ImmunoGuide®

Instructions for Use

Antibody to Ustekinumab ELISA

Enzyme immunoassay for the semi-quantitative determination of free antibodies to Ustekinumab in serum and plasma

REF: IG-BB121

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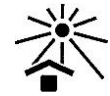

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Version.-No.: BB121E
2018/1 WZ

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1. INTENDED USE

Enzyme immunoassay for the semi-quantitative determination of free antibodies to Ustekinumab in serum and plasma.

2. SUMMARY AND EXPLANATION

The drug Ustekinumab (trade nameStelara®) is a humanised immunoglobulin G1κ monoclonal antibody that binds with 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 in Ustekinumab concentration over time when given s**ubcutaneously every 8 weeks. According to the prescribing information, approximately** 6% of patients treated with Ustekinumab in psoriasis and psoriatic arthritis clinical studies developed antibodies to Ustekinumab, which were generally low-titer. In Crohn's disease clinical studies, less than 3% of patients treated with Ustekinumab developed antibodies to Ustekinumab. The *ImmunoGuide* Antibody to Ustekinumab ELISA kit has been designed for the measurement of free antibodies against this drug. It does not detect such antibodies which already are bound to the drug.

3. PRINCIPLE OF THE TEST

This *ImmunoGuide* anti-drug antibody(ies) (ADA) kit is a bridging type ELISA for the determination of free antibodies against the drug Ustekinumab in serum and plasma samples. During the first incubation period, ADA in serum or plasma samples are captured by the drug coated on the microtiter wells. After washing away the unbound components from samples, a peroxidase-labelled drug conjugate is added and then incubated. ADA, if present in sample, will make a bridge, with its identical Fab arms, between the drug coated on the well and the other drug molecule labelled with peroxidase. After a second washing step, the bound enzymatic activity is detected by addition of tetramethylbenzidine (TMB) chromogen-substrate. Finally, the reaction is terminated with stop solution. The positive reaction is expected to be related to the presence of ADA in the sample.

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 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 has 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 sun light. 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)*

<table>
<tr><td colspan="4">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.</td></tr>
<tr><td>Storage:</td><td>2-8°C</td><td>≤-20°C (Aliquots)</td><td rowspan="2">Keep away from heat or direct sun light
Avoid repeated freeze-thaw cycles</td></tr>
<tr><td>Stability:</td><td>3 d</td><td>6 mon</td></tr>
</table>

***** Drug administration/infusion may camouflage/mask the presence of anti-drug antibodies (ADA) in serum/plasma samples. Therefore, blood sampling time is also critical for detection of ADA. It is proposed to obtain blood sample just before administration of the drug.

7. CONTENTS OF THE KIT

QUANTITY	COMPONENT
1 x 12 x 8	**Microtiter Plate** Break apart strips pre-coated with the drug Ustekinumab.
1 x 1 mL	**Negative Control** Ready to use. Contains serum and <15 mM NaN_3.
1 x 1 mL	**Positive Control** Ready to use. Contains Ustekinumab-specific antibody and <15 mM NaN_3.
1 x 15 mL	**Dilution Buffer** Ready to use. Contains orange dye, proteins and <15 mM NaN_3.
1 x 12 mL	**Assay Buffer** Blue colored. Ready to use. Contains proteins and <15 mM NaN_3.
1 x 12 mL	**Enzyme Conjugate** Red colored. Ready to use. Contains horseradish peroxidase(HRP)-conjugated Ustekinumab, 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 microtiter plate during incubation.

8. MATERIALS REQUIRED BUT NOT SUPPLIED

1. Micropipettes (< 3% CV) and tips to deliver 5-1000 µL.
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

***** Prepare Wash Buffer before starting the assay procedure.

10.2. Dilution of Samples[a,b]

Sample	To be diluted	With	Remarks
Serum/ Plasma	1:10	Dilution Buffer	For dilution at 1:10; 10 µL Sample + 90 µL Dilution Buffer

[a] Negative and Positive Controls are ready-to-use and should NOT be diluted with the dilution buffer.
[b] Incubate 1:10 diluted serum/plasma samples for 15 min at room temperature (20-25°C) before pipetting 50 µL of each 1:10 diluted sample per well for analysis.

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.
11.1.6. The total pipetting time needed for dispensing all samples into the wells should not exceed 5 minutes. If this is difficult to achieve the samples should be pre-dispensed in a separate neutral polypropylene microplate and then transferred into the reaction ELISA plate by a multi channel pipette. Such neutral plates are available from the kit manufacturer upon request.

11.2. ASSAY PROCEDURE

1.	Pipette **100 µl** of **Assay Buffer** into each of the wells to be used.
2.	Pipette **50 µL** of each **Ready-to-Use Negative Control, Ready-to-Use Positive Control, and 1:10 Diluted Samples (as described in section 10.2)** into the respective wells of the microtiter plate. Bubble formation during the pipetting of standards and samples must be avoided. Wells A1: Negative Control B1: Negative Control C1: Positive Control D1 and so on: Diluted samples (Serum/Plasma)
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 **Enzyme Conjugate** (HRP-drug) into each well.
6.	Cover the plate with adhesive seal. Shake plate carefully by tapping several times. **Incubate the plate on bench top for 60 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 Ready-to-Use **TMB** Substrate Solution into each well.
9.	**Incubate 15 min** at RT. Avoid exposure to direct sunlight**.**.
10.	Stop the substrate reaction by adding **100 µL** of **Stop Solution** into each well. Briefly mix contents by gently shaking the plate. Color changes from blue to yellow.
11.	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 below 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

For the run to be valid, the OD450 nm of the Positive Control should be ≥ 0.500 and the OD450 nm of each Negative Control should be ≤0.150. If not, improper technique or reagent deterioration may be suspected and the run should be repeated.

The results are evaluated by dividing each individual OD results by the Cut-off OD value. The results are expressed in arbitrary units (AU/mL).

Cut-off value = 2 x the mean OD450 nm of Negative Control = 3 AU/mL

64 different naive samples have been measured for estimating the cut-off value. In order to avoid a sample from being reported as false positive the cut-off value was determined by 2 times of the mean of Negative Control. All 64 screened naive samples show ODs (ranged between 0.051 and 0.107) lower than the cut-off value.

Samples which have an equal and higher OD than the cut-off value are considered to be positive.

Range	Interpretation
≥ Cut-off (3 AU/mL)	POSITIVE
< Cut-off (3 AU/mL)	NEGATIVE

An example for semi-quantitative calculation for a positive sample
OD of patient‘s sample = 0.770
The mean OD of Negative Control = 0.077
Cut-off value (3 AU/mL) = 2 x 0.077 = 0.154
Result for the patient‘s sample = 0.770/0.124 × 3 AU/mL = 15 AU/mL

The results themselves should not be the only reason for any therapeutical consequences. They have to be correlated to other clinical observations. In addition, the positive reaction should be clarified whether it is true or false positive following the confirmatory assay as mentioned in section “12.1. Specificity” below.

12. ASSAY CHARACTERISTICS

12.1. SPECIFICITY

The detection of ADA formation is highly dependent on the sensitivity and specificity of the assay used.

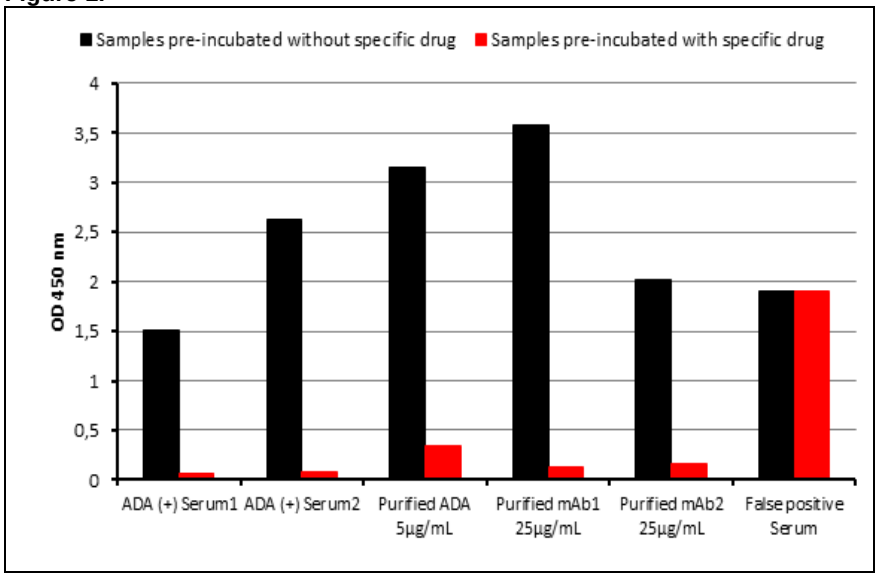
The initial screening assay should be sensitive to low and high-affinity ADA. Endogenous and exogenous components in serum or plasma may influence assay results. Measuring immune responses to therapeutic protein products that possess Ig tails, such as mAb and Fc-fusion proteins, may be particularly difficult when RF is present in serum or plasma. RF is generally an IgM antibody that recognizes IgG, although other Ig specificities have been noted. Therefore, there is frequently a need to dilute patient samples and to make approach for minimizing interference from RF to maintain a reasonable ability to detect ADA. However, dilution and/or addition of RF-blocking reagents may not solve all potential interference related with matrix components contributing to non-specific signal in samples.

If the serum/plasma sample of a patient, a candidate for a specific monoclonal drug treatment, is negative at the time before starting drug therapy and become positive during treatment, it could be concluded that the induction of the specific ADA is suspected in this individual. As shown in Figure 1, the true positive reaction of the sample is inhibited by pre-incubation of the sample with the specific drug itself (samples are spiked with certain amount of drug). However, inhibition was not observed in false positive reaction related with other components in sample (Figure 1).

Based on the approach used in calculating the assay cut-off value of anti-drug antibody (ADA) ELISAs, approximately 1-5% of tested samples are expected to generate false-positive ADA response during initial screening analysis. Therefore, further confirmation of the specificity of the ADA activity in the samples, identified as positive, is also recommended by recent publications. In addition, the FDA released a guidance paper on immunogenicity testing of therapeutic proteins: (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM192750.pdf)

In order to confirm the specificity of the ADA signal, a Confirmatory Assay, (competitive drug inhibition test), is proposed by *ImmunoGuide*. The Confirmation Test is performed with the Confirmatory Reagent. (coded IG-CR121) This Confirmatory Reagent is available as a separate product and can be obtained from *ImmunoGuide* upon request.

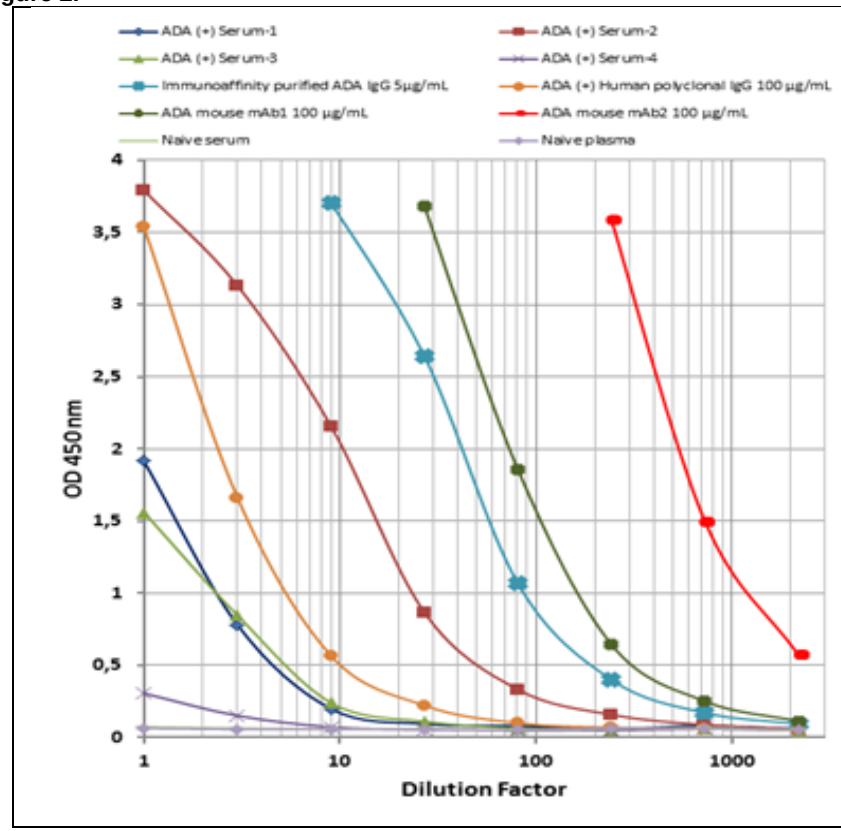
Figure 1.



12.2. SENSITIVITY

Generally, the sensitivity of the ADA assay is calculated as the lowest concentration of the positive control that can consistently generate a positive signal. However, as shown in Figure 2 below, data obtained at the *ImmunoGuide* Laboratory, the assay sensitivity also differs significantly depending on the high vs low affinity antibody used for the construction of the standard curve in the assay system. For example, when the immunoaffinity-purified ADA was exogenously added into naive serum, it was observed that the lowest detectable level that can be clearly distinguished from the negative control value is 7ng/mL by the *ImmunoGuide* bridging ELISA. It is much lower when mAb2 is used instead. Therefore, it is clear that the assay sensitivity will be highly dependent on the properties of a particular positive control to be used in the assay (Figure 2).

Figure 2.



12.3. PRECISION
Intra-assay CV: <10%.
Inter-assay CV: <10%

13. AUTOMATION
The *ImmunoGuide* Anti Drug Antibody (ADA) ELISA kits are suitable also for being used by an automated ELISA processor.

14. REFERENCES

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