Anti-RSV ELISA (IgG)
Test instruction

ORDER NO.	ANTIBODIES AGAINST	IG-CLASS	SUBSTRATE	FORMAT
EI 2670-9601 G	Respiratory syncytial virus (RSV)	IgG	Ag-coated microplate wells	96 x 01 (96)

Principles of the test: The ELISA test kit provides a semiquantitative or quantitative in vitro assay for human antibodies of the IgG class against RSV in serum or plasma. The test kit contains microtiter strips each with 8 break-off reagent wells coated with RSV antigens. 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: 12 microplate strips each containing 8 individual break-off wells in a frame, ready for use	---	12 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. Quality control certificate	---	1 protocol	

LOT	Lot description		Storage temperature
IVD	In vitro diagnostics	CE	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 shoud 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.

- **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 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 controls used have been tested negative for HBsAg, anti-HCV, anti-HIV-1 and anti-HIV-2 using enzyme immunoassays or 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 are contain the toxic agent sodium azide. Avoid skin contact.

Preparation and stability of the serum or plasma 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 should 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 **semiquantative analysis** incubate **calibrator 2** along with the positive and negative controls and patient samples. For **quantitative analysis** incubate **calibrators 1, 2 and 3** along with the positive and negative controls and patient samples.

(Partly) manual test performance

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).

Washing:
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 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.

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 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).

Washing:
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.

Test performance using fully automated analysis devices

Sample dilution and test performance are carried out fully automatically using the analysis device. The incubation conditions programmed in the respective software authorised by EUROIMMUN may deviate slightly from the specifications given in the ELISA test instruction. However, these conditions were validated in respect of the combination of the EUROIMMUN Analyzer I, Analyzer I-2P or the DSX from Dynex and this EUROIMMUN ELISA. Validation documents are available on inquiry.
Automated test performance using other fully automated, open system analysis devices is possible, however, the combination should be validated by the user.

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

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:

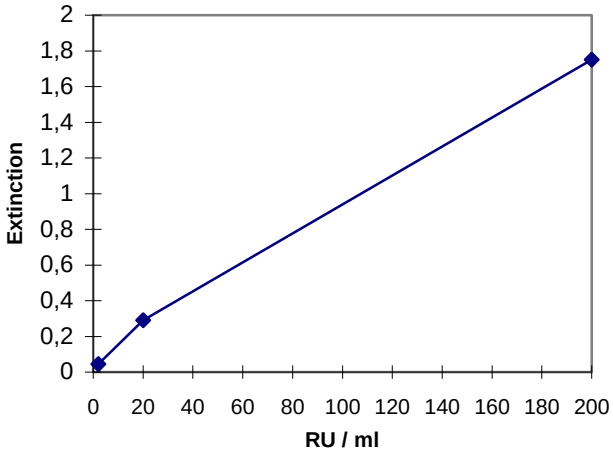
$$\frac{\textbf{Extinction of the control or patient sample}}{\textbf{Extinction of calibrator 2}} = \textbf{Ratio}$$

EUROIMMUN recommends interpreting results as follows:

Ratio <0.8:	**negative**
Ratio ≥0.8 to <1.1:	**borderline**
Ratio ≥1.1:	**positive**

In cases of borderline test results, an additional patient sample should be taken 7 days later and re-tested in parallel with the first patient sample. The results of both samples allow proper evaluation of titer changes.

Quantitative: The standard curve from which the concentration of antibodies in the patient 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 reference range of non-infected persons (**cut-off value**) recommended by EUROIMMUN is **20 relative units (RU/ml)**. EUROIMMUN recommends interpreting results as follows:

<16 RU/ml:	**negative**
≥16 to <22 RU/ml:	**borderline**
≥22 RU/ml:	**positive**

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 RSV, 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 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 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 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 largelycompensated in the calculation of the result.

Antigen: The reagent wells were coated with inactivated purified cell lysates from vero cells infected with RS virus from strain "Long".

Linearity: The linearity of the Anti-RSV ELISA (IgG) was determined by assaying 4 serial dilutions of 6 serum samples. The linear regression was calculated and R^2 amounts to > 0.95 in all samples. The Anti-RSV ELISA (IgG) is linear in the measurement range 2 - 200 RU/ml.

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 detection limit of the Anti-RSV ELISA (IgG) is 0.4 RU/ml.

Cross reactivity: The quality of the antigen used ensures high specificity of the ELISA. For this ELISA cross reactions of antibodies against other respiratory viruses are not barred.

Interference: No interference was observed with haemolytic, lipaemic or icteric samples for concentrations of up to 10 mg/ml for haemoglobin, 20 mg/ml for triglyceride and 0.4 mg/ml for bilirubin.

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	15	4.0
2	89	4.4
3	122	4.3

Inter-assay-variation, n = 4 x 6		
Serum	**Mean value (RU/ml)**	**CV (%)**
1	17	11.1
2	97	6.6
3	134	8.6

Specificity and sensitivity: Samples from 28 patients were investigated using the EUROIMMUN Anti-RSV ELISA (IgG) and another commercial Anti-RSV ELISA (IgG) as reference method. The EUROIMMUN ELISA showed a specificity of 100% and a sensitivity of 96.2% with reference to the other ELISA.

Reference range: The levels of the anti-RSV antibodies (IgG) were analyzed with this EUROIMMUN ELISA in a panel of 300 healthy blood donors. With a cut-off of 20 RU/ml, 95.7% of the blood donors were anti-RSV positive (IgG) which reflects the known percentage of infections in adults.

Clinical significance

Respiratory syncytial virus (RSV) is a polymorphic RNA virus which belongs to the paramyxovirus family. It was first identified and characterised in 1956 by Robert M. Chanock [1]. The nucleocapsid contains the components nucleoprotein N, phosphoprotein P and polymerase L. Between these and the viral coat are the non-glycosylated matrix proteins M and M2. Glycoprotein F is responsible for fusion of the viral coat and cell membrane, and glycoprotein G for attachment to the host cell [2, 3]. RSV does not have either haemaglutinin or neuraminidase. The two groups of RSV, named A (with 18 subgroups) and B (with 2 subgroups), differ in glycoprotein G, whereby there are indications that group A may possess a stronger pathogenicity [2, 3, 4, 5]. In cell culture RSV generates a characteristic syncytium formation with eosinphilic cytoplasmic inclusions. Humans are the sole RSV reservoir [6].

RSV causes predictable outbreaks of infection every year, which occur particularly in winter and early spring in temperate zones. It is the most significant pathogen of respiratory infections in infants and

young children [6, 7, 8]. 50% acquire an RSV infection in the first year of life, and almost 100% within the first three years [4, 6, 7, 9, 10]. 40% of these infections cause diseases of the lower respiratory tract.

The highly contagious RSV infection is transmitted by droplets with inoculation via the eyes, nose or mouth [6, 8]. The incubation time is 2-8 days, on average 4 days until pulmonary disease. RSV infections can lead to clinical symptoms similar to whooping cough. Transplacentally transferred antibodies do not provide protection from an RSV infection [5, 7]. On the contrary, the most severe disease course occurs in the first months of life when maternal protection should be at its highest [5, 7, 11]. In young children a febrile infection of the upper respiratory tract develops initially [6]. After several days this leads to bronchiolitis, pneumonia with tachy- and dyspnea and hypoxemie. In older children the most frequent manifestation is tracheobronchitis, characterised by reduced general wellbeing, linked to an infection of the upper respiratory tract and vomiting with dehydration [6]. Fever over 39°C is only noted in 20% of cases [6]. The most frequent complications are pneumonia, which occurs in up to 40% of inpatient cases. Further complications are acute otitis media or otitides caused by bacterial superinfections [6].

A life-threatening disease course is most frequent during the first four months of life and affects mostly boys [6]. High-risk patients are premature babies with damaged lungs (e.g. bronchopulmonary dysplasia), children with cardiac defects (in particular with cor pulmonale) and children with immune defects or under immunosuppression [6, 9, 10]. Its lethality lies under the current intensive care specifications at around 1% [6, 12, 13]. Severe courses are, however, not limited to the defined high-risk groups [14]. A severe RSV infection of the lower respiratory tract can lead to hyperreactivity, which can last up to several years and which probably represents a transient virus-triggered form of childhood bronchial asthma [6, 10, 13, 15, 16]. It is currently under discussion whether RSV infections can trigger a predisposition to chronic lung diseases [10, 13]. In elderly adults (e.g. often in old peoples' homes) and in patients with immune weaknesses severe infections of the lower respiratory tract up to pneumonia can occur [9, 14].

Recurrent infections are common and can be as severe as primary infections, since an infection does not confer long-lasting immunity. Re-infections can occur throughout life. These generally proceed mildly in healthy people, often just as "silent bronchiolitis" with tachypnea [6]. Relative protection develops only after time. RSV is the cause of 50% - 90% of all bronchiolitis cases, 5% - 40% all pneumonia cases, and < 10% of croup cases in young children [10, 12]. Complications are otitis media and apnea in 20% of hospitilized cases.

Since RSV causes a wide range of respiratory diseases and leads to the same disease symptoms as various other pathogens, diagnosis cannot be made solely on clinical symptoms [17]. Consequently, laboratory diagnostics are particular valuable.

Viral culture for detection of RSV requires qualified personnel and is time-consuming, since the cytopathic effect only occurs after 4-7 days. It is also necessary to use fresh material which is not contaminated with other pathogens (e.g. fungi). The detection of viral RNA using RT-PCR is considered a fast and reliable detection procedure, but is only carried out in a few specialized laboratories. A frequently used procedure is the complement fixation assay (CFA). In this assay a high antibody titer indicates infection, without a serological control having to be performed. The CFA is increasingly criticised because of its low sensitivity and its inability to differentiate antibody classes [18].

In clinical practice, alongside fast diagnostics by viral antigen detection in respiratory secretions using ELISA or IIFT (virus isolation on monkey kidney cells takes 3-5 days until a syncytium is visible), sensitive and specific immunological detection techniques have become established, which are simple to perform and readily available [19, 20, 21]. The anti-RSV ELISA or anti-RSV IIFT are the serum diagnostic methods of choice for the immunoglobulin classes IgA, IgG and IgM [6, 18, 19, 20, 22]. It is generally recommended that a diagnosis be made based on the analysis of two serum samples taken 2-3 weeks apart. Specific IgG antibodies in infants and small children can be acquired transplacentally [17]. Consequently, a single sample is not considered as sufficiently meaningful. Therefore, in ELISA a titer course is determined, which in correlation with clinical symptoms delivers the most reliable diagnostic result [5, 6, 11, 18, 19]. Selective IgM and IgA titers play a minor role except in young children. Here an isolated positive IgM result in correlation with clinical symptoms can indicate the presence of an RSV infection [11, 17, 22].

Although the infection is generally only contagious for around 1-5 days, premature babies, immunodeficient or immunosuppressed patients can secrete the virus over several weeks, in individual cases even over months [9]. Insufficient hand hygiene and autoinoculation of the mucosa leads to

contact people rapidly becoming vectors for rapid nosocomial distribution [4, 6, 8, 13, 23]. Nosocomial RSV infection is the most common nosocomial infection (also the most common type of pneumonia acquired in hospital) in inpatient children's care [6, 8, 12, 23]. Up until now, efforts to develop an active vaccine have been unsuccessful [12].

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