Instructions for use

Sanquin Reagents B.V.
Plesmanlaan 125
1066 CX Amsterdam
The Netherlands

Phone: +31 20 5123599
Fax: +31 20 5123570
Reagents@sanquin.nl
www.sanquin.org/biologics

MabTrack level infliximab

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For professional use only

ELISA for quantitative measurement of infliximab drug levels

Ab	BUF	CAL	CONJ	HRP
Antibodies against	Buffer	Calibrator	Conjugate	HRP

HUM	IFX	MOU	REK	TNF
Human	Infliximab	Mouse	Recombinant	TNF

WELL	≥ - ≤
Well	Range

Intended use
MabTrack level infliximab is an enzyme linked immunoassay (ELISA) for fast, reproducible and specific quantitative determination of all pharmaceuticals containing the active substance infliximab in human plasma and serum samples.

General information
The therapeutic chimeric antibody infliximab affects tumour necrosis factor alpha (TNF) and is frequently administered to patients who suffer from rheumatic arthritis, intestinal disorders, dermatological diseases and cancer. TNF plays an important role in inflammation; it causes for example pain, swollen joints and stiffness in rheumatoid arthritis patients. Inhibition of TNF is therefore believed to relieve some of these symptoms and thus to improve quality of life of patients. Plasma and serum levels of TNF inhibitors are highly variable between patients, and clearly correlate to the clinical symptoms in patients. In approximately 8%-43% of the patients treated with infliximab, antibodies are formed directed towards infliximab. This can partially hamper the function of the TNF inhibitor and can cause a reduction in plasma concentration of the TNF inhibitor. Identification of drug levels can be important for patient adjusted treatment schedules, as low drug levels are frequently an indication for antibody formation against infliximab. In addition, low drug levels may be a sign of ineffectiveness of infliximab before rebound of clinical symptoms. Alternatively, it is proposed that in patients that respond well to infliximab, the dosing of infliximab can be reduced according to serum concentrations. Drug level tests can therefore help to adapt patient medication or to switch to an alternative TNF inhibitor. This infliximab level ELISA has been developed for fast, reproducible and specific quantification of infliximab concentrations in plasma and serum.

Clinical optimal ranges, e.g. therapeutic cut-off, were determined at Sanquin Diagnostic Services with the infliximab ELISA for rheumatoid arthritis to be minimally 3.0 µg/mL (Van de Bemt *et al*. 2013). For inflammatory bowel disease, the optimal range is 3.0–7.0 µg/mL (Vande Casteele *et al*.).

The MabTrack level infliximab kit is calibrated on the WHO International Standard sold by the National Institute for Biological Standards and Control (#16/170).

Principle of the test
The MabTrack level infliximab ELISA is a "sandwich-type" of enzyme immunoassay. In the microtiter plates, TNF is captured by monoclonal antibodies coated to polystyrene microtiter wells. The infliximab, present in the patient sample, the calibrator or the controls, binds to the TNF on the microtiter plate. Non-bound material is then removed by washing. Subsequently, a horseradish peroxidase-labeled monoclonal anti-drug antibody is added. This antibody binds to the infliximab/TNF/anti-TNF complex present in the microtiter well. After removal of non-bound HRP conjugate by washing, substrate solution is added to the wells. A coloured product is formed in proportion to the amount of infliximab present in the sample, calibrator and controls. After the reaction has been terminated by the addition of a stop solution, absorbance is measured in a microtiter plate reader. From the absorbance of samples and those of the calibrator curve, the concentration of infliximab can be determined by interpolation with the calibrator curve.

Package contents

Mouse-anti-TNF/recombinant TNF pre-coated microtiter plate	12 x 8 wells	-	REF M2911	ready for use
Calibrator 1 - 6	6 x 1 mL	black caps	REF M2922	ready for use
Control 1	1 x 1 mL	clear cap	REF M2923	ready for use; therapeutic range
Control 2	1 x 1 mL	clear cap	REF M2924	ready for use; therapeutic range
Human anti-infliximab HRP-conjugate	1 x 12.5 mL	brown bottle	REF M2925	ready for use
Wash buffer stock solution	1 x 50 mL	white bottle	REF M1805	dilute 1:20 in distilled water
HPE dilution buffer	1 x 50 mL	white bottle	REF M2940	ready for use
TMB substrate solution	1 x 12.5 mL	brown bottle	REF M1821	ready for use
Stop solution 0,18 M H_2SO_4	1 x 13.0 mL	white bottle	REF M1823	ready for use
Plate seals	10 x	-	-	-

- The flat-bottom microtiter plate consists of 12 strips of 8 wells ready for use. All the wells are coated with TNF-specific mouse monoclonal antibody and recombinant TNF. The microtiter plate is vacuum sealed in a plastic pouch containing desiccant. The kit provides the flexibility to use the microtiter plate on separate occasions. Determine the number of strips required to test the desired number of samples plus 8 wells needed for running calibrators and controls. Remove strips that will not be used from the microtiter plate-frame and re-pack them in the plastic pouch containing the desiccant.
- After opening, all reagents and the microtiter plate strips may be used for ≤ 6 weeks if stored at 2–8 °C.
- Consult the enclosed information leaflet for the kit specific infliximab concentrations in calibrator 1-6 and in control 1 and control 2.

Additional materials and/or equipment
- Distilled or deionised water.
- Calibrated pipettes (5-1000 µL).
- Multichannel pipette (30–300 µL).
- Beakers, flasks, cylinders, and liquid containers necessary for preparation of reagents.
- Microtiter plate reader (for reading OD at 450 nm).

Precautions
For in vitro diagnostic use only. Reagents should be stored at 2–8°C. Leaking or damaged vials must not be used. Reagents (unopened or opened) should not be used beyond the expiration date, which is printed on the label of the vial. The reagent cannot be assumed to be free from infectious agents. Care must be taken in the use and disposal of each container and its contents. Waste-disposal, after completion of the test, should be performed according to your laboratory regulations.

Test procedure
Specimen collection and preparation
1. Trough samples must be used to measure the concentration of infliximab, thus samples must be taken within 24 hours BEFORE the drug is injected to make sure that the indicated expected levels reflect the trough level of the patient.
2. Only serum and EDTA plasma can be used in the assay.
3. Separate plasma or serum from the blood cells within 4 hours after collection and perform the analyses immediately. If testing of the samples is delayed, they can be stored at 2-8 °C for 72 hours. If samples are not analysed within 72 hours, the samples must be stored frozen, they can be stored at ≤ -18 °C for 12 months.
4. Aliquot samples to avoid freeze-thaw cycles.
5. Prior to the assay, frozen samples must be thawed at room temperature. Do not use 37 °C or 56 °C water baths for thawing.
6. Mix the samples just before preparing the dilutions.

Dilution of the samples
1. Singular determinations of patient samples can be used when measurements are performed on validated protocols on automated ELISA systems. When tested manually, it is recommended to perform duplicate determinations for each sample.
2. A dilution of 1:1500 can be used in this assay to measure infliximab levels in patients.
3. If the concentration of the sample is too high to obtain an exact concentration, repeat the test with a sample dilution of 1:2000 to obtain a reliable result.
4. If the concentration of the sample is too low to obtain an exact concentration, repeat the test with a sample dilution of 1:200 to obtain a reliable result.

Dilution	Sample type	HPE volume
1:50	5 µL undiluted patient sample	245 µL
1:200	50 µL 1:50 pre-diluted patient sample	150 µL
1:1500	10 µL 1:50 pre-diluted patient sample	290 µL
1:2000	5 µL 1:50 pre-diluted patient sample	195 µL

Preparation of the working-strength solution of the wash buffer
Prepare a working-strength solution by adding 50 mL of the wash stock solution (this is the total volume of one bottle) to 950 mL of distilled water. The working-strength solution can be stored up to 2 months at 2-8 °C.

Preparation for the ELISA test procedure

1. Allow all reagents to reach room temperature (18–25 °C).
2. The complete assay must be performed at room temperature (18-25 °C) without shaking.
3. Do not allow wells to stand uncovered or dry for extended periods between incubation steps.
4. Carefully remove all air bubbles from the wells before incubation.
5. To avoid cross contamination use disposable pipette tips for each transfer and use new plate seals for each incubation/fixation step in the ELISA experiment.
6. Mix all reagents thoroughly but gently before use (without foaming).

Performance of the ELISA test procedure

1. Remove the microtiter plate with the required number of microtiter plate strips from the pouch. The unused strips can be stored in the plastic pouch with the desiccant.
2. Prepare the wash buffer and the samples according to protocol.
3. Add 100 µL per well of calibrators, controls or diluted patient samples according to the proposed microtiter plate lay-out or your own lay-out. Close the vials of the calibrators and controls after use, to prevent evaporation.
4. Cover the microtiter plate with adhesive seal and incubate for 1 hour.
5. Aspirate supernatants from wells and fill each well with 250 µL of diluted wash buffer. - Leave the wash buffer in each well for 30 to 60 seconds per washing cycle, then empty the wells. After washing (manual and automated washing), thoroughly dispose of all liquid from the microtiter plate by tapping it on absorbent paper with the openings facing downwards to remove all residual wash buffer. Repeat this four times. After the final wash the wells must be dry!
6. Add 100 µL of the anti-infliximab HRP-conjugate to each well.
7. Cover microtiter plate with adhesive seal and incubate for 1 hour.
8. Aspirate supernatants from wells and fill each well with 250 µL of diluted wash buffer. - Leave the wash buffer in each well for 30 to 60 seconds per washing cycle, then empty the wells. After washing (manual and automated washing), thoroughly dispose of all liquid from the microtiter plate by tapping it on absorbent paper with the openings facing downwards to remove all residual wash buffer. Repeat this four times. After the final wash the wells must be dry!
9. Add 100 µL of TMB substrate solution to each well.
10. Incubate the microtiter plate in the dark and do not shake. Check the colour formation every 5 minutes, when the blue colour has developed in the positive wells and the blank is still colourless the reaction must be stopped. The average incubation time is 10 ± 1 minutes.
11. Stop the reaction by adding 100 µL of stop solution per well.
12. Measure the microtiter plate in an ELISA reader at A450 nm. Read the plate within 30 minutes after the stop solution is added. It is allowed to use a second, reference wavelength of 540–620 nm during measurement.

Proposed microtiter plate lay-out

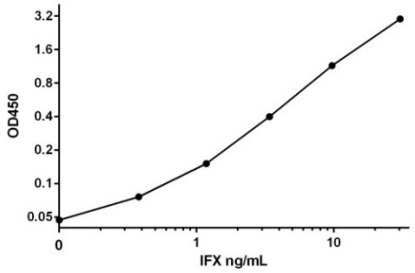
The calibration curve and controls must be included for each quantitative analysis run, they can be performed in one single row. The reagents provided give the user the possibility to use the microtiter plate in one to maximally four runs. A proposed microtiter plate lay-out is given for use in one single run.

	1	2	3	4	5	6	7	8	9	10	11	12
A	CAL1	sample 1 DIL 1	sample 5 DIL 1	sample 9 DIL 1	sample 13 DIL 1	sample 17 DIL 1	Sample21 DIL 1	sample 25 DIL 1	sample 29 DIL 1	sample 33 DIL 1	sample 37 DIL 1	sample 41 DIL 1
B	CAL2	sample 1 DIL 1	sample 5 DIL 1	sample 9 DIL 1	sample 13 DIL 1	sample 17 DIL 1	sample 21 DIL 1	sample 25 DIL 1	sample 29 DIL 1	sample 33 DIL 1	sample 37 DIL 1	sample 41 DIL 1
C	CAL3	sample 2 DIL 1	sample 6 DIL 1	sample 10 DIL 1	sample 14 DIL 1	sample 18 DIL 1	sample 22 DIL 1	sample 26 DIL 1	sample 30 DIL 1	sample 34 DIL 1	sample 38 DIL 1	sample 42 DIL 1
D	CAL4	sample 2 DIL 1	sample 6 DIL 1	sample 10 DIL 1	sample 14 DIL 1	sample 18 DIL 1	sample 22 DIL 1	sample 26 DIL 1	sample 30 DIL 1	sample 34 DIL 1	sample 38 DIL 1	sample 42 DIL 1
E	CAL5	sample 3 DIL 1	sample 7 DIL 1	sample 11 DIL 1	sample 15 DIL 1	sample 19 DIL 1	sample 23 DIL 1	sample 27 DIL 1	sample 31 DIL 1	sample 35 DIL 1	sample 39 DIL 1	sample 43 DIL 1
F	CAL6 = blank	sample 3 DIL 1	sample 7 DIL 1	sample 11 DIL 1	sample 15 DIL 1	sample 19 DIL 1	sample 23 DIL 1	sample 27 DIL 1	sample 31 DIL 1	sample 35 DIL 1	sample 39 DIL 1	sample 43 DIL 1
G	CTRL 1	sample 4 DIL 1	sample 8 DIL 1	sample 12 DIL 1	sample 16 DIL 1	sample 20 DIL 1	sample 24 DIL 1	sample 28 DIL 1	sample 32 DIL 1	sample 36 DIL 1	sample 40 DIL 1	sample 44 DIL 1
H	CTRL 2	sample 4 DIL 1	sample 8 DIL 1	sample 12 DIL 1	sample 16 DIL 1	sample 20 DIL 1	sample 24 DIL 1	sample 28 DIL 1	sample 32 DIL 1	sample 36 DIL 1	sample 40 DIL 1	sample 44 DIL 1

Results

1. Every in-house or on-line available software method for calculation of concentrations may be used. Use non-linear regression analysis for curve fitting. Four-parameter (4PL) logistic regression analysis is recommended, but five-parameter (5PL) logistic regression or third order polynomial curve fitting can be used as well. A general method for calculation by hand is given.
2. Record the absorbance at 450 nm for each well containing calibrator.
3. Plot the absorbance on the Y-axis on a linear scale and plot the infliximab concentration of the calibrator sample on the X-axis on a log scale and draw the best fitting curve.
4. Record the absorbance at 450 nm for each well containing a specific sample.
5. Locate the net average absorbance value found for each sample on the vertical axis and follow a horizontal line intersecting the calibrator curve.
6. Draw a vertical line from the intersection of the calibrator curve towards the X-axis.
7. At the intersection with the X-axis, read the infliximab concentration from the horizontal axis.
8. Multiply the obtained infliximab concentration with the dilution factor of the sample, this is the actual concentration of infliximab in the sample. For control 1 and control 2 a dilution factor of 1:1500 must be used.
9. Calculate the average of the duplicate values when the sample is performed in duplicate.

Example of standard curve after 10 minutes of colour formation:



Interpretation
The optimal therapeutic concentration of infliximab depends on the disease in combination with patient specific characteristics. When performing the level test for diagnostic purposes and/or to determine the patient's treatment protocol, the concentration found can never provide a definite diagnosis, but must be considered as an indication of the clinical situation possibly requiring further diagnostic investigation. Clinical parameters must be used together with the infliximab drug concentration in the process of decision making. Moreover, for patients with a low concentration of infliximab, anti-infliximab antibodies must be measured and added to the results of the immunogenicity test and the clinical parameters in the process of decision making.

Indication of therapeutic levels for patients treated with infliximab*	
Healthy donors and patients not treated with infliximab	negative
Sub-therapeutic levels of infliximab	< 3.0 µg/mL
Normal therapeutic levels of infliximab	3.0 – 7.0 µg/mL**
Elevated levels of infliximab	> 7.0 µg/mL

*Only indications for therapeutic values are given, each laboratory must define its own cut-off values for diagnostic purposes.
**The normal therapeutic level is the concentration at which a patient has a higher probability of a good to moderate clinical response. The normal therapeutic level is not necessarily equal to the optimal therapeutic level. The optimal therapeutic level is related to the disease and the patient's individual parameters.

Specifications
The values cited for specific performance characteristics of the test represent typical results and are not to be viewed as specifications for this kit. Consult the enclosed information leaflet for the kit specific assay range and for the infliximab concentration in the calibrators.

Recovery	:	94% at 2 µg/mL (1:1500)	
Limit of quantification			
lower	:	0.08 µg/mL (1:200)	
higher (antigen excess)	:	no antigen access observed (47 µg/mL at 1:2000)	
Precision		Total precision	between-run precision
0.30 µg/mL (1:200)	:	11.0%	9.5%
2.14 µg/mL (1:1500)	:	8.8%	5.8%
17.3 µg/mL (1:2000)	:	7.4%	6.5%
Linear range	:	0.22–39.7 µg/mL	
No cross reactivity with	:	TNF inhibitors adalimumab, etanercept and golimumab	
Interference factors	:	interference < 20% with:	
		haemoglobin - 5 and 40 mg/mL bilirubin conjugated - 0.02 and 0.5 mg/mL bilirubin unconjugated - 0.1 and 1.5 mg/mL triglycerides - 15 and 50 mg/mL human serum albumin - 60 and 80 mg/mL rheumatoid factor (RA) - 1600 U/mL	
Method comparison			
assay	:	70 patient samples were compared to the in-house validated method of Sanquin Diagnostic Services	
analysis	:	Passing and Bablock: y = 0.91x + 0.04 Spearman correlation: 0.99	

Limitations
- The kit has been designed for professional use only, the user must be trained and familiar with ELISA test procedures.
- For optimal performance of ELISA make sure that all pipets and systems are checked and under full maintenance service according to described procedures of the manufacturers.
- Only manual testing of this kit, as described in this IFU, is validated by Sanquin. All claims in this IFU are validated with the manual testing procedure. When using the kit on an ELISA automate, the test must be validated by the user before use. The claims in this IFU are not valid for the performance of this kit on an ELISA machine.
- Do not use the reagents after the expiry date that is mentioned on the labels.
- When the controls are not within the indicated range, the results are not valid and the test must be repeated.
- As the controls are pre-diluted, they cannot be used to check sample and reagent preparation by the user.
- Samples that have an OD450 nm outside the calibrator curve are not valid and cannot be used for calculations; extrapolation of the results is not acceptable. These samples must be measured in lower or higher dilutions.

- False positive or negative results can be obtained when samples are used with interference factors higher than indicated in the specifications.
- Only plates, HRP-conjugate, calibrators and controls supplied with the kit must be used, do not use these components from different batches , these are not interchangeable. However, the HPE sample buffer, TMB, Stop and Wash buffer may be used from other MabTrack kits, provided that the materials are within their shelf life, the materials were stored in closed bottles at 2-8 °C and are opened no longer than 6 months earlier. These components can be mixed before performing the ELISA, e.g. to overcome dead volume issues. Batch numbers and expiry dates can be found on each label of the separate components.
- Reagents or remnants of reagents (e.g. dead volume) cannot be mixed with contents of freshly opened vials.
- Caps and vials are not interchangeable, caps must be replaced on the corresponding vials.
- NaN_3 cannot be added to the reagents, this affects the performance of the test.
- Do not use aluminium foil during the incubation steps.
- The concentrated buffer may contain salt crystals. Before preparing the working-strength buffer, warm the concentrated buffer BRIEFLY to 37°C to dissolve the crystals.

References

1. van den Bemt B.J.F.; Annals of the Rheumatic Diseases. 2008;67:1697-1701.
2. Aarden L.; Current Opinion in Immunology. 2008;20(4):431-435.
3. de Vries M.K.; Annals of the Rheumatic Diseases. 2007;66(9):1252-1254.
4. Wolbink G.J.; Arthritis & Rheumatology. 2006;54(3):711-715.
5. Van der Bemt B.J.F.; British Journal of Clinical Pharmacology. 2013;76(6):939-945.
6. Vande Casteele N.; Gastroenterology. 2015;148:1320-1329.

For a list of more publications by Sanquin on infliximab see www.sanquin.org/biologics.

Sanquin products are guaranteed to perform as described in the original manufacturer's instructions for use. Strict adherence to the procedures, test layouts and recommended reagents and equipment is essential. Sanquin declines all responsibility arising from any deviation thereof.