Automating a Linear Density Gradient for Purification of a Protein:Ligand Complex

Introduction

Proteins have a variety of cellular functions, structures, and mechanisms of action. Routinely, proteins bind other biomolecules, or ligands, in order to complete a task. Researchers gain valuable knowledge on how proteins work in a cellular environment by purification of proteins bound to their appropriate substrate. Typically, protein:ligand complexes are further analyzed by cryo-EM, NMR, and/or x-ray crystallography for structural information. However, purification of protein:ligand complexes remains challenging due to the lack of robust, reproducible separation techniques.

Linear (also known as continuous), rate-zonal density gradients are formed in several ways, but the process always starts with layering a discontinuous (also known as step) gradient first. In the most popular technique, an aliquot of a less dense solution is first pipetted into a centrifuge tube and successively denser solutions are introduced to the bottom of the tube by a long syringe as to not disturb the previous layer, leaving a sharp interface between the different density layers. Another approach layers decreasingly dense solutions gently on top of a more dense solution. In order to generate a continuous gradient from a discontinuous gradient, 3 main techniques are used: 1) incubating at 4° to 8°C for 16 hours or overnight; 2) spinning in a centrifuge for a set speed and duration; or 3) using a commercial gradient maker that spins the tube at a specific angle for a set speed and duration. In all techniques, the solution diffuses such that a gradual increase in density is produced from the top to the bottom of the tube. Both layering techniques are tedious and time-consuming and are often not reproducible among researchers, requiring practice and a whole lot of patience to generate strong interfaces between densities.

Here, we present a simplified, fourth method for gradient preparations. To eliminate user variability, Beckman Coulter's Biomek 4000 Workstation provides consistent and reproducible results in layering discontinuous density gradients (see Application Notes DS-18638A, IB-18433A, and CENT-447APP08.14-A). The Biomek 4000 Workstation offers ease of use and outstanding precision in liquid handling. Additionally, the Biomek 4000 Workstation is easily equipped with a cooling, static peltier Automated Labware Position (ALP) that provides an incubation platform on-deck for linear gradient formation overnight without the need for a refrigerator or cold room.

After centrifugation, gradients are typically fractionated by puncturing a hole at the bottom of a tube and collecting a specific number of drops per aliquot, or by manually pipetting from the meniscus. There are commercially available systems that are capable of automatically fractionating gradients, but these systems are typically expensive and not compatible with all tube types. Since the Biomek 4000 Workstation offers solutions to accurate liquid handling, the instrument was tested to determine whether or not it was also suitable to fractionate a sucrose gradient.

The ATPase of phi29 and DNA ligand

Double stranded (ds) DNA viruses package their genomic dsDNA into a pre-formed protein shell, called procapsid, during maturation.[1,2] This entropically unfavorable process is accomplished by a nanomotor which uses ATP as an energy source.[3-6] Bacteriophage phi29 is an extensively

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investigated phage due to its simplistic design, comprised of an ATPase packaging enzyme—deemed gp16—a connector portal protein (gp10), and packaging RNA (pRNA). Guo et al.[7] first proposed that the mechanism by which dsDNA is packaged into the procapsid resembles the action of other AAA+ (ATPases Associated with Diverse Cellular Activities) proteins using ATP as energy. Recently, it has been determined that gp16 utilizes a sequential action mechanism with dsDNA and ATP to accomplish packaging.[8] Furthermore, it was validated that gp16 exists as a hexamer—similar to other AAA+ proteins—on the viral packaging motor[9] and that cooperativity exists among the ATPase and ATP, generating a high affinity state for dsDNA after binding a non-hydrolyzable ATP substrate, γ-S-ATP.[10] A revolution mechanism for DNA packaging was proposed[10] and subsequently substantiated.[11-13] This motor is of particular interest as it has been extensively shown to be utilized in several applications of nanotechnology.[14-17]

In the study of this motor, it was critical to research how certain components interact with others in cellular conditions to better understand the phage's mechanism and biology. One such assay involved isolating the gp16/dsDNA complex by rate-zonal centrifugation. In a previous experiment, published in *Nucleic Acids Research*,[8] complexes were purified in a 5–20% sucrose gradient in a Beckman Coulter SW-55 rotor at 35,000 rpm and subjected to further kinetic analysis to determine rate of ATP hydrolysis. Additional experiments were also performed on the purified complex, gaining valuable information that allowed researchers to elucidate the mechanism of DNA packaging in phi29 phage maturation.

In the example to follow, purification of the gp16/dsDNA complex will be assayed by mimicking the previous published experiment but in a larger rotor and using an automated layering and fractionating method with the Biomek 4000 Workstation.

Methods

Sucrose was diluted at 5% and 20% (w/v) using a dilution buffer (50 mM NaCl, 25 mM Tris pH 8.0, 2% glycerol, 0.01% Tween-20, 2 mM MgCl2, 0.15 mM γ-S-ATP) and a gradient was made either manually by layering 5% solution on top of 20% solution with a pipette, or automatically using the Biomek 4000 Workstation. Solutions were incubated at 4°C overnight in a refrigerator or on the pre-cooled peltier ALP of the Biomek 4000 Workstation. In the Biomek 4000 Workstation method, 15 mL conical tubes containing either 5% or 20% sucrose solution in a holding rack were placed on-deck along with a pre-chilled static peltier holding up to six, 13.2 mL Beckman Coulter polypropylene centrifuge tubes (P/N 331372). The Biomek 4000 Workstation was first prompted to add 5.5 mL of 5% solution to the bottom of the centrifuge tube using a P1000SL tool in 916.6 µl aliquots. Next, the Biomek Workstation was asked to add 5.5 mL of 20% sucrose underneath the 5% solution at the bottom of the tube. Beckman Coulter Span-8 P1000 pre-sterile tips (P/N B01124) are long and cylindrical, causing minimal damage to the sucrose interface during this step. The peltier ALP was then set to hold temperature overnight, allowing the sucrose to generate a linear gradient.

Gp16 was re-engineered in 2009 to contain a fluorescent arm, enhanced green fluorescent protein (eGFP), which did not affect the protein's folding or activity[18] but provided an easily identifiable marker for *in vitro* and single molecule assays. Additionally, it has been shown that eGFP-gp16 binds non-specifically to dsDNA, and fluorescent tags—such as cy3—are easily conjugable for further identification. Ultrapurified 40 bp cy3-conjugated dsDNA was purchased from Integrated DNA Technologies (IDT) and resuspended in DEPC-H_2O. γ-S-ATP was purchased from Roche Diagnostics.

Samples were prepared by mixing eGFP-gp16, cy3-dsDNA, and γ-S-ATP in final concentrations of 1 µM, 250 nM, and 1.25 mM, respectively. Samples were subsequently gently added to the top of the gradient as not to disrupt the formed gradient, balanced, and then placed in a Beckman Coulter Optima XPN Ultracentrifuge and spun for 7 hours at 40,000 rpm at 4°C.

Samples were then either fractionated directly from the top of the tube using a pipette set at 250 µl into an opaque microplate, or using the Biomek 4000 Workstation

to liquid level track the meniscus and automatically transfer fractions of the same volume to the microplate sitting on the deck. In the Biomek 4000 Workstation method, a rack holding the spun centrifuge tubes was placed on-deck along with a black-bottom microplate. P1000 tips were used to fractionate 250 µl from the very top of the meniscus and added directly to consecutive wells of the 96-well microplate. Based on user-defined parameters for the geometry of the tube, the Biomek 4000 Workstation is capable of precisely tracking the liquid level of the tube as fractions are removed. The fractions were subsequently analyzed by a Molecular Devices SpectraMax® i3 Multi-Mode Detection Platform at both GFP and cy3 wavelengths (488 nm and 540 nm, respectively) and data was exported to a Microsoft® Excel® file for analysis. The data was then transferred into Origin Pro v9.0 for plotting.

Results

The data from the Molecular Devices SpectraMax® i3 microplate reader was plotted and overlaid for both wavelengths and methods in Figure 1. The direction of sedimentation is from left to right on the graphs as fractions were taken from the top of the tube. The black cy3 line represents cy3-DNA and the red GFP line denotes eGFP-gp16. In Figure 1a, the manual layering and manual fractionation technique was able to resolve free protein and DNA (fraction 2–6) from the protein-DNA complex (fractions 17–32). Using the Biomek 4000 Workstation for both layering and fractionation (Figure 1b), again the free protein and free DNA (fractions 3–6) was separated from the gp16/DNA complex (fractions 17–27). However, in the Biomek technique, two distinct peaks exist in the complex region, especially evident looking at the cy3 signal. Fractions 16–21 are clearly an independent peak from fractions 24–27, suggesting that 2 separate conformations or oligomeric state of the protein exist bound to DNA. In fact, this phenomenon has previously been discussed in a recent *Virology* paper[10] and is a profound finding that provides relevant information to the packaging mechanism. It is believed that gp16 first binds to dsDNA as a dimer and then assembles into a hexamer to complete the packaging function. It is hypothesized that fractions 16–21 represent the dimeric state, whereas fractions 24–27 consist of the hexamer.

The same data was plotted in Figure 2 but compared the 2 methods in individual graphs for each signal. The overall plot shape is very similar, suggesting that the automation method is robust and can replace the storied manual method. Again, it appears the resolution is greater for the Biomek 4000 Workstation method (red line), which can be attributed to better pipetting techniques and less physical movement after layering. The standard deviation is also comparable between both techniques, signifying reproducibility.

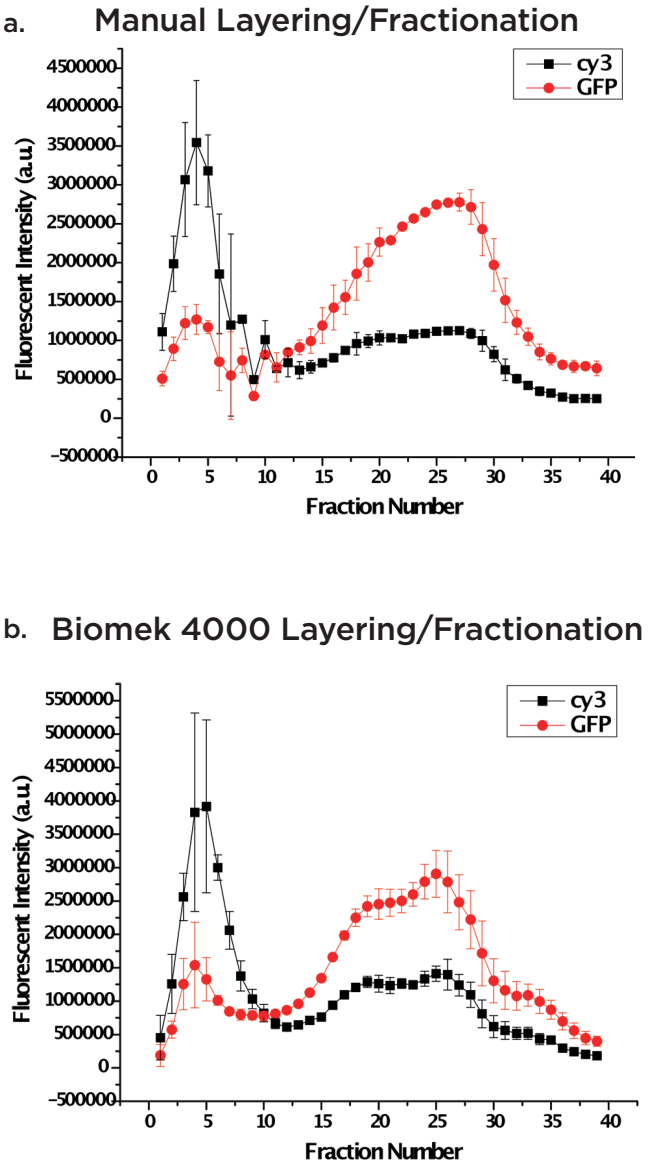
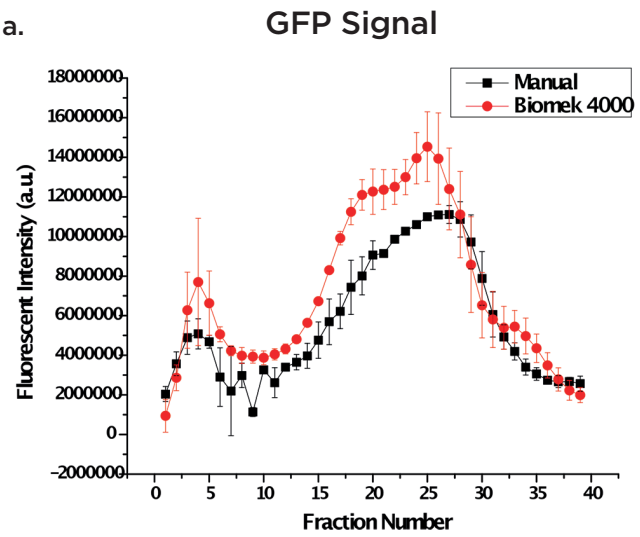


Fig. 1a and 1b. Manual versus Biomek 4000 Workstation preparation of a 5–20% linear sucrose gradient.

a.



b.

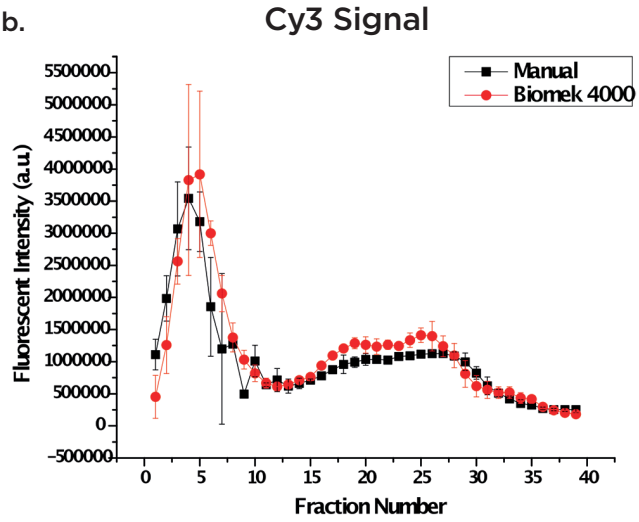


Fig. 2a and 2b. Overlaid images of different preparation techniques for eGFP-gp16 (a) and cy3-dsDNA (b).

Discussion

Purification of protein:ligand complexes are important to understanding biological processes. Often times, purified complexes are used in downstream analyses such as high-resolution imaging, sequencing, or crystallography for discovery of protein-based therapeutics. In the previous example, the purified gp16/dsDNA complex revealed the mechanism of a unique biological function known as DNA packaging in a bacteriophage. Lack of a robust, reproducible method for protein:ligand purification has hindered research for many years. Chromatography has several advantages; however, often times, the method requires large dilution factors, protein incompatibilities, and low resolution. Density gradient centrifugation allows the user to quickly modify parameters, offering efficient separations, and is governed by the laws of thermodynamics.

Here, we described an automated method for purifying protein:ligand complexes by rate-zonal centrifugation. It is important to note that this method is amenable to almost all proteins, after optimization of spin time, speed, and gradient conditions. The protocol offers significant advantages over manual preparations as the following outlines.

Reproducibility

Human error is common in an array of scientific experiments and compounded in difficult tasks, such as layering and fractionating a density gradient by different users in a lab group. Layering a gradient manually, using either the needle and syringe method or by pipette, requires a steady hand and patience. By automating the process, the Biomek 4000 Workstation provides a distinct interface and consistent fraction every time. No more worrying about adding samples to the same well twice or manually counting irregular drops out of the bottom of a centrifuge tube. Additionally, the automated approach for layering includes a chilled peltier step that reduces the jostling of tubes that occur when moving gradients to the cold room or refrigerator. This advantage is important as this movement often causes the interface to become turbid.

Ease of Use

It is diligent, tedious work to manually layer and fractionate a density gradient. Let the Biomek do the brunt of the work by just pressing a button.

Walk-Away Approach

The automated methods took just about the same amount of time to layer and fractionate a gradient than doing so manually. In fact, layering 2 gradients took less than 20 minutes and fractionating 2 tubes took less than 50 minutes. The difference is that a researcher can simply walk-away from the machine and perform other work during these methods. Lastly, the Biomek Automated Liquid Handling

product line is capable of being integrated with several types of downstream analysis equipment, including microplate readers such as Molecular Devices SpectraMax® i3 Multi-Mode Detection Platform. This allows researchers to focus on more important matters, such as data analysis and grant writing.

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