Distribuito in ITALIA da
Li StarFish S.r.l.
Via Cavour, 35
20063 Cernusco S/N (MI)
telefono 02-92150794
info@listarfish.it
www.listarfish.it

Manual

IDK® α_1-Antitrypsin Clearance ELISA

For the in vitro determination of α_1-antitrypsin in serum, plasma and stool

Valid from 2023-07-27

REF **K 6752**

Σ 96

+2 °C ±8 °C

IVD

CE

Immundiagnostik AG, Stubenwald-Allee 8a, 64625 Bensheim, Germany
Tel.: +49 6251 70190-0 Fax: + 49 6251 70190-363
e.mail: info@immundiagnostik.com www.immundiagnostik.com

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1. INTENDED USE

This Immundiagnostik AG assay is an enzyme immunoassay intended for the quantitative determination of α_1-Antitrypsin in serum, plasma and stool.

For *in vitro* diagnostic.

2. INTRODUCTION

Intestinal protein loss is a serious consequence of various systemic or local gastrointestinal diseases (e. g. allergies, chronic inflammation, malignancies). These pathologies damage the mucosal integrity and/or cause lymphostasis, thereby leading to an increased transfer of plasma proteins into the bowel lumen. Subsequently, hypoproteinemia accompagnied with edema may develop. This condition is diagnosed by exclusion of other sources of protein loss and by proof of an elevated α_1-antitrypsin concentration in stool.

In serum, α_1-antitrypsin represents the majority of serine protease inhibitors and protects tissues from protease damages during inflammation. The protein is synthesised primarily in the liver but also to a small extent in intestinal macrophages, monocytes, and intestinal epithelial cells. Since α_1-antitrypsin is relatively resistent against enzymatic digestion, the secreted amount in stool reflects the internal concentration of the protein. An elevated α_1-antitrypsin stool concentration is therefore a widely recognised marker for intestinal protein loss and for an increased mucosal permeability.

In clinical routine, the α_1-antitrypsin clearance (ratio of the α_1-antitrypsin ELISA values of stool and serum samples) has been established along with the sole determination of the 24h α_1-antitrypsin secretion in stool. Thus the group of J. S. Fordtran reports that the sole determination of the α_1-antitrypsin concentration in stool yielded false positive or false negative results in 21 % of the patients compared to the α_1-antitrypsin clearance measurement (Strygler *et al*. 1990).

The combination of two specific antibodies in our *IDK*® α_1-antitrypsin ELISA widely excludes the possibility of false negative results thereby enabling a reliable diagnostics of enteral protein loss.

Indications

- Suspected enteric protein loss
- Crohn's disease
- Necrotic enterocolitis
- Chronic mesenterial ischemia
- Viral, bacterial, allergic, or autoimmune-induced gastrointestinal inflammation

3. MATERIAL SUPPLIED

Cat. No.	Label	Kit components	Quantity
K 6752	PLATE	Microtiter plate, pre-coated	12 x 8 wells
K 0001.C.100	WASHBUF	Wash buffer concentrate, 10x	2 x 100 ml
K 6752	CONJ	Conjugate concentrate, (goat-anti-α_1-antitrypsin, peroxidase-labelled)	1 x 200 µl
K 6752	STD	Standards, lyophilised (0; 3.3; 10; 30; 90 µg/l)*	2 x 5 vials
K 6752	CTRL 1	Control, lyophilised (see specification for range)	2 x 1 vial
K 6752	CTRL 2	Control, lyophilised (see specification for range)	2 x 1 vial
K 0002.15	SUB	Substrate (tetramethylbenzidine), ready-to-use	1 x 15 ml
K 0003.15	STOP	Stop solution, ready-to-use	1 x 15 ml
K 6999.C.100	IDK Extract®	Extraction buffer concentrate *IDK Extract*® 2.5x	2 x 100 ml
K 6752	SAMPLEBUF	Sample dilution buffer, ready-to-use	2 x 70 ml

* The used standards have been calibrated on the WHO reference material CRM 470.

For reorders of single components, use the catalogue number followed by the label as product number.

4. MATERIAL REQUIRED BUT NOT SUPPLIED

- Ultrapure water*
- Calibrated precision pipettors and 10–1 000 µl single-use tips
- Foil to cover the microtiter plate
- Horizontal microtiter plate shaker
- Multi-channel pipets or repeater pipets
- Centrifuge, 3 000 *g*
- Vortex
- Standard single-use laboratory glass or plastic vials, cups, etc.
- Microtiter plate reader (required filters see chapter 7)

* Immundiagnostik AG recommends the use of ultrapure water (water type 1; ISO 3696), which is free of undissolved and colloidal ions and organic molecules (free of particles > 0.2 µm) with an electrical conductivity of 0.055 µS/cm at 25 °C (≥ 18.2 MΩ cm).

5. STORAGE AND PREPARATION OF REAGENTS

- To run the assay more than once, ensure that reagents are stored at the conditions stated on the label. **Prepare only the appropriate amount necessary for each run.** The kit can be used up to 4 times within the expiry date stated on the label.
- Reagents with a volume less than **100 µl** should be centrifuged before use to avoid loss of volume.
- **Preparation of the wash buffer:** The **wash buffer concentrate (WASHBUF)** has to be diluted with ultrapure water **1:10** before use (100 ml WASHBUF + 900 ml ultrapure water), mix well. Crystals could occur due to high salt concentration in the concentrate. Before dilution, the crystals have to be redissolved at room temperature or in a water bath at 37 °C. The **WASHBUF** is stable at **2–8 °C** until the expiry date stated on the label. **Wash buffer** (1:10 diluted WASHBUF) can be stored in a closed flask at **2–8 °C for 1 month**.
- **Preparation of the extraction buffer:** The **extraction buffer concentrate *IDK Extract*®** has to be diluted with ultrapure water **1:2.5** before use (100 ml ***IDK Extract***® + 150 ml ultrapure water), mix well. Crystals could occur due to high salt concentration in the concentrate. Before dilution, the crystals have to be redissolved at 37 °C in a water bath. The ***IDK Extract***® is stable at **2–8 °C** until the expiry date stated on the label. Extraction buffer (1:2.5 diluted ***IDK Extract***®) can be stored in a closed flask at **2–8 °C for 4 months**.

- The **lyophilised standards (STD)** and **controls (CTRL)** are stable at **2–8 °C** until the expiry date stated on the label. Before use, the STD and CTRL have to be reconstituted with **500 µl of ultrapure water** and mixed by gentle inversion to ensure complete reconstitution. Allow the vial content to dissolve for 10 minutes and then mix thoroughly. **Standards and controls** (reconstituted STD and CTRL) **can be stored at 2–8 °C for 4 weeks.**
- **Preparation of the conjugate:** Before use, the **conjugate concentrate (CONJ)** has to be diluted **1:101** in wash buffer (100 µl CONJ + 10 ml wash buffer). The CONJ is stable at **2–8 °C** until expiry date stated on the label. **Conjugate** (1:101 diluted CONJ) **is not stable and cannot be stored.**
- All other test reagents are ready-to-use. Test reagents are stable until the expiry date (see label) when stored at **2–8 °C**.

6. STORAGE AND PREPARATION OF SAMPLES

Storage

The **sample stability** is as follows:

Raw stool: 3 days at room temperature (15–30 °C), 3 days at 2–8 °C or at least 4 weeks at -20 °C

Stool extracts: 9 days at room temperature, 2–8 °C or -20 °C, maximum 3 freeze-thaw cycles

Serum and plasma samples

Fresh collected blood should be centrifuged within one hour. Store samples at -20 °C if not assayed on the same day. Lipemic or hemolytic samples may give erroneous results. Samples should be mixed well before assaying.

Extraction of the stool samples

Extraction buffer (1:2.5 diluted ***IDK Extract***®) is used as a sample extraction buffer. We recommend the following sample preparation:

Stool Sample Application System (SAS) (Cat. No.: K 6998SAS)

Stool sample tube – Instructions for use

Please note that the dilution factor of the final stool suspension depends on the amount of stool sample used and the volume of the buffer.

SAS with 1.5 ml sample extraction buffer:

Applied amount of stool:	15 mg
Buffer Volume:	1.5 ml
Dilution Factor:	1:100

Please follow the instructions for the preparation of stool samples using the SAS as follows:

a) The raw stool sample has to be thawed. For particularly heterogeneous samples we recommend a mechanical homogenisation using an applicator, inoculation loop or similar device.

b) Fill the empty stool sample tube with 1.5 ml sample extraction buffer (1:2.5 diluted IDK Extract®) before using it with the sample. Important: Allow the sample extraction buffer to reach room temperature

c) Unscrew the tube (yellow part of cap) to open. Insert the yellow dipstick into the 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, leaving 15 mg of sample to be diluted. Screw tightly to close the tube.

d) Shake 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 sample extraction buffer for ~ 10 minutes improves the result.

e) Allow sample to stand for ~10 minutes until sediment has settled. Floating material like shells of grains can be neglected.

f) 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.

Dilution I: 1:100

Dilution of samples

Stool samples

The supernatant of the **sample** preparation procedure (dilution I) is further diluted **1:250 in wash buffer**. For example:

- **20 µl** supernatant (dilution I) + **980 µl** wash buffer, mix well = **dilution II** (1:50)
- **200 µl** dilution II + **800 µl** wash buffer, mix well = **dilution III** (1:5)

This results in a **final dilution of 1:25 000**.

For analysis, pipet **100 µl** of **dilution III** per well.

Serum and plasma samples

Normal samples are diluted **1:40 000** with sample dilution buffer (SAMPLEBUF). Samples from patients with Morbus Crohn etc. are diluted **1:250 000 and 1:1 000 000**. Use the corresponding dilution factor to calculate the α_1-antitrypsin concentration.

1:40 000 dilution

For example:

- **25 µl** serum + **975 µl** SAMPLEBUF, mix well = **1:40** (**dilution Ia**)
- **25 µl** dilution Ia + **975 µl** SAMPLEBUF, mix well= **1:40** (**dilution IIa**)
- **40 µl** dilution IIa + **960 µl** SAMPLEBUF, mix well= **1:25** (**dilution IIIa**).
  This results in a final dilution of **1:40 000.**

1:250 000 dilution

For example:

- **10 µl** serum + **990 µl** SAMPLEBUF, mix well = **1:100** (**dilution Ib**)
- **10 µl** dilution Ib + **990 µl** SAMPLEBUF, mix well= **1:10** (**dilution IIb**)
- **40 µl** dilution IIb + **960 µl** SAMPLEBUF, mix well= **1:40** (**dilution IIIb**).
  This results in a final dilution of **1:250 000.**

1:1 000 000 dilution

For example:

- **125 µl** dilution IIIb + **375 µl** SAMPLEBUF, mix well= **1:40** (**dilution Ic**).
  This results in a final dilution of **1:1 000 000.**

For analysis, pipet **100 µl** of the final dilution step (IIIa/IIIb/Ic) per well.

7. ASSAY PROCEDURE

Principle of the test

This ELISA is designed for the quantitative determination of α_1-Antitrypsin in serum, plasma and stool.

The assay utilises the sandwich technique with two selected antibodies that bind to human α_1-antitrypsin.

Standards, controls and prediluted samples which are assayed for human α_1-antitrypsin are added into the wells of a micro plate coated with a high affine anti-human α_1-antitrypsin antibody. During the first incubation step, α_1-antitrypsin is bound by the immobilised antibody. Then a peroxidase-conjugated polyclonal anti-human α_1-antitrypsin antibody is added into each microtiter well and a sandwich of capture antibody – human α_1-antitrypsin – peroxidase-conjugate is formed. Tetramethylbenzidine is used as peroxidase substrate. Finally, an acidic stop solution is added to terminate the reaction. The colour changes from blue to yellow. The intensity of the yellow colour is directly proportional to the concentration of α_1-antitrypsin. A dose response curve of the absorbance unit (optical density, OD at 450 nm) vs. concentration is generated, using the values obtained from the standard. α_1-antitrypsin present in the samples is determined directly from this curve.

Test procedure

Bring all **reagents and samples to room temperature** (15–30 °C) and mix well.

Mark the positions of standards/controls/samples on a protocol sheet.

Take as many microtiter strips as needed from the kit. Store unused strips together with the desiccant bag in the closed aluminium 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 your supplier or Immundiagnostik AG.

We recommend to carry out the tests in duplicate.

1.	**Before use**, wash the wells **5 times** with **250 µl wash buffer**. After the final washing step, remove residual wash buffer by firmly tapping the plate on absorbent paper.
2.	Add each **100 µl standards/controls/diluted samples** into the respective wells.

3.	Cover the strips and incubate for **1 hour** at room temperature (15–30 °C) on a **horizontal shaker**.**
4.	Discard the content of each well and wash **5 times** with **250 µl wash buffer**. After the final washing step, remove residual wash buffer by firmly tapping the plate on absorbent paper.
5.	Add **100 µl conjugate** (diluted CONJ) into each well.
6.	Cover the strips and incubate for **1 hour** at room temperature (15–30 °C) on a **horizontal shaker**.**
7.	Discard the content of each well and wash **5 times** with **250 µl wash buffer**. After the final washing step, remove residual wash buffer by firmly tapping the plate on absorbent paper.
8.	Add **100 µl substrate** (SUB) into each well.
9.	Incubate for **10–20 minutes*** at room temperature (15–30 °C) in the **dark**.
10.	Add **100 µl stop solution** (STOP) into each well and mix well.
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 colour change is temperature sensitive. We recommend observing the colour change and stopping the reaction upon good differentiation.

** We recommend shaking the strips at 550 rpm with an orbit of 2 mm.

8. RESULTS

The following algorithms can be used alternatively to calculate the results. We recommend using 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 standard must be specified with a value less than 1 (e.g. 0.001).

2. Point-to-point calculation

We recommend a linear ordinate for the optical density and a linear abscissa for the concentration.

3. Spline algorithm

We recommend a linear ordinate for the optical density and a linear abscissa for the concentration.

The plausibility of the duplicate values should be examined before the automatic evaluation of the results. If this option is not available with the programme used, the duplicate values should be evaluated manually.

Stool samples

The obtained results have to be multiplied by the **dilution factor of 25 000** to get the actual concentrations.

Serum and plasma samples

The obtained results have to be multiplied by the **dilution factor of 40 000, 250 000 or 1 000 000** and **additionally by a factor of 3** to get the actual concentrations.

In case **another dilution factor** has been used, multiply the obtained result by the dilution factor used.

Clearance

Use the following formula to calculate the clearance:

Clearance (ml/day) = (V * F) / S

V = volume of faeces in ml/day, mean value from 3 days (1 ml stool=1 g)

F = mean faeces α_1-antitrypsin concentration from 3 days, calculated from the standard curve and multiplied by the dilution factor (µg/l or mg/dl)

S = mean serum α_1-antitrypsin concentration from 3 days calculated from the standard curve and multiplied by the dilution factor (µg/l or mg/dl)

9. LIMITATIONS

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:

Analytical sensitivity × *sample dilution factor to be used*

Analytical sensitivity see chapter "Performance Characteristics".

10. QUALITY CONTROL

Immundiagnostik AG recommends the use of external controls for internal quality control, if possible.

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

Reference range

Based on Immundiagnostik AG studies of stool samples of apparently healthy persons (n = 76) the following reference range was estimated:

cut-off value:	**< 26,8 mg/day**
α1-Antitrypsin-Clearance:	**< 27,5 ml/day**
α_1-Antitrypsin concentration (serum and plasma):	**90–180 mg/dl***

*L. Thomas 5. Auflage; Labor und Diagnose

We recommend each laboratory to establish its own reference range.

11. PERFORMANCE CHARACTERISTICS

Accuracy – Precision

Repeatability (Intra-Assay); n = 35

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

Sample	Mean value [µg/l]	CV [%]
1	11.97	5.4
2	32.36	9.1

Reproducibility (Inter-Assay); n = 0

The reproducibility was assessed with 2 stool-samples under **varying** parameters (different operators, instruments, days and kit lots).

Sample	Mean value [µg/l]	CV [%]
1	13.36	9.6
2	41.99	11.9

Analytical sensitivity

The following values have been estimated based on the concentrations of the standard without considering possibly used sample dilution factors.

Limit of blank, LoB 0.359 µg/l

Accuracy – Trueness

The trueness states the closeness of the agreement between the result of a measurement and the true value of the measurand. Therefore, α1-antitrypsin spikes with known concentrations were added to 2 different stool-samples.

Sample [µg/l]	Spike [µg/l]	Expected [µg/l]	Obtained [µg/l]	Recovery [%]
6.67	15.00	21.67	20.37	93.98
	5.00	11.67	11.48	98.39
	1.65	8.32	7.39	88.77
7.87	45.00	52.87	45.06	85.24
	15.00	22.87	21.49	94.00
	5.00	12.87	11.67	90.68
	1.65	9.52	7.25	76.14
5.95	45.00	50.95	44.69	87.71
	15.00	20.95	18.42	87.90
	5.00	10.95	10.35	94.49
	1.65	7.60	6.72	88.38
< LoB	22.50	22.50	23.56	104.72
< LoB	22.50	22.50	22.64	100.63

Analytical specificity

The specificity of the antibody was tested by measuring the cross-reactivity against a range of compounds with structural similarity to calprotectin. There was no cross-reactivity observed.

Substance tested	Concentration added	Concentration obtained [ng/ml]	Conclusion
sIgA	600 µg/l	0.23	< LoB
Albumin	800 µg/l	0.63	0.08 %
PMN elastase	40 µg/l	0.39	0.98 %
Pankreatic amylase	28 333 mU/l	0.26	< LoB
Chymotrypsin	1 000 µg/l	0.34	< LoB

Linearity

The linearity states the ability of a method to provide results proportional to the concentration of analyte in the test sample within a given range. This was assessed according to CLSI guideline EP06-A with a serial dilution of 5 different stool-samples.

For α_1-antitrypsin in stool, the method has been demonstrated to be linear from 3.76 to 54.38 µg/l, showing a non-linear behaviour of less than ±20 % in this interval. The following values have been determined without considering possibly used sample dilution factors:

Sample	Dilution	Expected [µg/l]	Obtained [µg/l]	Recovery [%]
A	1:25 000	40.59	40.59	100.00
	1:50 000	20.29	15.64	77.05
	1:100 000	10.15	7.67	75.56
	1:200 000	5.07	4.45	87.77
B	1:25 000	30.08	30.08	100.00
	1:50 000	15.04	13.01	86.50
	1:100 000	7.52	6.30	83.75
	1:200 000	3.76	3.26	86.69
C	1:25 000	28.67	28.67	100.00
	1:50 000	14.49	14.34	101.04
	1:100 000	7.33	7.17	102.28
	1:200 000	3.83	3.58	106.86
D	1:25 000	23.12	23.12	100.00
	1:50 000	11.56	12.66	109.48
	1:100 000	5.78	7.11	122.99
E	1:25 000	54.38	54.38	100.00
	1:50 000	27.19	22.63	83.21
	1:100 000	13.60	11.20	82.39
	1:200 000	6.80	5.52	81.20

12. PRECAUTIONS

- All reagents in the kit package are for *in vitro* diagnostic use only.
- Human materials used in kit components were tested and found to be negative for HIV, Hepatitis B and Hepatitis C. However, for safety reasons, all kit components should be treated as potentially infectious.
- Kit reagents contain sodium azide or ProClin as bactericides. Sodium azide or ProClin are hazardous to health and the environment. Substrates for enzymatic colour 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 Immundiagnostik AG on request.
- The 10x Wash buffer concentrate (WASHBUF) 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.
- The stop solution consists of diluted sulphuric acid, a strong acid. Although diluted, it still should 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 breath vapour and avoid inhalation.

13. TECHNICAL HINTS

- Do not interchange different lot numbers of any kit component within the same assay. Furthermore we recommend not assembling wells of different microtiter plates for analysis, even if they are of the same batch.
- Control samples should be analysed with each run.
- Reagents should not be used beyond the expiration date stated on the kit label.
- Substrate solution should remain colourless 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.

14. GENERAL NOTES ON THE TEST AND TEST PROCEDURE

- This assay was produced and distributed according to the IVD guidelines of 98/79/EC.
- The guidelines for medical laboratories should be followed.
- *IDK*® and *IDK Extract*® are trademarks of Immundiagnostik AG.
- Incubation time, incubation temperature and pipetting volumes of the components are defined by the producer. Any variation of the test procedure, which is not coordinated with the producer, may influence the results of the test. Immundiagnostik AG can therefore not be held responsible for any damage resulting from incorrect use.
- Warranty claims and complaints regarding deficiencies must be logged within 14 days after receipt of the product. The product should be send to Immundiagnostik AG along with a written complaint.

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Used symbols:


Temperature limitation

Catalogue number

In Vitro Diagnostic Medical Device

To be used with


Manufacturer

Contains sufficient for <n> tests

Lot number


Use by


Attention


Consult instructions for use

Consult specification data sheet


Irritant