Anti-AChR (Acetylcholine Receptor) ELISA

Catalog Number:
ACR31-K01
1 x 96 well ELISA kit
For Research Use Only
v. 1.0

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Intended Use:

The Eagle Biosciences Anti-AChR (Acetylcholine Receptor) ELISA Assay kit is intended for the quantitative determination of Anti-AChR in serum by enzyme linked immunoassay (ELISA). The Anti-AChR ELISA Assay kit is for research use only and not to be used in diagnostic procedures. Autoantibodies to the acetylcholine receptor (AChR) are responsible for failure of the neuromuscular junction in myasthenia gravis, a neuromuscular disease leading to fluctuating muscle weakness and fatigability. Measurement of these antibodies can be of considerable value in disease diagnosis and management.

Principle of the Test:

The Anti-AchR ELISA Assay kit is an enzyme immunoassay for the quantitative determination of autoantibodies to acetylcholine receptor (AChR Abs) in human serum.

The assay system depends on the ability of AChR Abs 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 labeled with biotin). In the absence of AChR Abs 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), substrate (TMB) and stop solution. In the presence of AChR Abs 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 450nm. The higher the concentration of AChR Abs in the test serum the greater the inhibition of MAb biotin binding.

Samples:

Specimen collection and storage

Blood is taken by venipuncture. Serum is separated after clotting by centrifugation. Do not use lipaemic and hemolytic samples. Plasma is not suitable.

The samples may be kept at 2 - 8 °C for up to three 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. 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.

Materials required:

- Precision pipettes 10 - 100 µl
- Multi-channel pipette
- Disposable pipette tips
- Eppendorf tubes (1.5 ml)
- 8 channel wash comb or microplate washer

- Micro plate shaker (> 500 / min), not orbital shaker
- Micro plate reader with optical filters for 450 nm and 620 or 690 nm
- Graduated cylinders
- Distilled or de-ionized water
- Absorbent paper or paper towel
- foil

Size and storage:

The Anti-AChR ELISA Assay kit has been designed for 96 determinations. This is sufficient for the analysis of 43 unknown samples as well as for calibrators and control serum, assayed in duplicates.
The expiry date of each component is reported on its respective label, that of the complete Anti-AChR ELISA Assay kit on the box label.
Upon receipt, all components of the Anti-AChR ELISA Assay kit have to be kept at 2 - 8 °C, preferably in the original Anti-AChR ELISA Assay kit box.

Preparation before use:

Allow samples to reach room temperature prior to assay. Take care to agitate serum samples gently in order to ensure homogeneity.
Allow all reagents to stand at room temperature (20-25 °C) for at least 30 minutes prior to use.

Please, handle carefully with the following components:

A Allow the sealed microplate to reach room temperature before opening. Unused wells should be stored refrigerated and protected from moisture in the original bag carefully resealed for 16 weeks.

B Prepare a sufficient amount of washing solution by diluting the concentrated wash buffer (B) 1 + 9 with distilled or de-ionized water. For example, dilute 50 ml of the concentrate with 450 ml of distilled water. B should be free of crystals before dilution, otherwise dissolve by warming up to max. 37 °C. The diluted washing solution can be stored at 2 - 8 °C up to 30 days.

D Prepare a sufficient amount of Streptavidin-peroxidase solution by diluting SA-POD concentrate (D) 1 + 19 with diluent for SA-POD (G); eg. dilute 0.25 ml SA-POD concentrate with 4.75 ml diluent. The SA-POD solution prepared is stable up to 16 weeks at 2 - 8 °C.

E Avoid exposure of substrate solution (E) to light.

H Prepare a sufficient amount of MAb-Biotin solution directly prior to use by reconstitution of one vial lyophilized MAb-Biotin (H) with the volume of diluent for MAb-Biotin (J) shown on the leaflet enclosed. The MAb-Biotin solution can be store at 2 - 8 °C for up to 16 weeks.

K Reconstitute each vial of fetal type AchR (K) with 0.7 ml buffer for AChR (M). Mix gently, and leave to stand at room temperature for 5 minutes before use. Pool the vials when more than one vial is required. Use on day of reconstitution.

K+L Reconstitute each vial of adult type ACHR (L) with 0.5 ml of the solution of reconstituted fetal type AChR (K) to give a mixture of fetal and adult AChR (K+L). Mix gently, and leave to stand at room temperature for 5 minutes before use. Pool the vials when more than one vial is required. Use on day of reconstitution.

Assay Procedure:

Duplicates are recommended.

1. Pipette **100 µl** of negative control (CI), calibrators (1 - 4), positive controls (CII, CIII) and test sera into individual 1.5 ml Eppendorf tubes, labelled accordingly.
2. Pipette **25 µl** of fetal and adult type AChR mix (K+L) 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 10-15,000g). Vortex gently and incubate **overnight (16 - 20 hrs)** at 2 - 8°C.
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 corresponding wells (A) according to assay scheme.
4. Cover the plate and incubate for **60 min** at room temperature (18 - 25 °C) while shaking >500 rpm.
5. Aspirate the plate wells by use of a microplate washer or discard by briskly inverting the frame of stripwells over a suitable receptacle. Wash the wells **3 times** with **300 µl** washing solution (diluted from B). Tap the inverted wells gently on a clean dry absorbent surface to remove excess wash.
6. Add **50 µl** of reconstituted MAb-Biotin solution (prepared from H and J) to each well.
7. Cover the plate and incubate for **60 min** at room temperature (18 - 25 °C) while shaking >500 rpm.
8. Aspirate the plate wells by use of a microplate washer or discard by briskly inverting the frame of stripwells over a suitable receptacle. Wash the wells **3 times** with **300 µl** washing solution (diluted from B). Tap the inverted wells gently on a clean dry absorbent surface to remove excess wash.
9. Add **100 µl** reconstituted SA-POD (prepared from D and G) to each well.
10. Cover the plate and incubate for **30 min** at room temperature (18 - 25 °C) while shaking > 500 rpm.
11. Aspirate the plate wells by use of a microplate washer or discard by briskly inverting the frame of stripwells over a suitable receptacle. Wash the wells **3 times** with **300 µl** washing solution (diluted from B), in the case of washing manually, use an additional final wash step with pure water to remove any foam. Tap the inverted wells gently on a clean dry absorbent surface to remove excess wash.
12. Add **100 µl** substrate solution (E) to each well and shake shortly.
13. Incubate for **30 min** in the **dark** at room temperature.

14. Add **50 µl** stop solution (F) to each well. Shake the plates for 5 seconds.
15. Read the optical density **at 450 nm** versus **620 or 690 nm within 5 min** after adding the stop solution.

Please note that the washing procedure is crucial. Insufficient washing will result to poor precision and falsely elevated OD readings. Without shaking the ODs will be measured about 20 % lower with a loss of sensitivity.

Data Processing:

The standard curve is established by plotting the mean OD values of the calibrators 1 - 4 on the ordinate, y-axis, versus their respective AChR Ab concentrations on the abscissa, x-axis. In addition negative control (CI) should be used (see below), the assigned value of CI is 0.2 nmol/l toxin bound.

The AChR Ab concentrations of the controls and the unknown samples are directly read off in nmol/l toxin bound from the measured OD_{450} values. **Anti-AChR ELISA Assay kit** may be used also with Computer Assisted Analysis using software able to curves with 4 parameter curve fit.

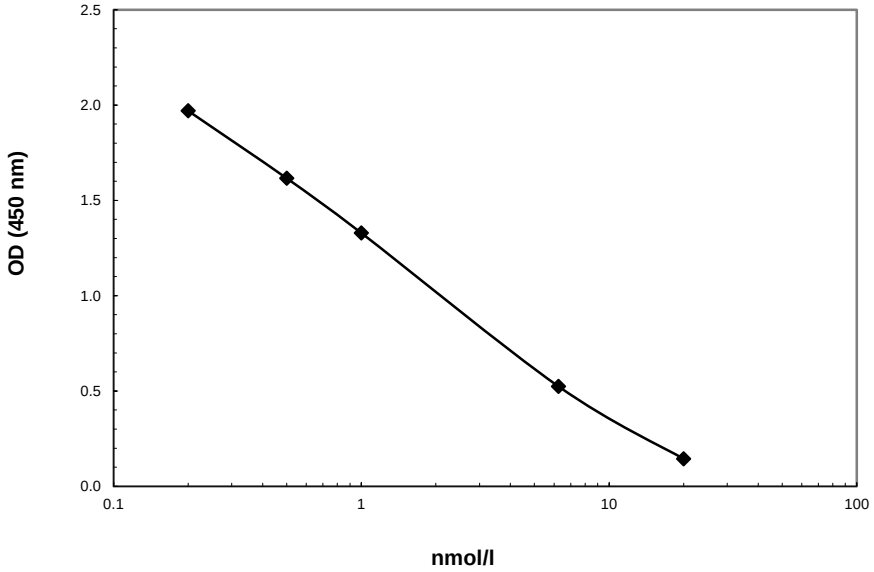
Typical Example:

Do not use for evaluation!

Wells	OD (a)	OD (b)	OD (mean)	IU/ml
Control I	1.951	1.990	1.970	**0.2**
Calibrator **1**				
Calibrator **2**	1.598	1.634	1.616	**0.5**
Calibrator **3**	1.318	1.340	1.329	**1.0**
Calibrator **4**	0.517	0.531	0.524	**6.5**
	0.131	0.157	0.144	**20**
Control II				
	0.460	0.478	0.469	7.5
Control III	1.088	1.160	1.124	1.6

Standard Curve:

Typical Example:



Samples with high AChR Ab concentrations can be diluted in kit negative control (CI). For example: 10 µl of sample plus 90 µl of negative control leading to a 10x dilution. Other dilutions (e.g. 100 x) can be prepared from a 10x dilution or otherwise as appropriate. Dilution factor has to be considered when calculating results for these samples. 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 AChR Ab concentration.

Results can also be expressed as inhibition (%I) of AChR binding calculated by comparison of the OD of a sample and of the negative control CI using the formula:

$\%I = 100 \times (1-OD_{sample}/OD_{CI})$

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

$nmol/l = 0.2 \times 2^{(0.067 \times \%I)}$

Typical results using % inhibition:

	OD 450 nm	% inhibition	calculated nmol/l
negative control CI	1.970	0	0.2
positive control CII	0.469	76.2	6.9
positive control CIII	1.124	42.9	1.5

Reference Values:

anti-AChR	
negative	< 0.45 nmol/l
positive	≥ 0.45 nmol/l

It is recommended that each laboratory establishes its own normal and pathological reference ranges for serum anti-AChR antibodies as usually done for other diagnostic parameters, too. Therefore, the above mentioned reference values provide only a guide.

Characteristic Assay Data:

Clinical Specificity

302 individual healthy blood donors were assayed in The Anti-AChR ELISA Assay kit. All (100%) were identified as being negative for AChR autoantibodies.

Clinical Sensitivity

Samples from 83 patients diagnosed with myasthenia gravis were assayed in the anti-AChR. 76 (92%) were identified as being positive for AChR autoantibodies.

Lower Detection Limit

The analytical sensitivity (lower detection limit, 0 ± 3 SD) was established to be 0.23 nmol/l.

Intra- and inter-assay variation

Intra-assay (n = 24)			Inter-assay (n = 21)		
Sample no.	Mean Concentration (nmol/l)	CV (%)	Sample no.	Mean Concentration (IU/ml)	CV (%)
1	14	2.6	5	7.3	9.1
2	1.7	5.2	6	1.9	13.0
3	0.67	8.4			

Limitations of the Method:

Analysis of 90 sera from patients with autoimmune diseases other than myasthenia gravis indicated no interference from autoantibodies to thyroglobulin; thyroid peroxidase; dsDNA; TSH receptor, glutamic acid decarboxylase, 21-hydroxylase or rheumatoid factor. No interference was observed when samples were spiked with the following materials; hemoglobin up to 500 mg/dl, bilirubin up to 20 mg/dl or intralipid up to 3000 mg/dl. Little assay drift was observed in the AChR Ab ELISA. It is important that the incubation times and all other conditions specified in the instructions are adhered to for optimum assay performance.

Anti-AchR Assay Scheme

Bring all reagents to room temperature. Gently mix all reagents to ensure homogeneity.

Step	Activity	Material	CI / CAL 1-4	CII / CIII	Patients 1, 2 etc.
1	Pipette into Eppendorf tubes	Samples	100 µl	100 µl	100 µl
	Add	AChR preparation (K+L)	25 µl	25 µl	25 µl
2	Incubate	Eppendorf tubes	**16 - 20 hours (overnight)** at 2 - 8 °C		
Day 2					
3	Pipette into plate	Samples-AChR mixture	50 µl	50 µl	50 µl
4	Incubate	Plate	**1 hour** at room temperature **while shaking (> 500 rpm)**		
5	Aspirate or decant		put sharply onto absorbent tissue		
	Pipette	Washing solution	3 x 300 µl	3 x 300 µl	3 x 300 µl
6	Pipette	MAb-Biotin solution (H+J)	50 µl	50 µl	50 µl
7	Incubate	Plate	**1 hour** at room temperature **with shaking (> 500 rpm)**		
8	Aspirate or decant		put sharply onto absorbent tissue		
	Pipette	Washing solution	3 x 300 µl	3 x 300 µl	3 x 300 µl
9	Pipette	SA-POD solution (D+G)	100 µl	100 µl	100 µl

10	Incubate	Plate	**30 min** at room temperature **with shaking (> 500 rpm)**		
11	Aspirate or decant		put sharply onto absorbent tissue		
	Pipette	Washing solution	3 x 300 µl	3 x 300 µl	3 x 300 µl
12	Pipette	Substrate (E)	100 µl	100 µl	100 µl
13	Incubate	Plate	**30 min** at room temperature **in the dark**		
14	Pipette and mix	Stop solution (F)	50 µl	50 µl	50 µl
15	Measure OD		at 450 nm versus 620 nm (or 690 nm) within 5 min		

Safety Precautions:

- This Anti-AchR ELISA Assay kit is for Research use only. Follow the working instructions carefully. This instruction manual is valid only for the present kit with the given composition. An exchange of single components is not in agreement with CE regulations.
- The expiration dates stated on the respective labels are to be observed. The same relates to the stability stated for reconstituted reagents.
- Do not use or mix reagents from different lots.
- Do not use reagents from other manufacturers.
- Avoid time shift during pipetting of reagents.
- All reagents of the Anti-AchR ELISA Assay kit should be kept at 2 - 8 °C before use in the original shipping container.
- Some of the reagents contain small amounts (< 0.1 % w/v) of sodium azide as a preservative. They must not be swallowed or allowed to come into contact with skin or mucosa.
- 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.
- In any case GLP should be applied with all general and individual regulations to the use of this kit.

Warranty Information

Eagle Biosciences, Inc. warrants its Product(s) to operate or perform substantially in conformance with its specifications, as set forth in the accompanying package insert. This warranty is expressly limited to the refund of the price of any defective Product or the replacement of any defective Product with new Product. This warranty applies only when the Buyer gives written notice to the Eagle Biosciences within the expiration period of the Product(s) by the Buyer. In addition, Eagle Biosciences has no obligation to replace Product(s) as result of a) Buyer negligence, fault, or misuse, b) improper use, c) improper storage and handling, d) intentional damage, or e) event of force majeure, acts of God, or accident.

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Product(s) may not be resold, modified, or altered for resale without prior written approval from Eagle Biosciences, Inc.

*For further information about the **Anti-AChR** ELISA Assay kit, its application or the procedures in this kit insert, please contact the Technical Service Team at Eagle Biosciences, Inc. at info@eaglebio.com or at 866-411-8023.*