Letter to the Editor

Maxim De Schaepdryver, Janne Goossens, Andreas Jeromin, Britta Brix, Philip Van Damme and Koen Poesen*

Analytical performance of a CE-marked immunoassay to quantify phosphorylated neurofilament heavy chains

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To the Editor,

The phosphorylated neurofilament heavy chain (pNfH) in cerebrospinal fluid (CSF) is a shortlisted diagnostic biomarker in amyotrophic lateral sclerosis (ALS) [1, 2]. Here, we report the analytical performances of a CE-marked enzyme-linked immunosorbent assay (ELISA) for pNfH (Cat. # EQ 6561-9601, LOT. # E161124CN, Euroimmun AG, Germany), now released for clinical applications. The ELISA, performed according to the manufacturer's protocol, consisted of a polyclonal anti pNfH capturing antibody (CPCA-NF-A, EnCor Biotechnology Inc., USA), a peroxidase-labelled monoclonal anti pNfH detection antibody (MCA-NAP4, EnCor Biotechnology Inc., USA) and a ready to use (RTU) calibrator set, ranging from 0 to 10,000 pg/mL, prepared with bovine pNfH (EnCor Biotechnology Inc., USA). Also, a low (200 pg/mL) and a high (1000 pg/mL) quality control were included. Two different calibration methods were applied. First, a standard calibration method where a distinct calibrator set was measured each analysis to construct a four parameter logistic (4-PL) calibration curve (GraphPad Prism 6, USA). Second, an experimental master calibration curve method where a 4-PL fixed calibration curve, obtained during the first analysis, was used to convert every optical density value generated in all analyses [3]. Leftovers of CSF, obtained via lumbar puncture for routine diagnostic purposes, were used. The study has been approved by the local Ethical Committee of the University Hospitals Leuven (S50354).

The analytical sensitivity was assessed in terms of limit of blank (LOB), limit of detection (LOD) and limit of quantification (LOQ) according to the Clinical and Laboratory Standards Institute (CLSI) guideline EP17-A2 [4]. Briefly, the LOB was determined by measuring 72 replicates of sample diluent over three runs. The 95th percentile of the calculated concentrations corresponded to a LOB of 33 pg/mL. Next, three CSF samples (93, 139 and 185 pg/mL) were measured over 5 days in triplicate to assess the LOD. The LOD was defined as the 95th percentile of the normally distributed LOB. This was calculated by adding to the LOB, the pooled standard deviation (SD_L) of the CSF measurements multiplied by a Z-value (1.652). Using the standard calibration method, the SD_L was 21.72 pg/mL resulting in a LOD of 69 pg/mL. Alternatively, using the master calibration curve method, the SD_L was 15.16 pg/mL leading to a LOD of 58 pg/mL. Three additional CSF samples (232, 278 and 370 pg/mL) were measured in triplicate over 5 days to determine the LOQ. For all six CSF samples, the total error, which is the combined effect of bias and precision on a result, was calculated according to the root mean squared model. The intersect of the curve fit using all data couples (pNfH; total error) and the

***Corresponding author: Koen Poesen,** PharmD, PhD, Department of Neurosciences, Laboratory for Molecular Neurobiomarker Research, KU Leuven (University of Leuven), Leuven, Belgium; and Laboratory Medicine, University Hospitals Leuven, Leuven, Belgium, Phone: 003216347013, E-mail: koen.poesen@uzleuven.be
Maxim De Schaepdryver: Department of Neurosciences, Laboratory for Molecular Neurobiomarker Research, KU Leuven (University of Leuven), Leuven, Belgium; and Laboratory Medicine, University Hospitals Leuven, Leuven, Belgium. https://orcid.org/0000-0003-2480-863X
Janne Goossens: Department of Neurosciences, Laboratory for Molecular Neurobiomarker Research, KU Leuven (University of Leuven), Leuven, Belgium
Andreas Jeromin: Iron Horse Diagnostics, Inc., Scottsdale, AZ, USA
Britta Brix: Euroimmun AG, Lübeck, Germany
Philip Van Damme: Department of Neurosciences, Laboratory of Neurobiology, KU Leuven (University of Leuven) and Center for Brain and Disease Research VIB Leuven, Leuven, Belgium; and Department of Neurology, University Hospitals Leuven, Leuven, Belgium

predefined allowable total error, which is chosen by the user, corresponded to the LOQ. Thus, a pNfH concentration above the LOQ guarantees the user not only to detect the protein but also to quantify it with a predefined precision and bias. Using the master calibration curve method, the LOQ corresponded to 150 pg/mL with a predefined allowable total error of 15%. The standard calibration method did not allow to calculate a LOQ as the best curve fit remained above the allowable total error of 15%.

The analytical precision in terms of optical densities and back-calculated concentrations per RTU calibrator, measured in duplicate over 10 runs using the standard calibration method, are listed in Table 1. The mean repeatability, total coefficient of variance and between day coefficient of variance (CVs), defined and calculated as previously described [5], of the back-calculated concentrations were 6.27%, 7.56% and 5.42%, respectively. In accordance with an LOQ of 150 pg/mL, a clear variability in the precision of the lowest RTU calibrator (target value of 125 pg/mL) was seen in comparison to the higher RTU calibrators (Table 1).

Repeatability and within-laboratory precision reported by the manufacturer in terms of CVs were verified in patient samples. For this purpose, five CSF samples were measured in triplicate over 5 days according to the CLSI guideline EP15-A2 [6]. The obtained CVs were converted into a standard deviation (SD). If the repeatability and the within-laboratory SDs were less than the SDs reported by the manufacturer, the claim of the manufacturer was verified. However, if the repeatability or within-laboratory SD was higher, the verification value was calculated to determine whether this difference was significant. The variance term for the daily means, the within-laboratory variance term and the verification value were calculated as previously described [5, 6]. The repeatability and within-laboratory precision of all individual CSF samples are listed in Table 2. Using the standard calibration method, the repeatability SD of 127.5 pg/mL

Table 1: Precision profile of the ready to use calibrators.

RTU calibrator[a], pg/mL	Optical densities				Back-calculated concentration, pg/mL			
	Mean (±SD)	$CV(\%)_r$	$CV(\%)_{bd}$	$CV(\%)_{total}$	Mean (±SD)	$CV(\%)_r$	$CV(\%)_{bd}$	$CV(\%)_{total}$
0	0.050 (±0.005)	4.54	7.31	9.26	–	–	–	–
125	0.086 (±0.006)	3.42	5.95	6.48	154 (±26)	10.36	15.38	16.75
500	0.177 (±0.016)	5.09	6.42	9.22	535 (±51)	5.70	8.90	9.60
2000	0.600 (±0.043)	5.01	8.68	7.23	1981 (±75)	5.06	1.07	3.82
5000	1.506 (±0.105)	5.37	6.15	6.98	4998 (±197)	5.34	0.77	3.94
10,000	2.688 (±0.204)	3.69	8.89	7.60	9972 (±366)	4.88	1.00	3.67

RTU, ready to use; SD, standard deviation; CV, coefficient of variance; r, repeatability; bd, between day. [a]Target values provided by the company.

Table 2: Assessment of the analytical precision of the immunoassay using CSF samples.

CSF sample	Conc., pg/mL	Repeatability		Within-laboratory variability			
		S_r	$CV(\%)_r$	B	S_{total}	CV, %	Verification value
SC							
1	398±54	54.43	13.67	1406	58.14	14.94	220.35
2	710±63	58.39	8.23	3193	73.93	10.65	253.77
3	761±50	27.11	3.56	4244	68.80	9.23	356.09
4	1939±113	105.22	5.43	7489	121.94	6.34	235.09
5	5430±255	194.88	3.59	39,877	255.33	4.68	261.11
MCC							
1	348±46	46.56	13.37	630	45.56	13.09	–
2	613±55	51.69	8.44	1352	55.98	9.14	–
3	657±28	23.01	3.50	468	28.65	4.36	–
4	1720±88	91.39	5.31	1991	86.95	5.06	–
5	4951±334	175.29	3.54	104,644	353.75	7.14	–

SC, standard calibration; MCC, master calibration curve; Conc., concentration (mean ± standard deviation); S_r, repeatability variance term; CV, coefficient of variance; B, variance term for the daily means; S_{total}, within-laboratory variance term; verification value, if S_{total} is less than or equal to the verification value, the manufacturer's claim can be considered as verified.

(CV: 6.9%, pNfH: 1848 pg/mL) was lower than the SD of 152.0 pg/mL (CV: 5.5%, pNfH: 2764 pg/mL) mentioned in the manufacturer's validation report. Likewise, using the master calibration curve method, a repeatability SD of 112.7 pg/mL (CV: 6.8%, pNfH: 1658 pg/mL) made our findings consistent with the manufacturer's claim given a higher SD of 152.0 pg/mL reported by the manufacturer.

Using the standard calibration and the master calibration curve method, the within-laboratory SD was 169.4 pg/mL (CV: 9.17%, pNfH: 1848 pg/mL) and 128.6 pg/mL (CV: 7.76%, pNfH: 1658 pg/mL), respectively. As the within-laboratory SD of 164.5 pg/mL (CV: 5.95%, pNfH: 2764 pg/mL) using the standard calibration method was higher than the SD reported by the manufacturer, we tested whether the within-laboratory SD was significantly larger. As the within-laboratory variance term was lower than the verification value for each sample (Table 2), our findings were consistent with the within-laboratory precision reported by the manufacturer. For the master calibration curve method a within-laboratory SD of 128.6 pg/mL was found, which was below the manufacturer's within-laboratory SD, meaning that their claim about the within-laboratory precision was verified.

Conform the CLSI guideline EP6-A, parallelism was assessed by performing a serial dilution of nine equidistant dilutions measured in quadruplicate using three CSF samples (373, 4637 and 20,604 pg/mL) [7]. The percentage difference between the best fit and the linear curve fit was less than the predefined allowable deviation of 20%, except for the highest dilution of the lowest pNfH sample, which resulted in a concentration below the LOD. Therefore, parallelism was shown for three independent CSF pNfH samples.

Our study confirmed the repeatability and within-laboratory precision reported by the manufacturer. Though, the precision did not improve when using the master calibration curve method, despite the existing variability of the calibrator set when using the standard calibration method. The increase of the variability as the concentration decreased was likely related to the fact that the targeted concentration was situated on the lower flat part of the 4-PL curve meaning a high variability of the calculated concentration related to small differences in optical densities. Next, our results can only be partially compared with previously published assays because different materials and methods have been used [8, 9]. Finally, our study assessed the detection capabilities of a CE-marked assay. When evaluating our previously reported results on pNfH in CSF, all patients with ALS or a disease mimicking ALS had a pNfH concentration above the LOQ of 150 pg/mL, except for one mimic with a CSF pNfH concentration below the LOD [1]. Therefore, we provide evidence that the herein described CE-marked ELISA is technically sound to quantify pNfH in CSF for diagnostic purposes in motor neuron diseases.

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