

User Manual proteoCHIP 12*16 for Multiplexed Single Cell Proteomics workflow using the cellenONE®

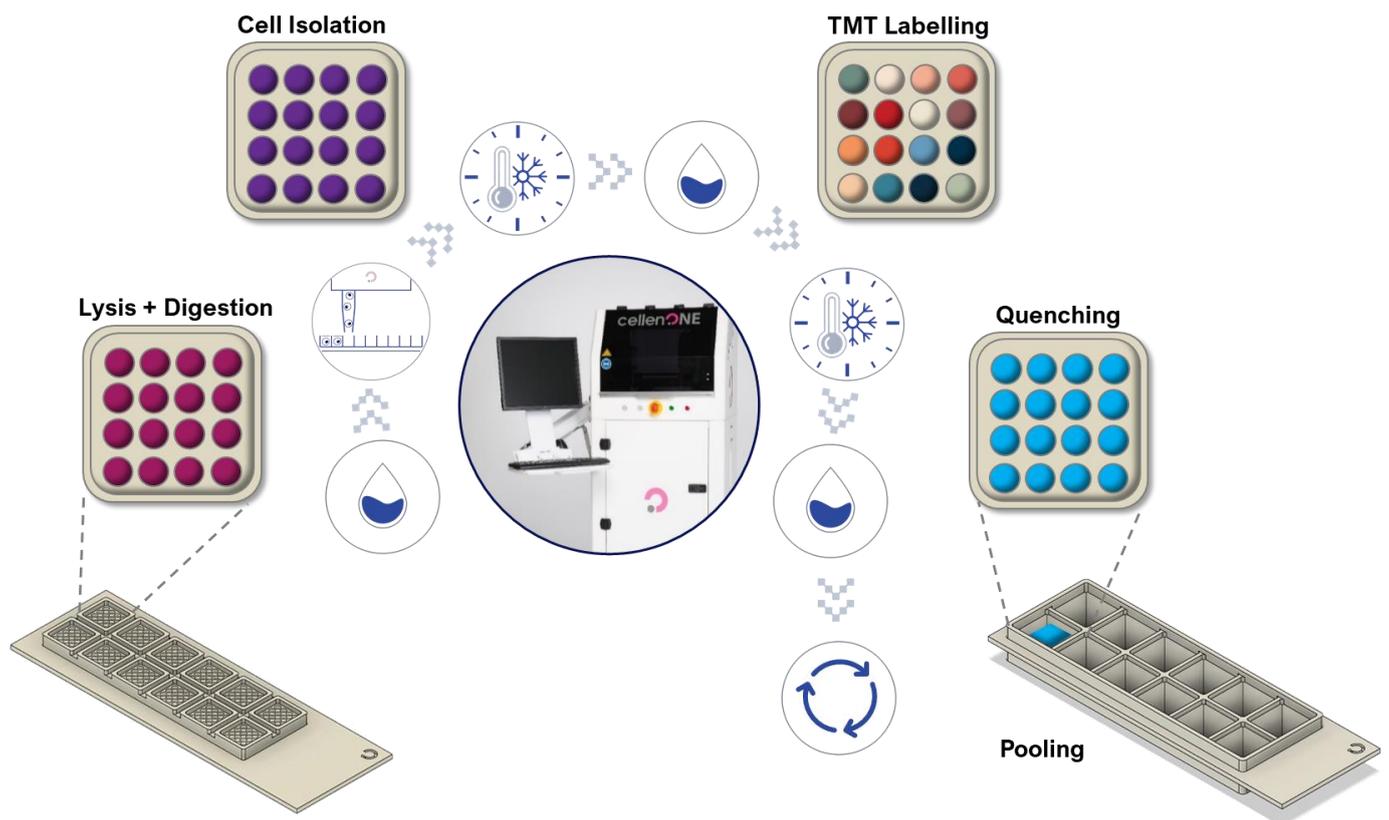


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Document history

Revision	Author(s)	Changes	Effective date
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Highlights layout

Warnings will be highlighted in framed boxes.

Important **notes** will be highlighted in green boxes.

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CELLENION SASU
Bioserra 2
60 av. Rockefeller
69008 Lyon
France
support@cellenion.com

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Warning

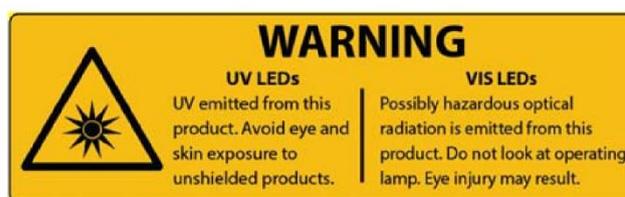
The instrument control computer comes equipped with Windows operating system and the cellenONE software along with other standard software programs related to the basic operation of the cellenONE instrument. **This computer should be dedicated to control cellenONE** robotics and is not intended for data analysis, office applications, web surfing or other non-instrument related actions. Users are warned **not to install any additional software**, including but not limited to OneDrive, Google Drive, Dropbox, other synchronization utilities, or antivirus software. Such software programs or synchronization utilities will significantly slow down the performance of any computer and may impact the rate of cell isolation.

Cellenion and its affiliates cannot be held responsible for system functionality if recommendations regarding third-party software programs like those aforementioned are not followed

If **antivirus** software is a requirement for instrument control computers in your lab, this should be configured to have an **exception for the cellenONE** software and related folders ('PROG', 'USER', and data path i.e., 'Run'), **real-time protection** should be **switched off**, and scan/activity hours should be set to out of cellenONE operation hours. Ensure that a 'System Restore' point is created prior to the installation of any software.

The instrument control computer is not equipped with internet connectivity and is not recommended due to antivirus incompatibilities. In the case that remote support is needed, please utilize a Wi-Fi dongle to establish a **temporary** connection. Windows updates are not recommended due to the risk of unforeseen incompatibilities and have been configured not to occur. Enabling windows updates may put your computer at risk. If required for instrument control computers in your lab, scan/download/install hours should be set to out of cellenONE operation hours. **Feature updates must be disabled.** It is highly recommended to regularly move images from the instrument control computer with the aim of securing the data and free up space for further experiments.

It is strongly recommended to perform post-run image analysis on a separate computer in order to prevent installing other third-party software programs on the instrument computer, which is dedicated to the instrument control.



Warnings and Safety for LED lights

Inappropriate use of the product may result in permanent eye damage. To prevent injury, use this product in accordance with the IEC 62471:2006 standard, Photobiological Safety of Lamps & Lamp Systems.

Inappropriate use of a product containing LEDs may result in permanent eye damage.

The cellenONE instrument contains an LED which falls into Risk Group RG0 – Exempt Group according to the IEC 62471:2006 standard.

The cellenONE instrument contains two LEDs which fall into Risk Group RG1 – Low Risk Group according to the IEC 62471:2006 standard.

The cellenONE instrument contains an LED which falls into Risk Group RG2 – Moderate Risk Group according to the IEC 62471:2006 standard.

UV / Visible Radiation Warning Statement: This LED emits intense UV / visible radiation during operation. **DO NOT** look directly into the UV / visible light or look through the optical system during operation, as this can be harmful to the eyes, even for brief periods of exposure, due to the high intensity of the light. If viewing the UV / visible light directly is necessary, protective glasses must be worn to avoid eye damage.

1 Introduction

The cellenONE software monitors cells inside the Piezo Dispense 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 cellenONE User Manual before performing any experiment with the cellenONE instrument.

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

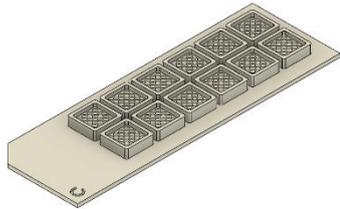
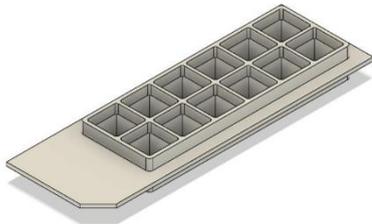
IBSCI™: Image-Based Single Cell Isolation

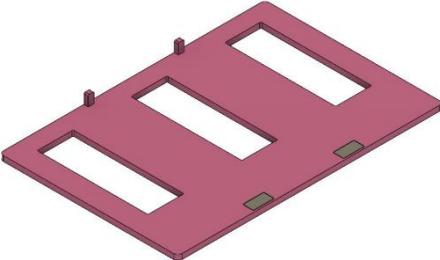
IBSCI™ is a high resolution-based technology consisting of automated image acquisition, processing and advanced algorithms to automatically isolate single cells from a cell suspension.

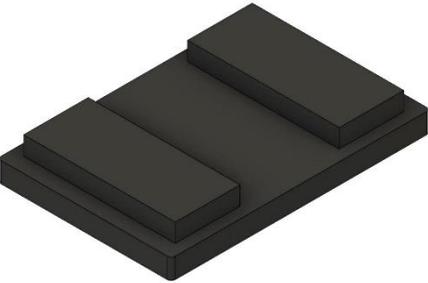
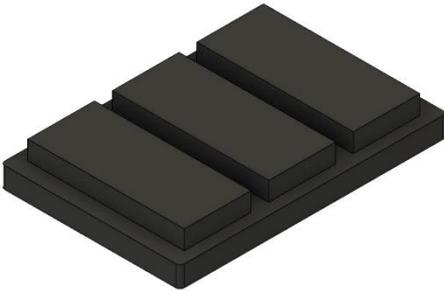
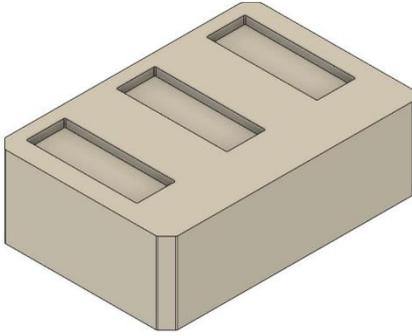
FIBSCI™: Fluorescence Image-Based Single Cell Isolation

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 Equipment

Part number	Description	
P-20-CL	cellenONE PDC L Piezo Dispense Capillary (Fixed drop volume between 450-600 pl) 4x manifold	
P-20-CM	cellenONE PDC M Piezo Dispense Capillary (Fixed drop volume between 300-450 pl) 4x manifold	
CPS-1216-3	proteoCHIP 12*16 Set The proteoCHIP 12*16 Set contains: <ul style="list-style-type: none"> • 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). <i>(1 piece/pack)</i>	

<p>CHT-1216</p>	<p>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). <i>(1 piece/pack)</i></p>	
<p>CAB-1216</p>	<p>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. <i>(1 piece/pack)</i></p>	
<p>CAT-1216</p>	<p>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. <i>(1 piece /pack)</i></p>	
<p>CCA-1216</p>	<p>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. <i>(1 piece/pack)</i></p>	

<p>CCB-1216</p>	<p>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. <i>(1 piece/pack)</i></p>	
<p>CCC-1216</p>	<p>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. <i>(1 piece/pack)</i></p>	
<p>CAS-1216-50</p>	<p>proteoCHIP 12*16 Seal Adhesive aluminum foil, dedicated to proteoCHIP 12*16, for all reactions and storage. Pierceable, peelable, and free of contamination. <i>(50 pieces/pack)</i></p>	
<p>CAH-1216</p>	<p>proteoCHIP 12*16 Autosampler Holder proteoCHIP holder for the LC-MS/MS system Thermo Fisher Dionex Ultimate 3000.</p>	

3 cellenONE setup

3.1 General remarks

- Wipe the inside of the enclosure and door with isopropanol or 70% ethanol as well as the proteoCHIP 12*16 holder.
- When manipulating inside the cellenONE, make sure that you are wearing gloves and a lab-coat. No skin should be exposed inside the enclosure to avoid contamination.
- Avoid opening the door as much as possible during the experiment.
- Make sure the PDC is aligned to the red cross and produces a stable drop.
- Measure the drop volume.
- Set the temperature to 10 °C.
- Place the proteoCHIP(s) inside the cellenONE holder. If processing only one chip, insert it on the spot closest to you.
- Place the 'proteoCHIP holder top' on top of the 'holder' and insert the assembled unit on the target holder inside the instrument.
- All solutions should be prepared in a biosafety cabinet.

Note. Please make sure that the 14 days wash have been performed previously (every 14 days), clean the humidifier with 70% ethanol every 14 days if possible, clean the PDC with sciClean at the end or beginning of every experiment followed by 3 flushes (Flush_PDC).

3.2 Nozzle Setup

Before starting your experiment, you will need to make sure that your Nozzle (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 optimization.

3.3 Humidity and Temperature Setup

Throughout the workflow, the humidity and temperature need to be set to a specific value to avoid evaporation and minimize condensation.

Note. The runs already contain specific tasks controlling humidity and temperature for each run. The following section describes how to change humidity/temperature “manually” if you wish to change it.

- Humidity setup (Figure 2):
Main >> File >> Humidity Control >> Pop-up window >> Set humidity >> Click on Set button.

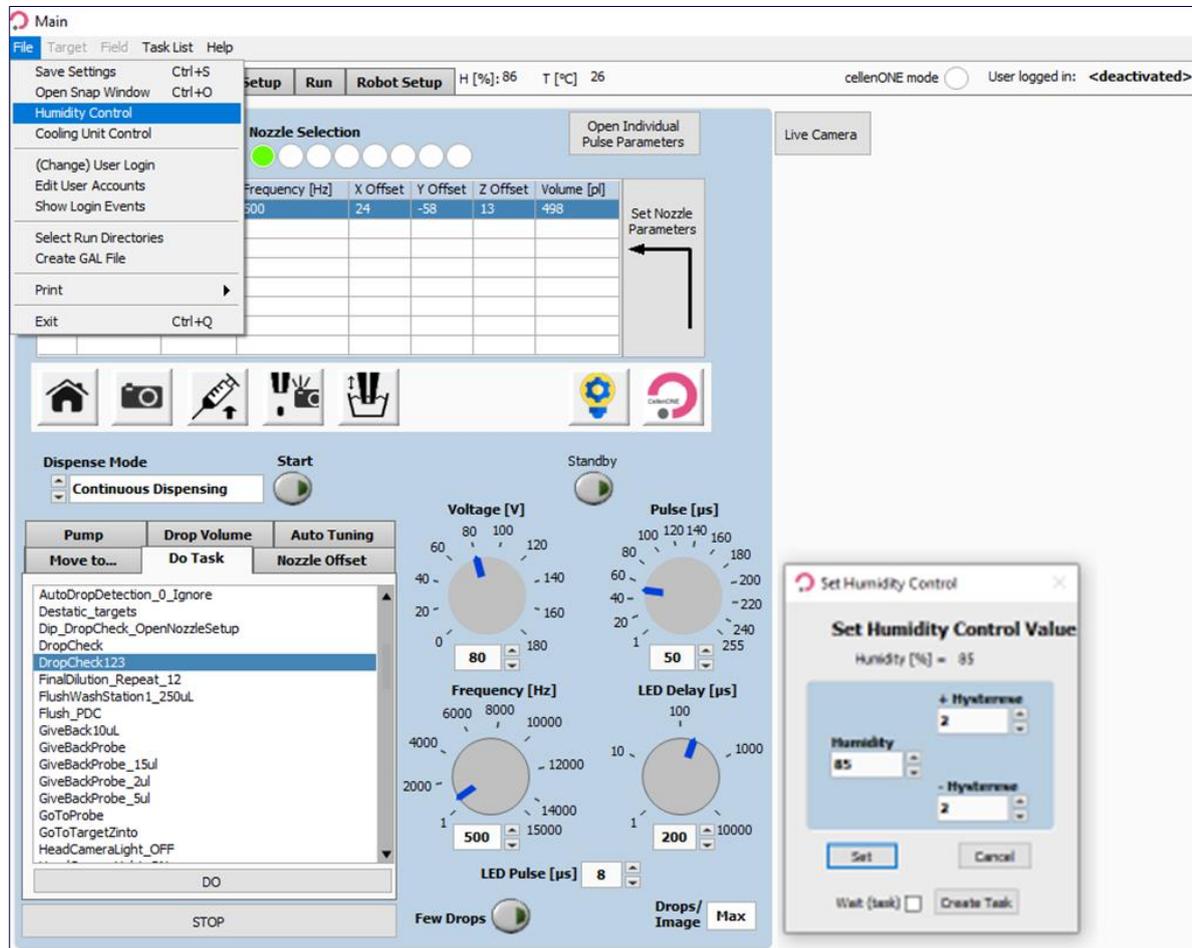


Figure 2. Humidity control.

- Temperature setup:
Main >> File >> Cooling Unit Control >> Pop-up window >> Set the temperature to fixed control and turn the cooling unit ON (Figure 3).



Figure 3. Temperature control.

Note 1. During a long cell isolation run, the low temperature can result in some condensation on the plate. However, this is not affecting the Master Mix efficiency nor is it leading to cross contamination.

Note 2. When performing a proteoCHIP workflow, it is best to start by setting the temperature to 10 °C.

3.4 Target Setup

To display your target, go to:

- **Target Setup >> Target**

For the proteoCHIP 12*16, you will always have the following configuration in **Target** subtab (Figure 4):

- 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

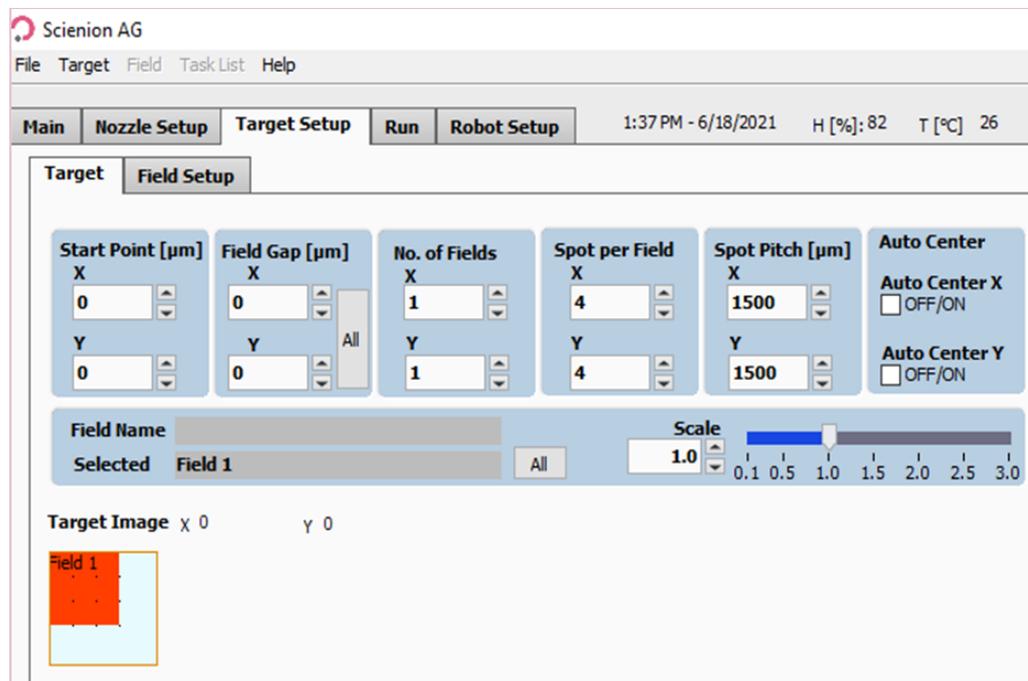


Figure 4. Target subtab.

3.5 Field Setup

The **Field Setup** subtab 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 this protocol).
- Number of drops/volume to be dispensed in which well of the Target.

Note 1. To change from Volume to drops and vice versa:

- **Target Setup >> Field Setup >> Field** (at the top) >> Volume entry

Note 2. For every run, fields are saved in the software.

You can edit these fields using **Edit Field Table** item 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 Table** pop-up window can be found at: **Field Setup >> Field >> Edit Field table** (Figure 5).

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 (Figure 5.3) to the column C, select whole “column” (Figure 5.4), and then select column C (Figure 5.5) and click on **Change all** button (Figure 5.6).

You will have all the wells from the probe that will be switched to column C as seen in Figure 6.

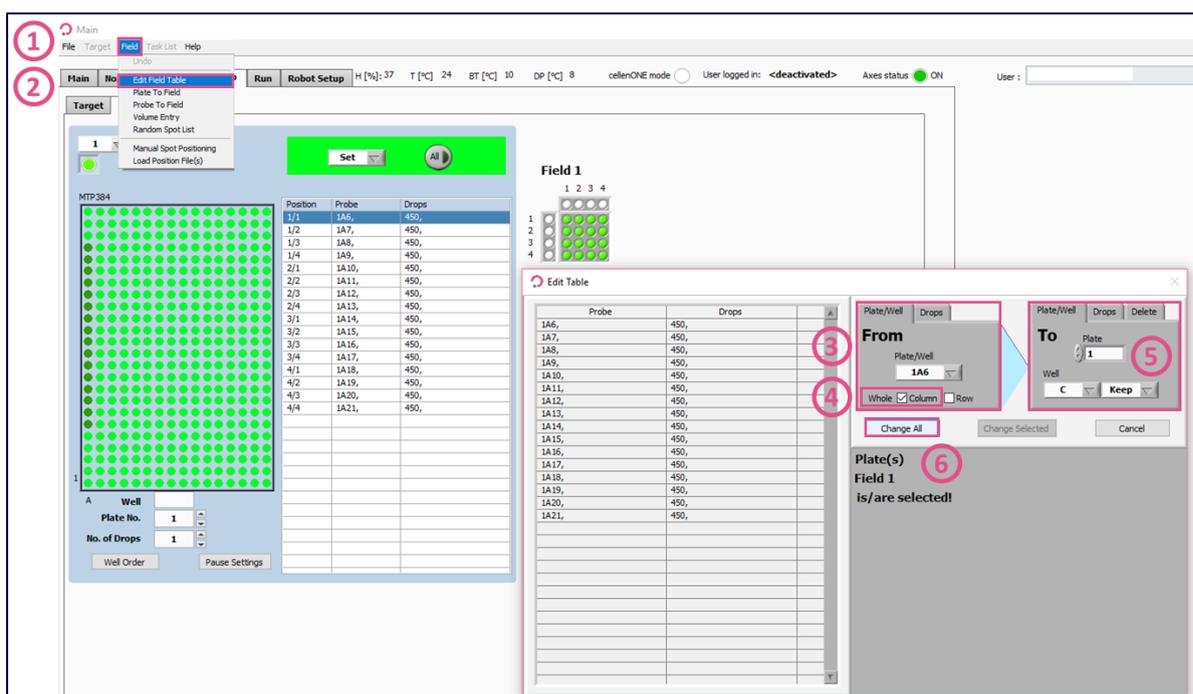


Figure 5. Edit Field Table.

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, H₂O in the rehydration steps, quenching solution and 0.1% TFA/FA.

To modify the number of drops in the Field setup refer to Figure 5 and change the number of drops by clicking on the **Drops** subtab in displays (4) and (5) (Figure 5.4).

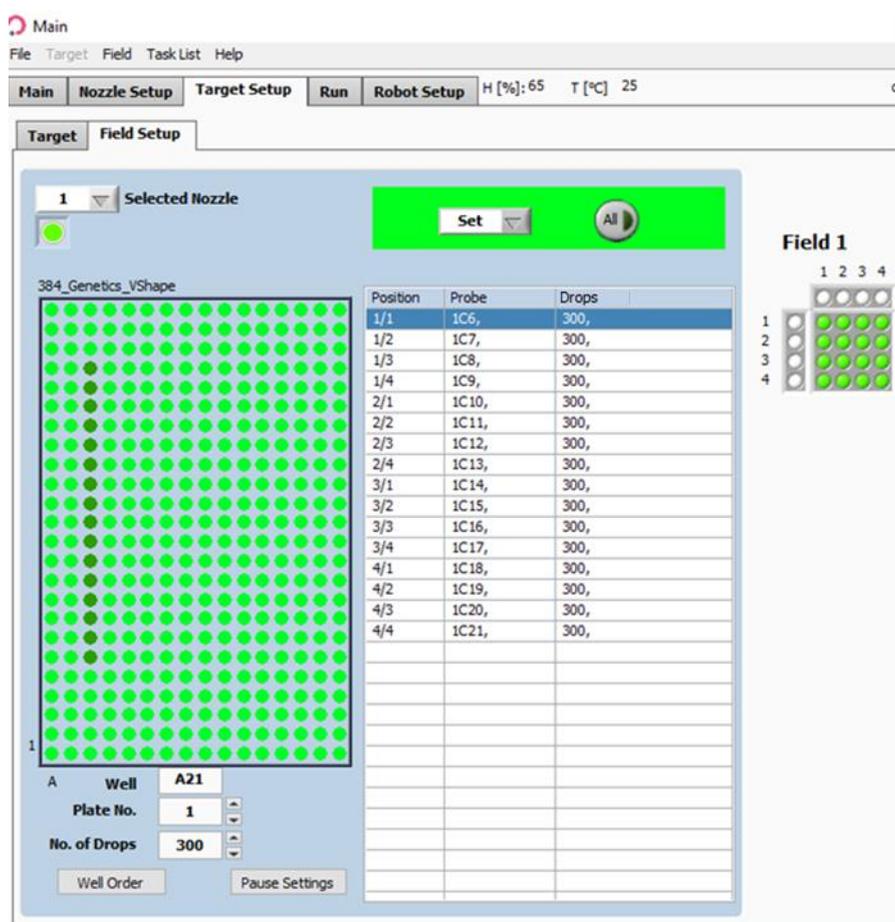


Figure 6. Field Setup.

3.6 Drop optimization

Each dispensing run is built with the same logic, once the run starts (Figure 7).

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

- Sample should be loaded in a chosen well of the 384-well source plate (the well is chosen in the **Field Setup** tab, see section 3.5).
- The volume to be aspirated is predefined inside the run by the **TakeProbe** task (*required volume is mentioned on the message that pops up at the beginning of the run*).
- The Piezo Dispense 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 automatically checks that the dispensing parameters are correct by performing a **DropCheck** task. 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 7.2), are correct by pressing the **Continuous Dispensing** button (Figure 7.3) 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 7.4) and press the **Drop Check** button (Figure 7.5) to check if the drop is identified by the software, circled in red (Figure 7.6)
- If needed, you can also perform troubleshooting tasks (**AirEx**, **Flush_PDC**, etc.) and uptake more sample.
- Once droplet generation is stabilized, click on **Continue** to resume the run (Figure 7.7).

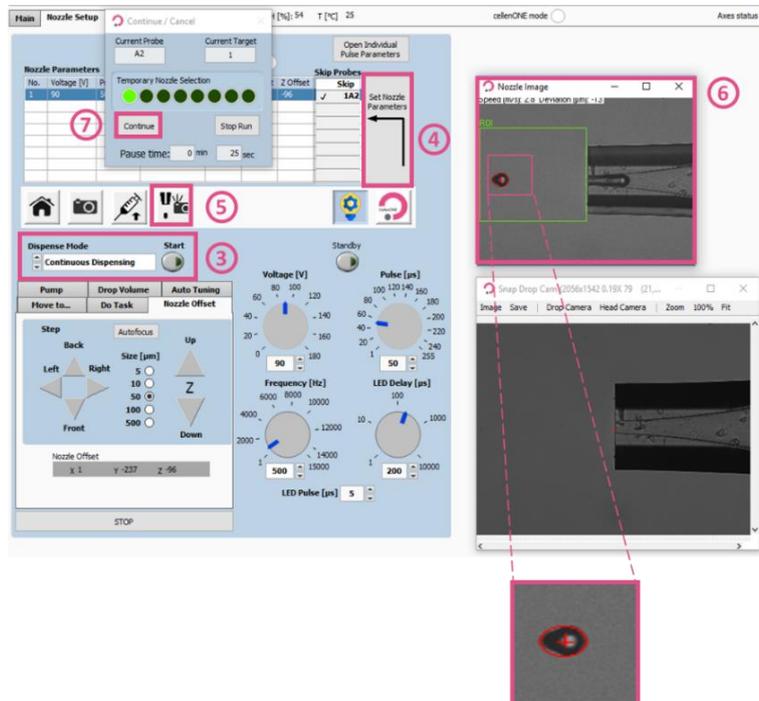
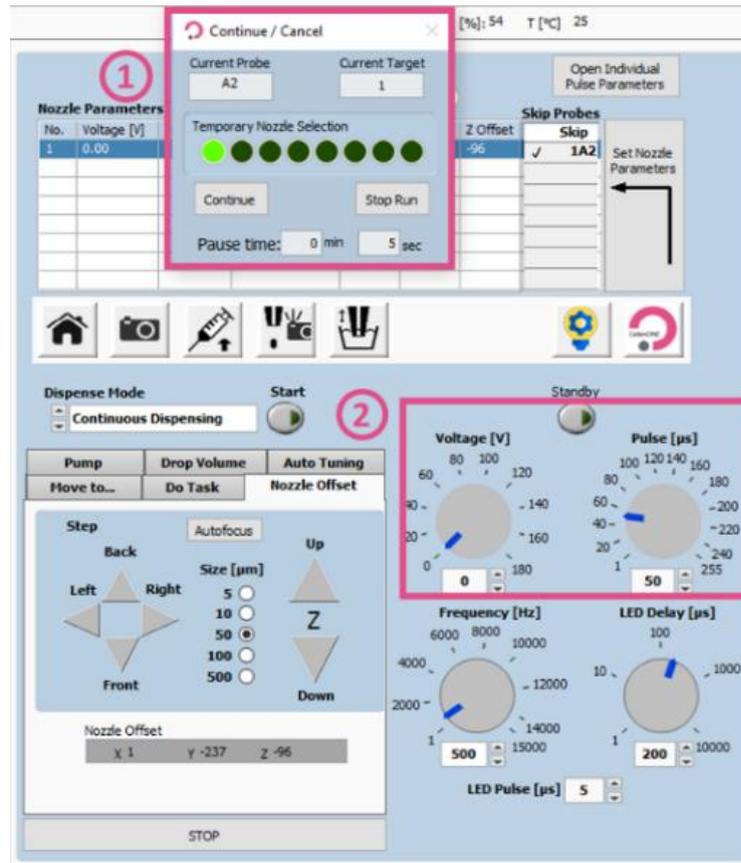


Figure 7. Drop optimization.

3.7 proteoCHiPs inside the cellenONE

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



*Figure 8. proteoCHIP 12*16 inside the cellenONE holder.*

- Place the proteoCHIP(s) 12*16 inside the proteoCHIP 12*16 holder (ref. CHB-1216) (Figure 8).

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 the target holder inside the instrument.

Note. 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 need to repeat Run 8 for each proteoCHIP 12*16.

E.g., If you are running 3 proteoCHiPs you 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, turn the temperature to 10 °C and the humidity to 45% to speed up the process.

Note 1. 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 H₂O + 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 2. 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 3. 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

The Master Mix dispensing happens at 10 °C and at a humidity set to 45%.

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

- Probe: Select **MTP384** (Figure 9.1).
- Run: Select **12x16_Run_1_MasterMix** (Figure 9.2).
- Target: Select **proteoCHIP_1216** (Figure 9.3).

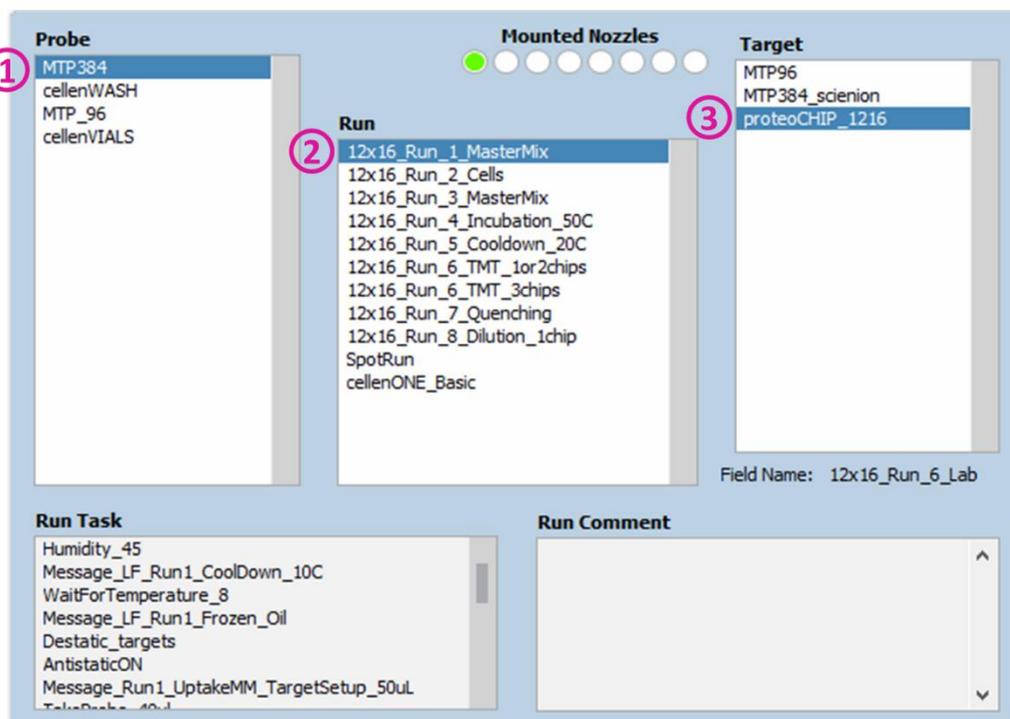


Figure 9. Probe, Run and Target Selection for Run 1 – Master Mix dispensing.

4.1.2 Field Selection

Load the **_Run_1_MasterMix** field which will spot 135 drops (= 50 nL) per well.

To load the field:

Target (Figure 10.1) >> **Load Field** (Figure 10.2) >> Select the field corresponding to the run (Figure 10.3) and you will have the field corresponding to Figure 11.

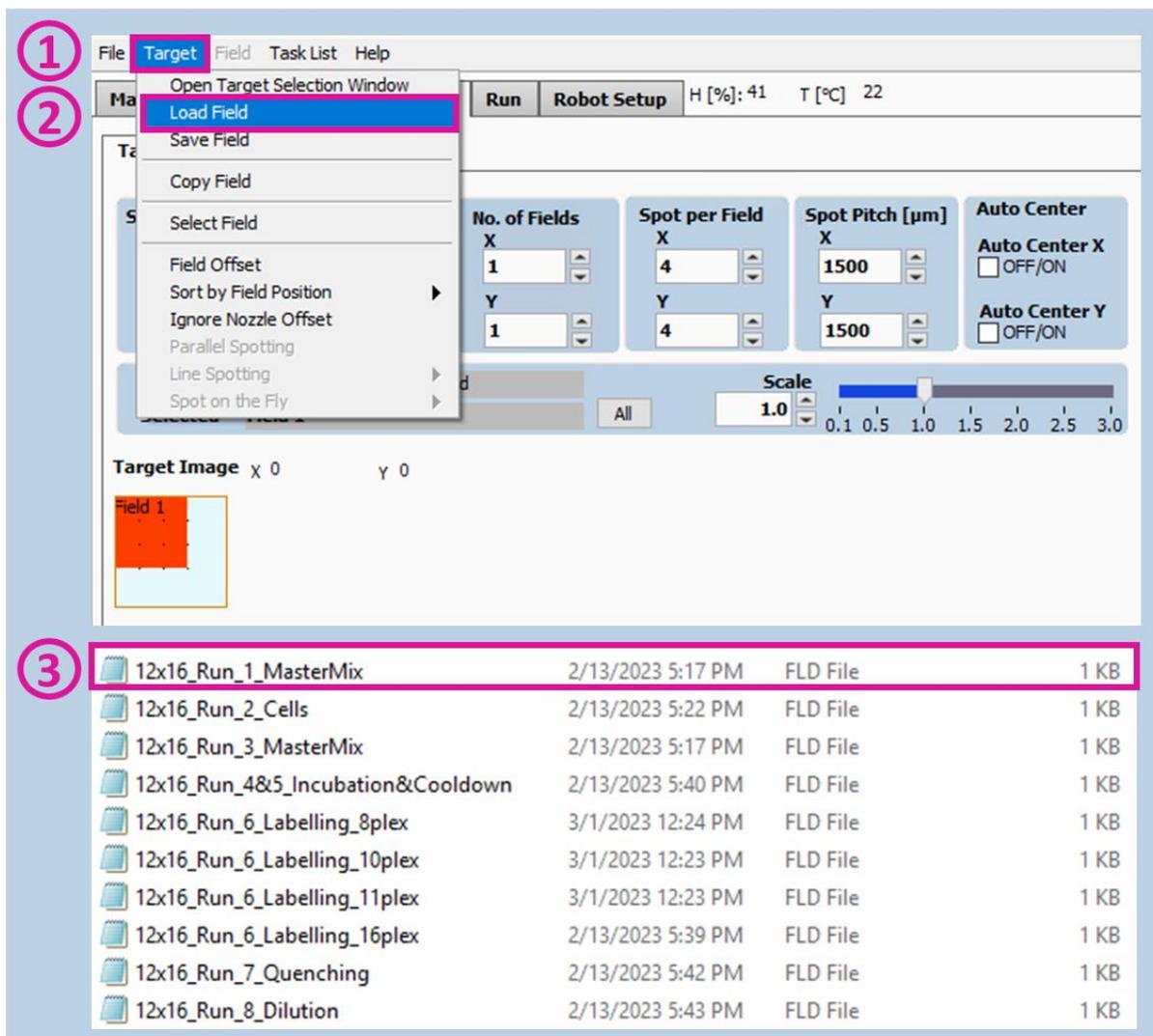


Figure 10. Field Selection for Run 1 – Master Mix dispensing.

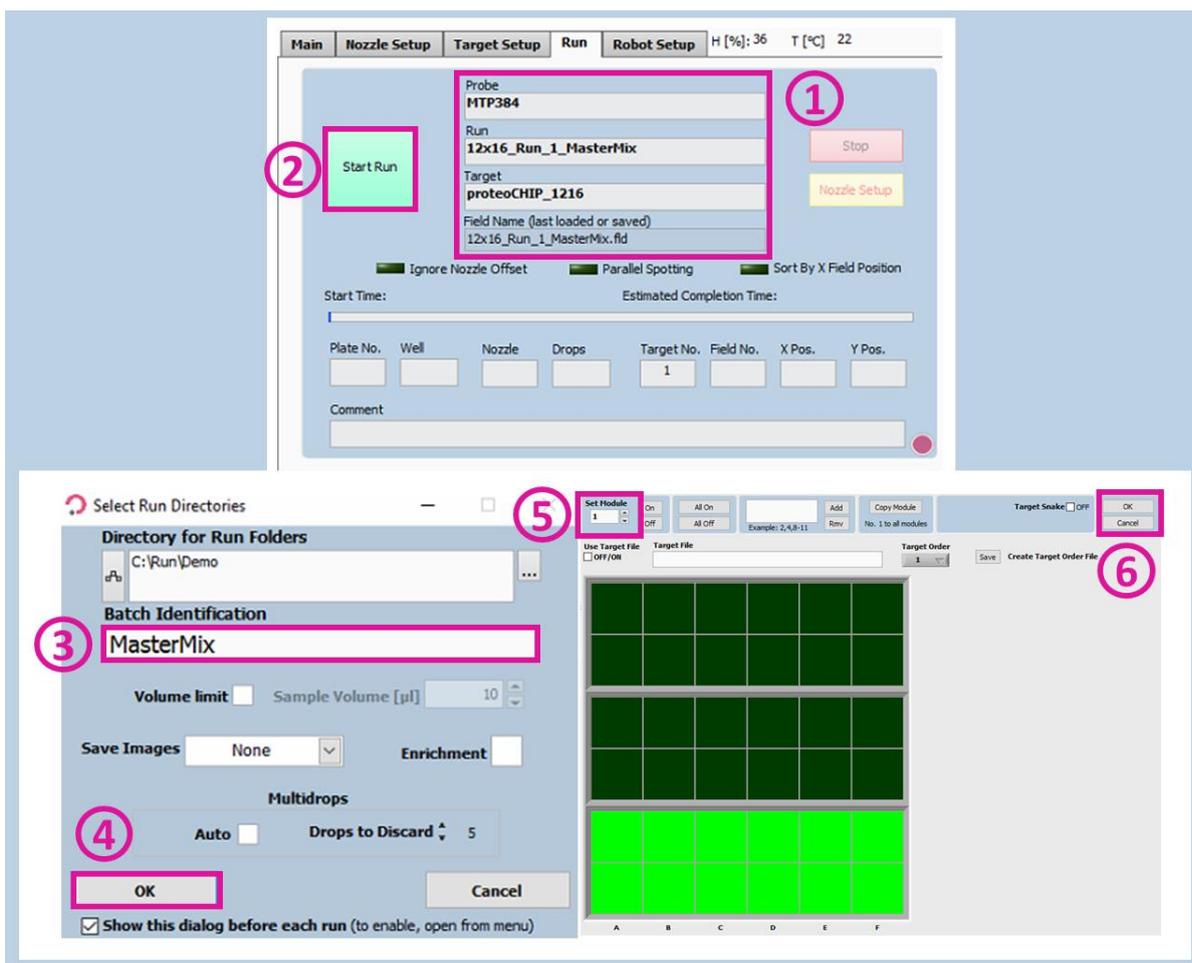


Figure 12. Run Start for Run 1 – Master Mix dispensing.

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

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

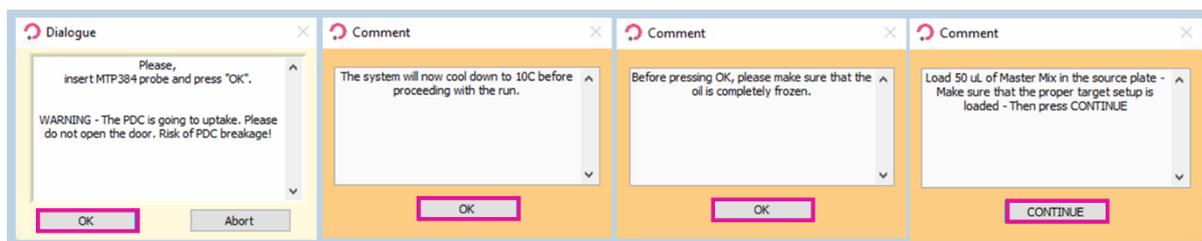


Figure 13. Pop-up messages for Run 1 - Master Mix Dispensing.

4.2 Run 2 – Cell isolation

Prepare your cells at a concentration of 100-200 cells/ μ L 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 14).

- Probe: Select **MTP384**.
- Run: Select **12x16_Run_2_Cells**.
- Target: Select **proteoCHIP_1216**.

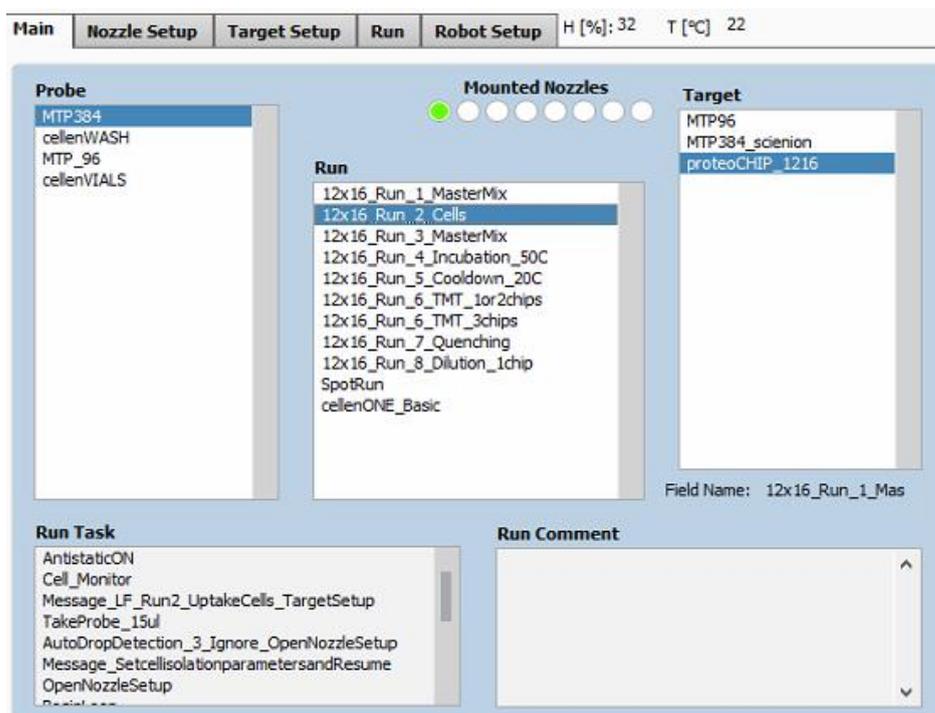


Figure 14. Probe, Run and Target Selection for Run 2 – Single cell isolation.

4.2.2 Field Setup

Click on the **Target Setup** tab, and the 1st subtab, **Target**. **Target** subtab 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 **12x16_Run_2_Cells** field which will spot one drop per well (which equals to one cell per well). To load the field, click on **Target** (Figure 15.1) >> **Load Field** (Figure 15.2) and select the field corresponding to the run (Figure 15.3, Figure 16).

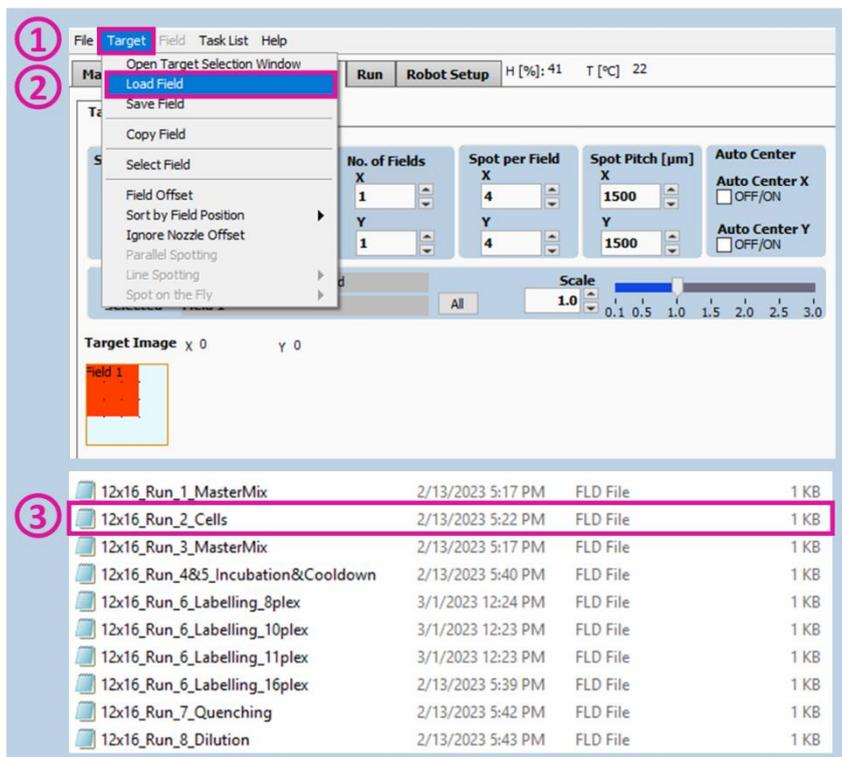


Figure 15. Field Selection for Run 2 – Cell isolation.

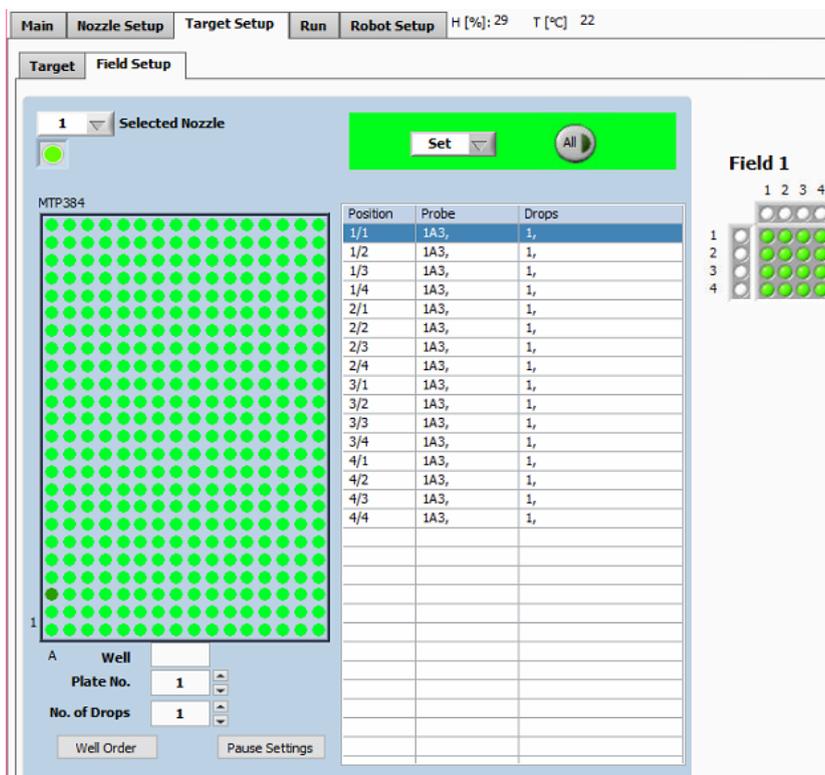


Figure 16. Field Setup for Run 2 – Cell isolation.

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 need to **open the cellenONE window** and start the run in this same window. Once the **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 17.1) and click on the cellenONE logo (Figure 17.2) to open the **cellenONE mode** module for single cell isolation.

Please refer to the cellenONE User Manual, in **Single cell isolation** section, to understand all the options and features for single cell dispensing.

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

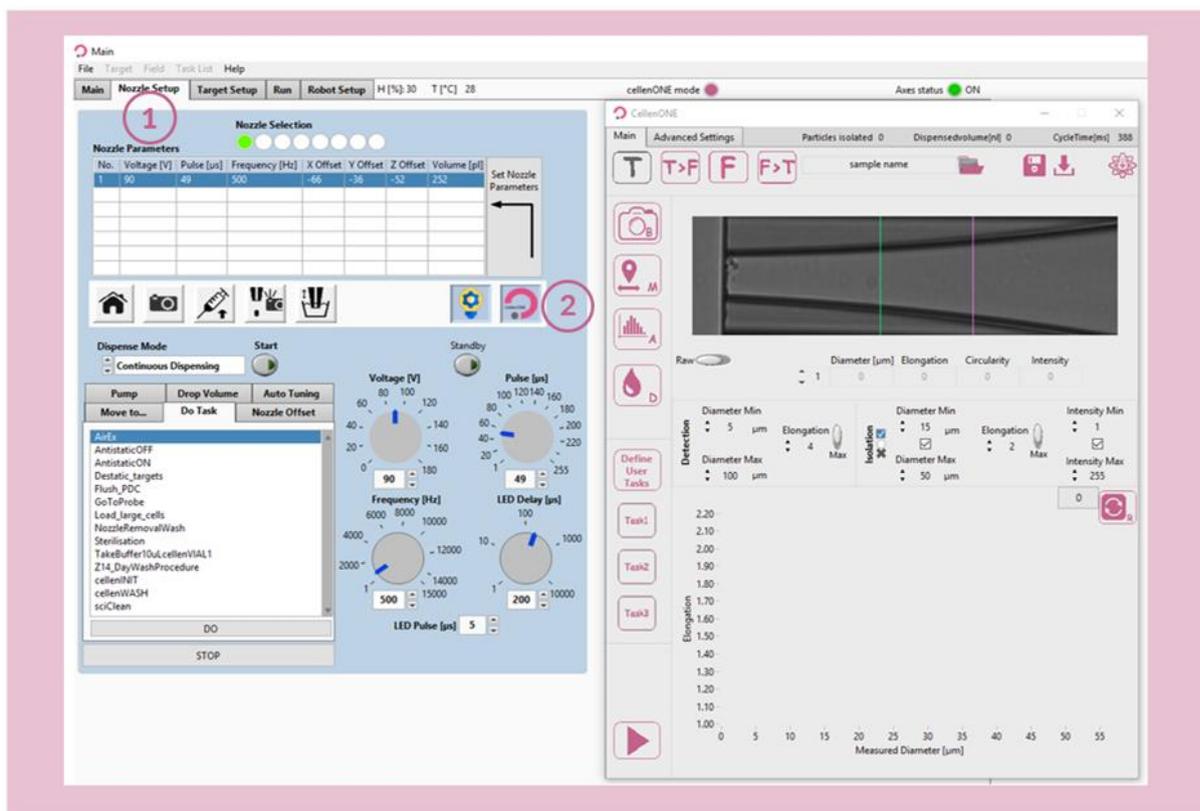


Figure 17. cellenONE module for single cell isolation (right window).

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.

When ready, go to the **Run** tab, and press Start Run. In the pop-up window, modify the run name if needed (Figure 18.1), check that the options are correct (Saved images, Multidrops), and then press OK (Figure 18.2).

Select the proteoCHIP positions that will be used (Figure 18). You can use the **On** button in **Set Module** item (Figure 18.3) to set a full proteoCHIP in one click. Then click **OK** (Figure 18.4).

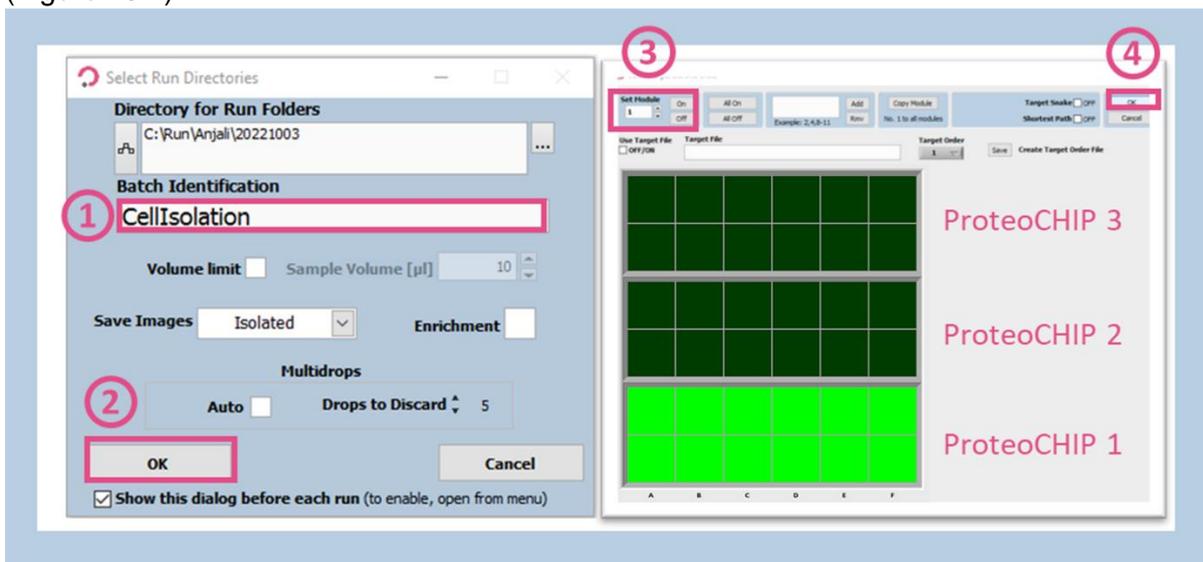


Figure 18. Starting Run 2 – Cell isolation.



Figure 19. Pop-up messages for Run 2 – Cell isolation.

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 Detection parameters

The **Main** tab of cellenONE module (Figure 20) displays images of the region of interest of the PDC in which cells are detected. During dispensing, cells travel 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. If you want to see the same information for cells located further right, you can navigate between cells using the small arrows on the left.



Figure 20. Main tab of cellenONE® module.

Note. 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 21). These parameters are crucial to successfully isolate and dispense single cells.

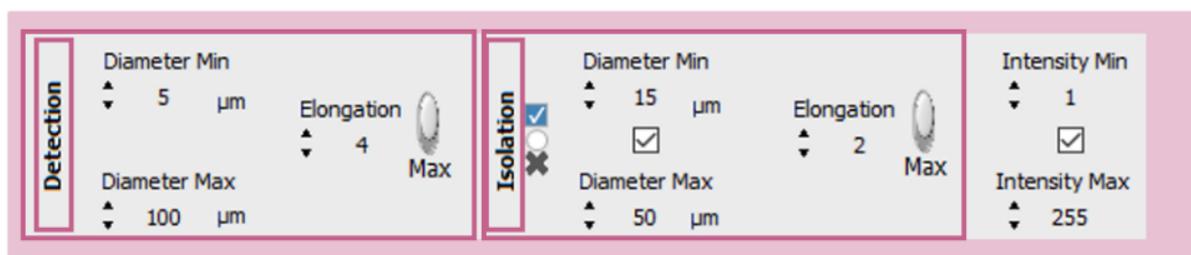


Figure 21. Detection and isolation parameters.

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**.

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

Note 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 is detected but does not fulfil the isolation parameters and would not be isolated.

Note 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 with 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 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 20). Pressing this button again 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 22). 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 cell within the PDC (i.e., upon next droplet generation these cells were ejected from the PDC). Once done, click **Save & close** button.

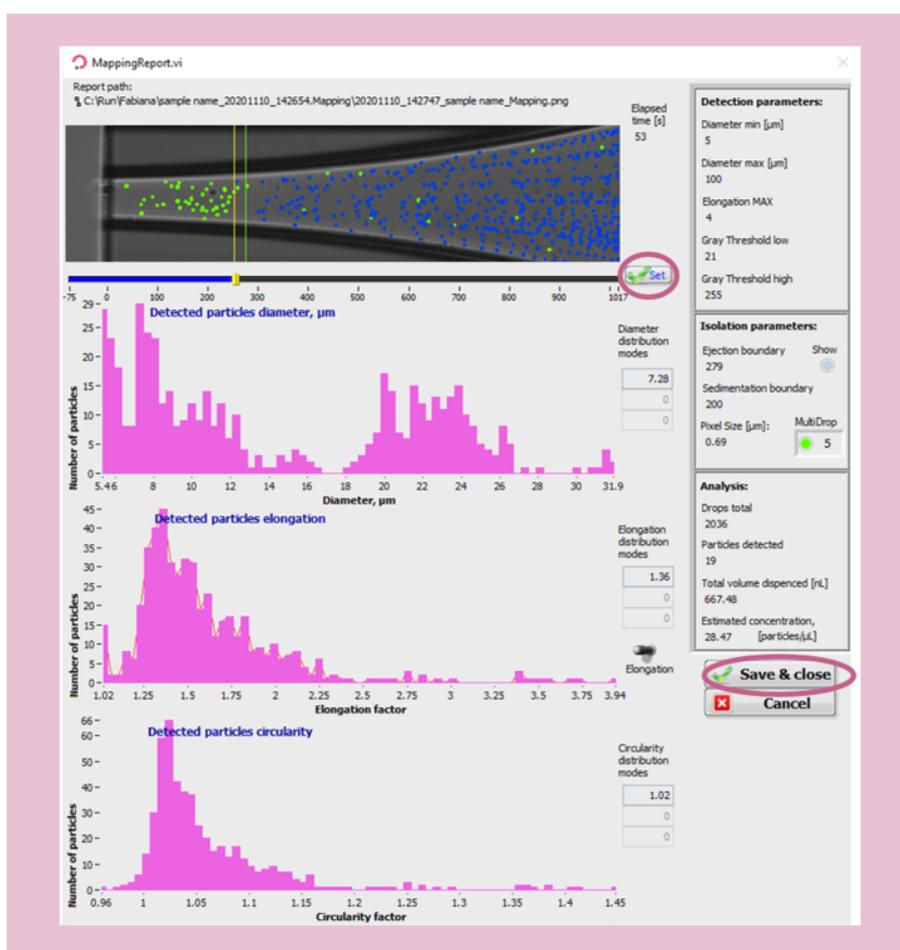


Figure 22. Mapping.

Note. 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 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 22) 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 generated from the mapping data 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 23). The number of analyzed cells can be set in the **Advanced Settings** tab. You can choose new isolation parameters by drawing a gate around your population of interest, and then clicking the **i** button (the letter, not the tick box) on the left of the plot.

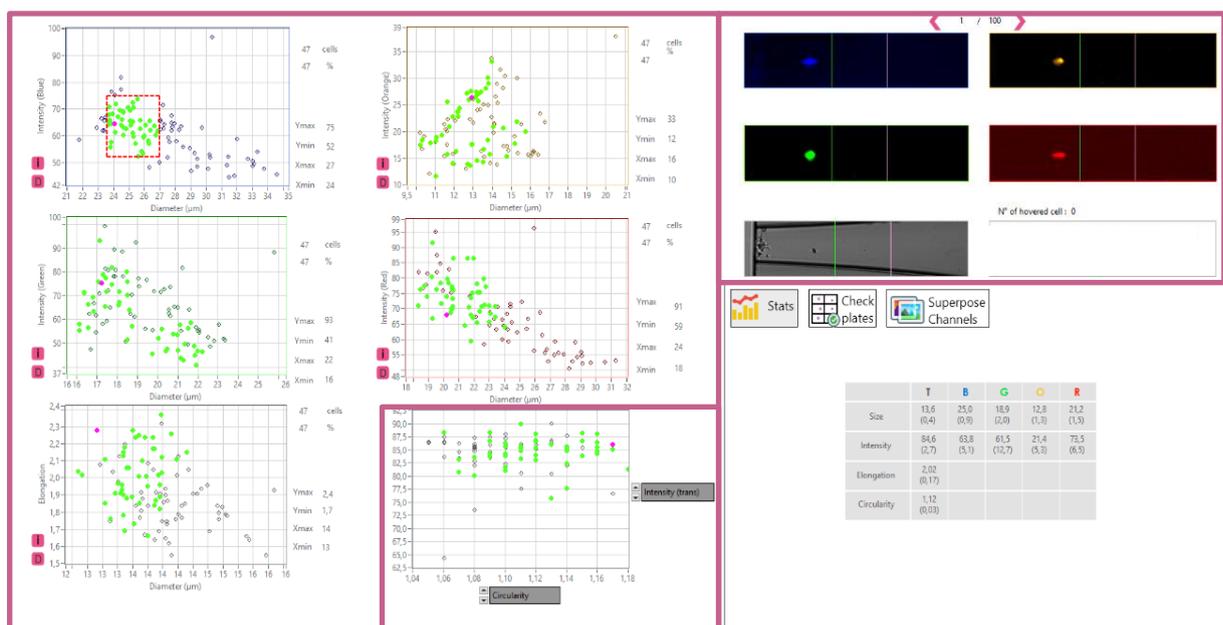


Figure 23. Scatter Plot.

4.2.7 Setup check

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 cells/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 24). After the last cell is isolated, a pop-up message informs you that the isolation is complete (Figure 24). If you are done with your cell isolation, do not forget to flush your PDC with the task Flush_PDC before proceeding to the next run.

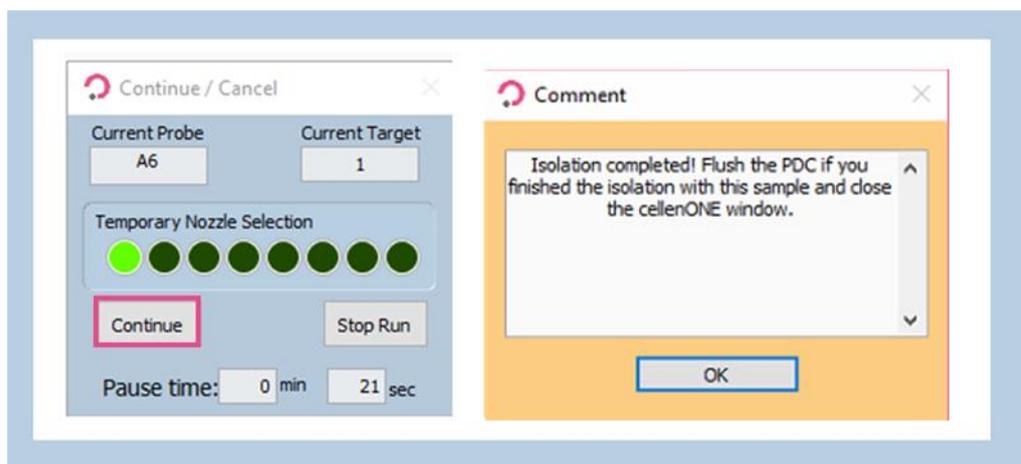


Figure 24. Run Start for Run 2 – Cell isolation.

4.3 Run 3 – Master Mix 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 been 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 **MTP384** (Figure 25.1).
- Run: Select **12x16_Run_3_MasterMix** (Figure 25.2).
- Target: Select **proteoCHIP_1216** (Figure 25.3).

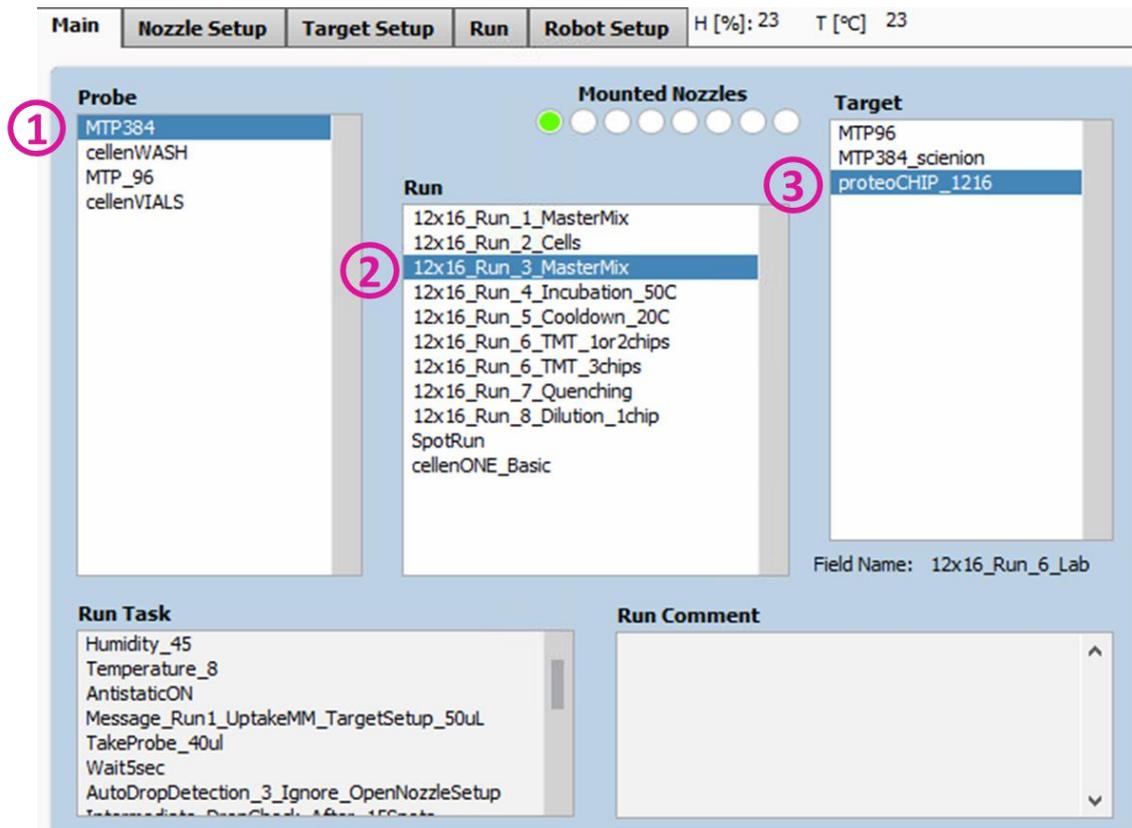


Figure 25. Probe, Run and Target Selection for Run 3 – Master Mix dispensing.

4.3.2 Field Setup

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

To load the field:

Target (Figure 26.1) >> **Load Field** (Figure 26.2) >> Select the field corresponding to the run (Figure 26.3) and you will have the field corresponding to the Figure 27.

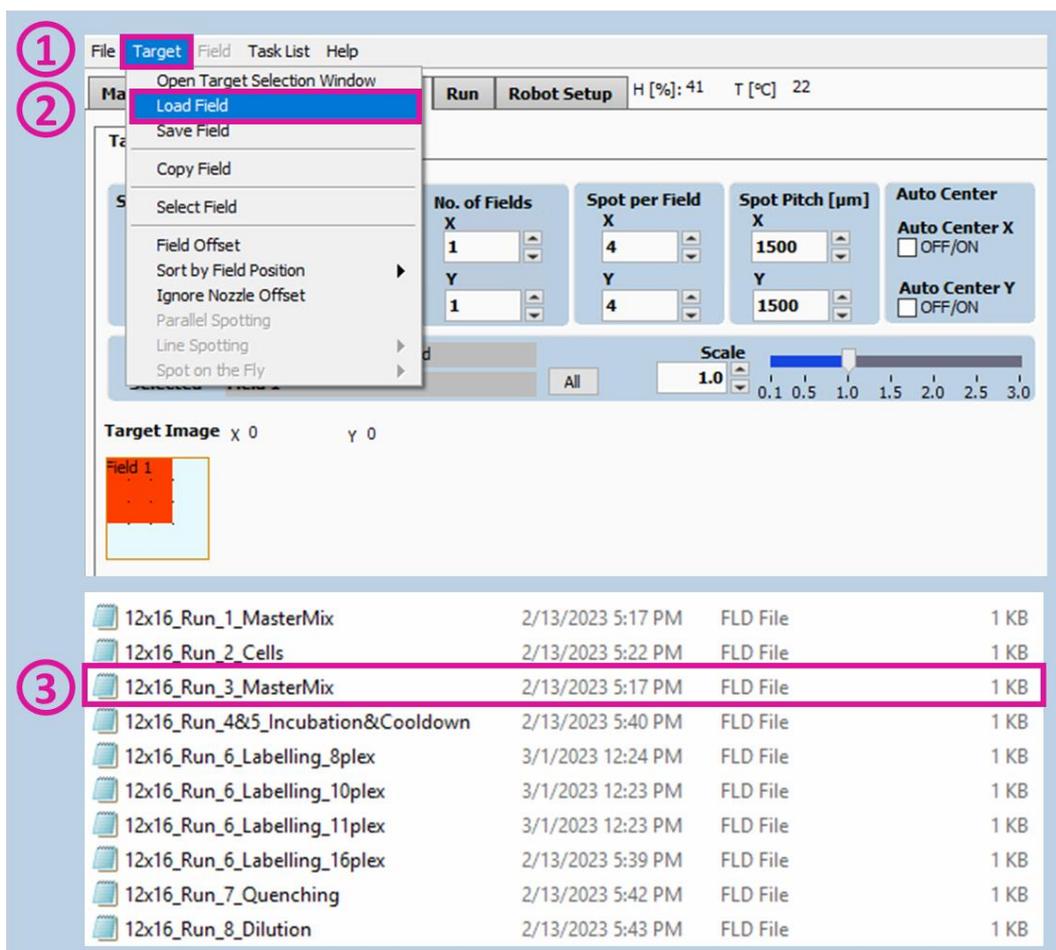


Figure 26. Field Selection for Run 3 – Master Mix Dispensing.

4.3.3 Run Start

Check that you selected the right Probe, Run and Target in the **Run** tab (Figure 28.1). Start the run (Figure 28.2), set the run name (Figure 28.3) and click **OK** (Figure 28.4). Then select the number of proteoCHiPs that you want to dispense in (Figure 28.5) and click **OK** (Figure 28.6).

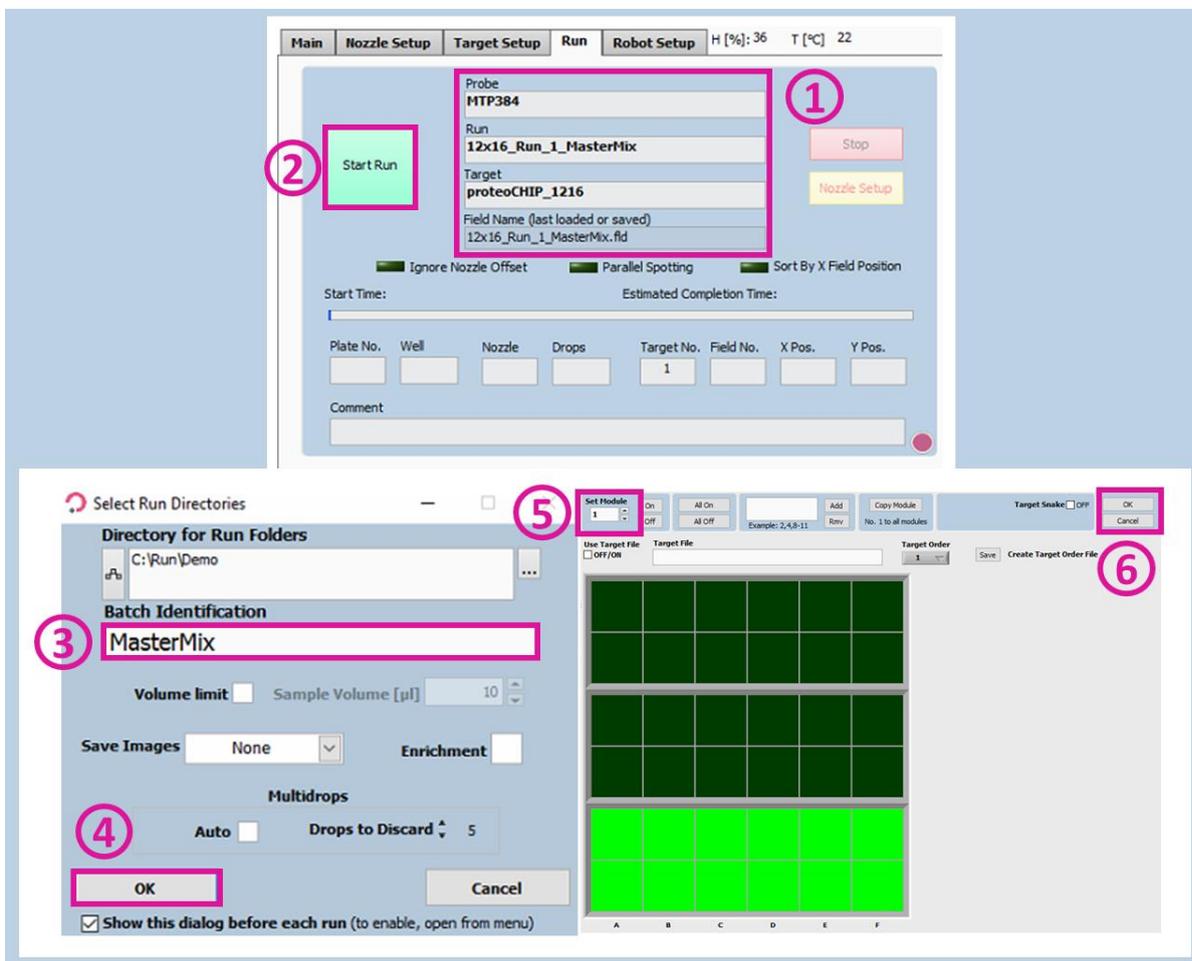


Figure 28. Run Start for Run 3 – Master Mix dispensing.

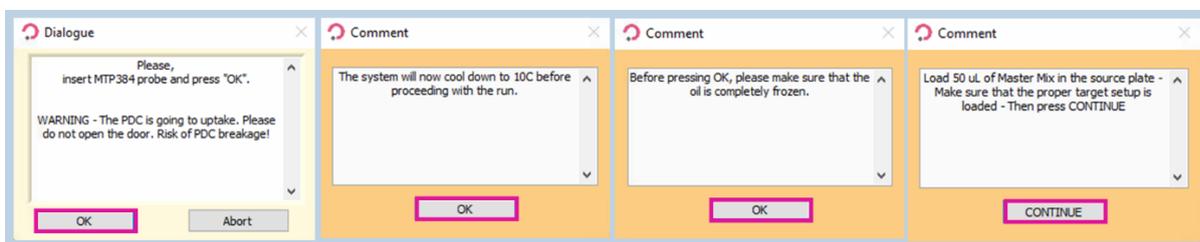


Figure 29. Pop-up messages for Run 3 – Master Mix dispensing.

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 lasts 2 hours at a temperature of 50 °C. In addition to an increased ambient humidity (85%, the PDC will regularly dispense Ultrapure 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 30).

- Probe: Select **MTP384**.
- Run: Select **12x16_Run_4_Incubation_50C**.
- Target: Select **proteoCHIP_1216**.

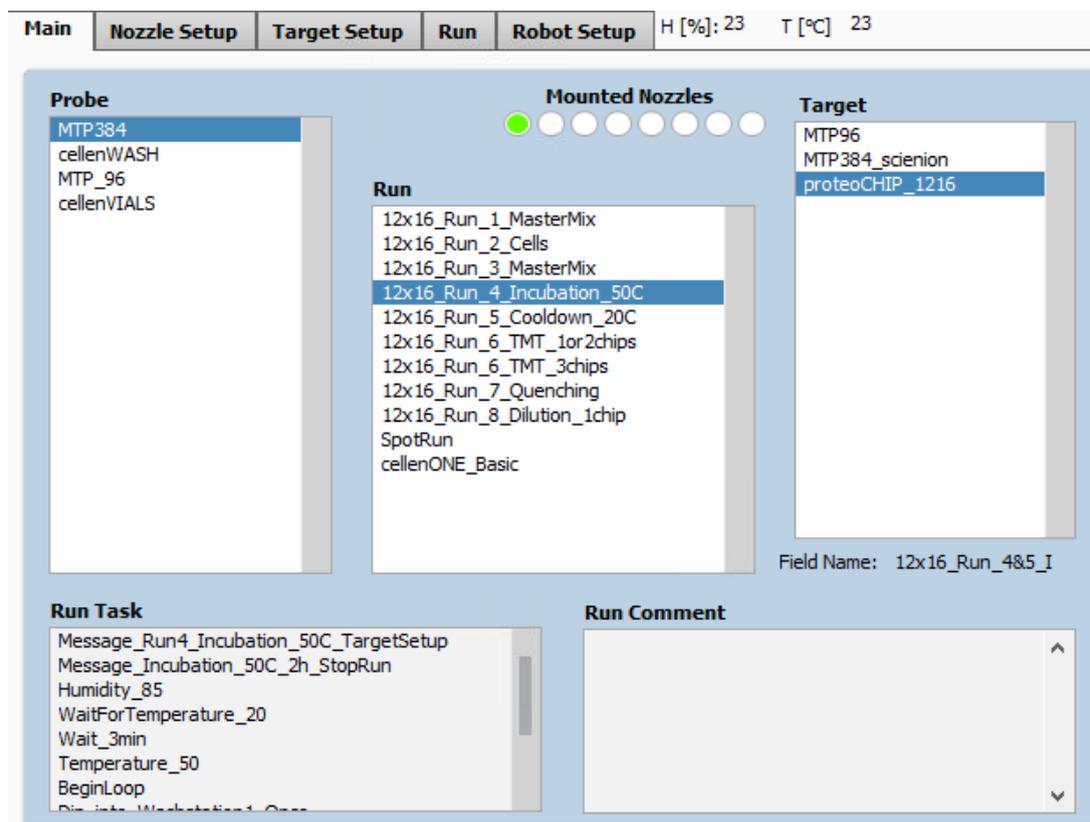


Figure 30. Probe, Run and Target Selection for Run 4 – Incubation.

4.4.2 Field Setup

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 on **OK** (Figure 33).

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

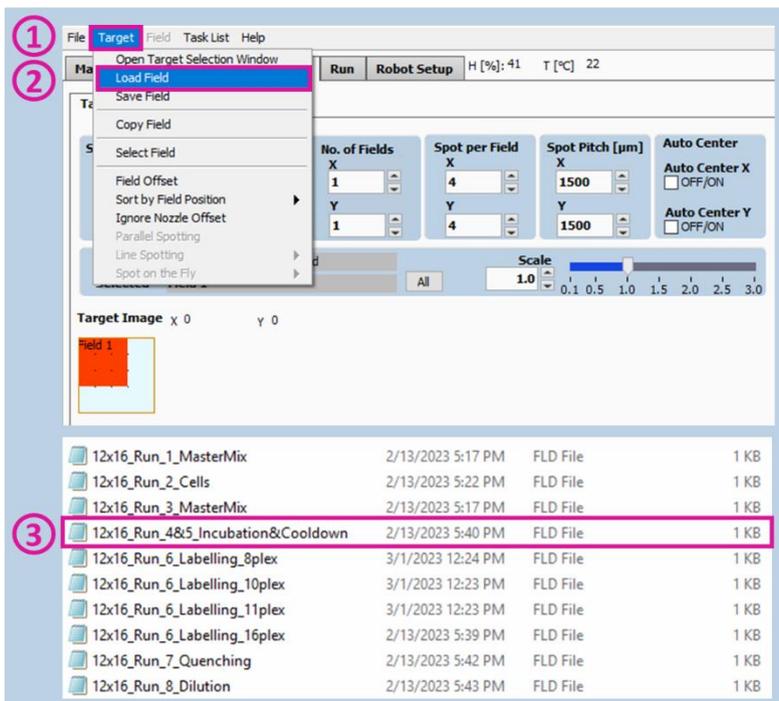


Figure 31. Field Selection Run 4 – Incubation.

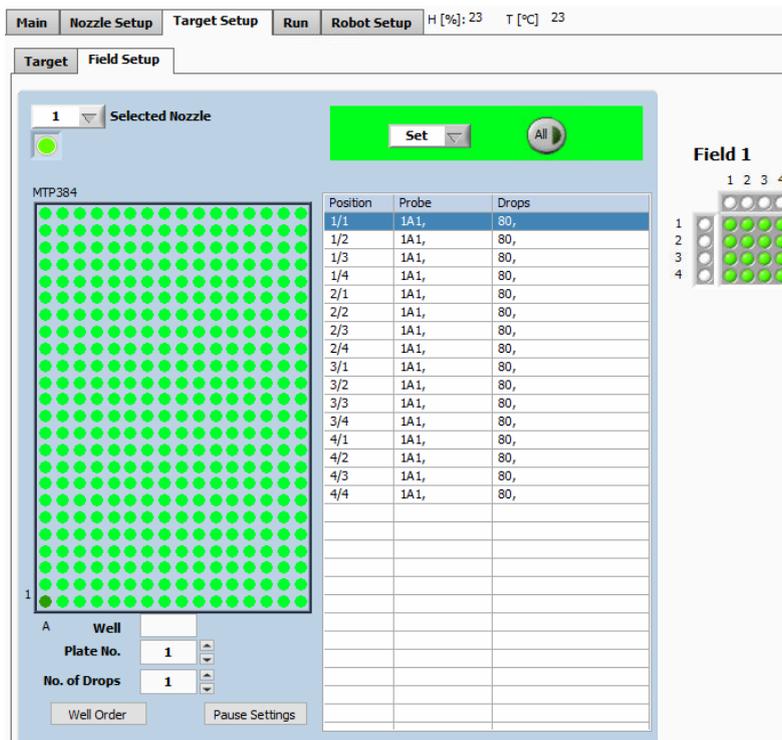


Figure 32. Field Setup for Run 4 – Incubation.

4.4.3 Run Start

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

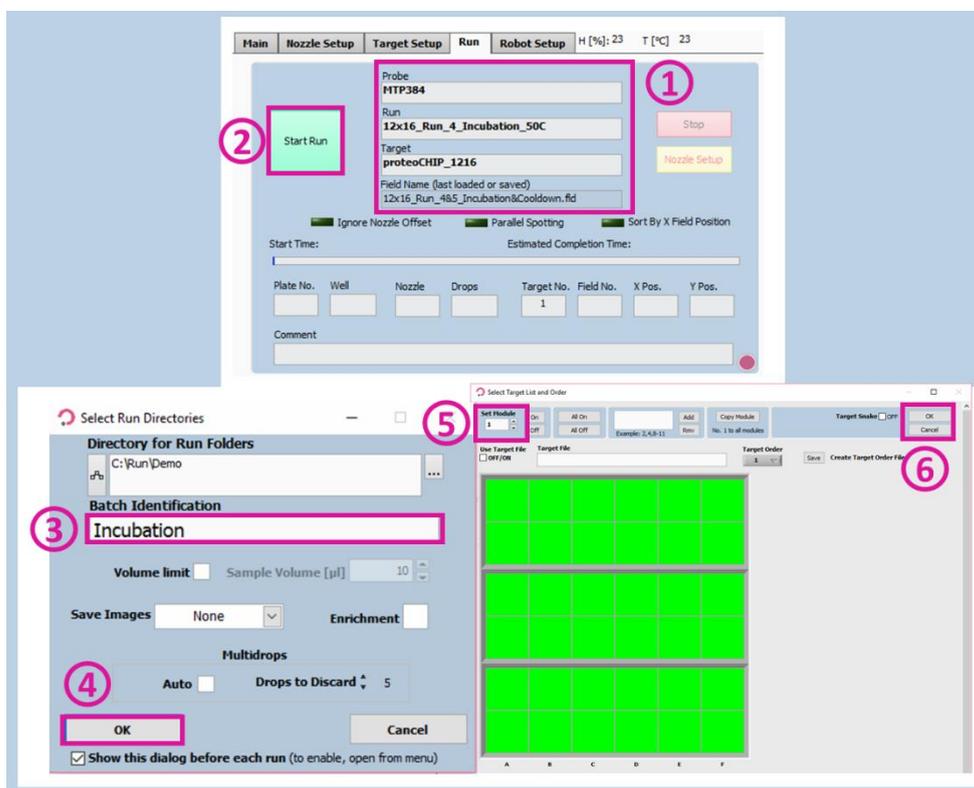


Figure 33. Run Start for Run 4 – Incubation.

Once the Run has started, the humidity and temperature are automatically set. A pop-up message will be displayed, click on **OK** to start the 2 hours incubation (Figure 34).



Figure 34. Pop-up messages for Run 4 – Incubation.

After 2 hours, once you are ready to proceed to the next step, click on the red **Stop** button. Dismiss the pop-ups by clicking on **Exit Run** and then **Partially spotted** (Figure 35).



Figure 35. Run exit for Run 4 – Incubation.

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 36).

- Probe: Select **MTP384**.
- Run: Select **12x16_Run_5_Cooldown_20C**.
- Target: Select **proteoCHIP_1216**.

No changes need to be made in the **Field Setup** subtab, so you can proceed to start the run immediately.

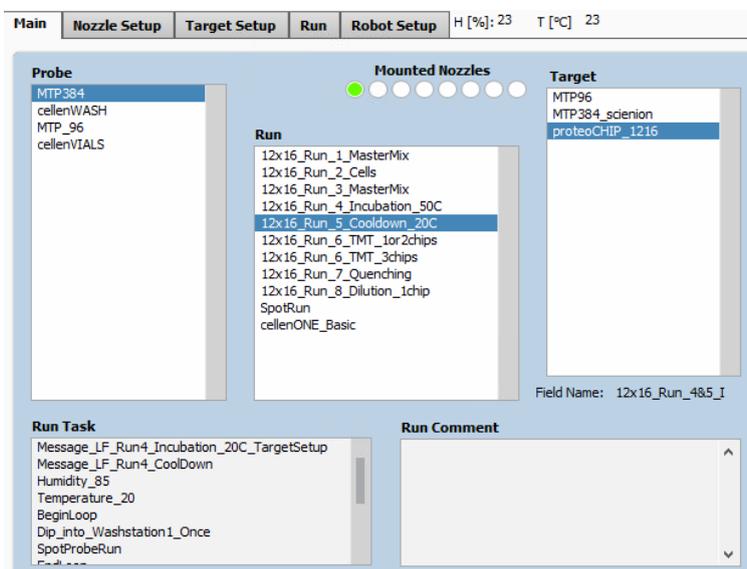


Figure 36. Probe, Run and Target Selection for Run 5 – Cooldown.

4.5.2 Run Start

Once the run has been selected, click on the **Start Run** button in the **Run** tab (Figure 37.2). 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 on **OK** (Figure 37.6). The run finishes once the temperature of 20 °C is reached. This should take approximately 30 minutes (Figure 38).

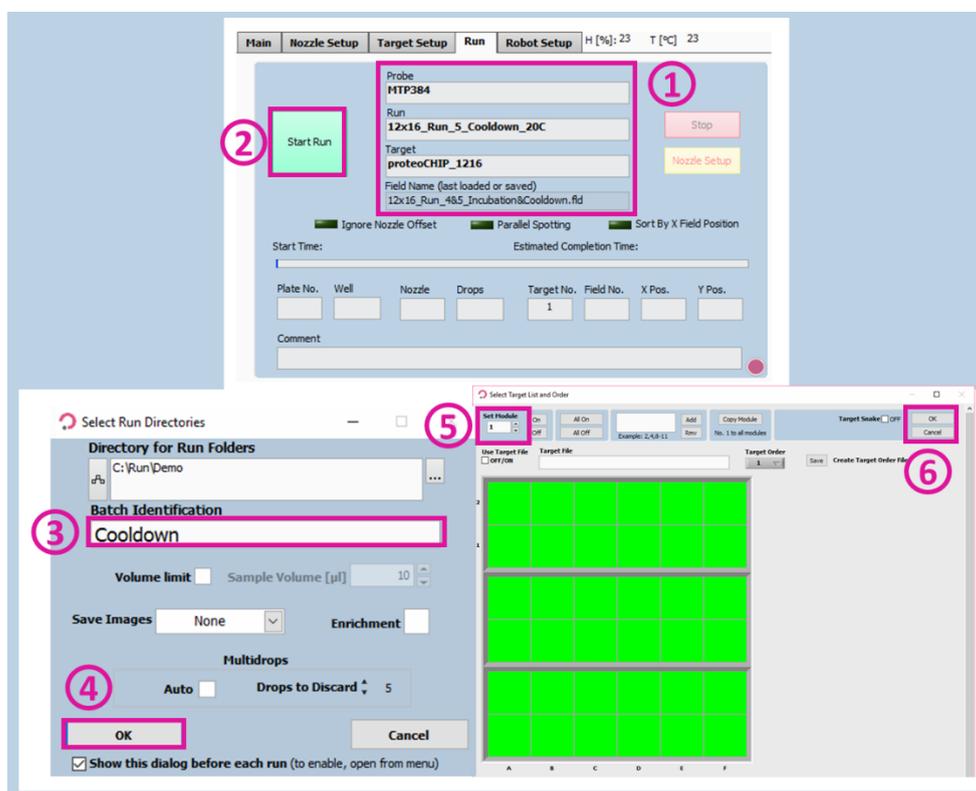


Figure 37. Run Start for Run 5 – Cooldown.

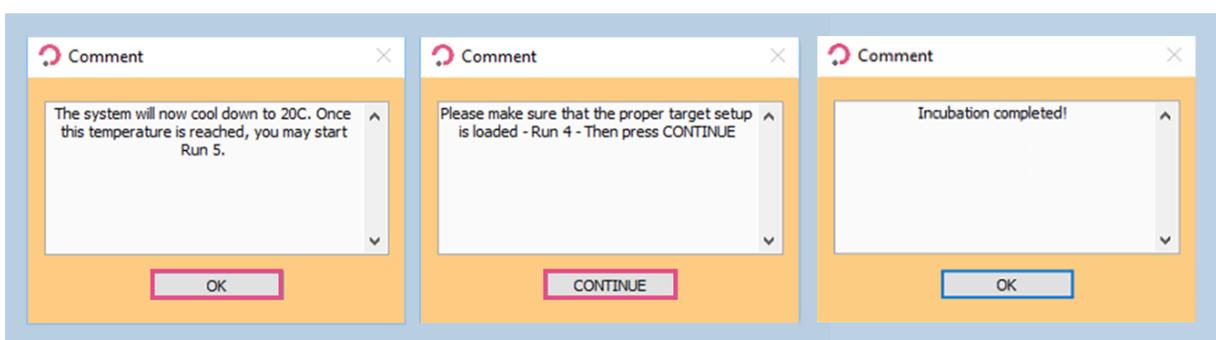


Figure 38. Pop-up messages for Run 5 – Cooldown.

4.6 Run 6 – TMT Labelling

Warning. 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 optimized for TMT labelling, however, adaptations using iTRAQ or mTRAQ are possible by switching the solvent and/or the reagent concentration.

Note 1. 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 water-free acetonitrile can be added to each tube containing 0.5 mg of lyophilized 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 PCR tube will be needed. 15 µL will be needed per TMT label.

Note 2. This workflow was optimized for TMT labels, however, adaptations using other labels like iTRAQ are possible by adapting the reagent concentration and/or the solvent according to respective single cell proteomics literature.

Note 3. Before starting the run, you need to optimize the Voltage and Pulse parameters for acetonitrile dispensing. You can do this with the AirGapTakeProbe_40ul task and perform 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.

Warning. 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 **MTP384** (Figure 39.1).
- Run: Select **12x16_Run_6_TMT_1or2chips** or **12x16_Run_6_TMT_3chips** (Figure 39.2).
- Target: Select **proteoCHIP_1216** (Figure 39.3).

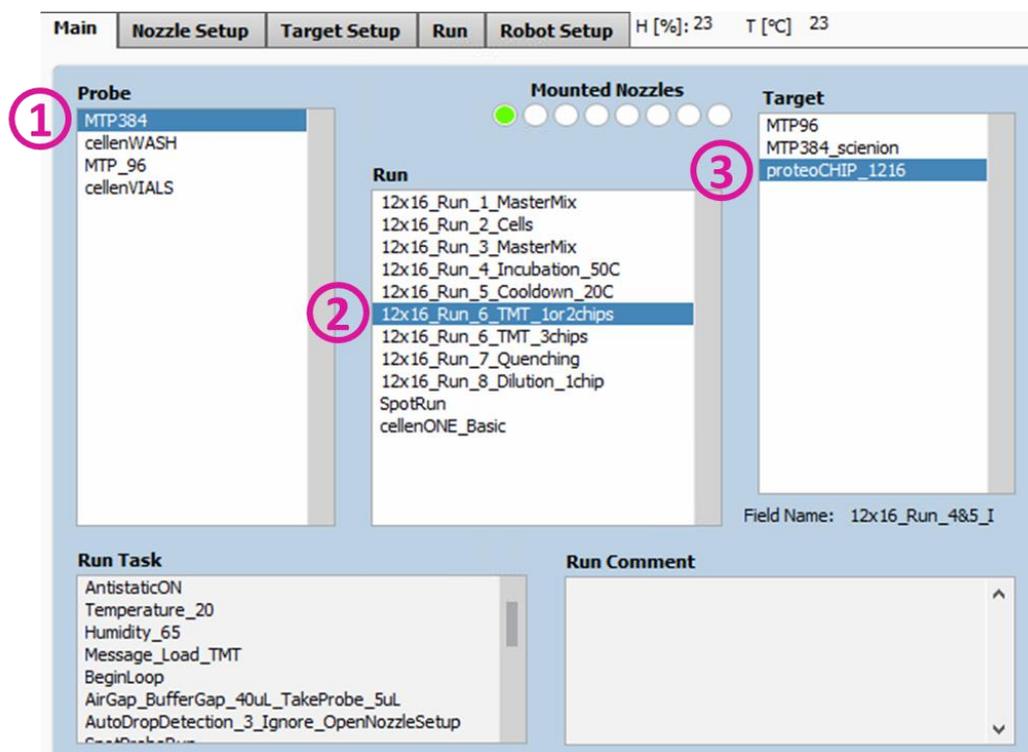


Figure 39. Probe, Run and Target Selection for Run 6 – TMT Labelling.

4.6.2 Field Setup

Load the **12x16_Run_6_Labelling_Nplex** field 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 40, Figure 41, Figure 42).

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

To load the field, click on **Target setup** (Figure 40.1) >> **Target** (Figure 40.2) >> **Load Field** (Figure 40.3).

During this TMT labelling run, a pre-programmed task is 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 μ L of air to ensure no mixing between water and acetonitrile.
2. A buffer gap of 40 μ L 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 μ L of TMT will be aspirated before dispensing.

Warning. Throughout the run, you need to load:

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

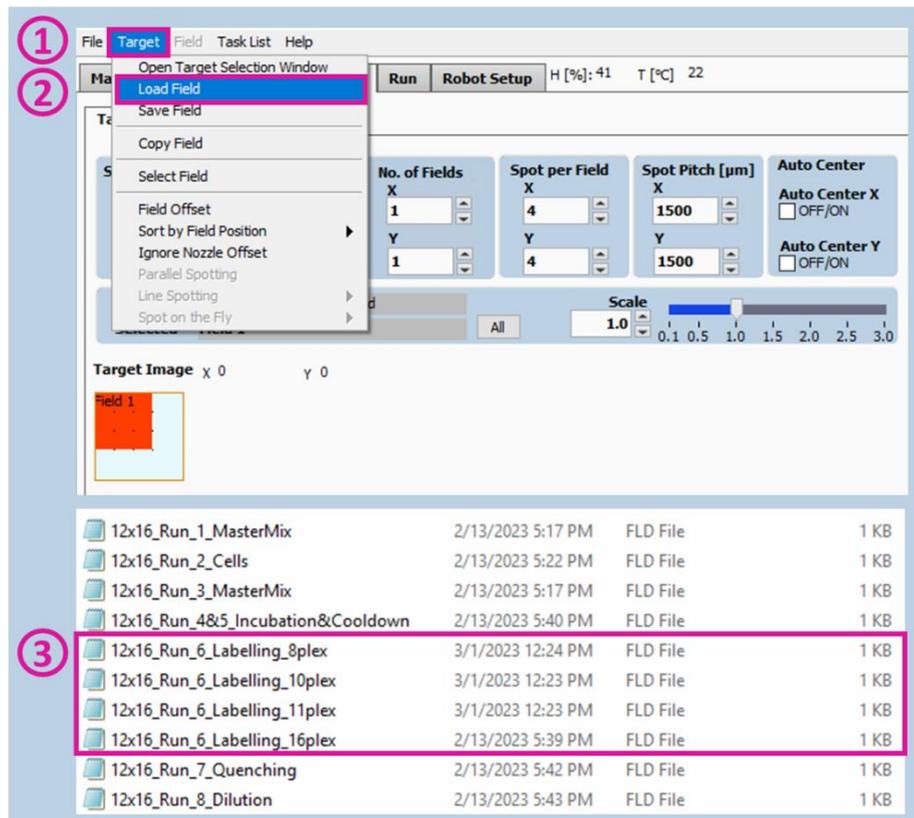


Figure 40. Field Selection for Run 6 – TMT labelling.

Different number of labels can be chosen:

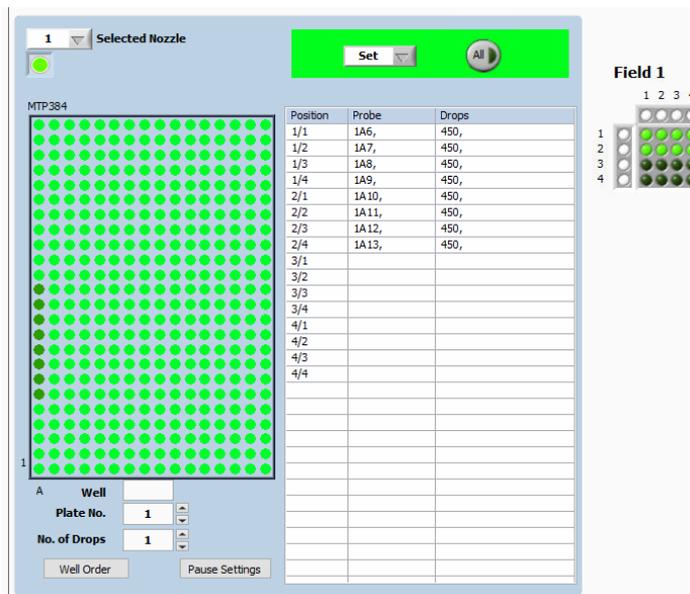


Figure 41. Field Setup for 8-plex labelling for Run 6 – TMT labelling.

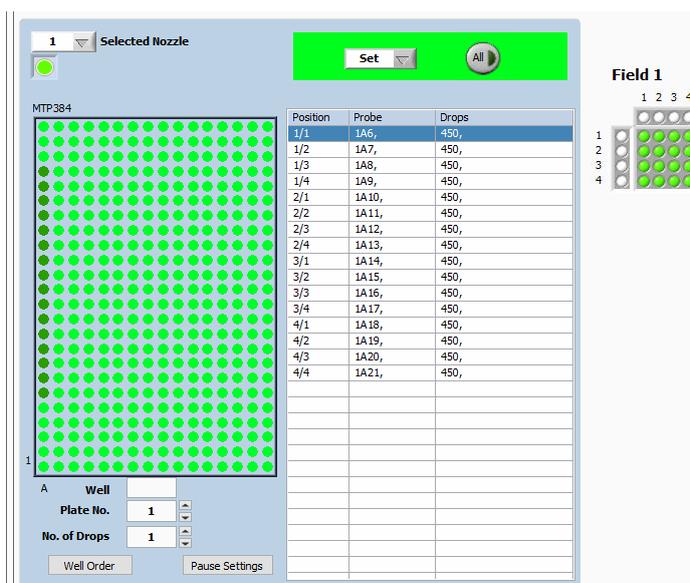


Figure 42. Field Setup for 16-plex labelling for Run 6 – TMT labelling.

Warning. 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 Run Start

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

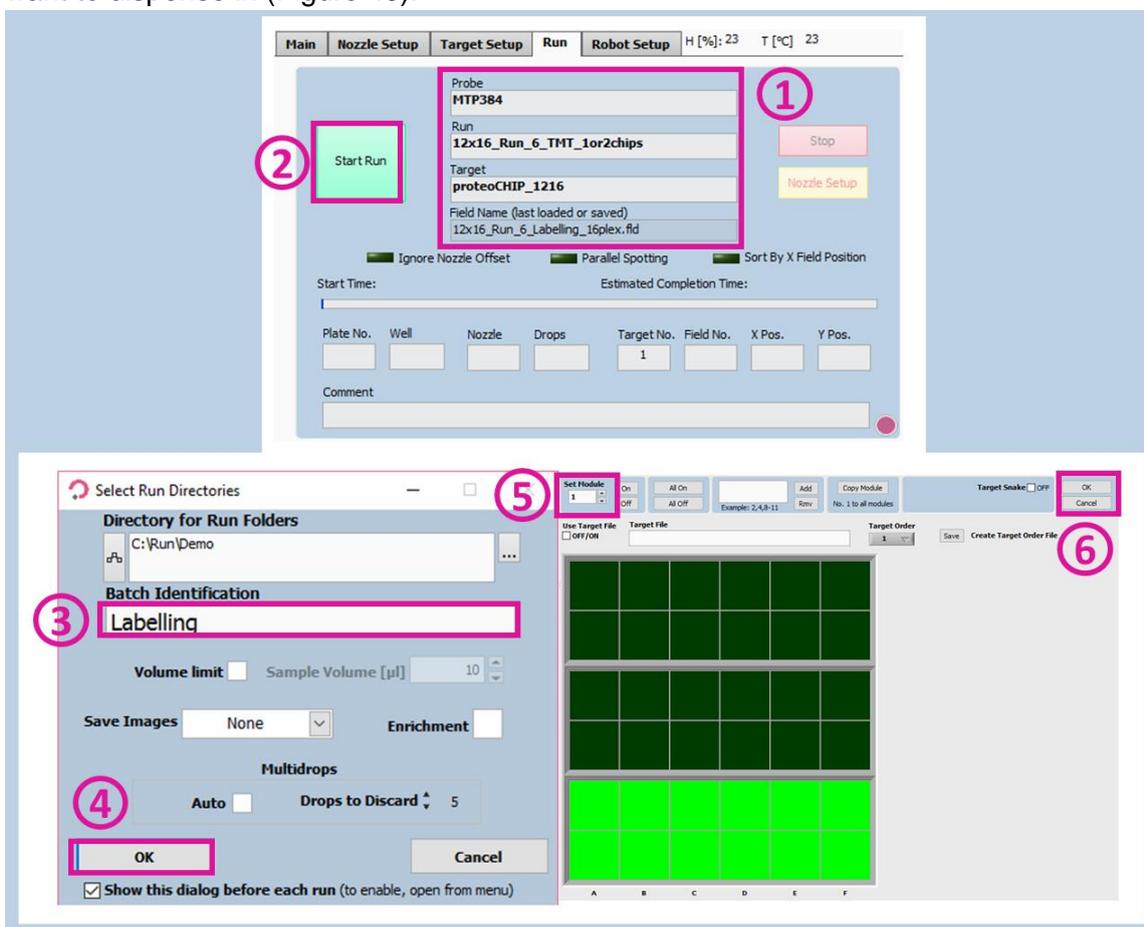


Figure 43. Run Start for Run 6 – TMT labelling.

Note. Before the aspiration of each label, a pop-up window is displayed. Click on **OK** after completing the loading of 55 μ L of ACN (left well) and 15 μ L of TMT (right well) (Figure 44).

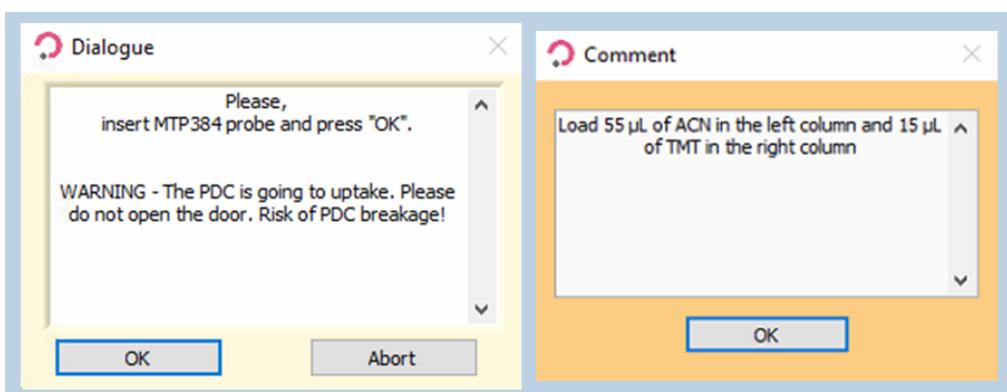


Figure 44. Pop-up messages for Run 6 – TMT labelling.

Note. At the end of this run, an incubation of 30 minutes at RT starts.

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 H₂O + 3 µL 37% HCl + 1 µL 50% Hydroxylamine

Load 50 µL of the Quenching solution in well A23.

Warning. Remember 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 **MTP384**.
- Run: Select **12x16_Run_7_Quenching**.
- Target: Select **proteoCHIP_1216**.

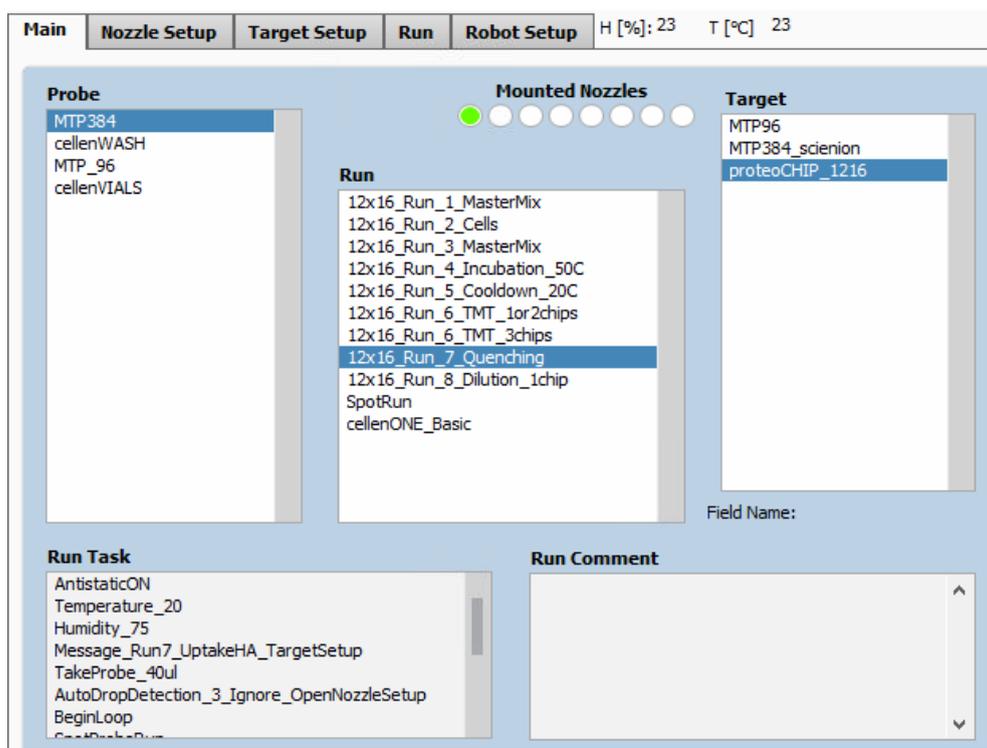


Figure 45. Probe, Run and Target Selection for Run 7 – Quenching.

4.7.2 Field Setup

To dispense the quenching solution, load the **12x16_Run_7_Quenching** field 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 **12x16_Run_7_Quenching** run (Figure 46.3). The field associated with quenching run will be displayed in the Field setup (Figure 47).

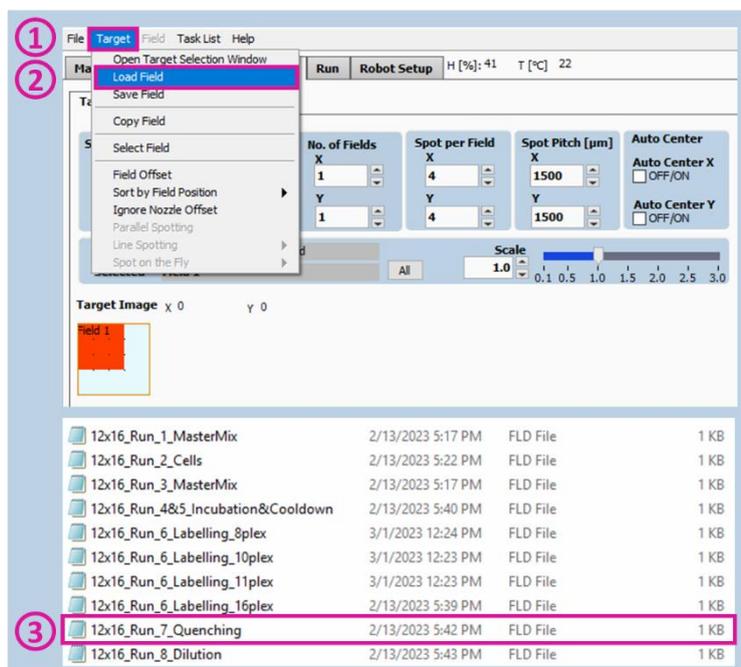


Figure 46. Field Selection for Run 7 – Quenching.

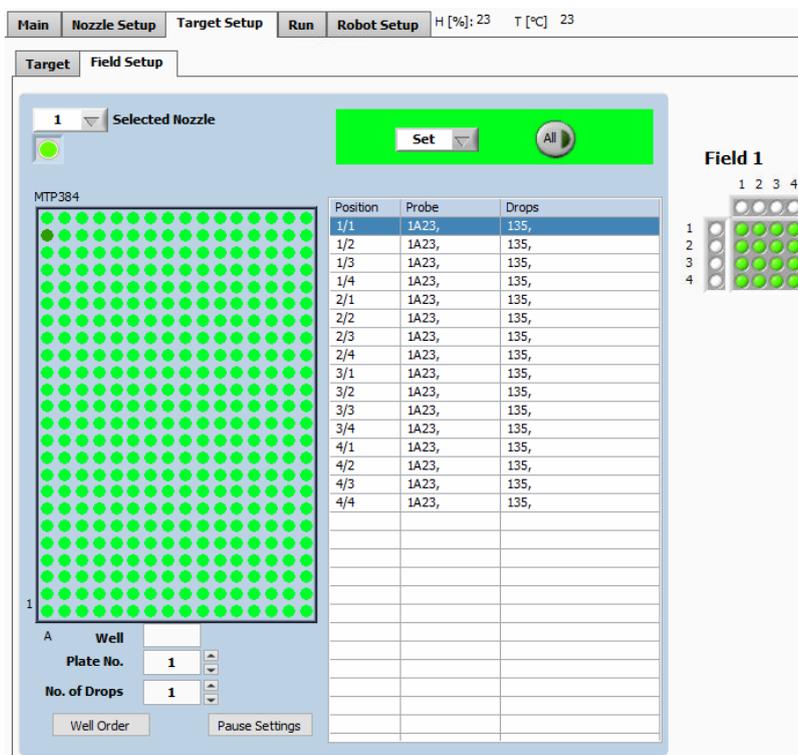


Figure 47. Field setup for Run 7 – Quenching.

4.7.3 Run Start

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 on **OK** (Figure 48.6).

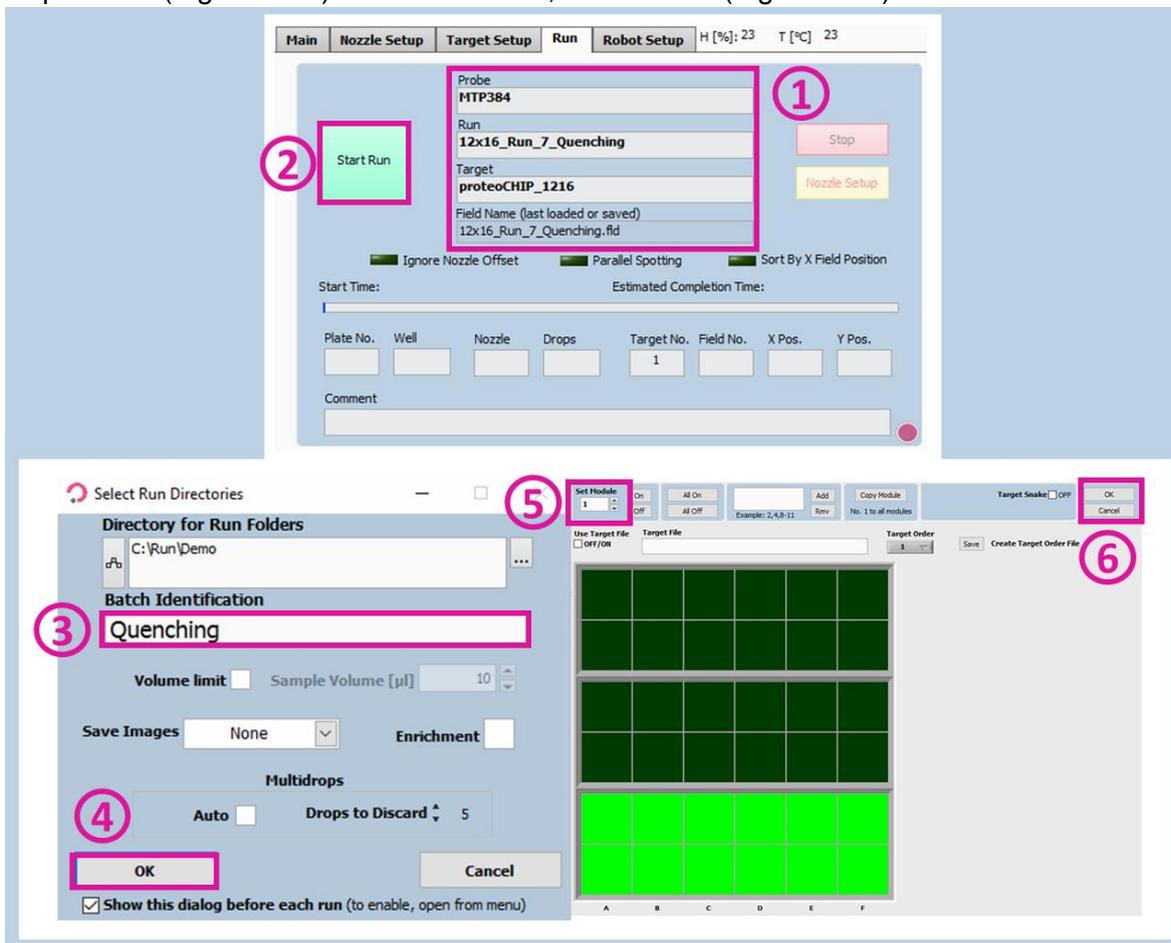


Figure 48. Run Start for Run 7 – Quenching.

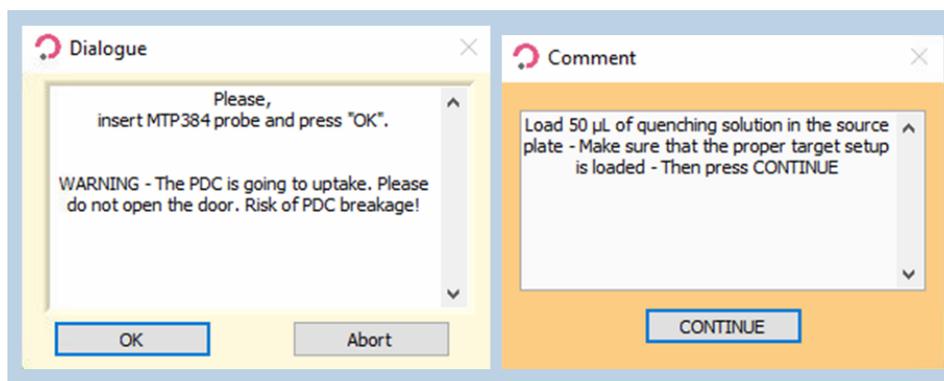


Figure 49. Pop-up messages for Run 7 – Quenching.

Note. At the end of this run, an incubation of 15 minutes at RT starts.

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 µL of 0.1% TFA/FA into each funnel and proceed to the next step: 4.9 Pooling.

4.8.2 cellenONE automated dilution

4.8.2.1 Probe, Run and Target Selection

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

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

- Probe: Select **MTP384**.
- Run: Select **12x16_Run_8_Dilution_1chip**.
- Target: Select **proteoCHIP_1216**.

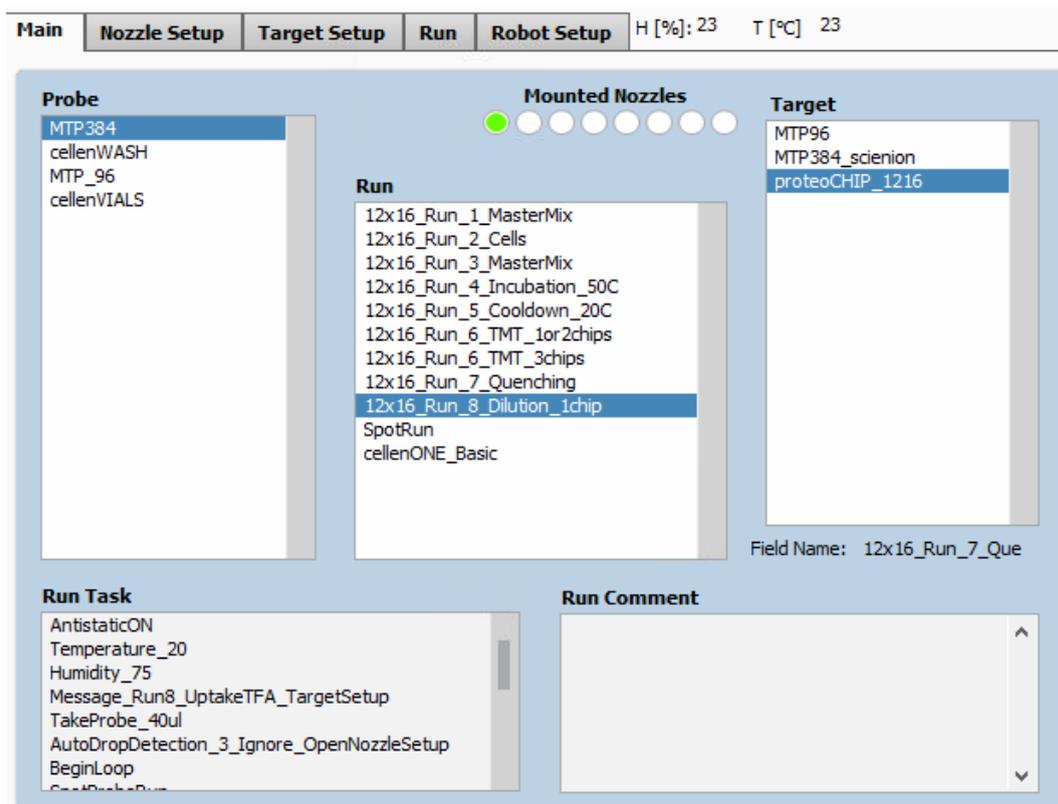


Figure 50. Probe, Run and Target Selection for Run 8 – Dilution.

4.8.2.2 Field Selection

Load the **12x16_Run_8_Dilution** field which will dispense ~150 nL TFA or 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).

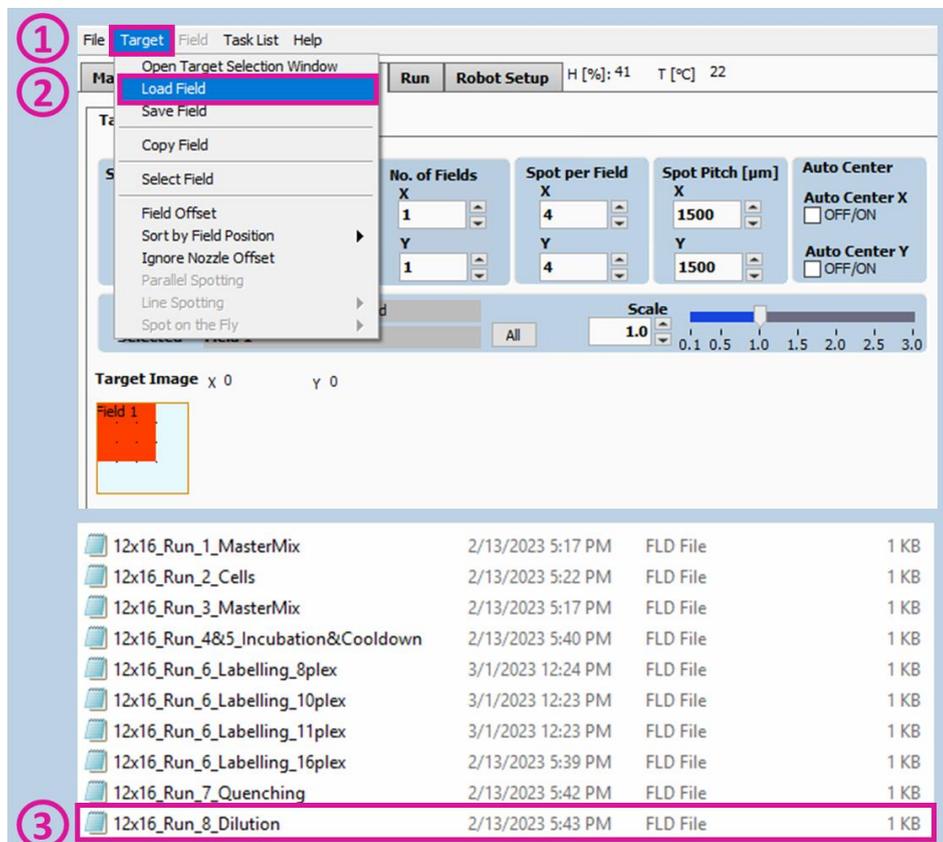


Figure 51. Field Selection for Run 8 – Dilution.

4.8.2.3 Starting 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 on **OK** (Figure 53.4).

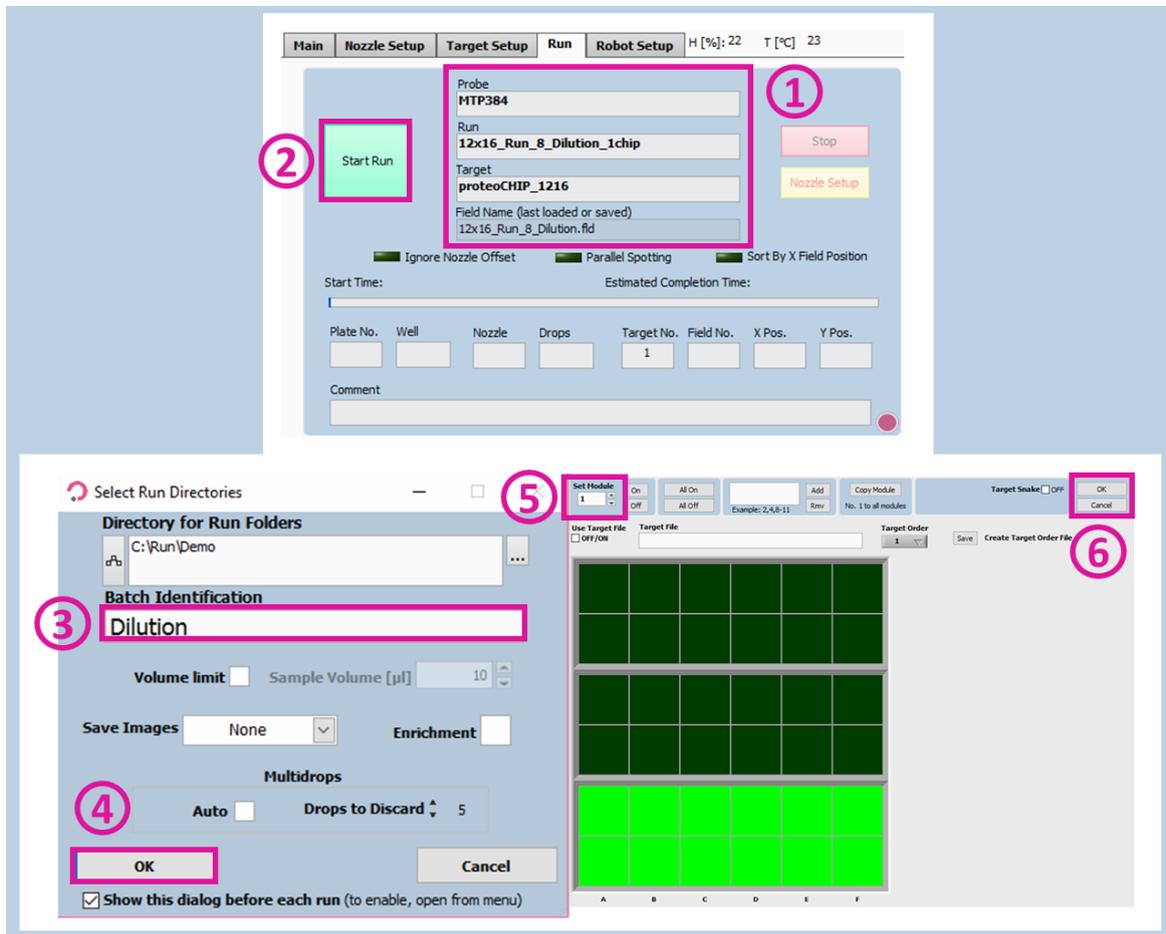


Figure 53. Run Start for Run 8 – Dilution.

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).

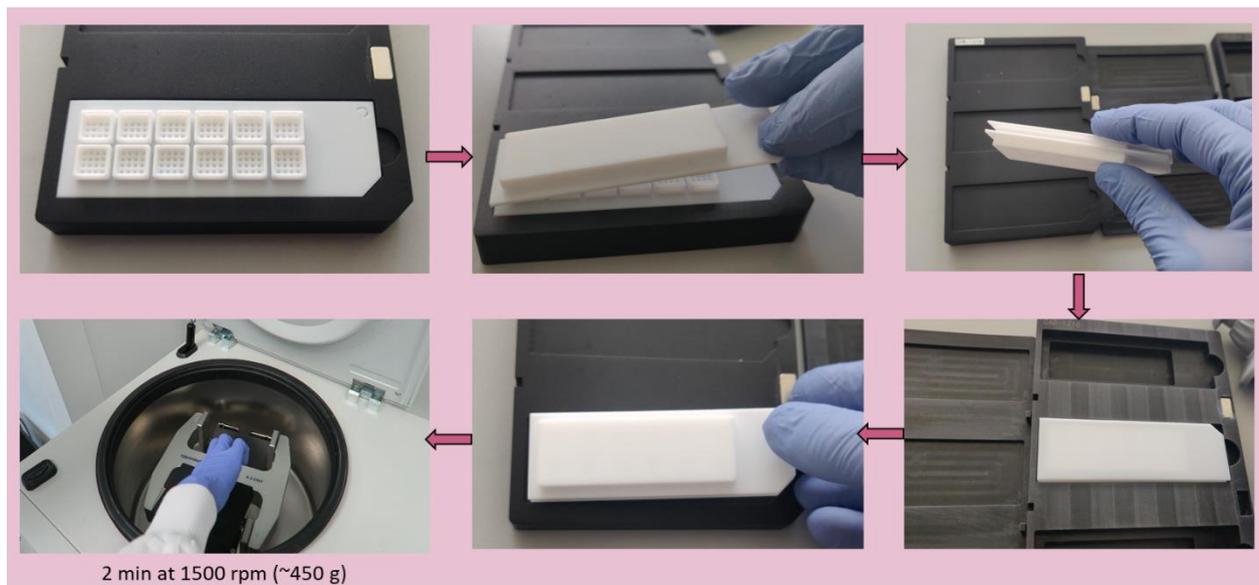


Figure 54. Pooling (Step 2).



Figure 55. Pooling (Step 3).

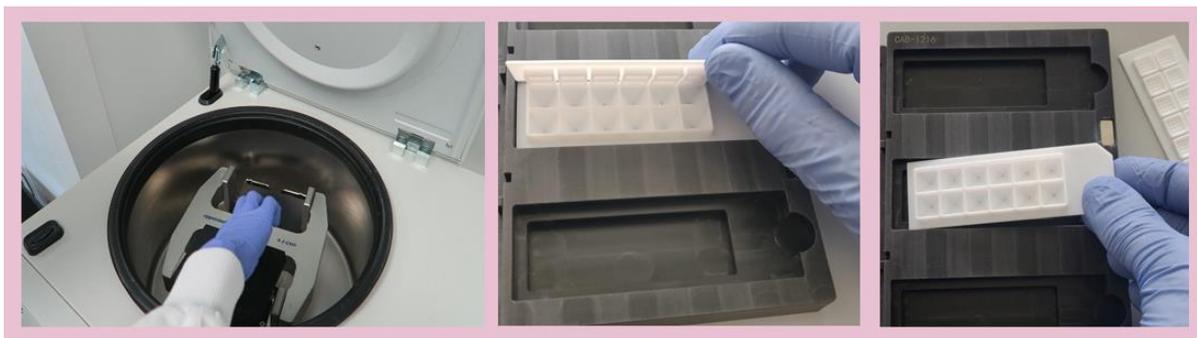


Figure 56. Pooling (Step 4).

- 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 aluminum foil and store them 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 the 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 μ L 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.

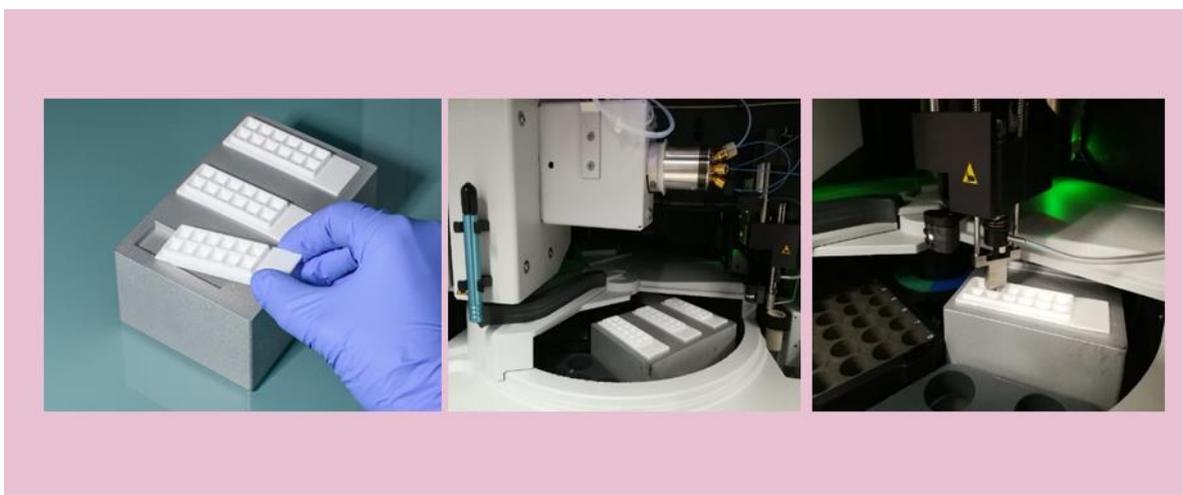


Figure 60. Autosampler.

5 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 nanowell-array workflow for quantitative multiplexed single-cell proteomics sample preparation at high sensitivity

Claudia Ctortecka, David Hartlmayr, Anjali Seth, Sasha Mendjan, Guilhem Tourniaire, Namrata D. Udeshi, Steven A. Carr, Karl Mechtler 1 3 4 5

2023, Mol Cell Proteomics 22(12), 100665

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6 Service and Support

6.1 Service Contracts

A cellenONE should receive preventative maintenance (PMI) annually to replace the system fluid and wash lines and inspect / replace wear-and-tear items.

Scienion will automatically be in contact when a service contract is active, and a PMI is due. We look forward to providing your lab with outstanding service and support.

Service contracts are provided through SCIENION AG and SCIENION US to all customers worldwide with varying levels of coverage. Please contact ticket@scienion.com to request a quote.

6.2 Service Ticket

For service or application support, contact ticket@scienion.com with your request. Only certified users, those who attended basic training provided by a Scienion or Cellenion representative, may submit a service/support ticket. Training of additional users or re-training may be provided.

If your institution possesses multiple cellenONES or sciFLEXARRAYERS please include the serial number of the instrument in your request to facilitate the support. The serial number of the cellenONE may be found on a sticker on the back panel of the system for integrated units or within the software under “Help” and the option “Info”.

Tickets are tracked within our service system and dispatched to the most appropriate representative. Please do not email only your field service engineer or application specialist as a timely response is not guaranteed.

6.3 Remote support

Prior to dispatching a service engineer, we will try to solve a problem remotely. We can do this via phone call, web-conference, or remote access to your instrument control computer. In the instance that a remote connection is needed, we will ask you to establish a Wi-Fi connection via an external Wi-Fi dongle. Please remove this dongle to disconnect the Wi-Fi connection after the service is complete.

The remote connection is established through an external program pre-installed on the instrument control computer and requires the user to provide their service representative with the computer ID and password displayed within the application. Please adjust the computer sleep settings so that the screen does not power off (no screensavers) as this will disconnect the remote connection.