INTRODUCTION:
Atypical pneumonia produced by *M. pneumoniae* is most frequently found in children and adolescents. The isolation in culture is tedious and consequently serological diagnosis is most frequently performed. IgM can be detected a week after the appearance of symptoms and IgG a week later. IgM does not appear in all patients and very rarely in reinfections. Thus, 56% of patients over 40 show no IgM response. In children and adolescents, IgM detection is more suitable. IgM is a good marker of acute illness because it persists for only 3-4 months, while IgG antibodies are found during years after infection. The most traditional serological method has been the complement fixation reaction whereas enzyme-linked immunosorbent assay (ELISA) and immunofluorescence assay (IFA) are now more often used.

PRINCIPLE OF THE TEST:
The ELISA method is based upon the reaction of antibodies in the sample tested with the antigen adsorbed on the polystyrene surface. Unbound immunoglobulins are washed off. An enzyme-labelled anti-human globulin binds the antigen-antibody complex in a second step. After a new washing step, bound conjugate is developed with the aid of a substrate solution (TMB) to render a blue coloured soluble product which turns into yellow after adding the acid stopping solution.

KIT FEATURES:
All reagents, except for the washing solution, are supplied ready to use. Serum dilution solution and conjugate are coloured to help in the performance of the technique. Sample predilution is not necessary. Break-apart individual wells are supplied, so that the same number of wells is consumed than the number of tests performed.

KIT CONTENTS:
- VIRCELL MYCOPLASMA PNEUMONIAE PLATE: 1 96-wells plate coated with detergent-soluble antigens (containing P1 protein) of *M. pneumoniae*, strain FH (ATCC 15531).
- VIRCELL IgG POSITIVE CONTROL: 500 µl of positive control serum containing Proclin.
- VIRCELL IgG CUT OFF CONTROL: 500 µl of cut off control serum containing Proclin.
- VIRCELL IgG NEGATIVE CONTROL: 500 µl of negative control serum containing Proclin.
- VIRCELL IgG CONJUGATE: 15 ml of anti-human IgG peroxidase conjugate dilution in a red-coloured Proclin-containing buffer. Ready to use.
- VIRCELL TMB SUBSTRATE SOLUTION: 15 ml of substrate solution containing tetramethylbenzidine (TMB). Ready to use.
- VIRCELL STOP REAGENT: 15 ml of stopping solution: 0.5 M sulphuric acid.
- VIRCELL WASH BUFFER: 50 ml of 20x washing solution: a phosphate buffer containing Tween®-20 and Proclin.

Store at 2-8°C and check expiration date.

Materials required but not supplied:
- Precision micropipettes 5 and 100 µl.
- Eight channel micropipette 100 µl.
- ELISA plate washer.
- Thermostatted incubator/water bath.
- ELISA plate spectrophotometer with a 450 nm measuring filter and a 620 nm reference filter.
- Alternatively, an ELISA automated processor.
- Distilled water.

STORAGE REQUIREMENTS:
Store at 2-8°C. Do not use the kit reagents beyond the expiration date. This will be valid only if reagents are stored closed and at 2-8°C.

STORAGE OF REAGENTS ONCE OPENED:

<table>
<thead>
<tr>
<th>REAGENT</th>
<th>STABILITY AND STORAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1x washing solution</td>
<td>4 months at 2-8°C</td>
</tr>
<tr>
<td>Rest of reagents</td>
<td>Refer to package label for expiration date (at 2-8°C)</td>
</tr>
</tbody>
</table>

STABILITY AND HANDLING OF REAGENTS:
Handle reagents in aseptic conditions to avoid microbial contaminations. Do not let the plate dry between washing and reagent addition. Substrate solution is light sensitive. Avoid light exposure and discard if blue colour develops during storage. Substrate solution should not get in contact with oxidizers such as bleach solutions or metals. Make sure that no metal components come in contact with the substrate. Use only the amount of washing, serum dilution, conjugate and TMB solutions required for the test. Do not return the excess solution into the bottles.

VIRCELL, S.L. does not accept responsibility for the mishandling of the reagents included in the kit.

RECOMMENDATIONS AND PRECAUTIONS:
1. For *in vitro* diagnosis use only. For professional use only.
2. Use kit components only. Do not mix components from different kits or manufacturers. Only the serum dilution, washing, stopping and substrate solutions are compatible with the equivalents in other VIRCELL ELISA references and lots.
3. Clean pipette tips must be used for every assay step. Use only clean, preferably disposable material.
4. Do not use in the event of damage to the package.
5. Never pipette by mouth.
6. Serum dilution solution, plate, conjugates and controls in this kit include substances of animal origin. Controls include as well substances of human origin. Although the human serum controls of this kit have been tested and found negative for Hepatitis B Surface Antigen (HBsAg), Hepatitis C antibodies and Human Immunodeficiency Virus antibodies, control sera and patient
specimens should be handled as potentially infectious. The wells are coated with inactivated *M. pneumoniae* antigen. Nevertheless, they should be considered potentially infectious and handled with care. No present method can offer complete assurance that these or other infectious agents are absent. All material should be handled and disposed as potentially infectious. Observe the local regulations for clinical waste disposal. 

7. Substrate solution may be irritant to skin and mucus. In case of contact with this solution, rinse thoroughly with water and seek medical attention. For further information a Material Safety Data Sheet is available.

8. Before incorporating this product onto an automatic processing system, we strongly recommend the performance of a pre-evaluation assay. To this purpose, VIRCELL counts with sets of samples reserved for evaluation in parallel with the manual technique. These sets of samples are available on request, as well as a list of commercial systems which have already been validated for use with the VIRCELL ELISA range.

9. During incubation times, an adequate sealing of the plates with the adhesive film included in the kit avoids the desiccation of the samples, and guarantees the repeatability of the results.

**SPECIMEN COLLECTION AND HANDLING:**

Blood should be collected aseptically using venipuncture techniques by qualified personnel. Use of sterile or aseptic techniques will preserve the integrity of the specimen. Serum samples are to be refrigerated (2-8ºC) upon collection or frozen (-20ºC) if the test cannot be performed within 7 days. Samples should not be repeatedly frozen and thawed. Do not use hyperlipemic, hemolysed or contaminated sera. Samples containing particles should be clarified by centrifugation.

**Do not use plasma.**

**PRELIMINARY PREPARATION OF THE REAGENTS:**

Only the washing solution must be prepared in advance. Fill 50 ml of 20x washing solution up to 1 litre with distilled water. Should salt crystals form in the washing concentrate during storage, warm the solution to 37ºC before diluting. Once diluted, store at 2-8ºC.

**ASSAY PROCEDURE:**

1. Set incubator/water bath to 37±1ºC.
2. Bring all reagents to room temperature before use (approximately 1 hour), without removing the plate from the bag.
3. Shake all components.
4. Remove the plate from the package. Determine the numbers of wells to be employed counting in four wells for the controls: two for the cut off serum and one each for the negative and positive sera. Wells not required for the test should be returned to the pouch, which should then be sealed.
5. Add 100 µl of serum diluent to all wells. Add 5 µl of each sample, 5 µl of positive control, 5 µl of cut off control (in duplicate) and 5 µl of negative control into the corresponding wells. If the assay is performed manually, shake the plate in a plate shaker (2 min) in order to achieve a homogeneous mixture of the reagents. If for some reason correct shaking cannot be guaranteed, a pre-dilution of the sample in a separate tube or plate should be made, using double volume of serum diluent and sample. Mix homogenously with the pipette and dispense 105 µl of each diluted sample to the wells.
6. Cover with a sealing sheet and incubate at 37±1ºC for 45 min.
7. Remove the seal, aspirate liquid from all wells and wash five times with 0.3 ml of washing solution per well. Drain off any remaining liquid.
8. Immediately add 100 µl of IgG conjugate solution into each well.
9. Cover with a sealing sheet and incubate in incubator/water bath at 37±1ºC for 30 min.
10. Remove the seal, aspirate liquid from all wells and wash five times with 0.3 ml of washing solution per well. Drain off any remaining liquid.
11. Immediately add 100 µl of substrate solution into each well.
12. Incubate at room temperature for 20 minutes protected from light.
13. Add immediately 50 µl of stopping solution into all wells.
14. Read with a spectrophotometer at 450/620 nm within 1 hour of stopping.

**INTERNAL QUALITY CONTROL:**

Each batch is subjected to internal quality control (Q.C.) testing before batch release complying with specifications stricter than validation protocol for users. Final Q.C. results for each particular lot are available. The control material is traceable to reference sera panels internally validated.

**VALIDATION PROTOCOL FOR USERS:**

Positive, negative and cut off controls must be run with each test run. It allows the validation of the assay and kit. Optical densities (O.D.) must fall in the following ranges. Otherwise, the test is invalid and must be repeated.

<table>
<thead>
<tr>
<th>CONTROL</th>
<th>O.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>POSITIVE CONTROL</td>
<td>&gt;0.9</td>
</tr>
<tr>
<td>NEGATIVE CONTROL</td>
<td>&lt;0.55</td>
</tr>
<tr>
<td>CUT OFF CONTROL</td>
<td>&lt;0.7 x(O.D. POSITIVE CONTROL)</td>
</tr>
<tr>
<td></td>
<td>&gt;1.5 x(O.D. NEGATIVE CONTROL)</td>
</tr>
</tbody>
</table>

**INTERPRETATION OF RESULTS:**

Calculate the mean O.D. for cut off serum. 

**Antibody index=(sample O.D./ cut off serum mean O.D.) x 10**

<table>
<thead>
<tr>
<th>INDEX</th>
<th>INTERPRETATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 5</td>
<td>Negative</td>
</tr>
<tr>
<td>9-11</td>
<td>Equivocal</td>
</tr>
<tr>
<td>&gt;11</td>
<td>Positive</td>
</tr>
</tbody>
</table>

Samples with equivocal results must be retested and/or a new sample obtained for confirmation. Samples with indexes below 9 are considered as not having IgG specific antibodies against *M. pneumoniae*. Samples with indexes above 11 are considered as having IgG specific antibodies against *M. pneumoniae*.

**LIMITATIONS:**

1. This kit is intended to be used with human serum.
2. The user of this kit is advised to carefully read and understand the package insert. Strict adherence to the protocol is necessary to obtain reliable test results. In particular, correct sample and reagent pipetting, along with careful washing and timing of the incubation steps are essential for accurate results.
3. The results of samples should be used in conjunction with clinical evaluation and other diagnostic procedures. A definitive diagnosis should be made by isolation techniques.
4. This test will not indicate the site of infection. It is not intended to replace isolation.
5. Lack of significant rise in antibody level does not exclude the possibility of infection.
6. Samples collected very early in the course of an infection may not have detectable levels of IgG. In such cases, it is recommended an IgM assay be performed or a second serum sample be obtained 14 to 21 days later to be tested in parallel with the original sample to determine seroconversion.
7. Results in IgG detection in neonates must be interpreted with caution, since maternal IgG is transferred passively from the mother to the foetus before birth. IgM assays are generally more useful indicators of infection in children below 6 months of age.
8. The results of a single-specimen antibody determination should not be used to aid in the diagnosis of recent infection. Paired samples (acute and convalescent) should be collected and tested concurrently to look for seroconversion or a significant rise in antibody level.
PERFORMANCE

SENSITIVITY AND SPECIFICITY:

85 serum samples were assayed with MYCOPLASMA PNEUMONIAE ELISA IgG against another commercial ELISA kit. The results were as follows:

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>SENSITIVITY</th>
<th>SPECIFICITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG</td>
<td>85</td>
<td>98%</td>
</tr>
</tbody>
</table>

Indeterminate values were omitted from the final calculations. Discordant results were solved by means of an immunofluorescence test.

INTRA-ASSAY PRECISION:

3 sera were individually pipetted 10 times each serum in a single assay performed by the same operator in essentially unchanged conditions. The results were as follows:

<table>
<thead>
<tr>
<th>SERUM</th>
<th>N</th>
<th>%C.V.</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC</td>
<td>10</td>
<td>2.13</td>
</tr>
<tr>
<td>NC</td>
<td>10</td>
<td>10.64</td>
</tr>
<tr>
<td>CO</td>
<td>10</td>
<td>5.87</td>
</tr>
</tbody>
</table>

C.V. Coefficient of variation

INTER-ASSAY PRECISION:

3 sera were individually pipetted on 5 consecutive days by 2 different operators. The results were as follows:

<table>
<thead>
<tr>
<th>SERUM</th>
<th>N</th>
<th>%C.V.</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC</td>
<td>10</td>
<td>2.20</td>
</tr>
<tr>
<td>NC</td>
<td>10</td>
<td>8.32</td>
</tr>
<tr>
<td>CO</td>
<td>10</td>
<td>4.71</td>
</tr>
</tbody>
</table>

C.V. Coefficient of variation

CROSS REACTIVITY AND INTERFERENCES:

6 samples known to be positive for other bacteria of the syndromic group (Legionella pneumophila, Chlamyphila pneumoniae and Coxiella burnetii) were assayed. The negative results of the test demonstrated the specific reaction of the kit with no cross-reaction or interferences with the referred specimens.

SYMBOLS USED IN LABELS:

**IVD** In vitro diagnostic medical device

Use by (expiration date)

Store at 2-8ºC

Contains sufficient for <X> tests

Batch code

Catalogue number

Consult instructions for use

<X> wells

SUMMARY OF THE ASSAY PROCEDURE

100 µl serum diluent

5 µl serum and controls

45 min. at 37ºC

Wash 5x (washing solution)

100 µl conjugate

30 min. at 37ºC

Wash 5x (washing solution)

100 µl substrate

20 min. at room temperature

50 µl stopping solution

Read at 450/620 nm
LITERATURE:


For any question please contact:
technicalservice@vircell.com

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