Simplifying Protein Analysis

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“I love running Westerns.”

- no one, ever.

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Welcome

Proteins are the heart and soul of functional biology and understanding proteins is central to understanding disease. However, proteins are difficult to interrogate because they are large, complex, and unique. Here at ProteinSimple, we believe that traditional protein analysis tools can be overly complex or inadequate, and our goal is to make protein analysis simpler, more quantitative, and affordable. Ultimately, we want to help researchers gain a better understanding of proteins and their role in disease. The most widely used protein analysis technique in existence today is the Western blot, or Western, which detects whether a specific protein is present in a sample. The Western workflow, unchanged since its invention in 1979, requiresmany manual steps, can take up to 24 hours to complete, and can lead to variable and semi-quantitative results. Our Simple Western platform is a complete reinvention of the Western blot that we believe is a historic breakthrough for protein research. Our Simple Western automates the entire assay workflow and transforms the Western into an analytical tool, enabling researchers to determine precisely how much of a specific protein exists in a given sample. Protein-based therapeutics are transforming the pharmaceutical industry and the treatment of many diseases. The development and production of biologics requires a variety of analytical tools to ensure the quality and efficacy of these complex drugs. Our iCE and MFI tools help researchers analyze protein purity and identify contaminants during biologics development and production. Finally, many people today are asking how drug treatment affects various biological pathwaysand processes, and they want to look at it using a holistic systems biology approach. Such tools are rare and traditional tools like ELISA and even the availble multiplex platforms have sensitivity and specificity limitations. Our Simple Plex platform takes a different approach to look at multiple analytes simultaneously in a truly simple format. In this eBook, we hope you can get some valuable information from the articles about these new protein analysis methods. Protein analysis is our passion and along the way, we hope you get to know us a little bit too. We hope you enjoy the book.

John Proctor, Ph.D.
Vice President of Marketing

Contents

Westerns and Total Protein Analysis:
No Gels or Blots Required

Patricia Piatti, Ph.D.

When we introduced the Simple Western in 2011, we made a giant leap forward by automating an age-old process—30 years old to be precise: Western blotting. A proteomics workhorse, it's plagued by poor reproducibility, lack of accurate quantitation, extensive time to result, and reliability issues. Simple Westerns are about protein analysis made simple without compromising quality and reliability. With automation of all steps from protein loading and separation, immunoprobing, washing, detection, and quantitative analysis of data, it finally gives researchers a complete, walk-away solution.

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The Basics

Simple Western is based on capillary electrophoresis (CE) and offers two assays: A size assay that combines CE-SDS with immunodetection to separate proteins by molecular weight and a charge assay that combines cIEF with immunodetection where proteins are resolved based on their pI. In both assays, the separated proteins are attached to the wall of capillary by a proprietary photo activated chemical crosslink. Subsequent immunodetection is done automatically by incubating and washing the capillary with primary and secondary antibodies conjugated with horseradish peroxidase and detected via chemiluminescence (Figure1). Molecular weight or pI and signal for immunodetected proteins are automatically reported. Depending on the system used, throughput can be up to 96 data points/assay with only 5 µL of sample in <16 hours (Sally Sue or Peggy Sue), or up to 25 data points/assay in < 3 hours (Wes).

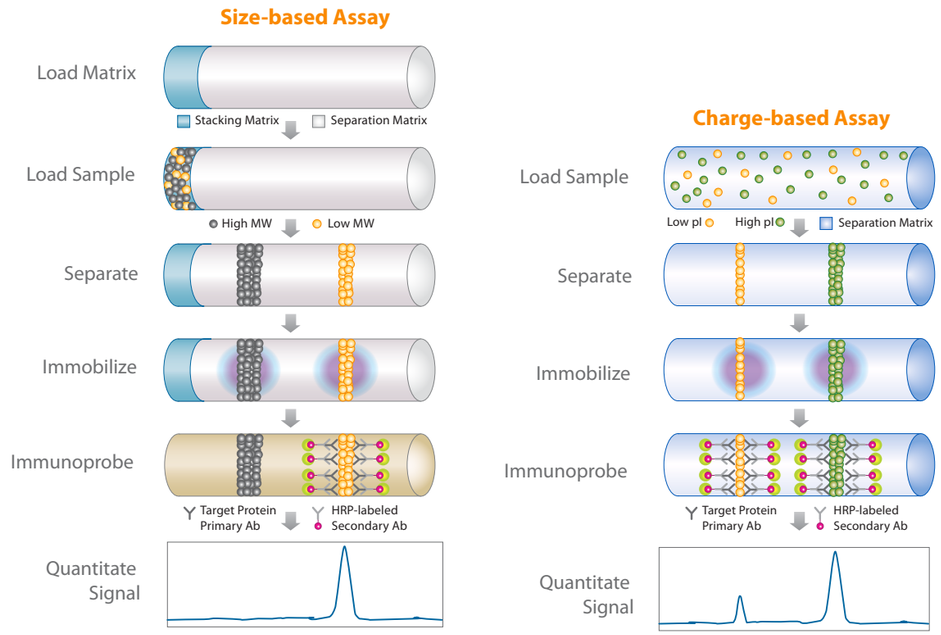


Figure 1. Simple Western assays combine a familiar separation technique, capillary electrophoresis, with a well-known detection technique, an immunoassay, to give you quantifiable, highly reproducible data.

The Incredible Data

Reproducibility is something Simple Western does very well. Not only intra-assay (**Figure 2**) but thanks to a system control that is included in every capillary, the size assays can achieve inter-assay CV <20%. Our large systems Sally Sue and Peggy Sue can analyze an entire cell signaling pathway in a single run. In this example (**Figure 3**), HeLa cells were left untreated or treated with TNFα to stimulate the NFκB signaling pathway. Using only 5 µL/well (1 µg/µL) of either a whole cell lysate (WC) or nuclear extract (NE) from those cells, Sally Sue was able to quantitate seven different proteins from the pathway plus a loading control (αTubulin). And how about multiplexing you may ask? Well, we've got that covered too as long as your targets differed either by at least 10% difference in molecular weight or 0.1 pI units.

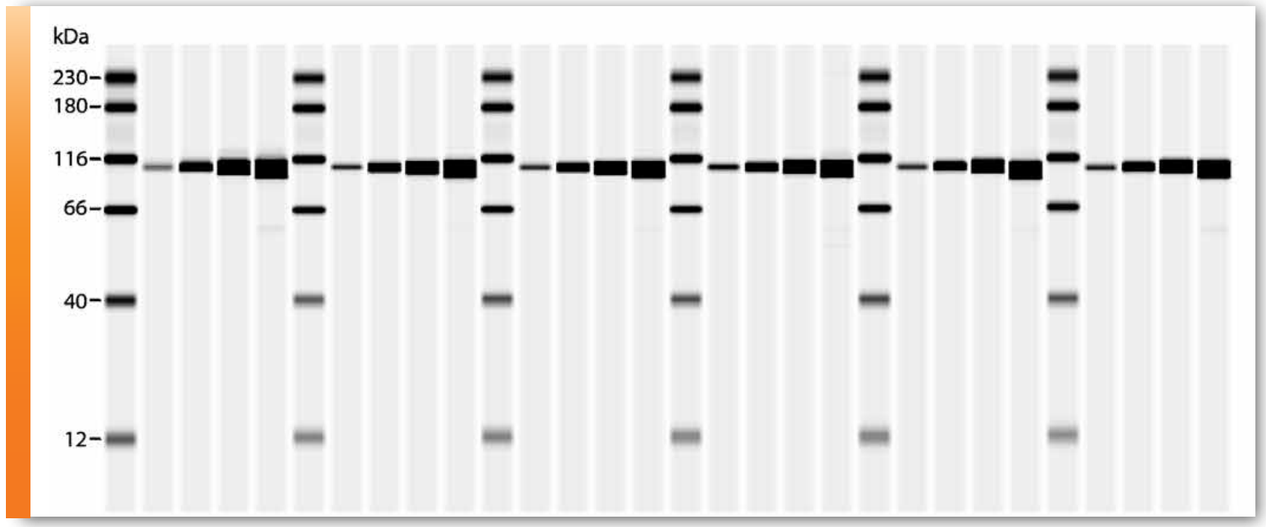


Figure 2. Detection of PI3 Kinase in a serial dilution of HeLa lysate (1000 ng/µL—37 ng/µL) in Sally Sue. All eight cycles showed similar results (CV <15%). Linearity of the dilution series (R2=0.989) is maintained even at very low protein concentrations.

Expanding Simple Western Applications

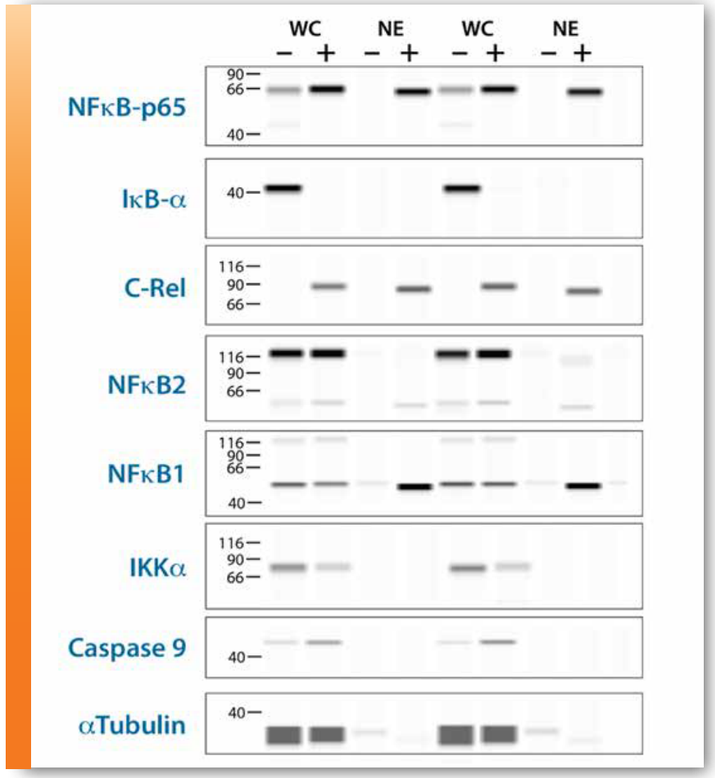


Figure 3. An entire cell signaling pathway analyzed in one run by Sally Sue.

We at ProteinSimple like challenges and are always on the lookout for problem areas in protein research that need a little (or a lot of) simplifying, like total protein analysis. We found a way to simplify that too with the Simple Western Total Protein Assay. It lets you detect all proteins in your sample without running a single gel, and does it with Simple Western simplicity. Without the need forantibodies, a biotin reagent binds to the proteins and is detected by chemiluminescence using streptavidin-HRP (**Figure 4**).

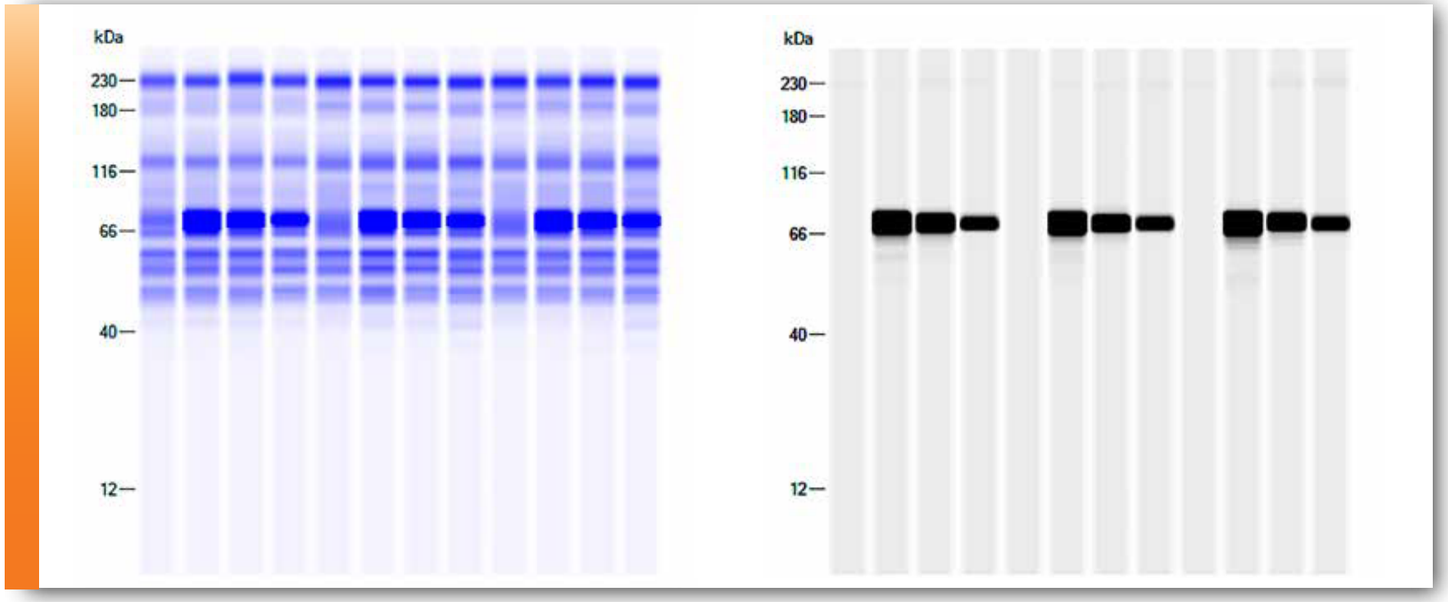
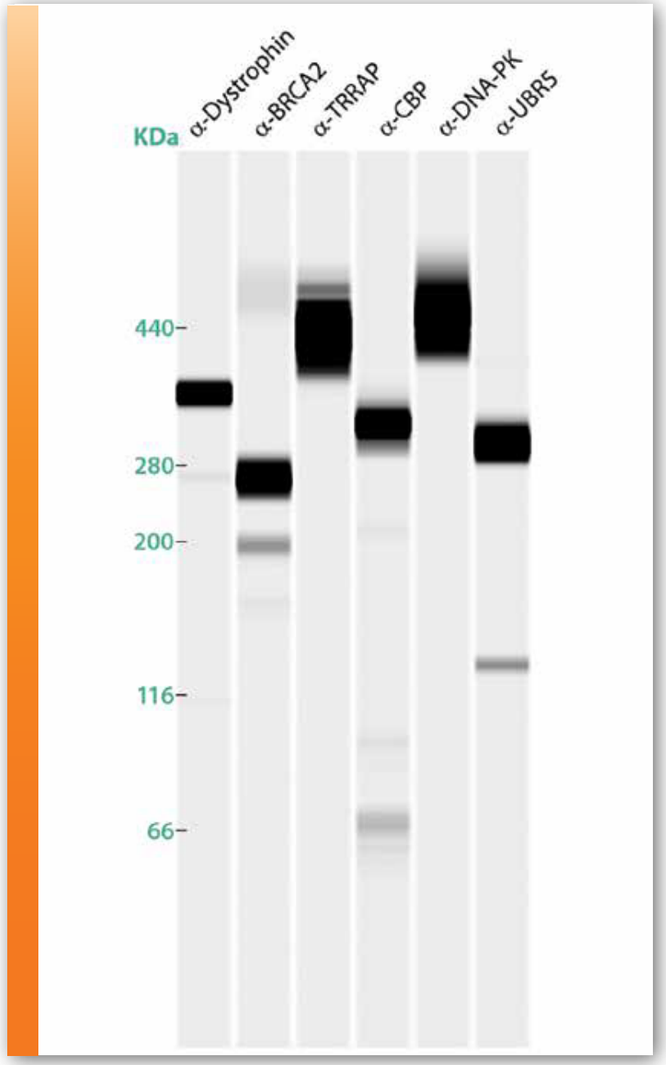


Figure 4. Total protein and immunoassay detection of decreasing concentrations of DNAK in HeLa lysate (15, 7.5 and 3.75 µg/mL in the Total Protein Assay, 0.015, 0.0075 and 0.00375 µg/mL in the immunoassay) performed in Wes.



Big proteins are a BIG challenge with a traditional Western blot. If you're spending too much time troubleshooting the transfer of a high molecular weight protein, you're not alone! The process of optimizing gels and buffer systems and figuring out whether to use a tank or semi-dry transfer is a real pain. And judging by the number of online forums dealing with this topic, it's clear Western blotting of high molecular weight proteins is an obstacle in the road to publication-worthy data for many. Our Simple Western assay for high molecular weight proteins leaves no big protein behind! Wes, Peggy Sue and Sally Sue have extended their molecular weight range and can now detect proteins up to 440 kDa. And they do it with the same simplicity, sensitivity, and reproducibility they're known for (**Figure 5**).
We promised it was going to be simple, right? Whichever instrument or assay you choose, you'll get the separation you need, identification of your target protein, and truly quantitative data that enables you to make accurate experimental decisions.

With 21CFR11 option, and a choice of throughput Simple Western is the platform of choice of many major pharma, CROs and academics laboratories. Go ahead, move that mountain of gel apparatuses, transfer tanks, and shakers off your bench and Simple your Western. It's time.

Figure 5. Panel of large proteins run with the 66–440 kDa pre-filled plate on Wes. We used 0.2 mg/mL of A10 lysate to detect dystrophin, 2 µg/µL of A431 lysate to detect BRCA2, 1.0 µg/µL of A431 lysate to detect TRRAP, 0.25 µg/µL of K562 lysate to detect CBP, and 1.0 µg/µL of K562 to detect DNA-PK and UBR5. All samples were denatured at 95 °C for 5 minutes. Analysis on Wes was complete in under three hours.

Mastering Charge Heterogeneity
Analysis of Therapeutic Proteins

Analyzing charge variants of biopharmaceuticals is a critical component of product development and quality control. Charge variants commonly occur as a result of both chemical and posttranslational modifications including deamidation, oxidation, glycosylation, and glycation. These changes can affect biological activity, patient safety, and drug stability.

Charge variants have traditionally been monitored by ion exchange chromatography (IEC), but newer CE-based techniques, such as imaged capillary isoelectric focusing (icIEF), offer the advantages of generic methods for multiple products and faster analysis times. Analysis of biopharmaceuticals using icIEF on the iCE3 system provides high-resolution charge heterogeneity peak profiles in 10 minutes. In this tutorial, we describe a simple and easy approach for icIEF method development on the iCE3 along with tips to improve method robustness.

Imaged cIEF Principle

Significantly different from traditional cIEF, the iCE3 performs capillary IEF with wholecolumn detection, which eliminates the need for a lengthy mobilization step—this both increases sample throughput and reduces assay complexity. Two electrolytic tanks at each end of the cartridge are filled with acid (anolyte) and base (catholyte). Prepared samples for icIEF contain a mixture of

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the protein of interest, carrier ampholytes, and pI markers. When this sample mixture is injected, it fills the entire capillary cartridge, where separation takes place. Samples are focused by applying voltage across the cartridge, and during the focusing step, a pH gradient forms across the capillary. The pI markers and protein of interest migrate through the capillary until they reach a pH value where their net charge is zero—this is their isoelectric point. The iCE3 then uses whole-column imaging detection at 280 nm to capture the separation within the capillary. Finally, the capillary is washed to ready it for the next sample injection. The full process from sample injection through final wash takes place in 10–12 minutes.

Method Development

A successfully defined and optimized iCE icIEF method gives you a highly reproducible peak profile and satisfactory peak resolution for the targeted application. This is quickly done on the iCE3 as only a few parameters need to be optimized by following a simple workflow as outlined in Figure 1. The first step in method development is to screen new compounds with a generic method employing a Pharmalyte 3–10 pH gradient as shown in **Figure 2A.** For many molecules, methods with this broad pH range provide sufficient performance and do not require further development. For more challenging molecules with complex peak profiles and/or limited solubility, method optimization can be accomplished using the following simple strategies.

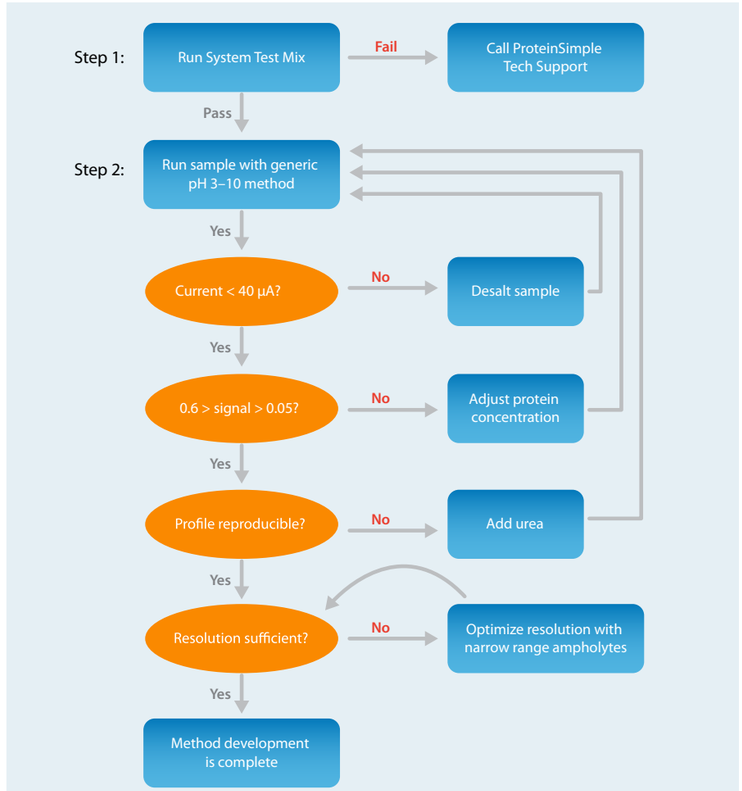

Figure 1. Method optimization workflow

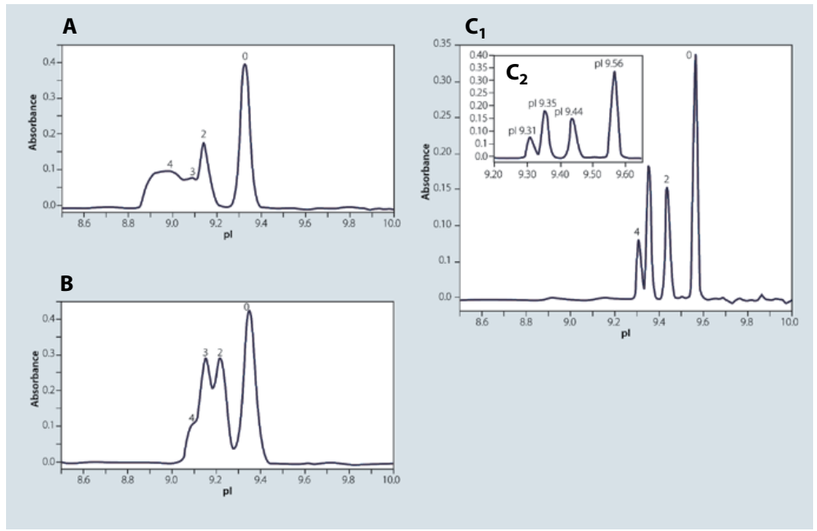

Figure 2. The complete icIEF method development process—from the screeningof compounds in a pH gradient (A), to the addition of solubilizers (B), to theaddition of narrow-range ampholytes (C)—can be completed in 2.5 hours.

In icIEF, proteins simultaneously lose surface charge while being focused into very concentrated sample zones. Under these conditions, hydrophobic regions may aggregate or interact, which can in turn affect the resolution and reproducibility of a charge heterogeneity profile. Addition of solubilizers such as urea into the sample eliminates aggregation effectively and improves separation as shown in **Figure 2B**.

After a protein's peak profile has been stabilized, resolution can be addressed by adding narrow pH range ampholytes to the sample matrix. In **Figure 2C**[1] the addition of narrow-range ampholytes results in near-baseline resolution of all isoforms. Triplicate run overlays shown in **Figure 2C**[2] demonstrate the separation is very reproducible while providing high resolution of 0.04 pH units. The complete icIEF method development process, from compound screening (**Figure 1**) to obtaining a final analytical method (**Figure 2C**), was completed in only 2.5 hours.

Once developed, an analytical method can be further optimized for robustness by implementing computational tools such as Central Composite Design of Experiment. A step-by-step description of executing a DOE for iCE3 method fine-tuning and characterizing is available online at www.proteinsimple.com.

Considerations for Method Robustness

Sample components, especially salts, can compromise the resolution and robustness of icIEF methods. During the focusing process, ions that do not have a zwitterionic or neutral charge state are driven out of the capillary by electrokinesis. As these charged

compounds leave the capillary, they are replaced by the anolyte's hydronium and catholyte's hydroxyl ions to maintain electroneutrality. This results in a high separation current along with compression of the pH gradient.

The separation of IgG 1 Kappa in **Figure 3A** clearly demonstrates salt's adverse effects on icIEF analysis. The resulting pH gradient compression can be observed by both the loss in resolution of IgG 1 Kappa charge isoforms and the pH shift of the pI 9.46 marker. Replicate runs at the highest salt concentration shown in Figure 3B illustrate the combined impact of salt-related high separation current and gradient compression on IgG 1 Kappa charge isoforms. The distribution of IgG 1 Kappa charge variants migrates toward lower pH, and forms an unresolved mound as it either degrades and/or aggregates in this extreme separation environment.

Separation artifacts due to high salt concentration can be easily avoided by reducing the concentration of salt components in the sample prior to analysis. In the case of formulations with high protein concentration, the act of diluting the protein down to the final working concentration in sample solution, typically in the range of 200–250 µg/mL for a mAb, will eliminate enough ionic strength to allow for successful iCE analysis. For formulations with low protein concentrations (<10× dilution to final sample concentration), a buffer exchange step may be needed to achieve best results. As with all separation techniques, high-quality reagents should be used with icIEF methods to ensure consistent results

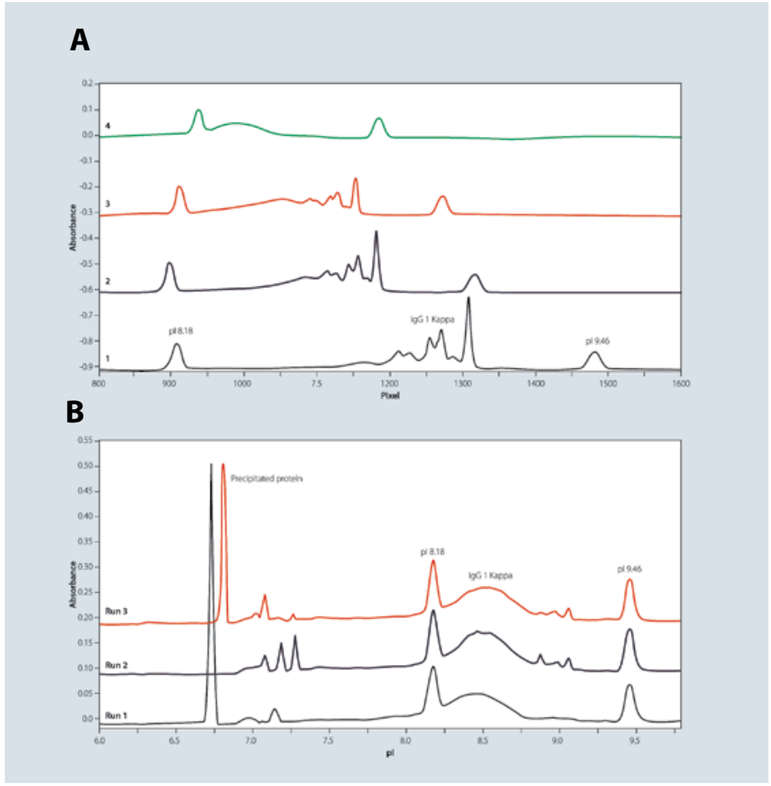

Figure 3. The separation of IgG 1 Kappa demonstrates the adverse effects of salts (A). Triplicate runs at 100 mM NaCl show that salt also affects reproducibility (B).

(**Figure 4B**). Using improperly stored or expired consumables and reagents can also have a profound effect on performance. This is especially true for methods that employ urea to eliminate aggregation. Urea solutions should be made fresh and kept away from heat to avoid thermal degradation. One of the thermal degradation products of urea, isocyanic acid, will rapidly react with amine groups and artificially increase a protein's acidic species' percent composition (Figure 4A).

Conclusion

The iCE3 system's quick and easy icIEF method development lets even those analysts new to icIEF develop robust charge heterogeneity methods in an afternoon by following some simple procedures and guidelines. Potential issues that can arise with commonly interfering sample matrix components are easily resolved through addition of solubilizers, dilution, or buffer exchange. In addition, the high-resolution, 10-minute separations obtained are ideal for the characterization and monitoring of charge variants in biopharmaceutical formulations.

Scott Mack, Ph.D., Senior Scientist and
Alpana Prasad, Ph.D., Product Manager
ProteinSimple

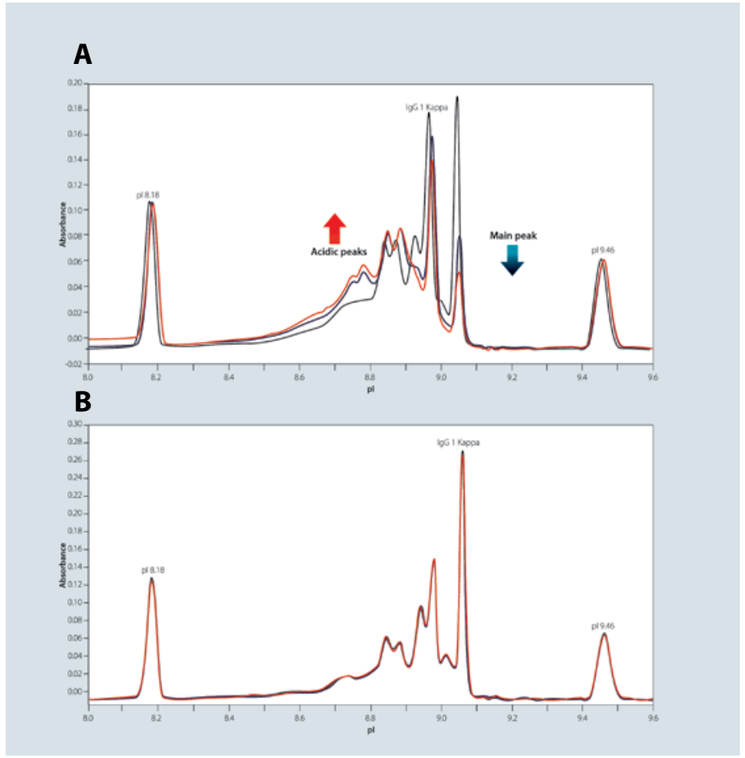

Figure 4. High-quality reagents help ensure consistent results. (B) For example, if urea solutions are used, they should be made fresh and kept away from heat. Doing so can avoid artificial increases in a protein's acidic species' percent composition (A).

Application Note

Comparability Study of Manual and Automated Particle Characterization with MFI

Dan Bach Kristensen, and
Trine Meiborg-Sloth, Takeda Pharma

Scott Mack, Ph.D., Angelica Olcott,
and Erik Gentalen, ProteinSimple

Introduction

Micro-Flow Imaging (MFI) has become a standard application for particle analysis of protein formulations[1] because of its ability to easily detect particle size and morphology for a diverse range of particle contaminants. Now you can monitor changes in translucent protein particles and silicone micro-droplets, which older compendial methods like light obscuration and coulter counter cannot do, since they only provide size and count[2,3]. To meet the increasing demand for biopharmaceutical testing of sub-visible particle characterization, higher throughput and standardization are needed, and MFI 5000 series easily meets that demand through automated, walk-away particle analysis with the addition of the Bot1 autosampler. In collaboration with Takeda Denmark, we outlined the process for transferring a manual

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MFI makes it easy to detect sub-visible particles like protein aggregates early, before they ruin your formulation. Plus if offers information that compendial methods can't.

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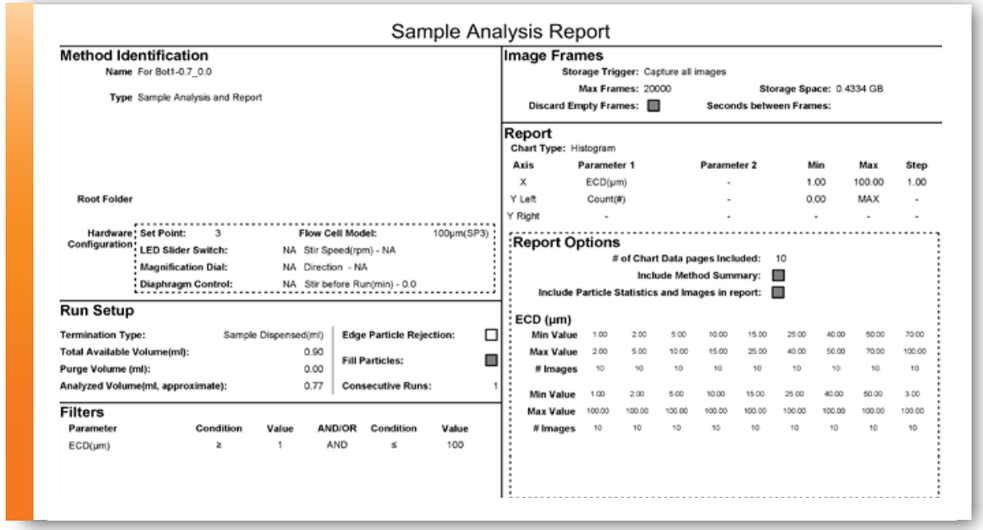
protocol to an automated format with the Bot1 autosampler and the MFI 5000 series, and demonstrated comparability of the two modes using a model protein system composed of 1% BSA at the two sites (ProteinSimple and Takeda Denmark). No statistically relevant differences were observed between manual and automated runs at either facility.

Materials and Methods

Sample Preparation and Instrument Operation

- 1% BSA sample was prepared, heated at 60 °C overnight (at least 12 hours) with shaking at 1200 rpm at the ProteinSimple facility. The sample was then aliquotted and stored at –80 °C. Frozen aliquots were shipped to the Takeda facility on dry ice and stored at–80 °C until use.
- Flow cell was washed with 1 mL of 10% Triton-X 100 in distilled, deionized (DDI) water (0.2 micron filtered) and rinsed 4X with 1 mL water before each run.
- A fourth stirring step was added to the protocol to ensure particles did not settle during the sampling process on the Bot1 (**Table 1**).
- Manual pipetting steps were performed by turning off the Bot1 autosampler and manually pipetting sample into the MFI 5200 system via the sample inlet port. The same analysis method was used for both the automated and manual protocols (**Figure 1**).


Sample Analysis Report

Method Identification
Name For Bot1-0.7_0.0
Type Sample Analysis and Report
Root Folder
Hardware Configuration: Set Point: 3 | Flow Cell Model: 100µm(SP3) | LED Slider Switch: NA | Stir Speed(rpm) - NA | Magnification Dial: NA | Direction - NA | Diaphragm Control: NA | Stir before Run(min) - 0.0

Run Setup
Termination Type: Sample Dispensed(ml)
Total Available Volume(ml): 0.90
Purge Volume (ml): 0.00
Analyzed Volume(ml, approximate): 0.77
Edge Particle Rejection:
Fill Particles:
Consecutive Runs: 1

Filters

Parameter	Condition	Value	AND/OR	Condition	Value
ECD(µm)	≥	1	AND	≤	100

Image Frames
Storage Trigger: Capture all images
Max Frames: 20000 Storage Space: 0.4334 GB
Discard Empty Frames: Seconds between Frames:

Report
Chart Type: Histogram

Axis	Parameter 1	Parameter 2	Min	Max	Step
X	ECD(µm)	-	1.00	100.00	1.00
Y Left	Count(#)	-	0.00	MAX	-
Y Right	-	-	-	-	-

Report Options
# of Chart Data pages Included: 10
Include Method Summary:
Include Particle Statistics and Images in report:

ECD (µm)

Min Value	1.00	2.00	5.00	10.00	15.00	25.00	40.00	50.00	70.00
Max Value	2.00	5.00	10.00	15.00	25.00	40.00	50.00	70.00	100.00
# Images	10	10	10	10	10	10	10	10	10
Min Value	1.00	2.00	5.00	10.00	15.00	25.00	40.00	50.00	3.00
Max Value	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
# Images	10	10	10	10	10	10	10	10	10

Figure 1. Analysis method for manual and automated samples at both ProteinSimple and Takeda sites. This analysis method was used with the batch protocol for modified Bot1 Protocol D. All samples were analyzed under the sameconditions, with an analysis volume of 0.77 µL, and Optimize Illumination volumeof 0.22 µL. Particle baseline was established with 0.2 µm-filtered DDI water.

OPERATION	LIQUID	VOLUME (ML)
Flush	Filtered water or filtered buffer	0.90
Flush	Filtered water or filtered buffer	0.90
Flush	Filtered water or filtered buffer	0.90
Dry system		
Flush	Filtered water or filtered buffer	0.90
Optimize Illumination	Filtered water or filtered buffer	0.22
Baseline	Filtered water or filtered buffer	0.70
Flush	Filtered water or filtered buffer	0.90
Dry system		
Stir 4 cycles, speed 5	Sample (well 1)	0.90
Flush	Sample (well 1)	0.50
Stir 4 cycles, speed 5	Sample (well 1)	0.90
Optimize Illumination	Sample (well 1)	0.22
Stir 4 cycles, speed 5	Sample (well 1)	0.70
Analysis Run	Sample (well 1)	0.70
Flush	Filtered water or filtered buffer	0.90
Flush	Filtered water or filtered buffer	0.90
Flush	Filtered water or filtered buffer	0.90

Method Transfer

Translating a manual method to an automated method requires modification of the protocol to ensure comparable results due to differences in sampling handling between the two methods.

Method transfer considerations include:

- Changes in sample introduction—manual pipetting or stirrer with syringe barrel mix differently than an automated pipettor. The user can control the duration and speed of mixing with the automated pipettor.
- Suitability of sample type—manual or automated sample requirements are similar; highly viscous or extremely concentrated samples may not be suited for automation.
- Sample volumes—sample and purge volumes may change due to new labware and fluid path.
- Ambient temperature—samples should be stable at room temperature during an automated run. Sample volumes should be chosen based on particle concentration in the sample, as described in the next section. Sample volumes should also be adjusted further to accommodate the sample delivery format of the instrument configuration. Thus, moving from a manual to automated protocol may require changes to sample volume and flush volume initially to achieve accurate results. This is due to the fixed sample delivery format which includes 1–2 mL deep well plates, the 1 mL pipette tips and the interface to the inlet port (**Table 2**).

Table 1. Description of automated Protocol D used for 1% BSA solution. Original version of Protocol D uses 0.90 mL for Flush step. Steps from Flush after Baseline through Analysis Run are repeated for each sample in the run. Stir of four cycles was used prior to each sample analysis to ensure particles remained in suspension.

Optimization of the automated protocol for the 1% BSA solution was performed as follows. We used a larger volume initially in Protocol D and then modified pipetting parameters as described in the modified Protocol D to reduce the volume requirements (**Table 2**). Additional stirring steps counteracted the effects of settling time during the automated run, so that samples are well mixed at the time of analysis. Sample types used with the MFI system in manual or automated mode should also be evaluated for suitability, as outlined in **Table 3**.

Guidelines for Sample Volume

Particle concentration in the sample can aid in determining required volume for accurate sizing and concentration measurements, as shown in **Figure 2.** Optimal measurements are achieved for values at the 0.00% line of each graph. For example, in samples with particle concentrations of 10,000 P/mL or greater, volumes of at least 500-900µL should be used. If samples contain particle concentrations of 1,000 P/mL, volumes of 1 mL or greater should be used.

CRITERIA	DESCRIPTION
Volume	Minimum 900 µL*
Throughput	>10 samples/day (or unattended operation)
Labware	1 or 2 mL deepwell plates
Mixing format	Aspirate and dispense steps using automated pipettor
Ambient temperature	Samples can be tested at room temperature (ambient)
Viscosity	Some viscosity, not highly viscous (≤10 cP units)
Concentration	>50–150 mg/mL for protein formulations
MFI 5000 Series	Bot1 is used with MFI 5000 series

Table 3. Recommended criteria for MFI 5000 series with Bot1 autosampler.

*Required sample volume may be reduced following method optimization.

	MANUAL OPERATION	BOT1 PROTOCOL D	MODIFIED BOT1 PROTOCOL D
Bot1 flush with sample	N/A	0.90 mL*	0.50 mL
Sample volume dispensed (defined in Method)	0.90 mL	0.70 mL	0.77 mL
Sample purge volume (defined in Method)	0.20 mL	0.00 mL	0.00 mL
Optimized Illumination volume and liquid type	0.22 mL of buffer (MFI 5200)	0.22 mL of sample (MFI 5200)	0.22 mL of sample (MFI 5200)
Dead volume	0.10 mL	0.03 mL	0.03 mL
Total Sample Volume	**1.20 mL**	**1.85 mL**	**1.52 mL**

Table 2. Method comparison of manual to modified Bot1 Protocol D. The Bot1 Protocol D was optimized further to reduce flush volume, resulting in a total sample volume of 1.52 mL for Modified Bot1 Protocol D, which was the automated protocol used in this study.

*Volume may be reduced by optimizing the batch protocol for a specific sample type. Optimize Illumination step is equivalent to a purge step, so additional purge step is not required.

Accounting for Differences in Sample Handling and Volume Requirements

The automated protocol uses 1.52 mL of sample as compared to the manual protocol's 1.20 mL. Volume and mixing steps for the automated protocol were optimized to ensure consistent counts and concentrations between replicates. As shown in **Table 2**, the initial total volume requirements of 1.85 mL for Protocol D were reduced to 1.52 mL in the modified version by adding automated mixing steps, reducing the sample flush volume, and increasing the stir speed from a setting of 3 to 5 (users can control the speed of this setting from 117 to 700 µL/sec). With this change, the actual sample flush volume required was reduced to 500 µL from 900 µL with no additional variation due to precipitation or aggregation. In each case, two runs of four replicates each were averaged and analyzed for count and concentration, with standard statistical tests as outlined in the next section.

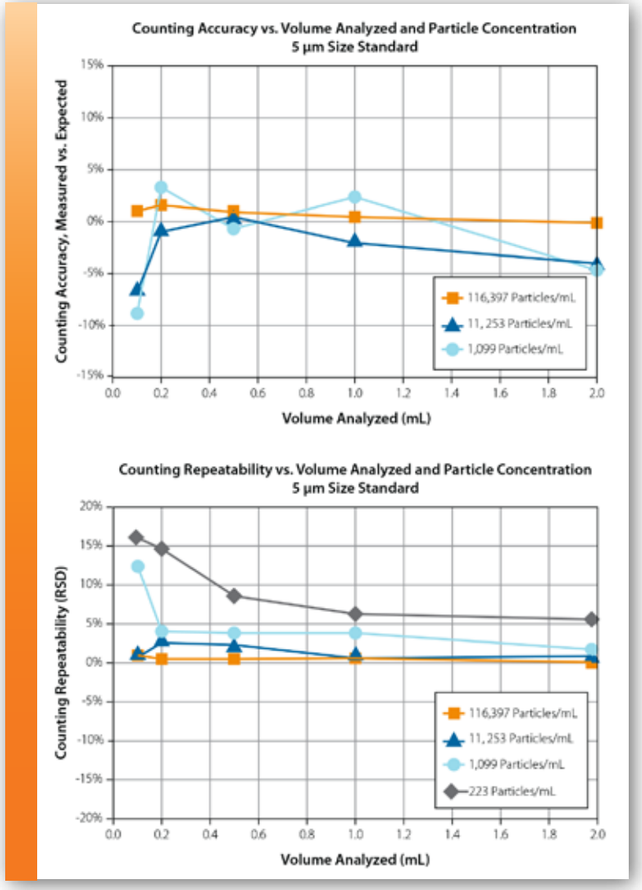


Figure 2. Impact of volume on counting accuracy for differing particle concentrations (top). Impact of volume on counting reproducibility for differing particle concentrations (bottom).

```
Predictor      Coef  SE Coef      T      P
Constant   10534.6    519.9  20.26  0.000
Automated    172.3    410.2   0.42  0.678
Set 2        279.3    404.0   0.69  0.496
Day 2        224.6    410.2   0.55  0.589
Run 2        107.0    553.1   0.19  0.848
Run 3       -266.9    553.1  -0.48  0.634
Run 4        164.7    605.9   0.27  0.788


S = 1106.28   R-Sq = 6.8%   R-Sq(adj) = 0.0%

PRESS = 48120295   R-Sq(pred) = 0.00%


Analysis of Variance

Source          DF        SS       MS     F      P
Regression       6   2064274   344046  0.28  0.940
Residual Error  23  28148679  1223856
Total           29  30212953


Source      DF  Seq SS
Automated    1  203214
Set 2        1  585203
Day 2        1  463322
Run 2        1  153389
Run 3        1  568725
Run 4        1   90420
```

```
Predictor      Coef  SE Coef      T      P
Constant   8885.2    249.4  35.62  0.000
Automated   398.8    245.6   1.62  0.136
Set 2        41.4    229.8   0.18  0.861
Run 2      -162.4    313.1  -0.52  0.615
Run 3      -590.6    319.1  -1.85  0.094
Run 4      -189.8    319.1  -0.59  0.565


S = 442.818   R-Sq = 36.9%   R-Sq(adj) = 5.3%

PRESS = 5405327   R-Sq(pred) = 0.00%


Analysis of Variance

Source          DF       SS      MS     F      P
Regression       5  1145756  229151  1.17  0.388
Residual Error  10  1960880  196088
Total           15  3106636


Source      DF  Seq SS
Automated    1  401933
Set 2        1   15025
Run 2        1   21197
Run 3        1  638197
Run 4        1   69403
```

Figure 3. Linear regression analysis was used with dummy coding to evaluate relevant variables (manual method, automated method, day) as a group. Analysis shows no significant impact of system inputs (automation, day to day variance) on particle counts. The various system inputs could account for 0.0% (ProteinSimple, left) and 5.3% (Takeda, right) of the variation in the data. An R-Sq (adj) of >30% would indicate a significant impact.

Results

At least eight manual and automated runs were performed at each site, using the same manual and automated protocols for pipetting and analysis (**Figure 1**). Data were analyzed to assess normality of data set, and were not found to significantly deviate from normal distribution. P-values were 0.072 and 0.053 for ProteinSimple and Takeda, respectively. Variances between automated and manual methods were not statistically different by f-test. Employing a standard analysis for statistical variance(ANOVA) indicated that only between 0 and 4% of variance in particle counts can be attributed to method type which is not significant. Regression analysis showed no relationship between other input variables and variance in the data (**Figure 3)**. Comparison of data showed results at Takeda were approximately 2000 counts/mL lower than those at ProteinSimple. This difference is likely related to sample quality in terms of sample stress or degradation during shipment. Neither site showed any significant difference in counts or concentration between the manual and automated method, confirming that similar results can be obtained using either format. Count and concentration for automated versus manual format for 1% BSA, ProteinSimple. Data from tests performed at ProteinSimple are listed in **Table 4**. The automated format of 1% BSA protein solution produced consistent counts and concentration data, compared to the manual format. The modification to Protocol D reduces the sample flush volume without impacting the consistency of results between replicates in the automated method. The standard deviation for both methods was 5% or less for all samples tested.

MANUAL		
RUN	**COUNTS**	**COUNTS/ML**
1	8783	11184
2	9745	12409
3	8530	10862
4	8238	10490
Average	8824.00	11236.25
Std Dev	565.63	720.25
%CV	6.41%	6.41%
RUN	**COUNTS**	**COUNTS/ML**
5	7918	10083
6	7703	9809
7	7815	9951
8	7403	9427
Average	7709.75	9817.50
Std Dev	192.73	245.40
%CV	2.50%	2.50%
AVERAGE OF ALL 8 MEASUREMENTS		
Average	8227.83	10477.17
Std Dev	875.47	1114.73
%CV	10.64%	10.64%

AUTOMATED		
RUN	**COUNTS**	**COUNTS/ML**
1	8463	10780
2	8444	10773
3	8483	10812
4	9003	11479
Average	8598.25	10961.00
Std Dev	234.09	299.43
%CV	2.72%	2.73%
RUN	**COUNTS**	**COUNTS/ML**
5	8840	11274
6	9124	11633
7	8905	11343
8	10572	13471
Average	9360.25	11930.25
Std Dev	707.47	899.69
%CV	7.56%	7.54%
AVERAGE OF ALL 8 MEASUREMENTS		
Average	9058.00	11545.67
Std Dev	787.21	1001.27
%CV	8.69%	8.67%

Table 4. Comparison of the Manual Versus Automated Method, ProteinSimple

Comparison of the Manual Versus Automated Method, Takeda

Tests performed at Takeda were comparable to the ProteinSimple site, and the automated format of 1% BSA protein solution produced consistent counts and concentration data similar to the manual format (data not shown). The standard deviation for both methods was 6% or less for all samples tested. Takeda results were similar to ProteinSimple's in terms of successful transfer of the 1% BSA assay to an automated format.

Conclusion

The MFI 5200 produces the same high-quality particle characterization in manual or automated mode. This study showed that method optimization could further reduce the sample volume required without impairing concentration accuracy. Statistical analysis confirmed that these protocols are robust and provide an example of standardization of methods across instrument configurations.

In the Takeda development laboratory, MFI was originally implemented due to its advantages over conventional techniques (HIAC-based light obscuration used according to Ph. Eur. 2.9.20/USP <788>) for measurement of sub-visible particles in the µm range (e.g. 2–10 µm).

Those advantages included:

- Lower sample consumption versus light obscuration
- 1–10 µm particle detection
- MFI's ability to distinguish between particle types based on morphology parameters

Reduction of hands-on time associated with manual operation was a primary goal, and became the main reason for upgrading to the automated MFI 5200 system with Bot1autosampler at an early stage. In addition, the improved reproducibility associated with the automated format further contributed to the decision to upgrade. The option to automate provides a key advantage for particle characterization of protein formulations, offering many benefits compared to more common techniques. Automated protocols allow for much greater throughput and less hands-on time, with up to 90 samples per unattended run, and address demand for more rapid and consistent screening methods in particle characterization. Moreover, the latest version of MFI's MVSS analysis software give you high throughput with the full benefits of multisample analysis. It's ideal for monitoring the impact of different conditions during stability testing. For more information on the new multisample analysis format, be sure to check the link to the Appendix section on the next page.

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Appendix

Want to speed through a lot of samples in no time? View a quick preview of the new MFI software MVSS 4.0 for Multi-Sample Analysis and Higher Throughput.

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Multi-Analyte, Automated, Microfluidic Immunoassay Platform

Danielle Svancara, Marty Putnam, and Patrick Smith

Introduction

Protein analytes or biomarkers have traditionally been measured individually in ELISAs, which can attain a high degree of analytical specificity by testing only a single analyte with a dedicated antibody pair. However, the clinical field is becoming increasingly aware that multiple markers are associated with complex, multimodal, multivariate diseases. Unfortunately, adoption of multianalyte biomarker tests in clinical research has been severely limited for many reasons, including technical concerns regarding assay reproducibility, cross-reactivity, decreased sensitivity, or increased variability at low concentrations, the time and labor-intensive nature of assay panel development, and non-correlation with conventional ELISA data. To address this issue, ProteinSimple developed Simple Plex™, a novel automated immunoassay platform that enabled

Ella runs hands-free, multiplex-free, ELISA-free Simple Plex assays

protein simple
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Additional Content

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simultaneous multi-analyte quantification, while retaining the sensitivity and specificity of single-analyte ELISAs. Simple Plex assays run on Ella™ enabled simultaneous quantitation of four analytes, in discrete channels, from 16 individual samples in a single disposable microfluidic cartridge in an hour. This eliminated potential negative interactions or interference from the antibody pairs for other assays, while simultaneously providing the benefits of a multiplexed antigen analysis and rapid microfluidic reaction kinetics. Glass nano reactors (GNRs) were developed as a solid-phase support for the capture antibodies, and were shown to possess enabling physical, optical, and chemical properties. GNRs were functionalized to allow antibody immobilization on the GNRs internal, but not external, surfaces (**Figure 1**). The composition of the GNR provided a highly uniform surface, supporting multiple well-characterized, stable immobilization chemistries, and exhibited low intrinsic fluorescence.

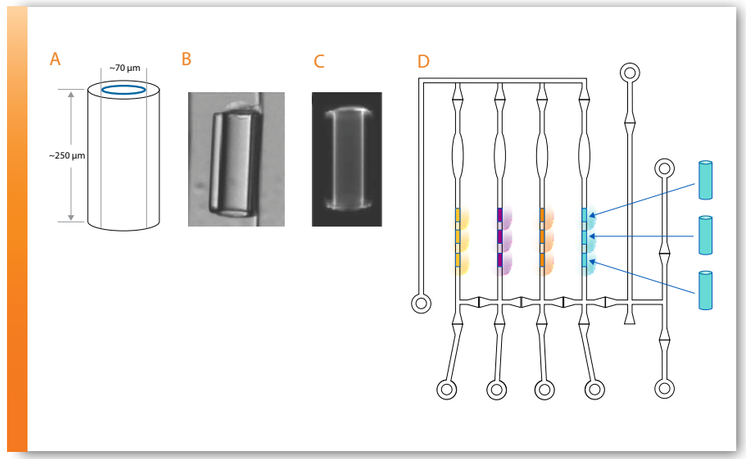


Figure 1. The Glass Nano Reactor (GNR). (A) Physical dimensions of a GNR. (B) Photograph of a GNR embedded in the channel of the Simple Plex cartridge. (C) Fluorescent image of a GNR revealing exclusive localization of antibodies in the center of the GNR, with no detectable signal on the outer surfaces. (D) Cartoon depicting placement of GNRs in discrete channels of one of the eight circuits on an 8-sample 4-plex cartridge. Different colors indicate GNRs specific for different analytes.

CARTRIDGE CONSTRUCTION

Cartridges were assembled with three analyte-specific GNRs, composed of glass capillary 250±25 µm (length) by150±6 µm (outer diameter) with an inner diameter of 75 ±3 µm, per channel in every circuit (**Figure 1**).

DAILY REAGENTS

Calibration curves were made daily over the course of five days with freshly thawed multi-analyte standard spiked and serially diluted into freshly thawed calibrator buffer. Curves consisted of eight points, including zero, spanning the biologically relevant range. The limit of detection (LOD)

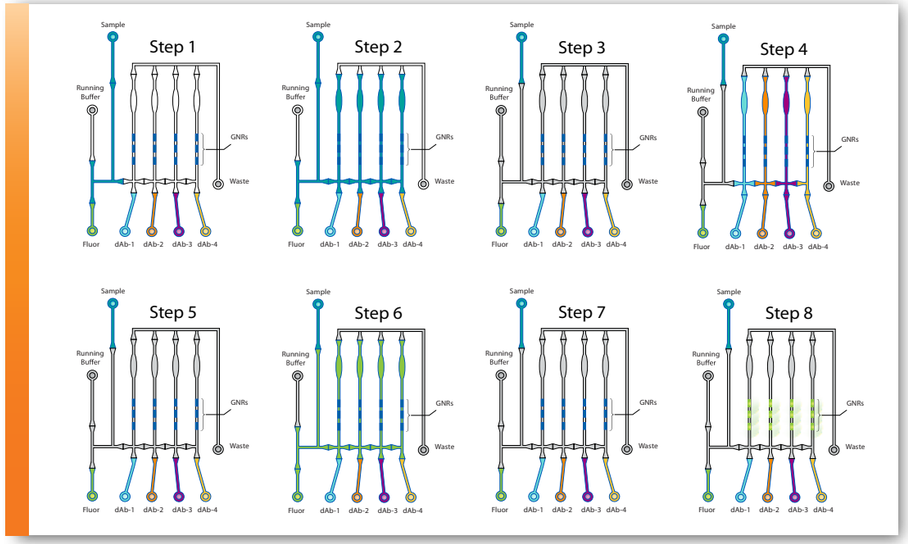


Figure 2. The Simple Plex asasy work flow, shown for one of the eight sample circuits. Step 1: The system is primed with sample. Step 2: Sample is pumped through the circuit and split evenly between the four channels containing analyte-specific GNRs. Step 3: After sample incubation, the circuit is cleaned with wash buffer. Step 4: Analyte-specific dAb's are individually pumped into their respective channels to bind analyte captured on the GNRs. Step 5: Unbound dAb's are removed with wash buffer. Step 6: Detect fluor is flowed into all channels, binding bound dAb's. Step 7: Residual detect fluor is removed with wash buffer. Step 8: Detect fluors are excited with a 631 nm laser and the signal is read with a CCD camera.

was determined by adding three standard deviations to the mean relative fluorescencen units (RFU) value of 20 zero standard replicates and calculating the corresponding concentration. The lower limit of quantification (LLOQ) was defined as greater than the LOD and the first concentration on the standard curve at which CVs were less than or equal to 20%. The upper limit of quantification (ULOQ) was defined as the highest concentration on the standard curve in which CVs were less than or equal to 20%. Selectivity was assessed with normal human serum samples obtained from Bioreclamation (Westbury, NY). Selectivity was assessed by spiking known concentrations, 7.5, 75, and 750 pg/mL (pmol/L), of freshly thawed multi-analyte standard into five lots of individual normal human serum. Samples were diluted 1:2, one part sample with one part freshly thawed sample buffer, for spiked and neat (unspiked) concentrations. Recovery was defined as the ratio percentage of the concentration measured from the curve fit, hereafter known as the backfit, divided by the sum of the amount spiked and the measured endogenous concentration from the unspiked sample.

SIMPLE PLEX ASSAY PROTOCOL

Assays were conducted by pipetting a minimum of 35 µL of sample and/or calibration point into each sample inlet, and a minimum of 300 µL of wash buffer in corresponding inlets. The user initiated the run with software designed to facilitate proper data organization and execution of automated scripts without user intervention; cartridge run steps are outlined in **Figure 2**, and took one hour from start to finish. Once an analyte-specific calibration curve was generated in associated software, all subsequent cartridges run were automatically backfit to generate concentration results. Further analysis, including calculating mean recovery and CVs, was done in Microsoft® Excel®.

SIMPLE PLEX MODULARITY

In order to show the modularity of the Simple Plex assay, calibration curves were run on Simple Plex cartridges containing constant assays in channels one, two and three of IL-1β, IL-6, and IL-10 respectively, and with channel four variable between TNF-α, IL-2, IL-5, and IL-12. A known concentration of 40 pg/mL (pmol/L) made in calibration buffer was run in conjunction with standard curves for each panel composition and was backfit to each panel's specific curve.

SIMPLE PLEX VS. TRADITIONAL ELISA PLATE

ELISA plates were obtained from R&D Systems for IL-1β (DLB50), IL-5 (D5000B), IL-10 (D1000B), and IL-12 (D1200) and were run according to manufacturer's instructions with the exception that lyophilized standards were the same standard used in the Simple Plex assay.

Cartridges containing IL-1β, IL-5, IL-10, and IL-12 were run in alongside the ELISA plates and data were converted from RFU/ms or ocular density to relative units to directly compare formats.

SIMPLE PLEX VS. MULTIPLEX ASSAY

Calibration curves for IL-5 were run as a traditional Simple Plex assay format and as a traditional multiplex with increased complexity of cocktailed dAbs. For the cocktailed detect mixtures, dAbs were added to IL-5 in order of IL-1β, IL-10, and IL-1α, where IL-1β was present in all cocktails and IL-1α present only in the final 4-plex cocktail.

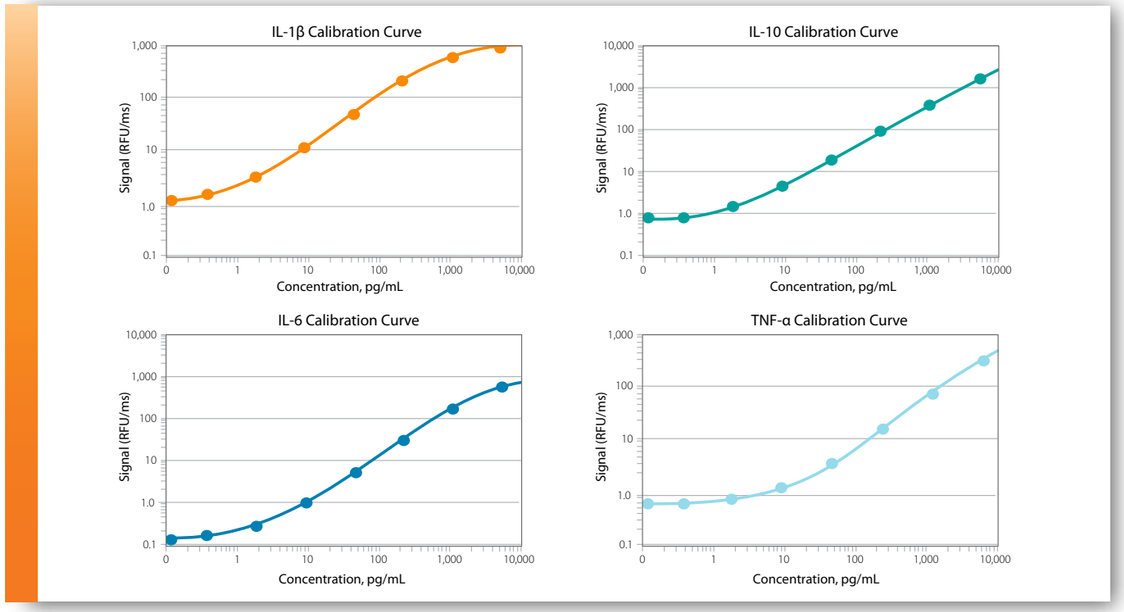


Figure 3. Calibration curves for the individual biomarkers run in the Simple Plex assay format. Data points are the average of a minimum of four data points per day over five days with standard deviation error bars.

Results and Discussion

STANDARD CURVES

Specific capture and detect antibody pairs were tested in the Simple Plex assay format for LOD, LLOQ, ULOQ, and intra-/inter-assay variation. The standard curve for each biomarker showed dynamic ranges >2.8 to >3.5 orders of magnitude (**Figure 3**), with LLOQs and ULOQs (**Table 1**) such that concentrations in the biologically range can be detected. Intra-assay variation was calculated for concentration within the quantifiable range, between LLOQ and ULOQ, as the mean percent CV of the CV per cartridge run for each biomarkers standard curve (**Table 1**). Inter-assay variation was calculated as the percent CV of all concentrations of the standard curve within the quantifiable range (**Table 1**).

BIOMARKER	LOD (pg/mL)	LLOQ (pg/mL)	ULOQ (pg/mL)	MEAN PERCENT RECOVERY	INTRA-ASSAY VARIATION (%CV)	INTER-ASSAY VARIATION (%CV)
IL-1β	0.3	0.32	1,000	102%	5.90%	4.20%
IL-6	0.35	1.6	>5,000	98%	6.00%	6.00%
IL-10	0.71	1.6	>5,000	100%	7.90%	9.90%
TNF-α	1.98	8	>5,000	102%	6.80%	6.80%

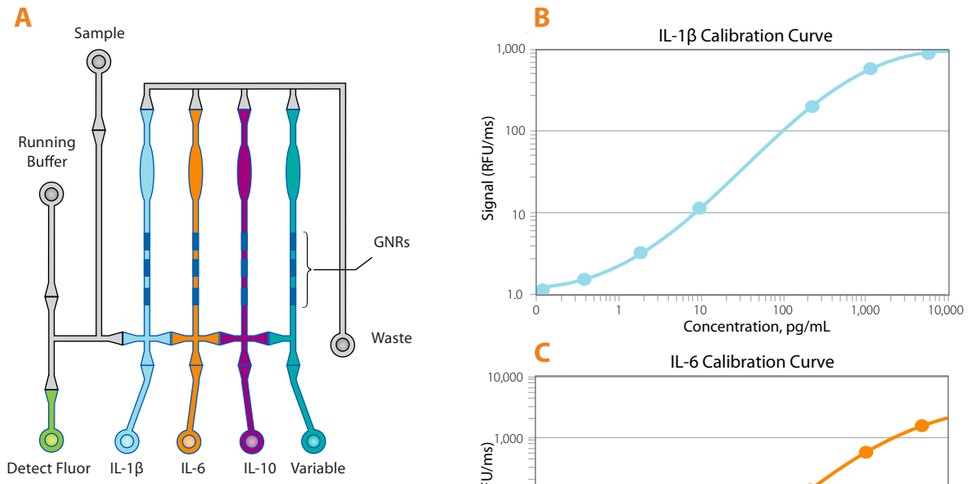
Table 1. Characteristics of Simple Plex biomarker assays.

NORMAL HUMAN SERUM ID	IL-1B		IL-6		IL-10		TNF-α	
	MEAN RECOVERY	CV	MEAN RECOVERY	CV	MEAN RECOVERY	CV	MEAN RECOVERY	CV
BRH687933	102%	9%	116%	4%	98%	9%	109%	7%
BRH687937	95%	9%	115%	3%	103%	2%	125%	4%
BRH687939	94%	5%	125%	4%	89%	11%	109%	6%
BRH687943	86%	14%	113%	2%	87%	20%	112%	5%
BRH687945	79%	16%	109%	4%	89%	20%	91%	7%

Table 2. Selectivity of Simple Plex assays. Mean recovery per individual normal human serum samples spiked at three concentrations of multianalyte standard, 7.5, 75 and 750 pg/mL (pmol/L), for IL-1β, IL-6, IL-10, and TNF-α.

SELECTIVITY

Selectivity of each biomarker was assessed at concentrations near LLOQ, below ULOQ, and midway between the two. Acceptance criteria for selectivity during the validation of an immunoassay was mean biomarker recovery of 75–125% with ≤25% CV and 70–130% recovery with ≤25% CV for a minimum 83% of the samples tested21. Mean percent recovery and percent CV of three spiked concentrations per biomarker for individual samples were all between 70–130% mean recovery with ≤25% CV (Table 2). Mean recovery of five individual samples at three spiked concentrations tested was 91% (14% CV) for IL-1β, 116% (6% CV) for IL-6, 92%



	PANEL 1	PANEL 2	PANEL 3	PANEL 4
Channel 1	IL-1β	IL-1β	IL-1β	IL-1β
Channel 2	IL-6	IL-6	IL-6	IL-6
Channel 3	IL-10	IL-10	IL-10	IL-10
Channel 4	TNF-α	IL-2	IL-5	IL-12

Figure 4. Assay uniformity as influenced by panel composition. (A) Schematic of circuit composition across the four panels. (B–D) Calibration curve stability for each of the constant assays. Each graph contains the mean standard curve with standard deviation error bars; the maximum CV between standard curve points was 4%,7%,and 7% for IL-1β, IL-6, and IL-10, respectively. (E) Recovery of a 40 pg/mL calibration point; the results were averaged across the four panels IL-l, IL-6, and IL-10; and single measurements for TNF-α, IL-2, IL-5, and IL-12.

(14% CV) for IL-10, and 107% (11% CV) for TNF-α. The accuracy and precision of each biomarker shows selectivity for each assay's specific analyte run in the Simple Plex assay format.

SIMPLE PLEX MODULARITY

Mean recovery for calibration concentrations in the detectable range (between LLOQ and ULOQ) for the constant assays in channels 1–3 with four different panels represented in channel 4 was 99% (7.3% CV) for IL-1β, 100% (6.2% CV) for IL-6, and 100% (9.8% CV) for IL-10. Mean recovery for the 40 pg/mL (pmol/L) point, made in calibration buffer, for the constant assays back-fit to each panels specific calibration curve was 104% (4% CV) for IL-1β, 101% (5% CV) for IL-6, and 106% (7% CV) for IL-10. These data show that for the immunoassays studied the accuracy and precision of an individual assay is not negatively impacted by changing the immunoassay in neighboring channels within the Simple Plex cartridge.)

SIMPLE PLEX VS. TRADITIONAL PLATE ELISA

Calibration curves run in the ELISA format showed dynamic ranges around 2 to 2.5 orders of magnitude, whereas in the Simple Plex assay format dynamic ranges with the same standard were all greater than four orders of magnitude showing a twofold increase over the ELISA format (Figure 5). Furthermore, theincrease in dynamic range of the Simple Plex assay format spanned

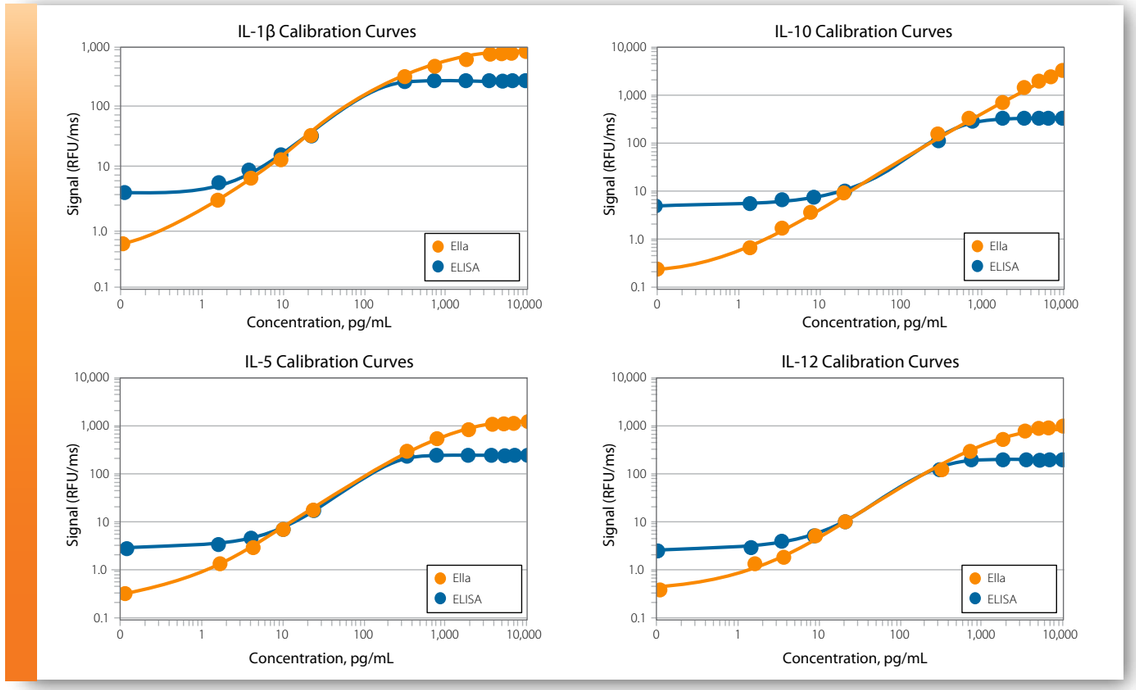


Figure 5. Simple Plex assay vs. traditional ELISA. Calibration curves graphed with relative units.

both ends of the curve showing improved sensitivity for measuring low concentrations andexpanded range for elevated samples. In particular, the increased sensitivity isespecially crucial for cytokines as most healthy levels range in the single digitpg/mL range.

SIMPLE PLEX VS. TRADITIONAL MULTIPLEX ELISA

Calibration curves for IL-5 were run as a traditional Simple Plex assay format and as a traditional multiplex with increased complexity of cocktailed dAbs (Figure 6). The data revealed that as the number of dAbs in the cocktail increased,sensitivity declined. For the purposes of this analysis, an arbitrary point of comparison was defined as the concentration at which the signal to noise (signal at concentration divided by signal of zero) was equal to five. Comparison of the cocktailed dAbs to the traditional Simple Plex assay showed that the arbitrary point of comparison increases 3.4, 15.2, and 44.3 fold for two dAbs, three dAbs, and four dAbs respectively, which directly corresponds to decreased sensitivity equal to the fold increase observed for the arbitrary point.

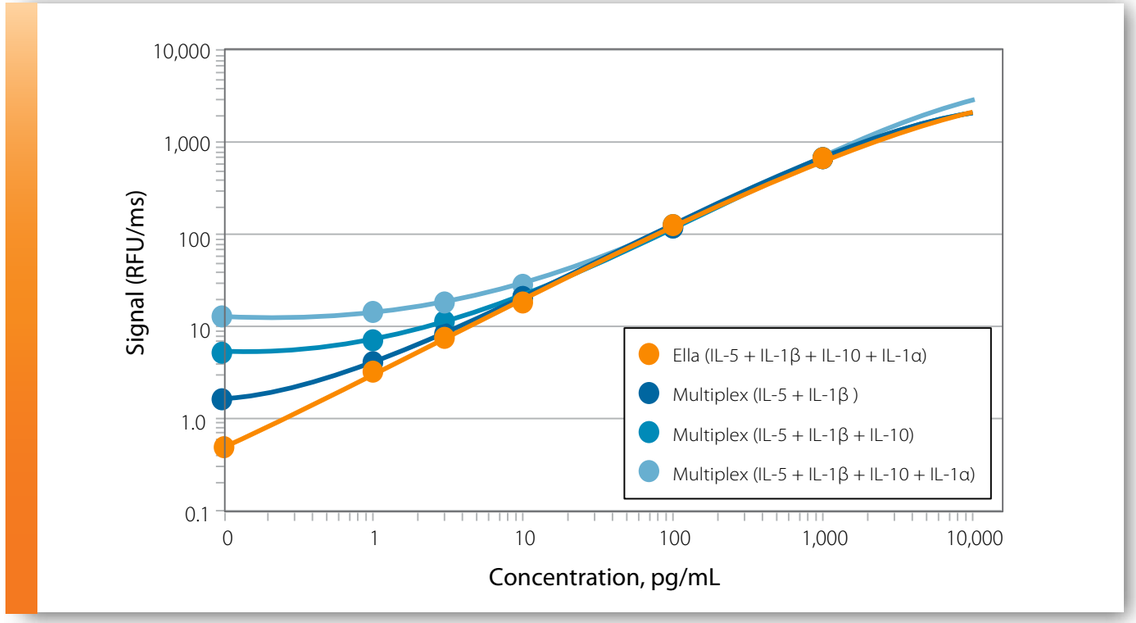


Figure 6. Simple Plex vs. multiplex format. IL-5 calibration curves for Simple Plex format with microfluidically separated detection steps vs. increasingly complex cocktailed detect calibration curves, mimicking traditional multiplex ELISA.

Conclusion

ProteinSimple has developed a microfluidic multi-analyte detection platform, Simple Plex, that allows guided diagnosis and treatment of life-threatening diseases by quantifying indicative biological molecules found in the body known as biomarkers. Effective detection of complex diseases or conditions often requires the analysis of multiple biomarkers, unfortunately, typical multiplexed assays suffer from several limitations; the most severe limitation is that the detection of multiple biomarkers in a single sample requires the simultaneous use of multiple capture and detection antibodies in the same reaction. This often results in cross-reactivity between noncompatible antibodies, where the antibody for a given biomarker also reacts to, and detects, any number of additional antibodies (**Figure 6**).

The Simple Plex assay run on Ella is a fully automated, multi-analyte platform with the accuracy and precision of a single-analyte assay due to its unique microfluidic architecture, which separates the analyte-specific assays into discrete channels. The utilization of GNRs, with low surface area, results in the use of very low sample volumes with a short assay time due to the high surface area to volume ratio. Finally, due to the use of materials with very low intrinsic fluorescence in conjunction with the laser excitation of fluors, the resulting low background increases the low end sensitivity of the platform when compared with traditional ELISA.

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