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For Individual Laboratory to Complete:

**Anti-Gliadin
IgG
Enzyme Immunoassay**

Laboratory Name		
Adopted		
Reviewed		
Reviewed		
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

Celiac disease is a gluten enteropathy occurring in both adults and children. The condition is characterized by a sensitivity to gluten that results in inflammation and atrophy of the mucosa of the small intestine. Clinical manifestations include malabsorption with symptoms of diarrhea, steatorrhea and nutritional and vitamin deficiencies. Secondary immunologic illnesses, such as atopic dermatitis, dermatitis herpetiformis, alopecia and aphthous ulcers may be the primary presentation. It is thought that the prevalence of celiac disease in the United States of approximately 1:250 may be greatly underestimated and underdiagnosed. In addition, the incidence of celiac disease in relatives of celiac patients is significantly greater than the incidence in the control population. Untreated celiac disease can significantly increase the risk of gastrointestinal cancer as well as being implicated in thyroid disease and several illnesses (1-6).

The diagnosis of celiac disease can be difficult considering the broad range of symptoms. Recently serological testing has been increasingly used to test patients with suspected gluten-sensitive enteropathy as well as for monitoring dietary compliance. Both IgG and IgA antibodies are detected in sera of patients with gluten-sensitive enteropathy. IgA antibodies are less sensitive but more specific markers of the disease and their measurement is useful in following disease activity and monitoring maintenance of a gluten-free diet. IgG antibodies appear to be more sensitive but less specific markers of disease than IgA. It is recommended that both antibodies should be measured due to the high incidence of IgA deficiency among celiac patients which may mask the disease. Antibody testing is also important in detecting individuals who are at risk for having celiac disease but have no symptomology, in individuals with atypical symptoms or extraintestinal manifestations of celiac disease and in individuals with presumed celiac disease who fail to respond to a gluten-free diet. Patients with positive antibody tests must undergo small intestine biopsy to confirm the diagnosis and assess the degree of mucosal involvement. Treatment for celiac disease is a strict gluten-free diet which leads to a complete resolution of symptoms in most patients. After a gluten-free diet IgA anti-gliadin antibody levels become undetectable. However, the time to achieve remission can vary (1-6).

The immunosimplicity Is-anti-Gliadin IgA test and the immunosimplicity Is-anti-Gliadin IgG test are EIA procedures for the semi-quantitative detection of gliadin antibodies. Both tests can be used either manually or in conjunction with one of the Diamedix Automated EIA Systems.

Principle of the Procedure

Purified gliadin antigen is bound to microwells. Diluted patient sera, standards, and controls are placed in the microwells and incubated. Anti-gliadin 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) 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 an end product. The absorbance of this end product can be read spectrophotometrically at 450 nm (reference 600-630 nm) and is directly proportional to the concentration of IgG antibodies to gliadin 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.
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Reagents

Each Is-anti-Gliadin IgG Test Kit contains reagents for 96 tests.

Antigen Wells	Twelve, 8-well microwell breakapart strips, color-coded dark green, coated with purified gliadin.
Standard A (0 U/ml)	One vial with yellow cap containing 1.8 ml of pre-diluted human serum or defibrinated plasma. Color-coded light yellow. The assigned value is printed on the label.
Standard B (25 U/ml)	One vial with green cap containing 1.8 ml of pre-diluted human serum or defibrinated plasma. Color-coded light pink. The assigned value is printed on the label.
Standard C (50 U/ml)	One vial with brown cap containing 1.8 ml of pre-diluted human serum or defibrinated plasma. Color-coded pink. The assigned value is printed on the label.

Standard D (100 U/ml)	One vial with purple cap containing 1.8 ml of pre-diluted human serum or defibrinated plasma. Color-coded light purple. The assigned value is printed on the label.
Standard E (200 U/ml)	One vial with white cap containing 1.8 ml of pre-diluted human serum or defibrinated plasma. Color-coded mid-purple. The assigned value is printed on the label.
Standard F (400 U/ml)	One vial with red cap containing 1.8 ml of pre-diluted human serum or defibrinated plasma. Color-coded dark purple. 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. Color-coded green. The assigned range is printed on the label.
Positive Control	One vial with blue cap containing 1.8 ml of pre-diluted human serum or defibrinated plasma. Color-coded blue. The assigned range is printed on the label.
Sample Diluent	One bottle with a blue cap containing 60 ml Tris buffer with protein stabilizers and preservative. Color-coded blue.
Wash Concentrate (20X)	Two bottles with clear caps containing 50 ml of Tris buffer with detergent and preservative. Each bottle is sufficient to make 1 liter of wash solution. of water.
Conjugate	One bottle with a red cap containing 25 ml rabbit anti-human immunoglobulin G labeled with horseradish peroxidase and preservative. Color-coded pink.
Substrate	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 N Solution	One bottle with white cap containing 30 ml of 1N Sulfuric acid. CAUTION: Solution is corrosive. Avoid contact with skin or eyes. If contact is made, flush area with copious amounts of water.

<p>Store these reagents at 2 to 8° C.</p>
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Other Materials Required

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. Never pipette by mouth.
3. Avoid contact with open skin and mucous membranes.
4. 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.
5. Sodium azide inhibits horseradish peroxidase activity. Care must be taken to ensure that azide is not carried over from other reagents into immunocomplex and substrate steps.
6. Avoid contamination of the TMB substrate solution with conjugate or other oxidants which will cause the solution to change color prematurely.
7. 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 internal Standard reference sera. The Highest Standard (Std F) has been assigned a value of 400 U/ml. The other Standards have been assigned values of: Std E(200 U/ml), Std D(100 U/ml), Std C(50 U/ml), Std B(25 U/ml), Std A(0 U/ml). 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 > 55 U/ml are considered positive for IgG antibodies to gliadin and samples with values < 45 U/ml are considered negative for IgG antibodies to gliadin. To account for the inherent variations in enzyme immunoassays an equivocal range of from 45 to 55 U/ml has 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 < 0.100.
- (c) The absorbance of Standard F(400 U/ml) must be greater than 2.5 times the absorbance of Standard C(50 U/ml).
- (d) The absorbance of Standard F(400 U/ml) must be higher than the absorbance of the Positive Control.
- (e) The absorbance of Standard C(50 U/ml) must be lower than the absorbance of the Positive Control.
- (f) The absorbance of Standard C(50 U/ml) must be greater than the absorbance of Standard B (25 U/ml).
- (g) The absorbance of Standard D(100 U/ml) must be greater than the absorbance of Standard C(50 U/ml).
- (h) The absorbance of Standard E(200 U/ml) must be greater than the absorbance of Standard D(100 U/ml).

If any of these criteria is not met, the results are invalid and the test should be repeated.

(For 3-point calibration, f, g and h 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 50 ml of Wash Concentrate (20X) to one liter with deionized or distilled H₂O.

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. A Standard curve must be generated each time the assay is run.

1. Prepare 1:51 dilutions of the patient samples in Sample Diluent. (e.g., by addition of 10 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 Standards (three or six), 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 if necessary. 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 of 600-630 nm reading 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 and procedures.

Calculation of Results

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). 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.

U/ml Value

< 45	Negative, anti-gliadin IgG antibodies below clinical threshold
45-55	**Equivocal for presence of anti-gliadin IgG antibodies. Samples should be retested. If retest results are equivocal, the sample should be reported as equivocal, tested by another method or a new sample should be tested.
> 55	Positive anti-gliadin IgG antibodies detected.

** Equivocal samples that give positive results on retest should be reported as positive. Equivocal samples that give negative results on retest should be reported as negative.

Samples which yield absorbances greater than that of Standard F (400 U/ml) may be reported as 'greater than 400 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. Sample Diluent, Wash Concentrate and Substrate from anti-Gliadin Kits may be interchanged. With the exception of Stop **N** Solution, do not interchange reagents from other Diamedix Is assays.
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.*
8. The reported concentration of anti-gliadin IgG in a given specimen determined with assays from different manufacturers can vary due to differences in assay methods and reagent specificity.

Limitations

1. The results obtained with the Is-anti-Gliadin IgG Test Kit serve only as an aid to diagnosis and should not be interpreted as diagnostic in themselves.
2. Assay performance characteristics have not been established for visual result determination or for spectrophotometry using a single wavelength.
3. The test should be performed on serum. The use of whole blood or plasma has not been established.
4. Performance characteristics of the Is anti-Gliadin IgG Test Kit with automated equipment other than the MAGO[®] Plus Automated EIA Processor have not been established.
5. False positives can occur as other gastrointestinal disorders, such as Crohn's disease and food protein intolerance, may induce circulating antibodies to gliadin.
6. Gliadin IgA negative results in untreated patients does not rule out gluten-sensitive enteropathy when associated with high levels of gliadin IgG antibodies. The finding can often be explained by selective IgA deficiencies, a relatively frequent finding in celiac disease.
7. Values for the pediatric population have not been established with this assay.

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