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Poor Man's Dot-Blot Apparatus

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Dot-blot hybridization is a simple, fast and efficient method for the quantification of nucleic acids. I describe a protocol for the rapid construction of a functional dot-blot apparatus from a sealed-bottom pipet box, autoclave tape, a pipet tip and Parafilm® (American National Can, Greenwich, CT, USA). Assembly is as follows: (i) remove the lid from a pipet box; (ii) cover the top surface of the pipet box with autoclave tape except for the holes that are to be used during the blot; (iii) using a heated glass rod, puncture the side of the box, enlarging the hole to allow the snug fit of a 1-mL pipet tip; (iv) cut off the small end of a 1-mL pipet tip and insert the remaining piece into the newly formed hole; and (v) seal any remaining openings with Parafilm or tape.

Operation of the apparatus simply involves: (i) connecting a vacuum to the pipet tip on the side of the box; (ii) placing a pre-wetted piece of Whatman chromatography paper (Clifton, NJ, USA) over the top surface of the box; (iii) piercing small corresponding holes in the Whatman paper with a gel-loading pipet tip, thus allowing the vacuum to act directly on the membrane; (iv) placement of the membrane over the Whatman paper; and (v) slow and careful application of a nucleic acid solution using a gel-loading pipet tip. Sites for application of nucleic acids are characterized by slight indentations in the membrane.

Although crude, this dot-blot apparatus provides sensitive dot blotting comparable to commercial devices. With

careful use, the described apparatus adds little to nothing in background and provides quantitative, reproducible results. Although it does not provide the durability found in a commercial dot-blot apparatus, it does provide a quick, inexpensive and reliable alternative.

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Gradient-Based Automated Protein Folding

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There is an ever-increasing demand for the rapid characterization of small amounts of proteins. This is especially true for recombinant proteins because high-level expression often results in folding errors. Systematic examinations of folding pathways are time-consuming and therefore rarely performed. Smith et al. (6) address this problem with an HPLC-based system that makes folding analyses easier to perform, permits exploration of an array of refolding conditions and affords reproducibility at a preparative scale. In this method, continuous-flow injection into a narrow-bore, open-channel tube is used for both equilibrium and time-resolved kinetic folding experiments. With appropriate in-line detectors (UV, fluorescence, circular dichroism [CD]), it is possible to measure nearly simultaneously the changes in protein quaternary, tertiary and secondary structure in response to chemical denaturants and solvent additives. Experiments using this method consume approximately 1.0 mg/100-min run. The purpose of this paper is to introduce a gradient-based system for smaller-scale applications to obtain similar information. The setup described below requires only 10–20 µg protein, and the basic principle can be extended to even lower amounts for special purposes. The method thus serves to bridge the gap between preparative and micro-scale levels.

To this end, we fabricated a small, closed Plexiglas® container containing an inlet, an outlet and a magnetic stirrer (Figure 1), within which a gradient is produced in the solution under investigation. The outlet is a fused-silica capillary that can be connected to the detector of one's choice. A continuous flow of an appropriate solvent into the chamber changes the solution conditions. Although the concentration of the protein can be maintained constant by infusing it into the chamber along with the changed solvent, a more material-saving approach is simply to allow dilution as the chemical environment of the protein is changed. The concentra-

Benchmarks

tion change of the substance in the chamber is then a function of run time and is accessible by a mathematical procedure. This experimental principle has a history in studies of surfactants and detergents using flow calorimetry (5) and conductivity (3) to obtain enthalpies of micellization or critical micelle concentrations, and it is certainly applicable in other fields as well. A more general description of the mathematical background, which allows the change of mass of the solution inside the chamber (in the case of more open chambers), was given earlier (3). For the present study, the small, tightly closed container (Figure 1) allows calculation using a fixed amount of solution (neglecting density changes), leading to an exponential dependence of the concentration on the run time:

$$c(t) = m_{S0}/m_0 e^{(-vt/m_0)}$$

where $c(t)$ = concentration at time t (allowance made for dead time); v = pump rate; m_{S0} = initial mass of the substance

Figure 1. Plexiglas chamber for the formation of a time-dependent concentration gradient. It consists of two parts that are screwed together and sealed with an O-ring. The lower part contains the inlet opening and the magnetic stirrer. The upper part of the chamber has a conical shape to force unwanted air bubbles out through the top opening. The chamber has an approximate volume of 200 μ L. The inlet consists of a 0.5-mm optical density (OD) stainless steel tube accommodating the fused-silica capillary, which is in turn glued to Teflon[®] tubing, providing both a seal and a means of easy removal for cleaning. The outlet is threaded for a fingertight fitting.

in question; and m_0 = mass of the solution inside the chamber.

As one example, we have used the chamber to study the folding and unfolding of proteins using guanidinium hydrochloride as an unfolding agent as well as changing pH. The sample typically contains 0.1–0.2 g/L of protein in a solvent promoting either the native or the denatured state. The chamber is filled to capacity with this solution, identifying the initial conditions. The unfolding agent is infused by a syringe pump (KD Scientific, Boston, MA, USA), while inside the chamber, a magnetic stirrer ensures satisfactory mixing. The chamber itself is placed on a temperature-controlled surface (in our case, a flow chamber connected to a thermostat [Neslab Instruments, Newington, NH, USA]) because stirrers tend to become hot during use. The outlet is a fused-silica line connected directly to a low-volume variable-wavelength detector (VWD HP 1050; Hewlett-Packard, Wilmington, DE, USA; data recovery with Radio Shack Digital Multimeter with PC interface), although addition of other detectors in-line is possible.

Figure 2 shows the results obtained in a study of the unfolding of horse skeletal muscle myoglobin (ICN Biochemicals, Aurora, OH, USA) using acetic acid (Mallinckrodt Baker, Paris, KY, USA). In the case shown, the

chamber was filled with 175 mg of protein solution (1.6 g/L of myoglobin in 25 mM ammonium acetate [Advanced Biotechnologies, Columbia, MD, USA], HPLC-grade water [Fisher Scientific, Pittsburgh, PA, USA]) to avoid absorption changes due to the dilution of the protein. However, we have found that the experiment works as well using 0.1 g/L myoglobin solution if the gradient parameters (run time, dilution rate) are adjusted accordingly. The solution was continuously diluted with 0.5% acetic acid for up to 200 min using a pump rate of 5 μ L/min. Since protein absorption peaks generally undergo a small shift to lower wavelengths on unfolding due to their increasing access to the more polar surrounding medium, it is possible to monitor the absorbance at a single wavelength in a region of maximal slope to follow the process (4). In the case of myoglobin, the heme complicates the simple increase or decrease in absorption expected, which would in turn depend on which side of the absorption band was being monitored. At 410 nm, the absorption is mostly due to the heme (2,7), and as expected, a simple downward trend is observed simply because of dilution. The fact that it is not an exponential decrease shows that the pH change that accompanies dilution is affecting the protein structure as well. The structural effect is very

Figure 2. Acid-induced unfolding of horse skeletal muscle myoglobin at 280 and 410 nm. The change in time corresponds to a change in pH (pH analyzer 350; Corning Costar, Cambridge, MA, USA).

Benchmarks

clearly evident at 280 nm, where the influence of the heme is minimized. The acid-induced unfolding of myoglobin has been followed by mass spectrometry where the folded and unfolded states give rise to two different m/z envelopes as the heme is released (1,2). Other authors (2) have found that the unfolding of the protein is more rapid than the destruction of the heme-protein interactions. Our experiment supports this observation because, especially at 280 nm, the absorption curve indicates two processes.

Of course, one must always allow for the very slow folding that some proteins exhibit, but considering this and the limited equilibration time, the method should be a useful tool for systematic studies of folding pathways.

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