Olink® Explore HT

Formulatrix® F.A.S.T.™

Laboratory Instructions

1548, v1.0, 2024-10-07

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1.Introduction

Olink® Explore HT is a high-multiplex, high-throughput protein biomarker platform intended to measure the relative concentration of proteins in liquid biopsies. The platform uses Olink's PEA™ technology coupled to an innovative new readout methodology based on Next Generation Sequencing (NGS). The protocol is semi-automated, meaning that most pipetting steps are performed by robots. Plate sealings and plate transfers are performed manually.

Actionable protein profiles that are identified by the assays may provide relevant insights into real-time human biology and facilitate development of more effective, targeted therapies. The results are typically used by scientists involved in drug development, clinical research or basic life science research who are looking to run large-scale discovery studies focusing on the low abundant plasma proteome.

1.1 Intended use

Olink® Explore is a multiplex immunoassay platform for human protein biomarker discovery. The product is intended for Research Use Only. Not for use in diagnostic procedures. The laboratory work shall only be run by trained laboratory staff. Data processing shall only be performed by trained staff. The results are meant to be used by researchers in conjunction with other clinical or laboratory findings.

1.2 About this manual

This manual provides the instructions needed to run Olink Explore HT using Formulatrix® F.A.S.T.™. Before performing the protocol for the first time, please read the Olink® Explore HT Overview User Manual.

For optimal results, the instructions must be strictly and explicitly followed. Any deviations throughout the laboratory steps may result in impaired data.

NOTE: *Olink controls are added to each sample plate in either fixed or randomized positions.*

2.Preparations

2.1 Plan the study

A successful study requires careful planning. Perform the following before starting the experiment:

- Decide the number of samples required to get the data that you want from the study.
- When running more than one Sample Plate, make sure that the samples are appropriately randomized across all plates and that necessary steps for normalizing and combining data are taken. If you need assistance, consult a statistician or contact Olink Support before running the study.
- The 3 replicates of sample control are optional.
- Consult the Olink white paper "Strategies for design of protein biomarker studies" and the sample randomization guidelines. For links, refer to *3. Associated documentation and resources.*

NOTE: *Sample randomization helps to ensure that technical variation does not overlap with biological variation.*

2.2 Important information

Reagent lots

Before starting each step of the workflow, make sure that the lot number of each reagent matches the lot numbers indicated in the Lot Configuration document provided with the kit.

384-well PCR plates

Make sure to use the twin.tec 384-well skirted PCR plate from Eppendorf (art nr. 0030128508). All instrument protocols have been calibrated for this specific plate. Other models should not be used.

Vortexing

Vortexing is performed using the MixMate, with settings according to:

Plate type	No adaptor	Tube Holder PCR 96	Tube Holder 25/50
96-wells, semi-skirted	–	2,000 rpm, 30 sec	–
96-wells, skirted	2,500 rpm, 30 sec	–	–
384-wells	3,000 rpm, 30 sec	–	–
8-well strip	–	2,000 rpm, 30 sec	–
50 mL tube	–	–	1,000 rpm, 30 sec

2.3 Prepare the samples

During this step, samples are manually transferred to the Sample Plate. It is recommended to use the Sample Plate within the same day of preparation to minimize the number of freeze-thaw cycles.

IMPORTANT: *The Olink Explore HT protocol is optimized and validated for plasma and serum samples. If using other sample matrices than plasma or serum, please contact support@olink.com before proceeding with the sample preparation as the positions of the external controls in the Sample Source Plate differ.*

Prepare bench

- Samples (provided by the user)
- 2x 96-well PCR plate, preferably with full skirt
- Manual single- or multichannel pipette (10 µL)
- Filter pipette tips
- Adhesive films
- Temperature-resistant labels or marker pen

Before you start

- Select the samples to be included in the study.

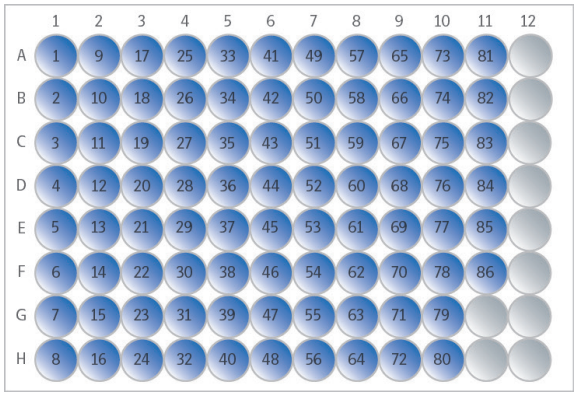
IMPORTANT: *The samples must be undiluted, as dilution of samples may result in lower detectability.*

- Thaw the samples at room temperature if frozen.
- Vortex and spin down samples.
- Mark the Sample Plates: "Sample Plate 1" and "Sample Plate 2".

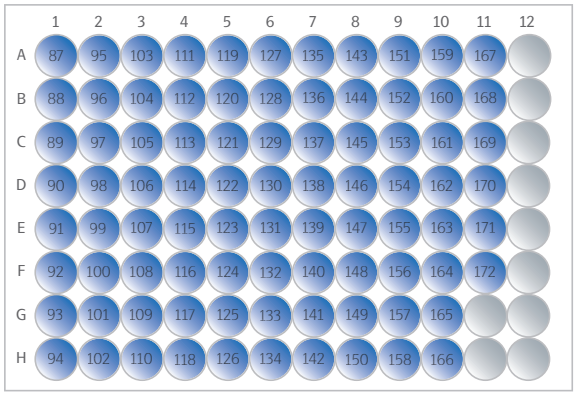
Instructions

1. Transfer the samples into the Sample Plates, at least 10 µL. Make sure that samples are added to every applicable well.

Sample Plate 1



Sample Plate 2



NOTE: *Make sure there is enough sample in each well to perform the dilution. 10 µL will be transferred to the Sample Source plate.*

2. Seal the Sample Plates using an adhesive film or individual seals.
3. Store the Sample Plates at 4 °C if used the same day, otherwise at -80 °C.

IMPORTANT: *Avoid subjecting the samples to multiple freeze-thaw cycles.*

3.Prepare Sample Source Plate

During this step, samples are automatically transferred from two Sample Plates to one Sample Source Plate using the F.A.S.T. and controls are added to the Sample Source Plate. The Sample Source Plate must be used within the same day of preparation.

IMPORTANT: *Using the correct combination of reagents is essential for the downstream data analysis. Before starting each step of the workflow, make sure that the lot number of each reagent matches the lot numbers indicated in the Lot Configuration document provided with the kit.*

Prepare bench

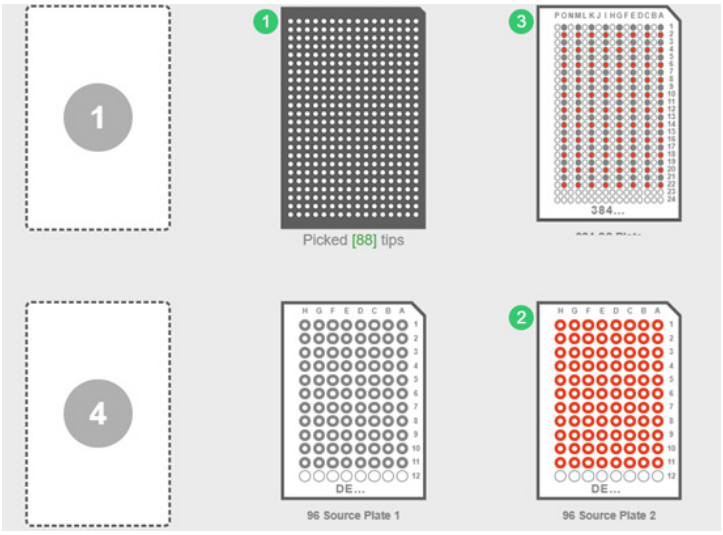
- Sample Plates, prepared in previous step
- Olink® Explore Negative Control
- Olink® Explore Plate Control
- Olink® Explore Sample Control (optional)
- 1x 384-well PCR Plate (skirted)
- 1x 8-well PCR strip
- Manual pipette (10 µL)
- Filter pipette tips
- F.A.S.T.
- 1x box of Formulatrix F.A.S.T. positive displacement tips (13 µL)
- Adhesive films

Before you start

- Ensure that the Sample Plates have been prepared according to section *2.3 Prepare the samples.*
- If frozen, thaw the Sample Plates at room temperature.
- Thaw the Negative Control, Plate Control and Sample Control at room temperature.

Instructions

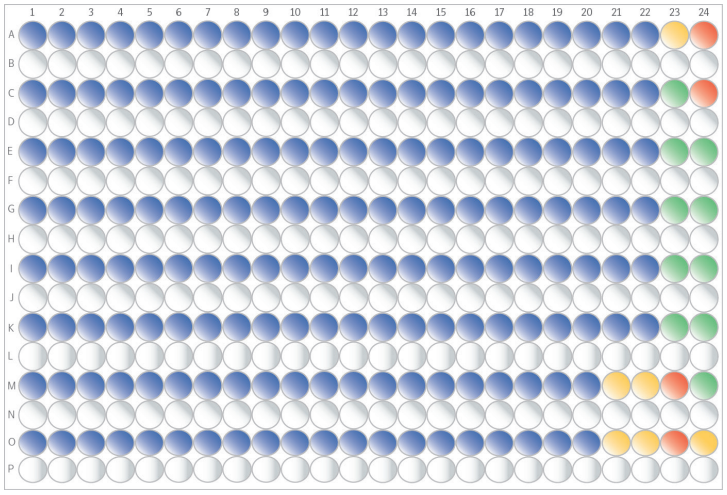
1. Vortex the 96-well Sample Plates and spin at 400–1000 x g for 1 minute at room temperature.
2. Turn on the F.A.S.T. and open the web application interface.
3. Open the protocol `0_Olink_ExploreHT_SS_Plate_Creation` and load the tray according to:



4. Click **Start** in the upper right corner to execute the protocol.
   Result:
   F.A.S.T. transfers 10 µL from Sample Plate 1 to every second column and every second row of the Sample Source Plate (column 1, 3, etc and row A, C, etc).
   F.A.S.T. changes tips and transfers 10 µL from Sample Plate 2 to every second column of the Sample Source Plate (column 2, 4, etc and row A, C, etc).
5. Vortex the Negative Control, Plate Control, and Sample Control and spin briefly.
6. Transfer 10 uL of Sample Control to the wells M21, M22, O21, and O22 of Sample Source Plate.
7. Using a single-channel pipette, transfer 26 ul of Sample Control, Plate Control and Negative Control to each well of a PCR strip, according to the figure below. Change pipette tip between each control sample.

8. Using an multichannel pipette, transfer 10 uL of the control samples from the PCR strip to each of the applicable wells in column 23 of the Sample Source Plate according to the figure below.
9. Flip around the PCR strip to get the mirror positioning of controls.
10. Change the tips in the multichannel pipette, and transfer 10 uL of the control samples from the PCR strip to each of the applicable wells in column 24 of the Sample Source Plate according to the figure below.
11. Seal the Sample Source Plate with a new adhesive film. Spin the Sample Source Plate at 400–1000 x g for 1 minute. Make sure that there are no bubbles trapped at the bottom of the wells. Store the plate at 4 °C until use (the same day).

12. Seal the Sample Plates using adhesive film and store at -80 ºC.



IMPORTANT: *All wells must be properly sealed to avoid evaporation of the samples.*

4.Sample Dilution

Sample Diluent is dispensed into two Sample Dilution Plates using the Dragonfly, and the samples are diluted using the F.A.S.T. instrument. The prepared Sample Dilution Plates must be used within three hours from preparation and stored at 4 °C until use.

4.1 Prepare Sample Dilution Plates

During this step, Sample Diluent is dispensed into each Sample Dilution Plate using the Dragonfly.

Prepare bench

- Olink® Target 96/Explore Sample Diluent
- 2x 384-well PCR Plates (skirted)
- Manual pipette (1,000 µL)
- 1x Disposable reservoir (10 mL)
- 1x Disposable ultra-low retention syringe with plunger
- Filter tips
- Adhesive films

Before you start

- Thaw the Sample Diluent at room temperature.
- Mark the 384-well PCR plates: "Sample Dilution Plate 1" and "Sample Dilution Plate 2".

NOTE: *For convenience, the Sample Diluent can be thawed at 4 °C overnight.*

Instructions part 1:

1. Prepare the Dragonfly according to manufacturer instructions.
   a. Use the protocol: `1_Olink_ExploreHT_Dilution_Plate1` to prepare Sample Dilution Plate 1.
   b. Attach one syringe in position B2.
2. Slide the reservoir tray to the filling position and place a new reservoir in position B2.
3. Vortex the Sample Diluent briefly, add 12 mL of Sample Diluent into the reservoir and carefully slide the reservoir tray back to the aspirate position.
4. Place the Sample Dilution Plate 1 in the plate position to the left, with well A1 in the top left corner.
5. Open the Explore HT Sample Dilution Plate 1 protocol and select the Run tab in the Constant layer view of the software, then press RUN to start the program.
   Result:
   Dragonfly dispenses 9 µL Sample Diluent into each well of Sample Dilution Plate 1.
6. When the Dragonfly has returned the Sample Dilution Plate 1 to the plate position and released the plate clamp, remove the Sample Dilution Plate 1 from the instrument and seal it with an adhesive film.
7. Repeat step 4–6 for Sample Dilution Plate 2. Use `2_Olink_ExploreHT_Dilution_Plate2`.
   Result:
   Dragonfly dispenses 9 µL Sample Diluent into each well of every other row (A, C, E etc.) and 29 µL into every other row (B, D, F etc.) of Sample Dilution Plate 2.

IMPORTANT: *The program will stop in position J22. It will reload and start again.*

Instructions part 2:

1. Spin the Sample Dilution Plates at 400–1,000 x g for 1 minute.
2. Visually inspect the Sample Dilution Plates to ensure that all wells contain the expected volumes. Make sure that there are no bubbles trapped at the bottom of the wells.
3. Clear the instrument and shut it down according to manufacturer's instructions.
4. Continue to *4.2 Perform Sample Dilution* or store the Sample Dilution Plates at 4 ºC until use (the same day).

4.2 Perform Sample Dilution

During this step, the samples are diluted in four sequential steps using the F.A.S.T. from 1:1 (undiluted) to 1:10, 1:100, 1:1,000 and approximately 1:100,000.

Prepare bench

- Sample Source Plate (prepared in previous step)
- Sample Dilution Plates 1 and 2 (prepared in previous step)
- 1x box of Formulatrix F.A.S.T. positive displacement tips (13 µL)
- Adhesive films

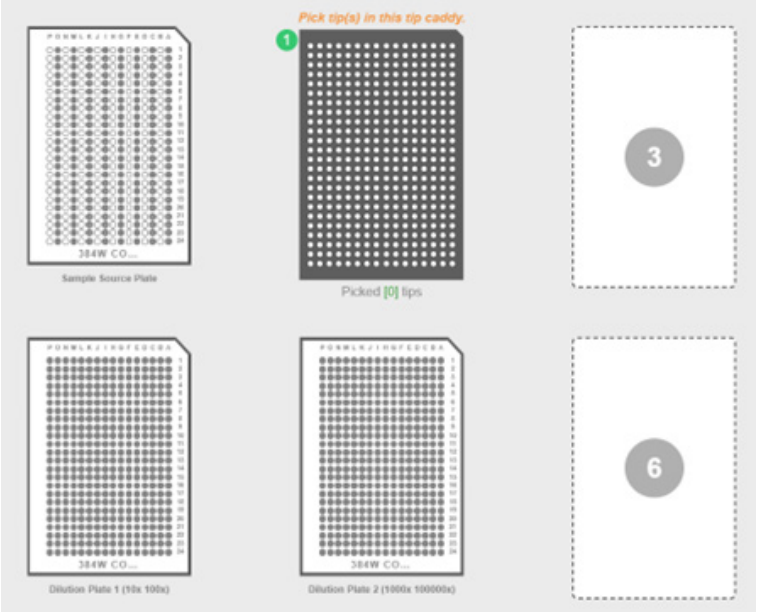
Before you start

- Turn on the F.A.S.T. and open the web application interface.

Instructions

IMPORTANT: *Make sure to vortex the Sample Dilution Plate(s) thoroughly with correct MixMate settings both between dilution and after the last dilution, as incorrect settings may lead to low-quality data. Refer to table in 2.2 Important information.*

1. Let the Sample Source Plate reach room temperature, vortex and spin at 400–1,000 x g for 1 minute at room temperature. Make sure that there are no bubbles trapped at the bottom of the wells.
2. Carefully remove the adhesive films from the Sample Source Plate and the Sample Dilution Plates.
3. Open the Protocol `1_Olink_ExploreHT_SampleDilution` and load the tray according to:



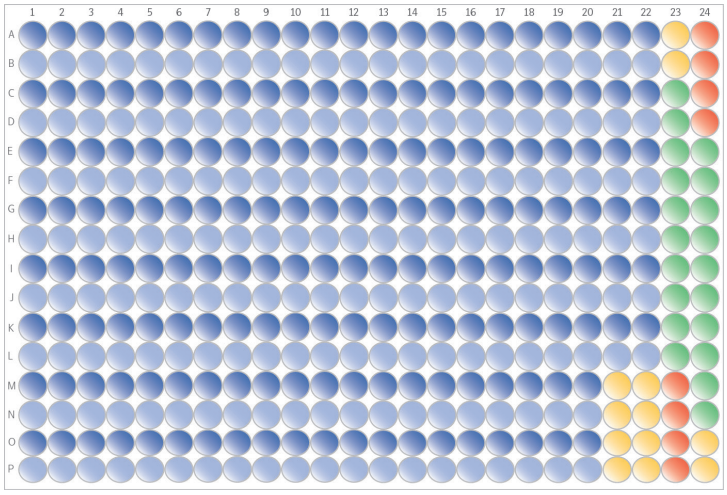
4. Press **Start** to run the protocol.
5. Between each dilution step, the program will pause, and a pop-up window will appear. When the protocol is paused:
   a. Remove the Sample Dilution Plate from the instrument as instructed in the pop-up.
   b. Seal the plate with adhesive film.
   c. Vortex the plate after each transfer, no adaptor needed.
   d. Spin at 400–1,000 x g for 1 minute. Make sure that no air bubbles are trapped at the bottom of the wells.
   e. Remove the adhesive film.

f. Return it to the correct position in the instrument.

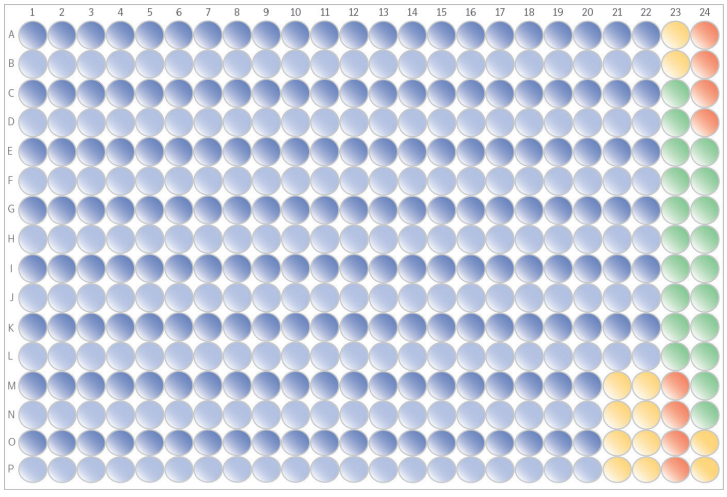
g. Press **Resume** to continue the program.

Result:

- *1 µL of undiluted (1:1) samples from the Sample Source Plate are transferred to every other row of the Sample Dilution Plate 1 (row A, C etc) resulting in a 1:10 dilution. Shown as dark blue in the figure below.*
- *1 µL of diluted 1:10 samples are transferred to every second row of Sample Dilution Plate 1 (row B, D etc.), resulting in a 1:100 dilution. Shown as light blue in the figure below.*



- *1 µL of diluted 1:100 samples from Sample Dilution Plate 1 are transferred to every other row of Sample Dilution Plate 2 (row A, C etc.), resulting in a 1:1,000 dilution. Shown as dark blue in the figure below.*
- *0.3 µL of diluted 1:1000 samples are transferred to every second row of Sample Dilution Plate 2 (row B, D etc.), resulting in a 1:100,000 dilution. Shown as light blue in the figure below.*



6. Once the protocol is finished, keep the F.A.S.T. on for later use.
7. Continue to *5. Incubation*, or place the Sample Dilution Plate at 4 ºC for up to 3 hours.

5.Incubation

During this step, eight incubation mixes are manually prepared, transferred to the Reagent Source Plate, and mixed with the samples. Incubation is then performed overnight.

5.1 Prepare Reagent Source Plate

During this step, eight incubation mixes are prepared manually and transferred to the Reagent Source Plate. Each mix contains a specific set of forward and reverse probes.

Prepare bench

- Olink Explore Incubation Solution
- Olink Explore HT Frw Probes (1 to 8)
- Olink Explore HT Rev Probes (1 to 8)
- 1x 384-well PCR Plate
- 1x 8-well PCR strip
- Multichannel pipette (20 µL)
- Manual pipettes (20 µL and 200 µL) Optional: Adjustable multichannel pipette (20 uL)
- 1x MixMate Tube Holder PCR 96
- Filter pipette tips
- Adhesive film

Before you start

- Allow the Incubation Solution, Forward Probes and Reverse Probes to reach room temperature.
- Mark the 384-well PCR plate: "Reagent Source Plate".
- Mark the wells of the 8-well PCR strip: "[1–8].

1	2	3	4	5	6	7	8
White	Red	Yellow	Blue	Green	Purple	Orange	Black

Instructions

1. Vortex the Incubation Solution, Forward Probes and Reverse Probes and spin down.
2. Prepare the eight incubation mixes in the PCR Strip:

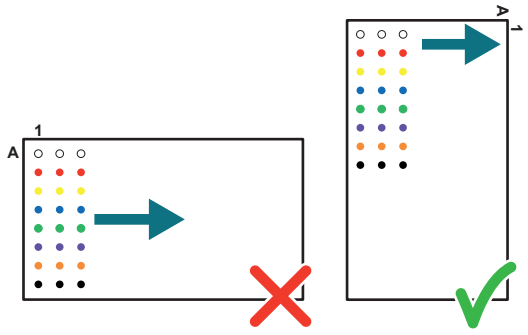
Addition order	Reagent	Volume (µL)
1	Incubation solution	160
2	Frw probes	20
3	Rev probes	20

   - Transfer the Incubation Solution to tubes 1–8 of the PCR strip. Use **reverse pipetting**. Pipette the Incubation Solution carefully to avoid foaming.
   - Add Forward Probes 1–8 to their applicable tubes.
     Optional: Use an adjustable multichannel pipette to transfer all Forward Probes in one step.
   - Add Reverse Probes 1–8 to their applicable tubes.
     Optional: Use an adjustable multichannel pipette to transfer all Reverse Probes in one step.

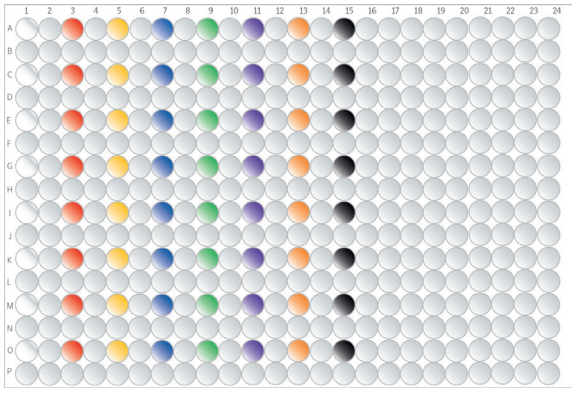
3. Seal the PCR strip with caps. Vortex the PCR strip using the Tube Holder PCR 96 and spin down. Store at room temperature until use.
4. Inspect the wells to make sure that they contain the expected volume of 200 µL.

TIME SENSITIVE STEP: *The incubation setup for Incubation plate 1 and 2 must be started within 30 minutes from preparation of the Incubation Mix.*

5. Rotate the Reagent Source Plate (384-well) 90 degrees clockwise to facilitate pipetting.



6. Using a multichannel pipette, transfer 20 µL from the PCR strip to each well in the Reagent Source Plate. Do not change tips between wells. Make sure to pipette to the bottom of the wells.



7. Seal the Reagent Source Plate with a new adhesive film and spin at 400–1,000 x g for 1 minute. Make sure that no air bubbles are trapped at the bottom of the wells.
8. Immediately continue to *5.2 Perform incubation*.

5.2 Perform incubation

During this step, the prepared Incubation Mixes are transferred to four incubation plates and mixed with the 192 samples and controls using the F.A.S.T. prior to incubation.

Prepare bench

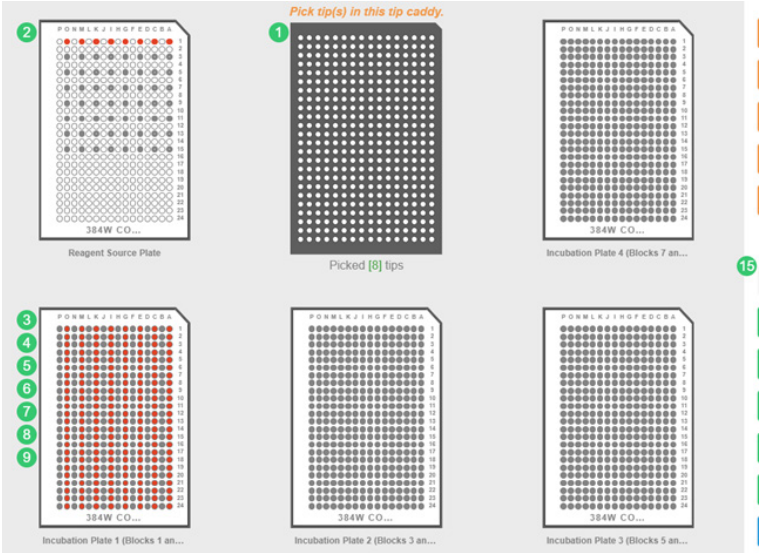
- Sample Source Plate, prepared in previous step
- Sample Dilution Plate 1 and 2, prepared in previous step
- Reagent Source Plate, prepared in previous step
- 4x boxes + 128 tips of Formulatrix F.A.S.T. positive displacement tips (13 µL)
- 4x 384-well PCR plates
- Adhesive films

Before you start

- Mark the 384-well PCR plates: "Incubation Plate [1–4]".
- Allow the Sample Source Plate and Sample Dilution Plate 1 and 2 to reach room temperature.

Instructions part 1: Transfer incubation mix

1. In the F.A.S.T. software, navigate to the Protocol Explorer and select the `2_Olink_ExploreHT_Incubation_ReagentTransfer` protocol from the Load Protocol Window.
2. Carefully remove the adhesive film from the Reagent Source Plate.
3. Place tips, the Reagent Source Plate, and the four Incubation Plates in the F.A.S.T. tray according to the picture below:



4. Press **Start**. The program will pause after each plate. Click **Next** to continue to next plate.
   Result:
   The F.A.S.T. transfers 0.6 µL of Incubation Mix from the Reagent Source Plate to the four Incubation Plates. Incubation mixes for two blocks will be transferred into each Incubation Plate:
   a. *Plate 1: Block 1 and 2*
   b. *Plate 2: Block 3 and 4*
   c. *Plate 3: Block 5 and 6*
   d. *Plate 4: Block 7 and 8*
5. Once the protocol is finished, remove the Reagent Source Plate.
6. Remove the Incubations Plates from the tray and visually inspect that there is liquid at the bottom of all wells and that no bubbles are present.

IMPORTANT: *It is critical to not vortex the Incubation Plates.*

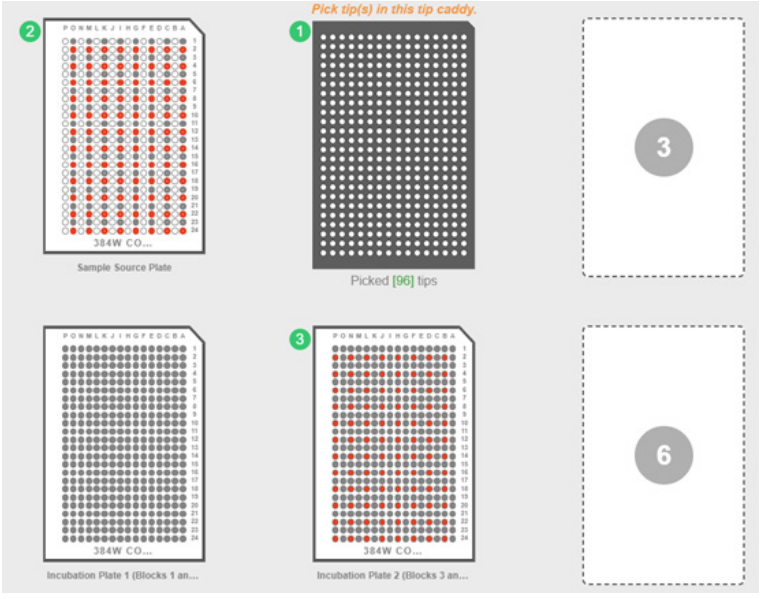
7. Discard the Reagent Source Plate.
8. Seal Incubation Plate 3 and 4 and keep them on the bench.

TIME SENSITIVE STEP: *Immediately proceed to the next step.*

NOTE: *If bubbles are present, seal the Incubation Plate(s) with new adhesive film(s) and spin at 400–1,000 x g for 1 minute, then carefully remove the adhesive film(s) and check again. Do not vortex. If bubbles are still present, puncture them using a syringe needle. Change needles between wells to avoid cross-well contamination.*

Instructions part 2: Transfer samples to block 1–4

1. In the F.A.S.T. software, select the `3_Olink_ExploreHT_Incubation_SampleTransfer_Block_1-4` protocol.
2. Spin down the Sample Source Plate at 400–1000 x g for 1 minute. Make sure that there are no bubbles trapped at the bottom of the wells and remove the adhesive seal.
3. Place the Sample Source Plate and Incubation Plate 1 and 2 in the F.A.S.T. tray according to:



4. Press **Start**.
   Result:
   F.A.S.T. transfers 0.3 µL undiluted samples from the Sample Source Plate to the Incubation Plate 1 and 2.
5. Once the protocol is finished, remove Incubation Plate 1 and 2 from the deck and seal them with new adhesive film. Spin down the plates at 400–1,000 x g for 1 minute.

IMPORTANT: *All wells must be properly sealed to avoid evaporation of the samples.*

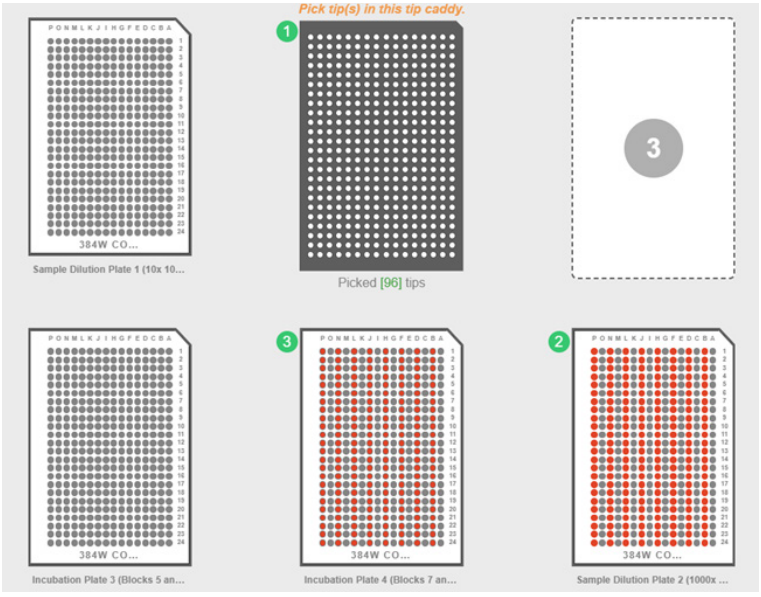
IMPORTANT: *It is critical to not vortex the Incubation Plates.*

6. Incubate Incubation Plate 1 and 2 for 16–24 hours at 4 °C.
7. Remove the Sample Source Plate from the deck, seal it with new adhesive film and store at -80 °C for potential reruns.

Instructions part 3: Transfer samples to block 5–8

IMPORTANT: *Empty the tip waste tray. Otherwise, the used tips will pile up and can cause an instrument error if tips cannot be ejected from the head.*

1. In the F.A.S.T. Software, Select the `4_Olink_ExploreHT_Incubation_SampleTransfer_Block_5-8` protocol from the Protocol Explorer.
2. Spin down Sample Dilution Plate 1 and 2 at 400–1,000 x g for 1 minute and remove the adhesive film.
3. Place Sample Dilution Plate 1 and 2 and Incubation Plate 3 and 4 on the F.A.S.T. deck, according to:



4. Press **Start**.
   Result:
   F.A.S.T. *transfers 0.3 µL diluted samples from Sample Dilution Plate 1 and 2 into the Incubation mixes on Incubation Plate 3 and 4.*
5. Once the protocol is finished, remove Incubation Plate 3 and 4 from the deck and seal them with new adhesive film. Spin down the plates at 400–1,000 x g for 1 minute.

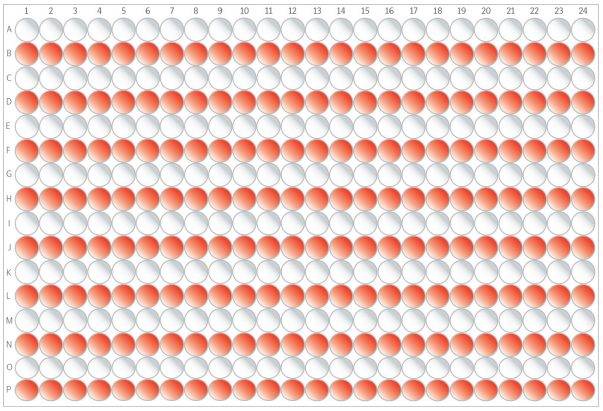
IMPORTANT: *All wells must be properly sealed to avoid evaporation of the samples.*

IMPORTANT: *It is critical to not vortex the Incubation Plates.*

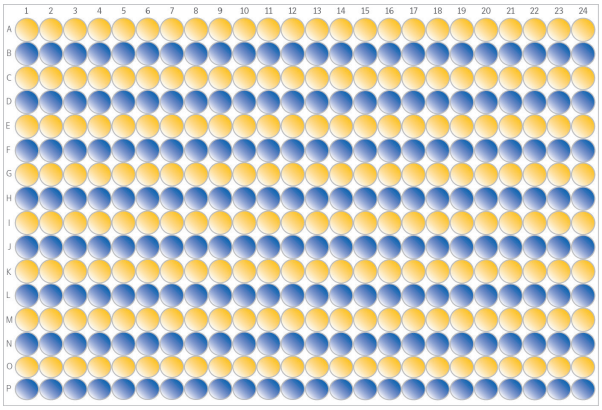
6. Incubate Incubation Plate 3 and 4 for 16–24 hours at 4 °C.
7. Remove Sample Dilution Plate 1 and 2 from the deck and discard them.

NOTE: *It is recommended to keep the incubation time within ±1 hour within a project. The incubation time starts when placing the Incubation Plate at 4 °C and ends when starting the PCR.*

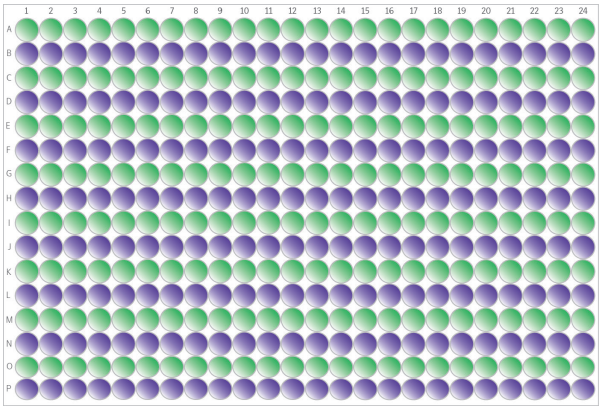
Incubation Plate 1



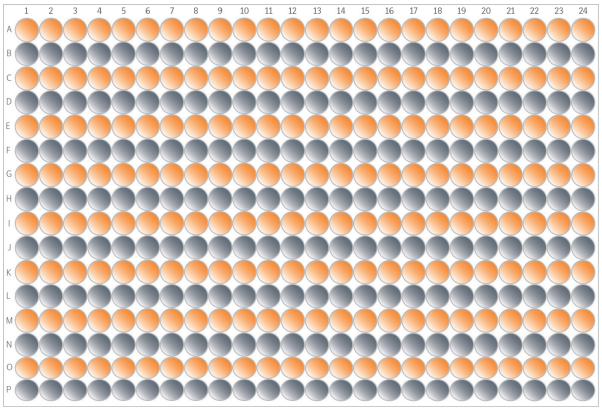
Incubation Plate 2



Incubation Plate 3



Incubation Plate 4



6.Prepare for day 2

Instructions

1. Put the 30 mL Milli-Q water in the fridge at 4 °C.
2. Thaw PCR Additive at room temperature overnight.
3. Thaw PCR Solution at 4 °C overnight or at room temperature before starting the PCR setup (may take several hours).

7.Index and PCR setup

During this step, index primers are added to Incubation Plates using the F.A.S.T. and a manually prepared PCR mix is added using the Dragonfly. The plates are renamed "PCR plate [1–4]" and subjected to a PCR reaction.

NOTE: *If you are using 96-well index plates, please contact support@olink.com for correct user manual.*

7.1 Index dispensation

During this step, the Index primers are added to the four Incubation Plates using the F.A.S.T. One single index plate is used for all 4 incubation plates. Each index is used 8 times, once for each sample in each block.

NOTE: *This section only covers instruction for using Index Plate C. If you are using Index Plate A and B, proceed to 7.1.1 Index dispensation with Index Plate A and B.*

Prepare bench

- Incubation Plate 1–4, prepared in previous step
- Olink Explore Index Plate C
- 4x boxes of Formulatrix F.A.S.T. positive displacement tips (13 µL)

Before you start

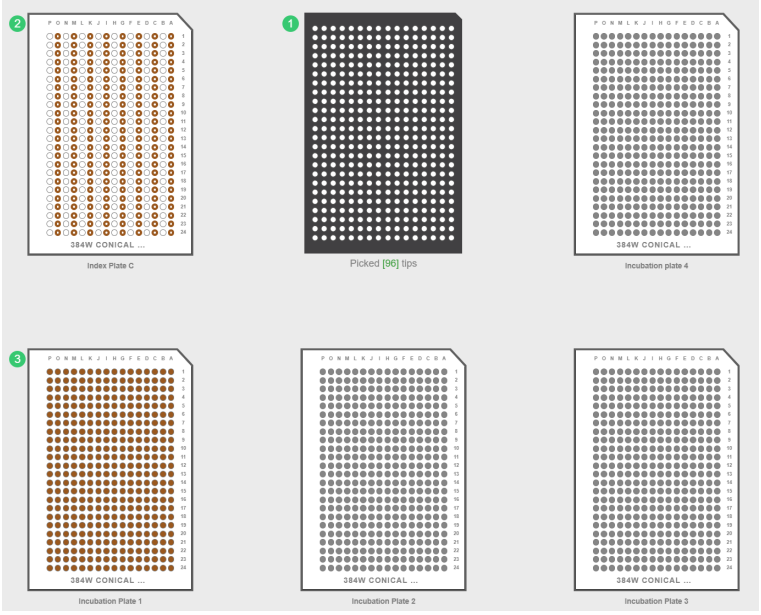
- Thaw Index Plate C at room temperature.
- Allow Incubation Plate 1–4 to reach room temperature.

Instructions

1. In the F.A.S.T. software, select the `5_Olink_ExploreHT_IndexTransfer` protocol.

NOTE: *Make sure that you are using **v3** of the protocol.*

2. Vortex Index Plate C, no adapter needed, and spin down at 400–1000 x g for 30 seconds.
3. Spin down the Incubation Plates at 400–1000 x g for 1 minute.
4. Place the Index Plate and the Incubation Plates in the F.A.S.T. tray according to picture below:



5. Press **Start**.
   Result:
   The F.A.S.T. transfers 1 µL of index primer from Index Plate C into the Incubation Plates.
6. Once the protocol is finished, remove the Incubation Plates from the deck and seal with adhesive films.
7. Spin the Incubation Plates with indexes at 400–1000 x g for 1 minute.
8. Visually inspect the Incubation Plates to ensure that all wells contain the expected volumes. Make sure that there are no bubbles trapped at the bottom of the wells.

IMPORTANT: *It is critical to not vortex the Incubation Plates.*

9. Remove Index plate C from the deck and discard it.
10. Immediately proceed to *7.2 Prepare PCR Mix*.

7.1.1 Index dispensation with Index Plate A and B

During this step, the Index primers are added to the four Incubation Plates using the F.A.S.T. The protocol is performed twice, once for Incubation Plate 1 and 2 and once for Incubation Plate 3 and 4. Each index plate is reused 8 times, once for each sample in each block.

Prepare bench

- Incubation Plate 1–4, prepared in previous step
- Olink Explore HT Index Plate A and B
- 4x boxes of Formulatrix F.A.S.T.™ positive displacement tips (13 µl)

Before you start

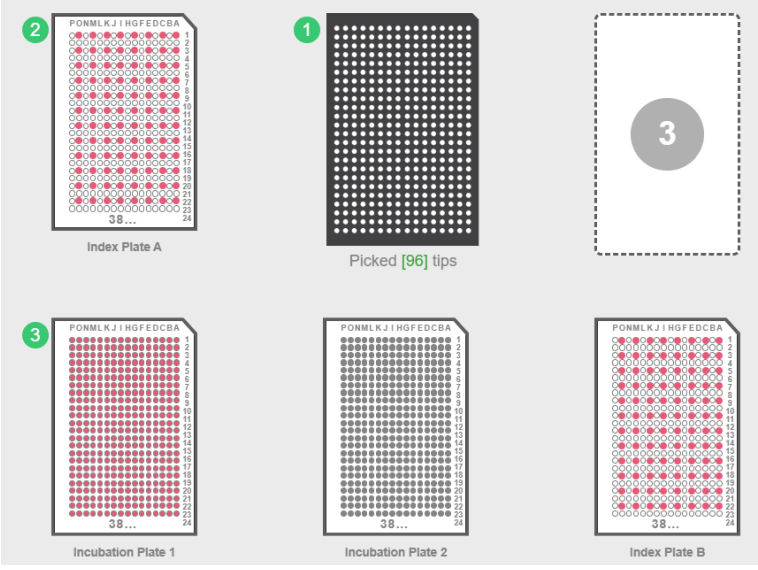
- Thaw Index Plate A and B at room temperature.
- Allow Incubation Plate 1–4 to reach room temperature.

Instructions

1. In the software, select the `5_Olink_ExploreHT_IndexTransfer` protocol.

NOTE: *Make sure that you are using **v2** of the protocol.*

2. Vortex Index Plate A and B and spin down at 400–1000 x g for 30 seconds.
3. Spin down Incubation Plate 1 and 2 at 400–1000 x g for 1 minute.Place the two Index Plates and Incubation Plate 1 and 2 in the F.A.S.T. tray according to picture below:



4. Press Start.
   Result:
   The F.A.S.T. transfers 1 µL of index primer from Index Plate A and B into Incubation Plate 1 and 2.
5. Once the protocol is finished, remove Incubation Plate 1 and 2 from the deck and seal with new adhesive films.
6. Spin Incubation Plate 1 and 2 with indexes at 400–1000 x g for 1 minute.
7. Visually inspect the Incubation Plates to ensure that all wells contain the expected volumes. Make sure that there are no bubbles trapped at the bottom of the wells.

IMPORTANT: *It is critical to not vortex the Incubation Plate.*

8. Keep Incubation Plates 1 and 2 at room temperature until further use (maximum 1 hour).
9. Keep Index Plate A and B in the F.A.S.T. tray and immediately proceed to the next step.
10. Repeat step 4–8 with Incubation Plates 3 and 4.
11. Remove Index plate A and B from the deck and discard them.
12. Immediately proceed to *7.2 Prepare PCR Mix*.

7.2 Prepare PCR Mix

During this step, a PCR Mix is prepared manually. The PCR mix must be used within 1 hour from preparation.

Prepare bench

- Explore HT PCR Solution
- Explore HT PCR Additive
- Explore HT PCR Enzyme A (keep on ice)
- Explore HT PCR Enzyme B (keep on ice)
- Milli-Q water (at 4 °C, preferably kept in the fridge until use)
- 1x Falcon tube, polypropylene (50 mL)
- 1x MixMate Tube Holder (25/50 mL)
- Manual pipette (5 or 10 mL)
- Manual pipette (1000 µL)
- Manual pipette (100 µL)
- Filter pipette tips

Before you start

- Ensure that the PCR Additive and the PCR Solution are fully thawed and have reached room temperature
- Mark the 50 mL Falcon tube: "PCR Mix".
- Switch on two ProFlex PCR instruments in the post-PCR room.
- Mount the Tube Holder 25/50 mL onto the MixMate instrument. Refer to *7.4 Vortexing.*

Instructions

1. Vortex PCR Solution and PCR Additive thoroughly and spin down briefly.

IMPORTANT: *Insufficient thawing and vortexing of the PCR Additive may lead to particles attaching to the syringe and run failure.*

2. Spin down PCR Enzyme A and B briefly. Do not vortex.
3. Prepare the PCR Mix in a 50 mL Falcon tube. The mix is enough for four incubation plates.

Addition order	Reagent	Volume (µL)
1	Milli-Q water	25,308
2	Explore HT PCR Additive	3,590
3	Explore HT PCR Solution	3,590
4	Explore HT PCR Enzyme A	722
5	Explore HT PCR Enzyme B	93
	Total	33,301

4. Pre-mix the PCR Mix by inverting the Falcon tube twice. Vortex using the Tube Holder 25/50 mL adaptor.
5. Keep the PCR Mix at room temperature until use.

TIME SENSITIVE STEP: *Dispensing of the PCR Mix using the Dragonfly must start within 1 hour from PCR Mix preparation.*

7.3 Prepare PCR Plates and perform PCR

During this step, the PCR Mix is added to the four Incubation Plates using the Dragonfly and the plates are subjected to a PCR reaction.

TIME SENSITIVE STEP: *The PCR Plates must be placed in the ProFlex exactly 10 minutes after the PCR Mix has been added to the first well of the first plate. Since each ProFlex can hold two plates, prepare two plates at a time.*

Perform the instructions in the following order:

1. Dispense PCR Mix into Incubation Plates 1 and 2 using the Dragonfly. Start a PCR run for these plates on one of the ProFlex instruments.
2. Dispense PCR Mix into Incubation Plates 3 and 4 plates using the Dragonfly. Start a PCR run for these plates on the second ProFlex instrument.

Prepare bench

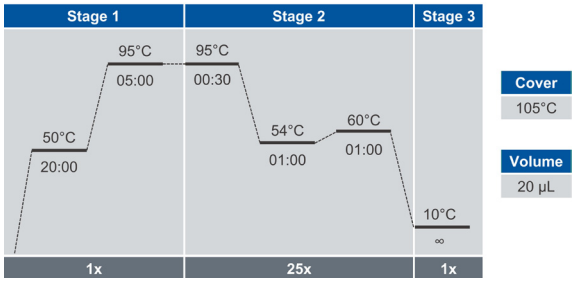
- Incubation Plates 1–4 with indices, prepared in previous step
- PCR Mix, prepared in previous step
- 2x Timer
- 3x Disposable reservoirs (10 mL)
- 3x Disposable ultra-low retention syringes with plungers
- Manual pipette (5 or 10 mL)
- Filter pipette tips
- Adhesive films

Before you start

- Set the timers to 10 minutes.
- Rename Incubation Plates 1–4: "PCR Plate [1–4]".

Instructions part 1: Prepare PCR plates

1. Start the PCR protocol `Olink_ExploreHT_PCR` on the two ProFlex instruments. Pause the program when the PCR block reaches 50 °C.



2. Prepare the Dragonfly according to manufacturer instructions.
   - Use the protocol `3_Olink_ExploreHT_PCRMix`.
   - Attach new syringes in position B2, B3 and B4.
3. Slide the reservoir tray to the filling position and place new reservoirs in position B2, B3 and B4.
4. Transfer 11 mL of PCR Mix into each of the three reservoirs.

IMPORTANT: *The syringes and PCR Mix are used to prepare all four PCR Plates. Do not discard until all PCR Plates are complete.*

5. Carefully slide the reservoir tray back to the aspirate position.
6. Place PCR Plate 1 in the plate position to the left, with well A1 in the top left corner.

7. Select the **Run tab** in the Constant layer view of the software. Press **Run.** Start the timer when PCR Mix is added to the first well of the plate.
   Result:
   Dragonfly dispenses 18 µL of PCR Mix into each well of PCR Plate 1.
8. When the Dragonfly has returned PCR Plate 1 to the plate position and released the plate clamp, remove the plate from the instrument and immediately seal it with adhesive film.
9. Vortex the plate without adaptor to ensure that all wells are mixed. Spin down at 400–1,000 x g for 1 minute.
10. Repeat steps 6–9 for PCR Plate 2.

Instructions part 2: Perform PCR

IMPORTANT: *Empty the tip waste tray. Otherwise, the used tips will pile up and can cause an instrument error if tips cannot be ejected from the head.*

1. In the Post-PCR room, centrifuge PCR Plate 1 and 2 at 400–1,000 x g for 1 minute.
2. Inspect PCR Plates 1 and 2 to ensure that all wells contain the same amount of liquid (19.8 µL). Note any deviations.
3. When the timer ends after 10 minutes, place PCR Plates 1 and 2 in one of the two pre-heated ProFlex instruments and click Resume to run the program.
4. Repeat step 7–14 for Incubation Plates 3 and 4. Place both plates in the second ProFlex.
5. Once the protocol is finished, clear the Dragonfly and shut it down according to manufacturer's instructions.
6. Once the PCR program is finished (2h), continue to *8. Pool PCR products using Hamilton Microlab® STAR* or *9. Pool PCR products using epMotion®*, or store the PCR Plates at 4 ºC if used the same day.

SAFE STOPPING POINT: *The plates can be stored at -20 °C for up to 2 weeks.*

8.Pool PCR products using Hamilton Microlab® STAR

During this step, PCR products from the four PCR Plates are pooled by block into one PCR Pooling Plate using Hamilton Microlab STAR. The libraries are then transferred from the PCR Pooling Plate to one microcentrifuge tube per block, either automatically or manually. Each tube contains PCR products corresponding to one block from the 192 samples.

This section includes two different instructions: one for automatic pooling and one for semi-manual pooling.

Prepare bench

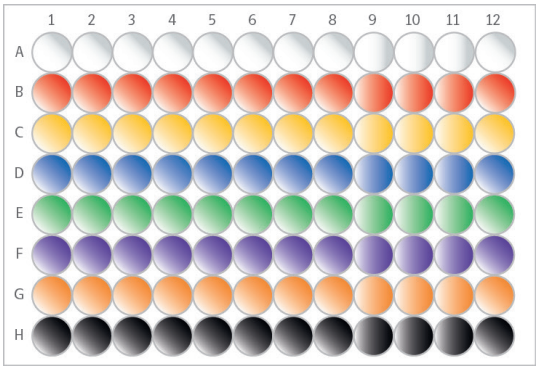
- PCR Plates 1–4, prepared in previous step
- 1x 96-well PCR plate (skirted)
- 8x Eppendorf tubes (1.5 mL)
- Hamilton STAR pipette tips (5 racks of 50 µL filtered conductive Hamilton Tips)
- Rack for 24 tubes, for 24 Safe-Lock tubes, 1.5/2.0 mL
- Waste bag
- Manual pipette (100 µL)
- Filter pipette tips
- Adhesive films

Before you start

- Thaw the PCR Plates 1–4 at room temperature if frozen. Allow to reach room temperature if stored at 4 ºC.
- Mark the 96-well PCR plate: "PCR Pooling Plate".
- Mark the eight microcentrifuge tubes: "PCR [1–8]".

Instructions: Automatic pooling

1. Open the Hamilton Run Control Application.
2. In the application, select the protocol `Olink_ExploreHT_PCRPooling`.
3. Click **Start** to initialize the instrument and run the protocol.
4. Make sure that the PCR Plates are properly sealed. Vortex the plates s without adaptor, and spin down at 400–1,000 x g for 1 minute.
5. Inspect the wells of the PCR Plates to make sure that no liquid has evaporated and that there are no bubbles in the wells. Remove the seals.
6. Pull out the carriers to the load position and prepare the STAR deck according to the software instructions.
   - Place PCR Plates on the carriers.
   - Place tubes in the tube rack carrier.
   - Carefully remove the adhesive films.
   - Fill tip carriers in the selected positions.
7. Select the correct input for the four source plates and click **Continue**.
8. Select the populated tip positions and click **Ok** to automatically load the carriers and begin the run.
   Result:
   - *STAR scans the tips automatically, pool 4 µL from each well of the PCR plates into the PCR Pooling Plate, keeping blocks separate in different rows. It will pause to allow for mixing, then pool subsequent rows to tubes.*
   - *20 µLfrom each well will be transferred to 1 Eppendorf tube per block.*



9. Once the protocol is finished, remove the PCR Pooling Plate from the deck. Inspect the PCR Pooling Plate to ensure that every applicable well contains the same amount of liquid. Discard the PCR Pooling Plate.
10. Remove the PCR Plates from the deck. Seal the plates with a new adhesive film and store at -20 ºC for potential reruns.
11. Close the Hamilton Run Control software and shut down the STAR.
12. Vortex PCR Tubes 1–8 and spin down briefly.
13. Continue to *11. Library purification* or store the PCR Tubes at 4 ˚C until use (the same day).

> **SAFE STOPPING POINT:** *The PCR Tubes can be stored at -20 ˚C for up to 2 weeks.*

Instructions: Manual pooling

1. Open the Hamilton Run Control Application.
2. In the application, select the protocol `Olink_ExploreHT_PCRPooling`.
3. Click **Start** to initialize the instrument and run the protocol.
4. Make sure that PCR Plates are properly sealed. Vortex the plates without adaptor, and spin down at 400–1,000 x g for 1 minute.
5. Inspect the wells of PCR Plates to make sure that no liquid has evaporated and that there are no bubbles in the wells. Remove the seals.
6. Pull out the carriers to the load position and prepare the STAR deck according to the software instructions.
   - Place PCR Plates on the carriers.
   - Place tubes in the tube rack carrier.
   - Carefully remove the adhesive films.
   - Fill tip carriers in the selected positions.
7. Select the correct input for the four source plates and click **Continue**.
8. Select the populated tip positions and click **Ok** to automatically load the carriers and begin the run.
   Result:
   STAR scans the tips automatically, pool 4 µL from each well of the PCR plates into the PCR Pooling Plate, keeping blocks separate in different rows.
9. Remove the PCR Pooling Plate from the worktable. Seal the plate with a new adhesive film, vortex the plate and spin down at 400 x g for 1 minute. Inspect the PCR Pooling Plate to ensure that every applicable well contains the same amount of liquid.
10. Carefully remove the adhesive film from the PCR Pooling Plate.
11. Manually transfer the pooled PCR products from the PCR Pooling Plate to the microcentrifuge tube(s) according to the table below. Use a single-channel pipette and **forward pipetting**, and change pipette tip after each well.

Volume (µL) /well	From column	To tube	Final volume in the tube (µL)
30	1	PCR 1	240
30	2	PCR 2	240
30	3	PCR 3	240
30	4	PCR 4	240
30	5	PCR 5	240
30	6	PCR 6	240
30	7	PCR 7	240
30	8	PCR 8	240

12. Vortex the PCR Tubes and spin down briefly.
13. Remove the PCR Plates from the deck. Seal the plates with a new adhesive film and store at -20 ºC for potential reruns.
14. Close the Hamilton Run Control software and shut down the STAR.
15. Continue to *11. Library purification* or store the PCR Tubes at 4 ˚C until use (the same day).

> **SAFE STOPPING POINT:** *The PCR Tubes can be stored at -20 ˚C for up to 2 weeks.*

9.Pool PCR products using ep*Motion*®

During this step, PCR products from the four PCR Plates are pooled by block into one PCR Pooling Plate using epMotion. The libraries are then transferred from the PCR Pooling Plate to one microcentrifuge tube per block, either automatically or manually. Each tube contains PCR products corresponding to one block from the 192 samples.

This section includes two different instructions: one for automatic pooling and one for semi-manual pooling.

Prepare bench

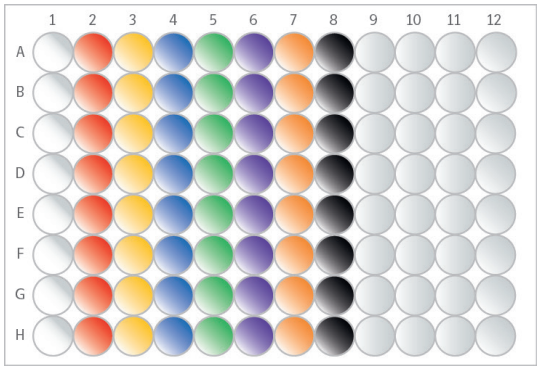
- PCR Plate 1–4, prepared in previous step
- 1x 96-well PCR plate (skirted)
- 8x Eppendorf tubes (1.5 mL)
- epT.I.P.S. Motion pipette tips (2boxes á 10 µL))
- epT.I.P.S. Motion pipette tips (50 µL)
- TS 50 single-channel dispensing tool (for automatic pooling)
- Manual pipette (100 µL or 200 µL) (for manual pooling)
- ep*Motion* TM 10-8 eight-channel dispensing tool
- ep*Motion* TS 50 single-channel dispensing tool
- Rack for 24 tubes, for 24 Safe-Lock tubes, 1.5/2.0 mL (for automatic pooling)
- Waste bag
- Manual pipette (100 µL)
- Filter pipette tips
- Adhesive films

Before you start

- Thaw PCR Plate 1–4 at room temperature if frozen. Allow to reach room temperature if stored at 4 ºC.
- Mark the 96-well PCR plate: "PCR Pooling Plate".
- Mark the eight microcentrifuge tubes: "PCR [1–8]".

Instructions: Automatic pooling

1. Open the EpBlue Application Runner.
2. In the application library, select user and the protocol `Olink_ExploreHT_PCRPooling`.
3. When the ID number of the instrument is shown in the software, click **Next** to continue.
4. Make sure that the PCR Plates are properly sealed. Vortex the plates without adaptor, and spin down at 400–1,000 x g for 1 minute.
5. Inspect the wells of the PCR Plate to make sure that no liquid has evaporated and that there are no bubbles in the wells. Remove the seals.
6. Prepare the ep*Motion* worktable according to the software instructions.
7. Click **Next** in the software, then enter the following settings:
   - Under **Volume settings:** Activate Input volumes manually.
   - Under **Worktable settings**: Deactivate Check tube lid removed.
8. Click **Next** until a Run button appears. Click **Run** to start the protocol.
   Result:
   - *epMotion pools 3 µL from each well of the same row in each PCR plate into a single column of the PCR Pooling Plate, keeping blocks separate in different columns.*
   - *30 uL from each well will be transferred to 1 Eppendorf tube.*

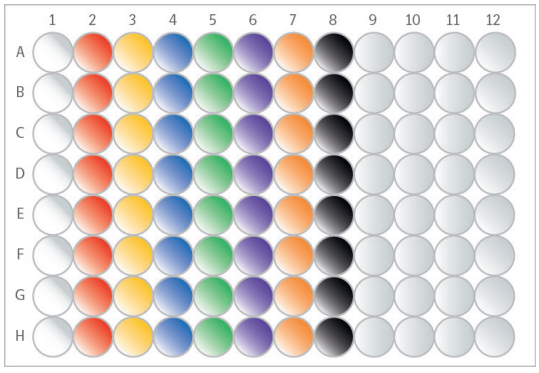


9. Once the protocol is finished, remove the PCR Pooling Plate from the worktable. Inspect the PCR Pooling Plate to ensure that every applicable well contains the same amount of liquid. Discard the PCR Pooling Plate.
10. Remove the PCR Plates from the worktable. Seal the plates with a new adhesive film and store at -20 ºC for potential reruns.
11. Clear the ep*Motion* and shut it down.
12. Vortex PCR Tubes 1–8 and spin down briefly.
13. Continue to *11. Library purification* or store the PCR Tubes at 4 °C until use (the same day).

> **SAFE STOPPING POINT:** *The PCR Tubes can be stored at -20 °C for up to 2 weeks.*

Instructions: Manual pooling

1. Open the EpBlue Application Runner.
2. In the application library, select user and the protocol `Olink_ExploreHT_PCRPooling_manual`.
3. When the ID number of the instrument is shown in the software, click **Next** to continue.
4. Make sure that the PCR Plates are properly sealed. Vortex the plates without adaptor, and spin down at 400–1,000 x g for 1 minute.
5. Inspect the wells of the PCR Plates to make sure that no liquid has evaporated and that there are no bubbles in the wells. Remove the seals.
6. Prepare the ep*Motion* worktable according to the software instructions.
7. Click **Next** in the software, then enter the following settings:
   - Under **Volume settings**: Activate Input volumes manually.
   - Under **Worktable settings**: Deactivate Check tube lid removed.
8. Click **Next** until a Run button appears. Click **Run** to start the protocol.
   Result:
   epMotion pools 3 µL from each well of the same row in each PCR plate into a single column of the PCR1 Pooling Plate, keeping blocks separate in different columns.



9. Remove the PCR Pooling Plate from the worktable. Seal the plate with a new adhesive film, vortex the plate and spin down at 400 x g for 1 minute. Inspect the PCR Pooling Plate to ensure that every applicable well contains the same amount of liquid.
10. Carefully remove the adhesive film from the PCR Pooling Plate. Make sure that every applicable well contain the same amount of liquid.
11. Manually transfer the pooled PCR products from the PCR Pooling Plate to the microcentrifuge tube(s) according to the table below. Use a single-channel pipette and **forward pipetting**, and change pipette tip after each well.

Volume (µL) /well	From column	To tube	Final volume in the tube (µL)
30	1	PCR 1	240
30	2	PCR 2	240
30	3	PCR 3	240
30	4	PCR 4	240
30	5	PCR 5	240
30	6	PCR 6	240
30	7	PCR 7	240
30	8	PCR 8	240

12. Vortex the tube(s) and spin down briefly.
13. Discard the PCR Pooling Plate.

14. Remove the PCR Plates from the worktable. Seal the plates with a new adhesive film and store at -20 ºC for potential reruns.
15. Clear the ep*Motion* and shut it down.
16. Vortex the PCR Tubes and spin down briefly.
17. Continue to *11. Library purification* or store the PCR Tubes at 4 ˚C until use (the same day).

> **SAFE STOPPING POINT:** *The PCR Tubes can be stored at -20 ˚C for up to 2 weeks.*

10.Pool PCR products using Formulatrix® F.A.S.T.™

During this step, PCR products from the four PCR Plates are pooled by block into one PCR Pooling Plate using Formulatrix F.A.S.T. The libraries are then transferred from the PCR Pooling Plate to tubes. Each tube contains PCR products corresponding to one block from the 192 samples.

This section includes two different instructions: one for automatic pooling to an 8-well strip tube and one for semi-manual pooling to 1.5 mL tubes.

Prepare bench

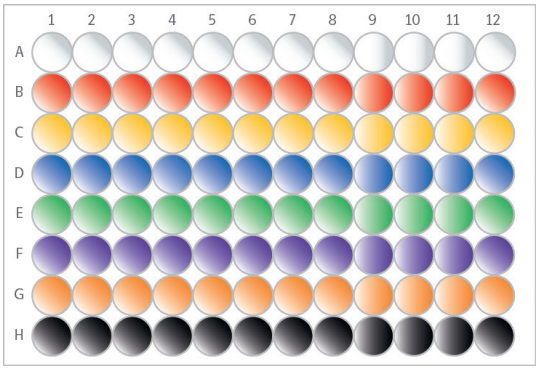
- PCR Plates 1–4, prepared in previous step
- 1x 96-well PCR plate (skirted)
- 1x 8-well strip tube
- 1x 8-well strip tube cap
- 8x Eppendorf tubes (1.5 mL)
- 104x F.A.S.T. Disposable Pipette Tips
- F.A.S.T. Plate adapter for 0.2 mL PCR Strip Tubes, 96 well format.
- Waste bag (for semi-manual pooling)
- Manual pipette (100 µL)
- Filter pipette tips
- Adhesive films

Before you start

- Thaw the PCR Plates 1–4 at room temperature if frozen. Allow to reach room temperature if stored at 4 ºC.
- Mark the 96-well PCR plate: “PCR Pooling Plate”.
- Mark the wells of the strip tube 1-8.
- Mark the eight microcentrifuge tubes: “PCR [1–8]”.
- Switch on the F.A.S.T. system and open the software.

Instructions: Automatic pooling

1. Make sure that the PCR Plates are properly sealed. Vortex the plates without adaptor, and spin down at 400–1,000 x g for 1 minute.
2. Inspect the wells of the the PCR Plates to make sure that no liquid has evaporated and that there are no bubbles in the wells. Remove the seals.
3. Open the protocol `Olink_Explore_HT_Plate_Pooling`.
4. Arrange the F.A.S.T. deck according to the layout in the protocol.
   - Place PCR Plates 1–4 on the tray.
   - Place PCR Pooling Plate on the tray.
   - Remove lid and place tips on the tray.
5. Click **Start** to begin the run.
   Result:
   - *F.A.S.T. pools 3 µL from each well of the same block into one row of the 96-well plate, each block in a different row.*

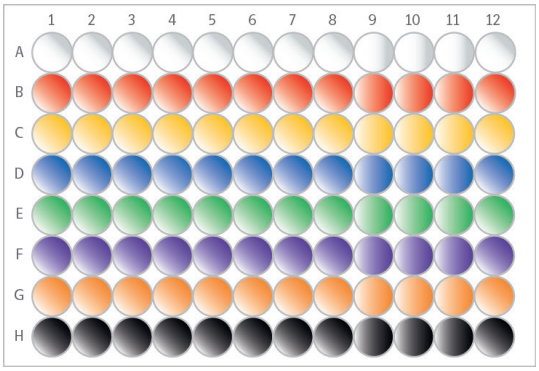


6. Once the protocol is finished, remove the PCR Pooling Plate from the deck. Seal the plate with a new adhesive film, vortex and spin down at 400 x g for 1 minute. Inspect the PCR Pooling Plate to ensure that every applicable well contains the same amount of liquid.
7. Remove the the PCR Plates from the deck. Seal the plates with a new adhesive film and store at -20 ºC for potential reruns.
8. Open the protocol `Olink_Explore_HT_Tube_Pooling`.
9. Arrange the F.A.S.T. deck according to the layout in the protocol.
   - Remove seal from PCR Pooling Plate and place on the tray.
   - Place 8-well strip tube in column 1 of the strip tube adapter and place on the tray.
   - Remove lid and place tips on the tray.
10. Click **Start** to begin the run.
    Result:
    - *F.A.S.T. pools 11 µL from each well of the same row of the PCR Pooling Plate into a single well of the PCR 1–8 strip tube, each block in a different well.*
11. Cap and vortex the PCR 1–8 strip tube and spin down briefly. Transfer each pooled library to its corresponding 1.5 mL microcentrifuge tube.
12. Clear the F.A.S.T. and shut it down.
13. Continue to *11. Library purification* or store the PCR Tubes at 4 °C until use (the same day).

> **SAFE STOPPING POINT:** *The PCR Tubes can be stored at -20 °C for up to 2 weeks.*

Instructions: semi-manual pooling

1. Make sure that the PCR Plates are properly sealed. Vortex the plates without adaptor, and spin down at 400–1,000 x g for 1 minute.
2. Inspect the wells of the PCR Plates to make sure that no liquid has evaporated and that there are no bubbles in the wells. Remove the seals.
3. Open the protocol `Olink_Explore_HT_Plate_Pooling`.
4. Arrange the F.A.S.T. deck according to the layout in the protocol.
   - Place PCR1 Plates on the tray.
   - Place PCR Pooling Plate on the tray.
   - Remove lid and place tips on the tray.
5. Click **Start** to begin the run.
   Result:
   - *F.A.S.T. pools 3 µL from each well of the same block into one row of the 96-well plate, each block in a different row.*



6. Once the protocol is finished, remove the PCR Pooling Plate from the deck. Seal the plate with adhesive film. Vortex the plate and spin down at 400 x g for 1 minute. Inspect the PCR Pooling Plate to ensure that every applicable well contains the same amount of liquid.
7. Remove the PCR Plates from the deck. Seal the plates with adhesive films and store at -20 ºC for potential reruns.
8. Clear the F.A.S.T. and shut it down.
9. Carefully remove the adhesive film from the PCR Pooling Plate. Make sure that every well contains the same amount of liquid.
10. Manually pipette PCR products from the PCR Pooling Plate to microcentrifuge tubes according to the table below. Use a single-channel pipette and **forward pipetting**, and change pipette tips after each well.

Volume (µL) /well	From row	To tube	Final volume in the tube (µL)
30	A	PCR 1	360
30	B	PCR 2	360
30	C	PCR 3	360
30	D	PCR 4	360
30	E	PCR 5	360
30	F	PCR 6	360
30	G	PCR 7	360
30	H	PCR 8	360

11. Vortex the tubes and spin down briefly.

12. Continue to *11. Library purification* or store the PCR Tubes at 4 °C until use (the same day).

> **SAFE STOPPING POINT:** *The PCR Tubes can be stored at -20 °C for up to 2 weeks.*

11.Library purification

During this step, the Olink Libraries are purified using magnetic beads and the eluates are transferred into one new microcentrifuge tube per block.

Prepare bench

- PCR Tubes 1–8, prepared in previous step
- Agencourt AMPure XP bottle
- 96% Ethanol (EtOH)
- Milli-Q water
- DynaMag-2 Magnet
- Timer
- 16x Microcentrifuge tubes (1.5 mL)
- 1x Falcon tube (15 mL)
- Manual pipettes (100 µL, 1,000 µL, and 5,000 µL)
- Filter pipette tips

Before you start

- Let the refrigerated AMPure XP bottle reach room temperature.
- Mark eight new microcentrifuge tubes: “BP [1–8]” (for “Bead Purification”).
- Mark eight new microcentrifuge tubes: “Lib [1–8]” (for "Library")
- Mark the Falcon tube: "70% EtOH".

Instructions

1. Prepare fresh 70% EtOH:

Addition order	Reagent	Volume (mL)
1	Milli-Q water	2.5
2	96% EtOH	6.5
	Total (70% EtOH)	9

2. Shake and vortex the AMPure XP bottle vigorously to resuspend the magnetic beads.
3. Transfer 80 µL from the AMPure XP bottle to each BP tube.
4. Transfer 50 µL from each PCR Tube to the corresponding BP Tube (PCR 1 to BP 1, PCR 2 to BP 2, and so on).

NOTE: *Store the PCR Tubes at -20 °C in case the purification needs to be repeated.*

5. Pipette-mix 10 times to thoroughly mix the Libraries with the beads. Change pipette tip between every tube.
6. Start the timer after the last tube has been mixed. Incubate the BP Tubes for 5 minutes at room temperature.
7. Place the Eppendorf tube on the Magnetic stand for 2 min to separate the beads from the solution.
8. With the tubes still on the magnetic stand, carefully open the lids and discard 125 µL supernatant and leave 5 µL behind. Use a single-channel pipette. Do not disturb the beads.

NOTE: *The appearance of the bead palettes may differ between block 1–4 and 5–8.*

9. With the tubes still on the magnetic stand, wash the beads:
    a. Add 500 µL of 70% EtOH to every BP Tube. Pipette onto the opposite wall from the beads.

NOTE: *Make sure not to disturb the beads.*

    b. Leave the tubes to incubate for 30 seconds.
    c. Using a single-channel pipette, aspirate the EtOH, without disturbing the beads. Discard the EtOH.
    d. Repeat steps a–c for a total of two washes.

IMPORTANT: *Make sure that no EtOH remains in the BP Tubes after this step. Use a smaller pipette to remove any residual EtOH.*

10. Leave the tubes with the lids open on the magnetic stand for 2 minutes for the beads to air dry.
11. Close the tubes and remove them from the magnetic stand.
12. Add 50 µL of Milli-Q water to each BP Tube and pipette-mix 10 times towards the beads to resuspend them. Change pipette tip between each tube.
13. Incubate the tubes for 2 minutes at room temperature.
14. Place the BP Tubes on the magnetic stand and leave them for 1 minute to separate the beads from the eluted Library solution.
15. With the BP Tubes still on the magnetic stand, transfer 45 µL of eluate from each BP Tubes to the corresponding Lib Tubes (BP1 to Lib 1, BP to Lib 2, and so on).

IMPORTANT: *Make sure not to disturb or aspirate the beads.*

16. Discard the BP Tubes.
17. Continue to *12. Quality control*.

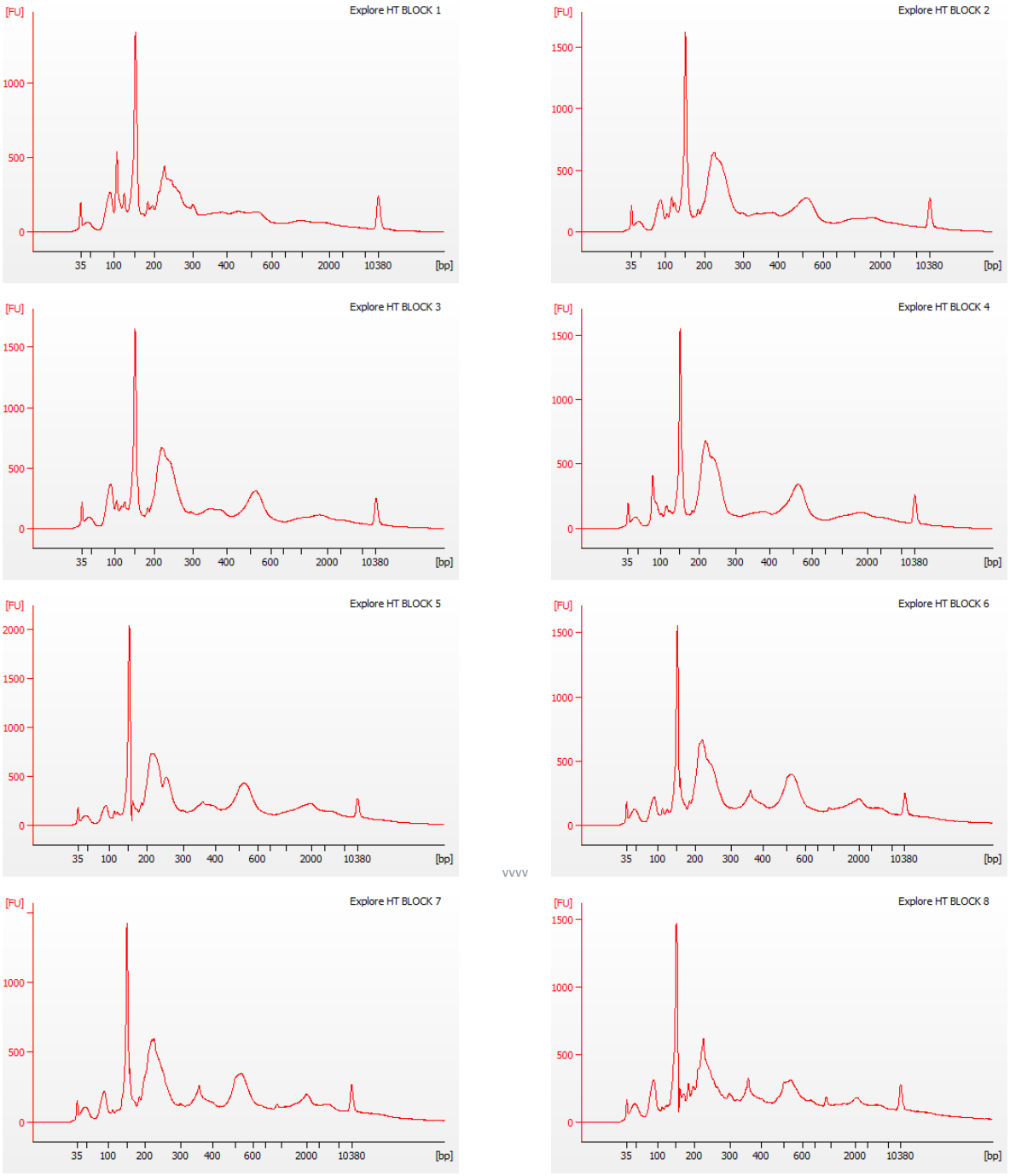
SAFE STOPPING POINT: *The Lib Tubes can be stored at -20 °C for up to 4 weeks.*

12. Quality control

During this step, the eight purified Olink Libraries are quality controlled on the Bioanalyzer using the High Sensitivity DNA kit according to manufacturer instructions.

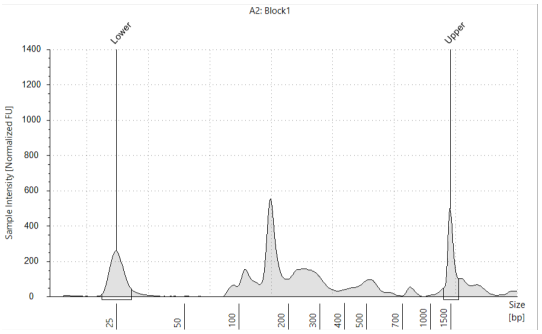
The electropherograms below display typical results for Olink Explore HT Library, one electropherogram per block. The purified Olink Library appears as a single or double peak around 150 bp. Peaks of larger sizes represents bubble products and do not have any impact on sequencing results, see emea.support.illumina.com/bulletins/2019/10/bubble-products-in-sequencing-libraries--causes--identification-.html for more information.

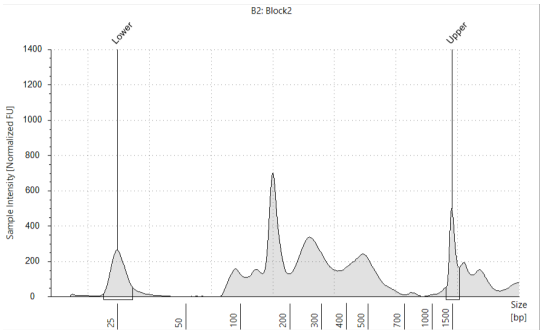
Electropherograms Bioanalyzer

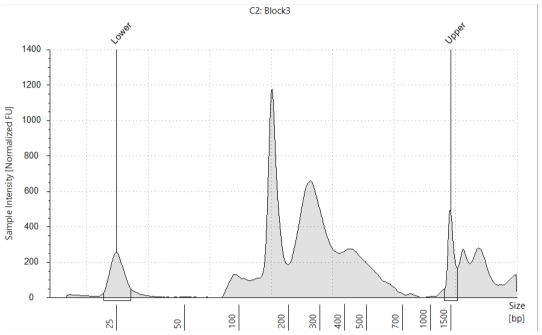


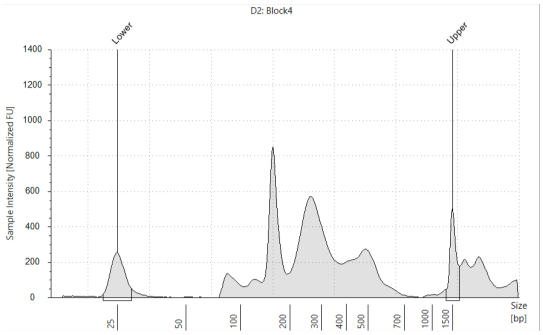
vvvv

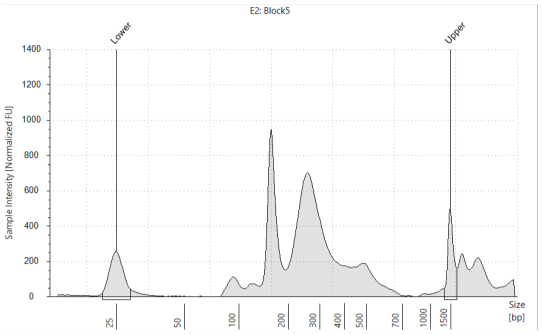
Electropherograms TapeStation

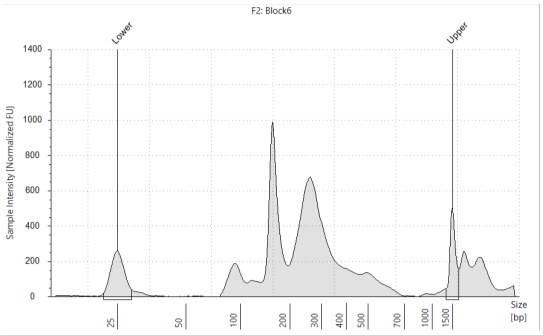


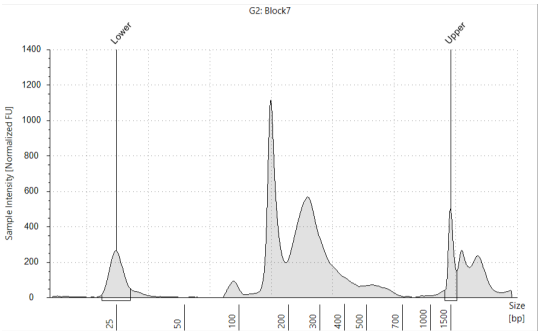


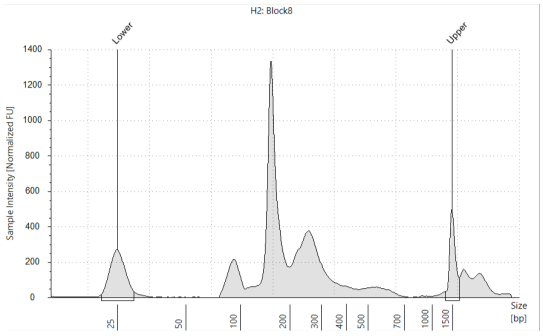












For expected results of a successful ladder run, refer to the manufacturer's manual. If the electropherograms do not look like expected, please contact *support@olink.com* for guidance.

13. Library pooling

Before you start

- Mark one new microcentrifuge tube: “PL” (for "Pooled Library").

Instructions

1. Transfer 10 µl from each of the 8 Lib Tubes to the PL tube.
2. Vortex the PL Tube and spin down briefly.

> **SAFE STOPPING POINT:** *The PL Tube can be stored at -20 °C for up to 4 weeks.*

14. Next generation sequencing

Next generation sequencing is performed using either of the following Illumina® instrument:

- NovaSeq™ 6000
- NovaSeq™ X Plus

As the workflow differs between instruments, refer to the applicable sequencing user manual for instructions on how to sequence Olink Libraries in *3.1 Olink documentation.*

15.Laboratory instructions for 86 samples

This section provides instructions on how to perform each step of the Olink Explore HT workflow for the preparation of up to 86 samples distributed across one 96-well Sample Plate.

Most steps are performed in a similar way as for the standard workflow. Changes specific to the 86-sample workflow are described in this section.

All deck positions are the same as for the standard protocols.

15.1 Required Olink® Explore HT reagents

The following reagents are intended for the preparation of 172 samples on two separate occasions (86 samples at a time), using the 86-sample workflow:

- Olink® Explore HT Reagent Kit (98100) (refer to *2. Olink® Explore HT Reagent Kit contents*)
- Olink® Explore HT Sample Prep Reagents (98101)

The Probe reagents included in the Olink® Explore HT Reagent Kit (98100) will be stored after preparing the first set of 86 samples and reused for preparation of the the second set of 86 samples. The Probe reagents can be stored at 4 °C for up to 3 months after opening.

15.1.1 Content of Olink® Explore HT Sample Prep Reagents

Content of Olink® Explore HT Sample Prep 2 (98004)

Art. No	Component	Cap color	Volume	Storage
88003	Olink® Explore HT PCR Additive	Brown	4,000 uL	4 °C
87004	Olink® Explore Incubation solution	Brown	1,400 uL	

Content of Olink® Explore HT Sample Prep (98023)

Art. No	Component	Cap color	Volume	Storage
88001	Olink® Explore HT PCR Solution	Natural	4,000 µL	-20 °C
88006	Olink® Explore HT PCR Enzyme A	Blue*	825 µL	
88007	Olink® Explore HT PCR Enzyme B	Violet*	115 µL	
87009	Olink® Explore Negative Control	Red	150 µL	
88016	Olink® Explore Index Plate C	Green	15 µL	
84032	Olink® Target 96/Explore Sample Diluent	Natural	2x 13 mL	

Content of Olink® Explore HT Controls (98003)

Art. No	Component	Cap color	Volume	Storage
87010	Olink® Explore Plate Control	Green	160 µL	-80 °C
88011	Olink® Explore Sample Control	Yellow	90 µL	

15.2 Preparations

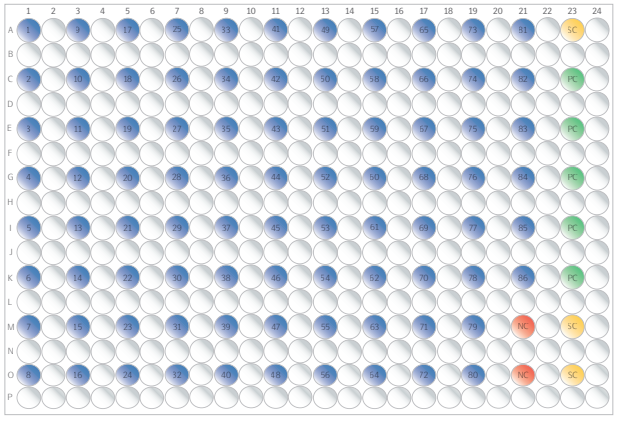
Perform this step according to standard instructions, refer to *2. Preparations.* Prepare only one Sample Plate.

15.3 Prepare Sample Source Plate

Perform this step according to standard instructions, refer to *3. Prepare Sample Source Plate*, with the modifications described below. Use only one Sample Plate, prepared in previous step.

Use the following F.A.S.T. protocol:
`0_Olink_ExploreHT_86_Samples_SS_Plate_Creation`

The protocol gives a Sample Source Plate with the following layout:



15.4 Sample Dilution

Perform this step according to standard instructions, refer to *4. Sample Dilution,* with the modifications described below.

15.4.1 Perform Sample Dilution

Use the following F.A.S.T. protocol:
`1_Olink_ExploreHT_86_Samples_SampleDilution`

15.5 Incubation

Perform this step according to standard instructions, refer to *5. Incubation,* with the modifications described below.

15.5.1 Prepare Reagent Source Plate

Prepare the eight incubation mixes in the PCR Strip according to standard instructions, but with the following volumes:

Addition order	Reagent	Volume (µL)
1	Incubation solution	80
2	Frw probes	10
3	Rev probes	10

When finished, inspect the wells to make sure that they contain the expected volume of 100 µL.

Transfer only 10 µL from the PCR strip to each well in the Reagent Source Plate.

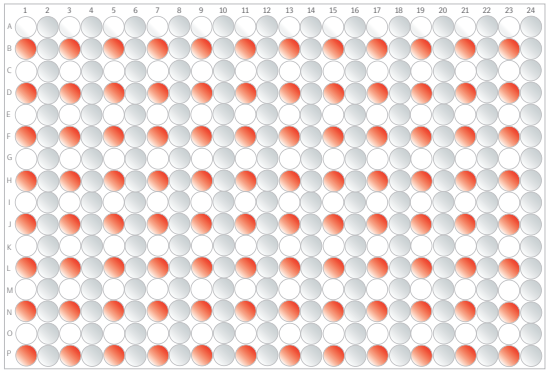
IMPORTANT: *Store the Probe reagents at 4 °C as they will be reused when preparing the second set of 86 samples. The Probe reagents can be stored at 4 °C for up to 3 months after opening.*

15.5.2 Perform incubation

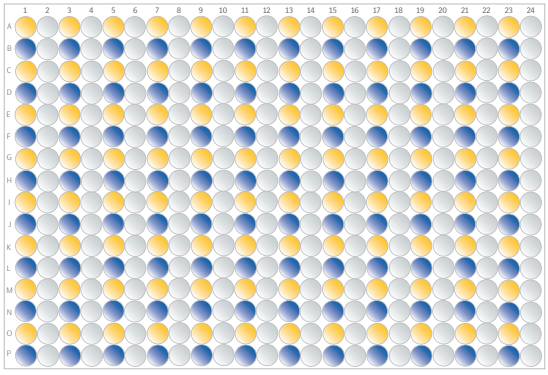
Use the following F.A.S.T. protocols:

```
2_Olink_ExploreHT_86_Samples_Incubation_ReagentTransfer
3_Olink_ExploreHT_86_Samples_Incubation_SampleTransfer_Block_1-4
4_Olink_ExploreHT_86_Samples_Incubation_SampleTransfer_Block_5-8
```

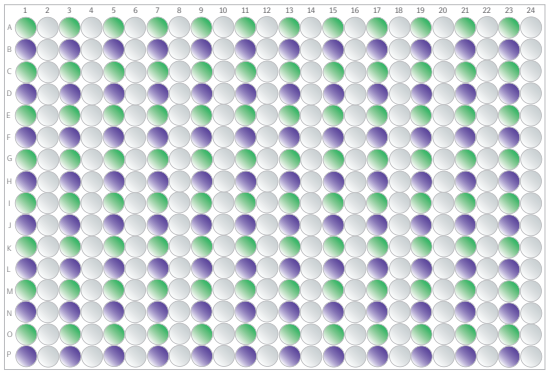
Incubation Plate Block 1–2



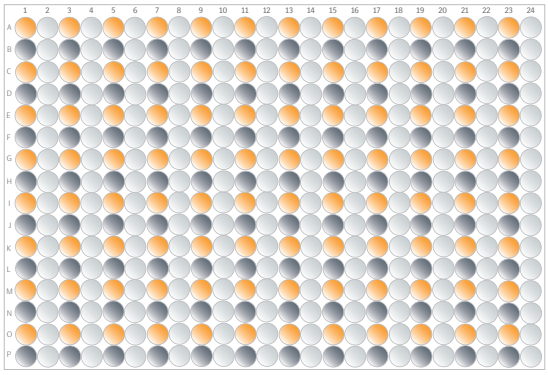
Incubation Plate Block 3–4



Incubation Plate Block 5–6



Incubation Plate Block 7–8



15.6 Prepare for day 2

Perform this step according to standard instructions, refer to *6. Prepare for day 2.*

15.7 Index and PCR setup

Perform this step according to standard instructions, refer to *7. Index and PCR setup* with the modifications described below.

15.7.1 Index dispensation

Use the protocol:
`5_Olink_ExploreHT_86_Samples_IndexTransfer`

NOTE: *Make sure that you are using **v3** of the protocol.*

Index dispensation using Index Plate A and B
The index dispensation only requires Index Plate A.

Use the protocol:
`5_Olink_ExploreHT_86_Samples_IndexTransfer`

NOTE: *Make sure that you are using **v2** of the protocol.*

15.8 Pool PCR Products using Hamilton Microlab® STAR

Perform this step according to standard instructions, refer to *8. Pool PCR products using Hamilton Microlab® STAR,* with the following modifications:

Use the following protocol:
`Pooling`

Select 86 as the number of samples at the initial prompt, and the number of PCR plates to process.

15.9 Pool PCR Products using ep*Motion*®

Perform this step according to standard instructions, refer to *9. Pool PCR products using epMotion®,* with the following modifications:

Use the following protocol:
`Olink_ExploreHT_86_Samples_PCRPooling`

15.10 Pool PCR Products using Formulatrix® F.A.S.T.™

Perform this step according to standard instructions, refer to *10. Pool PCR products using Formulatrix® F.A.S.T.™,* with the following modifications:

Use the following protocol:
`Olink_ExploreHT_86_Samples_PCRPooling`

15.11 Library purification

Perform this step according to standard instructions, refer to *11. Library purification.*

15.12 Quality control

Perform this step according to standard instructions, refer to *12. Quality control.*

15.13 Library pooling

Perform this step according to standard instructions, refer to *13. Library pooling.*

15.14 Next generation sequencing

Perform this step according to standard instructions, refer to *14. Next generation sequencing.*

16. Revision history

Version	Date	Description
4.0	2024-10-07	New - Part 2: laboratory section using Labtech Mosquito HT removed from the Olink® Explore Overview User Manual. *7.1, 15.1.1,* and *15.7.1* Index Plate A and B replaced by Index Plate C. Editorial changes.

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1548, v1.0, 2024-10-07