Anti-BP230-CF ELISA (IgG)
Test instruction

ORDER NO.	ANTIBODIES AGAINST	Ig CLASS	SUBSTRATE	FORMAT
EA 1502-4801-1 G	BP230	IgG	Ag-coated microplate wells	48 x 01 (48)

Indication: Bullous pemphigoid (BP)

Principle of the test: The ELISA test kit provides a semiquantitative or quantitative assay for the determination of human autoantibodies of the immunoglobulin class IgG against BP230. The test kit contains microtiter strips each with 8 individual break-off reagent wells coated with BP230. In the first reaction step, diluted patient samples (serum or EDTA, heparin or citrate plasma) are incubated in the wells. In the case of positive samples, the specific IgG antibodies (also IgA and IgM) will bind to the corresponding antigenic site. 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 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 description — CE — Storage temperature

IVD In vitro diagnostics — 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 reagents

Note: All reagents must be brought to room temperature (+18°C to +25°C) approx. 30 minutes before use. Unless stated otherwise, the reagents after initial opening are stable until the expiry date when stored between +2°C and 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 crystallisation occurs in the concentrated buffer, warm it to 37°C and mix well before diluting. The amount 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 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 bottle immediately after use, as the contents are sensitive to light. The 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 serum or plasma 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: The **serum or plasma samples** to be investigated are diluted **1:101** with sample buffer.
Example: Add 10 µl of serum to 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 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).

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 (programme 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.

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.

Stop:
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, carefully 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**	**P 25**			**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**	**P 25**		
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 **qualitative/semiquantitative analysis** of 25 patient sera (P 1 to P 25).
The pipetting protocol for microtiter strips 7-10 is an example for the **quantitative analysis** of 25 patient sera (P 1 to P 25).

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 further improved by duplicate determinations for each sample. 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

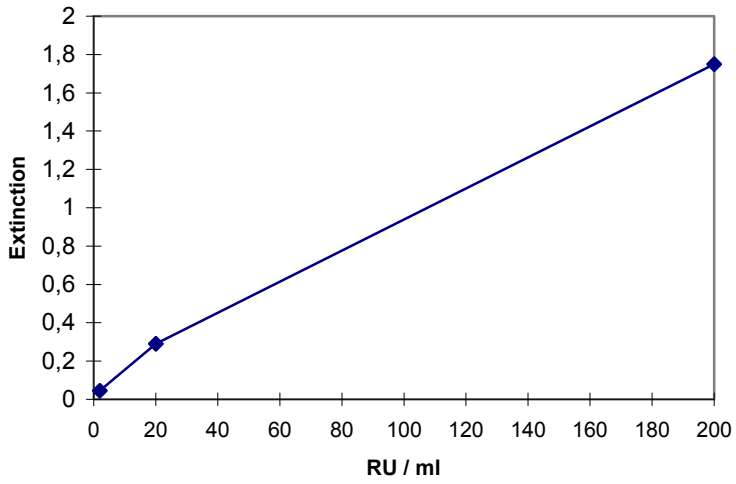
Qualitative/semiquantitative: Results can be evaluated semiquantitatively by calculating a ratio of the extinction value of the control or patient sample over the extinction value of calibrator 2. Calculate the ratio according to the following formula:

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

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 calibration sera 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 for a patient sample lies above that of calibrator 1 (200 RU/ml), the result should be reported as ">200 RU/ml". It is recommended that the sample be remeasured in a new test run at a dilution of e.g. 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**

The recommendation is based on data yielded in a ROC analysis using the results of 118 samples of patients with bullous pemphigoid and 729 control samples. According to the analysis, the specificity was 98% at a cut-off of 23.6 RU/ml. The 98th percentile based on 483 healthy blood donors was 20.4 RU/ml (q.v. respective paragraphs under "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 alongside the serological results.

Test characteristics

Calibration: As no international reference serum exists for antibodies against BP230, the calibration is performed in relative units (RU/ml).

For every group of tests performed, the relative units or ratio values 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 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 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: BP230 is a C-terminal section of the human 230 kDa bullous pemphigoid antigen, which was biochemically purified and expressed in E.coli.

Linearity: The linearity of the ELISA was determined by assaying four serial dilutions of 6 serum samples. The linear regression was calculated and R^2 amounted to > 0.95. The Anti-BP230 ELISA (IgG) is linear at least in the range of 17-190 RU/ml.

Detection limit: The detection limit is defined as a value of three times the standard deviation of an analyte-free sample and is the smallest detectable antibody titer. The lower detection limit of the Anti-BP230 ELISA is 1.0 RU/ml.

Cross reactivity: The ELISA presented here specifically detects IgG class antibodies directed against BP230 Cross-reactions with other autoantibodies were not observed in samples of patients with scleroderma (n = 12) and pemphigoid vulgaris (n =15).

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 using 4 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	24	5.0
2	61	4.6
3	97	3.4
4	114	3.0

Inter-assay variation, n = 4 x 6		
Serum	**Mean value (RU/ml)**	**CV (%)**
1	23	6.8
2	60	3.5
3	101	6.1
4	111	3.4

Clinical sensitivity and specificity: Sera from 118 patients with bullous pemphigoid, 20 patients with Pemphigoid gestationis, a control panel of 246 patients with other autoimmune diseases and 483 healthy blood donors were investigated using the EUROIMMUN Anti-BP230 ELISA. The sensitivity of the ELISA for bullous pemphigoid was 56.8%, with a specificity of 97.4%. In the pemphgoid gestationis panel 5% of patients were found positive.

Panel	**Anti-BP230 ELISA**	
	n	**positive**
Bullous pemphigoid	**118**	**67 (56.8%)**
Pemphigoid gestationis	20	1 (5.0%)
Sensitivity for bullous pemphigoid	**118**	**67 (56.8%)**
Asymptomatic blood donors	483	10 (2.1%)
Rheumatoid arthritis and other arthritis	170	3 (1.8%)
Systemic lupus erythematosus	56	5 (8.9%)
Linear IgA dermatosis	20	1 (5.0%)
Specificity for bullous pemphigoid	**729**	**19 (97.4%)**

In a ROC analysis using the results of 118 samples of patients with bullous pemphigoid and 729 control samples listed in the above table the following characteristics were determined:

Cut-off	Specificity:	Sensitivity:
16.0 RU/ml	95%	61%
23.6 RU/ml	98%	56%

Reference range: Levels of anti-BP230 antibodies were determined in 483 sera from healthy blood donors of between 18 and 67 years of age (177 women, 306 men) using the EUROIMMUN ELISA. The mean concentration of antibodies against BP230 was 4.9 RU/ml, and the values ranged from 1.0 to 94.5 RU/ml. With a cut-off of 20 RU/ml, 2.1% of blood donors were anti-BP230 positive.

Cut-off	Percentile
14.1 RU/ml	95th
20.4 RU/ml	98th

Clinical significance

Bullous autoimmune dermatoses belong to the organ-specific autoimmune diseases. They are characterised by the formation of autoantibodies against structural proteins of the skin. These structural proteins establish the cell-to-cell contact in ceratinocytes within the epidermis and the adhesion of the epidermis to the dermis. Bullous autoimmune dermatoses are divided into 4 main groups based on their target antigens and the localisation of the blisters: pemphigoid and pemphigus diseases, epidermolysis bullosa acquisita and Duhring's dermatitis herpetiformis [1]. In pemphigus diseases the blisters form intraepidermally, whereas in all other bullous autoimmune dermatoses they occur subepidermally [2, 3, 4, 5, 6, 7, 8].

Table. Target antigens of blister-forming autoimmune dermatoses

Disease	Target antigen
Pemphigoid disease	
Bullous pemphigoid	**BP180**, **BP230**
Pemphigoid gestationis	**BP180**, BP230
Linear IgA dermatosis	**BP180**, BP230
Mucosal pemphigoid	**BP180, laminin 332**, $\alpha 6\beta 4$ integrin, laminin 311, BP230
Lichen planus pemphigoides	**BP180**, BP230
Anti-laminin $\gamma 1$/p200 pemphigoid	Laminin $\gamma 1$ chain (p200 protein)
Epidermolysis bullosa acquisita	**Collagen type VII**
Dermatitis herpetiformis Duhring	Epidermal/tissue **transglutaminase**

Main target antigens are printed in bold.

With an incidence of 1.3 to 4.2 new cases per year per 100,000 inhabitants, bullous pemphigoid (BP) is the most frequent subepidermal blister-forming autoimmune dermatosis in central Europe and north America [9, 10, 11]. The disease mainly affects elderly people. The manifestation of BP is bulging blisters at the integument. However, BP may proceed without blisters for weeks or months. Therefore, all elderly patients with irritating skin disorders persisting for long periods should be tested for BP in differential diagnosis [1, 2, 12].

Autoantibodies in BP are directed against two hemidesmosomal proteins, BP180 (type XVII collagen) and BP230 [1, 2, 7, 8, 13, 14].

BP180 is a transmembrane glycoprotein with an intracellularly localised C-terminus and an extracellular N-terminus. The ectodomain consists of 15 collagenous and 16 non-collagenous domains [15, 16, 17, 18]. The 16th non-collagenous domain (**NC16A**) directly flanking the ceratinocyte membrane presents the immunogenic epitope [19]. The majority of BP patients have autoantibodies against BP180 NC16A. BP180 NC16A is, moreover, the immunodominant region in patients with pemphigoid gestationis and lichen planus pemphigoides and is found in around half of patients with mucosal pemphigoid and antibodies against BP180.

BP230 is a glycoprotein of the hemidesmosomal inner cytoplasmic plaque and contributes to the anchoring of the keratin filament system via its C-terminal domain [20, 21]. The N-terminal end of BP230 is important for its integration into the hemidesmosomes and interacts with BP180 and the β4-subunit of α6β4-integrin [17].

The **Anti-BP180-4X ELISA** uses a tetramer of the immunogenic NC16A domain and is a reliable alternative to the indirect immunofluorescence test. The advantage of the ELISA is the clear characterisation of the autoantibody specificity when using the recombinant BP180 and the resulting differentiation from other bullous autoimmune dermatoses such as pemphigus diseases, epidermolysis bullosa acquisita and Duhring's dermatitis herpetiformis [6]. The multimer form of the autoantigen increases the immunoreactivity, thus improving the efficiency of the autoantibody test. The serum level of autoantibodies against BP180 correlates with BP activity [6, 24].

The **Anti-BP230 ELISA** supplements the serological diagnosis of BP via the detection of a second, independent autoantigen in the genetic sense. In this test a recombinant BP230 is used, namely a biochemically purified C-terminal piece of the human "230 kDa bullous pemphigoid antigen" expressed in E. coli. Anti-BP230 develop with a time lag compared to Anti-BP180 [25, 26].

The diagnostic gold standard is the detection of tissue-bound autoantibodies using direct immunofluorescence. In pemphigoid disease and epidermolysis bullosa acquisita deposits of IgG/IgA/C3 are found in perilesional sample biopsies. Further differentiation is achieved in indirect immunofluorescence on human skin sheets [6, 19, 22, 23]. Final diagnosis is based on a combination of the clinical picture with the detection of autoantibodies against the individual target antigens (table) using monospecific ELISA or immunoblot analysis [5, 22].

Patients who suffer from **bullous pemphigoid** exhibit antibodies against BP180 and BP230 [2, 27, 28, 29]. The use of recombinant BP180 and recombinant BP230 in the Anti-BP180-4X-ELISA (IgG) and the Anti-BP230-ELISA (IgG) significantly improves the immunoreactivity in the serological diagnosis of bullous pemphigoid, namely to the level of an autoantibody specificity of almost 100% and a sensitivity of 96-100% [2, 3, 5, 14, 19, 27, 28]. The serum level of autoantibodies against BP180 correlates with the disease activity of BP, the serum level of autoantibodies against BP230 with the duration of the disease [3, 6, 24, 27]. Hence, the Anti-BP180-ELISA (IgG) and the Anti-BP230-ELISA (IgG) are not only suited to reliably serologically identifying autoimmune dermatosis bullous pemphigoid, but also to assessing the activity of the disease before and during treatment (e.g. with corticoseroids or in therapy-refractory cases with rituximab) [12, 26, 27, 30].

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