Anti-AtherOx® IgG Test Kit
(anti-oxLDL-β_2GPI Antibody)

For *In Vitro* Diagnostic Use

INTENDED USE

An enzyme-linked immunoassay (ELISA) for the detection of IgG antibodies to complexes formed by oxidized low-density lipoprotein (oxLDL) with β2-glycoprotein I (β2GPI) in individuals with systemic lupus erythematosus (SLE) and lupus-like disorders (antiphospholipid syndrome).

SUMMARY AND EXPLANATION OF THE ASSAY

The antiphospholipid syndrome (APS) is one of the most common causes of acquired hypercoagulability (thrombophilia) [1,2]. It is frequently diagnosed in the context of a systemic autoimmune disorder such as SLE (secondary APS), however, it may also occur in the absence of an obvious underlying disease (primary APS) [3]. APS is characterized by high titers of antiphospholipid antibodies with thromboembolic events of venous and arterial vasculatures, or with pregnancy morbidity (miscarriages and fetal loss). High titers of antiphospholipid antibodies in secondary APS increase the risk of thrombosis by at least 2-fold [2]. In both primary and secondary APS, recurrence rates of thrombosis up to 30% and mortality up to 10% in 10 years have been reported [4,5].

Antiphospholipid antibodies are a heterogeneous family of immunoglobulins [6]. Most of these antibodies do not directly recognize phospholipids but instead recognize phospholipid-binding plasma proteins such as β_2GPI and prothrombin. β_2GPI is the most relevant antigenic target for antiphospholipid antibodies clinically associated with thrombosis [7,8]. β_2GPI-dependent anti-cardiolipin (aCL) antibodies may be detected by ELISA tests using immobilized cardiolipin (CL) in the presence of β_2GPI [9,10]. These antibodies also recognize β_2GPI immobilized on oxygenated microtiter plates but not when β_2GPI is immobilized on plain polystyrene plates [11]. These findings suggested that β_2GPI-dependent aCL antibodies recognize an altered conformation of β_2GPI when it is bound to negatively charged phospholipids.

Oxidative stress and oxLDL formation are common in patients with SLE and APS [12] suggesting an important relationship between lipid peroxidation and clotting activation (hypercoagulability). β_2GPI specifically binds to oxLDL [13,14]. OxLDL/β_2GPI complexes have been characterized [15], demonstrated in patients with APS and SLE and implicated as pro-atherothrombotic autoantigens [16]. The physiologic relevance of IgG antibodies to oxLDL/β_2GPI complexes was demonstrated in vitro by the enhanced macrophage uptake of IgG immune complexes containing oxLDL/β_2GPI. The participation of macrophage Fcγ receptors in the uptake of these complexes seems to be particularly important in the development of foam cells, atherosclerotic plaques and arterial thrombosis [13-15]. IgG anti-oxLDL/β_2GPI antibodies in autoimmune patients may further accelerate the development of atherothrombosis [17,18].

Previously, IgG anti-oxLDL/β_2GPI antibodies have been detected in SLE, systemic sclerosis (SSc) and rheumatoid arthritis (RA) patients. SLE and SSc patients had significantly higher anti-oxLDL/β_2GPI antibody levels compared to healthy controls [18-20]. Also, in those studies RA patients had higher antibody levels than the controls but this difference was not statistically significant. IgG anti-oxLDL/β_2GPI antibodies were significantly higher in SLE patient with APS compared to SLE controls without APS. Thus, the presence of circulating IgG anti-oxLDL/β2GPI antibodies seem to be etiologically important and a useful serologic marker for venous and arterial (atherothrombotic) risk in autoimmune patients [21-23].

PRINCIPLE OF THE TEST

This test is an indirect ELISA detecting IgG anti-oxLDL/β_2GPI antibodies. Diluted serum samples, calibrator(s), and controls are incubated in microwells coated with the oxLDL-β_2GPI complex. Incubation allows the IgG anti-oxLDL-β_2GPI antibody present in the samples to react with the immobilized antigen complex. After the removal of unbound serum proteins by washing, anti-human IgG antibodies, labeled with horseradish peroxidase (HRP), are added forming complexes with the bound IgG anti-oxLDL-β_2GPI 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 IgG anti-oxLDL-β_2GPI antibody.

Results are obtained by reading the OD (optical density or absorbance) of each well in a spectrophotometer. Calibrator sera are provided, with the IgG anti-oxLDL-β_2GPI antibody concentration expressed in G 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® IgG Test Kit contains the following reagents:
(**volumes may vary depending on kit size and configuration**):

- 12x8 stabilized oxLDL-β_2GPI antigen (human) coated microwells, with frame.
- 2 bottles (60 mL) Sample Diluent* (green solution).
- 3 vials (0.250 mL) IgG Calibrator Sera* (human) (1-high, 2-moderate, 3-low) - refer to vial label for the assigned value in G Units.
- 1 vial (0.250 mL) IgG Positive Control Serum* (human) - refer to vial label for expected range in G Units.
- 1 vial (0.250 mL) IgG Normal Control Serum* (human) - refer to vial label for expected range in G Units.
- 1 bottle (15 mL) IgG anti-human (goat) HRP-Conjugated Antibody Solution (blue 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 In Vitro Diagnostic Use

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, HIV I & 2 and Syphilis 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. Wear disposable gloves while handling kit reagents and wash hands thoroughly afterwards.
5. 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.
6. 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.
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, flush affected areas with copious amounts of water and seek medical advice (S 26). Wear suitable protective clothing (S 36). If swallowed, seek medical advice immediately and show this 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℃. If specimens are to be stored for more than 72 hours, they should be frozen at –20℃ 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:

IgG Anti-AtherOx® 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. Careful 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 IgG anti-human HRP-conjugated antibody solution (blue) to the wells corresponding to the IgG calibrator(s), controls, patient samples, and reagent blank.
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 G Units) on the y-axis against the log of the OD's for each calibrator on the x-axis.
3. The calibration 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 0.150 when the spectrophotometer has been properly blanked against air. Blank readings greater than 0.150 may indicate possible reagent contamination or inadequate plate washing.
3. The IgG anti-OxLDL-β_2GPI 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 G Units". Alternatively, the sample may be additionally diluted 1:10 and re-run in the assay. The resulting value in G Units would then be multiplied by a factor of 10.

NORMAL RANGE

Serum samples from 75 healthy blood donors were tested using the IgG anti-AtherOx® Test Kit. The mean IgG anti-oxLDL-β_2GPI antibody value of this population was determined to be 8.8 G Units, with a standard deviation of 3.0 G Units. The 95th percentile was 13.8 G Units and the mean + 3SD was 17.7 G Units. A normal range of 0-20 G Units was established based on both calculations from the normal population.

IgG anti-OXLDL-B_2GPI antibody normal range = 0–20 G Units

PERFORMANCE CHARACTERISTICS

Testing of Healthy Normal Patients

Serum samples from 205 healthy individuals were tested on the IgG Anti-AtherOx® Test Kit and the REAADS IgG Anti-Cardiolipin Test. The mean IgG anti-oxLDL-β_2GPI antibody value of this population was determined to be 11.7 G Units. Relative to the established normal range of 0–20 G Units, 6 serum samples were found to be positive.

Healthy Controls		REAADS IgG Anti-Cardiolipin Pos	REAADS IgG Anti-Cardiolipin Neg
Corgenix IgG Anti-AtherOx®	Pos	0	6
	Neg	0	199

Positive Percent Agreement = N/A

Negative Percent Agreement = 97.1%
(95% CI = 94.8 – 99.4%)

Overall % Agreement = 97.1%
(95% CI = 94.8 – 99.4%)

Clinical sensitivity

Autoimmune patients

Serum samples from 143 SLE patients were tested on the IgG Anti-AtherOx® Test Kit and the REAADS IgG Anti-Cardiolipin Test. The mean IgG anti-oxLDL-β_2GPI antibody level of this group was determined to be 24.4 G Units. Relative to the established normal range of 0–20 G Units, 35 serum samples were positive.

SLE		REAADS IgG Anti-Cardiolipin Pos	REAADS IgG Anti-Cardiolipin Neg
Corgenix IgG Anti-AtherOx®	Pos	19	16
	Neg	6	102

Positive Percent Agreement = 76.0%
(95% CI = 59.3 – 92.7%)

Negative Percent Agreement = 86.4%
(95% CI = 80.3 – 92.6%)

Overall % Agreement = 84.6%
(95% CI = 78.7 – 90.5%)

Of these 143 SLE patients, 94 patients were diagnosed as having secondary APS. Sera from this 94-patient cohort were tested on the Corgenix IgG Anti-AtherOx® Test. The mean IgG anti-oxLDL-β_2GPI antibody level of this population was 28.8 G Units. Relative to the established normal range of 0–20 G Units, 25 serum samples were positive. A matched group of 19 SLE patients without APS (controls) had a mean value of 12.0 G Units, with one sample being above the 20-G unit cutoff.

The APS patients were then further divided according to their clinical history of APS manifestations: arterial thrombosis, venous thrombosis or pregnancy morbidity. Results, including the SLE controls are summarized in the following table:

APS Clinical History	Number of patient samples	Mean IgG anti-oxLDL-β_2GPI antibody value of sample	Number (%) of positive samples	Number (%) of negative samples
Arterial Thrombosis	42	30.7	12 (28.6%)	30 (71.4%)
Venous Thrombosis	37	32.6	12 (32.4%)	25 (67.6%)
Pregnancy Morbidity	15	14.0	1 (6.7%)	14 (93.3%)
Controls (SLE only)	19	12.0	1 (5.3%)	18 (94.7%)

Pregnancy Morbidity		REAADS IgG Anti-Cardiolipin Pos	REAADS IgG Anti-Cardiolipin Neg
Corgenix IgG Anti-AtherOx®	Pos	0	1
	Neg	2	12

Positive Percent Agreement = 0%

Negative Percent Agreement = 92.3%
(95% CI = 77.8 – 100%)

Overall % Agreement = 80.0%
(95% CI = 59.8 – 100%)

Arterial Thrombosis		REAADS IgG Anti-Cardiolipin Pos	REAADS IgG Anti-Cardiolipin Neg
Corgenix IgG Anti-AtherOx®	Pos	11	1
	Neg	3	27

Positive Percent Agreement = 78.6%
(95% CI = 57.1 -100%)

Negative Percent Agreement = 96.4%
(95% CI = 89.6 – 100%)

Overall % Agreement = 90.5%
(95% CI = 84.7 – 100%)

Venous Thrombosis		REAADS IgG Anti-Cardiolipin Pos	REAADS IgG Anti-Cardiolipin Neg
Corgenix IgG Anti-AtherOx®	Pos	6	6
	Neg	1	24

Positive Percent Agreement = 85.7%
(95% CI = 59.8 – 100%)

Negative Percent Agreement = 80.0%
(95% CI = 65.7 – 94.3%)

Overall % Agreement = 81.1%
(95% CI = 68.5 – 93.7%)

In addition, serum samples from 99 rheumatoid arthritis patients were tested on the IgG Anti-AtherOx® Test Kit and the REAADS IgG Anti-Cardiolipin Test. The mean IgG value of this population was determined to be 16.4 G Units. Relative to the established normal range of 0–20 G Units, 18 serum samples were positive.

Rheumatoid Arthritis		REAADS IgG Anti-Cardiolipin Pos	REAADS IgG Anti-Cardiolipin Neg
Corgenix IgG Anti-AtherOx®	Pos	1	17
	Neg	0	81

Positive Percent Agreement = 100%

Negative Percent Agreement = 82.7%
(95% CI = 75.2 – 90.2%)

Overall % Agreement = 82.8%
(95% CI = 75.4 – 90.3%)

Precision

The precision of the Corgenix IgG Anti-AtherOx® Test Kit was assessed as specified in CLSI EP5-A2 Evaluation of Precision Performance of Quantitative Measurement Methods. Serum samples with concentrations spanning the range of the assay were tested in the Corgenix laboratory by 2 operators in duplicate on each of 20 days over 30 calendar days. One reagent lot was tested, and assays were calibrated each day.

Sample	**Mean Concentration (G Units)**	**Reproducibility as %CV**	**Within-Laboratory Precision as %CV**
Low	9.6	3%	13%
Med-Low	20.2	4%	12%
Med	28.1	7%	13%
Med-High	38.4	5%	12%
High	76.8	4%	10%

Limit of Blank (LOB)/Limit of Detection (LOD)

Limit of Blank and Limit of Detection were assessed according to CLSI Guideline EP17-A *Protocols for Determination of Limits of Quantitation; Approved Guideline*. The Limit of Blank (LoB) was defined nonparametrically.at the 95th percentile of the negative results at 6.1 G units. The Limit of Detection (LoD) was defined the lowest level where 5% or fewer of the observed measurements are below the LoB. Since none of the results for the positive data set were at 7.0 G units or below, so the LoD was set as 7 G units.

Linearity

For IgG anti-oxLDL-β_2GPI antibody measurements using the Corgenix IgG Anti-AtherOx® Test Kit, the method has been demonstrated to be linear from 10 - 100 G units with less than $\pm$ 5 G units error within this interval.

Interference

The materials in the following list were tested for their potential to interfere in measurements with the Anti-AtherOx® Test Kit by adding the materials to five clinical samples with a range of IgG anti-oxLDL-β_2GPI antibody concentrations up to the levels listed in the table.

Hemoglobin	5 mg/mL
Triglycerides	30 mg/mL
Conjugated Bilirubin	0.2 mg/mL
Rheumatoid Factor	500 units/mL

The results of these interference studies indicated that the presence of hemoglobin, conjugated bilirubin, triglycerides, and rheumatoid factor (RF) had no effect on the IgG Anti-AtherOx® test kit results.

LIMITATIONS OF THE TEST

IgG anti-oxLDL-β_2GPI antibody levels obtained with this assay may be used to assess the risk for the development of vascular thrombotic events in patients with systemic autoimmune disorders and APS. Each physician must interpret these results in light of the patient's history, physical findings and other diagnostic procedures.

IgG anti-oxLDL-β_2GPI antibodies may be produced when oxidative stress caused by various underlying inflammatory conditions form oxLDL-β_2GPI complexes. For example, oxLDL-β2GPI complexes may be present in other autoimmune diseases such as Rheumatoid Arthritis and Systemic Sclerosis. Also, other transient conditions such as certain bacterial infections may cause oxidative stress, formation of oxLDL-β_2GPI complexes, and IgG antibodies to these complexes. The exact clinical significance of these IgG anti-oxLDL-β_2GPI antibodies is under further investigation.

While the presence of Rheumatoid Factor (RF) in patients' samples was found not to interfere with assay performance, excess RF may interfere with ELISA methods by binding to antibodies. The presence of RF should be considered when evaluating results.

Though the presence of excess hemoglobin, lipids, and/or bilirubin was found not to affect assay performance, testing of grossly hemolyzed, lipemic or icteric samples is not recommended as these substances may interfere with the results of the assay.

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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SYMBOL LEGEND

	IVD	LOT			Xi		REF	
Manufacturer	In vitro diagnostic medical device	Batch Code	Use by/ Expiry Date	Temperature Limitation	Irritant	Biological Risk	Catalog Number	Consult Instructions for Use/ Package Insert
Hersteller	In-vitro-Diagnostikum	Chargennummer	Verfallsdatum	Temperatur-beschränkungen	Reizend	Biologisches Risiko	Katalognummer	Gebrauchsanweisung im Inneren der Verpackung beachten
Fabriqué par	Dispositif de diagnostic in vitro	Code de Lot	Utiliser jusqu' à/ Date de péremption	Limites de température	Irritant	Risque biologique	Numéro de catalogue	Consulter le mode d'emploi/ notice jointe au conditionnement
Fabricado por	Dispositivo médico para diagnóstico in vitro	Código de Lote	Usar antes de/ Fecha de caducidad	Limitación de temperatura	Irritante	Riesgo biológico	Número de catálogo	Consultar las instrucciones de uso/ prospecto del envase
Prodotta da	Dispositivo medico-diagnostico in vitro	Codice del lotto	Scade il/ data di scadenza	Limite di temperatura	Irritante	Rischio biologico	Numero di catalogo	Consultare le istruzioni per l'uso/ il foglietto illustrativo

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