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Instructions for Use

Ipilimumab (Yervoy®)ELISA

SHIKARI ®
Q-IPI

Enzyme immunoassay for the quantitative determination of Ipilimumab (Yervoy®) in serum and plasma

REF **TR-IPIv1** Σ 12 x 8 2-8°C

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	Free ipilimumab (Yervoy®) quantitative analyses
Required Volume (µl)	10
Total Time (min)	70
Sample	Serum, plasma
Sample Number	96
Detection Limit (ng/mL)	100
Spike Recovery (%)	Between 85-115
Shelf Life (year)	1

Intended Use

Enzyme immunoassay for the quantitative determination of ipilimumab (Yervoy®) in serum and plasma. *Matriks Biotek® ipilimumab ELISA* has been especially developed for the quantitative analysis of ipilimumab in serum and plasma samples between the Cmin and Cmax range of concentrations indicated in the pharmacokinetics section of prospectus.

Summary and Explanation

Ipilimumab, a recombinant human monoclonal antibody (IgG1 kappa immunoglobin), is an antineoplastic agent. Ipilimumab is indicated for the treatment of unresectable or metastatic melanoma in adults. It is also used to reduce the risk of the deadly skin cancer returning after surgery.

The pharmacodynamics of Ipilimumab are not completely understood. In melanoma patients receiving Ipilimumab, the mean peripheral blood absolute lymphocyte counts (ALC) increased throughout the induction dosing period. This increase occurred in a dose-dependent fashion in Phase 2 studies.

Ipilimumab given with or without gp100 at 3 mg/kg increased ALC throughout the induction dosing period, but no meaningful change in ALC occurred in the control group who received an investigational peptide vaccine alone.

Furthermore, ipilimumab binds to CTLA-4 with high affinity (Kd = 5.24 ± 3.62 nM). As a result, ligands CD80 and CD86 are blocked from binding to CTLA-4 with a minimum EC50 value of 0.2 µg/mL.

Ipilimumab is a fully human IgG1κ antibody that binds to CTLA-4 (cytotoxic T lymphocyte-associated antigen 4), a molecule on T-cells that is indicated for unresectable or metastatic melanoma. The absence or presence of CTLA-4 can augment or suppress the immune system's T-cell response in fighting disease. Ipilimumab is designed to block the activity of CTLA-4, thereby sustaining an active immune response in its attack on cancer cells. The proposed mechanism of action is indirect, and may be through T-cell - mediated anti-tumor immune responses.

In one pharmacokinetic study of patients with unresectable or metastatic melanoma peak concentrations, trough concentrations, and area under the curve (AUC) were found to be dose proportional in the dosage range examined (0.3, 3, or 10mg/kg every 3 weeks for four doses).

The metabolism of ipilimumab does not involve the cytochrome P450 enzyme system. Because ipilimumab is a protein it is expected to be degraded into small peptides and amino acids by proteolytic enzymes.

Clearance was measured to be 15.3mL/hr-16.8 mL/hr.

In one pharmacokinetic study examining ipilimumab administered every 3 weeks, clearance was found to be time invariant. Minimal systemic accumulation was observed (accumulation index of 1.5 fold or less).
Steady state concentrations was reached by the third dose.

Clearance will increase with increasing body weight; however, no dose adjustment is needed if administration occurs on a mg/kg basis. The following had no clinically meaningful influence on clearance: Age (range 26-86 years), gender, creatinine clearance (if ≥29ml/min), baseline AST, total bilirubin, ALT levels, concomitant use of budesonide, performance status, HLA-A2*0201 status, positive anti-ipilimumab antibody status, prior use of systemic anticancer therapy, baseline lactate dehydrogenase levels.

Test Principle

Solid phase enzyme-linked immunosorbent assay (ELISA) based on the sandwich principle. Standards and samples (serum or plasma) are incubated in the microtitre plate coated with the reactant specific for ipilimumab (Yervoy®). Following incubation wells are washed and then horse radish peroxidase (HRP) is added and binds to ipilimumab. After incubation, the wells are washed and the bound enzymatic activity is detected by addition of chromogen-substrate. The color developed is proportional to the amount of ipilimumab (Yervoy®)in the sample or standard. Results of samples can be determined directly using the standard curve.

Warnings and Precautions

1. For professional use only.
2. 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 please refer to the local distributor.
3. In case of severe damage of the kit package please contact Matriks biotek or your supplier in written form, latest one week after receiving the kit. Do not use damaged components in test runs, but keep safe for complaint related issues.
4. Obey lot number and expiry date. Do not mix reagents of different lots. Do not use expired reagents.
5. Follow good laboratory practice and safety guidelines. Wear lab coats, disposable latex gloves and protective glasses where necessary.
6. Reagents of this kit containing hazardous material may cause eye and skin irritations. See MATERIALS SUPPLIED and labels for details.
7. Chemicals and prepared or used reagents have to be treated as hazardous waste according the national biohazard safety guidelines or regulations.
8. Avoid contact with Stop solution. It may cause skin irritations and burns.

9. All reagents of this kit containing human serum (i.e. standards) have been tested and were found negative for HIV I/II, HBsAg and HCV. However, a 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.

10. 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.

Storage and Stability

The kit is shipped at ambient temperature and should be stored at 2-8°C. Keep away from heat or direct sun light. The strips of microtiter plate is stable up to the expiry date of the kit in the broken, but tightly closed bag when stored at 2–8°C.

Specimen Collection 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	Keep away from heat or direct sun light Avoid repeated freeze-thaw cycles
Stability:	2 d	6 mon	

*. Ipilimumab (Yervoy®) infusion camouflages/masks the presence of antibody to ipilimumab in serum/plasma samples. Therefore, blood sampling time is critical for detection of Ipilimumab. Matriks Biotek. Laboratories propose to obtain blood sample just before the infusion of ipilimumab (Yervoy®) or at least 2 weeks after the infusion of ipilimumab (Yervoy®).

Materials Supplied

1 x 12 x 8	MTP	**Microtiter Plate** Break apart strips. Microtiter plate with 12 rows each of 8 wells coated with reactant.
7 x 0.3 mL	STND A-E HIGH CNTRL LOW CNTRL	**Ipilimumab Standards A-E, High Level Control, Low Level Control** 3000, 1000, 300, 100 and 0 ng/mL Ready to use. Used for construction of the standard curve. Contains ipilimumab (Yervoy®), human serum, stabilizer and <0.1% NaN_3.
1 x 50 mL	ASSAY BUF	**Assay Buffer** Blue colored. Ready to use. Contains animal serum, proteins and <0.1% NaN_3.
1 x 12 mL	HRP CONJ	**Peroxidase Conjugate** Red colored. Ready to use. Contains horse radish peroxidase (HRP) and stabilizers.
1 x 12 mL	TMB SUBS	**TMB Substrate Solution** Ready to use. Contains TMB
1 x 12 mL	TMB STOP	**TMB Stop Solution** Ready to use. 1N HCl.
1 x 50 mL	WASHBUF CONC	**Wash Buffer**, Concentrate (20x) Contains Buffer with Tween 20.
2 x 1	ADH FILM	**Adhesive Film** For covering of Microtiter Plate during incubation.

Materials Required but not Supplied

1. Micropipettes (< 3% CV) and tips to deliver 5-1000 µL.
2. Calibrated measures.
3. Tubes (1 mL) for sample dilution.

4. Wash bottle, automated or semi-automated microtiter plate washing system.

5. Microtiter plate reader capable of reading absorbance at 450/650 nm.

6. Bidistilled or deionised water, paper towels, pipette tips and timer.

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 pretreatment 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 ready at the appropriate time. Allow all reagents and specimens to reach room temperature (18-25 °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 caps. Always cap not used vials. Do not reuse wells/tubes 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 micropipette 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/tubes. Do not open the pouch until it reaches room temperature. Unused wells/tubes should be returned immediately to the resealed pouch including the desiccant.

Pre-Test Setup Instructions

1. Preparation of Components

Dilute/ disolve	Component	with	Diluent	Relation	Remarks	Storage	Stability
10 mL	Wash Buffer*	Up to 200 mL	bidist. Water	1:20	Warm up at 37°C to dissolve crystals. Mix vigorously.	2-8 °C	2 w

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

2.Dilution of Samples

Sample	To be diluted	With	Relation	Remarks
Serum/ Plasma	1:50	Assay Buffer	1:50	For dilution at 1:50; 10µl Sample + 490µl Assay Buffer

Patient samples with a concentration of infliximab above the measuring range are to be rated as > "Highest Standard (Standard A)". The result must not be extrapolated. The patient sample in question should be further diluted with Assay Buffer and retested.

Test Procedure

1	Pipette 100µl of Assay Buffer non-exceptionally into each of the wells to be used
2	Pipette 10 µL of **Standards, High Level Control, Low Level Control and Diluted Samples** into the respective wells of microtiter plate. Wells A1: Standard A B1: Standard B C1: Standard C D1: Standard D E1: Standard E F1: High Level Control G1: Low Level Control H1 and on: Sample (Serum / Plasma)
3	Cover the plate with adhesive foil. Incubate 30 min at room temperature (18-25°C).
4	Remove adhesive foil. Discard incubation solution. Wash plate 3 times each with 300 µL of diluted Wash Buffer. Remove excess solution by tapping the inverted plate on a paper towel.
5	Pipette 100 µL of ready-to use Peroxidase into each well.
6	Cover the plate with adhesive foil. Incubate 30 min at room temperature (18-25°C).
7	Remove adhesive foil. Discard incubation solution. Wash plate 3 times each with 300 µL of diluted Wash Buffer. Remove excess solution by tapping the inverted plate on a paper towel.
8	Pipette 100 µL of TMB Substrate Solution into each well.
9	Incubate 10 min (without adhesive foil.) at room temperature (18-25°C) in the dark.
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 with a photometer at 450/650 nm within 30 min after pipetting of the Stop Solution.

Quality Control

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

Calculation & Interpretation of Results

1. Using the standards (3000; 1000; 300; 100; 0 ng/mL) disregarding zero standard, construct a standard curve by plotting the OD450/650 nm for each of 4 standards on the vertical (Y-axis) axis versus the corresponding ipilimumab concentration on the horizontal (X-axis) axis, thus creating a standard curve by 4 points obtained.

2. The concentration of the samples can be read directly from this standard curve. Using the absorbance value for each sample, determine the corresponding concentration of ipilimumab from the standard curve. Find the absorbance value on the Y-axis and extend a horizontal line to the curve. At the point of intersection, extend a vertical line to the X-axis and read the ipilimumab concentration for the unknown sample.

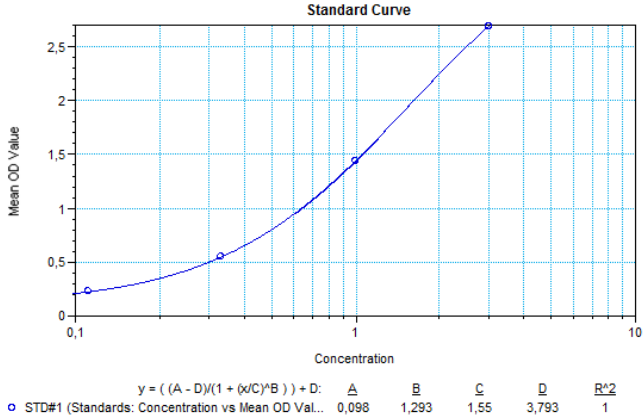
3. If computer data regation is going to be used, we recommend primarily "4 Parameter Logistic (4PL)" or secondly the "point-to-point calculation".

4. To obtain the exact values of the samples, the concentration determined from the standard-curve must be multiplied by the dilution factor (50x). Any sample reading greater than the highest standard should be further diluted appropriately with Assay Buffer and retested. Therefore, if the pre-diluted samples have been further diluted, the concentration determind from the standard curve must be multiplied by the further dilution factor.

   E.g.; *If the pre-diluted sample further diluted in a ratio of 1:10 then results should be multiplied by 500.*

5. Automated method: Computer programs can also generally give a good fit.

Typical Calibration Curve
(Example. Do not use for calculation!)



Standard	Concentration (ng/mL)	Mean OD450/650
A	3000	2,690
B	1000	1,436
C	300	0,544
D	100	0,217
E	0	0,090

Assay Characteristics

1. **Specificity:** Except for ipilimumab, there is no cross reaction with other therapeutic antibodies and native serum immunoglobins.

2. **Sensitivity:** The lowest detectable level that can be distinguished from the zero standard is 100 ng/mL.

3. **Precision Of Kit:**
   Intra-assay CV: <15% for ipilimumab range 3000-100 ng/mL
   Inter-assay CV: <15% for ipilimumab range 3000-100 ng/mL

4. **Recovery:** Recovery rate was found to be between 85-115% with normal human serum samples with known concentrations.

Automation

Experiments have shown that the ***Matriks Biotek®*** **SHIKARI® Ipilimumab ELISA** is also suitable to run on an automated ELISA processor.

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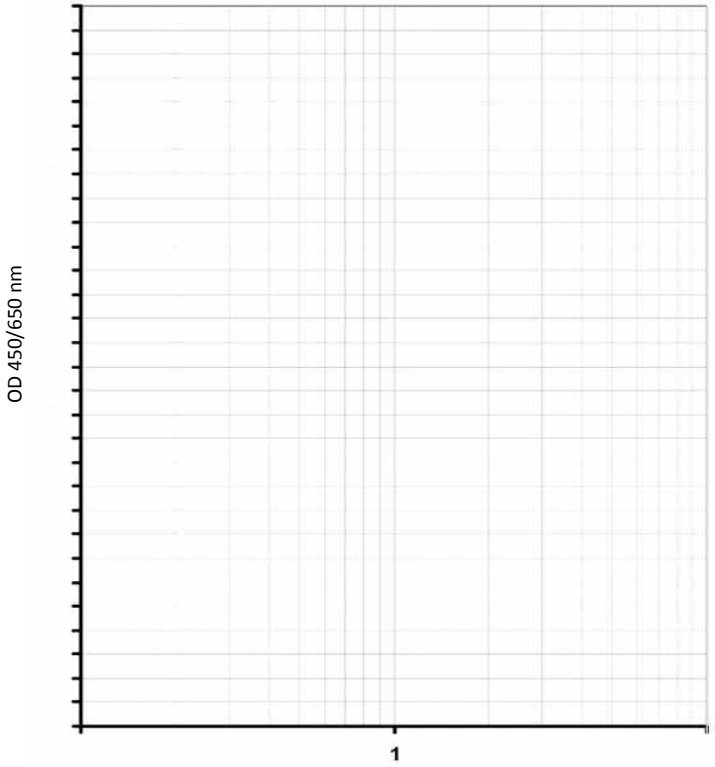
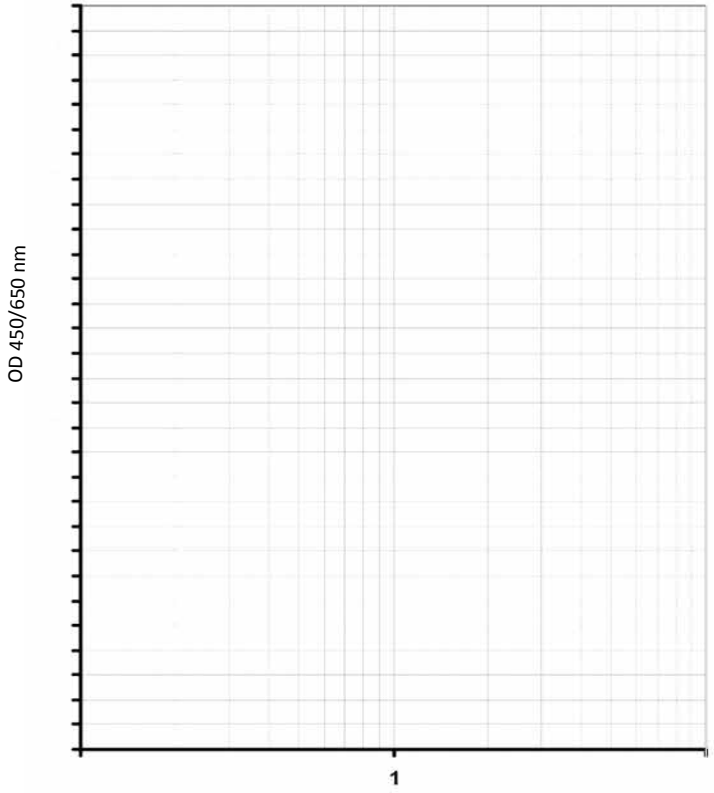

OD 450/650 nm
1
ipilimumab concentration (ng/mL)


OD 450/650 nm
1
ipilimumab concentration (ng/mL)