Acid Labile Subunit ELISA
For the quantitative determination of ALS in serum and plasma.

For Research use Only. Not For Use In Diagnostic Procedures.

Catalog Number: 22-ALSHU-E01
Size: 96 Wells
Version: 2 - 010212- ALPCO February 28, 2014
INTENDED USE
This enzyme immunoassay kit is suited for measuring Acid Labile Subunit (ALS) in human serum or EDTA-/heparin-/citrate plasma for research use only. For research use only. Not for use in diagnostic procedures.

INTRODUCTION
The Insulin-like Growth Factors (IGF) – I and II are bound to specific binding proteins in circulation (IGFBP). Until today seven different proteins have been identified IGFBP-1 to 7 [1, 2]. IGF bioavailability, transport and storage is regulated or facilitated by these binding proteins which are expressed differentially according physiological and developmental requirements. The most abundant IGFBP in circulation is IGFBP-3. Together with IGFBP-5 it is able to form the so called ternary complex with IGF and the acid-labile subunit (ALS) [3-5]. In the circulation nearly all IGF is bound in this ternary complex and thus not able to cross the endothelial barrier. Only very small amounts of IGF or IGFBP-3 exist outside this complex [6, 7]. The acid-labile subunit is an important part of the IGF-storage mechanism in circulation. In ALS deficiency or in ALS knock-out mice the concentration of IGF and IGFBP-3 in the circulation is significantly decreased resulting in impaired growth [10].

The acid-labile Subunit, is a synthezised as propeptide of 605 amino acids. The signal peptide, necessary for ALS secretion (AA 1-27) cleaved off enduring the transport process (Swiss-Prot P35858 Version 82). The mature protein consists of 578 amino acids and contains about 20 leucin rich sequence repeats. Beside the leucin-rich repeats several potential N-linked glycosylation sides have been described. Miller BS et al. were able to demonstrate that incomplete glycolysation of IGFs, ALS and IGFBP-3 results in a decreased serum concentration of these proteins. Oral mannose therapy resulted in a partial normalization of the glycosylation pattern and went along with improved growth [8]. Mutations in or the complete knock out of the ALS gene result in IGF / IGFBP-3 deficiency and therewith in disturbance of growth [9,10]. Beside growth also other endocrine axes may be involved. In primary ALS deficiency hypoinsulinemia could be observed [11, 12]. Further, the ALS-IGF-IGFBP-system seems to be of relevance in coronary disease [13].

The first ALS immunoassay was described by Baxter RC in 1990 [6]. By this in-house radioimmunoassay it was shown that ALS is present in high concentrations in serum (50µg/mL) of healthy humans. But not detectable in other body fluids like amniotic fluid, cerebrospinal fluid or seminal plasma – in spite of the fact that these body fluids contain high level IGFBP-3.

PERFORMANCE CHARACTERISTICS AND VALIDATION ELISA
The ALS is a so-called Sandwich-Assay. It utilizes two specific and high affinity antibodies for this protein. These antibodies were created by immunization of rabbits with specific peptides as previously described by Khosravi and Stadler [16, 17].

The ALS in the sample binds to the immobilized first antibody on the microtiter plate. In the following step, the biotinylated and Streptavidin-Peroxidase conjugated second specific anti-ALS-Antibody binds in turn to the immobilized ALS. In the closing substrate reaction the turn of the colour will be high specific catalysed, quantitatively depending on the ALS-level of the samples.

This Assay was calibrated against a serum containing arbitrary 480 mU/mL human ALS. This serum is used as Standard in the following concentrations: 0; 1.5; 6.25; 12.5; 25 and 40 mU/mL in this Kit. Calibration with recombinant ALS (procaryotic expressed, not glycosylated, Prod. Code: H3483-P01, Abnova (Taiwan) Corporation) resulted in 17.8 mU/µg (according to 0.0561 µg/mU).

The analytical sensitivity of the ELISA yields 0.23 mU/mL (2 SD of zero standard in 18fold determination).
The Inter- and Intra-Assay variation coefficients were found less than 8% and 6.8%. Exemplary determinations are shown in table 3 and table 4.

**Table 3: Inter-Assay-Variation (n=10)**

<table>
<thead>
<tr>
<th>mU/mL</th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:50</td>
<td>2106</td>
<td>1234</td>
<td>810</td>
</tr>
<tr>
<td>1:100</td>
<td>1997</td>
<td>1117</td>
<td>790</td>
</tr>
<tr>
<td>1:200</td>
<td>1667</td>
<td>1065</td>
<td>774</td>
</tr>
<tr>
<td>1:400</td>
<td>1572</td>
<td>985</td>
<td>696</td>
</tr>
<tr>
<td>1:500</td>
<td>1782</td>
<td>1180</td>
<td>812</td>
</tr>
</tbody>
</table>

**Table 4: Intra-Assay-Variation (n=16)**

<table>
<thead>
<tr>
<th>Mean Value (mU/mL)</th>
<th>Standard Deviation (mU/mL)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>931.35</td>
<td>80.97</td>
</tr>
<tr>
<td>Sample 2</td>
<td>1061.38</td>
<td>80.97</td>
</tr>
<tr>
<td>Sample 3</td>
<td>1926.1</td>
<td>157.96</td>
</tr>
</tbody>
</table>

**Table 2: Interference:**

Serum samples were enriched with the indicated amount of potentially interfering substances and measured. Results are shown as % of control.

<table>
<thead>
<tr>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triglycerides [100 mg/mL]</td>
</tr>
<tr>
<td>Hemoglobin [1mg/mL]</td>
</tr>
<tr>
<td>Bilirubin [200µg/mL]</td>
</tr>
</tbody>
</table>

**Specificity**

250 ng/mL IGF-I or 3000 ng/mL IGFBP-3 do not exert any influence on ALS measurement.

**SPECIMEN COLLECTION, PREPARATION AND STORAGE**

Serum samples are suitable. Further, 30 IU/mL Heparin, 6.8 mM EDTA or 0.015M Sodium Citrate did not interfere with ALS measurement.

Serum Samples can be stored and transported at room temperature (20-25°C) for up to 3 days. 3 freeze/thaw cycles do not influence the ALS determination.

Samples should be handled as recommended in general: chilled as soon as possible. In case there will be a longer period between the sample withdrawal and determination store the undiluted samples frozen -20°C or below in tightly closable plastic tubes. Avoid on principal repeated freeze-thaw cycles of serum/plasma (if required, please subaliquote).
In most determinations (e.g. Serum- or Plasma samples and no extreme values expected) the dilution of 1:150 with Sample Buffer PP is suitable, respectively the assay covers the range from 0 - 6000 mU/mL. If required, the dilution with Sample Buffer PP could be performed higher or lower.

Suggestion for dilution protocol:
Pipette 1490 µL Sample Buffer PP (red colored) in PE-/PP-Tubes (application of a multi-stepper is recommended in larger series), add 10 µL Serum- or Plasma (dilution 1:150) and mix each tube immediately. After mixing use 50 µL of this solution within 1 hour per determination in the assay (pipetting control = red coloring of the solution in the wells).

REAGENTS PROVIDED

<table>
<thead>
<tr>
<th>No.</th>
<th>Name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MTP</td>
<td>Microtiter plate, ready for use, Microtiter plate with 96 wells. 12 x 8 well strips (separately breakable). Coated with anti-human ALS Antibody. Packed in a laminate bag.</td>
</tr>
<tr>
<td>2</td>
<td>CAL</td>
<td>Standards A-F, 1 mL, lyophilised, contain human ALS. Standard values are 0; 1.5; 6.25; 12.5; 25 and 40 mU/mL ALS. Standards are reconstituted with 1000 µL Sample Buffer PP each. Use 50 µL per well in the assay. Standards can be stored at 4°C overnight or at -20°C for up to three months after reconstitution. Avoid repeated freeze thaw cycles.</td>
</tr>
<tr>
<td>3</td>
<td>BUF PP</td>
<td>Sample Buffer PP, 125 mL, ready for use, red colored, please use for the reconstitution of Standards A-F and Controls KS1/KS2 and for the dilution of Samples and Controls KS1/KS2.</td>
</tr>
<tr>
<td>4</td>
<td>BUF VP</td>
<td>Dilution Buffer VP, 7 mL, ready for use, please use for the dilution of Antibody Conjugate AK.</td>
</tr>
<tr>
<td>5</td>
<td>Control</td>
<td>Control Sera KS1 and KS2, 250 µL, lyophilized, contains human Serum and should be reconstituted in 250 µL Sample Buffer PP each. The ALS target values and the respective ranges are given on the vial labels. The dilutions should be according to the dilution of the respected samples. Use 50 µL per well in the assay.</td>
</tr>
<tr>
<td>6</td>
<td>Ab</td>
<td>Antibody Conjugate AK, 140 µL, 50-fold concentrate, contains the biotinylated anti-human ALS Antibody. Dilute before use 1:50 in Dilution Buffer VP and use 50 µl for each well in the assay. Attention: Please dilute Antibody Conjugate AK freshly according to daily requirements.</td>
</tr>
<tr>
<td>7</td>
<td>CONJ</td>
<td>Enzyme Conjugate EK, 12 mL, ready-to-use, contains HRP (Horseradish-Peroxidase)-labelled Streptavidin. Use 100 µl for each well in the assay.</td>
</tr>
<tr>
<td>8</td>
<td>WASHBUF 20x</td>
<td>Wash Buffer (WP), 50 mL, 20-fold concentrated solution dilute in A. dest. (e.g. add the complete contents of the flask 50 mL into a graduated flask and fill with A.dest. to 1000 mL). Please dilute only according to requirements. Please dilute Wash Buffer WP according to the requirements, use max. 4 weeks.</td>
</tr>
<tr>
<td>9</td>
<td>SUBST</td>
<td>Substrate (S), 12 mL, ready for use, horseradish-peroxidase-(HRP)-substrate stabilised H₂O₂ Tetramethylbenzidine.</td>
</tr>
<tr>
<td>10</td>
<td>H₂SO₄</td>
<td>Stop Solution (SL), 12 mL, ready for use, 0.2 M sulphuric acid. Caution acid!</td>
</tr>
<tr>
<td>11</td>
<td>Sealing tape</td>
<td>Sealing tape for covering of the microtiter plate, 2 x, adhesive.</td>
</tr>
</tbody>
</table>

MATERIALS REQUIRED BUT NOT PROVIDED

- Precision pipettes and multichannel pipettes with disposable plastic tips
- Distilled or deionized water for dilution of the Wash Buffer (WP)
- Vortex-mixer
- Microtiter plate shaker (350 rpm)
- Microtiter plate washer (recommended)
- Micro plate reader ("ELISA-Reader") with filter for 450 and ≥590 nm
- Polyethylene PE/Polypropylene PP tubes for dilution of samples
TECHNICAL NOTES
The assay has to be conducted strictly according the test protocol herein.
Reagents with different lot numbers cannot be mixed. The microtiterplate and reagents are stable until the indicated expiry, if stored unopened and protected from sunlight at 2 – 8°C.
The shelf life of the components after opening is not affected, if used appropriately.

Bring all reagents to room temperature (20 - 25°C) before use. Possible precipitations in the buffers have to be resolved before usage by mixing and / or warming.

Incubation at room temperature means: 20-25°C
The incubation steps should be performed at mean rotation frequency of a particularly suitable microtiter plate shaker. 350 rpm is recommended. Due to certain technical differences deviations may occur, in case the rotation frequency must become adjusted. Insufficient shaking may lead to inadequate mixing of the solutions and thereby to low optical densities, high variations and/or false values, excessive shaking may result in high optical densities and/or false values.

Proper washing is of basic importance for a secure, reliable and precise performance of the test. Incomplete washing is common and will adversely affect the test outcome. Possible consequences may be uncontrolled unspecific variations of measured optical densities, potentially leading to false results calculations of the examined samples. Effects like high background values or high variations may indicate washing problems.

All washing must be performed with the provided wash buffer diluted to usage concentration. Washing volume per washing cycle and well must be 300 µl at least.

The danger of handling with potentially infectious material must be taken into account.
When using an automatic microtiter plate washer, the respective instructions for use must be carefully followed. Device adjustments, e.g. for plate geometry and the provided washing parameters, must be performed. Dispensing and aspirating manifold must not scratch the inside well surface. Provisions must be made that the remaining fluid volume of every aspiration step is minimized. Following the last aspiration step of each washing cycle, this could be controlled, and possible remaining fluid could then be removed, by inverting the plate and repeatedly tapping it dry on non fuzzy absorbent tissue.

Manual washing is an adequate alternative option. Wash Buffer may be dispensed via a multistepper device, a multichannel pipette, or a squirt bottle. The fluid may be removed by dynamically swinging out the microtiter plate over a basin. If aspirating devices are used, care has to be taken that the inside well surface is not scratched. Subsequent to every single washing step, the remaining fluid should be removed by inverting the plate and repeatedly tapping it dry on non fuzzy absorbent tissue.

Standards and Controls
The Standards A – F and Control Sera KS1/KS2 are reconstituted with the Sample Buffer PP provided in the kit. It is recommended to keep the reconstituted reagents at room temperature for 15 minutes and then to mix them thoroughly but gently (no foam should result) with a Vortex mixer.

Reconstituted Components (Standards A – F and Control Sera KS1/KS2) should be stored at -20°C (or below) for not longer than 3 months. When using the standards anew, please thaw them rapidly but gently (no temperature rise over the room temperature and no powerful vortexing). Avoid repeated freeze-thaw cycles. In case of a plan to perform multiple determinations over a longer period with one kit, aliquot the components prior to freezing into suitable smaller volumes.
**Antibody Conjugate**
The required volume of Antibody Conjugate is prepared by 1:50 dilution of the provided 50-fold concentrate with Dilution Buffer VP. Please dilute Antibody Conjugate freshly according to daily requirements.

**Wash Buffer**
The required volume of wash buffer is prepared by 1:20 dilution of the provided 20-fold concentrate with deionised water. The diluted Wash Buffer is stable for max. 4 weeks at 2-8°C.

**Substrate Solution**
The Substrate Solution S, stabilised H₂O₂-Tetramethylbencidine, is photosensitive – store and incubate in the dark.

**Microtiter plate**
Store the once unused microtiter strips and wells together with the desiccant in the tightly closed clip lock bag at 2-8°C use in the frame provided. The labelled expiry is not influenced in case of proper storage.

**WARNINGS AND PRECAUTIONS**
For Research Use Only. Not for Use in Diagnostic Procedures.
Before starting the assay, read the instructions completely and carefully. Use the valid version of the package insert provided with the kit. Be sure that everything is understood. The GmbH is not liable for any loss or harm caused by non-observance of the instructions, as far as no law withstands.

Temperature WILL affect the absorbance readings of the assay. However, values for the *-samples will not be affected.
Do not use expired reagents.
Use separate pipette tips for each sample, control and reagent to avoid cross contamination. Use reservoirs only for single reagents. This especially applies to the substrate reservoirs. Using a reservoir for dispensing a substrate solution that had previously been used for the conjugate solution may turn solution colored. Do not pour reagents back into vials as reagent contamination may occur. Mix the contents of the microplate wells thoroughly to ensure good test results. Do not reuse microwells. Do not let wells dry during assay; add reagents immediately after completing the rinsing steps.
Caution: This kit contains material of human and/or animal origin.

**Human Serum**
Contained in following components: Control Serum KS1, KS2 and Standards
The sources of human sera were tested and found non-reactive for Hepatitis-B surface antigen (HBsAg), Hepatitis C virus (HCV), and Human Immunodeficiency Virus 1 and 2 (HIV) antibodies. No known test methods can offer total assurance of the absence of infectious agents; therefore all components and patient’s specimens should be treated as potentially infectious.

**Stop solution contains 0.2 M Sulfuric Acid (H₂SO₄)**
R36/38 Irritating to eyes and skin
S26 In case of contact with eyes, rinse immediately with plenty of water and seek medical advice
S28.1 After contact with skin, wash immediately with plenty of water
S36/37 Wear suitable protective clothing and gloves
2-Methyl-4-Isothiazolin-3-one
contained in following components: AK, EK, VP, PP
< 0.01% 2-Methyl-4-isothiazolin-3-one Solution
R34 Irritating to eyes and skin
R43 Sensibilisation through skin contact possible
S26 In case of contact with eyes, rinse immediately with plenty of water and seek medical advice
S36/37 Wear suitable protective clothing and gloves
S45 In case of accident or feeling unwell seek medical advice

5-chloro-2-methyl 2H isothiazol-3-one and 2-methyl-2H-Isothiazol-3-one
contained in following components: AK, EK, WP, VP, PP
< 0.01% (w/w) 5-chloro-2-methyl 2H isothiazol-3-one and 2-methyl-2H-Isothiazol-3-one Solution
R36/38 Irritating to eyes and skin
R43 Sensibilisation through skin contact possible
S26 In case of contact with eyes, rinse immediately with plenty of water and seek medical advice
S28.1 After contact with skin, wash immediately with plenty of water

TMB-Substrate (S) contains 3,3’5,5’ Tetramethylbenzidine
R20/21/R22 Harmful by inhalation, in contact with skin and if swallowed
R36/37/38 Irritating to eyes, respiratory system and skin
S26 In case of contact with eyes, rinse immediately with plenty of water and seek medical advice
S28.1 After contact with skin, wash immediately with plenty of water
S36/37 Wear suitable protective clothing and gloves

General first aid procedures:
Skin contact: Wash affected area thoroughly with water. Discard contaminated cloths and shoes.
Eye contact: In case of contact with eyes, rinse immediately with plenty of water at least 15 minutes.
In order to assure an effectual rinsing spread the eyelids.
Ingestion: If swallowed, wash out mouth thoroughly with water. Immediately see a physician.
Do not eat, drink or smoke in these areas.
Never pipette the materials with the mouth.
Spilled material must be wiped off immediately and should become disinfected. Clean contaminated areas and equipment with a suitable detergent.
ASSAY PROCEDURE
NOTES: All determinations (Standards, Control Sera and samples) should be assayed in duplicate. For optimal results, accurate pipetting and adherence to the protocol are recommended.
When performing the assay, the Standards, the Control Sera and the samples should be pipetted as fast as possible (e.g. within<15 minutes). To avoid distortions due to differences in incubation times the Enzyme Conjugate EK, Substrate Solution S and as well the Stop Solution SL should be added to the plate in the same order and in the same time interval each, respectively.

1) Please pipette on before in all needed wells 50 µL 1:50 diluted Antibody Conjugate (AK).
2) Pipette in positions A1/2 50µL each Standard A (0 mU/mL),
   pipette in positions B1/2 50µL each Standard B (1.5 mU/mL),
   pipette in positions C1/2 50µL each Standard C (6.25 mU/mL),
   pipette in positions D1/2 50µL each Standard D (12.5 mU/mL),
   pipette in positions E1/2 50µL each Standard E (25 mU/mL),
   pipette in positions F1/2 50µL each Standard F (40 mU/mL).
   To control the correct accomplishment 50 µL each of the 1:150 (or in respective dilution rate of the sample) in Sample Buffer PP diluted Control Sera KS1 and KS2 can be pipetted in positions G1/2 and H1/2.
   Pipette 50 µL each of the diluted sample (generally 1:150 diluted in Sample Buffer PP) in the rest of the wells according to requirements. Please mix the dilutions immediately after sample addition and use within 60 minutes.
3) Cover the wells with the sealing tape and incubate the plate for 2 hours at room temperature (shake at 350 rpm).
4) After incubation aspirate the contents of the wells and wash the wells 5 times with 300 µL Wash Buffer WP.
5) Following the last washing step pipette 100 µL of the Enzyme Conjugate EK in each well.
6) Cover the wells with the sealing tape and incubate 0.5 hours at room temperature (shake at 350 rpm).
7) After incubation wash the wells 5 times with Wash Buffer WP as described in step 4)
8) Pipette 100 µL of the Substrate Solution S in each well.
9) Incubate the plate for 30 minutes in the dark at room temperature.
10) After incubation pipette 100 µL Stop Solution SL in each well.
11) Measure the absorbance within 30 minutes at 450 nm (Reference filter ≥590 nm)
CALCULATION OF RESULTS

For the evaluation of the assay it is required that the absorbance values of the blank should be below 0.25 and the absorbance of standard F should be above 1.00. Samples, which yield higher absorbance values than Standard F, thus are beyond the standard curve. For reliable determinations such samples should be retested at a higher dilution.

Establishing the Standard Curve

The standards provided contain the following concentration of ALS

<table>
<thead>
<tr>
<th>Standard</th>
<th>mU/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>1.5</td>
</tr>
<tr>
<td>C</td>
<td>6.25</td>
</tr>
<tr>
<td>D</td>
<td>12.5</td>
</tr>
<tr>
<td>E</td>
<td>25</td>
</tr>
<tr>
<td>F</td>
<td>40</td>
</tr>
</tbody>
</table>

1) Calculate the mean absorbance value for the blank from the duplicated determination (well A1/A2).
2) Subtract the mean absorbance of the blank from the mean absorbances of all other values.
3) Plot the standard concentrations on the x-axis versus the mean value of the absorbance of the standards on the y-axis.
4) Recommendation: Calculation of the standard curve should be done by using a computer program because the curve is in general (without respective transformation) not ideally described by linear regression. A higher-grade polynomial, or four parametric logistic (4-PL) curve fit or non-linear regression are usually suitable for the evaluation (as might be spline or point-to-point alignment in individual cases).
5) The ALS concentration in mU/mL of the samples can be calculated by multiplication with the respective dilution factor.
**SUMMARY - ALS ELISA**

<table>
<thead>
<tr>
<th>Reconstitution/ Dilution of Reagents</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Standards A-F</strong></td>
</tr>
<tr>
<td>Reconstitution in Sample Buffer PP</td>
</tr>
<tr>
<td><strong>Control Serum KS1</strong></td>
</tr>
<tr>
<td>Reconstitution in Sample Buffer PP</td>
</tr>
<tr>
<td><strong>Control Serum KS2</strong></td>
</tr>
<tr>
<td>Reconstitution in Sample Buffer PP</td>
</tr>
<tr>
<td><strong>Antibody Conjugate AK</strong></td>
</tr>
<tr>
<td>dilute before use: 1:50 in Dilution Buffer VP</td>
</tr>
<tr>
<td><strong>Wash Buffer WP</strong></td>
</tr>
<tr>
<td>dilute in A. dest. (e.g. add the complete contents of the flask 50 mL into a graduated flask and fill with A.dest. to 1000 mL)</td>
</tr>
</tbody>
</table>

**Sample and Control Sera KS1 &KS2 Dilution: 1:150 in Sample Buffer PP** (red colored; e.g. 10 µL in 1490 µL PP). mix directly and use within max. 60 min.

Use **50 µL per determination** (pipetting control= red coloration)

Before assay procedure bring all reagents to room temperature

**Proposal of Assay Procedure for Double Determination:**

<table>
<thead>
<tr>
<th>Pipette</th>
<th>Reagents</th>
<th>Well Positions</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 µL</td>
<td>1:50 diluted Antibody Conjugate</td>
<td>Pipette in all required number of wells</td>
</tr>
<tr>
<td>50 µL</td>
<td>Standard A (0 mU/mL)</td>
<td>A1 and A2</td>
</tr>
<tr>
<td>50 µL</td>
<td>Standard B (1.5 mU/mL)</td>
<td>B1 and B2</td>
</tr>
<tr>
<td>50 µL</td>
<td>Standard C (6.25 mU/mL)</td>
<td>C1 and C2</td>
</tr>
<tr>
<td>50 µL</td>
<td>Standard D (12.5 mU/mL)</td>
<td>D1 and D2</td>
</tr>
<tr>
<td>50 µL</td>
<td>Standard E (25 mU/mL)</td>
<td>E1 and E2</td>
</tr>
<tr>
<td>50 µL</td>
<td>Standard F (40 mU/mL)</td>
<td>F1 and F2</td>
</tr>
<tr>
<td>50 µL</td>
<td>Diluted Control Serum KS1</td>
<td>G1 and G2</td>
</tr>
<tr>
<td>50 µL</td>
<td>Diluted Control Serum KS2</td>
<td>H1 and H2</td>
</tr>
<tr>
<td>50 µL</td>
<td>Diluted Sample</td>
<td>Pipette sample in the rest of the wells according to requirements</td>
</tr>
</tbody>
</table>

Cover the wells with the sealing tape

**Incubation: 2 h at RT, 350 rpm**

| 5x 300 µL | Aspirate the contents of the wells and wash 5x with 300 µL each WP/ well |
| 100 µL    | Enzyme Conjugate EK | each well |

**Incubation: 0.5 h at RT, 350 rpm**

| 5x 300 µL | Aspirate the contents of the wells and wash 5x with 300 µL each WP/ well |
| 100 µL    | Substrate Solution S | each well |

**Incubation: 30 min in the dark at RT**

| 100 µL   | Stop Solution SL | each well |

Measure the absorbance within 30 min at **450 nm** (≥590 nm Reference)