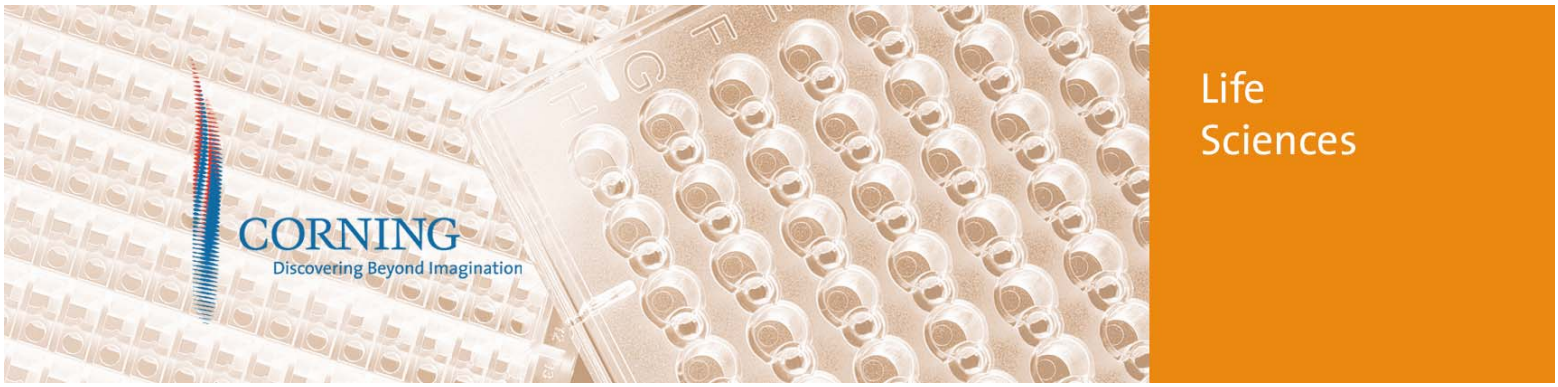
Automated High-Throughput Microdrop Crystallization Using Corning® Crystal*EX*™ 96 Well Protein Crystallization Microplates

Technical Note



Arezou Azarani
Protogen Consulting

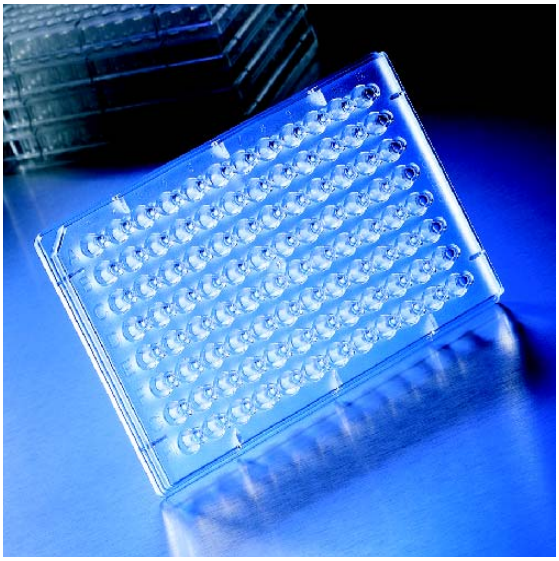


Figure 1. Corning Crystal*EX* 96 Well Protein Crystallization Microplate

Introduction

In this thriving proteomics era, X-ray crystallography provides important structural and biological insights into the nature and function of proteins, facilitating the design of more efficient clinical drugs. However, there are some major economic disadvantages associated with the setup of large-scale crystallization trials by the three most popular protein crystallization methods – sitting drop, hanging drop, and microbatch. These are:

a) lack of suitable automation for the many lengthy and labor-intensive setup steps,

b) irreproducibility due to manual intervention,

c) waste of precious and scarce protein caused by the absence of precise nanoliter dispensers and appropriate microplate technology, and

d) costs in both time and money (1, 2).

To address these limitations, a novel automated microdrop crystallization platform has been designed that is composed of an advanced high performance, 96 well, sitting-drop crystallization microplate (Figure 1) and a highly accurate nanodispenser robot. This microplate and dispenser technology facilitates automation and throughput by increasing reproducibility and accuracy while reducing time, sample consumption, and cost.

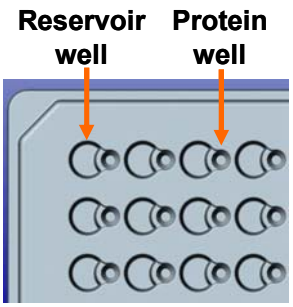


Figure 2. Each Crystal*EX* crystallization microplate has 96-round buffer reservoir wells and 96 conical flat bottom protein wells.

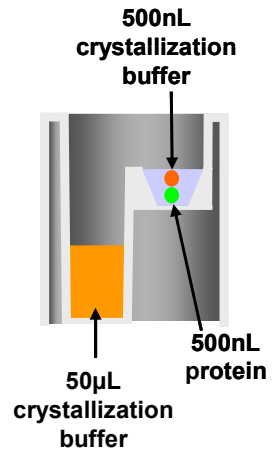


Figure 3. Crystallization by sitting drop vapor diffusion technology in a well from the 96 well Crystal*EX* microplate

The new Corning® Crystal*EX*™ 96 well protein crystallization microplate (Corning Cat. No. 3785), designed for automated high-throughput crystal screening by the sitting-drop vapor diffusion method, has 96 round buffer reservoirs, each with a corresponding conical 1.5mm diameter flat-bottomed protein well (Figure 2). The buffer reservoirs (filled with mother liquor) can contain up to 210µL of reagents while the protein wells have a maximum volume of 7.5µL (Figure 3). In vapor diffusion experiments, the starting concentration of the protein drop in the protein well is half the concentration of the mother liquor. The resulting diffusion of the solvent from the protein well gradually increases the protein concentration and thus drives protein crystallization (2, 3).

This new crystallization microplate, made of a proprietary polymer, is treated to have a hydrophilic surface to prevent static forces from separating the protein and the buffer drops in the protein wells upon dispensing. This protein-buffer separation problem, which is very pronounced in polystyrene and polypropylene plates, leads to protein denaturation at lower protein volumes (<1µL), thus increasing consumption of scarce protein samples and therefore leading to protein waste. In addition, an essential, difficult to automate centrifugation step, which is required to prevent the separation of the buffer and protein drops by static forces in polystyrene and polypropylene plates, is eliminated in these treated microplates. This is true even when the total volume of the drop in the protein well is as low as 1µL (500nL of protein plus 500nL of buffer). Another advantage of the treated polymer used in these microplates is its low water absorption characteristics that prevent very rapid volume changes in the protein drops and subsequent denaturing during the dispensing phase. Consequently, this new microplate reduces protein consumption significantly (nanoliters instead of microliters).

This microplate has a high chemical resistance toward the most commonly used crystallization buffers, such as DMSO, acetone, acids, alcohols, and ammonia. It also provides high optical clarity, with no background interference, enabling easy detection of protein crystals under polarized light. In addition, this microplate is designed with an SBS footprint, making it compatible with new automated imaging and liquid-handling equipment.

The Hydra®-Plus-One system is an automated high-throughput nanodispenser composed of the 96-channel Hydra-PP system, and the NanoFill® single-channel, noncontact, microsolenoid dispenser (4, 5). The Hydra system uses positive-displacement syringe technology that can accurately dry dispense volumes as low as 200nL, even when containing viscous crystallization buffers (up to 50% glycerol and 30% PEG 8000) with coefficients of variance (CVs) of less than 10% (5). The NanoFill microsolenoid dispensing system uses helium under positive pressure to dispense protein samples (up to 100 mg/ml) as low as 100nL with a precision variation of less than 10% (CVs of < 10%) (4, 5).

In this study we demonstrate an accurate, fast, and economical procedure to set up automated high-throughput microdrop crystallization by the sitting-drop vapor diffusion technology, using a new microplate technology and a nanoliter liquid dispenser.

Materials and Methods

The Crystal*EX*™ 96 well protein crystallization microplates (Cat. No. 3785) were provided by Corning Incorporated, Acton, MA. The Hydra®-Plus-One system (composed of the Hydra-PP dispenser equipped with 96, 100µL syringes with Teflon® coated stainless steel needles and the Nanofill® single-channel, noncontact microsolenoid dispenser) was purchased from Apogent Discoveries, Sunnyvale, CA.. Micro-90® soap (Cat. No. Z28, 156-5) and Coulter Clenz® detergent (Cat. No. 8546930) were purchased from Sigma-Aldrich, Milwaukee, WI, and Beckman Coulter, Fullerton, CA, respectively. Crystal Screens™ I, II, Index HT™, and ClearSeal Film™ were purchased from Hampton Research, Aliso Viejo, CA. The image-acquisition system, VersaScan™, was purchased from Velocity 11, Palo Alto, CA.

Protein Crystallization

Crystals of an unknown protein (unknown function, a generous gift by Lawrence Berkeley National Laboratories, Berkeley, CA) called protein 1139 were obtained by the sitting-drop vapor diffusion method (3) in 1µL drops (500nL of the protein and 500nL of the mother liquor in each protein well) suspended over 50µL buffer reservoirs at room temperature (Figure 3). The Hydra-PP part of the Hydra-Plus-One system was used for dispensing the mother liquor, first into the buffer reservoir and then into the protein well, while the NanoFill single-channel dispenser part of the system was used to dispense the protein into each of the 96 protein wells of the Crystal*EX* protein crystallization microplates (Figure 4).

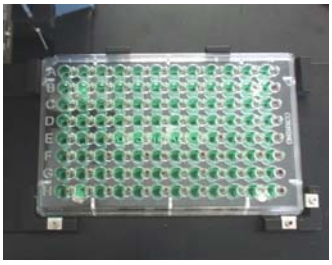


Figure 4. The 96 different crystallization buffers are simultaneously dispensed into the 96 well Crystal*EX* microplate using the Hydra system while the protein is dispensed with the NanoFill dispenser.

Preparing the Hydra-Plus-One System

The Hydra-PP component of the Hydra-Plus-One system is equipped with fixed needles. To prevent sample-to-sample carry-over, cross-contamination, and syringe clogging, efficient syringe-cleaning procedures have been developed (4, 6, and 7). In short, prior to the setup of experiments, the Hydra syringes were washed with water for six cycles ("one wash cycle" was defined as one filling and emptying of the syringes at full syringe volume). After the last use of the system for the day, the Hydra syringes were washed three times with Coulter Clenz (a detergent containing proteases) and then rinsed with water for an additional six wash cycles (5).

The microsolenoid dispenser component of the Hydra-Plus-One system was washed automatically with 1500µL of water through the execution of wash commands before experiment setup. An air gap (1µL) was then aspirated into the microsolenoid dispenser, followed by aspiration of the sample. The preceding wash step was repeated between aspirations of different protein samples. After the last use of the system for the day, the microsolenoid dispenser was washed with Micro-90 soap and then rinsed with water (through the execution of the software command called Daily Wash). The outside of the microsolenoid dispenser was rinsed and wiped clean (4, 5).

Both components of the Hydra-Plus-One system were used to set up the crystallization microplates. The crystallization buffers, which varied from one well to another, were dispensed using the Hydra-PP component of the system, while the protein was dispensed using the noncontact microsolenoid dispenser component. Using the Hydra syringes, 5µL of air followed by 51µL of mother liquors (from Crystal Screens I, II, and Index HT) were aspirated from the 96

deep well screen microplates. After a triple 500nL trial dispensing back into the deep-well screen microplate (to increase dispensing accuracy), 50µL of the crystallization buffer was dispensed into the buffer reservoirs of the Crystal*EX*™ microplates. This was followed by a 500nL dispense of the same buffer into the protein wells. After a triple 500nL trial dispensing back into the protein tube, 1µL of air followed by 50µL of protein was aspirated by the NanoFill® microsolenoid dispenser from a 0.5mL microtube. This was then dispensed (500nL per well) into the protein wells. Microplates were sealed (manually or automatically) using ClearSeal™ films and kept at room temperature. This approach eliminated the need for a centrifugation step.

Determining the Dispensing Precision of the Hydra®-Plus-One System

Prior to the use of the Hydra-Plus-One system, the dispensing uniformity and consistency of the Hydra-PP syringes and the single-channel microsolenoid dispenser were determined by measuring the CV's (detailed protocols reported in references 4 and 8). A high uniformity for dispensing volumes equal to and greater than 100nL was evident, with CVs of less than 10% for both components.

Results and Discussion

An innovative platform, composed of a new high-throughput 96-well sitting drop microplate technology and a highly precise, automated, nanoliter dispenser, was set up to perform large-scale microdrop protein crystallization. To determine the effectiveness of this new procedure in overcoming the limitations of current methods of crystallization parameters such as reproducibility, time and sample consumption, and cost factors were analyzed in depth.

Reproducibility in Setting Up Crystallization Trials

The Hydra-PP part of the Hydra-Plus-One system was used to dispense crystallization buffers (Crystal Screens™ I, II, Index HT™) and the NanoFill single channel microsolenoid dispenser component to dispense the protein 1139 sample into the Crystal*EX* microplates. Crystals were then successfully grown using the sitting-drop vapor diffusion technology. Dispensing volumes as low as 500nL of protein (10 mg/mL) and 500nL of crystallization buffers into the protein wells suspended over 50µL buffer reservoirs resulted in highly reproducible crystal formation (Figure 5). The protein crystallized after 48 hours of incubation (room temperature) in more than one condition. After a month of incubation, the drops remained well hydrated.

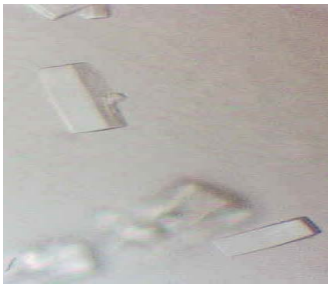


Figure 5. Crystals of an unknown protein (1139) grown by the sitting drop vapor diffusion method using Crystal*EX* microplates.

The conical design and the hydrophilic treatment of the Crystal*EX* microplate prevented static forces from separating the protein and the buffer drops in the protein wells upon dispensing. Consequently, no centrifugation step was necessary to ensure mixing, allowing immediate sealing of the microplates, thus increasing throughput speed. In addition, the special polymer made the protein wells less permeable to aqueous solutions, therefore preventing decreases in the volume of the small protein drops (as low as 500nL) by keeping them hydrated longer. These microplate characteristics (as well as the speed of the nanodispenser; times are shown below in Table 1) allowed the dispensing of very low volumes of proteins, reducing protein consumption significantly without requiring use of expensive humidifying chambers.

Table 1. Setup time using the Hydra®-Plus-One system to prepare a 96 well Crystal*EX*™ microplate

Function Performed	Process Speed
Aspirating 96 crystallization buffers and simultaneously dispensing 50µL of samples, first into the buffer wells and then into the protein wells (500nL), using the Hydra-PP component of the Hydra-Plus-One system	20 seconds
Aspirating the protein sample from a microtube and dispensing the protein sample (500nL) into the 96 protein wells using the NanoFill® microsolenoid dispenser	58 seconds
Total setup time excluding the washes	2 minutes
Total syringe washing time	2 minutes
Total setup time including washes	4 minutes

The advantages associated with the use of the Hydra-Plus-One dispenser in setting up crystallization trials were:

a) its highly precise nanoliter-dispensing capabilities,
b) speed,
c) the ability to dispense samples without any wash requirements between dispenses of the same buffer or protein samples,
d) elimination of large dead volumes (in the milliliter range) associated with aspirating the protein from reservoirs or troughs (the protein is aspirated from a 0.5mL microtube by the microsolenoid dispenser) and therefore reducing protein waste, and
e) full protein recovery from the single-channel microsolenoid dispenser.

The protein waste volume associated with this experiment was 500nL or approximately 1%. In addition, the use of fixed syringes, instead of disposable tips in the Hydra-PP component of the Hydra-Plus-One system was a more cost-efficient (no need to purchase expensive tips) (9) and environmentally friendly (less waste) method of dispensing. Precision glass syringes are robust, inexpensive, easily replaceable, and simple to use. After the samples have been aspirated into the syringes or the microsolenoid dispenser, they do not evaporate or dry up.

Plate Processing Time

The dispensing speed of the Hydra-PP component of the Hydra-Plus-One system for dispensing 96 crystallization buffers simultaneously into the buffer and protein wells was approximately 20 seconds. The dispensing speed of the NanoFill microsolenoid dispenser for dispensing 96 protein drops was 58 seconds. The total time for setting up a crystallization microplate of 96 samples was 2 minutes excluding syringe washes. The syringe washing process can be carried out separately after the sample microplates are sealed during the changing of the protein and the crystallization buffer microplates (Table 1).

The throughput of the system allowed the setup of thirty 96 well microplates per hour with no washes or fifteen 96 well microplates per hour with washes. In comparison, the traditional manual setup procedure allows the preparation of 2 to 3 microplates per day. The speed of this system therefore prevented sample

evaporation and facilitated the use of very low volumes of protein samples, thereby preventing protein waste.

Conclusion

The new technology provided by the Crystal*EX*™ microplates, together with the nanoliter-dispensing capabilities of the Hydra®-Plus-One system, allowed the generation of automated large-scale protein crystallization experiments in the microliter range. This new platform enabled precise and reliable high-throughput protein crystallization in hours significantly reducing time, protein consumption, and cost.

References

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