CE 0483

# IPAzyme™ Chlamydia TRUE IgM

Indirect Immunoperoxidase Assay (IPA) for the detection of specific IgM antibodies ***Chlamydia*** in human serum

**Instruction Manual**

Test kit for 96 determinations
(Catalog No. 012-01)

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

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## Intended Use

IPAzyme Chlamydia TRUE-IgM assay of Savyon Diagnostics is intended for the determination of specific IgM anti Chlamydia antibodies in human serum.

For ***In Vitro*** Diagnostic Use Only

## Introduction

Chlamydia is an energy parasitic bacterium with a genome of 660 x $10^6$ daltons (1-5). The genus Chlamydia comprises three species: Chlamydia *trachomatis*, Chlamydia *psittaci* and recently described Chlamydia *pneumoniae* (TWAR) strain (6, 7). All members of Chlamydia share a common genus specific antigen which is a glycolipid (8). C.*pstitaci* is recognized as the causative agent of psittacosis (12). Chlamydia *trachomatis* serovars share species and type specific antigens to a varying degree. Four of 15 serovars (A, B, A, Ba, and C) cause endemic binding trachoma; eight serovars (D through K) are responsible for sexually transmitted Chlamydia infections; three serovars ($L_1$, $L_2$ and $L_3$) are responsible for lymphogranuloma venereum (LGV) (9). It is now evident that Chlamydia *trachomatis* (serovars D through K) is the major cause of sexually transmitted infections, such as non-gonococcal urethritis (NGU), post gonococcal urethritis (PGU), epididymitis, cervicitis, endometritis, salpingitis, perihepatitis, peritonitis, Reiter's syndrome, conjunctivitis and pneumonia (reviewed by Ladany and Sarov (10).

Specific IgM serum antibodies are accepted as a diagnostic marker of acute and/or recent viral and bacterial infections (11).
It was found that Chlamydia IgM has diagnostic value in pneumonia caused by Chlamydia *trachomatis*, Chlamydia *psittaci* and Chlamydia *pneumoniae* (TWAR) (12, 26). The serological pattern of Chlamydia IgM in Chlamydia pneumonitis is as follows: Chlamydia antibodies are produced in the early stages of an infection, peak after 1-2 weeks and then decline moderately to undetectable levels within 2-3 months (13).
This pattern has been observed in 20-50% of infants born to mothers' culture positive for Chlamydia *trachomatis* and/or with elevated levels of specific IgG and IgA anti Chlamydia antibodies, who developed Chlamydia pneumonitis during the first 6 months (14, 15). Some studies indicate that IgM anti Chlamydia antibodies have been found in cases of non-gonococcal urethritis (NGU), post gonococcal urethritis (PGU), epididymitis, cervicitis, endometritis, salpingitis, perihepatitis, arthritis and some respiratory diseases in pediatric patients (16-21).
Consequently, Chlamydia IgM is considered a most useful and sensitive marker of Chlamydia infections. It is an indispensable marker in pneumonitis caused by Chlamydia *psittaci*, Chlamydia *trachomatis* or Chlamydia *pneumoniae* (TWAR) strain. However, most infants with chlamydial inclusion conjunctivitis do not develop significant titers of anti Chlamydia IgM (22). Since IgM is present only in active disease, the Chlamydia IgM test requires only a single serum specimen, positive sera do not need to be titrated and results can be reported in terms of presence or absence of immune IgM.
High titers of immune IgG which compete with immune IgM for the same antigenic sites may produce false negative IgM results. Rheumatoid factor (Rf, auto-immune activity) causes false positive (IgM results (23). Therefore, IgG/Rf stripping of serum is essential before IgM assay.
IPAzyme Chlamydia TRUE-IgM of Savyon consists of all reagents necessary for the accurate, sensitive and specific determination of IgM anti Chlamydia by indirect immunoperoxidase assay (IPA). Savyon Diagnostics Ltd. proposes an indirect immunoperoxidase assay comprising Chlamydia *trachomatis* $L_2$ serovar infected cells as antigen for detection of anti specific IgM antibodies. $L_2$ serovar has been found to have a broad reacting antigen for anti Chlamydia *trachomatis* antibodies (24), Chlamydia *psittaci* (25) and Chlamydia *pneumoniae* (TWAR) (26).
This test can be performed in almost any clinical laboratory, since its IgG/Rf Stripping Solution, stabilized liquid components and ordinary light microscopy provide a reliable, easy-to-perform and economical procedure.

## Principle of TRUE-IgM IPA

In step one; human serum to be tested is treated by a solution which removes IgG and Rf anti IgG related complexes to a non interfering level. If present IgM in the IgG/Rf stripped serum is specific to Chlamydia, when determined by the following steps. In step two, human stripped serum to be tested in brought into contact with antigenic material. (Chlamydia *trachomatis* infected cells). Anti Chlamydia IgM, if present in the serum, will bind to the antigen, forming an antigen-antibody complex. If the serum being examined contains no antibody for this particular antigen, no complex is formed and all serum components are

washed away during the rinse phase. The third step involves an addition of horseradish peroxidase (HRP) conjugate of anti-human IgM (μ-chain specific) to the test. If IgM antibody antigen complex was formed in step two, the HRP labeled antibody will bind to this complex in step three. A positive reaction, a deep blue precipitate inside the infected cells can be observed with the aid of an ordinary light microscope following the enzymatic reaction of the peroxidase moiety with hydrogen peroxide/chromogen reactant.

## Summary of Steps

1. Human Serum + IgG/Rf Stripping Solution positive for IgM anti Chlamydia
   ↓
   IgG/Rf stripped serum positive for IgM anti Chlamydia

2. IgG/Rf stripped serum positive for IgM anti Chlamydia ($Ab_1$) + Chlamydia *trachomatis* infected cells (Ag)
   ↓
   $Ab_1Ag$ complex

3. $Ab_1Ag$ complex + HRP con. Anti human IgM ($Ab_2$)
   ↓
   $Ab_1AgAb_2$ complex

4. $Ab_1AgAb_2$ complex + Substrate / Chromogen
   ↓
   Insoluble colored precipitate

### Notes:

1. **Warning:** THE ANTIGENIC MATERIAL IN THIS KIT HAS BEEN FIXED AND CONTAINS NO DETECTABLE LIVE ORGANISMS. HOWEVER, SLIDES SHOULD BE HANDLED AND DISPOSED OF AS WOULD ANY POTENTIALLY BIO-HAZARDOUS LABORATORY MATERIAL.
2. **Precaution:** The human serum components have been tested by FDA approved methods and found to be negative for Hepatitis B Surface Antigen (HBsAg) and Human Immunodeficiency Virus (HIV) antibody. This does not ensure the absence of HBsAg or HIV. Therefore, human serum components and patients' serum specimens should be handled and disposed of as would any potentially biohazardous laboratory material.
3. All components in this kit have been tested and standardized as a unit. Do not mix components from different kit lots or other manufacturers' kits.
4 For *in vitro* use only.

## Materials Supplied

1. 8 x 12 Well Slides with Chlamydia *Trachomatis* Infected Cells.
2. 1 x 0.5ml Vial Positive Control – Human Serum Positive for IgM Anti Chlamydia Antibody. Ready for Use
3. 1 x 0.5ml Vial Negative Control – Human Serum Negative for IgM Anti Chlamydia Antibody. Ready for Use
4. 1 x 1.0ml Vial HRP Conjugated Anti-Human IgM (μ chain specific) Ready for Use
5. 1 x 2.0ml Vial Substrate/Chromogen Ready for Use
6. 1 x 4.0ml Mounting Medium. Ready for Use
7. 1 x 2.5ml Concentrated IgG/Rf Stripping Solution (x10)
8. 1 x 4.0ml Concentrated Strip-Stop Reagent (x10)
9. 1 x 4.0ml concentrated Chlamydia-IgM Diluent (x10)
10. 1 x 100ml Concentrated IPAzyme Buffer (x20)
11. 15 Slide Cover Slips
12. Instruction Manual

## Materials Required but not Supplied

1. Test tubes and rack for IgG/Rf stripping and dilution of serum specimens.
2. Adjustable micropipettes (5-50, 50-200 and 200-1000 microliters ranges) and disposable tips.
3. Safety pipetting devices
4. 1000ml volumetric flasks
5. 50ml volumetric cylinders
6. A 37ºC water bath with a lid, or a moist chamber placed in a 37ºC incubator
7. A 4ºC chamber or refrigerator
8. Refrigerated centrifuge (4ºC, 3000 RPM)
9. Slide holder tray
10. Light microscope, x200 magnification and blue filter
11. Wash bottle
12. Vortex mixer
13. Staining dish for immersion of slides
14. Compressed air generator (or a fan, hairdryer, etc.)
15. Double deionized or distilled water for the dilution of Concentrated IPAzyme Buffer, Concentrated Chlamydia-IgM Diluent and Concentration Strip-Stop Reagent

## Storage and Shelf-Life of Reagents

All materials supplied, except for Slide Cover Slips, should be stored at 2º - 8ºC. However, an exposure of a few hours to an ambient temperature will not cause any damage to reagents. Do not freeze. If a constant storage temperature (2º-8ºC) is maintained, reagents in vials originally stoppered will be stable until the expiry date stated on each label. Matched reagents in a kit will be stable until the dated indicated on the kit package. When a kit is in use, the shelf life to consider is sixty days for the day first opened.

## Specimen Collection

One serum specimen, taken as close as possible to onset of suspected illness is sufficient.
Serum specimens should be collected aseptically and stored at 2º - 8ºC with 0.1% sodium azide ($NaN_3$) as preservative, if they are to be tested within a few days. For longer periods aliquots of serum specimens should be stored at -20ºC.
Although hemolysis and/or turbidity effects on the assay have not been elucidated, testing of clear non-hemolytic serum specimens is highly recommended.

## Assay Procedure

**Notes:**

a) The person performing the assay should be familiar with laboratory practices and routines.
b) All the procedure steps should be read carefully and understood before starting the test.
c) All reagents should reach room temperature before use.
d) Centrifuge should reach 4ºC prior to procedure.
e) Positive and Negative Controls should be run each time the test is performed.
f) Tap vial lightly on a hard surface to free liquid that might be entrapped in the cap.
g) Use disposable pipette tips. Avoid cross contamination by replacing tips between different reagents.

**Procedure**
IPAzyme Chlamydia TRUE-IgM test consists of two stages:

I. IgG/Rf Strip-Treatment of Serum Specimens and Controls
II. IPA (Indirect Immunoperoxidase Assay)

All steps within each stage should be performed sequentially and without intermissions.

**I. IgG/Rf Strip-Treatment of Serum Specimens and Controls**

1. Dilute the Concentrated IPAzyme Buffer according to instructions on the label.

2. Dilute the CONCENTRATED IgG/Rf STRIPPING SOLUTION 1:10 with IPAzyme BUFFER (one part of Concentrated IgG/Rf Stripping Solution with nine parts of IPAzyme Buffer) to obtain 50 microliters for each serum and control to be tested.
   Freshly diluted IgG/Rf Stripping Solution should be prepared prior to each run. Excess should be discarded.

3. To 50 microliters serum and to 50 microliters of each positive and negative control add 50 microliters of each positive and negative control add 50 microliters IgG/Rf Stripping Solution. Vortex briefly at low speed. Serum and controls dilution obtained at this step is 1:2.

4. Incubate the diluted serum and controls at 0-4º for 30 minutes.

5. Dilute the CONCENTRATED STRIP-STOP REAGENT 1:10 with DISTILLED WATER (one part of Concentrated Strip-Stop Reagent plus nine parts of distilled water) to obtain 100 microliters for each strip-treated serum and control.
   Freshly diluted Strip-Stop Reagent should be prepared prior to each run. Excess should be discarded.

6. To the 100 microliters diluted strip-treated serum specimens and controls add 100 microliters Strip-Stop Reagent. Vortex briefly at low speed. Serum and controls dilution obtained at this step is 1:4.

7. Centrifuge the cold incubated serum specimens and controls at 4ºC, at 3000 RPM, for 15 minutes.

8. Transfer each supernatant carefully to an empty test tube. At this stage each control supernatant and each serum specimen supernatant is ready for further dilution for IPA.

**Note:**
Supernatants may be stored for up to 24 hours at 4ºC prior to testing by IPA.

**II. IPA**

1. Remove required number of slides from aluminum foil pouches and place them in a slide-holder tray.
   One well is required for testing each serum, an additional two wells for the positive control and one well for the negative control in each run.

2. Dilute the CONCENTRATED CHLAMYDIA-IgM Diluent 1:10 with DISTILLED WATER (one part of Concentrated Chlamydia –IgM Diluent with nine parts of distilled water) to obtain 150 microliters for each serum and 100 microliters for each control.
   Freshly diluted Chlamydia-IgM Diluent should be prepared prior to each run. Excess should be discarded.

3. **Preparation of Controls Dilutions:**
   Dilute the supernatants of the Positive and Negative CONTROLS to obtain 1:8 dilution as follows: To
   50 microliters supernatant add 50 microliters Chlamydia-IgM Diluent.
   Dilute the 1:8 dilution to obtain 1:16 dilution as follows: To 50 microliters of 1:8 dilution and 50 microliters Chlamydia IgM- Diluent.

4. **Preparation of Sera Dilutions:**
   Dilute the supernatant of each SERUM to obtain 1:16 dilution as follows: To each 50 microliters supernatant add 150 microliters Chlamydia-IgM Diluent

5. Pipette into separate wells 10 microliters of each one of the controls dilutions and in other wells 10 microliters of serum specimens' dilutions.

6. Place slides in a moist chamber, Incubate at 37°C for 120 minutes. Careful attention should be paid to proper closure of moist chamber during incubation, otherwise the pipetted reagents will dry during incubation. Avoid dripping of condensed water drops from the lid onto the slides.

7. Rinse slides thoroughly with a light stream of IPAzyme Buffer using a wash bottle, and then immerse in IPAzyme Buffer in a staining dish for 5 minutes.

8. Remove slides from IPAzyme Buffer and dry with stream of compressed air.

9. Pipette 10 microliters of HRP-Conjugated anti-Human IgM into each well. Place slides in a moist chamber. Incubate at 37°C for 45 minutes.

10. Rinse and dry as in steps 7 and 8.

11. Pipette 10 microliters of Substrate/Chromogen solution into each well. Incubate for 10 minutes at room temperature.

12. Rinse and dry as in steps 7 and 8.

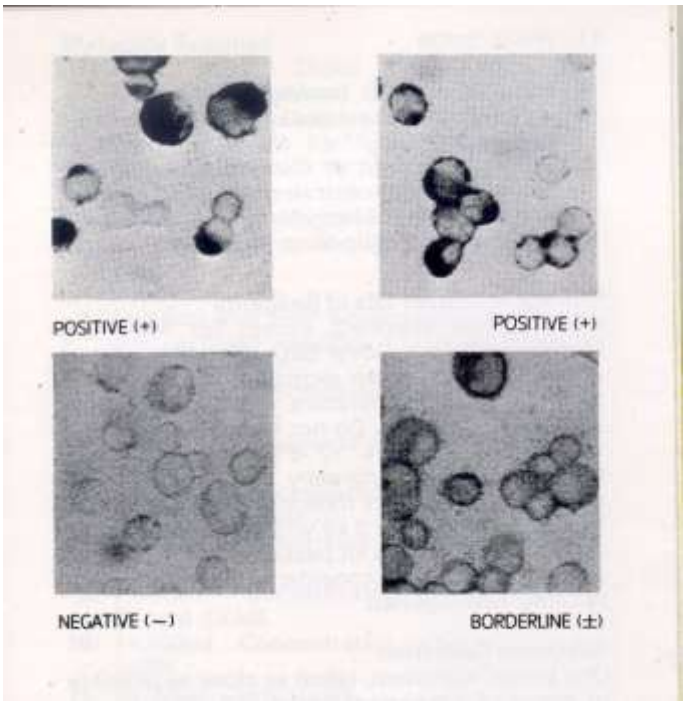
13. Place four drops of Mounting Medium on each slide and cover with a cover slip. Avoid entrapping air bubbles between slide and the cover slip.

14. Read slides within the same day at a x200 magnification. It is recommended that a blue filter be used when observing results.

## Display of Results

The cell population is composed of infected and uninfected cells. Approximately 25% of cells exhibit Chlamydia *trachomatis* antigenic activity Presence of a dark blue precipitate inside infected cells indicates a positive reaction. In the positive Chlamydia IgM tests a part of the infected cells display the positive reaction by inclusion in the infected cells. The major part of the positive reactions appear in crescent forms of different dimensions close to the inner part of the infected cells membrane.
Cells along the edges of a well stained blue should be ignored. Absence of a blue precipitate inside infected cells indicates a negative reaction in this test. Grey shades should be considered as a negative reaction. Should ALL CELLS exhibit a positive reaction – the test result is not valid (See Test Limitations item 2).

**Note:**
There may be a difference in the results between the appearance of positives in IPAzyme Chlamydia IgG/IgA and the appearance of positives in Chlamydia IgM. This phenomenon is due to the high molecular weight of the IgM molecule (approx. 950,000 Daltons) and is relative low affinity constant.




## Suggested Interpretation of Results

| Serum Dilution | Possible Results | | |
|---|---|---|---|
| | 1 | 2 | 3 |
| 1:16 | + | ± | - |

1. **Indication of acute or recent infection.**
2. **Negative – testing of a subsequent serum sample is recommended.**
3. **Negative**

## Test Limitations

1. If serial dilutions are carried out, the titer of human IgM anti Chlamydia can be determined by this IPA test. However, no serological testing should be used as the sole criterion for diagnosis. All clinical and laboratory data must be taken into account.
2. Should all the cells exhibit a positive reaction, the test result is not valid. In IPA performed with IPAzyme Chlamydia, this phenomenon is rare and serum related, possibly caused by a disease state unrelated to or in addition to Chlamydia infections, e.g. antinuclear antibody, antimitochondrial antibody or unexplained nonspecific reaction (27, 28).
3. The significance of specimen titers must be established in relation to the characteristics of the population being tested. These characteristics would include age, geographical location and sexual behavior, among other factors.

4. This test will not indicate the site of infection(s). It is not intended to replace cell cultures.

5. The test is a single server inclusion ($L_2$) immunoperoxidase assay. $L_2$ contains antigenic determinants existing in the other serovars of C.*trachomatis* as well as the group antigen. Although the cells have been infected by Chlamydia *trachomatis* of the $L_2$ serovar, antibodies against C. *psittaci*, C. *pneumoniae* (TWAR) strains and Acinebacter calcoaceticus may be revealed by this IPA.
6. Excess lipids in the serum may produce a "filming" reaction, which is caused by lipids which stick nonspecifically to the glass and are difficult to remove. This reaction, although rare, can be observed along the edges of wells where the serum comes into contact with the slide coating. This reaction, being nonspecific, should be ignored. Only the reaction observed in the inner area of the well should be considered as test results.
7. Since infection with Chlamydia may not produce any immediate symptoms, the acute stage may be missed and there may be no presence of IgM titers.

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