RIDASCREEN® Calprotectin

REF G09036

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1. Intended use

For *in-vitro* diagnostic use. RIDASCREEN® Calprotectin is an enzyme immunoassay for the quantitative detection of human calprotectin in stool samples.

2. Summary and explanation of the test

Calprotectin, a calcium-binding protein produced in cells such as neutrophils, is released during inflammation.
Fecal calprotectin serves as a biomarker of gastrointestinal inflammation and neoplasia in the gastrointestinal tract.
Calprotectin (also known as MRP-8/14, calgranulin A/B and S100A8/A9) is a calcium- and zinc-binding protein of the S100 protein family.
Calprotectin binds calcium under physiological conditions, making it a stable protein that is highly resistant to heat and proteolysis.
Calprotectin is produced by both neutrophils (neutrophil granulocytes) and monocytes. In neutrophils, the calprotectin concentration in the cytosol ranges from 5 to 15 mg/l, which accounts for about 60 % of the soluble cytosolic protein fraction and 5 % of the total protein fraction of these cells.
The biological function of calprotectin is not completely understood. It is assumed that calprotectin serves a biological function in protecting cells from leukocytic and bacterial proteases. Calprotectin is also presumed to have antibacterial properties by virtue of its zinc-binding capacity. In addition, calprotectin exhibits both intra- and extracellular regulatory functions in inflammatory processes.
In the presence of an inflammatory bowel disease, granulocytes migrate into the intestinal lumen and release calprotectin, which is excreted in the feces. The concentration of calprotectin in the feces correlates with the number of neutrophilic granulocytes that migrated into the intestinal lumen and released calprotectin. Consequently, the fecal calprotectin concentration can be used as a measure of the number of granulocytes in the bowel lumen and as an indicator of the severity of intestinal inflammation.
Measuring fecal calprotectin is of special clinical relevance because it allows reliable diagnosis of inflammation in the intestinal tract. In many cases, chronic inflammatory bowel disease (CIBD) produces symptoms that are very difficult to distinguish from those of irritable bowel syndrome (IBS). Fecal calprotectin measurement provides a reliable indication of the presence of inflammation in the bowel. Through the use of this biomarker, many patients with irritable bowel syndrome can therefore be spared an unnecessary colonoscopy.
In a number of publications, fecal calprotectin concentrations have been shown to be strongly correlated with histological and endoscopic parameters of disease activity in CIBD patients. Therefore, measuring fecal calprotectin provides a means of objectively assessing the response to treatment of chronic inflammatory bowel disease and for monitoring these patients during clinical remission to enable the early detection and treatment of relapses.

Fecal calprotectin is measured:
- To reliably differentiate between chronic inflammatory bowel disease and irritable bowel syndrome
- To detect flares of inflammatory bowel disease early
- To objectively document the severity of inflammation for response monitoring during the course of treatment
- To provide a marker of acute intestinal inflammation

3. Test principle

RIDASCREEN® Calprotectin employs specific antibodies in a sandwich-type method. The well surface of the microtiter plate is coated with monoclonal antibodies against human calprotectin. A suspension of the stool sample to be tested is pipetted into a well of the microtiter plate and incubated. This is followed by a washing step and a second incubation together with a monoclonal antibody that is conjugated with horseradish peroxidase. In the presence of calprotectin, the immobilized antibody, calprotectin, and conjugated antibody form a sandwich complex. Unbound enzyme-marked antibodies are removed in a subsequent washing step. In positive samples, after the addition of a substrate, the bound enzyme changes the colorless solution in the wells of the microtiter plate to a blue solution. The addition of a stop solution changes the color from blue to yellow. The measured absorbance is proportional to the calprotectin concentration present in the sample.

4. Reagents provided

The reagents in the kit are sufficient for 96 determinations.

Plate	96 assays	Microtiter plate; 12 microtiter strips (breakable) in a strip holder; coated with monoclonal antibodies (mouse) against human calprotectin
Extract	2x 100 ml	Extraction buffer (3x conc.); Tris buffer; contains 0.05 % NaN_3
Diluent | 3	100 ml	Sample dilution buffer 3 (for final dilution), protein-buffered NaCl solution; contains 0.1 % NaN_3; ready for use, dyed red
Wash buffer	100 ml	Wash buffer 10x (10x concentration); phosphate-buffered NaCl solution; contains 0.1 % thimerosal
Calibrator	1 ml	Calibrator (1-point calibration procedure); contains 0.1 % ml NaN_3; ready for use
Standard 1 to Standard 5	1 ml	5 standards (standard curve procedure); calprotectin concentrations of standards 1 to 5: 19.5 / 56 / 95 / 275 / 800 mg/kg; contains 0.1 % NaN_3; ready for use
Control | +	1 ml	Positive control; contains 0.1 % NaN_3; ready for use
Low control | +	1 ml	Low positive control; contains 0.1 % NaN_3; ready for use
Conjugate	12 ml	Peroxidase conjugated, monoclonal antibody (mouse) in stabilized protein solution; ready for use
SeroSC	12 ml	Substrate; hydrogen peroxide/TMB; ready for use
Stop	12 ml	Stop solution; 1 N sulfuric acid; ready for use

Dangerous substances are indicated according to labeling obligations. For further details, please refer to the safety data sheets (SDSs) at www.r-biopharm.com.

5. Storage instructions

All reagents must be stored at 2 - 8 °C and can be used until the expiry date printed on the respective label. Providing the diluted wash buffer is stored at 2 - 8 °C, it can be used for a maximum of 4 weeks. The diluted extraction buffer can be used for 6 months when stored at 2 - 8 °C, at maximum until the expiration date of the concentrate. Microbial contamination must be prevented. After the expiration date, the quality guarantee is no longer valid. Use scissors to open the aluminum bag containing the microtiter strips, but take care to not tear off the clip seal. Any microtiter strips which are not required must immediately be returned to the aluminum bag and stored at 2 - 8 °C. The colorless substrate must also be protected from direct light to prevent it from decomposing or turning blue due to auto-oxidation. Once the substrate has turned blue, it must not be used.

6. Reagents required but not provided

6.1. Reagents

-Distilled or deionized water

6.2. Equipment

-Microbalance or RIDA®TUBE Calprotectin (item no. GZ3016)
-Vortex mixer (optional, see 9.4.)
-Micropipette for 20 - 100 µl and 1 ml volumes
-Graduated cylinder (1000 ml)
-Stopwatch
-Washing device for microtiter plates or multichannel pipette (300 µl)
-Microtiter plate photometer (450 nm; reference filter 620 nm)
-Filter paper (lab wipes)
-Waste container containing 0.5 % hypochlorite solution

7. Precautions for users

For *in-vitro* diagnostic use only.

This test must be carried out only by trained laboratory personnel. The guidelines for working in medical laboratories must be followed. Always adhere strictly to the user instructions for carrying out this test.
The stool samples should be handled as if potentially infectious, pursuant to the national safety requirements.
Specimens or reagents must not be pipetted by mouth, and contact with injured skin or mucous membranes must be prevented. When handling the samples, wear disposable gloves and, when the test is finished, wash your hands. Do not smoke, eat, or drink in areas where samples or test reagents are being processed.

For further details, see the safety data sheets (SDSs) at www.r-biopharm.com.

The wash buffer contains 0.1 % thimerosal as preservative. This substance must not be allowed to come into contact with skin or mucous membranes.
Calibrator, standards, positive control, low positive control, extraction and sample dilution buffer contain 0.0 % or 0.1 % NaN_3 as a preservative. The extraction buffer also contains guanidinium chloride as a renaturation and denaturation agent. This substance must not be allowed to come into contact with skin or mucous membranes.
Hydrogen peroxide (substrate) can cause chemical burns. Handle with care.
The Stop solution contains 1 N sulphuric acid. Avoid contact with the skin and clothing. If the reagent comes into contact with your skin, rinse your skin off with water.
All reagents and materials coming into contact with potentially infectious samples must be treated with suitable disinfectants or autoclaved at 121 °C for at least one hour. CAUTION: To prevent the formation of poisonous gases, any liquid waste containing the Stop solution must be neutralized before it is added to hypochlorite solution.

8. Collection and storage of samples

The stool samples should be transported chilled if possible and stored at 2 - 8 °C before testing. If the samples are not used immediately after receipt (within 3 days), we recommend storage at -20 °C or lower. When stool samples are frozen, neutrophilic granulocytes can burst and release calprotectin. This can slightly increase the calprotectin concentration in frozen compared with fresh samples. Avoid repeated freezing and thawing of the sample. Stool samples should not be collected in transport containers that contain transport media with preservatives, animal sera, metal ions, oxidizing agents, or detergents since these may interfere with the RIDASCREEN® Calprotectin test.

9. Test procedure

9.1. General information

All reagents and the microtiter plate Plate must be brought to room temperature (20 - 25 °C) before use. Once they have reached room temperature, remove the microtiter strips from the aluminum bag. Mix the reagents well immediately before use. After use, the microtiter strips (placed in sealed bags) and the reagents must be stored again at 2 - 8 °C. Once used, the microtiter strips must not be re-used. Do not use reagents or microtiter strips if the packaging is damaged or the containers are not tightly sealed. In order to prevent cross-contamination, the samples must be prevented from coming into direct contact with the kit components. The test must not be carried out in direct sunlight. We recommend covering the microtiter plate or sealing with plastic wrap to prevent evaporation losses.

9.2. Preparing the wash buffer

Mix 1 part wash buffer concentrate Wash with 9 parts distilled water (1:10 AM). For this step, add 100 ml concentrate to a 1,000 ml measuring cylinder, and fill with distilled water to 1,000 ml. Any crystals present in the concentrate should be dissolved beforehand through heating (water bath at 37 °C).

9.3. Preparing the extraction buffer

Mix 1 part extraction buffer Extract (100 ml) with 2 parts distilled water (200 ml) (1:3).

9.4. Sample preparation

The stool samples should be stored continuously if possible at 2 - 8°C before use. For longer storage (> 3 days), keep them at -20 °C or lower. Frozen samples should be slowly adapted to room temperature.

9.4.1 Sample preparation and suspension by weighing

Weigh 100 mg of stool sample into a labeled test tube, and then add 5 ml of the diluted extraction buffer using a pipette (1:50 dilution).
Alternatively, place between 80 and 130 mg of the stool in the test tube and suspend it in a proportionally smaller or larger volume of diluted extraction buffer (see Table 1) to maintain a constant dilution ratio (1:50).

Table 1: Data on the required amounts of diluted extraction buffer depending on the amount of stool weighed

Sample weight [mg]	Volume [ml]
80	4.00
85	4.25
90	4.50
95	4.75
100	5.00
105	5.25
110	5.50
115	5.75
120	6.00
125	6.25
130	6.50

During re-suspension in a constant extraction buffer volume of 5 ml, the variable dilution factor must be taken into consideration in the calculation (see Table 2).

Table 2: Data on the dilution factor with constant addition of diluted extraction buffer (5 ml), depending on the stool amount weighed out

Sample weight [mg]	Volume [ml]
80	62.50
85	58.82
90	55.55
95	52.63
100	50.00
105	47.62
110	45.45
115	43.45
120	41.66
125	40.05
130	38.46

The stool suspension is homogenized by thoroughly mixing it on a vortex mixer, regardless of the method used to weigh the stool sample. If the stool is in liquid form, use a pipette to take exactly 100 µl and suspend it in exactly 5 ml diluted extraction buffer.

The samples should be incubated for 5 minutes at room temperature after the addition of the extraction buffer and then thoroughly vortexed again. Then the homogenized suspension must be centrifuged for 10 minutes at least 3,000xg to settle out rough stool particles. We recommend further diluting the supernatant of the extract immediately for use in the test.

a. Manual sample dilution

20 µl of the clarified supernatant are further diluted in 980 µl RIDASCREEN® sample dilution buffer Diluent | 3 (1:50). 100 µl of the final diluted stool sample are then used in the test.
Alternatively, samples can be prepared and homogenized using the RIDASCREEN®TUBE Calprotectin (item no. GZ3016, see 9.4.2.).

b. Sample dilution in an automated system

If the assay is to be run on the DSX automated ELISA system (Dynex Technologies, Inc.), the specific assay protocol required for this should be requested from R-Biopharm AG and applied to the system. The sample is then automatically diluted as described below.
20 µl of the clarified supernatant is pipetted by the automatic ELISA system into a deep-well plate and diluted with 980 µl of diluted Diluent | 3 (1:50). Two mixing cycles follow.
The required number of microtiter strips is placed in the strip holder for the RIDASCREEN® Calprotectin microtiter plate Plate. From the deep-well plate, 100 µl of the sample diluent are transferred to the RIDASCREEN® calprotectin microtiter plate Plate (final dilution 1:2,500).
For instructions on how to perform the test with other ELISA automated pipetting systems, please contact R-Biopharm AG.

9.4.2 Sample preparation and suspension with RIDA®TUBE Calprotectin (item no. GZ3016)

RIDA®TUBE Calprotectin (item no. GZ3016) is a fast and clean alternative to weighing stool samples for preparation and suspension as described in 9.4.1. This is offered as an accessory to RIDASCREEN® Calprotectin and simplifies sample preparation considerably.
10 mg stool sample is added to RIDA®TUBE Calprotectin pre-filled with 2.5 ml ready-to-use extraction buffer. If the stool sample is liquid, 10 µl of the stool sample can be taken using the pipette and pipetted directly into the extraction buffer.
The sample collection and extraction procedure is described in detail in the instructions included with the RIDA®TUBE Calprotectin and can also be downloaded from www.r-biopharm.com.
We recommend using the stool extracts in the test immediately after dilution.

a. Manual sample dilution

As described under item 8 in the instructions for use of GZ3016, 100 µl of the stool suspension obtained using RIDA®TUBE Calprotectin (item no. GZ3016) are further diluted in 900 µl RIDASCREEN® sample dilution buffer Diluent | 3. 100 µl of the final diluted stool sample are immediately used in the test.

b. Sample dilution in an automated system

If the assay is to be run on the DSX automated ELISA system (Dynex Technologies, Inc.), the specific assay protocol required for this should be requested from R-Biopharm AG and applied to the system.
The required number of microtiter strips is placed in the strip holder for the RIDASCREEN® Calprotectin microtiter Plate. The stool suspension extracted with the stool tube is automatically diluted 1:10 in the ELISA system on the microtiter Plate (final dilution 1:2,500). 10 µl of the stool suspension from RIDA®TUBE Calprotectin (item no. GZ3016) is pipetted directly into the calprotectin microtiter plate and further diluted with 90 µl of Diluent | 3.
For instructions on how to perform the test with other ELISA automated pipetting systems, please contact R-Biopharm AG.

9.5. First incubation

After placing a sufficient number of wells in the holder, add 100 µl of calibrator Calibrator (1-point calibration procedure, recommended in duplicate), or 100 µl Standard 1 to Standard 5 Standard 1 – Standard 5 (standard curve procedure, recommended in duplicate), and 100 µl RIDASCREEN® sample dilution buffer Diluent | 3 (=negative control), positive control Control | + and the final stool sample dilution to the respective wells. If a low positive control is used Low control | +, also add 100 µl to the test. Next, incubate the plate for one hour at room temperature (20 - 25°C).

9.6. First washing

Careful washing is important in order to achieve the correct results and should therefore be carried out strictly in accordance with the instructions. The incubated substance in the wells must be emptied into a waste container containing the hypochlorite solution for disinfection. Next tap the plate over absorbent paper to remove the remaining moisture. Then wash the plate 5 times using 300 µl diluted wash buffer each time (see 9.2). Make sure that the wells are emptied completely by tapping them out after each wash onto a part of the absorbent paper which is still dry and unused.

Note: When using a microplate washer, make sure that the machine is correctly adjusted to the type of microtiter plate being used. Furthermore, a stool suspension that is not completely particle-free before the first wash should be removed manually by centrifuging in order to avoid blocking the wash needles.

Note: Also ensure that all of the liquid is aspirated during each washing stage. After washing for the last time, tap out the plate thoroughly onto clean, absorbent paper or laboratory towels in order to remove any residual moisture.

9.7. Second incubation

Add 100 µl conjugate Conjugate to each well. Next, incubate the plate for one hour at room temperature (20 - 25 °C).

9.8. Second washing

Following the incubation period, empty the conjugate in the wells into a waste container containing hypochlorite solution for disinfection. Next, tap the plate over absorbent paper to remove the remaining moisture. Then wash the plate five times using 300 µl diluted wash buffer each time. Make sure that the wells are emptied completely by tapping them out after each wash onto a part of the absorbent paper which is still dry and unused.

9.9. Third incubation

Add 100 µl substrate SeroSC to each well. Then incubate the plate for 15 minutes in darkness at room temperature (20 - 25°C). Subsequently, stop the reaction by adding 50 µl stop solution Stop to each well.
After mixing carefully (by tapping lightly on the side of the plate), measure the absorbance at 450 nm and at a reference a wavelength of 620 nm.

Note: High-positive patient samples may cause black-colored precipitates of the substrate. These samples should be diluted 1:10 and used in the test again (see 9.5.) (final dilution of the stool sample: 1:25000).

10. Quality control — Indication of deterioration of reagents

For quality control purposes, the calibrator Calibrator (1-point calibration, recommended in duplicate) or Standard 1 to Standard 5 Standard 1 – Standard 5 (standard curve, recommended in duplicate), the RIDASCREEN® sample dilution buffer Diluent | 3 as a negative control, and the positive control Control | + must be used every time the test is carried out to ensure the stability of the reagents and correct performance of the test. The use of the low positive control is optional.
The test has been performed correctly when the extinction mean (OD) of the negative control at 450 nm/620 nm is less than 0.05, and the averaged extinction mean (OD) of the calibrator (if 1-point calibration is used) is within the range indicated on the batch-specific data sheet. When the calibrator and the five standards are used, the positive control provides information on the validity of the test. The concentration range must be within the lot-specific range indicated on the data sheet. When RIDASCREEN® Calprotectin is processed on open, fully automated ELISA systems, the measured OD of the calibrator Calibrator (when 1-point calibration is used) may deviate from the range indicated on the batch-specific certificate depending on the system. Even on fully automated ELISA systems, the positive control Control | + is decisive for the validity of the test results and must

therefore always be tested. It is not absolutely essential to use the low positive control Low control | +.
Deviation from the required values, reagent turbidity, or blue coloring of the colorless substrate before adding to the wells may indicate that the reagents have expired. If the stipulated values are not met, the following points must be checked before repeating the test:

- Expiration date of the reagents used
- Functional performance of the equipment used (e.g., calibration)
- Correct test procedure
- Visual inspection of the kit components for contamination or leaks; a substrate solution which has turned blue must not be used.

If the conditions are still not met after repeating the test, please consult the manufacturer or your local R-Biopharm distributor.

11. Evaluation and interpretation

The concentration of calprotectin in mg/kg stool is determined with the RIDASCREEN® Calprotectin ELISA either via the 1-point calibration procedure (see 11.1.) according to the 4-parameter logistic log model (4PL) or via a standard curve procedure (see 11.2.).
The RIDA®SOFT Win.NET evaluation software is required to calculate the results. RIDA®SOFT Win.NET or updates can be obtained on request by contacting R-Biopharm AG or your local R-Biopharm distributor.
Other evaluation software that provides the 4-parameter logistic log model can also be used instead of RIDA®SOFT Win.net.

11.1. Single-point quantification according to the 4-parameter logistic log model

The parameters (A - D) required for the 4PL calculation of the standard curve and the target value for the calibrator, positive control, and low positive control can be found on the batch-specific data sheet that comes with the test kit, and they must be compared with the values in the evaluation software before measurement.
In the final quality control, R-Biopharm AG calculates the standard curve (including parameters A - D) under optimum test conditions for each kit batch, as well as a target value and a permissible concentration range for the OD of the calibrator, positive control, and low positive control. The calibrator is also tested to compensate for test fluctuations and to check the quality of the test procedure. The positive control provides information on the validity of the test.
RIDA®SOFT Win.net calculates correction factor F internally from the mean of the calibrator duplicate analysis and its target value. This correction factor is then reconciled with the absorbances for the stool samples. The test results can be confidently and reliably evaluated within the limits of the standard curve.

11.2. Quantification using the standard curve

RIDASCREEN® Calprotectin is evaluated with a standard curve that must be included with each run. An example of a standard curve can be taken from the lot-specific data sheet included.
In final quality control, R-Biopharm AG determines a reference value under optimum test conditions for each kit lot and a permitted concentration range for the positive control and low positive control. For quantification via the standard curve, the positive control provides information on the validity of the test. The positive control must reach the concentration range specified.

11.3. Test result

As of a cut-off value of > 50 mg/kg of human calprotectin in the stool, the result must be considered positive. If the results lie below the cut-off value, the samples must be considered negative.
The cut-off-value of 50 mg/kg suggested for adults can also be used for children aged from 4 - 17.
We advise each laboratory to establish its own standard value range.

Note: Samples that show calprotectin concentrations of more than 600 mg/kg when using 1-point calibration or the standard curve are recommended to be further diluted (1:5 in Diluent 3) and re-analyzed in the test.

Note: Samples that show OD values higher than 3.0 when quantified via 1-point calibration are recommended to be further diluted (1:5 in Diluent 3) and re-analyzed in the test.

12. Limitations of the method

RIDASCREEN® Calprotectin detects epitopes of human calprotectin in stool samples. A diagnosis should not be based solely on the determined calprotectin concentrations, but should always take the patient’s medical history into account.

13. Performance characteristics

13.1. Precision

13.1.1 Intra-assay precision

Intra-assay precision was tested in a single run using 4 references in 40 replicates each. The OD values from these measurements were used to determine the calprotectin concentrations via the calibrator or the standard curve, from which the mean value (MV), the standard deviations (SDs), and the coefficients of variation (CVs) of the measurements were calculated for each sample.

Table 3: Intra-assay precision of RIDASCREEN® Calprotectin

	Calibrator			Standard curve		
	MV (mg/kg)	SD (mg/kg)	**CV (%)**	MV (mg/kg)	SD (mg/kg)	**CV (%)**
Reference A	53.8	2.9	**5.4**	56.6	3.0	**5.3**
Reference B	96.6	5.5	**5.7**	102.4	6.3	**6.2**
Reference C	136.5	5.6	**4.1**	130.5	5.2	**4.0**
Reference D	246.7	14.1	**5.7**	267.6	15.0	**5.6**

13.1.2 Inter-assay precision

The inter-assay precision was determined in duplicate in 20 runs (2 runs per day) using 4 references on different days by 3 technicians. The OD values from these measurements were used to determine the calprotectin concentrations via the calibrator or the standard curve, from which the mean value (MV), the standard deviations (SDs), and the coefficients of variation (CVs) of the measurements were calculated for each sample.

Table 4: Inter-assay precision of RIDASCREEN® Calprotectin

	Calibrator			Standard curve		
	MV (mg/kg)	SD (mg/kg)	**CV (%)**	MV (mg/kg)	SD (mg/kg)	**CV (%)**
Reference A	55.7	4.3	**7.7**	57.5	4.1	**7.1**
Reference B	106.5	7.2	**6.8**	112.8	8.3	**7.3**
Reference C	129.6	7.8	**6.0**	138.5	7.6	**5.5**
Reference D	260.9	28.3	**10.9**	284.7	22.7	**8.0**

13.2. Extraction precision

Extraction precision was determined in duplicate in 10 runs (5 days, 2 runs a day) on different days by 2 technicians using 3 stool samples above the cut-off. The samples were extracted and diluted as required before each run. The OD values from these measurements were used to determine the calprotectin concentrations via the calibrator or the standard curve, from which the mean value (MV), the standard deviations (SDs), and the coefficients of variation (CVs) of the measurements were calculated for each sample.

Table 5: Inter-assay extraction precision

	Calibrator			Standard curve		
	MV (mg/kg)	SD (mg/kg)	**CV (%)**	MV (mg/kg)	SD (mg/kg)	**CV (%)**
Reference A	64.0	5.0	**7.9**	67.4	5.0	**7.5**
Reference B	114.1	10.3	**9.0**	123.2	10.7	**8.7**
Reference C	264.0	33.3	**12.6**	284.8	22.1	**7.8**

14. Version history

Version number	Chapter and designation
2019-07-01	General revision 14. Version history 15. Explanation of symbols

15. Explanation of symbols

General symbols

IVD	For in vitro diagnostic use
	Consult instructions for use
LOT	Lot number
	Expires
	Store at
REF	Article number
	Number of tests
	Date of manufacture
	Manufacturer

Test-specific symbols

Plate	Microtiter plate
Extract 10x	Extraction and dilution buffer
Diluent | 3	Sample dilution buffer
Wash buffer	Wash buffer 10x
Calibrator	Calibrator
control	Positive control
Low control	Low positive control
Conjugate	Conjugate
SeroSC	Substrate
Stop	Stop solution

16. References

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