Olink®

Olink® Explore 4 x 384

User Manual

1189, v3.1, 2023-06-28

Document note

The Olink® Explore User manual, doc nr 1153, is obsolete, and has been replaced by the following documents:

- Olink® Explore Overview User Manual, doc nr 1187
- Olink® Explore 384 User Manual, doc nr 1188
- Olink® Explore 4 x 384 User Manual, doc nr 1189
- Olink® Explore 1536 & Expansion User Manual, doc nr 1190
- Olink® Explore 3072 User Manual, doc nr 1191
- Olink® Explore Sequencing using NextSeq 550 User Manual, doc nr 1192
- Olink® Explore Sequencing using NextSeq 2000 User Manual, doc nr 1193
- Olink® Explore Sequencing using NovaSeq 6000 User Manual, doc nr 1194

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1. Introduction

1.1 Intended use

Olink® Explore is a multiplex immunoassay platform for human protein biomarker discovery,, based on Olink's proprietary PEA™ technology. The product is intended for Research Use Only, and 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 4 x 384 Reagent Kits, refer to *2. Laboratory instructions for 96 samples using Hamilton Microlab® STAR* and *3. Laboratory instruction for 96 samples using Eppendorf epMotion®*
- Olink® Explore 4 x 384 Reagent Kits (384 samples), refer to *4. Laboratory instruction for 384 samples.*

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

Prior to starting the laboratory workflow, consult the Olink® Explore Overview User Manual for an introduction to the Explore platform, including information about equipment and documentation needed, an overview of the workflow, as wells as laboratory guidelines.

For instructions on how to prepare and sequence Olink® Libraries using Illumina® NextSeq™ 550 , NextSeq™ 2000 or NovaSeq™ 6000, refer to the applicable Olink® Explore Sequencing User Manual.

For data processing and analysis of the Olink® Explore sequence results, refer to the Olink® NPX Explore User Manual or the Olink® MyData Cloud User Manual.

1.3 Reagents included

1.3.1 Olink® Explore 4 x 384 Reagent Kit

Olink® Explore 4 x 384 Reagent Kit consists of a mixed 4-pack of Olink® Explore 384 panels in any combination, as described in *Table 1*. It contains reagents for up to 90-360 user samples and 6-24 Olink controls, and is intended for the simultaneous preparation of the four panel libraries. The number of samples and controls to be run depends on how the panels are combined. See *2.1.3 Prepare the samples* for more details.

Table 1. Content of Olink® Explore 4 x 384 Reagent Kit.

Art. No	Products included	Components included	Cap color	Storage
Combination of any 4 of the following Probe Kits:		**Each 384 Probe Kit contains:**		+4 °C
97500	Olink® Explore 384 Inflammation Probe Kit	Olink® Explore 384 [Panel] Frw probes A	White	
97600	Olink® Explore 384 Oncology Probe Kit	Olink® Explore 384 [Panel] Rev probes A	White	
97700	Olink® Explore 384 Cardiometabolic Probe Kit	Olink® Explore 384 [Panel] Frw probes B	Purple	
97800	Olink® Explore 384 Neurology Probe Kit	Olink® Explore 384 [Panel] Rev probes B	Purple	
97510	Olink® Explore 384 Inflammation II Probe Kit	Olink® Explore 384 [Panel] Frw probes C	Blue	
97610	Olink® Explore 384 Oncology II Probe Kit	Olink® Explore 384 [Panel] Rev probes C	Blue	
97710	Olink® Explore 384 Cardiometabolic II Probe Kit	Olink® Explore 384 [Panel] Frw probes D	Black	
97810	Olink® Explore 384 Neurology II Probe Kit	Olink® Explore 384 [Panel] Rev probes D	Black	
97041	Olink® Explore Sample Prep 1.0	Olink® Explore Negative Control	Red	-20 °C
		Olink® Explore PCR 1 Solution (x2)	Transparent	
		Olink® Explore PCR 1 Enzyme	Black	
		Olink® Explore PCR 2 Solution	White	
		Olink® Explore PCR 2 Enzyme	Yellow	
		Olink® Explore Index Plate 1		
97011	Olink® Explore Sample Prep 1.1			
97004	Olink® Target96/Explore Sample Diluent (x2)	Olink® Target96/Explore Sample Diluent	Transparent	-20 °C
97003	Olink® Explore Sample Prep 2	Olink® Explore Incubation Solution	Brown	+4 °C
		Olink® Explore PCR 1 Enhancer (x2)	Black	
97002	Olink® Explore Plate Control	Olink® Explore Plate Control	Green	-80 °C

If the four libraries are not prepared at the same time, additional Olink Explore Sample Prep Reagents will be required (*Table 2*). One Olink Explore Sample Prep Reagents is needed for each additional library preparation run.

Table 2. Content of Olink® Explore Sample Prep Reagents.

Art. No	Products included	Components included	Cap color	Storage
97041	Olink® Explore Sample Prep 1.0	Olink® Explore Negative Control Olink® Explore PCR 1 Solution (x2) Olink® Explore PCR 1 Enzyme Olink® Explore PCR 2 Solution Olink® Explore PCR 2 Enzyme Olink® Explore Index Plate 1	Red Transparent Black White Yellow –	-20 °C
97004	Olink® Target96/Explore Sample Diluent	Olink® Target96/Explore Sample Diluent	Transparent	-20 °C
97003	Olink® Explore Sample Prep 2	Olink® Explore Incubation Solution Olink® Explore PCR 1 Enhancer (x2)	Brown Black	+4 °C
97002	Olink® Explore Plate Control	Olink® Explore Plate Control	Green	-80 °C

1.3.2 Olink® Explore 4 x 384 Reagent Kit (384 samples)

Olink® Explore 4 x 384 Reagent Kit (384 samples) consists of a mixed 4-pack of Olink® Explore 384 panels in any combination, as described in *Table 3*. It contains reagents for preparing and pooling up to 360 user samples and 24 Olink controls, and is intended for the simultaneous preparation of the four panel libraries.

Table 3. Content of Olink® Explore 4 x 384 Reagent Kit (384 samples)

Art. No	Products included	Components included	Cap color	Storage
Combination of any 4 of the following Probe Kits (4x):		**Each 384 Probe Kit contains:**		+4 °C
97500	Olink® Explore 384 Inflammation Probe Kit	Olink® Explore 384 [Panel] Frw probes A	White	
97600	Olink® Explore 384 Oncology Probe Kit	Olink® Explore 384 [Panel] Rev probes A	White	
97700	Olink® Explore 384 Cardiometabolic Probe Kit	Olink® Explore 384 [Panel] Frw probes B	Purple	
97800	Olink® Explore 384 Neurology Probe Kit	Olink® Explore 384 [Panel] Rev probes B	Purple	
97510	Olink® Explore 384 Inflammation II Probe Kit	Olink® Explore 384 [Panel] Frw probes C	Blue	
97610	Olink® Explore 384 Oncology II Probe Kit	Olink® Explore 384 [Panel] Rev probes C	Blue	
97710	Olink® Explore 384 Cardiometabolic II Probe Kit	Olink® Explore 384 [Panel] Frw probes D	Black	
97810	Olink® Explore 384 Neurology II Probe Kit	Olink® Explore 384 [Panel] Rev probes D	Black	
97041	Olink® Explore Sample Prep 1.0	Olink® Explore Negative Control	Red	-20 °C
		Olink® Explore PCR 1 Solution (x2)	Transparent	
		Olink® Explore PCR 1 Enzyme	Black	
		Olink® Explore PCR 2 Solution	White	
		Olink® Explore PCR 2 Enzyme	Yellow	
		Olink® Explore Index Plate 1		
97011	Olink® Explore Sample Prep 1.1	Olink® Explore Negative Control	Red	-20 °C
		Olink® Explore PCR 1 Solution (x2)	Transparent	
		Olink® Explore PCR 1 Enzyme	Black	
		Olink® Explore PCR 2 Solution	White	
		Olink® Explore PCR 2 Enzyme	Yellow	
		Olink® Explore Index Plate 2		
97021	Olink® Explore Sample Prep 1.2	Olink® Explore Negative Control	Red	-20 °C
		Olink® Explore PCR 1 Solution (x2)	Transparent	
		Olink® Explore PCR 1 Enzyme	Black	
		Olink® Explore PCR 2 Solution	White	
		Olink® Explore PCR 2 Enzyme	Yellow	
		Olink® Explore Index Plate 3		
97031	Olink® Explore Sample Prep 1.3	Olink® Explore Negative Control	Red	-20 °C
		Olink® Explore PCR 1 Solution (x2)	Transparent	
		Olink® Explore PCR 1 Enzyme	Black	
		Olink® Explore PCR 2 Solution	White	
		Olink® Explore PCR 2 Enzyme	Yellow	
		Olink® Explore Index Plate 4		
97004	Olink® Target96/ Explore Sample Diluent (8x)	Olink® Target96/ Explore Sample Diluent	Transparent	-20 °C
97003	Olink® Explore Sample Prep 2 (4x)	Olink® Explore Incubation Solution	Brown	+4 °C
		Olink® Explore PCR 1 Enhancer (x2)	Black	
97002	Olink® Explore Plate Control (4x)	Olink® Explore Plate Control	Green	-80 °C

1.4 Associated documentation

- Olink® Explore Overview User Manual, doc nr 1187
- Olink® Explore Sequencing using NextSeq 550 User Manual, doc nr 1192
- Olink® Explore Sequencing using NextSeq 2000 User Manual, doc nr 1193
- Olink® Explore Sequencing using NovaSeq 6000 User Manual, doc nr 1194
- Olink® NPX Explore User Manual, doc nr 1078
- Olink® MyData Cloud User Manual, doc nr 1152

All relevant Olink documentation is available from the Olink website: *https://www.olink.com/*downloads.

1.5 Technical support

For technical support, contact Olink Proteomics at *support@olink.com.*

2. Laboratory instructions for 96 samples using Formulatrix F.A.S.T.™ and Hamilton Microlab® STAR

This chapter provides instructions on how to perform each step of the Olink Explore 4 x 384 laboratory workflow, using the Microlab® STAR from Hamilton Company. The four panels should be prepared simultaneously. If the panels are prepared separately, additional Olink Explore Sample Prep Reagents will be required, refer *Table 2*.

NOTE: *If running the combination CARDIO, INF, NEURO and ONC **or** the combination CARDIO II, INF II, NEURO II and ONC II it is possible to follow the instructions in Olink Explore 3072 User Manual. The instructions in Olink Explore 3072 User Manual provide a faster workflow by allowing for preparation of two incubation plates simultaneously using different instrument protocols.*

2.1 Preparations

2.1.1 Plan the study

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

- Decide the number of samples and controls required to get the data that you want from the study.
- Optional Sample Control: It is recommended to include a pooled plasma sample in duplicate on each plate. These samples are used to assess potential variation between runs and plates, for example to calculate inter-assay and intra-assay CV, as well as for troubleshooting. When including the Sample Controls, 88 to 352 user samples can be processed simultaneously.
- 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.
- Consult the Olink white paper "Strategies for design of protein biomarker studies" (*www.olink.com/whitepapers*) and the sample randomization guidelines (*www.olink.com/faq/sample-randomization*).

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

2.1.2 Important information

Panel nomenclature

As the panels can be combined in different ways, the panel names are not mentioned and are instead referred to as Panel 1, 2, 3, and 4 throughout the instructions. Before running Olink Explore 4 x 384, decide which panel shall be Panel 1, 2, 3 and 4, respectively.

IMPORTANT: *It is crucial to keep this order throughout the entire workflow. Mixing up the panels will lead to a failed run.*

Throughout the instructions, plates and tubes are marked with names and numbers, for example "Incubation Plate 1".

When using the marked plates and tubes, make sure that the number on the plate or tube corresponds to the number of the applicable panel, i.e when running Panel 1, use XX Plate 1, and when running Panel 4, use XX Plate 4.

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.

Vortexing
Correct vortexing is essential to generate reproducible results. Review instructions in the Olink Explore Overview User Manual prior to starting the laboratory workflow.

384-well PCR plates
Make sure to use the twin.tec 384-well skirted PCR plate from Eppendorf® (art nr. 0030128508) as stated in the Olink® Explore Overview User Manual. All instrument protocols have been calibrated for this specific plate. Other models should not be used.

2.1.3 Prepare the samples

During this step, samples are manually transferred to the Sample Plate(s) (*Figure 1*). The Sample Plate(s) must be used within the same day of preparation.

The number of Sample Plate(s) required depends on how the panels are combined. One Sample Plate contains a sufficient sample volume for the preparation of four panels, but when running multiple panels of the same type (e.g 4x Olink Explore Inflammation panel), separate Sample Plates are required in order to not perform the same analysis several times on the same samples.

NOTE: *The Olink Explore protocol is optimized and validated for plasma and serum samples. Contact support@olink.com for more information regarding alternative sample types.*

Prepare bench
- Sample Plates (provided by the user)
- 1-4x 96-well PCR plate(s), preferably with full skirt
- Manual pipette (0.5-10 µL)
- Filter pipette tips
- Adhesive Film
- 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.
- Mark the 96-well Sample Plate(s) with a simple alphanumeric code that you can later identify (“A, B, C”, “1, 2, 3”, or “A1, A2, A3”) using temperature-resistant labels or marker pen. Use unique sample identification names or numbers.

Instructions
1. Transfer the samples into the Sample Plate(s), according to the plate layout shown in *Figure 1*. Make sure that samples are added to every applicable well.
2. Seal the Sample Plate(s) using an adhesive film or individual seals.

3. Store the Sample Plate(s) at +4 °C if used the same day, otherwise at -80 °C.

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

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	9	17	25	33	41	49	57	65	73	81	
B	2	10	18	26	34	42	50	58	66	74	82	
C	3	11	19	27	35	43	51	59	67	75	83	
D	4	12	20	28	36	44	52	60	68	76	84	
E	5	13	21	29	37	45	53	61	69	77	85	
F	6	14	22	30	38	46	54	62	70	78	86	
G	7	15	23	31	39	47	55	63	71	79	87	
H	8	16	24	32	40	48	56	64	72	80	88	

Figure 1. Sample Plate layout.
The numbers indicate the sample numbers.

2.2 Prepare Sample Source Plate (day 1)

There is one sample source plate per sample plate. During this step, samples are manually transferred from the prepared Sample Plate(s) into the Sample Source Plate(s), and controls are added to the Sample Source Plate. The Sample Source Plate must be used within the same day of preparation.

The number of Sample Plate(s) required depends on how the panels are combined in the run, as explained in *2.1.3 Prepare the samples.* The preparation of more than one Sample Plate must be performed in separate runs. Perform the instruction once for every Sample Plate required, i.e in one to four runs.

IMPORTANT: *Using the correct combination of Plate Control and Probes lots is essential for the downstream data analysis. Before starting the workflow, ensure that the lot number for each Probe kit and for the Plate Control matches the lot numbers stated in the Lot Configuration document provided with the kit.*

Prepare bench

- Sample Plate, prepared in previous step
- Olink Explore Negative Control
- Olink Explore Plate Control
- Sample Control (optional, provided by the user)
- 1–4x 384-well PCR plate(s) (skirted): same as the number of 96-well Sample Plates used
- Manual pipette (0.5–10 µL)
- Manual multichannel pipette (0.5–10 µL)
- Filter pipette tips
- Adhesive films

Before you start

- Ensure that the Sample Plate(s) have been prepared according to *2.1.3 Prepare the samples*.
- If frozen, thaw the Sample Plate(s) at room temperature and mark the plates: "Sample Plate [Panel no:s]".
- Thaw the Negative Control, Plate Control and recommended Sample Control at room temperature.
- Mark one 384-well PCR plate per run: “Sample Source Plate [Panel no:s]”.

NOTE: *If preparing more than one 384-well Sample Source Plate, make sure that the 96-well Sample*

Plate and 384-well Sample Source Plate are marked with the same number, and keep track of which Panel(s) each 384-well Sample Source Plate shall be used for.

Instructions

1. Using the MixMate® or manual vortexing, vortex the applicable 96-well Sample Plate and spin at 400-1000 x g for 1 minute at room temperature.
2. Using a multichannel pipette, transfer 10 µL of each sample into the applicable 384-well Sample Source Plate according to the plate layout shown in (*Figure 2*). Use forward pipetting, change pipette tips between every column, and make sure that no air bubbles are trapped at the bottom of the wells.

IMPORTANT: *The plate layout of the Sample Source Plate has changed and is only compatible with version 4 of the Olink Explore instrument protocols. Make sure that you have version 4 of all instrument protocols installed before proceeding. Please contact support@olink.com if you need assistance.*

3. Vortex the Negative Control, Plate Control and Sample Control and spin briefly.
4. Using a single-channel pipette, transfer 10 µL of Negative Control (NC), Plate Control (PC) and Sample Control (SC) to each of the applicable wells in column 23 of the 384-well Sample Source Plate according to the plate layout shown in *Figure 2*. Change pipette tip between every control sample.
5. Seal the Sample Source Plate with a new adhesive film.

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

6. 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).
7. Seal the Sample Plate with a new adhesive film. Store at -80 °C.
8. Repeat the instruction as necessary to prepare any additional Sample Source Plate(s) (384-well) to be used the same day.

NOTE: *Freeze thawing of Sample Source Plates (384-well) for future use is not recommended.*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	1		9		17		25		33		41		49		57		65		73		81		SC	
B																								
C	2		10		18		26		34		42		50		58		66		74		82		SC	
D																								
E	3		11		19		27		35		43		51		59		67		75		83		NC	
F																								
G	4		12		20		28		36		44		52		60		68		76		84		NC	
H																								
I	5		13		21		29		37		45		53		61		69		77		85		NC	
J																								
K	6		14		22		30		38		46		54		62		70		78		86		PC	
L																								
M	7		15		23		31		39		47		55		63		71		79		87		PC	
N																								
O	8		16		24		32		40		48		56		64		72		80		88		PC	
P																								

Figure 2. Sample Source Plate layout.
The numbers indicate the sample numbers. SC = Sample Control, NC = Negative Control, PC = Plate Control.

2.3 Sample Dilution

During this step, Sample Diluent is dispensed into Sample Dilution Plate(s) using the Dragonfly® and the samples are diluted using the F.A.S.T.. The prepared Sample Dilution Plate(s) must be used within one hour from preparation.

Perform the instruction once for every Sample Source Plate prepared in the previous step, i.e. one to four times. It is not possible to prepare multiple plates at the same time.

2.3.1 Prepare Sample Dilution Plate

During this step, Sample Diluent is dispensed into Sample Dilution Plate(s) using the Dragonfly®.

Prepare bench

- Olink Target 96/Explore Sample Diluent
- 1-4x 384-well PCR plate(s) (skirted)
- 1x Disposable reservoir (10 mL)
- 1x Disposable ultra-low retention syringe with plunger
- Manual pipette (100–1000 µL)
- Filter pipette tips
- Adhesive films

Before you start

- Thaw the Sample Diluent at room temperature.
- Mark one new 384-well PCR plate per run: “Sample Dilution Plate [Panel no:s]”.

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

Instructions

1. Prepare the Dragonfly® according to instructions in the Olink Explore Overview User Manual.
   - Use the protocol Olink Sample Dilution Plate v4.

IMPORTANT: *Make sure that the correct protocol version is used. The plate layout has changed and using an older version of the protocol will result in unusable data. Please contact support@olink.com if you need assistance.*

   - Attach one syringe in position B2.
2. Slide the reservoir tray to the filling position (A) and place a new reservoir in position B2 (B) (*Figure 3*).

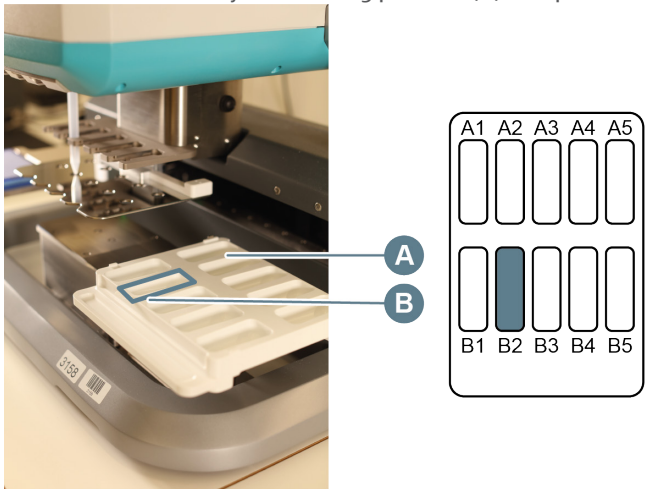

Figure 3. Disposable reservoir in reservoir tray of the Dragonfly.

3. Vortex the Sample Diluent briefly, add 6 mL of Sample Diluent into the reservoir and carefully slide the reservoir tray back to the aspirate position.
4. Place the Sample Dilution Plate in the plate position to the left, with well A1 in the top left corner.
5. Select the Run tab in the Constant layer view of the software (*Figure 4*), then click RUN to start the program.
   Result: Dragonfly® dispenses 9 µL Sample Diluent into each well of quadrant 1-3 and 29 µL in quadrant 4 of the Sample Dilution Plate. Filled wells turn purple in the plate map. The run stops automatically when the Dragonfly® has dispensed Sample Diluent into all applicable wells.

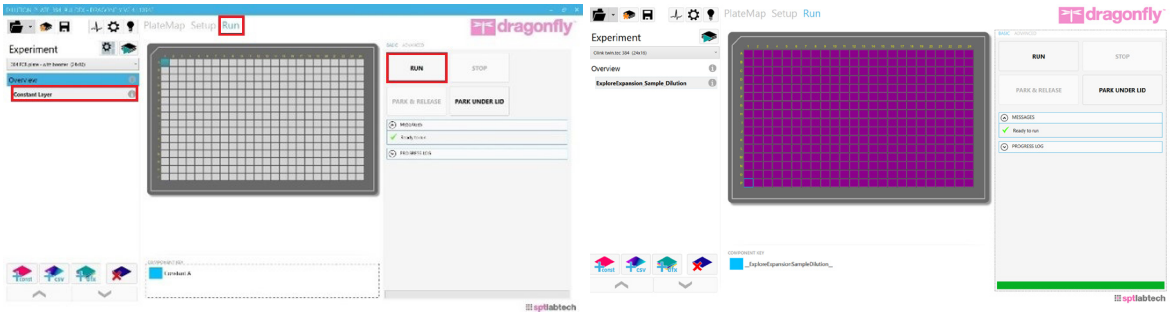

Figure 4. Run tab and Run button (left). Filled Plate (right).

6. When the Dragonfly® has returned the Sample Dilution Plate to the plate position and released the plate clamp, remove the Sample Dilution Plate from the instrument and seal it with an adhesive film.
7. Spin the Sample Dilution Plate at 400–1000 x g for 1 minute.
8. Visually inspect the Sample Dilution Plate to ensure that all wells in quadrant 1-3 contain 9 µL of liquid, and that wells in quadrant 4 contain 29 µL. Make sure that there are no bubbles trapped at the bottom of the wells.
9. Repeat the steps 4–8 as necessary to prepare any additional Sample Dilution Plate(s).
10. When finished, clear the instrument and shut it down according to instructions in the Olink Explore Overview User Manual.
11. Continue to *2.3.2 Perform Sample Dilution*, or store the Sample Dilution Plate(s) at +4 °C until use (the same day).

2.3.2 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, 1:1000, and approximately 1:100 000 (*Figure 6*).

Perform the instruction once per Sample Dilution Plate prepared in the previous step, i.e. one to four times. It is not possible to prepare multiple plates at the same time.

Prepare bench

- Sample Source Plate(s), prepared in previous step
- Sample Dilution Plate(s), prepared in previous step
- Adhesive films

Before you start

- Prepare the F.A.S.T.™ according to the manufacturer's instructions. Use the protocol *Olink Sample Dilution 3072.*
- Make sure that F.A.S.T..™ pipette tips have been loaded into the F.A.S.T. according to the manufacturer's instructions.

Instructions

IMPORTANT: *Make sure to vortex the Sample Dilution Plate(s) thoroughly both between dilutions and after the last dilution.*

1. Let the Sample Source Plate reach room temperature, vortex it briefly and spin down at 400–1000 x g for 1 minute. 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 Plate.
3. Place the Sample Source Plate and the Sample Dilution Plate on the F.A.S.T. deck, refer to the software for the correct position.

IMPORTANT: *Risk of instrument damage! If the plates are not correctly placed, they can collide with the internal parts of the instrument during the run.*

4. Click Run to start the F.A.S.T. program Olink Sample Dilution 3072.
   Result: F.A.S.T. performs a 1:10 dilution by transferring 1 µL of sample from the Sample Source Plate into quadrant 1 in the Sample Dilution Plate. The run can be monitored in the protocol tab.
5. When the F.A.S.T. protocol is paused and a pop-up window appears on the screen, remove the Sample Source Plate and the Sample Dilution Plate from the F.A.S.T. deck.
6. Seal both plates with new adhesive films and store the Sample Source Plate at +4 °C for later use.
7. Using the MixMate® or manual vortexing, vortex the Sample Dilution Plate thoroughly to ensure that all wells are mixed.
8. Spin at 400–1000 x g for 1 minute. Make sure that there are no bubbles trapped at the bottom of the wells.
9. Carefully remove the adhesive film from the Sample Dilution Plate.
10. Place the Sample Dilution Plate back in the correct position of the F.A.S.T. deck.
11. Click Resume to continue the program.
    Result: F.A.S.T. performs a 1:100 sample dilution by transferring 1 µL of diluted samples (1:10) into quadrant 2 of the Sample Dilution Plate.
12. When the F.A.S.T. protocol is paused, and a pop-up window appears on the screen, remove the Sample Dilution Plate from the F.A.S.T. deck. Seal with a new adhesive film.
13. Repeat steps 7–12 to perform the third dilution (1:1000).
    Result: F.A.S.T. performs a 1:1000 sample dilution by transferring 1 µL of diluted samples (1:100) into quadrant 3 of the Sample Dilution Plate.
14. Repeat steps 7-12 again to perform the last dilution (1:100 000).
    Result: F.A.S.T. performs a 1:100 000 sample dilution by transferring 0.3 µL of diluted samples (1:1000) into quadrant 4 of the Sample Dilution Plate.
15. Vortex the Sample Dilution Plate thoroughly using the MixMate® or manual vortexing, and ensure that all wells are mixed.
16. Spin at 400–1000 x g for 1 minute. Make sure that there are no bubbles trapped at the bottom of the wells.
17. When finished, click Resume to end the F.A.S.T. program.
18. Repeat the instruction as necessary to prepare any additional Sample Dilution Plate(s). Keep the F.A.S.T. on for later use.
19. Continue to *2.10 Library purification*, or place the Sample Dilution Plate at +4 °C for up to 1 hour.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	1	1	9	9	17	17	25	25	33	33	41	41	49	49	57	57	65	65	73	73	81	81	SC	SC
B	1	1	9	9	17	17	25	25	33	33	41	41	49	49	57	57	65	65	73	73	81	81	SC	SC
C	2	2	10	10	18	18	26	26	34	34	42	42	50	50	58	58	66	66	74	74	82	82	SC	SC
D	2	2	10	10	18	18	26	26	34	34	42	42	50	50	58	58	66	66	74	74	82	82	SC	SC
E	3	3	11	11	19	19	27	27	35	35	43	43	51	51	59	59	67	67	75	75	83	83	NC	NC
F	3	3	11	11	19	19	27	27	35	35	43	43	51	51	59	59	67	67	75	75	83	83	NC	NC
G	4	4	12	12	20	20	28	28	36	36	44	44	52	52	60	60	68	68	76	76	84	84	NC	NC
H	4	4	12	12	20	20	28	28	36	36	44	44	52	52	60	60	68	68	76	76	84	84	NC	NC
I	5	5	13	13	21	21	29	29	37	37	45	45	53	53	61	61	69	69	77	77	85	85	NC	NC
J	5	5	13	13	21	21	29	29	37	37	45	45	53	53	61	61	69	69	77	77	85	85	NC	NC
K	6	6	14	14	22	22	30	30	38	38	46	46	54	54	62	62	70	70	78	78	86	86	PC	PC
L	6	6	14	14	22	22	30	30	38	38	46	46	54	54	62	62	70	70	78	78	86	86	PC	PC
M	7	7	15	15	23	23	31	31	39	39	47	47	55	55	63	63	71	71	79	79	87	87	PC	PC
N	7	7	15	15	23	23	31	31	39	39	47	47	55	55	63	63	71	71	79	79	87	87	PC	PC
O	8	8	16	16	24	24	32	32	40	40	48	48	56	56	64	64	72	72	80	80	88	88	PC	PC
P	8	8	16	16	24	24	32	32	40	40	48	48	56	56	64	64	72	72	80	80	88	88	PC	PC

Figure 5. Sample Dilution Plate layout.
The numbers indicate the sample numbers. SC = Sample Control, NC = Negative Control and PC = Plate Control.

2.4 Incubation

During this step, four Incubation Mixes are prepared manually for each panel, transferred to Reagent Source Plates, and mixed with the samples. Incubation is then performed overnight.

Since one Reagent Source Plate is used per panel, perform the instructions four times. As the Incubation Mixes must be used within 30 minutes from preparation. Prepare one Reagent Source Plate and two Incubation Plates at a time.

1. Run 1: Prepare Incubation Mixes for Panel 1, transfer to Reagent Source Plate 1, then prepare Incubation Plate 1 and start incubation.
2. Run 2: Prepare Incubation Mixes for Panel 2, transfer to Reagent Source Plate 2, then prepare Incubation Plate 2 and start incubation.
3. Run 3: Prepare Incubation Mixes for Panel 3, transfer to Reagent Source Plate 3, then prepare Incubation Plate 3 and start incubation.
4. Run 4: Prepare Incubation Mixes for Panel 4, transfer to Reagent Source Plate 4, then prepare Incubation Plate 4 and start incubation.

2.4.1 Prepare Reagent Source Plate

During this step, four Incubation Mixes are prepared manually for each panel and transferred to a Reagent Source Plate. Each mix contains a specific set of Forward and Reverse probes.

IMPORTANT: *Using the correct combination of Plate Control and Probes lots is essential for the downstream data analysis. Before starting the workflow, ensure that the lot number for each Probe kit and or the Plate Control matches the lot numbers stated in the Lot Configuration document provided with the kit.*

Prepare bench

- Olink Explore Incubation Solution
- Olink Explore Forward Probes A-D (Frw probes)
- Olink Explore Reverse Probes A-D (Rev probes)
- 4x 8-well strip
- 4x 384-well PCR plate (skirted)
- Manual pipettes (0.5–10, 10–100 µL)
- Manual multichannel pipette (8-channel, 0.5–10 µL)
- Filter pipette tips
- Adhesive films

Before you start

- Allow the Incubation Solution, Forward Probes and Reverse Probes to reach room temperature.
- Mark four new 384-well PCR plates: "Reagent Source Plate [1–4]".
- Mark the wells of four 8-well strips according to *Table 4.*

Table 4. PCR strips

Run	Well							
	1	2	3	4	5	6	7	8
1	1A	1B	1C	1D	-	-	-	-
2	2A	2B	2C	2D	-	-	-	-
3	3A	3B	3C	3D	-	-	-	-
4	4A	4B	4C	4D	-	-	-	-

Instructions

1. Vortex the Incubation Solution, as well as Forward Probes and Reverse Probes and spin down.
2. Prepare four Incubation Mixes in the PCR Strip 1: Using forward pipetting, first transfer the Incubation Solution to tubes 1–4 of the strip, then all Frw Probes to the applicable tubes of the strip, and last all Rev Probes to the applicable tubes of the strip according to *Table 5*.

NOTE: *Pipette the Incubation Solution carefully to avoid foaming.*

IMPORTANT: *The Incubation Solution will be used to prepare Incubation Mixes for the other panels. Do not discard.*

Table 5. Incubation Mix preparation

Addition order	Reagent	Well (of the applicable strip 1-4)							
		A	B	C	D	-	-	-	-
1	Incubation Solution	80 µL	80 µL	80 µL	80 µL	–	–	–	–
2	Frw probes	10 µL	10 µL	10 µL	10 µL	–	–	–	–
		[PANEL] Frw Probes A	[PANEL] Frw Probes B	[PANEL] Frw Probes C	[PANEL] Frw Probes D	–	–	–	–
3	Rev probes	10 µL	10 µL	10 µL	10 µL	–	–	–	–
		[PANEL] Rev Probes A	[PANEL] Rev Probes B	[PANEL] Rev Probes C	[PANEL] Rev Probes D	–	–	–	–

3. Seal the PCR Strip 1 with caps. Vortex and spin down briefly. Store at room temperature until use.

IMPORTANT: *Make sure that all applicable reagents are added to each Incubation Mix. The final volume*

shall be 100 µL

TIME SENSITIVE STEP: *The incubation setup using the F.A.S.T. must be started within 30 minutes from preparation of the Incubation Mix.*

4. Using a multichannel pipette, transfer 10 µL of each Incubation Mix from the PCR Strip 1 to the Reagent Source Plate 1, according to the plate layout in *Figure 8.* Use reverse pipetting and do not change pipette tips between wells. Make sure to pipette to the bottom of the wells.

NOTE: *Rotate the Sample Reagent Plate (384-well) 90 degrees clockwise to facilitate pipetting (shown to the right of Figure 7).*

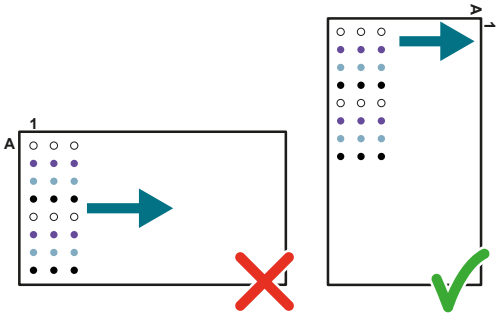

Figure 6. Rotating the Sample Reagent Plate

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	1A		1B		1C		1D																	
B																								
C	1A		1B		1C		1D																	
D																								
E	1A		1B		1C		1D																	
F																								
G	1A		1B		1C		1D																	
H																								
I	1A		1B		1C		1D																	
J																								
K	1A		1B		1C		1D																	
L																								
M	1A		1B		1C		1D																	
N																								
O	1A		1B		1C		1D																	
P																								

Figure 7. Reagent Source Plate layout.
The numbers indicates the Panel number and the letters indicate the different blocks within the panel.

5. Seal the Reagent Source Plate 1 with a new adhesive film and spin at 400–1000 x g for 1 minute. Make sure that there are no bubbles trapped at the bottom of the wells.
6. Immediately continue to *2.4.2 Prepare Incubation Plates and perform incubation*.

2.4.2 Prepare Incubation Plates and perform incubation

During this step, the prepared Incubation Mixes are mixed with the samples in one Incubation Plate per panel, using the F.A.S.T. prior to incubation (*Figure 9*).

As the F.A.S.T. deck can only hold a limited number of plates, perform the instruction four times, once for each Run (one F.A.S.T. run per Reagent Source Plate).

Prepare bench

- Sample Source Plate(s), prepared in previous step
- Sample Dilution Plate(s), prepared in previous step
- Reagent Source Plates 1-4, prepared in previous step
- 4x 384-well PCR plates (skirted)
- Adhesive films

Before you start

- Allow the refrigerated Sample Source Plate(s) and Sample Dilution Plate(s) to reach room temperature.
- Mark four new 384-well PCR plates: “Incubation Plate [1–4]”.

Instructions

1. In the open F.A.S.T. software, Select *File/Open* and choose the applicable protocol from the Explore Window:
   - CARDIO: *Olink Incubation CARDIO*
   - INF*: Olink Incubation INF*
   - NEURO: *Olink Incubation NEURO*
   - ONC: *Olink Incubation ONC*
   - CARDIO II: *Olink Incubation CARDIO II*
   - INF II: *Olink Incubation INF II*
   - NEURO II: *Olink Incubation NEURO II*
   - ONC II: *Olink Incubation ONC II*

IMPORTANT: *Make sure to select the correct F.A.S.T.™ program. Selecting the wrong program will result in unusable data..*

NOTE: *Make sure that all the plates are marked with the same panel number.*

2. Place the Incubation Plate 1 on the F.A.S.T. deck, refer to the software for the correct position.
3. Carefully remove the adhesive film from the Reagent Source Plate 1.
4. Place the Reagent Source Plate 1 in the F.A.S.T. deck. Refer to the software for the correct position.
5. Click *Run*.
   Result: The F.A.S.T. transfers 0.6 µL of each Incubation Mix from the Reagent Source Plate into the Incubation Plate. The run takes approximately 10 minutes to be completed and can be monitored in the protocol tab.
6. When the F.A.S.T. protocol is paused, and a pop-up window appears on the screen, remove the Incubation Plate 1 from the F.A.S.T. deck.
7. Inspect the Incubation Plate to ensure that there is liquid at the bottom of all wells, and that there are no bubbles present.

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

NOTE: *If bubbles are present, seal the Incubation Plate(s) with new adhesive film(s) and spin at 400–1000 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.*

8. Place Incubation Plate 1 back in the F.A.S.T. deck.
9. Vortex the applicable Sample Source Plate and Sample Dilution Plate briefly and spin down at 400–1000 x g for 1 minute. Make sure that there are no bubbles trapped at the bottom of the wells and carefully remove the adhesive films.

NOTE: *Make sure that all plates are marked with the same panel number.*

10. Place the Sample Source Plate and Sample Dilution Plate in the F.A.S.T. deck. Refer to the software for the correct positions.
11. Click *Resume* to continue the protocol.
    Result: The F.A.S.T. transfers 0.2 µL of samples from the Sample Source Plate and Sample Dilution Plate into the Incubation Plate. The run takes approximately 15 min to be completed.
12. When the F.A.S.T. protocol is finished, remove Incubation Plate 1 from the F.A.S.T. deck. Seal with a new adhesive film.

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

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

13. Spin Incubation Plate 1 at 400–1000 x g for 1 minute.
14. Inspect Incubation Plate 1 to ensure that all wells contain the same amount of liquid (0.8 µL). Note if there are any wells containing bubbles.
15. Write the time of the incubation starting point on the plate seal and incubate Incubation Plate 1 for 16 to 24 hours at +4 °C.

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 PCR1 in the ProFlex™ PCR instrument.

16. Remove the Sample Source Plate, Sample Dilution Plate, and Reagent Source Plate from the F.A.S.T. deck. Treat the plates as described in *Table 6*.

Table 6. Plates handling

Incubation Run	Sample Source Plate	Sample Dilution Plate	Reagent Source Plate
Run 1	Seal with adhesive film Store at +4 °C for later use	Seal with adhesive film Store at +4 °C for later use	Discard
Run 2	Seal with adhesive film Store at +4 °C for later use	Seal with adhesive film Store at +4 °C for later use	Discard
Run 3	Seal with adhesive film Store at +4 °C for later use	Seal with adhesive film Store at +4 °C for later use	Discard
Run 4	Seal with adhesive film Store at -80 °C for later use	Discard	Discard

17. Repeat *2.4.1 Prepare Reagent Source Plate* and *2.4.2 Prepare Incubation Plates and perform incubation* for Run 2, 3 and 4.
18. When finished, clear the F.A.S.T. and shut it down according to the manufacturer's instructions.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	1	1	9	9	17	17	25	25	33	33	41	41	49	49	57	57	65	65	73	73	81	81	SC	SC
B	1	1	9	9	17	17	25	25	33	33	41	41	49	49	57	57	65	65	73	73	81	81	SC	SC
C	2	2	10	10	18	18	26	26	34	34	42	42	50	50	58	58	66	66	74	74	82	82	SC	SC
D	2	2	10	10	18	18	26	26	34	34	42	42	50	50	58	58	66	66	74	74	82	82	SC	SC
E	3	3	11	11	19	19	27	27	35	35	43	43	51	51	59	59	67	67	75	75	83	83	NC	NC
F	3	3	11	11	19	19	27	27	35	35	43	43	51	51	59	59	67	67	75	75	83	83	NC	NC
G	4	4	12	12	20	20	28	28	36	36	44	44	52	52	60	60	68	68	76	76	84	84	NC	NC
H	4	4	12	12	20	20	28	28	36	36	44	44	52	52	60	60	68	68	76	76	84	84	NC	NC
I	5	5	13	13	21	21	29	29	37	37	45	45	53	53	61	61	69	69	77	77	85	85	NC	NC
J	5	5	13	13	21	21	29	29	37	37	45	45	53	53	61	61	69	69	77	77	85	85	NC	NC
K	6	6	14	14	22	22	30	30	38	38	46	46	54	54	62	62	70	70	78	78	86	86	PC	PC
L	6	6	14	14	22	22	30	30	38	38	46	46	54	54	62	62	70	70	78	78	86	86	PC	PC
M	7	7	15	15	23	23	31	31	39	39	47	47	55	55	63	63	71	71	79	79	87	87	PC	PC
N	7	7	15	15	23	23	31	31	39	39	47	47	55	55	63	63	71	71	79	79	87	87	PC	PC
O	8	8	16	16	24	24	32	32	40	40	48	48	56	56	64	64	72	72	80	80	88	88	PC	PC
P	8	8	16	16	24	24	32	32	40	40	48	48	56	56	64	64	72	72	80	80	88	88	PC	PC

Figure 8. Incubation Plate layout.
The numbers indicate the sample numbers. The colors indicate the different blocks within the panel: white = block A, purple = block B, blue = block C, black = block D.

2.5 Preparation of reagents for day 2

Prepare bench

- MilliQ water
- Olink® Explore PCR1 Enhancer
- Olink® Explore PCR1 Solution

Instructions

1. Place 30 mL of MilliQ water at +4 °C overnight.
2. Thaw the PCR1 Enhancer at room temperature overnight.
3. Optional: Thaw the PCR1 Solution at +4 °C overnight.

2.6 Extension and pre-amplification (PCR1) (day 2)

During this step, a PCR1 Mix is prepared manually and dispensed into the Incubation Plates using the Dragonfly®. The plates are renamed “PCR1 Plates” and subjected to a PCR reaction.

2.6.1 Prepare PCR1 Mix

During this step, a PCR1 Mix is prepared manually. The PCR1 Mix must be used within 30 minutes from preparation.

Prepare bench

- Incubation Plates 1–4, prepared in previous step
- Olink® Explore PCR1 Enhancer
- Olink® Explore PCR1 Solution
- Olink® Explore PCR1 Enzyme (when brought to the lab bench, keep the PCR1 Enzyme in a freezing block (-20 °C))
- MilliQ water (at +4 °C, preferably kept in the fridge until use)
- 1x Falcon tube (50 mL)

Before you start

- Thaw the PCR1 Solution at +4 °C overnight (optional), or at room temperature before starting the PCR1 Setup (it may take several hours) and ensure that it is fully thawed and has reached room temperature.
- Ensure that the PCR1 Enhancer is fully thawed and has reached room temperature.
- Let the refrigerated Incubation Plates 1–4 reach room temperature.
- Mark the new 50 mL Falcon tube: "PCR1 Mix".
- Switch on two ProFlex™ PCR instruments in the post-PCR room.

Instructions

1. Vortex the PCR1 Solution and PCR1 Enhancer thoroughly and spin down briefly.

IMPORTANT: *Insufficient thawing and vortexing of the PCR1 Enhancer may lead to particles attaching to the syringe and run failure.*

2. Spin the PCR1 Enzyme briefly. Do not vortex.

NOTE: *Always keep the PCR1 Enzyme at -20 °C. When brought to the lab bench, keep it in a freezing block (-20 °C).*

3. Spin Incubation Plates 1–4 down for 1 minute at room temperature.
4. Prepare a PCR1 Mix in a Falcon tube in the following order, using the volumes indicated in *Table 7*:
    1. Add MilliQ water and PCR1 Enhancer and vortex thoroughly.
    2. Add the PCR1 Solution and PCR1 Enzyme, and vortex for 3 seconds.

Table 7. PCR1 Mix

Addition order	Reagent	Volume (µL)
1	MilliQ water (+4 °C)	27 000
2	PCR1 Enhancer	3 510
3	PCR1 Solution	3 510
4	PCR1 Enzyme	351
	Total	34 371

    3. Keep at room temperature until use.

TIME SENSITIVE STEP: *Dispensing of the PCR1 Mix using the Dragonfly® must start within 30 minutes from PCR1 Mix preparation.*

2.6.2 Prepare PCR1 Plates and perform PCR1

During this step, the prepared PCR1 Mix is dispensed into the Incubation Plates using the Dragonfly®, and the plates are subjected to a PCR reaction (*Figure 13*).

The PCR1 Plates must be placed in the ProFlex™ exactly 10 minutes after the PCR1 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 PCR1 Mix into Incubation Plates 1 and 2 using the Dragonfly®. Start a PCR1 run for these plates on one of the ProFlex™ instruments.
2. Dispense PCR1 Mix into Incubation Plates 3 and 4 plates using the Dragonfly®. Start a PCR1 run for these plates on the second ProFlex™ instrument.

Prepare bench

- Incubation Plates 1–4 at room temperature, prepared in previous step
- PCR1 Mix, prepared in previous step
- 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 a timer to 10 minutes.

Instructions

1. Start the PCR program *Olink PCR1* on the two ProFlex™ PCR instruments (*Figure 10*). Pause when the PCR block temperature reaches 50 °C.

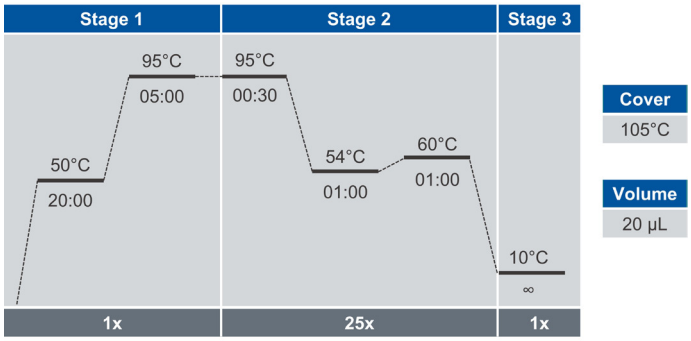

Figure 9. Olink PCR1 program

2. Prepare the Dragonfly® according to instructions in the Olink Explore Overview User Manual.
   - Use the protocol *Olink PCR1 Setup plate v4.*
   - Attach three syringes in position B2, B3, and B4.
3. Slide the reservoir tray to the filling position (A) and place three new reservoirs in position B2, B3 and B4 (B) (*Figure 11*).

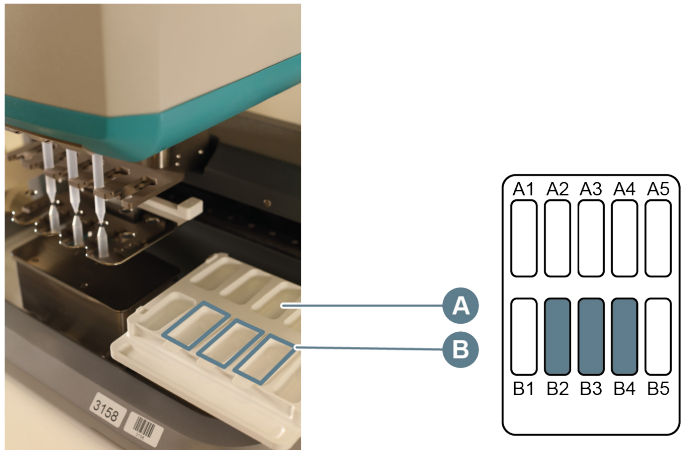

Figure 10. Reservoir tray in filling position

4. Transfer 11 mL of PCR1 Mix into each of the three reservoirs.

IMPORTANT: *The syringes and PCR1 Mix are used to prepare all four PCR1 Plates. Do not discard until all PCR1 Plates are complete.*

5. Carefully slide the reservoir tray back to the aspirate position (*Figure 12*).

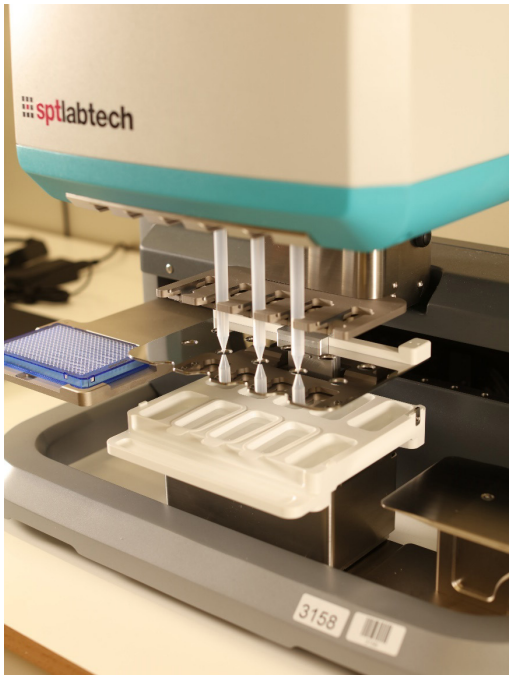

Figure 11. Reservoir tray in aspirate position

6. Rename Incubation Plate 1 “PCR1 Plate 1” and Incubation Plate 2 “PCR1 Plate 2”. Spin down the plates.
7. Carefully remove the adhesive film from PCR1 Plate 1 and place it in the Dragonfly® plate position (to the left), with well A1 in the top left corner.
8. Select the *Run* tab in the *Constant layer* view of the software, then click *RUN* to start the program. Start the timer when PCR1 Mix is added to the first well of the plate.
   Result: Dragonfly® dispenses 19 µL of PCR1 Mix into each well of the PCR1 Plate 1.

TIME SENSITIVE STEP: *The PCR1 Plate must be placed in the ProFlex™ exactly 10 minutes after the PCR1 Mix has been added to the first well of the first plate.*

9. When the Dragonfly® has returned the PCR1 Plate 1 to the plate position and released the plate clamp, remove the plate from the instrument and immediately seal it with a new adhesive film.
10. Inspect the PCR1 Plate 1 to ensure that all wells contain the same amount of liquid (19.8 µL). Using the MixMate® or manual vortexing, vortex the plate thoroughly to ensure that all wells are mixed.
11. Repeat steps 7–10 for PCR1 Plate 2.
12. In the Post-PCR room, centrifuge the two PCR1 Plates 1-2 at 400–1000 x g for 1 minute.
13. Inspect PCR1 Plates 1–2 to ensure that all wells contain the same amount of liquid (19.8 µL). Note any deviations.
14. When the timer ends after 10 minutes, place PCR1 Plates 1–2 in one of the two pre-heated ProFlex™ instruments and click *Resume* the run the *Olink PCR1* program.
15. Repeat steps 6-14 for Incubation Plates 3–4, except rename Incubation Plate 3 as "PCR Plate 3, and Incubation Plate 4 as "PCR Plate 4". Place both plates in the second ProFlex™.
16. When finished, clear the Dragonfly® and shut it down according to instructions in the Olink Explore Overview User Manual.
17. When the *Olink PCR1* programs are finished (~1 hour 55 minutes), continue to *3.7 Pool PCR1 products*, or store the four PCR1 Plates at +4 °C if used the same day.

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

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	1	1	9	9	17	17	25	25	33	33	41	41	49	49	57	57	65	65	73	73	81	81	SC	SC
B	1	1	9	9	17	17	25	25	33	33	41	41	49	49	57	57	65	65	73	73	81	81	SC	SC
C	2	2	10	10	18	18	26	26	34	34	42	42	50	50	58	58	66	66	74	74	82	82	SC	SC
D	2	2	10	10	18	18	26	26	34	34	42	42	50	50	58	58	66	66	74	74	82	82	SC	SC
E	3	3	11	11	19	19	27	27	35	35	43	43	51	51	59	59	67	67	75	75	83	83	NC	NC
F	3	3	11	11	19	19	27	27	35	35	43	43	51	51	59	59	67	67	75	75	83	83	NC	NC
G	4	4	12	12	20	20	28	28	36	36	44	44	52	52	60	60	68	68	76	76	84	84	NC	NC
H	4	4	12	12	20	20	28	28	36	36	44	44	52	52	60	60	68	68	76	76	84	84	NC	NC
I	5	5	13	13	21	21	29	29	37	37	45	45	53	53	61	61	69	69	77	77	85	85	NC	NC
J	5	5	13	13	21	21	29	29	37	37	45	45	53	53	61	61	69	69	77	77	85	85	NC	NC
K	6	6	14	14	22	22	30	30	38	38	46	46	54	54	62	62	70	70	78	78	86	86	PC	PC
L	6	6	14	14	22	22	30	30	38	38	46	46	54	54	62	62	70	70	78	78	86	86	PC	PC
M	7	7	15	15	23	23	31	31	39	39	47	47	55	55	63	63	71	71	79	79	87	87	PC	PC
N	7	7	15	15	23	23	31	31	39	39	47	47	55	55	63	63	71	71	79	79	87	87	PC	PC
O	8	8	16	16	24	24	32	32	40	40	48	48	56	56	64	64	72	72	80	80	88	88	PC	PC
P	8	8	16	16	24	24	32	32	40	40	48	48	56	56	64	64	72	72	80	80	88	88	PC	PC

Figure 12. PCR1 plate layout.
The numbers indicate the sample numbers. The colors indicate the different blocks within the panel: white = block A, purple = block B, blue = block C, black = block D.

2.7 Pool PCR1 products

During this step, the PCR1 products from the PCR1 Plates are pooled into one PCR1 Pooling Plate using the Microlab STAR (*Figure 14*).

Prepare bench

- PCR1 Plates, prepared in previous step
- MilliQ water (at +4 °C, preferably kept in the fridge until use)
- 1x 384-well PCR plate (skirted)
- 1x water reservoir (300 mL)
- 50 µL Conductive Filter Tips (1x rack per panel, 1x rack for water)
- Adhesive films
- Temperature-resistant labels or marker pen

Before you start

- Thaw PCR1 Plates at room temperature if frozen.
- Mark the new 384-well PCR plate: “PCR1 Pooling Plate”.
- Switch on the Microlab STAR system, and open the Hamilton Run Control software.

Instructions

18. Make sure that PCR1 Plates are thawed and properly sealed, then vortex the plates and spin down at 400–1000 x g for 1 minute at room temperature.
19. Inspect the wells of PCR1 Plates to make sure that no liquid has evaporated and that all the liquid is at the bottom of the wells. Spin down the plates again if necessary.
20. In Run Control, select the protocol *PCR1 Pooling* and click the **Start** button.
21. Select the number of Destination Pooling Plates and the number of Panels being Pooled for each, then click **OK**.

22. Pull out all carriers to the load position and prepare the Microlab STAR deck according to the software instructions.
    - Add MilliQ water to the reservoir.
    - Place PCR1 Plates on the carrier.
    - Carefully remove the adhesive films.
    - Fill Tip carrier in selected positions.
    - Push in Water Reservoir manually.
23. Click **OK** in the software to load the tip and plate carriers automatically and begin the run.
    Result: The Microlab STAR automatically scans the tips, dispenses 12 µL MilliQ water into each well of the PCR1 Pooling Plate, and pools 3 µL of each PCR1 product from each sample into one well per panel. The run takes approximately 6 minutes to be completed.
24. When the protocol is finished, remove the PCR1 Pooling Plate and seal it with a new adhesive film. Keep the Microlab STAR®on for later use.
25. Vortex the PCR1 Pooling Plate and spin down at 400–1000 x g for 1 minute.
26. Inspect the PCR1 Pooling Plate to ensure that all wells contain the same amount of liquid (24 µL).
27. Remove PCR1 Plates containing the remaining PCR products and seal them with new adhesive films. Store them at 20 °C for up to 2 weeks in case of potential reruns.
28. Continue to *2.8 Amplification and sample indexing (PCR2)*, or store the PCR1 Pooling Plate at +4 °C until ready to use (the same day).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	1	1	9	9	17	17	25	25	33	33	41	41	49	49	57	57	65	65	73	73	81	81	SC	SC
B	1	1	9	9	17	17	25	25	33	33	41	41	49	49	57	57	65	65	73	73	81	81	SC	SC
C	2	2	10	10	18	18	26	26	34	34	42	42	50	50	58	58	66	66	74	74	82	82	SC	SC
D	2	2	10	10	18	18	26	26	34	34	42	42	50	50	58	58	66	66	74	74	82	82	SC	SC
E	3	3	11	11	19	19	27	27	35	35	43	43	51	51	59	59	67	67	75	75	83	83	NC	NC
F	3	3	11	11	19	19	27	27	35	35	43	43	51	51	59	59	67	67	75	75	83	83	NC	NC
G	4	4	12	12	20	20	28	28	36	36	44	44	52	52	60	60	68	68	76	76	84	84	NC	NC
H	4	4	12	12	20	20	28	28	36	36	44	44	52	52	60	60	68	68	76	76	84	84	NC	NC
I	5	5	13	13	21	21	29	29	37	37	45	45	53	53	61	61	69	69	77	77	85	85	NC	NC
J	5	5	13	13	21	21	29	29	37	37	45	45	53	53	61	61	69	69	77	77	85	85	NC	NC
K	6	6	14	14	22	22	30	30	38	38	46	46	54	54	62	62	70	70	78	78	86	86	PC	PC
L	6	6	14	14	22	22	30	30	38	38	46	46	54	54	62	62	70	70	78	78	86	86	PC	PC
M	7	7	15	15	23	23	31	31	39	39	47	47	55	55	63	63	71	71	79	79	87	87	PC	PC
N	7	7	15	15	23	23	31	31	39	39	47	47	55	55	63	63	71	71	79	79	87	87	PC	PC
O	8	8	16	16	24	24	32	32	40	40	48	48	56	56	64	64	72	72	80	80	88	88	PC	PC
P	8	8	16	16	24	24	32	32	40	40	48	48	56	56	64	64	72	72	80	80	88	88	PC	PC

Figure 13. PCR1 Pooling Plate layout.
The numbers indicate the sample numbers. The colors indicate the panel numbers: light blue = Panel 1, green = Panel 2, dark blue = Panel 3, grey = Panel 4.

2.8 Amplification and sample indexing (PCR2)

During this step, a PCR2 Mix is prepared manually and then mixed with the samples along with index primers (unique for each sample) using the Microlab STAR. The samples are then subjected to a second PCR reaction.

2.8.1 Prepare PCR2 Mix

During this step, a PCR2 Mix is prepared manually. The PCR2 Mix must be used within 15 minutes from preparation.

Prepare bench

- PCR1 Pooling Plate, prepared in previous step
- Olink® Explore PCR2 Solution
- Olink® Explore PCR2 Enzyme (when brought to the lab bench, keep the PCR2 Enzyme in a freezing block (-20 °C))
- Olink® Explore Index Plate 1
- MilliQ water (at +4 °C, preferably kept in the fridge until use)
- Falcon tube (15 mL)
- Manual pipettes (10–100 µL, 100–1000 µL)
- Filter pipette tips
- Temperature-resistant labels or marker pen

Before you start

- Thaw the PCR2 Solution and the Index Plate 1 at room temperature.
- If stored at +4 °C, allow the PCR1 Pooling Plate to reach room temperature.
- Mark the new 15 mL tube: "PCR2 Mix".
- Switch on one ProFlex™ PCR instrument. No preheating is required.

Instructions

1. Vortex the PCR2 Solution and spin it briefly.
2. Vortex the Index Plate 1 and spin it at 400–1000 x g for 1 minute.
3. Make sure that the PCR1 Pooling Plate is properly sealed, then vortex it and spin at 400–1000 x g for 1 minute.
4. Spin the PCR2 Enzyme briefly. Do not vortex.

NOTE: *Always keep the PCR2 Enzyme at -20 °C. When brought to the lab bench, keep it in a freezing block (-20 °C).*

5. Prepare a PCR2 Mix in a Falcon tube following the order and volumes indicated in *Table 8*.

Table 8. PCR2 Mix

Addition order	Reagent	Volume (µL)
1	MilliQ water (+4 °C)	7 076
2	PCR2 Solution	1 015
3	PCR2 Enzyme	21
	Total	8 112

5. Vortex the PCR2 Mix thoroughly. Aliquot to 96 well PCR plate with at least 72 uL/well. Keep at room temperature until use.

TIME SENSITIVE STEP: *Start the dispensing of the PCR2 Mix within 15 minutes from preparation.*

2.8.2 Prepare PCR2 Plate and perform PCR2

During this step, the prepared PCR2 Mix is mixed with the samples along with index primers using the Microlab STAR. The samples are then subjected to a second PCR reaction (*Figure 16*).

Prepare bench

- PCR1 Pooling Plate (at room temperature), prepared in previous step
- PCR2 Mix, prepared in previous step
- Olink® Explore Index Plate 1
- 1x 384-well PCR plate (skirted)
- 1x 96-well PCR plate with PCR2 Master Mix (skirted), prepared in previous step
- 50 µL Conductive Filter Tips (6x rack per PCR2 plate)
- Adhesive films
- Temperature-resistant labels or marker pen

Before you start

- Mark the new 384-well PCR plate: "PCR2 Plate".

Instructions

1. Open Microlab Run Control and select the protocol *PCR2_Setup* and click **Start.**
2. Select the number of PCR2 plates being prepped and the number of Panels in each plate, and click **OK**.
3. Centrifuge Index Plate 1 at 400–1000 x g for 1 minute and inspect the plate to ensure that all wells contain the same amount of liquid (20 µL). If a well is empty or contains a lower volume, contact Olink Support.
4. Carefully remove the adhesive films from the Index Plate 1 and the PCR1 Pooling Plate. Make sure that no air bubbles are trapped at the bottom of the wells of the PCR1 Pooling Plate.
5. Pull out all carriers and prepare the Microlab STAR deck according to the software instructions.
6. Click **OK** once the deck is loaded, then the system will automatically load the carriers and begin the protocol.
   Result: Microlab STAR transfers 16 µL of PCR2 Mix, 2 µL of Index Primers (from the Index Plate 1) and 2 µL of PCR1 products (from the PCR1 Pooling Plate) into the applicable wells of the PCR2 Plate (Figure 16). The run takes approximately 8 minutes to be completed.
7. When the protocol is finished (indicated by a message in the software), remove the PCR2 Plate and seal it with a new adhesive film. Keep the Microlab STAR on for later use.

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

TIME SENSITIVE STEP: *Start the PCR2 program within 5 minutes from end of Microlab STAR protocol.*

8. Vortex and spin down at 400–1000 x g for 1 minute at room temperature.
9. Inspect the PCR2 Plate to ensure that all wells contain the same amount of liquid (20 µL).
10. Place the PCR2 Plate in the ProFlex and add a balance plate to the other side of the ProFlex.
11. Click Open and select the program *Olink Index PCR2* (*Figure 15*). Click **Start**.

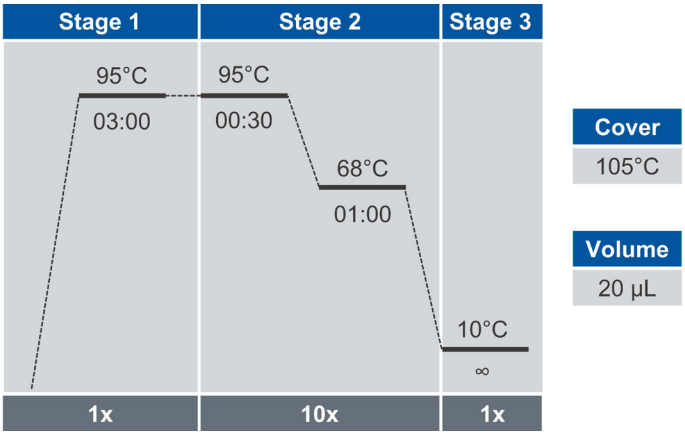

Figure 14. Olink Index PCR2 program

12. Remove the PCR1 Pooling Plate containing the remaining PCR products from the Microlab STAR and seal it with a new adhesive film. Store it at -20 °C for up to 2 weeks in case of potential reruns.
13. Discard the Index Plate 1.
14. When the PCR program is finished (~25 minutes), continue to *2.9 Pool PCR2 products*, or store the PCR2 Plate at +4 °C until use (the same day).

> **SAFE STOPPING POINT:** *The PCR2 Plate can be stored at -20 °C for up to 2 weeks.*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	1	1	9	9	17	17	25	25	33	33	41	41	49	49	57	57	65	65	73	73	81	81	SC	SC
B	1	1	9	9	17	17	25	25	33	33	41	41	49	49	57	57	65	65	73	73	81	81	SC	SC
C	2	2	10	10	18	18	26	26	34	34	42	42	50	50	58	58	66	66	74	74	82	82	SC	SC
D	2	2	10	10	18	18	26	26	34	34	42	42	50	50	58	58	66	66	74	74	82	82	SC	SC
E	3	3	11	11	19	19	27	27	35	35	43	43	51	51	59	59	67	67	75	75	83	83	NC	NC
F	3	3	11	11	19	19	27	27	35	35	43	43	51	51	59	59	67	67	75	75	83	83	NC	NC
G	4	4	12	12	20	20	28	28	36	36	44	44	52	52	60	60	68	68	76	76	84	84	NC	NC
H	4	4	12	12	20	20	28	28	36	36	44	44	52	52	60	60	68	68	76	76	84	84	NC	NC
I	5	5	13	13	21	21	29	29	37	37	45	45	53	53	61	61	69	69	77	77	85	85	NC	NC
J	5	5	13	13	21	21	29	29	37	37	45	45	53	53	61	61	69	69	77	77	85	85	NC	NC
K	6	6	14	14	22	22	30	30	38	38	46	46	54	54	62	62	70	70	78	78	86	86	PC	PC
L	6	6	14	14	22	22	30	30	38	38	46	46	54	54	62	62	70	70	78	78	86	86	PC	PC
M	7	7	15	15	23	23	31	31	39	39	47	47	55	55	63	63	71	71	79	79	87	87	PC	PC
N	7	7	15	15	23	23	31	31	39	39	47	47	55	55	63	63	71	71	79	79	87	87	PC	PC
O	8	8	16	16	24	24	32	32	40	40	48	48	56	56	64	64	72	72	80	80	88	88	PC	PC
P	8	8	16	16	24	24	32	32	40	40	48	48	56	56	64	64	72	72	80	80	88	88	PC	PC

Figure 15. PCR2 Plate layout
The numbers indicate the sample numbers. The colors indicate the panel numbers: light blue = Panel 1, green = Panel 2, dark blue = Panel 3, grey = Panel 4.

2.9 Pool PCR2 products

During this step, all PCR2 products belonging to the same panel are pooled into one column of the PCR2 Pooling Plate using the Microlab STAR (*Figure 17*). The Olink libraries are then manually transferred to one microcentrifuge tube per panel. Each tube contains amplicons from 96 samples, including controls.

Prepare bench

- PCR2 Plate, prepared in previous step
- 1x 96-well PCR plate (skirted)
- Microcentrifuge tubes (1.5 mL, 1x tube per panel)
- 50 µL Conductive Filter Tips (1x rack per pooling plate)
- Manual pipette (10–100 µL)
- Filter pipette tips
- Adhesive films
- Temperature-resistant labels or marker pen

Before you start

- Thaw PCR2 Plate at room temperature if frozen.
- Mark the new 96-well PCR plate: ”PCR2 Pooling Plate”.

- Mark four new microcentrifuge tubes: ”PCR2 [1–4, depending on the number of panels]”.

Instructions

1. Open Hamilton Run Control and select the applicable protocol *PCR2_Final_Pooling* and click **Start**.
2. Select the number of Destination Pooling Plates and the number of Panels being pooled in each, then click **OK**.
3. Make sure that the PCR2 Plate is thawed and properly sealed, then vortex and spin it down at 400–1000 x g for 1 minute at room temperature to ensure that all wells are mixed.
4. Inspect the wells of the PCR2 Plate to make sure that no liquid has evaporated and that all the liquid is at the bottom of the wells.
5. Pull out all carriers an prepare the Microlab STAR deck according to the software instructions.
6. Once loaded, click **OK** to automatically load the carriers and begin the protocol.
   Result: Microlab STAR pools 3 µL from each row of a given Panel into a single well of the PCR2 Pooling Plate. The result is one column of pooled PCR2 products per panel in the PCR2 Pooling Plate. (Figure 17).
7. When finished, remove the PCR2 Pooling Plate from the worktable. Seal the plate with a new adhesive film, vortex the plate and spin down at 400–1000 x g for 1 minute.
8. Inspect the PCR2 Pooling Plate to ensure that every applicable well contains the same amount of liquid (36 µL in columns 1, 3, 5 and 7).
9. Clear the Microlab STAR and shut it down.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1		2		3		4					
B	1		2		3		4					
C	1		2		3		4					
D	1		2		3		4					
E	1		2		3		4					
F	1		2		3		4					
G	1		2		3		4					
H	1		2		3		4					

Figure 16. PCR2 Pooling Plate layout.
The numbers and colors indicate the panel numbers: light blue = Panel 1, green = Panel 2, dark blue = Panel 3, grey = Panel 4.

10. Carefully remove the adhesive film from the PCR2 Pooling Plate.
11. Using a single-channel pipette transfer the pooled PCR2 products from the PCR2 Pooling Plate to the PCR2 Tubes as described in *Table 9*. Use forward pipetting and change pipette tip after each well.

Table 9. Transfer PCR2 products. If running a single panel, only transfer from column 1 to PCR2 tube 1 will be applicable.

Volume (µL)	From column	To tube
30	1	PCR2 1
30	3	PCR2 2
30	5	PCR2 3
30	7	PCR2 4

12. Vortex the PCR2 Tubes and spin down briefly.
13. Remove the PCR2 Plate containing the remaining PCR2 products from the instrument, seal it with a new adhesive film and store it at -20 °C for up to 2 weeks in case of potential reruns.
14. Discard the PCR2 Pooling Plate.
15. Continue to the Library purification step in the workflow manual, or store the PCR2 Tubes at +4 °C until use (the same day).

SAFE STOPPING POINT: *The PCR2 Tubes can be stored at –20 °C for up to 2 weeks.*

2.10 Library purification

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

Prepare bench

- PCR2 Tubes 1–4, prepared in previous step
- Agentcourt AMPure XP bottle
- 96% Ethanol (EtOH)
- MilliQ water
- DynaMag™-2 Magnet
- Timer
- 8x Microcentrifuge tubes (1.5 mL)
- Falcon tube (15 mL)
- Manual pipettes (10–100 µL, 100–1000 µL)
- Pipette (5 mL)
- Filter pipette tips

Before you start

- Let the refrigerated Agentcourt AMPure XP bottle reach room temperature.
- Mark four new microcentrifuge tubes: “BP [1–4]” (for “Bead Purification”).
- Mark four new microcentrifuge tubes: “Lib [1–4]”.
- Mark the new 15 mL Falcon tube: "70% EtOH".
- Set a timer to 5 minutes.

Instructions

1. Freshly prepare a sufficient amount of 70% EtOH for the number of Libraries to purify according to *Table 10*. Manually transfer first the MilliQ water and then the 96% EtOH into the Falcon tube.

Table 10. Prepare 70% EtOH

Reagent	Volume (mL) for							
	1 library	2 Libraries	3 Libraries	4 Libraries	5 Libraries	6 Libraries	7 Libraries	8 Libraries
MilliQ water	0.5	0.8	1.1	1.4	1.6	1.9	2.2	2.5
96% EtOH	1.5	2.2	2.9	3.6	4.4	5.1	5.8	6.5
Total (70% EtOH)	2	3	4	5	6	7	8	9

2. Shake and vortex the Agentcourt AMPure XP bottle vigorously to resuspend the magnetic beads.
3. Transfer 80 µL from the Agentcourt AMPure XP bottle to each BP Tube.
4. Transfer 50 µL from each PCR2 Tube to the corresponding BP Tube according to *Table 11*.

Table 11. Transfer PCR2 products to BP tubes

Volume (µL)	From tube	To tube
50	PCR2 1	BP 1
50	PCR2 2	BP 2
50	PCR2 3	BP 3
50	PCR2 4	BP 4

NOTE: *Store the PCR2 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 and incubate BP Tubes 1–4 for 5 minutes at room temperature.
7. After the incubation, place the BP Tubes on the DynaMag™-2 Magnetic stand and leave them for 2 minutes to separate the beads from the solution (*Figure 18*).

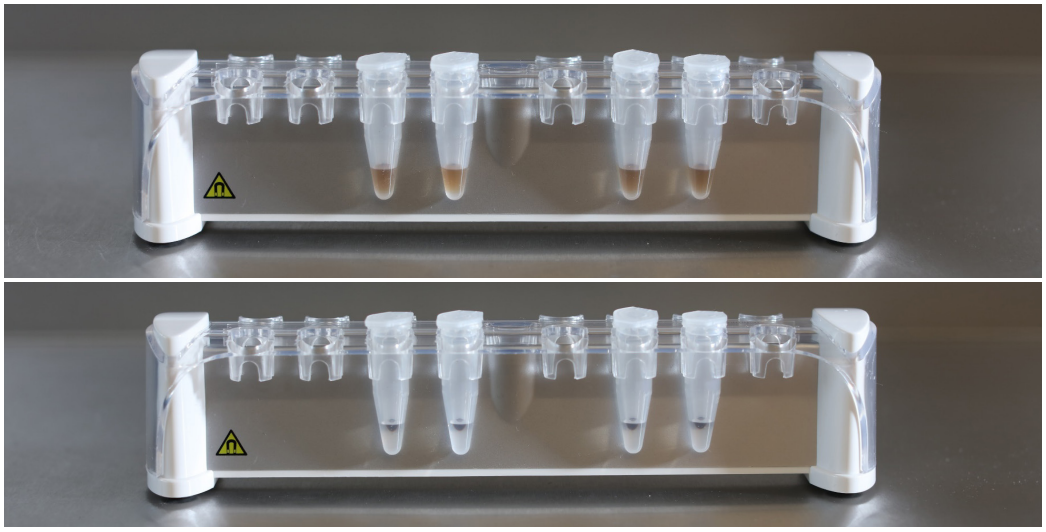

Figure 17. Tubes in DynaMag™-2 Magnetic stand before (upper) and after (lower) bead separation.

8. With the tubes still on the magnetic stand, carefully open the lid and discard 125 µL supernatant using a single-channel pipette. Do not disturb the beads.
9. With the tubes still in the magnetic stand, wash the beads:
   a. Add 500 µL of 70 % EtOH to the 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) once.

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 MilliQ water to the BP Tubes and pipette-mix 10 times towards the beads to elute the purified Libraries

from the beads.

13. Incubate the tubes for 2 minutes at room temperature.
14. Place BP Tubes 1–4 on the magnetic stand and leave them for 1 minute to separate the beads from the eluted Library solution.
15. With BP Tubes 1-4 still on the magnetic stand, transfer 45 µL of eluate from the BP Tubes to the corresponding Lib Tubes according to *Table 12.*

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

Table 12. Transfer eluat

Volume (µL)	From tube	To tube
45	BP 1	Lib 1
45	BP 2	Lib 2
45	BP 3	Lib 3
45	BP 4	Lib 4

16. Discard BP Tubes 1-4.
17. Continue to *2.11 Quality control*.

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

2.11 Quality control

During this step, the four purified Olink Libraries are quality controlled on the Bioanalyzer using the High Sensitivity DNA kit or on the TapeStation using the High Sensitivity D5000 kit, according to the Manufacturer's instructions.

The electropherograms in *Figure 19* and *Figure 20* displays typical results for an Olink Library. 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 https://emea.support.illumina.com/bulletins/2019/10/bubble-products-in-sequencing-Libraries--causes--identification-.html for more information.

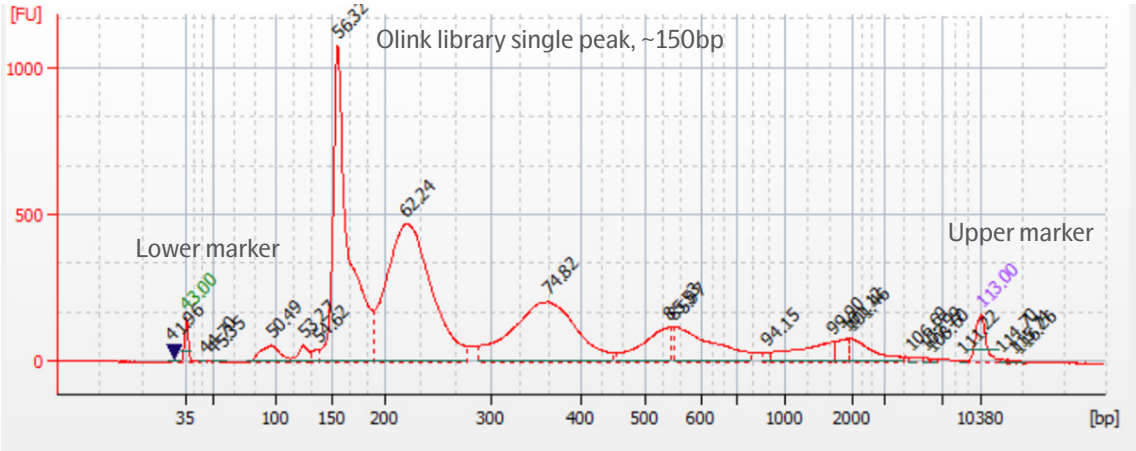

Figure 18. Bioanalyzer's electropherogram.

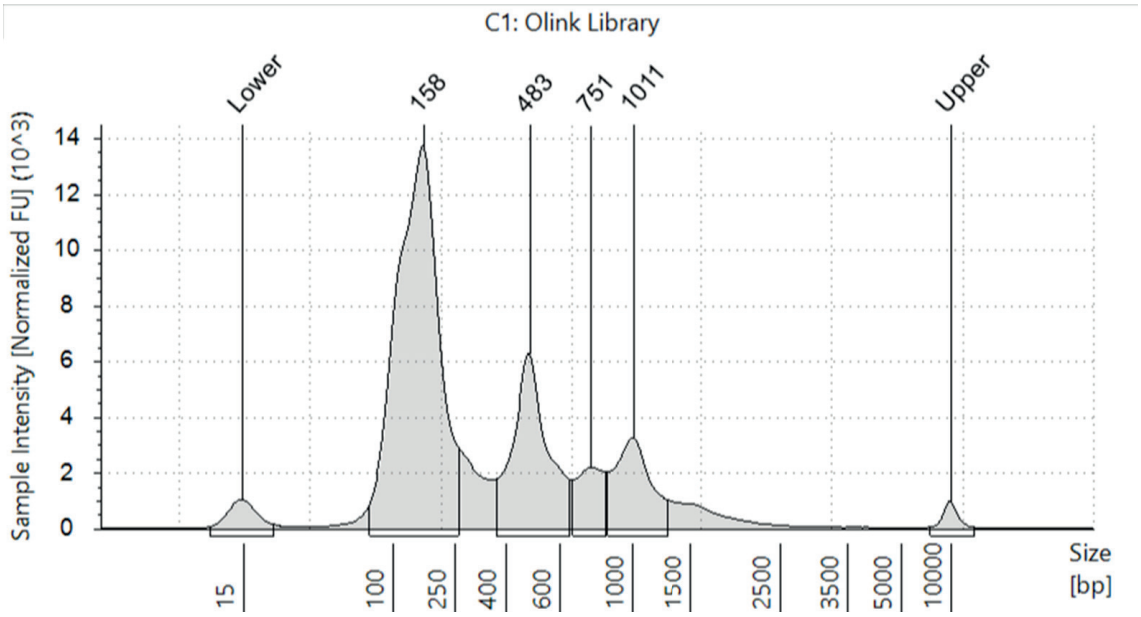

Figure 19. TapeStation's electropherogram.

For expected results of a successful ladder run, refer to the Manufacturer's Manual. If any of the deviations in *Table 13* should occur in the electropherogram, do not continue to sequencing. Instead, perform the suggested corrective action.

Table 13. Deviations in electropherogram.

Peak deviation	Possible cause	Action
No Library peak present at 150bp.	1) No PCR1 amplification has taken place, and therefore there are no PCR2 products and no Library 2) The bead purification failed	Rerun the quality control with unpurified Library. If a peak is present at 150bp: purify a new aliquot of the unpurified Library and rerun the quality control. If successful, proceed to sequencing. If there is no 150bp peak present in the unpurified Library, no PCR product has been generated. Repeat the Library preparation, quality control and sequencing.
No Library peak present at 150bp, but a smaller peak at 120bp	The index primers were not included in the PCR2 reaction (the 120bp peak corresponds to the PCR1 product) 1) The Index plate was empty 2) There was no transfer of index primers from the Index Plate to the PCR2 Plate due to ep*Motion*® failure	Contact Support. Make sure to centrifuge and visually inspect the Index Plate before starting the PCR2 setup. Make sure that the ep*Motion*® is calibrated, functional and that the correct protocol is used.

2.12 Next generation sequencing

Next generation sequencing is performed using either an Illumina® NextSeq™ 550, NextSeq™ 2000 or NovaSeq™ 6000 instrument. As the workflow differs between instruments, refer to the applicable Olink Explore Sequencing User Manual for instructions on how to sequence Olink Libraries.

3. Laboratory instructions for 96 samples using SPT Labtech Mosquito® and Hamilton Microlab® STAR

This chapter provides instructions on how to perform each step of the Olink Explore 4 x 384 laboratory workflow, using the Microlab® STAR from Hamilton Company. The four panels should be prepared simultaneously. If the panels are prepared separately, additional Olink Explore Sample Prep Reagents will be required, refer *Table 2*.

NOTE: *If running the combination CARDIO, INF, NEURO and ONC **or** the combination CARDIO II, INF II, NEURO II and ONC II it is possible to follow the instructions in Olink Explore 3072 User Manual. The instructions in Olink Explore 3072 User Manual provide a faster workflow by allowing for preparation of two incubation plates simultaneously using different instrument protocols.*

3.1 Preparations

3.1.1 Plan the study

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

- Decide the number of samples and controls required to get the data that you want from the study.
- Optional Sample Control: It is recommended to include a pooled plasma sample in duplicate on each plate. These samples are used to assess potential variation between runs and plates, for example to calculate inter-assay and intra-assay CV, as well as for troubleshooting. When including the Sample Controls, 88 to 352 user samples can be processed simultaneously.
- 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.
- Consult the Olink white paper "Strategies for design of protein biomarker studies" (*www.olink.com/whitepapers*) and the sample randomization guidelines (*www.olink.com/faq/sample-randomization*).

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

3.1.2 Important information

Panel nomenclature

As the panels can be combined in different ways, the panel names are not mentioned and are instead referred to as Panel 1, 2, 3, and 4 throughout the instructions. Before running Olink Explore 4 x 384, decide which panel shall be Panel 1, 2, 3 and 4, respectively.

IMPORTANT: *It is crucial to keep this order throughout the entire workflow. Mixing up the panels will lead to a failed run.*

Throughout the instructions, plates and tubes are marked with names and numbers, for example "Incubation Plate 1". When using the marked plates and tubes, make sure that the number on the plate or tube corresponds to the number of the applicable panel, i.e when running Panel 1, use XX Plate 1, and when running Panel 4, use XX Plate 4.

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.

Vortexing

Correct vortexing is essential to generate reproducible results. Review instructions in the Olink Explore Overview User Manual prior to starting the laboratory workflow.

384-well PCR plates

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

3.1.3 Prepare the samples

During this step, samples are manually transferred to the Sample Plate(s) (*Figure 1*). The Sample Plate(s) must be used within the same day of preparation.

The number of Sample Plate(s) required depends on how the panels are combined. One Sample Plate contains a sufficient sample volume for the preparation of four panels, but when running multiple panels of the same type (e.g 4x Olink Explore Inflammation panel), separate Sample Plates are required in order to not perform the same analysis several times on the

same samples.

NOTE: *The Olink Explore protocol is optimized and validated for plasma and serum samples. Contact support@olink.com for more information regarding alternative sample types.*

Prepare bench

- Sample Plates (provided by the user)
- 1-4x 96-well PCR plate(s), preferably with full skirt
- Manual pipette (0.5-10 µL)
- Filter pipette tips
- Adhesive Film
- 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.
- Mark the 96-well Sample Plate(s) with a simple alphanumeric code that you can later identify (“A, B, C”, “1, 2, 3”, or “A1, A2, A3”) using temperature-resistant labels or marker pen. Use unique sample identification names or numbers.

Instructions

18. Transfer the samples into the Sample Plate(s), according to the plate layout shown in *Figure 1*. Make sure that samples are added to every applicable well.
19. Seal the Sample Plate(s) using an adhesive film or individual seals.
20. Store the Sample Plate(s) at +4 °C if used the same day, otherwise at -80 °C.

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

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	9	17	25	33	41	49	57	65	73	81	
B	2	10	18	26	34	42	50	58	66	74	82	
C	3	11	19	27	35	43	51	59	67	75	83	
D	4	12	20	28	36	44	52	60	68	76	84	
E	5	13	21	29	37	45	53	61	69	77	85	
F	6	14	22	30	38	46	54	62	70	78	86	
G	7	15	23	31	39	47	55	63	71	79	87	
H	8	16	24	32	40	48	56	64	72	80	88	

Figure 20. Sample Plate layout. The numbers indicate the sample numbers.

3.2 Prepare Sample Source Plate (day 1)

There is one sample source plate per sample plate. During this step, samples are manually transferred from the prepared Sample Plate(s) into the Sample Source Plate(s), and controls are added to the Sample Source Plate. The Sample Source Plate must be used within the same day of preparation.

The number of Sample Plate(s) required depends on how the panels are combined in the run, as explained in *2.1.3 Prepare the samples.* The preparation of more than one Sample Plate must be performed in separate runs. Perform the instruction once for every Sample Plate required, i.e in one to four runs.

IMPORTANT: *Using the correct combination of Plate Control and Probes lots is essential for the downstream data analysis. Before starting the workflow, ensure that the lot number for each Probe kit and for the Plate Control matches the lot numbers stated in the Lot Configuration document provided with the kit.*

Prepare bench

- Sample Plate, prepared in previous step
- Olink Explore Negative Control
- Olink Explore Plate Control
- Sample Control (optional, provided by the user)
- 1–4x 384-well PCR plate(s) (skirted): same as the number of 96-well Sample Plates used
- Manual pipette (0.5–10 µL)
- Manual multichannel pipette (0.5–10 µL)
- Filter pipette tips
- Adhesive films

Before you start

- Ensure that the Sample Plate(s) have been prepared according to *2.1.3 Prepare the samples*.
- If frozen, thaw the Sample Plate(s) at room temperature and mark the plates: "Sample Plate [Panel no:s]".
- Thaw the Negative Control, Plate Control and recommended Sample Control at room temperature.
- Mark one 384-well PCR plate per run: “Sample Source Plate [Panel no:s]”.

NOTE: *If preparing more than one 384-well Sample Source Plate, make sure that the 96-well Sample Plate and 384-well Sample Source Plate are marked with the same number, and keep track of which Panel(s) each 384-well Sample Source Plate shall be used for.*

Instructions

1. Using the MixMate® or manual vortexing, vortex the applicable 96-well Sample Plate and spin at 400-1000 x g for 1 minute at room temperature.
2. Using a multichannel pipette, transfer 10 µL of each sample into the applicable 384-well Sample Source Plate according to the plate layout shown in (*Figure 2*). Use forward pipetting, change pipette tips between every column, and make sure that no air bubbles are trapped at the bottom of the wells.

IMPORTANT: *The plate layout of the Sample Source Plate has changed and is only compatible with version 4 of the Olink Explore instrument protocols. Make sure that you have version 4 of all instrument protocols installed before proceeding. Please contact support@olink.com if you need assistance.*

3. Vortex the Negative Control, Plate Control and Sample Control and spin briefly.
4. Using a single-channel pipette, transfer 10 µL of Negative Control (NC), Plate Control (PC) and Sample Control (SC) to each of the applicable wells in column 23 of the 384-well Sample Source Plate according to the plate layout shown in *Figure 2*. Change pipette tip between every control sample.
5. Seal the Sample Source Plate with a new adhesive film.

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

6. 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).
7. Seal the Sample Plate with a new adhesive film. Store at -80 °C.
8. Repeat the instruction as necessary to prepare any additional Sample Source Plate(s) (384-well) to be used the same day.

NOTE: *Freeze thawing of Sample Source Plates (384-well) for future use is not recommended.*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	1		9		17		25		33		41		49		57		65		73		81		SC	
B																								
C	2		10		18		26		34		42		50		58		66		74		82		SC	
D																								
E	3		11		19		27		35		43		51		59		67		75		83		NC	
F																								
G	4		12		20		28		36		44		52		60		68		76		84		NC	
H																								
I	5		13		21		29		37		45		53		61		69		77		85		NC	
J																								
K	6		14		22		30		38		46		54		62		70		78		86		PC	
L																								
M	7		15		23		31		39		47		55		63		71		79		87		PC	
N																								
O	8		16		24		32		40		48		56		64		72		80		88		PC	
P																								

Figure 21. Sample Source Plate layout.
The numbers indicate the sample numbers. SC = Sample Control, NC = Negative Control, PC = Plate Control.

3.3 Sample Dilution

During this step, Sample Diluent is dispensed into Sample Dilution Plate(s) using the Dragonfly® and the samples are diluted using the Mosquito®. The prepared Sample Dilution Plate(s) must be used within one hour from preparation.

Perform the instruction once for every Sample Source Plate prepared in the previous step, i.e. one to four times. It is not possible to prepare multiple plates at the same time.

3.3.1 Prepare Sample Dilution Plate

During this step, Sample Diluent is dispensed into Sample Dilution Plate(s) using the Dragonfly®.

Prepare bench

- Olink Target 96/Explore Sample Diluent
- 1-4x 384-well PCR plate(s) (skirted)
- 1x Disposable reservoir (10 mL)
- 1x Disposable ultra-low retention syringe with plunger
- Manual pipette (100–1000 µL)
- Filter pipette tips
- Adhesive films

Before you start

- Thaw the Sample Diluent at room temperature.
- Mark one new 384-well PCR plate per run: “Sample Dilution Plate [Panel no:s]”.

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

Instructions

1. Prepare the Dragonfly® according to instructions in the Olink Explore Overview User Manual.

– Use the protocol Olink Sample Dilution Plate v4.

IMPORTANT: *Make sure that the correct protocol version is used. The plate layout has changed and using an older version of the protocol will result in unusable data. Please contact support@olink.com if you need assistance.*

– Attach one syringe in position B2.

2. Slide the reservoir tray to the filling position (A) and place a new reservoir in position B2 (B) (*Figure 3*).

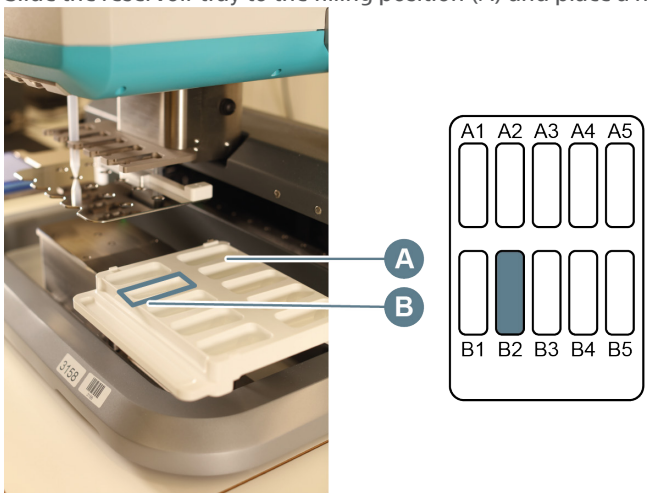

Figure 22. Disposable reservoir in reservoir tray of the Dragonfly.

3. Vortex the Sample Diluent briefly, add 6 mL of Sample Diluent into the reservoir and carefully slide the reservoir tray back to the aspirate position.
4. Place the Sample Dilution Plate in the plate position to the left, with well A1 in the top left corner.
5. Select the Run tab in the Constant layer view of the software (*Figure 4*), then click RUN to start the program.
   Result: Dragonfly® dispenses 9 µL Sample Diluent into each well of quadrant 1-3 and 29 µL in quadrant 4 of the Sample Dilution Plate. Filled wells turn purple in the plate map. The run stops automatically when the Dragonfly® has dispensed Sample Diluent into all applicable wells.

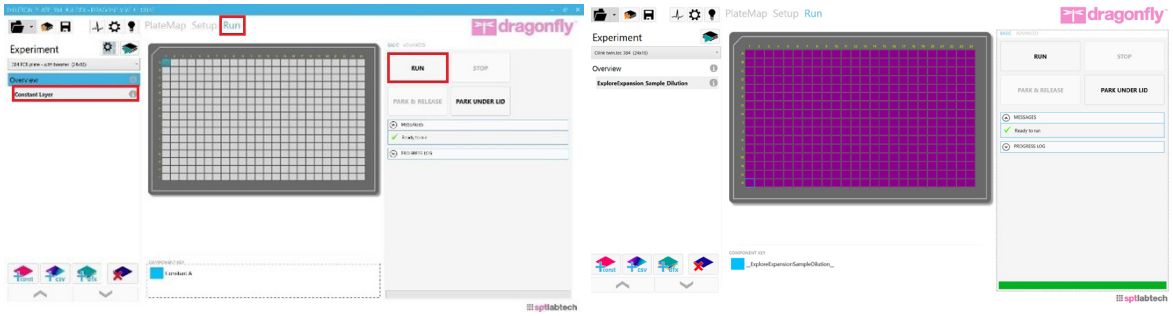

Figure 23. Run tab and Run button (left). Filled Plate (right).

6. When the Dragonfly® has returned the Sample Dilution Plate to the plate position and released the plate clamp, remove the Sample Dilution Plate from the instrument and seal it with an adhesive film.
7. Spin the Sample Dilution Plate at 400–1000 x g for 1 minute.
8. Visually inspect the Sample Dilution Plate to ensure that all wells in quadrant 1-3 contain 9 µL of liquid, and that wells in quadrant 4 contain 29 µL. Make sure that there are no bubbles trapped at the bottom of the wells.
9. Repeat the steps 4–8 as necessary to prepare any additional Sample Dilution Plate(s).
10. When finished, clear the instrument and shut it down according to instructions in the Olink Explore Overview User Manual.
11. Continue to *2.3.2 Perform Sample Dilution*, or store the Sample Dilution Plate(s) at +4 °C until use (the same day).

3.3.2 Perform Sample Dilution

During this step, the samples are diluted in four sequential steps using the Mosquito®: from 1:1 (undiluted) to 1:10, 1:100, 1:1000, and approximately 1:100 000 (*Figure 6*).

Perform the instruction once per Sample Dilution Plate prepared in the previous step, i.e. one to four times. It is not possible to prepare multiple plates at the same time.

Prepare bench

- Sample Source Plate(s), prepared in previous step
- Sample Dilution Plate(s), prepared in previous step
- Adhesive films

Before you start

- Prepare the Mosquito® according to instructions in the Olink Explore Overview User Manual. Use the protocol Olink Sample Dilution 3072 v4.

IMPORTANT: *Make sure that the correct protocol version is used. The plate layout has changed and using an older version of the protocol will result in unusable data. Please contact support@olink.com if you need assistance.*

- Make sure that a spool of Mosquito® pipette tips has been loaded into the Mosquito® according to the manufacturer's instructions.
- Make sure that the knobs at the back of the two Mosquito® humidifiers are set to maximum level.

Instructions

IMPORTANT: *Make sure to vortex the Sample Dilution Plate(s) thoroughly both between dilutions and after the last dilution.*

1. Let the Sample Source Plate reach room temperature, vortex it briefly and spin down at 400–1000 x g for 1 minute. 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 Plate.
3. Place the Sample Source Plate in a magnetic clamp booster according to instructions in the Olink Explore Overview User Manual.
4. Place the Sample Source Plate on the Mosquito® deck, refer to the software for the correct position. Insert the corner of the magnetic clamp booster under the small metal holder of the deck as described in the Olink Explore Overview User Manual (this applies to all plate positions).
5. Place the Sample Dilution Plate in a magnetic clamp booster and place it on the Mosquito® deck, refer to the software for the correct position.

IMPORTANT: *Risk of instrument damage! If the magnetic clamp booster is not correctly placed it may collide with the internal parts of the instrument during the run.*

6. Click Run to start the Mosquito® program Olink Sample Dilution 3072.
   Result: Mosquito® performs a 1:10 dilution by transferring 1 µL of sample from the Sample Source Plate into quadrant 1 in the Sample Dilution Plate. The run can be monitored in the protocol tab.
7. When the Mosquito® protocol is paused and a pop-up window appears on the screen (*Figure 5*), remove the Sample Source Plate and the Sample Dilution Plate from the Mosquito® deck and from the magnetic clamp boosters.
8. Seal both plates with new adhesive films and store the Sample Source Plate at +4 °C for later use.

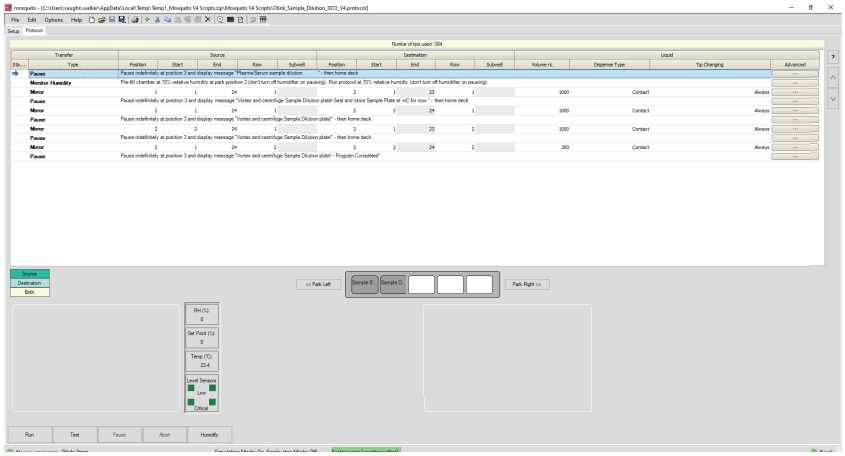

Figure 24. Pop-up window during Mosquito dilution protocol.

9. Using the MixMate® or manual vortexing, vortex the Sample Dilution Plate thoroughly to ensure that all wells are mixed.
10. Spin at 400–1000 x g for 1 minute. Make sure that there are no bubbles trapped at the bottom of the wells.
11. Carefully remove the adhesive film from the Sample Dilution Plate.
12. Place the Sample Dilution Plate back in the magnetic clamp booster and return it to the correct position of the Mosquito® deck. All other positions shall remain empty.
13. Click Resume to continue the program.
    Result: Mosquito® performs a 1:100 sample dilution by transferring 1 µL of diluted samples (1:10) into quadrant 2 of the Sample Dilution Plate.
14. When the Mosquito® protocol is paused, and a pop-up window appears on the screen, remove the Sample Dilution Plate from the Mosquito® deck and the magnetic clamp booster. Seal with a new adhesive film.
15. Repeat steps 9–14 to perform the third dilution (1:1000).
    Result: Mosquito® performs a 1:1000 sample dilution by transferring 1 µL of diluted samples (1:100) into quadrant 3 of the Sample Dilution Plate.
16. Repeat steps 9-14 again to perform the last dilution (1:100 000).
    Result: Mosquito® performs a 1:100 000 sample dilution by transferring 0.3 µL of diluted samples (1:1000) into quadrant 4 of the Sample Dilution Plate.
17. Vortex the Sample Dilution Plate thoroughly Using the MixMate® or manual vortexing, and ensure that all wells are mixed.
18. Spin at 400–1000 x g for 1 minute. Make sure that there are no bubbles trapped at the bottom of the wells.
19. When finished, click Resume to end the Mosquito® program.
20. Repeat the instruction as necessary to prepare any additional Sample Dilution Plate(s). Keep the Mosquito® on for later use.
21. Continue to *2.10 Library purification*, or place the Sample Dilution Plate at +4 °C for up to 1 hour.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	1	1	9	9	17	17	25	25	33	33	41	41	49	49	57	57	65	65	73	73	81	81	SC	SC
B	1	1	9	9	17	17	25	25	33	33	41	41	49	49	57	57	65	65	73	73	81	81	SC	SC
C	2	2	10	10	18	18	26	26	34	34	42	42	50	50	58	58	66	66	74	74	82	82	SC	SC
D	2	2	10	10	18	18	26	26	34	34	42	42	50	50	58	58	66	66	74	74	82	82	SC	SC
E	3	3	11	11	19	19	27	27	35	35	43	43	51	51	59	59	67	67	75	75	83	83	NC	NC
F	3	3	11	11	19	19	27	27	35	35	43	43	51	51	59	59	67	67	75	75	83	83	NC	NC
G	4	4	12	12	20	20	28	28	36	36	44	44	52	52	60	60	68	68	76	76	84	84	NC	NC
H	4	4	12	12	20	20	28	28	36	36	44	44	52	52	60	60	68	68	76	76	84	84	NC	NC

I	5	5	13	13	21	21	29	29	37	37	45	45	53	53	61	61	69	69	77	77	85	85	NC	NC
J	5	5	13	13	21	21	29	29	37	37	45	45	53	53	61	61	69	69	77	77	85	85	NC	NC
K	6	6	14	14	22	22	30	30	38	38	46	46	54	54	62	62	70	70	78	78	86	86	PC	PC
L	6	6	14	14	22	22	30	30	38	38	46	46	54	54	62	62	70	70	78	78	86	86	PC	PC
M	7	7	15	15	23	23	31	31	39	39	47	47	55	55	63	63	71	71	79	79	87	87	PC	PC
N	7	7	15	15	23	23	31	31	39	39	47	47	55	55	63	63	71	71	79	79	87	87	PC	PC
O	8	8	16	16	24	24	32	32	40	40	48	48	56	56	64	64	72	72	80	80	88	88	PC	PC
P	8	8	16	16	24	24	32	32	40	40	48	48	56	56	64	64	72	72	80	80	88	88	PC	PC

Figure 25. Sample Dilution Plate layout.
The numbers indicate the sample numbers. SC = Sample Control, NC = Negative Control and PC = Plate Control.

3.4 Incubation

During this step, four Incubation Mixes are prepared manually for each panel, transferred to Reagent Source Plates, and mixed with the samples. Incubation is then performed overnight.

Since one Reagent Source Plate is used per panel, perform the instructions four times. As the Incubation Mixes must be used within 30 minutes from preparation. Prepare one Reagent Source Plate and two Incubation Plates at a time.

1. Run 1: Prepare Incubation Mixes for Panel 1, transfer to Reagent Source Plate 1, then prepare Incubation Plate 1 and start incubation.
2. Run 2: Prepare Incubation Mixes for Panel 2, transfer to Reagent Source Plate 2, then prepare Incubation Plate 2 and start incubation.
3. Run 3: Prepare Incubation Mixes for Panel 3, transfer to Reagent Source Plate 3, then prepare Incubation Plate 3 and start incubation.
4. Run 4: Prepare Incubation Mixes for Panel 4, transfer to Reagent Source Plate 4, then prepare Incubation Plate 4 and start incubation.

3.4.1 Prepare Reagent Source Plate

During this step, four Incubation Mixes are prepared manually for each panel and transferred to a Reagent Source Plate. Each mix contains a specific set of Forward and Reverse probes.

IMPORTANT: *Using the correct combination of Plate Control and Probes lots is essential for the downstream data analysis. Before starting the workflow, ensure that the lot number for each Probe kit and or the Plate Control matches the lot numbers stated in the Lot Configuration document provided with the kit.*

Prepare bench

- Olink Explore Incubation Solution
- Olink Explore Forward Probes A-D (Frw probes)
- Olink Explore Reverse Probes A-D (Rev probes)
- 4x 8-well strip
- 4x 384-well PCR plate (skirted)
- Manual pipettes (0.5–10, 10–100 µL)
- Manual multichannel pipette (8-channel, 0.5–10 µL)
- Filter pipette tips
- Adhesive films

Before you start

- Allow the Incubation Solution, Forward Probes and Reverse Probes to reach room temperature.

- Mark four new 384-well PCR plates: “Reagent Source Plate [1–4]”.
- Mark the wells of four 8-well strips according to *Table 4.*

Table 14. PCR strips

Run	Well							
	1	2	3	4	5	6	7	8
1	1A	1B	1C	1D	-	-	-	-
2	2A	2B	2C	2D	-	-	-	-
3	3A	3B	3C	3D	-	-	-	-
4	4A	4B	4C	4D	-	-	-	-

Instructions

1. Vortex the Incubation Solution, as well as Forward Probes and Reverse Probes and spin down.
2. Prepare four Incubation Mixes in the PCR Strip 1: Using forward pipetting, first transfer the Incubation Solution to tubes 1–4 of the strip, then all Frw Probes to the applicable tubes of the strip, and last all Rev Probes to the applicable tubes of the strip according to *Table 5*.

NOTE: *Pipette the Incubation Solution carefully to avoid foaming.*

IMPORTANT: *The Incubation Solution will be used to prepare Incubation Mixes for the other panels. Do not discard.*

Table 15. Incubation Mix preparation

Addition order	Reagent	Well (of the applicable strip 1-4)							
		A	B	C	D	-	-	-	-
1	Incubation Solution	80 µL	80 µL	80 µL	80 µL	–	–	–	–
2	Frw probes	10 µL	10 µL	10 µL	10 µL	–	–	–	–
		[PANEL] Frw Probes A	[PANEL] Frw Probes B	[PANEL] Frw Probes C	[PANEL] Frw Probes D	–	–	–	–
3	Rev probes	10 µL	10 µL	10 µL	10 µL	–	–	–	–
		[PANEL] Rev Probes A	[PANEL] Rev Probes B	[PANEL] Rev Probes C	[PANEL] Rev Probes D	–	–	–	–

3. Seal the PCR Strip 1 with caps. Vortex and spin down briefly. Store at room temperature until use.

IMPORTANT: *Make sure that all applicable reagents are added to each Incubation Mix. The final volume shall be 100 µL*

TIME SENSITIVE STEP: *The incubation setup using the Mosquito® must be started within 30 minutes from preparation of the Incubation Mix.*

4. Using a multichannel pipette, transfer 10 µL of each Incubation Mix from the PCR Strip 1 to the Reagent Source Plate 1, according to the plate layout in *Figure 8.* Use reverse pipetting and do not change pipette tips between wells. Make sure to pipette to the bottom of the wells.

NOTE: *Rotate the Sample Reagent Plate (384-well) 90 degrees clockwise to facilitate pipetting*

(shown to the right of Figure 7).

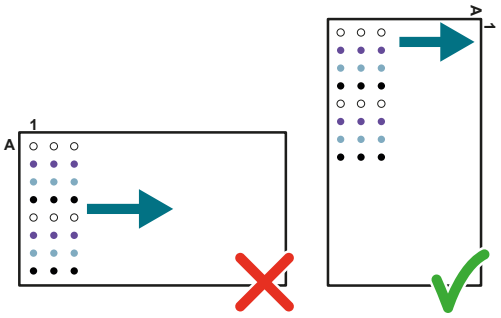

Figure 26. Rotating the Sample Reagent Plate

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	1A		1B		1C		1D																	
B																								
C	1A		1B		1C		1D																	
D																								
E	1A		1B		1C		1D																	
F																								
G	1A		1B		1C		1D																	
H																								
I	1A		1B		1C		1D																	
J																								
K	1A		1B		1C		1D																	
L																								
M	1A		1B		1C		1D																	
N																								
O	1A		1B		1C		1D																	
P																								

Figure 27. Reagent Source Plate layout.
The numbers indicates the Panel number and the letters indicate the different blocks within the panel.

5. Seal the Reagent Source Plate 1 with a new adhesive film and spin at 400–1000 x g for 1 minute. Make sure that there are no bubbles trapped at the bottom of the wells.

6. Immediately continue to *2.4.2 Prepare Incubation Plates and perform incubation*.

3.4.2 Prepare Incubation Plates and perform incubation

During this step, the prepared Incubation Mixes are mixed with the samples in one Incubation Plate per panel, using the Mosquito® prior to incubation (*Figure 9*).

As the Mosquito® deck can only hold a limited number of plates, perform the instruction four times, once for each Run (one Mosquito® run per Reagent Source Plate).

Prepare bench

- Sample Source Plate(s), prepared in previous step
- Sample Dilution Plate(s), prepared in previous step
- Reagent Source Plates 1-4, prepared in previous step
- 4x 384-well PCR plates (skirted)
- Adhesive films

Before you start

- Allow the refrigerated Sample Source Plate(s) and Sample Dilution Plate(s) to reach room temperature.
- Mark four new 384-well PCR plates: “Incubation Plate [1–4]”.

Instructions

1. In the open Mosquito® software, Select *File/Open* and choose the applicable protocol from the Load Protocol Window:
   - CARDIO: *Olink Incubation CARDIO v4*
   - INF*: Olink Incubation INF v4*
   - NEURO: *Olink Incubation NEURO v4*
   - ONC: *Olink Incubation ONC v4*
   - CARDIO II: *Olink Incubation CARDIO II v4*
   - INF II: *Olink Incubation INF II v4*
   - NEURO II: *Olink Incubation NEURO II v4*
   - ONC II: *Olink Incubation ONC II v4*

IMPORTANT: *Make sure that the correct protocol and version is used. The plate layouts of the sample source plate and sample dilution plate have changed and using an older version of the protocol will result in unusable data. Please contact support@olink.com if you need assistance.*

NOTE: *Make sure that all the plates are marked with the same panel number.*

2. Place the Incubation Plate 1 in a magnetic clamp booster and place it on the Mosquito® deck, refer to the software for the correct position. Make sure to insert the corner of the magnetic clamp booster under the small metal holder of the deck.

IMPORTANT: *Risk of instrument damage! If a magnetic clamp booster is not correctly placed it may collide with the internal parts of the instrument during the run.*

3. Carefully remove the adhesive film from the Reagent Source Plate 1.
4. Place the Reagent Source Plate 1 in a magnetic clamp booster and place it on the Mosquito® deck. Refer to the software for the correct position.
5. Click *Run*.
   Result: The Mosquito® transfers 0.6 µL of each Incubation Mix from the Reagent Source Plate into the Incubation Plate. The run takes approximately 10 minutes to be completed and can be monitored in the protocol tab.

6. When the Mosquito® protocol is paused, and a pop-up window appears on the screen, remove the Incubation Plate 1 from the Mosquito® deck and from the magnetic clamp booster.
7. Inspect the Incubation Plate to ensure that there is liquid at the bottom of all wells, and that there are no bubbles present.

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

NOTE: *If bubbles are present, seal the Incubation Plate(s) with new adhesive film(s) and spin at 400–1000 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.*

8. Place Incubation Plate 1 back in the magnetic clamp boosters and return it to its previous position on the Mosquito® deck.
9. Vortex the applicable Sample Source Plate and Sample Dilution Plate briefly and spin down at 400–1000 x g for 1 minute. Make sure that there are no bubbles trapped at the bottom of the wells and carefully remove the adhesive films.

NOTE: *Make sure that all plates are marked with the same panel number.*

10. Place the Sample Source Plate and Sample Dilution Plate in magnetic clamp boosters and place them on the Mosquito® deck. Refer to the software for the correct positions.
11. Click *Resume* to continue the protocol.
    Result: The Mosquito® transfers 0.2 µL of samples from the Sample Source Plate and Sample Dilution Plate into the Incubation Plate. The run takes approximately 15 min to be completed.
12. When the Mosquito® protocol is finished, remove Incubation Plate 1 from the Mosquito® deck and from the magnetic clamp boosters. Seal with a new adhesive film.

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

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

13. Spin Incubation Plate 1 at 400–1000 x g for 1 minute.
14. Inspect Incubation Plate 1 to ensure that all wells contain the same amount of liquid (0.8 µL). Note if there are any wells containing bubbles.
15. Write the time of the incubation starting point on the plate seal and incubate Incubation Plate 1 for 16 to 24 hours at +4 °C.

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 PCR1 in the ProFlex™ PCR instrument.*

16. Remove the Sample Source Plate, Sample Dilution Plate, and Reagent Source Plate from the Mosquito® deck. Treat the plates as described in *Table 6*.

Table 16. Plates handling

Incubation Run	Sample Source Plate	Sample Dilution Plate	Reagent Source Plate
Run 1	Seal with adhesive film Store at +4 °C for later use	Seal with adhesive film Store at +4 °C for later use	Discard
Run 2	Seal with adhesive film Store at +4 °C for later use	Seal with adhesive film Store at +4 °C for later use	Discard
Run 3	Seal with adhesive film Store at +4 °C for later use	Seal with adhesive film Store at +4 °C for later use	Discard
Run 4	Seal with adhesive film Store at -80 °C for later use	Discard	Discard

17. Repeat *2.4.1 Prepare Reagent Source Plate* and *2.4.2 Prepare Incubation Plates and perform incubation* for Run 2, 3 and 4.
18. When finished, clear the Mosquito® and shut it down according to instructions in the Olink Explore Overview User Manual.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	1	1	9	9	17	17	25	25	33	33	41	41	49	49	57	57	65	65	73	73	81	81	SC	SC
B	1	1	9	9	17	17	25	25	33	33	41	41	49	49	57	57	65	65	73	73	81	81	SC	SC
C	2	2	10	10	18	18	26	26	34	34	42	42	50	50	58	58	66	66	74	74	82	82	SC	SC
D	2	2	10	10	18	18	26	26	34	34	42	42	50	50	58	58	66	66	74	74	82	82	SC	SC
E	3	3	11	11	19	19	27	27	35	35	43	43	51	51	59	59	67	67	75	75	83	83	NC	NC
F	3	3	11	11	19	19	27	27	35	35	43	43	51	51	59	59	67	67	75	75	83	83	NC	NC
G	4	4	12	12	20	20	28	28	36	36	44	44	52	52	60	60	68	68	76	76	84	84	NC	NC
H	4	4	12	12	20	20	28	28	36	36	44	44	52	52	60	60	68	68	76	76	84	84	NC	NC
I	5	5	13	13	21	21	29	29	37	37	45	45	53	53	61	61	69	69	77	77	85	85	NC	NC
J	5	5	13	13	21	21	29	29	37	37	45	45	53	53	61	61	69	69	77	77	85	85	NC	NC
K	6	6	14	14	22	22	30	30	38	38	46	46	54	54	62	62	70	70	78	78	86	86	PC	PC
L	6	6	14	14	22	22	30	30	38	38	46	46	54	54	62	62	70	70	78	78	86	86	PC	PC
M	7	7	15	15	23	23	31	31	39	39	47	47	55	55	63	63	71	71	79	79	87	87	PC	PC
N	7	7	15	15	23	23	31	31	39	39	47	47	55	55	63	63	71	71	79	79	87	87	PC	PC
O	8	8	16	16	24	24	32	32	40	40	48	48	56	56	64	64	72	72	80	80	88	88	PC	PC
P	8	8	16	16	24	24	32	32	40	40	48	48	56	56	64	64	72	72	80	80	88	88	PC	PC

Figure 28. Incubation Plate layout.
The numbers indicate the sample numbers. The colors indicate the different blocks within the panel: white = block A, purple = block B, blue = block C, black = block D.

3.5 Preparation of reagents for day 2

Prepare bench

- MilliQ water
- Olink® Explore PCR1 Enhancer
- Olink® Explore PCR1 Solution

Instructions

1. Place 30 mL of MilliQ water at +4 °C overnight.
2. Thaw the PCR1 Enhancer at room temperature overnight.
3. Optional: Thaw the PCR1 Solution at +4 °C overnight.

3.6 Extension and pre-amplification (PCR1) (day 2)

During this step, a PCR1 Mix is prepared manually and dispensed into the Incubation Plates using the Dragonfly®. The plates are renamed “PCR1 Plates” and subjected to a PCR reaction.

3.6.1 Prepare PCR1 Mix

During this step, a PCR1 Mix is prepared manually. The PCR1 Mix must be used within 30 minutes from preparation.

Prepare bench

- Incubation Plates 1–4, prepared in previous step
- Olink® Explore PCR1 Enhancer
- Olink® Explore PCR1 Solution
- Olink® Explore PCR1 Enzyme (when brought to the lab bench, keep the PCR1 Enzyme in a freezing block (-20 °C))
- MilliQ water (at +4 °C, preferably kept in the fridge until use)
- 1x Falcon tube (50 mL)

Before you start

- Thaw the PCR1 Solution at +4 °C overnight (optional), or at room temperature before starting the PCR1 Setup (it may take several hours) and ensure that it is fully thawed and has reached room temperature.
- Ensure that the PCR1 Enhancer is fully thawed and has reached room temperature.
- Let the refrigerated Incubation Plates 1–4 reach room temperature.
- Mark the new 50 mL Falcon tube: "PCR1 Mix".
- Switch on two ProFlex™ PCR instruments in the post-PCR room.

Instructions

1. Vortex the PCR1 Solution and PCR1 Enhancer thoroughly and spin down briefly.

IMPORTANT: *Insufficient thawing and vortexing of the PCR1 Enhancer may lead to particles attaching to the syringe and run failure.*

2. Spin the PCR1 Enzyme briefly. Do not vortex.

NOTE: *Always keep the PCR1 Enzyme at -20 °C. When brought to the lab bench, keep it in a freezing block (-20 °C).*

3. Spin Incubation Plates 1–4 down for 1 minute at room temperature.
4. Prepare a PCR1 Mix in a Falcon tube in the following order, using the volumes indicated in *Table 7*:
    1. Add MilliQ water and PCR1 Enhancer and vortex thoroughly.
    2. Add the PCR1 Solution and PCR1 Enzyme, and vortex for 3 seconds.

Table 17. PCR1 Mix

Addition order	Reagent	Volume (µL)
1	MilliQ water (+4 °C)	27 000
2	PCR1 Enhancer	3 510
3	PCR1 Solution	3 510
4	PCR1 Enzyme	351
	Total	34 371

    3. Keep at room temperature until use.

TIME SENSITIVE STEP: *Dispensing of the PCR1 Mix using the Dragonfly® must start within 30 minutes from PCR1 Mix preparation.*

3.6.2 Prepare PCR1 Plates and perform PCR1

During this step, the prepared PCR1 Mix is dispensed into the Incubation Plates using the Dragonfly®, and the plates are subjected to a PCR reaction (*Figure 13*).

The PCR1 Plates must be placed in the ProFlex™ exactly 10 minutes after the PCR1 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 PCR1 Mix into Incubation Plates 1 and 2 using the Dragonfly®. Start a PCR1 run for these plates on one of the ProFlex™ instruments.
2. Dispense PCR1 Mix into Incubation Plates 3 and 4 plates using the Dragonfly®. Start a PCR1 run for these plates on the second ProFlex™ instrument.

Prepare bench

- Incubation Plates 1–4 at room temperature, prepared in previous step
- PCR1 Mix, prepared in previous step
- 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 a timer to 10 minutes.

Instructions

1. Start the PCR program *Olink PCR1* on the two ProFlex™ PCR instruments (*Figure 10*). Pause when the PCR block temperature reaches 50 °C.

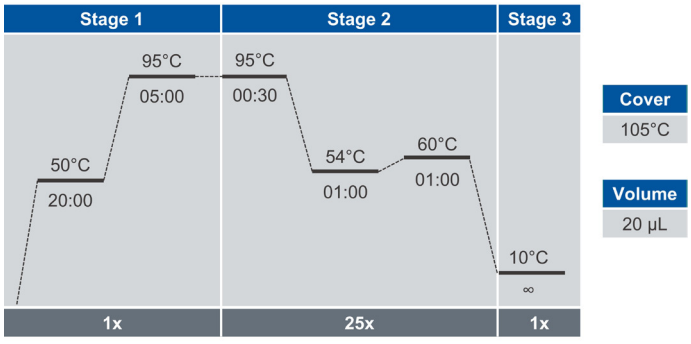

Figure 29. Olink PCR1 program

2. Prepare the Dragonfly® according to instructions in the Olink Explore Overview User Manual.
   - Use the protocol *Olink PCR1 Setup plate v4.*
   - Attach three syringes in position B2, B3, and B4.
3. Slide the reservoir tray to the filling position (A) and place three new reservoirs in position B2, B3 and B4 (B) (*Figure 11*).

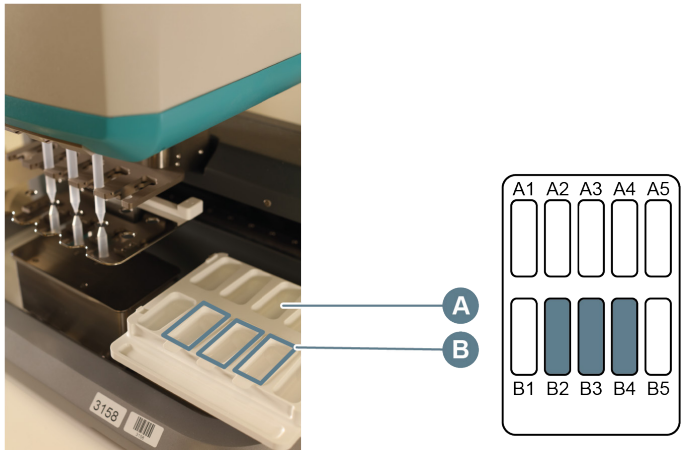

Figure 30. Reservoir tray in filling position

4. Transfer 11 mL of PCR1 Mix into each of the three reservoirs.

IMPORTANT: *The syringes and PCR1 Mix are used to prepare all four PCR1 Plates. Do not discard until all PCR1 Plates are complete.*

5. Carefully slide the reservoir tray back to the aspirate position (*Figure 12*).

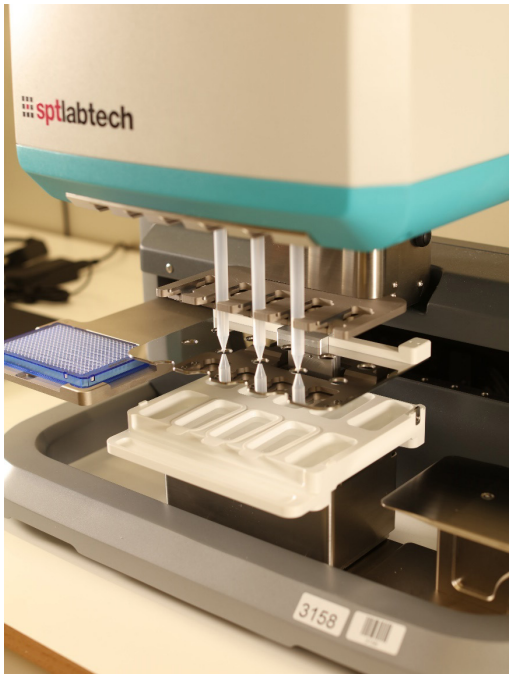

Figure 31. Reservoir tray in aspirate position

6. Rename Incubation Plate 1 “PCR1 Plate 1” and Incubation Plate 2 “PCR1 Plate 2”. Spin down the plates.
7. Carefully remove the adhesive film from PCR1 Plate 1 and place it in the Dragonfly® plate position (to the left), with well A1 in the top left corner.
8. Select the *Run* tab in the *Constant layer* view of the software, then click *RUN* to start the program. Start the timer when PCR1 Mix is added to the first well of the plate.
   Result: Dragonfly® dispenses 19 µL of PCR1 Mix into each well of the PCR1 Plate 1.

TIME SENSITIVE STEP: *The PCR1 Plate must be placed in the ProFlex™ exactly 10 minutes after the PCR1 Mix has been added to the first well of the first plate.*

9. When the Dragonfly® has returned the PCR1 Plate 1 to the plate position and released the plate clamp, remove the plate from the instrument and immediately seal it with a new adhesive film.
10. Inspect the PCR1 Plate 1 to ensure that all wells contain the same amount of liquid (19.8 µL). Using the MixMate® or manual vortexing, vortex the plate thoroughly to ensure that all wells are mixed.
11. Repeat steps 7–10 for PCR1 Plate 2.
12. In the Post-PCR room, centrifuge the two PCR1 Plates 1-2 at 400–1000 x g for 1 minute.
13. Inspect PCR1 Plates 1–2 to ensure that all wells contain the same amount of liquid (19.8 µL). Note any deviations.
14. When the timer ends after 10 minutes, place PCR1 Plates 1–2 in one of the two pre-heated ProFlex™ instruments and click *Resume* the run the *Olink PCR1* program.
15. Repeat steps 6-14 for Incubation Plates 3–4, except rename Incubation Plate 3 as "PCR Plate 3, and Incubation Plate 4 as "PCR Plate 4". Place both plates in the second ProFlex™.
16. When finished, clear the Dragonfly® and shut it down according to instructions in the Olink Explore Overview User Manual.

17. When the *Olink PCR1* programs are finished (~1 hour 55 minutes), continue to *3.7 Pool PCR1 products*, or store the four PCR1 Plates at +4 °C if used the same day.

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

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	1	1	9	9	17	17	25	25	33	33	41	41	49	49	57	57	65	65	73	73	81	81	SC	SC
B	1	1	9	9	17	17	25	25	33	33	41	41	49	49	57	57	65	65	73	73	81	81	SC	SC
C	2	2	10	10	18	18	26	26	34	34	42	42	50	50	58	58	66	66	74	74	82	82	SC	SC
D	2	2	10	10	18	18	26	26	34	34	42	42	50	50	58	58	66	66	74	74	82	82	SC	SC
E	3	3	11	11	19	19	27	27	35	35	43	43	51	51	59	59	67	67	75	75	83	83	NC	NC
F	3	3	11	11	19	19	27	27	35	35	43	43	51	51	59	59	67	67	75	75	83	83	NC	NC
G	4	4	12	12	20	20	28	28	36	36	44	44	52	52	60	60	68	68	76	76	84	84	NC	NC
H	4	4	12	12	20	20	28	28	36	36	44	44	52	52	60	60	68	68	76	76	84	84	NC	NC
I	5	5	13	13	21	21	29	29	37	37	45	45	53	53	61	61	69	69	77	77	85	85	NC	NC
J	5	5	13	13	21	21	29	29	37	37	45	45	53	53	61	61	69	69	77	77	85	85	NC	NC
K	6	6	14	14	22	22	30	30	38	38	46	46	54	54	62	62	70	70	78	78	86	86	PC	PC
L	6	6	14	14	22	22	30	30	38	38	46	46	54	54	62	62	70	70	78	78	86	86	PC	PC
M	7	7	15	15	23	23	31	31	39	39	47	47	55	55	63	63	71	71	79	79	87	87	PC	PC
N	7	7	15	15	23	23	31	31	39	39	47	47	55	55	63	63	71	71	79	79	87	87	PC	PC
O	8	8	16	16	24	24	32	32	40	40	48	48	56	56	64	64	72	72	80	80	88	88	PC	PC
P	8	8	16	16	24	24	32	32	40	40	48	48	56	56	64	64	72	72	80	80	88	88	PC	PC

Figure 32. PCR1 plate layout.
The numbers indicate the sample numbers. The colors indicate the different blocks within the panel: white = block A, purple = block B, blue = block C, black = block D.

3.7 Pool PCR1 products

During this step, the PCR1 products from the PCR1 Plates are pooled into one PCR1 Pooling Plate using the Microlab STAR (*Figure 14*).

Prepare bench

- PCR1 Plates, prepared in previous step
- MilliQ water (at +4 °C, preferably kept in the fridge until use)
- 1x 384-well PCR plate (skirted)
- 1x water reservoir (300 mL)
- 50 µL Conductive Filter Tips (1x rack per panel, 1x rack for water)
- Adhesive films
- Temperature-resistant labels or marker pen

Before you start

- Thaw PCR1 Plates at room temperature if frozen.
- Mark the new 384-well PCR plate: “PCR1 Pooling Plate”.
- Switch on the Microlab STAR system, and open the Hamilton Run Control software.

Instructions

18. Make sure that PCR1 Plates are thawed and properly sealed, then vortex the plates and spin down at 400–1000 x g for 1 minute at room temperature.
19. Inspect the wells of PCR1 Plates to make sure that no liquid has evaporated and that all the liquid is at the bottom of the wells. Spin down the plates again if necessary.
20. In Run Control, select the protocol *PCR1 Pooling* and click the **Start** button.

21. Select the number of Destination Pooling Plates and the number of Panels being Pooled for each, then click **OK**.
22. Pull out all carriers to the load position and prepare the Microlab STAR deck according to the software instructions.
    - Add MilliQ water to the reservoir.
    - Place PCR1 Plates on the carrier.
    - Carefully remove the adhesive films.
    - Fill Tip carrier in selected positions.
    - Push in Water Reservoir manually.
23. Click **OK** in the software to load the tip and plate carriers automatically and begin the run.
    Result: The Microlab STAR automatically scans the tips, dispenses 12 µL MilliQ water into each well of the PCR1 Pooling Plate, and pools 3 µL of each PCR1 product from each sample into one well per panel. The run takes approximately 6 minutes to be completed.
24. When the protocol is finished, remove the PCR1 Pooling Plate and seal it with a new adhesive film. Keep the Microlab STAR®on for later use.
25. Vortex the PCR1 Pooling Plate and spin down at 400–1000 x g for 1 minute.
26. Inspect the PCR1 Pooling Plate to ensure that all wells contain the same amount of liquid (24 µL).
27. Remove PCR1 Plates containing the remaining PCR products and seal them with new adhesive films. Store them at 20 °C for up to 2 weeks in case of potential reruns.
28. Continue to *2.8 Amplification and sample indexing (PCR2)*, or store the PCR1 Pooling Plate at +4 °C until ready to use (the same day).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	1	1	9	9	17	17	25	25	33	33	41	41	49	49	57	57	65	65	73	73	81	81	SC	SC
B	1	1	9	9	17	17	25	25	33	33	41	41	49	49	57	57	65	65	73	73	81	81	SC	SC
C	2	2	10	10	18	18	26	26	34	34	42	42	50	50	58	58	66	66	74	74	82	82	SC	SC
D	2	2	10	10	18	18	26	26	34	34	42	42	50	50	58	58	66	66	74	74	82	82	SC	SC
E	3	3	11	11	19	19	27	27	35	35	43	43	51	51	59	59	67	67	75	75	83	83	NC	NC
F	3	3	11	11	19	19	27	27	35	35	43	43	51	51	59	59	67	67	75	75	83	83	NC	NC
G	4	4	12	12	20	20	28	28	36	36	44	44	52	52	60	60	68	68	76	76	84	84	NC	NC
H	4	4	12	12	20	20	28	28	36	36	44	44	52	52	60	60	68	68	76	76	84	84	NC	NC
I	5	5	13	13	21	21	29	29	37	37	45	45	53	53	61	61	69	69	77	77	85	85	NC	NC
J	5	5	13	13	21	21	29	29	37	37	45	45	53	53	61	61	69	69	77	77	85	85	NC	NC
K	6	6	14	14	22	22	30	30	38	38	46	46	54	54	62	62	70	70	78	78	86	86	PC	PC
L	6	6	14	14	22	22	30	30	38	38	46	46	54	54	62	62	70	70	78	78	86	86	PC	PC
M	7	7	15	15	23	23	31	31	39	39	47	47	55	55	63	63	71	71	79	79	87	87	PC	PC
N	7	7	15	15	23	23	31	31	39	39	47	47	55	55	63	63	71	71	79	79	87	87	PC	PC
O	8	8	16	16	24	24	32	32	40	40	48	48	56	56	64	64	72	72	80	80	88	88	PC	PC
P	8	8	16	16	24	24	32	32	40	40	48	48	56	56	64	64	72	72	80	80	88	88	PC	PC

Figure 33. PCR1 Pooling Plate layout.
The numbers indicate the sample numbers. The colors indicate the panel numbers: light blue = Panel 1, green = Panel 2, dark blue = Panel 3, grey = Panel 4.

3.8 Amplification and sample indexing (PCR2)

During this step, a PCR2 Mix is prepared manually and then mixed with the samples along with index primers (unique for each sample) using the Microlab STAR. The samples are then subjected to a second PCR reaction.

3.8.1 Prepare PCR2 Mix

During this step, a PCR2 Mix is prepared manually. The PCR2 Mix must be used within 15 minutes from preparation.

Prepare bench

- PCR1 Pooling Plate, prepared in previous step
- Olink® Explore PCR2 Solution
- Olink® Explore PCR2 Enzyme (when brought to the lab bench, keep the PCR2 Enzyme in a freezing block (-20 °C))
- Olink® Explore Index Plate 1
- MilliQ water (at +4 °C, preferably kept in the fridge until use)
- Falcon tube (15 mL)
- Manual pipettes (10–100 µL, 100–1000 µL)
- Filter pipette tips
- Temperature-resistant labels or marker pen

Before you start

- Thaw the PCR2 Solution and the Index Plate 1 at room temperature.
- If stored at +4 °C, allow the PCR1 Pooling Plate to reach room temperature.
- Mark the new 15 mL tube: "PCR2 Mix".
- Switch on one ProFlex™ PCR instrument. No preheating is required.

Instructions

1. Vortex the PCR2 Solution and spin it briefly.
2. Vortex the Index Plate 1 and spin it at 400–1000 x g for 1 minute.
3. Make sure that the PCR1 Pooling Plate is properly sealed, then vortex it and spin at 400–1000 x g for 1 minute.
4. Spin the PCR2 Enzyme briefly. Do not vortex.

NOTE: *Always keep the PCR2 Enzyme at -20 °C. When brought to the lab bench, keep it in a freezing block (-20 °C).*

5. Prepare a PCR2 Mix in a Falcon tube following the order and volumes indicated in *Table 8*.

Table 18. PCR2 Mix

Addition order	Reagent	Volume (µL)
1	MilliQ water (+4 °C)	7 076
2	PCR2 Solution	1 015
3	PCR2 Enzyme	21
	Total	8 112

5. Vortex the PCR2 Mix thoroughly. Aliquot to 96 well PCR plate with at least 72 uL/well. Keep at room temperature until use.

TIME SENSITIVE STEP: *Start the dispensing of the PCR2 Mix within 15 minutes from preparation.*

3.8.2 Prepare PCR2 Plate and perform PCR2

During this step, the prepared PCR2 Mix is mixed with the samples along with index primers using the Microlab STAR. The samples are then subjected to a second PCR reaction (*Figure 16*).

Prepare bench

- PCR1 Pooling Plate (at room temperature), prepared in previous step
- PCR2 Mix, prepared in previous step
- Olink® Explore Index Plate 1
- 1x 384-well PCR plate (skirted)
- 1x 96-well PCR plate with PCR2 Master Mix (skirted), prepared in previous step

- 50 µL Conductive Filter Tips (6x rack per PCR2 plate)
- Adhesive films
- Temperature-resistant labels or marker pen

Before you start

- Mark the new 384-well PCR plate: “PCR2 Plate”.

Instructions

1. Open Microlab Run Control and select the protocol *PCR2_Setup* and click **Start.**
2. Select the number of PCR2 plates being prepped and the number of Panels in each plate, and click **OK**.
3. Centrifuge Index Plate 1 at 400–1000 x g for 1 minute and inspect the plate to ensure that all wells contain the same amount of liquid (20 µL). If a well is empty or contains a lower volume, contact Olink Support.
4. Carefully remove the adhesive films from the Index Plate 1 and the PCR1 Pooling Plate. Make sure that no air bubbles are trapped at the bottom of the wells of the PCR1 Pooling Plate.
5. Pull out all carriers and prepare the Microlab STAR deck according to the software instructions.
6. Click **OK** once the deck is loaded, then the system will automatically load the carriers and begin the protocol.
   Result: Microlab STAR transfers 16 µL of PCR2 Mix, 2 µL of Index Primers (from the Index Plate 1) and 2 µL of PCR1 products (from the PCR1 Pooling Plate) into the applicable wells of the PCR2 Plate (Figure 16). The run takes approximately 8 minutes to be completed.
7. When the protocol is finished (indicated by a message in the software), remove the PCR2 Plate and seal it with a new adhesive film. Keep the Microlab STAR on for later use.

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

TIME SENSITIVE STEP: *Start the PCR2 program within 5 minutes from end of Microlab STAR protocol.*

8. Vortex and spin down at 400–1000 x g for 1 minute at room temperature.
9. Inspect the PCR2 Plate to ensure that all wells contain the same amount of liquid (20 µL).
10. Place the PCR2 Plate in the ProFlex and add a balance plate to the other side of the ProFlex.
11. Click Open and select the program *Olink Index PCR2* (*Figure 15*). Click **Start**.

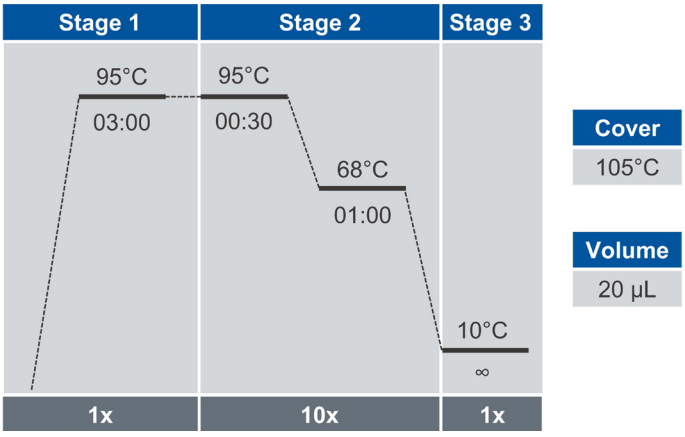

Figure 34. Olink Index PCR2 program

12. Remove the PCR1 Pooling Plate containing the remaining PCR products from the Microlab STAR and seal it with a new adhesive film. Store it at -20 °C for up to 2 weeks in case of potential reruns.
13. Discard the Index Plate 1.
14. When the PCR program is finished (~25 minutes), continue to *2.9 Pool PCR2 products*, or store the PCR2 Plate at +4 °C until use (the same day).

SAFE STOPPING POINT: *The PCR2 Plate can be stored at -20 °C for up to 2 weeks.*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	1	1	9	9	17	17	25	25	33	33	41	41	49	49	57	57	65	65	73	73	81	81	SC	SC
B	1	1	9	9	17	17	25	25	33	33	41	41	49	49	57	57	65	65	73	73	81	81	SC	SC
C	2	2	10	10	18	18	26	26	34	34	42	42	50	50	58	58	66	66	74	74	82	82	SC	SC
D	2	2	10	10	18	18	26	26	34	34	42	42	50	50	58	58	66	66	74	74	82	82	SC	SC
E	3	3	11	11	19	19	27	27	35	35	43	43	51	51	59	59	67	67	75	75	83	83	NC	NC
F	3	3	11	11	19	19	27	27	35	35	43	43	51	51	59	59	67	67	75	75	83	83	NC	NC
G	4	4	12	12	20	20	28	28	36	36	44	44	52	52	60	60	68	68	76	76	84	84	NC	NC
H	4	4	12	12	20	20	28	28	36	36	44	44	52	52	60	60	68	68	76	76	84	84	NC	NC
I	5	5	13	13	21	21	29	29	37	37	45	45	53	53	61	61	69	69	77	77	85	85	NC	NC
J	5	5	13	13	21	21	29	29	37	37	45	45	53	53	61	61	69	69	77	77	85	85	NC	NC
K	6	6	14	14	22	22	30	30	38	38	46	46	54	54	62	62	70	70	78	78	86	86	PC	PC
L	6	6	14	14	22	22	30	30	38	38	46	46	54	54	62	62	70	70	78	78	86	86	PC	PC
M	7	7	15	15	23	23	31	31	39	39	47	47	55	55	63	63	71	71	79	79	87	87	PC	PC
N	7	7	15	15	23	23	31	31	39	39	47	47	55	55	63	63	71	71	79	79	87	87	PC	PC
O	8	8	16	16	24	24	32	32	40	40	48	48	56	56	64	64	72	72	80	80	88	88	PC	PC
P	8	8	16	16	24	24	32	32	40	40	48	48	56	56	64	64	72	72	80	80	88	88	PC	PC

Figure 35. PCR2 Plate layout
The numbers indicate the sample numbers. The colors indicate the panel numbers: light blue = Panel 1, green = Panel 2, dark blue = Panel 3, grey = Panel 4.

3.9 Pool PCR2 products

During this step, all PCR2 products belonging to the same panel are pooled into one column of the PCR2 Pooling Plate using the Microlab STAR (*Figure 17*). The Olink libraries are then manually transferred to one microcentrifuge tube per panel. Each tube contains amplicons from 96 samples, including controls.

Prepare bench

- PCR2 Plate, prepared in previous step
- 1x 96-well PCR plate (skirted)
- Microcentrifuge tubes (1.5 mL, 1x tube per panel)
- 50 µL Conductive Filter Tips (1x rack per pooling plate)
- Manual pipette (10–100 µL)
- Filter pipette tips
- Adhesive films
- Temperature-resistant labels or marker pen

Before you start

- Thaw PCR2 Plate at room temperature if frozen.
- Mark the new 96-well PCR plate: ”PCR2 Pooling Plate”.
- Mark four new microcentrifuge tubes: ”PCR2 [1–4, depending on the number of panels]”.

Instructions

1. Open Hamilton Run Control and select the applicable protocol *PCR2_Final_Pooling* and click **Start**.
2. Select the number of Destination Pooling Plates and the number of Panels being pooled in each, then click **OK**.
3. Make sure that the PCR2 Plate is thawed and properly sealed, then vortex and spin it down at 400–1000 x g for 1 minute at room temperature to ensure that all wells are mixed.

4. Inspect the wells of the PCR2 Plate to make sure that no liquid has evaporated and that all the liquid is at the bottom of the wells.
5. Pull out all carriers an prepare the Microlab STAR deck according to the software instructions.
6. Once loaded, click **OK** to automatically load the carriers and begin the protocol.
   Result: Microlab STAR pools 3 µL from each row of a given Panel into a single well of the PCR2 Pooling Plate. The result is one column of pooled PCR2 products per panel in the PCR2 Pooling Plate. (Figure 17).
7. When finished, remove the PCR2 Pooling Plate from the worktable. Seal the plate with a new adhesive film, vortex the plate and spin down at 400–1000 x g for 1 minute.
8. Inspect the PCR2 Pooling Plate to ensure that every applicable well contains the same amount of liquid (36 µL in columns 1, 3, 5 and 7).
9. Clear the Microlab STAR and shut it down.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1		2		3		4					
B	1		2		3		4					
C	1		2		3		4					
D	1		2		3		4					
E	1		2		3		4					
F	1		2		3		4					
G	1		2		3		4					
H	1		2		3		4					

Figure 36. PCR2 Pooling Plate layout.
The numbers and colors indicate the panel numbers: light blue = Panel 1, green = Panel 2, dark blue = Panel 3, grey = Panel 4.

10. Carefully remove the adhesive film from the PCR2 Pooling Plate.
11. Using a single-channel pipette transfer the pooled PCR2 products from the PCR2 Pooling Plate to the PCR2 Tubes as described in *Table 9*. Use forward pipetting and change pipette tip after each well.

Table 19. Transfer PCR2 products. If running a single panel, only transfer from column 1 to PCR2 tube 1 will be applicable.

Volume (µL)	From column	To tube
30	1	PCR2 1
30	3	PCR2 2
30	5	PCR2 3
30	7	PCR2 4

12. Vortex the PCR2 Tubes and spin down briefly.
13. Remove the PCR2 Plate containing the remaining PCR2 products from the instrument, seal it with a new adhesive film and store it at -20 °C for up to 2 weeks in case of potential reruns.
14. Discard the PCR2 Pooling Plate.
15. Continue to the Library purification step in the workflow manual, or store the PCR2 Tubes at +4 °C until use (the same day).

> **SAFE STOPPING POINT:** *The PCR2 Tubes can be stored at –20 °C for up to 2 weeks.*

3.10 Library purification

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

Prepare bench

- PCR2 Tubes 1–4, prepared in previous step
- Agentcourt AMPure XP bottle
- 96% Ethanol (EtOH)
- MilliQ water
- DynaMag™-2 Magnet
- Timer
- 8x Microcentrifuge tubes (1.5 mL)
- Falcon tube (15 mL)
- Manual pipettes (10–100 µL, 100–1000 µL)
- Pipette (5 mL)
- Filter pipette tips

Before you start

- Let the refrigerated Agentcourt AMPure XP bottle reach room temperature.
- Mark four new microcentrifuge tubes: “BP [1–4]” (for “Bead Purification”).
- Mark four new microcentrifuge tubes: “Lib [1–4]”.
- Mark the new 15 mL Falcon tube: "70% EtOH".
- Set a timer to 5 minutes.

Instructions

1. Freshly prepare a sufficient amount of 70% EtOH for the number of Libraries to purify according to *Table 10*. Manually transfer first the MilliQ water and then the 96% EtOH into the Falcon tube.

Table 20. Prepare 70% EtOH

Reagent	Volume (mL) for							
	1 library	2 Libraries	3 Libraries	4 Libraries	5 Libraries	6 Libraries	7 Libraries	8 Libraries
MilliQ water	0.5	0.8	1.1	1.4	1.6	1.9	2.2	2.5
96% EtOH	1.5	2.2	2.9	3.6	4.4	5.1	5.8	6.5
Total (70% EtOH)	2	3	4	5	6	7	8	9

2. Shake and vortex the Agentcourt AMPure XP bottle vigorously to resuspend the magnetic beads.
3. Transfer 80 µL from the Agentcourt AMPure XP bottle to each BP Tube.
4. Transfer 50 µL from each PCR2 Tube to the corresponding BP Tube according to *Table 11*.

Table 21. Transfer PCR2 products to BP tubes

Volume (µL)	From tube	To tube
50	PCR2 1	BP 1
50	PCR2 2	BP 2
50	PCR2 3	BP 3
50	PCR2 4	BP 4

NOTE: *Store the PCR2 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 and incubate BP Tubes 1–4 for 5 minutes at room temperature.
7. After the incubation, place the BP Tubes on the DynaMag™-2 Magnetic stand and leave them for 2 minutes to separate the beads from the solution (*Figure 18*).

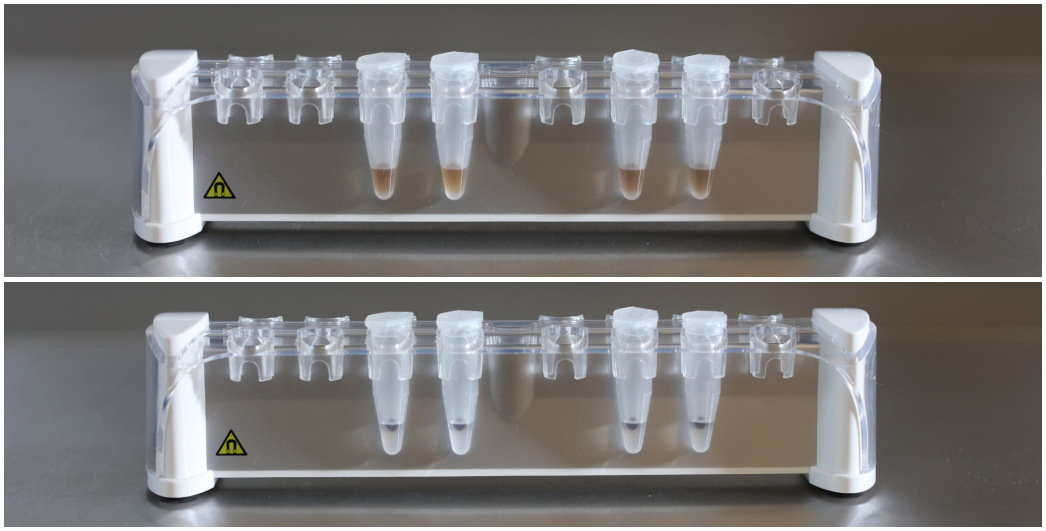

Figure 37. Tubes in DynaMag™-2 Magnetic stand before (upper) and after (lower) bead separation.

8. With the tubes still on the magnetic stand, carefully open the lid and discard 125 µL supernatant using a single-channel pipette. Do not disturb the beads.
9. With the tubes still in the magnetic stand, wash the beads:
   a. Add 500 µL of 70 % EtOH to the 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) once.

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 MilliQ water to the BP Tubes and pipette-mix 10 times towards the beads to elute the purified Libraries from the beads.
13. Incubate the tubes for 2 minutes at room temperature.
14. Place BP Tubes 1–4 on the magnetic stand and leave them for 1 minute to separate the beads from the eluted Library solution.
15. With BP Tubes 1-4 still on the magnetic stand, transfer 45 µL of eluate from the BP Tubes to the corresponding Lib Tubes according to *Table 12.*

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

Table 22. Transfer eluat

Volume (µL)	From tube	To tube
45	BP 1	Lib 1
45	BP 2	Lib 2
45	BP 3	Lib 3

45	BP 4	Lib 4

16. Discard BP Tubes 1-4.
17. Continue to *2.11 Quality control*.

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

3.11 Quality control

During this step, the four purified Olink Libraries are quality controlled on the Bioanalyzer using the High Sensitivity DNA kit or on the TapeStation using the High Sensitivity D5000 kit, according to the Manufacturer's instructions.

The electropherograms in *Figure 19* and *Figure 20* displays typical results for an Olink Library. 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 https://emea.support.illumina.com/bulletins/2019/10/bubble-products-in-sequencing-Libraries--causes--identification-.html for more information.

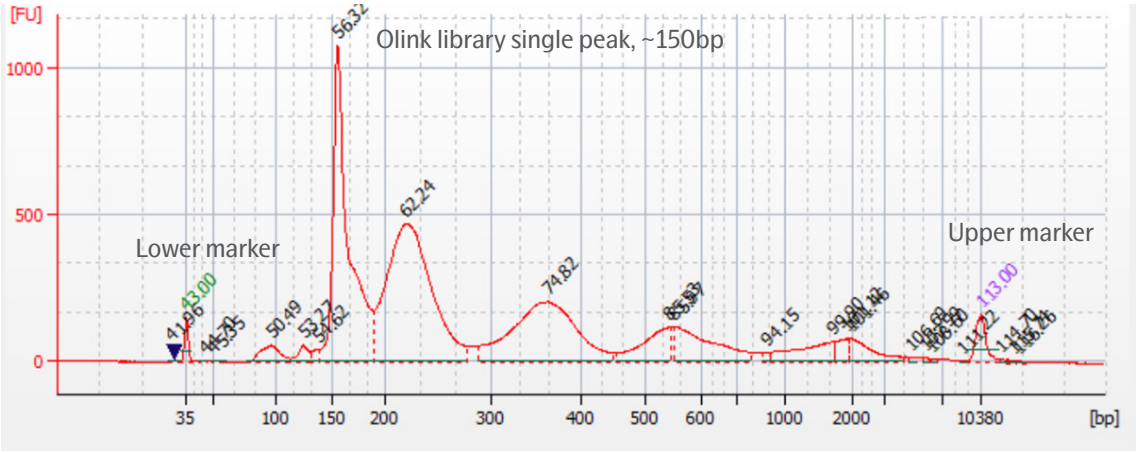

Figure 38. Bioanalyzer's electropherogram.

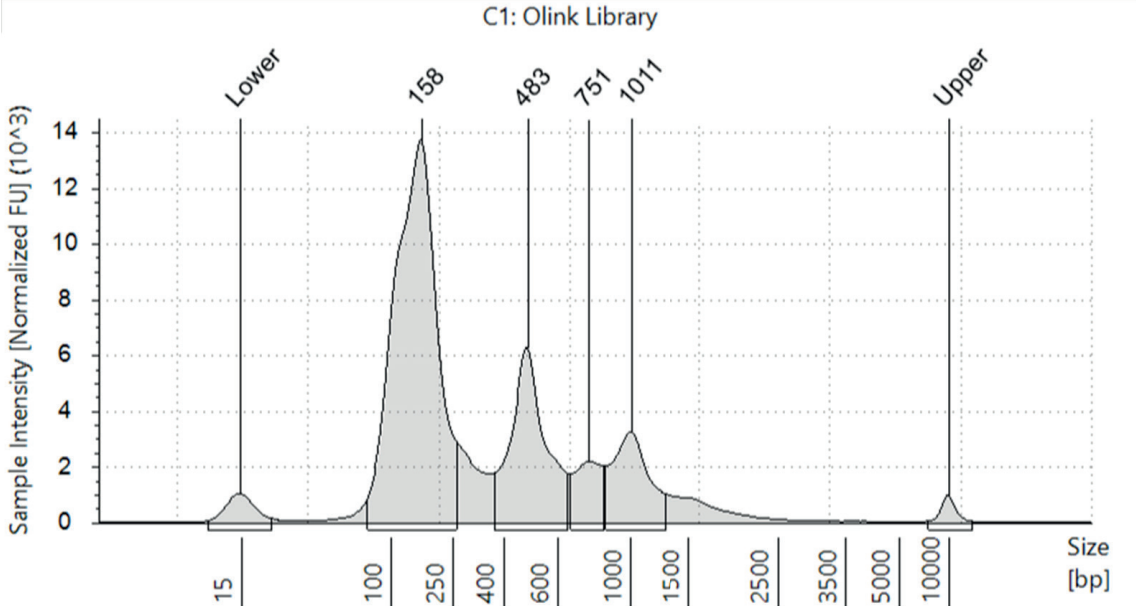

Figure 39. TapeStation's electropherogram.

For expected results of a successful ladder run, refer to the Manufacturer's Manual. If any of the deviations in *Table 13* should occur in the electropherogram, do not continue to sequencing. Instead, perform the suggested corrective action.

Table 23. Deviations in electropherogram.

Peak deviation	Possible cause	Action
No Library peak present at 150bp.	1) No PCR1 amplification has taken place, and therefore there are no PCR2 products and no Library 2) The bead purification failed	Rerun the quality control with unpurified Library. If a peak is present at 150bp: purify a new aliquot of the unpurified Library and rerun the quality control. If successful, proceed to sequencing. If there is no 150bp peak present in the unpurified Library, no PCR product has been generated. Repeat the Library preparation, quality control and sequencing.
No Library peak present at 150bp, but a smaller peak at 120bp	The index primers were not included in the PCR2 reaction (the 120bp peak corresponds to the PCR1 product) 1) The Index plate was empty 2) There was no transfer of index primers from the Index Plate to the PCR2 Plate due to ep*Motion*® failure	Contact Support. Make sure to centrifuge and visually inspect the Index Plate before starting the PCR2 setup. Make sure that the ep*Motion*® is calibrated, functional and that the correct protocol is used.

3.12 Next generation sequencing

Next generation sequencing is performed using either an Illumina® NextSeq™ 550, NextSeq™ 2000 or NovaSeq™ 6000 instrument. As the workflow differs between instruments, refer to the applicable Olink Explore Sequencing User Manual for instructions on how to sequence Olink Libraries.

4. Laboratory instructions for 96 samples using SPT Labtech Mosquito® and Eppendorf ep*Motion*®

This chapter provides instructions on how to perform each step of the Olink Explore 4 x 384 laboratory workflow, using the ep*Motion*® from the Eppendorf Company. The four panels should be prepared simultaneously. If the panels are prepared separately, additional Olink Explore Sample Prep Reagents will be required, refer *Table 2*.

NOTE: *If running the combination CARDIO, INF, NEURO and ONC **or** the combination CARDIO II, INF II, NEURO II and ONC II it is possible to follow the instructions in Olink Explore 3072 User Manual. The instructions in Olink Explore 3072 User Manual provide a faster workflow by allowing for preparation of two incubation plates simultaneously using different instrument protocols.*

4.1 Preparations

4.1.1 Plan the study

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

- Decide the number of samples and controls required to get the data that you want from the study.
- Optional Sample Control: It is recommended to include a pooled plasma sample in duplicate on each plate. These samples are used to assess potential variation between runs and plates, for example to calculate inter-assay and intra-assay CV, as well as for troubleshooting. When including the Sample Controls, 88 to 352 user samples can be processed simultaneously.
- 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.
- Consult the Olink white paper “Strategies for design of protein biomarker studies” (*www.olink.com/whitepapers*) and the sample randomization guidelines (*www.olink.com/faq/sample-randomization*).

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

4.1.2 Important information

Panel nomenclature

As the panels can be combined in different ways, the panel names are not mentioned and are instead referred to as Panel 1, 2, 3, and 4 throughout the instructions. Before running Olink Explore 4 x 384, decide which panel shall be Panel 1, 2, 3 and 4, respectively. It is crucial to keep this order throughout the entire workflow. Mixing up the panels will lead to a failed run.

Throughout the instructions, plates and tubes are marked with names and numbers, for example “Incubation Plate 1”. When using the marked plates and tubes, make sure that the number on the plate or tube corresponds to the number of the applicable panel, i.e when running Panel 1, use XX Plate 1, and when running Panel 4, use XX Plate 4.

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.

Vortexing
Correct vortexing is essential to generate reproducible results. Review instructions in the Olink Explore Overview User Manual prior to starting the laboratory workflow.

384-well PCR plates
Make sure to use the twin.tec 384-well skirted PCR plate from Eppendorf® (art nr. 0030128508) as stated in chapter 4 of the Olink® Explore Overview User Manual. All instrument protocols have been calibrated for this specific plate. Other models should not be used.

4.1.3 Prepare the samples

During this step, samples are manually transferred to the Sample Plate(s) (*Figure 21*). The Sample Plate(s) must be used within the same day of preparation.

The number of Sample Plate(s) required depends on how the panels are combined. One Sample Plate contains a sufficient sample volume for the preparation of four panels, but when running multiple panels of the same type (e.g 4x Olink Explore Inflammation panel), separate Sample Plates are required in order to not perform the same analysis several times on the same samples.

NOTE: *The Olink Explore protocol is optimized and validated for plasma and serum samples. Contact support@olink.com for more information regarding alternative sample types.*

Prepare bench
- Sample Plates *(provided by the user)*
- 1-4x 96-well PCR plate(s), preferably with full skirt
- Manual pipette (0.5-10 µL)
- Filter pipette tips
- Adhesive Film
- 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.
- Mark the 96-well Sample Plate(s) with a simple alphanumeric code that you can later identify (“A, B, C”, “1, 2, 3”, or “A1, A2, A3”) using temperature-resistant labels or marker pen. Use unique sample identification names or numbers.

Instructions
1. Transfer the samples into the Sample Plate(s), according to the plate layout shown in *Figure 21*. Make sure that samples are added to every applicable well.
2. Seal the Sample Plate(s) using an adhesive film or individual seals.
3. Store the Sample Plate(s) at +4 °C if used the same day, otherwise at -80 °C.

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

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	9	17	25	33	41	49	57	65	73	81	
B	2	10	18	26	34	42	50	58	66	74	82	
C	3	11	19	27	35	43	51	59	67	75	83	
D	4	12	20	28	36	44	52	60	68	76	84	
E	5	13	21	29	37	45	53	61	69	77	85	
F	6	14	22	30	38	46	54	62	70	78	86	
G	7	15	23	31	39	47	55	63	71	79	87	
H	8	16	24	32	40	48	56	64	72	80	88	

Figure 40. Sample Plate layout.
The numbers indicate the sample numbers.

4.2 Prepare Sample Source Plate (day 1)

There is one sample source plate per sample plate. During this step, samples are manually transferred from the prepared Sample Plate(s) into the Sample Source Plate(s), and controls are added to the Sample Source Plate. The Sample Source Plate must be used within the same day of preparation.

The number of Sample Plate(s) required depends on how the panels are combined in the run, as explained in *3.1.3 Prepare the samples.* The preparation of more than one Sample Plate must be performed in separate runs. Perform the instruction once for every Sample Plate required, i.e in one to four runs.

IMPORTANT: *Using the correct combination of Plate Control and Probes lots is essential for the downstream data analysis. Before starting the workflow, ensure that the lot number for each Probe kit and for the Plate Control matches the lot numbers stated in the Lot Configuration document provided with the kit.*

Prepare bench

- Sample Plate, prepared in previous step
- Olink Explore Negative Control
- Olink Explore Plate Control
- Sample Control *(optional, provided by the user)*
- 1–4x 384-well PCR plate(s) (skirted): same as the number of 96-well Sample Plates used
- Manual pipette (0.5–10 µL)
- Manual multichannel pipette (0.5–10 µL)
- Filter pipette tips
- Adhesive films

Before you start

- Ensure that the Sample Plate(s) have been prepared according to *3.1.3 Prepare the samples*.
- If frozen, thaw the Sample Plate(s) at room temperature and mark the plates: "Sample Plate [Panel no:s]".
- Thaw the Negative Control, Plate Control and recommended Sample Control at room temperature.
- Mark one 384-well PCR plate per run: “Sample Source Plate [Panel no:s]”.

NOTE: *If preparing more than one 384-well Sample Source Plate, make sure that the 96-well Sample Plate and 384-well Sample Source Plate are marked with the same number, and keep track of which Panel(s) each 384-well Sample Source Plate shall be used for.*

Instructions

1. Using the MixMate® or manual vortexing, vortex the applicable 96-well Sample Plate and spin at 400-1000 x g for 1 minute at room temperature.
2. Using a multichannel pipette, transfer 10 µL of each sample into the applicable 384-well Sample Source Plate according to the plate layout shown in (*Figure 22*). Use forward pipetting, change pipette tips between every column, and make sure that no air bubbles are trapped at the bottom of the wells.

IMPORTANT: *The plate layout of the Sample Source Plate has changed and is only compatible with version 4 of the Olink Explore instrument protocols. Make sure that you have version 4 of all instrument protocols installed before proceeding. Please contact support@olink.com if you need assistance.*

3. Vortex the Negative Control, Plate Control and Sample Control and spin briefly.
4. Using a single-channel pipette, transfer 10 µL of Negative Control (NC), Plate Control (PC) and Sample Control (SC) to each of the applicable wells in column 23 of the 384-well Sample Source Plate according to the plate layout shown in *Figure 22*. Change pipette tip between every control sample.
5. Seal the Sample Source Plate with a new adhesive film.

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

6. 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).
7. Seal the Sample Plate with a new adhesive film. Store at -80 °C.
8. Repeat the instruction as necessary to prepare any additional Sample Source Plate(s) (384-well) to be used the same day.

NOTE: *Freeze thawing of Sample Source Plates (384-well) for future use is not recommended.*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	1		9		17		25		33		41		49		57		65		73		81		SC	
B																								
C	2		10		18		26		34		42		50		58		66		74		82		SC	
D																								
E	3		11		19		27		35		43		51		59		67		75		83		NC	
F																								
G	4		12		20		28		36		44		52		60		68		76		84		NC	
H																								
I	5		13		21		29		37		45		53		61		69		77		85		NC	
J																								
K	6		14		22		30		38		46		54		62		70		78		86		PC	
L																								
M	7		15		23		31		39		47		55		63		71		79		87		PC	
N																								
O	8		16		24		32		40		48		56		64		72		80		88		PC	
P																								

Figure 41. Sample Source Plate layout.
The numbers indicate the sample numbers. SC = Sample Control, NC = Negative Control, PC = Plate Control.

4.3 Sample Dilution

During this step, Sample Diluent is dispensed into Sample Dilution Plate(s) using the Dragonfly® and the samples are diluted using the Mosquito®. The prepared Sample Dilution Plate(s) must be used within one hour from preparation.

Perform the instruction once for every Sample Source Plate prepared in the previous step, i.e. one to four times. It is not possible to prepare multiple plates at the same time.

4.3.1 Prepare Sample Dilution Plate

During this step, Sample Diluent is dispensed into Sample Dilution Plate(s) using the Dragonfly®.

Prepare bench

- Olink Target 96/Explore Sample Diluent
- 1-4x 384-well PCR plate(s) (skirted)
- 1x Disposable reservoir (10 mL)
- 1x Disposable ultra-low retention syringe with plunger
- Manual pipette (100–1000 µL)
- Filter pipette tips
- Adhesive films

Before you start

- Thaw the Sample Diluent at room temperature.
- Mark one new 384-well PCR plate per run: “Sample Dilution Plate [Panel no:s]”.

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

Instructions

1. Prepare the Dragonfly® according to instructions in the Olink Explore Overview User Manual.
   - Use the protocol Olink Sample Dilution Plate v4.

IMPORTANT: *Make sure that the correct protocol version is used. The plate layout has changed and using an older version of the protocol will result in unusable data. Please contact support@olink.com if you need assistance.*

   - Attach one syringe in position B2.
2. Slide the reservoir tray to the filling position (A) and place a new reservoir in position B2 (B) (*Figure 23*).

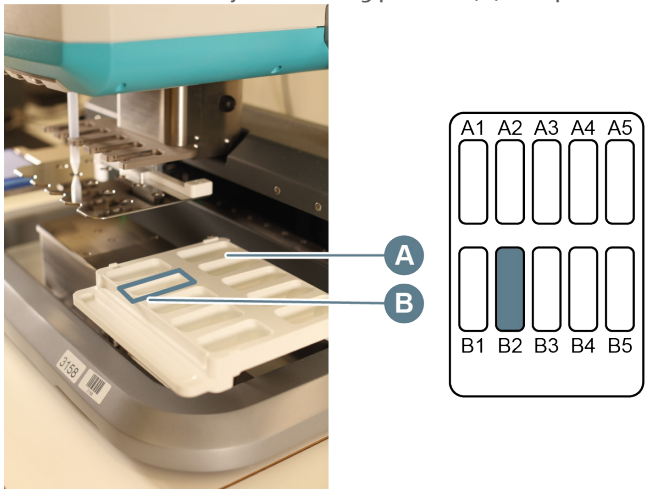

Figure 42. Disposable reservoir in reservoir tray of the Dragonfly.

3. Vortex the Sample Diluent briefly, add 6 mL of Sample Diluent into the reservoir and carefully slide the reservoir tray back to the aspirate position.
4. Place the Sample Dilution Plate in the plate position to the left, with well A1 in the top left corner.
5. Select the Run tab in the Constant layer view of the software (*Figure 24*), then click RUN to start the program.
   Result: Dragonfly® dispenses 9 µL Sample Diluent into each well of quadrant 1-3 and 29 µL in quadrant 4 of the Sample Dilution Plate. Filled wells turn purple in the plate map. The run stops automatically when the Dragonfly® has dispensed Sample Diluent into all applicable wells.

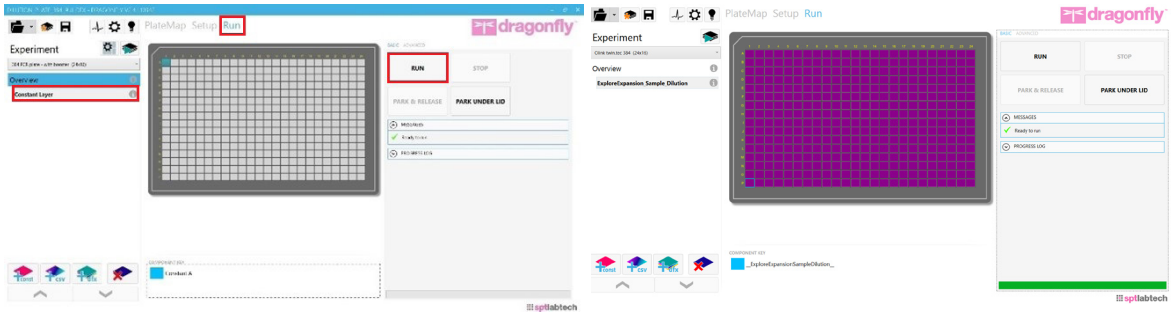

Figure 43. Run tab and Run button (left). Filled Plate (right).

6. When the Dragonfly® has returned the Sample Dilution Plate to the plate position and released the plate clamp, remove the Sample Dilution Plate from the instrument and seal it with an adhesive film.
7. Spin the Sample Dilution Plate at 400–1000 x g for 1 minute.
8. Visually inspect the Sample Dilution Plate to ensure that all wells in quadrant 1-3 contain 9 µL of liquid, and that wells in quadrant 4 contain 29 µL. Make sure that there are no bubbles trapped at the bottom of the wells.
9. Repeat the steps 4–8 as necessary to prepare any additional Sample Dilution Plate(s).
10. When finished, clear the instrument and shut it down according to instructions in the Olink Explore Overview User Manual.
11. Continue to *3.3.2 Perform Sample Dilution*, or store the Sample Dilution Plate(s) at +4 °C until use (the same day).

4.3.2 Perform Sample Dilution

During this step, the samples are diluted in four sequential steps using the Mosquito®: from 1:1 (undiluted) to 1:10, 1:100, 1:1000, and approximately 1:100 000 (*Figure 26*).

Perform the instruction once per Sample Dilution Plate prepared in the previous step, i.e. one to four times. It is not possible to prepare multiple plates at the same time.

Prepare bench

- Sample Source Plate(s), prepared in previous step
- Sample Dilution Plate(s), prepared in previous step
- Adhesive films

Before you start

- Prepare the Mosquito® according to instructions in the Olink Explore Overview User Manual. Use the protocol Olink Sample Dilution 3072 v4.

IMPORTANT: *Make sure that the correct protocol version is used. The plate layout has changed and using an older version of the protocol will result in unusable data. Please contact support@olink.com if you need assistance.*

- Make sure that a spool of Mosquito® pipette tips has been loaded into the Mosquito® according to the manufacturer's instructions.
- Make sure that the knobs at the back of the two Mosquito® humidifiers are set to maximum level.

Instructions

IMPORTANT: *Make sure to vortex the Sample Dilution Plate(s) thoroughly both between dilutions and after the last dilution.*

1. Let the Sample Source Plate reach room temperature, vortex it briefly and spin down at 400–1000 x g for 1 minute. 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 Plate.
3. Place the Sample Source Plate in a magnetic clamp booster according to instructions in the Olink Explore Overview User Manual.
4. Place the Sample Source Plate on the Mosquito® deck - refer to the software for the correct position. Insert the corner of the magnetic clamp booster under the small metal holder of the deck as described in the Olink Explore Overview User Manual (this applies to all plate positions).
5. Place the Sample Dilution Plate in a magnetic clamp booster and place it on the Mosquito® deck - refer to the software for the correct position.

IMPORTANT: *Risk of instrument damage! If the magnetic clamp booster is not correctly placed it may collide with the internal parts of the instrument during the run.*

6. Click Run to start the Mosquito® program Olink Sample Dilution 3072.
   Result: Mosquito® performs a 1:10 dilution by transferring 1 µL of sample from the Sample Source Plate into quadrant 1 in the Sample Dilution Plate. The run can be monitored in the protocol tab.
7. When the Mosquito® protocol is paused and a pop-up window appears on the screen (*Figure 25*), remove the Sample Source Plate and the Sample Dilution Plate from the Mosquito® deck and from the magnetic clamp boosters.
8. Seal both plates with new adhesive films and store the Sample Source Plate at +4 °C for later use.

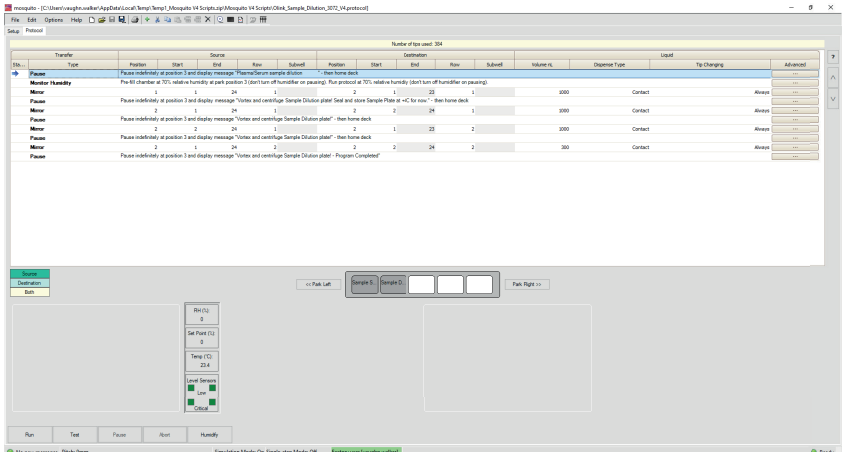

Figure 44. Pop-up window during Mosquito dilution protocol.

9. Using the MixMate® or manual vortexing, vortex the Sample Dilution Plate thoroughly to ensure that all wells are mixed.
10. Spin at 400–1000 x g for 1 minute. Make sure that there are no bubbles trapped at the bottom of the wells.
11. Carefully remove the adhesive film from the Sample Dilution Plate.
12. Place the Sample Dilution Plate back in the magnetic clamp booster and return it to the correct position of the Mosquito® deck. All other positions shall remain empty.
13. Click Resume to continue the program.
    Result: Mosquito® performs a 1:100 sample dilution by transferring 1 µL of diluted samples (1:10) into quadrant 2 of the Sample Dilution Plate.
14. When the Mosquito® protocol is paused, and a pop-up window appears on the screen, remove the Sample Dilution Plate from the Mosquito® deck and the magnetic clamp booster. Seal with a new adhesive film.

15. Repeat steps 9–14 to perform the third dilution (1:1000).
    Result: Mosquito® performs a 1:1000 sample dilution by transferring 1 µL of diluted samples (1:100) into quadrant 3 of the Sample Dilution Plate.
16. Repeat steps 9-14 again to perform the last dilution (1:100 000).
    Result: Mosquito® performs a 1:100 000 sample dilution by transferring 0.3 µL of diluted samples (1:1000) into quadrant 4 of the Sample Dilution Plate.
17. Vortex the Sample Dilution Plate thoroughly Using the MixMate® or manual vortexing, and ensure that all wells are mixed.
18. Spin at 400–1000 x g for 1 minute. Make sure that there are no bubbles trapped at the bottom of the wells.
19. When finished, click Resume to end the Mosquito® program.
20. Repeat the instruction as necessary to prepare any additional Sample Dilution Plate(s). Keep the Mosquito® on for later use.
21. Continue to *3.10 Library purification*, or place the Sample Dilution Plate at +4 °C for up to 1 hour.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	1	1	9	9	17	17	25	25	33	33	41	41	49	49	57	57	65	65	73	73	81	81	SC	SC
B	1	1	9	9	17	17	25	25	33	33	41	41	49	49	57	57	65	65	73	73	81	81	SC	SC
C	2	2	10	10	18	18	26	26	34	34	42	42	50	50	58	58	66	66	74	74	82	82	SC	SC
D	2	2	10	10	18	18	26	26	34	34	42	42	50	50	58	58	66	66	74	74	82	82	SC	SC
E	3	3	11	11	19	19	27	27	35	35	43	43	51	51	59	59	67	67	75	75	83	83	NC	NC
F	3	3	11	11	19	19	27	27	35	35	43	43	51	51	59	59	67	67	75	75	83	83	NC	NC
G	4	4	12	12	20	20	28	28	36	36	44	44	52	52	60	60	68	68	76	76	84	84	NC	NC
H	4	4	12	12	20	20	28	28	36	36	44	44	52	52	60	60	68	68	76	76	84	84	NC	NC
I	5	5	13	13	21	21	29	29	37	37	45	45	53	53	61	61	69	69	77	77	85	85	NC	NC
J	5	5	13	13	21	21	29	29	37	37	45	45	53	53	61	61	69	69	77	77	85	85	NC	NC
K	6	6	14	14	22	22	30	30	38	38	46	46	54	54	62	62	70	70	78	78	86	86	PC	PC
L	6	6	14	14	22	22	30	30	38	38	46	46	54	54	62	62	70	70	78	78	86	86	PC	PC
M	7	7	15	15	23	23	31	31	39	39	47	47	55	55	63	63	71	71	79	79	87	87	PC	PC
N	7	7	15	15	23	23	31	31	39	39	47	47	55	55	63	63	71	71	79	79	87	87	PC	PC
O	8	8	16	16	24	24	32	32	40	40	48	48	56	56	64	64	72	72	80	80	88	88	PC	PC
P	8	8	16	16	24	24	32	32	40	40	48	48	56	56	64	64	72	72	80	80	88	88	PC	PC

Figure 45. Sample Dilution Plate layout.
The numbers indicate the sample numbers. SC = Sample Control, NC = Negative Control and PC = Plate Control.

4.4 Incubation

During this step, four Incubation Mixes are prepared manually for each panel, transferred to Reagent Source Plates, and mixed with the samples. Incubation is then performed overnight.

Since one Reagent Source Plate is used per panel, perform the instructions four times. As the Incubation Mixes must be used within 30 minutes from preparation. Prepare one Reagent Source Plate and two Incubation Plates at a time.

1. Run 1: Prepare Incubation Mixes for Panel 1, transfer to Reagent Source Plate 1, then prepare Incubation Plate 1 and start incubation.
2. Run 2: Prepare Incubation Mixes for Panel 2, transfer to Reagent Source Plate 2, then prepare Incubation Plate 2 and start incubation.
3. Run 3: Prepare Incubation Mixes for Panel 3, transfer to Reagent Source Plate 3, then prepare Incubation Plate 3 and start incubation.
4. Run 4: Prepare Incubation Mixes for Panel 4, transfer to Reagent Source Plate 4, then prepare Incubation Plate 4 and start incubation.

4.4.1 Prepare Reagent Source Plate

During this step, four Incubation Mixes are prepared manually for each panel and transferred to a Reagent Source Plate. Each mix contains a specific set of Forward and Reverse probes.

IMPORTANT: *Using the correct combination of Plate Control and Probes lots is essential for the downstream data analysis. Before starting the workflow, ensure that the lot number for each Probe kit and or the Plate Control matches the lot numbers stated in the Lot Configuration document provided with the kit.*

Prepare bench

- Olink Explore Incubation Solution
- Olink Explore Forward Probes A-D (Frw probes)
- Olink Explore Reverse Probes A-D (Rev probes)
- 4x 8-well strip
- 4x 384-well PCR plate (skirted)
- Manual pipettes (0.5–10, 10–100 µL)
- Manual multichannel pipette (8-channel, 0.5–10 µL)
- Filter pipette tips
- Adhesive films

Before you start

- Allow the Incubation Solution, Forward Probes and Reverse Probes to reach room temperature.
- Mark four new 384-well PCR plates: “Reagent Source Plate [1–4]”.
- Mark the wells of four 8-well strips according to *Table 14.*

Table 24. PCR strips

Run	Well							
	1	2	3	4	5	6	7	8
1	1A	1B	1C	1D	-	-	-	-
2	2A	2B	2C	2D	-	-	-	-
3	3A	3B	3C	3D	-	-	-	-
4	4A	4B	4C	4D	-	-	-	-

Instructions

1. Vortex the Incubation Solution, as well as Forward Probes and Reverse Probes and spin down.
2. Prepare four Incubation Mixes in the PCR Strip 1: Using forward pipetting, first transfer the Incubation Solution to tubes 1–4 of the strip, then all Frw Probes to the applicable tubes of the strip, and last all Rev Probes to the applicable tubes of the strip according to *Table 15*.

NOTE: *Pipette the Incubation Solution carefully to avoid foaming.*

IMPORTANT: *The Incubation Solution will be used to prepare Incubation Mixes for the other panels. Do not discard.*

Table 25. Incubation Mix preparation

Addition order	Reagent	Well (of the applicable strip 1-4)							
		A	B	C	D	-	-	-	-
1	Incubation Solution	80 µL	80 µL	80 µL	80 µL	–	–	–	–
2	Frw probes	10 µL	10 µL	10 µL	10 µL	–	–	–	–
		[PANEL] Frw Probes A	[PANEL] Frw Probes B	[PANEL] Frw Probes C	[PANEL] Frw Probes D	–	–	–	–
3	Rev probes	10 µL	10 µL	10 µL	10 µL	–	–	–	–
		[PANEL] Rev Probes A	[PANEL] Rev Probes B	[PANEL] Rev Probes C	[PANEL] Rev Probes D	–	–	–	–

3. Seal the PCR Strip 1 with caps. Vortex and spin down briefly. Store at room temperature until use.

IMPORTANT: *Make sure that all applicable reagents are added to each Incubation Mix. The final volume shall be 100 µL*

TIME SENSITIVE STEP: *The incubation setup using the Mosquito® must be started within 30 minutes from preparation of the Incubation Mix.*

4. Using a multichannel pipette, transfer 10 µL of each Incubation Mix from the PCR Strip 1 to the Reagent Source Plate 1, according to the plate layout in *Figure 28*. Use reverse pipetting and do not change pipette tips between wells. Make sure to pipette to the bottom of the wells.

NOTE: *Rotate the Sample Reagent Plate (384-well) 90 degrees clockwise to facilitate pipetting (shown to the right of Figure 27).*

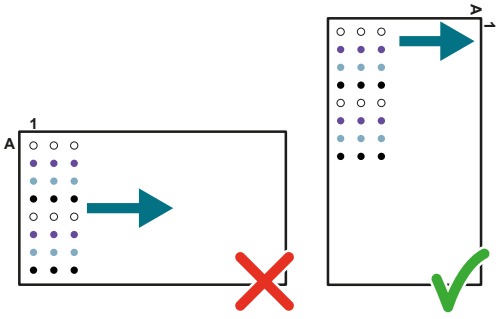

Figure 46. Rotating the Sample Reagent Plate

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	1A		1B		1C		1D																	
B																								
C	1A		1B		1C		1D																	
D																								
E	1A		1B		1C		1D																	
F																								
G	1A		1B		1C		1D																	
H																								
I	1A		1B		1C		1D																	
J																								
K	1A		1B		1C		1D																	
L																								
M	1A		1B		1C		1D																	
N																								
O	1A		1B		1C		1D																	
P																								

Figure 47. Reagent Source Plate layout.
The numbers indicates the Panel number and the letters indicate the different blocks within the panel.

5. Seal the Reagent Source Plate 1 with a new adhesive film and spin at 400–1000 x g for 1 minute. Make sure that there are no bubbles trapped at the bottom of the wells.
6. Immediately continue to *3.4.2 Prepare Incubation Plates and perform incubation*.

4.4.2 Prepare Incubation Plates and perform incubation

During this step, the prepared Incubation Mixes are mixed with the samples in one Incubation Plate per panel, using the Mosquito® prior to incubation (*Figure 29*).

As the Mosquito® deck can only hold a limited number of plates, perform the instruction four times, once for each Run (one Mosquito® run per Reagent Source Plate).

Prepare bench

- Sample Source Plate(s), prepared in previous step
- Sample Dilution Plate(s), prepared in previous step
- Reagent Source Plates 1-4, prepared in previous step
- 4x 384-well PCR plates (skirted)
- Adhesive films

Before you start

- Allow the refrigerated Sample Source Plate(s) and Sample Dilution Plate(s) to reach room temperature.
- Mark four new 384-well PCR plates: “Incubation Plate [1-4]”.

Instructions

1. In the open Mosquito® software, Select *File/Open* and choose the applicable protocol from the Load Protocol Window:
   - CARDIO: *Olink Incubation CARDIO v4*
   - INF*: Olink Incubation INF v4*
   - NEURO: *Olink Incubation NEURO v4*
   - ONC: *Olink Incubation ONC v4*
   - CARDIO II: *Olink Incubation CARDIO II v4*

- INF II: *Olink Incubation INF II v4*
- NEURO II: *Olink Incubation NEURO II v4*
- ONC II: *Olink Incubation ONC II v4*

IMPORTANT: *Make sure that the correct protocol and version is used. The plate layouts of the sample source plate and sample dilution plate have changed and using an older version of the protocol will result in unusable data. Please contact support@olink.com if you need assistance.*

NOTE: *Make sure that all the plates are marked with the same panel number.*

2. Place the Incubation Plate 1 in a magnetic clamp booster and place it on the Mosquito® deck, refer to the software for the correct position. Make sure to insert the corner of the magnetic clamp booster under the small metal holder of the deck.

IMPORTANT: *Risk of instrument damage! If a magnetic clamp booster is not correctly placed it may collide with the internal parts of the instrument during the run.*

3. Carefully remove the adhesive film from the Reagent Source Plate 1.
4. Place the Reagent Source Plate 1 in a magnetic clamp booster and place it on the Mosquito® deck. Refer to the software for the correct position.
5. Click *Run*.
   Result: The Mosquito® transfers 0.6 µL of each Incubation Mix from the Reagent Source Plate into the Incubation Plate. The run takes approximately 10 minutes to be completed and can be monitored in the protocol tab.
6. When the Mosquito® protocol is paused, and a pop-up window appears on the screen, remove the Incubation Plate 1 from the Mosquito® deck and from the magnetic clamp booster.
7. Inspect the Incubation Plate to ensure that there is liquid at the bottom of all wells, and that there are no bubbles present.

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

NOTE: *If bubbles are present, seal the Incubation Plate(s) with new adhesive film(s) and spin at 400–1000 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.*

8. Place Incubation Plate 1 back in the magnetic clamp boosters and return it to its previous position on the Mosquito® deck.
9. Vortex the applicable Sample Source Plate and Sample Dilution Plate briefly and spin down at 400–1000 x g for 1 minute. Make sure that there are no bubbles trapped at the bottom of the wells and carefully remove the adhesive films.

NOTE: *Make sure that all plates are marked with the same panel number.*

10. Place the Sample Source Plate and Sample Dilution Plate in magnetic clamp boosters and place them on the Mosquito® deck. Refer to the software for the correct positions.
11. Click *Resume* to continue the protocol.
    Result: The Mosquito® transfers 0.2 µL of samples from the Sample Source Plate and Sample Dilution Plate into the Incubation Plate. The run takes approximately 15 min to be completed.
12. When the Mosquito® protocol is finished, remove Incubation Plate 1 from the Mosquito® deck and from the magnetic clamp boosters. Seal with a new adhesive film.

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

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

13. Spin Incubation Plate 1 at 400–1000 x g for 1 minute.
14. Inspect Incubation Plate 1 to ensure that all wells contain the same amount of liquid (0.8 µL). Note if there are any wells containing bubbles.
15. Write the time of the incubation starting point on the plate seal and incubate Incubation Plate 1 for 16 to 24 hours at +4 °C.

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 PCR1 in the ProFlex™ PCR instrument.*

16. Remove the Sample Source Plate, Sample Dilution Plate, and Reagent Source Plate from the Mosquito® deck. Treat the plates as described in *Table 16.*

Table 26. Plates handling

Incubation Run	Sample Source Plate	Sample Dilution Plate	Reagent Source Plate
Run 1	Seal with adhesive film Store at +4 °C for later use	Seal with adhesive film Store at +4 °C for later use	Discard
Run 2	Seal with adhesive film Store at +4 °C for later use	Seal with adhesive film Store at +4 °C for later use	Discard
Run 3	Seal with adhesive film Store at +4 °C for later use	Seal with adhesive film Store at +4 °C for later use	Discard
Run 4	Seal with adhesive film Store at -80 °C for later use	Discard	Discard

17. Repeat *3.4.1 Prepare Reagent Source Plate* and *3.4.2 Prepare Incubation Plates and perform incubation* for Run 2, 3 and 4.
18. When finished, clear the Mosquito® and shut it down according to instructions in the Olink® Explore Overview User Manual.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	1	1	9	9	17	17	25	25	33	33	41	41	49	49	57	57	65	65	73	73	81	81	SC	SC
B	1	1	9	9	17	17	25	25	33	33	41	41	49	49	57	57	65	65	73	73	81	81	SC	SC
C	2	2	10	10	18	18	26	26	34	34	42	42	50	50	58	58	66	66	74	74	82	82	SC	SC
D	2	2	10	10	18	18	26	26	34	34	42	42	50	50	58	58	66	66	74	74	82	82	SC	SC
E	3	3	11	11	19	19	27	27	35	35	43	43	51	51	59	59	67	67	75	75	83	83	NC	NC
F	3	3	11	11	19	19	27	27	35	35	43	43	51	51	59	59	67	67	75	75	83	83	NC	NC
G	4	4	12	12	20	20	28	28	36	36	44	44	52	52	60	60	68	68	76	76	84	84	NC	NC
H	4	4	12	12	20	20	28	28	36	36	44	44	52	52	60	60	68	68	76	76	84	84	NC	NC
I	5	5	13	13	21	21	29	29	37	37	45	45	53	53	61	61	69	69	77	77	85	85	NC	NC
J	5	5	13	13	21	21	29	29	37	37	45	45	53	53	61	61	69	69	77	77	85	85	NC	NC
K	6	6	14	14	22	22	30	30	38	38	46	46	54	54	62	62	70	70	78	78	86	86	PC	PC
L	6	6	14	14	22	22	30	30	38	38	46	46	54	54	62	62	70	70	78	78	86	86	PC	PC
M	7	7	15	15	23	23	31	31	39	39	47	47	55	55	63	63	71	71	79	79	87	87	PC	PC
N	7	7	15	15	23	23	31	31	39	39	47	47	55	55	63	63	71	71	79	79	87	87	PC	PC
O	8	8	16	16	24	24	32	32	40	40	48	48	56	56	64	64	72	72	80	80	88	88	PC	PC
P	8	8	16	16	24	24	32	32	40	40	48	48	56	56	64	64	72	72	80	80	88	88	PC	PC

Figure 48. Incubation Plate layout.
The numbers indicate the sample numbers. The colors indicate the different blocks within the panel: white = block A, purple = block B, blue = block C, black = block D.

4.5 Preparation of reagents for day 2

Prepare bench

- MilliQ water
- Olink® Explore PCR1 Enhancer
- Olink® Explore PCR1 Solution

Instructions

1. Place 30 mL of MilliQ water at +4 °C overnight.
2. Thaw the PCR1 Enhancer at room temperature overnight.
3. Optional: Thaw the PCR1 Solution at +4 °C overnight.

4.6 Extension and pre-amplification (PCR1) (day 2)

During this step, a PCR1 Mix is prepared manually and dispensed into the Incubation Plates using the Dragonfly®. The plates are renamed “PCR1 Plates” and subjected to a PCR reaction.

4.6.1 Prepare PCR1 Mix

During this step, a PCR1 Mix is prepared manually. The PCR1 Mix must be used within 30 minutes from preparation.

Prepare bench

- Incubation Plates 1–4, prepared in previous step
- Olink® Explore PCR1 Enhancer
- Olink® Explore PCR1 Solution
- Olink® Explore PCR1 Enzyme (when brought to the lab bench, keep the PCR1 Enzyme in a freezing block (-20 °C))
- MilliQ water (at +4 °C, preferably kept in the fridge until use)
- 1x Falcon tube (50 mL)

Before you start

- Thaw the PCR1 Solution at +4 °C overnight (optional), or at room temperature before starting the PCR1 Setup (it may take several hours) and ensure that it is fully thawed and has reached room temperature.
- Ensure that the PCR1 Enhancer is fully thawed and has reached room temperature.
- Let the refrigerated Incubation Plates 1-4 reach room temperature.
- Mark the new 50 mL Falcon tube: "PCR1 Mix".
- Switch on two ProFlex™ PCR instruments in the post-PCR room.

Instructions

1. Vortex the PCR1 Solution and PCR1 Enhancer thoroughly and spin down briefly.

IMPORTANT: *Insufficient thawing and vortexing of the PCR1 Enhancer may lead to particles attaching to the syringe and run failure.*

2. Spin the PCR1 Enzyme briefly. Do not vortex.

NOTE: *Always keep the PCR1 Enzyme at -20 °C. When brought to the lab bench, keep it in a freezing block (-20 °C).*

3. Spin Incubation Plates 1–4 down for 1 minute at room temperature.
4. Prepare a PCR1 Mix in a Falcon tube in the following order, using the volumes indicated in *Table 17*:
    1. Add MilliQ water and PCR1 Enhancer and vortex thoroughly.
    2. Add the PCR1 Solution and PCR1 Enzyme, and vortex for 3 seconds.

Table 27. PCR1 Mix

Addition order	Reagent	Volume (µL)
1	MilliQ water (+4 °C)	27 000
2	PCR1 Enhancer	3 510
3	PCR1 Solution	3 510
4	PCR1 Enzyme	351
	Total	34 371

3. Keep at room temperature until use.

TIME SENSITIVE STEP: *Dispensing of the PCR1 Mix using the Dragonfly® must start within 30 minutes from PCR1 Mix preparation.*

4.6.2 Prepare PCR1 Plates and perform PCR1

During this step, the prepared PCR1 Mix is dispensed into the Incubation Plates using the Dragonfly®, and the plates are subjected to a PCR reaction (*Figure 33*).

The PCR1 Plates must be placed in the ProFlex™ exactly 10 minutes after the PCR1 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 PCR1 Mix into Incubation Plates 1 and 2 using the Dragonfly®. Start a PCR1 run for these plates on one of the ProFlex™ instruments.
2. Dispense PCR1 Mix into Incubation Plates 3 and 4 plates using the Dragonfly®. Start a PCR1 run for these plates on the second ProFlex™ instrument.

Prepare bench

- Incubation Plates 1–4 at room temperature, prepared in previous step
- PCR1 Mix, prepared in previous step
- 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 a timer to 10 minutes.

Instructions

1. Start the PCR program *Olink PCR1* on the two ProFlex™ PCR instruments (*Figure 30*). Pause when the PCR block temperature reaches 50 °C.

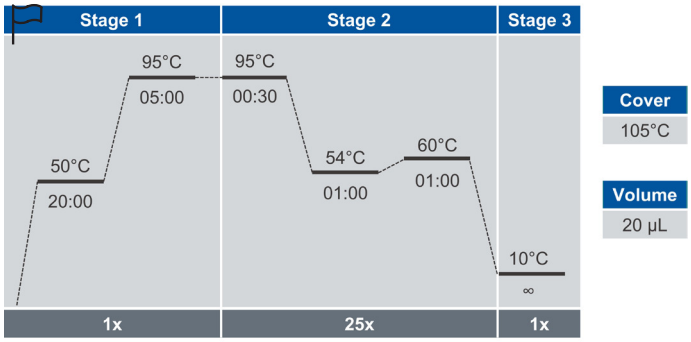

Figure 49. Olink PCR1 program

2. Prepare the Dragonfly® according to instructions in the Olink Explore Overview User Manual.
   - Use the protocol *Olink PCR1 Setup plate v4.*
   - Attach three syringes in position B2, B3, and B4.
3. Slide the reservoir tray to the filling position (A) and place three new reservoirs in position B2, B3 and B4 (B) (*Figure 31*).

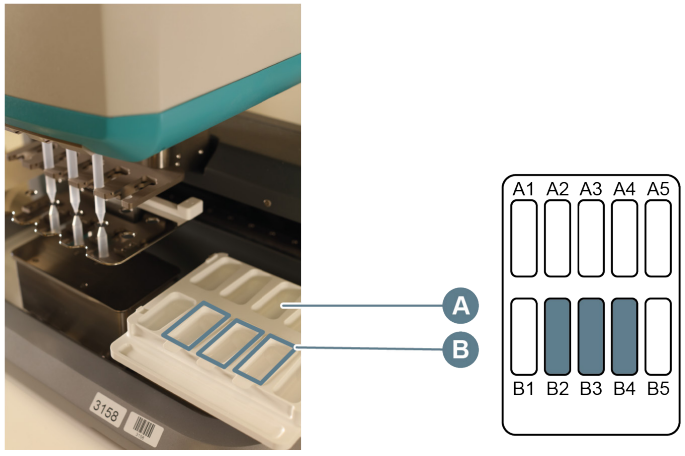

Figure 50. Reservoir tray in filling position

4. Transfer 11 mL of PCR1 Mix into each of the three reservoirs.

IMPORTANT: *The syringes and PCR1 Mix are used to prepare all four PCR1 Plates. Do not discard until all PCR1 Plates are complete.*

5. Carefully slide the reservoir tray back to the aspirate position (*Figure 32*).

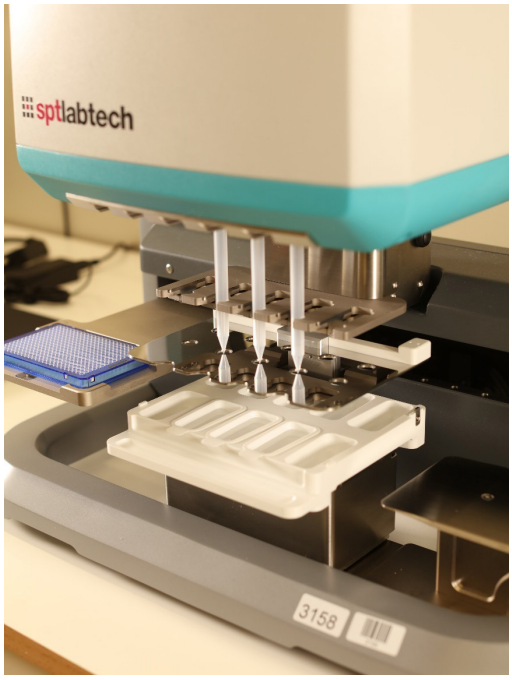

Figure 51. Reservoir tray in aspirate position

6. Rename Incubation Plate 1 “PCR1 Plate 1” and Incubation Plate 2 “PCR1 Plate 2”. Spin down the plates.
7. Carefully remove the adhesive film from PCR1 Plate 1 and place it in the Dragonfly® plate position (to the left), with well A1 in the top left corner.
8. Select the *Run* tab in the *Constant layer* view of the software, then click *RUN* to start the program. Start the timer when PCR1 Mix is added to the first well of the plate.
   Result: Dragonfly® dispenses 19 µL of PCR1 Mix into each well of the PCR1 Plate 1.

TIME SENSITIVE STEP: *The PCR1 Plate must be placed in the ProFlex™ exactly 10 minutes after the PCR1 Mix has been added to the first well of the first plate.*

9. When the Dragonfly® has returned the PCR1 Plate 1 to the plate position and released the plate clamp, remove the plate from the instrument and immediately seal it with a new adhesive film.
10. Inspect the PCR1 Plate 1 to ensure that all wells contain the same amount of liquid (19.8 µL). Using the MixMate® or manual vortexing, vortex the plate thoroughly to ensure that all wells are mixed.
11. Repeat steps 7–10 for PCR1 Plate 2.
12. In the Post-PCR room, centrifuge the two PCR1 Plates 1-2 at 400–1000 x g for 1 minute.
13. Inspect PCR1 Plates 1–2 to ensure that all wells contain the same amount of liquid (19.8 µL). Note any deviations.
14. When the timer ends after 10 minutes, place PCR1 Plates 1–2 in one of the two pre-heated ProFlex™ instruments and click *Resume* the run the *Olink PCR1* program.
15. Repeat steps 6-14 for Incubation Plates 3–4, except rename Incubation Plate 3 as "PCR Plate 3, and Incubation Plate 4 as "PCR Plate 4". Place both plates in the second ProFlex™.
16. When finished, clear the Dragonfly® and shut it down according to instructions in the Olink Explore Overview User Manual.
17. When the *Olink PCR1* programs are finished (~1 hour 55 minutes), continue to *3.7 Pool PCR1 products*, or store the four PCR1 Plates at +4 °C if used the same day.

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

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	1	1	9	9	17	17	25	25	33	33	41	41	49	49	57	57	65	65	73	73	81	81	SC	SC
B	1	1	9	9	17	17	25	25	33	33	41	41	49	49	57	57	65	65	73	73	81	81	SC	SC
C	2	2	10	10	18	18	26	26	34	34	42	42	50	50	58	58	66	66	74	74	82	82	SC	SC
D	2	2	10	10	18	18	26	26	34	34	42	42	50	50	58	58	66	66	74	74	82	82	SC	SC
E	3	3	11	11	19	19	27	27	35	35	43	43	51	51	59	59	67	67	75	75	83	83	NC	NC
F	3	3	11	11	19	19	27	27	35	35	43	43	51	51	59	59	67	67	75	75	83	83	NC	NC
G	4	4	12	12	20	20	28	28	36	36	44	44	52	52	60	60	68	68	76	76	84	84	NC	NC
H	4	4	12	12	20	20	28	28	36	36	44	44	52	52	60	60	68	68	76	76	84	84	NC	NC
I	5	5	13	13	21	21	29	29	37	37	45	45	53	53	61	61	69	69	77	77	85	85	NC	NC
J	5	5	13	13	21	21	29	29	37	37	45	45	53	53	61	61	69	69	77	77	85	85	NC	NC
K	6	6	14	14	22	22	30	30	38	38	46	46	54	54	62	62	70	70	78	78	86	86	PC	PC
L	6	6	14	14	22	22	30	30	38	38	46	46	54	54	62	62	70	70	78	78	86	86	PC	PC
M	7	7	15	15	23	23	31	31	39	39	47	47	55	55	63	63	71	71	79	79	87	87	PC	PC
N	7	7	15	15	23	23	31	31	39	39	47	47	55	55	63	63	71	71	79	79	87	87	PC	PC
O	8	8	16	16	24	24	32	32	40	40	48	48	56	56	64	64	72	72	80	80	88	88	PC	PC
P	8	8	16	16	24	24	32	32	40	40	48	48	56	56	64	64	72	72	80	80	88	88	PC	PC

Figure 52. PCR1 plate layout.
The numbers indicate the sample numbers. The colors indicate the different blocks within the panel: white = block A, purple = block B, blue = block C, black = block D.

4.7 Pool PCR1 products

During this step, the PCR1 products from the four PCR1 Plates are pooled into one PCR1 Pooling Plate using the ep*Motion*® (*Figure 36*).

Prepare bench

- PCR1 Plates 1–4, prepared in previous step
- MilliQ water (at +4 °C, preferably kept in the fridge until use)
- 1x 384-well PCR plate (skirted)
- 1x ep*Motion*® reservoir (30 mL)
- Dispensing tool TM50-8
- Dispensing tool TM10-8
- epT.I.P.S.® Motion pipette tips (10 µL)
- epT.I.P.S.® Motion pipette tips (50 µL)
- Waste bag
- Adhesive films

Before you start

- Thaw PCR1 Plates 1–4 at room temperature if frozen.
- Mark the new 384-well PCR plate: “PCR1 Pooling Plate”.
- Switch on the ep*Motion*® system, open the EpBlue™ software and log in.

Instructions

1. Make sure that PCR1 Plates 1–4 are thawed and properly sealed, then vortex the plates and spin down at 400–1000 x g for 1 minute at room temperature.
2. Inspect the wells of PCR1 Plates 1–4 to make sure that no liquid has evaporated and that all the liquid is at the bottom of the wells. Spin down the plates again if necessary.

3. Open the EpBlue™ Application Runner. In the application library, select user and the protocol: *1 Olink PCR1 Pooling 4 panels.*
4. When the ID number of the instrument is shown in the software, click *Next* to continue.
5. Prepare the ep*Motion*® worktable according to the software instructions. Add 6 mL MilliQ water to the reservoir.
   - Place PCR1 Plates 1–4 on the thermoadapters (*Figure 34*).
   - Carefully remove the adhesive films.

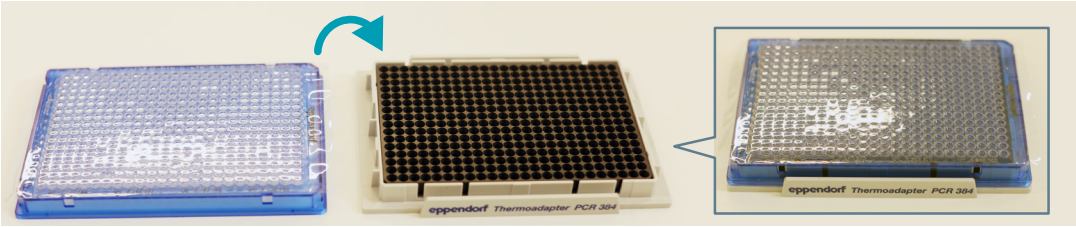

Figure 53. Thermoadapter

NOTE: *Make sure to place tip boxes and plates in the correct position and orientation according to the layout presented in the software, and that empty positions in the software are also empty on the worktable. Failure to follow the layout in the software can lead to instrument damage.*

6. Place a waste bag in the waste container. Pull the edge of the bag tightly downwards to ensure that the path of the dispensing tool and access to the rack are not obstructed (*Figure 35*).

Figure 54. Waste container

7. Close the front hood.
8. 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*.
9. Click *Next* until a *Run* button appears, then click *Run* to start the protocol.
   Result: The ep*Motion*® *automatically scans the worktable, dispenses 12 µL MilliQ water into each well of the PCR1 Pooling Plate, and pools 3 µL of each PCR1 product from each sample into one well per panel. The run takes approximately 50 minutes to be completed.*
10. When the protocol is finished (indicated by a sound signal and a message in the software), remove the PCR1 Pooling Plate and seal it with a new adhesive film. Keep the ep*Motion*® on for later use.
11. Vortex the PCR1 Pooling Plate and spin down at 400–1000 x g for 1 minute.
12. Inspect the PCR1 Pooling Plate to ensure that all wells contain the same amount of liquid (24 µL).
13. Remove PCR1 Plates 1-4 containing the remaining PCR products and seal them with new adhesive films. Store it at -20 °C for up to 2 weeks in case of potential reruns.
14. Continue to *3.8 Amplification and sample indexing (PCR2)*, or store the PCR1 Pooling Plate at +4 °C until ready to use (the same day).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	1	1	9	9	17	17	25	25	33	33	41	41	49	49	57	57	65	65	73	73	81	81	SC	SC
B	1	1	9	9	17	17	25	25	33	33	41	41	49	49	57	57	65	65	73	73	81	81	SC	SC
C	2	2	10	10	18	18	26	26	34	34	42	42	50	50	58	58	66	66	74	74	82	82	SC	SC
D	2	2	10	10	18	18	26	26	34	34	42	42	50	50	58	58	66	66	74	74	82	82	SC	SC
E	3	3	11	11	19	19	27	27	35	35	43	43	51	51	59	59	67	67	75	75	83	83	NC	NC
F	3	3	11	11	19	19	27	27	35	35	43	43	51	51	59	59	67	67	75	75	83	83	NC	NC
G	4	4	12	12	20	20	28	28	36	36	44	44	52	52	60	60	68	68	76	76	84	84	NC	NC
H	4	4	12	12	20	20	28	28	36	36	44	44	52	52	60	60	68	68	76	76	84	84	NC	NC
I	5	5	13	13	21	21	29	29	37	37	45	45	53	53	61	61	69	69	77	77	85	85	NC	NC
J	5	5	13	13	21	21	29	29	37	37	45	45	53	53	61	61	69	69	77	77	85	85	NC	NC
K	6	6	14	14	22	22	30	30	38	38	46	46	54	54	62	62	70	70	78	78	86	86	PC	PC
L	6	6	14	14	22	22	30	30	38	38	46	46	54	54	62	62	70	70	78	78	86	86	PC	PC
M	7	7	15	15	23	23	31	31	39	39	47	47	55	55	63	63	71	71	79	79	87	87	PC	PC
N	7	7	15	15	23	23	31	31	39	39	47	47	55	55	63	63	71	71	79	79	87	87	PC	PC
O	8	8	16	16	24	24	32	32	40	40	48	48	56	56	64	64	72	72	80	80	88	88	PC	PC
P	8	8	16	16	24	24	32	32	40	40	48	48	56	56	64	64	72	72	80	80	88	88	PC	PC

Figure 55. PCR1 Pooling Plate layout.
The numbers indicate the sample numbers. The colors indicate the panel numbers: light blue = Panel 1, green = Panel 2, dark blue = Panel 3, grey = Panel 4.

4.8 Amplification and sample indexing (PCR2)

During this step, a PCR2 Mix is prepared manually and then mixed with the samples along with index primers (unique for each sample) using the ep*Motion*®. The samples are then subjected to a second PCR reaction.

4.8.1 Prepare PCR2 Mix

During this step, a PCR2 Mix is prepared manually. The PCR2 Mix must be used within 15 minutes from preparation.

Prepare bench

- PCR1 Pooling Plate, prepared in previous step
- Olink Explore PCR2 Solution
- Olink Explore PCR2 Enzyme (when brought to the lab bench, keep the PCR2 Enzyme in a freezing block (-20 °C))
- Olink Explore Index Plate 1
- MilliQ water (at +4 °C, preferably kept in the fridge until use)
- Falcon tube (15 mL)
- Manual pipettes (10–100 µL, 100–1000 µL)
- Filter pipette tips

Before you start

- Thaw the PCR2 Solution and the Index Plate 1 at room temperature.
- If stored at +4 °C , allow the PCR1 Pooling Plate to reach room temperature.
- Switch on one ProFlex™ PCR instrument. No preheating is required.
- Mark the new 15 mL tube: "PCR2 Mix".

Instructions

1. Vortex the PCR2 Solution and spin it briefly.
2. Vortex the Index Plate 1 and spin it at 400–1000 x g for 1 minute.
3. Make sure that the PCR1 Pooling Plate is properly sealed, then vortex it and spin at 400–1000 x g for 1 minute.

4. Spin the PCR2 Enzyme briefly. Do not vortex

NOTE: *Always keep the PCR2 Enzyme at -20 °C. When brought to the lab bench, keep it in a freezing block (-20 °C).*

5. Prepare a PCR2 Mix in a Falcon tube following the order and volumes indicated in *Table 18*.

Table 28. PCR2 Mix

Addition order	Reagent	Volume (µL)
1	MilliQ water (+4 °C)	6 065
2	PCR2 solution	870
3	PCR2 Enzyme	18
	Total	6 953

6. Vortex the PCR2 Mix thoroughly. Keep at room temperature until use.

TIME SENSITIVE STEP: *Start the dispensing of the PCR2 Mix within 15 minutes from preparation.*

4.8.2 Prepare PCR2 Plate and perform PCR2

During this step, the prepared PCR2 Mix is mixed with the samples along with index primers using the epMotion®. The samples are then subjected to a second PCR reaction (*Figure 38*).

Prepare bench

- PCR1 Pooling Plate (at room temperature), prepared in previous step
- PCR2 Mix, prepared in previous step
- Olink Explore Index Plate 1
- 1x 384-well PCR plate (skirted)
- ep*Motion*® reservoir (30 mL)
- epT.I.P.S.® Motion pipette tips (10 µL)
- epT.I.P.S.® Motion pipette tips (50 µL)
- Dispensing tool TM50-8
- Dispensing tool TM10-8
- Waste bag
- Adhesive films

Before you start

- Mark the new 384-well PCR plate: “PCR2 Plate”.

Instructions

1. Pour the PCR2 Mix into a 30 mL ep*Motion*® reservoir. Make sure that no air bubbles are trapped at the bottom of the reservoir.
2. Open the *EpBlue ™Application Runner*. In the application library, select user and the protocol: *2 Olink PCR2 Setup 4 panels.*
3. When the ID number of the instrument is shown in the software, click *Next* to continue.
4. Centrifuge Index Plate 1 at 400–1000 x g for 1 minute and inspect the plate to ensure that all wells contain the same amount of liquid (20 µL). If a well is empty or contains a lower volume, contact Olink Support.
5. Carefully remove the adhesive films from the Index Plate 1 and the PCR1 Pooling Plate. Make sure that no air bubbles are trapped at the bottom of the wells of the PCR1 Pooling Plate.
6. Prepare the ep*Motion*® worktable according the software instructions.

NOTE: *Make sure to place tip boxes and plates in the correct position and orientation according to the layout presented in the software, and that empty positions in the software are also empty on the worktable. Failure to follow the layout in the software can lead to instrument damage.*

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, then click *Run* to start the protocol.
   Result: ep*Motion*® *transfers 16 µL PCR2 Mix, 2 µL Index Primers (from the Index Plate 1) and 2 µL PCR1 products (from the PCR1 Pooling Plate) into the applicable wells of the PCR2 Plate (Figure 38). The run takes approximately 40 minutes to be completed.*
9. When the protocol is finished (indicated by a sound signal and a message in the software), remove the PCR2 Plate and seal it with a new adhesive film. Keep the ep*Motion*® on for later use.

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

TIME SENSITIVE STEP: *Start the PCR2 program within 5 minutes from end of* ep*Motion*® *protocol.*

10. Vortex and spin down at 400–1000 x g for 1 minute at room temperature.
11. Inspect the PCR2 Plate to ensure that all wells contain the same amount of liquid (20 µL).
12. Place the PCR2 Plate in the ProFlex™ and add a balance plate to the other side of the ProFlex™.
13. Click *Open* and select the program *Olink Index PCR2 (Figure 37)*. Click *Start.*

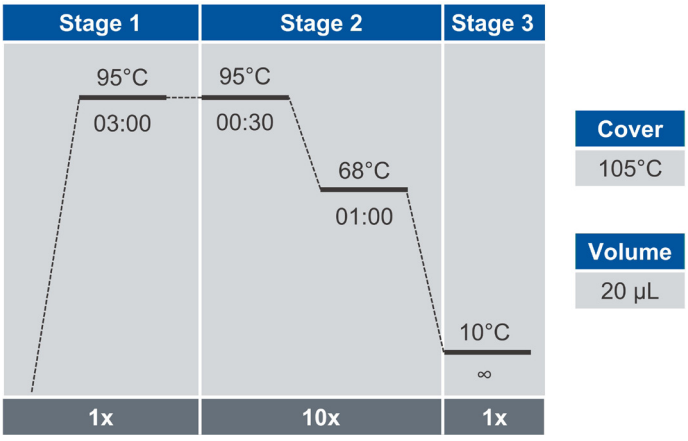

Figure 56. Olink Index PCR2 program

14. Remove the PCR1 Pooling Plate containing the remaining PCR products from the ep*Motion*® and seal it with a new adhesive film. Store it at -20 °C for up to 2 weeks in case of potential reruns.
15. Discard the Index Plate 1.
16. When the PCR program is finished (~25 minutes), continue to *3.9 Pool PCR2 products*, or store the PCR2 Plate at +4 °C until use (the same day).

SAFE STOPPING POINT: *The PCR2 Plate can be stored at -20 °C for up to 2 weeks.*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	1	1	9	9	17	17	25	25	33	33	41	41	49	49	57	57	65	65	73	73	81	81	SC	SC
B	1	1	9	9	17	17	25	25	33	33	41	41	49	49	57	57	65	65	73	73	81	81	SC	SC
C	2	2	10	10	18	18	26	26	34	34	42	42	50	50	58	58	66	66	74	74	82	82	SC	SC
D	2	2	10	10	18	18	26	26	34	34	42	42	50	50	58	58	66	66	74	74	82	82	SC	SC
E	3	3	11	11	19	19	27	27	35	35	43	43	51	51	59	59	67	67	75	75	83	83	NC	NC
F	3	3	11	11	19	19	27	27	35	35	43	43	51	51	59	59	67	67	75	75	83	83	NC	NC
G	4	4	12	12	20	20	28	28	36	36	44	44	52	52	60	60	68	68	76	76	84	84	NC	NC
H	4	4	12	12	20	20	28	28	36	36	44	44	52	52	60	60	68	68	76	76	84	84	NC	NC
I	5	5	13	13	21	21	29	29	37	37	45	45	53	53	61	61	69	69	77	77	85	85	NC	NC
J	5	5	13	13	21	21	29	29	37	37	45	45	53	53	61	61	69	69	77	77	85	85	NC	NC
K	6	6	14	14	22	22	30	30	38	38	46	46	54	54	62	62	70	70	78	78	86	86	PC	PC
L	6	6	14	14	22	22	30	30	38	38	46	46	54	54	62	62	70	70	78	78	86	86	PC	PC
M	7	7	15	15	23	23	31	31	39	39	47	47	55	55	63	63	71	71	79	79	87	87	PC	PC
N	7	7	15	15	23	23	31	31	39	39	47	47	55	55	63	63	71	71	79	79	87	87	PC	PC
O	8	8	16	16	24	24	32	32	40	40	48	48	56	56	64	64	72	72	80	80	88	88	PC	PC
P	8	8	16	16	24	24	32	32	40	40	48	48	56	56	64	64	72	72	80	80	88	88	PC	PC

Figure 57. PCR2 Plate layout.
The numbers indicate the sample numbers. The colors indicate the panel numbers: light blue = Panel 1, green = Panel 2, dark blue = Panel 3, grey = Panel 4.

4.9 Pool PCR2 products

During this step, all PCR2 products belonging to the same panel are pooled into one column of the PCR2 Pooling Plate using the ep*Motion*® (*Figure 39*), then manually transferred to one microcentrifuge tube per panel. Each tube contains amplicons from 96 samples, including controls.

Prepare bench

- PCR2 Plate, prepared in previous step
- 1x 96-well PCR plate (skirted)
- 4x microcentrifuge tubes (1.5 mL)
- epT.I.P.S.® Motion pipette tips (10 µL)
- Dispensing tool TM10-8
- Waste bag
- Manual pipette (10–100 µL)
- Filter pipette tips
- Adhesive films

Before you start

- Thaw PCR2 Plate at room temperature if frozen
- Mark the new PCR plate: “PCR2 Pooling Plate”.
- Mark the four new microcentrifuge tubes: “PCR2 [1–4]”.

Instructions

1. Open the *EpBlue™ Application Runner*.
2. In the application library, select user and the applicable protocol: *3 Olink PCR2 Pooling 4 panels.*
3. When the ID number of the instrument is shown in the software, click *Next* to continue.
4. Make sure that PCR2 Plate is thawed and properly sealed, then vortex and spin it down at 400–1000 x g for 1 minute at room temperature to ensure that all wells are mixed.

5. Inspect the wells of the PCR2 Plate to make sure that no liquid has evaporated and that all the liquid is at the bottom of the wells.
6. Prepare the ep*Motion*® worktable according to the software instructions.

IMPORTANT: *Make sure to place tip boxes and plates in the correct position and orientation according to the layout presented in the software, and that empty positions in the software are also empty on the worktable. Failure to follow the layout in the software can lead to instrument damage.*

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, then click Run to start the protocol.
   Result: ep*Motion*® *pools 3 µL from each well in a row of the PCR2 Plate into a single column of the PCR2 Pooling plate. The result is one PCR2 Pooling Plate column of pooled PCR2 products per panel.*
9. When finished, remove the PCR2 Pooling Plate from the worktable. Seal the plate with a new adhesive film, vortex the plate and spin down at 400–1000 x g for 1 minute.
10. Inspect the PCR2 Pooling Plate to ensure that every applicable well contains the same amount of liquid (36 µL in columns 1, 3, 5 and 7).
11. Clear the ep*Motion*® and shut it down according to instructions in the Olink® Explore Overview User Manual.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1		2		3		4					
B	1		2		3		4					
C	1		2		3		4					
D	1		2		3		4					
E	1		2		3		4					
F	1		2		3		4					
G	1		2		3		4					
H	1		2		3		4					

Figure 58. PCR2 Pooling Plate layout.
The numbers and colors indicate the panel numbers: light blue = Panel 1, green = Panel 2, dark blue = Panel 3, grey = Panel 4.

12. Carefully remove the adhesive film from the PCR2 Pooling Plate.
13. Using a single-channel pipette, transfer the pooled PCR2 products from the PCR2 Pooling Plate to the PCR2 Tubes as described in *Table 19*. Use forward pipetting, and change pipette tip after each well.

Table 29. Transfer PCR2 products

Volume (µL) /well	From column	To tube
30	1	PCR2 1
30	3	PCR2 2
30	5	PCR2 3
30	7	PCR2 4

14. Vortex PCR2 Tubes 1-4 and spin down briefly.
15. Remove the PCR2 Plate containing the remaining PCR2 products from the instrument, seal it with a new adhesive film and store it at -20 °C for up to 2 weeks in case of potential reruns.
16. Discard the PCR2 Pooling Plate.
17. Continue to *3.10 Library purification*, or store the PCR2 Tubes at +4 °C until use (the same day).

SAFE STOPPING POINT: *The PCR2 Tubes can also be stored at –20 °C for up to 2 weeks.*

4.10 Library purification

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

Prepare bench

- PCR2 Tubes 1–4, prepared in previous step
- Agentcourt AMPure XP bottle
- 96% Ethanol (EtOH)
- MilliQ water
- DynaMag™-2 Magnet
- Timer
- 8x Microcentrifuge tubes (1.5 mL)
- Falcon tube (15 mL)
- Manual pipettes (10–100 µL, 100–1000 µL)
- Pipette (5 mL)
- Filter pipette tips

Before you start

- Let the refrigerated Agentcourt AMPure XP bottle reach room temperature.
- Mark four new microcentrifuge tubes: “BP [1–4]” (for “Bead Purification”).
- Mark four new microcentrifuge tubes: “Lib [1–4]”.
- Mark the new 15 mL Falcon tube: "70% EtOH".
- Set a timer to 5 minutes.

Instructions

1. Freshly prepare a sufficient amount of 70% EtOH for the number of Libraries to purify according to *Table 20*. Manually transfer first the MilliQ water and then the 96% EtOH into the Falcon tube.

Table 30. Prepare 70% EtOH

Reagent	Volume (mL) for							
	1 library	2 Libraries	3 Libraries	4 Libraries	5 Libraries	6 Libraries	7 Libraries	8 Libraries
MilliQ water	0.5	0.8	1.1	1.4	1.6	1.9	2.2	2.5
96% EtOH	1.5	2.2	2.9	3.6	4.4	5.1	5.8	6.5
Total (70% EtOH)	2	3	4	5	6	7	8	9

2. Shake and vortex the Agentcourt AMPure XP bottle vigorously to resuspend the magnetic beads.
3. Transfer 80 µL from the Agentcourt AMPure XP bottle to each BP Tube.
4. Transfer 50 µL from each PCR2 Tube to the corresponding BP Tube according to *Table 21*.

Table 31. Transfer PCR2 products to BP tubes

Volume (µL)	From tube	To tube
50	PCR2 1	BP 1
50	PCR2 2	BP 2
50	PCR2 3	BP 3
50	PCR2 4	BP 4

NOTE: *Store the PCR2 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 and incubate BP Tubes 1–4 for 5 minutes at room temperature.
7. After the incubation, place the BP Tubes on the DynaMag™-2 Magnetic stand and leave them for 2 minutes to separate the beads from the solution (*Figure 40*).

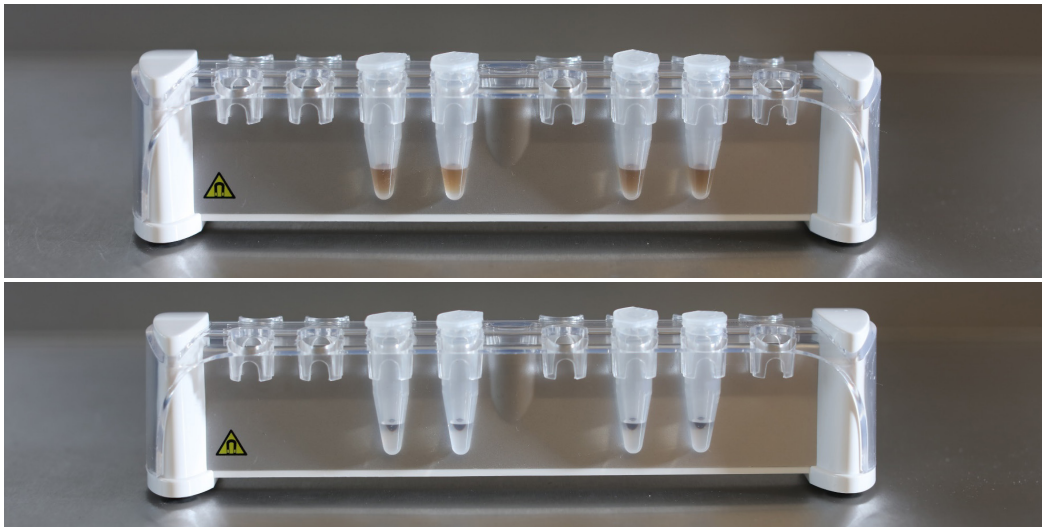

Figure 59. Tubes in DynaMag™-2 Magnetic stand before (upper) and after (lower) bead separation.

8. With the tubes still on the magnetic stand, carefully open the lid and discard 125 µL supernatant using a single-channel pipette. Do not disturb the beads.
9. With the tubes still in the magnetic stand, wash the beads:
   a. Add 500 µL of 70 % EtOH to the 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) once.

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 MilliQ water to the BP Tubes and pipette-mix 10 times towards the beads to elute the purified Libraries from the beads.
13. Incubate the tubes for 2 minutes at room temperature.
14. Place BP Tubes 1–4 on the magnetic stand and leave them for 1 minute to separate the beads from the eluted Library solution.

15. With BP Tubes 1-4 still on the magnetic stand, transfer 45 µL of eluate from the BP Tubes to the corresponding Lib Tubes according to *Table 22.*

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

Table 32. Transfer eluat

Volume (µL)	From tube	To tube
45	BP 1	Lib 1
45	BP 2	Lib 2
45	BP 3	Lib 3
45	BP 4	Lib 4

16. Discard BP Tubes 1-4.
17. Continue to *3.11 Quality control*.

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

4.11 Quality control

During this step, the four purified Olink Libraries are quality controlled on the Bioanalyzer using the High Sensitivity DNA kit or on the TapeStation using the High Sensitivity D5000 kit, according to the Manufacturer's instructions.

The electropherograms in *Figure 41* and *Figure 42* displays typical results for an Olink Library. 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 https://emea.support.illumina.com/bulletins/2019/10/bubble-products-in-sequencing-Libraries--causes--identification-.html for more information.

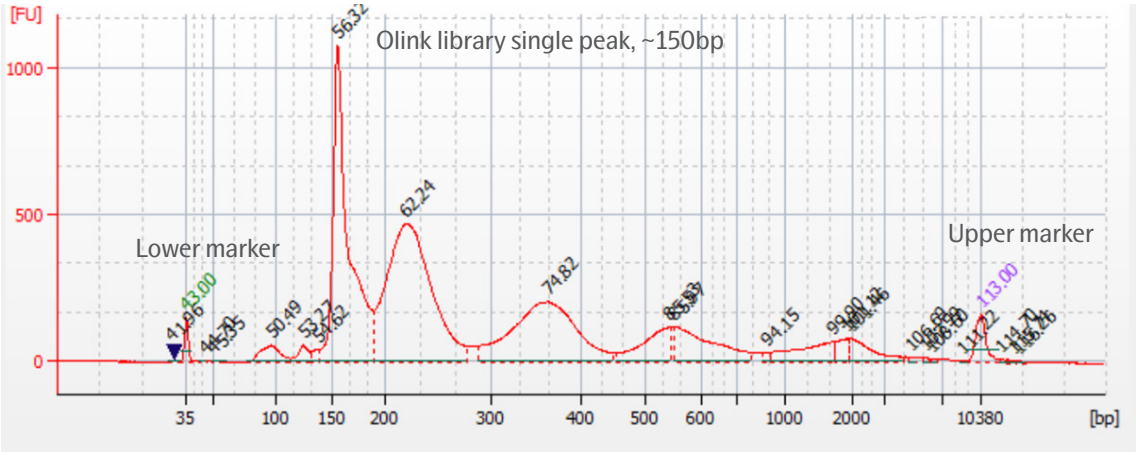

Figure 60. Bioanalyzer's electropherogram.

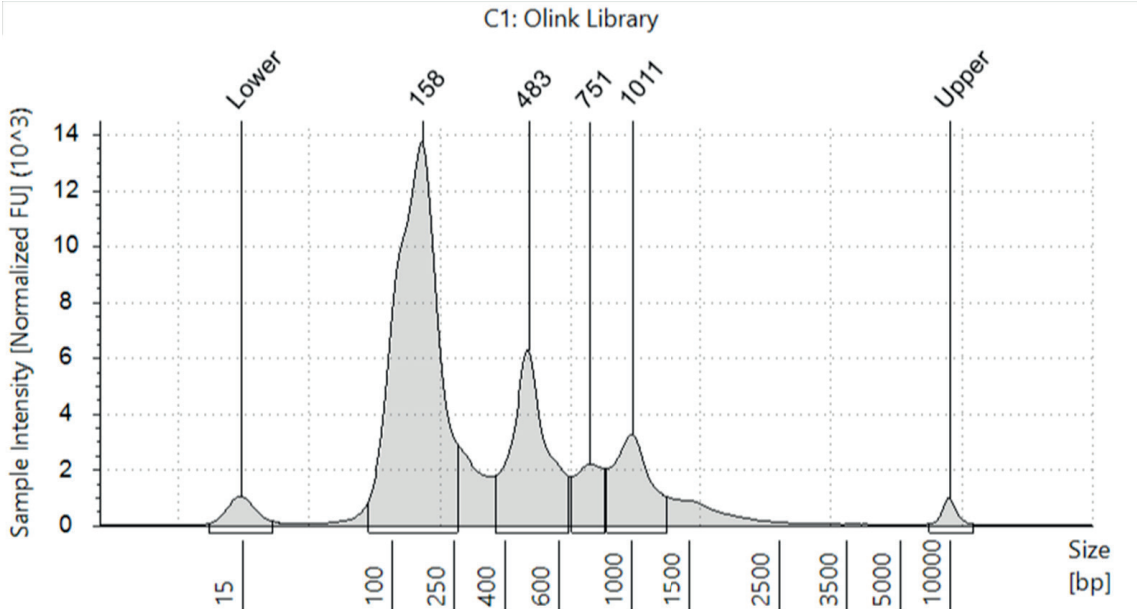

Figure 61. TapeStation's electropherogram.

For expected results of a successful ladder run, refer to the Manufacturer's Manual. If any of the deviations in *Table 23* should occur in the electropherogram, do not continue to sequencing. Instead, perform the suggested corrective action.

Table 33. Deviations in electropherogram.

Peak deviation	Possible cause	Action
No Library peak present at 150bp.	1) No PCR1 amplification has taken place, and therefore there are no PCR2 products and no Library 2) The bead purification failed	Rerun the quality control with unpurified Library. If a peak is present at 150bp: purify a new aliquot of the unpurified Library and rerun the quality control. If successful, proceed to sequencing. If there is no 150bp peak present in the unpurified Library, no PCR product has been generated. Repeat the Library preparation, quality control and sequencing.
No Library peak present at 150bp, but a smaller peak at 120bp	The index primers were not included in the PCR2 reaction (the 120bp peak corresponds to the PCR1 product) 1) The Index plate was empty 2) There was no transfer of index primers from the Index Plate to the PCR2 Plate due to ep*Motion*® failure	Contact Support. Make sure to centrifuge and visually inspect the Index Plate before starting the PCR2 setup. Make sure that the ep*Motion*® is calibrated, functional and that the correct protocol is used.

4.12 Next generation sequencing

Next generation sequencing is performed using either an Illumina® NextSeq™ 550, NextSeq™ 2000 or NovaSeq™ 6000 instrument. As the workflow differs between instruments, refer to the applicable Olink Explore Sequencing User Manual for instructions on how to sequence Olink Libraries.

5. Laboratory instruction for 384 samples

This chapter provides instructions on how to perform each step of the Olink Explore 4 x 384workflow for the preparation of 384 samples distributed across four different 96-well Sample Plates. The instructions below describe how to perform the workflow for the preparation of one Sample Plate at a time.

Most steps are performed in a similar way as for the 96-sample workflow described in *2. Laboratory instructions for 96 samples using Formulatrix F.A.S.T.™ and Hamilton Microlab® STAR*, *3. Laboratory instructions for 96 samples using SPT Labtech Mosquito® and Hamilton Microlab® STAR* and *4. Laboratory instructions for 96 samples using SPT Labtech Mosquito® and Eppendorf epMotion®*. Changes specific to the 384-sample workflow are highlighted in this chapter.

5.1 Preparations

During this step, the study is planned according to the *Plan the study* and *Important information* sections and one 96-well Sample Plate is prepared according to the *Prepare the samples* section in the relevant chapter.

5.2 Prepare Sample Source Plate (day 1)

During this step, samples are manually transferred from the Sample Plate into the Sample Source Plate, and controls are added to the Sample Source Plate according to the section *Prepare Sample Source Plate (day 1)* in the relevant chapter.

5.3 Sample Dilution

During this step, Sample Diluent is dispensed into the Sample Dilution Plate using the Dragonfly® according to the *Prepare Sample Dilution Plate* in the relevant chapter. The samples are then diluted according to the *Perform Sample Dilution* section.

NOTE: *If performing the workflow for multiple Sample Plates, note that it is not possible to prepare multiple Sample Dilutions Plates at the same time.*

5.4 Incubation

During this step, four Incubation Mixes are prepared manually for each panel, transferred to Reagent Source Plates, and mixed with the samples according to the *Incubation* section in the relevant chapter. Incubation is then performed overnight.

5.5 Preparation of reagents for day 2

During this step, the reagents are prepared for day 2 according to the *Preparation of reagents for day 2* section in the relevant chapter.

5.6 Extension and pre-amplification (PCR1) (day2)

During this step, a PCR1 Mix is prepared manually according to the section *Prepare PCR1 Mix* in the relevant chapter The PCR1 Mix is then dispensed into the Incubation Plates using the Dragonfly® and the plates are subjected to a PCR reaction according to the section *Prepare PCR1 Plates and perform PCR1* in the relevant chapter.

NOTE: *If performing the workflow for multiple Sample Plates, note that one PCR1 Mix is enough for four Incubation Plates.*

5.7 Pool PCR1 products

During this step, the PCR1 products from the four PCR1 Plates are pooled into one PCR1 Pooling Plate according to the *Pool PCR1 products* section in the relevant chapter.

5.8 Amplification and sample indexing (PCR2)

During this step, the PCR2 products belonging to the same panel are pooled into one column of the PCR2 Pooling Plate, then manually transferred to one microcentrifuge tube per panel according to the section *Pool PCR2 products* in the relevant chapter. Each of the four tubes contain amplicons from 96 samples, including controls. During this step, the prepared PCR2 Mix is mixed with the samples along with index primers according to the section *Prepare PCR2 Mix* in the relevant chapter. However, one of the four Index Plates (Index Plates 1, 2, 3, or 4) is used. The samples are then subjected to a second PCR reaction using a ProFlex™ instrument according to the section *Prepare PCR2 Plate and perform PCR2* in the relevant chapter

NOTE: *Using four different Index Plates allows each of the 384 samples to be indexed with a unique index, and to be sequenced together in the same flow cell.*

5.9 Pool PCR2 products

During this step, the PCR2 products belonging to the same panel are pooled into one column of the PCR2 Pooling Plate, then manually transferred to one microcentrifuge tube per panel according to the section *Pool PCR2 products* in the relevant chapter.

5.10 Library purification

During this step, the four Olink Libraries are purified using magnetic beads and the eluates are transferred into one new microcentrifuge tube per panel according to the *Library purification* section in the relevant chapter.

5.11 Quality control

During this step, the purified Olink Libraries are quality controlled as described in the *Quality control* section in the relevant chapter.

5.12 Pool Libraries with different indices

Performing the workflow for all four Sample Plates results in a total of 16 purified and quality controlled Olink Libraries: four different 96-sample Libraries per panel. During this step, the four different 96-samples Libraries from the same panel are pooled together, resulting in four pooled Libraries containing one panel and 384 samples each.

Prepare bench

- 16x Lib Tubes, prepared previously
- 4x Microcentrifuge tubes (1.5 mL)
- Manual pipette (0.5-10 µL)
- Filter pipette tips

Before you start

- Mark four new microcentrifuge tubes: “PL [1-4]” (for "Pooled Library")

Instruction

1. Transfer 5 µl from the four Lib Tubes containing the same panel Library to the corresponding PL Tube.
2. Vortex PL Tubes 1-4 and spin down briefly.

IMPORTANT: *Make sure to pool Libraries generated using the four different Index Plates. Pooling Libraries that have the same indices will result in unusable data.*

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

5.13 Next generation sequencing

Next generation sequencing is performed using the Illumina® NovaSeq™ 6000 instrument. Refer to chapter 3 of the Olink® Explore Sequencing using NovaSeq 6000™ User Manual for instructions on how to sequence 384-sample Olink Libraries.

6. Revision history

Version	Date	Description
3.1	2023-06-28	Added instructions for F.A.S.T.™.
3.0	2023-05-12	New trademarks and disclaimer. *2* added.
2.0	2023-02-16	TapeStation added. *1.2* updated. *1.3* updated. *Table 3* added. *1.3.2* added. *2:* Note added. Columns, quadrants and protocol versions edited. *Figure 2* and *Figure 6* corrected. *2.3.1* and *2.3.2* Important added. *2.4.2* important edited. *2.11* edited. *Figure 20* added. *4* added.
1.4	2022-12-21	*1.4* added. References to Microlab STAR® added.
1.3	2022-09-28	*2.2* Important edited. *2.4.1* Important added and changed name of Sample Source Plate to Sample Reagent Plate. Editorial changes
1.2	2022-05-13	*2.3.2* Step 17 and step 18 added. *2.4.2* Note edited. *2.6.1* Note ediied. *3.8.1* and *3.8.2* Clarified the time limit for using the PCR2 Mix: Time Sensitive Step edited. Time Sensitive Step added. *Figure 9*, *Figure 13* and *Figure 36* corrected.
1.1	2021-12-13	*2.5* Volume of MilliQ water changed to 30 mL. *2.6.1* and*2.6.2* "Vortex the PCR1 Mix thoroughly" removed.
1.0	2021-12-01	New

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