KRIBIOLISA® Certolizumab pegol (CIMZIA™) Inhibition ELISA

REF : KBI1060

Ver 1.0

RUO

Enzyme Immunoassay for the Quantitative Determination of Certolizumab pegol in human serum and plasma

RUO	**For Research Use**	**REF**	**Catalog Number**
	Store At	**LOT**	**Batch Code**
	Manufactured By		**Biological Risk**
	Expiry Date		**Consult Operating Instructions**

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REF KBI1060 **Σ 96 tests**

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Introduction:

Certolizumab pegol is a humanised, PEGylated antigen-binding fragment (Fab′) monoclonal antibody that selectively neutralises tumour necrosis factor-alpha (TNF-α), a key pro-inflammatory cytokine involved in the pathogenesis of several autoimmune and inflammatory disorders. Unlike full-length monoclonal antibodies, certolizumab pegol lacks an Fc region and is conjugated to polyethylene glycol (PEG), a modification that prolongs its plasma half-life and reduces renal clearance while minimising Fc-mediated immune effector functions such as antibody-dependent cellular cytotoxicity (ADCC) and complement activation. By binding with high affinity to both soluble and membrane-bound TNF-α, certolizumab pegol inhibits TNF-α–mediated signalling pathways, thereby reducing inflammation, immune cell activation, and tissue damage. Certolizumab pegol has been extensively evaluated in clinical studies and is approved in multiple major markets, including the United States and the European Union, for the treatment of conditions such as rheumatoid arthritis, Crohn's disease, ankylosing spondylitis, psoriatic arthritis, and axial spondyloarthritis.

Intended Use:

The KRIBIOLISA® Certolizumab pegol (CIMZIA™) Inhibition ELISA is intended for use as an analytical tool for the quantitative determination of Certolizumab pegol in human serum and plasma.

Principle:

The method employs an indirect enzyme-linked immunosorbent assay (ELISA) technique. Recombinant Human TNF-alpha antigen is pre-coated onto microwells. Samples or standards are added to the microwells, and Certolizumab pegol present in the samples binds specifically to the immobilized antigen. After incubation, unbound components are removed by washing, and an HRP (horseradish peroxidase)-conjugated with Anti-Human Kappa antibody is added, which binds to the captured Certolizumab pegol. Following a second washing step to remove any unbound conjugate, the ready-to-use substrate 3,3',5,5'-Tetramethylbenzidine (TMB) is added, producing a color reaction proportional to the amount of Certolizumab pegol in the sample. The reaction is stopped with a stop solution, and absorbance is measured at 450 nm to determine the concentration of Certolizumab pegol.

Materials Provided:

Part	Description	Qty
Recombinant Human TNF-alpha protein (His Tag) Coated Microtiter Plate	96 well polystyrene microplate (12 strips of 8 wells) coated with Recombinant Human TNF-alpha protein (His Tag)	1 x 96 wells
Certolizumab pegol Standard	Certolizumab pegol Standard in a buffered protein base with protein stabilizer and preservatives 0.02% Methylisothiazolinone and 0.02% bromonitrodioxane (lyophilized, 1 ug/ml)	2 vials
Anti-Human Kappa antibody:HRP Conjugate concentrated	Anti-human Kappa antibody:HRP conjugated to Horseradish Peroxidase, concentrated.	1 vial
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	2 x 50 ml
(1X) Standard Diluent	Buffered protein base with 1:100 dilution human serum and preservatives 0.02% Methylisothiazolinone and 0.02% bromonitrodioxane.	10 ml
(20X) Wash Buffer	20-fold concentrated solution of buffered surfactant with preservative thiomersol < 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 stores the Anti-human Kappa antibody: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-human Kappa antibody: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. 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.

Preparation Before Use:

Allow assay. The samples may be kept at 2 - 8°C for up to three days. Long-term storage requires the samples to be kept at -20°C.samples to reach room temperature prior to assay. Take care to agitate patient samples gently in order to ensure homogeneity.

Test Sample preparation - Serum samples have to be diluted 1:1000 (v/v), e.g. for 1:1000 (1 ul sample + 999 ul sample diluent) prior to

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 to obtain a concentration of 1000 ng/ml. Keep the vial for 15 mins with gentle agitation before making further dilutions. Prepare **Standards** as per the below table. Use the Standard Diluent as the Zero Standard (Standard No.0).

Standard Concentration	Standard Vial	Dilution Particulars
1000 ng/ml	Reconstituted Standard	Lyophilized Standard provided in the Kit + 1 ml of Standard Diluent (1X)
500 ng/ml	Standard No.6	500 ul Reconstituted Standard + 500 ul Standard Diluent (1X)
250 ng/ml	Standard No.5	500 ul Standard No.6 + 500 ul Standard Diluent (1X)
125 ng/ml	Standard No.4	500 ul Standard No.5 + 500 ul Standard Diluent (1X)
62.5 ng/ml	Standard No.3	500 ul Standard No.4 + 500 ul Standard Diluent (1X)
31.25 ng/ml	Standard No.2	500 ul Standard No.3 + 500 ul Standard Diluent (1X)
15.6 ng/ml	Standard No.1	500 ul Standard No.2 + 500 ul Standard Diluent (1X)
0 ng/ml	Standard No.0	Only Standard Diluent (1X)

5. Working Anti-human Kappa antibody: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 Certolizumab pegol. High Dose Hook Effect is due to excess of antibody for very high concentrations of Certolizumab pegol present in the sample.
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 Certolizumab pegol.
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 Standards and Samples be run in duplicates or triplicates. All steps must be performed at 37ºC.

2. Pipette **100 ul** of **prepared Standards and diluted Samples** to the respective wells.

3. Cover the plate and incubate for 60 minutes at 37ºC.

4. 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 as any residue can interfere in the reading step.

5. Pipette **100 ul** of **Working Anti-human Kappa antibody:HRP Conjugate to** all wells.

6. Cover the plate and incubate for 60 minutes at 37ºC.

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

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

9. Incubate the plate at 37ºC 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.

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

11. Read the absorbance at 450 nm with a microplate reader.

Interpretation of Results:

Determine the Mean Absorbance for each set of duplicate or triplicate Standards and Samples. Using 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 Certolizumab pegol , 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 Certolizumab pegol Concentration. If samples were diluted, multiply by the appropriate dilution factor.

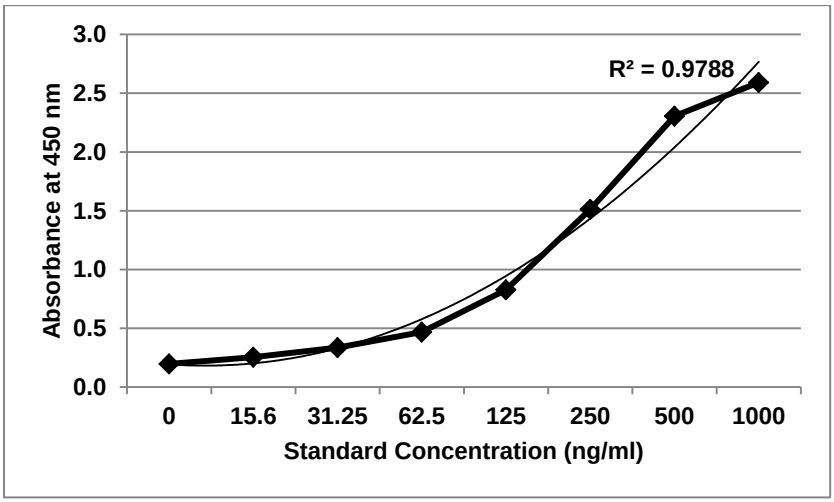
Software which is able to generate a linear regression is best recommended for automated results.

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 1000 ng/ml standard.

Typical Data (representative only)

Standards (ng/ml)	Absorbance	Interpolated Concentration	% Recovery
0	0.198		
15.6	0.255	14.0	90.0
31.25	0.336	40.2	128.6
62.5	0.468	67.0	107.2
125	0.828	124.5	99.6
250	1.512	242.1	96.8
500	2.305	536.8	107.4
1000	2.591	922.3	92.2

Typical Graph (representative only)



Abs = absorbance at 450nm

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 Quantification: It is defined as the lowest concentration of an analyte that can be determined with an acceptable repeatability and the LOQ was found to be 14 ng/ml.
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 14 ng/ml.

Specificity:
The capture reagent used in the kit is a recombinant human tumour necrosis factor-alpha (TNF-α) protein, expressed in a mammalian expression system and representing the biologically active form of the cytokine recognised by certolizumab pegol. The assay standard consists of a certolizumab pegol biosimilar-grade reference material, produced in a controlled expression system and exhibiting high-affinity and specific binding to human TNF-α. The assay is highly specific for certolizumab pegol and does not show cross-reactivity with

unrelated human immunoglobulins, other anti-TNF therapeutic monoclonal antibodies or Fc-containing antibodies, owing to the unique Fab′ structure of certolizumab pegol. This specificity ensures accurate and selective quantification of certolizumab pegol in the presence of endogenous serum components and other biologic therapeutics.

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 (15.6 ng/ml), medium (125 ng/ml) and high (1000 ng/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
Low	<12%
Medium	<10%
High	<10%

Pool	Inter Assay %CV
Low	<15%
Medium	<10%
High	<10%

Safety Precautions:

- **This kit is 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.
- 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

SCHEMATIC ASSAY PROCEDURE

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

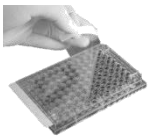
Components (2-8ºC) → 30 mins RT Before Use → 

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

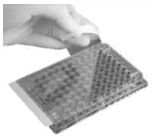
3.  Pipette **100 ul prepared Standards** / **diluted Samples** to the respective wells.

4. Cover plate  and incubate for 

at 37ºC.

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

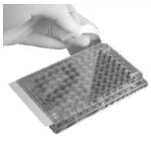
6.  Pipette **100 ul Working Anti-human Kappa antibody:HRP Conjugate** into each well.

7. Cover plate  and incubate for 

at 37ºC.

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 37ºC.

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

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

of stopping reaction.

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SYMBOLS KEY

MTP	**Recombinant Human TNF-alpha Protein (His Tag) Coated Microtiter Plate (12x8 wells)**
STD	**Recombinant Certolizumab pegol Standard, Lyophilized**
HRP CONJ	**Anti-human Kappa antibody:HRP Conjugate Concentrated**
DETN DIL	**Detection Diluent**
1X SAMP DIL	**(1X) Sample Diluent**
1X STD DIL	**(1X) Standard Diluent**
20X WASH BUF	**(20X) Wash Buffer**
SUB TMB	**TMB Substrate**
SOLN STOP	**Stop Solution**
	**Consult Instructions for Use**
REF	**Catalog Number**
	**Expiration Date**
	**Storage Temperature**