Olink® Target 96

User Manual

0935, v4.1, 2024-03-x

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1. Introduction

1.1 Intended use

Olink high-multiplex immunoassay panels provide an efficient and innovative tool for targeted human protein biomarker discovery, development and validation.

The disease- or biology-focused panels enable rapid, high-throughput analysis, with exceptional data quality and minimal sample consumption. Using just 1 µl of sample, 92 biomarkers are assayed simultaneously on each panel, with results in only 24 hours.

1.2 About this manual

This manual provides you with the instructions needed to run an Olink® Target 96 panel using Olink® Signature Q100. If using the Standard Biotools® BioMark system, refer to *9. Standard Biotools® BioMark system.*

1.2.1 Definition of alert levels

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.*

1.3 Important user information

1.3.1 Sample preparation

To reduce sample-handling time, samples should be distributed in 8-well strips or on a 96-well plate prior to the start of the run.

1.3.2 Sample types

Olink panels have been validated using EDTA plasma and serum samples. A range of additional sample types are compatible with the technology. For example citrate plasma, heparin plasma, tissue and cell lysates, CSF and saliva. Different sample matrices are expected to affect the detection of specific proteins in different ways. In addition, extreme levels of IgG or flourescent particles can interfere with the Olink assay. For more information on sample types, please see the Data Validation documents corresponding to each panel, or contact Olink support at support@olink.com.

1.3.3 Pipettes

A multichannel pipette and a reverse pipetting technique must be used in the reagent transfer step (refer to *5.2.2 Pipetting techniques*). Maintain and calibrate the pipettes regularly.

1.4 Associated documentation

1.4.1 Olink documentation

The Olink documents are available from the Olink website: www.olink.com/downloads.

User Manuals

- Olink® Signature Q100 User Manual
- Olink® NPX Signature User Manual

White papers

- "Strategies for design of protein biomarker studies"
- "Data normalization and standardization"

1.4.2 Other documents

For information on the Standard Biotools IFC Controller HX and BioMark System, read the following User Guides that can be found on www.standardbio.com:

- IFC Controller User Guide - PN 68000112
- Real-Time PCR Analysis User Guide - PN 68000088
- Data Collection Software User Guide - PN 68000127

For information on the MixMate® read the Operating Manual that can be found on www.eppendorf.com:

- MixMate® Operating Manual - 5353 900.058-00/092018

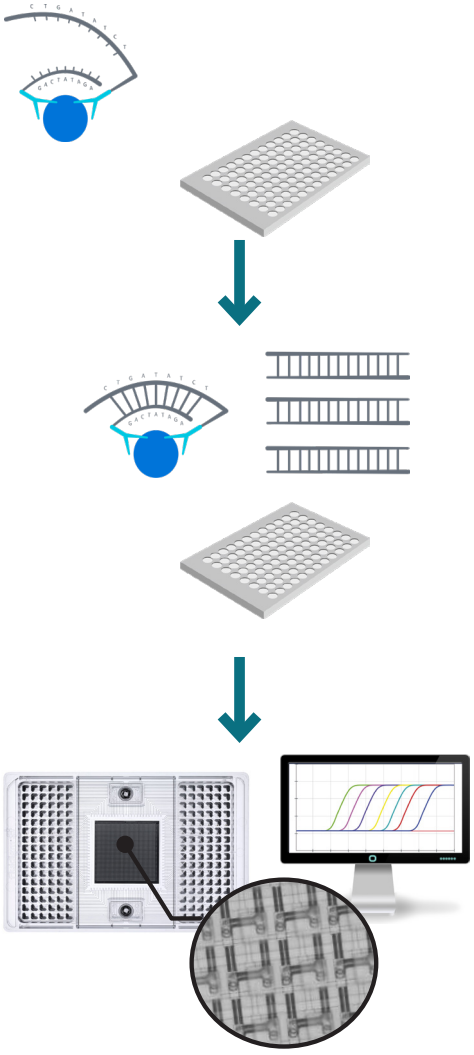
1.5 Technical support

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

2. Technology description

2.1 About PEA

The technology behind the Olink panels is called Proximity Extension Assay (PEA). The three core steps of the technology are illustrated below.



1. Incubation step

Duration: Overnight 16-22 hours

The 92 antibody pairs, labelled with DNA oligonucleotides, bind to their respective protein in the samples.

2. Extension and amplification

Duration: 2 hours

Oligonucleotides that are brought into proximity hybridize, and are extended using a DNA polymerase. This newly created piece of DNA barcode is amplified by PCR.

3. Detection

Duration: 4.5 hours

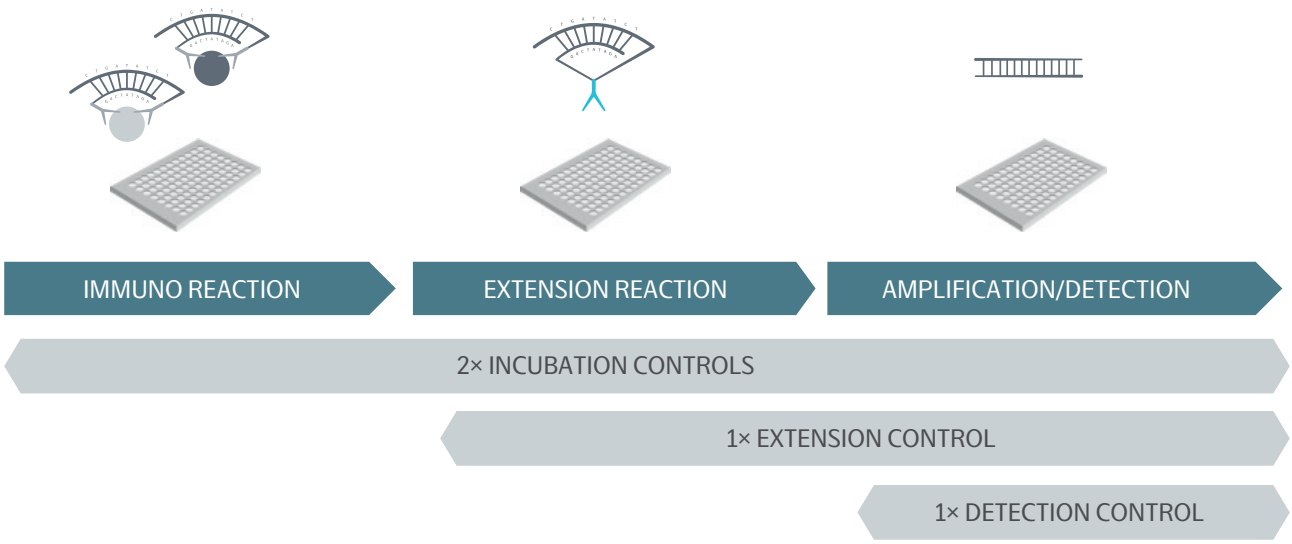
The amount of each DNA barcode is quantified by microfluidic qPCR.

2.2 Quality control

Olink has developed a built-in QC system using internal controls, for its multiplex biomarker panels. This system enables full control over the technical performance of assays and samples.

2.2.1 Internal controls

The QC system consists of four internal controls that are spiked into every sample and designed to monitor the three main steps of the Olink protocol: Immuno reaction (incubation step), extension and amplification/detection.



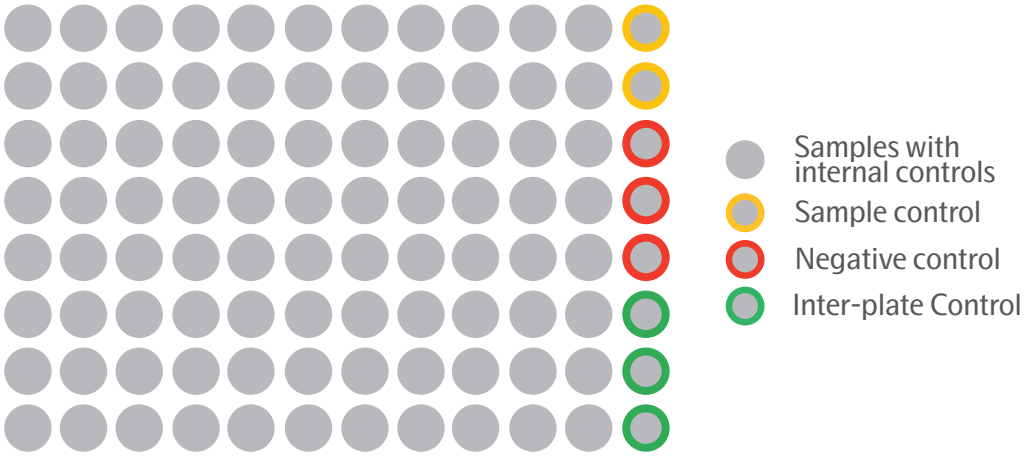
Incubation controls: Incubation Control 1 and 2 are two different non-human antigens measured with PEA. These controls monitor potential technical variation in all three steps of the reaction.

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/detection step and is used to adjust the signal from each sample with respect to extension and amplification.

Detection control: The Detection 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/detection step.

2.2.2 External controls

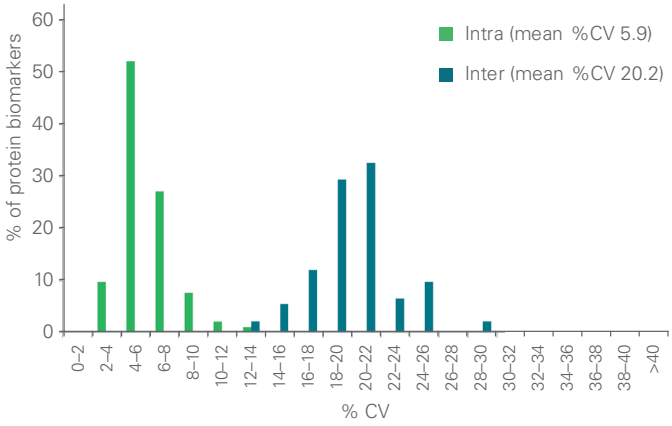
There are six required and two recommended external controls that are added to separate wells on each sample plate. On the illustrated plate there are 88 samples, 2 pooled plasma samples called External sample controls, 3 Negative controls and 3 Inter-plate controls.



Inter-plate Control: Inter-plate Control (IPC) is included in triplicate on each plate and these are run as normal samples. The IPC is a pool of 92 antibodies, each with one pair of unique DNA-tags positioned in fixed proximity and can be seen as a synthetic sample, expected to give a high signal for all assays. The median of the IPC triplicates is used to normalize each assay, to compensate for potential variation between runs and plates.

Negative Control: Negative Control is also included in triplicate 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 set the background levels for each protein assay and are used to calculate the limit of detection (LOD).

Sample Control: On each plate, it is recommended to run a pooled plasma sample in duplicate. These are used to assess potential variation between runs and plates, for example to calculate inter-assay and intra-assay CV, as well as troubleshooting. Validation data for all panels is availabe on the Olink website. An example of inter- and intra-assay CV is illustrated here:



2.3 Data analysis

2.3.1 NPX values

Olink reports protein expression levels using an arbitrary unit called Normalized Protein eXpression (NPX). NPX is a relative quantification unit on log2 scale that allows users to identify changes for individual protein levels across their sample set, and then use this data to establish protein signatures. NPX is derived from the Ct values obtained from the qPCR using the following equations:

Extension Control:

$$Ct_{Analyte} - Ct_{Extension\ Control} = dCt_{Analyte}$$

Inter-plate Control:

$$dCt_{Analyte} - dCt_{Inter\text{-}plate\ Control} = ddCt_{Analyte}$$

Adjustment against a correction factor:

$$Correction\ factor - ddCt_{Analyte} = NPX_{Analyte}$$

The data processing (normalization) of the Ct-values is performed to minimize both intra- and inter-assay variation and make the data more intuitive and easy to interpret. Quality control and normalization is achieved using the Olink NPX Signture software (refer to *4.1 Olink® NPX Signature*).

The NPX unit is unique to each protein assay, meaning that even if two different proteins have the same NPX values, their absolute concentrations may differ. Due to the relative nature of the unit, NPX should not be compared between runs without proper inter-plate normalization

For more information, refer to the Olink white paper "Data normalization and standardization", available on the Olink website: www.olink.com/downloads.

3. Product description

3.1 Reagents supplied in Olink kits

Each Olink kit contains reagents for 96 wells, sufficient for 90 samples and 6 controls. The reagents are supplied in two individual boxes. Storage temperature and expiry date for the components are stated on the label on each box.

NOTE: *All reagents for Olink Target 96 kits are lot specific and reagents from different kit lots cannot be combined. It is also not possible to use reagents from Olink Target 96 kits with reagents from the Olink Target 48 kits.*

3.1.1 Small box

The small box includes the Olink Probe kit and should be stored at +4 °C. The Olink Probe kit includes:

Part	Description
Incubation Solution	Contains components needed for the incubation reaction
A-probes	Contains 96 antibody probes labeled with A oligos
B-probes	Contains 96 antibody probes labeled with B oligos

3.1.2 Large box

The large box contains the Olink Detection and Control kit. It should be stored at -20 °C and includes:

Part	Description
PEA Solution	Contains components needed for the extension reaction
PEA Enzyme	For extension of A and B probes bound to their target
PCR Polymerase	For pre-amplification of the extension product created by the PEA Enzyme, also used in the Detection step
Detection Solution	Contains components needed for the detection reaction
Detection Enzyme	For qPCR amplification
Primer Plate	96-well plate with ready-to-use primers for amplification of extension product
Inter-plate Control	For normalization of each assay, to compensate for potential variation between runs and plates
Negative Control	For determination of background levels
Incubation Stabilizer	For stabilization of the incubation reaction
Sample Diluent	Only included for panels that require pre-dilution of samples

4. Additional requirements

4.1 Olink® NPX Signature

Olink NPX Signature software is an easy to use data import and pre-processing tool developed by Olink Proteomics. The software lets you import data, validate data quality and normalize Olink data for subsequent statistical analysis. See the Olink® NPX Signature User Manual for more information.

4.2 Required consumables (not supplied)

- Pipette filter tips
- Microcentrifuge tubes (1-1.5 mL)
- Centrifuge tube (>11 mL)
- 8-well strips with lids
- 96-well PCR plate (à 0.2 mL)
- Multichannel pipette reservoir
- Adhesive plastic film (heat-resistant)
- High purity water (sterile filtered, MilliQ® or similar)
- Olink® 96.96 IFC for Protein Expression (Olink product code 95007).
- External Sample Controls (Pooled plasma samples)

Contact Olink support at support@olink.com for specific recommendations.

4.3 Required equipment (not supplied)

- Pipettes (covering the range from 1 µL to 1000 µL) and compatible pipette tips
- Multichannel pipettes (recommended range 1-10 µL and 50-100 µL and/or 50-200 µL) and compatible multichannel pipette tips
- Vortex
- MixMate®
- 96 tube holder for MixMate®
- Centrifuge for plates
- Microcentrifuge for tubes
- Freezing block (-20 °C) for enzyme handling
- Thermal cycler with:
  - Heated lid
  - Temperature range from +50 °C to +95 °C
  - Validated for 0.1 mL volumes (important)
  - 96-well format (recommended)
- Refrigerator or cold room (+2 °C to +8 °C) where the light is off when the door is closed
- Freezer (-20 °C) where the light is off when the door is closed
- Olink Signature Q100
  - alternatively Standard Biotools Biomark or Biomark™ HD system

5. Laboratory work safety and guidelines

5.1 Safety considerations

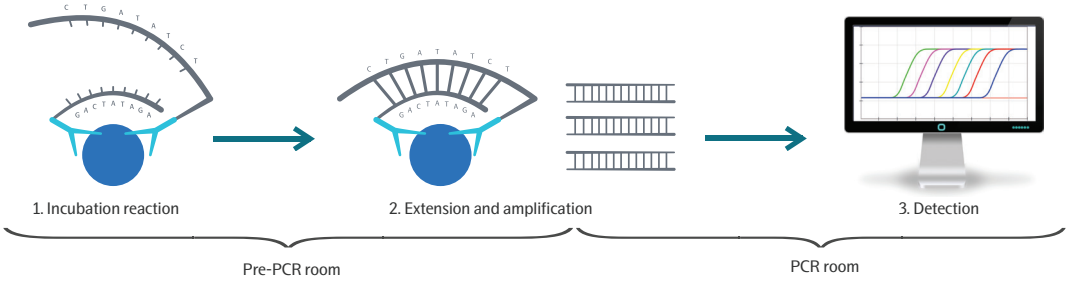
5.1.1 Safety

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.

5.1.2 PCR technology

PCR technology is sensitive to contaminations. Perform the Detection step in a PCR room, separate from the room where the Incubation and Extension steps are performed. Maintain and calibrate the PCR and BioMark™ instruments regularly.



5.2 Pipetting guidelines

5.2.1 Tips and tricks for pipetting

- Calibrate pipettes regularly (at least a 6 month interval)
- Pipette near the liquid surface
- Let the reagents and liquids reach room temperature before use to maximize accuracy.
- Pre-rinsing the tip 1 to 3 times with the liquid to be pipetted improves accuracy when using reverse pipetting
- Do not turn the pipette on its side when there is liquid in the tip as liquid might contaminate the interior of the pipette
- Keep pipettes vertical while pipetting and pipette to the bottom of the wells.

5.2.2 Pipetting techniques

Both forward and reverse pipetting is used in the Olink protocol.

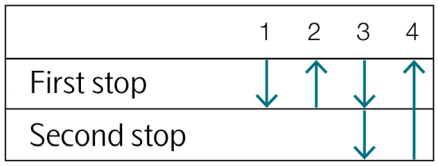
Forward pipetting

Forward pipetting is the most commonly used pipetting technique and is performed as described in the following instruction:

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

1. Press the operating button to the first stop.
2. Dip the tip into the liquid just below the liquid surface, and slowly release the operating button all the way up.

3. Press the operating button to the first stop to dispense the liquid into the receiving vessel and then press the operating button to the second stop. This action will empty the tip. Remove the tip from the vessel.
4. Release the operating button all the way up and discard the empty pipette tip.



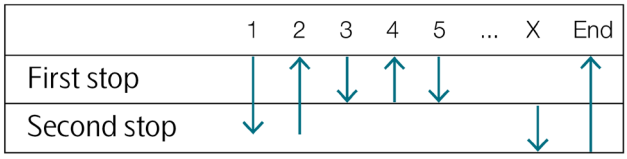
Reverse pipetting

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

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

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.
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.

X. 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.



5.3 Vortexing guidelines

Correct vortexing is critical when running Olink panels.

5.3.1 Vortexing tips and tricks

- Visually inspect the wells during vortexing to ensure complete mixing. The liquid should swirl in the wells.

5.3.2 Vortexing instruction

Correct vortexing is essential for generating reproducible results. To vortex thoroughly, follow these steps:

Vortexing using MixMate®

- The MixMate should be used for the vortexing of PCR-plates in the pre-PCR steps. For the Detection step it is recommended but not required.
- Start with inserting the 96 tube holder in the plate holder:
  - Hold the 96 tube holder up against the back edge of the plate holder.
  - Engage the 96 tube holder by pressing gently on the front.

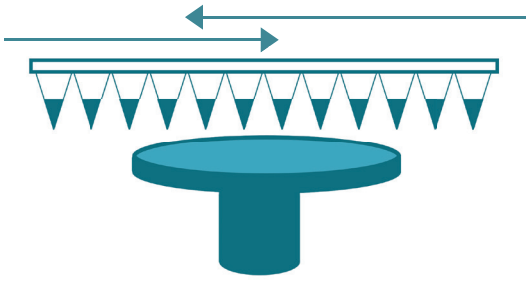
- Insert the plate into the tube holder and press the PCR plate into the bores of the tube holder and make sure that the plate is seated evenly. Note that misplacement of the plate will result in uneven mixing of the wells and low-quality data.
- Set mixing speed at 2000 rpm and mixing time at 30 seconds.
- Start mixing by pressing the start/stop key. After 30 seconds, MixMate will automatically stop.
- Take out the plate by pulling it out from the front side of the plate holder.
- For fully skirted plates the MixMate® should be operated without the plate holder. Please see the MixMate Operating Manual for further instructions.

Manual vortexing

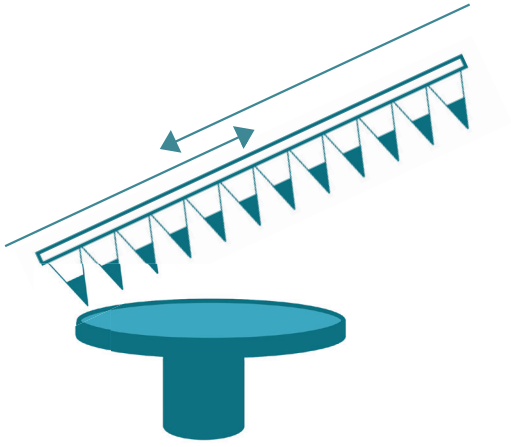
A manual vortex is needed to vortex all separate tubes throughout the protocol. A manual vortex may also be used for the vortexing of plates during the detection step, if so, follow the below instructions. You can also see our vortexing video for further guidance on manual vortexing.

- When using a manual vortex, cover the rubber platform of the vortex with adhesive plastic film to make it easier to slide the 96-well plate during vortexing.
- Vortex for 20-30 seconds at full speed.

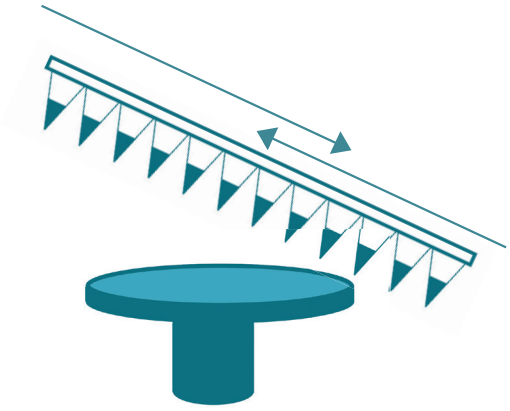
1. Move the plate back and forth over the vortex in horizontal direction. Make sure that the wells at the outside edges of the plate (columns 1 and 12) are also vortexed.



2. Tilt the plate away from you and move it back and forth over the vortex.



3. Tilt the plate towards you and move it back and forth over the vortex.



4. Turn the plate 180° and repeat steps 1-3.

5.4 Sterile lab environment

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. The following recommendations are intended to simplify the workflow in the lab and improve data quality.

- Keep all consumables (tubes, pipette tips, PCR plates etc.) in closed bags or boxes, preferably in a closed storage unit.
- Always wear gloves, including when bringing reagents in and out from fridge or freezer.
- Change gloves when needed and always when going from pre-PCR to PCR operations.
- Always wear a long-sleeved lab coat.
- Use a separate lab coat for working with the PCR steps.
- Use separate rooms for pre-PCR and PCR operations. If this is not possible, keep separate benches.
- Use separate consumables and equipment for pre-PCR and PCR operations.

6. Preparation

6.1 Experimental design

Decide how many samples, replicates and controls that are needed to get the data you want from the study. When running more than one plate it is important that the samples are appropriately randomized across all plates necessary steps for normalizing and combining data are taken.

It may be wise to consult a statistician or Olink Support (support@olink.com) prior to running the study. For more information, refer to the Olink white paper "Strategies for design of protein biomarker studies" available on the Olink website: www.olink.com/downloads.

6.2 Create programs

6.2.1 Olink® extension and pre-amplification program

Create a PEA program on the thermal cycler with the following settings:

Step	Temperature	Duration
Extension	50 °C	20 min
Hot start	95 °C	5 min
PCR cycle (17 cycles)	95 °C	30 s
	54 °C	1 min
	60 °C	1 min
Maintain the reaction at	10 °C	∞, hold

NOTE: *Enable the heated lid function.*

6.3 Bench setup

Before you start working it is important to know that you have everything you need and that all reagents are ready for pipetting. Do the following before you start the experiment:

1. Clean the lab bench, hood, racks and pipettes with 70% ethanol.
2. Bring out all reagents, consumables and samples needed for the specific lab step. Potential contamination from the surrounding environment is minimized by reducing physical movements in the lab.
3. Organize equipment, consumables and samples in the work station, in a way that enables clean work.
4. Label pipette boxes with column number to more easily monitor where you are on the plate.
5. Note that all consumables and reagents are single use only.

6.4 Time indications and limits

Step	Duration	Comment
Dilution (for diluted panels)	15-30 min	The diluted panels Metabolism, CVD III, Development and Cardiometabolic need a dilution step.
Incubation setup	30-45 min	
Incubation	16-22 hours (overnight)	Keep incubation times consistent when running multiple IFCs for the same project (variation < 2 hours).
Extension	30 min	From preparing mix to start of PCR machine. **IMPORTANT:** *A maximum of 5 minutes can pass between adding extension mix to the incubation plate until the start of the PCR. This is important for sensitivity.*
Priming	24 min	Prepare sample plate during priming of the IFC. **NOTE:** *The primed IFC must be used within 60 minutes for the pressure to be maintained.*
Load/qPCR	4 hours	After priming and pipetting the diluted PCR product and primers into the IFC chip, the Signature Q100 does the loading and qPCR steps automatically. **NOTE:** *We do not recommend setting up more than 3 plates at a time.*

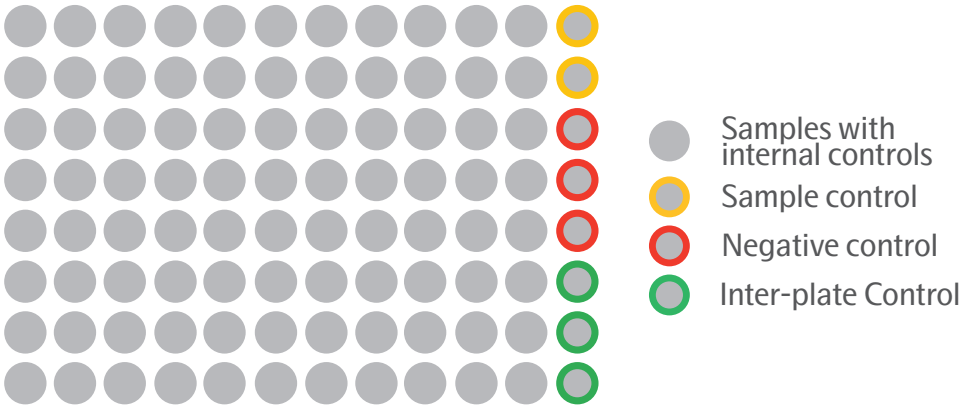
6.5 Sample preparation

Sample preparation recommendations are listed below:

- Use a 96-well PCR plate format, preferably with full skirt. All plates need to be able to withstand -80° C, be dry-ice resistant and easily re-sealable.
- Ensure that the samples are randomized, or in the order ready to be run and compatible with the plate layout.
- Ensure that each well is separately sealed using an adhesive film or individual seals.
- Clearly mark sample plates or tubes with a simple alphanumeric code that you can later identify (“A, B, C”, “1, 2, 3”, or “A1, A2, A3”) using temperature-resistant labels or marker pen.
- Use unique sample identification names or numbers.

6.6 Plate layout

Below is an illustration of the plate layout. The twelfth column consists of controls. The negative controls and inter-plate controls are always supplied by Olink. The pooled sample controls are provided by Olink if the samples are run at an Olink Analysis Service lab.



7. Dilution step

7.1 Overview

For selected panels, a sample dilution step is required prior to running the assay protocol. For Olink panels that require pre-dilution of samples, this information is indicated on the kit boxes as well as on the lot configuration sheet. During this step, the samples are diluted so that the target proteins are in an optimal concentration range for the assay.

NOTE: *Dilutions are optimized for serum and plasma only. Other sample matrices may require different dilutions. Contact support@olink.com for more information.*

IMPORTANT: *Always double check that you have the correct volume in ALL pipette tips.*

Dilution	Panels
1:10	Metabolism
1:100	CVD III and Development
1:2025	Cardiometabolic

7.2 Sample dilution step for 1:10 panels

Prepare bench

For the Dilution step for 1:10 panels, you will need:

- Prepared 96-well plate with samples
- 1 (one) 96-well PCR plate
- 1 Sample Diluent
- 1 multichannel pipette reservoir
- 1 multichannel pipette (10 µL)
- 98 multichannel pipette tips (10 µL)
- 2 adhesive films

Before you start

- Decide how many samples you will include in the experiment.
- Use the 96-well plate layout and select a location for each sample. Make sure the samples are distributed randomly. The negative control and inter-plate control samples should not be diluted.
- Sample dilutions should be made in a 96-well plate (0.2 mL per well) using a multichannel pipette.

NOTE: *Pipette near the surface of the samples to prevent liquid from sticking to the outside of the pipette tip.*

- Prepare the dilutions freshly, as close to the start time of the assay as possible, no more than 1 hour before the start of incubation. Store the dilutions in the fridge.

7.2.1 Dilution step instruction

1. Thaw the Sample Diluent, vortex and empty the bottle into a multichannel pipette reservoir (minimum volume 15 mL).
2. Mark a 96-well plate as "Dilution Plate".
3. Pipette up and down in the Sample Diluent a few times in the sample diluent to pre-condition the pipette tips.
4. Transfer 9 µL of the Sample Diluent to each well of columns 1-11 and positions A-B in column 12 on the 96-well plate, using **reverse pipetting**. Pipette the Sample Diluent carefully to avoid foaming.

NOTE: *Do not change pipette tips and use the same multichannel pipette throughout the entire dilution series.*

5. Vortex the sample plate using the MixMate® at 2000 for 30 seconds and spin down the liquid at 400-1000 x g, for 1 minute at room temperature. Carefully transfer 1 µL of your samples and pooled sample controls according to your plate layout to the Dilution Plate using **forward pipetting**.

NOTE: *Use the same multichannel pipette throughout the entire plate, also for samples A-B of column 12. Change tips between each pipetting step.*

6. Seal both the original sample plate and the Dilution Plate with adhesive plastic film.
7. Vortex the Dilution Plate thoroughly using the MixMate® at 2000 rpm for 30 seconds. Refer to *5.3 Vortexing guidelines* for more information.
8. Spin down the content at 400-1000 x g for 1 minute at room temperature.
9. Double check that all wells in the plate contain the same volume. Note any deviations. This information is needed to interpret the data in the statistical analysis step.

7.3 Sample dilution step for 1:100 panels

Prepare bench

For the Dilution step for 1:100 panels, you will need:

- Prepared 96-well plate with samples
- 1 (one) 96-well PCR plate
- 1 Sample Diluent
- 1 multichannel pipette reservoir
- 1 multichannel pipette (200 µL)
- 1 multichannel pipette (10 µL)
- 8 multichannel pipette tips (200 µL)
- 90 multichannel pipette tips (10 µL)
- 2 adhesive films

Before you start

- Decide how many samples you will include in the experiment.
- Use the 96-well plate layout and select a location for each sample. Make sure the samples are distributed randomly.

NOTE: *The negative control and inter-plate control samples should* **not** *be diluted.*

- Sample dilutions should be made in a 96-well plate (0.2 mL per well) using a multichannel pipette.

NOTE: *Pipette near the surface of the samples to prevent liquid from sticking to the outside of the pipette tip.*

- Prepare the dilutions freshly, as close to the start time of the assay as possible, no more than 1 hour before the start of incubation. Store the dilutions in the fridge.

7.3.1 Dilution step instruction

1. Thaw the Sample Diluent, vortex and empty the bottle into a multichannel pipette reservoir (minimum volume of 15 mL).
2. Mark a 96-well plate as "Dilution Plate".
3. Pipette up and down in the Sample Diluent a few times to pre-condition the pipette tips.
4. Transfer 99 µL of the Sample Diluent to each well of columns 1-11 and positions A-B in column 12 on the 96-well plate, by using **reverse pipetting**. Pipette the Sample Diluent carefully to avoid foaming.

NOTE: *Do not change pipette tips and use the same multichannel pipette throughout the entire dilution series.*

5. Vortex the samples using the MixMate® at 2000 rpm for 30 seconds and spin down the liquid at 400-1000 x g, for 1 minute at room temperature. Carefully transfer 1 µL of your samples and pooled sample controls according to your plate layout using **forward pipetting**.

NOTE: *Use the same multichannel pipette throughout the entire plate, also for samples A-B of column 12. Change tips between each pipetting step.*

6. Seal both the original sample plate and the Dilution Plate with adhesive plastic film.
7. Vortex the Dilution Plate plate using the MixMate® thoroughly at 2000 rpm for 30 seconds. Refer to *5.3 Vortexing guidelines* for more information.
8. Spin down the content at 400-1000 x g for 1 minute at room temperature.
9. Double check that all wells in the plate contain the same volume. Note any deviations. This information is needed to interpret the data in the statistical analysis step.

7.4 Sample dilution step for 1:2025 panels

Prepare bench

For the Dilution step for 1:2025 panels, you will need:

- Prepared 96-well plate with samples
- 2 (two) 96-well PCR plates
- 1 Sample Diluent
- 1 multichannel pipette reservoir
- 1 multichannel pipette (200 µL)
- 1 multichannel pipette (10 µL)
- 16 multichannel pipette tips (200 µL)
- 180 multichannel pipette tips (10 µL)
- 3 adhesive films

Before you start

- Decide how many samples you will include in the experiment.
- Use the 96-well plate layout and select a location for each sample. Make sure the samples are distributed randomly.

NOTE: *The negative control and inter-plate control samples should* **not** *be diluted.*

- A 2-step sample dilution should be performed in order to reach the desired dilution factor of 1:2025, and made in 2×96-well plates (0.2 mL per well) using a multichannel pipette. Pipette near the surface of the samples to prevent liquid from sticking to the outside of the pipette tips.
- Prepare the dilutions freshly, as close to the start time of the assay as possible, no more than 1 hour before the start of incubation. Store the dilutions in the fridge.

7.4.1 Dilution step instruction

1. Thaw the Sample Diluent, vortex and empty the bottle into a multichannel pipette reservoir (minimum volume of 15 mL).
2. Mark two 96-well plates as "Dilution Plate 1" and "Dilution Plate 2".
3. Pipette up and down in the Sample Diluent a few times to pre-condition the pipette tips.
4. Transfer 44 µL of the Sample Diluent into each well of columns 1–11 and positions A–B in column 12 on Dilution Plate 1 and Dilution Plate 2 using **reverse pipetting**.

NOTE: *Do not change pipette tips and use the same multichannel pipette throughout the entire dilution series.*

NOTE: *Pipette the Sample Diluent carefully to avoid foaming.*

5. Vortex the sample using the MixMate® at 2000 rpm for 30 seconds and spin down the liquid at 400-1000 x g, for 1 minute at room temperature. Carefully transfer 1 µL of your samples and pooled sample controls into Dilution Plate 1 according to your plate layout using **forward pipetting**. Refer to *6.6 Plate layout* for more information.

NOTE: *Use the same multichannel pipette throughout the entire plate, also for samples A-B of column 12. Change tips between each pipetting step.*

6. Seal both the original sample plate and Dilution Plate 1 with an adhesive plastic film.
7. Vortex Dilution Plate 1 using the MixMate® thoroughly at 2000 rpm for 30 seconds and ensure that all wells are mixed. Spin down the contents at 400-1000 x g for 1 minute at room temperature.
8. Transfer 1 µL of your diluted samples and pooled sample controls from Dilution Plate 1 to the previously prepared Dilution Plate 2 using **forward pipetting**.

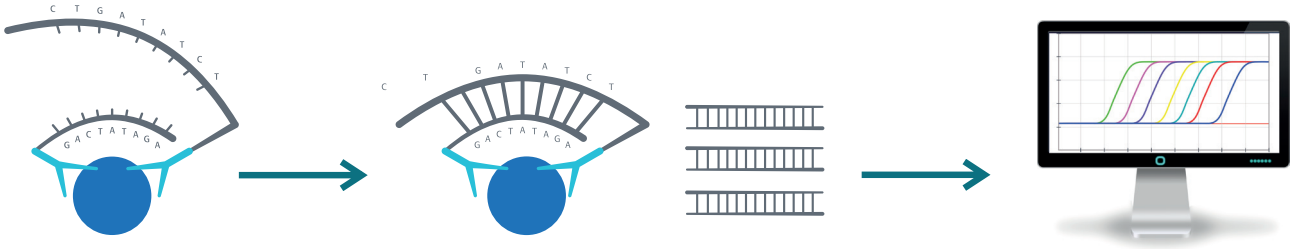
NOTE: *Use the same multichannel pipette throughout the entire plate, also for samples A-B of column 12. Change tips between each pipetting step.*

9. Seal Dilution Plate 2 with adhesive plastic film.
10. Vortex Dilution Plate 2 using the MixMate® thoroughly at 2000 rpm for 30 seconds and ensure that all wells are mixed. Spin down the contents at 400-1000 x g for 1 minute at room temperature.
11. Double check that all wells in the plate contain the same volume. Note any deviations. This information is needed to interpret the data in the statistical analysis step.

8. Operation

8.1 Overview

The Olink protocol consists of three core steps. These are the Incubation step, the Extension and amplification step and the Detection step. Panels that target high abundant proteins require an additional Sample dilution step. Refer to *7. Dilution step*.



1. Incubation

Duration: Overnight 16-22 hours

Location: Pre-PCR room

Needed for this step:

- A-probes
- B-probes
- Incubation Solution
- Incubation Stabilizer
- Negative Controls
- Inter-plate Controls
- Pooled sample controls

2. Extension and amplification

Duration: 2 hours

Location: Pre-PCR and PCR room

Needed for this step:

- PEA Solution
- PEA Enzyme
- PCR Polymerase

3. Detection

Duration: 2.5 hours

Location: PCR room

Needed for this step:

- Detection Solution
- Detection Enzyme
- PCR Polymerase
- Primer plate
- Olink IFC (96.96GE)

8.2 Incubation step

The Incubation step is where the antibody-pairs with attached DNA tags are added to the samples, and allowed to bind to their target proteins during an overnight incubation.

Prepare bench

Prepare the lab space with pipettes and consumables needed for this step and let the reagents and controls reach room temperature. For the Incubation step, you will need:

- Incubation Solution
- Incubation Stabilizer
- A- and B-probes
- Negative and Inter-plate Controls
- Pooled sample controls
- Two 8-well strips
- Prepared 96-well plate with samples
- 1 (one) 96-well plate
- 1 microcentrifuge tube (1-1.5 mL)

- 3 pipette tips (10 µL)
- 4 pipette tips (100/200 µL)
- 1 pipette tip (1000 µL)
- 104 multichannel pipette tips (10 µL)
- 2 adhesive films

Vortex and spin all reagents before use.

8.2.1 Incubation step instruction

TIP: *Practice reverse pipetting before you start. Refer to 5.2.2 Pipetting techniques.*

IMPORTANT: *Always double check that you have the correct volume in ALL pipette tips.*

1. Thaw the samples.
2. Vortex the Negative Control, Inter-plate Control and Sample Control and spin briefly. Add 5 µL of the controls to an 8-well strip according to the following order:

Sample control

Negative control

Inter-plate Control

NOTE: *If running a diluted panel, the two sample controls will be placed on the sample plate and not in the control strip.*

3. Prepare the Incubation Mix in a microcentrifuge tube according to the table below. Vortex and spin each reagent before adding it to the mix. The Incubation Mix must be used within 45 minutes from preparation.

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

Incubation Mix	Per 96-well plate (µL)
Incubation Solution	280.0
Incubation Stabilizer	40.0
A-probes	40.0
B-probes	40.0
Total	400.0

4. Vortex and spin down the Incubation Mix. Transfer 47 µL of the Incubation Mix to each well of a new 8-well strip.
5. Pre-condition the pipette tips and transfer 3 µL of Incubation Mix to the bottom of the wells of a new 96-well plate by reverse pipetting and name it Incubation Plate. Use the same pipette tips for the entire plate. Pipette near the surface of the Incubation Mix to prevent liquid from sticking to the outside of the pipette tips.
6. Vortex the plate with samples using the MixMate® at 2000 rpm for 30 seconds and spin down the liquid at

400-1000 x g, for 1 minute at room temperature. If samples are located in tubes, use a regular pipette to transfer 1 µL of each sample, using a multichannel pipette, to the bottom of the wells of the Incubation plate according to your sample plate layout.

NOTE: *For diluted panels, transfer from the final Dilution Plate.*

Use **forward pipetting** and change pipette tips between every column.

Use a multichannel pipette to transfer 1 µL of Negative Control and Inter-plate Control from the prepared 8-well strip with controls, to the last 6 wells of column 12 of the Incubation Plate, according to the plate layout. Refer to *6.6 Plate layout* for more information. Use **forward pipetting**.

7. Seal the Incubation Plate thoroughly with an adhesive plastic film, spin at 400-1000 x g for 1 minute at room temperature.

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

8. Double check that all wells in the plate contain the same volume. Note any deviations. This information is needed to interpret the data in the statistical analysis step.
9. Incubate the Incubation Plate overnight at +4 °C for 16-22 hours in a refrigerator or cold room where the light is off when the door is closed.

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

8.3 Extension and amplification step

In the morning of the following day, the extension and amplification steps take place. Unique DNA reporter sequences for each target protein are generated and pre-amplified using regular PCR.

Prepare bench

Prepare the lab space with pipettes and consumables needed for the Extension and amplification step. You will need:

- High purity water
- PEA Solution
- PEA Enzyme
- PCR Polymerase

NOTE: *Keep the PEA Enzyme and the PCR Polymerase in a freezing block or on ice.*

- 1 centrifuge tube (> 11 mL)
- 1 multichannel pipette reservoir
- 2 pipette tips (1000 µL)
- 2 pipette tips (100/200 µL)
- 8 multichannel pipette tips (200 µL)
- 1 adhesive film

8.3.1 Extension and amplification step instruction

IMPORTANT: *Always double check that you have the correct volume in ALL pipette tips.*

1. Allow the PEA Solution to reach room temperature. Vortex and spin down briefly before use.
2. Pre-heat the PCR machine to 50 °C and pause the program.

3. Spin down the Incubation Plate at 400-1000 x g for 1 minute at room temperature.
4. Prepare the Extension Mix in a 15 mL tube according to the table below. The Extension Mix must be used within 30 minutes from preparation.

Extension Mix	Per 96-well plate (µL)
High Purity Water	9385
PEA Solution	1100
PEA Enzyme	55
PCR Polymerase	22
Total	10 562

NOTE: *Vortex and spin down all the Extension Mix components tubes.*

Save the remainder of the PCR Polymerase for the final detection step.

5. Vortex the Extension Mix and pour it into a multichannel pipette reservoir.
6. Carefully remove the adhesive film from the Incubation Plate.

TIME SENSITIVE STEP: *Perform steps 7-11 during 5 minutes.*

7. Start a 5 minute timer and transfer 96 µL of Extension Mix to the upper parts of each of the well walls of the Incubation Plate using **reverse pipetting**. Use the same pipette tips throughout the plate.

IMPORTANT: *Do not let the tips come in contact with the content of a well.*

8. Seal the plate with a new adhesive plastic film.

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

9. Use the MixMate® to vortex the plate thoroughly at 2000 rpm for 30 seconds to ensure that all wells are mixed before spinning it down. Refer to *5.3 Vortexing guidelines* for more information.
10. Double check that all wells in the plate contain the same volume. Note any deviations. This information is needed to interpret the data in the statistical analysis step.
11. Take the Incubation Plate to the PCR room.
12. Immediately place the Incubation Plate in the PCR instrument and resume the PEA program. The PEA program takes approximately 1 hour and 30 minutes. Refer to *6.2.1 Olink® extension and pre-amplification program* for more information.

NOTE: *If the thermal cycler requires a silicon cover for plates covered with plastic film, use one to avoid evaporation.*

TIP: *You can start the preparations for the detection step during the last 10 minutes of the PEA program.*

13. When the PCR PEA program is finished (~1 hour 55 minutes), continue to detection step.

SAFE STOPPING POINT: *The extension products can be stored in the Incubation Plate for up to one week at +4 °C or up to four weeks at -20 °C in a refrigerator or freezer when the light is off when the door is closed.*

8.4 Detection step

The final Detection step quantifies the DNA reporters for each biomarker using high throughput real-time qPCR on the Olink Signaure Q100 system.

Prepare bench

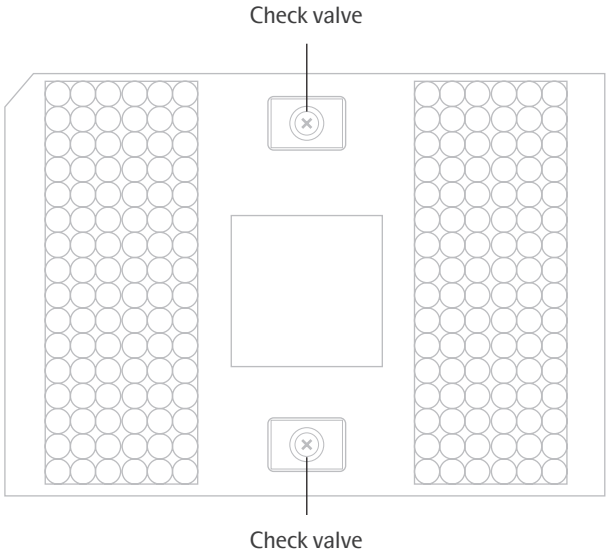
For the priming of the chip, you need:

- 96.96 Dynamic Array IFC
- Two syringes with control line fluid

For the Detection step, you need:

- Detection Solution
- High purity water
- Detection Enzyme
- PCR Polymerase
- Primer Plate
- Extension products
- 1 (one) 8-well strip
- 1 (one) 96-well plate
- 1 microcentrifuge tube (1-1.5 mL)
- 2 pipette tips (10 µL)
- 1 pipette tip (100/200 µL)
- 2 pipette tips (1000 µL)
- 3 boxes + 8 multichannel pipette tips (10 µL)
- 2 adhesive films

8.4.1 Prime IFC



1. Unpack the syringes and actuate both check valves of the IFC with gentle pressure using a syringe. Ensure that the poppets of the check valves can move freely up and down.
2. Tilt the IFC and insert the syringe tip into the check valve opening. The tip should be fully inserted. Confirm that the valve is fully open with the O-ring seal pushed down and moved to the side.

3. Slowly inject one control line fluid syringe into each accumulator on the IFC.
4. Remove the protective film from the bottom of the IFC.
5. On the **Home** screen of the Signature Olink Q100 instrument, tap **Target 96.** Place the IFC containing control line fluid and the interface plate on the drawer. Align the notched corner of the IFC to the notch on the drawer and face the barcoded edges of the IFC and interface plate forward. Tap **Close Drawer**. On the Ready to start chip preparation screen, tap **Start**. The screen shows the remaining time. For further details, refer to the Olink® Signature Q100 User Manual.

NOTE: *During the priming of the IFC, you should prepare the Detection Mix.*

8.4.2 Detection step instruction

IMPORTANT: *Always double check that you have the correct volume in ALL pipette tips.*

1. Remove the Extension products from the PCR instrument. Vortex and spin down the liquid.
2. Double check that all wells in the plate contain the same volume. Note any deviations. This information is needed to interpret the data in the statistical analysis step.
3. Thaw the Primer Plate and Detection Solution. Vortex and spin down the Detection Solution and Primer Plate. Keep the Detection Enzyme and PCR Polymerase in a freezing block or on ice.
4. Prepare the Detection Mix in a microcentrifuge tube. The Detection Mix must be used within 30 minutes from preparation.

Detection Mix	Per 96-well plate (µL)
Detection Solution	550.0
High purity water	230.0
Detection Enzyme	7.8
PCR Polymerase	3.1
Total	790.9

NOTE: *The maximum time to perform all steps from preparation of the Detection Mix until start of IFC loading (chip run) in the Signature Q100 is 60 minutes. Do not exceed that time.*

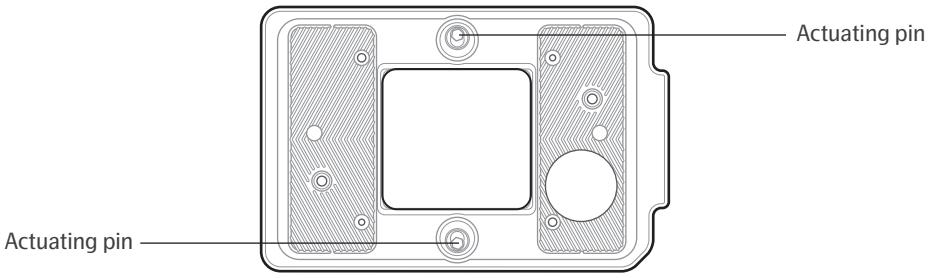
5. Vortex the Detection Mix and spin briefly. Transfer 95 µL of the mix to each well of an 8-well strip.
6. Use a multichannel pipette to transfer 7.2 µL of the Detection Mix to each well of a new 96-well plate by **reverse pipetting**. Use the same pipette tips throughout the plate. Name this plate Sample Plate.
7. Carefully remove the adhesive film from the Incubation Plate.
8. Transfer 2.8 µL from the extension products in the Incubation Plate to the Sample Plate using a multichannel pipette and **forward pipetting**. Change tips between each column.
9. Seal both the Sample Plate and the plate with extension products with adhesive plastic film.

TIP: *The plate with extension products can be saved for 1 week at +4 °C.*

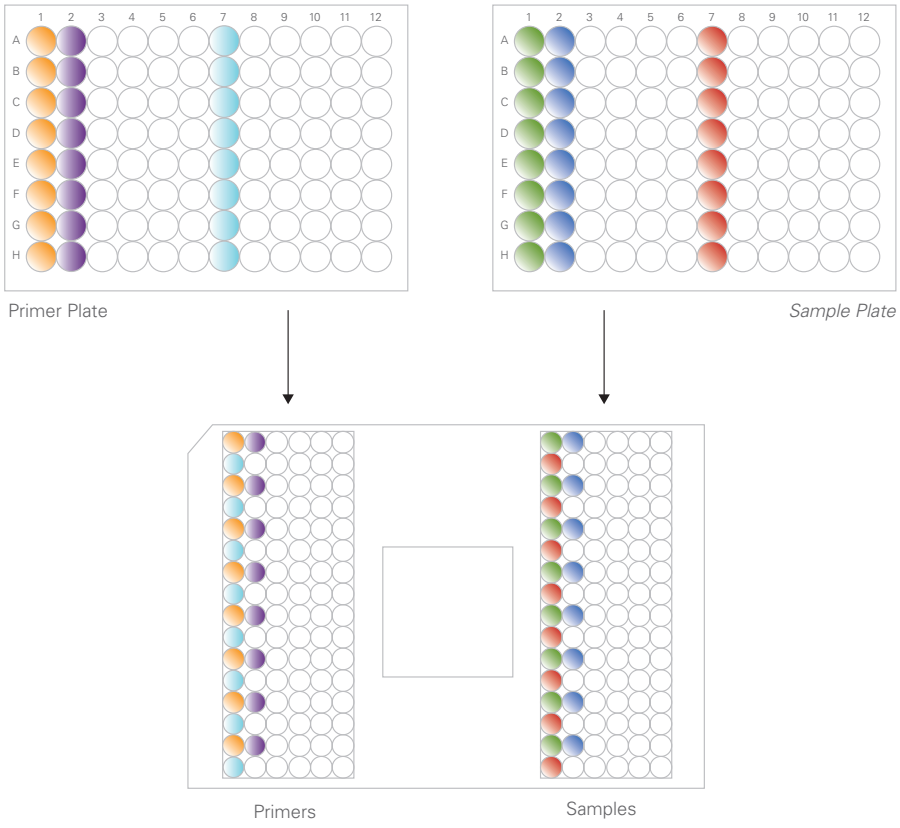
10. Vortex and spin the Sample Plate at 400-1000 x g, for 1 minute at room temperature together with the Primer Plate.

11. Double check that all wells in the Sample Plate contain the same volume. Note any deviations. This information is needed to interpret the data in the statistical analysis step.
12. Remove the primed IFC chip from the Olink Signature Q100.

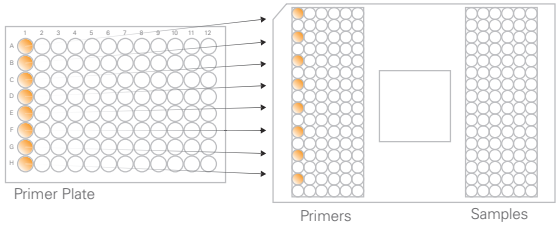
IMPORTANT: *Do not touch the interface plate actuating pins once affixed to the IFC, since that could result in depressurizing of the accumulators.*



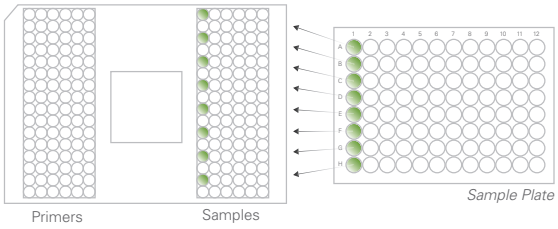
NOTE: *The IFC should be oriented so that the cut corner of the IFC is placed on the upper left side. In the following steps, the primers will be loaded to the left and samples to the right on the 96.96 Dynamic Array IFC. Here follows an overview illustration of the loading:*



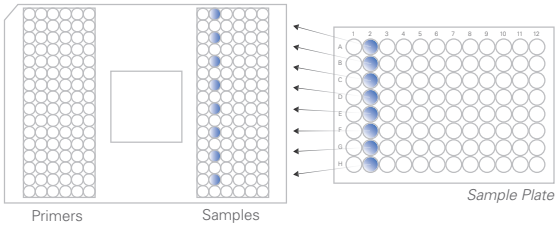
13. Carefully remove the adhesive film from the Primer Plate to avoid contamination between wells.
14. Transfer 5 µL using **reverse pipetting** from each well in the Primer Plate to the inlets on the left side of the IFC. Change pipette tips after each primer.



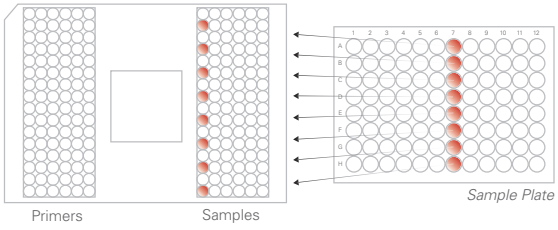
15. Transfer 5 µL of each sample using **reverse pipetting** from each well in position 1 A-H (green) to the inlets in the first column on the right side of the IFC (green). Change pipette tips after each sample. When using an eight-channel pipette every other inlet will be filled according to the image.



16. Transfer 5 µL from each well in position 2 A-H (blue) to the second column of inlets (blue) according to the image. Continue with columns 3-6. Use **reverse pipetting** and change tips between each column.

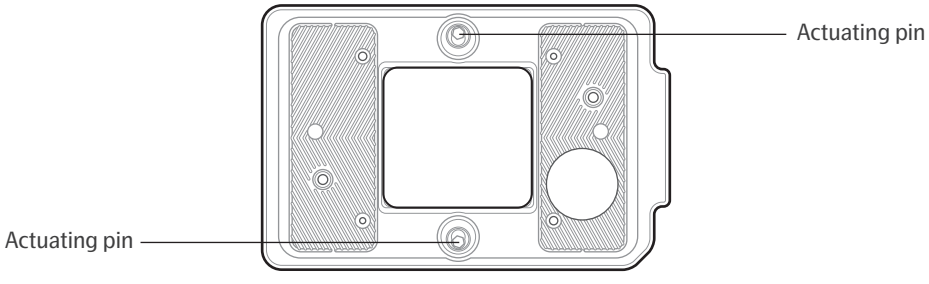


17. Transfer 5 µL from each well in position 7 A-H (red) to the inlets in the first column on the right side of the IFC (red), start on the second row according to image. Continue with columns 8-12. Use **reverse pipetting** and change tips between each column.



18. When loading is finished, inspect the wells and remove any bubbles using a syringe needle. Change needle between wells to avoid contamination.
19. Use a piece of adhesive tape to remove dust from the top of the middle section of the IFC. Let the sticky part lightly touch the surface of the IFC.
20. On the screen on Olink Signature Q100, tap **Open drawer**.

IMPORTANT: *Do not touch the interface plate actuating pins once affixed to the IFC, since that could result in depressurizing of the accumulators.*



Place the correct interface plate on the IFC, put the IFC with the interface plate in the open drawer of the Olink Signature Q100. Make sure that the Interface plate is placed properly on the IFC and in level with the drawer. Align the notched corner of the IFC with the notch on the drawer and face the barcoded edges of the IFC and interface plate forward. Tap **Close Drawer**. On the Add run details screen, confirm the Chip ID, then enter either

Run Name or Run Notes. Tap **Start Run**. The screen shows the remaining time.

21. When the run is complete, remove the IFC and interface plate from the instrument.

9. Standard Biotools® BioMark system

9.1 Additional requirements

The Olink Target 96 can be run both on the Olink Signature Q100 instrument as well as on the Standard Biotools BioMark system. if using the BioMark system the additional requirements and operational steps of this chapter should be followed.

9.1.1 Analysis software

The Biomark™ Data Collection software needs to be installed on the Biomark computer, and Real-Time PCR Analysis software is required for the analysis of Olink data in the Olink NPX Signature software.

9.2 Preparation - Create program

9.2.1 Olink® Protein Expression 96x96 v2 program

Create a BioMark System program named "Olink Protein Expression 96×96 v2" with the following steps:

Step	Temperature	Duration
Thermal mix	50°C	120 s
	70°C	1800 s
	25°C	600 s
Hot start	95°C	300 s
PCR cycle (35 cycles)	95°C	15 s
	60°C	60 s

Verify that the program has the following settings:

Variable	Setting
Application	Gene Expression
Passive Reference	5-Carboxy-x-Rhodamine (abbreviation ROX in Standard BioTools software)
Assay	Single probe
Probes	FAM-MGB

9.3 Operation

9.3.1 Detection step

Prime IFC

After injected one control line fluid syringe, place the IFC with barcoding facing you in the Olink Signature Q100. First select **Load Chip** and then **Prime** on the IFC Controller. The program takes approximately 20 minutes.

Detection step instruction

When finished with step 18, continue with:

1. Place the IFC with its barcoding facing you in the IFC Controller. Select **Load Chip** and **Load Mix** followed by **Run Script** to load the assay and sample mixes into the central part of the IFC. The loading program takes approximately 90 minutes. See the manufacturer's instructions for more details.
2. When the IFC loading is completed, eject the IFC from the IFC controller.
3. Use a piece of adhesive tape to remove dust from the top of the middle section of the IFC. Let the sticky part lightly touch the surface of the IFC.
4. Remove the protective film from underneath the chip.
5. Load the IFC in the BioMark with the barcode facing outwards and start the Olink Protein Expression protocol. Refer to *9.2.1 Olink® Protein Expression 96x96 v2 program*. The Biomark program takes approximately 2 hours and 10 minutes.

10. Other resources

10.1 Olink FAQs

The answers to the most common questions asked by our customers can be found on the Olink website: www.olink.com/faq.

10.2 Data analysis troubleshooting

Regarding issues detected during the quality control of the data or statistical analysis, see the troubleshooting chapter in the Olink NPX Signature User Manual for solutions. The manual is available from the Olink website: www.olink.com/downloads.

10.3 Lab instruction video

Learn from our experienced Analysis Service team how to run Olink panels by watching the Olink Lab Instruction video on the Olink youtube channel.

10.4 PEA technology video

For an animated description of how our innovative dual recognition, DNA-coupled methodology provides exceptional readout specificity, watch the PEA overview on the Olink youtube channel.

11. Revision history

Version	Date	Description
4.1	2024-03-14	Added notes about not touching the interface plate actuating pins once affixed to the IFC, in section 8.4.2. Removed information about *.plt files.
4.0	2023-05-31	Replaced references to the old membership site. Updated trademarks. Updated section 5.2.2. Updated 6.4 Editorial changes
3.5	2022-11-24	Changed the centrifugation speed for MixMate® to 2000 rpm. Changed last page footer.
3.4	2022-08-16	*8.4.2* Removed the sentence ”Vortex and spin down the enzymes before adding them to the mix.” *8.1* Changed PEA polymerase to PCR polymerase.
3.3	2022-05-18	*8.4.1* Added step 4. *8.4.2* Referred to Olink® Signature Q100.
3.2	2022-05-13	*9.2.1* Corrected number of PCR cycles.
3.1	2022-04-12	Adjusted the instructions to Olink® Signature Q100, and moved Fluidigm® BioMark to *9.* Added MixMate® and changed centrifugation speed. Revision history added. Changed logotype. Editorial changes.

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0935, v4.1, 2024-03-14