ADNI Alzheimer's Disease Neuroimaging Initiative

Plasma samples analysis by AlzoSure® Predict

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Introduction

AlzoSure® Predict is an *in vitro* diagnostic (IVD) assay performed using LC-MS/MS or Ion Trap, on protein-depleted human plasma samples from Individuals and patients greater than or equal to 50 years of age and performed in a regulated clinical laboratory environment.

This test can be used for the general screening of individuals over 50 years of age, asymptomatic individuals with an Alzheimer's disease (AD) family history or documented genetic predisposition and for patients demonstrating signs of mild cognitive impairment (MCI), subjective cognitive impairment (SCI), or subjective memory complaints (SMC).
The AlzoSure® Predict test is able to diagnose whether a patient will or won't progress to AD.

Summary (or Abstract)

593 blinded human plasma samples from ADNI were received by Diadem SpA to be analysed by AlzoSure® Predict method (see below for details).

The samples were analysed over 8 analytical runs from 16th June, 2022 to 12nd July, 2022. Each analytical session included from 54 to 77 plasma samples from ADNI and at least 3 QC samples from AIBL biobank, previously analysed and used as QC-samples and selected in order to cover the whole range of U-p53^{AZ} expression over AD progression from preclinical and prodromal stage. 9 out 593 samples were analysed two times and discard from data release as even after a second run the corresponding result was not obtainable.

The quality control analysis was successful and in compliance with criteria described below (see Paragraph "Quality control for analysis of clinical samples". QC samples from qualified and tested longitudinal biological samples with and without Alzheimer's disease i.e., AIBL and provided data in compliance with previous data with a deviation of less than 20%.

Methodology

1. Performing the test

Note: The AlzoSure® Predict test is only validated for the detection of the AZ284® peptide from plasma that has been immune-precipitated using the 2D3A8 antibody, specific to detect the biomarker U-p53^{AZ}

a) ***Overview***

The AlzoSure® Predict test is a two-day laboratory process, requiring a human blood plasma sample.

Laboratory Procedure

Day 1.

- Preparation of fresh blood plasma or defrosting.
- Preparation of buffers and kit standards.
- Preparation of the LC-MS/MS equipment and standardisation of AZ284® detection.
- Preparation of complex Protein L-2D3A8. Incubation of U-p53^{AZ} with complex Protein L-2D3A8 Ab for immune-precipitation reaction.

Day 2.

- Completion of U-p53^{AZ} immune-precipitation from plasma with 2D3A8 antibody.
- Digestion of U-p53^{AZ}.
- Analysis on LC-MS/MS.

Mass Spectrometer LC-ESI-MS/MS: TSQ Vantage Thermofisher heated-ESI Ion Source HPLC Surveyor Thermofisher Quaternary Pump.The analyses were carried out using a two-phase gradient: Phase A (H2O-0.2 % Formic Acid (HCOOH)) and Phase C methanol (CH3OH). The column used is a Phenomenex Kinetex PFP 50x4.1 mm 2.6 m. The volume of the injected sample is equal to 10 µL AZ 284®.

AlzoSure® Predict has been validated on multiple other platforms in addition to one above described.

Main Parameters of AZ 284® detection

Source: ESI positive

Scan type: MRM

Capillary Temp °C: 320

Collision Energy: 5eV

Sheath gas flow: 30.00

Aux gas flow: 2.00

Sweep gas flow: 15.00

Calculation of the results

The results of the assay for the concentration of AZ284® are calculated comparing the peak/area of internal standard (labelled AZ284®) with pick area of each sample.

The quantization of AZ284® peptide in the clinical sample is obtained taking into consideration the peak area of the analytes vs peak area of the labelled standard peptide spikes in the sample. In particular it is calculated using the following steps:

1. Calculate the ratio between the peak area of the peptide in the clinical sample and the peak area of labelled standard peptide spiked in the sample (Value "A").
2. Perform the calibration curve.
3. Use the standard curve formula to calculate the concentration of AZ284® in each sample.

AZ284® quantization in the clinical sample is obtained by the interpolation of the area ratio ("A") of the sample in the standard curve (formula: Y=mX + c): the Area ratio between the peptide in the sample and the labelled internal standard ("A") must be replaced to the "Y" value in the formula. The concentration of peptide corresponds to the "X" value.

Quality control for analysis of clinical samples

Before starting the analysis of any clinical samples, a calibration curve by negative plasma spiked with AZ284® unlabelled peptide covering the range from 0.08 fmol/10 µl - 5 fmol/10µl by at least 4 different points must be done (e.g., 4 concentrations selected among: 0.08- 0.2- 0.4- 0.8- 2- 5/10µl). This calibrant is included in the AlzoSure® Predict kit components for use with every 20 samples.

For each analytical session it is recommended to check the immunoprecipitation reaction efficacy by labelled U-p53 protein spiked at three different concentrations (0.5-1-2 fmol/10 µl) to negative plasma samples. Ion fragment from AZ284® peptide derived from labelled U-p53 has m/z 448.5 and its ion son is at 658.5. The labelling of the protein occurs at ([U- 13C6, 15N2]-L-Lysine). These standards are included in the AlzoSure® Predict kit components for use with every 20 samples.

Acceptance Criteria of QC testing by AlzoSure® Predict Protocol

The QC data generated by each analysis session will be considered acceptable if in compliance with the following criteria:

a) LC/MS-MS ; ESI-Source :

1) R^2 of standard curve R^2>0.980.

2) Labelled U-p53 Recovery>90% (at each concentration tested)

3) The variability of labelled internal standard (AZ284®) area over the full set of analysis must not overcome 15% of its average value.

4) Matrix effect: The deviation of the ME at each single concentration level must be within 15% (except at the LLOQ, CV <20%), as suggested by EMA guidelines and described by De Nicolò et al. (2017). Particularly the deviation must be calculated taking into account the Area Ratio between unlabelled AZ284®/ IntSt (labelled AZ284®) between same level of concentration.

AlzoSure® Predict Quality Criteria over ADNI-samples analysis.

The outcome from each quality criteria above described and applied to the analysis of ADNI samples is hereafter described:

Quality Criteria		Outcome from ADNI samples Analysis
1. Standard curve	R^2>0.980	R^2>0.990 over the full analysis
2. Recovery	Recovery of Labelled U-p53>90%	Labelled U-p53>90% over the full analysis
3. Internal Labelled standard (AZ284®) area CV%	CV≤15%	CV<10% over the full analysis
4. Matrix Effect	Point divergency ≤15% for all concentrations except at LLOQ <20%	Point divergency CV<5% for all concentrations
5. QC-samples	CV≤15% for all concentration except at LLOQ CV≤20%	33 AIBL samples included over the full analysis (at least 3 samples/ analytical session) CV% <10% over **all** analytical sessions of the full ADNI samples analysis

Tab 1. QC AlzoSure® Predict quality Criteria

An additional set of 4 plasma samples from AIBL has been analysed multiple times by different analytical sessions in the last year to show to further confirm the repeatability of AlzoSure® Predict over time:

AIBL Samples	Date of Analysis	CV%-Average
Sample "A"	Session 1	**3%**
	Session 2	
	Session 3	
	Session 4	
	Session 5	
	Session 6	
Sample "B"	Session 1	**11%**
	Session 2	
	Session 3	
	Session 4	
	Session 5	
	Session 6	
Sample "C"	Session 1	**4%**
	Session 2	
	Session 3	
	Session 4	
	Session 5	
	Session 6	
Sample "D"	Session 1	**4%**
	Session 2	
	Session 3	
	Session 4	
	Session 5	
	Session 6	

Tab 2. Repeatability of AlzoSure® Predict by QC- Samples from AIBL biobank.

The above table 1 summarises the information below:

1. **Standard Curve**: 6 different concentrations (as mentioned in "Quality control for analysis of clinical samples") were performed at each analytical session, and the value of R^2 was above than 0.99 at all sessions;
2. **Recovery:** all 3 different concentrations (as above described in "Quality control for analysis of clinical samples") of synthetic U-p53 were used to prove the effectiveness of 2D3A8 to immunoprecipitated the protein and for each analytical session it was above than 90%
3. **-Internal Labelled Standard (AZ284®)** performance: it was evaluated taking into consideration all values (Area (counts/s)) carried out over the full analytical session for each criteria (as linearity; recovery; matrix effect) and each sample analysed (including both QC-samples; ADNI clinical samples) per analytical session. The Internal Label Standard (AZ284®) showed high repeatability over the different analytical sessions and over the analysis period (about 45 days from first analytical session to the last) showing a CV% less than 10% and the same for all analysis.
4. **Matrix Effect:** all 3 different AZ284® concentrations (as above described in "Quality control for analysis of clinical samples") were used to evaluate the effect of plasma matrix on the peptide. The Divergency between the concentration of AZ284® spiked to plasma sample and water was less than 15% at each analytical session, the Slope divergency between the standard curve carried out from AZ284® in plasma and water was <10% at each session. The total Average Divergency calculated considering each AZ284® concentration and the Slope Divergency were both <5%.
5. **QC-samples:** in total 33 samples from AIBL biobank previously analysed were included over ADNI samples analysis (at least 3 samples/ analytical session). The difference between AZ284® concentration carried out from AIBL samples and the re-testing during ADNI analysis was calculated as also applying the criteria of "Incurred samples" reanalysis as described by FDA guideline about the Validation of Analytical Method (Bioanalytical Method Validation Guidance for Industry, 2018; https://www.fda.gov/files/drugs/published/Bioanalytical-Method-Validation-Guidance-for-Industry.pdf). The difference average between AZ284® concentration carried out from AIBL samples was <10% over each analytical session over the whole ADNI samples analysis.

6. **Additional evaluation of the repeatability and reliability of AlzoSure® Predict test,** 5 samples from each analytical session were selected by chance and analysed all in the same analytical session. As the results from this additional analytical session were used

only to confirm the repeatability of the test and are not included in the data release. The first data carried out from each sample as part of analytical session in compliance with AlzoSure® Predict quality criteria was considered reliable and uniquely used for data release. The divergency between the first AZ284® concentration value and which carried out over the retesting session was calculated as mentioned about QC-samples and in all cases (as divergency%, as "Incurred samples" criteria and CV% as well) was <7% with an average of value of approximately 2.5%. The standard curve R^2 value, Internal standard CV% and Matrix Effect criteria confirmed the values obtained over the full set of analysis as well.

Sample ID	**Divergency %**	**Incurred Samples criteria**	**CV%**
HA806SBK 03	0%	0%	0%
DA806SLL-03	2.9%	3%	2%
JA806SMP-02	3.8%	4%	3%
KA806SZ6-04	4.76%	5%	3%
KA806TW6-03	2.99	3%	2%
CA80730Y-03	2.13%	2%	1%
JA8073ZF-07	4.76%	5%	3%
DA8074XZ-05	5.48%	5%	4%
KA8076KN-12	4.92%	5%	3%

AA807M7K-10	0%	0%	0%
JA807NBJ-05	1.35%	1%	1%
AA807NWG-02	1.18%	1%	1%
HA807P2V-02	2.55%	3%	2%
DA80CQ3W-06	1.08%	1%	1%
DA807R5B02	0.01%	0%	0%
CA808BR3-11	0%	0%	0%
CA808BPR-04	2.5%	2%	2%
HA808DR7-08	0.85%	1%	1%
CA808F12-09	1.83%	2%	1%
BA808K7L-03	1.72%	2%	1%
HA808RGV-09	1.61%	2%	1%
CA808TW8-07	1.54%	2%	1%
HA808V4B-13	1.45%	1%	1%

HA808WXK-04	2.22%	2%	2%
AA808WZT-08	5.19%	5%	4%
BA8091HF-07	3.39%	3%	2%
BA8080VC-02	3.75%	4%	3%
JA8093ZM-04	5.48%	5%	4%
EA8093YC-07	1.47%	1%	1%
AA809FLR-03	1.16%	1%	1%
BA809FKT-02	0%	0%	0%
HA8095SB-06	5.26%	5%	4%
KA80972M-03	2.56%	3%	2%
BA809C72-10	2.06%	2%	1%
FA80BRJ5-03	5.56%	5%	4%
DA80C26Q-11	0%	0%	0%
HA80C57T-06	1.79%	2%	1%

FA80C8H9-03	6.15%	6%	4%
JA80CH9L-04	4.30%	4%	3%
DA80CH6B-04	1.02%	1%	1%
JA80BN5V-04	3.7%	4%	3%

References

1. Bioanalytical Method Validation Guidance for Industry, 2018; https://www.fda.gov/files/drugs/published/Bioanalytical-Method-Validation-Guidance-for-Industry.pdf)
2. Piccirella S et al, A conformational variant of p53 (U-p53AZ) as blood-based biomarker for the prediction of the onset of symptomatic Alzheimer's disease; 2022, JPAD.

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Note: The above data has not been confirmed or compared to clinical data to enable full interpretation of the clinical relevance or prognostic performance of the above analysis, this will be done once the samples are un blinded and the data will then be published