

hsAtherOx® Test Kit (oxLDL-β₂GPI Antigen Complex)

For Research Use Only
Not for use in diagnostic procedures.

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 β₂GPI to form oxLDL-β₂GPI complexes. The interaction between oxLDL and β₂GPI is initially mediated by electrostatic forces, producing unstable or dissociable complexes. This initial interaction is followed by the formation of stable (non-dissociable) complexes mediated by covalent bonds. Stable complexes are regarded as pathogenic and more clinically relevant.¹⁴⁻¹⁷ Both oxLDL and β₂GPI have been demonstrated in atherosclerotic lesions by immunostaining, along with immunoreactive lymphocytes and immunoglobulins.^{6,18} The *in vitro* macrophage uptake of oxLDL-β₂GPI complexes is mediated by scavenger receptors, and this uptake is significantly enhanced in the presence of anti-β₂GPI antibodies, a process likely mediated by Fcγ receptors.¹⁷ This mechanism is physiologically relevant in explaining the development of foam cells within the atherosclerotic lesions or plaques. Thus, oxLDL-β₂GPI complexes have been implicated as pro-atherogenic antigens and may represent a serologic risk factor and/or a significant contributor to the development of 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-β₂GPI complexes have been demonstrated in patients with SLE, SSc and APS, disorders characterized by a pro-thrombotic predisposition and significant vascular complications.²⁶⁻²⁹ Atherosclerotic cardiovascular disease is also common in patients with type 2 DM.^{12,13} About half of type 2 DM patients showed elevated serum levels of oxLDL-β₂GPI complexes, compared to age and sex matched healthy controls. Thus, the presence of circulating oxLDL-β₂GPI complexes in patients with autoimmune diseases and DM suggest a possible pathogenic role in the development of athero-thrombotic complications.

PRINCIPLE OF THE TEST

The test is performed as an indirect ELISA. Diluted serum or plasma samples, calibrators, and controls are incubated in microwells coated with purified anti-human monoclonal antibody directed only to complexed β₂GPI. Incubation allows the oxLDL-β₂GPI antigen complex present in the samples to react with the immobilized antibody. After the removal of unbound serum or plasma proteins by washing, anti-human apoB100 (LDL) monoclonal antibodies, conjugated to biotin, are added to form complexes with the bound antigen. Following another washing step, horseradish peroxidase (HRP) conjugated Streptavidin (SA) is added to form complexes with the bound biotin conjugated antibody. Following another washing step, the bound HRP-SA conjugate is assayed by the addition of tetramethylbenzidine (TMB) and hydrogen peroxide (H₂O₂) chromogenic substrate. Color develops in the wells at an intensity proportional to the serum or plasma concentration of oxLDL-β₂GPI antigen complex. Results are calculated against a calibration curve prepared from the calibrator provided in the kit.

REAGENTS

Store at 2–8°C. Do Not Freeze.

Each hsAtherOx® Test Kit (oxLDL-β₂GPI antigen complex) contains the following reagents. **(volumes may vary depending on kit size and configuration):**

- 96 Stabilized Antibody (murine) Coated Microwells, with frame.
- 2 Bottles (50 mL) Sample Diluent (yellow solution).
- 2 Vials (0.2 mL) hsAtherOx Calibrator Serum (human); refer to vial label for the assigned value in Units/mL.*
- 2 Vials (0.2 mL) High Control Positive Serum (human); see vial label for expected range in Units/mL.*
- 2 Vials (0.2 mL) Low Control Positive Serum (human); see vial label for expected range in Units/mL.*
- 1 Bottle (15 mL) Biotin Conjugated Antibody (murine) Solution (green solution); may contain 0.1% ProClin® 150 as a preservative.
- 1 Bottle (15 mL) Horseradish Peroxidase Conjugated Streptavidin (HRP-SA) Solution (blue solution).
- 1 Bottle (20 mL) Substrate (TMB and H₂O₂); ready to use.
- 1 Bottle (20 mL) Stopping Solution (0.25 M sulfuric acid).
- 2 Bottles (50 mL) Wash Concentrate (20X).

*CAUTION: May Contain Sodium Azide as a Preservative

WARNINGS AND PRECAUTIONS

For Laboratory or Investigational Use Only. Not for use in diagnostic procedures. The performance characteristics of this product have not been established.

1. 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, and HIV 1 & 2 by FDA required tests. However, all human blood derivatives, including patient samples, should be handled as potentially infectious material.
2. Do not pipette by mouth.
3. Do not smoke, eat, or drink in areas where specimens or kit reagents are handled.
4. Certain components of this product may 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 Sodium Azide must be thoroughly flushed with copious amounts of water to prevent the build-up of explosive metal azides in the plumbing system.
5. Wear disposable gloves while handling kit reagents and wash hands thoroughly afterwards.
6. The 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.
7. 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 . Biological Risk .

SPECIMEN COLLECTION AND PREPARATION

Serum, EDTA plasma, or citrated plasma (3.2%) are the preferred sample matrixes. Blood should be collected by venipuncture, allowed to clot, and the serum separated from the cells by centrifugation. If not tested immediately, specimens should be stored at 2–8°C. If specimens are to be stored for more than 1 week, then freeze at -20°C or below. Avoid repeated freezing and thawing. Do not use hemolyzed, icteric, or lipemic serum as these conditions may cause aberrant results. Specimens containing visible particulate matter should be clarified by centrifugation before testing.

If EDTA or citrated plasma is to be used, blood should be collected by venipuncture and the plasma separated from the cells immediately by centrifugation at 1500g for 10 minutes. The supernatant must be carefully removed after centrifugation to avoid contamination with platelets. Repeating the centrifugation and separation steps may be advisable to minimize platelet contamination. If not tested immediately, plasma samples should be stored as described for serum.

INSTRUCTIONS FOR USE

Materials Provided:

hsAtherOx® Test Kit; see “Reagents” for a complete listing.

Materials Required but not Provided:

- Reagent grade water to prepare 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 or 12 wells simultaneously

Procedural Notes

1. Bring serum or plasma 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. The plate reader should be programmed to air blank.
3. 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.
4. **IMPORTANT:** Failure to adequately remove residual Wash Solution can cause inconsistent color development of the Substrate Solution.
5. Use a multichannel pipettor capable of delivering to 8 or 12 wells simultaneously when possible. This speeds the process and provides more uniform incubation and reaction times for all wells.
6. Careful controlled timing of all steps is critical. All Calibrator, 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.
7. For all incubations, the start of the incubation period begins with the completion of reagent or sample addition.
8. Addition of all samples and reagents should be performed at the same rate and in the same sequence.
9. Incubation temperatures above or below normal room temperature (18-26°C) may contribute to inaccurate results.
10. Avoid contamination of reagents when opening and removing aliquots from the primary vials.
11. Do not use kit components beyond expiration date.
12. Do not use kit components from different kit lot numbers.

Reagent Preparation

Wash Solution: Measure 50 mL of Wash Concentrate (20X) and dilute to 1 liter with reagent grade water. The pH of the final solution should be 7.35 ± 0.1 . Store unused Wash Solution in the refrigerator at 2–8°C . Discard if the solution shows signs of microbial contamination.

Assay Procedure

1. Remove any microwell strips that will not be used from the frame. Store them with the desiccant pouch in the resealable bag provided.
2. Prepare a six-point calibration curve: Label six tubes for Calibrators 1 – 6.
In tube #1, prepare a 1:100 dilution of Calibrator in Sample Diluent by adding 10 µL Calibrator to 1000 µL Sample Diluent.
Add 500 µL of Sample Diluent (yellow) to tubes # 2-6.
Remove 250 µL from tube # 1, transfer to tube # 2 and mix well.
Repeat this 3-fold serial dilution series through tube 5.
Tube # 6 containing only Sample Diluent (yellow) to be used as reagent blank. A reagent blank control should be run on each plate. These wells will be treated the same as sample wells in subsequent assay steps.

The value of Calibrator 1 is indicated on the vial label. The value of Calibrator dilutions 2 – 5 are calculated by dividing Calibrator 1 value by each Calibrator dilution factor (DF).

Example:

| Calibrator # | DF | Volume to Add | | Volume of Sample Diluent | | Calibrator value |
|-------------------|----|---------------------|---|--------------------------|---|------------------|
| 1 | - | 10 µL Calibrator | | 1000 µL | = | 5.00 (example) |
| 2 | 3 | 250 µL Calibrator 1 | + | 500 µL | = | 1.67 |
| 3 | 9 | 250 µL Calibrator 2 | + | 500 µL | = | 0.56 |
| 4 | 27 | 250 µL Calibrator 3 | + | 500 µL | = | 0.19 |
| 5 | 81 | 250 µL Calibrator 4 | + | 500 µL | = | 0.06 |
| 6 (Reagent Blank) | | ----- | | 500 µL | = | 0.00 |

3. Duplicate determinations are recommended. Prepare a 1:100 dilution of the controls and patient samples in Sample Diluent (yellow), e.g., 10 µL sample added to 1000 µL Sample Diluent equals a 1:100 sample dilution.
4. Mix thoroughly, and add 100 µL of the dilutions (6 Calibrators which include the reagent blank, controls and patient samples) to the appropriate microwells.
5. Incubate 60 minutes at room temperature (18-26°C). 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. Each well should be completely filled with Wash Solution per wash. 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 Biotin Conjugated Antibody Solution (green) to each well.
8. Incubate for 30 minutes at room temperature (18-26°C). After the incubation is complete, carefully invert the microwells and empty the conjugate solution.
9. Wash 4 times as in step 6.
10. Add 100 µL HRP Conjugated Streptavidin solution (blue) to each well.
11. Incubate for 30 minutes at room temperature (18-26°C) as in step 8.

12. Wash 4 times as in step 6.
13. Add 100 μ L Substrate (TMB, H₂O₂) to each well and incubate for 30 minutes at room temperature (18-26°C). Add the substrate to the wells at a steady rate. Blue color will develop in wells with positive samples.
14. Add 100 μ L Stopping Solution (0.25 M 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. Air blank or zero the plate reader. Read the O.D. of each well at 450 nm (and 650 nm reference if dual beam). The O.D. values should be measured within 5 minutes after the addition of Stopping Solution.

Results

1. Calculate the mean O.D. values for the duplicates of the Calibrator Solution dilutions, Reagent Blank, Controls and patient samples.
2. Plot the mean O.D. obtained for each Calibrator (x axis) against the corresponding Calibrator Level value (y axis) using a 4-parameter curve fit calculation.
3. Ensure that all quality control parameters have been met (see Quality Control) before reporting test results.
4. A new calibration curve should be prepared with every test run.

QUALITY CONTROL

1. The mean O.D. of the reagent blank (zero point) should be less than 0.100. Readings greater than 0.100 may indicate possible reagent contamination or inadequate plate washing.
2. The oxLDL- β_2 GPI antigen complex values obtained for the control sera should be within the ranges indicated on the container labels. Occasional small deviations outside these ranges are acceptable.
3. O.D. values for the duplicates of the controls or patient samples should be within 20% CV of the mean O.D. value for samples with absorbance readings greater than 0.200.
4. Each laboratory should periodically determine their own normal range for the appropriate population of patients.
5. Samples with oxLDL- β_2 GPI antigen complex values greater than 5 Units/mL may be reported as “greater than 5 Units/mL” or diluted and re-assayed. Correct the values obtained multiplying by the dilution factor.

LIMITATIONS OF THE TEST – FOR LABORATORY OR INVESTIGATIONAL USE ONLY

oxLDL- β_2 GPI complexes may result from oxidative stress caused by various underlying inflammatory conditions. Chronic vascular inflammation characteristic of systemic autoimmune diseases and chronic hyperglycemia of DM are known causes of oxidative stress, however, other transient conditions such as certain systemic bacterial infections may cause oxidative stress and elevated oxLDL- β_2 GPI complexes. oxLDL- β_2 GPI complexes have been detected in some patients with syphilis and infective endocarditis.

The capture monoclonal antibody used in the assay only binds β_2 GPI complexed with oxLDL. The assay does not detect native or unmodified LDL, as this form of LDL does not bind to β_2 GPI.

The assay uses Mg⁺⁺ in the Sample Diluent to dissociate electrostatically bound complexes, thus favoring the detection of stable (covalently bound) and possibly pathogenic oxLDL- β_2 GPI complexes.

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

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Symbol Legend

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

Warranty

This product is warranted to perform as described in this package insert. Corgenix, Inc. disclaims any implied warranty of merchantability or fitness for a particular use, and in no event shall Corgenix, Inc. be liable for consequential damage.

For Technical or Customer Service, phone 1-800-729-5661. Outside the United States, phone 1-303-457-4345, fax 1-303-457-4519, email techsupport@Corgenix.com, or contact a Corgenix authorized distributor.



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