Anti-Desmoglein 3 ELISA (IgG)
Test instruction

ORDER NO.	ANTIBODIES AGAINST	IG-CLASS	SUBSTRATE	FORMAT
EA 1496-4801 G	Desmoglein 3	IgG	Ag-coated microplate wells	48 x 01 (48)

Indications: Pemphigus vulgaris.

Principle of the test: The ELISA test kit provides a semiquantitative or quantitative in vitro assay for human autoantibodies of the immunoglobulin class IgG against desmoglein 3. The test kit contains microtiter strips each with 8 individual break-off reagent wells coated with desmoglein 3. In the first reaction step, diluted patient samples (serum, EDTA, heparin or citrate plasma) 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, 6 microplate strips each containing 8 individual break-off wells in a frame, ready for use	---	6 x 8	STRIPS
2. Calibrator 1 200 RU/ml (IgG, human), ready for use	dark red	1 x 2.0 ml	CAL 1
3. Calibrator 2 20 RU/ml (IgG, human), ready for use	red	1 x 2.0 ml	CAL 2
4. Calibrator 3 2 RU/ml (IgG, human), ready for use	light red	1 x 2.0 ml	CAL 3
5. Positive control (IgG, human), ready for use	blue	1 x 2.0 ml	POS CONTROL
6. Negative control (IgG, human), ready for use	green	1 x 2.0 ml	NEG CONTROL
7. Enzyme conjugate peroxidase-labelled anti-human IgG (rabbit), ready for use	green	1 x 12 ml	CONJUGATE
8. Sample buffer ready for use	light blue	1 x 100 ml	SAMPLE BUFFER
9. Wash buffer 10x concentrate	colourless	1 x 100 ml	WASH BUFFER 10x
10. Chromogen/substrate solution TMB/H_2O_2, ready for use	colourless	1 x 12 ml	SUBSTRATE
11. Stop solution 0.5 M sulphuric acid, ready for use	colourless	1 x 12 ml	STOP SOLUTION
12. Test instruction	---	1 booklet	
13. Protocol with reference values	---	1 protocol	

LOT	Lot		Storage temperature
IVD	In vitro determination		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 are to be disposed of according to official 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.

- **Calibrators 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 crystallization 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 deionized 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 wash buffer is stable for 1 month 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: Calibrators and controls used have been tested negative for HBsAg, anti-HCV, anti-HIV-1 and anti-HIV-2 using enzyme immunoassays and indirect immunofluorescence methods. Nonetheless, all materials should be treated as being a potential infection hazard and should be handled with care. Some of the reagents contain the toxic agent sodium azide. Avoid skin contact.

Preparation and stability of the patient samples

Sample material: 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 serum in 1.0 ml sample buffer and mix well by vortexing (sample pipettes are not suitable for mixing).

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

Incubation

For **qualitative/semiquantative analysis** incubate **calibrator 2** along with the positive and negative controls and patient samples. For **quantitative analysis** incubate **calibrators 1 to 3** along with the positive and negative controls and patient samples.

Sample incubation: (1. step)
Transfer 100 µl of the calibrators, 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).

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

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.

Attention: Residual liquid (> 10 µl) 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 reaction 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: (2. 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).

Wash:
Empty the wells. Wash as described above.

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

The pipetting protocol for microtiter strips 1-4 is an example for the **semiquantitative analysis** of 24 patient samples (P 1 to P 24).
The pipetting protocol for microtiter strips 7-10 is an example for the **quantitative analysis** of 24 patient samples (P 1 to P 24).

The calibrators (C 1 to C 3), the positive (pos.) and negative (neg.) controls, and the patient samples have each been incubated in one well. The reliability of the ELISA test can be improved by duplicate determinations for each sample.
The reagent wells are break off format. Therefore, the number of tests performed can be matched to the number of samples, minimizing 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

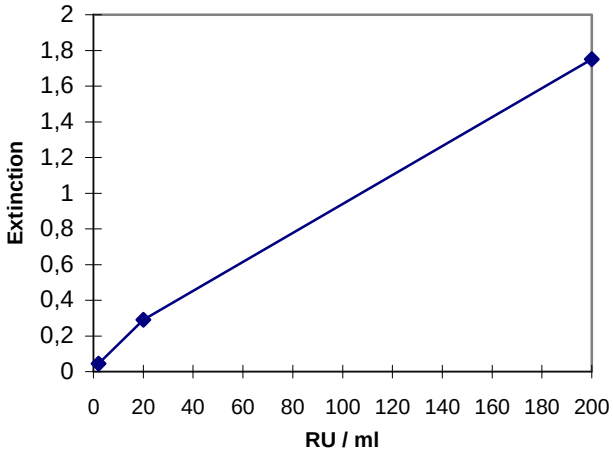
Qualitative/Semiquantitative: Results can be evaluated semiquantitatively by calculating a ratio of the extinction value of the control or patient sample over the extinction value of calibrator 2. Calculate the ratio according to the following formula:

$$\frac{\text{Extinction of the control or patient sample}}{\text{Extinction of calibrator 2}} = \text{Ratio}$$

EUROIMMUN recommends interpreting results as follows:

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

Quantitative: The standard curve from which the concentration of antibodies in the serum samples can be taken is obtained by point-to-point plotting of the extinction values measured for the 3 calibrators against the corresponding units (linear/linear). Use “point-to-point” plotting for calculation of the standard curve by computer. The following plot is an example of a typical calibration curve. Please do not use this curve for the determination of antibody concentrations in patient samples.



If the extinction of a serum sample lies above the value of calibrator 1 (200 RU/ml), the result should be given as ">200 RU/ml". It is recommended that the sample be re-tested at a dilution of 1:400. The result in RU/ml read from the calibration curve for this sample must then be multiplied by a factor of 4.

The upper limit of the normal range **(cut-off)** recommended by EUROIMMUN is 20 relative units (RU) /ml. EUROIMMUN recommends interpreting results as follows:

<20 RU/ml:	**negative**
≥20 RU/ml:	**positive**

This recommendation is based on data attained from a ROC analysis using the results from 71 patient samples with pemphigus vulgaris and 470 control samples. A specificity of 99,4 % at a cut-off of 15.9 RU/ml was obtained. The 99th percentile determined with 401 healthy blood donors was at 11.4 RU/ml (see relevant information in section „Test characteristics").

For duplicate determinations the mean of the two values should be taken. If the two values deviate substantially from one another the sample should be retested.

For diagnosis, the clinical symptoms of the patient should always be taken into account along with the serological results.

Test characteristics

Calibration: As no international reference serum exists for antibodies against Desmoglein 3, the calibration is performed in relative units (RU).

For every group of tests performed, the extinction values of the calibrators and the relative units and/or ratios determined for the positive and negative controls must lie within the limits stated for the relevant test kit lot. A protocol 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 activity of the enzyme used is temperature-dependent and the extinction values may vary if a thermostat is not used. The higher the room temperature (+18°C to +25°C) during substrate incubation, the greater will be the extinction values. Corresponding variations apply also to the incubation times. However, the calibrators are subject to the same influences, with the result that such variations will be largely compensated in the calculation of the result.

Antigen: The reagent wells are coated with an extracellular domain of desmoglein 3 (5 subdomains). The protein based on human cDNA was produced recombinantly in mammalian cells.

Linearity: The linearity of the ELISA was determined by assaying 4 serial dilutions of 6 serum samples. The calculated linear regression R^2 was > 0.95 for all samples. The Anti-Desmoglein 3 ELISA (IgG) is linear at least in the tested concentration range (14 RU/ml to 195 RU/ml) linear.

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-Desmoglein 3 ELISA (IgG) is 0.3 RU/ml.

Cross reactivity: The ELISA specifically detects IgG class antibodies directed against desmoglein 3. There were no cross reactions with other autoantibodies in samples of patients with bullous pemphigoid (n = 20) and linear IgA dermatosis (n = 20).

Interference: Haemolytic, lipaemic and icteric samples showed no influence at 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 using 3 sera. The intra-assay CVs are based on 20 determinations and the inter-assay CVs on 4 determinations performed in 6 different test runs.

Intra-assay variation, n = 20		
Serum	**Mean value (RU/ml)**	**CV (%)**
1	42	4.4
2	63	2.6
3	155	5.9

Inter-assay variation, n = 4 x 6		
Serum	**Mean value (RU/ml)**	**CV (%)**
1	44	6.1
2	69	5.3
3	163	3.3

Clinical sensitivity and specificity: Sera from 71 patients with pemphigus vulgaris, 50 patients with Pemphigus foliaceus, a control panel of 69 patients with autoimmune diseases and from 401 healthy blood donors were investigated using the EUROIMMUN Anti-Desmoglein 3 ELISA. The sensitivity of the ELISA for pemphigus vulgaris was 100 %, at a specificity 99.6 %. In a panel of patients with pemphigus foliaceus none of the patients were found positive.

Panel	**Anti-Desmoglein 3 ELISA**	
	n	**positive**
Pemphigus vulgaris	71	71 (100 %)
Pemphigus foliaceus	50	0 (0.0 %)
Asymptomatic blood donors	401	1 (0.2 %)
Bullous pemphigoid	48	1 (2.1 %)
Linear IgA-dermatosis	21	0 (0.0 %)
Sensitivity for pemphigus vulgaris	**71**	**71 (100 %)**
Specificity for pemphigus vulgaris	**470**	**2 (99.6 %)**

A ROC analysis (AUC value: 1.0) based on the results of 71 samples from patients with pemphigus vulgaris and 470 control samples shown in the table yielded the following data:

Cut-off	**Specificity**	**Sensitivity**
15.9 RU/ml	99,4 %	100 %
21.8 RU/ml	99,6 %	100 %

Reference range: Levels of anti-desmoglein 3 antibodies were analysed in 401 sera from healthy blood donors of between 18 and 68 years of age (151 women, 250 men) using the EUROIMMUN ELISA. The mean concentration of antibodies against desmoglein 3 was 1.8 RU/ml and ranged from 0.4 to 25.3 RU/ml. With a cut-off value of 20 RU/ml, 0.2 % of blood donors were anti-desmoglein 3 positive.

Cut-off	Percentile
11.4 RU/ml	99.
25.3 RU/ml	100.

Clinical significance

Autoantibodies against desmoglein 1 and 3 are markers for pemphigus diseases, which can be clinically and immunopathologically subdivided into 4 different forms: pemphigus vulgaris, pemphigus foliaceus, paraneoplastic pemphigus and IgA pemphigus. Paraneoplastic pemphigus is always associated with neoplasia. The resulting immune response is not only directed against desmoglein 3 but also against other proteins of desmosomal plaques such as envoplakin, periplakin, desmoplakin and plektin. IgA pemphigus is characterised by IgA antibodies primarily against desmocollin 1, and secundarily against desmoglein 1 or desmoglein 3 [1, 2, 3].

In Europe, Connecticut (USA) and Kuwait the incidence of pemphigus diseases is reported to be 1 to 5 new cases per 1 million inhabitants per year [4, 5, 6]. In Malaysia and China the incidence appears to be even higher [7, 8]. Around 65% to 80% of patients with pemphigus suffer from pemphigus vulgaris. For pemphigus foliaceus, incidences of 3.4 and 6.6 new cases per 1 million inhabitants per year have been reported in South America and Tunisia, respectively [5, 9]. Epidemiological data for IgA and paraneoplastic pemphigus are not yet available since they are very rare.

Pemphigus vulgaris always affects the mucous membranes. The majority of patients initially only develop lesions in the mucosa of the mouth. Blisters are rarely visible because of mechanical irritation. They burst early and cause slightly bleeding, painful sores. In the course of the disease some patients show flaccid blisters at the integument, particularly on parts of the body which are exposed to pressure and friction. In patients with pemphigus foliaceus, blisters are rarely found due to a very superficial cleft formation in the stratum granulosum. The disease is rather characterised by scaly crusts, especially in the seborrheic areals. The mucosa is never affected. In pemphigus with high disease activity, superficial sores can be produced on clinically healthy skin by exerting tangential pressure (Nikolski's sign). The clinical picture is generally defined by the autoantibody response. Patients with pemphigus vulgaris who show damage exclusively to the mouth mucosa exhibit IgG antibodies only against desmoglein 3, whereas patients with lesions of the skin and mucosa produce antibodies against desmoglein 1 and 3 [10, 11, 12]. Congruently, pemphigus foliaceus is only associated with desmoglein 1.

Desmoglein 1 and 3 are cadherins, which are calcium-dependent transmembrane glycoproteins of epidermal desmosomes. They are components of the maculae adherentes and permit the cell-to-cell contact in the epidermis and the surface mucosa via homophilic and heterophilic extracellular binding. The pathogenetic relevance of autoantibodies against desmoglein 1 and 3 is well proven. For example, the injection of serum from pemphigus patients into neonatal mice leads to blister formation [13]. The relevance of antibodies specific for desmoglein 1 and 3 could be shown by the fact that injection of IgG from patient sera, which was purified using recombinant desmoglein 1 or 3, into neonatal mice causes pemphigus lesions. However, after preadsorption of the patient sera with recombinant desmoglein 1 or 3 the pathogenic effect is lost [14]. The exact mechanisms which cause the blister formation are still unknown [11, 15]. There are data pointing to direct inhibition of desmoglein transinteraction in the form of steric hindrance by depletion of desmosomal proteins from the keratinocyte surface, which results from weakening of the interaction between cells [16]. Signal-transduced processes, observed for the first time by Kitjima et al., alter the composition of the desmosomes, disrupt the build-up of the cytoskeleton and affect the cell cycle [12, 17, 18, 19, 20, 21].

Clinical diagnosis of the disease is made on the basis of the typical dermatological symptoms and the presence of Nikolski's sign. Histopathological results show suprabasally located, acantholytic blisters [23]. Basal keratinocytes remain intact, but the cutis is infiltrated by leukocytes. The blister lumen contains "pemphigus cells". Autoantibodies bound in the skin can be detected in skin biopsies using direct immunofluorescence, and in biopsies of healthy skin or in primate oesophagus in the space between epithelial cells after incubation with patient serum using indirect immunofluorescence [1, 22, 23].

Gold standard in the diagnosis of bullous autoimmune dermatoses is the determination of tissue bound autoantibodies and/or C3 in the skin or mucosa of pemphigus patients. The epidermis or mucosa epithelium usually shows an intercellular, reticular pattern. Subepidermal, blistering autoimmune dermatoses are characterised by linear binding of IgG, IgA and/or C3 along the basal membrane zone.

In the diagnosis of pemphigus the determination of circulating autoantibodies using indirect immunofluorescence (on primate oesophagus as sensitive substrate) has proven successful. ELISA using desmoglein 1 and 3 offers the same sensitivity and specificity as IIFT [23, 24, 25, 26, 27, 28, 29]. In most cases the performance of Anti-Desmoglein 1 ELISA and Anti-Desmoglein 3 ELISA is sufficient for diagnosis [30, 31, 32]. In suspected pemphigus cases with a negative ELISA result IIFT should be carried out in addition. The Anti-Desmoglein 3 ELISA is of particular importance in lesions of the mouth mucosa for differentiatiating pemphigus vulgaris from Lichen ruber mucosae, benign aphtha, Behcet's disease and Steven-Johnson syndrome.

The Anti-Desmoglein 1 ELISA and Anti-Desmoglein 3 ELISA are highly sensitive and specific test systems for the determination of IgG antibodies against desmoglein 1 (100% and 94.7%) and desmoglein 3 (85% and 99.1%) [27, 30]. The clinical diagnosis of pemphigus can be confirmed by the detection of autoantibodies against desmoglein 1 and/or desmoglein 3. In untreated patients a positive result in the Anti-Desmoglein 3 ELISA alone suggests the presence of pemphigus vulgaris with only mucosa involvement. If both the Anti-Desmoglein 3 ELISA and the Anti-Desmoglein 1 ELISA are positive, this indicates pemphigus vulgaris with mucosa and skin involvement. A positive Anti-Desmoglein 1 ELISA result alone is indicative of pemphigus foliaceus [30]. The antibody levels of desmoglein 1 and 3 in the serum correlate with the severity and activity of the disease as well as with the therapy success [12, 21, 22, 23, 24, 25, 26, 33, 34, 35, 36, 37].

Before corticosteroids were available, bullous autoimmune dermatoses were mostly lethal. Today, immunosuppressive treatment is in the foreground. In the future, recombinant autoantigens are likely to be used in new and more specific therapy concepts such as immunoadsorption, generation of toxin-conjugated autoantigens and immunological tolerance [1, 38].

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