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A reproducible and quantitative CE-immunoassay for eNOS protein expression measurements directly in human brain tissue homogenates

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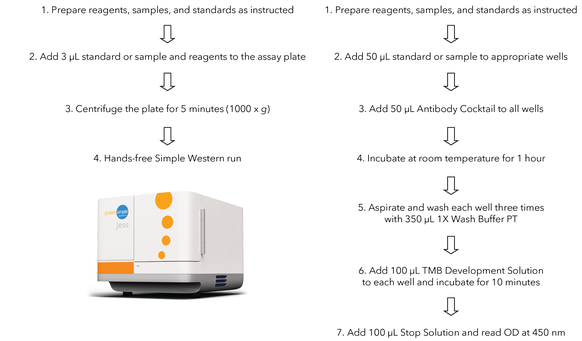
ABSTRACT

Detailed proteomic characterization of human brain tissue is needed to identify potential novel biomarkers and drug targets for a variety of neurological diseases. The enzyme-linked immunosorbent assay (ELISA) offers quantitative protein expression measurements with high specificity and sensitivity. However, obtaining reliable results from ELISA assays can be challenging with complex sample types like brain tissue , which can have significant interference by matrix effects due to the high content of lipids and lipoproteins, as well as the sheer protein intricacy that results from the brain's complexity. As a result, ELISAs for studying protein biomarkers of neurological diseases are limited primarily to peripheral blood and CSF samples.

Here, we developed a capillary electrophoresis (CE) immunoassay using Simple Western, a hands-free CE-immunoassay platform, to measure a biomarker of the cerebrovascular system, eNOS, directly in human brain whole tissue homogenates. We show that the size-based separation provided by the CE-immunoassay identified differential expression of eNOS isoforms in brain tissue compared to cultured endothelial and cervical cancer cells which could not be detected by ELISA. Compared to a commercial ELISA kit, the CE-immunoassay demonstrated increased sensitivity and dynamic range of detection. Furthermore, differences in tissue homogenization and storage buffer conditions impacted the ability of ELISA to detect eNOS in brain tissue samples but had no observable effect on the CE-immunoassay. Finally, the CE-immunoassay consumed less brain tissue for analysis, needing only 3 µL of homogenate compared to 50 µL for ELISA. Because the CE-immunoassay requires only one target-validated antibody for detection, we anticipate that the CE-immunoassay will enable the analysis of additional protein biomarkers in brain tissue samples that historically have been challenging to detect by traditional ELISAs.

METHODS

CE-Immunoassay (Simple Western) Vs. ELISA



	CE-immunoassay	ELISA
Sample Requirement	3 µL	50 µL
Hands-on time	30-60 min upfront sample prep followed by hands-free run	>90 min with intermissions throughout
Liquid waste	No	Yes
Size separation	Yes	No
Matrix effect	No	Yes

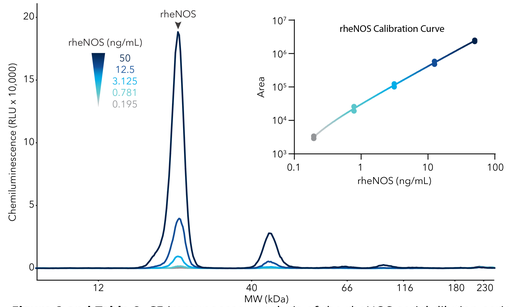
Figure 1 and Table 1. Comparison of the CE-immunoassay and ELISA methods for eNOS quantification used in this study.

Scan the QR code to access and complete materials and methods for this study in the App Note entitled: "Stuck in the Matrix? Escape Matrix Effects in complex Samples With Simple Western"

RESULTS

Establishing the CE-Immunoassay for Human eNOS Biomarker Testing

- To establish a CE-immunoassay for human eNOS quantification, a serial dilution series of recombinant human eNOS (rheNOS) was prepared and analyzed by the Simple Western CE-immunoassay instrument (Jess instrument, ProteinSimple). The electropherograms from this analysis showed a strong peak corresponding to rheNOS with a molecular weight (MW) of approximately 22 kDa, and less abundant secondary peak at a MW of roughly 45 kDa, possibly caused by rheNOS dimerization (**Fig. 2**).
- To generate a calibration curve for eNOS quantification, rheNOS peak areas were plotted against the rheNOS concentration, and a 4-parameter logistic (4PL) regression was applied to create a line of fit, resulting in a quantitative range of approximately 2.5 logs (**Fig. 2, inset**).
- We calculated the recovery of rheNOS spiked in each sample in the serial dilution series, which closely matched expected values with 99.9% to 100% recovery (**Table 2**).

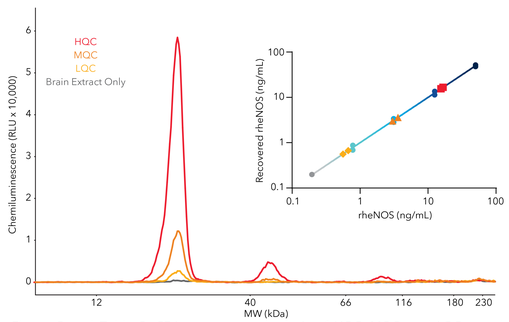


rheNOS (ng/mL)	Recovery (%)
50	100.0
12.5	100.0
3.125	99.9
0.781	100.0
0.195	100.0

Table 2. Recovery of rheNOS standards (*n*=2).

Figure 2 and Table 2. CE-immunoassay analysis of the rheNOS serial dilution series and generation of a calibration curve. Immunodetection was performed using a mouse anti-eNOS antibody and an HRP-conjugated anti-mouse secondary antibody. The rheNOS peak areas (*n*=2) were plotted against rheNOS concentration to generate a calibration curve with a 4PL line of fit.

- For quality control (QC) of the CE-immunoassay assay, samples of rheNOS were prepared at low (LQC), medium (MQC), and high (HQC) final concentrations of 0.6, 3, and 15 ng/mL, respectively (**Fig. 3**).
- Each QC sample was subtracted by the matrix-only control and then the percent recovery of each sample was calculated and plotted against expected rheNOS concentrations (**Fig. 3, inset**). The percent recovery of each QC sample fell within ±20% of expected values (**Table 3**).
- To determine assay linearity, we diluted the HQC sample to 1:2, 1:4, and 1:8 from the original concentration, and each sample dilution was analyzed by the CE-immunoassay. Each sample dilution resulted in a percent recovery within ±20% of expected values (**Table 3**).



Sample	Recovery (%)
LQC	102.6
MWC	110.6
HQC	107.5
1:2 HQC	117.3
1:4 HQC	103.7
1:8 HQC	98.4

Table 3. Recovery of QC samples (*n*=2).

Figure 3 and Table 3. CE-immunoassay analysis of HQC, MQC, and LQC samples. HQC, MQC, and LQC samples contain rheNOS at final concentrations of 15, 3, and 0.6 ng/mL, respectively. Each sample was probed with the anti-eNOS antibody. Each QC sample (*n*=2) was subtracted by the matrix-only control and then the percent recovery was calculated and plotted against expected rheNOS concentrations.

Specificity of the CE-Immunoassay in Human Brain Tissue Homogenates

- CE-immunoassay analysis of serially diluted brain tissue samples showed similar expression profiles in both homogenates, with a major eNOS peak and several minor peaks, indicating the presence of eNOS isoforms (**Fig. 4**).
- The eNOS signal in both tissue samples decreased with decreasing sample concentration in a linear fashion and all sample dilutions recovered within ±20% (**Table 4**).
- As expected, CE-immunoassay analysis detected eNOS expression in the endothelial whole-cell lysate and no expression was observed in the cervical cancer whole-cell lysate (**Fig. 5**).
- Lower MW isoforms were more prevalent in cervical cancer cells, possibly due to differences in gene regulation, post-translational modifications, and/or protease activity (**Fig. 5**).

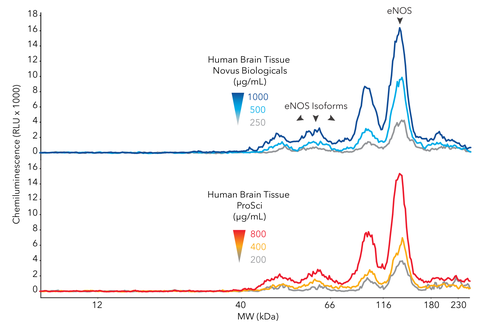


Figure 4. CE-immunoassay analysis of endogenous eNOS expression in human brain tissue homogenates.

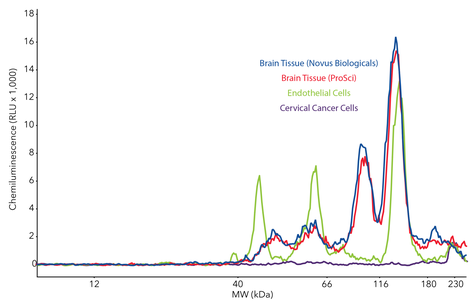


Figure 5. Tissue biodistribution of eNOS in human brain tissue, endothelial cells, and cervical cancer cells.

Sample Dilution	Recovery (%) in Brain Tissue Lysate (Novus Biologicals)	Recovery (%) in Brain Tissue Lysate (ProSci)
1:2	118.1	84.3
1:4	118.8*	109.8

Table 4. Recovery of eNOS in dilutions of human brain tissue samples from the undiluted sample. (*n*=2, **n*=1)

Comparing the CE-Immunoassay to ELISA: Specificity, Sensitivity, and Range

- ELISA only detected eNOS in the brain tissue homogenate from Novus Biologicals and was not able to detect eNOS in the brain tissue homogenate from ProSci with differing homogenization and buffer conditions, suggesting the CE-immunoassay is less susceptible to matrix effects in brain tissue samples than ELISA.
- ELISA appeared to underestimate the concentration of eNOS and demonstrated a narrow assay range and poor sensitivity compared to CE-immunoassay (**Table 5**). Again, these results suggest that the CE-immunoassay is less susceptible to matrix effect in brain tissue samples than ELISA.

	ELISA	CE-immunoassay
Quantification of eNOS (ng/mL)	1.56	7.9
Assay range (ng/mL)	0.234 – 15	0.195 – 50
Assay sensitivity (pg/well)	11.7	0.6

Table 5. Comparison of ELISA and CE-immunoassays for eNOS quantification in brain tissue.

CONCLUSIONS

- We developed a CE-immunoassay on the Simple Western platform that reproducibly quantifies eNOS in human brain tissue homogenates & whole-cell lysates.
- While the CE-immunoassay measured eNOS in two different whole brain tissue homogenates from different vendors and different homogenization and/or storage buffer conditions, ELISA could only detect brain tissue biomarker eNOS in one of the two brain tissue homogenates, suggesting the CE-immunoassay is less susceptible to matrix effects.
- The CE-immunoassay outperformed ELISA in assay accuracy, range, and sensitivity for eNOS quantification in human brain whole tissue homogenate. Due to size-based separation profiles, CE-immunoassay was able to distinguish between specific targets like other eNOS isoforms and nonspecific background cross-reactivity, which would go unnoticed by ELISA.

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