use
Contents: 0.25 M sulfuric acid
Volume: 1 x 12 ml/vial, light grey cap

BA E-2831 CIT **Citrulline Microtiter Strips** - Ready to use
Contents: 1 x 96 well (12x8) antigen precoated microwell plate in a resealable foil pouch with desiccant

BA E-2810 AS CIT **Citrulline Antiserum** - Ready to use
Contents: Rabbit anti-Citrulline antibody, blue coloured
Volume: 1 x 6 ml/vial, blue cap

BA E-2413 ASSAY-BUFF **Assay Buffer** - Ready to use
Contents: Buffer with alkaline pH
Volume: 1 x 20 ml/vial, yellow cap

BA E-2428 EQUA-REAG **Equalizing Reagent** - Lyophilized

Contents: Lyophilized protein

Volume: 1 vial, brown cap

Standards and **Controls** - Ready to use

Cat. no.	Component	Colour/Cap	Concentration µg/ml	Concentration µmol/l	Volume/ Vial
BA E-2801	STANDARD A	white	0	0	4 ml
BA E-2802	STANDARD B	light yellow	0.6	3.4	4 ml
BA E-2803	STANDARD C	orange	2	11.4	4 ml
BA E-2804	STANDARD D	dark blue	6	34.3	4 ml
BA E-2805	STANDARD E	light grey	20	114	4 ml
BA E-2806	STANDARD F	black	60	343	4 ml
BA E-2851	CONTROL 1	light green	Refer to QC-Report for expected value and acceptable range!		4 ml
BA E-2852	CONTROL 2	dark red			4 ml

Conversion: Citrulline (µg/ml) x 5.71 = Citrulline (µmol/l)

Content: Acidic buffer with non-mercury preservative, spiked with defined quantity of Citrulline

BA E-2446 D-REAGENT **D-Reagent** - Ready to use

Contents: Crosslinking agent in dimethylsulfoxide

Volume: 1 x 4 ml/vial, white cap

Hazards identification:

H318 Causes serious eye damage.
H334 May cause allergy or asthma symptoms or breathing difficulties if inhaled.
H332 Harmful if inhaled.
H315 Causes skin irritation.
H317 May cause an allergic skin reaction.

BA E-2458 Q-BUFFER **Q-Buffer** - Ready to use

Contents: Tris containing buffer

Volume: 1 x 20 ml/vial, white cap

BA E-2460 DILUENT **Diluent** - Ready to use

Contents: Buffer with acidic pH

Volume: 2 x 20 ml/vial, dark green cap

BA E-2787 NAOH **NaOH** - Ready to use

Contents: Sodium hydroxide solution

Volume: 1 x 2 ml/vial, purple cap

Hazards identification:

H314 Causes severe skin burns and eye damage.

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

- Calibrated precision pipettes to dispense volumes between 20 µl – 300 µl; 10 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
- Microtiter plate shaker (shaking amplitude 3 mm; approx. 600 rpm)
- Absorbent material (paper towel)
- Water (deionized, distilled, or ultra-pure)
- Vortex mixer

5. Sample collection and storage

Urine
Spontaneous urine or 24-hour urine, collected in a bottle containing 10 - 15 ml of 6 M HCl, can be used.
If 24-hour urine is used please record the total volume of the collected urine.
Storage: for longer periods (up to 6 month) at -20 °C.
Repeated freezing and thawing should be avoided. Avoid exposure to direct sunlight.

Plasma
Whole blood should be collected into centrifuge tubes (Monovette™ or Vacuette™) containing an anti-coagulant and centrifuged (according to manufacturer's instructions) immediately after collection.
Haemolytic and lipemic samples should not be used for the assay.
Storage: for a longer period (up to 6 month) at -20 °C.
Repeated freezing and thawing should be avoided.

Serum
Collect blood by venipuncture (Monovette™ or Vacuette™ for serum), allow to clot, and separate serum by centrifugation according to manufacturer's instruction. Do not centrifuge before complete clotting has occurred. Patients receiving anticoagulant therapy may require increased clotting time.
Haemolytic and lipemic samples should not be used for the assay.
Storage: for a longer period (up to 6 month) at -20 °C.
Repeated freezing and thawing should be avoided.

6. Test procedure

Allow all reagents to reach room temperature and mix thoroughly by gentle inversion before use. Number the extraction plate and microwell plate (microtiter strips which are removed from the frame for usage should be marked accordingly to avoid any mix-up). Duplicate determinations are recommended.

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

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

Equalizing Reagent
Reconstitute the Equalizing Reagent with **10 ml** of **Assay Buffer**.
Reconstituted Equalizing Reagent which is not used immediately has to be stored in aliquots for max 1 month at -20 °C and may be thawed only once.

D-Reagent
The D-Reagent has a freezing point of 18.5 °C. To ensure that the D-Reagent is liquid when being used, it must be ensured that the D-Reagent has reached room temperature and forms a homogeneous, crystal-free solution.

6.2 Preparation of samples

Serum and plasma samples
Serum and plasma samples have to be diluted 1:4 with Standard A prior to the test, for example:
50 µl sample + 150 µl Standard A.

Other sample types
The Citrulline ELISA is a flexible test system for various biological sample types. It is not possible to give a general advice how to prepare the samples. However, the following basics should help the researcher to adapt the protocol to his specific needs:

- It is advisable to perform a **Proof of Principle** to determine the recovery of Citrulline from the samples. Prepare a stock solution of Citrulline. 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.
- Avoid excess of acid: excess of acid might exceed the buffer capacity of the dilution buffer. A **pH of 5.0** during the extraction is mandatory.
- Samples with concentrations above the measuring range can be diluted with the included diluent.
- Samples with an interfering sample matrix can also be diluted with the included diluent.

6.3 Extraction

1.	Pipette **50 µl** of **standards**, **controls, urines** and **diluted serum and plasma samples** (refer to 6.2) into the respective wells of the **Extraction Plate.**
2.	Add **300 µl** of **Diluent** to all wells and shake for **10 min** at **RT** (20 - 25 °C) on a **shaker** (approx. 600 rpm).
⚠	Take **100 µl** of the **supernatant** for the **derivatization**.

6.4 Derivatization

1.	Pipette **100 µl** of the **standards, controls** and **samples** into the appropriate wells of the **Macrotiter Plate.**
2.	Add **20 µl** of the **NaOH** to all wells and mix shortly.
2.	Add **100 µl** of the **Equalizing Reagent** (refer to 6.1) to all wells.
4.	Add **20 µl** of the **D-Reagent** to all wells.
5.	Cover plate with **Adhesive Foil** and shake for **2 h** at **RT** (20 - 25 °C) on a **shaker** (approx. 600 rpm).
6.	Pipette **150 µl Q-Buffer** into all wells and shake for **10 min** at **RT** (20 - 25 °C) on a **shaker** (approx. 600 rpm).
⚠	Take **50 µl** for the **ELISA**!

6.5 Citrulline ELISA

1.	Pipette **50 µl** of the **prepared standards, controls** and **samples** into the appropriate wells of the **Citrulline Microtiter Strips.**
2.	Pipette **50 µl** of the **Citrulline Antiserum** into all wells and mix shortly.
3.	Cover plate with **Adhesive Foil** and incubate for **15 - 20 h** (overnight) at **2 - 8 °C**.
4.	Remove the foil. Discard or aspirate the contents of the wells. Wash the plate **3 x** by adding **300 µl** of **Wash Buffer**, **discarding** the content 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.	Incubate for **30 min** at **RT** (20 - 25 °C) on a **shaker** (approx. 600 rpm).
7.	Discard or aspirate the contents of the wells. Wash the plate **3 x** by adding **300 µl** of **Wash Buffer**, **discarding** the content 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 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 (LOD – highest standard)	**Citrulline**
	0.23 – 60 µg/ml

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). Use non-linear regression for curve fitting (e.g. spline, 4- parameter, 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 have to be reported as being positive.*

The concentrations of the **controls and urine samples** can be read directly from the standard curve.

The concentrations of the **serum and plasma** samples have to be **multiplied by 4**.

The total amount of Citrulline excreted in urine during 24 h is calculated as following:
µg/24h = µg/ml x ml/24h

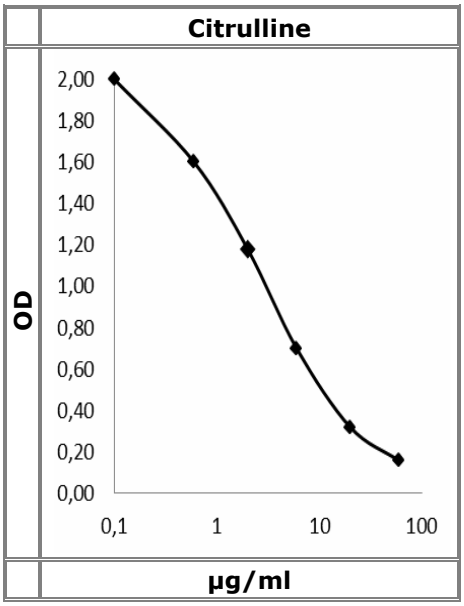
Conversion
Citrulline (µg/ml) x 5.71 = Citrulline (µmol/l)

7.1 Quality control

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

7.2 Typical standard curve

⚠ *Example, do not use for calculation!*



8. Assay characteristics

Analytical Sensitivity (Limit of Detection)	Citrulline
	0.23 µg/ml

Analytical Specificity (Cross Reactivity)	Substance	Cross Reactivity (%)
	DL-Citrulline	100
	L-Arginin	<0.001
	L-Glutamine	<0.001
	L-Ornithin	<0.001

Precision: Intra Assay CV		Sample	Mean concentration (µg/ml)	Intra Assay CV (%)
	Urine	1	0.80	10.8
		2	1.54	12.8
		3	5.23	9.6
		4	10.6	6.1
	Plasma	1	1.45	13.2
		2	2.28	11.2
		3	6.18	7.0
		4	10.6	7.9
	Serum	1	1.33	12.0
		2	2.19	10.3
		3	5.79	7.4
		4	10.8	6.4

		Sample	Mean concentration (µg/ml)	Inter Assay CV (%)
Precision: Inter Assay CV	Urine	1	1.3	13.4
		2	4.9	6.9
		3	10.1	5.4
	Plasma	1	2.2	6.9
		2	5.5	8.3
		3	10.9	5.9
	Serum	1	2.1	10.8
		2	5.4	17.0
		3	11.1	14.1

		Serial dilution up to	Mean Linearity (%)	Range Linearity (%)
Linearity	Urine	1:32	87	80 – 97
	Plasma	1:32	93	86 – 95
	Serum	1:32	93	86 – 101

		Sample	Mean concentration (µg/ml)	Mean Recovery (%)
Recovery	Urine	1	1.54	85
		2	5.23	90
		3	10.6	98
	Plasma	1	2.28	93
		2	6.18	96
		3	10.6	93
	Serum	1	2.19	94
		2	5.79	92
		3	10.8	95

⚠ ***For literature or any other information please contact your local supplier.***

Symbols:

	Storage temperature		Manufacturer		Contains sufficient for <n> tests
	Expiry date	LOT	Batch code	I V D	For in-vitro diagnostic use only!
	Consult instructions for use	CONT	Content	CE	CE labelled
	Caution	REF	Catalogue number	RUO	For research use only!