研究用試薬

Anti-Envoplakin ELISA (IgG)
Test instruction

ORDER NO.	ANTIBODIES AGAINST	IG CLASS	SUBSTRATE	FORMAT
EA 1491-4801 G	Envoplakin	IgG	Ag-coated microplate wells	48 x 01 (48)

Indication: The ELISA test kit provides semiquantitative in vitro determination of human antibodies of the immunoglobulin class IgG against envoplakin in serum or plasma and supports the delimitation of a paraneoplastic pemphigus from bullous pemphigoid, pemphigus vulgaris, or pemphigus foliaceus.

Application: The determination of autoantibodies against envoplakin using the Anti-Envoplakin ELISA (IgG) supports the diagnosis of paraneoplastic pemphigus (PNP). In PNP, envoplakin antibodies can occur alone or in combination with autoantibodies against various desmosomal and hemidesmosomal proteins.

Principles of the test: The test kit contains microtiter strips each with 8 break-off reagent wells coated with human envoplakin. 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	dark 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		Storage temperature
IVD	In vitro diagnostic medical device	CE	Unopened usable until

Storage and stability: The test kit has to be stored at a temperature between +2°C and +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.

Modifications to the former version are marked in grey.

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 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 must be incubated within one working day.

Sample dilution: Patient samples are diluted **1:101** in sample buffer.
For example: dilute 10 µl sample in 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 or negative control 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 readings. 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 readings.
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 above pipetting protocol is an example of the semiquantitative determination of antibodies in 24 patient samples (P 1 to P 24).

Calibrator (C), positive (pos.) and negative (neg.) control 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 of the calibrator defines the upper limit of the reference range of 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 semiquantita- tive 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

Information on the detection of antibodies against envoplakin:
The cut-off recommended for the Anti-Envoplakin ELISA was calculated on the basis of results obtained for samples from 28 patients with paraneoplastic pemphigus (PNP), 30 patients with bullous pemphigoid and 20 patients with pemphigus vulgaris. The specificity was determined to be 98%.
Diagnosis of PNP is based on the clinical picture, the histopathology result, the detection of tissue- bound and circulating autoantibodies and the underlying neoplasia. The consideration of all these data is necessary because the reliability of the diagnostic value of the anti-envoplakin results is limited within the scope due to the low pre-test probability and low prevalence of antibodies against envoplakin.
Autoantibodies against envoplakin, which are diagnostically relevant, often occur in PNP patients in combination with antibodies against desmoglein. However, case examples of isolated anti-envoplakin autoantibodies in PNP patients have also been described in literature.
Due to the therapeutic consequences of a positive anti-envoplakin antibody result we recommend always verifying the plausibility of a positive finding and, if necessary, to confirm the result using further methods (e.g. detection of autoantibodies against envoplakin on urinary bladder by indirect immunofluorescence).

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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 the medical diagnosis, the clinical symptoms of the patient and, if available, further findings should always be taken into account alongside the serological result. A negative serological result does not exclude the presence of a disease.

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.

For every group of tests performed, the extinction readings 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. 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 envoplakin. The corresponding cDNA was expressed in E. coli.

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-Envoplakin ELISA (IgG) is ratio 0.07.

Cross reactivity: This ELISA specifically detects autoantibodies of class IgG against envoplakin. Cross reactions with other autoantibodies were not found in samples from patients with the following diseases: bullous pemphigoid (n = 30), pemphigus vulgaris (n = 20).

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 determina- tions and the inter-assay CVs on 4 determinations performed in 6 different test runs.

Intra-assay variation, n = 20		
Sample	**Mean value (Ratio)**	**CV (%)**
1	2.2	2.9
2	2.9	1.6
3	3.4	2.4

Inter-assay variation, n = 4 x 6		
Sample	**Mean value (Ratio)**	**CV (%)**
1	2.2	3.9
2	3.0	2.8
3	3.4	3.9

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Clinical sensitivity and specificity: Samples from 28 patients with paraneoplastic pemphigus, a control panel of 50 patients with other diseases and 100 healthy blood donors were analysed using the EUROIMMUN Anti-Envoplakin ELISA.
The sensitivity of the ELISA for paraneoplastic pemphigus was 85.7% with a specificity of 98.0%.

Panel	Anti-Envoplakin ELISA	
	n	positive
Paraneoplastic pemphigus	**28**	**24 (85.7%)**
Asymptomatic blood donors	100	1 (1.0%)
Bullous pemphigoid	30	2 (6.7%)
Pemphigus vulgaris	20	0 (0.0%)
Sensitivity for paraneoplastic pemphigus	**28**	**24 (85.7%)**
Specificity for paraneoplastic pemphigus	**150**	**3 (98.0%)**

In a ROC analysis (AUC value: 0.96) using the results of 28 samples from patients with paraneoplastic pemphigus and 150 control samples the following characteristics were determined:

Cut-off	Specificity	Sensitivity
0.95 Ratio	98.0%	85.7%
1.15 Ratio	98.0%	82.1%

Reference range: The levels of anti-envoplakin antibodies were determined in 100 samples from healthy blood donors of between 22 and 67 years of age (47 women, 53 men) this EUROIMMUN ELISA using. No differences with respect to age or gender were observed. The mean concentration of antibodies against envoplakin corresponds to the ratio value of 0.23. With a cut-off of ratio 1.0, 1.0% of the blood donors were anti-envoplakin positive (IgG)

Cut-off	Percentile
0.60 ratio	98th
1.72 ratio	100th

Clinical significance

The Anti-Envoplakin ELISA is designed for the specific serological detection of paraneoplastic pemphigus (PNP). The high diagnostic value of the test is due to the use of the N-terminal fragment of envoplakin in the form of a recombinant antigen .

PNP is a life-threatening intraepidermal blister-forming skin disease, which occurs in all age groups. It is regarded as a separate disease from pemphigus vulgaris (PV) and pemphigus foliaceus (PF). The skin lesions of PNP do not correspond to classic PV, but are polymorphic, i.e. the clinical picture is a combination of PV symptoms and skin changes resembling erythema exsudativum multiforme, usually combined with pronounced stomatitis or other mucosal changes (oral mucositis). Histological characteristics are acantholysis and dyskeratosis.

The skin changes are accompanied by malignant diseases, in 84% on average by haematological neoplasia. Of these, 39% are non-Hodgkin lymphoma, 19% lymphatic leukaemia (mostly CCL), 19% Castleman's tumour (predominantly in children and adolescents; due to the hypertrophy of one or more lymph nodes with angiofollicular hyperplasia) and 6% benign thymoma. PNP can also be associated with sarcoma.

The disease mechanism encompasses immunological, endocrinological and cytokine-mediated reactions.
Criteria for PNP are:
1. Presence of a malignant disease or a benign neoplasia,
2. Unusual therapy resistance for dermatosis,
3. Parallel course of tumour and neoplasia (the paraneoplasia disappears after removal of the tumour and returns with recurrence of the tumour).

In contrast to the autoantigens in PV and PF, the antigen complex in PNP is expressed in the epithelium and on the lymphocytes of the associated tumour. In PNP autoantibodies against desmosomes (desmoglein 3, desmoplakin I and II, envoplakin, plectin, periplakin), hemidesmosomes (bullous pemphigoid antigen 230) and against an unknown anitgen with 170 kD are detected. Autoantibodies against envoplakin (seroprevalence of approx. 80%) are considered a diagnostic marker for PNP.

The Anti-Envoplakin ELISA based on the N-terminal fragment of envoplakin not only identifies circulating autoantibodies against envoplakin in PNP, but also contributes to differential diagnostic clarification through differentiation from bullous pemphigoid (BP), PV and PF.

Note:
Reliable diagnosis of BP can be made using the Anti-BP180-NC16A-4X ELISA (IgG) and Anti-BP230-CF ELISA (IgG). The Anti-Desmoglein 1 ELISA and Anti-Desmoglein 3 ELISA can be used to confirm a clinical pemphigus diagnosis. These highly sensitive and specific test systems show the following:
- In untreated patients a positive result in the Anti-Desmoglein 3 ELISA alone suggests the presence of PV with only mucosa involvement.
- If both the Anti-Desmoglein 3 ELISA and the Anti-Desmoglein 1 ELISA are positive, this indicates PV with mucosa and skin involvement.
- A positive Anti-Desmoglein 1 ELISA result alone is indicative of PF.

Furthermore, prima vista diagnosis of BP, PV, PF, epidermolysis bullosa acquisitia and Duhring's dermatitis herpetiformis is facilitated using the IIFT Dermatology Mosaic with an autoantibody specificity of almost 100% and a sensitivity of 96% to 100%.

Statistics for serological PNP detection using the Anti-Envoplakin ELISA confirm the high diagnostic value of this test. The specificity is 96.3% to 99% (depending on the study) and the sensitivity 80.6% to 86%. In comparison, the specificity of the Anti-Periplakin ELISA is approx. 75%. Envoplakin autoantibodies are detected in less than 1% of BP, PV and PF patients. In some studies, envoplakin autoantibodies correlate with the extent of the PNP or the associated tumour disease, respectively. Also, individual findings of increased autoantibody titers against particular epitopes in accompanying diseases or complications of the PNP were reported. In contrast, the epitope specificity of the envoplakin autoantibodies was associated neither with a particular prognosis, nor with a particular neoplasia type.

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