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M2960 Lot: Expiration:

ELISA for the measurement of antibodies to infliximab

General information

The ELISA for the measurement of antibodies to infliximab is an enzyme linked immunoassay (ELISA) for fast, reproducible and specific semi-quantitative determination of antibodies against infliximab in human plasma and serum samples. Low drug levels are frequently an indication for antibody formation against infliximab. In approximately 25 to 30% of the rheumatoid arthritis patients treated with infliximab, antibodies are formed directed towards the idiotype of infliximab. This can hamper the function of the TNF inhibitor and can cause a reduction in plasma concentration of the TNF inhibitor. By closely monitoring drug levels and antibody formation in combination with disease activity, it is possible for the physician to objectively determine the drug activity in an individual patient and to design patient specific treatment schedules. The therapeutic antibody infliximab affects tumor necrosis factor (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 may cause pain, swollen joints and stiffness in rheumatoid arthritis patients and ulcers in patients with intestinal bowel disease. 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.

Principle of the test

The ELISA for infliximab antibodies is a "sandwich-type" of enzyme immunoassay. In the microtiter plates, infliximab is captured to polystyrene microtiter wells. Free anti-infliximab antibodies, present in the patient sample, level 1-2 and the positive and negative control, binds to the infliximab on the microtiter plate. Non-bound material is then removed by washing. Subsequently, horseradish peroxidase-labelled infliximab is added. HRP-labelled infliximab binds to the infliximab/ anti-infliximab complex present on the surface of the microtiter well. After removal of non-bound HRP conjugate by washing, substrate solution is added to the wells. A colored product is formed in proportion to the amount of anti-infliximab antibodies present in the sample 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 levels, the samples can be qualified negative (-), (weak) positive (+), strong positive (++) for anti-infliximab antibodies.

Package contents

Infliximab pre-coated microtiter plate	6 x 8 wells	-	M2961	ready for use
Level 1	3 x 0.50 mL	clear caps	M296201	ready for use
Level 2	3 x 0.50 mL	clear caps	M296202	ready for use
Control +	3 x 0.50 mL	clear caps	M296203	ready for use
Control	3 x 0.50 mL	clear caps	M296204	ready for use
Infliximab HRP-conjugate	3 x 2.0 mL	brown bottle	M2965	ready for use
Wash buffer stock solution	1 x 50 mL	white bottle	M1805	dilute 1:20 in distilled water
HPE dilution buffer	1 x 50 mL	white bottle	M2940	ready for use
TMB substrate solution	1 x 12.5 mL	brown bottle	M1821	ready for use
Stop solution 0,18 M H_2SO_4	1 x 13.0 mL	white bottle	M1823	ready for use
Plate seals	10 x	- -		-

The flat-bottom microtiter plate consists of 6 strips of 8 wells ready for use. All the wells are coated with infliximab. The microtiter plate is vacuum sealed in a plastic pouch containing desiccant. The kit provides the flexibility to use the microtiter plate on 3 separate occasions. Determine the number of strips required to test the desired number of samples plus 4 wells needed for running the controls. Remove strips that will not be used from the microtiter plate-frame and re-pack them in the plastic pouch containing the desiccant.

-Vials of level 1, level 2, positive control, negative control and HRP-conjugate once opened need to be discarded after use.

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Additional materials and/or equipment

- Distilled or deionized 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 RESEARCH 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 analyzed 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. The serum samples are each incubated in one well. To improve the reliability of the ELISA test, it is recommended to perform duplicate determinations for each sample.
2. A dilution of 1:10 must be used in this assay to measure anti-infliximab antibodies in patients.

Dilution	Sample type	HPE volume
1:10	20 µL undiluted patient sample	180 µL

Preparation of the working-strength solution of the wash buffer

Prepare a working-strength solution by adding 50 mL of the wash buffer 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 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.

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Performance of the ELISA test procedure, continued

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 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 color formation every 5 minutes, when the blue color has developed in the positive wells and the blank is still colorless 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

	1	2	3	4	5	6	7	8	9	10	11	12
A	CTRL	Sample 3	Sample 7	Sample 11	Sample 15	Sample 19						
B	Level 1	Sample 3	Sample 7	Sample 11	Sample 15	Sample 19						
C	Level 2	Sample 4	Sample 8	Sample 12	Sample 16	Sample 20						
D	CTRL +	Sample 4	Sample 8	Sample 12	Sample 16	Sample 20						
E	Sample 1	Sample 5	Sample 9	Sample 13	Sample 17	Sample 21						
F	Sample 1	Sample 5	Sample 9	Sample 13	Sample 17	Sample 21						
G	Sample 2	Sample 6	Sample 10	Sample 14	Sample 18	Sample 22						
H	Sample 2	Sample 6	Sample 10	Sample 14	Sample 18	Sample 22						

The levels and controls must be included for each semi-quantitative analysis run. The reagents provided give the user the possibility to use the microtiter plate in one to maximally 3 runs. A proposed microtiter plate lay-out is given for use in one single run.

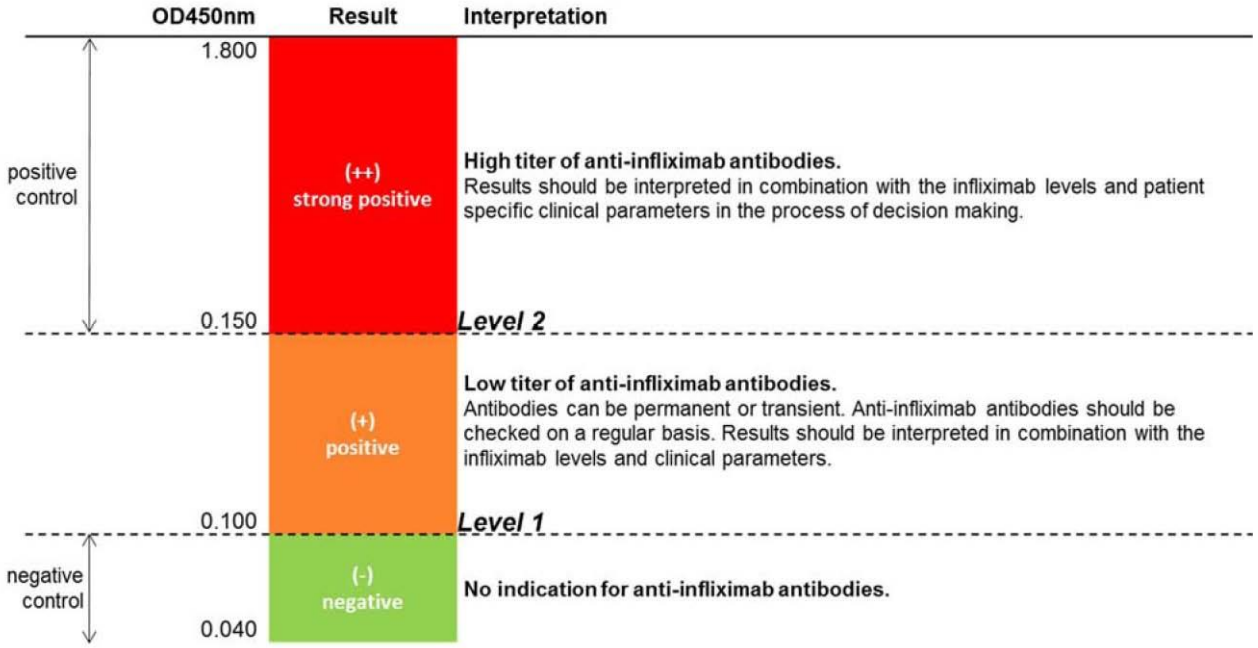
Results

1. Record the absorbance at 450 nm for each well containing the levels and the controls.
2. Record the absorbance at 450 nm for each well containing a specific sample.
3. Calculate the average of the duplicate values for the sample.
4. Compare the outcome of the samples with the levels.

Interpretation

The anti-infliximab ELISA provides 2 semi-quantitative ranges for the presence of anti-infliximab antibodies, e.g. negative (-) and positive (+ and ++), indicated by level 1. Level 2 is a secondary and arbitrary cut-off that can be used to classify patients in (weak) positive (+) and strong positive (++) for the presence of anti-infliximab antibodies. This arbitrary cut-off is based on studies. Level 2 can only be used as an indication of the amount of antibody, it cannot be used as an official diagnostic cut-off. Levels 1 & 2 contain fixed concentrations of anti-drug antibodies. The concentrations of anti-drug antibodies were measured in the in-house Radio Immunoassay. Level 1 contained 25 AU/mL (SD 3, n = 8) and Level 2 59 AU/mL (SD 3, n = 8). For the interpretation of the results, the OD450 nm of the samples should be compared with the OD450 nm of the cut-offs as described in the figure below (the OD450 nm results given in the figure are typical results and should be considered indicative). Interpretation of the results is only valid when the OD450 nm of the positive control is > level 2 and the OD450 nm of the negative control is < level 1.

The analysis of the semi-quantitative range of anti-infliximab antibodies of a patient strongly depends on the IgG subclass of the antibodies and the patients' infliximab level. IgG4 anti-infliximab antibodies cannot be detected, therefore a negative result in this test doesn't exclude the presence of anti-infliximab antibodies of the IgG4 subclass. In addition, anti-infliximab antibodies cannot be determined reliably when the infliximab level is > 0,5 µg/ml. When performing the anti-infliximab test for diagnostic purposes and/or to determine the patient's treatment protocol, the semi-quantitative range found can never provide a definite diagnosis, but must be considered as an indication of the clinical situation possibly requiring further diagnostic investigation. Patient specific characteristics and clinical parameters must be used together with the infliximab drug concentration and the anti-infliximab range in the process of decision making.



Specifications

Precision	:	High (cut-off + 30%) and low (cut-off - 30%) serum and EDTA plasma samples were measured in duplicate in 20 runs. All results were correctly measured in at least 19/20 results.
Interference factors	:	Serum and EDTA plasma samples were spiked 30% below and above the cut off (level 1). No false positive or false negative results are obtained with: hemoglobin - 1 and 50 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 - 80 mg/mL rheumatoid factor (RA) - 1600 U/ml infliximab - 0,1 µg/mL
Cut-off		EDTA plasma samples of healthy blood bank donors (n = 50) free of anti-infliximab antibodies or infliximab were measured. Cut-off level 1 is mean + five times standard deviation.
Method comparison		Concordance of 89%, tested with 50 positive samples (OD450 ≥ level 1) and 40 negative samples (OD450 < level 1). Kappa 0,75; strength of the agreement is 'good'.

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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. 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.
-Only reagents supplied with the kit must be used, do not use reagents from different batches or from different kit lots, these are not interchangeable. However, the HPE, TMB, stop and wash buffer may be used from other Cell Sciences kits for biologics, provided that the materials are within their shelf life and the materials were stored in closed bottles at 2-8 °C.
-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.
-NaN3 cannot be added to the reagents, this affects the performance of the test.
-Do not use aluminum foil during the incubation steps.
-Through levels of infliximab should be measured with the infliximab ELISA (M2920) before anti-infliximab is determined. The interpretation of the results can only be performed which both infliximab levels and antibodies are taken into account.
-The anti-infliximab ELISA is drug sensitive. Infliximab levels ≥ 0,5 µg/mL may interfere with the outcome and cause negative results. The provided cut-off of 0,5 µg/mL is, however just an indication, the amount of interference depends on the affinity of the anti-infliximab antibody for the drug. The cut-off was determined with the infliximab ELISA (M2920) and one high affinity monoclonal anti-infliximab antibody.
-The infliximab antibody is a bridging ELISA. Anti-infliximab antibodies of the IgG4 subclass cannot be detected with this type of ELISA because in IgG4 the f(ab') arms of one molecule recognize different epitopes and therefore are unable to 'bridge' between two infliximab molecules.
-As the controls are pre-diluted, they cannot be used to check sample and reagent preparation by the user.
-False positive or negative results can be obtained when samples are used with interference factors higher than indicated in the specifications.
-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

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