Calprotectin ELISA

For the quantitative determination of calprotectin in human stool.

For Research Use Only in the United States. Not for Use In diagnostic procedures.

Health Canada Licensed.

Catalog Number: 30-CALPHU-E01

Size: 96 Wells

Version: 2022-02-15– ALPCO 3.1

INTENDED USE

The Calprotectin ELISA is intended for the quantitative determination of calprotectin (MRP8/14, S100A8/A9) in human stool samples. For Research Use Only in the United States. Health Canada Licensed.

PRINCIPLE OF THE ASSAY

The assay utilizes the two-site "sandwich" technique with two selected monoclonal antibodies that bind to human calprotectin. Standards, controls, and diluted samples, which are assayed for human calprotectin, are added to wells of a microplate coated with a high affinity monoclonal anti-human calprotectin antibody. During the first incubation step, calprotectin in the samples is bound by the immobilized antibody. Then, a peroxidase labeled conjugate is added to each microtiter well and a "sandwich" complex of capture antibody-human calprotectin-peroxidase conjugate is formed. Tetramethylbenzidine (TMB) is used as peroxidase substrate. Finally, an acidic stop solution is added to terminate the reaction. The color changes from blue to yellow. The intensity of the yellow color is directly proportional to the calprotectin concentration in the sample. A standard curve is generated using the values obtained from the standards. The calprotectin present in the samples is determined directly from this curve.

MATERIALS SUPPLIED

Component	Quantity	Preparation
Calprotectin Microplate (96 wells)	12 strips of 8 wells	Ready to use
Standards (1-5) [0, 13, 52, 210, 840 ng/mL] [0, 32.5, 130, 525, 2100 µg/g*]	1 vial each	Ready to use
Control Levels 1 and 2	1 vial each	Ready to use
Wash Buffer Concentrate	2 x 100 mL	10X
Extraction Buffer Concentrate	100 mL	2.5X
Conjugate	15 mL	Ready to use
Sample Dilution Buffer	100 mL	Ready to use
TMB Substrate	15 mL	Ready to use
Stop Solution	15 mL	Ready to use

* Concentrations calculated based on formula in Example Calculation section below.

MATERIALS RECOMMENDED BUT NOT SUPPLIED

- Precision pipettes for dispensing up to 1000 µL (with disposable tips)
- Repeating or multi-channel pipette for dispensing up to 1000 µL
- Volumetric containers and pipettes for reagent preparation
- Distilled/Deionized/UltraPure water for reagent preparation
- Microplate washer or wash bottle
- Microplate reader
- Centrifuge (1,000 x g to 3,000 x g)
- Vortex for sample preparation
- Laboratory Balance
- Foil to cover the microplate
- Timer

PRECAUTIONS

1. The human blood products incorporated into this kit have been tested for the presence of HIV (Human Immunodeficiency Virus), HBV (Hepatitis B Virus), and HCV (Hepatitis C Virus). Test methods for these viruses do not guarantee the absence of a virus; therefore, all reagents should be treated as potentially infectious. Handling and disposal should be in accordance with all appropriate national and local regulations for the handling of potentially biohazardous materials.
2. Kit reagents contain sodium azide or Proclin as bactericides. Sodium azide and Proclin are hazardous to health and the environment. Substrates for the enzymatic color reactions may also cause skin and/or respiratory irritation. Any contact with the substances must be avoided. Further safety information can be found in the safety data sheet, which is available from ALPCO upon request.
3. The 10x Wash Buffer concentrate contains surfactants which may cause severe eye irritation in case of eye contact.

Warning: Causes serious eye irritation
If in Eyes: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. If eye irritation persists, get medical advice/ attention.

4. The stop solution consists of diluted sulfuric acid, a strong acid. Although diluted, it still must be handled with care. It can cause burns and should be handled with gloves, eye protection, and appropriate protective clothing. Any spill should be wiped up immediately with copious quantities of water. Do not breathe vapor and avoid inhalation.
5. This product is not for internal use.
6. Avoid eating, drinking, or smoking when using this product.
7. Do not pipette any reagents by mouth.
8. Reagents from this kit are lot-specific and must not be substituted.
9. Do not use reagents beyond the expiration date.
10. Variations to the test procedure are not recommended and may influence the test results.

STORAGE CONDITIONS

The kit should be stored at 2-8°C. The unopened kit is stable until the expiration date on the box label. The kit can be used up to 4 times within the expiry date stated in the certificate of analysis.

SAMPLE HANDLING

Stool samples are appropriate for use in this assay.

Raw Stool: Calprotectin in stool is stable for at least 3 days at room temperature. Nevertheless, it is recommended to store the samples for no more than 48 hours at 2–8 °C and transporting samples at room temperature for a maximum of 2 days. Long term storage (up to 12 months) is recommended at -20 °C. Allow frozen samples to thaw slowly, preferably at 2–8 °C, and warm the samples to room temperature before analysis. Avoid repeated freezing and thawing of the sample. Freezing can cause neutrophil granulocytes in the stool sample to burst and release calprotectin. Therefore, frozen samples can be expected to contain slightly elevated concentrations of calprotectin compared to fresh samples.

Chemical or biological additives in stool sample tubes may interfere with the measurement of calprotectin. Therefore, use only empty tubes or Easy Extraction Devices.

Stool Extracts: Stool extracts are stable for nine days at room temperature, 2–8 °C or -20 °C. Avoid more than three freeze-thaw cycles.

Extraction of the Stool Sample: 1X working extraction buffer is used as a sample extraction buffer. It is recommended to perform the following sample preparation:

1. The raw stool sample must be thawed. For particularly heterogeneous samples it is recommended to perform a mechanical homogenization using an applicator, inoculation loop or similar device.
2. Fill the empty sample tube with 1.5 mL of 1X working extraction buffer (diluted 1:2.5) before using it with the sample. Important: Allow the extraction buffer to reach room temperature. If using the Easy Extraction Device, the device is provided in a prefilled format and does not require any additional extraction buffer.
3. Unscrew the tube (yellow part of cap) to open. Insert yellow dipstick into sample. The lower part of the dipstick has notches which need to be covered completely with stool after inserting it into the sample. Place dipstick back into the tube. When putting the stick back into the tube, excess material will be stripped off and leave 15 mg of sample to be diluted. Screw tightly to close the tube.
4. Vortex the tube well until no stool sample remains in the notches. Important: Please make sure that you have a maximally homogenous suspension after shaking. Especially with more solid samples, soaking the sample in the tube with buffer for approximately 10 minutes improves the result.
5. Allow sample to stand for approximately 10 minutes until sediment has settled. Floating material like shells of grains can be neglected.
6. Carefully unscrew the complete cap of the tube including the blue ring plus the dipstick. Discard cap and dipstick. Make sure that the sediment will not be dispersed again. This will result in a dilution of 1:100 (Dilution I).

Dilution of samples

The extracted sample (Dilution I) is diluted a second time at 1:25 with sample dilution buffer. For example:

> 40 µL suspension (Dilution I) + 960 µL Sample Dilution Buffer = 1:25 (Dilution II)
> This results in a final dilution of 1:2500.

For analysis, pipet 100 µL of Dilution II per well.

REAGENT PREPARATION

All reagents must be equilibrated to room temperature prior to preparation and subsequent use in the assay. Prepare enough reagents for only the number of strips used. Reagents with a volume less than 100 µL should be centrifuged before use to avoid loss of volume.

Wash Buffer Concentrate (10X) must be diluted with distilled water 1:10 before use (100 mL WASHBUF + 900 mL distilled water) and mixed well. Crystals could occur due to high salt concentration in the concentrate. Before dilution, the crystals must be dissolved at room temperature or in a water bath at 37 °C. The undiluted wash buffer is stable at 2–8 °C until the expiry date stated on the label. 1X working wash buffer (1:10 diluted wash buffer) can be stored in a closed flask at 2–8 °C for 1 month.

Extraction Buffer Concentrate (2.5X) must be diluted with distilled water 1:2.5 before use (100 mL Extraction Buffer + 150 mL distilled water), mix well. Crystals could occur due to high salt concentration in the concentrate. Before dilution, the crystals must be dissolved at 37°C in a water bath. The Extraction Buffer concentrate is stable at 2–8 °C until the expiry date stated on the label. 1X working extraction buffer (1:2.5 diluted) can be stored in a closed flask at 2–8 °C for 4 months.

All other test reagents are ready to use. Test reagents are stable until the expiry date (see label of test package) when stored at 2–8 °C.

QUALITY CONTROL

Control samples should be analyzed with each run. Results generated from the analysis of control samples, should be evaluated for acceptability using appropriate statistical methods. The results for the samples may not be valid if within the same assay one or more values of the quality control samples are outside the acceptable limits.

ASSAY PROCEDURE

All reagents and microplate strips (while sealed in foil pouch) should be equilibrated to room temperature prior to use. Gently mix all reagents before use. A standard curve must be performed with each assay run and with each microplate if more than one is used at a time. All standards, controls, and samples should be run in duplicate. Take as many microtiter strips as needed from kit. Store unused strips together with the desiccant bag in the closed aluminum packaging at 2–8 ° C. Strips are stable until expiry date stated on the label.

For automated ELISA processors the given protocol may need to be adjusted according to the specific features of the respective automated platform. For further details, please contact ALPCO.

1. Bring all reagents and diluted samples to room temperature (15–30°C) and mix well.
2. Add 100 µL of standards, controls, and diluted samples into respective wells. A suggested plate layout is included.
3. Cover plate tightly and incubate for 30 minutes at room temperature (15–30°C).
4. Discard the contents of each well. Wash each well 5 times with 250 µL of wash buffer. After the final washing step, remove residual wash buffer by firmly tapping the plate on absorbent paper.
5. Add 100 µL of conjugate into each well.
6. Cover plate tightly and incubate for 30 minutes at room temperature (15–30°C).
7. Discard the contents of each well. Wash each well 5 times with 250 µL of wash buffer. After the final washing step, remove residual wash buffer by firmly tapping the plate on absorbent paper.
8. Add 100 µL of TMB substrate into each well.
9. Incubate for 10–20 minutes at room temperature (15–30°C) in the dark. *
10. Add 100 µL of stop solution into each well, mix thoroughly.
11. Determine absorption immediately with an ELISA reader at 450 nm against 620 nm (or 690 nm) as a reference. If no reference wavelength is available, read only at 450 nm. If the extinction of the highest standard exceeds the range of the photometer, absorption must be measured immediately at 405 nm against 620 nm as a reference.

*The intensity of the color change is temperature sensitive. It is recommended to observe the color change and to stop the reaction upon good differentiation.

CALCULATION OF RESULTS

The following algorithms can be used alternatively to calculate the results. It is recommended to use the 4-parameter algorithm.

1. 4-parameter algorithm: It is recommended to use a linear ordinate for the optical density and a logarithmic abscissa for the concentration. When using a logarithmic abscissa, the zero calibrator must be specified with a value less than 1 (e.g. 0.001).
2. Point-to-point calculation: It is recommended to use a linear ordinate for the optical density and a linear abscissa for the concentration.

3. Spline algorithm: It is recommended to use a linear ordinate for the optical density and a linear abscissa for the concentration.

The plausibility of the pairs of values should be examined before the automatic evaluation of the results. If this option is not available with the program used, a comparison of the paired values should be done manually.

Stool samples

The obtained calprotectin levels of the stool samples must be multiplied by the dilution factor of 2500 (Dilution I × Dilution II) to get the actual concentration. In the case where another dilution factor has been used, multiply the obtained result by the dilution factor used.

1g stool is equivalent to 1mL.

CONVERSION
1 ng/mL = 1000 µg/g

Example Calculation

$$\frac{100\ ng}{mL} \times \frac{1\ mL}{1\ g} \times \frac{0.001\ \mu g}{1\ ng} = \frac{0.1\ \mu g}{g} \times 2500\ (\text{dilution factor}) = \frac{250\ \mu g}{g}$$

Conversion of Standard Curve Values from ng/mL to µg/g

ng/mL without dilution factor	µg/g not including dilution factor	µg/g including dilution factor
0	0	0
13	0.013	32.5
52	0.052	130
210	0.210	525
840	0.840	2100

LIMITATIONS

Samples with concentrations above the measurement range (see definition below) must be further diluted and re-assayed. Please consider this greater dilution when calculating the results. Samples with concentrations lower than the measurement range (see definition below) cannot be clearly quantified.

The upper limit of the measurement range can be calculated as:
highest concentration of the standard curve × sample dilution factor to be used

The lower limit of the measurement range can be calculated as:
LoB × sample dilution factor to be used

PERFORMANCE CHARACTERISTICS

Analytical Sensitivity

Limit of Blank, LoB: 0.957 ng/mL (2.393 µg/g)
Limit of Detection, LoD: 2.27 ng/mL (5.675 µg/g)
Limit of Quantitation, LoQ: 4.77 ng/mL (11.93 µg/g)

Precision: Within run (intra-assay) variation

The repeatability was assessed with 2 stool samples under constant parameters (same operator, measurement system, day, and kit lot).

	Sample 1	Sample 2
Mean	89.2 ng/mL	229.1 ng/mL
CV (%)	5.6	3.2
n	20	20

Precision: Between run (inter-assay) variation

The reproducibility was assessed with 3 stool samples under varying parameters (different operators, measurement systems, days, and kit lots).

	Sample 1	Sample 2	Sample 3
Mean	59.2 ng/mL	253.3 ng/mL	490.3 ng/mL
CV (%)	11.6	8.8	9.1
n	25	25	25

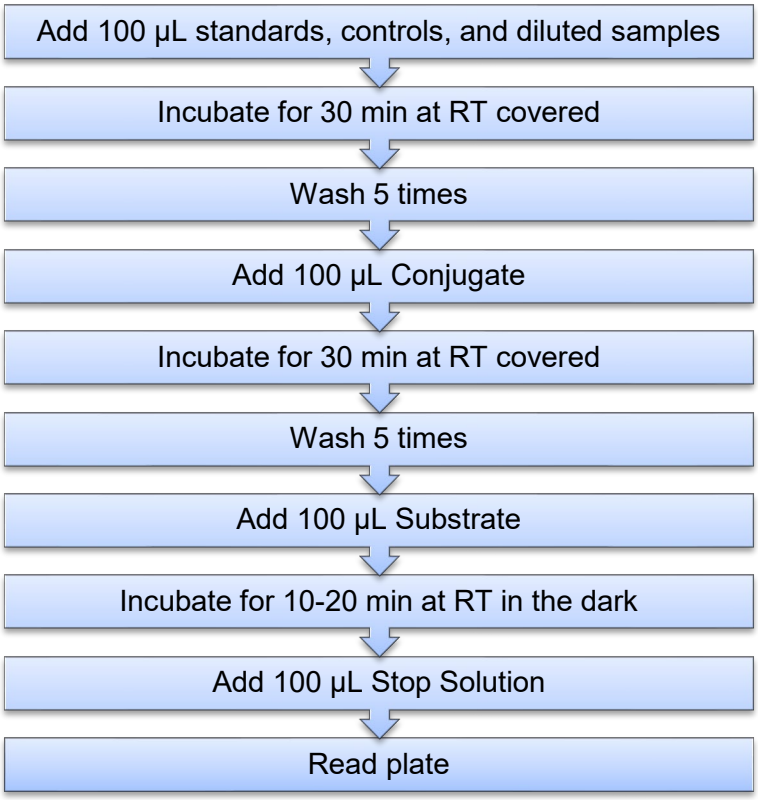
TECHNICAL HINTS

- Do not interchange different lot numbers of any kit component within the same assay. Furthermore, it is not recommended to assemble wells of different microtiter plates for analysis, even if they are of the same batch.
- Control samples should be analyzed with each run.
- Reagents should not be used beyond the expiration date stated on kit label.
- Substrate solution should remain colorless until use.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- Avoid foaming when mixing reagents.
- Do not mix plugs and caps from different reagents.
- The assay should always be performed according to the enclosed manual.

GENERAL NOTES ON THE TEST AND TEST PROCEDURE

- The guidelines for medical laboratories should be followed.
- Incubation time, incubation temperature and pipetting volumes of the components are defined by the vendor. Any variation of the test procedure, which is not coordinated with the vendor, may influence the results of the test.

SHORT ASSAY PROTOCOL



Total Incubation Time = 1 hour 20 minutes

SUGGESTED PLATE LAYOUT

Below is a suggested plate layout for running standards, controls, and up to 41 samples in duplicate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std 1	Std 1	2	2	10	10	18	18	26	26	34	34
B	Std 2	Std 2	3	3	11	11	19	19	27	27	35	35
C	Std 3	Std 3	4	4	12	12	20	20	28	28	36	36
D	Std 4	Std 4	5	5	13	13	21	21	29	29	37	37
E	Std 5	Std 5	6	6	14	14	22	22	30	30	38	38
F	Ctrl 1	Ctrl 1	7	7	15	15	23	23	31	31	39	39
G	Ctrl 2	Ctrl 2	8	8	16	16	24	24	32	32	40	40
H	1	1	9	9	17	17	25	25	33	33	41	41

Std= Standard
Ctrl = Control
Numbered wells = Samples