Anti-Adalimumab ELISA

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The apDia Anti-Adalimumab ELISA is an enzyme linked immunosorbent assay intended for the quantitative determination of antibodies to adalimumab in human serum and plasma.

1. BACKGROUND AND DIAGNOSTIC VALUE

Adalimumab

Adalimumab (ADM) is a fully human antibody that targets the pro-inflammatory cytokine TNF-alpha. The introduction of therapeutic antibodies has revolutionized the treatment of chronic inflammatory diseases like inflammatory bowel disease (IBD), rheumatoid arthritis (RA), spondyloarthritis and plaque psoriasis. It has been shown that adalimumab can induce deep remission and improve the patient's quality of life[1]. Some patients do not respond to adalimumab therapy upon induction (primary non-responders), while others lose response over time (secondary non-responders)[2].

Immunogenicity

Secondary loss of response is often due to the development of anti-adalimumab antibodies (ATA), because of the immunogenic character of the drug. ATA can develop in any patient undergoing adalimumab therapy and are primarily neutralizing the activity of adalimumab through immunocomplex formation[3].
In addition, these immunocomplexes are rapidly cleared from the system[4]. Analytically, they are responsible for subtherapeutic adalimumab concentrations. Therefore, in the case of very low trough concentrations of adalimumab (< 1 µg/mL), subsequent measurement of ATA may be helpful to determine the optimal treatment strategy [5].

Diagnostic Value

The diagnostic value of the Anti-Adalimumab ELISA lies in its ability to stratify patients with subtherapeutic adalimumab concentrations (< 1 µg/mL) in patients who need dose intensification or a drug (class) switch. Patients with low adalimumab concentrations (< 1 µg/mL) and low ATA titers can benefit from adalimumab dose intensification, as shown in several studies[5]. However, the ATA titers of patients with low ATA titers undergoing a dose intensified treatment regimen must be adequately monitored. Patients that have high ATA titers are preferably switched to another drug, both within class or out of class. The course of ATA titers, based on the measurement of antibodies in at least two consecutive samples, is an important basis for the evaluation of the anti-drug antibody titer of the patient, rather than the absolute value of the titer.

Note. The Anti-Adalimumab ELISA is not capable of measuring ATA in the presence of high concentrations of adalimumab. It should only be used when < 1 µg/mL adalimumab is quantified in the sample using the apDia Adalimumab ELISA.

2. PRINCIPLE OF THE ANTI-ADALIMUMAB ELISA

The apDia Anti-Adalimumab ELISA uses a highly specific monoclonal antibody – clone 6A10, developed at the KU Leuven – that only bridges adalimumab[6,7].

Microtiter strips coated with adalimumab (Humira®) are incubated with calibrators, controls and diluted patient samples. During this incubation step ATA binds specifically to the adalimumab on the solid phase. After removal of the unbound serum proteins by a washing procedure, the strips are incubated with biotin conjugated adalimumab (Humira®), binding directly to the antigen-antibody complex. After removal of the unbound biotin conjugate, the strips are incubated with peroxidase conjugated streptavidin. After removal of the unbound peroxidase conjugate, the strips are incubated with a chromogenic solution containing tetramethylbenzidin and hydrogen peroxide: a blue colour develops in proportion to the amount of immunocomplex bound to the wells of the strips. The enzymatic reaction is stopped by the addition of 0.5M H_2SO_4 and the absorbance values at 450 nm are determined.
A standard curve is obtained by plotting the absorbance values versus the corresponding calibrator values. The concentration of ATA in patient samples is determined by interpolation from the calibration curve.

3. REAGENTS

Component	Name SYMBOLI
1 coated microtiter plate (12 x 8 strips)	Precoated Strips
Strips coated with adalimumab.	MTPI

6 vials, 1300 µl, ready-to-use	Calibrator CAL NI
Each vial contains a ready-to-use calibrator solution, N having following values: CAL 0: 0 ng/ml; CAL 0,1: 0,1 ng/ml; CAL 0,5: 0,5 ng/ml; CAL 1: 1 ng/ml; CAL 2,5: 2,5 ng/ml; CAL 5: 5 ng/ml anti-ADM clone 6A10. Contains 0,09 % NaN_3.	
1 vial, 1300 µl, ready-to-use	Control 1 CTL1I
Control for ATA, level 1; contains 0,375 ng/ml anti-ADM clone 6A10. Contains 0.09% NaN_3.	
1 vial, 1300 µl, ready-to-use	Control 2 CTL2I
Control for ATA, level 2; contains 3 ng/ml anti-ADM clone 6A10. Contains 0.09% NaN_3.	
1 bottle, 100 ml, ready-to-use	Sample Diluent DILSAMI
Sample dilution buffer Contains 0.09% NaN_3 and an inert orange dye.	
1 bottle, 12 ml, ready-to-use	Conjugate 1 CONJ 1I
Contains biotin conjugated adalimumab. Contains antimicrobial agents and an inert blue dye.	
1 bottle, 12 ml, ready-to-use	Conjugate 2 CONJ 2I
Contains peroxidase conjugated streptavidin. Contains antimicrobial agents and an inert red dye.	
1 vial, 12 ml, ready-to-use	Chromogen Solution CHROM
Contains a solution of substrate (H_2O_2) and chromogen (tetramethylbenzidin).	
1 bottle, 50 ml, 20x concentrated	Wash Solution WASH 20x I
Contains detergent in phosphate buffered solution and antimicrobial agents.	
1 bottle, 6 ml, ready-to-use	Stop Solution STOPI
Consists of 0.5 M H_2SO_4.	
4 plate covers	-

4. MATERIALS REQUIRED BUT NOT SUPPLIED

1. Precision micropipettes and standard laboratory pipettes.
2. Clean standard laboratory volumetric glassware.
3. Clean glass or plastic tubes for the dilution of the samples.
4. A microplate reader capable of measuring absorbances at 450 nm with reference filter at 600-650 nm.

5. WARNINGS AND PRECAUTIONS FOR USERS

1. For *in vitro* diagnostic use only.
2. Do not mix reagents or coated microtiter strips from kits with different lot numbers.
3.. Chromogen Solution contains the hazardous ingredient N-Methyl-2-pyrrolidone at a concentration $> 0,3$ %. It is classified as a Reproductive Toxicant Category 1B.
   Following hazard statements are applicable:
   H360D: May damage the unborn child.
   Following precautionary statements are applicable:
   P280: Wear protective gloves/protective clothing/eye protection/face protection.
   P308+P313: IF exposed or concerned: Get medical advice/attention.
4. Some kit components contain sodium azide as a preservative. In order to prevent the formation of potentially explosive metal azides in laboratory plumbing, flush drains thoroughly after disposal of these solutions.
5. Although it might be advised to run calibrators/controls and samples in duplicate, reliable results are equally obtained by doing the analysis in singlicate.

6. STORAGE CONDITIONS

1. Store the microtiter strips in their original package with the desiccant until all the strips have been used.
2. Opened components should be stored at 2-8°C until next use and can be maintained for 6 months.
3. Never use any kit components beyond the expiration date.

7. SPECIMEN COLLECTION AND PREPARATION

Serum and plasma (EDTA, citrate) samples may be used in this assay. Remove serum from clot as soon as possible to avoid haemolysis. Transfer the serum to a clean storage tube. Specimens may be stored at 2-8 °C for 3-4 days, or they can be stored frozen for at least one year. Avoid repeated freezing and thawing. Samples must be diluted in sample diluent, see chapter 9.

8. ASSAY PROCEDURE

8.1 General Remarks

1. Use a separate disposable tip for each sample transfer to avoid cross-contamination.
2. All reagents must be allowed to come to room temperature before use. All reagents must be mixed without foaming.
3. Once the assay has been started, all steps should be completed without interruption.
4. The use of an ELISA washer is recommended, however depending on the apparatus it may be necessary to adapt the washing procedure for obtaining optimal results.
5. The apDia Anti-Adalimumab ELISA may be used on any open ELISA automate after validation. Depending on the reader capacity of the instrument, it might be required to reduce the incubation time for the Chromogen Solution from 10 to 6 minutes (applicable for the Dynex DS2 and Dynex DSX instruments). For instructions on how to perform the assay with ELISA instruments, please contact apDia.

8.2 Reconstitution of Reagents

Wash Solution: dilute 50 ml of concentrated Wash Solution to 1000 ml with distilled water. Reconstituted solution can be stored at least 1 month, store at 2-8°C. At higher temperatures, the concentrated Wash Solution may appear cloudy without affecting its performance. Upon dilution, the solution will be clear.

8.3 Assay Procedure

Before starting the assay, dilute the patient samples according to the guidelines in chapter 9.

1. Pipette 100 µl of the calibrators, controls and diluted samples into the wells.
2. Incubate the covered microtiter strips for 60 ± 2 min at 37 °C (± 2 °C).
3. Empty the wells entirely by aspiration. Fill the wells completely with 350-400 µl of reconstituted wash solution, avoiding overflow of buffer from one well to another. Repeat the wash procedure two more times for a total of three washes. Finally, aspirate the content of the wells and remove any residual liquid by gently tapping the inverted wells on clean absorbent paper. Incomplete washing will adversely affect the test outcome.
4. Add 100 µl of Conjugate 1 and incubate the covered microtiter strips for 30 ± 2 min at 37 °C (± 2 °C).
5. Repeat the washing procedure as described in 3.
6. Add 100 µl of Conjugate 2 and incubate the covered microtiter strips for 15 ± 1 min at 37 °C (± 2 °C).
7. Repeat the washing procedure as described in 3.
8. Add 100 µl of Chromogen Solution to each well.
9. Incubate the covered microtiter strips for 10 ± 1 min at 37 °C (± 2 °C). Avoid light exposure during this step.
10. Add 50 µl of Stop Solution to each well.
11. Determine the absorbance of each well at 450 nm with reference filter 600 – 650 nm within 30 min following the addition of Stop Solution.

9. SAMPLE DILUTION FACTOR

Prepare for each patient sample a dilution of 1:25 and 1:200.

By diluting the samples 1:25, ATA concentrations between 2.5 and 125 ng/ml can be determined.
Example: add 25 µl patient sample to 600 µl Sample Diluent

By diluting the samples 1:200, ATA concentrations between 20 and 1000 ng/ml can be determined.
Example: add 100 µl of dilution 1:25 to 700 µl Sample Diluent

The dilution factor must be taken into account when calculating ATA concentration in the samples by multiplying the measured concentration by the dilution factor. Concentration is expressed in ng/ml.

10. RESULTS

The average absorbance value of each calibrator is plotted against the corresponding ATA concentration value and the best calibration curve (e.g. polygonic, quadratic regression) is constructed.
Use the average absorbance of each patient sample obtained in the ATA ELISA to determine the corresponding concentration value by simple interpolation from the curve. Multiply the obtained value by the dilution factor.
Depending on the experience and/or availability of software, other methods of data analysis may be used.

For sample dilution 1:25
Multiply the obtained concentration by factor 25.
If the obtained concentration is lower than 2.5 ng/ml, the results must not be extrapolated and concentration is reported as < 2.5 ng/ml.
If the obtained concentration is higher than 125 ng/ml, the results must not be extrapolated and concentration is reported as > 125 ng/ml.

For sample dilution 1:200
Multiply the obtained concentration by factor 200.
If the obtained concentration is lower than 20 ng/ml, the results must not be extrapolated and concentration is reported as < 20 ng/ml.
If the obtained concentration is higher than 1000 ng/ml, the results must not be extrapolated and concentration is reported as > 1000 ng/ml.

Only if both 1:25 and 1:200 dilution result in a measurable concentration value, the mean of both values is calculated and reported.

11. PERFORMANCE CHARACTERISTICS

Example of typical optical density (O.D.) values:

CALIBRATOR	O.D.
CAL 0	0.020
CAL 0,1	0.097
CAL 0,5	0.410
CAL 1	0.829
CAL 2,5	1.584
CAL 5	2.251

Precision

Intra-assay variation (n=20; 1 run)

	Level 1	Level 2	Level 3	Level 4
Mean (ng/ml)	0.45	0.87	1.69	3.90
SD	0.05	0.07	0.22	0.51
% CV	**11.3**	**7.9**	**12.8**	**13.1**

Inter-assay variation (n=12; 3 runs, two levels)

	Level 1	Level 2
Mean (ng/ml)	0.36	2.61
SD	0.04	0.35
% CV	**10.5**	**13.2**

Specificity – normal human serum/plasma

Specificity has been evaluated by testing 100 healthy donor samples from Dutch origin. None of the samples showed a detectable concentration of ATA, resulting in a specificity of 100%.

Specificity – interference

A panel of 34 potentially interfering samples was tested consisting of HAMA positive, lipemic, high bilirubin, high cholesterol, haemolysed, high total protein and 1st semester pregnant woman samples. No interaction with the investigated factors was observed.

Specificity – RF interference

The potential interference of rheumatoid factor (RF) in a clinical sample panel of patients suffering from auto-immune diseases and positive for RF, was evaluated in the apDia ATA ELISA. The results indicated that RF does not interfere in the assay.

Diagnostic sensitivity

A clinical sample panel of 20 specimens was analysed using the apDia Anti-Adalimumab ELISA and results were compared with data obtained using the ATA ELISA developed at the KU Leuven which served as reference assay. All samples having measurable ATA levels (17 samples) according to the reference assay, were detected positive, resulting in a diagnostic sensitivity of 100 %.

Limit of quantification

Based on a serial dilution of calibrator CAL 0,1, the limit of quantification of ATA is lower than 0.06 ng/ml.
Taking into account a dilution factor of 1:25 this corresponds to 1.5 ng/ml.
Taking into account a dilution factor of 1:200 this corresponds to 12 ng/ml.

For a 1:25 dilution a concentration lower than 2.5 ng/ml, corresponding to the lowest calibrator, should be reported as < 2.5 ng/ml.
For a 1:200 dilution a concentration lower than 20 ng/ml should be reported as < 20 ng/ml.

Test validity

The following specifications must be met for each run to be valid:
O.D. value for the zero calibrator: < 0.080
O.D. value for the highest calibrator: > 1.400

Concentration value for positive control CTL1: 0.375 ng/ml, range 0.25-0.50 ng/ml
Concentration value for positive control CTL2: 3 ng/ml, range 2-4 ng/ml

If one of the specifications is not met, the test run should be repeated.

12. TROUBLE SHOOTING

In case of high background signal (OD CAL 0 > 0.080), the washing was insufficient. Repeat the test with more vigorous washing (increased number of cycles, soak time).

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