Multiplexed Immunoassays for Brain Injury Markers

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1 Abstract

The development of effective therapies for traumatic brain injury (TBI) has been hindered by the lack of biomarkers to measure injury severity, identify subtypes of pathology, and provide evidence of therapeutic effect. Our goal was to develop a panel of TBI-related biomarker assays to help support research in this field. Multiplexed immunoassay panels were developed to measure plasma levels of 12 TBI-related biomarkers representing neuronal injury, astrocyte activation, vascular injury, and inflammation. The panels included Tau, GFAP, S100β, UCH-L1, BDNF, NSE, MMP-9, CKBB, NRGN, WVF, MCP-1 and VILIP-1. The assays were performed in 96-well plates using MSD’s MULTI-ARRAY® technology with sensitive electrochemiluminescence detection.

The assays were qualified through analytical testing and by measuring biomarker levels in normal plasma and archived TBI patient sets (mild, moderate and severe). Some biomarkers were detectable in normal plasma while others were detectable only after TBI. Proper sample processing was important for accurate measurements, as some of the markers were also present in blood cells.

In summary, a set of sensitive, multiplexed immunoassays for TBI-related biomarkers has been developed that should serve as effective tools for investigating TBI.

2 Methods

MSD’s electrochemiluminescence detection technology uses SULFO-TAG™ labels that emit light upon electrochemical stimulation initiated at the electrode surfaces of MULTI-ARRAY and MULTI-SPOT® microplates.

Electrochemiluminescence Technology

- Minimal non-specific background and strong responses to analyte yield high signal-to-background ratios.
- The stimulation mechanism (electrochemistry) is decoupled from the response (light signal), minimizing matrix interference.
- Only labels bound near the electrode surface are excited, enabling non-washed assays.
- Labels are stable, non-radioactive, and directly conjugated to biological molecules.
- Emission at ~420 nm eliminates problems with color quenching.
- Multiple rounds of label excitation and emission enhance light levels and improve sensitivity.
- Carbon electrode surface has 10X greater binding capacity than polystyrene wells.
- Surface coatings can be customized.

General Assay Protocols

One Incubation Step:
1. Wash plate with PBS-T.
2. Add 25 µL of assay diluent containing detection reagents and 25 µL of neat plasma, diluted plasma, or Calibrator.
3. Incubate for 1 hour at room temperature (RT) with shaking.
4. Wash with PBS-T: Add 150 µL of Read Buffer T. Read on an MSD® imager.

Two Incubation Steps:
1. Wash plate with PBS-T.
2. Add 25 µL of assay diluent and 25 µL of neat plasma, diluted plasma, or Calibrator.
3. Incubate for 1 hour at RT with shaking.
5. Incubate for 1 hour at RT with shaking.
6. Wash with PBS-T: Add 150 µL of Read Buffer T. Read on an MSD imager.

Clinical Samples

1. Healthy & Pre-Analytical. Healthy controls were obtained from a commercial vendor as K2EDTA plasma prepared using standard centrifuge conditions. Four samples from healthy/from healthy donors were spiked with three levels of Calibrator. Average recovery for the three spikes is shown for each sample along with an overall average across all samples.

2. Mild TBI. Mild TBI plasma samples from healthy donors were diluted 2X, 4X, and 8X with Calibrator diluent. Average recovery over the three dilutions is shown for each sample along with an overall average across all samples.

3. Moderate and Severe TBI. Blood samples were obtained upon admission (within an average of 2 hours post injury) from TBI patients at St. Michael’s Hospital, Toronto ON. TBI was classified using a combination of neuro-psychological testing and imaging. Plasma was prepared using standard procedures (1,600 g for 15 minutes) and therefore samples likely contained blood cells. All samples were frozen.

4. TBI Plasma Testing

Representative data from TBI plasma samples are shown below. Samples with undetectable biomarker levels are plotted at the assay LOD. Moderate and Severe TBI Data: Data for moderate and severe TBI samples are shown along with healthy control samples (tan circles) collected under the same protocol.

5. Pre-Analytical Factors

Pre-analytical effects were demonstrated by testing plasma prepared using standard centrifuge conditions (≥ 2,000 g for ≥ 20 minutes), plasma prepared using a low centrifuge speed (200 g for 15 minutes). Plasma samples lysed via a freeze-thaw cycle are shown. All samples were from healthy donors. Pre-analytical factors affect all biomarkers to varying degrees.

6. Conclusion

- Prototype assay panels for 12 TBI-related biomarkers were developed and characterized on MSD’s MULTI-ARRAY® platform.
- The high sensitivity, dynamic range, and multiplexing of MSD assays allowed all 12 biomarkers to be measured (in duplicate) in 200 µL of plasma. These assays will be used for screening large TBI sample sets under an ongoing DoD funded study.
- Artificially elevated levels of BDNF, NRGN, NSE, and MCP-1 were observed in plasma due to the presence of residual blood cells that contained substantial amounts of these markers. Careful control over sample collection and processing can minimize these effects. When collecting TBI samples, inclusion of identically-processed healthy controls is recommended to help control for any pre-analytical effects.

Assay Characterization

Dilution Linearity. Four individual plasma samples from healthy donors were diluted 2X, 4X, and 8X with Calibrator diluent. Average recovery over the three dilutions is shown for each sample along with an overall average across all samples. Samples used for GFAP, CKBB, VILIP-1, NRGN, and Tau dilution linearity were first spiked with Calibrator to provide measurable levels.

Spike Recovery. Four individual samples from healthy donors were spiked with three levels of Calibrator. Average recovery for the three spikes is shown for each sample along with an overall average across all samples.

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Pre-analytical factors were demonstrated by testing plasma prepared using standard centrifuge conditions (≥ 2,000 g for ≥ 20 minutes); Standard plasma), plasma prepared using a low centrifuge speed (200 g for 15 minutes; Platelet-rich plasma), and whole blood plasma lysed via a freeze-thaw cycle. All samples were from healthy donors. Pre-analytical factors affect all biomarkers to varying degrees.

Assay Characterization

Dilution Linearity. Four individual plasma samples from healthy donors were diluted 2X, 4X, and 8X with Calibrator diluent. Average recovery over the three dilutions is shown for each sample along with an overall average across all samples. Samples used for GFAP, CKBB, VILIP-1, NRGN, and Tau dilution linearity were first spiked with Calibrator to provide measurable levels.

Spike Recovery. Four individual samples from healthy donors were spiked with three levels of Calibrator. Average recovery for the three spikes is shown for each sample along with an overall average across all samples.