



SeroMP™ Recombinant IgG

Enzyme -Linked Immunosorbent Assay (ELISA)
for the semi-quantitative detection
of specific IgG antibodies to *Mycoplasma pneumoniae* in human serum

Instruction Manual

Test kit for 96 determinations
(Catalog No. 1261-01)

For *In Vitro* Diagnostic Use
For professional use only
Store at 2-8°C. **Do Not Freeze**



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Intended Use

SeroMP™ Recombinant IgG kit is a semi-quantitative Enzyme Linked Immunosorbent assay (ELISA) for the determination of species specific IgG antibodies to *Mycoplasma pneumoniae* in human serum.

The Savyon® SeroMP™ Recombinant IgG is used as an aid in the diagnosis of *Mycoplasma pneumoniae* infection. The test also enables the diagnosis of current infection by determining the rise of IgG antibodies in paired sera taken 2-4 weeks apart.

For *In Vitro* Diagnostic Use.

Introduction

M. pneumoniae is a common cause of community-acquired pneumonia, often characterized by gradual onset of headache, fever, malaise and, most typically, dry cough. *M. pneumoniae* is common in all age groups, however, it is most common in the first two decades of life and is rare in children under the age of four. It has been reported as the cause of up to 30% of all pneumonia cases (2).

M. pneumoniae has also been associated with non respiratory diseases as meningitis, encephalitis, pancreatitis, sensorineural hearing loss, and acute brainstem syndrome (5).

Due to its common occurrence, one should consider *M. pneumoniae* in all cases of pneumonia, but being the same symptoms for different agents, additional diagnostic tools, such as serological tests, are required (3).

The ELISA technique is sensitive, specific and enables a differential determination of specific IgG, IgA and IgM antibodies (6).

In respect to diagnosis and treatment, the most prominent structural feature of MP is the lack of a cell wall. It has been shown that surface-exposed polypeptides elicit an immunogenic response, in particular those that are involved in the attachment organelle of MP. This attachment organelle is composed of a complex of polypeptides, in which P1 Cytoadhesin Protein has a major role. (1; 4; 10) Due to its high immunogenicity P1 is a paradigm for utilizing a definitive antigen in serology-based diagnostic systems, attempting to improve various parameters of assay performance. A common way to improve test performances by using highly immunogenic polypeptides like the P1 is incorporating these polypeptides in the tests as recombinant antigens. Indeed, several polypeptides have been identified in the literature as good candidates for this purpose. (9)

M. pneumoniae specific IgM antibodies rise early after onset of the disease, reach peak levels in one to four weeks, then decline to diagnostically insignificant levels within a few months (7). Due to the early appearance and relatively short lifetime of IgM antibodies, their detection allows the diagnosis of acute infection using a single serum sample. Young patients tend to have higher IgM levels than adults (8). IgG levels rise slower than IgM, but remain elevated much longer, so a significant increase in two consecutive samples taken at least 2 weeks apart, may indicate current infection or re-infection even in the absence of IgM. IgA antibodies are seen at higher levels in elderly patients (7) and may be more useful than IgM for the diagnosis of current infection in adults (8).

Savyon® Diagnostics Ltd. has developed semi-quantitative kits utilising recombinant antigens in IgG, IgA ELISA tests and a qualitative kit utilising mixture of recombinant and native antigen in the IgM ELISA test which enable to follow the change of antibody levels in human sera.

The SeroMP™ Recombinant IgG, IgA and IgM tests enable early and accurate detection of *M. pneumoniae* infection.

Principle of the Test

- SeroMP™ Recombinant microtiter plates are coated with *M. pneumoniae* recombinant antigens.
- The serum to be tested is diluted and incubated in the SeroMP™ Recombinant plate. In this step, *M. pneumoniae* specific antibodies are bound to the immobilized antigens.
- Non-specific antibodies are removed by washing.
- Anti-human IgG conjugated to horseradish peroxidase (HRP) is added. In this step the HRP-conjugate is bound to the prebound antigen-antibody complex.
- Unbound conjugate is removed by washing.
- Upon the addition of TMB-substrate, the substrate is hydrolyzed by the peroxidase, yielding a blue solution of the reduced Substrate.
- Upon the addition of the stop solution, the blue color turns yellow and should be read by an ELISA reader at a wavelength of 450/620nm.
- The absorbance is proportional to the levels of the specific antibodies that are bound to the coated antigens.

Assay Procedure

Wells of microtiter plate coated with *M.pneumoniae* antigens
 ↓
 Add 50µl of Negative Control, 50µl of Positive Control, 50µl of each calibrator: (P10, P50, P75), and diluted specimens
 ↓
 Cover plate and incubate 1h at 37°C at 100% humidity
 ↓
 Wash 3 times with Wash Buffer
 ↓
 Add 50µl of 1/300 diluted HRP Conjugate
 ↓
 Cover plate and incubate 1 h at 37°C at 100% humidity
 ↓
 Wash 3 times with Wash buffer
 ↓
 Add 100µl of TMB-Substrate
 ↓
 Cover plate and incubate 15min at room temperature
 ↓
 Add 100µl of Stop Solution
 ↓
 Read absorbance at 450/620nm
 ↓
 Calculate and interpret results

Kit contents:

Test kit for 96 Determinations

- M. pneumoniae* antigen coated microtiter plate:** 96 break apart wells (8x12) coated with *M. pneumoniae* antigens, packed in an aluminum pouch containing a desiccant card.
1 Plate
- Concentrated Wash Buffer (20X):** A PBS - Tween buffer.
1 Bottle, 100 ml
- UniDiluent™ (yellow):** A ready-to-use buffer solution. Contains less than 0.05% Proclin as a preservative.
1 Bottle, 60 ml
- Positive Control:** A ready-to-use *M.pneumoniae* IgG positive human serum. Contains less than 0.05% Proclin and less than 0.1% sodium azide as preservatives.
1 Vial, 2.0 ml
- Negative Control:** A ready-to-use *M.pneumoniae* IgG negative human serum. Contains less than 0.05% Proclin and less than 0.1% sodium azide as preservatives.
1 Vial, 2.0 ml
- P10-calibrator:** A ready-to-use *M. pneumoniae* IgG low positive human serum. Contains 10 BU/ml of IgG (arbitrary binding units). Contains less than 0.1% Sodium Azide and less than 0.05% Proclin as preservatives.
1 Vial, 2.0 ml
- P50-calibrator:** A ready-to-use *M. pneumoniae* IgG medium positive human serum. Contains 50 BU/ml of IgG (arbitrary binding units) Contains less than 0.1% Sodium Azide and less than 0.05% Proclin as preservatives.
1 Vial, 2.0 ml
- P75-calibrator:** A ready-to-use *M. pneumoniae* IgG high positive human serum. Contains 100 BU/ml of IgG (arbitrary binding units). Contains less than 0.1% Sodium Azide and less than 0.05% Proclin as preservatives.
1 Vial, 2.0 ml

- Concentrated HRP-Conjugate (300 X):** Horseradish peroxidase (HRP) conjugated anti-human IgG (γ chain specific).
1 Vial, 0.2 ml
- TMB-Substrate:** A ready-to-use solution. Contains 3, 3', 5, 5' - tetramethylbenzidine as a chromogen and peroxide as a substrate.
1 Bottle, 14 ml
- Stop Solution:** A ready-to-use solution. Contains 1M H_2SO_4 .
1 Bottle, 15 ml
- Plate Cover:**
1 Unit
- Instruction Manual:**
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Materials Required But Not Supplied:

- Clean test tubes for dilution of patients sera.
- Disposable plastic vial for dilution of the concentrated HRP- conjugate.
- Adjustable micropipettes and multichannel pipettes (5-50, 50-200 and 200-1000µl ranges) and disposable tips.
- One liter volumetric flask.
- One 50ml volumetric cylinder.
- Wash bottle.
- Absorbent paper.
- Vortex mixer
- A 37°C water bath with a lid, or a moisture chamber placed in a 37°C incubator.
- ELISA-reader with a 450 and 620nm filters.
- Distilled or double deionized water.

Warning and Precautions

For In Vitro Diagnostic Use

- This kit contains human sera which have been tested by FDA approved techniques, and found to be negative for HBV antigen and for HCV and HIV 1 and 2 antibodies. Since no known method can offer complete assurance that products derived from human blood do not transmit infection, all human blood components supplied in this kit must be handled as potentially infectious serum or blood according to the recommendations published in the CDC/NIH manual "Biosafety in Micro Biological and Biomedical Laboratories, 1988".
- TMB-Substrate solution is an irritant material to skin and mucous membranes. Avoid direct contact.
- All the components of this kit have been calibrated and tested by lot. It is not recommended to mix components from different lots since it might affect the results.
- Diluted sulfuric acid (1M H_2SO_4) is an irritant agent for the eyes and skin. In case of contact with eyes, immediately flush area with water and consult a physician.

Storage and Shelf -Life of Reagents

- All the reagents supplied should be stored at 2-8°C. The unopened reagents vials are stable until the expiration date indicated on the kit pack. Exposure of originally stoppered or sealed components to ambient temperature for a few hours will not cause damage to the reagents.
DO NOT FREEZE!
- Once the kit is opened, it's shelf life is 90 days.
- Unused strips must be resealed in the aluminum pouch with the desiccant card, by rolling the open end and

sealing tightly with tape over the entire length of the opening.

4. Crystals may form in the 20x concentrated Wash Buffer during cold storage, this is perfectly normal. Redissolve the crystals by warming the buffer to 37°C before diluting. Once diluted, the solution may be stored at 2-8°C up to twenty one days.

Serum Collection

Prepare sera from aseptically collected samples using standard techniques. Heat inactivated sera should not be used. The use of lipemic, turbid or contaminated sera is not recommended. Particulate material and precipitates in sera may cause erroneous results. Such specimens should be clarified by centrifugation or filtration prior to the test.

Storage

Specimens should be stored at 2-8°C and tested within 7 days (adding of 0.1% Sodium Azide is highly recommended). If longer storage period is anticipated, aliquot and store the specimens below -20°C. Avoid repeated thawing and freezing.

Test Procedure - Manual

Automation protocol available upon request

A. Preparation of Reagents

1. Bring all components and the clinical specimens to be tested to room temperature. Mix well the calibrators (P10, P50, P75), Negative Control, Positive Control and the clinical specimens before use.
2. Determine the total number of specimens to be tested. In addition to the specimens, the following must be included in each test: One well of Negative Control, Positive Control and three wells of calibrators (P10, P50, P75).
3. Withdraw the microtiter plate from its aluminum pouch by cutting one end near the seal. Leave the required number of strips (according to the number of specimens to be tested) in the 96 well frame.
4. Dilute the Concentrated Wash Buffer 1/20 with double-deionized or distilled water. For example, in order to prepare one liter of wash buffer, add 50ml of the Concentrated Wash Buffer to 950ml of double-deionized or distilled water.

B. Incubation of sera samples and controls

5. Dilute each patient serum 1:105 with the supplied UniDiluent™ as follows: Add 10µl of patient serum to 200µl of UniDiluent™ (1/21), and then dilute further by adding 25µl of 1/21 dilution to 100µl of UniDiluent™.
6. Dispense 50µl of Negative Control, Positive Control, three calibrators (P10, P50, P75), and 1:105 diluted serum samples into separate wells of the test strip.
7. Cover the strips with a plate cover and incubate for 1h at 37°C in a moisture chamber.
8. Discard the liquid content of the wells.
9. **Washing step:** Fill each well with wash buffer up to the end of the well and discard the liquid, repeat this step for a total of three times.

10. Dry the strips and frame by gently tapping them over clean absorbent paper.

C. Incubation with conjugate

11. Concentrated HRP-conjugated anti-human IgG should be diluted to working solution shortly before use. Dilute the concentrated HRP-conjugated anti-human IgG 1/300 with UniDiluent™. For example: for two strips prepare a minimum of 3ml conjugate as follows: 10µl of Concentrated HRP-conjugated anti-human IgG is mixed with 3ml of UniDiluent™.
12. Dispense 50µl of diluted conjugate into each well.
13. Cover the strips with a plate cover and incubate for 1h at 37°C in a moisture chamber.
14. Discard the liquid content and wash as described in steps 9-10.

D. Incubation with TMB - Substrate

15. Dispense 100µl TMB-Substrate into each well, cover the strips with a plate cover and incubate at room temperature for **15 minutes**.
16. Stop the reaction by adding 100µl of stop solution (1M H₂SO₄) to each well.

E. Determination of Results

17. Determine the absorbance at 450/620nm and record the results. Determination should not exceed 30 minutes following stopping of chromogenic reaction.
 - **Note:** Any air bubbles should be removed before reading. The bottom of the ELISA plate should be carefully wiped.

Test Validation

The following criteria must be met for the test to be valid. If these criteria are not met, the test should be considered invalid and should be repeated.

1. O.D._{P75} > **0.9**
2. Ratio: O.D._{P10} / O.D._{NC} > **1.5**
3. Ratio: O.D._{P50} / O.D._{NC} > **4**
4. Ratio: O.D._{P75} / O.D._{NC} > **5**
5. PC should be ≥ **40 BU/ml**

Calculation of Test Results

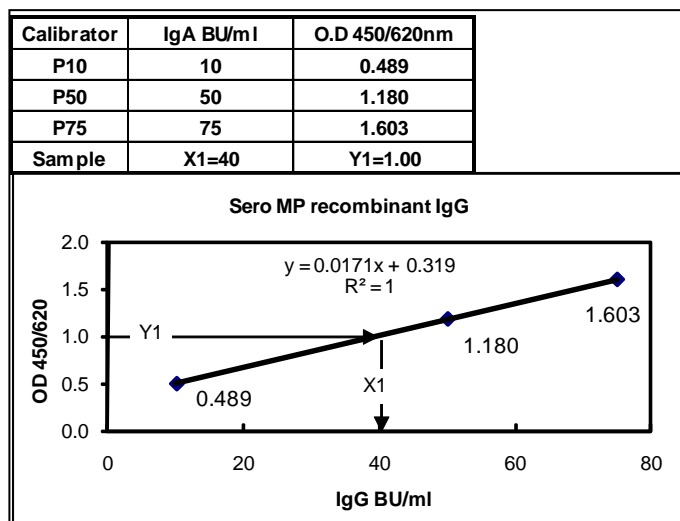
Manual method, using a squared graph paper:

1. Plot the absorbance values (OD) of the 3 calibrators (P10, P50 and P75) on Y axis versus their concentration (BU/ml) on X axis.
2. Draw the best fitted linear curve through the points.
3. Using the standard curve, interpolate the concentration of the tested sample values (in BU/ml) from each absorbance measured (see example 1).

Example 1: Interpolation of results:

On the Y-axis read the absorbency value of the sample and draw a horizontal line to the calibration curve. From the intercept, draw a vertical line to the X-axis.

Read the concentration in BU/ml of the sample.



BU2 = Concentration in BU/ml of the 2nd sample
If $R \geq 1.55$, the difference is statistically significant ($p=0.005$)

² Interpretation based on profile of IgG, IgM, IgA antibodies

In view of the common pattern of appearance of IgG, IgM, and IgA antibodies, the absence of a specific antibody should be taken in the context of its expected timing of appearance. For example, a case in which IgM is detected but IgG is negative may indicate a clear distinction between the times of appearance of these antibodies.

In order to achieve a comprehensive profile of antibodies, IgM and IgA should also be tested

Interpretation of results based on the combination of IgM and IgG and IgA antibodies detection.

Level of <i>M. pneumoniae</i> antibodies			
IgG	IgM	IgA	
Negative	Negative	Negative	No indication of <i>M. pneumoniae</i> infection
Negative or Positive	Positive	Negative or Positive	Indication of current infection
Positive	Negative	Negative	Indication of past infection
Negative or Positive	Negative	Positive	Indication of current infection or re-infection

Interpretation of Results based on single serum

IgG BU/ml	Result	Diagnostic Interpretation
< 10 BU/ml	Negative No detectable IgG antibodies	No indication of <i>M. pneumoniae</i> Infection To verify the interpretation it is recommended to test a second sample ¹ , and/or test IgM and IgA antibodies ² .
≥ 10 BU/ml	Positive Relevant level of IgG antibodies	Indication of current or past <i>M. pneumoniae</i> infection To verify the interpretation it is recommended to test a second sample ¹ , and/or test IgM and IgA antibodies ² .

¹ Interpretation based on paired sera

In cases of **negative results**, in which distinction is required between no infection and current infection and in cases of **positive results**, in which distinction is required between past and current infection, it is recommended to take a second sample after 2-4 weeks. If the BU/ml value of the second sample significantly increases current infection is indicated.

In order to evaluate if the difference between the 2 measurements is significant, at least one of the paired sera samples must be **positive**.

The ratio between the paired sera should be calculated as follows:

$$R = \frac{BU2 + 15}{BU1 + 15}$$

BU1 = Concentration in BU/ml of the 1st sample

Test Performance

The effect of recombinant based-antigen compared to native based-antigen in IgG, in pneumonia patients

	Group	Native Antigen %	Recombinant Antigen %
IgG N=91	Positive	35	45
	Borderline	12	0
	Negative	53	55

The effect of recombinant based-antigen compared to native based-antigen in IgG, in a healthy population

	Group	Native Antigen %	Recombinant Antigen %
IgG N=91	Positive	27	10
	Borderline	30	0
	Negative	43	90

- In the pneumonia patients – rate of positive is higher and borderline samples are eliminated in the recombinant test.
- In the healthy population – rate of positive is lower and borderline samples are eliminated in the recombinant test

Performance of Savyon® SeroMP™ Recombinant test based on IgG, IgA and IgM detection compared to the performance of a respective commercial recombinant based tests*			
	Group (N)	SeroMP Recombinant % POS.	Commercial MP% POS.
IgG	Healthy Population (30)	20	40
	Pneumonia Patients (61)	32.8	36.1
IgA	Healthy Population (30)	6.6	3.3, 6.6 (BL)
	Pneumonia Patients (61)	55.7	42.6
IgM	Healthy Population (30)	6.6	3.3
	Pneumonia Patients (56)	64.2	44.6, 18 (BL)

- Higher rates of antibodies representing states of acute/current infections detected by Savyon SeroMP Recombinant kit in pneumonia patients, versus higher rates of IgG representing past infections detected by competitor's test
- The prevalence within the healthy population is lower in Savyon SeroMP Recombinant kit.

*In house study

Cross Reaction

Hospitalized patients, infected with respiratory tract pathogens: *Chlamydia pneumoniae* and *EBV* who were diagnosed by commercial serology kits, were also tested with the SeroMP kit. Most of the sera were found negative, there was no significant cross-reaction detected.

Precision

Inter-assay (within-run) precision:

Sample	No. of Replicates	Mean Value	CV%
Positive	10	1.516	4.2
Negative	10	0.180	9.9

Inter-assay (between-run) precision:

Sample	No. of Replicates	Mean Value	CV%
Positive	10	0.978	4.1
Negative	10	0.202	6.5

Test Limitations

1. No single serological test should be used for final diagnosis. All clinical and laboratory data should be taken into account.
2. Samples obtained too early during primary infection may not contain detectable antibodies.
3. Interfering substances: The use of lipemic, turbid or contaminated sera is not recommended. Particulate material and precipitates in sera may cause erroneous results. Such specimens should be clarified by centrifugation or filtration prior to the test.

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