

# A comparison of protein detection and quantification techniques

Enabling researchers to make scientific discoveries using the best tools for specific needs

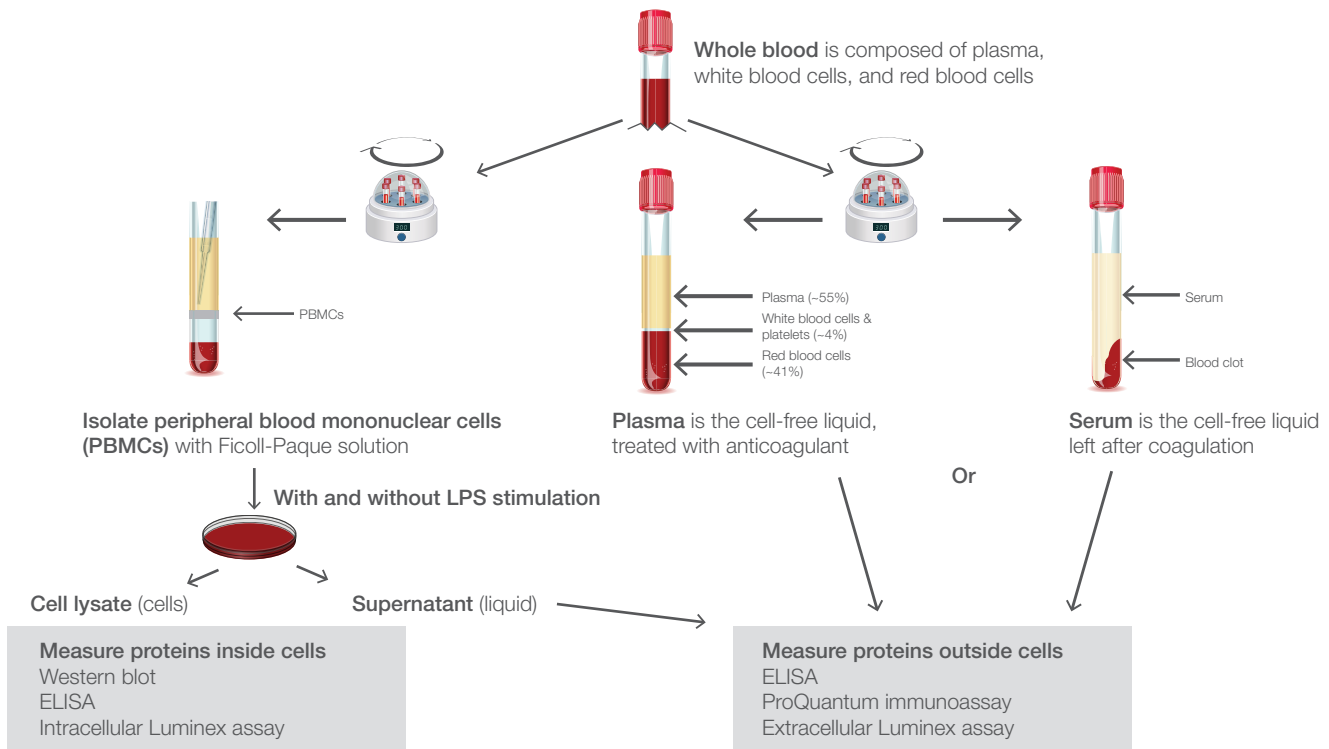
## Introduction

Understanding the complex, coordinated protein interactions that sustain life may enable us to unlock mysteries of disease mechanisms and thereby discover treatments. Protein identification and quantification have become extremely valuable in advancing our understanding and finding cures. As there are many different methodologies available to scientists today, it can be challenging to determine which tools are the most effective to answer specific questions. Thermo Fisher Scientific provides a vast selection of protein quantitation solutions, from simple reagents to validated assay kits.

Here we describe these platforms and show expected results using whole blood from three donors. Starting with these samples, we separated the whole blood into common constituent sample types: serum, plasma, peripheral blood mononuclear cell (PBMC) supernatant, and PBMC lysate. We stimulated the PBMCs with lipopolysaccharide (LPS) to demonstrate how natural cytokine expression can be measured. Protein detection and quantitation systems featured in this application note are western blots, enzyme-linked immunosorbent assays (ELISAs), Invitrogen™ ProQuantum™ high-sensitivity immunoassays, and multiplex assays on the Luminex® platform.

## Experimental design

Three healthy human donors were selected, and one sample of whole blood was drawn from each, per normal standards (>20 mL) using EDTA tubes for plasma and serum tubes for serum (Figure 1). Red blood cells were separated via centrifugation, and plasma or serum was aliquoted. To isolate PBMCs, equal volumes of freshly drawn whole blood and PBS were mixed in a 50 mL conical tube, and then one volume of Ficoll-Paque™ solution equal to that of the whole blood was added (i.e., 1:1:1 by volume of whole blood, PBS, and Ficoll-Paque solution); the tubes were centrifuged at 2,000 x g for 20–25 minutes. The visible PBMC band was collected by gentle aspiration with a 5 mL or smaller transfer pipette and dispensing into a separate conical tube containing PBS. The conical tube was then centrifuged and the supernatant was discarded. The cells were washed 3 times with 45 mL of 1X PBS, followed by centrifugation at 1,200 x g for 5 minutes. The PBMCs were incubated for one day at a starting density of 1–2 x 10<sup>6</sup> cells/mL in growth medium at 37°C, 5% CO<sub>2</sub>. A final concentration of 10 µg/mL LPS was used to stimulate the PBMCs for 4 days. After stimulation, the PBMCs were lysed and intracellular proteins isolated using Invitrogen™ Cell Extraction Buffer (Cat. No. FNN0011). Both the lysate and cell culture supernatant were aliquoted and stored at –80°C for future testing. These processed samples were used in various assays (Figure 1).



**Figure 1. Sample collection and analysis workflow.** Beginning with whole blood collection from 3 donors, samples were processed into plasma, serum, and isolated PBMCs for stimulation. Aliquots were collected from each fraction and stored frozen at  $-80^{\circ}\text{C}$ . The processed samples were then analyzed using the indicated techniques.

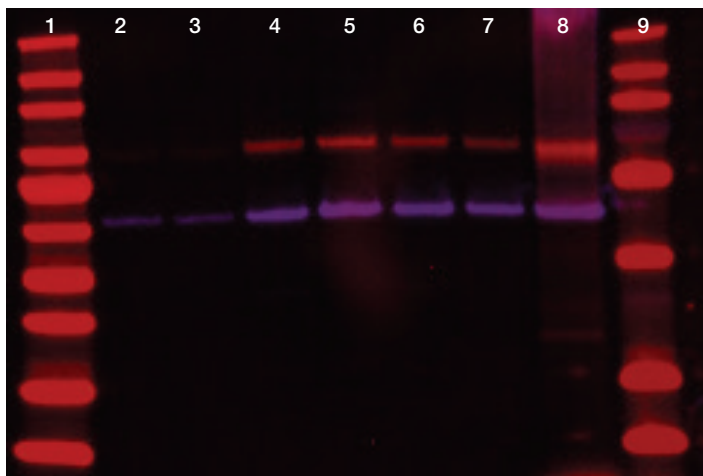
## Western analysis

Western blotting is the most widely used method to detect specific protein expression levels in cells or tissues using highly specific antibodies and molecular weight markers for protein verification. Only one antibody is required to detect the protein. Detection methods can include colorimetry, fluorescence, and chemiluminescence to match experimental needs and instrument compatibility. Similar detection methods are used with other technologies that include readout methods, like ELISAs.

PBMC lysates were denatured and electrophoresed on Invitrogen™ Bolt™ 4–12% Bis-Tris Plus Gels (10-well). These gels require as little as 22 minutes to run and are ideal for western blotting and analysis. To transfer the proteins from the gel to a membrane (nitrocellulose or PVDF), the fast 7-minute transfer of the Invitrogen™ iBlot™ 2 Gel Transfer Device was utilized. The iBlot 2 device offers fast, convenient, high-quality transfer that produces sharp and

straight bands with exceptional efficiency. For the detection portion of the experiment, the Invitrogen™ iBind™ Flex Western Device was used. The iBind device automatically performs all immunodetection steps using sequential lateral flow technology, a simple form of capillary action, in about 2.5 hours. Final imaging was performed using the Invitrogen™ iBright™ FL1000 Imaging System.

Using the fluorescence multiplexing capabilities of the iBright™ system, the intracellular proteins AKT and GAPDH (protein control) were measured using two primary antibodies and two secondary Invitrogen™ Alexa Fluor™ antibodies for a fluorescence readout (Figure 2). The advantage of western blotting over other protein detection methods is being able to visualize a band corresponding to the protein and confirm the protein by molecular weight. The trade-off is that western blotting is only semiquantitative.



**Lanes:**

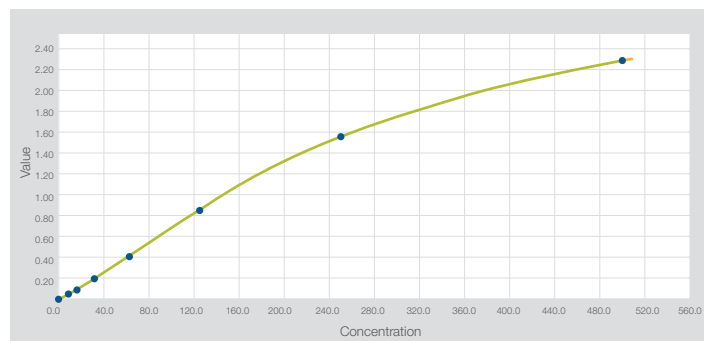
1. Invitrogen™ PageRuler™ Prestained NIR Protein Ladder
2. Donor A (unstimulated)
3. Donor A (stimulated)
4. Donor B (unstimulated)
5. Donor B (stimulated)
6. Donor C (unstimulated)
7. Donor C (stimulated)
8. AKT positive control
9. Invitrogen™ PageRuler™ Plus Prestained Protein Ladder

**Figure 2. Western blot showing levels of AKT (red) and GAPDH (purple).** No significant differences were detected between stimulated and unstimulated PBMCs. The blot shows the signal of the AKT band relative to the signal of the GAPDH internal positive control.

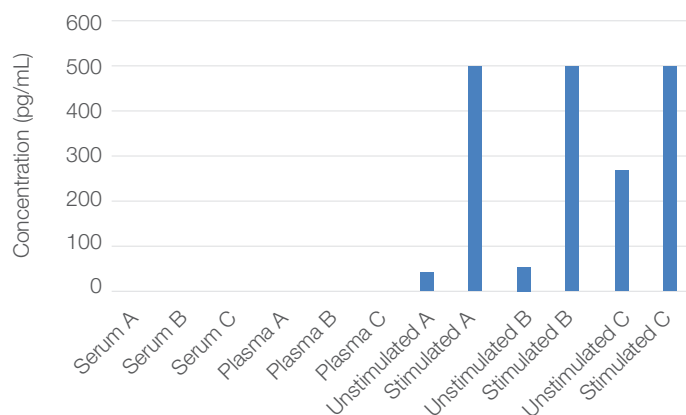
**ELISA analysis**

ELISA is the most common choice for quantitation of secreted, circulating proteins in bodily fluid samples such as serum and plasma, or cell culture supernatant in cell model systems. The assay is a proven, gold-standard method for quantifying protein concentrations using a microplate reader such as the Thermo Scientific™ Multiskan™ GO Microplate Spectrophotometer. The most typical type of ELISA utilizes a capture antibody adsorbed to the bottom surface of a 96-well plate and a matching detection antibody used for quantitation. The antibodies are used to “sandwich” the antigen for analysis. Typically, a biotin–streptavidin system is used to link the protein–antibody complex to horseradish peroxidase, which reacts with a substrate and results in a color change. The degree of color change is proportional to the amount of protein measured.

In this example, the secreted cytokine IL-6 was selected for measurement in serum, plasma, and cell culture supernatant (stimulated and unstimulated) using the Invitrogen™ Human IL-6 ELISA Kit (Cat. No. KHC0061). This is a typical result, where basal levels of cytokines in serum samples may not be detectable and only diseased samples may show increased levels of cytokines. Because the dynamic range of the ELISA kit is relatively small (Figure 3), it is common that serial dilutions of the sample are needed to fall within the range of the standard curve. The advantage of ELISA kits is that a protein standard is provided to generate a standard curve so that unknown samples can be quantified by interpolation (Figure 4).



**Figure 3. IL-6 standard curve generated using a logistic 5-parameter weighted curve-fitting algorithm.** Standard concentrations were determined based on the information from the product insert. The resulting curve is typical for a quantitative ELISA. The dynamic range of the standard curve is 7.8–500 pg/mL.

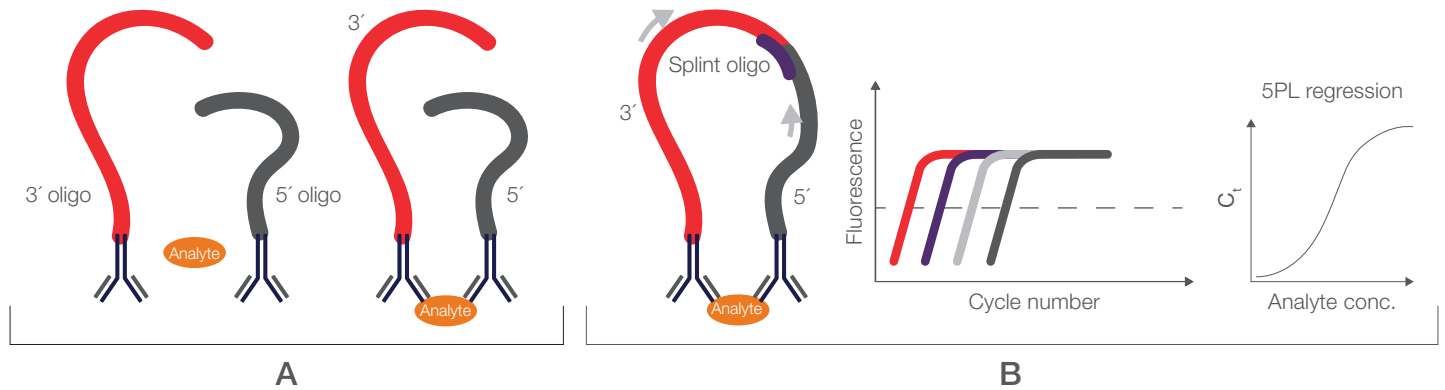


**Figure 4. Sample results interpolated from the standard curve shown in Figure 3.** As expected, levels of IL-6 in serum and plasma were undetectable and fell between the last standard point and the background. Unstimulated PBMC supernatant showed low levels of IL-6; however, LPS-stimulated PBMCs had IL-6 levels above the upper limit of quantitation (500 pg/mL) of the assay. The values plotted for samples above and below the range of the assay are shown for illustration only.

## ProQuantum high-sensitivity immunoassays

ProQuantum immunoassays are a new line of ready-to-use kits that enable an easy method to measure target-specific proteins with higher sensitivity and larger dynamic range than ELISA while consuming only a very small amount of sample (2–5  $\mu\text{L}$ ). The assay workflow is simplified, with only one antibody incubation step and no washing prior to placing the plate in any qPCR instrument for signal detection.

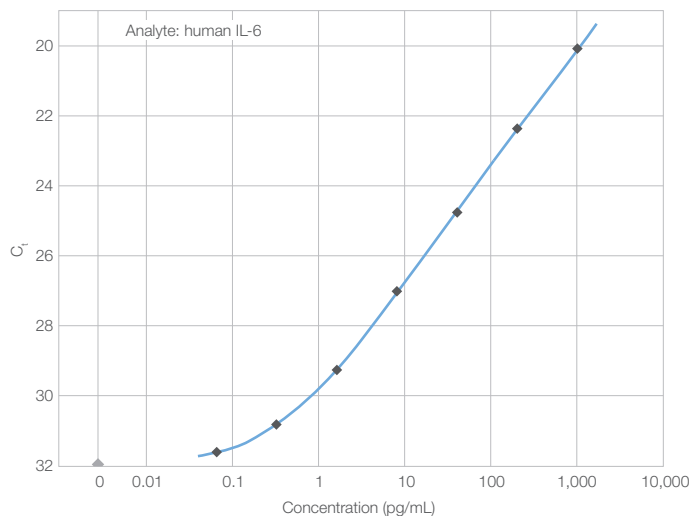
ProQuantum immunoassays utilize a proximity-based immunoassay technology to combine antigen-antibody binding for analyte detection with qPCR signal amplification and readout (Figure 5).



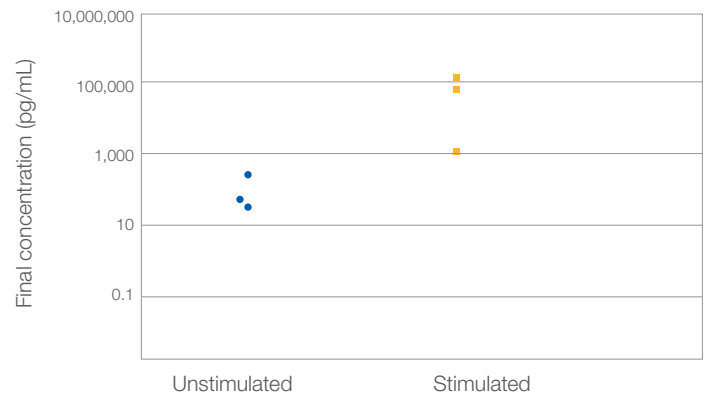
**Figure 5. ProQuantum assay workflow. (A)** Bind antigen (1 hr incubation). **(B)** Ligation and qPCR.

Serum, plasma, and supernatant were tested using the Invitrogen™ ProQuantum™ Human IL-6 Immunoassay Kit (Cat. No. A35573) and Applied Biosystems™ StepOnePlus™ Real-Time PCR System using parameters outlined in the protocol. Analysis was performed using a logistic 5-parameter weighted regression analysis method supported by Invitrogen™ ProQuantum™ cloud-based software ([apps.thermofisher.com/apps/proquantum](https://apps.thermofisher.com/apps/proquantum)).

Using the standard curve for each sample type (Figure 6), IL-6 concentrations were calculated. Samples for the ProQuantum assay are diluted 1:10 in Assay Dilution Buffer, so all final concentration results were adjusted for this dilution factor. The unstimulated vs. stimulated PBMC supernatant results are displayed using the groupwise comparison function in ProQuantum software (Figure 7).

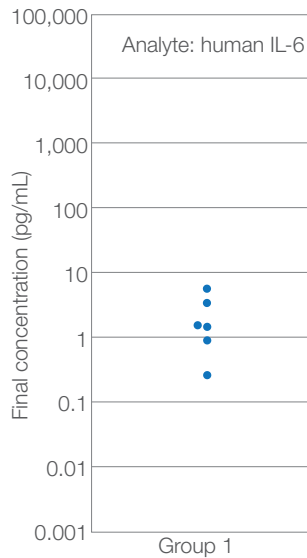


**Figure 6. Example of a 7-point standard curve with 5-fold dilutions of IL-6.** The curve shows a large assay range from 0.064 pg/mL to >1,000 pg/mL.



**Figure 7. Groupwise comparison of samples.** Unstimulated PBMC supernatant (blue) vs. stimulated supernatant (yellow) show large differences in IL-6 concentrations.

As expected, the concentrations of IL-6 in serum and plasma were very low (Figure 8). Using ELISA, IL-6 was not quantifiable within the range of the standard curve; however, the ProQuantum immunoassay is sensitive enough to determine the concentrations. The advantages of ProQuantum immunoassays are the large dynamic range to measure low through high concentrations, only 2  $\mu$ L of sample consumption, and a fast workflow.

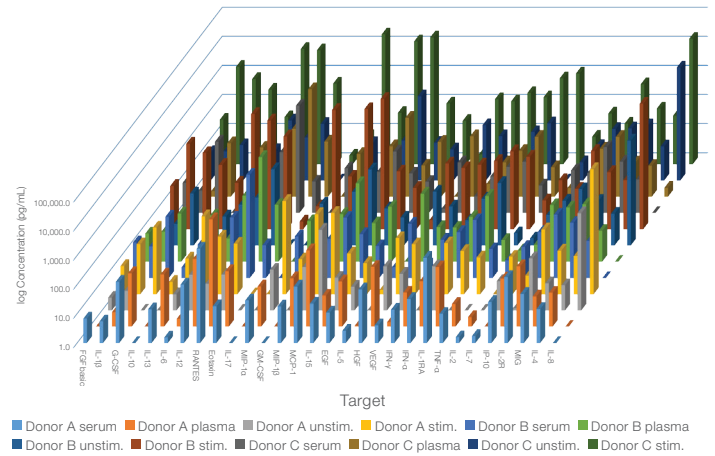


**Figure 8. Quantitation of concentrations of IL-6 in normal serum and plasma samples, using the ProQuantum Human IL-6 Immunoassay Kit.**

### Multiplex protein analysis using the Luminex platform

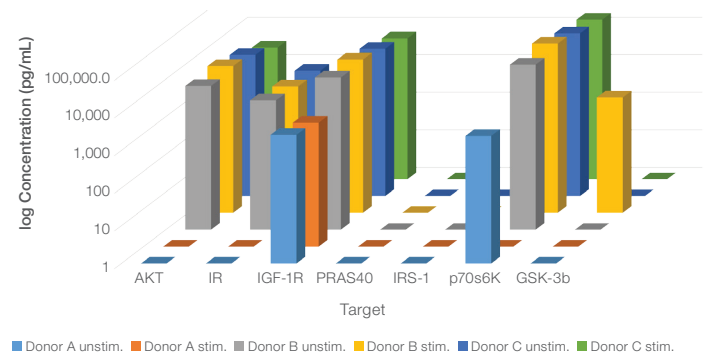
ELISAs and single-analyte quantitative assays like ProQuantum immunoassays can be a valuable tool for measuring one protein at a time, but when there are multiple proteins of interest, multiplex methods such as Luminex® xMAP® assays can save on cost, sample, and time. Multiplex analysis also provides a much broader, holistic view than what would be expected from a single-protein experiment. Luminex assays use differentially dyed beads, with each specific bead type conjugated with specific capture antibodies. Detector antibodies are used like in sandwich ELISA to detect the protein of interest. The distinction of the Luminex platform is that there are multiple specific bead-conjugated antibodies in suspension within a plate well. The assays require specific instrumentation to select and detect the beads as well as their concentrations.

In this experiment, the Invitrogen™ Human Cytokine Magnetic 30-Plex Panel (Cat. No. LHC6003M) was used to measure secreted proteins in plasma, serum, and cell culture supernatant. Like the ELISA and ProQuantum immunoassay data for IL-6, it was expected that most soluble cytokines would be low for normal plasma and serum but would be expressed at higher concentrations when the PBMCs were stimulated with LPS (Figure 9).



**Figure 9. Multiplex analysis using the Human Cytokine Magnetic 30-Plex Panel.** This plot shows the extensive amount of data that can be obtained with a multiplex assay. Like the IL-6 ELISA, protein measurements are derived through the use of standard curves, and the resulting concentrations are displayed. Most cytokine levels were low, but some markers had a very strong response to the LPS stimulation, such as IL-8, IL-6, MCP-1, MIP-1 $\beta$ , and IL-12.

Additionally, the PBMC lysates were analyzed using the Invitrogen™ AKT Pathway (Total) 7-Plex Multispecies Panel (Cat. No. LHO0002M). To demonstrate the versatility of the Luminex platform, an intracellular assay was performed to show how key pathway markers can be quantitated in cell lysates (Figure 10).



**Figure 10. Intracellular assay using the AKT Pathway (Total) 7-Plex Multispecies Panel.** Several markers showed a positive result (AKT, IR, IGF-1R, p70s6K). As expected, differences observed between the LPS-stimulated and unstimulated PBMC lysates were minimal.

**Table 1. Advantages of protein analysis platforms.**

Researcher need	Western blot	ELISA	ProQuantum immunoassay	Luminex assay
Large breadth of protein selections	•	•		
Protein band visualization	•			
Quantitation		•	•	•
Small sample consumption			•	•
Easy workflow			•	
Screening tool	•			•

## Conclusion

In this study, we have demonstrated how much data can be extracted from only three whole blood samples using common protein detection and quantification techniques, as well as how those results provide insight into a particular response that otherwise might be overlooked. Overall, the results between the various methods are in agreement. For example, the western analysis for intracellular AKT showed trends similar to those revealed in the AKT Luminex multiplex immunoassay, with minimal differences between treated and untreated groups. The human IL-6 results were similar in the ELISA, ProQuantum, and Luminex assays,

with low levels in normal serum and high levels in LPS-stimulated PBMCs. The consistency between the different platforms provides confidence in the expected results as well as options for researchers depending on the specific need (Table 1). The ability to measure extracellular soluble cytokines and intracellular proteins can provide researchers with a more holistic view of the immune response. With a wide variety of protein detection and quantitation tools available in the market, researchers are now better equipped than ever to make advances in proteomics.

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