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Application Note

Stuck in the Matrix? Escape Matrix Effects in Complex Samples With Simple Western

The Unmet Need for Analytical Precision and Reproducibility in Tissue Homogenates

Analytical development groups need to consider FDA guidelines when developing quantitative, validated assays.[1] Researchers in these groups need quantitative assays using inherently complex sample types, often with limited material available for analysis. While ELISAs have the advantages of sensitivity and specificity, matrix effects can occur when analyzing complex sample types like tissue homogenates. In addition, custom development of sandwich ELISAs can also be challenging owing to the need to identify and validate a pair of two antibodies rather than just a single antibody reactive against the target of interest.

Overestimation of analyte molecules due to cross-reactivity is still a problem with ELISA[2]. ELISA lacks the separation profiles needed to characterize off-target binding and protein isoforms, like full-length and cleaved protein targets, or non-functional and enzymatically activated prodrugs. ELISAs also use capture antibodies to bind sample analytes directly in the presence of the sample matrix which can increase matrix effect.

Introducing orthogonal separation-based immunoassays like Western blot alleviates many of the limitations of ELISA.[3] However, separation-based immunoassays like the traditional Western blots are only semi-quantitative at best. This is due in part to the gel-to-membrane transfer of the protein sample. As a result, reproducibility is lacking in Western blot workflows, with extensive manual labor and a long time to results.

Get Real Tissue Biodistribution Results Without Matrix Effects

Simple Western™ assays allow biopharma groups to develop and validate quantitative assays to support preclinical and clinical studies as well as upstream and downstream bioprocessing workflows. Automated Simple Western removes the manual steps involved in traditional Western blot workflows, leading to high-quality data and reproducible quantitation.

Insane in the Membrane! Simple Western Eliminates Unreliable Membrane Transfers

- Simple Western's high sensitivity and specificity allow you to easily analyze complex sample types like tissue homogenates with minimal background and using a limited amount of precious sample volume (3 µL).
- Separation by size or charge on Simple Western combined with the specificity of immunodetection allow you to discriminate between different isoforms of target proteins with high resolution.
- Simple Western uses proprietary chemistry to bind sample analytes to the capillary wall after separation. Covalent binding even occurs within a complex matrix, reducing matrix effect that can occur when sample analytes are captured using a specific capture antibody in an ELISA.
- Following covalent sample immobilization, Simple Western flushes the sample matrix from the capillary before immunodetection in an optimized buffer environment, further reducing matrix effect.
- Simple Western only needs one target-specific antibody to give specific detection which simplifies assay development and accelerates development time compared to custom ELISA development.

A Clearer Picture of Tissue Biodistribution and Biomarker Protein Expression in Human Cells

Brain tissue samples are known to have significant interference by matrix effect in immunoassays caused by the high level of lipids, lipoproteins, and protein intricacy that results from the brain's complexity.[4] As a result, matrix effects in brain tissue and other complex samples have limited the availability of preclinical and clinical biomarkers in drug discovery and development.

Despite its name, endothelial nitric oxide synthase (eNOS or NOS3) is expressed in both endothelial cells and brain tissue where eNOS plays an important role in vascular regulation. The lack of functional eNOS contributes to vascular disease, chronic inflammation, and even cancer, making eNOS an important preclinical and clinical biomarker in drug discovery and development.[5,6] While a commercial ELISA kit provided eNOS biomarker testing in human endothelial cell lysates, when this kit was applied to human brain tissue samples following homogenization, a high matrix effect was observed and the kit has since been discontinued.[4]

In this Application Note, we show that Simple Western analysis of human eNOS in brain whole tissue lysate is less susceptible to matrix effect than a leading commercial eNOS ELISA kit,[7] allowing for more accurate quantification in brain tissue homogenates. Unlike the ELISA kit, Simple Western is fully automated following sample preparation and plate loading (**FIGURE 1**) and provides other assay advantages like low sample requirements and no liquid waste (**TABLE 1**). With size separation, Simple Western can detect protein isoforms and off-target antibody binding that go unnoticed by ELISA, skewing results.

FIGURE 1. Comparison of ELISA and Simple Western workflows for eNOS quantification.

TABLE 1. Comparison of ELISA and Simple Western assays for eNOS quantification.

	ELISA[7]	Simple Western
Sample requirement	50 µL	3 µL
Hands-on time	1.5 hours with intermissions throughout the entire run	1 hour of upfront sample preparation and plate loading, followed by full automation
Liquid waste	Yes	No
Size separation	No	Yes
Matrix effect	Yes	No

Materials and Methods

Simple Western Analysis

All materials used in this study are listed in **TABLE 2** and Simple Western assays were performed with the Simple Western instrument Jess™. Samples were denatured under reducing conditions with 1X Master Mix for 5 minutes at 95 ˚C, as described in the 12-230 kDa Separation Module Product Insert. Antibody Diluent 2 was used as the blocking buffer. The mouse primary anti-eNOS (6H2) antibody was diluted at 1:10 in Antibody Diluent 2 and the Anti-Mouse Secondary Antibody was used at the ready-to-use stock concentration.

To create a calibration curve for eNOS quantification by Simple Western, recombinant human eNOS (rheNOS) was prepared in a 5-point, 4-fold titration at final concentrations of 50, 12.5, 3.125, 0.781, and 0.195 ng/ml. Each sample in this titration series was prepared in HeLa lysate background at a final concentration of 100 µg/mL.

To calculate the percent recovery of rheNOS spiked in tissue homogenate, samples were prepared independently at final rheNOS concentrations of 15, 3, and 0.6 ng/mL for high, medium, and low controls, respectively. To calculate the linearity of the percent recovery of high spike samples, the high spike sample was diluted to 1:2, 1:4, and 1:8. Each spike sample was prepared in human brain extract at a final concentration of 250 µg/mL.

The tissue lysate from Novus Biologicals was prepared at final concentrations of 1000, 500, and 250 µg/mL. The tissue lysate from ProSci was prepared at final concentrations of 800, 400, and 200 µg/ml. As a positive control, endothelial (HUV-EC-C) whole-cell lysate was prepared at a final concentration of 200 µg/mL. As a negative control, cervical cancer (HeLa) whole-cell lysate was prepared at a final concentration of 100 µg/mL.

ELISA Analysis

To create a calibration curve for eNOS quantification by ELISA, a 7-point, 2-fold titration at final concentrations of 15 ng/mL to 0.234 ng/mL was prepared according to the manufacturer's instructions (Human eNOS ELISA Kit, Abcam). The same samples described above for analysis on Simple Western were evaluated using the Human eNOS ELISA kit, including the calibration curve samples, high spike linearity samples, and human tissue homogenate and whole-cell lysate samples.

TABLE 2. Materials used in this study.

Item	Vendor	Part Number
12-230 kDa Separation Module	ProteinSimple and Novus Biologicals Bio-Techne brands	SM-W004
rheNOS (aa 1030 1203)		In House
HeLa Lysate Control		042-488
Human Brain Whole Tissue Lysate (Adult Whole Normal)		NB820-59177
Anti-Mouse Detection Module		DM-002
Human Brain Lysate	ProSci	1303
Human Brain Extract	Santa Cruz Biotechnology	sc-364375
HUV E-C-C Whole Cell Lysate		sc-364180
eNOS (6H2) Mouse mAb	Cell Signaling Technology	5880S
Human eNOS ELISA Kit	Abcam	ab263878

Establishing the Simple Western Assay for Human eNOS Biomarker Testing

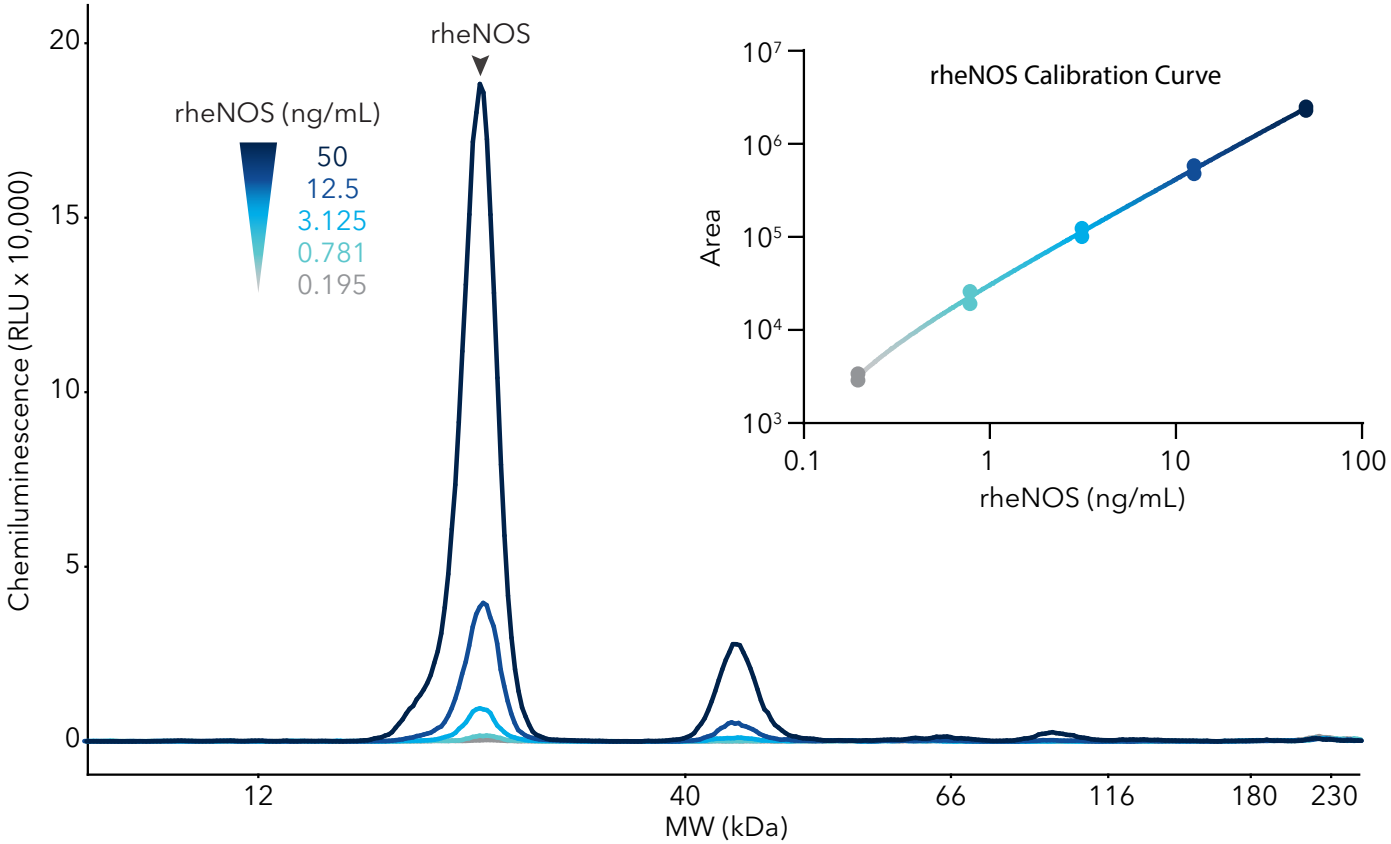
To establish a Simple Western assay for human eNOS quantification, a serial dilution series of rheNOS was prepared as described in the Materials and Methods and analyzed by Simple Western. The electropherograms from this analysis showed a strong peak corresponding to rheNOS with a molecular weight (MW) of approximately 22 kDa, and less abundant secondary peak at a MW of roughly 45 kDa, possibly caused by rheNOS dimerization (**FIGURE 2**).

To generate a calibration curve for eNOS quantification, rheNOS peak areas were subtracted by no-rheNOS background control. Then, the resulting values 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 (**FIGURE 2**, inset). Next, 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 3**).

TABLE 3. Recovery of rheNOS standards. (*n*=2)

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

FIGURE 2. Simple Western 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.

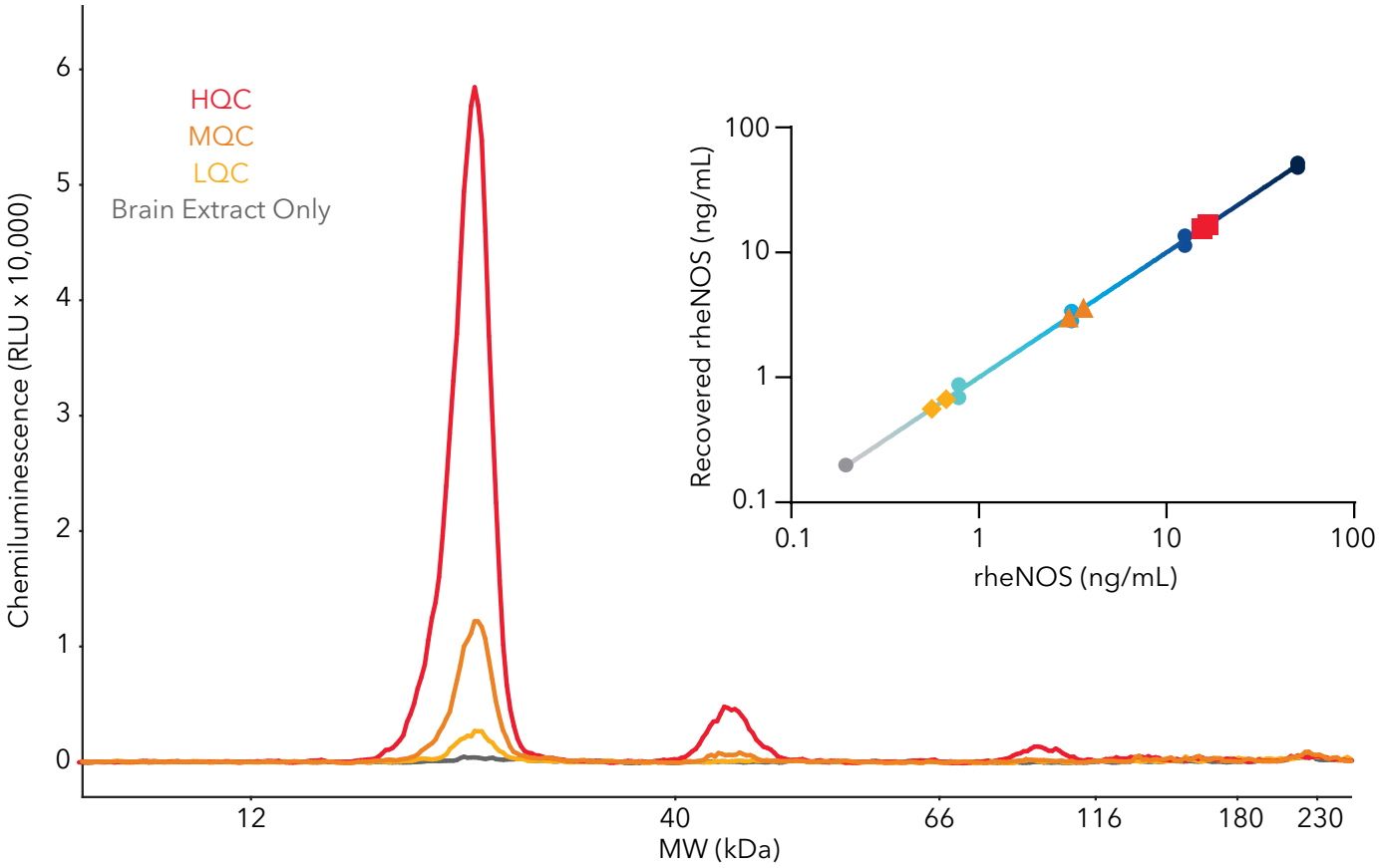
For quality control (QC) of the Simple Western assay, samples of rheNOS were prepared at low (LQC), medium (MQC), and high (HQC) final concentrations of 0.6, 3, and 15 ng/mL, respectively. Each QC sample was prepared in a background of brain extract as described in the Materials and Methods. When these samples were analyzed by Simple Western, a clear peak corresponding to rheNOS was observed (**FIGURE 3**) with a similar separation profile as before (**FIGURE 2**). The matrix-only controls lacking rheNOS showed little to no detectable non-specific binding (**FIGURE 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 (**FIGURE 3**, inset). The percent recovery of each QC sample fell within ±20% of expected values (**TABLE 4**).

Next, we sought to determine the linearity of HQC recovery. To do so, we diluted the HQC sample to 1:2, 1:4, and 1:8 from the original concentration, and each sample dilution was analyzed by Simple Western. Each HQC sample dilution was subtracted by the matrix-only control and then the percent recovery of each sample was calculated and plotted against expected rheNOS concentrations. Each sample dilution resulted in a percent recovery within ±20% of expected values (**TABLE 4**).

TABLE 4. Recovery of QC samples. (*n*=2)

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

FIGURE 3. Simple Western 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 of each sample was calculated and plotted against expected rheNOS concentrations.

Applying the Simple Western Assay to Human Brain Tissue Homogenates

With the Simple Western assay for eNOS quantification established, we sought to quantify endogenous expression levels of eNOS in human brain tissue homogenates. To do so, we acquired human brain tissue homogenates from two different commercial vendors that contain different homogenization and/or storage buffer conditions. Each tissue homogenate was titrated in a serial dilution series as described in the Materials and Methods and analyzed by Simple Western using the anti-eNOS antibody. The results from this analysis showed similar expression profiles in both homogenates, with a major eNOS peak and several minor peaks, indicating the presence of eNOS isoforms (**FIGURE 4**). It should be noted that these minor peaks would not be resolvable under these conditions by ELISA because ELISA lacks size separation.

The eNOS signal in both tissue samples decreased with decreasing sample concentration in a linear fashion and all sample dilutions recovered within ±20% (**TABLE 5**). Thus, Simple Western can detect and reproducibly quantify eNOS in human brain tissue lysates.

As expected, Simple Western detected eNOS expression in the endothelial whole-cell lysate positive control sample while no eNOS expression was observed in the cervical cancer whole-cell lysate negative control sample (**FIGURE 5**). Interestingly, eNOS isoforms of lower MW were more highly expressed in cervical cancer cells compared to brain tissue samples. This difference in eNOS tissue biodistribution could be due to differences in gene regulation, post-translational modification, and/or protease activity in these cell types.

FIGURE 4. Simple Western analysis of endogenous eNOS expression in human brain tissue homogenates.

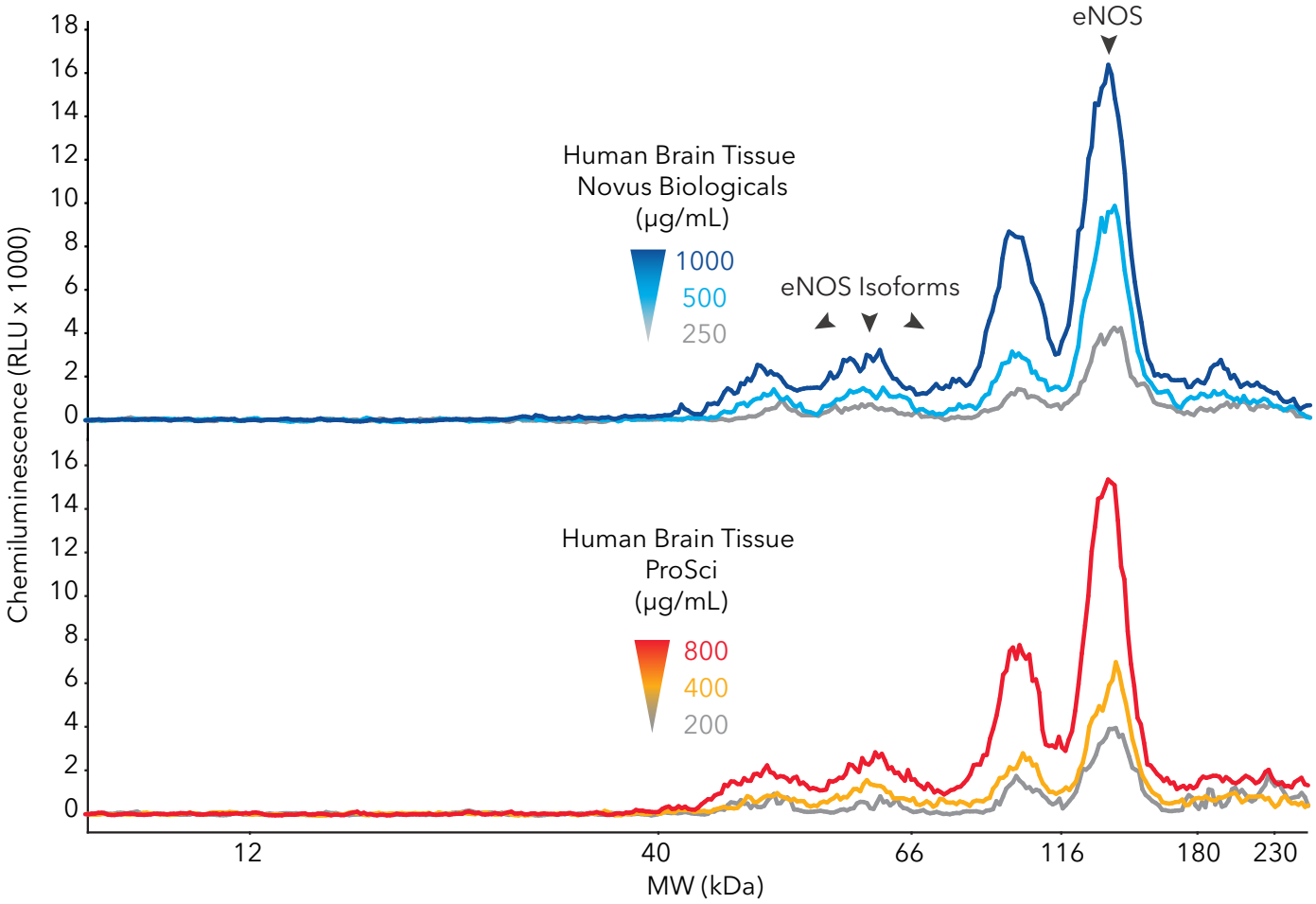
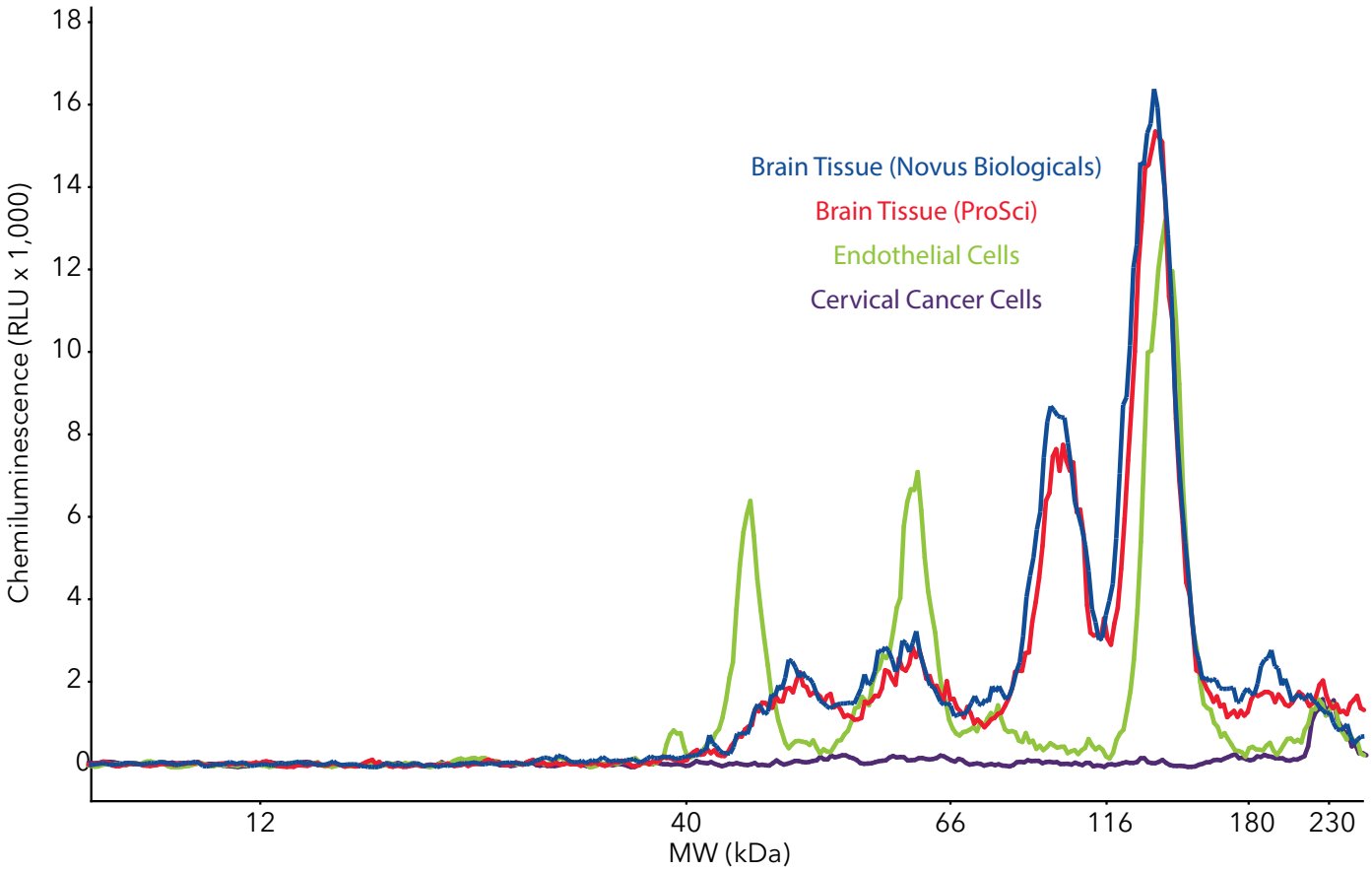


TABLE 5. Recovery of eNOS in dilutions of human brain tissue samples from the undiluted sample. (*n*=2, **n*=1)

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

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



Comparing the Simple Western Assay for eNOS Biomarker Testing to ELISA

We directly compared the Simple Western assay for eNOS quantification to a leading commercial ELISA Kit for eNOS biomarker testing.[7] In this comparison, we analyzed the endothelial and cervical cancer whole-cell lysates as positive and negative controls, respectively. As expected, ELISA did not detect eNOS in cervical cancer whole-cell lysate. In endothelial whole-cell lysate, ELISA quantified eNOS with similar results to Simple Western (3.13 ng/mL and 4.16 ng/mL, respectively). However, when we analyzed the same rheNOS standards and spiked samples used in the Simple Western assay, ELISA did not detect eNOS when the manufacturer's instructions were followed.[7]

When we analyzed the whole brain tissue samples from Novus Biologicals and ProSci, 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. Because these brain tissue homogenates are from different vendors and have different homogenization and/or storage buffer conditions, these results indicate that ELISA is limited to particular homogenization conditions and/or buffer compositions, while Simple Western was able to measure eNOS in both brain tissue samples. These results are consistent with the hypothesis that Simple Western is less susceptible to matrix effect in brain tissue samples than ELISA.

While ELISA detected eNOS in human brain tissue homogenate from Novus Biologicals, ELISA appeared to underestimate the concentration of eNOS and demonstrated a narrow assay range and poor sensitivity compared to Simple Western (**TABLE 6**). Again, these results suggest that Simple Western is less susceptible to matrix effect in brain tissue samples than ELISA.

TABLE 6. Comparison of ELISA and Simple Western assays for eNOS quantification in brain tissue.

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

A No-Brain Decision

Ditch ELISA for Automated Simple Western to Get the Clear Picture in Tissue Homogenates

Here, we developed a Simple Western Human eNOS Assay that reproducibly quantifies eNOS in human brain tissue homogenates and whole-cell lysates. While Simple Western 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. Therefore, Simple Western can handle variations in homogenization and/or storage buffer conditions compared to ELISA.

Simple Western outperformed ELISA in assay accuracy, range, and sensitivity for eNOS quantification in human brain whole tissue homogenate. Due to size-based separation profiles, Simple Western was able to distinguish between specific targets like other eNOS isoforms and nonspecific background cross-reactivity, which would go unnoticed by ELISA. While samples were prepared manually prior to Simple Western analysis, automated liquid handlers like the CyBio Felix from Analytik Jena may be implemented to automate sample preparation and plate loading to streamline workflows even further.[8]

Taken together, Simple Western is an automated high-performance protein characterization tool that provides biomarker protein testing and tissue biodistribution measurements in complex sample types like brain tissue homogenates and whole-cell lysates.

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