Application Note

Fully automated sitting drop protein crystallization with CyBi®-CrystalCreator using JBScreen Basic HTS Kit

Janet Kenklies, PhD and Ria Sachse, CyBio AG; Astrid Rau, PhD, Jena Bioscience

Abstract: Various proteins have been successfully crystallized with CyBio's CyBi®-CrystalCreator using the JBScreen Basic HTS Kit from Jena Bioscience. The high precision of the automated system and reliable choice of crystallization solutions were reflected in a very high rate of crystallization hits. The intuitive graphical software and the ready made buffer solutions facilitated a straightforward and extremely fast setup of the crystallization experiment.

Introduction: Knowledge of the three-dimensional structure of biological macromolecules can reveal essential information about their function, offering a unique opportunity to facilitate drug discovery. The most common experimental method of gaining a detailed picture of a protein, allowing the resolution of individual atoms, is single-crystal X-ray crystallography. Growing high-quality protein crystals is still a major bottleneck and very time consuming step in this process.

This note describes screening experiments using the fully automated CyBi®-CrystalCreator (Figure 1) in combination with a commercially available crystallization screen from Jena Bioscience. Since the composition of successful crystallization conditions cannot be predicted, it is usually necessary to perform a very large number of crystallization experiments.

Nucleation and crystal growth are influenced by the interaction of many variables, such as temperature, pH, precipitant and salt concentration. Testing all possible combinations would be prohibitively time consuming, and would require enormous amounts of sample. Thus, products of the JBScreen family are designed for efficient sampling of the crystallization parameter space, and the automation of this process is a prerequisite for high-throughput crystallization of biological macromolecules.

Figure 1: CyBi®-CrystalCreator, consisting of a 96-channel and an 8-channel automated pipettor mounted onto a 10-position deck equipped with an active tip wash station (left). The intuitive Graphical User Interface, which enables easy access to predefined programs (right).

E-mail: productinfo@cybio-ag.com www.cybio-ag.com

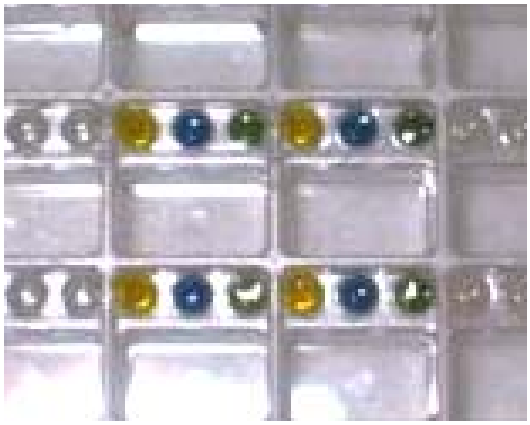
Application Note

Materials and Methods: The following proteins were diluted to a concentration of 10 mg/mL without further purification: Insulin, Alcohol Dehydrogenase, Phospholipase A2, Trypsin and Lysozyme (all Sigma-Aldrich Chemie GmbH). The experiments were conducted using the vapour-diffusion technique on 96 well CrystalQuick™ round bottom plates (Greiner bio-one, # 609120, 96 x 3 protein wells) sealed with transparent tape. This plate contains 96 reservoir wells and 96 times 3 proteins wells (Figure 2, left), which affords screening of 3 different proteins with 96 conditions each with one plate.

The screening for initial crystallization conditions was performed with the JBScreen Basic HTS Kit (Jena Bioscience # CS-203). The 96 unique reagents of this kit were supplied in a pre-filled deep well block and represent the most frequently used crystallization conditions based on the publication by Jancaric and Kim (*J. Appl. Cryst.*, 1991, 24, 409 – 411).

The crystallization protocol was set up on the CyBi®-CrystalCreator (Figure 1, left) composed of a 96- and an 8-channel pipettor, both with volume ranges of 0.1 to 25 µL. The pipettors are mounted onto a 10 position deck equipped with an active tip wash station. The CyBio system is operated through a Graphical User Interface (Figure 1, right). The precision data of both pipettors were determined by absorbance measurement at 405 nm with p-nitrophenol, which was dissolved in DMSO. Therefore 0.1 µL DMSO was pipetted into 96-well plates containing 100 µL 0.1 M NaOH.

The automated crystallization procedure comprises transfer of 96 crystallization solutions (100 µL) from a 96-well deep well block into 96 reservoir wells of the crystallization plate, followed by transfer of 3 proteins (0.1 µL) from PCR tubes (0.2 mL, Eppendorf) into the protein wells of the crystallization plate. Subsequently, 0.1 µL of crystallization solution in the reservoir well is transferred into the protein wells, where the protein drops are sitting. Plates were incubated at 4°C or room temperature and observed after 24 hours under a stereo-microscope.



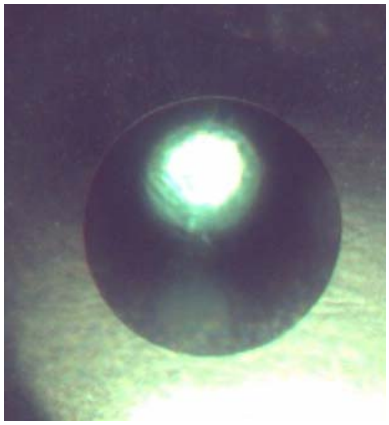


Figure 2: Section of crystallization plate, which shows square reservoir wells with 3 associated round protein wells. The plate contains 96 reservoir wells and 96 x 3 protein wells (left). A well-shaped drop, which was formed by pipetting 0.1 µL of colored liquid with the 8-channel, and subsequently with the 96-channel pipettor, onto the same position of a plain bottom protein well of a crystallization plate (right).

E-mail: productinfo@cybio-ag.com www.cybio-ag.com

Application Note

Automated Crystallization Procedure:

1. Add 4 times 25 µL crystallization solution from a 96-well deep well block into the reservoir well of the crystallization plate using the 96-channel pipettor.
2. Pick up new tips on the 8-channel pipettor. Transfer 0.1 µL of protein 1 from a row of the PCR tubes into the the first set of 96 protein wells of the crystallization plate
3. Transfer 0.1 µL buffer from the reservoir well into the protein well of the crystallization plate, where the protein drop is sitting, using the 96-channel pipettor. Wash tips using the active tip wash station.
4. Repeat steps 2 and 3 with proteins 2 and 3.

Results: All five proteins which were applied in the crystallization experiment yielded either micro crystals or well-shaped, small crystals. Each protein crystallized in at least two of the 192 different crystallization conditions (96 crystallization solutions and 2 temperatures). One protein (lysozyme) showed hits in 18 different conditions. The number of crystallization hits for each protein is summarized in Table 1. The formulations of the crystallization solutions which produced crystals are listed in Table 2.

The crystallization experiment was accomplished successfully using a very low volume of 0.1 µL. In order to show the reliability of the instrument at 0.1 µL, we determined the precision of the 8-channel and the 96-channel automated pipettors. The CVs of both pipettors were less than 10%. Furthermore, Figure 2 (right) shows that pipetting subsequently 0.1 µL colored liquid with the two different pipettors onto the same position forms a well-shaped drop. That indicates that both pipettors placed liquid at exactly matching positions.

The run time for setup of one crystallization plate with 3 different proteins, consisting of automated transfer of protein from tubes into the protein wells, and subsequent transfer of 0.1 µL crystallization solution from reservoir wells into protein wells, was only 4 minutes.

Table 1: Number of hits (micro crystals and well shaped small crystals) obtained from crystallization experiment (0.1 µL protein with 10 mg/mL concentration)

Protein	***Number of Hits in 96 Conditions***	
	Room Temperature	4 °C
Insulin	7	4
Alcohol Dehydrogenase	7	2
Phospholipase A2	0	2
Trypsin	1	1
Lysozyme	1	17

Discussion: All selected proteins were successfully crystallized within the first trial using the CyBi®-CrystalCreator in combination with the JBScreen Basic HTS Kit. Both, the reagent selection and the software-controlled robotic system with its Graphical User Interface (Figure 1) enabled easy and very efficient walk-away automation of the crystallization experiment.

The Graphical User Interface allows access to predefined crystallization protocols, which are optimized for precise pipetting at very low volumes. The application of this optimized pipetting protocol resulted in immediate success in the crystallization experiment.

E-mail: productinfo@cybio-ag.com www.cybio-ag.com

Application Note

Due to the high precision of the CyBi®-CrystalCreator, reliable results can be obtained utilizing very low protein volumes of only 0.1 µL per condition. This minimizes consumption of the valuable protein samples 20-fold compared to manual pipetting of typically 2 µL of protein per condition.

Both pipettors are closely related in terms of construction, which enables similar pipetting results regarding not only precision, but also pipetting at exactly matching positions. This produces well-shaped drops (Figure 2, right), thus facilitating optimal crystallization conditions.

The automated crystallization procedure is very fast. All steps, which are time-critical in terms of evaporation, are accomplished within 4 minutes. This high processing speed helps to avoid evaporation problems, thus leading to successful crystallization experiments.

E-mail: productinfo@cybio-ag.com www.cybio-ag.com

Application Note

Table 2: Formulation of crystallization solutions which produced crystals

Protein	Temp	Well	Precipitant 1	Precipitant 2	Buffer	pH	Additive
Insulin	RT	B9	8 % w/v PEG 4000	None	None		None
		D8	15 % w/v PEG 4000	None	100 mM Sodium Citrate	5.6	200 mM Ammonium Sulfate
		F6	25 % w/v PEG 6000	None	100 mM HEPES Sodium Salt	7.5	100 mM Lithium Cloride
		G5	10 % w/v PEG 8000	10 % w/v Ethylene Glycol	100 mM HEPES Sodium Salt	7.5	None
		G6	12 % w/v PEG 8000	10 % w/v Glycerol	None		500 mM Potassium Cloride
		G7	15 % w/v PEG 8000	None	None		200 mM Ammonium Sulfate
		H11	17 % w/v PEG 20000	None	100 mM Tris-HCl	8.5	100 mM Magnesium Chloride
	4°C	F7	28 % w/v PEG 6000	0.5 M Lithium Chloride	100 mM Tris-HCl	8.5	None
		G6	12 % w/v PEG 8000	10 % w/v Glycerol	None	7.5	500 mM Potassium Cloride
		G7	15 % w/v PEG 8000	None	None		200 mM Ammonium Sulfate
		G10	18 % w/v PEG 8000	None	100 mM HEPES Sodium Salt	7.5	200 mM Calcium Acetate
ADH	RT	E11	30 % w/v PEG 5000 MME	None	100 mM MES Sodium Salt	6.5	200 mM Ammonium Sulfate
		G9	15 % w/v PEG 8000	None	100 mM MES Sodium Salt	6.5	200 mM Sodium Acetate
		H9	30 % w/v PEG 10000	None	100 mM Tris-HCl	8.5	None
		H10	10 % w/v PEG 20000	None	100 mM MES Sodium Salt	6.5	None
		H11	17 % w/v PEG 20000	None	100 mM Tris-HCl	8.5	100 mM Magnesium Chloride
		H12	20 % w/v PEG 20000	None	None		None
	4°C	H11	17 % w/v PEG 20000	None	100 mM Tris-HCl	8.5	100 mM Magnesium Chloride
		H12	20 % w/v PEG 20000	None	None		None
PLP	4°C	H9	30 % w/v PEG 10000	None	100 mM Tris-HCl	8.5	None
		H12	20 % w/v PEG 20000	None	None		None
Trypsin	RT	H12	20 % w/v PEG 20000	None	None		None
	4°C	H9	30 % w/v PEG 10000	None	100 mM Tris-HCl	8.5	None
Lysozyme	RT	C12	30 % w/v PEG 4000	None	100 mM Tris-HCl	8.5	200 mM Sodium Acetate
	4°C	B11	10 % w/v PEG 4000	None	100 mM MES Sodium Salt	6.5	200 mM Magnesium Chloride
		C3	18 % w/v PEG 4000	None	100 mM Sodium Acetate	4.6	None
		C6	25 % w/v PEG 4000	None	100 mM Sodium Acetate	4.6	None
		C7	25 % w/v PEG 4000	None	100 mM MES Sodium Salt	6.5	200 mM Magnesium Chloride
		C8	25 % w/v PEG 4000	None	100 mM Tris-HCl	8.5	200 mM Calcium Chloride
		C10	30 % w/v PEG 4000	None	100 mM Sodium Acetate	4.6	100 mM Magnesium Chloride
		D3	8 % w/v PEG 4000	Lithium Chloride	100 mM Tris-HCl	8.5	None
		D8	15 % w/v PEG 4000	None	100 mM Sodium Citrate	5.6	200 mM Ammonium Sulfate
		D9	16 % w/v PEG 4000	10 % w/v 2-Propanol	100 mM HEPES Sodium Salt	7.5	200 mM Ammonium Sulfate
		D10	20 % w/v PEG 4000	None	None		200 mM Ammonium Sulfate
		F7	28 % w/v PEG 6000	0.5 M Lithium Chloride	100 mM Tris-HCl	8.5	None
		G5	10 % w/v PEG 8000	10 % w/v Ethylene Glycol	100 mM HEPES Sodium Salt	7.5	None
		H6	14 % w/v PEG 10000	None	100 mM Imidazole-HCl	8.0	None
		H7	18 % w/v PEG 10000	20 % w/v Glycerol	100 mM Tris-HCl	8.5	100 Mm Sodium Chloride
		H8	20 % w/v PEG 10000	None	100 mM HEPES Sodium Salt	7.5	None
		H9	30 % w/v PEG 10000	None	100 mM Tris-HCl	8.5	None
		H10	10 % w/v PEG 20000	None	100 mM MES Sodium Salt	6.5	None