Beta-Amyloid (1-42) ELISA
Test instruction

ORDER NO.	ANTIGEN	SUBSTRATE	FORMAT
EQ 6521-9601-L	Beta-amyloid (1-42)	Ab-coated microplate wells	96 x 01 (96)

Indication: This ELISA test kit is designed for the quantitative determination of beta-amyloid (1-42) in cerebrospinal fluid (CSF) of patients, to be used only as an additional diagnostic tool in suspected clinical amyloid pathology of the brain.

Application: Determination of beta-amyloid (1-42) in combination with total tau or p-tau in the CSF of patients can support the early diagnosis of suspected Alzheimer's disease.

Principles of the test: In the first analysis step, the calibrators and patient samples are diluted with biotin-labelled anti-beta-amyloid (1-x) and added to microplate wells coated with monoclonal anti-beta-amyloid (x-42) antibodies. In this process beta-amyloid (1-42) is bound in a complex. In a second incubation, this complex is labelled with peroxidase-labelled streptavidin. In a third incubation using the peroxidase substrate tetramethylbenzidine (TMB) the bound peroxidase promotes a colour reaction. The colour intensity is proportional to the beta-amyloid (1-42) concentration in the sample.

Contents of the test kit:

Component	Colour	Format	Symbol
1. Antibody-coated microplate wells 12 microplate strips each containing 8 individual break-off wells in a frame, ready for use	---	12 x 8	STRIPS
2. Calibrator 1, beta-amyloid (1-42), lyophilised	white	1 x 500 µl	CAL 1
3. Calibrator 2, beta-amyloid (1-42), lyophilised	white	1 x 500 µl	CAL 2
4. Calibrator 3, beta-amyloid (1-42), lyophilised	white	1 x 500 µl	CAL 3
5. Calibrator 4, beta-amyloid (1-42), lyophilised	white	1 x 500 µl	CAL 4
6. Calibrator 5, beta-amyloid (1-42), lyophilised	white	1 x 500 µl	CAL 5
7. Calibrator 6, beta-amyloid (1-42), lyophilised	white	1 x 500 µl	CAL 6
8. Control 1, beta-amyloid (1-42), lyophilised	white	1 x 500 µl	CONTROL 1
9. Control 2, beta-amyloid (1-42), lyophilised	white	1 x 500 µl	CONTROL 2
10. Biotin biotin-labelled beta-amyloid detection antibody, ready for use	green	1 x 12 ml	BIOTIN
11. Enzyme conjugate peroxidase-labelled streptavidin, ready for use	blue	1 x 12 ml	CONJUGATE
12. Wash buffer 10x concentrate	colourless	1 x 100 ml	WASH BUFFER 10x
13. Chromogen/substrate solution TMB/H_2O_2, ready for use	colourless	1 x 12 ml	SUBSTRATE
14. Stop solution 0.5 M sulphuric acid, ready for use	colourless	1 x 12 ml	STOP SOLUTION
15. Test instruction	---	1 booklet	
16. Quality control certificate	---	1 protocol	

LOT Lot description
IVD In vitro diagnostic medical device
CE
Storage temperature
Unopened usable until

Storage and stability: The test kit has to be stored at a temperature between +2°C to +8°C. Do not freeze. Unopened, all test kit components are stable until the indicated expiry date.

Waste disposal: Patient samples, calibrators, controls and incubated microplate strips should be handled as infectious waste. All reagents must be disposed of in accordance with local disposal regulations.

Preparation and stability of the reagents

Note: All reagents must be brought to room temperature (+18°C to +25°C) approx. 30 minutes before use. After first use, the reagents are stable until the indicated expiry date if stored at +2°C to +8°C and protected from contamination, unless stated otherwise below.

- **Coated wells:** Ready for use. Tear open the resealable protective wrapping of the microplate at the recesses above the grip seam. Do not open until the microplate has reached room temperature to prevent the individual strips from moistening. Immediately replace the remaining wells of a partly used microplate in the protective wrapping and tightly seal with the integrated grip seam (Do not remove the desiccant bag).
  Once the protective wrapping has been opened for the first time, the wells coated with antibodies can be stored in a dry place and at a temperature between +2°C and +8°C for 4 months.

- **Calibrators and controls:** Reconstitute calibrators and controls with 500 µl deionised or distilled water approximately 10 minutes before use and mix thoroughly upside down. Before use, confirm that the lyophilisate is completely dissolved in the water. If necessary, shortly centrifuge vials to get remaining liquid from the cap into tube. The reconstituted calibrators and controls must be frozen at -20°C immediately after use. The calibrators and controls can be frozen and thawed up to three times. Longer residence times at room temperature must be avoided at all costs.

- **Biotin:** Ready for use. Mix thoroughly before use. Contains an indicator. In the microplate, the color changes from grey to green after adding the calibrators, controls or CSF samples.

- **Enzyme conjugate:** Ready for use. Mix thoroughly before use.

- **Wash buffer:** The wash buffer is a 10x concentrate. If crystallisation occurs in the concentrated buffer, warm it to +37°C and mix well before diluting. The quantity required should be removed from the bottle using a clean pipette and diluted with deionised or distilled water (1 part reagent plus 9 parts distilled water).
  For example: For 1 microplate strip, 5 ml concentrate plus 45 ml water.
  The working strength wash buffer is stable for 4 weeks when stored at +2°C to +8°C and handled properly.

- **Chromogen/substrate solution:** Ready for use. Close the bottle immediately after use, as the contents are sensitive to light. The chromogen/substrate solution must be clear on use. Do not use the solution if it is blue coloured.

- **Stop solution:** Ready for use.

Warning: Some of the reagents contain preserving agents in a non-declarable concentration. Avoid skin contact.

Preparation and stability of the patient samples

Samples: Cerebrospinal fluid (CSF).

Notes on sample handling: Standardised preanalytical sample handling, test systems and laboratory procedures are a prerequisite for accurate Alzheimer's-specific CSF diagnostics. Further information about this can be obtained from the recommendations of the Alzheimer's Biomarkers Standardisation Initiative (ABSI). Of particular importance is that CSF should be filled directly into polypropylene tubes. If the analysis is not to be performed immediately following puncture, the samples should be stored at -20°C and subjected to preferably no more than one, maximal two, freeze/thaw cycles.

Incubation

(Partly) manual test performance

Sample incubation: (1st step)
Pipette **100 µl** of biotin and **15 µl** of calibrators, controls and undiluted patient samples into each of the reagent wells. Slightly shake the microplate to ensure a homogeneous distribution of the solution.
Incubate for **180 minutes** at room temperature (+18°C to +25°C).

Washing:
Manual: Empty the wells and subsequently wash 5 times using 300 µl of working strength wash buffer for each wash.
Automatic: Wash reagent wells 5 times with 450 µl of working strength wash buffer (program setting: e.g. TECAN Columbus Washer "Overflow Modus").

Leave the wash buffer in each well for 30 to 60 seconds per washing cycle, then empty the wells. After washing (manual and automated tests), thoroughly dispose of all liquid from the microplate by tapping it on absorbent paper with the openings facing downwards to remove all residual wash buffer.

Note: Residual liquid (> 10 µl) in the reagent wells after washing can interfere with the substrate and lead to false low extinction values.
Insufficient washing (e.g. less than 5 wash cycles, too small wash buffer volumes, or too short residence times) can lead to false high extinction values.
Free positions on the microplate strip should be filled with blank wells of the same plate format as that of the parameter to be investigated.

Enzyme conjugate incubation: (2nd step)
Pipette **100 µl** of enzyme conjugate (streptavidin-peroxidase) into each of the microplate wells and incubate for **30 minutes** at room temperature (+18°C to +25°C).

Washing:
Empty the wells. Wash as described above.

Substrate incubation: (3rd step)
Pipette **100 µl** of chromogen/substrate solution into each of the microplate wells. Incubate for **30 minutes** at room temperature (+18°C to +25°C) (protect from direct sunlight).

Stopping:
Pipette **100 µl** of stop solution into each of the microplate wells in the same order and at the same speed as the chromogen/substrate solution was introduced.

Measurement:
Photometric measurement of the colour intensity should be made at a **wavelength of 450 nm** and a reference wavelength between 620 nm and 650 nm **within 30 minutes of adding the stop solution.** Prior to measuring, slightly shake the microplate to ensure a homogeneous distribution of the solution.

Test performance using fully automated analysis devices

Sample dilution and test performance are carried out fully automatically using an analysis device. The incubation conditions programmed in the respective software authorised by EUROIMMUN may deviate slightly from the specifications given in the ELISA test instruction. However, these conditions were validated in respect of the combination of the EUROIMMUN Analyzer I and the Analyzer I-2P and this EUROIMMUN ELISA. Validation documents are available on enquiry.
Automated test performance using other fully automated, open-system analysis devices is possible. However, the combination should be validated by the user.

Pipetting protocol

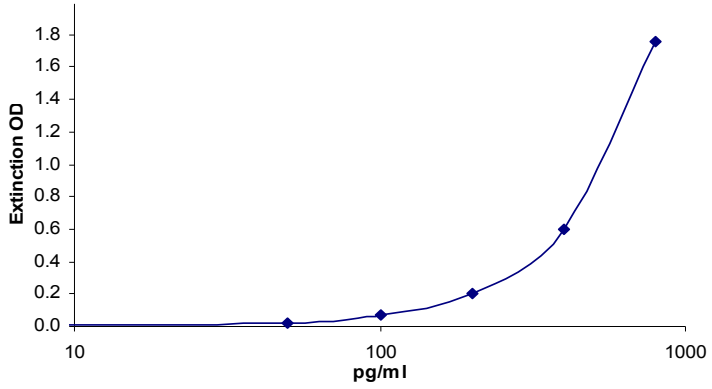
	1	2	3	4	5	6	7	8	9	10	11	12
A	**C 1**	**P 1**	**P 9**									
B	**C 2**	**P 2**	**P 10**									
C	**C 3**	**P 3**	**P 11**									
D	**C 4**	**P 4**	**P 12**									
E	**C 5**	**P 5**	**P 13**									
F	**C 6**	**P 6**	**P 14**									
G	**Co 1**	**P 7**	**P 15**									
H	**Co 2**	**P 8**	**P 16**									

The pipetting protocol for microtiter strips 1 to 3 is an example for the **quantitative analysis** of 16 patient samples (P 1 to P 16).

The calibrators (C 1 to C 6), the controls (Co 1, Co 2), and the patient samples have each been incubated in one well. The reliability of the ELISA test can be improved by duplicate determinations for each sample.
The controls serve as internal controls for the reliability of the test procedure. They should be assayed with each test run.

Calculation of results

Quantitative: The standard curve from which the beta-amyloid (1-42) concentrations in the CSF samples can be calculated by “5PL” plotting (alternatively “4PL”, “Akima” or “cubic spline” can be used) of the extinction values measured for the 6 calibrators against the corresponding units (linear/log).
For correct logarithmic representation it might be necessary to set the concentration of calibrator 1 from 0 to e.g. 0.1 pg/ml. The following plot is an example of a typical calibration curve. Please do not use this curve for the determination of concentrations in patient samples.



If the extinction for a patient sample lies above the value of calibrator 6, it is recommended to retest the sample at an initial dilution of 1:4 in calibrator 1 (or beta-amyloid (1-40) sample buffer, Cat# ZE 6511-0130) before following the test instruction.

Note: Only use polypropylene (PP) tubes for dilution. Pipette calibrator 1 (sample buffer) into tubes before adding sample. The result of sample in pg/ml read from the calibration curve for this sample must be multiplied by a factor of 4.

For duplicate determinations the mean of the two values should be taken. If the two values deviate substantially from one another, EUROIMMUN recommends retesting the samples.

Therapeutic decisions should not be made on the basis of results from this test, but only under consideration of clinical findings and further diagnostic values.

Test characteristics

Calibration: The concentrations of calibrators and the acceptance ranges of controls are lot-dependent and given on the quality control certificate enclosed with this test instruction.
For every group of tests performed, the values of the concentrations must lie within the limits stated for the relevant test kit lot. A quality control certificate containing these reference values is included. If the values specified for the controls are not achieved, the test results may be inaccurate and the test should be repeated.

Antibodies: The reagent wells are coated with monoclonal anti-beta-amyloid (x-42) antibodies.

Detection limit: The lower detection limit is defined as the mean value of an analyte-free sample plus three times the standard deviation and is the smallest detectable beta-amyloid (1-42) concentration. The detection limit of the beta-amyloid (1-42) ELISA calculated from 6 different runs is at an average of 6.5 pg/ml. The functional sensitivity defined as the lowest concentration of a real sample with a coefficient of variation (CV) <20% was found to be 14.3 pg/ml.

Cross reactivity: This ELISA detects beta-amyloid (1-42) specifically. Cross reactions with other beta-amyloid derivates are given in the following table.

Cross reactivity (%)	
Beta-amyloid (1-42)	100
Beta-amyloid (1-43)	4.1
Beta-amyloid (2-42)	2.1
Beta-amyloid (1-40)	1.0
Beta-amyloid (4-42)	0.6
Beta-amyloid (1-38)	<0.1
Beta-amyloid (1-39)	<0.1
Beta-amyloid (3-42)	<0.1
Beta-amyloid (11-42)	<0.1
Beta-amyloid (17-42)	<0.1

Interference: Contamination with blood up to a concentration of 1.0% (v/v) did not cause interference with the ELISA. Red tint of the sample indicates significant contamination with blood. The sample should not be used.

Reproducibility: The reproducibility of the test was investigated by determining the intra- and inter assay coefficients of variation using 3 CSF samples. The intra-assay CVs are based on 24 determinations and the inter-assay CVs on triplicates performed in 10 different test runs. The inter-lot CVs are based on duplicates in 4 different test runs performed for each of 3 kit lots.

Intra-assay variation, n = 24		
Sample	**Mean value (pg/ml)**	**CV (%)**
1	110	6.2
2	484	3.1
3	710	2.3

Inter-assay variation, n = 10 x 3		
Sample	**Mean value (pg/ml)**	**CV (%)**
4	104	6.9
5	328	5.7
6	940	4.4

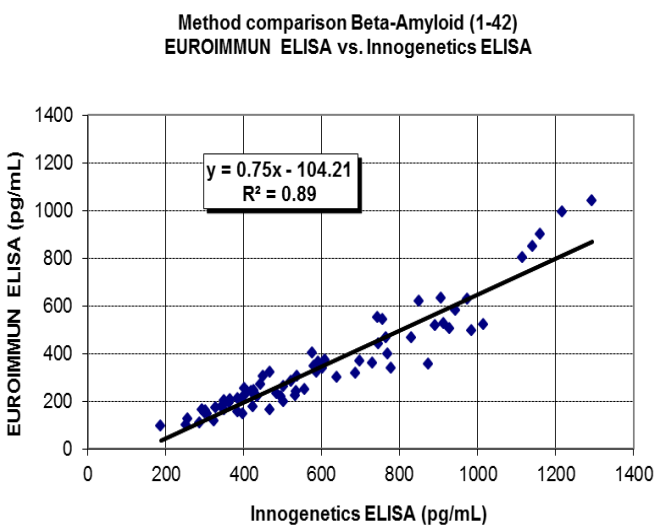
Inter-lot variation, n = 3 x 4 x 2		
Sample	**Mean value (pg/ml)**	**CV (%)**
1	111	8.1
2	359	11.0
3	635	8.9

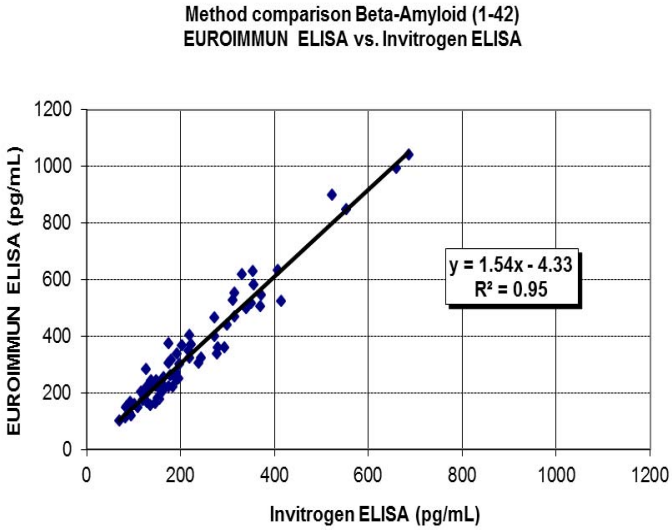
Linearity: The linearity of the test was investigated by diluting three samples (348 to 641 pg/ml) in nine steps up to 1:10 with sample buffer. The recovery of the expected concentrations ranged from 88 to 109% with a mean correlation coefficient of r = 0.994.

Sample	**Dilution (decimal)**	**Measured (pg/ml)**	**Expected (pg/ml)**	**Recovery (%)**
1	1.00	329	348	95
	0.90	297	314	95
	0.80	271	279	97
	0.70	244	244	100
	0.60	217	209	104
	0.50	182	174	104
	0.40	150	139	107
	0.30	110	105	105
	0.20	72	70	103
	0.10	31	35	89
2	1.00	598	602	99
	0.80	486	482	101
	0.60	339	361	94
	0.40	255	241	106
	0.20	119	120	99
	0.10	61	60	101
3	1.00	564	641	88
	0.90	542	577	94
	0.80	494	513	96
	0.70	436	449	97
	0.60	373	385	97
	0.50	350	320	109
	0.40	262	256	102
	0.30	202	192	105
	0.20	135	128	105
	0.10	68	64	106

Method comparison: The EUROIMMUN ELISA was compared with a commercially available ELISA from another manufacturer and showed the following correlation:

Innogenetics, Aβ (1-42) ELISA	EUROIMMUN = 0.75 x Inno - 104 pg/ml; n = 71; r2 = 0.89
Invitrogen, Hu Aβ42 ELISA	EUROIMMUN = 1.54 x Invitrogen - 4 pg/ml; n = 69; r2 = 0.95





Expected values: Clinically characterised CSF samples from 67 Alzheimer`s disease patients, 44 vascular dementia patients and 43 controls were measured with the EUROIMMUN Beta-amyloid (1-40) and Beta-amyloid (1-42) ELISA. 50 of 67 Alzheimer`s patients had Beta-amyloid (1-42) values of <550 pg/ml and 63 had a beta-amyloid-ratio (Aβ42/Aβ40) of <=0.1. 19 of 87 healthy controls and patients with vascular dementia however had Beta-Aymloid 1-42 <550 pg/ml and 66 had a higher beta-amyloid-ratio (Aβ42/Aβ40) ranging from 0.11 - 0.19.

n = 154	Beta-amyloid 1-42 ELISA			
clinical significance	+ (<550 pg/ml)	(+) (551 - 650 pg/ml)	- (>651 pg/ml)	Total
positive for AD +	50	11	6	67
negative for AD -	19	11	57	87

n = 154	Beta-amyloid 1-42/Beta-amyloid 1-40		
clinical significance	+ (<= 0.1)	- (> 0.1)	Total
positive for AD +	63	4	67
negative for AD -	21	66	87

Every laboratory should use their own normal values established under specific ambient conditions.

Clinical significance

In 2010 the number of people with dementia was estimated to be 36 million worldwide. Assuming a continuing lack of effective preventative measures and cures, an unchanging lethality and around 4.6 million new cases per year, the number of affected persons is projected to double every 20 years (2050: 115 million). Over the coming decades the increase in the disease prevalence is predicted to be three times higher in emerging countries (e.g. China, Brazil, India), where the majority of dementia patients live (2001: 60%, 2040: 70%), than in industrial nations.
Alzheimer's disease, which was first described in 1907, is with 60 to 70% the most common cause of dementia in old age. The prevalence and incidence of Alzheimer's disease increase with rising age. The prevalence doubles with about every five years of age, increasing from just over 1% in the age group 65 to 69 to more than 30% in those over 90.
In contrast to the age-dependent, sporadic form of Alzheimer's, the familial, genetically caused form can also occur in young adults from 30 years of age. The monogenetically induced form of Alzheimer's disease accounts for only 0.5% of cases.

Disease course
Alzeimer's disease is a progressive neurogenerative disease. The characteristic neuropathological features are extracellular β-amyloid (Aβ) deposits (plaques) and intracellular accumulation of neurofibrillary tangles in the cortical and limbic brain regions. The main components of the **neuritic plaques** are fibrillar aggregates of the peptides Aβ 1-40 and Aβ 1-42, which are produced from successive processing of the membrane-bound amyloid precursor protein (APP) by γ and β secretases. The amyloid core of the neuritic plaques is surrounded by dystrophic neurites. The hydrophobic Aβ 1-42 has a strong tendency to aggregate and the soluble, oligomeric form in particular is neurotoxic. The intracellular **neurofibrillary tangles** consist of pairs of helically arranged fibrils, composed of hyper-phosphorylated tau proteins. The frequency of neurofibrils in the brain and the associated extent of neurodegeneration correlate with the degree of dementia.
The disease course is divided into three consecutive phases, the preclinical stage, the MCI (mild cognitive impairment) stage and the dementia stage. The preclinical stage lasts for several years and is characterised initially by amyloidosis, followed by neurodegeneration and mild cognitive disorders. With clinical manifestation and an accelerated increase in cognitive deficits, the disease proceeds to the MCI stage. The final dementia stage is characterised in addition by an inability to work and carry out everyday activities.
Following a clinical diagnosis of Alzheimer's disease, the remaining life expectancy for the patient is on average seven to ten years.

Diagnosis
To date, the diagnosis of Alzheimer's disease requires an autopsy to confirm the neuropathological changes (plaques and neurofibrillary tangles) in patients' brains. Accordingly, *in vivo* diagnosis (probable Alzheimer's disease) is based predominantly on the **clinical verification** of dementia and its aetiological classificationon other investigations(e.g. endocrinopathies, vitamin deficiency syndromes, electrolyte disorders, meta-bolic encephalopathies, intoxication, haemolytical disorders, chronic infections, late forms of leuko-dystrophathy) by means of other analyses.
Clinical diagnosis is unreliable, particularly in the early and presymptomatic disease stages, and requires additional measurable biomarkers with high diagnostic reliability. Markers for monitoring the disease course and the efficacy of therapy are similarly required.

The concentration of soluble Aβ 1-42 in the **cerebrospinal fluid (CSF)** reflects the Alzheimer's-specific neuropathological changes in the brain (amyloid plaques). The CSF of persons who will later develop Alzheimer's disease exhibits a significant **decrease in the Aβ 1-42 concentration** already 5-10 years before the start of cognitive changes.

The **ratio of Aβ42/Aβ40** in the CSF is also diagnostically relevant. It shows a significant **decrease** already in patients in the preclinical or MCI stage. Diagnosis of Alzheimer's patients can therefore be improved by taking into consideration the individual amyloid expression levels. Imaging techniques such as MRT, SPECT, or PET (amyloid detection) can also be used to support diagnostics. Results from CSF-based neurochemical analyses and results from imaging procedures should only be assessed in the context of all available diagnostic information.

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