Intended Use

Enzyme immunoassay for the quantitative determination of **free infliximab** (Remicade®) in serum and plasma. *Matriks Biotek® Infliximab ELISA* has been especially developed for the quantitative analysis of free infliximab in serum and plasma samples at high specificity.

Summary and Explanation

Infliximab (Remicade®) is a chimeric monoclonal antibody and used to treat auto-immune disorders. Infliximab reduces the amount of active human tumour necrosis factor alpha (hTNFα) in the body by binding to it and preventing it from signaling the receptors for TNFα on the surface of various cell types. TNFα is one of the key cytokines that triggers and sustains the inflammatory reactions. Infliximab is used for the treatment of psoriasis, Crohn's disease, ankylosing spondylitis, psoriatic arthritis, rheumatoid arthritis, ulcerative colitis, and approved by FDA.

Single intravenous (IV) infusions of 3 mg/kg to 20 mg/kg showed a linear relationship between the dose administered and the maximum serum concentration. The volume of distribution at steady state was independent of dose and indicated that infliximab

was distributed primarily within the vascular compartment. Median pharmacokinetic results for doses of 3 mg/kg to 10 mg/kg in rheumatoid arthritis and 5 mg/kg in Crohn's disease indicate that the terminal half-life of infliximab is 8.0 to 9.5 days.

In controlled trials, clinical response rates of 20-40% have been achieved with above-mentioned regimens in Crohn's disease and rheumatoid arthritis. However, the therapeutic benefits must be balanced against the risk of a variety of severe adverse events (e.g. severe infections including tuberculosis, hepatotoxicity, infusion reactions, serum sickness-like disease and lymphoma). The volume of distribution of infliximab is low (3-6 L) and represents the intravascular space. Elimination of infliximab is most probably accomplished through degradation by unspecific proteases. It seemed that methotrexate delayed the decline in the serum concentrations of infliximab. When relating serum concentrations to the clinical response in patients, it can be assumed that through concentrations above 1㎎/mL could be used as a kind of therapeutic target. The rate of clinical remission was higher for patients with a detectable trough serum infliximab compared with patients in whom serum infliximab was undetectable, including those without antibodies. A detectable trough serum infliximab was also associated with a lower C-reactive protein and a higher rate of endoscopic improvement. For Crohn's disease patients treated with scheduled maintenance

infusions of infliximab, the serum concentration of infliximab seemed to predict clinical outcome. It was also proposed that, the surveillance of circulating infliximab concentration during maintenance therapy represents an indirect but reliable method to monitor anti-infliximab immunization.

In this context, identification of biomarkers for (non-)response and risk factors for adverse drug reactions relating to serum concentrations and therapeutic drug monitoring of infliximab would be very helpful.

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 for infliximab (Remicade®). After incubation, the wells are washed. A horse radish peroxidase (HRP) conjugated probe is added and binds to infliximab captured by the reactant on the surface of the wells. Following incubation wells are washed and the bound enzymatic activity is detected by addition of chromogen-substrate. The colour developed is proportional to the amount of infliximab 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 (clinical background, test performance, automation protocols, alternative applications, literature, etc.) 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 or plasma (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	

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

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.
5 x 0.3 mL	STND A-E	**Infliximab Standards A-E** 3; 1; 0.3; 0.1; 0 microgram/mL Ready to use. Used for construction of the standard curve. Contains infliximab (Remicade®), human serum, stabilizer and <0.1% NaN_3.
1 x 50 mL	ASSAY BUF	**Assay Buffer** Blue colored. Ready to use. Contains proteins and <0.1% NaN_3.
1 x 12 mL	HRP CONJ	**Horse radish peroxidase-Conjugated Probe** Red colored. Ready to use. Contains HRP-probe, stabilizer and ..... .
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	FOIL	**Adhesive Foil** For covering of Microtiter Plate during incubation.
2x 1	SLGP	**Semi-Log Graph Paper** For constructing standard curve and calculation of results.

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 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/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	4 w

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

2.Dilution of Samples

Sample	To be diluted	With	Relation	Remarks
Serum/ Plasma	Initially 1:20	Assay Buffer	1:20-1:100	For dilution at 1:20; 10µL Sample + 190µL Assay Buffer For dilution at 1:100; 5µLSample + 495µL 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 each ready-to use Standards, 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 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 HRP-Conjugated Probe 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.

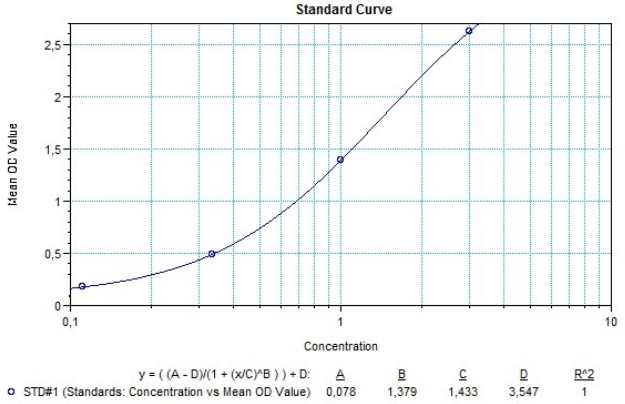
Calculation & Interpretation of Results

1. A standard curve should be calculated using the standard concentration (X-axis) versus the OD450 (we suggest that OD readings done at double wavelength at OD450/650 and OD value read at 650nm subtracted from 450nm OD VALUE) VALUES (Y-axis).In case of manual plot, we suggest semilog graph paper. If computer data regation is going to be used, we recommend primarily "4 Parameter Logistic (4PL)" or secondly the "point-to-point calculation".

2. 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 standard curve. This value always has to be multiplied by the dilution factor. If any diluted sample is reading greater than highest standard, it should be further diluted appropriately with Assay Buffer and retested. Also this second dilution has to be used for calculation the final results.

3. Any sample diluted at 1:20 and still reading greater than the highest standard should be further diluted appropriately with Assay Buffer and retested. **Because the samples have been diluted, the concentration determined from the standard- curve must be multiplied by the dilution factor.**

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



Standard	Concentration (µg/mL)	Mean OD450/650
A	3	2,627
B	1	1,390
C	0,3	0,487
D	0.1	0,177
E	0	0,044

Assay Characteristics

1. **Specificity:** Except for the other therapeutic anti-TNF antibodies such as etanercept (Enbrel®) and/or adalimumab (Humira®) with which cross reaction might occur to some extends, there is no cross reaction with native serum immunoglobulin.

2. **Sensitivity:** The lowest detectable level that can be distinguished from the zero standard is 30 ng/mL.

3. **Precision Of Kit:**
   Intra-assay CV: <20% for infliximab range 0.3-3 mg/mL.
   Inter-assay CV: <20% for infliximab range 0.3-3 mg/mL.

4. **Recovery:** Recovery rate was found to be equal and higher than 98% with normal human serum samples with known concentrations.

Automation

Experiments have shown that the infliximab ELISA is also suitable to run on an automated ELISA processor.

References

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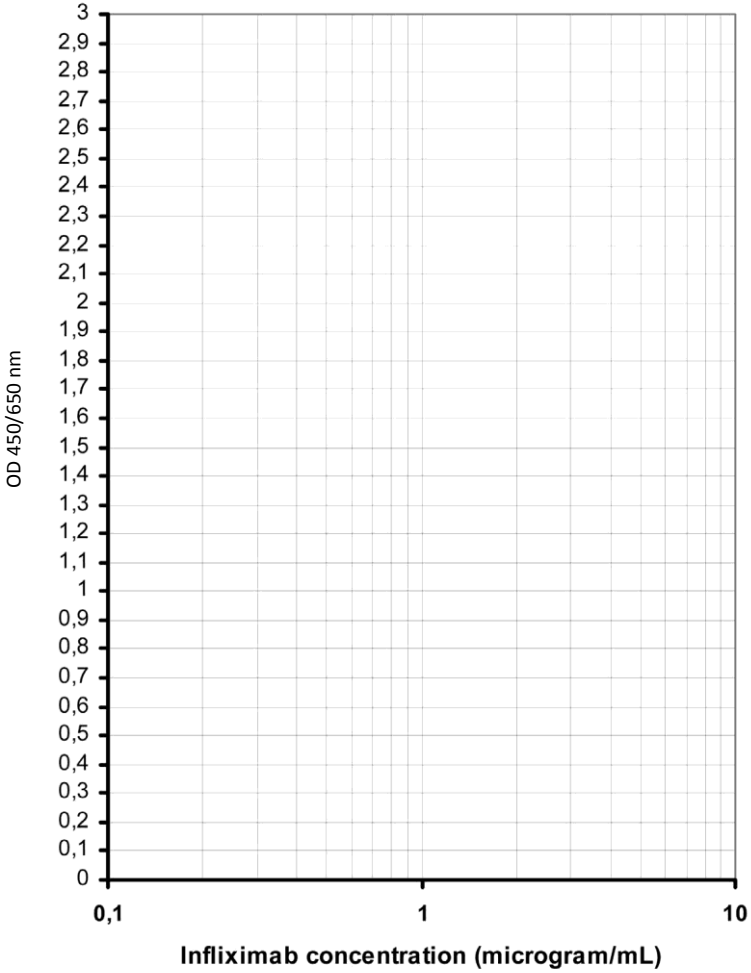
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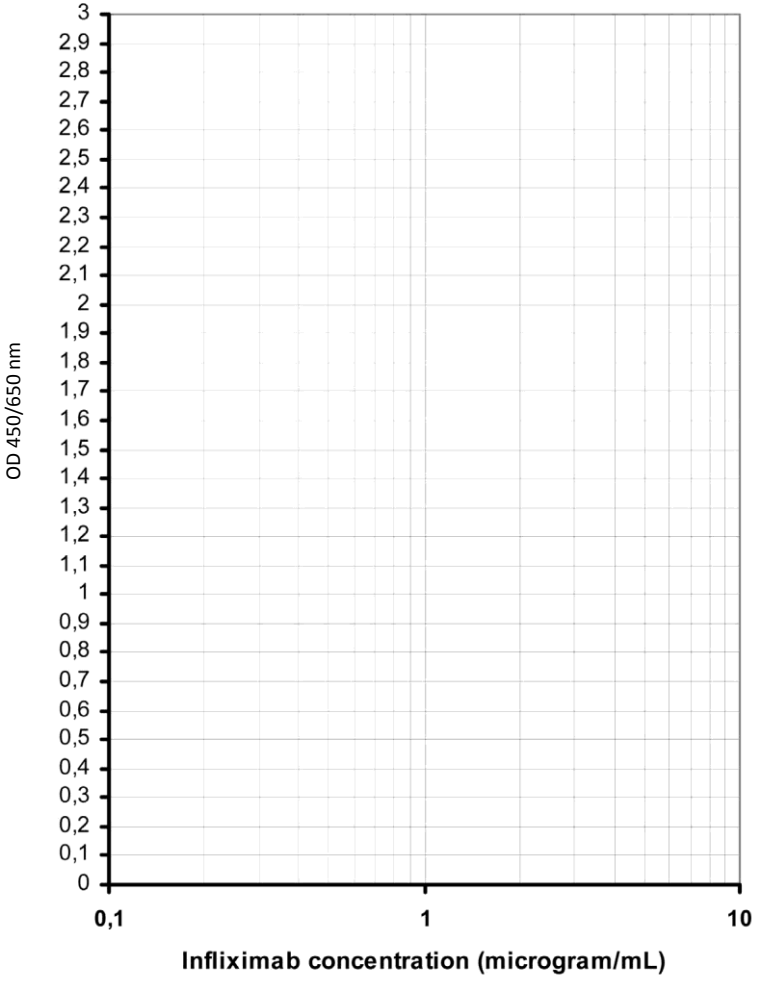
Infliximab concentration (microgram/mL)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Infliximab concentration (microgram/mL)