RIDASCREEN® α_1-Antitrypsin

Art. No. G09034

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1. Intended use

For *in vitro* diagnostic use. RIDASCREEN® α_1-antitrypsin is an enzyme immunoassay for 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 assist in the process of protein degradation. Proteolytic 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 of proteolytic enzymes is a 50-kilodalton glycoprotein known as alpha$_1$-antitrypsin (A1AT), also referred to as α_1-proteinase inhibitor. A1AT 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, A1AT 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 renders α_1-antitrypsin 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 α_1-antitrypsin. A1AT 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, α_1-antitrypsin is not subject to intestinal degradation and is thus well suited for use as a stool marker.

3. Test principle

RIDASCREEN® α_1-Antitrypsin is an α_1-antitrypsin-specific sandwich enzyme-linked immunosorbent assay (ELISA). The wells of its microtiter plate are coated with a specific antibody directed against epitopes of human α_1-antitrypsin.

An aliquot of fecal test suspension 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. A second washing step is performed to remove unbound enzyme-labeled antibodies. In α_1-antitrypsin-positive samples, the addition of substrate solution results in enzyme binding, transforming the colorless solution in the wells of the plate to a blue liquid. After the addition of stop solution, the color changes from blue to yellow. The subsequently measured extinction (optical density) is proportional to the concentration of α_1-antitrypsin in the sample.

4. Reagents provided

Each kit contains sufficient reagents for 96 tests.

Plate	96 tests	Microwell plate; 12 microwell strips (separable) in strip holder; Coated with (rabbit) polyclonal antibodies against human α_1-antitrypsin
Extract 10x	100 ml	Extraction/Dilution Buffer (for extraction and primary stool dilution); Phosphate-buffered NaCl solution; Contains 0.1 % NaN_3; 10 X concentrate
Diluent 3	100 ml	Sample Diluent (for final dilution); Protein-buffered NaCl solution; Contains 0.1 % NaN_3; Ready for use; Red in color
Wash 10x	100 ml	Wash Buffer; Phosphate-buffered NaCl solution; Contains 0.1 % thimerosal; 10 X concentrate
Calibrator	1 ml	1 Calibrator; Contains 0.1 % NaN_3; Ready for use; White cap
Control +	1 ml	1 Positive Control; Contains 0.1 % NaN_3; Ready for use; Red cap
Low Control +	1 ml	1 Low-Positive Control; Contains 0.1 % NaN_3; Ready for use; Green cap
Conjugate	12 ml	Peroxidase-conjugated (rabbit) polyclonal antibody against human α_1-antitrypsin in stabilized protein solution; Ready for use; Red cap
SeroSC	12 ml	Urea peroxide/tetramethylbenzidine (TMB); Ready for use
Stop	12 ml	Stop Solution; 1 N sulfuric acid; Ready for use

Details of hazardous substances according to labeling obligations. For more details see Material Safety Data Sheets (MSDS) at www.r-biopharm.com.

5. Storage instructions

All reagents must be stored at 2 to 8 °C and can be used until the expiration date printed on the product label. After reconstitution, diluted wash and extraction buffer can be stored for 4 weeks at 2 to 8 °C. Avoid microbial contamination! The quality of the product cannot be guaranteed after the expiration date. Use scissors to cut open the foil pouch without damaging the reclosable seal. Immediately return unused microwell strips to the foil pouch and store at 2 to 8 °C. Protect the colorless substrate solution from direct light exposure in order to prevent decomposition and discoloration (change to blue) due to auto-oxidation. If discolored, the substrate must be discarded.

6. Materials required but not provided

6.1. Reagents

- Distilled or deionized water

6.2. Equipment

- Test tubes
- Disposable pipettes (Art. No.: Z0001)
- Vortex mixer (optional; see 9.4.)
- Micropipettes for 50 - 100 µl and 1 ml volumes
- Graduated cylinder (1000 ml)
- Timer
- Washing unit for microwell plates or multichannel pipettes (300 µl)
- Photometer for microwell plates (450 nm/620 nm reference filter)
- Filter paper (laboratory tissues)
- Waste receptacle containing 0.5 % hypochlorite solution

7. Precautions

For in vitro diagnostic use only.

This test must only be carried out by trained laboratory personnel. The guidelines for working in medical laboratories have to be followed. The instruction manual for the test procedure has to be followed. Do not pipet samples or reagents by mouth. Avoid contact with bruised skin or mucosal membranes. During handling reagents or samples, wear appropriate safety clothing (appropriate gloves, lab coat, safety goggles) and wash your hands after finishing the test procedure. Do not smoke, eat or drink in areas where samples or reagents are being used.

For more details see Material Safety Data Sheets (MSDS) at www.r-biopharm.com.

Calibrator, Positive Control and Low-Positive Control contain human blood components which have tested negative for HIV and hepatitis infection. Nonetheless, these components and fecal samples must be treated as potentially infectious materials and handled in accordance with national safety regulations.

Wash Buffer contains 0.1 % thimerosal as a preservative. Avoid contact with skin and mucous membranes.

Calibrator, Positive Control, Low-Positive Control, Extraction Buffer and Sample Diluent contain 0.1 % NaN_3. Avoid contact with skin and mucous membranes.

Substrate contains urea peroxide/TMB and can cause burns. Handle with care!

Stop Solution contains 1 N sulfuric acid. Avoid contact with skin and clothing! Skin exposed to the substance should be rinsed with water.

All reagents and materials coming in contact with potentially infectious samples must be treated with appropriate disinfectants or autoclaved at 121 °C for at least 1 hour. WARNING: To prevent the formation of poisonous gases, any liquid waste containing Stop Solution must be neutralized prior to disposal in hypochlorite solution.

All reagents and materials used have to be disposed properly after use. Please refer to the relevant national regulations for disposal.

8. Specimen collection and storage

All test samples must be stored at 2 to 8 °C until processed. If the samples cannot be processed within 3 days, they should be frozen at –20 °C or colder. Repeated freezing and thawing of samples should be avoided. Stool samples or rectal swabs should not be collected in transport containers using transport media containing preservatives, animal sera, metal ions, oxidizing agents or detergents as these substances may interfere with the RIDASCREEN® α_1-Antitrypsin assay.

9. Test procedure

9.1. General information

Allow kit reagents and microwell plate Plate to reach room temperature before use (20 to 25 °C). Do not remove the microwell strips from the foil pouch until they have reached room temperature. The reagents are mixed thoroughly immediately prior to use. Return all unused microwell strips (in sealed pouch) and reagents to refrigerator and store at 2 to 8 °C. Once a microwell strip has been used, it may not be used again. Do not use reagents and microwell strips if the packaging is damaged or the vials are not sealed properly. To prevent cross-contamination, do not allow test samples to come in direct contact with the kit components.

Do not perform the test in direct sunlight. The microwell plate should be covered or taped during testing to avoid evaporation loss.

NOTE: Calibrator and Positive Control must be assayed with each batch of samples. Low-Positive Control may be assayed with the batch as needed.

9.2. Wash Buffer preparation

Mix 1 part Wash Buffer concentrate Wash 10x with 9 parts distilled water. Any crystals present in the concentrate must be dissolved by warming the vial in a warm water bath (37 °C) before reconstitution.

9.3. Extraction Buffer preparation

Mix 1 part Extraction Buffer concentrate Extract 10x with 9 parts distilled water (1:10). Any crystals present in the concentrate must be dissolved by warming the vial in a warm water bath (37 °C) before reconstitution.

9.4. Sample preparation

9.4.1. Weighing and suspension of samples

Place 100 mg of stool sample in a labeled test tube and add 5 ml of diluted Extraction Buffer (1:50) using a pipette.

Alternatively, one can place 80 to 130 mg of stool sample in the test tube and add a proportionally smaller or larger volume of diluted Extraction Buffer (see Table 1) to the sample (to maintain a constant dilution ratio).

Table1: Required volume of diluted extraction buffer as a function of stool sample weight

Sample weight [mg]	Buffer 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

Independent of the weighing method, all stool samples are homogenized by thoroughly mixing them on a vortex mixer. In case of liquid stools, exactly 100 µl of stool sample are aspirated into a pipette and suspended in exactly 5 ml of diluted extraction buffer.

After this, the homogenized sample must be centrifuged at a minimum of 3000 g for 10 minutes to ensure sedimentation of coarse fecal particles.

9.4.2. Manual sample dilution

Dilute 50 µl of clear supernatant with 950 µl of diluted extraction buffer and vortex (1:20). Final dilution of the sample is then performed by further diluting 50 µl of the first dilution with 950 µl RIDASCREEN® Sample Diluent Diluent | 3 (1:20) and vortexing. This final dilution of the stool sample is used for testing (see 9.5.).

9.4.3. Automated sample dilution

Users performing the assay with the fully automated DSX-ELISA system by Dynex can use the following sample dilution steps. As with manual dilution, particle-free supernatant must be used for automated sample dilution. Users with other ELISA automated pipetting systems should contact R-Biopharm for instructions.

The ELISA system automatically pipettes 25 µl of supernatant into a deep-well plate, which is diluted with 975 µl of diluted extraction buffer (1:40). Two mixing cycles are subsequently performed.

The required number of coated wells is then placed in the microwell holder of the RIDASCREEN® α_1-Antitrypsin microwell plate Plate.

Next, a 10 µl aliquot of diluted sample is removed from the deep-well plate and transferred to the RIDASCREEN® α_1 -Antitrypsin microwell plate Plate and further diluted using 90 µl of RIDASCREEN® Sample Diluent Diluent | 3 (1:10).

9.5. First incubation

After inserting the desired number of coated wells in the microwell holder, 100 µl of Calibrator Calibrator (in duplicate), 100 µl of Sample Diluent Diluent | 3 (= negative control), 100 µl of Positive Control Control | + and 100 µl of the diluted stool sample are added to the respective wells. 100 µl of Low-Positive Control Low Control | + can be assayed with the batch if necessary. The plate is then incubated at room temperature (20 - 25 °C) for 1 hour.

9.6. First washing

As thorough washing is essential for achieving correct test results, strict adherence to washing instructions is crucial. First, decant the incubated solutions in the microwells into a waste receptacle containing hypochlorite solution for disinfection. Next, tap the microplate on absorbent paper to remove the remaining liquid and wash the plate five times using 300 µl of Wash Buffer per washing step. Tap the plate on a dry, unused part of the absorbent paper after each washing step to ensure complete removal of liquids.

When using an automated washer, ensure that the washer is correctly set for the specific type of microtiter plate used. Stool sample suspensions that are not completely particle-free should be manually removed from the wells before the first washing step in order to prevent blockage of the washing needles. During the washing steps, it is important to ensure that the solutions are removed completely. After the final washing step, the plate should be tapped on clean absorbent paper or laboratory tissue to ensure removal of residual moisture.

9.7. Second incubation

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

9.8. Second washing

After second incubation, wash the plate five times using 300 µl of Wash Buffer per washing step. Tap the plate on a dry, unused part of the absorbent paper after each washing step to ensure complete removal of liquids.

9.9. Third incubation

Add 100 µl of Substrate Solution SeroSC to each well and allow the plate to incubate in the dark at room temperature (20 - 25 °C) for 15 minutes. Next, add 50 µl of Stop Solution Stop to each well to stop the reaction (mix cautiously by gently rocking the plate manually) and measure extinction/optical density (OD) in the wells at a wavelength of 450 nm (reference wavelength: 620 nm).

NOTE: Strong positive reactions can result in the production of black precipitates from the substrate solution.

10. Quality control – Signs of reagent deterioration

Calibrator (in duplicate) and Positive Control, and Low-Positive Control if needed, must be assayed with each batch of patient specimens to ensure reagent quality and proper performance of the assay. The test has been carried out correctly if extinction (OD) of the controls lies within the ranges specified in the lot-specific data sheet supplied with the kit. If the measured values lie outside the target ranges, the following variables should be checked before repeating the assay:

- Expiration date of the reagents used
- Functionality of the equipment being used (e.g. calibration)
- Correct performance of test procedure
- Visual inspection of kit components for contamination or leaks; Substrate Solution must not be used if discolored (blue)

If quality conditions still are 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 using the four-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.

RIDA®SOFT Win analytical software is needed to determine the test results. RIDA®SOFT Win or an update can be obtained from R-Biopharm AG or from your local R-Biopharm distributor.

Both the four parameters (A - D) of the standard curve required for 4PL calculations and the setpoint values for the Calibrator, Positive Control and Low-Positive Control are listed on the lot-specific data sheet supplied with the kit and must be compared to the values in the analytical software before each measurement using the corresponding information on the data sheet**.**

R-Biopharm AG determines the standard curve (including parameters A - D) as well as the setpoint value and the allowed ranges of standard deviation for the Calibrator, Positive Control and Low-Positive Control under optimal test conditions for each kit lot. Calibrator is used for quantitative analysis of samples. Positive Control and Low-Positive Control are used for internal test validation within a given laboratory.

RIDA®SOFT Win calculates a correction factor F from the mean value of the duplicate determination of Calibrator and its setpoint value, which is reconciled with the extinction (OD) values for the stool samples. Safe and reliable evaluation of the test results is possible within the range of the standard curve.

Other analytical software using the four-parameter logistic-log model can also be used as an alternative to RIDA®SOFT Win.

11.2. Test results

Measured values below the cut-off value of 400 µg/g α_1-antitrypsin in stool are interpreted as negative. We recommend that each laboratory establish its own standard value range.

12. Limitations of the method

The RIDASCREEN® α_1-Antitrypsin ELISA detects epitopes of α_1-antitrypsin in stool samples. Correlations between the level of the measured extinction (OD) values and the severity of clinical symptoms cannot be derived from this test. The test results must always be interpreted in combination 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 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 twice the 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 in stool was thus found to be 30.8 µg/g.

13.3. Linearity of test results

Dilution series of multiple α_1-antitrypsin-positive and negative stool samples were used to validate the linearity of the RIDASCREEN® α_1-Antitrypsin ELISA. The weighing and suspension of samples and the first dilution step were performed according to the directions in this instruction leaflet (see 9.4.). Graduated serial dilutions were then prepared using RIDASCREEN® Sample Diluent Diluent 3. Extinction (OD) 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 α_1-antitrypsin-negative sample is presented in Table 2.

Table 2: Determination of the linearity of the assay results

	RIDASCREEN® α_1-Antitrypsin	
Dilution	Positive sample [µg/g stool]	Negative sample [µg/g stool]
1:12500	526	241
1:20000	484	235
1:25000	525	255
1:30000	518	252
Mean	513	246
SD	19.8	9.4
CV %	3.9	4.8

13.4. Precision

Intra- and inter-assay reproducibility of the RIDASCREEN® α_1-Antitrypsin ELISA was validated in multiple determinations performed on different days under optimal test conditions. The results are presented in Tables 3 and 4.

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

Intra-Assay	RIDASCREEN® α_1-Antitrypsin	
	Concentration 1 [10 ng/ml]	Concentration 2 [30 ng/ml]
Mean (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® α_1-Antitrypsin	
	Concentration 1 [10 ng/ml]	Concentration 2 [30 ng/ml]
Mean (OD)	0.409	1.080
SD	0.032	0.057
CV %	7.9	5.3

14. References

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