Chimia 55 (2001) 40–42

ISSN 0009–4293

Nucleic Acid and Protein Quantitation in the Microplate Format

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Abstract: The quantitation of nucleic acids and protein are measurements common to many applications in basic science and clinical research. Several different assays for either total protein or nucleic acids that can be performed in microplates are described. In addition, the fundamental differences between conventional tube-based instrumentation and microplate readers and how these differences relate to the assays are discussed.

Keywords: Absorbance · Fluorescence · Microplates · Nucleic acids · Protein

The quantitation of nucleic acids and protein are measurements common to many applications in basic science and clinical research. These assays have used either absorbance or fluorescence as a means to measure the analyte in question. Regardless of the method employed, laboratories requiring high throughput have often adapted the described protocol to a 96- and more recently a 384-well microplate-based format. These standardized formats, in conjunction with instrumentation capable of recording measurements from them, allow for the rapid determination of large numbers of samples. With this in mind, we would like to describe several different assays for the quantitation of either total protein or nucleic acids that can be performed in microplates. In addition, the fundamental differences between conventional tube-based instrumentation and microplate readers, and how these differences relate to the assays, will be discussed.

There are fundamental differences between spectrophotometers, which utilize horizontal photometry, and microplate readers, which use vertical photometry. In horizontal photometry, the light path is 90° to the axis of the solution and, as such, the physical dimensions of the cuvette fixes the light pathlength of the sample. This light pathlength has been standardized to 1 cm, allowing easy comparison of data. As a result, the quantitation of compounds has been performed on the basis of constant values known as extinction coefficients, without standard curves. In vertical photometry, the light passes through the vertical axis of the absorbing solution. This results in a situation where the pathlength of the absorbing solution is dependent on the volume of the solution. In order for vertical photometric devices to be able to directly quantitate solutions, absorbance measurements need to be corrected to 1 cm.

Historically, microplate readers have been employed for the determination of colorimetric ELISA assays. These readers use a tungsten-halogen light source and band-pass filters to provide wavelength specificity. Recent advances in microplate absorbance reader technology have allowed microplate readers to be utilized on an equal basis with spectrophotometers. The use of diffraction-grating monochromaters in lieu of band-pass filters allows the user to select any wavelength within the spectrum of the reader rather than be limited to what filter(s) the reader contains. This also allows for spectral scans to be performed on samples. The replacement of the tungsten-halogen light source with a xenon-flash lamp allows for an expanded wavelength range. The tungsten-halogen lamp can be used effectively for wavelengths ranging from 340 nm to 800 nm, while xenon-flash lamps produce light over a much broader spectrum, allowing measurements from 200 nm to 1000 nm.

Pathlength correction in microplate wells takes advantage of the expanded wavelength range of the Bio-Tek microplate readers. Much like a dye solution, water has a small yet significant peak in absorbance at 977 nm [1]. Using this phenomenon, the pathlength of any aqueous solution in a microplate well can be determined by comparing the absorbance of that solution at a known pathlength (*e.g.* 1 cm) to the absorbance in the microplate well. The ratio of the microplate well absorbance determination to the 1-cm determination is equal to the pathlength in centimeters. The experimental wavelength absorbance divided by the pathlength results in absorbance corrected to 1 cm. The basis of this method is outlined by the Beer-Lambert Equation, which states:

$$OD = (\text{Extinction Coefficient}) \times (\text{Concentration}) \times (\text{Pathlength})$$

If the same solution is measured in a microplate and a fixed pathlength (*e.g.* a cuvette), then the differences in absorbance must be the result of differences in pathlength, as the extinction coefficient and concentration of the solution would be expected to be the same. This is valid when water is the only absorbing material in the near IR region of the spectrum. Fortunately, few aqueous buffer components have any significant absorption in this region.

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The quantitation of nucleic acids using absorbance can be accomplished by directly converting absorbance to concentration using extinction coefficients or by colorimetric assays. The direct quantitation of nucleic acids from UV absorbance is the *de facto* standard by which all other methods are judged. The physical properties of nucleic acid result in the absorption of light in the UV range (260 nm) of the light spectrum. This type of assay is very inexpensive and easy to perform, as it requires no other assay reagents and, with care, the measured samples can be reused for other purposes. The caveat with this method is its sensitivity (200 ng per well) and the requirement for UV transparent microplates. The formula for converting absorbance to nucleic acid concentration is 1 OD_{260} = 50 µg/ml for DNA; 40 µg/ml for RNA and 33.3 µg/ml for oligonucleotides [2]. These conversions are based on 1-cm pathlength absorbance values. The values obtained using a microplate would, of course, have to be corrected to 1 cm. The most commonly used colorimetric assay is that described by Burton *et al.* [3]. The assay employs a diphenylamine reaction with hydrolyzed DNA. As the assay is quite lengthy and uses corrosive agents, it is no longer used to any extent.

Protein, like nucleic acids, can be directly quantitated using UV absorbance (280 nm) or by colorimetric assay. The UV absorbance of specific proteins is a function of the amino acid content of the protein and, as a result, there can be protein-to-protein variability. An often used average conversion from absorbance to concentration is 1 OD (A_{280}) = 1 mg/ml solution. Purified proteins would each have a different extinction coefficient specific to that protein. This conversion is based on a 1cm pathlength, thus requiring pathlength correction when determined in microplates.

Total protein determination is most often performed using one of the several colorimetric assays available. The most frequently cited total protein assay is the method described by Lowry *et al.* [4]. This method is an improvement over the biuret reaction, but is sensitive to Tris buffer and reducing agent interference. The Lowry technique begins with the biuret reaction in which a protein in an alkaline medium containing Cu^{+2} forms a colored complex when copper ions are reduced to Cu^{+1}. The biuret reaction's sensitivity is then improved by the addition of Folin Ciocalteau reagent. The resultant color development is then measured at 750 nm. The method described by Bradford uses the color change at 595 nm that occurs when Coomassie Blue G-250 stain binds to peptides in an acidic medium [5]. This method is not affected by the presence of Tris buffer or reducing agents, but is sensitive to detergents and requires a disposable microplate, as it will stain quartz over time. The bicinchoninic acid (BCA) method was developed as an improvement to the Lowry assay. The BCA method has improved reagent stability and possesses a much broader tolerance to interfering compounds such as detergents and denaturing agents [6]. This assay is measured at a wavelength of 562 nm.

The need for improved sensitivity has driven the use of fluorescence as a means to quantitate nucleic acids and protein. Today's demand for 384-well microplates, with volumes of 100 µl, drives the need for assays with greater sensitivity. The geometry of microplates as compared to tubes results in fundamental differences in how fluorescence in microplate wells is measured. Tube-based fluorescence readers position the detector in the same plane, but at a 90° angle to the excitation source. Microplate readers are limited to having both the excitation source and the emission detector above or below the well. This is accomplished by the use of a mix of optic fibers that bifurcate to the excitation source and the detector.

There are several different fluorescent stains that target different species of nucleic acids, the basis of which is the creation or the enhancement of fluorescence of the stain when bound to nucleic acids. PicoGreen™ is a stain that is specific for dsDNA. The assay is linear for DNA concentrations from 0 to 1000 ng/ml with a detection level of 1–2 pg per well. This stain requires an excitation wavelength of 485 nm and fluoresces at 530 nm, shows little reactivity with RNA or ssDNA, and is commercially available from Molecular Probes (Eugene, Oregon). Another dsDNA specific stain is Hoechst dye 33258. This stain uses an excitation of 360 nm and has an emission of 460 nm [7]. While the detection limit of this stain is not as good as PicoGreen™ (5 ng/well), it is linear from 0 to 20 µg/ml and shows little reactivity towards RNA in the presence of high salt (2M NaCl). OliGreen™ stain can be used for the quantitation of ssDNA molecules, such as oligonucleotides, and is commercially available from Molecular Probes (Eugene, Oregon). While this stain has a sensitivity of 150 pg per well for oligonucleotides, it demonstrates significant fluorescence in the presence of dsDNA as well. Because it also stains RNA, OliGreen™ could be used to quantitate all nucleic acid species with the use of appropriate standards. However, OliGreen™ demonstrates a significant bias towards thymidine as compared to cytosine, making it important that the ssDNA source used for the standard curve be of a similar base composition as the ssDNA being measured. Regardless of the nucleic acid species, the excitation and emission wavelengths are 485 nm and 530 nm, respectively. RiboGreen™ is a fluorescent stain that preferentially binds RNA and fluoresces. It has a detection limit of 100 pg per well, but it will bind DNA as well, making it important that contaminating DNA be removed. This dye, also available from Molecular Probes, requires a 485-nm excitation filter and a 530-nm emission filter.

There are several compounds that can be used to quantitate protein using fluorescence. These compounds react with the specific amino acids to produce a fluorescent moiety. Fluorescamine, one of the first compounds developed, reacts with lysine to form a fluorescent moiety that has an excitation wavelength of 400 nm and emits at 460 nm. This compound is very water insoluble and needs to be added in an organic solvent such as acetonitrile [8]. The more watersoluble OPA, in the presence of 2-mercaptoethanol, reacts with protein to form a fluorescent moiety that absorbs at 360 nm and fluoresces at 460 nm [9]. CBQCA reacts with proteins to form a fluorescent compound with an excitation and emission wavelength of 460 nm and 545 nm respectively. CBQCA was developed to work in the presence of lipids making it appropriate for the determination of lipoproteins, but it requires cyanide as a cofactor [10]. NanoOrange™, which binds to proteins *via* SDS, is not amino acid specific, resulting in greater sensitivity and less protein-to-protein variability. However, NanoOrange™ requires that the protein be heat denatured, making the choice of microplate important as many polystyrene microplates warp when subjected to heat.

In this monograph we have described several different assays for nucleic acids and protein. In regard to absorbance measurements, direct quantitation of nucleic acids is most useful, while colorimetric assays are generally used to quantitate protein. Fluorescence detection has been used to provide increased sensitivity relative to absorbance-based measurements. In the case of nucleic acids, there

are different fluorescent stains available for different nucleic acid species. The detection of protein involves the reaction of a nonfluorescent compound with lysine to form a fluorescent moiety. For more information concerning these applications and others involving the use of microplates, please visit the Bio-Tek Instruments Web site at *www.biotek.com*.

Received: January 9, 2001

[1] E.L. McGown, J.W. Nelson, J. Williams, C. Fraser, *American Laboratory* **1997**, *29* (10), 21–24.
[2] T. Maniatis, E.F. Fritsch, J. Sambrook, 'Molecular Cloning A Laboratory Manual', Cold Spring Harbor Laboratory, Cold Springs Harbor, NY, **1982**.
[3] K. Burton, *Biochem. J.* **1965**, *62*, 315–322.
[4] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, *J. Biol. Chem.* **1951**, *193*, 265–275
[5] M.M. Bradford, *Anal. Biochem.* **1976**, *72*, 248–254.
[6] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, D.C. Klenk, *Anal. Biochem.* **1985**, *150*, 76–85.
[7] C. Labarca, K. Paigen, *Anal. Biochem.* **1980**, *102*, 344–352.
[8] S. Udenfriend, S. Stein, P. Böhlen, W. Dairman, W. Leimgruber, M. Weigele, *Science* **1972**, *178*, 871–872.
[9] J.R. Benson, P.E. Hare, *Proc. Nat. Acad. Sci. USA* **1975**, *72*, 619–622.
[10] W.W. You, R.P. Haugland, D.K. Ryan, R.P. Haugland, *Anal. Biochem.* **1997**, *244*, 277–282.

Chimia 55 (2001) 42–45

ISSN 0009–4293

Bioanalytische Festphasenextraktion: Ein Klassiker in einem neuen, massgeschneiderten Gewand

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Bioanalytical solid-phase extraction: A classic in a new, tailor-made garment

Abstract: In bioanalytical HPLC, optimization of sample pretreatment should be directed towards improved selectivity, high sample through-put and total automation with simultaneous cost-reduction and improvment of the analytical quality. These requirements can be accomplished by integrating the extractive sample clean-up process (Solid-Phase Extraction; SPE) into the HPLC-system. For this purpose, tailor-made adsorbents (Restricted Access Materials, RAM; Molecular Imprinted Polymers, MIP) are used as precolumn packings.

Keywords: Molecular Imprinted Polymers (MIP) · Restricted Access Materials (RAM) · Solid-Phase Extraction (SPE)

Trotz ausgereifter Instrumentierung, intelligenter Software und hoher Selektivität sowie Effizienz der analytischen Trennsäulen erfüllt die HPLC-Analytik von niedermolekularen Komponenten in komplexen biologischen Matrices bislang nur in eingeschränktem Masse die Anforderungen, die an ein routinetaugliches Hochleistungsanalysenverfahren gestellt werden.

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Biologische Proben – insbesondere proteinhaltige, wie z.B. Plasma, Serum, Milch, Speichel, Fermenterbrühe, Zell- und Gewebshomogenatüberstand – müssen vor der analytischen Trennung überwiegend noch manuell oder teilautomatisiert durch Extraktion, Denaturierung, Dialyse, Ultrazentrifugation oder unspezifische Adsorption aufbereitet werden. Diese Vorgehensweise ist mit einem hohen Arbeits- und Kostenaufwand sowie mit komplexen und somit fehlerträchtigen Mehrschrittoperationen verbunden.

Ziele einer optimierten Probenaufbereitung sind daher gesteigerte Selektivität, hoher Probendurchsatz und vollständige Automation bei gleichzeitiger Kosteneinsparung und Verbesserung der analytischen Qualität. Diese Anforderungen können – wie nachfolgend aufgeführt – durch die Kopplung oder Integration der Probenaufbereitung an bzw. in ein Trennsystem erfüllt werden. Beispielsweise kann die klassische Festphasenextraktion (Solid Phase Extraction, SPE) über spezielle Roboter (at-line Modus) oder SPE-Automaten (on-line Kartuschen-Modus) an ein HPLC-System gekoppelt werden (Abb. 1) [1].

Nachteile dieser Kopplungstechnik sind die komplexe Instrumentierung und die Verwendung von Einmalkartuschen. Darüber hinaus kann es bei der Aufbereitung von proteinhaltigen Proben zu einer unspezifischen Adsorption oder Denaturierung von Proteinen und somit zu