DIAGNOSTIKA GMBH

Instructions for Use

Aquaporin-4 (AQP4) Ab ELISA

Enzyme Immuno Assay
for the Quantitative Determination of
AQP4 Autoantibodies in Serum

CE

IVD

REF EA111/96

Σ 12 x 8

+2 +8 °C 2 – 8 °C

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1. Introduction and Principle of the Test

The AQP4 autoantibody ELISA assay kit is intended for use by professional persons only, for the quantitative determination of AQP4 autoantibodies in human serum. Neuromyelitis optica (NMO), also known as Devic's syndrome, is an immune-mediated neurologic disease that involves the spinal cord and optic nerves. It can be considered to be a disorder distinct from multiple sclerosis (MS). A serum immunoglobulin G autoantibody (NMO-IgG) has been shown to be a specific marker for NMO and the water channel aquaporin 4 (AQP4) has been identified as the antigen for NMO IgG. Measurement of AQP4 autoantibodies can be of considerable value in distinguishing NMO from MS when full clinical features may not be apparent and early intervention may prevent or delay disability.

In AQP4 autoantibody ELISA, AQP4 antibodies in patients' sera, calibrators and controls are allowed to interact with AQP4 coated onto ELISA plate wells and liquid phase biotinylated AQP4. AQP4 antibodies bound to the AQP4 coated on the well will also interact with AQP4-Biotin due to the divalent nature of antibodies. After incubation at room temperature for 2 hours with shaking, the well contents are discarded, leaving AQP4-Biotin bound to the well via an AQP4 autoantibody bridge. The amount of AQP4-Biotin bound is then determined in a second incubation step involving addition of streptavidin peroxidase (SA-POD), which binds specifically to biotin. Excess, unbound streptavidin peroxidase is then washed away and addition of the peroxidase substrate 3,3',5,5'-tetramethlybenzidine (TMB) results in formation of a blue colour. This reaction is stopped by the addition of a stop solution, causing the well contents to turn yellow. The absorbance of the yellow reaction mixture at 450nm and 405nm is then read using an ELISA plate reader. A higher absorbance indicates the presence of AQP4 autoantibody in the test sample. Reading at 405nm allows quantitation of high absorbances. Values below 10 U/ml should always be measured at 450nm. The measuring interval is 3.0 – 80 U/ml (arbitrary units).

Manufactured under licence to US patents 7,101,679 and 7,947,254, European patent 1700120, Chinese patent ZL200480040851.3, Japanese patent 4538464 and related patents and patents pending in other countries.

2. Precautions

- For in vitro use only.
- Some reagents contain sodium azide as preservative (<0.1%). Avoid skin contact.
- All reagents of human origin used in this kit are tested for HIV I/II antibodies, HCV and HBsAg and found to be negative. However, because no test method can offer complete assurance that infectious agents are absent, these reagents should be handled as potentially biohazardous materials.
- Material of animal origin used in the preparation of the kit have been obtained from certified healthy animals but these materials should be handled as potentially infectious.

3. Storage and Stability

On arrival, store the kit at 2-8 °C. Once opened the kit is stable until its expiry date. For stability of prepared reagents refer to Preparation of Reagents.

4. Contents of the Kit

4.1 **AQP4 Coated Wells** **STRIPS** 12 strips
8 wells per strip
coated with AQP4

4.2 **Calibrators A – E** **CAL A** – **CAL E** 5 vials
0.7 ml each, ready for use

Concentrations (arbitrary Units):

Calibrator	A	B	C	D	E
U/ml	1.5	5	20	40	80

4.3 **Positive Controls** **CONTROL I** + **CONTROL II** 2 vials
0.7 ml each, ready for use
values for the Controls are given on the QC certificate

4.4	**Negative Control** 0.7 ml, ready for use	**CON -**	1 vial
4.5	**AQP4-Biotin** 1.5 ml per vial, lyoph.	**AQP4-BIOTIN**	3 vials
4.6	**Reconstitution Buffer** 10 ml, ready for use for reconstituting AQP4-Biotin	**RECONST**	1 vial
4.7	**Streptavidin-Peroxidase (SA-POD)** 0.8 ml, 20 x concentrated	**SA-POD**	1 vial
4.8	**Diluent** 15 ml, ready for use for diluting SA-POD	**DIL**	1 vial
4.9	**Substrate** 15 ml, tetramethyl benzidine (TMB), ready for use	**SUB**	1 vial
4.10	**Wash Buffer** 120 ml, 10 x concentrated	**WASH**	1 vial
4.11	**Stop Solution** 14 ml, ready for use	**STOP**	1 vial

Additional materials and equipment required but not provided:

- Pipettes for 25 µl, 50 µl and 100 µl
- ELISA plate cover
- ELISA plate shaker capable of 500 shakes per min (not an orbital shaker)
- Pure water
- Microtiter plate reader (450 nm and 405 nm)

5. Specimen Collection and Storage

Sera to be analysed should be assayed soon after separation or stored (preferably in aliquots) at or below -20 ℃.
100 µl is sufficient for one assay. Subsequent freezing and thawing or increase in storage temperature should be avoided. Incorrect storage of serum samples can lead to loss of AQP4 autoantibodies. Do not use grossly haemolysed or lipaemic serum samples. Citrate, EDTA and heparin plasma may be used in the assay.

When required, thaw test sera at room temperature and mix gently to ensure homogeneity. Centrifuge the serum prior to assay (preferably for 5 min at 10-15,000 g in a microfuge) to remove any particulate matter. Please do not omit this centrifugation step if sera are cloudy or contain particulates.

6. Test Procedure

6.1. Preparation of Reagents

MT strips **STRIPS**
Before opening the packet of strip wells, allow it to stand at room temperature for 30 minutes. After opening, return any unused wells in the original foil packet (reseal with adhesive tape) and in the self-seal plastic bag with the desiccant provided. Store at 2-8 ℃ fo r up to 4 months.

AQP4-Biotin **AQP4-BIOTIN**
Immediately before use, reconstitute the contents of one vial with 1.5 ml Reconstitution Buffer. If more than 1 vial of AQP4-Biotin is going to be used, pool the contents of each vial after reconstitution and mix gently before use. Discard any unused reagent, storage and subsequent use of the prepared AQP4-Biotin is not possible.

Streptavidin-Peroxidase (SA-POD) **SA-POD**
Dilute the concentrate 1 in 20 with the Diluent provided (e.g. 0.5 ml SA-POD + 9.5 ml Diluent). Store at 2-8 ℃ for up to 16 wee ks after dilution.

Wash Buffer **WASH**
Dilute 1 in 10 with pure water before use. Store at 2-8 ℃ after dilution up to kit expiry date.

6.2. Assay Procedure

Calculate the number of individual ELISA plate wells needed for the assay. Allow all the reagents supplied, including the appropriate number of packets of strips, to reach room temperature (at least 30 min), remove the number of strip wells required and fit them firmly into the frame provided. Negative and Positive Controls in duplicate must always be included in each assay run.

1. Pipette each 50 µl (in duplicate) of Calibrators A - E, Negative and Positive Controls and test sera into the appropriate wells. Leave one well empty for blank.

2. Pipette 25 µl of reconstituted AQP4-Biotin into each well (except blank), cover the plate and incubate for 2 hours at room temperature (20 – 25 ℃) with shaking on an ELISA plate shaker (500 shakes per min).

3. After the 2 hour incubation with AQP4-Biotin, aspirate or discard the reagent from the wells, and wash the wells three times with diluted Wash Buffer. Tap the inverted wells gently on a clean dry absorbent surface to remove any droplets of Wash Buffer.

4. Pipette 100 µl of ready-for-use diluted SA-POD into each well (except blank). Cover the plate and incubate for 20 minutes at room temperature (20 – 25 ℃) with shaking on an ELISA plate shaker (500 shakes per min).

5. Aspirate or discard the reagent from the wells and wash twice with Wash Buffer followed by one wash with pure water to remove any foam from the wells. If a plate washing machine is used, wash 3 times with Wash Buffer only (i.e. omit water wash). Tap the inverted wells gently on a clean dry absorbent surface to remove any droplets of Wash Buffer.

6. Pipette 100 µl of Substrate (TMB) into each well (including blank) and incubate for 20 minutes at room temperature in the dark without shaking.

7. Stop the substrate reaction by addition of 100 µl of Stop Solution to each well (including blank) and shake the plate for about 5 seconds on a plate shaker to ensure uniformity of the solution in each well.

8. Read the absorbance at 450 nm and at 405 nm within 5 to 10 minutes after addition of Stop Solution using an ELISA plate reader blanked against a well containing 100 µl Substrate plus 100 µl of Stop Solution only.

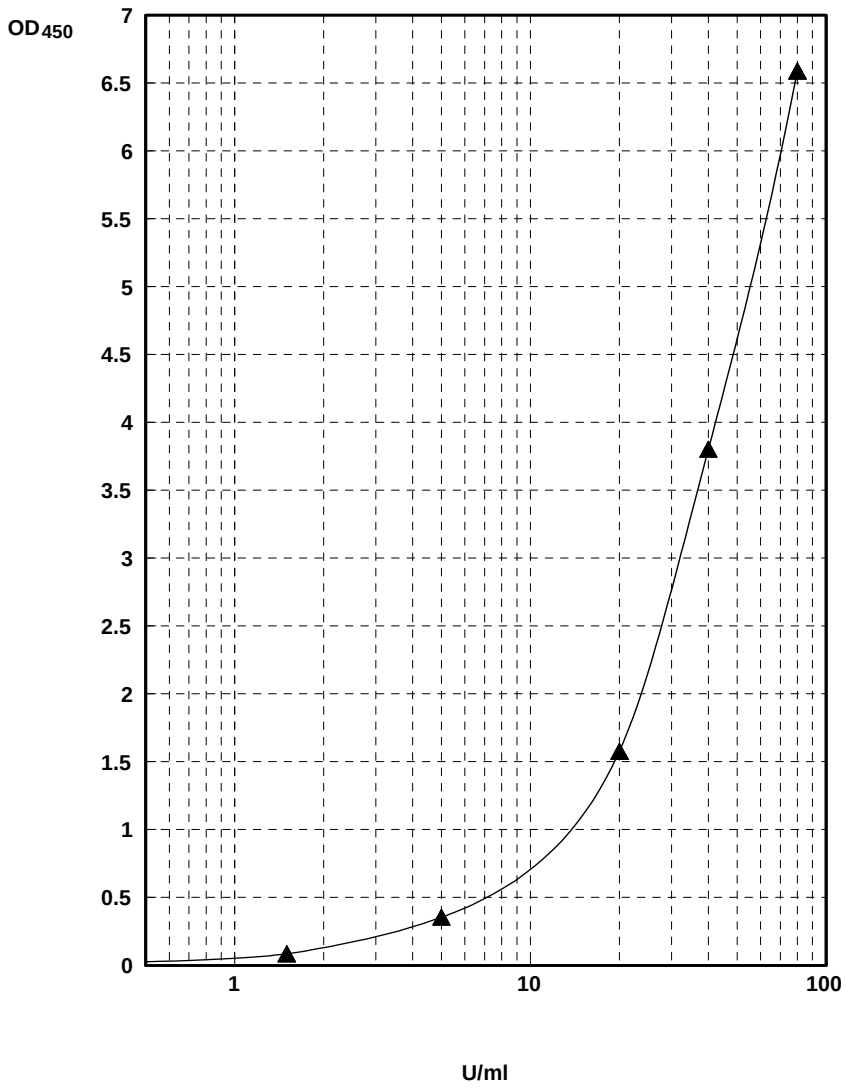
7. Calculation of Results

A calibration curve can be established by plotting calibrator concentration on the x-axis (log scale) against the absorbance of the calibrators on the y-axis (linear scale). The AQP4 autoantibody concentrations in patient sera can then be read off the calibration curve. Other data reduction systems can be used. The negative control can be assigned a value of 0.15 U/ml to assist in computer processing of assay results.
Samples with AQP4 autoantibody concentrations above 80 U/ml can be diluted (e.g 10 x and/or 100 x) in AQP4 antibody negative serum. Some sera will not dilute in a linear way

Typical Example

Typical results are shown in the following table.

	OD 450 nm	Conc. U/ml
Negative Control	0.021	
Calibrator A	0.085	1.5
Calibrator B	0.354	5
Calibrator C	1.577	20
Calibrator D	3.802	40
Calibrator E	6.588	80
Control I	0.755	12.0
Control II	2.506	28



8. Clinical Evaluation

Expected Values

Negative	< 3.0 U/ml
Positive	≥ 3.0 U/ml

Clinical Specificity

Samples from 358 individual healthy blood donors were analysed in the AQP4 Ab ELISA. All 356 (99%) healthy controls were identified as being negative for AQP4 autoantibodies.

Clinical Sensitivity
Of 62 sera from patients with neuromyelitis optica NMO or NMO spectrum disorder (NMOSD) 48 (77%) were positive for AQP4 Ab.

Clinical Accuracy
Analysis of 205 sera from patients with autoimmune diseases other than neuromyelitis optica spectrum disorders (NMOSD) indicated no interference from autoantibodies to TSH receptor (n=110), glutamic acid decarboxylase (n=26), 21-hydroxylase (n=12) or the acetylcholine receptor (n=10), thyroid peroxidase (n=15), thyroglobulin (n=10), IA2 (n=7) or from rheumatoid factor (n=15) in the AQP4 Ab ELISA.

Lower Detection Limit
The negative control was assayed 20 times and the mean and standard deviation calculated. The lower detection limit at 2 standard deviations was 0.17 U/ml.

Interference
No interference was observed when samples were spiked with the following materials; bilirubin up to 20 mg/dl or intralipid up to 3,000 mg/dl. Interference was seen from haemoglobin at 500 mg/dl.

Intra-Assay Precision (n=25)

sample	mean U/ml	cv (%)
1	3.9	7.7
2	7.0	8.6
3	28	3.2
4	58	3.1

Inter-Assay Precision (n=20)

sample	mean U/ml	cv (%)
1	5.0	15.6
2	13.3	10.5
3	35	7.9
4	59	7.5

9. Literature

V. A. Lennon et al.
A serum autoantibody marker of neuromyelitis optica: distinction from multiple sclerosis.
Lancet 2004; 364(9451): 2106 - 2112

V. A. Lennon et al.
IgG marker of optic-spinal multiple sclerosis binds to the aquaporin-4 water channel.
The Journal of Experimental Medicine 2005; 202: 473 - 477

N. Isobe et al.
Quantitative assays for anti-aquaporin-4 antibody with subclass analysis in neuromyelitis optica.
Multiple Sclerosis Journal 2012
DOI: 10.1177/1352458512443917

S. Jarius et al.
Testing for antibodies to human aquaporin-4 by ELISA: Sensitivity, specificity and direct comparison with immunohistochemistry.
Journal of the Neurological Sciences 2012 320: 32 - 37

Pipetting Scheme

		B_0	Calibrator A - E	Positive Control I/II	Patient Sample
Negative Control	µl	50			
Calibrators	µl		50		
Positive Controls	µl			50	
Patient Serum	µl				50
AQP4-Biotin	µl	25	25	25	25

Incubate for 2 hours at RT on a shaker 500 shakes/min

Aspirate / discard and wash three times with each 300 µl Wash Buffer

SA-POD	µl	100	100	100	100

Incubate for 20 min at room temperature on a shaker 500 shakes/min

Aspirate / discard and wash twice with each 300 µl Wash Buffer
Wash once with 300 µl pure water

TMB-Substrate	µl	100	100	100	100

Incubate for 20 minutes at RT in the dark without shaking

Stop Solution	µl	100	100	100	100

5 sec shaking on an ELISA plate shaker

Reading of absorbance at 450 nm and 405 nm within 5-10 minutes