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ImmuGlo™ Anti-Reticulin Antibody (ARA) Test System

For *in vitro* Diagnostic Use

CLIA Complexity: High

CDC Analyte Identification Code: 0444

CDC Test System Identification Code: 28331

PRODUCT INSERT

Catalog No. 1115

48 Determinations

INTENDED USE

An indirect immunofluorescence antibody test for the detection and semi-quantitation of anti-reticulin antibodies (ARA) in human serum.

SUMMARY AND EXPLANATION

The detection of anti-reticulin antibodies (ARA) is performed primarily by indirect immunofluorescence. ARA was first described by Seah et al.^{1,2} in 1971 in the sera of patients with gluten sensitive enteropathy: celiac disease (CD) or dermatitis herpetiformis (DH). ARA by definition are detected on rodent tissue. In 1973 Rizzetto and Doniach³ identified five different ARA reaction patterns. Of these, the R1 pattern, characterized by peri-glomerular, peri-tubular, and peri-vascular staining of mouse or rat kidney is associated with gluten sensitive enteropathy. Both IgG and IgA immunoglobulin class ARA occur. The IgA class ARA is the more specific and sensitive marker of gluten sensitive enteropathy. The specificity of IgA-ARA has been reported to be 59-100% and its sensitivity between 30-95%¹⁻¹¹. IgG-ARA occur less frequently, either in conjunction with IgA class ARA or in CD patients who are IgA deficient¹¹.

PRINCIPLES OF PROCEDURE

In the indirect immunofluorescence method used in this kit, patients' sera are incubated on tissue sections to allow binding of antibodies to the tissue substrate. Any antibodies not bound are removed by rinsing. Bound antibodies 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 ARA is demonstrated by an apple green fluorescence of the peri-tubular, peri-glomerular and peri-vascular fibers of the kidney. The titer (the reciprocal of the highest dilution giving a positive reaction) of the antibody is then determined by testing serial dilutions¹².

REAGENTS

Storage and Preparation

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

Materials Provided

Anti-Reticulin Antibody (ARA) Test System. *Catalog No. 1115*

Kit contains sufficient reagents to perform 48 determinations

- 8 x** 6-well Rat Kidney Substrate Slides.
 - 1 x 0.5 ml** ARA 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% NaN₃

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 materials¹³.

WARNING - Sodium azide (NaN₃) 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.

EXPECTED VALUES

As seen in the table below, ARA are highly specific markers of gluten sensitive enteropathy, i.e. celiac disease and dermatitis herpetiformis. The presence of ARA seems to be related to the intestinal pathology, both in celiac disease and dermatitis herpetiformis, rather than to the skin lesions in the latter.

Clinical Condition	No. Pos/Total	% Positive
Confirmed Celiacs		
On Gluten	32/38	84
On GFD	4/37	11
Suspected Celiacs		
On Gluten	18/30	60
On GFD	2/30	7
Dermatitis Herpetiformis	19/54	35
Family Member of CD	2/76	3
Disease Controls		
Ulcerative Colitis	0/11	0
Crohn's Disease	0/74	0
Pemphigus	0/38	0
Pemphigoid	0/60	0
Blood Donors	0/113	0

GFD = Gluten Free Diet; adapted from Kumar et al.¹¹

PERFORMANCE CHARACTERISTICS

The ImmuGlo™ Anti-Reticulin Antibody (ARA) Test kit was compared with a FDA cleared, commercially available endomysial antibody (EMA) kit which uses a polyvalent conjugate with a primate smooth muscle substrate. The comparison included a total of 100 sera: 50 from patients with clinically suspected celiac disease and 50 from normal controls. Sera were tested according to the method and quality control procedures recommended by the manufacturer. The results were as follows:

		ImmuGlo™ ARA		
		Positive	Negative	Total
Other	Positive	43	7	50
	Negative	0	50	50
	Total	43	57	100

Agreement: 93%
Sensitivity: 86%
Specificity: 100%

Quality Control

Both a Positive and Negative Control Serum should be included with each test run. The Negative Control should show no specific fluorescence of the peri-nuclear, peri-glomerular and peri-vascular reticulin fibers, 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 ARA should be reported as negative (< 2.5), positive (greater or equal to 40), or alternatively, positive with titer.

Read for specific peri-glomerular, peri-tubular and peri-vascular reticulin fiber staining.

Other detectable antibodies include antinuclear antibodies (ANA), anti-smooth muscle antibodies (ASMA) and anti-mitochondrial antibodies (AMA).

*Positive ARA staining reaction on rat kidney,
200X.*

Note staining of the reticulin fiber.

Negative ARA staining reaction

LIMITATIONS OF THE PROCEDURE

In some cases, sera positive for ARA 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 titers determined.

The presence of two or more antibodies in a serum which are reactive with the same substrate may cause an interference in their detection by immunofluorescence. This interference may cause either a failure to detect ARA or a suppression of its titer if the interfering antibody has a higher titer than ARA. The most common cause of the interference phenomenon in ARA tests is the co-existence of ASMA. It is recommended, that patient sera which also contain ASMA, be tested further at higher dilutions.

In some patients with celiac disease and dermatitis herpetiformis, ARA tests may be negative. In such cases repeat testing may yield additional positive results. A negative test for ARA does not rule out a diagnosis of gluten sensitive enteropathy. Celiac disease and dermatitis herpetiformis patients on a gluten free diet may have low ARA titers or even be negative.

In some patients with celiac disease and IgA deficiency, the IgA class reticulin antibodies are absent. However, these patients are usually positive for IgG class ARA.

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

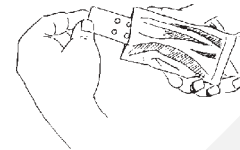
SPECIMEN COLLECTION AND PREPARATION

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.



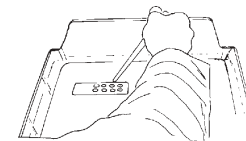
2. Place slide(s) into moisture chamber and add samples and controls. Cover and incubate 30 minutes.



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



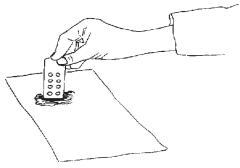
4. Blot edge of slide(s) on absorbent paper. Proceed immediately with next step.



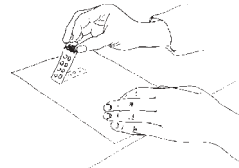
5. Apply Conjugate to each well. Cover and incubate 30 minutes.



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



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 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 μ l) 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 μ l) 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 wash bottle. Transfer slide immediately into Coplin jar and wash **10 minutes**. Repeat process with all remaining slides.
- 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 μ l) 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: End Point Determination (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 appropriate positive and negative controls. Make serial two-fold dilutions starting at 1:5 (see below). 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 for those sera still positive at a 1:320 dilution. 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.