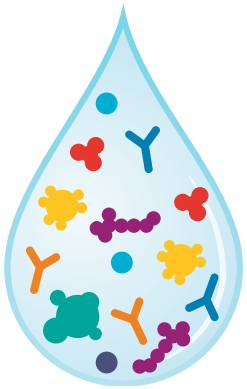
Accelerated Serum Biomarker Verification and Validation with Wes and Ella

Introduction



Serum biomarkers are measurable indicators that are used for the diagnosis and treatment of diseases like cancer, cardiac disease, autoimmune diseases, and neurodegenerative disorders. Using genomic, proteomic, and metabolomics techniques to identify molecules that play a critical role in early detection of disease helps researchers improve survival rates by 5-10X[1] and determine the best therapeutic course for a patient.

The proteomic biomarker development workflow starts with biomarker discovery which involves screening samples for the presence of many different proteins in order to identify candidate molecules. Next, the identity of potential protein biomarkers is traditionally verified using an immunoblot and then validated with an ELISA-based technique.[2]

We have faster, simpler solutions for you at every step of the process — from biomarker discovery to verification and validation. Luminex® systems are powerful discovery tools that let you simultaneously quantitate up to 50 proteins from a single sample. The great part is you can get Luminex instruments from Bio-Techne along with 600-plus assays to go with them. ProteinSimple, a Bio-Techne brand, then helps you verify promising targets with our Simple Western® assays using a detection kit optimized specifically for serum samples and then move on to quick validation of targets in lots of samples with our Simple Plex™ assays. Both techniques give you fast, reproducible results with just a fraction of the sample you'd use with traditional methods. Plus, both Luminex and Simple Plex assays are powered by R&D Systems® antibodies and validated across each platform, so your discovery data will correlate with your validation data.

In this application note, we're honing in on the biomarker verification and validation steps. Proof-of-concept data was generated to demonstrate how Simple Western and Simple Plex assays data give similar trends and work together to give you fast, sensitive, and precise information about your biomarkers of interest.

Quick biomarker verification with Simple Western Assays

Traditional Western blots are often used to verify candidate biomarkers. The antibody specificity confirms protein identity and the molecular weight provides information about protein processing events and confirms the protein integrity. But as we all know, traditional Westerns need a lot of hands on and take about two days to complete. They also lack reproducibility due to the many manual steps in the process.

Simple Western assays are automated, capillary-based immunoassays that solve many of the challenges that come with traditional Westerns. There are three Simple Western systems to choose from, all very reproducible with CVs <15%. All are open platforms, so you can screen for any biomarker as long as you have a primary antibody. Wes® gives you absolute or relative quantitation on 25 data points in just three hours with only 30 minutes of hands-on time for setup. You'll just need to dedicate capillaries for a standard curve if absolute quantitation is desired. If you need higher throughput, Sally Sue® and Peggy Sue® give you 96 data points overnight and only need about an hour of hands-on time. You'll also only use 3 or 5 µL of sample diluted in our master mix on Wes or Sally Sue/Peggy Sue respectively to get pg/mL level sensitivity data, so won't have to use as much of your precious serum samples that aren't always easy to get.

If you want to avoid secondary antibody cross-reaction with highly-abundant serum proteins like immunoglobulins or albumin, you can probe with a biotinylated primary antibody instead and detect with our Secondary Streptavidin-HRP in the Biotin Detection Module (DM-004) developed just for this purpose.[3] R&D Systems alone has over 6000 biotinylated primary antibodies to date that can really help you verify and narrow down the targets you want to take to validation.

Reproducible, high-throughput biomarker validation with Simple Plex Assays

Traditional ELISAs are often used to validate serum biomarkers because they provide absolute quantitation, offer high throughput, and are sensitive. Simple Plex assays and the Ella™ system let you do everything faster, with inter-assay reproducibility in the single-digit CVs and low pg/mL sensitivity.

Simple Plex assays are run on Ella, an automated immunoassay system, using either a 72 x 1 cartridge that generates triplicate data for one analyte in 72 samples, a 16 x 4 cartridge that generates triplicate data for four analytes in 16 samples, or a 32 x 4 cartridge that generates triplicate data for four analytes in 32 samples. Samples in the 16 x 4 and 32 x 4 cartridges are split into four different channels that each detect a distinct analyte, so you don't have to worry about the cross-reactivity you'd normally see when multiplexing. It only takes 10-15 minutes to set up a run and results are ready in just one hour — that's 3-6X faster than a traditional ELISA. That means you can screen a ton of serum samples in no time.

Cartridges come ready to go with the capture antibody already conjugated to glass nano reactors in the microfluidic channel and lyophilized detection antibody already in the circuit. All you have to do is add your sample and Wash Buffer. Plus, you only need 50 µL of sample diluted in sample buffer to generate triplicate data. That's 6X the sample savings compared to traditional ELISA which needs up to 300 µL of sample to generate triplicate data. Each cartridge comes with a factory-determined standard curve, which increases your throughput even further and reduces your hands-on time since you don't need to run your own standard curve to get absolute quantitation from the relative fluorescence units detected.

As of today, there are 156 fully validated Simple Plex assays for you to choose from and the list grows all the time. If we don't already have an assay for your biomarker of interest we'll work with you to build one using either your antibody or one of the 636 Quantikine® ELISA kits and 1000 DuoSet® ELISA Development Systems from R&D Systems.

Wes and Ella sample preparation

We ran the same samples to compare the average signal area and measured concentration on Wes and Ella respectively to see how well the data correlated. Six separate aliquots of normal human serum samples (Sigma, H3667) were each spiked with different concentrations of IL-6 (R&D Systems, 206-IL-10/CF), BDNF (R&D Systems, 248-BD-025/CF), and IL-1α (R&D Systems, 200-LA-010/CF). Samples were aliquoted at 5 µL volumes and stored at -20 °C before they were analyzed for the IL-6, BDNF, IL-1α, and endogenous PCSK9.

Samples for analysis with Simple Western assays on Wes were prepared by diluting the spiked serum into 5X Fluorescent Standard and DTT for a final serum concentration of 1.2 mg/mL. Samples were denatured for 5 minutes at 95 °C and then run on a 2-40 kDa Wes plate using default assay conditions. Triplicate samples were immunoprobed with 10 µL per capillary of 4 µg/mL biotinylated anti-IL-6 (R&D Systems, BAF206), 10 µg/mL biotinylated anti-BDNF (R&D Systems, BAF248), 10 µg/mL anti-IL-1α (R&D Systems, BAF200) or 2 µg/mL biotinylated anti-PCSK9 (R&D Systems, BAF3888) diluted in ProteinSimple Antibody Diluent 2 and then detected using the Biotin Detection Module (ProteinSimple, DM-004) to avoid secondary antibody cross-reaction.[3]

Simple Plex samples for analysis on Ella were prepared by serially diluting the spiked serum 1:10, 1:1000, 1:10000, and 1:100000 in Sample Buffer SD13. A 1:10 dilution is the minimum required to detect endogenous PCSK9 in normal serum. 50 µL of diluted sample was applied to the IL-6, BDNF, IL-1α, and PCSK9 16x4 cartridge and run right away on Ella.

Biomarker verification and validation

We first analyzed the six spiked serum samples using the Simple Western assay on Wes to generate triplicate data for our four targets of interest. Data was collected from four Wes runs which translates to a total of 12 hours to collect

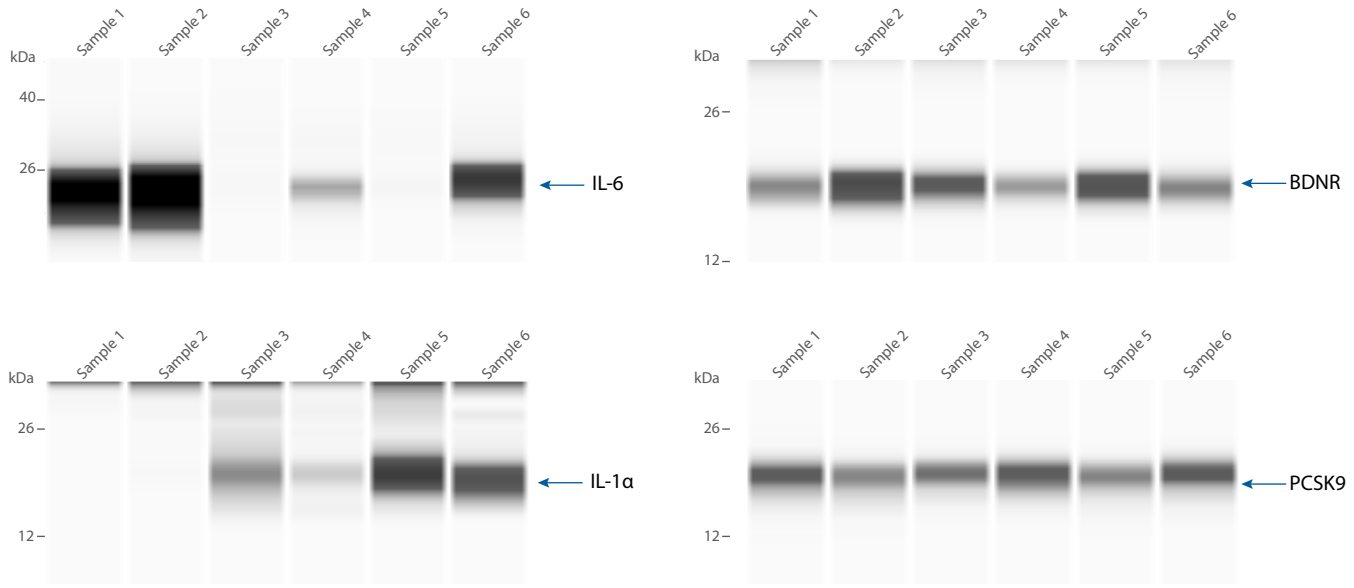


FIGURE 1. Wes detected single bands for IL-6, BDNR, IL-1α, and PCSK9, confirming the protein's identity and integrity. Non-specific bands around 33 kDa were detected with IL-1α and BDNR and were most likely due to cross-reaction between the biotinylated primary antibody and highly abundant IgG light chain.

72 data points using only 12 µL of serum per sample (3 µL per run x 4 runs). Data was visualized using Compass for Simple Western software and a single band that corresponded to each protein of interest was detected, confirming they weren't degrading, which would affect protein function (**Figure 1**). There were some non-specific bands detected that most likely corresponded to non-specific interactions between the biotinylated primary antibody and the highly-abundant IgG in the serum, based on the molecular weight of the peaks. The level observed was dependent on the abundance of the target protein as well as the affinity of the biotinylated antibody.

Further analysis with Compass for Simple Western software determined the average area and % CV from the relative chemiluminescent signal generated (**Table 1**). There was one sample with levels of IL-6 below the limit of quantitation (LOQ) and two samples with levels of IL-1a below the LOQ. For the remaining samples, reproducibility between technical replicates was very good with all CVs ≤ 13.4%.

	IL-6 (1:50)		BDNF (1:50)		IL-1A (1:50)		PCSK9* (1:50)	
Sample	Average Signal	% CV	Average Signal	% CV	Average Signal	% CV	Average Signal	% CV
1	145810	11.3	97837	13.4	ND	---	19170	12.9
2	191301	12.9	133003	9.6	ND	---	17340	9.1
3	ND	---	107789	1.3	8574	10.9	17334	10.2
4	35777	13.3	71049	12.8	4770	9.2	18513	4.6
5	8305	10.2	118950	10.8	14278	2.0	16780	12.1
6	77614	3.2	88094	5.4	12052	7.5	18692	8.6

TABLE 1. Relative quantitation of spiked IL-6, BDNF, IL-1α, and endogenous PCSK9 in six normal human serum samples using Wes. Samples were all diluted 1:50 in 0.1X Sample Buffer and 5X Fluorescent Standard and heat denatured at 95 °C for 5 minutes. Triplicate data was generated in one day and was extremely reproducible with CVs all under 13.4%. * Endogenous protein detected. ND = not detected.

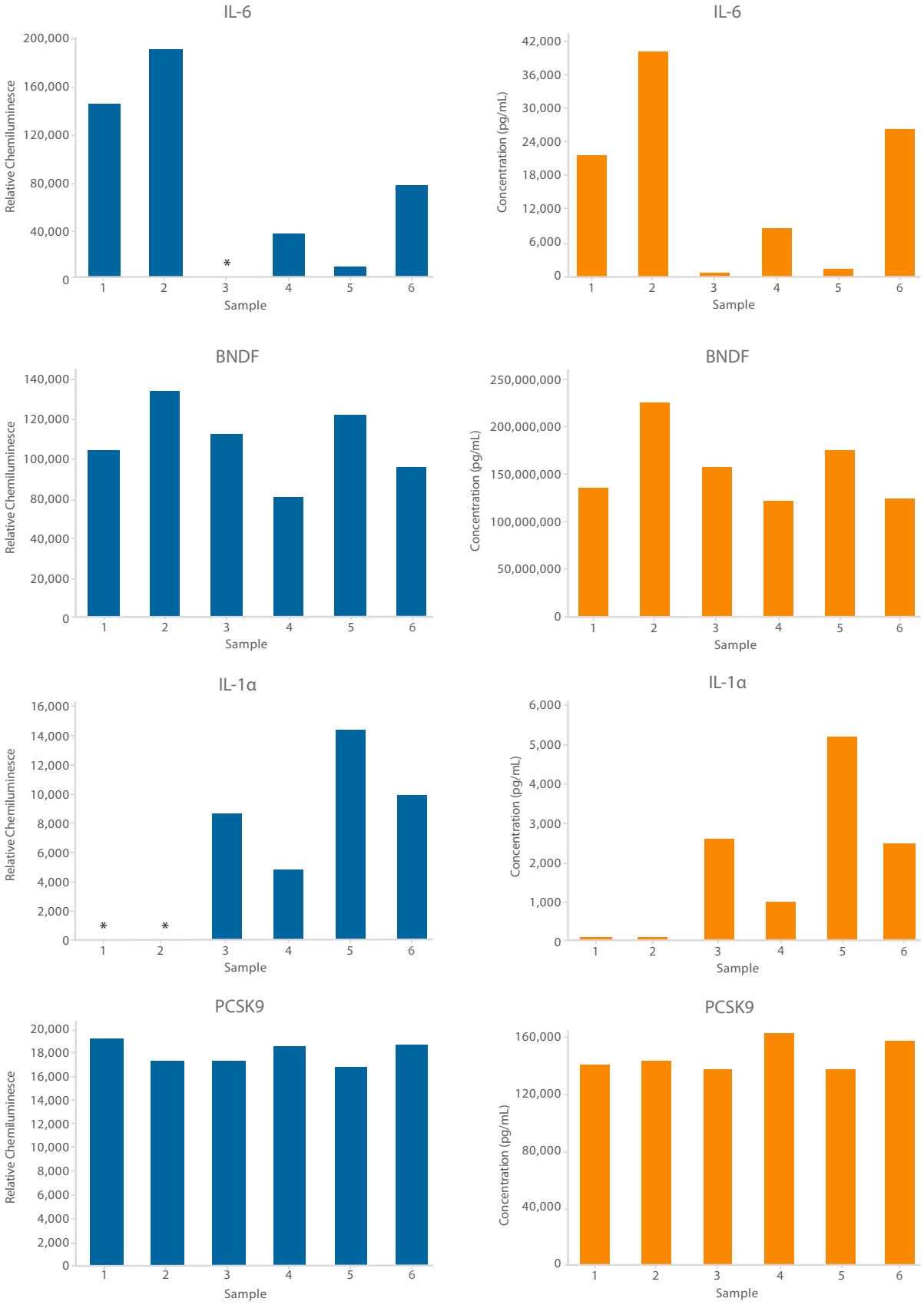


FIGURE 2. Comparison of relative quantitation on Wes (left, blue) and absolute quantitation on Ella (right, orange). Protein level trends were similar between both methods. * Signal not detected.

	IL-6 (1:1000)		BDNF (1:100,000)		IL-1A (1:10)		PCSK9* (1:10)	
Sample	Average Concentration (pg/mL)	% CV	Average Concentration (pg/mL)	% CV	Average Concentration (pg/mL)	% CV	Average Concentration (pg/mL)	% CV
1	21,693	5.2	136,074,206	2.0	83	1.6	140,052	2.0
2	39,905	2.0	227,185,347	2.5	26	1.5	143,325	1.0
3	809	9.5	156,588,495	2.1	2,562	2.3	137,668	2.1
4	8,770	9.0	123,014,794	2.8	970	6.6	162,587	2.8
5	1,377	1.2	175,274,056	1.3	5,205	0.8	137,478	1.3
6	26,153	6.2	125,021,865	1.8	2,463	0.5	157,494	0.8

TABLE 2. Absolute quantitation of spiked IL-6, BDNF, IL-1α, and endogenous PCSK9 in six normal human serum samples using a Simple Plex assay on Ella. Samples were diluted in SD13 Sample Buffer at 1:10, 1:1000, or 1:100,000 before loading on the 16 x 4 Simple Plex cartridge. Triplicate data with single-digit CVs were generated using two cartridges in in just two hours. * Endogenous protein detected.

We then ran the same samples with the Simple Plex assay on Ella using two 16 x 4 cartridges as different dilutions were needed to get all four targets in the linear, quantitative range. All the data was collected in only two hours and analyzed in Simple Plex Explorer software before exporting to Microsoft® Excel.

% CVs were all single-digit with an average across all six samples and four targets of less than 2.9% (**Table 2**). All targets in all sample runs were detected, so you can be sure Ella will measure your protein of interest. And when we compared the relative quantitation from Wes with the absolute quantitation from Ella, the trends were similar (**Figure 2**).

Conclusion

When it comes to fast biomarker development, we've got you covered — from discovery with Luminex assays, to verification with Simple Western assays, and validation with Simple Plex assays. We show that in normal human serum spiked with different concentrations of recombinant protein, relative quantitation with Simple Western assays on Wes and absolute quantitation with Simple Plex assays on Ella followed the same trends, giving you confidence in your results. Plus, the data was also extremely precise with Simple Western assays coming in with CVs under 13.4% and single-digit CVs on Simple Plex assays.

Most importantly, we generated high-quality, high-fidelity data in just a few days. Wes generated 72 data points (six samples, four analytes, triplicate results) in just 12 hours, and Ella gave us an astounding 288 data points (six samples, four dilutions, four analytes, triplicate results) in just two hours. With that kind of speed, you'll be on your way to novel biomarker discovery in no time!

References

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96-4005-00, Rev B