Supplementary Material

A Combination of Neurofilament Light, Glial Fibrillary Acidic Protein, and Neuronal Pentraxin-2 Discriminates Between Frontotemporal Dementia and Other Dementias

ELISA NPTX2 validation

Analytical validation and stability testing has been performed at the Department of Clinical Chemistry of the Amsterdam University Medical Center, location VUmc, according to international consensus criteria [1]. We evaluated the repeatability (intra-assay coefficient of variability (CV)), intermediate precision (inter-assay CV), sensitivity (lower limit of detection (LLOD)), parallelism, dilutional linearity, recovery, as well as the sample stability.

Inter- and intra-assay variation

The repeatability (intra-assay variability) and intermediate precision (inter-assay variability) of the assay were evaluated with 40 native CSF samples and three internal quality control (QC) CSF samples (QC low, QC medium and QC high) on six independent days. QC low, medium, and high samples were measured in duplicate in the beginning and the end of plate 2 to assess the within-plate reproducibility (intra-plate). The accepted range of variation was 15% for values within the linear range of the calibration curve.

The intra- and inter-assay coefficients of variation were QC low = 2.9%, QC medium = 3.4%, QC high = 6.6%, and QC low = 2.9%, QC medium = 3.9%, QC high = 6.6%, respectively.

Calibration curve

The standard calibration material from ADxNeuroSciences (recombinant human NPTX2) was used to prepare seven standards at the concentrations from 50 to 5000 pg/mL in sample dilution buffer. Unspiked sample dilution buffer was used as a buffer blank. Standard curves were fitted using 4-parameter logistic regression analysis.

Sensitivity

The sensitivity was measured using 16 blank samples. The blank (dilution buffer) was prepared from 45 mL DPBS (B. Braun), 5 mL Casein 1% (Thermo Scientific), and 50 µL Tween-20

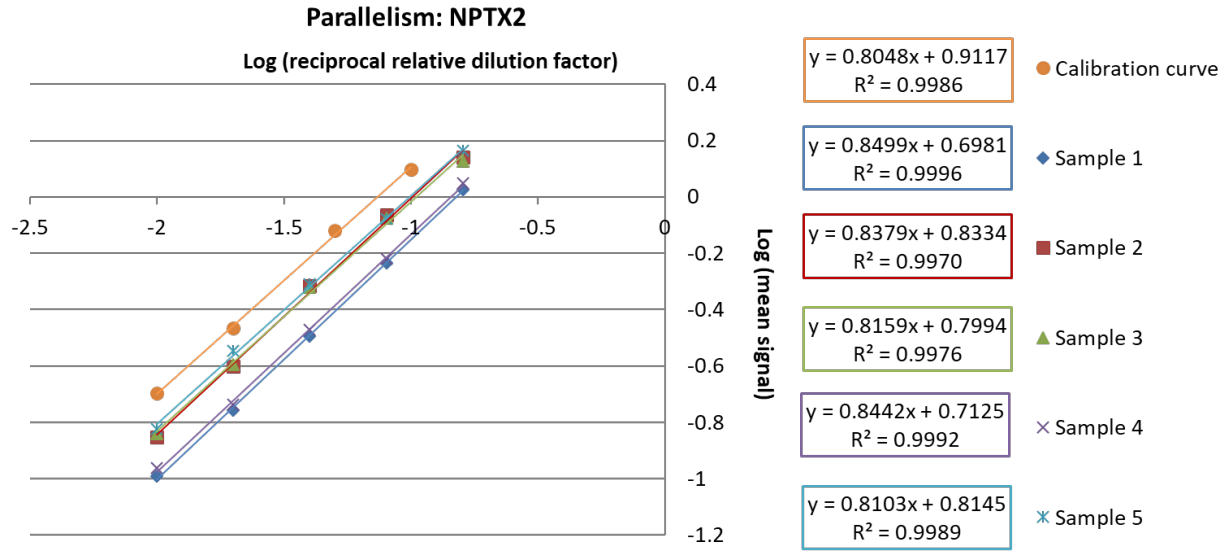
(DAKO). The LLOD was calculated as a signal of 10 standard deviations above the mean of the 16 blank samples.

The LLOD for was determined at 15.2 pg/mL.

Parallelism and linearity

Parallelism between the calibrator (spiked into sample diluent) and native protein in five neat CSF samples, was determined by comparing the signal of the different dilutions of the calibrator with the signal of the different dilutions of the neat CSF samples. The accepted range of deviation of the samples for parallelism was 85% to 115%. To assess linearity, three different CSF samples were spiked with 5000 pg/mL of calibrator and diluted at 3-fold until below the lowest calibration point (50 pg/mL). The spike solution amounted to 5% of the total volume. The acceptable range of variation of the analyte concentration for dilutional linearity was 85% to 115%.

All five neat CSF samples fell into the accepted range for parallelism and are thus parallel to the calibrator (Supplementary Figure 1). Spiked samples fall in the linear part of the standard curve with concentrations of 44 - 362 pg/mL. No hook effect was observed. These results indicate that the assay displays acceptable dilutional linearity within the quantitative range, which indicates the absence of a matrix effect within the limits of the concentrations tested.



Supplementary Figure 1. Parallelism of NPTX2 ELISA. Parallelism was assessed between recombinant calibrator (spiked into sample diluent) and five native CSF samples. Calibrator and CSF samples were diluted in similar concentration ranges. The acceptable range of parallelism was 85%-115%.

Recovery

Recovery was assessed by spiking four different CSF samples with a low (50 pg/mL), medium (250 pg/mL), and high (1000 pg/mL) concentration of the recombinant human NPTX2 calibrator. Dilution buffer that was spiked with the same amount of calibrator was measured as a reference. The measured values of the reference samples were subtracted from the results of the spiked samples, and recovery rates were calculated as a percentage of the spike level (50, 250, and 1000 pg/mL). The accepted range of variation for the recovery was 80% to 120%.

The reference concentrations of the low, medium and high spike were lower than theoretically calculated: 13.4 pg/mL, 129.8 pg/mL, and 642.7 pg/mL respectively. Mean recovery of CSF spiked with 50 pg/mL was 15%. Mean recovery of CSF spiked with 250 pg/mL was 98% and mean recovery for CSF spiked with 1000 pg/mL was 103%. Only the mean % recovery of the medium and high spike concentrations fell within the acceptable range of 80%-120%.

Sample stability

Aliquots of three samples were stored after centrifugation at -20°C, 4°C, 20°C, and 37°C during maximally 21 days. Upon measurement, the samples were transferred and stored at -80°C. The NPTX2 levels measured in the different conditions, were compared with a reference sample that was stored at -80°C. The effect of repeated freeze–thaw cycles was studied by freezing 3 samples directly after centrifugation and subsequently storing them at room temperature for 2 h before storing the samples at -80°C for a minimum of 24 h.

Concentrations in native CSF stored at all temperatures between -20°C and 37°C remained similar to the concentration in the reference samples stored at -80°C during 21 days and appeared not negatively affected by repeated freeze–thaw cycles.

Specificity

Cross-reactivity of capture and detection antibodies with other neuronal pentraxins (NPTX1 and NPTXR) were analyzed during the assay development by ADx Neurosciences (ADx Neurosciences NV, Belgium). The biotinylated detection antibody showed some cross-reactivity towards NPTX1. However, the capture antibody showed no cross-reactivity with NPTX1 or NPTXR. Thus, we can conclude that the final assay is specific for NPTX2 and does not show cross-reactivity to NPTX1 and NPTXR.

ELISA NPTXR validation

Analytical validation of the NPTXR ELISA from RayBiotech (RayBio® Human NPTXR ELSIA Kit; RayBiotech, USA) for the use in CSF has been performed at the Department of Clinical Chemistry of the Amsterdam University Medical Center, location VUmc, according to international consensus criteria [1]. We evaluated the reproducibility (intra-assay CV), repeatability (inter-assay CV), sensitivity (LLOD), parallelism, dilutional linearity, and recovery.

Inter-and intra-assay variation

The repeatability (intra-assay variability) and intermediate precision (inter-assay variability) of the assay were evaluated with three internal QC CSF samples (QC low, QC medium and QC high) on three independent days. QC low, medium, and high samples were measured in duplicate in the beginning and the end of plate 3 to assess the within-plate reproducibility (intra-plate). The accepted range of variation was 15% for values within the linear range of the calibration curve. The intra- and inter-assay coefficients of variation were QC low = 7.1%, QC medium = 6.7%, QC high = 6.2%, and QC low = 26.3%, QC medium = 28.0%, QC high = 33.4%, respectively. Intermediate precision exceeded the acceptance limits. During the sample runs for the current study, inter-assay CVs ranged from 4.6% to 8.7%.

Calibration curve

The calibration curve was prepared according to manufacturer's instructions, consisting of seven standards with concentrations ranging from 0.41 to 100 ng/mL in sample dilution buffer. Unspiked sample dilution buffer was used as a buffer blank. Standard curves were fitted using 4-parameter logistic regression analysis.

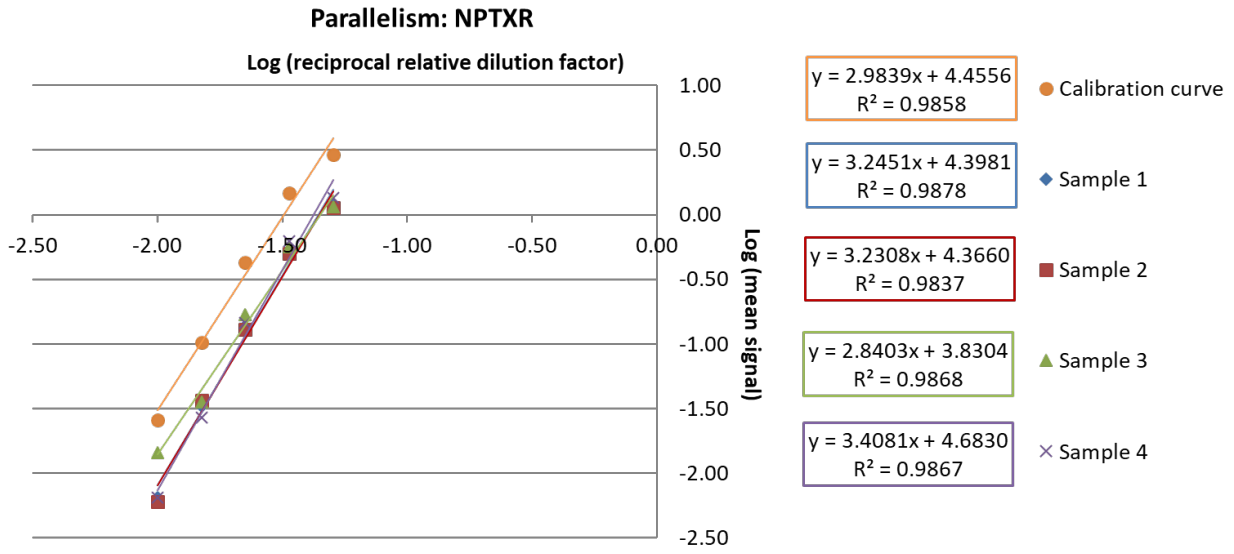
Sensitivity

Thirteen blank samples were measured to assess sensitivity. The LLOD was calculated as a signal of 10 standard deviations above the mean of the 13 blank samples.

The LLOD for was determined at 0.46 ng/mL.

Parallelism

Parallelism between the calibrator (spiked into sample diluent) and native protein in four neat CSF samples, was determined by comparing the signal of the different dilutions of the calibrator with the signal of the different dilutions of the neat CSF samples. The accepted range of deviation of the samples for parallelism was 85% to 115%. All four neat CSF samples fell within the accepted range for parallelism (95%-114%) and are thus parallel to the calibrator (Supplementary Figure 2). These results indicate equal binding characteristics of capture and detection antibodies to the calibrator and native protein, allowing to determine native protein concentration by applying the calibration curve.



Supplementary Figure 1. Parallelism of NPTXR ELISA. Parallelism was assessed between recombinant calibrator (spiked into sample diluent) and four native CSF samples. Calibrator and samples were diluted in similar concentration ranges. The acceptable range of parallelism was 85%-115%.

Dilutional linearity

To assess linearity, 3 different CSF samples were spiked with 1600 ng/mL of calibrator and diluted at 5-fold until below the lowest calibration point (0.41 ng/mL). The spike solution amounted to 5% of the total volume. The acceptable range of variation of the analyte concentration for dilutional linearity was 85% to 115%.

Spiked samples do not show linearity upon dilution. No hook effect was observed. These results indicate that the assay does not displays acceptable dilutional linearity within the

quantitative range, which could be caused by the presence of a matrix effect within the limits of the concentrations tested.

Recovery

Recovery was assessed by spiking four different CSF samples with a low (5 ng/mL), medium (10 ng/mL), and high (20 ng/mL) concentration of the recombinant human NPTXR calibrator. Dilution buffer that was spiked with the same amount of calibrator was measured as a reference. The measured values of the reference samples were subtracted from the results of the spiked samples, and recovery rates were calculated as a percentage of the spike level (5, 10, and 20 ng/mL). The accepted range of variation for the recovery was 80% to 120%.

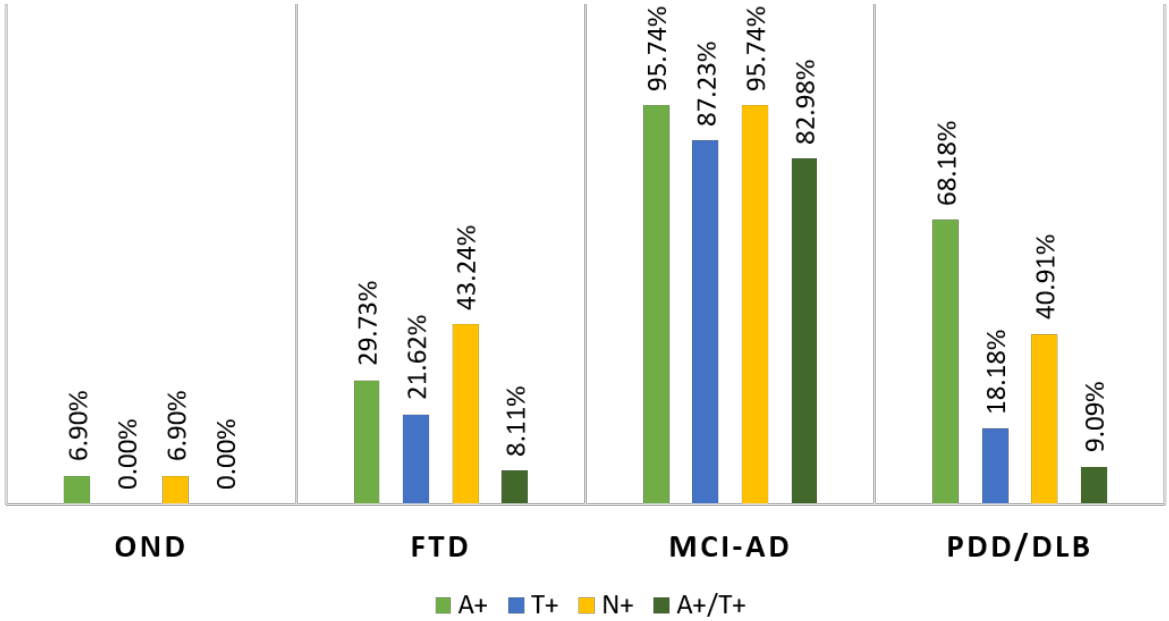
The reference concentrations of the low, medium, and high spike were slightly higher than theoretical calculated: 7 ng/mL, 12.8 ng/mL, 24.9 ng/mL, respectively. Mean recovery of CSF spiked to 5 ng/mL was 76%. Mean recovery of CSF spiked to 10 ng/mL was 95% and mean recovery for CSF spiked to 20 ng/mL was 101%. Only the mean % recovery of the low spike concentration falls slightly out of the acceptable range of 80%-120%.

Summary

The analytical validation of the NPTXR ELISA for CSF could be accepted for the parameters repeatability, parallelism and recovery. However, intermediate precision and dilutional linearity exceeded the acceptance limits. These results indicate that NPTXR measurements in CSF should be interpreted with caution.

A/T/(N) prevalence

A high prevalence (68.18%) of A+ was found in the Lewy body dementias group (PDD/DLB) and A+/T+ being present in 9.09% of cases. Among neurodegenerative diseases, FTD showed the lowest prevalence of A+ (29.73%), which was still significantly higher than in the OND group ($p = 0.004$) and significantly lower than in the PDD/DLB group ($p = 0.01$), with a concomitant T+ present only in three subjects (8.11%). Similar prevalences of N+ (40.91 and 43.24%) were observed across the FTD and PDD/DLB groups (Supplementary Figure 3).



Supplementary Figure 3. Prevalence of amyloidosis (A+), tauopathy (T+), neurodegeneration (N+) and concomitant amyloidosis and tauopathy (A+/T+) across the diagnostic groups.

Supplementary Table 1. Classical AD biomarker cut-off values and corresponding assays used for biomarker measurement

Time at LP	$A\beta_{42}$ cut-off	t-tau cut-off	p-tau cut-off	$A\beta_{42}/A\beta_{40}$ cut-off	Assay
August 2007 - March 2009	>1200	<200	<60	>0.076	INNOTEST ELISA ($A\beta_{42}$, t-tau, p-tau)
March 2010 - January 2016	>800	<300	<60	>0.076	INNOTEST ELISA ($A\beta_{42}$, t-tau, p-tau)
April 2016 - March 2018	>500	<400	<64.5	>0.076	EUROIMMUN ELISA ($A\beta_{40}$, $A\beta_{42}$), INNOTEST ELISA (t-tau, p-tau)
April 2018 - October 2018	>781	<400	<64.5	>0.077	Lumipulse G600-II ($A\beta_{40}$, $A\beta_{42}$, t-tau, p-tau)

The lists the date of lumbar puncture and the corresponding cut-off values of the classical AD CSF biomarkers, as well as the assay used for biomarker measurement at the time. Aβ, amyloid-β; t-tau, total tau; p-tau, tau phosphorylated at threonine 181; LP, lumbar puncture.

REFERENCE

[1] Andreasson U, Perret-Liaudet A, van Waalwijk van Doorn LJ, Blennow K, Chiasserini D, Engelborghs S, Fladby T, Genc S, Kruse N, Kuiperij HB, Kulic L, Lewczuk P, Mollenhauer B, Mroczko B, Parnetti L, Vanmechelen E, Verbeek MM, Winblad B, Zetterberg H, Koel-Simmelink M, Teunissen CE (2015) A practical guide to immunoassay method validation. *Front Neurol* **6**, 179.