A Highly Sensitive Point of Care Test for GFAP, A Brain Biomarker in Serum

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Abstract

Glial fibrillary acidic protein (GFAP) in serum has been proposed as a biomarker for traumatic brain injury (TBI). Many immunochemical methods have been developed for quantitating human GFAP in serum, including ELISA. However, a sensitive and quantitative assay which could be used as a point-of-care diagnostic test is not commercially available. Here we report the development of a sensitive, quantitative, and highly specific lateral flow assay for measuring GFAP in human serum. The assay utilizes a high affinity monoclonal antibody against human GFAP for capture and an europium (Eu, III) doped polystyrene nanoparticles conjugated to the $F(ab')_2$ fragment of a high affinity second monoclonal antibody against human GFAP for detection. The fluorescence of Eu signal on the lateral flow membrane was measured using a custom made portable lateral flow cassette scanner capable of time-resolved fluorescence measurement. The background fluorescence from the matrix and associated material (membrane and plastic) is greatly diminished in the time-resolved mode which significantly increased the signal window as well as detection limit to below pg/ml concentration of GFAP. The assay generates a linear calibration curve from 0 pg/ml to 250 pg/ml using human serum spiked with recombinant human GFAP. The assay performs equally well with the break-down product of GFAP (GFAP-BDP). The sensitivity of the assay is about 15 fold higher in the Time Resolved Fluorescence (TRF) mode when compared to the prompt fluorescence. The assay is highly reproducible, sensitive and fast, with an LOQ of 0.125 pg/ml.

Introduction

Traumatic brain injury (TBI) constitutes a major health and socioeconomic problem throughout the world. TBI is a leading cause of death and disability in children and young adults. It is currently diagnosed by neurological examination and radiographic imaging (CT, MRI, etc.,). As a result, proper and timely diagnosis of the TBI patient is limited. Basic science research has greatly advanced the knowledge and the mechanisms involved in the damage, and have led to the discovery of new biomarkers. Glial Fibrillary Acidic Protein (GFAP), which is a filament protein found in the cytoskeleton of astro-glial cells, (or its breakdown product GFAP-BDP), is one of the biomarkers released into the blood after a traumatic brain injury. Several studies using clinical blood and CSF samples have independently confirmed GFAP and GFAP-BDP as potential biomarkers for TBI. Currently there is no sensitive point of care test for quantitating the levels of GFAP. Here we report a lateral flow assay (LFA) format for measuring GFAP at sub-picogram levels in human serum and CSF samples.

Materials and Methods

Mouse monoclonal antibodies: mAb-1 and mAb-2 were developed in-house using full length human GFAP as antigen. Biotinylated F(ab)'2 fragment of mAb-2 was prepared and conjugated to the Streptavidin-Europium labeled latex nanoparticles. Recombinant human GFAP or GFAP-BDP were used as calibrators.

Preparation of cassettes: Test line (mAb-1) and control line (Ag) were stripped on the nitrocellulose membrane (1µl/cm) using Bio-dot dispenser. A 8mm wide glass fiber and a 19mm wide cellulose were used as sample pad and absorption pads, respectively. The nitrocellulose membrane was cut into 3.7mm wide bands and assembled along with sample, conjugate and absorbent pads into lateral flow cassettes. The membrane along with the cassette was dried at 37ºC and sealed individually in a desiccant containing pouch and stored at room temperature.

Preparation of calibrator solutions: Purified recombinant human GFAP-BDP was diluted in human serum to a final concentration of 100 ng/ml stock solution. The stock solution was serially diluted from 250 pg/ml up to 0.5 pg/ml. Neat serum was used as background (0 pg/ml) control.

Performing the assay: A 10 µl of 10% Triton X-100 was added to the sample port, followed by 100 µl of the samples containing indicated concentrations of GFAP-BDP. After 5 minutes, 50 µl of the 1X PBS containing 0.1% Tween 20 was added and the cassettes were incubated for 30-60 minutes at room temperature.

Signal detection: The amount of fluorescence signal on the test and the control lines were measured using a lateral flow time resolved fluorescence reader. For a comparative study, the cassettes were read in a prompt lateral flow fluorescence reader.

Results

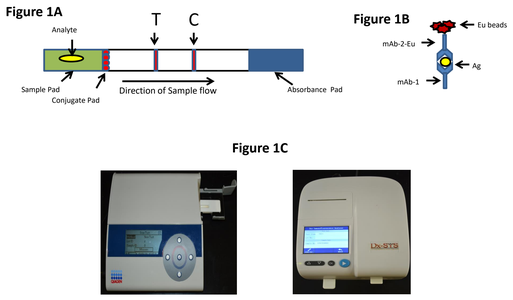

Figure 1A: Schematic illustration of lateral flow assay (LFA). **1B:** Principle of the Sandwich LFA. **1C:** Prompt fluorescence reader (Qiagen) and Time resolved fluorescence reader (Dx-Sys).

1. Assay sensitivity: The sensitivity of the assay was evaluated by generating dose response curves (DRC) using GFAP-BDP. The results are shown in Table 1 and Figure 2 below.

Concentration (pg/ml)	RFU @30 minutes
0	33
0.5	45
1	78
2	134
4	270
16	777
64	2978
256	10580

Table 1. RFU values for different concentrations of GFAP-BDP after 30 minutes incubation.

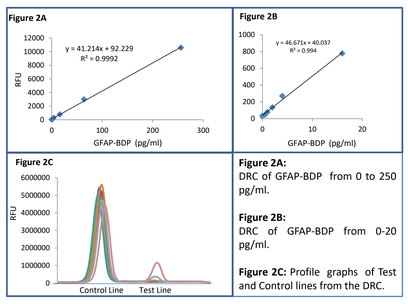

Figure 2A: DRC of GFAP-BDP from 0 to 250 pg/ml.

Figure 2B: DRC of GFAP-BDP from 0-20 pg/ml.

Figure 2C: Profile graphs of Test and Control lines from the DRC.

2. Time for maximum signal development: Time required for maximum signal development was evaluated by reading lateral flow cassette at 30, 60 ad 90 minutes after sample application. The results are shown below.

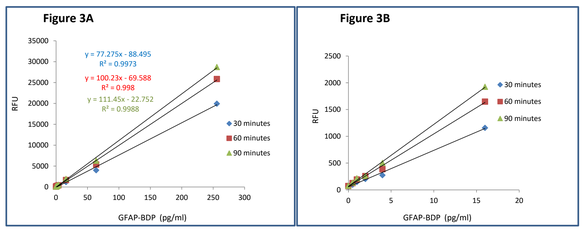

Figure 3: Dose response curves for GFAP-BDP using Time resolved fluorescence. **A:** RFU values for different concentrations of GFAP-BDP after 30,60 and 90 minutes incubation. **B:** DRC of GFAP-BDP from 0-20 pg/ml after 30, 60 and 90 minutes incubation. The RFU values for all the concentrations saturates by 60 minutes incubation.

3. Sensitivity of the assay using prompt fluorescence: To compare the sensitivity of the assay from the time resolved fluorescence with the prompt fluorescence, the same cassettes used for the time resolved fluorescent measurement were read using a prompt fluorescence reader. The results are shown below.

Table 2

RFU Values		
Concentration (pg/ml)	Peak Height	Peak Area
0	42.37	33.31
15.63	106.04	89.93
31.25	121.05	98.34
125	354.43	289.74
250	535.74	440.3
500	694.69	563.42
1000	910.38	727.88
2000	1576.69	1228.85

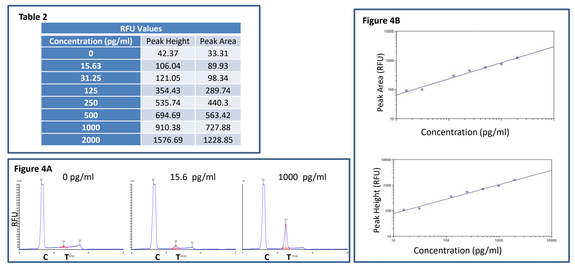

Table 2. Peak area and Peak height values calculated at 30 minutes incubation.
Figure 4: Dose response curve for GFAP-BDP using Prompt fluorescence reader. **A**: Control and test line profile for 0, 15.6 and 1000 pg/ml at 30 min. **B**: Standard curve with Peak area and Peak height RFU values at 30 min for varying concentration of GFAP-BDP (pg/ml).

4. Concentrations of GFAP/GFAP-BDP in clinical samples: Sera from a limited number of normal and TBI patients were analyzed using prompt florescence. While all the sera from normal subjects showed no detectable GFAP, the sera from the TBI patients showed high levels of GFAP including the one CSF sample from a TBI patient, suggesting that the current lateral flow format has no measurable matrix effect. Few of the TBI samples showed no detectable GFAP when analyzed using the prompt fluorescence, probably because of GFAP concentration below the detection limit (about 15 pg/ml) of the prompt fluorescence reader. These samples are currently being tested using the time resolved fluorescence.

Table 3

	Normal subjects		TBI patients	
	Sample ID	GFAP (pg/ml)	Sample ID	GFAP (ng/ml)**
1	Normal	Not detectable (ND)*	12163	8
2	Normal	ND	12250	3
3	Normal	ND	12147	8
4	Normal	ND	8694	4.8
5	Normal	ND	8532	1.2
6	Normal	ND	12122	ND
7	Normal	ND	12228	ND
*ND = Not detectable (<0.1 pg/ml)			8322	1.6
** = Concentration extrapolated from standard curve			3939 (CSF)	2500

Table 3. Concentration of GFAP in serum/CSF of normal and suspected TBI patients.

Summary

- During the assay development several parameters such as membrane porosity, capture and detection antibody concentrations, Eu-nanoparticles to antibody ratio, buffers, detergents, etc., were optimized to get low background, high signal, high dynamic range and shortest run time (data not shown).
- Using the optimized conditions, serum samples spiked with GFAP were tested. With increasing GFAP concentrations, there was a corresponding increase in the florescence intensity of the test line. In the absence of any GFAP in the sample, the fluorescence of the test line was <100 RFU. The LOQ obtained from several experiments was 0.125 pg/ml.
- When the same cassettes were read in the prompt fluorescence reader, the LOQ was 15 fold lower as compared to that obtained with the time resolved fluorescence reader.

Conclusions

- We have developed an ultra sensitive lanthanide based point of care lateral flow immunodiagnostic test for detecting sub-picogram per ml of GFAP, a potential biomarker for TBI.
- The same technology can be used for detecting other potential brain biomarkers such as UCH-L1, S100β, Myelin basic protein , SBDP, Tau protein, etc.
- Because of its high sensitivity, this POC technology will be very valuable for measuring any biomarker where high sensitivity is required and /or sample volume is limited.

References

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