"trace & catch"

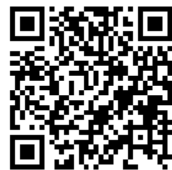
Instructions for Use

Etanercept (Enbrel®) ELISA

SHIKARI®
Q-ETA

Enzyme immunoassay for the quantitative determination of etanercept (Enbrel®) in serum and plasma

REF **TR-ETAv1** 12 x 8 2-8°C



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	SHIKARI Q-ETA
	Free etanercept (Enbrel®) monoclonal based quantitative analyses
Required Volume **(µl)**	20
Total Time (min)	160
Sample	Serum, plasma
Sample Number	96
Dedection Limit (µg/mL)	0,2
Spike Recovery (%)	97
Shelf Life (year)	1

Intended Use

Enzyme immunoassay for the quantitative determination of etanercept (Enbrel®) in serum and plasma. *Matriks Biotek etanercept ELISA* has been especially developed for the quantitative analysis of etanercept in serum and plasma samples between the Cmin and Cmax range of concentrations indicated in the pharmacokinetics section of prospectus.

The reliability of the data regarding the pharmacokinetics of etanercept (Enbrel®) is expected to be highly dependent on the specificity of the assay used and non specific assays might be misleading. The data reported in the literature were obtained with ELISA in which the capture and tracer antibodies were anti-human p75 TNF receptor monoclonal antibodies. However, it is well known that human sera contain soluble p75 TNF receptor in the range of nanogram levels. Therefore, it should be kept in mind that, at least some of the capture antibodies used in such an ELISA format will bind native p75 TNF receptors instead of etanercept (Enbrel®) and would be blocked and may lead to a possible underestimation. Because the level of native p75 TNF receptor is different between the sera of different individuals, using human sera for the preparation of standards would be not a real remedy for such an ELISA format. Matriks Biotek Q-ETA is especially developed for specific measurement of etanercept (Enbrel®) in sera and plasma by the advantage of using monoclonal antibodies specific for etanercept (Enbrel®) only. The user-friendly Matriks Biotek Q-ETA is the first and only ELISA kit in the market for the quantitative determination of etanercept (Enbrel®) ***at uppermost specificity***.

Summary and Explanation

According to the prospectus; etanercept (Enbrel®) is a dimeric fusion protein consisting of the extracellular ligand-binding portion of the human 75 kilodalton (p75) tumor necrosis factor receptor (TNFR) linked to the Fc portion of human IgG1. The Fc component of etanercept contains the CH2 domain, the CH3 domain and hinge region, but not the CH1 domain of IgG1. Etanercept consists of 934 amino acids and has an apparent molecular weight of approximately 150 kilodaltons. Etanercept binds specifically to tumor necrosis factor (TNF) and blocks its interaction with cell surface TNF receptors. Elevated levels of TNF are found in involved tissues and fluids of patients with rheumatoid arthritis (RA), psoriatic arthritis, ankylosing spondylitis (AS), and plaque psoriasis. Two distinct receptors for TNF (TNFRs), a 55 kilodalton protein (p55) and a 75 kilodalton protein (p75), exist naturally as monomeric molecules on cell

surfaces and in soluble forms. Biological activity of TNF is dependent upon binding to either cell surface TNFR. Etanercept is a dimeric soluble form of the p75 TNF receptor that can bind to two TNF molecules. Etanercept inhibits binding of both TNFα and TNFβ (lymphotoxin alpha [LTα]) to cell surface TNFRs, rendering TNF biologically inactive.

After administration of 25 mg of ENBREL® by a single subcutaneous (SC) injection to 25 patients with RA, a mean ± standard deviation half-life of 102 ± 30 hours was observed with a clearance of 160 ± 80 mL/hr. A maximum serum concentration (Cmax) of 1.1 ± 0.6 mcg/mL and time to Cmax of 69 ± 34 hours was observed in these patients following a single 25 mg dose. After 6 months of twice weekly 25 mg doses in these same RA patients, the mean Cmax was 2.4 ± 1.0 mcg/mL (N =23). Patients exhibited a two- to seven-fold increase in peak serum concentrations and approximately four-fold increase in AUC0-72 hr (range 1 to 17 fold) with repeated dosing. The pharmacokinetic parameters in patients with plaque psoriasis were similar to those seen in patients with RA. In another study, serum concentration profiles at steady state were comparable among patients with RA treated with 50 mg ENBREL® once weekly and those treated with 25 mg ENBREL® twice weekly. The mean (± standard deviation) Cmax, Cmin, and partial AUC were 2.4 ± 1.5 mcg/mL, 1.2 ± 0.7 mcg/mL, and 297 ± 166 mcg•h/mL, respectively, for patients treated with 50 mg ENBREL® once weekly (N = 21); and 2.6 ± 1.2 mcg/mL, 1.4 ± 0.7 mcg/mL, and 316 ± 135 mcg•h/mL for patients treated with 25 mg ENBREL® twice weekly (N = 16). No formal pharmacokinetic studies have been conducted to examine the effects of renal or hepatic impairment on ENBREL® disposition. Patients with juvenile idiopathic arthritis (JIA) (ages 4 to 17 years) were administered 0.4 mg/kg of ENBREL® twice weekly for up to 18 weeks. The mean serum concentration after repeated SC dosing was 2.1 mcg/mL, with a range of 0.7 to 4.3 mcg/mL. Limited data suggests that the clearance of ENBREL® is reduced slightly in children ages 4 to 8 years. Population pharmacokinetic analyses predict that administration of 0.8 mg/kg of ENBREL® once weekly will result in Cmax 11% higher, and Cmin 20% lower at steady state as compared to administration of 0.4 mg/kg of ENBREL® twice weekly. The predicted pharmacokinetic differences between the regimens in JIA patients are of the same magnitude as the differences observed between twice weekly and weekly regimens in adult RA patients.

Patients treated with ENBREL® are at increased risk for developing serious infections that may lead to hospitalization or death. Patients should be closely monitored for the development of signs and symptoms of infection during and after treatment with ENBREL®, including the possible development of tuberculosis in patients who tested negative for latent tuberculosis infection prior to initiating therapy.

Patients with RA, psoriatic arthritis, ankylosing spondylitis, or plaque psoriasis were tested at multiple timepoints for antibodies to ENBREL®. Antibodies to the TNF receptor portion or other protein components of the ENBREL® drug product were detected at least once in sera of approximately 6% of adult patients with RA, psoriatic arthritis, ankylosing spondylitis, or plaque psoriasis. These antibodies were all reported to be non-neutralizing. Results from JIA patients were similar to those seen in adult RA patients treated with ENBREL®. In PsO studies that evaluated the exposure of etanercept for up to 120 weeks, the percentage of patients testing positive at the assessed time points of 24, 48, 72, and 96 weeks ranged from 3.6%-8.7% and were all reported to be non-neutralizing. The percentage of patients testing positive increased with an increase in the duration of study, however, the clinical significance of this finding is unknown. However, the immunogenicity data of ENBREL® beyond 120 weeks of exposure is unknown.

Patients with RA had serum samples tested for autoantibodies at multiple timepoints. In RA Studies I and II, the percentage of patients evaluated for antinuclear antibodies (ANA) who developed new positive ANA (titer ≥ 1:40) was higher in patients treated with ENBREL® (11%) than in placebo-treated patients (5%). The percentage of patients who developed new positive anti-double-stranded DNA antibodies was also higher by radioimmunoassay (15% of patients treated with ENBREL® compared to 4% of placebo-treated patients) and by *Crithidia luciliae* assay (3% of patients treated with ENBREL® compared to none of placebo-treated patients). The impact of long-term treatment with ENBREL® on the development of autoimmune diseases is unknown. In addition, it was reported that patients receiving etanercept may develop antibodies that interfere with monoclonal antibody laboratory assays.

Serum concentration of ENBREL® might be related to predict some clinical outcome during maintenance therapy. It was also possible that the surveillance of circulating ENBREL® concentration during maintenance therapy represents a direct and/or indirect factor for immunogenicity and some other side effects.

In this context, identification of biomarkers for (non-)response and risk factors for adverse drug reactions that might be related to serum concentrations and maintaining the effective minimum concentration of ENBREL® in order to potentially avoid some side effects with a reliable method might be beneficial.

Test Principle

Solid phase enzyme-linked immunosorbent assay (ELISA) based on the sandwich principle. Standards and samples (serum or plasma) are incubated in the microtitre plate coated with the monoclonal antibody specific for etanercept (Enbrel®). After incubation, the wells are washed. A biotin labeled tracer monoclonal antibody against etanercept is added and binds to etanercept captured by the first monoclonal antibody on the surface of the wells. Following incubation wells are washed and then streptavidine–horse radish peroxidase (HRP) is added and binds to biotin conjugated probe. After incubation, the wells are washed and the bound enzymatic activity is detected by addition of chromogen-substrate. The color developed is proportional to the amount of etanercept (Enbrel®) in the sample or standard. Results of samples can be determined directly using the standard curve.

Warnings and Precautions

1. For professional use only.

2. 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. For further information please refer to the local distributor.

3. In case of severe damage of the kit package please contact Matriks biotek or your supplier in written form, latest one week after receiving the kit. Do not use damaged components in test runs, but keep safe for complaint related issues.

4. Obey lot number and expiry date. Do not mix reagents of different lots. Do not use expired reagents.

5. Follow good laboratory practice and safety guidelines. Wear lab coats, disposable latex gloves and protective glasses where necessary.

6. Reagents of this kit containing hazardous material may cause eye and skin irritations. See MATERIALS SUPPLIED and labels for details.

7. Chemicals and prepared or used reagents have to be treated as hazardous waste according the national biohazard safety guidelines or regulations.

8. Avoid contact with Stop solution. It may cause skin irritations and burns.

9. All reagents of this kit containing human serum (i.e. standards) have been tested and were found negative for HIV I/II, HBsAg and HCV. However, a presence of these or other infectious agents cannot be excluded absolutely and therefore reagents should be treated as potential biohazards in use and for disposal.

10. Some reagents contain sodium azide (NaN_3) as preservatives. In case of contact with eyes or skin, flush immediately with water. NaN_3 may react with lead and copper plumbing to form explosive metal azides. When disposing reagents, flush with large volume of water to avoid azide build-up.

Storage and Stability

The kit is shipped at ambient temperature and should be stored at 2-8°C. Keep away from heat or direct sun light. The strips of microtiter plate is stable up to the expiry date of the kit in the broken, but tightly closed bag when stored at 2–8°C.

Specimen Collection and Storage

Serum, Plasma (EDTA, Heparin)

The usual precautions for venipuncture should be observed. It is important to preserve the chemical integrity of a blood specimen from the moment it is collected until it is assayed. Do not use grossly hemolytic, icteric or grossly lipemic specimens. Samples appearing turbid should be centrifuged before testing to remove any particulate material.

Storage:	2-8°C	-20°C	Keep away from heat or direct sun light Avoid repeated freeze-thaw cycles
Stability:	2 d	6 mon	

Materials Supplied

1 x 12 x 8	MTP	**Microtiter Plate** Break apart strips. Microtiter plate with 12 rows each of 8 wells coated with monoclonal antibody specific for etanercept (Enbrel®).
5 x 0.3 mL 1 x 3 mL	STND A-F	**Etanercept Standards A-F** 4; 2; 1; 0.5; 0.25; and 0 microgram/mL Ready to use. Used for construction of the standard curve. Contains etanercept (Enbrel®), human serum, stabilizer and <0.1% NaN_3.
1 x 12 mL	ASSAY BUF	**Assay Buffer** Blue colored. Ready to use. Contains mouse serum, proteins and <0.1% NaN_3.
1 x 12 mL	BIOTIN CONJ	**Biotin-Labeled Tracer** Green colored. Ready to use. Contains biotin labeled tracer monoclonal antibody against etanercept (Enbrel®), stabilizer and <0.1% NaN_3.
1 x 12 mL	STREP-HRP	**Streptavidine-Peroxidase** Red colored. Ready to use. Contains streptavidine conjugated to horse radish peroxidase (HRP) and stabilizers.
1 x 12 mL	TMB SUBS	**TMB Substrate Solution** Ready to use. Contains TMB
1 x 12 mL	TMB STOP	**TMB Stop Solution** Ready to use. 1N HCl.
1 x 50 mL	WASHBUF CONC	**Wash Buffer**, Concentrate (20x) Contains Buffer with Tween 20.
2 x 1	ADH FILM	**Adhesive Film** For covering of Microtiter Plate during incubation.

Materials Required but not Supplied

1. Micropipettes (Multipette Eppendorf or similar devices, < 3% CV).
2. Calibrated measures.
3. Tubes for sample dilution.

4. Wash bottle, automated or semi-automated microtiter plate washing system
5. Microtiter plate reader capable of reading absorbance at 450 nm.
6. Bidistilled or deionised water, paper towels, pipette tips and timer.

Procedure Notes

1. Any improper handling of samples or modification of the test procedure may influence the results. The indicated pipetting volumes, incubation times, temperatures and pretreatment steps have to be performed strictly according to the instructions. Use calibrated pipettes and devices only.
2. Once the test has been started, all steps should be completed without interruption. Make sure that required reagents, materials and devices are prepared ready at the appropriate time. Allow all reagents and specimens to reach room temperature (18-25 °C) and gently swirl each vial of liquid reagent and sample before use. Mix reagents without foaming.
3. Avoid contamination of reagents, pipettes and wells/tubes. Use new disposable plastic pipette tips for each reagent, standard or specimen. Do not interchange caps. Always cap not used vials. Do not reuse wells/tubes or reagents.
4. Use a pipetting scheme to verify an appropriate plate layout.
5. Incubation time affects results. All wells should be handled in the same order and time sequences. It is recommended to use an 8-channel micropipettor for pipetting of solutions in all wells.
6. Microplate washing is important. Improperly washed wells will give erroneous results. It is recommended to use a multichannel pipette or an automatic microplate washing system. Do not allow the wells to dry between incubations. Do not scratch coated wells during rinsing and aspiration. Rinse and fill all reagents with care. While rinsing, check that all wells are filled precisely with Wash Buffer, and that there are no residues in the wells.
7. Humidity affects the coated wells/tubes. Do not open the pouch until it reaches room temperature. Unused wells/tubes should be returned immediately to the resealed pouch including the desiccant.

Pre-Test Setup Instructions

1. Preparation of Components

Dilute/ disolve	Component	with	Diluent	Relation	Remarks	Storage	Stability
10 mL	Wash Buffer*	Up to 200 mL	bidist. Water	1:20	Warm up at 37°C to dissolve crystals. Mix vigorously.	2-8 °C	4 w

***.** Prepare Wash Buffer before starting assay procedure.

2. Dilution of Samples*

Sample	To be diluted	With	Relation	Remarks
Serum/ Plasma	Initially no	Zero Standard	1:2	For dilution at 1:2; 20µl Sample + 20µl Zero Standard

*. Patient samples with a concentration of etanercept (Enbrel®) above the measuring range are to be rated as "> highest standard". The result must not be extrapolated. The patient sample in question ***should be diluted with Zero Standard*** and retested.

Test Procedure

1	Pipette 100µl of Assay Buffer non-exceptionally into each of the wells to be used.
2	Pipette 20 µL of each ready-to use Standards or Serum Samples into the respective wells of microtiter plate. Wells A1: Standard A B1: Standard B C1: Standard C D1: Standard D E1: Standard E F1: Standard F G1 and on: Sample (Serum/Plasma)
3	Cover the plate with adhesive foil. Incubate 60 min at room temperature (18-25°C).
4	Remove adhesive foil. Discard incubation solution. Wash plate 3 times each with 300 µL of diluted Wash Buffer. Remove excess solution by tapping the inverted plate on a paper towel.
5	Pipette 100 µL of ready-to use Biotin-Labeled Tracer into each well.
6	Cover the plate with adhesive foil. Incubate 60 min at room temperature (18-25°C).
7	Remove adhesive foil. Discard incubation solution. Wash plate 3 times each with 300 µL of diluted Wash Buffer. Remove excess solution by tapping the inverted plate on a paper towel.
8	Pipette 100 µL of ready-to use Streptavidine-Peroxidase into each well.
9	Cover the plate with adhesive foil. Incubate 30 min at room temperature (18-25°C).
10	Remove adhesive foil. Discard incubation solution. Wash plate 3 times each with 300 µL of diluted Wash Buffer. Remove excess solution by tapping the inverted plate on a paper towel.
11	Pipette 100 µL of TMB Substrate Solution into each well.
12	Incubate 10 min (without adhesive foil.) at room temperature (18-25°C) in the dark.
13	Stop the substrate reaction by adding 100 µL of Stop Solution into each well. Briefly mix contents by gently shaking the plate. Color changes from blue to yellow.
14	Measure optical density with a photometer at 450 nm (reference at 620 nm is optional) within 30 min after pipetting of the Stop Solution.

Quality Control

The test results are only valid only 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 standards/laws. All standards must be found within the acceptable ranges as stated above and/or label. If the criteria are not met, the run is not valid and should be repeated. In case of any deviation the following technical issues should be proven: Expiration dates of (prepared) reagents, storage conditions, pipettes, devices, incubation conditions and washing methods.

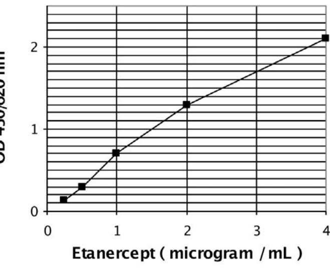
Calculation & Interpretation of Results

1. Construct a standard curve by plotting the absorbance obtained from each standard (***disregarding the zero standard***) against its concentration with absorbance value on the vertical(Y) axis and concentration on the horizontal (X) axis.
2. The concentration of the samples can be read directly from this standard curve. Using the absorbance value for each sample, determine the corresponding concentration of etanercept from the standard curve. Find the absorbance value on the Y-axis and extend a horizontal line to the curve. At the point of intersection, extend a vertical line to the X-axis and read the etanercept concentration for the unknown sample.
3. Any sample undiluted or diluted still reading greater than the highest standard should be further diluted appropriately with ***Zero Standard*** and retested. **If the samples have been diluted, the concentration determined from the standard-curve must be multiplied by the dilution factor.**

Typical Calibration Curve

(Example. Do not use for calculation!)

Standard	Concentration (µg/mL)	Mean OD450/620 nm
A	4	2,100
B	2	1,300
C	1	0,700
D	0,5	0,300
E	0,25	0,130
F	0	0,040



Assay Characteristics

1. **Specificity:** There is no cross reaction with any other proteins **including TNF receptors** present in native human serum. In addition, **no cross reaction** was observed with the other anti-TNF therapeutic immunoglobulins (i.e. infliximab (Remicade®) and adalimumab (Humira®)) tested at the concentrations up to 500 µg/mL.

2. **Sensitivity:** The lowest detectable level that can be distinguished from the zero standard is 0,2 µg/mL.

3. **Precision Of Kit:**
   Intra-assay CV: <8% for etanercept range 0.5-4 µg/mL.
   Inter-assay CV: <8% for etanercept range 0.5-4 µg/mL.

4. **Recovery:** Recovery rate was found to be equal and higher than 90% with normal human serum samples supplemented with known concentrations of etanercept.

Automation

Experiments have shown that the etanercept ELISA is also suitable to run on an automated ELISA processor.

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