

Adalimumab (Humira™) ELISA

USER GUIDE

4 x 96-wells plates: An enzyme immunoassay for the quantitative determination of Adalimumab drug levels

87-51885

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Product Information

	Contents: Human CCL19 (MIP-3 beta) Adalimumab (Humira™) ELISA
	Catalog Number: 87-51885
	Temperature Limitation: Store at $\leq -20^{\circ}\text{C}$
	Batch Code: Refer to Vial
	Use By: Refer to box label
	Caution, contains preservatives

Description

The therapeutic antibody Adalimumab affects tumour necrosis factor alpha (TNF) and is frequently administered to patients that 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 subsequently 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 25 to 30% of the patients treated with Adalimumab, antibodies are formed directed towards the idiotype of Adalimumab. This can 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 Adalimumab. In addition, low drug levels may be a sign of ineffectiveness of Adalimumab before rebound of clinical symptoms. Alternatively, it is proposed that in patients that respond well to Adalimumab, the dosing of Adalimumab 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 Adalimumab level ELISA has been developed for fast, reproducible and specific quantification of Adalimumab concentrations in plasma and serum.

Technical Principle

The Adalimumab level ELISA is a "sandwich-type" of enzyme immunoassay in which TNF is captured by monoclonal antibodies which are coated to polystyrene microtiter wells. The Adalimumab, present in a measured volume of sample or standard, binds to the TNF on the microtiter plate. Non-bound material is then removed by washing. Subsequently, a biotinylated anti-idiotype antibody is added. This antibody binds to the Adalimumab/TNF/anti-TNF complex present in the microtiter well. Excess biotinylated antibody is removed by washing, followed by addition of horseradish peroxidase (HRP) conjugated streptavidin, which binds onto the biotinylated side of the anti-idiotype antibody. 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 Adalimumab present in the sample or standard. 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 a standard curve, the concentration of Adalimumab can be determined by interpolation with the standard curve.

Components of 4-plate format (4x96 tests)

Coating Antibody (Red cap): 1 vial (60 µL) Coating Antibody Concentrate (1000x)

Recombinant TNF (Black cap): 1 vial (60 µL) Recombinant TNF Concentrate (1000x)

Biotinylated Antibody (Yellow cap): 1 vial (60 µL) Biotinylated Antibody Concentrate (1000x)

Streptavidin-poly-HRP (Brown cap): 1 vial (20 µL) Streptavidin-poly-HRP Concentrate (10,000x)

HPE-dilution Buffer: 1 bottle (55 mL) HPE-dilution Buffer Concentrate (5x)

Note: The kit does not contain the Adalimumab standard, a protocol is supplied how to prepare the Adalimumab standard curve from an Adalimumab syringe of 50 mg/mL.

Other Materials Needed

- Materials
 - Pipettes for accurate delivery of 1-10 µL, 50 µL, 100 µL and 1 mL volumes.
 - Polystyrene 96-wells microtiter plates (we recommend 96-wells ELISA plates Nunc Maxisorp™ Cat. No. 44-2404-21).
 - Beakers, flasks, cylinders necessary for preparation of reagents.
 - Microtiter plate reader 450 nm. We advise a reader with a dynamic range 0 - 6.0 OD.
 - One syringe with Adalimumab (50 mg/mL or a different concentration) within the expiry date.
 - Plate sealers

- Buffers and solutions

PBS: Cat. No. 00-0044-59 ELISA/ELISPOT Coating Buffer Powder

DO NOT use PBS tablets!

Wash Buffer: Cat. No. 00-0400-46 ELISA Wash Buffer - 10 x 1L Packets

Substrate solution: Cat. No. 00-4201-56 1X TMB ELISA Substrate Solution

Stop solution: 1.8 M H₂SO₄ solution in distilled water or 1M phosphoric acid

NOTES:

- In the concentrated PBS buffer salt crystals may appear, before preparing the working-strength buffer, first warm the concentrated buffer briefly to 37°C in a water bath to dissolve the precipitate.
- Avoid repeated freeze-thawing of the TNF and antibodies. Thaw the samples in tap water (18-25°C) and do not use 37°C or 56°C water baths for this purpose.
- Use calibrated pipettes.
- Prepare dilutions in tubes or in the U-shape microtiter plate.
- Prevent wells from drying.

Stability

This ELISA set is guaranteed to perform as defined if stored and handled as instructed according to this manual, which is included with the reagents. Expiration date is indicated on the box label.

Storage Instructions for Set Reagents

Store at $\leq -20^{\circ}\text{C}$.

Test Sample Handling

- Serum and EDTA anti-coagulated plasma are suitable for use in the assay. Samples should be taken within 24 hours before the drug is injected. The indicated expected levels reflect the trough level of the patient.
- Separate plasma or serum from the blood cells within 4 hours after collection.
- If samples are not analysed within 24 hours, the samples should be stored frozen ($< -18^{\circ}\text{C}$, preferably $< -70^{\circ}\text{C}$). Aliquot samples to avoid excessive freeze-thaw cycles.
- **Fresh samples:** If samples are to be run within 24 hours, they may be stored at $2-8^{\circ}\text{C}$.
- **Frozen samples:** Prior to the assay, frozen samples should be thawed as quickly as possible in tap water ($18-25^{\circ}\text{C}$), do not use 37°C or 56°C water baths for this purpose.

CAUTION: DO NOT use grossly haemolysed or lipaemic specimens.

Assay Protocol

1. Reagents

- All reagents have to be at room temperature (RT: $18-25^{\circ}\text{C}$) before use, with the exception of the streptavidin-HRP which has to be kept at $\leq -20^{\circ}\text{C}$ to ensure stability.
- All steps should be performed at RT.
- In all steps, a volume of $100\text{ }\mu\text{L}$ /well should be used.
- All incubations, except for the coating step, should be performed in HPE buffer.
- All incubation steps, except for the coating step, should be performed on a horizontal plate shaker (maximal size $30 \times 30\text{ cm}$) $200 \pm 100\text{ rpm}$.
- Before each incubation, cover plate(s) with adhesive seal, gently agitate the microtiter plate by tapping the edge of the plate for a few seconds to mix contents of each well.

2. Washing

- Aspirate supernatants from wells and completely fill the wells ($> 250\text{ }\mu\text{L}$) with washing buffer.
- Aspirate washing buffer and completely fill the wells with washing buffer. Repeat this four times.

After the final aspiration the wells should be dry!

3. Experimental Procedure

Day 0

- Prepare the PBS as described in **Test Sample Handling**.
- Dilute the coating antibody (anti-TNF antibody) 1:1000 in PBS.
- Add 100 µL to all wells, cover microtiter plate(s) with lid and incubate overnight at RT.

Day 1

- At the start of the day, bring all reagents to 18 to 25°C, with the exception of the streptavidin-HRP which has to be kept at ≤-20°C at all times to ensure stability.
- Prepare the following solutions.

HPE-dilution buffer:

The kit contains one bottle with 5X HPE-dilution buffer concentrate. For optimal assay results, dilute samples and standard in working-strength (1X) HPE-dilution buffer (also called: **HPE buffer**).

Per microtiter plate add 13 mL 5-fold concentrated HPE buffer to 52 mL distilled water to prepare HPE buffer.

The opalescent HPE buffer can be stored for one week at 2-8°C.

Adalimumab standard of 50 ng/mL in HPE buffer:

Dilute the Adalimumab from the syringe directly into HPE buffer in 3 steps as described below.

NOTE: For the example below, we assume a concentration of 50 mg/mL of Adalimumab. Please refer to your Adalimumab vial for concentration, as the concentration may vary. Adjust the dilutions of your standard accordingly.

Step 1: Take 10 µL of the drug and dilute the drug into 990 µL HPE buffer (= 500 µg/mL)

Step 2: Take 10 µL of the solution from step 1 and dilute the drug into 990 µL HPE buffer (= 5 µg/mL)

Step 3: Take 10 µL of the solution from step 2 and dilute the drug into 990 µL HPE buffer (= 50 ng/mL)

The dilutions from each step can be stored for one week at 2-8°C.

IMPORTANT:

Use calibrated pipettes and prepare the Adalimumab dilutions in tubes. Close the tubes after each step and mix the dilutions by **GENTLY** vortexing for ~1 minute. Prevent foam formation.

Just before each washing procedure, prepare the next incubation reagent.

- Take the coated plate(s) from day 0 and wash plate(s) with washing buffer according to the washing procedure from 8 Assay Protocol, Step 2.
- Dilute the recombinant TNF 1:1000 in HPE buffer. Add 100 µL of diluted recombinant TNF per well, seal plate with plate sealer and incubate for 1 hour on a plate shaker (200±100 rpm) at RT.
- Prepare the Adalimumab standard of 50 ng/mL in HPE buffer as described previously.
- The Adalimumab standard curve can be prepared using a 2-fold dilution series in HPE buffer to generate a 7-point standard curve with 25 ng/mL Adalimumab as the top standard concentration (**standard curve range: 25, 12.5, 6.3, 3.1, 1.6, 0.8, 0.4 ng/mL**). It is recommended to run each standard point in duplicates. (See also **Proposed Plate Plan**)
- Dilute patient samples 1:100, 1:500, 1:2500 and 1:12500 in HPE buffer. It is recommended to run each sample in duplicates.

- Wash plate(s) 5-times with washing buffer.
- Add 100 μ L of diluted standard or patient sample per well, seal plate with plate sealer, and incubate for 1 hour on a plate shaker (200 $\pm$ 100 rpm) at RT. Use 100 μ L of HPE Buffer per well in duplicate to the blank wells.
- Dilute the biotinylated antibody (rabbit anti-Adalimumab antibody) 1:1000 in HPE buffer.
- Wash plate(s) 5-times with washing buffer.
- Add 100 μ L of diluted anti-Adalimumab antibody per well, seal plate with plate sealer, and incubate for 1 hour on a plate shaker (200 $\pm$ 100 rpm) at RT.
- Dilute the streptavidin-polyHRP 1:10,000 in HPE buffer.
- Wash plate(s) 5-times with washing buffer.
- Add 100 μ L of diluted streptavidin-polyHRP per well and incubate 30 minutes on a plate shaker (200 $\pm$ 100 rpm) at RT.
- Prepare the substrate solution as described in **Other Materials Needed**. The substrate must be at 18-25°C and must be protected from exposure to light.
- Wash plate(s) 5-times with washing buffer.
- Add 100 μ L of substrate solution per well and wait until the blue color has developed in the positive wells and the blank is still colorless (average incubation time is 10 $\pm$ 3 min, the total time needed for development of the color is dependent on the quality of the substrate solution).
- Stop the reaction by adding 100 μ L of 1.8M H₂SO₄ (1M phosphoric acid) to the colored substrate solution.
- Measure the plate(s) in an ELISA reader at A450 nm.

Read the plates within 30 minutes after the stop solution is added.

Results

Any in-house software capable of logistic regression analysis to calculate concentrations may be used. We recommend the four-parameter logistic equation (4PL) or five-parameter logistic equation (5PL).

An adequate, but less precise fit of the data can be generated by plotting the log of the standard concentrations on the x-axis and the value of the standard's mean OD on the y-axis. The best fit line can be generated by regression analysis. Below is a general protocol for manual calculation using the log-linear method.

Standard curve

Record the absorbance at 450 nm for each well containing standard and calculate the average of the duplicate values. Plot the net average absorbance (ordinate) versus the Adalimumab concentration (abscissa) on log-linear scale and draw the best fitting curve.

Samples

Record the absorbance at 450 nm for each well containing a specific sample, and calculate the average of the duplicate values. Locate the net average absorbance value found for each sample on the vertical axis and follow a horizontal line intersecting the standard curve. At the point of intersection, read the Adalimumab concentration from the horizontal axis. Multiply the obtained Adalimumab concentration with the dilution factor of the sample and record this figure.

Assay Ranges

The assay detects a concentration range of 0.8 to 50 ng/mL. Adalimumab values in fresh serum and plasma samples of individuals not treated with Adalimumab are below 20 ng/mL. Concentrations in individuals treated with Adalimumab usually range between 0.02–20 µg/mL. Trough samples in patients without antibodies contain median concentrations of 12 µg/mL (IQR 9-16 µg/mL).

Specificity

No cross reactivity was observed with other plasma proteins or with other TNF inhibitors.

Proposed Plate Plan

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1										
B	S2	S2										
C	S3	S3										
D	S4	S4										
E	S5	S5										
F	S6	S6										
G	S7	S7										
H	B	B										

B: substrate blank S1-S7: Adalimumab standard curve Empty: samples

Restrictions

1. User should be trained and familiar with ELISA assays and test procedure.
2. The method described in this manual is specific for manual performance of the test, (fully) automated procedures have to be validated by the user.
3. Grossly haemolysed or lipaemic samples should not be used.
4. Samples with OD values out of the standard curve range should be repeated using different dilutions.
5. Only use the reagents supplied with the kit.
6. Reagents from different batches are not interchangeable; do not mix reagents from different kit lots.
7. Leftover reagents (e.g. dead volume) should not be mixed with contents of freshly opened vials.
8. Caps and vials are not interchangeable. Caps should be replaced on the corresponding vials.
9. Sodium azide inactivates HRP, do not use sodium azide-containing solutions, nor add sodium azide to the supplied materials.
10. Mix all reagents thoroughly but gently before use (without foaming).
11. Centrifuge all vials before use (1 minute 3000 xg).

12. Do not allow wells to stand uncovered or dry for extended periods between incubation steps.
13. All samples should be considered as potentially infectious. Handle all plasma and serum samples with care to prevent transmission of blood-borne infections.
14. Preservative: contains merthiolate (0.001% w/v).
15. Use new plate seals for each incubation/fixation step in the ELISA-experiment to avoid cross contamination. Do not use aluminium foil.
16. Use disposable pipette tips for each transfer to avoid cross contamination.
17. The waste-disposal should be performed according to your laboratory regulations.

Documentation and support

Obtaining support

Technical support	For the latest services and support information for all locations, visit www.thermofisher.com. At the website, you can: • Access worldwide telephone and fax numbers to contact Technical Support and Sales facilities • Search through frequently asked questions (FAQs) • Submit a question directly to Technical Support (thermofisher.com/support) • Search for user documents, SDSs, vector maps and sequences, application notes, formulations, handbooks, certificates of analysis, citations, and other product support documents • Obtain information about customer training • Download software updates and patches
Safety Data Sheets (SDS)	Safety Data Sheets (SDSs) are available at thermofisher.com/support.
Limited product warranty	Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale found on Life Technologies' website at www.thermofisher.com/us/en/home/global/terms-and-conditions.html. If you have any questions, please contact Life Technologies at www.thermofisher.com/support.

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