KRIBIOLISA® Etarnacept (ENBREL™) ELISA

REF : KBI1013

Ver 2.2

RUO

Enzyme Immunoassay for the quantitative determination of Etarnacept in serum, plasma and cell culture supernatant

RUO	For Research Use only	REF	Catalog Number
	Store At	LOT	Batch Code
	Manufactured By		Biologi isk
	Expiry D		Consu rating Instructions

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REF KBI1013 Σ 96 tests

Krishgen Biosystems Private Limited
For US/Europe Customers: toll free +1(888)-970-0827 | tel +1(562)-568-5005
For Asia/India Customers: tel +91(22)-49198700
Email: sales1@krishgen.com | http://www.krishgen.biz / www.krishgenbio.com

Introduction:

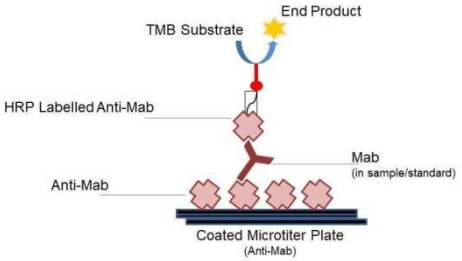
Etarnacept is a biopharmaceutical that treats autoimmune diseases by interfering with tumor necrosis factor (TNF, a soluble inflammatory cytokine) by acting as a TNF inhibitor. It has U.S. F.D.A. approval to treat rheumatoid arthritis, juvenile rheumatoid arthritis and psoriatic arthritis, plaque psoriasis and ankylosing spondylitis. TNF-alpha is the "master regulator" of the inflammatory (immune) response in many organ systems. Autoimmune diseases are caused by an overactive immune response. Etarnacept has the potential to treat these diseases by inhibiting TNF-alpha.

Intended Use:

The KRIBIOLISA® Etarnacept (ENBREL™) ELISA is used as an analytical tool for quantitative determination of Etarnacept in serum, plasma and cell culture supernatant.

Principle:

The method employs the quantitative sandwich enzyme immunoassay technique. Antibodies to Etarnacept are pre-coated onto microwells. Samples and standards are pipetted into microwells and human Etarnacept present in the sample are bound by the capture antibody. Then, a HRP (horseradish peroxidase) conjugated anti-Etarnacept antibody is pipetted and incubated. After washing microwells in order to remove any non-specific binding, the ready to use substrate solution 3, 3', 5, 5' Tetra Methyl Benzidine (TMB) is added to microwells and color develops proportionally to the amount of Etarnacept in the sample. Color development is then stopped by addition of stop solution. Absorbance is measured at 450 nm.



Materials Provided:

Part	Description	Qty
Anti-Etarnacept Coated Microtiter Plate	96 well polystyrene microplate (12 strips of 8 wells) coated with Anti-Etarnacept monoclonal antibody	1 x 96 wells
Etarnacept Standard	Etarnacept Injection in a buffered protein with protein stabilizer and preservatives 0.02% methylisothiazolone and 0.02% bromonitrodioxane.– lyophilized (1 ug/ml)	2 vials
Anti-Etarnacept:HRP Conjugate concentrated	Anti-Etarnacept conjugated to Horseradish Peroxidase concentrated (0.5 mg/ml)	2 vials
(1X) Standard Diluent	Buffered protein base with protein stabilizer and preservatives 0.02% methylisothiazolone and 0.02% bromonitrodioxane with 1:100 dilution human serum protein	10 ml
Detection Diluent	Buffered protein base with protein stabilizer and preservatives 0.02% methylisothiazolinone and 0.02% bromonitrodioxane	12 ml
(1X) Sample Diluent	Buffered protein base with protein stabilizer and preservatives 0.02% methylisothiazolone and 0.02% bromonitrodioxane	50 ml
(20X) Wash Buffer	20-fold concentrated solution of buffered surfactant with preservative thiomersal < 0.01%. May turn yellow over time	25 ml
TMB Substrate	Stabilized Chromogen	12 ml
Stop Solution	0.73M Phosphoric Acid	12 ml
Instruction Manual		1 no

Materials to be provided by the End-User:

1. Microtiter Plate Reader able to measure absorbance at 450 nm.
2. Adjustable pipettes and multichannel pipettor to measure volumes ranging from 25 ul to 1000 ul.
3. Deionized (DI) water.

4. Wash bottle or automated microplate washer.
5. Graph paper or software for data analysis.
6. Timer.
7. Absorbent Paper.

Handling/Storage:

1. It is advisable to aliquot and store the Anti-Etarnacept:HRP Conjugate concentrated at -20°C upon receipt. Rest of the kit components should be stored at 2-8°C. Immediately discard any excess working Anti-Etarnacept:HRP Conjugate after running your assay.
2. All the reagents and wash solutions should be used within 12 months from manufacturing date.
3. Before using, bring all components to room temperature (18-25ºC). Upon assay completion ensure all components of the kit are returned to appropriate storage conditions.
4. The Substrate is light-sensitive and should be protected from direct sunlight or UV sources.

Health Hazard Warnings:

1. Reagents that contain preservatives may be harmful if ingested, inhaled or absorbed through the skin.
2. For Research Use Only.



Sample Preparation and Storage:

Blood is taken by venipuncture. Serum is separated after clotting by centrifugation. Plasma can be used, too. Lipaemic, hemolytic or contaminated samples should not be run. Repeated freezing and thawing should be avoided. If samples are to be used for several assays, initially aliquot samples and keep at - 20°C.

For Cell Culture Supernatant **–** If necessary, centrifuge to remove debris prior to analysis. Samples can be stored at -20°C or -80ºC. Avoid repeated freeze-thaw cycles.

Preparation before Use:
Allow samples to reach room temperature prior to assay. Take care to agitate patient samples gently in order to ensure homogeneity.

Test Sample preparation - Samples have to be diluted 1:100 (v/v), e.g. for 1:100 (1 ul sample + 99 ul sample diluent) prior to assay. The samples may be kept at 2 - 8°C for up to three days. Long-term storage requires -20°C.

Reagent Preparation (all reagents should be diluted immediately prior to use):

1. Label any aliquots made with the kit Lot No and Expiration date and store it at appropriate conditions mentioned.
2. Bring all reagents to Room temperature before use.
3. To make Wash Buffer (1X); dilute 25 ml of 20X Wash Buffer in 475 ml of DI water.
4. **Standards Preparation**: Reconstitute the concentrated Standard lyophilized vial with 1 ml of Standard Diluent (1X) to obtain a concentration of 1000 ng/ml. Keep the vial for 15 mins with gentle agitation before making further dilutions. Dilute 80 ul of original **Standard (1000 ng/ml)** with 420 ul of Standard Diluent (1X) to generate a **160 ng/ml Standard Solution**. Prepare further **Standards** by serially diluting the Standard Solution as per the below table. Use the Standard Diluent (1X) as the Zero Standard (Standard No.0).

Standard Concentration	Standard Vial	Dilution Particulars
1000 ng/ml	Original Standard	Original Standard provided in the Kit + 1ml Standard Diluent (1X)
160 ng/ml	Standard No.5	80 ul Original Standard (1000 ng/ml) + 420 ul Standard Diluent (1X)
80 ng/ml	Standard No.4	250 ul Standard No.5 + 250 ul Standard Diluent (1X)
40 ng/ml	Standard No.3	250 ul Standard No.4 + 250 ul Standard Diluent (1X)
20 ng/ml	Standard No.2	250 ul Standard No.3 + 250 ul Standard Diluent (1X)
10 ng/ml	Standard No.1	250 ul Standard No.2 + 250 ul Standard Diluent (1X)

0 ng/ml	Standard No. 0	Only Standard Diluent (1X)

Use the Standards immediately upon reconstitution. Discard balance standard after use. Do not store them for further experiments.

5. **Working Anti-Etarnacept:HRP Conjugate – Refer to the Reagent Preparation sheet attached with the IFU and COA (enclosed in the kit).**

Procedural Notes:

1. In order to achieve good assay reproducibility and sensitivity, proper washing of the plates to remove excess un-reacted reagents is essential.
2. High Dose Hook Effect may be observed in samples with very high concentrations of Etarnacept. High Dose Hook Effect is due to excess of antibody for very high concentrations of Etarnacept present in the sample. High Dose Hook effect is most likely encountered from samples early in the purification process. If Hook Effect is possible, the samples to be assayed should be diluted with a compatible diluent. Thus if the Etarnacept concentration of the undiluted sample is less than the diluted sample, this may be indicative of the Hook Effect.
3. Avoid assay of Samples containing sodium azide (NaN_3), as it could destroy the HRP activity resulting in under-estimation of the amount of Etarnacept.
4. It is recommended that all Standards and Samples be assayed in duplicates.
5. Maintain a repetitive timing sequence from well to well for all the steps to ensure that the incubation timings are same for each well.
6. If the Substrate has a distinct blue color prior to use it may have been contaminated and use of such substrate can lead to compromisation of the sensitivity of the assay.
7. The plates should be read within 30 minutes after adding the Stop Solution.
8. Make a work list in order to identify the location of Standards and Samples.

Assay Procedure:

1. It is strongly recommended that all Controls and Samples be run in duplicates or triplicates. A standard curve is required for each assay. All steps must be performed at 37°C.

2. Pipette out **100 ul** of **Standards** or **Samples** in each well and incubate at 37°C for 60 minutes.

3. Aspirate and wash plate 4 times with **Wash Buffer (1X)** and blot residual buffer by firmly tapping plate upside down on absorbent paper. Wipe of any liquid from the bottom outside of the microtiter wells.

4. Add **100 ul** of **Working Anti-Etarnacept:HRP detection conjugate** into the respective wells and incubate at 37°C for 60 minutes.

5. Aspirate and wash plate 4 times with **Wash Buffer (1X)** same as mentioned in Step 3.

6. Add **100 ul** of **TMB Substrate** in each well.

7. Incubate the plate at 37°C for 30 minutes in dark. DO NOT SHAKE or else it may result in higher backgrounds and worse precision. Positive wells should turn bluish in color.

8. Pipette out **100 ul** of **Stop Solution**. Wells should turn from blue to yellow in color.

9. Read the absorbance at 450 nm with a microplate within 10-15 minutes after addition of Stop solution.

Calculation of Results:

Determine the Mean Absorbance for each set of duplicate or triplicate Standards and Samples. Using Semi-Log graph paper, plot the average value (absorbance 450nm) of each standard on the Y-axis versus the corresponding concentration of the standards on the X-axis. Draw the best fit curve through the standard points. To determine the unknown Etarnacept concentrations, find the unknown's Mean Absorbance value on the Y-axis and draw a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the

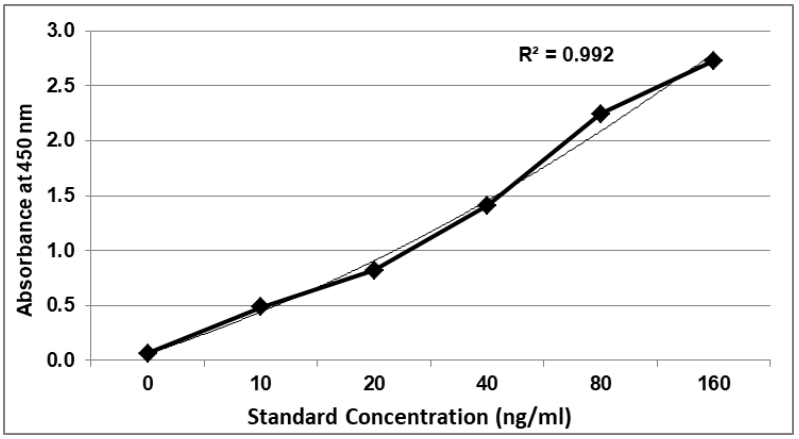
X-axis and read the Etarnacept Concentration. If samples were diluted, multiply by the appropriate dilution factor. Software which is able to generate a cubic spline curve-fit is best recommended for automated results.

Note:
It is recommended to repeat the assay at a different dilution factor in the following cases:
- If the sample absorbance value is below the first standard.
- If the absorbance value is equivalent or higher than the 160 ng/ml standard.

Typical Data (representative only)

Standard Concentration (ng/ml)	Abs A	Abs B	Mean Abs	Interpolated Concentration	% Interpolated Concentration against Actual Concentration
0	0.063	0.061	0.062	--	--
10	0.480	0.493	0.486	11.2	111.7
20	0.826	0.816	0.821	19.7	98.3
40	1.424	1.392	1.408	38.0	94.9
80	2.221	2.266	2.243	85.2	106.6
160	2.690	2.772	2.731	154.4	96.5

Typical Graph (representative only)



Abs = absorbance @450 nm

Quality Control:

It is recommended that for each laboratory assay appropriate quality control samples in each run to be used to ensure that all reagents and procedures are correct.

Performance Characteristics of the Kit:

This kit has been validated as per EMA/FDA guidelines in line with ICH Code for Harmonization of Biological Assays.

Sensitivity:
Limit Of Detection: It is defined as the lowest detectable concentration corresponding to a signal of Mean of '0' standard plus 2* SD.
10 replicates of '0' standards were evaluated and the LOD was found to be less than 4 ng/ml

Linearity:
Standards provided in the kit will be used for measuring the linearity range of Etarnacept present in matrix.

Precision:
Precision is defined as the percent coefficient of variation (%CV) i.e. standard deviation divided by the mean and multiplied by 100. Assay precision was determined by both intra (n=5 assays) and inter assay (n=5 assays)

reproducibility on two pools with low (10ng/ml), medium (40ng/ml) and high (160ng/ml) concentrations. While actual precision may vary from laboratory to laboratory and technician to technician, it is recommended that all operators achieve precision below these design goals before reporting results.

Pool	Intra Assay %CV	Inter Assay %CV
Low	<10%	<10%
Medium	<5%	<5%
High	<5%	<5%

Safety Precautions:

- **For Research Use Only**. Follow the working instructions carefully.
- The expiration dates stated on the kit are to be observed. The same relates to the stability stated for reagents.
- Do not use or mix reagents from different lots.
- Do not use reagents from other manufacturers.
- Avoid time shift during pipetting of reagents.
- All reagents should be kept in the original shipping container.
- Some of the reagents contain small amount of sodium azide (< 0.1 % w/w) as preservative. They must not be swallowed or allowed to come into contact with skin or mucosa.
- Source materials maybe derived from **human body fluids** or organs used in the preparation of this kit were tested and found negative for HBsAg and HIV as well as for HCV antibodies. However, no known test guarantees the absence of such viral agents. Therefore, handle all components and all patient samples as if potentially hazardous.
- Since the kit contains potentially hazardous materials, the following precautions should be observed
  - Do not smoke, eat or drink while handling kit material
  - Always use protective gloves
  - Never pipette material by mouth
  - Wipe up spills promptly, washing the affected surface thoroughly with a decontaminant.
- In any case GLP should be applied with all general and individual regulations to the use of this kit.

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SCHEMATIC ASSAY PROCEDURE

1. Remove all components, 30 minutes before adding into the assay plate.

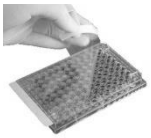
Components (2-8ºC) → 30 mins RT Before Use → 

Thaw at Room Temperature (18-24ºC) → USE NOW

2. Avoid repeated cool-thaw of the components as there will be a loss of activity and this can affect the results.

Kit Components → AVOID → COOL (2-8ºC) → THAW (22±4ºC) → THAW (22±4ºC) → COOL (2-8ºC) → AVOID → NO CHANGE IN RESULTS

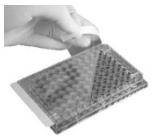
3.  Pipette **100 ul Standards** / **Samples** into the respective wells.

4. Cover plate  and incubate for 

at 37°C.

5.  Aspirate and wash wells 4 times with **Wash Buffer (1X).**

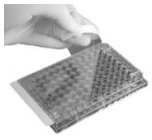
6.  Pipette **100 ul working Anti Etarnacept:HRP** into each well.

7. Cover plate  and incubate for 

at 37°C.

8.  Aspirate and wash wells 4 times with **Wash Buffer (1X).**

9.  Pipette **100 ul TMB Substrate** into each well.

10. Cover plate  and incubate for 

at 37°C.

11.  Pipette **100 ul Stop Solution** into each well.

12. Read absorbance at 450 nm with a  microplate reader within 

of stopping reaction.

Typical Example of a Work List

Well #	Contents	Absorbance at 450nm	Mean Absorbance	ng/ml Etarnacept equivalent
1A 2A	zero std zero std			
1B 2B	10 ng/ml 10 ng/ml			
1C 2C	20 ng/ml 20 ng/ml			
1D 2D	40 ng/ml 40 ng/ml			
1E 2E	80 ng/ml 80 ng/ml			
1F 2F	160 ng/ml 160 ng/ml			
1G 2G	Sample			
1H 2H	Sample			
3A 4A	Sample			
3B 4B	Sample			

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SYMBOLS KEY

	MTP
	STD
	HRP CONJ
	DETN DIL
	1X SAMP DIL
	1X STD DIL
	20X WASH BUF
	SUB TMB
	SOLN STOP
	Consult Instructions for Use
	REF
	Expiration Date
	Storage Temperature