ALS - ELISA

KAPME35

Enzyme Immunoassay for quantitative Determination of human Acid Labile Subunit

For Research Use Only!

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1. INTENDED USE

This enzyme immunoassay kit is suited for measuring ALS in human serum or EDTA-/heparin-/citrate plasma for scientific purposes.

2. 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 and 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 concentrations of IGF and IGFBP-3 in the circulation are significantly decreased thus resulting in impaired growth [10].

The acid-labile subunit is a synthesized 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). Thus 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 are described. Miller BS et al. were able to demonstrate that incomplete glycolsylation 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 hyperinsulinemia could be observed [11, 12]. Further, the ALS-IGF-IGFBP-system seems to be of relevance in coronary disease [13].

The results of this test system can be used as supplementary information in GH-diagnostics together with IGF-I and IGFBP-3 measurements. Thus, it is of use in evaluation of GH-deficiency and excess [14, 15]

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 (50 µg/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 levels of IGFBP-3.

3. ASSAY PRINCIPLE

The DIAsource ELISA for ALS KAPME35 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. The biotinylated second anti-ALS-Antibody binds also to the immobilized ALS. In the following step the Streptavidin-POD-Conjugate binds to the biotinylated antibody and in the closing substrate reaction the turn of the colour will be catalysed, quantitatively depending on the ALS-level of the samples.

Initially the test system was calibrated against an internal serum containing arbitrary 480 standard and measurement results were expressed as DIAsource mU/mL. After successful production of eukaryotic recombinant ALS the calibration was transferred to mass units **(see Calibration / Traceability)**.
Additionally recombinant material was used to quantify the ALS content of the calibrators in mass units. Thorough analysis revealed that **1 mU ALS is equivalent to 5 ng ALS** and all previous assay data describing the assay performance were accordingly transferred to ng/mL.

4. WARNINGS AND PRECAUTIONS

For research and professional use only.
The DIAsource kit is suitable only for in vitro use and not and not for internal use in humans and animals. Follow strictly the test protocol. Use the valid version of the package insert provided with the kit. Be sure that everything has been understood. DIAsource will not be held responsible for any loss or damage (except as required by statute) howsoever caused, arising out of noncompliance with the instructions provided. A Material Safety Data sheet is available on request.

Do not use obviously damaged or microbial contaminated or spilled material.

Caution: This kit contains material of human and/or animal origin. Therefore all components and patient´s specimens should be treated as potentially infectious.

Appropriate precautions and good laboratory practices must be used in the storage, handling and disposal of the kit reagents. The disposal of the kit components must be made according to the local regulations.

Human Serum
Following components contain human serum: **Control 1 and 2, and Standards 0-5**
Source human serum for the control sera provided in this kit was tested and found non-reactive for Hepatitis-B surface antigen (HBsAg), Hepatitis C virus (HCV), and Human Immunodeficiency Virus 1 and 2 (HIV). No known methods can offer total security of the absence of infectious agents; therefore all components and patient´s specimens should be treated as potentially infectious.

Reagents, Antibody Conjugate, Dilution buffer, Washing buffer
Contain as preservative a mixture of **5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one** (<0.015%)

H317	May cause an allergic skin reaction.
P280	Wear protective gloves/ protective clothing/ eye protection/ face protection.
P272	Contaminated work clothing should not be allowed out of the workplace.
P261	Avoid breathing dust/ fume/ gas/ mist/ vapours/ spray.
P333+P313	If skin irritation or rash occurs: Get medical advice/ attention.
P302+P352	IF ON SKIN: Wash with plenty of soap and water.
P501	Dispose of contents/ container in accordance with local/ regional/ national/ international regulations.

Substrate Solution
The TMB-Substrate contains 3,3´,5,5´ Tetramethylbencidine (<0.05%)

H315	Causes skin irritation.
H319	Causes serious eye irritation.
H335	May cause respiratory irritation.
P261	Avoid breathing dust/ fume/ gas/ mist/ vapours/ spray.
P305+P351+	IF IN EYES: Rinse cautiously with water for several minutes.
P338	Remove contact lenses, if present and easy to do. Continue rinsing.

Stopping Solution
The Stopping solution contains 0.2 M acid sulphur acid (H_2SO_4)

H290	May be corrosive to metals.
H314	Causes severe skin burns and eye damage.
P280	Wear protective gloves/ protective clothing/ eye protection/ face protection.
P301+P330+	IF SWALLOWED: rinse mouth.
P331	Do NOT induce vomiting.
P305+P351+	IF IN EYES: Rinse cautiously with water for several minutes.
P338	Remove contact lenses, if present and easy to do. Continue rinsing.

P309+P310 IF exposed or if you feel unwell: Immediately call a POISON CENTER or doctor/physician.

4.1 General first aid procedures:
Skin contact: Wash affected area rinse immediately with plenty of water at least 15 minutes. Remove 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: After swallowing the product, if the affected person is conscious, rinse out the mouth with plenty of water: seek medical advice immediately.

5. SAMPLES

5.1 Sample type
Serum and Plasma

30 IU/mL Sodium Heparin, 3,8 g/L Sodium Citrate or 0,0068 mol/L EDTA did not interfere with ALS measurement.

5.2 Specimen collection
The blood sample for serum preparation should be gained according to standardized venipuncture procedure. Hemolytic reactions have to be avoided.

5.3 Required sample volume: 10 µL

5.4 Sample stability
In firmly closable sample vials
- Storage at 20-25°C: 3 days
- Storage at -20° C: min. 2 years
- Freeze-thaw cycles max. 5

The storage of samples over a period of 2 years at -20°C, showed no influence on the reading. Freezing and thawing of samples should be minimized, 5 freeze-thaw cycles showed no effect on the measured ALS concentration.

5.5 Interference
Hemoglobin, triglyceride and bilirubin in the sample do not interfere to a concentration of
1 µg/mL, **100 mg/mL** and **200 µg/mL**, respectively. However, the use of hemolytic, lipemic or icteric samples should be validated by the user.

5.6 Sample dilution
- Dilution: **1:150** with Sample Diluent
- Pipette **1490 µL** Sample Diluent (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).
- Sample stability after dilution of the sample: maximum 1 hour at 20-25°C.
- In most determinations (e.g. Serum- or Plasma samples and no extreme values expected) the dilution of **1:150** with Sample Diluent is suitable, respectively the assay covers the range from **0.53 ng/mL - 30 µg/mL**. 1:50 is the minimal tested sample dilution.
- If required, the dilution with Sample Diluent could be performed lower or higher

6. Materials

6.1 Materials provided

The reagents listed below are sufficient for 96 wells including the standard curve.

1)	**Microtiter plate**, ready for use, Microtiter plate with 96 wells. Divided up in 12 strips with 8 wells separately breakable. Coated with anti-human ALS Antibody. Packed in a laminate bag.
2)	**Standards 0-5, 1 mL**, lyophilized, contain human ALS. The concentrations are given on vial labels and on quality certificate in ng/mL.
3)	**Sample Diluent, 125 mL**, ready for use, red colored, please use for the reconstitution of Standards 0-5 and Controls and for the dilution of Samples and Controls.
4)	**Dilution Buffer, 7 mL**, ready for use, please use for the dilution of **Antibody Conjugate**.
5)	**Controls 1 and 2, 250 µL**, lyophilized, contain human Serum. The concentration is given on quality certificate in ng/mL.
6)	**Antibody Conjugate**, 50-fold concentrate, contains the biotinylated anti-human rabbit ALS Antibody. Dilute before use 1**:50 in Dilution Buffer** and use **50 µl** for each well in the assay. **Attention:** Please dilute **Antibody Conjugate** freshly according to daily requirements.
7)	**Enzyme Conjugate, 12 mL**, ready-to-use, contains HRP (Horseradish-Peroxidase)-labelled Streptavidin.
8)	**Washing Buffer, 50 mL, 20-fold concentrated** solution dilute in A. dest or in deionized Water
9)	**Substrate, 12 mL**, ready for use, horseradish-peroxidase-(HRP)-substrate stabilized Tetramethylbencidine.
10)	**Stopping Solution, 12 mL,** ready for use, 0.2 M sulphuric acid.
11)	Sealing tape for covering of the microtiter plate, 2 x.

6.2 Materials required, but not provided

- Distilled (Aqua destillata) or deionized water for dilution of the Washing Buffer (A. dest.), 950 mL.
- Precision pipettes and multichannel pipettes with disposable plastic tips
- Polyethylene PE/Polypropylene PP tubes for dilution of samples
- Vortex-mixer
- Microtiter plate shaker (350 rpm)
- Microtiter plate washer (recommended)
- Micro plate reader ("ELISA-Reader") with filter for 450 and ≥ 590 nm

7. TECHNICAL NOTES

Storage Conditions

Store the kit at 2-8°C after receipt until its expiry date. The lyophilized reagents should be stored at –20 °C after reconstitution. Avoid repeated thawing and freezing.

Storage Life

The shelf life of the components **after initial opening** is warranted for **4 weeks**, store the unused strips and microtiter wells **airtight** together with the desiccant at 2-8°C in the clip-lock bag, use in the frame provided. The **reconstituted components** standards **0-5** and Control Sera must be stored at –20°C (max. 4 weeks). For further use, thaw quickly but gently (avoid temperature increase above room temperature and avoid excessive vortexing). Up to 3 of the freeze-thaw cycles did not influence the assay. The 1:20 diluted Washing Buffer is 4 weeks stable at 2-8°C

Preparation of reagents

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. Reagents with different lot numbers cannot be mixed.

Reconstitution
The Standards **0 – 5** and Control Sera are reconstituted with the Sample Diluent. It is recommended to keep reconstituted reagents at room temperature for 15 minutes and then to mix them thoroughly but gently (no foam should result) with a Vortex mixer.

Dilution
After reconstitution **dilute** the Controls with the Sample Diluent in the same ratio **(1:150)** as the sample.

The required volume of **Antibody Conjugate** is prepared by **1:50** dilution of the provided 50-fold concentrate with **Dilution Buffer.** Please dilute Antibody Conjugate freshly according to daily requirements.

The required volume of Washing Buffer is prepared by 1:20 dilution of the provided 20fold concentrate with Aqua dest.

Assay Procedure
When performing the assay Standards **0-5**, Controls and the samples should be pipette as fast as possible (e.g. <15 minutes). To avoid distortions due to differences in incubation times, Enzyme Conjugate as well as the succeeding Substrate Solution should be added to the plate in the same order and in the same time interval as the samples. Stopping Solution should be added to the plate in the same order as Substrate Solution.
All determinations (Standards **0-5**, Controls and samples) should be assayed in duplicate. For optimal results, accurate pipetting and adherence to the protocol are recommended.

Incubation
Incubation at room temperature means: Incubation at 20 - 25°C. The Substrate Solution, stabilised H_2O_2-Tetramethylbencidine, is photosensitive–store and incubation in the dark.

Shaking
The incubation steps should be performed at mean rotation frequency of a particularly suitable microtiter plate shaker. We are recommending 350 rpm. Due to certain technical differences deviations may occur, in case the rotation frequency must be 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.

Washing
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 Washing 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 fur 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. Washing Buffer may be dispensed via a multistepper device, a multichannel pipette, or a squirt bottle. The fluid may be removed by dynamical 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.

8. ASSAY PROCEDURE

Preparation of reagents	**Reconstitution:**	**Dilution**
Antibody-conjugate	-	Dilute before use with **1:50** Dilution Buffer
Standards 0-5	in **1 mL** Sample Diluent	-
Control Serum 1-2	In **250 µL** Sample Diluent	**1:150** with Sample Diluent
Washing Buffer	-	**1:20** with **Aqua dest.**

Sample and Control Sera 1 & 2 Dilution: 1:150 in Sample Diluent (red colored; e.g. **10 µL** in **1490 µL**). **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 **20-25°C**.

Assay Procedure in Double Determination:

Pipette	**Reagents**	**Well Position**
50 µL	**1:50 diluted Antibody Conjugate**	**Pipette in <u>all</u> required number of wells**
50 µL	Standard **0** (**0 ng/mL**)	A1/A2
50 µL	Standard **1** (**7.5 ng/mL**)	B1/B2
50 µL	Standard **2** (**31.25 ng/mL**)	C1/C2
50 µL	Standard **3** (**62.5 ng/mL**)	D1/D2
50 µL	Standard **4** (**125 ng/mL**)	E1/E2
50 µL	Standard **5** (**200 ng/mL**)	F1/F2
50 µL	Control Serum **1** (1:150 diluted)	G1/G2
50 µL	Control Serum **2** (1:150 diluted)	H1/H2
50 µL	Sample (1:150 diluted)	in the rest of the wells according the requirements
Cover the wells with the sealing tape.		
Sample Incubation: **2 h at 20-25°C**, **350 rpm**		
5 x 300 µL	Aspirate the contents of the wells and **wash** 5 x with 300 µL each Washing Buffer **/ well**	In each well
100 µL	**Enzyme Conjugate**	In each well
Cover the wells with the sealing tape.		
Incubation: 30 Minutes at 20-25°C, 350 rpm		

5 x 300 µL	Aspirate the contents of the wells and **wash** 5 x with 300 µL each Washing Buffer **/ well**	In each well
100 µL	Substrate Solution	In each well
Incubation: 30 Minutes in the Dark at 20-25°C		
100 µL	Stopping Solution	In each well
	Measure the absorbance within 30 min at **450 nm** with ≥ 590 nm as reference wavelength.	

9. QUALITY CONTROL

Good laboratory practice requires that controls are included in each assay. A statistically significant number of controls should be assayed to establish mean values and acceptable ranges to assure proper performance. The test results are only valid if the test has been performed following the instructions. Moreover the user must strictly adhere to the rules of GLP (Good Laboratory Practice) or other applicable federal, state or local standards/laws. All standards and kit controls must be found within the acceptable ranges as stated on the QC Certificate. If the criteria are not met, the run is not valid and should be repeated. Each laboratory should use known samples as further controls.

9.1 Quality criteria

For the evaluation of the assay it is required that the absorbance values of the Standard 0 should be below 0.25, and the absorbance of **Standard 5** should be above **1.00**.
Samples, which yield higher absorbance values than **Standard 5**, should be re-tested with a higher dilution.

10. EVALUATION OF RESULTS

10.1 Establishing of the standard curve

Standard	**0**	**1**	**2**	**3**	**4**	**5**
ng/mL	0	7.5	31.25	62.5	125	200
mU/mL	0	1.5	6.25	12.5	25	40

1) Calculate the **mean absorbance** value for the Standard 0 from the duplicated determination (well A1/A2).

2) Subtract the mean absorbance of the Standard 0 from the mean absorbance of all other samples and standards

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 ng/mL of the samples and controls **1** and **2** can be calculated by **multiplication** with the respective **dilution factor.**

10.2 Example of a typical standard curve

The following data is for demonstration only and cannot be used in place of data generation at the time of assay.

	0	1	2	3	4	5
ng/mL	0	7.5	31,25	62,5	125	200
$OD_{(450\text{-}620\ nm)}$	0.049	0.282	1.248	1.929	2.54	2.934

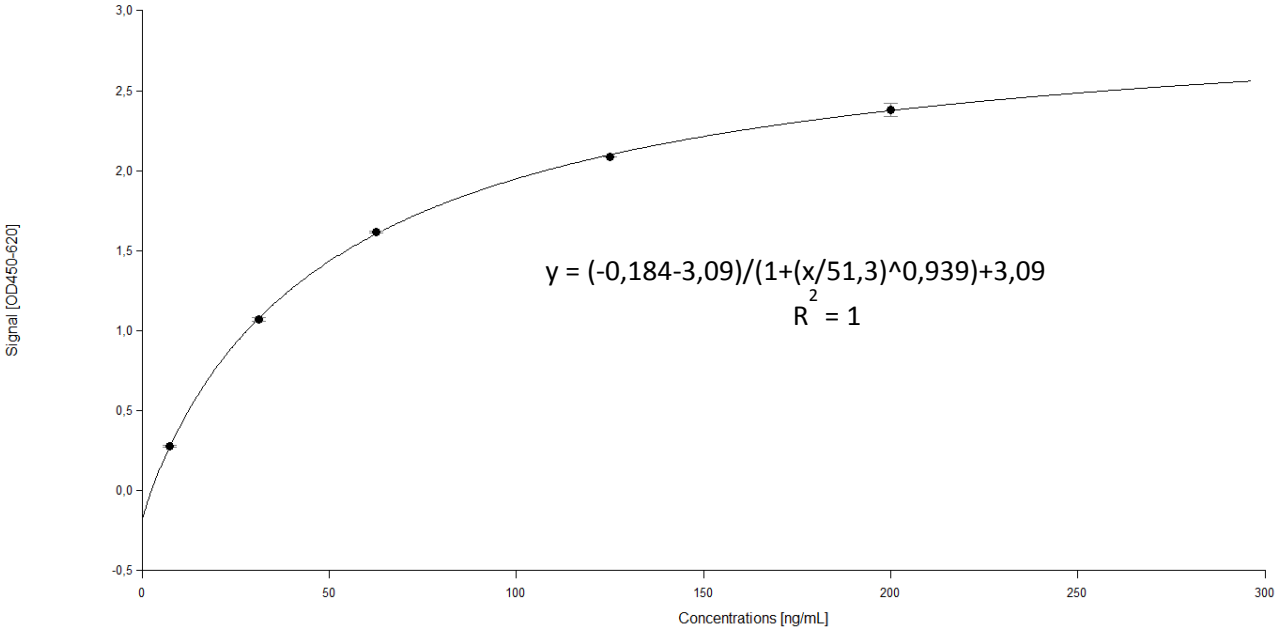

Figure 1 Examplary standard curve

The exemplary shown standard curve in Figure 1 **cannot** be used for calculation of your test results. You have to establish a standard curve for each test you conduct!

10.3 Exemplary calculation of ALS concentrations

Sample dilution: 1:150

Measured extinction of your sample 1.5
Measured extinction of the Standard 0 0.049

Your measurement program will calculate the ALS concentration of the diluted sample automatically by using the difference of extinction values of sample and Standard 0 for the calculation. You only have to determine the most suitable curve fit.

In this exemplary case the following equation is solved by the program to calculate the ALS concentration in the sample:

$$1.451 = (-0.184\text{-}3.09)/(1+(x/51.3)^{0.939})+3.09$$

$$51.201 = x$$

If the dilution factor (1:150) is taken into account the ALS concentration of the undiluted sample is

$$51.201 \times 150 = 7680 \text{ ng/mL}$$

10.4 Limitation of procedure

The DIAsource sensitive human **ALS ELISA**, KAPME35 is based on antibodies. Generally, this technique could be sensible to heterophilic antibodies or rheumatic factors in the sample. Their influence is reduced by assay design, but cannot be excluded completely.

11. REFERENCE VALUES

Serum samples of healthy blood donors were used to assess concentration in healthy adult humans. Significant differences between sexes were not detected and an age dependency was not evaluated.

Table 1 Reference values for adults in serum.

	male [ng/mL]	female [ng/mL]
Mean	**7095**	**8413**
SD	**1252**	**1956**
Median	7162	8236
Min	4525	5332
Max	10031	11981
n	39	35

12. PERFORMANCE CHARACTERISTICS

12.1 Calibration – Traceability

No international standard or reference preparation of ALS is available. Initially, the DIAsource ALS ELISA was calibrated against a human serum standard. In a second step the test system was recalibrated with eukaryotic, recombinant ALS. The recombinant ALS was measured in three different DIAsource KAPME35 lots. A comparison of the measured results is shown in Figure 2. The analysis revealed a factor of 5 to transfer DIAsource Units in mass units (ng/mL).
According to the function y = 4,997x, the factor of 5 is used in the conversion of DIAsource Units (mU/mL) in mass units (ng/mL):
1 DIAsource Unit ALS ≈ 5 ng rec. ALS

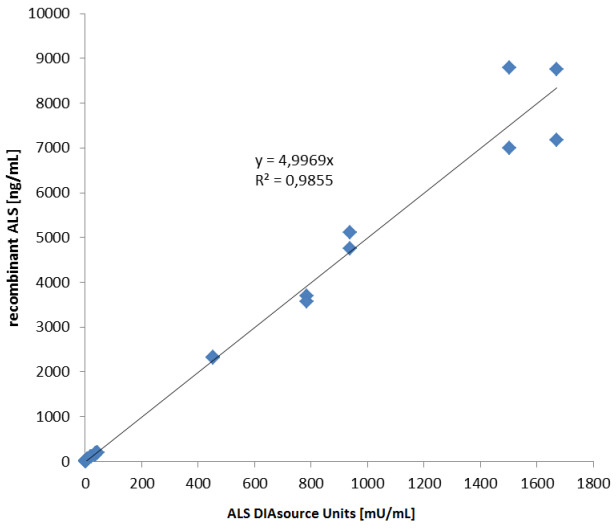

Figure 2 Assay Calibration, relation of DIAsource Units and mass units.

A previously conducted comparative analysis of serum samples demonstrates that the DIAsource KAPME35 measures comparable results referring to an in-house assay used by an academic group (see Figure 3).

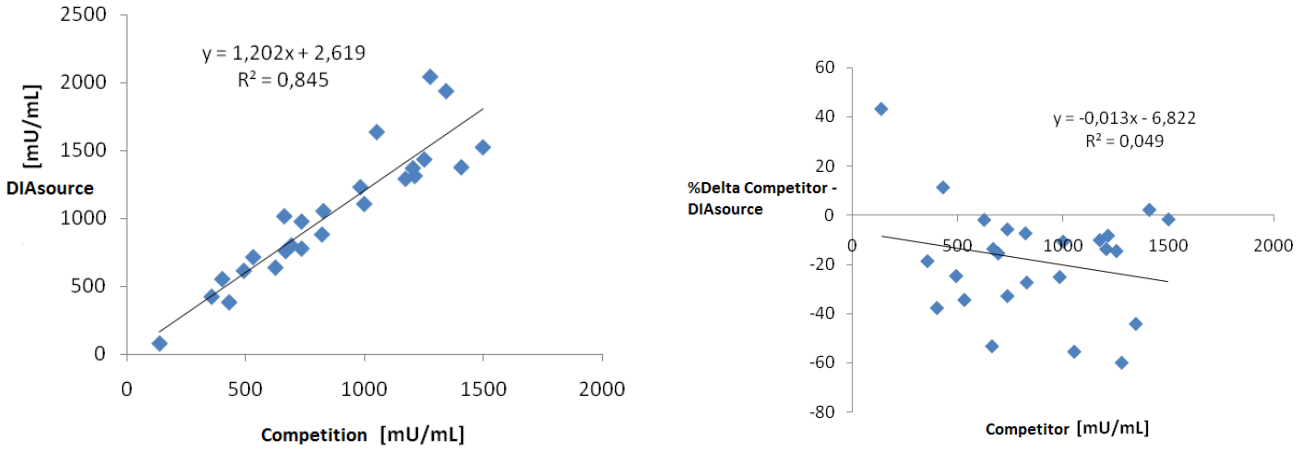

Figure 3 Comparative analysis of a competitive immunoassay with the DIAsource KAPME35 (serum samples: n=25).

12.2 Sensitivity

Sensitivity was assessed by measuring the blank and calculating the theoretical concentration of the blank + 2SD. In three measurements a range of 0.15 – 1.15 ng/mL with a mean sensitivity of 0.53 ng/mL was detected.

12.3 Precision Data

Intra-Assay Variance

Two samples have been measured 22 times in the same assay. The results are shown in Table 2. The measured coefficient of variation (CV) is 6.7% on average.

Table 2 Intra-Assay Variation

	Number of determinations	Mean value [ng/mL]	Standard deviation [ng/mL]	CV [%]
Sample 1	22	4556	298	6,55
Sample 2	22	6694	458	6,84

Inter-Assay Variance

Serum samples where measured in independent assays. On average the coefficient of variation was 8.96% (SD 6.11). Exemplary results are shown in table 3.

Table 3 Inter-Assay Variation

	Number of single determinations	Mean value [ng/mL]	Standard deviation [ng/mL]	CV [%]
Sample 1	39	4980	485	10
Sample 2	45	5530	525	9
Sample 4	12	3225	230	7

12.4 Linearity

Linearity was tested by dilution of three native serum samples with high ALS content. The optical density of each dilution was measured and the results are shown in Figure 4. Serial dilution of three samples within a range of 1:50 – 1:500 revealed a good linearity measured by linear regression analysis (R2>0.95).

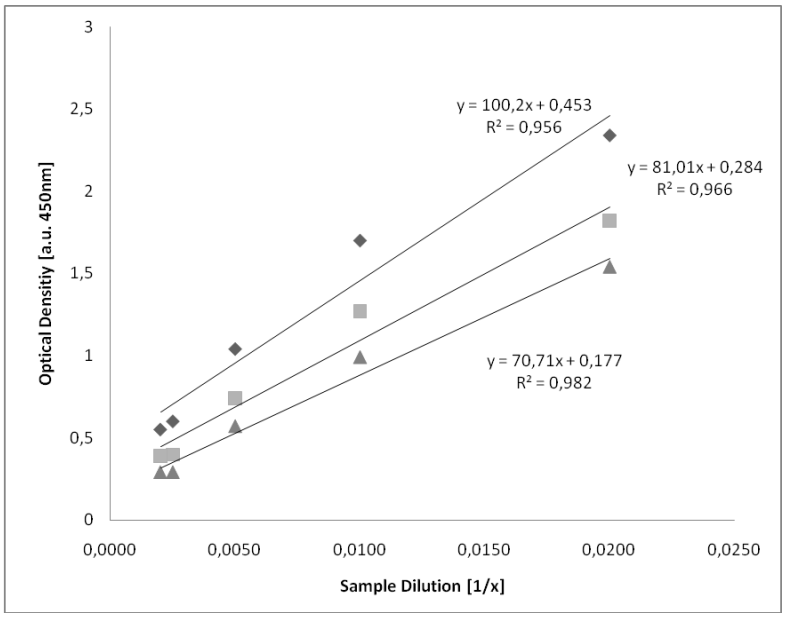

Figure 4 Linearity, measured signal intensity [OD_{450}] of differentially diluted samples. The recommended dilution is 1:150 (0.007).

12.5 Interference

Interference of bilirubin and triglycerides was tested by adding different amounts of these substances to human serum containing ALS. For comparison the same amount of buffer without any substance was also added to the serum. Table 4 demonstrates that none of the tested substances exert any influence on the measurement of ALS in human serum.

Table 4 Interference

Triglyceride [mg/mL]	**0**	**12,5**	**25**	**50**	**100**
ALS [ng/mL]	5809	5403	5780	5383	5813
Bilirubin [µg/mL]	**0**	**25**	**50**	**100**	**200**
ALS [ng/mL]	5809	5283	5431	5771	5439
Hemoglobin [µg/mL]	**0**	**0.125**	**0.25**	**0.5**	**1**
ALS [ng/mL]	5809	5667	5315	6015	6100

12.6 Species Cross-Reactivity

Several commercially available animal sera have been diluted 1:10 and the diluted specimens were used as samples in this assay. Only light signals were detected in serum samples of chicken, cattle, dog, rat, donkey, mouse, goat, sheep, guinea pig, fetal calve serum. On average signal intensity was about 0.1 (corresponding Standard 0 value: 0.04).

13. References

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