RIDASCREEN® α_1-Antitrypsin

REF G09034

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1. Intended use

For *in vitro* diagnostic use. RIDASCREEN® α_1-antitrypsin is an immunoassay for the quantitative determination of α_1-antitrypsin in stool samples.

2. Summary and explanation of the test

Proteins are essential constituents of food and essential building blocks of the body. The body must be able to process proteins, that is, to synthesize and break down proteins as needed.

Proteolytic enzymes such as trypsin and chymotrypsin help break down proteins. These enzymes not only digest food but also help to fight bacterial infections and inflammatory diseases of the gastrointestinal tract. Proteolytic inhibitors ensure that the action of proteolytic enzymes is stopped before they destroy healthy tissues. One of the most important inhibitors is α_1-antitrypsin (also known as α_1-proteinase inhibitor), a glycoprotein with a molecular weight of 50 kilodaltons (kDAs). The protein is a primary inhibitor that forms reversible complexes with serine proteases such as polymorphonuclear neutrophil (PMN) elastase, trypsin, chymotrypsin and active inflammatory immune cells.

Thus, α_1-antitrypsin also has an important regulatory effect on inflammatory processes, primarily inhibiting PMN elastase, a protease released by leukocytes. The body releases PMN elastase in response to inflammatory stimuli. As a regulator of protease activity, α_1-antitrypsin ensures that the effects of PMN elastase remain limited to the inflammation, thus protecting healthy tissues from proteolytic damage. This makes the inhibitor useful as an indicator of activity of chronic inflammatory bowel diseases. Patients with Crohn's disease, ulcerative colitis and other bowel disorders such as polyps, colon cancer, diverticulitis, celiac disease or severe food allergies have sharply elevated levels of α1-antitrypsin. α_1-antitrypsin is also used as a fecal marker of intestinal protein loss and increased mucosal permeability in patients with a non-intact intestinal mucosa.

Generally, it is assumed that α_1-antitrypsin is synthesized primarily in the liver but also in intestinal cells, and that it is excreted in the feces without tryptic cleavage or resorption. Consequently, it is not subject to intestinal degradation and is thus well suited for use as a stool marker.

3. Test principle

RIDASCREEN® α_1-antitrypsin employs a sandwich method to test for specific antibodies. The surface of the well of the microtiter plate is coated with a specific antibody against epitopes of human α_1-antitrypsin.
A suspension of the stool sample to be tested is pipetted into a well of the microtiter plate and incubated. The plate is then washed and incubated a second time with a polyclonal anti-α_1-antitrypsin antibody conjugated to horseradish peroxidase. If α_1-antitrypsin is present in the sample, this results in the formation of a sandwich complex consisting of the immobilized antibody, α_1-antitrypsin antigen and the conjugated antibody. 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 extinction is proportional to the concentration of α_1- antitrypsin found 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 polyclonal antibodies (from rabbits) against human α_1-antitrypsin
Extract 10x	100 ml	Extraction and dilution buffer (for extraction and primary dilution of stool samples); phosphate-buffered NaCl solution; contains 0.1 % NaN_3; 10x concentrate
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; 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 (from rabbit) against human α_1-antitrypsin 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 more details, refer to Safety Data Sheets (SDS) at www.r-biopharm.com.

5. Storage instructions

All reagents must be stored at 2 – 8 °C and can be used until the date printed on the label. The diluted wash buffer and extraction buffer can be used for a maximum of 4 weeks when stored at 2 – 8 °C. Microbial contamination must be prevented. After the expiration date, the quality guarantee is no longer valid. The aluminum bag must be opened with scissors in such a way that the clip seal is not separated. Any microtiter strips which are not required must immediately be sealed in 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. Additional necessary reagents and necessary equipment

6.1. Reagents

- Distilled or deionized water

6.2. Equipment

- Sample vials
- Vortex mixer (optional, see 9.4.)
- Micropipette for 50 - 100 µl, 5 and 1 ml volumes
- Graduated cylinder (1000 ml)
- Stopwatch
- Washing device for microtiter plates or multichannel pipettes (300 µl)
- Microtiter plate photometer (450 nm; reference filter 620 nm)
- Filter paper (lab wipes)
- Waste container containing 0.5 % hypochlorite solution

7. Warnings and precautions for the 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. Do not pipette samples or reagents using your mouth. Avoid contact with broken skin or mucous membranes. Wear personal protective equipment (appropriate gloves, lab coat, safety glasses) when handling reagents and samples, and wash hands after completing the test. Do not smoke, eat, or drink in areas where samples are handled.

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

The calibrator, the positive control, and the low-positive control contain human blood components that have tested negative for HIV, HCV, and HBsAg infection. Nonetheless, these components and fecal samples must be treated as potentially infectious and handled in accordance with national safety regulations.

The wash buffer contains 0.1 % thimerosal as preservative. This substance must not be allowed to come into contact with skin or mucous membranes.

The calibrator, positive control, low-positive control, extraction and sample dilution buffer contain 0.1 % NaN_3. This substance must not be allowed to come into contact with skin or mucous membranes.

Hydrogen peroxide (TMB) 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 toxic gases, any liquid waste containing stop reagent must be neutralized before adding to hypochlorite solution.
Users are responsible for proper disposal of all reagents and materials after use. For disposal, please adhere to national regulations.

8. Collection and storage of samples

Until it is used, store the test material at 2 - 8 °C. If the material cannot be used for a test within three days, we recommend storage at -20 °C or colder. Avoid freezing and thawing the sample repeatedly. Stool samples and rectal smears should not be collected in transport containers which contain transport media with preservatives, animal sera, metal ions, oxidizing agents, or detergents since these may interfere with the RIDASCREEN® α_1-antitrypsin test.

9. Test procedure

9.1. General information

All reagents and the microtiter 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.

Note: The calibrator and positive control must be added in every test run. The use of the low-positive control is optional.

9.2. Preparing the wash buffer

Mix 1 part wash buffer concentrate Wash 10x with 9 parts distilled water. 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 10x with 9 parts distilled water (1:10). Any crystals present in the concentrate should be dissolved beforehand through heating (water bath at 37 °C).

9.4. Sample preparation

9.4.1 Sample weighing and suspension

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.

Table 1: Required volume of diluted extraction buffer as a function of stool sample weight

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

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.

Then the homogenized suspension must be centrifuged for 10 minutes at at least 3,000 g to settle out rough stool particles.

9.4.2 Manual sample dilution

Dilute 50 µl of the clarified supernatant in 950 µl diluted extraction buffer (1:20) and vortex the sample. For final dilution of the sample, dilute 50 µl of the first dilution with 950 µl of RIDASCREEN® sample dilution buffer Diluent | 3 (1:20), and then vortex. This final dilution of the stool sample is used for testing (see 9.5.).

9.4.3 Automatic sample dilution

If the test is run on a DSX automated ELISA system from Dynex, use the following sample dilution steps. As with manual dilution, particle-free supernatant must be used for automatic sample dilution. For instructions on using other ELISA automated pipetting systems, please contact R-Biopharm AG.
The automatic ELISA system pipettes 25 µl of the supernatant into a deep-well plate, which is diluted with 975 µl of diluted extraction buffer (1:40). Two mixing cycles follow.
Place the required number of microtiter strips in the holder of the RIDASCREEN® α_1-antitrypsin microtiter plate Plate .Transfer 10 µl of the sample dilution from the deep-well plate into the RIDASCREEN® α_1-antitrypsin microtiter plate Plate and further dilute with 90 µl RIDASCREEN® sample dilution buffer Diluent | 3 (1:10).

9.5. First incubation

After inserting a sufficient number of wells into the holder, add 100 µl of calibrator Calibrator (in duplicate), 100 µl sample dilution buffer Diluent | 3 (= negative control), 100 µl of positive control Control | +, and 100 µl of the final stool sample dilution being analyzed to the respective wells. If needed, 100 µl of low-positive control Low control | + can be added. Next incubate the plate for one (1) hour at room temperature (20 – 25 °C).

9.6. First washing

Careful washing is important in order to obtain the correct results and should therefore be carried out strictly according to the instructions. The incubated substance in the wells must be emptied into a waste container containing hypochlorite for disinfection. Next, tap the plate over absorbable paper to remove the remaining moisture. Then wash the plate 5 times using 300 µl 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.
When using a microplate washer, make sure that the machine is correctly set to the type of microtiter plate being used. Furthermore, a stool suspension which is not completely particle-free before the first wash should be removed manually by centrifuging in order to avoid blocking the wash needles. 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 (1) hour at room temperature (20 – 25 °C).

9.8. Second washing

After the incubation time has elapsed, again wash the plate 5 times using 300 µl wash buffer each time. Make sure that the wells are completely empty by tapping them out after each wash onto a dry and unused area of absorbent paper.

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 wavelength of 620 nm.

Note: High-positive patient samples may cause black-colored precipitates of the substrate.

10. Quality control — Indication of deterioration of reagents

For quality control purposes, the calibrator (in duplicate), positive and possibly low-positive controls must be added each time the test is carried out to ensure that the reagents are stable and that the test is conducted correctly. The test has been carried out correctly if the controls lie within the ranges specified in the lot-specific data sheet supplied with the kit.

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 fulfilled after repeating the test, please consult the manufacturer or your local R-Biopharm distributor.

11. Evaluation and interpretation

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

RIDASCREEN® α_1-antitrypsin ELISA uses a four-parameter logistic-log model (4PL) to determine the concentration of α_1-antitrypsin in stool in units of µg/g.

The RIDA®SOFT Win.NET evaluation software is required to calculate the results. RIDA®SOFT Win or updates can be obtained on request by contacting R-Biopharm AG or your local R-Biopharm distributor.

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 range for the standard deviation for the calibrator, positive control, and low-positive control. The calibrator is used for quantitative analysis of samples. The positive control and low-positive control are used for internal test validation in a given laboratory.

RIDA®SOFT Win calculates a 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.

Other evaluation software that uses the 4-parameter logistic log model can also be used instead of RIDA®SOFT Win.

11.2. Test result

Measured values < 400 µg/g in stool are considered negative. We advise each laboratory to establish its own standard value range.

12. Limitations of the method

The RIDASCREEN® α_1-antitrypsin test detects epitopes of α_1-antitrypsin in stool samples. A correlation between the level of the determined extinction mean with the severity of clinical symptoms cannot be derived from such. The obtained results must always be interpreted in connection with the clinical signs and symptoms.

13. Performance characteristics

13.1. Test quality

In a study performed by an independent laboratory, the sensitivity and specificity of the RIDASCREEN® α_1-antitrypsin ELISA was tested in 153 stool samples and compared with that of the corresponding immunochemiluminometric assay (α_1-antitrypsin ILMA) routinely used there. Based on the study results, the performance characteristics are as follows:

Sensitivity: 96.3 %

Specificity: 83.0 %

13.2. Detection limit

The detection limit of the RIDASCREEN® α_1-antitrypsin ELISA was calculated as the sum of B_0 and the two-fold standard deviation of B_0. B_0 is the mean of multiple determinations (n=36) of the negative control (= Diluent | 3).

The detection limit for α_1-antitrypsin was thus found to be 30.8 µg/g.

13.3. Linearity of test results

To check the linearity of the RIDASCREEN® α_1-antitrypsin ELISA, serial dilution series of several α_1-antitrypsin-positive and negative stool samples were prepared. The weighing and suspension of samples and the first dilution step were performed according to the directions in these instructions (see 9.4.). Serial dilutions were then prepared using RIDASCREEN® sample dilution buffer Diluent | 3. Extinction of the individual concentrations was back-calculated to the starting concentrations using the respective dilution factor. The dilution series for a representative α_1-antitrypsin-positive and negative sample is presented in Table 2.

Table 2: Determination of linearity of the test results

	RIDASCREEN® α1-antitrypsin	
Dilution	**Pos. sample [µg/g stool]**	**Neg. sample [µg/g stool]**
1:12,500	526	241
1:20,000	484	235
1:25,000	525	255
1:30,000	518	252
MV	513	246
SD	19.8	9.4
CV %	3.9	4.8

13.4. Precision

The intra- and inter-assay reproducibility of RIDASCREEN® α1-antitrypsin ELISA was determined multiple times on different days while maintaining optimal conditions. Tables 3 and 4 show the results.

Table 3: Intra-assay reproducibility (n = 20)

Intra-assay	RIDASCREEN® α1-antitrypsin	
	1. Concentration [10 ng/ml]	**2. Concentration [30 ng/ml]**
MV (OD)	0.404	1.081
SD	0.020	0.051
CV %	4.9	4.8

Table 4: Inter-assay reproducibility (n = 16)

Inter-assay	RIDASCREEN® α1-antitrypsin	
	1. Concentration [10 ng/ml]	**2. Concentration [30 ng/ml]**
MV (OD)	0.409	1.080
SD	0.032	0.057
CV %	7.9	5.3

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
Diluent | 3	Sample dilution buffer
Wash buffer	Wash buffer 10x
Calibrator	Calibrator
Control | +	High control
Low control | +	Low control
Conjugate	Conjugate
SeroSC	Substrate
Stop	Stop solution

16. References

1. Arndt et al., 1993, *Crohn; Clin. Lab*. 11: 867-876
2. Stein, J., 1996, 3. *Post-graduiertenkurs der DGVS*
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