ElisaRSR™ Fast AChRAb

Fast Acetylcholine Receptor Autoantibody ELISA Kit – Instructions for use

RSR Limited
Parc Ty Glas, Llanishen, Cardiff
CF14 5DU United Kingdom
Tel.: +44 29 2068 9299 Fax: +44 29 2075 7770
Email: info@rsrltd.com Website: www.rsrltd.com

EC REP Advena Ltd. Tower Business Centre, 2nd Flr., Tower Street, Swatar, BKR 4013 Malta.

INTENDED USE

The RSR Fast Acetylcholine Receptor Autoantibody (AChRAb) ELISA kit is intended for use by professional persons only, for the quantitative determination of AChRAb in human serum.

Autoantibodies to the acetylcholine receptor (AChR) are responsible for failure of the neuromuscular junction in myasthenia gravis. Measurement of these antibodies can be of considerable value in disease diagnosis and management.

REFERENCES

R. Hewer et al
A sensitive non-isotopic assay for acetylcholine receptor autoantibodies
Clinica Chimica Acta 2006 **364**: 159 – 166

ASSAY PRINCIPLE

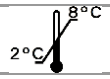
RSR's Fast AChRAb ELISA depends on the ability of AChRAb in human serum to bind to similar sites on the AChR as various monoclonal antibodies such as MAb1 (coated on ELISA plate wells) and/or MAb2 and/or MAb3 (which are labelled with Biotin). In the absence of AChRAb a complex is formed between MAb1 coated on the plate wells, the AChR and MAb2- and MAb3-Biotin. MAb2- and MAb3-Biotin bound are then detected by addition of Streptavidin Peroxidase (SA-POD), which binds specifically to Biotin. Excess, unbound SA-POD is then washed away and addition of the peroxidase substrate 3,3',5,5' – tetramethylbenzidine (TMB) results in formation of a blue colour. This reaction is stopped by addition of stop solution causing the well contents to turn yellow. The absorbance of the yellow reaction mixture at 450 nm is then read using an ELISA plate reader. In the presence of AChRAb the formation of the MAb1-AChR-MAb2-/MAb3-Biotin complex is inhibited, resulting in less SA-POD being bound and a reduction in final absorbance at 450 nm. The higher the concentration of AChRAb in the test serum, the greater the inhibition of MAb-Biotin binding.

This Fast AChRAb ELISA kit assay can be performed in a day with incubation time of 5 hours and without refrigeration. It may be particularly suitable for users with automated ELISA processors.

STORAGE AND PREPARATION OF TEST SERUM SAMPLES

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

SYMBOLS

Symbol	Meaning
CE	EC Declaration of Conformity
IVD	In Vitro Diagnostic Device
REF	Catalogue Number
LOT	Lot Number
	Consult Instructions
	Manufactured by
	Sufficient for
	Expiry Date
	Store
CONTROL -	Negative Control
CONTROL +	Positive Control

MATERIALS REQUIRED AND NOT SUPPLIED

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

PREPARATION OF REAGENTS SUPPLIED

Store unopened kit and components (A – P) at 2–8°C

A	**AChR MAb1 Coated Wells** 12 breakapart strips of 8 wells (96 in total) in a frame and sealed in a foil bag. Allow foil bag to stand at room temperature (20–25 °C) for 30 minutes before opening.
	Ensure wells are firmly fitted in the frame provided. After opening return any unused wells to the original foil bag and seal with adhesive tape. Then place foil bag in the self-seal plastic bag with desiccant provided, and store at 2-8°C for up to kit expiry date.

B	**Foetal Type AChR** 3 vials Lyophilised
	Reconstitute each vial with 0.7 mL reconstitution buffer for AChR (D). Mix gently, and leave to stand at room temperature (20–25 °C) for 5 minutes before use. Pool the vials when more than one vial is required, and then use immediately to reconstitute adult type AChR.
C	**Adult Type AChR** 3 vials Lyophilised
B+C	Reconstitute each vial of C with 0.5 mL of reconstituted foetal type AChR (B) to give a mixture of foetal and adult AChR (B+C). Mix gently, and leave to stand at room temperature (20–25 °C) for 5 minutes before use. Pool the vials when more than one vial is required. Use up to 6 hours after reconstitution if stored at 2-8°C[1].
D	**Reconstitution Buffer for AChR** 5 mL Ready for use
E	**AChR MAb–Biotin (MAb2+MAb3)** 3 vials Lyophilised
	Reconstitute each vial with the volume of reconstitution buffer for MAb-Biotin (F) shown on the vial label. Mix gently, and leave to stand at room temperature (20–25 °C) for 5 minutes before use. Pool the vials when more than one vial is required. Store at 2-8°C for up to kit expiry date after reconstitution.
F	**Reconstitution Buffer for MAb-Biotin** 15 mL Ready for use
G	**Streptavidin Peroxidase (SA-POD)** 0.7 mL Concentrated
	Dilute 1 in 20 with diluent for SA-POD (H). For example, 0.5 mL (G) + 9.5 mL (H). Store at 2–8°C for up to 16 weeks after dilution.
H	**Diluent for SA-POD** 15 mL Ready for use
J	**Peroxidase Substrate (TMB)** 15 mL Ready for use
K	**Stop Solution** 10 mL Ready for use
L	**Concentrated Wash Solution** 100 mL Concentrated
	Dilute 10 X with pure water before use. For example, 100 mL (L) + 900 mL pure water. Use up to kit expiry date after dilution.
M1-4	**Calibrators** 0.5, 1.0, 6.5 and 20 nmol/L toxin bound 4 x 0.7 mL Ready for use
N	**Negative Control** 3 mL Ready for use
P1-2	**Positive Controls I & II** **(see label for concentration range)** 2 x 0.7 mL Ready for use

[1]The absorbance at 450 nm will be 10-15% lower when reconstituted receptors have been stored for 6 hours at 2-8°C.

ASSAY PROCEDURE

Allow all reagents to stand at room temperature (20-25°C) for at least 30 minutes prior to use. A repeating Eppendorf type pipette is recommended for steps 2, 5, 7, 9 and 10.

1	Pipette **100 µL** of samples [calibrators (M 1-4 – optional), positive controls (P 1-2), and negative control (N) and test sera] into individual 1.5 mL Eppendorf tubes, labelled accordingly.
2	Pipette **25 µL** of foetal and adult type AChR mixture (B+C) into each Eppendorf tube (from step 1) and seal the tubes. Make sure that all liquid is in the bottom of each tube (if in doubt centrifuge the tubes in a microfuge for 10 seconds at 10–15,000 rpm). Vortex gently and incubate for 2 hours at room temperature.
3	Gently mix each tube of sample-AChR mixture from step 2 using a vortex mixer. Pipette duplicate **50 µL** of each sample-AChR mixture into the AChR MAb1 coated wells (A), (in duplicate is recommended), leaving 2 wells empty for blanks. Cover the frame and incubate at room temperature on an ELISA plate shaker (500 shakes per min) for 1 hour.
4	Aspirate the wells by use of a plate washing machine or discard by briskly inverting the frame of wells over a suitable receptacle. Wash the wells three times with diluted wash solution (L). For manual washing tap the inverted wells gently on a clean dry absorbent surface to remove excess wash.
5	Pipette **50 µL** of reconstituted AChR MAb-Biotin (E) into each well (except blanks). Cover the frame and incubate at room temperature on an ELISA plate shaker (500 shakes per min) for 1 hour.
6	Repeat wash step 4.
7	Pipette **100 µL** of diluted SA-POD (G) into each well (except blanks). Cover the frame and incubate at room temperature on an ELISA plate shaker (500 shakes per min) for 30 minutes.
8	Repeat wash step 4. For manual washing, wash once more with pure water to remove any foam. Tap the inverted wells gently on a clean, dry, absorbent surface to remove excess wash.
9	Pipette **100 µL** of TMB (J) into each well (including blanks). Cover the frame and incubate in the dark at room temperature for 30 minutes without shaking.

10	Pipette **50 µL** stop solution (K) to each well (including blanks), cover the frame and shake for approximately 5 seconds on a plate shaker. Ensure substrate incubations are the same for each well.
11	Within 30 minutes, read the absorbance of each well at 450 nm using an ELISA plate reader, blanked against the wells containing **100 µL** of TMB (J) and **50 µL** stop solution (K) only.

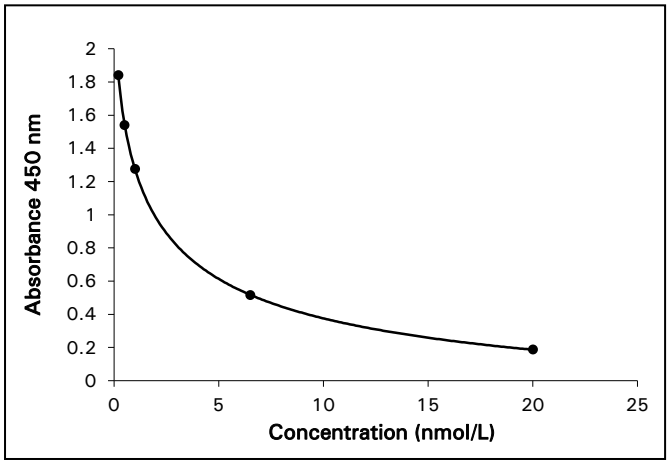
RESULT ANALYSIS

A calibration curve can be established by plotting calibrator concentration (including a value of 0.2 nmol/L for the negative control) on the x-axis (linear scale) against the absorbance of the calibrators on the y-axis (linear scale). The AChRAb concentrations in patients' sera can then be read off the calibration curve. The data in these instructions are based on a 4 parameter curve fit. Samples with high AChRAb concentrations can be diluted in negative control (N). For example 10 µL of sample plus 90 µL of negative control (N) to give a 10x dilution. Other dilutions (e.g. 100x) can be prepared from a 10x dilution or otherwise as appropriate. Some sera will not dilute in a linear way and we suggest that the dilution giving a value closest to 50% inhibition is used for calculation of AChRAb concentration.

TYPICAL RESULTS WITH THE STANDARD CURVE (Example only, not for calculation of actual results)

Sample	Abs. 450 nm	Conc. nmol/L
Negative Control N	1.842	0.2[2]
M1	1.540	0.5
M2	1.276	1.0
M3	0.516	6.5
M4	0.187	20
Positive Control P1	0.568	5.6
Positive Control P2	1.132	1.4

[2] See Result Analysis above



Results can also be expressed as inhibition (%I) of AChR binding calculated using the formula:

$$100 \times \left(1 - \frac{\text{test sample absorbance at 450 nm}}{\text{negative control (N) absorbance at 450 nm}}\right)$$

This % inhibition value can then be converted to nmol/L toxin bound using the formula;

$$0.2 \times 2^{(0.067 \times \%\,\text{Inhibition of test sample})}$$

This formula has been established empirically using a comparison of AChRAb measurements by the RSR ELISA and RIA methods. Close agreement between nmol/L values obtained in the AChRAb ELISA using the calibration curve and using this formula should not be expected in the case of all individual sera.

TYPICAL RESULTS USING % INHIBITION

Sample	Abs. 450 nm	% Inhibition	Calculated nmol/L
Negative Control N	1.842	0	0.2
Positive Control P1	0.568	69.2	5.0
Positive Control P2	1.132	38.5	1.2

ASSAY CUT OFF

Negative	< 0.45 nmol/L
Positive	≥ 0.45 nmol/L

This cut off has been validated at RSR. However each laboratory should establish its own normal and pathological reference ranges for AChRAb levels. Also it is recommended that each laboratory include its own panel of control samples in the assay.

ASSAY EVALUATION

Clinical Specificity

Sera from 50 individual healthy blood donors were assayed in the Fast AChRAb ELISA. All 50 samples (100%) were identified as being negative for AChRAb.

Clinical Sensitivity

Sera from 48 patients diagnosed with myasthenia gravis were assayed in the Fast AChRAb ELISA. 43 (90%) were identified as being positive for AChRAb.

Limit of Blank and Limit of Detection

The kit negative control and a low analyte sample were assayed 20 times in 3 different kit lots and the limit of blank and limit of detection calculated.

Limit of Blank at 2 standard deviations was 0.25 nmol/L.

Limit of Detection was 0.30 nmol/L.

Inter Assay Precision (n = 20)

Sample	% Inhibition	CV (%)	nmol/L	CV (%)
1	12	21.1	0.35	11.6
2	52	2.7	2.3	6.8
3	67	2.0	4.5	6.7
4	84	1.3	10.0	4.8

Intra Assay Precision (n = 25)

Sample	% Inhibition	CV (%)	nmol/L	CV (%)
1	15	12.6	0.40	8.3
2	54	2.5	2.4	5.8
3	69	2.1	5.0	6.7
4	85	0.6	10.5	1.9

Clinical Accuracy

Analysis of sera from patients with autoimmune diseases other than myasthenia gravis indicated no interference from autoantibodies to glutamic acid decarboxylase (n = 10), rheumatoid factor (n = 13), 21-OH (n = 10) and TSH receptor Ab (n = 31).

SAFETY CONSIDERATIONS

Streptavidin Peroxidase (SA-POD)

Signal word: Warning

Hazard statement(s)

H317: May cause an allergic skin reaction

Precautionary statement(s)

P280: Wear protective gloves/protective clothing/ eye protection/face protection

P302 + P352: IF ON SKIN: Wash with plenty of soap and water

P333 + P313: If skin irritation or rash occurs: Get medical advice/attention

P362 + P364: Take off contaminated clothing and wash it before reuse

Peroxidase Substrate (TMB)

Signal word: Danger

Hazard statement(s)

H360: May damage fertility or the unborn child

Precautionary statement(s)

P280: Wear protective gloves/protective clothing/ eye protection/face protection

P308 + P313: IF exposed or concerned: Get medical advice/attention

Diluent for SA-POD

Hazard statement(s)

EUH208: Contains 2-Chloroacetamide. May produce an allergic reaction.

This kit is intended for *in vitro* use by professional persons only. Follow the instructions carefully. Observe expiry dates stated on the labels and the specified shelf life for coated wells, diluted or reconstituted reagents. Refer to Safety Data Sheet for more detailed safety information. With all kit components, avoid ingestion, inhalation, injection or contact with skin, eyes or clothing. Wear protective clothing. 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. Avoid formation of heavy metal azides in the drainage system by flushing any kit components away with copious amounts of water.

ASSAY PLAN

Allow all reagents and samples to reach room temperature (20-25 °C) before use	
Pipette:	**100 µL**, Calibrators (M 1-4 optional), controls (N and P 1-2), and test sera into Eppendorf tubes
Pipette:	**25 µL** AChR (foetal and adult mixture B+C) (centrifuge if necessary) and vortex mix
Incubate:	2 hours at room temperature
Pipette:	**50 µL** Sample-AChR mixture (in duplicate) from each tube into wells (except blanks)
Incubate:	1 hour 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
Pipette:	**50 µL** AChR MAb-Biotin (E) (reconstituted) into each well (except blanks)
Incubate:	1 hour 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
Pipette:	**100 µL** SA-POD (G) (diluted 1:20) into each well (except blanks)
Incubate:	30 minutes at room temperature on an ELISA plate shaker at 500 shakes/min
Aspirate/Decant:	Plate
Wash:	Plate three times and rinse with pure water[3] and tap dry on absorbent material
Pipette:	**100 µL** TMB (J) into each well (including blanks)
Incubate:	30 minutes **in the dark** at room temperature without shaking
Pipette:	**50 µL** Stop solution (K) into each well (including blanks) and shake for 5 seconds
Read absorbance at 450 nm, within 30 minutes of adding stop solution [3]Omit water wash if a plate washing machine is used	