HT SEEDING OF PROTEIN CRYSTALS

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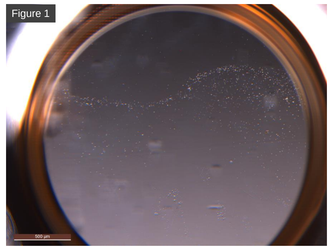
ABSTRACT

To explore methods of optimizing crystal growth, we manipulated salt, protein, and seed concentrations in seeded and non-seeded droplets. All drops presented no crystal nuclei and were treated with seed solution such that each successive column received less seed than the previous. Recording observations after specific timed increments, we analyzed and measured properties of the crystals such as size and abundance. After evaluating the images, we could conclude that seeding is the most prominent factor in influencing the crystal size for proteinase K.

Figure 1



INTRODUCTION

Protein concentration, precipitant concentration, and nucleation influence the growth of proteinase K crystals. Increasing the concentration of protein shifts the equilibrium in favor of crystal formation. Following Le Chatlier's principle, the equilibrium favors the precipitation of solids as the concentration of soluble material (proteinase K) is increased [1]. The solvent shell surrounding the protein is removed as precipitant (ammonium sulfate) draws water molecules from the protein. In traditional optimizations of crystallization conditions an ideal concentration of stock solutions and protein are tested to produce the best crystal [2]. Here we demonstrate that the condition that produces the best crystal is not necessarily determined by the concentration of components, but also the controlled introduction of nuclei. In fact, the condition which produces the best result otherwise may not have grown.

Figure 2

Figure 3

In a previous time trial experiment (Figures 2 and 3) we determined that the optimal condition for large single crystals occurs with 50mg/ml Proteinase K and 0.4 M ammonium sulfate. Over a period of 24 hours our largest crystal grew. Small crystals appeared within minutes. However, when attempting to reproduce the 50mg/ml and 0.4M ammonium sulfate condition, only several crystals grew in a setup of 96 identical drops.

The inconsistent behavior of the sample led us to visit the idea that a condition that works best may also need the introduction of or induction of nucleation. We chose to introduce nuclei through manual seeding, as described by Hampton [3]. In Figure 1, we observed crystal appearing in dilute protein and ammonium sulfate. This casual test inspired the automation of the process. We designed a platform which makes possible a high-throughput attempt to seed any sample using the following stocks:

- 40 uL protein stock at same concentration as the original crystallization experiment (50 mg/mL proteinase K here)
- 10 mL of the crystallization condition at the concentrations reported in a scoring sheet (0.4 M ammonium sulfate here)
- Original crystals to crush and use for seed material
- Water

METHODS

We began by weighing 10.0 mg of lyophilized proteinase K (VWR 97062-238) into 200 uL of water forming a 50 mg/mL solution. Each drop in rows E-H received 150 nL of the 50 mg/mL protein solution, and 150 nL of 0.4 M, 0.3 M, 0.2 M, 0.1 M ammonium sulfate respectively. We filled rows A-D with 0.2 M ammonium sulfate and a 50 mg/mL, 40 mg/mL, 30 mg/mL, and 20 mg/mL protein gradient. Two drops were placed above each well in a hanging-drop format (Figure 6), top and bottom drops received the same protein and salt combination. The bottom drops were subsequently seeded with 5 nL of proteinase K crystal in a serial dilution, each successive drop receiving less seed. To generate the seed stock two 0.2 mm proteinase K crystals were crushed and vortexed into 100 uL of 1.0 M ammonium sulfate until no fragments larger than 10 microns were observed. Drops were observed over time using a brightfield microscope and the average size of crystals that appeared in each drop was recorded. Crystal size versus seed dilution were plotted.

Figure 4

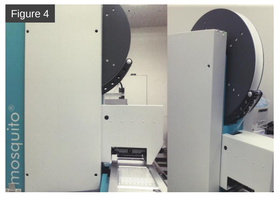


Figure 5

A TTP LabTech Mosquito Crystal nanodispenser (Figures 4 and 5) was used to setup the tray in under four minutes after the loading plates were prepared manually. A Greiner V-bottom 651101 plate was used to hold a protein dilution (Column 7) row A-H, the seed stock in a separate column (Column 8), and water to wash the seed in four additional columns. The loading plate is detailed in Table 1.

Table 1. Arrangement of protein and seed in loading plate

	1	2	3	4	5	6	7	8	9	10	11	12
A							100%	4 uL Seed 10 uL H2O	10 uL H2O	10 uL H2O	10 uL H2O	10 uL H2O
B							80%	4 uL Seed 10 uL H2O	10 uL H2O	10 uL H2O	10 uL H2O	10 uL H2O
C							60%	4 uL Seed 10 uL H2O	10 uL H2O	10 uL H2O	10 uL H2O	10 uL H2O
D							40%	4 uL Seed 10 uL H2O	10 uL H2O	10 uL H2O	10 uL H2O	10 uL H2O
E							100%	4 uL Seed 10 uL H2O	10 uL H2O	10 uL H2O	10 uL H2O	10 uL H2O
F							100%	4 uL Seed 10 uL H2O	10 uL H2O	10 uL H2O	10 uL H2O	10 uL H2O
G							100%	4 uL Seed 10 uL H2O	10 uL H2O	10 uL H2O	10 uL H2O	10 uL H2O
H							100%	4 uL Seed 10 uL H2O	10 uL H2O	10 uL H2O	10 uL H2O	10 uL H2O

Percentages reflect the concentration of protein in the loading plate (50mg/ml Proteinase K in this case)

A reservoir, Greiner F-bottom 65501, was loaded with a dilution of ammonium sulfate detailed in Table 2.

Table 2. Concentration of ammonium sulfate in reservoir

	1	2	3	4	5	6	7	8	9	10	11	12
A	50%	50%	50%	50%	50%	50%	50%	50%	50%	50%	50%	50%
B	50%	50%	50%	50%	50%	50%	50%	50%	50%	50%	50%	50%
C	50%	50%	50%	50%	50%	50%	50%	50%	50%	50%	50%	50%
D	50%	50%	50%	50%	50%	50%	50%	50%	50%	50%	50%	50%
E	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
F	75%	75%	75%	75%	75%	75%	75%	75%	75%	75%	75%	75%
G	50%	50%	50%	50%	50%	50%	50%	50%	50%	50%	50%	50%
H	25%	25%	25%	25%	25%	25%	25%	25%	25%	25%	25%	25%

Percentages reflect the concentration of the condition being optimized (0.4M ammonium sulfate in this case)

The nanodispenser set up hanging drops by combining conditions from the loading plate and reservoir. The stocks combined are detailed in Table 3. Both drops received 150 nL of each stock. The resulting drop contains one half of the values reported in the table.

Table 3. Concentration of protein (mg/ml) and ammonium sulfate (M) in each drop

	1	2	3	4	5	6	7	8	9	10	11	12
A	50 mg/ml 0.2 M	50 mg/ml 0.2 M	50 mg/ml 0.2 M	50 mg/ml 0.2 M	50 mg/ml 0.2 M	50 mg/ml 0.2 M	50 mg/ml 0.2 M	50 mg/ml 0.2 M	50 mg/ml 0.2 M	50 mg/ml 0.2 M	50 mg/ml 0.2 M	50 mg/ml 0.2 M
B	40 mg/ml 0.2 M	40 mg/ml 0.2 M	40 mg/ml 0.2 M	40 mg/ml 0.2 M	40 mg/ml 0.2 M	40 mg/ml 0.2 M	40 mg/ml 0.2 M	40 mg/ml 0.2 M	40 mg/ml 0.2 M	40 mg/ml 0.2 M	40 mg/ml 0.2 M	40 mg/ml 0.2 M
C	30 mg/ml 0.2 M	30 mg/ml 0.2 M	30 mg/ml 0.2 M	30 mg/ml 0.2 M	30 mg/ml 0.2 M	30 mg/ml 0.2 M	30 mg/ml 0.2 M	30 mg/ml 0.2 M	30 mg/ml 0.2 M	30 mg/ml 0.2 M	30 mg/ml 0.2 M	30 mg/ml 0.2 M
D	20 mg/ml 0.2 M	20 mg/ml 0.2 M	20 mg/ml 0.2 M	20 mg/ml 0.2 M	20 mg/ml 0.2 M	20 mg/ml 0.2 M	20 mg/ml 0.2 M	20 mg/ml 0.2 M	20 mg/ml 0.2 M	20 mg/ml 0.2 M	20 mg/ml 0.2 M	20 mg/ml 0.2 M
E	50 mg/ml 0.4 M	50 mg/ml 0.4 M	50 mg/ml 0.4 M	50 mg/ml 0.4 M	50 mg/ml 0.4 M	50 mg/ml 0.4 M	50 mg/ml 0.4 M	50 mg/ml 0.4 M	50 mg/ml 0.4 M	50 mg/ml 0.4 M	50 mg/ml 0.4 M	50 mg/ml 0.4 M
F	50 mg/ml 0.3 M	50 mg/ml 0.3 M	50 mg/ml 0.3 M	50 mg/ml 0.3 M	50 mg/ml 0.3 M	50 mg/ml 0.3 M	50 mg/ml 0.3 M	50 mg/ml 0.3 M	50 mg/ml 0.3 M	50 mg/ml 0.3 M	50 mg/ml 0.3 M	50 mg/ml 0.3 M
G	50 mg/ml 0.2 M	50 mg/ml 0.2 M	50 mg/ml 0.2 M	50 mg/ml 0.2 M	50 mg/ml 0.2 M	50 mg/ml 0.2 M	50 mg/ml 0.2 M	50 mg/ml 0.2 M	50 mg/ml 0.2 M	50 mg/ml 0.2 M	50 mg/ml 0.2 M	50 mg/ml 0.2 M
H	50 mg/ml 0.1 M	50 mg/ml 0.1 M	50 mg/ml 0.1 M	50 mg/ml 0.1 M	50 mg/ml 0.1 M	50 mg/ml 0.1 M	50 mg/ml 0.1 M	50 mg/ml 0.1 M	50 mg/ml 0.1 M	50 mg/ml 0.1 M	50 mg/ml 0.1 M	50 mg/ml 0.1 M

Figure 6 (right) shows the drop configuration. The control drop on the top receives no seed. Two separate experiments were performed. In the first, the same tips on the nanodispenser added 5 nL of seed to drops assuming that a natural dilution would occur as we omitted tip changing between contact with the drops. In the second experiment, tips were washed by aspirating 5 nL of water every four columns to reduce the amount of seeds. Contact corresponding author for a protocol file for the Mosquito.

RESULTS

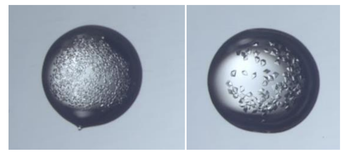
Out of 192 unseeded conditions **one** crystal appeared.
Crystals of various quality and size appeared in nearly every seeded condition, and followed a pattern of increasing crystal quality and size as the amount of seed added was reduced.

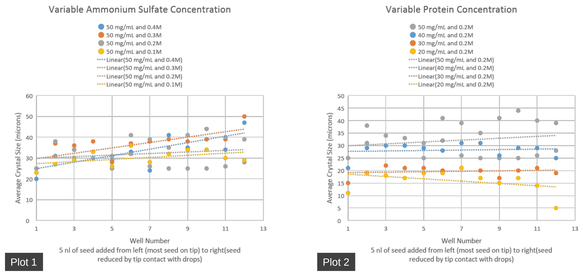
ATTEMPT 1: The amount of seed is reduced by contact with drops. A trend in the crystal size is observed as seed is diluted (left to right). Table 4 below records the average sizes of crystals that appeared after 40 hours in microns.

Table 4. Average crystal size in each well when seed concentration was reduced by contact only (no wash)

	1	2	3	4	5	6	7	8	9	10	11	12
A control												
A seeded	25	31	30	30	25	32	26	25	25	25	26	28
B control												
B seeded	21	29	30	30	29	28	31	31	26	29	29	25
C control						230						
C seeded	15	19	22	21	21	20	21	20	17	20	21	19
D control												
D seeded	11	19	18	17	19	19	21	17	15	17	14	5
E control												
E seeded	20	31	34	30	29	33	24	41	35	44	34	47
F control												
F seeded	23	37	36	38	28	37	38	39	38	39	39	50
G control												
G seeded	25	38	34	33	31	41	39	35	41	44	40	39
H control												
H seeded	23	27	29	33	26	36	28	32	34	34	30	29

5 nL of seed added from left (most seed on tip) to right (seed reduced by tip contact with drops)





Plot 1

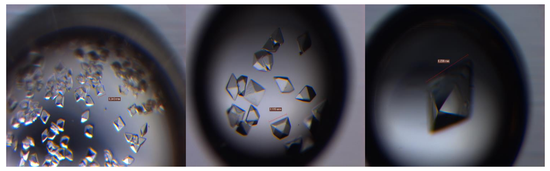
Plot 2

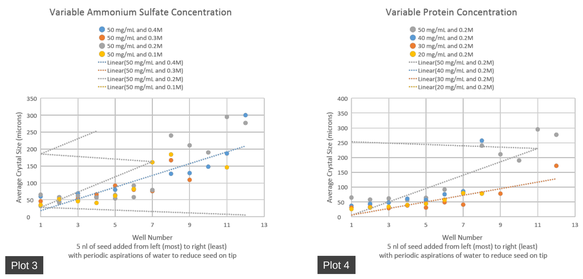
ATTEMPT 2: The amount of seed reduced by successive washes in water. A dramatic influence on crystal size is observed. Table 5 shows the average size of crystals that appeared after 57 hours in microns.

Table 5. Average crystal size when seed concentration is reduced by washing tips between contact

	1	2	3	4	5	6	7	8	9	10	11	12
A control												
A seeded	65	58	62	57	64	92	79					277
B control												
B seeded	37	44	48	61	57	76	86	257				
C control												
C seeded	31	33	29	38	31	49	41	79	78	105		172
D control												
D seeded	26	31	34	40	44	57	80	78		57	77	
E control												
E seeded	60	44	69	60	80	82	76	127	129	148	187	300
F control												
F seeded	46	52	54	66	92	80	76	167	109	104		
G control												
G seeded	32	42	49	57	54	58	79	240	211	190	295	
H control												
H seeded	35	52	46	41	62	82	161	184			146	

5 nL of seed added from left (most seed) to right (least) with periodic aspirations of water to reduce seed on tip



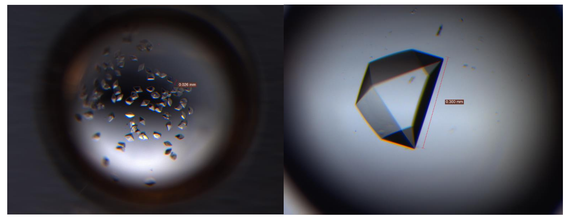


Plot 3

Plot 4

DISCUSSION

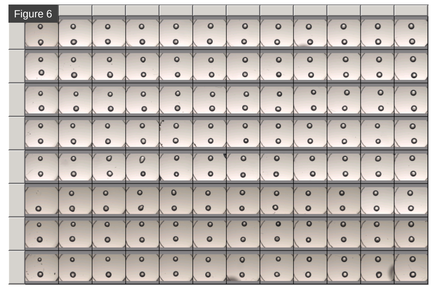
After observing whether crystals had grown in the seeded and unseeded drops of each well, we decided to plot only bottom drop crystal size for each condition, as the unseeded drops yielded only one crystal out of 96 attempts. As illustrated by the data plot of average crystal size versus ammonium sulfate concentration, these variables produce no correlation in the first experiment where seed was reduced by contact only (Plot 1). However, plotting average crystal size against Proteinase K concentration a positive correlation is observed in the same experiment (Plot 2). Furthermore, the table displaying average crystal sizes in control (Table 4) and seeded (Table 5) drops suggests that the execution of seeding contributes most significantly to crystal development and growth. Moreover, seeding with periodic water aspirations in the second attempt led to a decrease in amount of seed injected into each successive drop and larger crystals overall (Plot 3 and Plot 4). Comparing measurements of drops whose seeds were periodically aspirated to drops containing seeds whose pipette tip had been washed before each injection, we discovered that a low seed concentration produces greater crystal size.



CONCLUSION

We determined that for proteinase K seeding is crucial to the development of large single crystals. Furthermore, the technique can be automated to optimize the amount of seed deposited using only 40 uL of the protein of interest. The seeding process can be easily adapted using the TTP LabTech Mosquito nanodispenser. The concentration of seed, protein concentration, and concentration of precipitant can be tested using a single plate, however is it possible to easily adapt this protocol to test countless additional variables such as pH, drop size, drop ratio, temperature, salt concentration or buffer. In summary, we have adapted a traditional method of optimizing crystals by seeding using the TTPLabtech Mosquito Crystal automated liquid handler and achieved a means of generating large single crystals with minimal effort.

Figure 6



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