Application Note

The Direct Detect™ Biomolecular Quantitation System

Exploits Membrane Technology to Enable Accurate, Infrared-based Detection.

Abstract

The Direct Detect™ system, an infrared (IR)-based spectrometry system, represents an innovation in biomolecular quantitation. The key to this advance lies in a new membrane technology for preparing and presenting aqueous biological samples to make them compatible with infrared analysis. Built upon EMD Millipore's extensive experience in membrane technology, it employs a hydrophilic polytetrafluoroethylene (PTFE) membrane that is designed to be transparent in most of the infrared spectral region and enables application of biomolecule solutions directly onto the membrane. The Direct Detect™ system has been optimized for detection and quantitation of proteins. By measuring amide bonds in protein chains, the system accurately determines an intrinsic component of every protein without relying on amino acid composition, dye binding properties or redox potential.

Introduction

Limitations of traditional methods for protein quantitation. Classical methods for protein quantitation rely on colorimetric assays, such as those involving protein-copper chelation (bicinchinonic acid (BCA) and Lowry assays) and dye-binding based detection (Bradford and "660 Assay") or ultraviolet/visible (UV-Vis) spectroscopy. Each of these methods has limitations. In colorimetric assays, protein concentration is determined by comparing signals from samples of unknown composition to signals from reference standards (composed of common proteins such as Bovine Serum Albumin (BSA)) which are prepared for every measurement. Standard curve determinations differ considerably from assay to assay, affecting reproducibility of protein concentration estimations.

The UV based method relies on absorbance at 280 nm by a protein's aromatic amino acids, predominantly tryptophan with minor contributions from tyrosine. Therefore, protein extinction coefficients can vary widely (greater than two-fold difference between extinction coefficients of albumin and immunoglobulin G). Those proteins that do not contain tryptophan, such as Protein A, cannot be quantified based on 280 nm absorbance.

Amino acid analysis delivers possibly the most accurate protein quantitation; however, it is expensive and slow if samples are sent to a third party for analysis. If amino acid analysis is performed in-house, it requires time-consuming sample manipulation and specialized equipment.

The Direct Detect™ system provides more universally applicable, less limited and faster protein quantitation that requires minimal amounts of often precious samples. Thanks to its new membrane technology for sample handling and presentation, the Direct Detect™ method is convenient, requires minimal sample preparation and can be used under diverse sample conditions.

EMD Millipore is a division of Merck KGaA, Darmstadt, Germany

How IR spectroscopy provides universal, accurate quantitation. IR spectroscopy is one of the oldest and most well established experimental techniques for the analysis of protein structure[1]. IR spectroscopy exploits the fact that molecules absorb radiation at specific frequencies characteristic of their structure.

Protein primary structure (common to all proteins) is formed by a long chain of amide (peptide) bonds. Each Amide bond is made up of chemical substructures that absorb IR radiation in nine areas of the spectrum (Table 1).

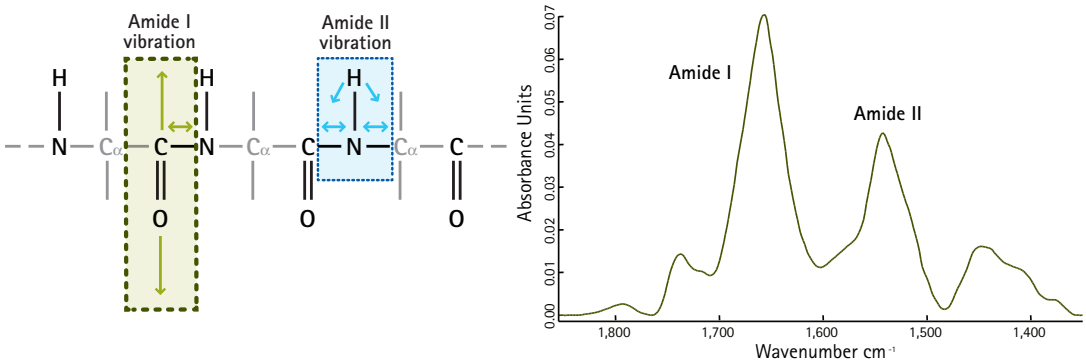
Table 1.
Characteristic infrared bands of the peptide bond.

Designation	Approximate Frequency (cm^{-1})	Description
Amide A	3,300	NH stretching
Amide B	3,100	NH stretching
Amide I	1,600 – 1,690	C=O stretching
Amide II	1,480 – 1,575	CN stretching, NH bending
Amide III	1,229 – 1,301	CN stretching, NH bending
Amide IV	625 – 767	OCN bending
Amide V	640 – 800	Out-of-plane NH bending
Amide VI	537 – 606	Out-of-plane C=O bending
Amide VII	200	Skeletal torsion

In order to determine protein concentration, the Direct Detect™ system calculates the intensity of the amide I band, about 80% of which is assigned to the C=O stretching vibration of the peptide bond, with a minor contribution from C-N stretching vibration (Figure 1)[2].

Amide bond quantitation is not subject to interference from many common buffer components such as detergents, reducing agents and chelators. As a result, the Direct Detect™ system can be used to measure protein concentrations from 0.2 mg/mL to 5 mg/mL within minutes, without any bio- or immuno-chemical staining, directly from samples, including buffered solutions.

Figure 1.
Vibrations responsible for the Amide I and Amide II bands in the infrared spectra of proteins.



Methods

Sample presentation. All concentration estimations were performed using the Direct Detect™ assay-free sample card (Cat. No. DDAC00010-8P) and the Direct Detect™ quantitation system (Cat. No. DDHW00010-00). The card contains four hydrophilic PTFE membrane positions, sized for easy sample application and surrounded by a hydrophobic ring to retain analyzed sample within the IR beam. All measurements were performed using 2 µL of sample solution per membrane position.

Instrument calibration. Sample concentration was determined in reference to a calibration method. The Direct Detect™ system requires a single-time standard curve generation after the instrument is installed. The system used in this study was calibrated using National Institute of Standards & Technology (NIST) -certified BSA SRM927d in phosphate-buffered saline (PBS). A series of ten concentration points from 0.125 mg/mL to 5 mg/mL was used to generate the instrument calibration curve.

Proteins used for quantitation. We assessed the performance of the Direct Detect™ system within the claimed dynamic range using pure protein solutions as well as protein mixtures. Pure protein solutions were prepared with lysozyme (Cat. No. 5990) solubilized in Milli Q® Water and protein A (RepliGen, Cat. No. 10-2001-00) in PBS. Protein mixtures consisted of BSA (Cat. No. 126609), cytochrome C (Cat. No. 250600), alcohol dehydrogenase (ADH), (Sigma-Aldrich, Cat. No. A8656), human transferrin (Cat. No. 616395), concanavalin A (Cat. No. 234567), lysozyme (Cat. No. 5990), ϒ-globulins from rabbit (Sigma-Aldrich, Cat. No. G2018) and protein A (RepliGen, Cat. No. 10-2001-00) in PBS.

For reference, the concentration of all protein solutions was determined by amino acid analysis. To obtain a 1 mg/mL solution, lysozyme sample (AAA determination at 68 mg/mL) was diluted with PBS at a 1 to 68 ratio. Protein A (AAA determination at 52 mg/mL) was diluted, also with PBS, 1 to 13 to obtain a 4 mg/mL sample. Protein mixture (AAA determination at 1.98 mg/mL) was used both at AAA estimated concentration and as a 1 to 8 dilution.

Effect of additives on protein quantitation. Potential interference from detergents and reducing agents was investigated using known concentrations of BSA in PBS (from 0.25 to 2 mg/mL) spiked with increasing concentrations of investigated additive. The influence of Tween® 20 and Triton® X on protein quantitation was tested within range of 0.1 - 5% while NP40 was analyzed up to 1% only. β-mercaptoethanol (BME) and DL-dithiothreitol (DTT) were analyzed up to 150 mM and 50 mM, respectively. Possible obstruction of protein quantitation due to the presence of sodium dodecyl sulfate (SDS) was analyzed using buffers containing up to 1% of the detergent.

Tissue lysate analysis. Rat liver lysate was prepared by homogenization of frozen rat liver in RIPA buffer containing phosphatase and protease inhibitor cocktails followed by a series of centrifugal spins that allowed collection of clear lysate solution. For Direct Detect™ analysis, lysates were diluted 40-fold with PBS, eliminating any probable buffer interference.

Results

Standard Curve Generation. The Direct Detect™ biomolecular quantitation system was calibrated using NIST-certified BSA. A series of ten dilutions (in triplicates) spanning the range from 0.125 mg/mL to 5 mg/mL was used to prepare a robust calibration curve (Figure 2).

The intensity of the amide signal delivered at each concentration point was fitted to a regression line represented by linear equation y = 0.01379x + 0.00267. The equation was further used by Direct Detect™ software to determine protein concentration in the analyzed samples.

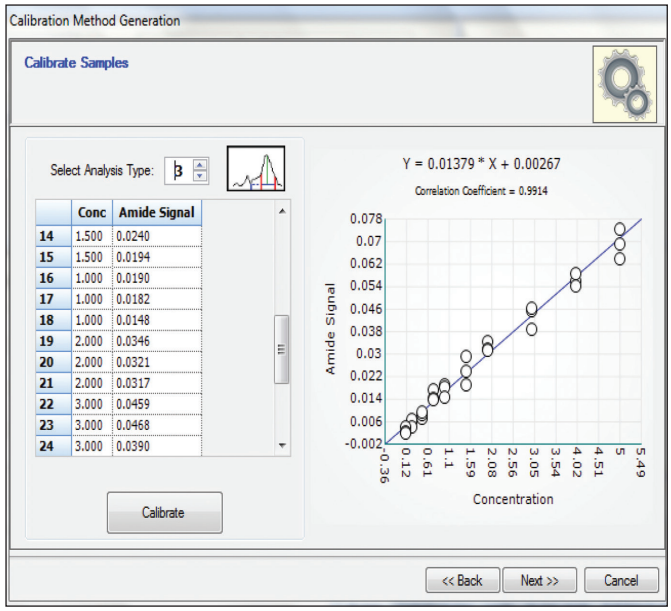


Figure 2.
Example of instrument calibration method generation.

Figure 3.

The Direct Detect™ system provides protein quantitation in agreement with amino acid analysis (AAA). Direct Detect™ concentration determination performed within system dynamic range and compared to reference method (AAA). Error bars represent standard deviations of triplicate sample spots (Direct Detect™) or readings (AAA).

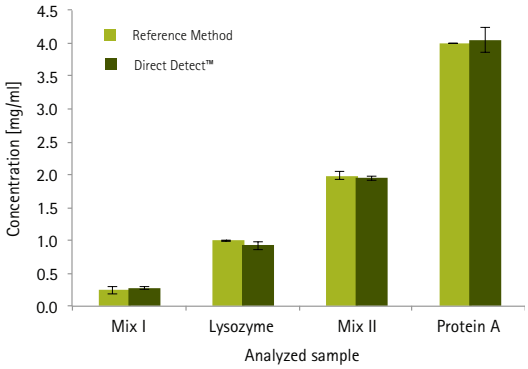
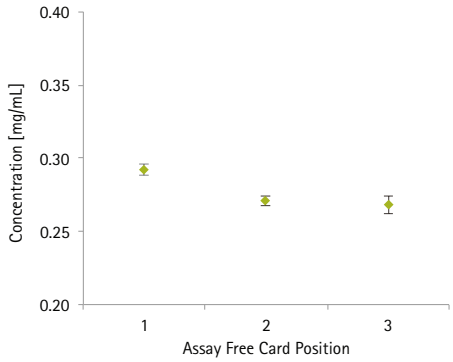


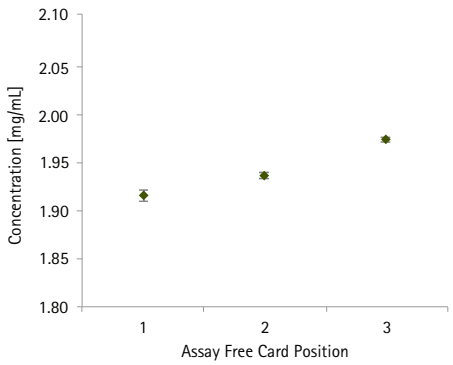
Figure 4.

The Direct Detect™ system delivers precise concentration analysis of protein mixtures at 0.25 mg/mL (A) and 1.98 mg/mL (B). Each data point is represented by four independent measurements. Error bars represent standard deviation.

A. Protein Mixtures at 0.25 mg/mL



B. Protein Mixtures at 1.98 mg/mL



Dynamic concentration range and instrument accuracy. The Direct Detect™ system is recommended for use with protein samples within the range 0.2 mg/mL - 5 mg/mL. Although the instrument can measure protein concentrations from 0.1 mg/mL to around 30 mg/mL, the most accurate results are achieved within the recommended concentration range.

Accuracy of concentration estimation within instrument's dynamic range was determined using single-protein solutions as well as a protein mixture. Results (see Figure 3) were compared to the data from AAA. The Direct Detect™ system estimated the concentration of the lysozyme sample at 0.922 ± 0.061 mg/mL and the protein A sample at a concentration of 4.047 ± 0.184 mg/mL. The Direct Detect™ system determined the concentrations of the protein mixtures at 0.273 ± 0.016 mg/mL and 1.944 ± 0.028 mg/mL, respectively.

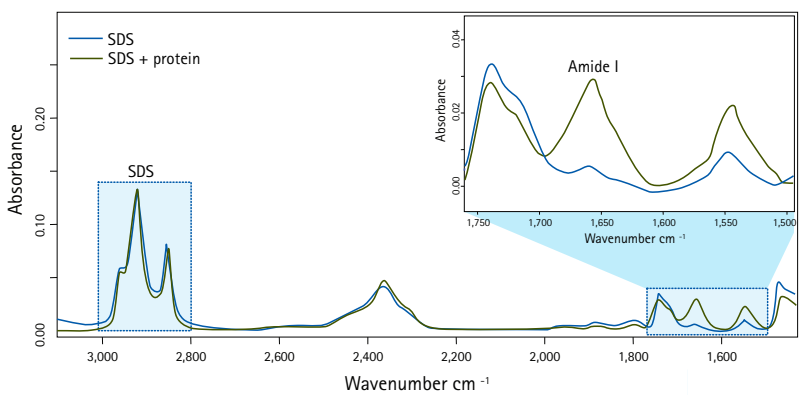
Reproducibility and precision. In contrast to other currently available methods for estimating protein concentration, Direct Detect™ enables the user to perform multiple measurements without additional sample requirement. The assay free sample card permits multiple analyses of the same sample that can be separated by hours or days.

Sample cards prepared using protein mixtures at 0.25 and 1.98 mg/mL, were analyzed multiple times at each position on the card (3 positions) to determine measurement reproducibility. Each card was measured four times and the concentrations obtained for each position as well as for an entire card were compared. The average concentration was 0.277 mg/mL with a CV = 4.9% for the first sample and 1.942 mg/mL with a CV = 1.5% for the second. Precision of the measurement for each card position is shown in Figure 4. For the 0.25 mg/mL sample, the coefficient of variation (CV) ranged from 1.23 to 2.34%. The measurement for the 1.98 mg/mL sample was even more precise, ranging from 0.1 to 0.3%.

Measurement in buffers containing detergents and reducing agents. Direct Detect™ accurately quantified protein in the presence of up to 50 mM DTT. Higher concentrations of the reducing agent were not investigated, because the majority of protocols describing use of DTT in biological samples preparation call for concentrations from 0.5 to 10 mM. Experiments analyzing possible interference of BME with protein quantitation showed no obstruction within the analyzed range (up to 150 mM). Infrared absorption of SDS (used up to 1%) does not overlap with the protein region (Figure 5), allowing unimpeded protein quantitation in the presence of this detergent. Our experiments also showed that Direct Detect™ quantitation retained its accuracy and precision in the presence of up to 5% of Tween® 20 and Triton® X (data not shown).

Figure 5.

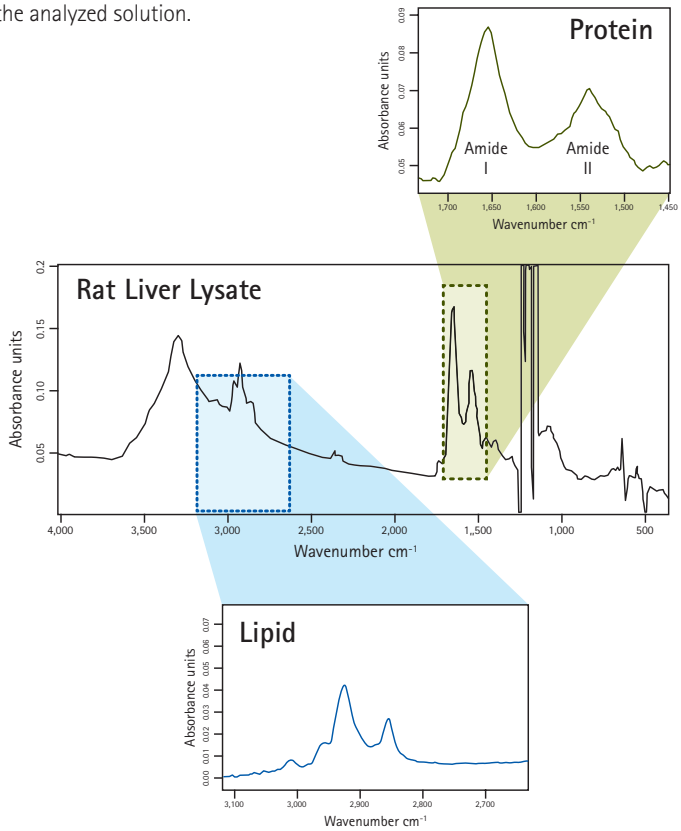
SDS does not interfere with Direct Detect™ quantitation. The IR spectrum of SDS alone (blue) does not contribute appreciably to the intensity of the Amide I peak in the IR spectrum of SDS + protein (green).



Analysis of non-protein components. Virtually every organic compound absorbs infrared radiation at frequencies that correspond in energy to stretching and bending of its functional groups. The resulting spectral profiles of many biochemical compounds are distinct, enabling the Direct Detect™ system to detect a broad range of biomolecules. The spectral range of the Direct Detect™ system is suitable for analysis of proteins, lipids, nucleic acids, carbohydrates and many other molecules. For example, examination of a complex cell lysate sample (Figure 6) not only provided accurate determination of protein concentration, but it also provided information on lipids present in the analyzed solution.

Figure 6.

Protein quantitation in presence of lipids within a complex cell lysate is possible because the most intense regions of lipid absorbance are spectrally distinct from the protein's Amide I and Amide II signals.



Conclusions

The Direct Detect™ biomolecular quantitation represents innovative protein quantitation by providing accurate, sequence-independent analysis that requires minimal amounts of analyte. System accuracy and precision are comparable with results obtained by amino acid analysis, placing the Direct Detect™ system among the most reliable protein quantitation tools available today. Because the system relies on IR-based detection of biomolecules, users can obtain accurate and reproducible protein quantitation in presence of reducing agents and detergents. The Direct Detect™ system also provides information on non-protein sample components such as lipids.

In addition to its analytical powers, the Direct Detect™ system provides a groundbreaking departure from traditional sample prep requirements involved in routine biomolecular quantitation. After samples are spotted, the Direct Detect™ assay-free cards can be stored in ambient conditions without appreciable change in readout. The Direct Detect™ calibration standard curve needs to be generated only once, providing additional time savings and ease of use.

Because accurate protein quantitation in complex mixtures has the potential to dramatically improve the reproducibility of biological and biochemical data, a streamlined, benchtop quantitation system such as Direct Detect™ is likely to be quickly integrated into the workflow of life scientists in virtually all research environments.

References

1. Kong, J., Yu, S., Fourier Transform Infrared Spectroscopic Analysis of Protein Secondary Structure. Acta Biochimica et Biophysica Sinica 2007; 39 (8): 549–559.
2. Jackson, M., Mantsch, H.H., The use and misuse of FTIR spectroscopy in the determination of protein structure. Critical Reviews in Biochemistry and Molecular Biology 1995, 30 (2): 95-120.

Ordering Information

Description	Qty	Catalogue No.
Direct Detect™ Assay-free Sample Card	1	DDAC00010-8P
Direct Detect™ Quantitation System	1	DDHW00010-00

Related Products

Description	Size	Catalogue No.
OmniPur® Lysozyme, Egg White	5GM 10GM 1KG	5990-OP*
Albumin, Bovine Serum, Fraction V, Fatty Acid-Free, Nuclease- and Protease-Free	5GM 10GM 100GM	126609
Cytochrome c, Equine Heart	1MG 100MG	250600
Transferrin, Apo-, Human Plasma	100MG	616395
Concanavalin A, *Canavalia ensiformis*	1GM 250MG	234567

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