VIROTECH Mycoplasma pneumoniae IgG/IgM ELISA
(M. pneumoniae IgG/IgM ELISA)

Order No.: EC114.00

M. pneumoniae IgA-Set

Order No.: EC114.08

Color Coding: dark blue

FOR IN VITRO DIAGNOSIS ONLY

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Contents

1. Intended Use

The *Mycoplasma pneumoniae* ELISA is used for the semiquantitative and qualitative detection of IgG, IgM and IgA antibodies in human serum. The detection of IgG antibodies is set so that it mainly detects fresh infections.

2. Diagnostic Relevance

The bacteria *Mycoplasma pneumoniae*, which is lacking cell wall components, is the cause of atypical pneumonia and tracheobronchitis of humans and affects mostly children, young adults and immunodeficient people (1,2,3,4). So called adhesins (6), enable the bacteria to attach to the epithelial cells, against which the host develops antibodies. Studies made by Foy show, that in the USA 15 to 20% of all pneumonia cases are caused by *Mycoplasma pneumoniae* (8). The ELISA detects Mycoplasma-antibodies with a defined antigen fraction of the strain M129, which is defined via monospecific sera. It includes membrane proteins, cytoskeleton proteins and recombinant proteins.

The incubation time during an infection with *Mycoplasma pneumoniae* is 10 – 21 days:

- Specific IgM-antibodies occur 6-10 days after infection. Basically, about 80% of the patients younger than 20 years develop IgM-antibodies and 40% of the patients that are older than 20 years. This means a specific IgM-response can be missing especially in older patients. IgM-antibodies may be detected, referring to literature, still at least one year after beginning of the symptoms.
- Specific IgG-antibodies appear 9-14 days after infection. They may persist up to 4 years.
- Specific IgA-antibodies appear one week after start of the infection and decrease about 5 weeks after start of the infection again. As a rule, the IgA-titer exceeds the IgM-titer.

Considering the fact that IgM-antibodies persist very long in some persons and are missing in others completely, it is important to detct beside the IgM- also the specific IgG- and IgA-titer. Re-infections often take place without any production of IgM-antibodies but under significant increase of IgG- and IgA-antibody titers. Two patient sera, taken at an interval of 5-10 days allow a proper statement concerning the rise of the antibody titer (5). It is important to consider that a first attack of Mycoplsma pneumoniae does not leave a sufficient protection against a new colonization. For diagnosis it is necessary in any case to consider the clinical picture in addition to the serological results. Mycoplasma infections are generally treated successfully with antibiotics like Tetracycline and Macrolide. The treatment with non-suitable, w.g. cell-wall-specific antibiotics (penicillin) leads to a serological advantage for Mycoplasma against all Penicillin-sensitive microorganisms.

3. Test Principle

The antibody searched for in the human serum forms an immune complex with the antigen coated on the microtiter-plate. Unbound immunoglobulins are removed by washing processes. The enzyme conjugate attaches to this complex. Unbound immunoglobulins are again removed by washing processes. After adding the substrate solution (TMB), a blue dye is produced by the bound enzyme (peroxidase). The color changes to yellow when the stopping solution is added.

4. Package Contents

4.1 IgG/IgM Testkit

1. **1 Microtiter-Plate** consisting of 96 with antigen coated, breakable single wells, lyophilised
2. **PBS-Dilution Buffer, (blue, ready to use), 2x50ml**, pH 7,2, with preservative and Tween 20
3. **PBS-Washing Solution, (20x concentrated) 50ml**, pH 7,2, with preservative and Tween 20
4. **IgG negative Control, 1300µl,** human serum with protein-stabilizer and preservative, ready to use
5. **IgG cut-off Control, 1300µl**, human serum with protein-stabilizer and preservative, ready to use
6. **IgG positive Control, 1300µl**, human serum with protein-stabilizer and preservative, ready to use
7. **IgM negative Control, 1300µl,** human serum with protein-stabilizer and preservative, ready to use
8. **IgM cut-off Control, 1300µl**, human serum with protein-stabilizer and preservative, ready to use
9. **IgM positive Control, 1300µl**, human serum with protein-stabilizer and preservative, ready to use
10. **IgG-Conjugate (anti-human), 11ml**, (sheep or goat)-horseradish-peroxidase-conjugate with protein-stabilizer and preservative in Tris-Buffer, ready to use
11. **IgM-Conjugate (anti-human), 11ml,** (sheep or goat)-horseradish-peroxidase-conjugate with FCS and preservative in Tris Buffer, ready to use
12. **Tetramethylbenzidine substrate solution (3,3',5,5'-TMB), 11ml**, ready to use

13. **Citrate-Stopping Solution, 6ml**, contains an acid mixture

4.2 IgA-Set

1. **IgA negative Control, 1300µl,** human serum with protein stabilizer and preservative, ready to use
2. **IgA cut-off Control, 1300µl,** human serum with protein stabilizer and preservative, ready to use
3. **IgA positive Control, 1300µl,** human serum with protein stabilizer and preservative, ready to use
4. **IgA-Conjugate 2 (anti-human), 11ml,** (sheep or goat)-horseradish-peroxidase with FCS and preservative in Tris-Buffer, ready to use

5. Storage and Shelflife of the testkit and the ready to use reagents

Store the testkit at 2-8°C. The shelf life of all components is shown on each respective label; for the kit shelf life please see Quality Control Certificate.

1. Microtiter strips/single wells are to be resealed in package after taking out single wells and stored with desiccant at 2-8°C. Reagents should immediately be returned to storage at 2-8°C after usage.
2. The ready to use conjugate and the TMB-substrate solution are sensitive to light and have to be stored in dark. Should there be a color reaction of the substrate dilution due to incidence of light, it is not useable anymore.
3. Take out only the amount of ready to use conjugate or TMB needed for the test insertion. Additional conjugate or TMB taken out may not be returned but must be dismissed.

Material	Status	Storage	Shelflife
Test Samples	Diluted	+2 to +8°C	max. 6h
	Undiluted	+2 to +8°C	1 week
Controls	After Opening	+2 to +8°C	3 months
Microtitreplate	After Opening	+2 to +8° (storage in the provided bag with desiccant bag)	3 months
Rheumatoid factor - Absorbent	Undiluted, After Opening	+2 to +8°C	3 months
	Diluted	+2 to +8°C	1 week
Conjugate	After Opening	+2 to +8°C (protect from light)	3 months
Tetramethylbenzidine	After Opening	+2 to +8°C (protect from light)	3 months
Stop Solution	After Opening	+2 to +8°C	3 months
Washing Solution	After Opening	+2 to +8°C	3 months
	Final Dilution (ready-to-use)	+2 to +25°C	4 weeks

6. Precautions and Warnings

1. Only sera which have been tested and found to be negative for HIV-1 antibodies, HIV-2 antibodies, HCV antibodies and Hepatitis-B surface-antigen are used as control sera. Nevertheless, samples, diluted samples controls, conjugate and microtiter strips should be treated as potentially infectious material. Please handle products in accordance with laboratory directions.
2. Those components that contain preservatives, the Citrate Stopping Solution and the TMB have an irritating effect to skin, eyes and mucous. If body parts are contacted, immediately wash them under flowing water and possibly consult a doctor.
3. The disposal of the used materials has to be done according to the country-specific guidelines.

7. Material required but not supplied

1. Aqua dest./demin.
2. Eight-channel pipette 50µl, 100µl
3. Micropipettes: 10µl, 100µl, 1000µl
4. Test tubes
5. Paper towels or absorbent paper
6. Cover for ELISA-plates
7. Disposal box for infectious material
8. ELISA handwasher or automated EIA plate washing device
9. ELISA plate spectrophotometer, wavelength = 450nm, reference length = 620nm (Reference Wavelength 620-690nm)
10. Incubator

8. Test Procedure

Working exactly referring to the VIROTECH Diagnostics user manual is the prerequisite for obtaining correct results.

8.1 Examination Material

Either serum or plasma can be used as test material, even if only serum is mentioned in the instructions. Any type of anticoagulant can be used for plasma.

Always prepare patient-dilution freshly.

For a longer storage the sera must be frozen. Repeated defrosting shall be avoided.

1. Only fresh non-inactivated sera should be used.
2. Hyperlipaemic, haemolytic, microbially contaminated and turbid sera should not to be used (false positive/negative results).

8.2 Preparation of Reagents

The VIROTECH Diagnostics System Diagnostica offers a high degree of flexibility regarding the possibility to use the dilution buffer, washing solution, TMB, citrate stopping solution as well as the conjugate for all parameters and for all different lots. The ready to use controls (positive control, negative control, cut-off control) are **parameter specific** and **only to use** with the plate lot indicated in the Quality Control Certificate.

1. Set incubator to 37°C and check proper temperature setting before start of incubation.
2. Bring all reagents to room temperature before opening package of microtiter strips.
3. Shake all liquid components well before use.
4. Make up the washing solution concentrate to 1 L with distilled or demineralised water. If crystals have formed in the concentrate, please bring the concentrate to room temperature before use and shake well before use.
5. High IgG-titers or rheumatoid-factors may disturb the specific detection of IgM-antibodies and lead to false positive respetively false negative results. **For a correct IgM-determination it is therefore necessary to treat the sera with RF-SorboTech** (VIROTECH adsorption). The IgM-controls must not be treated with the pre-absorption.

8.3 VIROTECH ELISA Test Procedure

1. For each test run, pipette 100µl each of ready to use dilution buffer (blank), IgG-, IgM- and IgA-positive, negative and cut-off controls as well as diluted patient sera. We propose a double insertion (blank, controls and patient sera); for cut-off control a double insertion is absolutely necessary. Working dilution of patient sera: 1+100; e.g. 10µl serum + 1ml dilution buffer.
2. After pipetting start incubation for 30 min. at 37°C (with cover).
3. End incubation period by washing microtiter strips 4 times with 350 – 400µl washing solution per well. Do not leave any washing solution in the wells. Remove residues on a cellulose pad.
4. Pipette 100µl of ready to use conjugate into each well.
5. Incubation of conjugates: 30 min. at 37°C (with cover).
6. Stop conjugate incubation by washing 4 times (pls. refer to point 3 above).
7. Pipette 100µl of ready to use TMB into each well.
8. Incubation of substrate solution: 30 min. at 37°C (with cover, keep in dark).
9. Stopping of substrate reaction: pipette 50µl of citrate stop solution into each well. Shake plate carefully and thoroughly until liquid is completely mixed and a homogeneous yellow color is visible.
10. Measure extinction (OD) at 450/620nm (Reference Wavelength 620-690nm). Set your photometer in such a way that the blank value is deducted from all other extinctions. Extinctions should be measured within 1 hour after adding the stopping solution!

Pls. refer to last page for Test Procedure Schemata

8.4 Usage of ELISA processors

All VIROTECH Diagnostics ELISAs can be used on ELISA processors. The user is bound to proceed a validation of the devices (processors) on a regular basis.

VIROTECH Diagnostics recommends the following procedure:

1. VIROTECH Diagnostics recommends to proceed the validation of device referring to the instructions of the device manufacturer during the implementation of the ELISA processor respectively after bigger reparations.
2. It is recommended to check the ELISA-processor with the Validationkit (EC250.00) afterwards. A regular check using the Validationkit shall be proceeded minimum once a quarter to test the accuracy of the processor.
3. The release criteria of the Quality Control Certificate of the product must be fulfilled for each testrun.

With this procedure, your ELISA processor will function properly and this will support quality assurance in your laboratory.

9. Test Evaluation

The ready to use controls serve for a semiquantitative determination of specific IgG-, IgM- and IgA-antibodies. Their concentration can be expressed in VIROTECH units = VE. Fluctuations resulting from the test procedure can be balanced with this calculation method and a high reproducibility is achieved in this way. Use the means of the OD values for calculation of the VE.

9.1 Test function control

a) OD-values

The OD of the blank should be < 0.15.

The OD-values of the negative controls should be lower than the OD-values mentioned in the Quality Control Certificate. The OD-values of the positive controls as well as of the cut-off controls should be above the OD-values mentioned in the Quality Control Certificate.

b) VIROTECH Units (VE)

The VIROTECH Units (VE) of the cut-off controls are defined as 10. The calculated VE of the positive controls should be within the ranges mentioned in the Quality Control Certificate.

If those requirements (OD-values, VE) are not fulfilled, the test has to be repeated.

9.2 Calculation of the VIROTECH Units (VE)

The extinction of the blank value (450/620nm) has to be subtracted from all other extinctions.

$$VE\ (\text{positive control}) = \frac{OD\ (\text{positive control})}{OD\ (\text{cut-off control})} \times 10$$

$$VE\ (\text{patient serum}) = \frac{OD\ (\text{patient serum})}{OD\ (\text{cut-off control})} \times 10$$

9.3 Interpretation of Results

a) In IgM and IgA for all patients, in IgG for patients > 14 years

Result (VE) (IgG > 14 years, IgM and IgA)	**Evaluation**
< 9,0	negative
9,0 – 11,0	borderline
> 11,0	positive

b) In IgG for children (0-14 years), if IgM and/or IgA positive

For children between 0 and 14 years, the threshold (cut off) in the IgG can be reduced, as the VIROTECH ELISA for IgG is adjusted so that it mainly detects acute infections. However, the condition for using this scheme is that the serum gives a positive IgM and /or IgA result.

Result (VE) (IgG 0-14 years)	Evaluation
< 7.0	negative
7.0 – 8.0	threshold
> 8.0	positive

1. If the measured values are above the defined borderline range, they are considered to be positive.
2. For the secure detection of an infection it is necessary to determine the antibody concentration of two serum samples.One sample shall be taken directly at the beginning of the infection and a second sample 5 – 10 days later (convalescent serum). The antibody concentration of both samples have to be tested in parallel, that means in one test run. A correct diagnosis based on the evaluation of a single serum sample is not possible. The highest sensitivity is reached if all 3 antibody classes (IgG, IgM and IgA) are tested, as it has to be considered that some persons do not develop IgM.
3. If the measured values are below the defined borderline range, no measurable antigen specific antibodies are present in the sample. The samples are considered to be negative.

9.4 Interpretation Scheme

IgG	IgA	IgM	
-	-	-	No contact with *Mycoplasma pneumoniae* or antibody level has already decreased below the c.o. level
-	+	+	Very early stage of an acute infection or re-infection
-	+	-	Very early stage of an acute infecction; either primary infection or reinfection without IgM or IgM-titer is still to come
+	+	+	Acute infection, primary infection as a rule; later stage, IgG- and IgM already developed, IgA not yet decreased
+	-	+	Acute infection, primary infection as a rule; late stage, IgG and IgM already developed, IgA already decreased
+	+	-	Re-infection, very late stage, IgA still present, IgM no longer present, or reactivation or infection without development of IgM
+	-	-	Re-infection, very late stage, IgA already decreased or not developed at all (happens in some adults) or reactivation or infection without development of IgM or persistent IgG-titer after past infection
-	-	+	Acute early infection, IgA still missing or already decreased, IgG-titer still too low.

Important note: Isolated false positive IgA or IgM results can never be totally excluded. As confirmation, it is recommended that the IgG titer should be checked in 5-10 days or that a control should be performed using Immunoblot (LINE).

9.5 Limits of the Test

1. The interpretation of serological results shall always include the clinical picture, epidemiological data and all further available laboratory results.
2. Even if a medical history is taken and a clinical examination is performed, with standard clinical chemistry and X-rays, it is difficult to distinguish a *Mycoplasma* infection from other infections of the upper and lower respiratory tracts or from atypical pneumonias. In unclear cases, or if the symptoms persist in spite of a negative finding, we recommend that the serological diagnosis should be supported by a molecular biological test.
3. Cross-reactions with *M. genitalium* or *M. hominis* can not be excluded. Also EBV-positive sera can cross-react.

10. Performance Data

10.1 Sensitivity and Specificity

To determine the sensitivity, 99 sera were tested for IgG, IgA and IgM in comparison to the VIROTECH Mycoplasma pneumoniae LINE. The serum panel was made up as follows: 49 clinically characterized sera from patients with confirmed *Mycoplasma pneumoniae*–induced atypical pneumonia (*M. pneumoniae* PCR positive; provided by the CAPNETZ

Foundation), 34 sera from children aged 1 to 14 years suspected of having a *Mycoplasma pneumoniae* infection; 16 sera from adults suspected of having a *Mycoplasma pneumoniae* infection.

To determine the specificity, 161 sera were tested for IgG, IgA and IgM in comparison to the VIROTECH Mycoplasma pneumoniae LINE. The serum panel was made up as follows: 71 blood donor sera, 26 sera from clinically confirmed community-acquired pneumonia not caused by *Mycoplasma pneumoniae* (CAP: community-acquired Pneumonia) – with a positive PCR result for the corresponding pathogen and a negative PCR result for *Mycoplasma pneumoniae*, 26 sera from neonates aged 0-3 months and 38 sera from patients with other respiratory diseases (21 *B. pertussis* positive sera and 17 *Legionella pneumophila* positive sera).

	Sensitivity - Reference: Mycoplasma pneumoniae LINE -	**Specificity** - Reference: Mycoplasma pneumoniae LINE -
IgG	97 %	98 %
IgA	95 %	97 %
IgM	94 %	98 %

10.2 Diagnostic Sensitivity

To determine the diagnostic sensitivity, 49 clinically characterized sera were tested from patients with established atypical pneumonia. These sera came from the stocks of the CAPNETZ Foundation. All patient samples had previously given a positive PCR result for *Mycoplasma pneumoniae*. The previous serological findings with ELISA gave a positive finding for IgM in 34 sera and a negative finding in 15 sera (7, 9). Although *Mycoplasma pneumoniae*-DNA was detectable in these patient samples, antibodies may not be detectable if the immune response is delayed. This explains the low sensitivity of the previous findings.

	Diagnostic Sensitivity Previous findings with the CAPNETZ sera: PCR positive and 69% serologically positive
IgG	85 %
IgA	91 %
IgM	79 %
Total: IgG, IgA and IgM	96 %

If an overall evaluation is performed with IgG, IgA and IgM for this critical serum panel, the sensitivity is clearly raised, to a value of 96 %.

10.3 Diagnostic Specificity

To determine the diagnostic specificity, the following sera were tested from the stocks of the CAPNETZ Foundation:
26 sera from patients with clinically confirmed community-acquired pneumonia not caused by *Mycoplasma pneumoniae* (CAP: Community-acquired Pneumonia), with a positive PCR result for the corresponding pathogen and a negative PCR result for *Mycoplasma pneumoniae*.

	Diagnostic Specificity Previous findings with the CAPNETZ sera: *M. pneumoniae* PCR negative
IgG	100 %
IgA	92 %
IgM	100 %

10.4 Prevalence (Expected Values)

71 blood donor sera were tested for IgG, IgA and IgM.

	IgG		**IgA**		IgM	
negative	65	92 %	68	96 %	70	99 %
threshold	5	7 %	1	1 %	1	1%
positive	1	1 %	2	3 %	0	0%

10.5 Intra-Assay Coefficient of Variation (Repeatability)

In a single assay, strips from different plates from a batch were tested in a chessboard pattern. The resulting coefficients of variation were under 9% (n=2x48).

10.6 Inter-Assay Coefficient of Variation (Reproducibility)

3 sera were tested in 10 independent test batches on 3 different test days. This gave a coefficient of variation of < 15%.

11. Literature

1. Clyde WA.J.: Clinical overview of typical Mycoplasma pneumoniae infections. J. Clin Infect. Dis. 1993, 17 (suppl. 1) 32-37
2. Hu, P.-C., Collier, A.M. and Baseman, J.B. (1977): Surface parasitism by Mycoplasma pneumoniae of respiratory epithelium. J. of Experimental med. 145, 1328-13343.
3. Razin, S. (1992): Peculiar properties of mycoplasmas: the smallest self-replicating prokaryotes. FEMS Microbiol. Lett. 100, 423-432.
4. Taylor-Robinson, D. (1996): Infections due to species of Mycoplasma and Ureaplasma: an update. Clin. Infect. Dis. 23, 671-684.
5. Jacobs, E.: Mycoplasmen-Infektionen. mta. 1997, 12: 236-239
6. Jacobs, E.: Das Adhäsin von Mycoplasma pneumoniae: Seine Bedeutung als Virulenzfaktor in der Pathogenese und in der Diagnostik. Klin. Lab. 1994: 40: 228-229
7. Dumke, R., A. Strubel, C. Cyncynatus, H. Nuyttens, R. Herrmann, C. Lück, and E. Jacobs. 2012. Optimized serodiagnosis of Mycoplasma pneumoniae infections. Diagnostic microbiology and infectious disease. Elsevier Inc. 73:200-203.
8. Foy, HM: Infections caused by Mycoplasma pneumoniae and possible carrier state in different populations of patients. J Clin Infect Dis 1993, 17(suppl. 1) 37-47.
9. Baum, H. v. et.al.: Mycoplasma pneumoniae pneumonia revisited within the German Competence Network for Community-acquired pneumonia (CAPNETZ), BMC Infectious Diseases 2009, 9:62

Preparation of Patient Samples and Washing Solution

▼ **Washing Solution:** Fill up concentrate to 1 liter with aqua dest./demin.

▼ IgG-/IgA-Samples – Dilution 1:101

e.g.:
10 µl serum/plasma + 1000 µl Dilution Buffer
(Serum Dilution Buffer is ready to use)

▼ IgM-Samples – Dilution 1:101
Rheumafactor-absorption with RF-SorboTech

e.g.:
5 µl serum/plasma + 450 µl Dilution Buffer +
1 drop RF-SorboTech, incubate for 15 min. at room temperature.

Testprocedure

Samples Incubation	**30 minutes at 37°C**	**100 µl Patient Samples** blank value (Dilution Buffer) and controls
⇩		
Wash 4times		**400 µl Washing Solution** Remove Residues on a Cellulose Pad
⇩		
Conjugate Incubation	**30 minutes at 37°C**	**100 µl Conjugate** IgG, IgM, IgA
⇩		
Wash 4times		**400 µl Washing Solution** Remove Residues on a Cellulose Pad
⇩		
Substrate Incubation	**30 minutes at 37°C**	**100 µl Substrate**
⇩		
Stopping		**50 µl Stop Solution** shake carefully
⇩		
Measure Extinctions		**Photometer at 450/620nm** **(Reference Wavelength 620-690nm)**