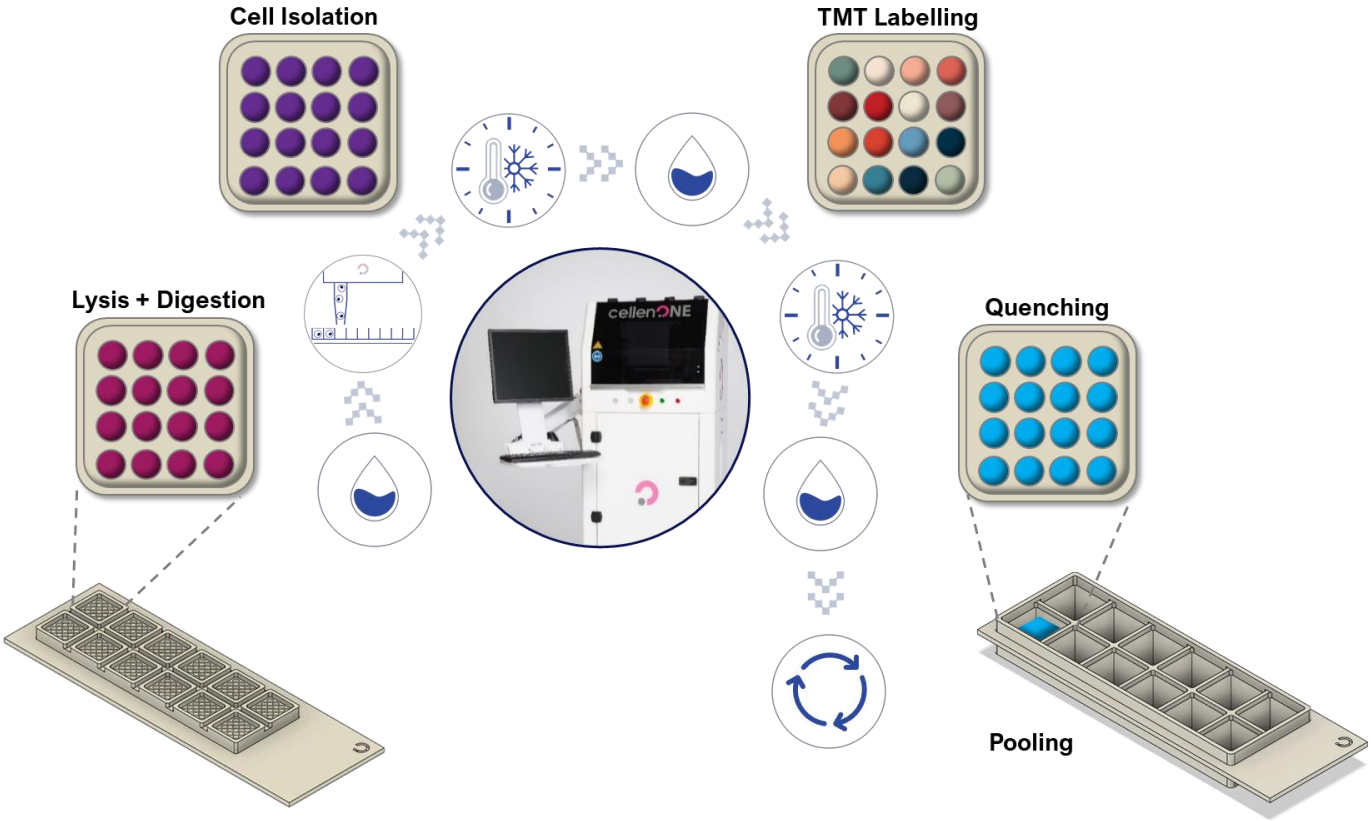
CELLENION
A BICO COMPANY

MANUAL

*proteoCHIP 12*16*

for the multiplexing
Single Cell Proteomics workflow
using the cellenONE®



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1 Introduction

The cellenONE software monitors cells inside the piezo dispensing capillary (PDC) before dispensing. All steps associated with single-cell isolation and dispensing experiments are presented in the Software User Guide.

Note: It is highly recommended to read the Software User Guide before performing any experiment with the cellenONE.

This Manual will guide you through all the steps of the workflow dedicated to single cell proteomics for multiplexing experiments using proteoCHIP 12*16.

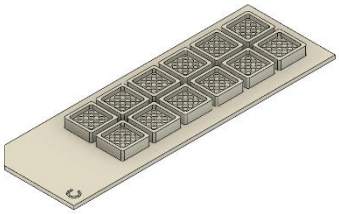
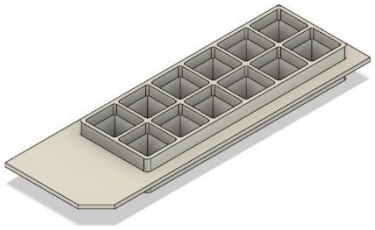
IBSCI™: Image-Based Single Cell Isolation

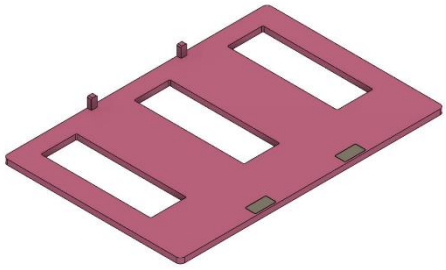
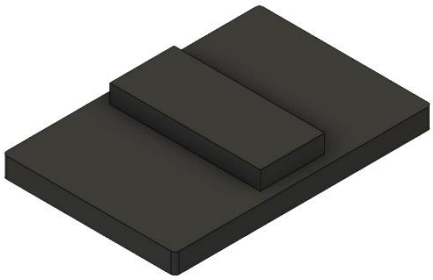
IBSCI™ is a high resolution-based technology consisting of automated image acquisition, processing and advanced algorithms to automatically isolate single cells from a cell suspension.

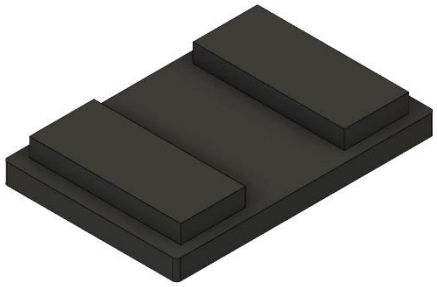
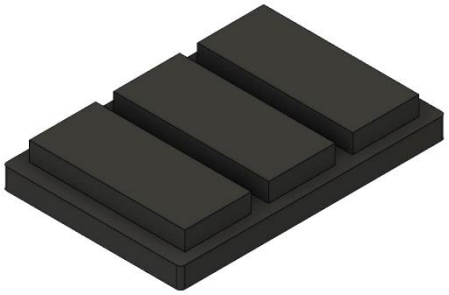
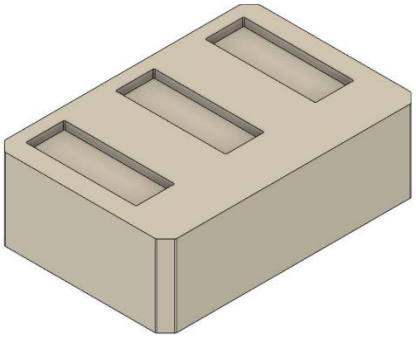
FIBSCI™: Fluorescence Image-Based Single Cell Isolation

FIBSCI™ utilizes multichannel fluorescence-based images to identify and isolate single cells of interest from subpopulations in mixed cell samples based on the presence, absence and intensity of fluorescent signal.

2 Materials

Part number	Description	
P-20-CL	**cellenONE PDC L Piezo Dispensing Capillary** (Fixed drop volume between **450-600** pl) **4x manifold**	
P-20-CM	**cellenONE PDC M Piezo Dispensing Capillary** (Fixed drop volume between **300-450** pl) **4x manifold**	
CPS-1216-3	**proteoCHIP 12*16 Set** The proteoCHIP 12*16 Set contains: • 3 (three) proteoCHIP 12*16 • 3 (three) proteoCHIP 12*16 Funnel	
	The proteoCHIP 12*16 is a conical bottom nanowell chip with 12 arrays of 16 nanowells, prefilled with oil, enabling sample preparation for single cell proteomics.	
	The proteoCHIP 12*16 Funnel is used to pool samples from the proteoCHIP 12*16.	
CHB-1216	**proteoCHIP 12*16 cellenONE Holder** Holder to accommodate up to 3 proteoCHIP 12*16 on the deck of the cellenONE throughout sample preparation (cell isolation, reagent dispensing and incubation steps). *(1 piece/pack)*	

CHT-1216	**proteoCHIP 12*16 cellenONE Holder Top** Top to maintain the proteoCHIP 12*16 inside the cellenONE Holder on the deck of the cellenONE throughout sample preparation (cell isolation, reagent dispensing and incubation steps). *(1 piece/pack)*	
CAB-1216	**proteoCHIP 12*16 Centrifuge Adaptor** Centrifuge adaptor enabling pooling of each set of 16 cells of the 12 arrays of the proteoCHIP 12*16 by spinning down into the proteoCHIP 12*16 Funnels. The proteoCHIP 12*16 Centrifuge Adaptor can accommodate up to 3 proteoCHIP 12*16 per run. **Warning**: For centrifugation the weight needs to be carefully balanced using the centrifuge counterweight 1, 2 or 3. *(1 piece/pack)*	
CAT-1216	**proteoCHIP 12*16 Centrifuge Adaptor Top** Top to maintain the proteoCHIP 12*16 and proteoCHIP 12*16 Funnels tightly together inside the proteoCHIP 12*16 Centrifuge Adaptor during the pooling step in the centrifuge. *(1 piece /pack)*	
CCA-1216	**proteoCHIP 12*16 Centrifuge Counterweight 1** Counterweight for proteoCHIP 12*16 to be used during centrifugation. **Warning:** Use this counterweight if the centrifuge adaptor holds 1 proteoCHIP 12*16 and 1 proteoCHIP 12*16 Funnel. *(1 piece/pack)*	

CCB-1216	**proteoCHIP 12*16 Centrifuge Counterweight 2** Counterweight for proteoCHIP 12*16 to be used during centrifugation. **Warning:** Use this counterweight if the centrifuge adaptor holds 2 proteoCHIP 12*16 and proteoCHIP 12*16 Funnels. *(1 piece/pack)*	
CCC-1216	**proteoCHIP 12*16 Centrifuge Counterweight 3** Counterweight for proteoCHIP 12*16 to be used during centrifugation. **Warning:** Use this counterweight if the centrifuge adaptor holds 3 proteoCHIP 12*16 and proteoCHIP 12*16 Funnels. *(1 piece/pack)*	
CAS-1216-50	**proteoCHIP 12*16 Seal** Adhesive aluminium foil, dedicated to proteoCHIP 12*16, for all reactions and storage. Pierceable, peelable, and free of contamination. *(50 pieces/pack)*	
CAH-1216	**proteoCHIP 12*16 Autosampler Holder** proteoCHIP holder for the LC-MS/MS system Thermo Fisher Dionex Ultimate 3000.	

3 cellenONE setup

3.1 Nozzle setup and drop parameters setup

Before starting your experiment, you will need to make sure that your PDC is correctly positioned and produces stable droplets.

After doing the PRIME, the PDC should be centered to the red cross. Make sure that this is the case, otherwise re-align your PDC using:

- Nozzle Setup (Figure 1) >> Nozzle Offset >> Up, Down, Back, Front, Left, Right controls.

After entering the Voltage and Pulse parameters (indicated on the PDC box), click on:

- Set Nozzle Parameters
- Start Continuous Dispensing

If needed, adjust the Voltage and Pulse until you have a stable drop at about 450 µm (+/-25 µm) distance from the PDC tip and then press:

- Set Nozzle Parameters

Figure 1: Drop optimisation

3.2 Humidity and temperature setup

Throughout the workflow, humidity and temperature need to be set to a specific value.

Note. *The runs already contain specific tasks controlling humidity and temperature for each run, but you should verify that the dew point temperature (DP) is above the temperature in the enclosure (BT) to avoid evaporation (BT<DP). We recommend setting -1 °C dew point correction (Figure 4).*

The following describes how to change humidity/temperature "manually" if you wish to change it.

- Humidity setup (Figure 2): Main >> File >> Humidity Control >> Popup window >> Set humidity >> Click on "Set".

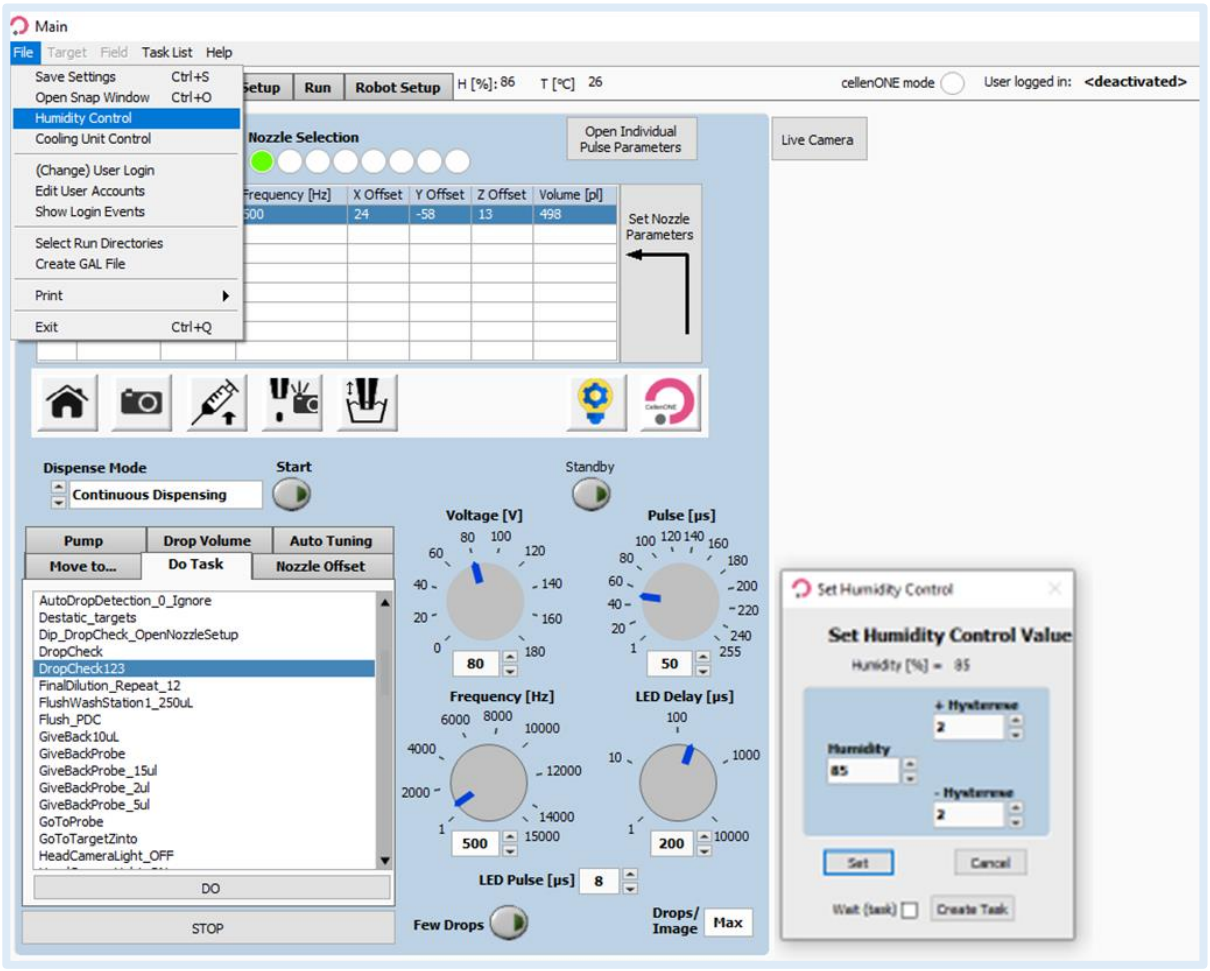


Figure 2: Humidity control

- Temperature setup (Figure 3): Main >> File >> Cooling Unit Control >> Popup window >> Set the temperature to a Fixed control or at Dew point and turn the cooling unit ON.

Please also make sure that the Dew Point Correction Value (Cooling Unit Control >> Popup window >> Settings tab) is set to -1 (Figure 4).

Figure 3: Temperature control

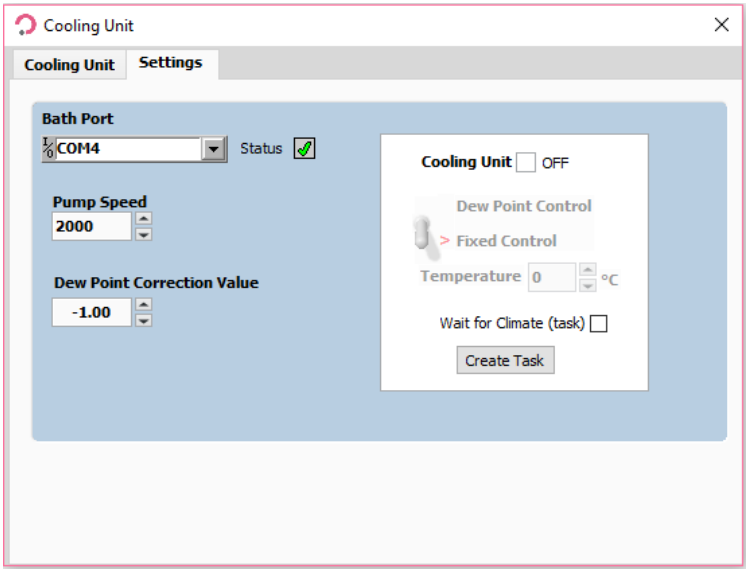


Figure 4: Dew Point Correction Value

Note. *During a long cell isolation run, the -1 °C Dew Point Correction Value can result in some condensation on the plate. However, this is not affecting the Master Mix efficiency neither is it leading to cross contamination.*

Note. *When performing a proteoCHIP workflow it is best to start by setting the temperature to 10 °C.*

3.3 Target Setup

Note. *To teach the proteoCHIP_1216, please refer to the manual "proteoCHIP_User Manual_proteoCHIP_1216_Teaching".*

To display your target, go to:

- Target Setup >> Field

For the proteoCHIP 12*16, you will always have the following configuration in Target Setup (Figure 5):

- Start point: 0,0
- Field Gap: 0,0
- Number of Fields: X=1, Y=1
- Spot per field: X=4, Y=4
- Spot pitch: X=1500 µm, Y=1500 µm

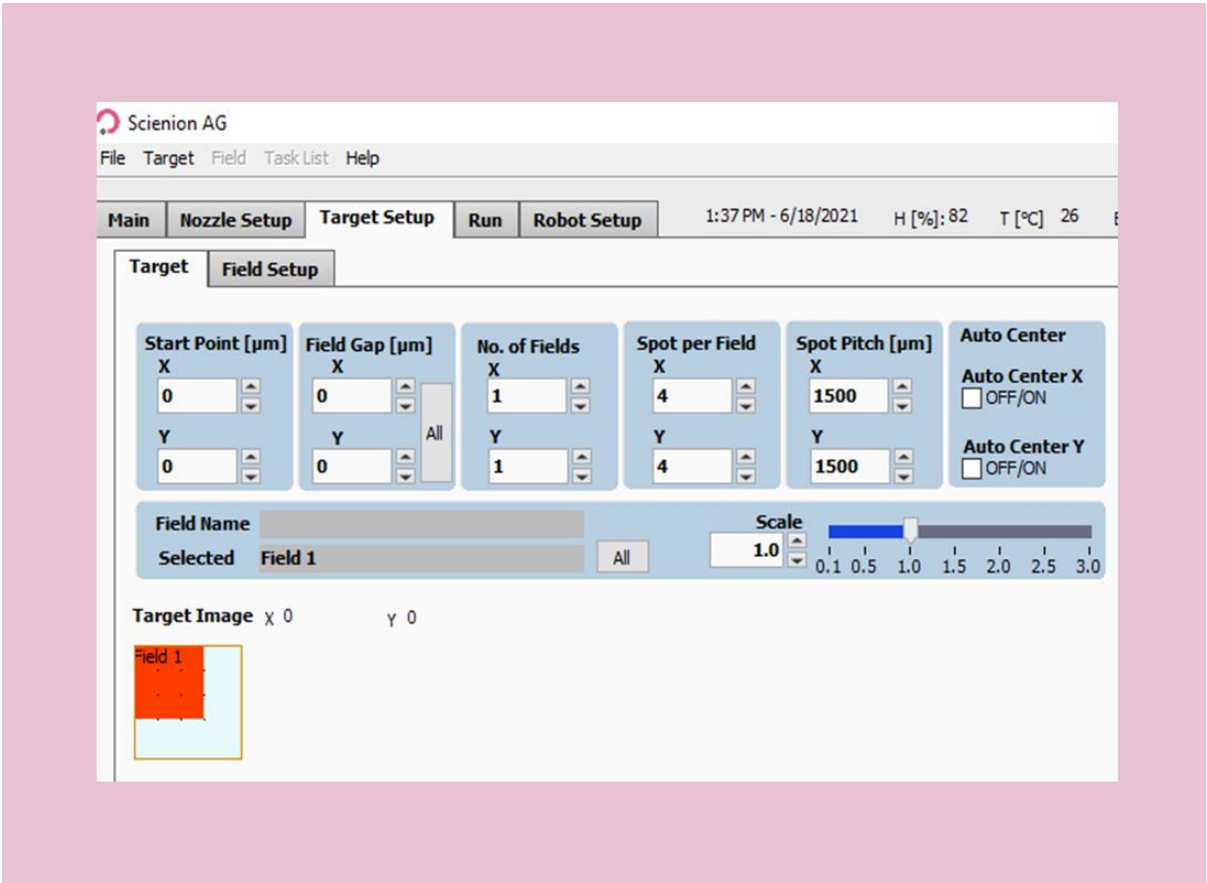


Figure 5: Target Setup

3.4 Field Setup

The “Field Setup” sub-tab is used to define:

- From which well the sample must be aspirated
  ONLY if the run contains a “TakeProbe” task (This is the case for all Runs of the labelling protocol).
- Number of drops/volume to be dispensed in which well of the Target.

Note. *To change from Volume to drops and vice versa:*
- Target Setup >> Field Setup >> “Field” at the top >> Volume entry

Note. *For every RUN, fields are saved in the software.*

You can edit these Fields using “Edit Field Table” to change:

- *From which well the sample must be aspirated.*
- *Number of drops/volume to be dispensed in which well of the Target.*

- The “Edit Field Table” can be found at: Field setup >> Edit Field table >> Popup window will appear (*Figure 6* ③).

You can then modify single wells or entire rows or columns out of the list. Wells used as “probes” can be shifted to another well/row/column.

Note. *If you want to shift the entire column from A to the column C, select whole “column” (Figure 6 ④) and then select column C (Figure 6 ⑤) and “Change all” (Figure 6 ⑥).*

You will have all the wells from the probe that will be switched to column C as seen in Figure 7.

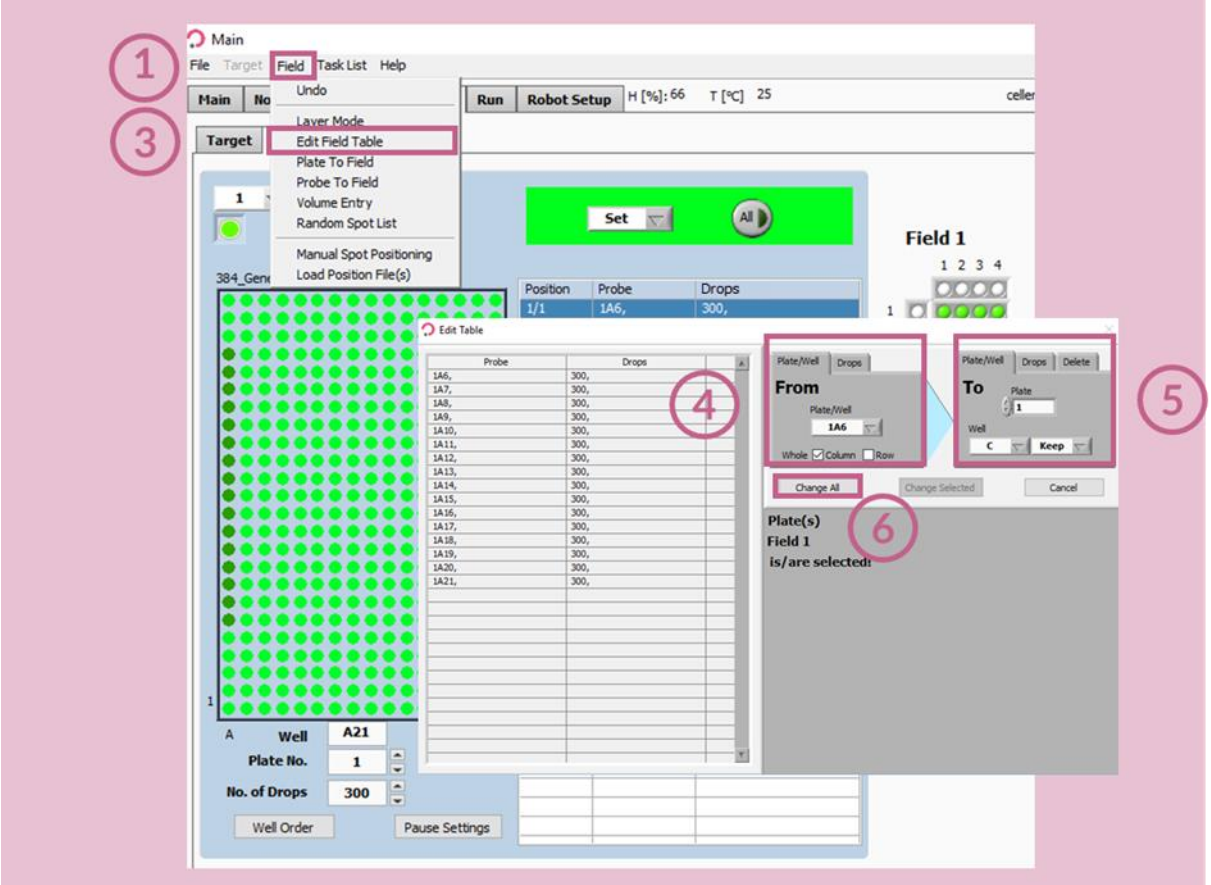


Figure 6: Edit Field Table

Note. *To assure that the exact suggested volume of each reagent is dispensed we recommend performing a Drop volume to calculate the corresponding number of drops before each liquid dispensing run.*

This should be done before dispensing the Master Mix, acetonitrile, H_20 in the rehydration steps, quenching solution and 0.1% TFA/FA.

To modify the number of drops in the Field setup refer to Figure 6 and change the number of drops by clicking on the Drops subtab (Figure 6 ④).

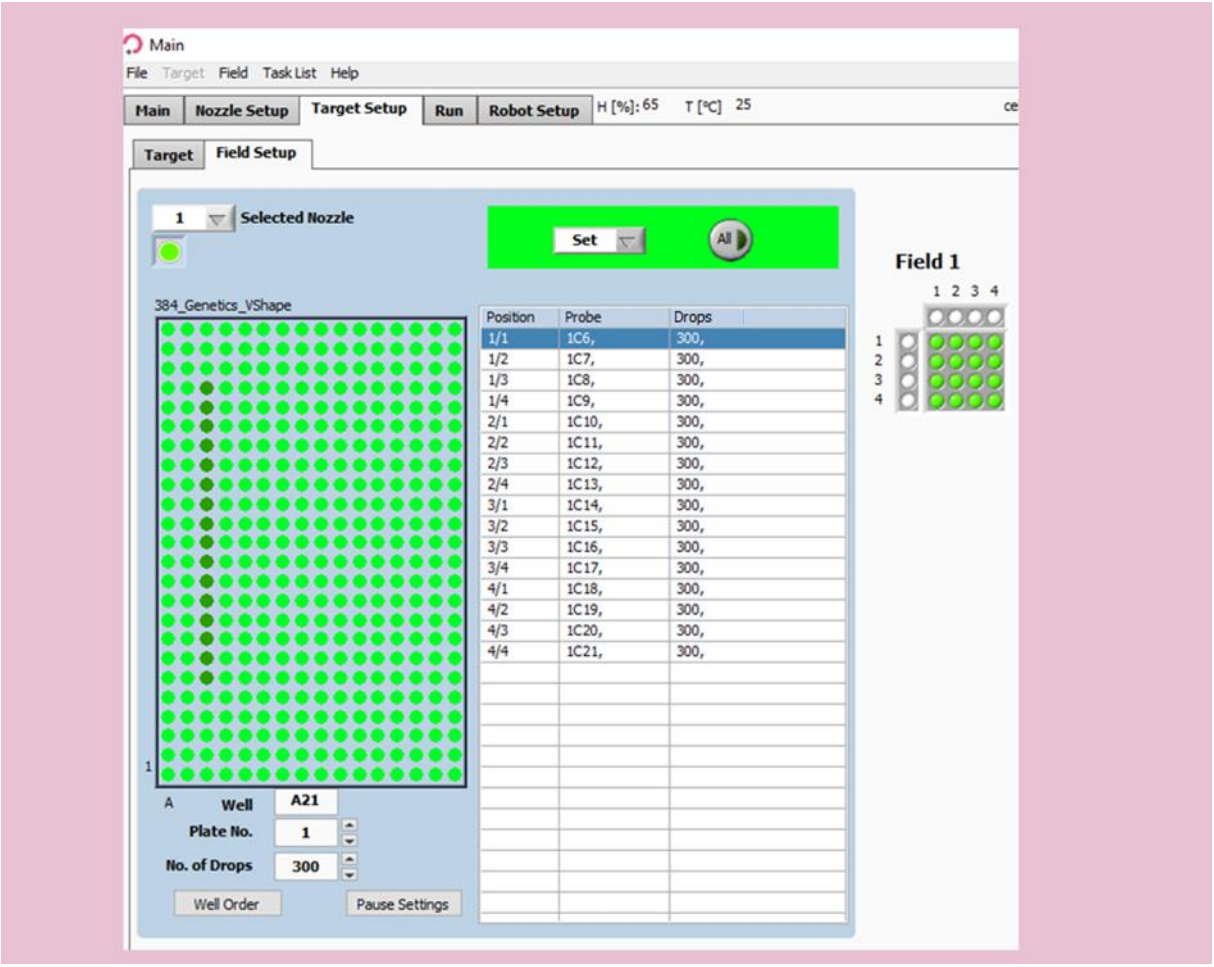


Figure 7: Field Setup

3.5 Drop setup & Start dispensing

Each dispensing run is built with the same logic, once the run starts (Figure 8):

Several pop-up messages will prompt you to load the correct amount of the desired solution in the well specified in the Target Setup section.

- Sample should be loaded in a chosen well of the 384-well source plate (The well is chosen in the Field Setup tab, see paragraph 3.4).
- Volume to be aspirated is predefined inside the Run by the “TakeProbe” task. (*Required volume will be mentioned on the message that will pop up at the beginning of the run*).
- Capillary (PDC) moves to the chosen well, aspirates the chosen volume and moves back to do a wash cycle and the camera takes a picture of the drop.
- The software will automatically check that the dispensing parameters are correct by performing a “DropCheck”. Should the system fail to detect a suitable drop, the run will pause, and the user will be able to modify the parameters to restore a stable droplet generation.
- The user must check that the dispensing parameters (Voltage and Pulse) (Figure 8 ①) are correct by pressing the “Continuous Dispensing” button (Figure 8 ②) until a drop is stable without any satellite (if any).
- The user must save the new settings if the Voltage and Pulse were modified (Figure 8 ③) and press the “Drop Check” button (Figure 8 ④) to check if the drop is identified by the software (circled in red) (Figure 8 ⑤)
- If needed, you can also perform troubleshooting tasks (AirEx, Flush_PDC, …) and uptake more sample.
- Once droplet generation is stabilized, click “Continue” to resume the run (Figure 8 ①).

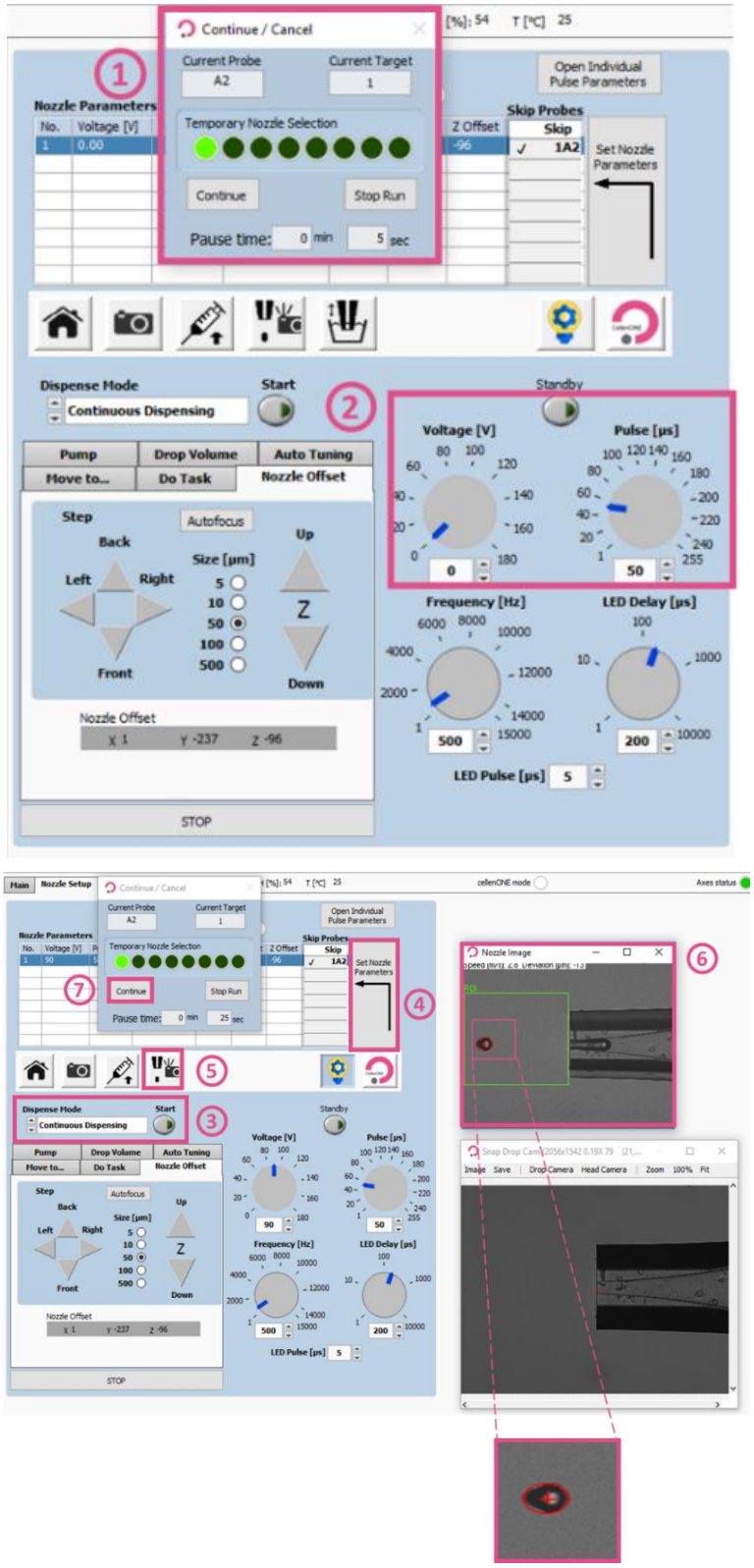


Figure 8: Drop Setup and start dispensing

3.6 proteoCHIPs inside the cellenONE

- To speed up the process, change the cooling plate temperature to 10 °C right after starting the software.

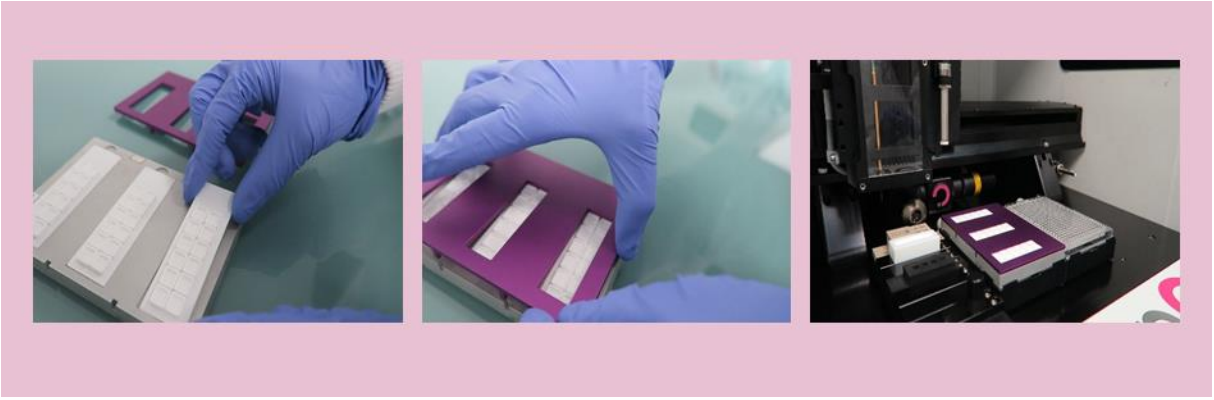


*Figure 9: proteoCHIP 12*16 inside the cellenONE holder*

Place the proteoCHIP(s) 12*16 inside the proteoCHIP 12*16 holder (ref. CHB-1216) (Figure 9).

Note. *If processing only one chip, insert it on the spot closest to you.*

- Place the cellenONE holder top (ref. CHT-1216) on top of the holder and insert on the target holder inside the instrument.

Note. *The Run 8 of the experiment is a standardized workflow for only one proteoCHIP 12*16. If you are doing more than one proteoCHIP 12*16 in parallel, you will need to repeat Run 8 for each proteoCHIP 12*16.*

E.g., If you are running 3 proteoCHIPs you will need to perform:

1X Run 1

1X Run 2

1X Run 3

1X Run 4

1X Run 5

1X Run 6

1X Run 7

3X Run 8

4 Multiplexed Single Cell Proteomics Workflow

4.1 Run 1: Master Mix dispensing

Before starting the run, already turn the temperature to 10 °C and the humidity to 45% to speed up the process.

Note.
We recommend using this Master Mix (Lysis + Digestion):
0.2% DDM, 10 ng/µL Enzyme(s), 100 mM TEAB
For each experiment 100 µL of Master Mix is needed.

To prepare 100 µL of Master Mix add the reagents in the following order:
10 µL 1M TEAB + 60 µL H_2O + 20 µL 1% DDM + 10 µL 100 ng/µL Enzyme(s)

Do not vortex the mixture, this will introduce air bubbles. *Mix by gently pipetting the solution up and down.*

Tip: *Aliquot 20 µL 1% DDM and 10 µL 100 ng/µL Enzyme(s) at -20 °C for short term usage. For long term storage of enzymes, we recommend storage at -80 °C. TEAB should be stored at 4 °C.*

Note.
We recommend using MS-grade enzymes (e.g., Trypsin Gold, Promega or Trypsin/LysC Mix, Thermo). For enzyme mixtures we recommend to also use a 10 ng/µL mixture of the enzymes.

Note.
For the Master Mix, you will probably need higher Voltage (+5-10%) to achieve an optimal drop formation. You can also optimize that during the run by clicking Nozzle Setup.

4.1.1 Probe, Run and Target selection

Master Mix dispensing (at temperature 10 °C and humidity 45%).

In the Main tab of the software, select the Probe, Run and Target to be used.

- Probe: Select the “MTP384” (Figure 10 ①)
- Run: Select “12x16_Run_1_MasterMix” (Figure 10 ②).
- Target: Select “proteoCHIP_1216” (Figure 10 ③).

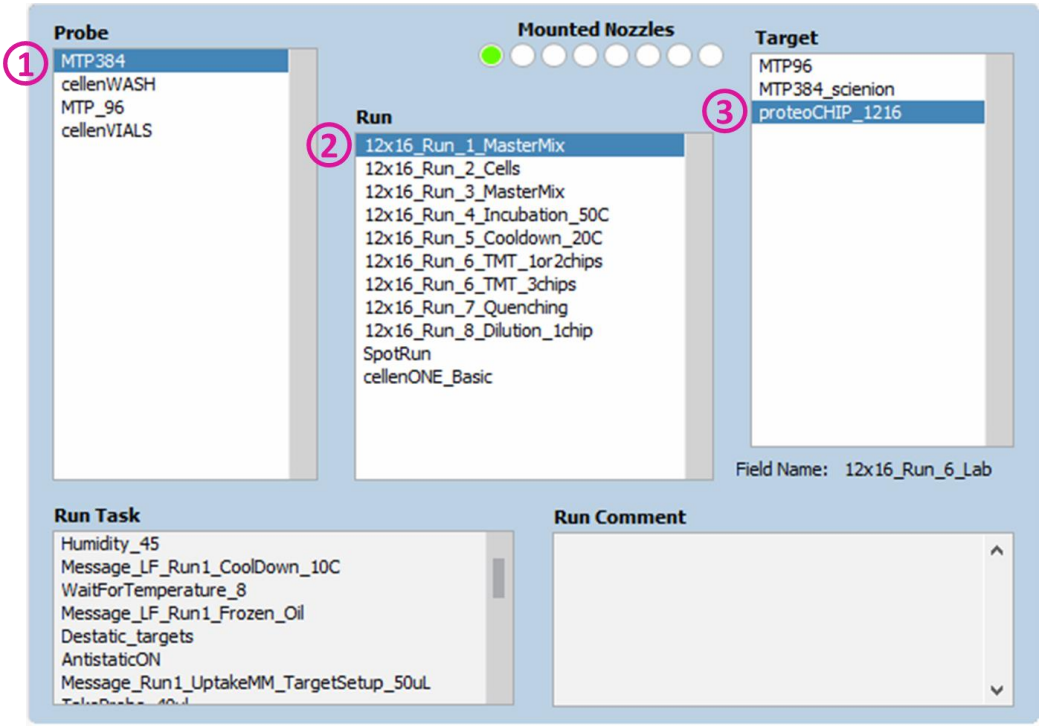


Figure 10: Master Mix run selection

4.1.2 Field selection

Load the field “12x16_Run_1_MasterMix” which will spot 135 drops (= 50 nL) per well.

To load the field:

“Target” (Figure 11 ①) >> “Load Field” (Figure 11 ②) >> Select the field corresponding to the run (Figure 11 ③) and you will have the field corresponding to Figure 12.

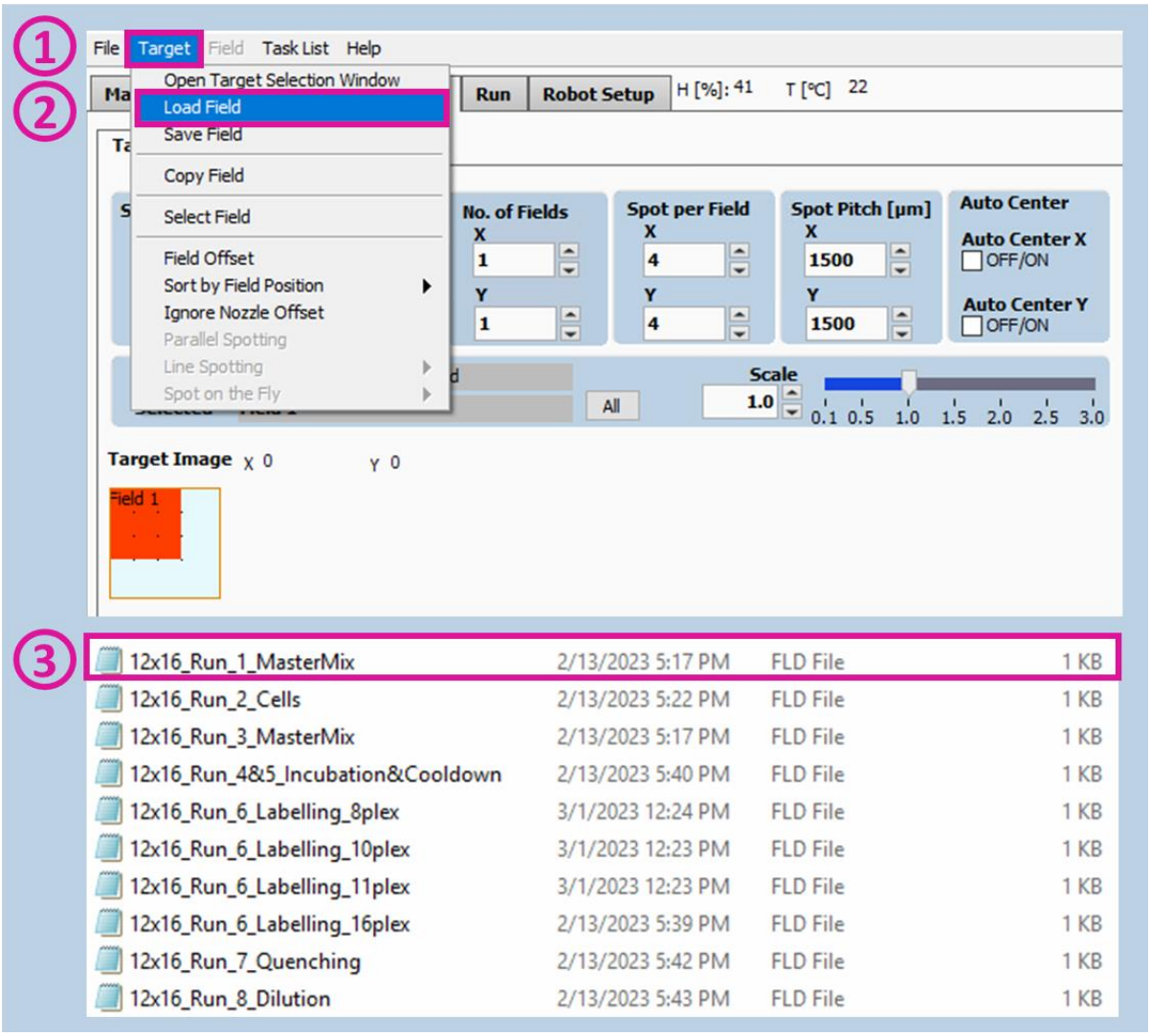


Figure 11: Load Field Run 1

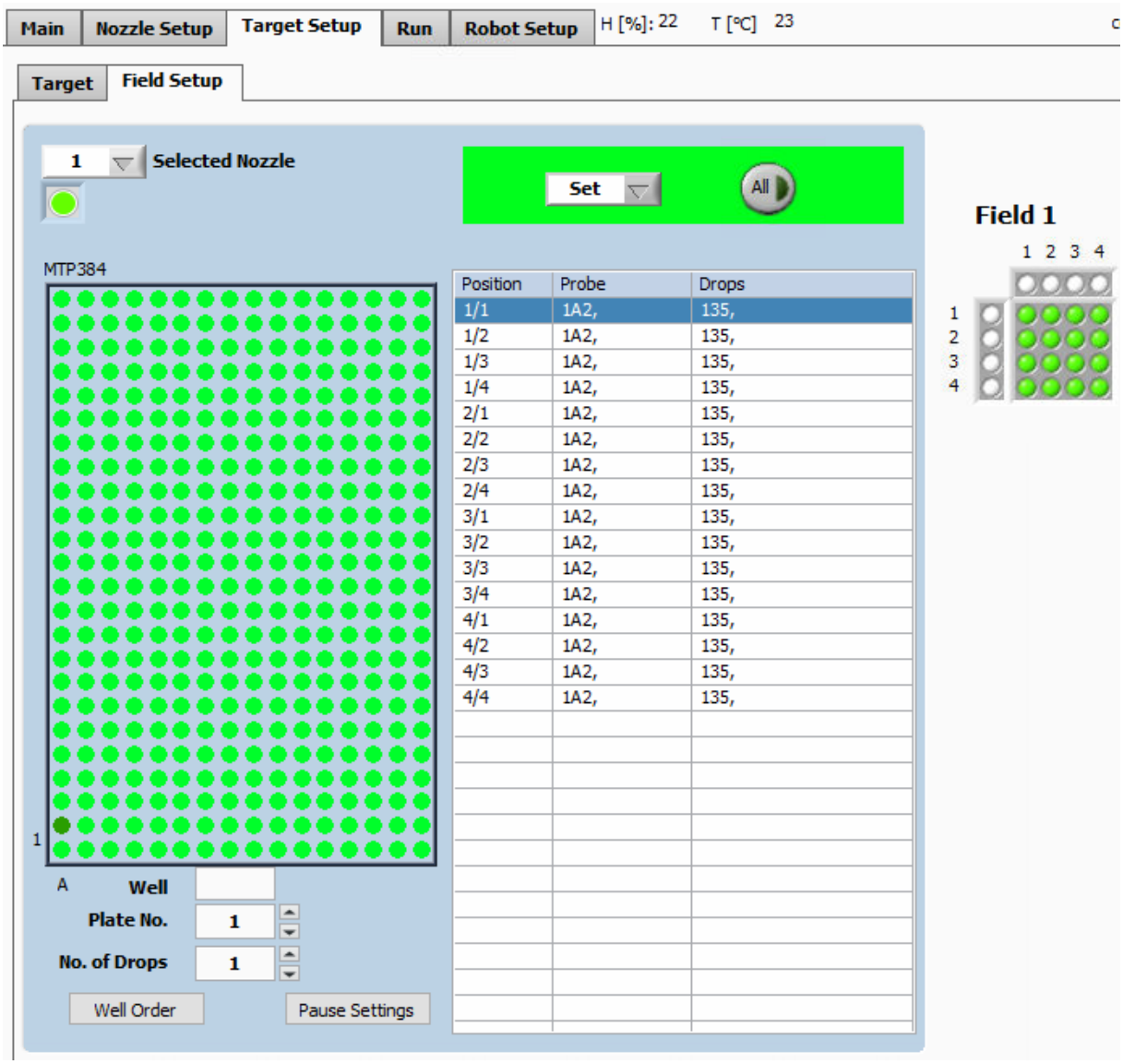


Figure 12: Field Run 1

4.1.3 Start the Run

Once the proteoCHIPs are loaded in the cellenONE as seen in Chapter 3.6., check that you selected the right Probe, Run and Target in the run window (Figure 13 ①). Start the run (Figure 13 ②), select the number of proteoCHIPs that you want to dispense in (Figure 13 ⑤ and click “OK” (Figure 13 ⑥).

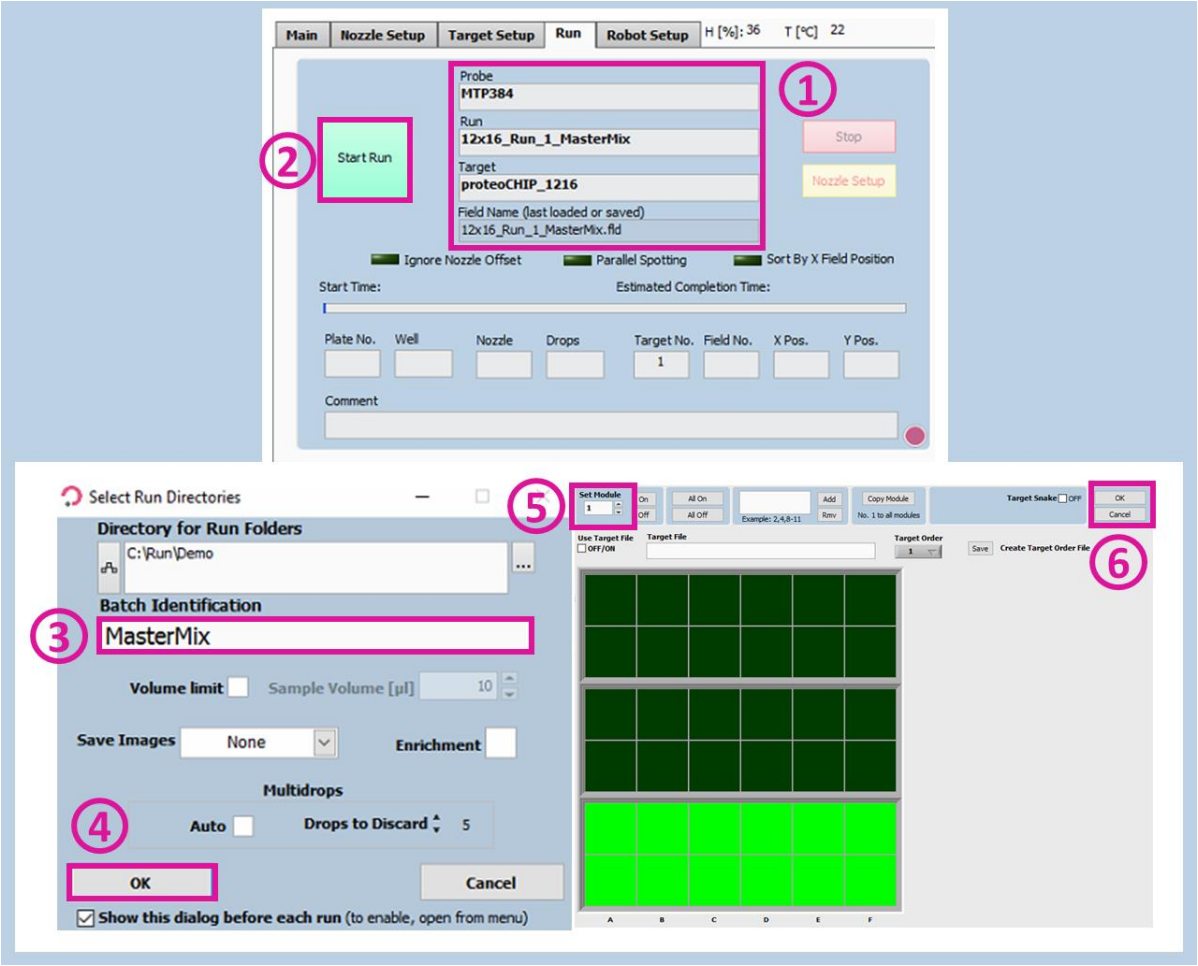


Figure 13. Start Run 1

Note.
When loading the Master Mix, make sure, that no air bubbles are introduced.

Caution. *ALWAYS use at least the target 1 to make the "AutoDropDetection" work (first proteoCHIP, as shown above).*

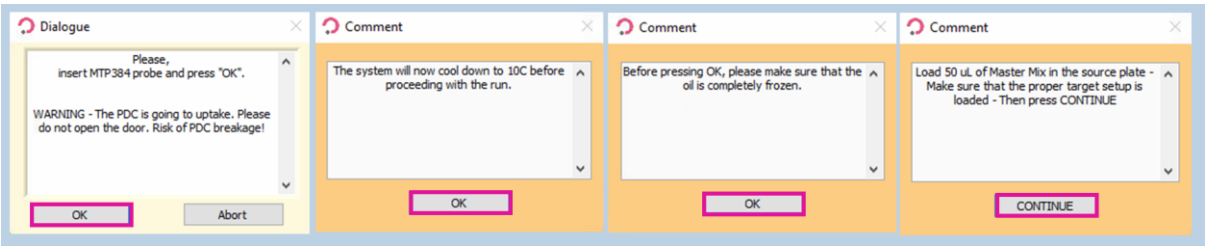


Figure 14: Messages for Run 1 Master Mix

4.2 Run 2: Cell isolation

Prepare your cells at a concentration of 100-200 cells/µL in degassed PBS.

4.2.1 Probe, Run and Target selection

In the Main tab of the software, select the Probe, Run and Target to be used (Figure 15).

- Probe: Select the “MTP384”.
- Run: Select “12x16_Run_2_Cells”.
- Target: Select “proteoCHIP_1216”.

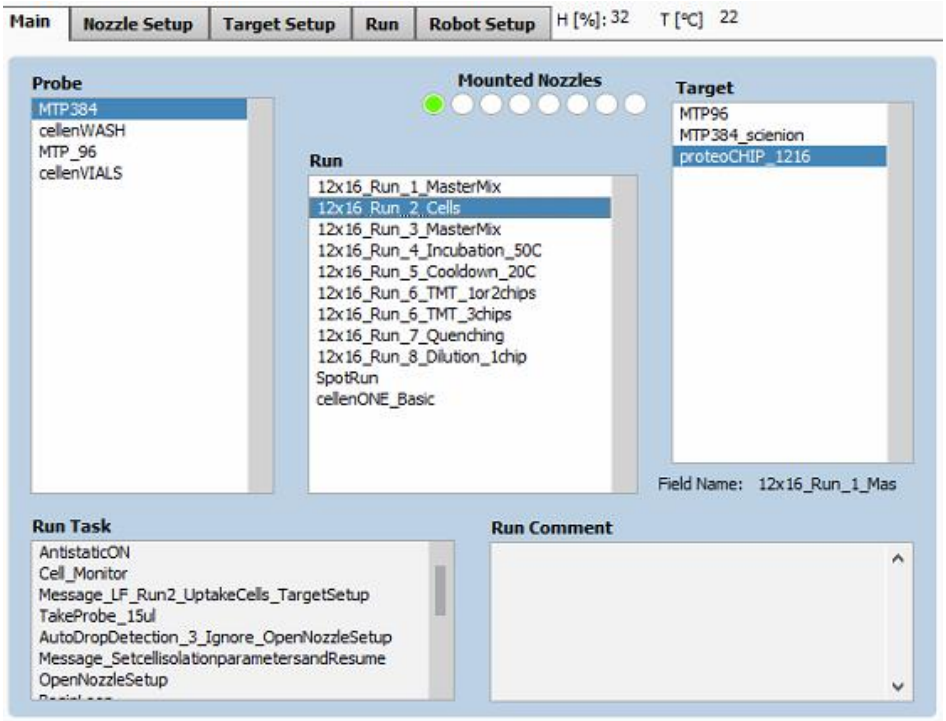


Figure 15. Run 2 Single cell isolation

4.2.2 Field selection

Click on the “Target Setup” tab, and the 1st sub-tab, “Target”. Target is the same for the whole workflow, but the field setup will need to be changed at every step.

Note. *To isolate and dispense single cells, positions defined in the field setup must contain only one drop per well.*

Load the field “12x16_Run_2_Cells” which will spot one drop per well (which equals to one cell per well). To load the field, click in “Target” (Figure 16 ①) >> “Load Field” (Figure 16 ②) and select the field corresponding to the run (Figure 16 ③, Figure 17).

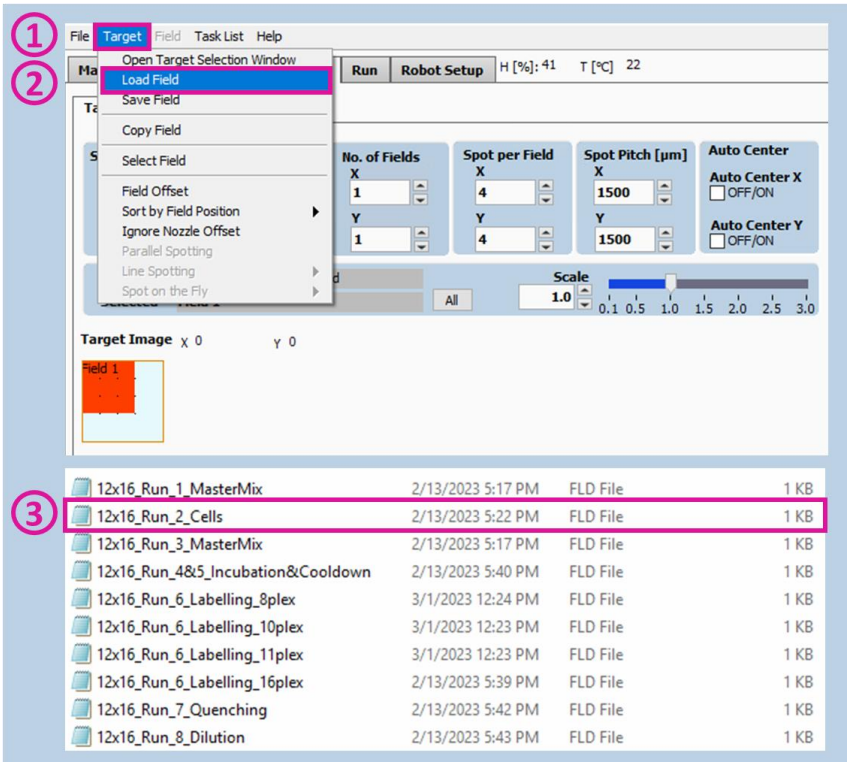


Figure 16: Load Field for cell isolation

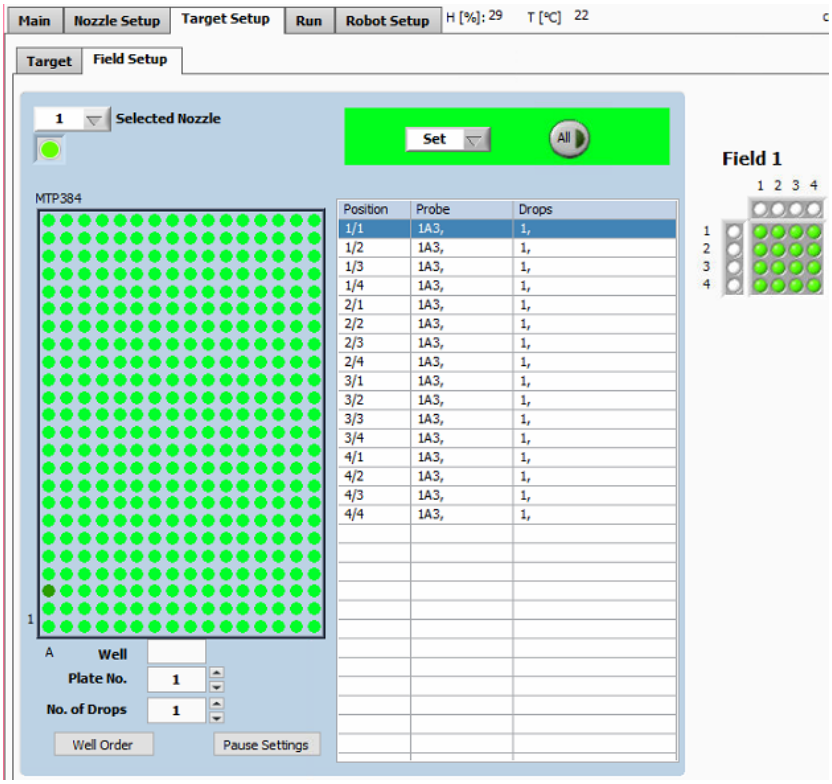


Figure 17: Field Setup for cell isolation

Note. *If you wish to dispense more than one single cell, the following syntax must be used: 1, 1 (for isolation of two single cells); 1, 1, 1 (for isolation of three single cells); 1,1,1,1 (for isolation of four single cells) etc. You can do so by clicking multiple times (n) on the well where you want to have 1+n cells isolated.*

4.2.3 cellenONE module

Note. *Load 30 µL of cell suspension (100-200 cells/µL) in a chosen well of the 384-well source plate.*

For this run you will need to **open the cellenONE window** and start the run in this same window. Once **cellenONE module** is opened, it **should not be closed** before the end of the run (nevertheless the window can be minimized)**.**

Select the "Nozzle Setup" tab (Figure 18 ①) and click on the cellenONE logo (Figure 18) to open the "cellenONE mode" module for single cell isolation.

Please refer to the Software reference Guide Chapter 7 "Single cell isolation" to understand all the options and features for single cell dispensing.

The cell isolation run is still performed at 10 °C and ~45% humidity.

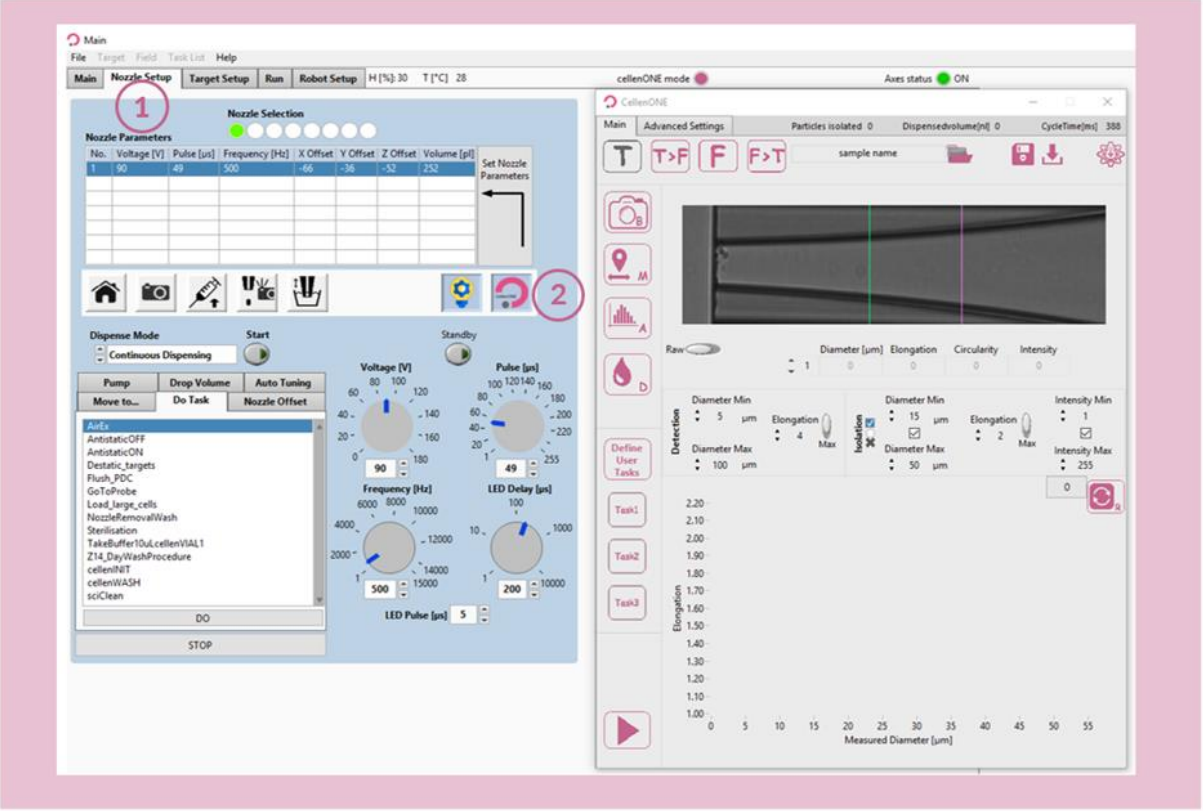


Figure 18: cellenONE module for single cell isolation (right window)

Before starting, define the folder location and name your experiment by clicking the following button:

All files associated with your experiment will be saved in this location. By default, data is saved in the last folder chosen and named “samplename_date”. Start the run by clicking on:

Please note that you can always pause the run with the Nozzle setup shortcut:

You will be able to modify the parameters if wanted.

Select the proteoCHIP positions that will be used (Figure 19) and click “OK” (Figure 19 ②).

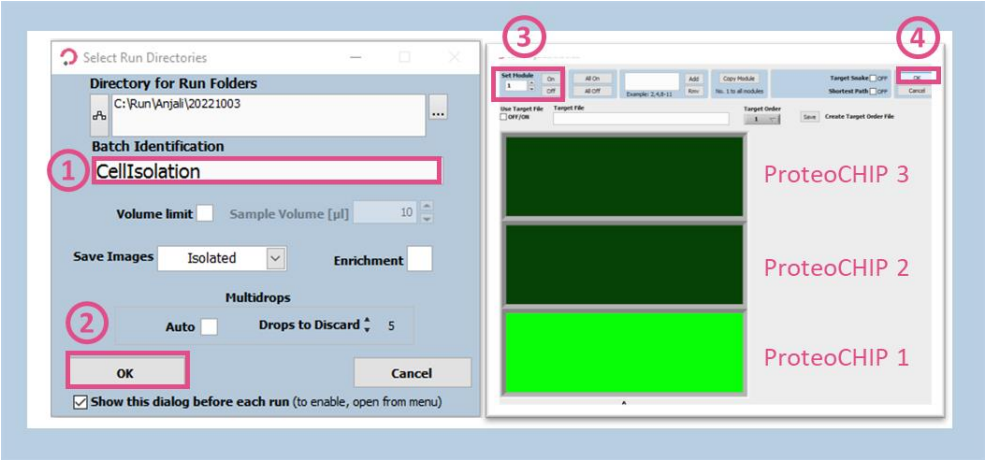


Figure 19: Start the run for single cell isolation

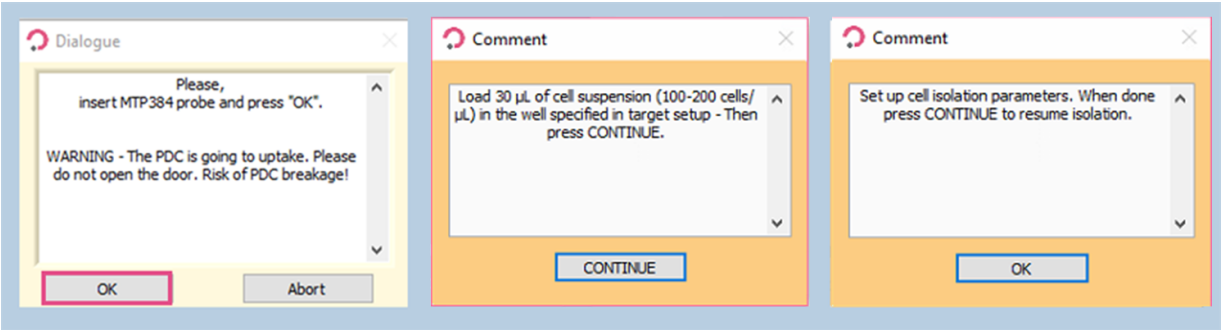


Figure 20: Pop-up messages

After uptaking the desired volume of the cell sample, the PDC comes back to the camera station so you can define the cell parameters used for cell isolation.

4.2.4 Set the detection parameters

The “Main” tab of cellenONE module (Figure 21) displays images of the region of interest of the PDC in which cells are detected. During dispensing, cells are travelling from right (upper part of the nozzle) to left (nozzle tip). Information below the image (i.e., Diameter, Elongation, Circularity, and Intensity) is given for the leftmost detected cell, i.e., the closest to the nozzle tip.

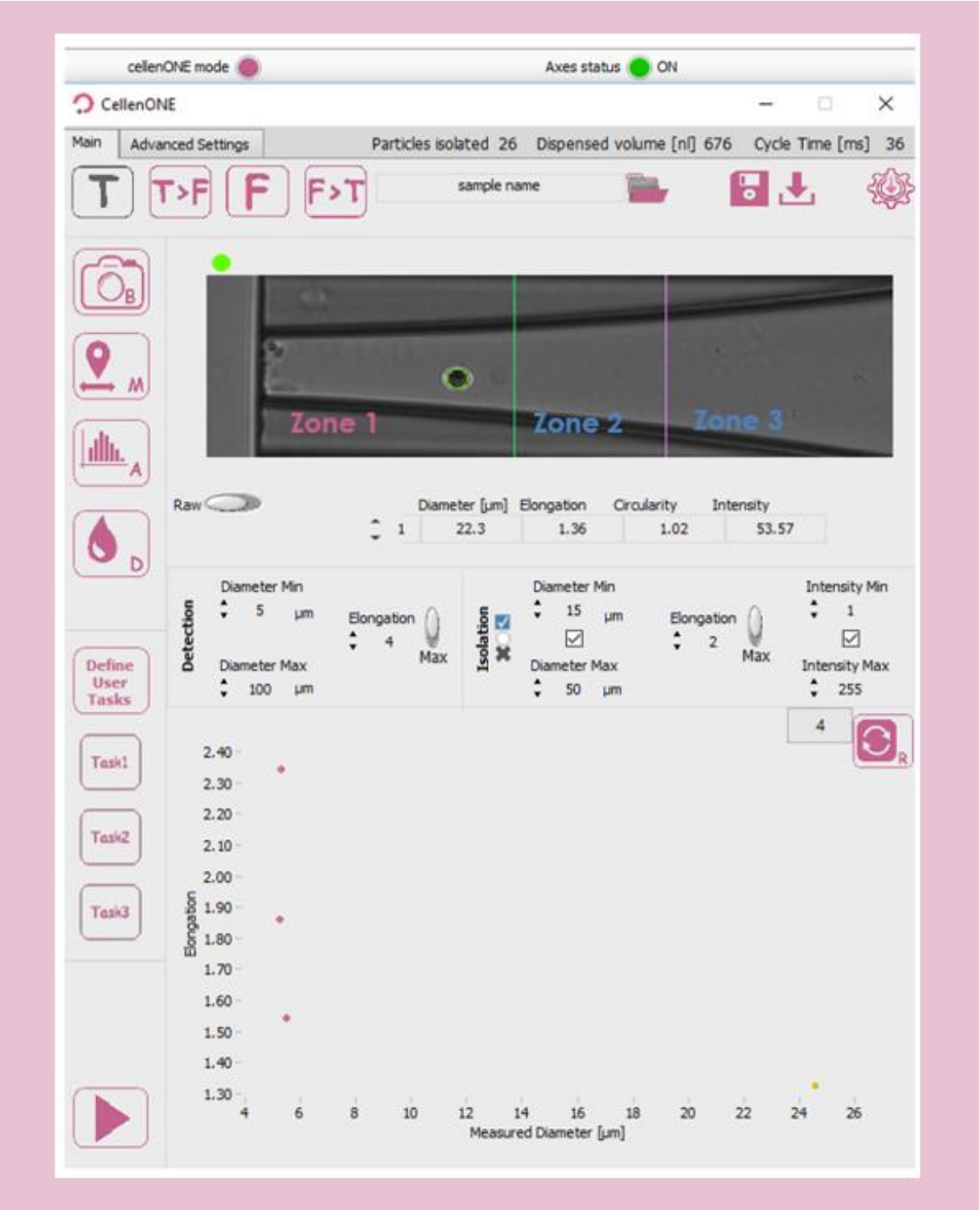


Figure 21: Main tab of cellenONE® module.

Hint. *The elongation factor can be used to effectively differentiate two aggregated cells from a single cell as these will have different elongation factors.*

Round single cells: ***<1.6****; Doublets* ***>2.5***

This section is used to define detection and isolation parameters (Figure 22). These parameters are crucial to successfully isolate and dispense single cells.

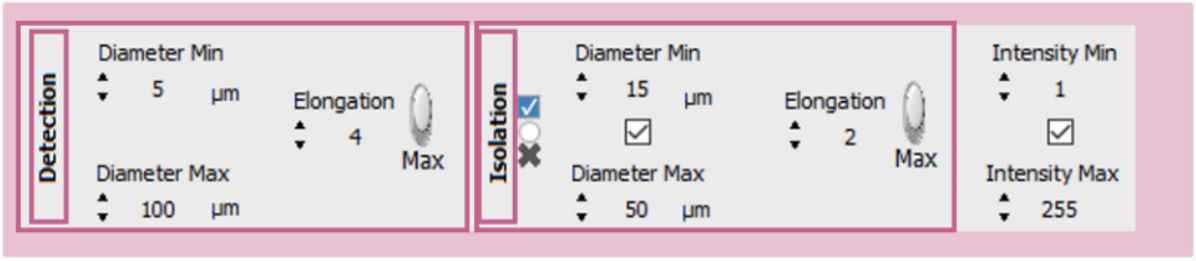


Figure 22: Detection and isolation parameters

Detection parameters: These parameters will determine what the software will detect and consider as an object (e.g., cell or particle). As a result, these parameters should be "large" enough to ensure that all cells and cell clusters are detected as objects.

- **Min diameter:** detected diameter of the smallest object that should be detected. An object with a diameter lower than the entered value will **not be detected**.

- **Max diameter:** detected diameter of the largest object that should be detected. Beware that cell aggregates will typically be detected as a single large object. An object with a diameter bigger than the entered value will **not be detected**.

- **Elongation:** an object with an elongation above (for MAX) or below (for MIN) the entered value will **not be detected**.

HINT 1 The detected diameter is typically about 10% larger than real diameter.

HINT 2 A detected object is surrounded by a green or a red circle:

- a **green circle** means that the object fulfils the isolation parameters and would be isolated.
- a **red circle** means that the object does not fulfil the isolation parameters and would not be isolated.

HINT 3 To start, set wide detection parameters and then, restrict them if needed.

- Diameter min: 5 µm
- Diameter max: 100 µm
- MAX Elongation: 4

After loading the sample, do a background:

Get background button: Takes a reference background image. This button should only be pressed when the nozzle is free of cells and/or particles and when the PDC is in front of the camera.

Make several drops:

Test droplet button**:** Ejects a single droplet.

Check visually by eye that every particle inside the PDC is detected (i.e., that the particle is circled by a red or green circle).

4.2.5 Set the ejection boundary

Once detection parameters are set, do a mapping:

Start mapping button**:** Starts the mapping process.

This process maps the position of cells inside the PDC during the dispensing of droplets in front of the camera. This will determine **the size of the ejection zone** (Zone 1 on Figure 21). Pressing this button during mapping will interrupt the mapping process.

At the end of the mapping process, the ejection boundary is set automatically according to the blue and green dot distribution inside the PDC (Figure 23). Cells that were detected in the location of blue dots were subsequently detected again inside the PDC upon next droplet generation. Whereas green dots correspond to the last seen location of a cells within the PDC (i.e., upon next droplet generation these cells were ejected from the PDC). Once done, click “Save & close”.

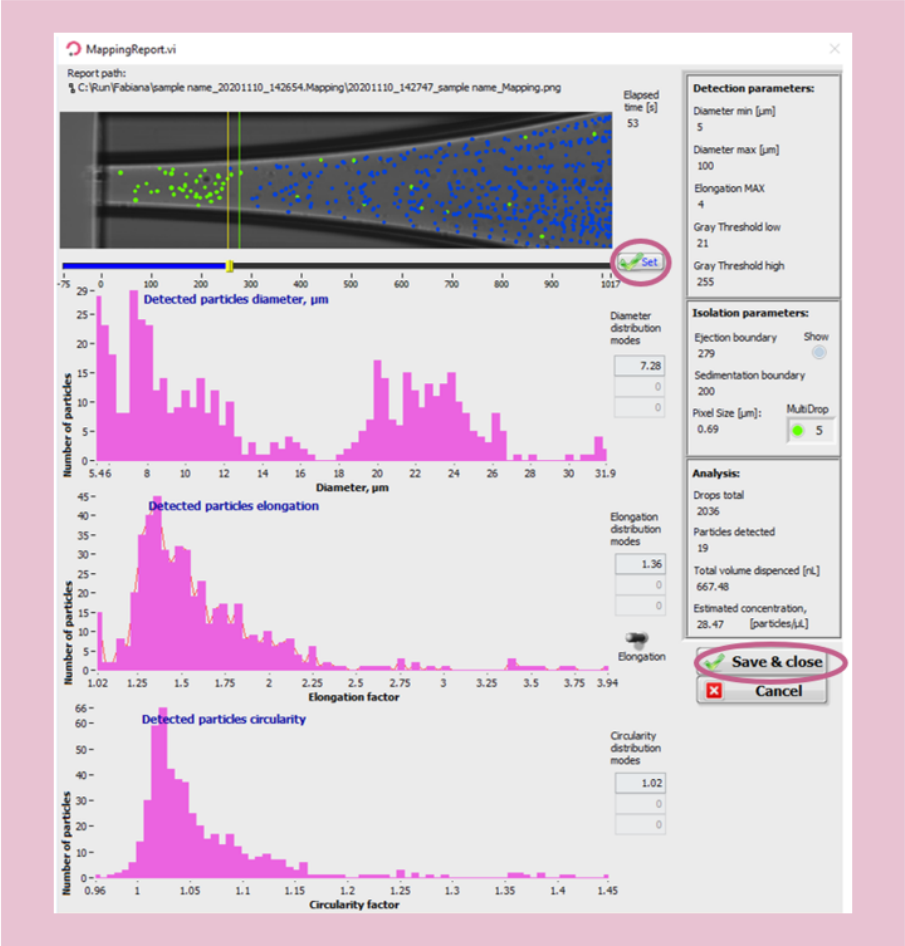


Figure 23: Mapping

Hint. *At the end of the mapping process, the ejection boundary is set automatically according to the blue and green dots distribution inside the PDC. To* ***adjust the ejection zone manually****,* ***move the slider*** *by clicking and dragging the positioner on the ruler below the image and* ***press "Set" to validate****.*

4.2.6 Set the isolation parameters

Once Mapping is done, **set up your isolation parameters**.

Isolation parameters: These parameters can be determined from the characteristics of the cells shown in the mapping report (Figure 23) and adjusted as needed to include only cells of interest. These values are critical to ensure satisfactory single cell isolation. All objects that fulfil the isolation parameters and single cell condition will be isolated.

- **Min diameter:** detected diameter of the smallest object that should be isolated.
- **Max diameter:** detected diameter of the largest object that should be isolated.
- **Elongation:** an object with an elongation above (for MAX) or below (for MIN) the entered value will **not be isolated**.

Three options are available here:

- Draw a gate right after the mapping from the scatter plot that was just performed or enter values manually into the isolation parameters based on the mapping.
- Open the mapping (in the folder you chose earlier, folder name.mapping) and interpret the different scatter plots.
- Perform an analysis of your sample.

Analyze sample button: Analyzes 100 cells from your sample.

This can be used to estimate the heterogeneity of your sample. At the end of the analysis, a window will pop up with scatter plots (Figure 24). The number of analysed cells can be set in the “Advanced Settings” tab. You can do a gate to choose new values for isolation parameters.

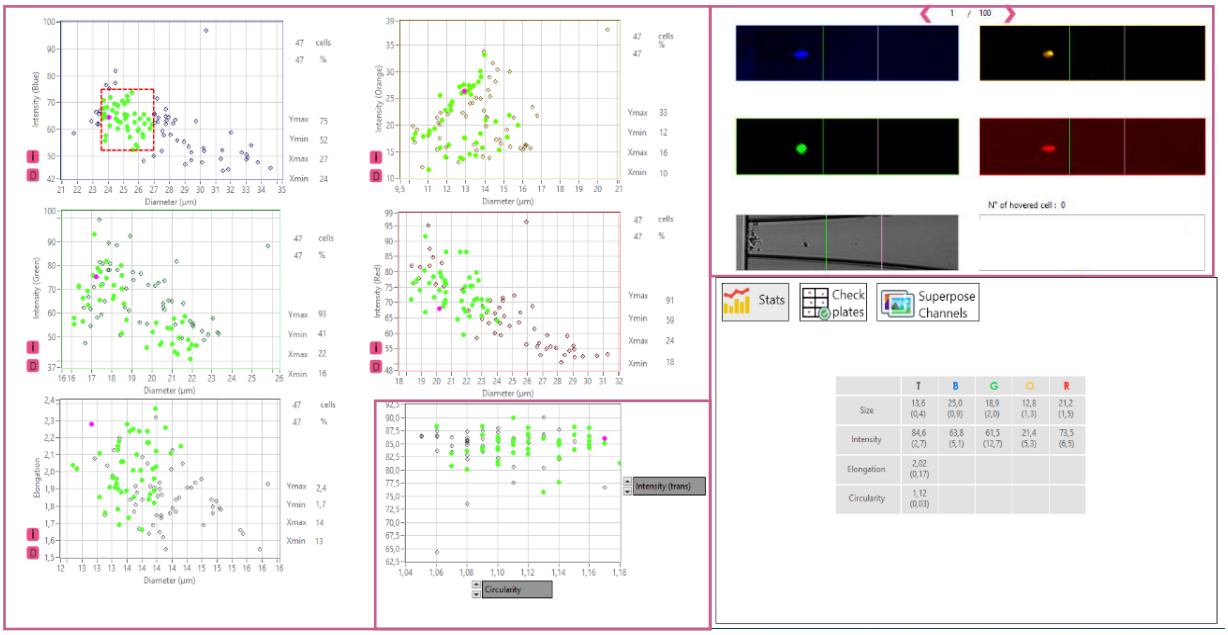


Figure 24: Scatter Plot

4.2.7 Check the setup of your experiment

Check the ejection boundary:

Test droplet button**:** Press it manually multiple times to verify the ejection boundary. When a particle is inside the ejection zone, it should be ejected within the next droplet when pressing the droplet button once more.

Check the isolation parameters:
cells (or particles) of interest should be circled with a green circle and the other particles with a red circle.

Note. *Continuous drop generation can be activated by simultaneously clicking on the left mouse button and Shift button and is interrupted by clicking once again on the test droplet button.*

Once you are satisfied with your parameters you can click Continue to start dispensing your cells (Figure 25).

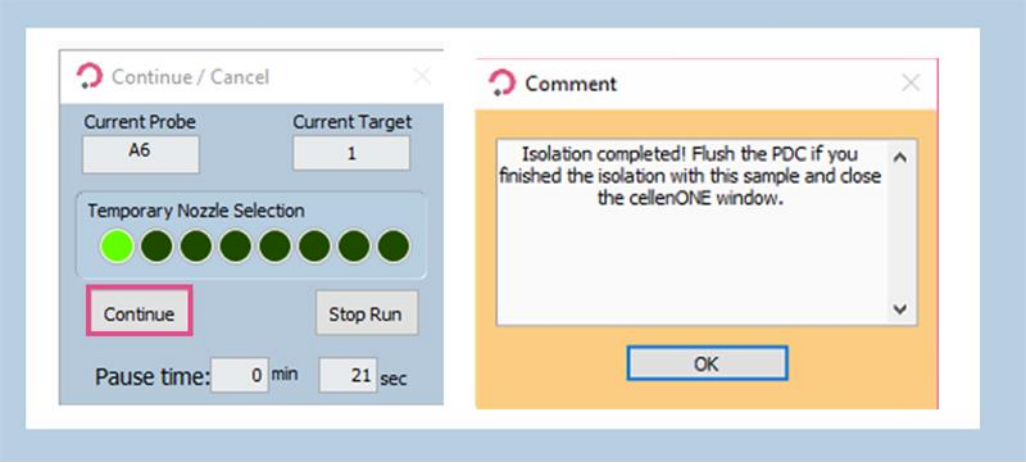


Figure 25: Starting the cell isolation run

4.3 Run 3: MasterMix dispensing

In this task, 50 nL of Master Mix is dispensed again. Splitting 100 nL of Master Mix dispensing in two separate steps (one before and one after cell dispensing) has shown to improve overall quality of the protocol.

4.3.1 Probe, Run and Target selection

Master Mix dispensing (at temperature 10 °C and humidity 45%).

In the Main tab of the software, select the probe, run and target to be used.

- Probe: Select the “MTP384” (Figure 26 ①).
- Run: Select “12x16_Run_3_MasterMix” (Figure 26 ②).
- Target: Select “proteoCHIP_1216” (Figure 26 ③).

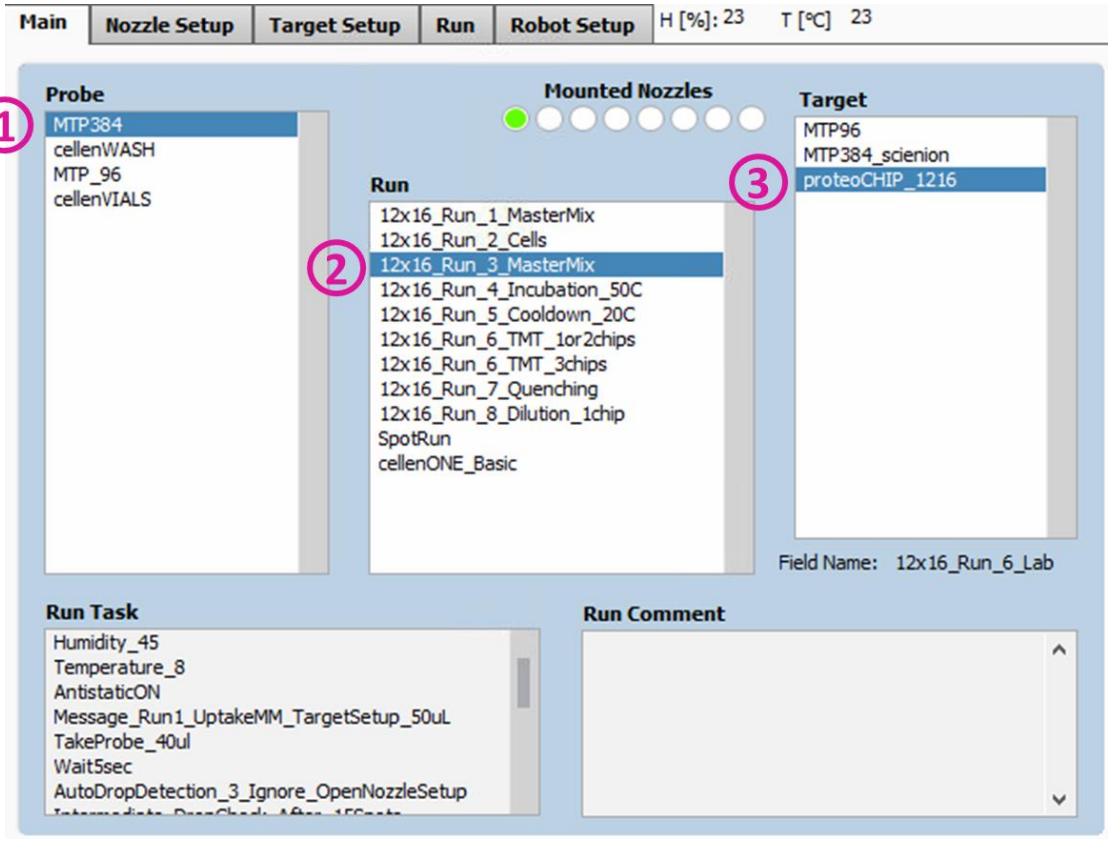


Figure 26: Master Mix Run 3 selection

4.3.2 Field selection

Load the field “12x16_Run_3_MasterMix” which will spot 135 drops (= 50 nL) per well.

To load the field:

“Target” (Figure 27 ①) >> “Load Field” (Figure 27 ②) >> Select the field corresponding to the run (Figure 27 ③) and you will have the field corresponding to the Figure 28.

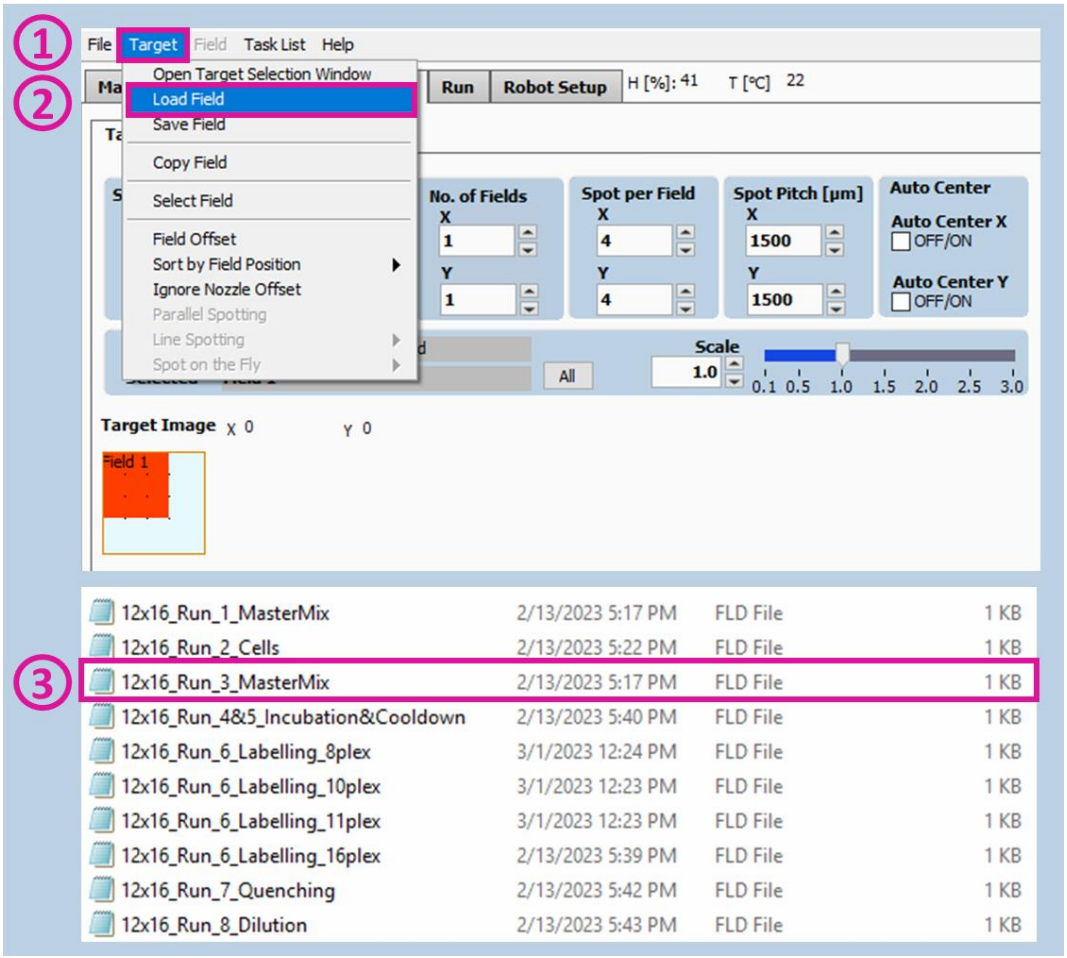


Figure 27. Load Field Run 3

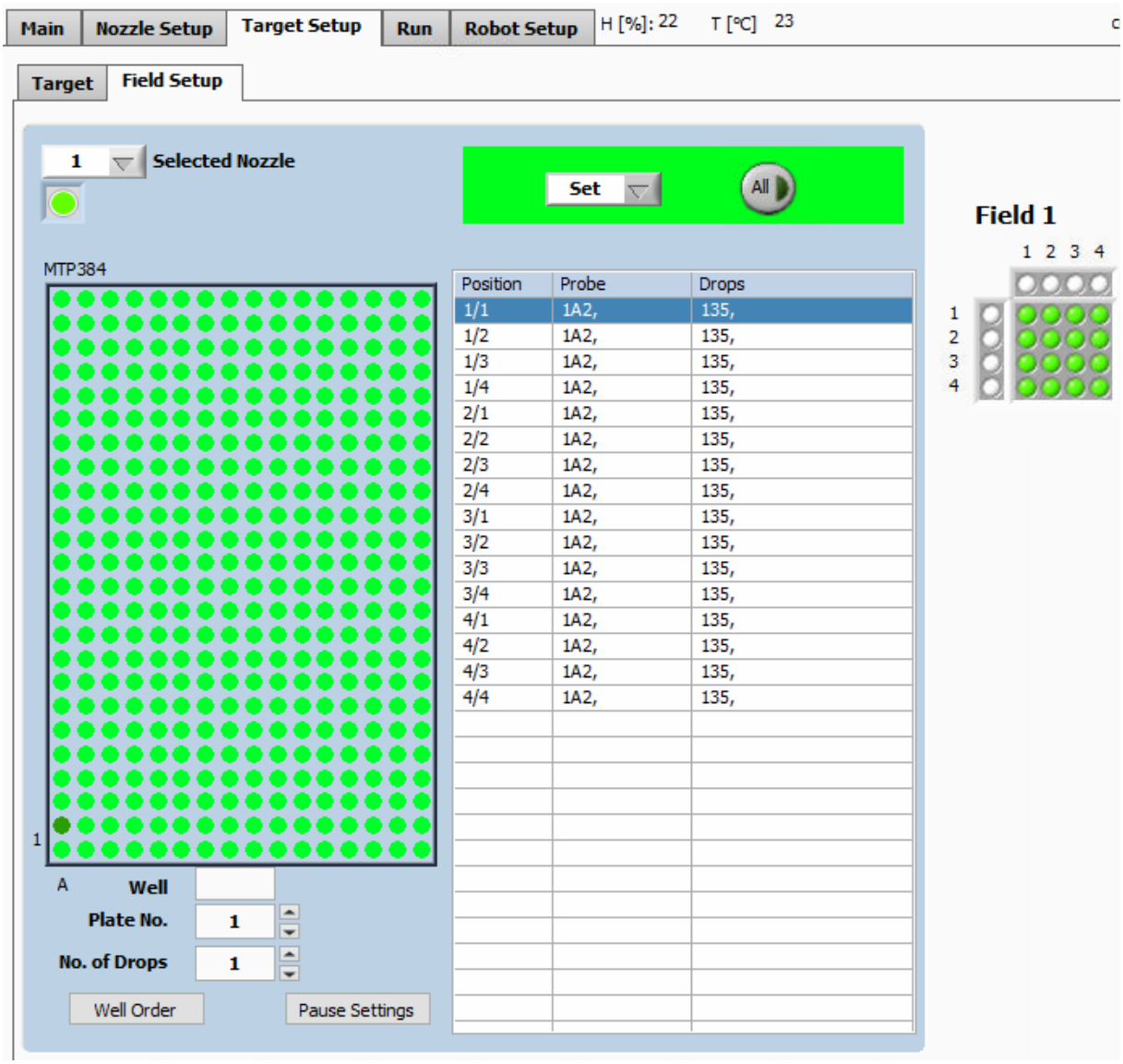


Figure 28: Field Run 3

4.3.3 Start the Run

Check that you selected the right Probe, Run and Target in the Run tab (Figure 29 ①). Start the run (Figure 29 ②), select the number of proteoCHIPs that you want to dispense in (Figure 29 ⑤) and click “OK” (Figure 29 ⑥).

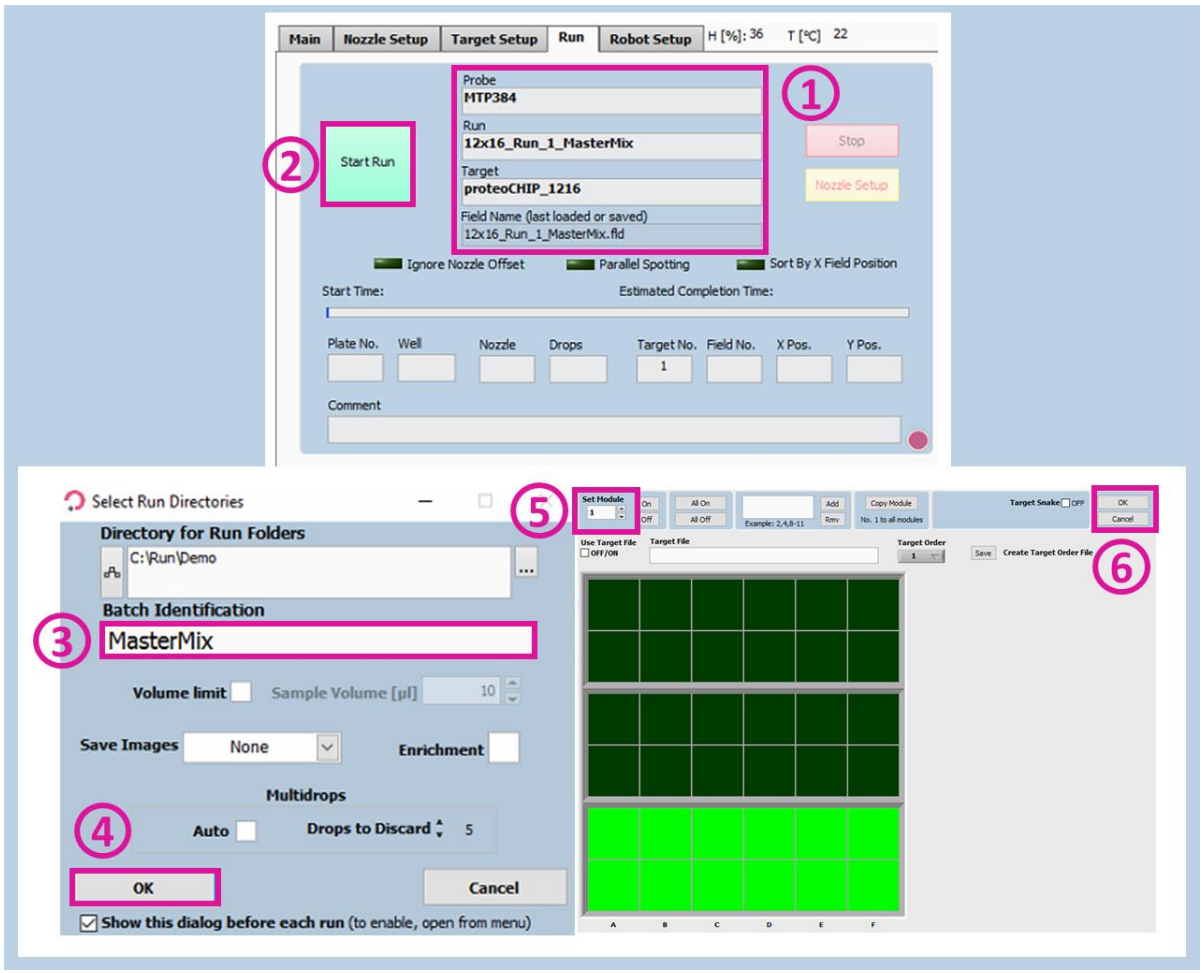


Figure 29: Start Run 3

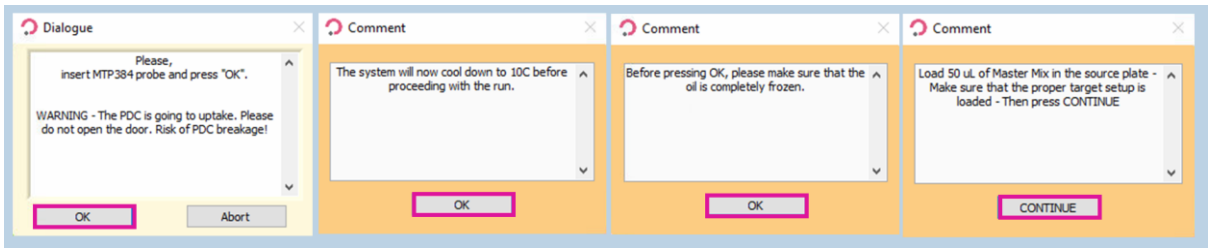


Figure 30: Messages for Run 3 Master Mix

4.4 Run 4: Incubation at 50 °C

Single cells have been isolated into wells containing lysis and digestion buffer. The next step is to perform the incubation for:

- Cell lysis
- Digestion of the proteins

This step can be performed directly on deck of the cellenONE. This incubation step will last 2 hours at a temperature of 50 °C. In addition to an increased ambient humidity (85%, the PDC will regularly dispense milliQ water in each well to maintain a constant water content and create a mechanical mixing phenomenon to help achieving homogeneous cell lysis and protein digestion.

4.4.1 Probe, Run and Target selection

In the Main tab of the software, select the probe, run and target to be used (Figure 31).

- Probe: Select the “MTP384”.
- Run: Select “12x16_Run_4_Incubation_50C”.
- Target: Select “proteoCHIP_1216”.

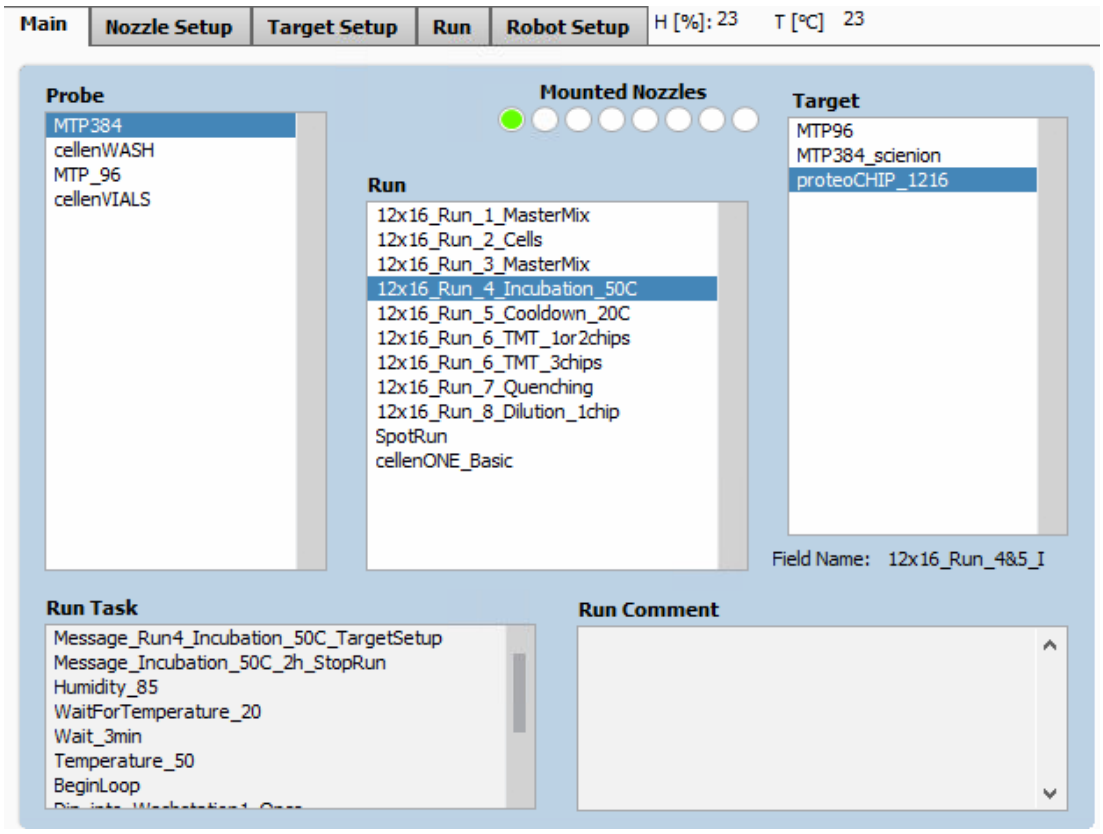


Figure 31: Run selection for incubation

4.4.2 Field selection

Load the correct field setup, then start the run and select all 3 chips (regardless of the number of chips you are using), then click OK (Figure 34).

During the incubation the cellenONE will continuously spot 80 drops (= 30 nL) per well.

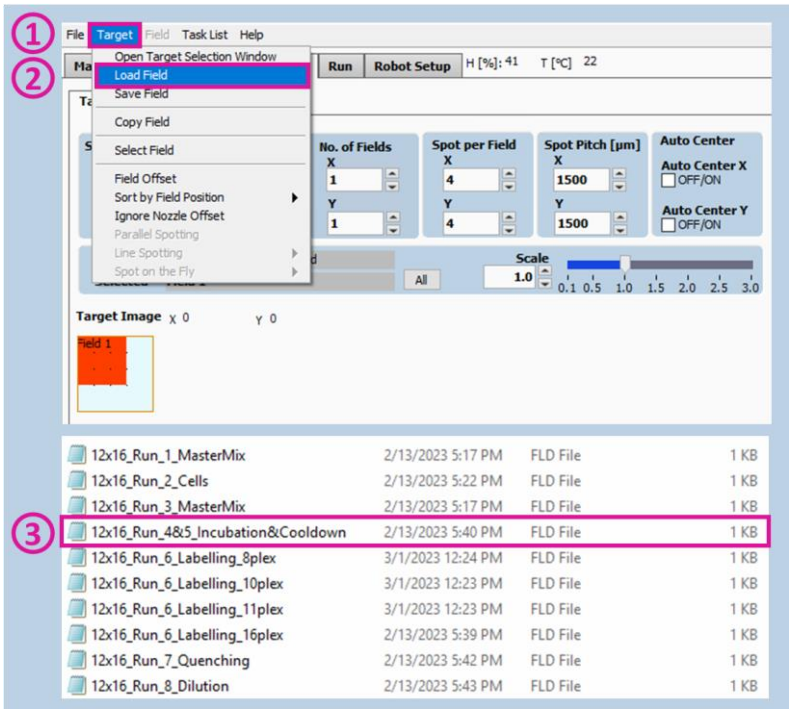


Figure 32: Load field for incubation

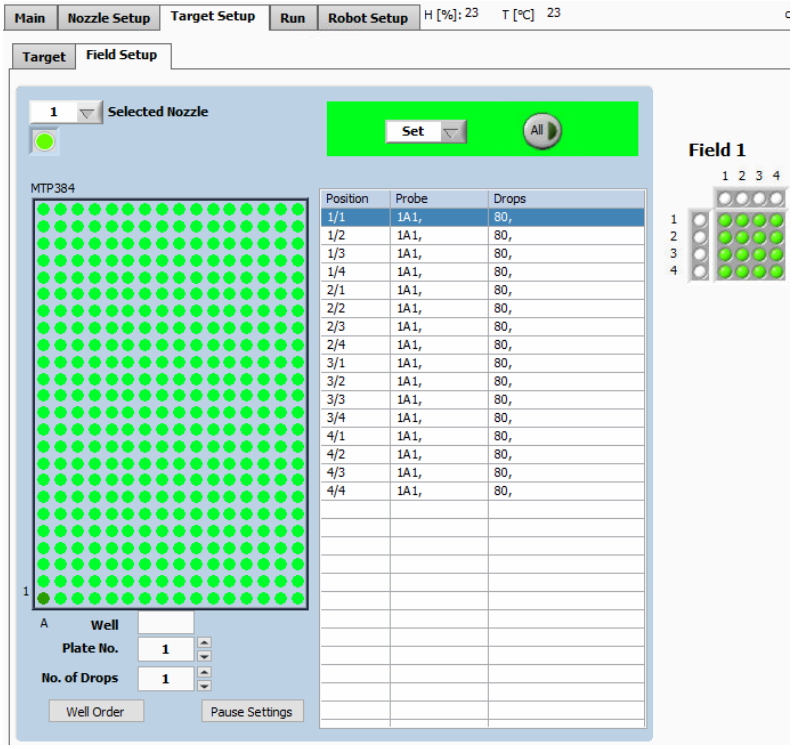


Figure 33: Field setup for incubation

4.4.3 Start the Run

Warning. *Please make sure that the 3 proteoCHIPs are selected regardless of the number of chips processed.*

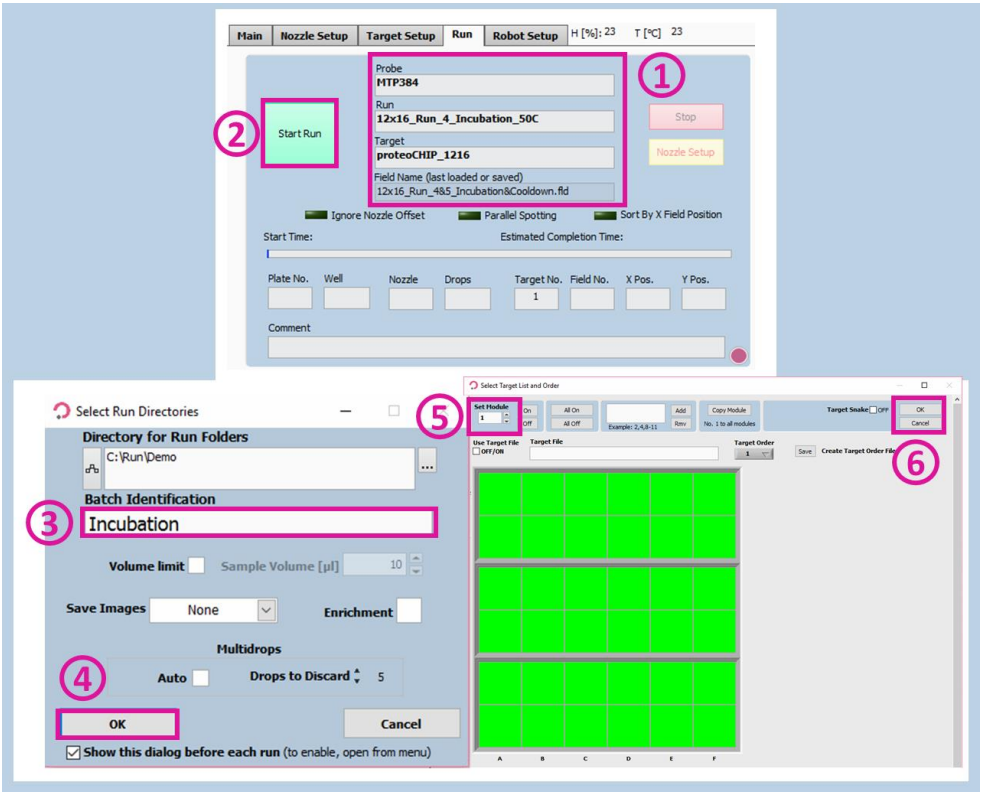


Figure 34: Start the Run for Incubation

Once the Run has started, the humidity and temperature are automatically set. A pop-up message will be displayed, click “OK” to start the 2 hours incubation (Figure 35).

Figure 35: Incubation message

After 2 hours, once you are ready to proceed to the next step, click the red “Stop” button. Dismiss the pop-ups by clicking “Exit Run” and then “Partially spotted” (Figure 36).

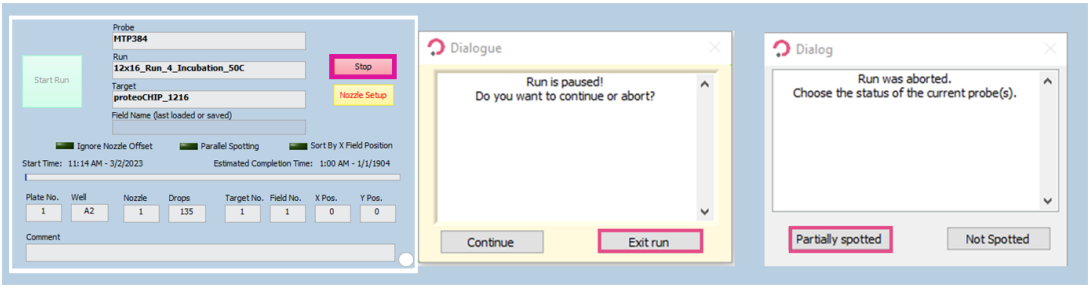


Figure 36: Exit the run

4.5 Run 5: Cooldown to 20 °C

Following incubation at 50 °C, we need to bring the plate temperature back to 20 °C. During this process, the system will keep on rehydrating the wells continuously. The cooldown step takes approximately 30 minutes to complete.

This run needs to be launched **immediately** after stopping the previous run, as in the meantime rehydration cycles are interrupted but the temperature setting is still 50 °C.

4.5.1 Probe, Run and Target selection

In the Main tab of the software, select the probe, run and target to be used (Figure 37).

- Probe: Select the “MTP384”.
- Run: Select “12x16_Run_5_Cooldown_20C”.
- Target: Select “proteoCHIP_1216”.

No changes need to be made in the field setup section, so you can proceed to start the run immediately.

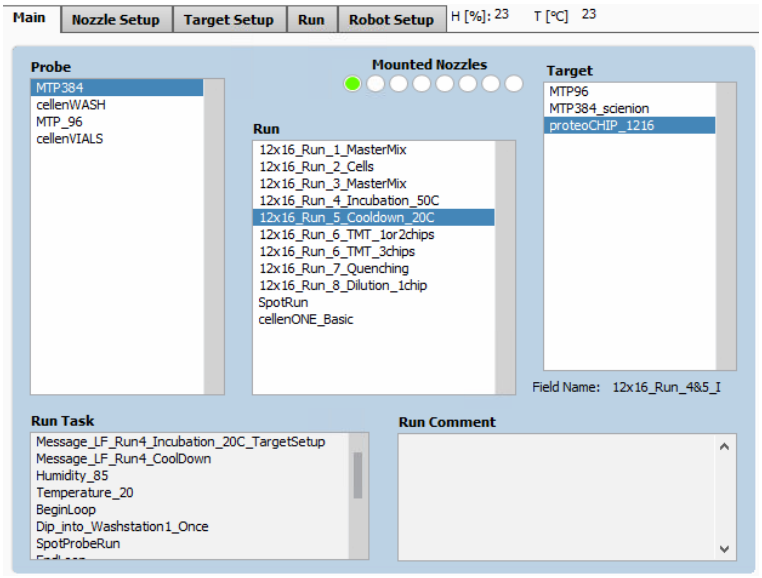


Figure 37: Run 5: Cooldown to 20 °C

4.5.2 Start the Run

Once the run has been selected, click “Start Run” in the Run tab (Figure 38). The field setup is the same for both incubation and cooldown runs. Select all 3 chips regardless of the real number of loaded chips and click “OK” (Figure 38).

The run will finish once the temperature of 20 °C is reached. This should take approximately 30 minutes (Figure 39).

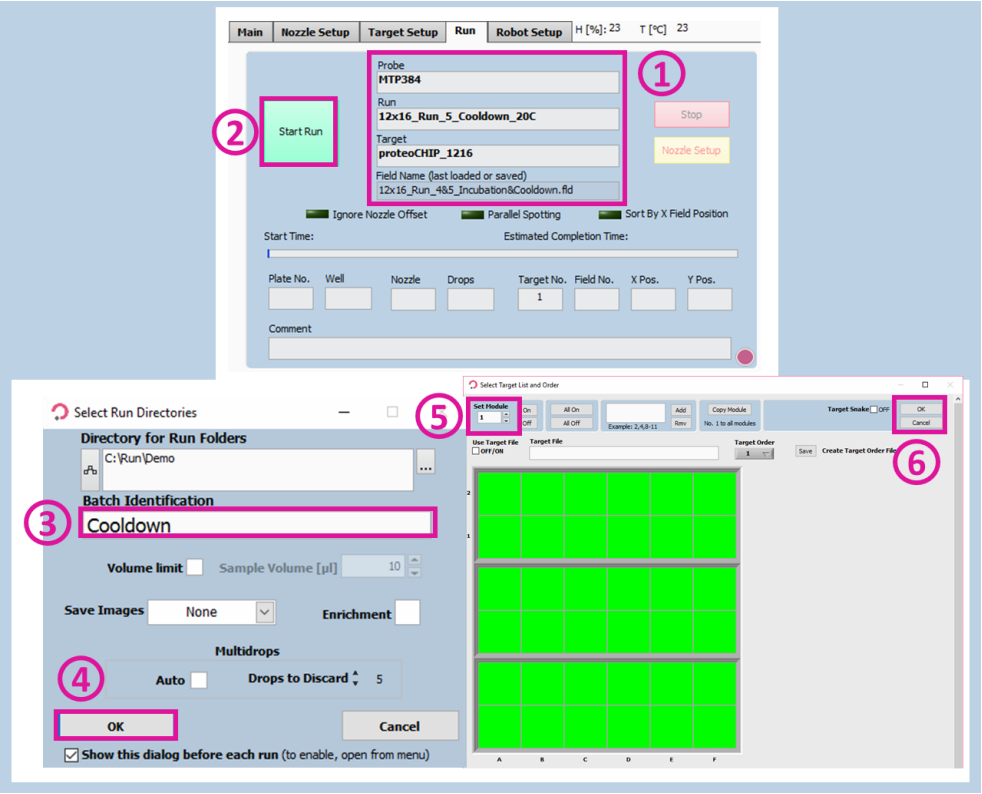


Figure 38: Start cooldown

Figure 39: Pop-up messages

4.6 Run 6: Labelling

Caution. *When starting this run, please make sure that the chiller has cooled down to 20 °C.*

For labelling, the probe uptake is programmed to first do a 6 µL air gap (ensuring no mixing of water and solvent) then an aspiration of 40 µL of acetonitrile (buffer gap to ensure drop stability) and then label aspiration (5 µL). This enables to dispense the labels correctly, without mixing with water when it is aspirated by the PDC and without excessive consumption of TMT labels. This workflow was optimised for TMT labelling, however, adaptions using iTRAQ or mTRAQ are possible by switching the solvent and/or the reagent concentration.

Note. *For TMT labelling using the proteoCHIP, we recommend using TMT 5x more diluted compared to Thermo recommendations.*

TMTpro

- *Thermo – 0,5 mg in 20 µL = 25 µg/µL*
- *proteoCHIP protocol = 5 µg/µL*

Suggestion. *When preparing TMT labels, 100 µL of waterfree acetonitrile can be added to each tube containing 0.5 mg of lyophilised TMT label. Mix by pipetting several times, close the lid and let the labels resolubilize for 5 minutes. Mix again several times by pipetting and transfer 15 µL to 0.2 mL PCR tubes, put a respective set of PCR tubes (6 sets) in a 50 mL Falcon tube and seal it with parafilm. If the labels need to be stored for several months, preparation using argon gas is strongly recommended.*
For each experiment a single falcon tube will be needed. 15 µL will be needed per TMT label.

Note. *This workflow was optimised for TMT labels, however, adaptions using other labels like iTRAQ are possible by adapting the reagent concentration and/or the solvent according to respective single cell proteomics literature.*

Note. *Before starting the run, you will need to optimize the Voltage and Pulse parameters for acetonitrile dispensing. You can do this with the AirGapTakeProbe_40ul task and performing Continuous Dispensing. The general starting point should be decreasing the Voltage by 10 and increasing the Pulse by 5.*

If you do not have a stable drop with these new values, play with Voltage, Pulse and you can also decrease the frequency to 350 Hz.

Caution. *Write down the default Voltage and Pulse values so you can change them back after the TMT labelling run.*

4.6.1 Probe, Run and Target selection

In the Main tab of the software, select the probe, run and target to be used.

- Probe: Select the "MTP384" (Figure 40 ①).
- Run: Select "Run_6_TMT_1or2chips" or "Run_6_TMT_3chips" (Figure 40 ②).
- Target: Select "proteoCHIP_1216" (Figure 40 ③).

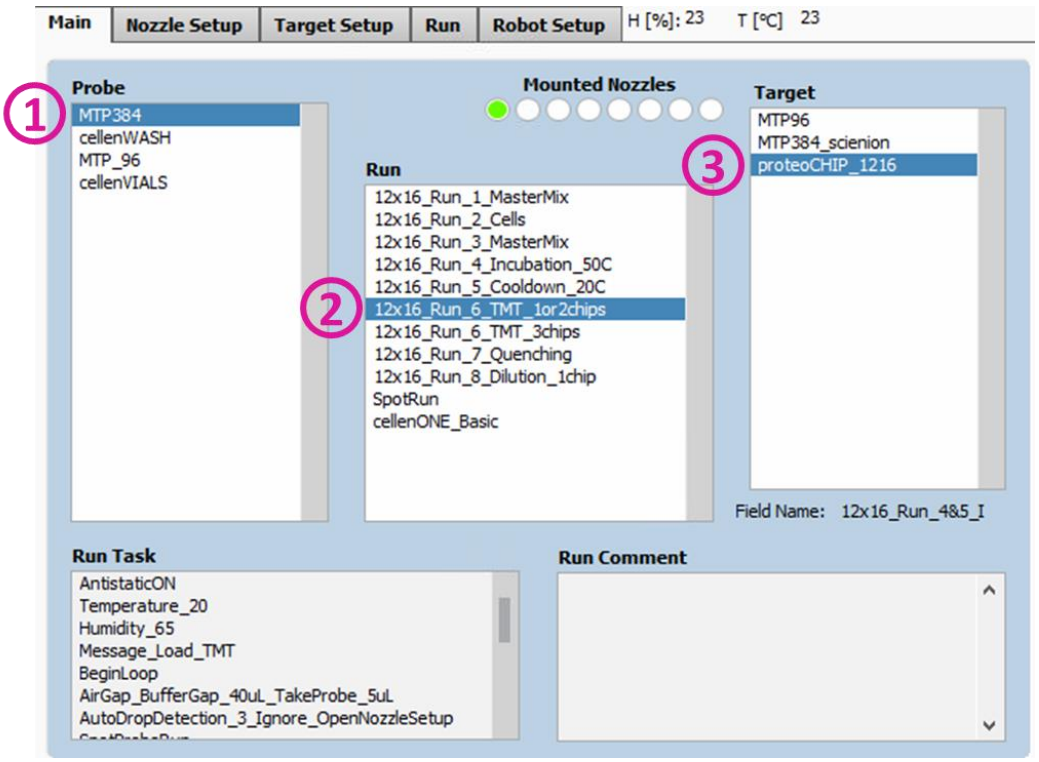


Figure 40: Run for TMT labelling

4.6.2 Field selection

Load the field "12x16_Run_6_Labelling_Nplex" with N corresponding to the number of TMT labels that are used. We provide pre-programmed fields for 8-, 10-, 11- and 16-plex labelling (Figure 41, Figure 42, Figure 42).

During the run the cellenONE will spot 450 drops (=120 nL) per well.

To load the field, click on

- "Target setup" (Figure 41 ①) >>" Target" (Figure 41 ②) >> "Load Field" (Figure 41 ③).

During this TMT labelling run, a pre-programmed task is going to be used to uptake the TMT labels.

Note. *Because acetonitrile needs to stay anhydrous, aspiration of TMT labels requires a specific pre-programmed task:*

1. *An air gap is going to be created by aspiration of 6 µL of air to ensure no mixing between water an acetonitrile.*
2. *A buffer gap of 40 µL of pure acetonitrile is then going to be aspirated in the nozzle to ensure drop stability and prevent excessive consumption of TMT labels.*
3. *5 µL of TMT will be aspirated before dispensing.*

Caution. *Throughout the run, you will need to load:*

- *55 µL of pure acetonitrile (well shown in the field setup, e.g., A6).*
- *15 µL of TMT label (well on the right of the one displayed in the field setup, e.g., B6).*

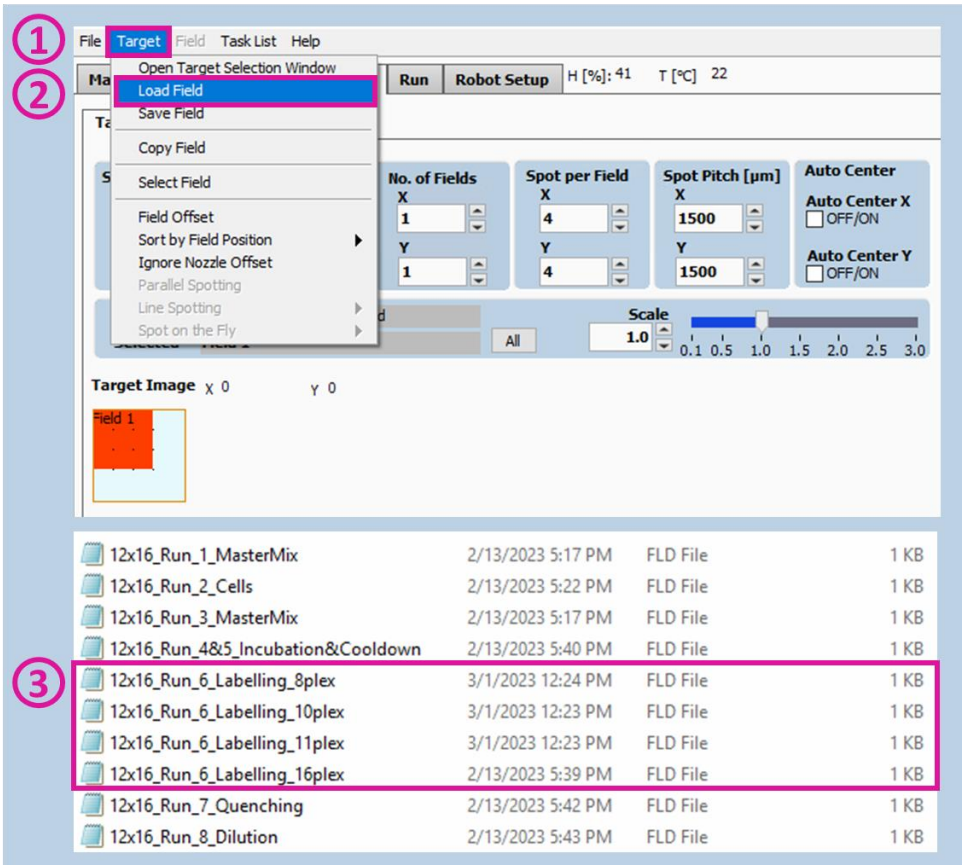


Figure 41: Load Field for TMT labelling

Different number of labels can be chosen:

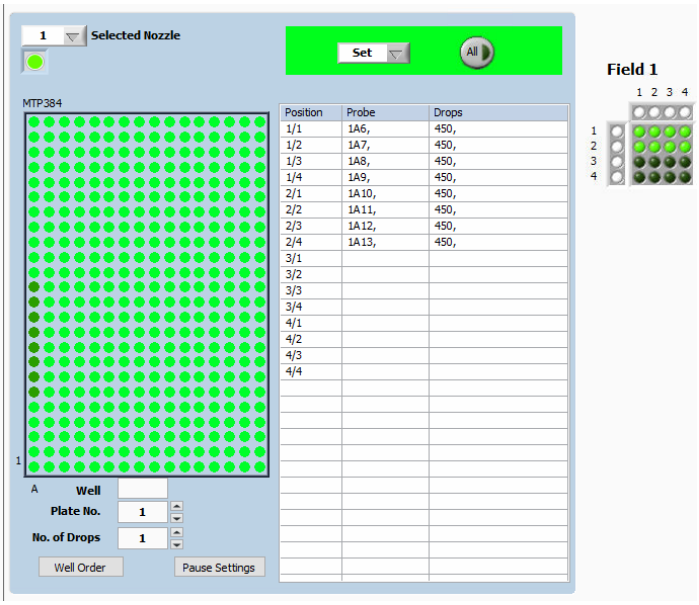


Figure 42: Run 6 for 8-plex labelling

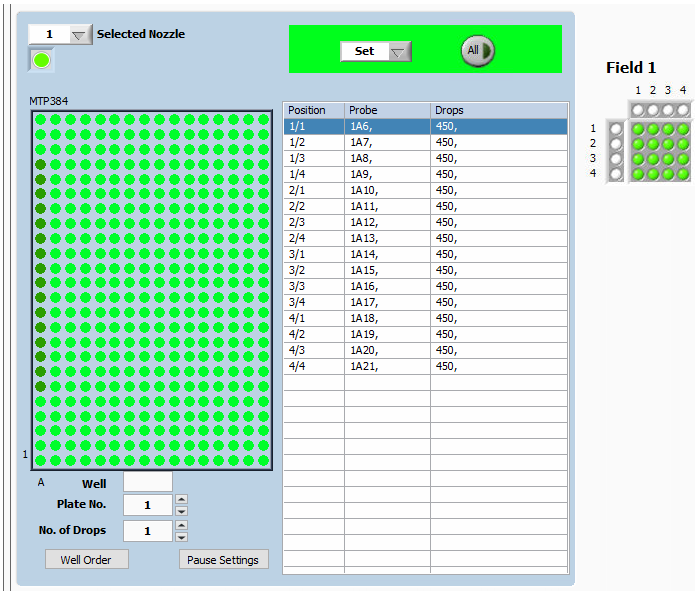


Figure 42: Run 6 for 16-plex labelling

***Caution**. Remember that you can change the column that is going to be used for the probe uptake by going to:*

- *"Target setup" >> "Field setup" >> "Field" >> "Edit Field Table"*
- ***From** Plate/Well X6 > **To** Y Keep*
  - ☑ *Column*
- *>> Change All*

4.6.3 Start the Run

You can start the run in the “Run” window and select the number of proteoCHIPs that you want to dispense in (Figure 43).

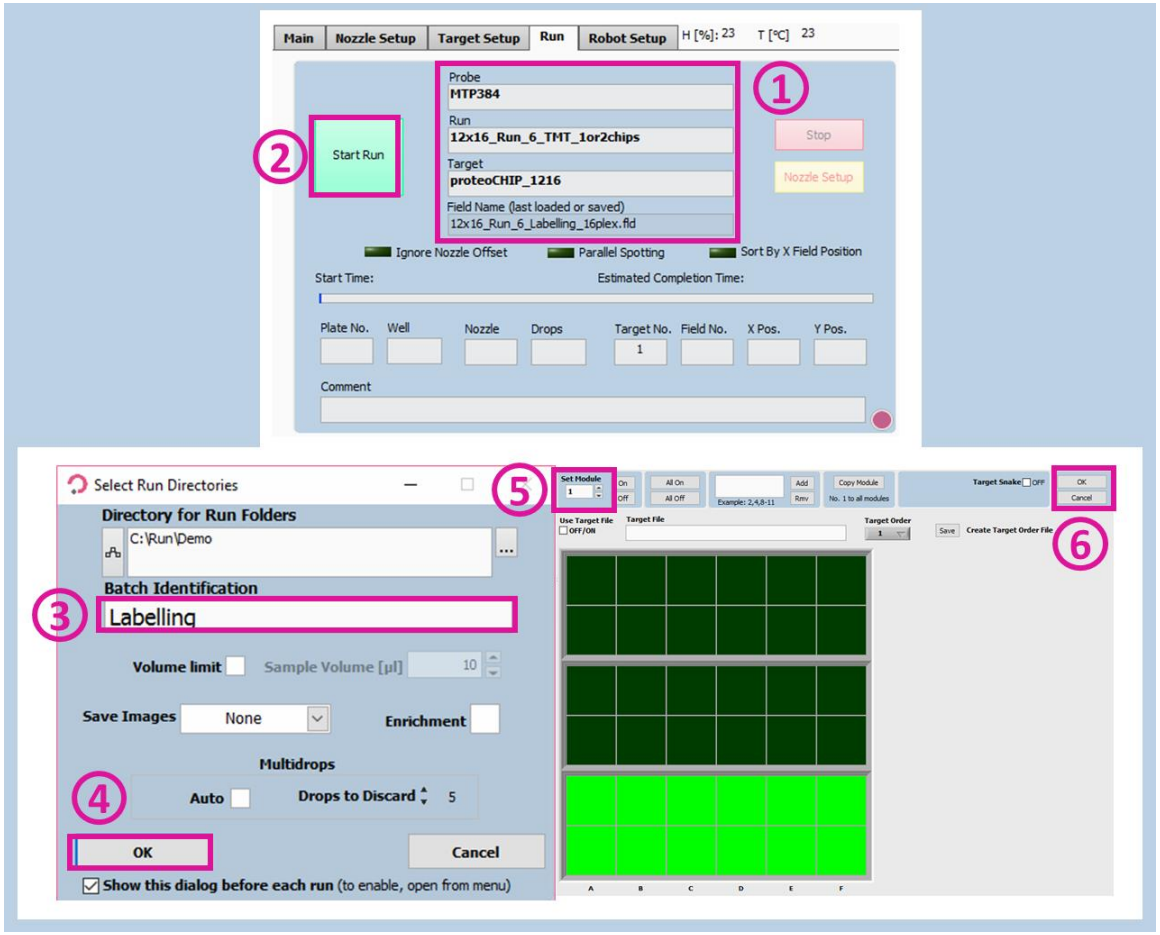


Figure 43: Start the run for labelling

Note. *Before uptaking of each label, a pop-up window will be displayed. Click “OK” after completing the loading of 55 µL of ACN (left well) and 15 µL of TMT (right well) (Figure 44).*

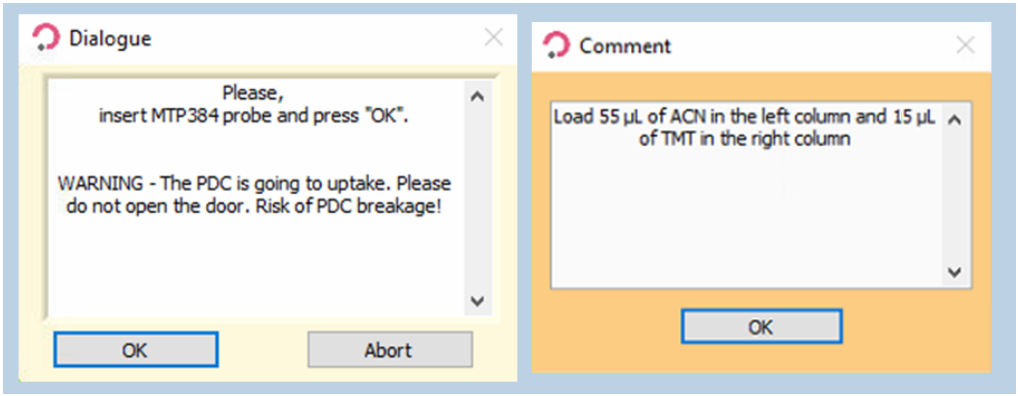


Figure 44: Message "Load TMT" and Start

Note. *At the end of this run, an incubation of 30 minutes at RT will start.*

4.7 Run 7: Quenching

Note. *The quenching solution consists of Hydroxylamine (final conc. 0.5%) and HCl (final conc. 1%)*

Before the run starts, prepare 100 µL of this mixture:

- *96 µL H_2O + 3 µL 37% HCl + 1 µL 50% Hydroxylamine*

Load 50 µL of the Quenching solution in well A23.

Caution. *Rember to put the Voltage and Pulse values back as before the TMT run.*

4.7.1 Probe, Run and Target selection

In the Main tab of the software, select the probe, run and target to be used (Figure 45).

- Probe: Select the “MTP384”.
- Run: Select “12x16_Run_7_Quenching”.
- Target: Select “proteoCHIP_1216”.

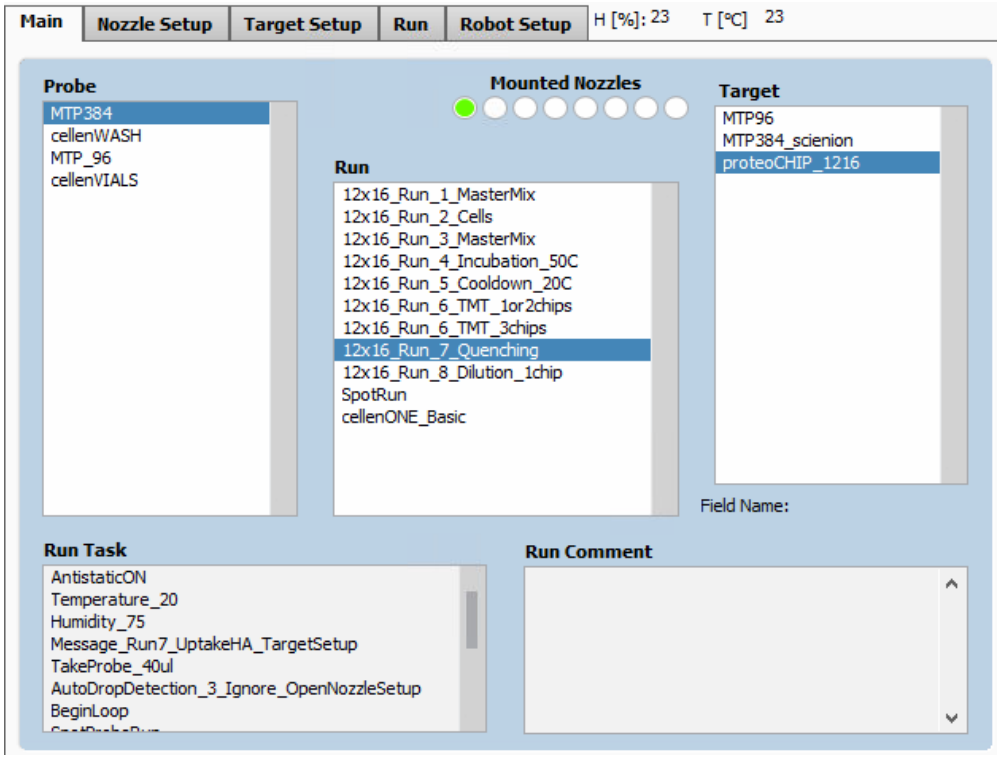


Figure 45: Run for Quenching

4.7.2 Field selection

To dispense the quenching solution, load the field “12x16_Run_7_Quenching” which will spot 50 nL per well. To load the field, click on “Target” (Figure 46 ①) >> “Load Field” (Figure 46 ②) and select the field corresponding to the run “12x16_Run_7_Quenching” (Figure 46 ③). The field associated with quenching run will be displayed in the Field setup (Figure 47).

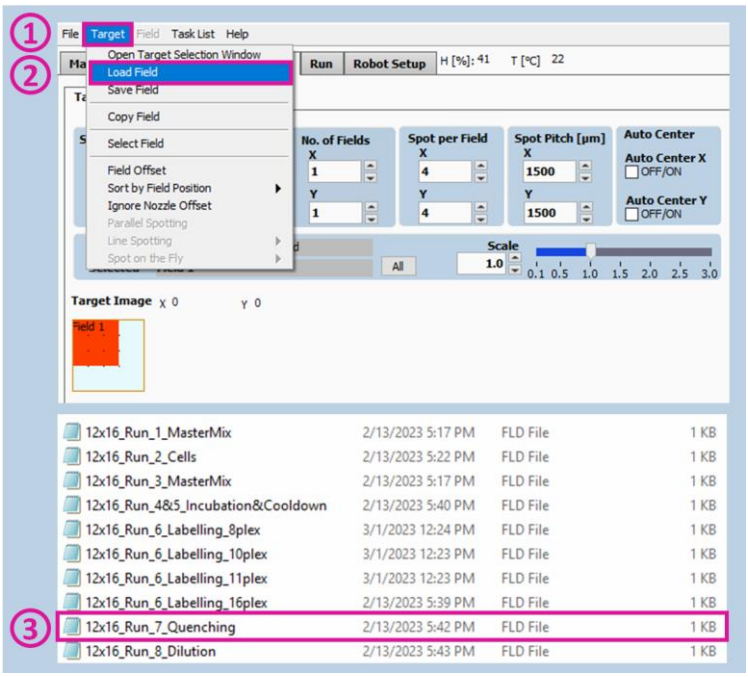


Figure 46: Load Field for Quenching

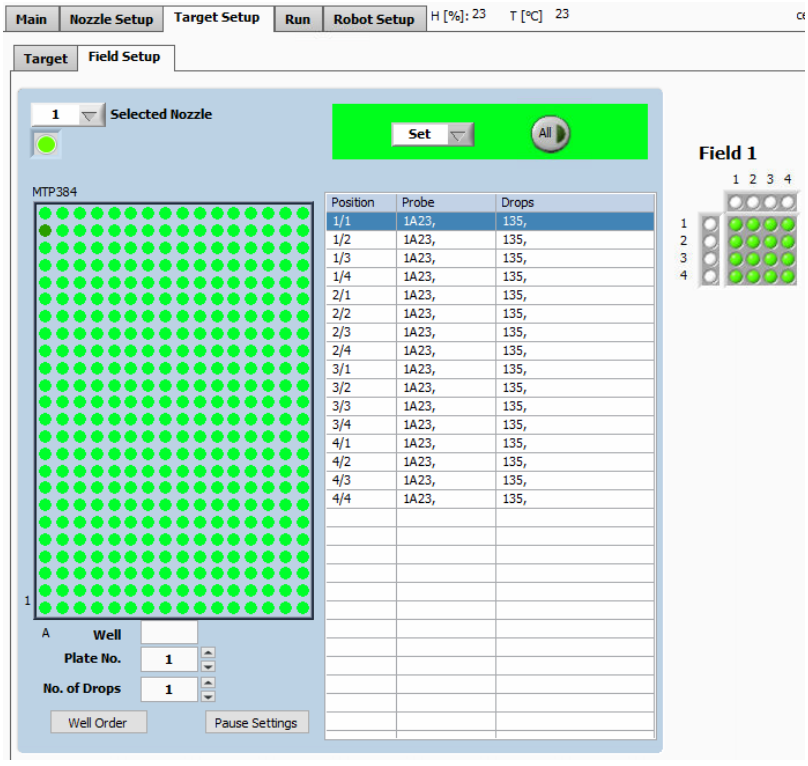


Figure 47. Field setup for Quenching

4.7.3 Start the Run

Check that you selected the right Probe, Run and Target in the Run window (Figure 48 ①). Start the run (Figure 48 ②) and select the number of proteoCHIPs that you want to dispense in (Figure 48 ⑤). To start the run, click OK (Figure 48 ⑥).

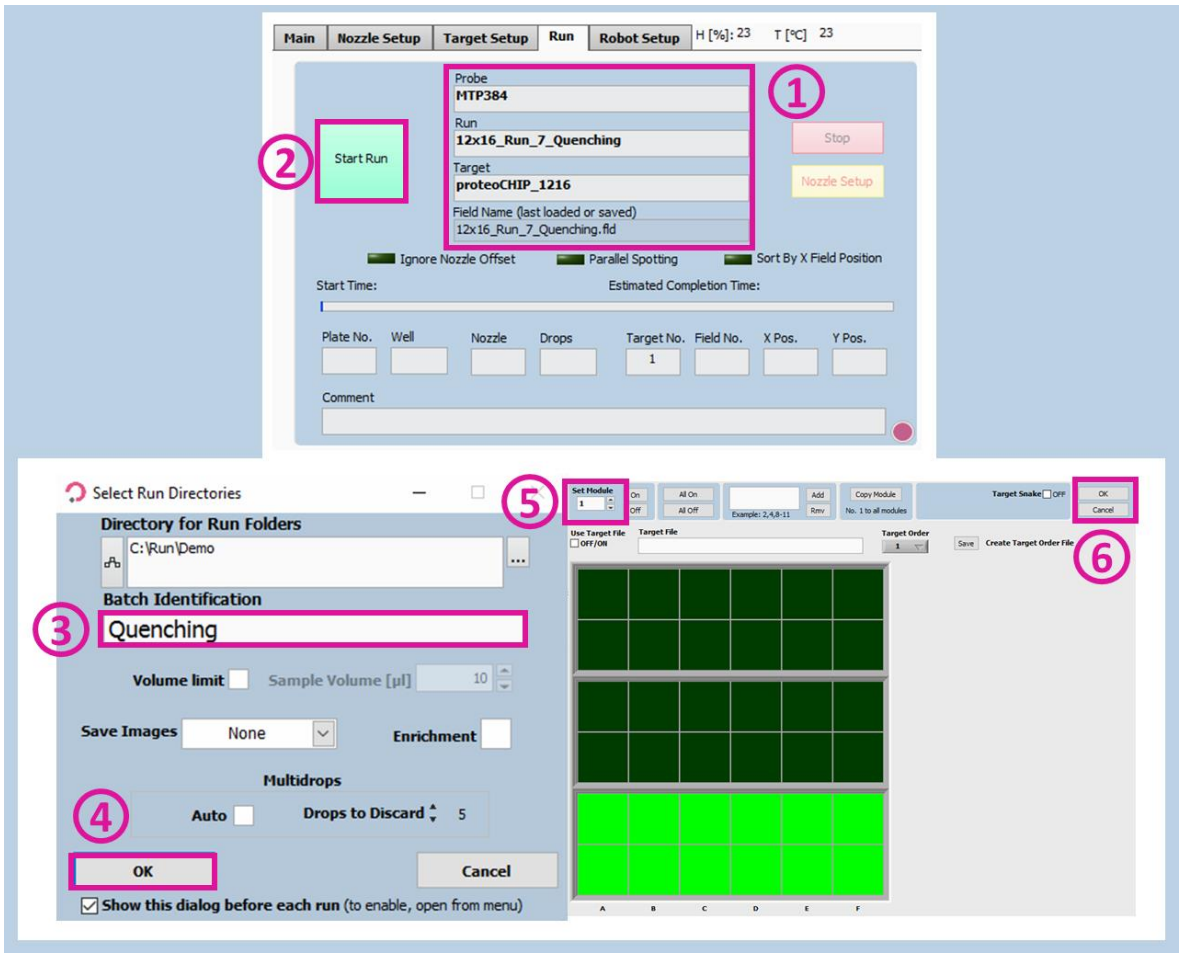


Figure 48. Start the Run for Quenching

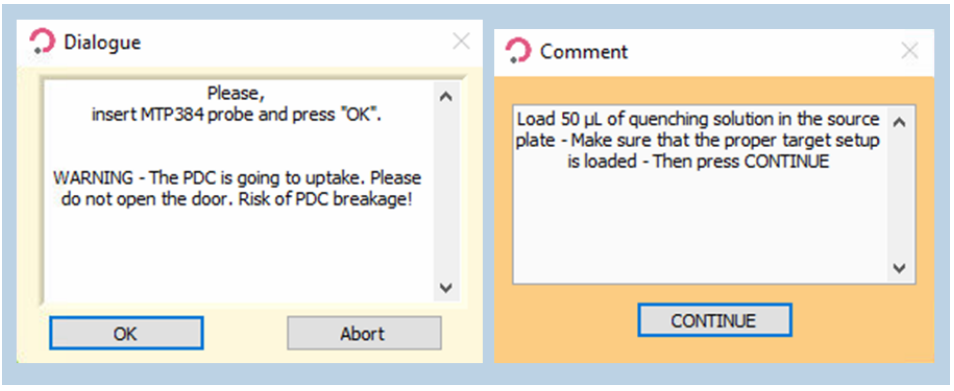


Figure 49: Quenching Run Pop-Up messages

Note. *At the end of this run, an incubation of 15 minutes at RT will start.*

4.8 Run 8: Dilution

The dilution step is used to adjust the final volume of the sample that should be around 3.5 µL to be correctly injected into LC-MS. Both 0.1% TFA or FA can be used for dilution.

This step can either be performed manually or by an automated cellenONE run.

4.8.1 Manual dilution

Pipette 2.5 ul 0.1% TFA/FA into each funnel and proceed to the next step: 4.9 Pooling.

4.8.2 cellenONE automated dilution

Note. *For one proteoCHIP load 50 µL 0.1% TFA/FA into a well and repeat this process for each additional proteoCHIP.*

4.8.2.1 Probe, Run and Target selection

In the Main tab of the software, select the probe, run and target to be used (Figure 50).

- Probe: Select the “MTP384”.
- Run: Select “12x16_Run_8_Dilution_1chip”.
- Target: Select “proteoCHIP_1216”.

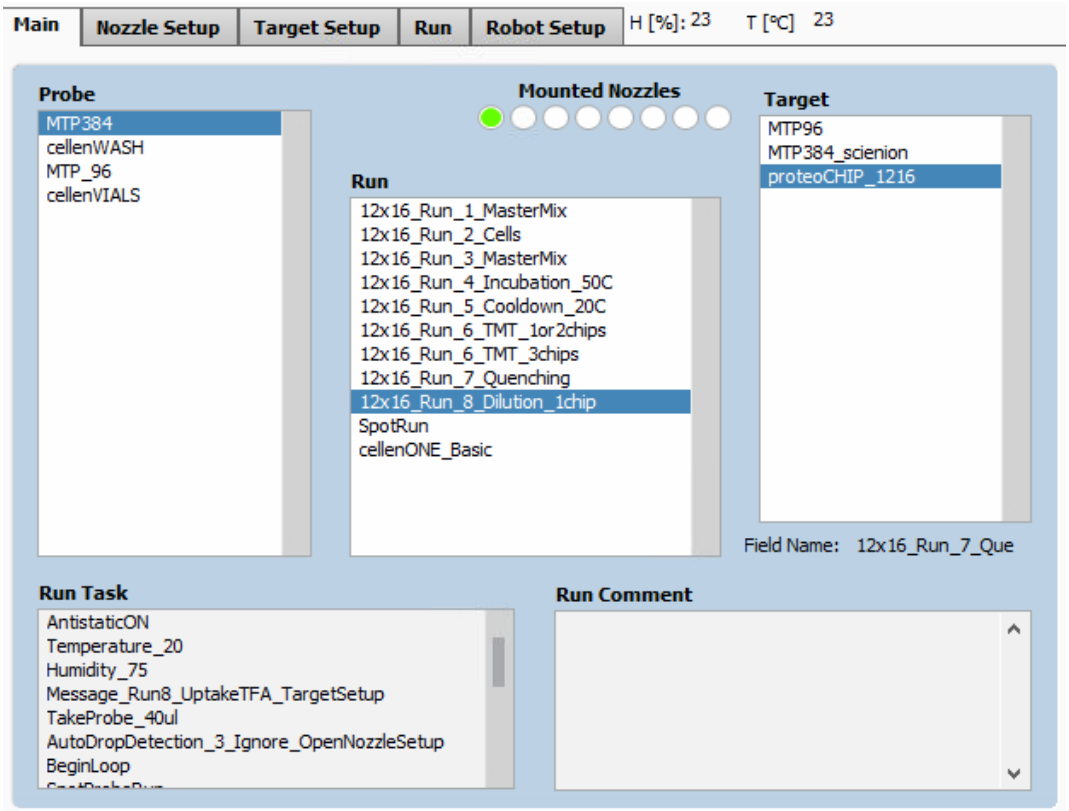


Figure 50: Run selection for dilution

4.8.2.2 Field selection

Load the field “12x16_Run_8_Dilution” which will dispense ~150 nL TFA/FA per well. To load the field, click on “Target” (Figure 51 ①) >> “Load Field” (Figure 51 ②) and select the field corresponding to the run (Figure 51 ③).

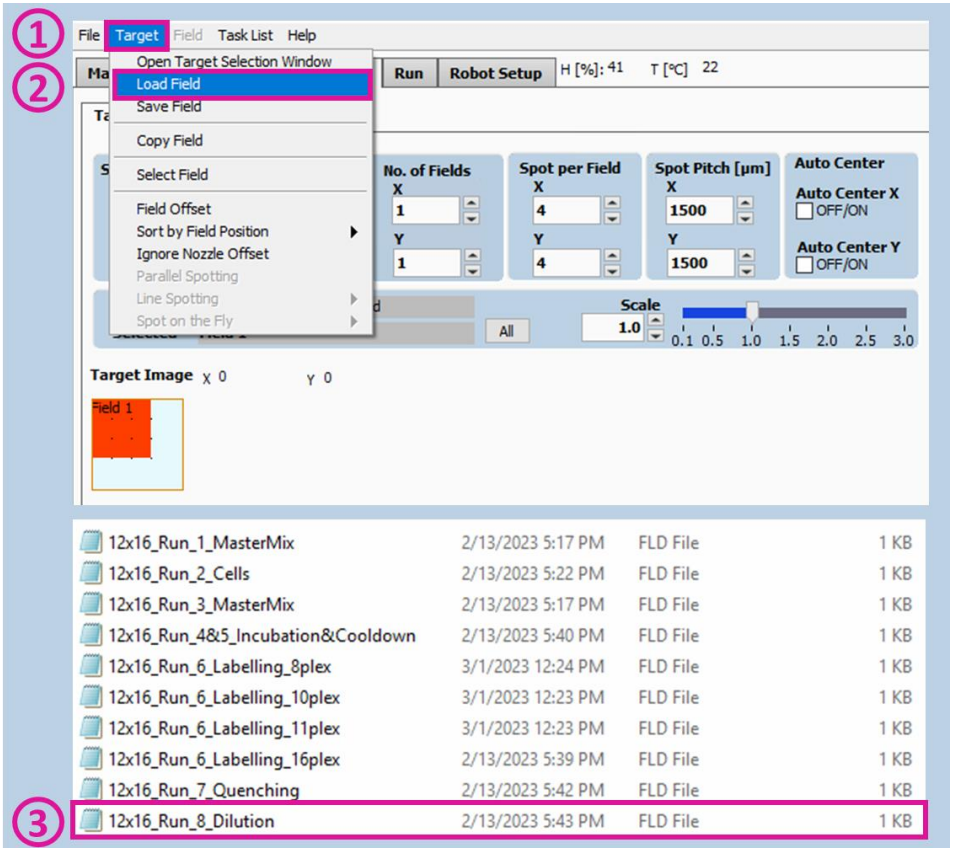


Figure 51: Load Field for Dilution

The field corresponding to the dilution run will be displayed in the “Target Setup” >> “Field Setup” (Figure 52).

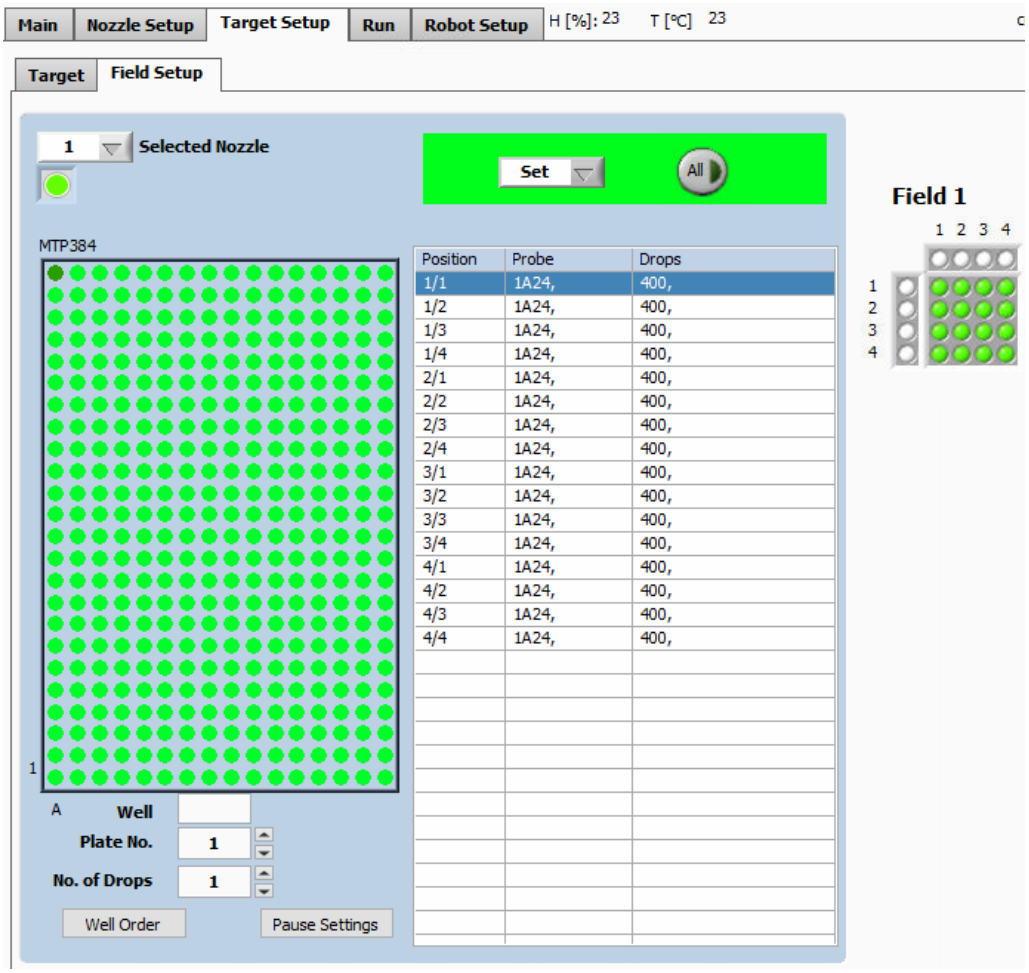


Figure 52: Field setup for dilution

Caution. *For this run, you must dispense 150 nL in every well of the array even if you did an experiment of 8-plex, 10-plex or 11-plex. If not, the volume is not going to be sufficient for injection.*

4.8.2.3 Start the Run

Check that you selected the right Probe, Run and Target in the Run window (Figure 53 ①). Start the run (Figure 53 ②), select the number of proteoCHIPs that you want to dispense in (Figure 53 ③) and click OK (Figure 53 ④).

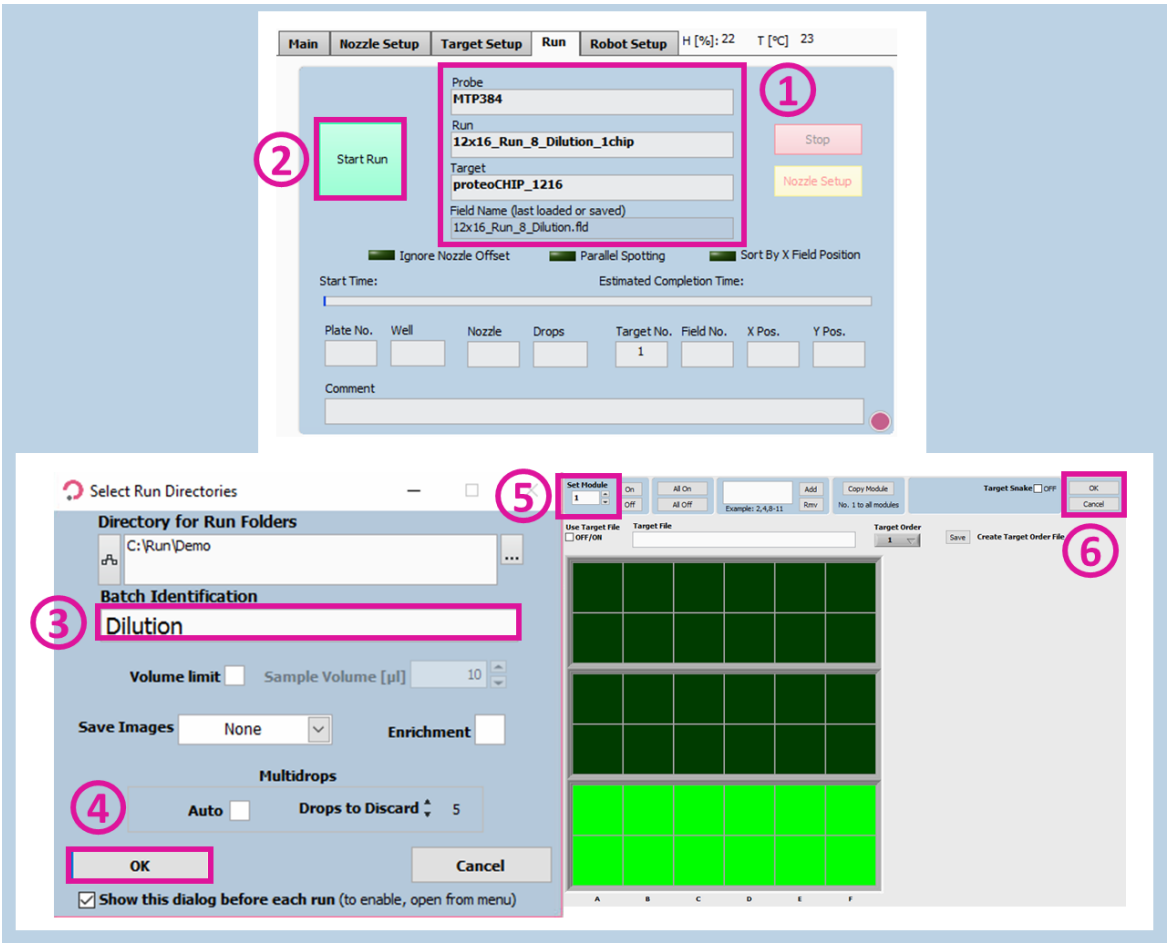


Figure 53: Start dilution run

4.9 Pooling

After completion of the sample preparation on the cellenONE, the next step is “sample pooling”. During that step, each array of 16 single cells is going to be pooled into a Funnel (One array per funnel).

- Step 1: Place the proteoCHIP Funnels on top of the proteoCHIPs and take the assembled unit out of the cellenONE
- Step 2: Transfer the proteoCHIPs+Funnels into the centrifuge adaptor with the Funnels at the bottom of the centrifuge holder and the proteoCHIP on top. Then close the lid of the centrifuge holder (Figure 54).

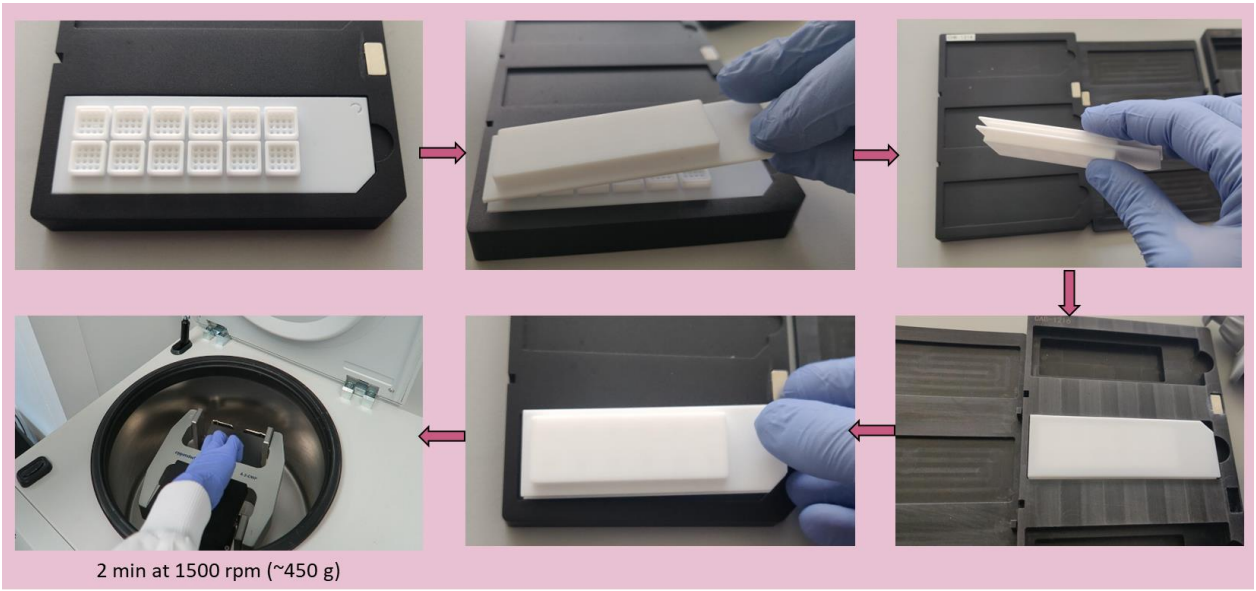


Figure 54: Pooling (Step 2)

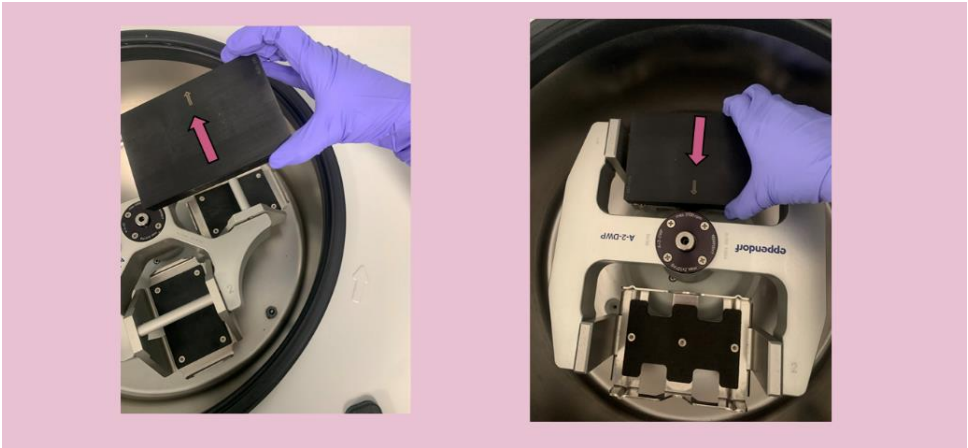


Figure 55: Pooling (Step 3)

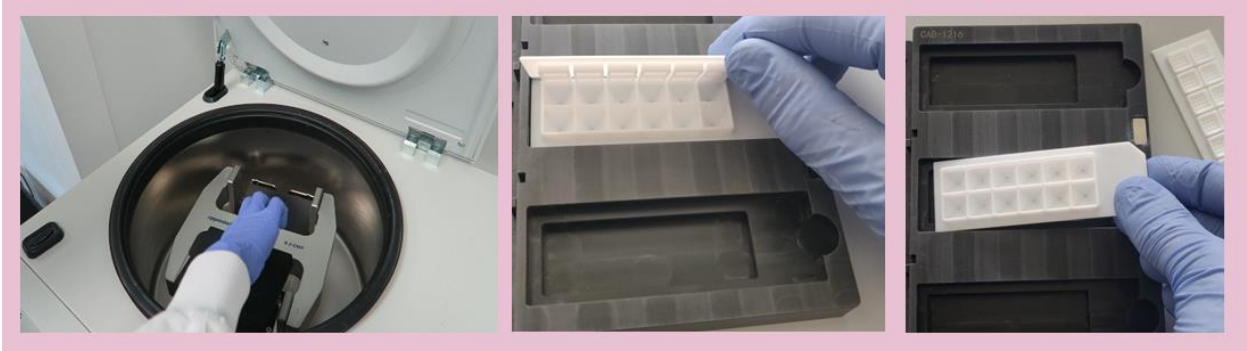


Figure 56. Pooling (Step 4)

- Step 3: Use a plate centrifuge to pool the arrays into the funnel at 1500 rpm for 2 minutes at room temperature (Figure 55).
- Step 4: Take out the proteoCHIP with the funnel from the centrifuge adaptor and keep the funnel without the proteoCHIP (Figure 56).
- After pooling, the 8- to 16-plex samples are in each funnel and can be directly injected into the LC from the funnels (refer to the Autosampler and direct injection manual for instructions). In that case, seal the funnels with aluminium foil and store at 4 °C (can be done directly inside the autosampler) for at least 10 minutes before starting injection (to allow the oil layer to become solid).

The funnel layout will look like in Figure 57 below:

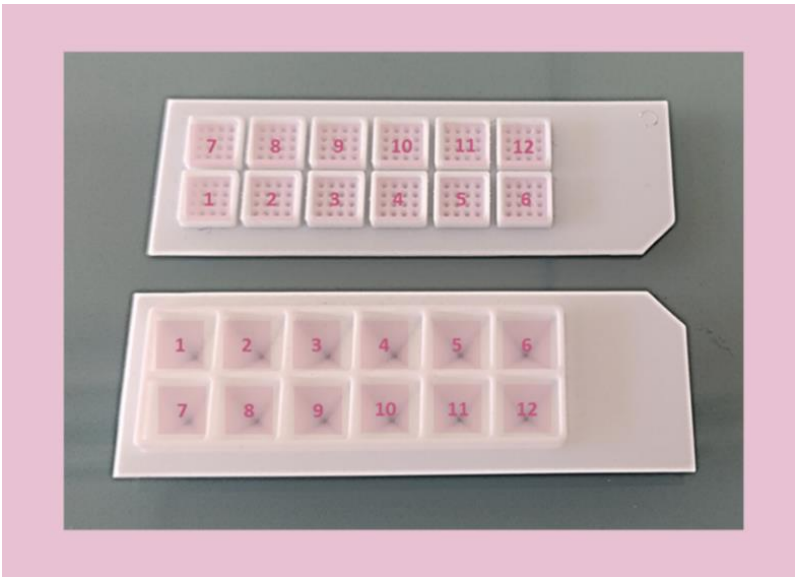


Figure 57: Funnel layout after pooling

4.10 Sample transfer

The sample can be transferred from the funnels either manually to any substrate used for injection or can be directly injected with an autosampler.

4.10.1 Manual sample transfer

In case of long-term storage of the sample or injection from another substrate (low-binding PCR tubes, Eppendorf, plate, etc.), the sample must be transferred from the funnels to the other substrate manually. In that case, place the funnels in the fridge for 10 minutes. Once the oil layer is solidified take the proteoCHIP funnels out of the fridge and use a pipette to collect the samples. To do so, set your pipette to 5 µL volume to aspirate. While holding the pipette ready to aspirate (plunger down), place it in the middle of the sample vertically and once the bottom is reached, release the plunger gently to aspirate your sample. The oil is a solid layer so it will not be aspirated in the pipette tip (Figure 58, Figure 59).

Figure 58: Manual sample uptake

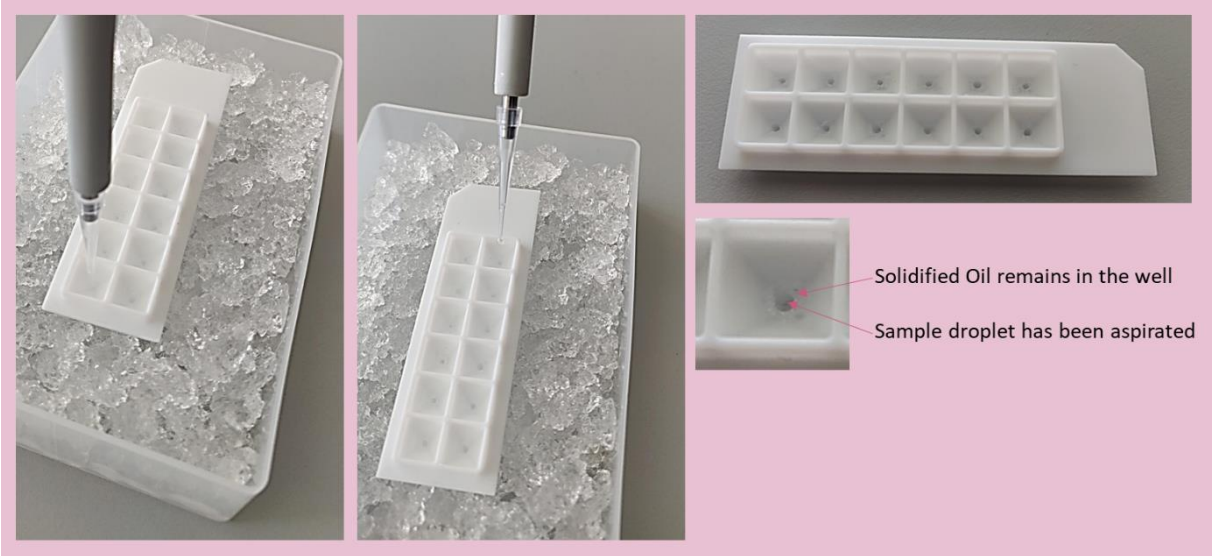


Figure 59: Sample transfer

4.10.2 Autosampler

The wells of the proteoCHIP funnels inside the Autosampler holder correspond exactly to certain positions within a 96-well plate. Currently, direct injection is only possible using the Thermo Scientific UltiMate 3000 UHPLC, with which the injection height is easily adjustable.

Note. *To set your autosampler for direct injection from the proteoCHIP funnels (Figure 60), please refer to the "Autosampler User Manual".*

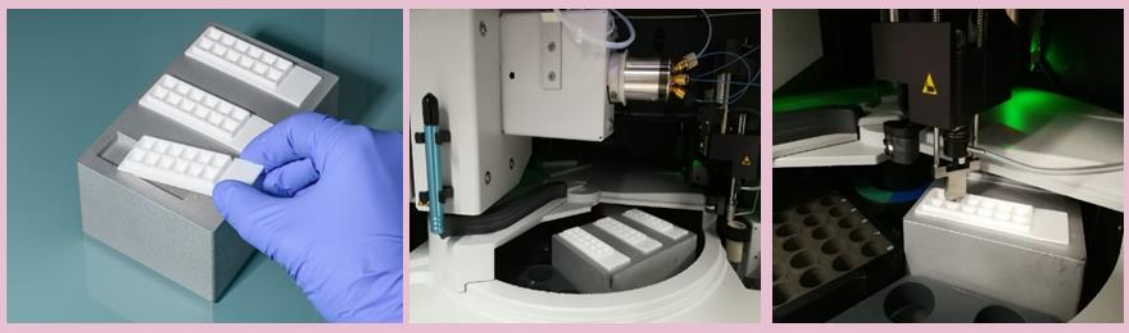


Figure 60: Autosampler

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References

Please note that this protocol has been developed in collaboration with the Institute of Molecular pathology, Vienna.

For reference, please cite this paper:

An automated workflow for label-free and multiplexed single cell proteomics sample preparation at unprecedented sensitivity

Claudia Ctortecka, David Hartlmayr, Anjali Seth, Sasha Mendjan, Guilhem Tourniaire, Karl Mechtler

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