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**Intended Use**

The described Enzyme-Linked-Immuno-Sorbent-Assay (ELISA) is intended for the quantitative determination of α-1-Antitrypsin in serum, plasma, and stool. **It is for research use only.**

**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, hypoproteinememia accompanied with edema may develop. This condition is diagnosed by exclusion of other sources of protein loss and by proof of an elevated α1-antitrypsin concentration in stool.

In serum, α1-antitrypsin represents the majority of serine protease inhibitors and protects tissues from protease damages during inflammation. The protein is synthesized primarily in the liver but also to a small extent in intestinal macrophages, monocytes, and intestinal epithelial cells. Since α1-antitrypsin is relatively resistant against enzymatic digestion, the secreted amount in stool reflects the internal concentration of the protein. An elevated α1-antitrypsin stool concentration is therefore a widely recognized marker for intestinal protein loss and for an increased mucosal permeability.

In clinical routine, the α1-antitrypsin clearance (ratio of the α1-antitrypsin ELISA values of stool and serum samples) has been established along with the sole determination of the 24h α1-antitrypsin secretion in stool. Thus, the group of J. S. Fordtran reports that the sole determination of of the α1-antitrypsin concentration in stool yielded false positive or false negative results in 21 % of the patients compared to the α1-antitrypsin clearance measurement (Strygler et al. 1990).

The analytical quality of PromoCell’s α1-antitrypsin ELISA surpasses by far the conventional radial immunodiffusion (RID) technique in the determination of serum, stool and tissue culture supernatants. In direct comparison, the concentrations measured with the ELISA were approximately 30 % above the corresponding RID levels. Cell culture
supernatants of an intestinal cell line as well as fecal samples of lymphostasis patients yielded negative results with RID. Our ELISA could detect α1-antitrypsin in all of these samples, in some of them even in very high concentrations. These results clearly prove that the α1-antitrypsin ELISA is far more sensitive than the conventional method and that it recognizes not only hepatic but also enteral α1-antitrypsin. The discrepancy of both methods and hence the superiority of the ELISA to RID is especially striking in the analysis of extremely high enteral protein losses. The combination of two specific antibodies in our α1-antitrypsin ELISA widely excludes the possibility of false negative results thereby enabling a reliable determination 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
### Material Supplied

<table>
<thead>
<tr>
<th>Content</th>
<th>Kit Components</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLATE</td>
<td>One holder with precoated strips</td>
<td>12 x 8 wells</td>
</tr>
<tr>
<td>WASHBUF</td>
<td>ELISA wash concentrate 10x</td>
<td>2 x 100 ml</td>
</tr>
<tr>
<td>SAMPLEBUF</td>
<td>Sample buffer, ready-to-use</td>
<td>2 x 70 ml</td>
</tr>
<tr>
<td>CONJ</td>
<td>Conjugate, (goat anti α-1-antitrypsin, peroxidase-labelled)</td>
<td>200 µl</td>
</tr>
<tr>
<td>STD</td>
<td>Standards, lyophilized (0; 3.3; 10; 30; 90 µg/l)*</td>
<td>2 x 5 vials</td>
</tr>
<tr>
<td>CTRL1</td>
<td>Control, ready-to-use</td>
<td>1 vial</td>
</tr>
<tr>
<td>CTRL2</td>
<td>Control, ready-to-use</td>
<td>1 vial</td>
</tr>
<tr>
<td>SUB</td>
<td>TMB substrate (Tetramethylbenzidine), ready-to-use</td>
<td>1 x 15 ml</td>
</tr>
<tr>
<td>STOP</td>
<td>ELISA stop solution, ready-to-use</td>
<td>1 x 15 ml</td>
</tr>
<tr>
<td>IDK Extract®</td>
<td>Extraction Buffer Concentrate, IDK Extract® 2.5x</td>
<td>2 x 100 ml</td>
</tr>
</tbody>
</table>

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

### Material Required but not Supplied

- Ultra-pure water*
- Laboratory balance
- Precision pipettors and disposable tips to deliver 10-1000 µl
- Foil to cover the microtiter plate
- Horizontal microtiter plate shaker
- A multi-channel dispenser or repeating dispenser
- Centrifuge capable of 3000 x g
- Vortex-Mixer
- Standard laboratory glass or plastic vials, cups, etc.
- Microtiter plate reader at 450 nm (reference wavelength 620 or 690 nm)

* We recommend the use of Ultra Pure 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).

**Preparation and Storage 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 ultra pure water 1:10 before use (100 ml WASHBUF + 900 ml ultra pure water), mix well. Crystals could occur due to high salt concentration in the stock solutions. The crystals must be redissolved at room temperature or in a water bath at 37 °C before dilution of the buffer solutions. 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 one month.
- **Preparation of the extraction buffer:** The extraction buffer concentrate IDK Extract® has to be diluted with ultra pure water 1:2.5 before use (100 ml IDK Extract® + 150 ml ultra pure water), mix well. Crystals could occur due to high salt concentration in the stock solutions. Before dilution, the crystals must 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 three months.
- The lyophilized standards (STD) and controls (CTRL) are stable at 2–8 °C until the expiry date stated on the label. Before use, the standards and controls have to be reconstituted with 500 μl of ultra-pure water. Allow the vial content to dissolve for 10 minutes.
and mix thoroughly by gentle inversion to ensure complete reconstitution. **Reconstituted standards and controls can be stored at 2–8 °C for four 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 of test package) when stored at 2–8 °C.

### Storage and Preparation of Samples

#### Storage

**Stool samples**

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)** (available at Immundiagnostik, Germany)
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 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 sample tube with 1.5 ml of ready to use IDK Extract® extraction buffer before using it with the sample. Important: Allow the extraction buffer to reach room temperature.

c) Unscrew the tube (orange part of cap) to open. Insert the orange 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 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/plasma samples
Normal samples are diluted 1:40,000. Samples from patients with Morbus Crohn etc. are diluted 1:250,000 and 1:1000,000. Use the corresponding dilution factor to calculate the α1-antitrypsin concentration.

1:40,000 dilution
For example:

- 10 μl serum + 990 μl SAMPLEBUF (sample buffer), mix well = 1:100 (dilution Ia)
- 100 μl dilution Ia + 900 μl SAMPLEBUF (sample buffer), mix well = 1:10 (dilution IIa)
- 25 μl dilution IIa + 975 μl SAMPLEBUF (sample buffer), mix well = 1:40 (dilution IIIa).
- This results in a final dilution of 1:40,000.

1:250,000 dilution
For example:

- 10 μl serum + 990 μl SAMPLEBUF (sample buffer), mix well = 1:100 (dilution Ib)
- 10 μl dilution Ib + 990 μl SAMPLEBUF (sample buffer), mix well = 1:10 (dilution IIb)
• 20 μl dilution IIb + 480 μl SAMPLEBUF (sample buffer), mix well = 1:40 (dilution IIIb).
• This results in a final dilution of 1:250,000.
• For analysis, pipet 100 μl of the final dilution step (IIIa/IIIb) per well.

Assay Procedure

**Principle of the Test**
The assay utilizes the sandwich technique with two selected polyclonal antibodies that bind to human α1-antitrypsin. Standards, controls and prediluted samples which are assayed for human α1-antitrypsin are added into the wells of a micro plate coated with a high affine polyclonal anti-human α1-antitrypsin antibody. During the first incubation step, α1-antitrypsin is bound by the immobilized antibody. Then a peroxidase-conjugated polyclonal anti-human α1-antitrypsin antibody is added into each microtiter well and a sandwich of capture antibody – human α1-antitrypsin – peroxidase-conjugate is formed. Tetramethylbenzidine (TMB) 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 α1-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. α1-antitrypsin present in the samples is determined directly from this curve.

1. **Bring all reagents and samples to room temperature** (15–30 °C) and mix well.
2. Take as many microtiter strips as needed from kit. Store unused strips covered at 2–8 ° C. Strips are stable until expiry date stated on the label.
3. Mark the positions of **STD** (Standard) **SAMPLE** (Sample) **CTRL** (Controls) on a protocol sheet.
4. 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 us.
5. We recommend carrying out the tests in duplicate.

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Wash each well <strong>5 times</strong> by dispensing <strong>250 μl wash buffer</strong> into each well. After the final washing step, remove residual buffer by tapping the plate on absorbent paper.</td>
</tr>
<tr>
<td>2.</td>
<td>Add <strong>100 μl of STD</strong> (standard), <strong>SAMPLE</strong> (sample) and <strong>CTRL</strong> (controls) into respective well.</td>
</tr>
<tr>
<td>3.</td>
<td>Cover the plate tightly and incubate for <strong>1 hour</strong> at room temperature (15–30 °C) on a horizontal shaker.</td>
</tr>
<tr>
<td>4.</td>
<td>Discard the contents of each well. Wash each well <strong>5 times</strong> by dispensing <strong>250 μl wash buffer</strong> into each well. After the final washing step remove residual buffer by tapping the plate on absorbent paper.</td>
</tr>
<tr>
<td>5.</td>
<td>Add <strong>100 μl conjugate</strong> into each well.</td>
</tr>
<tr>
<td>6.</td>
<td>Cover the plate tightly and incubate for <strong>1 hour</strong> at room temperature (15–30 °C) on a horizontal shaker.</td>
</tr>
<tr>
<td>7.</td>
<td>Discard the contents of each well. Wash each well <strong>5 times</strong> by dispensing <strong>250 μl wash buffer</strong> into each well. After the final washing step remove residual buffer by tapping the plate on absorbent paper.</td>
</tr>
<tr>
<td>8.</td>
<td>Add <strong>100 μl of SUB</strong> (TMB substrate) into each well.</td>
</tr>
<tr>
<td>9.</td>
<td>Incubate for <strong>10–20 minutes</strong> at room temperature (15–30 °C) in the dark*.</td>
</tr>
<tr>
<td>10.</td>
<td>Add <strong>100 μl of STOP</strong> (stop solution) into each well, mix thoroughly.</td>
</tr>
<tr>
<td>11.</td>
<td><strong>Determine absorption immediately</strong> with an ELISA reader at <strong>450 nm</strong> 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 <strong>405 nm</strong> against 620 nm as a reference.</td>
</tr>
</tbody>
</table>

*The intensity of the colour change is temperature sensitive. We recommend observing the colour change and stopping the reaction upon good differentiation.*
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. When using a logarithmic abscissa, the zero calibrator must be specified with a value less than 1 (e.g. 0.001).

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, the duplicate values should be evaluated manually.

Stool samples
Multiply the obtained results by the dilution factor of 25,000 to get the real concentration.

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

Serum and plasma samples
For the calculation of the α1-antitrypsin concentration in serum and plasma samples, the result must be multiplied by 40,000 or 250,000 and additionally by a factor of 3 for each sample.

In case another dilution factor has been used, multiply the obtained result with the dilution factor used.
Clearance
Use the following formula to calculate the clearance:

\[
\text{Clearance (ml/day)} = \frac{V \times F}{S}
\]

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

\(F\) = mean faeces α1-antitrypsin concentration from 3 days, calculated from the standard curve and multiplied by the dilution factor (μg/l or mg/dl)

\(S\) = mean serum α1-antitrypsin concentration from 3 days (mg/dl), calculated from the standard curve and multiplied by the dilution factor (μg/l or mg/dl)

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:

\[
\text{highest concentration of the standard curve} \times \text{sample dilution factor to be used}
\]

The lower limit of the measurement range can be calculated as:

\[
\text{detection limit} \times \text{sample dilution factor to be used}
\]

Quality Control

PromoCell recommends the use of external/commercial control samples for internal quality control if available.
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 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 studies of stool samples of apparently healthy persons (n = 76) the following reference range was estimated:

- $\alpha$-1-Antitrypsin-Clearance: < 27.5 ml/day
- Cut off-value: < 26.8 ml/dl
- $\alpha$-1-Antitrypsin concentration (serum and plasma): 90 - 180 mg/dl*

*(L. Thomas 5. edition; Labor und Diagnose)

These normal ranges should be used as a guideline only. It is recommended that each laboratory establishes an own expected range for its patient population.

Performance Characteristics

Precision and Reproducibility

Intra-Assay-Variation
The precision (intra-assay variation) of the PromoKine $\alpha$-1-Antitrypsin ELISA test was calculated from 20 replicate determinations on each one of two samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$\alpha$-1-Antitrypsin mean value [mg/dl]</th>
<th>Intra-Assay CV [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15.2</td>
<td>4.5</td>
</tr>
<tr>
<td>2</td>
<td>42.2</td>
<td>13.1</td>
</tr>
</tbody>
</table>
Inter-Assay (n=20)
The total precision (inter-assay variation) of the PromoKine α-1-Antitrypsin ELISA test was calculated from data on 2 samples obtained in 20 different assays by three technicians on two different lots of reagents over a period of three months.

<table>
<thead>
<tr>
<th>Sample</th>
<th>α-1-Antitrypsin mean value [mg/dl]</th>
<th>Inter-Assay CV [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16.15</td>
<td>9.8</td>
</tr>
<tr>
<td>2</td>
<td>54.46</td>
<td>14.8</td>
</tr>
</tbody>
</table>

**Analytical Sensitivity**
The detection limit was set as B0 + 2 SD. The Zero-standard was measured 20 times. The values were estimated in relation to the concentration of the calibration curve and resulted in a detection limit of 0.72 µg/l without consideration of the sample dilution factor.

**Specificity**
No cross reactivity with other plasma proteins in stool was observed. No cross reactivity with alpha-1-antitrypsin in mouse serum was observed.

**Spiking Recovery**
Two samples were spiked with different α-1-antitrypsin calibrator amounts and measured with the assay.
### Linearity

Two patient serum samples were diluted with wash buffer. The results are shown below (n= 2):

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dilution</th>
<th>Expected [mg/dl]</th>
<th>Measured [mg/dl]</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1:12,500</td>
<td>48</td>
<td>48.88</td>
</tr>
<tr>
<td></td>
<td>1:25,000</td>
<td>24.5</td>
<td>23.25</td>
</tr>
<tr>
<td></td>
<td>1:50,000</td>
<td>12.3</td>
<td>11.9</td>
</tr>
<tr>
<td></td>
<td>1:100,000</td>
<td>6.1</td>
<td>6</td>
</tr>
<tr>
<td>B</td>
<td>1:12,500</td>
<td>158.4</td>
<td>158.4</td>
</tr>
<tr>
<td></td>
<td>1:25,000</td>
<td>79.3</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>1:50,000</td>
<td>39.6</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>1:100,000</td>
<td>19.8</td>
<td>22.1</td>
</tr>
</tbody>
</table>
Precautions

- Human material used in the kit components was 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.
- Reagents of the kit package contain sodium azide or ProClin as bactericides. Sodium azide and ProClin are toxic. The substrates for the enzymatic colour reactions are toxic and carcinogenic. Avoid contact with skin or mucous membranes.
- Stop solution is composed of sulphuric acid, which is a strong acid. Even diluted, it still must be handled with care. It can cause acid burns and should be handled with gloves, eye protection, and appropriate protective clothing. Any spill should be wiped out immediately with copious quantities of water.

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 analyzed with each run.
- Reagents should not be used beyond the expiration date shown 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 the enclosed manual.
General Notes on the Test and Test Procedure

- All reagents in the kit package are for research use only.
- The quality control guidelines should be followed.
- 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. PromoCell can therefore not be held responsible for any damage resulting from wrong use.
- Warranty claims and complaints in respect of deficiencies must be lodged within 14 days after receipt of the product. The product should be sent to PromoCell together with a written complaint.

References


Ordering Information

<table>
<thead>
<tr>
<th>Product Name</th>
<th>Product Description</th>
<th>Size</th>
<th>Catalog Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1-Antitrypsin ELISA Kit, human</td>
<td>Human alpha1-Antitrypsin (alpha1-Proteinase Inhibitor) ELISA Kit</td>
<td>96 Tests</td>
<td>PK-EL-K6750</td>
</tr>
</tbody>
</table>

For in vitro research use only. Not for diagnostic or therapeutic procedures.

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