Ustekinumab ELISA

REF: KBI1014
Ver 2.0
RUO

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

For Research Use Only. Purchase does not include or carry the right to resell or transfer this product either as a stand-alone product or as a component of another product. Any use of this product other than the permitted use without the express written authorization of Eagle Biosciences, Inc. is strictly prohibited.

Eagle Biosciences, Inc.
20A NW BLVD., Suite 112
Nashua, NH 03063
Phone: +1 (617) 419-2019
Fax: +1 (617) 419-1110
Info@eaglebio.com

KBI1014, Ver2.0
www.eaglebio.com
Introduction:
Ustekinumab is a human monoclonal antibody. It is directed against interleukin 12 and interleukin 23, naturally occurring proteins that regulate the immune system and immune-mediated inflammatory disorders. In two Phase III trials for moderate to severe psoriasis, the longest >76 weeks, ustekinumab was safe and effective. A third Phase III trial, ACCEPT, compared the efficacy and safety of ustekinumab with etanercept in the treatment of moderate to severe plaque psoriasis. This trial found a significantly higher clinical response with ustekinumab over the 12-week study period compared to high-dose etanercept. It also demonstrated the clinical benefit of ustekinumab among patients who failed to respond to etanercept. Ustekinumab is approved in Canada, Europe and the United States to treat moderate to severe plaque psoriasis.

Intended Use:
The Eagle Biosciences Ustekinumab ELISA is used as an analytical tool for quantitative determination of Ustekinumab in serum, plasma and cell culture supernatant.

Principle:
The method employs the quantitative sandwich enzyme immunoassay technique. Antibodies to Ustekinumab are pre-coated onto microwells. Samples and standards are pipetted into microwells and human Ustekinumab present in the sample are bound by the capture antibody. Then, a HRP (horseradish peroxidase) conjugated anti-Ustekinumab antibody 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 Ustekinumab in the sample. Color development is then stopped by addition of stop solution. Absorbance is measured at 450 nm.

Materials Provided:
1. Anti-Ustekinumab Coated Microtiter Plate (12x8 wells) – 1 no
2. Ustekinumab Standard, (0.5 ml/vial) – 0, 5, 10, 20, 40, 80, 160 and 320 ng/ml
3. Anti-Ustekinumab:HRP Conjugate – 3 ml
4. Assay Diluent – 3 ml
5. Sample Diluent – 50 ml
6. Wash Buffer (20X) – 25 ml
7. TMB Substrate – 12 ml
8. Stop Solution – 12 ml

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 25µl to 1000µl
3. Deionized (DI) water
4. Wash bottle or automated microplate washer
5. Semi-Log 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.
Ustekinumab ELISA

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 -20ºC.

For Cell Culture Supernatant – If necessary, centrifuge to remove debris prior to analysis. Samples can be stored at -20ºC 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 1:500 to 1:1000 (v/v), e.g. for 1:500 (1 µl sample + 499 µl sample diluent) prior to assay. The samples may be kept at 2 - 8ºC for up to three days. Long-term storage requires -20ºC.

**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 50 ml of 20X Wash Buffer in 950 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 Ustekinumab. High Dose Hook Effect is due to excess of antibody for very high concentrations of Ustekinumab 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 Ustekinumab 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 (NaN₃), as it could destroy the HRP activity resulting in under-estimation of the amount of Ustekinumab.
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 compromised covering 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 25 µl of Assay Diluent in each well.
3. Pipette 25 µl of Anti-Ustekinumab: HRP Conjugate into each well.

4. Add 100 µl of Standards or Samples into the respective wells.

5. Cover the plate and incubate for 120 minutes at 37°C

6. 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.

7. Add 100 µl of TMB Substrate in each well.

8. 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.

9. Pipette out 100 µl of Stop Solution. Wells should turn from blue to yellow in color.

10. 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 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 Ustekinumab 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 Ustekinumab 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 320 ng/ml standard.

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 5ng/ml

Linearity:

Standards provided in the kit will be used for measuring the linearity range of Ustekinumab 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 (5ng/ml), medium (40ng/ml) and high (320ng/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.
Limitations of Method

Healthy individuals should be tested negative by the Ustekinumab. This kit is for research use only and should not be used as a diagnostic tool.

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.
- 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:

Comparison of the pharmacokinetics of subcutaneous ustekinumab between Chinese and non-Chinese healthy male subjects across two phase 1 studies. Y Zhu, Q Wang, B Frederick, E Bouman-Thio… - Clinical drug …, 2013 - Springer

Ustekinumab: a review in moderate to severe Crohn's disease. YN Lamb, ST Duggan - Drugs, 2017 - Springer;


Switching biologics in the treatment of psoriatic arthritis. JF Merola, B Lockshin, EA Mody - Seminars in arthritis and rheumatism, 2017 - Elsevier

The association between clinical response to ustekinumab and immunogenicity to ustekinumab and prior adalimumab. HY Chiu, TW Chu, YP Cheng, TF Tsai - PloS one, 2015 - journals.plos.org


Prolongation of biologic dosing intervals in patients with stable psoriasis: a feasibility study. JS van Bezooijen, MBA van Doorn… - Therapeutic drug …, 2017 - journals.lww.com


SCHEMATIC ASSAY PROCEDURE

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

Components (2-8°C) → 30 mins → Thaw at Room Temperature (18-24°C) → 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 → NO CHANGE IN RESULTS

3. Pipette 25 µl Assay Diluent into each well.

4. Pipette 25 µl Anti Ustekinumab: HRP into the respective wells.

5. Pipette 100 µl Standards / Samples into the respective wells.

6. Cover plate and incubate for 120 mins at 37°C.

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

8. Pipette 100 µl TMB Substrate into each well.

9. Cover plate and incubate for 30 mins at 37°C.

10. Pipette 100 µl Stop Solution into each well.

11. Read absorbance at 450nm with a microplate reader within 30 mins of stopping reaction.
## Typical Example of a Work List

<table>
<thead>
<tr>
<th>Well #</th>
<th>Contents</th>
<th>Absorbance at 450nm</th>
<th>Mean Absorbance</th>
<th>ng/ml Ustekinumab equivalent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>zero std</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2A</td>
<td>zero std</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1B</td>
<td>5 ng/ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2B</td>
<td>5 ng/ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1C</td>
<td>10 ng/ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2C</td>
<td>10 ng/ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1D</td>
<td>20 ng/ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2D</td>
<td>20 ng/ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1E</td>
<td>40 ng/ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2E</td>
<td>40 ng/ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1F</td>
<td>80 ng/ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2F</td>
<td>80 ng/ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1G</td>
<td>160 ng/ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2G</td>
<td>160 ng/ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1H</td>
<td>320 ng/ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2H</td>
<td>320 ng/ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3A</td>
<td>Sample</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4A</td>
<td>Sample</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3B</td>
<td>Sample</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4B</td>
<td>Sample</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### LIMITED WARRANTY

Eagle Biosciences, Inc. warrants its Product(s) to operate or perform substantially in conformance with its specifications, as set forth in the accompanying package insert. This warranty is expressly limited to the refund of the price of any defective Product or the replacement of any defective Product with new Product. This warranty applies only when the Buyer gives written notice to the Eagle Biosciences within the expiration period of the Product(s) by the Buyer. In addition, Eagle Biosciences has no obligation to replace Product(s) as result of a) Buyer negligence, fault, or misuse, b) improper use, c) improper storage and handling, d) intentional damage, or e) event of force majeure, acts of God, or accident.

Eagle Biosciences makes no warranties, either expressed or implied, except as provided herein, including without limitation thereof, warranties as to marketability, merchantability, fitness for a particular purpose or use, or noninfringement of any intellectual property rights. In no event shall the company be liable for any indirect, incidental, or consequential damages of any nature, or losses or expenses resulting from any defective product or the use of any product. Product(s) may not be resold, modified, or altered for resale without prior written approval from Eagle Biosciences, Inc.

For further information about this kit, its application or the procedures in this kit insert, please contact the Technical Service Team at Eagle Biosciences, Inc. at info@eaglebio.com or at 866-411-8023.

**THANK YOU FOR USING EAGLE BIOSCIENCES!**