

INTENDED USE

The ZEUS ELISA Gliadin IgA Test System is intended for the qualitative and semi-quantitative detection of IgA-class antibodies to gliadin in human serum. This test is intended to be used as an aid in the diagnosis of gastrointestinal disorders, mainly Celiac Disease. This test is for *In Vitro* diagnostic use.

SIGNIFICANCE AND BACKGROUND

Celiac disease is an inflammatory disorder of the small intestine. The prolamines of certain cereals, mainly the gliadins of wheat, induce Celiac disease. This permanent intolerance to gliadin results in intestinal villous flattening and crypt hyperplasia in susceptible individuals. Immune reactions to gliadin are likely to play a role in the pathogenesis of the disease since research demonstrates that both humoral and cell-mediated responses occur in the peripheral blood and in the gut of celiac patients (1).

Classic signs of Celiac Disease in adults include, malabsorption characterized by weight loss, abdominal distension, diarrhea, and steatorrhea (which occurs because of the loss of absorptive area and the immaturity of surface epithelial cells). By the early 1980s, the clinical features of Celiac Disease had changed (2, 3). A shift towards milder symptoms, such as indigestion in adults and recurrent abdominal pain in children, occurred and made the classic symptoms and signs of Celiac Disease a rarity. Despite the manifestation of mucosal lesions, the disease can even be symptom-free and clinically silent. It has become evident that the disease exists or appears late in children, even though classical forms with malabsorption are not apparent (4).

PRINCIPLE OF THE ASSAY

The ZEUS ELISA Gliadin IgA Test System is designed to detect IgA class antibodies to Gliadin in human sera. Wells of plastic microwell strips are sensitized by passive adsorption with Gliadin antigen. The test procedure involves three incubation steps:

1. Test sera (properly diluted) are incubated in antigen coated microwells. Any antigen specific antibody in the sample will bind to the immobilized antigen. The plate is washed to remove unbound antibody and other serum components.
2. Peroxidase Conjugated goat anti-human IgA is added to the wells and the plate is incubated. The Conjugate will react with Gliadin antigen immobilized on the solid phase in step 1. The wells are washed to remove unreacted Conjugate.
3. The microwells containing immobilized peroxidase Conjugate are incubated with peroxidase Substrate Solution. Hydrolysis of the Substrate by peroxidase produces a color change. After a period of time the reaction is stopped and the color intensity of the solution is measured photometrically. The color intensity of the solution depends upon the antibody concentration in the original test sample.

TEST SYSTEM COMPONENTS

Materials Provided:

Each Test System contains the following components in sufficient quantities to perform the number of tests indicated on the packaging label. **NOTE: The following components contain Sodium Azide as a preservative at a concentration of <0.1% (w/v): Controls, Calibrator, and SAVE Diluent®.**

PLATE	1. Plate: 96 wells configured in twelve, 1x8-well, strips coated with inactivated antigen. The strips are packaged in a strip holder and sealed in an envelope with desiccant.
CONJ	2. Conjugate: Conjugated (horseradish peroxidase) goat anti-human IgA. One, 15mL, white-capped bottle. Ready to use.
CONTROL +	3. Positive Control (Human Serum): One, 0.35mL, red-capped vial.
CAL	4. Calibrator (Human Serum): One, 0.5mL, blue-capped vial.
CONTROL -	5. Negative Control (Human Serum): One, 0.35mL, green-capped vial.
DIL SPE	6. SAVE Diluent®: One, 30mL, green-capped, bottle containing Tween-20, bovine serum albumin and phosphate-buffered-saline, (pH 7.2 ± 0.2). Ready to use. NOTE: The SAVE Diluent® will change color when combined with serum.
SOLN TMB	7. TMB: One, 15mL, amber-capped, amber bottle containing 3, 3', 5, 5' - tetramethylbenzidine (TMB). Ready to use.
SOLN STOP	8. Stop Solution: One, 15mL, red-capped, bottle containing 1M H ₂ SO ₄ , 0.7M HCl. Ready to use.
WASHBUF 10X	9. Wash Buffer Concentrate (10X): Dilute 1 part concentrate + 9 parts deionized or distilled water. One, 100mL, clear-capped, bottle containing a 10X concentrated phosphate-buffered-saline and Tween-20 solution (blue solution). NOTE: 1X solution will have a pH of 7.2 ± 0.2.

NOTES:

1. The following components are not Test System Lot Number dependent and may be used interchangeably with the ZEUS ELISA Test Systems: TMB, Stop Solution, and Wash Buffer. SAVE Diluent® may be used interchangeably with any ZEUS ELISA Test System utilizing Product No. 005CC.
2. Test System also contains a Component Label containing lot specific information inside the Test System box.

PRECAUTIONS

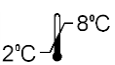
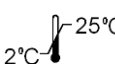
1. For *In Vitro* diagnostic use.
2. Follow normal precautions exercised in handling laboratory reagents. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. Wear suitable protective clothing, gloves, and eye/face protection. Do not breathe vapor. Dispose of waste observing all local, state, and federal laws.
3. The wells of the ELISA plate do not contain viable organisms. However, consider the strips **potentially biohazardous materials** and handle accordingly.
4. The Controls are **potentially biohazardous materials**. Source materials from which these products were derived were found negative for HIV-1 antigen, HBsAg and for antibodies against HCV and HIV by approved test methods. However, since no test method can offer complete assurance that infectious agents are absent, these products should be handled at the Biosafety Level 2 as recommended for any potentially infectious human serum or blood specimen in the Centers for Disease Control/National Institutes of Health manual "Biosafety in Microbiological and Biomedical Laboratories": Current Edition; and OSHA's Standard for Bloodborne Pathogens (5).
5. Adherence to the specified time and temperature of incubations is essential for accurate results. **All reagents must be allowed to reach room temperature (20 - 25°C) before starting the assay.** Return unused reagents to refrigerated temperature immediately after use.
6. Improper washing could cause false positive or false negative results. Be sure to minimize the amount of any residual wash solution; (e.g., by blotting or aspiration) before adding Conjugate or Substrate. Do not allow the wells to dry out between incubations.
7. The SAVE Diluent®, Controls, and Calibrator contain Sodium Azide at a concentration of <0.1% (w/v). Sodium Azide has been reported to form lead or copper azides in laboratory plumbing which may cause explosions upon hammering. To prevent, rinse sink thoroughly with water after disposing of solution containing Sodium Azide.
8. The Stop Solution is TOXIC if inhaled, has contact with skin or if swallowed. It can cause burns. In case of accident or ill feelings, seek medical advice immediately.

9. The TMB Solution is HARMFUL. It is irritating to eyes, respiratory system and skin.
10. The Wash Buffer concentrate is an IRRITANT. It is irritating to eyes, respiratory system and skin.
11. Wipe the bottom of the plate free of residual liquid and/or fingerprints that can alter optical density (OD) readings.
12. Dilution or adulteration of these reagents may generate erroneous results.
13. Do not use reagents from other sources or manufacturers.
14. TMB Solution should be colorless, very pale yellow, very pale green, or very pale blue when used. Contamination of the TMB with Conjugate or other oxidants will cause the solution to change color prematurely. Do not use the TMB if it is noticeably blue in color.
15. Never pipette by mouth. Avoid contact of reagents and patient specimens with skin and mucous membranes.
16. Avoid microbial contamination of reagents. Incorrect results may occur.
17. Cross contamination of reagents and/or samples could cause erroneous results.
18. Reusable glassware must be washed and thoroughly rinsed free of all detergents.
19. Avoid splashing or generation of aerosols.
20. Do not expose reagents to strong light during storage or incubation.
21. Allowing the microwell strips and holder to equilibrate to room temperature prior to opening the protective envelope will protect the wells from condensation.
22. Collect the wash solution in a disposal basin. Treat the waste solution with disinfectant (i.e.: 10% household bleach - 0.5% Sodium Hypochlorite). Avoid exposure of reagents to bleach fumes.
23. Caution: Neutralize any liquid waste at an acidic pH before adding to a bleach solution.
24. Do not use ELISA plate if the indicator strip on the desiccant pouch has turned from blue to pink.
25. Do not allow the Conjugate to come in contact with containers or instruments that may have previously contained a solution utilizing Sodium Azide as a preservative. Residual amounts of Sodium Azide may destroy the Conjugate's enzymatic activity.
26. Do not expose any of the reactive reagents to bleach-containing solutions or to any strong odors from bleach-containing solutions. Trace amounts of bleach (sodium hypochlorite) may destroy the biological activity of many of the reactive reagents within this Test System.

MATERIALS REQUIRED BUT NOT PROVIDED

1. ELISA microwell reader capable of reading at a wavelength of 450nm. **NOTE: Use of a single (450nm), or dual (450/620 - 650nm), wavelength reader is acceptable. Dual wavelength is preferred, as the additional reference filter has been determined to reduce potential interference from anomalies that may absorb light.**
2. Pipettes capable of accurately delivering 10 - 200µL.
3. Multichannel pipette capable of accurately delivering 50 - 200µL.
4. Reagent reservoirs for multichannel pipettes.
5. Wash bottle or microwell washing system.
6. Distilled or deionized water.
7. One liter graduated cylinder.
8. Serological pipettes.
9. Disposable pipette tips.
10. Paper towels.
11. Laboratory timer to monitor incubation steps.
12. Disposal basin and disinfectant (i.e.: 10% household bleach - 0.5% Sodium Hypochlorite).

STORAGE CONDITIONS

	Coated Microwell Strips: Immediately reseal extra strips with desiccant and return to proper storage. After opening - strips are stable for 60 days, as long as the indicator strips on the desiccant pouch remains blue.
	Conjugate – DO NOT FREEZE.
Unopened Test System, Calibrator, Positive Control, Negative Control, TMB, SAVE Diluent®	
	Stop Solution: 2 - 25°C
	Wash Buffer (1X): 20 - 25°C for up to 7 days, 2 - 8°C for 30 days.
	Wash Buffer (10X): 2 - 25°C

SPECIMEN COLLECTION

1. ZEUS Scientific recommends that the user carry out specimen collection in accordance with CLSI document M29: [Protection of Laboratory Workers from Infectious Disease \(Current Edition\)](#).
2. No known test method can offer complete assurance that human blood samples will not transmit infection. Therefore, consider all blood derivatives potentially infectious.
3. Use only freshly drawn and properly refrigerated sera obtained by approved aseptic venipuncture procedures in this assay (6, 7). Do not use if there are any added anticoagulants or preservatives. Avoid using hemolyzed, lipemic, or bacterially contaminated sera.
4. Store sample at room temperature for no longer than 8 hours. If testing is not performed within 8 hours, sera may be stored between 2 - 8°C, for no longer than 48 hours. If a delay in testing is anticipated, store test sera at -20°C or lower. Avoid multiple freeze/thaw cycles which may cause loss of antibody activity and give erroneous results. It is the responsibility of the individual laboratory to use all available references and/or its own studies to determine stability criteria for its laboratory (8).

ASSAY PROCEDURE

1. Remove the individual components from storage and allow them to warm to room temperature (20 - 25°C).
2. Determine the number of microwells needed. Allow for six Control/Calibrator determinations (one Reagent Blank, one Negative Control, three Calibrators and one Positive Control) per run. Run a Reagent Blank on each assay. Check software and reader requirements for the correct Controls/Calibrator configurations. Return unused strips to the resealable pouch with desiccant, seal, and return to storage between 2 - 8°C.

EXAMPLE PLATE SET-UP		
	1	2
A	Blank	Patient 3
B	Negative Control	Patient 4
C	Calibrator	Etc.
D	Calibrator	
E	Calibrator	
F	Positive Control	
G	Patient 1	
H	Patient 2	

3. Prepare a 1:21 dilution (e.g.: 10µL of serum + 200µL of SAVE Diluent®) of the Negative Control, Calibrator, Positive Control, and each patient serum. **NOTE: The SAVE Diluent® will undergo a color change confirming that the specimen has been combined with the diluent.**

4. To individual wells, add 100µL of each diluted Control, Calibrator and patient specimen. Ensure that the samples are properly mixed. Use a different pipette tip for each sample.
5. Add 100µL of SAVe Diluent® to well A1 as a Reagent Blank. Check software and reader requirements for the correct Reagent Blank well configuration.
6. Incubate the plate at room temperature (20 - 25°C) for 25 ± 5 minutes.
7. Wash the microwell strips 5 times.
 - a. **Manual Wash Procedure:**
 1. Vigorously shake out the liquid from the wells.
 2. Fill each microwell with Wash Buffer. Make sure no air bubbles are trapped in the wells.
 3. Repeat steps 1. and 2. for a total of 5 washes.
 4. Shake out the wash solution from all the wells. Invert the plate over a paper towel and tap firmly to remove any residual wash solution from the wells. Visually inspect the plate to ensure that no residual wash solution remains. Collect wash solution in a disposable basin and treat with disinfectant at the end of the day's run.
 - b. **Automated Wash Procedure:**
If using an automated microwell wash system, set the dispensing volume to 300 - 350µL/well. Set the wash cycle for 5 washes with no delay between washes. If necessary, the microwell plate may be removed from the washer, inverted over a paper towel and tapped firmly to remove any residual wash solution from the microwells.
8. Add 100µL of the Conjugate to each well, including the Reagent Blank well, at the same rate and in the same order as the specimens.
9. Incubate the plate at room temperature (20 - 25°C) for 25 ± 5 minutes.
10. Wash the microwells by following the procedure as described in step 7.
11. Add 100µL of TMB to each well, including the Reagent Blank well, at the same rate and in the same order as the specimens.
12. Incubate the plate at room temperature (20 - 25°C) for 10 - 15 minutes.
13. Stop the reaction by adding 50µL of Stop Solution to each well, including the Reagent Blank well, at the same rate and in the same order as the TMB. Positive samples will turn from blue to yellow. After adding the Stop Solution, tap the plate several times to ensure that the samples are thoroughly mixed.
14. Set the microwell reader to read at a wavelength of 450nm and measure the optical density (OD) of each well against the Reagent Blank. Read the plate within 30 minutes of the addition of the Stop Solution.

ABBREVIATED TEST PROCEDURE

1. Dilute Serum 1:21.
2. Add diluted sample to microwell - 100µL/well.
3. —————→ *Incubate 25 ± 5 minutes.*
4. Wash.
5. Add Conjugate - 100µL/well.
6. —————→ *Incubate 25 ± 5 minutes.*
7. Wash.
8. Add TMB - 100µL/well.
9. —————→ *Incubate 10 - 15 minutes.*
10. Add Stop Solution - 50µL/well - Mix.
11. READ within 30 minutes.

QUALITY CONTROL

1. Each time the assay is performed, the Calibrator must be run in triplicate. A Reagent Blank, Negative Control, and Positive Control must also be included.
2. Calculate the mean of the three Calibrator wells. If any of the three values differ by more than 15% from the mean, discard that value and calculate the mean using the remaining two wells.
3. The mean OD value for the Calibrator, Positive Control, and Negative Control should fall within the following ranges:

	<u>OD Range</u>
Negative Control	≤0.250
Calibrator	≥0.300
Positive Control	≥0.500

 - a. The OD of the Negative Control divided by the mean OD of the Calibrator should be ≤0.9.
 - b. The OD of the Positive Control divided by the mean OD of the Calibrator should be ≥1.25.
 - c. If the above conditions are not met the test should be considered invalid and should be repeated.
4. The Positive Control and Negative Control are intended to monitor for substantial reagent failure, but will not ensure precision at the assay Cutoff.
5. Additional Controls may be tested according to guidelines or requirements of local, state, and/or federal regulations or accrediting organizations.
6. Refer to CLSI document C24: Statistical Quality Control for Quantitative Measurement Procedures for guidance on appropriate QC practices.

INTERPRETATION OF RESULTS

The Calibrator within this Test System has been assigned both a Correction Factor for the generation of Index Values and a Calibrator Value for the generation of Unit Values. Based upon testing of normal and disease-state specimens, a maximum normal Unit Value has been determined by the manufacturer and correlated to the Calibrator.

1. **Calculations:**

- a. **Correction Factor:** The manufacturer determined a Cutoff OD Value for positive samples and correlated it to the Calibrator. The Correction Factor (CF) allows for the determination of the Cutoff Value for positive samples. It will also correct for slight day-to-day variations in test results. The Correction Factor is determined for each lot of components and is printed on the Component Label located in the Test System box.
- b. **Cutoff OD Value:** To obtain the Cutoff OD Value, multiply the CF by the mean OD of the Calibrator determined above.
(CF x Mean OD of Calibrator = Cutoff OD Value)
- c. **Index Values/OD Ratios:** Calculate the Index Value/OD Ratio for each specimen by dividing its OD Value by the Cutoff OD from step b.

Example: Mean OD of Calibrator	= 0.793
Correction Factor (CF)	= 0.25
Cutoff OD	= 0.793 x 0.25 = 0.198
Unknown Specimen OD	= 0.432
Specimen Index Value/OD Ratio	= 0.432/0.198 = 2.18

- d. **Conversion of Optical Density to Autoantibody Units (AAU)/mL:** The conversion of OD to Unit Value (AAU/mL) can be represented by the following equation:

Test Specimen AAU/mL = (A x B)/C, Where: AAU/mL = Unknown Unit Value to be determined; A = OD of the test specimen in question; B = Unit Value of the Positive Calibrator (AAU/mL) & C = The mean OD of the Calibrator.

Example: Test Specimen OD = 0.946	Test Specimen AAU/mL = (0.946 x 155) / 0.435
Calibrator OD = 0.435	Test Specimen = 337 AAU/mL

Calibrator Unit Value = 155 AAU/mL

2. **Interpretations:** Index Values/OD Ratios are interpreted as follows.

	Unit Values	Index Value/OD Ratio
Negative Specimens	< 150 AAU/mL	≤0.90
Equivocal Specimens	150 to 180 AAU/mL	0.91 to 1.09
Positive Specimens	> 180 AAU/mL	≥1.10

Retest specimens with OD Ratio Values in the equivocal range (0.91 – 1.09) in duplicate. Report any two of the three results which agree. Evaluate repeatedly equivocal specimens using an alternate serological method and/or re-evaluate by drawing another sample one to three weeks later.

LIMITATIONS OF THE ASSAY

- Do not make a diagnosis based solely on the results of the ZEUS ELISA Gliadin IgA Test System. Use the results from the ZEUS ELISA Gliadin IgA Test System in conjunction with the clinical evaluation and the results of other diagnostic procedure.
- Crohn's disease and other food protein intolerance/gastrointestinal disorders may induce circulating antibodies to gliadin and cause a false positive.
- The clinical significance of any test result depends upon its relationship to other medical patient data. Doctors must base all disease diagnosis and management on an evaluation of all relevant patient information.
- ZEUS Scientific, Inc. did not establish values for the pediatric population with this assay.
- A Gliadin IgA negative result in an untreated patient does not rule out gluten-sensitive enteropathy when associated with high levels of gliadin IgG antibodies. Selective IgA deficiency is a relatively frequent finding in celiac disease.

EXPECTED RESULTS

Three hundred and five (305) specimens were tested to establish, or estimate, the expected reactivity rate with the assay. This represented two groups of specimens; 255 clinical specimens which were either sent to the lab for routine gliadin serological analysis or were part of an external gliadin study, and 50 random normal donor specimens. With respect to the clinical population, 107/255 (42.0%) were positive, 140/255 (54.9%) were negative, and 8/255 (3.1%) were equivocal. With respect to the normal population, 49/50 (98.0%) were negative, 1/50 (2.0%) was positive.

PERFORMANCE CHARACTERISTICS

1. **Comparative Study:**

An in-house comparative study was conducted to demonstrate the equivalence of the ZEUS ELISA Gliadin IgA Test System to another commercially available ELISA Gliadin IgA Test System. Performance was evaluated using 305 specimens and the results are summarized below:

		ZEUS ELISA Gliadin IgA Test System			
		Negative	Equivocal**	Positive	Total
Commercial ELISA Test System	Negative	175	2	7	184
	Equivocal**	8	2	9	19
	Positive	6	4	92	102
	Total	189	8	108	305

Relative Sensitivity = 92/98 = 93.9% 95% Confidence Interval = 89% to 99% ** Data Excluded From Calculation
 Relative Specificity = 175/182 = 96.2% 95% Confidence Interval = 93% to 99%
 Relative Agreement = 267/280 = 95.4% 95% Confidence Interval = 93% to 98%

2. **Reproducibility:**

Technicians tested eight specimens in-house, to determine intra-assay and inter-assay variation: two strong positive specimens, two specimens near the cut off zone, two low negative specimens and the kit's positive and negative controls. On each of three days, a technician tested each specimen once a day, eight times each, resulting in 24 test points. A responsible party then calculated the mean OD ratio and coefficient of variation from the resulting data. Depiction of the results of the experiment is below

Kit One	Intra-Assay (n=8)						Inter-Assay (n=24)	
	Day One		Day Two		Day Three			
	Sample (N)	Mean AAU/mL	% CV	Mean AAU/mL	% CV	Mean AAU/mL	% CV	Mean AAU/mL
Sample 1	8	32.4	11	17.8	7	15.8	9	29.1
Sample 2	81	5.0	99	2.0	87	4.3	89	9.3
Sample 3	160	4.6	178	4.2	168	2.8	169	5.8
Sample 4	178	9.0	203	3.4	182	8.1	187	9.0
Sample 5	165	5.1	171	3.6	175	7.8	170	6.1
Sample 6	269	7.8	333	3.2	318	5.1	307	10.5
NC	11	18.7	15	9.2	11	11.2	12	18.7
HPC	973	6.8	1139	2.9	1050	6.1	1054	8.3

Kit Two	Intra-Assay (n=8)						Inter-Assay (n=24)	
	Day One		Day Two		Day Three			
	Sample (N)	Mean AAU/mL	% CV	Mean AAU/mL	% CV	Mean AAU/mL	% CV	Mean AAU/mL
Sample 1	5	30.2	6	21.4	7	20.6	6	26.5
Sample 2	81	6.1	77	4.3	83	5.4	80	6.1
Sample 3	153	3.1	158	4.5	159	7.0	157	5.3
Sample 4	179	6.3	162	2.2	177	3.9	173	6.3
Sample 5	174	10.0	154	6.9	171	6.4	166	9.4
Sample 6	321	4.4	282	4.1	304	3.3	302	6.6
NC	10	10.9	11	28.8	11	9.1	11	17.8
HPC	1175	8.6	1185	8.4	1089	5.4	1150	8.3

3. **Cross Reactivity:**

To investigate the potential for positive reactions due to cross reactive antibodies, 26 specimens which were reactive for various auto antibodies (ANA, PR3, MPO, cardiolipin, dsDNA, ENA, Jo-1, RF, Scl-70, Sm, Sm/RNP, SSA and SSB) were tested on the ZEUS ELISA Gliadin IgA Test System. All 26 were negative for gliadin IgA activity. The results of this study indicate that the potential for interference due to cross reactivity with such auto antibodies is unlikely.

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