Version 05-11/24

User's Manual

Acid-labile subunit (ALS) ELISA Kit

REF **DEIA4478**

Σ **96T**

RUO

This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

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PRODUCT INFORMATION

Intended Use

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

General Description

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. 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). 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. 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.

The acid-labile subunit is 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. Mutations in or the complete knock out of the ALS gene result in IGF / IGFBP-3 deficiency and therewith in disturbance of growth. Beside growth also other endocrine axes may be involved. In primary ALS deficiency hyperinsulinemia could be observed. Further, the ALS-IGF-IGFBP-system seems to be of relevance in coronary disease.

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.

The first ALS immunoassay was described by Baxter RC in 1990. 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.

Principles of Testing

The ALS ELISA Kit 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. 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 standard and measurement results were expressed as 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.

Reagents And Materials Provided

1. **Microtiter plate:** ready for use, coated with rabbit-anti-ALS-antibody. Wells are separately breakable. (8x12) wells
2. **Sample Buffer (PP):** ready for use, red colored, please use for the reconstitution of Standards and Controls KS1/KS2 and for the dilution of Samples and Controls KS1/KS2. 1 x 125 mL
3. **Standards (A-F):** lyophilized, (native human ALS), concentrations are given on vial labels and on quality certificate in ng/mL. 6 x 1 mL
4. **Control Serum 1 (KS1):** lyophilized, (human serum), concentration is given on quality certificate in ng/mL. 1 x 250 µL
5. **Control Serum 2 (KS2):** lyophilized, (human serum), concentration is given on quality certificate in ng/mL. 1 x 250 µL
6. **Dilution Buffer (VP):** ready for use, please use for the dilution of Antibody Conjugate. 1 x 7 mL
7. **Antibody Conjugate (AK):** 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. 1 x 140 µL

   Attention: Please dilute Antibody Conjugate freshly according to daily requirements.
8. **Enzyme Conjugate (EK):** ready for use, contains HRP (Horseradish-Peroxidase)-labeled Streptavidin. 1 x 12 mL
9. **Washing Buffer (WP):** 20-fold concentrated solution, dilute 1:20 in A.dest. or in deionized Water. 1 x 50 mL
10. **Substrate (S):** ready for use, horseradish-peroxidase-(HRP) substrate, stabilised Tetramethylbencidine. 1 x 12 mL
11. **Stopping Solution (SL):** ready for use, 0.2 M sulfuric acid. 1 x 12 mL
12. **Sealing Tape:** for covering the microtiter plate. 2

Materials Required But Not Supplied

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

Storage

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.

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 A-F and Control Sera KS1 and KS2 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 WP is 4 weeks stable at 2-8°C

Specimen Collection And Preparation

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.

2. Specimen collection

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

3. Required sample volume: 10 µL

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.

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. 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.

6. Sample dilution

• Dilution: 1:150 with Sample Buffer PP

• 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).

• 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 Buffer PP is suitable, respectively the assay covers the range from 0.53 ng/mL - 30 µ

g/mL. 1:50 is the minimal tested sample dilution.

• If required, the dilution with Sample Buffer PP could be performed lower or higher

Reagent Preparation

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.

1. The Standards A – F is reconstituted with 1mL Sample Buffer PP. After resuspension, the standard is diluted according to a gradient - A (0 ng/mL), B (7.5 ng/mL), C (31.25 ng/mL), D (62.5 ng/mL), E (125 ng/mL) and F (200 ng/mL), which are prepared for immediate use.
2. The Control Sera KS1 and KS2 are reconstituted with 250 µL Sample Buffer PP. After reconstitution dilute the Controls KS1 and KS2 with the Sample Buffer PP in the same ratio (1:150) as the sample. (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)

Note: 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.

3. The required volume of Antibody Conjugate AK 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.
4. The required volume of Washing Buffer WP is prepared by 1:20 dilution of the provided 20-fold concentrate with Aqua dest.

Assay Procedure

Note

When performing the assay, Standards A-F, Controls KS1, KS2 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 EK as well as the succeeding Substrate Solution S should be added to the plate in the same order and in the same time interval as the samples. Stopping Solution SL should be added to the plate in the same order as Substrate Solution S. All determinations (Standards A-F, Controls KS1, KS2 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 S, 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 WP 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. 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.

Assay Step

1. Set Standard A-F, test samples (1:150 diluted), Control Serum KS1 and KS2 (1:150 diluted) wells on the pre-coated plate respectively, and then, records their positions. It is recommended to measure each standard and sample in duplicate.
2. Prepare Standards: Aliquot 50ul of Standard A-F, Control Serum KS1/KS2 or test samples into wells.
3. Incubate: Seal the plate with a cover and incubate at 20-25°C for 2 hours.
4. Wash: Aspirate the contents of the wells, and wash plate 5 times with 300 µL Washing Buffer WP. Do not let the wells dry completely at any time.
5. Add 100ul Enzyme Conjugate EK into above wells (standard, control serum and test samples). Add the solution at the bottom of each well without touching the sidewall, cover the plate and incubate at 20-25°C, 350 rpm for 30 minutes.
6. Wash: Aspirate the contents of the wells, and wash plate 5 times with 300 µL Washing Buffer WP. Do not let the wells dry completely at any time.
7. Add 100ul of Substrate Solution S into each well, cover the plate and incubate at 20-25°C in dark within 30 minutes.
8. Stop: Add 100ul Stopping Solution SL into each well.
9. Measure the absorbance within 30 min at 450 nm, with ≥ 590 nm as reference wavelength.

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.

Quality criteria

For the evaluation of the assay it is required that the absorbance values of the Standard A 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, should be re-tested with a higher dilution.

Calculation

1. Calculate the mean absorbance value for the Standard A from the duplicated determination (well A1/A2).
2. Subtract the mean absorbance of the Standard A 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 KS1 and KS2 can be calculated by multiplication with the respective dilution factor.

Standard	A	B	C	D	E	F
ng/mL	0	7.5	31.25	62.5	125	200
mU/mL	0	1.5	6.25	12.5	25	40

Exemplary calculation of ALS concentrations

Sample dilution: 1:150

Measured extinction of your sample.....1.5

Measured extinction of the Standard A.....0.049

Your measurement programm will calculate the ALS concentration of the diluted sample automatically by using the difference of extinction values of sample and Standard A 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:

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

$$51.201= x$$

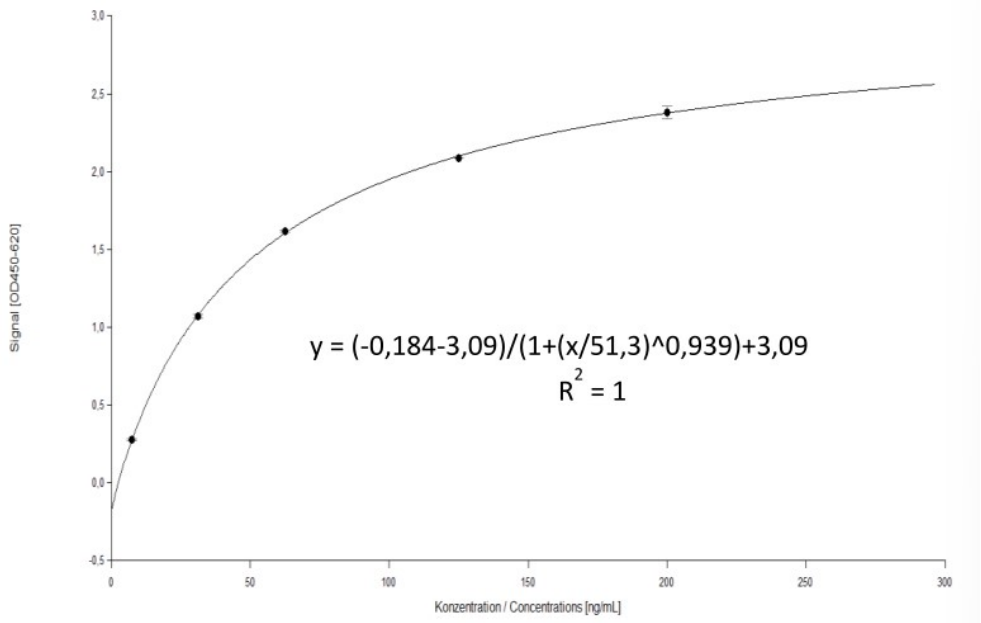
If the dilution factor (1:150) is taken into account the ALS concentration of the undiluted sample is

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

Typical Standard Curve

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

	A	B	C	D	E	F
ng/mL	0	7.5	31,25	62,5	125	200
OD(450-620 nm)	0.049	0.282	1.248	1.929	2.54	2.934



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

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.

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

Performance Characteristics

Calibration - Traceability

No international standard or reference preparation of ALS is available. Initially, the ALS ELISA Kit 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 lots. A comparison of the measured results is shown in Figure below. The analysis revealed a factor of 5 to transfer Units in mass units (ng/mL). According to the function y = 4,997x, the factor of 5 is used in the conversion of Units (mU/mL) in mass units (ng/mL): 1 Unit ALS ≈ 5 ng rec. ALS

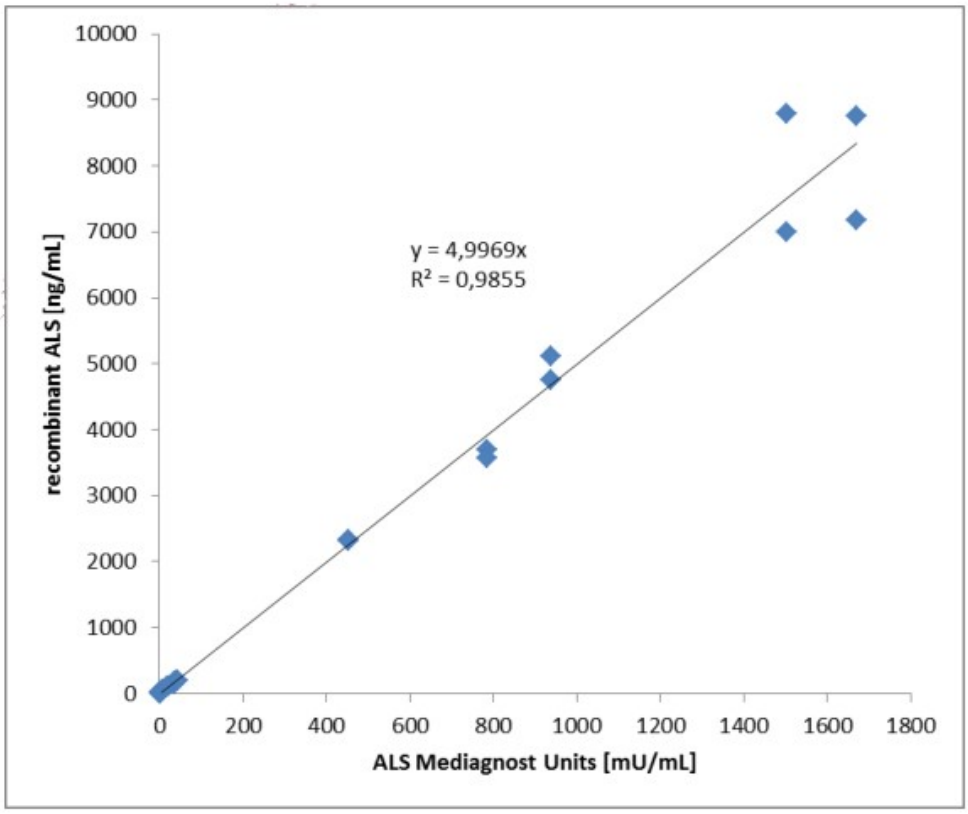


Figure. Assay Calibration, relation of Units and mass units.

A previously conducted comparative analysis of serum samples demonstrates that the ALS ELISA Kit measures comparable results referring to an in-house assay used by an academic group.

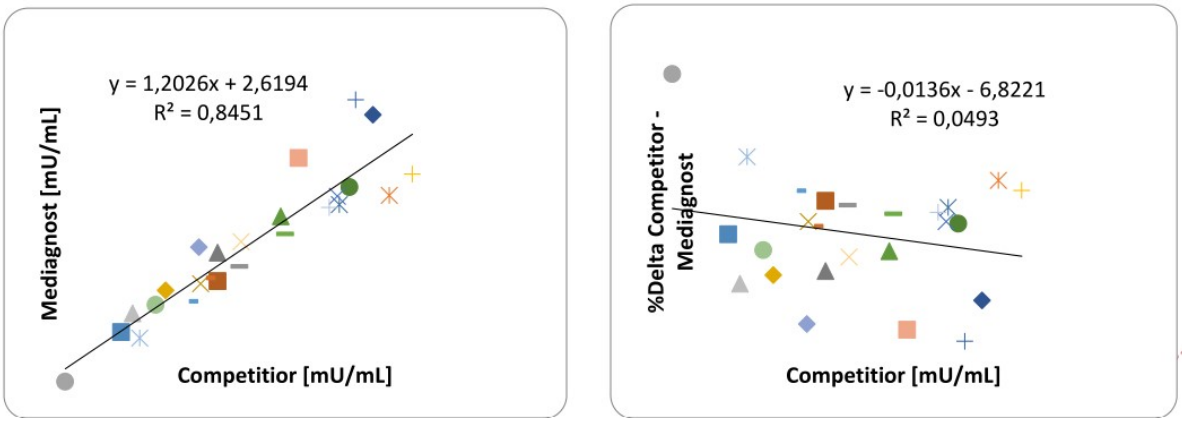


Figure. Comparative analysis of a competitive immunoassay with the ALS ELISA Kit (serum samples: n=25).

Precision

Intra-Assay Variance

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

	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 were measured in independent assays. On average the coefficient of variation was 8.96% (SD 6.11).

	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 3	12	3225	230	7

Sensitivity

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

Specificity

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 A value: 0.04).

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 below. Serial dilution of three samples within a range of 1:50 – 1:500 revealed a good linearity measured by linear regression analysis ($R^2$0.95).

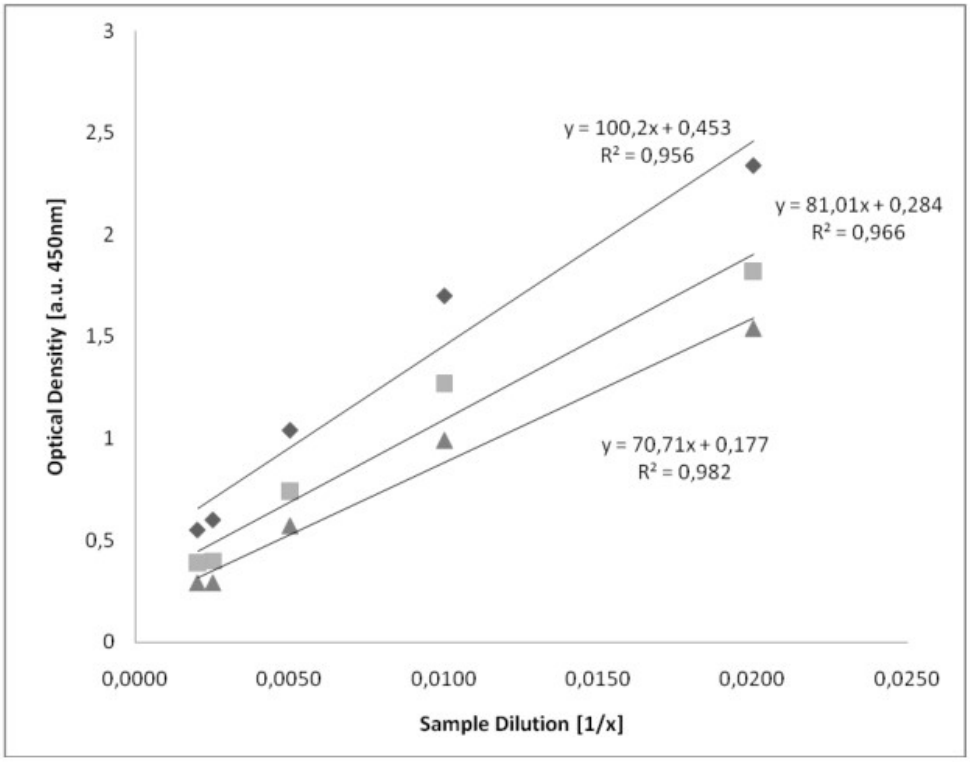


Figure. Linearity, measured signal intensity [OD450] of differentially diluted samples. The recommended dilution is 1:150 (0.007).

Interferences

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 below demonstrates that none of the tested substances exert any influence on the measurement of ALS in human serum.

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

Precautions

1. For research and professional use only. The Creative Diagnostics kit is suitable only for in vitro use and not for internal use in humans and animals.
2. Follow strictly the test protocol. Use the valid version of the package insert provided with the kit. Be sure that everything has been understood. Creative Diagnostics 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.
3. Do not use obviously damaged or microbial contaminated or spilled material.

4. This kit contains material of human and/or animal origin. Therefore all components and patient´s specimens should be treated as potentially infectious.
5. 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.
6. Following components contain human serum: Control Sera KS1 and KS2, and Standards A-F. 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.

Limitations

The ALS ELISA Kit 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.