Etanercept Drug Level ELISA

For the quantitative determination of free ENBREL® concentration in human serum and EDTA plasma.

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

Catalog Number: 30-ETLHU-E01

Size: 96 Wells

Version: 2016-11-10 – ALPCO May 3, 2017

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INTENDED USE

The ALPCO Etanercept Drug Level ELISA is intended for the quantitative determination of free etanercept (e.g. ENBREL®) concentration in human serum and EDTA plasma. For Research Use Only. Not for diagnostic use.

PRINCIPLE OF THE ASSAY

This ELISA is designed to determine the quantity of free etanercept (therapeutic antibody against TNFα, ENBREL®) in EDTA plasma or serum samples. In a first incubation step, the free etanercept from the sample is bound to the specific monoclonal anti-etanercept antibody coated on the plate. To remove all unbound substances, a washing step is carried out. In a further incubation step, peroxidase-labeled antibody is added. Tetramethylbenzidine (TMB) is used as a substrate for peroxidase. Finally, an acidic stop solution is added to terminate the reaction. The color changes from blue to yellow. The intensity of the yellow color is directly proportional to the concentration of free etanercept in the sample. A dose response curve of the absorbance unit (optical density, OD) vs. concentration is generated, using the values obtained from the standards. The concentrations of free etanercept in the samples are determined directly from this curve.

MATERIALS SUPPLIED

Component	Quantity	Preparation
Microplate (96 wells)	12 x 8 strips	Ready to use
Control Levels 1 + 2	2 vials each	Lyophilized
Wash Buffer Concentrate	100 mL	10X
Standards 1-6 (0, 4.15, 8.3, 25, 75, 225 ng/mL)	2 vials each	Lyophilized
Sample Buffer	2 x 100 mL	Ready to use
Conjugate Concentrate	200 µL	101X
TMB Substrate	15 mL	Ready to use
Stop Solution	15 mL	Ready to use

MATERIALS REQUIRED

- Precision pipettes for dispensing up to 1000 µL (with disposable tips)
- Repeating or multi-channel pipette for dispensing up to 1000 µL
- Volumetric containers and pipettes for reagent preparation
- Distilled/Deionized water for reagent preparation
- Microplate washer or wash bottle
- Microplate shaker capable of 700-900 rpm
- Microplate reader
- Centrifuge (1,000 xg to 3,000 xg)
- Vortex for sample preparation
- Laboratory Balance
- Foil to cover the microplate
- Timer

PRECAUTIONS

1. The human blood products incorporated into this kit have been tested for the presence of HIV (Human Immunodeficiency Virus), HBV (Hepatitis B Virus), and HCV (Hepatitis C Virus). Test methods for these viruses do not guarantee the absence of a virus; therefore, all reagents should be treated as potentially infectious. Handling and disposal should be in accordance with all appropriate national and local regulations for the handling of potentially biohazardous materials.
2. All materials derived from animal sources are bovine spongiform encephalopathy (BSE) negative. However, all materials should be treated as potentially infectious.
3. Kit reagents contain sodium azide or Proclin as bactericides. Sodium azide and Proclin are toxic. Substrates for the enzymatic color reactions are toxic and carcinogenic. Avoid contact with skin or mucous membranes.
4. The stop solution consists of diluted sulfuric acid, a strong acid. Although diluted, it still must be handled with care. It can cause burns and should be handled with gloves, eye protection, and appropriate protective clothing. Any spill should be wiped up immediately with copious quantities of water. Do not breathe vapor and avoid inhalation.
5. Avoid direct contact with skin.
6. This product is not for internal use.
7. Avoid eating, drinking, or smoking when using this product.
8. Do not pipette any reagents by mouth.
9. Reagents from this kit are lot-specific and must not be substituted.
10. Do not use reagents beyond the expiration date.
11. Variations to the test procedure are not recommended and may influence the test results.

STORAGE CONDITIONS

The kit should be stored at 2-8°C. The kit is stable until the expiration date on the box label.

SAMPLE HANDLING

Serum and EDTA plasma are appropriate for use with this assay.

EDTA plasma or serum samples must be diluted 1:50 before performing the assay.
e.g. 10 µL sample + 490 µL Sample Buffer, mix well. For testing in duplicates, pipet 2x 100 µL per well of each prepared sample.

Sample storage

Freshly collected EDTA plasma or serum can be stored for seven days at room temperature (15–30°C) or for longer storage at -20°C. Diluted EDTA plasma or serum samples can be stored for two weeks at 2–8°C and at -20°C for at least four weeks.

REAGENT PREPARATION

All reagents must be equilibrated to room temperature prior to preparation and subsequent use in the assay. Prepare enough reagents for only the number of strips used. Reagents with a volume less than 100 µL should be centrifuged before use to avoid loss of volume.

Wash Buffer Concentrate (10X) must be diluted with distilled water 1:10 before use (100 mL wash buffer + 900 mL distilled water) and mixed well. Crystals could occur due to high salt concentration in the stock solutions. The crystals must be dissolved at room temperature or in a water bath at 37 °C before dilution of the buffer solutions. The undiluted wash buffer is stable at 2–8 °C until the expiry date stated on the label. Wash buffer (1:10 diluted wash buffer) can be stored in a closed flask at 2–8 °C for one month.

Controls (Levels 1 and 2) are provided in a lyophilized form. Reconstitute in 500 µL of distilled water. Allow the vial content to dissolve for 10 minutes at room temperature (15–30°C), and mix thoroughly by gentle inversion to ensure complete reconstitution. Reconstituted controls can be stored at -20 °C for 3 months. Avoid repeated thawing and freezing.

Standards are provided in a lyophilized form. Reconstitute in 500 µL of distilled water. Allow the vial content to dissolve for 10 minutes at room temperature (15–30°C), and mix thoroughly by gentle inversion to ensure complete reconstitution. Reconstituted standards can be stored at -20 °C for 3 months. Avoid repeated thawing and freezing.

Conjugate Concentrate must be diluted 1:101 in wash buffer (100 µL conjugate concentrate + 10 mL wash buffer). The undiluted conjugate is stable at 2–8 °C until expiry date stated on the label. Diluted conjugate (1:101 diluted) 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.

QUALITY CONTROL

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 samples may not be valid if within the same assay one or more values of the quality control sample are outside the acceptable limits.

ASSAY PROCEDURE

Bring all reagents and samples to room temperature (15–30 °C) and mix well.
Take as many microtiter strips as needed from kit. Store unused strips covered at 2–8 ° C. Strips are stable until the expiry date stated on the label. Gently mix all reagents before use. A standard curve must be performed with each assay run and with each microplate if more than one is used at a time. All standards, controls, and samples should be run in duplicate.

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 ALPCO.

1. Wash each well 5x by dispensing 250 µL of wash buffer into each well. After the final washing step remove residual buffer by tapping the plate on absorbent paper.
2. Add 100 µL of standard, samples, and controls into the respective wells.
3. Cover the plate tightly and incubate for 1 hour at room temperature (15–30 °C) on a horizontal shaker set at 550 rpm, amplitude 2 mm.
4. Discard the contents of each well. Wash each well 5x by dispensing 250 µL of wash buffer into each well. After the final washing step remove residual buffer by tapping the plate on absorbent paper.
5. Add 100 µL conjugate into each well.
6. Cover the plate tightly and incubate for 1 hour at room temperature (15–30 °C) on a horizontal shaker set at 550 rpm, amplitude 2 mm.
7. Discard the contents of each well. Wash each well 5x by dispensing 250 µL of wash buffer into each well. After the final washing step remove residual buffer by tapping the plate on absorbent paper.
8. Add 100 µL substrate into each well.
9. Incubate for 10–20 min* at room temperature (15–30 °C) in the dark.
10. Add 100 µL stop solution into each well, mix.
11. Determine absorption immediately with an ELISA reader at 450 nm 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 405 nm against 620 nm as a reference.

*The intensity of the color change is temperature sensitive. It is recommended to observe the color change and to stop the reaction upon good differentiation.

CALCULATION OF RESULTS

The following algorithms can be used to calculate the results. It is recommended to use a 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 calibrator must be specified with a value less than 1 (e.g. 0.001).
2. Point-to-point calculation: It is recommended to use a linear ordinate for the optical density and a linear abscissa for the concentration.
3. Spline algorithm: It is recommended to use a linear ordinate for the optical density and a linear abscissa for the concentration.

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, a comparison of the paired values should be done manually.

EDTA plasma and serum samples.
The obtained etanercept levels of EDTA plasma and serum samples have to be multiplied with the dilution factor of 50. In case another dilution factor has been used, multiply the obtained result with the dilution factor used.

LIMITATIONS

Samples with concentrations above the measurement range must be further diluted and re-assayed. Please consider this greater dilution when calculating the results. Samples with concentrations lower than the measurement range cannot be clearly quantified.

The upper limit of the measurement range can be calculated as:
highest concentration of the standard curve × sample dilution factor to be used

The lower limit of the measurement range can be calculated as:
LoB × sample dilution factor to be used

PERFORMANCE CHARACTERISTICS

Analytical Sensitivity

Limit of blank, LoB 1.65 ng/mL
Limit of detection, LoD 2.88 ng/mL
Limit of quantitation, LoQ 2.88 ng/mL

The evaluation was performed according to the CLSI-Guideline:EP-17-A2. The specified accuracy goal for the LoQ was 20 % CV.

Precision: Within run (intra-assay) variation
The within run precision (intra-assay variation) was calculated from 20 replicate determinations on two samples (n = 20).

Sample	Etanercept (µg/mL)	CV %
1	1.66	6.53
2	5.53	7.60

Precision: Between run (inter-assay) variation
The total precision (inter-assay variation) was calculated from data on two samples obtained in 12 different assays (n = 12).

Sample	Etanercept (µg/mL)	CV %
1	2.25	8.00
2	6.62	8.72

Spiking Recovery

The following values have been estimated based on the concentrations of the standard curve without considering possibly used sample dilution factors.
Two samples were spiked with different Etanercept concentrations and measured using this assay (n = 2).

Sample	Unspiked sample (ng/mL)	Spike (ng/mL)	Expected (ng/mL)	Measured (ng/mL)
1	0.42	6.69	7.11	7.59
	0.42	13.38	13.8	13.63
	0.42	26.35	26.77	25.49
	0.42	37.73	38.15	36.32
2	< LOB	6.69	6.69	8.38
	< LOB	13.38	13.38	14.67
	< LOB	26.35	26.35	27.62
	< LOB	37.73	37.73	34.62

Dilutional Linearity

Probe	Dilution	Expected (µg/mL)	Measured (µg/mL)
1	1:50		2.903
	1:100	1.451	1.347
	1:200	0.725	0.617
	1:400	0.362	0.301
2	1:50		4.019
	1:100	2.009	1.892
	1:200	1.005	0.964
	1:400	0.502	0.498
3	1:50		9.591
	1:100	4.796	3.934
	1:200	2.398	1.929
	1:400	1.199	0.955
	1:800	0.599	0.488
	1:1600	0.300	0.234
4	1:50		2.688
	1:100	1.344	1.369
	1:200	0.672	0.726
	1:400	0.336	0.382

Specificity
The specificity of the antibody was tested by measuring the cross-reactivity against a range of compounds with structural similarity. The specificity is calculated in percent:

Infliximab 0 %
Adalimumab 0 %
Golimumab 0 %

TECHNICAL HINTS

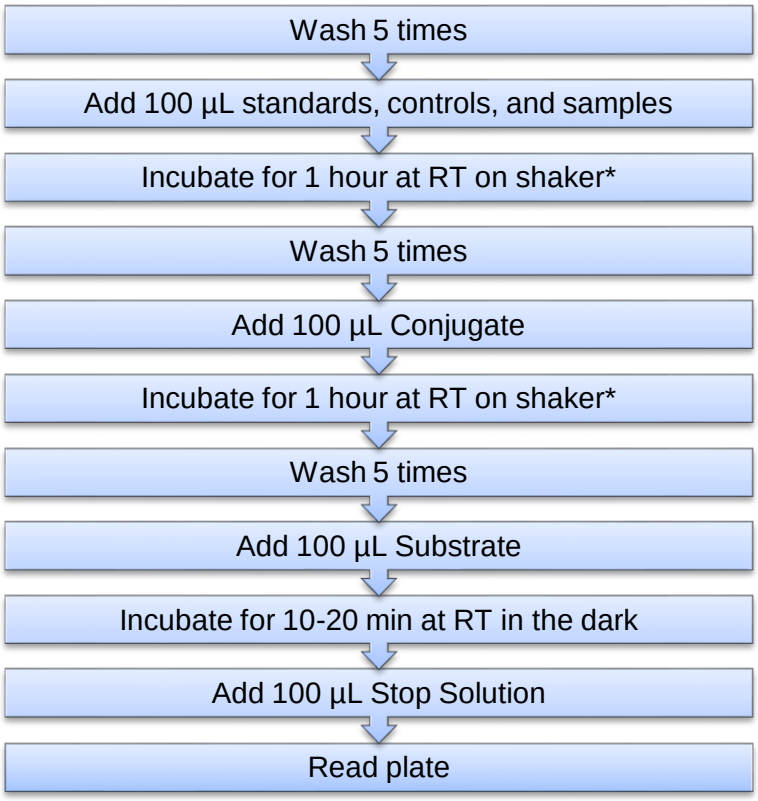
- Do not interchange different lot numbers of any kit component within the same assay. Furthermore, it is recommended not to assemble wells of different microtiter plates for analysis, even if they are of the same batch.
- Reagents should not be used beyond the expiration date stated on kit label.
- Substrate solution should remain colorless 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

- This assay was produced and distributed according to the IVD guidelines of 98/79/EC.
- 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.

SHORT ASSAY PROTOCOL



Total Incubation Time = 2 hours 20 minutes

*Plate Shaker Settings: 550 rpm, amplitude 2 mm

SUGGESTED PLATE LAYOUT

Below is a suggested plate layout for running standards, controls, and up to 40 samples in duplicate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std 1	Std 1	1	1	9	9	17	17	25	25	33	33
B	Std 2	Std 2	2	2	10	10	18	18	26	26	34	34
C	Std 3	Std 3	3	3	11	11	19	19	27	27	35	35
D	Std 4	Std 4	4	4	12	12	20	20	28	28	36	36
E	Std 5	Std 5	5	5	13	13	21	21	29	29	37	37
F	Std 6	Std 6	6	6	14	14	22	22	30	30	38	38
G	Ctrl 1	Ctrl 1	7	7	15	15	23	23	31	31	39	39
H	Ctrl 2	Ctrl 2	8	8	16	16	24	24	32	32	40	40

Std= Standard
Ctrl = Control
Numbered wells = Samples