Anti-AtherOx® IgM Test Kit
(anti-oxLDL/β_2GPI Antibody)
For Laboratory or Investigational Use Only

INTENDED USE

An enzyme-linked immunoassay (ELISA) for the determination of IgM antibodies to complexes formed by the interaction of oxidized low-density lipoprotein (oxLDL) with β_2-glycoprotein I (β_2GPI) in human serum.

SUMMARY AND EXPLANATION OF THE ASSAY

Atherosclerosis is characterized by a gradual thickening of arterial walls due to the excessive accumulation of lipids, a process that leads to reduced elasticity, decreased blood supply, thrombus (clot) formation and tissue or organ damage. Experimental evidence indicates that pro-inflammatory factors and dyslipidemia are the main contributors to the development of atherosclerosis.[1-5] Low-density lipoprotein (LDL) is the principal form of cholesterol that accumulates in atherosclerotic lesions or plaques, but LDL must be first modified into an oxidized structure (oxLDL). The most significant pro-atherogenic mechanism for modifying LDL into oxLDL is oxidative stress.[6,7] In addition, chronic oxidative stress produces endothelium and platelet dysfunction, which in combination with the accumulation of oxLDL in the arterial wall, leads to the development of an arterial pro-thrombotic state referred to as athero-thrombosis.[8-13]

Unlike native LDL, oxLDL binds to β_2GPI to form oxLDL/β_2GPI complexes.[14-17] Both oxLDL and β_2GPI have been demonstrated in atherosclerotic lesions by immunostaining, along with immunoreactive lymphocytes and immunoglobulins. OxLDL/β_2GPI complexes are immunogenic and antibodies to this complex have been demonstrated in patients with SLE and APS.[6,18] The *in vitro* macrophage uptake of oxLDL/β_2GPI complexes is mediated by scavenger receptors, and this uptake is significantly enhanced in the presence of anti-β_2GPI antibodies, a process likely mediated by Fcγ receptors.[17] This mechanism is physiologically relevant to explain the accelerated development of foam cells within the atherosclerotic lesions or plaques. Thus, autoantibodies to pro-atherogenic oxLDL/β_2GPI complexes may represent a serologic risk factor and/or a significant contributor to the development of autoimmune-mediated athero-thrombosis.[19-21]

The premature (or accelerated) development of clinical atherosclerosis in patients with systemic autoimmune diseases cannot be fully explained by the traditional risk factors or disease treatment i.e. steroids.[22-25] Elevated serum levels of oxLDL/β_2GPI complexes and anti-oxLDL/β_2GPI antibodies have been demonstrated in patients with SLE and APS, disorders characterized by a pro-thrombotic predisposition and significant vascular complications.[26-28] Anti-oxLDL/β_2GPI antibodies were strongly associated with arterial thrombosis. These antibodies also showed a positive predictive value for APS and arterial thrombosis of 90%, compared to 77% for venous thrombosis, while the predictive value of anti-cardiolipin (aCL) antibodies was 71% for APS and 64% for arterial thrombosis.[29] Thus, the presence of circulating autoantibodies to oxLDL-β_2GPI complexes are etiologically important in the development of autoimmune-mediated athero-thrombotic complications.

PRINCIPLE OF THE TEST

This test is an indirect ELISA detecting IgM anti-oxLDL/β_2GPI antibodies. Diluted serum samples, calibrator(s), and controls are incubated in microwells coated with the oxLDL/β2GPI complex. Incubation allows the IgM anti-oxLDL/β2GPI antibody present in the samples to react with the immobilized antigen complex. After the removal of unbound serum proteins by washing, anti-human IgM antibodies, labeled with horseradish peroxidase (HRP), are added forming complexes with the bound IgM anti-oxLDL/β2GPI antibody. Following another washing step, the bound enzyme-antibody conjugate is assayed by the addition of a solution containing tetramethylbenzidine (TMB) and hydrogen peroxide (H_2O_2) as the chromogenic substrate. Color develops in the wells at an intensity proportional to the serum concentration of IgM anti-oxLDL/β2GPI antibody.

Results are obtained by reading the OD (optical density or absorbance) of each well in a spectrophotometer. Calibrator sera are provided, with the IgM anti-oxLDL/β2GPI antibody concentration expressed in M Units. A log-log regression analysis is performed with calibrator values plotted against calibrator mean ODs. Controls and patient results are determined from the calibration curve.

REAGENTS

Store at 2–8°C. Do Not Freeze.

Each Anti-AtherOx® IgM Test Kit contains the following reagents:
(volumes may vary depending on kit size and configuration):

- 12x8 stabilized oxLDL/β2GPI antigen (human) coated microwells, with frame.
- 2 bottles (60 mL) Sample Diluent* (green solution).
- 3 vials (0.250 mL) IgM Calibrator Sera* (human) (1-high, 2-moderate, 3-low) - refer to vial label for the assigned value in M Units.
- 1 vial (0.250 mL) IgM Positive Control Serum* (human) - refer to vial label for expected range in M Units.
- 1 vial (0.250 mL) IgM Normal Control Serum* (human) - refer to vial label for expected range in M Units.
- 1 bottle (15 mL) IgM anti-human (goat) HRP-Conjugated Antibody Solution (red solution).
- 1 bottle (15 mL) One-Component Substrate (TMB and H_2O_2); ready to use.
- 1 bottle (15 mL) Stopping Solution (0.36 N sulfuric acid).
- 2 bottles (30 mL) Wash Concentrate (33X PBS/Tween 20).

*** CAUTION: Contains sodium azide**

WARNINGS AND PRECAUTIONS

For Laboratory or Investigational Use Only

1. Not for use in diagnostic procedures. The performance characteristics of this product have not been established
2. Human source material used to prepare the calibrators and controls included in this kit has been tested and shown to be negative for antibodies to HBsAg, HCV, HIV I & 2, and Syphilis by FDA required tests. However, all human blood derivatives, including patient samples, should be handled as potentially infectious material.
3. Do not pipette by mouth.
4. Do not smoke, eat, or drink in areas where specimens or kit reagents are handled.
5. Wear disposable gloves while handling kit reagents and wash hands thoroughly afterwards.
6. Certain components of this product contain sodium azide as a preservative. Sodium azide has been reported to form lead and copper azides when left in contact with these metals. These metal azides are explosive. Any solutions containing azide must be thoroughly flushed with copious amounts of water to prevent the build-up of explosive metal azides in the plumbing system.
7. One-component substrate can cause irritation to the eyes and skin. Absorption through the skin is possible. Use gloves when handling substrate and wash thoroughly after handling. Keep reagent away from ignition sources. Avoid contact with oxidizing agents.
8. Certain components are labeled with the following: Irritating to eyes (R 36). Avoid contact with skin and eyes (S 24/25). In case of contact with eyes, rinse immediately with plenty of water and seek medical advice (S 26). If swallowed, seek medical advice immediately and show container or label (S 46).

   Irritant Xi. Biological Risk

SPECIMEN COLLECTION AND PREPARATION

Serum is the preferred sample matrix. Blood should be collected by venipuncture, and the serum separated from the cells by centrifugation after clot formation. If not tested immediately, specimens should be stored at 2–8°C. If specimens are to be stored for more than 72 hours, they should be frozen at –20°C or below. Avoid repeated freezing and thawing. Do not use grossly hemolyzed, icteric, or lipemic serum as these conditions may cause aberrant results. Specimens containing visible particulate matter should be clarified by centrifugation before testing.

INSTRUCTIONS FOR USE

Materials Provided:

Anti-AtherOx® IgM Test Kit; see "Reagents," for a complete listing.

Materials Required but not Provided:

- Reagent grade water to prepare PBS/Tween 20 wash solution (1L)
- Graduated cylinders
- Precision pipettors capable of delivering between 10 µL and 1000 µL, with appropriate tips
- Miscellaneous glassware appropriate for small volume handling
- Flask or bottle, 1 liter
- Wash bottles, preferably with the tip partially cut back to provide a wide stream, or an automated or semi-automated washing system
- Disposable gloves
- Plate reading spectrophotometer capable of reading absorbance at 450 nm (with a 650 nm reference if available)
- Multichannel pipettors capable of delivering to 8 wells simultaneously

Procedural Notes

1. Bring serum samples and kit reagents to room temperature (18–26°C) and mix well before using; avoid foaming. Return all unused samples and reagents to refrigerated storage as soon as possible.
2. All dilutions of calibrators, controls, and test sera must be made just prior to use in the assay.
3. The plate reader should be programmed to blank or zero against air.
4. Good washing technique is critical for optimal performance of the assay. Adequate washing is best accomplished by directing a forceful stream of wash solution from a plastic squeeze bottle with a wide tip into the bottom of the microwells. An automated microtiter plate washing system can also be used.
5. IMPORTANT: Failure to adequately remove residual PBS/Tween 20 can cause inconsistent color development of the Substrate Solution.
6. Use a multichannel pipettor capable of delivering to 8 wells simultaneously when possible. This speeds the process and provides more uniform incubation and reaction times for all wells.
7. Carefully controlled timing of all steps is critical. All calibrators, controls, and samples must be added within a five minute period. Batch size of samples should not be larger than the amount that can be added within this time period.
8. For all incubations, the start of the incubation period begins with the completion of reagent or sample addition.
9. Addition of all samples and reagents should be performed at the same rate and in the same sequence.
10. Incubation temperatures above or below normal room temperature (18–26°C) may contribute to inaccurate results.
11. Avoid contamination of reagents when opening and removing aliquots from the primary vials.
12. Do not use kit components beyond expiration date.
13. Do not use components from different kit lot numbers.

Reagent Preparation

Wash Solution (PBS/Tween 20): Measure 30 mL of Wash Concentrate (33X PBS/Tween 20) and dilute to 1 liter with reagent grade water. Unused PBS/Tween 20 solution may be stored in the refrigerator or at room temperature. Discard if the solution shows signs of microbial growth or other contamination.

Assay Procedure

1. The assay is performed using a three-point calibration curve. A reagent blank control must be run with each assay in which Sample Diluent without serum is added to the well. This well is then treated the same as sample wells in subsequent assay steps.
2. Remove any microwell strips that will not be used from the frame and store them in the bag provided.
3. Prepare a 1:100 dilution of the calibrator(s), controls and patient samples in Sample Diluent; e.g., 10 µL sample added to 1000 µL Sample Diluent equals a 1:100 sample dilution.
4. Add 100 µL of prepared calibrator(s), diluted controls, diluted patient samples, and reagent blank sample to the appropriate microwells.
5. Incubate 60 minutes at room temperature. After the incubation is complete, carefully invert the microwells and empty the sample fluid. Do not allow samples to contaminate other microwells.
6. Wash 4 times with wash solution. Invert microwells between each wash to empty fluid. Use a snapping motion of the wrist to shake the liquid from the wells. The frame must be squeezed at the center on the top and bottom to retain microwell modules during washing. Blot on absorbent paper to remove residual wash fluid. Do not allow wells to dry out between steps.
7. Add 100 µL IgM anti-human HRP-conjugated antibody solution (red) to the wells.
8. Incubate for 60 minutes at room temperature. After the incubation is complete, carefully invert the microwells and empty the conjugate solution.
9. Wash 4 times with wash solution as in step 6. Use a snapping motion to drain the liquid and blot on absorbent paper after the final wash. Do not allow the wells to dry out.
10. Add 100 µL One-Component Substrate to each well and incubate for 30 minutes at room temperature. Add the substrate to the wells at a steady rate. Blue color will develop in wells with positive samples.
11. Add 100 µL Stopping Solution (0.36 N sulfuric acid) to each well to stop the enzyme reaction. Be sure to add Stopping Solution to the wells in the same order and at the same rate as the Substrate was added. Blue substrate will turn yellow and colorless substrate will remain colorless. Blank or zero the plate reader against air. Read the OD of each well at 450 nm (650 nm reference, if available). The OD values should be measured within 5 minutes after the addition of Stopping Solution.

Calculation of Results

1. Calculate the mean OD values if duplicate wells of the calibrators, controls, and patient samples were performed.
2. Perform a log-log regression analysis with the log of the three calibrator values (see vial labels for assigned M Units) on the y-axis against the log of ODs for each calibrator on the x-axis.
3. The calibrator curve can be plotted either automatically using a validated software program or manually with graph paper. When generating the curve manually, draw a best-fit line through the plotted points on log-log graph paper.
4. Determine the control and patient sample values from the calibrator curve.
5. Assure that all quality control parameters have been met (see Quality Control section) before reporting test results.

QUALITY CONTROL

1. The OD value or mean OD value of Calibrator 2 should be at least 0.400 to assure that the kit is functioning properly. Calibrator 2 OD readings of less than 0.400 may indicate that the kit is no longer suitable for use.
2. The OD value, or mean OD value of the reagent blank should be less than 0.100 when the spectrophotometer has been properly blanked against air. Blank readings greater than 0.100 may indicate possible reagent contamination or inadequate plate washing.
3. The IgM anti-OxLDL/β2GPI antibody values obtained for the control sera should be within the ranges indicated on the container labels.
4. Each laboratory should periodically determine their own normal range for the appropriate population of patients.
5. Samples with values greater than 100 Units may be reported as “greater than 100 M Units”. Alternatively, the sample may be additionally diluted 1:10 and re-run in the assay. The resulting value in M Units would then be multiplied by a factor of 10.

NORMAL RANGE

Serum samples from 100 healthy blood donors were tested across 4 lots of the Anti-AtherOx® IgM Test Kit. The mean IgM anti-oxLDL/β2GPI antibody value of this population was determined to be 11 M Units, with a standard deviation of 6.4 Units. The 95th percentile was 24.3 M Units and the mean + 2SD was 23.7 M Units. A normal range of 0–20 M Units was established based on both calculations from the normal population.

IgM ANTI-oxLDL/β_2GPI ANTIBODY NORMAL RANGE = 0–20 M UNITS

Precision:

The Anti-AtherOx® IgM Test Kit was evaluated for intra-assay (16 replicates per assay) and inter-assay (3 assays, 16 replicates per assay) value precision across 4 kit lots. The mean coefficients of variation (CV%) obtained are summarized in the following table:

Mean IgM anti-oxLDL/β2GPI antibody value of sample	Intra-assay mean CV%	Inter-assay mean CV%
56.5 Units	3.8%	2.1%
41.9 Units	4.5%	2.6%
4.8 Units	6.0%	3.5%

LIMITATIONS OF THE TEST

IgM anti-oxLDL/β2GPI antibody levels obtained with this assay are not for use in diagnostic procedures.

IgM anti-oxLDL/β2GPI antibodies may be produced when oxidative stress caused by various underlying inflammatory conditions form oxLDL/β2GPI complexes. However, other transient conditions such as certain bacterial infections may cause oxidative stress, formation of oxLDL/β2GPI complexes, and IgM antibodies to these complexes. The exact clinical significance of these IgM anti-oxLDL/β2GPI antibodies is under further investigation.

The presence of Rheumatoid Factor (RF) in patients' samples may interfere with ELISA methods by binding to antibodies. The presence of RF should be considered when evaluating results.

Testing patients' samples containing excess hemoglobin, lipids, and/or bilirubin is not recommended as these substances may interfere with the results of the assay.

REFERENCES

1. Steinberg D. Low-density lipoprotein oxidation and its pathobiological significance. *J Biol Chem* 1997; 272: 20963-6.
2. Berliner JA, Heinecke JW. The role of oxidized lipoproteins in atherogenesis. *Free Radic Biol Med* 1996; 20: 707-27.
3. Ross R. Atherosclerosis: an inflammatory disease. *N Engl J Med* 1999; 340: 115-126.
4. Steinberg D. Atherogenesis in perspective: Hypercholesterolemia and inflammation as partners in crime. *Nature Med* 2002; 8: 1211-7.
5. Rose N, Afanasyeva M. Autoimmunity: busting the atheorsclerotic plaque. *Nat Med* 2003; 9: 641-2.
6. Yla-Herttuala S, Palinski W, Rosenfeld ME, Parthasarathy S, Carew TE, Butler S, Witztum JL, Steinberg D. Evidence for presence of oxidatively modified low-density lipoprotein in atherosclerotic lesions of rabbit and man. *J Clin Invest* 1989; 84: 1086-95.
7. McMurray HF, Parthasarathy S, Steinberg D. Oxidatively modified low-density lipoprotein is a chemoattractant for human T lymphocytes. *J Clin Invest* 1993; 92: 1004-8.
8. Pratico D, Ferro D, Iuliano, Rokach J, Conti F, Valesini G, Fitzgerald GA, Violi F. Ongoing prothrombotic state in patients with antiphospholipid antibodies: a role for increased lipid peroxidation. *Blood* 1999; 93: 3401-7.
9. Ames PRJ, Alves J, Murat I, Isenberg DA, Nourooz-Zadeh J. Oxidative stress in systemic lupus erythematosus and allied conditions with vascular involvement. *Rheumatology* 1999; 38: 529-34.
10. Bruckdorfer KR, Hillary JB, Bunce T, Vancheeswaran R, Black CM. Increased susceptibility to oxidation of low-density lipoproteins isolated from patients with systemic sclerosis. *Arthritis Rheum* 1995; 38: 1060-7.
11. Stein CM, Tanner SB Awad JA Roberts LJ, Morrow JD. Evidence of free radical-mediated injury (isoprostane overproduction) in scleroderma. *Arthritis Rheum* 1996; 39: 1146-50.
12. De Cristofaro R, Rocca B, Vitacolonna E, Falco A, Marchesani P, Ciabattoni G, Landolfi R, Patrono C, Davi G. Lipid and protein oxidation contribute to a prothrombotic state in patients with type 2 diabetes mellitus. *J Thromb Haemost* 2003; 1: 250-6.
13. Tsuzura S, Ikeda Y, Suehiro T, Ota K, Osaki F, Arii K, Kumon Y, Hashimoto K. Correlation of plasma oxidized low-density lipoprotein levels to vascular complications and human serum paraoxonase in patients with type 2 diabetes. *Metabolism* 2004; 53: 297-302.
14. Hasunuma Y, Matsuura E, Makita Z, Katahira T, Nishi S, Koike T. Involvement of β_2-glycoprotein I and anticardiolipin antibodies in oxidatively modified low-density lipoprotein uptake by macrophages. *Clin Exp Immunol* 1997; 107: 569-73.
15. Kobayashi K, Matsuura E, Liu Q, Furukawa J, Kaihara K, Inagaki J, Atsumi T, Sakairi N, Yasuda T, Voelker DR, Koike T. A specific ligand for β_2-glycoprotein I mediates autoantibody-dependent uptake of oxidized low-density lipoprotein by macrophages. *J Lipid Res* 2001; 42: 697-709.
16. Liu Q, Kobayashi K, Inagaki J, Sakairi N, Iwado A, Yasuda T, Koike T, Voelker DR, Matsuura E. ω-carboxyl variants of 7-ketocholesteryl esters are ligands for β_2-glycoprotein I and mediate antibody-dependent uptake of oxidized LDL by macrophages. *J Lipid Res* 2002; 43: 1486-95.
17. Kobayashi K, Kishi M, Atsumi T, Bertolaccini ML, Makino H, Sakairi N, Yamamoto I, Yasuda T, Khamashta MA, Hughes GRV, Koike T, Voelker DR, Matsuura E. Circulating oxidized LDL forms complexes with β_2-glycoprotein I: implication as an atherogenic autoantigen. *J Lipid Res* 2003; 44: 716-26.
18. George J, Harats D, Gilburd B, Afek A, Levy Y, Schneiderman J, Barshack I, Kopolovic J, Shoenfeld Y. Immunolocalization of β_2-glycoprotein I (apolipoprotein H) to human atherosclerotic plaques: potential implications for lesion progression. *Circulation* 1999; 99: 2227-30.
19. Matsuura E, Lopez LR. Are oxidized LDL/β_2-glycoprotein I complexes pathogenic antigens in autoimmune-mediated atherosclerosis? *Clin Dev Immunol* 2004; 11:103-11.
20. Matsuura E, Lopez LR. Oxidized low-density lipoprotein complexed with β_2-glycoprotein I as a common metabolic form in atherogenesis and autoimmune-mediated atherosclerosis. In “Focus on Atherosclerosis Research”, Editor Leon V Clark, Nova Science Publishers (NY), pp.91-113 (2004).
21. Lopez LR, Matsuura E. Autoimmune-mediated atherosclerosis: Pathogenic role of oxLDL/β_2GPI complexes. In “Recent Research Development Lipids”, Transworld Research Network, 7: 1-26 (2004).
22. Vaarala O. Antiphospholipid antibodies and atherosclerosis. *Lupus* 1996; 5: 442-7.
23. Ward MM. Premature morbidity from cardiovascular and cerebrovascular diseases in women with systemic lupus erythematosus. *Arthritis Rheum* 1999; 42: 338-46.
24. Esdaile JM, Abrahamowicz M, Grodzicky T, Li Y, Panaritis C, du Berger R, Cote R, Grover SA, Fortin PR, Clarke AE, Senecal JL. Traditional Framingham risk factors fail to fully account for accelerated atherosclerosis in systemic lupus erythematosus. *Arthritis Rheum* 2001; 44: 2331-7.
25. Schattner A, Liang MH. The cardiovascular burden of lupus; a complex challenge. *Arch Intern Med* 2003; 163: 1507-10.
26. Lopez D, Kobayashi K, Merrill JT, Matsuura E, Lopez LR. IgG Autoantibodies against β_2-glycoprotein I complexed with a lipid ligand derived from oxidized low-density lipoprotein are associated with arterial thrombosis in antiphospholipid syndrome. *Clin Dev Immunol* 2003; 10:203-11.
27. Lopez LR, Dier, KJ, Lopez D, Merrill JT, Fink CA. Anti-β_2-glycoprotein I and antiphosphatidylserine antibodies are predictors of arterial thrombosis in patients with antiphospholipid syndrome. *Am J Clin Pathol* 2004; 121: 142-9.
28. Lopez D, Garcia-Valladares I, Palafox-Sanchez C, Garcia De La Torre I, Kobayashi K, Matsuura E, Lopez LR. Oxidized low-density lipoprotein/β_2-glycoprotein I complexes and autoantibodies to oxLig-1/β_2-glycoprotein I in patients with systemic lupus erythematosus and antiphospholipid syndrome. *Am J Clin Pathol* 2004; 121: 426-36.
29. Lopez LR, Kuca J, Hurley B, Fink C, Matsuura E. IgG and IgM anti-oxLDL/β_2GPI antibodies in patients with autoimmune disorders and anti-phospholipid syndrome. *Autoimmunity Reviews* 2004; 3 (Suppl 2): 84.

							
Manufacturer	Batch Code	Use by/ Expiry Date	Temperature Limitations	Irritant	Biological Risk	Catalog Number	Consult Instructions for Use (Package Insert)

Warranty

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