RSR **ElisaRSR™ AQP4 Ab**

Aquaporin-4 (AQP4) Autoantibody ELISA Kit - Instructions for use

CE

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INTENDED USE

The RSR 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.

REFERENCES

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

B. G. Weinshenker et al.
Neuromyelitis optica IgG predicts relapse after longitudinally extensive transverse myelitits.
Annals of Neurology 2006 59: 566 - 569

ASSAY PRINCIPLE

In RSR's 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 3,3', 5,5'-tetramethlybenzidine substrate (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 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. Low values (below 10 u/mL) should always be measured at 450nm. The measuring range is 5 – 160 u/mL (arbitrary RSR units).

STORAGE AND PREPARATION OF SERUM SAMPLES

Sera to be analysed should be assayed soon after separation or stored, preferably in aliquots, at or below –20°C. 100 µL is sufficient for one assay (duplicate 50 µL determinations). Repeated freeze thawing or increases in storage temperature should be avoided. Do not use lipaemic or haemolysed serum samples. Do not use plasma in the assay. When required, thaw test sera at room temperature and mix gently to ensure homogeneity. Centrifuge serum prior to assay (preferably for 5 min at 10-15,000 g in a microfuge) to remove particulate matter. Please do not omit this centrifugation step if sera are cloudy or contain particulates.

IFU SYMBOLS

Symbol	Meaning
CE	EC Declaration of Conformity
IVD	In Vitro Diagnostic Device
REF	Catalogue Number
LOT	Lot Number
	Consult Instructions
	Manufactured By
	Expiry Date
	Store

MATERIALS REQUIRED AND NOT SUPPLIED

Pipettes capable of dispensing 25 µL, 50 µL and 100 µL.
Means of measuring various volumes to reconstitute or dilute reagents supplied.
Pure water.
ELISA Plate reader suitable for 96 well formats and capable of measuring at 450nm and 405nm.
ELISA Plate shaker, capable of 500 shakes/min (not an orbital shaker).
ELISA Plate cover.

PREPARATION OF REAGENTS SUPPLIED

Store unopened kit and components at 2-8°C.

A	**AQP4 Coated Wells** 12 breakapart strips of 8 wells (96 in total) in a frame and sealed in foil bag. Allow foil bag to stand at room temperature (20-25°C) for 30 minutes before opening.
	Ensure stripwells are firmly fitted in the frame provided. After opening return any unused wells to the original foil packet with desiccant provided and seal with adhesive tape. Place foil bag in the self-seal plastic bag and store at 2-8°C for up to kit expiry date.
B1-4	**Calibrators** 5, 30, 75, 160 u/mL (arbitrary RSR units) 4 x 0.7 mL Ready for use
C1-2	**Positive Controls** **(see label for concentration range)** 2 x 0.7 mL Ready for use
D	**Negative Control** 1 x 0.7 mL Ready for use
E	**AQP4–Biotin** 3 vials Lyophilised
	Immediately before use, reconstitute with AQP4-Biotin reconstitution buffer (F), 1.5 mL per vial. When more than one vial is to be used, pool the vials and mix gently.
F	**AQP4-Biotin Reconstitution Buffer** 1 x 10 mL Ready for use
G	**Streptavidin Peroxidase (SA-POD)** 1 x 0.8 mL Concentrated
	Dilute 1 in 20 with diluent for diluting SA-POD (H). For example, 0.5 mL (G) + 9.5 mL (H). Store for up to 16 weeks at 2-8°C after dilution.
H	**Diluent for SA-POD** 1 x 15 mL Ready for use
I	**Peroxidase Substrate (TMB)** 1 x 15 mL Ready for use
J	**Concentrated Wash Solution** 1 x 120 mL Concentrated
	Dilute 1 in 10 with pure water before use. Store at 2-8°C up to kit expiry date.
K	**Stop Solution** 1 x 14 mL Ready for use

ASSAY PROCEDURE

Allow all reagents to stand at room temperature (20-25°C) for at least 30 minutes prior to use. Do not reconstitute AQP4-Biotin until step 2 below. An Eppendorf type repeating pipette is recommended for steps 2, 5, 8, and 9.

1.	Pipette **50 µL** (in duplicate) of patient sera, calibrators (B1-4) and controls (C1-2 and D) into respective plate wells. Leave one well empty for blank.
2.	Reconstitute AQP4-Biotin and pipette **25 µL** into each well (except blank).
3.	Cover the frame and shake the wells for 2 hours at room temperature on an ELISA plate shaker (500 shakes per min).
4.	Use an ELISA plate washer to aspirate and wash the wells three times with diluted wash solution (J). If a plate washer is not available, discard the well contents by briskly inverting the frame of stripwells over a suitable receptacle, wash three times manually and tap the inverted wells gently on a clean dry absorbent surface to remove excess wash.
5.	Pipette **100 µL** of diluted SA-POD (G) into each well (except blank).
6.	Cover the plate and incubate for 20 minutes at room temperature on an ELISA plate shaker (500 shakes per min).
7.	Repeat wash step 4. If manual washing is being carried out, use pure water for the final wash step (to remove any foam) before tapping the wells dry.
8.	Pipette **100 µL** of TMB (I) into each well (including blank) and incubate for 20 minutes in the dark at room temperature without shaking.
9.	Pipette **100 µL** stop solution (K) to each well (including blank) and shake the plate for approximately 5 seconds on a plate shaker. Ensure substrate incubations are the same for each well.
10.	Read the absorbance of each well at 450nm and 405nm (within 5-10 minutes of completing step 9.) using an ELISA plate reader, blanked against a well containing **100 µL** of TMB substrate (I) and **100 µL** Stop solution (K) **only**.

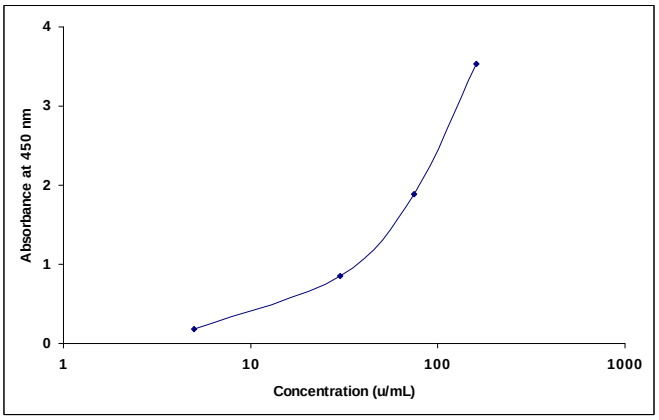
RESULT ANALYSIS

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. Samples with AQP4 autoantibody concentrations above 160 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 RESULTS (Example only; not for calculation of actual results)

	Abs. 450 nm	Conc. u/mL
Negative Control (D)	0.005	
B1	0.177	5
B2	0.854	30
B3	1.888	75
B4	3.539	160
Positive Control (CI)	0.439	12.4
Positive Control (CII)	1.095	40.6

Absorbance readings at 405nm can be converted to 450nm absorbances by multiplying by the appropriate factor (3.4 in the case of equipment used at RSR).



ASSAY CUT OFF

Negative	< 5.0 u/mL
Positive	≥ 5.0 u/mL

CLINICAL EVALUATION

Clinical Specificity

Samples from 216 individual healthy blood donors were analysed in the AQP4 Ab ELISA. All 216 (100%) healthy controls were identified as being negative for AQP4 autoantibodies.

Clinical Sensitivity

Samples from 62 patients with positive NMO IgG in the immunofluorescence test were assayed in the AQP4 Ab ELISA. 61 (98%) were identified as being positive for AQP4 autoantibodies.

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 0.16 u/mL.

Inter Assay Precision (n=20)

Sample	u/mL	CV (%)
1	55.7	8.6
2	9.8	6.4

Intra Assay Precision (n=25)

Sample	u/mL	CV (%)
1	57.3	4.5
2	10.5	8.0

Clinical Accuracy

None of 26 multiple sclerosis patients, nor 216 patients with autoimmune diseases other than NMO except 1 (out of 102) patient with Graves' disease and 1 (out of 36) patient with myasthenia gravis were seropositive for AQP4 Ab. This indicated no interference from autoantibodies to TSH receptor, glutamic acid decarboxylase, 21-hydroxylase, acetylcholine receptor or dsDNA in the AQP4 Ab ELISA.

Interference

No interference was observed when samples were spiked with the following materials; haemoglobin up to 500 mg/dL, bilirubin up to 20 mg/dL or intralipid up to 3000 mg/dL.

SAFETY CONSIDERATIONS

This kit is intended for use by professional persons only. Follow the instructions carefully. Observe expiry dates stated on the labels. Refer to Materials Safety Data Sheet for more detailed safety information. Material of human origin used in the preparation of the kit has been tested and found non-reactive for HIV1 and 2 and HCV antibodies and HBsAg but should, none-the-less, be handled as potentially infectious. Wash hands thoroughly if contamination has occurred and before leaving the laboratory. Sterilise all potentially contaminated waste, including test specimens before disposal. Material of animal origin used in the preparation of the kit has been obtained from animals certified as healthy but these materials should be handled as potentially infectious. Some components contain small quantities of sodium azide as preservative. With all kit components, avoid ingestion, inhalation, injection and contact with skin, eyes and clothing. Avoid formation of heavy metal azides in the drainage system by flushing any kit component away with copious amounts of water.

ASSAY PLAN

Allow all reagents and samples to reach room temperature (20-25 ^{o}C) before use	
Pipette:	**50 µL** calibrators, controls and patient sera.
Pipette:	**25 µL** AQP4-Biotin (reconstituted) into each well (except blank).
Incubate:	2 hours at room temperature on an ELISA plate shaker at 500 shakes/min.
Aspirate/Decant:	Plate.
Wash:	Plate three times and tap dry on absorbent material.[1]
Pipette:	**100 µL** SA-POD (diluted 1:20) into each well (except blank).
Incubate:	20 minutes at room temperature on a ELISA plate shaker at 500 shakes/min.
Aspirate/Decant:	Plate
Wash:	Plate three times and tap dry on absorbent material.[1, 2]
Pipette:	**100 µL** TMB into each well (including blank).
Incubate:	20 minutes at room temperature **in the dark** without shaking.
Pipette:	**100 µL** stop solution into each well (including blank) and shake for 5 seconds.
Read absorbance at 450nm and 405nm promptly (i.e. within 5-10 minutes).	

[1]It is not necessary to tap the plates dry after washing when an automatic plate washer is used.

[2]Use pure water for the final wash when washing manually.