Anti-Mycoplasma pneumoniae ELISA (IgG)
Test instruction

ORDER NO.	ANTIBODIES AGAINST	IG CLASS	SUBSTRATE	FORMAT
EI 2202-9601 G	Mycoplasma pneumoniae	IgG	Ag-coated microplate wells	96 x 01 (96)

Indication: The ELISA test kit provides a semiquantitative or quantitative in vitro assay for human antibodies of the immunoglobulin class IgG against Mycoplasma pneumoniae in serum or plasma for the diagnosis of diseases of the upper and lower respiratory tract: rhinitis, pharyngitis, otitis media, bronchitis, pneumonia.

Application: The anti-Mycoplasma pneumoniae ELISA is excellently suited for the serological detection of a Mycoplasma pneumoniae infection and is a useful supplement to the direct detection method. A positive IgM and/or IgA detection, together with a significant IgG titer increase in a sample taken after 2-8 weeks indicates an acute infection. Moreover, serological investigations may give information about the epidemiology of Mycoplasma pneumoniae infections.

Principle of the test: The test kit contains microtiter strips each with 8 break-off reagent wells coated with Mycoplasma pneumoniae 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
IVD In vitro diagnostic medical device
CE
Storage temperature
Unopened usable until

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 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 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.

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.

Warning: The calibrators 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. 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 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: (1st 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 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) 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.

Test performance using fully automated analysis devices

Sample dilution and test performance are carried out fully automatically using an 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 enquiry.
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 to 4 is an example for the **semiquantitative analysis** of 24 patient samples (P 1 to P 24).
The pipetting protocol for microtiter strips 7 to 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 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 minimizes 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

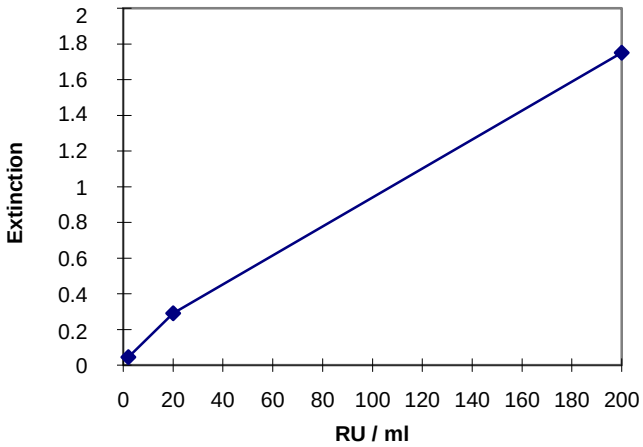
Semiquantitative: Results can be evaluated semiquantitatively by calculating a ratio of the extinction value of the control or patient sample over the extinction value of the calibrator 2. Calculate the ratio according to the following formula:

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

EUROIMMUN recommends interpreting results as follows:

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

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 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 the value of calibrator 1 (200 RU/ml), the result should be reported as “>200 RU/ml“. It is recommended that the sample be re-tested 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 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, EUROIMMUN recommends retesting the samples.

A negative serological result does not exclude an infection. Particularly in the early phase of an infection, antibodies may not yet be present or are only present in such small quantities that they are not detectable. In case of a borderline result, a secure evaluation is not possible. If there is a clinical suspicion and a negative test result, we recommend clarification by means of other diagnostic methods and/or the serological investigation of a follow-up sample. A positive result indicates that there has been contact with the pathogen. In the determination of pathogen-specific IgM antibodies, polyclonal stimulation of the immune system or antibody persistence may affect the diagnostic relevance of positive findings. Significant titer increases (exceeding factor 2) and/or seroconversion in a follow-up sample taken after 7-10 days can indicate an acute infection. To investigate titer changes, sample and follow-up sample should be incubated in adjacent wells of the ELISA microplate within the same test run. For diagnosis, the clinical picture of the patient always needs to be taken into account along with the serological findings.

Test characteristics

Calibration: As no international reference serum exists for antibodies against Mycoplasma pneumoniae, 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 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 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 antigen is a detergent extract of Mycoplasma pneumoniae, strain MAC ATCC 15531. The extract was further purified to prevent cross reactivity.

Linearity: The linearity of the Anti-Mycoplasma pneumoniae ELISA (IgG) was determined by assaying 4 serial dilutions of different patient samples. The coefficient of determination R^2 for all sera was > 0.95. The Anti-Mycoplasma pneumoniae ELISA (IgG) is linear at least in the tested concentration range (9 RU/ml to 153 RU/ml).

Detection limit: The lower detection limit is defined as 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-Mycoplasma pneumoniae ELISA (IgG) is 1 RU/ml.

Cross reactivity: The quality of the antigen used ensures a high specificity and sensitiviy of the ELISA. Sera from patients with infections caused by various agents were investigated with the Anti-Mycoplasma pneumoniae ELISA (IgG). This ELISA showed no cross reactivity.

Antibodies against	n	Anti-Mycoplasma pneumoniae ELISA (IgG) positive
Adenovirus	12	0%
Chlamydia pneumoniae	6	0%
CMV	7	0%
EBV-CA	12	0%
Helicobacter pylori	8	0%
HSV-1	9	0%
Influenza virus A	11	0%
Influenza virus B	12	0%
Measles virus	12	0%
Mumps virus	12	0%
Parainfluenza virus Pool	12	0%
Rubella virus	12	0%
RSV	12	0%
Toxoplasma gondii	3	0%
VZV	11	0%

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 (CV) using 3 sera with values at different points on the calibration curve. The intra-assay CVs are based on 20 determinations and the inter-assay CVs on 4 determinations performed on 6 different runs.

Intra-assay variation, n = 20		
Serum	**Mean value (RU/ml)**	**CV (%)**
1	23	4.4
2	38	6.3
3	71	7.1

Inter-assay variation, n = 4 x 6		
Serum	**Mean value (RU/ml)**	**CV (%)**
1	22	9.2
2	42	7.0
3	71	8.5

Specificity and sensitivity: 35 clinically pre-characterised patient samples (INSTAND and Labquality) were investigated with the EUROIMMUN Anti-Mycoplasma pneumoniae ELISA (IgG). The sensitivity amounted to 100%, with a specificity of 100%. Borderline results were not included in the calculation.

n = 35		**INSTAND/Labquality**		
		positive	borderline	negative
EUROIMMUN Anti-Mycoplasma pneumoniae ELISA (IgG)	positive	26	3	0
	borderline	0	1	0
	negative	0	0	5

Reference range: The levels of the anti-Mycoplasma pneumoniae antibodies (IgG) were analysed with this EUROIMMUN ELISA in a panel of 500 healthy blood donors. With a cut-off of 20 RU/ml, 78.6% of the blood donors were anti-Mycoplasma pneumoniae positive (IgG).

Clinical significance

Mycoplasma bacteria belong to the class of Mollicutes, along with five other genera. Mycoplasma are the smallest autonomous self-replicating bacteria and have the smallest genome of self-replicating prokaryotes. They depend on a host cell or a host organism and live intra- or extra-cellularly as parasites. Mycoplasma do not possess a cell wall and are therefore resistant to antibiotics that act on the cell wall.

Alongside the medically significant species Mycoplasma hominis, a pathogenic agent of urethral infections, and Mycoplasma fermentans, a potential trigger of fulminant systemic infections, the worldwide distributed species Mycoplasma pneumoniae is of particular significance as the cause of approximately 10% to 15% of all acute respiratory tract infections, including sometimes severe infections of the upper respiratory tract, bronchia and lungs. Humans are the only reservoir for Mycoplasma pneumoniae. The pathogen is trans-mitted aerogenically via droplets. Endemic and sometimes epidemic spread can occur. Children and young people are mostly affected (approx. 40% are younger than 5 years old).The incidence is higher in males than in females.

Following an incubation time of 10 to 20 days symptoms such as persistent cough, fever and headaches can occur. Furthermore, some cases of throat infections and middle ear infections have been documented. Alongside general symptoms of an infection of the respiratory organs, primary atypical pneumonia occurs in around 70% of serologically diagnosed Mycoplasma pneumoniae infections. From these patients 22% on average develop severe pneumonia, 3% interstitial pneumonia and 75% prolonged tracheobronchitis, which affects predominantly school children and young adults. Usually several members of a family or people who live in close contact fall ill. Around 10% of Mycoplasma pneumoniae infections are observed in patients over 40 years old. Histologically it is notable that Mycoplasma binds to the epithelial of the trachea, bronchia and bronchioles. This cyto-adhesion is mediated in Mycoplasma pneumoniae by surface lipoproteins (adhesins), which are at the same time significant as antigens.

A severe disease course up to ARDA (acute respiratory distress syndrome) has been described in immunosuppressed patients, following previous bacterial pulmonary infection and in patients with sickle cell anaemia. Accompanying myocarditis or pericarditis can occur, as well as erythema nodosum, erythema multiforme, reactive arthritides (e.g. juvenile spondylarthropathy), spleen infection, glomerulonephritis, meningitis, encephalitis, myelitis and polyradiculitis. Alongside the severe disease course and reinfections, some infections proceed inapparently, while others follow a mild disease course, healing spontaneously without antibiotic treatment.

Infections with Mycoplasma pneumoniae are asymptomatic in 20% of cases. An infection can also cause disease symptoms other than primary atypical pneumonia and general infection of the respiratory organs, which can also be caused by a variety of other pathogens. Therefore, laboratory diagnostic procedures have a valuable role to play.

There is no generally recognised gold standard. Often direct pathogen detection in secretions taken by bronchoalveolar lavage forms the basis. Pathogen cultivation is only performed by a few specialised laboratories, and is difficult, time-consuming (6 to 15 days) and subject to errors. From the literature it is known that that measurement of antibodies does not necessarily correlate with pathogen detection. A frequently used method is CFA (complement fixation assay). In this test only titer increases of 4 levels are diagnostically significant. The CFA is increasingly criticised due to its lack of sensitivity and inability to differentiate antibody classes. Pathogen detection (ribosomal RNA) using RT-PCR (reverse transcriptase polymerase chain reaction) is considered a fast and reliable detection procedure, but is only performed in a few specialised laboratories, since commercial tests of high quality are hardly available. In at least half of infections cold agglutinins are detectable in blood. These can be attributed to oligoclonal antibodies against the I antigen of erythrocytes. Serum cold agglutination is not specific, even though it is found in 50% to 70% of patients between the 7th and 10th day after the start of clinical symptoms of infection. CFA and agglutination test are not very helpful, since they cannot differentiate the different Ig classes.

Sensitive and specific immunological detection techniques such as ELISA and IIFT have now become established in clinical practice. They are simple to perform and readily available. The Anti-Mycoplasma pneumoniae ELISA and the Anti-Mycoplasma pneumoniae IIFT for the immunoglobulin classes IgA, IgG and IgM are the tests of choice for serum diagnostics.

Specific antibodies of class IgM are detectable 4 days after the start of illness. IgG follows a few days later. A continuous increase in IgM titer and especially in IgG titer is usually observed. IgG is significant for confirming an acute Mycoplasma pneumoniae infection. It increases 3 to 4 fold by the 10th or at the latest by the 21st day after the start of illness. Specific IgA antibodies, which can occur together with IgG antibodies, are almost exclusively found in adults. In children the evaluation of the specific IgM titer is indicated. The IgG level can remain the same over several months. In general, it is currently recommended that a diagnosis be based on the analysis of two serum samples taken two to three weeks apart. A single sample is not considered as meaningful enough. Potential cross reactions with other Mycoplasma species cannot be totally excluded with this ELISA or with IIFT.

Mycoplasma pneumoniae infections can be responsible or partly responsible for around 10% of cases of CAP (community acquired pneumonia) and ARDS where artificial ventilation and antibiotic treatment are required. Mycoplasma pneumoniae is sensitive to macrolides and tetracyclines.

Literature references

1. Accomando S, Trizzino A, Montaperto D, Barcellona R, Amato GM. **Mycoplasma pneumonias distribution, epidemiology and prevalence in a triennial survey.** Pediatr Med Chir 26 (2004) 434-438.
2. Espinola-Klein C, Rupprecht HJ, Blankenberg S, Bickel C, Kopp H, Victor A, Hafner G, Prellwitz W, Schlumberger* W, Meyer J. (*EUROIMMUN AG). **Impact of Infectious Burden on Progression of Carotid Atherosclerosis.** Stroke 33 (2002) 2581-2586.
3. EUROIMMUN AG. **Testkit für die Labordiagnostik.** Deutsches Gebrauchsmuster DE 20 2012 004 404 (angemeldet 2012).
4. Guleria R, Nisar N, Chawla TC, Biswas NR. **Mycoplasma pneumoniae and central nervous system complications: a review.** J Lab Clin Med 146 (2005) 55-63.
5. Harjacek M, Ostojic J, Djakovic Rode O. **Juvenile spondyloarthropathies associated with Mycoplasma pneumoniae infection.** Clin Rheumatol 25 (2006) 470-475.
6. Kenri T, Horino A, Matsui M, Sasaki Y, Suzuki S, Narita M, Ohya H, Okazaki N, Shibayama K. **Complete genome sequence of Mycoplasma pneumoniae type 2a strain 309, isolated in Japan.** J Bacteriol 194 (2012) 1253-1254.
7. Kottayam R, Rozenberg G, Cohn RJ. **Unusual haematologic manifestations of Mycoplasma pneumoniae infection.** J Paediatr Child Health 43 (2007) 80-82.

8. Krunkosky TM, Jordan JL, Chambers E, Krause DC. **Mycoplasma pneumoniae host-pathogen studies in an air-liquid culture of differentiated human airway epithelial cells.** Microb Pathog 42 (2007) 98-103.

9. EUROIMMUN AG. Morrin M. **Vorrichtung und Verfahren zur automatischen Fokussierung für die Mikroskopie schwach leuchtender Substrate.** Deutsche und Internationale Patentanmeldung DE 10 2010 035 104.0 (angemeldet 2010) und WO 2012/025220 (angemeldet 2011).

10. Narita M. **Two unexpected phenomena in macrolide-resistant Mycoplasma pneumoniae infection in Japan and the unique biological characteristics of Mycoplasma pneumoniae.** J Infect Chemother 17 (2011) 735-736.

11. EUROIMMUN AG. Stöcker W, Ehling T. **Vorrichtung und Verfahren zur Untersuchung einer biologischen Probe.** Deutsche Patentanmeldung DE 10 2011 011 795.4 (angemeldet 2011).

12. Stöcker* W, Fauer* H, Krause* C, Barth E, Martinez A. (*EUROIMMUN AG). **Verfahren zur Optimierung der automatischen Fluoreszenzerkennung in der Immundiagnostik.** Deutsche Patentanmeldung (Offenlegungsschrift) DE 10 2006 027 516.0 und WO2007140952 (2006).

13. Witmer CM, Steenhoff AP, Shah SS, Raffini LJ. **Mycoplasma pneumoniae, splenic infarct, and transient antiphospholipid antibodies: a new association?** Pediatrics 119 (2007) 292-295.