WHITEPAPER

Rapid Conversion of NSE ELISA to Diffraction Based Immunoassay

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Abstract

Neuron-Specific Enolase (NSE), a homodimer of the gamma form of enolase, is localized in the cytoplasm of neurons and cells of neuronal origin. Its presence in cerebrospinal fluid and blood is attributed to cell destruction. Serum NSE level is a marker for neuronal damage after traumatic brain injury, tumor, and neurodegenerative diseases.

Serum NSE levels can be measured with commercial ELISA kits. Though quite sensitive, ELISA has disadvantages in near patient testing. It is time consuming and inconvenient, not suitable for individually/ randomly available patient samples.

Here we describe the transfer of the NSE ELISA to the Axela dotLab® System, to demonstrate its use as a rapid alternative to ELISA analysis. The dotLab® System provides comprehensive information on the interaction between the ligand and the analyte of interest in complex sample matrix in a matter of minutes. Its inherent advantages include short assay time, the use of small sample volume, the direct use of complex biological samples with little or no sample preparation, and minimal non-specific binding. Capabilities including a wide choice of assay formats, direct detection, quantitative measurement, and extended dynamic range with real-time data makes the dotLab System ideal for routine immunoassays. The system can serve as a common platform for rapid proof-of-concept, reagent qualification, assay optimization, and routine quantitation. This example illustrates general principles in dotLab assay development, which can be directly adapted to other applications.

Background

Inflicted traumatic brain injury is a leading cause of death for infants in many countries.[1] Timely diagnosis of the injury is critical for the proper treatment of patients. Because the diagnosis of the condition is often impeded by subjective parameters, the design of a rapid and sensitive assay to detect the primary biomarkers of the condition is of paramount importance in the attempts to save lives.

Previously, a group of researchers from Children's Hospital of Pittsburgh of UPMC discovered that the presence of NSE in patient serum could be an indication of brain injury. This biomarker was used in the study because it showed potential as a screening test for the diagnosis of traumatic brain injury in infants.[1] Determination of a threshold level of serum NSE, suggesting brain trauma, was also established.

Existing methods to determine the quantity of NSE in patients are limited to standard ELISA techniques using a pair of monoclonal anti-NSE antibodies, which recognize non-overlapping epitopes on the antigen. One of the antibodies is labeled with biotin for immobilization onto avidin-coated ELISA plates and is thus designated as the capture antibody. NSE from samples binds to the capture antibody on the plate. The other antibody is tagged with HRP and designated as the detector antibody, which binds to NSE captured on the plate. The bindings are visualized by appropriate enzymatic reaction and detected by a spectrophotometer. This method requires a calibration curve for each plate and is time and labor consuming. It is best suited for a large number of samples to be analyzed simultaneously.

Optical biosensors with diffractive patterns were shown to be useful in the design of immunochemical assays to detect and quantify both antigens and antibodies in complex matrices.[2,3] Biosensors with a series of discrete diffraction spots using patterned avidin layers on the surface of the prism are used to generate a diffraction signal. The diffraction signal changes with each additional layer of reagents specifically bound to the pattern on the surface.

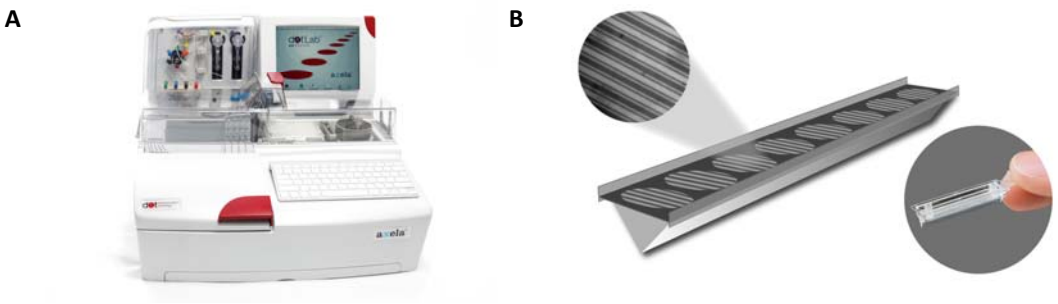
In the current study, we applied the principles of the ELISA method to the dotLab System using avidin sensors to simplify the assay and improve the throughput characteristics and robustness of the analysis.

The dotLab® mX System

The dotLab® mX System utilizes diffraction-based optical sensing for the real time, label-free measurement of molecular interactions. The system uses inexpensive, disposable biosensors with coupling reagents (eg: avidin, amine reactive substrates or unique oligonucleotide-based addressing reagents to allow multiplexing) pre-patterned on the surface of 10 µL flow channels forming a diffraction grating (Figure 1). The dotLab® mX instrument illuminates the grating with a laser generating a diffraction image which is monitored by a photodiode detector. Diffractional efficiency increases as molecules bind to the surface resulting in an increase in image intensity. Conversely, molecular dissociation from the surface results in a decrease in image intensity. Therefore, the real time monitoring of molecular interactions through changes in diffractional efficiency provides information on the quantity and rate of binding and dissociation events. The dotLab® mX System simplifies and automates this analysis using a fully integrated, easy to use, bench top instrument.

Figure 1

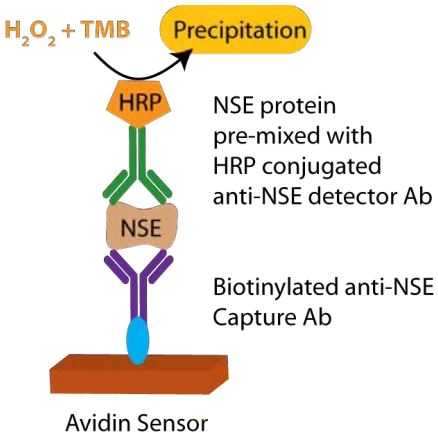
(A) The dotLab mX Instrument: a fully automated, bench-top instrument for real time molecular interaction analysis. (B) Schematic of a dotLab sensor with a contiguous array of capture surfaces (spots) with coupling reagent pre-patterned on the surface forming diffraction gratings.



The NSE quantitative assay using the dotLab® mX System takes only 20 minutes thus is particularly suited for use in a fast-paced setting of an emergency room. The ease and robustness of this method and instrumentation also allows for quick screening of additional biomarkers as well. Figure 2 shows NSE immunoassay developed for the dotLab® mX System. Signal of the antigen binding is amplified by

Figure 2

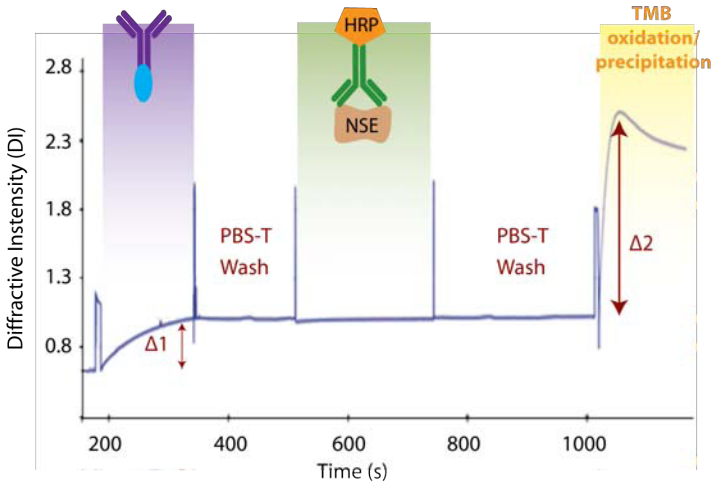
Biotinylated monoclonal anti-NSE capture antibody (CAb) binds strongly to the avidin surface. An HRP-conjugated detector monoclonal anti-NSE antibody (DAb) is pre-incubated with NSE to form a detection complex. The resulting complex binds to the capture antibody on the surface of the sensor through the antigen as a link, as the DAb and the CAb recognize non overlapping epitopes of the antigen.



the HRP/TMB reaction on the sensor surface. Interfering factors from crude samples such as serum are washed away before the TMB amplification, resulting in significantly reduced background level and avoiding any matrix effects. Figure 3 shows the profile of a typical assay.

Figure 3

Real time monitoring of each reagent loading step. Length of the assay time can be adjusted as needed by users. Spikes represent air gaps used to separate reagents.



Materials & Methods

dotLab Sensors: Polystyrene sensors with avidin molecules immobilized in a diffraction pattern were used for all the experiments (BM-000002).

Assay Platform: All experiments were performed on the Axela dotLab System.

Antibodies: Monoclonal anti-Human neuron specific enolase antibody, (United Biotech Inc. cat. #CM-901 A) as the capture antibody. Monoclonal anti-Human neuron specific enolase antibody, (United Biotech Inc. cat.# CM-901 B) as the detector antibody.

Labeling Kits: SureLink HRP conjugation kit 6 x 1 mg rxn (KPL, cat # 84-00-02). FluoReporter Mini-Biotin-XX Protein Labeling kit (Molecular Probes, cat # F-6347)

Antigen: NSE protein from CanAg NSE EIA kit (FUJIREBIO Diagnostics, Inc, cat # 420-85) was used for calibration curves.

Human Serum: Normal human serum (Jackson ImmunoResearch, cat # 009-000-121).

Buffer System: PBS-Tween 20 (0.1% (v/v) Tween 20).

Blocking Agents: Bovine serum albumin (BSA) 5mg/mL in PBST.

TMB Membrane Peroxidase Substrate: KPL, Product Code 50-77-18.

Protease Inhibitors: Protease inhibitor cocktail (SIGMA, cat # P8340). Complete, Mini Protease Inhibitor Cocktail Tablets (Roche, cat # 11 836 153 001).

Procedures

Antibody Labeling: Labeling of antibodies was performed according to manufacturer's instructions, except in the case of the detector antibody, excess HRP was used to increase the overall amplified signal compared to ELISA antibodies (CanAg NSE EIA kit, data not shown).

Sample Preparation: NSE standards were prepared by serial dilution in either PBST-BSA or in normal human serum. Biotin-CAb antibody - diluted to 10 µg/mL in PBST-BSA. HRP-DAb - diluted to 1 µg/mL in PBST-BSA.

Binding: 80 µL HRP-DAb was mixed with 20 µL of NSE standard solution and incubated at room temperature for 30 min.

Assay Run:

Step 1 - Avidin sensor block with 5 mg/ml BSA in PBST buffer, 1 min in mixing mode
Step 2 - Binding of biotin-anti NSE capture antibody, 2 min in mixing mode
Step 3 - Wash with PBST in mixing mode
Step 4 - Binding of Dab-NSE complex, 3 min in mixing mode
Step 5 - Wash with PBST in mixing mode
Step 6 - TMB substrate load in static mode

Results

Optimization of Conditions

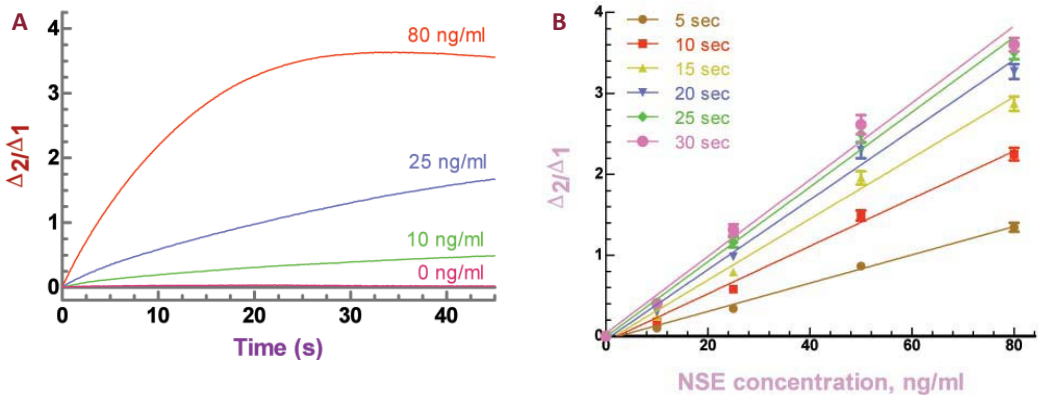
The concept of the ELISA assay was directly transferred onto the dotLab® System with antibodies and reagents from the kit (data not shown). However, custom modification of the antibodies conjugated to biotin or HRP provided increased signal and dynamic range of the assay, thus improving the linearity and sensitivity. Once optimized, the total assay time was reduced to 20 minutes. This short runtime was possible because of the dotLab® System's liquid handling and mixing modes abilities.

Calibration Curve

The normalized real time trace of the HRP-conjugated antibody reaction with TMB membrane substrate exhibited linear dependence vs. the actual concentration of NSE (Figures 4a and 4b). The R2 for linear regression at time points 5 – 25 seconds was 0.98. Standard deviations for each point were calculated from at least 3 different assays and represented by error bars (Figure 4b).

Figure 4

(A) Overlaid Signal for NSE Levels used on Generation of Calibration Curve. **(B)** NSE concentration vs. D2/D1 ratio for the signal amplification of the binding of NSE complex with HRP conjugated DAb to CAb, immobilized on avidin sensors. Assays performed in PBST buffer with BSA 5 mg/ml added for blocking.



Statistical Analysis and LOQ

Limit of Detection (LOD) was calculated with statistical analysis of zero control assays at 1.9 ng/mL NSE, N=8 (Δ2/Δ1 for zero control + 3 σ = 0.081 at 25 seconds). The average CV for all points was 9.3% performed on different days.

Serum Matrix Effect

Matrix effect on the results of the assay was tested by diluting the NSE standards in normal human serum. Longer incubation times for high concentrations of NSE resulted in uncharacteristically low signal. The hypothesis tested was that serum NSE is subjected to protease degradation in a concentration-dependent manner.

Sequence analysis of NSE demonstrated multiple protease cleavage sites (data not shown). Addition of the protease inhibitor significantly improved the signal at higher concentrations of NSE (Figure 5). We demonstrated that the presence of the inhibitor does not influence the signal of the assay and protects NSE from significant degradation.

Calculations and Statistical Analysis

For the calculation of NSE concentration:

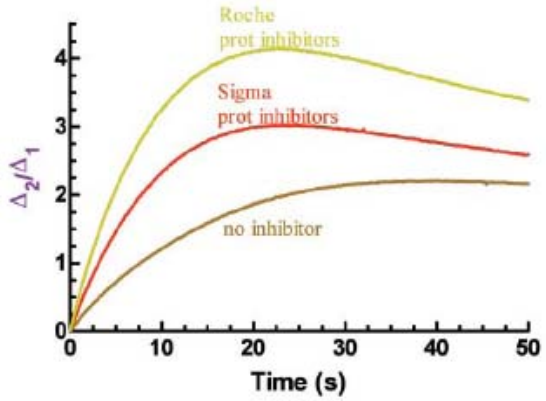
1. Δ1 was determined as the difference between the highest and the lowest points of CAb binding (Figure 3).

2. Δ2 of TMB amplification at different time points were determined as the difference between the TMB signal at a particular time point and the starting level of TMB signal.

3. Δ1 was normalized to Δ2 by the ratio of Δ2/Δ1.

Figure 5

Addition of protease inhibitors on the diffraction signal.



4. The Δ2/Δ1 ratios are calibrated against that of the blank control (Δ2/Δ1 of the blank control is subtracted from all data sets).

5. Normalized and calibrated Δ2/Δ1 ratios were plotted as a function of the NSE concentration. Results from at least three assays performed on different days were used to analyze the intermediate reproducibility.

6. Statistical analysis was performed to compare normalized ratio Δ2/Δ1 without the zero control baseline subtracted and NSE at 10 ng/mL at N=8, using dotLab Data manager and Prizm GraphPad package. LOD was calculated as the mean of Δ2/Δ1 for zero control + 3 σ for time points from 5 to 25 seconds.

Conclusion

- The NSE detection quantitative assay was successfully transferred onto the dotLab System. Assay time was reduced to 20 minutes. Dynamic range was optimized to suit its application to the detection of NSE as a potential marker of traumatic brain injury in a research trial.
- Optimized assay was linear in the range of interest (10 – 80 ng/mL NSE) with R2 of 0.98 up to 25 seconds of TMB amplification (up to 250 data points at 10 Hz data acquisition).
- The LOD was calculated to be 1.9 ng/mL NSE. Statistical analysis showed that difference between the normalized signal for 10 ng/mL and 0 ng/mL NSE was significant with $p < 0.05$ using two-tailed paired t-test for the same time points.
- Assay was demonstrated to be reproducible within the same system (Figure 5) and robust for transfer to another operator (data not shown).
- The assay was demonstrated to be matrix independent (when PBST buffer was substituted by normal human serum) in the presence of protease inhibitors.
- The NSE serum assay described here has been shown to be sensitive, linear within the designed working range of concentrations, robust and rapid. This platform transfer can be generally applied to the development of novel biomarker assays using complex biological samples.

References:

1. R.P. Berger et al, Pediatrics 2006; 117; 325-332.
2. J.B. Goh et al, Analytical Biochemistry 2003; 313, 262-266.
3. V. Borisenko et al, Clinical Chemistry 2006; 52, 2168-2172.

About Axela, Inc.

Axela's platforms provide powerful new approaches to multiplexed protein and nucleic acid analysis designed to greatly simplify biomarker testing in clinical research and diagnostics. Axela's commercial research products significantly improve the amount and quality of information derived from traditional assays. This approach shortens time to result and provides access to unique categories of markers that form a pipeline of future diagnostic offerings.

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