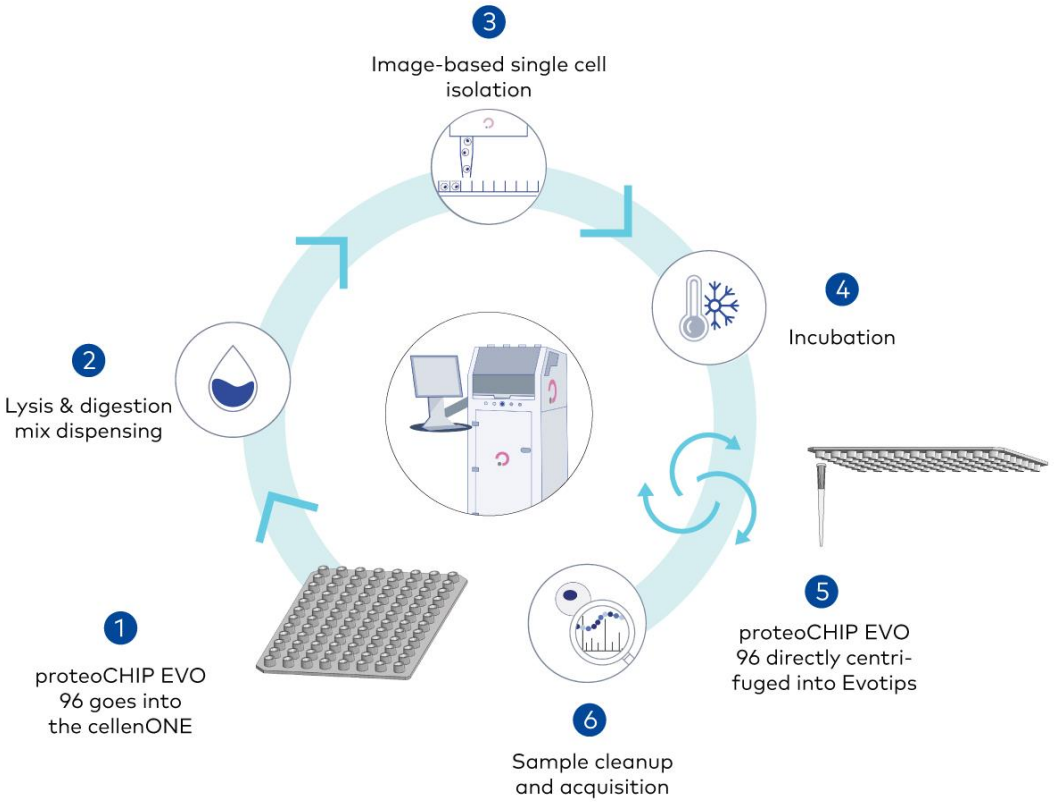
# User Manual
# proteoCHIP EVO 96 for SCP
# (Single Cell Proteomics)



# Table of contents



# Document history

| Revision | Author(s) | Changes | Effective date |
|---|---|---|---|
| 0.0 | D. Hartlmayr | Creation | 07/12/2023 |
| 1.0 | D. Hartlmayr | Transfer speed to Evotips Pure, Volumes instead of N° of drops | 16/02/2024 |

# Highlights layout

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

# 1 Introduction

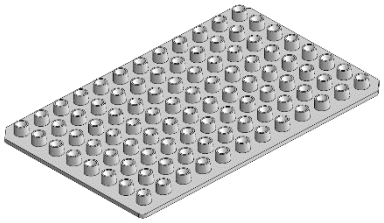
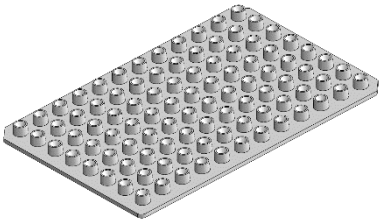
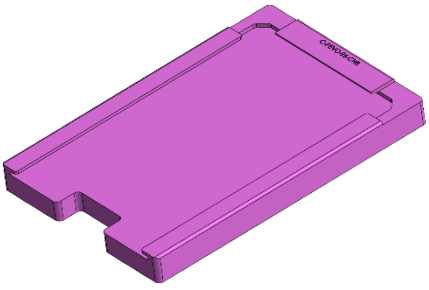
All basic functions of the cellenONE associated with dispensing or cell sorting are presented in great details in the User Manual for cellenONE. Furthermore, the User Manual for Proteomics Workflows provides step-by-step instructions with software screenshots through the most basic procedures referenced in this manual.

This manual serves as a check-list for the label-free Single Cell Proteomics (SCP) experiments using cellenONE software and proteoCHIP EVO 96. The protocol starts with placing the proteoCHIP(s) EVO 96 into the proteoCHIP EVO 96 holder(s) and ends with the direct transfer step to Evotips via centrifugation.

**Note.** It is highly recommended to read the User Reference Manual for cellenONE and the User Manual for Proteomics Workflows, before performing proteomics experiments with the cellenONE instrument.

# 2 Equipment and reagents

## 2.1 Equipment needed

| Product code | Description | |
|---|---|---|
| **P-20-CM/L** | **cellenONE PDC M Piezo Dispensing Capillary, cellenONE PDC L Piezo Dispensing Capillary** (Fixed **Drop Volume** between **300-450 pL or 450-600 pL respectively**) **4x manifold** | |
| **C-PEVO-96-10** | **proteoCHIP_EVO_96** The proteoCHIP EVO 96 Set containing:<br>• Ten proteoCHIP EVO 96 | |
| | The **proteoCHIP EVO 96** is a nanowell chip with 96 nanowells, enabling label-free single cell proteomics sample preparation for direct transfer to Evotip and Evotip Pure™. |  |
| **As part of the Accessory kit:**<br><br>**C-PEVO-96-AK** | The **proteoCHIP EVO 96 Centrifugation Dummy** is a counterweight for centrifugation when transferring samples from proteoCHIP EVO 96 to Evotips. |  |
| **C-PEVO-96-CHB**<br><br>**or**<br><br>**As part of the Accessory kit:**<br><br>**C-PEVO-96-AK** | **proteoCHIP_EVO_96 cellenONE Holder** Holder to accommodate up to one proteoCHIP EVO 96 on the deck of the cellenONE throughout sample preparation (reagent dispensing, cell isolation and incubation steps). *(1 piece/pack)* |  |

## 2.2 Reagents needed

Use only MS-grade reagents and prepare dilutions with purified water (>18 MΩ, <3 ppb TOC at 25 °C) See the full recipe in section 4.1.

- Master mix protease: 100 ng/µL Trypsin or Trypsin-LysC mixture in 50 mM acetic acid. Store at -20 °C (*see* **Note 1**).
- Master mix lysis reagent: 1% n-Dodecyl-β-D-maltoside (DDM) in purified water. Store at -20 °C (*see* **Note 2**).
- Master mix buffer: 1M Triethylammonium bicarbonate (TEAB). Store at 4 °C (*see* **Note 3**).
- 0.1% Trifluoroacetic acid (TFA) or formic acid (FA) in purified water.
- For adherent cell culture: 0.05% Trypsin-EDTA.
- 1x Phosphate-buffered saline (PBS).
- For reagent aliquoting: Low-binding 0.2 mL PCR tubes.

**Note 1.** Prepare 20 µL aliquots of 100 ng/µL enzyme solution (i.e., 200 µL Mastermix). Store the aliquots short term at -20 °C or for prolonged storage of enzymes (more than 1 month) dissolve the enzymes in 50 mM acetic acid and store at -80 °C.

**Note 2.** Prepare 40 µL aliquots of 1% DDM solution (i.e., 200 µL Mastermix). Store the aliquots at -20 °C.

**Note 3.** Test buffer stability every 6 months and exchange if needed.

## 2.3 Cell culture

When using adherent cell culture, use 0.05% Trypsin-EDTA to detach your cells. Afterwards, wash your cell suspension 3x by centrifuging it at 250 g for 2 minutes and then resuspending the cell pellet with PBS (equal to the starting volume). Bring the cell suspension to a final concentration of 100-200 cells/µL. Keep the cell suspension on ice until use. Make sure to isolate single cells within 3h after detaching them.

# 3 Preparation of the cellenONE

## 3.1 Preparation of the system

- Exchange Water of the **System Liquid** bottle: Degas fresh ultrapure water for 15 min by applying a vacuum and placing the **System Liquid** bottle inside the ultrasonic water bath.
- Check if purified water needs to be added to the **Fresh Water** bottle at the bottom of the cellenONE or if the waste needs to be emptied.
- Insert the PDC in the black PDC cassette and place the cassette in the cellenONE (but don't connect the tubing yet). Align the red cross with the tip of the capillary and focus the PDC (press the light bulb symbol to turn the blue LED on if needed). Click Prime and follow the steps.
- Connect the small flush bottle tubing to the outlet for PDCs inside the cellenONE.
- When the specific message is displayed, disconnect the tubing of the small flush bottle and click on **Continue**. Wait until the first drop comes out and then connect the PDC tubing to the outlet for PDCs inside the cellenONE.
- When the Prime Run is finished, check the drop. If there is no drop or the drop is not stable, make sure that the Voltage and Pulse settings match the settings written on the PDC box. Otherwise, try the tasks **FlushWashstation1_250ul** or **AirEx** several times until stable drops are formed.
- Adjust the PDC settings (Voltage and Pulse) for optimal drop shape.
- Measure the **Drop Volume**.

## 3.2 Cleaning of the cellenONE interior and preparation of reagents

- Wipe the top of the cellenONE door and the inside of the enclosure as well as the proteoCHIP EVO 96 holder with isopropanol or 70% ethanol.
- 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.
- Set the temperature to 10 °C.
- All solutions should be prepared in a biosafety cabinet.

**Note.** Please make sure that the 14-days wash is regularly performed (suggested every 14 days), clean the humidifier with 70% ethanol every 14 days if possible, and clean the PDC with 0.5% sciClean solution at the end of every experiment followed by 2 flushes (Flush_PDC): Pipette 2 mL of 0.5% sciClean solution into the cleaned white wash tray, then perform the **sciClean** task.

# 4 proteoCHIP EVO 96 – cellenONE Runs for SCP

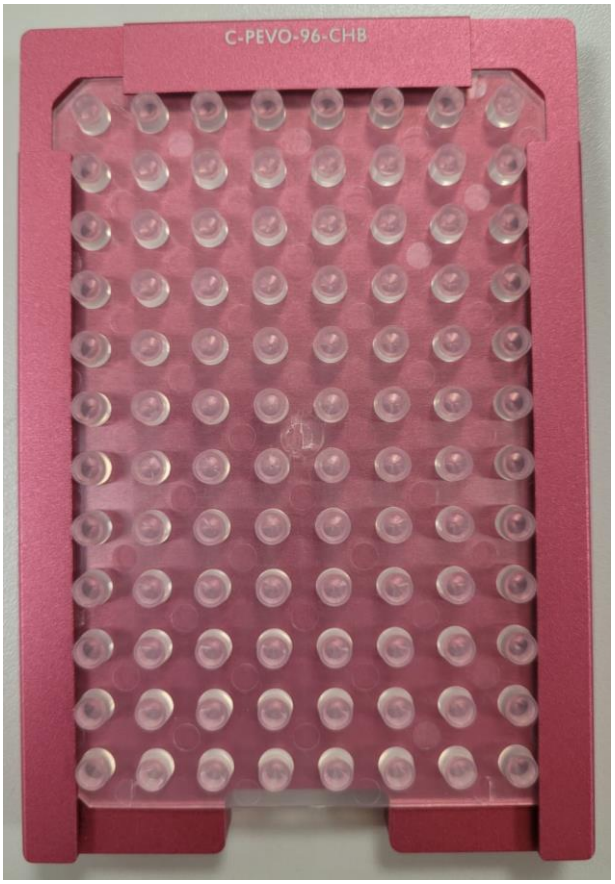



*Figure 1. proteoCHIP EVO 96 inside the cellenONE holder*

Place the proteoCHIP(s)_EVO_96 inside the cellenONE holder(s) (ref. **C-PEVO-96-CHB**) and insert the holder(s) containing the proteoCHIP inside the instrument on top of the cooling plates (*see* Figure 1).

**Note. 1.** For some Runs you will be required to select the Field files for either processing one or two proteoCHIPs EVO 96.

**Note 2.** When processing two proteoCHIPs EVO 96, the cellenWASH positions are designated for sample aspiration. The cellenVIALS that are used in these positions hold a dead volume of ~45-50 µL. Please keep in mind, that therefore you will need at least ~65-70 µL of cell suspension for the cell isolation Run. When processing only one proteoCHIP EVO 96, **MTP384** can be selected as **Probe** on the right cooling plate position with a dead volume of only 3 µL.

## 4.1 Run 1: Master Mix dispensing

Prepare 200 µL of Master Mix: 20 µL 1M TEAB + 120 µL $H_2O$ + 40 µL 1% DDM + 20 µL 100 ng/µL Trypsin (or Trypsin/LysC). Do not vortex the sample, but gently pipette the solution up and down several times to avoid bubbles.

**Main tab**

- Probe: **cellenWASH**
- Run: **EVO_96_SCP_Run_1_MasterMix**
- Target: **proteoCHIP_EVO_96**

**Nozzle Setup tab**

- For dispensing the Lysis Buffer make note of the original Voltage setting, then increase it by 5-10%. Press **Set Nozzle Parameters**.
- Check if the drop is stable by performing a **Drop Check**. Start **Continuous Dispensing** and check if the drop is still stable after 10 seconds.
- Measure the **Drop Volume**. Drop recognition will be needed during the Run, so make sure that the drop is detected with a red circle. If not, slightly adjust the Voltage and Pulse until the red circle is shown when performing the **Drop Check**.

**Target Setup tab**

- Load the Field (*Target → Load Field*) **EVO_96_SCP_Run_1_MasterMix_1Chip** or **EVO_96_SCP_Run_1_MasterMix_2Chips** depending on the use of one or two proteoCHIPs EVO 96.
- Update the **Field table** if needed: Adjust volume or Probe position (*Field → Edit Field table*). The volume dispensed per well is 300 nL.
- Place one or two fresh cellenVIALs in the first or first two cellenWASH positions (i.e., A1 or A1 & B1) depending on the use of one or two proteoCHIPs EVO 96. Fill the cellenVIAL(s) with 100 µL of Master Mix without introducing any bubbles.

**Run tab**

- Start the Run.
- Select the module position(s) of your proteoCHIP(s) EVO 96.

## 4.2 Run 2: Cell Isolation

Prepare your cell suspension as described in section 2.3.

**Main tab**

- Probe: **cellenWASH**
- Run: **EVO_96_SCP_Run_2_Cell_Isolation** or **EVO_96_SCP_Run_2_Cell_Isolation_NoUptake**
- Target: **proteoCHIP_EVO_96**

**Nozzle Setup tab**

- For dispensing the Cell suspension decrease the Voltage back to its original value and press **Set Nozzle Parameters**.
- Check if the drop is stable by performing a **Drop Check**.
- Measure the **Drop Volume**.

**Target Setup tab**

- Load the Field (*Target → Load Field*): **EVO_96_SCP_Run_2_Cells**.
- There should be only 1 drop per well selected if single cells are to be isolated. If multiple cells are to be isolated in a well, then add 1 drop multiple times to the same well, **NOT** X drops (the table should show “1,1,1,1,1” for 5 cells in one well, **NOT** “5”).
- Load at least 70 µL (*see* **Note 2** on page 7 of this manual) of the cell suspension into a cellenVIAL and place it in the first position of the cellenWASH station (i.e., A1).

**Run tab**

- Start the Run.
- Select the module position(s) of your proteoCHIP(s) EVO 96.
- If you want to continue sorting with the already aspirated cells, select and start the **EVO_96_SCP_Run_2_Cell_Isolation_NoUptake** Run.
- **Flush the PDC before continuing with Run 3 by performing the *Flush_PDC* task and close the *cellenONE Module.***

## 4.3 Run 3: Incubation at 50 °C (1.5 h)

**Main tab**

- Probe: **cellenWASH**
- Run: **EVO_96_SCP_Run_3_Incubation_50C**
- Target: **proteoCHIP_EVO_96**

**Nozzle Setup tab**

- When processing two proteoCHIPs EVO 96 in parallel, **increase the Frequency from 500 Hz to 1000 Hz** and press **Set Nozzle Parameters**. **Otherwise keep it at 500 Hz**.
- Start **Continuous Dispensing** and check if the drop is still stable after 10 seconds.
- Measure the **Drop Volume**.

**Target Setup tab**

- Load the Field (*Target → Load Field*): **EVO_96_SCP_Run_3&4_Incubation& Cooldown**.
- The volume dispensed per well should be 260 nL.

**Run tab**

- Start the Run.
- **Select one module position** when using one proteoCHIP EVO 96 and **select both module positions** when processing two chips.
- After 1.5 h, when ready, stop the Run and **immediately** proceed to Run 4.

## 4.4 Run 4: Cooldown to 20 °C (~0.5 h)

**Main tab**

- Probe: **cellenWASH**
- Run: **EVO_96_SCP_Run_4_Cooldown_20C**
- Target: **proteoCHIP_EVO_96**

**Nozzle Setup tab**

- Keep all the settings from the previous run.

**Target Setup tab**

- Keep the Target Setup from the previous run.

**Run tab**

- Start the Run.
- **Select one module position** when using one proteoCHIP EVO 96 and **select both module positions** when processing two chips.
- The cooling plate will now cool down to 20 °C and it will continue to rehydrate until a temperature of 25 °C. A message will pop up when this temperature is reached.

## 4.5 Run 5: Dilution

For dilution use 0.1% TFA or 0.1% FA (Mass Spec grade). In the end 3.2 µL of solution is added to each well resulting in a total volume of ~3.5 µL.

**Main tab**

- Probe: **cellenWASH**
- Run: **EVO_96_SCP_Run_5_Dilution**
- Target: **proteoCHIP_EVO_96**

**Nozzle Setup tab**

- If the frequency was changed prior to Run 3, **decrease it from 1000 Hz back to 500 Hz** and press **Set Nozzle Parameters**.
- Check if the drop is stable by performing a **Drop Check**. In this Run the pump and not the Piezo element will be used for dispensing the 0.1% TFA/FA. To make sure that the pumping step works properly, please move to camera position, press the light bulb symbol and go to the **Pump** subtab in **Nozzle setup** tab. Insert the parameters 3.2 µL for volume and 20 µL/s for speed and press **Start**. If you see a jet of water being ejected out of the PDC, the pumping step works perfectly fine. Repeat 2-3 times (*see* **Note 1**).

**Target Setup tab**

- Load the Field (*Target → Load Field*): **EVO_96_Run_5_Dilution_1Chip** or **EVO_96_Run_5_Dilution_2Chips** depending on the use of one or two chips.
- Place two or four cellenVIALs into the first two or four positions of the cellenWASH station, respectively.
- Load 250 µL of the 0.1% TFA/FA into each of the cellenVIALs.

**Run tab**

- Start the Run.
- Select the module position of your proteoCHIP(s) EVO 96.

**Note. 1.** If you observe that the dilution droplet does not properly fall to the target, please gently bring the white silica-part of the sciTIPCLEANER in contact with the tip of your PDC in the Home Position. It might be needed to perform the **FlushPDC** task multiple times afterwards if a lot of silica particles have accumulated on the tip of the PDC. You can also remove the particles by gently cleaning the tip with a disposable wiper soaked in 70% EtOH. TFA accumulating on the side of the PDC is automatically washed away during the Run.

**Note 2.** Alternatively, pipette manually 3.2 µL 0.1% TFA/FA to each well (preferably by using a multichannel pipette).

## 4.6 Sample transfer

### 4.6.1 Option 1 – Transfer to Evotips

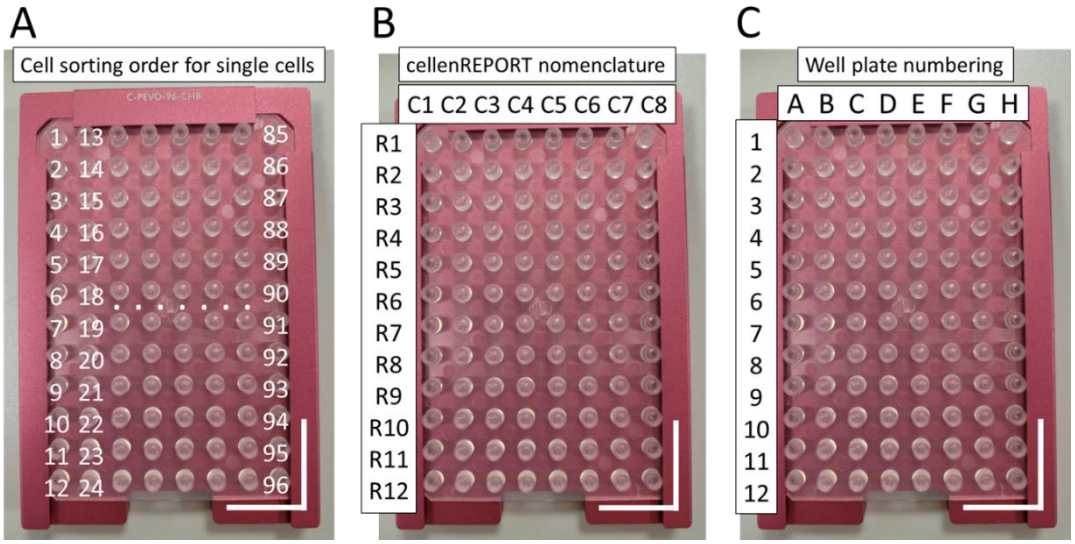



*Figure 2: proteoCHIP EVO 96 inside of the corresponding Holder. The sharp corner of the chip is shown in the bottom right corner.* ***(A)*** *Cell sorting order within a single cell isolation Run.* ***(B)*** *Nomenclature of each cell image presented in the cellenREPORT.* ***(C)*** *Well plate numbering corresponding to the Evotip box.*

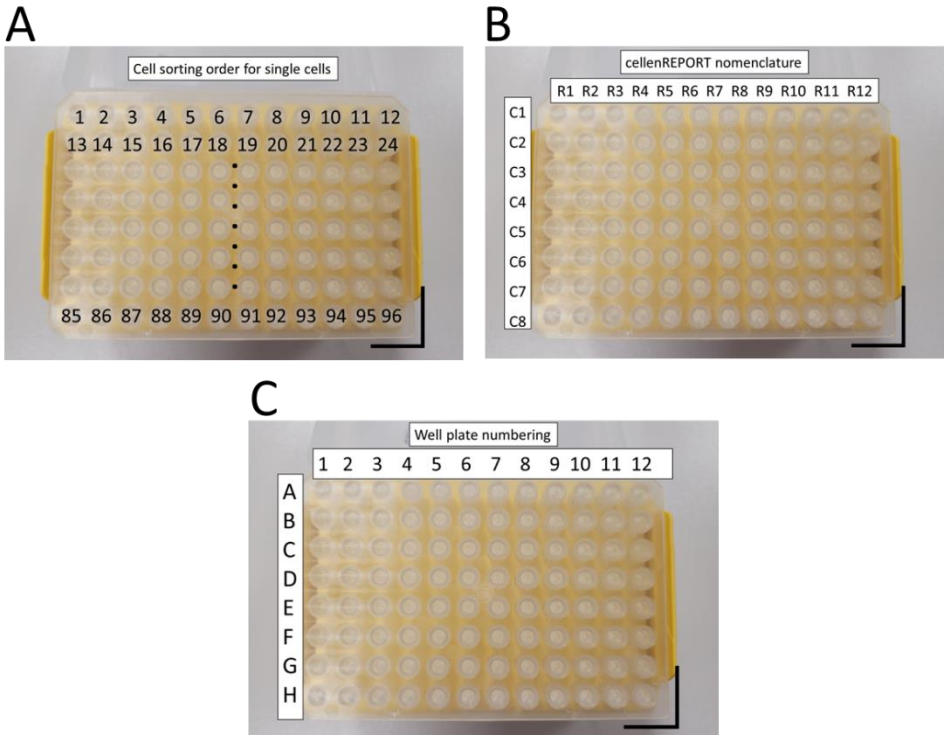



*Figure 3: proteoCHIP EVO 96 on-top of a Evotip box with the mirrored naming schemes of single wells. The sharp corner of the chip is shown on the bottom right corner.* ***(A)*** *Cell sorting order within a single cell isolation Run.* ***(B)*** *Nomenclature of each cell image presented in the cellenREPORT.* ***(C)*** *Well plate numbering corresponding to the Evotip box.*

- Since only ~3 µL of sample is transferred, preload the Evotips with only 17 µL Solvent A instead of 20 µL at the correct Evotip protocol step (**Loading**).
- Gently place the proteoCHIP EVO 96 with the diluted samples turning it upside down on top of the Evotips and close the Evotip box lid. **Keep in mind that the sample injection scheme of your Evosep system will be mirrored compared to your cell sorting scheme after the transfer.** See Figure 2 and Figure 3 for the cell sorting order, naming scheme within the cellenREPORT, the well plate numbering and mirroring effect after placing the chip on top of the Evotips. Make sure to orient the chip in a way that the samples represent the injection scheme of your Evosep system (e.g., Figure 3C).
- Transfer the Evotip box with the chip into a centrifuge and make sure that the counterweight is adjusted accordingly (e.g., using the proteoCHIP EVO 96 Centrifugation Dummy on a Evotip box containing used Evotips).
- Centrifuge the samples into Evotips at 800 rcf for 60 seconds according to the Evotip Pure protocol.

### 4.6.2 Option 2 – 96-Well plate

Alternatively, gently place the proteoCHIP EVO 96 with the diluted samples turning it upside down on top of a low-binding 96-well plate and make sure that the counterweight is adjusted accordingly. Centrifuge the samples into the 96-well plate at 700 rcf for 30 seconds.