

## Instructions for use

# L-Citrulline ELISA

## Citrulline ELISA

### 1. Intended use and principle of the test

Enzyme Immunoassay for the quantitative determination of L-Citrulline (CIT) in plasma samples.

After extraction and 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 processed 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

#### 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 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 certain types of samples 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.
- (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<sub>2</sub>SO<sub>4</sub>. 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 Safety Data Sheet (SDS). The 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) The results obtained with this test kit should not be taken as the sole reason for any therapeutic consequence but have to be correlated to other diagnostic tests and clinical observations.
- (20) 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.

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

### 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 Contents of the kit

**BA D-0090**  **Adhesive Foil** - Ready to use

Contents: Adhesive Foils in a resealable pouch

Volume: 1 x 4 foils

**IS I-0324**  **Reaction Plate** - Ready to use

Contents: 1 x 96 well plate, empty in a resealable pouch

**BA E-0030**  **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**  **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** - 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 Solution** - Ready to use

Contents: 0.25 M sulfuric acid

Volume: 1 x 12 mL/vial, light grey cap

Hazards  
identification:



H290 May be corrosive to metals.

**IS I-1531**  **Citruiline Microtiter Strips** - Ready to use

Contents: 1 x 96 well (12x8) antigen precoated microwell plate in a resealable pouch with desiccant, blue bag

**IS I-1510**  **Citruiline Antiserum** - Ready to use

Contents: Rabbit anti-Citruiline antibody, green coloured

Volume: 1 x 10 mL/vial, green cap

**BA E-2413**  **Assay Buffer** - Ready to use

Contents: Buffer with alkaline pH

Volume: 1 x 20 mL/vial, yellow cap

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

Contents: Lyophilized protein

Volume: 1 vial, brown cap

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

Contents: Crosslinking agent in dimethylsulfoxide

Volume: 1 x 3 mL/vial, white cap

**BA E-0429**  **Reducing Concentrate** - Concentrated 100X

Contents: Reducing agent in sodium hydroxide

Volume: 1 x 1 ml/vial, pink cap

Hazards  
identification  
:



H290 May be corrosive to metals.

H301 Toxic if swallowed.

H314 Causes severe skin burns and eye damage.

H360FD May damage fertility. May damage the unborn child

**IS I-0488** **PBS** **PBS** - Ready to use

Contents: Phosphate Buffered Saline

Volume: 1 x 50 ml/vial, orange cap

**BA E-2721** **PRECIPITATING REAGENT** **Precipitating Reagent** - Ready to use

Contents: Acidic reagent for precipitation of plasma/serum proteins, red coloured

Volume: 2 x 4 mL/vial, white cap

**Standards and Controls** - Ready to use

| Cat. no.         | Component       | Colour/Cap   | Concentration<br>µg/mL                                      | Concentration<br>µmol/L | Volume/<br>Vial |
|------------------|-----------------|--------------|---|-------------------------|-----------------|
| <b>IS I-1501</b> | <b>STANDARD</b> | white        | 0   | 0                       | 4 mL            |
| <b>IS I-1502</b> | <b>STANDARD</b> | light yellow | 1,12  | 6,4                     | 4 mL            |
| <b>IS I-1503</b> | <b>STANDARD</b> | orange       | 2,80  | 16                      | 4 mL            |
| <b>IS I-1504</b> | <b>STANDARD</b> | dark blue    | 7,01  | 40                      | 4 mL            |
| <b>IS I-1505</b> | <b>STANDARD</b> | light grey   | 17,52   | 100                     | 4 mL            |
| <b>IS I-1506</b> | <b>STANDARD</b> | black        | 43,80   | 250                     | 4 mL            |
| <b>IS I-1551</b> | <b>CONTROL</b>  | light green  | Refer to QC-Report for expected value and acceptable range! |                         | 4 mL            |
| <b>IS I-1552</b> | <b>CONTROL</b>  | dark red     |   |                         | 4 mL            |

Conversion: Citrulline (µg/mL) x 5,708 = Citrulline (µmol/L)

Contents: Acidic buffer with non-mercury stabilizer, spiked with defined quantity of Citrulline

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

- Calibrated precision pipettes to dispense volumes between 10 – 300 µL; 12.5 mL
- Polystyrene or polypropylene tubes (0.5 mL) 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 (shaking amplitude 3 mm; approx. 600 rpm)
- Absorbent material (paper towel)
- Water (deionized, distilled or ultra-pure)
- Vortex mixer

#### 5. Sample collection and storage

##### EDTA Plasma

Whole blood should be collected into centrifuge tubes containing EDTA as anti-coagulant (Monovette™ or Vacuette™ for plasma) and centrifuged according to manufacturer's instructions at room temperature immediately after collection.

Haemolytic and especially lipemic samples should not be used for the assay.

Storage: up to 48 hours at 2 - 8 °C, for longer period (up to 6 month) at -20 °C.

Repeated freezing and thawing should be avoided.

## 6. Test procedure

Allow all reagents and samples to reach room temperature and mix thoroughly by gentle inversion before use. Duplicate determinations are recommended. To do that, perform duplicates after derivatization (1 derivatization well = 2 competition wells).

It is recommended to number the strips of the microwell plate before usage to avoid any mix-up.

The binding of the antisera and of the enzyme conjugate and the activity of the enzyme are temperature dependent, and the absorbance values may vary if a thermostat is not used. The higher the temperature, the higher the extinction values will be. Corresponding variations also apply to the incubation times. The optimal temperature during 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 **12.5 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. It must be ensured that the D-Reagent has reached room temperature and forms a homogeneous, crystal-free solution.

#### Reducing Solution

Dilute Reducing Concentrate 1:100 with water (deionized, distilled, or ultra-pure) and mix thoroughly. Use immediately!


Examples for the preparation of Reducing Solution:

|                             |         |         |         |          |
|-----------------------------|---------|---------|---------|----------|
| <b>Reducing Concentrate</b> | 40 µL   | 50 µL   | 80 µL   | 160 µL   |
| <b>Water</b>                | 3.96 mL | 4.95 mL | 7.92 mL | 15.84 mL |

#### Citrulline 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 Precipitation

|   |  |
|---|--|
| 1.  | Pipette <b>20 µL</b> of <b>standards, controls</b> and <b>samples</b> into the respective tubes.   |
| 2.  | Add <b>400 µL</b> of <b>PBS</b> to the standards, controls and samples.                            |
| 3.  | Add <b>50 µL Precipitating Reagent</b> to all tubes.   |
| 4.  | Mix the <b>tubes</b> thoroughly (vortex) and centrifuge for <b>15 minutes</b> at <b>3000 x g</b> . |
|  | Take <b>25 µL</b> of the clear supernatant for the <b>derivatization</b> .                         |

### 6.3 Derivatization

|    |   |
|----|---|
| 1. | Pipette <b>25 µL</b> of the <b>precipitated standards, controls</b> and <b>samples</b> into the appropriate wells of the <b>Reaction Plate</b> .                                      |
| 2. | Add <b>50 µL</b> of the <b>Equalizing Reagent</b> into all wells (2 min at RT under stirring at 700 rpm)  |
| 3. | Add <b>10 µL</b> of the <b>D-Reagent</b> into all wells (immerse the tips into the reaction medium).  |
| 4. | Cover plate with <b>Adhesive Foil</b> and incubate for <b>2 h</b> at <b>RT</b> (20 – 25 °C) on a <b>shaker</b> (approx. 500 rpm).   |
| 5. | Prepare <b>Reducing Solution 1X</b> from Reducing Concentrate 100X in <b>sterile water</b> (see 6.1)<br><b><i>The Reducing Solution should be prepared directly prior to use!</i></b> |
| 6. | Add <b>100 µL</b> of the <b>Reducing Solution 1X</b> into all wells.  |

7. Incubate for **10 min** at **RT** (20 – 25 °C) on a **shaker** (approx. 500 rpm).



**Use 25 µL for the ELISA!**


#### 6.4 Citrulline ELISA

1. Pipette **25 µL** of the **prepared standards, controls and samples** into the appropriate wells of the **Citrulline Microtiter Strips**.  
**Be carefull not to pipette bubbles!**
2. Add **100 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 content of the wells. Wash the plate **4 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. 500 rpm).
7. Discard or aspirate the content of the wells. Wash the plate **4 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 **15 - 25 min** at **RT** (20 – 25 °C) on a **shaker** (approx. 500 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 | Citrulline   |
|-----------------|--------------|
|                 | 6,4 – 250 µM |

The calibration 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.*

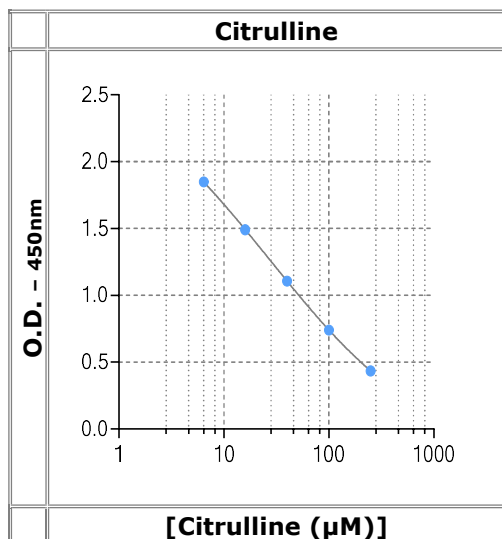
The concentrations of the samples and controls can be read directly from the standard curve.

##### 7.1 Quality control

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

##### 7.2 Typical standard curve

 *Example, do not use for calculation!*



## 8. Assay characteristics

|  |                   |
|--|-------------------|
| <b>Analytical Sensitivity<br/>(Limit of Detection)</b> | <b>Citrulline</b> |
|  | 1,47 µM           |

|  |                      |                             |
|--|----------------------|-----------------------------|
| <b>Analytical Specificity<br/>(Cross Reactivity)</b> | <b>Substance</b>     | <b>Cross Reactivity (%)</b> |
|  | L-Citrulline         | 100%                        |
|  | L-Arginine           | <0,04%                      |
|  | L-Ornithine          | <0,04%                      |
|  | L-Glutamine          | <0,04%                      |
|  | L-Glutamate          | <0,08%                      |
|  | Agmatine             | <0,04%                      |
|  | L-Argino-succinate   | <0,05%                      |
|  | NG-Methyl-L-Arginine | <0,1%                       |

| Plasma Sample | Mean<br>(µM) | SD<br>(µM) | CV (%) |
|---------------|--------------|------------|--------|
| 1 (n = 8)     | 30           | 1          | 5      |
| 2 (n = 8)     | 64           | 2          | 4      |
| 3 (n = 8)     | 101          | 3          | 3      |











|                  |   |                     |                    |
|------------------|---|---------------------|--------------------|
| <b>Linearity</b> | Plasma Sample<br>(Serial dilution up to 1:32) | Range Linearity (%) | Mean Linearity (%) |
|                  | 1   | 96 - 110            | 103                |
|                  | 2   | 90 - 103            | 97                 |
|                  | 3   | 97 - 105            | 101                |

|                 |               |                    |                   |
|-----------------|---------------|--------------------|-------------------|
| <b>Recovery</b> | Plasma Sample | Range Recovery (%) | Mean Recovery (%) |
|                 | 1             | 92 - 108           | 98                |
|                 | 2             | 90 - 101           | 94                |
|                 | 3             | 96 - 107           | 101               |

|   |                |  |                          |
|---|----------------|--|--------------------------|
| <b>Methode Comparison:<br/>ELISA vs LC/MS</b> | Plasma samples | $[L\text{-Citrulline}]_{\text{ELISA}} = 0,9803 * [L\text{-Citrulline}]_{\text{LC-MS}} + 5,018$ | $R^2 = 0,9932$<br>N = 40 |
|---|----------------|--|--------------------------|

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

**Symbols:**

|   |                              |   |                  |   |                                   |
|---|------------------------------|---|------------------|---|-----------------------------------|
|  | Storage temperature          |  | Manufacturer     |  | Contains sufficient for <n> tests |
|  | Expiry date                  |  | Batch code       |  | For research use only!            |
|  | Consult instructions for use |  | Content          |   |                                   |
|  | Caution                      |  | Catalogue number |   |                                   |