ttplabtech

labCrystal 18

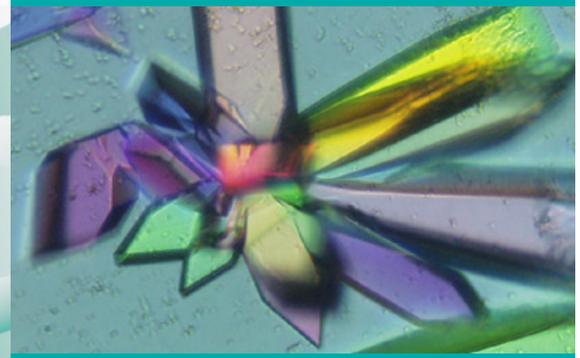
news from the world of protein crystallography

in this issue

welcome to labCrystal

Another exciting year in X-ray crystallography with many new structures solved! This edition of labCrystal encompasses a diverse selection of research from areas such as potassium channels involved in pain to natural sugars broken down by gut bacteria.

TTP Labtech have been a part of the protein crystallography community for over 15 years now. Over that time, we've developed alongside those growing protein crystals not only by delivering useful instruments into labs, but also by engaging closely with the protein crystallography community via lab visits, workshops, trade shows and on-going scientific collaborations.



We're listening - join one of our user group meetings

Scientists from a broad spectrum of organisations such as blue chip Pharma, successful contract research organisations and renowned academic institutions joined us for a fun day of talks, sneaky previews, future ideas and relaxing with some delicious food!

The talks were wonderfully varied with one presentation from Bhamini Vaidialingam (Agios Pharmaceuticals) explaining how our instruments are readily applied to other biophysical applications to improve sample characterisation and analysis.

This edition of labCyrstal includes highlights from a stunningly visual presentation by Art Laganowsky (Texas A&M University) on the potential of allostery within membrane lipids studied using native mass spectrometry (page 10).

Following the success of this year's event we aim to host the next meeting on the US West Coast. If you'd be interested in coming along and taking part in these fun events email us on **discover@ttplabtech.com** and we'll keep you informed.

Good luck with the crystals!

I hope you enjoy reading our labCrystal and look forward to seeing you soon,

Paul Thaw PhD
Structural Biology

Digesting sugar differently with a little help from gut bacteria!

Obesity is a major cause of health complaints in western developed countries, resulting in a wide selection of products promoted for weight-loss. In this area, there is a great need to supply safe products, such as digestive enzyme inhibitors. One such inhibitor is L-arabinose. This sugar is broken down by arabinofuranosidases (Arafs) which can be found in the gut microbiome. In this article, Professors Logan and Nordberg-Karlsson (Lund University, Sweden) describe how the mosquito® liquid handler was used to determine the structure of two Arafs from two different bacteria.

references

[1] Osaki S et al. L-arabinose feeding prevents increases due to dietary sucrose in lipogenic enzymes and triacylglycerol levels in rats. (2001) J Nutr 131: 796-799.

[2] Seri K et al. L-arabinose selectively inhibits intestinal sucrase in an uncompetitive manner and suppresses glycemic response after sucrose ingestion in animals. (1996) Metabolism, 45: 1368-1374.

[3] Linares-Pastén JA et al. Three-dimensional structures and functional studies of two GH43 arabinofuranosidases from Weissella sp. strain 142 and Lactobacillus brevis. (2017) FEBS J 284: 2019–2036.

substituting sucrose

L-arabinose is a common, naturally occurring sugar with a sweet taste that has been shown to inhibit the digestive enzyme sucrase [1,2]. Sucrase catalyses conversion of sucrose (refined white sugar) to glucose and fructose, which in turn increases the levels of lipogenic enzymes and triacylglycerol in the liver. When arabinose is co-administered with sucrose, sucrase is inhibited and a decrease in adipose tissue has been observed [1,2] making it potentially useful in the food industry.

The sugar residues of L-arabinose are released after degradation by arabinofuranosidases (Arafs). Interestingly, Arafs are part of the glycoside hydrolase 43 family (GH43) which is one of the most common GH-families in the gut microbiome.

the ‘good’ bacteria

The gut microbiome has been extensively investigated, revealing a close relationship between gut bacteria and many key processes in our bodies. Understanding the mechanisms of action of gut enzymes, in this case those that are involved with sugar digestion, could provide valuable tools and medical interventions for preventing or treating conditions such as obesity.

crystals of sugar enzymes in bacteria

In a recent collaborative study led by Professors Derek Logan and Eva Nordberg-Karlsson, the crystal structures of homologous GH43 enzyme candidates from two gut bacteria (Weissella strain 142 and Lactobacillus brevis DSM1269) were solved and analysed [3].

This work involved isolating and purifying recombinant Araf proteins from Weissella and L. brevis, (WAraf43 and LbAraf43 respectively). These proteins were then used to set up crystallization trials where drops of protein solution were mixed with reservoir solution in varying ratios of 100 or 200 nL and various combinations of reagents were added to identify the best conditions for crystal formation.

better research with confident results

The small volumes used in the crystallization screen were pipetted using TTP Labtech’s mosquito crystal robot which uses automated positive displacement pipetting. This method of aspirating and dispensing reduces error rates and increases consistency in drop sizes which ensures greater confidence in the results. Setting up this type of screen by hand is a tedious task with low rates of reproducibility!

optimal crystals

Optimal crystals grew in Greiner low-profile 96-well plates with drops of 100 nL of protein solution and 100 nL of reservoir at 4°C. The crystals produced in these small drops were already good enough to provide the data for solving the structure.

These structures were solved at a resolution of 2 Å for WAaf43 and 1.9 Å for LbAraf43. Although there were structural similarities, as expected with 74% sequence homology, there were significant differences. WAraf43 crystallised as a dimer (Fig 1A) stabilised by several hydrogen bonds and salt bridges at the dimer interface (Fig 1B).

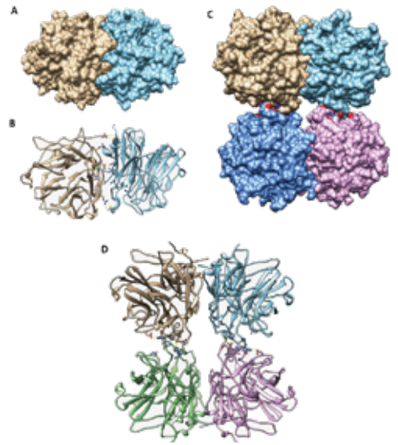
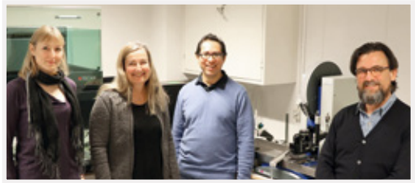


Fig 1. Crystallographic structures. The dimeric WAraf43 (1.9 Å resolution) displayed in surface representation (A) and as ribbons (B) with the hydrogen bonds and salt bridges between the monomers displayed as sticks. Tetramer stabilisation (C, D) of LbAraf43.

However, analysis of the crystal contacts in LbAraf43 identified additional interactions between the dimers that could result in stabilisation of a tetrameric structure (Fig 1C and D).

To study the substrate-interacting residues in the enzymes, α-L-1,5-arabinofuranotriose was modelled into the respective binding pockets of WAraf43 and LbAraf43. Both enzymes showed activity with similar catalytic efficiency on 1,5-α-L-arabinooligosaccharide substrates with a low degree of polymerisation (DP) of 2-3, although the activity was restricted to substrates of low DP. The reason for this is believed to be an extended loop at the entrance to the active site, creating interactions in the +2 subsite.

Dr. Derek Logan



Dr. Derek Logan (far right) is an Associate Professor of Biochemistry and Structural Biology at Lund University, Sweden. Derek’s group study the structure-function relationships in a wide variety of biological systems using X-ray and neutron crystallography, SAXS and cryoEM. These range from allosteric regulation in ribonucleotide reductases, through cell-surface proteoglycans to protein-carbohydrate interactions.

> “It is extremely user-friendly and requires minimal maintenance.”

Regulating the painful potassium channel

K^+ channels from the K2P class generate 'leak' currents that regulate neuronal excitability, and respond to lipids, temperature and mechanical stretch. These influence pain, temperature perception and anaesthetic responses. Despite recent advances, poor pharmacological profiles of K2P channels limit mechanistic and biological studies. This article describes Professor Minor's (University of California, San Francisco, USA) work to determine the structural mechanism of a class of molecular activators that directly stimulate a K2P channel. TTP Labtech's mosquito® crystal was essential to grow reproducible crystals of the channel/activator complexes.

K2P2.1 (TREK-1) is a two-pore-domain background potassium channel protein. This type of potassium channel is formed by a homodimer of subunits that create a channel that leaks potassium out of the cell to control resting membrane potential. The channel is found in mammalian neurons, in the central and peripheral nervous systems and heart. As TREK-1 has a key role in neuroprotection, ischemia, and pain, it is being evaluated as a potential target for new developments of therapeutic agents for neurology and anesthesiology.

customised crystallization screening

The crystal structures of K2P2.1 (TREK-1) and K2P2.1 (TREK-1) activator complexes were determined using recombinant K2P2.1cryst (TREK-1) in a recent Nature article [1]. K2P2.1 crystal leads could not be obtained from any of the commercial screens, therefore a custom screen was devised based on other K2P crystallization studies.

TTP Labtech's dragonfly® liquid handler ensured rapid screening by enabling various 96-well formatted screens to be created that were directly compatible with subsequent smaller volume crystallization studies using mosquito crystal.

In the final crystallization trails, purified K2P2.1cryst (TREK-1) was crystallized alone or with activators ML335 and ML402 by hanging-drop vapour diffusion at 4 °C using a mixture of 200 nL of protein and 100 nL of precipitant over 100 µL of reservoir. Crystals appeared in 12 h and grew to full size (200–300 µM) in about a week before being flash-frozen in liquid nitrogen.

Crystals of K2P2.1cryst (TREK-1) alone and complexed with ML335 and ML402 diffracted X-rays to 3.1 Å, 3.0 Å and 2.8 Å, respectively enabling structure determination (Fig 1).

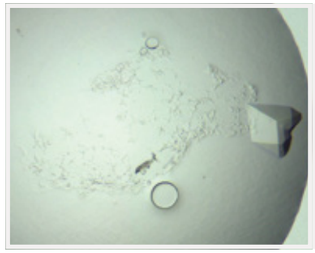


Fig 1. Optimised crystal of K2P2.1 (TREK-1) apo form (no modulators present).

using the best tools for the job

Prof. Minor and Dr. Marco Lolicato, the lead author on the study, stated, "mosquito crystal is essential for optimising and producing sufficient numbers of reproducible quality crystals, which in this case, was crucial for obtaining complexes with K2P2.1 (TREK-1) small molecule activators."

new approach for pain control

ML335 and ML402 directly stabilised the channel's gate which is formed by the selectivity filter (called the C-type gate) acting like molecular wedges reducing the dynamics at a key interface that supports the selectivity filter. This stabilisation causes the filter to enter the leak mode, bypassing modulation mechanisms that may involve other channel regions (Fig 2). The K2P modulator pocket properties revealed by these studies raise the possibility that natural processes or native signalling molecules may also target this site. It also suggests that this site could be modified by specific drugs and provide new avenues for neuroprotection and pain control.

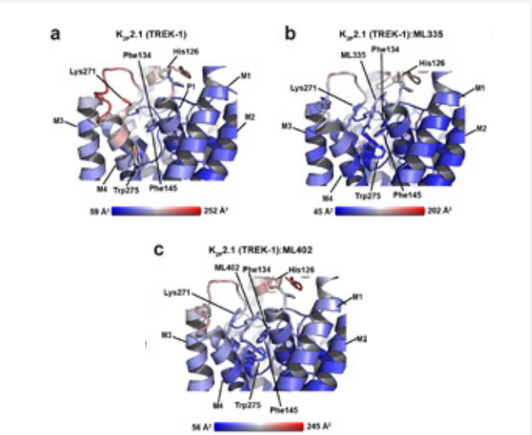


Fig 2. K2P structure comparisons [1]. K2P modulator pocket views coloured by B-factor for K2P2.1 (a), K2P2.1–ML335 (b) and K2P2.1–ML402 (c). Bars show B-factor scale.

Prof. Daniel Minor

Prof. Daniel Minor (left with Marco Lolicato) is a Professor at the Cardiovascular Research Institute and Departments of Biochemistry and Biophysics and Cellular and Molecular Pharmacology, UCSF, US. His laboratory focuses on using functional, chemical, and structural approaches to uncover the molecular mechanisms by which diverse types of ion channels work and to develop new reagents that can manipulate ion channel function. They are particularly interested in ion channels that respond to physical forces and that are important in pain and sensory physiology.

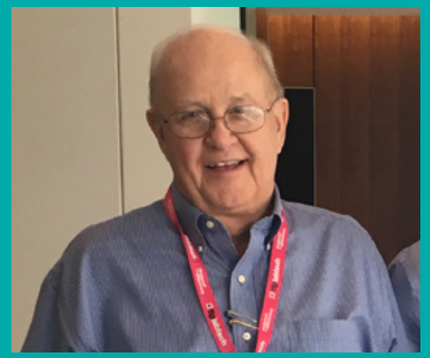
> We tested a number of liquid handlers for the UCSF Macromolecular Structure Group (MSG)'s shared facility and found the mosquito crystal to be the best, most robust, and most reliable.

references

[1] Lolicato M et al. K2P2.1 (TREK-1)–activator complexes reveal a cryptic selectivity filter binding site (2017) Nature 547: 364 - 368

walking the halls

with Chuck Luke



Our veteran account manager, Chuck Luke, has witnessed first-hand the development of protein crystallography. Here he shares his thoughts and experiences over the last 30 years in the industry.

tell us how you first got involved in the protein crystallography industry?

I became involved in crystallography in the early 1960s because I was a part of the supply community to laboratories as president of a small company called Mallinckrodt; who produced HPLC solvents. Prior to that I was selling high purity chemicals to labs. As we evolved as a company, we identified crystallography (nearly all chemical crystallography back in those days) as a niche market and began to focus on it more and more. We were bringing in pipettors at that time and we realised that the early protein crystallography labs such as those run by Harker at Buffalo and Wyckoff & Richards over at Yale needed these and all kinds of supplies to go with them. You have to remember that around that time when Perutz and Kendrew were doing the early protein structures, even things like SDS-Page and some biological buffers were relatively new to labs.

Ultimately, I ended up in the protein crystallography space because the people I met were great and I realised I could deliver real value to the bench scientists.

what is it about crystallography that keeps you going and enables you to still enjoy being part of the community?

What I enjoy most is working with all the labs that still pipette by hand and over many years I have made friends in lots of labs all over the US, Canada and South America... I feel inspired and grow when I introduce an individual who knows nothing about TTP Labtech to our technology and see how surprised and delighted they are when they realise how it can help them day to day in the lab.

what techniques, moments or advances in the industry have made you think 'wow!' during your career?

To me, in the early 2000's when drop-setters and positive displacement emerged and were good enough to really increase the throughput of crystallization labs – The 'rise of the machines' meant that precise automated liquid handling made the lives of those in the lab considerably easier and really fed into the whole era of structural genomics which drove huge advances in automation in many aspects of the workflow.

Crystallography started to become one of the most important disciplines for many branches of science, especially for chemistry, biology and biomedicine.

x-ray crystallography currently plays an important role in structural biology, but as cryogenic electron microscopy (CryoEM) comes into the frame, will it become a dying art?

One of the greatest advantages of CryoEM relative to conventional structural biology techniques is its ability to analyze large, complex and flexible structures. These are very challenging to crystallize for use in X-ray crystallography or are too large and complex. I may be the only man on the planet that thinks there will still be a place for conventional crystallography and in many ways CryoEM is allowing people to go back and tackle some of the projects that perhaps failed in the past, which has to be a good thing for us all. We all want the structures that give us the answers to take us forward

Walking the halls Chuck (far right) with the TTP Labtech team at ACA 2018

tell us one thing you've seen or experienced over the years working in the field that would make us smile?

It always makes me smile when people come up to me at a conference not knowing who TTP Labtech are and what we represent, but then discover during the conversation they've been using one of our mosquito instruments for years and not actually made the connection.

what one thing do you wish other people knew about TTP Labtech?

Unlike many other companies out there, we have an amazing applications team dedicated to helping customers develop protocols they can use on their specific projects. The relationship with us continues long after purchase and that's the way it should be.

> "
>
> ...we have an amazing applications team dedicated to helping customers develop protocols they can use on their specific projects.

when you're not out visiting labs what do you most enjoying doing?

Helping out with church events and spending time on my boat fishing and scuba diving. Being out on the ocean in the boat just gives me a bit of a break.

and lastly, what are you looking forward to this year?

The launch of mosquito® Xtal3 – it's going to be so accessible for so many labs and really speed up the research for the those that perhaps don't have a whole lot of budget.

Defining the lipid environment by allostery modification of protein-lipid interactions

The diverse environment of cellular membranes presents many challenges in understanding the roles that lipids play in modulating membrane protein structure and function. Lipids have been shown to allosterically modulate protein activity affecting physiological processes, but the molecular mechanisms are not well understood. This article describes how Dr. Arthur Laganowsky's group has demonstrated specific lipid–protein interactions that can act as allosteric modulators for the binding of different lipid types.

references

[1] Fabelo N et al. (2014) Altered lipid composition in cortical lipid rafts occurs at early stages of sporadic Alzheimer's disease and facilitates APP/BACE1 interactions. Neurobiology of Aging 35(8): 1801-1812

[2] Patrick JW et al. (2018) Allostery revealed within lipid binding events to membrane proteins. PNAS online http://www.pnas.org/lookup/doi/10.1073/pnas.1719813115

[3] Laganowsky A et al. (2014) Membrane proteins bind lipids selectively to modulate their structure and function. Nature 510:172–175

dissecting lipid and bacterial Ammonia channel (AmtB) interaction

A recent paper from Dr. Laganowsky's group set out to determine the allosteric nature of heterogeneous lipid binding events to the Ammonia channel (AmtB) from Escherichia coli (*E.coli*) using native mass spectrometry (MS) [2]. From Dr. Laganowsky's previous work, a specific binding site for phosphatidylglycerol (PG) was identified that bound and stabilised the AmtB channel [3]. Building on these previous findings, the latest work studied the molecular interaction of AmtB with a heterogeneous mixture of two lipid types or lipid pairs.

Although some of the lipids only differed by their headgroup composition, there was a significant difference in their ability to bind to AmtB. TFCDL (1,1',2,2'vv-tetraoleoyl cardiolipin) and POPE (1-palmitoyl-2-oleoyl phosphatidylethanolamine) lipid pairs were selected for further structural analysis as they exhibited the largest potent allosteric modulation when bound to AmtB.

To gain structural insight into the allosteric effect observed for TFCDL and POPE, the crystal structure was determined in the presence of AmtB. Initial cocrystallisation trials were carried out using mosquito® LCP in hanging drop plates at 20°C for 8 µM to 95 µM of AmtB combined with POPE and TFCDL in various molar ratios. The best diffracting crystals grew within 2 - 3 days at 79 µM AmtB mixed with 1:1.5:3 molar excess of POPE and TFCDL and resolved to 2.45 Å.

Dr. Laganowsky remarked, "The opportunity to use low sample volumes with the mosquito LCP enabled us to rapidly identify many different conditions to yield the best diffracting crystals."

mutational analysis reveals significant allosteric effect

It was shown that mutation of residues of AmtB involved in binding to the lipids abolished the observed allosteric effect. These findings are of significance as they contribute to our general knowledge of how lipids modulate protein structure and function and how membrane proteins may recruit, through allostery, their own lipid microenvironment.

It was concluded that lipid-protein interactions can allosterically modulate remote binding sites for lipids of different types. This allosteric binding could be utilised in the biological membrane to effectively recruit a defined lipid microenvironment.

managing low sample volumes

Crystallization trials rely on good X-ray diffraction which is often challenging to achieve. In this study, the sample volume was low which would normally limit the number of conditions and lipid-protein ratios that would be screened. With the use of the mosquito LCP liquid handler, large hanging drop crystallization screens were rapidly set up of AmtB mixed with different ratios of different lipids. Due to the advantages of automation, the accuracy and reliability of the trials was also improved.

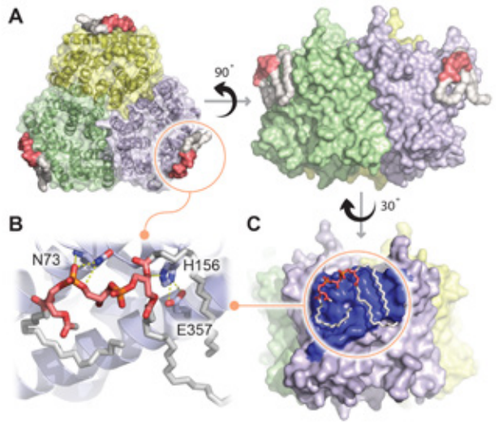
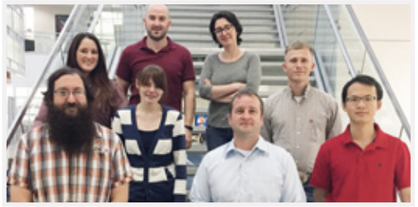


Fig 1. Crystal structure of AmtB Bound to TFCDL. (A) Top view of the periplasmic face of AmtB (Left) and parallel view (Right) to the transmembrane portion of the trimeric AmtB·TFCDL3 assembly. TFCDL is shown as a surface representation, with the headgroup (phosphoglycerol bridge) coloured light red and the acyl chains in white. (B) Molecular interactions formed between TFCDL and AmtB. Hydrogen bonds are shown as yellow dashed lines. (C) A large interacting surface (dark blue) is generated by TFCDL bound to AmtB. TFCDL is shown as sticks and coloured as in A.

Dr. Arthur Laganowsky



Dr. Arthur Laganowsky (front row, centre right) is an assistant professor in the Department of Chemistry at Texas A&M University, Texas. The long-term research goal of his group is to determine the molecular basis behind protein-lipid interactions and how these interactions can modulate the structure and function of membrane proteins, including their interactions with signaling molecules.

> The low sample volume requirements of the mosquito LCP enabled us to rapidly identify conditions that yield the best diffracting crystals.

new

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protein crystallization without compromise

mosquito® Xtal3 is our latest research enhancing liquid handler for protein crystallization screening. With structural biology becoming more multi-disciplinary, labs need a robust solution that's easy-to-use by all. Delivering the same repeatable and reliable performance with our proven true positive displacement technology, **mosquito® Xtal3 delivers more performance at a lower price point than ever before…**

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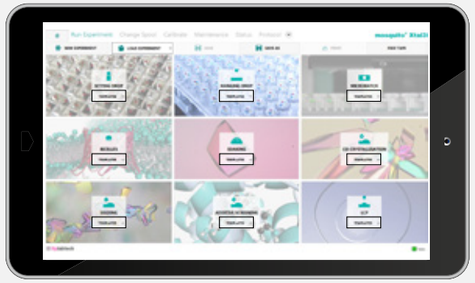

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improving your crystallization workflow

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> mosquito (crystal) has been very reliable and robust for us and has added a great deal of value to our lab
>
> Brandon Collins, Boehringer Ingelheim, Germany

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- **no configuration** changes needed for different experiments
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Applications include: hanging drop, sitting drop, bicelle, seeding, additive screening, soaking, microbatch

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membrane protein crystallization made easy

mosquito LCP allows you to fully automate LCP set-ups accurately and reproducibly. Dispensing lipidic cubic phase (LCP) volumes as low as 25 nL, while automated calibration of syringe and pipette positioning ensures precise drop-on-drop placement to facilitate automated imaging.

- **versatility** ability to set up both LCP and all traditional protein crystallization experiments
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- **speed** rapid and no washing
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Applications include: hanging drop, sitting drop, bicelle, seeding, additive screening, soaking, microbatch, LCP

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+

25 nL–1200 nL	pipetting range	25 nL–1200 nL
10 nL	minimal accessible volume	10 nL
96-, 384-	primary SBS plate format	96-, 384-
< 2 mins/ 96-well plate 4 mins/ 288 drops	throughput	2 mins/ 96-drop plate for vapour diffusion 5 mins/ 96-drop LCP plate
active humidity chamber	optional extras/ accessories	active humidity chamber, LCP mixer, LCP syringe coupling and needles

dragonfly®

optimisation optimised

TTP Labtech's dragonfly enhances protein crystal screen optimisation. Once the initial crystal 'hits' are identified, dragonfly automates the set-up of optimised conditions to grow better diffracting crystals. It is the ideal system to complement TTP Labtech's mosquito in the protein crystallization workflow.

- **focused** specifically designed to deliver highly accurate and reproducible optimisation screens
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reservoir capacity	10 mL
throughput	4–6 ingredient, 96-well plate in 4–8 mins, irrespective of viscosity
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> A breath of fresh air in the world of crystallization hits optimisation!
>
> Dr. Dimitri Chirgadze, University of Cambridge, UK speaking about dragonfly

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