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# Anti-Tocilizumab ELISA

**REF** : KBI2022

Ver 1.1

**RUO**

Enzyme Immunoassay for the quantitative determination of Antibodies to Tocilizumab in serum, plasma and cell culture supernatant

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

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**REF** KBI2022 Σ 96 tests

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**Introduction:**

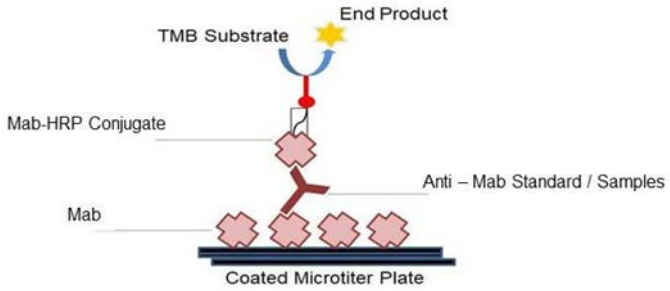
Tocilizumab, also known as atlizumab, is an immunosuppressive drug, mainly for the treatment of rheumatoid arthritis (RA) and systemic juvenile idiopathic arthritis, a severe form of arthritis in children. It is a humanized monoclonal antibody against the interleukin-6 receptor (IL-6R). Interleukin 6 (IL-6) is a cytokine that plays an important role in immune response and is implicated in the pathogenesis of many diseases, such as autoimmune diseases, multiple myeloma and prostate cancer. It was developed by Hoffmann–La Roche and Chugai.

**Intended Use:**

The Anti-Tocilizumab ELISA is used as an analytical tool for quantitative determination of antibodies to Tocilizumab in serum, plasma and cell culture supernatant.

**Principle:**

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




**Materials Provided:**

1. Tocilizumab Coated Microtiter Plate (12 x 8 wells) – 1 no
2. Anti-Tocilizumab Standard, (0.5 ml/vial) – 0, 10, 20, 40, 80, 160, 320 and 640 ng/ml
3. Tocilizumab:HRP Conjugate – 6 ml
4. Sample Diluent – 50 ml
5. Wash Buffer (20X) – 25 ml
6. TMB Substrate – 12 ml
7. Stop Solution – 12 ml
8. Instruction Manual

**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. Standard graph paper or software for data analysis
6. Timer
7. Absorbent Paper

**Handling/Storage:**

1. All reagents should be stored at 2ºC to 8ºC for stability.

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 In-Vitro Diagnostic 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.

Samples should be diluted 1:1000 (v/v), i.e. 1 ul sample + 999 ul sample diluent prior to assay. The samples may be kept at 2 - 8°C for up to three days. For long-term storage please store at -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.

**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 Anti-Tocilizumab antibodies. High Dose Hook Effect is due to excess of Tocilizumab for very high concentrations of antibodies against Tocilizumab 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 Anti-Tocilizumab antibody 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 Anti-Tocilizumab.
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 **50 ul of Anti-Tocilizumab: HRP Conjugate** into each well.
3. Add **100 ul** of **Standards** or **Samples** into the respective wells.
4. Cover the plate and incubate for 45 minutes at 37°C
5. 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.
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 reader.

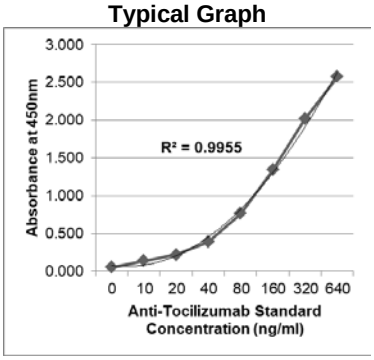
**Calculation of Results:**

Determine the Mean Absorbance for each set of duplicate or triplicate Standards and Samples. Using standard 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 Anti-Tocilizumab antibodies concentration, 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 Anti-Tocilizumab antibodies 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 640 ng/ml standard.

**Typical Graph**




| Standards (ng/ml) | Abs1 | Abs 2 | Mean | Net Abs |
|---|---|---|---|---|
| 0 | 0.051 | 0.046 | 0.048 | 0.000 |
| 10 | 0.137 | 0.136 | 0.137 | 0.088 |
| 20 | 0.217 | 0.218 | 0.218 | 0.169 |
| 40 | 0.395 | 0.380 | 0.388 | 0.339 |
| 80 | 0.771 | 0.754 | 0.762 | 0.714 |
| 160 | 1.365 | 1.322 | 1.343 | 1.295 |
| 320 | 2.040 | 1.998 | 2.019 | 1.971 |
| 640 | 2.566 | 2.584 | 2.575 | 2.527 |

**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 10 ng/ml

**Specificity:**
The kit uses two antigens specific for Tocilizumab (RoActemra/Actemra) which were commercially procured. A full traceability certificate for standards/calibrator standardization is available on request.

**Traceability:**
There are no reference standards for Anti-Tocilizumab. The results are reported in ng/mL and the method has been standardized in the laboratories of KRISHGEN BIOSYSTEMS.

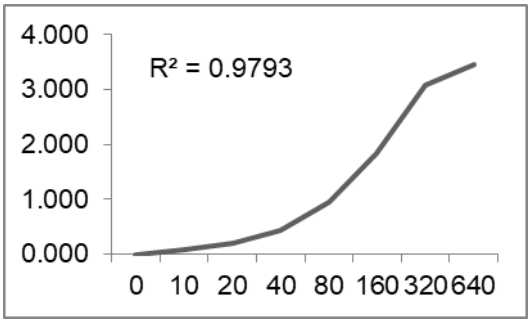
**Linearity:**
Standards provided in the kit will be used for measuring the linearity range of Anti-Tocilizumab present in matrix. The standards / calibrator range is 0 - 640 ng/ml.

**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 (10 ng/ml), medium (60 ng/ml) and high (640 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 | Inter Assay %CV |
|---|---|---|
| Low | <10% | <10% |
| Medium | <5% | <5% |
| High | <5% | <5% |

**Recovery:**
Human sera and plasma were measured with two replicates and two runs per day for 20 days (n = 80). The human sera and plasma were pooled patient and single donor spiked samples. Samples were measured using one lot of reagent, in two laboratories of KRISHGEN BIOSYSTEMS. All data met our acceptance criteria for % CV and 95% confidence intervals for % CV.

| Standards (ng/ml) | Absorbance of 1:1000 Diluted and Spiked in Serum | Net Abs | Interpolated Concentration | Serum Recovery % |
|---|---|---|---|---|
| 0 | 0.066 | 0.000 | | --- |
| 10 | 0.160 | 0.094 | 10.27 | 86.39 |
| 20 | 0.255 | 0.189 | 22.88 | 92.10 |
| 40 | 0.496 | 0.430 | 44.23 | 97.86 |
| 80 | 1.016 | 0.950 | 81.30 | 99.49 |
| 160 | 1.896 | 1.830 | 149.96 | 94.20 |
| 320 | 3.142 | 3.076 | 358.46 | 100.90 |
| 640 | 3.526 | 3.460 | 575.23 | 109.17 |




** Recov Conc = Recovery Concentration*
*Abs = Absorbances at 450 nm*
*Std = Standards*

Note: Serum was diluted using Standard Diluent provided with the kit

**Safety Precautions:**

- **This kit is For In-Vitro Diagnostic 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.

References:

Intravenous tocilizumab: a review of its use in adults with rheumatoid arthritis…Dhillon S…BioDrugs…2014…Springer

Clinical pharmacology of tocilizumab for the treatment of patients with rheumatoid arthritis… Zhang X, Peck R…Expert Rev Clin Pharmacol…2011…Taylor & Francis

A phase I trial combining carboplatin/doxorubicin with tocilizumab, an anti-IL-6R monoclonal antibody, and interferon-α2b in patients with recurrent epithelial ovarian cancer…Dijkgraaf EM, Santegoets SJ…Ann Oncol…2015…Oxford

Tocilizumab in early progressive rheumatoid arthritis: FUNCTION, a randomised controlled trial… Burmester GR, Rigby WF, van Vollenhoven RF, Kay J, Rubbert-Roth A, Kelman A, Dimonaco S, Mitchell N…Ann Rheum Dis…2015…BMJ

## SCHEMATIC ASSAY PROCEDURE

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

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

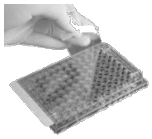
Thaw at Room Temperature (18-24ºC) → USE NOW

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 **50 ul Tocilizumab:HRP Conjugate** into the wells.

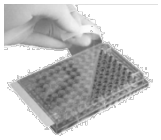
4.  Pipette **100 ul Samples / Standards** into respective wells.

4. Cover plate  and incubate for 

t 37°C.

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

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

7. Cover plate  and incubate for 

at 37°C.

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

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

of stopping reaction.

## Typical Example of a Work List

| Well # | Contents | Absorbance at 450nm | Mean Absorbance | ng/ml Tocilizumab equivalent |
|---|---|---|---|---|
| 1A<br>2A | zero std<br>zero std | | | |
| 1B<br>2B | 10 ng/ml<br>10 ng/ml | | | |
| 1C<br>2C | 20 ng/ml<br>20 ng/ml | | | |
| 1D<br>2D | 40 ng/ml<br>40 ng/ml | | | |
| 1E<br>2E | 80 ng/ml<br>80 ng/ml | | | |
| 1F<br>2F | 160 ng/ml<br>160 ng/ml | | | |
| 1G<br>2G | 320 ng/ml<br>320 ng/ml | | | |
| 1H<br>2H | 640 ng/ml<br>640 ng/ml | | | |
| 3A<br>4A | *Sample* | | | |
| 3B<br>4B | *Sample* | | | |

## LIMITED WARRANTY

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