ABSTRACT
To explore methods of optimizing crystal growth, we manipulated salt, protein, and seed concentrations in seeded and non-seeded droplets. All droplets presented no crystal nuclei and were treated with seed solution such that each successive column received less seed than the previous. Noting objectively, we added protein at 0.5 M ammonium sulfate and a 50 mg/mL protein concentration. Each droplet was plated 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 mL of proteinase K crystals and a small dilution, each droplet receiving less seed. To generate the seed stock two 0.2 mL proteinase K crystals were crushed and vortexed into 100 mL of 1 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 was plotted.

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 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.

METHODS
We began by weighing 10.0 mg of lyophilized proteinase K (VWR, 97002-238) into 200 mL of water forming a 50 mg/mL solution. Each drop in rows E-H received 150 mL of the 50 mg/mL protein solution, and 150 mL of 0.4 M, 0.3 M, 0.2 M, 0.1 M ammonium sulfate solution 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 mL of proteinase K crystals and a small dilution, each droplet receiving less seed. To generate the seed stock two 0.2 mL proteinase K crystals were crushed and vortexed into 100 mL of 1 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 was plotted.

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.

ATTENTION 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.

ATTENTION 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.

CONCLUSION
We determined that for proteinase K seeding is crucial to the development of large single crystals. In addition, 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. Thus, it is possible to easily adopt this protocol to test countless additional variables such as pH, drop size, drop rate, temperature, salt concentration or buffer. In summary, we have adapted a traditional method of optimizing crystals by seeding using the TTP LabTech Mosquito automated liquid handler and achieved a means of generating large single crystals with minimal effort.

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REFERENCES

FIGURE 3
Figure 3 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 mL of seed to drops assuming that a natural dilution would occur as we omitted tip spacing changing contact with the drops. In the second experiment, tips were washed by aspirating 5 mL of water every four columns to reduce the amount of seeds. Contact corresponding author for a protocol file for the Mosquito.