ImmunoGuide®

Instructions for Use

Infliximab ELISA

**Enzyme immunoassay for the determination
of free Infliximab in serum and plasma**

REF: TM09001

12X8 **2-8°C**

For Research Use Only - Not for Use in Diagnostic Procedures

Manufactured for
Immuno-Biological Laboratories Inc. (IBL-America)
8201 Central Avenue NE,
Suite P Minneapolis,
MN 55432 USA
Tel: 763-780-2955 Toll Free: 1-888-523-1246
www.ibl-america.com

1

<u>Contents</u>   **<u>Page</u>**

1. INTENDED USE

Enzyme immunoassay for the determination of free Infliximab in serum and plasma. For research use only - not for use in diagnostic procedures.

2. SUMMARY AND EXPLANATION

The drug Infliximab (trade name Remicade®, Remsima®, Inflectra®) is a chimeric monoclonal antibody and used to treat auto-immune disorders. It reduces the amount of active tumor necrosis factor alpha (hTNFα) 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. The drug is used for the treatment of psoriasis, Crohn's disease, ankylosing spondylitis, psoriatic arthritis, rheumatoid arthritis, ulcerative colitis.

Single intravenous (IV) infusion 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.

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

3. PRINCIPLE OF THE TEST

This ELISA assay is based on sandwich type ELISA. Diluted standards and samples (serum or plasma) are incubated in the microtiter plate coated with recombinant human TNF-α (rh TNF-α). After incubation, the wells are washed. A horseradish peroxidase (HRP) conjugated anti-human IgG monoclonal antibody is added and binds to the Fc part of Infliximab pre-captured by the rhTNF-α on the surface of the wells. Following 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 free Infliximab in the sample or standard. Results of samples can be determined by 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 **IBL-America** 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 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 preservatives. In case of contact with eyes or skin, flush immediately with water.

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)

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 sun light 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 recombinant human TNF-α (rhTNF-α)
5 x 0.5 mL	**Infliximab Standards A-E, Concentrate (10X)** 2000; 600; 200; 60; and 0 ng/mL Used for construction of the standard curve. Contains Infliximab, proteins, preservative and stabilizers.
1 x 12 mL	**Assay Buffer** Blue colored. Ready to use. Contains proteins and preservative.
1 x 60 mL	**Dilution Buffer,** Concentrate (5X) Contains orange dye, proteins and preservative.
1 x 12 mL	**Enzyme Conjugate** Red colored. Ready to use. Contains horseradish peroxidase(HRP)-conjugated anti-human IgG mouse monoclonal antibody, 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*

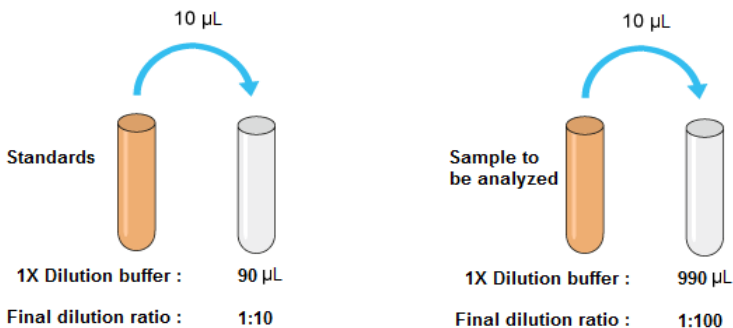| Dilute/ dissolve	Component		Diluent	Relation	Remarks	Storage	Stability
10 mL	Wash Buffer	up to 200 mL	Distilled Water	1:20	Warm up at 37°C to dissolve crystals. Mix vigorously.	2-8 °C	4 w
10 mL	Dilution Buffer	up to 50 mL	Distilled Water	1:5		2-8 °C	4 w

* 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. We are recommending that each laboratory determines the best initial dilution for their samples in order to minimize retesting.

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 990 µL of 1X dilution buffer, giving the total volume of 1000 µL and a dilution of 1:100.
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.

Standard/Sample Dilution

	10 µL			10 µL
Standards	→	Sample to be analyzed	→	
1X Dilution buffer :	90 µL	1X Dilution buffer :	990 µL	
Final dilution ratio :	1:10	Final dilution ratio :	1:100	

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.

11.2. ASSAY PROCEDURE

1.	Pipette **100 μl** of **Assay Buffer** into each of the wells to be used.
2.	Pipette **75 μL** of each **1:10 Diluted Standard, and 1:100 Diluted Samples** into the respective wells of the microtiter plate. Bubble formation during the pipetting of standards and samples must be avoided. Wells A1: Standard A B1: Standard B C1: Standard C D1: Standard D E1: Standard E F1 and so on: Samples (Serum/Plasma)
3.	Cover the plate with adhesive seal. Shake plate carefully by tapping several times. **Incubate the plate on a 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 **5 X 350 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-anti human IgG mAb) 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 **5 X 350 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 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 constructed 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 100). If any diluted sample is reading greater than the highest standard, it should be further diluted (in such a case, a 1:1000 final dilution of the sample is proposed) appropriately with 1X Dilution Buffer and retested. Also this second dilution has to be used for calculation of the final result. We are recommending that each laboratory determines the best initial dilution for their samples in order to minimize retesting.

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)	200	60	20	6	0
Mean OD450/620 nm	2.781	0.953	0.354	0.121	0.042

Fig. A

Fig. B
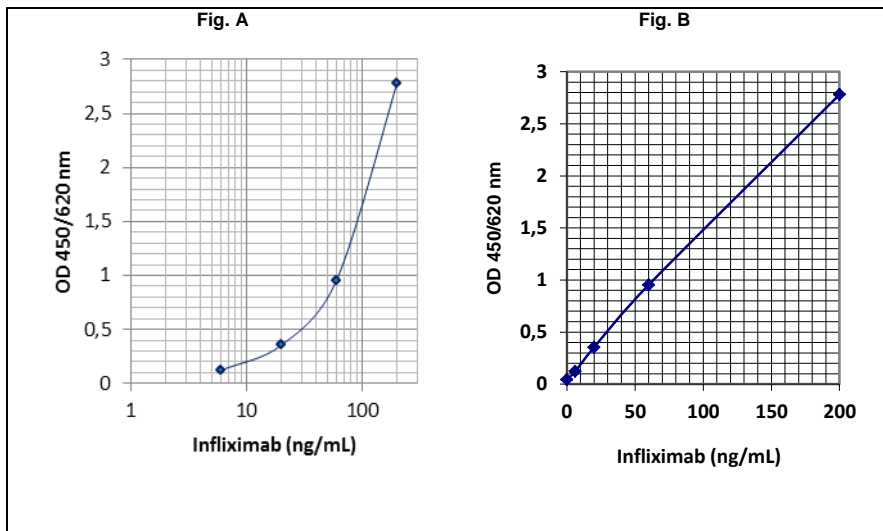
12. ASSAY CHARACTERISTICS
12.1. SPECIFICITY
There is no cross reaction with any other proteins present in naïve serum. Because the solid phase is coated with rhTNF-α, other therapeutic anti-TNF antibodies cause full cross reaction. However, a quantification of other therapeutic antibodies is possible only by using the drug-specific standards, which are available as separate kits from *ImmunoGuide*.

12.2. SENSITIVITY
The lowest detectable level that can be clearly distinguished from the zero standard is 5 ng/mL (zero standard +2SD read from the curve) under the above-described conditions: Analytical sensitivity is 5 ng/mL, and corresponding to the detection limit (limit of quantification) of 0.5 µg/mL for undiluted clinical samples because the serum or plasma samples are instructed to be diluted at 1:100 before starting the assay.

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 Infliximab.

13. AUTOMATION
The *ImmunoGuide* Infliximab ELISA is suitable also for being used by an automated ELISA processor.

14. REFERENCES
1. Lee YS, Baek SH, Kim MJ, Lee YM, Lee Y, Choe YH. Efficacy of Early Infliximab Treatment for Pediatric Crohn's Disease: A Three-year Follow-up. Pediatr Gastroenterol Hepatol Nutr. 2012; 15(4):243-9.
2. Bortlik M, Machkova N, Duricova D, Malickova K, Hrdlicka L, Lukas M, Kohout P, Shonova O, Lukas M. Pregnancy and newborn outcome of mothers with inflammatory bowel diseases exposed to anti-TNF-α therapy during pregnancy: three-center study. Scand J Gastroenterol. 2013; 48(8):951-8.
3. Grosen A, Julsgaard M, Kelsen J, Christensen LA. Infliximab concentrations in the milk of nursing mothers with inflammatory bowel disease. J Crohns Colitis. 2014; 8(2):175-6.
4. Krajcovicova A, Hlavaty T, Zelinkova Z, Letkovsky J, Huorka M. Delayed hypersensitivity reaction after initial dose of infliximab: a case report. Eur J Gastroenterol Hepatol. 2014; 26(4):485-7.
5. Ducourau E, Mulleman D, Paintaud G, Chu Miow Lin D, Lauféron F, Antibodies toward infliximab are associated with low infliximab concentration at treatment initiation and poor infliximab maintenance in rheumatic disease. Arthritis Research & Therapy 2011; 13:R105.
6. Farkas K, Rutka M, Bálint A, Nagy F, Bor R, Milassin Á, Szepes Z, Molnár T. Efficacy of the new infliximab biosimilar CT-P13 induction therapy in Crohn's disease and ulcerative colitis - experiences from a single center. Expert Opin Biol Ther. 2015; 15(9):1257-62.
7. Lee YM, Kang B, Lee Y, Kim MJ, Choe YH. Infliximab "Top-Down" Strategy is Superior to "Step-Up" in Maintaining Long-Term Remission in the Treatment of Pediatric Crohn Disease. J Pediatr Gastroenterol Nutr. 2015;60(6):737-43.
8. Gecse KB, Végh Z, Lakatos PL. Optimizing biological therapy in Crohn's disease. Expert Rev Gastroenterol Hepatol. 2015 Oct 16:1-9.
9. Choe JY, Prodanovic N, Niebrzydowski J, Staykov I, Dokoupilova E, Baranauskaite A, Yatsyshyn R, Mekic M, Porawska W, Ciferska H, Jedrychowicz-Rosiak K, Zielinska A, Choi J, Rho YH, Smolen JS. A randomised, double-blind, phase III study comparing SB2, an infliximab biosimilar, to the infliximab reference product Remicade in patients with moderate to severe rheumatoid arthritis despite methotrexate therapy. Ann Rheum Dis. 2015. pii: annrheumdis-2015-207764.
10. Thomas SS, Borazan N, Barroso N, Duan L, Taroumian S, Kretzmann B, Bardales R, Elashoff D, Vangala S, Furst DE. Comparative Immunogenicity of

TNF Inhibitors: Impact on Clinical Efficacy and Tolerability in the Management of Autoimmune Diseases. A Systematic Review and Meta-Analysis. BioDrugs. 2015; 29(4):241-58.

11. Plasencia C, Jurado T, Villalba A, Peitedado D, Casla MT, Nuño L, Bonilla MG, Martínez-Feito A, Martín-Mola E, Pascual-Salcedo D, Balsa A. Effect of Infliximab Dose Increase in Rheumatoid Arthritis at Different Trough Concentrations: A Cohort Study in Clinical Practice Conditions. Front Med (Lausanne). 2015 Oct 8;2:71. doi: 10.3389/fmed.2015.00071.

12. Minar P, Saeed SA, Afreen M, Kim MO, Denson LA. Practical Use of Infliximab Concentration Monitoring in Pediatric Crohn's Disease. J Pediatr Gastroenterol Nutr. 2015 Nov 5.doi: 10.1097/MPG.0000000000001029.

13. Plasencia C, Jurado T, Villalba A, Peitedado D, Casla MT, Nuño L, Bonilla MG, Martínez-Feito A, Martín-Mola E, Pascual-Salcedo D, Balsa A. Effect of Infliximab Dose Increase in Rheumatoid Arthritis at Different Trough Concentrations: A Cohort Study in Clinical Practice Conditions. Front Med (Lausanne). 2015 Oct 8;2:71. doi: 10.3389/fmed.2015.00071.

14. Van Stappen T, Billiet T, Vande Casteele N, Compernolle G, Brouwers E, Vermeire S, Gils A. An Optimized Anti-infliximab Bridging Enzyme-linked Immunosorbent Assay for Harmonization of Anti-infliximab Antibody Titers in Patients with Inflammatory Bowel Diseases. Inflamm Bowel Dis. 2015; 21(9):2172-7.

15. Elberdín L, Outeda M, Salvador P, Paradela S, Fernández-Torres RM, Iglesias R, Fonseca E, Martín I. Infliximab drug and antibody levels in patients with dermatological conditions. Int J Clin Pharm. 2015; 37(2):320-6.

16. Van Stappen T, Brouwers E, Tops S, Geukens N, Vermeire S, Declerck PJ, Gils A Generation of a Highly Specific Monoclonal Anti-Infliximab Antibody for Harmonization of TNF-Coated Infliximab Assays. Ther Drug Monit. 2015; 37(4):479-85.

17. Steenholdt C, Bendtzen K, Brynskov J, Thomsen OØ, Ainsworth MA Clinical implications of measuring drug and anti-drug antibodies by different assays when optimizing infliximab treatment failure in Crohn's disease: post hoc analysis of a randomized controlled trial. Am J Gastroenterol. 2014; 109(7): 1055-64.

18. Marits P, Landucci L, Sundin U, Davidsdottir L, Nilsson J, Befrits R, Wikström AC, Eberhardson M Trough s-infliximab and antibodies towards infliximab in a cohort of 79 IBD patients with maintenance infliximab treatment. J Crohns Colitis. 2014; 8(8):881-9.

19. Steenholdt C, Ainsworth MA, Tovey M, Klausen TW, Thomsen OO, Brynskov J, Bendtzen K Comparison of techniques for monitoring infliximab and antibodies against infliximab in Crohn's disease. Ther Drug Monit. 2013; 35(4):530-8.

20. Wang SL, Ohrmund L, Hauenstein S, Salbato J, Reddy R, Monk P, Lockton S, Ling N, Singh S Development and validation of a homogeneous mobility shift assay for the measurement of infliximab and antibodies-to-infliximab levels in patient serum. J Immunol Methods. 2012; 382(1-2):177-88.