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AUG2001 Document No. PI4114

ImmuGlo™

Anti-Endomysial Antibody (EMA) Test System

For *in vitro* Diagnostic Use
CLIA Complexity: High
CDC Analyte Identification Code: 0497
CDC Test System Identification Code: 28281

PRODUCT INSERT

Catalog No. 1114 *48 Determinations*

INTENDED USE

An indirect immunofluorescence antibody test for the qualitative and semi-quantitative detection of endomysial antibodies (EMA) in human serum as an aid in the diagnosis of celiac disease and dermatitis herpetiformis.

SUMMARY AND EXPLANATION

Endomysial antibodies (EMA), as reported in the literature, are detected primarily on the smooth muscle of monkey esophagus by indirect immunofluorescence. The detection of EMA aids in the diagnosis of *gluten sensitive enteropathy*, i.e. *celiac disease* (CD) and *dermatitis herpetiformis* (DH). Patients with CD and DH are reported to have antibodies to endomysium, reticulin and gliadin[1-12]. These serological markers have recently been incorporated into the revised criteria for the diagnosis of CD by the European Society of Pediatric Gastroenterology and Nutrition[13]. Of the various antibody markers of CD and DH, EMA of the IgA class seem to be the most sensitive and specific marker. EMA of the IgG class also occur when IgA class EMA are in high titer or in individuals who are IgA deficient. A rapid decrease in EMA levels results with adherence to a gluten free diet. A gluten challenge or a failure to maintain a gluten free diet leads to the appearance or an increase in endomysial antibody titers. Patients on a gluten free diet >9 months have reduced or negative EMA titers if they adhere to their diet restrictions[1,6-8,10].

PRINCIPLES OF PROCEDURE

In the indirect immunofluorescence method, patient serum is incubated on tissue sections to allow binding of antibodies to the substrate. Any antibodies not bound are removed by rinsing. Bound antibodies of the IgA and IgG class are detected by incubation of the substrate with fluorescein-labeled, anti-human immunoglobulin conjugate. Reactions are observed under a fluorescence microscope equipped with appropriate filters. The presence of EMA is demonstrated by an apple green fluorescence of the endomysial lining of smooth muscle bundles. The titer (the reciprocal of the highest dilution giving a positive reaction) of the antibody is then determined by testing serial dilutions[14]

REAGENTS

Storage and Preparation

Store all reagents at 2°-8°C. Ready for use after equilibration to room temperature.

Materials Provided

Anti-Endomysial Antibody (EMA) Test System. *Catalog No. 1114*

Kit contains sufficient reagents to perform 48 determinations

8 x 6-well - Primate Smooth Muscle Tissue Substrate Slides.

1 x 0.5 ml EMA Positive Control*.

1 x 0.5 ml Negative Control*.

1 x 5.0 ml Goat anti-human polyvalent FITC Conjugate. Ready to use. **Protect from light***.

1 x 60 ml Buffered Diluent. Ready to use*.

2 vials Phosphate Buffered Saline. **Dissolve each vial to 1 liter.**

1 x 5.0 ml Mounting Medium. **Do not freeze***.

1 x 1.0 ml Evans Blue Counterstain

1 x 12 Cover Slips

*CAUTION - Contains <0.1% NaN_3

Precautions

For *in vitro* Diagnostic Use. All human derived components used have been tested for HBsAg, HCV, HIV-1 and 2 and HTLV-I and found negative by FDA required tests. All human serum specimens and human derived products should be treated as potentially hazardous, regardless of their origin. Follow good laboratory practices in storing, dispensing and disposing of these materials[15].

WARNING - Sodium azide (NaN_3) may react with lead and copper plumbing to form highly explosive metal azides. Upon disposal of liquids, flush with large volumes of water to prevent azide buildup. Sodium azide may be toxic if ingested. If ingested, report incident immediately to laboratory director or poison control center.

Instructions should be followed exactly as they appear in this insert to ensure valid results. Do not interchange kit components with those from sources other than the same catalog number from IMMCO Diagnostics. Do not use beyond expiration date.

REFERENCES

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12. Peters MS and McEvoy MT. IgA antiendomysial antibodies in dermatitis herpetiformis. J Am Acad Dermatol; 1989, 21: 1225-1231.
13. Walker-Smith JA, Quandalini S, Schmitz J et al. Revised criteria for diagnosis of celiac disease. Report of working group of European Society of Paedriatric Gastroenterology and Nutrition. Arch Dis Child; 1990, 65:909-911.
14. Beutner EH, Kumar V, Krasny SA and Chorzelski TP. Defined immunofluorescence in immunodermatology. In “Immunopathology of the Skin”, Beutner EH, Chorzelski Tp and Kumar V, Eds, John Wiley and Sons New York; 1987, 3rd Ed, 3-40.
15. Biosafety in Microbiological and Biomedical Laboratories. Centers for Disease Control, National Institutes of Health, 1993 (HHS Pub. No. {CDC} 93-8395).

Table 1. Incidence of IgA Class EMA

Clinical Condition	No. Tested	% Positive
Confirmed Celiacs		
On gluten	185	99
On gluten free diet	190	9
Suspected Celiacs		
On gluten	82	83
On gluten free diet	30	16
Dermatitis Herpetiformis (DH)	253	80
DH with Subtotal Villous Atrophy	42	100
DH on gluten free diet	36	3
Disease Controls (GI)		
Infectious Diarrhea	210	0
Recurrent Diarrhea	124	0
Toddlers Diarrhea	170	0
Milk Protein Intol.	69	0
Ulcerative Colitis	69	0
Crohn's Disease	65	0
Liver Diseases	21	0
Disease Controls (Skin)		
Linear IgA Bullous Dermatosis	4	0
Other Skin Diseases	180	0

Compiled from the literature as per Chorzelski TP et al[1].

PERFORMANCE CHARACTERISTICS

The ImmuGlo™ Anti-Endomysial Antibody (EMA) Test kit, using primate smooth muscle substrate and a polyvalent conjugate, was compared with another commercially available kit also using a polyvalent conjugate and monkey esophagus as a substrate. The comparison included a total of 68 sera: 20 from patients with clinically suspected celiac disease and 48 from normal controls. Sera were tested according to the procedure recommended by the manufacturer. A screening dilution of 2.5 was used and all sera positive for EMA were titrated to endpoint. The results were as follows:

		ImmuGlo™ EMA		
		Positive	Negative	Total
Other	Positive	18	0	18
	Negative	2	48	50
	Total	20	48	68

Agreement: 97%
Sensitivity: 100%
Specificity: 96%

Materials Required but not Provided

- Fluorescence microscope
- Micropipette or Pasteur pipette
- Serological pipettes
- Staining dish (e.g. Coplin jar)
- Small test tubes (e.g. 13 x 75 mm) and test tube rack
- Distilled or deionized water
- 1 liter container
- Wash bottle
- Paper towels
- Incubation chamber

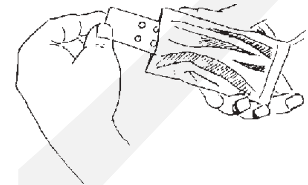
SPECIMEN COLLECTION AND PREPARATION

Only serum specimens should be used for this procedure. Grossly hemolyzed, lipemic or microbially contaminated specimens may interfere with the performance of this test and should not be used. Store specimens at 2°- 8°C for no longer than one week. For longer storage, serum should be frozen at -20°C. Avoid repeated freezing and thawing of samples.

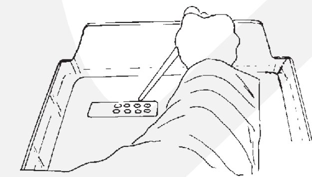
PROCEDURE

Test Method

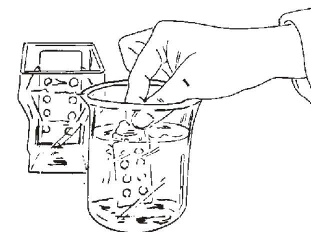
The indirect immunofluorescence staining procedure is illustrated in the following figures:



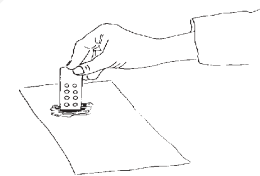
1. Let pouch equilibrate to room temperature, then remove slide(s) from pouch.



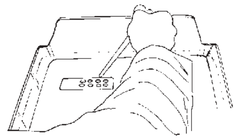
2. Place slide(s) into moisture chamber and add samples and controls. Cover and incubate 30 minutes.



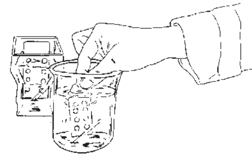
3. Rinse slide(s) by dipping into beaker with PBS. Transfer slide(s) into Coplin jar and wash 10 minutes.



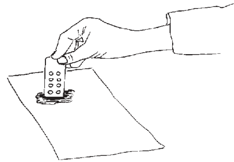
4. Blot edge of slide(s) on absorbent paper. Proceed immediately with Conjugate step.



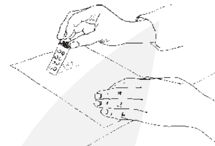
5. Apply Conjugate to each well. Cover and incubate 30 minutes.



6. Rinse slide(s) by dipping into beaker with PBS. Transfer slide(s) into Coplin jar and wash 10 minutes.



7. Blot edge of slide(s) on absorbent paper. Proceed immediately with next step.



8. Mount cover slip and read under fluorescent microscope.

A. Screening:

Step 1. Dilute each patient serum **1:2.5** with the Buffered Diluent provided (0.2 ml serum + 0.3 ml Diluent). **Do not dilute the Positive or Negative controls**. Save the undiluted sera to determine antibody titers if screening tests are found to be positive.

Step 2 Allow pouches containing substrate slides to equilibrate to room temperature for **10-15 minutes**. Carefully remove the slides without touching the substrate.

Step 3 Label the slides and place them in an incubation chamber lined with paper towels moistened with water to prevent drying.

Step 4 Invert dropper vial and gently squeeze to apply **1 drop** (approximately 50 µl) of the **Negative Control** to well #1. Similarly apply **1 drop** of **Positive Control** to well #2. Avoid overfilling the wells.

Step 5 Using a micropipette or Pasteur pipette, apply **1 drop** of patient's diluted serum (approximately 50 µl) to the other wells. Avoid overfilling the wells.

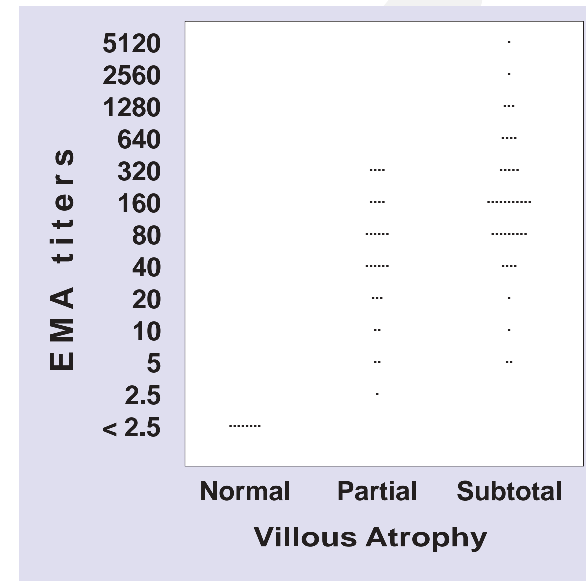
Step 6 Place the lid on the incubation chamber and incubate slides **30 minutes** at room temperature.

Step 7 Remove a slide from the incubation chamber. Hold slide at tab end and rinse gently with approximately **10 ml** of PBS using a pipette, or rinse slide in a beaker filled with PBS. Do not use a wash bottle. Transfer slide immediately into Coplin jar and wash **10 minutes**. Repeat process with all remaining slides.

EXPECTED VALUES

As seen in Table 1, EMA, as detected on primate smooth muscle are highly specific markers for celiac disease and dermatitis herpetiformis. The presence of EMA seems to be related to the intestinal pathology both in celiac disease and dermatitis herpetiformis rather than to the skin lesions in the latter, as depicted in Figure 2:

Figure 2: Correlation of IgA-EMA titers in Villous Atropy



From Chorzelski TP et al[1] and Kumar V et al[8]

LIMITATIONS OF THE PROCEDURE

The ImmuGlo™ EMA IFA Test System: CLIA Complexity: High; CDC Analyte Identification Code: 0497; CDC Test System Identification Code: 2828.

In some cases, sera positive for EMA may either be very weak or negative at the initial screening dilution (prozone phenomenon). In such doubtful cases the sera should be screened at higher dilutions and, if positive, antibody titer determined.

The presence of two or more antibodies in a serum which are reactive with the same tissue may cause an interference in their detection by immunofluorescence. This interference may cause either a failure to detect EMA or a suppression of its titer if the interfering antibody has a higher titer than EMA. The most common cause of the interference phenomenon in EMA tests is the coexistence of smooth muscle antibodies. It is recommended that patients sera which also contain ASMA be tested further at higher dilutions. IgA class ASMA are not a common occurrence. IgG class ASMA do not block IgA-EMA as the former react with the sarcoplasm of smooth muscle bundles and the latter react with the endomysium of the sarcolemma around the smooth muscle bundles. Anti-reticulin antibodies do not interfere with the reaction of EMA because they do not react with primate smooth muscle tissue. The coexistence of IgG class EMA may interfere with the detection of IgA class EMA. However, this rarely occurs as:

1. IgG class - EMA are present in only 25% of celiac disease patients,
2. IgG class - EMA titers are usually much lower than IgA-EMA titers and
3. IgA antibodies are usually of higher avidity than IgG antibodies.

In some patients with celiac disease and IgA deficiency, the IgA-class endomysial antibodies are absent. However, such patients are usually positive for IgG class EMA.

Patients with celiac disease on a gluten free diet for >9 months invariably are negative for EMA.

When making a diagnosis, results of all laboratory testing must always be evaluated along with the total clinical history of the patient.

Step 8 Remove slide(s) from Coplin jar. Blot the edge of the slide on a paper towel to remove excess PBS. Place the slide in the incubation chamber. Immediately invert the **Conjugate** dropper vial and gently squeeze to apply **1 drop** (approximately 50 µl) to each well.

Step 9. Repeat **Steps 7 and 8** for each slide.

Step 10 Replace the lid on the incubation chamber. Incubate **30 minutes** at room temperature.

Step 11 Remove a slide from incubator. Hold the slide at the tab end and dip the slide in a beaker containing PBS to remove excess conjugate. Place slide(s) in a staining dish filled with PBS for **10 minutes**. If desired, 2-3 drops of Evans blue counterstain may be added to the final wash. Repeat for the remaining slides. **NOTE:** Improper washing may lead to increased background fluorescence.

Step 12 Remove a slide from the staining dish. Blot the edge of the slide on a paper towel to remove excess PBS. **To prevent slide from drying, proceed immediately with Step 13 while the slide is still wet.**

Step 13 Mount the coverslip. Place **3 drops** of the Mounting Medium evenly spaced on a coverslip and invert the slide onto the coverslip. To remove any air bubbles gently apply pressure along the edge of the coverslip. Avoid any movement of the coverslip.

Step 14 Repeat **Steps 12 and 13** for each slide.

Step 15 Examine for specific fluorescence under a fluorescence microscope at a magnification of **200x** or greater.

Slides may be read as soon as prepared. However, because of the presence of antifading agent in the mounting medium, no significant loss of staining intensity occurs if reading is delayed. Slides should be stored in the dark at 2-8°C.

B: <u>End Point Determination</u> (Titration)

A serum positive in the screening test may be further tested following **Steps 5 through 13** to determine the titer. Each test run should include the Positive and Negative Controls. Make serial twofold dilutions starting at 1:5. Using one slide, a serum may be tested at dilutions ranging from 1:5 to 1:40. If positive at a 1:40 dilution, the titer is reported as greater or equal to 40, or additional slides may be used to obtain endpoints. The reciprocal of the highest dilution producing a positive reaction is the titer.

Preparation of Serial Dilutions

Number four tubes 1 through 4. Add 0.4 ml of Buffered Diluent to tube 1 and 0.2 ml to tubes 2 through 4. Pipette 0.1 ml of undiluted serum to tube 1 and mix thoroughly. Transfer 0.2 ml from tube 1 to tube 2 and mix thoroughly. Continue transferring 0.2 ml from one tube to the next after mixing to yield the dilutions depicted in the following table:

Tubes	1	2	3	4
Serum	0.1 ml +			
Buffered Diluent	0.4 ml	0.2 ml	0.2 ml	0.2 ml
Transfer	0.2 ml	0.2 ml	0.2 ml	
Final dilution	1:5	1:10	1:20	1:40 etc.

Quality Control

Both the Positive and Negative Controls should be included with each test run. The Negative Control should show no specific fluorescence of the endomysium lining of the smooth muscle bundles, whereas the Positive Control should have 2+ or greater staining intensity of the tubules of these structures.

If expected results are not obtained, the run should be repeated. If inadequate results continue to occur with the controls, these may be due to:

- If signs of turbidity is seen, discard and use another control.
- Problems with the optical system of the fluorescence microscope. These may include: improper alignment, use of the bulb beyond the expected performance life, etc.
- Allowing the slide to dry during the procedure.

RESULTS

The results of the tests for endomysial antibodies should be reported as negative (<2.5), positive greater or equal to 40, or preferably, positive with titer.

Read for specific staining of the endomysium lining of the smooth muscle bundles. **See Figure 1a.** Endomysial antibodies react as a network of thin, irregular lines around the sarcolemma of the individual smooth muscle fibrils. This is in a sharp contrast to anti-smooth muscle antibodies which react with the sarcoplasm. **See Figure 1b.**

Other detectable antibodies besides anti-smooth muscle antibodies (ASMA) include antinuclear antibodies (ANA). The presence of ASMA is known to cause false negative results for endomysial antibodies. If ASMA are detected, then the sample should be tested at higher dilutions[1]. ANA reactions on smooth muscle tissue, when they occur, are usually weak and sparsely distributed and, therefore, unlikely to cause false negative results.

Figure 1a. EMA staining reaction on primate smooth muscle, 200X. Note staining of lining of the smooth muscle bundles.

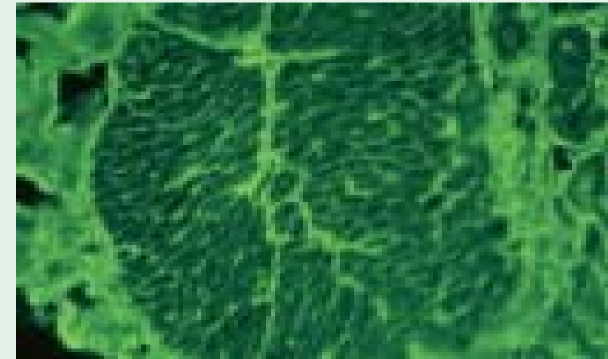


Figure 1b. ASMA staining reaction on primate smooth muscle, 200X. Note staining of the smooth muscle sarcoplasm.