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Review

Robotic nanolitre protein crystallisation at the MRC Laboratory of Molecular Biology

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Abstract

We have set up high-throughput robotic systems to screen and optimise crystallisation conditions of biological macromolecules with the aim to make difficult structural biology projects easier. The initial screening involves two robots. A Tecan Genesis liquid handler is used to transfer commercially available crystallisation reagents from 15 ml test tubes into the reservoirs of 96-well crystallisation plates. This step is fully automated and includes a carousel for intermediate plate storage, a Beckman plate sealer and a robotic arm, which transfers plates in between steps. For adding the sample, we use a second robot, a 17-tip Cartesian Technologies PixSys 4200 SynQuad liquid handler, which uses a syringe/solenoid valve combination to dispense small quantities of liquid (typically 100 nl) without touching the surface of the plate. Sixteen of the tips are used to transfer the reservoir solution to the crystallisation wells, while the 17th tip is used to dispense the protein. The screening of our standard set of 1440 conditions takes about 3 h and requires 300 μl of protein solution. Once crystallisation conditions have been found, they are optimised using a second Tecan Genesis liquid handler, which is programmed to pipette gradients from four different corner solutions into a wide range of crystallisation plate formats. For 96-well plates, the Cartesian robot can be used to add the sample. The methods described are now used almost exclusively for obtaining diffraction quality crystals in our laboratory with a throughput of several thousand plates per year. Our set-up has been copied in many institutions worldwide.

Keywords: Protein crystallization; High throughput; Robotics; Automation; Nanolitre dispensing

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1. Introduction

Ever since the beginning of protein crystallography, determining X-ray structures has become easier, faster and more automated, while the initial crystallisation step has remained a time consuming and erratic process. As protein structures can now often be solved within a few hours, crystallisation has evolved into a major bottleneck of X-ray crystallography.

When Max Perutz and colleagues set off to solve the first protein structure in the 1930s, the situation was strikingly different. While determining the atomic structure of haemoglobin turned into an enormous endeavour, which continued over more than 30 years (Perutz et al., 1968), the researchers never had to worry about finding crystallisation conditions. In fact, the first haemoglobin crystals had been reported almost 100 years before they started (by F.L. Hünefeld, 1840; cited in Lehmann, 1853). Most protein crystals obtained in those days had been grown serendipitously—by leaving a concentrated protein solution on the shelf or in the refrigerator. Haemoglobin was crystallised in 2 ml batch mode under high phosphate conditions in sealed tubes (Perutz, 1968), which produced crystals of several millimetres in diameter. These crystals are so stable that they are still intact today and a complete dataset to 1.5 Å could be collected when a crystal was mounted on a rotating anode X-ray source (Fig. 1).

Similar criteria determined the choice of the next few proteins to be probed by X-ray crystallography, namely myoglobin (Kendrew et al., 1960), lysozyme (Blake et al., 1965), and ribonuclease (Kartha et al., 1967). Only when X-ray structure determination had evolved into a standard technique, which researchers wanted to apply to their favoured proteins, crystallisation started to become a problem and soon turned into a science in itself. A growing number of crystallisation reagents became known that needed to be tested on a limited supply of protein (McPherson, 1985a–c, 1990). Consequently, methods were developed so that the amount of

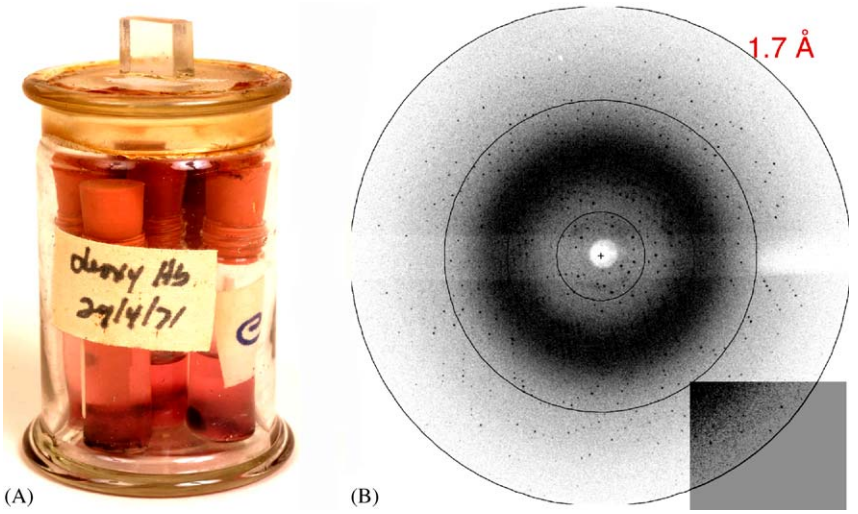

Fig. 1. First generation haemoglobin crystals. (A) Typical set-up of haemoglobin crystals from 1971: a few millilitres of concentrated protein solution were mixed with ammonium phosphate buffer and incubated, which produced crystals of several millimetres in diameter. (B) Diffraction image of one of these crystals. After more than 30 years, they still diffract to beyond 1.5 Å Bragg spacing on a rotating anode X-ray source.

protein spent *per* individual crystallisation condition continued to decrease. Around 1985, 24-well plates for vapour diffusion techniques were introduced, which allowed fast and efficient screening with drop sizes down to 1 μl, which represents the minimum volume for manual handling. At about the same time, a further advance in protein crystallisation came with the introduction of sparse matrix screens (Carter and Carter, 1979; Jancarik and Kim, 1991). Before, the multidimensional space of crystallisation parameters had routinely been sampled in grid screens, which probe parameter space as continuously as possible. An exhaustive screen however, is not feasible, because the number of possible parameters is astronomically high. Factorial or sparse matrix screens statistically sample crystallisation space at random points thus increasing the probability to find suitable conditions from a smaller number of trials. The first widely used screen of this type was developed by Jancarik and Kim (1991), and consisted of a selection of the 50 most successful crystallisation conditions, which had been accumulated by the authors. These developments along with advances in structure determination techniques (mainly the use of synchrotrons, area detectors and crystal freezing) led to an enormous increase in new protein structures: in 1990, about 500 structures had been deposited in the Protein Data Bank (PDB), almost exclusively solved by X-ray crystallography. Five years later, that number had grown to almost 4000.

In recent years, technologies at synchrotrons and algorithms for structure analysis have continued to advance in tremendous leaps (Hendrickson, 2000; Lamzin and Perrakis, 2000), while new developments in protein crystallisation were mostly restricted to the introduction of further sparse matrix screens. In 2000, the number of commercially available screens had increased to about 1000 individual solutions. Most of them are sold in 15 ml test tubes that have to be unscrewed and closed again for each individual condition, making it a day-filling job to screen only a fraction of the conditions for one individual sample.

At the MRC Laboratory of Molecular Biology (LMB) about 15–20 groups are more or less intensely involved in protein crystallisation and/or structure determination. Typical projects include very large protein complexes, membrane protein structures and structures of protein-DNA or protein-ligand complexes. Some of these structures are particularly difficult to crystallise and require many more rounds of initial screening with variations of the organism, detergent, construct, sub-complexes, etc., than a soluble single subunit protein would normally require. To increase our chances of success, we decided to set up a communal crystallisation facility at the LMB. The requirements for the facility were manifold: the aim was to pipette as many conditions as possible, as fast as possible and using as little sample as possible, while minimising manual handling. In addition, the procedure had to be simple enough to allow a large number of users to operate the system with very little training.

At the time two types of robots dedicated for crystallisation set-ups were commercially available: the IMPAX and ORYX systems from Douglas Instruments Ltd., UK, and the Cyberlab 200, distributed by Gilson Inc., USA. Both systems have only low to medium throughput and a minimal drop size of 2 μl.

The main difficulty in designing an ideal robot for crystallisation screens is in combining a nanolitre dispensing capability with the ability to handle millilitre volumes of very viscous solutions. We therefore decided to divide the problem into two parts and to purchase two different robots for the initial screening: one for the re-distribution of commercially obtained crystallisation reagents from 15 ml tubes into crystallisation plates, and a second one for the dispensing of nanolitre drops into the crystallisation wells. In 2000, the first structural genomics projects had come into operation (see Abbott, 2000; and Gershon, 2000 for overviews), which required high-throughput crystallisation screens. In general, they proposed to build their own custom made robots. Fortunately for us, these developments resulted in the release of 96-well crystallisation plates by several companies.

Once initial crystallisation conditions have been identified, the optimisation step puts yet different requirements on a liquid handler. We therefore purchased a third robot dedicated to this purpose, which came into operation at the beginning of this year. It is typically used to dispense gradients of precipitants into a wide choice of crystallisation plate formats.

2. Preparation of crystallisation plates

2.1. Design and operation of the robot

For the re-formatting of commercially obtained crystallisation reagents into 96-well crystallisation plates, we required a robot with a set of at least eight tips for simultaneous transfer of the solutions. As the spacing between the 15 ml test tubes containing the reagents is much wider than the spacing between the wells in the plate, we needed tips with adjustable distances. To reduce cost and plastic consumption, we decided to use fixed tips, which need to be washed in between steps to prevent cross contamination. As we were aiming for as little manual handling as possible, the set-up needed a device for intermediate plate storage, a robotic arm to transfer plates and an automated plate sealer. We were concerned that some of the reagents might be heat-sensitive, which restricted the choice of plate sealers to those that use adhesives instead of heat. The system

that met our requirements best was the Tecan Genesis 150 liquid handler (Tecan, Switzerland), supplemented with an adhesive plate sealer from Beckman (Sagian Sealer, Beckman, USA) (Fig. 2(A)). This set-up required almost no custom made parts or changes from the factory design.

Our Tecan Genesis 150 set-up contains an optional carousel, a robotic manipulator arm, and eight adjustable tips mounted on a moveable liquid handling arm (Fig. 2(B–E)). The tips are made of Teflon coated stainless steel to minimise interference with the crystallisation reagents. They are connected to 1 ml syringes, which operate with de-mineralised water as system liquid. By measuring the conductance, the tips can automatically detect the liquid level, which allows minimal insertion into the reagents thus preventing contamination and spillage. The 96 reagent tubes needed for one set of plates are held in a custom made cooling block. The cooling block is equilibrated to 14 °C to prevent evaporation of volatile reagents during operation. Before starting the programme, a few manual steps are necessary: opening and loading the 96 tubes into the cooling block, loading 72 empty crystallisation plates (CrystalEX 96-well round bottom protein crystallisation plate #3773, Corning, USA) into the carousel, manually labelling them and flushing the tubing with system liquid. After starting the programme, the robot fills 72 identical plates with 96 different crystallisation reagents, 85 μl per well. The Beckman plate sealer seals the plates with clear tape by using a pneumatic mechanism. After sealing, the plates are transferred back into the carousel. Once all plates have been filled, they are manually transferred into an incubator for storage and the system liquid is replaced by flushing the syringes and tubes in 20% ethanol/water. Currently, we offer a choice of 15 different plates, termed LMB1–LMB15 (see Table 1 for compositions).

2.2. *Optimisation of liquid handling—Tecan*

In addition to the physical movements of the tips, the aspiration and dispensing steps need to be optimised to allow accurate handling of viscous solutions. To achieve this, the robot allows definition of different liquid classes, from volatile to very viscous, which requires optimisation of a number of parameters, such as the size of the air gap, aspiration speed, excess volume, delay after aspiration and the same for dispensing (Fig. 3). As the crystallisation reagents from the commercially obtained kits are not ordered according to their liquid properties and reordering would have created logistic problems, we decided to optimise the pipetting for the worst case (i.e. the most viscous solution) and to use this liquid class for all reagents. The most critical problem for the dispensing is to avoid mixing of system liquid with crystallisation reagent during dispensing, which would lead to random dilutions of the crystallisation reagents. The only possibility to avoid this is to aspirate relatively large excess volumes (approximately 40% more than what is needed for dispensing). Using this aspirated volume, four plates are dispensed in a multi-dispensing mode where each of the eight tips dispenses 4×85 μl. Four plates seemed to be the maximum number that could be handled at a time, since we wanted to keep the opening times for each plate below 15 min. After four plates are filled, the robotic arm takes each plate to the plate sealer and then returns it to the carousel. Our initial idea for long-term storage of the plates was to store them at −20 °C to prevent microbial growth and deterioration of some less stable compounds. However, the freezing and thawing process produced considerable condensation on the seal, which could not be prevented by careful freezing and thawing procedures. Opening of the

plates inevitably led to cross-contamination and to spillage of the crystallisation reagent into the crystallisation wells. As the throughput of the plates turned out to be higher than we had anticipated, preservation of the reagents became less of a concern.

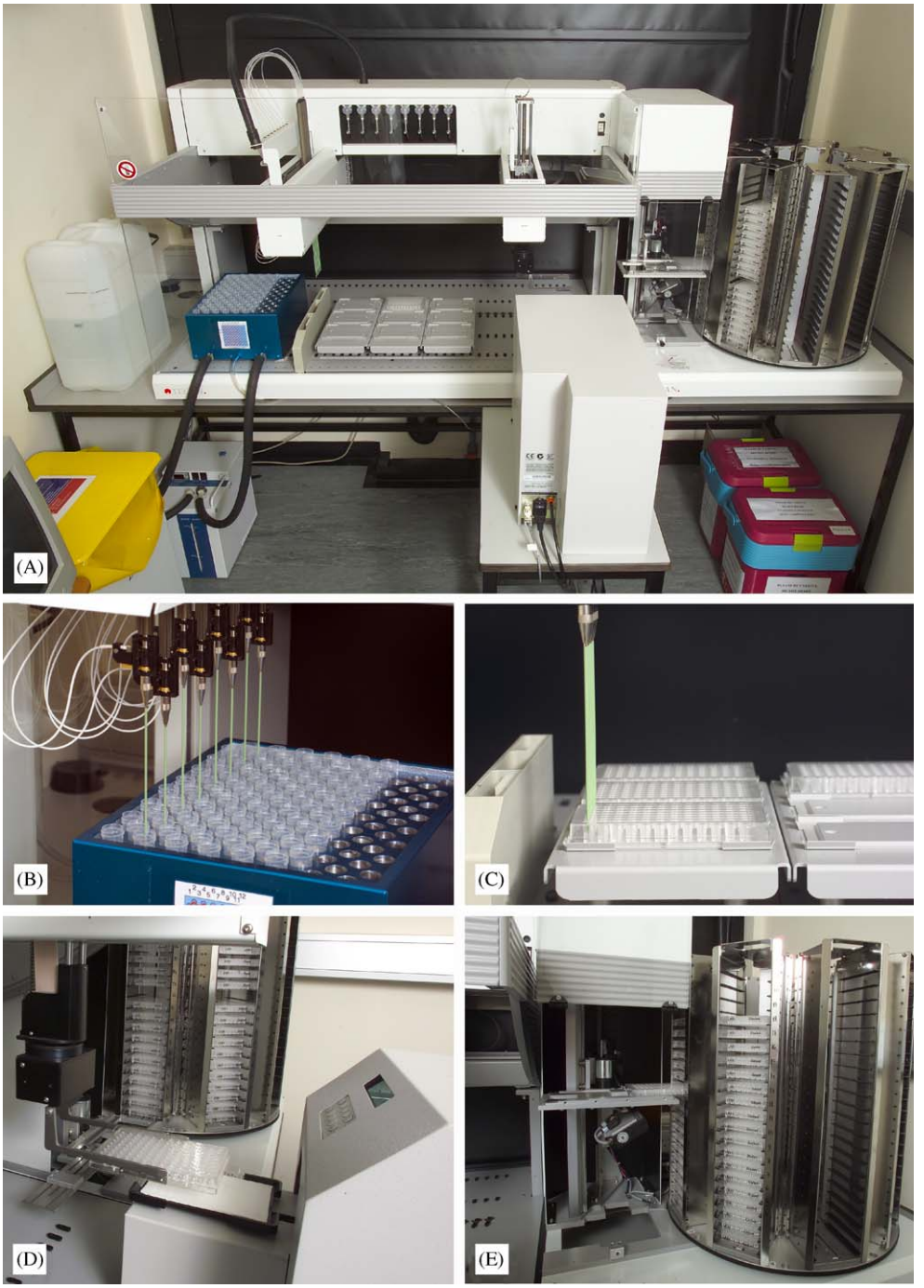

Table 1
LMB crystallisation plates

	Name of screen	Number of conditions	Supplier
LMB1	Crystal screen 1	48	Hampton Research
	Crystal screen 2	48	Hampton Research
LMB2	Wizard 1	48	Emerald BioStructures
	Wizard 2	48	Emerald BioStructures
LMB3	Ammonium sulphate grid screen	24	Hampton Research
	PEG 6000/LiCl grid screen	24	Hampton Research
	Quik phosphate grid screen	24	Hampton Research
	NaCl grid screen	24	Hampton Research
LMB4	PEG 6000 grid screen	24	Hampton Research
	MPD grid screen	24	Hampton Research
	MembFac	48	Hampton Research
LMB5	PEG-Ion screen	48	Hampton Research
	Natrix	48	Hampton Research
LMB6	Crystal Screen Lite	48	Hampton Research
	Crystal Screen Cryo	48	Hampton Research
LMB7	Wizard Cryo 1	48	Emerald BioStructures
	Wizard Cryo 2	48	Emerald BioStructures
LMB8	JBS1 (PEG 400-3000)	24	JenaBioScience
	JBS2 (PEG 4000)	24	JenaBioScience
	JBS3 (PEG 4000 plus)	24	JenaBioScience
	JBS4 (PEG 6000-8000)	24	JenaBioScience
LMB9	JBS 5 (PEG 8000-20000)	24	JenaBioScience
	JBS 6 (Ammonium sulphate)	24	JenaBioScience
	JBS 7 (MPD)	24	JenaBioScience
	JBS 8 (MPD/alcohols)	24	JenaBioScience
LMB10	JBS9 (alcohols/salt)	24	JenaBioScience
	JBS10 (salt)	24	JenaBioScience

Fig. 2. Tecan Genesis 150. (A) Overview. (B) Aspiration of reagents from 15 ml test tubes held in a cooled rack. (C) Dispensing of reagents into 96-well crystallisation plates. (D) Robotic arm and plate sealer in operation. (E) Plate carousel with gripper in operation. The Tecan Genesis 150 set-up shown here is 1426 mm wide (plus an additional 800 mm for the carousel), 780 mm deep (plus an additional 600 mm for the Beckman plate sealer, which is kept on a fixed table in front of the robot and needs to be connected to pressurised air) and 830 mm high. A PC running the software is kept next to the robot. Additional space close to the robot is needed for a 25 l water container, that holds the system liquid. The outlet of the wash station is directly connected to the drainage.

Table 1 (*continued*)

	Name of screen	Number of conditions	Supplier
	Clear Strategy Screen 1 pH 4.5[a]	24	Molecular Dimensions Limited
	Clear Strategy Screen 1 pH 5.5[a]	24	Molecular Dimensions Limited
LMB11	Clear Strategy Screen 1 pH 6.5[a]	24	Molecular Dimensions Limited
	Clear Strategy Screen 1 pH 7.5[a]	24	Molecular Dimensions Limited
	Clear Strategy Screen 1 pH 8.5[a]	24	Molecular Dimensions Limited
	Clear Strategy Screen 2 pH 4.5[a]	24	Molecular Dimensions Limited
LMB12	Clear Strategy Screen 2 pH 5.5[a]	24	Molecular Dimensions Limited
	Clear Strategy Screen 2 pH 6.5[a]	24	Molecular Dimensions Limited
	Clear Strategy Screen 2 pH 7.5[a]	24	Molecular Dimensions Limited
	Clear Strategy Screen 2 pH 8.5[a]	24	Molecular Dimensions Limited
LMB13	Index	96	Hampton Research
LMB14	SaltRX	96	Hampton Research
LMB15	MemStart	48	Molecular Dimensions Limited
	MemSys	48	Molecular Dimensions Limited

[a] 1 ml of buffer (provided by Molecular Dimensions) is added to each tube.

Sealing and plate storage is now optimised to facilitate adhesion of the tape and to prevent any condensation using the following protocol: plates are transferred manually from the carousel into insulated picnic coolers and stored in a 4 °C cold room overnight. This ensures very slow equilibration to 4 °C, which decreases the vapour pressure, leading to a very tight sealing. At the same time, the temperature gradient between the chamber and the tape is never large enough to induce condensation. After this, the plates are transferred to a 10 °C incubator, where they are stored until further use. Using this procedure, we have had neither problems with condensation, nor with microbial growth.

3. Setting up nanolitre crystallisation experiments

3.1. Design and operation of the robot

For the second step, we required a robot that could transfer nanolitre amounts of the reservoir solutions into the small crystallisation wells attached to the reservoirs and that could add equal amounts of protein to each well. This robot needed to be fast and precise for very small volumes. The Cartesian Technologies PixSys 4200 SynQuad liquid handler (Genomic Solutions, USA) seemed to fulfil these requirements. SynQuad technology uses a syringe/solenoid valve combination to dispense very quickly small quantities of liquid without touching the surface of the plate (Fig. 4).

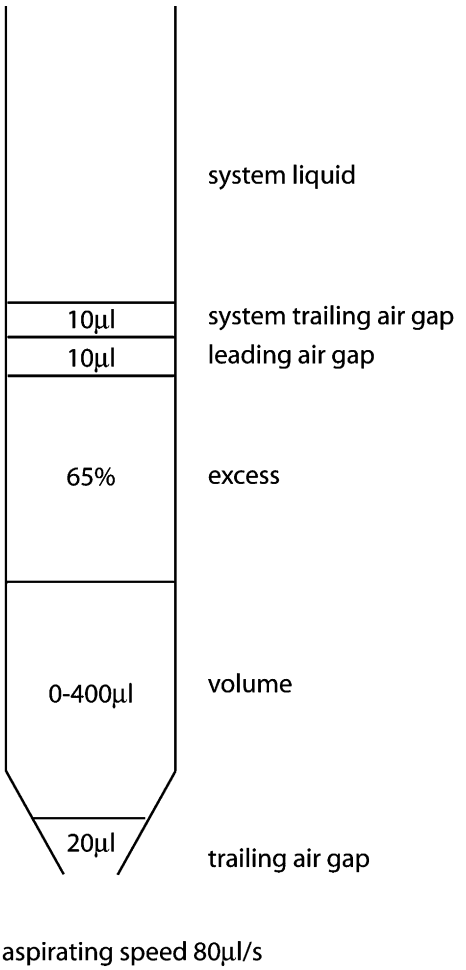

Fig. 3. Tecan tips. Universal liquid class used for setting up optimisation plates (Tecan Genesis 100, Tecan, Switzerland). Approximately 65% excess of the dispensed volume is needed to ensure correct pipetting of solutions with viscosities up to 40% PEG 8000. For the Tecan Genesis 150, which is used to dispense the LMB screens, the excess volume is 40%, while the aspirated volume is 340 µl/s and no trailing air gap.

The dispensing from the fixed tips is synchronised with a translation stage that holds the plates. The robot can operate in two modes: an aspirate/dispense mode, which is used to transfer crystallisation reagents from the reservoir wells to the crystallisation wells and a multi-dispense mode, which we use to add the sample. The multi-dispense mode operates ''on the fly'' and dispenses 96 protein drops without touching the wells, thus eliminating the need to wash the tips in between steps. The aspiration and dispensing of the reservoir solutions would ideally require a set of 96 tips for simultaneous transfer of all solutions in one plate. However, although this option is available, our budget restrained the number of tips for reservoir transfer to 16, which allows simultaneous transfer of two rows in a plate (Fig. 4(B)). After this, the tips need to be washed in a wash station. For each plate this needs to be repeated six times. In the last step, the protein is aspirated from a 17th tip (Fig. 4(C)) and distributed without stopping or washing (''on-the-fly'')

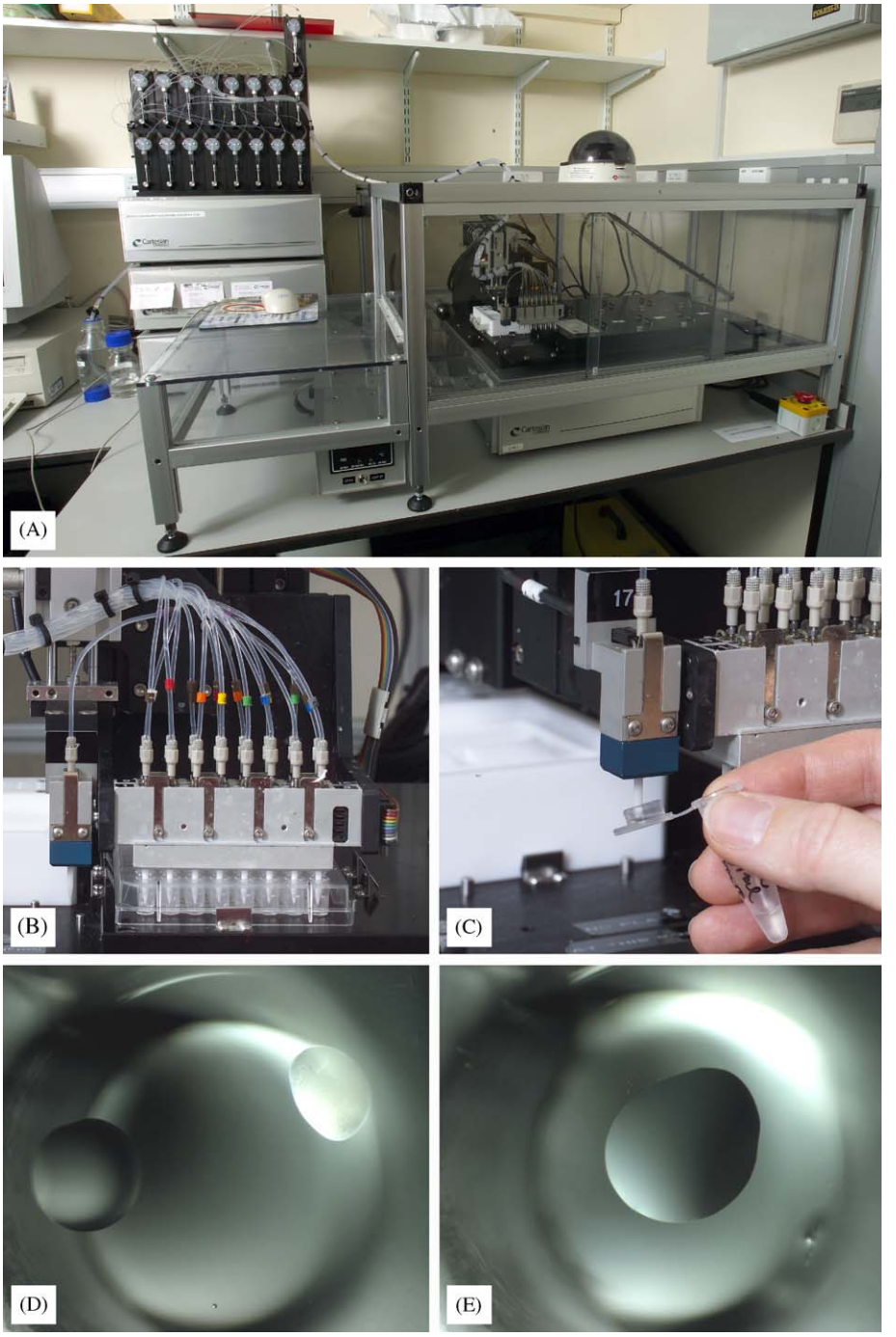

Fig. 4. Cartesian PixSys 4200. (A) Overview. (B) Tips during aspiration of crystallisation reagents from wells. (C) Aspiration of protein solution from the lid of an Eppendorf tube by the 17th head. (D) 40 nl drops before and (E) after centrifugation of the plate. The Cartesian PixSys 4200 set-up including the humidity chamber is 1210 mm wide, 890 mm deep and 490 mm high, plus a PC that runs the programme. The humidifier, a 10 l water container for the wash station, a vacuum pump and a compressor needed to pressurise the system are kept underneath the robot. The outlet from the wash station is directly connected to the drainage.

to the crystallisation wells. The plates are then manually sealed and transferred to crystallisation rooms or incubators. Our set-up has an optional humidity chamber that encloses the pipetting platform, which is kept at 85% humidity to prevent evaporation. The wash station contains a basin for inside and outside washes, and a tip drying station, which is connected to a vacuum pump.

Setting up individual plates requires initialisation of the instrument with system liquid, which is necessary to eliminate air bubbles from the syringes and the tubing. This is followed by tip testing to ensure that all tips are operational and unblocked. After this, the instrument is ready for crystallisation screens. One plate at a time is manually unsealed and placed onto the working platform of the robot. The programme is started with the hit of one button and the reservoirs and the protein are dispensed as explained above. After each run, which takes about 6 min, the plate needs to be manually removed from the platform and sealed. Typically, users do all 15 plates, containing 1440 conditions in one go, which takes about 3 h.

3.2. Optimisation of the robot—Cartesian

Aspirating and dispensing of viscous crystallisation reagents does not pose a serious problem for the Cartesian robot, because a large excess volume can be aspirated from the reservoirs (5 μl), whereas the dispensed volumes are very small (typically 100 nl). For the dispensing, the syringe and valve are synchronized such that when the syringe displaces a measured volume of fluid the valve is opened long enough for the drop to be dispensed. Dispensing is achieved by actuating the solenoid, which creates an acoustic shock wave through the fluid such that the velocity of the fluid increases as it is forced through the small orifice of the ceramic tip. To achieve the highest possible precision an optimal pressure at the tip needs to be built up before dispensing the drop. This is achieved by pre-dispensing several microlitres of reagent back into the reservoir well before the actual drop is set into the crystallization well. Pre-dispensing is also needed before the multi-dispensing of the sample, but only a few pre-dispensing steps are needed followed by the continuous dispensing of 96 sample drops. The major problem of the bulk dispensing is to avoid mixing of the sample and system liquid, which can be achieved by aspirating an air gap before aspirating the sample. Alas, if the air gap travels through the valve, which opens and closes at a speed of about 100 Hz, air bubbles, mixing, and inaccuracies do occur. We therefore have to ensure that the air gap stays behind the valve at all times, thus wasting a few microlitres of sample for each plate. Currently, we offer a choice of 40, 100, and 500 nl drops (Table 2), of which the 100 nl option is the most popular. As the 40 and the 100 nl drops often do not combine in the wells, the plates are spun in a bench top centrifuge for 1 min at 3000 rpm immediately after the set-up. Due to the round bottom of the crystallisation wells, the drop is concentrated in the centre of

Table 2
Protein consumption

nl	96 × (μl)	Aspirated (μl)	Wasted (μl)	Whole screen (15 plates)
40	3.84	9	5.16	135 μl (1.35 mg at 10 mg ml^{-1})
100	9.6	20	10.4	300 μl (3.0 mg at 10 mg ml^{-1})
500	48	70	22	1.05 ml (10.5 mg at 10 mg ml^{-1})

the well. The 40 nl crystallisation experiments require the instrument to be very clean and free of air bubbles. After the cleaning, the machine is initialised as usual with 2-propanol and water and is ready to use for 40 nl dispensing. Though successful screens with the 40 nl option have been reported, the reproducibility of the 40 nl drops is not optimal and might be hampered by evaporation.

3.3. Additive and detergent screens

Some initial crystallisation conditions only lead to usable crystals after the addition of one or two more compounds with subsequent optimisation. We have automated additive and detergent screens using the Cartesian robot. The screens are distributed by Hampton Research in 1.5 ml reaction tubes, each typically containing 100 µl of solution. Currently, three sets of 24 different additives (#HR2-428) and three sets of 24 different detergents (#HR2-416) are available. We manually transfer these solutions into 96-well assay plates and freeze the plates in between usage. For screening, 500 nl reservoir plus 500 nl protein drops are dispensed and 25, 50, 100, and 200 nl of reagent are added. This procedure gives four different concentrations for each additive, which greatly increases the success rate of the additive screens.

4. Optimisation of crystallisation conditions—Tecan Genesis 100

After the robots for the preliminary screens were in operation, optimisation of initial crystallisation conditions started to become increasingly time consuming. Emulating the manual optimisation process with a robot is difficult, because a very wide range of concentrated stock solutions needs to be pipetted into the crystallisation plates in a wide volume range.

Typically two sets of parameters are optimised against each other per plate. We prepare the four extreme conditions manually (the so-called corner solutions A, B, C, and D for top-left, top-right, bottom-left and bottom-right of the crystallisation plate) and use a Tecan Genesis 100 liquid handler to set up a linear gradient in between these extreme conditions (Fig. 5(A)). This strategy is both easy to perform for a robot and is very versatile. For calculating the composition of the corner solutions and for individual wells, we have set-up an Excel sheet for general use (Table 3). The four corner solutions are aspirated from four different troughs on the platform of the robot (Fig. 5(B)). The strategy also works for narrow pH screens around the pK_a of a buffer, but is not suitable for wider pH screens using different buffers. For this case and for the option to test a larger variety of chemicals at a constant concentration, we set up corner solutions that are 10% more concentrated and add 10% of the desired chemical/buffer from 15 ml test tubes held in a rack on the robot platform. The platform also accommodates most commercially available crystallisation plates (24-well Linbro (Hampton Research, #HR3-110), 24-well Cryschem (Hampton Research, #HR3-158), 24-well Cryschem (Charles Supper, #MVD/24), 24-well Q-plate (Hampton Research, #HR3-124) and 96-well Corning plates (Corning, #3773) but does not need a carousel, plate sealer or robotic arm. The plates are usually sealed immediately after filling and put on a shaker to mix the chemicals in the wells. Sample is added manually or, in case of 96-well plates, can be added by the Cartesian nanolitre robot. As grid screens in a 96-well plate would in many cases become narrower than necessary, we have programmed the option to include two

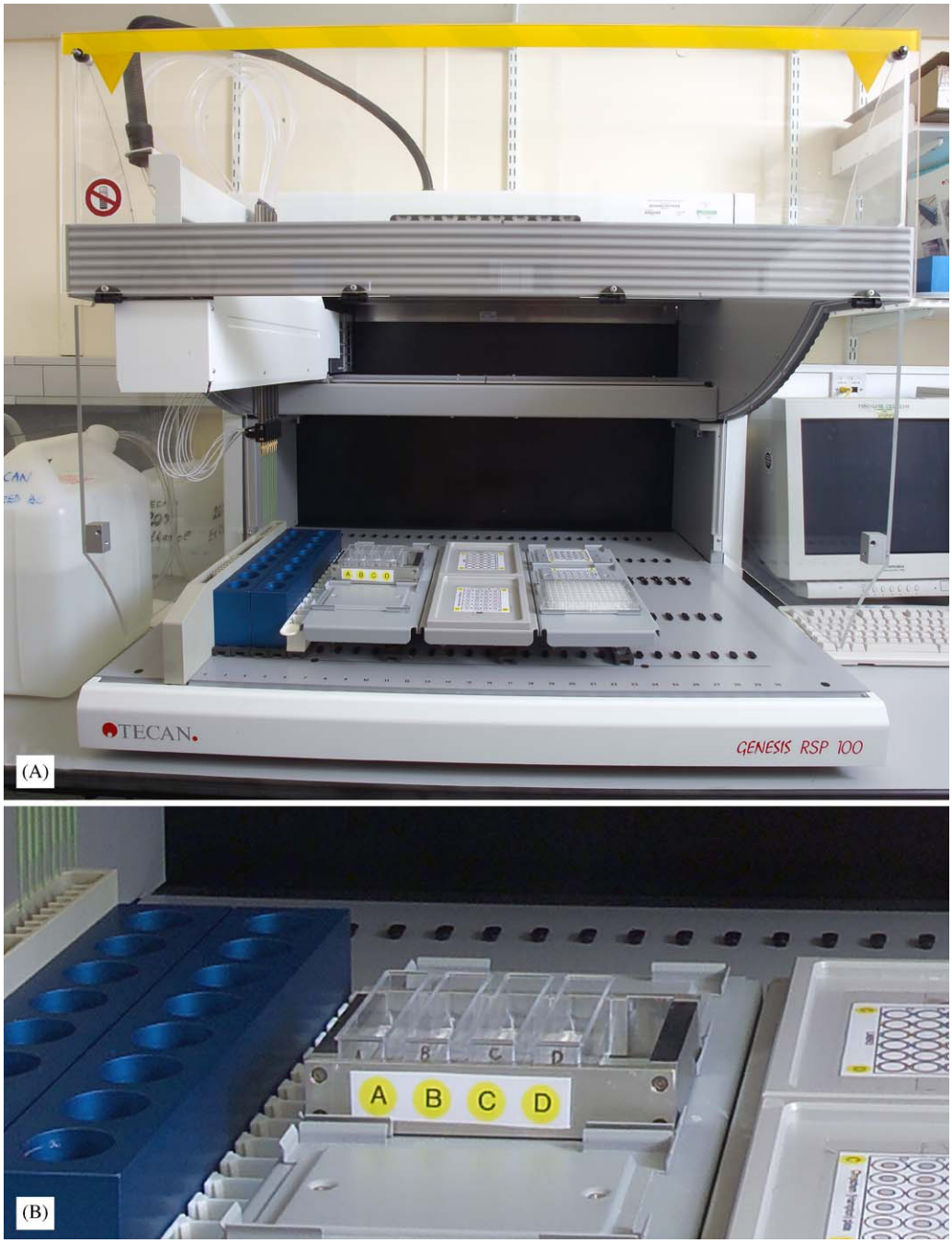

Fig. 5. Tecan Genesis 100 liquid handler. (A) The robot used to set up optimisation screens includes a liquid handling arm with eight adjustable tips, a wash station, racks for 50 and 15 ml test tubes, a rack to hold troughs for simultaneous aspiration of the same solution by eight tips (shown enlarged in (B)) and a platform for a wide range of crystallisation plates. The set-up is 1060 mm wide, 800 mm deep and 840 mm high and requires additional space for a container (about 10 l) holding the system liquid and for a PC running the software.

Table 3
Excel sheet for setting up optimization screens

Volume for corners (ml):		15			
Variable comp.		***Low***	***High***	**Step**	***Stock***
Vary across:	***Precipitant 1***	***10.000***	***20.000***	2.000	***50.000***
Vary Down:	***Precipitant 2***	***0.100***	***1.000***	0.300	***4.000***
Fixed components		***Conc.***	**Volume**		
	Buffer pH 7.0	***0.100***	1.500		***1.000***
	chemical 1	***0.001***	0.015		***1.000***
	chemical 2		0.000		

Change only the numbers and chemicals in the shaded areas above and nothing below!!!!!!!

Plate No	
Date:	
Protein:	
Plate type	
Seeded from:	
Drop size:	
Set by:	

Corner	Comp.	Conc.	Volume	Corner	Comp.	Conc.	Volume
A	Precipitant 1	10.000	**3.000**	**B**	Precipitant 1	20.000	**6.000**
	Precipitant 2	0.100	**0.375**		Precipitant 2	0.100	**0.375**
	Buffer pH 7.0	0.100	**1.500**		Buffer pH 7.0	0.100	**1.500**
	chemical 1	0.001	**0.015**		chemical 1	0.001	**0.015**
	chemical 2	0.000	**0.000**		chemical 2	0.000	**0.000**
	Water		**10.110**		Water		**7.110**
C	Precipitant 1	10.000	**3.000**	**D**	Precipitant 1	20.000	**6.000**
	Precipitant 2	1.000	**3.750**		Precipitant 2	1.000	**3.750**
	Buffer pH 7.0	0.100	**1.500**		Buffer pH 7.0	0.100	**1.500**
	chemical 1	0.001	**0.015**		chemical 1	0.001	**0.015**
	chemical 2	0.000	**0.000**		chemical 2	0.000	**0.000**
	Water		**6.735**		Water		**3.735**

	1	Conc.	2	Conc.	3	Conc.	4	Conc.	5	Conc.	6	Conc.
A	Precipitant 1	10.000	Precipitant 1	12.000	Precipitant 1	14.000	Precipitant 1	16.000	Precipitant 1	18.000	Precipitant 1	20.000
	Precipitant 2	0.100	Precipitant 2	0.100	Precipitant 2	0.100	Precipitant 2	0.100	Precipitant 2	0.100	Precipitant 2	0.100
	Buffer pH 7.0	0.100	Buffer pH 7.0	0.100	Buffer pH 7.0	0.100	Buffer pH 7.0	0.100	Buffer pH 7.0	0.100	Buffer pH 7.0	0.100
	chemical 1	0.001	chemical 1	0.001	chemical 1	0.001	chemical 1	0.001	chemical 1	0.001	chemical 1	0.001
	chemical 2		chemical 2		chemical 2		chemical 2		chemical 2		chemical 2	
B	Precipitant 1	10.000	Precipitant 1	12.000	Precipitant 1	14.000	Precipitant 1	16.000	Precipitant 1	18.000	Precipitant 1	20.000
	Precipitant 2	0.400	Precipitant 2	0.400	Precipitant 2	0.400	Precipitant 2	0.400	Precipitant 2	0.400	Precipitant 2	0.400
	Buffer pH 7.0	0.100	Buffer pH 7.0	0.100	Buffer pH 7.0	0.100	Buffer pH 7.0	0.100	Buffer pH 7.0	0.100	Buffer pH 7.0	0.100
	chemical 1	0.001	chemical 1	0.001	chemical 1	0.001	chemical 1	0.001	chemical 1	0.001	chemical 1	0.001
	chemical 2		chemical 2		chemical 2		chemical 2		chemical 2		chemical 2	
C	Precipitant 1	10.000	Precipitant 1	12.000	Precipitant 1	14.000	Precipitant 1	16.000	Precipitant 1	18.000	Precipitant 1	20.000
	Precipitant 2	0.700	Precipitant 2	0.700	Precipitant 2	0.700	Precipitant 2	0.700	Precipitant 2	0.700	Precipitant 2	0.700
	Buffer pH 7.0	0.100	Buffer pH 7.0	0.100	Buffer pH 7.0	0.100	Buffer pH 7.0	0.100	Buffer pH 7.0	0.100	Buffer pH 7.0	0.100
	chemical 1	0.001	chemical 1	0.001	chemical 1	0.001	chemical 1	0.001	chemical 1	0.001	chemical 1	0.001
	chemical 2		chemical 2		chemical 2		chemical 2		chemical 2		chemical 2	
D	Precipitant 1	10.000	Precipitant 1	12.000	Precipitant 1	14.000	Precipitant 1	16.000	Precipitant 1	18.000	Precipitant 1	20.000
	Precipitant 2	1.000	Precipitant 2	1.000	Precipitant 2	1.000	Precipitant 2	1.000	Precipitant 2	1.000	Precipitant 2	1.000
	Buffer pH 7.0	0.100	Buffer pH 7.0	0.100	Buffer pH 7.0	0.100	Buffer pH 7.0	0.100	Buffer pH 7.0	0.100	Buffer pH 7.0	0.100
	chemical 1	0.001	chemical 1	0.001	chemical 1	0.001	chemical 1	0.001	chemical 1	0.001	chemical 1	0.001
	chemical 2		chemical 2		chemical 2		chemical 2		chemical 2		chemical 2	

different optimisation screens in one plate using 2×4 corner solutions. Inversely, we can also use two 6×4 plates to generate a continuous matrix. These options allow a wide range of optimisation conditions to be screened with a small amount of protein.

5. Conclusions and future perspectives

We have been running the automated protein crystallisation facility at the LMB for more than two years. After the initial set-up and optimisation period, all three robots have required very little maintenance. Before the facility was released for public access in April 2002, tests with previously crystallised proteins gave the first encouraging results. Crystals generally appeared under the same conditions both in drops set up by the robot and manually. Most encouragingly, crystals in the smaller drops set by the robot grew faster, due to faster equilibration, whereas the final size of the crystals was about the same. This seems to be a unanimous observation made by several laboratories running nanolitre facilities (Kuhn et al., 2002; Stevens, 2000).

Soon after the launch of our facility, crystallisation conditions for previously uncrystallised proteins were obtained. Since then, the robots have become increasingly popular with currently about 80 registered users. The throughput of plates is gradually increasing. At the moment, each set of 72 plates for a given screen lasts about eight weeks, corresponding to a turnover of about 1000 plates (96 000 conditions) per month. The Cartesian robot has reached the limit of its capacity. We estimate that the invested time per person per experiment is several times lower with the robot and costs are 5–10 times lower, because one crystallisation well holds only 85 μl compared to the 500–1000 μl of reservoir solutions in 24-well plates. The 96-well plates also take only about 5% of the space of four 24-well plates. On the other hand, because of the speed and convenience, users do many more crystallisation set-ups now, resulting in an overall larger expenditure for crystallisation trials. Nevertheless, the time spent per person on crystallisation set-ups is still much smaller. As the facility is shared among many different groups with different projects without centralised documentation, the exact rate of success is difficult to estimate. A survey at the end of March 2004 brought up roughly 30 structures that have been solved using crystals identified by the robot, although many of the larger structures and the more difficult cases are pending. At the same time 11 000 crystallisation plates were purchased for the robot, corresponding to slightly more than 1 million trials.

Several trends have become apparent. If a protein crystallises relatively easily, using standard precipitants, our 1440 condition screen will result in many drops with crystals. It is then possible to pick the very best condition and optimisation time is greatly reduced. If a protein crystallises more reluctantly (and our experiments suggest that this is the most likely outcome), only very few positive conditions will be found and careful viewing of the trials at high magnification ($1000 \times$) is required not to miss anything. If the protein is available as seleno-methionine substituted protein, we now routinely re-screen because seleno-methionine substituted proteins often produce different crystal forms. Unfortunately, many proteins still do not crystallise and increasing the number of conditions will probably not significantly change this. However, we have had several proteins that crystallised in only a single condition, maybe indicating that the number of conditions we use is still too low.

All three robots require only a minimal amount of training, are easy to use and quick to set up before each run. In fact, it is now so convenient to set up crystallisation screens that a considerable

fraction of users who had never been trained in protein crystallisation routinely set up crystallisation trials. Because not much protein is needed, many more groups are including X-ray crystallography among their objectives. This has encouraged several in-house collaborations between biochemists and structural biologists. A further advantage of the facility is that it can be operated in complete darkness, which is crucial for light-sensitive samples.

The bottleneck has now shifted to sample preparation on the one side, and to monitoring of the crystallisation screens on the other. Many efforts are being made by structural genomics groups worldwide to automate these steps and the first specialised systems are on the market. We are currently investigating and hope to incorporate such automated systems in the near future.

We would like to implement further improvements in the next generation high-throughput facility such as better temperature and evaporation controls during sample set-up, faster transfer of reservoir solutions to the wells, less sample loss, even smaller drop sizes, and possibly smaller reservoir volumes. The option to pre-cool plates to 4 °C and to maintain them at this temperature would allow crystallisation of particularly sensitive samples. Better evaporation control can be achieved by the design of a sliding lid that covers the crystallisation plate, while leaving a gap for the tips, as exemplified by the Oxford set-up for the Cartesian liquid handler (Walter et al., 2003). The problem could also be solved by a very fast reservoir transfer, which could be achieved by using 96 tips. To reduce the amount of excess sample, the protein sample tip could be re-designed such that a sample loop could be inserted in front of the valve (“extended tip”). This would avoid passage of the air gap through the valve and should enable us to reduce the dead volume to a few percent.

Since we designed and started our high-throughput crystallisation facility, many other crystallisation facilities were launched (e.g. Brown et al., 2003; Heinemann et al., 2003; Rupp et al., 2002; Stevens, 2000; Sulzenbacher et al., 2002; Walter et al., 2003). In general, researchers have become very reluctant to set up crystallisation trials manually. This has pushed the development of techniques and high-throughput supplies enormously. Pre-filled 96-well crystallisation plates have become available (e.g. from Nextal Biotechnologies, Canada). In addition, more dedicated robots are emerging like the Mosquito (TTP Labtech, UK), the Topaz free-interface diffusion system (Fluidigm Corp., USA) and Innovadyne’s Screenmaker 96, which uses 96 tips.

In our laboratory, automated crystallisation set-up has almost entirely replaced manual liquid handling. Although crystallisation of biological macromolecules is still as unpredictable as it used to be, automation has greatly reduced the time and effort invested into this step.

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