ab213972
Human ADMA ELISA kit

For the quantitative determination of Human ADMA in plasma and serum samples.

This product is for research use only and is not intended for diagnostic use.
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1. Overview

The Human ADMA Enzyme-Linked Immunosorbent Assay (ELISA) kit (ab213972) is a complete kit for the quantitative determination of Human ADMA in human plasma and serum samples. It is recommended that you read the entire kit insert before proceeding with the assay.

Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of NO- synthase. It is formed during proteolysis of methylated proteins and removed by renal excretion or metabolic degradation by the enzyme dimethylarginine dimethylaminohydrolase (DDAH). Several cell types, including human endothelial and tubular cells are capable of synthesizing and metabolizing ADMA. Elevated ADMA concentrations in the blood are found in numerous diseases associated with endothelial dysfunction. For example, elevated ADMA levels in blood of dialysis patients correlate significantly with the degree of arteriosclerosis and cardiovascular risk. Furthermore, elevated ADMA levels are found in patients with hypercholesterolemia, hypertension, arteriosclerosis, chronic renal failure and chronic heart failure, and are associated with restrictions in endothelial vasodilatation.

During the last years, the important clinical relevance of the regulation of vascular tone and structure by nitric oxide (NO) has been shown. Moreover, there were reports that human endothelial cells produce ADMA as well as nitric oxide, which points to an endogenous endothelial NO- regulation by ADMA. Therefore, it was assumed that hypertension, arteriosclerosis and immunological dysfunction in patients with chronic renal failure are connected to a dysfunction of the L-arginin/NO- metabolism and to ADMA accumulation. The reasons for the deregulation of the L-arginin/NO- metabolism could only partially be elucidated. Certainly, there are multiple factors involved in the L-arginin/NO- metabolism regulation as for example elevation of free superoxide radicals (O2-), ADMA accumulation and reduced NO-synthase activity.
Prospective clinical studies of the last years demonstrate the increased importance of ADMA as a novel cardiovascular risk factor. Indication: Arteriosclerosis, Hypertension, Chronic heart failure, Coronary artery disease, Hypercholesterolemia, Chronic renal failure, Diabetes mellitus, Peripheral arterial occlusive disease.

This assay is based on the method of competitive enzyme linked immunoassays. The sample preparation includes the addition of a derivatization-reagent for ADMA coupling. Afterwards, the treated samples and the polyclonal ADMA-antiserum are incubated in wells of microplate coated with ADMA-derivative (tracer). During the incubation period, the target ADMA in the sample competes with the tracer immobilized on the wall of the microtiter wells for the binding of the polyclonal antibodies. The ADMA in the sample displaces the antibodies out of the binding to the tracer. Therefore, the concentration of the tracer-bound antibody is inverse proportional to the ADMA concentration in the sample. During the second incubation step, a peroxidase-conjugated antibody is added to each microtiter well to detect the anti-ADMA antibodies. After washing away the unbound components, tetramethylbenzidine (TMB) is added as a substrate for peroxidase. Finally, the enzymatic reaction is terminated by an acidic stop solution. The color changes from blue to yellow and the absorbance is measured in the photometer at 450 nm. The intensity of the yellow color is inverse proportional to the ADMA concentration in the sample; this means high ADMA concentration in the sample reduces the concentration of tracer-bound antibodies and lowers the photometric signal.

A dose response curve of absorbance unit (optical density, OD at 450 nm) vs. concentration is generated using the values obtained from the standard. ADMA present in the patient samples is determined directly from this curve.
2. Protocol Summary

Treat standards, controls and samples

↓
Wash plate

↓
Add Standards, controls and Samples to wells

↓
Add ADMA antibody to wells

↓
Incubate (+2-8 °C)

↓
Wash plate

↓
Add POD antibody to wells

↓
Incubate (RT)

↓
Wash

↓
Add TMB substrate

↓
Add Stop solution

↓
Read plate at 450 nm
3. Precautions

Please read these instructions carefully prior to beginning the assay.

- All ELISA kit components have been formulated and quality control tested to function successfully as a kit.
- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Reagents should be treated as possible mutagens and should be handled with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipette by mouth. Do not eat, drink or smoke in the laboratory areas.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

4. Storage and Stability

Store standard and controls at -20°C. Store rest of the kit at +2-8°C.

immediately upon receipt

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Materials Supplied section.

Aliquot components in working volumes before storing at the recommended temperature.
5. Limitations

- ELISA kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.
### 6. Materials Supplied

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity</th>
<th>Storage Condition (Before prep)</th>
<th>Storage Condition (After prep)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADMA coated microplate (12x 8 well strips)</td>
<td>96 wells</td>
<td>+2-8°C</td>
<td>+2-8°C</td>
</tr>
<tr>
<td>Standard 1 (0.001 µmol/L)</td>
<td>1 Vial</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>Standard 2 (0.1 µmol/L)</td>
<td>1 Vial</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>Standard 3 (0.12 µmol/L)</td>
<td>1 Vial</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>Standard 4 (0.15 µmol/L)</td>
<td>1 Vial</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>Standard 5 (1.25 µmol/L)</td>
<td>1 Vial</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>Standard 6 (2.0 µmol/L)</td>
<td>1 Vial</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>Control 1</td>
<td>1 Vial</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>Control 2</td>
<td>1 Vial</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>Wash buffer concentrate (10X)</td>
<td>2x 100 mL</td>
<td>+2-8°C</td>
<td>+2-8°C</td>
</tr>
<tr>
<td>ADMA antibody (lyophilized)</td>
<td>2 Vials</td>
<td>+2-8°C</td>
<td>+2-8°C</td>
</tr>
<tr>
<td>POD antibody concentrate</td>
<td>120 µL</td>
<td>+2-8°C</td>
<td>+2-8°C</td>
</tr>
<tr>
<td>Conjugate stabilizing buffer</td>
<td>24 mL</td>
<td>+2-8°C</td>
<td>+2-8°C</td>
</tr>
<tr>
<td>Reaction buffer</td>
<td>15 mL</td>
<td>+2-8°C</td>
<td>+2-8°C</td>
</tr>
<tr>
<td>Derivatization reagent</td>
<td>2x 50 mg</td>
<td>+2-8°C</td>
<td>+2-8°C</td>
</tr>
<tr>
<td>Dimethylsulfoxid (DMSO)</td>
<td>7 mL</td>
<td>+2-8°C</td>
<td>+2-8°C</td>
</tr>
<tr>
<td>Dilution buffer for coupling</td>
<td>28 mL</td>
<td>+2-8°C</td>
<td>+2-8°C</td>
</tr>
<tr>
<td>TMB substrate</td>
<td>25 mL</td>
<td>+2-8°C</td>
<td>+2-8°C</td>
</tr>
<tr>
<td>Stop solution</td>
<td>15 mL</td>
<td>+2-8°C</td>
<td>+2-8°C</td>
</tr>
</tbody>
</table>
7. Materials Required, Not Supplied

These materials are not included in the ELISA kit, but will be required to successfully perform this assay:

- Deionized or distilled water
- Precision pipettes for volumes between 10 μL and 1,000 μL
- Repeater or multichannel pipette
- Centrifuge capable of 10,000 x g
- Vortex mixer
- Disposable beakers for diluting buffer concentrates
- Aluminum foil
- Graduated cylinders
- A microplate shaker
- Absorbent paper for blotting
- Microplate reader capable of reading a 450 nm (reference wave length 620 or 690 nm)
- Software for calculating sample values from optical density readings utilizing a four parameter logistic curve fit.
8. Technical Hints

- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Complete removal of all solutions and buffers during wash steps is necessary to minimize background.
- All samples should be mixed thoroughly and gently.
- Avoid multiple freeze/thaw of samples.
- When generating positive control samples, it is advisable to change pipette tips after each step.
- This ELISA kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.
9. Reagent Preparation

- Equilibrate all reagents to room temperature (18-25°C) prior to use. The kit contains enough reagents for 96 wells.
- Prepare only as much reagent as is needed on the day of the experiment.

9.1 ADMA coated microplate (12x 8 well strips):
One plate (break-apart strips) of 96 wells. Ready to use. Store at +2-8°C.

9.2 Human ADMA Standards:
6 vials of ready to use standard. Store at -20°C. Thaw before use in the test, and re-freeze immediately after use. Standards can be re-frozen up to 3 times. (0.001, 0.1, 0.12, 0.15, 1.25, 2.0 µmol/L).

9.3 Controls:
1 vial each of ready to use Control 1 and Control 2. Store at -20°C. Thaw before use in the test, and re-freeze immediately after use. Controls can be re-frozen up to 3 times.

9.4 Wash Buffer Concentrate, 10X:
2 x 100 mL. Store at +2-8°C. Dilute 100 mL of the Wash Buffer concentrate 10X with 900 mL of deionized water.

\(\text{\Delta Note: Crystals could occur due to high salt concentration in the stock solutions. The crystals must be dissolved at room temperature or at 37°C using a water bath before dilution of the buffer solutions. Diluted buffer solution can be stored at +2-8°C for one month.}\)

9.5 ADMA antibody (lyophilized):
2 vials. Store at +2-8°C. Dissolve ADMA antibody (lyophilized) with 0.6 mL of 1X Wash Buffer for 5 minutes. Once reconstituted, transfer to a fresh tube and add a further 5 mL of 1X Wash Buffer.

\(\text{\Delta Note: The diluted ADMA antibody can be stored for 4 weeks at +2-8°C from preparation. The ELISA kit can be separated into two performances by the two ADMA antibody (lyophilized) vials.}\)

9.6 POD antibody concentrate:
1 vial of 120 µL. Store at +2-8°C. Dilute 1:200 by adding 110 µL of POD antibody concentrate to 22 mL of Conjugate stabilizing buffer.
△Note: The diluted POD antibody can be stored for 5 days at +2-8°C from preparation.

9.7 Conjugate stabilizing buffer:
1 vial of 24 mL. Ready to use. Store at +2-8°C.

9.8 Reaction buffer:
2 vials of 50 mg. Ready to use. Store at +2-8°C.

9.9 Derivatization reagent:
1 vial of 120 µL. Store at +2-8°C. Dissolve Derivatization reagent with 3 mL of Dimethylsulfoxid (DMSO) for 5 minutes. Place on a horizontal shaker for 5 minutes.
△Note: The dissolved Derivatization reagent cannot be stored for later use and should be prepared immediately before use.

9.10 Dimethylsulfoxid (DMSO):
1 vial of 7 mL. Ready to use. Store at +2-8°C.
△Note: DMSO could crystallize at 4°C. Dissolve the crystals at 20-25°C in a water bath.

9.11 Dilution buffer for coupling:
1 vial of 28 mL. Ready to use. Store at +2-8°C.

9.12 TMB substrate:
1 vial of 25 mL. Ready to use. Store at +2-8°C.

9.13 Stop solution:
1 vial of 15 mL. Ready to use. Store at +2-8°C.
10. Sample Preparation

- Venous fasting blood is suited for this test system. Samples are stable for one week at +2-8°C. For longer storage samples should be frozen at -20°C up to the measurement.
- Lipemic or hemolytic samples may give erroneous results and should not be used for analysis.
- The serum and plasma samples are analyzed without any dilution. However, if the sample volume is less than 50 µL, a 1:1 dilution in Reaction buffer is recommended (for example: 25 µL sample + 25 µL Reaction buffer). This dilution factor should be considered for data evaluation.
- Samples with visible amounts of precipitates should be centrifuged at 1,000 x g for 5 minutes, collect supernatant for testing.

\textbf{Note:} Standards and Sample Diluent are prepared from human-derived serum and should be handled accordingly. Materials have been tested and found negative for anti-Human Immunodeficiency Virus (HIV 1 and 2), anti-Hepatitis C virus and anti-Hepatitis B surface antigen. Since no test offers complete assurance that infectious agents are absent, the reagents should be handled in accordance at Biosafety Level 2.

10.1 Plasma:
10.1.1 Collect Venous fasting blood using Citrate or EDTA.
10.1.2 Within 30 minutes of collection, centrifuge samples at 1,000 x g for 15 minutes and collect plasma.
10.1.3 Transfer the serum to labeled polypropylene tubes for analysis. Store un-diluted serum at -20°C or below. Avoid repeated freeze-thaw cycles.

10.2 Serum:
10.2.1 Samples should be collected into a serum separator tube using established methods.
10.2.2 Allow clot to form for 30 minutes at room temperature.
10.2.3 After clot formation, centrifuge samples at 2,700 x g for 10 minutes and collect serum.
10.2.4 Transfer the serum to labeled polypropylene tubes for analysis. Store un-diluted serum at -20°C or below. Avoid repeated freeze-thaw cycles.
10.3 Standards, Control and Sample Preparation:
   - Equilibrate all materials and prepared reagents to room temperature prior to use.
   - Label an appropriate number of test tubes:
     10.3.1 Pipette 200 µL of Standards to corresponding tubes.
     10.3.2 Pipette 200 µL of Controls 1 and 2 to corresponding tubes.
     10.3.3 Pipette 50 µL of samples to corresponding tubes.
     10.3.4 Pipette 150 µL of Reaction buffer to sample tubes only.
     10.3.5 Pipette 50 µL of Derivatization reagent to each tube, mix well and place on a shaker (180-240 rpm) to incubate for 45 minutes at room temperature.
     10.3.6 Pipette 250 µL of Dilution buffer for coupling to each tube, mix well and place on a shaker (180-240 rpm) to incubate for 45 minutes at room temperature.

11. Plate Preparation
   - The 96 well plate strips included with this kit are supplied ready to use. It is not necessary to rinse the plate prior to adding reagents.
   - Unused plate strips should be immediately returned to the foil pouch containing the desiccant pack, resealed and stored at +2-8°C.
   - For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).
   - Differences in well absorbance or “edge effects” have not been observed with this assay.
12. Assay Procedure

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- It is recommended to assay all standards, controls and samples in duplicate.

12.1 Wash each well with an automated plate washer by adding 250 μL of 1X Wash Buffer to every well. Aspirate wells and repeat 4 more times for a total of 5 washes. After the final wash, empty or aspirate the wells and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.

12.2 Pipette 100 μL of the standards, controls and samples into corresponding wells on microtiter plate.

12.3 Pipette 100 μL of the diluted ADMA antibody into each well.

12.4 Seal the plate. Incubate for 15-20 hours at +2-8°C.

12.5 Empty the contents of the wells and wash each well with an automated washer by adding 250 μL of 1X Wash Buffer to every well. Aspirate wells and repeat 4 more times for a total of 5 washes. After the final wash, empty or aspirate the wells and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.

12.6 Pipette 200 μL of the prepared POD antibody into each well.

12.7 Seal the plate. Incubate for 1 hour with mixing on a plate shaker at room temperature*

12.8 Wash plate as described in section 12.5.

12.9 Pipette 200 μL of the TMB substrate into each well.

12.10 Seal the plate. Incubate for 6-10 minutes in the dark, at room temperature.

12.11 Pipette 100 μL of Stop Solution into each well, mix thoroughly.

12.12 After blanking the plate reader against the substrate, read optical density at 450nm (against 620 nm as a reference). If the plate reader is not capable of adjusting for the blank, manually subtract the mean OD of the substrate blank from all readings.

*The optimal speed for each shaker will vary and may range from 180-240 rpm. The speed must be set to ensure adequate mixing of the wells, but not so vigorously that the contents of the wells splash out and contaminate other wells.
13. Calculations

If the test is performed in strict compliance with the assay procedure, e.g. with the exact volumes for standards, controls and samples/sample treatment, standards, controls and samples are equally diluted. No dilution factor is required for calculation of the results.

**Note:** If it was necessary to dilute a sample 1:1 as described in section 10, then the dilution factor should be considered.

Several options are available for the calculation of the concentration of Human ADMA in the samples. We recommend that the data be handled by an immunoassay software package utilizing a 4 parameter logistic (4PL) curve fitting program.

The results generated from blank, standard and control samples, should be evaluated for acceptability using appropriate statistical methods.

The concentration of Human ADMA can be calculated as follows:

13.1 Calculate the average net OD for each standard and sample by subtracting the average NSB OD from the average OD for each standard and sample.

\[
\text{Average Net OD} = \text{Average OD} - \text{Average NSB OD}
\]

13.2 Plot the Net OD versus concentration of Human ADMA for the standards. Fit a curve through the data points (4PL curve fit is suggested). The concentration of Human ADMA in the unknown samples is then determined by interpolation from the standard curve. Be sure to account for the dilution factor during the sample dissociation step.

13.3 Samples with concentrations outside of the standard curve range will need to be re-analyzed using an additional dilution step.
14. Typical data

**Typical standard** curve – Data provided for demonstration purposes only. A new standard curve must be generated for each assay performed.

![Graph showing standard curve](image)

**Figure 1.** Human ADMA ELISA kit (ab213972) Standard Curve

**Expected values:**
Based on internal studies of evidently healthy persons (n=70) a mean value of 0.45 µmol/L was estimated.

**Normal range:**
Serum/Plasma mean value ± 2 standard variations:
0.45 ± 0.19 µmol/L.

We recommend each laboratory to develop its own normal range. The values mentioned above are only for orientation and can deviate from other published data.
15. Typical sample values

SENSITIVITY –
The sensitivity was determined by interpolation at 1 standard deviation below the mean signal at a concentration of 0 µmol/L (n=6).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Human ADMA (OD)</th>
<th>SD (%)</th>
<th>Detection limit (µmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.78</td>
<td>0.12</td>
<td>0.05</td>
</tr>
</tbody>
</table>

RECOVERY –
Recovery was determined by spiked with different ADMA concentrations and measured using this assay. The analytical recovery rate was determined by the expected and measured ADMA levels. The expected levels were calculated as the sum of the measured ADMA concentration in the original sample and the spiked ADMA amount. The mean recovery rate for all concentrations was 104 % (n=5).

<table>
<thead>
<tr>
<th>Spike (µmol/L)</th>
<th>Expected ADMA (µmol/L)</th>
<th>Measured ADMA (µmol/L)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>X</td>
<td>X = 0.419</td>
<td>100</td>
</tr>
<tr>
<td>0.25</td>
<td>0.25 + X = 0.699</td>
<td>0.694</td>
<td>104</td>
</tr>
<tr>
<td>0.5</td>
<td>0.50 + X = 0.919</td>
<td>0.948</td>
<td>103</td>
</tr>
</tbody>
</table>

LINEARITY OF DILUTION –
The linearity of the ELISA was determined by the dilution of a spiked patient sample. The mean linearity was 103 %.

<table>
<thead>
<tr>
<th>Spike (µmol/L)</th>
<th>Measured ADMA (µmol/L)</th>
<th>Expected ADMA (µmol/L)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.468</td>
<td>0.468</td>
<td>100</td>
</tr>
<tr>
<td>1:1</td>
<td>0.255</td>
<td>0.234</td>
<td>109</td>
</tr>
<tr>
<td>1:3</td>
<td>0.114</td>
<td>0.117</td>
<td>97</td>
</tr>
</tbody>
</table>
**PRECISION –**

**Intra-assay precision:**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Number of measures</th>
<th>ADMA (µmol/L)</th>
<th>SD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>26</td>
<td>0.27</td>
<td>0.021</td>
</tr>
<tr>
<td>2</td>
<td>26</td>
<td>0.78</td>
<td>0.041</td>
</tr>
</tbody>
</table>

**Inter-assay precision:**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Number of measures</th>
<th>ADMA (µmol/L)</th>
<th>SD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6</td>
<td>0.33</td>
<td>0.029</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>0.79</td>
<td>0.045</td>
</tr>
</tbody>
</table>

**INTERFERENCE –**

**Cross Reactivity:**

<table>
<thead>
<tr>
<th>Substance</th>
<th>Reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDMA</td>
<td>&lt;0.5%</td>
</tr>
<tr>
<td>NMMA</td>
<td>&lt;0.5%</td>
</tr>
<tr>
<td>L-Arginin</td>
<td>&lt;0.02%</td>
</tr>
</tbody>
</table>
### 16. Troubleshooting

<table>
<thead>
<tr>
<th>Problem</th>
<th>Cause</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poor standard curve</td>
<td>Inaccurate Pipetting</td>
<td>Check Pipettes</td>
</tr>
<tr>
<td></td>
<td>Improper standard dilution</td>
<td>Prior to opening, briefly spin the stock standard tube and dissolve the</td>
</tr>
<tr>
<td></td>
<td></td>
<td>powder thoroughly by gentle mixing</td>
</tr>
<tr>
<td>Low Signal</td>
<td>Incubation times too brief</td>
<td>Ensure sufficient incubation times standard/sample incubation</td>
</tr>
<tr>
<td></td>
<td>Inadequate reagent volumes or improper dilution</td>
<td>Check Pipettes and ensure correct preparation</td>
</tr>
<tr>
<td></td>
<td>Incubation times with TMB too brief</td>
<td>Ensure sufficient incubation time until blue color develops prior addition of Stop solution</td>
</tr>
<tr>
<td>Large CV</td>
<td>Plate is insufficiently washed</td>
<td>Review manual for proper wash technique. If using a plate washer, check all ports for obstructions.</td>
</tr>
<tr>
<td></td>
<td>Contaminated wash buffer</td>
<td>Prepare fresh wash buffer</td>
</tr>
<tr>
<td>Low sensitivity</td>
<td>Improper storage of the ELISA kit</td>
<td>All components 4°C. Keep TMB substrate solution protected from light.</td>
</tr>
</tbody>
</table>
17. Notes
Technical Support

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