Olink® Explore 384

User manual

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Part 1: Olink® Explore 384 overview

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

The Olink® Explore 384 User Manual provides the instructions needed to run any of the Olink® Explore 384 Reagent Kits.

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® NovaSeq™ 550, NextSeq™ 2000, NextSeq™ 6000, or NovaSeq™ X Plus, refer to the applicable Olink® Explore Sequencing User Manual.

For data processing and analysis of the Olink® Explore sequence results, refer to the software manuals for the both Olink NPX Explore softwares and CLI listed in *3. Associated documentation.*

2.Reagents included

Olink Explore 384 Reagent Kit consists of one of the Olink Explore 384 panels described in the table below. It contains reagents for up to 90 user samples and 6 Olink controls and is intended for the preparation of one single panel library.

2.1 Content of Olink® Explore 384 Reagent Kit.

Art. No	Products included	Components included	Cap color	Storage
One of the following 384 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	–	
97004	Olink® Target96/Explore Sample Diluent	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

3. Associated documentation

- Olink® Explore Overview User Manual
- Olink® Explore Sequencing using NextSeq™ 550 User Manual
- Olink® Explore Sequencing using NextSeq™ 2000 User Manual
- Olink® Explore Sequencing using NovaSeq™ 6000 User Manual
- Olink® Explore 384/3072 Sequencing using NovaSeq™ X Plus User Manual
- Olink® NPX Explore 3072 User Manual
- NPX™ Explore HT & 3072 User Manual
- Olink® Explore CLI Technical Information
- NPX™ Explore CLI HT & 3072 Technical Information

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

4. Technical support

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

Part 2: Laboratory instructions for 96 samples using Formulatrix F.A.S.T.™

This chapter provides instructions on how to perform each step of the Olink® Explore 384 laboratory workflow, using the Formulatrix F.A.S.T. These instructions apply to running one Sample Plate on one Olink Explore 384 panel. If more Sample Plates or panels are processed, repeat the instructions the applicable number of times.

5.Preparations

5.1 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 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.*

5.2 Important information

Reagent lots

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

Vortexing

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

Plate type	Speed (rpm)	Time (s)
96-wells	2500	30
384-wells	3000	30

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.

5.3 Prepare the samples

During this step, samples are manually transferred to the Sample Plate. The Sample Plate must be used within the same day.

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

Prepare bench

- Samples (provided by the user)
- 1x 96-well PCR plate, 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 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.

Instruction

1. Transfer the samples into the Sample Plate, according to the plate layout shown in the figure below. Make sure that samples are added to every applicable well.

Sample Plate layout.
The numbers indicate the sample numbers.

	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	

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

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

6. Prepare Sample Source Plate

During this step, samples are manually transferred from the prepared Sample Plate into the Sample Source Plate, and controls are added into the Sample Source Plate. The Sample Source Plate must be used within the same day of preparation.

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, prepared in the previous step)
- 1x 384-well PCR plate (skirted)
- 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 has been prepared according to previous step.
- Thaw the Sample Plate at room temperature if frozen.
- Thaw the Negative Control, Plate Control and recommended Sample Control at room temperature.
- Mark the new 384-well PCR plate: “Sample Source Plate”.

Instructions

1. Using the MixMate or manual vortexing, vortex the 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 384-well Sample Source Plate according to the plate layout shown in the figure below. 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 the figure below. 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.

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

	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																								

7.Sample Dilution

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

7.3.1 Prepare Sample Dilution Plate

Prepare bench

- Olink® Target 96/Explore Sample Diluent
- 1x 384-well PCR plate (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 the new 384-well PCR plate: "Sample Dilution Plate".

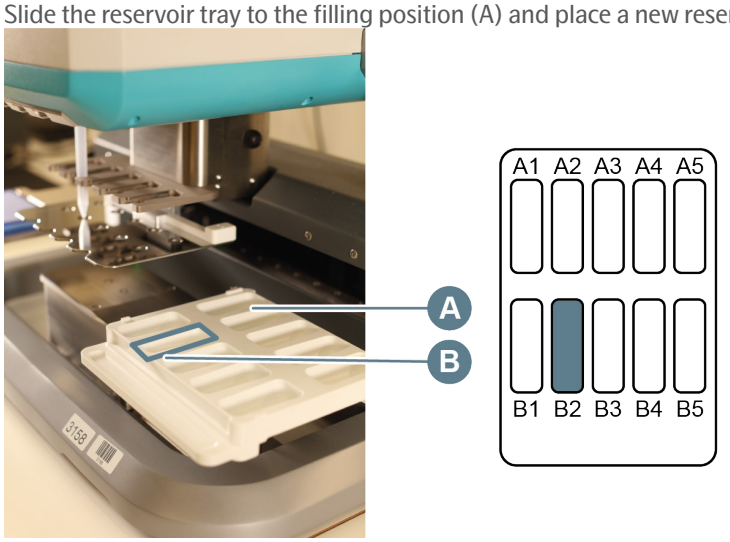
NOTE: *For convenience, the Sample Diluent can be thawed at +4 °C overnight.*

Instructions

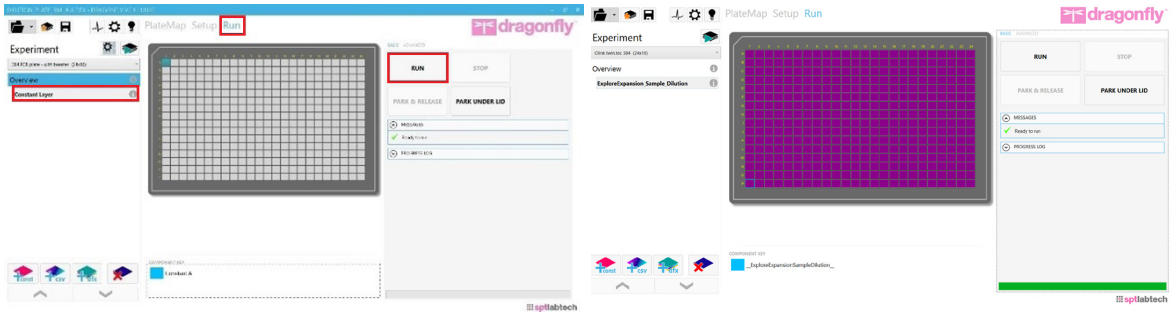
1. Prepare the Dragonfly according to instructions in the Olink® Explore Overview User Manual.
   - Use the protocol `Olink Sample Dilution Plate v4.`
   - Attach one syringe in position B2.

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.*

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



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, then click *RUN* to start the protocol.
   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.



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. When finished, clear the instrument and shut it down according to instructions in the Olink® Explore Overview User Manual.
10. Continue to *7.1 Perform Sample Dilution*, or store the Sample Dilution Plate at +4 °C until use (the same day).

7.1 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.

Prepare bench

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

Before you start

- Prepare the F.A.S.T. according to the manufacturer's instructions.
- 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. If using MixMate, make sure to vortex the Sample Dilution Plate thoroughly with correct MixMate settings, as incorrect settings may lead to low-quality data. Refer to table in 5.2 Important information.*

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 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. protocol `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 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 protocol.
    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 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 protocol. Keep the F.A.S.T. on for later use.
18. Continue to *8. Incubation*, or place the Sample Dilution Plate at +4 °C for up to 1 hour.

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

	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

8.Incubation

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

8.1 Prepare Reagent Source Plate

During this step, four Incubation Mixes are prepared manually and transferred to one 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 for 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)
- 1x 8-well strip
- 1x 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 the new 384-well PCR plate: “Reagent Source Plate”.
- Mark the wells of an 8-well strip according to:

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

Instructions

1. Vortex the Incubation Solution, Forward Probes and Reverse Probes and spin down.
2. Prepare four Incubation Mixes: Using **forward pipetting**, first transfer 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:

Addition order	Reagent	Well							
		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	–	–	–	–
		Frw Probes A	Frw Probes B	Frw Probes C	Frw Probes D	–	–	–	–
3	Rev probes	10 µL	10 µL	10 µL	10 µL	–	–	–	–
		Rev Probes A	Rev Probes B	Rev Probes C	Rev Probes D	–	–	–	–

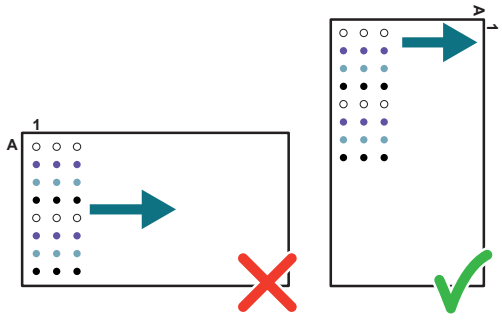
NOTE: *Pipette the Incubation Solution carefully to avoid foaming.*

3. Seal the PCR strip 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. Rotate the Sample Reagent Plate (384-well) 90 degrees clockwise to facilitate pipetting



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

Reagent Source Plate layout.
The letters indicate the different blocks within the panel.

	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	A		B		C		D																	
B																								
C	A		B		C		D																	
D																								
E	A		B		C		D																	
F																								
G	A		B		C		D																	
H																								
I	A		B		C		D																	
J																								
K	A		B		C		D																	
L																								
M	A		B		C		D																	
N																								
O	A		B		C		D																	
P																								

6. Seal the Reagent Source Plate 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.
7. Immediately continue to *8.2 Prepare Incubation Plates and perform incubation*.

8.2 Prepare Incubation Plates and perform incubation

During this step, the prepared Incubation Mixes are mixed with the samples in one Incubation Plate using the F.A.S.T., prior to incubation.

Prepare bench

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

Before you start

- Allow the refrigerated Sample Source Plate and Sample Dilution Plate to reach room temperature
- Mark the new 384-well PCR plate: “Incubation Plate".

Instructions

1. In the open F.A.S.T. software, Select *File/Open* and choose the protocol for the applicable panel 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. protocol. Selecting the wrong protocol will result in unusable data.*

2. Place the Incubation Plate 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.
4. Place the Reagent Source Plate on 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 protocol is paused, and a pop-up window appears on the screen, remove the Incubation Plate from the 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 with a new adhesive film and spin at 400–1000 x g for 1 minute, then carefully remove the adhesive film 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 the Incubation Plate back in its previous position on the F.A.S.T. deck.
9. Vortex the 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.
10. Place the Sample Source Plate and Sample Dilution Plate on 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 protocol is finished, remove the Incubation Plate from the 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 the Incubation Plate at 400–1000 x g for 1 minute.
14. Inspect the Incubation Plate to ensure that all wells contain the same amount of liquid (0.8 µL). Note if there is any wells containing bubbles.
15. Write the time of the incubation starting point on the plate seal and incubate Incubation Plate 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 deck. Treat the plates as follows:
    - Sample Source Plate: Seal with adhesive film and store at -80 °C for potential reruns.
    - Sample Dilution Plate: Discard
    - Reagent Source Plate: Discard
17. When finished, clear the F.A.S.T. and shut it down according to the manufacturer's instructions.

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.

	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

9.Prepare 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.

10. Extension and pre-amplification (PCR1)

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

10.1 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 Plate, 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 Plate reach room temperature.
- Mark the new 50 mL Falcon tube: "PCR1 Mix"
- Switch on one ProFlex PCR instrument in the post-PCR room.
- Ensure that the PCR1 Enhancer is fully thawed and has reached room temperature.

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 the Incubation Plate down for 1 minute at room temperature.
4. Prepare a PCR1 Mix in a Falcon tube in the following order, using the volumes according to:

Addition order	Reagent	Volume (µL)
1	MilliQ water (+4 °C)	8 250
2	PCR1 Enhancer	1 073
3	PCR1 Solution	1 073
4	PCR1 Enzyme	107
	Total	10 502

   - First add MilliQ water and PCR1 Enhancer and vortex thoroughly.
   - Then add the PCR1 Solution and PCR1 Enzyme, and vortex for 3 seconds.

5. 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.*

10.2 Prepare PCR1 Plate and perform PCR1

During this step, the prepared PCR1 Mix is dispensed into the Incubation Plate using the Dragonfly, and the plate is subjected to a PCR reaction.

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 plate.

Prepare bench

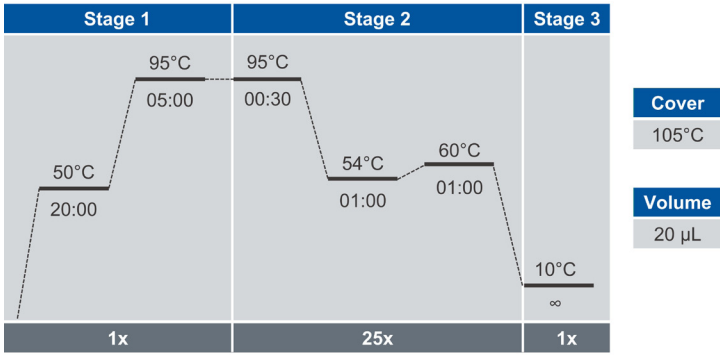
- Incubation Plate at room temperature, prepared in previous step
- PCR1 Mix, prepared in previous step
- Timer
- 3x Disposable reservoirs (10 mL)
- 3x Disposable ultra-low retention syringes with plungers
- Manual pipette (5 or 10 mL)
- Filter pipette tips
- Adhesive films

Before you start

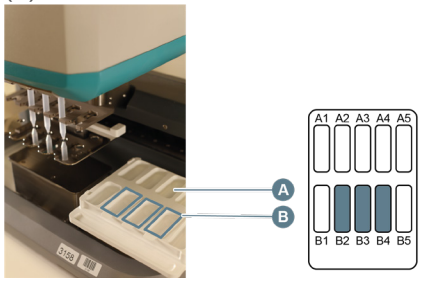
- Set a timer to 10 minutes.

Instructions

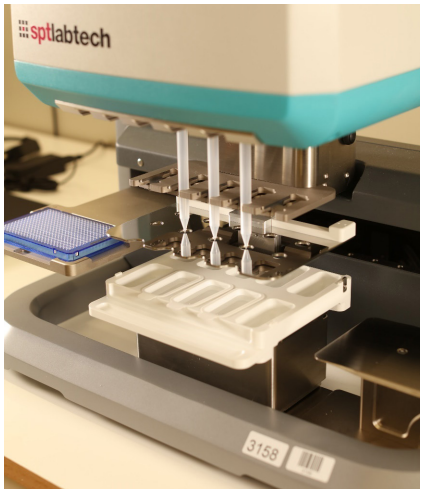
1. Start the PCR protocol `Olink PCR1` on the ProFlex™ PCR instrument. Pause when the PCR block temperature reaches 50 °C.



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). Place three new reservoirs in positions B2, B3, and B4 (B).



4. Transfer 3.3 mL of PCR1 Mix into each of the three reservoir.
5. Carefully slide the reservoir tray back to the aspirate position.



6. Rename Incubation Plate “PCR1 Plate” and spin down the plate.
7. Carefully remove the adhesive film from PCR1 Plate 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 protocol. 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.

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 plate.*

9. When the Dragonfly has returned the PCR1 Plate 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. Using the MixMate or manual vortexing, vortex the plate thoroughly to ensure that all wells are mixed.
11. In the Post-PCR room, centrifuge the PCR1 Plate at 400–1000 x g for 1 minute.
12. Inspect the PCR1 Plate to ensure that all wells contain the same amount of liquid (19.8 µL). Note any deviations.
13. When the timer ends after 10 minutes, place PCR1 Plate in the pre-heated ProFlex and click *Resume* the run the protocol.
14. When finished, clear the Dragonfly and shut it down according to instructions in the Olink® Explore Overview User Manual.
15. When the protocol is finished (~1 hour 55 minutes), continue to *11. Pool PCR1 products*, or store the PCR1 Plate at +4 °C if used the same day.

SAFE STOPPING POINT: *The PCR1 Plate can be stored at -20 °C for up to 2 weeks.*

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.

	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

11.Pool PCR1 products

During this step, the PCR1 products from the PCR1 Plates are pooled into one PCR1 Pooling Plate using the SPT Dragonfly and Formulatrix F.A.S.T. instrument.

Prepare bench

- PCR1 Plate, prepared previously
- MilliQ water (at +4 °C, preferably kept in the fridge until use)
- 1x 384-well PCR plate (skirted)
- 1x Dragonfly® Disposable ultra-low retention syringe with plunger
- 1x Disposable Dragonfly® reservoir (10 mL)
- F.A.S.T.™ Disposable Pipette Tips (384 tips per panel)
- Adhesive films
- Temperature-resistant labels or marker pen

Before you start

- Thaw PCR1 Plate at room temperature if frozen.
- Mark the new 384-well PCR plate: “PCR1 Pooling Plate”.
- Switch on the Dragonfly system and open the software.
- Switch on the F.A.S.T. system and open the software.

Instructions SPT Dragonfly®

1. Prepare the Dragonfly according to instructions in the Olink® Explore Overview User Manual.
   - Use the protocol `Olink_Explore_384_1panel_PCR1_Pooling_Water`
2. Attach one syringe in position B3.
3. Slide the reservoir tray to the filling position and place a new reservoir in position B3.
4. Transfer 10 mL of water into the reservoir and carefully slide the reservoir tray back to the aspirate position .
5. Place the PCR1 Pooling Plate in the Dragonfly plate position (to the left), with well A1 in the top left corner.
6. Select the Run tab in the Constant layer view of the software, then click *RUN* to start the protocol.

IMPORTANT: *Do not stop the instrument until the run is completed*

NOTE: *The Dragonfly will dispense water in the 96 wells corresponding to the first quadrant of the plate.*

7. When the Dragonfly has returned the PCR1 Pooling Plate to the plate position and released the plate clamp, remove the plate from the instrument and immediately seal it with a new adhesive film.

Instructions Formulatrix F.A.S.T.

1. 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.
2. 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.
3. In Explorer, select the protocol `Olink_Explore_3072_PCR1_Pooling` In the task list, untick the checkboxes for Pooling Panel 2, 3 and 4.
4. Carefully remove the adhesive films from the PCR1 Plates, PCR1 Pooling Plate, and the lid from the tip box. Place on the tray according to the protocol diagram.
5. Click the *START* button to begin the run.
   Result:
   The F.A.S.T. pools 3 µL of each PCR1 product from each sample into one well per panel. The run takes approximately 6 minutes.
6. When the protocol is finished, remove the PCR1 Pooling Plate and seal it with a new adhesive film. Keep the F.A.S.T. on for later use.
7. Vortex the PCR1 Pooling Plate and spin down at 400–1000 x g for 1 minute.
8. Inspect the PCR1 Pooling Plate to ensure that all wells contain the same amount of liquid (24 µL).
9. 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.
10. Continue to *12. Amplification and sample indexing (PCR2)*, or store the PCR1 Pooling Plate at +4 °C until ready to use (the same day).

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.

	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

12. Amplification and sample indexing (PCR2)

During this step, a PCR2 Mix is prepared manually and then aliquoted into a new 384-well plate with the Dragonfly. The F.A.S.T. then adds pooled PCR1 products and index primers (unique for each sample). The samples then undergo a second PCR reaction.

12.1 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))
- 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 according to

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.

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

12.2 Prepare PCR2 Plate and perform PCR2

During this step, the prepared PCR2 Mix is mixed with the samples along with index primers. PCR2 Mix is aliquoted into a new 384 well plate by the Dragonfly, then samples and index are added using the F.A.S.T. The samples are then subjected to a second PCR reaction.

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 Dragonfly® Disposable ultra-low retention syringe with plunger
- 1x Disposable Dragonfly® reservoir (10 mL)
- F.A.S.T.™ Disposable Pipette Tips (480 tips per four panels)
- Adhesive films
- Mark the new 384-well PCR plate: “PCR2 Plate”.
- Temperature-resistant labels or marker pen

Before you start

- Mark the new 384-well PCR plate: “PCR2 Plate”.
- Switch on the Dragonfly system and open the software.
- Switch on the F.A.S.T. system and open the software.

Instructions STP Dragonfly®

1. Prepare the Dragonfly according to instructions in the Olink® Explore Overview User Manual.
   - Use the protocol `Olink_Explore_384_1panel_PCR2_Set-Up_MM`
2. Attach one syringe in position B3.
3. Slide the reservoir tray to the filling position and place a new reservoir in position B3.
4. Transfer the entire volume of the PCR2 Mix into the reservoir and carefully slide the reservoir tray back to the aspirate position.
5. Place the PCR2 Plate in the Dragonfly plate position (to the left), with well A1 in the top left corner.
6. Select the Run tab in the Constant layer view of the software, then click *RUN* to start the protocol.

IMPORTANT: *The mastermix must be used within one hour, proceed without interruptions*

NOTE: *The Dragonfly will dispense into the 96 wells corresponding to the first quadrant of the plate*

7. When the Dragonfly has returned the PCR2 Plate to the plate position and released the plate clamp, remove the plate from the instrument and immediately seal it with a new adhesive film.
8. When finished, clear the Dragonfly and shut it down according to instructions in the Olink® Explore Overview User Manual.

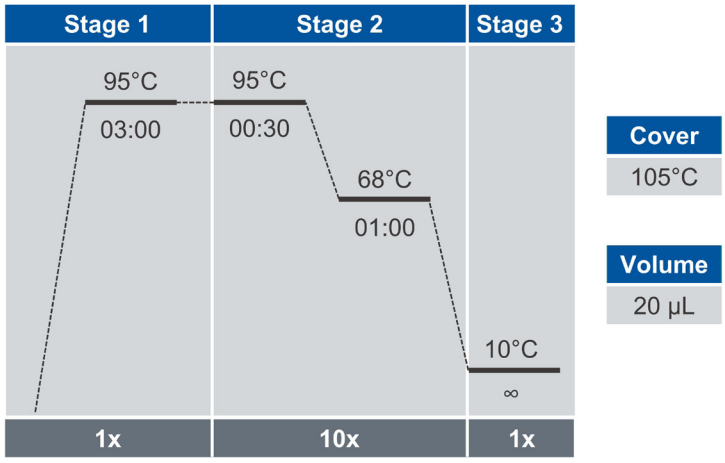
Instructions Formulatrix F.A.S.T.®

1. Open the F.A.S.T. software and select the protocol `Olink_Explore_3072_PCR2_Set-Up`.
2. In the Task list, deactivate the Indices transfer steps relative to the second, third and fourth quadrant by unticking the checkboxes next to the transfers names
3. In the Task list, deactivate the sample transfers relative to the second, third and fourth quadrant by unticking the checkboxes next to the transfers names
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. Remove the lid of the tip box. 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. Arrange the F.A.S.T. deck according to the layout in the protocol.
7. Click START to begin the run.
   Result:
   The F.A.S.T. transfers 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. The run takes approximately 4 minutes to complete.
8. When the protocol is finished, remove the PCR2 Plate and seal it with a new adhesive film.

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

   TIME SENSITIVE STEP: *Start the PCR2 protocol within 5 minutes from end of the F.A.S.T. protocol.*

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



13. Remove the PCR1 Pooling Plate containing the remaining PCR products from the F.A.S.T. and seal it with a new adhesive film. Store it at -20 °C for up to 2 weeks in case of potential reruns.
14. Discard the Index Plate 1.
15. When the PCR protocol is finished (~25 minutes), continue to *13. 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.*

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.

	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

13.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 F.A.S.T. 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)
- F.A.S.T.™ Disposable Pipette Tips (32 tips per four panels)
- 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]”. Thaw PCR2 Plate at room temperature if frozen.
- Switch on the F.A.S.T. system and open the software.

Instructions

1. Open the F.A.S.T. software and select the protocol `Olink_Explore_3072_PCR2_Pooling`.
2. In the Task list, deactivate the transfers relative to the second, third and fourth quadrant by unticking the checkboxes next to the transfers names.
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. Carefully unseal the plate.
5. Arrange the F.A.S.T. deck according to the layout in the protocol.
6. Click *START* to begin the run.
   Result:
   F.A.S.T 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. The run takes approximately 13 minutes.
7. When finished, remove the PCR2 Pooling Plate. 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. 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.
10. Clear the F.A.S.T. and shut it down.

CR2 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.

	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					

11. Carefully remove the adhesive film from the PCR2 Pooling Plate.
12. Using a single-channel pipette transfer the pooled PCR2 products from the PCR2 Pooling Plate to the PCR2 Tubes as described in the table below. If running a single panel, only transfer from column 1 to PCR2 tube 1 will be applicable. Use **forward pipetting** and change pipette tip after each well.

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

13. Vortex the PCR2 Tubes and spin down briefly.
14. Discard the PCR2 Pooling Plate.
15. Continue to *14. Library purification*, 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.*

14. 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 Timer
- 8 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 the table below. Manually transfer first the MilliQ water and then the 96% EtOH into the Falcon tube.

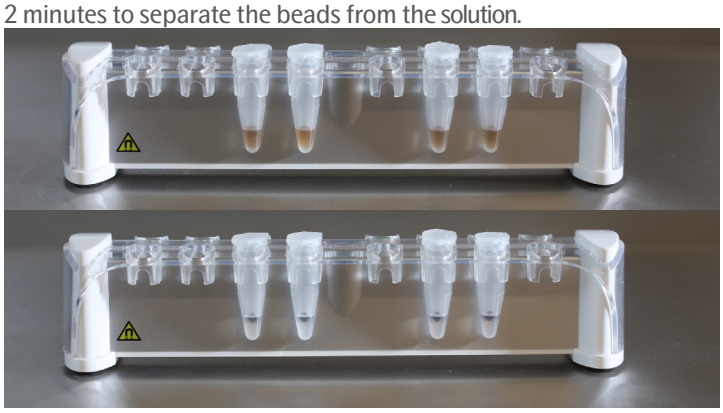
Reagent	Volume (mL) for							
	1 library	2 Libraries	3 Libraries	4 Libraries	5 Libraries	6 Libraries	7 Libraries	8 Libraries
MilliQ water	0.5	0.8	1.1	1.4	1.6	1.9	2.2	2.5
96% EtOH	1.5	2.2	2.9	3.6	4.4	5.1	5.8	6.5
Total (70% EtOH)	2	3	4	5	6	7	8	9

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:

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 the BP Tubes 1–4 for 5 minutes at room temperature.
7. After the incubation, place the BP Tubes 1–4 on the DynaMag-2 Magnetic stand and leave them for 2 minutes to separate the beads from the solution.



With the tubes still on the magnetic stand, carefully open the lids and discard 125 µL supernatant using a single-channel pipette. Do not disturb the beads.

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

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

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

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

9. Leave the tubes with the lids open on the magnetic stand for 2 minutes for the beads to air dry.
10. Close the tubes and remove them from the magnetic stand.
11. Add 50 µL of MilliQ water to the BP Tubes 1–4 and pipette-mix 10 times towards the beads to elute the purified Libraries from the beads.
12. Incubate the tubes for 2 minutes at room temperature.
13. Place BP Tubes 1–4 on the magnetic stand and leave them for 1 minute to separate the beads from the eluted Library solution.
14. 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:

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

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

15. Discard BP Tubes 1–4.
16. Continue to *15. Quality control*.

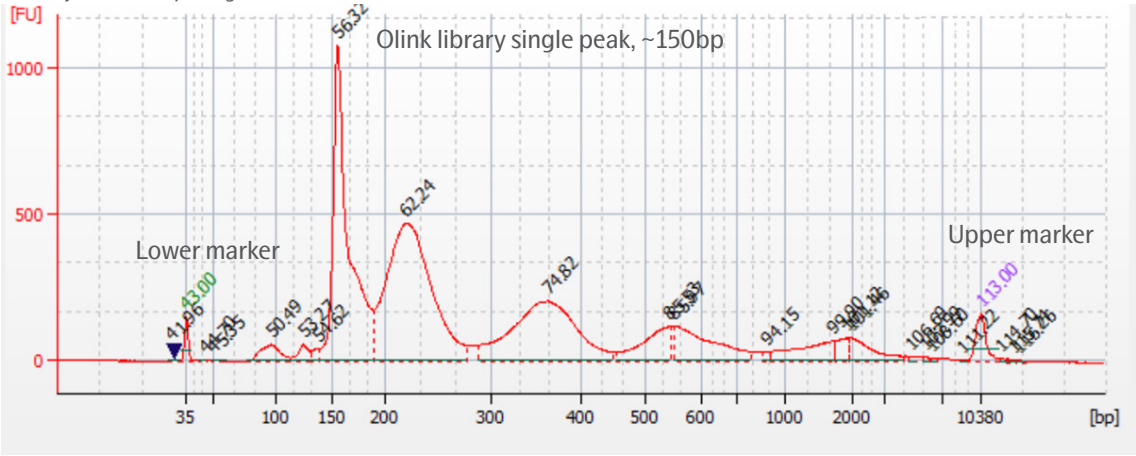
SAFE STOPPING POINT: *The Lib Tubes can be stored at -20 °C for up to 4 weeks.*

15.Quality control

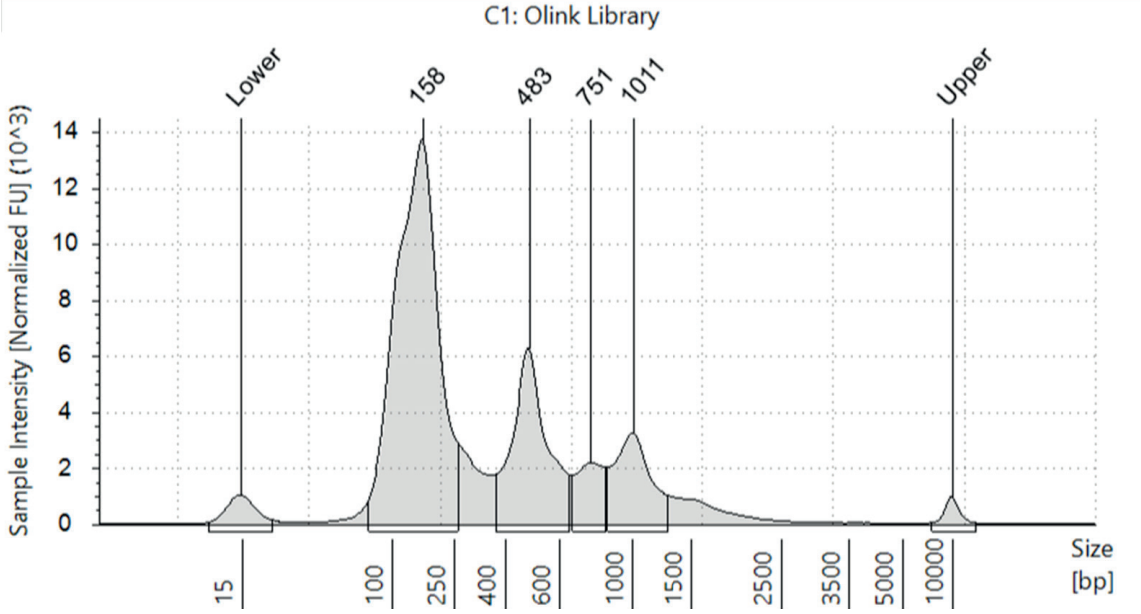
During this step, the four purified Olink Libraries are quality controlled on the Bioanalyzer using the High Sensitivity DNA kit or on the TapeStation using the High Sensitivity D5000 kit, according to the Manufacturer's instructions.

The electropherograms in the figures below display 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.

Bioanalyzer's electropherogram.



TapeStation's electropherogram.



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

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.

16. Next generation sequencing

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

- NextSeq™ 550
- NextSeq™ 2000
- NovaSeq™ 6000
- NovaSeq™ X Plus

As the workflow differs between instruments, refer to the applicable Olink® Explore Sequencing User Manual for instructions on how to sequence Olink Libraries.

Part 3: 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 384 laboratory workflow, using the Microlab STAR from Hamilton Company. These instructions apply to running one Sample Plate on one Olink Explore 384 panel. If more Sample Plates or panels are processed, repeat the instructions the applicable number of times.

17.Preparations

17.2.1 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 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.*

17.1 Important information

Reagent lots

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

Vortexing

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

Plate type	Speed (rpm)	Time (s)
96-wells	2500	30
384-wells	3000	30

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.

17.2 Prepare the samples

During this step, samples are manually transferred to the Sample Plate. The Sample Plate must be used within the same day.

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

Prepare bench

- Samples (provided by the user)
- 1x 96-well PCR plate, 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 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.

Instruction

1. Transfer the samples into the Sample Plate, according to the plate layout shown in the figure below. Make sure that samples are added to every applicable well.

Sample Plate layout.
The numbers indicate the sample numbers.

	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	

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

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

18.Prepare Sample Source Plate

During this step, samples are manually transferred from the prepared Sample Plate into the Sample Source Plate, and controls are added into the Sample Source Plate. The Sample Source Plate must be used within the same day of preparation.

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, prepared in the previous step)*
- 1x 384-well PCR plate (skirted)
- 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 has been prepared according to *17.2 Prepare the samples.*
- Thaw the Sample Plate at room temperature if frozen.
- Thaw the Negative Control, Plate Control and recommended Sample Control at room temperature.
- Mark the new 384-well PCR plate: “Sample Source Plate”.

Instructions

1. Using the MixMate or manual vortexing, vortex the 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 384-well Sample Source Plate according to the plate layout shown in the figure below. 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 the figure below. 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.

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

	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																								

19.Sample Dilution

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

19.1 Prepare Sample Dilution Plate

Prepare bench

- Olink® Target 96/Explore Sample Diluent
- 1x 384-well PCR plate (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 the new 384-well PCR plate: "Sample Dilution Plate".

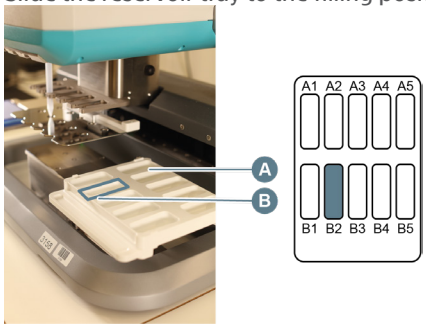
NOTE: *For convenience, the Sample Diluent can be thawed at +4 °C overnight.*

Instructions

1. Prepare the Dragonfly according to instructions in the Olink Explore Overview User Manual.
   - Use the protocol `Olink Sample Dilution Plate v4`.
   - Attach one syringe in position B2.

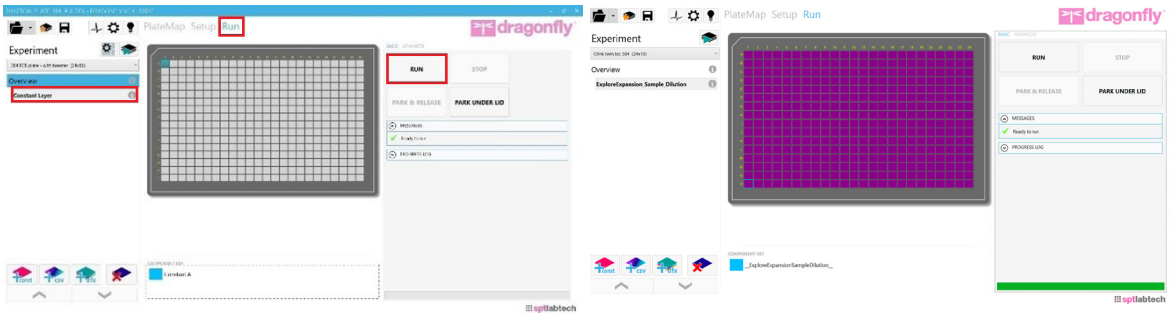
IMPORTANT: *Make sure that the correct protocol version is used. The plate layout has changed and using an older version of the protocol will result in unusable data. Please contact support@olink.com if you need assistance.*

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



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, then click *RUN* to start the protocol.
   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



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. When finished, clear the instrument and shut it down according to instructions in the Olink Explore Overview User Manual.
10. Continue to *19.2 Perform Sample Dilution*, or store the Sample Dilution Plate at +4 °C until use (the same day).

19.2 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.

Prepare bench

- Sample Source Plate, prepared in previous step
- Sample Dilution Plate, 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. If using MixMate, make sure to vortex the Sample Dilution Plate thoroughly with correct MixMate settings, as incorrect settings may lead to low-quality data. Refer to table in 17.1 Important information.*

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 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. protocol `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 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 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 protocol.
    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 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. protocol. Keep the F.A.S.T. on for later use.
18. Continue to *20. Incubation*, or place the Sample Dilution Plate at +4 °C for up to 1 hour.

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

	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

20.Incubation

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

20.1 Prepare Reagent Source Plate

During this step, four Incubation Mixes are prepared manually and transferred to one 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 for 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)
- 1x 8-well strip
- 1x 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 the new 384-well PCR plate: “Reagent Source Plate”.
- Mark the wells of an 8-well strip according to:

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

Instructions

1. Vortex the Incubation Solution, Forward Probes and Reverse Probes and spin down.
2. Prepare four Incubation Mixes: Using forward pipetting, first transfer 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:

Addition order	Reagent	Well							
		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	–	–	–	–
		Frw Probes A	Frw Probes B	Frw Probes C	Frw Probes D	–	–	–	–
3	Rev probes	10 µL	10 µL	10 µL	10 µL	–	–	–	–
		Rev Probes A	Rev Probes B	Rev Probes C	Rev Probes D	–	–	–	–

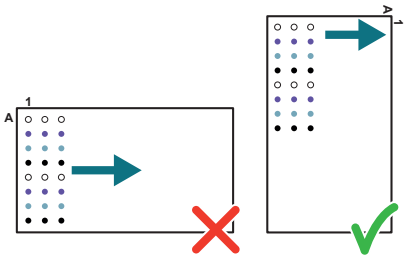
NOTE: *Pipette the Incubation Solution carefully to avoid foaming.*

3. Seal the PCR strip 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. Rotate the Sample Reagent Plate (384-well) 90 degrees clockwise to facilitate pipetting.



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

Reagent Source Plate layout.
The letters indicate the different blocks within the panel.

	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	A		B		C		D																	
B																								
C	A		B		C		D																	
D																								
E	A		B		C		D																	
F																								
G	A		B		C		D																	
H																								
I	A		B		C		D																	
J																								
K	A		B		C		D																	
L																								
M	A		B		C		D																	
N																								
O	A		B		C		D																	
P																								

6. Seal the Reagent Source Plate 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.
7. Immediately continue to *20.2 Prepare Incubation Plates and perform incubation*.

20.2 Prepare Incubation Plates and perform incubation

During this step, the prepared Incubation Mixes are mixed with the samples in one Incubation Plate using the F.A.S.T., prior to incubation.

Prepare bench

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

Before you start

- Allow the refrigerated Sample Source Plate and Sample Dilution Plate to reach room temperature
- Mark the new 384-well PCR plate: “Incubation Plate".

Instructions

1. In the open F.A.S.T. software, Select *File/Open* and choose the protocol for the applicable panel 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. protocol. Selecting the wrong protocol will result in unusable data.*

2. Place the Incubation Plate 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.
4. Place the Reagent Source Plate on 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 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 with a new adhesive film and spin at 400–1000 x g for 1 minute, then carefully remove the adhesive film 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 the Incubation Plate back in its previous position on the F.A.S.T. deck.
9. Vortex the 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.
10. Place the Sample Source Plate and Sample Dilution Plate on 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 the Incubation Plate 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 the Incubation Plate at 400–1000 x g for 1 minute.
14. Inspect the Incubation Plate to ensure that all wells contain the same amount of liquid (0.8 µL). Note if there is any wells containing bubbles.
15. Write the time of the incubation starting point on the plate seal and incubate Incubation Plate 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 follows:
    - Sample Source Plate: Seal with adhesive film and store at -80 °C for potential reruns.
    - Sample Dilution Plate: Discard
    - Reagent Source Plate: Discard
17. When finished, clear the F.A.S.T. and shut it down according to the manufacturer's instructions.

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.

	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

21. Prepare 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.

22.Extension and pre-amplification (PCR1)

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

22.1 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 Plate, 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 Plate reach room temperature.
- Mark the new 50 mL Falcon tube: "PCR1 Mix"
- Switch on one ProFlex™ PCR instrument in the post-PCR room.
- Ensure that the PCR1 Enhancer is fully thawed and has reached room temperature.

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 the Incubation Plate down for 1 minute at room temperature.
4. Prepare a PCR1 Mix in a Falcon tube in the following order, using the volumes according to:

Addition order	Reagent	Volume (µL)
1	MilliQ water (+4 °C)	8 250
2	PCR1 Enhancer	1 073
3	PCR1 Solution	1 073
4	PCR1 Enzyme	107
	Total	10 502

   - First add MilliQ water and PCR1 Enhancer and vortex thoroughly.
   - Then add the PCR1 Solution and PCR1 Enzyme, and vortex for 3 seconds.

5. 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.*

22.2 Prepare PCR1 Plate and perform PCR1

During this step, the prepared PCR1 Mix is dispensed into the Incubation Plate using the Dragonfly, and the plate is subjected to a PCR reaction.

The PCR1 plate must be placed in the ProFIe exactly 10 minutes after the PCR1 Mix has been added to the first well of the plate.

Prepare bench

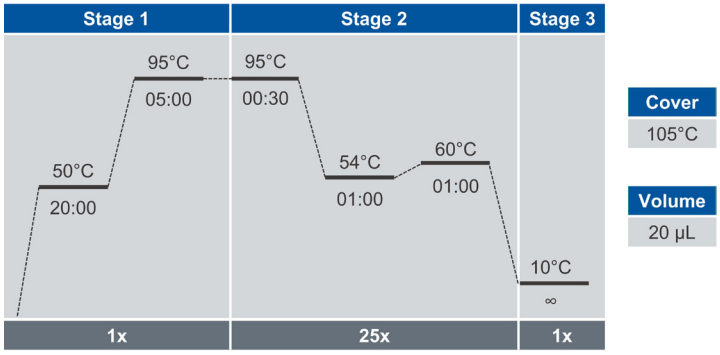
- Incubation Plate at room temperature, prepared in previous step
- PCR1 Mix, prepared in previous step
- Timer
- 3x Disposable reservoirs (10 mL)
- 3x Disposable ultra-low retention syringes with plungers
- Manual pipette (5 or 10 mL)
- Filter pipette tips
- Adhesive films

Before you start

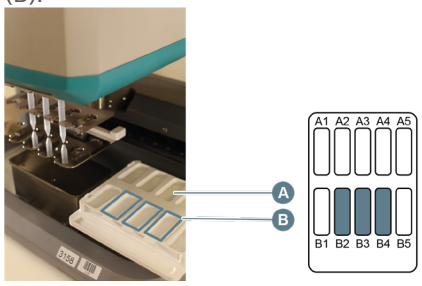
- Set a timer to 10 minutes.

Instructions

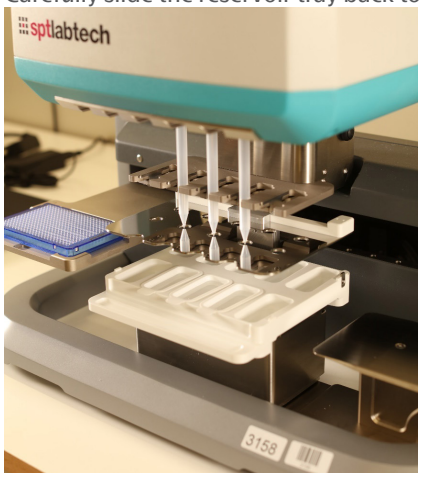
1. Start the PCR protocol `Olink PCR1` on the ProFlex PCR instrument. Pause when the PCR block temperature reaches 50 °C.



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).



4. Transfer 3.3 mL of PCR1 Mix into each of the three reservoir.
5. Carefully slide the reservoir tray back to the aspirate position.



6. Rename Incubation Plate “PCR1 Plate” and spin down the plate.
7. Carefully remove the adhesive film from PCR1 Plate 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 protocol. 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.

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 plate.*

9. When the Dragonfly has returned the PCR1 Plate 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. Using the MixMate or manual vortexing, vortex the plate thoroughly to ensure that all wells are mixed.
11. In the Post-PCR room, centrifuge the PCR1 Plate at 400–1000 x g for 1 minute.
12. Inspect the PCR1 Plate to ensure that all wells contain the same amount of liquid (19.8 µL). Note any deviations.
13. When the timer ends after 10 minutes, place PCR1 Plate in the pre-heated ProFlex and click *Resume*.
14. When finished, clear the Dragonfly and shut it down according to instructions in the Olink Explore Overview User Manual.
15. When the protocol is finished (~1 hour 55 minutes), continue to *23. Pool PCR1 products*, or store the PCR1 Plate at +4 °C if used the same day.

SAFE STOPPING POINT: *The PCR1 Plate can be stored at -20 °C for up to 2 weeks.*

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.

	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

23.Pool PCR1 products

During this step, the PCR1 products from the PCR1 Plates are pooled into one PCR1 Pooling Plate using the Microlab STAR instrument.

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

1. 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.
2. 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.
3. In Run Control, select the protocol `PCR1 Pooling` and click the *Start* button.
4. Select the number of Destination Pooling Plates and the number of Panels being Pooled for each, then click *OK*.
5. 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.
6. 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.
7. 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.
8. Vortex the PCR1 Pooling Plate and spin down at 400–1000 x g for 1 minute.
9. Inspect the PCR1 Pooling Plate to ensure that all wells contain the same amount of liquid (24 µL).
10. 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.
11. Continue to *24. Amplification and sample indexing (PCR2)*, or store the PCR1 Pooling Plate at +4 °C until ready to use (the same day).

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.

	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

24. 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.

24.1 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 according to:

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

6. 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.*

24.2 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.

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

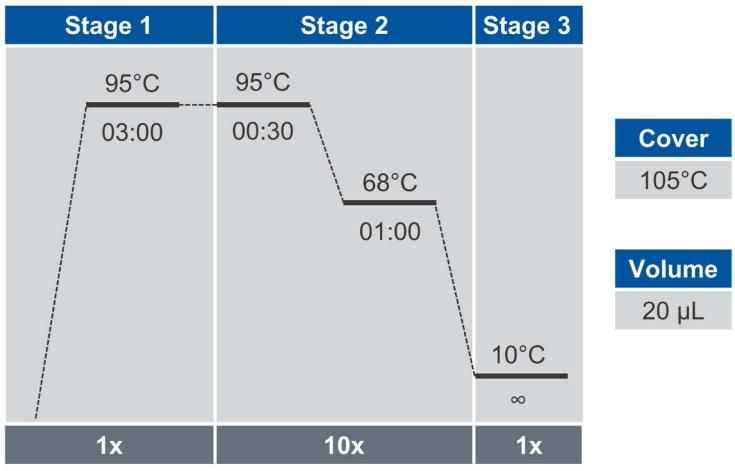
1. Open Hamilton Run Control and select the protocol *PCR2_Setup* and click *Start*.
2. Select the number of PCR2 plates being prepped and the number of Panels in each plate, and click *OK*.
3. Centrifuge Index Plate 1 at 400–1000 x g for 1 minute and inspect the plate to ensure that all wells contain 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. 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 protocol within 5 minutes from end of the 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 protocol `Olink Index PCR2`. Click *Start*.



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 protocol is finished (~25 minutes), continue to *24.3 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.*

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.

	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

24.3 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. 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.

   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.

	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					

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.
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 according to the table below. If running a single panel, only transfer from column 1 to PCR2 tube 1 will be applicable. Use **forward pipetting** and change pipette tip after each well.

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.*

25.Library purification

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

Prepare bench

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

Before you start

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

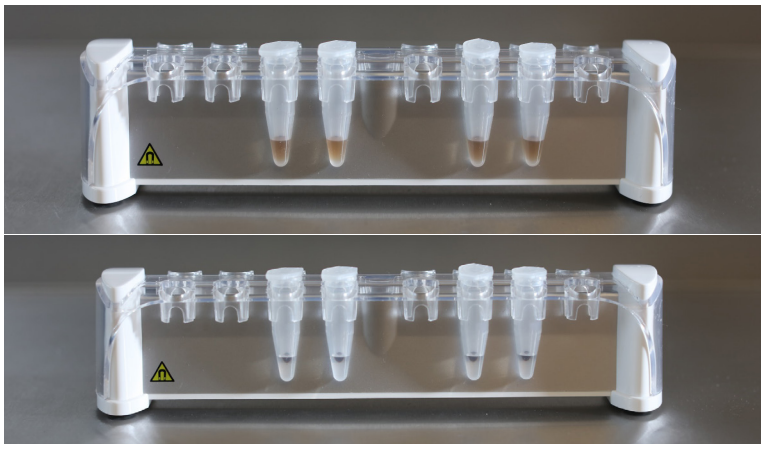
Instructions

1. Freshly prepare 2 mL of 70% EtOH by adding 0.5 mL MilliQ water and 1.5 mL 96% EtOH into the 70% EtOH Tube.
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 the BP Tube.
4. Transfer 50 µL from the PCR2 Tube to the BP Tube.

NOTE: *Store the PCR2 Tubes at -20 °C in case the purification step needs to be repeated.*

5. Pipette-mix 10 times to thoroughly mix the Library with the beads.
6. Start the timer and incubate the BP Tube for 5 minutes at room temperature.

7. After the incubation, place the BP Tube on the DynaMag-2 Magnetic stand and leave it for 2 minutes to separate the beads from the solution.



8. With the tube 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 tube still on 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 tube 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 Tube after this step. Use a smaller pipette to remove any residual EtOH.*

10. Leave the tube with the lid open on the magnetic stand for 2 minutes for the beads to air dry.
11. Close the tube and remove it from the magnetic stand.
12. Add 50 µL of MilliQ water to the BP Tube and pipette-mix 10 times towards the beads to elute the purified Library from the beads.
13. Incubate the tube for 2 minutes at room temperature.
14. Place the PB tube on the magnetic stand and leave it for 1 minute to separate the beads from the eluted Library solution.
15. With the PB tube still on the magnetic stand, transfer 45 µL of eluate from the BP Tube to the Lib Tube.

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

16. Discard the BP Tube.
17. Continue to *26. Quality control.*

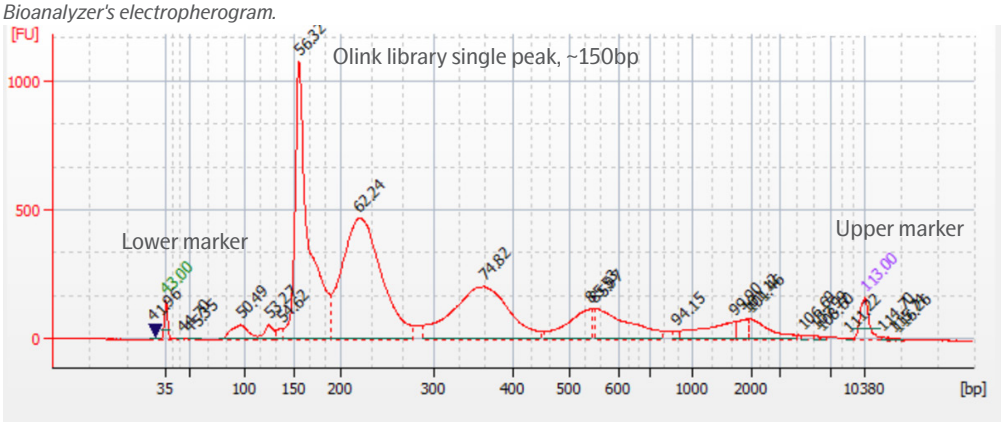
SAFE STOPPING POINT: *The Lib Tube can be stored at -20 °C for up to 4 weeks.*

26.Quality control

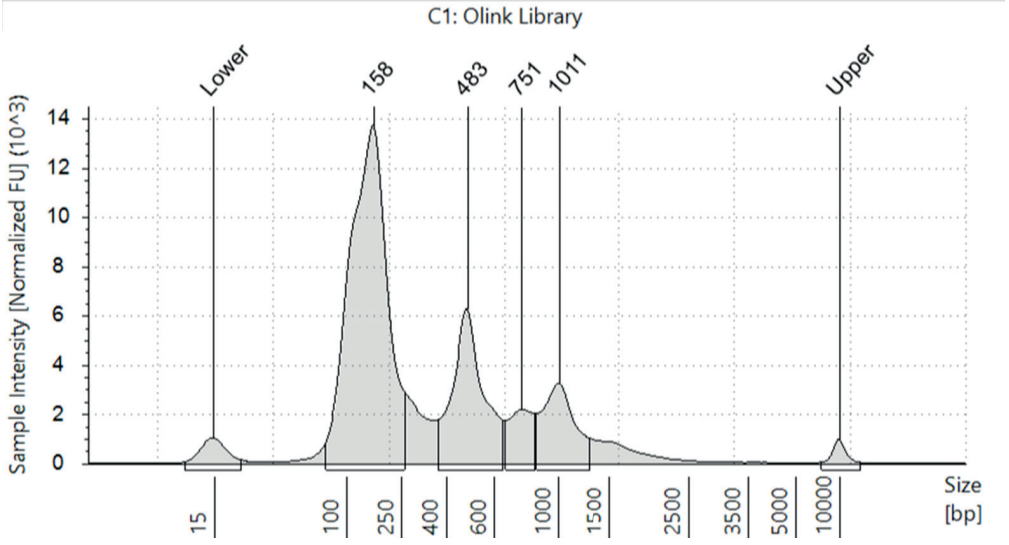
During this step, the purified Olink Libraries are quality controlled on the Bioanalyzer using the High Sensitivity DNA kit or on the TapeStation using the High Sensitivity D5000 kit, according to the Manufacturer's instructions.

The electropherograms in the figures below 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.

Bioanalyzer's electropherogram.



TapeStation's electropherogram.



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

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.

27.Next generation sequencing

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

- NextSeq™ 550
- NextSeq™ 2000
- NovaSeq™ 6000
- NovaSeq™ X Plus

As the workflow differs between instruments, refer to the applicable Olink® Explore Sequencing User Manual for instructions on how to sequence Olink Libraries.

Part 4: 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 384 laboratory workflow, using the Microlab® STAR from Hamilton Company. These instructions apply to running one Sample Plate on one Olink Explore 384 panel. If more Sample Plates or panels are processed, repeat the instructions the applicable number of times.

28.Preparations

28.1 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 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.*

28.2 Important information

Reagent lots

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

Vortexing

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

Plate type	Speed (rpm)	Time (s)
96-wells	2500	30
384-wells	3000	30

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.

28.3 Prepare the samples

During this step, samples are manually transferred to the Sample Plate. The Sample Plate must be used within the same day.

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

Prepare bench

- Samples (provided by the user)
- 1x 96-well PCR plate, 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 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.

Instruction

1. Transfer the samples into the Sample Plate, according to the plate layout shown in the figure below. Make sure that samples are added to every applicable well.

Sample Plate layout.
The numbers indicate the sample numbers.

	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	

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

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

29.Prepare Sample Source Plate

During this step, samples are manually transferred from the prepared Sample Plate into the Sample Source Plate, and controls are added into the Sample Source Plate. The Sample Source Plate must be used within the same day of preparation.

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, prepared in the previous step)*
- 1x 384-well PCR plate (skirted)
- 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 has been prepared according to *28.3 Prepare the samples.*
- Thaw the Sample Plate at room temperature if frozen.
- Thaw the Negative Control, Plate Control and recommended Sample Control at room temperature.
- Mark the new 384-well PCR plate: “Sample Source Plate”.

Instructions

1. Using the MixMate or manual vortexing, vortex the 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 384-well Sample Source Plate according to the plate layout shown in the figure below. 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 the figure below. 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.

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

	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																								

30.Sample Dilution

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

30.1 Prepare Sample Dilution Plate

Prepare bench

- Olink® Target 96/Explore Sample Diluent
- 1x 384-well PCR plate (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 the new 384-well PCR plate: "Sample Dilution Plate".

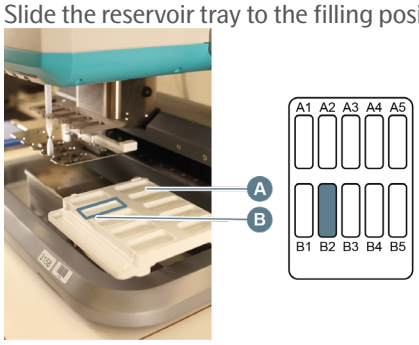
NOTE: *For convenience, the Sample Diluent can be thawed at +4 °C overnight.*

Instructions

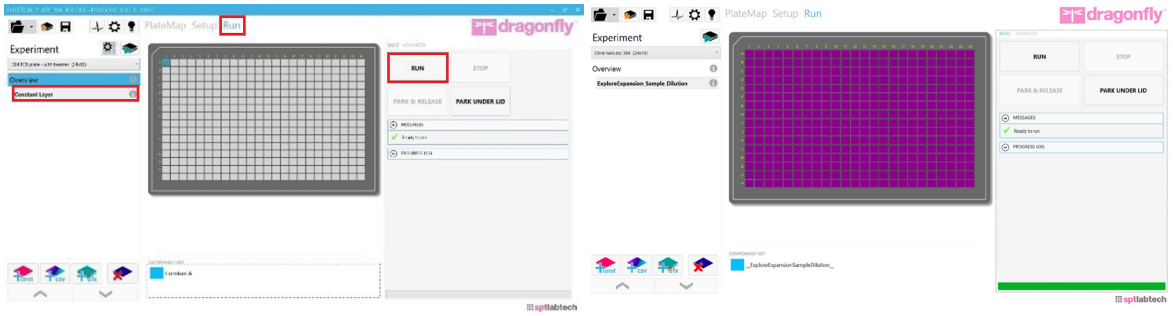
1. Prepare the Dragonfly according to instructions in the Olink Explore Overview User Manual.
   - Use the protocol `Olink Sample Dilution Plate v4.`
   - Attach one syringe in position B2.

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.*

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



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, then click *RUN* to start the protocol.
   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.



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. When finished, clear the instrument and shut it down according to instructions in the Olink Explore Overview User Manual.
10. Continue to *30.2 Perform Sample Dilution*, or store the Sample Dilution Plate at +4 °C until use (the same day).

30.2 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.

Prepare bench

- Sample Source Plate, prepared in previous step
- Sample Dilution Plate, 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.

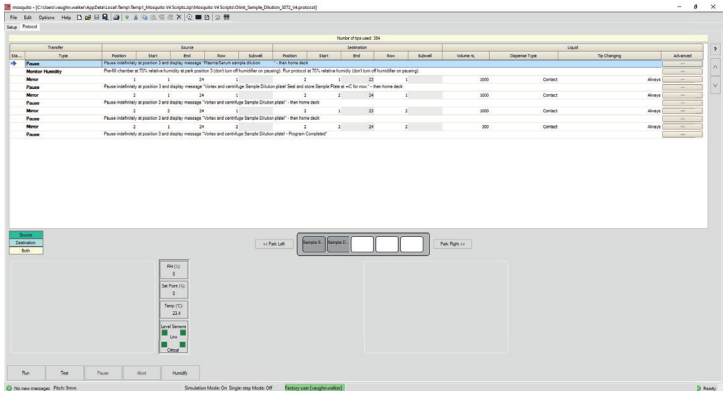
Instructions

IMPORTANT: *Make sure to vortex the Sample Dilution Plate(s) thoroughly both between dilutions and after the last dilution. If using MixMate, make sure to vortex the Sample Dilution Plate thoroughly with correct MixMate settings, as incorrect settings may lead to low-quality data. Refer to table in 28.2 Important information.*

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 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 protocol `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 Sample Dilution Plate. The run can be monitored in the protocol tab.
7. When the protocol is paused and a pop-up window appears on the screen, 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.
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 protocol.
    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 protocol is paused and a pop-up window appears on the screen, remove the Sample Dilution Plate from the 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 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 protocol. Keep the Mosquito on for later use.
20. Continue to *31. Incubation*, or place the Sample Dilution Plate at +4 °C for up to 1 hour.

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

	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

31.Incubation

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

31.1 Prepare Reagent Source Plate

During this step, four Incubation Mixes are prepared manually and transferred to one 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 downstreamdata 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

- Olink® Explore Incubation Solution
- Olink® Explore Forward Probes A–D (Frw probes)
- Olink® Explore Reverse Probes A–D (Rev probes)
- 1x 8-well strip
- 1x 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 the new 384-well PCR plate: “Reagent Source Plate”.
- Mark the wells of an 8-well strip according to:

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

Instructions

1. Vortex the Incubation Solution, Forward Probes and Reverse Probes and spin down.
2. Prepare four Incubation Mixes: Using forward pipetting, first transfer 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:

Addition order	Reagent	Well							
		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	–	–	–	–
		Frw Probes A	Frw Probes B	Frw Probes C	Frw Probes D	–	–	–	–
3	Rev probes	10 µL	10 µL	10 µL	10 µL	–	–	–	–
		Rev Probes A	Rev Probes B	Rev Probes C	Rev Probes D	–	–	–	–

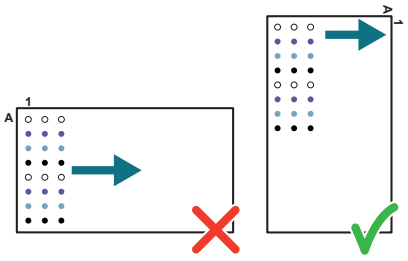
NOTE: *Pipette the Incubation Solution carefully to avoid foaming.*

3. Seal the PCR strip 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. Rotate the Sample Reagent Plate (384-well) 90 degrees clockwise to facilitate pipetting.



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

Reagent Source Plate layout.
The letters indicate the different blocks within the panel.

	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	A		B		C		D																	
B																								
C	A		B		C		D																	
D																								
E	A		B		C		D																	
F																								
G	A		B		C		D																	
H																								
I	A		B		C		D																	
J																								
K	A		B		C		D																	
L																								
M	A		B		C		D																	
N																								
O	A		B		C		D																	
P																								

6. Seal the Reagent Source Plate 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.
7. Immediately continue to *31.2 Prepare Incubation Plates and perform incubation*

31.2 Prepare Incubation Plates and perform incubation

During this step, the prepared Incubation Mixes are mixed with the samples in one Incubation Plate using the Mosquito, prior to incubation.

Prepare bench

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

Before you start

- Allow the refrigerated Sample Source Plate and Sample Dilution Plate to reach room temperature
- Mark the new 384-well PCR plate: “Incubation Plate".

Instructions

1. In the open Mosquito software, Select *File/Open* and choose the protocol for the applicable panel 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.*

2. Place the Incubation Plate 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.
4. Place the Reagent Source Plate 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 protocol is paused, and a pop-up window appears on the screen, remove the Incubation Plate from the 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 with a new adhesive film and spin at 400–1000 x g for 1 minute, then carefully remove the adhesive film 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 the Incubation Plate back in the magnetic clamp booster and return it to its previous position on the Mosquito deck.
9. Vortex the 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.
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 protocol is finished, remove the Incubation Plate from the deck and from the magnetic clamp booster. 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 the Incubation Plate at 400–1000 x g for 1 minute.
14. Inspect the Incubation Plate to ensure that all wells contain the same amount of liquid (0.8 µL). Note if there is any wells containing bubbles.
15. Write the time of the incubation starting point on the plate seal and incubate Incubation Plate 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 deck. Treat the plates as follows:
    - Sample Source Plate: Seal with adhesive film and store at -80 °C for potential reruns.
    - Sample Dilution Plate: Discard
    - Reagent Source Plate: Discard
17. When finished, clear the Mosquito and shut it down according to instructions in the Olink® Explore Overview User Manual.

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.

	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

32.Prepare 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.

33. Extension and pre-amplification (PCR1)

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

33.2.1 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 Plate, 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 Plate reach room temperature.
- Mark the new 50 mL Falcon tube: "PCR1 Mix"
- Switch on one ProFlex™ PCR instrument in the post-PCR room.
- Ensure that the PCR1 Enhancer is fully thawed and has reached room temperature.

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 the Incubation Plate down for 1 minute at room temperature.
4. Prepare a PCR1 Mix in a Falcon tube in the following order, using the volumes according to:

Addition order	Reagent	Volume (µL)
1	MilliQ water (+4 °C)	8 250
2	PCR1 Enhancer	1 073
3	PCR1 Solution	1 073
4	PCR1 Enzyme	107
	Total	10 502

   - First add MilliQ water and PCR1 Enhancer and vortex thoroughly.
   - Then add the PCR1 Solution and PCR1 Enzyme, and vortex for 3 seconds.

5. Keep at room temperature until use.

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

33.2.2 Prepare PCR1 Plate and perform PCR1

During this step, the prepared PCR1 Mix is dispensed into the Incubation Plate using the Dragonfly, and the plate is subjected to a PCR reaction.

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 plate.

Prepare bench

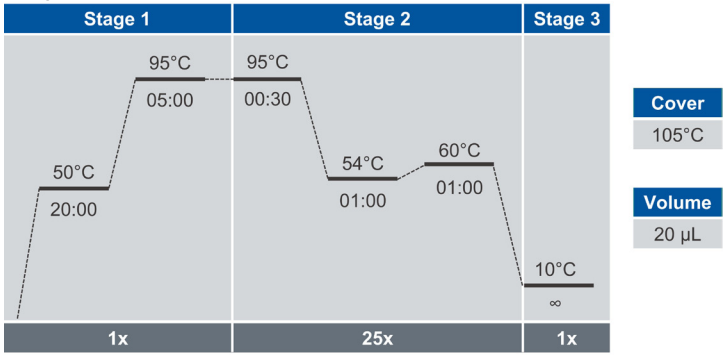
- Incubation Plate at room temperature, prepared in previous step
- PCR1 Mix, prepared in previous step
- Timer
- 3x Disposable reservoirs (10 mL)
- 3x Disposable ultra-low retention syringes with plungers
- Manual pipette (5 or 10 mL)
- Filter pipette tips
- Adhesive films

Before you start

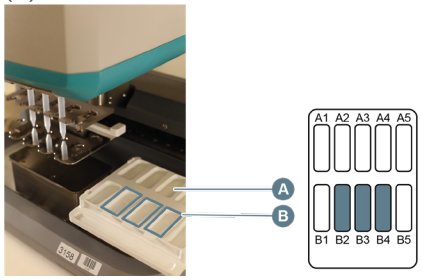
- Set a timer to 10 minutes.

Instructions

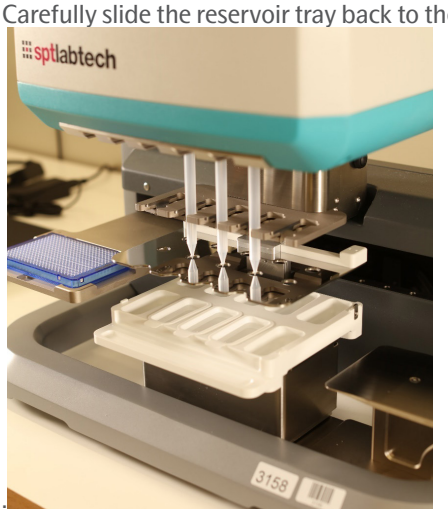
1. Start the PCR protocol `Olink PCR1` on the ProFlex PCR instrument. Pause when the PCR block temperature reaches 50 °C.



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)



4. Transfer 3.3 mL of PCR1 Mix into each of the three reservoir.
5. Carefully slide the reservoir tray back to the aspirate position



6. Rename Incubation Plate “PCR1 Plate” and spin down the plate.
7. Carefully remove the adhesive film from PCR1 Plate 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 protocol. 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.

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 plate.*

9. When the Dragonfly has returned the PCR1 Plate 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. Using the MixMate or manual vortexing, vortex the plate thoroughly to ensure that all wells are mixed.
11. In the Post-PCR room, centrifuge the PCR1 Plate at 400–1000 x g for 1 minute.
12. Inspect the PCR1 Plate to ensure that all wells contain the same amount of liquid (19.8 µL). Note any deviations.
13. When the timer ends after 10 minutes, place PCR1 Plate in the pre-heated ProFlex and click *Resume*.
14. When finished, clear the Dragonfly and shut it down according to instructions in the Olink Explore Overview User Manual.
15. When the protocol is finished (~1 hour 55 minutes), continue to *34. Pool PCR1 products*, or store the PCR1 Plate at +4 °C if used the same day.

SAFE STOPPING POINT: *The PCR1 Plate can be stored at -20 °C for up to 2 weeks.*

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.

	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

34.Pool PCR1 products

During this step, the PCR1 products from the PCR1 Plates are pooled into one PCR1 Pooling Plate using the Microlab STAR instrument.

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

1. 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.
2. 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.
3. In Run Control, select the protocol `PCR1 Pooling` and click the **Start** button.
4. Select the number of Destination Pooling Plates and the number of Panels being Pooled for each, then click *OK*.
5. 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.
6. 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.
7. 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.
8. Vortex the PCR1 Pooling Plate and spin down at 400–1000 x g for 1 minute.
9. Inspect the PCR1 Pooling Plate to ensure that all wells contain the same amount of liquid (24 µL).
10. 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.
11. Continue to *35. Amplification and sample indexing (PCR2)*, or store the PCR1 Pooling Plate at +4 °C until ready to use (the same day).

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.

	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

35.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.

35.2.1 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 according to:

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

6. 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.*

35.1 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.

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

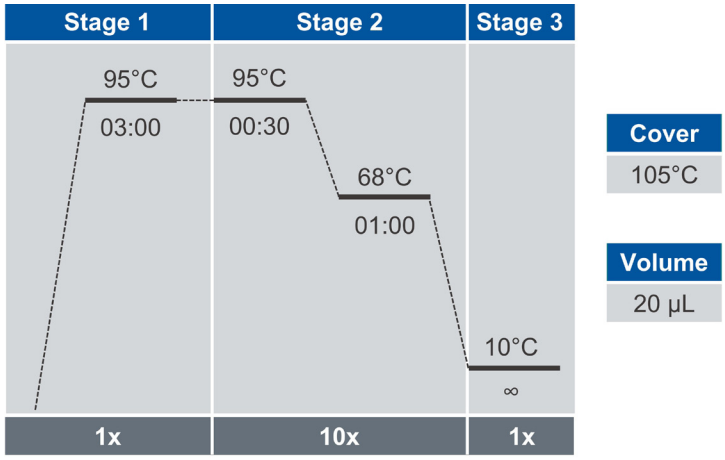
1. Open Hamilton Run Control and select the protocol `PCR2_Setup` and click *Start*.
2. Select the number of PCR2 plates being prepped and the number of Panels in each plate, and click *OK*.
3. Centrifuge Index Plate 1 at 400–1000 x g for 1 minute and inspect the plate to ensure that all wells contain 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. 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 protocol within 5 minutes from end of the 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 protocol `Olink Index PCR2`. Click *Start*.



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 protocol is finished (~25 minutes), continue to *36. 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.*

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.

	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

36.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. 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.
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.

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

	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					

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 the table below. If running a single panel, only transfer from column 1 to PCR2 tube 1 will be applicable. Use **forward pipetting** and change pipette tip after each well.

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.*

37.Library purification

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

Prepare bench

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

Before you start

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

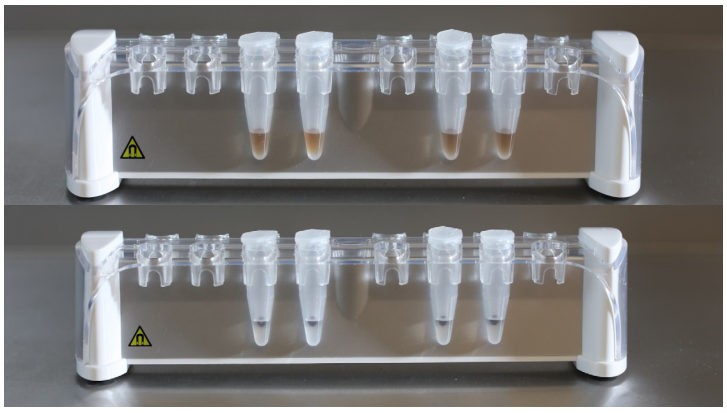
Instructions

1. Freshly prepare 2 mL of 70% EtOH by adding 0.5 mL MilliQ water and 1.5 mL 96% EtOH into the 70% EtOH Tube.
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 the BP Tube.
4. Transfer 50 µL from the PCR2 Tube to the BP Tube.

NOTE: *Store the PCR2 Tubes at -20 °C in case the purification step needs to be repeated.*

5. Pipette-mix 10 times to thoroughly mix the Library with the beads.
6. Start the timer and incubate the BP Tube for 5 minutes at room temperature.

7. After the incubation, place the BP Tube on the DynaMag-2 Magnetic stand and leave it for 2 minutes to separate the beads from the solution.



8. With the tube 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 tube still on 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 tube 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 Tube after this step. Use a smaller pipette to remove any residual EtOH.*

10. Leave the tube with the lid open on the magnetic stand for 2 minutes for the beads to air dry.
11. Close the tube and remove it from the magnetic stand.
12. Add 50 µL of MilliQ water to the BP Tube and pipette-mix 10 times towards the beads to elute the purified Library from the beads.
13. Incubate the tube for 2 minutes at room temperature.
14. Place the PB tube on the magnetic stand and leave it for 1 minute to separate the beads from the eluted Library solution.
15. With the PB tube still on the magnetic stand, transfer 45 µL of eluate from the BP Tube to the Lib Tube.

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

16. Discard the BP Tube.
17. Continue to *38. Quality control.*

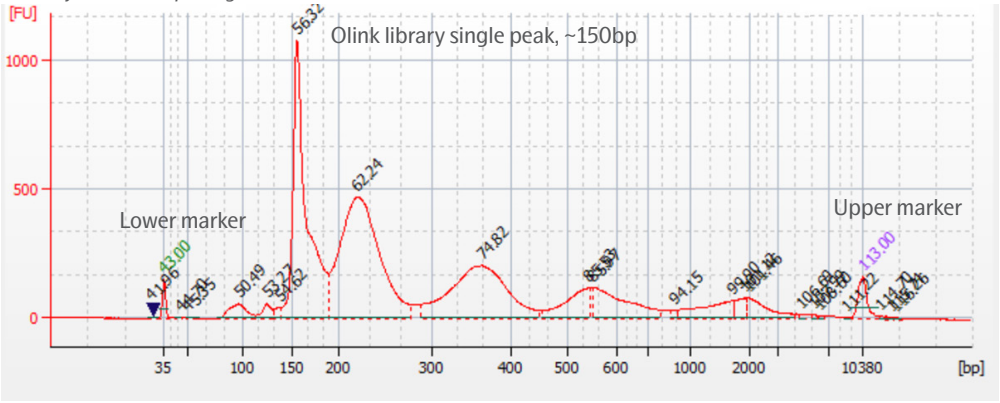
SAFE STOPPING POINT: *The Lib Tube can be stored at -20 °C for up to 4 weeks.*

38.Quality control

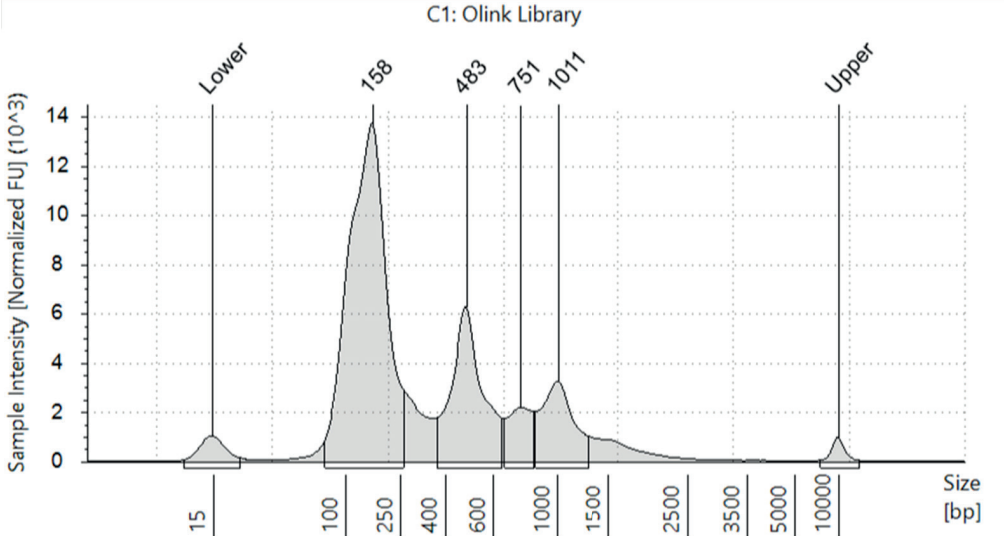
During this step, the purified Olink Libraries are quality controlled on the Bioanalyzer using the High Sensitivity DNA kit or on the TapeStation using the High Sensitivity D5000 kit, according to the Manufacturer's instructions.

The electropherograms in the figures below 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.

Bioanalyzer's electropherogram.



TapeStation's electropherogram.



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

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.

39. Next generation sequencing

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

- NextSeq™ 550
- NextSeq™ 2000
- NovaSeq™ 6000
- NovaSeq™ X Plus

As the workflow differs between instruments, refer to the applicable Olink® Explore Sequencing User Manual for instructions on how to sequence Olink Libraries.

Part 5: 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 384 laboratory workflow, using the ep*Motion* from Eppendorf Company. These instructions apply to running one Sample Plate on one Olink Explore 384 panel. If more Sample Plates or panels are processed, repeat the instructions the applicable number of times.

40.Preparations

40.1 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 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.*

40.2 Important information

Reagent lots

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

Vortexing

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

Plate type	Speed (rpm)	Time (s)
96-wells	2500	30
384-wells	3000	30

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.

40.3 Prepare the samples

During this step, samples are manually transferred to the Sample Plate. The Sample Plate must be used within the same day.

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

Prepare bench

- Samples *(provided by the user)*
- 1x 96-well PCR plate, 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 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.

Instruction

1. Transfer the samples into the Sample Plate, according to the plate layout shown in the figure. Make sure that samples are added to every applicable well.

Sample Plate layout.
The numbers indicate the sample numbers.

	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	

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

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

41.Prepare Sample Source Plate

During this step, samples are manually transferred from the prepared Sample Plate into the Sample Source Plate, and controls are added into the Sample Source Plate. The Sample Source Plate must be used within the same day of preparation.

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, prepared in the previous step)*
- 1x 384-well PCR plate (skirted)
- 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 has been prepared according to *40.3 Prepare the samples.*
- Thaw the Sample Plate at room temperature if frozen.
- Thaw the Negative Control, Plate Control and recommended Sample Control at room temperature.
- Mark the new 384-well PCR plate: “Sample Source Plate”.

Instructions

1. Using the MixMate or manual vortexing, vortex the 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 384-well Sample Source Plate according to the plate layout shown in the figure below. 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 the figure below. 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.

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

	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																								

42.Sample Dilution

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

42.1 Prepare Sample Dilution Plate

Prepare bench

- Olink® Target 96/Explore Sample Diluent
- 1x 384-well PCR plate (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 the new 384-well PCR plate: "Sample Dilution Plate".

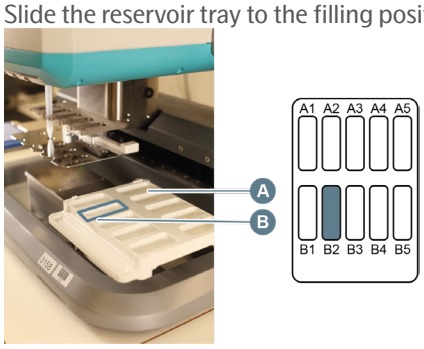
NOTE: *For convenience, the Sample Diluent can be thawed at +4 °C overnight.*

Instructions

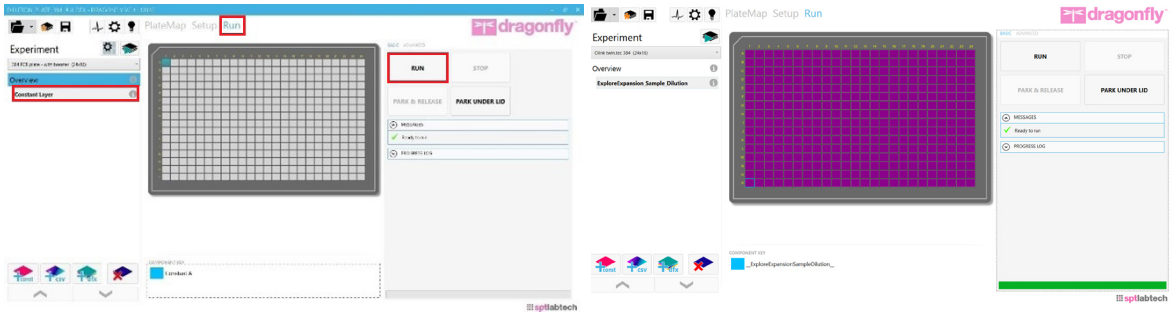
1. Prepare the Dragonfly according to instructions in the Olink® Explore Overview User Manual.
   - Use the protocol `Olink Sample Dilution Plate v4`.
   - Attach one syringe in position B2.

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.*

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



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, then click *RUN* to start the protocol.
   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.



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. When finished, clear the instrument and shut it down according to instructions in the Olink Explore Overview User Manual.
10. Continue to *42.2 Perform Sample Dilution*, or store the Sample Dilution Plate at +4 °C until use (the same day).

42.2 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.

Prepare bench

- Sample Source Plate, prepared in previous step
- Sample Dilution Plate, 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.

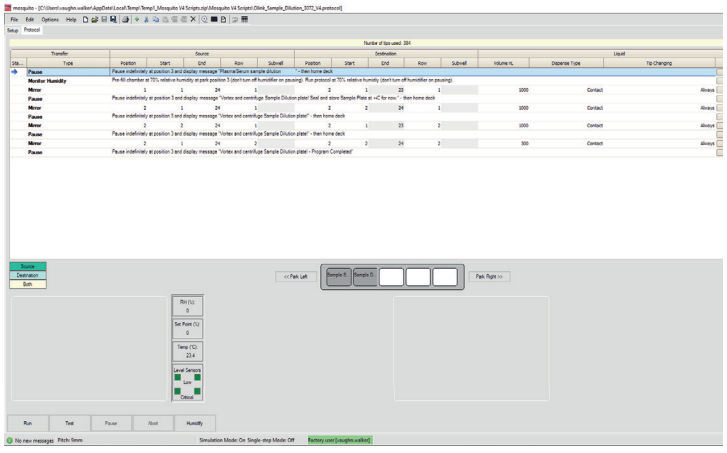
Instructions

IMPORTANT: *Make sure to vortex the Sample Dilution Plate(s) thoroughly both between dilutions and after the last dilution. If using MixMate, make sure to vortex the Sample Dilution Plate thoroughly with correct MixMate settings, as incorrect settings may lead to low-quality data. Refer to table in 5.2 Important information.*

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 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 protocol `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 Sample Dilution Plate. The run can be monitored in the protocol tab.
7. When the protocol is paused and a pop-up window appears on the screen, remove the Sample Source Plate and the Sample Dilution Plate from the 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.
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 protocol.
    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 protocol is paused and a pop-up window appears on the screen, remove the Sample Dilution Plate from the 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 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 protocol. Keep the Mosquito on for later use.
20. Continue to *43. Incubation*, or place the Sample Dilution Plate at +4 °C for up to 1 hour.

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

	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

43.Incubation

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

43.1 Prepare Reagent Source Plate

During this step, four Incubation Mixes are prepared manually and transferred to one 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 for 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)
- 1x 8-well strip
- 1x 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 the new 384-well PCR plate: “Reagent Source Plate”.
- Mark the wells of an 8-well strip according to:

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

Instructions

1. Vortex the Incubation Solution, Forward Probes and Reverse Probes and spin down.
2. Prepare four Incubation Mixes: Using forward pipetting, first transfer 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:

Addition order	Reagent	Well							
		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	–	–	–	–
		Frw Probes A	Frw Probes B	Frw Probes C	Frw Probes D	–	–	–	–
3	Rev probes	10 µL	10 µL	10 µL	10 µL	–	–	–	–
		Rev Probes A	Rev Probes B	Rev Probes C	Rev Probes D	–	–	–	–

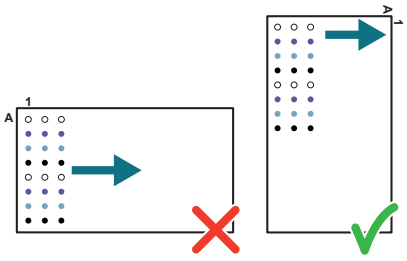
NOTE: *Pipette the Incubation Solution carefully to avoid foaming.*

3. Seal the PCR strip 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. Rotate the Sample Reagent Plate (384-well) 90 degrees clockwise to facilitate pipetting.



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

	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	A		B		C		D																	
B																								
C	A		B		C		D																	
D																								
E	A		B		C		D																	
F																								
G	A		B		C		D																	
H																								
I	A		B		C		D																	
J																								
K	A		B		C		D																	
L																								
M	A		B		C		D																	
N																								
O	A		B		C		D																	
P																								

6. Seal the Reagent Source Plate 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.
7. Immediately continue to *43.2 Prepare Incubation Plates and perform incubation*.

43.2 Prepare Incubation Plates and perform incubation

During this step, the prepared Incubation Mixes are mixed with the samples in one Incubation Plate using the Mosquito, prior to incubation.

Prepare bench

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

Before you start

- Allow the refrigerated Sample Source Plate and Sample Dilution Plate to reach room temperature
- Mark the new 384-well PCR plate: “Incubation Plate".

Instructions

1. In the open Mosquito software, Select *File/Open* and choose the protocol for the applicable panel 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.*

2. Place the Incubation Plate 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.
4. Place the Reagent Source Plate 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 protocol is paused, and a pop-up window appears on the screen, remove the Incubation Plate from the 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 with a new adhesive film and spin at 400–1000 x g for 1 minute, then carefully remove the adhesive film 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 the Incubation Plate back in the magnetic clamp booster and return it to its previous position on the Mosquito deck.
9. Vortex the 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.
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 protocol is finished, remove the Incubation Plate from the deck and from the magnetic clamp booster. 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 the Incubation Plate at 400–1000 x g for 1 minute.
14. Inspect the Incubation Plate to ensure that all wells contain the same amount of liquid (0.8 µL). Note if there is any wells containing bubbles.
15. Write the time of the incubation starting point on the plate seal and incubate Incubation Plate 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 deck. Treat the plates as follows:
    - Sample Source Plate: Seal with adhesive film and store at -80 °C for potential reruns.
    - Sample Dilution Plate: Discard
    - Reagent Source Plate: Discard
17. When finished, clear the Mosquito and shut it down according to instructions in the Olink® Explore Overview User Manual.

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.

	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

44.Prepare 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.

45.Extension and pre-amplification (PCR1)

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

45.1 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 Plate, 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 Plate reach room temperature.
- Mark the new 50 mL Falcon tube: "PCR1 Mix"
- Switch on one ProFlex PCR instrument in the post-PCR room.
- Ensure that the PCR1 Enhancer is fully thawed and has reached room temperature.

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 the Incubation Plate down for 1 minute at room temperature.
4. Prepare a PCR1 Mix in a Falcon tube in the following order, using the volumes according to:

Addition order	Reagent	Volume (µL)
1	MilliQ water (+4 °C)	8 250
2	PCR1 Enhancer	1 073
3	PCR1 Solution	1 073
4	PCR1 Enzyme	107
	Total	10 502

   - First add MilliQ water and PCR1 Enhancer and vortex thoroughly.
   - Then add the PCR1 Solution and PCR1 Enzyme, and vortex for 3 seconds.

5. 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.*

45.2 Prepare PCR1 Plate and perform PCR1

During this step, the prepared PCR1 Mix is dispensed into the Incubation Plate using the Dragonfly, and the plate is subjected to a PCR reaction.

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 plate.

Prepare bench

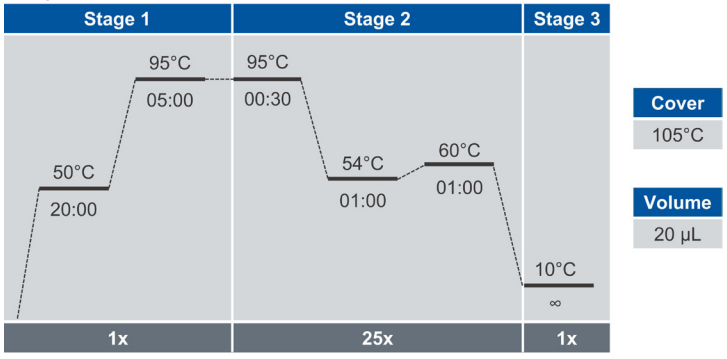
- Incubation Plate at room temperature, prepared in previous step
- PCR1 Mix, prepared in previous step
- Timer
- 3x Disposable reservoirs (10 mL)
- 3x Disposable ultra-low retention syringes with plungers
- Manual pipette (5 or 10 mL)
- Filter pipette tips
- Adhesive films

Before you start

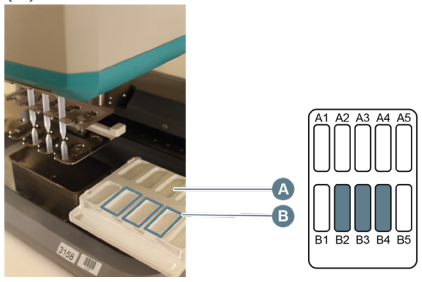
- Set a timer to 10 minutes.

Instructions

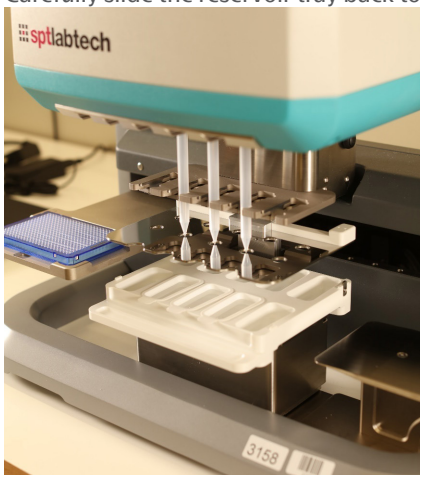
1. Start the PCR protocol `Olink PCR1` on the ProFlex PCR instrument. Pause when the PCR block temperature reaches 50 °C.



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).



4. Transfer 3.3 mL of PCR1 Mix into each of the three reservoir.
5. Carefully slide the reservoir tray back to the aspirate position.



6. Rename Incubation Plate “PCR1 Plate” and spin down the plate.
7. Carefully remove the adhesive film from PCR1 Plate 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 protocol. 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.

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 plate.*

9. When the Dragonfly has returned the PCR1 Plate 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. Using the MixMate or manual vortexing, vortex the plate thoroughly to ensure that all wells are mixed.
11. In the Post-PCR room, centrifuge the PCR1 Plate at 400–1000 x g for 1 minute.
12. Inspect the PCR1 Plate to ensure that all wells contain the same amount of liquid (19.8 µL). Note any deviations.
13. When the timer ends after 10 minutes, place PCR1 Plate in the pre-heated ProFlex and click *Resume*.
14. When finished, clear the Dragonfly and shut it down according to instructions in the Olink Explore Overview User Manual.
15. When the *Olink PCR1* protocol is finished (~1 hour 55 minutes), continue to *46. Pool PCR1 products*, or store the PCR1 Plate at +4 °C if used the same day.

SAFE STOPPING POINT: *The PCR1 Plate can be stored at -20 °C for up to 2 weeks.*

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.

	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

46.Pool PCR1 products

During this step, the PCR1 products are pooled into one PCR1 Pooling Plate using the ep*Motion*.

Prepare bench

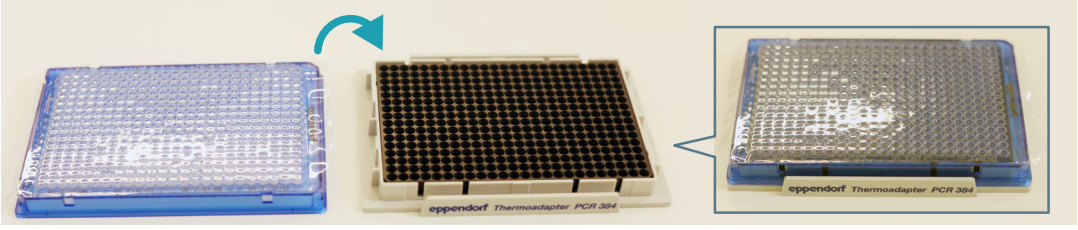
- PCR1 Plate, prepared in previous step
- MilliQ water (at +4 °C, preferably kept in the fridge until use)
- 1x 384-well PCR plate (skirted)
- 1x ep*Motion*® reservoir (30 mL)
- Dispensing tool TM50-8
- Dispensing tool TM10-8
- epT.I.P.S.® Motion pipette tips (10 µL)
- epT.I.P.S.® Motion pipette tips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

Before you start

- Thaw the PCR1 Plate 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 the PCR1 Plate is thawed and properly sealed, then vortex it and spin down at 400–1000 x g for 1 minute at room temperature.
2. Inspect the wells of the PCR1 Plate 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 protoco `1 Olink PCR1 Pooling 1 panel.`
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 3 mL MilliQ water to the reservoir.
   - Place the PCR1 Plate on the thermoadapter.
   - Carefully remove the adhesive film.



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.

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.*
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 the PCR1 Plate containing the remaining PCR1 products and seal it with a new adhesive film. Store it at -20 °C for up to 2 weeks in case of potential reruns.
14. Continue to *47. Amplification and sample indexing (PCR2)*, or store the PCR1 Pooling Plate at +4 °C until ready to use (the same day).

PCR1 Pooling Plate layout.
The numbers indicate the sample numbers.

	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																								

47.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.

47.1 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.
- 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 according to:

Addition order	Reagent	Volume (µL)
1	MilliQ water (+4 °C)	1800
2	PCR2 solution	258
3	PCR2 Enzyme	5
	Total	2063

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.*

47.2 Prepare PCR2 Plate and perform PCR2

During this step, the prepared PCR2 Mix is mixed with the samples along with index primers using the ep*Motion*. The samples are then subjected to a second PCR reaction.

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 1 panel.`
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.

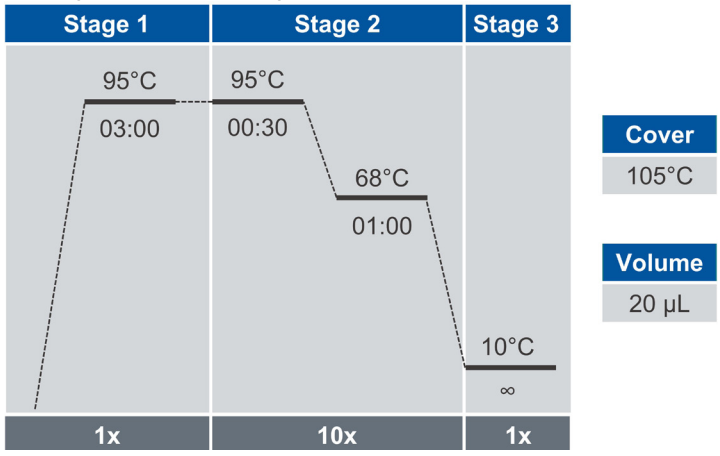
NOTE: *Make sure to place tip boxes and plates in the correct position and orientation according to the layout presented in the software, and that empty positions in the software are also empty on the worktable. Failure to follow the layout in the software can lead to instrument damage.*

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, see fiigure below. 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 protocol 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 protocol `Olink Index PCR2`. Click *Start*.



14. Remove the PCR1 Pooling Plate containing the remaining PCR1 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 protocol is finished (~25 minutes), continue to *48. 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.*

PCR2 Plate layout.
The numbers indicate the sample numbers.

	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																								

48.Pool PCR2 products

During this step, all PCR2 products are pooled into one PCR2 Pooling Plate using the ep*Motion*. The Olink libraries are then manually transferred to one microcentrifuge tube containing amplicons from 96 samples, including controls.

Prepare bench

- PCR2 Plate, prepared in previous step
- 1x 96-well PCR plate (skirted)
- 1x 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 96-well PCR plate: “PCR2 Pooling Plate”.
- Mark the new microcentrifuge tube: “PCR2”.

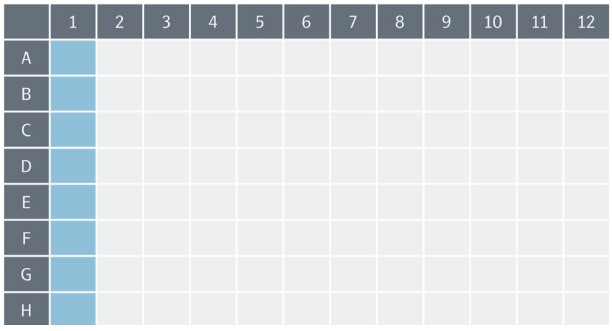
Instructions

1. Open the *EpBlue Application Runner*.
2. In the application library, select user and the protocol: `3 Olink PCR2 Pooling 1 panel`.
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.*
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 wells contain sthe same amount of liquid (36 µL in column 1).
11. Clear the ep*Motion* and shut it down according to instructions in the Olink® Explore Overview User Manual.

PCR2 Pooling Plate layout



12. Carefully remove the adhesive film from the PCR2 Pooling Plate.
13. Using a single-channel pipette, transfer the 30 µL pooled PCR2 products from each well in column 1 of the PCR2 Pooling Plate to the PCR2 Tube. Use forward pipetting, and change pipette tip after each well. Vortex the PCR2 Tube and spin down briefly.
14. 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.
15. Discard the PCR2 Pooling Plate.
16. Continue to *49. Library purification*, or store the PCR2 Tube at +4 °C until use (the same day).

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

49.Library purification

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

Prepare bench

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

Before you start

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

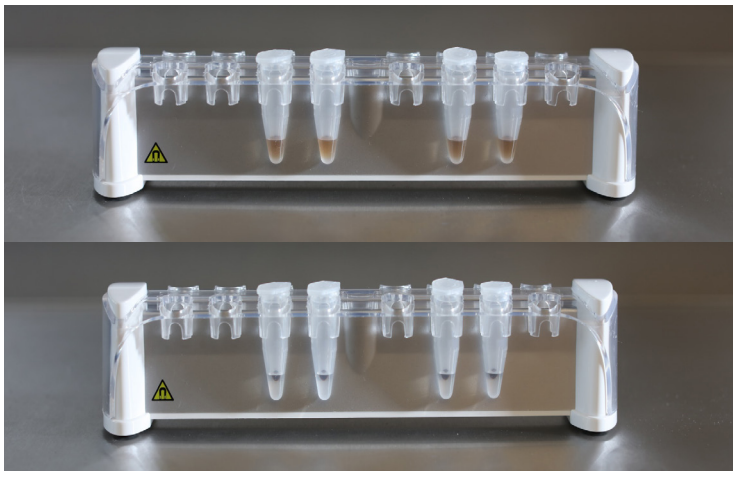
Instructions

1. Freshly prepare 2 mL of 70% EtOH by adding 0.5 mL MilliQ water and 1.5 mL 96% EtOH into the 70% EtOH Tube.
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 the BP Tube.
4. Transfer 50 µL from the PCR2 Tube to the BP Tube.

NOTE: *Store the PCR2 Tubes at -20 °C in case the purification step needs to be repeated.*

5. Pipette-mix 10 times to thoroughly mix the Library with the beads.
6. Start the timer and incubate the BP Tube for 5 minutes at room temperature.

7. After the incubation, place the BP Tube on the DynaMag-2 Magnetic stand and leave it for 2 minutes to separate the beads from the solution.



8. With the tube 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 tube still on 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 tube 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 Tube after this step. Use a smaller pipette to remove any residual EtOH.*

10. Leave the tube with the lid open on the magnetic stand for 2 minutes for the beads to air dry.
11. Close the tube and remove it from the magnetic stand.
12. Add 50 µL of MilliQ water to the BP Tube and pipette-mix 10 times towards the beads to elute the purified Library from the beads.
13. Incubate the tube for 2 minutes at room temperature.
14. Place the PB tube on the magnetic stand and leave it for 1 minute to separate the beads from the eluted Library solution.
15. With the PB tube still on the magnetic stand, transfer 45 µL of eluate from the BP Tube to the Lib Tube.

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

16. Discard the BP Tube.
17. Continue to *50. Quality control.*

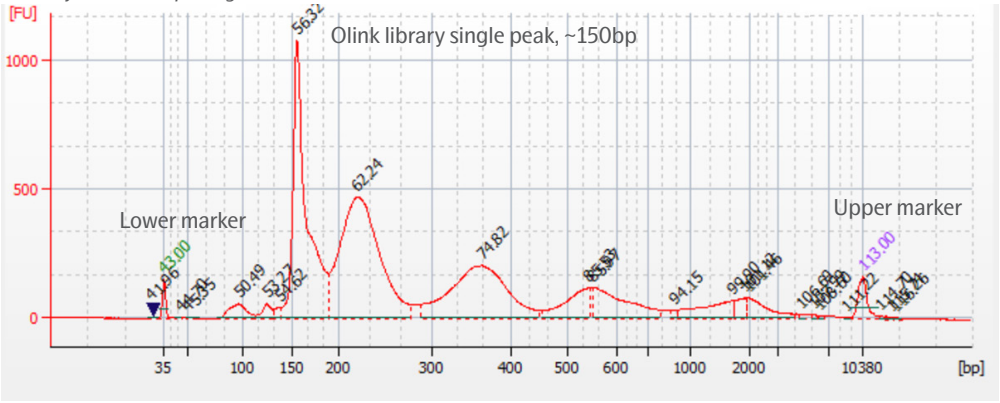
SAFE STOPPING POINT: *The Lib Tube can be stored at -20 °C for up to 4 weeks.*

50.Quality control

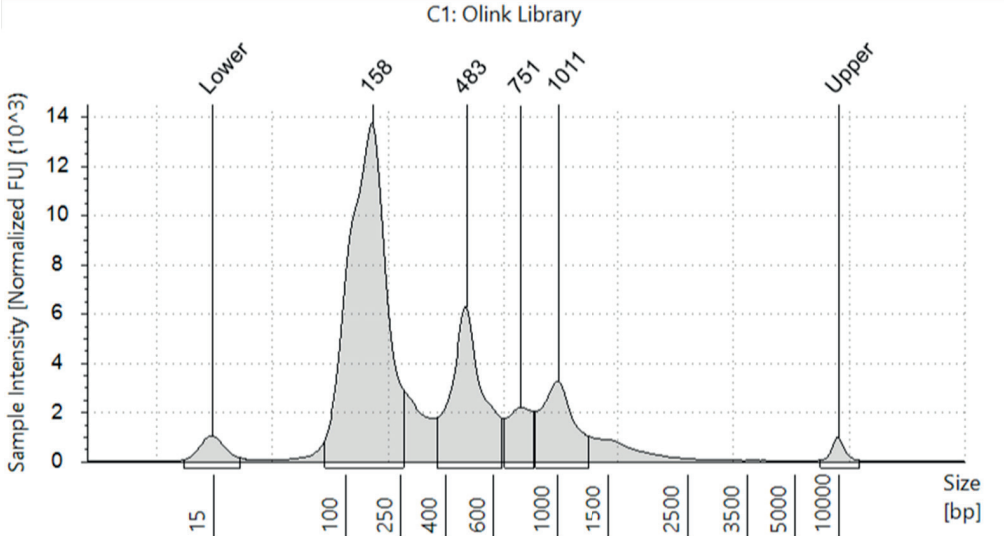
During this step, the purified Olink Libraries are quality controlled on the Bioanalyzer using the High Sensitivity DNA kit or on the TapeStation using the High Sensitivity D5000 kit, according to the Manufacturer's instructions.

The electropherograms in the figues below 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.

Bioanalyzer's electropherogram.



TapeStation's electropherogram.



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

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 Bioanalyzer 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.

51. Next generation sequencing

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

- NextSeq™ 550
- NextSeq™ 2000
- NovaSeq™ 6000
- NovaSeq™ X Plus

As the workflow differs between instruments, refer to the applicable Olink® Explore Sequencing User Manual for instructions on how to sequence Olink Libraries.

52.Revision history

Version	Date	Description
4.0	2024-04-16	*3* updated. Vortex table added to all "important information" sections. IMPORTANT regarding vortex with MixMate added to all "Perform sample dilution" sections. *Part 2:* Laboratory instructions for 96 samples using Formulatrix F.A.S.T.™ as Post-PCR added. Illumina® NovaSeq™ X Plus as sequencing instrument added.
3.1	2023-06-26	*Part 3:* Laboratory instructions for 96 samples using Formulatrix F.A.S.T.™ and Hamilton Microlab® Star added.
3.0	2023-05-12	New trademarks and disclaimer. *Part 4* added.
2.0	2023-02-16	Columns, quadrants and protocol versions edited. Sample Source Plate and Reagent Source Plate layout edited. Sample Dilution: Important added. Prepare Incubation Plates and perform incubation: Important edited. Pool PCR2 products: figure added. Quality control edited.
1.4	2022-12-21	*3* added. References to Microlab STAR® added.
1.3	2022-09-28	Prepare Sample Source Plate: Important edited. Prepare Reagent Source Plate: Important added and changed name of Sample Source Plate to Reagent Source Plate. Editorial changes.
1.2	2022-05-13	Perform Sample Dilution: Step 17 and 18 added. Prepare Incubation Plates and perform incubation: Note edited. Prepare PCR1 Mix: Note clarified. Amplification and sample indexing (PCR2): Time limit for using the PCR2 Mix clarified. Time Sensitive Step changed. Time Sensitive Step added. Step 16 corrected. Plate layout corrected.
1.1	2021-12-13	Prepare for day 2: Volume of MilliQ water changed to 30 mL Extension and pre-amplification (PCR1): "Vortex the PCR1 Mix thoroughly" removed.
1.0	2021-12-01	New

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