1. Intended use

The Eagle Biosciences Alpha-1 Antitrypsin ELISA Kit is intended for the quantitative determination of alpha-1-antitrypsin in serum, plasma and stool. The Alpha-1 Antitrypsin ELISA Kit is for research use only and not to be used in diagnostic procedures.

2. Introduction

Alpha-1-Antitrypsin is a 52 kD glycoprotein, which is produced by the liver, intestinal macrophages, monocytes and mucous membrane cells of the gut. It belongs to the group of acute phase proteins and is one of the most important proteinase inhibitors. Alpha-1-antitrypsin inhibits, beside others, the proteinases trypsin and the elastase of neutrophils. A lack of α-1-AT leads to an enhanced proteolysis. Only a very small amount of alpha-1-antitrypsin is cleaved or resorbed in the gut. Therefore the measurement of α-1-AT in stool reflects the permeability of the gut during inflammatory processes.

The Eagle Biosciences Alpha-1 Antitrypsin ELISA Kit allows an easy, rapid and precise quantitative determination of alpha-1-antitrypsin in biological samples. The kit includes all reagents ready to use for preparation of the samples.

3. Warnings and precautions

- All reagents of this kit are strictly intended for Research Use Only.
- This assay was produced and put on the market according to the IVD guidelines of 98/79/EC.
- Do not interchange kit components from different lots.
- The stop solution (STOP) contains acid and has to be handled carefully. It is corrosive and causes burns. It should be handled with gloves, eye protection, and appropriate protective clothing in a hood. Any spill should be wiped out immediately with copious quantities of water. Do not breathe vapor and avoid inhalation. In case of an accident or indisposition contact immediately a physician.
- The substrate TMB (tetramethyl benzidine) is toxic by ingestion and contact with the skin. Any spill should be wiped out immediately with copious quantities of water.
- Wear disposable gloves while handling specimens or kit reagents and wash hands thoroughly afterwards.
- Do not pipette by mouth.
- Do not eat, drink, smoke or apply makeup in areas where specimens or kit reagents are handled.
- The reagents of the Alpha-1 Antitrypsin ELISA Kit contain bactericides to protect against bacterial growth. Avoid the contact with the skin or mucous membrane.
- Reagents should not be used beyond the expiration date shown on kit label.
- Observe the guidelines for performing quality control in medical laboratories by assaying controls and/or pooled sera. During handling of all kit reagents, controls and serum samples observe the existing legal regulations.

4. Materials Provided

<table>
<thead>
<tr>
<th>Catalog #</th>
<th>Component</th>
<th>Description</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC6200mtp</td>
<td>MTP</td>
<td>Microtiter plate coated</td>
<td>12 x 8 wells</td>
</tr>
<tr>
<td>IC6200wp</td>
<td>WASHBUF</td>
<td>ELISA wash buffer conc. 10 fold</td>
<td>100 ml</td>
</tr>
<tr>
<td>IC6200st</td>
<td>STD</td>
<td>Standard (1 ml) (0; 3.3; 10; 30; 90 ng/ml)</td>
<td>5 vials</td>
</tr>
<tr>
<td>IC6200ko</td>
<td>CTRL</td>
<td>Control 1 and 2 (1 ml)</td>
<td>1 vial each</td>
</tr>
<tr>
<td>IC6200kg</td>
<td>CONJ</td>
<td>Conjugate, peroxidase labeled antibody</td>
<td>15 ml</td>
</tr>
<tr>
<td>IC6200su</td>
<td>SUB</td>
<td>TMB substrate (tetramethylbenzidine)</td>
<td>15 ml</td>
</tr>
<tr>
<td>IC6200sp</td>
<td>STOP</td>
<td>Stop solution</td>
<td>7 ml</td>
</tr>
</tbody>
</table>

5. Additional special equipment

- Laboratory balance
- Centrifuge, 3000xg
- Glass or plastic vials
- Various pipettes
- Foil to cover the microtiter plate
- Multichannel or multi-pipette
- ELISA reader with filter 450 nm (reference filter 620 or 690 nm)
- Microtiter plate shaker
- Vortex mixer

6. Reagent preparation

**Microtiter Plate (MTP):** Take the needed strips out of the bag and mount them on the holder. Please take care that the package has reached room temperature before opening the bag. Strips which are not needed could be stored at 2-8°C. Please dispose the holder when all strips are used.
**Wash buffer (WASHBUF):** Dilute the wash buffer concentrate 1:10 with deionized or distilled water (1 part buffer + 9 parts water). The dilution is stable for 14 days at 2-8°C.  
*Important: When storing the wash buffer concentrate at 2-8°C crystallization could occur. Before dilution all crystals must be dissolved.*

It is recommended to dilute only the amount of buffer which is used to process the given samples. All other test reagents are stable at 2-8 °C, up to the date of expiry stated on the label.

### 7. Specimen

**Stool samples**

Alpha-1-antitrypsin is extracted by the sample dilution buffer out of the stool sample.

**Extraction in glass or plastic vials**

- 100 mg stool are mixed with 5 ml WASHBUF on a vortex mixer until the mixture is homogenous.
- 1 ml of the mixture is transferred into an “Eppendorf” reaction vial and centrifuged for 10 min at 10000xg.
- Dilute the supernatant 1:250 with WASHBUF (4 µl + 996 µl WASHBUF)
- 100 µl of the dilution are used in the test per well.

**Extraction in stick vials**

- Alternatively stick vials can be used for extraction.
- We recommend using 20 mg stool per ml extraction buffer (WASHBUF). In case of using a 15 mg stick vial 0.75 ml of WASHBUF should be filled in the vials.
- When the top of the stick is submersed in the buffer it can be left over night at 2-8 °C to improve solution.
- The suspension is mixed on a vortex mixer and centrifuged for 10 min at 3000xg.
- Dilute the supernatant 1:250 with WASHBUF (4 µl + 996 µl WASHBUF)
- 100 µl of the dilution are used in the test per well.

**Serum/plasma samples**

Serum or EDTA-plasma drawn from venous fasting blood could be used in this test system. The sample should be centrifuged (3000 g, 10 min., 2-8°C) within 60 min after venipuncture and stored directly at 2-8°C. For long term storage the samples should be frozen at -20°C.

- Samples suffering Morbus Crohn may show very high concentrations of α-1-AT in blood. These samples should be diluted 1:1,000,000 with wash buffer.
- All other samples should be diluted 1: 250,000 with wash buffer.
- 100 µl of the dilution are used in the test per well.
8. Procedure

Principle of the Assay

The Alpha-1 Antitrypsin ELISA assay determines human alpha-1-antitrypsin according to the “sandwich”-principle. Alpha-1 Antitrypsin in sample, standard and controls binds to antibodies, which are coated to the microtiter plate. After a washing step a peroxidase labeled detection antibody is added. A second washing step is followed by the addition of the substrate which is converted to a colored product by the peroxidase. The reaction is terminated by the addition of an acidic stop solution. The optical densities are read at 450 nm (against the reference wavelength 620 nm) in a microtiter plate reader. The α-1-AT concentration can be calculated from the standard curve.

Sample preparation

All reagents and samples should have room temperature (18-26°C) and mixed well before use. The position of standards, controls and samples are noted on a protocol sheet.

1. Washing step
   - Take out the needed strips of the microtiter plate and wash 1x with 250 µl diluted WASHBUF. Remove residual buffer by tapping the plate on absorbent paper after the washing step.

2. Incubation samples
   - Pipette 100µl STD, CTRL and samples in duplicate in the microtiter plate.
   - The strips are covered and incubated by shaking for 60 min at room temperature (18-26 °C).
   - The reaction in each well starts immediately. Pipetting should be performed as quickly as possible. When processing many samples at once the samples should be pipetted to a separate microtiter plate (150 µl) and transferred simultaneously using a multichannel pipette.

3. Washing step
   - Discard the content of the microwells and wash 5x with 250 µl diluted WASHBUF. Remove residual buffer by tapping the plate on absorbent paper after the last washing step.

4. Incubation conjugate
   - Pipette 100 µl CONJ in each microwell.
   - The strips are covered and incubated by shaking for 60 min at room temperature (18-26 °C).

5. Washing step
- Discard the content of the microwells and wash 5x with 250 µl diluted WASHBUF. Remove residual buffer by tapping the plate on absorbent paper after the last washing step.

6. Incubation substrate
   - Pipette **100 µl** SUB in each microwell.
   - Incubate for **10-15 min** at room temperature (18-26 °C) in the dark.

7. Stopping reaction
   - Pipette **50 µl** STOP in each microwell, mix well.

8. Reading
   - Read the absorbance at 450 nm. If the microtiter plate reader allows to use a reference wavelength use 620 or 690 nm as reference wavelength.
   - Reading should be done within 5 min after stopping reaction.
   - In case that the highest standard exceeds the range of the reader the reading should be done at 405 nm against 620 nm (690 nm).

9. Calculation of analytical results

For calculating the results we recommend to use the 4-parameter algorithm. Is this algorithm not available a “point to point” or a “spline” function can be used.

**Stool samples**
- The obtained α-1-AT concentration is multiplied by **12.5**
- Dilution 1: 100 mg in 5 ml corresponds to a factor **50** (assumption: 1 g stool = 1 ml)
- Dilution 2: Factor **250**

  Calculation: Conc. Sample [µg/ml] = obtained conc. [ng/ml] x 50 x 250 / 1000

**Serum/plasma samples**
- The obtained sIgA concentration [ng/ml] is multiplied by the used dilution factor (250,000 or 1,000,000).
The curve given above is only for demonstration. It must not be used for calculation of your samples.

10. Internal quality control

Reference values

Stool: < 0.27 mg/g stool

Ref: G. Beckmann (Hrsg.). Mikroökologie des Darmes
ISBN 3-87706-521-X; S.263

We recommend that each laboratory develop their own normal range. The values mentioned above are only for orientation and can deviate from other published data.

11. Validation data

Precision and reproducibility

**Intra-Assay CV:**
- 9.6 % (77.0 ng/ml) [n = 6]
- 8.7 % (42.6 ng/ml) [n = 6]
- 11.2 % (13.0 ng/ml) [n = 6]

**Inter-Assay CV:**
- 11.9 % (75.1 ng/ml) [n = 6]
- 9.7 % (44.3 ng/ml) [n = 6]
- 14.4 % (14.6 ng/ml) [n = 6]
Linearity

The dilution of the samples was done with WASHBUF.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dilution factor</th>
<th>Expected [ng/ml]</th>
<th>Measured [ng/ml]</th>
<th>Recovery [%]</th>
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<tr>
<td>1</td>
<td>--</td>
<td>--</td>
<td>72.8</td>
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</tr>
<tr>
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<td>18.2</td>
<td>15.1</td>
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<td>1:16</td>
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<td>1:4</td>
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<td>3.2</td>
<td>82.1</td>
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</table>

Detection limit

1.5 ng/ml

For the determination of the detection limit 20 replicates of the standard 0 were measured. After addition of the twofold standard deviation to the mean value the concentration was read from the standard curve.

Recovery

The recovery was found between 80.3 and 105.2 %

Cross reactivity

Cross reactivity to other plasma proteins could not be detected in stool and serum/plasma samples.

12. Limitations of the method

Stool samples with Alpha-1 Antitrypsin concentrations above the standard curve should be diluted with wash buffer (WASHBUF) and measured again. In case of strong diarrhea it is possible that even patients with an inflammation in the gut show normal values.
Blood samples with Alpha-1 Antitrypsin concentrations above the standard curve should be diluted with wash buffer (WASHBUF) and measured again. Hemolytic and lipemic samples should not be measured.

13. Disposal

The substrate (SUB) must be disposed as non-halogenated solvent. The stop solution (STOP) could be neutralized with NaOH and if the pH value is neutral it can be disposed as salt solution. (Important: Reaction will produce heat, be careful)

Please refer to the appropriate national guidelines.

14. Literature references

G. Beckmann (Hrsg.). Mikroökologie des Darmes
ISBN 3-87706-521-X;

For further information about this kit, its application or the procedures in this insert, please contact the Technical Service Team at Eagle Biosciences, Inc. at info@eaglebio.com or at 866-411-8023.