研究用試薬

Anti-cN-1A ELISA (IgG)
Test instruction

ORDER NO.	ANTIBODIES AGAINST	IG CLASS	SUBSTRATE	FORMAT
EA 1675-4801 G	cytosolic 5´-nucleotidase 1A (cN-1A)	IgG	Ag-coated microplate wells	48 x 01 (48)

Indication: The ELISA test kit provides a semiquantitative in vitro assay for human antibodies of the immunoglobulin class IgG against cytosolic 5´-nucleotidase 1A (cN-1A; synonyms: Mup44, NT5C1A, NT5c1A, NT5C1a) in serum or plasma for the diagnosis of inclusion body myositis (IBM).

Application: Antibodies against cN-1A are the first and only serological marker for inclusion body myositis. In cases with a suspicious clinical picture, antibodies against cN-1A should be investigated using the Anti-cN-1A ELISA.

Principles of the test: The test kit contains microtiter strips each with 8 break-off reagent wells coated with cytosolic 5´-nucleotidase 1A. In the first reaction step, diluted patient samples are incubated in the wells. In the case of positive samples, specific IgG antibodies (also IgA and IgM) will bind to the antigens. To detect the bound antibodies, a second incubation is carried out using an enzyme-labelled anti-human IgG (enzyme conjugate) catalysing a colour reaction.

Contents of the test kit:

Component	Colour	Format	Symbol
1. Microplate wells coated with antigens 6 microplate strips each containing 8 individual break-off wells in a frame, ready for use	---	6 x 8	STRIPS
2. Calibrator (IgG, human), ready for use	red	1 x 1.0 ml	CAL
3. Positive control (IgG, human), ready for use	blue	1 x 1.0 ml	POS CONTROL
4. Negative control (IgG, human), ready for use	green	1 x 2.0 ml	NEG CONTROL
5. Enzyme conjugate peroxidase-labelled anti-human IgG (rabbit), ready for use	green	1 x 12 ml	CONJUGATE
6. Sample buffer ready for use	light blue	1 x 100 ml	SAMPLE BUFFER
7. Wash buffer 10x concentrate	colourless	1 x 100 ml	WASH BUFFER 10x
8. Chromogen/substrate solution TMB/H_2O_2, ready for use	colourless	1 x 12 ml	SUBSTRATE
9. Stop solution 0.5 M sulphuric acid, ready for use	colourless	1 x 12 ml	STOP SOLUTION
10. Test instruction	---	1 booklet	
11. Quality control certificate	---	1 protocol	

LOT Lot description
IVD In vitro diagnostic medical device
CE
Storage temperature
Unopened usable until

Storage and stability: The test kit has to be stored at a temperature between +2°C to +8°C. Do not freeze. Unopened, all test kit components are stable until the indicated expiry date.

Waste disposal: Patient samples, calibrators, controls and incubated microplate strips should be handled as infectious waste. All reagents must be disposed of in accordance with local disposal regulations.

Preparation and stability of the reagents

Note: All reagents must be brought to room temperature (+18°C to +25°C) approx. 30 minutes before use. After first use, the reagents are stable until the indicated expiry date if stored at +2°C to +8°C and protected from contamination, unless stated otherwise below.

- **Coated wells:** Ready for use. Tear open the resealable protective wrapping of the microplate at the recesses above the grip seam. Do not open until the microplate has reached room temperature to prevent the individual strips from moistening. Immediately replace the remaining wells of a partly used microplate in the protective wrapping and tightly seal with the integrated grip seam (Do not remove the desiccant bag).
  Once the protective wrapping has been opened for the first time, the wells coated with antigens can be stored in a dry place and at a temperature between +2°C and +8°C for 4 months.

- **Calibrator and controls:** Ready for use. The reagents must be mixed thoroughly before use.

- **Enzyme conjugate:** Ready for use. The enzyme conjugate must be mixed thoroughly before use.

- **Sample buffer:** Ready for use.

- **Wash buffer:** The wash buffer is a 10x concentrate. If crystallisation occurs in the concentrated buffer, warm it to +37°C and mix well before diluting. The quantity required should be removed from the bottle using a clean pipette and diluted with deionised or distilled water (1 part reagent plus 9 parts distilled water).
  For example: For 1 microplate strip, 5 ml concentrate plus 45 ml water.
  The working strength diluted wash buffer is stable for 4 weeks when stored at +2°C to +8°C and handled properly.

- **Chromogen/substrate solution:** Ready for use. Close the bottle immediately after use, as the contents are sensitive to light. The chromogen/substrate solution must be clear on use. Do not use the solution if it is blue coloured.

- **Stop solution:** Ready for use.

Warning: The calibrator and controls of human origin have tested negative for HBsAg, anti-HCV, anti-HIV-1 and anti-HIV-2. Nonetheless, all materials should be treated as being a potential infection hazard and should be handled with care. Some of the reagents contain the agent sodium azide in a non-declarable concentration. Avoid skin contact.

Preparation and stability of the patient samples

Samples: Human serum or EDTA, heparin or citrate plasma.

Stability: Patient samples to be investigated can generally be stored at +2°C to +8°C for up to 14 days. Diluted samples should be incubated within one working day.

Sample dilution: Patient samples are diluted **1:101** in sample buffer.
Example: Add 10 µl sample to 1.0 ml sample buffer and mix well by vortexing (sample pipettes are not suitable for mixing).

NOTE: The calibrator and controls are prediluted and ready for use, do not dilute them.

Incubation

Sample incubation: (1st step)
Transfer 100 µl of the calibrator, positive and negative controls or diluted patient samples into the individual microplate wells according to the pipetting protocol. Incubate for **30 minutes** at room temperature (+18°C to +25°C).

Washing:
Manual: Empty the wells and subsequently wash 3 times using 300 µl of working strength wash buffer for each wash.
Automatic: Wash the reagent wells 3 times with 450 µl of working strength wash buffer (program setting: e.g. TECAN Columbus Washer "Overflow Mode").

Leave the wash buffer in each well for 30 to 60 seconds per washing cycle, then empty the wells. After washing (manual and automated tests), thoroughly dispose of all liquid from the microplate by tapping it on absorbent paper with the openings facing downwards to remove all residual wash buffer.

Note: Residual liquid (> 10 µl) remaining in the reagent wells after washing can interfere with the substrate and lead to false low extinction values. Insufficient washing (e.g., less than 3 wash cycles, too small wash buffer volumes, or too short residence times) can lead to false high extinction values. Free positions on the microplate strip should be filled with blank wells of the same plate format as that of the parameter to be investigated.

Conjugate incubation: (2nd step)
Pipette 100 µl of enzyme conjugate (peroxidase-labelled anti-human IgG) into each of the microplate wells. Incubate for **30 minutes** at room temperature (+18°C to +25°C).

Washing:
Empty the wells. Wash as described above.

Substrate incubation: (3rd step)
Pipette 100 µl of chromogen/substrate solution into each of the microplate wells. Incubate for **15 minutes** at room temperature (+18°C to +25°C) protect from direct sunlight.

Stopping:
Pipette 100 µl of stop solution into each of the microplate wells in the same order and at the same speed as the chromogen/substrate solution was introduced.

Measurement:
Photometric measurement of the colour intensity should be made at a **wavelength of 450 nm** and a reference wavelength between 620 nm and 650 nm **within 30 minutes of adding the stop solution**. Prior to measuring, slightly shake the microplate to ensure a homogeneous distribution of the solution.

Pipetting protocol

	1	2	3	4	5	6	7	8	9	10	11	12
A	**C**	**P 6**	**P 14**	**P 22**								
B	**pos.**	**P 7**	**P 15**	**P 23**								
C	**neg.**	**P 8**	**P 16**	**P 24**								
D	**P 1**	**P 9**	**P 17**									
E	**P 2**	**P 10**	**P 18**									
F	**P 3**	**P 11**	**P 19**									
G	**P 4**	**P 12**	**P 20**									
H	**P 5**	**P 13**	**P 21**									

The pipetting protocol for microtiter strips is an example of the **semiquantitative analysis** of 24 patient samples (P 1 to P 24).

The calibrator (C), the positive (pos.) and negative (neg.) controls as well as the patient samples have been incubated in one well each. The reliability of the ELISA test can be improved by duplicate determinations of each sample.
The wells can be broken off individually from the strips. This makes it possible to adjust the number of test substrates used to the number of samples to be examined and minimises reagent wastage.
Both positive and negative controls serve as internal controls for the reliability of the test procedure. They should be assayed with each test run.

Calculation of results

Semiquantitative: The extinction value of the calibrator defines the upper limit of the reference range for healthy subjects **(cut-off)** recommended by EUROIMMUN. Values above the indicated cut-off are to be considered as positive, those below as negative. Besides this qualitative interpretation, a semiquantitative evaluation of results is possible by calculating a ratio according to the following formula:

$$\frac{\textbf{Extinction of control or patient sample}}{\textbf{Extinction of the calibrator (cut - off)}} = \textbf{Ratio}$$

EUROIMMUN recommends interpreting results as follows:

Ratio <1.0:	**negative**
Ratio ≥1.0:	**positive**

For duplicate determinations the mean of the two values should be taken. If the two values deviate substantially from one another, EUROIMMUN recommends retesting the samples.

For diagnosis, the clinical picture of the patient always needs to be taken into account along with the serological findings.

Test characteristics

Calibration: There is no international reference serum for the determination of these antibodies. The results are given in ratios, which are a relative measure of the antibody concentration in the patient sample.

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For every group of tests performed, the extinction values of the calibrator and the ratios of the positive and negative controls must lie within the limits stated for the relevant test kit lot. A quality control certificate containing these reference values is included. If the values specified for the controls are not achieved, the test results may be inaccurate and the test should be repeated.

The binding activity of the antibodies and the activity of the enzyme used are temperature-dependent. It is therefore recommended using a thermostat in all three incubation steps. The higher the room temperature (+18°C to +25°C) during the incubation steps, the greater will be the extinction values. Corresponding variations apply also to the incubation times. However, the calibrator is subject to the same influences, with the result that such variations will be largely compensated in the calculation of the result.

Antigen: The microplate wells were coated with recombinant cytosolic 5'-nucleotidase 1A (cN-1A).

Detection limit: The lower detection limit is defined as the mean value of an analyte-free sample plus three times the standard deviation and is the smallest detectable antibody titer. The lower detection limit of the Anti-cN-1A ELISA (IgG) is ratio 0.03.

Cross reactivity: This ELISA specifically detects autoantibodies of class IgG against cN-1A. Cross reactions with other autoantibodies were not found in samples from patients with PBC (n = 10) and non-IBM myopathy (n = 10).

Interference: Haemolytic, lipaemic and icteric samples showed no influence on the result up to a concentration of 10 mg/ml for haemoglobin, 20 mg/ml for triglycerides and 0.4 mg/ml for bilirubin in this ELISA.

Reproducibility: The reproducibility of the test was investigated by determining the intra- and inter-assay coefficients of variation (CV) using 3 samples. The intra-assay CVs are based on 20 determinations and the inter-assay CVs on 3 determinations performed in 10 different test runs.

Intra-assay variation, n = 20		
Sample	**Mean value (Ratio)**	**CV (%)**
1	1.4	1.5
2	2.5	1.0
3	4.3	1.4

Inter-assay variation, n = 3 x 10		
Sample	**Mean value (Ratio)**	**CV (%)**
1	1.3	2.3
2	2.7	4.6
3	4.3	2.6

Clinical sensitivity and specificity: The samples from 64 European and US-American patients with inclusion body myositis (IBM), a control panel of 66 samples from non-IBM myopathy patients and 202 samples from apparently healthy blood donors were investigated using the EUROIMMUN Anti-cN-1A ELISA (IgG). The sensitivity of the ELISA for the detection of IBM was 39.1%, with a specificity of 95.9%.

Panel	**Anti-cN-1A ELISA (IgG)**	
	n	**positive**
IBM (Europe)	51	20 (39.2%)
IBM (USA)	13	5 (38.5%)
Sensitivity for IBM	**64**	**25 (39.1%)**
Asymptomatic blood donors	202	7 (96.5%)
Non-IBM myopathies (e.g. myositis, dermatomyositis, polymyositis and necrotising myositis)	66	4 (93.9%)
Specificity for IBM	**268**	**11 (95.9%)**

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In a ROC analysis using the results of 51 samples of patients with IBM, 158 patients with other autoimmune diseases and 102 healthy blood donors the following characteristics were determined:

Cut-off	Specificity	Sensitivity
0.9	94.6%	39.2%
1.0	95.0%	39.2%
1.2	95.0%	37.3%

Reference range: Levels of anti-cN-1A antibodies were determined in 100 samples from healthy blood donors of between 18 and 64 years of age (46 women and 54 men) using the EUROIMMUN ELISA. The mean concentration of antibodies against cN-1A was 0.3 ratio, and the values ranged from 0.0 to 2.8 ratio. With a cut-off of 1.0 ratio, 3.0% of blood donors were anti-cN-1A positive.

Cut-off	Percentile
0.7 Ratio	96.0%
0.9 Ratio	97.0%
2.6 Ratio	98.0%

Clinical significance

The serological test Anti-cN-1A ELISA (IgG) aids the diagnosis of inclusion body myositis (IBM), a degenerative autoimmune disease of the muscles. It is distinguished from the sporadically occurring hereditary (inherited, non-inflammatory) form of inclusion body myositis (hIBM compared to sIBM). The test shows a sensitivity of around 40% and a specificity of over 90% for the detection of anti-cN-1A antibodies.

Anti-cN-1A antibodies attack/destroy structures of the muscle cells/fibres and initiate inflammatory reactions with infiltration by cytotoxic T-cells. The target of the attack is the autoantigen cN-1A (synonyms: Mup44, cN1A, NT5C1A, NT5c1A, NT5C1a, sporadic inclusion body myositis autoantigen, 44 kDa IBM autoantigen). The complex pathogenesis encompasses degenerative mechanisms in the muscle in addition to the distinct inflammatory reactions. These are based on an accumulation of aberrant molecules, especially β-amyloid, induced by IL-1β, and on several cellular molecular mechanisms which are responsible for multiprotein aggregation and accumulation within the muscle fibres. These are:
a) Enrichment of several proteins including amyloid-β42 and its oligomers and phosphorylated tau in the form of paired helical filaments within the muscle fibres.
b) Local protein deficiency as a result of incorrect protein folding, abnormal myoproteostatis or abnormal posttranslational modification of proteins.

IBM is the most frequent chronic inflammatory myopathy in elderly patients. It leads to muscle weakness and amyotrophia of muscles near to and distant from the trunk. In the European Union around 500 persons and in the USA around 750 persons per 1,000,000 inhabitants are affected. Typically, distal muscle groups are preferentially attacked, in particular finger benders in the upper extremities and the dorsal flexor of the lower leg. The muscle atrophy is often asymmetrical with emphasis on the knee extensors on the legs and the flexor muscles on the arms. This causes patients to fall easily and to have difficulties performing fine-motor tasks. During the course of disease the muscle weakness increases continually, so that patients need a wheelchair within a few years. Swallowing difficulties can occur as an accompanying symptom. Diagnosis is based on assessment of the clinical signs, MRT, histology, EMG (EMG pattern mixed myopathic-neurogen) and immunological serodiagnostics using the Anti-cN-1A ELISA (IgG). Anti-cN-1A-positive IBM patients show particularly severe courses of this autoimmune disease with distinctive motor impairments, including of the eyes, face and respiratory muscles.

Depending on the study, the prevalence of anti-cN-1A antibodies is 33% to 76% in IBM, 0% to 14% in polymyositis, 0% to 21% in dermatomyositis, 0% to 23% in Sjögren's syndrome and 0% to 14% in systemic lupus erythematosus. While antibodies against cN-1A are a diagnostic marker for IBM, their positivity or negativity is not significant in the other diseases. Anti-cN-1A antibodies are also found in 5% of healthy persons.

Current therapy strategies for IBM encompass targeted antibody-mediated blockade of T-cells (using e.g. alemtuzumab) and B cells (using e.g. rituximab). Moreover, treatment in the future will involve blocking of inflammatory mediators and blocking of β-amyloid-generating mechanisms.

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