



INSTRUCTION MANUAL

REF 86048

December 28, 2007

EmA IFA

- 48 determinations -



IVD *In vitro* diagnostic device

Indirect immunofluorescence assay for the determination of IgA antibodies to endomysium in human serum

Substrate: monkey esophagus

REF	Catalogue number	LOT	Batch code
	Consult accompanying documents		Manufactured by
	Temperature limitation		Use by
	Consult operating instruction		Biological risk



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INTENDED USE

EmA IFA is used for the qualitative and semi-quantitative determination of anti-endomysial antibodies of IgA class (EmA IgA) in human serum on monkey esophagus for the diagnosis of celiac disease.

Celiac disease, or gluten-sensitivity, is a gluten intolerance which causes chronic enteral disorder. The disease manifests from the second half of the first year of life. Its frequency is 1:950, with boys being affected more often than girls. Toxic-allergic reactions of the intestine mucous membrane against gliadin occur (gliadin being the gluten fraction of wheat and rye) with damage of the enterocytic membrane as well as a decrease in activity of the brush border enzyme. This process in turn causes the activation of the local immune defence leading to villous atrophy.

Clinical symptoms of the disease include massive, fatty, fermented stools, a large gassy abdomen and a dystrophy caused by resorption disorder.

Dermatitis herpetiformis is a skin manifestation of celiac disease which occurs in phases accompanied by severe itching. Hives, small nodules an predominantly blisters occur which, being close together, form round herpes-like foci. The chronic dermatosis lasts for years and may occur at any age.

Beside this a large number of subclinical cases is reported (latent or silent form of celiac disease). Individuals suffering from prolonged celiac disease additionally face an elevated risk of developing T cell lymphoma.

In indirect immunofluorescent assay sera of patients suffering from celiac disease show a reaction with the endomysium, the collagenous connective tissue covering the muscle fibres. A useful substrate is the lower third of the esophagus where the mucosa is surrounded by a smooth muscle layer.

EmA IgA show a diagnostic specificity of about 98% and a diagnostic sensitivity of greater 95%. Although diagnostic sensitivity is limited in children below two.

PRINCIPLE of the TEST

EmA IFA is an indirect immunofluorescence assay for the qualitative and semi-quantitative determination of IgA antibodies to endomysial antigens.

The antibodies of the diluted patient samples and controls react specifically with the antigens of the tissue sections immobilized on the slides. After an incubation period of 30 min at room temperature (RT), unbound serum components are removed by a wash step.

The bound antibodies react specifically with anti-human IgA conjugated to Fluorescein-isothiocyanat (FITC). After an incubation period of 30 min at RT excessive conjugate is separated from the solid-phase immune complexes by an additional wash step.

Stained slides are read using a fluorescence microscope (excitation wavelength 490 nm, emission wavelength 520 nm). According to the histologic alignment of endomysial antigens in the tissue a specific fluorescent staining can be detected: a honeycomb structure of the connective tissue surrounding the muscle fibres in the muscularis mucosae.

PATIENT SAMPLES

Specimen collection and storage

Blood is taken by venipuncture. Serum is separated after clotting by centrifugation. The samples may be kept at 2 - 8 °C for up to two days. Long-term storage requires - 20 °C. Repeated freezing and thawing should be avoided. If samples are to be used for several assays, initially aliquot samples and keep at - 20 °C.

Lipaemic samples could bring about a film covering the cell substrate and should not be used. Contaminated samples should be avoided as they may contain proteolytic enzymes which might digest the cell substrate.

Preparation before use

Allow samples to reach room temperature prior to assay. Take care to agitate serum samples gently in order to ensure homogeneity.

Screening: Patient samples have to be diluted **1:10** (v/v) prior to the assay, e.g. **10 µl sample + 90 µl PBS buffer (made of C)**.

Titration: prepare a 4-fold serial dilution based on the **1:10** (v/v) dilution using **PBS buffer solution (made of C)**, e.g. **100 µl sample dilution + 300 µl PBS (made of C)**, resulting the following dilutions: 1:10, 1:40, 1:160, 1:640, etc.

Preparation before use

Allow all components to reach room temperature prior to use in the assay.

The substrate slides are individually covered in a sealed pouch. Allow the slides to reach room temperature before opening.

PBS buffer preparation:

Place content of a one-liter PBS packet into one-liter volumetric flask, add distilled water to the mark. Dissolve dry substance by stirring or shaking. Reconstituted buffer solution should have a pH of 7.4 ± 0.2 .

Store the solution in a clean bottle at 25°C or lower. Stable for at least two months. Do not use if pH changes, if the solution turns cloudy, or if a precipitate forms.

Avoid exposure of the conjugate to light.

TEST COMPONENTS for 48 determinations

A Ag 4	Substrate slides 4 wells coated with cryostat sections of monkey esophagus	12 sealed in a foil pouch
C BUF PBS	PBS Buffer for 2 x 1000 ml PBS solution	2 x 10 g dry substance
D CONJ	Conjugate anti-human IgA (sheep), labeled to FITC	3.0 ml ready for use dropper bottle capped white
E MOUNT	Mounting medium glycerol solution, PBS buffered, pH 7.4 ± 0.2	3.0 ml ready for use dropper bottle capped white
F TEMPL	Blotting templates	12
G COVER	Coverslips (22 x 70 mm) 12	1
P CONTROL	Positive control antigen specificity and titer on the label (diluted human serum)	1.0 ml ready for use dropper bottle capped red
N CONTROL	Negative control (diluted human serum)	1.0 ml ready for use dropper bottle capped green

Materials required

- micropipettes (10, 100, 1000 µl)
- disposable pipette tips
- disposable test tubes and rack
- graduated cylinders, volumetric flasks
- moist chambers
- plastic squeeze wash bottle
- coplin jars or staining dishes with slide racks
- distilled (or de-ionized) water
- fluorescence microscope (excitation wavelength 490 nm, emission wavelength 520 nm)

Size and storage

EmA IFA (86048) has been designed for 48 determinations.

The expiry date of each component is reported on its respective label, that of the complete kit on the box label.

Upon receipt, all components of the EmA IFA have to be kept at 2 - 8 °C, preferably in the original kit box.

After opening all kit components are stable for at least 2 months, provided proper storage.

ASSAY PROCEDURE

- Dilute patient sera according to test demands (screening, titration)
- Do not allow the substrate slides to dry during the test procedure

1. Bring all reagents to room temperature (18...25°C) before use. Mix gently without causing foam. Remove slides from pouch immediately before use and identify slides using a permanent marking pen.
2. Apply **1 - 2 drops (30 - 50 µl)** controls (P, N) **30 - 50 µl** diluted patient samples onto the respective wells. Completely cover the immobilised tissue section. Do not touch antigen surface.
3. Incubate **30 min** at RT (20...25°C) in a moist chamber.
4. Rinse gently with PBS solution (made of C) using a squeeze wash bottle. Do not focus the PBS stream directly onto the wells. To prevent cross contaminations avoid rinsing from one well across other wells. For multi-row slides run PBS stream from the midline of the slide successively along both rows to the edge of the slide.
5. Wash **2 x 5 min** in changing PBS solution in Coplin jars or staining dishes, agitate gently at entry and prior to removal.
6. Remove slides from the wash one at a time, shake off excess PBS tapping the edge of the slide onto absorbent towel, carefully dry around the wells using a blotting template (F). Apply **one drop** of conjugate (D) to each well of the slides, making sure each well is completely covered.
7. Incubate **30 min** at RT (20-25°C) in a moist chamber, protected from direct light.
8. Rinse gently with PBS solution (made of C) using a squeeze wash bottle as described in 4.
9. Wash **2 x 5 min** in changing PBS solution in Coplin jars or staining dishes, agitate gently at entry and prior to removal.
10. Remove slides from the wash one at a time, shake off excess PBS tapping the edge of the slide onto absorbent towel, carefully dry around the wells using a blotting template (F), apply **2-4 drops** of mounting medium (E) across the slide. Rest the edge of a coverslip (G) against the bottom of the slide allowing the mounting medium to form a continuous bead between coverslip and slide. Gently lower the coverslip from the bottom to the top of the slide, avoid air bubbles. Drain excess mounting medium from the edge of the slide with absorbent paper.
11. Read stained slides using a fluorescence microscope. Avoid longer exposition of one field of vision to minimize bleaching of FITC fluorescence.

Preservation of slides

It is recommended that slides are examined at the same day they are stained. If any delay is anticipated, store slides in a refrigerator (2-8°C) for some days. For long-term preservation, seal edges of slides using nail-varnish, store slides at -20°C.

READING of the RESULTS

Fluorescence intensity

Fluorescence intensity may be semi-quantitated following the guidelines established by the CDC, Atlanta, USA (5):

- 4+ = maximal fluorescence, brilliant yellow-green
- 3+ = less brilliant yellow-green fluorescence
- 2+ = definite but dull yellow-green fluorescence
- 1+ = very dim subdued fluorescence

The degree of intensity is not of clinically relevance and has only limited value as an indicator of titer. Differences in microscope optics, filters and light source may result in differences of +1 or more in intensity.

Negative result

A serum dilution is considered negative for EmA IgA if the fluorescence intensity is less than 1+ and the tissue lacks the specific endomysial fluorescence pattern. Tissue will appear reddish-orange due to Evans blue counterstain.

Positive result

A serum dilution is considered positive for EmA IgA if the fluorescent staining is at an intensity of 1+ or greater with a clearly discernable pattern of fluorescence in the muscularis mucosae (staining of the endomysium around the smooth muscle fibers).

Titration

If semi-quantitative titration is performed, the result should be reported as the reciprocal of the last dilution in which 1+ apple-green fluorescent intensity with a clearly discernable staining pattern is detected.

Using the recommended fourfold serial dilution the endpoint titer can be extrapolated:

1:10	=	3+	
1:40	=	2+	
1:160	=	+/-	
1:640	=	-	The extrapolated titer is 80.

REFERENCE VALUES

EmA IFA	Titer
negative	< 10
positive	≥ 10

It is recommended that each laboratory establishes its own normal and pathological IgA EmA reference ranges for serum levels as usually done for other diagnostic parameters, too.

Test validity

Both the positive and negative control provided in the test kit must be included in each test run. These controls must be examined prior to reading test samples and should demonstrate the following results:

Negative control: The cells should exhibit less than 1+ fluorescence and appear reddish-orange due to the counterstain.

Positive control: Fluorescence of the muscularis mucosae and the muscle layer around the esophagus with an intensity of 3+ to 4+.

A titered positive control allows to check the test sensitivity as well as the reactivity of the reagents and microscope optical system. The endpoint titer stated on the label should be reproduced within one twofold difference in titer (+/-).

If the above mentioned quality criteria are not met, repeat the test and make sure that the test procedure is followed correctly (incubation times and temperatures, sample and wash buffer dilution, wash steps etc.). In case of repeated failure of the quality criteria contact your supplier. A troubleshooting guide is available to check laboratory procedure.

Limitations of Method

The detection of EmA IgA largely depends on the oesophagus part used. While little antigen activity may be detected in the upper and middle oesophagus parts due to the lack of smooth musculature, the lower part of the oesophagus employed in this test reacts with the endomysium antibodies.

False negative results may be caused in patients with a chronic lack of IgA. In infants also a retarded IgA formation can be observed.

Smooth muscle antibodies (SMA) should be considered and eliminated before reporting a positive EmA. SMA stains only the myofibril and not the network between them in which the endomysial antigen is found.

Antibodies to other parts of the tissue section could lead to a respective fluorescence pattern (e.g. cell nuclei, intercellular substance). These patterns are to be judged negative in relation to endomysial antibodies but can indicate other autoimmune diseases.

Endpoint titer determination may vary depending on type and condition of the fluorescence microscope used and depending on subjective judgement of different observers.

Samples and wash solutions contaminated with bacteria or fungi could cause unspecific staining of the cell culture substrate.

Proteolytic enzymes in patient samples could result in a damage or loss of the tissue sections fixed on the slide.

Any clinical diagnosis should not be based on the results of in vitro diagnostic methods alone. Physicians are supposed to consider all clinical and laboratory findings possible to state a diagnosis.

CHARACTERISTIC ASSAY DATA

Cross-reactivity

Cross-reactivity of other antibodies to the characteristic antigen structure are unknown.

Precision and Reproducibility

With this immunofluorescence assay, no difference in the interassay and Interlot variability by using the controls could be detected.

Remarks:

INCUBATION SCHEME

EmA IFA (86048)

Dilute patient sera: screening dilution / endpoint titration using PBS solution (made of C)

1	Bring all test reagents and slides to room temperature (20...25°C)		
		Controls	Patient samples
2	Dispense	Controls P, N	1 - 2 drops (30 - 50 µl)
		Diluted patient samples	30 - 50 µl
3	Incubate 30 minutes, room temperature (20...25°C)		
4	Rinse with PBS solution (made of C)		
5	Wash 2 x 5 minutes in changing PBS solution (made of C)		
6	Dispense Conjugate (D)	1 - 2 drops (30 - 50 µl)	1 - 2 drops (30 - 50 µl)
7	Incubate 30 minutes, room temperature (20-25°C)		
8	Rinse with PBS solution (made of C)		
9	Wash 2 x 5 minutes in changing PBS solution (made of C)		
10	Place coverslip; 3-4 drops Mounting medium (E) per slide, lower the coverslip (G) gently		
11	Read using a fluorescence microscope		

SAFETY PRECAUTIONS

- **This kit is for in vitro use only.** Follow the working instructions carefully. GA GENERIC ASSAYS GmbH and its authorized distributors shall not be liable for damages indirectly or consequentially brought about by changing or modifying the procedure indicated. The kit should be performed by trained technical staff only.
- The expiration dates stated on the respective labels are to be observed. The same relates to the stability stated for reconstituted reagents.
- The substrate slides are individually covered in a sealed pouch. Do not use if pouch has been punctured.
- Mixing of reagents from different kit lots and from other manufacturers could lead to differences in assay results.
- Avoid time shift during pipetting of reagents.
- All reagents should be kept at 2 - 8 °C before use in the original shipping container.
- Some of the reagents contain small amounts of Sodium azide (< 0.1 %) as preservative. They must not be swallowed or allowed to come into contact with skin or mucosa. Sodium azide may react with lead and copper plumbing building highly explosive metal azides. Flush with sufficient water when disposing of reagents to prevent potential residues in plumbing.
- Source materials derived from human body fluids or organs used in the preparation of this kit were tested and found negative for HBsAg and HIV as well as for HCV antibodies. However, no known test guarantees the absence of such viral agents. Therefore, handle all components and all patient samples as if potentially hazardous.
- Since the kit contains potentially hazardous materials, the following precautions should be observed:
 - Do not smoke, eat or drink while handling kit material,
 - Always use protective gloves,
 - Never pipette material by mouth,
 - Wipe up spills promptly, washing the affected surface thoroughly with a decontaminant.

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