

Instructions for Use

Ustekinumab ELISA (mAb-based)

Enzyme immunoassay for the specific quantitative determination
of free Ustekinumab in serum and plasma

REF: IG-AB121



12X8



2-8°C



IVD

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1. INTENDED USE

Enzyme immunoassay for the specific and quantitative determination of free Ustekinumab in serum and plasma.

2. SUMMARY AND EXPLANATION

The drug Ustekinumab (trade name Stelara[®]) is a humanized immunoglobulin G1 κ monoclonal antibody that binds with high specificity to the p40 protein subunit used by both the IL-12 and IL-23 cytokines.

Ustekinumab is indicated for the treatment of adult patients with:

- *moderate to severe plaque psoriasis (Ps)* who are candidates for phototherapy or systemic therapy.
- *active psoriatic arthritis (PsA)*, alone or in combination with methotrexate.
- *moderately to severely active Crohn's disease (CD)* who have
 - failed or were intolerant to treatment with immunomodulators or corticosteroids, but never failed a tumor necrosis factor (TNF) blocker or
 - failed or were intolerant to treatment with one or more TNF blockers.

Steady state Ustekinumab concentration was achieved by the start of the second maintenance dose. There was no apparent accumulation of Ustekinumab concentration over time when given subcutaneously every 8 weeks. According to the prescribing information the mean steady-state trough concentration was 2.51 \pm 2.06 μ g/mL for 90 mg Ustekinumab administered every 8 weeks. In a phase II study it was reported that the median trough serum levels of Ustekinumab reached steady state by week 12 (1.59 μ g/mL) in the every-8-week group and by week 16 (0.61 μ g/mL) in the every-12-week group, and were maintained through week 28 (1.77 μ g/mL and 0.54 μ g/mL, respectively).

Identification of biomarkers for (non-) response and risk factors might be beneficial for adverse drug reactions that might be related to serum drug levels and maintaining the effective concentration in order to potentially avoid some side effects with a reliable method.

3. PRINCIPLE OF THE TEST

This ELISA is based on Ustekinumab-specific monoclonal antibody (catcher Ab, *ImmunoGuide* clone 9C7). Diluted standards and samples are incubated in the microtiter plate coated with IG-9C7 mAb. After incubation, the wells are washed. A biotinylated anti-human IgG monoclonal antibody (clone *ImmunoGuide* 1B5; specific for the Fc part of all human IgG i.e. IgG1, IgG2, IgG3 and IgG4) is added and binds to the Fc part of Ustekinumab. Following incubation, wells are washed and the horseradish peroxidase (HRP)-conjugated streptavidin is added and binds to the biotinylated 1B5 mAb. Following incubation, wells are washed and the bound enzymatic activity is detected by addition of chromogen-substrate. The color developed is proportional to the amount of Ustekinumab in the sample or standard. Results of samples can be determined by using the standard curve. Preincubation of Ustekinumab with recombinant human interleukin-12 (IL-12), contains p40 protein subunit as that of IL-23, inhibited the reaction. Therefore, the *ImmunoGuide* Ustekinumab ELISA (mAb-based) measures the free form of Ustekinumab.

4. WARNINGS AND PRECAUTIONS

1. 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 (clinical background, test performance, automation protocols, alternative applications, literature, etc.) please refer to the local distributor.
2. In case of severe damage of the kit package, please contact *Tani Medikal* or your supplier in writing, latest one week after receiving the kit. Do not use damaged components in test runs, but keep safe for complaint related issues.
3. Obey lot number and expiry date. Do not mix reagents of different lots. Do not use expired reagents.
4. Follow good laboratory practice and safety guidelines. Wear lab coats, disposable latex gloves and protective glasses where necessary.
5. Reagents of this kit containing hazardous material may cause eye and skin irritations. See MATERIALS SUPPLIED and labels for details.
6. Chemicals and prepared or used reagents have to be treated as hazardous waste according to the national biohazard safety guidelines or regulations.
7. Avoid contact with Stop solution. It may cause skin irritations and burns.
8. If any component of this kit contains human serum or plasma it is indicated and if so, it have been tested and were found to be negative for HIV I/II, HBsAg and HCV. However, the 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.
9. 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

5. STORAGE AND STABILITY OF THE KIT

The kit is shipped at ambient temperature and should be stored at 2-8°C. Keep away from heat or direct sunlight. The storage and stability of specimen and prepared reagents is stated in the corresponding chapters. The microtiter strips are stable up to the expiry date of the kit in the broken, but tightly closed bag when stored at 2-8°C.

6. SPECIMEN COLLECTION, HANDLING 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 (Aliquots)	Keep away from heat or direct sunlight Avoid repeated freeze-thaw cycles
Stability:	3 d	6 mon	

7. CONTENTS OF THE KIT

QUANTITY	COMPONENT
1 x 12 x 8	Microtiter ELISA Plate Break apart strips coated with anti-Ustekinumab monoclonal antibody.
5 x 0.5 mL	Ustekinumab Standards A-E, Concentrate (10X) 600; 200; 60; 20; and 0 ng/mL Used for construction of the standard curve. Contains Ustekinumab, proteins, stabilizer and <15 mM NaN ₃ .
1 x 12 mL	Assay Buffer Blue colored. Ready to use. Contains proteins, mouse IgG and <15 mM NaN ₃ .
1 x 60 mL	Dilution Buffer, Concentrate (5X) Orange colored. Contains proteins and <15 mM NaN ₃ .
1 x 12 mL	Biotinylated α-hIgG Green colored. Ready to use. Contains biotinylated anti-human IgG monoclonal antibody, proteins, stabilizers and <15 mM NaN ₃ .
1 x 12 mL	Enzyme Conjugate Red colored. Ready to use. Contains horseradish peroxidase(HRP)-conjugated streptavidin (HRP-Streptavidin), Proclin [®] and stabilizers.
1 x 12 mL	TMB Substrate Solution Ready to use. Contains 3,3',5,5'-Tetramethylbenzidine (TMB).
1 x 12 mL	Stop Solution Ready to use. 1 N Hydrochloric acid (HCl).
1 x 50 mL	Wash Buffer, Concentrate (20x) Contains buffer, Tween [®] 20 and Kathon [™] .
2 x 1	Adhesive Seal For sealing the microtiter plate during incubation.

8. MATERIALS REQUIRED BUT NOT SUPPLIED

1. Micropipettes (< 3% CV) and tips to deliver 5-1000 μ L.
2. Bidistilled or deionised water and calibrated glasswares (e.g. flasks or cylinders).
3. Wash bottle, automated or semi-automated microtiter plate washing system.
4. Microtiter plate reader capable of reading absorbance at 450 nm (reference wavelength at 600-650 nm is optional).
5. Absorbent paper towels, standard laboratory glass or plastic vials, and a timer.

9. 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 pre-treatment 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 readily at the appropriate time. Allow all reagents and specimens to reach room temperature (20-25 °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 the caps of vials. Always cap not used vials. Do not reuse wells 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. Do not open the pouch until it reaches room temperature. Unused wells should be returned immediately to the resealed pouch including the desiccant.

10. PRE-TEST SETUP INSTRUCTIONS

10.1. Preparation of Components*

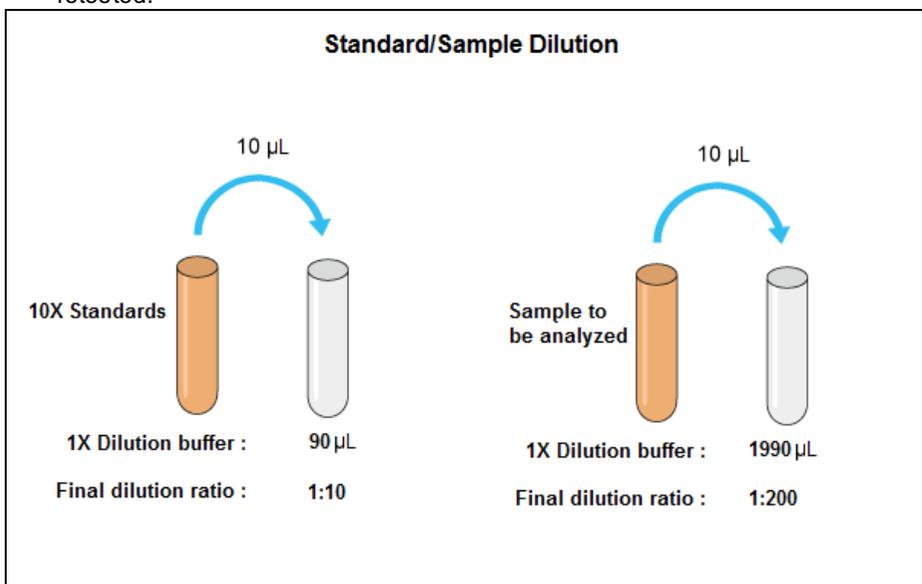
Dilute/dissolve	Component		Diluent	Relation	Remarks	Storage	Stability
10 mL	Wash Buffer	up to 200 mL	Distilled Water	1:20	Warm up at 37°C to dissolve crystals. Mix vigorously.	2-8 °C	4 w
10 mL	Dilution Buffer	up to 50 mL	Distilled Water	1:5		2-8 °C	4 w

* Prepare Wash and Dilution Buffers before starting the assay procedure.

10.2. Dilution of Standards and Samples

The dilutions depicted below are examples of how to obtain final dilutions for standards and samples. Standards and samples should be properly diluted as homogenous mixture before starting the assay procedure. It is recommended mixing the standards and samples well to homogenous solution at each dilution step.

1. 10 μL of standard added to 90 μL of 1X dilution buffer, giving the total volume of 100 μL and a dilution of 1:10.
2. 10 μL of sample added to 1990 μL of 1X dilution buffer, giving the total volume of 2000 μL and a dilution of 1:200.
3. Samples with a drug concentration above the measuring range should be rated as ">highest standard". The result should not be extrapolated. The sample in question should be further diluted with 1X Dilution Buffer and then retested.



11. TEST PROCEDURE

11.1. GENERAL REMARKS

11.1.1. Before performing the assay, samples and assay kit should be brought to room temperature (about 30 minutes beforehand) and ensure the homogeneity of the solution.

11.1.2. All Standards should be run with each series of unknown samples.

11.1.3. Standards should be subject to the same manipulations and incubation times as the samples being tested.

11.1.4. All steps of the test should be completed without interruption.

11.1.5. Use new disposable plastic pipette tips for each reagent, standard or specimen in order to avoid cross contamination.

11.2. ASSAY PROCEDURE

1.	Pipette 100 μl of Assay Buffer into each of the wells to be used.
2.	<p>Pipette 75 μl of each 1:10 Diluted Standard, and 1:200 Diluted Samples into the respective wells of the microtiter plate. Bubble formation during the pipetting of standards and samples must be avoided.</p> <p><u>Wells</u></p> <p>A1: Standard A B1: Standard B C1: Standard C D1: Standard D E1: Standard E F1 and so on: Samples (Serum/Plasma)</p>
3.	Cover the plate with adhesive seal. Shake plate carefully by tapping several times. Incubate the plate on bench top for 60 min at room temperature (RT, 20-25°C).
4.	Remove adhesive seal. Aspirate or decant the incubation solution. Wash the plate 3 X 300 μl of Diluted Wash Buffer per well. Remove excess solution by tapping the inverted plate on a paper towel.
5.	Pipette 100 μl of Biotinylated α-hlgG into each well.
6.	Cover plate with adhesive seal. Shake plate carefully by tapping several times. Incubate the plate on a bench top for 30 min at RT.
7.	Remove adhesive seal. Aspirate or decant the incubation solution. Wash the plate 3 X 300 μl of Diluted Wash Buffer per well. Remove excess solution by tapping the inverted plate on a paper towel.
8.	Pipette 100 μl of Enzyme Conjugate (HRP-Streptavidin) into each well.
9.	Cover plate with adhesive seal. Shake plate carefully by tapping several times. Incubate the plate on a bench top for 30 min at RT.
10.	Remove adhesive seal. Aspirate or decant the incubation solution. Wash the plate 3 X 300 μl of Diluted Wash Buffer per well. Remove excess solution by tapping the inverted plate on a paper towel.
11.	Pipette 100 μl of Ready-to-Use TMB Substrate Solution into each well.
12.	Incubate 20 min at RT. Avoid exposure to direct sunlight.
13.	Stop the substrate reaction by adding 100 μl of Stop Solution into each well. Color changes from blue to yellow. Briefly mix contents by gently shaking the plate.
14.	Measure optical density (OD) with a photometer at 450 nm (Reference at OD620 nm is optional) within 15 min after pipetting the Stop Solution.

11.3. QUALITY CONTROL

The test results are only valid 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/controls 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 reviewed: Expiration dates of (prepared) reagents, storage conditions, pipettes, devices, incubation conditions and washing methods.

11. 4. CALCULATION OF RESULTS

A standard curve should be calculated using the standard concentration (X-axis) versus the OD450 (or OD450/620) values (Y-axis). This can be done manually using graph paper or with a computer program. Concerning the data regression by computer, it is recommended to primarily use the “4 Parameter Logistic (4PL)” or alternatively the “point-to-point calculation”. In case of manual plot there are 2 options: Semilog graph (see Fig. A) or linear graph (see Fig. B). Semilog graph paper is available at <http://www.papersnake.com/logarithmic/semilogarithmic/>.

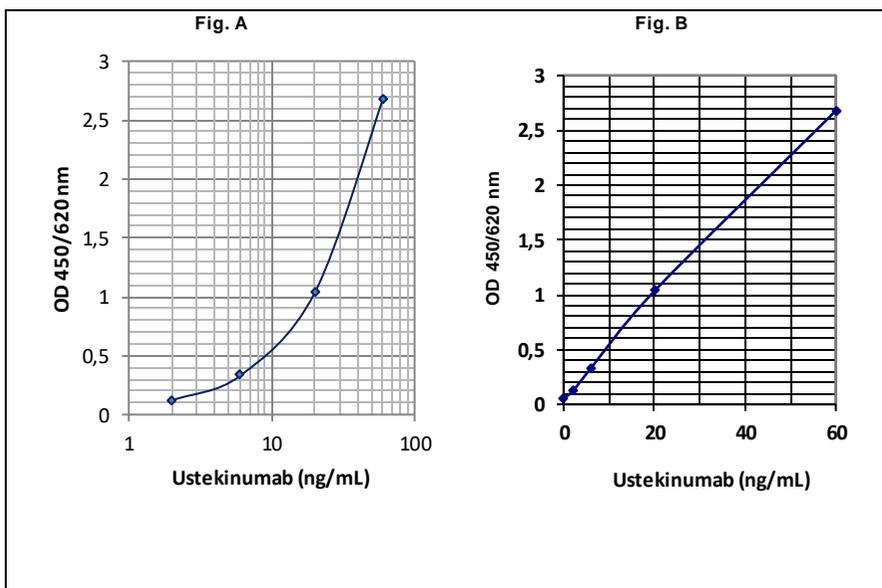
The concentration of the samples can be read from this standard curve as follows. Using the absorbance value for each sample, determine the corresponding concentration of the drug from the standard curve. This value always has to be multiplied by the individual dilution factor (usually 200). If any diluted sample is reading greater than the highest standard, it should be further diluted appropriately with 1X Dilution Buffer and retested. Also this second dilution has to be used for calculation of the final result.

It is proposed that a sample with an OD lower than the standard D should NOT be plotted in the standard curve, instead should be reported as “below detection limit”.

Typical Calibration Curve

(All steps were performed at 23°C. Just an example. Do not use it for calculation!)

1:10 Diluted Standard	A	B	C	D	E
Concentration (ng/mL)	60	20	6	2	0
Mean OD450/620 nm	2.678	1.033	0.329	0.120	0.054



12. ASSAY CHARACTERISTICS

12.1. SPECIFICITY

There is no cross reaction with any other proteins present in native human serum. A screening test was performed with 21 different native human sera. All produced OD_{450/620 nm} values (ranged from 0.036 to 0.068) less than the mean OD (0.120) of standard D (2 ng/mL). In addition, binding of Ustekinumab to the solid phase is inhibited by p40-containing recombinant human interleukin-12 (hIL-12) protein. Therefore, the *ImmunoGuide* Ustekinumab ELISA (mAb-Based) measures the biologically active free form of Ustekinumab, i.e. not pre-occupied by human IL-12 or IL-23 antigen. No cross reaction was observed with sera spiked with the other therapeutic antibodies including Infliximab, Rituximab, Cetuximab, Vedolizumab, Tocilizumab, Trastuzumab, Nivolumab and Bevacizumab at concentrations tested up to 40 µg/mL. All produced mean OD_{450/620 nm} values (ranging from 0.035 to 0.058) less than standard D.

12.2. SENSITIVITY

The lowest detectable level that can be clearly distinguished from the zero standard is 1,5 ng/mL (zero standard +2SD read from the curve) under the above-described conditions.

12.3. PRECISION

Intra-assay CV: <10%.

Inter-assay CV: <10%.

12.4. RECOVERY

Recovery rate was found to be >95% with native serum and plasma samples when spiked with exogenous Ustekinumab.

13. AUTOMATION

The *ImmunoGuide* Ustekinumab ELISA (mAb-based) is suitable also for being used by an automated ELISA processor.

14. REFERENCES

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