Anti-β_2-Glycoprotein I IgG/IgM Enzyme immunoassay

Laboratory Name		
Adopted		
Reviewed		
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Revised		
Supercedes		

Method: **Diamedix Corp., Immunosimplicity®**
Manual or in conjunction with one of the Diamedix Automated EIA Systems such as the MAGO Plus, the DSX, or the DS2. For *In Vitro* Diagnostic Use.

Clinical Significance

Anti-phospholipid antibodies are autoantibodies that react with most negatively-charged phospholipids including cardiolipin. Autoantibodies directed against phospholipids, and anti-cardiolipin in particular, have been associated with recurrent venous and arterial thrombosis, thrombocytopenia and spontaneous abortions. The term 'anti-phospholipid syndrome' is used to describe patients with these clinical manifestations. Autoantibodies to cardiolipin are described in many autoimmune diseases. They are frequently found in patients with SLE, in patients with other autoimmune diseases as well as in some individuals with no apparent underlying disease (1,2,3,4,5).

Recently, β2-glycoprotein I has become well established as a co-factor required for the binding of cardiolipin antibodies (6). β2-glycoprotein I, also known as apolipoprotein H, is a 50 kDa β2-globulin occurring in plasma at a level of 200 ug/ml. It has been found that β2-glycoprotein I inhibits the intrinsic coagulation pathway and thus is involved in the regulation of blood coagulation (7).

Detailed investigations about the nature of the cardiolipin-β2-glycoprotein I complex have shown that epitopes on the fifth domain of β2-glycoprotein I are the real target of the anti-cardiolipin antibodies. β2-glycoprotein I is not only a pre-requisite for the binding of anti-cardiolipin antibodies; it has now been identified as the primary antigen for these antibodies. In addition, ELISA tests for β2-glycoprotein I antibodies appear to show better overall specificity since cross-reactivity with other phospholipids, e.g. in syphilis positive samples, is eliminated (8,9,10,11).

Anti-β2-glycoprotein I antibodies are found in the immunoglobulin classes IgG, IgM and/or IgA. The Diamedix immunosimplicity (Is)Anti-β2-Glycoprotein I IgG/IgM Test Kit is an enzyme immunoassay intended to measure IgG and/or IgM antibodies to β2-glycoprotein I in human serum. The β2-glycoprotein I antibody test is a useful and specific assay to be used in conjunction with

the traditionally used anti-cardiolipin kits for aiding in the diagnosis of thrombosis in at-risk patients. The test can be performed manually or in conjunction with one of the Diamedix Automated EIA Systems.

Principle of the Procedure

Highly purified human β_2-glycoprotein I is bound to microwells. Diluted patient sera, Standards and Controls are placed in the microwells and incubated. Anti-β_2-glycoprotein I antibodies, if present, will bind to the antigen forming antigen-antibody complexes. Residual sample is eliminated by aspirating and washing. Conjugate (horseradish peroxidase-labeled anti-human IgG or IgM) is added and will bind to these complexes. Unbound conjugate is removed by aspirating and washing. Substrate is then added and incubated. In the presence of bound enzyme, the substrate is converted to a colored end product. Stop solution is added and the absorbance of this end product is then read spectrophotometrically at 450 nm (reference 600-630 nm) and is directly proportional to the concentration of IgG or IgM antibodies to β_2-glycoprotein I present in the sample.

Specimen Collection

Whole blood should be collected by accepted medical techniques. Separated serum should remain at 22°C for no longer than 8 hours. If assays are not completed within 8 hours, serum should be refrigerated (2-8°C). If assays are not completed within 48 hours, or the separated sample is to be stored beyond 48 hours, samples should be frozen at -20°C. Avoid multiple freeze-thaw cycles. Prior to testing, bring frozen sera to room temperature slowly and mix gently, avoiding foam formation. Specimens containing visible particulate matter should be clarified by centrifugation before testing. Grossly contaminated, hemolyzed, lipemic, or icteric specimens should not be used. The NCCLS provides recommendations for collecting and storing blood specimens (12).

CAUTION: Serum samples must not be heat-inactivated prior to use.

Reagents

Each anti-β_2-Glycoprotein Screen Test Kit contains reagents for 96 tests.

Antigen Wells	Twelve, 8-well microwell breakapart strips, color-coded red, coated with highly purified human β_2-glycoprotein I.
Standard A(0 U/ml)	One vial with yellow cap containing 1.8 ml of prediluted human serum or defibrinated plasma in a PBS/BSA matrix. The assigned value is printed on the label.
Standard B(6.3 U/ml)	One vial with green cap containing 1.8 ml of combined IgG and IgM Standard composed of prediluted human serum or defibrinated plasma in a PBS/BSA matrix. The assigned value is printed on the label.

Standard C(12.5 U/ml)   One vial with brown cap containing 1.8 ml of combined
                         IgG and IgM Standard composed of prediluted human serum
                         or defibrinated plasma in a PBS/BSA matrix. The
                         assigned value is printed on the label.

Standard D(25 U/ml)   One vial with purple cap containing 1.8 ml of combined
                         IgG and IgM Standard composed of prediluted human serum
                         or defibrinated plasma in a PBS/BSA matrix. The
                         assigned value is printed on the label.

Standard E(50 U/ml)   One vial with white cap containing 1.8 ml of combined
                         IgG and IgM Standard composed of prediluted human serum
                         or defibrinated plasma in a PBS/BSA matrix. The
                         assigned value is printed on the label.

Standard F(100 U/ml)   One vial with red cap containing 1.8 ml of combined
                         IgG and IgM Standard composed of prediluted human serum
                         or defibrinated plasma in a PBS/BSA matrix. The
                         assigned value is printed on the label.

Negative Control   One vial with black cap containing 1.8 ml of pre-
                         diluted human serum or defibrinated plasma in a
                         PBS/BSA matrix, negative for β_2-glycoprotein I IgG and
                         IgM antibodies. The assigned value is printed on the
                         label.

Positive Control   One vial with blue cap containing 1.8 ml of pre-
                         diluted human serum or defibrinated plasma in a PBS/BSA
                         matrix, moderately reactive for β_2-glycoprotein I IgG
                         and IgM antibodies. The assigned range is printed on
                         the label.

Sample **F** Diluent   One bottle with blue cap containing 60 ml phosphate
                         buffer with protein stabilizers. Color-coded blue.

Wash **X** Concentrate   Two bottles with clear caps containing 20 ml. Each
    (50X)   bottle is sufficient to make 1 liter of wash solution.

IgG Conjugate   One bottle with red cap containing 25 ml rabbit anti-
                         human IgG labeled with horseradish peroxidase, diluted
                         in a PBS/BSA matrix. Color-coded pink.

IgM Conjugate   One bottle with red cap containing 25 ml rabbit anti-
                         human IgM labeled with horseradish peroxidase, diluted
                         in a PBS/BSA matrix. Color-coded pink.

Substrate **H**   One amber bottle with brown cap containing 25 ml
                         buffered TMB solution (3,3',5,5'
                         tetramethylbenzidine). The substrate solution may
                         develop a slight blue color upon storage.

Stop **P** Solution   One bottle with white cap containing 30 ml 1M
                             Hydrochloric acid. **CAUTION:** Solution is corrosive.
                             Avoid contact with skin or eyes. If contact is made,
                             flush area with copious amounts of water.

Other Materials Required   Store these reagents at 2 to 8° C.

Manual Users:
1.   Wash bottle or automated microplate washer
2.   Pipettors capable of dispensing appropriate volumes
3.   Timer
4.   One liter graduated cylinder
5.   One liter wash solution reservoir
6.   Deionized or distilled water
7.   Absorbent toweling
8.   Tubes or microwell plate for sample dilution
9.   Reader capable of reading absorbance at 450nm, reference at 600-
      630 nm

Diamedix Automated EIA System Users:
1.   One liter graduated container
2.   Deionized or distilled water
3.   Dilution containers as appropriate to system
4.   Sample and Reagent tips required by system
5.   Reagent containers required by system

Warnings and Precautions:

   REAGENTS: For in vitro Diagnostic Use

1. Handle samples, standards, controls and the materials that contact them as
   potential biohazards. Each donor unit in the Calibrator and controls has
   been found negative for Hepatitis B surface antigen and HIV-1 and –2
   antibodies by FDA-approved third generation tests. However, because no
   method can offer complete assurance that HIV-1 and -2, Hepatitis B virus, or
   Hepatitis C virus, or other infectious agents are absent, these materials
   should be handled at the Biosafety Level 2 as recommended for any potentially
   infectious serum or blood specimen in the Centers for Disease
   Control/National Institutes of Health Manual, "Biosafety in Microbiological
   and Biomedical Laboratories", 1993.
2. The concentrations of anti-β_2-glycoprotein I IgG and IgM antibodies in a
   given specimen determined with assays from different manufacturers can vary
   due to differences in assay methods and reagent specificity.
3. Never pipette by mouth.
4. Avoid contact with open skin and mucous membranes.
5. Certain components contain sodium azide as preservative. Azides are reported
   to react with lead and copper in plumbing to form compounds that may become
   explosive. When disposing of solutions containing sodium azide, flush with
   copious amounts of water to minimize the build up of metal azide compounds.
6. Sodium azide inhibits horseradish peroxidase activity. Care must be taken to
   ensure that azide is not carried over from other reagents into conjugate and
   substrate steps.

> 7. Avoid contamination of the TMB substrate solution with conjugate or other oxidants which will cause the solution to change color prematurely.
> 8. The substrate contains 3,3' 5,5' Tetramethylbenzidine (TMB) which has shown possible mutagenic effects in laboratory experiments.

Calibration

This test uses Standards that are traceable to in-house reference Standards. The Highest Standard (Std F) has been assigned a value of 100 U/ml. The other Standards have been assigned values of: Std E(50 U/ml), Std D(25 U/ml), Std C(12.5 U/ml), Std B(6.3 U/ml), Std A(0 U/ml) respectively. Semi-quantitative results may be obtained from the point to point curve fit or 4-parameter logistic curve fit using all six Standards or from the point to point curve fit using three Standards (A,C and F). Samples with values $\geq$ 12 U/ml are considered positive for IgG antibodies to β_2-glycoprotein I; or sample values $\geq$ 7 U/ml are considered positive for IgM. Samples whose values are < 10 are considered negative for IgG antibodies to β_2-glycoprotein I and sample values < 5 U/ml are considered negative for IgM. To account for the inherent variations in enzyme immunoassays equivocal ranges of from 10 to 11.9 U/ml (for IgG), and from 5.0 to 6.9 U/ml (for IgM) have been included just below the assay cut-off.

Quality Control

(a)   The Positive and Negative Controls must be included in each test run and must be within their assigned ranges.

(b)   The absorbance of Standard A (0 U/ml) must be < 0.200.

(c)   The absorbance of Standard F(100 U/ml) must be greater than 3 times the absorbance of Standard C (12.5 U/ml).

(d)   The absorbance of Standard C (12.5 U/ml) must be greater than the absorbance of Standard B (6.3 U/ml).

(e)   The absorbance of Standard D(25 U/ml) must be greater than the absorbance of Standard C(12.5 U/ml).

(f)   The absorbance of Standard E (50 U/ml) must be greater than the absorbance of Standard D (25 U/ml).

If any of these criteria is not met, the results are invalid and the test should be repeated.
(For 3-point calibration, d, e and f do not apply).

Note: Additional controls may be tested according to guidelines or requirements of local, state, or federal regulations or accrediting organizations. For guidance on appropriate Quality Control practices please refer to NCCLS C24-A, Internal Quality Control Testing: Principles and Definitions.

Procedure

Allow all test components and patient samples to warm to room temperature before use. Invert reagent bottles gently several times before use. Return promptly to the refrigerator after use.

Prepare Wash Solution by adding 20 ml of Wash Concentrate (50X) to one liter with deionized or distilled H_2O.

Manual Users:

The Standards and Controls are provided ready to use: **DO NOT DILUTE FURTHER.**

The assays can be performed either using all six Standards and a 6-point Calibration system or by using three Standards, namely Stds. A, C and F, and a 3-point Calibration system. Positive and Negative Controls must be run for either assay option.

1. Prepare 1:101 dilutions of the patient samples in Sample Diluent. (e.g., by addition of 5 µl sample to 500 µl Sample Diluent).

2. Mix sample dilutions gently by withdrawing and expelling in a pipette tip 2 or 3 times or by vortex mixing for 2 or 3 seconds. Transfer 100 µl of controls and diluted patient samples, to the antigen wells. Avoid formation of bubbles when transferring diluted samples.

3. Allow the wells to incubate at room temperature (18-30°C) for 30 ± 5 minutes.

4. Aspirate or discard the contents of the wells. Remove excess moisture in the wells by tapping on paper toweling. Wash the wells by rinsing 3 times with at least 300 µl of Wash Solution. Remove excess moisture from the wells after washing. When using an automated washer, follow the manufacturer's instructions.

5. Place 100 µl of Conjugate into each well, avoiding bubble formation.

6. Allow the wells to incubate uncovered at room temperature (18-30°C) for 30 ± 5 minutes.

7. Wash the wells as described in Step 4 above.

8. Place 100 µl of Substrate into each well, avoiding bubble formation.

9. Allow the wells to incubate uncovered at room temperature (18-30°C) for 30 ± 5 minutes.

10. Place 100 µl of Stop Solution into each well, avoiding bubble formation.

11. Mix well contents thoroughly.

12. Read the absorbance of each well at 450 nm. A suitable reference wavelength (e.g., 600-630 nm) should be used.

NOTE: The developed color is stable for 30 minutes. Read the absorbances during this time.

Diamedix Automated EIA System Users:

When using one of Diamedix's Automated EIA Systems, please refer to the corresponding Operating Manual for the test setup, procedure, and accessories/consumables needed.

Calculation of Results

Semi-quantitative results may be obtained from the point to point or 4-parameter logistic curve fit using all six Standards or from the point to point curve fit using three Standards (A, C and F). The Diamedix Automated EIA Systems will calculate and print results automatically for either assay option.

Reference Ranges

The following is only a guide to interpretation. Each laboratory can establish its own "normal" ranges based on populations encountered.

IgG U/ml Value	Interpretation
< 10.0	Negative, no detectable IgG antibodies to β_2-glycoprotein I.
$\geq$ 12.0	Positive, IgG antibody to β_2-glycoprotein I detected.
10.0 to 11.9	**Equivocal for IgG antibodies to β_2-glycoprotein I.

IgM U/ml Value	Interpretation
< 5.0	Negative, no detectable IgM antibodies to β_2-glycoprotein I.
$\geq$ 7.0	Positive, IgM antibody to β_2-glycoprotein I detected.
5.0 to 6.9	**Equivocal for IgM antibodies to β_2-glycoprotein I.

** Equivocal samples can be retested by this method, tested by another method or a new sample tested.

Samples which yield absorbances greater than that of Standard F (100 U/ml) may be reported as 'greater than 100 U/ml'. Alternatively, such samples may be pre-diluted in Sample Diluent and retested. The resulting U/ml value must be multiplied by the dilution factor for reporting.

Example: If the specimen was pre-diluted 1:5 before testing, the resulting U/ml should be multiplied by 5.

Procedure Notes

1. Do not interchange reagents from different reagent lots except for Sample **F** Diluent, Wash **X** Concentrate, Substrate **H** and Stop **P** Solution.

2. Do not use reagents beyond their expiration date.

3. Store unused reagents at 2 to 8°C.

4. Incubations above or below the recommended temperatures or times may give erroneous results.

5. The EIA method is a very sensitive technique. Maintain consistent pipetting technique, incubation times and temperature conditions throughout the test procedure. Cross contamination between reagents can invalidate the test.

6. Coated microwells should be stored with the desiccant in the resealable bag provided and returned to the refrigerator immediately after use.

7. *(Manual Procedure Only)* The washing procedure is very important and requires special attention. (Please refer to the Procedure section)

   NOTE: *Improperly washed* wells *may* give *erroneous results.*

Limitations

1. The results obtained with the *Is*-anti-β_2-Glycoprotein I IgG/IgM Kit serve only as an aid to diagnosis and should not be interpreted as diagnostic in themselves. Results must be interpreted in conjunction with the patient history, clinical symptoms, physical findings as well as other diagnostic procedures.

2. The clinical significance of elevated anti-β_2-glycoprotein I antibody levels in diseases other than SLE is still under investigation.

3. When a normal anti-β_2-glycoprotein I antibody level is found in the presence of clinical manifestations, a lupus anti-coagulant or other additional testing is indicated.

4. Some samples may be positive for anti-cardiolipin antibodies and negative for anti-β_2-glycoprotein I antibodies. The Is-anti-β_2-Glycoprotein I IgG/IgM Test Kit is a more specific marker of thrombotic risk. Anti-cardiolipin tests can produce false positive results due to cross-reactions with dsDNA or certain infectious disease antibodies.

5. Assay performance characteristics have not been established for visual result determination or for spectrophotometry using a single wavelength.

6. The test should be performed on serum. The use of whole blood or plasma has not been established.

7. Performance characteristics of the Diamedix *Is*-anti-β_2-Glycoprotein I IgG/IgM Test Kit with automated equipment other than Diamedix Automated EIA Systems have not been established.

References

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