PROTOCOL: **producing two linear gradients of concentrations into the reservoirs of a crystallization plate (the 4-corner method).**

1. First, ensure that the liquid handler is on and initialized with its software open.

2. On the 'GRADIENT' tab: Open the required program, select the crystallization plate type and the final volume in the reservoirs (**Table 1**). *The advanced setting for 'max shot vol' should be lowered from 6,000 to 3,000 when using solutions containing [isopropanol] > 10% v/v and [MPD] > 20% v/v.*

3. Prepare the syringes. Place a piston in each syringe (pointed ends down) and insert the back of the syringes into the designated grooves underneath the robot head. Twist a syringe clockwise to lock it in position (The program will start only with all the required syringes attached correctly).

4. Prepare the troughs. Remove the stainless-steel frame and insert the troughs. The 4 positions on the left correspond to the 4 corners A, B, C, D. Switch to 'SET UP' tab which displays the volumes of solutions required in each syringe (on **Table 1**, 0.5 mL dead volume was added to the volumes displayed). Pour the corner solutions into their respective troughs and place the frame back on the deck (the frame holds in position with 2 small magnets located at the front of the deck). *An alternative way to proceed with this step is to pour the solutions into the troughs when they are already placed on the deck.*

5. Place the crystallization plate on the motorized SBS carrier.

6. Click 'ASPIRATE' and wait for this step to be completed (when pistons stopped on their way up).

7. Switch to the 'RUN' tab and run program.

8. Upon completion of the program, go back to the SET UP tab and click 'REMOVE': the system purges the syringes from leftover solutions, and then lifts the pistons all the way up. *'PURGE' may be requested instead of 'REMOVE'; this will leave the pistons at the bottom position, ready to aspirate more solutions with the same syringes.*

9. Remove the syringes by twisting them anticlockwise.

10. Discard syringes and troughs in the appropriate bin (or rinse them with deionized water and then 20% v/v ethanol solution for reuse).

11. Seal the plate and place it onto the microplate mixer for 3 min at 1,000 rpm, or 10 min when highly viscous solutions are being mixed. The plate is ready for setting up crystallization droplets on the nanoliter dispenser.

Table 1: Programs available on the DRAGONFLY for preparing optimization screens according to the 4-corner method. The MRC 96-well plate can be used to prepare 96- or 48-condition optimization screen (when two 48-condition screens are prepared simultaneously, the robot must be equipped with 8 syringes and 8 troughs). Other programs enable the use of the MAXI 48-well plates. The listed volumes in troughs include the required dead volume (0.5 mL).

Plate type	No. of conditions	Vol. in plate (reservoirs, µL)	Vol. in trough (mL)	Duration
MRC	48	80	1.6	2 min 5 sec
MRC	96	80	2.5	3 min 50 sec
MRC	2 x 48	80	1.6	2 min 50 sec
MAXI	24	200	1.8	2 min 25 sec
MAXI	48	200	3	4 min 20 sec