EV Profiler 2 Tetraspanin Detection

Aplo Flow preparation protocol

This protocol is intended for use with the Application Kit™: EV Profiler 2 - Tetraspanin Profiling.

This protocol is meant for use with CODI System App (CSA) version 19.5 and later.

For the characterization of extracellular vesicles (EVs) using the Nanoimager and Aplo Flow automated sample preparation system.

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EV Profiler 2 Tetraspanin Profiling Component List

Component	Quantity	Volume	Hazard	Storage
Assay Chip	3	-	N/A	-20°C †
Surface Reagent	3	55 µL	N/A	-20°C †
Wash Buffer	1	12 mL	N/A	4°C
PS Capture	1 x	55 µL	N/A	-20°C †
PS Capture Supplement	1 x	16 µL	N/A	4°C
anti-CD81 Capture	1 x	55 µL	N/A	4°C
anti-CD63 Capture	1 x	55 µL	N/A	4°C
anti-CD9 Capture	1 x	55 µL	N/A	4°C
Tetraspanin Trio Capture	1 x	55 µL	N/A	4°C
EV Standard Control	1	30 µg	N/A	-20°C *
Fixative	1	1.1 mL		4°C
Staining Buffer	1	400 µL	N/A	4°C
anti-CD81 Detection (647)	1	15 µL	N/A	4°C
anti-CD63 Detection (561)	1	10 µL	N/A	4°C
anti-CD9 Detection (488)	1	10 µL	N/A	4°C
dSTORM Imaging Buffer Part A	3	400 µL		-20°C
Part B Resuspension Buffer	1	30 µL	N/A	-20°C
dSTORM Imaging Buffer Part B	1	25 µL	N/A	-20°C

-20°C Store at -20°C

4°C Store at 4°C

† Single-use

x Dependent on customer-selected configuration

* Aliquoting recommended after first use (aliquots only stable for 5 days at 4°C)

EV Profiler 2 Automation Accessory Kit Component List

Component	Step/Use	Quantity
0.5 mL Skirted Screw Cap Tube, Amber	Detection Antibodies and fixative	15
0.5 mL Skirted Screw Cap Tube, Clear	Tubes for custom capture	7
Standard Cap for Screw Cap Microtubes, White	Capping reagents during preparation	25
0.2 mL 12 Sample Strip Tube with caps	EV samples	1
MilliQ® Water	For Aplo Flow Instrument	3
Tip Box	For Aplo Flow Instrument	1

Overview

The Application Kit™: EV Profiler 2 provides a fast, user-friendly system for the characterization of EVs. The EV Profiler 2 uses a combination of functionalized surface chemistry, biochemical capture and detection molecules, and is compatible with the ONI Nanoimager and ONI's cloud-based analysis platform, Collaborative Discovery software (CODI), to streamline sample preparation, imaging, and analysis.

Intended use

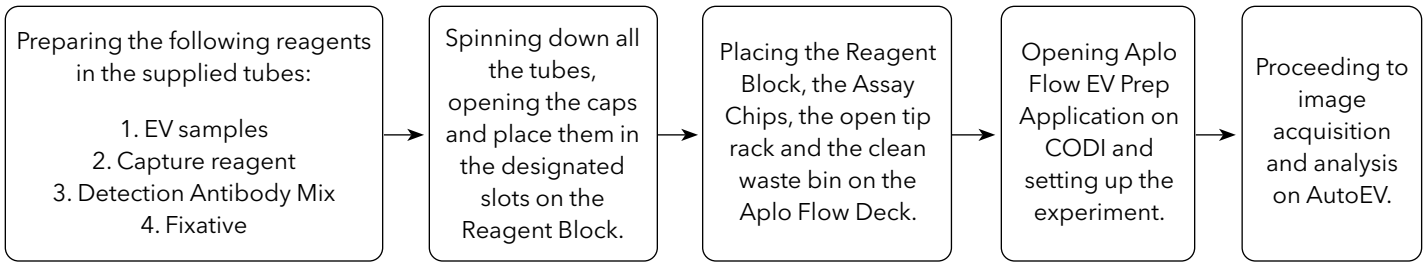
EV Profiler 2 kits are intended for Research Use Only. The kits are intended for use by professional users with training in basic laboratory techniques. Care should be taken in the handling of products. We recommend that users adhere to the MISEV guidelines provided by the International Society of Extracellular Vesicles.

Tetraspanin detection Workflow

The protocol for Tetraspanin Detection (3-color) is designed to characterize CD81, CD63, and CD9 expression on human-derived EVs. It can be used with either tetraspanin-specific EV capture (Tetraspanin Capture) or phosphatidylserine EV capture (PS Capture).

Workflow overview

The Aplo Flow is a fully featured liquid handler that has validated workflows for preparing ONI consumables without the need for continuous monitoring by lab personnel. The instrument is controlled through CODI and is fully integrated with the ONI's Auto-EV application.



Resources

Equipment and consumables listed below are not included with the purchased EV Profiler 2 - Tetraspanin Profiling kit, but are needed to prepare working reagents and samples.

Equipment needed

- ☐ Personal protective equipment: gloves, safety glasses, lab coat, biohazardous waste vessel as required by local health and safety regulations
- ☐ Pipettes (p2.5, p20, and p200)
- ☐ Vortex mixer
- ☐ Benchtop microcentrifuge compatible with strips of 0.2 mL tubes and 1.5/2.0 mL tubes at 5,000 RPM
- ☐ Standard lab timer
- ☐ Microcentrifuge tube rack
- ☐ Aspirator (optional)

Components to be provided by the user

1. Purified EV samples to be tested
2. Bead slide for channel mapping (ONI- 800-00016)
3. Pipette tips (10 µL, 20 µL 200 µL)
4. 0.5 mL tubes
5. 0.2 mL tubes
6. Lens cleaning paper
7. Objective oil
8. Kimwipes

Advice before you start

Note: This kit was optimized for a room temperature of 18˚C - 30˚C and humidity >25%. Please contact ONI's technical support if the working environment does not meet these criteria.

Reagent selection considerations

Before you begin, decide which capture and detection method is ideal for your experiment.

Capture reagents

- Select anti-CD81, anti-CD63, or anti-CD9 capture if you want to enrich in a specific tetraspanin. If the EV source is known to be enriched in an individual tetraspanin, isolating with that individual tetraspanin for capture can help isolate that EV sub-population. Otherwise, combined tetraspanin capture can be used to isolate a broad range of EVs.
- PS Capture may be used with EVs enriched in phosphatidyl-serine (e.g., as is typically seen in cancer-derived EVs). PS Capture is valuable when you do not want to enrich a protein biomarker.
- If phosphatidylserine (PS) content is unknown, we suggest comparing PS Capture to anti-CD81, anti-CD63, and anti-CD9 (individual or combined) capture.
- When using a user-defined capture molecule, ensure the target of interest is membrane-associated and accessible. In order to check for compatibility with EV Profiler 2, it is recommended to test a lane with the selected staining protocol but use a representative negative control without EVs: analyze with the predefined analysis parameters and confirm fewer than 100 clusters per field of view in negative control lanes. For more detailed information regarding capture molecule selection, see the "capture molecule alternatives" section. If using a user-defined capture, final volume to be loaded on the instrument is 10 µL per lane plus 25% overage volume (i.e. 150 µL if using the same user-defined capture molecule across 12 lanes: 12*10 + 25%). One tube of user-defined capture can be used across multiple chips.

Capture molecule alternatives

Any biotinylated molecule can be used to capture EVs onto the EV Profiler 2 Assay Chip. An alternative biotinylated capture molecule can be selected to isolate EV subpopulations via a capture modality not provided in the kit. The molecule should detect a protein marker known to be found on the external surface of EVs. Capture cannot succeed if the capture molecule targets a protein marker that is primarily expressed on the internal surface of the EVs.

Optimization of user-defined capture molecules should include the following steps:

- **Capture molecule specificity assessment**
  To determine specificity, stain cultured cells that express the target, as well as negative control (e.g. knockout or peptide blocking) with a fluorescent-labeled version of the capture molecule. Ideally, the negative controls should include less than 10% of the density of the positive control samples, however, this threshold should be predetermined by the user.
- **Capture molecule compatibility with EV Profiler 2 assay-**
  When using a new biotinylated capture molecule, ensure that at least one lane with the capture molecule does not contain EVs and that there are less than 100 clusters detected in the EV-negative lane when stained in the same manner as EVs.
- **Background optimization**
  If negative control lanes with the new capture molecule result in high background, 0.5%- 2.5% BSA can be included in the biotinylated capture molecule dilution.
- **Capture efficiency and reproducibility optimization**
  For the most efficient capture, start with 70 nM of biotiny-lated capture molecule and test 20% higher and lower concentrations for ideal capture efficiency. A 75-minute incubation time is recommended, but 20% shorter and longer incubation times can be tested for ideal capture efficiency and reproducibility.

Detection reagents

This kit is designed for EV biotyping with anti-CD81 (647), anti-CD63 (561), and anti-CD9 (488), and it can aid in understanding of EV populations. ONI also offers an alternative EV Profiling 2 kit configuration, containing reagents for the detection of a user-defined biomarker.

General guidance

Important reagent handling considerations

Prepared EV samples should be purified prior to deposition onto the Assay Chip. If assistance is needed regarding suggested EV purification for dSTORM imaging, please reference this webinar on EV preparation or contact ONI support. Size exclusion chromatography or differential centrifugation are recommended for purification.

CAUTION: Do not vortex EVs

EV standard control handling and storage

If using the EV Standard Control:

- Reconstitute the lyophilized EV Standard Control by gently adding 30 µL MilliQ H2O dropwise on the tube sides, taking care not to introduce bubbles to the solution. Allow to rehydrate for 10 minutes at room temperature.
- Centrifuge to ensure all EVs are in the bottom of the tube.
- Dilute reconstituted EVs 20-fold in Wash Buffer (e.g., 1.25 µL EV Standard Control into 23.75 µL Wash Buffer per lane). PS Capture Supplement is not required for the EV Standard Control.
- The reconstituted EV Standard Control will produce the best results when used within 6 hours of reconstitution; however, it can be stored at 4°C for up to 5 days.

  CAUTION: Storage of reconstituted EV standard control for longer than 5 days at 4°C can compromise the reagent and cause failures with the positive control.

Reagent preparation

The next sections provide detailed steps of the EV Profiler 2 assay with Aplo Flow.

Thawing Assay Chip and frozen reagents

Kit components are stored at different temperatures. Those that are frozen should be removed from the kit first and allowed to equilibrate to room temperature.

1. Decide how many chips will be processed.
2. Locate the following boxes and remove them from storage:
   a. EV Profiler 2 kit box 1 (4°C storage)
   b. EV Profiler 2 kit box 2 (-20°C storage)
   c. EV Profiler 2 Automation Accessory Kit (Ambient Temperature Storage)
3. Allow these reagents to equilibrate to room temperature for at least 10 minutes as the following reagents are being prepared.

   Note: Sample processing should begin no more than 45 minutes after all reagents are prepared. If further delays occur, cap the tubes and store them at 4°C for up to 3 hours.

Component	Storage Temperature	Quantity	Notes
Assay Chip	❄	1-3 (Depending on experimental design)	Keep in sealed pouch until step: "Loading the EV Profiler 2 Reagent Block"
Surface Reagent	❄	1-3 (One per chip)	Place in tube rack
Optional: PS Capture	❄	1-3 (One per chip)	Place in tube rack

Tetraspanin Detection Antibody Mix preparation

This section details the unique preparation steps required for the Tetraspanin Detection modality. This detection method is used for biotyping EVs by probing for CD81, CD63, and CD9.

Prepare Tetraspanin Detection Antibody Mix in a provided 0.5 mL amber tube. Scale as necessary for the amount of lanes using Tetraspanin Detection Antibody Mix. Use the table below to calculate the appropriate volumes.

	Number of lanes mix will be added to (≥2 lanes*)	Volume per lane	Volume to be added
Staining Buffer	____ X	10.5 µL	= _____
anti-CD81 Detection (647)	____ X	1 µL	= _____
anti-CD63 Detection (561)	____ X	0.5 µL	= _____
anti-CD9 Detection (488)	____ X	0.5 µL	= _____
Final Volume	____ X	12.5 µL	= _____

*Note: *If using Tetraspanin Detection in only a single lane, please prepare 25 µL to allow sufficient dead volume.*

1. Once the Detection Antibody Mix is prepared, perform a quick spin (e.g. 5 seconds) with a microcentrifuge to ensure that all liquid is at the bottom of the tube.
2. Label and cap the tubes to avoid evaporation. Set aside at room temperature until ready to load the Reagent Block ("Loading the EV Profiler 2 Reagent Block" section).
3. Return unused antibody dilution reagents to 4°C. The remaining Staining Buffer will be loaded on the Reagent Block in "Loading the EV Profiler 2 Reagent Block" section.

Transferring Fixative

Fixative is provided in an amber glass 1.1 mL vial with a gray rubber septa with and a red perforated metal seal to maximize shelf life. The required working volume of fixative should be transferred to one of the provided automation-compatible 0.5 mL amber tubes:

Component	Volume to add		
	1 chip	2 chips	3 chips
Fixative	150 µL	300 µL	450 µL

1. Remove the red metal cap from the fixative vial by peeling away the perforated tab, then removing the gray stopper from the glass vial.
2. For each chip being prepped, transfer 150 µL of the Fixative into a provided amber 0.5 mL tube.
3. Briefly spin the tube down to ensure that all liquid is at the bottom of the tube.
4. Cap the tube to avoid evaporation and set it aside at room temperature until ready to load the Reagent Block (Loading the EV Profiler 2 Reagent Block Section).

   Note: Keep the remaining Fixative reagent in the original glass vial and store at 4°C.

Preparing EV samples

This section outlines how to prepare EV samples to be characterized using the EV Profiler 2 assay. Follow the steps below for EV sample preparation. If choosing to use the EV Standard Control provided by ONI, please see the "Important EV Sample Preparation Considerations" Section.

1. Calculate the volume of EVs needed for the assay. 10 µL will be added to each lane and at least 25% of overage volume is required (example: provide 150 µL of sample if the same sample will be loaded in 12 lanes). If an EV sample will only be used in one lane, please provide a minimum of 25 µL of sample.

Note: Minimum sample volume of 25 µL is to ensure minimal impact to the assay due to evaporative loss. If the lab is in a particularly arid environment, consider increasing minimum EV sample volume to 40 µL.

Calculator for sample volume needed

Number of lanes the sample will be added to (≥2 lanes)	Volume per lane	Overage factor	Final Volume
____ X	10 µL	X 1.25 =	_____

2. Dilute EVs to 10^8-10^{10} EVs / mL in Wash Buffer.
3. Prepare EVs according to the selected capture method:
   a. If using PS Capture and user-supplied EVs, PS Capture Supplement is required. Add PS Capture Supplement at a 1:10 ratio, e.g. 2.5 µL of supplement for a final volume of 25 µL EVs.
   b. If using Tetraspanin Capture, no PS Capture Supplement is required.
   c. If preparing EV Standard Control, see "Important EV Sample Preparation Considerations" section for instructions on reconstitution. Dilute reconstituted EVs 20-fold in Wash Buffer (e.g., 1.25 µL EV Standard into 23.75 µL Wash Buffer per lane). PS Capture Supplement is not required for the EV Standard Control.

1. Load the required volume of EV samples into one well of the 0.2 mL 12 strip tube. If using multiple samples, each specific sample will be its own well.
2. Cap the tubes to avoid evaporation and quickly spin (e.g. 5 seconds) the tube strip. Set aside until ready to load the tube block.

Note: The 0.2 mL 12 strip tube can be cut down to size to accommodate the number of EV samples as well as spinning down on 8-strip compatible microfuges.

Loading the EV Profiler 2 Reagent Block

Once Detection Reagents and EV samples are prepared, place them on the block in their supplied tubes. For reagents that are supplied 3 tubes per kit, one tube will need to be loaded for each chip to be processed. If processing only one or two chips, use the required reagents and keep the spare tubes in the appropriate storage conditions.

Load one tube for the following reagents for each chip

- Surface Reagent
- The selected capture reagent
  - For PS capture, load tube prepared in instructions from prior section "Thawing Assay Chip and frozen reagents" on page 8'.

  - For TS capture, load tube(s) directly from the kit.

The following reagents go into one specific Reagent Block section, regardless of the number of chips.

- Wash buffer
- Staining buffer
- MilliQ® Water
- Fixative

Load the appropriate number of tubes of Antibody Dilution Mix.

Note: Each tube of Staining Buffer contains sufficient volume for making Detection Reagents and subsequent steps on the Aplo Flow for up to Three Chips. Only use one tube of Staining Buffer per Aplo Flow run to ensure optimal stability.

Perform the following steps for each tube:

1. Ensure the solution is at the bottom of each tube and spin down again if needed.
2. Remove the caps from all the tubes.
3. Load the Reagent Block immediately prior to starting a run. Follow the block map provided by CODI software in the Aplo Flow EV Prep App.

Loading the Aplo Flow system deck

The deck is divided into five positions labeled A through E, plus a sixth position for the waste.

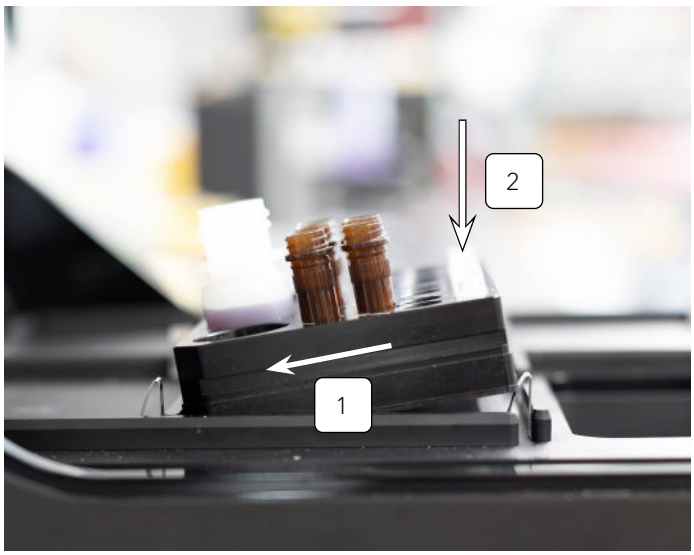
Deck Position	Description
A	Open deck position for future workflows
B	Reagent Block
C	Tip rack
D	Optional section tip rack position for certain workflows
E	4-position chip holder
F	Waste tub

Position A (Unused)

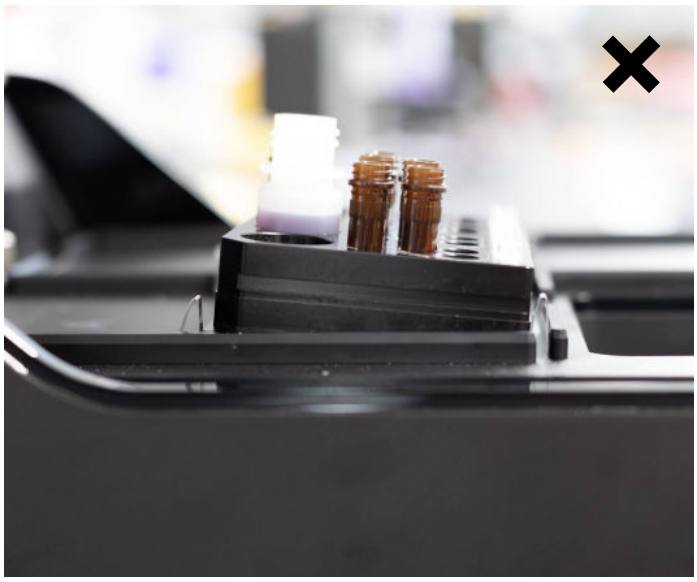
Deck position A is left empty.

Position B- Reagent Block

1. Push the Reagent Block into the back of Position B.
2. Firmly press the front of the Reagent Block down against the steel retaining clips to insert the block into the deck position.
3. View the reagent block from the side to visually confirm it is fully seated flat against the deck.



Incorrect



Correct

Deck Positions C & D (Pipette Tip Boxes)

1. Load the pipette tip box into position C of the Aplo Flow deck.

   a. Remove the lid from the the pipette tip box.

   b. If using a partial tip box, ensure there are at least 8 full columns of tips (128 tips) for each chip to be run. If processing 3 chips, ensure the tip box is full and make sure "Reset Tips" is set to 'YES' as shown in the section of this guide on Configuring the Aplo Flow EV Prep Protocol.

Deck Position E (Assay Chips)

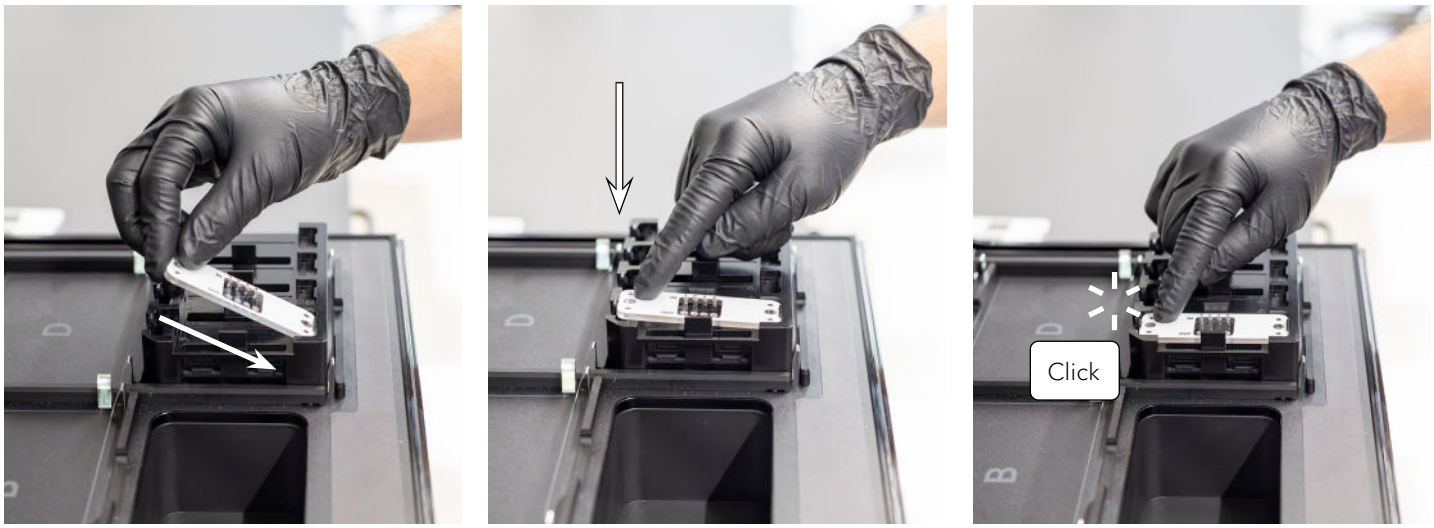
1. Remove the Assay Chips (previously equilibrated to room temperature) from pouches and load into the chip holder in Position E of the Aplo Flow deck.

   a. Position the chips to match the orientation indicated on the chip holder block. The "IN" label should always be on the right side of an Assay Chip when facing the machine.

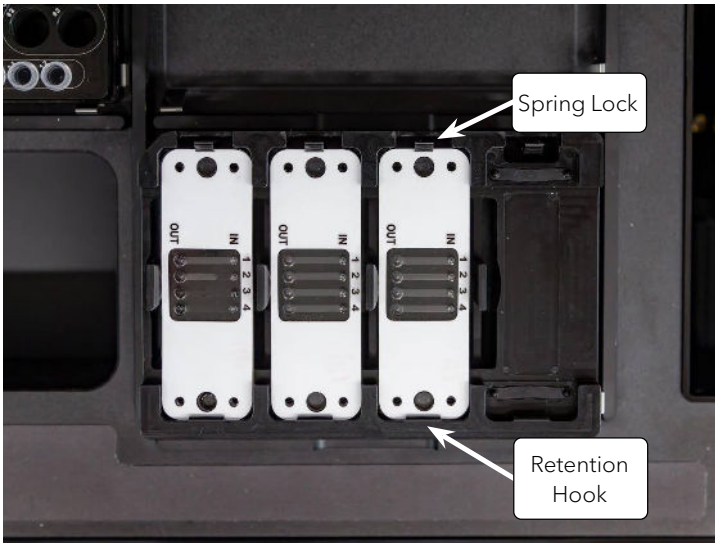
   b. Load Assay Chips from left to right (the first chip will always be in slot A).

   c. Slide the end closest to Lane 4 of the Assay Chip under the retention hook of the chip holder (toward the front of the instrument).

   d. Push down on the opposite end of the Assay Chip until it lies flat and the spring loaded lock clicks into place.

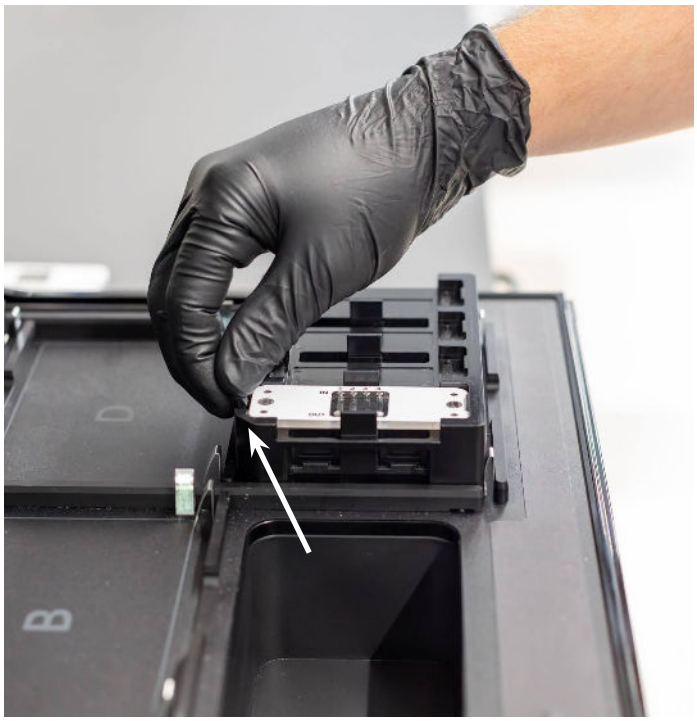


Loading the Assay Chip

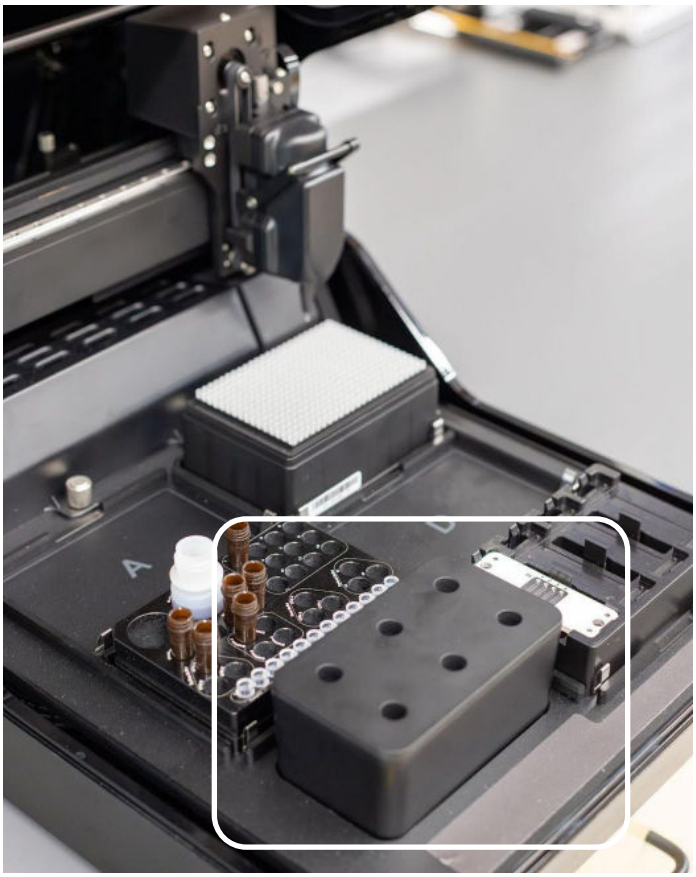


Three chips loaded in 4-chip holder on deck

Note: The EV Profiler kit is only compatible with up to 3 chips per run. Slot D is not used for the EV Profiler kit.



To remove the Assay Chip after run completion, push the spring lock toward the rear of the instrument. Lift the Assay Chip from the end that was under the spring lock.



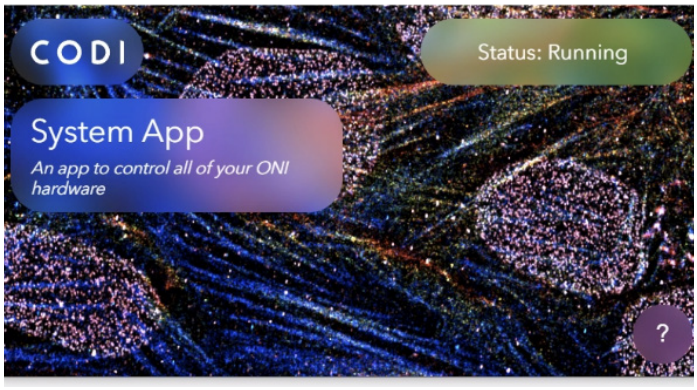
Deck Position F

1. Empty the contents of the waste tub before each run and in accordance with your local health and safety guidance for pipette tips exposed to potential biological hazards
2. Insert the empty waste tub in the recessed position on the deck, position F, with the lid on top.

   CAUTION: Failure to empty the waste bin before a run can result in damage to the pipette head or sample loss if a collision occurs when the system attempts to dispose of a tip. Always empty the waste bin before initiating a run.

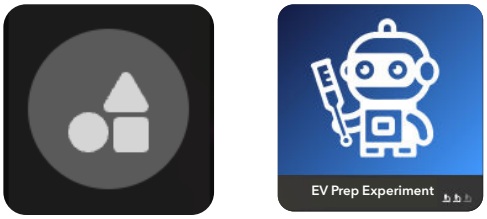
Aplo Flow Application Experimental Set-up

Once all reagents have been prepared and placed on the Aplo Flow Deck, proceed to the computer connected to the Aplo Flow system, and begin the experimental set-up.



Opening the Aplo Flow EV Prep Application

1. To start an Aplo Flow automated chip preparation protocol, ensure Aplo Flow is connected to the ONI computer and CSA version v0.19.5 or newer is running. From there, navigate in CODI via a web-browser and click on the "Acquisition Apps" tab on the left pane. Ensure the hardware is connected and powered on.
2. Click the Aplo Flow EV Prep application to open the experiment setup screen. The Aplo Flow Experiment application has a logo of a Robot holding a pipette.



Top: CODI System App (CSA) is opened and in a running state.
Left: Acquisition Apps logo
Right: EV Prep Experiment application logo

Verifying the Aplo Flow hardware is connected

1. Before beginning, start by verifying that the Aplo Flow system is connected and seen by the software. This can be done by verifying the System Status icon in the top right-hand corner of CODI (A).

A green checkmark within the System Status icon indicates that the Aplo Flow system is connected to the software and running. It is normal to see the red X briefly before the green checkmark appears because the connection takes a bit to connect.

A red X within the System Status icon indicates that the Aplo Flow System is not connected to the software or an error has occurred in detecting the Aplo Flow System. Should this occur, close the browser window, restart the CODI System App, and open a new browser window. If the problem persists, contact ONI support.

Changing the Experiment Name

An experiment name can be set in the textbox in the top left-hand corner of the Experiment Setup Screen (B). Click the Edit button to change the experiment name.

Configuring the EV Aplo Flow EV Kit

Next, select the version of the Aplo Flow EV Prep kit being run from the dropdown menu (C). Reference the kit purchased and ensure that the EV Aplo Flow Kit chosen in the dropdown menu matches the assay kit provided.

Note: If using an Antibody Dilution Mix with only user-defined antibodies select Tetraspanin Profiling Kit.

Configure the number of chips to prepare

To change the number of chips in the experiment, modify the "Number of chips to prepare." (D) This number should match the number of chips on the deck. The tool can prepare up to three chips. The chips can be toggled between Chips A, B, and C. They are ordered from left to right on the chip holder on-deck as well. A white outline appears on the chip currently being edited (E).

Switching between chip settings

1. Each chip's settings can be viewed using the selection menu at the top of the screen (E).
2. Chips can be selected by either:
   a. Using the left and right arrows.
   b. By clicking on each chip individually.
3. The active Chip Position will be indicated by a gray outline around the chip image and will be displayed at the top of the lane selection menu (F).
4. The lane settings can be changed by clicking on each chip icon (E). In the example below, Chip A has been selected, and the Sample Position is displayed as 1 through 4.

Reset tips to first position

From the 'Reset tips to first position' dropdown menu (F), choose desired behavior:

a. 'Yes' will instruct the Aplo Flow system to start the chip preparation using tip #1 from deck position C. Please select this option when using a new box of tips.

b. 'No' will allow the Aplo Flow system to start the chip preparation using a tip box used in the previous run. This will occur when processing less than 3 chips at a time.

Aplo Flow System control

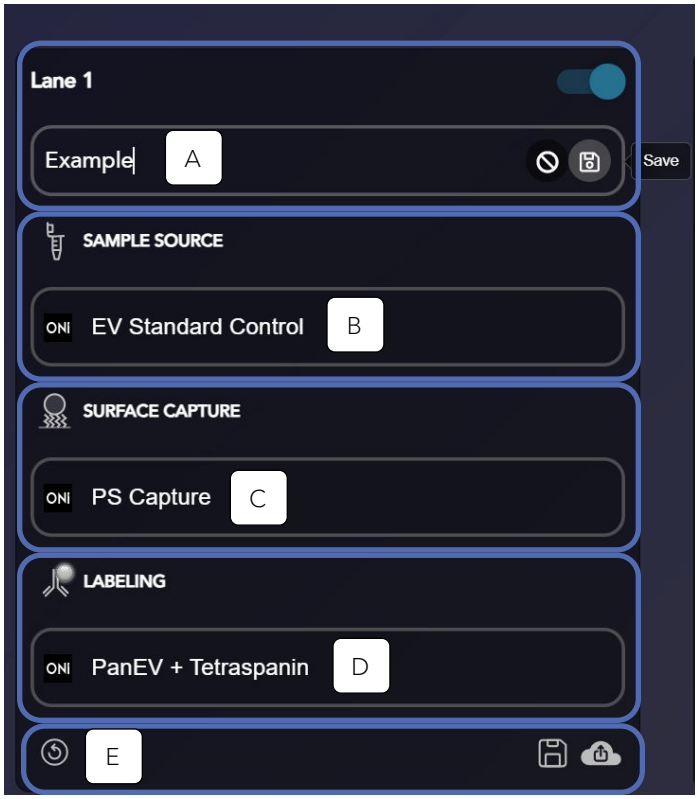
The pipette head can be moved to the default location (termed Re-homed) or the tip can be ejected from the device using the commands on the left-hand pane (G).

The 'Re-home' button will re-home the Aplo Flow System. Use this in the case when a run has been aborted to move the pipette head to the default location.

The 'Eject Tip' button will run the system's tip ejection procedure. This can be used when the system is stopped and a tip has been left on the device.

Configuring the lane specific settings

The Aplo Flow EV Prep software allows for the flexibility to modify settings on a lane to lane and chip-to-chip basis within the same assay kit selected. There are default ONI-provided reagents in the dropdown menus as well as the ability to save user-provided and user-defined reagents and lane configurations.

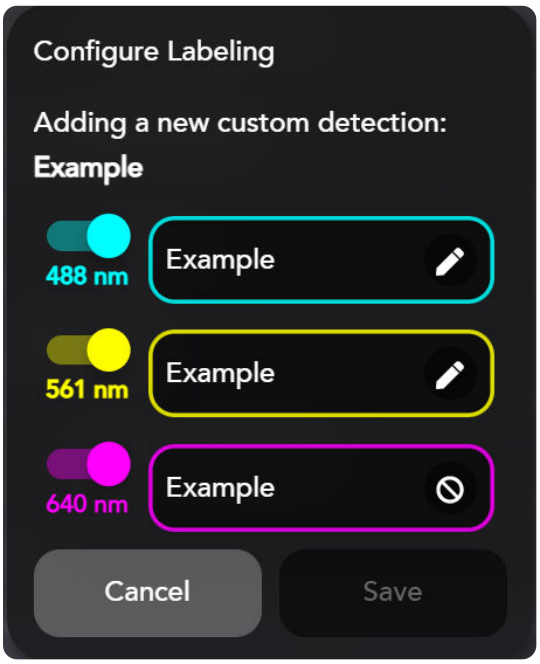


A. Lane Title: the lane labels can be changed and saved

B. Sample Source: the physical sample for the assay, such as which source of extracellular vesicles. For example, ONI-provided sample source: EV Standard Control

C. Surface Capture: the desired protein capture method: ONI-provided surface captures: CD9, CD63, CD81, No Capture, PS Capture, Tetraspanin Trio

D. Labeling: Antibody Detection Mixes: ONI-provided labeling: dropdown menu options will depend on the EV Aplo Flow Kit chosen:



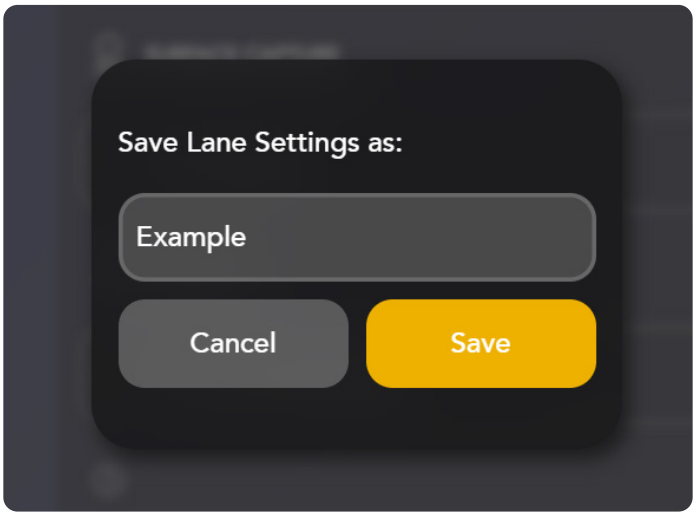
In the Labeling section, a 'Configure Labeling' prompt will appear. The three different channels, 488 nm, 561 nm, and 640 nm can be named as unique user-defined antibodies. This combination can be saved and applied to other lanes and chips. For this kit configuration the default values should be CD9-488, CD63-561, CD81-647.

E. Lane configuration buttons

The ‘Reset to default values’ button will replace anything user-defined and replace it with the default ONI-provided reagents for the EV Aplo Flow Kit chosen.

The ‘Save lane configuration’ button will prompt a “Save Lane Settings as:” popup, which can be tilted and saved to use for other lanes, chips, or future Aplo Flow EV Prep runs.

The “Load a saved lane configuration” button will prompt a “Load Lane Settings” popup which will have a drop down menu with previously saved lane configurations.



Once the configured custom labeling has been applied, a “Save Lane Settings as:” prompt will appear. This allows a unique name for the lane configuration to be saved, which can then be applied to other lanes or chips.

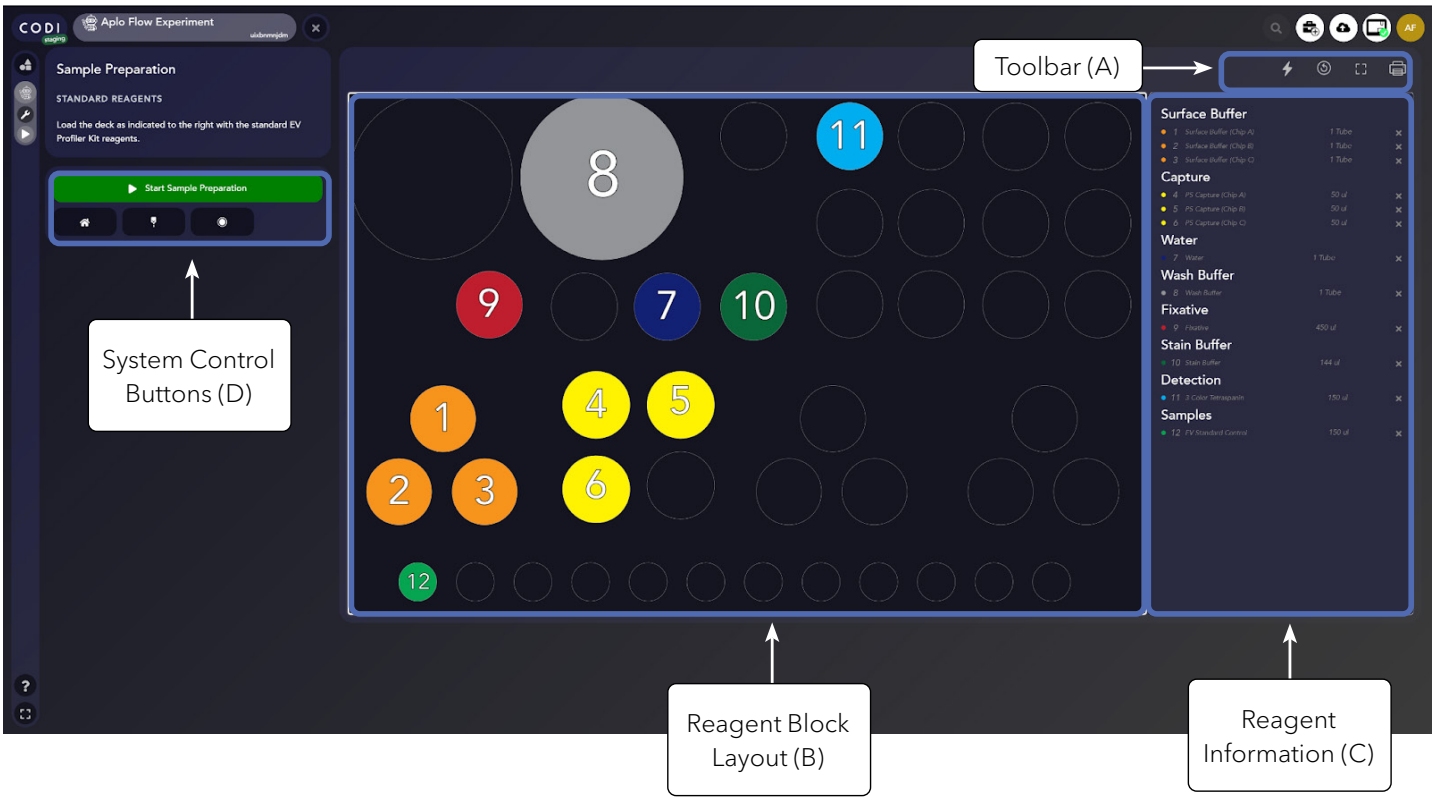
The Sample Preparation screen

5. Once experimental settings are completed, click the Next button.

6. Pending the experimental configuration, a default layout of reagents will load. Or, the reagent deck can be customized to a user-specific layout.

   Note: If the full list of reagents cannot be seen in the window then use the sidebar or mouse wheel to scroll down. The Reagent Block is Block B on the deck. The Reagent Block layout will differ depending on the Aplo Flow EV Prep kit being run.

Example Sample Preparation screen



Toolbar buttons (A)

The "Use default deck values" button will move reagents back to their default locations. The "Customizing reagent block layout" section details which reagents can be user-defined and moved around in the reagent block.

The "Reset to default values" button will reset to a fully blank reagent block layout. This is helpful when a user wants to drag and drop all reagents to fixed or user-defined locations.

The "See deck in full screen" button will allow a user to view and edit the deck layout in full screen mode.

The "Print deck layout" button will download a PDF titled "Aplo Flow Experiment_deckLayout" in which the "Aplo Flow Experiment" title gets replaced with a user chosen experiment title.

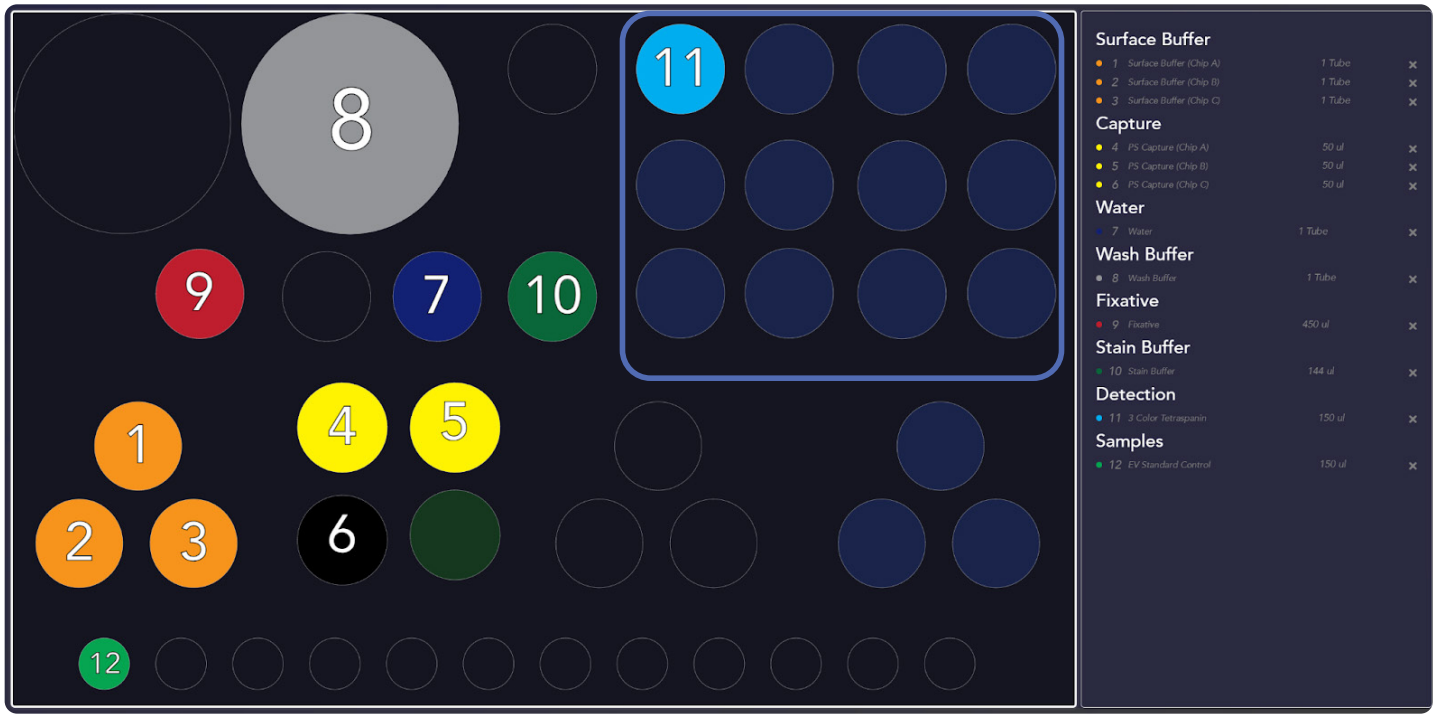
Customizing reagent block layout (B)
The reagent block will default to prefilled positions. Depending on how the experiment is set up the tube block can be user-defined. Reagents can be dragged and dropped into positions pointed by the circle with blue infill, indicating it is an acceptable location for placement. Placing the cursor over the acceptable location will turn the infill green. Let go of the mouse click and the system will update with that reagent in that position.

- For Capture Reagents, Detection, and Samples, there are more allowable positions. Drag and drop reagents into desired positions or click the "x" next to the tube number. This will move it back into the selection window on the right hand side of the screen and will need to be dragged and dropped back into position.
- Reagents such as Water, Wash Buffer, Fixative, and Stain, need to be placed into the labeled position on the tube block and are not custom locations.

Note: The "Detection" section (labeled C) will differ depending on the Aplo Flow EV Prep kit being run.

Starting a run (D)
Once the deck has been prepared and all reagents have been placed, the experiment is ready to start. To start a run, press the 'Start Sample Preparation' button (see below).

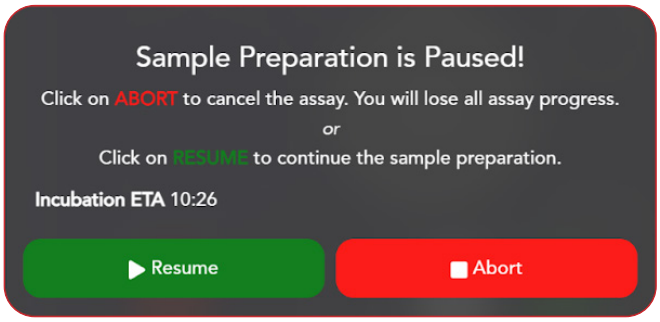
Once the run has been started, the chip completion times will populate in the bottom left corner.



Pausing/Aborting a Run

Once a run has been started, the "Start Sample Preparation" button (D) will turn into a pause button. Pressing this button will immediately pause the system.

When the system is paused, the 'Pause/Abort' screen will be displayed.



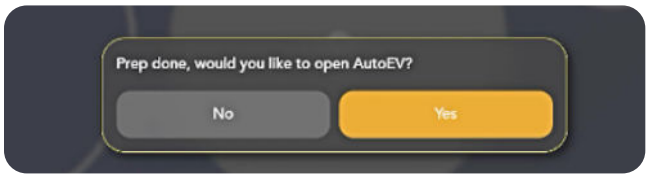
Pressing "Resume" will resume the run. Please ensure the instrument lid is closed before pressing Resume. Pressing "Abort" will abort the run. When aborted, the current tip will be ejected and the system will return home.

Error Handling

Clicking the Retry button will re-run the step that the error occurred on and Abort will end the run entirely. For more details on Aplo Flow error handling and troubleshooting, please contact your local ONI representative.

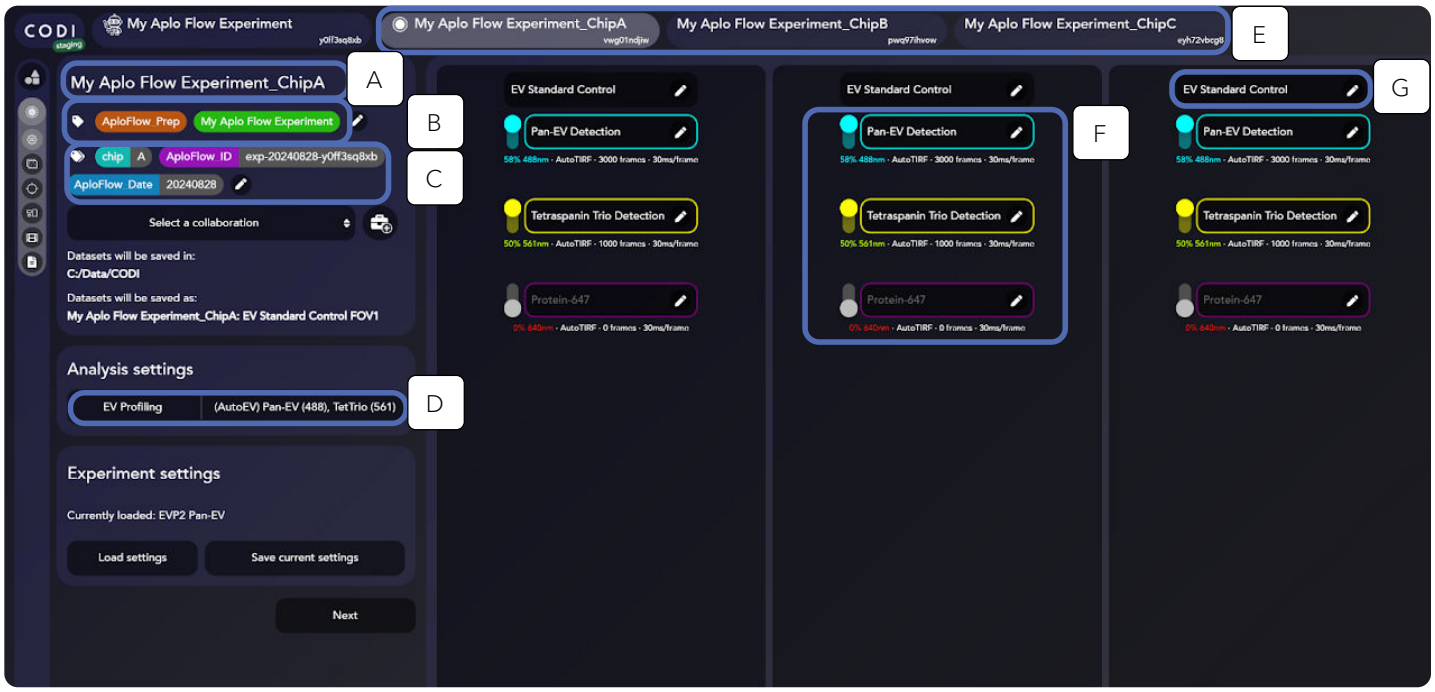
Completion of EV Prep

When the run completes, the tool will display the "Prep Done" message. At this point the user will have the option of moving on the AutoEV tool for imaging.



- Select 'No' if finished chips are to be stored overnight at 4°C for a later imaging session or the Aplo Flow system is not connected to a Nanoimager.
- Select 'Yes' if the intent is to perform an imaging session using AutoEV on the current Aplo Flow system that is attached to a Nanoimager.

AutoEV can be accessed by the "Launch Auto EV" button to manually launch AutoEV (D).



When opening AutoEV, this CODI landing page is the chosen option.

Configuration settings that transfer from Aplo Flow EV Prep to AutoEV:

A. Experiment ID

B. Tags: AploFlow_Prep, Experiment Title

C. Key-value tags: [(chip, [A/B/C]), (AploFlow_ID, aploflow id), (Aploflow_Date, date)]

D. Analysis settings depending on the kit selected

E. Experiment title_Chip [A/B/C]

F. Channels picked with its corresponding label

G. Lane name

Once automated chip preparation has completed

Due to the low volume of liquid in the lanes of prepared chips, they are very sensitive to evaporation. Therefore, leaving prepared chips exposed to open air for extended periods can result in the loss of one or multiple samples. Relative humidity and temperature vary from lab to lab, so the following guidance should be followed to ensure the highest possible imaging quality from prepared chips:

- It is recommended to image the chip immediately for best results. Chip A can be removed from the instrument before Chip B and C are complete.

  Or

- Leave the prepared chips on the deck, unsealed, for no more than one (1) hour before imaging.

  Or

- Prepared chips can be immediately sealed using the provided stickers from the EV Profiler 2 kit and stored at room temperature for up to four (4) hours, or stored overnight at 4°C. If storing chips overnight, make sure they are protected from light, and wash them with 100 µL of Wash Buffer immediately prior to adding the dSTORM Imaging Buffer.

  CAUTION: Storing the prepared chips for more than 24 hours before imaging is not recommended due to potential degradation of the sample.

Preparing chips for imaging

ONI's proprietary dSTORM Imaging Buffer has been specially formulated for extended imaging time to enable processing of multi-lane fluidic chips.

Note: The prepared dSTORM Imaging Buffer is stable for 90 minutes, so mix enough dSTORM Imaging Buffer for one chip at a time and inject the mixed solution into the lanes of a single chip immediately prior to imaging. After 90 minutes of imaging, the dSTORM imaging buffer should be replenished.

Prepare dSTORM Imaging Buffer

1. Remove one vial of dSTORM Imaging Buffer Part A from –20°C storage.
2. Allow dSTORM Imaging Buffer Part A to reach room temperature and thaw completely. This should take approximately 10 minutes if incubated at room temperature. Part A can sit at room temperature for up to 4 hours.
3. Centrifuge the tube of dSTORM Imaging Buffer Part A for 5 seconds on a standard benchtop microcentrifuge.
4. Add 99 µL of dSTORM Imaging Buffer Part A to a fresh tube.
5. Remove dSTORM Imaging Buffer Part B from –20°C and let come to room temperature. 25 µL of Part B Resuspension Buffer to the vial. Put on ice for 5 minutes. Then, gently mix by pipetting up and down. Do not introduce bubbles or air. Do not shake or vortex vial. Immediately add 1 µL of dSTORM Imaging Buffer Part B to the fresh tube of dSTORM Imaging Buffer Part A.
6. Return dSTORM Imaging Buffer Part B to –20°C storage promptly.
7. Carefully mix the now combined solution by gentle pipetting with a P100, making sure not to introduce bubbles.

   CAUTION: Never flick, shake, invert, or vortex dSTORM Imaging Buffer. Introducing excess oxygen will negatively affect its performance

8. If chips have been stored, remove sealing stickers from all 4 lanes.
9. Manually add 20 µL of prepared dSTORM Imaging Buffer to the prepared chip lanes that are ready to be imaged.
   a. When pipetting liquid into the inlet hole, the liquid will flow through the lane and exit through the outlet marked "OUT". We suggest removing excess liquid as it flows out of the outlet using a vacuum aspirator by placing it gently next to the outlet hole. If an aspirator is not available, use a laboratory dust-free wipe to remove excess liquid. Regardless of method, ensure there is minimal spillover from one lane outlet to another.

      Note: Do not insert the aspirator directly into the outlet hole, as this will result in liquid being completely removed from the lane.

   b. If there are bubbles within the lane, or liquid has been inadvertently removed, wash the lane with 100 µL of Wash Buffer to dislodge bubbles and reintroduce the Imaging Buffer.
10. Seal lanes with inlet/outlet sealing stickers. Let each full sealed chip with Imaging Buffer incubate for 10 minutes prior to image acquisition. The AutoEV setup steps (Experiment Setup, Channel Mapping, System Calibration & Sample Check, and Field of View Selection) can be started at this time.
11. To start an AutoEV automated image acquisition protocol, ensure the Nanoimager is connected to the same computer and CSA version 0.19.5 or newer is running. From there, navigate to CODI via a web-browser and click on the "Acquisition Apps" tab on the left pane.
12. Search for the AutoEV Experiment application with a logo of an EV.

AutoEV Application logo

13. Use the protocol for AutoEV imaging to set up an image acquisition and analysis