INSTRUCTION MANUAL

REF **8049**

April, 01. 2014

Anti-MuSK IFA

- 60 determinations -

IVD *In-vitro* diagnostic device

CE

Indirect immunofluorescence test for the determination of antibodies against muscle specific tyrosine kinase (MuSK) in human serum.

REF	Catalog No.	**LOT**	Lot No.
	Consult accompanying documents		Manufacturer
	Storage temperature		Expiry date
	See instruction manual		Biological hazard

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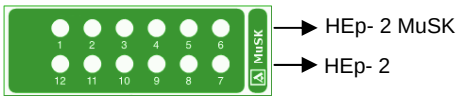
INTENDED USE

Anti-MuSK IFA is a reagent set for the qualitative and semi-quantitative measurement of antibodies against the MuSK antigen in human serum. For this HEp-2 cells, which have been transfected with MuSK antigens.

Myasthenia gravis (MG) belongs to the group of neurological diseases which are characterized by disturbed signal transmission between nerves and muscles. It is an autoimmune disease, caused by autoantibodes directed against the striated muscle of the motor end-plate. The disease picture is characterized by a load-dependant weakening of the skeletal muscle, which typically increases through the course of the day and goes through recovery phases. The cause of MG is antibodies directed against structures of the post-synaptic membrane, in the area of the neuromuscular end-plate. In the vast majority of cases (~ 85%), acetylcholine receptor antibodies are detectable. In 1 – 10% of cases, antibodies against muscle specific tyrosine kinase (MuSK) are detectable, and in some affected people the so-called low affinity acetylcholine receptor antibodies, or antibodies against the lipoprotein receptor-related protein (LRP4) are seen. There is a subset of patients, who are most likely suffering from myasthenia gravis, in whom no antibodies are detectable (seronegative myasthenia gravis) (1).

PRINCIPLE of the TEST

Anti-MuSK IFA is an indirect immunofluorescence test for the qualitative and semi-quantitative measurement of anti-MuSK antibodies, in which the top row is coated with HEp-2 MuSK cells (transfection rate ~ 40%), and the bottom row is coated with untransfected HEp-2 cells. To rule out unspecific reactions, every sample is tested in parallel on the HEp-2 MuSK and untransfected HEp-2 cells.



Antibodies in the diluted patient sample and controls specifically react in the first step with MuSK antigens in the HEp-2 MuSK cells fixed onto the slide. Unbound components are removed with a wash step following 30 minutes incubation at room temperature.

In the second reaction step the bound antibodies react specifically with anti-human antibodies (IgG and light chain specific) coupled with biotin. Excess conjugate molecules are separated from immunocomplexes bound to the fixed phase through a further wash step, following 30 minutes incubation at room temperature.

A third reaction step allows the detection of the biotin coupled anti-human antibodies using streptavidin Alexa Fluor 488. Excess streptavidin-fluorochrome molecules are separated from immunocomplexes bound to the fixed phase through a further wash step, following 30 minutes incubation at room temperature.

After covering, the slides are read using a fluorescence microscope (excitation wavelength 490nm, emission wavelength 520nm).

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.

Lipemic 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 use in the Anti-MuSK IFA assay. Take care to agitate serum samples gently in order to ensure homogeneity.

Screening: Patient samples are to be diluted 1:20 (v/v) with PBS-BSA buffer (from B+C) e.g. **10 µl sample + 190 µl PBS-BSA (B+C) buffer**.

Titration: **1:20** (v/v) diluted samples are to be further diluted, 2-fold, with PBS-BSA (B+C), e.g. **100 µl diluted sample + 100 µl PBS-BSA (B+C)** giving the following titration: 1:20, 1:40, 1: 80, 1:160 etc.

TEST COMPONENTS for 60 determinations

A (8041) Ag 12	**Slides** 6 wells coated with HEp-2 MuSK cells and 6 wells coated with HEp-2 cells		10 Vacuum sealed, with dessicant
B (8210) BUF BSA	**BSA** For 2 x 1000 ml solution		2 x 10 g Dry substance
C (9018) BUF PBS	**PBS Buffer** For 2 x 1000 ml solution		2 x 10 g Dry substance
D1 (8042) CONJ 1	**Conjugate 1** Anti-human-IgG (heavy and light chain specific), biotin labeled		2 x 2.5 ml Ready for use dropper bottle capped yellow
D2 (8048) CONJ 2	**Conjugate 2** Streptavidin-Alexa Fluor 488		2 x 2.5 ml Ready for use dropper bottle capped blue
E (8008) MOUNT	**Mounting Medium** Glycerine solution, phosphate buffered, pH 7.4 ± 0.2		3.0 ml Ready for use dropper bottle capped white
F (8046) TEMPL	**Blotting Templates**		10
P (8043) CONTROL	**Positive Control** Specification on the label (diluted human serum)	+	0.5 ml Ready for use dropper bottle capped red
N (8047) CONTROL	**Negative Control** (diluted human serum)	–	0.5 ml Ready for use dropper bottle capped green

Additional tools and materials required

- Adjustable micropipettes (10, 100, 1000 µl)
- Disposable ipette tips
- Sample dilution tubes
- Destilled (or deionized) water
- pH meter
- 1L measuring cylinder or measuring beaker
- Moist chamber
- Plastic squeeze wash bottle
- Coplin jar or staining dish
- Coverslips 24 x 60 mm
- Fluorescence microscope with excitation wavelength 490 nm and emission wavelength 520 nm, 10-fold objective (recommended)

Size and storage

Each Anti-MuSK IFA (8049) kit contains reagents sufficient for 60 determinations (10x6).

The expiry date of the complete kit is found on the label on the outside of the box. The expiry dates of individual reagents can exceed this, in some cases, and are displayed on the respective reagents. Until use, store all HEp-2 MuSK reagents at 2 - 8 °C. The dry substance PBS can be stored at room temperature.

After opening all kit components are stable for at least 2 months, provided proper storage at 2 - 8 °C.

Preparation and use

Allow slides to reach room temperature before opening their packaging.

To make up the PBS-BSA solution (B+C), empty the dry substances PBS (10g) and BSA (10g) into a beaker or measuring cylinder (1000ml) and fill to the mark with distilled or deionized water (**destilled water is recommended**). Dissolve dry substances fully by stirring or shaking. The dissolved solution must have a pH value of 7.4 ± 0.2 (adjust if necessary). The resulting PBS-BSA (B+C) buffer solution can be stored at room temperature in a sealed glass container. Cloudy, contamination solutions or those with changed pH values are to be discarded.
Protect the conjugate from light.

ASSAY PROCEDURE

- **Dilute patient samples as needed (screening, titration)**
- **Do not allow slides to dry out during processing**

1. Bring all test reagents to room temperature (RT, 20-25°C), remove slides from packaging immediately before use and label.
2. Pipette **25 µl** of the PBS-BSA solution (B+C) to each well. Do not touch the surface of the wells with the pipette tip.
3. Incubate slides for **10 Minuten** at RT in a moist chamber.
4. Remove excess PBS-BSA (B+C) by tapping onto absorbent paper.
5. Apply **1 drop (25 - 30 µl)** of the controls (P, N), and pipette **25 µl** of diluted patient serum to one well of the transfected cells (top row), and one well of the untransfected cells (bottom row). Do not touch the surface of the wells with the pipette tip.
6. Incubate slides for **30 minutes** at RT in a moist chamber.
7. Using a squeeze bottle, rinse slides with PBS-BSA (B+C) solution, without focusing the stream directly on the wells. To avoid cross contamination, the fluid should not be allowed to run over other wells. To achieve this with 12 well slides, run the stream along the centre of the slide, over first one row then the other.
8. Wash for **3 x 3 min** with fresh PBS-BSA (B+C) solution in a Coplin jar or staining dish, agitating the container lightly at the beginning and at each change.
9. Remove slides **individually**, tap onto absorbent paper to remove excess PBS-BSA (B+C), and carefully dry around the wells with blotting templates (F). Apply **1 drop (25 - 30 µl)** of conjugate 1 (D1) to each well, ensuring the surface is completely covered.
10. Incubate slides for **30 minutes** at RT in a moist chamber.
11. Wash slides as described in points 7 and 8.
12. Remove slides **individually**, tap onto absorbent paper to remove excess PBS-BSA (B+C), and carefully dry around the wells with blotting templates (F). Apply **1 drop (25 - 30 µl)** of conjugate 2 (D2) to each well, ensuring the surface is completely covered.
13. Incubate slides for **10 minutes** at RT in a moist chamber. Protect from direct light.
14. Wash slides as described in points 7 and 8.
15. Remove slides **individually**, tap onto absorbent paper to remove excess PBS-BSA (B+C), and carefully dry around the wells with blotting templates (F). Apply **1 small drop** of mounting medium (E) to each well of the slide. Carefully place a coverslip onto the slide, so that the mounting medium forms a continuous layer with no bubbles. Wipe any excess mounting medium from the bottom of the slide.
16. Read slides under a fluorescence microscope.

Preservation of slides

If it is not possible to read the slides immediately, they can be stored for a few days at 2-8°C. For conservation over a longer period, seal the edges of the coverslip with nail polish and store slides at–20°C.

READING / INTERPRETATION of the RESULTS

Fluorescence intensity

The fluorescence intensity is classified as follows:
3+ = brilliant yellow-green fluorescence
2+ = clear, but matt, yellow-green fluorescence
1+ = very weak, supressed fluorescence

Positive Result

A sample dilution is classified as positive if the Hep-2 MuSK cells demonstrate a fluorescence of 1+ or more of the cell membrane, visible as fluorescence staining of the whole cytoplasm of the cell (fig. 1). In addition there must be no fluorescence staining of the untransfected HEp-2 cells.

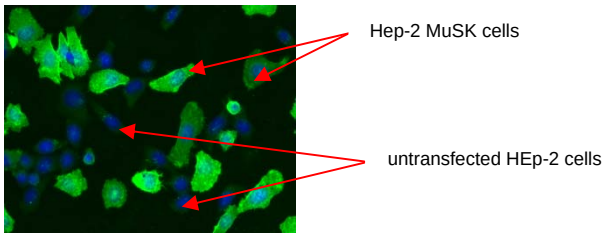


Fig. 1: Antibodies against MuSK on HEp-2 MuSK cells (green), counterstaining of the nuclei with DAPI (blue) (40x objective).

Figure 2 shows example images of sera from a myasthenia gravis patient and a healthy blood donor on HEp-2 MuSK and HEp-2 cells. The patient serum shows a clear fluorescence of the whole HEp-2 MuSK cell surface. Observe that not all cells carry the MuSK antigen. Conversely, the patient serum shows no fluorescence on the untransfected cells. The blood donor serum shows no or only very weak fluorescence on either cell type.

Fig. 2.: Example images of one MuSK antibody positive and one negative serum on HEp-2 MuSK (upper row of the slide) and untransfected HEp-2 cells (lower row of the slide) (10x objective).

The intensity of the fluorescence does not reflect the antibody concentration, and has no clinical relevance. Differences in optics, filters and light sources between various microscopes can give rise to differences of more than one classification stage in the fluorescence intensity.

Negative Result

A sample dilution is classified as negative if the HEp-2 MuSK cells demonstrate fluorescence less than 1+ and have no detectable pattern.

The presence of antibodies against other components of HEp-2 cells besides the MuSK antigen (eg. ANA, AMA) gives rise to fluorescence on both the transfected and untransfected cells. Such images are not indicative of the presence of MuSK antibodies.

Titration

With a semi-quantitative titration, the result is given as the last dilution step where 1+ fluorescence is detectable. The titer is the reciprocal value of this dilution.

1:20 = 3+
1:40 = 2+
1:80 = +
1:160 = - the titer is 80.

REFERENCE VALUES

Anti-MuSK IFA	Titer
Negative	**< 20**
Positive	**≥ 20**

Due to differences between populations, it is recommended that each laboratory establishes its own normal and pathological reference ranges for anti-MuSK antibody levels. The values given above are intended only as a guide.

Test validation

A positive and a negative control must be included in each test run. The controls included in test kits must show the following results:

Negative control: Fluorescence less than 1+
Positive control: positive staining of the HEp-2 MuSK cells with fluorescence 2+.

If the controls do not display the expected results, the test is invalid and must be repeated. Make sure that the test is executed exactly according to the instructions (correct reagent preparation, incubation times and temperatures, careful washing). If the validation criteria are not met with the repeated test, please contact your supplier. An information sheet for troubleshooting is available on request.

Limitations of the method

A clinical diagnosis should not be made based on the results of in-vitro diagnostic methods alone. Clinicians should consider all clinical and laboratory findings possible to state a diagnosis.

The determination of titer end points is dependent on the type and condition of the fluorescence microscope used the magnification of the objective, and the subjective judgment of the observer.

Samples or wash buffer solutions contaminated with bacteria can give rise to unspecific staining of the cell substrate.

TEST CHARACTERISTICS

The relative sensitivity and specificity of the novel recombinant cell assay in our cohort (MuSK antibody positive patients = 38; AChR antibody positive patients = 46, healthy controls = 60) was assessed in comparison to MuSK ELISA as 95 % and 99.1%, respectively

ASSAY SCHEME

Anti-MuSK IFA (8049)

Dilute patient samples according to requirements:
Screening dilution / titration dilution with PBS-BSA (B+C) solution

1	Bring all test reagents and slides to room temperature (20-25°C)		
2	Pipette 25 µl PBS-BSA solution (B+C) to all wells		
3	Incubate	10 minutes, room temperature (20-25°C)	
4	Tap on absorbant paper		
		Controls	Patient samples
5	Pipette Controls P, N	1 drop (25 – 30 µl)	
	Prediluted sera		25 µl
6	Incubate	30 minutes, room temperature (20-25°C)	
7	Wash with PBS-BSA (B+C) solution		
8	Wash	3 x 3 min in fresh PBS-BSA (B+C) solution	
9	Pipette conjugate 1 (D1)	1 drop (25 - 30 µl)	1 drop (25 - 30 µl)
10	Incubate	30 minutes, room temperature (20-25°C)	
11	Wash with PBS-BSA (B+C) solution		
12	Wash	3 x 3 min in fresh PBS-BSA (B+C) solution	
13	Pipette conjugate 2 (D2)	1 drop (25 - 30 µl)	1 drop (25 - 30 µl)
14	Incubate	10 minutes, room temperature (20-25°C)	
15	Wash with PBS-BSA (B+C) solution		
16	Wash	3 x 3 min in fresh PBS-BSA (B+C) solution	
17	Mount; 1 drop mounting medium (E) per well, carefully place coverslip		
18	Read using a fluorescence microscope		

SAFETY PRECAUTIONS

- This kit is exclusively for *in vitro* use and should only be performed by trained laboratory personnel. Follow the instructions strictly. The test kit and its individual reagents must be used before their stated expiry dates.
- Mixing of components from different lots, or using reagents from other manufacturers, can lead to altered results.
- Some reagents contain small amounts of thimerosal (< 0.005 %) or sodium azide (< 0.1%) as preservatives. Do not swallow reagents, and avoid contact with mucus membranes.
- The recommended storage temperature of opened reagents until their next use is 2 - 8 °C.
- All reagents from this test kit of human origin have been tested for HBsAg (Hepatitis B surface Antigen) as well as antibodies against HIV (Human Immunodeficiency Virus) and HCV (Hepatitis C Virus) with negative results. Nevertheless, no test can completely exclude the presence of infectious agents. Therefore, all reagents should be treated as potentially infectious.
- When handling the components of the test kit, patient samples and controls, all health and safety regulations, as well as those covering the handling of potentially infectious material and hazardous chemicals must be observed. The following regulations in particular should be followed:
  - Do not eat, drink or smoke!
  - Do not pipette by mouth!
  - Wear gloves to prevent contact with reagents and sera!
  - Observe safety warnings on individual components!
- We recommend that laboratories perform their own quality assurance using internal control sera (see regulation of German federal law gazette I, p. 1657, as well as the recommendations of the German Medical Association, Dt. Ärzteblatt 98, issue 42, p. A2747-59, 2001).

LITERATURE

(1) N. E. Gilhus: *Myasthenia and the neuromuscular junction.* In: *Current opinion in neurology.* Volume 25, Number 5, October 2012, S. 523–529