**mosquito® crystal**
application note

# the benefits gained from using a high throughput microseeding technique for protein crystallisation

## introduction

Protein crystallisation is a major bottleneck for protein structure determination, and it still remains the least standardised part of the protein structure determination process by X-ray crystallography.

Nucleation is the first and critical step to succeed with protein crystallisation. Crystal nuclei (seeds) can be transferred from drop to drop to increase success with the 3D growth of crystals. This seeding technique was mentioned in 1976 by Blundell and Johnson [1], where the standard microseeding technique was performed with a 10-fold dilution of crushed crystals.

In 1991, Enrico Stura introduced streak seeding and perfected macroseeding for setting up drops and the concept of using seeding to maneuver in the phase diagram without needing to calculate a phase diagram. This method revolutionised the protein crystallisation process.

Microseed Matrix Seeding (MMS) was introduced in 2004 by C.G. Ireton and B. Stoddard [2]. Allan D'Arcy and co-workers expanded this method by automating the procedure and seeding directly into crystallisation screens [3]. The method has been successfully demonstrated to be applicable for general use and has shown success on different classes of proteins in generating new space groups, improving diffraction quality, and finding useful hits when there were none before. The number of hits generated by MMS is much greater than when crystallisation screening is done without seeding, as shown by using the same sparse matrix (Fig 1).

## bottlenecks

The most expensive part of crystallisation and optimisation of medically relevant human proteins is the cost of the protein itself. Thus, it is essential to optimise the use of protein as far as possible. If the quantities used can be accurately miniaturised, then vapor diffusion is proportionally faster. When microseeding these small drops, the challenge is to directly introduce the seeds while avoiding any cross-contamination between the drops.

TTP Labtech's mosquito® Crystal and LCP robots have proven highly effective for automating MMS set-ups in a miniaturised format due to three key reasons:

1. mosquito uses disposable tips to perform the contact dispensing essential for seeding, to avoid cross-contamination.
2. mosquito offers a multi-aspirate then dispense mode, so it is able to sample very small quantities of seed stock, followed by screen, and then dispense the drop combining the two solutions directly onto the protein drop.
3. mosquito is very accurate at handling low volumes of different viscosity solutions (e.g. 20-100 nL) because it uses true positive displacement pipetting.

Additionally, TTP Labtech's mosquito crystal is proven to be an extremely reliable and robust instrument. Therefore, experiments performed by mosquito are highly reproducible, which is essential for maintaining high throughput processes, especially when one instrument is used by many people, which tends to be common in crystallisation labs.

## methods

At Sanofi R&D, a high throughput MMS method is used in setting up all the sitting drop plates. TTP Labtech's mosquito is used to first set the protein (100 nL) into the crystallisation plate subwells. mosquito's multiaspirate and then dispense function enables the pipetting of two (and more) solutions in conjunction. Solution 1 is a seed stock and solution 2 is from the crystallisation solution reservoir. Routinely, this is 20 nL of seeds + 80 nL of reservoir aspirated together but not mixed inside of the mosquito tips. Both solutions are then dispensed together on top of the protein solution drop in the subwells.

mosquito provides extremely fast plate set-up times suitable for high throughput laboratories: a 96-well plate MMS set-up with a single protein takes about 90 seconds, and a three protein plate set-up is complete in under 5 minutes. This results in 288 individual seeded crystallisation conditions in less than 5 minutes.

## key benefits

- **uncouple crystal nucleation from growth**
- **produce new hits, space groups and improve diffraction quality**
- **use very low volumes of seed stock - screen more crystallisation space, stocks go further**
- **no cross-contamination of stocks due to disposable tips**
- **high reproducibility due to positive disposable pipettes**
- **speed - very quick to set up**

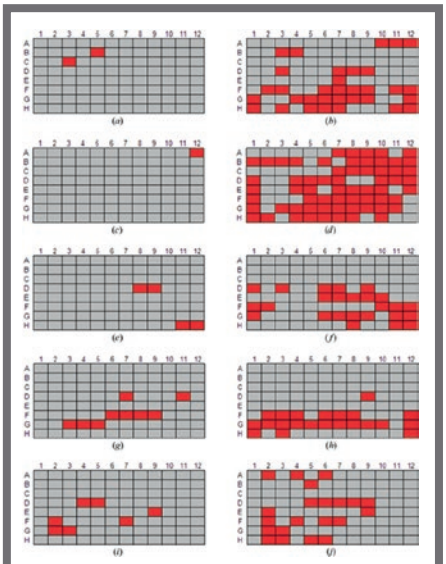

**Fig 1. MMS success rate demonstrated by Allan D'Arcy and co-workers. The left column shows sparse matrix screening, the right shows the same sparse matrix screening but with MMS seeding.[3]**

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## results

Crystallisation optimisation by seeding-enforced screening using MMS has been shown to be extremely successful at generating new crystal forms and hits. In one protein screen set up at Sanofi R&D, only one crystallisation hit was obtained, which is shown in Fig 2.

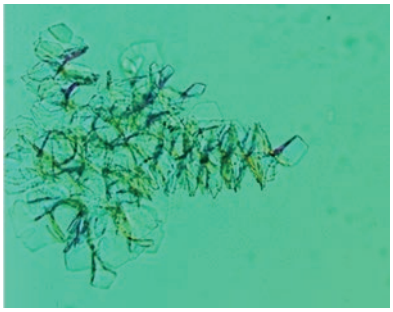

**Fig 2. The only hit obtained from initial protein screen of a protein at Sanofi R&D. It was not suitable for X-ray diffraction.**

The resultant hit was further optimised manually and this generated the crystal shown in Fig 3. This optimisation took ~6 weeks with a Full Time Employee (FTE) working on it one third of their time, and used a total of 4.5 mg of protein. The crystal diffracted to 3.2 A resolution.

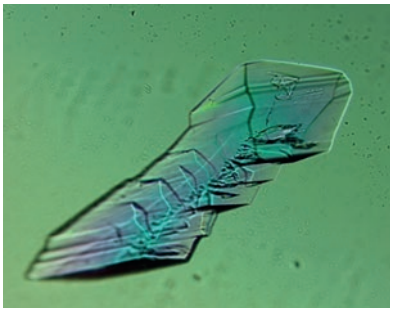

**Fig 3. Best crystal obtained from manual optimisation with microseeding.**

In contrast, MMS in 4 x 96 conditions gave the crystals shown in Fig 4. Two sparse matrix and two grid screens were used. This took only 1 day of a FTE, and it used a total of only 0.6 mg of protein. The crystals diffracted to 1.7-2.2 A resolution. Interestingly, this approach resulted in 26 new conditions yielding crystals of better than initial quality.

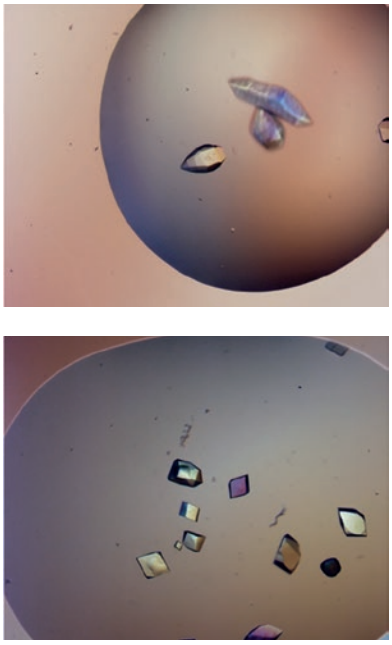

**Fig 4. Best crystals obtained from automated MMS**

At Sanofi R&D, automated MMS is also used for generating crystals of otherwise non-crystallisable targets, such as:

- new, shorter or extended construct of the crystallised protein
- new ligand bound crystal forms of the apo crystallised protein
- new proteins using similar but not identical protein crystals (including cross-seeding among the same protein family)
- protein complexes using crystal seeds of one of the complex components
- individual proteins using crystals of protein complexes, where the target protein was complexed with another partner

## conclusions

The bottleneck in protein crystallisation is to obtain proteins at high enough concentration for the crystallisation trials. This could end up being a very tedious, time-consuming and costly process. Crystallisation optimisation by MMS takes a lot less time than the classical manual crystallisation optimisation but also consumes much less protein:

- ~14 µL of protein solution can be dispensed by TTP Labtech's mosquito as 96 crystallisation conditions with automated microseeding in the form: (20 nL of seeds + 80 nL of reservoir) + 100 nL of protein
- ~14 µL of protein is enough to set up ~14 crystallisation conditions with manual microseeding
- it is much quicker to set up 96 conditions for MMS experiments using the mosquito robot than 14 crystallisation/seeding drops performed manually
- the vapor diffusion rate for 100 nL + 100 nL drops is proportionally higher than for 1 µL + 1 µL, therefore crystal hits will be obtained faster

Thus crystallisation optimisation by MMS is more time and cost-effective than classical manual crystallisation optimisation

## references


1. Blundell, T.L and Johnson, L.N (1976). *Protein Crystallography*. Academic Press, London, pp 67-68.

2. Ireton, C.G and Stoddard, B. (2004) Microseed matrix screening to improve crystals of yeast cytosine deaminase. *Acta Cryst*. D60, 601-605.

3. D'Arcy, A. et al. (2007) An automated microseed matrix screening method for protein crystallization. *Acta Cryst*. D63, 550-4.