Olink® Explore HT

User Manual

1293, v2.0, 2024-04-16

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Part 1: Olink® Explore HT overview

1.Introduction

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

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

1.1 Intended use

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

1.2 About this manual

This manual provides an introduction to Olink® Explore HT, including information about reagents, equipment and documentation needed, an overview of the workflow, laboratory guidelines as well as instructions needed to run the Olink® Explore HT Reagent Kit.

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

2.Olink® Explore HT Reagent Kit contents

Olink® Explore HT Reagent Kit contains reagents for 172 user samples and 20 Olink controls spread over two sample plates. Storage temperature and expiry date for the components are stated on the label on each box.

2.1 Content of Olink® Explore HT Probes (98001)

Art. No	Component	Cap color	Volume	Storage
88030	Olink® Explore HT Frw Probes 1	White	25 uL	+4 ºC
88031	Olink® Explore HT Rev Probes 1	White	25 uL	
88032	Olink® Explore HT Frw Probes 2	Red	25 uL	
88033	Olink® Explore HT Rev Probes 2	Red	25 uL	
88034	Olink® Explore HT Frw Probes 3	Yellow	25 uL	
88035	Olink® Explore HT Rev Probes 3	Yellow	25 uL	
88036	Olink® Explore HT Frw Probes 4	Blue	25 uL	
88037	Olink® Explore HT Rev Probes 4	Blue	25 uL	
88038	Olink® Explore HT Frw Probes 5	Green	25 uL	
88039	Olink® Explore HT Rev Probes 5	Green	25 uL	
88040	Olink® Explore HT Frw Probes 6	Purple	25 uL	
88041	Olink® Explore HT Rev Probes 6	Purple	25 uL	
88042	Olink® Explore HT Frw Probes 7	Orange	25 uL	
88043	Olink® Explore HT Rev Probes 7	Orange	25 uL	
88044	Olink® Explore HT Frw Probes 8	Black	25 uL	
88045	Olink® Explore HT Rev Probes 8	Black	25 uL	
88003	Olink® Explore HT PCR Additive	Brown	4000 uL	
87004	Olink® Explore Incubation solution	Brown	1400 uL	

2.2 Content of Olink® Explore HT Sample Prep (98005)

Art. No	Component	Cap color	Volume	Storage
88001	Olink® Explore HT PCR Solution	Natural	4000 µL	-20 ºC
88006	Olink® Explore HT PCR Enzyme A	Blue*	825 µL	
88007	Olink® Explore HT PCR Enzyme B	Violet*	115 µL	
87009	Olink® Explore Negative Control	Red	150 µL	
88014	Olink® Explore HT Index Plate A	Yellow	15 µL	
88015	Olink® Explore HT Index Plate B	Blue	15 µL	
84032	Olink® Target 96/Explore Sample Diluent	Natural	2x13 mL	

*Some lots may have different colors (black and yellow respectively).

2.3 Content of Olink® Explore HT Controls (98003)

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

3.Associated documentation and resources

3.1 Olink documentation

User Manuals

- Olink Explore HT Sequencing using NovaSeq 6000 S4
- Olink Explore HT Sequencing using NovaSeq X Plus
  - For sequencing of Olink Explore HT libraries
- NPX™ Explore HT & 3072 User Manual
- NPX™ CLI Explore HT & 3072 Technical Information
  - For data analysis of Olink Explore HT libraries

The user manuals can be downloaded from *olink.com/downloads.*

3.2 Other resources

The following resources are available from the Olink website for technology information, protocol guidance and lists of available biomarker assays.

Guidelines

- Olink guidelines for sample randomization
  - Describes the importance of sample randomization and provides guidance on how to perform optimal randomization. *olink.com/faq/sample-randomization*

White papers

- PEA - a high multiplex immunoassay technology with qPCR or NGS readout
  - Describes the PEA technology including the internal and external controls, QC and its advantages for medium-to-large-scale protein biomarker studies.
- Pre-analytical variation in protein biomarker research
  - Provides guidance on factors to consider when collecting, storing and handling samples for use in protein biomarker studies.
- Strategies for design of protein biomarker studies
  - Describes important aspects of study design to consider, such as adequate sample size, control of confounding factors and biases, and appropriate statistical analysis.

The white papers can be downloaded from *olink.com/downloads.*

3.3 Videos

- The Promise of Proteomics
  - Introduction to Olink Explore and how it can help to accelerate proteomics research. *olink.com/our-platform/promise-of-proteomics/*
- PEA technology animation video
  - Explains the principles of PEA with NGS and qPCR readout. */olink.com/our-platform/our-pea-technology/.*

4.Technical support

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

5.Safety

This chapter contains important safety information. Make sure to read and understand the information before performing lab work.

5.1 Definition of alert levels

The following alert levels are used in the Olink Explore manuals:

WARNING: Indicates a potentially hazardous situation which, if not avoided, could result in injury.

IMPORTANT: *Indicates an important action that may impair the results if not performed correctly.*

NOTE: *Contains information that can make it easier to understand or perform a certain task.*

SAFE STOPPING POINT: *Indicates a step where the protocol can be safely paused and restarted at a later time.*

TIME SENSITIVE STEP: *Indicates a step that must be performed within a limited time period. Results may be impaired if not performed correctly.*

5.2 Safety instructions

Follow general laboratory safety procedures:

- Use gloves, safety goggles and protective clothing when performing the experiments.
- Handle and dispose of hazardous sample material according to local regulations.

5.3 Hazardous chemicals

See safety data sheet (SDS), available from the Olink website: *olink.com/downloads*.

6. Technology description

6.1 About PEA™

The technology behind the Olink panels is called Proximity Extension Assay (PEA). The Olink Explore HT platform (PEA with NGS readout) is comprised of a sample preparation that generates an Olink library per block, followed by quality control and a readout using Next Generation Sequencing (NGS). The process consists of five core steps (the stated durations do not include preparation time):

Day 1:

1. Sample preparation and dilution

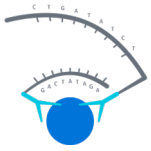
Duration of instrument protocols: 36 min

Samples and controls are transferred to one sample source plate and samples are diluted.

2. Immuno reaction (Incubation)

Duration of instrument protocols: 50 min
Duration of incubation: 16-24h (performed overnight)

High multiplexed PEA™ probes, matched pairs of antibodies with unique DNA oligonucleotides, bind to their respective proteins in the samples.

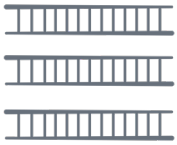


Day 2:

3. PCR

Duration of PCR program: 2 h

Oligonucleotides that are brought into proximity hybridize and are extended using a DNA polymerase. The piece of DNA barcode that is created is then amplified by a Polymerase Chain Reaction (PCR). Unique sample indexes are added to every sample, to allow pooling of the DNA amplicons for all samples.

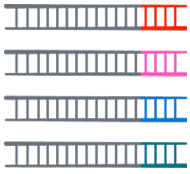


The final DNA amplicons in the Olink Explore HT libraries include:

- Specific barcode sequences for each assay

- Sample specific indexes

- Required sequences for Illumina sequencing (P5 and P7 Adapters and Sequencing Primer Binding Site Rd1SP)



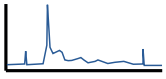
4. PCR pooling

Duration of instrument protocols: 40 min

Samples from the same dilution block are pooled together, resulting in one pool per dilution block, each containing 192 samples/controls.

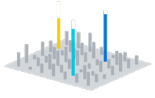
5. Library purification and quality control

Each pool is purified using magnetic beads. The quality of each Olink Explore HT library is assessed through automated electrophoresis.



6. Sequencing

The Olink Explore HT library is sequenced by NGS using Illumina® platform. The relative concentration of each biomarker, based on matched counts (the number of reads for each specific combination of sample and assay), is calculated using the NPX™ Explore HT & 3072 or Olink Explore CLI software.

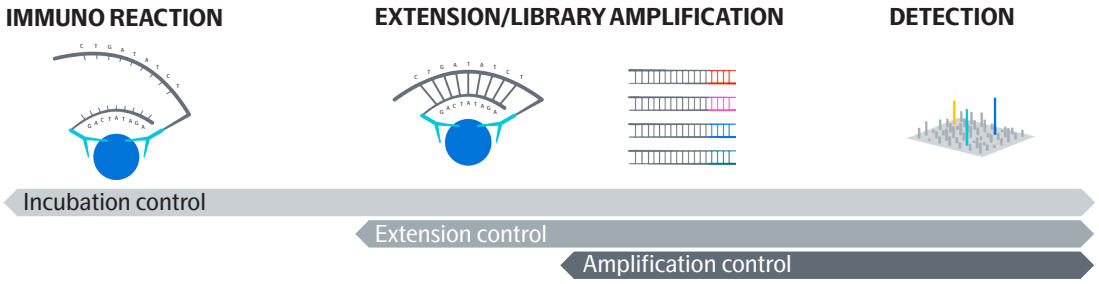


6.2 Quality control

Olink Explore HT contains a built-in quality control system using internal and external controls, which enables full control over the technical performance of assays and samples.

6.2.1 Internal controls

Three internal controls are spiked into every sample for each block. The internal controls are designed to monitor the quality of assay performance, as well as the quality of individual samples:



Incubation Control (Immuno Control): The Immuno Control is a non-human antigen measured with PEA. This control is included in the immuno reaction (incubation) and monitors potential technical variation in all three steps of the reaction.

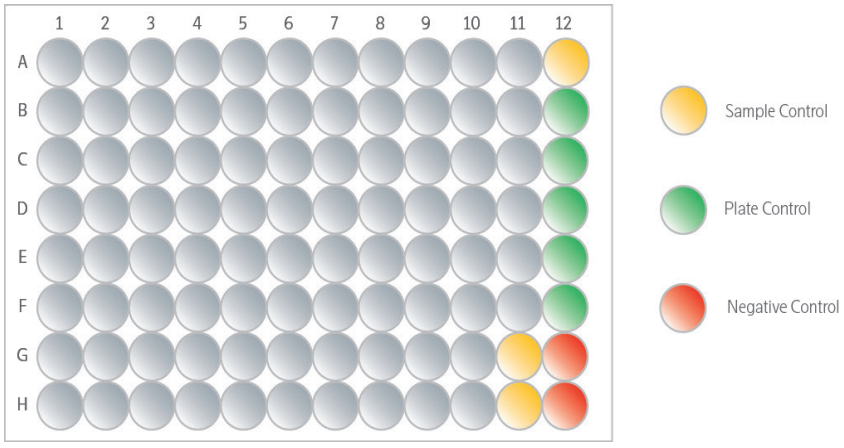
Extension Control: The Extension Control is composed of an antibody coupled to a unique pair of DNA-tags. These DNA-tags are always in proximity, so that this control is expected to give a constant signal independently of the immuno reaction. This control monitors variation in the extension and amplification step.

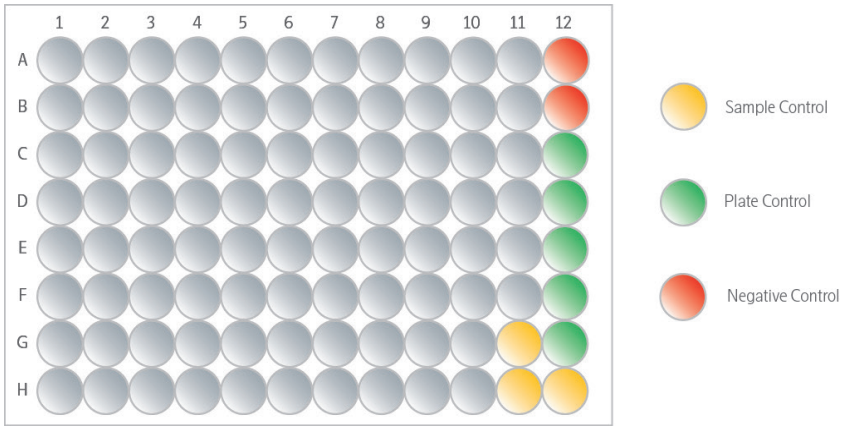
Amplification Control: The Amplification Control is a complete double stranded DNA amplicon which does not require any proximity binding or extension step to generate a signal. This control monitors the amplification/sample indexing step.

6.2.2 External controls

External controls are separate samples that are used for different purposes. There are seven required and three recommended external controls that are added to separate wells on each of the two sample plates. The figures below shows the two sample plate layouts, with 86 samples, 2 Negative Controls, 3 Sample Controls (optional), and 5 Plate Controls (required).

IMPORTANT: *If using other sample matrices than plasma or serum, please contact support@olink.com before proceeding with the sample preparation as the positions of the external controls in the Sample Source Plate differ.*





Sample Control: Sample Control is included in triplicates 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 troubleshooting.

Plate Control: Plate Control is included in 5 replicates on each plate. The median of the Plate Control replicates is used to normalize each assay and to compensate for potential variation between runs and plates.

Negative Control: Negative Control is included in duplicate on each plate and consists of buffer run as a normal sample. These are used to monitor any background noise generated when DNA-tags come in close proximity without prior binding to the appropriate protein. The Negative Controls assess potential contamination of assays.

7.Guidelines

7.1 Laboratory setup

The high sensitivity of the Olink assays requires a clean laboratory environment. Particles from the surroundings, such as dust, hair, saliva and skin flakes are common sources of contamination.

A well-recognized risk with molecular detection methods is contamination from PCR products. Therefore, make sure to organize the workspace so that the workflow occurs in one direction: from clean areas free from PCR products (pre-PCR) to areas containing PCR products (post-PCR). Olink recommends setting up at least two separate rooms: one pre-PCR and one post-PCR room. If this is not possible, keep separate benches and equipment.

7.2 Clean laboratory environment

The following recommendations are intended to reduce the risk of contamination, simplify the workflow in the laboratory and improve data quality. Make sure to follow these recommendations at all times.

- Use separate rooms for pre-PCR and post-PCR operations.
- Use separate consumables and equipment for pre-PCR and post-PCR operations.
- Always work from clean areas free from PCR products (pre-PCR) to areas containing PCR products (post-PCR).
- Fit ultra-violet (UV) lamps in closed working areas such as working cabinets or pipetting robots to enable decontamination by irradiation.
- Always wear a long-sleeved lab coat.
- Always wear gloves, including when bringing reagents in and out from fridge or freezer. Change gloves when needed.
- Wash your hands and change gloves and lab coat when moving between pre-PCR and post-PCR.
- Regularly decontaminate bench spaces with 10% sodium hypochlorite (followed by water to remove residual bleach), or a validated commercially available DNA-degrading decontaminant.
- Decontaminate pipettes on a regular basis according to the manufacturer's instructions.
- Clean instruments and pipetting robots on a regular basis according to the manufacturer's instructions.
- Keep all consumables (tubes, pipette tips, PCR plates etc.) in closed bags or boxes, preferably in a closed storage unit, until use.
- Clean the lab bench, hood, racks and pipettes with 70% ethanol.
- Bring out all reagents, consumables and samples needed for the specific lab step, as stated in the "Prepare the bench" list at the beginning of each instruction. Leave enzymes in the freezer until use.
- Organize equipment, consumables and samples at the workstation in a way that enables clean work.
- Label pipette boxes with column numbers to more easily monitor where you are on the plate.
- Briefly centrifuge tubes and plates before opening to avoid the generation of aerosols that may contaminate other samples.
- Pipette all reagents and samples using filter tips, and use a unique set of pipettes for each working station.

NOTE: *All consumables and reagents are for single use only.*

7.3 Pipetting techniques

Both forward and reverse pipetting are used in the Olink Explore HT workflow. Forward pipetting is the most commonly used pipetting technique. Reverse pipetting improves precision with smaller volumes and viscous solutions. Both techniques are described in this section, along with general guidelines for pipetting.

7.3.1 General pipetting guidelines

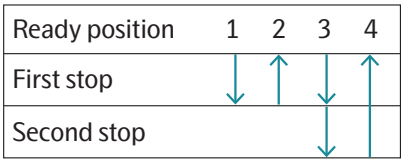
- Calibrate all pipettes regularly (at least with a 6-month interval).
- Let the reagents and liquids reach room temperature before use to maximize accuracy.
- Pipette near the liquid surface.
- Do not turn the pipette on the side when there is liquid in the tip, as liquid might contaminate the interior of the pipette.
- Keep the pipettes vertical while pipetting, and pipette to the bottom of the wells.

7.3.2 Forward pipetting

1. Press the operating button to the first stop.
2. Dip the tip into the solution to a depth in accordance with the set volume, and slowly release the operating button. Remove the tip from the liquid.

NOTE: *If using a multichannel pipette, ensure that all tips contain the exact same volume.*

3. Dispense the liquid into the receiving vessel by gently pressing the operating button to the first stop and then to the second stop. This action will empty the tip. Remove the tip from the vessel.
4. Release the operating button to the ready position.



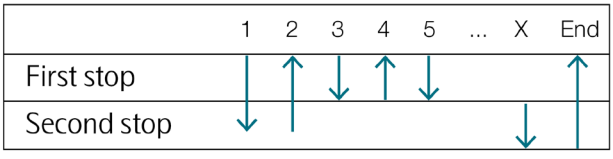
7.3.3 Reverse pipetting

Reverse pipetting improves precision with smaller volumes and viscous solutions, and is performed as follows:

1. Before filling the pipette with liquid, push the operating button past the first stop and then dip the tip into the liquid, just below the liquid surface.
2. Slowly release the operating button all the way up. This will fill the pipette tip with a volume larger than the set volume.

NOTE: *Ensure that all tips contain the exact same volume if a multichannel pipette is used.*

3. Press the operating button to the first stop to dispense the liquid into the recieving vessel. This volume is equal to the set volume, and a small volume will remain in the pipette tip. Hold the button pressed down to the first stop if the same pipette tip is going to be used to transfer the same liquid to another well.
4. Dip the pipette tip in the liquid again, and slowly release the operator button all the way up.
5. Continue pipetting into the recieving vessel.
   Repeat steps 4 and 5 as shown in the illustration, until liquid has been transferred into all wells.
6. Discard the small volume left in the pipette tip once the pipetting of the liquid is done. Release the operating button all the way up and discard the empty pipette tip.



7.4 Vortexing

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

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

7.4.1 96-well semi-skirted plates vortexing

1. Insert the Tube Holder PCR 96 in the plate holder by holding it up against the back edge of the plate holder so that the stop pins fit in the holes.
2. Press the plate into the bores of the holder and make sure they sit evenly. Note that misplacement of the plate will result in uneven mixing of the wells and low-quality data.
3. Set mixing speed and time according to the table above.
4. Start mixing by pressing the start/stop key. After 30 seconds, MixMate will automatically stop.
5. Take the plate out of the holder.
6. Remove the holder by lightly pressing the Push release button.

7.4.2 384- and 96-well skirted Plates vortexing

1. Insert the appropriate plate into the plate holder by placing it at the rear of the plate holder and pressing it down until it is firmly seated. Note that misplacement of the plate will result in uneven mixing of the wells and low-quality data.
2. Set mixing speed and time according to the table above.
3. Start mixing by pressing the start/stop key. After 30 seconds, MixMate will automatically stop.
4. Take out the plate by pulling it out from the front side of the plate holder.

7.4.3 8-well strip vortexing

1. Insert the Tube Holder PCR 96 in the plate holder by holding it up against the back edge of the plate holder so that the stop pins fit in the holes.
2. Push the 8-well strip fully into the bores of the holder. Make sure that the strip sits tight and flat. Note that uneven placement of the strip will result in uneven mixing and low quality-data.
3. Set mixing speed and time according to the table above.
4. Start mixing by pressing the start/stop key. After 30 seconds, MixMate will automatically stop.
5. Gently take the 8-well strip out of the holder. The rubber on top of the holder is sticky, so make sure that the lids do not open while removing the strip.
6. Remove the holder by lightly pressing the Push release button.

7.4.4 50 mL tube vortexing

1. Insert the Tube Holder 25/50 in the plate holder by holding it up against the back edge of the plate holder so that the stop pins fit in the holes.
2. Push the 50 mL tube fully into one of the bores of the holder.
3. Set mixing speed and time according to the table above.
4. Start mixing by pressing the start/stop key. After 30 seconds, MixMate will automatically stop.
5. Take the 50 mL tube out of the holder.
6. Remove the holder by lightly pressing the Push release button.

7.5 Plate centrifugation

Common centrifuges only have the speed setting in RPM. As the centrifugation force depends on the size of the rotor, the corresponding g force will vary between different centrifuges. Contact the centrifuge vendor to get help converting RPM to g.

8.Required equipment and consumables

This chapter lists everything that is required to perform an experiment using the Olink Explore HT protein biomarker platform, excluding the Olink Explore HT Reagent Kits. Where applicable, it is clearly stated if the items shall be used in the pre-PCR or post-PCR room. This is to facilitate preparation of separate rooms.

For information about the Olink Explore HT Reagent Kits, refer to *2. Olink® Explore HT Reagent Kit contents.*

8.1 Important information

The Olink Explore HT protocol has been optimized and validated using the instruments, accessories and consumables listed in this chapter. Comparable performance is not guaranteed when using alternative instruments, accessories, or consumables. In case of support, Olink may be the initial point of contact, but for any hardware related issue, Olink refers to the support of the respective vendors.

8.2 Olink softwares

8.2.1 NPX™ Explore HT & 3072 software

NPX Explore HT & 3072 is an analysis software specifically designed for the Olink Explore HT analysis platform. It comes with an accompanying pre-processing software and is required for the generation and analysis of counts files for completed Olink Explore HT runs.

For further information, refer to the NPX™ Explore HT & 3072 User Manual.

8.2.2 NPX™ Explore CLI HT & 3072 software

NPX Explore CLI HT & 3072 is a command-line interface (cli) for the Olink Explore HT product. The application is capable of performing normalization, quality control (QC) and CV computations on NGS data and exporting the results on several supported formats.

8.3 Instruments

This section contains specifications for all instruments and accessories that are required to perform an Olink Explore HT run. Either one of the instruments marked with the same letter in the figure and the table below can be used.

For detailed instructions regarding the instruments listed in this manual, refer to the documentation provided by the applicable manufacturer.

NOTE: *All robot protocols are subject to changes and registered in different versions (Vx). Before running the experiment, make sure the latest robot protocols are installed.*

Instrument specifications

Pos	Instrument	Room	Supplier	Article number
A1	**F.A.S.T.™ Instrument, 96-channel head, transfer range 0.1–13.0 µL**	**Pre-PCR/ Post-PCR**	**Formulatrix®**	**814091A**
	F.A.S.T.™ Plate adapter for 0.2 mL PCR Strip Tubes, 96 well format.	Pre-PCR/ Post-PCR	Formulatrix®	813647B
	F.A.S.T.™ Adapter block for semi-skirted and non skirted PCR plates.	Pre-PCR/ Post-PCR	Formulatrix®	816409B
A2	**Mosquito® LV (low volume)**	**Pre-PCR**	**SPT Labtech**	**3019-0036**
	5 way Mosquito® Precise Humidity Chamber (PHC)	Pre-PCR	SPT Labtech	3210-01002
	Mosquito® Application Software	Pre-PCR	SPT Labtech	3019-06101
	Mosquito® Software licence & comms installed in PC controller	Pre-PCR	SPT Labtech	3019-0030
	Pipette loader	Pre-PCR	SPT Labtech	3019-03020
	Pipette tape spool cover	Pre-PCR	SPT Labtech	3019-04134
	Calibration Block	Pre-PCR	SPT Labtech	3019-05104
	Magnetic PCI plate clamp (x 5)	Pre-PCR	SPT Labtech	3085-01035 / 1x
B	**Dragonfly® discovery 3 head**	**Pre-PCR**	**SPT Labtech**	**3152-10006**

Pos	Instrument	Room	Supplier	Article number
C	**Proflex™ 2 x 384-well PCR System (x 2) Software version 2.0.0 or later NOTE: 2 instruments required**	**Post-PCR**	**Thermo Fisher Scientific**	**4484077 /1x**
D1	**ep*Motion*® 5075lc**	**Post-PCR**	**Eppendorf®**	**5075006019**
	CleanCap and completely contained housing	Post-PCR	Eppendorf®	5075006019
	MultiCon PC complete	Post-PCR	Eppendorf®	5075006019
	TM 50-8 eight-channel dispensing tool, 1–50 µL volume range (x 2)	Post-PCR	Eppendorf®	5280000215 / 1x NA: 960001044
	TM 10-8 eight-channel dispensing tool, 0.2–10 µL (x 2)	Post-PCR	Eppendorf®	5280000304 / 1x
	epMotion® TS 50 single-channel dispensing tool, 1–50 uL (x 2) (Optional, needed if running fully automated PCR pooling)	Post-PCR	Eppendorf®	5280000010 NA: 960001010
	Reservoir rack	Post-PCR	Eppendorf®	5075754002 NA: 9600002148
	epMotion® Rack for 24 tubes, 1.5/2.0 mL (Optional, needed if running fully automated PCR pooling)	Post-PCR	Eppendorf®	5075751275
	Thermoadapter for PCR (for temperature control of PCR plates) 384 wells skirted (x 5)	Post-PCR	Eppendorf®	5075788004 / 1x NA: 9600002202
D2	**Hamilton Microlab® STAR**	**Post-PCR**	**Hamilton**	
	8 Channels with 1000 µL Pipetting Channels	Post-PCR	Hamilton	173081
	CO-RE 96 Probehead II 1000uL	Post-PCR	Hamilton	199090
	Venus Four V4.5 Base Package	Post-PCR	Hamilton	911264-USB
	Modular Arm for 4 / 8 / 12 Ch. / MPH	Post-PCR	Hamilton	173051
	Waste Chute, MPH, Left-Side Front	Post-PCR	Hamilton	92573-01
	Tip Carrier, Landscape (x2-4)	Post-PCR	Hamilton	182085
	Tall Tip Pickup Adapter MPH96	Post-PCR	Hamilton	6608647-01
	3 SMP-CAR-32, Set of 3 carriers	Post-PCR	Hamilton	173410
	Tube Carrier Insert Eppendorf 1.5mL, Set of 3	Post-PCR	Hamilton	182238
E	**MixMate®**	**Pre- and Post-PCR**	**Eppendorf®**	**5353000510 (230V Version) 5353000529 (110V Version)**
	Tube Holder PCR 96		Eppendorf®	5353040113
	Tube Holder 25/50 mL		Eppendorf®	5353040156
F	**2100 Bioanalyzer System including chip priming station and IKA Vortex mixer**	**Post-PCR**	**Agilent**	**G2939BA**
F2	**4200 TapeStation System including IKA Vortex mixer**	**Post-PCR**	**Agilent**	**G2991BA**

Pos	Instrument	Room	Supplier	Article number
G1	**NovaSeq™ 6000**	**Post-PCR**	**Illumina**	**20012850**
G2	**NovaSeq™ X Plus**	**Post-PCR**	**Illumina**	**20084804**

8.4 Equipment and consumables: Pre-PCR

This section lists all equipment and consumables required in the pre-PCR room.

8.4.1 Sample dilution, Incubation and PCR

Equipment

Equipment	Supplier	Article number
F.A.S.T.™ instrument including accessories	For specifications, refer to *8.3 Instruments*	
Mosquito® LV including accessories	For specifications, refer to *8.3 Instruments*	
Dragonfly® discovery 3 head including accessories	For specifications, refer to *8.3 Instruments*	
Manual pipettes: • 0.5–10 µL • 10–100 µL • 20–200 µL • 100–1000 µL	Any	–
Manual multichannel pipettes (8-channel): 0.5–10 µL, 5–50 µL (Optional: adjustable)	Any	–
Plate centrifuge	Any	–
MixMate® controlled plate vortex with holders	Eppendorf®	5353000510 NA: 5353000529
Plate vortex	Any	–
Tube vortex	Any	–
Microcentrifuge (high speed not necessary)	Any	–
Pipetboy / Pipette Controller	Any	–
Cooler rack for microcentrifuge tubes	Any	–
Timer	Any	–
Freezing block	Any	–

Consumables

Consumables	Supplier	Article number
96-well PCR plate, preferably with full skirt*		–
Filter pipette tips (compatible with manual pipettes)		–
MicroAmp™ Clear Adhesive Film	Thermo Fisher Scientific	4306311 /100x
Plate sealer	Any	–
Twin.tec 384-well PCR plate (skirted)**	Eppendorf®	0030128508 /25x NA: 951020702
Sample Control (pooled plasma sample)		
Dragonfly® reservoirs	SPT Labtech	4150-07103
Dragonfly® discovery ultra low retention syringes	SPT Labtech	4150-07208 /100x
F.A.S.T.™ Disposable positive displacement pipette tips (case of 38,400)***	Formulatrix®	233590
Spool of Mosquito® pipette tips at 9 mm pitch (26,000 per spool)	SPT Labtech	4150-03030 /1x
MilliQ water	Any	–
8-well strips with lids	Any	–
50 mL Falcon tubes made of polypropylene	Any	–

* The plates must be able to withstand -80 °C, be dry-ice resistant and easily re-sealable.
** All instrument protocols have been calibrated for this specific plate. Other models should not be used.
***Either F.A.S.T. or Mosquito LV can be used.

8.5 Equipment and consumables: Post-PCR

This section lists all equipment and consumables required in the post-PCR room.

8.5.1 Pooling of PCR products

Equipment

Equipment	Supplier	Article number
2 x ProFlex™ 2 x 384-well PCR System	For specifications, refer to *8.3 Instruments*	
Formulatrix F.A.S.T.™ including accessories*	For specifications, refer to *8.3 Instruments*	
epMotion® 5075l including accessories*	For specifications, refer to *8.3 Instruments*	
Hamilton Microlab STAR® including accessories*	For specifications, refer to *8.3 Instruments*	
Manual pipettes: 10–100 µL (Optional)	Any	–
MixMate® controlled plate vortex	Eppendorf®	5353000510
Plate centrifuge	Any	–
Plate vortex	Any	–
Microcentrifuge with inserts for both tubes and 8-strip (high speed not necessary)	Any	–

* Either F.A.S.T.™, ep*Motion*® 5075lc or Hamilton Microlab STAR® can be used.

Consumables

Consumables	Supplier	Article number
MicroAmp™ Clear Adhesive Film	Thermo Fisher Scientific	4306311 /100x
Plate sealer	Any	–
Filter pipette tips (compatible with manual pipettes) (Optional)	Any	–
Microcentrifuge tubes, 1.5 mL	Any	–

F.A.S.T.™ consumables

Consumables	Supplier	Article number
F.A.S.T.™ Disposable positive displacement pipette tips (case of 38,400)*	Formulatrix®	233590

*Either F.A.S.T. or Mosquito LV can be used.

epMotion® consumables

Consumables	Supplier	Article number
ep Dualfilter T.I.P.S.® pipette tips:	Eppendorf®	951020702
• 10 µL		003 0014.391 (with boxes) /10x96 003 0014.553 (refills)
• 50 µL		003 0014.413 (with boxes) /10x96 003 0014.430 (refills)
Waste bags bio. for ep*Motion*®, up to 7 L volume	Eppendorf®	5075751763 /50x

Hamilton STAR® consumables

Consumables	Supplier	Article number
50 µL Conductive Filter Tips (Case of 5,760 tips)	Hamilton	235948

8.5.2 Library purification

Equipment

Equipment	Supplier	Article number
DynaMag™-2 Magnet (magnetic stand for Eppendorf® tubes)	Thermo Fisher Scientific	12321D
Manual pipettes: • 10–100 µL • 100–1000 µL	Any	–
Tube vortex	Any	–
Microcentrifuge (high speed not necessary)	Any	–
Timer	Any	–

Consumables

Consumables	Supplier	Article number
Agencourt AMPure XP beads	Beckman Coulter	A63880 / 5 mL
96% Ethanol	Any	–
MilliQ water	Any	–
15 mL Falcon tube	Any	–
Filter pipette tips (compatible with manual pipettes)	Any	–
Disposable serological pipettes • 5 mL • 10 mL	Any	–
Microcentrifuge tubes, 1.5 mL	Any	–

8.5.3 Quality control of Olink® libraries

Equipment

Equipment	Supplier	Article number
2100 Bioanalyzer System including accessories	For specifications, refer to *8.3 Instruments*	
4200 TapeStation System including accessories*	For specifications, refer to *8.3 Instruments*	
Microcentrifuge (> 13000 x g needed for 2100 Bioanalyzer)	Any	–
Tube vortex	Any	–
Manual pipettes: • 0.5–10 µL • 10–100 µL • 100–1000 µL	Any	–

* Either 2100 Bioanalyzer System or 4200 TapeStation System can be used.

2100 Bioanalyzer consumables

Consumables	Supplier	Article number
Agilent High Sensitivity DNA Kit (includes reagents and 10 chips)	Agilent	5067–4626 /10 chips
Microcentrifuge tubes, 1.5 mL	Any	–
Filter pipette tips (compatible with manual pipettes)	Any	–
MilliQ water	Any	–

4200 TapeStation consumables

Consumables	Supplier	Article number
High Sensitivity D5000 Reagents	Agilent	5067-5593
High Sensitivity D5000 ScreenTape	Agilent	5067-5592
Loading Tips, 1 Pk	Agilent	5067-5598
Optical tube strip caps (8x Strip)	Agilent	401425
Optical tube strips (8x Strip)	Agilent	401428

8.5.4 Next generation sequencing using NovaSeq™ 6000

Equipment

Equipment	Supplier	Article number
NovaSeq™ 6000 including accessories	For specifications, refer to *8.3 Instruments*	
Tube vortex	Any	–
Microcentrifuge (high speed not necessary)	Any	–
Manual pipettes: • 0.5–10 µL • 10–100 µL • 20–200 µL • 100–1000 µL	Any	–
Laboratory bottle 1 L	Any	–
Pipetboy/Pipette Controller	Any	–

Flow cell specific consumables

Flow cell specific consumables		Supplier	Article number
S4	NovaSeq™ 6000 S4 Reagent Kit v1.5 (35 cycles)	Illumina	20044417/ 1x

Other consumables

Consumables	Supplier	Article number
Sodium Hypochlorite (NaOCl), 5 % active chlorine, Acros Organics	Thermo Fisher Scientific	419552500/ 250 mL
Tween®20	Sigma-Aldrich	P7949/ 100 mL
Sodium Hydroxide (NaOH), 1.0 N	Sigma-Aldrich	S2770/ 100 mL
Trizma® hydrochloride solution, 1 M (pH 8.0)	Sigma-Aldrich	T2694/ 100 mL
Low linting Wipes (ex. KIMTECH SCIENCE® KIMWIPES™)	Any	–
Used buffer cartridge	Illumina	–
Cluster wash cartridges	Illumina	–
SBS wash cartridges	Illumina	–
MilliQ water, large volumes	Any	–
Filter pipette tips (compatible with manual pipettes)	Any	–
Wash Flow cell or S4 flow cells	Illumina	20016005/ 1x
Microcentrifuge tubes, 1.5 mL	Any	–
Disposable serological pipettes, 5 mL	Any	–

For assistance, consult a statistician or contact Olink Support before running the study.

- The 3 replicates of sample control are optional.
- Consult the Olink white paper "Strategies for design of protein biomarker studies" and the sample randomization guidelines. For links, refer to *3. Associated documentation and resources.*

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

8.5.5 Next generation sequencing using NovaSeq™ X Plus

Equipment

Equipment	Supplier	Article number
NovaSeq™ X Plus including accessories	For specifications, refer to *8.3 Instruments*	
Tube vortex	Any	–
Microcentrifuge (high speed not necessary)	Any	–
Manual pipettes: • 0.5–10 µL • 10–100 µL • 20–200 µL • 100–1000 µL	Any	–

Flow cell specific consumables

Flow cell specific consumables	Supplier	Article number
NovaSeq™ X Series 10B Reagent Kit (100 cycles)	Illumina	20085596

Other consumables

Consumables	Supplier	Article number
Sodium Hypochlorite (NaOCl), 5 % active chlorine, Acros Organics	Thermo Fisher Scientific	419552500/ 250 mL
Sodium Hydroxide (NaOH), 1.0 N	Sigma-Aldrich	S2770/ 100 mL
Contec Polynit Heatseal wipes	VWR	68310-176
MilliQ water, large volumes	Any	–
Filter pipette tips (compatible with manual pipettes)	Any	–
Microcentrifuge tubes, 1.5 mL	Any	–
Reagent or spectrophotometric-grade isopropyl alcohol (70%), 100 ml bottle	Any	–

Part 2: Laboratory instructions using SPT Labtech Mosquito®

9.Preparations

9.1 Plan the study

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

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

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

9.2 Important information

Reagent lots

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

384-well PCR plates

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

Vortexing

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

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

9.3 Prepare the samples

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

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

Prepare bench

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

Before you start

- Select the samples to be included in the study.

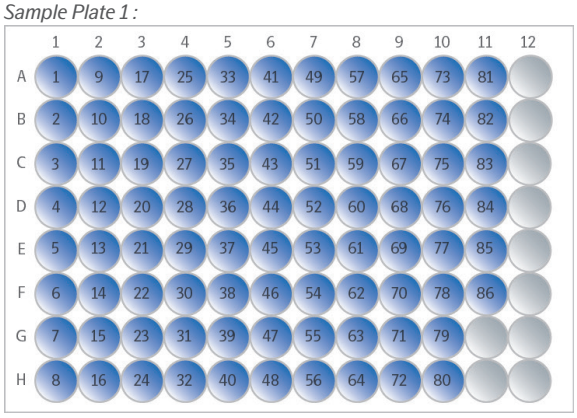
IMPORTANT: *The samples must be undiluted, as dilution of samples may result in lower detectability.*

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

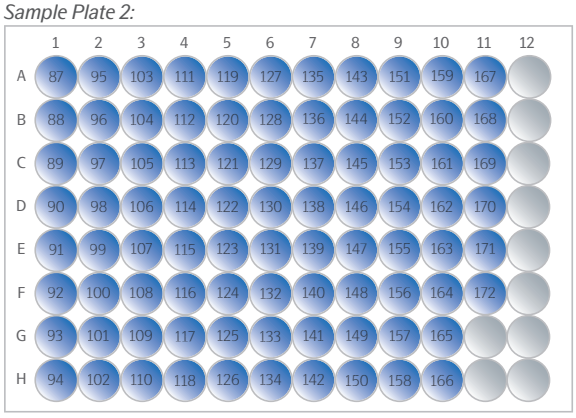
Instructions

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

Sample Plate 1 :



Sample Plate 2:



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

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

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

10. Prepare Sample Source Plate

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

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

Prepare bench

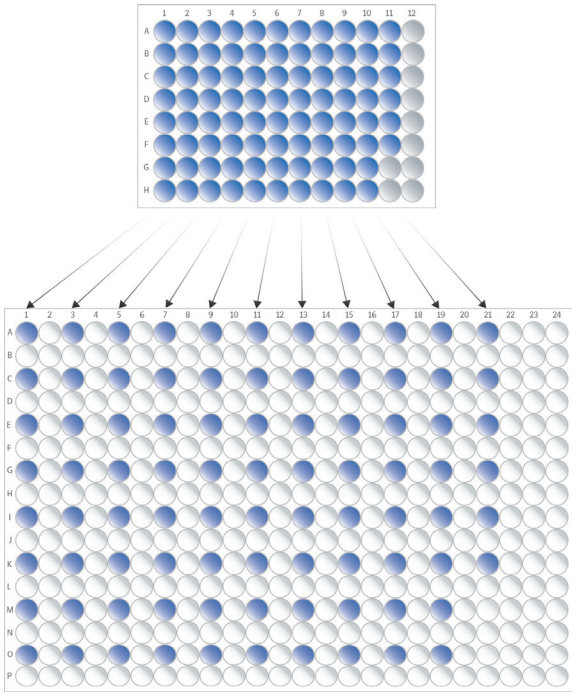
- Sample Plates, prepared in previous step
- Olink® Explore Negative Control
- Olink® Explore Plate Control
- Olink® Explore Sample Control (optional)
- 1x 384-well PCR Plate (skirted)
- 1x 8-well PCR strip
- Manual pipette (10 µL)
- Manual multichannel pipette (10 µL)
- Filter pipette tips
- Adhesive films

Before you start

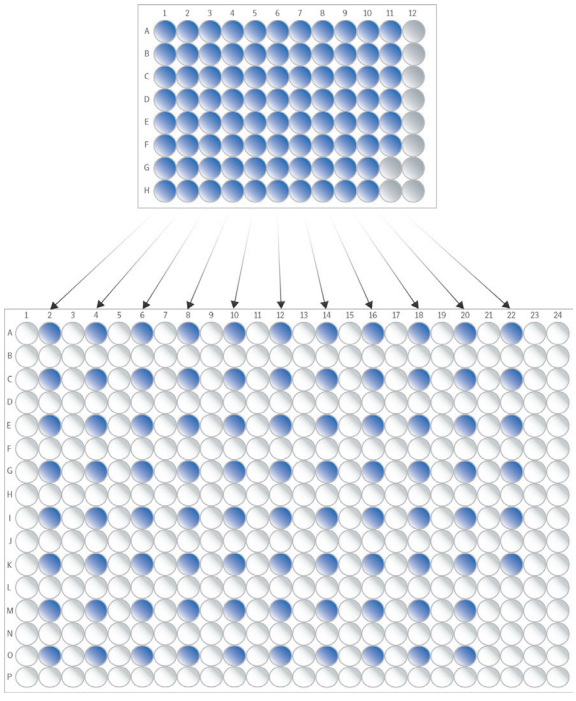
- Ensure that the Sample Plates have been prepared according to section *9.3 Prepare the samples*.
- If frozen, thaw the Sample Plates at room temperature.
- Thaw the Negative Control, Plate Control and Sample Control at room temperature.

Instructions

1. Vortex the 96-well Sample Plates and spin at 400–1000 x g for 1 minute at room temperature.
2. Using a multichannel pipette, transfer 10 µL from Sample Plate 1 to odd numbered columns and rows starting at 1A. Use **forward pipetting** and change pipette tips between every column. Make sure that no air bubbles are trapped at the bottom of the wells.

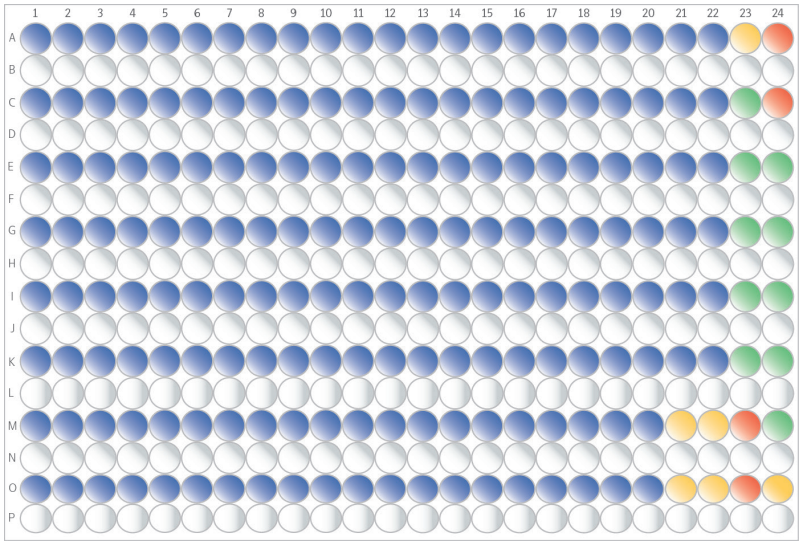


3. Visually check that the sample has been transferred to the correct wells.
4. Using a multichannel pipette, transfer 10 µL from Sample Plate 2 to even numbered columns and rows starting at 2B. Use **forward pipetting** and change pipette tips between every column. Make sure that no air bubbles are trapped at the bottom of the wells.



5. Vortex the Negative Control, Plate Control, and Sample Control and spin briefly.
6. Transfer 10 ul of Sample Control (SC) to the wells M21, M22, O21 and O22 (see figure below) of Sample Source Plate.
7. Using a single-channel pipette, transfer 26 ul of Sample Control, Plate Control and Negative Control to each well of a PCR strip, according to the figure below. Change pipette tip between each control sample.

8. Using an multichannel pipette, transfer 10 ul of the control samples from the PCR strip to each of the applicable wells in column 23 of the Sample Source Plate according to the figure below.
9. Flip around the PCR strip to get the mirror positioning of controls.
10. Change the tips in the multichannel pipette, and transfer 10 ul of the control samples from the PCR strip to each of the applicable wells in column 24 of the Sample Source Plate according to the figure below.
11. Seal the Sample Source Plate with a new adhesive film. Spin the Sample Source Plate at 400–1000 x g for 1 minute. Make sure that there are no bubbles trapped at the bottom of the wells. Store the plate at +4 °C until use (the same day).
12. Seal the Sample Plates using adhesive film and store at -80 ºC.



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

11.Sample Dilution

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

11.1 Prepare Sample Dilution Plates

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

Prepare bench

- Olink® Target 96/Explore Sample Diluent
- 2x 384-well PCR Plates (skirted)
- Manual pipette (1000 µL)
- 1x Disposable reservoir (10 mL)
- 1x Disposable ultra-low retention syringe with plunger
- Filter tips
- Adhesive films

Before you start

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

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

Instructions part 1:

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

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

Instructions part 2:

1. Spin the Sample Dilution Plates at 400–1000 x g for 1 minute.
2. Visually inspect the Sample Dilution Plates to ensure that all wells contain the expected volumes. Make sure that there are no bubbles trapped at the bottom of the wells.
3. Clear the instrument and shut it down according to manufacturers instructions.
4. Continue to *11.2 Perform Sample Dilution* or store the Sample Dilution Plates at +4 ºC until use (the same day).

11.2 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 Plates 1 and 2 (prepared in previous step)
- Adhesive films

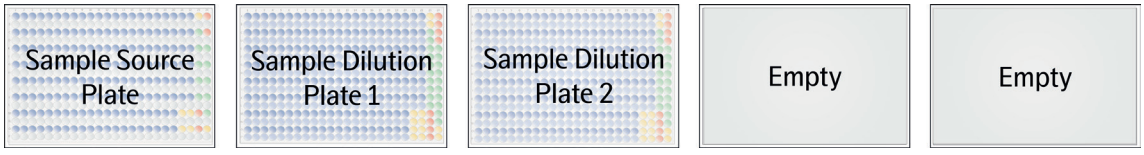
Before you start

- Prepare the Mosquito according to manufacturer's instructions.
- Make sure that the reservoirs for the Mosquito active humidity chambers have sufficient water in them.

Instructions

> **IMPORTANT:** *Make sure to vortex the Sample Dilution Plate(s) thoroughly with correct MixMate settings, both between dilution and after the last dilution, as incorrect settings may lead to low-quality data. Refer to table in 9.2 Important information.*

1. Let the Sample Source Plate reach room temperature, vortex and spin at 400–1000 x g for 1 minute at room temperature. Make sure that there are no bubbles trapped at the bottom of the wells.
2. Carefully remove the adhesive films from the Sample Source Plate and the Sample Dilution Plates.
3. Place the Sample Source Plate and the Sample Dilution Plates in magnetic clamp boosters and place the plates on the Mosquito deck according to the picture below:

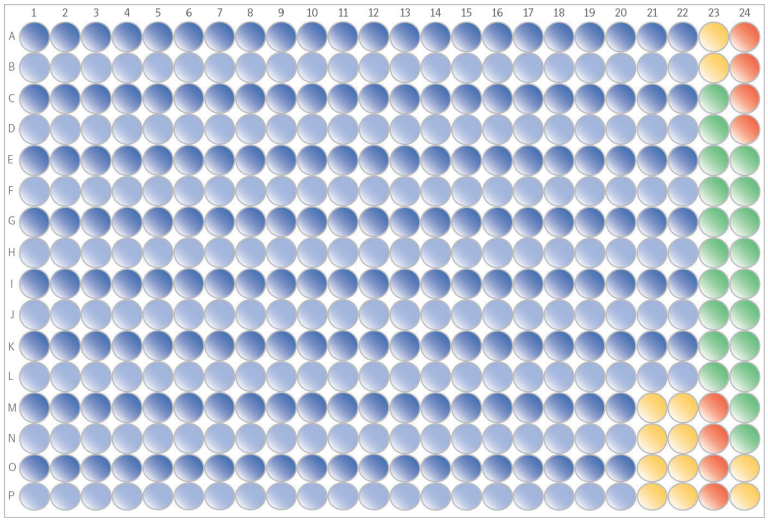


4. Press Run to start the Mosquito program `1_Olink_ExploreHT_SampleDilution`.
5. Between each dilution step, the program will pause, and a pop-up window will appear. When the protocol is paused:
   a. Remove the Sample Dilution Plate from the instrument and magnetic clamp booster as instructed in the pop-up.
   b. Seal the plate with new adhesive film.
   c. Vortex the plate after each transfer with the MixMate at 3000 rpm for 30 seconds, no adaptor needed.
   d. Spin at 400–1000 x g for 1 minute. Make sure that no air bubbles are trapped at the bottom of the wells.
   e. Remove the adhesive film.
   f. Place the plate in the magnetic clamp booster and return it to the correct position in the instrument.
   g. Press Resume to continue the program. The run takes approximately 24 minutes.

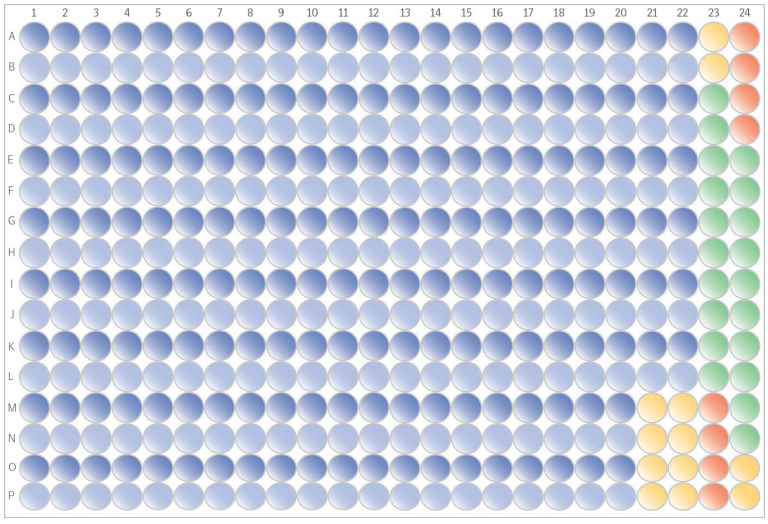
> **IMPORTANT:** *Risk of instrument damage! If the magnetic clamp booster is not correctly placed it may collide with the internal parts of the instrument during the run.*

Result:

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



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



1. Once the protocol is finished, press Resume to end the program. Keep the Mosquito on for later use.
2. Continue to *12. Incubation*, or place the Sample Dilution Plate at +4 ºC for up to 3 hours.

12.Incubation

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

12.1 Prepare Reagent Source Plate

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

Prepare bench

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

Before you start

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

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

Instructions

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

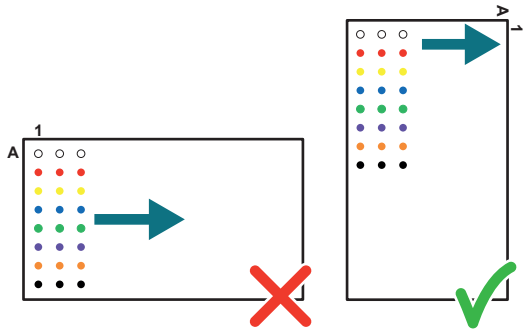
Addition order	Reagent	Volume (µL)
1	Incubation solution	160
2	Frw probes	20
3	Rev probes	20

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

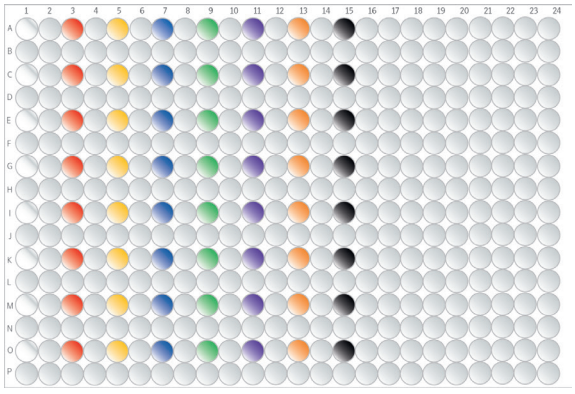
3. Seal the PCR strip with caps, vortex with the MixMate at 2000 rpm for 30 seconds (use the Tube Holder PCR 96 to accommodate the PCR strip) and spin down. Store at room temperature until use.
4. Inspect the wells to make sure that they contain the expected volume of 200 µL.

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

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



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



7. Seal the Reagent Source Plate with a new adhesive film and spin at 400–1000 x g for 1 minute. Make sure that no air bubbles are trapped at the bottom of the wells.
8. Immediately continue to *12.2 Perform incubation*.

12.2 Perform incubation

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

Prepare bench

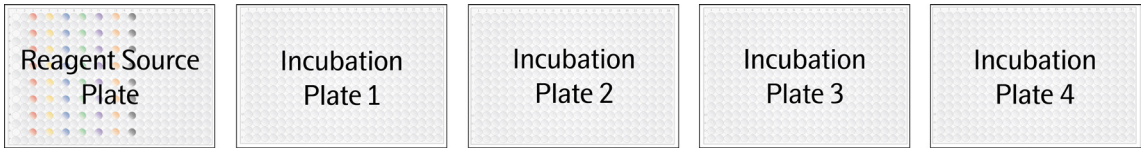
- Sample Source Plate, prepared in previous step
- Sample Dilution Plate 1 and 2, prepared in previous step
- Reagent Source Plate, prepared in previous step
- 4x 384-well PCR plates
- Adhesive films

Before you start

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

Instructions part 1: Transfer incubation mix

1. In the Mosquito software, select File/Open and choose the `2_Olink_ExploreHT_Incubation_ReagentTransfer` protocol from the Load Protocol Window.
2. Carefully remove the adhesive film from the Reagent Source Plate.
3. Place the Reagent Source Plate and the four Incubation Plates in magnetic clamp booster and place them on the Mosquito deck according to the picture below:



4. Press Run.
   Result:
   The Mosquito transfers 0.6 µL of Incubation Mix from the Reagent Source Plate to the four Incubation Plates. Incubation mixes for two blocks will be transferred into each Incubation Plate:
   a. *Plate 1: Block 1 and 2*
   b. *Plate 2: Block 3 and 4*
   c. *Plate 3: Block 5 and 6*
   d. *Plate 4: Block 7 and 8*

   The run takes approximately 19 minutes.
5. Once the protocol is finished, remove the Reagent Source Plate from the Mosquito and magnetic clamp booster.
6. Remove the Incubations Plates 1–4 from the Mosquito and magnetic clamp boosters and visually inspect that there is liquid at the bottom of all wells and that no bubbles are present.

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

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

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

NOTE: *If bubbles are present, seal the Incubation Plate(s) with new adhesive film(s) and spin at 400–1000 x g for 1 minute, then carefully remove the adhesive film(s) and check again. Do not vortex. If bubbles are still present, puncture them using a syringe needle. Change needles between wells to avoid cross-well contamination.*

Instructions part 2: Transfer samples to block 1–4

1. In the Mosquito software, select File/Open and choose the `3_Olink_ExploreHT_Incubation_SampleTransfer_Block_1-4` protocol from the Load Protocol Window.
2. Spin down the Sample Source Plate at 400–1000 x g for 1 minute. Make sure that there are no bubbles trapped at the bottom of the wells and remove the adhesive seal.
3. Place the Sample Source Plate and Incubation Plate 1 and 2 in magnetic clamp boosters on the Mosquito deck according to the picture below:

4. Press Run.
   Result:
   The Mosquito will transfer 0.2 µL of undiluted samples from the Sample Source Plate to the Incubation Plate 1 and 2. The run takes approximately 15 minutes.
5. Once the protocol is finished, remove Incubation Plate 1 and 2 from the deck and magnetic booster plates and seal them with new adhesive film. Spin down the plates at 400–1000 x g for 1 minute.

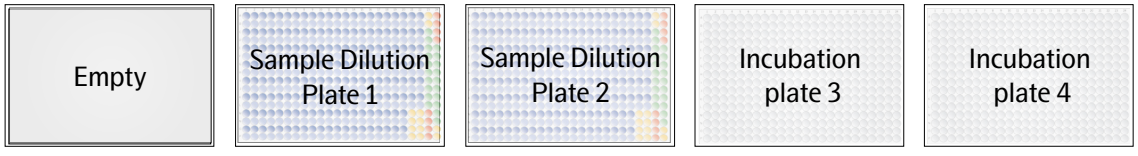
IMPORTANT: *All wells must be properly sealed to avoid evaporation of the samples*

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

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

Instructions part 3: Transfer samples to block 5–8

1. In the Mosquito Software, Select File/Open and choose the `4_Olink_ExploreHT_Incubation_SampleTransfer_Block_5-8` protocol from the Load Protocol Window.
2. Spin down Sample Dilution Plate 1 and 2 at 400–1000 x g for 1 minute and remove the adhesive film.
3. Place Sample Dilution Plate 1 and 2 and Incubation Plate 3 and 4 in magnetic clamp boosters on the Mosquito deck, according to the picture below:



4. Press Run.
   Result:
   The Mosquito will transfer 0.2 µL of diluted samples from Sample Dilution Plate 1 and 2 into the Incubation mixes on Incubation Plate 3 and 4. The run takes approximately 16 minutes.
5. Once the protocol is finished, remove Incubation Plate 3 and 4 from the deck and magnetic booster plates and seal them with new adhesive film. Spin down the plates at 400–1000 x g for 1 minute.

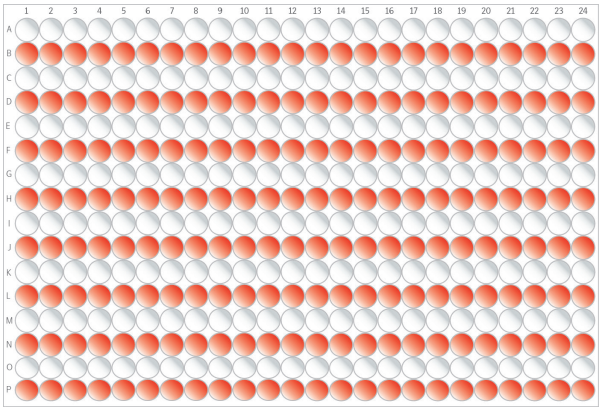
IMPORTANT: *All wells must be properly sealed to avoid evaporation of the samples*

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

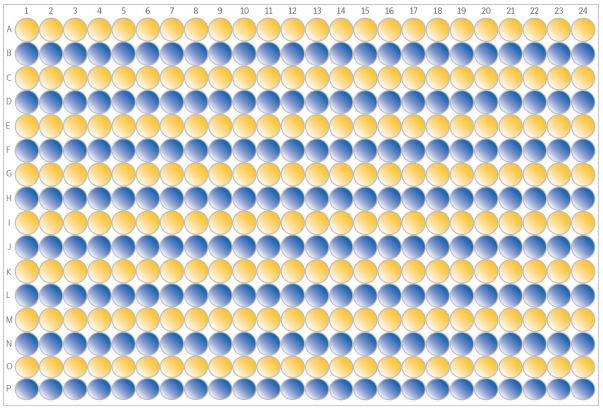
6. Incubate Incubation Plate 3 and 4 for 16–24 hours at +4 °C.
7. Remove Sample Dilution Plate 1 and 2 from the deck and magnetic booster plates and discard them.

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

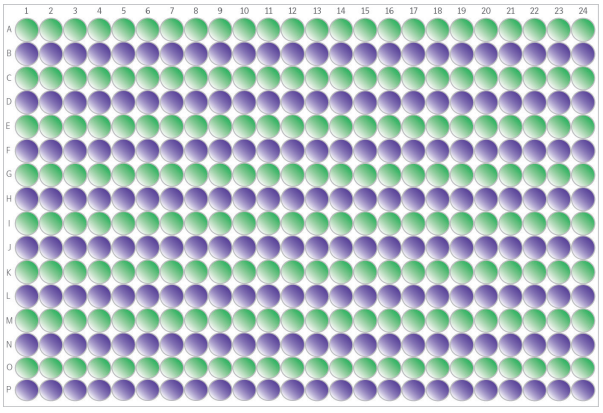
Incubation Plate 1



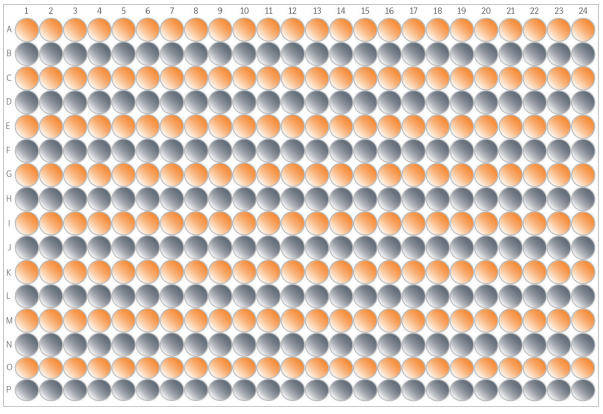
Incubation Plate 2



Incubation Plate 3



Incubation Plate 4



13.Prepare for day 2

Instructions

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

14. Index and PCR setup

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

14.1 Index dispensation

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

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

Prepare bench

- Incubation Plate 1–4, prepared in previous step
- Olink Explore HT Index Plate A and B

Before you start

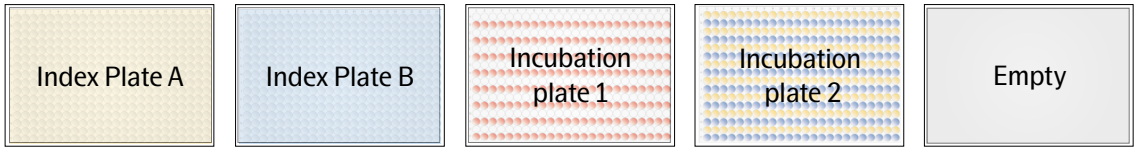
- Thaw Index Plate A and B at room temperature.
- Allow Incubation Plate 1–4 to reach room temperature.

Instructions

1. In the Mosquito software, select File/Open and choose the `5_Olink_ExploreHT_IndexTransfer` protocol from the Load Protocol Window.

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

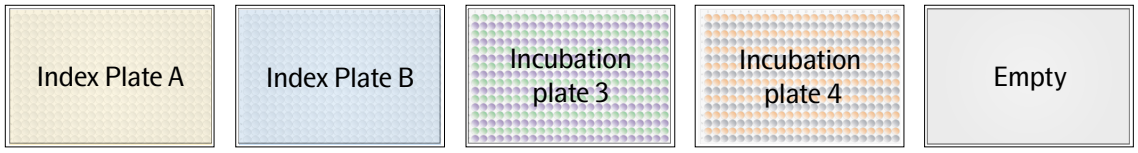
2. Vortex Index Plate A and B with the MixMate at 3000 rpm for 30 seconds, no adapter needed, and spin down at 400–1000 x g for 30 seconds.
3. Spin down Incubation Plate 1 and 2 at 400–1000 x g for 1 minute.
4. Place the two Index Plates in magnetic booster plates on the Mosquito deck, according to the picture in step 5.
5. Place Incubation Plate 1 and 2 in magnetic booster plates on the Mosquito deck, according to:



6. Press Run.
   Result:
   The Mosquito will transfer 1 µL of index primer from Index Plate A and B into Incubation Plate 1 and 2.
   The run takes approximately 18 minutes.
7. Once the protocol is finished, remove Incubation Plate 1 and 2 from the deck and magnetic booster plates and seal with new adhesive films.
8. Spin Incubation Plate 1 and 2 with indexes at 400–1000 x g for 1 minute.
9. Visually inspect the Incubation Plates to ensure that all wells contain the expected volumes. Make sure that there are no bubbles trapped at the bottom of the wells.

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

10. Keep Incubation Plates 1 and 2 at room temperature until further use (maximum 1 hour).
11. Keep Index Plate A and B on the deck and immediately proceed to the next step.
12. Repeat step 5–9 with Incubation Plates 3 and 4. Place the plates in the Mosquito according to the picture below:



13. Remove Index plate A and B from the deck and discard them.
14. Immediately proceed to PCR mix preparation and dispensation.

14.2 Prepare PCR Mix

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

Prepare bench

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

Before you start

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

Instructions

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

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

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

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

4. Pre-mix the PCR Mix by inverting the Falcon tube twice. Vortex with the MixMate at 1000 rpm for 30 seconds using the Tube Holder 25/50 mL adaptor.
5. Keep the PCR Mix at room temperature until use.

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

14.3 Prepare PCR Plates and perform PCR

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

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

Perform the instructions in the following order:

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

Prepare bench

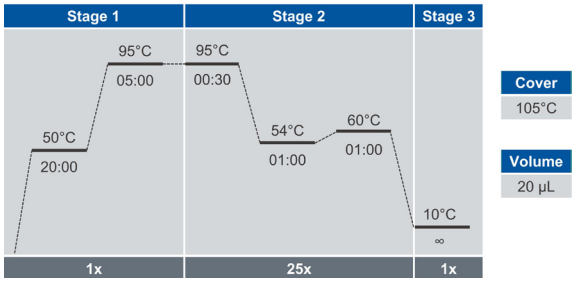
- Incubation Plates 1–4 with indices, prepared in previous step
- PCR Mix, prepared in previous step
- 2x Timer
- 3x Disposable reservoirs (10 mL)
- 3x Disposable ultra-low retention syringes with plungers
- Manual pipette (5 or 10 mL)
- Filter pipette tips
- Adhesive films

Before you start

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

Instructions part 1: Prepare PCR plates

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



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

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

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

7. Select the Run tab in the Constant layer view of the software, then press RUN to start the program. Start the timer when PCR Mix is added to the first well of the plate.
   Result:
   Dragonfly dispenses 18 µL of PCR Mix into each well of PCR Plate 1.
8. When the Dragonfly has returned PCR Plate 1 to the plate position and released the plate clamp, remove the plate from the instrument and immediately seal it with a new adhesive film.
9. Using the MixMate, vortex the plate at 3000 rpm for 30 seconds without adaptor to ensure that all wells are mixed.
10. Repeat steps 6–9 for PCR Plate 2.

Instructions part 2: Perform PCR

11. In the Post-PCR room, centrifuge PCR Plate 1 and 2 at 400–1000 x g for 1 minute.
12. Inspect PCR Plates 1 and 2 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 PCR Plates 1 and 2 in one of the two pre-heated ProFlex instruments and click Resume to run the program.
14. Repeat step 6–13 for Incubation Plates 3 and 4. Place both plates in the second ProFlex.
15. Once the protocol is finished, clear the Dragonfly and shut it down according to manufacturer's instructions.
16. Once the PCR program is finished (2h), continue to *15. Pool PCR products using Hamilton Microlab® STAR* or *16. Pool PCR products using epMotion®*, depending on which instrument will be used, or store the PCR Plates at +4 ºC if used the same day.

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

15.Pool PCR products using Hamilton Microlab® STAR

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

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

Prepare bench

- PCR Plate 1–4, prepared in previous step
- 1x 96-well PCR plate (skirted)
- 8x Eppendorf tubes (1.5 mL)
- Hamilton STAR® pipette tips (5 racks of 50 µL filtered conductive Hamilton Tips)
- 1x Tube Carrier (for automatic pooling)
- 8x Tube Carrier Inserts for Eppendorf 1.5 mL (for automatic pooling)
- Waste bag
- Manual pipette (100 µL)
- Filter pipette tips
- Adhesive films

Before you start

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

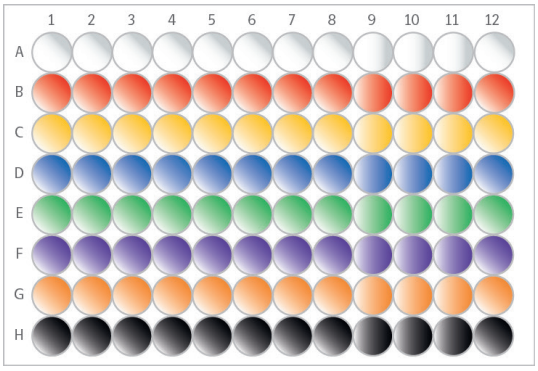
Instructions: Automatic pooling

1. Open the Hamilton Run Control Application.
2. In the application, select the protocol `Olink_ExploreHT_PCRPooling`.
3. Click the green Start button at the top to initialize the instrument and run the protocol.
4. Make sure that PCR1 Plates 1–4 are properly sealed, then vortex the plates with the MixMate at 3000 rpm for 30 seconds without adaptor, and spin down at 400–1000 x g for 1 minute.
5. Inspect the wells of PCR Plate 1–4 to make sure that no liquid has evaporated and that there are no bubbles in the wells. Remove the seals.
6. Pull out the carriers to the load position and prepare the Hamilton STAR deck according to the software instructions.
   - Place Plates on the carriers.
   - Place tubes in the tube rack carrier.
   - Carefully remove the adhesive films.
   - Fill tip carriers in the selected positions.
7. Select the correct input for the four source plates and click Continue.
8. Select the populated tip positions and click OK to automatically load the carriers and begin the run.
   Result:
   - *Hamilton Microlab STAR will automatically scan tips, pool 4 µL from each well of the PCR plates into the PCR Pooling Plate, keeping blocks separate in different rows. It will pause to allow for mixing, then pool subsequent rows to tubes. 20 µLfrom each well will be transferred to 1 Eppendorf tube per block.*
9. Once the protocol is finished, remove the PCR Pooling Plate from the deck. Inspect the PCR Pooling Plate to ensure that every applicable well contains the same amount of liquid. Discard the PCR Pooling Plate.
10. Remove PCR Plate 1–4 from the deck. Seal the plates with a new adhesive film and store at -20 ºC for potential reruns.
11. Close the Hamilton Run Control software and shut down the Hamilton STAR.
12. Vortex PCR Tubes 1–8 and spin down briefly.
13. Continue to *18. Library purification* or store the PCR Tubes at +4 °C until use (the same day).

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

Instructions: Manual pooling

1. Open the Hamilton Run Control Application.
2. In the application, select the protocol `Olink_ExploreHT_PCRPooling`.
3. Click the green Start button at the top to initialize the instrument and run the protocol.
4. Make sure that PCR1 Plates 1–4 are properly sealed, then vortex the plates with the MixMate at 3000 rpm for 30 seconds without adaptor, and spin down at 400–1000 x g for 1 minute.
5. Inspect the wells of PCR Plate 1–4 to make sure that no liquid has evaporated and that there are no bubbles in the wells. Remove the seals.
6. Pull out the carriers to the load position and prepare the STAR deck according to the software instructions.
   - Place Plates on the carriers.
   - Carefully remove the adhesive films.
   - Fill tip carriers in the selected positions.
7. Select the correct input for the four source plates and click Continue.
8. Select the populated tip positions and click OK to automatically load the carriers and begin the run.
   Result:
   Hamilton STAR will automatically scan tips, pool 4 µL from each well of the PCR plates into the PCR Pooling Plate, keeping blocks separate in different rows.



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

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

12. Vortex PCR Tubes 1–8 and spin down briefly.
13. Remove PCR Plate 1–4 from the deck. Seal the plates with a new adhesive film and store at -20 ºC for potential reruns.
14. Close the Hamilton Run Control software and shut down the Hamilton STAR.
15. Continue to *18. Library purification* or store the PCR Tubes at +4 ˚C until use (the same day).

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

16. Pool PCR products using ep*Motion*®

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

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

Prepare bench

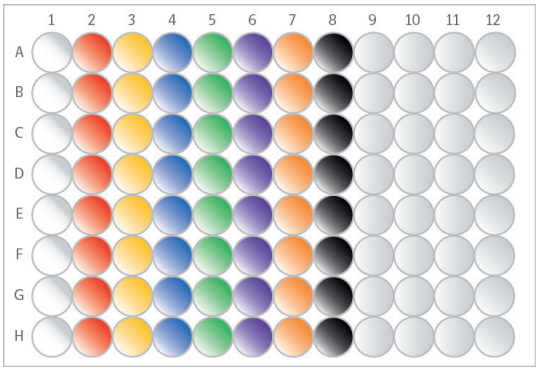
- PCR Plate 1–4, prepared in previous step
- 1x 96-well PCR plate (skirted)
- 8x Eppendorf tubes (1.5 mL)
- epT.I.P.S.® Motion pipette tips (2boxes á 10 µL))
- epT.I.P.S.® Motion pipette tips (50 µL)
- TS 50 single-channel dispensing tool (for automatic pooling)
- Manual pipette (100 µL or 200 µL) (for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

Before you start

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

Instructions: Automatic pooling

1. Open the EpBlue Application Runner.
2. In the application library, select user and the protocol `Olink_ExploreHT_PCRPooling`.
3. When the ID number of the instrument is shown in the software, click Next to continue.
4. Make sure that PCR1 Plates 1–4 are properly sealed, then vortex the plates with the MixMate at 3000 rpm for 30 seconds without adaptor, and spin down at 400–1000 x g for 1 minute.
5. Inspect the wells of PCR Plate 1–4 to make sure that no liquid has evaporated and that there are no bubbles in the wells. Remove the seals.
6. Prepare the ep*Motion* worktable according to the software instructions.
7. Click Next in the software, then enter the following settings:
   - Under Volume settings: Activate Input volumes manually.
   - Under Worktable settings: Deactivate Check tube lid removed.
8. Click Next until a Run button appears, then click Run to start the protocol.
   Result:
   - *epMotion will pool 3 µL from each well of the same row in each PCR plate into a single column of the PCR Pooling Plate, keeping blocks separate in different columns.*
   - *30 uL from each well will be transferred to 1 Eppendorf tube.*

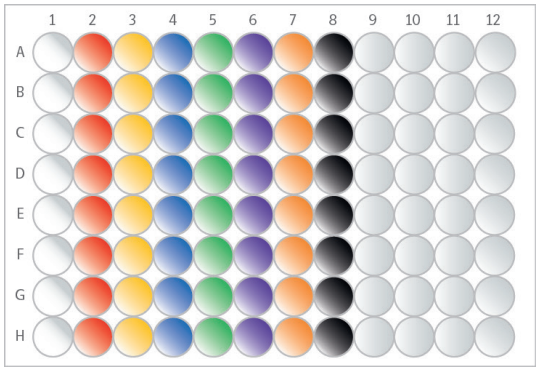


9. Once the protocol is finished, remove the PCR Pooling Plate from the worktable. Inspect the PCR Pooling Plate to ensure that every applicable well contains the same amount of liquid. Discard the PCR Pooling Plate.
10. Remove PCR Plate 1–4 from the worktable. Seal the plates with a new adhesive film and store at -20 ºC for potential reruns.
11. Clear the ep*Motion* and shut it down.
12. Vortex PCR Tubes 1–8 and spin down briefly.
13. Continue to *18. Library purification* or store the PCR Tubes at +4 °C until use (the same day).

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

Instructions: Manual pooling

1. Open the EpBlue Application Runner.
2. In the application library, select user and the protocol `Olink_ExploreHT_PCRPooling_manual`.
3. When the ID number of the instrument is shown in the software, click Next to continue.
4. Make sure that PCR1 Plates 1–4 are properly sealed, then vortex the plates with the MixMate at 3000 rpm for 30 seconds without adaptor, and spin down at 400–1000 x g for 1 minute.
5. Inspect the wells of PCR Plate 1–4 to make sure that no liquid has evaporated and that there are no bubbles in the wells. Remove the seals.
6. Prepare the ep*Motion* worktable according to the software instructions.
7. Click Next in the software, then enter the following settings:
   - Under Volume settings: Activate Input volumes manually.
   - Under Worktable settings: Deactivate Check tube lid removed.
8. Click Next until a Run button appears, then click Run to start the protocol.
   Result:
   epMotion will pool 3 µL from each well of the same row in each PCR plate into a single column of the PCR Pooling Plate, keeping blocks separate in different columns.



9. Remove the PCR Pooling Plate from the worktable. Seal the plate with a new adhesive film, vortex the plate and spin down at 400 x g for 1 minute. Inspect the PCR Pooling Plate to ensure that every applicable well contains the same amount of liquid.
10. Carefully remove the adhesive film from the PCR Pooling Plate. Make sure that every applicable well contain the same amount of liquid.
11. Manually transfer the pooled PCR products from the PCR2 Pooling Plate to the microcentrifuge tube(s) according to the table below. Use a single-channel pipette and **forward pipetting,** and change pipette tip after each well.

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

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

14. Remove PCR Plate 1–4 from the worktable. Seal the plates with a new adhesive film and store at -20 ºC for potential reruns.
15. Clear the ep*Motion* and shut it down.
16. Vortex PCR Tubes 1–8 and spin down briefly.
17. Continue to *18. Library purification* or store the PCR Tubes at +4 °C until use (the same day).

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

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

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

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

Prepare bench

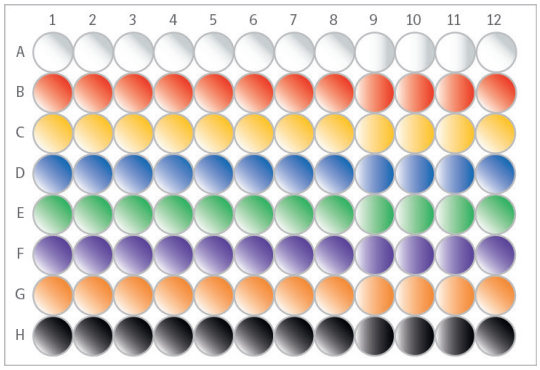
- PCR Plates 1–4, prepared in previous step
- 1x 96-well PCR plate (skirted)
- 1x 8-well strip tube
- 1x 8-well strip tube cap
- 8x Eppendorf tubes (1.5 mL)
- 104x F.A.S.T.™ Disposable Pipette Tips
- F.A.S.T.™ Plate adapter for 0.2 mL PCR Strip Tubes, 96 well format.
- Waste bag (for semi-manual pooling)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

Before you start

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

Instructions: Automatic pooling

1. Make sure that PCR1 Plates 1–4 are properly sealed, then vortex the plates with the MixMate at 3000 rpm for 30 seconds without adaptor, and spin down at 400–1000 x g for 1 minute.
2. Inspect the wells of PCR Plate 1–4 to make sure that no liquid has evaporated and that there are no bubbles in the wells. Remove the seals.
3. Open the protocol `Olink_ExploreHT_Pooling.`
4. Arrange the F.A.S.T. deck according to the layout in the protocol.
   - Place PCR1 Plates 1-4 on the tray.
   - Place PCR Pooling Plate on the tray.
   - Remove lid and place tips on the tray.
5. Click START to begin the run.
   Result:
   - *F.A.S.T. pools 3 µL from each well of the same block into one row of the 96-well plate, each block in a different row.*

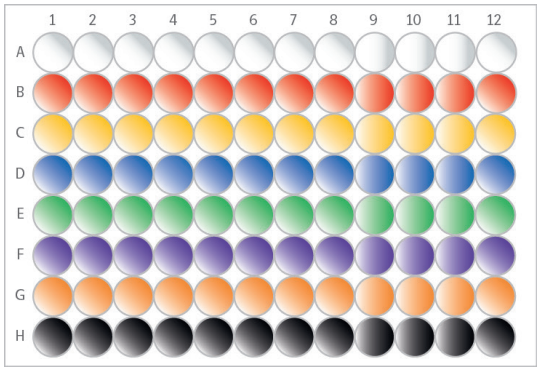


6. Once the protocol is finished, remove the PCR Pooling Plate from the deck. Seal the plate with a new adhesive film, vortex the plate and spin down at 400 x g for 1 minute. Inspect the PCR Pooling Plate to ensure that every applicable well contains the same amount of liquid.
7. Remove PCR Plate 1–4 from the deck. Seal the plates with a new adhesive film and store at -20 ºC for potential reruns.
8. Open the protocol `Olink_ExploreHT_TubePooling`.
9. Arrange the F.A.S.T. deck according to the layout in the protocol.
   - Remove seal from PCR Pooling Plate and place on the tray.
   - Place 8-well strip tube in column 1 of the strip tube adapter and place on the tray.
   - Remove lid and place tips on the tray.
10. Click START to begin the run.
    Result:
    - *The F.A.S.T. pools 11 µL from each well of the same row of the PCR Pooling Plate into a single well of the PCR 1-8 strip tube, each block in a different well.*
11. Cap and vortex the PCR 1–8 strip tube and spin down briefly. Transfer each pooled library to its corresponding 1.5 mL microcentrifuge tube.
12. Clear the F.A.S.T. and shut it down.
13. Continue to *18. Library purification* or store the PCR Tubes at +4 °C until use (the same day).

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

Instructions: semi-manual pooling

1. Make sure that PCR1 Plates 1–4 are properly sealed, then vortex the plates with the MixMate at 3000 rpm for 30 seconds without adaptor, and spin down at 400–1000 x g for 1 minute.
2. Inspect the wells of PCR Plate 1–4 to make sure that no liquid has evaporated and that there are no bubbles in the wells. Remove the seals.
3. Open the protocol `Olink_ExploreHT_PCRPooling`.
4. Arrange the F.A.S.T. deck according to the layout in the protocol.
   - Place PCR1 Plates 1–4 on the tray.
   - Place PCR Pooling Plate on the tray.
   - Remove lid and place tips on the tray.
5. Click START to begin the run.
   Result:
   - *F.A.S.T. pools 3 µL from each well of the same block into one row of the 96-well plate, each block in a different row.*



6. Once the protocol is finished, remove the PCR Pooling Plate from the deck. Seal the plate with a new adhesive film, vortex the plate and spin down at 400 x g for 1 minute. Inspect the PCR Pooling Plate to ensure that every applicable well contains the same amount of liquid.
7. Remove PCR Plates 1–4 from the deck. Seal the plates with new adhesive films and store at -20 °C for potential reruns.
8. Clear the F.A.S.T. and shut it down.
9. Carefully remove the adhesive film from the PCR Pooling Plate. Make sure that every well contains the same amount of liquid.
10. Manually pipette PCR products from the PCR Pooling Plate to microcentrifuge tubes according to the table below. Use a single-channel pipette and **forward pipetting**, and change pipette tips after each well.

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

11. Vortex the tubes and spin down briefly.
12. Continue to *18. Library purification* or store the PCR Tubes at +4 °C until use (the same day).

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

18. Library purification

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

Prepare bench

- PCR Tubes 1–8, prepared in previous step
- Agencourt AMPure XP bottle
- 96% Ethanol (EtOH)
- MilliQ water
- DynaMag™-2 Magnet
- Timer
- 16x Microcentrifuge tubes (1.5 mL)
- 1x Falcon tube (15 mL)
- Manual pipettes (100 µL, 1000 µL, and 5000 µL)
- Filter pipette tips

Before you start

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

Instructions

1. Prepare fresh 70% EtOH:

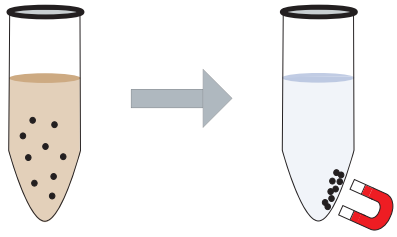
Addition order	Reagent	Volume (mL)
1	MilliQ water	2.5
2	96% EtOH	6.5
	Total (70% EtOH)	9

2. Shake and vortex the Agencourt AMPure XP bottle vigorously to resuspend the magnetic beads.
3. Transfer 80 µL from the Agencourt AMPure XP bottle to each BP tube.
4. Transfer 50 µL from each PCR Tube to the corresponding BP Tube:

Volume (µL)	From tube	To tube
50	PCR 1	BP 1
50	PCR 2	BP 2
50	PCR 3	BP 3
50	PCR 4	BP 4
50	PCR 5	BP 5
50	PCR 6	BP 6
50	PCR 7	BP 7
50	PCR 8	BP 8

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

5. Pipette-mix 10 times to thoroughly mix the Libraries with the beads. Change pipette tip between every tube.
6. Start the timer after the last tube has been mixed and incubate the BP Tubes 1–8 for 5 minutes at room temperature.
7. After the incubation, place the Eppendorf tube on the DynaMag-2 Magnetic stand for 2 min to separate beads from solution:



8. With the tubes still on the magnetic stand, carefully open the lids and discard 125 µL supernatant and leave 5 µL behind. Use a single-channel pipette. Do not disturb the beads.

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

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

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

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

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

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

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
45	BP 5	Lib 5
45	BP 6	Lib 6
45	BP 7	Lib 7
45	BP 8	Lib 8

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

16. Discard BP Tubes 1–8.
17. Continue to *19. Quality control*.

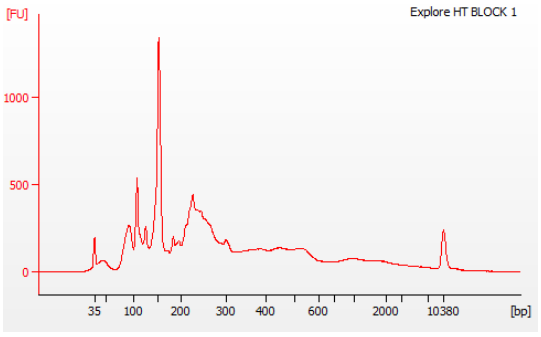
SAFE STOPPING POINT: *The Lib Tubes can be stored at -20 °C for up to 4 weeks.*

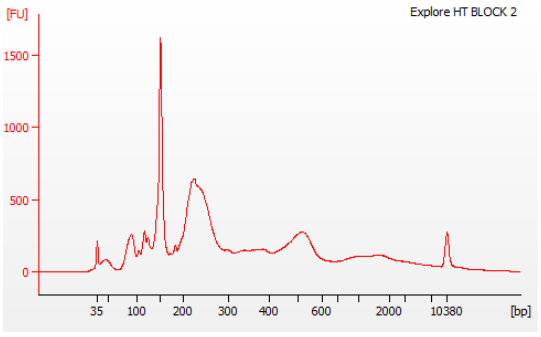
19.Quality control

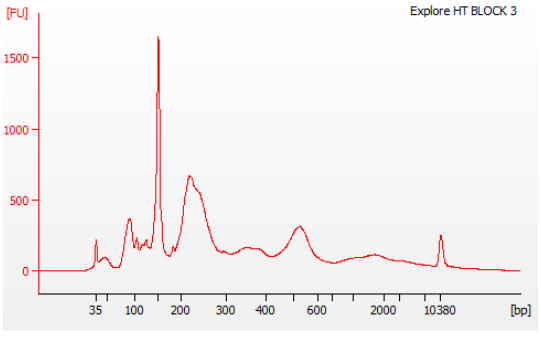
During this step, the eight purified Olink Libraries are quality controlled on the Bioanalyzer or the TapeStation using the High Sensitivity DNA kit according to manufacturer instructions.

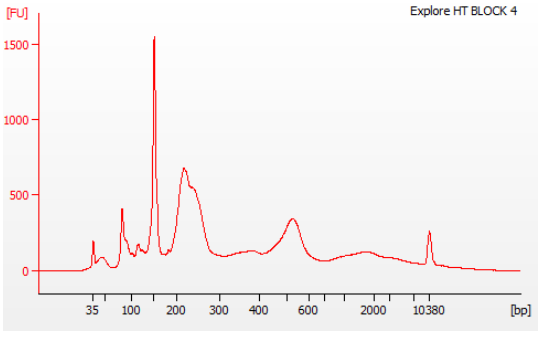
The electropherograms below display typical results for Olink Explore HT Library, one electropherogram per block. The purified Olink Library appears as a single or double peak around 150 bp. Peaks of larger sizes represents bubble products and do not have any impact on sequencing results, see emea.support.illumina.com/bulletins/2019/10/bubble-products-in-sequenc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

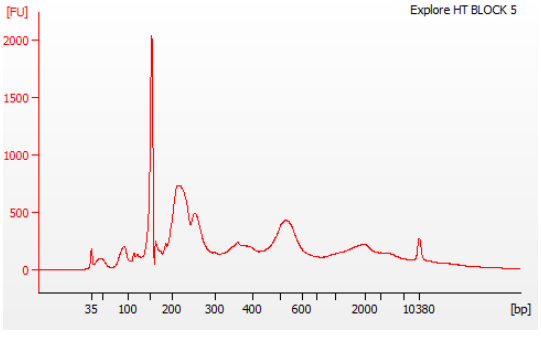
Electropherograms Bioanalyzer

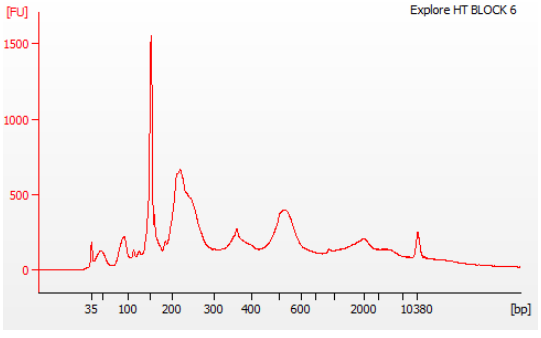




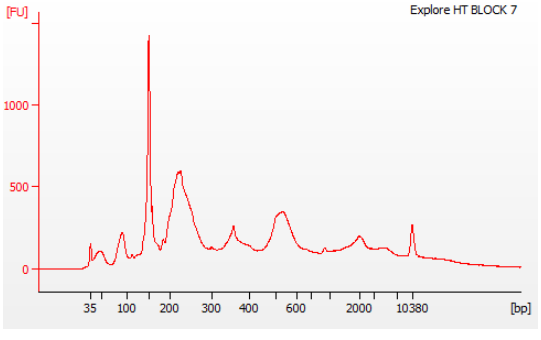


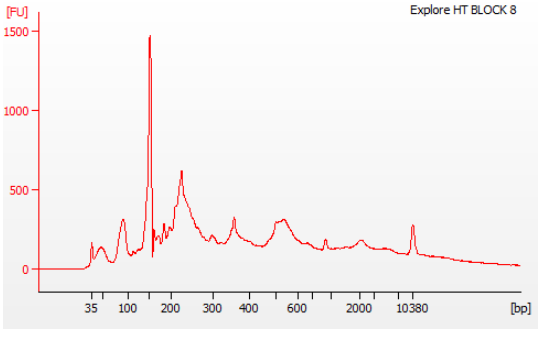




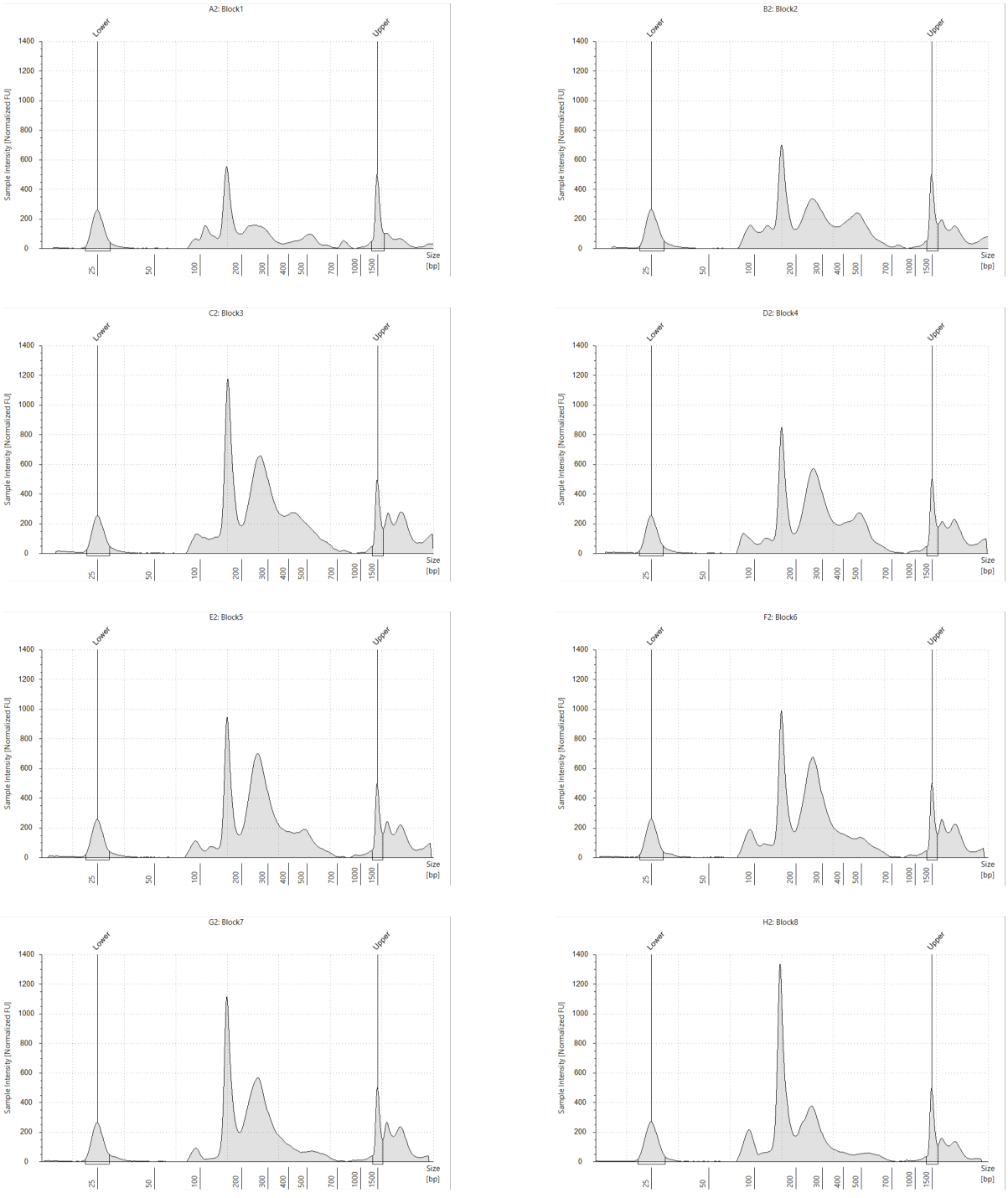


vvvv





Electropherograms TapeStation



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

20. Library pooling

Before you start

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

Instructions

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

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

21. Next generation sequencing

Next generation sequencing is performed using either the Illumina® NovaSeq™ 6000 or NovaSeq™ X Plus instrument. Refer to the following user manuals for instructions on how to sequence Olink Explore HT Libraries:

- Olink® Explore Sequencing using NovaSeq™ 6000 S4 User Manual
- Olink® Explore Sequencing using NovaSeq™ X Plus User Manual

Part 3: Laboratory instructions using Formulatrix F.A.S.T.™

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

22.Preparations

22.1 Plan the study

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

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

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

22.2 Important information

Reagent lots

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

384-well PCR plates

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

Vortexing

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

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

22.3 Prepare the samples

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

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

Prepare bench

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

Before you start

- Select the samples to be included in the study.

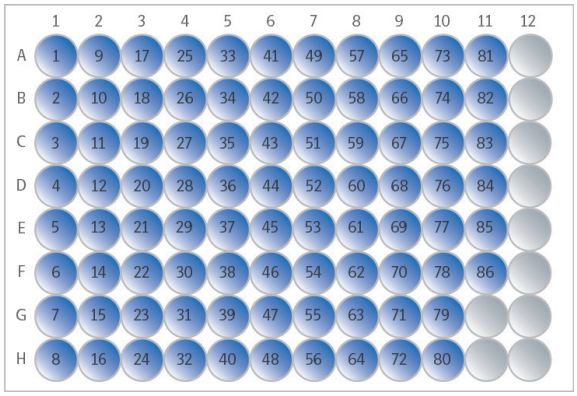
IMPORTANT: *The samples must be undiluted, as dilution of samples may result in lower detectability.*

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

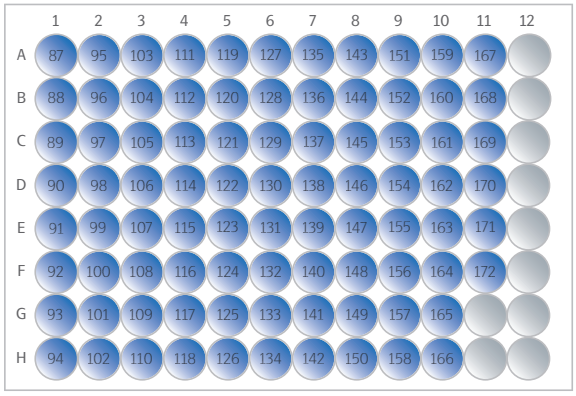
Instructions

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

Sample Plate 1



Sample Plate 2



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

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

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

23.Prepare Sample Source Plate

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

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

Prepare bench

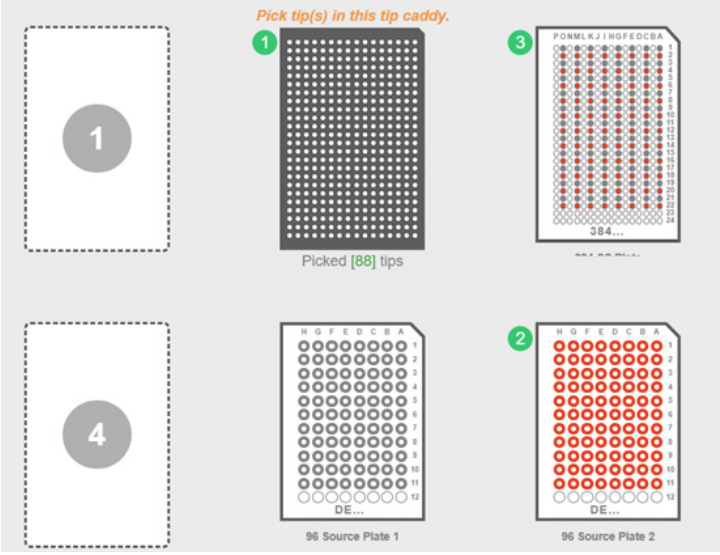
- Sample Plates, prepared in previous step
- Olink® Explore Negative Control
- Olink® Explore Plate Control
- Olink® Explore Sample Control (optional)
- 1x 384-well PCR Plate (skirted)
- 1x 8-well PCR strip
- Manual pipette (10 µL)
- Filter pipette tips
- F.A.S.T.™
- 1x box of Formulatrix F.A.S.T.™ positive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

Before you start

- Ensure that the Sample Plates have been prepared according to section *22.3 Prepare the samples.*
- If frozen, thaw the Sample Plates at room temperature.
- Thaw the Negative Control, Plate Control and Sample Control at room temperature.

Instructions

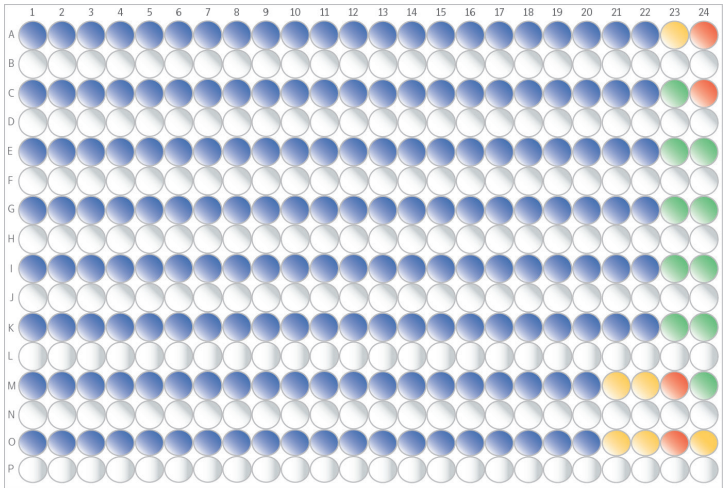
1. Vortex the 96-well Sample Plates and spin at 400–1000 x g for 1 minute at room temperature.
2. Turn on the F.A.S.T. and open the web application interface.
3. Open the protocol `0_Olink_ExploreHT_SS_Plate_Creation` and load the tray according to the diagram



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

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

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



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

24.Sample Dilution

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

24.1 Prepare Sample Dilution Plates

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

Prepare bench

- Olink® Target 96/Explore Sample Diluent
- 2x 384-well PCR Plates (skirted)
- Manual pipette (1000 µL)
- 1x Disposable reservoir (10 mL)
- 1x Disposable ultra-low retention syringe with plunger
- Filter tips
- Adhesive films

Before you start

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

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

Instructions part 1:

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

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

Instructions part 2:

1. Spin the Sample Dilution Plates at 400–1000 x g for 1 minute.
2. Visually inspect the Sample Dilution Plates to ensure that all wells contain the expected volumes. Make sure that there are no bubbles trapped at the bottom of the wells.
3. Clear the instrument and shut it down according to manufacturer's instructions.
4. Continue to *24.2 Perform Sample Dilution* or store the Sample Dilution Plates at +4 ºC until use (the same day).

24.2 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 Plates 1 and 2 (prepared in previous step)
- 1x box of Formulatrix F.A.S.T.™ positive displacement tips (13 µl)
- Adhesive films

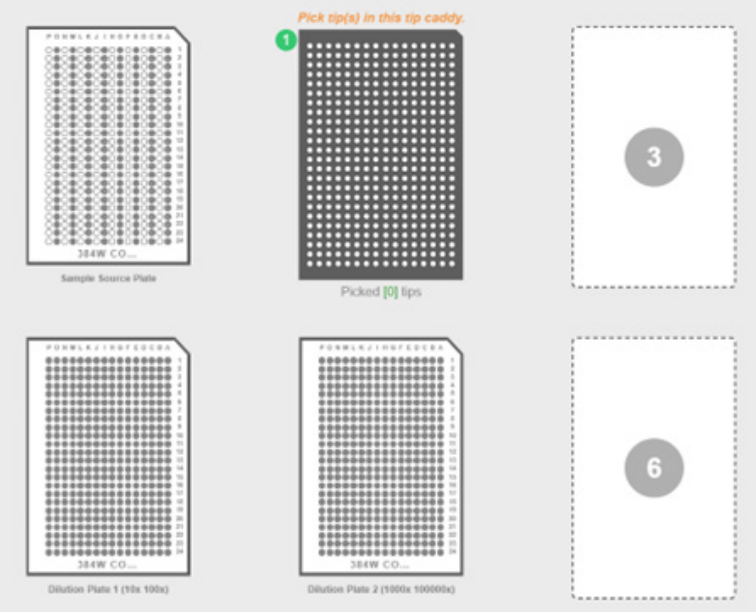
Before you start

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

Instructions

IMPORTANT: *Make sure to vortex the Sample Dilution Plate(s) thoroughly with correct MixMate settings both between dilution and after the last dilution, as incorrect settings may lead to low-quality data. Refer to table in 22.2 Important information.*

1. Let the Sample Source Plate reach room temperature, vortex and spin at 400–1000 x g for 1 minute at room temperature. Make sure that there are no bubbles trapped at the bottom of the wells.
2. Carefully remove the adhesive films from the Sample Source Plate and the Sample Dilution Plates.
3. Open the Protocol `1_Olink_ExploreHT_SampleDilution` and load the tray according to the picture.



4. Press START to run the protocol.
5. Between each dilution step, the program will pause, and a pop-up window will appear. When the protocol is paused:
   a. Remove the Sample Dilution Plate from the instrument as instructed in the pop-up.
   b. Seal the plate with new adhesive film.
   c. Vortex the plate after each transfer with the MixMate at 3000 rpm for 30 seconds, no adaptor needed.
   d. Spin at 400–1000 x g for 1 minute. Make sure that no air bubbles are trapped at the bottom of the wells.

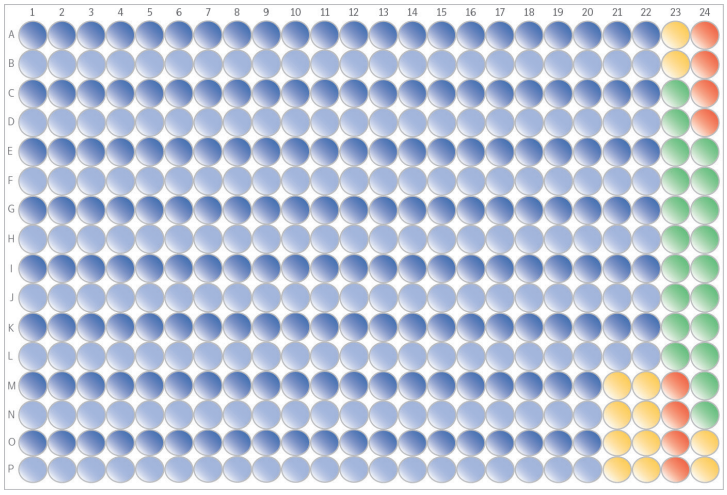
e. Remove the adhesive film.

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

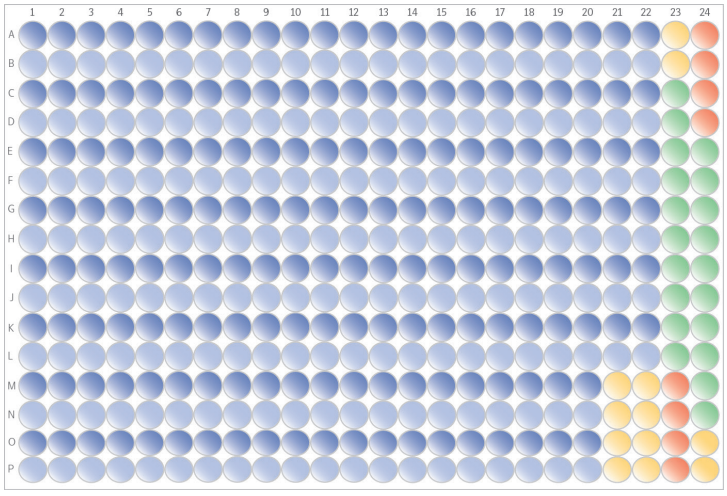
g. Press Resume to continue the program.

Result:

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



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



6. Once the protocol is finished, keep the F.A.S.T. on for later use.
7. Continue to *25. Incubation*, or place the Sample Dilution Plate at +4 ºC for up to 3 hours.

25.Incubation

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

25.1 Prepare Reagent Source Plate

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

Prepare bench

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

Before you start

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

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

Instructions

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

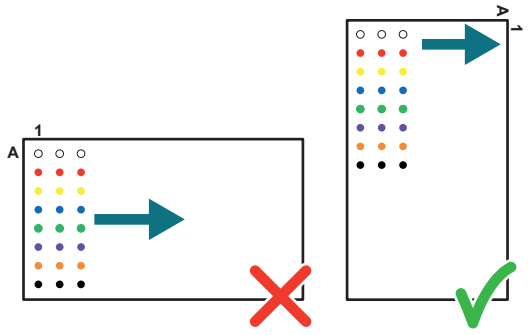
Addition order	Reagent	Volume (µL)
1	Incubation solution	160
2	Frw probes	20
3	Rev probes	20

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

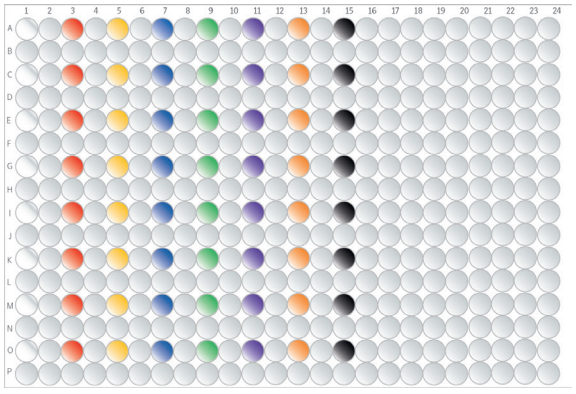
3. Seal the PCR strip with caps, vortex with the MixMate at 2000 rpm for 30 seconds (use the Tube Holder PCR 96 to accommodate the PCR strip) and spin down. Store at room temperature until use.
4. Inspect the wells to make sure that they contain the expected volume of 200 µL.

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

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



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



7. Seal the Reagent Source Plate with a new adhesive film and spin at 400–1000 x g for 1 minute. Make sure that no air bubbles are trapped at the bottom of the wells.
8. Immediately continue to *25.2 Perform incubation*.

25.2 Perform incubation

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

Prepare bench

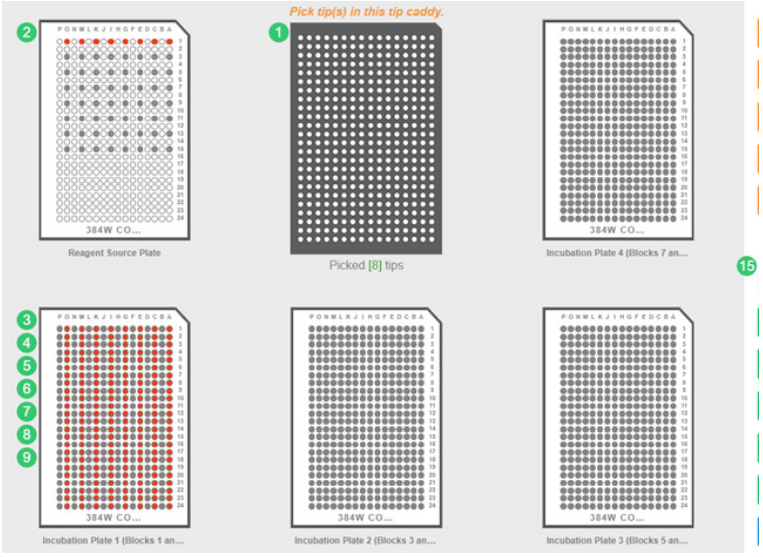
- Sample Source Plate, prepared in previous step
- Sample Dilution Plate 1 and 2, prepared in previous step
- Reagent Source Plate, prepared in previous step
- 4x boxes + 128 tips of Formulatrix F.A.S.T.™ positive displacement tips (13 µl)
- 4x 384-well PCR plates
- Adhesive films

Before you start

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

Instructions part 1: Transfer incubation mix

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



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

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

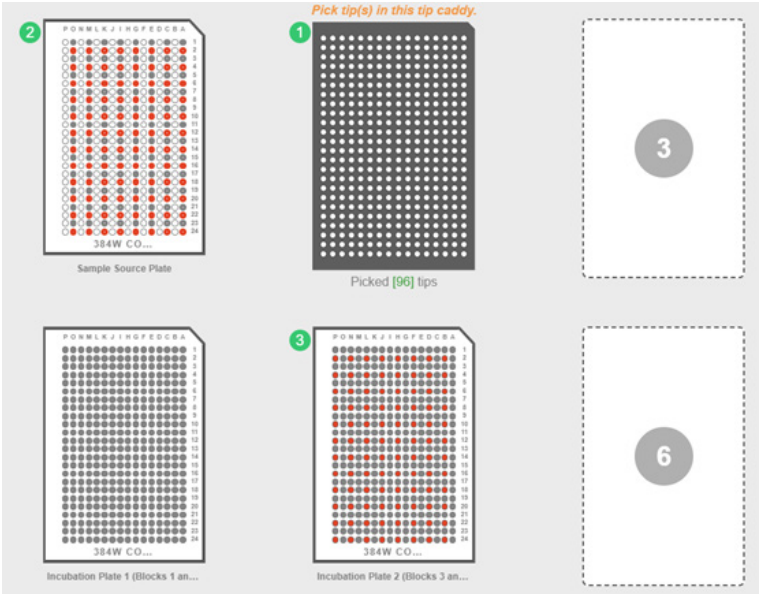
7. Discard the Reagent Source Plate.
8. Seal Incubation Plate 3 and 4 and keep them on the bench.

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

NOTE: *If bubbles are present, seal the Incubation Plate(s) with new adhesive film(s) and spin at 400–1000 x g for 1 minute, then carefully remove the adhesive film(s) and check again. Do not vortex. If bubbles are still present, puncture them using a syringe needle. Change needles between wells to avoid cross-well contamination.*

Instructions part 2: Transfer samples to block 1–4

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



4. Press Start.
   Result:
   The F.A.S.T. will transfer 0.3 µL of undiluted samples from the Sample Source Plate to the Incubation Plate 1 and 2.
5. Once the protocol is finished, remove Incubation Plate 1 and 2 from the deck and seal them with new adhesive film. Spin down the plates at 400–1000 x g for 1 minute.

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

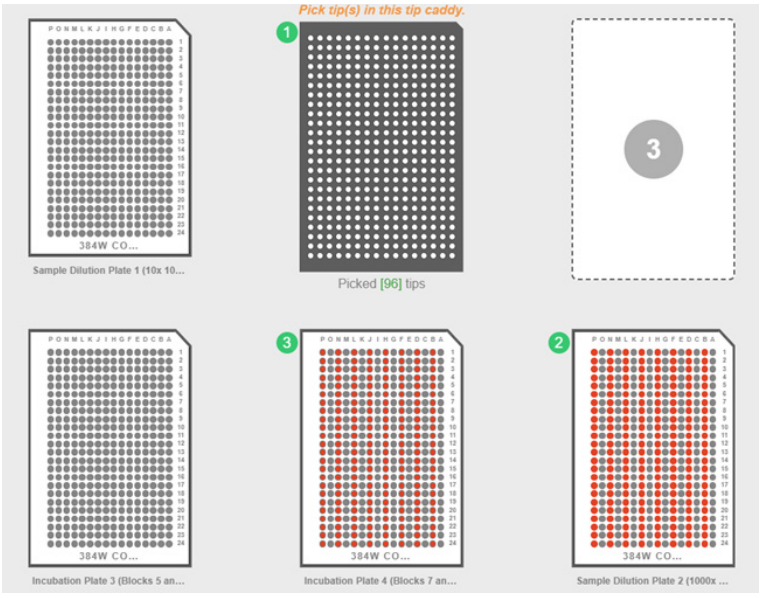
IMPORTANT: *It is critical to not vortex the Incubation Plates.*

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

Instructions part 3: Transfer samples to block 5–8

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

1. In the F.A.S.T. Software, Select the `4_Olink_ExploreHT_Incubation_SampleTransfer_Block_5-8` protocol from the Protocol Explorer.
2. Spin down Sample Dilution Plate 1 and 2 at 400–1000 x g for 1 minute and remove the adhesive film.
3. Place Sample Dilution Plate 1 and 2 and Incubation Plate 3 and 4 on the F.A.S.T. deck, according to the picture below:



4. Press START.
   Result:
   The F.A.S.T. *will transfer 0.3 µL of diluted samples from Sample Dilution Plate 1 and 2 into the Incubation mixes on Incubation Plate 3 and 4.*
5. Once the protocol is finished, remove Incubation Plate 3 and 4 from the deck and seal them with new adhesive film. Spin down the plates at 400–1000 x g for 1 minute.

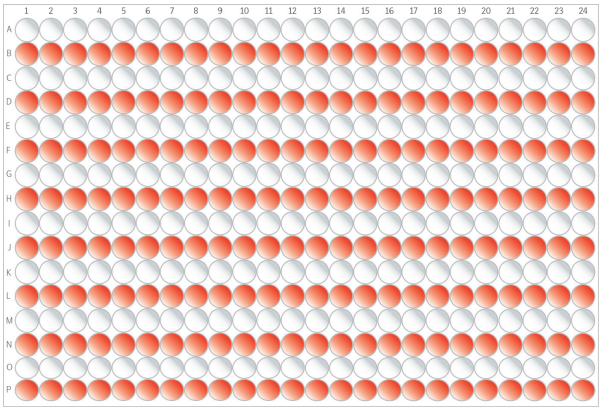
IMPORTANT: *All wells must be properly sealed to avoid evaporation of the samples.*

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

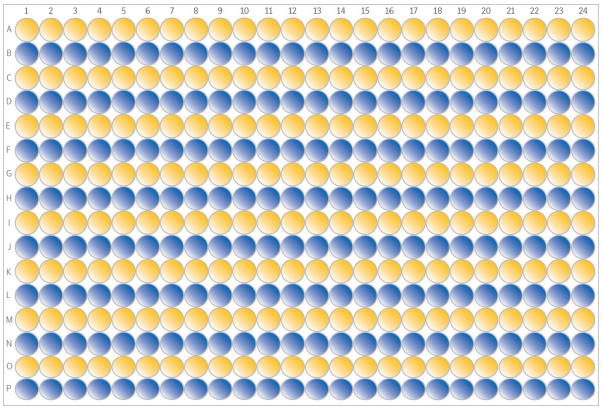
6. Incubate Incubation Plate 3 and 4 for 16–24 hours at +4 °C.
7. Remove Sample Dilution Plate 1 and 2 from the deck and discard them.

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

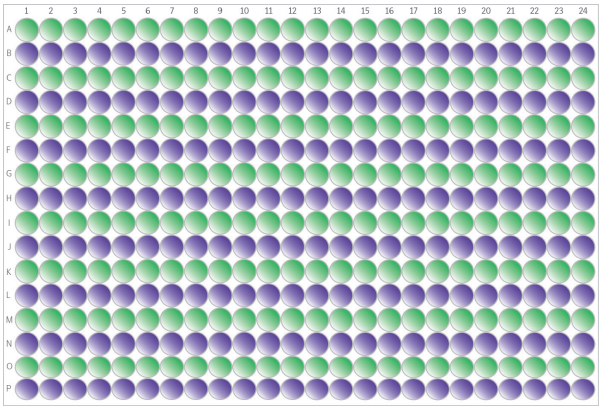
Incubation Plate 1



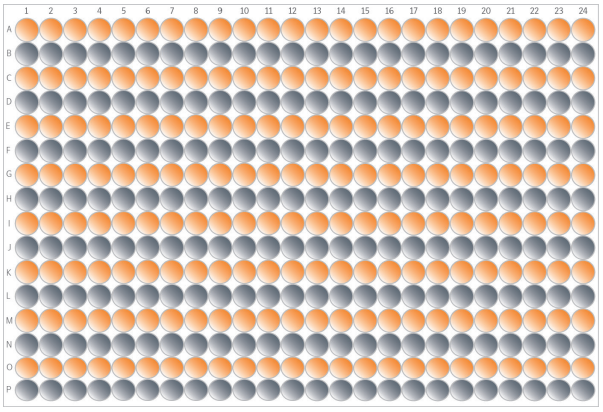
Incubation Plate 2



Incubation Plate 3



Incubation Plate 4



26.Prepare for day 2

Instructions

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

27.Index and PCR setup

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

27.1 Index dispensation

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

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

Prepare bench

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

Before you start

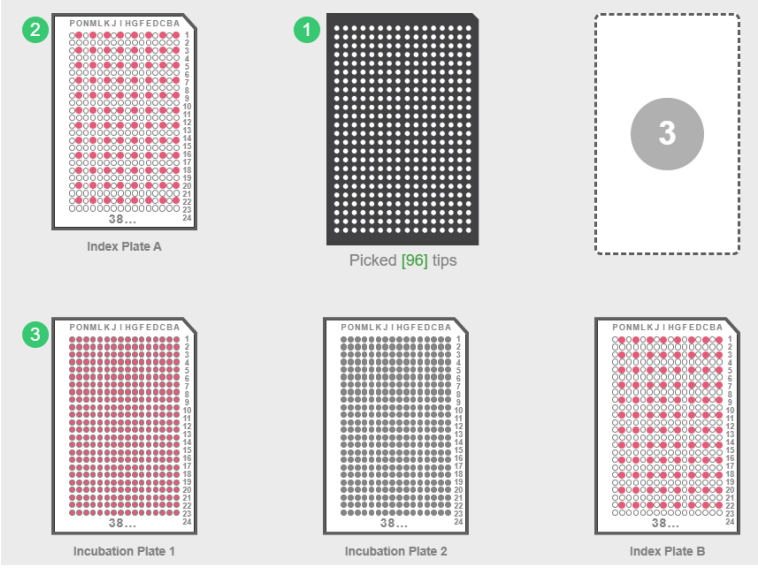
- Thaw Index Plate A and B at room temperature.
- Allow Incubation Plate 1–4 to reach room temperature.

Instructions

1. In the F.A.S.T.™ software, select the `5_Olink_ExploreHT_IndexTransfer` protocol from the Protocol Explorer.

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

2. Vortex Index Plate A and B with the MixMate at 3000 rpm for 30 seconds, no adapter needed, and spin down at 400–1000 x g for 30 seconds.
3. Spin down Incubation Plate 1 and 2 at 400–1000 x g for 1 minute.
4. Place the two Index Plates and Incubation Plate 1 and 2 in the F.A.S.T. tray according to picture below:



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

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

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

27.2 Prepare PCR Mix

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

Prepare bench

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

Before you start

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

Instructions

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

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

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

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

4. Pre-mix the PCR Mix by inverting the Falcon tube twice. Vortex with the MixMate at 1000 rpm for 30 seconds using the Tube Holder 25/50 mL adaptor.
5. Keep the PCR Mix at room temperature until use.

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

27.3 Prepare PCR Plates and perform PCR

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

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

Perform the instructions in the following order:

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

Prepare bench

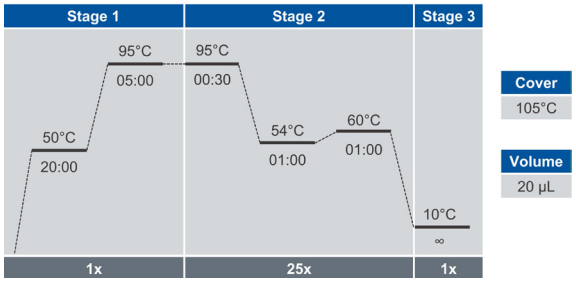
- Incubation Plates 1–4 with indices, prepared in previous step
- PCR Mix, prepared in previous step
- 2x Timer
- 3x Disposable reservoirs (10 mL)
- 3x Disposable ultra-low retention syringes with plungers
- Manual pipette (5 or 10 mL)
- Filter pipette tips
- Adhesive films

Before you start

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

Instructions part 1: Prepare PCR plates

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



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

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

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

7. Select the Run tab in the Constant layer view of the software, then press RUN to start the program. Start the timer when PCR Mix is added to the first well of the plate.
   Result:
   Dragonfly dispenses 18 µL of PCR Mix into each well of PCR Plate 1.
8. When the Dragonfly has returned PCR Plate 1 to the plate position and released the plate clamp, remove the plate from the instrument and immediately seal it with a new adhesive film.
9. Using the MixMate, vortex the plate at 3000 rpm for 30 seconds without adaptor to ensure that all wells are mixed.
10. Repeat steps 6–9 for PCR Plate 2.

Instructions part 2: Perform PCR

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

1. In the Post-PCR room, centrifuge PCR Plate 1 and 2 at 400–1000 x g for 1 minute.
2. Inspect PCR Plates 1 and 2 to ensure that all wells contain the same amount of liquid (19.8 µL). Note any deviations.
3. When the timer ends after 10 minutes, place PCR Plates 1 and 2 in one of the two pre-heated ProFlex instruments and click Resume to run the program.
4. Repeat step 7–14 for Incubation Plates 3 and 4. Place both plates in the second ProFlex.
5. Once the protocol is finished, clear the Dragonfly and shut it down according to manufacturer's instructions.
6. Once the PCR program is finished (2h), continue to *28. Pool PCR products using Hamilton Microlab® STAR* or *29. Pool PCR products using epMotion®*, or store the PCR Plates at +4 ºC if used the same day.

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

28.Pool PCR products using Hamilton Microlab® STAR

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

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

Prepare bench

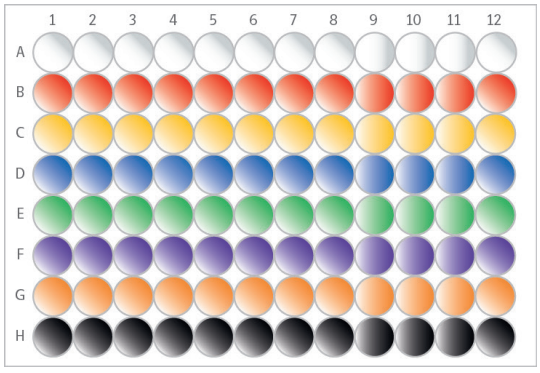
- PCR Plate 1–4, prepared in previous step
- 1x 96-well PCR plate (skirted)
- 8x Eppendorf tubes (1.5 mL)
- Hamilton STAR® pipette tips (5 racks of 50 µL filtered conductive Hamilton Tips)
- Rack for 24 tubes, for 24 Safe-Lock tubes, 1.5/2.0 mL
- Waste bag
- Manual pipette (100 µL)
- Filter pipette tips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

Before you start

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

Instructions: Automatic pooling

1. Open the Hamilton Run Control Application.
2. In the application, select the protocol `Olink_ExploreHT_PCRPooling`.
3. Click the green Start button at the top to initialize the instrument and run the protocol.
4. Make sure that PCR1 Plates 1–4 are properly sealed, then vortex the plates with the MixMate at 3000 rpm for 30 seconds without adaptor, and spin down at 400–1000 x g for 1 minute.
5. Inspect the wells of PCR Plate 1–4 to make sure that no liquid has evaporated and that there are no bubbles in the wells. Remove the seals.
6. Pull out the carriers to the load position and prepare the STAR deck according to the software instructions.
   - Place Plates on the carriers.
   - Place tubes in the tube rack carrier.
   - Carefully remove the adhesive films.
   - Fill tip carriers in the selected positions.
7. Select the correct input for the four source plates and click *Continue*.
8. Select the populated tip positions and click *OK* to automatically load the carriers and begin the run.
   Result:
   - *Hamilton STAR will automatically scan tips, pool 4 µL from each well of the PCR plates into the PCR Pooling Plate, keeping blocks separate in different rows. It will pause to allow for mixing, then pool subsequent rows to tubes.*
   - *20 µLfrom each well will be transferred to 1 Eppendorf tube per block.*



9. Once the protocol is finished, remove the PCR Pooling Plate from the deck. Inspect the PCR Pooling Plate to ensure that every applicable well contains the same amount of liquid. Discard the PCR Pooling Plate.
10. Remove PCR Plate 1–4 from the deck. Seal the plates with a new adhesive film and store at -20 ºC for potential reruns.
11. Close the Hamilton Run Control software and shut down the Hamilton STAR®.
12. Vortex PCR Tubes 1–8 and spin down briefly.
13. Continue to *31. Library purification* or store the PCR Tubes at +4 ˚C until use (the same day).

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

Instructions: Manual pooling

1. Open the Hamilton Run Control Application.
2. In the application, select the protocol `Olink_ExploreHT_PCRPooling`.
3. Click the green Start button at the top to initialize the instrument and run the protocol.
4. Make sure that PCR1 Plates 1–4 are properly sealed, then vortex the plates with the MixMate at 3000 rpm for 30 seconds without adaptor, and spin down at 400–1000 x g for 1 minute.
5. Inspect the wells of PCR Plate 1–4 to make sure that no liquid has evaporated and that there are no bubbles in the wells. Remove the seals.
6. Pull out the carriers to the load position and prepare the STAR deck according to the software instructions.
   - Place Plates on the carriers.
   - Place tubes in the tube rack carrier.
   - Carefully remove the adhesive films.
   - Fill tip carriers in the selected positions.
7. Select the correct input for the four source plates and click Continue.
8. Select the populated tip positions and click OK to automatically load the carriers and begin the run.
   Result:
   Hamilton STAR will automatically scan tips, pool 4 µL from each well of the PCR plates into the PCR Pooling Plate, keeping blocks separate in different rows.
9. Remove the PCR Pooling Plate from the worktable. Seal the plate with a new adhesive film, vortex the plate and spin down at 400 x g for 1 minute. Inspect the PCR Pooling Plate to ensure that every applicable well contains the same amount of liquid.
10. Carefully remove the adhesive film from the PCR Pooling Plate.
11. Manually transfer the pooled PCR products from the PCR Pooling Plate to the microcentrifuge tube(s) according to the table below. Use a single-channel pipette and **forward pipetting**, and change pipette tip after each well.

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

12. Vortex PCR Tubes 1–8 and spin down briefly.
13. Remove PCR Plate 1–4 from the deck. Seal the plates with a new adhesive film and store at -20 ºC for potential reruns.
14. Close the Hamilton Run Control software and shut down the Hamilton STAR®.
15. Continue to *31. Library purification* or store the PCR Tubes at +4 °C until use (the same day).

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

29.Pool PCR products using ep*Motion*®

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

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

Prepare bench

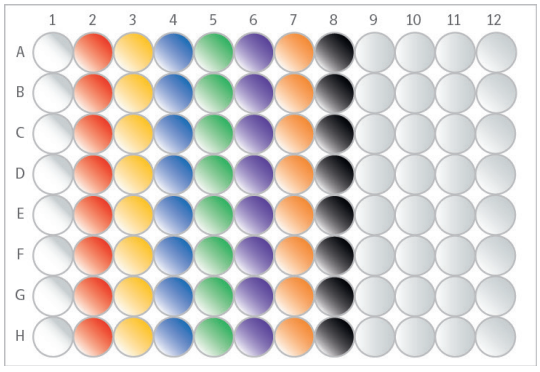
- PCR Plate 1–4, prepared in previous step
- 1x 96-well PCR plate (skirted)
- 8x Eppendorf tubes (1.5 mL)
- epT.I.P.S.® Motion pipette tips (2boxes á 10 µL))
- epT.I.P.S.® Motion pipette tips (50 µL)
- TS 50 single-channel dispensing tool (for automatic pooling)
- Manual pipette (100 µL or 200 µL) (for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

Before you start

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

Instructions: Automatic pooling

1. Open the EpBlue Application Runner.
2. In the application library, select user and the protocol `Olink_ExploreHT_PCRPooling`.
3. When the ID number of the instrument is shown in the software, click Next to continue.
4. Make sure that PCR1 Plates 1–4 are properly sealed, then vortex the plates with the MixMate at 3000 rpm for 30 seconds without adaptor, and spin down at 400–1000 x g for 1 minute.
5. Inspect the wells of PCR Plate 1–4 to make sure that no liquid has evaporated and that there are no bubbles in the wells. Remove the seals.
6. Prepare the ep*Motion* worktable according to the software instructions.
7. Click Next in the software, then enter the following settings:
   - Under Volume settings: Activate Input volumes manually.
   - Under Worktable settings: Deactivate Check tube lid removed.
8. Click Next until a Run button appears, then click Run to start the protocol.
   Result:
   - *epMotion will pool 3 µL from each well of the same row in each PCR plate into a single column of the PCR Pooling Plate, keeping blocks separate in different columns.*
   - *30 uL from each well will be transferred to 1 Eppendorf tube.*

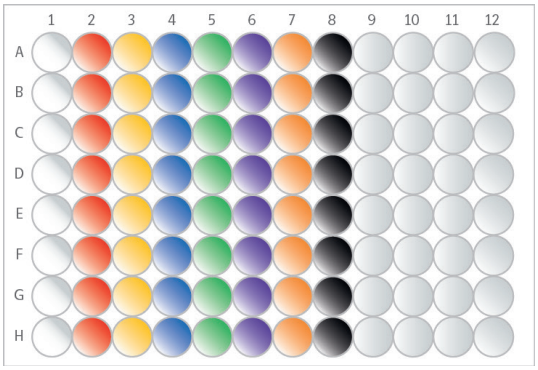


9. Once the protocol is finished, remove the PCR Pooling Plate from the worktable. Inspect the PCR Pooling Plate to ensure that every applicable well contains the same amount of liquid. Discard the PCR Pooling Plate.
10. Remove PCR Plate 1–4 from the worktable. Seal the plates with a new adhesive film and store at -20 ºC for potential reruns.
11. Clear the ep*Motion* and shut it down.
12. Vortex PCR Tubes 1–8 and spin down briefly.
13. Continue to *31. Library purification* or store the PCR Tubes at +4 °C until use (the same day).

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

Instructions: Manual pooling

1. Open the EpBlue Application Runner.
2. In the application library, select user and the protocol `Olink_ExploreHT_PCRPooling_manual`.
3. When the ID number of the instrument is shown in the software, click Next to continue.
4. Make sure that PCR1 Plates 1–4 are properly sealed, then vortex the plates with the MixMate at 3000 rpm for 30 seconds without adaptor, and spin down at 400–1000 x g for 1 minute.
5. Inspect the wells of PCR Plate 1–4 to make sure that no liquid has evaporated and that there are no bubbles in the wells. Remove the seals.
6. Prepare the ep*Motion* worktable according to the software instructions.
7. Click Next in the software, then enter the following settings:
   - Under Volume settings: Activate Input volumes manually.
   - Under Worktable settings: Deactivate Check tube lid removed.
8. Click Next until a Run button appears, then click Run to start the protocol.
   Result:
   epMotion will pool 3 µL from each well of the same row in each PCR plate into a single column of the PCR Pooling Plate, keeping blocks separate in different columns.



9. Remove the PCR Pooling Plate from the worktable. Seal the plate with a new adhesive film, vortex the plate and spin down at 400 x g for 1 minute. Inspect the PCR Pooling Plate to ensure that every applicable well contains the same amount of liquid.
10. Carefully remove the adhesive film from the PCR2 Pooling Plate. Make sure that every applicable well contain the same amount of liquid.
11. Manually transfer the pooled PCR2 products from the PCR2 Pooling Plate to the microcentrifuge tube(s) according to the table below. Use a single-channel pipette and **forward pipetting**, and change pipette tip after each well.

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

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

14. Remove PCR Plate 1–4 from the worktable. Seal the plates with a new adhesive film and store at -20 ºC for potential reruns.
15. Clear the ep*Motion* and shut it down.
16. Vortex PCR Tubes 1–8 and spin down briefly.
17. Continue to *31. Library purification* or store the PCR Tubes at +4 °C until use (the same day).

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

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

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

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

Prepare bench

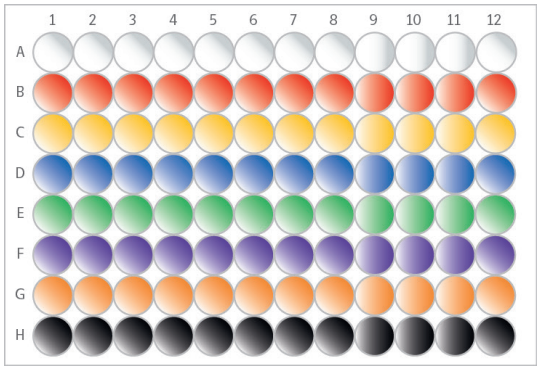
- PCR Plates 1–4, prepared in previous step
- 1x 96-well PCR plate (skirted)
- 1x 8-well strip tube
- 1x 8-well strip tube cap
- 8x Eppendorf tubes (1.5 mL)
- 104x F.A.S.T.™ Disposable Pipette Tips
- F.A.S.T.™ Plate adapter for 0.2 mL PCR Strip Tubes, 96 well format.
- Waste bag (for semi-manual pooling)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

Before you start

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

Instructions: Automatic pooling

1. Make sure that PCR1 Plates 1–4 are properly sealed, then vortex the plates with the MixMate at 3000 rpm for 30 seconds without adaptor, and spin down at 400–1000 x g for 1 minute.
2. Inspect the wells of PCR Plate 1–4 to make sure that no liquid has evaporated and that there are no bubbles in the wells. Remove the seals.
3. Open the proocol `Olink_Explore_HT_Plate_Pooling.`
4. Arrange the F.A.S.T. deck according to the layout in the protocol.
   - Place PCR1 Plates 1-4 on the tray.
   - Place PCR Pooling Plate on the tray.
   - Remove lid and place tips on the tray.
5. Click START to begin the run.
   Result:
   - *F.A.S.T. pools 3 µL from each well of the same block into one row of the 96-well plate, each block in a different row.*

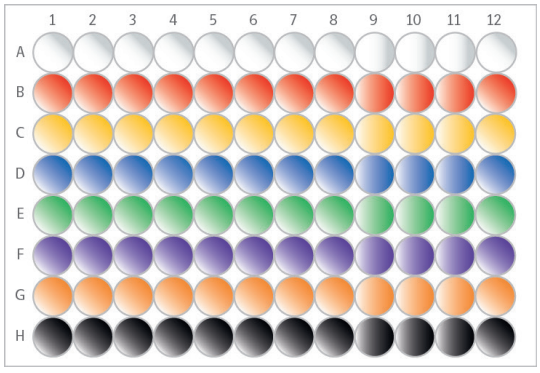


6. Once the protocol is finished, remove the PCR Pooling Plate from the deck. Seal the plate with a new adhesive film, vortex the plate and spin down at 400 x g for 1 minute. Inspect the PCR Pooling Plate to ensure that every applicable well contains the same amount of liquid.
7. Remove PCR Plate 1–4 from the deck. Seal the plates with a new adhesive film and store at -20 ºC for potential reruns.
8. Open the protocol `Olink_Explore_HT_Tube_Pooling`.
9. Arrange the F.A.S.T. deck according to the layout in the protocol.
   - Remove seal from PCR Pooling Plate and place on the tray.
   - Place 8-well strip tube in column 1 of the strip tube adapter and place on the tray.
   - Remove lid and place tips on the tray.
10. Click START to begin the run.
    Result:
    - *The F.A.S.T. pools 11 µL from each well of the same row of the PCR Pooling Plate into a single well of the PCR 1-8 strip tube, each block in a different well.*
11. Cap and vortex the PCR 1–8 strip tube and spin down briefly. Transfer each pooled library to its corresponding 1.5 mL microcentrifuge tube.
12. Clear the F.A.S.T. and shut it down.
13. Continue to *31. Library purification* or store the PCR Tubes at +4 °C until use (the same day).

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

Instructions: semi-manual pooling

1. Make sure that PCR1 Plates 1–4 are properly sealed, then vortex the plates with the MixMate at 3000 rpm for 30 seconds without adaptor, and spin down at 400–1000 x g for 1 minute.
2. Inspect the wells of PCR Plate 1–4 to make sure that no liquid has evaporated and that there are no bubbles in the wells. Remove the seals.
3. Open the protocol `Olink_Explore_HT_Plate_Pooling`.
4. Arrange the F.A.S.T. deck according to the layout in the protocol.
   - Place PCR1 Plates 1–4 on the tray.
   - Place PCR Pooling Plate on the tray.
   - Remove lid and place tips on the tray.
5. Click START to begin the run.
   Result:
   - *F.A.S.T. pools 3 µL from each well of the same block into one row of the 96-well plate, each block in a different row.*



6. Once the protocol is finished, remove the PCR Pooling Plate from the deck. Seal the plate with a new adhesive film, vortex the plate and spin down at 400 x g for 1 minute. Inspect the PCR Pooling Plate to ensure that every applicable well contains the same amount of liquid.
7. Remove PCR Plates 1–4 from the deck. Seal the plates with new adhesive films and store at -20 °C for potential reruns.
8. Clear the F.A.S.T. and shut it down.
9. Carefully remove the adhesive film from the PCR Pooling Plate. Make sure that every well contains the same amount of liquid.
10. Manually pipette PCR products from the PCR Pooling Plate to microcentrifuge tubes according to the table below. Use a single-channel pipette and **forward pipetting**, and change pipette tips after each well.

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

11. Vortex the tubes and spin down briefly.

12. Continue to *31. Library purification* or store the PCR Tubes at +4 °C until use (the same day).

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

31.Library purification

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

Prepare bench

- PCR Tubes 1–8, prepared in previous step
- Agencourt AMPure XP bottle
- 96% Ethanol (EtOH)
- MilliQ water
- DynaMag™-2 Magnet
- Timer
- 16x Microcentrifuge tubes (1.5 mL)
- 1x Falcon tube (15 mL)
- Manual pipettes (100 µL, 1000 µL, and 5000 µL)
- Filter pipette tips

Before you start

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

Instructions

1. Prepare fresh 70% EtOH:

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

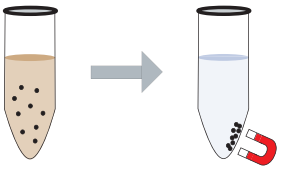
2. Shake and vortex the Agencourt AMPure XP bottle vigorously to resuspend the magnetic beads.
3. Transfer 80 µL from the Agencourt AMPure XP bottle to each BP tube.
4. Transfer 50 µL from each PCR Tube to the corresponding BP Tube:

Volume (µL)	From tube	To tube
50	PCR 1	BP 1
50	PCR 2	BP 2
50	PCR 3	BP 3
50	PCR 4	BP 4
50	PCR 5	BP 5
50	PCR 6	BP 6
50	PCR 7	BP 7
50	PCR 8	BP 8

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

5. Pipette-mix 10 times to thoroughly mix the Libraries with the beads. Change pipette tip between every tube.

6. Start the timer after the last tube has been mixed and incubate the BP Tubes 1–8 for 5 minutes at room temperature.
7. After the incubation, place the Eppendorf tube on the DynaMag-2 Magnetic stand for 2 min to separate beads from solution:



8. With the tubes still on the magnetic stand, carefully open the lids and discard 125 µL supernatant and leave 5 µL behind. Use a single-channel pipette. Do not disturb the beads.

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

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

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

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

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

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

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
45	BP 5	Lib 5
45	BP 6	Lib 6
45	BP 7	Lib 7
45	BP 8	Lib 8

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

16. Discard BP Tubes 1–8.
17. Continue to *32. Quality control*.

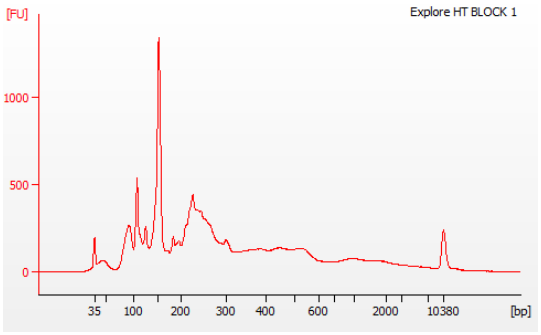
SAFE STOPPING POINT: *The Lib Tubes can be stored at -20 °C for up to 4 weeks.*

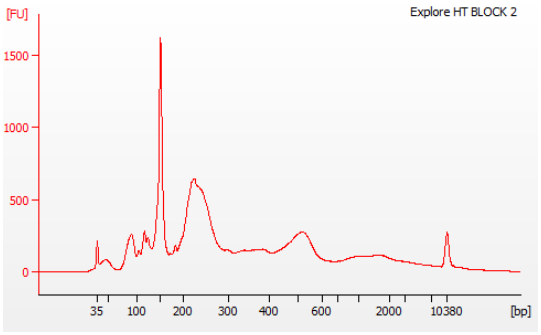
32. Quality control

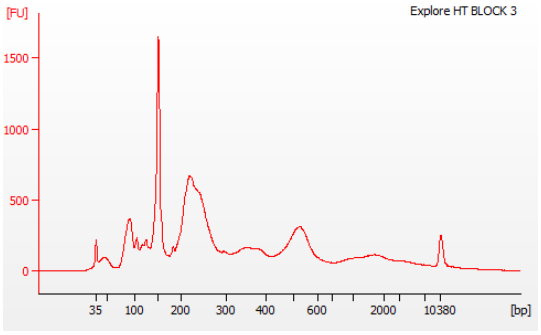
During this step, the eight purified Olink Libraries are quality controlled on the Bioanalyzer using the High Sensitivity DNA kit according to manufacturer instructions.

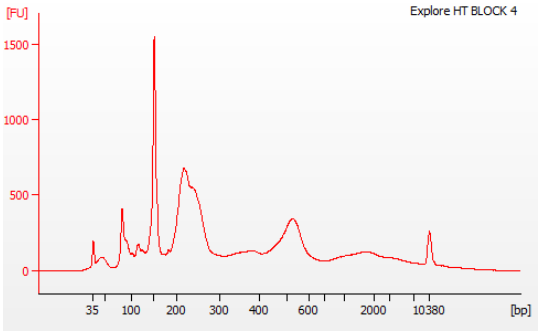
The electropherograms below display typical results for Olink Explore HT Library, one electropherogram per block. The purified Olink Library appears as a single or double peak around 150 bp. Peaks of larger sizes represents bubble products and do not have any impact on sequencing results, see emea.support.illumina.com/bulletins/2019/10/bubble-products-in-sequenc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

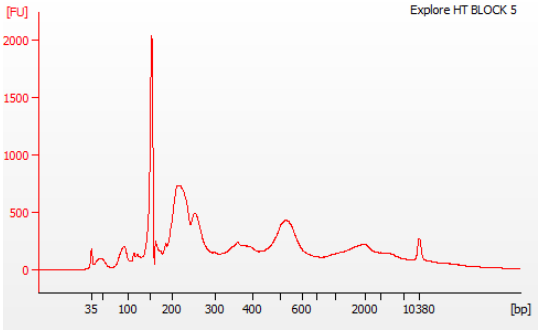
Electropherograms Bioanalyzer

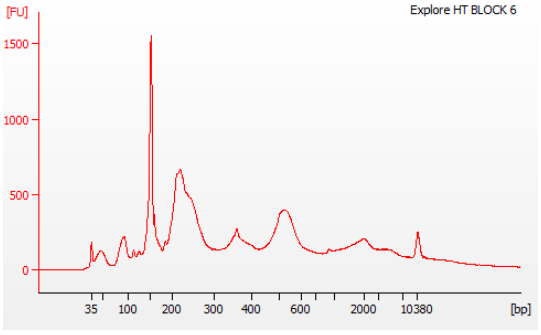




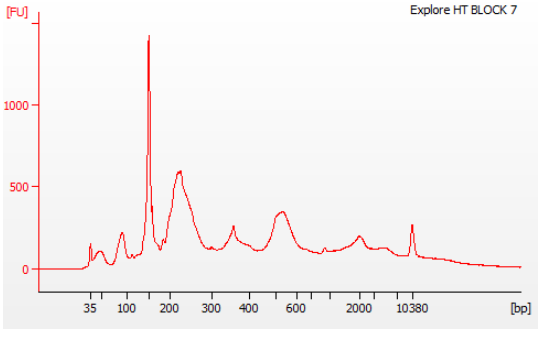


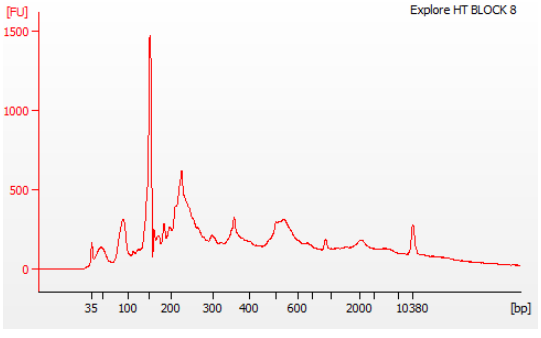




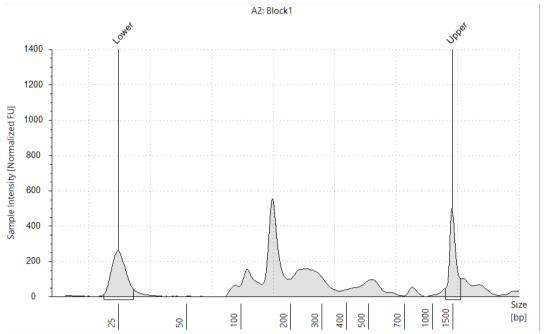


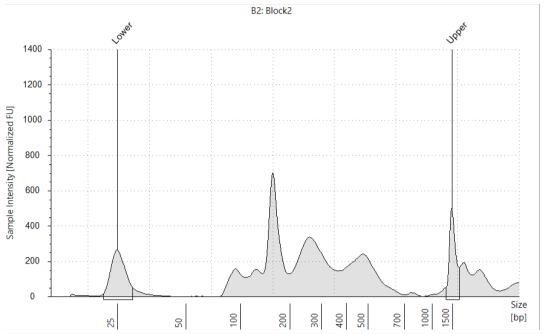
vvvv

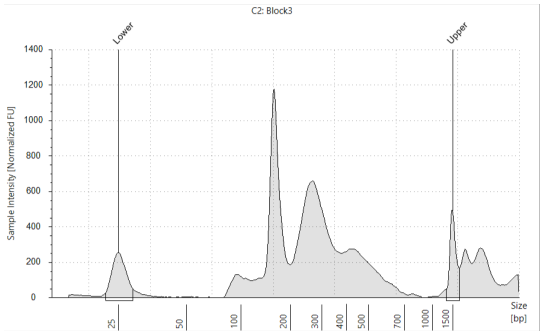


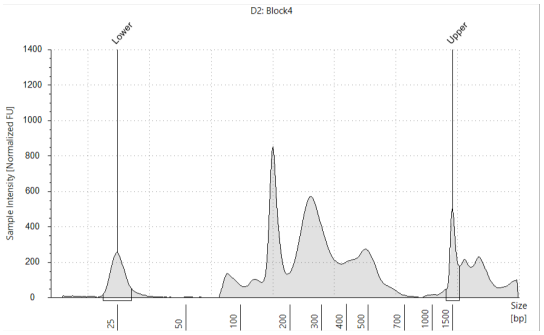


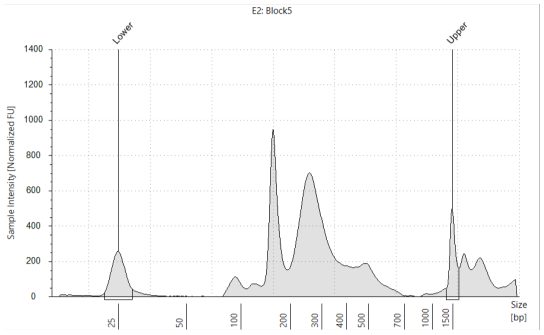
Electropherograms TapeStation

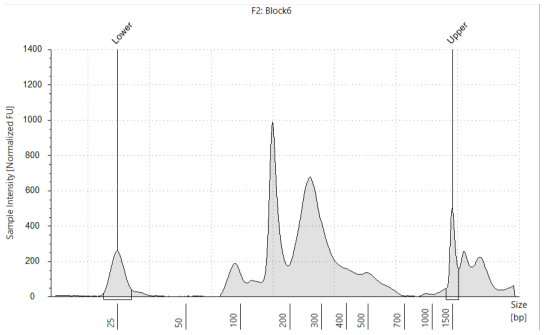


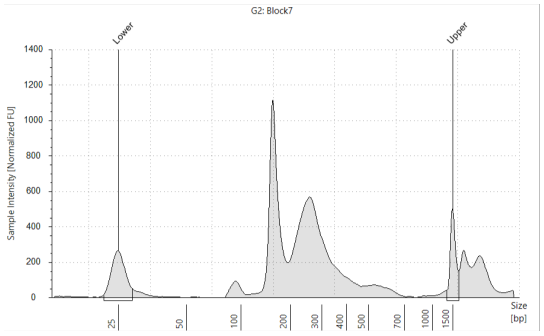


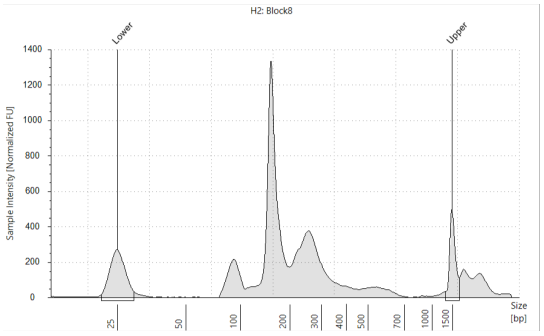












For expected results of a successful ladder run, refer to the manufacturer's manual. If the electropherograms do not look like expected, please contact *support@olink.com* for guidance.

33.Library pooling

Before you start

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

Instructions

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

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

34.Next generation sequencing

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

- NovaSeq™ 6000
- NovaSeq™ X Plus

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

35.Revision history

Version	Date	Description
2.0	2024-04-16	*3.1* updated. *8.2* updated. *8.3* Article number for MixMate Tube Holder PCR 96 changed. Precision Tab Carrier for Hamilton STAR deleted. *8.3, 8.4.1, and 8.5.1*: F.A.S.T. added as post-PCR instrument. *8.3, 8.5.3, 19* and *31*: TapeStation added. *8.5.5* added. *9.2* and *22.2*: Vortex table added. *17* and *30* F.A.S.T. added as post-PCR instrument. *11.2* and *24.2* Information added to Important: "Make sure to use the correct Mixmate settings as incorrect settings may lead to low-quality data". *21* and *34* Illumina® NovaSeq X Plus added. Safe stopping point adding at the end of the Pool PCR Products sections. Name change of Olink® NPX Explore HT to NPX™ Explore HT & 3072.
1.3	2024-01-26	*6.2.2, 9.3,* and *22.3*: Important regarding Plate layout for alternative plate matrices added. *11.2,* bullet 5: Information added to Important: "Make sure to use the correct Mixmate settings as incorrect settings may lead to low-quality data". *12.1* and *25.1,* Instructions bullet 2: reverse pipetting added. *14.1,* bullet 4: changed to "in the magnetic booster plates". *14.1* and *27.1,* bullet 2: rpm for MixMate changed to 3000. *24.2,* bullet 7: duration time changed to 3 hours.
1.2	2023-10-05	*2.2:* Olink Explore HT Sample Prep article number updated. PCR Enzyme and Index Plate Cap colors and article number updated and note added. *8.3:* A1 - both article numbers updated. D2 - Tall tip Pickup adapter, name and article number updated. *9.3* and *22.3:* well numbers in Sample Plate 2 updated. *11.2* and *24.2:* Volume of transferred diluted 1:1000 samples corrected (0.3 µL) *12.2:* Illustration for Instructions Part 3 corrected (left position empty). *13* and *26:* unit for milliQ water corrected (mL). *14* and *27:* updated for 384-well Index Plates.
1.1	2023-09-15	Part 4: Rerun removed.
1.0	2023-08-08	New

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1293, v2.0, 2024-04-16