DRG® Chlamydia pneumoniae IgM ELISA (EIA-2941)

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INTENDED USE

DRG's Chlamydia pneumoniae IgM test is developed for the detection of IgM antibodies specific to *Chlamydia pneumoniae* in human serum or plasma.

The positive result is an aid for the diagnosis of acute Chlamydia pneumoniae infection.

The test is recommended to be run and interpreted in parallel with the DRG's Chlamydia pneumoniae IgA and IgG EIA kits.

INTRODUCTION

Since the description in 1986 of *Chlamydia pneumoniae* as a pathogen (1) it has become recognized as a common infectious agent all over the world. *C. pneumoniae* is primarily a respiratory tract pathogen that causes approximately 10-20 % of acute bronchitis in adults (2, 3, 4). It also causes sinusitis, primary pharyngitis, and may trigger for asthma (5). Most infections with this micro-organism are in fact subclinical and asymptomatic and only rarely cause on overt disease. (3). Chronic infection with C. pneumoniae has been suggested as a factor in the development of atherosclerosis (6,7).

Seroepidemiologic studies (8, 9, 10, 11) in different populations suggest that the seroprevalence increases sharply in young children and adolescence. After adolescence the seroprevalence continues to increase and may achieve almost complete saturation for IgG and IgA-class antibodies in the senescence (11).Epidemic cycles of *C. pneumoniae* depends on the density of the population, and was reported to occur in 4-7 years' intervals (8).

To date most investigations have relied on serologic diagnosis, using modifications of a microimmunofluorescence (MIF) test (8). Early studies have been performed with a complement fixation (CF) test, which has been used for many years for the detection of psittacosis. This test is a genus-specific and is more likely to be positive in initial infection than during reinfection (8). The MIF method is a species-specific, but has a large subjective component, requires a skilled interpreter and is not suitable for automation and high volume testing.

The present EIA methods were developed to circumvent technical problems with MIF providing easy, fast and objective performance. The DRG EIAs were optimized to produce comparable results to those of respective DRG MIF assays.

PRINCIPLE OF THE TEST:

The principle of the DRG's Chlamydia pneumoniae IgM EIA kit is based on an indirect solid-phase enzyme immunoassay with horseradish peroxidase as a marker enzyme. The assay proceeds according to the following reactions.

1. *Chlamydia pneumoniae* IgM antibodies from the patient sample bind *to Chlamydia pneumoniae* antigen attached to the polystyrene surface of the Microstrip® wells.
2. Residual patient sample is removed by washing and horseradish peroxidase conjugated anti-human IgM (sheep) is added.
3. Unbound conjugate is washed off and a colorless enzyme substrate (H_20_2) containing the chromogen (TMB*) is added. The enzyme reaction with the chromogen results in a colored end product.
   *Tetramethylbenzidine, a non-mutagenic chromogen for horseradish peroxidase.

4. The color formation reaction is terminated by adding acid (H_2SO_4). The color intensity is directly proportional to the concentration of Chlamydia pneumoniae antibodies in a patient sample.

KIT CONTENTS

Note: Prewarm all reagents and Microstrips® to +20°C -+25°C and Incubator to +37°C before use.

- Weare disposable gloves while handling specimens and kit reagents. Wash hands carefully after handling of samples.
- Reagents are stored between +2°C and +8°C.
- The expiration date is printed on each component label and on the package.
- Avoid unnecessary exposure to light. This is merely a precaution. The light sensitive reagents are the chromogen (Tetramethylbenzidine, TMB), the conjugate and the substrate buffer, which are packed in brown glass or non-transparent plastic vials for protection.
- Once the Microstrip® foil-package is opened it should be resealed tightly and stored at +2°C to +8°C with a desiccant.
- Once opened the components must be resealed tightly.

1. MICROSTRIPS® 12 X 8 wells
   Coated Microstrips®.
2. SAMPLE DILUENT 100 ml
   Phosphate buffered saline with proprietary additives, a blue coloring reagent, and 0.05 % Bronidox® as preservative.

2a. IgG REMOVING REAGENT 20 ml
   anti-human IgG in phosphate buffered saline with proprietary additives and 0.05% Bronidox® as preservative.

3a. NEGATIVE CONTROL 1.0 ml
   Diluted human serum negative for *Chlamydia pneumoniae* antibodies with 0.05 % Bronidox® as preservative and a red coloring reagent.

3b. CUT-OFF CONTROL 1.0 ml
   Diluted human serum borderline in regard to *Chlamydia pneumoniae* IgM antibodies containing 0.05 % Bronidox® as a preservative and a red coloring reagent.

3c. POSITIVE CONTROL 1.0 ml
   Diluted human serum with 0.05 % Bronidox® as a preservative and a red coloring reagent.

4. CONJUGATE 30 ml
   Buffered salt solution with proprietary additives, a red coloring reagent, horseradish peroxidase conjugated anti-human IgM (sheep) with 0.1 % N-Methylisothiazolone as preservative

5. TMB-SUBSTRATE SOLUTION, ready to use 18 ml
   Citrate-acetate buffered solution of 3, 3', 5, 5'- Tetramethylbenzidine and hydrogen peroxide with proprietary additives and 0.01% Kathon CG as preservative.

6. STOPPING SOLUTION 25 ml
   0.45 M H_2SO_4

7. WASHING SOLUTION 100 ml
   Concentrated citrate buffered saline, with proprietary additives, and 0.05 % Bronidox® as preservative.

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INCUBATION COVER	2 pcs
REAGENT BASINS	6 pcs

REAGENT PREPARATION

Table 1

Reagent	Preparation	Stability of opened/diluted reagents (+2°C to 8 °C)
1. Coated Microstrips®	Ready for use	2 months
2. Sample diluent	Ready for use	6 months *)
2a. IgG removing reagent	Ready for use	6 months*)
3. Controls	Ready for use	6 months *)
4. Conjugate	Ready for use	6 months *)
5. TMB-Substrate solution	Ready for use	6 months *)
		Discard unused reagent from the reagent basin. A deep blue color present in the substrate solution indicates that the solution has been contaminated and must be discarded.
6. Stopping solution	Ready for use	6 months *)
7. Washing solution concentrate (10x) Washing solution	Dilute the concentrate (vial 7) 1+9 (1:10) with distilled water.	6 months *) 1 month at +4°C or 1 week at room temperature.

*) The stability of the opened reagents is maximum 6 months only if they are stored properly at +2°C to +8°C. However, high environmental temperature and contamination may decrease the stability.

The prediluted samples are stable at +4°C for at least 2 weeks.

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MATERIALS REQUIRED BUT NOT PROVIDED

- Distilled or deionized water, preferably sterile.
- Graduated cylinders for reagent dilution.
- Vials to store the diluted reagents.
- Precision pipettes.
- Paper towels or absorbent paper.
- Timer, 60 min range.
- Incubator
- Photometer (plate or Microstrip® reader), 450 nm
- Washer
- Sodium hypochlorite solution, free available chlorine 50-500 mg/l.
- Disposable gloves.

SPECIMEN COLLECTION AND HANDLING

- Serum and plasma samples should be refrigerated (+4°C) after collection or, if the test cannot be performed within 48 hours, frozen (-20°C or –70°C, which is preferred).
- **Samples should not be repeatedly frozen and thawed.**
- ***Do not use sodium azide as preservative because it inactivates horseradish peroxidase.***
- **Heat inactivation** of serum or plasma (+56°C, 30 min) may cause non-specific results.
- Microbially contaminated, grossly hemolyzed or hyperlipemic serum and plasma may give erroneous results.
- Long storage of serum (frozen over one year) may cause the formation of lipid aggregates. These aggregates may cause a non-specific result.

PRECAUTIONS

For in-vitro use only.

WARNING – POTENTIAL BIOHAZARDOUS MATERIAL:
Each donor unit used in the preparation of the control and calibrator sera in the kit has been tested for the presence of the antibodies to HIV (Human Immunodeficiency Virus) and HCV (Hepatitis C Virus) as well as Hepatitis B surface antigen (HBsAg) and found to be no-reactive. Because no test method can offer complete assurance that HIV, Hepatitis B virus HCV, or other infectious agents or absent, these calibrators and controls as well as specimens should be handled at the Biosafety level 2 as recommended for any potentially infectious human serum or blood specimen in the Centers for Disease Control/National Institutes for Health Manual, " Biosafety in Microbiological and Biomedical Laboratories", 1984.
Discard all materials and specimens as if capable of transmitting infection. The preferred method of disposal is autoclaving for a minimum of one hour at 121°C. Liquid wastes not containing acid and neutralized waste may be mixed with sodium hypochlorite in volumes such that the final mixture contains 50-500 mg/l free available chlorine. Allow 30 minutes for decontamination to be completed.

NOTE: Liquid waste containing acid must be neutralized with a proportional amount of base prior to the addition of sodium hypochlorite.

- Spills should be wiped up thoroughly using either an iodophor disinfectant or sodium hypochlorite solution. Materials used to wipe up spills should be added to biohazardous waste matter for proper disposal.

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- Wear disposable gloves while handling specimens and kit reagents. Afterwards, wash hands carefully.
- Stopping solution (vial 6) contains 0.45 M sulphoric acid. Avoid contact with skin and eyes.
- Salt crystals may form in the washing solution concentrate when kept at refrigerated temperatures. If necessary, redissolve the salt crystals before diluting by warming and mixing the solution.
- Avoid unnecessary exposure to light. The light sensitive reagents are the chromogen, the conjugate and the substrate buffer, which are packaged in non-transparent plastic vials for protection.
- Store all working solutions in clean containers to prevent contamination.
- Storage of reagents and samples in self-defrosting freezers in is not recommended.
- **All reagents and Microstrips® must be warmed up to +20°C-+25°C before use.**
- Do not use reagents after the expiration date printed on the label..
- Do not mix or interchange reagents from different lots. Cross contamination of reagents or samples could cause erroneous results.
- When removing aliquots from the reagent vials, use aseptic technique to avoid contamination, or erroneous results may occur.
- Do not interchange vial caps.
- Use a new pipette tip for each sample.
- Optimal results will be obtained by strict adherence to the test protocol. Accurate and precise pipetting, as well as following the exact time and temperature requirements, is essential.
- Once the assay has been started, all subsequent steps should be performed without interruption
- Do not reuse a Microstrip® even if some wells were not used.
- Do not touch the wells or splash reagents while pipetting.
- Do not let the wells dry once the assay has been started.
- Reusable glassware must be disinfected, washed out and rinsed free of detergents.
- Microbial contamination and presence of particulate matter are the signs of deterioration in diluents and control sera.

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TEST PROCEDURE

OUTLINE OF PROCEDURE

STEP I

Mix in a tube **500µl** sample diluent and **5µl** specimens (dilution 1:101)

Pipette by a multichannel pipette **100 µl** of IgG Removing reagent to the Microplate® wells.

Pipette into Removing reagent **10 µl** of the diluted specimens and ready for use controls. Do not pipette either controls or specimens into wells A1-B1 (=Blank!). While pipetting mix well.

Cover the plate and Incubate **1h at +37°C**

Wash **5 x 300 – 400 µl/well**

STEP II

Add **100µl** conjugate solution

Cover the plate and incubate **1h at +37°C**

Wash **5 x 300 – 400 µl/well**

STEP III

Add **100µl** TMB-substrate solution

Incubate **30 min at RT (+20°C- +25°C) in dark**

STEP IV

Add **100 µl Stopping solution**

Measure at **450nm**

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PRELIMINARY PREPARATIONS:

- Wear disposable gloves throughout the procedure!
- **Bring the reagents and Microstrips® to room temperature (+20°C - +25°C) before starting the assay.**
- Prewarm the incubator to +37°C.
- Do not let the wells dry once the assay has been started.

SPECIMEN DILUTION:

Dilute the specimen 1:101 in sample diluent (5µl serum or plasma sample and 500µl of sample diluent). Mix well.

NOTE: Do not dilute the calibrator and controls!

THE PROCEDURE:

STEP I

1. Reserve 2 empty wells for the blank (sample diluent is used for blank).
2. Pipette 100µl of transparent IgG removing reagent (vial 2a) into the wells.
3. Pipette 10 µl each of the diluted samples (1:101) and of the ready to use controls (vial 3a-c) into the wells containing the Removing reagent.
4. While pipetting mix well and cover the Microstrips with plastic sheet.
5. Incubate for 60 min. (±5 Min) at +37° C (± 1° C).

WASHING

Washing may be performed with a automated washer.

6. Empty the wells into a suitable biohazard container or aspirate the well contents with a washer.
7. Add 300-400µl of washing solution into each well.
8. Empty the wells.
9. Repeat the washing cycle five times in total. After the washing step tap the inverted Microstrip® a few times on the paper towel.

STEP II

1. Pipette 100µl of the conjugate (vial 4) into each well and cover the Microstrips® with plastic sheet.
2. Incubate for 1 hour (± 5 min) at +37°C (± 1°C).

NOTE: To avoid the contamination of the conjugate solution pour needed amount of the solution into a disposable reagent basin. Discard any unused conjugate solution, do not pour it back to the vial.

For this purpose the kit includes 6 disposable reagent basins. Disposable reagent basins can be used also for the sample diluent and the TMB-substrate solution.

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WASHING

Wash the wells five time in total as in items 6-9 in STEP I.

STEP III

1. Pipette 100µl of the TMB-substrate solution (vial 5) into each well.
   Avoid contamination of the substrate solution: do not touch the walls of the wells with pipette tips when adding substrate.
   See NOTE below.
2. Incubate for 30 minutes at room temperature in a dark place.

STEP IV

1. Stop the enzyme substrate reaction by adding 100µl of Stopping solution into each well. See NOTE below.

NOTE:
The use of an 8-channel pipette device is recommended for improved efficiency and precision.

MEASUREMENT

Measure the absorbency immediately at 450nm.

RESULTS

Table 2. Example of the OD values of the Controls.

QC Sample	Expected absorbency value at 450nm
Blank	0.050
Negative Control	0.078
Cut-Off Control	0.350
Positive control	1.255

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Calculation of the Results.

The results are expressed in Signal/Cut-Off (S/CO)

Use the formula for calculations:

$$S/CO_{sample} = \frac{(A_{sample} - A_{blank})}{(A_{cut\text{-}off} - A_{blank})}$$

where A_{sample} = absorbance of the sample
A_{blank} = absorbance of the Blank
$A_{cut\text{-}off}$ = absorbance of the Cut-off control

Equivocal zone

Based on the analysis of approx. 500 blood donor serum and plasma samples and calculation of parameters of distribution of S/CO-values the equivocal zone is 0.5-1.1 In other words, samples producing results that fall within 0.5-1.1 S/CO are considered equivocal and should be retested with a paired sample properly timed to monitor dynamics of IgM antibodies.

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Example 1

Expression of results in Signal/Cut-Off (S/CO) values

Sample	Mean A at 450 nm	S/CO-value
Blank	0.050	
Negative Control	0.078	0.09
Cut-Off control	0.350	1 per definition
Positive control	1.255	4.01
Sample 1	0.185	0.45
Sample 2	0.815	2.55

Acceptance criteria

The results of the run are accepted when :

- Blank ≤ 0.1 OD
- The Negative Control ≤ 0.15 OD*
- The cut-off Control ≥ 0.2 OD*
- The Positive Control is between 3.0 and 5.0 S/CO

* (after subtraction of the reagent blank)

Interpretation of results

S/CO < 0.5	Negative
0.5 ≤ S/CO ≤ 1.1	Equivocal
S/CO > 1.1	Positive

Equivocal zone:
Based on the analysis of approx. 500 Finnish blood donor serum and plasma samples and calculation of parameters of distribution of S/CO-values the equivocal zone is 0.5-1.1. In other words, samples producing results that fall within 0.5-1.1 S/CO are considered equivocal and should be retested with a paired sample properly timed to monitor dynamics of IgM antibodies.

Acute infection:
In primary acute infection the IgM response may be detected already in the first serum sample. In case of reinfections the IgM response may not be achieved. DRG's Chlamydia pneumoniae IgA and IgG EIAs provide additional information for the diagnosis of acute *Chlamydia pneumoniae* infection

Non acute infection:
Stable or decreasing levels of IgG and/or IgA with negative or equivocal IgM may indicate one of the following: past infection, recent infection, cured condition or persistent infection.

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LIMITATION OF THE PROCEDURE

Because no single method leads to the definitive diagnosis, the results of the present method should be interpreted in conjunction with the clinical condition, epidemiological situation and other laboratory methods.

A serum sample obtained during the acute phase of infection, when only IgM antibodies are present, may be negative by this procedure (8). On the other hand, in same rare cases "tail" IgM-antibodies may be detectable in asmyptomatic patients and can persist up to 3 years (9).

Due to the limited number of *Chlamydia psittaci* cases tested so far, the overall specificity has not been proven. However, since both conditions (*Chlamydia pneumoniae and Chlamydia psittaci*) require similar treatment this limitation will not violate the significance of the results.

PERFORMANCE CHARACTERISTICS

Specificity

Paired samples (n = 20) from infants and a few adults with proven by isolation *Bordetella pertussis* infection were analyzed in the EIA method. The cases were interpreted as negative for ongoing *Chlamydia pneumoniae* infection.

At the same time sera with IgM antibodies against EBV, Rubella, Toxoplasma gondii, CMV, Mycobacterium tuberculosis as well as sera with different autoantibodies have been determined with the C. pneumoniae IgM EIA. None of the sera showed cross reactivity in EIA.

Reproducibility

Table 3. Intra-Assay reproducibility.

Intra-assay reproducibility was tested using three sera with variable level of specific IgM antibodies. Each well represented an individual dilution.

Sample 1	Sample 2	Sample 3
S/CO = 4.2	S/CO = 1.9	S/CO = 1.1
SD = 0.61	SD = 0.24	SD = 0.12
Max = 5.3	Max = 2.3	Max = 1.3
Min = 3.5	Min = 1.6	Min = 0.9
n = 10	n = 10	n = 20
	Above the equivocal in all assays	Mostly equivocal

Table 4. Inter-Assay reproducibility

Inter-Assay reproducibility was tested with four samples representing a variable level of specific IgM antibodies. The samples were tested in 10 consecutive runs by 5 operators. Each run was performed in quadruplicates. For each quadruplicates serum, dilutions were prepared separately.

Sample 1	Sample 2	Sample 3	Sample 4
S/CO = 3.81	S/CO = 2.24	S/CO = 1.22	S/CO = 1.01
SD = 0.36	SD = 0.11	SD = 0.11	SD = 0.09
CV % = 9.6 %	CV % = 5.1%	CV % = 9.0 %	CV % = 8.7 %
n = 10	n = 10	n = 10	n = 10

Summary of the evaluation studies

Paired serum samples collected during the outbreak of C. pneumoniae epidemics in 1995 in Sweden were analyzed for the seroconversion. The rate of seroconversion detected by DRG methods was compared to the respective rates by a competitor's EIA's. an in-house MIFA and an in-house complement fixation methods. Seroconversion of IgG and IgA values and / or positive IgM was interpreted as an acute C. pneumoniae infection by DRG methods.

Originally, the samples were grouped as:
- positive pairs, meaning acute primary or reinfection (n = 106)
- negative pairs, meaning no infection or past infection (n = 134)

When the results of all methods were disclosed and the data was processed, the performance characteristics were calculated as shown in Table 5.

Table 5. Comparison of the four serological methods for detection of acute C. pneumoniae infection.

	MIF (%) in-house	EIA 1 (%) competitive	EIA 2 (%) competitive	EIA (%) DRG
Sensitivity	93/106 (88)	92/106 (87)	97/106 (92)	102/106 (96)
Specificity	133/134 (99)	132/134 (99)	127/134 (95)	133/134 (99)
PV pos	93/94 (99)	92/94 (98)	97/104 (93)	102/103 (99)
PV neg	133/146 (91)	132/146 (90)	127/136 (93)	133/137 (97)

The study shows that out of the 106 cases that were interpreted as acute infection by at least 2 methods, competitor's EIA 1 misinterpreted 14 cases, competitor's EIA2 9 cases, whereas DRG's EIAs only 4 cases.

Conclusion

Because of more reproducible and objective results EIA may better discriminate past infection from reinfection thus persons referred to the laboratory for analysis with reinfection during the epidemics will readily receive specific treatment.

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TROUBLE SHOOTING

Cause/Error	Remedy
BLANK IS TOO HIGH	
1. Contamination, spills from other wells	Avoid contamination
2. Washing solution concentrate was not diluted correctly	Should be diluted 1:10 (1+9)
3. Poor washing	Check your washer
4. Contamination of reaction basin for the substrate	Keep the residual substrate mixture until the test is completed. Check if the mixture in the reagent basin turns blue, This will indicate contamination.

Cause/Error	Remedy

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ALL ABSORBANCE VALUES ARE VERY LOW	
1. Reagents are deteriorated * due to contamination of HRP deterioration in the conjugate * due to improper storage	Protect reagents from excessive light When removing aliqouts from the reagent vials, use aseptic technique to avoid contamination or erroneous results may occur. Protectb reagents from excessive light. Store at +4°C
2. Reagent are not warmed up to room temperature before starting	Should be +20 to +25°C when starting the assay
3. Incubation time is too short or	Incubate 1h with a tolerance of ±5min.
4. Interchange for reagents	Do not mix or interchange reagents form different lots
5. Stopping solution is not mixed properly	Mix carefully before measurement

Cause/Error	Remedy
POOR PRECISION	
1. Liquid handling devices are not properly calibrated	Check calibration of the pipetting device
2. Improper washing due to contamination of washing tops head	Clean regularly tips of the washing head
3. The plate is allowed to stay too long after washing (drying of the plate)	Follow strictly the kit instructions
4. Uneven warming of the plate	Service iEMS Incubator/Shaker or incubator in use

Cause/Error	Remedy
BLANK WELLS	
1. Contamination of wells or conjugate with spills of human sera.	Only nanoliters or sera are enough to block the activity of the conjugate. Pay special attention to prevent contamination