

# *ImmunoGuide*<sup>®</sup>

## Instructions for Use

### Infliximab ELISA (Specific)

**Double-monoclonal antibody enzyme immunoassay for the quantitative determination of Infliximab in serum, plasma and other biological fluids**

**REF** IG1001-INFv1

 12X8



2-8°C

For research use only

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## 1. INTENDED USE

Double-monoclonal antibody enzyme immunoassay for the quantitative determination of biologically active form of infliximab (Remicade®)\* in serum, plasma and other biological fluids. The reliability of the data regarding the pharmacokinetics of infliximab (Remicade®) is expected to be highly dependent on the specificity of the assay used and non specific assays might be misleading. The data reported in the literature were almost totally obtained using ELISA in which the capture ligand, coated either directly or indirectly using anti-TNF antibody, was human tumor necrosis factor alpha (hTNF $\alpha$ ). However, it is well known that human serum contain soluble TNF receptors (TNF-RI (p55/60 kDa) and TNF-RII (p75/80 kDa) ). In addition, serum sample might contain other anti-TNF therapeutic immunoglobulins such as etanercept (Enbrel®) and/or adalimumab (Humira®). Although the affinities of these are lower compared to infliximab, the ligand, TNF $\alpha$  coated on the well, could be occupied by other molecules, mentioned above, to some extends and may lead to a potential underestimation. ImmunoGuide Infliximab ELISA is the **most specific** kit in the market for the measurement of Infliximab (Remicade®) in sera, plasma and other biological fluids by the advantage of using the site-directed **Peri-1 (for coating the wells) and Peri-2 (as tracer) monoclonal antibodies** specific for Infliximab (Remicade®) only. Binding of infliximab to solid phase, pre-coated with **Peri-1** mAb, is inhibited by TNF $\alpha$  in a concentration dependent manner. In addition, when the antibody to infliximab (ATI), immunoaffinity purified from various ATI-positive human sera, was pre-incubated with infliximab the reaction was inhibited in a concentration dependent manner. Hence, the ImmunoGuide Infliximab ELISA measures the active form of infliximab (that is, not infliximab molecules that are already bound to TNF $\alpha$  or ATI). The choice of measuring the active form allows investigators to analyze the concentration-effect relationship.

\* Remicade® is a registered trademark of Centacor Ortho Biotech Inc.

## 2. SUMMARY AND EXPLANATION

**Infliximab** (Remicade®) is a chimeric monoclonal antibody and used to treat auto-immune disorders. Infliximab reduces the amount of active human tumor necrosis factor alpha (hTNF $\alpha$ ) in the body by binding to it and preventing it from signaling the receptors for TNF $\alpha$  on the surface of various cell types. TNF $\alpha$  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. When relating serum concentrations to the clinical response in patients, it can be assumed that trough serum concentrations (serum concentrations of agents at the end of a dosing period) above

1 $\mu$ g/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 whose serum infliximab was undetectable, including those without antibodies. Although, the use of low-dose could be translated into few side effects and significant economic implications, several studies have shown that secondary response failure is associated with low trough levels of anti-TNF $\alpha$  biological agents and development of antibodies against these drugs.

There are many reports in the literature regarding the clinical response to anti-TNF $\alpha$  biological agents closely follows the trough drug levels and the presence of antibodies directed against the drugs. Indeed, trough level assessments of anti-TNF $\alpha$  drugs and measurements of antibodies against these biological agents are among the measures that best predict the response of individual patients with RA.

A good clinical response of ankylosing spondylitis to treatment with infliximab was correlated with the presence of high serum trough infliximab levels and the absence of anti-infliximab antibodies, and inefficacy with the reverse.

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.

Determination of the circulating concentration of anti-TNF $\alpha$  biological agents in patients with inflammatory disorders may contribute to understanding the mechanism of treatment failures with these biologic therapies. Monitoring trough serum levels of infliximab in patients with RA and those with psoriasis showed that an incomplete response is frequently associated with low circulating levels of infliximab.

Despite the potent efficacy of infliximab in these disorders, a minority of treated patients fail to improve. Among this minority of patients, monitoring trough serum levels of infliximab could be helpful to distinguish those patients with an insufficient circulation concentration of the antagonist from those with a form of the disease refractory to TNF $\alpha$  neutralization.

It was also proposed that the variable inter-individual response is explained at least in part by individual pharmacokinetics. Clinical response in RA is indeed influenced by infliximab serum concentration, and was shown that this concentration predicts long-term disease control in daily practice. The measurement of serum trough infliximab concentration modifies the therapeutic decision for RA patients and leads to improved control of disease activity. Thus, therapeutic drug monitoring of infliximab may improve the control of disease activity in RA. It was also suggested that, as in patients with RA, the inefficacy of infliximab in patients with AS is mostly attributable to an insufficient circulating concentration of the therapeutic mAb.

Recent studies showed that there are two types of "lack of efficacy" in the failure of infliximab therapy. One is the absence of any clinical responses (primary lack of efficacy), and the other is the disappearance of an initial favorable response during therapy (secondary lack of efficacy). Although the detailed mechanisms of losing efficacy, predictive factors, and therapeutic strategies to avoid the lack of efficacy are poorly understood, a characterization of "failure of infliximab therapy" by using pharmacokinetic data provides important information to determine an optimal treatment for individual patients with infliximab-refractory RA. For example the rapid clearance of infliximab from serum appears to be the main cause of unresponsiveness to infliximab. Maintaining the trough serum concentrations above therapeutic limit levels is beneficial to favorable clinical outcomes. It may also be useful to measure the trough serum concentration of infliximab in case of treatment failure, to evaluate the opportunity of increasing the infliximab dose. If

the serum level is quite high, as observed in some patients failing to respond to treatment, it is probably unnecessary to increase the dose.

In addition, patients with optimal disease control and high infliximab concentrations might benefit from a controlled reduction in infliximab dose or an increase of dosing intervals to decrease the risk of dose-related side effects by using the advantage of measuring the trough serum concentration of infliximab.

Dose in RA, like in other rheumatic diseases, of infliximab could be tailored (agents within a particular range that is sufficient to obtain effective clinical responses but that does not lead to an occurrence of adverse effects) using trough infliximab concentration to improve clinical status and maintenance of treatment. The measurement of infliximab concentration at least in a given patient may help the clinician to decide whether to intensify, reduce the dose, or switch to another biopharmaceutical.

In this context, identification of biomarkers for (non-)response and risk factors for adverse drug reactions relating to serum concentrations and therapeutic drug monitoring may help the researcher and/or clinician in various aspects.

### **3. TEST PRINCIPLE**

Solid phase enzyme-linked immunosorbent assay (ELISA) based on infliximab-specific Peri-1 and Peri-2 monoclonal antibodies. Standards and samples are incubated in the wells of microtitre plate coated with Peri-1 mAb. After incubation, the wells are washed. Biotin-labeled Peri-2 tracer monoclonal antibody specific for infliximab is added and binds infliximab captured by the first Peri-1 mAb on the surface of the wells. Following incubation wells are washed and then streptavidine–horse radish peroxidase (HRP) is added and binds to biotin conjugated tracer. After incubation, the 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 (Remicade®) in the sample or standard. Results of samples can be determined directly using the standard curve.

### **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 ImmunoGuide 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.
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. 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.

9. Some reagents contain sodium azide ( $\text{NaN}_3$ ) as preservatives. In case of contact with eyes or skin, flush immediately with water.  $\text{NaN}_3$  may react with lead and copper plumbing to form explosive metal azides. When disposing reagents, flush with large volume of water to avoid  $\text{NaN}_3$  build-up.

## 5. 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.

## 6. SPECIMEN COLLECTION AND STORAGE

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 days	6 months	

## 7. MATERIALS SUPPLIED

1 x 12 x 8	MTP	<b>Microtiter Plate</b> Break apart strips. Microtiter plate with 12 rows each of 8 wells <b>coated with Peri-1 monoclonal antibody specific for Infliximab only.</b>
3 x 0.3 mL 1 x 3 mL	STND A-D	<b>Infliximab Standards A-D</b> 3; 1; 0.3; 0 microgram/mL Ready to use. Used for construction of the standard curve. Contains infliximab, human serum, and <0.1% $\text{NaN}_3$ .
1 x 12 mL	ASSAY BUF	<b>Assay Buffer</b> Blue colored. Ready to use. Contains proteins, mouse serum (to inhibit any potential interferences of HAMA that might present in some sera) and <0.1% $\text{NaN}_3$ .
1 x 12 mL	BIOTIN CONJ	<b>Biotin-Labeled Tracer</b> Green colored. Ready to use. Contains <b>biotin-conjugated Peri-2 monoclonal antibody specific for Infliximab</b> , mouse serum, stabilizer and <0.1% $\text{NaN}_3$ .
1 x 12 mL	STREP-HRP	<b>Streptavidine-Peroxidase</b> Red colored. Ready to use. Contains streptavidine conjugated to horse radish peroxidase (HRP) and stabilizers.
1 x 12 mL	TMB SUBS	<b>TMB Substrate Solution</b> Ready to use. Contains TMB.
1 x 12 mL	TMB STOP	<b>TMB Stop Solution</b> Ready to use. 1N HCl.
1 x 50 mL	WASH BUF CONC	<b>Wash Buffer, Concentrate (20X)</b> Contains Buffer with Polysorbate 20.

<b>3 x 1</b>	<b>ADHSV FILM</b>	<b>Adhesive Film</b> For covering of Microtiter Plate during incubation.
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## 8. MATERIALS REQUIRED BUT NOT SUPPLIED

1. Micropipettes (Multipette, < 3% CV).
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 nm.
6. Bidistilled or deionised water, paper towels, pipette tips and 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 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.

## 10. PRE-TEST SETUP INSTRUCTIONS

### 10.1. Preparation of Components

Dilute/ Dissolve	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.

### 10.2. Dilution of Samples\*

Sample	To be diluted	With	Relation	Remarks
Serum/ Plasma	Initially No	Zero Standard	1:3	For dilution at 1:3; 20µl Zero Standard+10µl Sample

\*. Patient samples with a concentration of infliximab above the highest standard should be rated as "> highest standard". The result must not be extrapolated. The sample in question should be diluted with Zero Standard (Standard D), supplied with the Kit, and then retested.

## 11. TEST PROCEDURE

1.	Pipette <b>100µl</b> of <b>Assay Buffer</b> non-exceptionally into each of the wells to be used.
2	Pipette <b>5 µL</b> of each <b>ready-to use Standards, and Samples</b> into the respective wells of microtiter plate.  <u>Wells</u> A1: Standard A B1: Standard B C1: Standard C D1: Standard D E1 and on: Sample (Serum/Plasma)
3.	Cover the plate with adhesive film. <b>Incubate 60 min</b> at room temperature (18-25°C).
4.	Remove adhesive film. Discard incubation solution. Wash plate <b>3 times</b> each with <b>300 µL</b> of diluted <b>Wash Buffer</b> . Remove excess solution by tapping the inverted plate on a paper towel.
5.	Pipette <b>100 µL</b> of ready-to use <b>Biotin Conjugate</b> into each well.
6.	Cover the plate with adhesive film. <b>Incubate 60 min</b> at room temperature (18-25°C).
7.	Remove adhesive film. Discard incubation solution. Wash plate <b>3 times</b> each with <b>300 µL</b> of diluted <b>Wash Buffer</b> . Remove excess solution by tapping the inverted plate on a paper towel.
8.	Pipette <b>100 µL</b> of ready-to use <b>Streptavidine-Peroxidase</b> into each well.
9.	Cover the plate with adhesive foil. <b>Incubate 30 min</b> at room temperature (18-25°C).
10.	Remove adhesive foil. Discard incubation solution. Wash plate <b>3 times</b> each with <b>300µL</b> of diluted <b>Wash Buffer</b> . Remove excess solution by tapping the inverted plate on a paper towel.
11.	Pipette <b>100 µL</b> of <b>TMB Substrate Solution</b> into each well.
12.	<b>Incubate 15 min</b> (without adhesive film) at room temperature (18-25°C) in the dark.
13.	Stop the substrate reaction by adding <b>100 µL</b> of <b>Stop Solution</b> into each well. Briefly mix contents by gently shaking the plate. Color changes from blue to yellow.
14.	<b>Measure</b> optical density (OD) with a photometer at <b>450 nm</b> within <b>30 min</b> after pipetting of the Stop Solution.

## 12. QUALITY CONTROL

The test results are 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 in the lot specific QC sheet 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.

## 13. CALCULATION & INTERPRETATION OF RESULTS

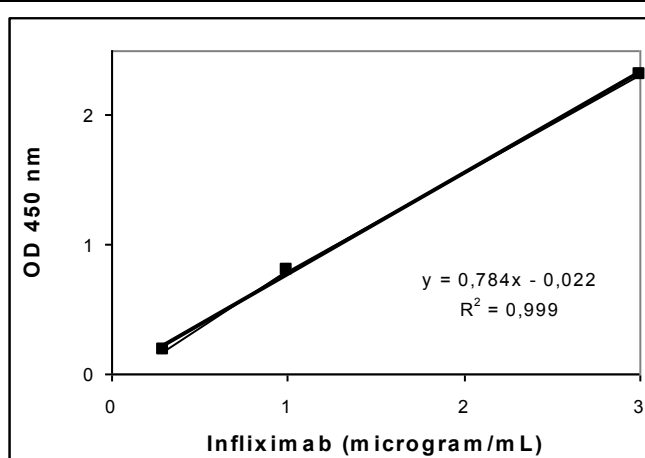
**13.1.** Construct a standard curve by plotting the absorbance obtained from each standard (**disregarding the zero standard**) against its concentration with absorbance value on the vertical (Y) axis and concentration on the horizontal (X) axis.

**13.2.** 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 Infliximab 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 Infliximab concentration for the unknown sample.

**13.3.** Any sample undiluted or diluted still reading greater than the highest standard should be further diluted appropriately with **Zero Standard** and retested. **If 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
A	3	2,320
B	1	0,810
C	0,3	0,185
D	0	0,054



## 14. ASSAY CHARACTERISTICS

### 14.1. SPECIFICITY

There is **no cross reaction** with any other proteins present in native human serum (85 different native human sera were screened and it was observed that all produced an OD below 0.080 at 450nm). In addition, **no cross reaction** was observed with the other anti-TNF therapeutic immunoglobulins (e.g. etanercept (Enbrel®) and adalimumab (Humira®)) tested at concentrations up to 500 µg/mL.

### 14.2. SENSITIVITY

The lowest detectable level that can be specifically distinguished from the zero standard is 0,250 µg/mL at the above-defined conditions. However, the sensitivity could be increased by increasing the volume of standard and sample.

### 14.3. PRECISION OF KIT

**Intra-assay CV:** <8% at the range of 1-3 µg/mL.

**Inter-assay CV:** <8% at the range of 1-3 µg/mL.

### 14.4. RECOVERY

Recovery rate was found to be equal and higher than 95% with human serum and plasma spiked with infliximab.

## 15. AUTOMATION

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

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