Technical Bulletin

Citrulline Fluorometric Assay Kit

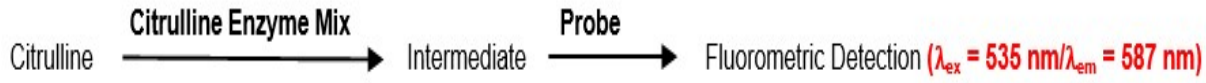
Catalog Number MAK423

Product Description

L-Citrulline is a non-proteogenic, semi-essential amino acid. Citrulline is formed either by ornithine carbamoyltransferase or as a by-product of Nitric Oxide Synthase (NOS) activity. Citrulline generated in the NOS reaction can be recycled to arginine by the two enzymes argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL) acting in the Urea Cycle. Citrulline concentrations have been shown to be elevated in patients with mutated ASS or ASL genes. Watermelon is one of the richest sources of citrulline. Additionally, citrulline has been advertised as a sports nutrition supplement due to its involvement in Nitric Oxide (NO) synthesis, which helps with vasodilation.

The Citrulline Assay Kit provides a rapid, specific, and easy method for the measurement of total citrulline concentrations in a wide variety of samples. In this enzymatic assay, citrulline is converted into a series of intermediates, which further reacts with a probe to produce a stable fluorescent signal (λ_{Ex} = 535 nm/ λ_{Em} = 587 nm). The assay is simple, easy to perform, sensitive and is suitable for high-throughput applications. The method can detect as little as 2 µM citrulline in biological samples.

The kit is suitable for the measurement of citrulline in beverages and biological samples (such as serum) and for the analysis of the urea cycle and NO cycle.



Components

The kit is sufficient for 100 fluorometric assays in 96-well plates.

- Citrulline Assay Buffer 25 mL
  Catalog Number MAK423A
- Citrulline Buffer Supplement 1 vial
  Catalog Number MAK423B
- Citrulline Converter Mix 1 vial
  Catalog Number MAK423C
- Citrulline Developer Mix 200 µL
  Catalog Number MAK423D
- Citrulline Cofactor Mix 200 µL
  Catalog Number MAK423E
- Citrulline Enzyme Mix 1 vial
  Catalog Number MAK423F
- Citrulline Probe 200 µL
  Catalog Number MAK423G
- Citrulline Standard 1 vial
  Catalog Number MAK423H

Reagents and Equipment Required but Not Provided

- Pipetting devices and accessories (e.g., multichannel pipettor)
- Fluorescence multiwell plate reader
- Black flat-bottom 96-well plates. Cell culture or tissue culture treated plates are **not** recommended.
- Refrigerated microcentrifuge capable of RCF ≥13,000 × *g*
- Corning® Spin-X® UF concentrators (Catalog Number CLS431478)

Precautions and Disclaimer

For Research Use Only. Not for use in diagnostic procedures. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

Storage/Stability

The kit is shipped on wet ice. Store components at -20 °C, protected from light.

Preparation Instructions

Briefly centrifuge small vials prior to opening.

Citrulline Assay Buffer: Warm to room temperature prior to use. Store at -20 °C.

Citrulline Developer Mix, Citrulline Cofactor Mix**:** Thaw on ice. Aliquot and store at -20 °C. Keep on ice while in use. Avoid freeze-thaw cycles. Use within two months.

Citrulline Buffer Supplement, Citrulline Converter Mix, Citrulline Enzyme Mix: Reconstitute each vial with 220 µL of Citrulline Assay Buffer. Aliquot and store at -20 °C. Keep on ice while in use. Avoid freeze-thaw cycles. Use within two months.

Citrulline Probe (in DMSO): Ready to use as supplied. Warm to room temperature before use. Store at 2-8 °C or -20 °C, protected from light.

Citrulline Standard: Reconstitute with 100 µL of purified water to make a 100 mM Citrulline Standard stock solution. Store at -20 °C.

Procedure

All samples and standards should be run in duplicate.

Sample Preparation

Note: Citrulline concentration varies over a wide range based on the sample type. For watermelon juice, the average citrulline concentration ranges from 10-20 mM. For normal human serum, the average citrulline concentration is 6-70 µM. Citrulline can range from 30-3000 µM for patients with Citrullinemia or Argininosuccinic Aciduria.

For unknown samples, perform a pilot experiment by testing several dilutions to ensure the readings are within the Standard Curve range.

Fruit juices and beverages

1. Centrifuge Samples at 13,000 × *g* to remove any insoluble precipitate.
2. Collect the supernatant and put 200-500 µL into a 10 kDa Spin Column such as Corning Spin-X UF concentrator.
3. Centrifuge the Sample at 13,000 × *g,* 4 °C for 10 minutes, and collect the filtrate.
4. Add 2-50 µL of the filtered Sample and label as "Sample" (S) and "Sample Background Control" (SBC)" into two parallel wells of a 96-well black plate. Adjust the total volume to 50 µL with Citrulline Assay Buffer.

Biological fluids

1. Centrifuge at 13,000 × *g,* 4 °C for 10 minutes to remove any insoluble precipitate in the biological fluids.
2. Add 200-500 µL of Sample into a 10 kDa Spin Column such as Corning Spin-X UF concentrator.

3. Centrifuge at 10,000 × *g*, 4 °C for 20 minutes, and collect the filtrate.
4. Due to the matrix effect in biological samples, an Internal Standard (Spike) is needed for each Test Sample. For each Sample, add 2-50 µL of Samples into 3 parallel wells of a 96-well black plate. Designate as "Sample" (S), "Sample Background Control" (SBC) and "Spiked Sample" (Sample + Citrulline Spike; SS).
5. Add 4 µL of 0.1 mM Citrulline Standard (400 pmol) to each Spiked Sample (SS) well (see Standard Curve Preparation section for preparation instructions).
6. Bring the total volume of all wells to 50 µL/well with Citrulline Assay Buffer.

Blank and Reagent Control

Prepare 2 additional wells with 50 µL of Citrulline Assay Buffer labeled as "Blank" (B) and "Reagent Control" (RC).

Standard Curve Preparation

Prepare a 1 mM Citrulline Standard solution by diluting 10 µL of the reconstituted 100 mM Citrulline Standard stock solution into 990 µL of purified water. Further dilute to 0.1 mM Citrulline Standard by adding 10 µL of the 1 mM Citrulline Standard solution into 90 µL of purified water. Prepare Standards according to Table 1. Mix well. Discard any remaining diluted standard solutions; do not store.

Table 1.
Preparation of Citrulline Standards

Well	0.1 mM Citrulline Standard	Citrulline Assay Buffer	Citrulline (pmol/well)
1	0 µL	50 µL	0
2	2 µL	48 µL	200
3	4 µL	46 µL	400
4	6 µL	42 µL	600
5	8 µL	38 µL	800
6	10 µL	34 µL	1000

Reaction Mixes

1. Prepare a 5-fold dilution of the Citrulline Probe with Citrulline Assay Buffer.
2. Mix enough reagents for the number of assays to be performed.
   a. For each well containing Blank (B), Standard, Sample (S), and Spiked Sample (SS), prepare 50 µL of Reaction Mix according to Table 2. Mix well.
   b. For each well containing Sample Background Control (SBC) and Reagent Control (RC), prepare 50 µL of Background Reaction Mix according to Table 2. Mix well.

Table 2.
Preparation of Reaction Mixes

Reagent	Reaction Mix	Background Reaction Mix
Citrulline Assay Buffer	38 µL	40 µL
Citrulline Buffer Supplement	2 µL	2 µL
Citrulline Converter Mix	2 µL	-
Citrulline Developer Mix	2 µL	2 µL
Citrulline Cofactor Mix	2 µL	2 µL
Citrulline Enzyme Mix	2 µL	2 µL
Diluted Citrulline Probe	2 µL	2 µL

3. Add 50 µL of the Reaction Mix to each well containing Blank (B), Standard, Sample (S), and Spiked Sample (SS).
4. Add 50 µL of the Background Reaction Mix to Sample Background Control (SBC) and Reagent Control (RC) wells. Mix well.

Measurement

Incubate the plate for 30 minutes at 37 °C, protected from light. Measure the fluorescence (RFU) of all wells at λ_{Ex} = 535 nm/λ_{Em} = 587 nm in endpoint mode.

Results

1. Subtract the 0 Standard RFU reading from all Standard readings.
2. Plot the Citrulline Standard Curve.
3. Subtract the Reagent Control (RC) RFU readings from the Blank RFU readings to determine corrected RFU fluorescence value (F_B).

   $$F_B = RFU_B - RFU_{RC}$$

4. Subtract the Sample Background Control (SBC) RFU readings from the Sample (S) RFU readings and the Spiked Sample (SS) readings, respectively, to determine corrected RFU fluorescence values (F).

   $$F_S = RFU_S - RFU_{SBC}$$

   $$F_{SP} = RFU_{SP} - RFU_{SBC}$$

5. For unspiked Samples (S), calculate the Citrulline amount (C) from the Citrulline Standard Curve using F_S.
6. For Spiked Samples (SS), calculate the amount of citrulline in Sample wells (C):

   $$\frac{F_S - F_B}{F_{SP} - F_S} \times 400 \text{ pmol}$$

   where 400 pmol is the amount of Citrulline added to the Spiked Sample (SS) per the procedure.

   Note: If calculated citrulline amount in the spiked well(s) is higher than 600 pmol dilute the sample further.

7. Calculate sample Citrulline concentration:

   Citrulline Concentration in Sample (pmol/μL or μM) =

   $$(C/V) \times D$$

where

C = Amount of Citrulline from Step 5 or 6 above (in pmol)

V = Sample volume added into reaction well (in μL)

D = Dilution factor (for undiluted samples, D=1)

Figure 1.
Typical Citrulline Standard Curve

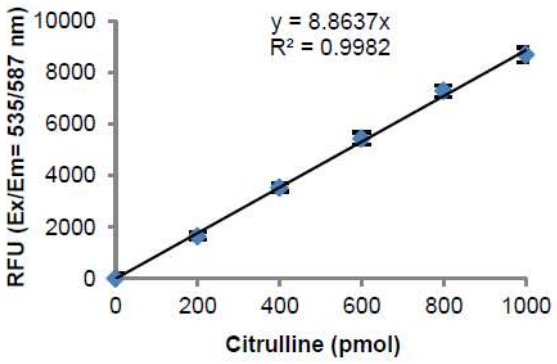


Figure 2.
Specificity of the detection of citrulline over other amino acids. Other amino acids were tested at a 10-fold molar excess (each AA: 10 nmol) vs citrulline (1 nmol).

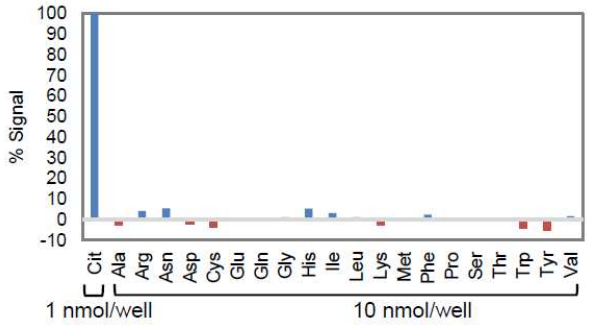
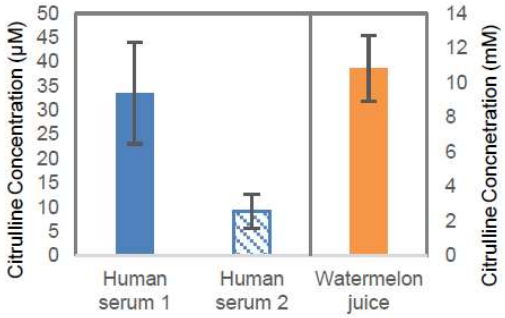


Figure 3.
Estimations of citrulline in two human serum samples (10 µL and 40 µL in each well respectively) and watermelon juice (4 µL of 100× dilution). Citrulline concentrations were 33.50 µM and 9.11 µM in human serum respectively and 10.81 mM in watermelon juice. Assays were performed following the kit protocol.



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