

PATHOZYME® MYCO IgG ^{Ref} OD167 / IgA ^{Ref} OD177 / IgM ^{Ref} OD187
Individual Enzyme-Immunoassays (EIA) For the detection of IgG, IgA and IgM
antibodies to *Mycobacterium* species in human serum.
Store at 2°C to 8°C. DO NOT FREEZE
For in-vitro use only

INTRODUCTION

PATHOZYME-MYCO are Enzyme-Immunoassays (EIA) for the detection of antibodies to *Mycobacterium* species in human serum. Individual assays are available for IgG, IgA and IgM. These tests utilise two highly purified antigens for maximum sensitivity and to ensure good specificity. The first antigen purified from *Mycobacterium tuberculosis*^{1,2}, is highly antigenic and present in all the members of the genus *Mycobacterium*³. Studies using this purified antigen in an EIA system found a high degree of specificity (91%) and sensitivity (72%) and concluded it to be a useful antigen for the serodiagnosis of Tuberculosis³. The second antigen utilised in this assay is a highly specific recombinant 38 kDa antigen from *M. tuberculosis* which has been expressed and purified from *Escherichia Coli*⁴. This antigen has been reported as the single most important antigen for the serodiagnosis of Tuberculosis⁵. It is a unique disease associated protein⁶ which appears to be completely specific to the *Mycobacterium tuberculosis* complex^{7,8,9}, which includes the three closely related species *M. tuberculosis*, *M.bovis* and *M. africanum*¹⁰. From limited studies, BCG vaccination has not been shown to elevate antibody levels^{11,12}. The **PATHOZYME- MYCO** EIA kits are therefore a highly sensitive screening assays for mycobacterial disease although due to the presence of the 38 kDa antigen, the assay has a bias for detecting *M. tuberculosis* infection.

INTENDED USE

PATHOZYME MYCO is an in-vitro diagnostic test for the detection of human IgG, IgA and IgM in response to infection with *Mycobacterium* species. For professional use only.

PRINCIPLE OF THE TEST

A recombinant 38 kDa protein and a highly purified antigen derived from *Mycobacterium tuberculosis* are bound to the surface of microtitration wells. Test sera diluted 1/100 are applied. Specific antibodies to *Mycobacterium* species bind to the antigens in the wells. Unbound material is washed away and anti-human IgG, IgA or IgM antibody conjugated to Horseradish Peroxidase is applied. The conjugate binds to the human antibodies bound to the antigen. Unbound material is again washed away. On addition of the substrate, stabilised 3,3', 5,5', Tetramethyl Benzidine (TMB), a colour will develop only in those wells in which the enzyme is present, indicating the presence of human anti-*Mycobacterium* species antibody. The enzyme reaction is stopped by the addition of dilute Sulphuric Acid and the absorbance is then measured at 450nm.

For IgG and IgA tests a standard curve may be constructed by plotting the optical densities of the references. The units/ml of the unknown sera are then determined from the standard curve.

For the IgM assay any result with an optical density (OD) greater than the cut off level is considered positive.

This test has been calibrated against in house standards. There is no International standard for this test.

^{Ref} OD167 ^{Ref} OD177 ^{Ref} OD167 ^{Ref} OD177

CONTENTS FOR IgG AND IgA

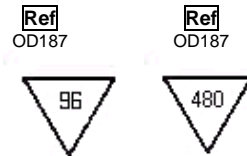
Microtitre Plate	12 x 8 wells	12 x 8 wells x 5
Breakable wells coated with specific antigens contained in a resealable foil bag with a desiccant.		
DIL SPE	100 ml	4 x 125 ml
Specimen Dilution Buffer: Tris based buffer containing stabilising proteins. Ready to use. (Orange)		
Control -	2 ml	4 ml
Negative Control. Clear solution, of human serum negative for IgG or IgA antibodies to <i>Mycobacterium</i> . Ready to use. (Blue)		
Control 2 U / ml	2 ml	4 ml
Clear solution, of human serum containing a low level of IgG or IgA antibodies to <i>Mycobacterium</i> . Ready to use. (light green)		



Control 4 U / ml	2 ml	4 ml
Clear solution, of human serum containing a low to moderate level of IgG or IgA antibodies to <i>Mycobacterium</i> . Ready to use. (light green)		
Control 8 U / ml	2 ml	4 ml
Clear solution, of human serum containing a moderate to high level of IgG or IgA antibodies to <i>Mycobacterium</i> . Ready to use. (light red)		
Control 16 U / ml	2 ml	4 ml
Clear solution, of human serum containing a high level of IgG or IgA antibodies to <i>Mycobacterium</i> . Ready to use. (red)		
Washbuf 20X	50 ml	2 X 125 ml
Wash Buffer concentrate: Tris based buffer containing detergents. (Colourless)		
Conj	11 ml	2 x 25 ml
Anti-human IgG or Anti-human IgA HRP Conjugate: Anti-human IgG or Anti-human IgA conjugated to Horseradish Peroxidase. Ready to use. (purple)		
Subs TMB	11 ml	5 X 11 ml
Substrate Solution: 3,3', 5,5' Tetramethyl Benzidine in a citrate buffer. Ready to use. (Colourless)		
Soln Stop H2SO4 0.2M	11 ml	50 ml
Stop Solution: Sulphuric Acid diluted in purified water. Ready to use. (Colourless)		
Instruction Leaflet and EIA Data Recording Sheet	1 + 1	1 + 3

CONTENTS FOR IgM

Microtitre Plate	12 x 8 wells	12 x 8 wells x 5
Breakable wells coated with specific antigens contained in a resealable foil bag with a desiccant.		
DIL SPE	100 ml	4 X 125 ml
Specimen dilution buffer: Tris based buffer containing stabilising proteins. Ready to use. (Orange)		
Control -	2 ml	4 ml
Negative Control. Clear solution, of human serum negative for IgM antibodies to <i>Mycobacterium</i> . Ready to use. (Blue)		
Control L	2 ml	4 ml
Low Positive Control. Clear solution, of human serum containing a low level of IgM antibodies to <i>Mycobacterium</i> . Ready to use. (Igreen)		
Control H	2 ml	4 ml
High Positive Control. Clear solution, of human serum containing a high level of IgM antibodies to <i>Mycobacterium</i> . Ready to use. (Red)		
Washbuf 20X	50 ml	2 X 125 ml
Wash Buffer concentrate: Tris based buffer containing detergents. (Colourless)		
Conj	11 ml	2 X 25 ml
Anti-human IgM Conjugate: Anti-human IgM conjugated to Horseradish Peroxidase. Ready to use. (purple)		
Subs TMB	11 ml	5 X 11 ml
Substrate Solution: 3,3', 5,5' Tetramethyl Benzidine in a citrate buffer. Ready to use. (Colourless)		
Soln Stop H2SO4 0.2M	11 ml	5 X 11 ml
Stop Solution: Sulphuric Acid diluted in purified water. Ready to use. (Colourless)		
Instruction Leaflet and EIA Data Recording Sheet	1 + 1	1 + 3



MATERIAL REQUIRED BUT NOT PROVIDED

- Micropipettes: 100µl, 200µl, 1000µl and 5000µl
- Disposable pipette tips
- Tubes for Sample Dilution
- Vortex Mixer
- Absorbent paper
- Microplate reader fitted with a 450nm filter
- Graph paper
- Thoroughly clean laboratory glassware.

PRECAUTIONS.

PATHOZYME MYCO contains materials of human origin which have been tested and confirmed negative for HCV, HIV I and II antibodies and HBsAg by FDA approved methods at single donor level. Because no test can offer complete assurance that products derived from human source will not transmit infectious agents it is recommended that the reagents within this kit be handled with due care and attention during use and disposal. All reagents should, however, be treated as potential Biohazards in use, and for disposal. Do not ingest.

PATHOZYME MYCO Reagents do not contain dangerous substances as defined by current UK Chemicals (Hazardous Information and Packaging for Supply) regulations. All reagents should, however, be treated as potential biohazards in use and disposal. Final disposal must be in accordance with local legislation.

PATHOZYME MYCO Stop Solution is dilute Sulphuric Acid and is therefore corrosive. Handle with care. In case of contact, rinse thoroughly with water.

PATHOZYME MYCO reagents contain 1% Proclin™ 300* as a preservative which may be toxic if ingested. In case of contact, rinse thoroughly with running water and seek medical advice.

Proclin™ 300 is a trade mark of ROHM & HAAS Limited.

STORAGE

Reagents must be stored at temperatures between 2°C to 8°C.

Expiry date is the last day of the month on the bottle and the kit label. The kit will perform within specification until the stated expiry date as determined from date of product manufacture and stated on kit and components. Do not use reagents after the expiry date.

Exposure of reagents to excessive temperatures should be avoided. Do not expose to direct sunlight.

DO NOT FREEZE ANY OF THE REAGENTS as this will cause irreversible damage.

SPECIMEN COLLECTION AND PREPARATION

Obtain a sample of venous blood from the patient and allow a clot to form and retract. Centrifuge clotted blood sample and collect clear serum. Fresh serum samples are required.

Do not use haemolysed, contaminated or lipaemic serum for testing as this will adversely affect the results.

Serum may be stored at 2°C to 8°C for up to 48 hours prior to testing. If longer storage is required, store at -20°C for up to 1 year. Thawed samples must be mixed prior to testing.

Do not use Sodium Azide as a preservative as this may inhibit the Peroxidase enzyme system.

Do not repeatedly freeze-thaw the specimens as this will cause false results.

Each test uses 100µl at a 1/100 dilution of the test serum. This can be achieved by adding 10µl of serum to 1000µl of Specimen Dilution Buffer. Do not store diluted sera samples, use within eight hours.

REAGENT PREPARATION

All reagents should be brought to room temperature (20°C to 25°C) and mixed gently prior to use. Do not induce foaming.

Wash Buffer:

Dilute the concentrated Wash Buffer using 1 part Wash Buffer concentrate with 19 parts distilled water. For every 8-well breakable strip, prepare 25ml of diluted Wash Buffer by adding 1.25ml of concentrated Wash Buffer to 23.75ml of distilled water. Prepare fresh diluted Wash Buffer prior to every assay run. Extra Wash Buffer is supplied to enable priming of automatic washing machines.

The washing procedure is critical to the outcome of this test. Insufficient washing will result in poor precision and falsely elevated absorbance readings.

LIMITATIONS OF USE

The use of samples other than serum has not been validated in this test. There is no reuse protocol for this product. When making an interpretation of the test it is strongly advised to take all clinical data into consideration. Diagnosis should not be made solely on the findings of one clinical assay.

A suspected positive result should be reassessed. A negative result does not rule out a recent or current infection. In cases of samples with a negative result, where a possible infection is suspected, an additional sample should be tested two to four weeks after the initial sample was taken.

ASSAY PROCEDURE

1. Bring all the kit components and the test serum to room temperature (20°C to 25°C) prior to the start of the assay.
2. One set of control serum should be run with each batch of test serum. Secure the desired number of coated wells in the holder. Record the position of the control serum and the test serum on the EIA Data Recording Sheet provided.
3. Unused strips should be resealed in the foil bag containing the desiccant, using the resealing zip-lock before being replaced at 2°C to 8°C.
4. Dilute each test serum 1/100 in Specimen dilution buffer by adding 10µl of serum to 1000µl of Specimen dilution buffer. DO NOT DILUTE THE CONTROL SERUM. Dispense 100µl of diluted samples and appropriate Control Serum into the appropriate wells. Gently shake for 5 seconds.
5. Cover the plate with the plate lid provided and place it on top of some moist absorbent paper at 37°C for 60 minutes.
6. At the end of the incubation period, discard the contents of the wells by flicking plate contents into a Biohazard container. Then strike the wells sharply against absorbent paper. Ensure adequate disinfectant is contained in the Biohazard container.
7. Hand Washing: Fill the wells with a minimum of 300µl of wash buffer per well. Flick plate contents into a Biohazard container. Then strike the wells sharply against absorbent paper. Wash the empty wells 3 times.
8. Strike the wells sharply onto absorbent paper or paper towel to remove all residual water droplets.
9. Machine Washing: Ensure that 300µl of wash buffer is dispensed per well and that an appropriate disinfectant is added to the waste collection bottle. Wash the empty wells 3 times. After washing remove excess fluid by striking the wells sharply onto absorbent paper or paper towel to remove all residual water droplets.
10. Dispense 100µl of Anti-Human HRP IgG or IgA or IgM Conjugate into each well. Gently shake for 5 seconds before replacing the lid onto the plate and ensuring that the plate is positioned on top of the moist absorbent paper. Incubate at 37°C for 30 minutes.
11. Wash plate as describe above.
12. Dispense 100µl of Substrate Solution into each well. Gently shake for 5 seconds before replacing the lid onto the plate and allow the reaction to develop in the dark at 37°C for 15 minutes.
13. Stop the reaction by adding 100µl of Stop Solution to each well. This will produce a colour change from blue to yellow in wells containing enzyme which indicates the presence of anti-*Mycobacterium* species antibodies. Blank the plate reader on air. Measure the absorbance of each well, at 450nm, IMMEDIATELY after stopping the reaction.

READING OF RESULTS

The plate reader should be set at a wavelength of 450nm and blanked on air. In determining the absorbances of each specimen and control. It is preferable not to use a reference filter as it will change the expected values of the controls.

TROUBLESHOOTING

For use by operatives with at least a minimum of basic laboratory training.

Do not use damaged or contaminated kit components.

Use a separate disposable tip for each sample to prevent cross contamination.

Duplication of all standards and specimens, although not required, is recommended.

Specimens and control serum should be run at the same time to keep testing conditions the same.

It is recommended that no more than 32 wells be used for each assay run if manual pipetting is used, since pipetting of all Standards and specimens should be completed within 3 minutes. A full plate of 96 wells may be used if automated pipetting is available.

Replace caps on all reagents immediately after use.

Avoid repeated pipetting from stock reagents as this is likely to cause contamination.

Do not mix reagents or antibody coated strips from different kits. When dispensing, care should be taken not to touch the surface of the well.

Do not allow reagent to run down the sides of the well. Prior to the start of the assay bring all reagents to room temperature (20°C to 25°C). Gently mix all reagents by gentle inversion or swirling.

Once an assay has been initiated, the wells should not be allowed to become dry during the assay.

Do not contaminate the Substrate Solution as this will render the whole kit inoperative.

Check the precision and accuracy of the laboratory equipment used during the procedure to ensure reproducible results.

The unused strips should be resealed in the foil bag, containing the desiccant, using the resealing zip-lock before being replaced at 2°C to 8°C.

CALCULATION OF RESULTS

For each test and Control Sera determine the OD obtained in the wells.

Assay Validation IgG: The average OD for the Negative Control should be less than 0.2, the Cut Off level (4 U/ml) should be between 0.6 and 1.1 and the 16 U/ml Control should be greater than 1.25 for the assay results to be valid.

Assay Validation IgA: The average OD for the Negative Control should be less than 0.35, the Cut Off level (Sum 2 U/ml and 4 U/ml divided by two) should be between 0.5 and 0.9 and the 16 U/ml Control should be greater than 1.25 for the assay results to be valid.

Assay Validation IgM: The average OD for the Negative Control should be less than 0.2, the Cut Off level (Low Positive Control should be between 0.4 and 0.8 and the High Positive Control should be greater than 1.00 for the assay results to be valid.

If levels of controls or users known samples do not give expected results, test results must be considered invalid.

FOR IgG AND IgA ASSAYS

Plot each control sera, except for the negative, on the semi-log graph paper provided. For the IgG assay draw the best fit straight line between 2, 4, 8 units and 16 units, as the standard curve is linear between these points. The IgA assay does not give a linear line and the points should be connected consecutively.

Using the OD for each sample determine the corresponding units/ml by finding the Y axis value and extend a horizontal line to the standard curve which has been plotted for the assay run. At the intersection point, extend a vertical line to the X axis and determine the samples units/ml. Samples with an OD greater than 16U/ml should be further diluted and reanalysed if a serum unit value is required.

The value for units/ml extrapolated from the standard curve are for samples diluted 1/100.

Therefore:

$$\text{Serounits} = \text{Standard Curve Units/ml} \times 100$$

Serounits	IgG	IgA
Negative Result:	Less than 400U/ml	Less than 300U/ml
Low Positive:	400U/ml to 900U/ml	300U/ml to 600U/ml
Positive Result:	Greater than 900U/ml	Greater than 600U/ml

Qualitative Methods

IgG Cut Off = OD of the average 4U/ml Control Sera.

$$\text{IgA Cut Off} = \frac{\text{Average OD of 2U/ml} + \text{Average OD of 4U/ml controls}}{2}$$

FOR IgM ASSAYS

IgM Cut Off = Average of the low positive control serum.

FOR BOTH IgG, IgA AND IgM ASSAYS

Negative Result: A negative result should have an OD less than the Cut Off.

Positive Result: A positive result should have an OD greater than the Cut Off.

For comparison between assays antibody indexes (AI) must be calculated:

$$\text{AI} = \frac{\text{OD of sample}}{\text{Cut Off}}$$

The use of paired sera samples conducted over a period of time can be useful in monitoring the efficiency of treatment and for identification of resistant strains. It may be necessary in the case of high titre sera to use greater than the recommended 1/100 dilution in order to bring the OD of the sample on to the linear part of the standard curve.

EVALUATION DATA

Calibrated to in house standards.

The co-efficient of variation of **PATHOZYME Myco** is less than or equal to 10%.

	SMEAR	Myco G	Myco A	Myco M	G, A, M Total
Overall Sensitivity n = 46	37% 17/46	63% 29/46	57% 26/46	33% 15/46	85% 39/46
Smear Negative Culture Positive n = 29	0% 0/29	52% 15/29	41% 12/29	34% 10/29	76% 22/29
Smear Positive Culture Positive N = 17	100% 17/17	82% 14/17	82% 14/17	29% 5/17	100% 17/17
Specificity n = 45	N/A	100% 45/45	98% 44/45	93% 42/45	91% 41/45

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QUICK REFERENCE TEST PROCEDURE

1. Dilute the test sera 1/100 by adding 10 μ l of serum to 1000 μ l of Serum Diluent.
2. Dispense 100 μ l of diluted test serum and appropriate working strength Control Serum to each well. Gently shake for 5 seconds.
3. Incubate for 60 minutes at 37°C
4. Discard well contents and wash 3 times.
5. Dispense 100 μ l of Anti-Human HRP IgG or IgA or IgM Conjugate to each well. Gently shake for 5 seconds.
6. Incubate for 30 minutes at 37°C
7. Discard well contents and wash 3 times.
8. Dispense 100 μ l of Substrate Solution to each well. Gently shake for 5 seconds.
9. Incubate in the dark for 15 minutes at 37°C.
10. Dispense 100 μ l of Stop Solution to each well.
11. Read the OD using an EIA reader with a 450nm filter.
12. Interpret results as described in the Interpretation of Results section.

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